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

M. Artico[a,b], C. Roux[a], F. Peruch[c], A.-F. Mingotaud[a], C.Y. Montanier[b]

[a] Laboratory IMRCP, CNRS UMR 5623, University Paul Sabatier, Toulouse, France, [b] TBI, Université de Toulouse, CNRS, INRAE, INSA, Toulouse, France, [c] Univ. Bordeaux, CNRS, Bordeaux INP, LCPO, UMR 5629, Pessac, France

cedric.montanier@insa-toulouse.fr, anne-francoise.mingotaud@cnrs.fr

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-

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

Keywords

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

1. Introduction

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

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

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

2. Specificity of grafting on synthetic polymers

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

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

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141 *Table 2. Main characteristics of polymeric matrices*

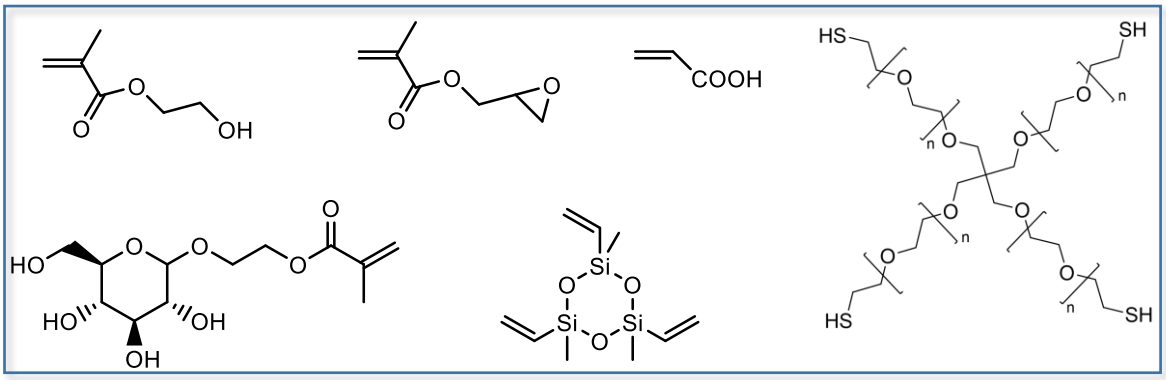
	Bulk polymers	Crosslinked polymers	Hydrogels
Network	1D	3D	3D
Morphology	Semi-crystalline or glassy, not porous	Semi-crystalline or glassy, porous or not	Amorphous, porous
Bonds	Covalent	Covalent and/or ionic and/or complexes	Covalent and /or ionic and/or complexes
Pristine chemical groups	Alcohols, 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

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

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



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

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

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

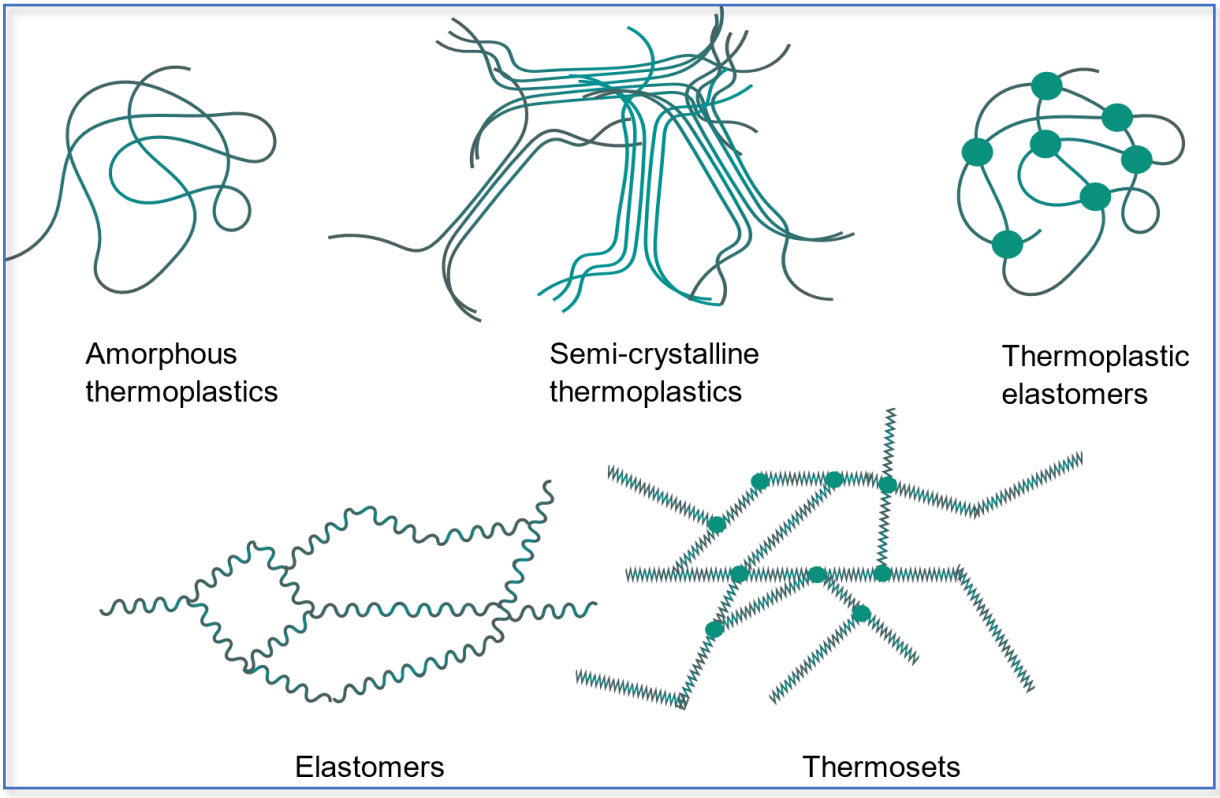
2.2 Physical properties

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

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

Table 3. Families of polymers from their macroscopic morphology

	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



Scheme 2. Schematic representation of the different polymer families

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

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

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

3. Non specific grafting

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

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

3.1 Epoxide groups

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

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

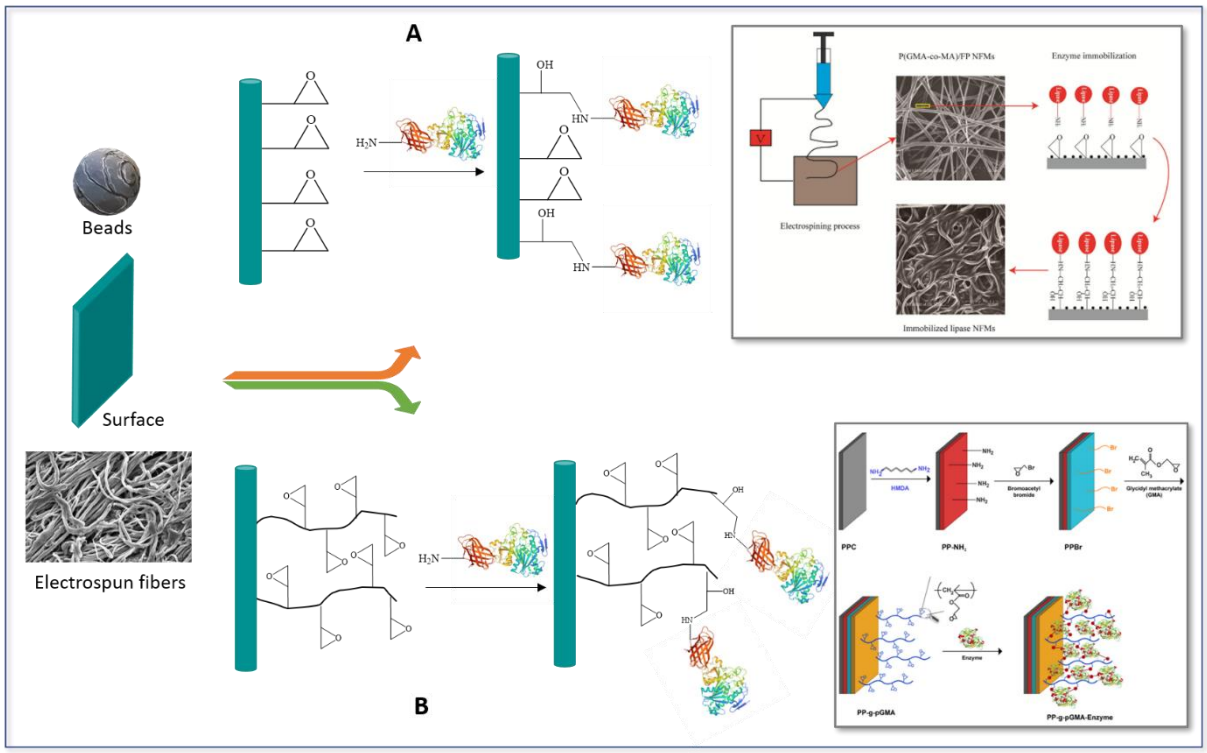


Figure 1. Grafting of proteins through epoxide groups. Illustration from(Arica et al., 2017) (A) and (Liu et al., 2018a) (B).

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

3.2 Aldehyde groups

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

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

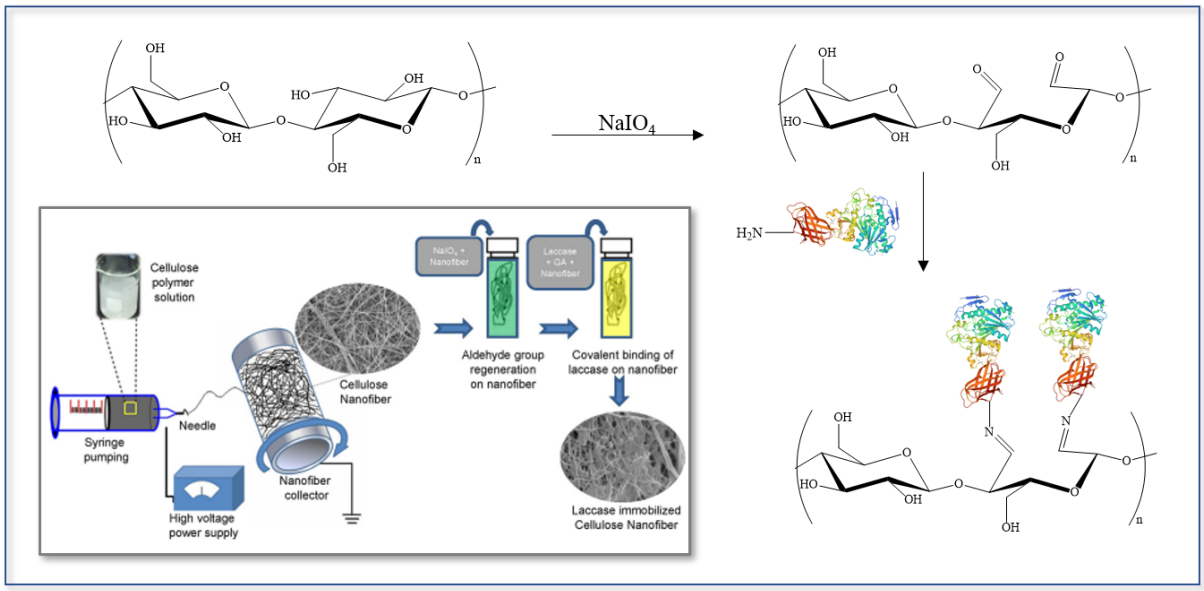


Figure 2. Grafting of proteins onto polysaccharides through partial degradation of polysaccharidic chain and aldehyde groups formation. Illustration from (Sathishkumar et al., 2014)

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

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

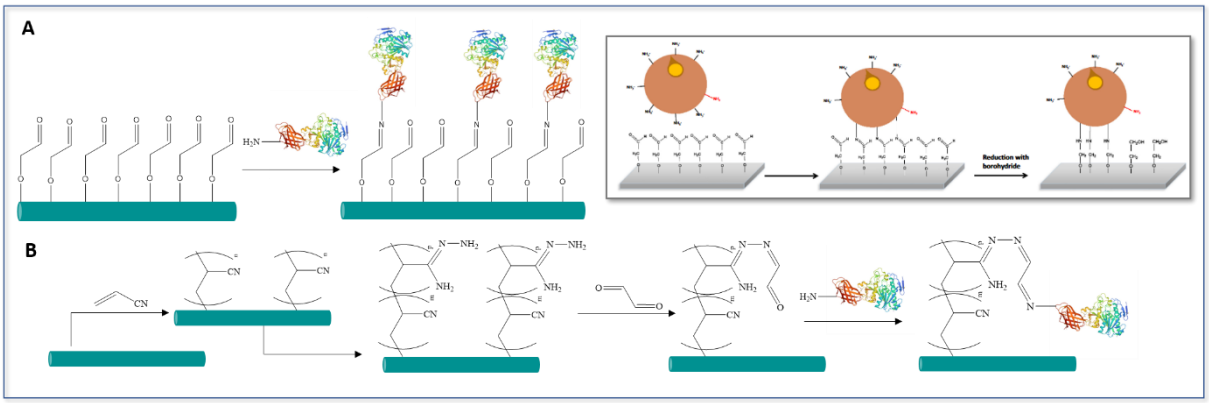


Figure 3. Grafting of proteins through glyoxyl route. Illustration from (Rodrigues et al., 2021)

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

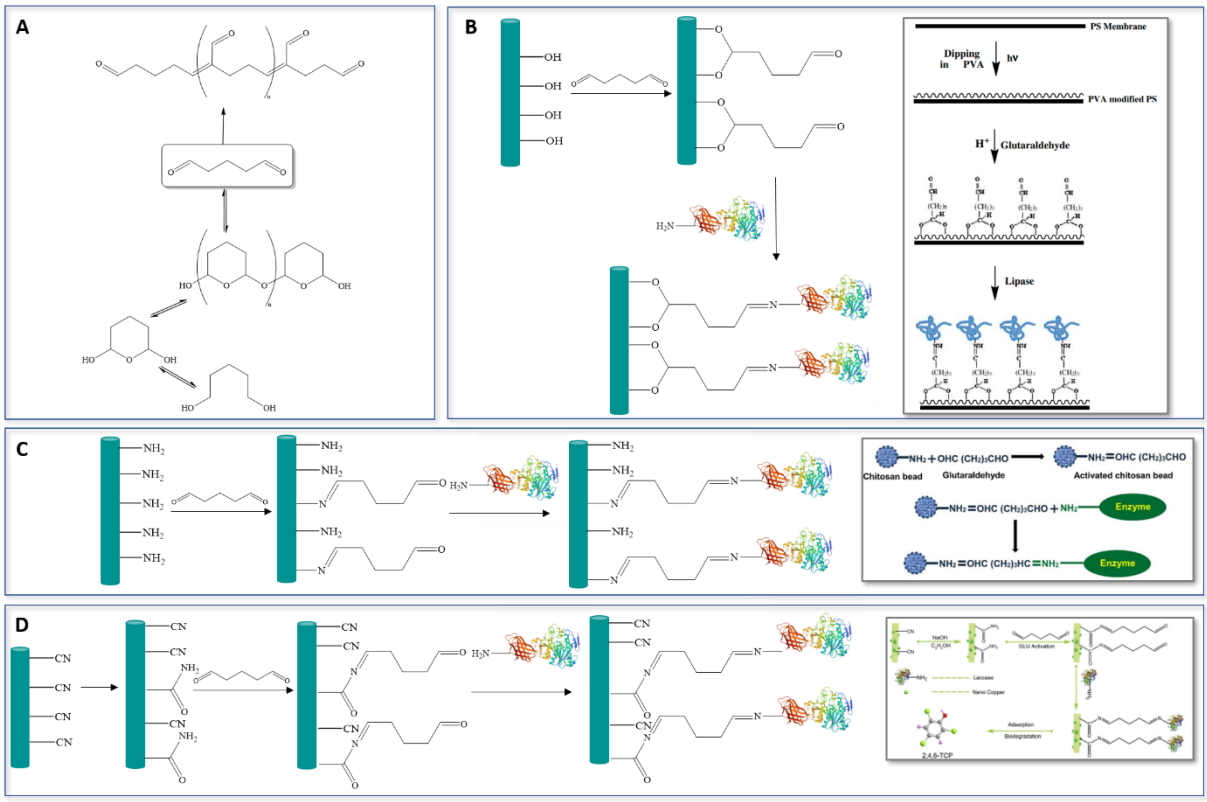


Figure 4. Grafting of proteins through glutaraldehyde route

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

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

Polymer support	Protein	Ref
P(AN-co-Am)	Lipase from Pseudomonas cepacia	(Lou et al., 2018)
Electrospun PAN/PVDF/Cu	Laccase from Trametes versicolor	(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 Trametes versicolor	(Wong et al., 2017) (Chen et al., 2020)
PA6 Partially hydrolyzed Electrospun with chitosan	Tyrosinase from Agaricus bisporus Laccase from Trametes versicolor	(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 Candida rugosa	(Gupta et al., 2010)
Electrospun chitosan/PVA with C nanotubes removal of PVA	Acetylcholinesterase Laccase from Trametes versicolor Laccase from Trametes versicolor Lipase from Candida rugosa	(El-Moghazy et al., 2016) (Xu et al., 2013) (Xu et al., 2015c) (Huang et al., 2007)
Chitosan beads beads hydrogel	Lipase B from Candida antarctica Inulase β-galactosidase from *Aspergillus oryzae* Lipase from Thermomyces lanuginosus	(dos Santos et al., 2017) (Singh et al., 2017) (Wahba, 2017)·(Urrutia et al., 2018) (Bonazza et al., 2018)
Agarose	Lipase B from Candida antarctica	(Barbosa et al., 2012)
Agar/κ-carrageenan hydrogels coated with PEI	β-galactosidase from *Aspergillus oryzae*	(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 Trametes versicolor	(Jhuang et al., 2020)

3.3 carboxylic acid functions

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

Table 5. Examples of protein grafting onto polymers through carboxylic acids or their derivatives

Polymer support	Protein	Ref
Electrospun poly(acrylonitrile-co-maleic acid)	Lipase from *Candida rugosa*	(Ye et al., 2006)
Electrospun partially hydrolyzed PAN/Fe_3O_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_3O_4	Laccase from *Trametes versicolor*	(Zdarta et al., 2020)
Electrospun PMMA/polyaniline	Laccase from *Trametes versicolor*	(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)

