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Review

From protein features to sensing surfaces

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Abstract: Proteins play a major role in biosensors in which they provide catalytic activity and specificity in molecular recognition. The immobilization process is however far from straightforward as it often affects the protein functionality. An extensive interaction of the protein with the surface or a significant surface crowding can lead to changes in the mobility and conformation of the protein structure. This review will provide an insight of how the analysis of the physico-chemical features of the protein surface features before the immobilization process can help to identify the optimal immobilization approach to preserve the functionality of the protein when on the surface of the biosensor.

Keywords: surface functionalization; biosensor functionalization; protein immobilization; protein structure analysis; protein immobilization.

1. Introduction

Proteins provide specific recognition for the analyte in biosensors and their immobilization is a crucial step as it can highly affect the performance of the device if electron transfer is not guaranteed, or if the protein undergoes major conformational changes that halter the functionality. As compared to small molecules that offer few chemical groups of clear position and solvent-accessibility, e.g. dyes in solar cells[1], DNA[2], or aptamers[3], proteins have sizes that can reach the tens of nanometers and complex three-dimensional structures that dynamically move during its bioactivity, with the environmental conditions, and especially after entering in contact with material surfaces.

Achieving the optimal immobilization in a biosensor can be a complex task often achieved with a try-and-error approach aiming at retaining the affinity for the analyte or the enzymatic activity, in the case of enzymes. Immobilization can in fact alter the enantioselectivity of enzymes as reported for lipase and acylase that undergo extensive conformational changes during catalysis[4]. Immobilization in a preferred orientation can guarantee the maximal exposure of biorecognition moieties, e.g. catalytic sites of enzymes and antigen-binding sites of antibodies, while the protein region interacting with the surface is minimized and limited to regions of the molecule that do not undergo conformational changes. These are critical aspects also when dealing with enzymes for industrial biocatalytic applications, as recently reviewed[5].

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The extensive conformational changes and partial denaturation of proteins adsorbed or chemically crosslinked to surfaces often leads to a significant loss of function [6,7]. However, the immobilization process, if well planned, can even provide an enhancement in bioactivity and stability, e.g. a 60000-fold increase in stability has been reported for chymotrypsin to aldehyde-agarose gels[4]. By carefully selecting the material, its coating, and by studying the properties of the protein to immobilize, it is possible to control their interaction through single or multiple points, using flexible or rigid linkers, in hydrophilic or hydrophobic environments, in order to protect the protein and to prolong its functionality through multiple cycles of use[4]. This review will provide an insight into different immobilization approaches and how the study of the protein structural and surface features can help to identify the optimal one ensuring the retention of the highest degree of functionality once assembled in the biosensing device.

2. Protein surface and function

Proteins differ widely in their biological functions and this is reflected in specific structural features[8]. Proteins are surface-active molecules and the distribution of charged and hydrophobic residues on their surface is often at the basis of their functionality. Hydrophobins, for example, are characterized by a well-defined hydrophobic patch on their surface that drives their interaction with surfaces and interfaces in a highly oriented manner (Figure 1a). Hydrophobin from *Schizophyllum commune* has been used to alter the properties of glassy carbon electrodes in a single self-assembly step prior to the immobilization of redox-active glucose oxidase and horseradish peroxidase by adsorption [9]. This process led to an adsorbed multi-layer assembly of glucose oxidase with thickness of 79 Å and of 173 Å for horseradish peroxidase, both permeable to the analytes and allowing an efficient electron transfer [9]. Fused at the gene level to glutathione-S-transferase (GST), hydrophobin has driven the enzyme to a hydrophobic polystyrene surface producing a biosensor for the detection of pesticides molinate and captan[10]. The hydrophobin-assisted immobilization of GST resulted in a higher affinity for the analytes and a higher catalytic activity, e.g. a lower K_M and an almost double k_{cat} [10]. Enzymes such as lipase (Figure 1b) and cholesterol oxidase that are active on hydrophobic substrates, often present an enrichment of hydrophobic residues in the proximity of the active site. Lipase B from *Candida antarctica* is strongly adsorbed to hydrophobic surfaces such as graphite[11] and porous styrene–divinylbenzene beads [12]. Immobilization of lipase from *Pseudomonas cepacia* into siliceous mesocellular foams with different degrees of hydrophobicity showed how an increased hydrophobicity led to an enhancement of the catalytic activity[13]. This activation of the enzyme is due to the interaction with the material that leads to an opening of the hydrophobic lid that covers the active site in many lipases[5,13]. Similarly, odorant-binding proteins (Figure 1c) are small 13-16 kDa proteins naturally secreted in vertebrate nasal cells to bind hydrophobic odorant molecules. These proteins have proven valuable to develop bioelectronic nose and odor biosensors. Immobilization of these proteins to nanomaterials has reached detection limits of 0.02 ppt molecules[14] and function in both gas and liquid phase[15]. The crystal structure

