Any sufficiently advanced technology is indistinguishable from magic
Arthur C. Clarke

Whole cell biosensors:
From new applications to novel sensing technology

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LIST OF ABBREVIATIONS

ACTB:	β actin coding gene
Ag (I):	Monovalent Silver
AMEGA:	Antigen MEdiated Genetically modified cell Amplification
AMV:	Avian Myeloblastosis Virus
ATP:	Adenosine Triphosphate
Au (I):	Monovalent Gold
APC:	Antigen Presenting Cell
AR:	Androgen Receptor
BaF3:	IL3 dependent pro B cell line
Ba/HE+LgIGFP:	BaF3 cells expressing the EPOR signaling chain coupled to the antibody heavy chain recognition domain and the gp130 signaling chain coupled to the antibody light chain recognition domain
Bcl-X_L:	Bcl 2-like 1
BCOP:	Bovine Corneal Opacity and Permeability
BGPA:	Bacterial Gene Profiling Assay
BM:	Bone Marrow
Bmp:	Bone Morfogenetic Protein
BVES:	Blood Vessel Epicardial Substance
BRET:	Bioluminescent Resonance Energy Transfer
CALUX ®:	Chemically Activated Luciferase eXpression assay
CANARY:	Cellular Analysis and Notification of Antigen Risk and Yield
Cd (II):	Bivalent Cadmium
cDNA:	complementary DNA
CEN:	European Committee for Standardization
CHR:	Cytokine Receptor Homology domain
CISH:	Cytokine inducible SH_2-containing protein
CFU:	Colony Forming Unit
CLP:	Classification, Labeling and Packaging system for chemicals in the EU
cTCR:	chimeric TCR
CTL:	Cutotoxic T Lymphocyte
c-Myc:	Myelocytomatosis oncogene
CRBN:	Cereblon
Cu (I):	Monovalent Copper
dATP:	Deoxyadenosine Triphosphate
dCTP:	Deoxycytidine Triphosphate
dGTP:	Deoxygunaosine Triphosphate
DHT:	Dihydrotestosterone
DNA:	Deoxyribonucleic acid
dNTP:	Deoxynucleoside Triphosphates
DMSO:	Dimethyl Sulfoxide
dTTP:	Deoxythymidine Triphosphate
EEA:	European Environmental Agency
EEC:	European Economic Community
EGF:	Early Growth Factor
EIS:	Electrical Impedance Sensing
ELISA:	Enzyme-Linked Immuno Sorbent Assay
ELS:	Early Life Stage

EPA:	Environmental Protection Agency
EPO:	Erythropoietin
EPOR:	Erythropoietin Receptor
ER:	Estrogen receptor
ERα:	Estrogen Receptor alfa
EST:	Embryonic Stem Cell Test
Etv5:	Ets variant gene 5
EU:	European Union
FCS:	Foetal Calf Serum
Fluc:	Firefly luciferase
FP:	Fluorescent Protein
FRET:	Fluorescent Resonance Energy Transfer
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GC:	Gas Chromatography
GFP:	Green Fluorescent Protein
GH:	Growth Hormone
gp130:	glycoprotein 130
Gpr34:	G protein-coupled receptor 34
GSH:	Glutathion
GR:	Glucocorticoid Receptor
Grb2/SOS:	Growthfactor receptor bound protein/Son Of Sevenless complex
HEL:	Hen Egg Lysozyme
HET-CAM:	Hens Egg Test - Chorioallantoic Membrane
HMBS:	Hydroxymethylbilane Synthase
HPRT:	Hypoxanthine-guanine Phosphoribosyl Transferase
HRP:	Horse Radish Peroxidase
HUVEC:	Human Umbilical Vein Endothelial Cells
HWD:	Hazardous Waste Directive
IFN:	Interferon
IGF-1:	Interleukin like growth factor 1
IgG:	Immonoglobin G
Igsf:	Immunoglobin Superfamily
IL x:	Interleukin x
IMS:	Ion Mobility Spectroscopy
ISE:	Ion Selective Electrode
ITAM:	Immunoreceptor Tyrosine based Activation Motif
JAK:	Janus Kinase
LAT:	Linker of Activated T cells
LB:	Luria Broth
LC:	Liquid Chromatography
Lck:	LeukocyteC-terminal Src Kinase
LDH:	Lactate DeHydrogenase
LIF:	Leucemia Inhibitory Factor
LGPA:	Liver Gene Profiling Assay
LN:	Lymph Nodes
MAPK:	Mitogen Activated Protein Kinase
MHC:	Major Histocompatibility Complex
MIP:	Molecular Imprinted Polymers
MIT:	Massachusetts Institute of Technology
MS:	Mass Spectrometry

MTL:	Maximum Toxic Load
MTT:	(3-(4,5-diMethylThiazol-2-yl)-2,5-diphenylTetrazolium bromide
Myc:	Myelocytomatosis oncogene
NEAA:	Non Essential Amino Acids
NRC:	National Research Council
OECD:	Organization for Economic Co-operation and Development
Olig:	Oligodendrocyte transcription factor
PAH:	Polycyclic Aromatic Hydrocarbon
PBS:	Phosphate Buffered Saline
PCB:	PoluChlorinated Biphenyls
PCR:	Polymerase Chain Reaction
Pb(II):	Bivalent Lead
PET:	PolyEthylene Terephthalate
PE:	PolyEthylene
PI3K:	Phosphoinositide 3 kinase
Pim:	Proto-oncogene serine/threonine protein kinase pim-1
PGK1:	Phosphoglycerate kinase Pgk1
PRL:	Prolactin
PSA:	Prostate Specific Antigen
Ptgs2:	Prostaglandin-endoperoxide synthase 2
PTK:	Protein Tyrosine Kinase
REACH:	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA:	Ribonucleic Acid
ROS:	Reactive Oxygen Species
RPM:	Rounds Per Minute
RPMI:	Roswell Park Memorial Institute
RT qPCR:	Real Time quantitative PCR
SAR:	Structure Active Relation
SAW:	Surface Acoustic Wave
scFv:	Single Chain Variable Fragment
SHP2:	SH_2 domain containing tyrosine Phosphatase
SLP-76:	SH_2 containing Leucocyte Protein of 76 kDa
Snap25:	Synaptosomal-Associated Protein 25
SOCSx:	Supressor Of Cytokine Signaling x
SOE:	Slpice by Overlap Extension
STAT x:	Signal Transducer and Activator of Transcription x
SPR:	Surface Plasmon Resonance
Spred x:	Sprouty-related, EVH1 domain containing x
Spry x:	Sprouty-homologue x
STAT:	Signal Transducer and Activator of Transcription
Syk:	Spleen Tyrosine Kinase
TBP:	TATA binding protein (reference gene)
TCDD:	2,3,7,8-Tetrachloordibenzo-p-dioxin
TCR:	T-Cell Receptor
TER:	Transepicutaneous Resistance Test
TNF-α:	Tumor Necrosis Factor α
TL:	Toxicity Limit
TR:	Thyroid Hormone Receptor
TYK2:	Interferon alpha/beta signaling pathway-related protein

	tyrosine kinase
UBA:	Environmental Agency of Germany
V_H:	Antibody Heavy chain Variable region
V_L:	Antibody Light chain Variable Region
WT:	Wild Type
WTP:	Water Treatment Plant
QCM:	Quartz Crystal Microbalance
YES:	Yeast Estrogen Screen
YWHAZ:	14-3-3 protein zeta/delta
ZAP-70:	ζ chain Associated Protein kinase 70
Zn(II):	Bivalent Zinc

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ABSTRACT

Living mammalian cells are considered to be the best alternative to animal tests, for the prediction of in vivo effects. Therefore, they are widely applied as effect sensors in (eco)toxicology, drug screening and food quality control. Aside from their application as effect sensors, increasing knowledge on cell receptors and their signaling pathways have enabled the development of targeted whole cell sensors, often reporter gene assays, for the detection of specific molecules or groups of (structurally related) molecules. Their capacity to react to very small concentrations of certain molecules (into the attomolar range) makes them excellent tools for detection of molecules in bodily fluids, allergens in foods and toxins in diluted water or soil samples. Cell based sensors are limited to the cell's natural targets, as opposed to affinity sensors, which are capable of detecting a much wider range of molecules, as long as a suitable affinity molecule (such as an antibody) exists.

In this project, two aspects of biosensor development are explored. First, we focus on the development of a cell based targeted biosensor prototype for detection of low concentrations of a wide range of molecules in complex mixtures. We combined the high sensitivity of cells with the versatility of antibody based sensing devices, by using chimeric receptors which combine antibody recognition domains with cell receptor signaling chains.

On the other hand the application of existing biosensors to the new field of waste toxicity assessment is explored. In this part, a novel strategy for fast waste classification is proposed, and a battery of tests is investigated in a pilot study.

Part I: Development of a novel, sensitive and widely applicable whole cell sensor

To build a biosensor prototype , two chimeric receptor cassettes are considered. The first links the T-cell receptor (TCR) ζ chain and a costimulatory CD28 molecule to an anti-PSA single chain antibody. The second is based on an

erythropoietin receptor (EPOR) /gp130 heterodimer and has hen egg lysozyme (HEL) for a proof of principle target.

To reach a proof of principle, the EPOR/gp130 chimeric receptor couple is chosen first, as these are naturally very sensitive receptors. The EPOR/gp130 based construct is expressed in the BaF3 cell line and cell proliferation experiments confirmed HEL-dependent proliferation of transformed cells. Activation of different promoters by the EPOR/gp130 signalling cascade was investigated by qPCR and three promoters, CISHp, SOCS1p and SOCS3p, have been selected for the development of a reporter gene assay. For the proof of principle, CISHp is coupled to a luciferase reporter gene and coexpressed with the anti-HEL EPOR/gp130 chimeric receptor couple. Results demonstrate a HEL induced expression of reporter protein. The sensitivity of the whole cell biosensor prototype is limited, however this may be improved by modifications to the chimeric receptor or to the reporter system. Also, cytokine induced cross-talk is investigated to evaluate the biosensor specificity. By combining the high sensitivity of whole cells with a label-free read-out system, we created a new and innovative biosensing detection applicable to a large range of potential targets. Sensitivity of the sensor must still be improved, however this proof of principle has demonstrated the potential of chimeric receptors for whole cell biosensing.

The development of a novel, versatile whole cell biosensor prototype is described in **chapters 2 to 4**.

Part II: Discriset: Development of a novel, fast waste classification strategy

The amount of waste produced in Europe (and in the rest of the world), is increasing every day. Therefore, the health and ecological consequences, as well as the economical consequences of correct management of hazardous waste, are enormous. The Hazardous Waste Directive (HWD, Council Directive 91/689/EC) provides a framework for classification of hazardous waste, based on 15 Hazard (H)-criteria. For complex wastes the HWD foresees the application of toxicity tests on the waste material itself to assess its toxic properties. However, these proposed test methods often involve mammalian testing, which

is not acceptable from an ethical point of view, nor is it feasible economically. The Discriset project has been initiated to investigate the use of alternative chemical and biological fast screening tests for waste hazard classification. In the first part of the project, different methods are reviewed and a testing strategy is proposed to minimize time and cost of analysis by a tiered approach. This includes, as a first tier, chemical analysis, followed by a general acute toxicity screen as a second tier, and as a third tier mechanistic toxicity tests to assess chronic toxicity (genotoxicity, hormone disturbance, teratogenic effects, immunologic activity). As a proof of application, selected methods are applied to sixteen different waste samples from various sources and industries in a pilot study. The first tier chemical tests are recommended for the full characterization of the leachate fraction (inorganics) but not for the organic fraction of samples. Here the chemical characterization is only useful if toxic content is known or suspected. As second tier the fast bacterial test Microtox® is validated as a general toxicity screen for the organic fraction (worst case organic extract). Samples that are not classified in tier 1 or 2 are then further investigated in the third tier by the mechanistic toxicity tests and tested for their potentially chronic toxicity: immune activity (TNF-α upregulation) is indicative for corrosive, irritating or sensitising effects (H4/H8/H15), reproductive effects (H10) are indicated by hormone disturbance and early life stage abnormalities in fish larvae when exposed to the extracts, and mutagenicity and carcinogenicity (H7, H11) are indicated by SOS reponse induction and increased mutation frequency in the Ames test when exposed to the extracts. Results indicate that the combination of chemical tests and bioassays allows important hazardous properties to be addressed and the tiered approach ensures that the tests are performed quickly and economically. The suggested strategy provides a solid and ethical alternative to the methods described in the HWD and is a vast improvement on the current, arbitrary classification.

The development of a new, tiered testing strategy for hazardous waste classification is described in **chapters 5 and 6**.

CHAPTER 1

GENERAL INTRODUCTION AND AIMS

General Introduction and Aims

The term biosensor is generally used for sensors which incorporate a biological element such as an enzyme, antibody, nucleic acid, microorganism or cell (Turner, 1996). Biosensors may detect a specific (group of) compound(s) or may be employed to monitor specific effects of samples on (higher) organisms. The development of biosensors has grown exponentially over the last decades, as the wide range of possibilities became clear, and the demand for new biosensors for specific applications increased. These devices, designed to allow the detection of specific molecules, groups of molecules, pathogens or (toxicological, environmental or drug) effects, have emerged from highly specialized and often interdisciplinary research. Indeed, biosensor technology unifies the knowledge and state-of-the-art technology from biotechnology, biology, chemistry, physics and engineering.

Biosensors are applicable in all sectors of the bioeconomy, including the white (industrial), green (agricultural), red (medical) and blue (aquatic) sectors. The applications of biosensors are as diverse as the sensors themselves. Examples include biosensors for process monitoring in industrial food processing plants, such as the lactose sensor utilized in dairy processing plants (Glithero et al., 2013) or pathogen and allergen detection (e.g. Ohk & Bhunia, 2013; Wang et al., 2011). Environmental applications include (eco)toxicity testing, reviewed by Pasco and colleagues (2011), and biomonitoring of specific pollutants, such as pesticides, and nutrients (potassium, phosphate,…) (e.g. Pundir & Chauhan, 2012; Warwick et al., 2013) both in soil or aqueous environments. In the medical sector, the use of biosensors has become essential. Some are used for detection of important biomarkers related to diseases including cancer, cardiovascular diseases and infectious diseases (e.g. Zhang & Ning, 2012), while others are employed to monitor patient health (e.g. blood glucose sensor) or recovery (e.g. wound healing sensor, Dargaville et al. 2012).

To meet with the wide range of applications and the continuously increasing demand for new biosensors, the development of innovative new biosensor technology is essential. These new developments may be aimed at new targets,

higher sensitivity, wide applicability, ease of use and low cost, depending on the application at hand. In this introductory section, general features of biosensors are discussed and the specific characteristics and applications of whole cell sensors are presented. Finally, the need for innovative new biosensing technologies, aimed at a wide applicability and high sensitivity, has led to the research presented in the following chapters.

1.1 Biosensors: the beginning

It is generally agreed that the history of biosensors begins with the development of the first glucose detector by Leland C. Clark Jr, shown in figure 1.1. The original paper from 1956 describes an oxygen (O_2) electrode: an O_2 reducing platinum (Pt) working electrode that allowed constant blood glucose monitoring. The sensor was improved in 1962 through the entrapment of enzymes (i.e. glucose oxidase) in a small volume of solution adjacent to the electrode. The membrane used to entrap the glucose oxidase is permeable to glucose, which is then detected by the change in the electrode potential that occurs when glucose reacts with the enzyme in this volume of solution (Clark et al. 1956, Clark & Lyons 1962).

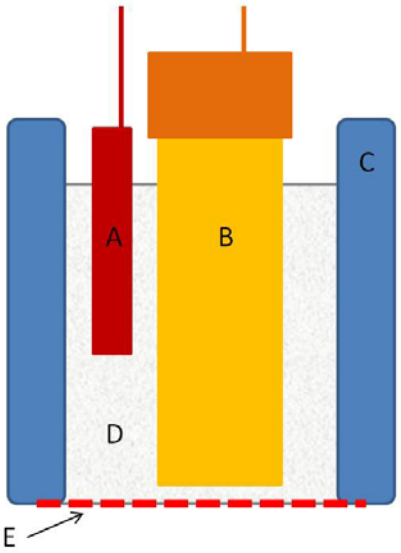


Figure 1.1 Schematic representation of the enzymatic glucose sensor designed by Prof. Clark (1962). The electrode cell contains a reference electrode (A) and a Pt working electrode (B), held in a cylinder (C), the end of which is covered in an O_2 permeable, multi-layered membrane (E). The cell is filled with an electrolyte (D) containing enzyme (i.e. glucose oxidase). The multi-layered membrane (E) also contains a layer of concentrated enzyme. (Adapted from Clark & Lyons 1962).

The principle behind the Clark electrode remains the core of modern glucose sensing technology, though new developments have improved both sensitivity and applicability of the glucose sensor. Table 1.1 lists the most important milestones in glucose sensing, and figure 1.2 depicts a commercial hand-held glucose sensor (1.2 A) and the state of the art in glucose sensing in an integrated closed loop system, also referred to as an artificial pancreas (Figure 1.2 B).

Table 1.1: Milestones in the history of glucose sensor development. From the glucose electrode to handheld devices, continuous glucose monitoring and an implantable closed loop system.

Date	Event	Reference
1956	Clarks glucose electrode	Clark 1956
1962	First glucose enzyme electrode	Clarck & Lyons, 1962
1973	Glucose enzyme electrode based on peroxide edetection	Guilbault et al., 1973
1975	Launch of the first comercial glucose sensor system	YSI Inc.
1982	Demonstration of in vivo glucose monitoring	Shichiri et al., 1982
1984	Development of ferrocene mediators	Cass et al., 1984
1987	Launch of the first personal glucose meter	Medisense Inc.
1987	Electrical wiring of enzymes	Degani et al., 1987
1999	Launch of a commercial in vivo glucose sensor	Minimed Inc.
2000	First wearable noninvasive glucose monitor	Cygnus Inc.
2008	Patient trials with a closed-loop sensor, integrating continuous glucose monitoring with an insulin pump system	Weinzimer et al., 2008
2013	Implantable, integrated glucose monitoring and insulin regulation?	Heo & Takeuchi, 2013

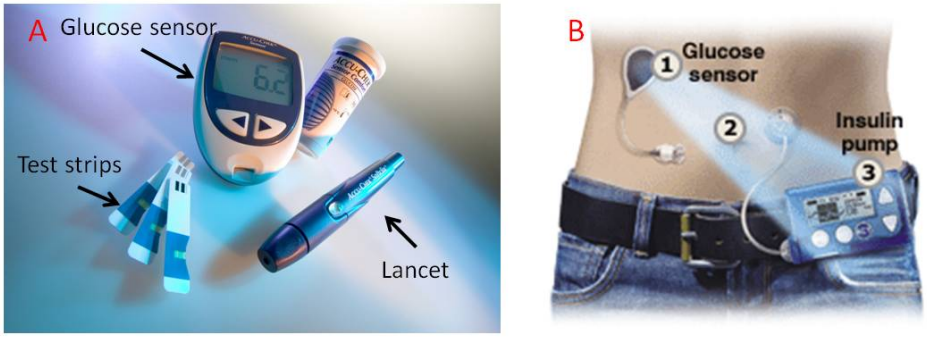


Figure 1.2 A: Commercial glucose sensor. A drop of blood is obtained using the lancet, and absorbed by the test strip. This is then inserted in the sensing device. Read out follows in a few seconds. (F. Hoffman – La Roche Ltd) **B: artificial pancreas:** the implanted glucose sensor continuously monitors blood glucose levels and communicates with an insulin pump in a closed system (based on Weinzimer et al., 2008).

This enzymatic sensor, while being of crucial importance in biosensor development and commercially still the most successful biosensor, is not an example of a typical biosensor as described in figure 1.3. Indeed, most biosensors, as opposed to the glucose electrode, are based on a biorecognition event between a ligand and a biological receptor molecule, which is then transduced into a readable (often electronic) signal. Biosensors based on biorecognition events between a receptor molecule and a target molecule are also referred to as targeted biosensors. They may be analytical devices (see figure 1.3 A) or whole cell sensors (see figure 1.3 B).

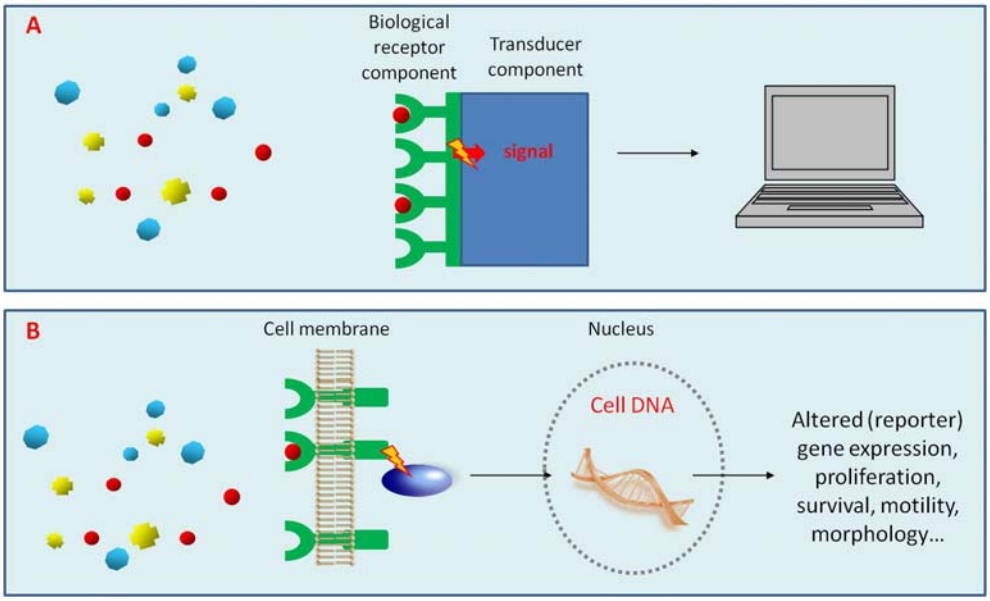


Figure 1.3: Targeted biosensors. (A) Typical representation of an analytical biosensor. Recognition molecules are immobilized on a transducer component, which turns the biological recognition event into a readable, often electronic, signal. (B) Typical example of a targeted whole cell sensor. The cell provides the biological receptor and turns the initial signal into a readable output.

An analytical biorecognition based biosensor consists of two main elements: a layer of biological receptor molecules, attached to a physical component that transduces the biological recognition event between the receptor molecules and their target into a readable signal (see figure 1.3 A). A whole cell biosensor can also be interpreted in this way, however the biological receptor and physical transducer are both replaced by a single living cell, as shown in figure 1.3 B.

Biorecognition occurs via a specific biological receptor molecule, which is expressed by the cell and which transduces a signal via cellular pathways resulting in changes in gene expression, cell viability, motility, shape and metabolism.

Aside from enzymatic biosensors as shown in figure 1.1 and the targeted biorecognition based biosensors presented in figure 1.3, another important class is formed by the effect biosensors. These are usually whole cell sensors (or possibly whole organism sensors), which are however not based on a single biorecognition event and show little similarity to either the enzymatic glucose sensor or to the analytical sensors presented above. Instead, they show more similarity to the canaries that were taken down into coal mines to warn mine workers for toxic gasses than to the glucose sensor which detects a specific chemical reaction. The effect sensor, much like the canary mentioned above, is subjected to its environment and translates the combined effects of all components of the environment (such as the presence of toxic carbon gas) into a detectable signal (e.g. cell death or canary death). This is in fact much like the assays performed with multicellular organisms. Figure 1.4 summarizes the types of biosensors introduced above in relation to each other. The first three types of biosensors which were discussed in the previous paragraph, the enzymatic glucose sensor, the biorecognition based analytical devices and the targeted whole cell sensors, all have a well-defined target, whereas the effect sensors are more closely related to tests on whole organisms and reflect the combined effect of all environmental components.

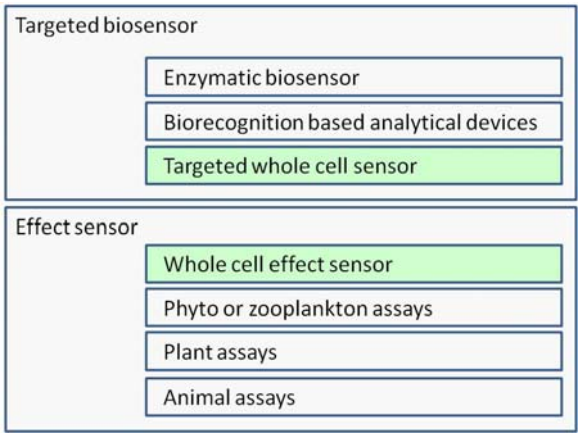


Figure 1.4: General classes of effect and targeted biosensor types in relation to each other. Whole cell biosensors may either belong to the targeted biosensors or to the effect biosensors.

Whole cell sensors form a rapidly evolving area of biosensor research both as effect sensors, and in a targeted approach. In this chapter two aspects of biosensor technology are tackled. First, the current state of the art in whole cell biosensing technology is described, focusing on targeted whole cell sensors in particular. Currently used techniques are discussed and possibilities for improvement and expansion are introduced. Also, existing and well validated (bio)sensors in the field of ecotoxicology are explored, for the specific goal of complex waste screening. In this part the focus is mainly on effect sensors. These two distinct approaches have led to two aims focusing on the development of an innovative whole cell biosensor on the one hand and on the application of existing biosensors in new, tiered testing strategy for the assessment of complex wastes.

1.2 Whole cell biosensors: a definition

Whole cell biosensors are in vitro tests that use living cells to evaluate a sample, either characterizing the sample by its effect on general cell metabolism or targeting specific pathways. The assay outcome is registered either by observing the general wellbeing of the cells (through proliferation rate, cell death, bioluminescence,) or is evaluated by the use of specific reporter genes that produce a readable signal (e.g. luciferase, Horse Radish Peroxidase or HRP).
Based on the cell type used and the output of the whole cell sensor, several types of whole cell sensors exist, described briefly in the following section.

1.2.1 Cell type

Whole cell sensors all have in common that they use living cells as primary transducers for signal generation. The signal is then converted by a secondary transducer for the purpose of detection, mostly by electrical or optical means. These types of biosensors can be based on yeast cells, bacteria or eukaryotic cells. The type of cell chosen partly dictates the conditions necessary for the bioassays and the possible applications, and vice versa.

1.2.1.1 Bacterial cells

Cell based biosensors employing bacterial cells are widely used in ecotoxicology. Most systems are based on the expression of a luminescent or fluorescent reporter protein. These bacterial biosensing systems can be categorized into two different types, depending on the mode of expression of the reporter protein (Gu et al. 2004). Expression of the reporter can either be constitutive or inducible. In constitutive expression systems, the reporter is expressed at high basal levels. An increase in the amount of compounds that are toxic to the cell causes its metabolism to slow down and ultimately leads to cell death, thus reducing and eventually stopping reporter protein expression and the generation of output signal. Whole-cell biosensing systems based on constitutive expression have been used to measure the general toxicity of a sample or test compound. A well-known example is Microtox® toxicity testing (Bulich & Isenberg, 1981), a standardized, commercially available toxicity testing system that uses the

bioluminescent marine bacteria Vibrio fischeri as bacterial sensor for detection of toxic compounds in (water) samples. When V. fischeri is exposed to a sample containing toxic compounds, a dose-dependent reduction in bioluminescence is observed, indicating the toxicity level of the sample. Microtox® is one of the effect assays used in Part II: Application of biosensors in waste toxicity screening.

Another class of whole cell bacterial biosensing systems comprises inducible expression systems in which the cells are genetically engineered to contain a plasmid in which an inducible promoter is fused to a reporter gene. Often, these assays use (modified) Escherichia coli bacteria, which have been studied for years. In-depth knowledge of its biochemistry and genetics makes it the most proficient prokaryote for the development of new toxicological assays (Robbens et al. 2010). These types of assays are amenable to multiplexing, which has led to the development of multiple endpoint whole cell biosensors such as the Bacterial Gene Profiling Assay (BGPA). In this biosensor system, 14 transgenic *E. coli* strains, each engineered to express a reporter gene upon activation of a specific (stress) pathway, are exposed to the sample which is under investigation. Different toxicological endpoints are thus measured in parallel, leading to a toxicological profile of the sample in question (Dardenne et al., 2008).

While most bacterial sensors employ reporter genes, there are also examples of widely used tests which function in a different way. The Ames test, a Salmonella (or sometimes *E. coli*) based test for mutagenesis, is a well validated example which has frequently been used to identify carcinogenic properties of commercial materials, food additives or pharmaceuticals. The bacterial strain used in this biosensor is histidine (His) deficient because of a mutation in the His gene. Only the bacteria which have undergone a back mutation are able to grow on histidine free substrates. Thus, exposure of the bacteria to a mutagenic substance increases the number of colony forming units grown (CFU) on such a substrate. The increase in CFU can be related directly to the mutagenicity of the sample under investigation.

Bacterial biosensors are extremely versatile and have found their way into many applications. Often, they are used for effect assays, such as the Microtox®, BGPA and Ames tests described in the previous paragraphs, which detect various toxic effects. But bacterial sensors have also been used for targeted sensing applications. One typical application is the detection of heavy metals and metalloids, a domain in which modified receptors have been used alongside natural receptors to broaden the range of targets (Hynninen & Virta, 2010). Most of the metal sensing bacterial sensors rely on two families of metal binding transcription factors, MerR and SmtB/ArsR, coupled to a reporter gene system. The characterized MerR receptors respond to structurally related metal ions with the same charge, for example, the monovalent metal receptor CueR recognizes copper (Cu(I)), silver (Ag(I)) or gold (Au(I)) ions with similar affinity; and the same occurs with ZntR that responds to zinc (Zn(II)), cadmium (Cd(II)) or lead (Pb(II)) (Binet et al., 2000; Stovanov, 2003). Both site-directed mutagenesis and motif swapping were employed to change metal preference on a number of transcriptional regulators, allowing for the generation of biosensors sharing some structural/functional receptor characteristics but with diverse specificities (e.g. Stoyanov et al., 2003; Checa et al., 2007; Cerminati et al., 2011). These types of biosensors, where bacterial signaling systems are employed as platforms for rational design of new whole cell biosensors, are reviewed extensively by Checa and colleagues (2012).

However the use of bacterial cells is also prone to some limitations: cellular responses to some molecules are different in prokaryotes from those in eukaryotes and this limits their application in toxicity evaluation and drug screening for higher organisms. Toxic levels of compounds disturbing metabolic pathways typical for eukaryotes or higher organisms (e.g. endocrine effects) often have a very different effect on bacteria. Aside from this important draw-back, limited pH, osmotic and temperature tolerances of individual species may also mean that the operating parameters of the sensor could be limited and bacterial cells may be relatively fragile in the sensor environment leading to short shelf and in-use lives (Raut et al. 2012)

1.2.1.2 Yeast cells

Yeast sensors have a number of advantages as opposed to the bacterial cell sensors discussed above. Like mammalian cell sensors, yeast cells are eukaryotes and as such they can provide information of direct relevance to other eukaryotes, which prokaryotes cannot. An important advantage of yeast cells is that they are more robust than bacteria, withstanding a broader pH, temperature and osmolarity range (Wajmsley and Keenan, 2000), while sharing some of the advantages of bacteria, such as ease of manipulation, high growth rate and growth on a wide range of substrates (Parry, 1999). The shelf life of yeast sensors is potentially very high compared to other cell sensors. This is demonstrated by the dried baker's yeast granules available for commercial and home baking: washed yeast cells are dried in air at 28–40 °C to a moisture content of between 7.5 and 8.3%. The dried cells have a useful shelf life of more than a year when stored in nitrogen or under vacuum at room temperature. The cells lose approximately 10% of their activity in this time (Ponte Jr. and Tsen, 1987). A yeast biosensor with a useful life of 1 year when stored at 4 °C has been reported (Preininger et al., 1994). Additionally, Baronian (2004) suggests that the potential of yeast as a biosensor resource is enormous, given the wide range of environments from which yeast species have been isolated and substrates they metabolize. Despite the advantages of yeast cells as opposed to bacteria, the use of yeast biosensors is as yet limited as compared to bacterial sensors. Baronian only cites 19 wild type yeast sensors and 13 biosensors based on modified yeast strains. The reason for this discrepancy may lie in the fact that, on the one hand, many yeasts species are thus far unknown (Walker, 1988), and that, on the other hand, there is a longer tradition and know-how in laboratories where bacteria are concerned as opposed to yeast.

1.2.1.3 Mammalian cells

have an unparalleled capacity to predict in vivo effects as compared to other whole cell sensors and as such they are considered the next best option compared to living animals as biosensors: while the best sensing system for detection of animal or human threat or effects would obviously be the living animals themselves, mammalian cell assays circumvent high cost and ethical issues coupled to the use of live animals while reflecting the effects of samples on mammals. This strategy is framed by the principles of Replacement,

Reduction and Refinement (three R's) proposed by Russel and Burch (1959) as the key strategies to achieve human experimental techniques. Although mammalian cell sensors are less robust and therefore require more specialized infrastructure than bacteria or yeast, their high predictive capacity has led to the widespread use of these types of sensors as tools in research and development in several areas, including drug discovery, toxicology, pharmacology, bio-assays, pathogen and toxin screening, environmental monitoring and biosecurity. Another important advantage of biosensing technology which employs mammalian cells is the vast knowledge which is available on mammalian cell signaling, which allows rewiring of this signaling to serve biosensing purposes. The identification of gene functions, signaling pathways and receptor functions has allowed mammalian cells to be used for targeted analysis alongside effect sensing and still holds great promise for the development of new biosensors.

Mammalian cell biosensors all have in common that they employ mammalian cells as primary transducers for signal generation. Different types of mammalian cell biosensors can be defined, based either on the means of signal generation, or on the mechanism of detection. These include sensors based on electrical responses, responses linked to cellular receptors, cellular metabolism, cytotoxicity or genomic responses (Banerjee & Bhunia 2004). Here, we will focus on cell based biosensors that generate a signal through the latter mechanism, i.e. genomic responses. For these types of sensors, the initial effect of the cell's environment or the effect of specific components of this environment leads to an altered expression profile, often inducing the expression of a reporter gene, which is registered by optical or electronic means.

Mammalian cell biosensors based on genomic responses are considered promising tools in several areas including drug discovery and pharmacology (reviewed by Michelini et al., 2010), bio-assays, pathogen screening (reviewed by Arora et al., 2011) and toxicity screening (reviewed by Banerjee & Bhunia, 2008), environmental monitoring and biosecurity. The following sections will focus on the means of signal generation and on the signal detection methods, considering the successes and remaining research challenges.

1.2.2 Signal generation in mammalian cells

Mammalian cell biosensors based on genomic responses can be categorized into two classes. The first type of mammalian sensors reflects general effects on cell metabolism and viability. The changes registered are the combined effect of the cell's environment on its metabolism and are often of great interest in ecotoxicological research. Examples of such effect sensors are further discussed in Part II: Application of biosensors in waste toxicity screening.

The second class of mammalian cell biosensors targets a specific pathway. These targeted whole cell sensors employ (recombinant) cellular receptors during the first biorecognition step, and use the associated receptor pathway to amplify the signal from this initial recognition event, ultimately leading to a readable signal which is often produced by the expression of an inducible reporter gene. As such the cell receptors dictate the biosensor target and are crucial for the development of new cell based biosensor systems.

Many natural cell receptors have been employed in whole cell sensors in environmental research, toxicological screening, food control and drug discovery, both in a laboratory setting and in commercial systems. An important example of a commercial whole cell based system which exploits natural cell receptors is the CALUX® system. The brand name is an acronym for Chemically Activated Luciferase Expression System. This system offers a whole battery of whole cell biosensor systems which test for endocrine effects, mediated via more than 15 hormone receptors, including the estrogen receptor (ER), androgen receptor (AR) (Sonneveld et al., 2005), glucocorticoid receptor (GR) and thyroid hormone receptor (TR) (Van der Linden et al. 2008), amongst others. The activation level of the receptor pathway reflects the sum of all agonistic, antagonistic or cross-talk inducing molecules in the cell's environment. When cross-talk is unwanted, the natural receptors may be expressed in cell types that normally lack this receptor: ERα CALUX® is an example of a whole cell sensor based on the U2OS cell line, a human osteosarcoma cell line that normally lacks the estrogen receptor (ER). Modification of this cell line with ERα leads to an estrogen responsive cell line, which lacks the cross-talk pathways normally present in ER expressing cells, thereby increasing the specificity of the sensor. Parallel assays with ER CALUX®, an endogenous ER expressing reporter cell line

(thus with cross-talk pathways), and ERα CALUX allow the ERα mediated effects to be discerned from cross-talk induced effects, leading to an even better interpretation of estrogenic effects upon exposure of the whole cell biosensors to (complex) samples (Van der Burg et al. 2010).

When the target is not a natural ligand of the cell receptors expressed in the cells, chimeric receptors provide a solution: engineering cell receptors offers the opportunity of a rational design of the cell based biosensor. Chimeric receptors couple a receptor signaling chain to the recognition domains of an antibody, thus inducing downstream signaling upon binding of the antibody target. This technology has already been used successfully in various applications, such as research into receptor signaling and also antigen mediated genetically modified cell amplification (AMEGA), where cytokine receptors were modified to elicit a growth signal upon exposure to the antibody ligand, thus allowing a positive selection of modified cells (as opposed to the negative selection using expensive antibiotics which is traditionally used for this purpose) (Kawahara et al., 2003).
This technique has already shown considerable promise in biosensor research in targeting bacteria and virus particles. The so-called "cellular analysis and notification of antigen risk and yields" (CANARY) sensor, developed by the Lincoln Laboratory at the Massachusetts Institute of Technology (MIT) is an example of a genetically engineered B-cell based biosensor that detects specific pathogens (Rider et al. 2003). In this system, a parental surface immunoglobulin M (IgM)-positive B-cell line (M12 g3R) was engineered to express a bioluminescent calcium-responsive protein – aequorin – from a jellyfish (which emits light when intracellular calcium concentration increases) and was cross-linked to the IgM. These B cells were further engineered to express variable regions of antibody light and heavy chains specific for molecules expressed on the surface of a particular pathogen. Binding of the target to the antibodies initiates a downstream signal transduction cascade, which can trigger an intracellular calcium flux; the increase in calcium concentration then causes the aequorin protein in the cytoplasm of the B cell to emit light almost instantaneously, which can be detected by using a portable luminometer (Rider et al. 2003).

Intelligent design of whole cell biosensors through the use of chimeric receptors holds great promise for novel developments in biosensor research. However, the pathogen targeting cell based biosensor described before only targets molecules expressed on the surface of bacteria or viruses. Being expressed on the surface of these pathogens, the target molecules are in fact preconcentrated. An interesting research question which arises from this is whether or not detection of low concentrations of molecules is also possible using chimeric cell receptors. Certain natural cell receptors, such as cytokine receptors, initiate a cell response even at very low concentration of target, and the question may arise wither a chimeric receptor, based on such a sensitive cell receptor combined to the variable parts of an antibody (targeting a molecule of choice) equally induces a measurable response at very low concentrations. Intelligent design of sensitive whole cell biosensors offers interesting possibilities for the detection of traces of certain substances, such as allergens, which are known to elicit serious effects in humans, even at very low concentrations.

1.2.3 Signal detection methods

Targeted mammalian cell sensors employ mammalian cells as primary transducers for signal generation as illustrated in figure 1.5. The initial biorecognition event between a cell receptor and its ligand leads to a readable signal. This signal may be a natural alteration in gene expression leading to changes in cell vitality, proliferation rate, motility or morphology, or it may be a

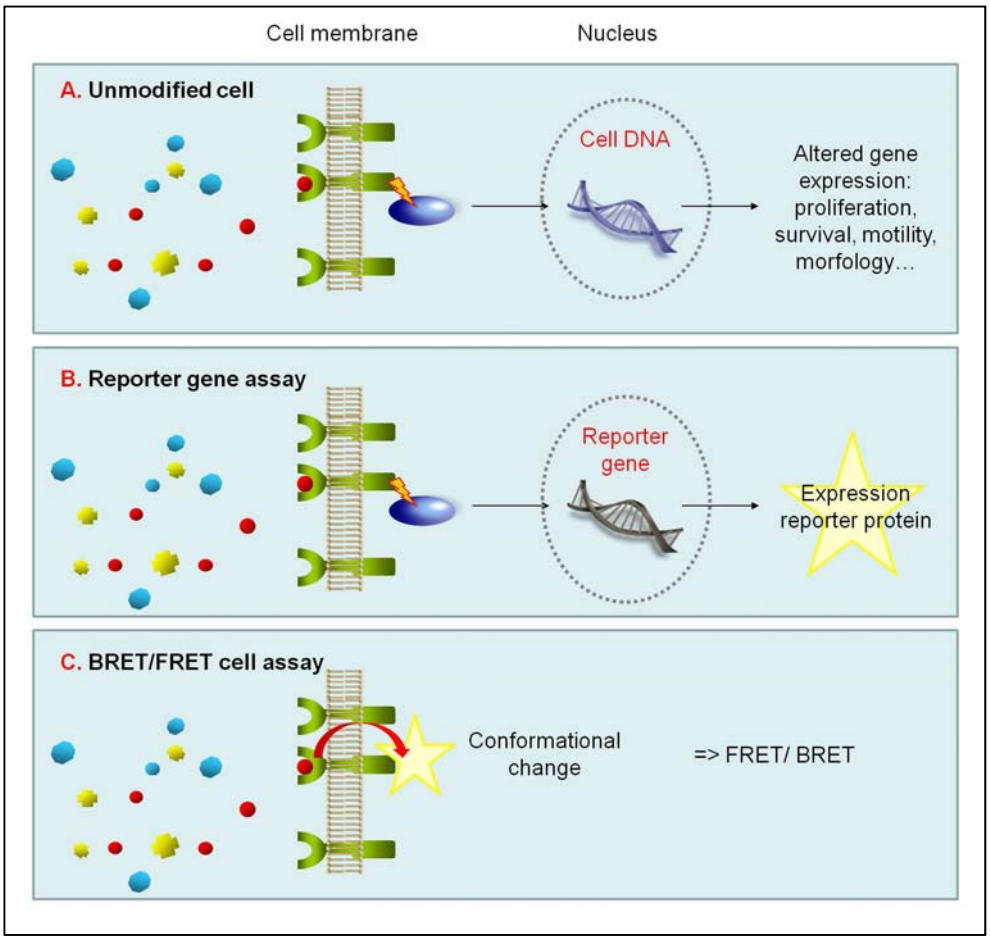


Figure 1.5 Whole cell sensor mechanisms exploiting a membrane bound receptor. A: In the unmodified cell, the binding event leads to an altered gene expression pattern, resulting in changes in proliferation rate, cell survival, motility or morphology. B: The binding event in the reporter gene assay leads to the expression of a recombinant reporter protein (e.g. luciferase), which is then detected by a secondary transducer. C: Upon binding of the target a conformational change of the receptor initiates fluorescent or bioluminescent energy transfer.

reporter protein which is then converted by a secondary transducer for the purpose of detection, usually by electrical or optical means. A third means of detection involves the use of fluorescence resonance energy transfer (FRET) or bioluminescent resonance energy transfer (BRET) to visualize the initial binding signal. In the following sections, the three read-out methods introduced above are discussed.

1.2.3.1 Detecting cellular signals of unmodified cells

Whole cell biosensors employing unmodified cells generally record such cellular events as proliferation, morphological changes, motility and cell viability. The signal output of these types of biosensors usually depend on some intrinsic property of the cell, such as metabolic events or electrical properties and, depending on the cell properties which are employed, read out may be optical or electronic.

One of the most widespread *optical tests* is the MTT test, a colorimetric assay for cellular growth and survival. This bioassay determines the metabolic rate of living cells by monitoring the reduction of tetrazolium salts (MTT) into formazan, a purple dye, by the cells. Read out of this assay is optical, using a spectrophotometer, and the results can be linked directly to the degree of activation of the cells (Mosmann, 1983; Carmichael et al., 1987). Other examples of tests which measure metabolics include the membrane leakage of lactate dehydrogenase (LDH) assay (Hussain and Frazier, 2002), glutathione (GSH) assay (Hussein et al., 2005) and reactive oxygen species (ROS) assay (Wang and Joseph, 1999). These tests allow cell viability, cell activity and cellular stress to be monitored, reflecting the effects of exposure of the cells to a particular sample, however without targeting a specific molecule.

Electronic biosensors utilizing (unmodified) whole cells generally depend on electrical impedance or dielectric permittivity in order to perform a measurement that translates into a determination of the number of cells in contact with the transducer surface, or more subtle measurement of the distribution of focal adhesion points with the transducer, leading to information on cell morphology.

A transducer for these types of biosensors may be prepared with a biomolecular surface that can be used to selectively capture specific cell populations through interaction with proteins that are expressed on the outer surface of cells, or the surface may be prepared with thin coatings of the extracellular matrix material to facilitate attachment. In order to be useful tools for cell-based biosensing, the transducer must be compatible with long-term operation in cell media at temperatures commonly used for incubation.

The technology employed for the secondary transducer, which translates the cellular effect (or signal) into a measurable, electronic or optical signal, includes acoustic devices, such as quartz crystal microbalance (QCM) and surface acoustic wave (SAW), electrical impedance sensing (EIS) and ion-selective Electrodes (ISE) (Saitakis et al., 2010).

QCM has been shown to be able to detect various cellular processes, including cell membrane rigidity, the number of binding events between a surface and the cell (Saitakis et al., 2010), aggregation of cells (Ergezen et al., 2007) and cell spreading (Galli Marxer et al., 2003, Hong et al., 2006, Saitakis et al., 2010). SAW offers the means to probe cell surface receptor/immobilized ligand interactions since the acoustic wave is confined where the actual binding occurs, i.e., within ~50nm from the surface, and does not extend further in the cell body (Saitakis et al., 2011).

EIS measures the resistance produced by growing cell monolayers over electrodes and can detect changes in resistance that may occur with changes in the cell layer after exposure to specific analytes or to complex samples. The EIS offers the ability to measure multiple samples simultaneously in real time, a critical feature in monitoring cell reactions such as cytotoxicity (Hondroulis et al., 2010).

ISE has been used to monitor cell monolayer permeability, and the changes which occur upon exposure of the cells to specific molecules such as growth factors. Gosh (2008) demonstrated that this type of biosensor, consisting of a confluent monolayer of human umbilical vein endothelial cells (HUVECs) on a potassium ion-selective electrode, takes advantage of cell monolayer permeability dysfunction to detect the presence of small quantities of cytokines.

The detection of cellular signals such as changes in morphology, viability and motility are mostly effect assays, investigating the cellular responses to either specific molecules or possibly complex samples. These assays are very well suited to applications in toxicology and drug screening assays. For quantitative approaches, aimed at a specific molecule (or group of structurally related molecules), other approaches are called for.

1.2.3.2 Reporter gene assays

Aside from cell proliferation, motility tests and morphology assays, a wide range of cell based sensors rely on the expression of reporter genes, coupled to specific signaling pathways to produce an interpretable signal. Luminescent or fluorescent reporter molecules are most commonly used in reporter gene assays. The key advantages of these reporter gene assays include high sensitivity, reliability, convenience, dynamic range and adaptability to high-throughput screening. The major weakness encountered in their use is the variability in cell response, mainly caused by sample aspecific effects on cell vitality. This can be countered by introducing an internal or external reference signal to correct the analytical response and separate the specific signal from aspecific interferences. To achieve this, a second reporter gene can be introduced which is constitutively expressed and whose activity thus parallels the cells vitality. The use of bioluminescent and fluorescent reporter proteins with altered emission properties facilitates the simultaneous monitoring of different reporter genes within one assay and expands the applicability of these reporters to multiplexed cell-based sensors (Roda et al., 2009). The choice of reporter protein, i.e. a fluorescent protein or a bioluminescent, depends on the application.

Bioluminescence involves the release of light energy following a chemical reaction catalyzed by a luciferase enzyme (Michelini et al. 2009). Luciferases generate visible light of a specific wavelength through the oxidation of their specific substrates. Unlike fluorescence, no external light source is necessary. Luciferase proteins have been isolated from a variety of insects, marine organisms and prokaryotes (Michelini et al. 2009). Firefly luciferase (FLuc)

isolated from Photinus pyralis, the North American firefly, is by far the most commonly used bioluminescence reporter currently exploited in biomedical research (Edinger 1999). The FLuc reaction, with its D-Luciferin substrate, produces light emission with a peak wavelength typically in the region of 560 nm in vitro. The chemical reaction associated with firefly luciferase is:

$$\text{D-Luciferin} \xrightarrow[\text{E-luciferase}]{\text{ATP / } Mg^{2+}\text{/ } O_2} \text{Oxyluciferin} + CO_2 + \text{Light}$$

Luciferases from the anthozoan sea pansy (Renilla reniformis) (Bhaumik et al. 2002) and the marine copepod (Gaussia princeps) (Tannous et al., 2005) metabolize the substrate coelerentarazine producing blue light with peak emission at around 480 nm. Despite the short emission wavelength, and short-lived kinetics of coelerentarazine, these luciferases have demonstrated high efficiency in gene therapy. Since there is no cross-reactivity between luciferin and coelerentarazine substrates, multiple luciferases can be imaged simultaneously (Roda et al., 2009).

Fluorescence involves the use of an external light source, which excites the fluorophore, converting it to an excited state, and the transferred light energy is emitted at a different wavelength as the fluorophore returns to its ground state. This principle is explained in a Jablonski diagram, shown in figure 1.6, reflecting the excitation state of the orbital electron of the fluorophore molecule (Guilbault 1990).

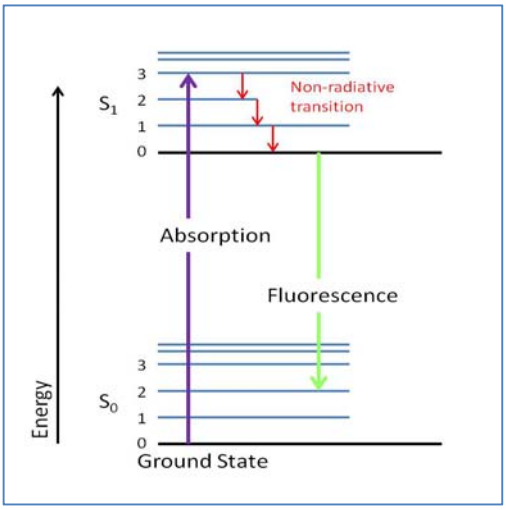


Figure 1.6: Jablonsky Diagram explaining the principle of fluorescence. Fluorescence occurs when an orbital electron of a molecule relaxes to its ground state by emitting a photon of light after being excited to a higher quantum state by absorption of energy from an external source (such as a photon from an external light source).

In contrast to bioluminescence, where light is emitted following enzyme catalyzed chemical energy release, with fluorescence, excitation light (of a

specific wavelength) shined on the subject, is wave shifted by the fluorophore and emitted back out for detection using filters unique to the emitted light wavelength. Reporter strategies based on delivery of genes coding for fluorescence proteins (FP) have been in use for many years, both in vitro and in vivo (Chuang et al. 2010). FP such as green fluorescent protein or GFP (from the Aequorea victoria jelly fish - the first isolated FP gene), have the powerful facet that they naturally fluoresce without the addition of substrates or enzymes, making this an excellent technique for tracking cellular interactions over time. On the down-side, the need for an external light source of a specific wavelength limits the use of this technique for many biosensor applications, where the necessary expensive infrastructure is often not feasible economically as well as being unpractical.

1.2.3.3 Resonance Energy Transfer

A major draw-back of cell based sensors is that these sensors are often based on signal transduction events that occur downstream of receptor activation, and while this allows smaller concentrations to be measured (since the cell amplifies the binding signal), this also results in a longer response time, ranging from hours to days. For this reason, alternative approaches relying on the monitoring of the initial activation step (i.e. receptor dimerization or changes in receptor conformation upon ligand binding) through Bioluminescent or Förster Resonance Energy Transfer (FRET or BRET) have been proposed. This technique, explained if figure 1.7, relies on the use of two chromophores in very close proximity to each other, for example as labels on different parts of a receptor.
One of the chromophores then acts as a donor, transferring energy to the acceptor chromophore, which in turn emits the energy in the form of fluorescence (FRET) or luminescence (Cheng 2006). If, through a conformational change of the receptor, the distance between both chromophores becomes larger, FRET (or BRET) no longer occurs, and the donor chromophore radiates the energy as fluorescence (or luminescence), at a different wavelength. An important advantage of BRET over FRET, is that the first is based on bioluminescence, and as such does not require an external light source, making it a better choice for biosensing applications. The predominant use of FRET and

BRET technology to date has been to measure membrane receptor interactions, (e.g. Ward & Milligan 2013, Navarro et al. 2013), interactions between growth factor receptors with their ligands (e.g. Almudi et al. 2013, Aleja et al. 2012) and nuclear receptor interactions, such as the glucocorticoid receptor (Robertson et al. 2013), the estrogen receptor (e.g. Huttunen et al. 2010, Koterba & Rowan 2006), and the androgen receptor (e.g. Abankwa et al. 2013).

