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# Summary

Worldwide, colorectal cancer is the third and second most common cancer in men and women with a mortality rate of 694,000 deaths in 2012 according to the World Health Organization, respectively. It is important to evaluate the potential risk of food compounds on human health. The food additive E171 is present as a whitening agent at significant levels mainly in sweets, cookies, icing and chewing gum. Depending on age, consumers are exposed between 1 and 2 mg/kg bw/day. E171 comprises titanium dioxide ($TiO_2$) both micro- (MPs) and nanoparticles (NPs) of $TiO_2$.$TiO_2$ is classified by the International Agency for Research on Cancer (IARC) as possible carcinogenic and could be a potential factor involved in colorectal cancer development. Contradictory data is available about health risks of $TiO_2$ which makes it important to investigate toxicity. The focus of this study was to evaluate *in vitro* toxic effects and its involved mechanisms of E171 ($TiO_2$) on the development of colorectal cancer.

Reactive oxygen species (ROS) were detected and quantified using electron spin resonance (ESR) spectroscopy either formed by E171, MPs or NPs in Caco-2 cells. In addition, intrinsic (particle-driven) ROS formation capacities of $TiO_2$ particles were investigated separately. With the alkaline comet assay, carcinogenic mechanisms of E171 and $TiO_2$ NPs were reviewed by studying effects of $TiO_2$ on ssDNA breakage also in combination with the genotoxic compound azoxymethane (AOM).

This study demonstrates that $TiO_2$, in particular nano-sized (10 – 15 nm) induce sufficient DNA damage as evident by the results of the alkaline comet assay. This finding was also supported by the fact that ROS formation was significantly enhanced in cell-free environments which proves the intrinsic (particle driven) ROS formation capacity of $TiO_2$ NPs. However, ROS formation in reaction to $TiO_2$ particle exposure is size dependent which is proven by the fact that MPs are able to affect Caco-2 cells, in all probability due to secondary toxicity mechanisms since they do not have intrinsic capacity to produce ROS, shown by acellular ESR assessments.

The findings of the present *in vitro* study demonstrate that $TiO_2$ as food additives coded as E171, even at the lowest concentration tested, have DNA damaging potential in human colorectal adenocarcinoma cells. The direct correlation between ROS formation and DNA damage further suggests that oxidative stress could act as an important route by which $TiO_2$ particles, induce DNA damage. Oxidative stress could therefore be one of the underlying mechanisms involved in the development of colorectal cancer induced by $TiO_2$ NPs in food products.

In conclusion, results discussed in this thesis provided us with new molecular insights in the *in vitro* effects of $TiO_2$ on colorectal cancer development. Overall, it is concluded that radical formation by $TiO_2$ particles taken up in the colon via food products may play an important role in health effects associated with colonic $TiO_2$ exposure. Future studies will focus on transcriptomic approaches to reveal mechanisms involved in potentially carcinogenic effects of the particles including pathways (e.g. oxidative stress, inflammatory pathways) induced by $TiO_2$.

# Samenvatting

Colorectale kanker is wereldwijd de derde (bij mannen) en tweede (bij vrouwen) meest voorkomende vorm van kanker met een sterftecijfer van 694.000 in 2012 volgens de Organisatie van Wereldgezondheid (WHO). Het is cruciaal om het potentiële risico van voedselcomponenten voor menselijke gezondheid te evalueren. Het additief E171 is in significante hoeveelheden aanwezig in snoep, koekjes, glazuur en kauwgom. Afhankelijk van leeftijd, wordt de consument, gemiddeld geschat, blootgesteld tussen 1 en 2 mg per kg lichaamsgewicht per dag. E171 bevat zowel micro- (MPs) en nanodeeltjes (NP) titaniumdioxide ($TiO_2$).$TiO_2$ is ingedeeld door het Internationaal Agentschap voor Kankeronderzoek (IARC) als mogelijk kankerverwekkende (carcinogeen) stof en daarbij een potentiële factor die betrokken kan zijn bij de ontwikkeling van colorectale kanker. Echter, tegenstrijdige onderzoeksgegevens zijn beschikbaar over de gezondheidsrisico's van $TiO_2$ waardoor het belangrijk is om de toxiciteit te onderzoeken. De focus van deze studie was om de *in vitro* toxische effecten en de betrokken mechanismen op de ontwikkeling van colorectale kanker te bepalen van E171.

Reactieve zuurstof species (ROS) zijn gedetecteerd en gekwantificeerd met behulp van electronen spin resonantie (ESR) spectroscopie ofwel gevormd door E171, MPs of NP in humane colorectale adenocarcinoom (Caco-2)-cellen. Bovendien werd de intrinsieke (deeltjes aangedreven) ROS vorming capaciteit van $TiO_2$ deeltjes afzonderlijk onderzocht. Met de alkaline comet assay zijn carcinogene mechanismen van E171 en $TiO_2$ NPs beoordeeld; dit via het bestuderen van $TiO_2$ geïnduceerde enkelstrengs DNA (ssDNA) breuken. Daarbij is ook de genotoxische verbinding azoxymethaan (AOM) onderzocht in combinatie met de $TiO_2$ deeltjes.

Deze studie toont aan dat $TiO_2$, met name NPs (10 - 15 nm), ssDNA schade induceert. Dit blijkt uit de resultaten van de alkaline comet assay. Deze bevinding wordt ook ondersteund door het feit dat de ROS vorming aanzienlijk is verhoogd in acellulaire omgevingen waarin de intrinsieke (deeltje aangedreven) ROS vorming capaciteit van $TiO_2$ NP is aangetoond. Echter, ROS vorming in reactie op $TiO_2$ deeltjes blootstelling is grootteafhankelijk. Dit blijkt uit het feit dat MPs Caco-2-cellen kunnen beïnvloeden. Hoogstwaarschijnlijk als gevolg van secundaire toxiciteitsmechanismen, omdat zij geen intrinsieke capaciteit ROS productie hebben, aangetoond door acellulaire ESR bepalingen.

De bevindingen van dit *in vitro* onderzoek tonen aan dat $TiO_2$ als levensmiddelenadditieven gecodeerd als E171, zelfs bij de laagste geteste concentratie, DNA-beschadigende potentieel hebben in Caco-2 cellen. De directe correlatie tussen ROS vorming en ssDNA-beschadiging wijst erop dat oxidatieve stress kan fungeren als een belangrijke route waarbij $TiO_2$ deeltjes, DNA-schade induceren. Oxidatieve stress zou dus een van de onderliggende mechanismen kunnen zijn betrokken bij de ontwikkeling van colorectale kanker, geïnduceerd door E171 in voedingsproducten.

De resultaten beschreven in deze thesis geven nieuwe moleculaire inzichten in de *in vitro* effecten van $TiO_2$ op colorectale kanker ontwikkeling. Toekomstig onderzoek zal zich richten op transcriptomische benaderingen van mechanismen die betrokken zijn in potentieel kankerverwekkende effecten van $TiO_2$.

## List of used abbreviations

| | |
|---|---|
| **AOM** | Azoxymethane |
| **BSA** | Bovine serum albumin |
| **Caco-2** | Colon carcinoma cell line |
| **$CO_2$** | Carbon dioxide |
| **CRC** | Colorectal cancer |
| **d.nm** | Dynamic nanometer |
| **DLS** | Dynamic light scattering |
| **DMEM** | Dulbecco's modified Eagle's medium |
| **DMPO** | 5,5-Dimethyl-1-Pyrroline N-oxide |
| **DNA** | deoxyribonucleic acid |
| **dsDNA** | Double strand DNA |
| **DSS** | Dextran sodium sulphate |
| **ESR** | Electron spin resonance |
| **EtBr** | Ethidium Bromide |
| **FBS** | Fetal bovine serum |
| **FDA** | Food and drugs administration |
| **$H_2O_2$** | Hydrogen peroxide |
| **IARC** | International Agency for Research on Cancer |
| **IBD** | Inflammatory bowel disease |
| **LDH** | Lactate dehydrogenase |
| **MAM** | Methylazoxymethanol |
| **MP** | Microparticle |
| **MTT** | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| **NP** | Nanoparticle |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PI** | Isoelectric point |
| **ROS** | Reactive oxygen species |
| **SOD** | Superoxide dismutase |
| **ssDNA** | Single strand deoxyribonucleic acid |
| **TEM** | Transmission electron microscopy |
| **$TiO_2$** | Titanium dioxide |

# 1. Introduction

Currently, **colorectal cancer** is one of the most prevalent cancer types observed in humans worldwide. Epidemiological studies estimate a yearly mortality rate of 228,000 deaths per year and predict 1,258,000 new cases in the coming years in Europe [1]. Since the colon is constantly exposed to a diverse range of compounds present in food, our diet is one of the most important risk factors in the development of colorectal cancer. Therefore it is important to evaluate the potential risk of food components on colorectal cancer development. E171, consisting of **titanium dioxide ($TiO_2$)** nano- and microparticles, is present as a whitening agent at significant levels as **food additive** in numerous products. Contradictory data in literature [2-5] necessitates the assessment of toxicity and possible **carcinogenicity** of $TiO_2$. Therefore in this study, we focus on the potential carcinogenic effects of food grade $TiO_2$, in particular the possible facilitation of colorectal cancer. It has been hypothesized by us that **oxidative stress** responses, formed by reactive oxygen species, are the underlying mechanisms of E171 toxicity and carcinogenicity.

In this thesis, a general background based on previous studies and preliminary results is summarized from paragraph 1.1, followed by materials and methods used in this study in chapter 2. The results of this study are outlined in chapter 3, which are discussed in chapter 4. Final conclusions are described in chapter 5. The follow up studies which are recommending based on the outcome of this *in vitro* study are outlined in chapter 6 followed by references in chapter 7.

## 1.1 Colorectal cancer

Millions of people are affected by cancer each year, it is one of the leading causes of death counting for 1 out of 4 deaths worldwide [6]. Every organ in the human body is susceptible to cancerous growth although some organs to a larger degree than others. The colon intestine is the major site for water absorption, maintaining body's fluid balance and production of vitamins which are subsequently absorbed. Next to this useful nutrients and vitamins, the human colon is continuously exposed to a wide variety of potentially hazardous compounds through the intake of food containing carcinogenic compounds. The combination of daily exposure, and thereby accumulation of potentially carcinogenic compounds in the intestinal lumen of the colon, with the high proliferative potential of the colon epithelium, creates an environment susceptible to mutagenic lesions with pro-carcinogenic potential. Colorectal cancer (CRC) is a malignant tumor in the colon or rectum. According to the World Health Organization, CRC is the third and second most common cancer in men (747,000 estimated cases in 2012, worldwide) and women (614,000 cases in 2012, worldwide) respectively [1].

### 1.1.1 Development of colorectal cancer

Colorectal cancer develops in multiple steps according to the well-known Vogelstein model [7]; initiation, promotion and progression [8]. Initiation of CRC results from exposure of carcinogenic compounds (e.g. benzo[a]pyrene present in cigarettes) leads to small cellular or tissue morphology (e.g. polyp formation). This alteration, although still innocent, confers to an irreversible alteration, also termed as mutation, in the DNA which gives rise to a permanent increase in cancer development susceptibility. The second stage, promotion, gives rise to clonal growth of initiated cell growth by a (non-)mutagenic stimulus. This stimulus could refer to inflammation or tissue disruption or either be an environmental carcinogenic ingested via food products. Finally, due to an accumulation of mutations and dysregulation of proto-oncogenes and tumor-suppressor genes, the pre-neoplastic growth transitions into a malignant tumor which is characterized by limitless and invasiveness growth of neoplastic cells[7].

### 1.1.2 Risk factors for colorectal development

Risk factors for CRC development include high body mass index, high intake of red meat and smoking [8]. Prevention of these risks factor is the best way to decrease the risk of CRC development. Around 90% of CRC is non-hereditary (sporadic) while 10% of patients have inherited CRC[8]. The etiology of sporadic CRC is complex. Progenitors in carcinogenesis are various environmental and lifestyle factors. As already mentioned, the colon is continuously exposed to numerous potentially hazardous components which are present in food. It has been estimated that 70% of sporadic CRC cases could be prevented by modifying dietary habits [9]. Identifying compounds that may be responsible for CRC incidence is therefore a high priority. By 2010, titanium dioxide is classified as possible carcinogenic [10] since numerous studies found certain risk factors involving the use of $TiO_2$ in consumer products [11]. $TiO_2$ could therefore be a potential factor involved in colorectal cancer development. Although these concerns, $TiO_2$ is still widely used in significant amounts in numerous products in the food industry.

## 1.2 Titanium dioxide ($TiO_2$) characteristics

Titanium dioxide is the natural oxide of titanium. It occurs in nature in several crystalline structures; rutile, anatase and brookite [12]. Rutile is the most common natural form of $TiO_2$ and contains around 98% $TiO_2$. Anatase and brookite are two more rare polymorps of the natural form of $TiO_2$ [13, 14]. Brookite is not commercially used [10]. Anatase $TiO_2$ does have higher photocatalytic activity and is therefore commonly used [15]. Disadvanteously, anatase $TiO_2$ could potentially induce more deleterious effects by producing radicals such as superoxide ($O_2\cdot^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($HO\cdot$) via Fenton reactions than rutile nanoparticles (NPs) [16, 17].

Moreover, it is known that due to their small size NPs can enter the nucleus of the cell [18] which could lead to particle agglomeration by accumulation in both cytoplasm and nucleus. Uptake mechanisms could include phagocytosis, pinocytosis and passive diffusion of the particles by cells which thereby occasionally could reach intracellular targets such as mitochondria and the nucleus [19].

This uptake mechanisms are rationally depending on the size and degree of agglomeration of the particles since small (nano-sized) particles could eventually form large agglomerates, while larger (micro sized) particles are not capable to penetrate the cell wall and interfere with the cell's DNA. Confirmatory data [20]showed that the uptake of small particles (~110- 120 nm) by the small intestine *in situ* is shown to be 15- to 250 fold higher in comparison to larger particles (~510- 550 nm), which could be explained by the fact that the size of a NP is similar to that of a typical cellular component which can travel throughout the human body [21].

Titanium dioxide is highly stable to heat, light, oxygen and pH, which makes it a perfect food additive as it stays unaffected during food processing. Advantageously, $TiO_2$ particles outperform in their brightness, high refractive index, and resistance to discoloration, while their industrial production is relatively low-cost [3]. Due to the unique properties of the nanoparticles, it is challenging to estimate their effects on humans and specifically on the development of cancer. To assess the effect of $TiO_2$, it is important that the particles are well dispersed in buffer or medium. In different toxicological studies, different surfactants have been tested to increase the dispersibility of nanomaterials. Bovine Serum Albumin (BSA) is shown to be the promising surfactant of nanomaterials including titanium dioxide in several studies [22, 23], and has been applied in larger harmonized studies on nanomaterials genotoxicity [24]. Furthermore, the assessment of the cytotoxicity of these particles cannot be performed with every viability assays. Indeed, there is interference between titanium dioxide particles and some viability assays like LDH, MTT and WST-1 [25-27] which were found assay- as well as NP-specific. Consequently, the results of these assays are mainly overestimated through interaction between $TiO_2$ and the assay due to its high refractive index.