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79 of protein 14 from *Apis mellifera* is available and show six alpha-helices whose hydrophobic
80 residues form a hydrophobic core that harbors the odorant molecule [16]. Immobilized to
81 reduced graphene oxide with a short 1-pyrenebutanoic acid succinimidyl ester (PBSE) linker,
82 protein 14 retained affinity for the aromatic molecules homovanillic acid, eugenol, and
83 methyl vanillate with K_d values in the micromolar range, although the binding provoked a
84 slight reorientation of the α-helices[17].
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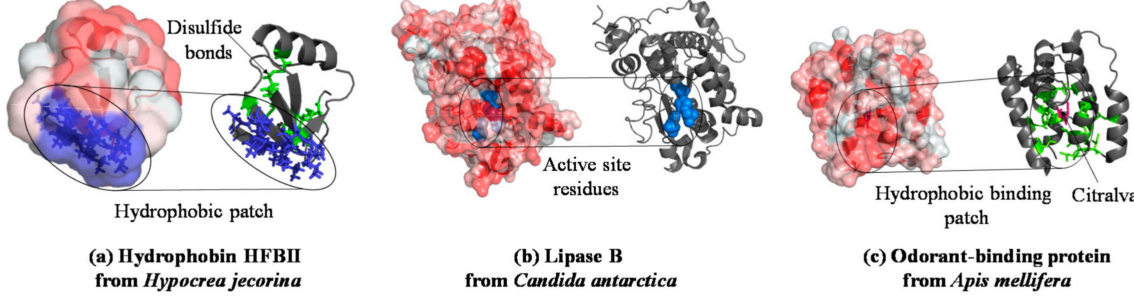
86 

87 **Figure 1** – Surface features play a crucial role in the function of proteins. Hydrophobins interact with
88 substrates in a specific region of the structure that is rich in hydrophobic residues (PDB ID: 2B97,
89 blue). Lipases are enzymes active on hydrophobic substrates, they catalyze the hydrolysis of
90 triacylglycerides and their active site (key residues in blue and spheres) and located in a hydrophobic
91 protein patch (PDB ID: 4k6g). Similarly, the odorant-binding protein from bee has a hydrophobic cleft
92 (residues in green as sticks) at the center of the molecule to bind the perfume-like water-insoluble
93 molecule citralva (in pink sticks, PDB ID: 3s0d).

