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In situ monitoring and optimization of CuAAC mediated protein functionalization of biosurfaces

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13 Abstract

14 With the current trend to miniaturize bioactive surfaces to micro- or nanometer scale, the strategy of immobilization becomes more important. Therefore, there is a growing need for 15 16 protein immobilization techniques that create both stable and homogeneously covered 17 surfaces in a reproducible way. One of the most promising methods to achieve this is the 18 combination of biological receptors with 'click' chemistry, like the Copper catalyzed Alkyne 19 Azide Cycloaddition (CuAAC). This work presents a full optimization of all aspects of the 20 'click' chemistry reaction between proteins and surfaces in order to create covalently and 21 homogeneously covered biosurfaces. The coupling procedure is monitored by in situ 22 ellipsometry, a unique characterization technique that offers the opportunity to quantify 23 minute amounts of surface coupled protein mass in real-time. The optimization involves the 24 azidification of a solid silicon support, the alkynation of two proteins, Staphylococcus aureus 25 Protein A (SpA) and Maltose Binding Protein (MBP), as well as the development of a highly 26 reproducible CuAAC 'click' coupling protocol. Using the here optimized protocol, active and 27 reproducible biolayers can be created rapidly. The proposed surface biofunctionalization method combined with ellipsometry forms a unique and promising platform towards the 28 29 development of highly sensitive, accurate biosensors.

30 Keywords

Protein surface functionalization, Bio-orthogonal chemistry, CuAAC 'Click' chemistry,
 Ellipsometry, *Staphylococcus aureus* Protein A, Maltose Binding Protein.

33

34 **1. Introduction**

35 During the last decade several exciting examples of innovative microarrays and biosensing 36 applications have been developed [1-6]. Typical sensing devices consist of three main parts, 37 *i.e.* the sensing target receptor, the transducer surface and the readout system. The current 38 trend of miniaturizing these devices is one of the key challenges in the field of advanced 39 biosensing, since downscaled biosensors can facilitate multiple parallel measurements, with 40 smaller amounts of expensive biological receptor material. Nowadays, a wide variety of target 41 receptors is described in literature, e.g. DNA, phospholipids, glycosaminoglycans, enzymes, 42 antibodies, cells and molecularly imprinted polymers (MIPs) [7-14]. Especially the quest for 43 durable coupling methods to attach proteins to surfaces is of considerable interest in 44 biomedical, biochemical and immunological research [15-18]. For miniaturized protein-based devices, an optimal and uniform coverage of the transducer surface with proteins becomes 45 even more crucial. The coupling reaction of choice must be highly efficient, selective, 46 47 reproducible, non-destructive, without side reactions and, if possible, rapid.

Many different strategies, such as physical adsorption, affinity-based interactions and covalent couplings have been reported to immobilize proteins to the transducer surfaces [19-21]. Coupling methods based on weak interactions (hydrogen bonds, electrostatic, hydrophobic and van der Waals interactions) can result in oriented immobilization but are reversible in nature, possibly leading to stability and reproducibility problems. On the other hand, covalent coupling could be formed using the naturally present amino acid chemistry such as, for example, amines in lysines or thiols in cysteines. Although this leads to stable coverage, there is generally no control over the orientation of the protein on the surface due to the presence of multiple copies of the same amino acids [22,23]. In either case, these methods often lead to sub-optimal sensitivity of the biosensing devices, due to lack of uniform biomolecule orientation, stability and/or reproducibility [24,25]. It is therefore important to develop methods that direct both orientation *and* stability.

One of the most promising methods for covalent protein immobilization is based on 'click' chemistry, *e.g.* the copper(I) catalyzed 1,3-dipolar cycloaddition of azides and alkynes (CuAAC) [21,26]. The CuAAC reaction is well known for its high specificity and efficiency, bioorthogonal properties (i.e. azides and alkynes do not interfere with native biochemical processes [27]) and lack of side reactions [28,29]. Furthermore, the coupling reaction can be accomplished in aqueous solution under mild physiological conditions and on a variety of biomolecules and transducer supports [30-34].

67 CuAAC has been extensively used for the conjugation, immobilization, and purification of 68 several biomolecules: DNA, peptides, proteins, oligosaccharides and glycoconjugates have 69 been labelled with various attachements [35,36]. With regard to the labeling in living 70 organisms, however, CuAAC suffers from the cytotoxicity of Cu(I) and has therefore mainly 71 been applied to labeling reactions in the extracellular space [37]. Since the proven biological 72 applicability and the fact that both azide and alkyne groups can be appended to biomolecules 73 without altering their function or metabolic processing, reactions between alkynes and azides 74 have been adapted to reduce the cell toxicity [38]. One approach to do this is by removing the 75 Cu(I) requirement in the reaction. By using cyclooctynes, for example, the reaction is 76 activated by ring strain in the so-called 'Strain-promoted azide-alkyne cycloaddition' or 77 SPAAC [39]. However, the relatively large size and hydrophobic nature of the cyclooctyne 78 components can affect the biological properties of the biomolecule to which it is attached 79 [40]. Copper-free SPAAC reactions are also 10-100 times slower than classical CuAAC 80 reaction [35,40]. In addition, strained cyclooctyne synthesis is difficult compared to terminal 81 alkynes and although available, commercial SPAAC components are still expensive. A 82 second approach to improve the biocompatibility of CuAAC is the use of ligands for Cu(I) 83 tris-(benzyltriazolylmethyl)amine (TBTA) [41] such as and tris(3hydroxypropyltriazolylmethyl)amine (THPTA) [42]. These ligands serve multiple purposes; 84 85 they not only accelerate the cycloaddition reaction, they also act as sacrificial reductants 86 protecting the copper from oxidation and help to protect cells and biomolecules [38,43]. 87 TBTA and the water-soluble THPTA are both used in the present study.

A secondary advantage of the use of Cu(I) as a catalyst in the present study is that it acts as a 'on-off' switch, making it possible to see a difference between immobilization through a click reaction reaction or by physical adsorption by respectively presence or absence of Cu(I) in the reaction. However, protein functionalized surfaces created with 'click' chemistry often still suffer from reproducibility issues and insufficient and/or non-homogeneous protein coverage.

94 This work therefore presents a full optimization of all aspects of the 'click' chemistry 95 reaction between proteins and surfaces. This is exemplified by the 'click' mediated 96 immobilization of two model proteins, Protein A (SpA, a 42 kDa immunoglobulin-binding 97 surface protein of Staphylococcus aureus) and Maltose Binding Protein (MBP, a 42 kDa 98 soluble periplasmic protein [44]). For this, SpA and MBP are both modified with an alkyne 99 linker via their endogenous surface lysines. Lysines are present in most proteins, and can 100 make up to over 10% of the overall amino acid sequence and are frequently located on the 101 surface of the protein [45]. However this method does not lead to single and site-specific 102 protein modification, for the purpose of this study, i.e. optimization of the 'click' mediated protein immobilization, lysines offer a relatively easy method to add bio-orthogonal chemistry to proteins. Conjugation with the amine group of lysine is very often done with N-Hydroxysuccinimide (NHS) esters resulting in the formation of stable peptide bonds. In the present study an alkyne-containing NHS-ester is used to alkynate SpA and MBP. The alkynemodified proteins are subsequently immobilized using CuAAC onto silica slides that are treated with the complementary reactive azides.

109 During the optimization of the reaction conditions, the binding efficiency was evaluated 110 using in situ ellipsometry. This technique is a non-destructive, optical technique based on 111 changes in the orientation of two polarizer prisms [46] and gives us a unique opportunity to 112 evaluate the CuAAC reaction in real-time with nanogram accuracy. To our knowledge the 113 real-time assessment of CuAAC-immobilized protein layers has never been performed before. 114 Ellipsometry offers the opportunity to not only detect and quantify the formed protein layer, 115 but also to observe the adsorption towards the surface while it happens. The target-binding 116 efficiency of this method is compared to commonly used immobilization techniques, *i.e.* 117 physical adsorption and EDC/NHS coupling using a direct reaction between the lysines and 118 carboxylated slides.