Nano sized particles are commonly used food additives as the refractive power of nanoscale particles is higher than in greater particles. Another property of the nanoparticles is their high contact surface [28]. Indeed, when particle size decreases, their contact surface areas becomes progressively larger, this can increase their reactivity and capacity to penetrate living cells. The widely application of $TiO_2$ in the food industry is likely to attract a significant public concern which leads to an urgent need to evaluate the carcinogenic potential and thereby the potential risks of exposure of $TiO_2$.

## 1.3 Titanium dioxide as food additive E171

Nowadays, food fabricants use an increasingly amount of food to improve and optimize the flavor, taste and appearance of their products. Titanium dioxide is accepted as food additive by the EU with the E number 171 and consists of both nano- and microparticles(MPs) [29]. Nanoparticles (NPs) are particles having at least one dimension smaller than 100 nm in size [30]. Micro- and nano-sized $TiO_2$ is generally perceived as a non-toxic and inert mineral and is widely applied in many different commodities and consumer goods, ranging from human food products like candies, gum and salad dressing, to others like paints, plastics, cosmetics (e.g. in sunscreen coded as Cl 77891) and medicines [2, 31]. E171 is primarily used as whitening agent whereas in sweets it can be used as physical barrier between layers of different colors or textures (e.g. the white barrier between the soft center of crisp and a chocolate coating in M&Ms® crispy).

Interestingly, controversial literature has been published about the average $TiO_2$ exposure in humans by food. Weir *et al.* [2]stated that consumers are exposed between 1 and 2 mg/kg bodyweight (bw) per day while Shi *et al.* [3] estimated that a typical diet may contribute to an average $TiO_2$ exposure of 300-400 µg per day. Presently, it is unknown to which extent ingested particles actually reach the intestine.with an estimated intake for an US adult of approximately 1 mg titanium/kg bw/day, while children consume approximately up to 2 mg titanium/kg bw/day[2]. Children consume more calories per kg body weight than adults which make them more sensitive for toxic compounds present in food.

### 1.3.1 Regulation and legislation for $TiO_2$ as food additive

Titanium dioxide was approved by the FDA with a regulation in 1966 and by the EU in 1994 (Official Journal of the European Communities)[10, 29]. In Europe, $TiO_2$ is being added as human food additives at *quantum satis* levels which states that the final product should not 'contain more than necessary'. Although $TiO_2$, under regulation of the FDA, as food color additive should not exceed 1% by total weight of the product [10].

A study by Lomer *et al.* [32] tested the amount of $TiO_2$ in foods using inductively coupled plasma optical emission spectrometry. In this study, only eight out of twelve foods contained detectable titanium indicated this on their labels. Based on these research it could be questioned whether food labels always provide complete information about food additives used in the final product, even though food labelling regulations state that food additives should always be described. An exception are individual catering pack and as long as they do not perform an additive function in the final product [32]. Despite these regulations the number of food compounds containing E171 is doubtless much larger than known. Currently, the production and hereby oral intake of $TiO_2$ nanoparticles as food additives is increasing globally since approximately 10,000 tons are produced worldwide per year (estimated in 2014 [3]) which have led to growing concerns about the consequences in humans [30].

## 1.4 Potential carcinogenic effects of $TiO_2$

Numerous studies report the potential risks of $TiO_2$ NPs exposure. The risks of cancer and inflammation by $TiO_2$ by inhalation are well known [4, 5, 33]. Some studies demonstrated that inhalation exposure of different concentrations $TiO_2$ induced lung tumor formation in rodents [34, 35]. For that reason, the International Agency for Research on Cancer (IARC) changed the $TiO_2$ classification from group 3 to group 2b as possible carcinogen for humans since 2010. Furthermore, it is also well-known that $TiO_2$ NPs can reach other organs (liver, spleen, lymph nodes [36] and ovary [37]) following oral consumption, and is even capable to penetrate the blood-brain, blood-testis and blood-placenta barriers [3]. Moreover, Iavicoli *et al.* established that $TiO_2$ NPs can cause considerable health effects on mammalian cells [38]. These effects could be explained by the potential of $TiO_2$ to disrupt cell membranes [39].

Results from different studies indicate the involvement of $TiO_2$ in CRC development and suggest a link between $TiO_2$ exposure and inflammatory bowel diseases. According to Al-Jubory *et al.,* exposure of 1 mg/L $TiO_2$ NPs for 4 hours causes infiltration across rat intestine with dysplasia in colon tissue due to accumulation of E171 [40]. Internalization of $TiO_2$ NPs through the cell membrane is hypothesized to induce inflammatory responses (i.e. production of pro-inflammatory cytokines, increased intracellular production of reactive oxygen species (ROS)) leading to DNA damage and increased risk of tumor growth. Furthermore, studies report immunomodulatory potency of $TiO_2$ NPs due to dermal exposure via sunscreen products [41, 42]. Studies have shown that $TiO_2$ has been linked to Crohn's disease and inflammatory bowel disease (IBD) from gastrointestinal intake [32, 43]. These inflammatory effects together with obtained DNA and chromosomal damage were also found by Trouiller *et al*., where mice were orally exposed to $TiO_2$ for five days [44].

An *in vivo* study in mice, performed by a collaborating research group Biomedicine from the National Autonomous University of Mexico is focused on the potential of oral exposure of E171 which could enhance tumor formation in colon in a mouse model with chemically induced colon cancer [45]. It was found that exposure of mice to E171 stimulated the onset and severity of colon cancer. In that model, colon cancer was induced by exposure to a genotoxicant (AOM: azoxymethane), followed by exposure to an irritant (DSS: dextran sodium sulphate)[45]. The results of additional exposure to titanium dioxide (E171) in terms of stimulation of tumor growth were remarkable (manuscript in preparation). Indeed, a significant difference of the number of tumors between the mice exposed to AOM+DSS only and the ones exposed to AOM+DSS and E171 was observed as clearly shown in figure 1.

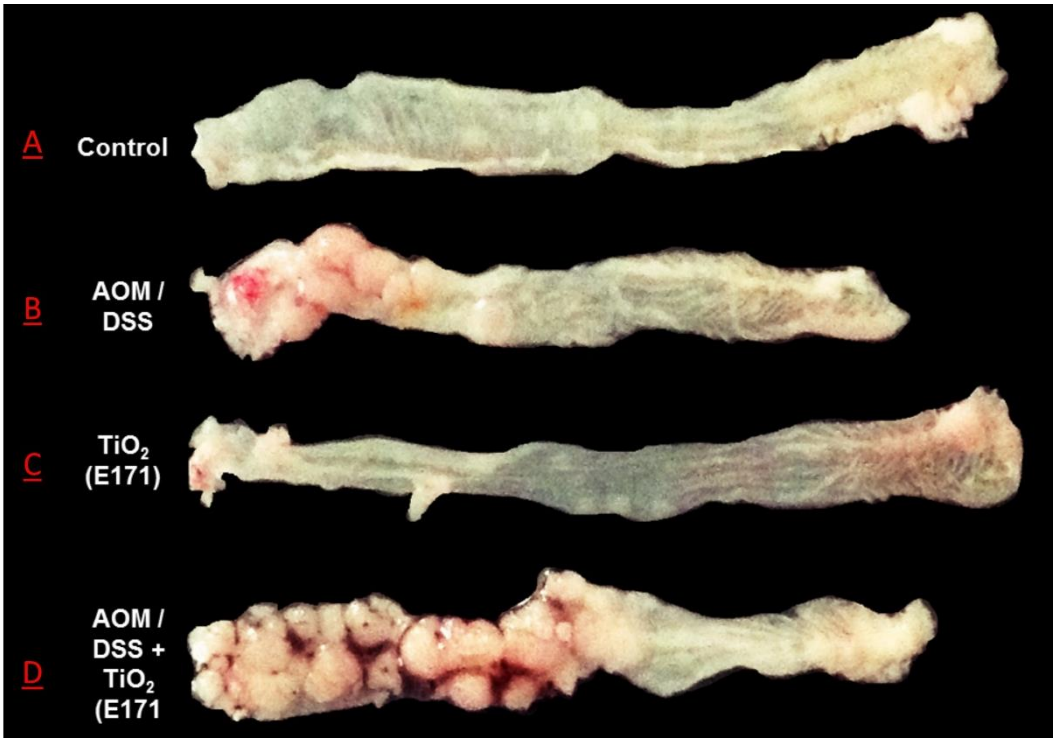



***Figure 1*** *In vivo study completed by the collaborating group of the National Autonomous University of Mexico. Photos shown colons of four groups:* ***A)*** *control group (intraperitoneal injection of saline solution),* ***B)*** *chemically induced colorectal cancer mouse group,* ***C)*** *E171 group (intragastrically exposed),* ***D)*** *Chemically induced CRC mouse group (intragastrically) exposed to E171. Colorectal cancer was induced with the genotoxicant AOM (azoxymethane) and an irritant DSS (Dextran sodium sulphane). These results show that E171, as a food additive, facilitates tumor formation in a mouse model of chemically induced colon cancer, evidenced by number of tumors. Photo received from University of Mexico (manuscript in preparation)*

Moreover, Zhang *et al*. investigated the active killing effect of human colon carcinoma cells by $TiO_2$ NPs as a potential new therapy for cancer [12]. The mechanism by which $TiO_2$ affects cells is not yet well-defined. Previous data [46] found that $TiO_2$ can cause considerable effects on mammalian cells such as increased ROS production [47], inflammation, decreased cell viability and proliferation, induction of cytotoxicity and genotoxicity, likely via apoptosis [48]. These data could give more insights in the potential toxic and carcinogenic mechanisms by which $TiO_2$ alter cells.

In contrast to the aforementioned studies, contradictory data is available which shows that $TiO_2$ NPs have no exposure-risk associations or carcinogenic effects [5, 22]. Prasad *et al.* evaluated the cytotoxicity, genotoxicity, agglomeration, cellular interaction, and effects on cell cycle of $TiO_2$ nanoparticles prepared in three media [49]. They measured various physicochemical characteristics thanks to two standard *in vitro* assays: the comet and the micronucleus assay. In this study, they found that $TiO_2$ nanoparticles were not cytotoxic at any concentration up to and including 0.1 mg/mL.

Taken together, these studies raise questions about the perceived harmless status of $TiO_2$. The molecular mechanisms by which $TiO_2$ could potentially induce or enhance cancer are unclear. For this reason, it is important to unravel the presence of toxicity and reveal the carcinogenic mechanism of $TiO_2$ in human colon cells to compose a reliable risk assessment of $TiO_2$ in food additives. The plausible carcinogenic mechanisms of $TiO_2$ are explained in the next paragraph.

## 1.5 $TiO_2$- induced carcinogenesis/ carcinogenicity

Three (of the several) important aspects in the development of (colon) cancer are inflammation, reactive oxygen species (ROS) production and alterations in cell signal transduction [50, 51]. It is well known that intracellular ROS formation can lead to oxidative stress, inflammation, immune response, (intra-) cellular damage and genotoxicity. In the following paragraph the main hypothesized carcinogenic mechanism of $TiO_2$ is outlined; oxidative stress.

### 1.5.1 Oxidative stress by formation of Reactive Oxygen Species (ROS)

Free radicals are chemical species with an odd number (unpaired) of electron(s) in the outer orbital which are extremely unstable and easily react with inorganic and organic chemicals. When free radicals are generated in cells, they can destructively attack various cell organelles and molecules such as mitochondria, DNA, cellular proteins and lipids. In addition, free radicals can initiate reactions where affected molecules can be converted into other types of free radicals, leading to a propagation chain of intracellular damage [52].

Reactive oxygen species (ROS) are intracellular produced oxygen derived free radicals what have an important role in cell injury. ROS are produced (in small amounts) in all cells during mitochondrial respiration and energy generation during redox reactions, where oxygen is reduced by the addition of four electrons to generate water. During this reaction, intermediates such as superoxide ($O_2$•) are spontaneously converted into hydrogen peroxide ($H_2O_2$) by the enzyme superoxide dismutase (figure 2). In contrast to •$O_2$, $H_2O_2$ is more stable and can cross cell membranes. In the presence of any metals (e.g. titanium), $H_2O_2$ is converted into highly reactive hydroxyl radical •OH[53].

During inflammation including production of various cytokines, ROS are intracellular produced in phagocytic leukocytes, mainly neutrophils and macrophages as protection mechanism for destroying invading pathogens. When the production of ROS increases (caused by (long-term) inflammation or absorption of radiant energy (x-rays, UV light)) or when the removal systems are ineffective (enzymatic (gluthatione (GSH) or catalase) and non-enzymatic (antioxidants like vitamins) systems do not work properly), excessed ROS levels will lead to a condition called oxidative stress [50, 51, 53]. Oxidative stress is defined as a disbalance of ROS and antioxidants [52].

ROS can cause cell damage by three main reactions (figure 2)[53];

1. Lipid peroxidation which leads to membrane damage; ROS attack double bounds in membrane polyunsaturated lipids which increases membrane permeability and inflammation. This lipid- radical interaction produces more unstable and reactive peroxides which automatically lead to a chain reaction.
2. Protein modifications such as cross-linking results in enhanced degradation or enzymatic activity loss by misfolding of the protein.
3. DNA damage by inducing single-strand breaks in nuclear and mitochondrial DNA which leads to cell death and malignant alterations of cells what may be involved in the initiation of cancer.

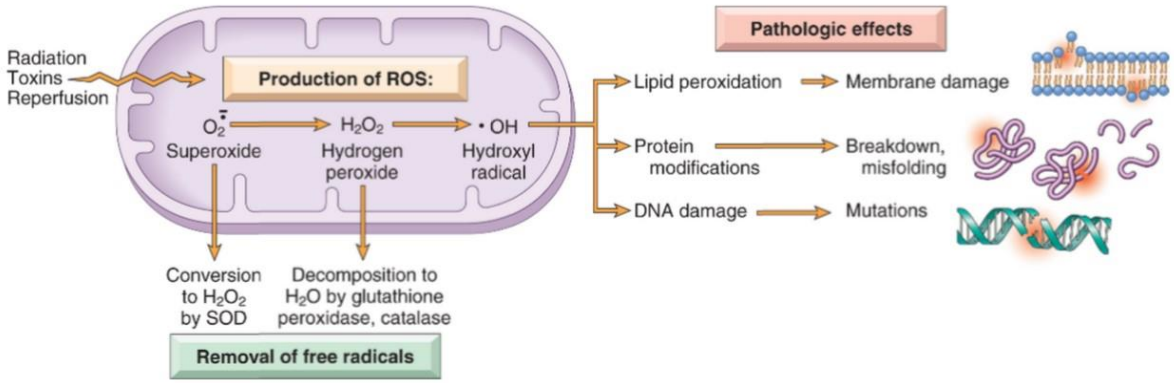



***Figure 2*** *Production, removal and pathologic effects of reactive oxygen species (ROS) in cell damage. The production of ROS is induced by various injurious stimuli like radiation, toxins or reperfusion. These produced radicals are removed spontaneously by decay or by (non-) enzymatic reactions. Excessive production or inadequate removal leads to free radical accumulation which may lead to several pathological effects resulting in cell damage. Abbreviations: SOD= superoxide dismutase. Source; Basic pathology, Robbins 9th edition[53].*

Thus, oxidative stress could lead to potential non-selective DNA damage and misfolded proteins accumulation by affecting important signaling (e.g. pro- or anti apoptotic or DNA repair) pathways and eventually cause genetic changes in (active) genes caused by reactive oxygen species (ROS) and free radicals. Production of ROS in reaction to $TiO_2$ exposure is believed to play an important role in many inflammatory disorders and cancer formation [54].