94 ## 3. Protein structure, surface and material surfaces

95 Based on their structural stability, proteins have long been divided in 'soft' or 'hard'
96 proteins. Proteins have been classified as ‘soft’ or ‘hard’ according to their structural
97 flexibility or rigidity, respectively[8]. Whereas soft proteins are characterized by a high
98 flexibility and are less thermodynamically stable, hard proteins are less structurally affected
99 by high temperatures, environmental conditions, and their conformation is mainly conserved
100 upon interaction with material surfaces. The application of a difference of potential to the
101 electrode can affect the behavior of the proteins at the surface as these contain dipoles and
102 charged residues and enhanced the degree of adsorption of proteins, especially of the ones
103 classified as hard[18]. Examples of soft proteins are myoglobin, α-lactalbumin, glucose
104 oxidase, and immunoglobulin G, and caseins, whereas hard proteins are often characterized
105 by multiple disulfide bonds that help to counteract the denaturation as in lysozyme,
106 ribonuclease A, and acetylcholinesterase. Bovine serum albumin (BSA), with its seventeen
107 disulfide bonds [19] is a hard protein and one of the most used model molecule to test the
108 interaction of a material with proteins and to mimic its behavior in physiological fluids. The
109 use of model proteins such as BSA is convenient but has limitations as the information can
110 hardly be directly applied to any other protein that we want to use for surface
111 functionalization such as a glucose oxidase for blood glucose monitoring or antibodies for
112 biomarkers detection.

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113 Tightly interacting secondary structure elements and disulfide bonds confer molecular
114 rigidity and help preserve the overall conformation, whereas hydrophobic or densely charged
115 surface patches can drive the interaction with specific surfaces. Protein surface can present a
116 highly heterogeneous distribution of charges and hydrophobicity that influence their
117 solubility, stability, and thus functionality in different environments. These are aspect of high
118 interest for the application of proteins in industry. Protein surface features play thus a crucial
119 role in the conformational stability of proteins. Moreover, it controls the interaction of
120 proteins not only with material surfaces, but also with other biomolecules that can introduce
121 rigidity features to the structure and thus tune their bioactivity. To understand and control the
122 interaction, the *in vitro* and *in silico* analysis of protein surface is a crucial step for future
123 engineering efforts. As an example, the green fluorescent protein could be selectively
124 adsorbed to the positively charged regions of a patterned coated surface [20] after analysis of
125 its surface features.

126 Adsorption of proteins to surfaces is driven, among other forces, by hydrophobicity and
127 ionic or electrostatic interactions. Analysis of the protein structural features, although
128 complex, can offer hints for selecting the optimal immobilization strategy. Surface
129 hydrophobic patches of soluble proteins are rich in Ala, Lys, and Pro residues and can have
130 areas of 400 A^2, they often drive multimerization or undesired aggregation and the interaction
131 with hydrophobic materials, e.g. cellulose-active enzymes and lignin[21]. Using
132 acetylcholinesterase (AchE) as a model, we how disulfide bonds (four) confer rigidity (Figure
133 2a) and hold the protein structure also during interaction with the material surface.
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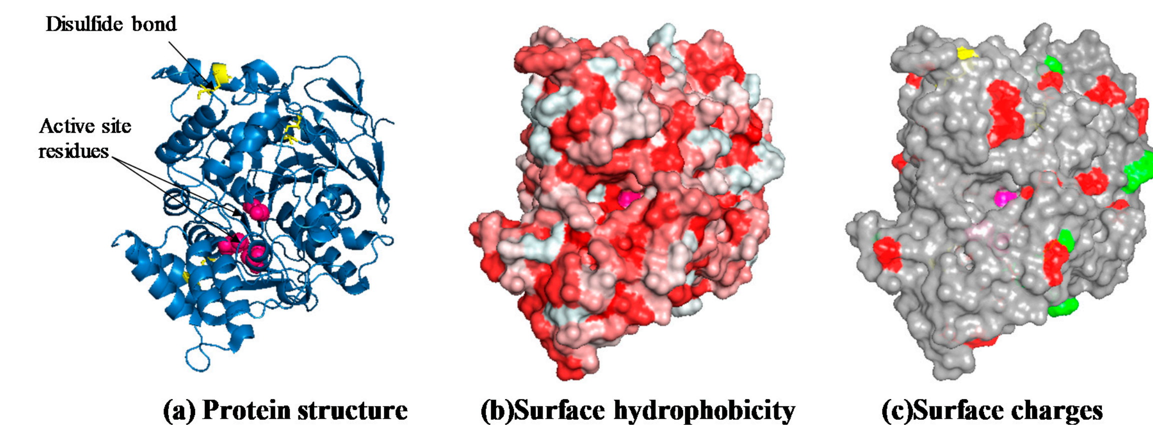