For obvious reasons, it is also important that the alkynation, the number of modifications on the protein and the immobilization process do not induce conformational changes or influence or block the active site(s) of the protein. Therefore, the activity of SpA and MBP after immobilization has been assessed using binding studies with human IgG (binding the Fc domain) and monoclonal anti-MBP (Fab domain), respectively. Again ellipsometry was used to quantify the amount of surface coupled antibody.

125 **2. Materials and Methods**

126 2.1. Materials

127 Zeba micro spin desalting columns (7K MWCO, 0.5 mL), SpA (Cowan strain, 128 recombinant, Staphylococcus aureus subsp. aureus strain NCTC 8325, expressed in E. coli) 129 and human IgG (hIgG) were obtained from Thermo Scientific. Carboxylated, hydrophilic 130 silicon slides, PVC coated slides and 'washing buffer' (WB) were developed by Synapse 131 B.V., Maastricht, The Netherlands. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 132 *N*-hydroxysuccinimide (NHS), bromopropylamine hydrobromide and sodium azide were 133 purchased from Acros. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA), tris(3-134 hydroxypropyltriazolylmethyl)amine (THPTA), diethylene glycolamine, sodium L-ascorbate 135 (NaAsc), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), monoclonal Anti-136 Maltose Binding Protein antibody (anti-MBP, clone-17), 2-(N-morpholino)ethanesulfonic 137 acid (MES), sodium acetate trihydrate and 5-hexynoic acid tris(2-carboxyethyl)phosphine 138 (TCEP) were obtained from Sigma-Aldrich. MBP was obtained from the pMXB10 vector 139 purchased from New England Biolabs.

140 2.2. Solutions

141 220 mM HEPES buffer pH 6.8; 10 mM alkyne NHS in acetonitrile; 1 M diethylene 142 glycolamine pH 7.5; PBS buffer pH 7.4: 137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄, 2 143 mM KH₂PO₄; 0.1 M glycine-0.2 M NaCl pH 2.5; 0.01 M sodium acetate buffer pH 4; 0.5% 144 SDS in H₂O; 0.01 M MES buffer pH 4; 0.05 M Tris-0.1 M NaCl buffer pH 7.5. Buffers were 145 prepared with Milli-Q water.

146 2.3. Instruments

147 Contact angle measurements were performed with a dataphysics OCA 15+ goniometer 148 (Filderstadt, Germany). Contour ellipse fitting of the water droplets was done by the SCA 1.0 149 software. The droplet size was 1 μ L dispensed at 0.1 μ L/s.

Ellipsometry was performed on an ellipsometer with eight cuvettes equipped with magnetic stirrers, monitoring time-dependent changes in polarizer angle, analyzer angle and reflected light intensity [46]. A reference surface mass was recorded for each slide before the start of the binding monitoring.

154 2.4. Azidification of the carboxylated silicon slides

155 The azide linker, 3-azido-1-aminopropane (AAP, (1) in figure 1), synthesized as described 156 by Hatzakis [47], was attached to the carboxylated silicon slides using EDC/NHS chemistry. 157 The slides were immersed into a mixture of EDC (0.2 M), NHS (0.045 M) and AAP (0.23 M) 158 in HEPES buffer for 3 hours. Remaining NHS esters on the surface of the slides were 159 neutralized with diethylene glycolamine for 45 min after which the slides were rinsed with 160 Milli-Q water and dried with nitrogen. The water droplet contact angles on the slides were 161 measured and compared to the contact angles on carboxylated slides before azidification. A 162 change in contact angle is an indication for the change in hydrophilicity of the surface caused 163 by the change of the chemical groups (carboxylic acid to azides) on the surface.



Figure 1. Reaction schemes of the azidification of the silicon slides (A), alkynation of proteins (B), CuAAC immobilization of the proteins (C) and EDC/NHS mediated immobilization of proteins (D).

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169 2.5. Alkynation of SpA and MBP

SpA and MBP were alkynated by the reaction of an NHS linker with lysine. The alkyne
NHS ester, 2,5-dioxopyrrolidin-1-yl-hex-5-ynoate ((2) in figure 1), was synthesized according
to Jagadish [48].

The proteins were alkynated by adding the alkyne NHS ester to an SpA solution of 17.7 μ M in PBS and an MBP solution of 3.5 μ M in PBS, yielding alkyne-SpA (A-SpA) and alkyne-MBP (A-MBP) respectively. Appropriate NHS ester concentrations were added to both proteins leading to a theoretical functionalization level of 16% of the present lysines (63 for SpA [49,50] and 36 for MBP [51]). In addition, a theoretically fully (100%) alkynated SpA (fA-SpA) and MBP (fA-MBP) were obtained by adding a 1.5 molar excess (to the total number of lysines) of the alkyne NHS ester. After 3 hours the reaction mixture was filtered using a Zeba micro spin desalting column. Chemical modification by alkynation was demonstrated by native PAGE, visualizing the changes in electrophoretic mobility caused by the alterations in the overall charge of the proteins.

183 2

2.6. CuAAC immobilization of A-SpA

184 A screening of optimal 'click' reaction conditions was performed by using different combinations of reducing agents (TCEP versus sodium L-ascorbate), buffers (PBS pH 7.4 185 186 versus sodium acetate buffer pH 4) and ligands, i.e. the commonly used non-polar TBTA or 187 the water-soluble THPTA. Since TBTA is not soluble in water, DMSO is added to the 188 reaction mixtures containing TBTA. The amount of immobilized SpA in the different reaction 189 conditions was measured as the average surface mass (ASM). All mixtures contained 1 μ M 190 A-SpA and 0.5 mM CuSO₄. 64 Azidified slides were placed in the 8 different reaction 191 mixtures for 18 hours at room temperature without stirring, resulting in 8 samples for each 192 immobilization condition. After the reactions, the ASM was determined by ellipsometry 193 analysis. The different reaction conditions are given in Table 1.

194

195 **Table 1.** Reaction mixtures used for the immobilization of SpA in either 196 sodium acetate buffer pH 4 or PBS pH 7.4. All mixtures contain 1μ M of protein.

Mixture	Reducing	gagent (mM)	Ligand ((mM)	Cu Catalys	st (mM)
1	NaAsc.	2.50	ТНРТА	1.00	CuSO ₄	0.50
2 *	TCEP	0.85	TBTA	1.00	CuSO ₄	0.50
3	TCEP	0.85	ТНРТА	1.00	CuSO ₄	0.50
4 *	NaAsc.	2.50	TBTA	1.00	CuSO ₄	0.50

197 *contains 2.5 % DMSO.

Additional to the coupling reaction of A-SpA to the azidified slides, three control experiments were simultaneously performed: 1) reaction between azidified slides and wildtype SpA, 2) carboxylated slides and A-SpA and 3) carboxylated slides and wildtype SpA. Reaction time and concentrations of the other reactants were left unchanged.

Washing of the samples after immobilization was performed in three subsequent steps using different buffers: PBS to remove the protein solution containing the reagents and the excess of protein on the surface, Washing Buffer to interrupt the electrostatic interactions and finally SDS to remove hydrophobic bonds.

To follow the 'click' reaction in real-time and to determine the rate of immobilization, the ASM of A-SpA using *Mixture 1* in acetate buffer and *Mixture 2* in PBS was monitored after 0 s, 1800 s (0.5h) and 65000 s (18h).