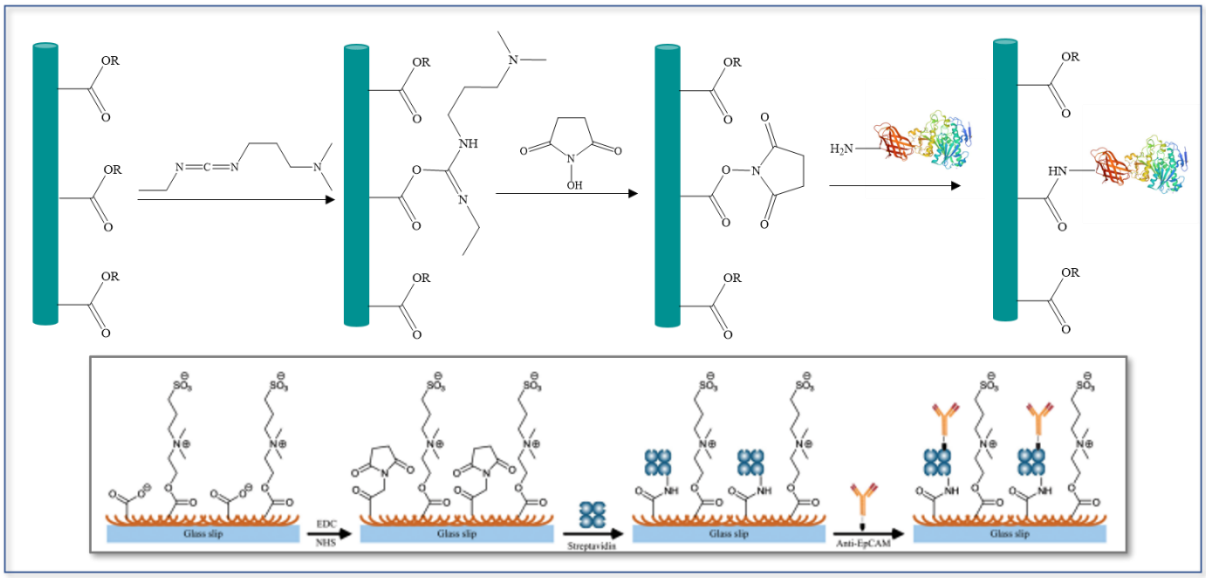


Figure 5. Grafting of proteins through carboxylic acid groups activated by EDC/NHS. Illustration from (Tseng et al., 2016)

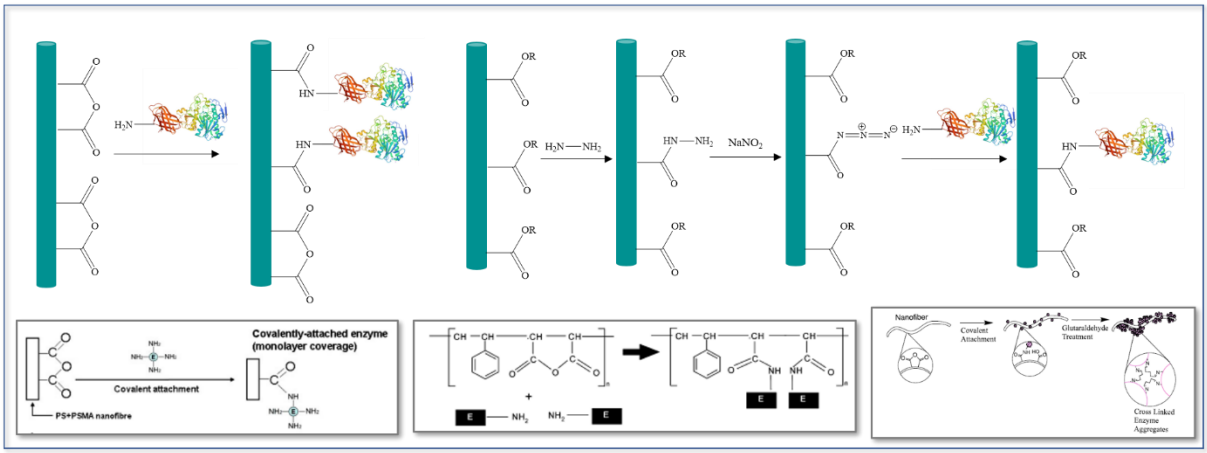


Figure 6. Grafting of proteins through anhydride groups or carboxylic groups. Illustration from (Kim et al., 2005) (left), (Nair et al., 2007) (middle) and (Smith et al., 2020a)

When carboxylic acid groups are not present on the surface, this latter can be first treated with plasma and then the same EDC/NHS coupling system is employed to covalently graft the proteins. Different kinds of plasma were used in the literature, like air, O_2, CO_2, N_2, micro-wave, etc. In some cases, the plasma treatment is used to polymerize monomers from the surface by free radical polymerization (Völcker et al., 2001). In other cases, the plasma treatment directly functionalizes the surface with functional groups (Guex et al., 2014; Heidari-Keshel et al., 2016; Khademi et al., 2017; Ma et al., 2005; Mahmoudifard et al., 2016; Teske et al., 2020; Vasile et al., 2011a; Wieland et al., 2020). This treatment can be applied to many kinds of surfaces and allowed the immobilization of a wide variety of proteins as indicated in the table below.

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)

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 *Rhyzopus 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.

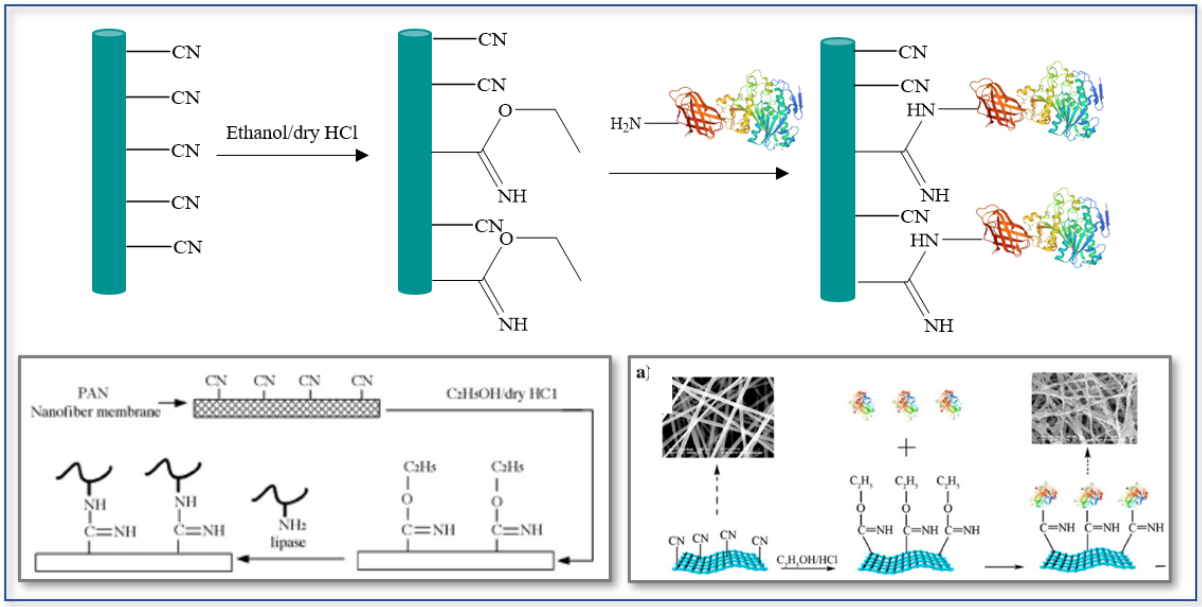


Figure 7. Grafting of proteins onto PAN chains. Illustrations from (Li et al., 2007) (left) and (Smith et al., 2020a) (right).

3.4 Hydroxyl group

The last functional groups which were used to graft protein onto polymeric surfaces are hydroxyl groups. As these latter are not able to react directly with the amino groups of the proteins, they have first to be activated through reaction with 1,1'-carbonyldiimidazole (CDI) (Baştürk et al., 2013; Çakıroğlu et al., 2018; Oktay et al., 2015b; Xu et al., 2015a, 2015b). This treatment is mainly applied to PVA containing surfaces. Thus, α-amylase from porcine pancreas (Baştürk et al., 2013) or Horseradish peroxidase (Xu et al., 2015a) could be grafted onto electrospun PVA/PAA fibers or PVA/PAA/SiO_2 fibers respectively. Horseradish peroxidase was also grafted onto electrospun PVA/NCC (nano-crystalline cellulose) or

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

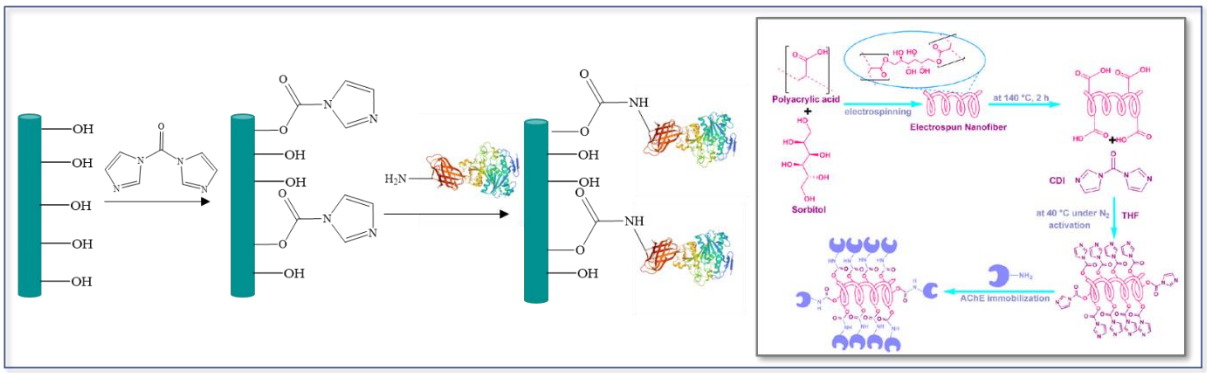


Figure 8. Grafting of proteins through hydroxyl groups activated by CDI. Illustration from (Çakıroğlu et al., 2018).

3.5 Other activations

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

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

3.6 "Click-chemistry" and/or other "coupling" route

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

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

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

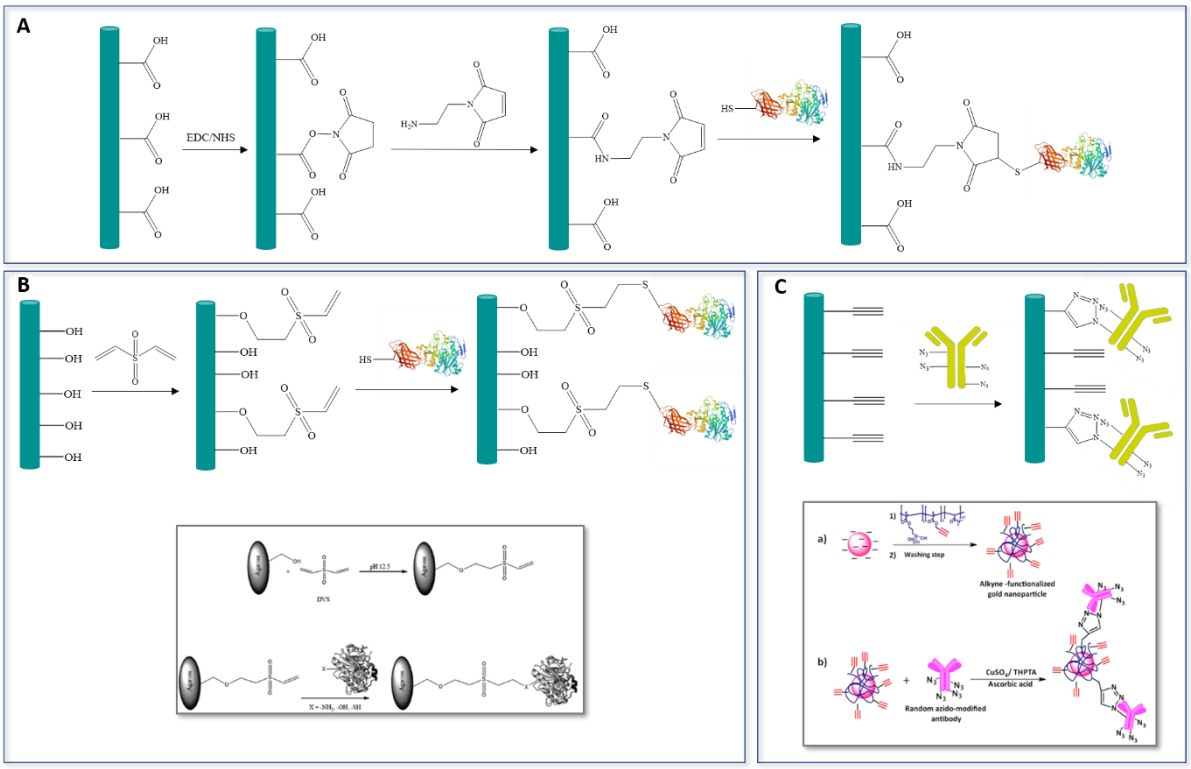


Figure 9. Grafting of proteins through click chemistries. Illustrations from (Jose C. S. dos Santos et al., 2015) (B) and (Shi et al., 2008) (C).

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

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

4 Regio- or chemoselective grafting

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

4.1 Immobilization via N-terminus

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

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

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

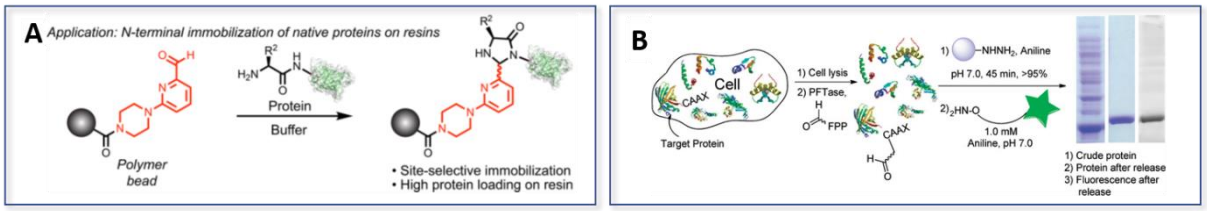


Figure 10. A) Application of the selective strategy to graft a protein onto polymer beads (Koo et al., 2019). B) Grafting of a protein to a hydrazine-functionalized bead using the CAAX motif, an aldehyde-bearing farnesyl group, and farnesyl transferase (Rashidian et al., 2012).

4.2 Immobilization via C-terminus

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

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

4.3 Immobilization via unnatural internal amino acids

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

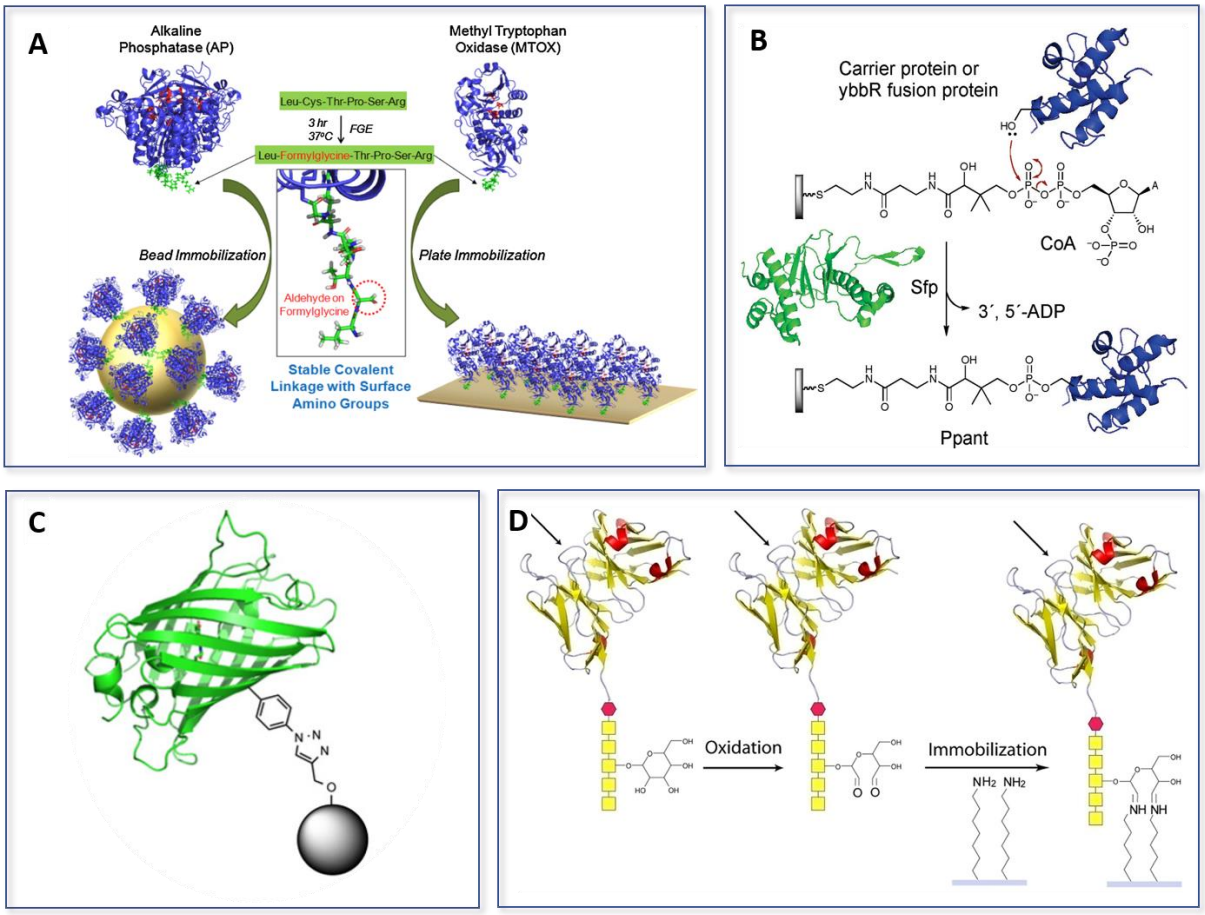


Figure 11. A) In Situ generation of the reactive protein (Cho and Jaworski, 2014). B) Enzyme-catalysed anchoring of protein on PEGA surfaces (Wong et al., 2008). C) Covalent anchoring of GFP via huisgen cyclisation on an unnatural p-azido phenylalanine (Raliski et al., 2014). D) Covalent attachment of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

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

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

In their 2015 paper, Yang et al. (Yang et al., 2015) described a method to tether TGF-β1, whose biochemical activity depends on whether it is free or attached, to pegylated surfaces. To achieve this, they used a variation of the Staudinger ligation, where surface-bound azido groups are reacted with Lysine-bound phosphine moieties. With this chemoselective anchoring strategy, the authors were able to prepare biofunctional beads that successfully converted $CD4^{+}$ $CD62L^{hi}$ T cells into functional regulatory T cells.

