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Cellulose-based biogenic supports, remarkably friendly biomaterials for proteins and biomolecules

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ABSTRACT

Cellulose has a long history dating back to ancient times in the evolution of humanity. It was a key material for basic needs, especially for the construction of shelters, paper making, which allowed our ancestors to perpetuate the valuable literary, philosophical or artistic works. In modern era, cellulose has acquired new dimensions of knowledge and scientific interest. This increased interest in cellulose is due to the need to exploit the still unknown resources that cellulose provides us, possibly due to the remarkable progress made lately in the field of fine characterization of the structure using sophisticated electron microscopes and other characterization techniques that have recently emerged. The growing demands of modern society in the direction of computerization and technology, have led the general interest to move from the classical writing paper to other types of "papers" that incorporates a high degree of ingenuity and intelligence, the so-called special papers, ranging from sensors, chips, motherboards, papers with a high degree of security, and many others. Among these, paper-based biosensors are of special interest, due to their high selectivity, simplicity, low price, and fast response. In this article we will review the new trends in the immobilization of biomolecules on various cellulose-based supports. In the first part, we will discuss the stages prior to the manufacture of a such support by specific chemical modification of the cellulosic substrate, followed by an overview of the most studied proteins, but also the most commonly used methods in monitoring protein adsorption on cellulosic substrates.

1. Introduction

Biosensors and biochips are essential devices in biomedical diagnostics, immunoassays as well as genomics for rapid, robust, sensitive and simultaneous detection of several different analytes in a biological mixture (Laib and MacCraith, 2007; Lee et al., 2009; Sassolas et al., 2008; Shabani et al., 2006). These devices consist of microarrays of different biological probes, like deoxyribonucleic acid (DNA), proteins and cells immobilized on solid substrates such as glass or silicon surfaces, but also synthetic polymers (Lee et al, 2007, 2009; Shabani et al., 2006). One of the most sensitive issue in the design of a biosensor is the strict control of the surface chemistry of the substrate, which can be achieved by an appropriate surface modification. This procedure aims to introduce a large number of reactive functional groups (e.g. aminoalkyl, carboxyl, aldehyde, mercaptoalkyl and epoxy) with the role of binding sites on the surfaces capable of recognizing their targets in a certain reaction mixture. Moreover, the unspecific interactions between the sample and the surface must be avoided, which reduces the exact quantification of the results (Sassolas et al., 2008). Surface modification can be done either by physical adsorption or direct conjugation, which are the most commonly used methods (Orelma et al., 2011; Sassolas et al., 2008; Sheldon, 2007). Among these, direct conjugation (covalent immobilization) is often the preferred method to enhance the specificity and immobilization efficiency of certain biomolecules. A major drawback of covalent modification employing chemical linkers is that the resulting functionalized surfaces have reduced shelf-life and additional

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blocking steps of free reactive groups are often required. In addition, the original structural properties of such substrates are altered to a larger extend. In physical adsorption, biomolecules are attached to the surface, preferably through intermolecular forces, including electrostatic (ionic), hydrophobic and polar interactions. This type of interaction strongly depends on the substrate's nature and the type of biomolecules (Rusmini et al., 2007; Sassolas et al., 2008). It is worth mentioning that physical adsorption of biological molecules on solid substrate can lead to low surface densities and high unspecific adsorption (Loscher et al., 1998). Therefore, it would be highly desirable to design a substrate that combines high binding capacity of biomolecules with increased shelf-life and a simple application procedure (Loscher et al., 1998). Until now, a variety of solid substrates, like nylon, nitrocellulose, polypropylene (PP), polystyrene (PS), cyclo olefin polymers (COP) (Saaem et al., 2010), polyethylene terephthalate (PET) (Zhou et al., 2004), glass and silicon wafers, have been used to construct biosensors. All the mentioned surfaces were either used directly or they were chemically functionalized, which at the same time results reduced shelf-life as mentioned above.