140 **Figure 2** –Protein feature to consider before selecting an immobilization strategy. As an example, the
141 protein acetylcholinesterase from the electric eel *Electrophorus electricus* (PDB ID: 1C2B) is shown. The
142 three-dimensional structure of acetylcholinesterase is shown as ribbons (a) with disulfide bonds
143 (yellow sticks) and active site residues highlighted (pink spheres, b), negatively (green) and positively
144 charged Lys residues (red) that are exposed on the surface (c).

145 AchE is an enzyme naturally involved in the synaptic signal transduction where it
146 hydrolyses acetylcholine to choline and acetate, and a widely used enzyme in biosensors for
147 its sensitivity towards pesticides and pharmacological molecules that are utilized in the
148 treatment of neurological disorders, e.g. Alzheimer's disease[22]. AchE is also reported as

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149 biocomponent in biosensors for the detection of aflatoxin B1 and organophosphate poisons
150 in general[23,24]. The AchE molecule is characterized by four disulfide bonds and can thus
151 be considered a relatively hard protein. The protein surface presents a certain degree of
152 hydrophobicity that can drive the interaction with hydrophobic substrates (Figure 2b). In
153 solution, the enzymes can undergo dynamic multimerization[25]. The immobilization of
154 acetylcholinesterase to a modified hydrophobic surface has been reported to not only not to
155 lead to denaturation and loss of functionality[26] but also to result in a higher-than-1000-
156 folds enhancement in the affinity for toxic organophosphor compounds and in a 110%-fold
157 increase in thermal protein stability[27].

158 The surface of AchE presents residues carrying carboxylic and amine groups (Figure 2c)
159 that can be used for protein immobilization by chemical means or drive adsorption. Lysine
160 residues carry amine groups that can be subject to chemical crosslinking with glutaraldehyde.
161 As a bifunctional crosslinker, glutaraldehyde covalently binds the protein to amino-decorated
162 surfaces, e.g. coated with polyethylenimine to covalently bind protein A and subsequently
163 antibodies by affinity[28]. AchE amine groups have been used for immobilization by
164 EDC/NHS chemistry to a carboxylate-modified silicon substrate to detect
165 organophosphorous pesticides. [29]. Immobilization of AchE has been performed to
166 modified carbon electrodes carrying dialdehyde moieties (covalent immobilization) or after
167 coating with polyethyleneimine (physisorption); both approaches resulted in a reduction of
168 the affinity for the analyte, i.e. an increase in K_m [30]. In an alternative approach, the
169 entrapment of AchE in the hydrophilic polymer chitosan protected the enzymatic activity and
170 provided functionality in the presence of methanol (25%), acetonitrile (15%) and
171 cyclohexane (100%) whereas an equivalent preparation with chemical crosslinking with
172 glutaraldehyde lost activity at a much lower concentration of organic solvents[31]. Surface-
173 exposed lysines are residues often used for fluorescent labelling of the protein for easier
174 tracking; the behavior of proteins whose surface has been modified with covalently-bonded
175 fluorescent dyes can however be quite different from the native one[32].

176 Modification of the surface of proteins is possible and it can significantly tune their
177 bioactivity[33,34], control their adsorption to material surfaces and interfaces[35], suggest
178 immobilization strategies to enhance enzymatic activity[36], allow specific protein
179 labelling[37], interact with smaller biomolecules such as peptides[38] and be used for
180 molecular detection[39]. The tuning of the degree of surface hydrophobicity of a protein is
181 possible. Reduction of surface hydrophobicity of AchE by individually substituting 14
182 solvent-exposed hydrophobic residues with arginine, resulted in many cases in an increased
183 stability to temperature and chemical denaturation[40]. In an opposite strategy for the lipase
184 from *Pseudomonas* sp., the introduction of hydrophobic surface patches by site-directed
185 mutagenesis increased the stability in organic solvents[41]. Glucose oxidase, especially from
186 *Aspergillus niger*, is widely used in biosensors for glucose monitoring and it has a dimeric
187 160 kDa structure whose units are held together by hydrophobic and hydrophilic interactions,
188 e.g. salt bridges and hydrogen bonds. Covalent bonds such as disulfide bonds are not the only
189 contributors to protein stabilization as the introduction of multiple weak interactions such as
190 salt bridges on the surface of the proteins is well known to counteract thermal denaturation.