4.4 Immobilization via saccharide units on proteins and antibodies

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

4.4.1 On oxidized glycosyl units

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

Another example was given by Yuan. First, stainless steel substrates were coated with ethylene vinyl acetate, then treated with O_2 plasma, and silanized with aminopropyl triethoxy silane (APTES) to create amine groups (labeled SCA-SS). Amines were then coupled with the oxidized carbohydrates and successful binding was assessed via cell uptake by the anti-CD34 antibody (Yuan et al., 2011).

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

4.4.2 Using boronic esters from glyco units and boronic acids

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

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

of the boronic ester formation.

4.5 On exogenous groups

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

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

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 or 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

5 Characterizations of the grafting on polymers

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

5.1 Revealing the presence of proteins on the surface

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

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

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

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

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

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

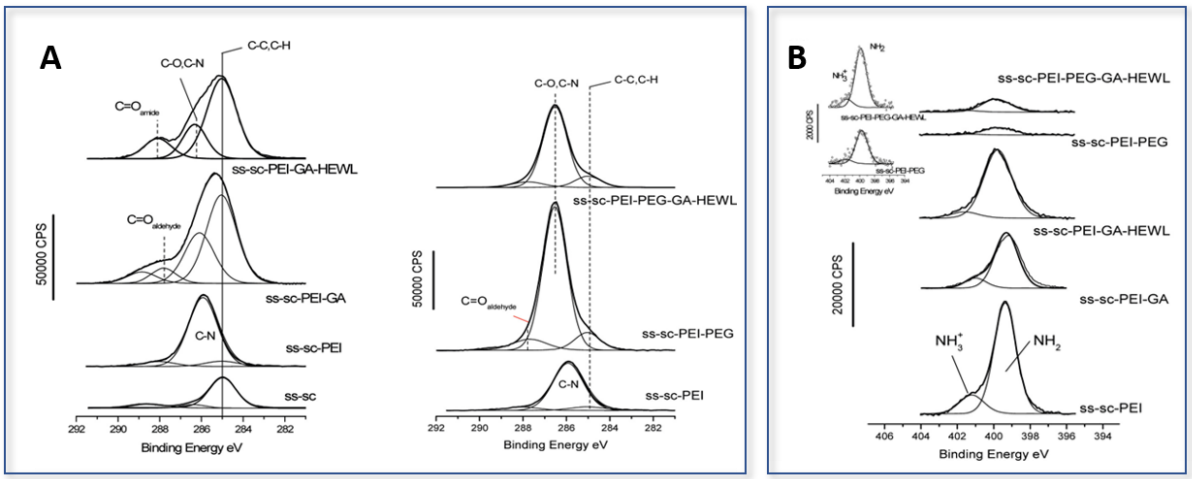


Figure 12. High Resolution XPS spectra C 1s A) and N 1s B) of coated stainless-steel surfaces. Spectra are used to identify and quantify the type of bonds represented on the surface in order to prove they efficiency of coating (Caro et al., 2009).

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

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

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

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

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

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

5.3 Describing the structure of the immobilized protein

Modifications of the structure of the protein/enzyme can completely modify their compatibility/activity. Usually, the only concern is about the functionality of the object so it could be possible to test it to get sufficient result (measurement of enzymatic activity on a surface for example). But sometimes, it can be important to get more information on the structure in order to be sure that no modifications happened. Circular Dichroism (CD) can be used to determine the structure of proteins adsorbed using a comparison of the results obtained on different kinds of surfaces such as quartz and Teflon for example (Vermeer and Norde, 2000). CD can also be used to observe modifications of the secondary and tertiary structure of enzymes such as horseradish peroxidase after it has been crosslinked with dendrimers to create nanoparticles (Khosravi et al., 2012).

Microcalorimetry can also be very specifically used to determine the effect of grafting on the structure of the proteins. It is for example possible to compare the effect on the structure of a single or multipoint grafting using glutaraldehyde on silica glass (Battistel and Rialdi, 2006).

5.4 Describing grafting density

It is often key to get knowledge of the grafting density in order to determine enzyme specific activity for example. It can also be important with proteins when they are used as an anchor for subsidiary reactions.

XPS can be used to solve this kind of problematics. As seen before, the method is very powerful and can be used to follow the different steps of functionalization and grafting (Abbas et al., 2009) on different types of surfaces such as metals functionalized with poly(ethylene glycol) (PEG) (Caro et al., 2009) or even on electroactive polymers (Loh et al., 1996). By studying the intensity of bands related to certain types of bonds, it becomes possible to quantify the surface density. To get solid results, it is mandatory to analyze different types of bonds because precision can vary in large proportions. In the case of enzymes, it can be very interesting to compare the results with those obtained with enzymatic activity measurements (Ghasemi et al., 2011; Zheng et al., 2015). This type of comparison shows how the use of different types of bonds can deeply modify the results obtained.

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

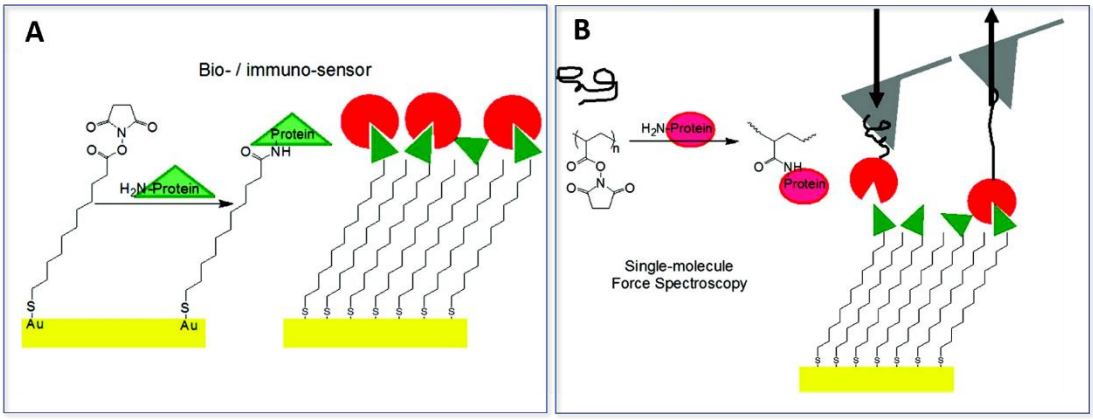


Figure 13. Schematic Representation of A) the Covalent Grafting of Proteins onto the Surface of the Biological Device and of B) a Biological Recognition Event Investigated by Single-Molecule Force Spectroscopy Experiment from (Cecchet et al., 2007).

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

5.5 Describing the distribution of the proteins on the surface

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

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

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

5.6 Describing enzymatic activity of the grafted surface

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

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

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

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

5.7 Transversal techniques

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

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

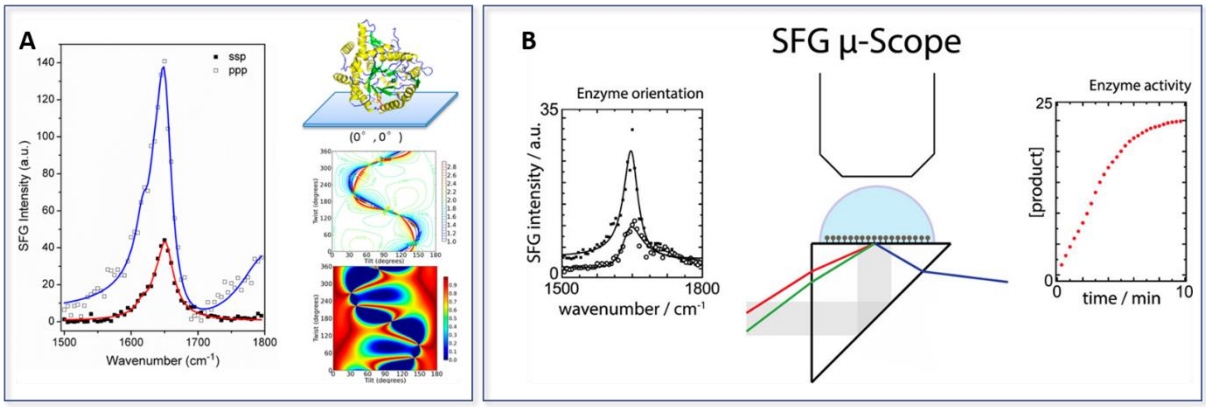


Figure 14. A) SFG spectra collected from β-Gal-V152C immobilized at the Mal-EG4 SAM–solution interface. (A right side) 1-Orientation of β-Gal with (tilt angle, twist angle) = (0°, 0°). 2-Dependence of the SFG χzzz/χxxz ratio on the tilt and twist angles of β-Gal-V152C calculated using the newly developed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

Enzymatic activity measurements can also be an interesting way to characterize the surface after grafting. The method gives access to multiple information such as the quantification of enzymes on the surface but also check their stability over time. It even can be used to obtain an idea of the grafting orientation (homogeneous or not) and also check if enzymatic activity is impacted by the grafting process. The measurement can be done following the evolution of the media due to substrate evolution. It is usually performed using UV-Vis spectroscopy measurements because they are very simple to set up. It is for example possible to follow, by absorbance measurements at 420 nm, the evolution of concentration of H_2O_2 in solution due to peroxidase activity (Amounas et al., 2000). Other substrates can be used with corresponding wavelength analysis for different types of enzymes such as lysozyme (450 nm) or trypsin (253 nm) (Caro et al., 2010).

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

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

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

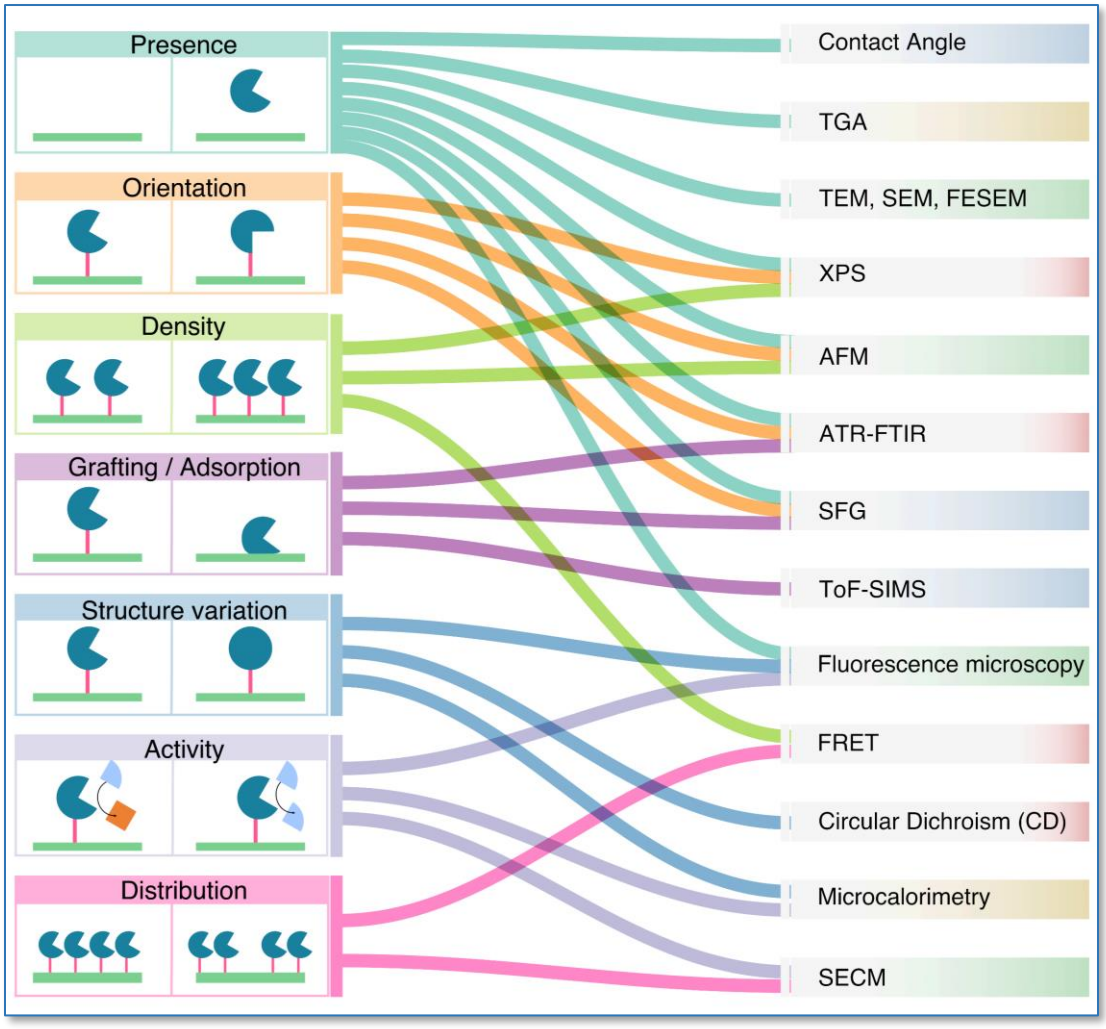


Figure 15. Sankey diagram of the analytical techniques used to answer biochemical questions. Analytical techniques are classified by colors with ● (Microscopy techniques), ● (Spectroscopy techniques), ● (Thermal analysis techniques), ● (Other types of techniques).

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 of the atom it comes from. XPS determines the atom composition on the surface with a maximum deepness 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

6 Applications

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

6.1 Industrial applications

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

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

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

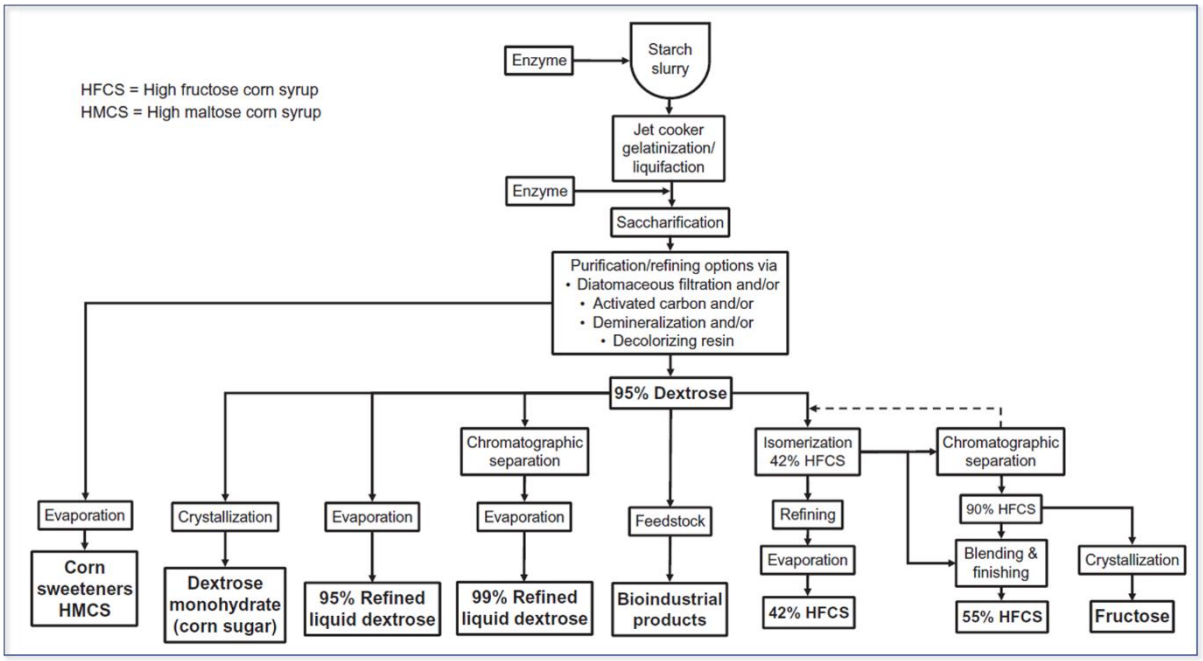


Figure 16. General process overview of enzyme/enzyme for production of corn syrup, from (Helstad, 2019). Maize or wheat starch is hydrolyzed through a total enzyme process, producing syrups such as dextrose, HMCs and HFCS mainly by the action of α-amylase, glucoamylase and glucose isomerase, respectively.