In the field of biosensor research, and important disadvantage has limited its use: the fluorescent or bioluminescent signal is not amplified as is the case for reporter gene assays. As a consequence, the signal is weaker and expensive optical imaging instrumentation is required, as compared to bioluminescent reporter gene assays.

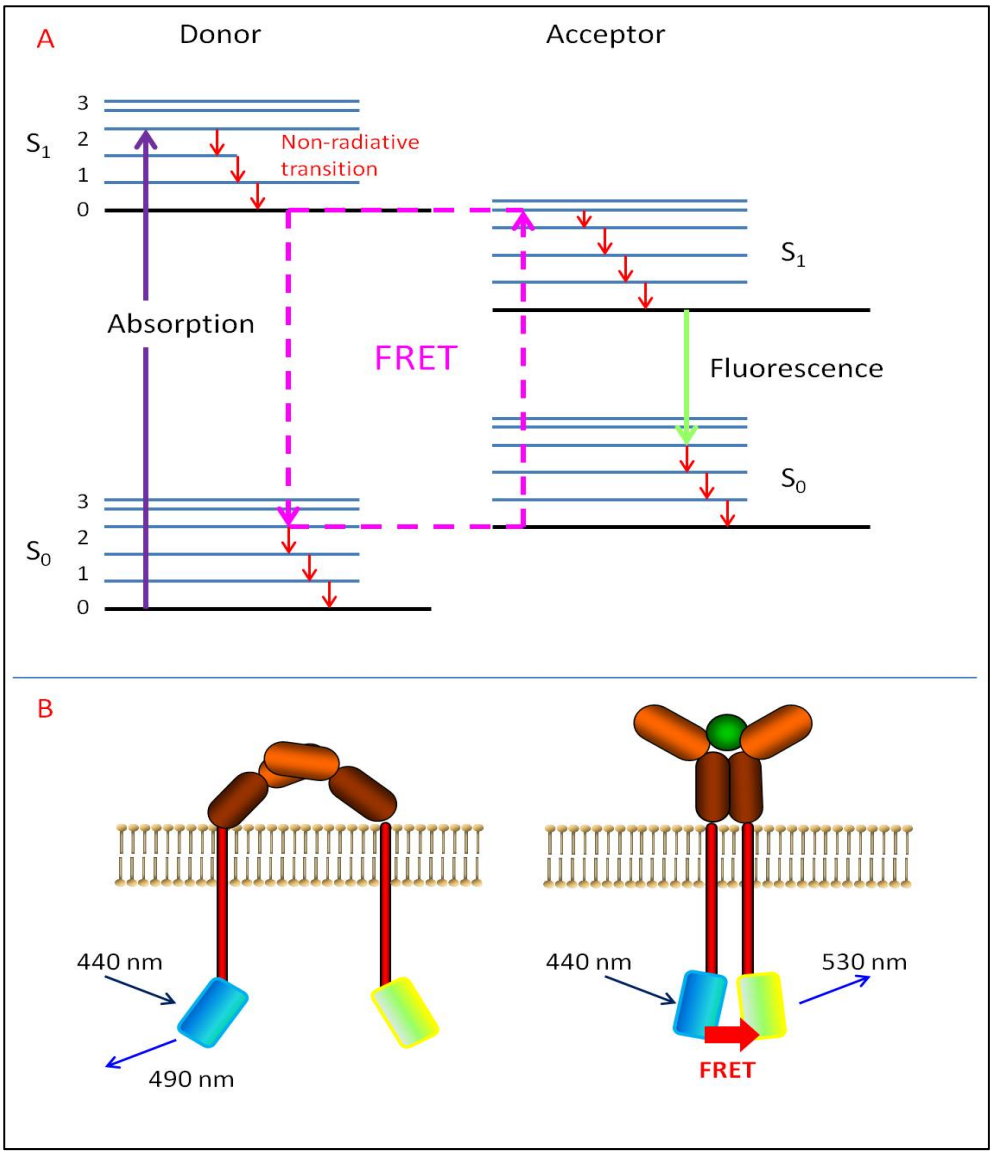


Figure 1.7: Principle of FRET. (A) Jablonski diagram showing the excitation state of the orbital electrons of resp. the donor and the acceptor fluorophore. FRET is the non-radiative energy transfer from donor to acceptor. The acceptor fluorophore then emits the excitation energy through fluorescence of a longer wavelength. (B) The use of FRET for visualization of molecular interactions, here the dimerization of a membrane receptor upon ligand binding. The donor fluorophore absorbs energy from an external light source at the absorption wavelength of 440 nm, and emits light of a 490 nm wavelength. When the acceptor fluorophore is far from the donor, no FRET occurs. However, when the receptor dimer binds its target, a conformational change brings donor and acceptor into close proximity, allowing FRET to take place. The donor no longer emits light, however the acceptor fluorophore now emits light of a 530 nm wavelength.

1.3 Analytical devices and mammalian whole cell biosensors: Combining the best of both worlds

Whole cell biosensor development has known a strong growth in recent years, as new applications led to new demands and as novel technologies expanded the possibilities of these types of sensors. Unmodified cells have been used as effect sensors to monitor cellular reaction to specific molecules or to complex samples in toxicology and drug development, whereas recombinant cells have led to targeted whole cell biosensors, where specific receptor pathways are coupled to (usually optical) output signals to detect the ligands of these receptors.

Mammalian cell based biosensors have a number of important strengths as opposed to analytical devices. On the one hand, they are unparalleled as predictors of effects on higher organisms and on the other hand, the extensive knowledge which is available on signaling pathways has led to a wide range of reporter gene assays. Also, the natural receptors expressed by mammalian cells often have a high sensitivity for specific ligands, which allows these sensors to detect very low concentrations. Because the cell amplifies the biorecognition event, even small concentrations of target can elicit a readable signal. However, the potential range of targets which are recognized by these whole cell biosensors is limited to the natural ligands of the cell receptors.

By contrast, *analytical biosensors*, such as Enzyme Linked Immuno Assays (ELISA) use biomolecules such as antibodies for biorecognition based biosensing. The versatility of antibodies, which can be produced to bind a wide range of target molecules, has meant that ELISA is now a well-known molecular technique, applied in a wide range of research areas and present in most biomolecular labs. However, the use of antibodies in this setting also has a number of draw-backs: the antibodies have to be produced in living animals and can only be produced in restricted quantities at one time, and for many analytical techniques such as ELISA, they must also be labeled, all adding to the cost of the assays. Moreover the stability of the antibodies is not optimal in complex matrices where they can be destroyed by aggressive or interfering molecules.

The use of chimeric cell receptors, which combine the recognition domains of antibodies with the signaling chain of a cell receptor, expressed in whole cell sensors, provides the opportunity to combine the advantages of whole cell based sensing with the versatility of antibody based techniques. This way, a wide range of targets can be recognized by the antibody recognition domains, whereas the receptor signaling chain initiates downstream signaling in the cell, thereby amplifying the original binding signal into a readable output signal. Especially receptor chains which naturally react to very low concentrations of ligand, such as cytokine receptors, are interesting candidates. As well as acting as a transducer, the cellular environment protects the biorecognition molecules from aggressive or interfering molecules, thus safe-guarding their stability. The use of chimeric cell receptors in whole cell biosensing has already been shown to be useful for the detection of pathogens (Rider et al. 2003). However, the detection of low concentrations of free target molecules (as opposed to pathogen-bound ligands) by means of cells expressing chimeric cell receptors has not yet been explored.

The high sensitivity and stability of cellular receptors, combined to the wide applicability of antibody recognition domains results in a novel class of *hybrid analytical whole cell biosensors*.

1.4 Novel applications of biosensors: Waste toxicity screening

In the previous paragraphs, whole cell sensor technology was explored and suggestions were made for novel developments uniting the sensitivity and stability of whole cell sensors and the versatility of analytical, antibody based sensors. The development of such new sensing technologies expands the potential applications of biosensors. As such, this may be considered a top down approach, providing the technology which is then applied in various fields such as allergen screening or screening for disease markers. Another approach is the bottom up approach, where a specific need is formulated by (potential) biosensor users, and existing or new technology is suggested as an answer. The following sections describe the need for biosensors in the specific application of complex waste toxicity screening, and formulates a potential answer to this need through the use of existing biosensors as well as identifying the gaps, where sensing technology has not yet offered a satisfactory tool. Such gaps may in turn be filled in through the development of new sensing technology, closing the circle between the top down and bottom up approach.

1.4.1 Complex waste treatment: an introduction

In the European Union, the amount of waste from both household and industrial sources is growing every day (e.g. household waste has increased 19% between 1995 and 2003 and hazardous waste has grown with 13% in the same period), and the European Environmental Agency expects a further increase of paper-, glass- and plastic waste of 40% by 2020. Health and environmental issues are associated with every step of the handling, treatment and disposal of waste, both directly (via recovery and recycling activities or other occupations in the waste management industry, by exposure to hazardous substances in the waste or to emissions from incinerators and landfill sites, vermin, odours and noise) or indirectly (e.g. via ingestion of contaminated water, soil and food) (Giusti 2009). As such, the correct treatment of potentially hazardous (complex) waste is of the uttermost importance (COM 2005, 666).

Depending on its composition, waste is treated using different techniques. For a number of specific types of waste, such as packaging plastics (PET and PE), paper or cardboard, mercurial waste and solvents, recycling is possible. Green waste is composted or may be used for the manufacture of biofuels or bioplastics. Inorganic wastes of a known composition are treated in physico-chemical plants, where the treatment is specifically aimed at the type of waste at hand: e.g. acids and bases are neutralized, cyanides oxidized, chromates reduced and heavy metals immobilized.

The treatment methods described above all depend on the known composition of specific types of waste, however a very large portion of produced waste, such as household waste and sewage sludge or wastewater treatment sludge has a complex composition, which also varies for each batch of waste. These types of waste cannot be treated based on the characteristics of a specific material they are composed of. Complex waste may be dumped in landfill sites (this should obviously only be the case for non-hazardous waste) or incinerated in one of several types of incinerators. Depending on the hazard characteristics of the waste, three incineration methods are used commonly. Below, two of these methods are introduced to illustrate the difference between treatment of non-toxic waste, where a cheaper and possibly less complete incineration is applied, and the treatment of toxic waste, where total incineration is of the utmost importance.

The *grate incinerator* is employed for the incineration of non-recyclable, non-hazardous household or commercial waste. In these incinerators, the waste is moved over a slope composed of several moving grates. The flue gas in the grate incinerator is kept at temperatures of 850 - 1 000°C, burning the waste as it is moved over the grates. The remaining ashes fall in a wet deslagger to be evacuated for recovery. Figure 1.8 shows a schematic representation of a grate incinerator. It is important to note that in this type of incinerators it is possible that not all the waste reaches the critical temperature of 850°C: it is common for some fine material (sometimes called riddlings or siftings) to fall through the grate. This material is recovered in the bottom ash remover. Sometimes it is recovered separately and may be recycled to the grate for repeated incineration

or removed directly for disposal. As the complete combustion of all waste material is not certain, this type of incineration is unsuitable to toxic waste types.

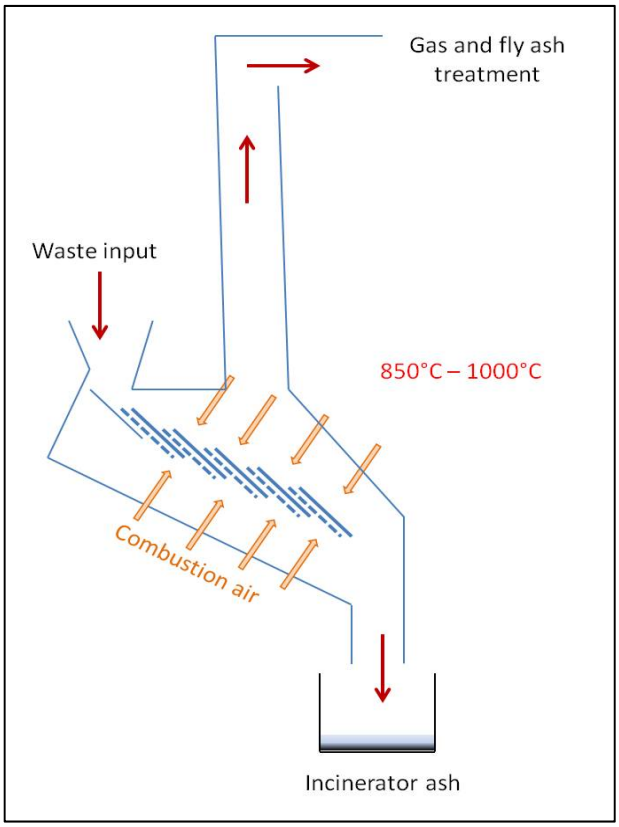


Figure1.8: Grate Incinerator. The waste is discharged into the feeding chute by an overhead crane, and then fed into the grate system. The grate moves the waste through the various zones of the combustion chamber in a tumbling motion. Ashes are collected at the bottom of the incinerator, as well as smaller (only partially combusted) particles which have fallen through the grates.

The *rotary kiln* (see figure 1.9) is proven to be one of the best available technologies to burn solid materials and sludge. It consists of a cylindrical vessel slightly inclined on its horizontal axis. The vessel is usually located on rollers, allowing the kiln to rotate or oscillate around its axis (reciprocating motion). The waste is conveyed through the kiln by gravity as it rotates. In order to increase the destruction of toxic compounds, a post-combustion chamber is usually added. In the cylindrical chamber the waste ignites and solid materials are burnt. The rotating movement of the cylinder around its own axis continually exposes new surfaces to be burnt and facilitates the movement of ashes to the end of the chamber, where they fall through a slot into an ash receptacle. The

smoke and volatile vapors and gases are drawn into the second, stationary chamber (i.e. the post combustion chamber). Due to the high temperatures and the secondary air introduction, the combustion of the exhaust gases is completed and organic compounds (e.g. PAHs, PCBs and dioxins) including low molecular weight hydrocarbons, are destroyed. Because complete combustion is guaranteed, this method is well suited to treatment of hazardous waste types.

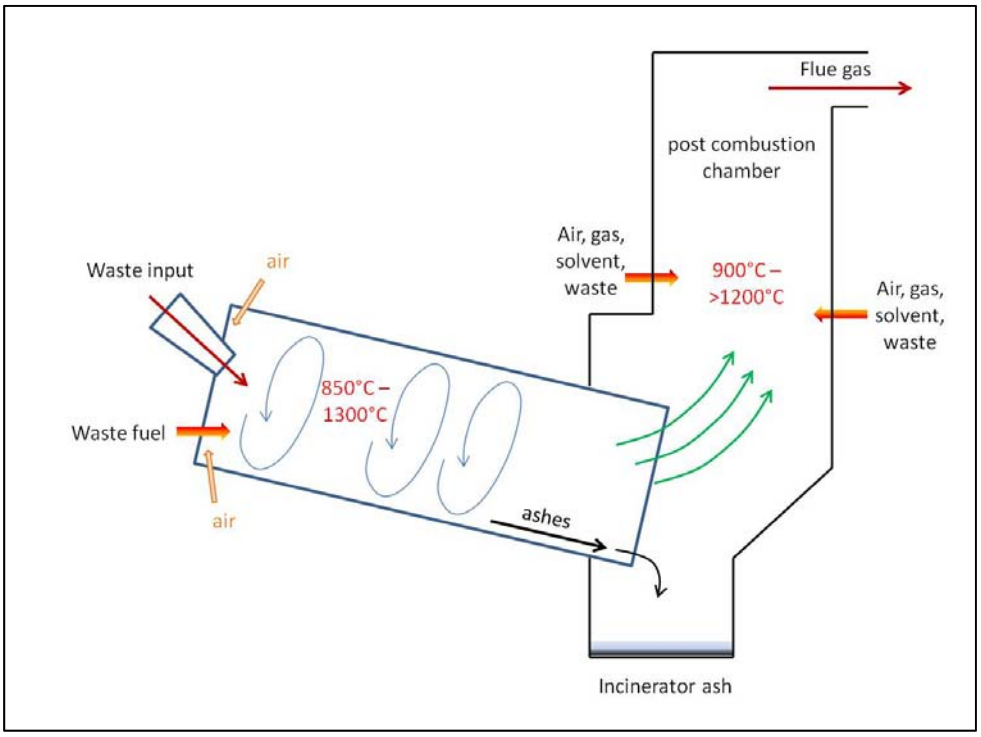


Figure 1.9: Rotary kiln with post-combustion chamber. Waste is conveyed through the kiln by gravity as it rotates, continuously exposing new surfaces. Ashes fall into the ash receptacle and vapours and gasses are completely combusted in the secondary, post combustion chamber, thanks to the high temperatures attained here and the secondary air introduction.

The costs of these different types of waste treatments are significantly different. According to the EU Reference Document on the Best Available Techniques for Waste Incineration (January 2006), treatment of hazardous waste is approximately 3 times more expensive than treatment of non-hazardous municipal waste (350 EUR/ton versus 115 EUR/ton). However, these figures are highly indicative, and in practice the difference may be even larger. The correct

classification of waste is therefore very important from an economical point of view as well as for ecological and health reasons.

The correct choice of treatment method is only possible if the intrinsic hazard risk of the waste has been evaluated, and the waste has been classified as hazardous or not. This however, is not straightforward where complex waste is concerned, because the waste content is unknown and often variable. Also, the combined effect of different components present in the waste is very difficult to predict.

1.4.2 Assessment and classification of complex waste

Currently, the Hazardous Waste Directive (HWD, Council Directive 91/689/EC) provides a framework for the hazard classification of waste. This classification is based on the hazardous properties (H1-15) of the waste: physical (H1 explosive, H2 oxidizing, H3 flammable) and toxicological hazard criteria (H4 Irritant, H5/6 harmful or toxic, H7 carcinogenic, H8 corrosive …). The hazard assessment is based upon information on the hazardous properties of identified individual waste compounds or - if not all compounds are identified - on results of hazard assessment tests on the waste material itself (direct testing). The recommended methods for the direct testing of toxicological properties of waste (HWD) are the acute and chronic animal tests that are used for hazard assessment of chemicals (CD 67/548/EC (dangerous substances), 726/2004/EC (pharmaceuticals), EC/1907/2006 (REACH), CD 98/8/EG (biocides)). These methods and test strategies are however specifically designed for profound human risk assessment for chemicals in applications where oral uptake, inhalation and skin contact are relevant exposure routes. Not only is human exposure to waste material different, also the waste test strategy is for purposes of hazard *classification* (which is a yes/no decision) and not for risk assessment. Moreover it is not ethical to use animal tests for waste classification. For these reasons no direct tests are applied at present and complex wastes are often arbitrarily classified.

Industrial companies facing this problem experience how these arbitrary measures can have serious financial consequences and are a threat to competitiveness. Moreover a false classification may lead to severe ecological and health consequences. There is an urgent need for a reliable and cost-effective testing strategy.

Effect based sensors, including whole cell sensors, may be very useful in this context, as their predictive capacity allows the combined toxic effects of wastes to be analyzed. Their potential is confirmed in other fields: aside from the well validated use of effect based assays (e.g. algae test, Daphnia test,…) in ecotoxicology, they have also been used for toxicological profiling of chemicals. The ToxCast program for example, is a research program initiated by EPA (United States Environmental Protection Agency) in 2007, with the purpose of developing the ability to forecast toxicity of specific chemicals based on bioactivity profiling. In this program computational chemistry, effect based high-throughput screening (HTS) and various toxicogenomic technologies are used instead of animal tests to predict potential for toxicity and prioritize limited testing resources towards chemicals that likely represent the greatest hazard to human health and the environment (Dix et al., 2007). This program has already explored some of the potential of in vitro tests for endocrine disruptive substances (e.g. Rotroff et al., 2012), teratogenic effects (e.g. Ducharme et al., 2013) and carcinogenic effects (e.g. Rotroff et al., 2013) amongst others, with very promising results.

The effectiveness of whole cell sensors, alongside chemical tests and other biosensors for waste assessment needs to be investigated. Also, endpoints which cannot yet be measured satisfactorily must be identified. These gaps in sensing technology represent an important challenge in biosensor innovation and development.

1.5 General Aims

The aims described here represent two different approaches in biosensor development and application. The first approach is aimed at the development of novel biosensor technology, widely applicable and providing a sensitive and specific outcome. In the second approach, the potential of existing biosensors is explored in a new field, i.e. complex waste assessment, in answer to the need for standardized, fast and cheap screening methods in this industry.

1.5.1 AIM 1: development of a novel whole cell biosensor prototype

As illustrated before, mammalian whole cell biosensors have an unparalleled ability to predict in vivo effects, and as such they are already well established as effect sensors. Reporter gene technology has also offered the possibility of developing targeted whole cell sensors, where a specific signaling pathway, which can be activated by a corresponding target molecule (or group of structurally related molecules), induces the (concentration dependent) expression of a reporter protein. However, these reporter gene assays are restricted to the natural ligands of cell receptors, limiting their applicability.

The use of chimeric receptors, which exploit the sensitivity and signaling of the cell receptor, combined with specific antibody recognition domains aimed at a target of choice, may lead to a new class of hybrid analytical whole cell sensors with a wider applicability then classical reporter gene assays, while retaining the high sensitivity of a whole cell sensor. The general aim of this project is the development of such an analytical mammalian cell based sensor in a proof of principle setting. To attain this goal, a number of challenges must be overcome:

1. Construction of a sensitive chimeric receptor

   The chimeric receptor needs the signaling domains of a chosen cellular receptor, preferably a sensitive natural receptor, and the recognition domains of an antibody aimed at a chosen target. The process leading to the construction of a chimeric receptor includes four defined steps, described below.

Structural design of the chimeric receptor: several domains are needed to form the chimeric receptor, the most important being the recognition domains derived from an antibody and the signaling chain of the chosen receptor. Besides these, a trans-membrane domain may be present (in the case of membrane bound receptors) and also cosignaling domains can be coupled to the receptor. To achieve a high versatility, the recognition domains must be easily interchangeable, so the incorporation of restriction sites between the recognition and signaling domains is preferred, resulting in a cassette-like structure of the chimeric receptor construct.

Choice of a suitable receptor molecule: the whole cell sensor depends for its sensitivity and signal initiation on the choice of a suitable receptor molecule. This choice will be based on the following criteria:

- The receptor must be as sensitive as possible,
- Receptor signaling (conformational changes upon ligand binding) must be compatible with the antibody recognition domains to ensure signaling upon binding of the target to the chimeric receptor,
- Availability of the necessary know-how to allow genetic engineering of the receptor: the receptor structure must be well known and recognition and signaling domains well defined to allow modifications without hampering the function of the receptor,
- Receptor signaling is preferably well described to allow the signal cascade to be coupled to a reporter gene system

Choice of target: the target is defined by the antibody recognition domains. These should preferably have a high affinity for the target, however because the recognition domains can be swapped due to the cassette-like structure we aim for, the choice of target for proof of principle purposes is quite wide, e.g. disease markers and allergens.

Construction and expression of the chimeric receptor cassette: molecular engineering techniques will be used to construct the final chimeric receptor cassette coding gene and to confirm the final sequence. Once this goal is

achieved, the receptor gene will be transfected into a suitable mammalian cell line, and expressed constitutively.

The results from the construction of the chimeric receptor cassette are presented in chapter 2.

2. Design and construction of the reporter system

Stimulation of the chimeric receptor pathway with the target must lead to the transcription of a reporter gene in a specific and dose-dependent way. The target inducible expression of a reporter gene is dependent on the promoter which is used to activate transcription of the reporter gene. This promoter should be activated by the chimeric receptor signaling pathway in a dose dependent manner, and preferably with as little background expression (leakage) as possible in the absence of target. The design of the reporter system is obtained in three steps, described below.

Selection of a panel of candidate promoters: based on the signaling pathway induced by the chimeric receptor signaling domains, a panel of potentially activated promoters must be identified.

Investigation of the activation profile of candidate promoters: The activation of the candidate promoters will be investigated in target exposure assays. By means of gene profiling techniques (see chapter 3), the most promising candidate promoters will be identified.

Cross-talk: false positive results through cross-talk between signaling pathways will be anticipated upon and possible solutions for cross-talk discussed (e.g. capturing the cross-talk inducing molecules or parallel assays that allow cross-talk to be discriminated from a true positive signal).

The results from this section are presented in chapter 3.

3. Assembling the whole cell biosensor prototype and developing a proof of principle

Once a suitable promoter has been chosen for the reporter system, all components of the whole cell sensor (i.e. the chimeric receptor, the cells and the reporter system) will be assembled to finalize the biosensor prototype and test its function, as described below.

Construction of a reporter plasmid: through molecular techniques, the chosen promoter will be inserted in a reporter plasmid to regulate the expression of a reporter gene. This plasmid should preferably contain a selectable marker, such as an antibiotic resistance gene, to allow selection of transfected cell lines.

Expression of the reporter plasmid: the cells already expressing the chimeric receptor cassette will subsequently be transfected with the reporter plasmid, and transfected cells will be selected using antibiotics.

Proof of principle: once the reporter plasmid is expressed in the same cell line that expresses the chimeric receptor, the biosensor prototype has been assembled. This whole cell biosensor will be investigated based on the following criteria:

- Does the biosensor show target driven expression of the reporter protein?
- Is the target driven induction of the reporter protein concentration dependent?
- What is the sensitivity of the biosensor?
- Is there cross-talk induced expression of the reporter protein (specificity)?

The aims described above lead to the construction and evaluation of a novel whole cell biosensor prototype, broadening the range of applications of targeted whole cell sensors for detection of low concentrations of targets, such as disease markers and allergens. The results are presented in chapters 2 to 4.

1.5.2 AIM II: Application of biosensors to waste assessment screening in a bottom-up approach

According to the European Environmental Agency, the complex waste volume in Europe as well as the rest of the world is increasing and will keep increasing in future years. The correct classification of complex waste types based on waste toxicity, followed by appropriate waste treatment, is extremely important to safe-guard human health and the ecology. However, present legislation does not provide an adequate testing strategy for complex waste: the recommended methods for waste hazard assessment are animal tests, which are impractical, unethical and economically unfeasible for this application. Therefore, there is an urgent need for an alternative testing strategy which can identify toxic waste quickly and efficiently, without the use of live animals.

The second general aim of this project is the development of such an alternative waste assessment strategy for complex waste, following a number of *a priori* conditions:

- The classification has to be in accordance with HWD principles i.e. based on *total* concentrations and based on the defined hazardous toxicological properties.
- Results need to be generated within short time (preferentially 48 hours) and at economically feasible prices. This is important to allow batch controls, and to prevent large volumes of waste piling up at the plant (occupation of space and/or odour problems).
- A high level of standardization is needed because the test results will be compared to preset limit values.

This goal can be reached in two phases. First, a number of candidate tests and assays will be investigated, and compared to the a priori conditions described above. From the tests and assays which are investigated, a panel of (bio)tests will be selected and a testing strategy developed. Finally, the new waste assessment strategy will be tested on a wide range of complex waste samples in a pilot experiment.

1. Development of a waste assessment strategy

For the development of a new waste assessment strategy, a number of testing tools will be reviewed, including targeted analyses, in vitro methods and Ecotoxicity tests, as described below.

Targeted analyses, such as chemical methods and affinity based biosensors, should be implemented to measure the concentrations of known toxicants. The outcome can then be compared to existing limit values, allowing immediate classification if the toxicity limit is exceeded.

In *vitro toxicity* tests will be implemented to assess the human health risk of complex waste, based on the hazard criteria described in the HWD. Hazard criteria which will be addressed are:

- General toxicity (H5/H6)
- Reproductive effects (H10)
- Corrosive, irritating or sensitizing effects (H4/H8/H15)
- Genotoxicity and mutagenicity (H7/H11)

The results from the in vitro tests will allow the toxicity of the waste to be evaluated directly, without the need to identify the culprit compound.

Ecotoxicity tests, which are also often whole cell sensors or tests which make use of lower organisms such as Algae and Daphnia, will allow the waste to be evaluated based on the HWD criterion H14.

The three groups of tests described above will be brought together in a *testing strategy* where the fastest and most general tests will be followed by more specific testing tools. The testing strategy will be developed to be as economical and as fast as possible (positive testing in the general tests will render the following tests redundant) while leading to a correct classification of the waste.
The (bio)tests are described and investigated in chapter 5. Also the new waste assessment strategy is also proposed and discussed.

2. Pilot test: application of the novel testing strategy

The battery of tests selected in the first phase of the project will be tested in the new waste assessment strategy, on a wide range of complex waste samples, preferably from as diverse sources as possible. The results will be compared to evaluate the predictive capacities of the general toxicity tests and of the targeted analyses, and the suitability for the bioassays for waste assessment discussed.

The results of the pilot experiment are presented in chapter 6.

CHAPTER 2

CONSTRUCTION OF CHIMERIC RECEPTORS

PART I: Development of a novel whole cell biosensor prototype

Chapter 2: Construction of chimeric receptors

2.1 Introduction:

Mammalian whole cell sensors are usually reporter gene assays which make use of the cells natural receptor pathways. In this chapter, natural cell receptors are modified to recognize targets of our own choice. Thus, we aim to develop a versatile sensor platform, capable of being implemented in a wide range of applications such as detection of biomarkers in disease or allergy and (eco)toxicological applications. Natural receptors which have been modified to recognize alternative targets in the past are the T-cell receptor (TCR) and the cytokine receptor. These receptors are now considered for biosensing applications for the first time.

2.1.1 TCR-based chimeric receptors

2.1.1.1 T-lymphocytes and the TCR: background and function

The TCR is a characteristic receptor for T-cells: these are white blood cells, which mature in the Thymus (hence "T"-cells). T-cells are involved in cell-mediated immunity in two major ways: some direct and regulate immune responses, whereas others directly attack infected or cancerous cells (Coico & Sunshine 2009). Helper T cells coordinate immune responses by communicating with other

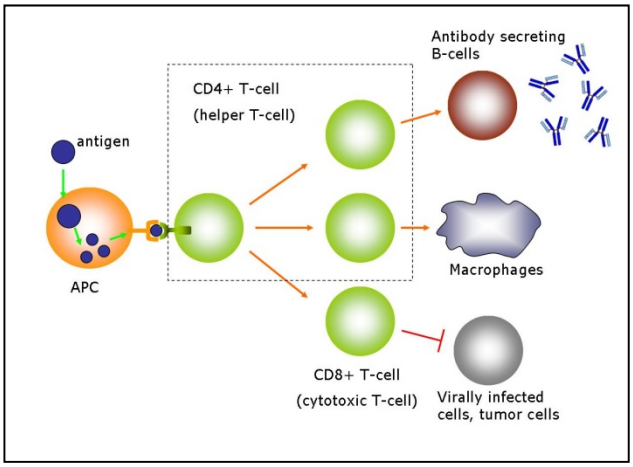


Figure 2.1: Function of CD4+ T-cells and CD8+ T-cells. The CD4+ T-cell coordinates the immune response by secreting cytokines. This activates B-cells which then produce antibodies against the antigen. Macrophages are activated to engulf antigens which have been marked by antibodies. CD8+ T-cells are activated to destroy virus infected cells or cancer cells.

cells, through the secretion of chemical messengers called cytokines (the function of cytokines is discussed further in section 2.1.2). Thus, some helper T cells stimulate nearby B cells to produce antibodies, others activate phagocytes, and still others activate other T cells (see figure 2.1). The helper T-cells are characterized by the presence of CD4, a specific receptor on the cell surface, and are therefore also called CD4+ T-cells. Cytotoxic T lymphocytes (CTLs) perform a different function. These cells directly attack virus infected cells or cancerous cells. They are characterized by the presence of CD8 on the cell surface and are therefore called CD8+ T-cells. The activation of CD4+ and CD8+ T-cells depends on the activation of the TCR mediated pathway. In the next section, the TCR signaling chain and the structure of the TCR are introduced (Coico & Sunshine 2009).

2.1.1.2 Structure of the TCR and structural similarity to antibodies

TCRs are multimeric receptors composed of seven subunits, two of which interact directly with the antigen (the α and β subunits). The remaining 5 subunits (γ, δ, ε, and two ζ subunits) are involved in intracellular signal transduction. Figure 2.2 shows the TCR structure and the cosignaling molecules which are present in the vicinity of the TCR (here CD28 and CD4, coreceptors in CD4+ T-cells). These cosignaling molecules interact with either Major Histocompatibility Complex (MHC) molecules or cosignaling molecules such as CD80 or CD86 on the surface of the antigen presenting cells (APCs). Co-interaction of these types of molecules simultaneously with antigen recognition is crucial for T-cell activation and lack thereof results in a stop of signal transduction and possibly even in T-cell apoptosis (Eshhar et al. 1993, Appleman et al. 2001).

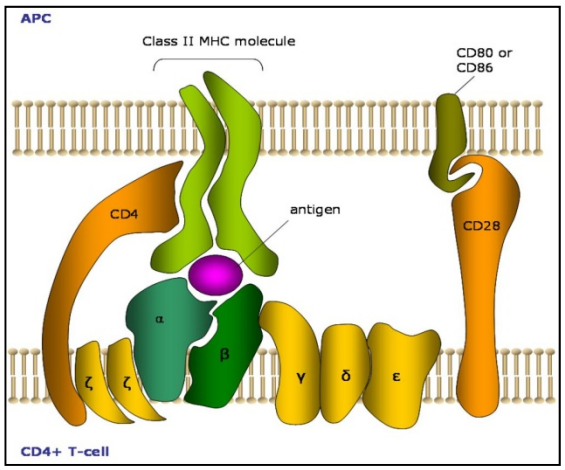


Figure 2.2: Target recognition by the TCR in a CD4+ T-cell.

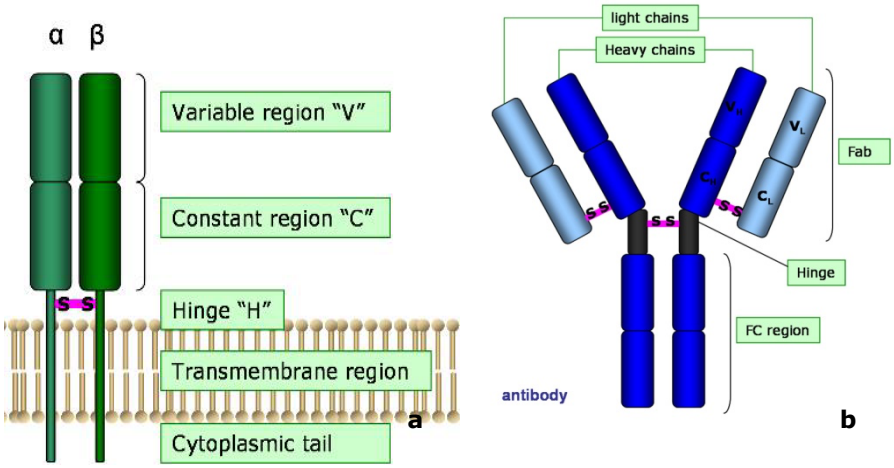


Figure 2.3: Graphical representation of (a) the structure of the TCR α and β chains, and (b) the related structure of the antibody Fab fragment

Figure 2.3 (a) depicts the TCR α and β chains. The extracellular parts of the α and β chain are strongly related to the antibody Fab fragments, shown in figure 2.3b. The α and β subunits of the TCR are linked to each other by a disulfide bond, as is also the case for the Fab fragment light chain and heavy chain. The extracellular components of the α and β subunits include a hinge region (H), a constant region (C) and a variable region (V). The two latter domains, V and C, are also highly similar to the antibody V and C domains. The V regions of both TCR and antibody interact with their ligand and render, respectively, the receptor and the antibody it's specificity (Coico & Sunshine 2009).

2.1.1.3 TCR signaling

The signaling cascade induced by interaction of the TCR with its ligand, and the costimulatory role of CD4 and CD28 is schematically shown in figure 2.4. The cytoplasmic portions of each of the CD3 chains contain sequence motifs called immunoreceptor tyrosine-based activation motifs (ITAM). When upon ligand binding, key tyrosines in the CD3 ITAMs are phosphorylated by the receptor-associated kinases Leukocyte C-terminal Src kinase (Lck) and the tyrosine-protein kinase Fyn, this initiates an activation cascade involving the ζ chain-associated protein kinase 70 (ZAP-70), and, farther downstream, Linker of Activated T cells (LAT) and SH2 domain containing leukocyte protein of 76kDa

(SLP-76). The initial phosphorylation by Lck is induced by binding of CD28 to CD80 or CD86 and by the interaction of MHC and CD4. Together, this complex of activation events leads to activation of genes that control lymphocyte proliferation and differentiation (Favero & Lafont 1998, Mustelin & Taské 2003, Chaplin 2010)

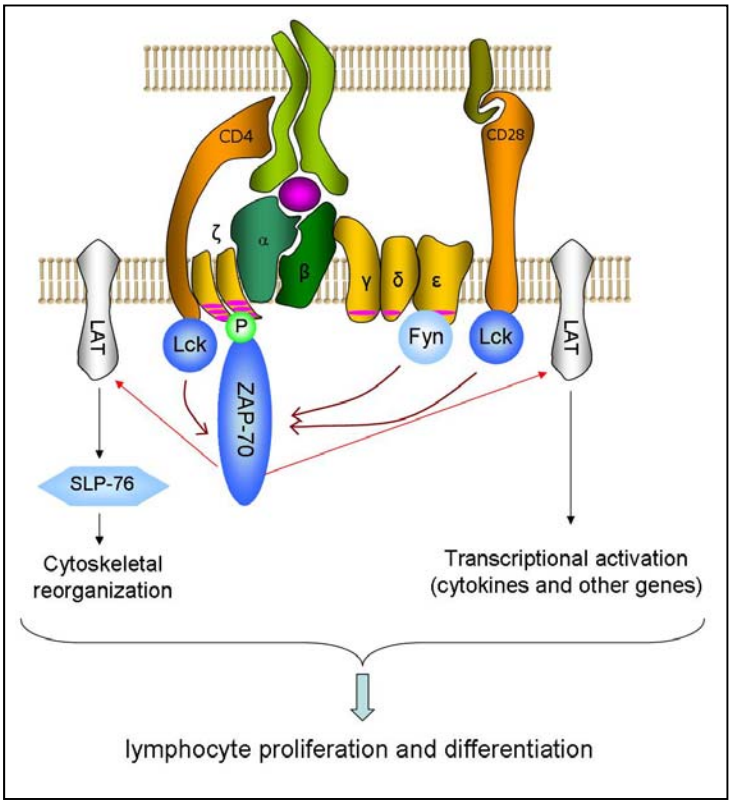


Figure 2.4: TCR signaling. CD3 ITAMs are shown in fuchsia

2.1.1.4 Versatile chimeric TCR (cTCR) target recognition

The structure of the TCR makes it a very good candidate for versatile target recognition: the recognition domain is strongly related to the recognition domain of antibodies. Thus, this domain can be exchanged with short antibody fragments, called single chain variable fragments (scFv), specific for targets of our own choice. Expression of such a chimeric TCR in T-cells will then elicit a TCR signaling cascade upon ligand binding, which in turn can be coupled to a

reporter gene response (see chapter 3). A challenge lays in the ease of interchangeability of the target recognition domains. This challenge can be met by incorporation of unique restriction sites within the chimeric TCR construct, resulting in a cassette-like structure (figure 2.5).

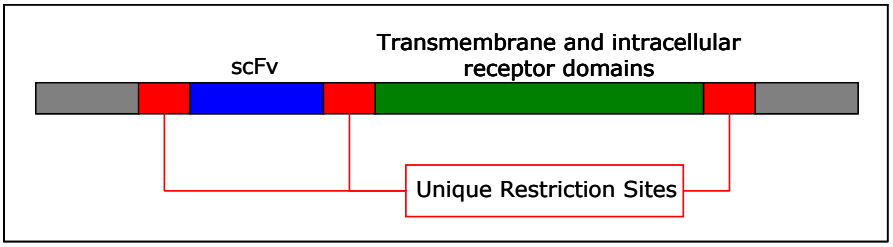


Figure 2.5: Cassette-like structure of the chimeric TCR

2.1.1.5 Structural requirements for successful cTCR signaling

Chimeric TCRs have successfully been constructed for over two decades (Eshhar 1998 to 2004, Finney et al. 2004, Willemsen 2005, Friedman-Morvinsky et al. 2005, Fitzer-Attaz et al. 1998 and others) and are traditionally used in cancer research. The recognition domains of these chimeric TCRs have been replaced by the recognition domains of antibodies, specific for tumor cells. T-cells expressing these tumor-specific receptors are called T-bodies (Eshar 2008). For a recent review on the use of chimeric TCRs in cancer research, see Shirasu and Kuroki, 2012.

However, although the recognition domain of the TCR is especially suited for replacement by a scFv, some other factors need to be taken into account when designing such a chimeric TCR. First, activation of the TCR is also dependent on the binding events of one or more coreceptors to cosignaling molecules on APCs (see figure 2.1). Therefore, the incorporation of cosignaling molecules is essential in the whole cell biosensor design.

In cancer research, the same challenge was faced, as tumor cells usually lack the costimulatory molecules necessary for T-cell stimulation. TCR costimulatory receptors which have been incorporated into TCR-based constructs are CD4, CD8, CD28, ICOS, OX40 (CD134), CD40L, PD-1 and 4-1BB (CD137). These molecules all induced MHC-independent antigen-specific signaling, however CD28 elicited the best response: inclusion of CD28 transmembrane and

intracellular regions induces the highest cytokine production (IL2, IFN-γ, TNF-α) and clonal expansion of stimulated T-cells (Finney et al. 1998 and 2004, Friedmann-Morvinsky et al. 2005, Eshhar et al. 1993, Gong et al. 1999; Hombach et al 2001; Haynes et al. 2002; Maher et al. 2002; Willemsen et al. 2005; Kowolik et al. 2006). Indications also exist that the addition of downstream signaling molecules, such as Lck and spleen tyrosine kinase (Syk, a tyrosine kinase related to ZAP-70), could improve signal transduction even further (Fitzer-Attaz et al. 1998, Geiger et al. 2001, Turner et al. 2000). Lck and Syk are both cytosolic protein tyrosine kinases (PTK) involved in signal propagation. The latter is closely related to ZAP70 but superior for intracellular signaling (Fitzer-Attas et al. 1998).

The combination of scFv, TCR components and costimulatory molecules leads to an MHC independent activation of the chimeric receptor. On the one hand, the scFv ensures target specificity and the affinity of the recognition domains for the ligand influences the sensor sensitivity. On the other hand, the threshold for signal initiation of the original receptor will also strongly influence the sensitivity of the biosensor. Most studies implementing chimeric TCRs have focused on stimulation of the receptor pathway by co incubation with tumor cells (e.g. Willemsen et al. 2005; Taylor et al. 2004; Rossig 2002), or with precipitated or plastic immobilized target (Finney et al. 1998 and 2004, Fitzer-Attaz et al. 1998, Geiger et al. 2001). As such, the concentration of free antigen necessary for TCR stimulation remains to be investigated.

2.1.2 Cytokine receptor based construct

Aside from the TCR, modified cytokine receptors are very promising tools for biosensing purposes. Cytokine receptors are known to be very sensitive: cytokines exert their effect in the pM range (e.g. Wang et al. (2011) found IL6 serum concentration in healthy controls of 0 – 0,7 pM, in rheumatoid arthritis patients of 0,7 – 4,5 pM). This high sensitivity combined to choice of target through modification of the extracellular component and a suitable reporter gene system may lead to a new generation of sensitive and versatile whole cell

sensors. In this chapter, the EPOR/gp130 receptor is considered as a receptor candidate for biosensing purposes for the first time.
For successful implementation of cytokine receptors in a whole cell sensor, it is important to understand the signaling cascade initiated by the activation of this receptor, as well as the structure of the receptor. Both are introduced in the following section.

2.1.2.1 Cytokine receptors: background information

The growth and differentiation of the hematopoietic lineages requires one or more of a variety of cytokines. These are polypeptides of diverse structure that exert pleotropic effects on target cells. Based on their structure and function, cytokines can be classified into five receptor families: the immunoglobulin cytokine receptor superfamily, the class I cytokine receptor family, the class II cytokine receptor family, the Tumor Necrosis Factor (TNF) receptor superfamily and the chemokine receptor family. The EPOR and gp130 receptors belong to the largest group of cytokine receptors, the class I cytokine receptor family (Coico & Sunshine, 2009).

2.1.2.2 Class I cytokine receptor family structure

Class I cytokine receptors are usually transmembrane receptors that bind to cytokines with four α helical domains. They share specific structural motifs in the extracellular membrane proximal domain, called the cytokine receptor homology (CHR) domain. The CHR usually contains two pairs of conserved cysteine residues and a conserved WSXWS motif. The cytosolic membrane proximal regions of the class I cytokine receptors have conserved BOX1 and BOX2 motifs (Bazan 1990, Cosman et al. 1990). These are proline-rich regions which bind to Janus Kinase (JAK). Figure 2.6 shows the positions of the common structural elements of the class I cytokine receptors.

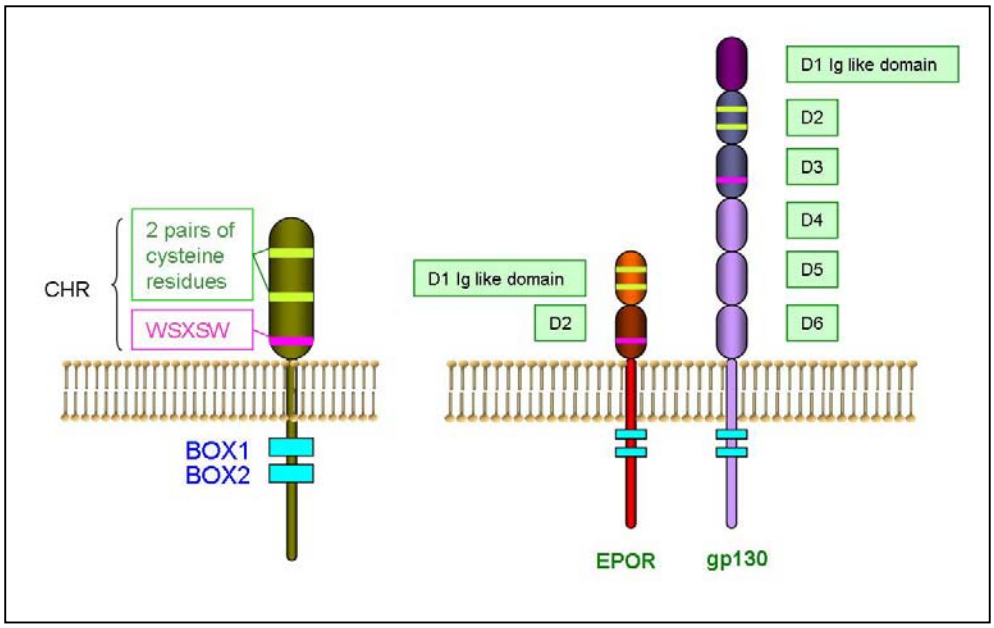


Figure 2.6: Position of the shared structural motifs of the class I cytokine receptors. Left: generic position of the cytokine receptor conserved domains. Right: structure of the EPOR and gp130 chains. D (distal) receptor domains are shown and the position of the conserved motifs.

2.1.2.3 Class I cytokine receptor family signaling

The similarities of the receptor structures within the class I receptor family is reflected in a similar mode of action. The receptors have been shown to be present as monomers or as pre-formed dimers (or oligomers) in the cell membrane of hematopoietic cells (Lu et al. 2006, Müller-Newen et al. 2000, Tenhumberg et al. 2006, Watowich 2011). Upon ligand binding, a conformational change occurs, bringing the cytoplasmic tails of the receptors into closer proximity, as well as the cytoplasmic kinases (such as JAK) associated with the tail. The result is a phosphorylation cascade ultimately leading to altered gene expression. Signal transduction involves the activation of JAK tyrosine kinase family members, leading to the activation of transcription factors of the STAT (signal transducers and activators of transcription) family. This results in the transcription of genes involved in growth, cell proliferation, fate determination of the receptor, development and immunity. Another major

signaling pathway which is activated is the MAPK (mitogen-activated protein kinase) cascade. Signal transduction to the MAPK pathway occurs through SHP2 (SH2-domain-containing tyrosine phosphatase), which is bound to a tyrosine residue of the cytosolic tail of the receptor and phosphorylated upon ligand binding. SHP2 links the Grb2–SOS (growth factor-receptor-bound protein/Son of Sevenless) complex to the cytokine receptor. Finally, recruitment of SOS to the receptor complex allows activation of the GTPase Ras, which in turn leads to the activation of the Ras–Raf (proto-oncogene c-Raf)-MEK (Mitogen activated protein kinase kinase)–MAPK cascade. The MAPK pathway activates transcription factors which initiate transcription of genes involved in antiapoptosis and the cell cycle. A third pathway involved in cytokine signaling is the PI3K (phosphoinositide 3-kinase) pathway. PI3K is phosphorylated by JAK, ultimately leading to antiapoptotic effects. A schematic view of EPOR signaling is provided as an example in figure 2.7 (Heinrich 2003, Kaczmarski & Mufti 1991, Hermanns 2005).

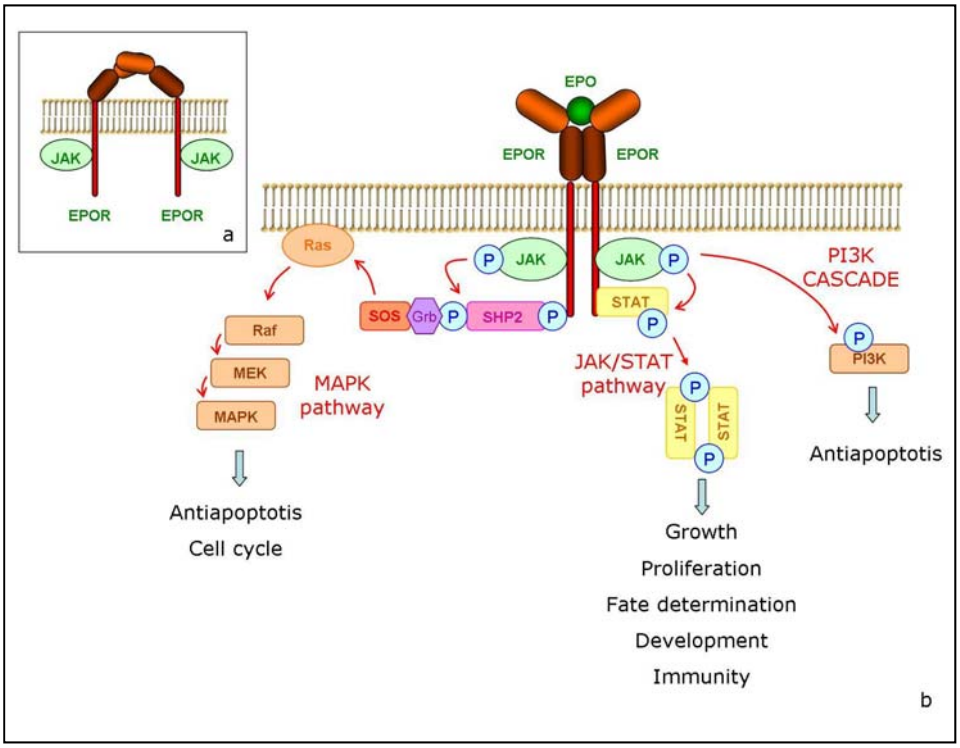


Figure 2.7: (a) the open, scissor-like conformation of the preformed dimer. (b) binding of EPO leads to a conformational change which initiates signaling through the JAK/STAT, MAPK and PI3K pathways.

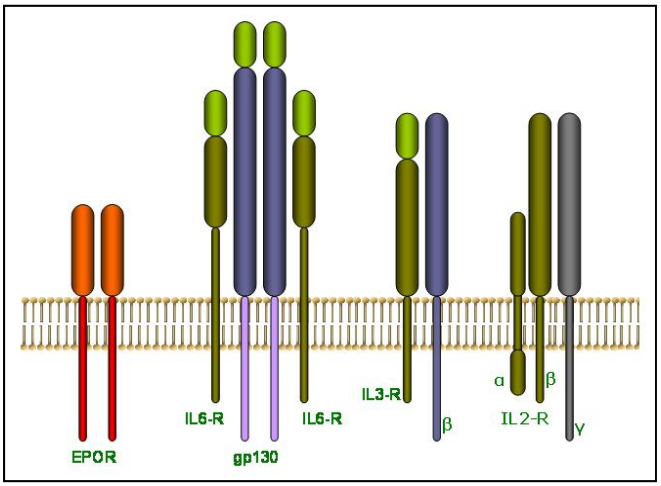


Figure 2.8: Dimerization and oligomerization of cytokine receptors, representatives of the class I cytokine receptor families. From left to right: the single chain family of receptors (e.g. EPOR), the gp130 family (e.g. IL6R), the common β chain (βc) family (e.g. IL3R), and the common γ chain (γc) family (e.g. IL2R)

Based on their interaction with either identical or other (common) receptor chains, the class I cytokines can be classified in the gp130 family, the common γ chain (γc) family, the common β chain (βc) family, and the single chain family of receptors (Schindler & Strehlow, 2000). Figure 2.8 represents different modes of action of the class I cytokine receptors.