Therefore, it is important to evaluate the toxicity associated with oxidative stress in the role of the initiation of multistage carcinogenesis of $TiO_2$ used as food additive.

## 1.6 Objectives

This research is focused on the evaluation of the *in vitro* toxicity of human food grade $TiO_2$ coded as E171 for human consumption, with emphasis on the possible facilitation of E171 to colon cancer. E171 consists of nano-sized and micro-sized $TiO_2$ particles [29]. During this study, cytotoxicity and its involved mechanisms of E171 on human colorectal adenocarcinoma (Caco-2) cells were mainly addressed. This cell line was chosen as model of the human intestinal barrier since it represents the *in vivo* structural characteristics and mirrors the differentiation of the brush border at molecular levels [19]. It was found that E171 resulted in intestinal brush border disruption at the lowest concentration tested (350 ng/mL i.e. 100 ng/cm$^2$ cell surface area) after exposure to an *in vitro* model of the human intestine [11], although molecular mechanisms which are responsible for the development of colorectal cancer via $TiO_2$ are still unclear.

With this, the following research question was raised; 'What are the effects of E171 (titanium dioxide micro- and nanoparticles) on the production of reactive oxygen species and the DNA damaging properties on Caco-2 cells at non-cytotoxic concentrations?' We hypothesized that via the production of reactive oxygen species (ROS) which could result in DNA damaging, E171 has a toxic effect on Caco-2 cells at non-cytotoxic concentrations and that these effects may be involved in carcinogenic activity that has been reported for E171(manuscript in preparation).

### 1.6.1 Scientific approaches

It is important to evaluate the toxicity associated with oxidative stress in the role of the initiation of multistage carcinogenesis of $TiO_2$ used as food additive. In this thesis, we raise the hypothesis that the NP fraction of the E171 is the most reactive fraction of the food additive on inducing inflammation and enhance the development of colorectal cancer via the production of ROS. For this reason, we compared E171 to NPs with a size range of 10 to 25 nm and MPs with an average size of 535 nm.

Firstly, colloidal stability of particles in aqueous solutions was determined. High colloidal stability causes less agglomeration in a way that particles are more likely to infiltrate cells or contain higher surface area which could lead to higher ROS production. Next to the colloidal stability, the mobility in solution is an important factor to characterize particles. The mobility indicates the potential stability of the colloidal system, which is in this study, solid particles dispersed in liquid (medium or buffer). Due to electrostatic repulsion particles will tend to repel to each other which will lead to less agglomeration. This agglomeration tendency in different dispersion solutions was measured by zeta potential.

Next, $TiO_2$ particles were tested for cytotoxicity on Caco-2 cells. This cell line was chosen as model of the human intestinal barrier since it represents the *in vivo* structural characteristics and mirrors the differentiation of the brush border at molecular levels [14]. Their intrinsic capacity to produce ROS in a cell-free as well as in a cellular environment was also evaluated since ROS production is termed as oxidative stress. With ESR spectroscopy, it could be revealed whether ROS formation is involved in carcinogenic mechanisms of $TiO_2$ and thereby determine which type of particles in E171 (micro,- or nanoparticles) has the highest capacity of producing ROS.

Carcinogenic mechanisms of $TiO_2$ particles (E171 and NPs) were also reviewed to address health concerns regarding carcinogenesis due to particle exposure. ssDNA breakage was measured with the alkaline comet assay to investigate the ability to enhance the genotoxicity of AOM in Caco-2 cells.

# 2. Materials and methods

## 2.1 $TiO_2$ particles

E171 was kindly donated by the Sensient Technologies company in Mexico. E171 contains 36% Nano-sized (average size 10-25 nm) particles and 67% micro-sized particles (average size 535 nm). Nanoparticles (NPs) were purchased at Io-Li-Tec (Germany) and are 99.5% anatase with particles size of 10-25 nm with a specific surface area of 50 – 150 $m^2/g$. Microparticles (MPs) were customarily made by PlasmaChem with an average size of 535 nm.

### 2.1.1 Dispersion of $TiO_2$ particles

To assess the effect of $TiO_2$ it is important that the particles are well dispersed in buffer or medium. In several toxicological studies, different surfactants have been tested to increase the dispersibility of nanomaterials. Bovine Serum Albumine (BSA) is found to be the best surfactant of nanomaterials including $TiO_2$ in several studies [22, 23] and has been applied in larger harmonized studies on nanomaterials genotoxicity [24]. $TiO_2$ particles (E171, NPs or MPs) were weighed into glass tubes and dispersed in 0.05% BSA Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich), Hank's Balanced Salt Solution (HBSS) supplemented with $Mg^{2+}$ and $Ca^{2+}$ (Life Technologies, The Netherlands) or PBS at a concentration of 1 mg/mL. Particles stock solutions were sonicated in a bath sonicator (Branson 2200, 40 KHz) for 30 minutes and further used to prepare the dilutions needed for exposure.

### 2.1.2 $TiO_2$ particles characterization with Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) was performed for characterization of hydrodynamic size distribution and zeta potential in dispersion solutions to determine the predictive toxicity of $TiO_2$ particles once introduced into the body. DLS analyzes the distribution acceleration of particle motion in solution by measuring dynamic fluctuations of light scattering intensity. $TiO_2$ particles were prepared as written in 2.1.1 and diluted in 0.05% BSA DMEM and HBSS supplemented with $Mg^{2+}$ and $Ca^{2+}$ at concentrations of 1.0 mg/mL and 0.001 mg/mL in duplicate. Samples were transferred into a disposable folded capillary (DTS 1060, Malvern Instruments) and placed in a Malvern Nano ZS (Malvern Instruments, UK) dynamic light scattering (DLS) instrument equipped with a 633 nm He-Ne laser. Measurements were performed at 25°C, with equilibration time set at 0 seconds, viscosity at 0.8872cP and a refracting index of 1.330.

## 2.2 Culturing of the human colon carcinoma cell line

The human colon carcinoma cell line Caco-2 was cultured in DMEM supplemented with glutamine, glucose, sodium pyruvate, penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS). Cells were grown and maintained at 37°C in a humidified incubator containing 5% $CO_2$. For experiments, Caco-2 cells were seeded in 21 $cm^2$ culture dishes and grown for three days to reach 80-100% confluency in 10% FBS medium in order to obtain ± $3.10^6$ cells/dish.

## 2.3 Cytotoxicity tests of $TiO_2$ particles

Prior exposure, cells were cultured for three days in 10% FBS medium and thereafter washed twice with HBSS. Subsequently, cells were exposed to a range of concentrations E171, MP or NP (1 – 0.001 mg/mL which is equivalent to $1.43.10^2$ – $1.43.10^{-1}$ µg/$cm^2$) dispersed in 0.05% BSA DMEM. After 24 hours, cells were washed twice with HBSS followed by trypsinization of the cells. It is known that NPs can react with the active agent of the viability or cytotoxicity tests [25-27]. Previous research revealed interactions of $TiO_2$ with LDH and MTT tests even at low concentrations (data not shown). Viability of the cells was assessed with Trypan blue assay and counted with an automatic cell-counter (Luna II, Logos Biosystems) after Trypan blue (0.4%) was added to the cell suspension (1:1). Non-cytotoxic concentrations of E171, $TiO_2$ NP or MP were selected based on viability (>80%) of the cells per condition compared to negative control. Triton-X-100 (Sigma-Aldrich) (1%) was added to the cells as positive control.

## 2.4 Identification and quantification of radical formation with electron spin resonance (ESR) spectroscopy

Electron spin resonance (ESR; also known as electron paramagnetic resonance) spectroscopy was chosen for detection and quantification of ROS and free radicals. As this is not a well- known and commonly used technique, the methodology of ROS detection and quantification is explained in the next paragraph.

### 2.4.1 Methodology of ESR

The base of ESR spectrometry lies in the configuration state of an electron and its associated magnetic moment. When a magnetic field is applied, radicals containing unpaired electrons behave like magnets which can have two possible spin states; $M_s$=+½ or $M_s$=-½ which represents the projection of the electron spin on the direction of the magnetic field (figure 3). These two possible spin states have different energies which is a result of the Zeeman Effect. When the magnetic moment of an electron is aligned (parallel) with the magnetic field a lower energy state occurs ($M_s$=-½), where a higher energy state occurs when the magnetic moment of the electron is aligned against (antiparallel) the magnetic field ($M_s$=+½).

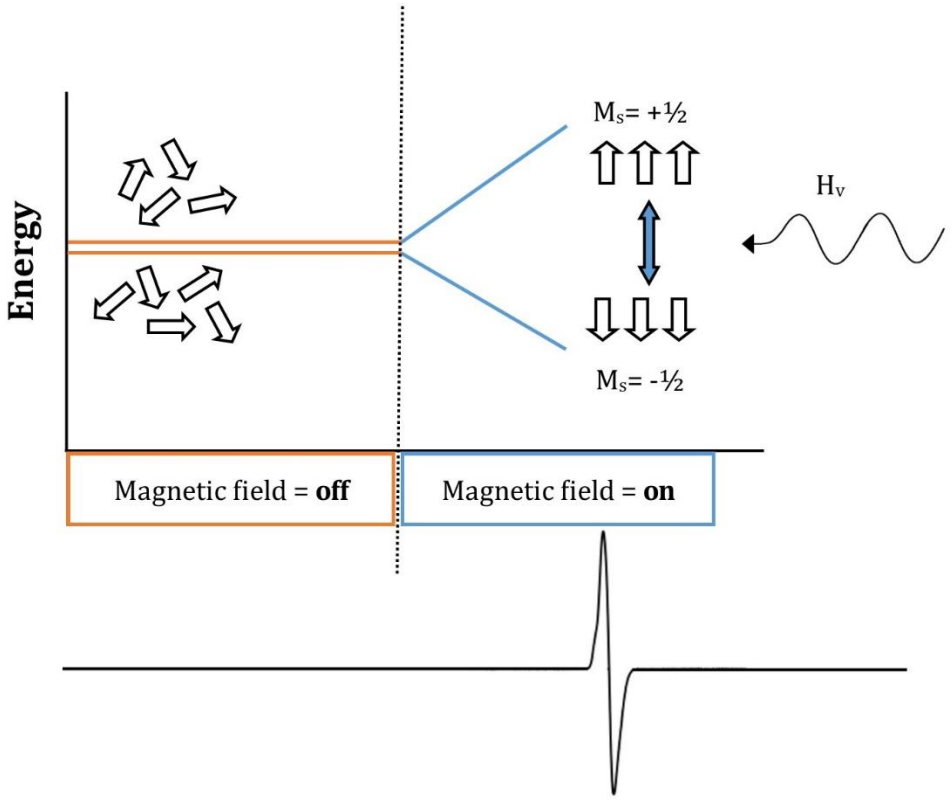



**Figure 3** Unpaired electrons irradiated with microwave radiation (Hv) will undergo transition state transitions between Ms= +½ and Ms= -½ (which represents the spin-up and spin-down state of the electron) when a magnetic field is applied. The change in electron state is the signal seen in the ESR spectra.

ESR can detect and quantify oxidation and reduction processes and is applicable for species with one or more unpaired electrons e.g. free radicals and transition metal compounds. Figure 4 illustrates a schematic setup of an ESR spectroscopy. The radiation source (figure 4; 'source') is a high power microwave source which is very stable with a high sensitivity. In ESR spectroscopy, the frequency of the radiation (Hv) is held constant while the magnetic field is varied in order to obtain an absorption spectrum.

For measurements, the capillary (which contains the sample) is placed into a resonant chamber which is located in between two electromagnets (figure 4). This sample cavity allows microwaves through an iris and facilitates to amplify the weak signals from the sample. In practice, most of the external components, such as the source and detector contain a microwave bridge control to stabilize the signals. Additionally, a field modulator, attenuator and amplifier contribute to a better performance of the instrument.

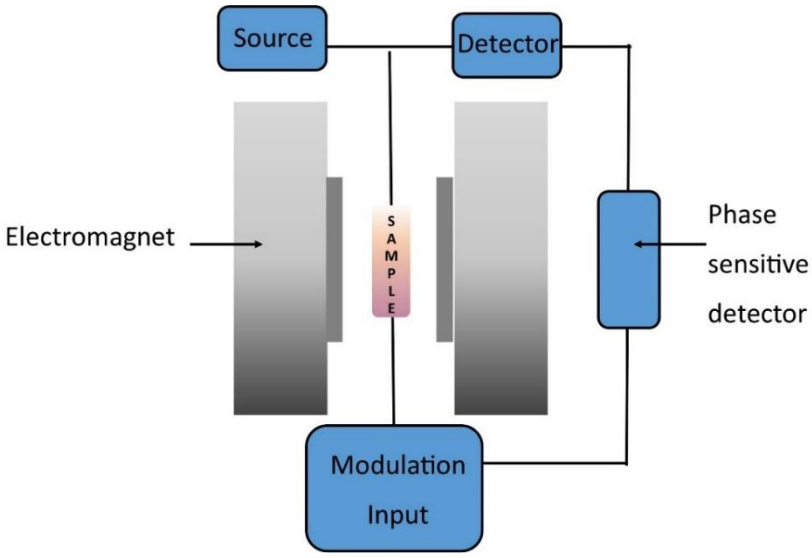
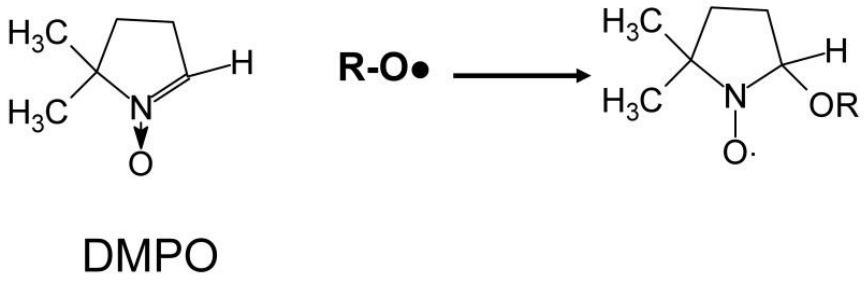



**Figure 4** Electron spin resonance setup

Due to short half life time (T½) of most of the free radical species (T½ OH• = $10^{-9}$ seconds), detection of these potential harmful causing oxidative stress free radicals is challenging since molecular stabilization is urgent. This stabilization of free radicals is accomplished with a diamagnetic spin trap (5,5-dimethyl-1-pyrolline N-oxide; DMPO) which reacts with reactive short-lived free radicals and form a more persistent nitroxide or spin adduct (figure 5).




DMPO

**Figure 5** 5,5-Dmethyl-1-purroline N-oxide (DMPO) spin trap reacts with a reactive short-lived free radical (= R) to form a more persistent nitroxide or spin adduct. From the spin adduct ESR spectrum, the structure of the reactive free radical can be deduced indirectly.

From the obtained ESR spectrum, the structure of the reactive free radical can be deduced indirectly.

### 2.4.1 Assessment of cell-free ROS formation by $TiO_2$ particles

Particles were dispersed as written in paragraph 2.1. Stock solutions of 1 mg/mL were serially diluted in 0.05% BSA medium, HBSS supplemented with $Ca^{2+}$ and $Mg^{2+}$ or PBS (1x). To assess the intrinsic capacity of ROS formation by E171, particles suspensions were incubated in a $CO_2$ incubator at 37°C with 50 mM DMPO (Sigma-Aldrich) for 30 minutes. $H_2O_2$ (1mM) was added to particles suspensions to assess the reactions which could take place in an alkali environment. After incubation, homogenized particles suspension was taken up into a 100 µL glass capillary. Radical formation was measured by ESR spectroscopy.