In this context, cellulose, as a natural hydrophilic polymer that combines the advantages of low fouling and enhanced affinity for various biological molecules (e.g., proteins) and structurally similar molecules, has received a huge interest in the field of diagnosis and biomedicine in recent years. Cellulose represents the prevalent biopolymer in the biosphere and plays a key role in nature, as a support and energy provider (Klemm et al., 2005). Even though it has been used for millennia for rudimentary purposes, for basic human needs, i.e., as a building material, energy producer or pulp and paper manufacturing material, cellulose today becomes a valuable resource providing derivatives with a high degree of built-in technology that have unpredictable applications, no more than twenty years ago. The main sources of cellulose are found in forests (e.g. wood), cotton, jute, straw, flax, hemp, and so on. Natural cotton contains the highest percentage of cellulose (about 90%) with high purity. Despite its technical applications, cellulose has been recognized as an inexhaustible source of versatile raw materials for the increasing demand of environmentally friendly, sustainable and biocompatible products (Klemm et al., 2006). Cellulose has gained a primary attention in the field of medical applications, particularly diagnosis, Pelton, named cellulose as "*particularly protein and biomolecule friendly*", suggesting its immense potential for the scientific community (Pelton, 2009). Cellulose itself offers many advantages, as follow: biocompatibility, biodegradability, non-toxicity, high selectivity for anchoring various biologically active molecules and low unspecific binding activity towards proteins. A typical support for immobilization of biomolecules (e.g. proteins, DNA, enzymes, etc.) should be stable and inert as well as resistant to mechanical changes, which makes the use of a cellulose ideal for both chemical and physical attachment (Sternberg et al., 1998; Yun et al., 2008). A fundamental issue in the development of cellulose-based biosensors is how to attach the biomolecules to the low-reactivity. Cellulose-based biosensors are characterized by several desirable properties, including fast accuracy, response, sensitivity, and low cost. Such sensors play an important role in various applications: Food stuff, forensic science, medical diagnostic instruments, and environmental monitoring (Tamayo et al., 2013). They can be divided into electrochemical, mechanical and optical biosensors based on their biotransducer type and can be used for detection methods. These includes fluorescent, colorimetric, conductometric, bioluminescent, electronic, gravimetric, quartz crystal microbalance, pyroelectric, etc. Due to the above characteristic advantages, cellulose-based optical biosensors are of huge interest for label-free and label-driven (fluorescent and colorimetric) biosensors. So far, various types of cellulose-based supports have been used in biosensors, such as nanocellulose, paper, bacterial cellulose, cellulose derivatives, hydrogels and meshes (Kamel and Khattab, 2020). These types of cellulose-based biosensors have been discussed in this review according to their fabrication methods and detection principle. Cellulose and its derivatives with their unique chemical structure have been found to be versatile materials that provide a high-quality platform for carrying out the immobilization process of biomolecules in biosensors. Cellulose-based biosensors exhibit several desirable properties, such as sensitivity, accuracy, convenience, rapid response, and low-cost. For example, cellulose paper-based biosensors are characterized by being low-cost and easy to use, while nano-cellulose biosensors are characterized by a good dispersion, high absorbance capacity, and large surface area (Chang et al., 2020). Cellulose and its derivatives are promising materials for biosensors that could be utilized to monitor various biomolecules, such as cells, proteins, amino acids, glucose, urea, cholesterol, hydroquinone and lactate. Future interest will focus on the design and construction of low-cost, miniaturized, multifunctional, environmentally friendly, and integrated biosensors (Chang et al., 2020). Therefore, the fabrication of cellulose-based biosensors or device is very important.

To manufacture a specific controllable device, such as implantable cellulose biosensors, it is important to strictly control the ability of proteins to interact. To do this, several methods are considered, mainly based on chemical functionalization, which allows the introduction of new moieties or on physical activation, a second polysaccharide bearing new functional groups, is adsorbed on the substrate. On the other hand, protein adsorption on various surfaces, has been paid a great interest in current research fields, especially those related with bio-nano medicine, including bio-nanotechnology, biocompatibility assessment and biological response between materials and biological organisms. There are many important factors that could be decisive and have a great influence on the behavior of adsorbed proteins on the surface used as a support. One of them, which greatly affects the adsorption process, is directly related to the chemical structure of the surfaces of the material used in the adsorption process. According to this key feature, an appropriate surface modification may provide providential behaviors of the needed protein-surface interactions (Orelma et al., 2012b; Roach et al., 2006). In order to prepare functional cellulose-based biomaterials, cellulose has to be subjected to several physical and chemical modifications (Heinze, 2009). Micro or nanofibrillated cellulose, cellulose nanocrystals (Habibi et al., 2010; Kostag et al., 2010), cellulose foils (cellophane) and composites of cellulose (Eichhorn et al., 2010) are only few examples of physical modification (Eichhorn et al., 2010; Habibi et al., 2010; Kostag et al., 2010). On the other hand, chemical modification has been widely used to synthesize functional cellulose derivatives, either from cellulose previously subjected to physical processes or from native cellulose (e.g. cellulose fibers, pulps). These include carboxymethyl cellulose (CMC), cellulose acetate, sulfated cellulose nanocrystals (SCN), trimethylsilyl cellulose (TMSC), 2,2,6,6-tetramethyl-4-piperidine-N-oxyl (TEMPO) -oxidized cellulose, and others (Heinze, 2009).