To determine the influence of the protein concentration on the CuAAC coupling, five additional A-SpA solutions were tested, *i.e.* 0.034 μ M, 0.068 μ M, 0.102 μ M, 0.136 μ M and 0.500 μ M. The immobilization was performed in *Mixture 1* in acetate buffer *in duplo* for every protein concentration for 30 minutes. The ASM after coupling as well as after the washing steps was determined by ellipsometry.

To test the effect of the reaction volume on the immobilization efficiency, immobilization was performed *via* a drop method. For this, eight azide functionalized slides were put horizontally in a water vapor saturated environment at room temperature. Subsequently one drop (30 μ L), containing 1 μ M A-SpA in *Mixture 1* in acetate buffer, was applied on each slide. A reference was measured before applying the protein-containing droplet. After 30 minutes, the ASM was measured during the different washings steps.

220 2.7. CuAAC immobilization of A-MBP

221	The CuAAC coupling of 1 μ M A-MBP was performed in <i>Mixture 1</i> in acetate buffe	er
222	during 30 minutes after which the ASM of immobilized MBP was determined.	

223 2.8. Physical adsorption and covalent EDC/NHS immobilization of SpA

For physical adsorption, PVC slides were put into a 1 μ M SpA solution in Tris-NaCl buffer for 1.5 hours. For covalent surface coupling by EDC/NHS, carboxylated silicon slides were put into a solution of 0.2 M EDC and 0.071 M NHS in MES buffer for 1 h. Next, the slides were put into MES buffer containing 1 μ M SpA for 1.5 h, followed by flushing with WB for several minutes and measuring the ASM in MES buffer. This procedure was performed in triplicates. After immobilization, the ASM on the slides was measured while flushing with Tris buffer. This procedure was again performed in triplicates.

231 2.9. Activity of immobilized SpA and MBP

232 It has been shown that adsorption can affect protein activity and due to random orientation 233 of the molecules on the surface, binding sites may not be reachable [52-56]. Therefore, the 234 effect of the immobilization methods on the activity, as measured by the interaction and 235 binding to an antibody, was determined. The SpA covered slides, obtained via physical 236 adsorption, EDC/NHS coupling or CuAAC chemistry were put into cuvettes containing PBS. 237 For each slide a reference surface mass was recorded and human IgG (hIgG) was added to a 238 final concentration of 0.33 μ M. The interaction between the immobilized SpA and hIgG was 239 monitored using real-time ellipsometry. Similarly, monoclonal anti-MBP (0.33 μ M) was 240 added to the cuvettes containing the MBP covered slides. The interaction between the 241 antibody and immobilized MBP was monitored in real-time by ellipsometry.

3. Results and Discussion

243 3.1. Azidification and Alkynation

A covalent surface protein immobilization via CuAAC 'click' chemistry requires the 244 245 introduction of two complementary functional groups, *i.e.* one on the silicon slide and the 246 other in the protein. After the azidification of the carboxylated slides using EDC/NHS 247 chemistry, the surface angle of water on the slides was measured. It was found that the contact angle changed from 0° (hydrophilic) to 32.4° (±2.9°) (more hydrophobic). This is in 248 249 agreement with the expected modification of carboxylic acids to azides on the surface. It 250 should be noted that at pH 4, parts of the remaining non-modified carboxylic groups will be 251 negatively charged, resulting in an electrostatic attraction of the positively charged SpA (SpA: 252 pI~5.4; theoretically calculated with Innovagen protein property calculator) and MBP (MBP: 253 pI~5; idem) to the surface at which the 'click' reaction takes place [57,58].

In addition to the surface azidification, the proteins SpA and MBP were complementary functionalized with alkynes *via* the endogenous lysines. By alkynating the lysines, the pI of the global protein will decrease and this change can be visualized by native polyacrylamide gel electrophoresis (PAGE). Figure 2 illustrates the difference between wild-type SpA (left) and A-SpA (right) after performing native PAGE. At pH 8.8 the more negatively charged A-SpA migrates faster than SpA towards the positive pole, demonstrating a successful alkynation.



261

Figure 2. Native polyacrylamide gel electrophoresis of wild-type SpA (left,
three replicate samples) and A-SpA (right, four replicate samples).

264 3.2. CuAAC Coupling with SpA

To find the optimal conditions for protein immobilization *via* CuAAC chemistry, SpA was used as a model protein. The reaction conditions were tested using different (combinations of) reducing agents (TCEP *versus* sodium L-ascorbate), ligands (water soluble THPTA *versus* the apolar TBTA) and buffers (PBS pH 7.4 *versus* sodium acetate buffer pH 4). Figures 3 and 4 summarize the increase in surface mass as measured by ellipsometry for the different reaction mixtures in PBS pH 7.4 and acetate buffer pH 4, respectively, after 18h. Each condition was repeated on 8 replicate slides.



272

Figure 3. Surface mass of four different mixtures in PBS pH 7.4. The dashed line marks the theoretical surface mass of a close-packed monolayer of SpA as estimated by Lahiri et al [59].





Figure 4. Surface mass of four different mixtures dissolved in sodium acetate buffer pH 4. The dashed line marks the theoretical surface mass of a closepacked monolayer of SpA as estimated by Lahiri et al [59].

281 When the CuAAC reaction was performed in PBS (pH 7.4) only reaction Mixture 2 shows a significant binding of 0.28 μ g/cm². However, the reproducibility was rather poor with a 282 283 standard deviation of 46 %. For the sodium acetate buffer (pH 4) reactions in *Mixture 1* and 284 *Mixture 4* both yielded significant protein surface coverages of 0.28 μ g/cm² and 0.47 μ g/cm², 285 with standard deviations of 11 % and 19 %, respectively. The better reproducibility in Mixture 1 and Mixture 4 at pH 4 can be explained by the attraction forces between the 286 287 positively charged proteins and the negative charges of the remaining carboxylate groups at the substrate surface at this pH [56]. 288

The maximum amount of protein that can be immobilized on a surface was estimated by a theoretical model described by Lahiri et al [59]. This model estimates the theoretical maximal number of protein molecules per mm² in a close-packed hexagonal monolayer arrangement. With this model, the maximum surface coverage of SpA was estimated to be $0.36 \,\mu g/cm^2$.

Although this is just a model estimate assuming the proteins are hard spheres of uniform density, the three reaction conditions yielding the highest surface mass are close to this theoretical value: *Mixture 2* in PBS and *Mixtures 1* and *Mixture 4* in acetate buffer. However, *Mixture 2* in PBS and *Mixture 4* in acetate buffer both contain DMSO, an organic solvent which might result in (partial) denaturation of proteins [60,61]. *Mixture 1* in acetate buffer is the DMSO-free alternative of *Mixture 4* in the acetate buffer and is therefore chosen as the optimal CuAAC reaction condition and is used for the subsequent reactions.

300 To test the stability of the CuAAC covalent coupling of A-SpA to the azidified surface in 301 *Mixture 1 in* acetate buffer, four experiments were simultaneously performed in which (non-) 302 alkynated SpA and/or non-azidified surfaces were used. Proteins were coupled to the surface 303 for 18 hours after which the surfaces were washed with different washing solutions. During 304 washing, the ASM was measured in real-time. Figure 5 shows that only A-SpA coupled to the 305 azidified silicon surface resisted the different washing steps. In the three control experiments 306 almost all protein is removed during washing. This suggests that the used CuAAC coupling 307 protocol of an alkynated protein to an azidified surface results in a stable and covalent bond.



Figure 5. Surface mass evolution as measured by ellipsometry during consecutive washing steps with acetate buffer (Ac.); washing buffer (WB); sodium dodecyl sulfate solution (SDS); washing buffer and acetate buffer: for A-SpA which is covalently coupled to azidified silicon slides by CuAAC with *Mixture 1* in acetate buffer (green) and for control experiments accomplished under identical conditions but with carboxylated plates and/or non-alkynated SpA.