2.1.2.4 Chimeric cytokine receptors

Cytokine receptors, like the TCR, are structurally related to antibodies, as they also have Ig like receptor domains. Replacing the recognition domains of a cytokine receptor couple with the heavy chain and the light chain V domains of a target-specific antibody, can result in a functional chimeric receptor couple. Cytokine receptors that have been modified in this way, include the EPOR (Kawahara et al. 2003), IL5 receptor (IL5R) α and β (Behrmann et al. 1996), the IL2 receptor (IL2R) (Sogo et al. 2008) and the common receptor chain gp130 (e.g. Behrmann et al. 1996, Kawahara et al. 2003). Until now, research has been focused on using chimeric cytokine receptors for positive selection of genetically modified cells (AMEGA: antigen-mediated genetically modified cell amplification) or to investigate cytokine receptor signaling. A successful example of AMEGA is the induction of cell expansion through an EPOR/gp130 modified receptor pair. Kawahara et al. (2003) coupled the extracellular D2 domain of the EPOR (containing the CHR) to either the light chain or the heavy chain V

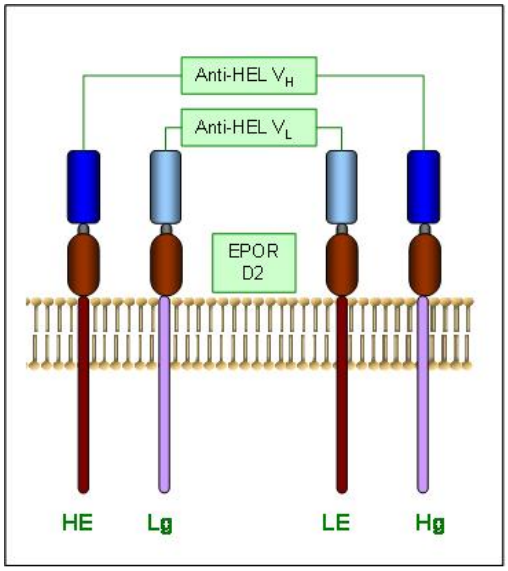


Figure 2.9: Anti-HEL scFV / cytokine receptor constructs. As a target recognition domain, the constructs include either the anti-HEL light chain V fragment (V_L, light blue) or the anti-Hel heavy chain V fragment (V_H, dark blue). The extracellular membrane proximal region of the constructs consists of the EPOR D2 fragment (burgundy ellipse). The transmembrane and intracellular regions of the construct are either derived from the EPOR (burgundy) or from gp130 (purple). **HE**: V_H / EPOR D2 / EPOR intracellular and transmembrane components. **Lg**: V_L / EPOR D2 / gp130 transmembrane and intracellular components. **LE**: V_L / EPOR D2 / EPOR intracellular and transmembrane components. **Hg**: V_H / EPOR D2 / gp130 transmembrane and intracellular components.

fragment of an anti-hen egg lysozyme (HEL) antibody. This was then coupled to the transmembrane and intracellular parts of either EPOR or gp130 (see figure 2.9). The D2 domains are necessary for successful dimerization, while the intracellular EPOR and gp130 domains initiate the phosphorylation cascade necessary for signaling and, ultimately, cell proliferation.

The anti-HEL EPOR/gp130 construct pairs are expressed in an IL3-dependent pro-B cell line (Ba/F3). The lower limit of HEL required for growth mediated by the construct combination HE+Lg is 1 ng/ml (70pM), however cells expressing this combination of receptors showed background proliferation in the absence of HEL. The receptor combination Hg+LE is less sensitive (minimal HEL concentration for growth is 10 ng/ml); however cell proliferation is strictly HEL dependent. Also, some anti-apoptotic effects were observed at a HEL concentration of 1 ng/ml (Kawahara 2003). The observed effects of low concentrations of HEL on the modified BaF3 cells imply that these cells are very good candidates for biosensing purposes, while the cassette like structure of the chimeric receptor couple means the sensor is applicable to a wide range of targets.

2.2 Aim

Whole cell biosensors classically use natural receptors to detect the natural ligands of these receptors. We propose to use cells expressing chimeric receptors, to create a versatile whole cell sensing platform, combining the sensitivity of a whole cell sensor to the versatility of antibody based chimeric receptors.

In this chapter, a TCR based receptor cassette is constructed which incorporates the CD3ζ chain and the CD28 coreceptor for signaling, and which is coupled to the anti-PSA (prostate specific antigen) scFv through an IgG hinge region and the CD28 spacer. The proof of principle antigen, PSA, is a known marker for prostate cancer and as such allows comparison of this sensor to other sensing platforms. The construct incorporates restriction sites, rendering it a cassette like structure, which allows the scFv (and as such the target specificity) to be exchanged easily.

As well as constructing a chimeric TCR based receptor, in this chapter the anti-HEL EPOR /gp130 constructs are presented as alternative chimeric receptors.

2.3 Materials and methods:

In this chapter, a chimeric receptor cassette is constructed combining the signaling components necessary for TCR signaling to the versatility of antibody based target recognition. This TCR based receptor cassette includes the anti-PSA scFv, the hinge region of the IgG coding sequence, the spacer region of CD28, the transmembrane and intracellular coding sequences of CD28 and the intracellular region of the TCRζ chain. The recognition domain, i.e. the anti-PSA scFv was present within the research facility (clone BWI395). The short IgG hinge region was incorporated in the forward primer for amplification of CD28 (see table 2.1). The coding sequences for CD28 and the TCRζ chains were

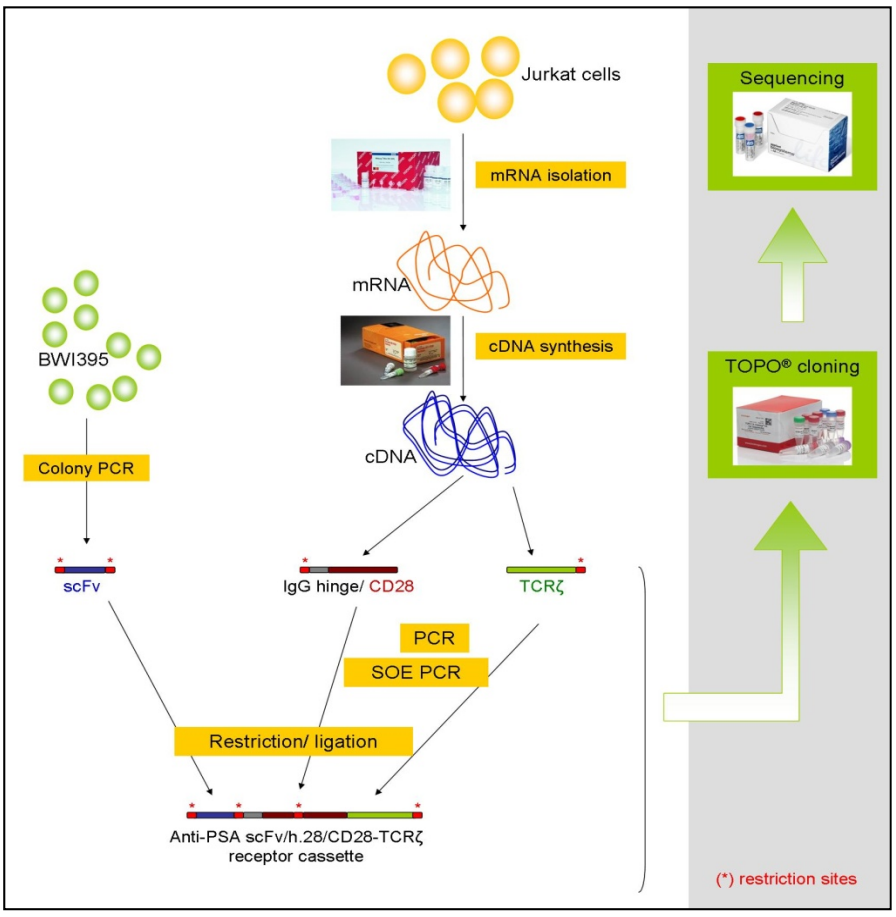


Figure 2.10: Workflow for the construction of the chimeric TCR-based construct

derived from Jurkat cDNA. The method for the isolation of the individual components and the construction of the receptor cassette is described below. Figure 2.10 schematically shows the overall workflow for the construction work.

2.3.1 Cell culture

Jurkat cell (ATCC E6-1) stocks were stored in liquid nitrogen. Cells were thawed in 50% Gibco® RPMI 1640 (Life Technologies Europe B.V., Belgium) and 50% Foetal Calf Serum (FCS, Life Technologies Europe B.V., Belgium) at 37°C and immediately pelleted at 800 rounds per minute (RPM). The cells were grown in complete growth medium (Gibco® RPMI 1640 supplemented with 1 mM Sodium Pyruvate, 0,1 mM NEAA and 10% FCS, all Life Technologies Europe B.V., Belgium) at 37°C and 5% CO_2. Cells were subcultured approximately every three days or when their number reached 10^6 cells/ml, in fresh complete growth medium at a density of 10^5 cells/ ml.

2.3.2 RNA extraction

RNA was isolated from the Jurkat cell pellets using the RNeasy extraction kit (Promega Benelux B.V., The Netherlands), according to manufacturer's instruction. In short, cells are lysed and homogenized by vigorously vortexing during one minute. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are washed away, and high-quality RNA is eluted in RNase-free water.

2.3.3 cDNA synthesis

The extracted RNA was converted to cDNA using the Reverse Transcription System (Promega Benelux B.V., The Netherlands) according to manufacturer's instructions. This kit uses avian myeloblastosis virus (AMV) reverse transcriptase to produce a DNA copy of the RNA template obtained in 2.3.2. cDNA quality was

confirmed by PCR of the housekeeping gene GAPDH (for PCR conditions, see §2.3.4).

2.3.4 Receptor cassette construction procedures

The CD28 and TCRζ cDNA was amplified by PCR from Jurkat cDNA, incorporating restriction sites where necessary. The anti-PSA scFv component was amplified from BWI395 (see §2.3.8) by colony PCR. The transmembrane and intracellular CD28 and TCRζ components were fused by Splice by Overlap Extension (SOE) PCR. An overview of the PCRs performed for the construction of the receptor construct is given in §2.4 (figure 2.12). Control reactions including colony PCR reactions and sequencing reactions were performed to confirm the lengths and sequences of the obtained fragments.

2.3.4.1 Primers

All PCR reactions were performed using the primers listed in table 2.1, supplied by Eurogentec Benelux S.A. (Belgium). cTCR primers were based on the publication by Finney et al. (1998) and adjusted for the required restriction sites, based on the pLenti vector which is foreseen for transfection of eukaryotic cells with the receptor cassette. The M13 primers are specific for the vector pCR2.1 (Invitrogen by Life Technologies Europe B.V., Belgium), used for Topo cloning of all of the components which were obtained in the course of the construction work.

Table 2.1: Primers used for the construction of the anti-PSA/ CD28/ TCRζ receptor cassette.

Component	Primers
Anti-PSA scFv	>001CTCRb (fw) CAC CAT GCG CG ATC GCA CCC AGT CTC CAG CCT CCC TA Asis I CD28
	>002CTCRb (rev) TGA GGA GAC ACG GCC GGC CTG GTC CCT TGG CCC C Fse I CD28
IgG Hinge-CD28	>003CTCR (fw) ACG GCC GGC CAC AAA ACT CAC ACA TGC CCA CCG TGC CCA AAA GGG AAA CAC CTT Fse I IgG hinge CD28 TGT CCA AGT CCC
	>004CTCR (rev) TATGAATTCTCAGGAGCGATAGGCTGCGAA EcoR I CD28
IgG hinge-CD28 spacer	>005CTCR(fw) ACG GCC GGC CAC AAA ACT CAC AC Fse I IgG hinge
	>006CTCR (rev) TTGGGATCCAGGGGCTTAGAAGGTCCCGGAAATAG BamH I CD28
TCR ζ chain	>007CTCR (fw) ATATAGGATCCCAAACTCTGCTACCTGCTG BamH I ZETA
	>008CTCR (rev) TATATGAATTCTTAGCGAGGGGGCAGGGCCTGCAT EcoR I ZETA
CD28-ZETA fusion cassette	>011CTCR (fw) CTGGATCCCAAATTTTGGGTGCTGGTGGTGGTTG BamH I CD28
	>012CTCR (rev) >GCTCCTGCTGAACTTCACTCTGGAGCGATAGGCTGCGAAGTCG ZETA CD28
	>013CTCR (fw) GCGACTTCGCAGCCTATCGCTCCAGAGTGAAGTTCAGCAGGAGCG CD28 ZETA
M13 primers	> M13 forward CTG GCC GTC GTT TTA C
	> M13 reverse CAG GAA ACA GCT ATG AC
GAPDH primers	> GAPDH forward AGGTCGGAGTCAACGGATTTG
	> GAPDH reverse GTGATGGCATGGACTGTGGT

2.3.4.2 PCR based construction steps

Amplification of the components of the receptor construct was performed using the Hotstar Highfidelity PCR kit (Qiagen Benelux B.V., The Netherlands). The reaction mix and cycling conditions were according to manufacturer's instructions. In short the reaction components were Hotstar PCR buffer (Tris·Cl, KCl, (NH4)2 SO4, 7.5 mM MgSO4, bovine serum albumin, Triton® X-100, Factor SB (patent pending); pH 8.7 (20°C), 1.5 mM dNTPs), 1 µM of both the forward and reverse primers and 2.5 units Hotstar Hifidelity Taq polymerase. Annealing temperatures were optimized for each primer pair, resulting in the annealing temperatures listed in table 2.2.

Table 2.2: Annealing temperatures

Target	Template	Primer pair	Annealing temperature
IgG hinge/ CD28	Jurkat cDNA	003CTCR; 004CTCR	55°C
TCRζ	Jurkat cDNA	007CTCR; 008CTCR	53°C
IgG hinge/ CD28 spacer	IgG hinge/ CD28	005CTCR; 006CTCR	55°C
TCRζ'	TCRζ	013CTCR; 008CTCR	63°C
CD28'	IgG hinge/ CD28	011CTCR; 012CTCR	64°C
h.28/ CD28/TCRζ	h.28 + CD28/TCRζ ligation product	005CTCR; 008CTCR	55°C
Anti-PSA scFv/ h.28/ CD28/TCRζ	Anti-PSA scFv + h.28/ CD28/TCRζ ligation product	001CTCR; 008CTCR	64°C
pCR2.1 insert	pCR2.1 with various inserts	M13 primers	55°C
GAPDH	Jurkat cDNA	GAPDH primers	57°C

PCR products were visualized by agarose gel electrophoresis according to standard techniques.

2.3.4.3 Splice by Overlap Extension PCR

The CD28/TCRζ fusion cassette was obtained by SOE PCR (see fig. 2.11). This involved 3 PCR reactions, performed with the Hotstar High Fidelity PCR kit from Qiagen, according to manufacturer's instructions. Cycling conditions were as described in §2.3.4.2, with an annealing temperature of 60°C.

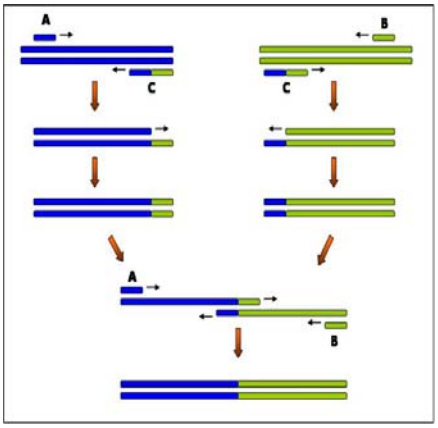


Figure 2.11: Graphical representation of SOE PCR. The two DNA sequences are represented as blue and green strands. Three PCRs are performed: one PCR amplifies the blue DNA sequence, while attaching a small green sequence complementary to the green DNA strand. By elongation, the new blue and green double strand is completed. A similar reaction results in a green double strand with a small blue sequence attached. The newly formed strands are now joined in a PCR where they act as primers to each other. Primers A and B are added to amplify the newly formed hybrid product.

2.3.4.4 Colony PCR reactions

Colony PCR was performed on colonies grown overnight on selective agar plates, to confirm the presence of the desired insert sequences, using appropriate primers. Reaction components are PCR buffer solution (10x concentrated, 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X), 0.2 mM dNTP mix (dATP, dCTP, dGTP and dTTP, each at 10mM in water at pH 7.5), 2 U Taq polymerase per 50 µl reaction (Roche Diagnostics Belgium nv). Both primers were added to a final concentration of $2 * 10^{-4}$ mM. Taq Polymerase, PCR buffer solution and the dNTP mix were used from Promega Benelux B.V (The Netherlands). The primers are described in §2.3.4.1. PCR cycling conditions include an initial lysis step (5' at 95°C) followed by 35 cycli of a three step amplification (20" at 94°C, 1' at 55°C, 1' per kb at 72°C) and a final extension step (10' at 72°C).

2.3.4.5 Sequencing reactions

Construction steps were verified using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, USA), according to manufacturer's instructions. Template input was between 50 and 100 ng per reaction. The sequencing product was purified using a Sephadex® G-50 column (GE Healthcare, UK), prior to capillary electrophoresis.

2.3.5 Cloning procedures

2.3.5.1 Restriction reactions:

Restriction reactions were performed according to manufacturer's instructions. Restriction enzymes (R.E.) and relevant conditions are specified in table 2.3. Restriction enzymes and buffers were purchased from New England Biolabs, UK.

Table 2.3: Restriction reactions

R.E.	Buffer	DNA input	Ratio construction fragments	Reaction conditions
Bam HI	NEB3	h.28 CD28-TCRζ; 1:2	1:2	37°C; 1 hour
Fse I	NEB4	anti-PSA scFv h.28/CD28/TCRζ;	9:11	37°C; 1 hour

After restriction, the reaction mixes were purified using the GFX purification kit (GE Healthcare, UK), according to manufacturer's instructions, to remove the smaller restriction fragments prior to ligation.

2.3.5.2 Ligation reactions:

Ligations were performed on 90 ng of restriction fragments, using T4 DNA ligase (Promega Benelux B.V, The Netherlands), according to manufacturer's instructions.

Ligation products were purified by use of a GFX purification kit (GE Healthcare, UK), according to manufacturer's instructions, and subsequently used as a template in a PCR reaction (using the Hotstar Highfidelity PCR kit), as described in §2.3.4.2.

2.3.5.3 Cloning reactions:

All components, intermediary constructs and the full construct were Topo® cloned into the pCR2.1 vector (Invitrogen, Life Technologies Europe B.V., Belgium). Cloning reactions were performed according to manufacturer's instructions. The resulting plasmid was transformed into chemically competent

Top10 E. coli cells (Invitrogen, Life Technologies Europe B.V., Belgium) through heat shock.

Transformed Top10 cells were investigated by colony PCR, as described in §2.3.4.4. and a glycerol stock was generated of the colonies which contained the wanted inserts, according to standard laboratory techniques.

2.3.6 Anti-PSA scFv

The anti-PSA scFv used for the construction of the chimeric receptor contains the V_H and V_L variable domains an anti-PSA antibody joined by a G4S linker. The variable domains were previously isolated from hybridoma mRNA using V_H and V_L specific primers and the scFv was constructed by SOE PCR (all primers are described in table 2.3).

Table 2.4: Primers used for the construction of the anti-PSA scFv

V_L forward primer ***V_L1-BACK***	GAC ATT CAG CTG ACC CAG TCT CCA PvuII
V_L reverse primer without linker ***V_L1-FOR***	GTT AGA TCT CCA GCT TGG TCC C
V_L reverse primer with linker ***GS-N-FOR***	CCA CCC GAC CCA CCA CCG CCC GAG CCA CCG CCA CCT TTG ATC ACC AGC TTG GTC CC
V_H forward primer without linker ***V_H1-BACK***	AGG TSM ARC TGC AGS AGT CWG G
V_H forward primer with linker ***GS-N-REV***	GGG CGG TGG TGG GTC GGG TGG CGG CGG ATC TCA GGT CCA ACT GCA GSA GTC WGG
V_H reverse primer ***V_H1-FOR(-2)***	TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC BstEII

2.4 Results

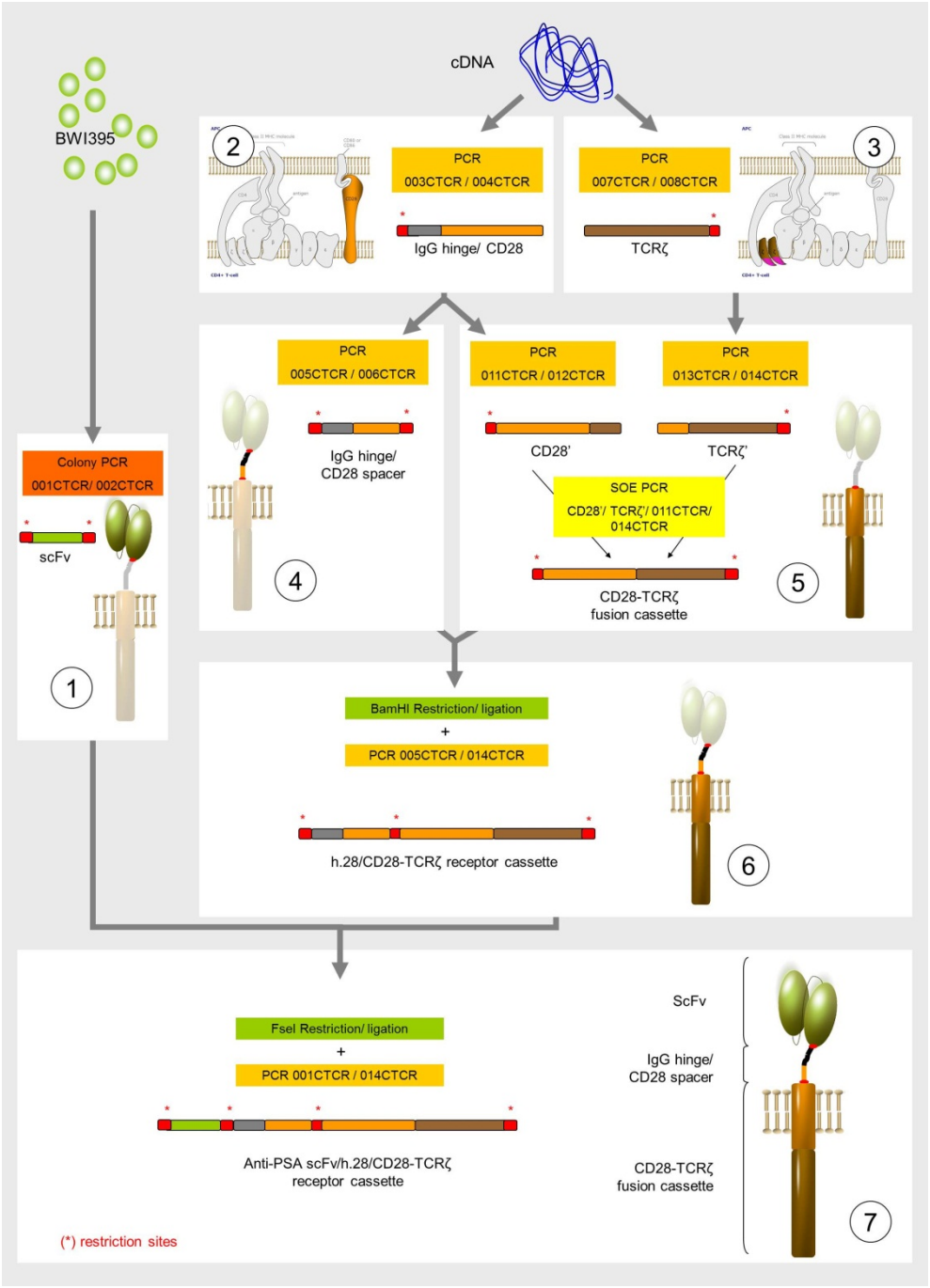


Figure 2.12: Overview of the construction of the chimeric receptor cassette.

This chapter reports on the construction of a TCR based receptor cassette, with PSA as a proof of principle target and incorporating parts of the coreceptor CD28 and the IgG hinge region for efficient signal initiation. The construction work is summarized in figure 2.12. Each construction step is represented by a figure of the final chimeric receptor, with the domain being constructed highlighted.
Construction was started with the isolation of parts of the CD28 and TCRζ coding sequences and the isolation of the anti-PSA scFv coding sequence. The separate components were then joined and the resulting fragments and constructs are described below.

2.4.1 Isolation of the anti-PSA scFv

The colony PCR on BWI395 colonies using primers 001CTCR and 002CTCR yielded a 714 bp sequence, with restriction sites for Asis I at the 5' and for Fse I at the 3' end. The sequence is shown below, with the restriction sites underlined and in red. Figure 2.13 shows the PCR product on gel, confirming the length of the product.

The sequence was conformed using M13 primers. In a later phase, the scFv fragment was joined to the IgG hinge region of the chimeric receptor (see §2.4.6).

>anti-PSA scFv/ AsisI/ FseI
CACCATG CGC GAT CGC ACC CAG TCT CCA GCC TCC CTA TCT GCA TCT
GTG GGA GAA ACT GTC ACC ATC ACA TGT CGA GCA AGT GGA AAT ATT
CAC GAT TAT TTA GCA TGG TAT CAG CAG AAA CAG GGA AAA TCT CCT
CAG CTC CTG GTC TAT AAT GCA AAA ACC TTA GCA GAT GGT GTG CCA
TCA AGG TTC AGT GGC AGT GGA TCA GGA ACA CAA TAT TCT CTC AAG
ATC AAC AGC CTG CAG CCT GAA GAT TTT GGG AGT TAT TAC TGT CAA
CAT TTT TGG AGT ACT ACA TTC ACG TTC GGC TCG GGG ACC AAG CTG
GTG ATC AAA GGT GGC GGT GGC TCC GGC GGT GGT GGG TCC GGT GGC
GGC GGA TCT CAG GTC CAA CTG CAG SAG TCW GGA GCT GAG CTG ATG
AAG CCT GGG GCC TCA GTG AAG ATA TCC TGC AAG TCT ACT GGS TAC
ACA TTC AGT GAC TAC TGG ATA GAG TGG GTA AAG CAG AGG CCT GGA
CAT GGC CTT GAG TGG ATT GGA CAG ATT TTA CCT GGA AGT GGT AGT
ACT AAC TTC AAT GAG AAG TTC AAG GGC AAG GCC ACA TTC ACT GCA
GAT ACA TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC AGC CTG ACA
TCT GAG GAC TCT GCC GTC TAT TTC TGT GCA AGA AGG AAA GTT GGT
ACG GTG GAC TAC TGG GGC CAA GGG ACC ACG GCC GGC CGT CTC CTC
A

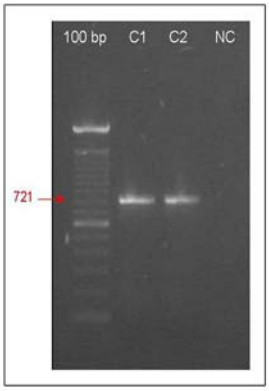
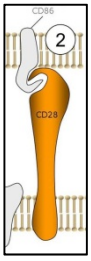


Figure 2.13: Anti-PSA scFv sequence and agarose gel with f.l.t.r.: 100 bp ladder, colony PCR product of 2 BWI395 colonies with primers 001CTCR and 002CTCR, negative template control.

2.4.2 Isolation of the IgG Hinge/ CD28 and TCRζ coding sequences

The scFv region isolated in §2.3.1 represents the recognition domain of the chimeric receptor which was constructed. The remaining components of the receptor, i.e. the hinge/spacer region and the transmembrane and intracellular parts of the receptor, were derived from the TCRζ chain and from CD28. First, parts of the coding sequences of these components were isolated from human cDNA, as described below in §2.3.2.1 and §2.3.2.2. These sequences were then used as templates for the following construction work, as described in detail in chapters §2.3.3 and on.



2.4.2.1 Isolation of the CD28 coding sequence (IgG hinge/CD28)

Jurkat mRNA was isolated and cDNA synthesized as described in the methods section (§2.2) and cDNA quality was assessed by PCR with GAPDH primers. Figure 2.14a shows the gel band corresponding to the GAPDH sequence. The IgG hinge region coupled to bp 628 to 886 of the

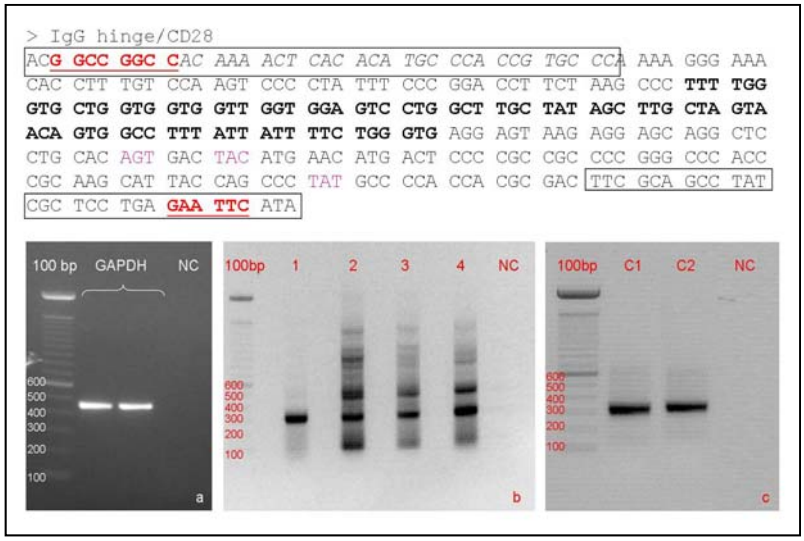
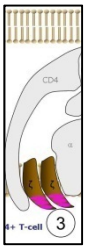


Figure 2.14: IgG hinge/CD28. Top: sequence of the PCR product amplified from Jurkat cDNA with primers 003CTCR and 004CTCR. Below:(a) from left to right: 100 bp ladder, GAPDH PCR products, negative control (no cDNA added to reaction). (b) Isolation of the 306 bp CD28 fragment. F.l.t.r.: 100 bp ladder, PCR products with (f.l.t.r.) 1.5 mM Mg^{2+}, 2.0 mM Mg^{2+}, 2.5 mM Mg^{2+}, 3 mM Mg^{2+} in the PCR mix, and the negative template control. (c) PCR product from colony PCR with (f.l.t.r.) 100 bp ladder, colony 1, colony 2 and the negative template control.

CD28 mRNA was amplified using primers 003CTCR and 004CTCR. The resulting 306 bp sequence is given in figure 2.14 (top). Fse I and Eco RI restriction sites are shown in red, the italic sequence is the IgG hinge sequence and the remaining sequence is part of the CD28 mRNA. The bold sequence codes for the transmembrane region of CD28 and the codons for the phosphorylation sites (one phosphoserin and two phosphotyrosin sites) are shown in fuchsia. The framed sequences indicate the priming sites.

The PCR product was loaded onto an agarose gel as shown in figure 2.14b to confirm the product length, and subsequently TOPO cloned into the pCR2.1 vector. The TOPO cloning reaction was transformed into Top10 cells. After plating the cells on selective plates and growing the bacteria overnight, a number of colonies were picked out for colony PCR. Two positive colonies were grown further and a glycerol stock was obtained for long-term storage of the fragment. Figure 2.14c shows the insert amplified by colony PCR of the two selected colonies. Finally, the sequence was confirmed by a PCR sequencing reaction using primer 004CTCR.

This 306 bp coding sequence of CD28 was used as a template for both the extracellular region of the chimeric receptor (the IgG hinge/CD28 spacer region), and the transmembrane and (part of the) intracellular regions (i.e. the CD28/TCRζ fusion cassette) of the receptor construct, as reported in §2.3.3 and 2.3.4 respectively.



2.4.2.2 Isolation of the TCRζ coding sequence (TCRζ)

The TCRζ chain was isolated from the jurkat cDNA by amplification using primers 007CTCR and 008CTCR, resulting in the 431 bp fragment given below. The isolated sequence spans bp 232 to bp 640 of the TCRζ mRNA (NCBI accession: NM_198053), including 7 phosphorylation sites (one phosphoserine and 6 phosphotyrosine sites), shown in fuchsia. The boxes indicate the priming sites and the restriction sites are shown in red.
The PCR product was TOPO cloned into the pCR2.1 vector and the TOPO cloning reaction was used to transform Top10 cells. Figure 2.15 (b) shows the insert amplified by colony PCR of colonies one to five. Finally, the colony PCR product

was sequenced using both the 007CTCR and 008CTCR primers. To confirm the sequence.

The coding sequence of the TCRζ signaling region isolated here was used as a template for the construction of the intracellular component of the chimeric receptor (the CD28-TCRζ fusion cassette), as reported in §2.3.4.

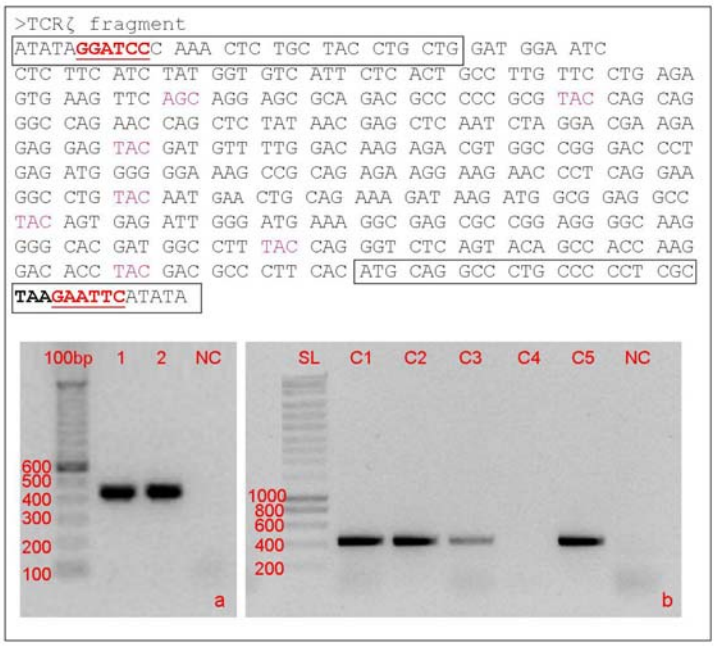
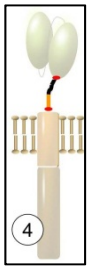


Figure 2.15: TCRζ. Top, TCRζ intracellular sequence isolated by PCR using primers 007CTCR and 008CTCR. Below, (a) PCR on Jurkat cDNA using primers 007CTCR and 008CTCR, with from left to right: 100 bp ladder, PCR product 1, PCR product 2, negative template control. (b) colony PCR using the same primers with from left to right: smart ladder (SL), colonies 1 to 5 and the negative template control.



2.4.3 Construction of IgG hinge/CD28 spacer (h.28)

The hinge/spacer region of the chimeric receptor, which allows the recognition domain of the receptor to be presented efficiently at the cell surface, was isolated form the IgG hinge/CD28 fragment (see 2.3.2.1) by using primers 005CTCR and 006CTCR. The 101 bp sequence obtained spans the IgG hinge region as well as the CD28 spacer region, but excludes the CD28 transmembrane and intracellular regions. The 3' end of the fragment now incorporates a Bam HI restriction site, which will be utilized to join h.28 to the CD28-TCRζ fusion cassette (see 2.4.4) and the 5' end incorporates a Fse I restriction site for the ligation of the hinge/spacer component to the scFv. The incorporation of this restriction site allows easier interchangeability of receptor cassette components. Figure 2.16 shows the hinge/spacer construct sequence and the PCR product on gel.

The h.28 component was TOPO cloned into the pCR2.1 vector. As described for the other components, the plasmid was transformed into Top10 cells and after colony PCR (see figure 2.16), a glycerol stock was made and stored at -70°C.

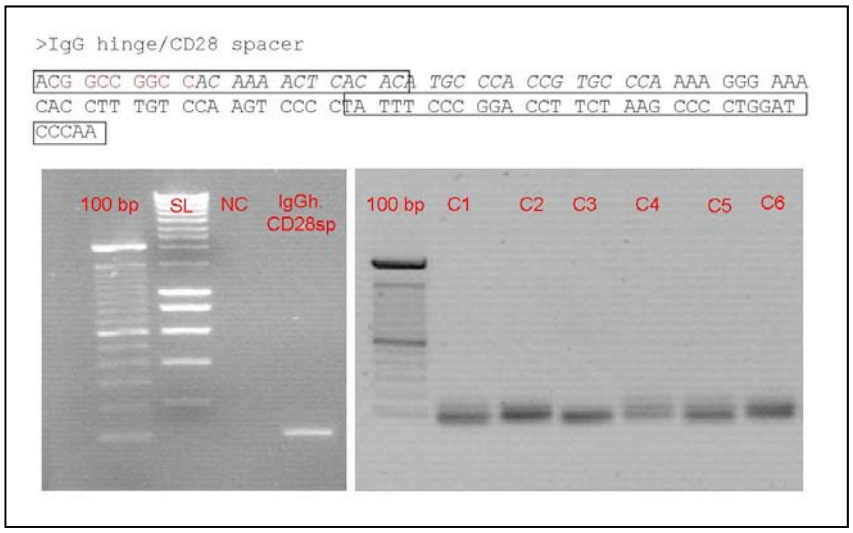
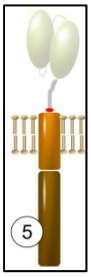


Figure 2.16: h.28. Top: IgG/CD28 spacer sequence. Below: (a) PCR with the IgG hinge/CD28 fragment as template, with primers 005CTCR and 006CTCR. F.l.t.r.: 100 bp ladder, smart ladder, negative template control, IgG hinge/CD28 spacer (b) Colony PCR products, f.l.t.r.: 100 bp ladder, colonies 1 to 6.



2.4.4 Construction of the CD28/TCRζ fusion cassette.

The CD28/TCRζ fusion cassette combines the transmembrane and intracellular signaling parts of CD28 with intracellular signaling domains of the TCRζ chain. The two components of the fusion cassette were joined by SOE PCR, a technique which circumvents the need for restriction sites. The SOE PCR technique is explained in detail in the methods chapter (§2.2). First, the two components (CD28 and TCRζ) were modified so that resp. the 3' and 5' ends of the sequences were complementary to each other. Then, the two fragments were joined in a single reaction where they act both as template and primers to each other. The resulting modified fragments and fusion cassette are described below.

2.4.4.1 Modification of CD28 and TCRζ

The modified CD28 fragment (CD28') was obtained by PCR with the IgG hinge/CD28 fragment (obtained in §2.3.2) as template, and with primers 011CTCR and 012CTCR. The reverse primer (012CTCR) has a 3' overhang complementary to the TCRζ 5' end, resulting in a 238 bp fragment composed of the CD28 fragment with at the 3' end, 22 nucleotides complementary to TCRζ.

Similarly, the TCRζ fragment obtained in §2.3.2 was amplified with primers 013CTCR and 008CTCR resulting in TCRζ'. The forward primer (013CTCR) has a 23 bp overhang complementary to the 3' end of the CD28 component, thus yielding a 376 bp fragment composed of the TCRζ chain with at the 5' end a 23 bp sequence complementary to CD28. Both CD28' and TCRζ' were visualized by gel electrophoresis, as shown in figure 2.17, which also shows the obtained sequence. Restriction sites and phosphorylation sites are indicated as before. The underlined black sequence is the overhang, complementary to either the TCRζ 5' or the CD28 3' region.

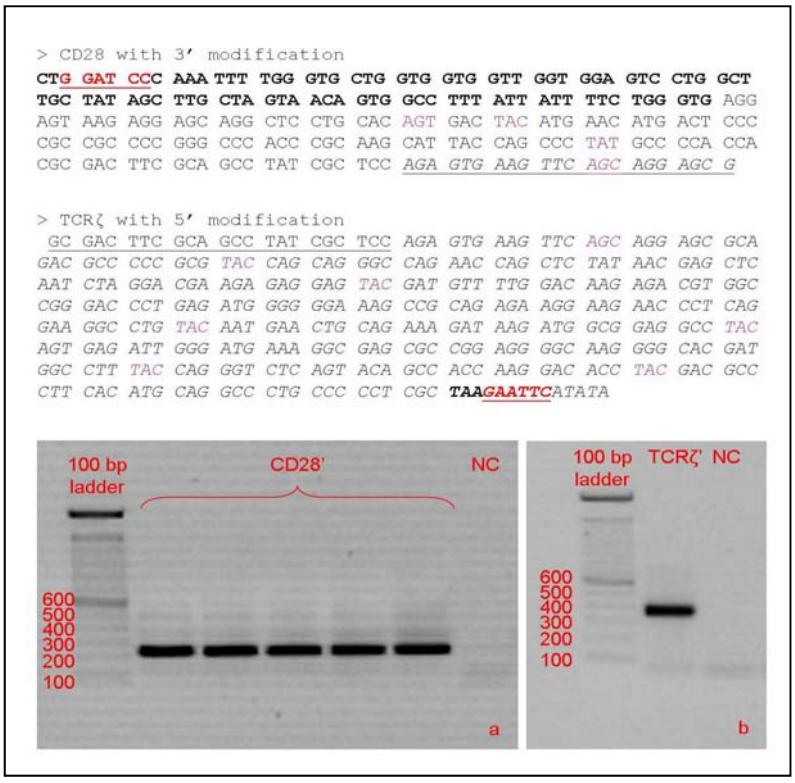


Figure 2.17: CD28′ and TCRζ′. Top: CD28′ and TCRζ′ sequence. Below: (a) CD28′ PCR with f.l.t.r.: 100 bp ladder, CD28′ PCR product (lanes 2 to 6), negative template control. (b) TCRζ′ PCR with f.l.t.r. 100 bp ladder, TCRζ′ PCR product, negative template control.

2.4.4.2 The CD28-TCRζ fusion cassette

CD28′ and TCRζ′ were joined in a single PCR, where they act both as template and primer, together with the primers 011CTCR and 008CTCR. The result of this reaction is the 569 bp CD28-TCRζ fusion cassette, shown in figure 2.18.

The SOE PCR product was cloned into the pCR2.1 vector and this plasmid was transformed into Top10 E. coli. The result of the colony PCR using M13 primers is shown in figure 2.18 b (product length is 569 bp insert + 200 bp vector sequence). All colonies except colony 1 and 5 have an insert of the correct size.

Inserts were confirmed by sequencing, as described in the methods section (§2.3). The CD28-TCRζ fusion cassette was then joined to the IgG hinge/ CD28 spacer region through the 5′ Bam HI site, as described in §2.4.5.

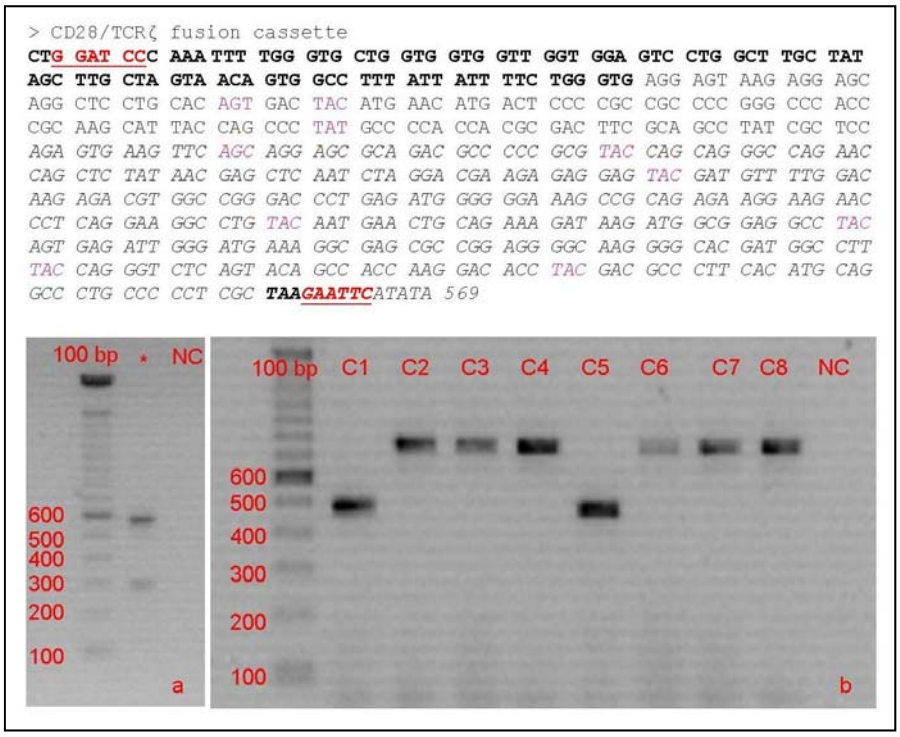
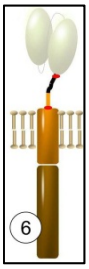


Figure 2.18: CD28/TCRζ fusion cassette: Top: sequence of the CD28/TCRζ fusion cassette. Below: (a) SOE PCR, with from left to right: 100 bp ladder, SOE PCR product (*), negative template control. (b) colony PCR with M13 primers. From left to right: 100 bp ladder, colony PCR products (C1 to C8), negative template control.



2.4.5 Ligation of h.28 and CD28/TCRζ fragments

The h.28 hinge/spacer region of the receptor cassette was obtained, as well as the transmembrane and intracellular CD28-TCRζ fusion cassette. In this chapter, the result of the restriction and ligation reaction is given, joining these components.

H.28 and CD28-TCRζ both incorporate a Bam HI restriction site (at resp. the 5' and the 3' end). These restriction sites were cut by Bam HI and the two fragments were ligated (as described in the methods chapter, 2.3). The result of this ligation, a 659 bp construct, is given in figure 2.19.

The ligation product was amplified with 005CTCR and 008CTCR and cloned into pCR2.1. This plasmid was transformed into Top 10 cells, colonies were grown on

selective plates and then picked up for colony PCR using M13 primers, the result of which, a 859 bp product, is given in figure 2.19. The insert sequence was confirmed using M13 primers.

The h.28/CD28-TCRζ construct obtained through ligation of the hinge/spacer region and the transmembrane and intracellular parts of the receptor cassette was then ligated to the recognition component, the anti-PSA scFv. This is described in detail in §2.4.6.

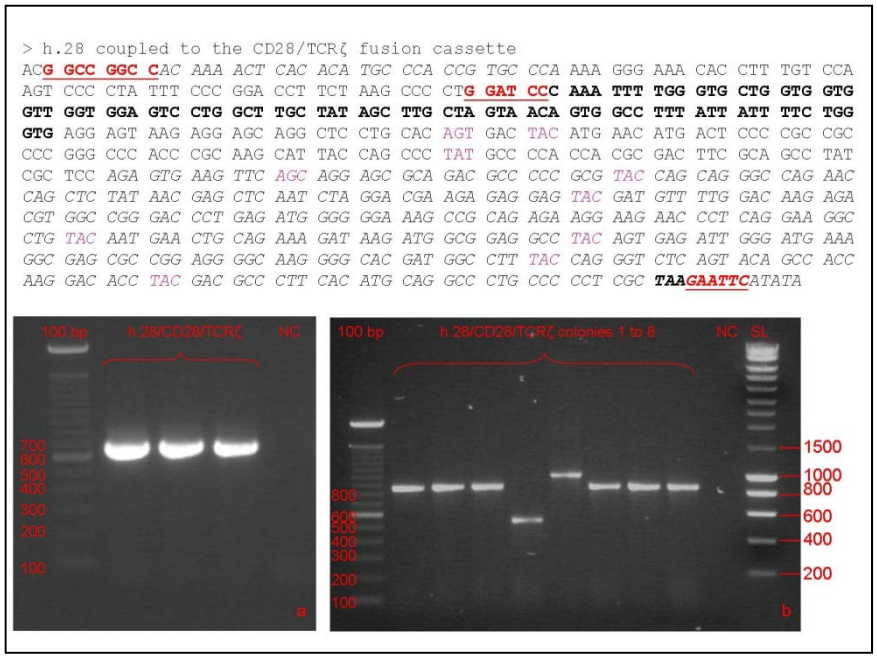
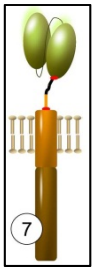


Figure 2.19: Ligation h.28 to CD28/TCRζ. Top: h.28/CD28-TCRζ construct sequence, with the restriction sites shown underlined and in red. The bold black sequence in the transmembrane region and the fuchsia codons code for the intracellular phosphorylation sites. Below: (a) PCR with the ligation product as template and primers 005CTCR and 008CTCR, f.l.t.r.: 100 bp ladder, h.28/CD28-TCRζ PCR product (lanes 2 to 5), negative template control. (b) Colony PCR using the M13 primers, f.l.t.r.: 100 bp ladder, colony PCR products (lanes 2 to 9), negative template control, smart ladder.



2.4.6 Ligation of the anti-PSA scFv and h.28/CD28/TCRζ

The final construction step joined the recognition domain of the receptor construct and the hinge/spacer region and the transmembrane and intracellular regions of the receptor, thus completing the receptor cassette. The Fse I restriction sites at the 3' end of the anti-PSA scFv and the 5' end of h.28/CD28/TCRζ were cut by Fse I as described in §2.3. and ligated (as described in §2.3) to render the 1362 bp full construct, which was then amplified by PCR using primers 001CTCR and 008CTCR. Figure 2.20 shows the full sequence of the anti-PSA TCR based receptor cassette and the PCR product.

The construct was TOPO cloned into the pCR2.1 vector, as described before, and the resulting plasmid was used to transform Top10 E. coli cells. The cells were streaked out on selective plates and grown overnight. A colony PCR was performed, using the vector M13 primers (adding 200 bp to the total length, resulting in a 1562 bp PCR product). Finally the inserts were sequenced using the M13 primers, as well as the internal primers 006CTCR and 005CTCR to confirm the correct sequence of the entire construct.

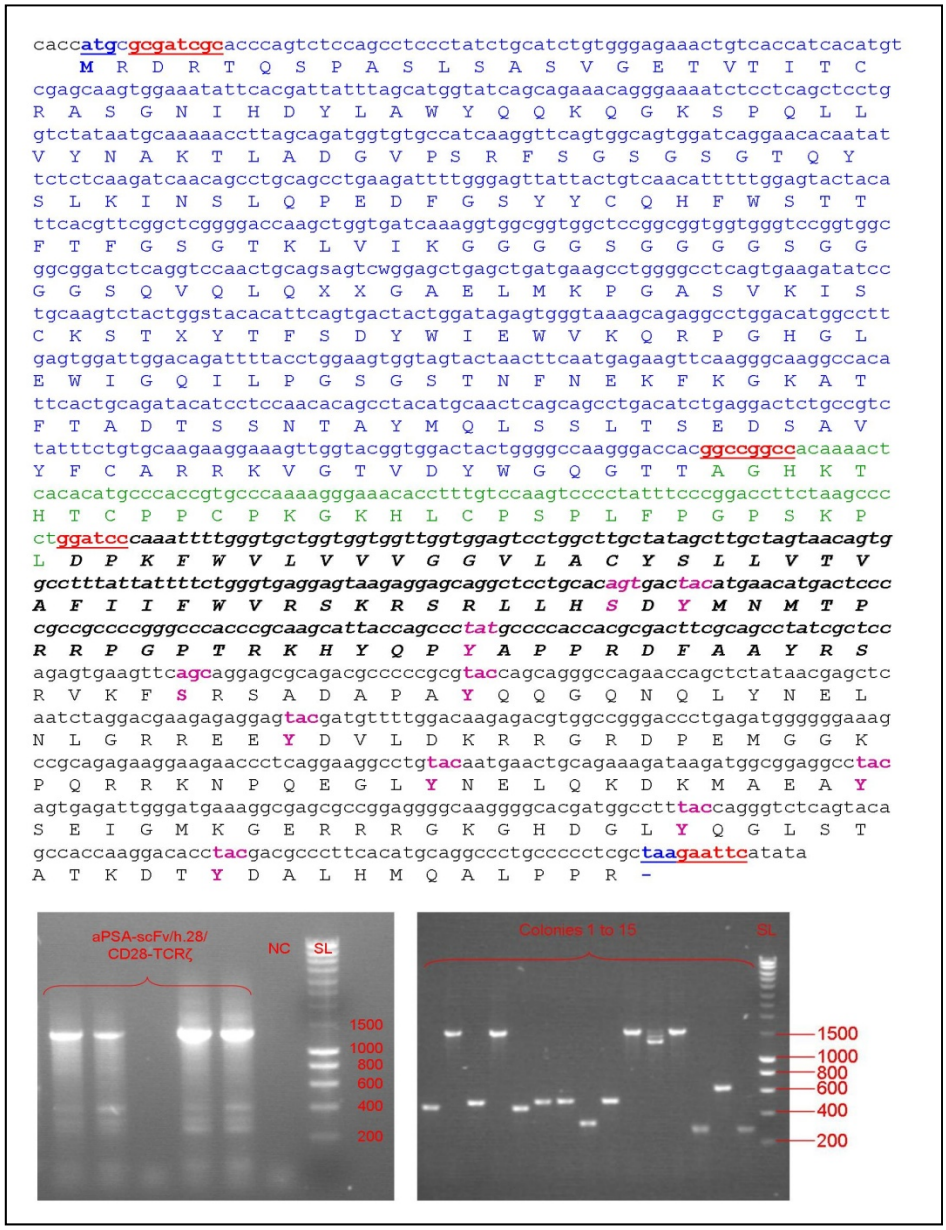


Figure 2.20: Ligation anti-PSA scFv to h.28/CD28/TCRζ top: TCR based anti-PSA receptor cassette nucleic acid and amino acid sequences. Blue: anti-PSA sequence, green: hinge/spacer sequence, bold/italic black: transmembrane sequence, normal black: intracellular sequence. Restriction and phosphorylation sites are shown as before. Below: (a) PCR on the ligation product with primers 001CTCR and 008CTCR, f.l.t.r.: PCR product (lanes 1 to 5), negative template control, smart ladder (b) Colony PCR with M13 primers, f.l.t.r.: colony PCR products C1 to C15, smart ladder.