### 2.4.2 Exposure conditions to assess cellular ROS formation

Caco-2 cells (passage 24-32) were cultured as written in paragraph 2.2. Particles were dispersed as written in paragraph 2.1. The stock solutions of 1 mg/mL were serially diluted in dispersion solutions to obtain non-cytotoxic concentrations of 0.01 and 0.001 mg/mL. Prior exposure, cells were washed twice with HBSS supplemented with $Ca^{2+}$ and $Mg^{2+}$. Cells were exposed to non-cytotoxic concentrations of E171, NPs or MPs in a $CO_2$ incubator at 37°C for several exposure times (30 minutes, 1, 2, 4, 6 and 24 hours, respectively). In addition, $H_2O_2$ was added at a non-cytotoxic concentration (20 µM, according to cytotoxicity tests by Briedé *et al*. [55]) to cells together with particles suspensions to mimic an inflammatory environment. Thirty minutes before ending the exposure, spin trap DMPO (50 mM) was added to the cells and incubated at 37°C. After exposure, cells were harvested by scraping and taken up into a 100 µL glass capillary and radical formation was measured by ESR spectrometry.

### 2.4.3 ESR spectroscopy measurements

Radical formation in response to (non-)cytotoxic concentrations $TiO_2$ exposure was measured by ESR spectroscopy in combination with spin trapping technique using 50 mM of DMPO with an incubation time of 30 minutes at 37°C. This incubation time was based on previous time-course experiments performed by Hebels *et al*. [56] where at 30 minutes the highest DMPO-radical adduct concentration generation was found. Before use, DMPO was purified according to the following protocol. DMPO was diluted in nitrogen-flushed Milli-Q water and purified by a charcoal treatment. The final concentration of DMPO was then established by spectrophotometry (λ =234 nm) and with an extinction coefficient of e=7700/M/cm. Stock solutions of DMPO were tested on forehand on •OH formation by $H_2O_2$ and $FeSO_4$ (0.75 mM) and scavenging by DMPO was tested and to verify the activity of DMPO (data not shown). After sealing, the capillary was immediately placed in the resonator in the ESR spectrometer. ESR spectra were recorded at room temperature on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS High sensitivity resonator and 12 kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were as follows: magnetic field 3450 G; scan range 200 G; modulation amplitude 1G; receiver gain $1x10^4$; microwave frequency 9.85 GHz; power 50 mW; time constant 40.96 ms; scan time 20.97 s; number of scans 35. Quantification of the spectra was performed by peak height measurements using the WIN-EPR spectrum manipulation program (Bruker). For comparison, spectra were visualized at identical intensity scale in this thesis.

## 2. 5 Genotoxicity assessment with alkaline comet assay

Cells (Passage 40-47) were grown for 3 days to reach confluency of 80-90% and were washed twice with HBSS supplemented with $Ca^{2+}$ and $Mg^{2+}$ before exposure. All the following steps are performed in the dark. Cells were exposed to non-cytotoxic concentrations E171 or NPs in a $CO_2$ incubator at 37˚C for 24 hours with or without 20 µg/mL azoxymethane (AOM) (Sigma Aldrich), a genotoxicant. Each condition was assessed in duplicate. As positive control, cells were exposed for 30 minutes to 200 µM $H_2O_2$. After washing with HBSS supplemented with $Ca^{2+}$ and $Mg^{2+}$, cells were harvested by trypsinization for 5-10 minutes and suspended in PBS (1x) at a concentration of $1x10^6$ cells/mL. A mixture of 25 µl cell suspension with 75 µl 0.6% low melting point agarose (Sigma Aldrich) was pipetted onto 1.5% agarose (Eurogentec) pre-coated slides in duplicates. Slides were lyzed for at least 1 hour at 4°C in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 250 mM NaOH, (all from Sigma Aldrich) pH 10, containing 1% Triton-X-100 (Sigma – Aldrich) and 10% DMSO (Merck, Germany) as final concentration). All slides were placed into an electrophoresis tank placed at 4°C. After DNA unwinding in alkaline electrophoresis buffer (300 mM NaOH, 1mM EDTA, pH >12) for 30 minutes, electrophoresis was performed at 300 mA, 25V for 20 minutes. Subsequently, slides were neutralized for 10 minutes in PBS (1x) and washed in pure ethanol. Before analysis, slides were stained with 30 µl of 10 µg/mL ethidium bromide (Etbr). Comet appearances were blindly analyzed using a fluorescence microscope (Zeiss, Germany) at 400x magnification using immersion oil. A total of randomly selected 50 cells were analyzed per slide per experiment. Comet image analysis software program was used for quantification of DNA damage. Comet tail and intensity were measured by using Comet IV software. The median tail intensity was used as DNA damage indicator [57].

## 2.6 Data analysis

Each (cellular) experiment was performed with three (biological) replicates with each unique sample in duplicate. Results were expressed as mean ± standard error (SE) except for cytotoxicity results which were expressed as mean ± standard deviation (SD). Differences between groups were evaluated using analysis of variance (ANOVA) except for Cytotoxicity assay results which were evaluated using unpaired two-tailed Student's T-test. Differences were considered significant with a P-value < 0.05.

# 3. Results

During this study the potential carcinogenic effects of food grade $TiO_2$, in particular E171, the possible facilitation of colorectal cancer was addressed. It was hypothesized that via oxidative stress responses by ROS production, E171 has toxic effects on Caco-2 cells at non-cytotoxic concentrations and that such mechanisms may be involved in the possible carcinogenic effects that have been reported for E171. In this *in vitro* study, E171, $TiO_2$ MPs and NPs were characterized with Dynamic Light Scattering (DLS) (paragraph 3.1) whereupon the non-cytotoxic concentration was determined with viability tests (paragraph 3.2). Involved mechanisms of possible carcinogenicity were assessed by ESR for ROS formation (paragraph 3.3). Finally, ssDNA damaging properties of E171 and $TiO_2$ NPs were addressed with the alkaline comet assay (paragraph 3.4).

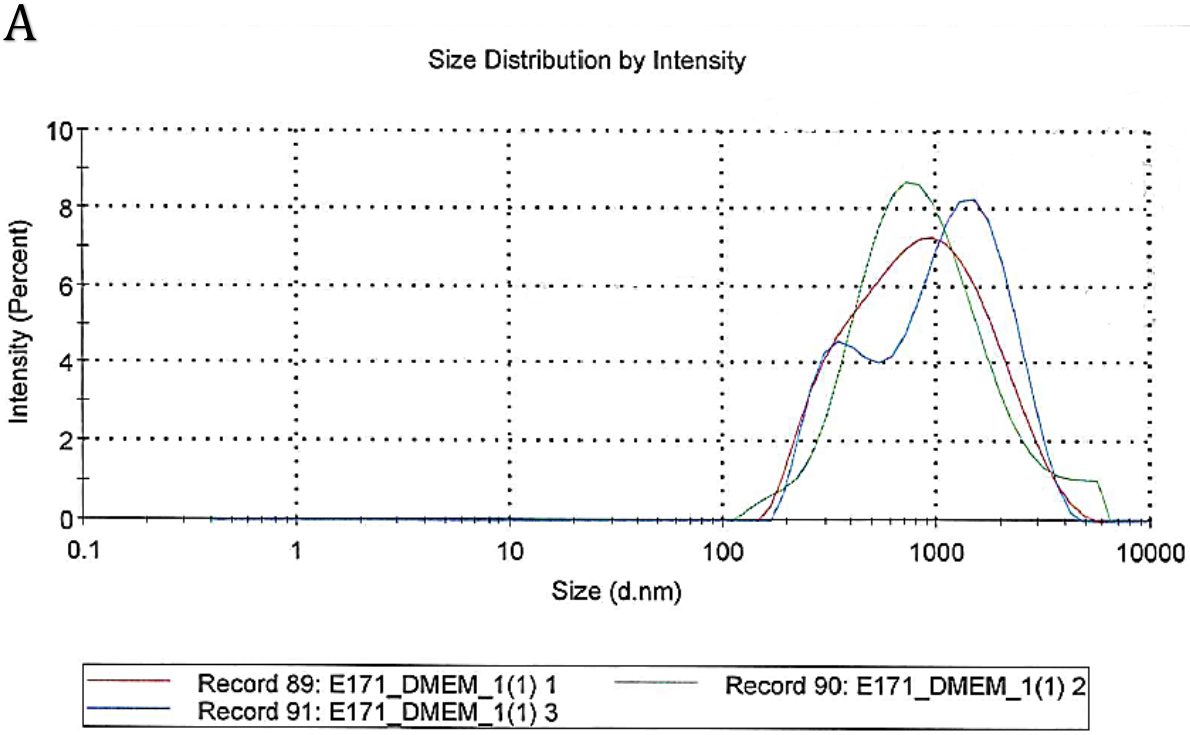
## 3.1 Characterization of E171, NPs and MPs in dispersion solutions

Hydrodynamic size and zeta potential of the three different types of particles (E171, $TiO_2$ NPs and MPs) were characterized by using DLS. Particles were dispersed in 0.05% BSA dissolved in DMEM and in HBSS buffer without BSA at two different concentrations: 1 and 0.001 mg/mL ($1.43.10^2$ and $1.43.10^{-1}$ µg/cm$^2$ respectively). The result of this characterization is shown in table 1.

Table 1 shows that even with the presence of BSA and after 30 minutes of sonication (40kHz), the dispersion results in particle agglomeration. Three different sizes (figure 6A) are found when E171 is dispersed in HBSS at 1 mg/mL while only one size (figure 6B) was found when dispersed in DMEM with BSA. The size differences caused by dispersion solutions indicates that E171, consisting of micro- and nano-sized particles, agglomerates to micro-sized particles with a size of 3085 d.nm when dispersed in HBSS without BSA.

There is a significant difference of size between the presence and the absence of BSA ($P < 0.001$). Indeed, in HBSS (buffer without BSA), the average hydrodynamic size of E171, $TiO_2$ MPs and NPs particles respectively is around $3.10^3$ d.nm whereas in medium with BSA the average hydrodynamic size is approximately $1.10^3$ d.nm at a concentration of 1.0 mg/mL. This shows the importance of BSA to improve the dispersion and the stability of the solution. The more the particles are diluted, the more polydispersed. Surprisingly, E171 is has the lowest hydrodynamic size in DMEM (669 d.nm) compared to MPs (1385 d.nm) and nanoparticles the highest size (1942 d.nm).

A




B

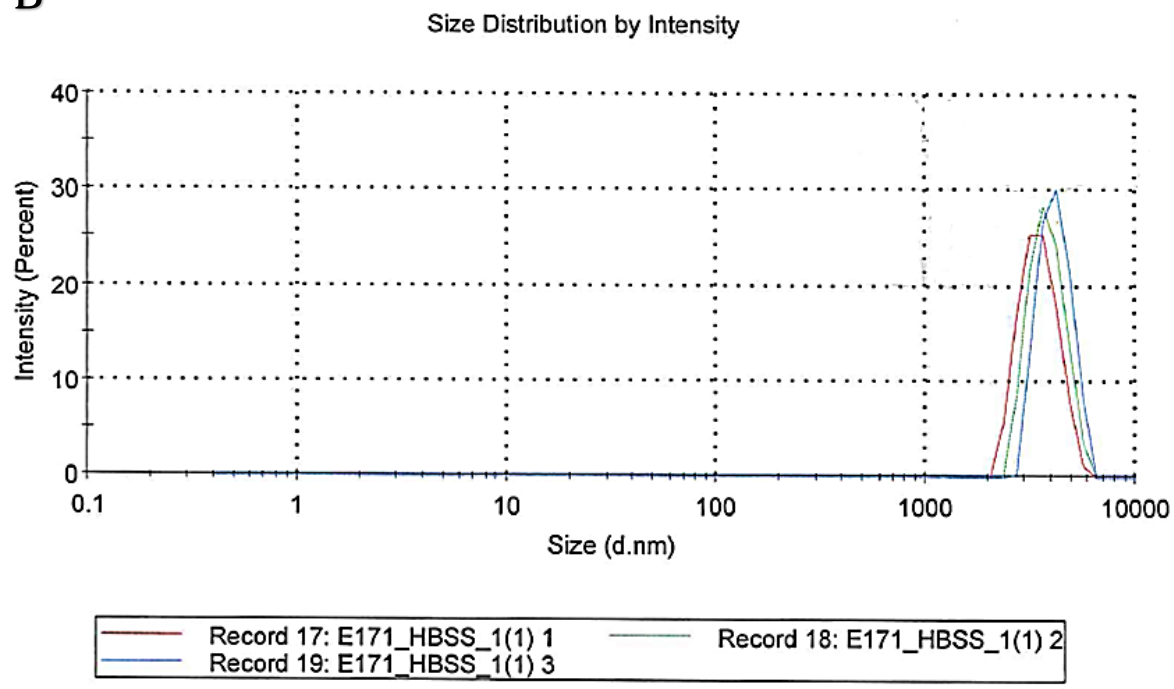



***Figure 6*** *DLS spectra of E171 (1.0 mg/mL) dispersed in* ***A)*** *DMEM with BSA or* ***B)*** *HBSS buffer. Hydrodynamic size is shown on the X-axis (shown in dynamic nanometers, d.nm). Intensity (%) is shown on the y-axis which represents the amount of particles detected relative to the total amount. Three independent measurements are performed per sample which is shown in red, blue and green. E171 in DMEM has three different hydrodynamic sizes* ***(A)*** *while E171 in HBSS has only one hydrodynamic size****(B)****. This indicates that E171 forms agglomerates in HBSS.*

***Table 1*** *DLS results of $TiO_2$ NPs, MPS and E171 (consisting of micro- and nano-sized $TiO_2$ particles).*

| Dispersant | Sample | Concentration (mg/mL) | Peak 1 (d.nm) | Peak 2 (d.nm) | Peak 3 (d.nm) | Peak 4 (d.nm) | Zeta potential (mV) |
|---|---|---|---|---|---|---|---|
| **HBSS** | E171 | 1.0 | 3085 | | | | -4.39 ± 0.12 |
| | | 0.001 | 5 | 644 | 5417 | | -15.03 ± 1.62 |
| | MP | 1.0 | 2565 | | | | -5.62 ± 0.09 |
| | | 0.001 | 640 | 5560 | | | -6.95 ± 0.62 |
| | NP | 1.0 | 2641 | | | | -6.08 ± 0.09 |
| | | 0.001 | 2160 | | | | -8.02 ± 0.69 |
| **DMEM + 0.05% BSA** | E171 | 1.0 | 670 | 821 | 3380 | | -12.97 ± 0.29 |
| | | 0.001 | 481 | 1220 | 5560 | | -12.78 ± 0.52 |
| | MP | 1.0 | 1386 | | | | -14.10 ± 0.56 |
| | | 0.001 | | | | | -13.40 ± 0.44 |
| | NP | 1.0 | 1942 | | | | -13.12 ± 0.44 |
| | | 0.001 | 1213 | | | | -10.66 ± 0.98 |
| | Control | | 11 | 156 | 664 | 5560 | -6.18 ± 1.35 |

The results obtained by the zeta potential are shown in table 1. The zeta potential is a measurement of the potential stability of the colloidal system which is in our study solid particles dispersed in liquid (0.05% BSA medium or buffer). When the zeta potential have large negative or positive potential (±30 mV), they will tend to repel each other where there is no tendency to agglomerate. In contrast, if the particles have low zeta potential values, there is no force to prevent the particles attaching to each other and agglomerating. Some results with the lowest concentrations tested (0.001 mg/mL) had an isoelectric point (PI) > 0.7 which means that these results are not reliable and therefore not included in the table. There is a significant difference between the dispersion in HBSS and DMEM ($P < 0.001$) except for E171 and NPs at a concentration of 0.001 mg/mL. The addition of BSA at low concentrations (0.05%) will lead to more colloidal stability what will lead to decreased agglomeration of the particles. However, the zeta potential values are between -30 mV and +30 mV which mean that the solutions still stay unstable.

