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Grafting of proteins onto polymeric surfaces: a synthesis and characterization challenge

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Abstract

This review aims at answering the following question: how can a researcher be sure to succeed in grafting a protein onto a polymer surface? Even if protein immobilization on solid supports has been used industrially for a long time, hence enabling natural enzymes to serve as a powerful tool, emergence of new supports such as polymeric surfaces for the development of so-called intelligent materials requires new approaches. In this review, we introduce the challenges in grafting protein on synthetic polymers, mainly because compared to hard surfaces, polymers may be sensitive to various aqueous media, depending on the pH or reductive molecules, or may exhibit state transitions with temperature. Then, the specificity of grafting on synthetic polymers due to difference of chemical functions availability or difference of physical properties are summarized. We present next the various available routes to covalently bond the protein onto the polymeric substrates considering the functional groups coming from the monomers used during polymerization reaction or post-modification of the surfaces. We also focus our review on a major concern of grafting protein, which is avoiding the potential loss of function of the immobilized protein. Meanwhile, this review considers the different methods of characterization used to determine the grafting efficiency but also the behavior of enzymes once grafted. We finally dedicate the last part of this review to industrial application and future prospective, considering the sustainable processes based on green chemistry.

Abbreviations

AA, Amino acid; AFM, Atomic Force Microscopy; AMP, antimicrobial peptides; 6-APA, 6-

36 aminopenicillanic acid; APTES, aminopropyl triethoxy silane; ATR-FTIR, Attenuated Total Reflection-
37 Fourier Transform Infrared Spectroscopy; CD, Circular Dichroism; CDI, 1,1'-carbonyldiimidazole;
38 COC, cyclic olefin copolymer; EDC, 1-ethyl-3-((dimethylamino)propyl)carbodiimide hydrochloride;
39 EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; EGF, Epidermal growth factor; EPS,
40 expanded polystyrene foam; FESEM, Field Emission Scanning Electron Microscopy; FRET, Förster
41 Resonance Energy Transfer; β -Gal, β -galactosidase; GFP, Green fluorescent protein; GI, glucose
42 isomerase; GOS, galacto-oligosaccharides; HA, hyaluronic acid; HFBI, hydrophobin; HFCS, high
43 fructose corn syrup; Ig, Immunoglobulins; IGI, immobilized D-glucose isomerase; LCST, Lower
44 Critical Solution Temperature; MOF, metal organic frameworks; NCC, nano-crystalline cellulose; NHS,
45 N-hydroxysuccinimide, PA6, polyamide 6; PA6,6, polyamide 6,6; PAA, poly(acrylic acid); Pam,
46 polyacrylamide; PAN, poly(acrylonitrile); P(AN-co-Am), poly(acrylonitrile-co-acrylamide); PCL,
47 poly(ϵ -caprolactone); PCL-PEO-PCL, poly(ϵ -caprolactone)-block-poly(ethyleneoxide)-block-poly(ϵ -
48 caprolactone); PDA, polydopamine; PDMS, polydimethylsiloxane; PE, polyethylene; PEG,
49 poly(ethylene glycol); PEGA, hydrophilic acrylamide-PEG commercial resin; PEI, polyethyleneimine;
50 PEM, polyelectrolyte multilayer; PET, poly(ethyleneterephthalate); PFTase, Protein Farnesyl
51 Transferase; PGMA, poly(glycidyl methacrylate); P(GMA-co-MA), poly(glycidyl methacrylate-co-
52 methyl methacrylate); PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; PHEA,
53 poly(hydroxyethyl acrylate); PHEMA, poly(hydroxyethyl methacrylate); PLL, poly(L-lysine); PLLA,
54 poly(L-lactic acid); PMMA, poly(methylmethacrylate); PNIPAM, poly(N-isopropyl acrylamide);
55 poly(S-co-MA), poly(styrene-co-maleic anhydride); PP, polypropylene; PPC, poly(propylene chloride);
56 PS, polystyrene; PSBMA, poly(sulfobetaine methacrylate); PVA, polyvinylalcohol; PVDF,
57 poly(vinylidene difluoride); SBS, poly(styrene)-block-poly(butadiene)-block-poly(styrene); SECM,
58 Scanning Electrochemical Microscopy; SEM, Scanning Electron Microscopy; SFG, Sum Frequency
59 Generation spectroscopy; SPR, Surface Plasmon Resonance; Tg, glass transition temperature; TEM,
60 Transmission Electron Microscopy; TG, triglycine; TGA, Thermogravimetric analysis; TGF,
61 Transforming Growth Factor; TNBS, 2,4,6-trinitrobenzene sulfonate; ToF-SIMS; Time of Flight
62 Secondary Ion Mass Spectroscopy; UCST, Upper Critical Solution Temperature; XPS, X-Ray
63 Photoelectron Spectroscopy.

64

65 **Keywords**

66 Synthetic polymer; Elastomer; Immobilization; Enzyme; Grafting; Biophysics; Covalent bond

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68

69 **1. Introduction**

70 Proteins immobilized on solid supports have been used industrially for a long time, the immobilization
71 bringing increased stability, ease of handling for multiple runs, and ease of recovery when linked to
72 magnetic beads(Bolivar et al., 2022; Pei et al., 2022), hence enabling natural enzymes to serve as a
73 powerful tool. This has been extensively described and reviewed, the reader is therefore encouraged to
74 consult this rich literature (Brena et al., 2013; Rodrigues et al., 2019; Santos et al., 2015; Wahab et al.,
75 2020a). Very early, different immobilization methods (Klibanov, 1979) were proposed: covalent
76 attachment, adsorption, covalent crosslinking or entrapment. Single enzyme immobilization started in
77 the sixties (Guisan, 2013) based on these methods, followed by the challenge of immobilizing multiple
78 enzymes (Ren et al., 2019). If the strategy has not evolved fundamentally, numerous parameters
79 (Boudrant et al., 2020) have been however assessed, with the objective of keeping or improving the
80 activity of the immobilized protein. Indeed, in order to keep the protein active, its conformation should
81 be maintained, its orientation controlled (especially for the active site of enzymes) and untimely protein
82 release should be avoided. The influence of the support itself has been particularly examined and found
83 important (Santos et al., 2015; Wahab et al., 2020b), either through its available chemical groups or its
84 mechanical property. It is noteworthy that a hydrophilic support is often considered as the best option
85 to keep the enzyme active. Controlling hydrophilicity around the enzyme helps keeping the enzyme in
86 a natural conformation. However, grafting on a solid support often introduces some hydrophobic groups
87 which then have to be counterbalanced by other hydrophilic groups (Santos et al., 2015). Compared to
88 early reviews describing in a very general way the different methods of grafting proteins or enzymes on
89 solid surfaces, recent reviews are more focused on specificities, such as grafting of proteins on
90 renewable polymers, supramolecular strategies (Finbloom and Francis, 2018), grafting on micro- or
91 nanostructured materials (Bilal and Iqbal, 2019a), specific applications such as membranes, biocatalysis
92 (Romero-Fernández and Paradisi, 2020) or water purification (Xu et al., 2013). A recent tutorial review
93 takes the original standpoint of the enzyme immobilization pitfalls, examining many different points
94 that could go wrong and lead to poor results, going furthermore from laboratory to industrial
95 environment(Bolivar et al., 2022). Among the various existing reviews, the lack of overview for the
96 grafting on polymeric surfaces is surprising, especially linked to the strong development of so-called
97 intelligent materials (Bratek-Skicki, 2021) designed for biological applications. A recent review
98 (Rodriguez-Abetxuko et al., 2020) presented an analysis of the use of polymer scaffolds for enzyme
99 immobilization, but it was mainly focused on polymer-enzyme hybrids either as new bioconjugates or
100 soluble assemblies. Only a very small part was dedicated to the polymer surfaces and there was no
101 mention of the desirable characterization techniques for such systems.

102 Among all assessed immobilization strategies, the covalent grafting of proteins presents the
103 asset of ensuring a strong attachment of the protein to its support, therefore avoiding untimely release.
104 However, covalent immobilization implies to control the molecular orientation of the enzyme in order
105 to preserve or improve its biological activity (Liu et al., 2013). The related constraints to this strategy,
106 such as protein structure resolution, dealing with unspecific enzyme-support interactions or enzyme

107 engineering, make it more challenging and economically costly. A tremendous progress has been made
108 by developing precise modification of proteins by protein engineering techniques which enables
109 introduction of non-standard amino acid in the sequence of the final enzyme (Pei et al., 2022).
110 Furthermore, covalent grafting on hard surfaces or polymeric ones does not constitute the same
111 challenge and this is most often overlooked in the literature. This review therefore aims at explaining
112 the specificities of covalent grafting of proteins onto polymeric surfaces, pointing at the different
113 grafting methods, the available characterizations and the existing or possible future industrial
114 applications. It is noteworthy that all polymeric systems are mentioned in this review, including beads
115 but also flat surfaces or fibers. The polymeric surface is thus the precise interface between the polymer
116 itself and its environment.

117
118

119 **2. Specificity of grafting on synthetic polymers**

120 As mentioned in the introduction, proteins have been often grafted onto hard inorganic materials. These
121 can be divided in two categories depending on the composition with only inorganic atoms present
122 (metallic surfaces, silicon) or other atoms (silicon dioxide, iron oxide, ceramics, metal organic
123 frameworks (MOFs), graphene). On such surfaces, the main point for a successful grafting is to be sure
124 of the chemical functions available and their density. If both are known, then the reactive chemical group
125 on the protein (if exposed) is expected to react and form the desired covalent bond. Chemical functions
126 on hard surfaces are often hydroxyl ones and they can be transformed into many reactive groups, using
127 functional silanes. Some cases imply specific processes, such as gold for which the main strategy is to
128 directly use the strong bond Au-S (Tähkä et al., 2019) or graphene-based systems where a chemical
129 modification of the aromatic rings is needed. Before describing the specificities of grafting proteins onto
130 synthetic polymers, it is useful to gather the properties of such pristine hard surfaces (table 1). Their
131 common characteristics are the presence of crystalline domains, the absence of any phase transition
132 close to room temperature, the immobility of the network and the fact that solvents or solutions have
133 either no influence on their structure or degrade them. Table 2 next presents the same characteristics for
134 polymeric systems in order to get a global overview. Their common properties are the variety of
135 chemical functions available and the possible presence of transitions near room temperature. In the next
136 paragraphs, we are going to compare these systems in more details in terms of chemical reactivity and
137 physical properties.

138
139

Table 1. Main characteristics of hard support materials.

	Inorganic matrix	Oxide matrix	Graphene-based systems
--	------------------	--------------	------------------------

Network	3D	3D	2D
Morphology	Crystalline, porous or not	Crystalline and/or glassy, porous or not	Partly crystalline, not porous
Bonds	Covalent	Covalent and/or ionic	Covalent
Pristine chemical groups	Mt-Mt	Mt-OH or Mt-O-Mt	C-C
Phase transition near room temperature	None	None	None
Mobility of the network atoms	None	None	None
Influence of solvents	None or degradation	None or degradation	None or degradation
Influence of pH	None or degradation	None or degradation	None or degradation
Influence of ionic strength	None	None	None

140

141 *Table 2. Main characteristics of polymeric matrices*

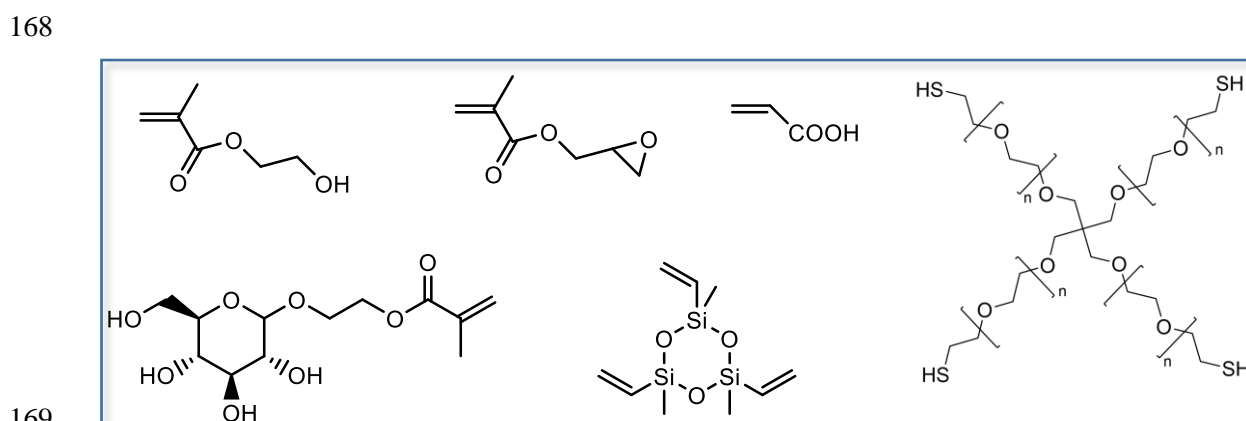
	Bulk polymers	Crosslinked polymers	Hydrogels
Network	1D	3D	3D
Morphology	Semi-crystalline or glassy, not porous	Semi-crystalline or glassy, porous or not	Amorphous, porous
Bonds	Covalent	Covalent and/or ionic and/or complexes	Covalent and /or ionic and/or complexes
Pristine chemical groups	Alcohols, esters, carboxylic acids, amines, ethers, amides, urethanes, siloxanes...	Alcohols, esters, carboxylic acids, amines, ethers, amides, urethanes, siloxanes...	Alcohols, esters, carboxylic acids, amines, ethers, amides, urethanes, siloxanes...
Phase transition near room temperature	Possible glass or melting temperature	Possible glass or melting temperature	Possible order-disorder transition
Mobility of the network atoms	Depending on the glass or melting temperature	Depending on the glass or melting temperature	Existing mobility by essence, linked to the high-water content
Influence of solvents	None, swelling or dissolution	None or swelling	Change of swelling
Influence of pH	None or degradation	None or degradation	Possible change of swelling or degradation

142 2.1 Chemical reactivity

143 In order to chemically graft proteins onto surfaces, the presence of available chemical functions is
144 essential. A very recent review presents all types of available functionalizations of nanomaterials
145 (Wieszczycka et al., 2021), which are also valid for any surface. Among inorganic systems, only oxide
146 support materials exhibit available reactive functions, in the form of either hydroxyl groups or Mt-O-
147 Mt bonds which can be broken and used for the grafting. The routine technique in this case is the use of
148 functional silanization (Liu et al., 2020). An alternative is the introduction of chemical functions by the

149 shell-by-shell method, a first layer of molecules is grafted on the surface, followed by another shell
150 entangled with the first one (Stiegler et al., 2020). For inorganic matrices such as metals or silicon, an
151 activation of the surface is mandatory, and this leads most of the time to the introduction of a thin layer
152 of oxide, thereby exhibiting a similar reactivity to the pure oxide matrices. The associated activation
153 methods will be subsequently described in the next part of this review, since some can also be used for
154 polymers. For the graphene-based systems, here also an activation is mandatory, implying the breaking
155 of very resistant C=C aromatic bonds (Al-Lolage et al., 2019; Wang and Jiang, 2019). Regarding the
156 available reactive functions, MOFs constitute an exception in this category. Indeed, their composition
157 enables the presence of a variety of chemical functions, such as amine, carboxyl, hydroxyl, epoxy, or
158 glyoxyl groups (Liang et al., 2020; Ye et al., 2020).

159
160 Compared to these systems, MOFs being the exception, polymers exhibit a very large variety of
161 chemical groups, coming directly from the range of functional monomers available. Indeed, based on
162 the different types of polymerizations (radical, ionic, coordination chain polymerizations or
163 polycondensations...), the panel of corresponding monomers spans from simple acrylates to
164 cyclosiloxanes or multifunctional molecules. Many of them are commercially available (scheme 1), but
165 the organic chemistry tools enable the synthesis and development of other functional monomers on
166 demand. This review focuses on synthetic polymers but including the possible use of the natural
167 polymers such as polysaccharides enlarges the variety of macromolecules even more.



170 *Scheme 1. Examples of commercially available functional monomers of interest for the grafting of proteins*

171 In some cases, the synthesis of desired functional polymers is however not immediate for people
172 not in the field or the desired functional monomers (or polymers) not commercially available. The
173 activation methods described in the next part of this review are then possible, in a similar manner than
174 for the previous inorganic systems. Even in this case, polymers can present the asset of leading to stable
175 activation groups, compared to inorganic surfaces yielding for instance Mt-O-C bonds which are known
176 to be sensitive to hydrolysis in some cases. It is also noteworthy here that in many instances, polymers
177 are used to bring functionalization on inorganic systems. By first grafting a polymer on the inorganic
178 surface, the inherent properties of the inorganic part can be maintained and used, together with the

179 tunability of the polymer layer (Dumri and Hung Anh, 2014; Kang et al., 2015; Malar et al., 2019; Wang
180 and Jiang, 2019).

181 2.2 Physical properties

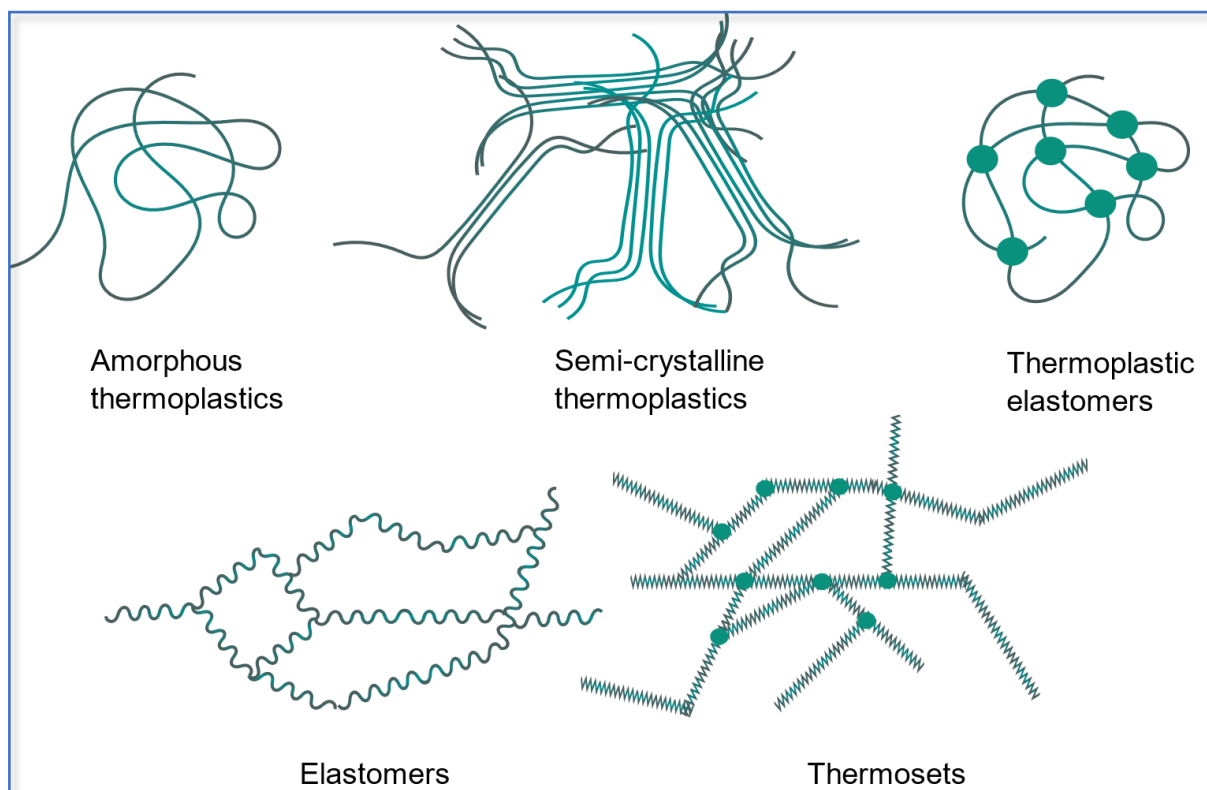
182 Beside the chemical reactivity, the physical behavior of the surface is also essential to ensure an efficient
183 protein grafting. At this point, it might be useful to present an overview of the physical properties of
184 polymers in general (table 3 and scheme 2). The common physical characteristics is the existence of a
185 glass transition temperature, below which the material is hard and breakable and above which
186 movements start to occur locally in the macromolecular chains. This glass transition is essential
187 considering the desired application. Indeed, depending on the desired temperature of usage, a certain
188 type of polymer should be favored. Polymers are generally divided in several families, depending on
189 their morphology and mechanical behaviour. Thermoplastics consist in linear polymer chains that can
190 be used in bulk. They are most often used below their glass transition temperature (T_g) to benefit from
191 good mechanical properties. Regular elastomers and thermosets are both crosslinked systems and as
192 such cannot be solubilized any more. Elastomers are used above the glass transition temperature to
193 obtain an elastic behaviour, whereas thermosets are below T_g and are by essence hard systems.
194 Thermoplastics elastomers, made of block copolymers of different T_g s, were developed later, and the
195 application between both T_g s enables to have a mixed behaviour between regular thermoplastics and
196 thermosets: they are rigid but can be easily processed and recycled which is not possible for thermosets.