In this review, we try to capture some general elements regarding the adsorption of proteins and other biomolecules using cellulose-based substrates, emphasizing the nature of cellulose support, the pretreatment of the support, the most common proteins studied on such processes, the adsorption mechanisms, summarizing the main techniques used to detect protein adsorption.

2. The surface modification of cellulose

Cellulose is a linear syndiotactic homopolymer formed by the repeated binding by β– (1 → 4) –glycosidic bonds of glucose units. It is known for its hydrophilicity, biodegradability, low-protein binding capacity and broad functional variability as well as its semicrystalline structure. Cellulose has limited reactivity, due to its supramolecular structure, characterized by the assembly of elementary microfibrils and highly ordered crystalline regions formed by H-bonds and hydrophobic interactions (Coseri, 2017). Further, the accessibility of hydroxyl groups within cellulose, plays a key role in determining its reactivity and solubility, which is a topic of constant interest for various chemical modifications, and its conversion into various products, such as multifunctional textile fibers and biosensors (Krassig, 1990). To

overcome the drawbacks related to poor reactivity and solubility, which are required in the fabrication of multifunctional cellulose-based sustainable materials, surface modification of cellulose is often carried out (Biliuta and Coseri, 2019). To obtain high sensitivity and selectivity, low non-specific adsorption is very important for the stability of the biological molecules immobilized on the cellulose surface. This can be done by choosing an appropriate method to modify the surface. The selectivity of the adsorption process is determined by both the physicochemical properties and the biomolecules of interest. To date, many different methods have been introduced to change the surface of cellulose. They can be classified into non-covalent and chemical, i.e. covalent immobilization (also called direct conjugation) (Rusmini et al., 2007; Sassolas et al., 2008).

2.1. Non-covalent modification of cellulose

Non-covalent modification is simpler and does not involve any harsh treatment steps for immobilization of biological molecules on the cellulose surface, thus maintaining the structural integrity of the matrix polymer. Such modification can be easily done by using charged components. An example of this modification is adsorption via electrostatic interactions, where the charged components are adsorbed onto an oppositely charged surface. This process in general, results in the release of counterions, followed by entropy gain, which is the key factor for adsorption of charged components onto oppositely charged surfaces (Gregory, 1995; Mohan et al., 2014). Another type of non-covalent surface modification is assisted conjugation/immobilization, where both the adsorbing components and the base substrate should have high affinity/selectivity, meaning that they should be either structurally and/or chemically similar. This process is carried out by increasing the electrolyte concentration (Zelin Liu et al., 2011) or by lowering the pH of the adsorbing components, resulting in irreversible deposition of similarly charged molecules (Kargl et al., 2012). A major advantage of non-covalent adsorption is that it is generally carried out in water under mild conditions, representing additional benefits in terms of cost and environmental impact. This ecological approach is preferred and can be a suitable alternative to surface modification by chemical methods. For effective surface modification, it is imperative that the interaction with the surfaces is strong enough to achieve irreversible adsorption of oppositely charged components. Cellulose loaded with a minimum content of anionic groups, was processed at pH values of 2 and 10 (Mohan et al., 2019) in various forms including thin films, particles, fibers, and papers, and studying the phenomena of interaction with other polyelectrolytes under different experimental conditions. The kinetics of polyelectrolyte adsorption have been studied using various analytical tools, such as a quartz crystal microbalance with dissipation (QCM-D), surface plasmon resonance (SPR), zeta potential, etc. The results obtained were then usedto immobilize various types of biological molecules such as proteins, antibodies and DNA.