316

To test whether the immobilization reaction time could be shortened, surface mass measurements were also performed at 1800 sec (30 minutes), since preliminary measurements of the immobilization in acetate buffer showed a fast increase in surface mass during the first 30 minutes. Reaction times under 30 minutes are too short to get complete covalent coupling and surface mass increase is mainly due to adsorption (data not shown). When using *Mixture*

322 1 in acetate buffer, it was found that the formation of the A-SpA layer was already complete 323 after 30 minutes, which is only 2.5% of the time that was presumed to be needed to obtain 324 sufficient protein layers. On the other hand when using Mixture 2 in PBS no signs of a 325 biolayer in development could be observed after 30 minutes and a reaction time of 18 hours 326 was needed to obtain a biolayer of comparable mass. In addition, Mixture 2 in PBS leads to 327 high standard deviations. This implies that *Mixture 1* in acetate buffer does not only enhances 328 the rate of formation of the biofilm on the azide functionalized substrate but also leads to 329 highly reproducible layers (table 2).

330 Table 2. Surface mass measurements and the corresponding standard 331 deviations after 30 min and 18 h of CuAAC reaction. At 30 min, a 5 min wash 332 step with WB was performed to see the difference in surface mass.

Time	Surface mass (µg/cm ²)				
	PBS	S.D. ¹	Acetate	S.D. ²	
0	0.00		0.00		
\downarrow		CuAAC co	oupling reaction		
30 min	0.00	0.01	0.35	0.02	
\downarrow		Wash s	step with WB		
35 min	0.00	0.01	0.33	0.01	
\downarrow		CuAAC co	oupling reaction		
18 h	0.38	0.18	0.37	0.03	

333 ¹n=3, ²n=4

The successful biofunctionalization of the silicon slide with proteins was supported by contact angle measurements. Whereas the azide functionalized slides exhibited an average water contact angle of 32.4° ($\pm 2.9^{\circ}$), this contact angle increased to 62.4° ($\pm 2.6^{\circ}$) after treatment with A-SpA using *Mixture 1* in acetate buffer. This is an indication of the chemical change caused by the formation of the A-SpA layer. Based on these experiments it can be concluded that *Mixture 1* in acetate buffer results in an optimal and reproducible coupling of
A-SpA to the azidified substrate in a short reaction time of 30 minutes.

341 3.3. Concentration Dependency of the CuAAC Coupling

342 Considering the future development of biofunctionalized surfaces based on antibodies, 343 reducing the amount of protein/antibody per sample is highly recommended. Therefore, five 344 additional protein concentrations (0.034, 0.068, 0.102, 0.136 and 0.500 μ M) were tested for 345 immobilization. After 30 minutes of immobilization in *Mixture 1* in acetate buffer, a surface 346 mass of 0.08 μ g/cm² was achieved for the 0.034 μ M solution. In case of the 0.068 μ M A-SpA 347 0.15μ g/cm² was achieved and 0.17μ g/cm² for the 0.102 μ M A-SpA. The 0.136 μ M A-SpA 348 solution reached a maximum of 0.20 μ g/cm² and 0.25 μ g/cm² was obtained by using a 0.5 μ M 349 A-SpA solution (Figure 6, top right).

The measurements confirm the effect of the protein concentration on the final protein mass of the biolayers (Figure 6). Varying the protein concentrations from 0 to 0.102 μ M results in a very steep increase in A-SpA surface mass. Further raising the A-SpA concentration results in a gradual increase in surface mass and apparent saturation around 0.30 μ g/cm². This amount is in agreement with the theoretical maximum amount of SpA that can be immobilized on a surface, i.e. 0.36 μ g/cm² as estimated by a theoretical model described by Lahiri et al. [59].



Figure 6. A-SpA Surface Mass evolution of CuAAC coupling using different protein concentrations, followed by subsequent washing steps with WB, SDS and PBS. The insert shows the concentration dependency of the final A-SpA surface mass.

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362 Lowering the concentration of the reaction mixture therefore results in a lower amount of 363 protein on the substrate. Higher protein concentrations are therefore preferred to create 364 maximally covered surfaces.

Another option to reduce protein consumption would be reducing the immobilization reaction volume. By using the drop method, a 1 μ M A-SpA solution in Mixture 1 in acetate buffer was used, but the reaction volume was reduced from 400 μ l (maximum volume cuvettes) to 30 μ l, the minimal volume to fully cover the same area of the azidefunctionalized silicon slides. Interestingly, this 13 times reduction in reaction volume still results in protein layers of $0.29 \pm 0.03 \,\mu g/cm^2$ A-SpA. This means a considerable reduction in protein usage to obtain dense A-SpA layers which gives the opportunity to efficiently use higher protein concentrations to get optimal surface coverage.

373 3.4. Comparison with other immobilizing methods

374 The optimized surface CuAAC coupling of A-SpA was compared to other commonly used 375 coupling procedures as shown in Figure 7. The ASM of 0.28 μ g/cm² resulting from the 376 CuAAC reaction matches a surface coverage of 78% of a close packed monolayer of 0.36 377 μ g/cm² (Lahiri et al. [59]). For EDC/NHS coupling and physical adsorption, this is only 61% 378 and 53%, respectively. Comparing the results of the EDC/NHS coupling of 1 μ M SpA (0.22) 379 +/- 0.02 μ g/cm²) with the results from Figure 6, it is remarkable that a 0.136 μ M solution used 380 in combination with the CuAAC coupling yields comparable surface mass (0.20 μ g/cm²), 381 even though the reaction time is 5 times less and the protein concentration is 7 times less. One 382 explanation for this immobilization efficiency and higher surface mass after click reaction 383 compared to EDC/NHS might be that when SpA is modified with an alkyne, a spacer was 384 also introduced (Figure 1). This linker creates a suitable separation between the surface and 385 the protein. This might reduce steric hindrance, leading to increased immobilization mass. 386 Hence, this indicates that CuAAC based immobilization leads to a considerable improvement 387 in reproducibility and surface mass.





Figure 7. Average Surface Mass (ASM) and standard deviation obtained from
 immobilizations of SpA on carboxylated silicon slides by physical adsorption and
 EDC/NHS coupling and of A-SpA on azidified slides with CuAAC.

393 3.5. Activity measurements of immobilized SpA

By measuring the binding of human IgG to the immobilized SpA, the activity of the proteins after surface coupling *via* CuAAC, EDC/NHS or physical adsorption has been evaluated (Figure 8).



Figure 8. Average Surface Mass (ASM) of antibody and corresponding
standard deviations obtained after binding of human IgG to different SpA
functionalized slides.

401

402 The physically adsorbed SpA is able to bind only minute amounts of hIgG, *i.e.* 0.08 403 μ g/cm². The hIgG density of EDC/NHS coupled SpA is 0.47 μ g/cm². The highest amount of hIgG binding, *i.e.* 0.65 μ g/cm², can be observed for A-SpA layers immobilized by CuAAC. 404 405 This mass increase of hIgG is two times higher than a side-on close packed hIgG monolayer, 406 *i.e.* 0.3 μ g/cm², and is more than 40% of fully end-on covered hIgG monolayer, *i.e.* 1.5 407 μ g/cm² [62]. This indicates that the surface consists of a mixture of sideway oriented and endon oriented hIgG molecules. After hIgG binding, the slides were flushed with an acidic 408 409 glycine solution, a procedure often used to only break the specific antibody-antigen 410 interactions in antibody purification columns [63]. After washing, the original layers of 0.28

411 μ g/cm² were restored demonstrating the very specific binding between A-SpA and hIgG. The 412 possibility of aspecific binding is reduced due to the obtained dense A-SpA layer (78% of 413 close-packed), which does not allow for much 'protein-free' spaces in the biofilm. These gaps 414 could be a possible cause for increased aspecific binding of the antibody.