197
198 When comparing the grafting of proteins onto hard inorganic surfaces and polymeric ones, the existence
199 of this glass transition is critical, because above T_g , as already mentioned, local movements exist in the
200 macromolecular chain. This implies that chemical groups which are exposed at one point to the outside
201 can move towards the inside and become hidden, and therefore not available any more for possible
202 reactions with a protein. This is particularly well known for contact angle measurements of elastomers:
203 the contact angle changes over several minutes to hours periods (Campeau et al., 2017; Zhang et al.,
204 2013) after a chemical or physical treatment. This means that any further grafting on the modified
205 surface should be performed as soon as possible. The influence of temperature can also lead to strong
206 changes of hydrophilicity of the polymer, this is known as Upper Critical Solution Temperature (UCST)
207 or Lower Critical Solution Temperature (LCST) when the polymer becomes hydrophobic respectively
208 below (UCST) or above (LCST) a critical temperature. The most popular LCST system is poly(N-
209 isopropyl acrylamide) PNIPAM (Sánchez-Moreno et al., 2018; Yang et al., 2020) for which the LCST
210 is at ca. 32°C, therefore close to temperature of biological experiments. This has led to numerous
211 systems with temperature-responsive behaviour, from drug release to control of cell attachment.

212

	Amorphous thermoplastics	Semi-crystalline thermoplastics	Thermoplastic elastomers	Elastomers	Thermosets
Mechanical properties at room temperature	Rigid, breakable	Can be slightly distorted Cold stretching possible	Rigid	Elastic	Rigid, even at high temperature
Preferred usage temperature	Below T_g	Between T_g and T_m	Between T_{g1} and T_{g2}	Above T_g	Below T_g
Solution behaviour	Soluble	Soluble	Soluble	Insoluble	Insoluble
Chain structure	Linear	Linear	Linear	Crosslinked	crosslinked
Melting	Fluidification	Melting	Melting	Infusible	Infusible
Crystallinity	Amorphous	Semi-crystalline	Possibly semi-crystalline	Amorphous	Amorphous
Recyclability	Yes	Yes	Yes	No	No
Examples	PMMA, PS	PE, Nylon	SBS	Silicone rubber, natural rubber	Polyurethanes, Epoxy resins

214



215

216 Scheme 2. Schematic representation of the different polymer families

217 Beside temperature response, polymers may also be sensitive to the chemical environment, this is

218 particularly true for polyelectrolytes, the solubility of which will depend on the pH or the ionic strength
219 of the solution. From this standpoint, polyelectrolytes exhibit a similar behaviour to proteins. The use
220 of polyelectrolyte as a protein support might be delicate because strong electrostatic attraction may lead
221 to the denaturation of the protein and on the other hand strong repulsion may lead to the absence of
222 grafting. To the best of our knowledge, very few cases of protein grafting onto polyelectrolytes exist.
223 Interestingly, the presence of a polymer, chitosan in the example reported by Kumar, has also been
224 already used to tune the accessibility of the enzyme and its activity (Malar et al., 2019).

225
226 In a global manner, the important points to keep in mind when grafting onto polymers is that they provide
227 the opportunity of a wide range of chemical functions, but that one should be careful about possible
228 transition occurring in the temperature range used. The next paragraphs will show different examples of
229 such chemical diversity, either for non specific or specific grafting of proteins.

230 **3. Non specific grafting**

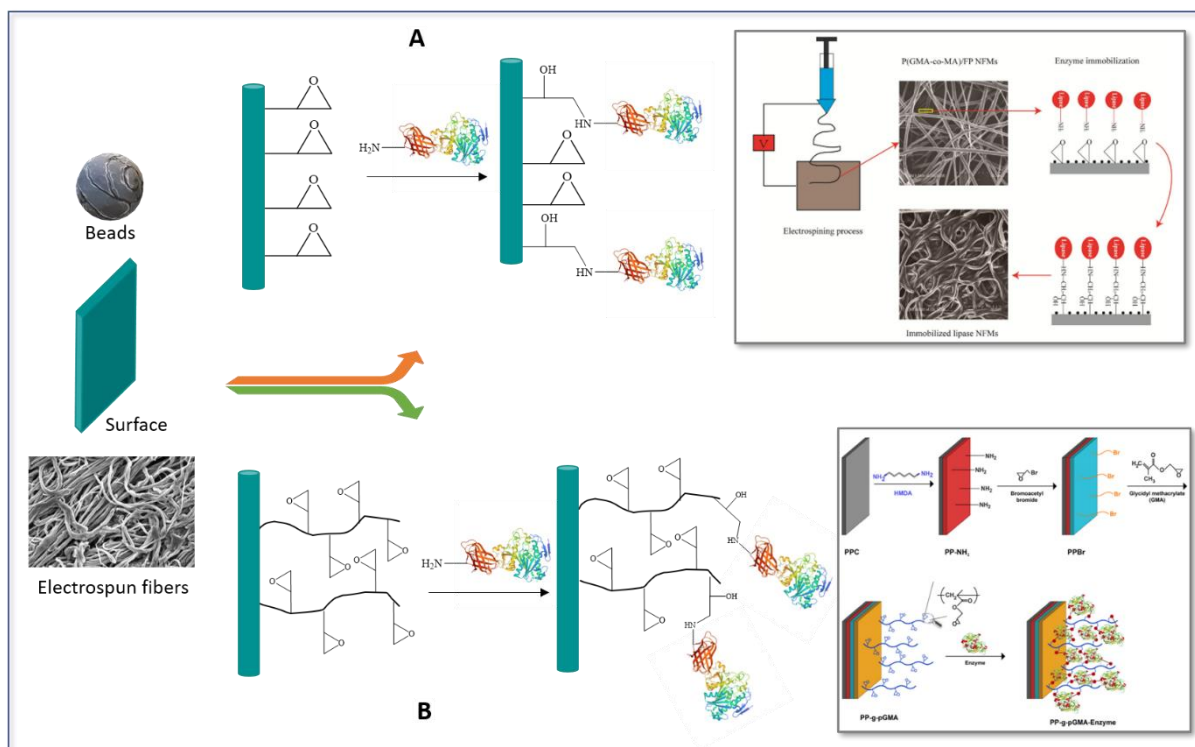
231 The non-specific grafting of proteins onto various substrates has already been extensively studied
232 (Barbosa et al., 2013, 2015; Bezerra et al., 2015; Bilal and Iqbal, 2019b; Cen et al., 2019; Delaittre et
233 al., 2015; Facin et al., 2019; Jochems et al., 2011; Lyu et al., 2021; Rodrigues et al., 2019, 2021; Smith
234 et al., 2020a; Tacias-Pascacio et al., 2021; Wahab et al., 2020b). Here, we will present the various
235 available routes if a “basic” grafting of the protein onto the polymeric substrates is sought. Most of the
236 time, in order to covalently link a protein to a polymeric surface, the latter has to be functional
237 (functional groups coming from the monomers used during polymerization reaction) or functionalized
238 (post-modification of the surfaces). The different strategies will thus be successively presented. The
239 nature of the functional groups is rather limited and only few different functional groups are used to
240 perform such grafting reactions.

241 One of the main strategies to covalently link proteins onto polymeric surfaces is to use the amino groups
242 present on the proteins (preventing thus tricky modification of proteins) and to make them react with
243 antagonist functional groups, such as epoxides, aldehydes, carboxylic acids or even hydroxyl groups
244 through a coupling agent. The targeted reacting group is dependent on the chemical nature of the surface.

245 3.1 Epoxide groups

246 Epoxide groups have extensively been studied as they present advantages to covalently link proteins
247 onto polymeric supports. Thus, in the case of epoxidized surfaces, the linkage of the proteins proceeds
248 through a two-step procedure: first the protein is adsorbed onto the surface via several interactions,
249 secondly the adsorption of the protein allows a multi-point covalent attachment of the protein through
250 regular epoxide chemistry (generally reaction of amino functions of the protein) (Mateo et al., 2000a,
251 2000b).

252 In the literature, many authors employed commercial epoxy-functionalized supports. For instance,
 253 Eupergit C beads (copolymer of methacrylamide, bisacrylamide and epoxy bearing monomer) were used
 254 to immobilize Penicillin G acylase from *Escherischia coli* or *Acetobacter turbidans*, β -galactosidase
 255 from *Aspergillus oryzae*, chymotrypsin and lipase from *Candida rugosa* (Mateo et al., 2000a, 2000b).
 256 Polymethylmethacrylate (PMMA) epoxy activated beads (sepabeads) served as support for laccase from
 257 *Myceliophthora thermophila* (Kunamneni et al., 2008).



258
 259 *Figure 1. Grafting of proteins through epoxide groups. Illustration from (Arica et al., 2017) (A) and (Liu et al., 2018a) (B).*

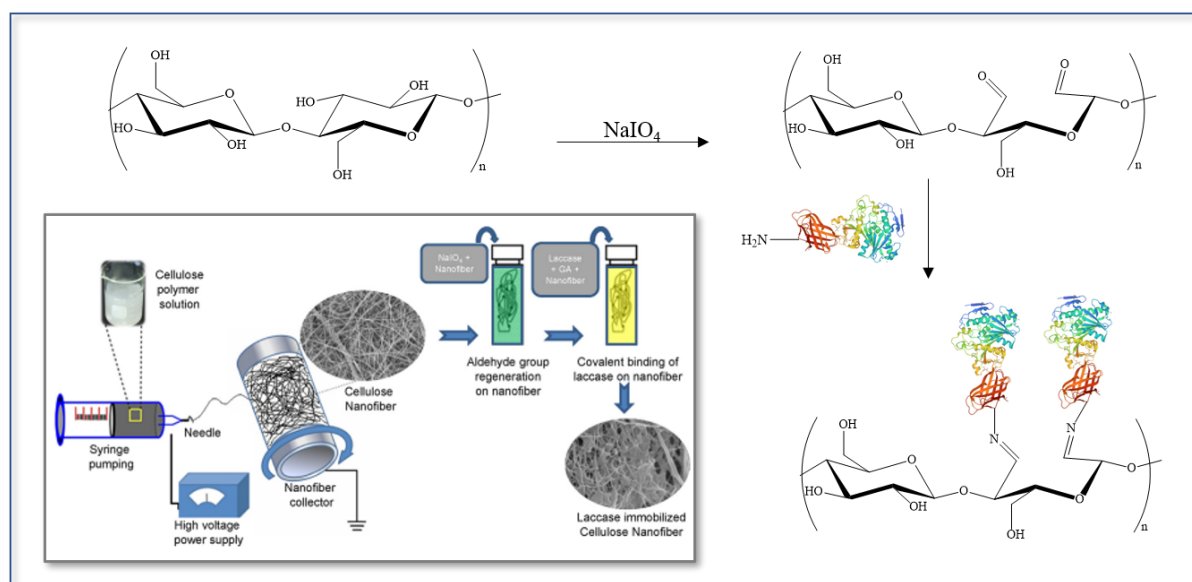
260 Apart from beads, (nano)fibers were also studied to support a variety of proteins. The epoxy units could
 261 be directly present onto the fiber thanks to the chemical nature of the polymer (Liu et al., 2018b, 2018a),
 262 or have to be added by a post-modification of the fibers (Arica et al., 2017; Huang et al., 2008; Oktay et
 263 al., 2015a). Thus nanofibrous membranes, bearing epoxidized functions, were obtained through electro-
 264 spinning of poly(glycidyl methacrylate-co-methyl methacrylate) (P(GMA-co-MA)) and were directly
 265 reacted with lipase B from *Candida antarctica* (Liu et al., 2018b, 2018a). On another hand, depending
 266 on the chemical nature of the polymer, different chemistries were employed for the introduction of oxide
 267 functional groups. For example, poly(acrylonitrile-co-2-hydroxyethylmethacrylate) (PANCHEMA)
 268 fibers obtained by electro-spinning were reacted with epichlorohydrin and lipase from *Candida rugosa*
 269 was thus covalently bonded (Huang et al., 2008). In another study, PGMA was grafted through free
 270 radical polymerization from polyvinylalcohol (PVA) nanofibers to allow the covalent immobilization
 271 of α -amylase from porcine pancreas (Oktay et al., 2015a), or from poly(propylene chloride) (PPC) fibers
 272 for the immobilization of laccase from *Trametes versicolor* (Arica et al., 2017).

273 3.2 Aldehyde groups

274 Another important functional group that is looked for onto surfaces is aldehyde as it can react easily
275 with the amino groups of proteins to yield imine function. As this reaction can be reversible, in order to
276 gain stability with time, it is sometimes necessary to reduce the imine function to a very stable secondary
277 amine. As aldehyde groups are almost never “naturally” present on polymers, the surfaces have to be
278 modified/activated following different routes, depending on the chemical nature of the polymer.

279 The method of choice for the modification of cellulosic surface is the use of sodium periodate that will
280 oxidize glucosidic rings to yield 2 aldehydes per oxidized saccharidic unit. Through this technique, many
281 proteins/enzymes were supported onto cellulosic fibers, like α -chymotripsin (Singh et al., 1979), papain
282 (Jin and Toda, 1988; Vasconcelos et al., 2020), glucoamylase (Varavinit et al., 2001), protein A/G (Ma
283 and Ramakrishna, 2008), lipase from *Candida rugosa* (Huang et al., 2011) or laccase from *Pleurotus*
284 *florida* (Sathishkumar et al., 2014).

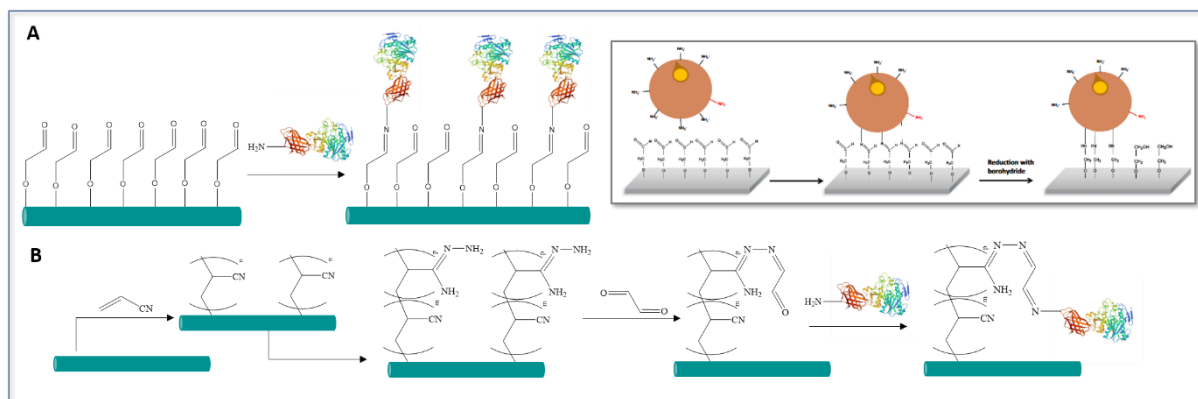
285



286
287 *Figure 2. Grafting of proteins onto polysaccharides through partial degradation of polysaccharidic chain and aldehyde groups*
288 *formation. Illustration from (Sathishkumar et al., 2014)*

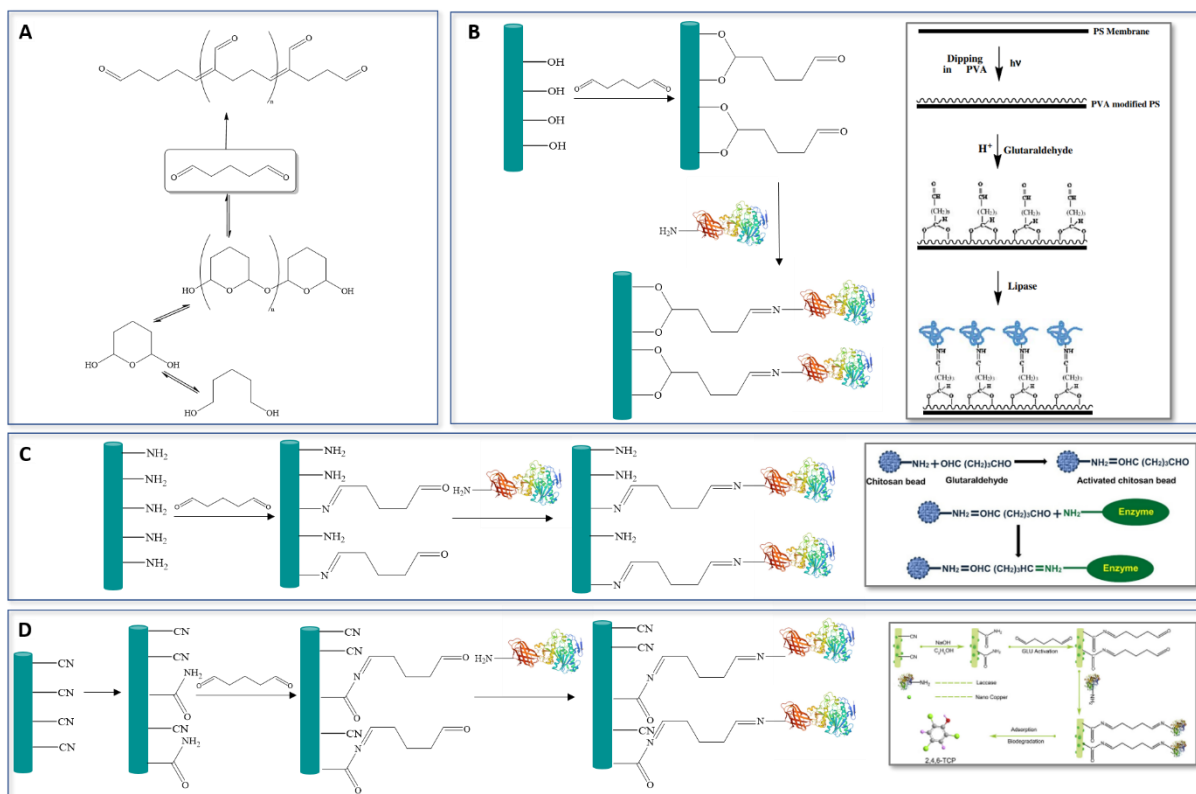
289 In the case of agarose/Sepharose supports, another route is preferred, namely the glyoxyl one. Those
290 supports can be commercially available or modified through a multi-step synthesis by reacting some
291 hydroxyl groups with glycidol followed by oxidation of the epoxide ring by sodium periodate to yield
292 aldehyde functions (Grazu et al., 2006; Guisán, 1988). On these activated supports the covalent
293 immobilization of various proteins was described: penicillin G acylase (Guisán, 1988; Mateo et al.,
294 2005), α -galactosidase from *Kluyveromyces lactis* (Mateo et al., 2005), bovine trypsin (Mateo et al.,
295 2005), glutamate racemase (Mateo et al., 2005), β -galactosidase from *Escherichia coli* (Grazu et al.,
296 2006), catalase from bovine liver (Grazu et al., 2006), IgG from rabbit (Grazu et al., 2006), glutamate
297 dehydrogenase from *Thermus thermophilus* (Bolivar et al., 2009), papain (Pessato and Tavano, 2015)

298 and lipases from *Candida Antarctica*, *Thermomyces lanuginosus* or *Rhizomucor miehei* (dos Santos et
 299 al., 2017; Rueda et al., 2016), Like for the epoxide functionalized surfaces, a multi-point attachment of
 300 the protein is observed (Mateo et al., 2006), A similar strategy was also developed to immobilize β -
 301 galactosidase from *Escherichia coli* onto silk fibers (Monier, 2013), To this end, poly(acrylonitrile)
 302 (PAN) was first grafted onto silk fiber, and all the cyano groups were reacted with hydrazine and in a
 303 second step with glyoxal.