2.5 Discussion

In this chapter, two types of chimeric receptors are introduced for biosensing purposes for the first time: one is based on the TCR with PSA as a proof of principle target, the other is a receptor couple based on the EPOR and the signaling chain gp130 and has HEL as a proof of principle target (Kawahara 2003).

The TCR based receptor cassette was constructed from the coding sequences of the TCRζ chain and incorporates parts of the CD28 coreceptor to ensure signal initiation upon ligand binding. The extracellular recognition domain is an anti-PSA scFv, coupled to a IgG hinge region and the CD28 spacer region, to ensure the efficient presentation of the recognition domain on the cell surface. The construct is now available for further steps: the construction of an expression vector and the transfection of TCR deficient Jurkat cells with the modified receptor for biosensing trials. For stable expression of the receptor cassette, a lentiviral transfection method is suggested.

The EPOR/gp130 based chimeric receptor couples were kindly offered by Dr. Kawahara. These receptor couples are expressed in the BaF3 cell line and have been shown to induce HEL-dependent proliferation at concentrations as low as 70 pM (Kawahara 2003).

The different chimeric receptors, one TCR based and the other based on cytokine receptors, pose different challenges due to the inherent characteristics of the wild type receptors they were derived from. The TCR based receptor is usually implemented in cancer research, and as such targets for these types of receptors are usually (tumor) cell bound. Cell bound targets are in fact pre-concentrated and this may raise the threshold for activation of the TCR signaling pathway. As opposed to the TCR, cytokine receptors react to very low concentrations of cytokines in blood so may be naturally more suited to detection of low concentration of target in complex solutions. As such, cytokine receptor based sensing has a great advantage over the TCR based system, and the cytokine receptor based constructs will be investigated first for biosensing applications in the following chapters.

In the following chapters, a suitable reporter gene system for the cytokine receptor based sensor is developed to transform the target recognition events

by the chimeric receptor into a quantifiable signal. The most crucial step in the development of the reporter system is the choice of a suitable promoter, based on three important criteria: the promoter should be strongly activated by the receptor signaling pathway, cross-talk should be avoided or possibly neutralized by the use of multiple promoters, and there should be a minimal promoter activation in the absence of target (i.e. promoter leakage). The choice of this promoter and of the reporter gene is investigated further in the following chapters.

2.6 Conclusion

Whole cell biosensors traditionally use a cells natural receptor pathways coupled to a reporter gene system to detect the natural targets of the chosen receptor. We aim to develop a whole cell biosensor with a receptor which is modified to detect a target of our own choice, thus using natural receptor pathways to detect a much wider range of targets at low concentrations in complex mixtures such as blood, serum and environmental samples.

Chimeric receptor based on two signaling pathways (the TCR signaling pathway and the cytokine signaling pathway) are now available for further steps in the development of a novel biosensing system. In the next chapters, suitable promoters are investigated and a reporter gene system is set up to translate the biological recognition events into a detectable system.

CHAPTER 3

DESIGN OF THE REPORTER SYSTEM

Data from chapters 3 and 4 to be published under the title:

CELL-BASED AFFINITY SENSORS: DEVELOPMENT OF A NOVEL TOOL FOR SENSING LOW CONCENTRATIONS IN COMPLEX MIXTURES

K. Deprez, V. Vermeeren, L. Michiels (in preparation)

Chapter 3: Design of the reporter system

3.1. Introduction

A typical targeted whole cell sensor is composed of a receptor which binds to its target and initiates intracellular signaling, coupled to a reporter gene which translates the cellular signaling events into a perceptible signal (e.g. light, an enzymatic reaction, cell proliferation…). In the previous chapter, chimeric receptors were introduced and constructed to fulfill the first requirement for the development of the biosensor. The next step, the design and construction of the reporter gene system, is reported in the following chapters.

The most important criterion for a reporter gene system is the choice of a suitable promoter. In this chapter, a panel of candidate promoters for the cytokine receptor based sensor is assembled based on the induction by cytokine receptor signaling pathways and on available microarray data of the IL3 dependent BaF3 cells. This panel of promoters is investigated in cell exposure assays to assess the activation of the candidate promoters by hen egg lysozyme (HEL) induction of the recombinant receptor pathway using real time quantitative PCR (RT qPCR). Finally the same approach was used to investigate the specificity of the most promising promoters in a cross-talk experiment.

3.1.1. Selection of the panel of candidate promoters

The Ba/HE+LgIGFP cell line introduced in the previous chapter expresses a recombinant anti-HEL EPOR/gp130 receptor couple, allowing cell proliferation of these cells to be induced by HEL. HEL dependent proliferation was demonstrated upon induction of 10 ng/ml (700 pM) HEL and anti-apoptotic effects have been observed upon stimulation with 1 ng/ml (70 pM) (Kawahara et al. 2003). The recombinant receptor successfully induces concentration dependent signaling upon HEL stimulation and by exchanging the receptor domain, can be adjusted to bind a wide range of possible targets, as need be. However, for biosensing purposes, cell proliferation is an unsatisfactory output: cell counts are cumbersome and can only be recorded after one or more days. The use of one

or more reporter genes is called for, allowing read-out in a matter of hours or depending on the read-out technology, may even be recorded in real-time. Other than speed, sensitivity may also be improved, as cell proliferation is a complex process which requires multiple factors and as such requires a higher threshold of activation as opposed to a single promoter.

The induction of cell proliferation by the modified EPOR/g130 receptors expressed in these cell lines is demonstrated by Kawahara (2003). These experiments also showed that cells expressing the receptor combination Hg+LE showed a stronger background proliferation than the Ba/HE+LgIGFP cell line (expressing the combination HE + Lg), implying higher promoter leakage in the former cell line and suggesting Ba/HE+LgIGFP as the better candidate for biosensing purposes.

In the following sections, candidate promoters are investigated and their expression profile in the Ba/HE+LgIGFP cell line is evaluated using RTqPCR.

3.1.2. Candidate promoters based on class I cytokine receptor signaling

For the selection of suitable promoters for biosensing purposes, the signaling pathway which is activated by the EPOR/gp130 chimeric receptor couple is investigated. The recombinant receptor couple initiates signaling through dimerization of the cytoplasmic tails of EPOR and gp130, resulting in a signaling cascade typical for class I cytokine receptors (as discussed in §3.1.2.3). Figure 3.1 shows the JAK/STAT signaling cascade induced by class I cytokine receptor family (KEGG database, Kanehisa et al. 2000, 2012). The genes targeted by cytokine signaling pathways include genes coding for transcriptional negative regulators involved in attenuation of EpoR-JAK2/STAT5 signaling, such as SH2-domain containing protein (CISH), SOCS1, SOCS2 and SOCS3 (Yoshimura et al, 1995; Jegalian and Wu, 2002; Sarna et al, 2003). Also, genes involved in the regulation of the cell cycle and proliferation are targeted, such as C-Myc (Mui et al., 1996) and the anti-apoptotic protein Bcl-xL (Catlett-Falcone et al., 1999). The promoters of the genes regulated by the JAK/STAT pathway are included in the panel of candidate promoters investigated further in the screening

experiment described in this chapter. These genes are also briefly described in §3.1.4.

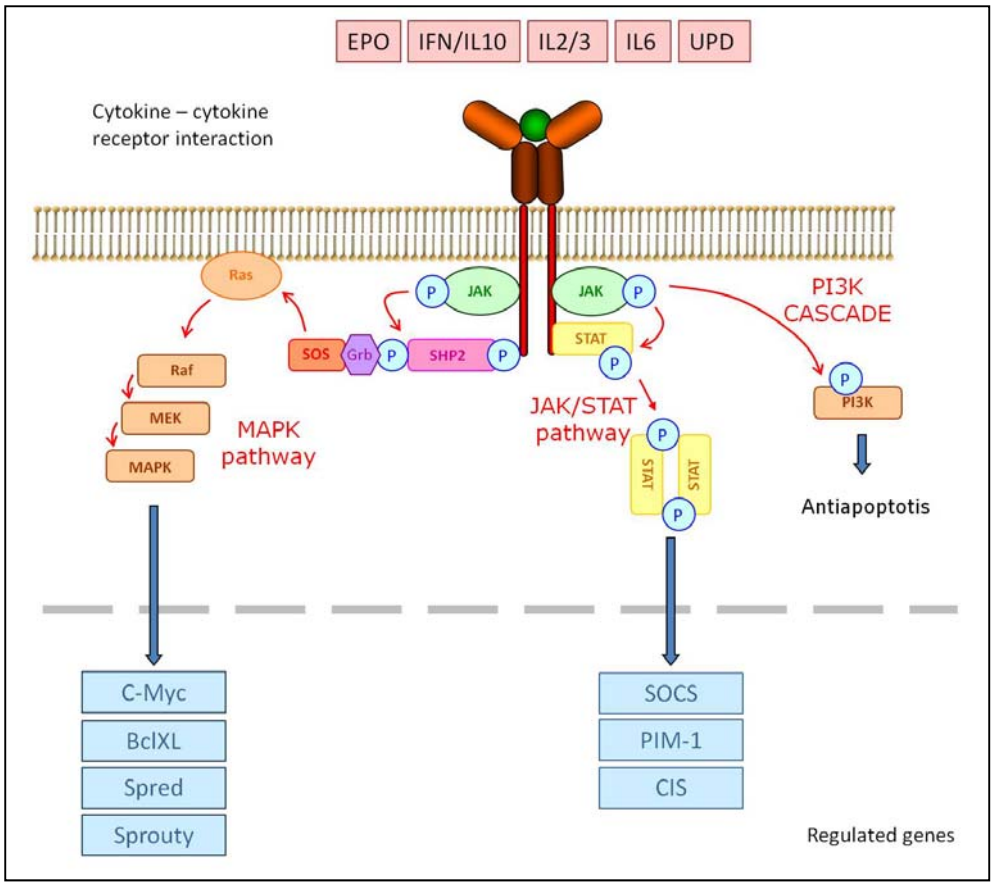


Figure 3.1: JAK/STAT signaling cascade. Leading to the expression of c-Myc, $BclX_L$, Spred, Sprouty, SOCS, Pim-1, CISH

3.1.3. Candidate promoters based on BaF3 microarray data

Aside from genes known to be induced by the JAK/STAT pathway (see §3.1.2), public microarray data available in the Geoprofiles database indicates that other, less obvious promoters are also activated by the cytokine induced pathway. IL3 induced BaF3 cells, the cell line which was used to express the recombinant receptor couple, show a large number of genes to be upregulated (Geoprofiles dataset GDS3349). Those showing a higher than 5-fold induction are shown in table 3.1. Some of these genes are known to be induced by the JAK/STAT

pathway, such as CISH, SOCS1, SOCS3 and Pim1. In the case of other induced genes, the signaling pathway is less obvious. IL3 signaling and EPOR/gp130 signaling share a similar signaling pathway, suggesting that at least some of the genes induced by IL3 are also induced by the EPOR/gp130 recombinant receptor pair. Therefore, the genes upregulated more than 5-fold by IL3 exposure of BaF3 cells were also included in the screening experiment presented here.

Table 3.1: Genes upregulated more than 5 fold by IL3 in BaF3 cells according to the geoprofiles dataset GDS3348

Gene		Fold induction
Socs3	Suppressor of cytokine signaling 3	14.97
CISH	cytokine inducible SH2-containing protein	14.43
Spred2	sprouty-related, EVH1 domain containing 2	11.30
Socs1	Suppressor of cytokine signaling 1	7.90
Gpr34	G protein-coupled receptor 34	7.04
Etv5	ets variant gene 5	6.54
Crbn	cereblon	6.22
Pim1	proto-oncogene serine/threonine-protein kinase pim-1	5.99

3.1.4. Candidate promoters selected for screening

Based on the cytokine signaling pathway (see §3.1.2) and on IL3 induced transcription derived from the public microarray database, a panel of 16 candidate promoters were screened by real-time qPCR. The selected promoters are listed in table 3.2 and relevant characteristics for biosensing applications are discussed in the addendum.

Table 3.2: Promoter panel assembled for screening

Candidate promoters	
CISH	Cytokine inducible SH2-containing protein
Socs1	Suppressor of cytokine signaling 1
Socs2	Suppressor of cytokine signaling 2
Socs3	Suppressor of cytokine signaling 3
Socs4	Suppressor of cytokine signaling 4
Socs5	Suppressor of cytokine signaling 5
Socs7	Suppressor of cytokine signaling 7
Pim1	Proto-oncogene serine/threonine-protein kinase pim-1
c-Myc	Myelocytomatosis oncogene
Spry1	Sprouty-homologue 1
Spry4	Sprouty-homologue 1
Spred2	Sprouty-related, EVH1 domain containing 2
Bcl-xl	Bcl 2-like 1
Gpr34	G protein-coupled receptor 34
Etv5	Ets variant gene 5
Crbn	Cereblon

3.1.5. Cross-talk

From the previous section it is clear that different cytokine receptors of the class I cytokine receptor family share common signaling pathways. While this allowed us to select promoters based on the related IL3 pathway (see §3.1.3), this also implies that some cytokines might cause cross-talk in the EPOR/gp130 based whole cell sensor, possibly leading to false positive results which should be addressed.

The class I cytokine receptors all activate the JAK/STAT pathway, however they differ in the kinases they activate. The JAK family of kinases is represented in mammals by JAK1 to 3 and by TYK2. Upon phosphorylation by one of the cytokine receptor signaling chains, one or more of these JAK kinases in turn phosphorylate members of the STAT family of proteins. In mammals, STAT1 to STAT6 may be activated, depending on the JAK kinase (Rawling et al. 2004). Each cytokine receptor may activate one or more of these pathways, as shown for the representatives of the four subfamilies of class I cytokine receptors in table 3.3. The activated pathway depends on the binding sites on the cytoplasmic tails of the various cytokine receptors. Table 3.3 shows that IL3R

signaling and gp130 signaling have both the activation of the TYK2/STAT3 and the JAK2/STAT5 pathways in common, while EPOR only activates the JAK2/STAT5 pathway and not TYK2/STAT3. This suggests that signaling through these receptors will lead to transcription of at least some of the same target genes, which will lead to false positive results in a biosensor setting. A condition for this cross-talk to occur is that all the components necessary for signaling (i.e. the receptors, JAK kinases and STATs) are expressed in the cells.

JAK/STAT pathway component activation					
Signaling components	gp130	Homo-dimeric	βc	γc	
	IL6R, LIFR	EPOR	IL3R, IL5R	IL4R	IL15R
JAK1					
JAK2					
JAK3					
TYK2					
STAT1					
STAT2					
STAT3					
STAT4					
STAT5					
STAT6					

Table 3.3: JAK/STAT component activation. Green cells indicate which JAK kinases are included in the cytokine receptor signaling, purple cells indicate that which STAT is part of the signaling cascade. (Nagata and Todokoro 1996, Rawling et al. 2004)

The BaF3 cell line which was modified to express the anti-HEL EPOR/gp130 receptor is an IL3 dependent cell line. This implies that all components for IL3 signaling are present in the cells and exposure to IL3 will activate the JAK2/STAT5 and the TYK2/STAT3 pathways and is expected to induce false positive results in a biosensor set-up.

Other obvious pathways which are implicated as potential cross-talk pathways are the EPOR pathway and gp130 mediated pathways, as these are the receptors the recombinant receptor couple is based on: the cytoplasmic tails of the recombinant receptor couple is derived from EPOR and gp130. Therefore, EPOR and gp130 signaling would probably induce the same effects as signaling elicited by the recombinant receptor couple. The occurrence of cross-talk through these receptors depends on the presence of the natural EPOR and

gp130 binding cytokine receptors (such as IL6R and LIFR). Also, because the extracellular membrane proximal region of the anti-HEL/gp130 construct (which in the wild type (WT) gp130 chain binds the gp130 binding cytokine receptors) consists of the EPOR D2 fragment (see chapter 3, 3.1.2.4.), the recombinant gp130 chain will not dimerize with cytokine receptors in the same way as WT gp130. Therefore, as well as the gp130 binding cytokine receptor, the WT gp130 signaling chain must also be expressed in the Ba/HE+LgIGFP cells for cross-talk to occur via this pathway. As gp130 is a signaling chain common to several cytokine receptors which define the receptor ligand, gp130 signaling is represented here by IL6R/gp130 signaling and LIFR/gp130 signaling. Cross-talk depends on the presence of the LIFR or IL6R and on the presence of WT gp130 in the cells. According to microarray data (GDS3349) from BaF3 cells, gp130 is expressed, confirming the need to investigate cross-talk via gp130 mediated pathways. However no expression was detected of the gp130 dimerizing cytokine receptors (including IL6R and LIFR).

The EPOR, a homodimer forming receptor, was shown to be expressed in BaF3 cells according to microarray data (GDS3349). This implies that EPO cross talk via this receptor is possible, and will be investigated.

Another pathway which is implicated is the IL5 receptor (IL5R) signaling cascade. The IL3R is closely related to the IL5R, and signaling for both of these receptors is mediated by the common β chain, providing the molecular basis for the functional redundancy of these cytokines (Miyajima et al. 1992). This implies that cross-talk through the IL5R, which may occur if this receptor is expressed in the modified cell line, leads to activation of the same promoters as IL3 signaling. Though IL5R was not detected in BaF3 cells according to microarray data (GDS3349), all other components for IL5 signaling are expressed and cross-talk via this signaling chain will be investigated.

Finally, cytokine receptors mediated by the common γ chain also may cause cross-talk. IL15R/γ signaling occurs through JAK1, JAK2 or TYK2 and through STAT1, STAT3 and STAT5, once more showing a high degree of overlap between pathways and demonstrating the need for cross-talk experiments. Signaling via

IL4R/γ activates JAK1 and JAK3, which phosphorylate STAT6, a pathway which does not overlap with the JAK2/STAT5 or TYK2/STAT3 pathways. However, IL4 is known to be an important cytokine in B-cell development and the IL4R is expressed in BaF3 cells according to microarray data (GDS3349). As such, IL4 was also included in the cross-talk assays and cytokine signaling mediated via the γ common chain was investigated for IL4R and IL15R.

The cytokine receptors discussed above are all representatives of the class I family of cytokine receptors which have some or all signaling components in common with the recombinant EPOR/gp130 receptor couple, except for the IL4R which was discussed because of its role in B-cell development. As such, the exposure of the Ba/HE+LgIGFP cell line to ligands of the discussed cytokine receptors will give an insight into possible problems which are to be expected regarding cross-talk in the cytokine receptor based whole cell biosensor. While IL3 cross-talk is to be expected, the effects of other cytokines cannot be predicted as easily, especially since some evidence exists that the IL4R and gp130 are expressed in the BaF3 cell line.

3.2. Aim

The whole cell sensor being developed constitutes of a receptor, in this case the recombinant anti-HEL EPOR/gp130 receptor, which renders the sensor it's specificity, and on the other hand a reporter gene which depends on its promoter for ligand induced expression and on the chosen reporter gene for fast read-out. In this chapter, a panel of promoters is selected and tested to single out the most promising promoters for use in a biosensor set-up.

The chosen promoter must comply with several conditions, the first and most important being that the promoter must be activated by the recombinant receptor pathway, leading to HEL dependent transcription of the reporter gene. Activation must occur at the lowest possible concentration of ligand and preferably little or no promoter leakage must be detected. Aside from activation of the promoter by the target, possible cross-talk must also be investigated. It is essential for the sensor that cross-talk events can be anticipated upon and dealt with.

The aim of the experiments presented here is the characterization of gene expression patterns due to HEL stimulation through the recombinant receptor pathway and the selection of one or more suitable promoters to be used in the whole cell biosensor set-up. Finally, the most promising promoters will be investigated regarding cross-talk via receptors with common signaling components.

3.3. Materials and Methods

3.3.1. Cell proliferation protocol

Ba/HE+LgIGFP cells were plated at a concentration of 10^4 cells/ml medium (Gibco® RPMI 1640 supplemented with 1 mM Sodium Pyruvate, 0,1 mM NEAA and 10% FCS, all Life Technologies Europe B.V., Belgium), either supplemented with 1µg/HEL (Sigma-Aldrich Co., Belgium) or unsupplemented. Cell density was monitored every 24 hours using a Fuchs-Rosenthal counting glass.

3.3.2. Gene expression profiling

Figure 3.2 shows the general workflow for the gene expression experiments. Ba/HE+LgIGFP cells were first starved of HEL for 24 hours, and then exposed for 30 minutes or 2 hours to HEL or to the panel of cytokines investigated for cross-talk. The different exposure times allow both fast induction and slower induction of genes to be recorded. After exposure, cells were snap frozen in liquid nitrogen (Air Liquide Benelux nv, Belgium) and stored until RNA extraction and cDNA synthesis. Finally, cDNA was used in the RT qPCR and data was analyzed.

3.3.2.1 Cell assays and RNA isolation/ cDNA synthesis

Freshly thawed Ba/HE+LgIGFP cells were plated at a density of 10^4 cells/ml total cell culture medium supplemented with 1µg/ml HEL and grown for at least three passages. Cells were then washed three times with sterile PBS (pH7.4, 1.06 mM KH_2PO_4, 155.17 mM NaCl, 2.97 mM Na_2HPO_4-$7H_2O$, all from GE Healthcare, UK) and plated in medium without HEL for 24 hours at a concentration of 10^5 cells/ml. HEL starved cells were then exposed to 1 µg/ml HEL and harvested after 30 min or 2 hours. Non-exposed cells (T0), cells exposed during 30 min (T1) and cells exposed for 2 hours (T2) were immediately washed in sterile ice-cold PBS, pelleted and snap frozen in liquid nitrogen. Cell pellets were kept at -70°C until RNA extraction. Cell assays were performed in duplicate for 7 samples per exposure group.

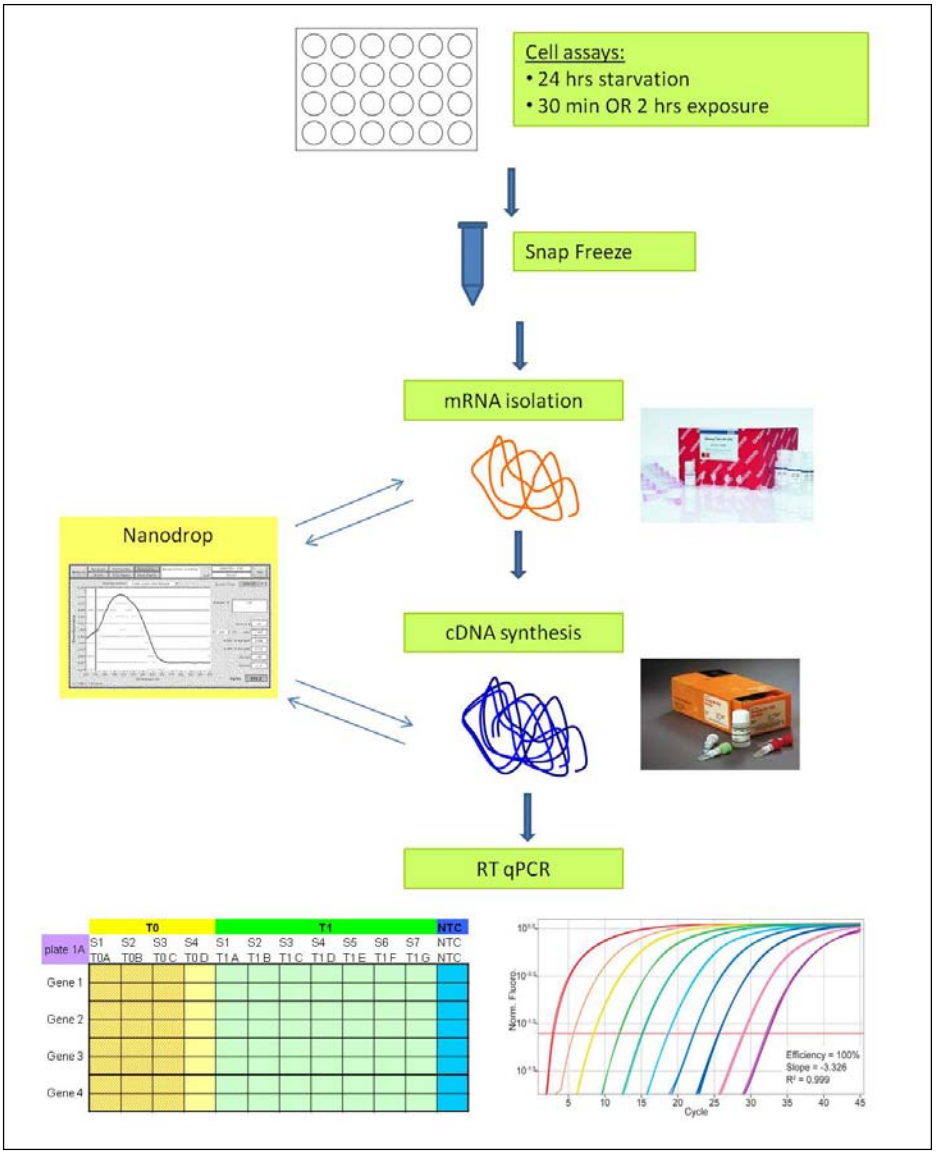


Figure 3.2: Gene expression profiling workflow

RNA was isolated from the cell pellet using the RNeasy extraction kit (Promega Benelux B.V., The Netherlands), according to manufacturer's instruction. The procedure is briefly described in chapter 2 (§ 2.3.2). The RNA concentration and purity was determined using the Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., USA). RNA purity was considered to be acceptable when the 260/280 nm ratio was approximately 1.8 and the 230/280 ratio was 1.8 to 2.2. The RNA concentration of the samples was typically between 200 and 700 ng/µl. Before cDNA synthesis, RNA concentration was adjusted to 200 ng/µl for all samples. RNA pellets were stored at -70°C until cDNA synthesis.
cDNA synthesis was performed using the Reverse Transcription System (Promega Benelux B.V., The Netherlands) according to manufacturer's instructions. Reverse Transcription reactions were set up in a 20 µl volume, processing 1 µg RNA per reaction. The resulting cDNA sample concentration was determined using the Nanodrop photospectrometer (concentration was usually approximately 190 ng/µl) and purity was once more confirmed as described for the RNA samples.

Table 3.4: Cytokine concentrations for cross-talk experiments

cytokine	Physiological concentration (ng/ml)	Reference
EPO	10	Kessler et al. 2012
IL6	10	März et al. 2002
LIF	5	
IL3	50	Gündogdu et al. 2010
IL5	40	Stathopoulos et al. 2010
IL4	50	Esen et al. 2012
IL15	10	

For the cross-talk experiments, cell assays and RNA isolation and cDNA synthesis were analogous to the HEL exposure assays, however HEL exposure was substituted for exposure to various cytokines (Sigma-Aldrich Co., Belgium), at physiological concentrations (see table 3.4).

3.3.2.2 RT qPCR experiments

RT qPCR was performed on cDNA samples from the T0, T1 and T2 exposure groups. Initially, the expression pattern of ten candidate reference genes was investigated to determine the best reference genes to normalize the gene expression levels of the genes of interest described in § 4.1.2. Once suitable

reference genes were selected, the activity of the candidate promoters was investigated. Finally, cross-activation of the most suitable candidate promoters was investigated upon stimulation by the cytokines described in §3.4.4.

RT qPCR is one of the most powerful and sensitive gene analysis techniques available. It is used for a broad range of applications including quantitative gene expression analysis, genotyping, copy number, drug target validation, biomarker discovery, pathogen detection, and measuring RNA interference.
RT qPCR measures PCR amplification as it occurs, so that it is possible to determine the starting concentration of template. In traditional PCR, which is based on end-point detection, results are collected after the reaction is complete, making it impossible to determine the starting concentration of nucleic acid.

Every RT qPCR contains a fluorescent reporter molecule such as a Taqman probe or SYBR Green dye, to monitor the accumulation of PCR product. As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore. Figure 3.3 shows SYBR green dye and a Taqman probe in the RT qPCR reaction. While SYBR Green dye binds to all double stranded DNA, thus increasing its fluorescence, Taqman probes are specific for a short sequence of DNA. The reporter dye of the Taqman probe is in close proximity to a quencher, and only emits fluorescence as the probe is degraded by DNA polymerase during the extension phase of the PCR, thereby releasing the fluorophore.

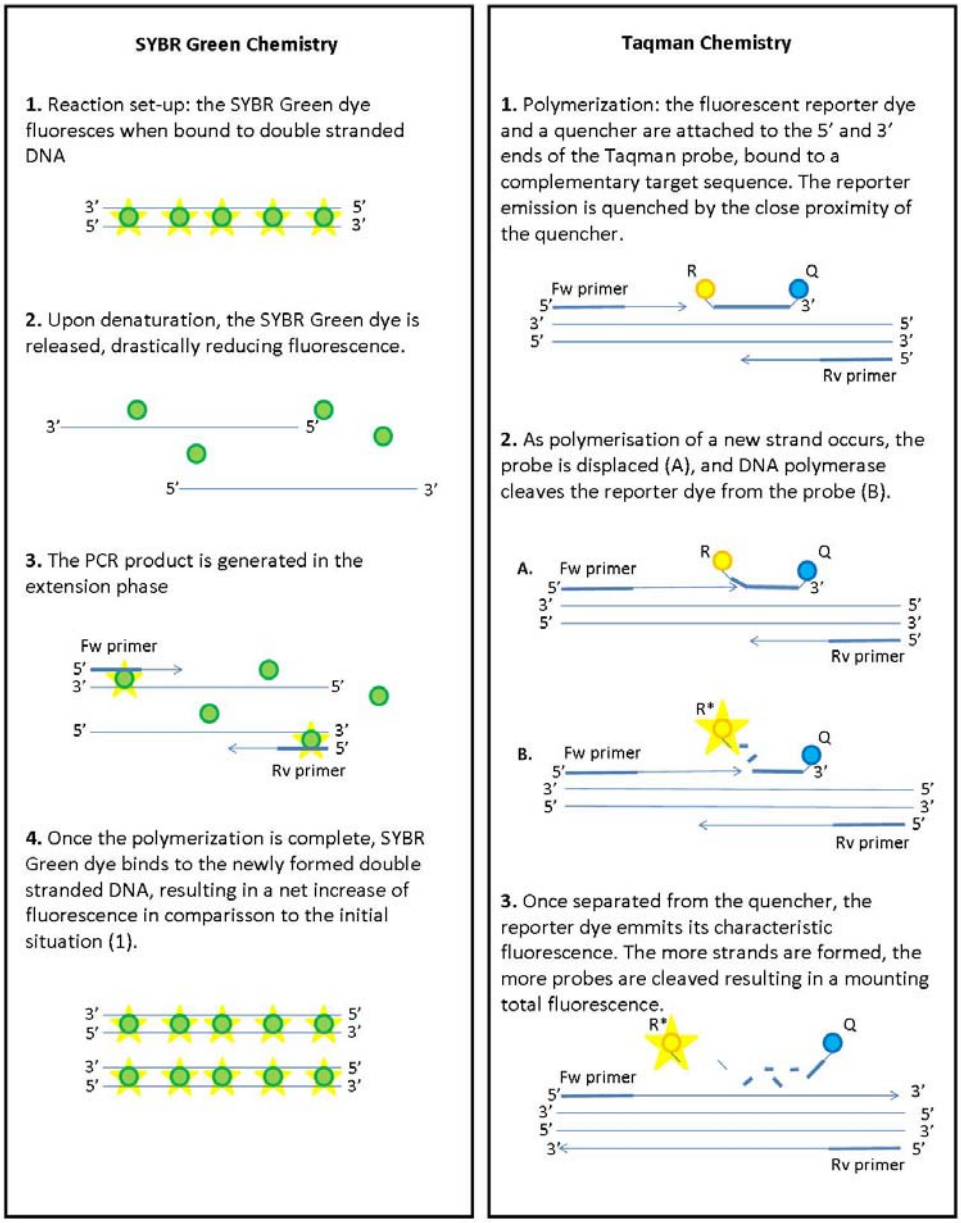


Figure 3.3: SYBR green dye and Taqman probe in a RT qPCR reaction

The accumulation of fluorescence over time depends on the amount of template originally present in the sample. Plotted in a graph (as shown in figure 4.4), this corresponds to faster or slower rise in fluorescence.

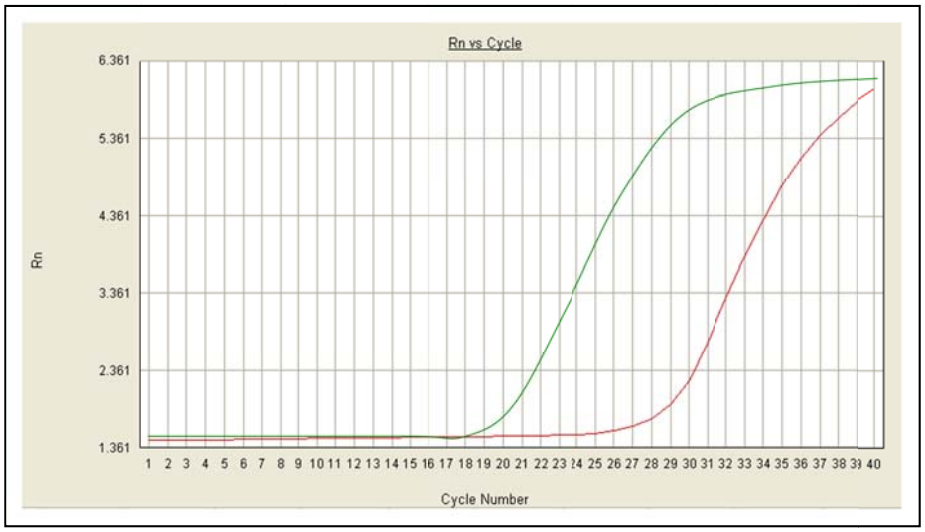


Figure 3.4: Example of an RT qPCR amplification plot. Fluorescence (Rn) versus cycle number. The green curve represents a RT qPCR with higher concentration of template and as such faster accumulation of fluorescence as opposed to the red curve.

SYBR green method

RT qPCR of the candidate reference genes was performed using the SYBR green method. Primer pairs for the candidate reference genes are specified in table 3.5. RT qPCR reactions were composed of 1 µM forward and reverse primer, 3 mM $MgCl_2$, and LightCycler DNA Master SYBR Green I according to manufacturer's instructions (Taq DNA Polymerase, reaction buffer, dNTP mix, SYBR Green I dye and 10mM $MgCl_2$, by Roche Diagnostics Gmbh, Germany). 10 ng cDNA was added to each reaction well and RT qPCR was performed on the Light Cycler 2.0 (Roche Diagnostics Gmbh, Germany) according to the following conditions: 10 min 95°C, 45 cycles (3 sec 95°C, 15 sec 58°C, 8 sec 72°C), melting curve (60 sec 70°C). Output was analyzed using GeNorm (see § 3.3.2.4).

Table 3.5: Mouse candidate reference gene primers

gene	Product length	Forward primer	Reverse Primer
PGK1	138 bp	GAA GGG AAG GGA AAA GAT GC	GCT ATG GGC TCG GTG TGC
RPL13a	131 bp	GGA TCC CTC CAC CCT ATG ACA	CTG GTA CTT CCA CCC GAC CTC
18S	310 bp	ACG GAC CAG AGC GAA AGC AT	TGT CAA TCC TGT CCG TGT CC
HPRT	123 bp	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC
YWHAZ	149 bp	GCA ACG ATG TAC TGT CTC TTT TGG	GTC CAC AAT TCC TTT CTT GTC ATC
TBP	197 bp	ATG GTG TGC ACA GGA GCC AAG	TCA TAG CTA CTG AAC TGC TG
GAPDH	452 bp	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA
ACTb	153 bp	GGC TGT ATT CCC CTC CAT CG	CAG TTG GTA ACA ATG CCA TGT
HMBS	168 bp	GAT GGG CAA CTG TAC CTG ACT G	CTG GGC TCC TCT TGG AAT G
CYCA	145 bp	GCG TCT CCT TCG AGC TGT T	AA GTC ACC ACC CTG GCA

Taqman method

For the analysis of the gene expression profiles of the Ba/HE+LgIGFP cell lines, optimized Taqman gene expression assays (Applied Biosystems, Life Technologies, USA) were used, thereby avoiding a time-consuming optimization of the PCR conditions of the 16 genes of interest (GOI). The Taqman assays used for the GOI and for the reference genes are specified in table 3.6.

Table 3.6: Taqman assay catalogue numbers (Applied Biosystems, Life Technologies, USA)

Gene	Taqman gene expression assay cat. n°		Gene	Taqman gene expression assay cat. n°
Bves	Mm00517902_m1		CISH	Mm00515488_m1
Igsf3	Mm01302150_m1		Socs1	Mm00782550_s1
Bmp2	Mm01340178_m1		Socs2	Mm00850544_g1
Gpr34	Mm00442229_s1		Socs3	Mm00545913_s1
Olig2	Mm01210556_m1		Socs4	Mm00466905_s1
Etv5	Mm00465816_m1		Socs5	Mm00465631_s1
Crbn	Mm01182416_m1		Socs7	Mm00507020_m1
Myc	Mm00487803_m1		Pim1	Mm00435712_m1
Ptgs2	Mm00478374_m1		**Reference genes:**	
Snap25	Mm00456921_m1			
Bcl X_L	Mm00437783_m1		ACTB	4352933E
Spry1	Mm01285700_m1		Pgk1	Mm00435617_m1
Spred2	Mm01223872_g1		Ywhaz	Mm03950126_s1

Before GOI were investigated, the selected reference genes (as described in §3.3.2.4) were confirmed using Taqman technology and GeNorm analyses (see §3.3.2.4). After confirmation of the reference genes, cDNA from the HEL exposure experiments and from the cross-talk experiments were investigated.
RT qPCR reaction mixes are composed of the Taqman gene expression assay (containing two unlabeled primers (900 nM), one 6-FAM dye-labeled Taqman MGB probe (250 nM), 6-VIC dye-labeled Taqman MGB probe (250 nM) and Taqman Gene expression mastermix (containing AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure) for dNTPs and a passive internal reference based on proprietary ROX™ dye) according to manufacturer's instruction. 10 ng cDNA was added to each reaction well, each sample was tested in duplicate. An interrun calibration was performed by repeating analyses of three samples on each plate.
Reactions were performed in MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems) and sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems). RT qPCR was performed on the *StepOnePlus*® instrument (Applied Biosystems), cycle conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec followed by 60°C for one minute.

3.3.2.3 GeNorm

RT qPCR results are normalized to account for technical or experimental variation due to differences in sample size, total RNA quantity and quality, efficiency of reverse transcription and PCR efficiency (Hugett et al. 2005). Reference gene normalization of the RT qPCR data captures most technical and experimental variation. However, the reference genes themselves may vary in expression between experimental groups, rendering the validation of reference genes essential and also making the use of multiple reference genes highly advisable. Vandesompele and colleagues (2002) evaluated the problems linked to the use of a single non-validated reference gene and developed a robust algorithm for assessment of expression stability of candidate reference genes. They propose that the geometric mean of at least two and preferably three or more validated reference genes should be used for reliable and accurate normalization.

The algorithm developed by Vandesompele and colleagues, named GeNorm, ranks candidate reference genes according to their stability. GeNorm calculates the pairwise variability (V) between each of the tested candidate reference genes and allocates a gene stability measure (M) to each candidate reference gene, which corresponds to the average pairwise variation V of that gene with all other candidate reference genes. The lower the M value, the more stable the gene. Genes with M < 0.5 are considered stable and suitable as reference genes (Vandesompele et al. 2002).
A panel of ten candidate reference genes (belonging to different functional classes, see table 3.6) were investigated in the different exposure groups (T0, T1, T2) using RT qPCR. Consecutive rounds of GeNorm calculations were performed on the data obtained from these experiments, removing the least stable gene (with the highest M value) after each round and finally resulting in three genes with M value < 0.5. These validated reference genes were then used in all following qPCR experiments.

3.3.2.4 RT qPCR data analysis

Before data analysis can be discussed, a number of basic RT qPCR principles must be explained. RT qPCR visualizes the amplification reaction by use of a fluorescent dye or reporter. Each reaction results in an amplification curve with the PCR cycle number (C) on the x axis and the fluorescence (ΔRn) on the y axis. ΔRn = Rnf – Rnb, where Rnf is the fluorescence emission of the product at each time point and Rnb is the fluorescence emission at the baseline (the baseline is defined as the PCR cycles in which reporter fluorescence is accumulating but is beneath the limits of detection of the instrument) (Heid et al. 1996, Gibson et al. 1996).
Figure 3.5 shows a representative amplification plot and defines the important terms associated with it. An arbitrary threshold fluorescence (T) is set in the region of exponential amplification of the amplification curve and is maintained across all amplification plots of the RT qPCR run. The fractional PCR cycle number at which the reporter fluorescence is equal to T, is defined as the C_T value. The presence of more template at the start of the PCR will lead to fewer

cycles before fluorescence reaches T. The C_T value will always occur in the exponential phase of the PCR, in the early cycles of the reaction. In this phase, none of the reaction components are limiting and therefore C_T values are very reproducible for replicate reactions with the same starting copy number.

Figure 3.5: RT qPCR amplification plot.

The C_T values may be used to calculate the relative expression levels of target genes relative to an internal calibrator (such as a non-treated sample) and normalized to one or more reference genes. The fold induction of target gene in a sample is then given by $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (calibrator), and $\Delta C_T = C_T$ (target) – mean C_T (reference genes). This method is referred to as the $\Delta\Delta C_T$ method (Livak & Schmittgen, 2001). Fold induction rates of each of the exposed groups (T1 and T2) were compared to the non-exposed control group (T0) using the two-tailed t-test.

3.4. Results

3.4.1. HEL exposure assay development

The BaF3 cells expressing the recombinant EPOR/gp130 receptor pairs are dependent on HEL for cell growth (Kawahara 2003) and as such, HEL is needed in the cell medium. However, in order to investigate variable gene expression induced by HEL, cells first need to be deprived of HEL for a period of time, allowing HEL-induced gene expression to attenuate. Only after this starvation time, induction of genes by HEL can be investigated. To determine the length of the starvation time required, cell proliferation experiments were conducted. Cells were plated at a density of 10^5 cells/ ml and either deprived of HEL or exposed to 1 µg/ml HEL. The cell densities observed in the following three days are shown in figure 3.6 (a). As well as live cells, the dead cells were also counted. The percentage of dead cells observed is shown in figure 3.6 (b). The peak in dead cells after 48 hours may partly be due to medium depletion (after 48 hours, medium was exchanged for all cells), however the difference in peak height between HEL exposed cells and deprived cells demonstrates the HEL dependency of the cells for survival.

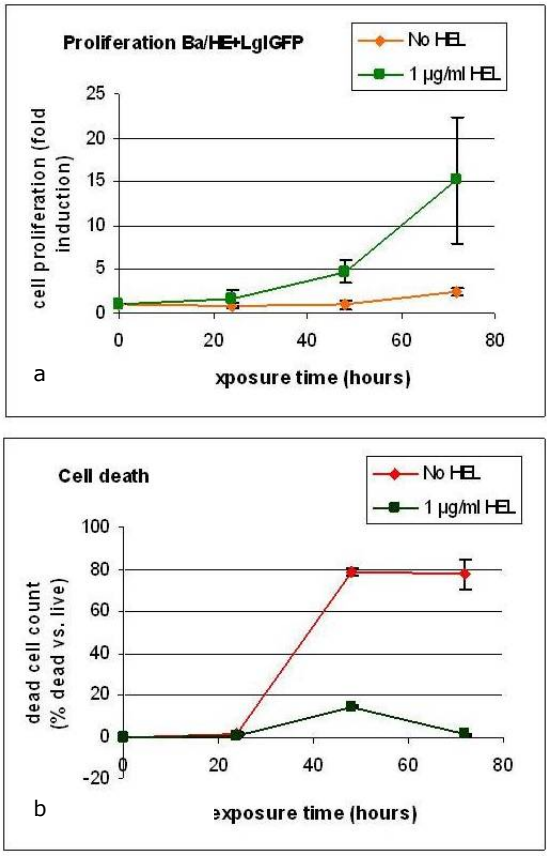


Figure 3.6: Cell proliferation rate (a) and percentage of dead cells (b) under the influence of HEL exposure

The proliferation experiments show that HEL deprived cells are stopped in their proliferation and

the lack of stimulation eventually leads to cell death. The first 24 hours, the difference in cell density is just under twofold (0.9 fold for HEL deprived cells versus 1.7 fold in HEL exposed cells), and after 48 hours there is a clear gap is cell densities. The percentage of dead cells follows a similar pattern, with small differences apparent after 24 hours (0.8 % dead cells versus 0.9%), after 48 hours the gap is very large (78.8 % versus 14.4% dead cells). These results demonstrate that a 24 hour starvation period leads to phenotypical changes and as such, this period of HEL deprivation is sufficient before each experiment.

3.4.2. Selection of reference genes

The HEL-induced gene expression was investigated by RT qPCR (see §3.4.3). Relative quantification of the RT qPCR data requires the use of well validated reference genes (see §3.3.2.5). In this chapter, a panel of ten candidate reference genes is tested to select the three most stable reference genes for further experiments using RT qPCR.

The expression patterns of the candidate reference genes were investigated for Ba/HE+LgIGFP cells, which were first starved of HEL for 24 hours, and subsequently exposed to 1 µg/ml HEL (see §3.3.1). Three experimental groups were investigated: T0 was not exposed to HEL, T1 was exposed during 30 minutes and T2 are cells exposed for 2 hours. The cells' RNA was converted to cDNA and this material was used in RT qPCR, using the primers described in the methods section (see table 3.5). RT qPCR failed for three out of the ten candidate reference genes, however the seven remaining candidates proved sufficient to deliver three validated stable reference genes. GeNorm output, shown in figure 3.7, resulted in three reference genes with $M < 0.5$: Pgk1 ($M = 0.425$), ACTB ($M = 0.412$) and YWHAZ ($M = 0.431$). These three validated reference genes were used in all subsequent RT qPCR experiments.

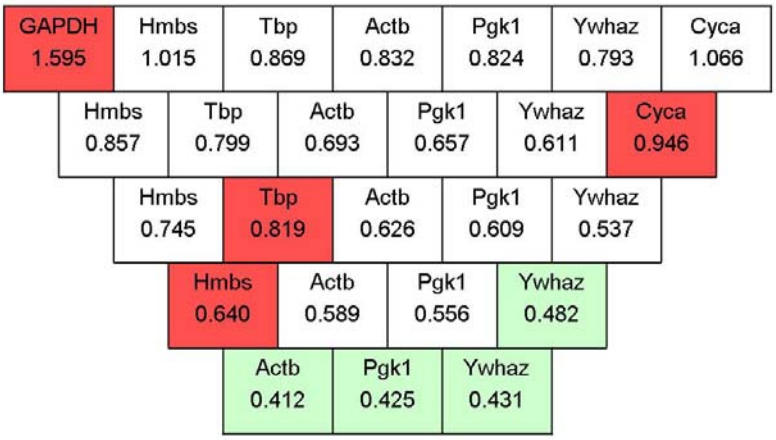


Figure 3.7: Evaluation of the reference genes using GeNorm. Each row represents the M-values calculated from RT qPCR data of all the candidate reference genes in that row (see §4.3.2.3). The least stable reference gene (with the highest M-value) is then removed and the calculation is repeated with RT qPCR data from the remaining reference genes, ultimately resulting tin three reference genes with M < 0.5 (highlighted in green).

3.4.3. Genes of interest: candidate promoters HEL induced activity

The choice of a suitable promoter for a reporter gene is crucial to the development of our whole cell biosensor. Leakage of the promoter (background induction) should be minimal and HEL dependent induction should be as strong as possible. Here, the twenty candidate promoters described in §3.1 are investigated using RT qPCR.

Ba/HE+LgIGFP cells were starved of HEL for 24 hours, then cells were exposed to 1 µg/ml HEL for either 30 minutes or 2 hours, and immediately snap frozen. RNA was extracted and converted to cDNA and the Taqman assays performed (see §3.2). Ba/HE+LgIGFP cells which were simultaneously starved but not exposed to HEL formed the control group. Results are represented in figure 3.8.

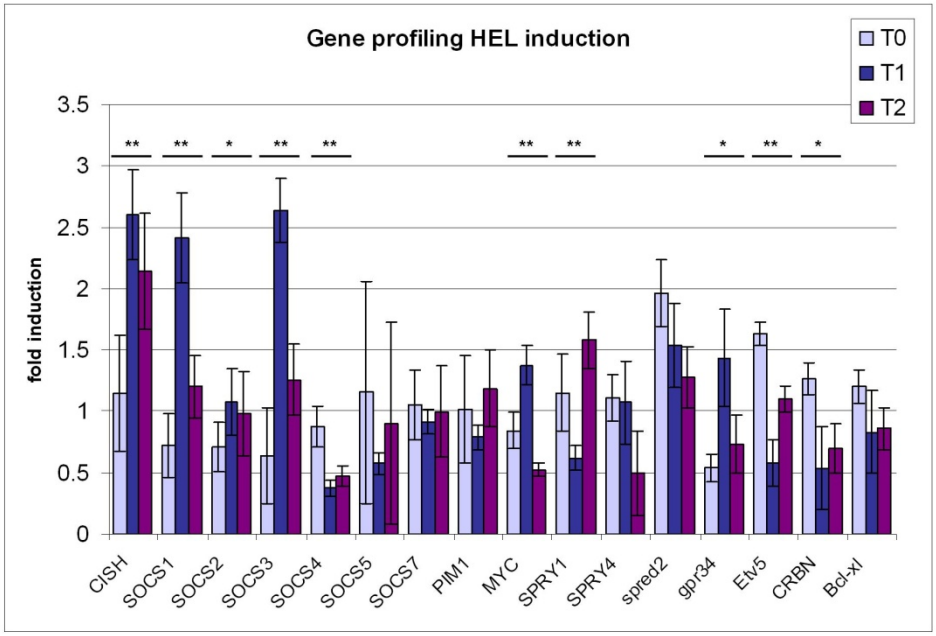


Figure 3.8: Gene expression profile of Ba/HE+LgIGFP cells starved of HEL for 24 hours (T0) and then exposed to 1 µg/ml HEL for 30 min (T1) to 2 hours (T2). Significant differences between T0 and either T1 and/or T2 are indicated by * ($0.01 < p < 0.05$) or by ** ($p < 0.01$).

Seven genes, CISH, SOCS1, SOCS2, SOCS3, c-Myc, Spry1, gpr34 and CRBN are significantly upregulated after either 30 min or 2 hours HEL exposure ($p<0.05$). For CISH, SOCS1, SOCS3, SOCS4, c-Myc, Spry1 and gpr34 the p-value was < 0.01. CISH, SOCS1 and SOCS3 were most strongly upregulated.
SOCS4 and Etv5 are significantly downregulated ($p < 0.01$).

These results, which are further discussed in §3.5, indicate that CISH, SOCS1 and SOCS3 are the most promising candidates for a biosensor setting. Based on these findings, the CISH, SOCS1 and SOCS3 promoters were investigated further to identify other pathways which might activate these promoters and as such anticipate on cross-talk which may lead to false positive results of the biosensor.