To further study the impact of surface coverage on the hIgG binding capacity of the A-SpA, different slides with increasing A-SpA surface mass was tested. The slides incubated with different A-SpA concentrations (figure 6) were incubated with hIgG and the antibody binding was followed (figure 9).



419

420 Figure 9. hIgG Surface Mass evolution after binding to surfaces with different
421 amounts of A-SpA. The binding of hIgG is followed by washing with glycine,
422 SDS and PBS.

423

424 The binding capacity towards hIgG increases with the increasing A-SpA surface mass, 425 indicating that the improved coverage with A-SpA has a direct positive impact on the binding 426 capacity of hIgG. Further it can be seen that the higher the initial amount of immobilized A-427 SpA on the azide surface is (before adding IgG), the less aspecific adsorbed IgG needs to be 428 removed by SDS after completion of the glycine washing step. This probably originates from 429 the fact that a lower density of the initial A-SpA layers can offer the opportunity for aspecific 430 adsorption of IgG molecules to the 'protein-free' substrate (vide supra). As a result, it can be 431 concluded that the increased SpA surface mass (0.28 μ g/cm²) obtained via CuAAC, not only 432 leads to an increased presence of receptor (SpA) molecules on the surface (see figure 6), but 433 also has the advantage of reducing aspecific binding of target molecules, in this case hIgG, to 434 the underlying substrate. This will have a positive impact on the signal-to-noise ratio when 435 applying this CuAAC coupling technique in sensing devices, thus improving their sensitivity 436 and decreasing the chance of possible errors.

437 3.6. Covalent coupling of MBP

438 To confirm that the results obtained for SpA are representative for and applicable to other 439 protein systems, experiments were repeated with MBP. The use of CuAAC with Mixture 1 in 440 acetate buffer for 30 minutes resulted in the following covalently coupled biolayers (Figure 441 10). For A-MBP, an average surface mass of 0.21 μ g/cm² ± 0.033 μ g/cm² was obtained (n=3). 442 Upon coupling fully alkynated MBP, an average surface mass of 0.25 μ g/cm² ± 0.07 μ g/cm² 443 was obtained (n=3). These results clearly demonstrate the covalent attachment of MBP. 444 Although higher surface mass was obtained for the fully alkynated MBP, this full alkynation 445 affects the antibody binding (vide infra).



Figure 10. Surface mass evolution for the coupling of standard alkynated MBP (green) and fully alkynated MBP (orange) to azidified silicon slides by CuAAC with *Mixture 1* and for consecutive washing steps with washing buffer (WB), sodium dodecyl sulfate solution (SDS) and PBS.

451 3.7. Activity measurements of MBP

452 The MBP covered slides were incubated with anti-MBP. Figure 11 shows a remarkable 453 difference between both immobilization types in terms of activity. Despite the fact that the 454 surface coverage of fA-MBP is almost 25% higher than that of A-MBP (Figure 10), the A-455 MBP coated slides bind significant higher amounts of anti-MBP antibody i.e. 0.13 μ g/cm² 456 compared to only 0.05 μ g/cm² for fA-MBP. This might indicate that the high number of 457 modifications causes conformational changes in the protein. This will lead to reduced 458 accessibility by anti-MBP antibodies and therefore less binding. Obviously, it is important to 459 minimize the number of modifications in order to prevent (conformational) changes that can 460 lead to reduced protein activity. Moving towards partial or even single and eventually to a 461 site-specific functionalization of the protein of interest can offer the possibility to modify and 462 immobilize the protein in a controllable way without interfering with its functioning. This 463 way, biomaterial with optimal biological activity can be created.



464

465 Figure 11. Surface mass evolution of the MBP-coupled slides during the 466 binding of monoclonal anti-MBP: standard alkynated A-MBP (green; average 467 increase: 0.13 μ g/cm²) and fully alkynated fA-MBP (orange; average increase: 468 0.05 μ g/cm²).

469

470 **4. Conclusions**

The covalent coupling of SpA and MBP on azide functionalized silicon slides has been
achieved *via* an optimized copper catalyzed azide-alkyne cycloaddition. For this optimization,

473 the immobilization of the proteins was followed in real-time with in situ ellipsometry. This 474 allowed us to study the click reaction in a time-dependent manner and to find the optimal 475 reaction time and conditions to create covalently coupled protein surfaces with an optimal 476 protein coverage and subsequent activity. An effective, reproducible and rapid method has 477 been developed by combining copper sulfate, sodium ascorbate and THPTA in 0.01 M sodium acetate buffer at pH 4 in 30 minutes. Furthermore, a considerably improved surface 478 479 mass was obtained as compared to other commonly used coupling techniques demonstrating 480 its innovative potential for future biofunctionalization of surfaces. After coupling, the 481 biolayers kept their characteristics, *i.e.* the bound SpA was still able to interact with human 482 IgG and the bound MBP with MBP antibody. These results confirm that this covalent 483 coupling method can be used for different proteins without inhibiting their corresponding 484 activity. At the same time the increased surface mass resulted in a reduction in aspecific 485 binding of target molecules. This rapid and covalent coupling strategy to a solid carrier offers promising prospects for a wide range of applications based on biofunctionalized surfaces, *i.e.* 486 487 biofunctionalized microarrays and biosensing devices.

488

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496 **Biographies**

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502 Erik Steen Redeker obtained his Master's degree in chemistry, with a specialism in 503 biochemistry, at the Utrecht University and his Ph.D. in Sciences at the Antwerp University. 504 In 2008 he was appointed as a postdoctoral fellow at the Hasselt University where he co-505 founded the Biomolecule Design Group. He is currently appointed at the Maastricht 506 University as a lecturer and member of the research group in Nano(Bio)Technology and Bio-507 Electronics. His research is focused on the rational (re)design, development and 508 (bio)synthesis of biopolymers. He uses synthetic biology approaches and other techniques 509 inspired by and derived from strategies found in nature.

Adam Miszta Since 2012 Dr Miszta has been working in the field of thrombosis and
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512 interest. Previously he worked in the field of Protein-membrane interactions at the J. 513 Heyrovsky Institute of Physical Chemistry of Czech Academy of Sciences in Prague. After 514 moving to Maastricht he worked as a Senior Scientist at the Maastricht University and at 515 Delbia bv. Currently he works as a scientist at the University of Maastricht and at Synapse 516 Research Institute. He published several papers in the field of protein-membrane interactions 517 measured with ellipsometry.

518 **Brecht Billen** received his Master Degree in Bioelectronics and Nanotechnology at Hasselt 519 University in 2012. Currently, he is a PhD-student at the Institute for Materials Research 520 (IMO) of Hasselt University. He is part of the Biomolecule Design Group and his current 521 research is focused on the site-specific functionalization of proteins.

522 Wim Hermens studied theoretical physics at the University of Amsterdam, where he 523 graduated in 1968. In 1971 he obtained his PhD degree at the same university for a thesis on 524 chemical reactions in neutron stars. In the meantime he worked at Leiden University on 525 ellipsometry, an optical technique for measurement of physico-chemical processes at 526 interfaces. In 1974 he moved to the medical faculty of the new Maastricht University, where 527 he worked on membrane-protein interactions in blood coagulation. In 1996 he became 528 professor of molecular physics at Maastricht University and he retired from active research in 529 2013.