304
 305 *Figure 3. Grafting of proteins through glyoxyl route. Illustration from (Rodrigues et al., 2021)*

306 Among the activation of surfaces with aldehyde, the use of glutaraldehyde (a bis-aldehyde) as a “sticker”
 307 between the protein and the surface is probably the most employed route to immobilize proteins onto
 308 polymeric surfaces. The chemistry of glutaraldehyde is not fully understood as it can lead to several
 309 kinds of structures (linear polymers, 6-membered units, etc.) (Barbosa et al., 2014). Nevertheless,
 310 literature examples have suggested that one moiety of the glutaraldehyde could react either with the
 311 amino groups present on the surface or 2 hydroxyl groups also present on the surface and the other
 312 aldehyde function could react with an amino group of the protein.



313

314 *Figure 4. Grafting of proteins through glutaraldehyde route*

315 This technique was employed either with synthetic or natural polymers or blends of the 2 types of
 316 polymers. Among the synthetic polymer utilized, one can cite PVA, polyamides or polyacrylamide. For
 317 the natural polymers, chitosan is the most described. Some examples of immobilization are presented in
 318 the following table showing that this technique is quite versatile and can be applied to many supports
 319 and many proteins.

320

321 *Table 4. Examples of protein grafting onto polymers through aldehyde groups*

Polymer support	Protein	Ref
P(AN-co-Am)	Lipase from <i>Pseudomonas cepacia</i>	(Lou et al., 2018)
Electrospun PAN/PVDF/Cu	Laccase from <i>Trametes versicolor</i>	(Xu et al., 2017)
Electrospun poly(S-co-MA) grafted Jeffamines	Acetylcholinesterase	(Stoilova et al., 2010)
Partially hydrolyzed PA6,6 films	β -glucosidase, trypsin	(Isgrove et al., 2001)
Electrospun PA6,6 partially hydrolyzed with C nanotubes6	α -chymotrypsin Laccase from <i>Trametes versicolor</i>	(Wong et al., 2017) (Chen et al., 2020)
PA6 Partially hydrolyzed Electrospun with chitosan	Tyrosinase from <i>Agaricus bisporus</i> Laccase from <i>Trametes versicolor</i>	(Harir et al., 2018) (Maryšková et al., 2016)

Casted P(VA-vinyl butyral)	Papain	(Zhuang and Allan Butterfield, 1992)
Polysulfone membranes coated with PVA	Lipase from <i>Candida rugosa</i>	(Gupta et al., 2010)
Electrospun chitosan/PVA with C nanotubes removal of PVA	Acetylcholinesterase Laccase from <i>Trametes versicolor</i> Laccase from <i>Trametes versicolor</i> Lipase from <i>Candida rugosa</i>	(El-Moghazy et al., 2016) (Xu et al., 2013) (Xu et al., 2015c) (Huang et al., 2007)
Chitosan beads beads hydrogel	Lipase B from <i>Candida antarctica</i> Inulase β -galactosidase from <i>Aspergillus oryzae</i> Lipase from <i>Thermomyces lanuginosus</i>	(dos Santos et al., 2017) (Singh et al., 2017) (Wahba, 2017)(Urrutia et al., 2018) (Bonazza et al., 2018)
Agarose	Lipase B from <i>Candida antarctica</i>	(Barbosa et al., 2012)
Agar/ κ -carrageenan hydrogels coated with PEI	β -galactosidase from <i>Aspergillus oryzae</i>	(Wahba and Hassan, 2017)
Cellulose membrane	Bovine serum albumine	(Shaimi and Low, 2016)
Carboxymethylcellulose beads grafted with PAm	Urease	(Alatawi et al., 2018)
Electrospun silk fibroin nanofibers	α -chymotrypsin	(Lee et al., 2005)
Electrospun Zein	Laccase from <i>Trametes versicolor</i>	(Jhuang et al., 2020)

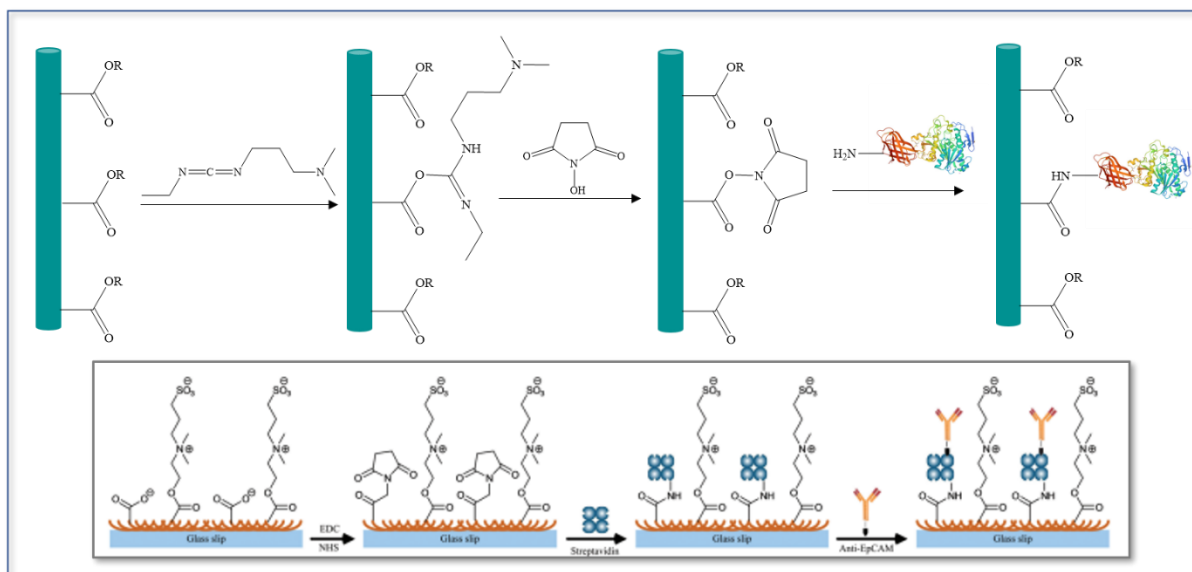
322 3.3 carboxylic acid functions

323 The third functional groups that can be useful to covalently bond proteins onto polymeric surfaces are
324 carboxylic acid and its derivatives (esters, anhydrides, etc.). They can either react directly with the
325 protein or be modified before reacting with the protein. Nevertheless, when carboxylic acid groups are
326 directly involved in the reaction with enzymes, the grafting yield is generally low. It is thus preferable
327 to activate those groups to allow a better yield. The most common activation system of carboxylic acid
328 is based on the use of 1-ethyl-3-((dimethylamino)propyl)carbodiimide hydrochloride (EDC) / *N*-
329 hydroxysuccinimide (NHS) coupling system. Like the use of glutaraldehyde, this synthetic strategy can
330 be applied to many polymeric supports and many proteins. Some examples are listed in the Table below.
331

Polymer support	Protein	Ref
Electrospun poly(acrylonitrile-co-maleic acid)	Lipase from <i>Candida rugosa</i>	(Ye et al., 2006)
Electrospun partially hydrolyzed PAN/Fe ₃ O ₄	Antibody	(Chauhan et al., 2018)
Electrospun partially hydrolyzed PCL	Matrigel (mixture of proteins)	(Ghasemi-Mobarakeh et al., 2010)
Electrospun partially hydrolyzed PHA	Collagen and neuromimetic peptides	(Masaeli et al., 2014)
Electrospun PMMA/Fe ₃ O ₄	Laccase from <i>Trametes versicolor</i>	(Zdarta et al., 2020)
Electrospun PMMA/polyaniline	Laccase from <i>Trametes versicolor</i>	(Jankowska et al., 2020)
Electrospun PA6/PSBMA/PAA	Antibodies	(Tseng et al., 2016)
Sodium alginate/graphene oxide beads	Pectinase	(Dai et al., 2018)
Alginate or chitosan coated with alginate beads	Acrylamidase	(Bedade et al., 2019)

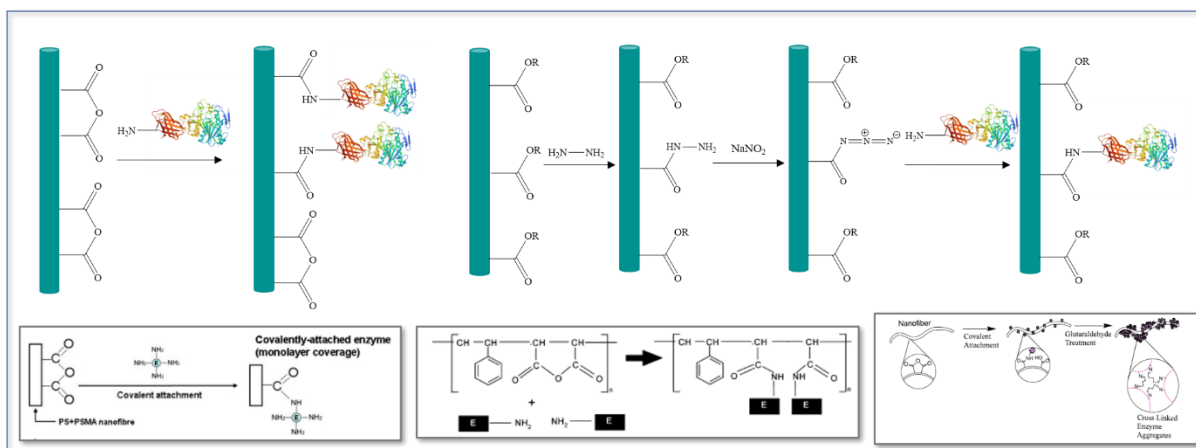
333

334



335

336 Figure 5. Grafting of proteins through carboxylic acid groups activated by EDC/NHS. Illustration from (Tseng et al., 2016)



337
 338 *Figure 6. Grafting of proteins through anhydride groups or carboxylic groups. Illustration from (Kim et al., 2005) (left), (Nair*
 339 *et al., 2007) (middle) and (Smith et al., 2020a)*

340 When carboxylic acid groups are not present on the surface, this latter can be first treated with plasma
 341 and then the same EDC/NHS coupling system is employed to covalently graft the proteins. Different
 342 kinds of plasma were used in the literature, like air, O₂, CO₂, N₂, micro-wave, etc. In some cases, the
 343 plasma treatment is used to polymerize monomers from the surface by free radical polymerization
 344 (Völcker et al., 2001). In other cases, the plasma treatment directly functionalizes the surface with
 345 functional groups (Guex et al., 2014; Heidari-Keshel et al., 2016; Khademi et al., 2017; Ma et al., 2005;
 346 Mahmoudifard et al., 2016; Teske et al., 2020; Vasile et al., 2011a; Wieland et al., 2020). This treatment
 347 can be applied to many kinds of surfaces and allowed the immobilization of a wide variety of proteins
 348 as indicated in the table below.

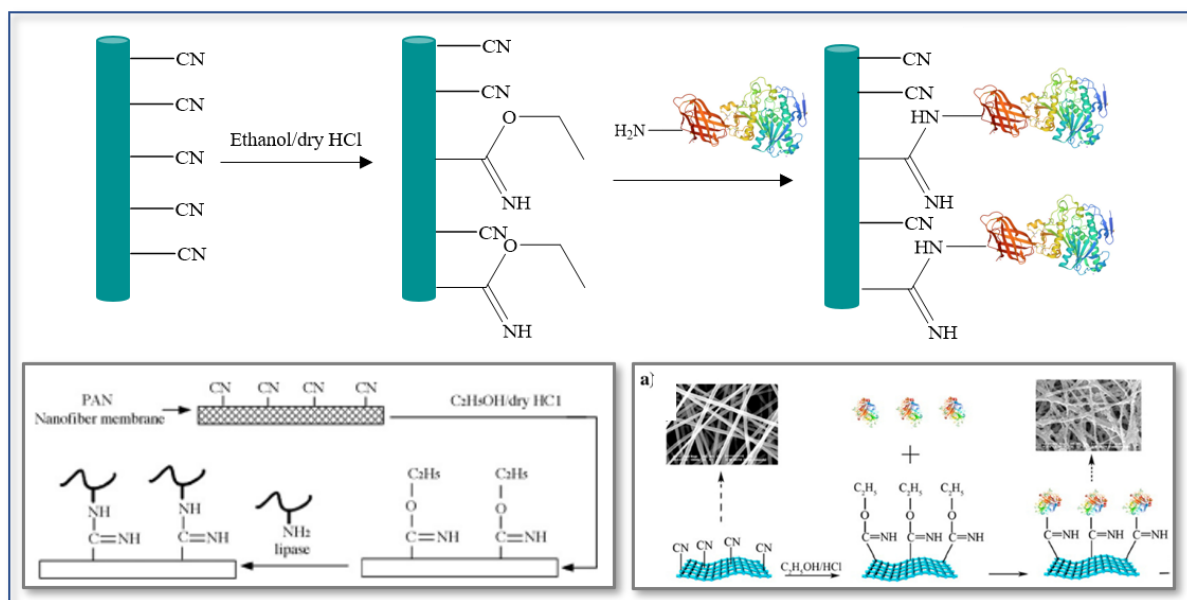
349
 350 *Table 6. Examples of protein grafting on polymeric supports after plasma treatment*

Polymer support	Protein	Ref
Silicone rubber grafted with (meth)acrylic acid	Human fibronectin	(Völcker et al., 2001)
PVDF films	Protein A, Triglycine	(Vasile et al., 2011a)
Electrospun PCL	Gelatin Growth factor	(Ma et al., 2005) (Guex et al., 2014)
Electrospun PHB	Collagen	(Heidari-Keshel et al., 2016)
PLLA films	Papain	(Teske et al., 2020)
Electrospun poly(ether sulfone)	Antigen, antibody Collagen	(Mahmoudifard et al., 2016) (Khademi et al., 2017)
PMMA or PA6 or PP films	IgC antibodies	(Wieland et al., 2020)

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Other activation procedures were also described in the literature. For instance, the esters pendant groups of poly(γ -methyl L-glutamate) beads were transformed into azido groups before reacting with papain (Hayashi et al., 1992). The anhydride groups of poly(styrene-*co*-maleic anhydride) fibers, obtained by electro-spinning, could react with the amino groups of different kinds of enzymes, α -chymotrypsin (Kim et al., 2005), lipase from *Mucor javanicus* (Nair et al., 2007) or carbonic anhydrase (Jun et al., 2020).

In the case of PAN support, a specific route was developed to graft proteins. PAN was reacted with HCl/EtOH to transform the cyano groups into imidoesters that can further react with the amino group of proteins (Handa et al., 1982, n.d.; Li et al., 2011, 2007; Li and Wu, 2009). Thus, glucoamylase from *Rhizopus niveus* (Handa et al., 1982), α -amylase from *Bacillus subtilis* (Handa et al., n.d.) and lipases from *Candida rugosa* (Li et al., 2007; Li and Wu, 2009) or *Pseudomonas cepacia* (Li et al., 2011) were grafted onto PAN beads or fibers.



365
366

Figure 7. Grafting of proteins onto PAN chains. Illustrations from (Li et al., 2007) (left) and (Smith et al., 2020a) (right).

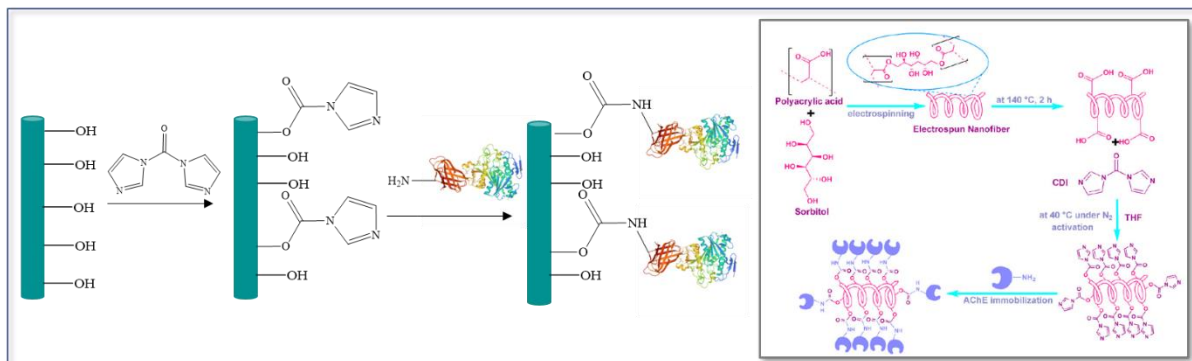
367

3.4 Hydroxyl group

368 The last functional groups which were used to graft protein onto polymeric surfaces are hydroxyl groups.
369 As these latter are not able to react directly with the amino groups of the proteins, they have first to be
370 activated through reaction with 1,1'-carbonyldiimidazole (CDI) (Baştürk et al., 2013; Çakıroğlu et al.,
371 2018; Oktay et al., 2015b; Xu et al., 2015a, 2015b). This treatment is mainly applied to PVA containing
372 surfaces. Thus, α -amylase from porcine pancreas (Baştürk et al., 2013) or Horseradish peroxidase (Xu
373 et al., 2015a) could be grafted onto electrospun PVA/PAA fibers or PVA/PAA/SiO₂ fibers respectively.
374 Horseradish peroxidase was also grafted onto electrospun PVA/NCC (nano-crystalline cellulose) or

375 Chitosan/NCC fibers (Xu et al., 2015b), Collagen was grafted onto electrospun crosslinked PVA fibers
376 (Oktay et al., 2015b). In a last example, acetylcholinesterase from *Electrophorus electricus* was grafted
377 onto electrospun PVA/sorbitol fibers (Çakıroğlu et al., 2018).