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

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

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

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

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

6.2 Laboratory scale applications

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

6.2.1 Rigid polymers

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

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

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

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

An efficient way to covalently graft protein to rigid polymers would be to benefit from synthetic polymers exhibiting surface chemical groups. Poly(glycidyl methacrylate-*co*-methylacrylate) (P(GMA-*co*-MA)) or poly(styrene-*co*-maleic anhydride) (PSMA) are strong electron acceptor polymers which can undergo rapid reaction at pHs compatible with protein or enzyme. Plant biomass conversion to biofuel is of increasing importance and is already a reality as a renewable and clean source of energy. It requires a large variety of carbohydrate active enzymes to deconstruct plant polymers to monosaccharides later fermented into ethanol by yeast (Mohd Azhar et al., 2017). Biodiesel, chemically consisting of methyl esters of long-chain fatty acids, is also a biofuel but is derived by using the catalytic transesterification of animal or plant oils with methanol (Sharma et al., 2008). Chemical industrial production of biodiesel, however, did not satisfy the increasing environmental concerns, and lipase, an esterase that catalyzes the hydrolysis of ester bonds of lipid, could address these hurdles. Xie and collaborators recently described the immobilization of a lipase from *Candida rugosa* on magnetic organic polymer (Xie and Huang, 2020). The support consisted in Fe_3O_4 nanoparticles with a synthetic

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

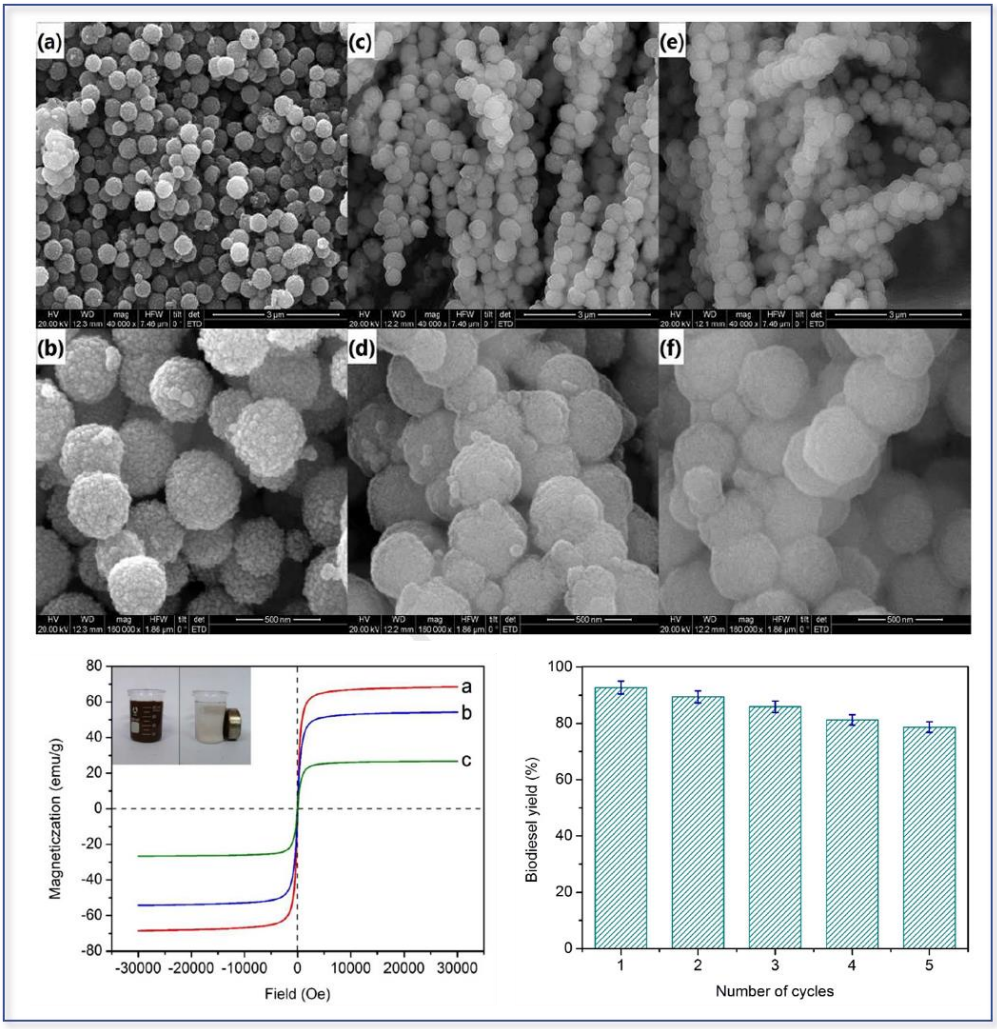


Figure 17. SEM images of Fe_3O_4 (a, b), Fe_3O_4-poly(GMA-co-MAA) (c, d) and the immobilized lipase (e,f). Bottom left, room temperature magnetization curves of Fe_3O_4 (a), Fe_3O_4-poly(GMA-co-MAA) (b) and the immobilized lipase (c). Bottom right, the recycling test results of the immobilized lipase for transesterification of soybean oil. From *(Xie and Huang, 2020)*

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

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

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

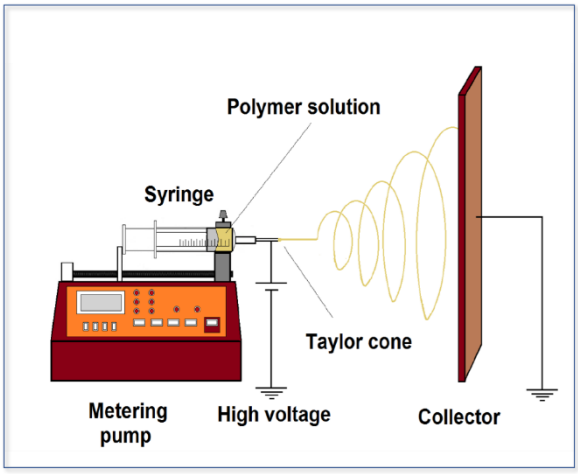


Figure 18. Schematic diagram of set up of electrospinning apparatus horizontal set up, from (Riazi et al., 2016).

6.2.2 Elastomers

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

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

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

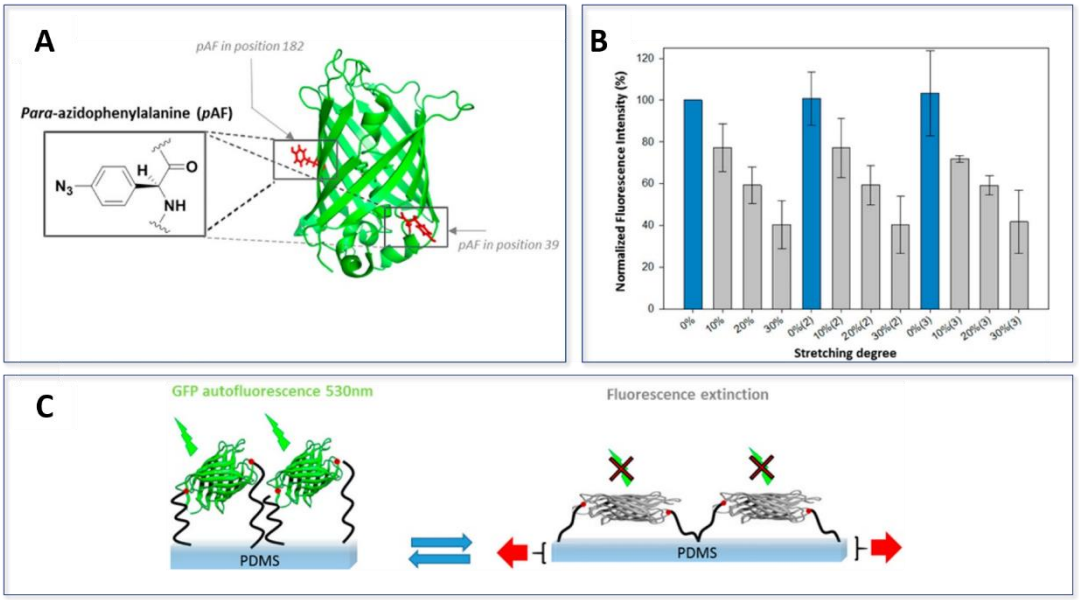


Figure 19. (a) GFP modified genetically at two opposite positions of the β-barrel with a unnatural amino acid bearing a para-azidophenyl group (pAF). (b) Schematic representation of the GFP covalently linked through POE linkers onto a modified PDMS sheet at rest and stretched. (c) Evolution of the normalized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

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

of the stretch.

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

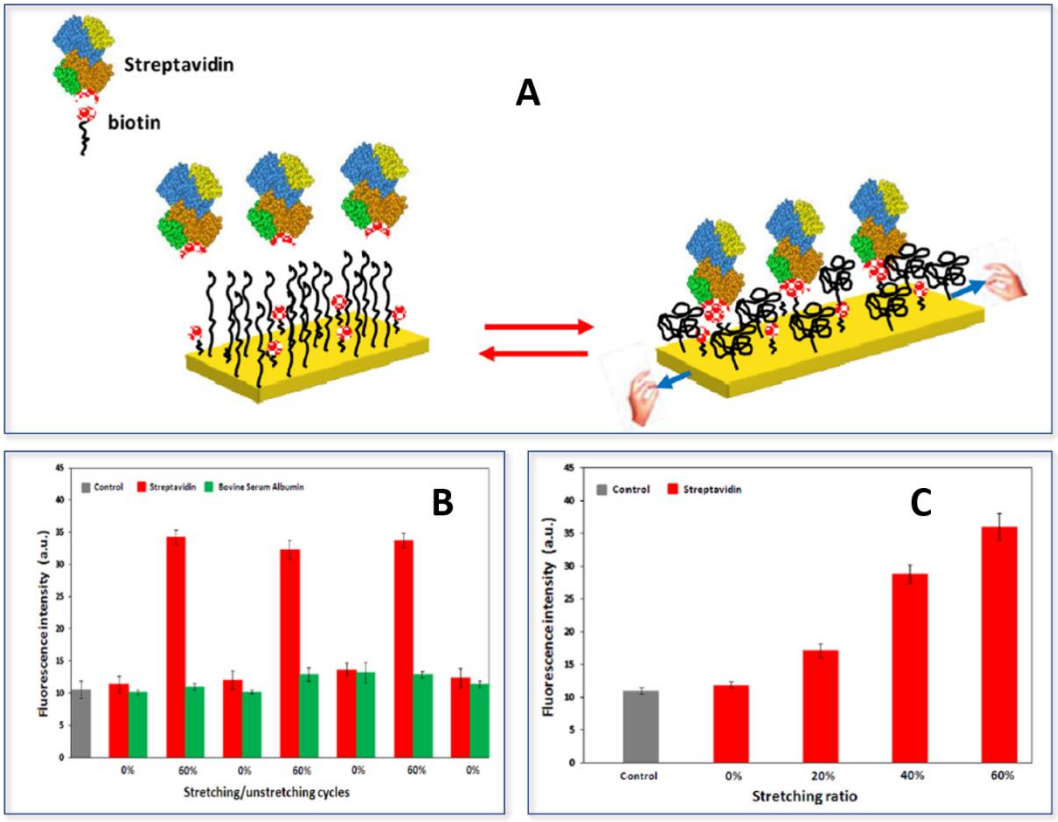


Figure 20. (A) Schematic representation of the fully reversible cryptic site mechanoresponsive surface. (B) Evolution of the fluorescence intensity for a series of three stretching (60%)/unstretching (0%) cycles. (C) Evolution of the fluorescence intensity at various stretching ratios: 0% (nonstretched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

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

6.2.3 Natural polymers

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

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

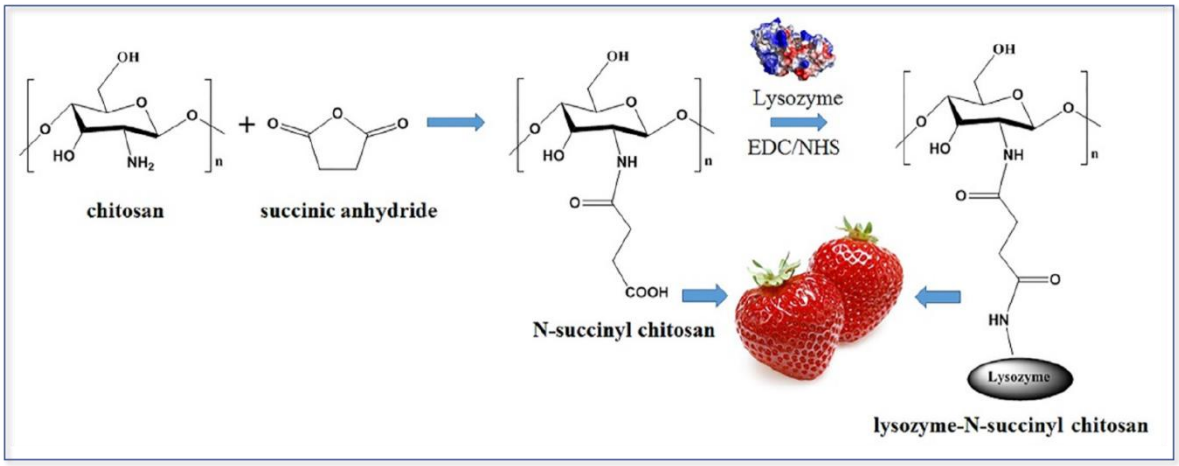


Figure 21. Illustration of the preparation of water-soluble N-succinyl chitosan and application as lysozyme-N-succinyl chitosan for fruit preservation, (from (Niu et al., 2020).

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

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

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

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

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

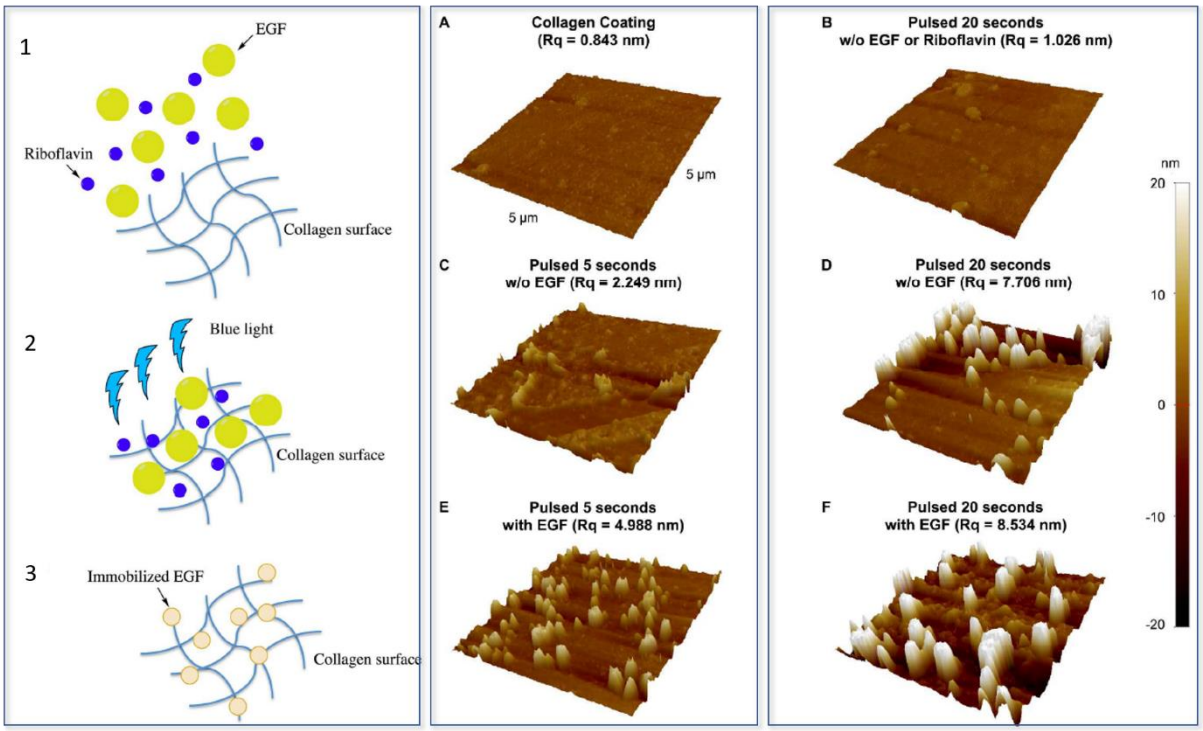


Figure 22. Photochemically immobilizing of epidermal growth factor (EGF) on collagen surface using riboflavin as a photosensitizer (step 1 to 3). Topography of dry collagen-coated surfaces measured by AFM. (A) Collagen coating without treatment, (B) after blue light exposure alone for 20 seconds without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

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

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

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

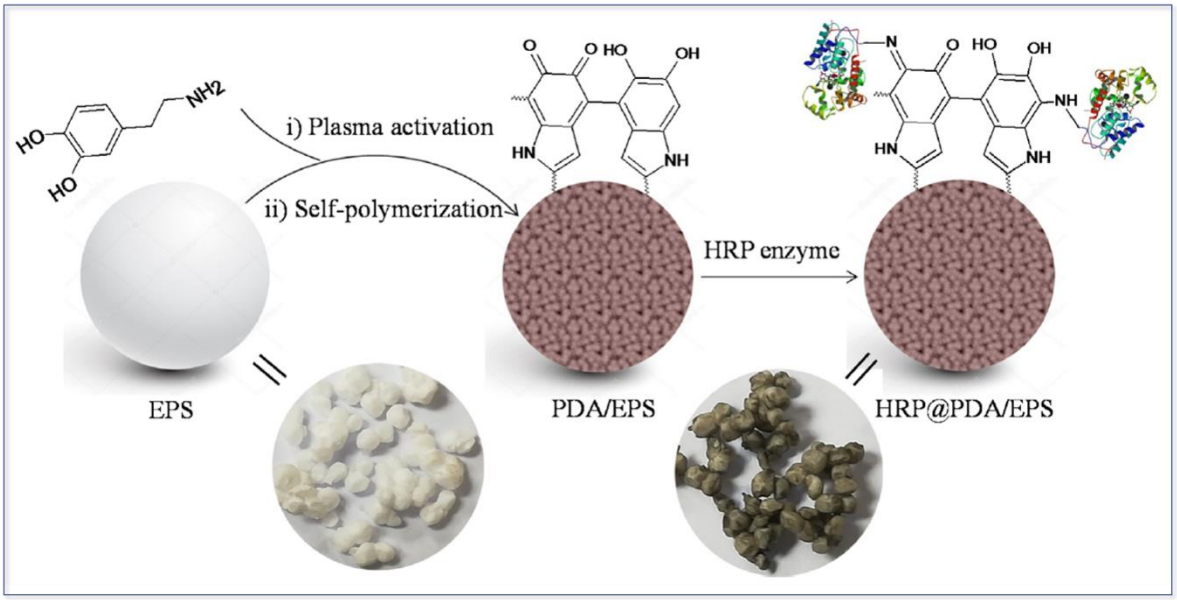


Figure 23. Scheme representing coating of expanded polystyrene foam (EPS) with polydopamine (PDA) layer followed by horseradish peroxidase (HRP) immobilization to realize HRP@PDA/EPS, from (Yassin and Gad, 2020).