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The modification of the surface of glucose oxidase to carry both a novel sulfur-pi interaction and a salt bridge led to a 3-fold increase in thermal stability[42]. These surface substitutions did not affect the glycosylation pattern of the enzyme that is also reported to enhance the thermal stability by introducing structural rigidity[43]. Accordingly, its covalent immobilization by entrapment into gelatin using 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide increased the melting temperature from 58°C to 76°C[43]. It is noteworthy that these substitutions can however affect not only the stability but also the catalytic activity.

The behavior of a protein towards charged surfaces can be estimated by the analysis of its surface for charged residues and their distribution, e.g. widespread or localized, by calculating its net charge *in silico*[44], or by experimentally determining its surface zeta potential under different pH conditions[45]. *In silico* simulations can even predict the orientation of the protein on the surface by evaluating the possible protein-surface electrostatic interactions[46]. Proteins are prone to aggregation when environmental conditions are close to their isoelectric point and they also tend to adsorb to surfaces in higher amounts under these conditions[47]. Willing to achieve immobilization based on electrostatic forces and charged amino acids, the addition of negatively or positively charged stretches of amino acids to one terminus of the protein might prevent be a valuable, yet reversible, approach [48,49]. A polyarginine tag has been attached to the green fluorescent protein that could reversibly be immobilized to mica surfaces[50] but the application of these protein engineering strategies has not yet found application in biosensor design.

4. Protein immobilization approaches

A thorough *in silico* and experimental study of the surface protein features can indicate the presence of exposed disulfide bonds or residues susceptible to immobilization by chemical enzymatic crosslinking. Deposition of proteins to the sensing surfaces can also rely on the intrinsic features of the protein, e.g. hydrophobicity and polarity, and thus the incubation conditions that promote attraction can be selected. Adsorption is however a reversible phenomenon and a more stable solution is provided by chemical crosslinkers that can give multi-point interaction with the surface and thus an additional degree of rigidity to the protein. When the deposition as single monolayer is desired, protein engineering can offer additional possibilities (Figure 3).