378



379

380 Figure 8. Grafting of proteins through hydroxyl groups activated by CDI. Illustration from (Çakıroğlu et al., 2018).

381 3.5 Other activations

382 Another method of activation, applied mainly for agarose supports, is the use of BrCN, which is a
383 method developed more than 50 years ago (Axén et al., 1967). Thanks to this method, various proteins
384 like α -chymotrypsin (Schnapp and Shalitin, 1976), catechol deoxygenase (Smith et al., 1990; Smith and
385 Ratledge, 1989), papain (Homaei et al., 2010) were successfully grafted. It was also coupled with the
386 EDC/NHS method to link papain or lipase onto agarose support (Kilara et al., 1977). Nevertheless,
387 nowadays, this method is less employed in the literature.

388

389 In few examples, γ -rays was used to activate polymeric surfaces (Beddows and Guthrie, 1980; Flores-
390 Rojas et al., 2018; Kumakura and Kaetsu, 1984). For instance, acrylic acid was polymerized from
391 polyethylene chains through irradiation with ^{60}Co source. BSA was then covalently bonded via the
392 activation of the carboxylic groups with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)
393 (Beddows and Guthrie, 1980). In the same vein, glycerol methacrylate was grafted onto silicone rubber
394 surfaces. It was then treated with sodium periodate to allow the grafting of lysozyme (Flores-Rojas et
395 al., 2018). In another study, papain was directly grafted onto PHEA or PHEMA during HEA or HEMA
396 polymerization initiated by irradiation with ^{60}Co source (Kumakura and Kaetsu, 1984).

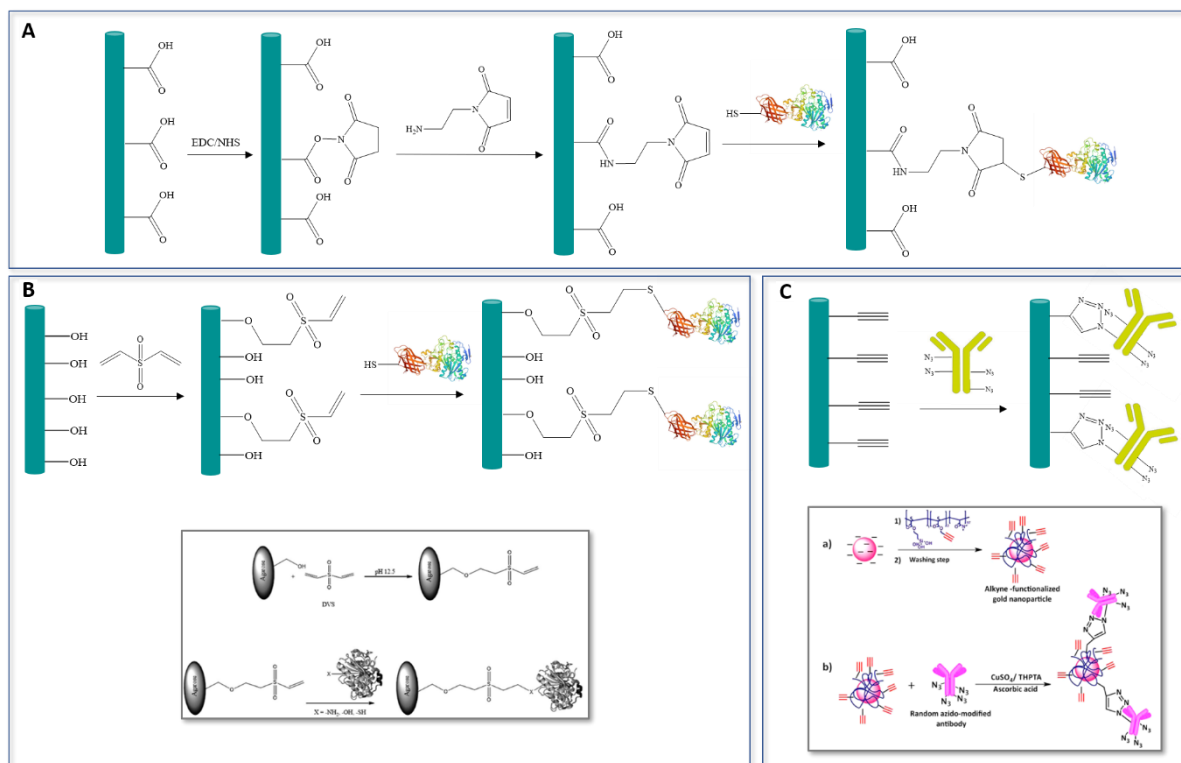
397

398 3.6 “Click-chemistry” and/or other “coupling” route

399 Instead of using amino groups of the proteins, it is also possible to use the thiol groups if cysteine amino
400 acids are present in the protein sequence. Thiol-maleimide coupling reactions are thus performed. To
401 this end, maleimido groups were first introduced on the polymeric surface. Through this synthetic route,
402 antimicrobial peptides were grafted onto silk fibers (Song et al., 2016). Divinyl sulfone was also

403 employed as a coupling agent between agarose supports and lipases from *Pseudomonas fluorescens*,
404 *Rhizomucor miehei*, *Thermomyces lanuginosus* or *Candida antarctica* (Jose C. S. dos Santos et al., 2015;
405 Jose C.S. dos Santos et al., 2015).

406 Finally, in few cases, proteins were modified in order to be grafted onto polymeric surfaces. For instance,
407 a growth factor protein was functionalized with an aldehyde group to achieve its coupling onto modified
408 hydrogels through oxime ligation (Batalov et al., 2021). There are also few examples of antibodies that
409 were modified with azido groups to be grafted onto alkyne-containing surfaces through copper-catalyzed
410 click chemistry (Finetti et al., 2016; Shi et al., 2008).



411
412 *Figure 9. Grafting of proteins through click chemistries. Illustrations from (Jose C. S. dos Santos et al., 2015) (B) and (Shi et al.,*
413 *2008) (C).*

414 Among all the strategies developed during the last decades, it appears nowadays that the multi-point
415 attachment of the enzymes onto their support is preferable as it allows a better stability in time but also
416 against heat or organic co-solvent. Different reviews have been published on this topic and the reader is
417 encouraged to consult them for a thorough evaluation ((Barbosa et al., 2013; Guisan et al., 2022;
418 Rodrigues et al., 2021). The most followed routes to achieve the multi-point attachment of enzymes is
419 the reaction of the amino groups of the enzyme with either epoxides or aldehydes (glyoxyl,
420 glutaraldehyde). To do so, the reaction must be performed in basic solution in order for the lysine
421 residues to be reactive ($pK_a \sim 10.5$). If the reaction is performed at neutral pH, only the amine at the
422 terminal of the enzyme will react, preventing thus the multi-point attachment. Main pros and cons are
423 summarized in table 7.

424

425 *Table 7. Pros and cons of the different methods developed for non-specific covalent grafting of proteins onto polymeric*
 426 *supports.*

Non specific grafting method	Pros	Cons
Through epoxides	Multi-point attachment	Multi-point attachment in basic solution
Through aldehydes	Multi-point attachment	Multi-point attachment in basic solution
Through carboxylic acid		Production of carboxylic acid onto the support needed. Activation of the carboxylic acid functions
Through hydroxyl		Activation of the hydroxyl functions
Click-chemistry		Modification of either the surface or the protein

427 **4 Regio- or chemoselective grafting**

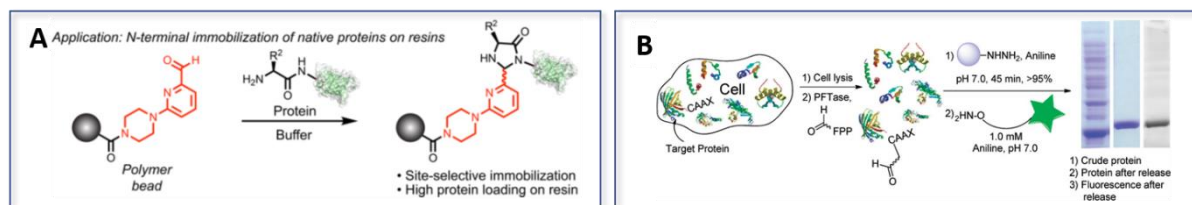
428 A major concern with either non-specific adsorption or random covalent grafting as discussed above is
 429 the potential loss of function of the immobilized protein. Although stable immobilization can be
 430 obtained by the covalent methods described above, they are typically non selective leading to random
 431 orientation of the protein on the surface with attenuated properties as recognized early by Rao et al. and
 432 Cha et al (Cha et al., 2005; Rao et al., 1998). To ensure uniform and optimal properties, the anchoring
 433 point should be precisely controlled so as to not interfere with the protein's intended function. Although
 434 most of the literature deals with immobilization onto glass slides for the preparation of microarrays,
 435 some interesting strategies were developed for polymer supports. Here we will show examples of
 436 selective immobilizations grouped according to the location of the anchor point on the biomolecule : N-
 437 terminus, C-terminus or internal amino acid, and finally on post-translational modifications such as
 438 carbohydrate chains.

439 4.1 Immobilization via N-terminus

440 Proteins' N termini are an attractive site for anchoring them onto solid substrate as they are accessible
 441 on most expressed proteins and are in most cases reactive as a primary amine. One can in principle
 442 exploit the reduced basicity of the N-terminal amine compared to lysine side chains, whose reactivity as
 443 nucleophiles can be reduced by precise pH control (Baker et al., 2006; Chan et al., 2012), but the
 444 abundance of lysine residues decreases the selectivity in most cases.

445 Many strategies have been proposed recently where the protein's N-terminus is first selectively
 446 modified, as reviewed recently (Jiang et al., 2022). Some of these modifications have been exploited to
 447 graft proteins in a chemo- and site-selective manner. The group of M. Francis is particularly active in
 448 this field. After demonstrating a highly selective method for N-term modification by carbonyl-bearing
 449 small molecules (MacDonald et al., 2015), they exploited this chemistry, with the most efficient modifier
 450 (2-pyridinecarboxyaldehyde) to graft functional proteins such as Rnase or a Green Fluorescent Protein
 451 (GFP) chimera via a N-terminal imidazolidinone linker, figure 10 A (Koo et al., 2019). Additionally,

452 this immobilization could be reversed and the protein liberated by addition of hydroxylamine. A
453 combination strategy is also possible since the authors showed that biotin could be efficiently attached
454 to the N-terminus of a protein, thereby allowing the highly-efficient binding with surface-coated avidin
455 (MacDonald et al., 2015).
456



457
458 *Figure 10. A) Application of the selective strategy to graft a protein onto polymer beads (Koo et al., 2019). B) Grafting of a*
459 *protein to a hydrazine-functionalized bead using the CAAX motif, an aldehyde-bearing farnesyl group, and farnesyl transferase*
460 *(Rashidian et al., 2012).*

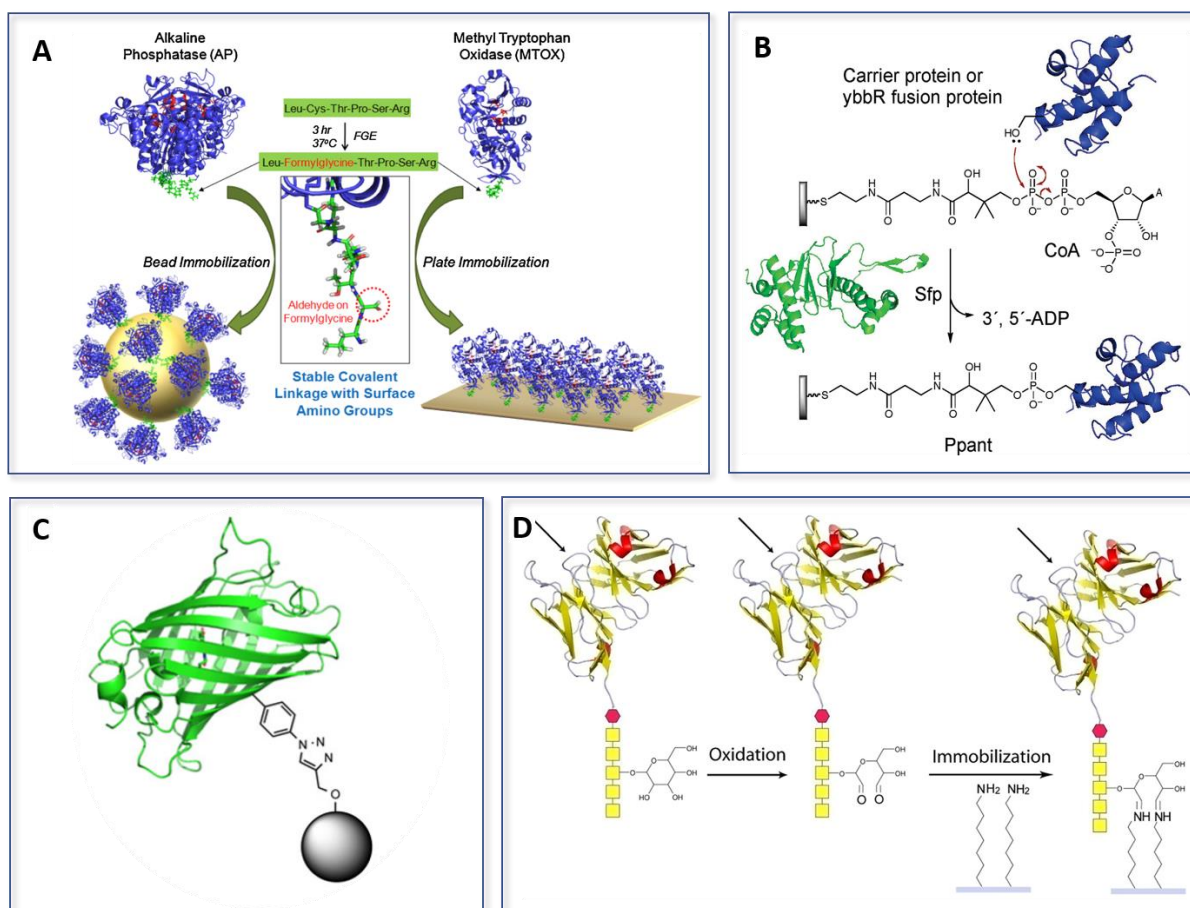
461 4.2 Immobilization via C-terminus

462 Selective immobilization of proteins onto solid support via the C-terminus can be achieved using the
463 native protein, or a modified version of the protein, obtained after either chemical or biochemical
464 modification. In 2017, Zhang et al. (Zhang et al., 2017) published a report on the site-selective
465 immobilization of recombinant protein A modified at the C-terminus with a cysteine residue, onto
466 agarose beads functionalized with maleimide moieties. This improved antibody capture capacity of the
467 beads when compared to the randomly attached protein A, with a two-fold increase for IgG, IgA and
468 IgM.

469 A number of groups have exploited the enzyme Protein Farnesyl Transferase (PFTase) to modify
470 proteins containing the CaaX motif (where a is an aliphatic AA and X is any AA depending on the
471 specific PFTase) on the C-terminus and graft a farnesyl motif. The latter can bear a reactive carbonyl
472 function, such as an aldehyde, and be used to anchor the protein onto a hydrazine-bearing surface, such
473 as described by the group of DiStefano (Rashidian et al., 2012) (figure 10 B). Their method is versatile
474 since the CaaX recognition box can be installed on virtually any protein's C-terminus, but this is also a
475 disadvantage since a recombinant protein is needed.

476 4.3 Immobilization via unnatural internal amino acids

477 Unnatural AA can be introduced in protein sequences to confer chemically orthogonal reactivities to
478 any other functional moieties. For example, Raliski et al. introduced *para*-azido phenylalanine in the
479 sequence of GFP to make it amenable to dipolar cycloaddition (Raliski et al., 2014). They applied this
480 functionalization strategy to regio- and chemo-selectively graft GFP onto solid supports such as
481 polystyrene and sepharose beads, with excellent retention of the GFP native fluorescence.



482
 483 *Figure 11. A) In Situ generation of the reactive protein (Cho and Jaworski, 2014). B) Enzyme-catalysed anchoring of protein on*
 484 *PEGA surfaces (Wong et al., 2008). C) Covalent anchoring of GFP via huisgen cyclisation on an unnatural p-azido phenylalanine*
 485 *(Raliski et al., 2014). D) Covalent attachment of proteins via oxidised glycosyl units and Schiff base formation (Hu et al., 2013).*

486 An astute method was described by Cho and Jaworski where the reactive version of a protein is generated
 487 in situ (Cho and Jaworski, 2014). The enzymes alkaline phosphatase or methyl tryptophan oxidase
 488 operate the site-selective oxidation of a cysteine residue into the aldehyde-bearing formylglycine, which
 489 can then be used to link with amines on the surface.

490 In their 2008 paper the group of Mickelfield (Wong et al., 2008) described the enzyme-catalyzed
 491 anchoring of a variety of proteins onto PEGA surfaces. They take advantage of the specificity of Sfp
 492 enzyme (Phosphopantetheinyl Transferase) to selectively graft proteins that either bear a specific ybbR
 493 helical peptide domain, or are fused to carrier protein. The surfaces to which they are attached have to
 494 be derivatized to bear Co-enzyme A.

495 In their 2015 paper, Yang et al. (Yang et al., 2015) described a method to tether TGF- β 1, whose
 496 biochemical activity depends on whether it is free or attached, to pegylated surfaces. To achieve this,
 497 they used a variation of the Staudinger ligation, where surface-bound azido groups are reacted with
 498 Lysine-bound phosphine moieties. With this chemoselective anchoring strategy, the authors were able
 499 to prepare biofunctional beads that successfully converted CD4⁺ CD62L^{hi} T cells into functional
 500 regulatory T cells.

501 4.4 Immobilization via saccharide units on proteins and antibodies

502 Glycosyl units can be found for example at the Fc region of antibodies, and provide chemical diversity
503 with respect to the main backbone, which can be exploited for site-selective immobilization. Many
504 reactions are available to create bioconjugates or to graft biomolecules by taking advantage of pendant
505 glycosyl units. The most common reactions are probably the formation of hydrazone or oximes using
506 oxidized glycosyl units, and the formation of boronic esters.

507 4.4.1 On oxidized glycosyl units

508 Shmanai et al. devised a rather elaborate system to chemoselectively graft antibodies onto polystyrene
509 substrate, later to be used in immunoassays (Shmanai et al., 2001). For this, they derivatized
510 poly(meth)acrylic acid with hydrazide functions, which they used to modify the surface of millimetric
511 polystyrene balls. Using the TNBS (2,4,6-trinitrobenzene sulfonate) test, they were able to determine
512 the surface density of hydrazide functions on the modified polymer balls. With this selective grafting
513 strategy, the authors were able to study the influence of spacer length on the capture of mildly oxidized
514 antibodies via an immunosorbent assay.

515 Another example was given by Yuan. First, stainless steel substrates were coated with ethylene vinyl
516 acetate, then treated with O₂ plasma, and silanized with aminopropyl triethoxy silane (APTES) to create
517 amine groups (labeled SCA-SS). Amines were then coupled with the oxidized carbohydrates and
518 successful binding was assessed via cell uptake by the anti-CD34 antibody (Yuan et al., 2011).