## 3.2 Cytotoxicity and dose selection of E171, $TiO_2$ MPs and NPs

Initially, viability assays were performed with MTT and LDH. The results showed an interaction between $TiO_2$ and the assay (data not shown) as shown in previous toxicological studies [22, 25-27]. For this reason, cytotoxicity was assessed with the Trypan blue assay.

In figure 7, cell viability after 24h exposure to several concentrations ranging from 1.0 to 0.001 mg/mL (which is equivalent to $1.43x10^{2}$ to $1.43x10^{-1}$ µg/cm$^2$) of E171 (black square), MP (blue dot), NP (grey triangle) is shown. Zero µg/cm$^2$ is stated as the control. Concentrations are considered as cytotoxic when average cell viability is below 80% and significantly lower than control (=0 mg/mL).

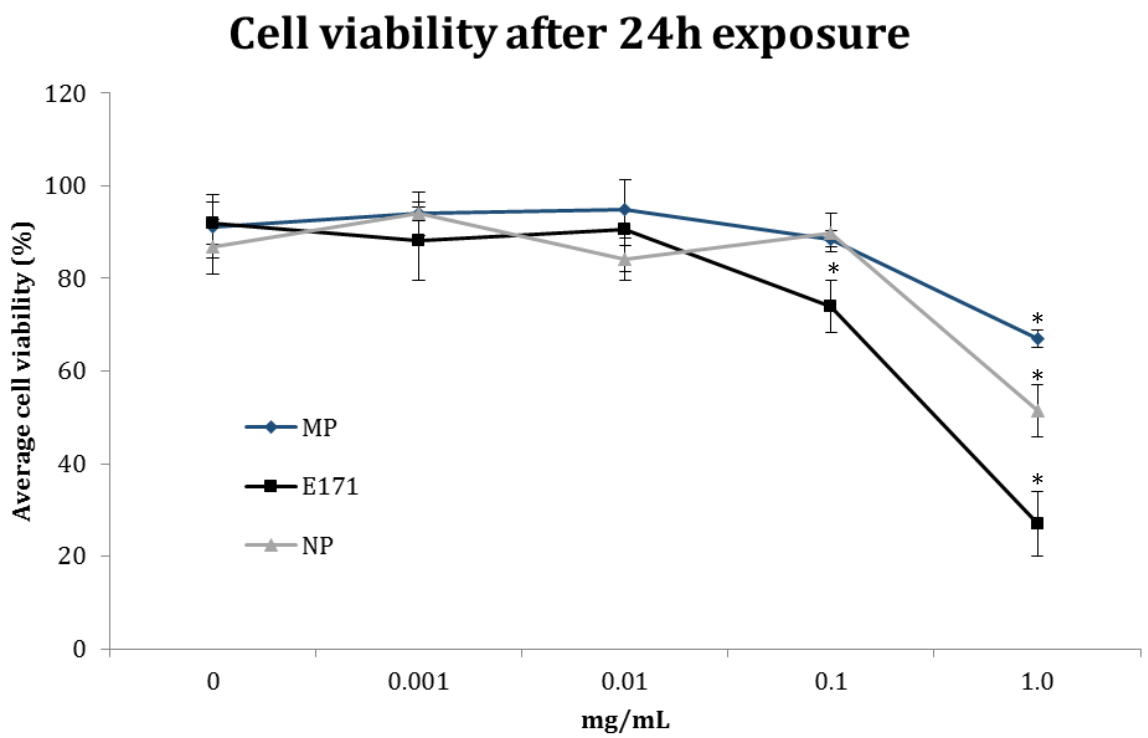



***Figure 7*** *Average viability of Caco-2 cells (shown at Y-axis in percentage) after 24 hours exposure to E171 (black square), microparticles (MP; blue dot) and nanoparticles (NP; grey triangle) at various concentrations (shown at X-axis in mg/mL) dispersed in DMEM supplemented with 0.05% BSA. Statistically significant differences compared to control (=0 mg/mL) are indicated by an asterisk. (*, P < 0.05). n=3 biological replicates. Mean ±SD*

Trypan blue viability test showed cytotoxicity after 24h exposure to Caco-2 cells with 1 mg/mL ($1.43x10^2$ µg/cm$^2$) of E171, MP and NP. E171 at a concentration of 0.1 mg/mL ($1.43x10^1$ µg/cm$^2$) is significant cytotoxic (average viability = 73.99%, P = 0.002) whereas this concentration of MPs and NPs is non-cytotoxic (average viability > 80%). Lowest concentrations 0.01 and 0.001 (1.43 and $1.43x10^{-1}$ µg/cm$^2$) shows no cytotoxicity for all three samples (all P > 0.1).

Based on these data a dose for further studies with these particles was selected. The following concentrations: $1.10^{-2}$ and $1.10^{-3}$ mg/mL (equivalent to $1.43x10^1$ and 1.43 µg/cm$^2$) corresponding to non-cytotoxic concentrations of all three different particles were selected.

## 3.3 ROS formation

In the following two paragraphs results of the assessment of hydroxyl radical formation from $TiO_2$ NPs, MPs or a combination of both (E171) in a cell-free (paragraph 3.3.1) or cellular (in Caco-2 cells, paragraph 3.3.2) environment are shown. In addition, cells were co-exposed to AOM together with $TiO_2$ NPs, MPs and E171 (paragraph 3.3.3).

### 3.3.1 Intrinsic ROS formation capacities of E171, $TiO_2$ NPs and MPs

Cell type specific pathways in cellular damage and carcinogenesis induced by oxidative stress may be the underlying risks for the development of chronic inflammation (e.g. IBD) and colorectal carcinogenesis [50]. For prevention and treatment of colorectal cancer, it is of importance to investigate cell type- specific changes at molecular levels. Oxidative stress related effects, one of the main molecular mechanisms involved in colorectal carcinogenesis also specified as ROS formation, are investigated with ESR spectroscopy in combination with spin trapping technique using DMPO.

We hypothesized that nano-sized $TiO_2$ particles in E171 are responsible for carcinogenic effects by inducing ROS formation. To investigate this, we first determined the intrinsic capacity of the particles to produce ROS. HBSS buffer was chosen as dispersion solution since no ROS formation as detected (DMPO could not trap radicals) when E171 particles were dispersed in 0.05% BSA medium which is shown in figure 8A. Figure 8B represents a spectrum obtained from ESR analysis of 1 mg/mL E171 dispersed in HBSS in absence of cells. Peaks are shown at 3463-3464, 3478-3479, 3493-3494 and 3508-3509 G respectively with a total peak height of 35,093. This spectrum shows a characteristic 4-line 1:2:2:1 ESR signal identified as a ROS-induced DMPO•-OH signal.

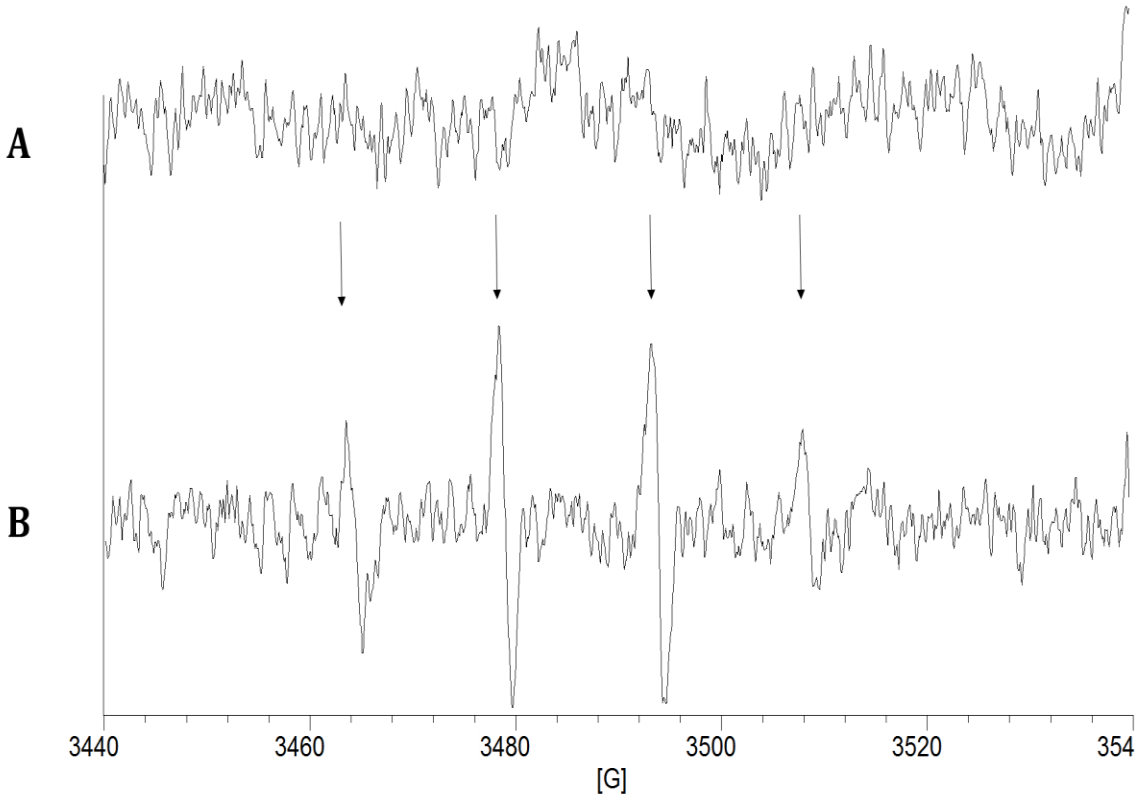



***Figure 8*** *ESR spectra of E171 at a concentration of 1 mg/mL dispersed in* ***A)*** *0.05% BSA medium or* ***B)*** *HBSS buffer in absence of cells. Figure* ***A*** *represent no DMPO•-OH signal whereas this is shown in figure* ***B****. Total peak height;* ***A)*** *8,765* ***B)*** *35,039*

Figure 9 illustrates the calculated peak heights obtained from ESR spectra of E171, $TiO_2$ MPs and NPs tested in cell-free environment. During these experiments, highest (1.0 mg/mL) and lowest concentrations (0.01 and 0.001 mg/mL) were tested with (black bars) or without (white bars) 1mM $H_2O_2$.

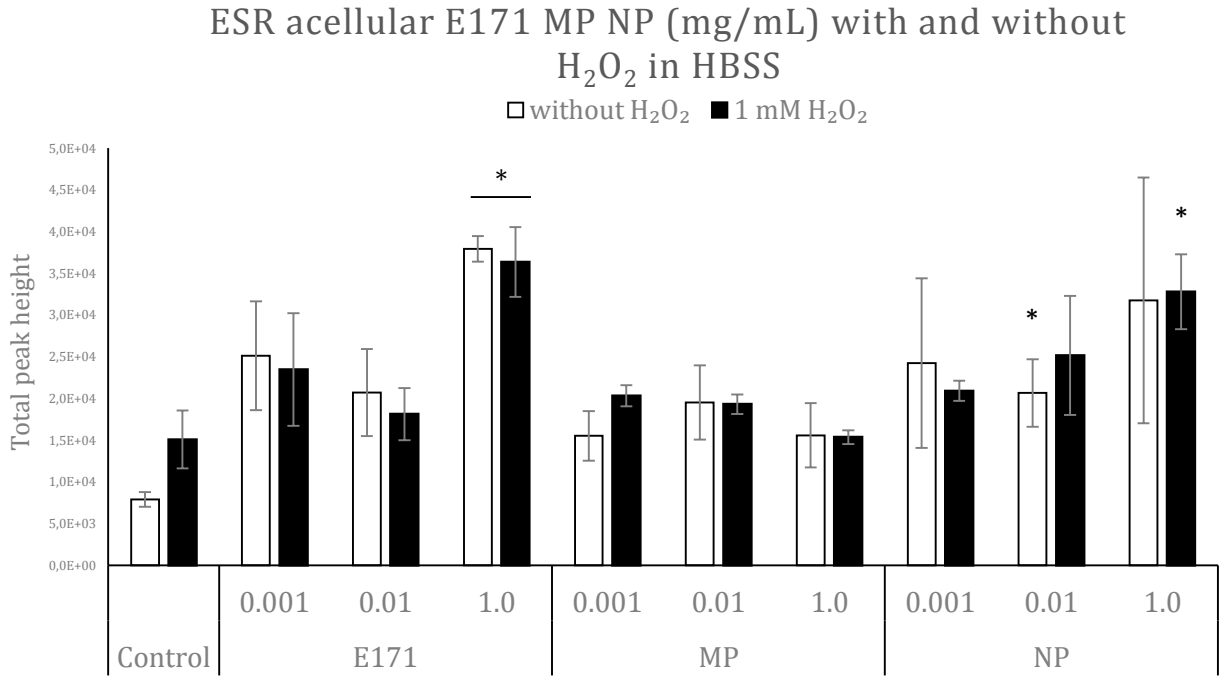



***Figure 9*** *Intensity of various concentrations E171, microparticles (MP) and nanoparticles (NP) in HBSS in cell-free radical formation presence (1 mM) or absence of $H_2O_2$ shown on x-axis. Y-axis represents the average total peak height of obtained ESR spectra calculated per sample (n≥3). Results include background levels and are shown as significant difference (indicated with an asterisk; *P < 0.05) compared to the control, Mean ± SE.*

The particles can be sorted by their intrinsic ROS formation capacity in the following order: E171 > NPs > MPs. E171 has the highest capacity to produce ROS in solutions with the highest total peak height of 37,926 with the concentration of 1 mg/mL which is followed by NPs (32,769) and MPs (15,583). As hypothesized, $TiO_2$ NPs were able to produce significant amounts of radicals ($P < 0.05$) but only without $H_2O_2$ at a concentration of 0.01 and with $H_2O_2$ at a concentration of 1.0 mg/mL. Other conditions of NPs do have intrinsic properties to produce sufficient ROS radicals although not significantly higher than controls. Next to $TiO_2$ NPs, E171 at highest concentration show ROS formation levels which are significant higher than control conditions ($P < 0.01$). A clear dose response reaction is seen in E171 and NPs whereas MPs show similar ROS formation levels for all concentrations. In general, MPs show lowest ROS formation levels which are relatively equal to control levels. These data show that the NP particles at non-cytotoxic concentrations have the highest ROS formation intrinsic capacity which is in line with DLS results were NPs had the highest hydrodynamic size when dispersed in HBSS. The high ROS formation capacity of E171 at a concentration of 1.0 mg/mL could be due to agglomeration by low colloidal stability of the particles, which was also found in DLS and zeta-potential results.