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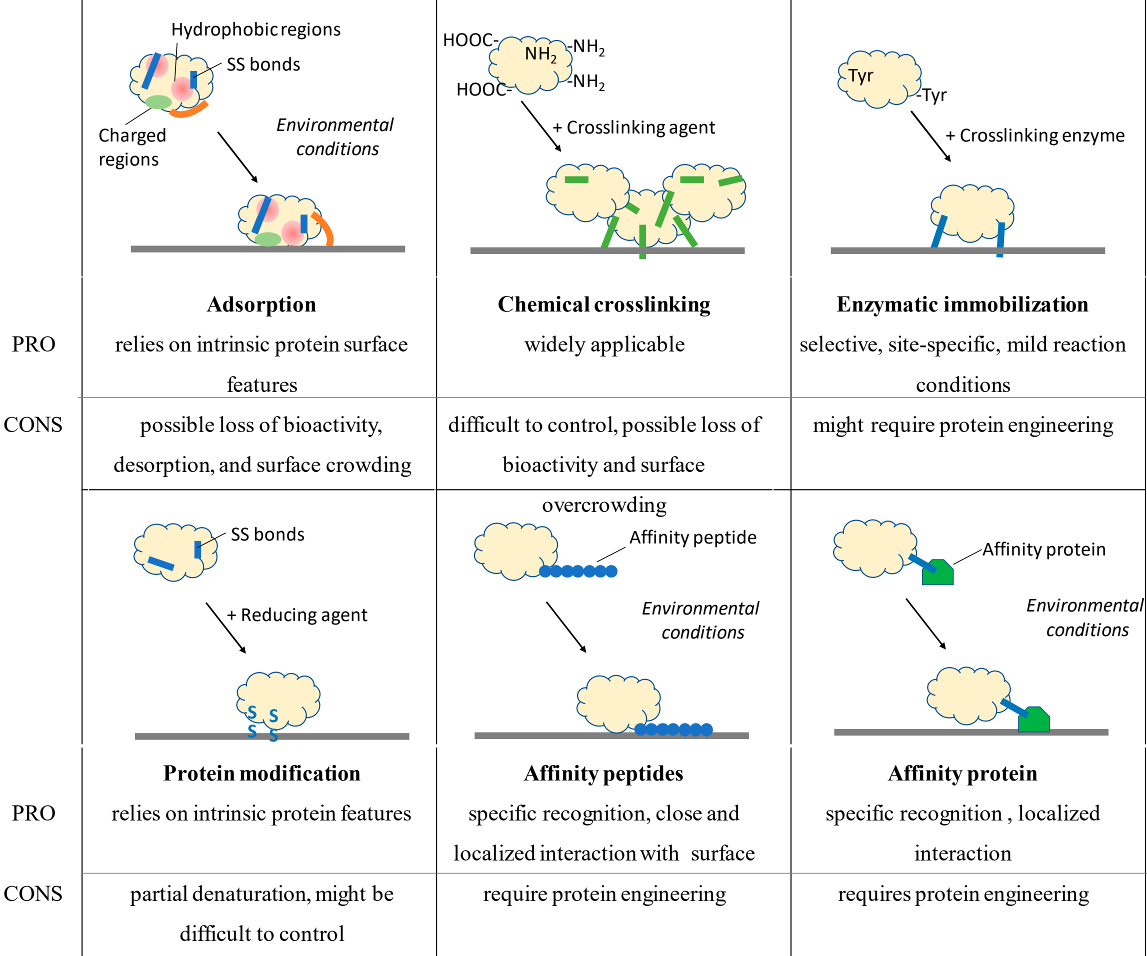

Figure 3 – Schematic view of selected immobilization strategies for proteins to surfaces and the advantages and disadvantages to consider after analyzing the protein structure and its surface features. Immobilization by adsorption and affinity rely on the environmental conditions and not on the presence of a catalyst or reactive molecule such as an enzyme, reducing agents or chemical crosslinkers.

Proteins carrying superficial cysteine can be immobilized directly to disulfide-containing materials or gold electrodes[51]. Protein disulfide bonds are reduced often chemically to form reactive thiols as in the case of antibody immobilization, e.g. using dithiothreithiol (DTT), dithiobutylamine (DTBA), tris(2-carboxyethyl)phosphine (TCEP) or 2-mercaptoethylamine (2-MEA) [52,53]. An alternative UV-light-based technique relying on the presence of aromatic residues in the proximity of disulfide bonds has also been developed to preserve the structure and functionality of the protein while allowing site-specific and space-resolved immobilization and applied to a wide range of proteins, e.g. hydrolytic enzymes, proteases (human plasminogen), alkaline phosphatase, antibody against PSA, major histocompatibility complex class I protein, pepsin, and trypsin[54].

Crosslinking by chemical means relies on the presence of functional groups on the surface of proteins, such as widespread amine groups of lysines and carboxylic groups of glutamate and aspartate residues (Figure 2c). Often used to link proteins to modified surfaces, glutaraldehyde introduces covalent linkages between amine groups and is also used to produce crosslinked protein aggregates. The stabilizing effect detected when proteins are

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chemically immobilized can be ascribed to the newly introduced molecular rigidity through the formation of multiple bonds between the protein and the surface, but also to newly introduced intramolecular bonds in the proteins, especially at low concentrations[55]. As an example lactate oxidase, immobilized by chemical crosslinking with (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (EDC) has proven a more stable arrangement not only in terms of enzyme retention at the surface and improved affinity but also by increasing the stability of the thermal/operational stability enzyme, as compared to the only physisorbed enzyme[56]. Chemical crosslinking similarly to physisorption, does not guarantee however control over the orientation of the protein at the surface. Concerning antibodies, I refer the readers to a recent focused review [52].