519 In a 2013 paper, the authors (Hu et al., 2013) prepared antibody-derived fragments on which the glycan
520 tag, conveniently located away from the binding pocket, was oxidized for covalent attachment with
521 amine functionalized beads (figure 12 D). Importantly, the grafting could take place at salt
522 concentrations that otherwise preclude nonspecific adsorption. Moreover, the fragments attached in this
523 oriented fashion exhibited 4-fold superior activity than the corresponding ionically adsorbed ones.

524

525 4.4.2 Using boronic esters from glyco units and boronic acids

526 In the field of molecular imprinting, particular attention has been given to the orientation of the proteins
527 to be used as templates. These efforts produced preferably oriented homogeneous protein constructs
528 with decreased changes in protein conformation during imprinting and maximal retention of protein
529 functionality. The field was reviewed recently by Kalecki et al. (Kalecki et al., 2020).

530 The surface grafting techniques have been employed including some non-covalent ones such as metal
531 coordination or aptamer binding. Covalent bonding using the specific reaction between boronic acids
532 and sugar units on proteins was described on a polymer coated sample by Wang et al. (Wang et al.,
533 2014). In this article, the authors designed a clever copolymer resulting from the copolymerisation of
534 dopamine and an aryl-boronic acid monomer. Good selectivity of the molecular imprinted polymer was
535 demonstrated with Horse Radish Peroxidase, and they could take advantage of the reversible character

536 of the boronic ester formation.

537 4.5 On exogenous groups

538 An elegant example of grafting a proteinaceous biomolecule onto a silicone surface was given by Pinese
539 et al. (Pinese et al., 2016). In this article, the authors used a silicon-containing group to graft site-
540 specifically onto a silicone surface, thereby preserving the antibacterial character of the peptide and
541 producing a material that could be used to make antiseptic catheter with superior activity compared to
542 silver-containing ones. For this, the peptide had to be derivatized prior to immobilization.

543
544 These selective grafting methods are of different difficulties, using the protein in its natural form or
545 implying chemical modification, up to specific recombinant protein synthesis. Incorporation of
546 unnatural functional groups presents the asset of excellent selectivity at the expense of enhanced
547 difficulty of the process. It is noteworthy that beyond these specific pros and cons (table 8), the problem
548 of single- or multiple point attachment also remains, as in part 3.

549
550 *Table 8. Pros and cons of the different methods developed for selective covalent grafting of proteins onto polymeric supports.*

grafting method	Pros	Cons
N-terminus	Easy access	Competition with internal Lys
C-terminus	Easy access	Competition with internal Glu and Asp. Need of chemical modification or production of a recombinant protein (e.g. CAAX motif)
unnatural internal amino acids	No competition with natural amino acids	Need of a recombinant protein
via saccharide units	Highly selective	Not possible for all proteins/expression systems. Variable amongst expression hosts.
Exogenous groups	Highly selective	Need of chemical modification

551 **5 Characterizations of the grafting on polymers**

552 Characterization after grafting is a key step to validate both the grafting itself on the surface and also
553 the behavior of the grafted enzymes. Different methods are available depending on what needs to be
554 proved (presence of proteins, orientation, structure of the grafted element, enzymatic activity, specificity
555 of grafting...). The main difficulties of the characterization remain in the possibly low amount of
556 material grafted and the low stability of proteins, often limiting the number of techniques available and
557 resulting in destructive analysis methods. The work becomes even more difficult when the used surface
558 is a polymer, because some of these techniques are not possible any more. In the subsequent paragraphs,
559 the focus is set on describing the techniques available for each question raised linked to the protein
560 grafting. When possible, examples based on grafting on polymers are provided.

561 5.1 Revealing the presence of proteins on the surface

562 Most of the methods described in this chapter could be used to show the presence of proteins on the
563 surface. But some methods are more efficient and simpler to use in order to make this characterization.
564 Contact Angle measurement using sessile drop method on a tensiometer is a very simple method that
565 does not require any prior preparation of the sample and is functional on any smooth surface. The method
566 requires at least 10 measurements to avoid high variance of the results. Hydrophilicity of the surface is
567 expected to be modified with immobilization of proteins on the surface. It is possible to differentiate the
568 proteins immobilized with this method because the hydrophilicity depends on the groups contained in
569 the protein (Motsa et al., 2022). An important limitation associated to this method is the necessity to
570 choose the good couple surface/protein. If the surface has a hydrophilicity close to the one of proteins
571 such as poly(L-lactic acid) (PLLA) (Kasálková et al., 2014), it becomes impossible to prove surface
572 modification with contact angle measurement. This technic can also be used to show the surface
573 modification prior to protein grafting like in the case of the silanization of a surface (Libertino et al.,
574 2008). Because contact angle strongly depends on the surface rugosity, the presence of proteins on the
575 surface can only be assessed by comparing the exact same surface before and after grafting.

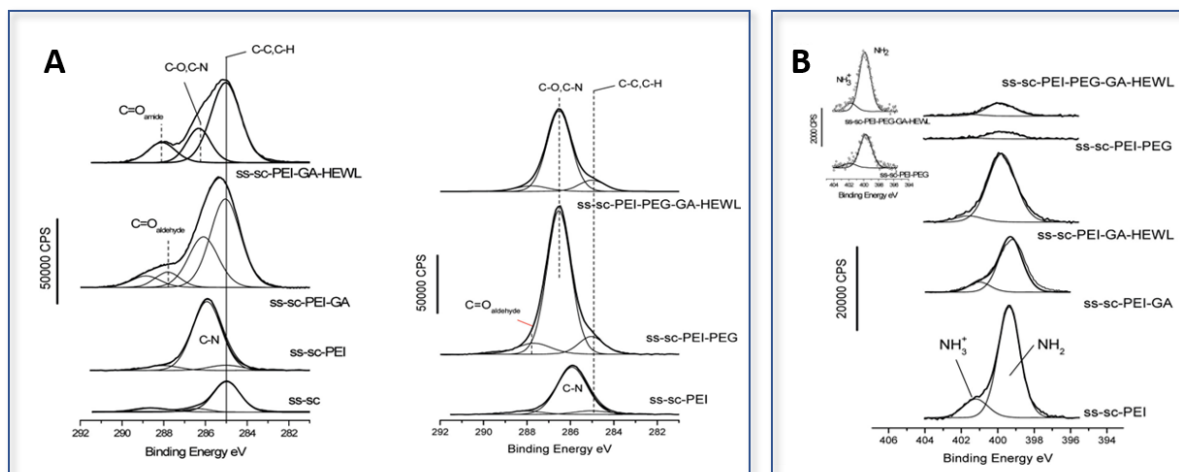
576 Electronic microscopy methods such as TEM, SEM and FESEM (Transmission Electron Microscopy,
577 Scanning Electron Microscopy, Field Emission Scanning Electron Microscopy) can be used to detect
578 the presence of proteins on some surfaces. Even if TEM and SEM are not precise enough to observe
579 single proteins, they can however be used to detect variations in the size of nanoparticles (Khosravi et
580 al., 2012; Ricco et al., 2014). FESEM is precise enough to detect the presence of proteins so it can be
581 used directly as an imaging method (Kamat et al., 2013).

582 Thermal Gravimetric Analysis (TGA) is also a pretty simple method allowing the detection of proteins
583 on a surface thanks to the evaporation of the water contained in the proteins at 100°C. This
584 characterization needs dry surfaces with no organic components which makes it of low interest on
585 polymers. TGA is mostly used on MOF (Gascón Pérez et al., 2018), silica surfaces such as monoliths
586 (Biggelaar et al., 2019) or nanotubes (Tully et al., 2016).

587 Fluorescence microscopy is also a straight forward method to identify the presence of proteins on a
588 surface. The biggest limitation is that it requires fluorescent proteins. For example, the presence of
589 triglycine (TG) was detected on a poly(vinylidene difluoride) (PVDF) surface using fluorescence (Vasile
590 et al., 2011b). In this case, fluorescence is also used to prove that grafting is a much more efficient
591 immobilization method than adsorption. It is also possible to graft antibodies to the immobilized protein
592 to detect it with fluorescence spectroscopy, Sum Frequency Generation spectroscopy (SFG), X-Ray
593 Photoelectron Spectroscopy (XPS), Atomic Force Microscopy (AFM), Surface Plasmon Resonance
594 (SPR) but it implies complicated steps for such a simple characterization.

595 XPS is one of the major characterization technics used on surfaces with grafted proteins. After

596 comparison of the spectra before/after immobilization, it is possible to observe the modification of the
 597 composition of the surface. For example, when enzymes are grafted on a stainless-steel surface
 598 functionalized with polyethyleneimine and glutaraldehyde, it is possible to detect every step by
 599 following the signal associated with specific types of bonds such as C-N, C=O, C-O, C-N (Caro et al.,
 600 2009).



601
 602 *Figure 12. High Resolution XPS spectra C 1s A) and N 1s B) of coated stainless-steel surfaces. Spectra are used to identify and*
 603 *quantify the type of bonds represented on the surface in order to prove they efficiency of coating (Caro et al., 2009).*

604 AFM is the second most common characterization method on protein immobilization on solid surfaces.
 605 The method requires a flat surface to work on. Once again, AFM can do much more than just detect the
 606 presence of proteins, but the tapping mode can be used to have a good definition and be able to see the
 607 grafting of fibronectin on a glass surface (Vallières et al., 2007).

608 5.2 Checking if the protein is grafted or adsorbed, assessing its orientation

609 It is important to differentiate adsorption from grafting because it has a massive influence on enzyme
 610 mobility and orientation. Sometimes, rinsing the surface with buffer is not enough to remove adsorption.
 611 In the case of hydrophobin (HFBI), when the adsorption is completed, a monolayer is formed and it
 612 makes HFBI resistant to desorption with buffer (Takatsuji et al., 2013). Routine method to rule out
 613 simple adsorption remains extensive washing with “extreme” solutions, such as surfactants followed by
 614 re-examination of the presence of proteins by the techniques mentioned in the last paragraph. However,
 615 in some cases, specific techniques can provide further evidence of the grafting.

616 Surface Plasmon Resonance (SPR) is often used to answer this problematic on surfaces such as
 617 chondroitin sulfate functionalized surfaces (Riahi et al., 2017) or even in association with Time of Flight
 618 Secondary Ion Mass Spectroscopy (ToF-SIMS) analysis on gold surfaces functionalized with NHS (Kim
 619 et al., 2007). It is however useless on polymer surfaces because the method needs surface conductivity
 620 to detect biological material.

621 ToF-SIMS can also be used to differentiate adsorption and grafting of proteins on the surface. The

622 surface can be treated with trealose subsequently to protein grafting and then be analyzed with ToF-
623 SIMS and SPR with or without treatment. When proteins are oriented, both results are correlated. When
624 proteins are randomly immobilized, the presence of trialose increases correlation between ToF-SIMS
625 and SPR showing a different behavior depending on protein orientation. In the case of specific
626 immobilization, proteins will be oriented on the surface which is not the case for adsorption (Kim et al.,
627 2007). It is also possible to show specificity of grafting by analyzing a patterned surface with affinity
628 for different proteins. When in presence of the two different types of proteins, the surface will be grafted
629 with only one protein depending of the functionalization of the surface of contact (Dubey et al., 2009).

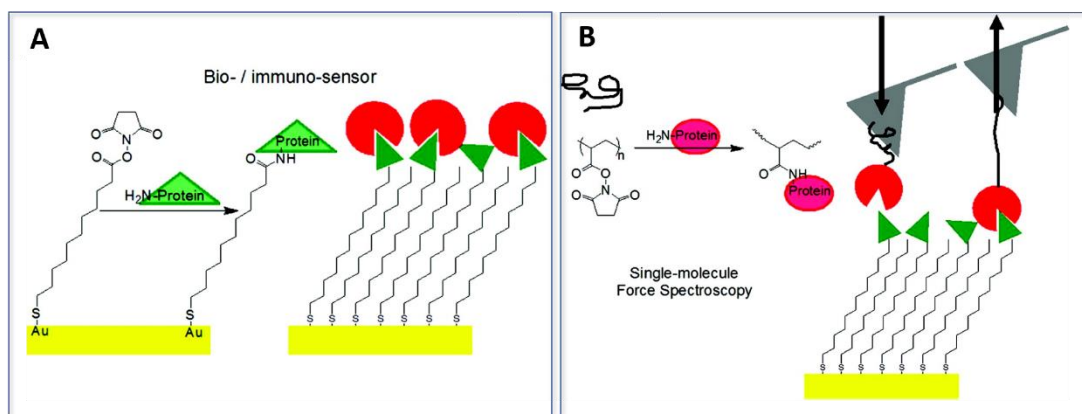
630 5.3 Describing the structure of the immobilized protein

631 Modifications of the structure of the protein/enzyme can completely modify their compatibility/activity.
632 Usually, the only concern is about the functionality of the object so it could be possible to test it to get
633 sufficient result (measurement of enzymatic activity on a surface for example). But sometimes, it can
634 be important to get more information on the structure in order to be sure that no modifications happened.
635 Circular Dichroism (CD) can be used to determine the structure of proteins adsorbed using a comparison
636 of the results obtained on different kinds of surfaces such as quartz and Teflon for example (Vermeer
637 and Norde, 2000). CD can also be used to observe modifications of the secondary and tertiary structure
638 of enzymes such as horseradish peroxidase after it has been crosslinked with dendrimers to create
639 nanoparticles (Khosravi et al., 2012).
640 Microcalorimetry can also be very specifically used to determine the effect of grafting on the structure
641 of the proteins. It is for example possible to compare the effect on the structure of a single or multipoint
642 grafting using glutaraldehyde on silica glass (Battistel and Rialdi, 2006).

643 5.4 Describing grafting density

644 It is often key to get knowledge of the grafting density in order to determine enzyme specific activity
645 for example. It can also be important with proteins when they are used as an anchor for subsidiary
646 reactions.
647 XPS can be used to solve this kind of problematics. As seen before, the method is very powerful and
648 can be used to follow the different steps of functionalization and grafting (Abbas et al., 2009) on different
649 types of surfaces such as metals functionalized with poly(ethylene glycol) (PEG) (Caro et al., 2009) or
650 even on electroactive polymers (Loh et al., 1996). By studying the intensity of bands related to certain
651 types of bonds, it becomes possible to quantify the surface density. To get solid results, it is mandatory
652 to analyze different types of bonds because precision can vary in large proportions. In the case of
653 enzymes, it can be very interesting to compare the results with those obtained with enzymatic activity
654 measurements (Ghasemi et al., 2011; Zheng et al., 2015). This type of comparison shows how the use
655 of different types of bonds can deeply modify the results obtained.

656 AFM can also be an interesting tool to describe grafting density on the surface. It is therefore used to
 657 detect the presence of aggregates on the surface (Abbas et al., 2009) resulting in the presence of objects
 658 bigger than the size of a single protein. Even if it is mostly used to study the global evolution of surface
 659 roughness after grafting (Kasálková et al., 2014), AFM can be used to detect single molecules on
 660 extremely flat surfaces. Using single molecule force spectroscopy in tapping mode, it is possible to scan
 661 a surface molecule by molecule in order to precisely study a surface after grafting of proteins (Cecchet
 662 et al., 2007).



663
 664 *Figure 13. Schematic Representation of A) the Covalent Grafting of Proteins onto the Surface of the Biological Device and of*
 665 *B) a Biological Recognition Event Investigated by Single-Molecule Force Spectroscopy Experiment from (Cecchet et al., 2007).*

666 FRET (Förster Resonance Energy Transfer) could also be used but since it is not a simple method to set
 667 up and it works only on very specific proteins, the method is not usually used to characterize grafting
 668 but more often to study interactions between proteins in biological materials (Rijn and Böker, 2011).

669 5.5 Describing the distribution of the proteins on the surface

670 When grafting density does not reach the saturation of the surface, it can be interesting to study the
 671 distribution of the protein/enzymes on the surface to verify if there are aggregates or if the distribution
 672 is homogeneous.

673 As described above, it is possible to use FRET on very specific types of proteins to detect it on the
 674 surface. It could be used to detect the presence of some proteins on a tumor after specific treatments in
 675 order to target it efficiently (Resnier et al., 2019).

676 SECM (Scanning Electrochemical Microscopy) is also a method that can be used to localize enzymes
 677 on a surface. It can be used on supports such as PVDF and poly(ethyleneterephthalate) (PET) but it will
 678 require a prior staining of the surface with copper (Carano et al., 2007). The method allows a mapping
 679 of adsorbed proteins on the surface without requirement of enzymatic activity or label affinity.

680 5.6 Describing enzymatic activity of the grafted surface

681 Enzymatic specific activity can be modified in the grafting process because of a modification of the
 682 secondary and tertiary structure of the protein but also because of a lack of mobility. It is therefore

683 paramount to study the activity in order to prove the grafting efficiency and the quality of the grafting
684 process.

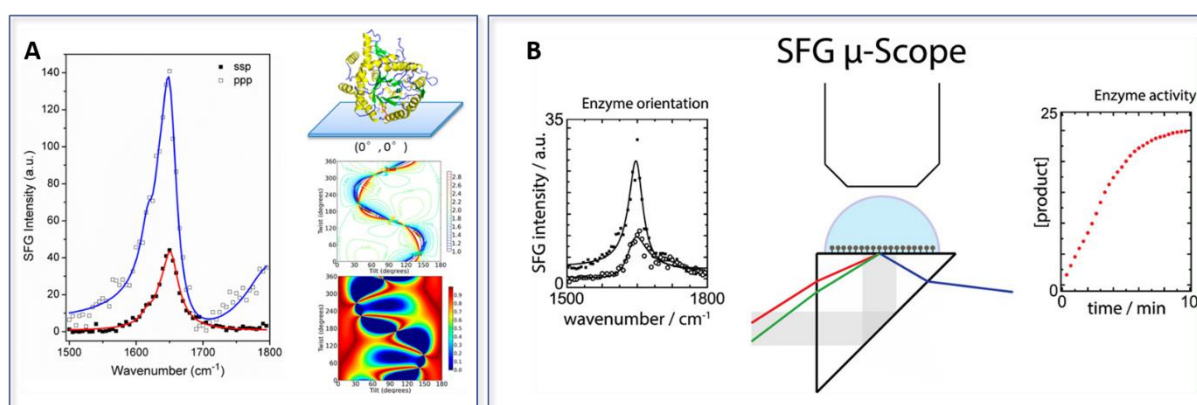
685 The most common way to detect enzymatic activity is to follow the substrate degradation by UV-Vis
686 spectroscopy. The analysis can easily be set up if there is a substrate available such as 4-nitrophenyl- β -
687 D-xylotrioside which will be hydrolyzed and release *para*-nitrophenol, absorbing at 401 nm (Montanier
688 et al., 2019). Sometimes, it is not possible to study enzymatic activity using such methods.

689 It is therefore interesting to use other systems such as SECM. The method can show enzymatic activity
690 but is not efficient in order to quantify it. However, it has the significant advantage to localize enzymatic
691 activity on the surface. It is the case for a biosensor non-homogeneously grafted with HRP on the
692 surface; by making a comparison between XPS and SECM results, it was possible to observe enzymatic
693 activity on determined areas of the surface (Gidle et al., 2003).

694 5.7 Transversal techniques

695 Some methods can give multiple information with a single analysis but are not applicable to every type
696 of surfaces and grafting.