Bas de Laat Since 2001 Dr. de Laat has been working in the field of thrombosis and haemostasis with the antiphospholipid syndrome, Von Willeband Factor and thrombin generation as special interest. After working at the University Medical Center Utrecht and Sanquin Blood Supply Amsterdam Dr. de Laat is currently associate professor at the University of Maastricht and general director of Synapse Research Institute. Dr. de Laat has received several grants in his career and his work is acknowledged by the Dutch Heart foundation as he received 2 consecutive fellowships. Dr. de Laat serves as reviewer for
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Peter Adriaensens Peter Adriaensens graduated in 1990 from UHasselt University, Belgium with a PhD on biopolymer NMR research. Subsequently, he was appointed as permanent researcher/lecturer. In 2007 he was appointed Professor at Hasselt University, where he joins the departments of Organic and (Bio)Polymer Chemistry and Applied and Analytical Chemistry. Generally, his research focuses on the site-specific "click" functionalization of nanobody proteins towards a covalent and oriented coupling to diverse surfaces for applications in the field of biohybrid particles and materials.

545 Wanda Guedens received her PhD degree in chemistry in 1999 from Hasselt University, 546 Belgium where she was appointed as assistant professor in 2004 at the Chemistry 547 Department. From 2015 she is a professor of (bio)chemistry. Currently, she is heading the 548 multidisciplinary research in biochemical and biomedical nanotechnology in the Biomolecule 549 Design Group at the Institute of Materials Research at Hasselt University. Her research 550 concentrates on the controlled biofunctionalization of surfaces for innovative biomaterials. 551 More specifically, the site-specific nanobody modification towards an oriented and covalent 552 coupling to functionalized substrates such as conjugated polymers, (nano) diamond, graphene 553 and nanoparticles using 'click' chemistry.

Thomas J. Cleij obtained his PhD in chemistry at Utrecht University. Subsequently he was appointed as assistant professor at Louisiana State University and associate and full professor at Hasselt University. Since 2012, he has been appointed at Maastricht University as a Full Professor of chemistry. Since 2015 he is also Dean Sciences at this university. The expertise of his research group in Nano(Bio)Technology and Bio-Electronics focuses on the combination and interplay of novel functional polymeric materials and advanced applications

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563References

564	[1]	C.A.K. Borrebaeck, C. Wingren, Design of high-density antibody microarrays for
565		disease proteomics: key technological issues, J Proteomics. 72 (2009) 928–935.
566		doi:10.1016/j.jprot.2009.01.027.
567	[2]	T.R.J. Holford, F. Davis, S.P.J. Higson, Recent trends in antibody based sensors,
568		Biosens. Bioelectron. 34 (2012) 12–24. doi:10.1016/j.bios.2011.10.023.
569	[3]	F. Li, W. Chen, S. Zhang, Development of DNA electrochemical biosensor based on
570		covalent immobilization of probe DNA by direct coupling of sol-gel and self-
571		assembly technologies, Biosens. Bioelectron. 24 (2008) 787–792.
572		doi:10.1016/j.bios.2008.06.052.
573	[4]	ZH. Wang, G. Jin, Covalent immobilization of proteins for the biosensor based on
574		imaging ellipsometry, J Immunol Methods. 285 (2004) 237-243.
575		doi:10.1016/j.jim.2003.12.002.
576	[5]	D. Van der Voort, C.A. McNeil, R. Renneberg, J. Korf, W.T. Hermens, J.F.C. Glatz,
577		Biosensors: basic features and application for fatty acid-binding protein, an early
578		plasma marker of myocardial injury, Sensor Actuat B-Chem. 105 (2004) 50–59.
579		doi:10.1016/j.snb.2004.02.035.
580	[6]	P.D. Skottrup, M. Nicolaisen, A.F. Justesen, Towards on-site pathogen detection
581		using antibody-based sensors, Biosensors and Bioelectronic. 24 (2007) 339–348.
582		doi:10.1016/j.bios.2008.06.045.
583	[7]	D. Chen, M. Davis, Molecular and functional analysis using live cell microarrays,
584		Curr. Opin. Chem. Biol. 10 (2006) 28–34. doi:10.1016/j.cbpa.2006.01.001.
585	[8]	M.P. Byfield, R.A. Abuknesha, Biochemical aspects of biosensors, Biosensors and
586		Bioelectronic. 9 (1994) 373-400. doi:10.1016/0956-5663(94)80038-3.
587	[9]	B.M. Paddle, Biosensors for chemical and biological agents of defence interest,
588		Biosensors and Bioelectronic. 11 (1996) 1079-1113. doi:10.1016/0956-
589		5663(96)82333-5.
590	[10]	Y. Lei, W. Chen, A. Mulchandani, Microbial biosensors, Anal. Chim. Acta. 568
591		(2005) 200–210. doi:10.1016/j.aca.2005.11.065.
592	[11]	S. Mun, SJ. Choi, Optimization of the hybrid bilayer membrane method for
593		immobilization of avidin on quartz crystal microbalance, Biosens. Bioelectron. 24
594		(2009) 2522–2527. doi:10.1016/j.bios.2009.01.006.
595	[12]	M.A. Skidmore, S.J. Patey, N.T.K. Thanh, D.G. Fernig, J.E. Turnbull, E.A. Yates,
596		Attachment of glycosaminoglycan oligosaccharides to thiol-derivatised gold surfaces,
597		Chem Commun (Camb). (2004) 2700–2701. doi:10.1039/b411726c.
598	[13]	F. Horemans, A. Weustenraed, D. Spivak, T.J. Cleij, Towards water compatible MIPs
599		for sensing in aqueous media, J. Mol. Recognit. 25 (2012) 344–351.
600		doi:10.1002/jmr.2191.
601	[14]	F. Horemans, H. Diliën, P. Wagner, T.J. Cleij, MIP-based Sensor Platforms for
602		Detection of Analytes in Nano- and Micromolar Range, in: S. Li, Y. Ge, S.A.
603		Piletsky (Eds.), Molecularly Imprinted Sensors, Elsevier B.V, 2012: pp. 91–124.
604		doi:10.1016/B978-0-444-56331-6.00005-0.