3.4.4. Cross-talk

In order to identify cytokine driven cross-talk between induction pathways, the Ba/HE+LgIGFP cell line was exposed to a selection of class one cytokines: representatives of four subfamilies of the type I class of cytokine receptors (i.e. the gp130 family, the common γ chain (γc) family, the common β chain (βc) family, and the single chain family of receptors) were chosen based on the common components in their signaling cascades or on their role in B-cell development (see §3.1.5). Table 3.7 lists the ligands tested.

Table 3.7: Cytokines used for cross-talk experiments and the specific and common receptor components

Common receptor	Specific receptor	Ligand
/	EPOR	EPO
gp130	IL6R	IL6
	LIFR	LIF
βc	IL3R	IL3 *
	IL5R	IL5
γc	IL4R	IL4
	IL15R	IL15

Induction of the CISH, SOCS1 and SOCS3 promoters by the listed cytokines was detected using RT qPCR (see materials & methods). Results are presented in figure 3.9.

These results confirm the activation of the three promoters by HEL, and as expected also show and IL3 induced activation of the pathways leading to CISH, SOCS1 and SOCS3 expression. Other cytokines which elicited a response were IL5 (a significant response is present for CISH and for SOCS3), IL6 (only significant for CISH), EPO (which only induces SOCS1) and IL4 (SOCS3).

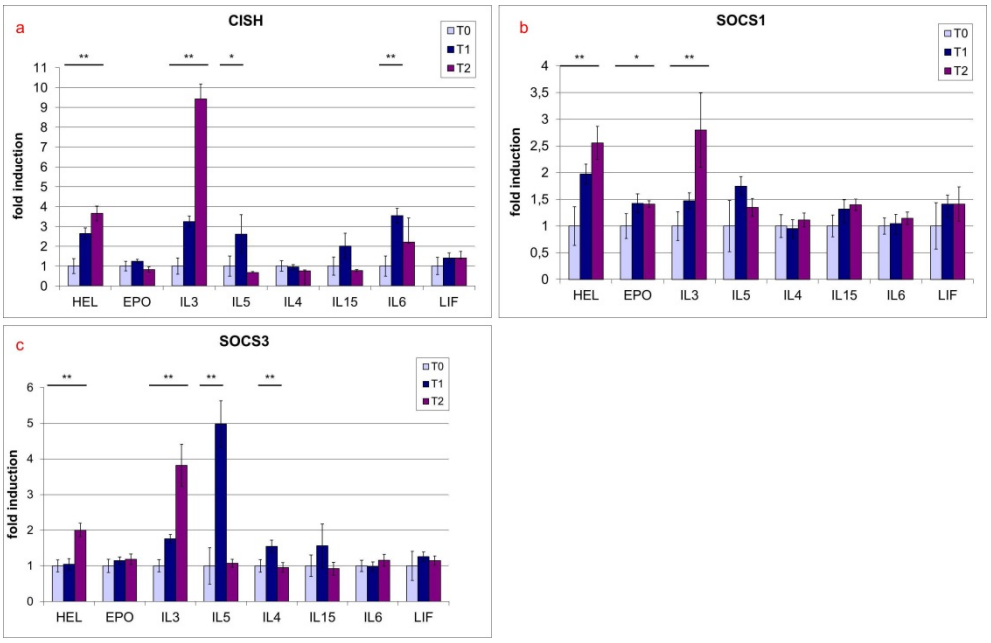


Figure 3.9: Cross-talk RT qPCR results for CISH (a), SOCS1 (b) and SOCS3 (c). Significant differences are indicated by * ($0.01 < p < 0.05$) or ** ($p < 0.01$).

3.5. Discussion

We designed a novel whole cell biosensor, based on a recombinant receptor, which must be coupled to a suitable reporter system. The target of the sensor is defined by the recognition domains of a recombinant receptor couple, here an anti-HEL EPOR/gp130 receptor. This receptor couple activates the JAK/STAT pathway, typical for class I family of cytokine receptors. Because the reporter gene should be expressed upon target binding, the choice of a suitable promoter is essential to the biosensor. In this chapter, 16 promoter candidates were screened for their activity upon HEL stimulation of the recombinant receptor couple using RT qPCR. The promoters showing the highest activation were withheld and investigated for cross-talk activity.

3.5.1. Promoter activation by HEL

Of the 16 promoters, three promoters were significantly ($p < 0.05$) downregulated (SOCS4, Etv5 and CRBN) and six were significantly ($p < 0.05$) upregulated (CISH, SOCS1, SOCS2, SOCS3, Spry1 and gpr34).
The activated promoters are all induced by the JAK/STAT pathway, a signaling pathway typically activated by class I cytokine receptor family, confirming the receptor dependent activation. However, not all promoters activated by IL3 signaling (see section 3.1.3) were activated by the EPOR/gp130 recombinant receptor pathway. This discrepancy in promoter activation may be explained by the difference in JAK/STAT pathways induced by these receptors: Nagata and Todokoro demonstrated in 1996 that IL3 signaling in BaF3 cells occurred through phosphorylation of JAK2 (which in turn phosphorylates STAT5) and TYK2, which phosphorylates STAT3. EPOR signaling was also shown to activate the JAK2/STAT5 pathway, the only difference which is seen between the EPOR and IL3 pathways is the phosphorylation of TYK2, leading to phosphorylation of STAT3 in the IL3 pathway. Possibly, the lack of TYK2 phosphorylation by the EPOR/gp130 recombinant receptor may lead to an altered gene expression upon ligand binding. This is confirmed by the lack of stimulation of the Bcl-xl promoter, which was shown to be STAT3 dependent in BaF3 cells (Muromoto et al. 2010).

CISH SOCS1 and SOCS3 are the promoters which are most strongly activated and as such the results presented here show that these three promoters will lead to the most sensitive reporter gene expression of the panel of promoters tested.

The basal promoter expression (which reflects the leakage of the promoters) is not significantly different between CISH, SOCS1 and SOCS3. Based on this criterion, none of these three promoters is preferable above the others, and therefore all three were included in the cross-talk experiments.

3.5.2. Cross-talk activation of CISHp, SOCS1p and SOCS3p

Ideally, the whole cell biosensor should be sensitive as well as only responding to the binding signal of the chosen target to the recombinant receptor couple. However, the class I cytokine receptor family of receptors all share a common signaling pathway and as such, the possibility that the chosen promoters might respond to stimulation by other cytokines needed to be investigated. The activity of the three most promising promoters, CISH, SOCS1 and SOCS3 were investigated upon stimulation with eight class I cytokine receptor ligands, representing the gp130 family, the common γ chain (γc) family, the common β chain (βc) family, and the single chain (homodimeric) family of receptors (see section 3.1.4). The results of these cross-talk experiments confirm that all three promoters are induced by HEL stimulation of the EPOR/gp130 pathway. As might be expected, the promoters are also strongly induced by IL3, the natural stimulant of the IL3 dependent BaF3 cell line. Aside from these expected observations, other cross-talk events were also observed.

Both CISH and SOCS3 were significantly induced by IL5. This was especially striking for SOCS3, where the induction by IL5 was more than two times the induction observed for HEL. This might be explained by the autocrine induction of IL5R expression in BaF3 cells: the β signaling chain is certainly expressed in the IL3 dependent BaF3 cells, and though the IL5R was not detectable according to the microarray data, it is possible that a basal expression of this receptor leads to an autocrine loop. IL5 may increase IL5R expression, enhancing its own

effect and inducing the CISH and SOCS3 promoters. The IL5R is closely related to the IL3R, which may explain why a response mediated by the IL5R is so large in the IL3 dependent cell line. A similar though smaller effect is observed for IL6 induction of CISH: while the IL6R was not shown to be expressed, the signaling chain gp130 is present in BaF3 cells according to microarray data, and an autocrine loop may induce IL6R expression.

Finally, IL4 activates the SOCS3 promoter in the recombinant cell line and EPO induces the SOCS1 promoter. This cross-talk is to be expected, as both the IL4R and the EPOR were detected in the BaF3 cell line according to microarray data (GDS3349). Additionally, IL4 is known to be an important cytokine in B-cell development. A question which may arise is why, while IL4 and EPO induced the activation of respectively the SOCS3 and SOCS1 promoter, the other promoters were not activated by these cytokines. One possible explanation is that the activation which was observed, though significant, was not very high and the induction of the other promoters was not detected because it was too faint.

From this data, it is clear that all three promoters, CISHp, SOCS1p and SOCS3p, are subject to cytokine driven cross-talk, aside from the expected IL3 cross-talk. CISHp is activated by IL5 and IL6, SOCS1 is activated by EPO and SOCS3 is induced by IL3 and IL4.

It is vital in the biosensor set-up that the promoter activity caused by cross-talk should be separated from promoter activation by the biosensor target to avoid false positive results. This can be achieved by depleting the cross-talk inducing cytokines. Alternatively, parallel assays should be set up to determine unknown concentrations of cross-reactive targets in the samples, compensating for this cross-reactivity.

3.6. Conclusion

We aim to develop a novel whole cell biosensor, capable of detecting low concentrations of target and with a versatile recognition domain which can be switched to different targets. The Ba/HE+LgIGFP cell line, which expresses a recombinant anti-HEL EPOR/gp130 receptor couple forms the basis of the novel whole cell biosensor. The recombinant receptor couple allows the target to be changed depending on the recognition domain of the recombinant receptor couple. However, while the activation of the anti-HEL EPOR/gp130 receptor was confirmed by cell proliferation experiments, for biosensing purposes a reporter gene system was called upon to allow faster and easier read-out. The first step in the development of such a reporter gene system is the choice of one or more suitable promoters which are activated upon HEL stimulation.

In this chapter, a panel of candidate promoters was assembled, and gene profiling experiments were conducted, exposing the Ba/HE+LgIGFP cell line to HEL and investigating the response of the candidate promoters. These experiments led to three promising promoters for the reporter gene system: CISH, SOCS1 and SOCS3. These three genes showed the highest activity upon stimulation of the EPOR/gp130 recombinant receptor pair, indicating that these promoters will lead to the most sensitive whole cell sensor.

Aside from the sensitivity of the promoter, the specificity is also essential. While the recombinant receptor couple expressed in the Ba/HE+LgIGFP cell line specifically binds HEL, cytokine induced cross-talk may still lead to false positive results. Indeed, cross-talk experiments presented in this chapter indicate that IL3, the cell line's natural stimulant, induces activation of the three selected promoters. Additionally, other cytokines such as IL5, IL4, IL15 and EPO are shown to elicit a cross-reaction. This challenge must be addressed in the final sensor set-up either by conducting parallel experiments or by depleting samples of cross-talk causing molecules.

In the next chapter, the selected promoters are coupled to a reporter gene, and the resulting reporter plasmids are expressed in Ba/HE+LgIGFP cells. HEL

exposure experiments coupled to luminescent read-out allow the whole cell sensor to be evaluated.

CHAPTER 4

TOWARDS A PROOF OF PRINCIPLE

Chapter 4: Towards a proof of principle

4.1 Introduction

The whole cell sensor being developed consists of a recombinant anti-HEL EPOR/gp130 receptor expressed in the BaF3 cell line. This modified cell line was shown to proliferate upon exposure to 10 ng/ml HEL, and a target concentration as low as 1 ng/ml still induced anti-apoptotic effects. The next step in the development is the construction and expression of a reporter gene coupled to a receptor activated promoter. RT qPCR experiments were conducted to select the most promising promoter(s) out of a panel of chosen promoters (see chapter 3), yielding three promoters: the CISH promoter (CISHp), SOCS1 promoter (SOCS1p) and SOCS3 promoter (SOCS3p). These promoters will now be coupled to a reporter gene and the sensitivity and specificity of the resulting whole cell sensor is investigated.

4.1.1 Promoter structure

To assure that the promoters are activated by the EPOR/gp130 pathway, all functional components of the promoters must be present in the promoter/ reporter gene construct. Below, the structure of the three selected promoters, CISHp, SOCS1p and SOCS3p are discussed.

4.1.1.1 CISH promoter

The 540 bp upstream region of the CISH gene was shown to be sufficient for EPO induced CISH expression. The major transcriptional start region is located 45 bp upstream of the translational start codon (see figure 4.1), and a minor transcriptional start site is located approx. 130 bp upstream. Four consensus sequences for STAT5 binding (sequence: TTCNNNGAA) were identified in the same region (Matsumoto et al. 1997), essential for efficient induction of CISH expression. These regions will therefore be included in the CISHp/ reporter gene expression plasmid.

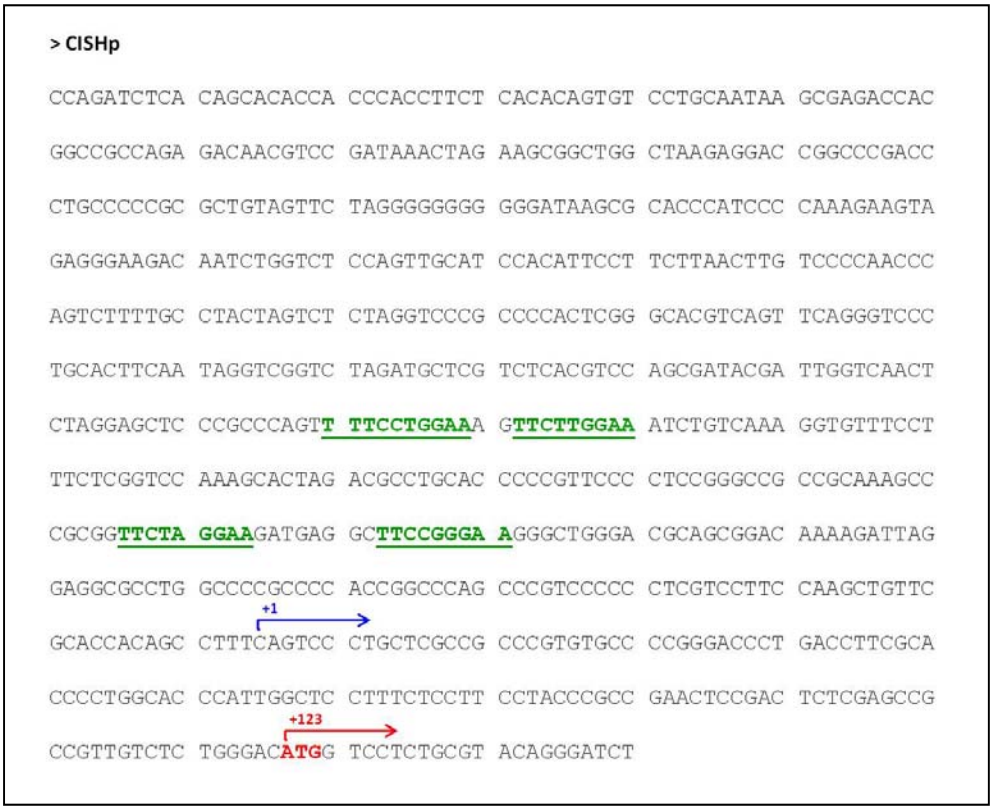


Figure 4.1: CISH promoter region. STAT binding sequences are indicated in green, bold font. The blue arrow indicated transcription start (+1), the red arrow indicated translation start.

4.1.1.2 SOCS1 promoter

Four consensus sequences for STAT binding (TTCNNN(N)GAA) are present between -645 and -443 of the promoter (indicated in green, underlined). These regions were shown not to be involved in IFNγ stimulation of CISHp, however their involvement in other stimuli is unknown. The three GAAA units indicated in orange are indispensable to IFNγ induced Socs1 expression, whereas the GC boxes and GC-like elements (bold, underlined) are involved in constitutive transcriptional activity. The entire promoter region (-735 to +122) described by Saito and colleagues (2000) will be included in a luciferase expression plasmid.

Figure 4.2: SOCS1 5' region. Green, underlined: STAT binding sequences; orange underlined: GAAA boxes involved in IFNγ induced expression; bold underlined: GC boxes and GC-like elements involved in constitutive expression. Blue arrow indicated transcription start (+1), red arrow indicated translation start.

4.1.1.3 SOCS3 promoter

SOCS3 gene expression is controlled by its 2.7 kb 5' flanking region. This region contains three TTNNNNNAA consensus sequences (located at nucleotides -95 to -87, at -72 to -64 and at -345 to -337) expected to be STAT binding sites. The -72 to -64 region is specifically identified as a STAT1 and STAT3 binding region. The entire 2.7 kb 5' region was cloned into the pGL3 luciferase plasmid and is known as clone 6 (Auernhammer et al. 1999).

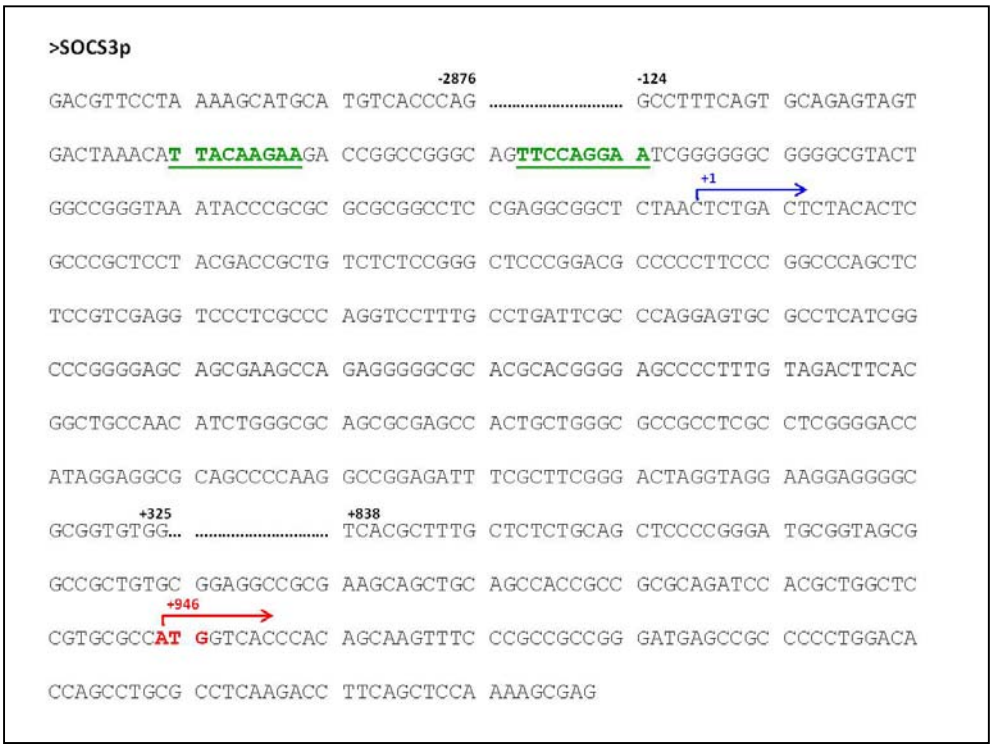


Figure 4.3: SOCS3 5' region. Green, underlined: STAT binding sequences. Blue arrow indicated transcription start (+1), red arrow indicated translation start.

4.2. Aim

The Ba/HE+LgIGFP cell line, expressing an anti-HEL EPOR/gp130 recombinant receptor pair was chosen as the basis for our whole cell sensor. Indeed, cell proliferation experiments showed that concentrations as low as 10 ng/ml induced cell proliferation, and anti-apoptotic effects were observed from 1 ng/ml, indicating that the recombinant receptor initiates a signaling cascade at very low target concentrations. To transform the EPOR/gp130 signaling into a fast and recordable output, a reporter gene system must be developed. In the previous chapter, HEL responsive promoters were investigated and selected, leading to a panel of three suitable promoters: CISHp, SOCS1p and SOCS3p. Each of these promoters must now be coupled to a reporter gene, such as luciferase, and expressed in the Ba/HE+LgIGFP cell line. The performance of the resulting whole cell biosensor sensor must then be evaluated based on its sensitivity, by exposure to a range of HEL concentrations, and its specificity, by evaluation of cytokine driven cross-talk. For proof of principle purposes, the CISH promoter was implemented in the whole cell biosensor prototype.

4.3 Materials and methods

4.3.1 Plasmids

The pGV/CISHp (Nippon Gene, Tokyo, Japan) luciferase expression plasmid, constructed by Matsumoto and colleagues (1997), was a kind gift of Dr. Haan (Life Sciences Research Unit, University of Luxembourg). Because pGV lacks a selectable marker, the CISHp insert was amplified from this plasmid for cloning into the hygromycin selectable luciferase expression plasmid, pGL4.14 (Invitrogen by Life Technologies Europe B.V., Belgium), as shown in figure 4.4.

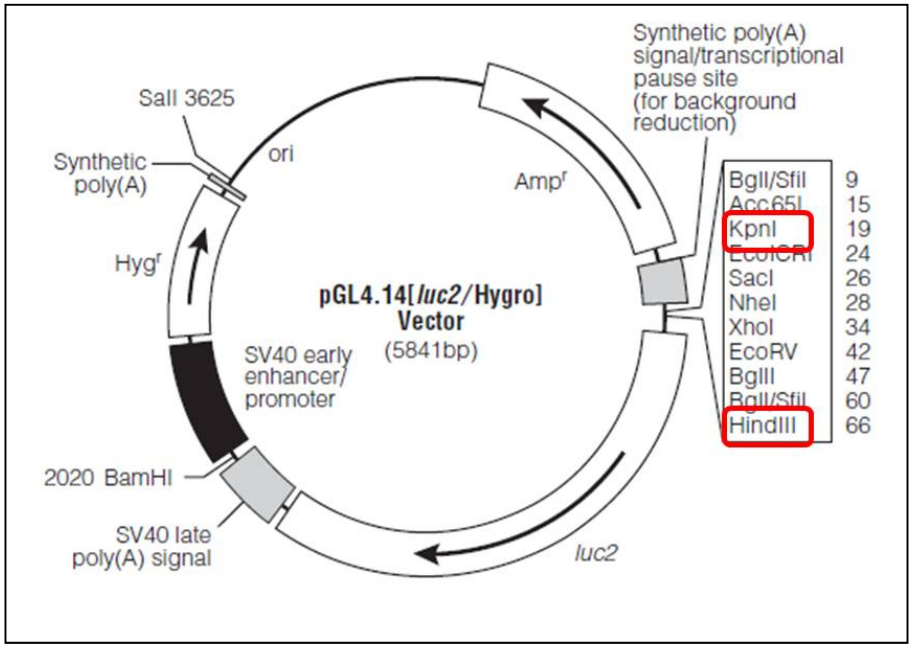


Figure 4.4: pGL4.14 plasmid. The pGL4.14 Vector encodes the luciferase reporter gene luc2 *(Photinus pyralis*) as well as the hygromycin resistance gene (Hyg^r). CISHp is cloned into the multiple cloning region (bp 0 – 70) via the specific restriction sites KpnI (bp 19) and HindIII (bp 66).

Cloning of CISHp into the pGL4.14 vector was however not possible (see § 4.3.4) and as a next best option, the original pGV/CISHp vector was cotransfected with the pREP9 selection plasmid (Invitrogen by Life Technologies Europe B.V., Belgium) containing the neomycin resistance gene, thus allowing

pGV/CISHp+/pREP9+ cells to be selected. Optimizations of the transfection reactions were performed with pCAGGS-RFP plasmid (Das et al., 2006), an RFP expression plasmid, to allow easy tracking of transfected cells (see §4.3.5).

4.3.2 PCR

PCR reactions were performed to isolate CISHp from the pGV vector, and to incorporate specific restriction sites in the 5' and 3' regions of the CISHp insert to allow directional cloning into pGL4.14. Basic PCRs, colony PCR and sequencing reactions were performed as described earlier in §3.3.4.2, §3.3.4.3 and §3.3.4.4. Primers are described in table 4.1. PCR with CP1 and CP2 has an annealing temperature of 65°C, yielding a 690 bp product.

Table 4.1: Primer sequences used for construction and sequencing of the pGL4.14/CISHp luciferase expression vector

Primer	Sequence
Construction primers	>CP1 GACTGGTACCCCAGATCTCACAGCACACCACCCAC
	>CP2 GACTAAGCTTAAGGAGAAAGGAGCCAATGGGTGCC
Sequencing primers	>pGL4.14 CTTAATGTTTTTGGCATCTTCCA
	>RV3 CTAGCAAAATAGGCTGTCC

4.3.3 Restriction and ligation reactions

The CISHp insert was isolated from pCR2.1 using FastDigest® (FD) HindIII and FD® KpnI (Fermentas, Life Sciences, USA). Reaction mix was according to manufacturer's conditions, 1 µl FD® restriction enzyme per µg of plasmid being digested, FD® Buffer according to manufacturer's instructions (composition not available) and pCR2.1/CISHp. Incubation was at 37°C for one hour. Restriction products were loaded on a 1% agarose gel (GE Healthcare, UK) supplemented with 0.5 µg/ml EtBr (VWR International). Purification of gel bands was performed using a Sephadex® G-50 column (GE Healthcare, UK) or using the

GFX purification kit (GE Healthcare, UK). Ligations were performed using T4 DNA ligase (Promega Benelux B.V, The Netherlands) as described in §3.3.6.

4.3.4 Transformation reactions

Transformation reactions were performed to transform pGV/CISHp, pGL4.14/CISHp, pREP9, pCR2.1/CISHp and pCAGGS-RFP into Top10 E. coli (Invitrogen by Life Technologies Europe B.V., Belgium) for storage and amplification purposes. Chemical transformations were successfully used for the transformation of pGV/CISHp, pREP9, pCR2.1/CISHp and pCAGGS-RFP according to standard conditions as described below. The transformation of pGL4.14/CISHp was attempted under a wide range of conditions, including both chemical transformation and electroporation. The standard conditions are described below and optimization conditions are described in tables 4.2 and 4.3.

4.3.4.1 Chemical transformation

Standard chemical transformation reaction: 10 ng plasmid is added to a 50µl vial of chemically competent Top10 cells. Heat shock is induced at 42°C for 30 seconds, followed by immediate cooling of the cells on ice. Top10 cells were then grown in non selective S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 10

Table 4.2: Chemical transformation reactions pGL4.14/CISHp. Central pane shows the conditions for the standard protocol, right pane shows the other tested conditions.

	Standard:	Other tested conditions:
pGL4.14/CISHp	• fresh ligation product	• GFX purified ligation product
Plasmid / 50 µl cell suspension	• 10 ng	• 1 ng • 50 ng • 100 ng • 1 µg
E. Coli strain	• chemically competent Top 10	• chemically competent TG1 *(Invitrogen by Life Technologies B.V., Belgium; LB: GE Healthcare, UK)*
Incubation time primary growth phase in non-selective medium	• 1 hour	• 3 hours
Incubation time plated cells	• overnight (12 hours)	• 36 hours

mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose; Invitrogen by Life Technologies Europe B.V., Belgium) for 1 hour, 37°C, 250 rpm. Cells were plated on selective Luria Broth (LB) agar plates (50 µg/ml ampicillin: Invitrogen by Life Technologies B.V., Belgium; LB: GE Healthcare, UK) and grown overnight. Colonies were picked out for colony PCR to confirm the presence of insert.

4.3.4.2 Electroporation

Electrocompetent cells: electrocompetent TG1 cells were prepared as follows. A TG1 preculture was grown at 37°C in 2 x Tryptone-Yeast extract (TY) medium (GE Healthcare, UK) until OD600 and then chilled on ice for 1 hour. Cells were pelleted (4000 rpm, 15 min, 4°C), resuspended in ice cold AD and chilled a further 15 min. Cells were pelleted (4000 rpm, 15 min, 4°C) and resuspended in ice cold 7% dimethyl sulfoxide (DMSO). Finally, after 15 min on ice, cells were pelleted and resuspended in sterile ice-cold 7% DMSO. Electrocompetent cells were used immediately or stored at -70°C.

Standard electroporation reaction: 50 µl of ice-cold electrocompetent cell suspension was pipetted into an ice-cold cuvette with 1 ng plasmid. Electroporation is performed in the Biorad Gene pulser II, on setting EC2 (pulse voltage is 2.5 kV, electric field E = 12.5 kV/cm) using cuvettes with 0.2 cm gap width. After electroporation the cells are grown at 37°C, 200 rpm in non-selective TY medium for 1 hour, then plated on selective TY agar plates (50µg/ml Ampicillin) and grown overnight.

Table 4.3: Electrical transformation reactions pGL4.14/CISHp. Central pane shows the conditions for the standard protocol, right pane shows the other tested conditions.

	Standard:	Other tested conditions:
Biorad setting and cuvette width	• EC2 (pulse voltage 2.5 kV, electric field E = 12.5 kV/cm) • 2 mm gap width	• EC1 (pulse voltage 1.8 kV, electric field E = 18 kV/cm)) • 1 mm gap width
Plasmid / 50 µl cell suspension	• 1 ng	• 0,1 ng • 10 ng • 100 ng
Incubation time primary growth phase in non-selective medium	• 1 hour	• 3 hours
Incubation time plated cells	• overnight (12 hours)	• 36 hours

4.3.5 Transfection reactions

For the transfection of luciferase expression plasmid into the Ba/HE+LgIGFP cell line, a wide range of conditions was explored using both lipofection and electroporation. The standard methodology is described below, and optimization conditions are shown in tables 4.4 and 4.5.

4.3.5.1 Lipofection

Lipofection is a DNA transfection protocol first introduced by Felgner and colleagues (1987) whereby liposomes interact spontaneously with plasmid DNA to form lipid-DNA complexes. The lipids in these complexes then fuse with the eukaryotic cell membrane, thus releasing the entrapped plasmid into the cells, resulting in both uptake and expression of the DNA. Lipofection was attempted using two different lipofection reagents: Lipofectamine LTX reagent (Invitrogen by Life Technologies Europe B.V., Belgium) and X-treme gene HP DNA transfection reagent (Roche Diagnostics Belgium nv), as described below.

Standard lipofection with Lipofectamine LTX: the standard protocol for lipofection with lipofectamine LTX was according to manufacturers guidelines. Brief description:

Prior to transfection, 10^5 Ba/HE+LgIGFP cells/ml are plated in 500 ml standard cell growth medium (Gibco® RPMI 1640 supplemented with 1 mM Sodium Pyruvate, 0,1 mM NEAA, all Life Technologies Europe B.V., Belgium) in each well of a 24 well plate. . Lipid DNA complexes were formed by adding 3 µl Lipofectamine™ to 1 µg plasmid DNA in 100 µl Opti-MEM Reduced Serum Medium. When required, Plus™ reagent is added prior to addition of Lipofectamine™ LTX (see table 4.4). Plus reagent™ enhances transfection for some cell lines. After incubation (room temperature, 30 min) 100 µl lipid DNA complexes is added to the Ba/HE+LgIGFP cells and cells are incubated in non selective complete growth medium (Gibco® RPMI 1640 supplemented with 1 mM Sodium Pyruvate, 0,1 mM NEAA, all Life Technologies Europe B.V., Belgium) supplemented with 1 µg/ml Hen Egg Lysozyme (HEL, Sigma-Aldrich Co.,

Belgium). After initial incubation (for incubation time see table 4.4), cells are selected with 300 µg/ml Neomycin (Sigma-Aldrich Co., Belgium).

Standard lipofection with X-treme gene HP DNA transfection reagent: 10^6 cells/ml were plated in complete cell growth medium prior to transfection. Xtreme gene HP reagent: DNA complexes are formed by addition of 3 µl Xtreme gene HP reagent per µg of plasmid DNA and per 100 µl Opti-MEML I Reduced serum Medium. The Xtreme gene HP reagent: DNA complex forming mixture is incubated 30 min at room temperature, and then added to 1 ml of cell suspension. The cells are incubated with the DNA lipid complexes for 24 hours (37°C, 5% CO_2).

Table 4.4: Lipid transfection methods and conditions tested for transfection of pGV/CISHp into Ba/HE+LgIGFP. Top: conditions tested using Lipofectamine LTX transfection reagent; Bottom: conditions tested using Xtreme gene HP reagent.

Method	Variables:	Tested conditions:
Lipofectamine LTX	• Lipofectamine / Plus reagent ratio	PLUS reagent (µl) | 0 | 0.5 | 1 | 1.5 Lipofectamine (µl): | | | | | 1.5 | A1 | B1 | C1 | D1 3.0 | A2 | B2 | C2 | D2 4.5 | A3 | B3 | C3 | D3 0 | A4 | B4 | C4 | D4
	• plasmid concentration	• 0.1 µg • 1 µg • 10 µg
	• incubation DNA-lipid complexes with cells	• 6 hours • 12 hours
	• post-transfection incubation time with non-selective medium:	• 12 hours • 24 hours • 48 hours
	• incubation time with selective medium: cells were tested for RFP expression (pCAGGS/ pREP9 cotransfection) or luciferase expression (pGV/CISHp/ pREP9 cotransfection) after 24 hours to 2 weeks selection period	
Lipofection with Xtreme gene	• 3 µl Xtreme gene HP agent + 1 µg DNA for 10^6 cells (12 well format) • 6µl Xtreme gene HP agent + 2 µg DNA for 10^6 cells (12 well format)	

4.3.5.2 Electroporation

Electrical transfection methods use electrical impulses to increase the permeability of the cell membrane: the interaction of the external electric field with the lipid dipoles of a pore configuration induces and stabilizes the permeation sites and thus enhances cross membrane transport of (for instance) plasmid DNA molecules, leading to the uptake and expressing of the DNA (Neuman et al., 1982). The method described here makes use of a square wave electroporation system as opposed to an exponential decay electroporation system. These two systems differ from each other in the waveform that is delivered, as illustrated in figure 4.5. An exponential decay wave is a waveform that is delivered (peak) and then exponentially decays. This waveform is ideal when transforming cells with tough cell walls such as bacteria and yeast. With the majority of the current being delivered immediately, the cell wall becomes permeable to allow the molecule of interest to enter. The square wave pulse on the other hand does not peak, but actually looks like a square. This waveform allows a period of homeostasis to be reached in the cells before the wave is removed. As a result, there is a lower mortality rate in cells while maintaining transfection efficiencies. While both waveforms are capable of electroporating bacterial, yeast and mammalian cells, each waveform has its benefits. For mammalian cells, the gentler approach of the square wave electroporation will result in less cell mortality then exponential decay electroporation, rendering this the method of choice.

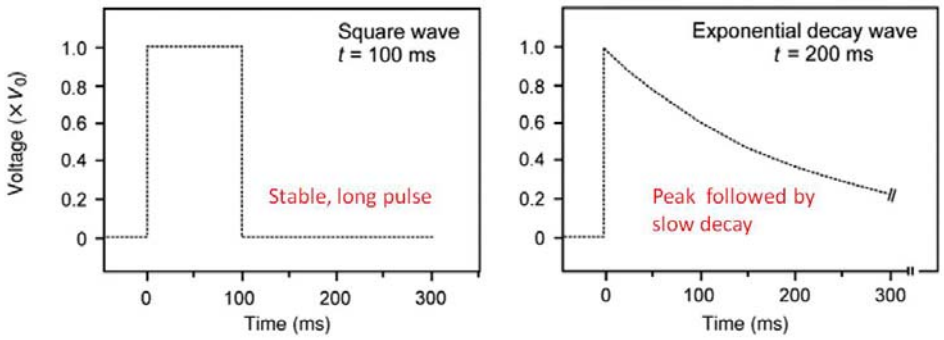


Figure 4.5: Voltage regime applied in different electroporation systems. Left: Square wave electroporation; Right: exponential decay wave electroporation.

The electroporation protocol BTX 410 (BTX Division of Genetronics, PR0410, 2000) for exponential decay wave electroporation of BaF3 cells was converted to square wave electroporation, performed on the ECM 830 Square Wave Electroporation System (BTX Division of Harvard Apparatus, USA). Cells were grown for at least three and maximum 10 passages and plated at $2*10^5$ cells/ml the night before electroporation. Electroporation is performed using an ice-cold BTX Disposable cuvette P/N 640 (4 mm gap, BTX Division of Harvard Apparatus, USA), with 500 µl cell suspension (2*106 cells/ml ice cold PBS) and 50 µg plasmid DNA per electroporation reaction. Charging voltage was optimized to 238 V and one 36 msec pulse was applied (optimization conditions are shown in table 5.5). In the cotransfection experiments, expression plasmid: selection plasmid ratios were 1:1, 3:1, 5:1. These ratio all yielded RFP expressing cells (when using the optimized electroporation conditions), so the highest and most stringent ratio (5:1) was used.

4.4 Results

The whole cell biosensor being developed is based on the recombinant anti-HEL EPOR/gp130 receptor couple on the one hand and on a luciferase reporter system on the other. For proof of principle purposes, a CISHp/luciferase reporter plasmid was used. However, the pGV/CISHp plasmid does not contain a selective marker, implying that the selection of transfected cells must either be accomplished through cotransfection with a selection plasmid which contains an antibiotic resistance gene or that the CISH promoter must be cloned into a different, selectable luciferase reporter plasmid. The latter option is more desirable, as this allows direct selection of the CISHp luciferase reporter plasmid, as opposed to the indirect selection via a cotransfected plasmid. The construction of a selectable CISHp reporter plasmid is described below.

4.4.1 Construction of the pGL4.14/CISHp luciferase expression plasmid

The first step in the construction of the pGL4.14/CISHp plasmid is the isolation of CISHp from the pGV/CISHp plasmid. This was achieved by high fidelity PCR using primers CP1 and CP2, fusing the Hind III and KpnI restriction sites to respectively the 5' and 3' ends of the promoter to allow the promoter to be ligated into the pGL4.14 expression plasmid via these specific restriction sites. Figure 4.6 shows the PCR product and the expected and confirmed sequence.

> CISHp / HindIII / KpnI

GACTGGTACCCCAGATCTCACAGCACACCACCCACCTTCTCACACAGT
GTCCTGCAATAAGCGAGACCACGGCCGCCAGAGACAACGTCCGATAAA
CTAGAAGCGGCTGGCTAAGAGGACCGGCCCGACCCTGCCCCCGCGCTG
TAGTTCTAGGGGGGGGGGGATAAGCGCACCCATCCCCAAAGAAGTAGA
GGGAAGACAATCTGGTCTCCAGTTGCATCCACATTCCTTCTTAACTTG
TCCCCAACCCAGTCTTTTGCCTACTAGTCTCTAGGTCCCGCCCCACTC
GGGCACGTCAGTTCAGGGTCCCTGCACTTCAATAGGTCGGTCTAGATG
CTCGTCTCACGTCCAGCGATACGATTGGTCAACTCTAGGAGCTCCCGC
CCAGTTTTCCTGGAAAGTTCTTGGAAATCTGTCAAAGGTGTTTCCTTT
CTCGGTCCAAAGCACTAGACGCCTGCACCCCCGTTCCCCTCCGGGCCG
CCGCAAAGCCCGCGGTTCTAGGAAGATGAGGCTTCCGGGAAGGGCTGG
GACGCAGCGGACAAAAGATTAGGAGGCGCCTGGCCCCGCCCCACCGGC
CCAGCCCGTCCCCCCTCGTCCTTCCAAGCTGTTCGCACCACAGCCTTT
CAGTCCCTGCTCGCCGCCCGTGTGCCCCGGGACCCTGACCTTCGCACC
CCTGGCACCCATTGGCTCCTTTCTCCTTAAGCTTAGTC

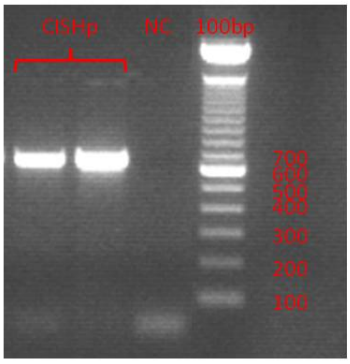


Figure 4.6: CISH promoter sequence with HindIII and KpnI restriction sites and PCR product on agarose gel

This PCR product was Topo cloned into the pCR2.1 amplification vector and used to transform E. coli Top10 cells for long term storage. Sequencing of the resulting clone confirmed the orientation and expected insertion of the promoter sequence. The CISH promoter was then isolated using the restriction enzymes KpnI and HindIII, and ligated into the pGL4.14 luciferase expression plasmid (which also contains a hygromycin resistance gene). Figure 4.7 shows the result of the restriction reactions on the CISHp/pCR2.1 plasmid and pGL4.14.

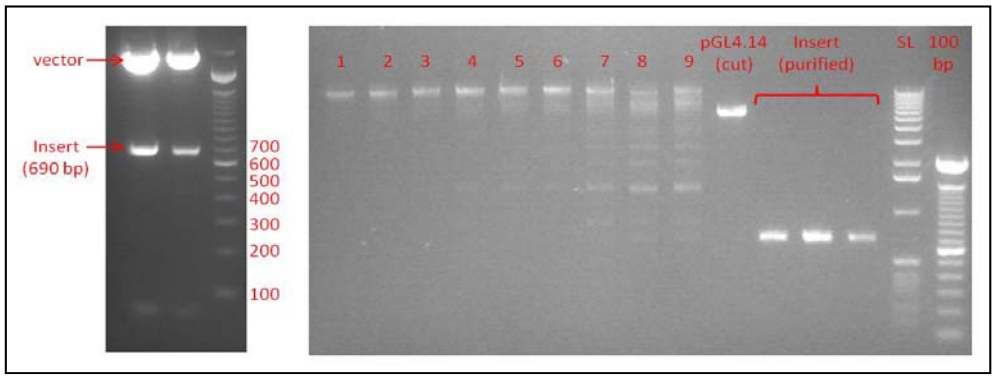


Figure 4.7: Construction of the pGL4.14/CISHp plasmid. Left**:** Isolation of CISHp from the pGV vector using primers CP1 and CP2. Right: Construction of the pGL4.14/CISHp plasmid using insert isolated from three different clone (K3, K4 and K8)and using different insert to vector ratios (insert/vector) and. *Left to right:* 1/1(insertK3); 1/1 (IK4); 1/1 (IK8); 3/1(insertK3); 3/1 (IK4); 3/1 (IK8) ; 10/1(insertK3); 10/1 (IK4); 10/1 (IK8); vector (cut); insert K3; I K4; I K8; kb ladder; 100bp ladder

Ligation products (pGL4.14/CISHp) were transformed into E. coli Top 10 cells and bacteria were plated out on selective plates. The transformation attempts, which are elaborated on in the methods section (§4.3.4) yielded few colonies, which either contained empty vector (colony PCR did not show any band) or plasmid with a mutated insert. As an example, figure 4.8 shows the sequencing output of an insert which seemed to have the correct length upon colony PCR, but was nevertheless mutated: restriction sites are reversed.

As multiple transformation attempts did not yield the desired colonies, pGL4.14/CISHp could not be used for transfection of the Ba/HE+LgIGFP cell line. Cotransfection of the original pGV/CISHp reporter plasmid with pREP9 (which contains the neomycin resistance gene) was opted for as an alternative method.

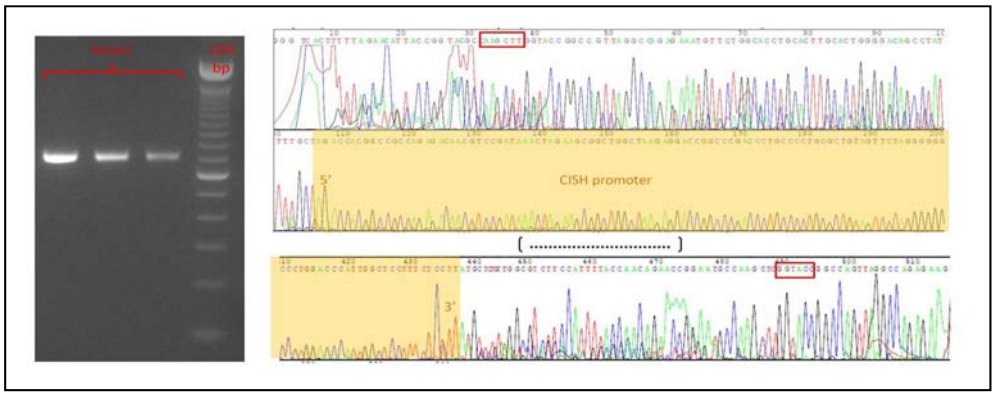


Figure 4.8: Insert isolated form transformed colonies. The agarose gel shows that three colonies contained the plasmid with an insert of the correct length. The sequencing output shows the promoter sequence to be reveresed

4.4.2 Transfection of the pGV/CISHp reporter plasmid

The next step in the development of the whole cell biosensor is the HEL dependent expression of luciferase reporter gene in the Ba/HE+LgIGFP cell line. As described earlier, this requires cotransfection of the pGV/CISHp reporter plasmid with pREP9 (for neomycin selection).

Figure 4.9 reflects the work flow leading to pGV/CISHp transfected Ba/HE+LgIGFP cells. Cotransfection conditions were optimized using the pCAGGS-RFP plasmid, an RFP expression plasmid, with pREP9. In the optimization phase both high voltage and low voltage electroporation protocols were tested, resulting in a successful electroporation protocol for square wave electroporation of Ba/HE+LgIGFP cells. Successful conditions as well as an example of the high voltage protocols tested are given in table 4.5. Cotransfections were then performed for pGV/pCISH + pREP9 in parallel with cotransfections of pCAGGS-RFP + pREP9 for monitoring of the transfection efficiency and the subsequent selection step. Transfected cells were then selected over a two week period and subsequently used in following

experiments. The pGV/CISHp + pREP9 transfected Ba/HE+LgIGFP cells will be referred to as the Ba/C9 cells in the following sections for convenience.

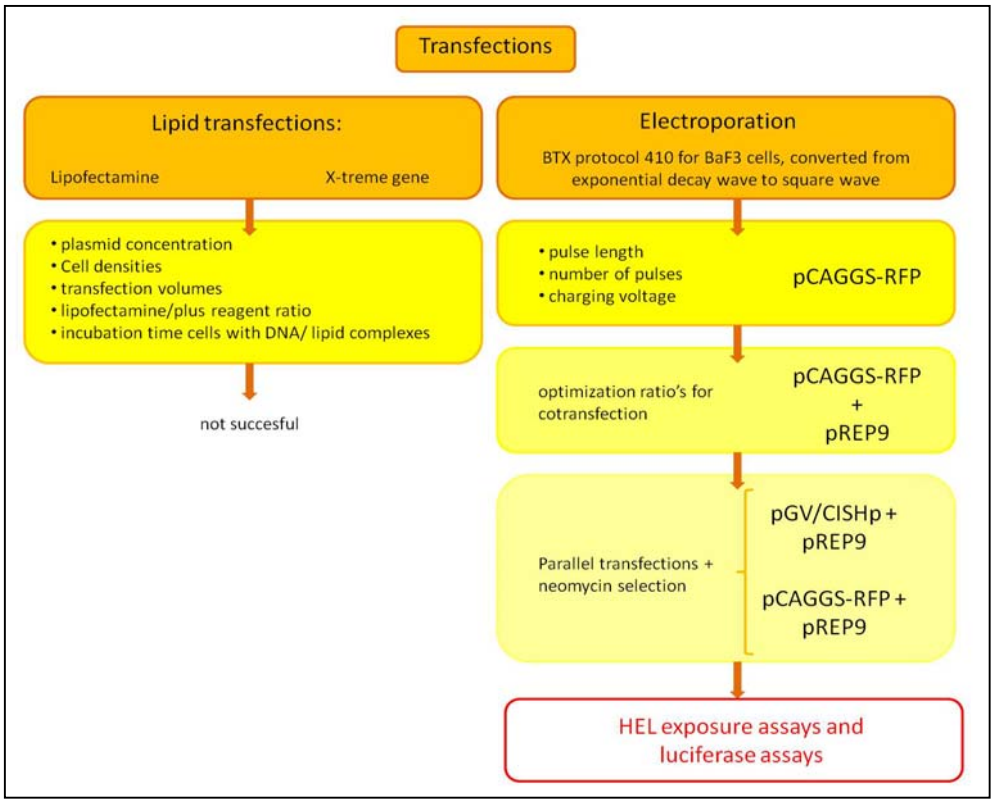


Figure 4.9: Transfection approaches used for transfection of the pGV/CISHp vector and the neomycin selectable pREP9 plasmid.

4.4.3. HEL driven luciferase expression: biosensor proof of principle

The whole cell sensor now contains both the recombinant HEL receptor couple and the CISHp dependent luciferase gene: all necessary components for HEL detection are present. As a proof of principle, the recombinant cells were used in HEL exposure experiments to test the sensitivity and the specificity of the sensor.

Table 4.5: Electroporation of Ba/HE+LgIGFP cells using high voltage (1000 V) or low voltage (238V) methods. The low voltage method (238 V) with one 36 ms pulse is the most efficient. (µs: microseconds, ms: milliseconds)

Charging Voltage	Number of pulses	Pulse length	Transfection efficiency
1000 V[1]	1	45 µs	Few RFP expressing cells
		90 µs	Dead cells
		180 µs	Dead cells
	3	45 µs	Cells are all damaged or dead and do not express RFP
		90 µs	Dead cells
		180 µs	Dead cells
238 V[2]	2	18 ms	Few cells express RFP
	3	18 ms	Dead cells
	1	36 ms	Transfection succesful, RFP expression visible

[1] High voltage protocol based on T820 Electroporation protocol for square wave electroporation of 38B9 Pre-B cells
[2] Low voltage protocol converted from BTX protocol 410 (BTX Division of Genetronics, PR0410, 2000) for exponential decay wave electroporation of BaF3 cells

Ba/C9 cells were deprived of HEL for 24 hours (see §4.4.1) and then exposed to HEL or to physiological concentrations of the cytokines investigated for cross-talk activity. Subsequently, cell lysates were tested for luciferase activity. The results of the various assays are presented in figure 4.10 A, B and C.
The top graph (A) shows the luminescence induced by 10 µg/ml HEL exposure for 2, 4 or 6 hours. The luminescence is higher for the exposed cells as opposed to the HEL deprived cells, also no significant difference is apparent between exposure times, all induce an increase of approximately 30% (t-test confidence interval (c.i.) of 93 to 94%). Therefore, the smallest exposure time of 2 hours was selected for further experiments. Graph (B) shows luminescence induced by 2 hours exposure to a dilution series of HEL, based on the concentrations which induce either proliferation or anti-apoptotic effects in Ba/HE+LgIGFP cells (see §3.1.2.4). A 45 to 50% increase in luciferase induction is observed upon exposure to 10 µg/ml or higher (c.i. 90%), however HEL concentrations below 10 µg/ml did not induce a significant effect and no concentration dependent effect is apparent.

Finally, cytokine driven cross-talk was investigated (see figure 4.10 C). IL3 is the only cytokine which induces a significant increase in luminescence: luciferase

activity is increased by 30% (c.i. 90%). The increase in luciferase expression induced by HEL is also confirmed in this experiment (a 25% increase was observed, c.i. 90%).

All three graphs show a high background luminescence from the unstimulated transfected cells, indicating a rather high promoter leakage (background luminescence from untransfected cells was subtracted).

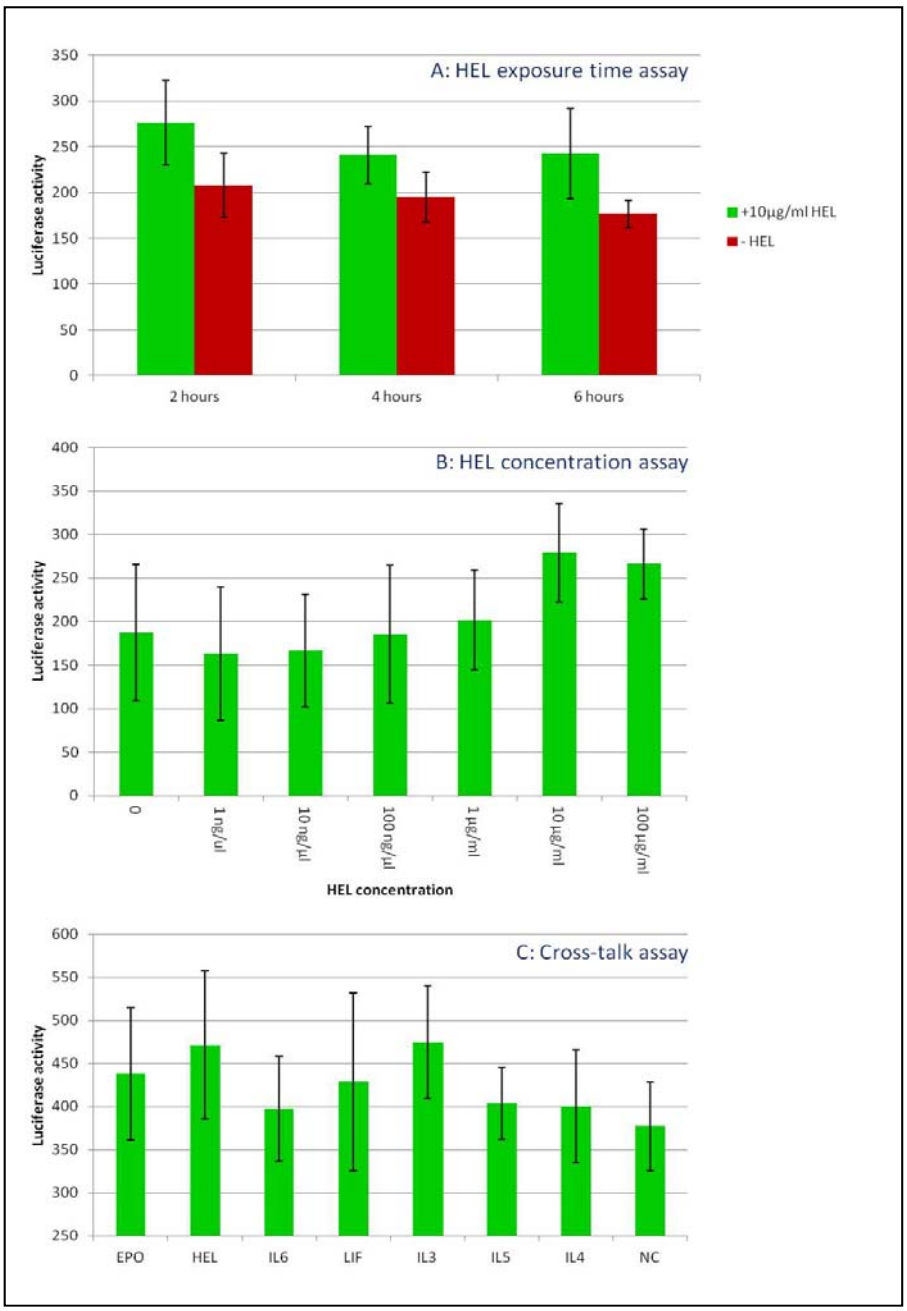


Figure 4.10: Luciferase expression profiles of HEL exposure experiments and cross-talk expertiments. (A) Exposure of BaC9 cells to 10 µg/ml HEL for 2, 4 or 6 hours, (B) exposure to 1 ng/ml to 100 µg/ml HEL, (C) exposure to cytokines (EPO: erythropoietin; HEL: Hen egg lysozyme; IL 6, 3, 5 and 4: interleukin 6, 3, 5 and 4; LIF: leukemia inhibitory factor; NC: negative control), at physiological concentrations (stated in table 4.4).