697 SFG coupled with ATR-FTIR (Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy)
698 can be used to determine the orientation of grafted enzymes on self-assembly monolayers. Orientation
699 was controlled using thiol-maleimide reaction. Two thiols were introduced in enzyme sequence in
700 specific areas. Results obtained with these two different enzyme populations were compared to prove
701 the control of enzyme orientation on the surface (Liu et al., 2013; Shen et al., 2014). A SFG microscope
702 was also developed allowing simultaneous SFG, ATR-FTIR and enzymatic activity measurements
703 (Jasensky et al., 2018). By gathering all results, data are obtained on enzymatic activity, enzyme
704 orientation, orientation, structure and stability.



705
706 *Figure 14. A) SFG spectra collected from β -Gal-V152C immobilized at the Mal-EG4 SAM-solution interface. (A right side) 1-*
707 *Orientation of β -Gal with (tilt angle, twist angle) = (0°, 0°). 2-Dependence of the SFG χ_{zzz}/χ_{xxz} ratio on the tilt and twist angles*
708 *of β -Gal-V152C calculated using the newly developed computer package.⁵⁶ 3-Possible orientation angle regions deduced on*
709 *the basis of the experimentally measured χ_{zzz}/χ_{xxz} ratio of β -Gal-V152C. Colors indicate the quality of the match. Adapted*
710 *from (Liu et al., 2013) B) Graphical abstract showing a scheme of the SFG microscope used in the experiment (middle) to obtain*
711 *informations on enzyme orientation (left) and enzyme activity (right). Adapted from (Chen et al., 2018)*

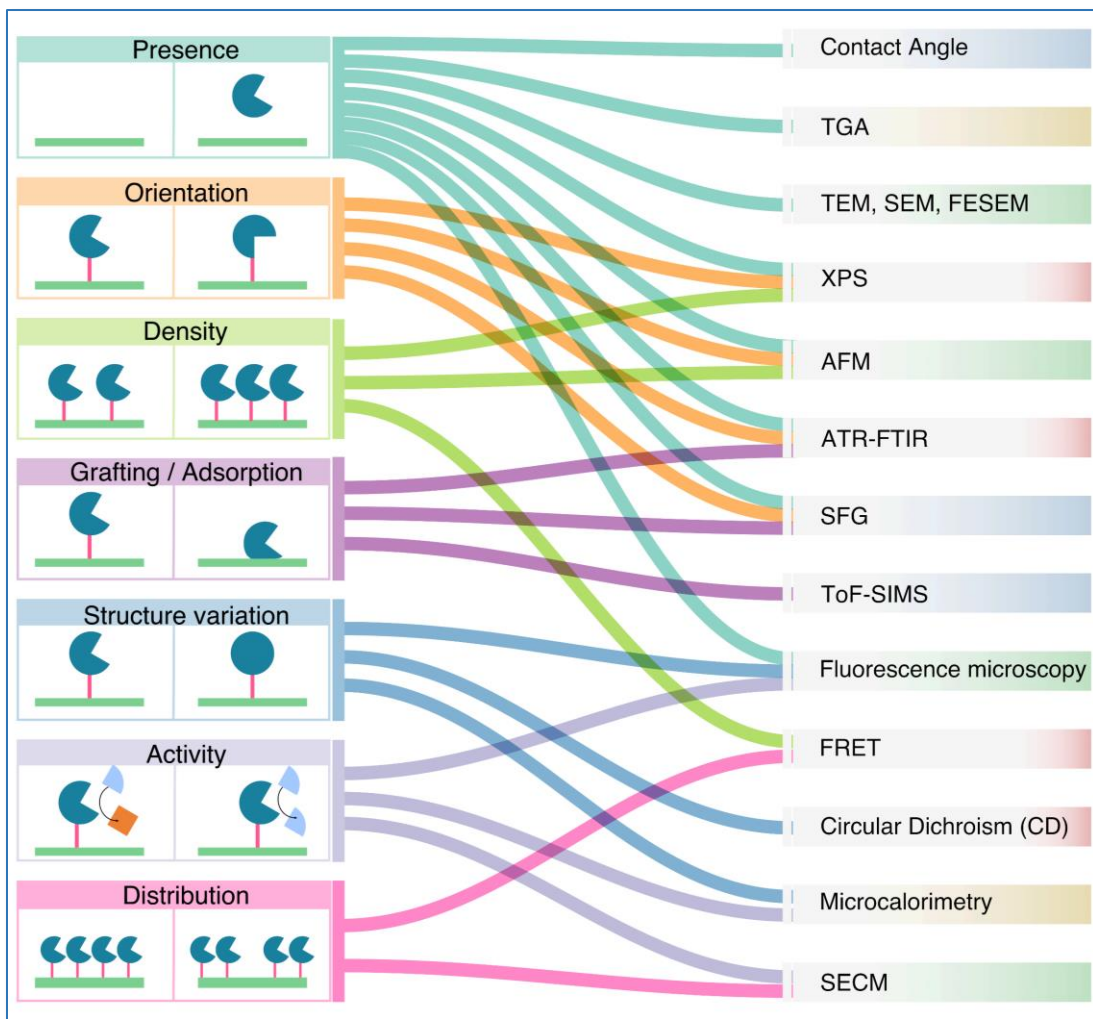
712 Enzymatic activity measurements can also be an interesting way to characterize the surface after
713 grafting. The method gives access to multiple information such as the quantification of enzymes on the
714 surface but also check their stability over time. It even can be used to obtain an idea of the grafting
715 orientation (homogeneous or not) and also check if enzymatic activity is impacted by the grafting
716 process. The measurement can be done following the evolution of the media due to substrate evolution.
717 It is usually performed using UV-Vis spectroscopy measurements because they are very simple to set
718 up. It is for example possible to follow, by absorbance measurements at 420 nm, the evolution of
719 concentration of H₂O₂ in solution due to peroxidase activity (Amounas et al., 2000). Other substrates
720 can be used with corresponding wavelength analysis for different types of enzymes such as lysozyme
721 (450 nm) or trypsin (253 nm) (Caro et al., 2010).

722 Last but not least, it is possible to characterize the surface with an indirect method by labelling the
723 proteins on the surface and analyzing the probe. The method can be applied to different techniques. It is
724 possible to characterize the grafting of antibodies on the surface using fluorescence by previously adding
725 fluorescent antigens that self-assemble with the antibodies (Grosjean et al., 2005). It is also possible to
726 measure zeta potential to follow Ig (Immunoglobulins) binding to a grafted ligand on the surface (Huan
727 and Shi, 2021).

728 The presence of antibody or different types of probes on the surface can also be detected using most of
729 the techniques described above.

730

731 Choices need to be made to characterize protein grafting on a surface. As shown above, there are no
732 perfect methods to complete characterization but there still are many options. The techniques used must
733 be adapted to the application and will be different almost every time, it is up to the experimenter to
734 figure out which methods fit the best with the application.



735

736 Figure 15. Sankey diagram of the analytical techniques used to answer biochemical questions. Analytical techniques are
 737 classified by colors with ● (Microscopy techniques), ● (Spectroscopy techniques), ● (Thermal analysis techniques), ● (Other
 738 types of techniques).

739

Table 9. Principle, advantages and disadvantages of each method described above

Method	Principle	Advantages	Drawbacks
Contact Angle	Characterization of the wettability of a solid surface by measuring the contact angle at the interface between a drop of liquid and a solid surface. It is a qualitative method.	-Simple access -No preparation needed -Not destructive	-Importance of repeated measurements
XPS	X-ray Photoelectron Spectroscopy consists in bombarding a surface with X-rays with a specific wavelength. From it, the retro-diffusion of core electrons will result. Each electron has a specific energy depending on the atom it comes from. XPS determines the atom composition on the surface with a maximum depth in the sample of 10 nm.	-Possibly -Quantitative method	-Need of expertise

AFM	Atomic Force Microscopy is used to determine the topography of the surface. It is based on a cantilever browsing or tapping the surface with its tip. The movement of the cantilever is detected thanks to a photodiode.	-Easy access -Large variety of analysis possible	-Caution needed for the interpretation of images -Expertise needed for non-basic methods
SFG	Sum Frequency Generation Spectroscopy is used to analyze surfaces and interfaces. It is based on two laser beams mixed at an interface generating a beam with a frequency equal to the sum of the two input lasers.	-Very simple sample preparation -Efficient on amorphous material -Non-Destructive -Efficient on monolayers	-Interpretation can be tricky
ATR-FTIR	Attenuated total reflectance-Fourier Transform Infrared Spectroscopy is used to measure the infrared absorption spectra of molecules. Different bonds between atoms will deliver different signals on the spectra.	-Simple access -Access to a chemical signature -Not destructive	-Low amounts of proteins will not be visible
Fluorescence spectroscopy	Fluorescence spectroscopy uses a beam of light to excite electrons and cause them to emit light. The signal goes through a filter and on a detector, which allows to detect the signal.	-Simple access -High sensitivity	-Need of fluorescent signal
TGA	Thermal Gravimetric Analysis consist in measuring variations of mass depending on time for a specific temperature or a temperature profile. The method shows the presence of enzymes on the surface but cannot prove the covalent grafting.	-Simple access -No preparation needed	-Destructive -Low amounts of proteins will not be visible

TEM, SEM FESEM	Transmission Electron Microscopy, Scanning Electron Microscopy, Field Emission Scanning Electron Microscopy are all based on electron microscopy. TEM consists in transmitting electrons through a sample imaging it using the resulting electrons. It can reach resolutions of 0.1 nm in the best cases. In SEM analysis, the sample is scanned using a focuses beam of electrons. It gives information on the topography of the sample but also on its compositions thanks to the back scattered electrons and characteristic X-rays. Resolution is between 1 and 20 nm. FESEM follows the same principle as SEM but it uses a single tungsten filament as electron source. This difference allows a better resolution (2-3 nm) and also a smaller penetration in the sample.	-Simple access -High sensitivity	-Preparation needed -Interpretation can be delicate in some cases
CD	Circular Dichroism is based on the differential absorption of left and right polarized light. Chiral molecules with optical activity absorb preferentially one of the two directions of the polarized light. CD with UV light can be used to determine the aspect of the secondary structure of proteins.	-Simple access -No preparation needed -Not destructive	-Low sensitivity
Micro-calorimetry	The method consists in measuring enthalpy. When chemical reactions occur on the surface, it induces changes in energy accompanied by heat release or absorption. Microcalorimetry can be used to observe fluctuations of energy following reactions catalyzed by enzymes on the surface of materials.	-Works with exo and endothermal reactions -Sensitive -Continuous monitoring of the catalysis	-Experiment planning must be very precise and well thought -Sample preparation can be difficult
FRET	Förster Resonance Energy Transfer consists in exciting a first chromophore, when it will relax, the energy can be received by the second chromophore which will also emit fluorescence. The method only gives information on distance between the molecules. Also, it can only be used on compatible chromophores. Last but not least, non-fluorescent molecules cannot be detected with this method.	-Very sensitive	-Applicable to a small range of proteins -pH sensitive -Requires a fluorescent tag

SECM	Scanning Electrochemical Microscopy is a technique used to measure the local electrochemical behavior between interfaces. By moving the tip of the electrode, it is possible to get an image of the topology on the surface. To analyze a surface, it is mandatory to have a liquid phase in contact with it. Thus it is only usable for hydrophilic polymer surfaces.	-Non-destructive -Quantitative	
ToF-SIMS	Time of Flight Secondary Ion Mass Spectroscopy The method is used to map the composition of the surface. A source of primary ions is used to bombard the surface. Secondary ions are emitted and analyzed with a Time-of-Flight analyzer to determine the element they came from.	-Sensitive -Mapping available	-Destructive -Data treatment can be complicated

740

741 6 Applications

742 As highlighted in the introduction, immobilization of enzymes has been largely used in industrial
743 processes for decades, meaning a significant amount of reviews are already found in the literature
744 (DiCosimo et al., 2013; Hassan et al., 2019; Madhu and Chakraborty, 2017; Yushkova et al., 2019).
745 However, such reviews generally did not restrain applications and are not exhaustive. We aspire to
746 dedicate this chapter to the immobilization of proteins, enzymes and peptide to polymeric materials
747 through a covalent bond. The reason is that the majority of immobilization is related to protein
748 adsorption on porous media, solid surface such as silica, or trapped in hydrogel *via* physisorption,
749 chemisorption, entrapment or cross-linked enzyme aggregates (Chapman et al., 2018). We draw the
750 reader's attention to the fact that industrial enzyme immobilization also lies in innovation such as new
751 carrier materials or protein engineering integrated to immobilization processes (Sheldon et al., 2021).
752 As covalent immobilization is more challenging and economically costly, such immobilization strategy
753 would thus be dedicated to high value-added processes or products, and still requires development of
754 innovating technology.

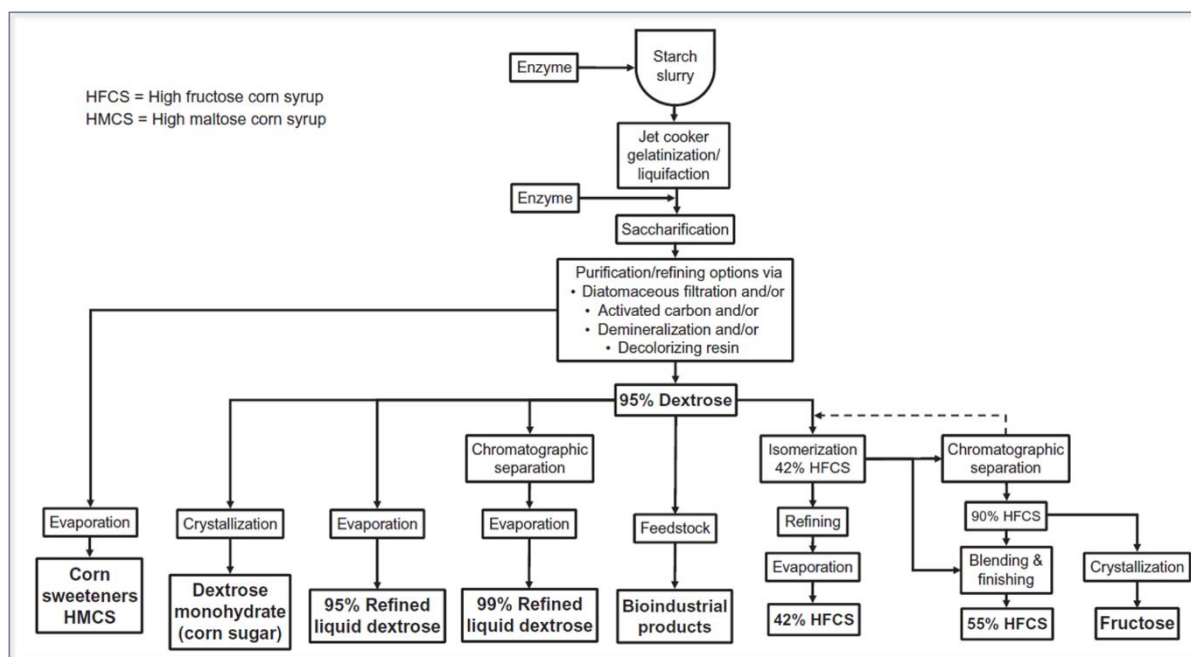
755 6.1 Industrial applications

756 The first industrial use of immobilized enzymes was reported in 1967 (Tosa et al., 1967). An
757 aminoacylase from *Aspergillus oryzae* was nonspecifically immobilized onto diethylaminoethyl-
758 cellulose for the resolution of synthetic racemic D,L amino acids. Today, the global enzymes market
759 represents USD 8,919 million and is expected to grow to USD 13,815 million by 2027 (“Enzymes
760 Market Size, Share, Growth | Global Report [2020-2027],” n.d.), where immobilized enzymes will
761 represent 20 % of the industrial enzyme sales (DiCosimo et al., 2013). Even if industrial applications of

762 immobilized enzymes cover a large variety of domains (animal feed, cosmetics, chemistry, paper
 763 industry, textile industry, laundry, diagnostic, biofuel), food and pharmaceutical industries represent
 764 more than 40 % of the total.

765 Among the largest scale industrial processes utilizing immobilized enzymes, production of high fructose
 766 corn syrup (HFCS) from corn syrup, used as sweetener for beverage, foodstuffs or directly as a food
 767 component, is by far the main product (DiCosimo et al., 2013). It is due to the high efficiency of the
 768 glucose isomerase (GI, also known as D-xylose ketol isomerase) to convert D-glucose from corn to D-
 769 fructose. Over 500 tons of immobilized D-glucose isomerase (IGI) are consumed annually, enabling the
 770 production of approximately 10 million tons of HFCS per annum (Tufvesson et al., 2010).

771



772
 773 Figure 16. General process overview of enzyme/enzyme for production of corn syrup, from (Helstad, 2019). Maize or wheat
 774 starch is hydrolyzed through a total enzyme process, producing syrups such as dextrose, HMCs and HFCS mainly by the action
 775 of α -amylase, glucoamylase and glucose isomerase, respectively.

776 Actually, IGI used to produce HFCS is proposed as an adsorbed form on inexpensive silica-based
 777 powder followed by cross-linking with glutaraldehyde, making the enzyme extremely stable when used
 778 in a packed bed reactor (Zittan et al., 1975). However, Tükel and collaborators reported a rare example
 779 of the covalent immobilization of GI using an epoxy support made with a copolymer of methacrylamide
 780 and N,N'-methylene-bis(acrylamide), demonstrating an improved catalytic efficiency and a better
 781 reusability of GI (Seyhan Tükel and Alagöz, 2008).

782 Prebiotic oligosaccharides are non-digestible food ingredients that beneficially affect the host by
 783 selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon,
 784 thereby improving host health (Roberfroid, 2007). Among them, galacto-oligosaccharides (GOS) are
 785 mainly produced commercially by a β -galactosidase (β -Gal) catalyzing the hydrolysis of lactose and

786 subsequently the synthesis of oligosaccharides as a reverse reaction (transgalactosylation) (Urrutia et
787 al., 2013). The main products are oligosaccharides composed by 3 or 4 molecules of β -D-galactose units
788 linked to each other *via* a β ,1-4 covalent bond. The world market is expected to reach USD 960 million
789 by the end of 2025 (360 Market updates, 2019). In this context, multi-point covalent immobilization of
790 the β -Gal was performed on an amino-epoxy solid support made with methacrylic polymer matrix and
791 was suitable for GOS synthesis in an industrial process (Benjamins et al., 2015).

792 The global antibiotic market size is expected to reach USD 57.4 billion by 2027 (Market Data Forecast,
793 2022). Among them, β -lactam antibiotics have become the most widely used class of antimicrobial drugs
794 (Pan et al., 2018). In order to slow down the emergence of drug-resistant bacteria, 6-aminopenicillanic
795 acid (6-APA) is now used as an intermediate to cope with semisynthetic penicillin produced by
796 derivatization of 6-APA. Nowadays, such industrial production occurs using two different penicillin G
797 acylases, one designed to produce 6-APA and another designed for the synthesis of semisynthetic β -
798 lactams as ampicillin or amoxicillin (Bruggink et al., 1998) that replaced the chemical route. During the
799 process, penicillin G acylases, among the most commonly industrial enzymes, are used covalently
800 immobilized on epoxy or amino methacrylate polymer in a sequential hydrolytic/synthetic process
801 (Katchalski-Katzir and Kraemer, 2000).