### 3.3.2 Cellular ROS formation in reaction to E171, $TiO_2$ MP and NP exposure

ROS formation in reaction to non-cytotoxic concentrations of $TiO_2$ was assessed with ESR spectroscopy in combination with spin trapping technique using DMPO. Non-cytotoxic concentrations of E171, $TiO_2$ MPs and NPs were selected based on cytotoxicity tests as described in paragraph 3.2. The results of this cellular ESR assessment are shown in this paragraph.

Since cellular oxidative stress-related effects differ in time, changes of ROS formation was observed over time. Firstly, time series analysis of ROS formation response in Caco-2 cells was performed with non-cytotoxic concentrations of E171, $TiO_2$ MPs and MPs with exposure times of 30 min, 1h, 2h, 4h, 6h and 24h. In the presence of Caco-2 cells, no radical formation was observed after 30 minutes exposure of E171, $TiO_2$ MPs nor NPs. E171 and $TiO_2$ MPs and NPs produced hydroxyl radicals (DMPO•-OH), solely at 1h exposure time. For all particles at continued cellular exposure (t>2h) ROS formation was not detectable anymore. From these results, it was decided that Caco-2 cells would be exposed for one hour prior ESR spectroscopy to observe ROS formation.

As previously found in cell-free experiments, HBSS buffer was chosen as dispersion solution since no ROS formation was detected when particles were dispersed in 0.05% BSA medium (data not shown). These findings were also confirmed in cellular experiments. Based on previous cellular time series analysis results, incubation time of 1h was selected for follow-up cellular ESR experiments with E171, $TiO_2$ MPs and NPs dispersed in HBSS buffer. Figure 10 illustrates the peak height calculated of spectra obtained from cellular radical formation by E171, MPs and NPs incubated 1h with Caco-2 cells and in presence (1 mM; black bars) or absence (white bars) of $H_2O_2$. Caco-2 cells were exposed to previously selected non-cytotoxic concentrations of E171, $TiO_2$ MPs and NPs (0.001 and 0.01 mg/mL respectively).

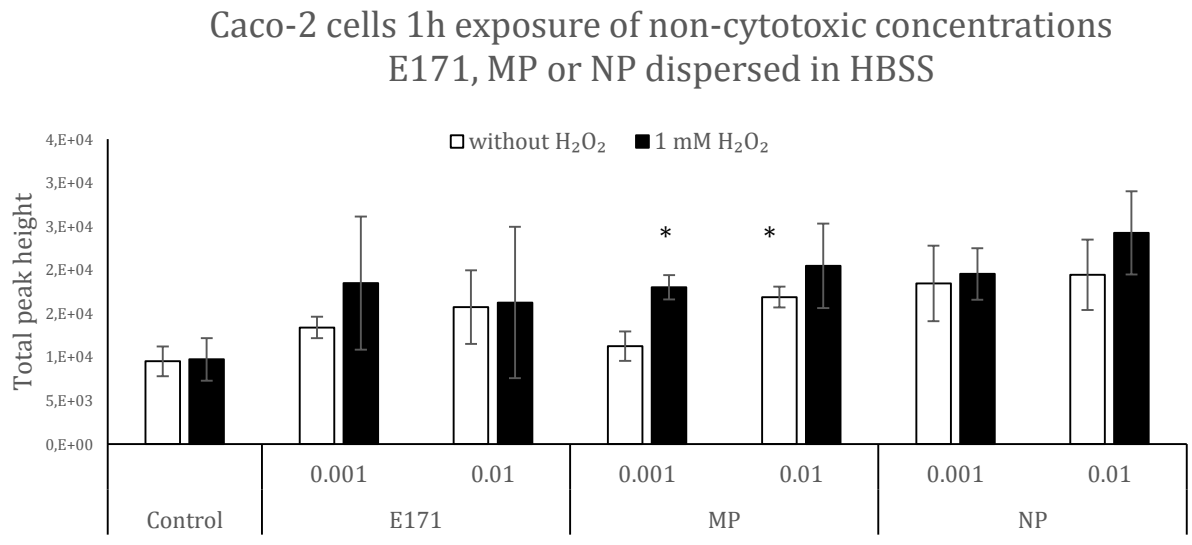



***Figure 10*** *Intensity of non-cytotoxic concentrations E171, microparticles (MP) and nanoparticles (NP) in HBSS in cellular (Caco-2) radical formation in presence (1 mM) or absence of $H_2O_2$ shown on x-axis. Y-axis represents the average total peak height of obtained ESR spectra calculated per sample (n≥3). Values include background levels and are shown as significant difference (indicated with an asterisk; *P < 0.05) compared to the control, mean ± SE.*

Caco-2 cells exposed to MPs at a concentration of 0.01 mg/mL produce significant (P = 0.01) amounts of ROS (DMPO•-OH). This significant ROS formation was also determined when cells were exposed to MPs at the lowest non-cytotoxic concentration (0.001 mg/mL) of MP co-exposed to $H_2O_2$ (P= 0.04). Either with or without $H_2O_2$, no significant ROS formation (P > 0.05) was found after E171 or NP exposure when compared to the control. In general, NPs show highest ROS formation levels with close significant difference compared to control conditions with a P value of 0.054. Consequently, results of the ROS production are particle dependent.

### 3.3.3 Cellular ROS formation in reaction to E171, $TiO_2$ MP and NP in co-exposure with AOM

We hypothesized that E171 ($TiO_2$ MPs and NPs) would enhance the genotoxic effects of AOM on Caco-2 cells, which was previously found *in vivo* by the collaborating research group Biomedicine from the National Autonomous University of Mexico. To this purpose, Caco-2 cells were exposed simultaneously to the genotoxic compound AOM and to $TiO_2$ particles (E171, MPs or NPs), both at non-cytotoxic concentrations. The concentration AOM was confirmed as non-cytotoxic (not significantly different from control, P= 0.80) with Trypan blue viability tests.

As shown in figure 11, co-exposure of AOM did not lead to significant increased ROS levels when compared to control (AOM alone). No significant difference is found between $TiO_2$ exposure and AOM co-exposure of Caco-2 cells.

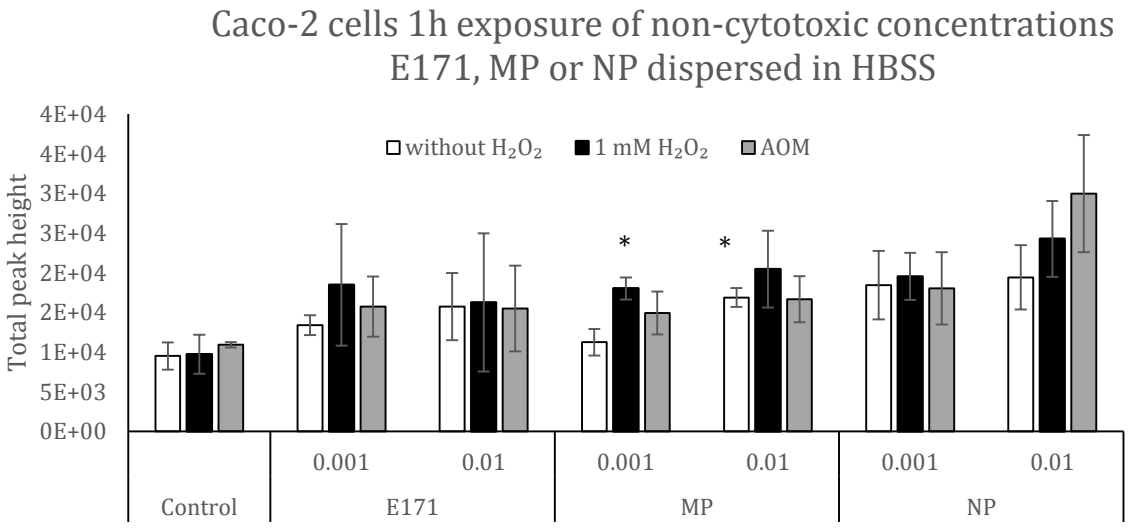



***Figure 11*** *Intensity of non-cytotoxic concentrations E171, microparticles (MP) and nanoparticles (NP) in HBSS in cellular (Caco-2) radical formation (white bars), co-exposed to 1 mM of $H_2O_2$ (black bars) or 20 µg/mL AOM (grey bars) shown on x-axis. Y-axis represents the average total peak height of obtained ESR spectra calculated per sample (n≥3). Results include background levels and are shown as significant difference (indicates with an asterisk; *P < 0.05) compared to the control, Mean ± SE.*

Remarkably, cells exposed MPs at non-cytotoxic concentrations and co-exposed to AOM have a supplementary peak (red arrow) besides the normally present peaks (black arrows) in its ESR spectra (figure 12A). This additional peak is present at 3487 – 3489 G which is identified as a vitamin-C antioxidant (figure 12B). This additional peak was solely found in MPs.

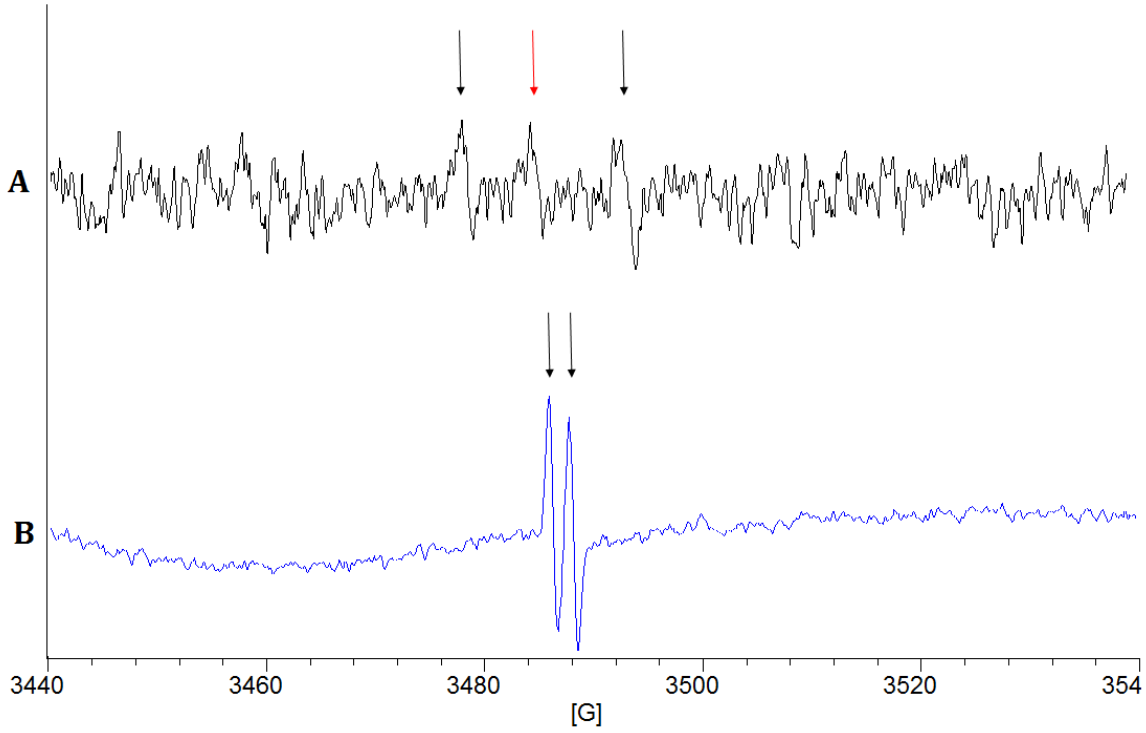



***Figure 12 A)*** *ESR spectra of MPs at a non-cytotoxic concentration of 0.001 mg/mL dispersed in HBSS buffer co-exposed with AOM (at non-cytotoxic concentration) for 1h to Caco-2 cells. Peaks are shown (from left to right) at 3478-3479(black), 3483-3484 (red) and 3493-3494 (black) G with a total peak height of 35,093.* ***B)*** *Representative spectrum from vitamin C radical. A signal was detected at ±3487-3490 G which was identified originating from Vitamin C radical. Vitamin C radical spectra obtained from Linschooten et al. [58].*

## 3.4 ssDNA strand breaks induced by E171, $TiO_2$ NPs and MPs

To investigate whether (hydroxyl) radicals formed in reaction to a non-cytotoxic concentration (paragraph 3.2; 0.001 mg/mL) of E171 or $TiO_2$ (nano- or micro) particles in absence or presence of cells have genotoxic properties, DNA damage in ssDNA isolated from treated and untreated Caco-2 cells was measured by using the alkaline comet assay. In this assay, after electrophoresis ssDNA damge is visible as a 'tail' of DNA fragments (figure 13) behind the cell center and is scored to determine the percentage of ssDNA fragments in the tail shown as tail intensity.

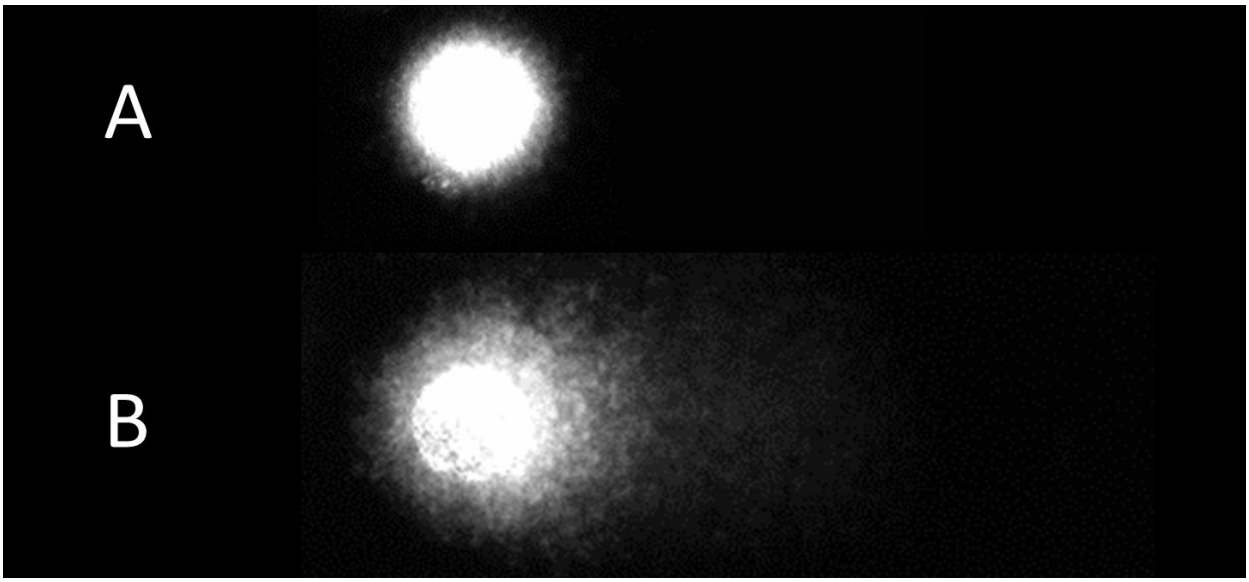



***Figure 13*** *Images of comets (from Caco-2 cells), stained with EtBr visualized with a fluorescence microscope at 400x magnification using immersion oil.* ***A)*** *Control; 24h of 0.05% BSA DMEM. Median tail intensity= 2.07%.* ***B)*** *24 exposure of 0.001 mg/mL. Median tail intensity=9.40%*

In order to verify the capacity of E171 and NPs to enhance ssDNA breaks, cells were co-exposed to the genotoxicant AOM at a non-cytotoxic concentration. Figure 14 shows mean results of the median tail intensity (%) of E171 (black bars) and $TiO_2$ NPs (grey bars) co-exposed with or without AOM.