605	[15]	D. Jiang, J. Tang, B. Liu, P. Yang, X. Shen, J. Kong, Covalently coupling the
606		antibody on an amine-self-assembled gold surface to probe hyaluronan-binding
607		protein with capacitance measurement, Biosensors and Bioelectronic. 18 (2003)
608		1183–1191. doi:10.1016/S0956-5663(02)00253-1.
609	[16]	S. Susmel, R. Toniolo, A. Pizzariello, N. Dossi, G. Bontempelli, A piezoelectric
610		immunosensor based on antibody entrapment within a non-totally rigid polymeric
611		film, Sensor Actuat B-Chem. 111 (2004) 331–338. doi:10.1016/j.snb.2004.11.052.
612	[17]	M. Köhn, Immobilization strategies for small molecule, peptide and protein
613		microarrays, J. Peptide Sci. 15 (2009) 393–397. doi:10.1002/psc.1130.
614	[18]	J.E. Gautrot, W.T.S. Huck, M. Welch, M. Ramstedt, Protein-resistant NTA-
615		functionalized polymer brushes for selective and stable immobilization of histidine-
616		tagged proteins, ACS Appl. Mater. Interfaces, 2 (2010) 193–202.
617		doi:10.1021/am9006484.
618	[19]	E. Briand, M. Salmain, C. Compère, CM. Pradier, Immobilization of Protein A on
619		SAMs for the elaboration of immunosensors. Colloids and Surfaces B: Biointerfaces.
620		53 (2006) 215–224. doi:10.1016/i.colsurfb.2006.09.010.
621	[20]	Y. Gao, I. Kyratzis, Covalent immobilization of proteins on carbon nanotubes using
622	[]	the cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimidea critical
623		assessment, Bioconiugate Chem, 19 (2008) 1945–1950, doi:10.1021/bc800051c.
624	[21]	E. Steen Redeker, D.T. Ta, D. Cortens, B. Billen, W. Guedens, Peter Adriaensens,
625		Protein engineering for directed immobilization, Bioconiugate Chem, 24 (2013)
626		1761–1777. doi:10.1021/bc4002823.
627	[22]	L.S. Wong, F. Khan, J. Micklefield, Selective covalent protein immobilization:
628		strategies and applications, Chem. Rev. 109 (2009) 4025–4053.
629		doi:10.1021/cr8004668.
630	[23]	L.S. Wong, J. Thirlway, J. Micklefield, Direct site-selective covalent protein
631		immobilization catalyzed by a phosphopantetheinyl transferase, J. Am. Chem. Soc.
632		130 (2008) 12456–12464. doi:10.1021/ja8030278.
633	[24]	S.H. North, E.H. Lock, C.J. Cooper, J.B. Franek, C.R. Taitt, S.G. Walton, Plasma-
634		based surface modification of polystyrene microtiter plates for covalent
635		immobilization of biomolecules, ACS Appl. Mater. Interfaces. 2 (2010) 2884–2891.
636		doi:10.1021/am100566e.
637	[25]	J.E. Butler, L. Ni, R. Nessler, K.S. Joshi, M. Suter, B. Rosenberg, et al., The physical
638		and functional behavior of capture antibodies adsorbed on polystyrene, J Immunol
639		Methods. 150 (1992) 77–90.
640	[26]	R.K. Iha, K.L. Wooley, A.M. Nyström, D.J. Burke, M.J. Kade, C.J. Hawker,
641		Applications of orthogonal "click" chemistries in the synthesis of functional soft
642		materials, Chem. Rev. 109 (2009) 5620–5686. doi:10.1021/cr900138t.
643	[27]	J.A. Prescher, C.R. Bertozzi, Chemistry in living systems, Nat. Chem. Biol. 1 (2005)
644		13-21. doi:10.1038/nchembioo605-13.
645	[28]	L. Nebhani, C. Barner-Kowollik, Orthogonal Transformations on Solid Substrates:
646		Efficient Avenues to Surface Modification, Adv. Mater. 21 (2009) 3442–3468.
647		doi:10.1002/adma.200900238.
648	[29]	L. Liang, D. Astruc, The copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC)
649		"click" reaction and its applications. An overview, Coordination Chemistry Reviews.
650		255 (2010) 2933–2945. doi:10.1016/j.ccr.2011.06.028.
651	[30]	A.C. Gouget-Laemmel, J. Yang, M.A. Lodhi, Functionalization of azide-terminated
652		silicon surfaces with glycans using click chemistry: XPS and FTIR study, J. Phys.
653		Chem. C. 117 (2013) 368–375. doi:10.1021/jp309866d.
654	[31]	B. Malvi, B.R. Sarkar, D. Pati, R. Mathew, "Clickable" SBA-15 mesoporous

655		materials: synthesis, characterization and their reaction with alkynes, J. Mater. Chem.
656		19 (2009) 140–14169. doi:10.1016/S1077-9108(08)79172-2.
657	[32]	P.K.B. Palomaki, P.H. Dinolfo, Structural analysis of porphyrin multilayer films on
658		ITO assembled using copper(I)-catalyzed azide-alkyne cycloaddition by ATR IR,
659		ACS Appl. Mater. Interfaces. 3 (2011) 4703–4713. doi:10.1021/am201125p.
660	[33]	JS. Seo, S. Lee, C.D. Poulter, Regioselective covalent immobilization of
661		recombinant antibody-binding proteins a. g. and L for construction of antibody
662		arrays I. Am. Chem. Soc. 135 (2013) 8973–8980. doi:10.1021/ja402447g.
663	[34]	F. Bally, K. Cheng, H. Nandiyada, X. Deng, A.M. Ross, A. Panades, et al., Co-
664	[6.]	immobilization of biomolecules on ultrathin reactive chemical vapor deposition
665		coatings using multiple click chemistry strategies ACS Appl Mater Interfaces 5
666		(2013) 9262–9268 doi:10.1021/am401875x
667	[35]	C Littamaninant A Tangpeerachaikul S Grecian S Clarke U Singh P Slade et
668	[55]	al Fast cell compatible click chemistry with conner chelating azides for
660		hismologular labeling Angew Chem. Int. Ed. 51 (2012) 5852-5856
670		doi:10.1002/apia.201108181
671	[26]	UCI.10.1002/alle.201100101.
672	[30]	J.E. Moses, A. Moonouse, The growing applications of click chemistry, Chem Soc
672	[27]	Kev. 30 (2007) 1249–1202. M. King, A. Wagner, Developments in the field of bigerthagenel hand forming
674	[37]	M. King, A. wagner, Developments in the netd of bioofulogonal bond forming
0/4		reactions-past and present trends, Bioconjugate Chem. 25 (2014) $825-859$.
013	[20]	dol:10.1021/0c300028d.
0/0 677	[38]	v.P. Hong, N.F. Steinmelz, M. Manchester, M.G. Finn, Labeling five cells by
0//		1012 1016 doi:10.1021/h.a100272
0/8	[20]	1912 - 1910. doi:10.1021/0c1002/2Z.
0/9	[39]	N.J. Agard, J.A. Prescher, C.R. Bertozzi, A strain-promoted [3 + 2] azide-alkyne
080		Cham See 126 (2004) 15046 15047 dai:10.1021/ie044006f
001	[40]	Chem. Soc. 120 (2004) 15040–15047. doi: 10.10217 a0449901.
082	[40]	L.S. MCKay, M.G. Finn, Click chemistry in complex mixtures: bioorthogonal
083		bioconjugation, Chem. Biol. 21 (2014) $1075-1101$.
084	E / 1]	dol:10.1010/j.cnemblo1.2014.09.002.
085	[41]	I.K. Chan, K. Hilgraf, K.B. Sharpless, V.V. Fokin, Polytriazoles as copper(1)-
686		stabilizing ligands in catalysis, Org Lett. 6 (2004) $2853-2855$.
68/	[40]	doi:10.1021/010493094.
688	[42]	V.P. Hong, S.I. Presolski, C. Ma, M.G. Finn, Analysis and optimization of copper-
689		catalyzed azide-alkyne cycloaddition for bioconjugation, Angew. Chem., Int. Ed. 48
690	5 4 9 3	(2009) 9879–9883. doi:10.1002/anie.200905087.
691	[43]	C.P. Ramil, Q. Lin, Bioorthogonal chemistry: strategies and recent developments,
692		Chem. Commun. 49 (2013) 11007–11022. doi:10.1039/c3cc44272a.
693	[44]	M.I. Austermuhle, J.A. Hall, C.S. Klug, A.L. Davidson, Maltose-binding protein is
694		open in the catalytic transition state for ATP hydrolysis during maltose transport, J
695		Biol Chem. 279 (2004) 28243–28250. doi:10.1074/jbc.M403508200.
696	[45]	E. Steen Redeker, D.T. Ta, D. Cortens, B. Billen, W. Guedens, Peter Adriaensens,
697		Protein Engineering For Directed Immobilization, Bioconjugate Chem. 24 (2013)
698		1761–1777. doi:10.1021/bc4002823.
699	[46]	C.W.N. Damen, H. Speijer, W.T. Hermens, J.H.M. Schellens, H. Rosing, J.H.
700		Beijnen, The bioanalysis of trastuzumab in human serum using precipitate-enhanced
701		ellipsometry, Anal. Biochem. 393 (2009) 73–79. doi:10.1016/j.ab.2009.06.006.
702	[47]	N.S. Hatzakis, H. Engelkamp, K. Velonia, J. Hofkens, P.C.M. Christianen, A.
703		Svendsen, et al., Synthesis and single enzyme activity of a clicked lipase-BSA hetero-
704		dimer, Chem Commun (Camb). 19 (2006) 2012–2014. doi:10.1039/b516551b.