4.5 Discussion

Whole cell sensors typically express a receptor, aimed at the target of interest, combined with a reporter gene such as luciferase which is expressed upon activation of a specific promoter. In this chapter, the Ba/HE+LgIGFP cell line expressing the recombinant anti-HEL EPOR/gp130 receptor was transiently transfected with a reporter gene plasmid for the expression of luciferase via the CISH promoter. The CISH promoter was inserted in the selectable luciferase reporter plasmid pGL4.14. However, pGL4.14/CISHp could not be transformed into bacteria for amplification. To establish a proof of principle, the non-selectable pGV/CISHp plasmid was cotransfected into the Ba/HE+LgIGFP cell line together with the selection plasmid pREP9. This allowed the whole cell biosensor based on a recombinant anti-HEL receptor couple and on a CISHp controlled reporter system to be evaluated.

4.5.1 Construction pGL4.14/CISHp

According to the results presented in §4.4.1 and §4.4.2, the amplification of the CISH promoter and the subsequent restriction reaction and ligation into pGL4.14 were successful. However the resulting plasmid could not be transformed into E. coli bacteria despite the wide range of conditions and the different techniques (see materials and methods) which were tested. Different approaches to transformation, including both chemical and electrical methods as well as a wide range of conditions only yielded colonies containing empty vector or vector with mutated insert plasmid. Possibly, the combination of the insert with the pGL4.14 plasmid leads to a growth disadvantage or even toxicity in E. coli bacteria, selecting out the plasmid containing the correctly inserted insert in favour of empty vectors and artifacts. To investigate this, the insert sequence was run through BLAST, but no similarities could be found with known toxic sequences. To test the suitability of the CISH promoter in the biosensor setting, a different approach was then used, cotransfecting the original non-selectable plasmid with a selection plasmid pREP9.

4.5.2 Evaluation of the anti-HEL CISHp mediated biosensor

The Ba/HE+LgIGFP cell line was cotransfected with pGV/CISHp and pREP9, and the resulting cells were used in exposure assays to establish the effects on luciferase expression in the cells. Initial assays where cells were exposed to a high HEL concentration (10 µg/ml) for two to six hours demonstrated that while the luciferase expression is significantly enhanced, there was no difference in luciferase expression observed between the exposure times. This implies that the shortest incubation time of two hours is sufficient for sensing and that the whole cell biosensor works fast, as opposed to some cell based sensors which require up to 48 hours before the signal is read out.

Exposure of the cells to a HEL dilution series showed a higher induction of luciferase at a concentration of 10 µg/ml or more, however no concentration dependent effect is observed, indicating that the dynamic range of the sensor is limited. Cross-talk experiments demonstrated that the cells' natural growth factor IL3 induced luciferase expression at the physiological concentration of 50 ng/µl. This implies that the IL3R, which is naturally expressed in BaF3 cells is much more sensitive than the recombinant HEL receptor couple, a phenomenon which may be explained by the affinity of the antibody which was used to construct the recombinant receptor couple, or possibly by the receptor structure. The antibody which was used for the construction of the recombinant receptor couple is the HyHEL-10 antibody (Kawahara et al. 2003), which has an affinity of 3.3 X 10^9 M^{-1} to 4.0 X 10^9 M^{-1} (Padlan et al, 1989, Li et al. 1996) and is considered a high affinity antibody. This indicates that a reduced signaling is not directly linked to an insufficient affinity for HEL, however the possibility that the incorporation of the antibody recognition domains in the receptor changes its affinity must also be considered.

The structure of the receptor is possibly a more likely candidate for the improvement of the biosensor sensitivity. The background luciferase activity is quite high, which is confirmed by Kawahara and colleagues. They showed that the background growth signal in unliganded chimeric EPO receptors may be partly due to the enforced substitution of EpoR D1 domain to the antibody variable regions. This effect could be reversed by mutations in the

transmembrane (TM) domain, which leads to a stricter cell growth switch (Kawahara et al. 2004).

Aside from causes linked to the sensor signaling, the low sensitivity of the biosensor could be linked to the cotransfection efficiency, which may not be as high as desired, leading to less pGV/CISHp positive cells and consequently to less luciferase expression. The use of cotransfection instead of the transfection of a single, selectable luciferase plasmid as was originally aimed for (see above, §4.5.1) means that selection of the luciferase expression plasmid is an indirect selection, dependent on the ratio of pREP9 and pGV/CISHp. Though the parallel cotransfections with pREP9 and pCAGGS-RFP indicate that this ratio was correct and that cotransfection was therefore successful, it remains possible that transfection of the pGV/CISHp plasmid was not as efficient as the pCAGGS-RFP plasmid, leading to lower luminescence. The use of a different selectable reporter plasmid should be investigated in the future to decisively answer this question.

Finally, the choice of promoter also influences the sensitivity of the biosensor. In this chapter, CISHp was incorporated in the whole cell biosensor, however other promoters such as the SOCS1p and SOCS3p may perform differently and better.

4.6 Conclusion and Future Prospects

We aimed to develop a novel whole cell sensor capable of sensing low concentrations of target, with an interchangeable antibody based receptor for versatile target recognition. In this chapter, the reporter system was developed based on the anti-HEL EPOR/gp130 induced CISH promoter. This biosensor principle was investigated using Ba/HE+LgIGFP cells cotransfected with a pGV/CISHp luciferase plasmid and pREP9 neomycin selection plasmid. Results indicate that this whole cell biosensor detected HEL concentrations of 10 µg/ml or more, however the observed expression profiles suggest a limited dynamic range and the biosensor sensitivity did not reach the low concentrations which were aimed for.
Possible modifications which could improve the sensitivity of the sensor include the incorporation of mutations in the transmembrane domain of the recombinant receptor, thus lowering background signal, or the use of alternative promoters such as SOCS1 and SOCS3. The use of another antibody may also have an effect, though the HyHEL antibody which was used in the recombinant receptor construction is a high affinity antibody (Padlan et al, 1989, Li et al. 1996), suggesting that this is a less promising alternative. Finally, a different selectable reporter plasmid should be tested to rule out possible problems with selection of cells through cotransfection.

The use of recombinant cytokine receptors in a whole cell biosensor remains an interesting area of research, however our results show that a lot of investigation remains to be done to explore all the possibilities of these types of sensors. The low concentrations of 10 ng/ml and even 1 ng/ml HEL which were shown to induce cell proliferation or at least anti-apoptotic signals, imply that it should remain possible to detect lower concentrations and this research deserves to be continued.

CHAPTER 5

BIOSENSORS IN WASTE TOXICITY SCREENING: SELECTION OF METHODS

Based on the publication:

SCREENING TESTS FOR HAZARD CLASSIFICATION OF COMPLEX WASTE MATERIALS - SELECTION OF METHODS

Weltens R., Vanermen G., Tirez K., Robbens J., Deprez K., Michiels L.
Waste Management Journal. 2012 Dec;32(12):2208-17

The own contribution includes literature study of methods (CALUX, Aptamers, MIP's) and participation in projectmeetings leading tot the proposed strategy and conclusions

Chapter 5:
Biosensors in waste toxicity screening: selection of methods

5.1 Introduction

The hazardous waste directive (HWD, Council Directive 91/689/EC) provides a framework for the classification of waste. Waste is classified by its hazardous properties as defined in the HWD-Hazard (H)-properties: physical (H1 explosive, H2 oxidising, H3 flammable) and toxicological hazardous criteria (H4 Irritant, H5/6 harmful or toxic, H7 carcinogenic, H8 corrosive,...) (table 6.1). These properties can be attributed to individual waste compounds, but for complex waste with unpredictable composition, the hazardous properties should be measured directly on (extracts of) the waste material (as recommended by HWD). The recommended methods in the HWD for the evaluation of toxicological and ecotoxicological properties are those used for the hazard assessment of chemicals (Council Directive 67/548/EC). However these involve mammalian testing which is not acceptable from an ethical point of view for hazard assessment of waste and is also not feasible from an economical point of view.

The application of many of the tests proposed in the following section is new in the field of waste management. Below, the principles of the available tests are described. The selected (bio) tests were performed on different types of waste material with good results in a pilot study, to evaluate their performance in a waste testing strategy. The results of these tests are presented in chapter 5.

5.2 Extraction methods

HWD limit values are based on *total* concentrations of compounds. The extraction methods therefore have to provide liquids that reflect as much as possible the total content of components that were present in the original sample. For practical reasons it is necessary to provide a universal extraction method to displace as many of the pollutants from the original (solid) waste into a liquid matrix that can be used for both chemical analyses and biological tests.

To achieve this two extraction methods in parallel are recommended: an aquatic extraction to retain the inorganic and ionic organic leachable components and an acetone extraction to retain (most of) the organic components.

5.2.1 Water leachable fraction:

In support of the Directive 91/689/EEC on hazardous waste, CEN, the European Committee for Standardization, has set up Technical Committee 292 for the "characterization of waste". CEN TC 292 issued several procedures to determine the characteristics of waste and waste behaviour, as sampling, pre-treatment, leaching properties, determination of total content of species, determination of sum parameters and assessment of ecotoxicity. For the preparation of test portions and water leachable fraction methods described in EN 15002:2006 and EN 12457-4:2002 are referred to.

5.2.2 Organic extract:

Acetone is both water soluble and dissolves organic components. It is able to remove also compounds out of porous materials. Aceton is therefore suitable as a worst case extraction solvent.

5.3 Targeted analyses

The most straightforward method to characterize and classify waste is to identify and measure the concentration of hazardous chemical components in waste directly and compare their concentrations to the limit values (HWD). For samples with known toxic components analytical methods should be used to measure their concentrations. For samples of unknown composition screening methods are needed to unravel their composition and/or their hazardous properties.

5.3.1 Targeted chemical analyses of inorganics

CEN TC 292 (in support of the Directive 91/689/EEC on hazardous waste) issued several procedures to determine the characteristics of waste. For inorganic characterization of waste a framework was already designed by CEN TC 292. The applied standardized methods for the chemical characterization of the inorganic species are listed in table 5.1.

Table 5.1: Applied standardized methods for the characterization of the inorganic parameters in the waste samples

Parameter	Method
pH	EN 12506:2003 Characterization of waste - Analysis of eluates - Determination of pH, As, Ba, Cd, Cl-, Co, Cr, Cr VI, Cu, Mo, Ni, NO2-, Pb, total S, SO42-, V and Zn
Hg	EPA 7473 Mercury in solids and solutions by thermal decomposition amalgamation and atomic absorption spectrometry
ammonium, cyanide	EN 13370:2003 Characterization of waste - Analysis of eluates - Determination of Ammonium, AOX, conductivity, Hg, phenol index, TOC, easily liberatable CN-, F-
Chromium VI	EN 15192:2006 Characterisation of waste and soil - Determination of Chromium(VI) in solid material by alkaline digestion and ion chromatography with spectrophotometric detection
Elemental composition	EN 15309:2007 Characterization of waste and soil - Determination of elemental composition by X-ray fluorescence
C, H, N, S	/TS 15407:2006 Solid recovered fuels - Method for the determination of carbon (C), hydrogen (H) and nitrogen (N) content

Most of these analytical methods measure individual elements or species such as anions (e.g. sulphate, chloride) and cations (e.g. metals). This complicates hazard classification, because different speciations of the same element can show very different toxic properties and it is difficult to link the analytical results to the limit values for toxic chemicals, as HWD requires.

The chemical methods for analyses of the inorganic fraction are very practical and fast, and suitable for batch analyses. HWD has to provide guidance on how to deal with the analytical information in terms of toxicity, and results can be used for hazard classification of the inorganic fraction.

5.3.2 Targeted chemical analyses of organics

Liquid or Gas chromatography (LC/GC) and Mass spectrometry (MS)), **Infrared Spectroscopy,** Ion Mobility Spectroscopy (IMS) can be used to screen for a wide variety of organic chemicals.

GC/MS is the best method for substance identification, but also has its limitations: coelution can complicate test results and not all compounds can be identified. LC/MS spectra are not library searchable and cannot be used for general screening. With the more advanced LC-amTOF-MS there is a possibility to obtain the molecular formulas of compounds, but a laborious study of isotope distributions and fragmentation patterns is necessary.

Complex waste samples can contain many hundreds of individual compounds. Figure 5.1 shows an example of a GC/MS spectrum of a waste material (sludge of a waste water treatment) where 396 peaks are present, however only 80 could be identified. Chemical analyses for complex samples is not useful because classification is hampered by the presence of many unidentified compounds and unpredictable mixture toxicity.

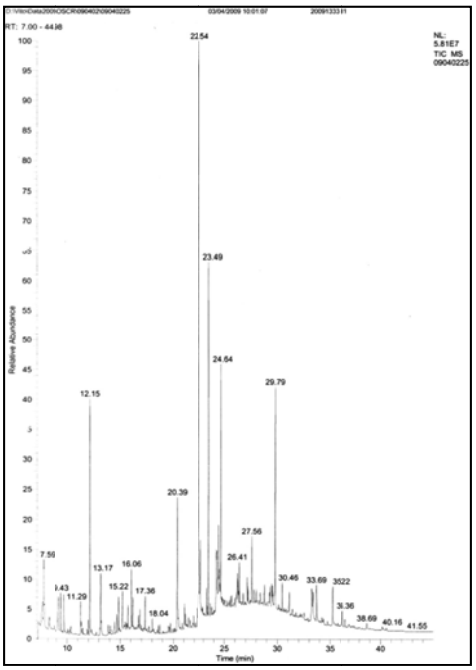


Figure 5.1: Example of a typical GC/MS spectrum of a complex waste sample

It is concluded that chemical methods for organics fail to distinguish hazardous from non-hazardous waste in case of complex materials. Only in case the source of waste indicates which specific hazardous compounds can be expected, targeted organic analysis is useful and several methods are available, including GC/MS, LC/MS and for some target compounds also specific test kits are available.

In general chemical methods are valuable for targeted analyses, because they are fast and specific, sensitive and easy to interpret. For an integrated evaluation of the hazardous properties of complex samples however they have important shortcomings such as unknown speciation (toxicity) of the inorganic compounds, the presence of unidentified organic compounds, and unpredictable combined toxicity of all the chemicals present in the sample.
In the pilot project the inorganic chemical parameters (heavy metals, anions) and pH and conductivity are measured in the aquatic leachable fraction, and GC/MS screening is performed on the organic extracts.

5.3.3 Targeted biological analyses

Biological targeted methods are available that might overcome some of the shortcomings of chemical analyses. They are based on bio-recognition: i.e. there is a match/affinity between the biological test system and specific (xenobiotic) ligand molecules or a part of the molecule (i.e. topical structure that is recognized by the biological component). In mixtures they will bind very specifically to their target. The biological binding between target and receptor can be translated into an easily measurable signal. Biorecognition sensors present the advantages of sensitivity and selectivity inherent to the use of immunochemical interactions (reviewed in Marquette and Blum, 2006). Limitations are the challenges originating from the regeneration of the immunosurface and cross-reactivity (Marquette and Blum, 2006).
Biorecognition instruments are widely available. Biomolecules capable of recognizing specific target structures are: Antibodies, Phages, Aptamers, DNA, RNA, (Cell) Receptors, Plastic antibodies: MIP (Molecularly Imprinted Polymers: see below).

For the purpose of hazard assessment recognition bioassays are useful when their target molecular structures are linked to toxic mechanisms. Experimental observation has led to the identification of several structural alerts that show mutagenic toxicity and can cause cancer (alkyl-, aryl- and benzyliccarbonium ions, nitrenium ions, epoxides, aldehydes…), or alerts associated with developmental toxicity (valproic acid, hydrazides, carbamates) (NRC, 2007).

This science involved in the prediction of biological activity from physical and chemical properties of molecules is called Structure Activity Relationship (SAR) and this will lead to further development of bio-recognition applications.

5.3.3.1 Enzyme Linked ImmunoSorbent Assay (ELISA)

ELISA is a well-known biochemical technique used to detect the presence of an antibody or an antigen in a sample. ELISA kits are commercially available for many substances and are often seen as a tool for chemical analyses. Some examples:

- PCB and coplanar PCBs (carcinogenic) (Laschi et al. , 2000; O'Neill et al.,2004)
- Pesticides (Vamvakaki et al., 2007; Cagnini et al., 1995; biosensor 2005; Hernandez et al., 2000)

Several other single endpoint biosensors are reported in literature. The Ah-IMMUNOASSAY® f.i. is used for screening for dioxin-like toxicity in environmental samples (e.g., soil, fly ash), and in biological samples (e.g. sera, tissue, food).

Antibody based assays have limited applicability. Antibodies are expensive: they have to be produced in living animals and only a restricted quantity can be produced at one time. Moreover the stability of the antibodies is not optimal in complex matrices where they can be destroyed by aggressive or interfering molecules. Often the antibody-based tests are not suitable for fast screening purposes due to complexity and test duration.

Promising biorecognition techniques are phage display, MIP (*Molecular Imprinted Polymers)* and aptamers: they are cheaper, easier to produce and their specificity is similar to antibody specificity.

5.3.3.2 Phage display (Pandea et al., 2010)

Filamentous bacteriophages (M13, fd...) are rather simple structures which consist of a protein coat that surrounds the phage genome. This protein coat is actually the phages membrane and consists of a number of major and minor proteins. It is possible to display a protein or peptide on one of these membrane proteins. The high phage titer (= number of different phages/ml) enables the display of a large number of different proteins on the phage membrane. This is ideal to display libraries - peptide, DNA or antibody libraries - on these phages. From these phage libraries those that interact specifically with a chosen ligand can be selected via "biopanning" (Jyoti et al., 2010).

No commercial applications are available yet, but in research programs the method is already used in combination with analytical methods such as Quartz Crystal Microbalance (QCM) and ELISA, as well as optical and electrochemical methods for detection of specific targets (Mao et al., 2009).
Although much cheaper, phages are - like antibodies – not inert enough for use in complex matrices as they are sensitive to the presence of disrupting compounds.

5.3.3.3 Aptamers

Aptamers are nucleic acid species and are more stable than phages, exhibiting a very high shelf-life. *Aptamers* can be selected with high affinities and specificities for their targets. The affinities are often comparable to those observed for antibodies. After selection, they can be produced by chemical synthesis with high accuracy and reproducibility. Denatured aptamers can be regenerated easily within minutes, which is important for many (high throughput) applications.
Although high affinity aptamers are not as widely available as antibodies at the moment, these molecules are very promising for future commercial biosensing applications (Stoltenburg et al. 2007).

5.3.3.4 Molecular Imprinted Polymers (MIP)

This technique leads to highly stable synthetic polymers that possess selective molecular recognition properties because of recognition sites within the polymeric matrix. Some of these polymers have high selectivity and affinity constants, comparable with naturally occurring recognition systems such as monoclonal antibodies or receptors (XU et al., 2011).

The technique of molecular imprinting allows the formation of specific recognition and catalytic sites in macromolecules by the use of templates. They are used in an increasing number of applications.

No commercial assays are available yet for detection of toxicants, but they are very promising tools in human and environmental diagnostics.

5.3.3.5 Surface Plasma Resonance (SPR) applications

SPR techniques allow the investigation of interactions of chemicals with small peptides to multiple sub-unit protein complexes (Abdlulahim et al., 2008). The extent to which different molecules interact with a single partner, immobilized on a sensor surface, reveals the specificity of the interaction. The system gives both simple yes/no answers**,** which may be interesting for a wide variety of highly toxic compounds, but also the concentration can be determined. The method can be used both for purified molecules and for molecules in complex mixtures. Results are achieved very fast (minutes). Most of the existing immunoassays, and cellular biorecognition assays, can be translated onto a plasma resonance platform, and also receptors and antibodies can be engineered for the construction of such biosensors (Hock et al, 2002).
Some examples of available SPR applications are sensors for endocrine disruptors (Rodriguez-Mozaz & Barcelo, 2004; Samsonova et al., 2004)), and for DDT and related compounds (Mauriz et al., 2007).

Although very fast and useful, and the availability of commercial applications, up to now no high throughput applications for environmental analyses are available yet. Matrix effects of environmental samples might be a problem. The method is expensive and has mainly been used for research of molecular interactions.

Recognition assays for the detection of hazardous compounds in mixtures seem a valuable tool: they are toxicologically relevant, fast and suitable for high throughput and lab on chips applications.

Within this group of assays the more robust applications such as aptamers and MIPs are the most suitable for complex matrices like waste. Up to now however no applications for hazard assessment of waste or for environmental diagnostics are available.

Like analytical methods, the biological affinity assays are useful for targeted analyses in the matrix, but also – as is the case for the biological in vitro methods described below - they are suitable for screening purposes for groups of toxic compounds with the same biological effects in mixtures.

5.4 Non targeted comprehensive analyses

Contrary to the targeted analyses - where the analyst knows exactly which compounds or group of compounds has to be analyzed – is the non-targeted comprehensive analysis where the analyst is not looking for individual compounds but for characteristics of the mixture. This is the case when exposing biological test systems to the test solutions: they will react to the combination of all compounds and their mutual biological impact. Without identifying the culprit compounds the test results are a measure for the "total hazardous content" of the sample.

5.4.1 In vitro cell based and effect based bioassays for general and mechanistic toxicity

Increased understanding of cellular pathways and cellular response mechanisms to specific hazardous compounds have led to the development of cellular biotests that enable hazard screening. This approach is put forward as *the* toxicology approach for the twenty-first century in a report by the US National Academy of Sciences on behalf of the Environmental Protection Agency (EPA) (NRC, 2007). This has already led to the formation of a revised toxicity testing strategy by the EPA with the EPA's ToxCast program being closest in terms of vision to the new process required (Hartung, 2009). ToxCast™ is profiling over 300 well-characterized chemicals in over 400 endpoints. Also within the REACH framework the use of alternative tests is promoted for toxicity screening to reduce animal testing (Poth & Jaeger, 2007).

In vitro bioassays are cell based - effect based biotests. The endpoints presented here include biochemical assays of protein function, transcriptional reporter assays, multi-cell interaction assays, transcriptomics on primary cell cultures, and developmental assays in zebra fish embryos.

A detailed review of mechanistic toxicology can be found in Boelsterli et al. (2007) and an extensive list of possible bioassays is available (see reference list). Here we focus on tests and methods that fit in the HWD Hazard-categories.

5.4.1.1 Bioassays for general toxicity (H5/H6)

General toxicity is defined as the measurement of acute toxic effects that kill or inhibit the biological test system, without looking at the underlying mechanism. All biological test systems are suitable for evaluation of general toxicity, because increasing concentrations will eventually kill the biosystem.

5.4.1.1.1 Cytotoxicity tests

In cytotoxicity tests cell cultures are exposed to the samples at different concentrations. When toxic compounds are present in the samples the cells will increasingly be affected at higher concentrations. Cytotoxicity tests can be very diverse: many cell types can be used and many endpoints can be measured to reflect the health condition of the cells.

Cytotoxicity tests are widely used in pharmacology to predict general toxicity and results are used as a prediction for LD50 values. The data sets comparing in vitro and in vivo results confirm the relevance of the cytotoxicity test for predicting acute toxicity as a first step in toxicity evaluation (Eisenbrand et al., 2002).

When using mammalian cell lines the exposure time needed to provoke the cellular effects is often relatively long. Physiological parameters in bacteria are therefore often used as an alternative: due to their short generation time they respond much faster than mammalian cell systems.

5.4.1.1.2 Bioluminescent bacterial toxicity test

Prokaryotic systems in general can indeed be considered as faster and cheaper than eukaryotic test systems. Often toxicological mechanisms are similar for both systems and therefore prokaryotic biosensors can also be used to predict effects on higher systems.

The bioluminescent bacterial toxicity test is one of the most widely used bioassays. A decrease of a measurable (light) signal of autoluminescent bacteria induced by the inhibition of the metabolism of the bacterial cell is an indication for general toxicity. The bacterial species can be either wild type or transgenic.

It has been demonstrated that there is a significant relationship between data from the bacterial toxicity test Microtox® and rodent LC50 values (Kaiser et al., 1994).
The measured effect can be considered as equivalent to cytotoxicity information of eukaryotic cells, but the response time is much shorter (hours instead of days), due to the short generation time.
Bacterial bioassay methods are commercially available, often as test kit with automated signal transducer and software for toxicity evaluation. They provide a solid and fast method for the evaluation of general toxicity and are also suitable for environmental applications.

Both cytotoxicity and bacterial assays are highly relevant as they measure the combined toxicity of all the toxic compounds present in a complex sample. They were both selected for the pilot study. Bacterial assays are more robust than eukaryotic systems and require much shorter exposure times. An automated, standardized and well validated bacterial test is therefore a good candidate to be used as an initial screening test for triage that enables the recognition of highly toxic samples already in a first stage of the test strategy.

5.4.1.2 Bioassays for carcinogens and mutagens

Mutations are changes in the constitution of the DNA, thereby affecting one or more cellular functions. They can affect one or more genes (gene mutation), entire parts of chromosomes (chromosome mutation) or even loss or gain of entire chromosomes (genomic mutations). When mutations take place in egg- or sperm cells, or at an early stage of development of the foetus, the mutation can lead to abnormalities in the offspring.

Mutations are an important mechanism in the development of cancer (carcinogenesis). Many mutations in the same cell are however needed before these cells turn into cancer cells Therefore mutation frequency is the most prominent parameter in increasing cancer incidence.

To evaluate genotoxicity, tests have to be combined that evaluate both the potential to induce gene and genomic mutations. Many *in vitro* tests are well known and validated tests could be useful for waste characterization.

- Ames test: bacterial test, measuring an increase in mutation frequency when exposed to genotoxic components. The Ames test is widely used and well validated for environmental applications. However the test duration is 48 hours.
- Comet assay: different cells can be used, measuring DNA fragmentation in response to exposure to genotoxic substances. However the test is elaborate and not validated for complex samples.
- Cytogenicity tests for chromosome and genome mutations: Chromosome Aberration test, Sister Chromatid Exchange test, micronucleus test. These tests require an exposure period of 48 hours and elaborate analyses, although automated image analyzing systems are available. The micronucleus test kit seems promising (Cellomics). This test is shorter (24 hours) and easier to perform, but needs an expensive infrastructure.
- There are also reporter gene assays for DNA damage.
  Promoters induced by the SOS response (response in reaction to DNA damage) and fused to a reporter gene are used to construct genotoxicity sensors (recA, sulA, umuCD, recN...). These systems - mostly in E. coli, some in Salmonella – are fast (in the range of hours), allow high throughput screening and are cost effective. Their sensitivity is often lower than the Ames test, and they are less robust. They are not commonly used for complex samples.

As yet no affinity bioassays for genotoxic substances are commercially available. NRC (2007) reports several structural alerts for molecules that are indicative for their potential to cause mutations and/or cancer (alkyl, aryl and benzylliccarbonium ions, nitrenium ions, epoxides, oxonium ions, aldahydes, polarized double bonds (alpha and beta unsaturated carbonyls or carboxylates, perosxides, free radicals, acylating intermediates).

For the present purpose of fast hazard identification of waste materials no suitable methods for the evaluation of genotoxic properties within the desired time range are available yet. The Ames test was selected in this project, but faster methods like affinity assays or more robust and sensitive gene reporter constructs for carcinogenics would be useful. Also bacterial assays with reporter gene for SOS response was selected for the pilot study (VITOTOX and BGPA: (see below)).

5.4.1.3 Biotests for reprotoxicity

The reproduction process is dependent upon 3 sub-elements that can be affected by xenobiotics (Reprotect project, EU Integrated Program 2004-2009): Fertility, Implantation of the embryo and embryo toxicity.

5.4.1.3.1 Biotests for fertility (hormonal disturbances)

Hormones exert their effect by receptor binding. Hormonal disturbance is caused by components that can compete for these hormone receptors, due to a common topical structure. As will be described below also direct assays have been developed based on recognition of these structures, but they are not commercially available yet.

The aim of the EU project MENDOS (2003-2007) was to develop a test battery for the detection of endocrine disrupting compounds in environmental samples. Different fields were explored: artificial receptor based optical sensor systems, SPR platform, MIPs, cell based assays... SPR application could not be fully developed but Molecularly imprinted polymers (MIPs) for 17 β- estradiol, benzo(a)pyrene, diethylhexylphtalate and atrazine, and antibodies against atrazine, dichloropheoxyacetic acid, BAP, 4-nonylphenol became available .
Also an aromatase whole cell assay for steroid activity was developed. A system for assessing the estrogenic activity of a sample *in situ* was established based on immobilized chemo-sensitive luminescent yeast cells. This hydrogel assay only takes 2.5 hours assay time. The method is however not commercially available yet;
Finally within this project DNA chips holding DNA probes for hormone responsive genes were developed and successfully used with human cells for (anti)

androgenic action, and estrogenic compounds. These developments are very promising and will be evaluated for waste assessment purposes when available.

Also other cellular effect assays for hormonal disturbance have been developed and are widely used. The tests described below are potentially of interest for waste assessment.

Cell Proliferation Experiments: MCF7 human breast cancer cells have been studied extensively as a model for hormonal effects on breast cancer cell-growth and specific protein synthesis. Because the proliferative effect of estrogen is considered to be the hallmark of estrogen action, it was proposed that this property to be used to determine estrogenic potency.

Genetically modified cell systems based on estrogen or androgen receptor binding: specific toxic pathways are selectively activated by specific toxic compounds. These cellular reactions can be measured when incorporating reporter genes in the cell's genome, which are under transcriptional control of the genes that are involved in the onset of the biological reaction.
Many test systems have been developed for detection of hormonal compounds (MELN test, YES assay and ER-Calux for estrogen activity, PALM, YAR assay and AR Calux for androgen activity). As Calux methods were available in the lab they were used for the pilot study. They use human cells and are very relevant for hazard assessment. The exposure time for full assays is usually 24 hours, though optimized assays can even be analyzed after 4 to 6 hours exposure. This shorter exposure time makes Calux a good candidate for waste hazard assessment.

5.4.1.3.2 Bioassays for Implantation (endometrium and placental toxicity)

No *in vitro* tests are available that can simulate the effect of xenobiotics on implantation of the embryo.

5.4.1.3.3 Bioassays for Prenatal development (embryo toxicity)

No *in vitro* tests are available, but promising results have been obtained in short term *in vivo* tests on the development of fish (48 hours ELS (early life stage assay)) and amphibian larvae (96 hours FETAX) as an indicator for teratogenesis.

EST (embryonic stem cell test), limb bud micromass culture and whole embryo culture are very promising *in vitro* cellular test for screening embryo toxicity (Reprotect project), but the duration is far too long for fast screening purposes.

Ongoing research in the field of gene expression involved in fetal development might lead to possible assays for early detection of teratogenic properties.

NRC (2007) listed 17 primary and intercellular signaling pathways that are known to be involved in normal developmental toxicology. Ongoing research is focusing on gene expression during normal development. These findings might lead to new effect bioassays for developmental toxicology.

Structure-Activity-Relation (SAR) - methodology has revealed some structural alerts (NRC, 2007) that might be useful for the construction of recognition bioassay for teratogenic compounds.

Also, within Toxcast, an American research program with the purpose of developing a testing strategy for chemicals (Dix et al., 2007), new tests and computational models for teratogenic effects are being developed. A computational model for blood vessel development was explored (Kleinstreuer et al., 2013). However, though the latter is very promising for risk assessment of chemicals, the model is based on data from 600 high throughput screening (HTS) assays, including biochemical assays (e.g., nuclear receptor binding, enzyme inhibition), cell based assays (e.g., cytotoxicity profiles, reporter gene assays), complex culture systems (e.g., embryonic stem cell differentiation, inflammatory/angiogenic signals), and chemical property information. While this may lead to a very accurate analysis of the chemicals under investigation, this is not feasible for complex waste samples and also surpasses the aim of waste classification.

At present no optimal methods for the overall evaluation of reproductive impairment within the desired time range are available. Many tests are available for the detection of hormone disturbing compounds, and also alternative developmental tests on fish and amphibians are available as a model for teratogenic effects. Fast screening tests are not for all relative endpoints involved in reproductive toxicology available. Based on this overview and the a priori requirements we selected ELS, CALUX and YES for the pilot study. Tests for early signs of developmental disturbance or affinity assays for teratogenic compounds are promising but still under development.

5.4.1.4 Bioassays for irritating, corrosive and sensitizing properties (H4, H8 and H13)

In this field many biotests have already been developed thanks to the urgent need for alternative tests in cosmetic industries where a complete ban on the use of animals for testing cosmetics will be a fact.

To evaluate the corrosive, irritating or sensitizing properties of a sample properly at least four tests are needed: skin irritation, eye irritation, corrosiveness and sensitization test

At present, validated *in vitro* alternatives for base-set tests are limited to tests for skin corrosiveness (OECD guidelines for testing chemicals 431, 435). The current guidelines for skin and eye irritation testing allow for the use of pH measurement: a substance with pH of <2.5 or >11 is assumed to be corrosive. When a substance is identified as corrosive, no further testing for eye irritation or acute dermal toxicity is needed. The current OECD guideline 404 (OECD, 2002) covers the assessment both of skin corrosiveness (classified as R34 or R35) and skin irritation (classified as R38). The severity of skin corrosion and the harm caused to the test animals, triggered significant international effort to develop and validate an *in vitro* method for skin corrosiveness. Three protocols are accepted up to now: SkinEthic (skin model), transepicutaneous resistance test (TER; EEC, 2000) and Corrositex™

Obviously these tests are designed for testing of chemicals and pharmaceuticals and have not been proven to be of use for complex samples of extracts in solvent.

No validated alternatives for eye and skin irritation potential are yet available. Certain *in vitro* methods such as the rabbit isolated eye test (York et al., 1998), the bovine corneal opacity and permeability (BCOP) assay and the hens egg chorioallantoic membrane (HET-CAM) assay are proven to be able to detect severe eye irritants. Positive results from both BCOP and HET-CAM assays are accepted by authorities as indicators for skin and eye irritation.

Also ELISA kits are available to measure specific interleukines that are produced in the cascade of immune reactions when immunologically active cells are exposed to allergens. And phenomena within the immune response like phagocytosis and antigen presentation can be measured. Interleukin patterns can be used to evaluate irritating and sensitizing properties of chemicals.

An alternative test is the in vivo slug mucosal test, where the slime production of slugs in response to skin contact with the sample is an indicator for irritation. This test was developed at Ghent University (Adriaens, 2000) and is being validated for testing of chemicals. The response time of the test is about 3-4 hours.

Up to now no officially validated tests are available to measure sensitization, but promising data have been generated by *in vitro* tests such as VitoSense (Basketter & Kimber, 2009; VitoSense: Hooyberghs, et al., 2008).

Not many alternative tests for irritating, corrosive and sensitizing properties are available that are suitable for testing on complex samples. From this overview and regarding the fast screening purposes we propose to measure first pH in the aquatic extracts (eluates): when pH values are below 2 and above 11 the waste contains irritating compounds. Also the upregulation of interleukin TNF alpha is measured in THP1 cells as an indicator for the on-set of inflammatory responses in reaction to immune disturbing compounds.

5.4.1.5 Biosensors that measure multiple endpoints in parallel

Multiple endpoint assessment is of high added value to speed up the screening process, because it allows the measurement of several toxic endpoints at the same time.

5.4.1.5.1 Whole cell multiple endpoint biosensors

Examples are the Bacterial Gene Profiling Assay (**BGPA;** 14 transgenic *E. coli* strains with single copy chromosomal inserts of different *promoter:lacZ* fusions), *hepG2* assay (13 different *promotor:cat* fusions) and the Liver Gene Profiling Assay (**LGPA**).

With these gene profiling assays different toxicological endpoints can be measured in parallel: in a 96 well plate a battery of different genetically modified reporter gene constructs are grown, each containing a different stress gene promoter fused to the reporter gene. These promoters respond to various stress types: e.g. osmotic stress, oxidative damage, DNA repair induction and protein perturbation (Dardenne et al., 2008).

5.4.1.5.2 DNA arrays

DNA arrays can contain thousands of different spots of DNA printed on a glass microscope slide, whereby each spot corresponds to a gene. By using differently coloured probes for control and treated group of cells, the extracted RNA will be stained differently. Hybridization of the extracted RNA to the spotted genes enables to distinguish by the colour which genes are differently activated in these two groups. As such each gene can be considered as an endpoint. The emerging field of 'toxicogenomics' exploits genomics approaches and sophisticated computational tools to deliver mechanistic understanding of traditional toxicological endpoints. Expression profiling has allowed researchers to decipher the mechanisms of target organ toxicities associated with a variety of compounds.

DNA arrays can be considered as the ultimate multi endpoint assay as each gene is considered as an endpoint. However the technology today cannot be used for high throughput assessment nor is it a cost effective method for toxicity screening. These techniques are therefore not described here.

Gene profiling assays have already proven their power in determining the mechanism of toxicity of pure compounds, environmental samples and food samples. The bacterial assays are in general more cost effective and best suited for the waste toxicity screening. BGPA was selected for the assessment of waste in the pilot study. The method is robust, but the quantification of the effects is not validated yet.

5.4.1.6 Bioassays for Ecotoxicity

Tests for the H14 criterion (ecotoxicity of waste) are already tackled at the European (EU) level, and guidelines are developed for testing the ecotoxicity of waste materials (CEN TC 292 WG 7). An EU Ringtest was organized within this CEN framework to provide information on the use of standardized ecotoxicity tests for solid waste and eluate fractions (UBA, 2009). The focus there was however on the evaluation of the potential risk of the bioavailable fractions of the waste materials, while in the Discriset project the hazard classification is based on total concentrations (intrinsic hazard). Standardized aquatic tests (OECD 201 (algal growth inhibition), OECD 202 (daphnia immobility test); acute tests) were shown to be suitable for the evaluation of both types of extracts.

In conclusion: A number of ecotoxicity tests were selected for further investigation in the Discriset project, including the algae growth inhibition test (72h), the Daphnia immobilization test (48h) and the fish larval mortality test (48h). As in ecotoxicity tests mortality or growth inhibition is measured, ecotoxicity tests are also a measure for general toxicity. Fast bacterial methods for general toxicity - such as Microtox - might be representative for ecotoxicity making additional (slower) tests superfluous.

5.5 Conclusions and summary

The HWD does not provide a transparent method for the classification of complex wastes. HWD recommends testing of the waste material itself and refers to the methods described in annex V of the dangerous substances directive. These methods are developed for pharmaceuticals and are well validated for hazard assessment, but as they often involve (long term) tests on mammals they are not suitable for hazard assessment of waste. The alternative *in vitro* methods are recognized in pharmacology as a very valuable alternative to evaluate hazardous properties in a fast (and much cheaper) and straightforward way and are increasingly introduced and used in the characterization of chemicals (Hartung, 2009, NRC, 2007, REACH).

In the Discriset project a tool is being developed for straightforward hazard identification of complex waste materials. The following *a priori* conditions were taken into account:

- The method should be based on the HWD requirements:
    - Total concentration
    - List of Toxicological Hazardous properties to be evaluated
- Rapid to allow batch analyses
- Economic
- Methods should be standardized and validated for the purpose

A screening of available methods was performed. Based on the criteria and requirements summarized in table 5.2, 16 tests were selected to be used in the next phase (listed in table 5.3). It was concluded that tests are available for most of the hazardous properties, but they do not always fulfill the *a priori* requirements yet.

Table 5.2: Overview of the conclusions of the literature review.

A priori conditions	Tools	Implication	Further needs
Based on total concentration	Worst case extraction	Extracts reflecting the contaminant mixture that is present in the original sample	Standardisation/automation of the extraction method
H-criteria	Chemical methods	These direct methods can be used to identify specific (groups) of toxic compounds in the waste extract itself and HWD limit values can be applied as such	Affinity assays for important groups of contaminants (hormones, dioxins,..)
	Affinity based sensors		
	Ecotoxicity	Standard tests are available	Limit values need to be defined
	General toxicity	Harmful, toxic and very toxic substances can be recognised by signs of toxicity in biotests (death, inhibition, up-regulation of genes involved in cell defence mechanisms..)	Selection of the most suitable test(s) + Limit value to be defined
	Mechanistic toxicity	Biotests are available for measurement of genotoxicity, teratogenicity, inflammatory responses, hormone disturbance	New tests are needed for specific end points Limit values to be defined for all end points
Short test duration	No unnecessary testing	Test strategy with screening tests that allow skipping further tests in case the response is already explicit	Select a representative screening test and define a limit value for this test
	Fast responding tests	Bacterial tests are faster than eukaryotic cell tests	Research to reduce exposure time
Economic testing	Routine tests	Routine methods are available for some tests	Standardisation of other selected tests
	Automation	For some bacterial tests and affinity tests kits and automated methods are available	Further automation for other tests
Standardisation	Protocols	Available for most tests and the extraction method	Prepare standard protocols for the whole evaluation procedure

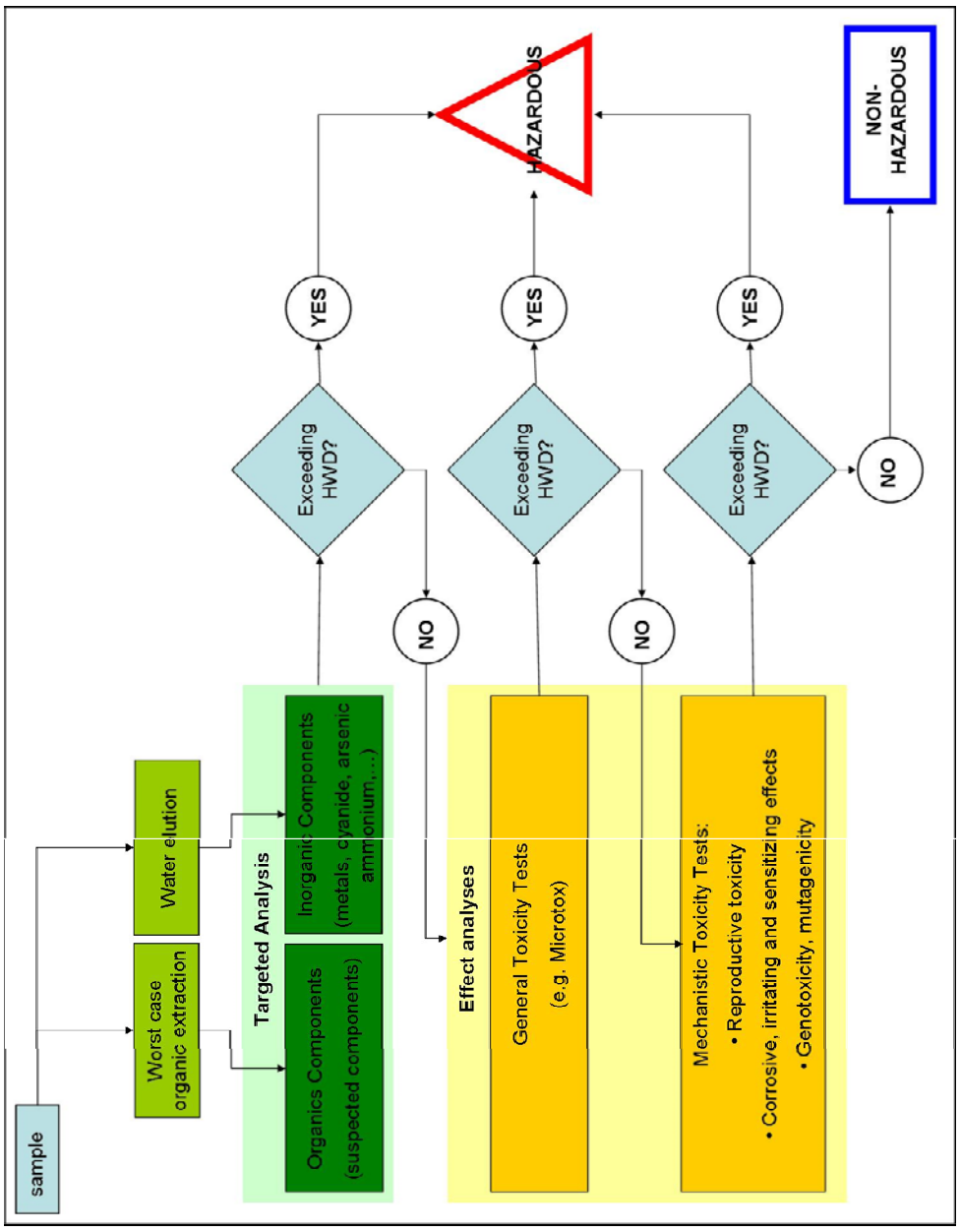


Figure 5.2: Proposed tiered testing strategy

Table 5.3: Summary of the selected tests (CLP: Classification, Labeling and Packaging system for chemicals in the EU)

Hazard	CLP classification	Bioassays
General Toxicity (H5/H6)	H302/312/332 H301/311/331 H300/310/330	Microtox Fish mortality (ELS) Cytotoxicity Vitotox toxicity Stress responses (BGPA)
Reproductive effects (H10)	H360F/360FD H361F/361D/361FD	Teratogenicity ELS Hormonal disturbance CALUX
Corrosive, irritating, sensitizing effects (H4/H8/H15)	H315/319/335 H317/334	TNFα upregulation
Genotoxicity, mutagenicity (H7/H11)	H340/350 H350i	Vitotox DNA damage (BGPA)
Ecotoxicity (H14)	H400-410	Algae growth inhibition Daphnia immobilisation Fish mortality Microtox

A test strategy is proposed (figure 5.2) that should allow waste to be classified in a stepwise way, thus avoiding unnecessary testing:

- Step 1: targeted chemical analyses and concentrations to be compared to the existing HWD limit values. If limits are exceeded, the waste is classified as hazardous, if not: proceed to step 2. This step is only useful if the potentially toxic compounds are known. If not, step 2 should be performed directly.
- Step 2: fast screening test for general toxicity of both extracts (eluate and organic extract) using a fast bacterial tests like Microtox that responds within 30 minutes. A limit value for maximum tolerable toxic load (MTL) has to be developed in accordance to H5 and H6 criteria. If MTL is exceeded the waste is classified as hazardous, if not: proceed to step 3.

- Step 3: different tests for mechanistic toxicity are performed in parallel to evaluate the H-criteria. Proper limit values for toxicity (TL, Toxicity Limit) are again needed for these tests in accordance to the H-criteria.

The proposed test strategy will identify waste materials with high (eco)toxicity and those containing genotoxic substances and hormonal disturbing compounds. These end points already represent very important hazardous properties. Teratogenic and irritation effects will also partly be recognized, but some reproductive/irritation/sensitization effects cannot yet be evaluated.

Toxicity profiling or hazard identification for chemicals/ pharmaceuticals/ cosmetics is more and more relying upon in vitro testing and mechanistic evaluation of toxic pathways (Hartung, 2009; NRC 2007). These modern methods are based on the scientific knowledge of cellular mechanisms and provide a strong tool to recognize, in complex mixtures, not only the presence of toxic compounds but also their mode of action (i.e. hazardous properties). The tests are applicable to (extracts of) products and complex materials, like environmental samples.

Finally, to our opinion this instrument for hazard assessment of complex samples is not only important for evaluation of waste materials but has wider applications when hazard of unknown mixtures has to be monitored e.g. as a quality control system for reused waste water, quality evaluation of contaminate soils.

CHAPTER 6

BIOSENSORS IN WASTE TOXICITY SCREENING: APPLICATION OF TESTS ON WASTE EXTRACTS

Based on the publication:

DISCRISET: A BATTERY OF TESTS FOR FAST WASTE CLASSIFICATION – APPLICATION OF TESTS ON WASTE EXTRACTS

Deprez K., Robbens J., Nobels I., Vanparys C., Vanermen G., Tirez K., Michiels L., Weltens R.
Waste Management Journal. 2012 Dec;32(12):2218-28.

The own contribution includes the CALUX study of endocrine effects, data analysis and overall interpretation and discussion of results.

Chapter 6:
Biosensors in waste toxicity screening: application of tests on waste extracts

6.1 Introduction

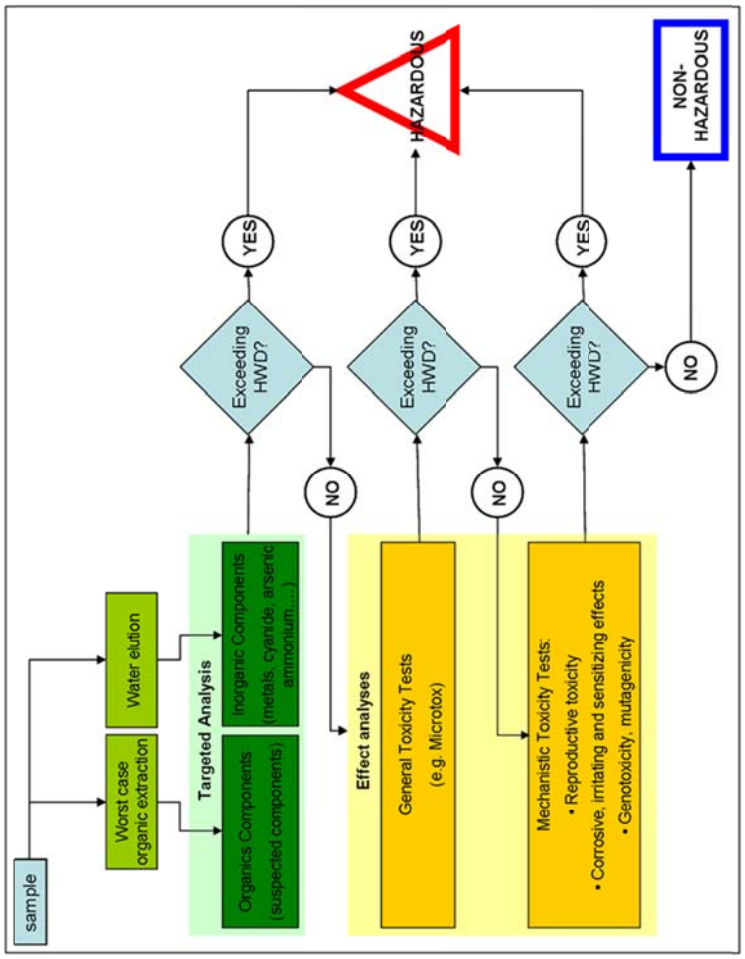
This project, named DISCRISET, was initiated to investigate the application of existing alternative tests for hazard assessment of chemicals to waste materials for classification purposes. In the previous phase of the project (see chapter 5), a number of existing assays were introduced and their suitability for assessment of complex waste samples was discussed (Weltens et al., 2012). This resulted in a list of tests (table 6.2) which comply with the following conditions:

(a) the classification has to be based on total concentrations and based on the 15 hazard properties described in HWD;
(b) assays should take only a minimum of time (preferentially less than 48 hours) and should be as cheap as possible to allow batch controls and to prevent waste from piling up on the site (avoiding odour and/or space problems);
(c) a high level of standardization is necessary to allow the results to be
(d) compared to preset limit values.