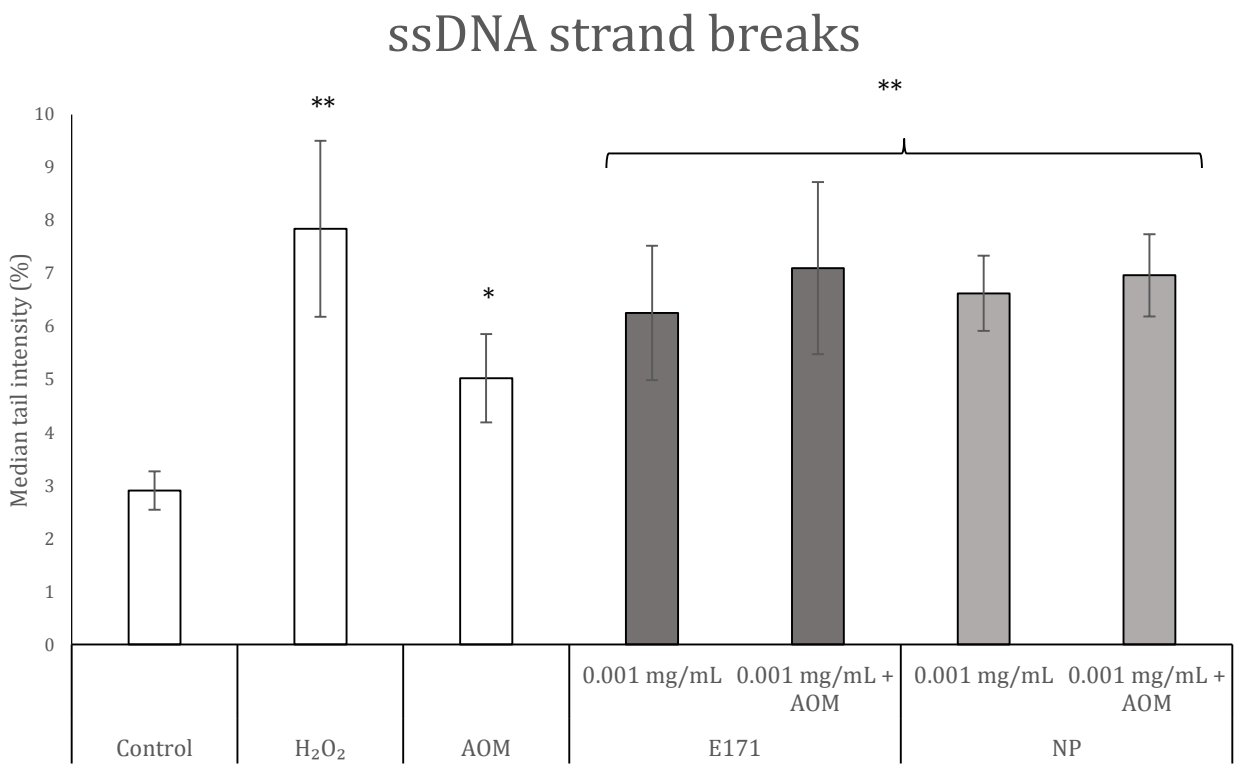



*Figure 14 Frequency of ssDNA strand breaks in Caco-2 cells after 24h exposure. Conditions are shown at X-axis (mg/mL). White bars; control; 0.05% BSA DMEM, $H_2O_2$; 30 minutes exposure of 200 µM $H_2O_2$ as positive control, AOM; 20 µg/mL AOM. Particles exposure of E171 (black bars), $TiO_2$ NP (grey bars), all at a non-cytotoxic concentration of 0.001 mg/mL (equals 1.43E$^{-01}$ µg/cm$^2$) with or without co-exposure of AOM. DNA damage is represented by median tail intensity shown at y-axis. All conditions are compared to control; *, P < 0.05, **, P < 0.001. n≥11, 4 biological replicates. Mean ±SE. $H_2O_2$= hydrogen peroxide, AOM= azoxymethane.*

Compared to the control, each condition shows significant higher ssDNA damage. The results indicate that E171 and NPs have a capacity to induce ssDNA strand break on Caco-2 cells whether AOM is present or not. There is a significant difference ($P < 0.05$) between the median tail intensity of $H_2O_2$ and with E171 only and NP only. With AOM the induction of ssDNA strand breaks is higher than in absence of AOM but not significantly.

There is no significant difference between exposure with or without AOM. No significant difference in ssDNA breaks is found when exposed to AOM in combination with particles when compared to AOM alone. These comet assay results suggest that E171 and NPs are genotoxic by inducing ssDNA strand breaks in Caco-2 cells.

# 4. Discussion

The colon is the major site for the uptake of foods and nutrition but also potentially hazardous compounds which can be present as food additive in the food. Titanium dioxide ($TiO_2$) is generally considered as non-toxic mineral and is widely used as nano-sized food additive coded as E171 consisting of 67% MPs and 33% NPs. Contradictory data is available about health risks of $TiO_2$ which makes it important to investigate the potential toxicity after intake. The focus of this study was to evaluate *in vitro* toxic effects and its involved mechanisms of E171 ($TiO_2$) on the development of colorectal cancer.

**Characteristics of $TiO_2$ NPs, MPs, and E171 in BSA DMEM and HBSS buffer**

Prior studying the genotoxicity and oxidative stress potential of $TiO_2$ particles, DLS was used to determine the hydrodynamic size and zeta potential of E171, MPs and NPs of $TiO_2$ in aqueous solutions. The mean hydrodynamic size determined with DLS was found to be significant greater ($P < 0.05$) when particles were dispersed in HBSS buffer than when dispersed in 0.05% BSA DMEM. This size dissimilarity might be due to agglomeration differences, which are caused by proteins present in the BSA. Proteins are charged amino acids with both negative and positive charges and may have either hydrophobic or hydrophilic characteristics. The adsorption of BSA on the surface will increase the net ionic charge, leading to a mild increase in zeta potential and electrostatic repulsions between other coated $TiO_2$ particles. The addition of BSA in aqueous dispersions of $TiO_2$ particles improved the dispersibility and colloidal stability which resulted in a significant increase ($P < 0.05$) of both the hydrodynamic diameter as well as the zeta potential. Here we confirm that the addition of 0.05% BSA in DMEM has shown a significant effect on the degree of aggregation of particles, as has also been observed by Shukla *et al.[42]* and shown by the NANOGENOTOX program [24]. However, the zeta potential values of our samples were between -30 mV and +30 mV, with or without BSA, which means that the solutions were unstable. A higher zeta potential leads to increased electrostatic repulsion between $TiO_2$ particles. Indeed, a higher colloidal stability leads to an increased total surface area of $TiO_2$ in solution. This was previously determined by Shukla *et al.* [42] which found a zeta potential of -17.6 and -11.5 mV for $TiO_2$ NPs dispersed in Milli-Q water and 10% FBS medium, respectively. In addition, the size of E171 was determined via TEM by a collaborating research group Biomedicine from the National Autonomous University of Mexico where they determined an average size of 535 nm. The size of NPs claimed by its commercial supplier is between 10 to 25 nm.

However, we need to stress that DLS conditions are not completely comparable to *in vitro* exposure conditions of cytotoxicity, ESR and comet assay experiments because of the use of a plastic capillary during DLS measurements .During exposure experiments, instable particles in solution have a tendency to sink to the bottom in such culture dishes. Exposure of cells is therefore additionally described as surface exposure (shown as μg/cm$^2$). Consistently, agglomeration of particles can also occur *in vivo* situations.

**Cytotoxicity and dose selection**

Since DNA damage is associated with cell death, it is important that cytotoxicity of particles is evaluated prior to genotoxicity testing. To determine non-cytotoxic concentrations of $TiO_2$ particles, cytotoxicity of E171, $TiO_2$ MPs and NPs was assessed with Trypan blue viability tests. This assay was chosen since MTT and LDH tests gave false positive reactions due to high refractive index of $TiO_2$ particles. This interference was also observed by Kroll *et al.* [25, 26]. Trypan blue viability test show cytotoxicity for E171 at 1.0 and 0.1 mg/mL while MP and NP show cytotoxicity only at a concentration of 1.0 mg/mL. With this, E171 is the most cytotoxic followed by MPs and NPs which are equivalent in cytotoxic properties. This could be explained by the fact that E171 comprises 67% MPs and 33% NPs by which we suggest that the combination of NPs and MPs are more cytotoxic to cells than NPs or MPs alone. Since 0.01 and 0.001 mg/mL ($1.43x10^{-1}$ and 1.43 μg/cm$^2$, respectively) show no cytotoxicity for all three types of particles this was selected as non-cytotoxic concentrations for follow up (cellular) experiments. To the best of our knowledge, this is the first study that compares the viability of cells exposed to the food additive E171 to MPs and NPs. Other studies [20, 42, 59], only focusses on $TiO_2$ NPs which accounts for only 33% of E171. Consistently, concentrations up to and including 0.1 mg/mL $TiO_2$ NPs were also found non-cytotoxic in human lung epithelial cells by Prasad *et al.* [49]with Trypan blue exclusion tests which is in line to our data.

**ESR spectroscopy for the detection of ROS**

Although DLS results showed more appropriate dispersibility and less aggregation when particles were dispersed in medium with BSA, HBSS was chosen as dispersion solution for cell-free and cellular experiments. The reason for this decision is based on the fact that when particles in 0.05% BSA show no ROS formation while without the presence of BSA (only HBSS) increased levels of ROS can be observed in both cellular and cell-free conditions. Therefore, we can conclude that BSA, even at low concentrations, scavenges ROS which is formed on the surface of the particles.

High refractive index of $TiO_2$ particles is not only an interfering problem during cytotoxicity assays but also with ROS formation assessment. Previous studies determined $TiO_2$ ROS formation capacity by fluorescent probe techniques [17, 42] which have high probability of false positive reactions due to the high refractive index of $TiO_2$ [25]. Due to these artefacts, ESR spectroscopy for the assessment of ROS formation capacities was chosen as technique. Numerous studies confirm the fact that $TiO_2$ particles can induce ROS production e.g. as reviewed in Iavicoli *et al .[38]*. To our knowledge, only one study has been performed to establish radical formation processes after $TiO_2$ NP exposure in a cell-free system using ESR spectroscopy [60].

Of note, ROS formation was only confirmed in a cell-free system with NPs suspensions while in this study both MPs and NPs were tested in both cell free and cellular environment.

To the best of our knowledge, $TiO_2$ induced ROS mechanisms are still unclear. Literature [17, 54] suggests that •OH is likely produced via Fenton processes which include the reaction of $H_2O_2$ with metal ions. Since titanium is not a precursor of Fenton reactions and characterized $TiO_2$ particles do not contain any metallic impurities, Fenton reactions could, in theory in our study, impossibly occur. Another reason for this is that all experiments were performed in the dark to prevent photocatalytic activity of the particles, by which radicals could possibly be formed. In cell-free environment, $H_2O_2$ was added at non-cytotoxic concentrations [55] to mimic an inflammatory environment to assess whether this addition led to higher ROS formation. Our results show that in none of the conditions (cell-free nor in cellular) the addition of $H_2O_2$ led to increased ROS formation which would be the case via Fenton reactions. In general, we therefore expect that the observed ROS formation is not formed by transition metal catalysis via Fenton reactions.

**Possible mechanism for ROS formation in cell free E171, NP or MP suspensions**

In a cell-free environment $TiO_2$ particles have intrinsic (particle driven) capacities to produce ROS independently on size although not all statistical different from controls due to high variation between ESR measurements. High variation is mostly seen in E171 and NP samples which could be explained based on DLS results which indicate an unstable solution with NPs in HBSS. Nevertheless, NPs have the highest (significant from control) intrinsic (particle driven) capacities to produce ROS in a cell-free environment. In contrast, MPs do not show significant ROS levels which indicates that NPs in E171 are responsible for significant ROS formation.

This data suggest that NPs have mechanical (particle driven) ROS formation capacities which are formed on the surface of the particles. Correspondingly to our data, previous studies have shown that NP exposure induces ROS production [61-64]. However, this is the first study investigating both MPs and NPs to establish the reactive element of E171 which is, confirmed by ESR spectroscopy, the nano-sized particles in E171 in cell-free environment.

**ROS formation in Caco-2 cells after E171, NP or MP exposure**

No significantly increased ROS levels are detected after cells are exposed to E171 and NPs. With this, we demonstrate that NPs have intrinsic (particle driven) capacities to produce ROS in cell-free environment, while this is not detected in a cellular environment. Contradictory data shows that $TiO_2$ NPs are capable of ROS induction in cells. For example, Huerta- Garcia *et al.* [65] did detect ROS in glial cells after cellular exposure to $TiO_2$ NPs while Shukla *et al. [42]* did detect ROS in human epidermal cells.

Therefore, we hypothesize that intrinsic (particle driven) ROS formation is taken up by the cell after infiltration of NPs into the nucleus and cytoplasm. For this reason we do not detect any ROS formation after cellular exposure of NPs. A number of researchers have reported that NPs can enter the nucleus of the cell shown by TEM in human lung carcinoma [17, 19], human epidermal [42] and Caco-2 [14] cells. According to these data, we hypothesize that ROS, produced by NPs, is not detected due to infiltration of the particles into the cells. Overall, this could be one of the

reasons why ROS is not detected with NPs and E171 (as it also contains NPs) when introduced in a cellular environment.

In contrast, MPs have the lowest intrinsic (particle driven) ROS formation capacity in cell free environment, while in cellular environment significant ROS production was detected. For this we hypothesize that MPs are inducing ROS formation in the cells via secondary responses (e.g. inflammatory responses). This response will lead to the production of intracellular $H_2O_2$ what accelerates ROS levels. This is in line with the results from Jugan *et al.* [17] who found that $H_2O_2$ was produced intranuclear in human lung carcinoma cells, which further reacted to form •OH. With this, MPs do not have intrinsic (particle driven) capacities to produce ROS while they can induce a (normal) cellular immune response leading to increased ROS formation.

Taken together, based on this ESR spectroscopy analysis with E171, NPs and MPs it has been found that ROS formation is particle size dependently. It can be suggested that NPs are the most reactive part in E171 since they induce ROS formation while MP induce inflammatory cellular responses including ROS formation. This could be one of the mechanisms involved in the potential carcinogenicity of $TiO_2$ particles.