Contrary to chemical crosslinkers, protein engineering and enzymatic bioconjugation might offer site-specific approaches to the functionalization of surfaces with proteins[57]. Although generally considered time-demanding, a first protein engineering step might provide advantages that benefit the later deposition to the material by providing a single-step process and by minimizing the amount of protein needed giving an ideal protein monolayer. It is often desired to achieve a controlled immobilization, giving a low surface crowding, optimally a monolayer, and an optimal orientation of the protein, that is often achieved in a site-specific immobilization. Proteins naturally offer features that can be used for this, however protein engineering is a powerful tool in this direction[58]. By protein engineering, it is for example possible to introduce selected chemical moieties at a specific location in the protein molecule by using unnatural amino acids without compromising the bioactivity; reactivity of the unnatural amino acid with the substrate can lead to site-specific point immobilization[59,60].

Peptides might be considered simpler structures than proteins, due to their smaller size, but they can also be valuable in biosensors and for protein immobilization. The possibility of synthesizing and designing peptides allows the insertion of desired chemical groups in specific position and thus functionality. A kinase biosensor has been assembled using a peptide labelled with a fluorescent tetramethylrhodamine (TAMRA) group, i.e. TAMRA-Leu-Arg-Arg-Ala-Ser-Leu-Gly, that produces a FRET signal *via* a Zn^{2+}-coordination with the COOH-rich surface of quantum dots only when phosphorylated by kinases[61]. Peptides can also be fused to proteins at the gene level and used to confer novel affinity features. Peptides of different length with affinity are available for a wide variety of substrates from polystyrene [62] to gold[63], from crystalline sapphire [64] to crystalline nanocellulose[65], from carbon nanotubes[66] to graphite[67]. Our group has engineered a bacterial laccase from *Bacillus pumilus* to carry a terminal affinity peptide for iron oxide that led to a higher protein loading on the surface and a doubling of the enzymatic turnover k_{cat} especially when in a monolayer assembly at the surface[68]. Similarly, carbonic anhydrase has been engineered to carry a single-walled-carbon-nanotube affinity peptide that provided not only binding but a 51% surface coverage while retaining the protein secondary structure elements and enzymatic activity [69]. Fused to carry multiple gold-binding peptides, alkaline phosphatase was immobilized to a gold patterned substrate giving a higher enzymatic activity per area than with the unmodified enzyme[70]. By engineering affinity motifs into the protein

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287 molecule and ensuring their exposure on the surface of the molecules, a controlled site-
288 specific immobilization can be achieved. With hexa-histidine tag (His-tag)[71] being one of
289 the most commonly used affinity tags, a wide range of proteins has been immobilized to
290 different surfaces as it also offers the advantage of reversibility once in the presence of
291 imidazole. After deposition of the nickel-chelator nitrilotriacetic acid (Ni-NTA) to gold
292 electrodes, the monomeric oxidase laccase[72] and even complex proteins such as
293 photosystem II (PSII) could be immobilized[73]. Similarly, His-tagged AChE was directly
294 immobilized to nickel nanoparticles to develop a biosensor detecting the insecticide paraoxon
295 even at a 10^{-13} M concentration[74]. By using cobalt instead of nickel, a more stable
296 immobilization of avidins and norovirus proteins on BLI biosensor surface was achieved and
297 stable even in the presence of 0.7 M imidazole[75]. His-tagged alanine racemase from
298 *Geobacillus stearothermophilus* was immobilized on a silica-coated plate that was modified
299 to contain cobalt ions and retained its activity unaltered after treatment of drying, freezing or
300 immersion in n-hexane[76]. His-tags bind also platinum and its deposition together with
301 graphene on paper allowed the functionalization with His-tagged odor-binding proteins and
302 the assembly of paper electrodes detecting neonicotinoid insecticides[77]. In a different
303 approach, peptides can also be designed or screened for binding specifically to the surface of
304 a selected proteins. Once immobilized, these peptides give a surface that specifically
305 recognizes a target protein. Peptides specifically binding β-galactosidase have been reported
306 to hold the enzyme at the surface while preserving a high specific activity, thermal stability,
307 and guaranteeing a controlled protein orientation[78].