Moreover, a tiered test strategy was proposed, aiming to avoid unnecessary testing and minimizing the time and costs of waste assessment by a tiered approach (figure 6.1). The first tier consists of a targeted chemical analysis of the inorganic fraction of the waste. This step is also useful for the organic fraction if the composition of the waste is known or if there is a strong indication about the potential toxic substances present in the waste. In these cases the analyses allow the concentrations of the analyzed elements and compounds to be compared to existing HWD limit values. Exceeding these limits results in immediate classification and further analysis is then no longer required. When targeted analytical approaches cannot provide the necessary chemical information bioassays are applied on extracts of the waste materials.

The second tier consists of a fast (bacterial) test for general toxicity that enables a first triage by recognizing the samples that have a very high intrinsic toxicity and can be classified as hazardous based on this test result alone. Microtox, a well validated bioassay which takes only 30 minutes, was suggested for this purpose. A validation study was started to confirm the screening abilities of this test (Weltens et al., in preparation).

The last tier of the strategy consists of a battery of biotests performed in parallel and evaluating different types of mechanistic toxicity. Selected tests identify samples with genotoxic content or endocrine disruptive substances as well as partially recognizing teratogenic and irritative effects.
Ecotoxicity tests are also included in this phase. Although these are not based on mechanistic toxicity but measure general toxicity, these tests tackle a specific hazardous property of waste (H14), hence forming a part of the last testing phase.



H	Criterion
H1	explosive
H2	Oxidising
H3a	highly flammable
H3b	Flammable
H4	Irritant
H5	Harmful
H6	Toxic
H7	Carcinogenic
H8	Corrosive
H9	Infectious
H10	Toxic for reproduction
H11	Mutagenic
H12	Release of (very) toxic gases
H13	Leachate with hazardous properties
H14	Ecotoxic
H15	substances and preparations capable by any means, after disposal, of yielding another substance, e.g. a leachate, which possesses any of the characteristics lieted above

Figure 6.1 (left): Proposed testing strategy for the hazard assessment and classification of complex waste materials
Table 6.1 (right): HWD - list of hazardous properties

Table 6.2: Bioassays used for waste toxicity assessment (CLP: Classification, Labeling and Packaging system for chemicals in the EU)

Hazard	General toxicity (H5/H6)	Reproductive effects (H10)	Corrosive, irritating, sensitising (H4/H8/H15)	Genotoxicity Mutagencity (H7/H11)	Ecotoxicity (H14)
CLP classification	H302/312/332 H301/311/331 H300/310/330	H360F/360FD H361F/361D/361FD	H315/319/335 H317/334	H400-410	R50 -58
Bioassays	Microtox Fish mortality (ELS) Cytotoxicity Vitotox toxicity toxicity Stress responses (BGPA) Daphnia immobilisation	Teratogenicity ELS Hormonal disturbance CALUX	TNFa upregulation	Vitotox damage (BGPA)	Algae growth inhibition Daphnia immobilisation Fish mortality microtox

6.2 Aim

In this study complex waste samples selected from a wide range of sources are used to test the robustness of the proposed testing strategy described above. For some of the industries represented here, such as the wood, paint and textile industries, toxic properties of waste or effluent have been described in literature (Giorgetti et al. 2011, Orrego et al. 2011, Ghisari et al. 2009). Often however, results indicate that toxicity varies between sites and samples, confirming the need for a waste assessment tool which can be implemented for routine waste classification. Analyses were performed on worst case extracts of the waste to comply to current legislation, which states that classification should be based on the intrinsic toxicity of the waste, as opposed to the bioavailable fraction, which is better evaluated using water eluates (Tigini et al. 2010, Vaajasaari et al. 2003, Charles et al. 2011, Ma 2010).

The results of the selected assay candidates on 16 waste samples from various sources are reported on. The suitability of the selected tests for waste assessment is discussed and the implementation in the proposed testing strategy is evaluated.

6.3 Materials and Methods

6.3.1 Samples

Initially seven filter press samples from the water treatment plant of a tank cleaning company were tested in a pilot study. In the next phase nine samples from a wider range of waste sources was used. Table 6.3 summarizes all samples.

Table 6.3: Samples (WTP= water treatment plant)

Sample	Source
fTEX	filter cake – input textile industry
fPAINT1	filter cake – input paint industry
fFOOD1	filter cake – input food industry
fFOOD2	filter cake – input food industry
fFOOD3	filter cake – input food industry
fPAINT2	filter cake – input paint industry
fPAINT3	filter cake – input paint industry
PSW	PCB containing shredder waste
SF	Shredder fluff
sDWTP	Sludge domestic WTP
FA	Fly ashes
WOOD	Wood
BA	Bottom ashes
sIWTP	Sludge industrial WTP
SA1	Soil additive
SA2	Soil additive

6.3.2 Extraction methods

Water elution and a worst case extraction were performed on all samples prior to the assays. Methods for the elution and preparation of test portions of the water leachable fraction of the samples were those issued by the Technical Committee 292 of the European Committee for Standardization (CEN TC 292). The procedures are described in EN 15002:2006 and EN 12457-4:2002. The

waste material was brought into water (demineralized, Liquid (g)/Solids (g) = 10) and stirred for 24 hours. The water fraction was used for inorganic analyses.

The organic fraction of the waste samples was obtained by acetone extraction: 300 g of waste material (grinded) was mixed with 300 ml of acetone and sonicated for 1 hour and then stirred for another hour. Then the solid material was allowed to sink. The liquid fraction was separated and filtered (glass fiber) and acetone was removed by evaporation. The remaining material was then diluted in the appropriate solvents. For the bioassays acetone was substituted by 33 g DMSO as a solvent that is suitable for use in bioassays. The final concentration of the extract was therefore approximately 10 geq/ml (gram equivalents of the original material). For GC-MS analyses 50µg dibromophenyl is added per ml acetone as an internal standard.

6.3.3 Chemical Analysis

6.3.3.1 Inorganics

In table 6.4 the applied standardized methods for the chemical characterization of the inorganics are listed. The majority of these chemical methods do not identify the components, but measure the individual species such as anions (e.g. sulphate, chloride) and cations (e.g. metals).

Table 6.4: Overview of applied standardized methods for the characterization of inorganic parameters of the waste samples

parameter	Method
pH	EN 12506:2003 Characterization of waste - Analysis of eluates - Determination of pH, As, Ba, Cd, Cl-, Co, Cr, Cr VI, Cu, Mo, Ni, NO2-, Pb, total S, SO42-, V and Zn
Hg	EPA 7473 Mercury in solids and solutions by thermal decomposition amalgamation and atomic absorption spectrometry
ammonium, cyanide	EN 13370:2003 Characterization of waste - Analysis of eluates - Determination of Ammonium, AOX, conductivity, Hg, phenol index, TOC, easily liberatable CN-, F-
Chromium VI	EN 15192:2006 Characterization of waste and soil - Determination of Chromium(VI) in solid material by alkaline digestion and ion chromatography with spectrophotometric detection
Elemental composition	EN 15309:2007 Characterization of waste and soil - Determination of elemental composition by X-ray fluorescence
C, H, N, S	/TS 15407:2006 Solid recovered fuels - Method for the determination of carbon (C), hydrogen (H) and nitrogen (N) content

6.3.3.2 Organics: Gas Chromatography – Mass Spectroscopy (GC-MS)

Organic compounds were monitored using Gas Chromatography – Mass Spectroscopy (GC-MS). 50 µg dibromobiphenyl internal standard was added to the extracts per ml acetone. 1 µl of the extract was then injected into an Agilent 5973 MSD in split or splitless mode, depending on the concentration. The compounds were separated on DB5-ms column (60m x 0.25 id x 0.25 µm df, helium carrier gas) and MS acquisition was carried out in full scan mode (mass range 40-500 amu). The mass spectrometric identification of the compounds was done by means of Chemstation software and NIST libraries.

6.3.4 Bioassays

6.3.4.1 Assays for General Toxicity (H5/H6/H14)

In this phase of the testing strategy effects of general toxicity are measured (as opposed to tests that measure specific toxicity: see below). General toxicity can be measured as effects on growth rate, cell metabolism and survival. These toxic effects result from the combined effects of all toxic components present in the extracts.
DMSO extracts were diluted in a twofold dilution series in DMSO.

6.3.4.1.1 Microtox toxicity assay

The acute general toxicity of waste extracts was measured using the Microtox toxicity assay according to ISO11348-3. The method is based on measured reduction of light emission in autoluminescent bacteria (i.e. *Vibrio fischeri*). Metabolism – and consequently light intensity - relates to the amount of toxic stress. The tests were carried out using a Microtox M500 analyzer (SDI Europe, UK) according to the Microtox Manual (1992) for the phenol controls. The procedure was adapted for the use of DMSO samples. *Vibrio fischeri* were exposed to 1 % of the diluted extracts in Microtox Diluent® (5µl sample in 500 µl Diluent; 1% DMSO was used as a negative control) and light emission was monitored after 5 min, 15 min and 30 min. Results after 30 minutes are reported.

For samples inducing a highly toxic response, the EC50 value was derived by linear regression (expressed in % of the original sample or geq of the original sample per liter). Tests are valid when EC50 for phenol is within the range of 13 to 26 mg/l.

6.3.4.1.2 VITOTOX

The Vitotox assay was originally developed as a genotoxicity assay designed for product testing. The test is based on SOS response reporting by a β-galactosidase reporter gene in the bacteria *E.coli*. The bacterial cultures are exposed to dilution series of the DMSO extracts at a concentration of 1% during 4 h. Results showed however that the test was not suitable for genotoxicity testing of these samples as toxicity was often very high. Therefore only the general toxicity is reported here. Six replicates were tested, EC50 values were calculated if a significant (unpaired t-test, $p < 0.05$) concentration dependent inhibition was measured.
The test was only performed on the first series of samples because results were comparable to the Microtox results and no extra information was provided by Vitotox.

6.3.4.1.3 Cytotoxicity test

In 96-well tissue culture plates, 10^4 cells in 200 µl DMEM per well were cultured and grown to sub-confluent monolayers for 24 h. Then cultures were exposed to 0.1 % of waste extract (and dilutions) by medium change, 6 replicates were used for each condition. Control groups were cells exposed to medium containing 0.1 % DMSO and medium without DMSO (n= 4). After 24h of incubation, the medium was removed and 1 ml solution of 10% Alamar Blue (Biosource, Camarilo, CA, USA) in DMEM was added to each well. After 5h incubation, fluorescence intensity was measured using a fluorescent plate reader (FLUOstar Galaxy, BMG Lab Technologies) for excitation at 530 nm and emission at 580 nm. Growth inhibition was determined from fluorescence intensity, the Dunnett test and t-test were used to identify significant differences ($p < 0,05$).

6.3.4.1.4 Ecotoxicity tests (H14)

Daphnia immobility test: the test is based on OECD guideline 202 (1984) - static acute toxicity test measuring the immobility of the water flea when exposed to a dilution series of the test substance. *Daphnia magna* were exposed to the dilution series of the waste extracts at 0,1 % in JP4 medium (n= 4 replicates, 6 for controls), 5 organisms per replicate; 20 ml per replicate; room temperature; 16/8 light/dark regime). 0,1 % DMSO was used as a control condition. Immobility (equal to mortality) was monitored after 24 and 48 hours. Tests are valid only when survival in control conditions is at least 90%.
When a significant concentration dependent mortality was measured (unpaired t-test, $p < 0.05$) the EC50 value (expressed in geq of the original sample per liter) was derived through linear interpolation.

Algae growth inhibition: the algae growth inhibition test was performed according to standard procedure (OECD guideline 201 (2006)). Inhibitory effects of the DMSO extracts on exponentially growing *Pseudokichneriella subcapitata* serve as an indicator for general toxicity. Algal cultures of 1500 to 5000 cells/ ml were exposed in a limit test to the DMSO extracts at 0.1% in OECD medium (n= 3 replicates, 6 for controls), 100 ml per replicate; incubator: 23°C, 100 rpm stirring rate, continuous light, appr. 4000 lux) 0.1% DMSO was used as a control condition. The number of cells was monitored using a Coulter counter at 24, 48 and 72 hours and growth rate was compared to the growth rate in control conditions. Significant differences were identified using an unpaired t-test ($p < 0.05$), the percent inhibition at 0.1% extract is reported.

6.3.4.2 Mechanistic Toxicity Tests

Toxic effects due to specific mechanisms were investigated using a battery of bioassays. The tests used here were selected as promising candidates for waste assessment and were described earlier (Weltens et al., in press). The intention was to select practical tests that cover the toxicological hazardous properties as described in table 6.1.

6.3.4.2.1 Genotoxicity / Mutagenicity (H7/H11): Ames test.

In the Ames test (Maron and Ames, 1983; Mortelmans and Zieger, 2000), histidine-deficient (his−) *Salmonella typhimurium* bacteria are inoculated on a histidine-poor growth medium. Only bacteria showing the reverse mutation (his−→his+ spontaneous or induced mutants) can grow to full colonies in the absence of histidine. A compound is considered mutagenic when a concentration-dependent increase in the number of mutant colonies is found that exceeds twice the background level. The Ames test (plate-incorporation assay) was performed in the absence and presence of an S9 fraction (mix of metabolizing enzymes from rat liver to mimic metabolism; Moltox, Boone, NC) so as to allow not only detection of a direct mutagenic effect, but also of an indirect mutagenic effect brought about by possible metabolites of the applied compound. Toxicity of the test compounds was assessed by inspecting the background layer of colonies on the agar medium according to standard protocols (e.g. Venitt et al. 1984).
There were three replicates per sample. When the test substance was toxic for the bacteria lower concentrations were used. When induction factor was > 2 the extract was assigned as genotoxic (+).

6.3.4.2.2 Reproductive effects (H10)

*CALUX®: C*hemically Activated Luciferase assay is a genetically modified cell system based on estrogen (ER and ERα Calux), androgen (AR Calux) and aryl hydrocarbon or "dioxin" receptor binding (DR Calux) (Garrison et al.1996; Murk et al. 1996). An overview of the different assays is given in table 6.5. Assays were performed according to the manufacturer's guidelines and all cell lines were provided by Biodetection Systems BV (BDS).

Cells were exposed to 0.1% of DMSO dilutions of the extracts according to the standard procedure implemented by BDS. After 24 h to 48 h cells were lysed and luciferase activity was measured. Each experimental point was performed in triplicate.

Table 6.5: Overview of implemented Calux methods

Assay		Cell line	Pathway	DMSO extract /dilution in medium	Reference compounds
ER Calux	agonist	T47d/luc	Estrogen receptor pathway	1 µl / ml medium	Estradiol (E2)
ER Calux	antagonist	T47d/luc		1 µl / ml medium[2]	Tamoxifen (Tam)
AR Calux	agonist	U2OS	Androgen receptor pathway	1 µl / ml medium	Dihydrotestosterone (DHT)
AR Calux	antagonist	U2OS		1 µl / ml medium[2]	Flutamide (Flut)
DR Calux	agonist	H4IIE	Aryl hydrocarbon receptor pathway[1]	4 µl/ml medium	2,3,7,8-tetrachlorine dibenzo-p-*dioxin* (TCDD)

[1]known cross-talk with estrogen receptor pathway (Safe, Wang et al. 1998)
[2]medium contains EC50 concentration of agonist (resp. E2 en DHT)

Concentration effect curves were fitted using the sigmoidal fit in Graphpad Prism (Graphpad Software, San Diego). For quality control purposes, only the results that reached the predefined parameters of r^2 of calibration curve >0.99, a concentration between the LOQ and EC50 (median effective concentration) and a relative standard deviation < 15% were used. Reference compounds for ER are estradiol (agonist), tamoxifen (antagonist), for AR Calux dihydrotestosterone (DHT, agonist) and flutamide (antagonist) are used. DR results were compared to the 2,3,7,8-Tetrachloordibenzo-p-dioxine (TCDD) standard curve.

Early Life Stage test (ELS): Zebrafish eggs were exposed to dilution series of the waste extracts in DMSO (50µl / 50 ml fish water, 0.1 %). Larval development of 12 replicates was monitored during 48 hours and scored for 4 parameters: presence of coagulation, somite development, tail release and heartbeat. The percentage of replicates displaying an abnormal development is an indicator for teratogenicity (H10) of the waste extracts. Teratogenic effects were considered significant if following criteria were fulfilled: (1) the percentage of replicates displaying abnormal development within the control group is zero, and (2) there is a dose-response relationship, confirming that the registered effects are indeed induced by the extract. Lethality (LC50) was derived by linear interpolation and evaluated as an indicator for general toxicity.

6.3.4.2.3 Corrosive, irritating, sensitizing effects (H4/H8/H15): TNFα assay

In 96-well tissue culture plates, 10^4 THP1 cells were cultured in 200 µl cell culture medium per well and grown to sub-confluent monolayers for 24 h. 1:2 dilution series of the waste extracts in DMSO were prepared (in duplicate) and cell cultures were exposed to 0.1% of the DMSO solutions. Negative control groups were cells exposed to medium containing 0.1% DMSO and cells exposed to medium without DMSO (n= 4). Positive control groups were exposed to 0.1, 0.5 or 1 mM Paraquat and cells exposed to Interferon Gamma (IFγ). After 24h incubation, the medium was removed and TNFα is measured via ELISA. Mean and standard deviation was calculated for each condition and the test concentration that induced 1000 pg/ml TNFα was reported.

6.3.4.3 Multiple endpoint assay: BGPA

The bacterial Gene Profiling assay (BGPA) comprises a battery of 16 bacterial reporter gene (β-galactosidase) assays allowing to gain insight in the mode of action and statistical grouping of samples based on their induction profile. Investigated genes and their function are summarized in table 6.6. Both genes involved in general toxicity (oxidative stress, membrane integrity, osmotic stress, growth arrest…) and specific toxicity (genotoxicity-SOS response, DNA damage) are involved.

The bacterial *E.coli* strains, and the procedure for the bacterial gene-profiling assay are described elsewhere (Dardenne et al., 2008). All strains were cultured in Luria Bertani Broth according to standard protocols and kept frozen in 15% glycerol at -80°C prior to use (Sambrook et al., 1989).
The bacteria were exposed at the onset of exponential growth for 90 min, after which cells were lysed and the β-galactosidase activity was determined.
Fold inductions were considered significant (Dardenne et al. 2008) based on the following criteria: (1) the presence of a dose-response relationship ($r^2 > 0.50$, significant at $p < 0.05$) and a positive slope different from 0 ($p < 0.05$) in a linear model; and (2) signal different from and higher than the blank is confirmed by Dunnett's test ($p < 0.05$).

Table 6.6: BGPA investigated responses

Main Group	Promoter	Gene product/Function	Responsive to
Oxidative stress	KatG	Hydrogen peroxidase I	Oxidative stress
	Zwf	Glucose-6-phosphate dehydrogenase	Oxidative stress
	Soi28	Superoxide inducible gene	Superoxide radical generating agents
	Nfo	Endonuclease IV	Ss and dsDNA breaks, oxidative DNA damage
	MerR	Regulation of the mercury resistance operon (mer)	oxidative DNA damage
Membrane damage	MicF	Antisense RNA to 5' OmpF	Membrane integrity, osmotic stress
	OsmY	Periplasmic Protein	Osmotic stress
Cellular stress	UspA	Universal stress protein	Growth arrest
	ClpB	Proteolytic activation of ClpP	Protein perturbation
Heavy metal stress	MerR	Regulation of the mercury resistance operon (mer)	Heavy metals
DNA Damage	Nfo	Endonuclease IV	Ss and dsDNA breaks, oxidative DNA damage
	RecA	General recombination and DNA repair	SOS response
	UmuDC	DNA repair	Radiation and/or chemically induced DNA damage
		Adaptive response to alkylation	DNA damage, mainly methyl adducts
	DinD	Unknown function within the DNA damage inducible response	DNA damage
	SfiA	Inhibitor of cell division	SOS response

6.4 Results and Discussion

In this section first the individual results for the different assays and analyses are reported. The obtained results are discussed in terms of the suitability of the assay in the tiered approach (figure 6.1).

6.4.1 Chemical Analysis

6.4.1.1 Inorganics

Results for inorganic parameters are shown in table 6.7. Many parameters were measured (table 6.4). Only compounds that were at concentrations above the background level in at least one of the samples, are shown in table 6.7. High concentrations (above median and/or mean) are highlighted for clarity.

It is clear from table 6.7 that in the first series of samples (fTEX-fPAINT3) the filter cake samples from the paint industry are the most contaminated with the measured inorganic compounds. In the second series PSW and SF are the most contaminated samples. FA and BA also show elevated levels for some inorganic elements.
The inorganic analyses can be performed in an efficient and economic way, providing the concentration levels of all relevant parameters: pH, heavy metals, anions, cyanide.

Table 6.7: pH and inorganic parameters above background. yellow: above the mean of the samples, orange: not above mean but higher than median (nm: parameter was not measured)

Element	Dimension	fTEX	fPAINT1	fFOOD1	fFOOD2	fFOOD3	fPAINT2	fPAINT3	PSW	SF	sDWTP	FA	WOOD	BA	sIWTP	SA1	SA2
pH		8.09	7.73	7.91	5.7	7.49	6.97	7.1	6.8	7.4	7.8	9.4	6	9.5	6.8	9.4	8.1
Al	%	0.34	3.71	0.12	0.27	<0.1	1.84	3.44	2.49	0.84	1.85	4.83	<0.05	3.14	2.84	2.8	9.17
Si	%	1.86	2.55	0.3	1.38	0.53	3.49	0.76	6.82	4.98	20.5	19.6	0.09	21.4	6.9	12.11	7.208
P	%	0.051	0.85	1.43	1.24	2.2	0.93	0.67	0.23	0.239	0.17	0.39	100	0.497	4.93	0.017	0.049
S	µg/g	6590	12370	9780	6520	8500	4840	7750	12220	27370	4460	7850	140	7190	8570	4483	4271
Cl	µg/g	2050	21100	6580	708	1730	6370	9810	15190	10890	8140	6320	190	4330	880	91.8	19680
V	µg/g	<20	<20	<20	26.2	<20	55.3	<20	87	37	28	23.4	nm	82.4	49.5	54.3	533
Cr	µg/g	13.2	906	29.5	38.5	37.9	181	746	465	414	142	408	200	304	127	50.4	404
Co	µg/g	26.3	146	<8	15.5	29	90.8	58	86	68.8	<9.6	89.9	nm	154	47.7	<9	<23
Ni	µg/g	24.2	692	74.2	31.6	52.4	765	794	574	554	18.7	144	nm	423	50	24.4	42.6
Cu	µg/g	16.4	1370	194	35.1	66.5	890	327	7700	1260	77.9	2130	2020	1630	609	19.1	15.8
Zn	µg/g	2130	5600	539	233	259	6410	4120	20820	15060	267	3190	36	2900	1960	46.3	26.2
Br	µg/g	11.8	13960	248	91.2	38.5	4200	67480	391	154	3	2.8	nm	16.3	49.7	1.4	171
Rb	µg/g	6.2	113	5.4	12.4	3.9	56.6	464	24.1	17.6	33.1	32.5	nm	26.3	32.8	nm	nm
Sr	µg/g	86.8	88	69.7	46.5	117	45	35.9	353	345	116	269	nm	413	345	70	96.5
Zr	µg/g	55.1	393	407	1140	667	830	664	521	172	148	174	nm	175	147	nm	nm
Nb	µg/g	1.7	1650	0.4	2.6	1.6	86.1	38.7	11.4	5.6	4.3	8.1	nm	9.1	6.13	nm	nm
Mo	µg/g	<2	85.2	4.6	<2	4.7	48.4	38.2	49.6	37.7	2.9	21.7	nm	13.3	21.6	nm	nm
Cd	µg/g	<3	24.4	<3	<3	<3	<3	39.5	76.1	48.8	6.3	6.7	nm	4.5	8.84	<3	<3
Sn	µg/g	<9	2670	<9	<9	<9	837	166	547	298	10.3	274	nm	169	58.3	<9	30.3
Sb	µg/g	<5	8540	<5	<5	<5	1570	10670	308	40.3	4.7	56.6	nm	65.3	21.6	<5	<5
Ba	µg/g	<18	481	62.9	54.7	<18	345	133	4150	961	234	1160	nm	1510	670		
Tl	µg/g	<2	19	<2	<2	<2	7.2	37.8	5	<4.3	<2	<3	nm	<2.4	<2	<2	<2
Pb	µg/g	6.9	1150	13.6	7.8	5.8	129	203	3330	2770	85.3	1490	nm	958	257	49.1	48.7

For classification according to HWD the potential toxicological risk for each individual compound should be taken into account as well as the sum of substances classified as hazardous for the same CLP class (H-numbers as specified in table 6.2). A cut-off value was appointed to each CLP class (ref. D/2013/5024/28). To determine if the waste is hazardous, the sum of all compound concentrations exceeding this cut-off is made, and this total concentration of hazardous substances is compared to a toxicity limit (specific for each CLP class).

For example, zinc chloride ($ZnCl_2$) corresponds to CLP classes H302, H314 en H410 (see table 6.8). The percentage of $ZnCl_2$ is first compared to the cut-off of these classes. If the cut-off is exceeded, the percentage of $ZnCl_2$ is added to the percentages of all other substances exceeding the cut-off (this addition is not always a straightforward sum, sometimes specific formulas are applied). This total concentration of hazardous substances is then compared to the limit value. A total concentration higher than the toxicity limit leads to classification as hazardous. For $ZnCl_2$ total percentage (once added to the concentrations of other hazardous substances exceeding the cut-off) should be compared to the limit values for H302 (= 25%), H314 (= 5%) and H410 (25%). Zinc oxide (ZnO_2), another zinc compound, only corresponds to the H-statement H410 (limit: 25%). Thus, ZnO_2 must only be taken into consideration for H410, and not for H302 and (the more stringent) H314.

Table 6.8: CLP classification, cut-offs and toxicity limits for $ZnCl_2$ and ZnO_2 (adapted from D/2013/5024/28)

Compound	CLP classification	Cut-off (%)	Toxicity Limit (%)
$ZnCl_2$	H302	1	25
	H314	1	5
	H410	0.1	25
ZnO_2	H410	0.1	25

The toxicity (i.e. the CLP class) of metal compounds depends on the solubility of the compound, as only the soluble compounds are bio-available and thus toxic. Metals are themselves not soluble, but metal compounds such as metal chlorides are. Table 6.9 shows the variable solubility of several Zn compounds.

Table 6.9: Solubility of different Zn compounds (adapted from D/2013/5024/28)

Compound	Solubility (mg/l)
$ZnCl_2$	28
$Ca_4Zn(PO_4)_3(OH)$	1.1
$Zn_3(PO_4)_2$	1.7
$CaZn_2(OH)_6.2H_2O$	28
$ZnSiO_4$	4.5
$ZnSiO_3$	0.06
ZnO_2	1.6

This example illustrates the difficulty of classifying complex waste based on its inorganic components: though XRF analysis quickly identifies and quantifies the elements present in the waste sample, no information is obtained regarding the speciation of these elements (and thus, the compounds that they form), making it impossible to directly compare these results with HWD cut-off values and toxicity limits.

Assuming worst case toxicity (i.e. the most toxic speciation for each element), all filter cake samples as well as samples PSW, FA and BA would be classified as hazardous.

Results demonstrate that the inorganic fraction can efficiently be characterized by chemical analyses, but the HWD should provide guidance on how to deal with the lack of information on the speciation of the compounds. It is not considered appropriate to perform biotests on the inorganic fraction as analytical information can be generated in a much faster and cost efficient way.

6.4.1.2 Organics

GC-MS analyses resulted in chromatograms showing 50 up to 396 product specific peaks, of which on average 22 % could be identified. Table 6.10 lists potentially toxic compounds that were detected in the samples. Results are only indicative. PCB content was further determined for SF (shredder fluff) and PSW (PCB containing shredder waste). The values were respectively 11 and 41 mg/kg DW (sum of seven PCB congeners).

GC-MS results provided non-quantitative information on the organic content of the waste extracts and results were clearly not suitable for classification purposes: samples often showed a high complexity, only compounds suitable for gas chromatography (i.e. sufficient volatility and thermal stability) can be detected, and no information is obtained on other organic (or inorganic) compounds present in the sample. It is estimated that only 10-20% of known compounds can be analyzed by GC (Weltens et al., in preparation). Additionally, the majority of peaks obtained after GC-MS analysis did not show sufficient similarity to known spectra to be identified, resulting in an incomplete output. GC-MS is a very efficient way of investigating samples with known or suspected toxic content but is not useful for the hazard characterization of unknown complex samples. Mixtures of compounds might enforce or compensate mutual toxicity, which cannot be measured. Therefore toxic effect assessment is necessary, making use of bioassays which require no prior knowledge of the waste content.

Table 6.10: Potentially hazardous substances identified by GC-MS

sample	Identified compounds
fTEX	n-Alkanes, phthalate esters, butylhydroxytoluene (BHT), sulfur, 2-methylthiobenzothiazole, mercaptobenzothiazole, N,N'-difenylguanidine
fPAINT1	Aliphatic hydrocarbons, naphthalene and methylnaphthalenes
fFOOD1	Alkanes, alkanoic acids and alkanoic acid esters, phenylethyl alcohol, phthalates, sterols and derivatives (cholesterol, cholestanol, cholestenone, stigmastane, ergostadineol, ergostenol, stigmasterol, sitosterol,..)
fFOOD2	Alkanes, alkanoic acids and alkanoic acid esters, sterols, cresol, indool, methylnaphthalene, chlorophene, amyrin
fFOOD3	Alkanes, alkanoic acids and alkanoic acid esters, sterols
fPAINT2	Alkanes, phthalates, glymes (butoxyethoxyethanol, butoxyethoxyethoxyethanol), alkylphenols (butylphenols, octylphenol), diphenylether
fPAINT3	Alkanes, aromatic petroleum hydrocarbons (methylnaphthalenes, methylphenanthrenes, methylanthracenes, biphenyl), phthalates, t-butylphenol, alkanoic acids
PSW	n-Alkanes, phthalate esters, butylhydroxytoluene (BHT), sulfur, 2-methylthiobenzothiazole, mercaptobenzothiazole, N,N'-difenylguanidine
SF	Aliphatic hydrocarbons, naphthalene and methylnaphthalenes
sDWTP	Alkanes, alkanoic acids and alkanoic acid esters, phenylethyl alcohol, phthalates, sterols and derivatives (cholesterol, cholestanol, cholestenon, stigmastane, ergostadineol, ergostenol, stigmasterol, sitosterol,..)
FA	Alkanes, alkanoic acids and alkanoic acid esters, sterols, cresol, indool, methylnaphthalene, chlorophene, amyrin
WOOD	Alkanes, alkanoic acids and alkanoic acid esters, sterols
BA	Alkanes, phthalates, glymes (butoxyethoxyethanol, butoxyethoxyethoxyethanol), alkylphenols (butylphenols, octylphenol), diphenylether
sIWTP	Alkanes, aromatic petroleum hydrocarbons (methylnaphthalenes, methylphenanthrenes, methylanthracenes, biphenyl), phthalates, t-butylphenol, alkanoic acids
SA1	Not measured
SA2	Very few organic compounds, only n-alkanes

6.4.2 Bioassays for general toxicity (H5/H6/14)

The bioassays implemented for general toxicity reflect how the over-all metabolism is affected by the (complex) samples. The samples elicit similar effects in the different tests used (table 6.11). From these results it can be concluded that one sensitive test can replace the battery and can be used as an initial screening tool. The toxicological profile of the waste samples can then be completed with results from mechanistic tests.
The bacterial tests Microtox and Vitotox show the highest sensitivity to the extracts, allowing the most samples to be ranked according to toxicity, even when the toxicity is low (e.g. samples FA, BA, and PSW). Since Microtox analysis takes only 30 minutes versus 4 hours for Vitotox, it was decided to continue with Microtox as the preferable screening instrument.

Microtox is also confirmed as the most representative test as compared to other general toxicity tests (see table 6.11): at least one of the other test organisms (ELS LC_{50}, Keratinocyte EC_{50}, Daphnia immobilization, Algae growth inhibition and Ames toxicity) were also affected by the highly toxic samples, corroborating Microtox results. The general toxicity effects measured by BGPA also corroborate Microtox results except for sample fPAINT1. This acutely toxic sample does not show any increased expression of the measured stress genes in BGPA, indicating that the toxic pathway involved here is not covered by the assay. Measuring the cellular response by gene expression has the advantage of being able to measure different endpoints in parallel and gain better insight in the mode of action. However, the up and down regulation cannot be quantified (yet) in terms of classification and not all pathways are covered (yet). Further validation by comparison to reference substances is therefore needed before implementing BGPA in this application.

Our results, as well as recent literature data (Ocampo-Duque et al. 2008) confirm that Microtox is a good initial screening instrument for the organic fraction of the waste, combining speed and low cost to sensitivity. Finally toxicity was also confirmed by the mechanistic toxicity assays (see table 6.15).

Table 6.11: General toxicity assessment of waste extracts (nm: not measured; >: EC50> highest concentration; CT: cytotoxic, N: not toxic). In yellow: samples with high toxicity (EC50<5geq/l or >50% effect in algae; Toxic in Ames, three highest readings for BGPA), NT: not tested

sample	Microtox	Vitotox	Daphnia	Algae (72h)	ELS	Cytotoxicity	Ames	BGPA		
	EC50 (30') (geq/l)	EC50(4h) (geq/l)	EC50 (48h) (geq/l)	% inhibition at 0.1%	LC50 (48h) (geq/L)	EC50 (72h) (geq/l)	EC50	Oxidative Stress	Membrane damage	Cellular Stress
fTEX	0.98	0.38	10.4	77.9	>	>	N	20.00	50.00	91.67
fPAINT1	0.19	0.1	0.05	97.5	0.19	0.2	CT	0.00	0.00	0.00
fFOOD1	16	-	>	0	>	>	N	2.86	0.00	0.00
fFOOD2	1.4	0.25	6.6	76.3	>	>	N	0.00	0.00	35.71
fFOOD3	2.02	1.65	8.2	61.3	>	>	N	5.71	21.43	35.71
fPAINT2	0.14	0.06	0.31	91	0.14	0. 46	CT	14.29	7.14	14.29
fPAINT3	0.08	0.06	0.21	85.3	0.18	0. 6	N	20.00	14.29	14.29
PSW	27	nm	>	0	>	>	N	NT	NT	NT
SF	1.98	nm	2.14	92.6	6.1	>	N	NT	NT	NT
sDWTP	5.12	nm	>	0	>	>	N	NT	NT	NT
FA	82	nm	>	0	>	>	N	NT	NT	NT
WOOD	0.6	nm	1.65	96	1.6	>	CT	NT	NT	NT
BA	34	nm	>	0	>	>	N	NT	NT	NT
sIWTP	1.1	nm	3.93	53	3.8	>	CT	NT	NT	NT
SA1	>	nm	>	0	>	>	N	NT	NT	NT
SA2	>	nm	>	0	>	>	N	NT	NT	NT

6.4.3 Mechanistic Toxicity Tests

As opposed to general acute toxicity tests, mechanistic toxicity tests detect toxic effects of (mixtures of) compounds on specific pathways within the cell. Though general toxicity is due to underlying mechanisms and as such also due to mechanistic toxicity (see table 6.15), not all mechanistic effects result in acute general toxicity (f.i. genotoxic substances only generate effects in the long run). Therefore the samples that have not been classified by the initial Microtox screening, when compared to a suitable and still to be determined limit, need to be assessed by mechanistic assays for investigating further hazardous properties (see figure 6.1). In this tier several bioassays were performed in parallel, resulting in a partial mechanistic toxicity profile for each sample, including information on the presence of carcinogenic or mutagenic substances, irritating or sensitizing effects and teratogenic or endocrine disturbing substances. Each of the mechanistic bioassays is discussed below in function of their suitability in a testing battery for assessment of these H properties.

6.4.3.1 Genotoxicity / Mutagenicity (H7/H11)

Mutagenicity was investigated by the frequency of reverse mutations in the Ames test and the induction of the SOS response in BGPA was used as an indicator for genotoxicity. Four samples out of sixteen were shown to be mutagenic in the Ames test and three out of seven samples showed SOS response (see table 6.12). The samples which test positive in the Ames test do not necessarily test positive for the BGPA SOS response and vice versa. As both tests measure different modes of action they address different types of interactions of chemicals with the genetic material.

Table 6.12: Mutagenicity (Ames test) and genotoxicity (BGPA SOS response) (+: mutagenic, -: non mutagenic, N: not tested)

sample	Ames mutagenicity	BGPA genotoxicity
fTEX	-	26.67
fPAINT1	-	14.29
fFOOD1	+	0.00
fFOOD2	-	0.00
fFOOD3	-	8.57
fPAINT2	+	5.71
fPAINT3	-	31.43
PSW	+	N
SF	+	N
sDWTP	-	N
FA	-	N
WOOD	-	N
BA	-	N
sIWTP	-	N
SA1	-	N
SA2	-	N

Mutagenicity was seen in the (acutely toxic) samples fPAINT2 and SF and in the (not acutely toxic) samples fFOOD1 and PSW. The SOS response (a measure for genotoxicity) was most upregulated after exposure to the acutely toxic samples fPAINT1 and fPAINT3, as well as the (not acutely toxic) sample fTEX.

The results confirm literature data on genotoxicity in paint (e.g. Cassini et al. 2011) and shredder waste composition (Gonzalez-Fernandez et al. 2007). Mutagenicity and genotoxicity biotests provide specific H7/H11 information that is not covered by acute toxic effect. However, the up and down regulation of the SOS response measured by BGPA cannot be quantified (yet) in terms of classification and further validation by comparison to reference substances is therefore needed.

6.4.3.2 Reproductive effects (H10)

Endocrine effects of the waste extracts were investigated using Calux. The ELS test was used to detect teratogenic effects. Results for both tests are summarized in table 6.13. Nine out of sixteen samples showed endocrine effects mediated through the estrogen and androgen receptors. From the remaining seven samples, SF induced a higher than average aryl hydrocarbon receptor activation (TCDD eq). Six samples did not show any endocrine activity above detection limits in either the ER or AR Calux assay and did not have a TCDD eq above average. Highest teratogenic effects were induced by all paint industry

samples, as well as samples SF, WOOD and sIWTP. Because of the small scale of the ELS test (4 fish embryo's tested per waste sample), anomalies in only one embryo (resulting in a score of 25% or less) are not considered positive for teratogenic effects (see also the summary of results in table 6.15). Positive samples are indicated in yellow.

Table 6.13: Results endocrine disruptive compounds (Calux) and teratogenic effects (ELS). Samples with high teratogenic activity (>50% ELS) and samples positive for endocrine disruptors (ER, AR Calux) are marked in yellow (LOD: limit of Detection) as well as samples with TCDD equivalent (DR Calux) above the mean of all samples.

Sample	ELS (% affected)	DR Calux ng TCDD eq.	ER Calux		AR Calux	
			Agonistic activity ng EEQ	Artagonist activity ng Tamoxifen eq.	Agonistic activity ng DHT eq.	Antagonistic activity µg Flutamide eq.
fTEX	25	0.403	0.12	7	<LOD	17
fPAINT1	100	2.119	<LOD	1885	<LOD	<LOD
fFOOD1	16.7	0.004	<LOD	<LOD	<LOD	<LOD
fFOOD2	25	0.033	<LOD	<LOD	<LOD	<LOD
fFOOD3	0	0.025	<LOD	<LOD	<LOD	<LOD
fPAINT2	100	3.812	4.21	<LOD	<LOD	748
fPAINT3	100	34.323	4.73	<LOD	<LOD	2429
PSW	0	51.33	<LOD	<LOD	<LOD	18
SF	100	64.41	<LOD	<LOD	<LOD	<LOD
sDWTP	0	<LOD	<LOD	<LOD	<LOD	230
FA	0	<LOD	<LOD	<LOD	<LOD	<LOD
WOOD	100	0.28	<LOD	<LOD	<LOD	<LOD
BA	8.3	0.91	<LOD	<LOD	<LOD	<LOD
sIWTP	100	25.32	7.93	<LOD	0.970	27
SA1	0	<LOD	<LOD	<LOD	<LOD	54
SA2	8.3	<LOD	<LOD	<LOD	<LOD	332

Substances with potential for reproductive impairment either by teratogenic effects or by hormonal disturbance, were detected in ten samples from varying sources such as the paint industry, shredder fluff, PCB containing shredder waste, wood and industrial WTP sludge. Earlier studies on wastes or effluent

from these industries and on PCB's have also shown reproductive effects, confirming the robustness of the bioassays (e.g. Li *et al*. 2011, Orrego *et al*. 2011, Tang-Péronard *et al.* 2011, Ghisari *et al. 2009*).

Again it is clear that these bioassays - addressing chronic endpoints – provide additional information next to assays for acute toxicity. Four not acutely toxic samples (i.e. PSW, sDWTP, SA1 and SA2) test positive for endocrine disturbing substances. This confirms that these mechanistic assays are needed to provide specific hazard information necessary for a complete assessment and classification of the waste. More validation is still needed and suitable limit values need to be further developed to allow these assays to be used as a tool in the tiered approach on waste classification. Especially samples such as SA1 and SA2, which unexpectedly test positive, illustrate the need for correct interpretation of the results.

6.4.3.3 Corrosive, irritating, sensitizing effects (H4/H8/H15): TNFα assay

Sample	pH	Concentration* (geq/l)
fTEX	8.09	1.33
fPAINT1	7.73	0.31
fFOOD1	7.91	9.24
fFOOD2	5.7	2.84
fFOOD3	7.49	5.78
fPAINT2	6.97	<0.29
fPAINT3	7.1	<0.28
PSW	6.8	0.2
SF	7.4	0.6
sDWTP	7.8	8.9
FA	9.4	9.2
WOOD	6	>>
BA	9.5	4.5
sIWTP	6.8	0.25
SA1	9.4	8.8
SA2	8.1	>>

Corrosive effects are expected to occur in cases of pH extremes (pH ≤ 2 or ≥ 11.5). Table 6.14 shows that none of the samples tested here have an extreme pH. However, while extreme pH indicates corrosive properties, the absence of extreme pH does not rule out corrosive, sensitizing or irritating effects. The TNFα test was used to investigate these properties further.

Table 6.14: Indicators for corrosive, irritating and sensitizing effects. pH and sample concentration(*) inducing an increase of TNF-α production of 1000 pg/ml. Results indicating high immunoreactivity are marked yellow (< 1geq/l).

Samples that increase TNF α production of cells are suspected to contain inflammatory compounds. This can be due to the presence of bacterial material or chemicals that act like antigens (potential allergens causing irritation and sensitization). Six samples already induce a high level of TNFα at low concentrations (see table 6.14): three paint industry samples, both shredder waste samples and sIWTP. Though no literature data are available on sensitizing effects of these types of wastes, studies have shown that PCB's induce inflammatory effects, supporting results from PSW (Hennig et al. 2002). Like the other mechanistic assays the TNFα assay also provides specific additional information on H4/H8/H15 next to the assays for acute toxicity (illustrated by sample PSW). More validation and development of suitable classification limit values is required.

6.4.3.4 General remarks on mechanistic toxicity assays

As compared to general toxicity, five out of seven not acutely toxic samples showed an effect in one or more of the mechanistic assays, confirming that mechanistic tests are needed to provide important additional toxicological information that is not apparent in acute tests like Microtox (table 6.15).

The responses in the mechanisitic tests clearly reflect the presence of hazardous substances with different modes of action, but the test results need to be translated into a classifier value. It would be useful to relate the observed effects to concentrations of a known reference substance, as is already the case for some toxic effects (e.g.: ER Calux results are already expressed as gram equivalents of estrogen or Tamoxifen, Microtox responses can be compared to DCP or benzene (Weltens *et al.* submitted)...). It is clear that the assays should be validated and additional studies should focus on suitable limit values for the proposed tests.

The incinerator ashes (FA and BA) showed no effects in any of the assays. According to the test strategy these sample are classified as non hazardous. Water eluates from incineration ashes also show low ecotoxicity, depending on incineration input (Barbosa *et al.* 2009), though the eluates only represent the bioavailable fraction and can therefore not be compared to the worst case extracts directly. Though the test battery presented here already provides a

hazard profile for most important potential risks, not all relevant toxicological endpoints are present yet. Therefore, the proposed testing strategy should be expanded as new tests become available to measure these missing endpoints (e.g. corrosive and infectious effects, specific carcinogenic mechanisms,…) meanwhile keeping in mind that some effects (e.g. embryonic implantation, long-term effects on fertility) may remain impossible to measure with fast in vitro tests.

Also practical issues need to be addressed. Test duration should decrease and automation will facilitate routine testing and batch screening of waste materials.

Table 6.15: Overview of sample toxicity

Samples	General toxicity (H5/H6/H14)	Reproductive effects (H10)		Corrosive, irritating, sensitizing (H4/H8/H15)	Genotoxicity, Mutagenicity (H7/H11)	
	Microtox	Teratogenicity (ELS > 25%)	Endocrine effects (ER, AR Calux)	TNFα upregulation	Mutagenicity	Genotoxicity
fTEX	+	-	+	-	-	+
fPAINT1	+	+	+	+	-	+
fFOOD1	-	-	-	-	+	-
fFOOD2	+	-	-	-	-	-
fFOOD3	+	-	-	-	-	-
fPAINT2	+	+	+	+	+	-
fPAINT3	+	+	+	+	-	+
PSW	-	-	+	+	+	Nm
SF	+	+	-	+	+	Nm
sDWTP	-	-	+	-	-	Nm
FA	-	-	-	-	-	Nm
WOOD	+	+	-	-	-	Nm
BA	-	-	-	-	-	Nm
sIWTP	+	+	+	+	-	Nm
SA1	-	-	+	-	-	Nm
SA2	-	-	+	-	-	Nm

6.5 Summary and Conclusions

A great improvement can be achieved in the assessment of complex waste using both chemical and biological in vitro methods. The proposed strategy is in line with modern toxicity testing (NRC 2007), replacing the current HWD methodology (where mammalian testing is referred to) by alternative effect based tests.

The proposed tiered approach involves as a first tier chemical analysis followed by a general toxicity screen as the second tier and subsequent mechanistic toxicity tests as the third tier. The first tier involving chemical analysis is recommended for the inorganic fraction of the waste, a framework for interpretation of inorganic results (also reckoning speciation of the elements) is called upon. For the organic fraction of the waste chemical analysis is recommended when known toxic substances are suspected. If not, chemical analysis may be limited to the inorganic fraction and assessment of the worst case organic extracts can be started directly with a general toxicity screen.

Microtox is a good screening test for the organic fraction: cheap, fast and transparent and it is proven to be the most sensitive bacterial test for acute toxicity of the organic contaminants. A validation report is in preparation (Weltens *et al.* in preparation). Although Microtox reflects acute general toxicity (H5/H6 and H14), these acute effects are often accompanied by chronic toxicity that is reflected in the mechanistic assays. These multiple pathway responses reflect the complexity of the toxic samples.

In the last tier, the selected mechanistic toxicity assays evaluate the presence of compounds with chronic effects: genotoxic substances, endocrine disruptive substances, teratogenic effects and immuno active substances. We demonstrated that these tests indeed provide important additional information needed for waste classification, next to the tier 2 acute toxicity tests. Further validation of these tests for waste assessment will allow the interpretation of test results to be simplified and assimilation of new biotests into the strategy as they are developed, will allow a number of missing endpoints to be addressed.

The tiered approach for waste assessment proposed here represents a vast improvement to the current often arbitrary hazardous waste classification and provides a solid and ethical alternative to the methods prescribed in the HWD.

CHAPTER 7

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

Chapter 7: Summary, Discussion and Future Perspectives

Biosensors are defined as sensors which incorporate a biological component, such as an enzyme, antibody, nucleic acid, microorganism or cell. As the wide range of possibilities of biosensors became clear and the demand for new biosensors for specific applications increased, the development of biosensors has grown tremendously.

To meet the continuously increasing demands in a wide range of applications, the development of innovative and versatile new biosensor technology (aimed at new targets, higher sensitivity, wide applicability, ease of use and/or low cost) is essential. A top down approach to biosensor development has the potential to provide new technology, applicable to a wide range of fields. On the other hand, present needs of (potential) biosensor users must also be addressed (i.e. bottom up approach). Thus the application of already validated technologies in new fields and the identification of gaps, where biosensor technology does not yet provide an answer, is of great importance.

In the work presented here, both the top down (Part I) and the bottom up (Part II) approach in biosensor development were addressed. In the first part, a novel whole cell sensing technology was explored to develop a versatile, widely applicable sensor aimed at the detection of very low concentrations of target. In the second part, existing (bio)tests were selected and evaluated for application in the field of waste assessment. This way well validated technologies are implemented in a testing strategy to attain fast and correct classification of complex waste, and missing endpoints were identified. In the following sections, the results and future prospects are summarized and discussed.

Part I:
Development of a novel whole cell biosensor prototype

In Part I a novel targeted whole cell sensor for detection of low concentrations and with a wide applicability was aimed for. This was achieved by coupling two intrinsic advantages of cells i.e. their sensitivity and the cellular signaling cascade which amplifies the binding signal to the wide applicability of antibody based biosensors.

We proposed to implement chimeric receptors, which couple the signaling chain of a cell receptor, to the recognition domains of antibodies, thus broadening the range of potential targets, while retaining the typical sensitivity of reporter gene assays. The development of this novel biosensor prototype was done in several phases:

1. Chapter 2 describes how the chimeric receptor was designed and constructed.
2. Second, the reporter system was designed and constructed, as described in chapter 3.
3. Finally, the reporter construct is coëxpressed with the chimeric receptor and the resulting whole cell biosensor is tested. The results of the integration experiments involving the completed whole cell biosensor prototype are presented in chapter 4.

In the following sections, chapters 2 to 4 are summarized. The advantages and remaining challenges for the whole cell sensor prototype are discussed and future prospects are suggested.

7.1 The chimeric receptor constructs

In chapter 2, two chimeric receptors are introduced for biosensing purposes for the first time. The first is a TCR based receptor cassette, with PSA as a proof of principle target. The second receptor is an EPOR/gp130 based receptor couple, with HEL as a ligand. The structure of the resulting recombinant receptors is summarized in table 7.1.

Each receptor is characterized by an antibody (Ab) derived recognition domain, either a single chain Ab (TCR based receptor construct) or the Ab heavy and light chain recognition domains, positioned in the extracellular region of a receptor couple (as is the case for the cytokine receptor constructs). The affinity of the antibody for the target strongly influences the sensitivity of the sensor, but also the accessibility of the recognition domains is of great importance. This is ensured by the spacer and hinge regions of the TCR based construct and the D2 domain of the EPOR/gp130 based construct (the latter is also necessary for dimerization of the receptor upon target recognition).

Table 7.1: Functional components of the chimeric receptors.

region	TCR based receptor cassette	Cytokine receptor based receptor couple	
		EPOR based monomer	EPOR/gp130 based monomer
Extracellular region	• Anti-PSA single chain antibody • IgG hinge region • CD28 spacer region	• anti-HEL heavy chain recogrition domain • EPOR D2 domain	• anti-HEL light chain recognition domain • EPOR D2 domain
Transmembrane (TM) region	CD28 TM domain	EPOR TM domain	gp130 TM domain
Intracellular region	•CD28 cosignaling domain • TCRζ signaling chain	EPOR signaling chain	gp130 signaling chain

The biorecognition domain is separated from the other parts of the receptor construct by single cutting restriction sites, thus allowing these domains to be exchanged according to the application of the sensor. The interchangeability of the recognition domains forms the key to the versatility of this biosensor prototype.

Transmembrane and signaling domains are included in the construct to ensure that the biorecognition event between the target and the recognition domain initiates downstream signaling. The TCR based receptor cassette relies on the CD28 and TCRζ signaling domains for signal propagation, whereas the cytokine receptor based construct employs gp130 and EPOR signaling domains. The initiation of the intracellular signal cascade by the cytoplasmic tails of the receptor constructs is essential for the whole cell biosensor, and the threshold of activation of this signaling cascade contributes strongly to the biosensor sensitivity.

The two types of chimeric receptors presented in chapter 2 have inherited certain specific characteristics from their wild type counterparts. Thus, for the TCR based constructs, the complex activation event necessary for wild type TCR signaling must be taken into account, as wild type TCR normally requires activation by the Major Histocompatibility Complex (MHC) of an antigen presenting cell, involving several possible cofactors. The CD28 coreceptor was incorporated to mimic natural activation of the TCR pathway and circumvent the need for MHC proteins. Even so, some concern remains that the threshold for activation of the chimeric TCR remains higher than the threshold expected for cytokine receptor based sensors. Indeed, the natural cytokine receptors have no need for co-activation, and are stimulated only by a free ligand to induce the receptor pathway. Additionally, cytokines, the natural ligands of the cytokine receptors elicit an effect at very low concentrations, in the pg/ml range (e.g. McDade 2012). These considerations led to the choice of the EPOR/gp130 based construct as a first choice for the development of the biosensor prototype.