**Antioxidant response found in Caco-2 cells exposed to $TiO_2$ MPs**

An *in vivo* study in mice, performed by the collaborating research group from the National Autonomous University of Mexico, found that exposure of mice to E171 stimulated the onset and severity of colon cancer when induced by AOM. The additional exposure of E171 led to excessive tumor growth compared to the control. To test the effect of the genotoxicant AOM on ROS formation, Caco-2 cells were co-exposed to AOM and E171, NPs and MPs. ROS levels were not significantly different when co-exposed to AOM compared to solely exposure of the particles. This suggests that AOM did not induce increased ROS formation. Interestingly, an extra peak corresponding to the antioxidant vitamin C was observed in samples with non-cytotoxic concentrations of MPs exposed to Caco-2 cells. Previously, this antioxidant production was also found by Petković *et al.* [66] during NP exposure in HepG2 (human liver carcinoma) cells. Notably, this was only found in ESR spectra obtained from cellular exposure to MP co-exposed to AOM which can be explained by the hypothesized cellular defence mechanism to the MP exposure. The presence of vitamin C radicals suggests that a higher oxidative stress status is present in the cells. We suggest that MPs act via secondary oxidative mechanisms by which a pro-inflammatory response is triggered and leads to the production of antioxidants. To our knowledge, this is the first time that vitamin C radical production is directly observed by ESR detection after $TiO_2$ exposure of cells.

**ssDNA damage after E171 and $TiO_2$ NP exposure**

In order to confirm the aforementioned *in vivo* results observed by the collaborative group in Mexico (manuscript in preparation) shown in figure 1, we co-exposed Caco-2 cells to AOM and $TiO_2$ particles (E171, NPs and MPs) and performed an alkaline comet assay to detect the presence of ssDNA breaks. Indeed, a significant induction of ssDNA strand breaks was visualized by the comet assay after 24h exposure of E171 and $TiO_2$ NPs in Caco-2 cells. A considerable amount of literature has been published about the genotoxic effects of $TiO_2$ NPs in other cell lines; adenocarcinoma human alveolar basal epithelial cells (A549) cells [17], human peripheral blood lymphocytes and cultured human embryonic kidney cells [67] where they found a significant increase of ssDNA damage using the comet assay. Contradictory, no DNA damage was found after $TiO_2$ exposure in Chinese hamster lung fibroblast cells [68], human fibroblasts and human bronchial epithelial cells [69] and Caco-2 cells [20]. Contradictory outcomes using the comet assay could be due to differences in cell lines, $TiO_2$ particle characteristics, exposure dosage, preparation method (sonication of particles) and dispersion solution (BSA- free). Moreover, it has been intensively questioned by Karlsson *et al.* [57] whether the comet assay is a reliable assay to detect nanoparticle-induced genotoxicity due to high photocatalytic activity of $TiO_2$ particles which, for example, could lead to interference during scoring with a fluorescent staining. Karlsson *et al.* [57] stated that the comet assay is a trustful method for genotoxic assessment of nanoparticles. When following the recommended guidelines including avoiding direct lab light and additional damage, the risk of producing false negative or false positive results can be limited. Consistently, in this study experiments have been performed in the dark to prevent photocatalytic effects of NPs.

Presently, no studies have been performed to assess the *in vitro* genotoxic effects of AOM in combination with $TiO_2$ particles on Caco-2 cells using the comet assay. Nevertheless, no significant difference was found between the addition of AOM and without AOM which indicates that $TiO_2$ particles induce ssDNA by themselves while this was not seen *in vivo.* Indeed, AOM is commonly used in *in vivo* models [45] to mimic sporadic colon cancer since this induces DNA mutations by changing the nucleotides from G:C to A:T which takes 14 weeks in mice or rats. AOM is catalysed in the liver into methylazoxymethanol (MAM) which causes colon cancer acting via MAPK and P13K/Akt pathways [70]. In this study, it was not tested whether this conversion took place since it is not known whether CYP2E1, the metabolite causing AOM conversion to MAM, is present. Nevertheless, AOM alone induced ssDNA strand breaks with significant increased tail intensity compared to the control which indicates the genotoxic activity of AOM *in vitro*.

The findings of the present *in vitro* study demonstrate that $TiO_2$ as food additives coded as E171, even at the lowest concentration tested, have DNA damaging potential in human colorectal adenocarcinoma cells. The direct correlation between ROS formation and DNA damage further suggests that oxidative stress could act as an important route by which $TiO_2$ particles, induce DNA damage. Oxidative stress could therefore be one of the underlying mechanisms involved in the development of colorectal cancer induced by $TiO_2$ NPs in food products.

# 5. Conclusion

In this study, we investigated the possible toxic effects and its involved mechanisms of E171 ($TiO_2$ MPs and NPs) on the development of colorectal cancer with respect to cytotoxicity, induction of oxidative stress via ROS formation and genotoxic potential in Caco-2 cells. Since E171 consists of a mixture of micro- and nano-sized particles, all three different (E171, $TiO_2$ MPs and NPs) were analyzed in detail by DLS with regard to their particle size, zeta potential and aggregation as well as their degree of aggregation in aqueous solutions.

This study demonstrates that $TiO_2$, in particular nano-sized (10 – 15 nm) induces sufficient DNA damage as evident by the results of the alkaline comet assay. This finding was also supported by the fact that ROS formation was significantly enhanced in cell-free environments which proves the intrinsic (particle driven) ROS formation capacity of $TiO_2$ NPs. However, ROS formation in reaction to $TiO_2$ particle exposure is size dependent which is proven by the fact that MPs are able to affect Caco-2 cells, in all probability due to secondary toxicity mechanisms since they do not have intrinsic capacity to produce ROS, shown by cell-free ESR assessments.

In conclusion, results discussed in this thesis provided us with new molecular insights in the *in vitro* effects of $TiO_2$ on colorectal cancer development. Overall, it can be concluded that radical formation by $TiO_2$ particles taken up in the colon via food products may play an important role in health effects associated with colonic $TiO_2$ exposure.

Overall, it is known that colon cancer that can be prevented by selecting the appropriate foods and lifestyle. The detected ROS formation and DNA damage potential raise concerns about the safety associated with applications of $TiO_2$ in numerous food products. However more studies need to be performed *in vitro* and *in vivo* to fully unravel and understand the potential carcinogenic mechanism of $TiO_2$ used as food additive and coded as E171. The studies which could be performed in the future are described in the following chapter.

# 6. Recommendations based on the *in vitro* evaluation of the effects of E171 on the development of colorectal cancer

Follow up studies are urgent to translate and understand the exact mechanism of $TiO_2$ NPs induced genotoxicity in order to improve safe and responsible use of nanoparticles in food. This study indicates that it might be prudent to control the application of $TiO_2$ as food additives and limit the ingestion of $TiO_2$ nanoparticles through nonessential foods.

To complete the *in vitro* observations in Caco-2 cells, it is of high priority to assess the ssDNA strand break capacity of $TiO_2$ MPs. For this reason an alkaline comet assay needs to be performed with $TiO_2$ MPs following the exact experimental setup which was used for E171 and $TiO_2$ NPs. Since the alkaline comet assay reveals only information of ssDNA strand breakages, the next experimental setup would be to gain insight into dsDNA breakage formation caused by E171 performing a y-H2AX staining. Moreover, ESR and comet assay results could be confirmed with an Fpg-modified comet assay to validate whether DNA damage is induced by oxidative stress. The link between ROS formation and transcriptomic response can be established by gene expression changes identification with transcriptomic approaches like micro-array whole genome gene expression analysis. Given the large amount of data that can be generated from this technology, micro-arrays can be helpful in the answering the question whether $TiO_2$ particles present in food are relevant in human colorectal carcinogenicity in a number of different ways.

Additionally, when using AOM as genotoxicant in these *in vitro* studies, it has to be taken into account that AOM is a precursor of the active compound methylazoxymethanol (MAM) which induces colon cancer o.a. via K-ras signaling pathways [70]. During this *in vitro* assay, it has not been tested whether AOM was metabolized by CYP2E1 into the active compound MAM. The presence of CYP2E1 could be tested with RT-qPCR or microarray to confirm the genotoxic results found with comet assay originated from AOM. Notably, another pathway besides CYP2E1 might participate in AOM activation.

Next to Caco-2 cells, other approaches could be recommended to establish the effects of E171 on the development of colorectal cancer. The flow chart in figure 15 represents the recommended follow up studies based on the *in vitro* evaluation of the effects of E171 on the development of colorectal cancer.

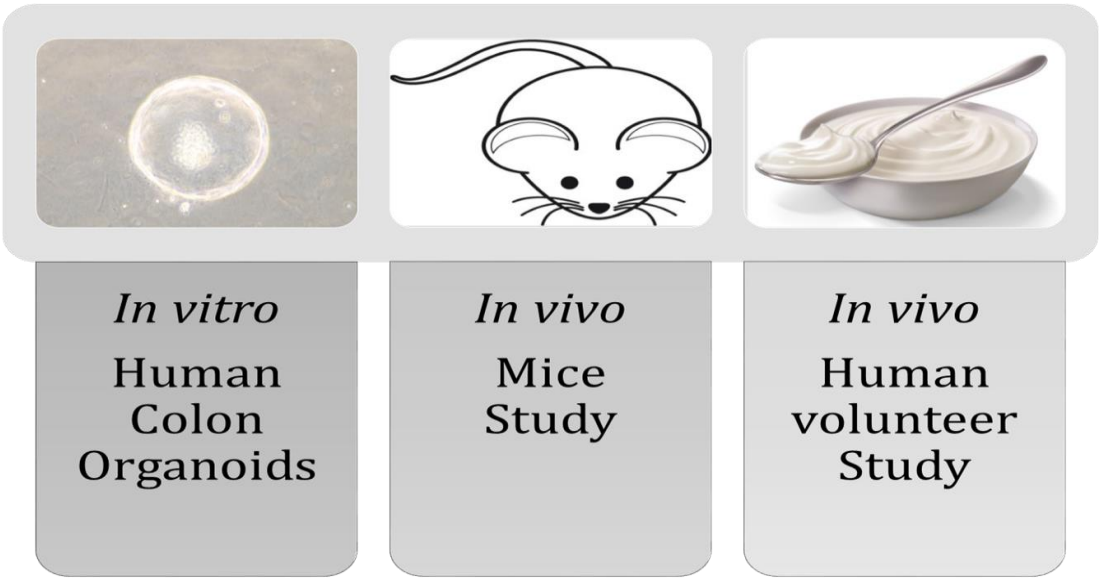



***Figure 15*** *Flowchart of recommending follow up studies based on the in vitro evaluation of the effects of E171 on the development of colorectal cancer. Photo of human colon organoid made by Carolyn Moonen.*

Since Caco-2 cells have multiple limitations, such as their polyclonal nature and limited monolayer growth, validation efforts in other *in vitro* colon models are of interest. Recently, a 'mini-gut'; human colon organoids are discovered and developed by the group of Prof. H. Clevers from Hubrecht Institute in Utrecht. Human colon Organoids are considered to represent the best *in vitro* system for colon studies since they represent the *in vivo* structure of the colon. This 3D cell model contains different types of cells including stem cells. However, the use of these spheroids in toxicity screening does encounter problems. Due to the complex structure of human colon organoids, culture medium contains 15 different supplements. Half of this culture medium supplements is Wnt3a which needs to be produced via another cell culture. When compared to Caco-2 cells, maintaining organoids *in vitro* is time consuming since they grow slowly; the doubling time of Caco-2 cells is approximately 32h whereas the doubling time of organoids is approximately 14 days. Disadvantageously, maintaining and culturing human colon organoids is an expensive and time consuming cell culture. Additionally, since this human colon organoid model is recently discovered there is not much known yet. Advantageously, this model should be the best model to mimic *in vivo* situations without using animals, thus this is a good alternative for *in vivo* animal experiments. Nevertheless, human colon organoids should be the perfect next step to confirm *in vitro* Caco-2 cell results.

Unfortunately, it is inevitable to assess the toxic effects of $TiO_2$ on colorectal carcinogenesis without using animals. For this, it would be best to expose mice to E171 to investigate the effects of E171 on colorectal cancer development *in vivo*. Animal bioassay data are key components of risk assessments. It is hypothesized that chemicals which cause tumors in animals can also cause tumors in humans. Accordingly, all human carcinogens that have been sufficiently tested in animals produce positive results in at least one animal model. Although this association cannot establish that all agents and mixtures that cause cancer in experimental animals also cause cancer in humans, nevertheless, in the absence of adequate data on humans, it is biological conceivable and thoughtful to regard chemicals for which there is sufficient evidence of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans for precautionary reasons.

The collaborating group from Mexico used a chemically induced colon cancer mice model. For this reason it could be interesting to investigate the effects in a mice model which spontaneously forms tumors in the colon, reflecting the normal pathophysiology of colon tumor formation. With this mice model, results from the collaborating group from Mexico can be confirmed by which organs can be obtained and comprehensively studied after sacrificing the animals.

Eventually, a human volunteer study could be performed where healthy volunteers are exposed to a minimum amount of E171 (control period) and exposed to a normal human daily amount of E171 which is 0.89 mg/kg bw/day. The latter is stated as an intervention. An option would be to provide this dose via dietary products like yoghurts, period. An option would be to provide E171 via dietary products like yoghurts which contain E171 (intervention period) or not (control period). Before and after intervention and control period (switched after 2 weeks, figure 16), colon biopsies could be taken for whole genome expression profiling with micro-array.

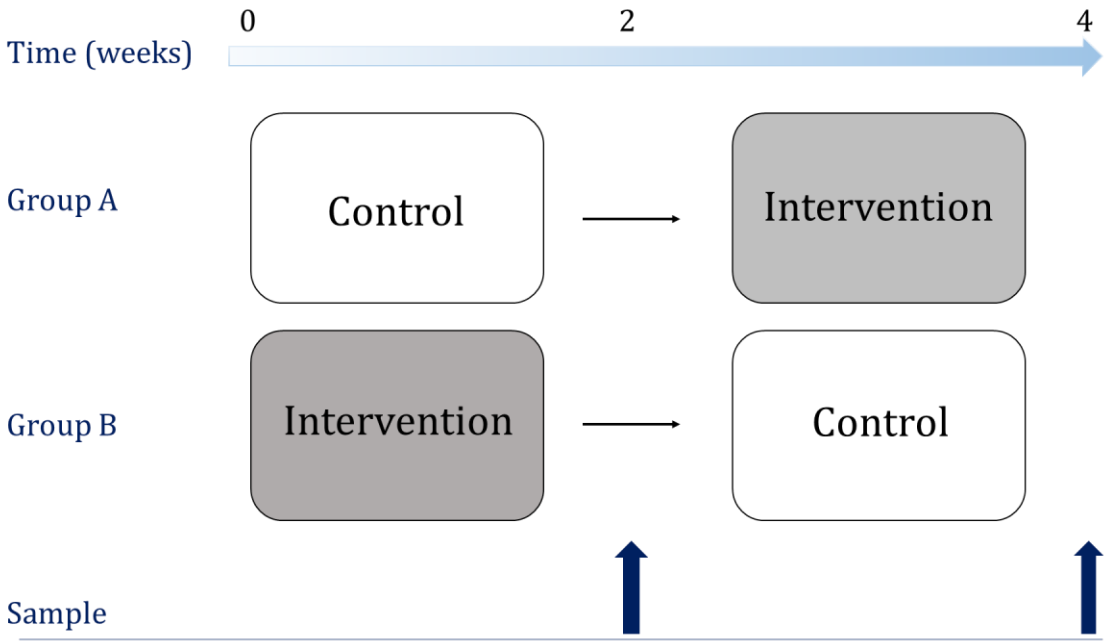



***Figure 16*** *Human volunteer protocol including 2 weeks intervention (normal daily amount exposure) and 2 weeks control (minimum exposure) period. Colon biopsy (sample) will be taken every 2 weeks.*

# 7. Literature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**In vitro Evaluation of the Effects of E171 (Titanium Dioxide) on the Development of Colorectal Cancer**

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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