308 Enzymatic immobilization approaches for site-directed protein immobilization are also
309 available and offer a high specificity, the need for small amounts of catalysts, and
310 environmentally-friendly reaction conditions[57]. The crosslinking enzyme sortase has been
311 used for both protein conjugation and protein immobilization, as it requires only amino/lysine
312 containing receiving surface, after introducing the pentapeptide Leu-Pro-Glu-Thr-Gly
313 (sortase tag) by genetic engineering of the protein to be immobilized[79-81]. Following this
314 strategy using the *Staphylococcus aureus* sortase A, a fibronectin-binding protein was
315 selectively and site-specifically immobilized to a sensor chips [82], the membrane-bound
316 glycosyltransferases ere covalently immobilized to amino-modified sepharose resin[81], and
317 a single-chain antibody recognizing to a flow cell biosensor recognizing the cancer biomarker
318 extracellular domain of the epidermal growth factor receptor[80]. Whereas the engineering
319 of a pentapeptide might seem an alteration possibly affecting the functionality of the protein,
320 all these studies show how its addition in terminal position of the primary structure does not
321 compromise the bioactivity of the enzyme or antibody subject of studies. An alternative
322 approach is the use of enzymes that recognize single residues such as tyrosinase and
323 transglutaminase that specifically attack surface-exposed tyrosines and glutamines,
324 respectively[57]. The enzyme tyrosinase has been used for the covalent immobilization of
325 fluorescent proteins and protein A carrying surface-exposed tyrosines to amino-modified
326 surfaces for subsequent antibody capture [83-85], i.e. surfaces treated with polyallylamine.
327 Transglutaminase has been used to immobilize alkaline phosphatase engineered to carry a
328 structurally exposed lysine within the tag Met-Lys-His-Lys-Gly-Ser to a glutamine-

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containing casein layer deposited on a polystyrene surface [86] and to agarose gel beads[87]. Crosslinking enzymes can also be used to achieve *in situ* entrapment of the proteins. Without genetic engineering, glucose oxidase and lactate oxidase were entrapped using translgutaminase into a network of lysine/glutamine-rich proteins and peptides, e.g. poly-lysine, poly-glutamine, fibrinogen, that has been produced directly on the electrode surface[88]. These enzymatically prepared electrodes retained more than double of the sensitivity upon immobilization and a more than 2-foldstability as compared to glutaraldehyde-prepared ones[88]. The polymerization of L-DOPA by tyrosinase has also been used to synthesize a melanin-like polymeric matrix for the entrapment of glucose oxidase and tyrosinase itself for amperometric biosensing reaching a 10 nM detection limit for phenol[89,90].

5. Conclusiom

The immobilization approach used to functionalize the surface of a biosensor is crucial to retain most of the bioactivity and multiple approaches are available. A thorough *in silico* and experimental analysis of the surface of the protein to identify pronounced regions of hydrophobicity or polarity, disulfide bonds, or residues susceptible of crosslinking with enzymes can suggest the most efficient immobilization approach. Protein engineering widens the possibilities and the fusion to affinity peptides or proteins provides a direct single-step immobilization process. In all cases, the study of the physico-chemical properties of not only the material surface but also of the protein, with its structural and surface features, is a crucial initial step towards the selection of the immobilization approach that can provide ease of assemble and optimal biosensor performance.

Conflicts of Interest: The author declares no conflict of interest.

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