The structure of the chimeric cell receptor determines the versatile character of the whole cell sensor which was developed, as well as strongly influencing the sensitivity of the sensor through the recognition domain affinity and through the signaling activation threshold. However the receptor by itself cannot amplify the initiated signal, nor can it produce an observable output signal. To achieve this, the chimeric receptor must be expressed in a suitable cell line and the cell needs a reporter system which is responsive to the receptors signaling cascade.

The EPOR/gp130 chimeric receptor is expressed in the IL3 dependent BaF3 cell line, resulting in the modified Ba/HE+LgIGFP cell line. This cell line forms the basis for the further development of the sensor.

The reporter system was designed and constructed as presented in chapter 3.

7.2 Design of the reporter system

The most important characteristic of the reporter system is that it should respond to the signaling cascade initiated by the anti-HEL EPOR/gp130 receptor construct. To achieve this, an EPOR/gp130 inducible promoter was selected and then coupled to a luciferase reporter gene.

Identification of a panel of candidate promoters

Suitable promoters for the reporter system were selected in two phases: first, a panel of candidate promoters was identified. The promoters in this panel are either known to be induced by the JAK/STAT signaling cascade, which is common to the type I family of cytokine receptors such as EPOR and gp130, or were shown to be induced by IL3 in the IL3 dependent BaF3 cell line. The latter promoters are highly relevant because IL3 receptor signaling in the BaF3 cell line overlaps with gp130 and EPOR signaling. This way, 13 candidate promoters were included in the panel and investigated further.

Selection of promoters showing the highest upregulation by HEL

After selecting the panel of candidate promoters, gene expression profiling experiments were conducted on the Ba/HE+LgIGFP cell line to identify the promoters showing the highest upregulation upon HEL induction from the panel of candidate promoters. The Ba/HE+LgIGFP cells were employed in exposure assays and the effects of HEL on the expression profiles of the candidate promoters were investigated. In total six genes were significantly upregulated by HEL: CISH, SOCS1, SOCS2, SOCS3, Spry1 and gpr34, however the strongest effects were observed for CISH, SOCS1 and SOCS3. There was no significant

difference in leakage between the three promoters, so all three were investigated further to establish cross-activation by other ligands.

Specificity on the transcriptional level

Cytokine induced cross-activation of the candidate promoters CISHp, SOCS1p and SOCS3p was investigated by RT qPCR, as discussed in chapter 3. Results indicated that none of the three promoters were solely activated by HEL. As was expected, the strongest cross-activation is elicited by IL3, the natural growth stimulant of the IL3 dependent BaF3 cell line. But other cross-talk events were also observed. CISHp is activated by IL5 and IL6, SOCS1 is activated by EPO and SOCS3 is induced by IL3 and IL4.

The observed cross-talk did not come as a surprise, as cytokine receptors often employ the same or similar signaling cascades, mediated by the JAK and STAT families of proteins. The IL5 induced activation of SOCS3p was the most remarkable: the effect of IL5 on SOCS3p was in fact higher than the activation by the chimeric anti-HEL receptor couple. This phenomenon may be explained by the autocrine induction of IL5 receptor (IL5R) in the Ba/HE+LgIGFP cell line. Also, the IL5R is closely related to the IL3R and signaling occurs through the same pathways, which may explain why a response mediated by the IL5R is so large in the IL3 dependent cell line.

Though cross-talk is essentially unwanted in a biosensor setting and affects the specificity of the sensor negatively, many cell based sensors encounter this problem, and it may be circumvented in the final biosensor set-up by conducting parallel experiments or by depleting samples of cross-talk causing molecules.

Selected promoters

The promoters of the three genes which were most strongly and quickly activated (i.e. CISHp, SOCS1p and SOCS2p) are most promising, and were shown to be sufficiently specific for biosensor purposes. In the proof of principle setting the CISH promoter was employed, leaving SOCS1p and SOCS3p for future explorations.

7.3 Proof of principle

Assemblage of the biosensor prototype

In chapter 4, the whole cell biosensor prototype is assembled, characterized by the coexpression of the chimeric anti-HEL EPOR/gp130 receptor and the CISHp/luciferase reporter plasmid.

The CISHp/luciferase plasmid is constructed, and then expressed in the Ba/HE+LgIGFP cell line. As initial attempts to use the selectable pGL4.14/CISHp luciferase expression plasmid failed, we turned to cotransfection of the pGL3/CISHp luciferase expression plasmid with pREP9, a selection plasmid containing the Neomycin resistance gene. Optimization of transfection conditions resulted in Ba/C9 cells, expressing both the anti-HEL chimeric receptor and the CISHp inducible luciferase reporter.

Thus all necessary components for detection of target (i.e. HEL) are present: the chimeric receptor allows biorecognition of the target and translates this biorecognition event into an intracellular signal. Subsequently, the intracellular signaling cascade amplifies the original signal and activates CISHp, which in turn initiates transcription of the luciferase gene. Ultimately, the concentration of luciferase reflects the concentration of target in the cells environment. Finally proof of principle assays were conducted to evaluate the sensitivity and the specificity of the Ba/C9 whole cell biosensor.

Biosensor sensitivity and specificity

The whole cell sensor showed an increase in luciferase expression upon exposure to 10µg/ml of target, within 2 hours of exposure. However the dynamic range of the sensor is small and it shows a limited sensitivity. Also, the background luciferase signal is quite high.

Cross-talk was observed for IL3, as was expected in the IL3 dependent cell line, but other cytokines did not induce a significant signal, implying that the specificity of the sensor is high.

Though we achieved the main goal of developing a whole cell biosensor which detects targets via a chimeric receptor, some challenges still remain before this biosensor meets all the requirements of a sensor for detection of very low concentrations. The results showed that the sensitivity of the sensor does not equal the sensitivity of unmodified cytokine receptors: target was only detected at a concentration of 10 µg/ml (70 nM), whereas natural cytokine receptors elicit a response in the pg/ml range (e.g. McDade 2012), four orders of magnitude lower. A sensitivity of two orders of magnitude lower is desirable for the detection of allergens. Current state of the art detection of peanut allergen (Ara h1) for example, reaches a sensitivity of 0.09 µg/ml, with a dynamic range of 0.1 to 2 µg/ml (Pollet et al., 2011). The dynamic range of our biosensor prototype was demonstrated to be very narrow and should be improved to facilitate quantitative detection of target.

The specificity of the biosensor was observed to be good, though IL3 is an important cross-talk ligand. This was to be expected, as the original cell line which was used for the development of the sensor is IL3 dependent. Other cytokines induced some cross-talk at the transcriptional level according to RT qPCR data, however this was not observed for the finished sensor. This discrepancy may be explained by the limited sensitivity of the sensor, and cross-talk should still be investigated when optimizing the biosensor.

7.4 Future Prospects

The shortcomings discussed above should be dealt with to improve the whole cell affinity sensor. The most important challenge is the limited sensitivity of the sensor as compared to the natural receptor. This may be caused by different parts of the constructed biosensor signaling chain: on the one hand the receptor and signaling domains which determine signal initiation and propagation, and on the other hand the promoter and reporter plasmid. We propose four suggestions for improvement of the sensor sensitivity and lowering the background signal:

1. *Mutations in the receptor transmembrane domain:* Kawahara and colleagues (2004) demonstrated that the background growth signal in unliganded chimeric EPO receptors may be partly due to the replacement of EpoR D1 domain by the antibody variable domains. Mutations in the transmembrane domain reverse this effect, leading to a stricter on/off switch of receptor signaling.
2. *Alternative promoters*: aside from CISHp, other promoters such as SOCS1p and SOCS3p are rapidly induced by the EPOR/gp130 pathway. Substituting CISHp with a stronger promoter would increase the sensitivity and this option certainly needs to be explored. Also, wider gene profiling of the modified Ba/HE+LgIGFP cells (e.g. by microarray) may yield other interesting promoters which were not included before.
3. *Switching antibody domains:* the affinity of the antibody recognition domains certainly has an important effect on the biosensor sensitivity. Thus, switching these domains with recognition domains from an antibody with a higher affinity for its ligand would be expected to yield a more sensitive biosensor. However, the HyHEL antibody which was used in the recombinant receptor construction is already high affinity antibody (Padlan et al, 1989, Li et al. 1996), suggesting that there is little room for improvement in this area.
4. *Improved transfection efficiency:* to circumvent the difficulties which were met concerning the construction of the reporter plasmid, we cotransfected a (non-selectable) reporter plasmid with a selection plasmid (containing the neomycin resistance gene). Though this allowed

the proof of principle to be reached, more efficient and more stable transfection may be reached using an alternative reporter plasmid including an antibiotic resistance gene. This would result in more efficient selection of transfected cells and thus in a higher ratio of cells expressing the reporter gene upon exposure to target.

Aside from the optimization of our cytokine receptor based whole cell biosensor prototype, other chimeric receptors should be considered, such as T-cell receptor based constructs, which are currently employed for cancer research. However cytokine receptors remain the more logical choice as these naturally respond to low concentrations of ligands.

Living cells have been proven to be a good choice in the search for new, sensitive sensing technologies. Reporter gene assays are already widely applied in laboratory settings as well as commercially (e.g. CALUX®, CANARY), and the development of these types of biosensors with a wider applicability remains a promising area of research. Though there is still need for improvement, the potential of these types of sensors is very large, and certainly worth exploring.

Part II

DISCRISET: Application of biosensors in waste toxicity screening

In the previous chapters, advances in technology have driven us to explore the limits of whole cell biosensing. This top-down approach may lead to widely applicable new developments. In the next section, instead of a top down approach, a bottom up approach is implemented to answer to a pressing need in one particular field, i.e. the waste treatment industry.

Current waste assessment legislation as prescribed by the Hazardous Waste Directive (HWD) for the classification of hazardous waste is based upon information on the hazardous properties of identified individual waste compounds or - if not all compounds are identified - on results of hazard assessment tests on the waste material itself (direct testing). The methods recommended in the HWD for the direct testing of toxicological properties of waste are the acute and chronic animal tests that are used for hazard assessment of chemicals (CD 67/548/EC (dangerous substances), 726/2004/EC (pharmaceuticals), EC/1907/2006 (REACH), CD 98/8/EG (biocides)). However, these methods are not suitable for the application of waste classification as they are unethical, costly, time consuming and aimed at irrelevant exposure routes. For these reasons, at present no direct tests are applied and complex wastes are often arbitrarily classified.

Keeping in mind the potential severe consequences for human health and the ecology in case of wrongful classification, as well as the economic implications of the current arbitrary classification, there is an urgent need for a more suitable and solid framework for waste assessment. The aim of the current project was the development of a fast, economical and solid waste assessment strategy for correct evaluation of waste toxicity. The development and evaluation of this strategy is described in chapters 5 and 6.

7.5 Development of a waste assessment strategy

For the development of a test strategy for hazard assessment of complex waste, alternative methods for waste screening are introduced and evaluated (see chapter 5).
The tools that are reviewed are (a) chemical methods and affinity based biosensors that identify (groups of) toxicants (targeted analyses), (b) in vitro methods that originally were developed for screening hazardous properties of pharmaceuticals/chemicals, and (c) ecotoxicity tests. (b) and (c) are effect analyses, usually employing whole cell sensors or lower organisms such as algae or Daphnia. It has become clear that the chemical and biological screening methodology is extensive (e.g. NATIBO, 2001, Eisenbrand et al., 2002; Krist et al., 2005, Witters et al., 2005; EPA, 2005; Allan et al., 2006) and new applications and techniques are emerging continuously (Riedel et al., 2003; NRC, 2007; Imec-LINC, 2008).

The evaluation of the waste is based on the following a priori conditions: (a) the classification has to be in accordance with HWD principles i.e. based on total concentrations and based on the defined hazardous toxicological properties, and (b) results need to be generated within short time (preferentially 48 hours) and at economically feasible prices. The latter conditions are important to allow batch controls, and to prevent large volumes of waste piling up at the plant (leading to occupation of space and/or odour problems). Also (c) a high level of standardization is needed because in practice, the test results will be compared to preset limit values. Based on these criteria, 16 biotests are selected to be tested in a pilot study on complex waste samples from a wide range of sources, alongside chemical analyses. Some of the 16 tests that do not always fulfill the (b) criterion are nevertheless selected for pragmatic reasons, i.e. to cover as many of the toxicological hazard properties as possible, but the needs for new or improved (faster) tests is also argued.

A tiered testing strategy is suggested to minimize time and cost of analysis, allowing classification of very toxic samples in the earliest stages of the testing strategy and moving on to chronic toxicity in the latter stages.

7.6 Pilot study: application of the novel testing strategy

In the pilot study, 16 waste samples from a wide range of sources were tested according to the new screening strategy. The tiered approach involves first, a chemical analysis, where both the organic and inorganic fractions were investigated, followed by a general toxicity screen employing a acute toxicity biotest. Finally, in the last tier, mechanistic toxicity (i.e. genotoxic substances, endocrine disruptive substances, teratogenic effects and immuno active substances) is investigated.

7.7 Discussion and future prospects

Results demonstrate that chemical analysis should be limited to the inorganic fraction, unless known organic toxic substances are suspected, because the organic chemical analysis is inconclusive: many of the molecules detected by GC-MS cannot be identified and additional steps are called upon to yield a quantitative result. Also, a framework for the interpretation of the inorganic fraction is needed, which brings into reckoning the speciation of elements, as this largely determines toxicity. For the general toxicity screen, Microtox® is shown to be a good biosensor for the organic fraction. It is the most sensitive of the tested bacterial assays, and the acute effects detected by Microtox® are predictive for chronic toxicity. Importantly, it is a cheap and transparent test and delivers results in only 30 minutes.

The mechanistic toxicity tests employed in the last tier of the strategy provide important additional information on chronic toxicity, needed for waste classification. Further validation of these tests is called upon to allow easy interpretation of results. Also a number of missing endpoint must still be addressed, such as sensitizing, irritating and corrosive effects, as well as teratogenic effects and effects on embryo development. Progress in understanding the mechanism of skin sensitization for example, such as effects on the production of cytokines by different cell types in the skin, provides the opportunity to develop in vitro tests as an alternative to in vivo sensitization testing (e.g. Galbiati & Corsini 2012, Hofmann et al., 2013). Also, tests for

teratogenic effects and effects on embryo development are being developed (e.g. Lazarri et al. 2008) but still need to be validated further before being assimilated into the waste assessment strategy. Projects aimed at the development of alternative, in vitro tests for these endpoints, such as the Sens-it-ive (www.sens-it-iv.eu) project and the ReProTect (www.reprotect.eu) project, have an important role to play in this field.

Alongside the development of tests for missing endpoints, there is still an urgent need for faster and more automated tests for all toxic endpoints. While some mammalian cell based biosensors can be optimized to deliver a result within a day or less (e.g. Calux, which can be read out after 4 to 6 hours), most of these types of cell based sensors remain slower than bacterial tests such as Microtox®. However bacterial tests are less representative for mechanistic toxicity than mammalian cells. Thus the development of faster methods, especially for mammalian cell based sensors, remains necessary. Real time monitoring of cells may lead to still faster mammalian cell based biosensors but requires specific technology. While state of the art microscopes enable real time monitoring of cell viability, motility and morphology, this requires an extremely expensive infrastructure. Impedimetric sensing platforms form an example of a much cheaper technology which enables real time monitoring of certain cellular characteristics such as cell motility, which may be used to investigate certain toxic effects (e.g. Tarantola et al., 2011; Hoffman et al., 2013). Recent research by Curtis and colleagues (2013) has even suggested a portable impedance and cell based toxicity sensor. This is just one example of technology which, though it still requires much research, has the potential of strongly reducing testing time and cost and which holds great promise for certain aspects of hazard assessment.

Alternatively, the validation and targeting of structural alerts (molecular structures which elicit specific biological effects, e.g. validation study by Rodriguez-Sanchez et al., 2013) may lead to fast, cheap and relevant biorecognition assays (as opposed to effect sensors). Such assays can make use of antibodies, aptamers (Sett et al., 2012) or MIPs (Molecular Imprinted Polymer; Mustafa & Lieberzeit 2013) as biorecognition tools. Moreover

aptamers and MIPs have the advantages of being cheap in production and also stable in more challenging environments such as waste samples. Not only cheap and stable biorecognition molecules are required, also the antibody based associated sensing platforms need to be fast and effective such as aptasensors (e.g. Tran et al., 2011) or MIP (Molecular Imprinted Polymer) based sensing platforms (e.g. Geerets et al., 2013).

Aside from the development of new biosensors and the validation of existing biotests, the most important remaining challenge is the development of a framework with new toxicity thresholds for the biotests. Microtox® has already been validated further and a reference value suggested (Weltens et al., submitted), providing the first important step in a solid and ethical alternative to the methods prescribed in the HWD.

According to Eurostat Statistics, we are producing 3 billion tons of waste per year in Europe, and this amount is increasing every day. The negative consequences of an insufficiently strong framework for waste classification is enormous. Health and ecological reasons are to be considered, as well as the severe economic impact of an arbitrary classification of (hazardous) waste. The tiered waste assessment strategy proposed here provides a solid, practical and ethical alternative to the methods prescribed by the HWD, and represents a vast improvement to waste classification today.

CONCLUDING REMARKS

The development of an innovative targeted whole cell sensor in the first chapters exploits the possibilities that advances in technology have offered and represents an attempt to optimize cells and cellular processes to expand the potential applications of whole cell sensing in a bottom up approach to biosensor development. Thus, we developed a whole cell biosensor prototype based on a chimeric cytokine receptor couple and expressing luciferase upon exposure to the target HEL. The sensor can be adapted to other targets by exchanging the antibody recognition domain of the chimeric receptor, implying a wide range of possible applications. In the future, this type of whole cell sensor may target markers for the missing endpoints discussed in part II. The biosensor must be optimized further as discussed in §7.4.4 before its full potential is reached. The whole cell sensor prototype developed here demonstrated the large potential of whole cell sensors, which certainly deserves further exploration.

In part 2, a different approach is introduced. Here, an important and urgent need expressed by the waste treatment industry is addressed in the DISCRISET project, by applying existing, often whole cell biotests in a new field. Where no tests (yet) exist or are suited to the a priori conditions coupled to this application, gaps are identified. These missing endpoints represent the future challenges in biosensor development and identifying them is of the utmost importance to fuel bottom up biosensor development.
The waste assessment strategy which was developed is a tiered testing strategy, involving first, a chemical analysis, where both the organic and inorganic fractions are investigated, followed by a general toxicity screen employing a acute toxicity biotest. Finally, in the last tier, mechanistic toxicity (i.e. genotoxic substances, endocrine disruptive substances, teratogenic effects and immuno active substances) is investigated. This strategy maximizes the identification of hazardous waste while minimizing the cost and time spent. Great care was taken to choose biotests suited to the a priori conditions coupled to routine waste assessment, and where no suitable tests existed gaps have been identified which may fuel bottom up biosensor development.

The development of whole cell biosensors holds great potential, both in a top down approach where sensor versatility implies a wide applicability, and in a bottom up approach where new applications create new needs, and point biosensor development in a specific direction. Whole cells are carefully orchestrated factories, their signaling has been fine-tuned by evolution and the speed and complexity of the signaling cascades are astounding. It stands to reason that science has not yet explored the full potential of living cells in the relatively new field of biosensor development, however cells are in effect natural biosensors, constantly detecting molecules in their environment and translating molecular interactions into physiological outputs. As such, cellular biosensors certainly deserve the unrelenting attention and admiration of biosensor researchers.

ADDENDUM

Description of genes selected for RT qPCR screening

CISH and SOCS

The CISH/SOCS family proteins are negative regulators of cytokine signaling, capable of blocking cytokine signaling by acting as (i) kinase inhibitors of JAK proteins, (ii) binding competitors against STATs and (iii) by acting as ubiquitin ligases, thereby promoting the degradation of their partners.
CISH was the first CISH/SOCS gene identified, and was shown to be a negative-feedback regulator of the STAT5 pathway (Yoshimura et al. 1995, Matsumoto 1999). CISH binds to phosphorylated tyrosine residues of cytokine receptors such as the erythropoietin receptor, IL-3 receptor β chain, IL-2 receptor β chain (Aman et al., 1990), growth hormone receptor (Hansen et al, 1999) and prolactin receptor (Pezet et al., 1999, Tonko-Geymayer et al., 2002, Endo et al., 2003) through the SH2 domain, thereby masking STAT5 docking sites.
Both SOCS1 and SOCS3 inhibit JAK activity. Both proteins contain a similar kinase inhibitory region (KIR) at the N-terminus that is essential for JAK inhibition (Yasukawa et al., 1999, Sasaki et al. 1999). SOCS1 binds directly to JAK through the SH2 domain, whereas SOCS3 has been shown to bind to Tyr757 of gp130 and Tyr401 of the erythropoietin receptor (Schmitz et al., 2000, Sasaki et al., 2000, Hortner et al. 2002). Extensive reviews of cytokine downregulation are given by Inagaki-Ohara (2003) and by Yoshimura (2005). CISH, SOCS1 and SOCS3 genes were all shown to be induced by IL3 in BaF3 cells (Basham 2008).

SOCS2 has been associated with the regulation of GH, IGF-1, PRL, IL-2, IL-3, EPO, LIF, EGF, leptin and IFN-a-dependent signaling pathways, either positive or negative (Rico-Bautista et al. 2005, Greenhalgh et al. 2002 and 2005). Expression is induced and/or enhanced by a wide number of hormones and cytokines in several biological systems, including IL3, EPO and gp130 mediated pathways such as IL6 and LIF (Starr et al. 1997, Dogusan et al. 2000). An elaborate review of SOCS2 function and induction in various tissues is given by Rico-Bautista et al. (2006).

Inhibition of cytokine pathways by CISH, SOCS1, SOCS2 and SOCS3 has been investigated extensively, whereas relatively few data have been generated to characterize SOCS4 to SOCS7. Human SOCS5 is expressed in many tissues, including heart, brain, placenta, and skeletal muscle, but its expression is especially high in lymphoid organs such as spleen, lymph nodes (LN), thymus, and bone marrow (BM) (Magrageas et al., 2000), indicating that SOCS5 might play a role in lymphocyte development or function. Brender and colleagues (2004) found elevated SOCS5 levels in B-cell development however the exact function was not discovered. In addition, SOCS4 and SOCS5 share significant homology and therefore may have similar or overlapping functions in some cell systems, which complicates research into their functions (Brender et al. 2004).

The CISH/SOCS family of proteins are normally induced by the JAK/STAT pathway, and as such these should be induced by HEL stimulation of the recombinant receptor pair. Therefore, the promoters of CISH and SOCS1 to 7 were included in the screening experiment.

c-Myc

According to microarray data, c-Myc upregulation by IL3 in BaF3 cells was only 1.43 fold (GDS3349). However, the c-myc gene is known to be induced in response to the proliferative signals elicited by cytokines, including EPO and IL-6, implying that the EPOR/gp130 receptor pathway activates the c-myc promoter (Henriksson and Luscher, 1996). Kiuchi et al. (1999) showed that STAT3 mediates the induction of the c-myc gene upon gp130 stimulation. As such, the c-myc promoter may still be an interesting candidate for the reporter system of the whole cell biosensor and was included in the promoter screening.

Spry and Spred

The Spry (Sprouty homolog) proteins were identified in mammals as negative feedback regulators of fibroblast growth factor signaling in angiogenesis (Minowada et al. 1999) and embryogenesis (de Maximy et al. 1999; Tefft et al.

1999). The closely related Spred (Sprouty-related EVH1 domaincontaining protein) protein family was identified by Wakioka and colleagues as negative feedback regulators of the ERK/MAP kinase pathway (Wakioka et al. 2001). Both these genes are induced by cytokines and though no specific information could be found on gp130 or EPOR induction of these promoters, Spred2 was upregulated 11.3 fold by IL3 stimulation according to microarray data (GDS3349, see table 3.1). Seeing as the signaling pathways of IL3R and the recombinant EPOR/gp130 receptor are similar, spred 2 as well as two other members of the sprouty family of proteins were included in the initial screening experiments.

Pim

Shirogane et al. (1999) identified the proto-oncogenes Pim-1 and Pim-2 as targets for the gp130-mediated signaling pathway. Pim-1 was also shown to be upregulated more than five fold by the IL3 pathway in BaF3 cells (GDS3349) and therefore included in the initial promoter screening.

gpr34

The X-chromosomal GPR34 gene encodes an orphan G1 protein coupled receptor that is highly conserved among vertebrates (Marchese 1999). Several publications suggested lysophosphatidylserine (lyso-PS) as the receptor ligand (Sugo et al. 2006, Iwashita et al. 2009, Kitamura et al. 2012), but recent evidence shows lyso-PS has only random agonistic activity at some GPR34 orthologues and the endogenous agonist is unknown as yet (Ritscher et al. 2012). Gpr34 function appears to be required for an adequate response of the immune system to antigen and pathogen contact (Liebscher et al. 2011). Baens and colleagues (2012) showed that Gpr34 is upregulate in B-cell lymphoma and overexpression of GPR34 in lymphoma and HeLa cells resulted increased cell proliferation (Ansell et al. 2012). Though the exact signaling pathway leading to gpr34 expression is not known, microarray data showed gpr34 expression to be induced 7-fold in IL3 stimulated BaF3 cells (GDS3349). This leads us to include gpr34 in the screening experiments.

Etv5

Etv5 is a member of Etv4 subfamily of E26 transformation-specific (Ets) transcription factors that influence a host of biological processes, including growth control, transformation, T-cell activation, and developmental programs in many organisms. Etv5 plays an important role in male reproduction (Chen et al. 2005), as well as female reproduction (Eo et al. 2008), but is also involved in processes as diverse as limb bud development in vertebrate foetusses (Mao et al. 2009), kidney development (Lu et al., 2009) and it is a marker for increase risk of obesity (Sandholt et al. 2011). Etv5 has not been linked to gp130, EPO or IL3 signalling before, however according to micro-array results, IL3 exposure of BaF3 cells induces 6.54 fold etv5 promoter activity (GDS3349). This indicates that etv5 might be induced by cytokine signaling pathways in BaF3 cells and the induction by HEL of the EPOR/gp130 pathway may also activate it's promoter. Therefore, etv5 is also investigated further in the promoter screening experiments.

Bcl-X_L

Bcl-XL is an anti-apoptotic member of the Bcl-2 of central regulators of apoptosis (Chan & Yu 2004). BclX_L is induced by a range of cytokines including IL6 (Lotem & Sachs 1995, Schwarze & Hawley 1995), IL3 (Leverrier et al. 1997) and EPO (Socolovsky et al. 1999). Therefore, Bcl-XL is also expected to be induced by the recomninant EPOR/gp130 pathway and was also included in the promoter screen.

CRBN

Cereblon is widely expressed in testis, spleen, prostate, liver, pancreas, placenta, kidney, lung, skeletal muscle, ovary, small intestine, peripheral blood leukocyte, colon, brain and retina (Xin et al. 2006, Jo et al. 2005, Higgins et al. 2010, Aizawa et al. 2011). It plays an important role in binding proteins for degradation, in cell survival, in memory and learning and in energy balance and

fetal development (Chang and Steward 2011). However, little is known of the pathways leading to CRBN expression. Microarray data showed a 6.22 fold increase in CRBN mRNA in IL3 treated BaF3 cells (GDS3349), indicating a cytokine induced activation of it's promoter. Therefore the induction of CRBN by the HEL induced pathway will be included in the screening experiments.

NEDERLANDSTALIGE SAMENVATTING

Biosensoren worden algemeen beschreven als sensoren met een biologische herkenningscomponent, een affiniteitsmolecule, gekoppeld aan een elektronisch of optisch uitleessysteem. Bij “whole cell” biosensoren is het biologische herkenningselement een levende cel, die bovendien het aanvankelijke herkenningssignaal vermenigvuldigt via een signalisatieketen binnen in de cel. Uiteindelijk wordt het cellulaire signaal omgezet in een waarneembare uitkomst via veranderingen in de viabiliteit, morfologie, motiliteit of het metabolisme van de cel, of via een reportereiwit. Naast deze traditionele detectie van een specifieke molecule (of eventueel een groep structureel verwante moleculen) worden cellulaire biosensoren ook ingezet voor effectmetingen. Effectsensoren vertalen het cumulatieve effect van alle omgevingsfactoren in een uitleesbaar outputsignaal. Dergelijke metingen worden veelvuldig ingezet voor toepassingen waar niet één doelwitmolecule van belang is maar het effect van een complex mengsel, zoals bij toxiciteitsmetingen, cosmeticatesten of drugscreening. Vooral zoogdiercellen zijn geschikt om in vivo effecten te voorspellen.

Deze beide types van cellulaire sensoren, effectsensoren en op een doelwitmolecule gerichte biosensoren, worden in de hier voorgestelde studie aangehaald. Enerzijds wordt de ontwikkeling van een nieuwe, hybride cellulaire affiniteitssensor beschreven, en anderzijds wordt de toepassing van bestaande effectsensoren in een nieuwe strategie voor afvalscreening voorgesteld.

DEEL I: Ontwikkeling van een nieuwe breed toepasbare cel gebaseerde biosensor voor detectie van lage doelwitconcentraties

Levende cellen worden al veelvuldig ingezet voor de detectie van lage concentraties aan natuurlijke liganden. Een dergelijke cel gebaseerde biosensor bindt met een celreceptor de doelwitmolecule, veroorzaakt zo een interne signalisatie cascade die uitmondt in een uitleessignaal. Aangezien celreceptoren voor hun functie vaak een hoge gevoeligheid hebben (cytokine receptoren bijvoorbeeld detecteren cytokines in de picomolaire grootteorde), zijn deze uitstekende mediatoren voor het bindingssignaal. Er is echter ook een nadeel

verbonden aan detectie via natuurlijke receptoren: in tegenstelling tot sensoren die gebaseerd zijn op affiniteitsmoleculen zoals antilichamen, zijn celgebaseerde sensoren die gebruik maken van natuurlijke receptoren, voor hun toepasbaarheid beperkt tot de natuurlijke liganden van deze receptoren.

Deze beperking werd echter in andere toepassingen van celreceptoren reeds omzeild: onder andere binnen het onderzoek naar celsignalisatie en in kankeronderzoek werden reeds chimere receptoren ingezet. De signalisatie domeinen van deze receptoren worden gekoppeld aan de herkenningsdomeinen van antilichamen, om zo de signalisatieketen te activeren door middel van een nieuwe doelwitstof. Deze technologie laat toe om de gevoeligheid van cellulaire sensoren te koppelen aan de brede toepasbaarheid van antilichaam gebaseerde sensoren.

De huidige studie beschrijft in het eerste deel de ontwikkeling van een dergelijke celgebaseerde biosensor, die gebruik maakt van chimere celreceptoren voor een brede toepasbaarheid. Om dit doel te bereiken worden drie fases doorlopen:

1. De chimere receptor werd ontworpen en geconstrueerd. De resultaten van deze fase zijn terug te vinden in hoofdstuk 2.
2. Er werd een reportersysteem ontwikkeld, zoals beschreven in hoofdstuk 3.
3. Als laatste werden de chimere receptor en het reporter construct samen tot expressie gebracht en de resulterende biosensor uitgetest. De resultaten van de experimenten met het cel gebaseerde biosensor prototype worden voorgesteld in hoofdstuk 4.

I. Chimere receptorconstructen

In hoofdstuk 2 werden twee chimere receptoren voorgesteld in de ontwikkeling van een nieuwe biosensor technologie. Enerzijds gaat het om een recombinante T-cel receptor (TCR), met Prostaat Specifiek Antigen (PSA) als doelwitmolecule, en anderzijds werden recombinante cytokine receptor constructen voorgesteld. Hier gaat het meer bepaald om een Erythropoietinereceptor (EPOR) gebaseerd construct en een glycoproteïne 130 (gp130) gebaseerd construct, die beide

nodig zijn om via heterodimerizatie de doelwitstof Hen Egg Lysozyme (HEL) te detecteren.

Beide receptoren worden gekenmerkt door het herkenningsdomein van een antilichaam (Ab), dat zich in de extracellulaire regio van het receptorconstruct bevindt. Deze regio bepaalt de specificiteit voor de doelwitmolecule van de biosensor en beïnvloedt ook de gevoeligheid, via de affiniteit van de antilichaamdomeinen. Het is dan ook van groot belang dat deze regio van het receptor construct goed toegankelijk is voor de moleculen in de omgeving van de cel. Dit wordt verzekerd door de "spacer" (een afstandhouderregio) en "hinge" (een scharnierdomein) domeinen van de TCR gebaseerde receptor en door de EPOR D2 regio van het EPOR/gp130 gebaseerde receptor koppel. Bij deze laatste staat het D2 domein niet alleen in voor de accesibiliteit van het herkenningsdomein, maar zorgt ook dat er dimerisatie optreedt bij binding van het doelwit.

region	TCR based receptor cassette	Cytokine receptor based receptor couple	
		EPOR based monomer	EPOR/gp130 based monomer
Extracellular region	• Anti-PSA single chain antibody • IgG hinge region • CD28 spacer region	• anti-HEL heavy chain recogrition domain • EPOR D2 domain	• anti-HEL light chain recognition domain • EPOR D2 domain
Transmembrane (TM) region	CD28 TM domain	EPOR TM domain	gp130 TM domain
Intracellular region	•CD28 cosignaling domain • TCRζ signaling chain	EPOR signaling chain	gp130 signaling chain

Tabel I.1: functionele componenten van de receptorconstructen.

Het bioherkenningsdomein is gescheiden van de overige regionen van het construct door specifieke restrictieplaatsen, waardoor deze domeinen vlot uitwisselbaar zijn om zo de specificiteit van de biosensor te wijzigen. Deze flexibiliteit op het gebied van doelwitten vormt de kern van de brede toepasbaarheid van de uiteindelijke biosensor.

De transmembranaire en intracellulaire delen van de receptorconstructen zorgen ervoor dat binding van doelwitmolecule omgezet wordt in een signalisatie-cascade, die uiteindelijk leidt tot veranderingen in genexpressie. Bij de TCR

gebaseerde chimere receptor verloopt deze signalisatie via de signalisatiedomeinen van de coreceptor CD28 en van de TCR. De EPOR/gp130 gebaseerde constructen maken gebruik van de signalisatieketens van gp130 en van EPOR. De activatiedrempel van de receptorsignalisatie bepaalt voor een groot deel de uiteindelijke gevoeligheid van de biosensor.

De structuur van de chimere celreceptor is bepalend voor de flexibiliteit en de brede inzetbaarheid van de celgebaseerde biosensor. Daarnaast beïnvloed deze structuur ook de gevoeligheid van de sensor via de Ab herkenningsdomeinen en via de activatiedrempel van de signaaldomeinen. Deze chimere constructen werden tot expressie gebracht in een geschikte cel om detectie van doelwitstoffen mogelijk te maken.

Het chimere EPOR/gp130 receptorkoppel komt tot expressie in de IL3 afhankelijke BaF3 cellijn, hetgeen resulteert in de Ba/HE+LgIGFP cellijn. Aangezien cytokinereceptoren van nature zeer gevoelig zijn (in de pM grootteorde) werd besloten in eerste instantie verder te gaan met deze constructen.

II. Ontwikkeling van het reportersysteem

Hoofdstuk 3 beschrijft de ontwikkeling van een reportersysteem, afgestemd op de EPOR/gp130 signalisatiecascade. Hiervoor werd een EPOR/gp130 induceerbare promotor geselecteerd die bijgevolg gekoppeld werd aan een luciferase reportergen.

De promotoren werden in twee fasen geselecteerd: eerst werd een panel van promotoren samengesteld op basis van de JAK/STAT signalisatiecascade, een signalisatie keten die alle type I familie van cytokinereceptoren (waar gp130 en EPOR deel van uitmaken) gemeen hebben. Anderzijds werd ook gekeken naar promotoren die volgens microarray data door IL3 geïnduceerd worden in de BaF3 cellijn. Deze laatsten zijn van belang aangezien IL3 signalisatie in BaF3 cellen (gedeeltelijk) samenvalt met gp130 en EPOR signalisatie in deze cellen. Op deze manier werden 13 potentiële promotoren geselecteerd voor verder onderzoek.

In de volgende fase werden genexpressie profileringsexperimenten uitgevoerd op de Ba/HE+LgIGFP cellen. Deze cellen werden aan de doelwitmolecule HEL blootgesteld en de effecten van deze blootstelling op de activatie van de betreffende promotoren werd onderzocht. Zes genen komen significant sterker tot expressie onder invloed van HEL: CISH, SOCS1, SOCS2, SOCS3, Spry1 en gpr34 en hiervan zijn CISH, SOCS1 en SOCS3 de beste. Ook werd geen significant verschil vastgesteld in achtergrondexpressie van deze genen (oftewel "leakage" van de promotoren), zodat zij alle drie verder onderzocht werden.

Naast HEL geïnduceerde activatie van de promotoren werd ook gekeken naar crosstalk, aangezien de biosensor zowel gevoelig als specifiek moet zijn. De specificiteit van de promotoractivatie werd onderzocht door de bootstelling van Ba/HE+LgIGFP cellen aan een panel cytokines. De resultaten van deze crosstalk experimenten demonstreren dat de drie promotoren (CISHp, SOCS1p en SOCS3p) allemaal aangeschakeld worden door IL3. Daarnaast is CISHp geactiveerd door IL5 en IL6, SOCS1 is geactiveerd door EPO en SOCS3 door IL3 en IL4. De waargenomen crosstalk is te wijten aan de gemeenschappelijke signalisatieketen van de onderzochte cytokines en moet in toekomstige experimenten in rekening gebracht worden. Dit kan bijvoorbeeld door parallelle experimenten om het onderscheid tussen activatie door doelwitmoleculen en activatie door kruisreactieve moleculen in kaart te brengen.

CISHp, SOCS1p en SOCS3p worden allemaal sterk en snel (na 30 min blootstelling) geactiveerd. Voor de verdere ontwikkeling van het prototype biosensor werd in de eerste plaats gebruik gemaakt van CISHp, de overige twee promotoren blijven beschikbaar voor verder onderzoek en ontwikkeling.

III. Ontwikkeling prototype whole cell biosensor

In hoofdstuk 4 werd een prototype van de celgebaseerde biosensor geassembleerd, gebruik makende van het chimere EPOR/gp130 receptorkoppel en van een CISHp/luciferase reporterplasmide.

Het CISHp/luciferase plasmide werd geconstrueerd en vervolgens tot expressie gebracht in de Ba/HE+LgIGFP cellijn. Aangezien de initiële pogingen om het

selecteerbare pGL4.14/CISHp luciferase plasmide te gebruiken mislukten, werd gekozen voor cotransfectie van het pGL3/CISHp luciferase plasmide met pREP9, een selectieplasmide dat het Neomycine resistentiegen bevat. Optimizatie van de cotransfectie resulteerde in de Ba/C9 cellijn, die zowel het anti-HEL receptorkoppel tot expressie brengt als de CISHp gereguleerde luciferase reporter.

De bekomen “whole cell” sensor bevat alle componenten voor detectie van de doelwitmolecule, HEL, namelijk: de chimere receptor kan de doelwitmolecule binden en een signalisatiecascade veroorzaken die resulteert in de expressie van luciferase (gecontroleerd door CISHp). Uiteindelijk geeft de luciferase productie de aanwezigheid van HEL in de omgeving van de cel weer. Finaal werden de gevoeligheid en de specificiteit van het biosensorprototype onderzocht.

De celgebaseerde biosensor vertoont een toename in luciferase productie na blootstelling aan HEL in de nM concentratierange, met een blootstellingstijd van slechts 2 uur. De dynamische range van de sensor is echter klein en de gevoeligheid dient nog verbeterd te worden. Daarenboven is er een hoge achtergrond productie van luciferase. Crosstalk wordt waargenomen voor IL3, zoals te verwachten is in de IL3 afhankelijke cellijn, maar niet voor andere cytokines. Dit kan echter te wijten zijn aan de gelimiteerde sensitiviteit en dient gecontroleerd te worden bij toekomstige onderzoeksdaden en ontwikkelingen.

IV. Conclusie

Celgebaseerde biosensortechnologie maakt optimaal gebruik van natuurlijke mechanismen voor detectie van doelwitstoffen. Reportergen assays worden reeds wijd toegepast, zowel in onderzoek als voor commerciële toepassingen (bv. CALUX). De ontwikkeling van dergelijke biosensoren met een toepassingsgebied dat niet gelimiteerd wordt door de natuurlijke liganden van cellen maar veel breder gaat is een veelbelovende onderzoekstak. Hoewel er nog steeds nood is aan verbeteringen en optimalisatie van deze technologie, wordt hier het bewijs geleverd dat het potentieel van dit type onderzoek zeer groot is.

DEEL II: Toepassing van biosensoren voor screening van complex afval

Het tweede luik van het onderzoek spitst zich toe op de toepassing van bestaande biosensoren in een nieuwe teststrategie voor afval analyse en de identificatie van hiaten, waar huidige biosensoren nog niet voldoen aan de vraag vanuit dit nieuwe toepassingsgebied.

De huidige wettelijke basis voor de classificatie van afval, zoals beschreven in de Hazardous Waste Directive (HWD), is gebaseerd op de toxiciteit van de individuele componenten van het afval of, indien de samenstellende componenten onbekend zijn, op de resultaten van toxiciteitstesten op het afval zelf. De test methodes die aangehaald worden in de HWD zijn acute en chronische dierproeven, die ook worden ingezet voor screening van chemicaliën (CD 67/548/EC (gevaarlijke stoffen), 726/2004/EC (farmaceutica), EC/1907/2006 (REACH), CD 98/8/EG (biociden)). Deze testen zijn echter niet geschikt voor toepassing op afvalstalen, aangezien de testtijd te lang is, ze een te hoge kost met zich meebrengen, op andere blootstellingsroutes toegespitst zijn en bovendien vanuit een ethisch standpunt onaanvaardbaar zijn voor afvalscreening. Omwille van deze redenen worden er in de praktijk geen testen toegepast op afval en is de indeling vaak arbitrair bepaald.

Aangezien de potentiële negatieve gevolgen voor het milieu en de gezondheid in het geval van foutieve classificatie zeer ernstig zijn en er bovendien ook aanzienlijke economische implicaties verbonden zijn aan deze arbitraire classificatie, is er een dringende nood aan een solide omkadering voor afval screening. Het DISCRISET project dat hier wordt voorgesteld is gericht op de ontwikkeling van een snelle, economisch haalbare en betrouwbare afval screening strategie die zal leiden tot de correcte classificatie van complexe afvalstalen. De ontwikkeling en evaluatie van deze strategie is beschreven in hoofdstukken 5 en 6.

I. Ontwikkeling van de afvalscreening strategie

Om tot een goede screening strategie te komen werden alternatieve testmethodes voor de evaluatie van de toxiciteit van afvalstalen voorgesteld en

geëvalueerd. De technieken die onderzocht werden omvatten (a) chemische testen en affiniteitssensoren die specifieke (groepen van) toxische stoffen identificeren (gerichte analyses), (b) in vitro testen die oorspronkelijk werden ontwikkeld voor het screenen van de toxiciteit van farmaceutica of chemicaliën, en (c) ecotoxiciteitstesten. (b) en (c) behoren tot de effectsensoren en maken vaak gebruik van levende cellen of lagere organismen zoals *Daphnia* of algen. De chemische en biologische screeningmethodologie is zeer uitgebreid (bv. NATIBO, 2001, Eisenbrand et al., 2002; Krist et al., 2005, Witters et al., 2005; EPA, 2005; Allan et al., 2006) en er ontstaan voortdurend nieuwe applicaties en technieken (Riedel et al., 2003; NRC, 2007; Imec-LINC, 2008).

De beoordeling van de toxiciteit van afval moet aan een aantal a priori voorwaarden voldoen: (a) de classificatie moet beantwoorden aan de principes van de HWD. Dit houdt in dat ze gebaseerd moet zijn op totale concentraties en op de in de HWD beschreven toxische eigenschappen (de zogenaamde H-criteria). Daarnaast moeten (b) de resultaten snel bekomen worden, liefst binnen 48 uur, en aan een economisch haalbare kostprijs. Deze voorwaarden zijn van belang om opstapeling van afval (en de daaruit voortvloeiende problemen inzake geurhinder en logistiek) te vermijden. Ook moet (c) een hoge graad van standaardisatie van de technieken mogelijk zijn omdat de resultaten in de praktijk vlot vergeleken moeten kunnen worden met vooraf bepaalde toxiciteitslimieten. Uitgaande van deze criteria werden, naast de chemische testen, 16 biotesten geselecteerd die in een piloot studie ingezet werden voor het screenen van afvalstalen van een brede waaier aan bronnen. Sommige van deze 16 testen voldoen momenteel niet aan criterium (b) maar werden toch ingezet voor pragmatische redenen, aangezien er geen alternatieven zijn voor bepaalde toxische eindpunten. De nood aan nieuwe testen werd in deze context echter aangekaart.

Er werd een gelaagde teststrategie voorgesteld om de tijd en kost verbonden aan afvalscreening tot een minimum te beperken. Op deze manier kunnen de acuut toxische stalen reeds vroeg in de strategie geïdentificeerd worden, en zonder meer geklasseerd worden, terwijl chronische toxiciteit alsnog in een later stadium vastgesteld kan worden.

II. Toepassing van de screening strategie in een pilootstudie

In de pilootstudie werden 16 afvalstalen uit diverse bronnen getest aan de hand van de voorgestelde gelaagde strategie. De eerste testlaag bestaat uit een chemische analyse. Hier werden zowel de organische als de anorganische fracties van het afval onderzocht. Uit de resultaten blijkt echter dat de organische chemische test enkel zin heeft indien er op voorhand een vermoeden bestaat van toxische organische componenten. Indien dit niet het geval is kan de teststrategie deze testen overslaan en dient hier enkel met de resultaten van de anorganische fractie rekening gehouden te worden. De chemische analyse werd gevolgd door een algemene toxiciteitsscreening waarbij een biotest voor acute toxiciteit werd ingezet. Microtox® blijkt hiervoor een zeer goede kandidaat te zijn aangezien deze test binnen 30 minuten voltooid is en het de meest gevoelige van de onderzochte bacteriële tests is. De resultaten worden bovendien bevestigd door de chronische toxiciteitstesten die op dezelfde stalen werden uitgevoerd.

Chronische toxiciteitstesten leveren belangrijke bijkomende informatie voor afvalclassificatie. Deze testen moeten verder gevalideerd worden en toxische limietwaarden moeten worden vastgelegd om de resultaten snel en correct te kunnen interpreteren, zodat ze als basis kunnen dienen voor afvalclassificatie. Daarnaast moeten een aantal ontbrekende eindpunten, zoals sensitiserende, irriterende en corrosieve effecten, alsook teratogene effecten en effecten op embryonale ontwikkeling aangepakt worden. Deze hiaten in de bestaande biosensortechnologie kunnen als voedingsbodem dienen voor de ontwikkeling van nieuwe biosensoren.

III. Conclusie

Volgens Eurostat statistieken produceert de EU 3 miljard ton afval per jaar en neemt dat volume dagelijks toe. De nadelige effecten van een ontoereikende omkadering voor classificatie van (gevaarlijk) afval zijn bijgevolg zeer ernstig. Naast gezondheid en ecologie, ondervindt ook de economie zware gevolgen van de foutieve behandeling van afval (arbitraire indeling kan leiden tot oneerlijke

praktijken en concurrentievervalsing). De gelaagde afvalscreening strategie die hier wordt voorgesteld reikt een ethisch, praktisch en economisch haalbaar alternatief aan voor de in de HWD beschreven methodes, en na validatie betekent dit een belangrijke vooruitgang tegenover de huidige classificatie van afval.

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Curriculum Vitae

Karolien Deprez was born on the 18th of August 1981 in Aalst.

In June 1999, she finished her secondary grade education (Sciences and mathematics) at the *Sint-Vincentius School,* Gijzegem.

She attended *Ghent University* from September 1999 to June 2004 and obtained a master's degree in Biology, with a specialty in plants.

From September 2003 to June 2004, she worked for Barry Callebaut Belgium as a laboratory technician and assistant "Vendor Assurance". In July 2005 she started working as project manager for Labo Van Vooren NV, where she performed soil and ground water pollution investigations for private persons, companies and governmental organizations. From September 2006 to June 2007, she stayed abroad in Brittany (France) and devoted herself to her family.
In April 2008, she joined the Biosensor research group at the *Biomedical Research Institute (BIOMED)* at *Hasselt University*, participating in two biosensor projects: developing an innovative novel whole cell biosensor prototype, and applying existing sensing systems in the field of complex waste screening. This will be completed in 2014, with a PhD thesis entitled 'Whole cell biosensors: from new applications to novel sensing technology'. She also had teaching responsibilities at the faculty of Medicine and Life Sciences at *Hasselt University*.
In her current position (since August 2013), at *Ghent University*, she is employed as coordinator for the Flemish Algae Platform (Vlaams Algenplatform).

Research stays abroad:

Oct. 15th to 17th 2008	Introduction to the CALUX biodetection system and application of the cell assays on complex waste extracts: pilot test phase *(BDS, Amsterdam, The Netherlands)*
May 27th to 29th 2009	Application of the CALUX cell assays on complex waste extracts: initial tests on an broader range of

	wastes *(BDS, Amsterdam, The Netherlands)*
Nov. 30th until Dec. 4th 2009	Application of the CALUX cell assays on complex waste extracts: fine-tuned detection ranges *(BDS, Amsterdam, The Netherlands)*

Attended workshops and courses:

15/08/2008:	Lab Demos of BioDetection Systems (BDS). *(Amsterdam, The Netherlands)*
15/12/2008:	Human Identification Traning/System 7500 (Applied Biosystems HID University) *(Hasselt, Belgium)*
27/05/2008:	Euron Course Immunology *(Hasselt, Belgium)*
26/06/2008:	Polylight training (Poli-Service BVBA) *(Hasselt, Belgium)*
18/02/2011:	Biogazelle course: qPCR experiment design and data-analysis *(Hasselt, Belgium)*
15/05/2012:	Summer school on Printed Biosensors and Electronics. *(Cancun, Mexico)*

Event organization

Member of the organizing committee of the **"***2nd NanoSensEU Symposium on Biosensor development: Trends and Technology"* on April 25th 2012. (Hasselt, Belgium)

Bibliography

Oral presentations:

09/03/2009:	Design of an innovative, novel whole cell biosensor using chimeric receptors*. Hasselt University Lunch Seminar (Hasselt, Belgium)*
24/09/2009:	DISCRISET: selecting a set of (biological/chemical) fast screening tests that allow discrimination between hazardous and non-hazardous waste, pilot study. *Biodetection Systems Workshop 2012 (Amsterdam, The*

Netherlands).

29/03/2010: Design of a novel whole cell biosensor using chimeric cell receptors. *Hasselt University Lunch Seminar (Hasselt, Belgium)*

14/10/2010: Biodetectors for Hazardous Waste. *BioDetectionSystems Workshop 2010 (Amsterdam, The Netherlands)*

14/11/2011: Development of innovative and versatile whole cell biosensor prototypes*. Hasselt University Lunch Seminar (Hasselt, Belgium)*

17/05/2012: Cell-based affinity sensors: Development of a novel tool for sensing low concentrations in complex mixture. *Biosensors 2012 (Cancun, Mexico)*

24/05/2012: DISCRISET: selecting a set of (biological/chemical) fast screening tests that allow discrimination between hazardous and non-hazardous waste, phase II. *Biodetection Systems Workshop 2012 (Amsterdam, The Netherlands)*

Poster presentations:

17/03/2010: Development of a novel whole cell biosensor prototype. *Biomedica 2010 (Achen, Germany)*

5/05/2011: Development of a novel Eukaryotic cell based biosensor prototype. *Knowledge for Growth(Ghent, Belgium)*

10/10/2011: Development of innovative and versatile whole cell biosensor prototypes. *2nd International Conference on Bio-Sensing Technology (Amsterdam, The Netherlands)*

25/04/2012: Development of an innovative and versatile whole cell biosensor prototype. *2nd NanoSensEU Symposium on Biosensor development: Trends and Technology (Hasselt, Belgium)*

Publications:

R. Weltens, G. Vanermen, K. Tirez a, J. Robbens, **K. Deprez** , L. Michiels. *Screening tests for hazard classification of complex waste materials – Selection of methods.* Waste Management 32 (2012) 2208–2217

K. Deprez, J. Robbens, I. Nobels, C. Vanparys, G. Vanermen, K. Tirez, L. Michiels, R. Weltens. *DISCRISET: A battery of tests for fast waste classification – Application of tests on waste extracts.* Waste Management 32 (2012) 2218–2228

K. Deprez, V. Vermeeren, L. Michiels. *Cell-based affinity sensors: Development of a novel tool for sensing low concentrations in complex mixtures* (In preparation)

R. Weltens, **K. Deprez**, L. Michiels. Discriset: **Validation of Microtox as a first screening tool for waste classification.** (Waste Management, submitted)

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