705	[48]	B. Jagadish, R. Sankaranarayanan, L. Xu, R. Richards, J. Vagner, V.J. Hruby, et al.,
706		Squalene-derived flexible linkers for bloactive peptides, Bloorg. Med. Chem. Lett. 17
707	5 4 6 3	(2007) 3310–3313. doi:10.1016/j.bmc1.2007.04.001.
708	[49]	S. Kalkhof, A. Sinz, Chances and pitfalls of chemical cross-linking with amine-
709		reactive N-hydroxysuccinimide esters, Anal. Bioanal. Chem. 392 (2008) 305–312.
710	[50]	E. Basle, N. Joubert, M. Pucheault, Protein Chemical Modification on Endogenous
711		Amino Acids, Chem. Biol. 17 (2010) 213–227. doi:10.1016/j.chembiol.2010.02.008.
712	[51]	P. Duplay, H. Bedouelle, A. Fowler, I. Zabin, W. Saurin, M. Hofnung, Sequences of
713		the malE gene and of its product, the maltose-binding protein of Escherichia coli
714		K12, J Biol Chem. 259 (1984) 10606–10613.
715	[52]	M. Aizawa, K. Yun, T. Haruyama, Y. Yanagida, Protein engineering for self-
716		assembling antibody molecules in an oriented manner, Supramolecular Science. 5
717		(1998) 761–764.
718	[53]	IH. Cho, EH. Paek, H. Lee, J.Y. Kang, T.S. Kim, SH. Paek, Site-directed
719		biotinylation of antibodies for controlled immobilization on solid surfaces, Anal.
720		Biochem. 365 (2007) 14–23. doi:10.1016/j.ab.2007.02.028.
721	[54]	R. Iwata, R. Satoh, Y. Iwasaki, K. Akiyoshi, Covalent immobilization of antibody
722		fragments on well-defined polymer brushes via site-directed method, Colloids and
723		Surfaces B: Biointerfaces. 62 (2008) 288–298. doi:10.1016/j.colsurfb.2007.10.018.
724	[55]	S.R. Ahmed, A.T. Lutes, T.A. Barbari, Specific capture of target proteins by oriented
725		antibodies bound to tyrosinase-immobilized Protein A on a polyallylamine affinity
726		membrane surface, Journal of Membrane Science. 282 (2006) 311-321.
727		doi:10.1016/j.memsci.2006.05.033.
728	[56]	K. Hernandez, R. Fernandez-Lafuente, Control of protein immobilization: Coupling
729		immobilization and site-directed mutagenesis to improve biocatalyst or biosensor
730		performance, Enzyme Microb. Technol. 48 (2011) 107–122.
731		doi:10.1016/j.enzmictec.2010.10.003.
732	[57]	W. Norde, J. Lyklema, The adsorption of human plasma albumin and bovine
733		pancreas ribonuclease at negatively charged polystyrene surfaces. III.
734		Electrophoresis, Journal of Colloid and Interface Science. 66 (1978) 277–284.
735		doi:10.1016/0021-9797(78)90304-1.
736	[58]	Z. Pei, H. Anderson, A. Myrskog, G. Dunér, B. Ingemarsson, T. Aastrup, Optimizing
737		immobilization on two-dimensional carboxyl surface: pH dependence of antibody
738		orientation and antigen binding capacity, Anal. Biochem. 398 (2010) 161-168.
739		doi:10.1016/j.ab.2009.11.038.
740	[59]	J. Lahiri, L. Isaacs, J. Tien, G.M. Whitesides, A strategy for the generation of
741		surfaces presenting ligands for studies of binding based on an active ester as a
742		common reactive intermediate: a surface plasmon resonance study, Anal. Chem. 71
743		(1999) 777–790. doi:10.1021/ac980959t.
744	[60]	T. Arakawa, Y. Kita, S.N. Timasheff, Protein precipitation and denaturation by
745		dimethyl sulfoxide, Biophysical Chemistry. 131 (2007) 62–70.
746		doi:10.1016/j.bpc.2007.09.004.
747	[61]	S. Bhattacharjya, P. Balaram, Effects of organic solvents on protein structures:
748		observation of a structured helical core in hen egg-white lysozyme in aqueous
749		dimethylsulfoxide, Proteins. 29 (1997) 492–507.
750	[62]	J.M. Bolívar, Latex immunoagglutination assays, Journal of Macromolecular Science
751		Part C - Polymer Reviews. 45 (2005) 59–98. doi:10.1081/MC-200045819.
752	[63]	M.L. Yarmush, K.P. Antonsen, S. Sundaram, D.M. Yarmush, Immunoadsorption:
753		strategies for antigen elution and production of reusable adsorbents, Biotechnol
754		Progr. 8 (1992) 168–178. doi:10.1021/bp00015a001.

Figure 1. Reaction schemes of the azidification of the silicon slides (A),
alkynation of proteins (B), CuAAC immobilization of the proteins (C) and
EDC/NHS mediated immobilization of proteins (D).

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Figure 2. Native polyacrylamide gel electrophoresis of wild-type SpA (left, three
replicate samples) and A-SpA (right, four replicate samples).

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Figure 3. Surface mass of four different mixtures in PBS pH 7.4. The dashed line marks the theoretical surface mass of a close-packed monolayer of SpA as estimated by Lahiri et al [59].

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Figure 4. Surface mass of four different mixtures dissolved in sodium acetate buffer pH 4.
The dashed line marks the theoretical surface mass of a close-packed monolayer of SpA as
estimated by Lahiri et al [59].

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Figure 5. Surface mass evolution as measured by ellipsometry during consecutive washing steps with acetate buffer (Ac.); washing buffer (WB); sodium dodecyl sulfate solution (SDS); washing buffer and acetate buffer: for A-SpA which is covalently coupled to azidified silicon slides by CuAAC with Mixture 1 in acetate buffer (green) and for control experiments accomplished under identical conditions but with carboxylated plates and/or non-alkynated SpA.

780 Figure 6. A-SpA Surface Mass evolution of CuAAC coupling using different protein 781 concentrations, followed by subsequent washing steps with WB, SDS and PBS. The insert 782 shows the concentration dependency of the final A-SpA surface mass. 783 784 Figure 7. Average Surface Mass (ASM) and standard deviation obtained from 785 immobilizations of SpA on carboxylated silicon slides by physical adsorption and EDC/NHS 786 coupling and of A-SpA on azidified slides with CuAAC. 787 788 Figure 8. Average Surface Mass (ASM) of antibody and corresponding standard deviations 789 obtained after binding of human IgG to different SpA functionalized slides. 790 791 Figure 9. hIgG Surface Mass evolution after binding to surfaces with different amounts of A-792 SpA. The binding of hIgG is followed by washing with glycine, SDS and PBS. 793 794 Figure 10. Surface mass evolution for the coupling of standard alkynated MBP (green) and 795 fully alkynated MBP (orange) to azidified silicon slides by CuAAC with Mixture 1 and for 796 consecutive washing steps with washing buffer (WB), sodium dodecyl sulfate solution (SDS) 797 and PBS. 798

Figure 11. Surface mass evolution of the MBP-coupled slides during the binding of monoclonal anti-MBP: standard alkynated A-MBP (green; average increase: $0.13 \ \mu g/cm^2$) and fully alkynated fA-MBP (orange; average increase: 0.05 $\mu g/cm^2$).

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Table 1. Reaction mixtures used for the immobilization of SpA in either sodium acetate buffer pH 4 or PBS pH 7.4. All mixtures contain 1μ M of protein.

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Table 2. Surface mass measurements and the corresponding standard deviations after 30 min
and 18 h of CuAAC reaction. At 30 min a 5 min wash step with WB was performed to see the
difference in surface mass.

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