7 Conclusion

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

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

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

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

Author contributions

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

Credit authorship contribution statement

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

Declaration of Competing Interest

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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References

360 Market updates, 2019. global galactooligosaccharides market. URL https://www.360marketupdates.com/global-galactooligosaccharides-gos-market-13729874

Abbas, A., Vercaigne-Marko, D., Supiot, P., Bocquet, B., Vivien, C., Guillochon, D., 2009. Covalent attachment of trypsin on plasma polymerized allylamine. Colloids and Surfaces B: Biointerfaces 73, 315–324. https://doi.org/10.1016/j.colsurfb.2009.06.007

Alatawi, F.S., Monier, M., Elsayed, N.H., 2018. Amino functionalization of carboxymethyl cellulose for efficient immobilization of urease. International Journal of Biological Macromolecules 114, 1018–1025. https://doi.org/10.1016/j.ijbiomac.2018.03.142

Al-Lolage, F.A., Bartlett, P.N., Gounel, S., Staigre, P., Mano, N., 2019. Site-Directed Immobilization of Bilirubin Oxidase for Electrocatalytic Oxygen Reduction. ACS Catal. 9, 2068–2078. https://doi.org/10.1021/acscatal.8b04340

Amounas, M., Innocent, C., Cosnier, S., Seta, P., 2000. A membrane based reactor with an enzyme immobilized by an avidin–biotin molecular recognition in a polymer matrix. Journal of Membrane Science 176, 169–176. https://doi.org/10.1016/S0376-7388(00)00441-5

Arica, M.Y., Salih, B., Celikbicak, O., Bayramoglu, G., 2017. Immobilization of laccase on the fibrous polymer-grafted film and study of textile dye degradation by MALDI–ToF-MS. Chemical Engineering Research and Design 128, 107–119. https://doi.org/10.1016/j.cherd.2017.09.023

Asakura, T., Kanetake, J., Demura, M., 1989. Preparation and Properties of Covalently Immobilized Alkaline Phosphatase on *Bombyx Mori* Silk Fibroin Fiber. Polymer-Plastics Technology and Engineering 28, 453–469. https://doi.org/10.1080/03602558908048608

Axén, R., Porath, J., Ernback, S., 1967. Chemical Coupling of Peptides and Proteins to Polysaccharides by Means of Cyanogen Halides. Nature 214, 1302–1304. https://doi.org/10.1038/2141302a0

Bacharouche, J., Badique, F., Fahs, A., Spanedda, M.V., Geissler, A., Malval, J.-P., Vallat, M.-F., Anselme, K., Francius, G., Frisch, B., Hemmerlé, J., Schaaf, P., Roucoules, V., 2013. Biomimetic Cryptic Site Surfaces for Reversible Chemo- and Cyto-Mechanoresponsive Substrates. ACS Nano 7, 3457–3465. https://doi.org/10.1021/nn400356p

Baker, D.P., Lin, E.Y., Lin, K., Pellegrini, M., Petter, R.C., Chen, L.L., Arduini, R.M., Brickelmaier, M., Wen, D., Hess, D.M., Chen, L., Grant, D., Whitty, A., Gill, A., Lindner, D.J., Pepinsky, R.B., 2006. N-Terminally PEGylated Human Interferon-β-1a with Improved Pharmacokinetic Properties and in Vivo Efficacy in a Melanoma Angiogenesis Model. Bioconjugate Chem. 17, 179–188. https://doi.org/10.1021/bc050237q

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., 2015. Strategies for the one-step immobilization–purification of enzymes as industrial biocatalysts. Biotechnology Advances 33, 435–456. https://doi.org/10.1016/j.biotechadv.2015.03.006

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R.C., Fernandez-Lafuente, R., 2014. Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. RSC Adv. 4, 1583–1600. https://doi.org/10.1039/C3RA45991H

Barbosa, O., Torres, R., Ortiz, C., Berenguer-Murcia, Á., Rodrigues, R.C., Fernandez-Lafuente, R., 2013. Heterofunctional Supports in Enzyme Immobilization: From Traditional Immobilization Protocols to Opportunities in Tuning Enzyme Properties. Biomacromolecules 14, 2433–2462. https://doi.org/10.1021/bm400762h

Barbosa, O., Torres, R., Ortiz, C., Fernandez-Lafuente, R., 2012. The slow-down of the CALB immobilization rate permits to control the inter and intra molecular modification produced by glutaraldehyde. Process Biochemistry 47, 766–774. https://doi.org/10.1016/j.procbio.2012.02.009

Basso, A., Serban, S., 2019. Industrial applications of immobilized enzymes—A review. Molecular Catalysis 479, 110607. https://doi.org/10.1016/j.mcat.2019.110607

Baştürk, E., Demir, S., Danış, Ö., Kahraman, M.V., 2013. Covalent immobilization of α-amylase onto thermally crosslinked electrospun PVA/PAA nanofibrous hybrid membranes. J. Appl. Polym. Sci. 127, 349–355. https://doi.org/10.1002/app.37901

Batalov, I., Stevens, K.R., DeForest, C.A., 2021. Photopatterned biomolecule immobilization to guide three-dimensional cell fate in natural protein-based hydrogels. Proc. Natl. Acad. Sci. U.S.A. 118, e2014194118. https://doi.org/10.1073/pnas.2014194118

Battistel, E., Rialdi, G., 2006. Characterization of Immobilized Enzymes by Microcalorimetry, in: Guisan, J.M. (Ed.), Immobilization of Enzymes and Cells, Methods in Biotechnology™. Humana Press, Totowa, NJ, pp. 295–310. https://doi.org/10.1007/978-1-59745-053-9_26

Becker, H., 2002. Polymer microfluidic devices. Talanta 56, 267–287. https://doi.org/10.1016/S0039-9140(01)00594-X
Bedade, D.K., Sutar, Y.B., Singhal, R.S., 2019. Chitosan coated calcium alginate beads for covalent immobilization of acrylamidase: Process parameters and removal of acrylamide from coffee. Food Chemistry 275, 95–104. https://doi.org/10.1016/j.foodchem.2018.09.090
Beddows, C.G., Guthrie, J.T., 1980. The use of graft copolymers as enzyme supports. Polymer Bulletin 3, 645–653.
Benjamins, F., Cao, L., Broekhuis, A.A., 2015. Production of Galacto-Oligosaccharides.
Bezerra, C.S., de Farias Lemos, C.M.G., de Sousa, M., Gonçalves, L.R.B., 2015. Enzyme immobilization onto renewable polymeric matrixes: Past, present, and future trends. J. Appl. Polym. Sci. 132, 42125. https://doi.org/10.1002/app.42125
Biggelaar, L. van den, Soumillion, P., Debecker, D., 2019. Continuous Flow Mode Biocatalytic Transamination Using Macrocellular Silica Monoliths: Optimizing Support Functionalisation and Enzyme Grafting. https://doi.org/10.26434/chemrxiv.7853552.v1
Bilal, M., Iqbal, H.M.N., 2019a. Chemical, physical, and biological coordination: An interplay between materials and enzymes as potential platforms for immobilization. Coordination Chemistry Reviews 388, 1–23. https://doi.org/10.1016/j.ccr.2019.02.024
Bilal, M., Iqbal, H.M.N., 2019b. Naturally-derived biopolymers: Potential platforms for enzyme immobilization. International Journal of Biological Macromolecules 130, 462–482. https://doi.org/10.1016/j.ijbiomac.2019.02.152
Bilal, M., Iqbal, H.M.N., 2019c. Naturally-derived biopolymers: Potential platforms for enzyme immobilization. International Journal of Biological Macromolecules 130, 462–482. https://doi.org/10.1016/j.ijbiomac.2019.02.152
Bolivar, J.M., Rocha-Martin, J., Mateo, C., Cava, F., Berenguer, J., Vega, D., Fernandez-Lafuente, R., Guisan, J.M., 2009. Purification and stabilization of a glutamate dehygrogenase from Thermus thermophilus via oriented multisubunit plus multipoint covalent immobilization. Journal of Molecular Catalysis B: Enzymatic 58, 158–163. https://doi.org/10.1016/j.molcatb.2008.12.010
Bolivar, J.M., Woodley, J.M., Fernandez-Lafuente, R., 2022. Is enzyme immobilization a mature discipline? Some critical considerations to capitalize on the benefits of immobilization. Chem. Soc. Rev. 51, 6251–6290. https://doi.org/10.1039/D2CS00083K
Bonazza, H.L., Manzo, R.M., dos Santos, J.C.S., Mammarella, E.J., 2018. Operational and Thermal Stability Analysis of Thermomyces lanuginosus Lipase Covalently Immobilized onto Modified Chitosan Supports. Appl Biochem Biotechnol 184, 182–196. https://doi.org/10.1007/s12010-017-2546-9
Boudrant, J., Woodley, J.M., Fernandez-Lafuente, R., 2020. Parameters necessary to define an immobilized enzyme preparation. Process Biochemistry 90, 66–80. https://doi.org/10.1016/j.procbio.2019.11.026
Bratek-Skicki, A., 2021. Towards a new class of stimuli-responsive polymer-based materials – Recent advances and challenges. Applied Surface Science Advances 4, 100068. https://doi.org/10.1016/j.apsadv.2021.100068
Brena, B., González-Pombo, P., Batista-Viera, F., 2013. Immobilization of Enzymes: A Literature Survey, in: Guisan, J.M. (Ed.), Immobilization of Enzymes and Cells. Humana Press, Totowa, NJ, pp. 15–31. https://doi.org/10.1007/978-1-62703-550-7_2
Bruggink, A., Roos, E.C., de Vroom, E., 1998. Penicillin Acylase in the Industrial Production of β-Lactam Antibiotics. Org. Process Res. Dev. 2, 128–133. https://doi.org/10.1021/op9700643
Çakıroğlu, B., Çiğil, A.B., Ogan, A., Kahraman, M.V., Demir, S., 2018. Covalent immobilization of acetylcholinesterase on a novel polyacrylic acid-based nanofiber membrane. Eng. Life Sci. 18, 254–262. https://doi.org/10.1002/elsc.201700130
Campeau, M.-A., Lortie, A., Tremblay, P., Béliveau, M.-O., Dubé, D., Langelier, È., Rouleau, L., 2017. Effect of manufacturing and experimental conditions on the mechanical and surface properties of silicone elastomer scaffolds used in endothelial mechanobiological studies. BioMedical Engineering OnLine 16, 90. https://doi.org/10.1186/s12938-017-0380-5
Carano, M., Lion, N., Girault, H.H., 2007. Detection of proteins on membranes and in microchannels using copper staining combined with scanning electrochemical microscopy. Journal of Electroanalytical Chemistry, Special Issue In Honour of David Schiffrin 599, 349–355.

https://doi.org/10.1016/j.jelechem.2006.06.019
Caro, A., Humblot, V., Méthivier, C., Minier, M., Barbes, L., Li, J., Salmain, M., Pradier, C.-M., 2010. Bioengineering of stainless steel surface by covalent immobilization of enzymes. Physical characterization and interfacial enzymatic activity. Journal of Colloid and Interface Science 349, 13–18. https://doi.org/10.1016/j.jcis.2009.12.001
Caro, A., Humblot, V., Méthivier, C., Minier, M., Salmain, M., Pradier, C.-M., 2009. Grafting of Lysozyme and/or Poly(ethylene glycol) to Prevent Biofilm Growth on Stainless Steel Surfaces. J. Phys. Chem. B 113, 2101–2109. https://doi.org/10.1021/jp805284s
Cecchet, F., Duwez, A.-S., Gabriel, S., Jérôme, C., Jérôme, R., Glinel, K., Demoustier-Champagne, S., Jonas, A.M., Nysten, B., 2007. Atomic Force Microscopy Investigation of the Morphology and the Biological Activity of Protein-Modified Surfaces for Bio- and Immunosensors. Anal. Chem. 79, 6488–6495. https://doi.org/10.1021/ac070155q
Cen, Y., Liu, Y., Xue, Y., Zheng, Y., 2019. Immobilization of Enzymes in/on Membranes and their Applications. Adv. Synth. Catal. 361, 5500–5515. https://doi.org/10.1002/adsc.201900439
Cha, T., Guo, A., Zhu, X.-Y., 2005. Enzymatic activity on a chip: the critical role of protein orientation. Proteomics 5, 416–419.
Chan, A.O.-Y., Ho, C.-M., Chong, H.-C., Leung, Y.-C., Huang, J.-S., Wong, M.-K., Che, C.-M., 2012. Modification of N-terminal α-amino groups of peptides and proteins using ketenes. Journal of the American Chemical Society 134, 2589–2598.
Chapman, J., Ismail, A., Dinu, C., 2018. Industrial Applications of Enzymes: Recent Advances, Techniques, and Outlooks. Catalysts 8, 238. https://doi.org/10.3390/catal8060238
Chauhan, D., Gupta, P.K., Solanki, P.R., 2018. Electrochemical immunosensor based on magnetite nanoparticles incorporated electrospun polyacrylonitrile nanofibers for Vitamin-D3 detection. Materials Science and Engineering: C 93, 145–156. https://doi.org/10.1016/j.msec.2018.07.036
Chen, H., Cheng, K., Hsu, R., Hsieh, C., Wang, H., Ting, Y., 2020. Enzymatic degradation of ginkgolic acid by laccase immobilized on novel electrospun nanofiber mat. J Sci Food Agric 100, 2705–2712. https://doi.org/10.1002/jsfa.10301
Cho, H., Jaworski, J., 2014. Enzyme directed formation of un-natural side-chains for covalent surface attachment of proteins. Colloids and Surfaces B: Biointerfaces 122, 846–850. https://doi.org/10.1016/j.colsurfb.2014.08.010
Cortina, M., Esplandiu, M.J., Alegret, S., del Valle, M., 2006. Urea impedimetric biosensor based on polymer degradation onto interdigitated electrodes. Sensors and Actuators B: Chemical 118, 84–89. https://doi.org/10.1016/j.snb.2006.04.062
Dai, X.-Y., Kong, L.-M., Wang, X.-L., Zhu, Q., Chen, K., Zhou, T., 2018. Preparation, characterization and catalytic behavior of pectinase covalently immobilized onto sodium alginate/graphene oxide composite beads. Food Chemistry 253, 185–193. https://doi.org/10.1016/j.foodchem.2018.01.157
Davis, D.A., Hamilton, A., Yang, J., Cremar, L.D., Van Gough, D., Potisek, S.L., Ong, M.T., Braun, P.V., Martínez, T.J., White, S.R., Moore, J.S., Sottos, N.R., 2009. Force-induced activation of covalent bonds in mechanoresponsive polymeric materials. Nature 459, 68–72. https://doi.org/10.1038/nature07970
Delaittre, G., Guimard, N.K., Barner-Kowollik, C., 2015. Cycloadditions in Modern Polymer Chemistry. Acc. Chem. Res. 48, 1296–1307. https://doi.org/10.1021/acs.accounts.5b00075
DiCosimo, R., McAuliffe, J., Poulose, A.J., Bohlmann, G., 2013. Industrial use of immobilized enzymes. Chem. Soc. Rev. 42, 6437. https://doi.org/10.1039/c3cs35506c
dos Santos, J.C.S., Bonazza, H.L., de Matos, L.J.B.L., Carneiro, E.A., Barbosa, O., Fernandez-Lafuente, R., Gonçalves, L.R.B., de Sant' Ana, H.B., Santiago-Aguiar, R.S., 2017. Immobilization of CALB on activated chitosan: Application to enzymatic synthesis in supercritical and near-critical carbon dioxide. Biotechnology Reports 14, 16–26. https://doi.org/10.1016/j.btre.2017.02.003
dos Santos, Jose C. S., Rueda, N., Sanchez, A., Villalonga, R., Gonçalves, L.R.B., Fernandez-Lafuente, R., 2015. Versatility of divinylsulfone supports permits the tuning of CALB properties during its immobilization. RSC Adv. 5, 35801–35810. https://doi.org/10.1039/C5RA03798K
dos Santos, Jose C.S., Rueda, N., Torres, R., Barbosa, O., Gonçalves, L.R.B., Fernandez-Lafuente, R., 2015. Evaluation of divinylsulfone activated agarose to immobilize lipases and to tune their