802
803 Currently, enzymatic immobilization is important for reducing the production cost of industrial
804 processes, mainly by facilitating the recovery and reuse of enzymes, improve the processes and reduce
805 the ecological costs. As previously exemplified, covalent enzyme immobilization could be justified
806 because it is well worth the cost (IGI formulation is largely unchanged since 1975) or because of a high
807 value-added product such as in pharmaceutical industries with antibiotics. As covalent immobilization
808 prevents regeneration of the support and is more expensive to develop, it is not surprising that most of
809 the immobilized enzymes used in industrial processes are nonspecifically bound to cheap support (Basso
810 and Serban, 2019). However, next generations of industrial processes still may lay at laboratory scale.

811 6.2 Laboratory scale applications

812 To facilitate reading, and to keep online with the topic of this review, we propose to exemplify our words
813 based on large class of mechanical properties, i.e. rigid, and elastic (Table 3). Furthermore, in a context
814 of sustainable processes based on green chemistry, this classification will also be compiled with grafting
815 of proteins on biobased soft material. The reader will find a large number of articles reflecting the
816 intensity of the worldwide research in this field. Here, we propose a limited number of non-exhaustive
817 examples of works published on protein immobilization to polymeric support with putative industrial
818 application.

819 6.2.1 Rigid polymers

820 Rigid synthetic polymers such as polyacrylates, or PS are by far the most widely used in biomolecule

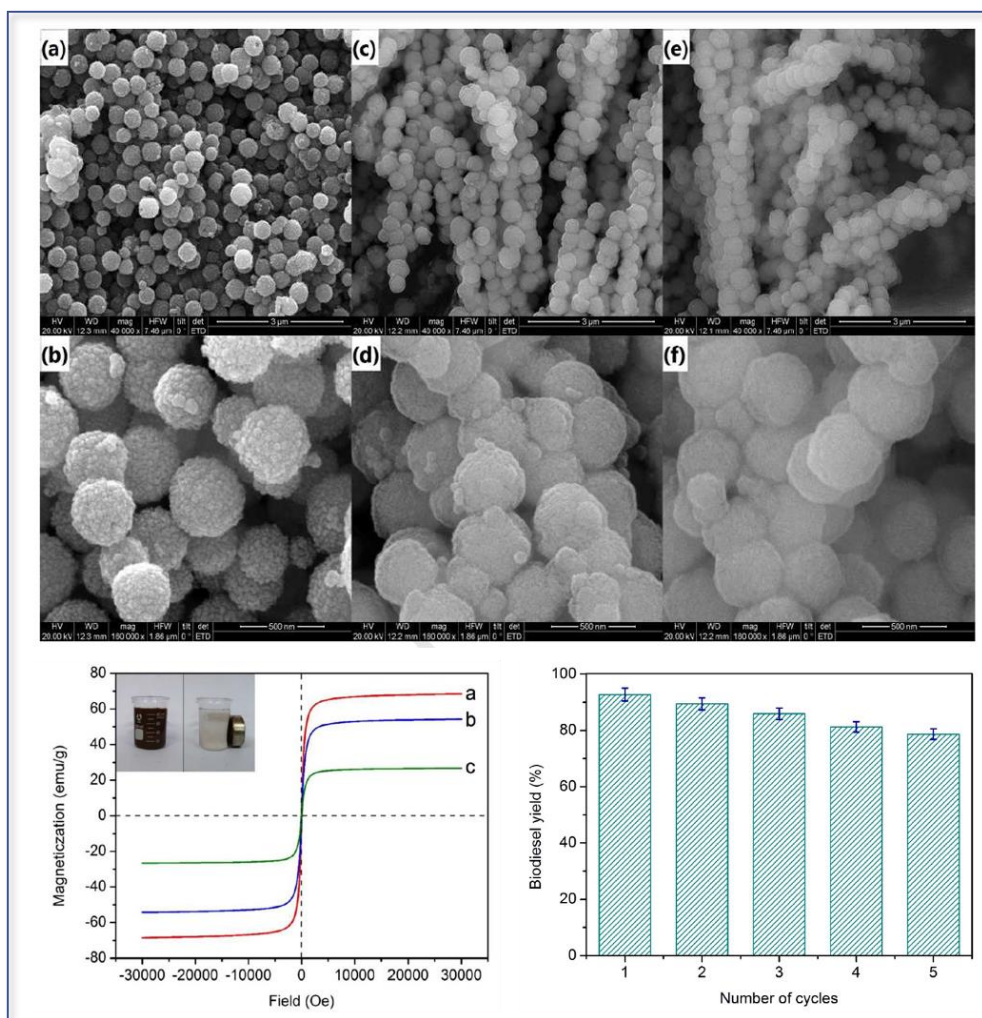
821 grafting due to low cost of fabrication, chemical resistance or optical transparency. They are generally
822 resistant to aqueous solvents and acids/bases, rigid but not fragile, and common in the marketplace
823 (Becker, 2002). However, direct covalent immobilization is not immediate due to the lack of reactive
824 chemical functions compatible with biomolecule grafting.

825 In pharmaceutical industries, hydrophobic PS is commonly used to carry out immunoassay in various
826 carriers, such as plates, balls or tubes. However, due to passive adsorption, immobilized proteins on PS
827 are often denatured. To circumvent this deleterious effect, Shmanai and collaborators modified a
828 formylated polystyrene sphere (see section 4.D.1). Hence, the activated PS surface allowed the covalent
829 immobilization of immunoglobulins G. Antibody molecules are known to display a Y-shape consisting
830 of one Fc fragment and two Fab fragments, the latter reacting specifically with antigens to yield immune
831 complexes. During the process, the spatial accessibility of the Fab is of importance to lead to efficient
832 immunosorbent (Shmanai et al., 2001). Orientated immobilized antibodies presented an increased
833 activity by 38 % compared to randomly immobilized antibodies to non-functionalized PS. This work
834 presents an effective tool for antibody immobilization on molded materials made with PS.

835 Simplified processes are also available to modify surface of synthetic polymers in order to make
836 covalent grafting easier. For medical applications, such polymers require suitable mechanical stability
837 and biodegradability. Rosellini and collaborators developed films of poly(ϵ -caprolactone)-*block*-
838 poly(ethylene oxide)-*block*-poly(ϵ -caprolactone) (PCL-PEO-PCL), which could be functionalized
839 following a four-step procedure (Rosellini et al., 2015) mainly as described in section 3.C. The authors
840 grafted on the surface two different pentapeptides, from fibronectin and laminin, using the primary
841 amine group of the *N*-terminus of each pentapeptide to orientate the immobilization. The presence of
842 such peptides enabled to promote specific cell adhesion of immortalized mouse skeletal muscle
843 myoblast cell line and *in vitro* experiments demonstrated their proliferation and differentiation as
844 possible source for cardiac tissue engineering on synthetic materials.

845 An efficient way to covalently graft protein to rigid polymers would be to benefit from synthetic
846 polymers exhibiting surface chemical groups. Poly(glycidyl methacrylate-*co*-methylacrylate) (P(GMA-
847 *co*-MA)) or poly(styrene-*co*-maleic anhydride) (PSMA) are strong electron acceptor polymers which
848 can undergo rapid reaction at pHs compatible with protein or enzyme. Plant biomass conversion to
849 biofuel is of increasing importance and is already a reality as a renewable and clean source of energy. It
850 requires a large variety of carbohydrate active enzymes to deconstruct plant polymers to
851 monosaccharides later fermented into ethanol by yeast (Mohd Azhar et al., 2017). Biodiesel, chemically
852 consisting of methyl esters of long-chain fatty acids, is also a biofuel but is derived by using the catalytic
853 transesterification of animal or plant oils with methanol (Sharma et al., 2008). Chemical industrial
854 production of biodiesel, however, did not satisfy the increasing environmental concerns, and lipase, an
855 esterase that catalyzes the hydrolysis of ester bonds of lipid, could address these hurdles. Xie and
856 collaborators recently described the immobilization of a lipase from *Candida rugosa* on magnetic
857 organic polymer (Xie and Huang, 2020). The support consisted in Fe₃O₄ nanoparticles with a synthetic

858 polymer shell of P(GMA-co-MA) displaying carboxylic groups (see section 3.C) used to attenuate the
 859 magnetic dipole-dipole attractions between the magnetic nanoparticles. Soybean oil was turned into
 860 biodiesel by the immobilized lipase with a yield of 92.8 %, magnetic immobilized lipase being easily
 861 recovered from the reaction. A yield of 79.4 % conversion was still achieved after reuse of five cycles.
 862

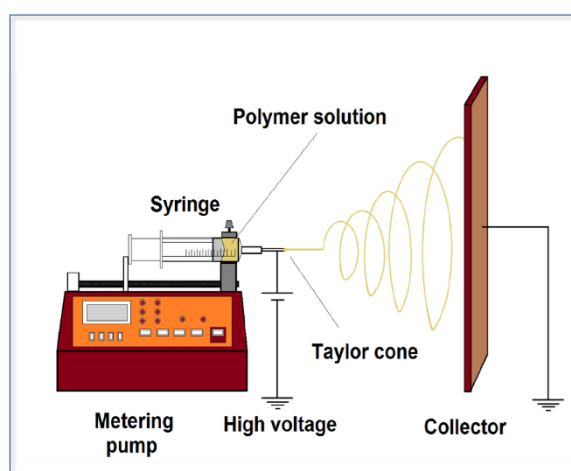


863
 864 *Figure 17. SEM images of Fe₃O₄ (a, b), Fe₃O₄-poly(GMA-co-MAA) (c, d) and the immobilized lipase (e,f). Bottom left, room*
 865 *temperature magnetization curves of Fe₃O₄ (a), Fe₃O₄-poly(GMA-co-MAA) (b) and the immobilized lipase (c). Bottom right,*
 866 *the recycling test results of the immobilized lipase for transesterification of soybean oil. From (Xie and Huang, 2020)*

867 Some enzyme immobilization-based processes required a signal-processing system through the use of a
 868 transducer. It is the basis of the enzyme-based biosensors, one of the most extensively studied
 869 biosensors. So far, the only industrial usage of enzyme-based biosensors is in clinical applications for
 870 diagnosis of diabetes mellitus, where a glucose oxidase is used to control over blood-glucose levels,
 871 even for usage at home (Mehrotra, 2016). Covalent immobilization of enzyme is required to offer stable
 872 complexes between enzymes and support, thus decreasing contamination and interference to the signal.
 873 The sensing principle is to detect the presence of molecules of interest by measuring their presence
 874 converted by the transducer into measurable signals. Electrochemical biosensors, using metallic

875 electrode, provide advantages such as simplicity, low cost and high sensitivity. Urea is an important
876 molecule as it is present in blood serum and is a marker for liver and kidney function (Taylor and
877 Vadgama, 1992). But urea analysis is also of importance in agricultural and food industries. Cortina and
878 collaborators developed an impedimetric biosensor consisting of an electrode covered with a pH-
879 sensitive methacrylic acid-methyl methacrylate copolymer that dissolves specifically at pH values
880 higher than 7 (Cortina et al., 2006). Urease, an enzyme catalyzing the hydrolysis of urea in ammonium
881 and bicarbonate ions, was immobilized to the polymer coating by carbodiimide coupling. Enzymatic
882 activity increased the pH of the medium, induced solubilization of the polymer whose degradation was
883 monitored by changes in impedance measurements.

884 Beyond the type of rigid synthetic polymers and the chemistry developed to covalently immobilize
885 proteins, readers have also to keep in mind that the shape and the surface properties of the solid support
886 are also to be considered. Actually, emerging supports are assessed as, for instance, electrospun
887 nanofibers (Smith et al., 2020b), or covalent organic frameworks (Gan et al., 2021). Their interest lies
888 in their large surface area to volume ratio, interconnectivity or even pre-designable structure. It is also
889 of interest to consider the technology developed with microfluidic chips, consisting in
890 microminiaturized devices containing chamber and tunnel surfaces made with PMMA, PS or cyclic
891 olefin copolymer (COC) (Kim and Herr, 2013). All the previous concept remains fully applicable for
892 operational considerations for protein immobilization in microfluidic systems.



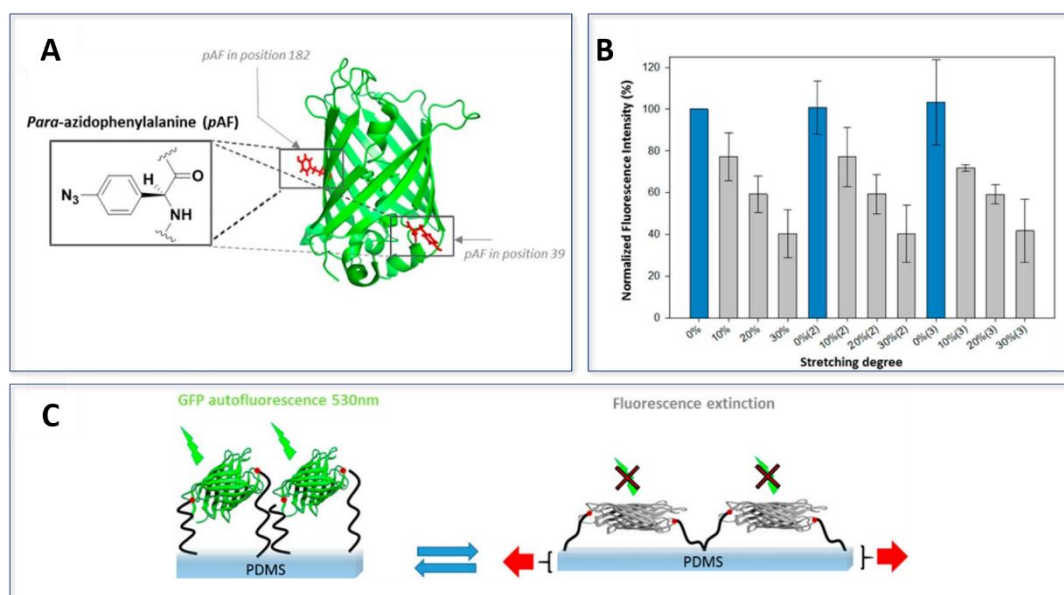
893
894 *Figure 18. Schematic diagram of set up of electrospinning apparatus horizontal set up, from (Riazi et al., 2016).*

895 6.2.2 Elastomers

896 Mechanochemistry is a relatively new field of research investigating the effect of mechanical forces to
897 chemical modification in macromolecules, involving irreversible rearrangement through bonds breaking
898 (Davis et al., 2009). This phenomenon is also involved in numerous vital processes in nature such as
899 cell growth, activation of ion channels, blood clotting or spatial orientation (Funtan et al., 2019), without
900 involving the breaking of covalent bond. These processes require much less energy and are collectively
901 termed soft-mechanochemistry (Lavalle et al., 2016). They often involve conformational change within
902 protein, converting the external force into a biochemical signal (Vogel, 2006). It is a reversible process,

903 leading the researchers investigating this area to develop covalent grafting of protein on elastic
 904 polymers. The aim here is to control, with an on/off signal, the activity of a protein or an enzyme through
 905 the stretching of an elastic support.

906 Longo and collaborators developed a reversible biomechano-responsive surface to induce
 907 conformational changes within protein (Longo et al., 2015). As proof of concept, GFP was covalently
 908 immobilized on polydimethylsiloxane (PDMS). Alpha-amino-omega-propargylpolyethylene glycol
 909 chains were grafted to the surface to provide antifouling properties. The alkyne groups were then used
 910 to control the immobilization of GFP by click-chemistry, as two non-natural amino acids carrying azide
 911 chemical function were genetically introduced at specific position in the GFP (see section 4.C).
 912 Stretching unidirectionally the PDMS surface with grafted GFP by 10 %, 20 % and 30 % led
 913 respectively to 23 %, 42 % and 60 % decrease of the initial fluorescence. Repeated stretching-relaxation
 914 cycles demonstrated a fully reversible recovering of the fluorescence. The decrease of fluorescence was
 915 explained by the modification of the GFP upon stretching.



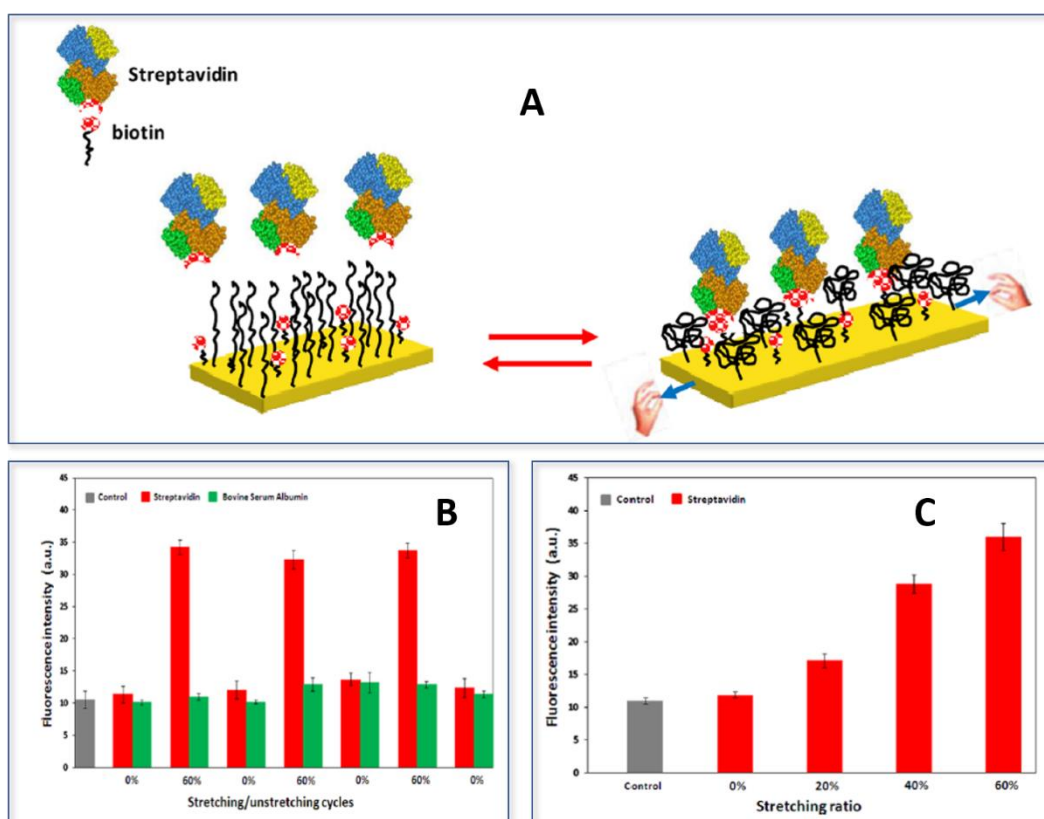
916
 917 *Figure 19. (a) GFP modified genetically at two opposite positions of the β -barrel with a unnatural amino acid bearing a para-*
 918 *azidophenyl group (pAF). (b) Schematic representation of the GFP covalently linked through POE linkers onto a modified PDMS*
 919 *sheet at rest and stretched. (c) Evolution of the normalized fluorescence of the GFP-modified PDMS as a function of the degree*
 920 *of stretching during three stretching-unstretching cycles, from (Lavalle et al., 2016).*

921 Enzymatic activity can also be modulated by stretching. Rios and collaborators covalently immobilized
 922 a β -Gal on elastic silicone (Longo et al., 2015). β -Gal is a tetrameric enzyme, comprising four
 923 polypeptide chains held together through non-covalent bonds, the catalytic site being composed of two
 924 different subunits. Silicone was covered with a poly(L-lysine)/hyaluronic acid (PLL/HA) exponentially
 925 growing polyelectrolyte multilayer (PEM) films using PLL chains chemically modified by thiopyridyl
 926 groups. The β -Gal, modified by maleimide groups, was immobilized by reaction of the maleimide
 927 groups to the thiopyridyl moieties of the elastomer. A decrease of 40 % of the enzymatic activity was
 928 observed by stretching the elastomer by 100 %, and 87 % of the initial rate was obtained by the release

929 of the stretch.