catalytic properties. Process Biochemistry 50, 918–927. https://doi.org/10.1016/j.procbio.2015.03.018
Dubey, M., Emoto, K., Takahashi, H., Castner, D.G., Grainger, D.W., 2009. Affinity-Based Protein Surface Pattern Formation by Ligand Self-Selection from Mixed Protein Solutions. Advanced Functional Materials 19, 3046–3055. https://doi.org/10.1002/adfm.200900809
Dumri, K., Hung Anh, D., 2014. Immobilization of Lipase on Silver Nanoparticles via Adhesive Polydopamine for Biodiesel Production. Enzyme Research 2014, e389739. https://doi.org/10.1155/2014/389739
El-Moghazy, A.Y., Soliman, E.A., Ibrahim, H.Z., Marty, J.-L., Istamboulie, G., Noguer, T., 2016. Biosensor based on electrospun blended chitosan-poly (vinyl alcohol) nanofibrous enzymatically sensitized membranes for pirimiphos-methyl detection in olive oil. Talanta 155, 258–264. https://doi.org/10.1016/j.talanta.2016.04.018
Enzymes Market Size, Share, Growth | Global Report [2020-2027] [WWW Document], n.d. URL https://www.fortunebusinessinsights.com/industry-reports/enzymes-market-100595 (accessed 7.20.22).
Facin, B.R., Melchiors, M.S., Valério, A., Oliveira, J.V., Oliveira, D. de, 2019. Driving Immobilized Lipases as Biocatalysts: 10 Years State of the Art and Future Prospects. Ind. Eng. Chem. Res. 58, 5358–5378. https://doi.org/10.1021/acs.iecr.9b00448
Fernandes-Cunha, G.M., Lee, H.J., Kumar, A., Kreymerman, A., Heilshorn, S., Myung, D., 2017. Immobilization of Growth Factors to Collagen Surfaces Using Pulsed Visible Light. Biomacromolecules 18, 3185–3196. https://doi.org/10.1021/acs.biomac.7b00838
Finbloom, J.A., Francis, M.B., 2018. Supramolecular strategies for protein immobilization and modification. Current Opinion in Chemical Biology 46, 91–98. https://doi.org/10.1016/j.cbpa.2018.05.023
Finetti, C., Sola, L., Pezzullo, M., Prosperi, D., Colombo, M., Riva, B., Avvakumova, S., Morasso, C., Picciolini, S., Chiari, M., 2016. Click Chemistry Immobilization of Antibodies on Polymer Coated Gold Nanoparticles. Langmuir 32, 7435–7441. https://doi.org/10.1021/acs.langmuir.6b01142
Flores-Rojas, G.G., López-Saucedo, F., Quezada-Miriel, M., Bucio, E., 2018. Grafting of glycerol methacrylate onto silicone rubber using γ-rays: derivatization to 2-oxoethyl methacrylate and immobilization of lysozyme. MRS Communications 8, 199–206. https://doi.org/10.1557/mrc.2018.16
Funtan, S., Michael, P., Binder, W.H., 2019. Synthesis and Mechanochemical Activity of Peptide-Based Cu(I) Bis(N-Heterocyclic Carbene) Complexes. Biomimetics 4, 24. https://doi.org/10.3390/biomimetics4010024
Gan, J., Bagheri, A.R., Aramesh, N., Gul, I., Franco, M., Almulaiky, Y.Q., Bilal, M., 2021. Covalent organic frameworks as emerging host platforms for enzyme immobilization and robust biocatalysis – A review. International Journal of Biological Macromolecules 167, 502–515. https://doi.org/10.1016/j.ijbiomac.2020.12.002
Gascón Pérez, V., Jiménez, M.B., Blanco Martín, R.M., Sánchez Sánchez, M., 2018. Semi-crystalline Fe-BTC MOF material as an efficient support for enzyme immobilization. https://doi.org/10.13039/501100003329
Ghasemi, M., Minier, M.J.G., Tatoulian, M., Chehimi, M.M., Arefi-Khonsari, F., 2011. Ammonia Plasma Treated Polyethylene Films for Adsorption or Covalent Immobilization of Trypsin: Quantitative Correlation between X-ray Photoelectron Spectroscopy Data and Enzyme Activity. J. Phys. Chem. B 115, 10228–10238. https://doi.org/10.1021/jp204097a
Ghasemi-Mobarakeh, L., Prabhakaran, M.P., Morshed, M., Nasr-Esfahani, M.H., Ramakrishna, S., 2010. Bio-functionalized PCL nanofibrous scaffolds for nerve tissue engineering. Materials Science and Engineering: C 30, 1129–1136. https://doi.org/10.1016/j.msec.2010.06.004
Glidle, A., Yasukawa, T., Hadyoon, C.S., Anicet, N., Matsue, T., Nomura, M., Cooper, J.M., 2003. Analysis of Protein Adsorption and Binding at Biosensor Polymer Interfaces Using X-ray Photon Spectroscopy and Scanning Electrochemical Microscopy. Anal. Chem. 75, 2559–2570. https://doi.org/10.1021/ac0261653
Grazu, V., Betancor, L., Montes, T., Lopez-Gallego, F., Guisan, J.M., Fernandez-Lafuente, R., 2006. Glyoxyl agarose as a new chromatographic matrix. Enzyme and Microbial Technology 38, 960–

966. https://doi.org/10.1016/j.enzmictec.2005.08.034
Grosjean, L., Cherif, B., Mercey, E., Roget, A., Levy, Y., Marche, P.N., Villiers, M.-B., Livache, T., 2005. A polypyrrole protein microarray for antibody–antigen interaction studies using a label-free detection process. Analytical Biochemistry 347, 193–200. https://doi.org/10.1016/j.ab.2005.09.033
Guex, A.G., Hegemann, D., Giraud, M.N., Tevaearai, H.T., Popa, A.M., Rossi, R.M., Fortunato, G., 2014. Covalent immobilisation of VEGF on plasma-coated electrospun scaffolds for tissue engineering applications. Colloids and Surfaces B: Biointerfaces 123, 724–733. https://doi.org/10.1016/j.colsurfb.2014.10.016
Guisan, J.M. (Ed.), 2013. Immobilization of Enzymes and Cells: Third Edition, Methods in Molecular Biology. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-62703-550-7
Guisan, J.M., Fernandez-Lorente, G., Rocha-Martin, J., Moreno-Gamero, D., 2022. Enzyme immobilization strategies for the design of robust and efficient biocatalysts. Current Opinion in Green and Sustainable Chemistry 35, 100593. https://doi.org/10.1016/j.cogsc.2022.100593
Guisán, JoséM., 1988. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. Enzyme and Microbial Technology 10, 375–382. https://doi.org/10.1016/0141-0229(88)90018-X
Gupta, S., Kumar, Y., Singh, K., Bhattacharya, A., 2010. Lipase immobilized on poly (vinyl alcohol) modified polysulfone membrane: application in hydrolytic activities for olive oil. Polym. Bull. 64, 141–158. https://doi.org/10.1007/s00289-009-0141-0
Handa, T., Hirose, A., Akino, T., Watanabe, K., Tsuchiya, H., n.d. Preparation of Immobilized a-Amylase Covalently Attached to Granular Polyacrylonitrile 12.
Handa, T., Hirose, A., Yoshida, S., Tsuchiya, H., 1982. The effect of methylacrylate on the activity of glucomylase immobilized on granular polyacrylonitrile. Biotechnol. Bioeng. 24, 1639–1652. https://doi.org/10.1002/bit.260240715
Harir, M., Bellahcene, M., Baratto, M.C., Pollini, S., Rossolini, G.M., Trabalzini, L., Fatarella, E., Pogni, R., 2018. Isolation and characterization of a novel tyrosinase produced by Sahara soil actinobacteria and immobilization on nylon nanofiber membranes. Journal of Biotechnology 265, 54–64. https://doi.org/10.1016/j.jbiotec.2017.11.004
Hassan, M.E., Yang, Q., Xiao, Z., Liu, L., Wang, N., Cui, X., Yang, L., 2019. Impact of immobilization technology in industrial and pharmaceutical applications. 3 Biotech 9, 440. https://doi.org/10.1007/s13205-019-1969-0
Hayashi, T., Hirayama, C., Iwatsuki, M., 1992. Papain immobilization onto porous poly(λ-methyl L-glutamate) beads. J. Appl. Polym. Sci. 44, 143–150. https://doi.org/10.1002/app.1992.070440115
Heidari-Keshel, S., Ahmadian, M., Biazar, E., Gazmeh, A., Rabiei, M., Adibi, M., Soufi M, A., Shabani, M., 2016. Surface modification of *Poly Hydroxybutyrate* (PHB) nanofibrous mat by collagen protein and its cellular study. Materials Technology 31, 799–805. https://doi.org/10.1080/10667857.2016.1258517
Helstad, S., 2019. Chapter 20 - Corn Sweeteners, in: Serna-Saldivar, S.O. (Ed.), Corn (Third Edition). AACC International Press, Oxford, pp. 551–591. https://doi.org/10.1016/B978-0-12-811971-6.00020-6
Homaei, A.A., Sajedi, R.H., Sariri, R., Seyfzadeh, S., Stevanato, R., 2010. Cysteine enhances activity and stability of immobilized papain. Amino Acids 38, 937–942. https://doi.org/10.1007/s00726-009-0302-3
Hsu, K.M., Sugar, J., 2016. Keratoconus and Other Corneal Diseases: Pharmacologic Cross-Linking and Future Therapy, in: Whitcup, S.M., Azar, D.T. (Eds.), Pharmacologic Therapy of Ocular Disease, Handbook of Experimental Pharmacology. Springer International Publishing, Cham, pp. 137–161. https://doi.org/10.1007/164_2016_23
Hu, X., Hortigüela, M.J., Robin, S., Lin, H., Li, Y., Moran, A.P., Wang, W., Wall, J.G., 2013. Covalent and Oriented Immobilization of scFv Antibody Fragments via an Engineered Glycan Moiety. Biomacromolecules 14, 153–159. https://doi.org/10.1021/bm301518p
Huan, L., Shi, Q.-H., 2021. Increasing immunoglobulin G adsorption in dextran-grafted protein A gels. Engineering in Life Sciences 21, 392–404. https://doi.org/10.1002/elsc.202000097
Huang, X.-J., Chen, P.-C., Huang, F., Ou, Y., Chen, M.-R., Xu, Z.-K., 2011. Immobilization of Candida

rugosa lipase on electrospun cellulose nanofiber membrane. Journal of Molecular Catalysis B: Enzymatic 70, 95–100. https://doi.org/10.1016/j.molcatb.2011.02.010
Huang, X.-J., Ge, D., Xu, Z.-K., 2007. Preparation and characterization of stable chitosan nanofibrous membrane for lipase immobilization. European Polymer Journal 43, 3710–3718. https://doi.org/10.1016/j.eurpolymj.2007.06.010
Huang, X.-J., Yu, A.-G., Xu, Z.-K., 2008. Covalent immobilization of lipase from Candida rugosa onto poly(acrylonitrile-co-2-hydroxyethyl methacrylate) electrospun fibrous membranes for potential bioreactor application. Bioresource Technology 99, 5459–5465. https://doi.org/10.1016/j.biortech.2007.11.009
Isgrove, F.H., Williams, R.J.H., Niven, G.W., Andrews, A.T., 2001. Enzyme immobilization on nylon–optimization and the steps used to prevent enzyme leakage from the support. Enzyme and Microbial Technology 28, 225–232. https://doi.org/10.1016/S0141-0229(00)00312-4
Jain, M., Vaze, R.G., Ugrani, S.C., Sharma, K.P., 2018. Mechanoresponsive and recyclable biocatalytic sponges from enzyme-polymer surfactant conjugates and nanoparticles. RSC Adv. 8, 39029–39038. https://doi.org/10.1039/C8RA08221A
Jankowska, K., Zdarta, J., Grzywaczyk, A., Kijeńska-Gawrońska, E., Biadasz, A., Jesionowski, T., 2020. Electrospun poly(methyl methacrylate)/polyaniline fibres as a support for laccase immobilisation and use in dye decolourisation. Environmental Research 184, 109332. https://doi.org/10.1016/j.envres.2020.109332
Jasensky, J., Ferguson, K., Baria, M., Zou, X., McGinnis, R., Kaneshiro, A., Badieyan, S., Wei, S., Marsh, E.Neil.G., Chen, Z., 2018. Simultaneous Observation of the Orientation and Activity of Surface-Immobilized Enzymes. Langmuir 34, 9133–9140. https://doi.org/10.1021/acs.langmuir.8b01657
Jhuang, J.-R., Lin, S.-B., Chen, L.-C., Lou, S.-N., Chen, S.-H., Chen, H.-H., 2020. Development of immobilized laccase-based time temperature indicator by electrospinning zein fiber. Food Packaging and Shelf Life 23, 100436. https://doi.org/10.1016/j.fpsl.2019.100436
Jiang, H., Chen, W., Wang, J., Zhang, R., 2022. Selective N-terminal modification of peptides and proteins: Recent progresses and applications. Chinese Chemical Letters 33, 80–88. https://doi.org/10.1016/j.cclet.2021.06.011
Jin, F., Toda, K., 1988. Preparation of immobilized papain covalently bound on natural cellulose for treatment of beer. Biotechnol Lett 10, 221–223. https://doi.org/10.1007/BF01134834
Jochems, P., Satyawali, Y., Diels, L., Dejonghe, W., 2011. Enzyme immobilization on/in polymeric membranes: status, challenges and perspectives in biocatalytic membrane reactors (BMRs). Green Chem. 13, 1609. https://doi.org/10.1039/c1gc15178a
Jun, S.-H., Yang, J., Jeon, H., Kim, H.S., Pack, S.P., Jin, E., Kim, J., 2020. Stabilized and Immobilized Carbonic Anhydrase on Electrospun Nanofibers for Enzymatic CO_2 Conversion and Utilization in Expedited Microalgal Growth. Environ. Sci. Technol. 54, 1223–1231. https://doi.org/10.1021/acs.est.9b05284
Kalecki, J., Iskierko, Z., Cieplak, M., Sharma, P.S., 2020. Oriented Immobilization of Protein Templates: A New Trend in Surface Imprinting. ACS Sens. 5, 3710–3720. https://doi.org/10.1021/acssensors.0c01634
Kamat, R.K., Ma, W., Yang, Y., Zhang, Y., Wang, C., Kumar, C.V., Lin, Y., 2013. Adsorption and Hydrolytic Activity of the Polycatalytic Cellulase Nanocomplex on Cellulose. ACS Appl. Mater. Interfaces 5, 8486–8494. https://doi.org/10.1021/am401916k
Kang, H.-J., Cha, E.J., Park, H.-D., 2015. Protein immobilization onto various surfaces using a polymer-bound isocyanate. Applied Surface Science 324, 198–204. https://doi.org/10.1016/j.apsusc.2014.10.117
Kasálková, N.S., Slepička, P., Kolská, Z., Hodačová, P., Kučková, Š., Švorčík, V., 2014. Grafting of bovine serum albumin proteins on plasma-modified polymers for potential application in tissue engineering. Nanoscale Research Letters 9, 161. https://doi.org/10.1186/1556-276X-9-161
Katchalski-Katzir, E., Kraemer, D.M., 2000. Eupergit® C, a carrier for immobilization of enzymes of industrial potential. Journal of Molecular Catalysis B: Enzymatic 10, 157–176. https://doi.org/10.1016/S1381-1177(00)00124-7
Khademi, F., Ai, J., Soleimani, M., Verdi, J., Mohammad Tavangar, S., Sadroddiny, E., Massumi, M., Mahmoud Hashemi, S., 2017. Improved human endometrial stem cells differentiation into

functional hepatocyte-like cells on a glycosaminoglycan/collagen-grafted polyethersulfone nanofibrous scaffold: ENDOMETRIAL STEM CELL TO HEPATOCYTE-LIKE CELL. J. Biomed. Mater. Res. 105, 2516–2529. https://doi.org/10.1002/jbm.b.33758
Khosravi, A., Vossoughi, M., Shahrokhian, S., Alemzadeh, I., 2012. Nano reengineering of horseradish peroxidase with dendritic macromolecules for stability enhancement. Enzyme and Microbial Technology 50, 10–16. https://doi.org/10.1016/j.enzmictec.2011.09.004
Kilara, A., Shahani, K.M., Wagner, F.W., 1977. Preparation and properties of immobilized papain and lipase. Biotechnol. Bioeng. 19, 1703–1714. https://doi.org/10.1002/bit.260191109
Kim, B.C., Nair, S., Kim, J., Kwak, J.H., Grate, J.W., Kim, S.H., Gu, M.B., 2005. Preparation of biocatalytic nanofibres with high activity and stability via enzyme aggregate coating on polymer nanofibres. Nanotechnology 16, S382–S388. https://doi.org/10.1088/0957-4484/16/7/011
Kim, D., Herr, A.E., 2013. Protein immobilization techniques for microfluidic assays. Biomicrofluidics 7, 041501. https://doi.org/10.1063/1.4816934
Kim, Y.-P., Hong, M.-Y., Kim, J., Oh, E., Shon, H.K., Moon, D.W., Kim, H.-S., Lee, T.G., 2007. Quantitative Analysis of Surface-Immobilized Protein by TOF-SIMS: Effects of Protein Orientation and Trehalose Additive. Anal. Chem. 79, 1377–1385. https://doi.org/10.1021/ac0616005
Klibanov, A.M., 1979. Enzyme stabilization by immobilization. Analytical Biochemistry 93, 1–25. https://doi.org/10.1016/S0003-2697(79)80110-4
Koo, B., Dolan, N.S., Wucherer, K., Munch, H.K., Francis, M.B., 2019. Site-Selective Protein Immobilization on Polymeric Supports through N-Terminal Imidazolidinone Formation. Biomacromolecules. https://doi.org/10.1021/acs.biomac.9b01002
Kumakura, M., Kaetsu, I., 1984. Properties of Immobilized Papain by Radiation Polymerization. Polym J 16, 113–117. https://doi.org/10.1295/polymj.16.113
Kunamneni, A., Ghazi, I., Camarero, S., Ballesteros, A., Plou, F.J., Alcalde, M., 2008. Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. Process Biochemistry 43, 169–178. https://doi.org/10.1016/j.procbio.2007.11.009
Lavalle, P., Boulmedais, F., Schaaf, P., Jierry, L., 2016. Soft-Mechanochemistry: Mechanochemistry Inspired by Nature. Langmuir 32, 7265–7276. https://doi.org/10.1021/acs.langmuir.6b01768
Lee, K.H., Ki, C.S., Baek, D.H., Kang, G.D., Ihm, D.-W., Park, Y., 2005. Application of electrospun silk fibroin nanofibers as an immobilization support of enzyme. Fibers and Polymers 6, 181–185.
Li, S.-F., Chen, J.-P., Wu, W.-T., 2007. Electrospun polyacrylonitrile nanofibrous membranes for lipase immobilization. Journal of Molecular Catalysis B: Enzymatic 47, 117–124. https://doi.org/10.1016/j.molcatb.2007.04.010
Li, S.-F., Fan, Y.-H., Hu, J.-F., Huang, Y.-S., Wu, W.-T., 2011. Immobilization of Pseudomonas cepacia lipase onto the electrospun PAN nanofibrous membranes for transesterification reaction. Journal of Molecular Catalysis B: Enzymatic 73, 98–103. https://doi.org/10.1016/j.molcatb.2011.08.005
Li, S.-F., Wu, W.-T., 2009. Lipase-immobilized electrospun PAN nanofibrous membranes for soybean oil hydrolysis. Biochemical Engineering Journal 45, 48–53. https://doi.org/10.1016/j.bej.2009.02.004
Liang, S., Wu, X.-L., Xiong, J., Zong, M.-H., Lou, W.-Y., 2020. Metal-organic frameworks as novel matrices for efficient enzyme immobilization: An update review. Coordination Chemistry Reviews 406, 213149. https://doi.org/10.1016/j.ccr.2019.213149
Libertino, S., Giannazzo, F., Aiello, V., Scandurra, A., Sinatra, F., Renis, M., Fichera, M., 2008. XPS and AFM Characterization of the Enzyme Glucose Oxidase Immobilized on SiO2 Surfaces. Langmuir 24, 1965–1972. https://doi.org/10.1021/la7029664
Liu, S., Yu, B., Wang, S., Shen, Y., Cong, H., 2020. Preparation, surface functionalization and application of Fe3O4 magnetic nanoparticles. Advances in Colloid and Interface Science 281, 102165. https://doi.org/10.1016/j.cis.2020.102165
Liu, X., Fang, Y., Yang, X., Li, Y., Wang, C., 2018a. Electrospun epoxy-based nanofibrous membrane containing biocompatible feather polypeptide for highly stable and active covalent immobilization of lipase. Colloids and Surfaces B: Biointerfaces 166, 277–285. https://doi.org/10.1016/j.colsurfb.2018.03.037

Liu, X., Fang, Y., Yang, X., Li, Y., Wang, C., 2018b. Electrospun nanofibrous membranes containing epoxy groups and hydrophilic polyethylene oxide chain for highly active and stable covalent immobilization of lipase. Chemical Engineering Journal 336, 456–464. https://doi.org/10.1016/j.cej.2017.12.048

Liu, Y., Ogorzalek, T.L., Yang, P., Schroeder, M.M., Marsh, E.N.G., Chen, Z., 2013. Molecular Orientation of Enzymes Attached to Surfaces through Defined Chemical Linkages at the Solid–Liquid Interface. J. Am. Chem. Soc. 135, 12660–12669. https://doi.org/10.1021/ja403672s

Loh, F.C., Tan, K.L., Kang, E.T., Kato, K., Uyama, Y., Ikada, Y., 1996. XPS Characterization of Surface Functionalized Electroactive Polymers. Surface and Interface Analysis 24, 597–604. https://doi.org/10.1002/(SICI)1096-9918(19960916)24:9<597::AID-SIA163>3.0.CO;2-2

Longo, J., Yao, C., Rios, C., Chau, N.T.T., Boulmedais, F., Hemmerlé, J., Lavalle, P., Schiller, S.M., Schaaf, P., Jierry, L., 2015. Reversible biomechano-responsive surface based on green fluorescent protein genetically modified with unnatural amino acids. Chem. Commun. 51, 232–235. https://doi.org/10.1039/C4CC07486F

Lou, L.-L., Qu, H., Yu, W., Wang, B., Ouyang, L., Liu, S., Zhou, W., 2018. Covalently Immobilized Lipase on a Thermoresponsive Polymer with an Upper Critical Solution Temperature as an Efficient and Recyclable Asymmetric Catalyst in Aqueous Media. ChemCatChem 10, 1166–1172. https://doi.org/10.1002/cctc.201701512

Lv, S., 2020. Silk Fibroin-Based Materials for Catalyst Immobilization. Molecules 25, 4929. https://doi.org/10.3390/molecules25214929

Lyu, X., Gonzalez, R., Horton, A., Li, T., 2021. Immobilization of Enzymes by Polymeric Materials. Catalysts 11, 1211. https://doi.org/10.3390/catal11101211

Ma, Z., He, W., Yong, T., Ramakrishna, S., 2005. Grafting of Gelatin on Electrospun Poly(caprolactone) Nanofibers to Improve Endothelial Cell Spreading and Proliferation and to Control Cell Orientation. Tissue Engineering 11, 1149–1158. https://doi.org/10.1089/ten.2005.11.1149

Ma, Z., Ramakrishna, S., 2008. Electrospun regenerated cellulose nanofiber affinity membrane functionalized with protein A/G for IgG purification. Journal of Membrane Science 319, 23–28. https://doi.org/10.1016/j.memsci.2008.03.045

MacDonald, J.I., Munch, H.K., Moore, T., Francis, M.B., 2015. One-step site-specific modification of native proteins with 2-pyridinecarboxyaldehydes. Nat Chem Biol 11, 326–331. https://doi.org/10.1038/nchembio.1792

Madhu, A., Chakraborty, J.N., 2017. Developments in application of enzymes for textile processing. Journal of Cleaner Production 145, 114–133. https://doi.org/10.1016/j.jclepro.2017.01.013

Mahmoudifard, M., Soudi, S., Soleimani, M., Hosseinzadeh, S., Esmaeili, E., Vossoughi, M., 2016. Efficient protein immobilization on polyethersolfone electrospun nanofibrous membrane via covalent binding for biosensing applications. Materials Science and Engineering: C 58, 586–594. https://doi.org/10.1016/j.msec.2015.09.007

Malar, C.G., Seenuvasan, M., Kumar, K.S., 2019. Improvisation of diffusion coefficient in surface modified magnetite nanoparticles: A novel perspective. Materials Science and Engineering: C 103, 109832. https://doi.org/10.1016/j.msec.2019.109832

Market Data Forecast, 2022. Global Antibiotics Market Size & Industry Analysis Report | 2022 to 2027 [WWW Document]. Market Data Forecast. URL http://www.marketdataforecast.com/ (accessed 9.15.22).

Maryšková, M., Ardao, I., García-González, C.A., Martinová, L., Rotková, J., Ševců, A., 2016. Polyamide 6/chitosan nanofibers as support for the immobilization of Trametes versicolor laccase for the elimination of endocrine disrupting chemicals. Enzyme and Microbial Technology 89, 31–38. https://doi.org/10.1016/j.enzmictec.2016.03.001

Masaeli, E., Wieringa, P.A., Morshed, M., Nasr-Esfahani, M.H., Sadri, S., van Blitterswijk, C.A., Moroni, L., 2014. Peptide functionalized polyhydroxyalkanoate nanofibrous scaffolds enhance Schwann cells activity. Nanomedicine: Nanotechnology, Biology and Medicine 10, 1559–1569. https://doi.org/10.1016/j.nano.2014.04.008

Mateo, C., Abian, O., Bernedo, M., Cuenca, E., Fuentes, M., Fernandez-Lorente, G., Palomo, J.M., Grazu, V., Pessela, B.C.C., Giacomini, C., Irazoqui, G., Villarino, A., Ovsejevi, K., Batista-Viera, F., Fernandez-Lafuente, R., Guisán, J.M., 2005. Some special features of glyoxyl supports to immobilize proteins. Enzyme and Microbial Technology 37, 456–462.

https://doi.org/10.1016/j.enzmictec.2005.03.020
Mateo, C., Abian, O., Fernandez–Lafuente, R., Guisan, J.M., 2000a. Increase in conformational stability of enzymes immobilized on epoxy-activated supports by favoring additional multipoint covalent attachment☆. Enzyme and Microbial Technology 26, 509–515. https://doi.org/10.1016/S0141-0229(99)00188-X
Mateo, C., Fernández-Lorente, G., Abian, O., Fernández-Lafuente, R., Guisán, J.M., 2000b. Multifunctional Epoxy Supports: A New Tool To Improve the Covalent Immobilization of Proteins. The Promotion of Physical Adsorptions of Proteins on the Supports before Their Covalent Linkage. Biomacromolecules 1, 739–745. https://doi.org/10.1021/bm000071q
Mateo, C., Palomo, J.M., Fuentes, M., Betancor, L., Grazu, V., López-Gallego, F., Pessela, B.C.C., Hidalgo, A., Fernández-Lorente, G., Fernández-Lafuente, R., Guisán, J.M., 2006. Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high stabilization of proteins. Enzyme and Microbial Technology 39, 274–280. https://doi.org/10.1016/j.enzmictec.2005.10.014
Mehrotra, P., 2016. Biosensors and their applications – A review. Journal of Oral Biology and Craniofacial Research 6, 153–159. https://doi.org/10.1016/j.jobcr.2015.12.002
Mohd Azhar, S.H., Abdulla, R., Jambo, S.A., Marbawi, H., Gansau, J.A., Mohd Faik, A.A., Rodrigues, K.F., 2017. Yeasts in sustainable bioethanol production: A review. Biochemistry and Biophysics Reports 10, 52–61. https://doi.org/10.1016/j.bbrep.2017.03.003
Monier, M., 2013. Immobilization of β-galactosidase from *Escherichia coli* onto modified natural silk fibers. J. Appl. Polym. Sci. 130, 2923–2931. https://doi.org/10.1002/app.39475
Montanier, C.Y., Fanuel, M., Rogniaux, H., Ropartz, D., Di Guilmi, A.-M., Bouchoux, A., 2019. Changing surface grafting density has an effect on the activity of immobilized xylanase towards natural polysaccharides. Scientific Reports 9, 5763. https://doi.org/10.1038/s41598-019-42206-w
Motsa, M., Mamba, P.P., Ogola, H.J., Msagati, T.A., Mamba, B.B., Nkambule, T.T., 2022. Laccase-Coated Polyethersulfone Membranes for Organic Matter Degradation and Removal. Journal of Membrane Science and Research 8. https://doi.org/10.22079/jmsr.2021.139576.1418
Nair, S., Kim, J., Crawford, B., Kim, S.H., 2007. Improving Biocatalytic Activity of Enzyme-Loaded Nanofibers by Dispersing Entangled Nanofiber Structure. Biomacromolecules 8, 1266–1270. https://doi.org/10.1021/bm061004k
Niu, X., Zhu, L., Xi, L., Guo, L., Wang, H., 2020. An antimicrobial agent prepared by N-succinyl chitosan immobilized lysozyme and its application in strawberry preservation. Food Control 108, 106829. https://doi.org/10.1016/j.foodcont.2019.106829
Oktay, B., Demir, S., Kayaman-Apohan, N., 2015a. Immobilization of α-amylase onto poly(glycidyl methacrylate) grafted electrospun fibers by ATRP. Materials Science and Engineering: C 50, 386–393. https://doi.org/10.1016/j.msec.2015.02.033
Oktay, B., Kayaman-Apohan, N., Erdem-Kuruca, S., Süleymanoğlu, M., 2015b. Fabrication of collagen immobilized electrospun poly (vinyl alcohol) scaffolds: Collagen Immobilized Electrospun PVA Scaffolds. Polym. Adv. Technol. 26, 978–987. https://doi.org/10.1002/pat.3512
Pan, X., Wang, L., Ye, J., Qin, S., He, B., 2018. Efficient synthesis of β-lactam antibiotics with very low product hydrolysis by a mutant Providencia rettgeri penicillin G acylase. Appl Microbiol Biotechnol 102, 1749–1758. https://doi.org/10.1007/s00253-017-8692-8
Pei, X., Luo, Z., Qiao, L., Xiao, Q., Zhang, P., Wang, A., Sheldon, R.A., 2022. Putting precision and elegance in enzyme immobilisation with bio-orthogonal chemistry. Chem. Soc. Rev. 51, 7281–7304. https://doi.org/10.1039/D1CS01004B
Pessato, T.B., Tavano, O.L., 2015. Hydrolysis of casein and β-lactoglobulin by immobilized papain after pre-treatment with immobilized trypsin. Acta Alimentaria 44, 570–577. https://doi.org/10.1556/066.2015.44.0029
Pinese, C., Jebors, S., Echalier, C., Licznar-Fajardo, P., Garric, X., Humblot, V., Calers, C., Martinez, J., Mehdi, A., Subra, G., 2016. Simple and Specific Grafting of Antibacterial Peptides on Silicone Catheters. Advanced Healthcare Materials 5, 3067–3073. https://doi.org/10.1002/adhm.201600757
Raliski, B.K., Howard, C.A., Young, D.D., 2014. Site-Specific Protein Immobilization Using Unnatural Amino Acids. Bioconjugate Chem. 25, 1916–1920. https://doi.org/10.1021/bc500443h

Rao, S.V., Anderson, K.W., Bachas, L.G., 1998. Oriented immobilization of proteins. Mikrochim Acta 128, 127–143. https://doi.org/10.1007/BF01243043
Rashidian, M., Song, J.M., Pricer, R.E., Distefano, M.D., 2012. Chemoenzymatic Reversible Immobilization and Labeling of Proteins without Prior Purification. J. Am. Chem. Soc. 134, 8455–8467. https://doi.org/10.1021/ja211308s
Ren, S., Li, C., Jiao, X., Jia, S., Jiang, Y., Bilal, M., Cui, J., 2019. Recent progress in multienzymes co-immobilization and multienzyme system applications. Chemical Engineering Journal 373, 1254–1278. https://doi.org/10.1016/j.cej.2019.05.141
Resnier, P., Lepeltier, E., Lucrezia Emina, A., Galopin, N., Bejaud, J., David, S., Ballet, C., Benvegnu, T., Pecorari, F., Chourpa, I., Benoit, J.-P., Passirani, C., 2019. Model Affitin and PEG modifications onto siRNA lipid nanocapsules: cell uptake and in vivo biodistribution improvements. RSC Advances 9, 27264–27278. https://doi.org/10.1039/C9RA03668G
Riahi, N., Murschel, F., Lerouge, S., Durocher, Y., Henry, O., De Crescenzo, G., 2017. Bioavailability of immobilized epidermal growth factor: Covalent versus noncovalent grafting. Biointerphases 12, 010501. https://doi.org/10.1116/1.4978871
Riazi, K., Kübel, J., Abbasi, M., Bachtin, K., Indris, S., Ehrenberg, H., Kádár, R., Wilhelm, M., 2016. Polystyrene comb architectures as model systems for the optimized solution electrospinning of branched polymers. Polymer, Rheology 104, 240–250. https://doi.org/10.1016/j.polymer.2016.05.032
Ricco, R., Doherty, C.M., Falcaro, P., 2014. Evaluation of Coupling Protocols to Bind Beta-Glucosidase on Magnetic Nanoparticles. Journal of Nanoscience and Nanotechnology 14, 6565–6573. https://doi.org/10.1166/jnn.2014.9353
Rijn, P. van, Böker, A., 2011. Bionanoparticles and hybrid materials: tailored structural properties, self-assembly , materials and developments in the field. Journal of Materials Chemistry 21, 16735–16747. https://doi.org/10.1039/C1JM11433F
Roberfroid, M., 2007. Prebiotics: The Concept Revisited. The Journal of Nutrition 137, 830S-837S. https://doi.org/10.1093/jn/137.3.830S
Rodrigues, R.C., Berenguer-Murcia, Á., Carballares, D., Morellon-Sterling, R., Fernandez-Lafuente, R., 2021. Stabilization of enzymes via immobilization: Multipoint covalent attachment and other stabilization strategies. Biotechnology Advances 52, 107821. https://doi.org/10.1016/j.biotechadv.2021.107821
Rodrigues, R.C., Virgen-Ortíz, J.J., dos Santos, J.C.S., Berenguer-Murcia, Á., Alcantara, A.R., Barbosa, O., Ortiz, C., Fernandez-Lafuente, R., 2019. Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions. Biotechnology Advances 37, 746–770. https://doi.org/10.1016/j.biotechadv.2019.04.003
Rodriguez-Abetxuko, A., Sánchez-deAlcázar, D., Muñumer, P., Beloqui, A., 2020. Tunable Polymeric Scaffolds for Enzyme Immobilization. Front. Bioeng. Biotechnol. 8, 830. https://doi.org/10.3389/fbioe.2020.00830
Romero-Fernández, M., Paradisi, F., 2020. Protein immobilization technology for flow biocatalysis. Current Opinion in Chemical Biology 55, 1–8. https://doi.org/10.1016/j.cbpa.2019.11.008
Rosellini, E., Cristallini, C., Guerra, G.D., Barbani, N., 2015. Surface chemical immobilization of bioactive peptides on synthetic polymers for cardiac tissue engineering. Journal of Biomaterials Science, Polymer Edition 26, 515–533. https://doi.org/10.1080/09205063.2015.1030991
Rueda, N., Santos, J.C.S. dos, Ortiz, C., Barbosa, O., Fernandez-Lafuente, R., Torres, R., 2016. Chemical amination of lipases improves their immobilization on octyl-glyoxyl agarose beads. Catalysis Today 259, 107–118. https://doi.org/10.1016/j.cattod.2015.05.027
Sánchez-Moreno, P., De Vicente, J., Nardecchia, S., Marchal, J.A., Boulaiz, H., 2018. Thermo-Sensitive Nanomaterials: Recent Advance in Synthesis and Biomedical Applications. Nanomaterials 8, 935. https://doi.org/10.3390/nano8110935
Santos, J.C.S. dos, Barbosa, O., Ortiz, C., Berenguer-Murcia, A., Rodrigues, R.C., Fernandez-Lafuente, R., 2015. Importance of the Support Properties for Immobilization or Purification of Enzymes. ChemCatChem 7, 2413–2432. https://doi.org/10.1002/cctc.201500310
Sathishkumar, P., Kamala-Kannan, S., Cho, M., Kim, J.S., Hadibarata, T., Salim, M.R., Oh, B.-T., 2014. Laccase immobilization of cellulose nanofiber: the catalytic efficiency and recyclic application for simulated dye effluent treatment. Journal of Molecular Catalysis B: Enzymatic 100, 111–