930 Proteins involved in mechanotransduction processes have the ability to exhibit specific active peptide
931 sequences under mechanical stretch (Vogel, 2006). These exposed cryptic sites are involved in specific
932 signaling pathways, as exemplified by cell adhesion. Bacharouche and collaborators developed a
933 reversible mechanoresponsive surface to mimic cryptic sites (Bacharouche et al., 2013). Silicone was
934 again used as elastomer support as described in section 3.C. A non-stretched state buried biotin
935 molecules into PEG brushes, preventing them from coming in contact with streptavidin, the receptor.
936 As the silicone was stretched, the PEG density decreased, allowing the biotin to become accessible to
937 streptavidin, in a fully reversible manner.

938



939
940 *Figure 20. (A) Schematic representation of the fully reversible cryptic site mechanoresponsive surface. (B) Evolution of the*
941 *fluorescence intensity for a series of three stretching (60%)/unstretching (0%) cycles. (C) Evolution of the fluorescence intensity*
942 *at various stretching ratios: 0% (nonstretched state), 20%, 40%, 60%. From (Bacharouche et al., 2013).*

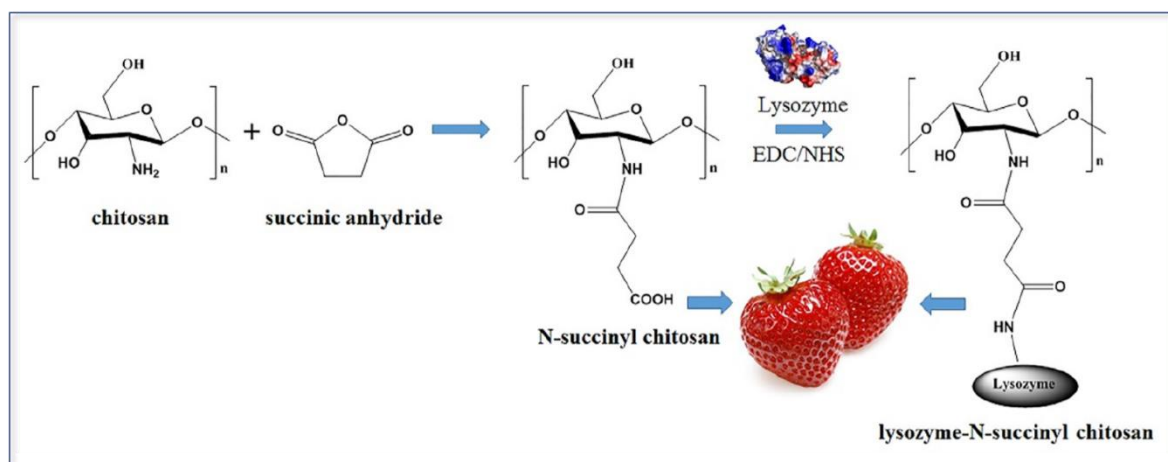
943 Elastomers could also be used to develop scaffold-like spongy material, and like a sponge, be squeezed
944 to induce an enzymatic mechanoresponse. Based on this mechanism, Jain and collaborators developed
945 enzyme-polymer surfactant core-shell conjugates supplemented with aqueous mixture of silica or silk
946 nanoparticles (Jain et al., 2018). The sponge presented a high level of porosity and ability to undergo
947 compression-decompression cycles without structural degradation. Furthermore, the amount of product
948 generated by the enzyme after 25 minutes was increased by around 8 times by compressing-
949 decompressing the sponge every 15 seconds.

950

6.2.3 Natural polymers

951 Anthropogenic climate change induces a transition toward a circular bio-based economy. It is partially
952 the case when industrial applications favor biocatalysis as an alternative to chemical catalysis (Wu et
953 al., 2021). But beyond the recyclability of some polymers such as PMMA, PS, PE, nylon or SBS (Table
954 3), it is also possible to develop applications using covalent immobilization of enzymes on renewable
955 carbon based polymers, polymers found in nature (Bilal and Iqbal, 2019c). They show unique
956 physicochemical properties and are massive-scale available, non-toxic and biocompatible.

957 Chitosan is a polysaccharide produced from chitin, sourced from marine exoskeleton of crustaceans
958 namely shrimps and crabs. It is composed of randomly distributed β -(1,4)-linked 2-amino-2-deoxy-D-
959 glucose and 2-acetamido-2-deoxy-D-glucose units, thus exhibiting numerous amine and hydroxyl
960 groups, enabling effective binding of protein without the involvement of cross-linking agents. Niu and
961 collaborators developed the covalent immobilization of an antimicrobial enzyme on activated chitosan
962 (Niu et al., 2020), whose chemistry is described in section 3.C. The antimicrobial agent is lysozyme, a
963 hydrolytic enzyme responsible for the lysis of Gram-positive bacteria. The authors demonstrated that
964 such lysozyme grafting was able to improve the thermal stability of the enzyme but also its activity by
965 256 %. They also demonstrated that strawberries treated with this antimicrobial chitosan had their shelf
966 life extended by 3 days.



967

968 *Figure 21. Illustration of the preparation of water-soluble N-succinyl chitosan and application as lysozyme-N-succinyl chitosan*
969 *for fruit preservation, (from (Niu et al., 2020).*

970 Urrutia and collaborators developed a two-step functionalization approach to graft a β -Galactosidase on
971 chitosan (Urrutia et al., 2018), mainly following the activation steps described in section 3.A. The
972 authors improved the performance of the β -Galactosidase and 10 sequential batch reactor operations,
973 they showed that the production of galacto-oligosaccharides was increased by a factor of 4.7 compared
974 to the soluble enzyme.

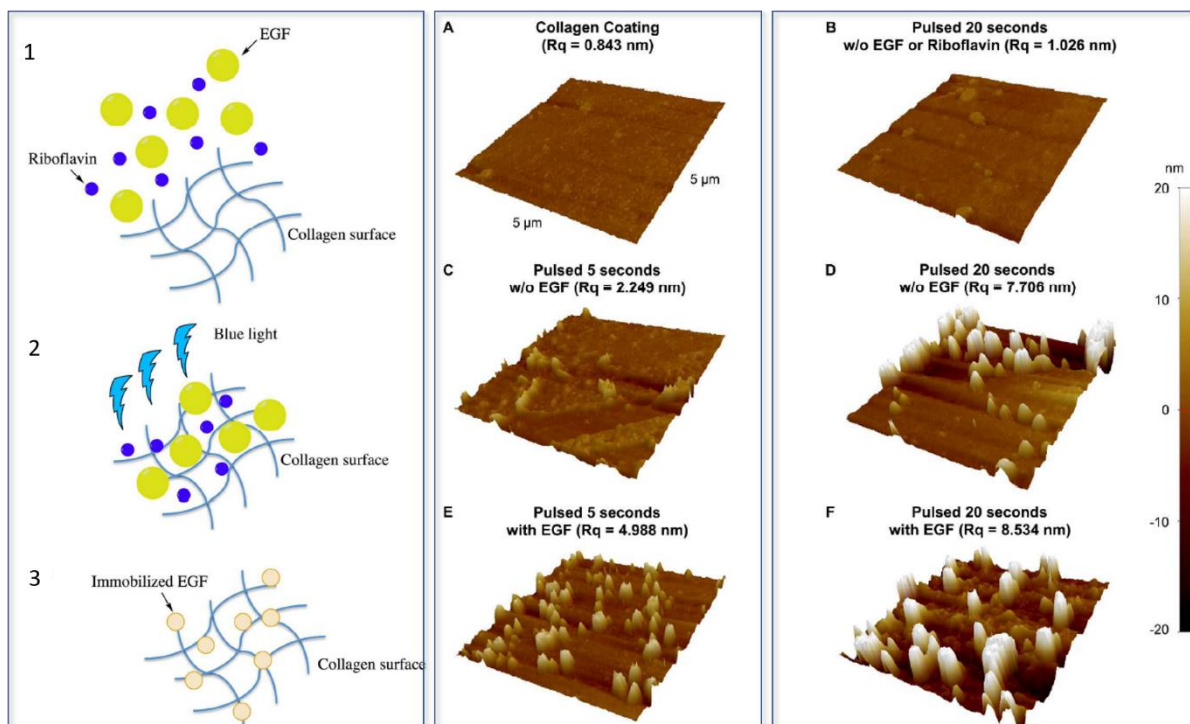
975 Cellulose is a natural polysaccharide, very similar to chitosan, found mainly in plant cell walls. It is
976 composed of repeated β -(1,4)-linked D-glucopyranosyl units, displaying a large amount of hydroxyl
977 groups which could be activated. It is inexpensive and commercially available. Common derivatives of

978 cellulose are cellulose acetate and diethylaminoethyl cellulose (DEAE-cellulose). Sharifi and
979 collaborators developed the immobilization of an organophosphorus hydrolase to cellulose microfibers
980 using epoxy groups (Sharifi et al., 2018). The methodology consisted in generating epoxy groups as
981 presented in section 3.A. Organophosphorus hydrolase is known to hydrolyze a wide range of
982 organophosphorus compounds, highly toxic molecules found in insecticides, pesticides and warfare
983 agents. The authors demonstrated a catalytic efficiency increase by about 4.85-fold on the degradation
984 of organophosphates compared to the free enzyme and improved the storage stability. Sperandeo and
985 collaborators immobilized antimicrobial peptides (AMP) onto microcrystalline cellulose, allowing
986 binding of the peptide through any desired position within the peptide chain or extremities (Sperandeo
987 et al., 2020). AMP are widely spread in all domains of life, used by organism to defend themselves from
988 external pathogens. These peptides are positively charged amphipathic molecules and mainly induce
989 bacterial membrane disruption, leading to cell lysis. To graft AMP to cellulose, the authors first modified
990 them both by adding thioester to AMP and cysteine to cellulose. Fmoc-cysteine was used to derivatize
991 cellulose to completion. The authors highlighted that cellulose conjugated to the AMP causes a
992 significant decrease in the concentration of viable bacterial cells compared to unmodified cellulose.

993 Silk fibroin is a polymer of amino-acids produced by domesticated *Bombyx mori* silkworm cocoon. It
994 contains large amounts of glycine, alanine, and serine as well as readily activated chemical groups, such
995 as tyrosyl/phenol, sulfhydryl, and imidazole groups, making this support suitable for catalyst
996 immobilization (Lv, 2020). Asakura and collaborators proposed to covalently immobilize an alkaline
997 phosphatase to silk fibroin using two different procedures (Asakura et al., 1989). Alkaline phosphatase
998 catalyzes the hydrolysis of organic phosphate and is widely used as model enzyme in biochemistry. The
999 grafting maintained also slightly the enzymatic activity over a long period of time. Monier described a
1000 new fibrous polymeric support based on natural worm silk fibers (Monier, 2013). It was prepared by
1001 means of graft copolymerization of polyacrylonitrile using a photo-initiator in order to create cyanide
1002 groups, which were converted in aldehydes using hydrazine. Finally, the fibers were activated with
1003 glyoxal to allow covalent bond with primary amine group at the surface of a β -Galactosidase. The
1004 resulting material was thoroughly characterized and the determination of the kinetic parameters of the
1005 immobilized enzyme as well as the reusability of the complex confirmed that this new fibrous support
1006 is of interest for enzymatic immobilization.

1007 Collagen is also a polymer of amino acids found in skin, tendons, cartilage, bones and tissues in general.
1008 It is sourced mainly from bovine horn and skin or fish scales and skin. Collagen is the main product in
1009 pharmaceuticals and food industry with a high demand. However, it is rarely used for immobilization of
1010 protein. However, Fernandes-Cunha and collaborators recently covalently immobilized growth factor to
1011 collagen (Fernandes-Cunha et al., 2017).

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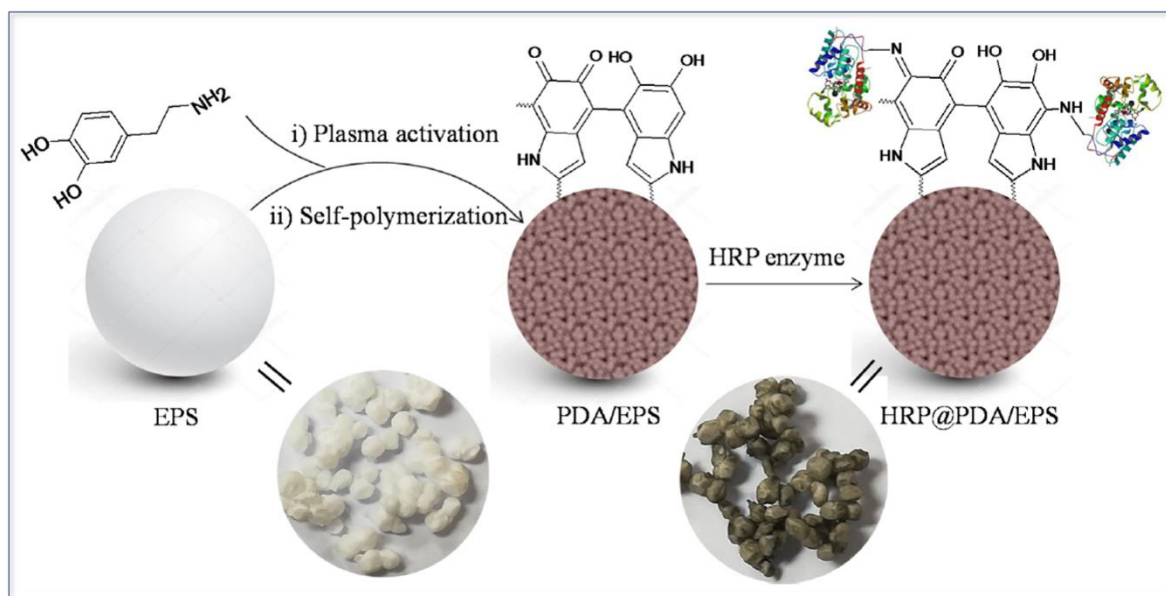


1013
 1014 *Figure 22. Photochemically immobilizing of epidermal growth factor (EGF) on collagen surface using riboflavin as a*
 1015 *photosensitizer (step 1 to 3). Topography of dry collagen-coated surfaces measured by AFM. (A) Collagen coating without*
 1016 *treatment, (B) after blue light exposure alone for 20 seconds without EGF or riboflavin, (C) pulsed blue light exposure with*
 1017 *riboflavin for 5 seconds but without EGF, (D) pulsed blue light exposure with riboflavin for 20 seconds without EGF, (E) pulsed*
 1018 *blue light exposure with riboflavin for 5 seconds with EGF, (F) pulsed blue light exposure with riboflavin for 20 seconds with*
 1019 *EGF. From (Fernandes-Cunha et al., 2017).*

1020
 1021 Epidermal growth factor (EGF) is a protein involved in regulation of cell proliferation and could be used
 1022 to enhance wound healing. The authors developed a strategy to immobilize EGF on collagen in order to
 1023 provide a cytocompatible substrate used as a cell scaffold or carrier for direct modification of tissue that
 1024 enhances cell proliferation, especially in the case of cornea injuries. Collagen was coated on the surface
 1025 of PS-well plates and the photosensitizer riboflavin was used as a highly reactive molecule to induce
 1026 the formation of covalent bonds between amino acids from EGF and collagen (Hsu and Sugar,
 1027 2016). The photo-immobilized EGF maintained its bioactivity by enhancing the proliferation and
 1028 spreading of corneal epithelial cells. Additionally, the photo-crosslinking reaction was not harmful to
 1029 cells and maintained viability at values near 100 %.

1030 Preserving our planet also includes the treatment of our waste or its reuse. Yassin and Gad proposed to
 1031 use expanded polystyrene foam (EPS) packaging waste as a support to immobilize a horseradish
 1032 peroxidase (Yassin and Gad, 2020). The inert EPS was at first coated with polydopamine (PDA). This
 1033 polymer is originated from mussel foot proteins and provides a large amount of catechol (as in L-Dopa)
 1034 and primary amine groups that can be functionalized at will. Indeed, functional molecules containing
 1035 nucleophilic groups (thiols, amines) can be easily immobilized onto quinones present in the structure of

1036 PDA to obtain synthetic derivatives. The peroxidase used in this work was an enzyme that catalyzes the
 1037 oxidation of diaminobenzidine and was used as a bleaching of dye wastewater agent. This strategy
 1038 provided a noteworthy tolerance of the grafted enzyme to higher and elevated temperature compared to
 1039 the free enzyme, compatible with industrial process. Under this condition, the immobilized enzyme
 1040 achieved almost complete oxidation of the dye within 120 min. After ten cycles of reusability, the
 1041 enzyme still provided 80 % of efficiency.
 1042



1043
 1044 *Figure 23. Scheme representing coating of expanded polystyrene foam (EPS) with polydopamine (PDA) layer followed by*
 1045 *horseradish peroxidase (HRP) immobilization to realize HRP@PDA/EPS, from (Yassin and Gad, 2020).*

1046 7 Conclusion

1047 By analyzing the specificities of grafting proteins onto polymeric surfaces, the following points could
 1048 be pointed out:

- 1049 - Keep always in mind the needed compatibility of the chemical conditions both for proteins and
 1050 polymers. Many polymers can be exposed to different reactants in order to provide desirable
 1051 functions, but a lot of conditions would lead to the degradation or denaturation of the proteins.
 1052 Reversely, proteins most often need aqueous buffers which might lead in extreme cases to
 1053 degradation of the polymer.
- 1054 - Keep in mind that polymers may adapt to their environment (temperature, solvent, ionic
 1055 strength, pH...). This may lead to a complete change of the surface morphology or type.
- 1056 - Be aware of possible changing orientation of the chemical groups, both on proteins and
 1057 polymers, especially above their glass transition temperature. This means that working with
 1058 proteins on polymers should always be considered as a system with possible evolution in time

1059 - “Seeing” proteins signals is not sufficient to prove a covalent grafting. This is a long-lasting
1060 problem, already known naturally on inorganic surfaces. However, the presence of similar
1061 chemical groups and atoms on both the proteins and its polymeric support renders this point
1062 particularly challenging to examine.

1063

1064 Finally, from a general standpoint, inorganic surfaces present the advantages of being well known with
1065 a lot of examples in the literature, being quite resistant and compatible with the proteins. However, they
1066 suffer from poor versatility if used by themselves. Polymers on the other hand exhibit a wide diversity
1067 of structures, chemical functions, hydrophilicity and mechanical properties. This thus constitutes an
1068 extremely versatile tool for the grafting of proteins. However, one should keep in mind their specificities
1069 to ensure that no misinterpretation of the experiments occurs.

1070

1071 **Author contributions**

1072

1073 All authors have read and agreed to the published version of the manuscript.

1074

1075 **Credit authorship contribution statement**

1076

1077 M. Artico: writing, review and editing; C. Roux: writing, illustration, review and editing; F. Péruch:
1078 writing, illustration, review and editing; A.-F. Mingotaud: conceptualization, writing, review, editing
1079 and supervision. C.Y. Montanier: writing, review and editing.

1080

1081 **Declaration of Competing Interest**

1082

1083 The authors declared no potential conflicts of interest concerning the research, authorship, and/or
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1085

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1087

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1091

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1093

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