120.
Schnapp, J., Shalitin, Y., 1976. IMMOBILIZATION OF ENZYMES BY COVALENT BINDING TO AMINE SUPPORTS VIA CYANOGEN BROMIDE ACTIVATION. Biochemical and Biophysical Research Communications 70, 8–14. https://doi.org/10.1016/0006-291X(76)91101-3
Seyhan Tükel, S., Alagöz, D., 2008. Catalytic efficiency of immobilized glucose isomerase in isomerization of glucose to fructose. Food Chemistry 111, 658–662. https://doi.org/10.1016/j.foodchem.2008.04.035
Shaimi, R., Low, S.C., 2016. Prolonged protein immobilization of biosensor by chemically cross-linked glutaraldehyde on mixed cellulose membrane. Journal of Polymer Engineering 36, 655–661. https://doi.org/10.1515/polyeng-2015-0308
Sharifi, M., Robatjazi, S.-M., Sadri, M., Mosaabadi, J.M., 2018. Covalent immobilization of organophosphorus hydrolase enzyme on chemically modified cellulose microfibers: Statistical optimization and characterization. Reactive and Functional Polymers 124, 162–170. https://doi.org/10.1016/j.reactfunctpolym.2018.01.019
Sharma, Y.C., Singh, B., Upadhyay, S.N., 2008. Advancements in development and characterization of biodiesel: A review. Fuel 87, 2355–2373. https://doi.org/10.1016/j.fuel.2008.01.014
Sheldon, R.A., Basso, A., Brady, D., 2021. New frontiers in enzyme immobilisation: robust biocatalysts for a circular bio-based economy. Chem. Soc. Rev. 50, 5850–5862. https://doi.org/10.1039/D1CS00015B
Shen, L., Schroeder, M., Ogorzalek, T.L., Yang, P., Wu, F.-G., Marsh, E.N.G., Chen, Z., 2014. Surface Orientation Control of Site-Specifically Immobilized Nitro-reductase (NfsB). Langmuir 30, 5930–5938. https://doi.org/10.1021/la5016862
Shi, Q., Chen, X., Lu, T., Jing, X., 2008. The immobilization of proteins on biodegradable polymer fibers via click chemistry. Biomaterials 9.
Shmanai, V.V., Nikolayeva, T.A., Vinokurova, L.G., Litoshka, A.A., 2001. Oriented antibody immobilization to polystyrene macrocarriers for immunoassay modified with hydrazide derivatives of poly(meth)acrylic acid. BMC Biotechnol 1, 4. https://doi.org/10.1186/1472-6750-1-4
Singh, M., Ray, A.R., Verma, P.V.K., Guha, S.K., 1979. Potential Biosoluble Carriers: Biocompatibility and Biodegradability of Oxidized Cellulose. Biomaterials, Medical Devices, and Artificial Organs 7, 495–512. https://doi.org/10.3109/10731197909118964
Singh, R.S., Singh, R.P., Kennedy, J.F., 2017. Immobilization of yeast inulinase on chitosan beads for the hydrolysis of inulin in a batch system. International Journal of Biological Macromolecules 95, 87–93. https://doi.org/10.1016/j.ijbiomac.2016.11.030
Smith, M.R., Ratledge, C., 1989. Quantitative biotransformation of catechol to cis,cis-muconate. Biotechnology Letters 11, 105–110. https://doi.org/10.1007/BF01192183
Smith, M.R., Ratledge, C., Crook, S., 1990. Properties of cyanogen bromide-activated, Agarose-immobilized catechol 1,2-dioxygenase from freeze-dried extracts of Nocardia sp. NCIB 10503. Enzyme and Microbial Technology 12, 945–949. https://doi.org/10.1016/0141-0229(90)90114-6
Smith, S., Goodge, K., Delaney, M., Struzyk, A., Tansey, N., Frey, M., 2020a. A Comprehensive Review of the Covalent Immobilization of Biomolecules onto Electrospun Nanofibers. Nanomaterials 10, 2142. https://doi.org/10.3390/nano10112142
Smith, S., Goodge, K., Delaney, M., Struzyk, A., Tansey, N., Frey, M., 2020b. A Comprehensive Review of the Covalent Immobilization of Biomolecules onto Electrospun Nanofibers. Nanomaterials 10, 2142. https://doi.org/10.3390/nano10112142
Song, D.W., Kim, S.H., Kim, H.H., Lee, K.H., Ki, C.S., Park, Y.H., 2016. Multi-biofunction of antimicrobial peptide-immobilized silk fibroin nanofiber membrane: Implications for wound healing. Acta Biomaterialia 39, 146–155. https://doi.org/10.1016/j.actbio.2016.05.008
Sperandeo, P., Bosco, F., Clerici, F., Polissi, A., Gelmi, M.L., Romanelli, A., 2020. Covalent Grafting of Antimicrobial Peptides onto Microcrystalline Cellulose. ACS Appl. Bio Mater. 3, 4895–4901. https://doi.org/10.1021/acsabm.0c00412
Stiegler, L.M.S., Luchs, T., Hirsch, A., 2020. Shell-by-Shell Functionalization of Inorganic Nanoparticles. Chemistry – A European Journal 26, 8483–8498.

https://doi.org/10.1002/chem.202000195
Stoilova, O., Ignatova, M., Manolova, N., Godjevargova, T., Mita, D.G., Rashkov, I., 2010. Functionalized electrospun mats from styrene–maleic anhydride copolymers for immobilization of acetylcholinesterase. European Polymer Journal 46, 1966–1974. https://doi.org/10.1016/j.eurpolymj.2010.08.005
Tacias-Pascacio, V.G., Morellon-Sterling, R., Castañeda-Valbuena, D., Berenguer-Murcia, Á., Kamli, M.R., Tavano, O., Fernandez-Lafuente, R., 2021. Immobilization of papain: A review. International Journal of Biological Macromolecules 188, 94–113. https://doi.org/10.1016/j.ijbiomac.2021.08.016
Tähkä, S., Sarfraz, J., Urvas, L., Provenzani, R., Wiedmer, S.K., Peltonen, J., Jokinen, V., Sikanen, T., 2019. Immobilization of proteolytic enzymes on replica-molded thiol-ene micropillar reactors via thiol-gold interaction. Anal Bioanal Chem 411, 2339–2349. https://doi.org/10.1007/s00216-019-01674-9
Takatsuji, Y., Yamasaki, R., Iwanaga, A., Lienemann, M., Linder, M.B., Haruyama, T., 2013. Solid-support immobilization of a "swing" fusion protein for enhanced glucose oxidase catalytic activity. Colloids and Surfaces B: Biointerfaces 112, 186–191. https://doi.org/10.1016/j.colsurfb.2013.07.051
Taylor, A.J., Vadgama, P., 1992. Analytical Reviews in Clinical Biochemistry: The Estimation of Urea. Ann Clin Biochem 29, 245–264. https://doi.org/10.1177/000456329202900301
Teske, M., Kießlich, T., Fischer, J., Bahl, H., Wulf, K., Eickner, T., Grabow, N., Illner, S., 2020. Immobilizing hydrolytic active Papain on biodegradable PLLA for biofilm inhibition in cardiovascular applications. Current Directions in Biomedical Engineering 6, 172–175. https://doi.org/10.1515/cdbme-2020-3044
Tosa, T., Mori, T., Fuse, N., Chibata, I., 1967. Studies on continuous enzyme reactions. IV. Preparation of a DEAE-sephadex-aminoacylase column and continuous optical resolution of acyl-DL-amino acids. Biotechnol. Bioeng. 9, 603–615. https://doi.org/10.1002/bit.260090413
Tseng, H.-C., Lee, A.-W., Wei, P.-L., Chang, Y.-J., Chen, J.-K., 2016. Clinical diagnosis of colorectal cancer using electrospun triple-blend fibrous mat-based capture assay of circulating tumor cells. J. Mater. Chem. B 4, 6565–6580. https://doi.org/10.1039/C6TB01359G
Tufvesson, P., Fu, W., Jensen, J.S., Woodley, J.M., 2010. Process considerations for the scale-up and implementation of biocatalysis. Food and Bioproducts Processing 88, 3–11. https://doi.org/10.1016/j.fbp.2010.01.003
Tully, J., Yendluri, R., Lvov, Y., 2016. Halloysite Clay Nanotubes for Enzyme Immobilization. Biomacromolecules 17, 615–621. https://doi.org/10.1021/acs.biomac.5b01542
Urrutia, P., Bernal, C., Wilson, L., Illanes, A., 2018. Use of chitosan heterofunctionality for enzyme immobilization: β-galactosidase immobilization for galacto-oligosaccharide synthesis. International Journal of Biological Macromolecules 116, 182–193. https://doi.org/10.1016/j.ijbiomac.2018.04.112
Urrutia, P., Rodriguez-Colinas, B., Fernandez-Arrojo, L., Ballesteros, A.O., Wilson, L., Illanes, A., Plou, F.J., 2013. Detailed Analysis of Galactooligosaccharides Synthesis with β-Galactosidase from *Aspergillus oryzae*. J. Agric. Food Chem. 61, 1081–1087. https://doi.org/10.1021/jf304354u
Vallières, K., Chevallier, P., Sarra-Bournet, C., Turgeon, S., Laroche, G., 2007. AFM Imaging of Immobilized Fibronectin: Does the Surface Conjugation Scheme Affect the Protein Orientation/Conformation? Langmuir 23, 9745–9751. https://doi.org/10.1021/la701323q
Varavinit, S., Chaokasem, N., Shobsngob, S., 2001. Covalent immobilization of a glucoamylase to bagasse dialdehyde cellulose. World Journal of Mircobiology & Biotechnology 17, 721–725.
Vasconcelos, N.F., Cunha, A.P., Ricardo, N.M.P.S., Freire, R.S., Vieira, L. de A.P., Brígida, A.I.S., Borges, M. de F., Rosa, M. de F., Vieira, R.S., Andrade, F.K., 2020. Papain immobilization on heterofunctional membrane bacterial cellulose as a potential strategy for the debridement of skin wounds. International Journal of Biological Macromolecules 165, 3065–3077. https://doi.org/10.1016/j.ijbiomac.2020.10.200
Vasile, C., Baican, M.C., Tibirna, C.M., Tuchilus, C., Debarnot, D., Pâslaru, E., Poncin-Epaillard, F., 2011a. Microwave plasma activation of a polyvinylidene fluoride surface for protein immobilization. J. Phys. D: Appl. Phys. 44, 475303. https://doi.org/10.1088/0022-

3727/44/47/475303
Vasile, C., Baican, M.C., Tibirna, C.M., Tuchilus, C., Debarnot, D., Pâslaru, E., Poncin-Epaillard, F., 2011b. Microwave plasma activation of a polyvinylidene fluoride surface for protein immobilization. J. Phys. D: Appl. Phys. 44, 475303. https://doi.org/10.1088/0022-3727/44/47/475303
Vermeer, A.W.P., Norde, W., 2000. CD Spectroscopy of Proteins Adsorbed at Flat Hydrophilic Quartz and Hydrophobic Teflon Surfaces. Journal of Colloid and Interface Science 225, 394–397. https://doi.org/10.1006/jcis.2000.6769
Vogel, V., 2006. MECHANOTRANSDUCTION INVOLVING MULTIMODULAR PROTEINS: Converting Force into Biochemical Signals. Annu. Rev. Biophys. Biomol. Struct. 35, 459–488. https://doi.org/10.1146/annurev.biophys.35.040405.102013
Völcker, N., Klee, D., Höcker, H., Langefeld, S., 2001. Functionalization of silicone rubber for the covalent immobilization of fibronectin. J Mater Sci Mater Med 12, 111–119. https://doi.org/10.1023/a:1008938525489
Wahab, R.A., Elias, N., Abdullah, F., Ghoshal, S.K., 2020a. On the taught new tricks of enzymes immobilization: An all-inclusive overview. Reactive and Functional Polymers 152, 104613. https://doi.org/10.1016/j.reactfunctpolym.2020.104613
Wahab, R.A., Elias, N., Abdullah, F., Ghoshal, S.K., 2020b. On the taught new tricks of enzymes immobilization: An all-inclusive overview. Reactive and Functional Polymers 152, 104613. https://doi.org/10.1016/j.reactfunctpolym.2020.104613
Wahba, M.I., 2017. Porous chitosan beads of superior mechanical properties for the covalent immobilization of enzymes. International Journal of Biological Macromolecules 105, 894–904. https://doi.org/10.1016/j.ijbiomac.2017.07.102
Wahba, M.I., Hassan, M.E., 2017. Agar-carrageenan hydrogel blend as a carrier for the covalent immobilization of β-D-galactosidase. Macromol. Res. 25, 913–923. https://doi.org/10.1007/s13233-017-5123-8
Wang, D., Jiang, W., 2019. Preparation of chitosan-based nanoparticles for enzyme immobilization. International Journal of Biological Macromolecules 126, 1125–1132. https://doi.org/10.1016/j.ijbiomac.2018.12.243
Wang, S., Ye, J., Bie, Z., Liu, Z., 2014. Affinity-tunable specific recognition of glycoproteins via boronate affinity-based controllable oriented surface imprinting. Chem. Sci. 5, 1135–1140. https://doi.org/10.1039/C3SC52986J
Wieland, F., Bruch, R., Bergmann, M., Partel, S., Urban, G.A., Dincer, C., 2020. Enhanced Protein Immobilization on Polymers—A Plasma Surface Activation Study. Polymers 12, 104. https://doi.org/10.3390/polym12010104
Wieszczycka, K., Staszak, K., Woźniak-Budych, M.J., Litowczenko, J., Maciejewska, B.M., Jurga, S., 2021. Surface functionalization – The way for advanced applications of smart materials. Coordination Chemistry Reviews 436, 213846. https://doi.org/10.1016/j.ccr.2021.213846
Wong, D.E., Senecal, K.J., Goddard, J.M., 2017. Immobilization of chymotrypsin on hierarchical nylon 6,6 nanofiber improves enzyme performance. Colloids and Surfaces B: Biointerfaces 154, 270–278. https://doi.org/10.1016/j.colsurfb.2017.03.033
Wong, L.S., Thirlway, J., Micklefield, J., 2008. Direct site-selective covalent protein immobilization catalyzed by a phosphopantetheinyl transferase. Journal of the American Chemical Society 130, 12456–12464. https://doi.org/10.1021/ja8030278
Wu, S., Snajdrova, R., Moore, J.C., Baldenius, K., Bornscheuer, U.T., 2021. Biocatalysis: Enzymatic Synthesis for Industrial Applications. Angew. Chem. Int. Ed. 60, 88–119. https://doi.org/10.1002/anie.202006648
Xie, W., Huang, M., 2020. Fabrication of immobilized Candida rugosa lipase on magnetic Fe3O4-poly(glycidyl methacrylate-co-methacrylic acid) composite as an efficient and recyclable biocatalyst for enzymatic production of biodiesel. Renewable Energy 158, 474–486. https://doi.org/10.1016/j.renene.2020.05.172
Xu, R., Cui, J., Tang, R., Li, F., Zhang, B., 2017. Removal of 2,4,6-trichlorophenol by laccase immobilized on nano-copper incorporated electrospun fibrous membrane-high efficiency, stability and reusability. Chemical Engineering Journal 326, 647–655. https://doi.org/10.1016/j.cej.2017.05.083

Xu, R., Si, Y., Li, F., Zhang, B., 2015a. Enzymatic removal of paracetamol from aqueous phase: horseradish peroxidase immobilized on nanofibrous membranes. Environ Sci Pollut Res 22, 3838–3846. https://doi.org/10.1007/s11356-014-3658-1

Xu, R., Tang, R., Liu, S., Li, F., Zhang, B., 2015b. An environmentally-friendly enzyme-based nanofibrous membrane for 3,3′,5,5′-tetrabromobisphenol removal. RSC Adv. 5, 64091–64097. https://doi.org/10.1039/C5RA09090C

Xu, R., Tang, R., Zhou, Q., Li, F., Zhang, B., 2015c. Enhancement of catalytic activity of immobilized laccase for diclofenac biodegradation by carbon nanotubes. Chemical Engineering Journal 262, 88–95. https://doi.org/10.1016/j.cej.2014.09.072

Xu, R., Zhou, Q., Li, F., Zhang, B., 2013. Laccase immobilization on chitosan/poly(vinyl alcohol) composite nanofibrous membranes for 2,4-dichlorophenol removal. Chemical Engineering Journal 222, 321–329. https://doi.org/10.1016/j.cej.2013.02.074

Yang, E.Y., Kronenfeld, J.P., Gattás-Asfura, K.M., Bayer, A.L., Stabler, C.L., 2015. Engineering an "infectious" Treg biomimetic through chemoselective tethering of TGF-β1 to PEG brush surfaces. Biomaterials 67, 20–31.

Yang, L., Fan, X., Zhang, J., Ju, J., 2020. Preparation and Characterization of Thermoresponsive Poly(N-Isopropylacrylamide) for Cell Culture Applications. Polymers 12, 389. https://doi.org/10.3390/polym12020389

Yassin, M.A., Gad, A.A.M., 2020. Immobilized Enzyme on Modified Polystyrene Foam Waste: a Biocatalyst for Wastewater Decolorization. Journal of Environmental Chemical Engineering 8, 104435. https://doi.org/10.1016/j.jece.2020.104435

Ye, N., Kou, X., Shen, J., Huang, S., Chen, G., Ouyang, G., 2020. Metal-Organic Frameworks: A New Platform for Enzyme Immobilization. ChemBioChem 21, 2585–2590. https://doi.org/10.1002/cbic.202000095

Ye, P., Xu, Z.-K., Wu, J., Innocent, C., Seta, P., 2006. Nanofibrous Membranes Containing Reactive Groups: Electrospinning from Poly(acrylonitrile- *c o* -maleic acid) for Lipase Immobilization. Macromolecules 39, 1041–1045. https://doi.org/10.1021/ma0517998

Yuan, Y., Yin, M., Qian, J., Liu, C., 2011. Site-directed immobilization of antibodies onto blood contacting grafts for enhanced endothelial cell adhesion and proliferation. Soft Matter 7, 7207–7216. https://doi.org/10.1039/C1SM05086A

Yushkova, E.D., Nazarova, E.A., Matyuhina, A.V., Noskova, A.O., Shavronskaya, D.O., Vinogradov, V.V., Skvortsova, N.N., Krivoshapkina, E.F., 2019. Application of Immobilized Enzymes in Food Industry. J. Agric. Food Chem. 67, 11553–11567. https://doi.org/10.1021/acs.jafc.9b04385

Zdarta, J., Jankowska, K., Bachosz, K., Kijeńska-Gawrońska, E., Zgoła-Grześkowiak, A., Kaczorek, E., Jesionowski, T., 2020. A promising laccase immobilization using electrospun materials for biocatalytic degradation of tetracycline: Effect of process conditions and catalytic pathways. Catalysis Today 348, 127–136. https://doi.org/10.1016/j.cattod.2019.08.042

Zhang, J., Chen, Y., Brook, M.A., 2013. Facile Functionalization of PDMS Elastomer Surfaces Using Thiol–Ene Click Chemistry. Langmuir 29, 12432–12442. https://doi.org/10.1021/la403425d

Zhang, X., Duan, Y., Zeng, X., 2017. Improved Performance of Recombinant Protein A Immobilized on Agarose Beads by Site-Specific Conjugation. ACS Omega 2, 1731–1737. https://doi.org/10.1021/acsomega.7b00362

Zheng, M., Griveau, S., Dupont-Gillain, C., Genet, M.J., Jolivalt, C., 2015. Oxidation of laccase for improved cathode biofuel cell performances. Bioelectrochemistry, Special Issue on "Biological fuel cells" 106, 77–87. https://doi.org/10.1016/j.bioelechem.2015.06.004

Zhuang, P., Allan Butterfield, D., 1992. Structural and enzymatic characterizations of papain immobilized onto vinyl alcohol/vinyl butyral copolymer membrane. Journal of Membrane Science 66, 247–257. https://doi.org/10.1016/0376-7388(92)87015-P

Zittan, L., Poulsen, P.B., Hemmingsen, St.H., 1975. Sweetzyme - A New Immobilized Glucose Isomerase. Starch/Stärke 27, 236–241. https://doi.org/10.1002/star.19750270705