2.1.1. Surface modification with cationic components

Cellulose thin solid films regenerated from trimethylsilyl cellulose under hydrochloric acid atmosphere were primed with chitosan (Chi) for controlled attachment of human immunoglobulin G (hIgG) and bovine serum albumin (BSA) (Orelma et al., 2011). Chitosan, a polysaccharide composed of *N*-acetyl-D-glucosamine, randomly linked via β-(1–4) bonds to D-glucosamine units, has usually a degree of deacetylation between 70 and 80%, and a pK_a value of ∼ 6.5 (Mishima et al., 1998). Chitosan, however, was employed as a cationic charge's provider that can control and enrich protein adsorption. The adsorption of chitosan onto a weakly charged cellulose surface, due to electrostatic attraction, was monitored *in-situ* by QCM-D and SPR. As Fig. 1 reveals, chitosan adsorbs irreversibly but in a lower extent (Δf of −12 Hz).

Cellulose thin films, prepared from the deacetylation of spin coated acetate films, were decorated with multilayers of anionic CMC and cationic chitosan at different pH values (2–5.5) by Mohan et al. (2015a).

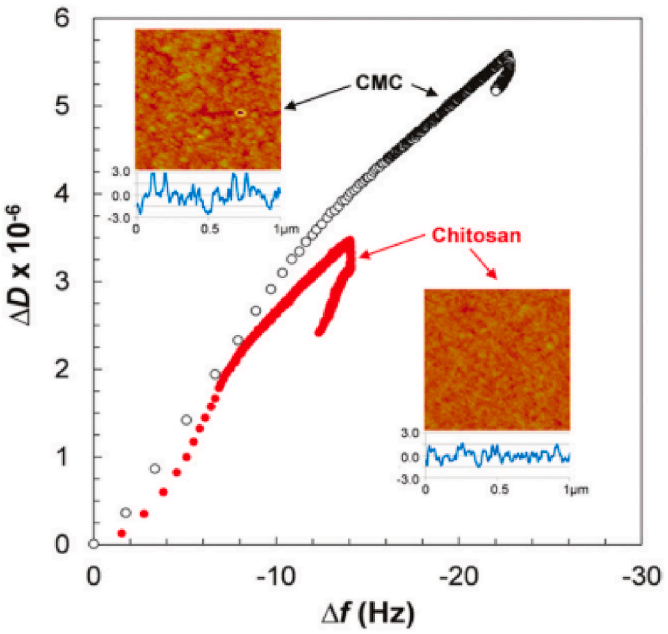

Fig. 1. ΔD-Δf fifth normalized overtone profiles, in QCM-D, upon adsorption from 0.5 mg/mL of chitosan or carboxymethyl cellulose (CMC) (aqueous solutions) on Langmuir-Schaeffer cellulose films (50 mM ionic strength). Insets: 1 × 1 μm^2 AFM height images and corresponding roughness profiles. Reproduced from (Orelma et al., 2011) with permission from American Chemical Society.

Such films were used to control the adsorption behavior of blood proteins, namely bovine serum albumin (BSA) (Mohan et al., 2015a). Another cellulosic film cationized with *N,N,N*-trimethylchitosan chlorides (TMC) was prepared using two types of TMC differing in the degree of substitution (DS). These films exhibited good control over the amount of BSA deposition at three different pH values: 4, 5, and 7 (Mohan et al., 2014). Beside cellulose films, Lyocel fibers were modified in the same manner by using TMC, where Payerl et al. (2017) monitored the zeta-potential value of the TMC-coated fibers, with the aim to control the adsorption behaviour of BSA (Mohan et al., 2015a). For both cellulose thin films and fibers, the results proved that TMC with lower DS yielded higher adsorption, presumably due to the reduced cationic charges and solubility of TMC in solution, as evidenced by QCM-D and zeta-potential measurements (Mohan et al, 2013a, 2014; Payerl et al., 2017). Chitosan is a readily available precursor, which is especially valuable for applications where the amino groups to be introduced on the surface of cellulose. The amount of introduced amino groups on the cellulosic surface can be manipulated by adjusting the degree of substitution of the OH groups in position 2 and 3 of the anhydroglucose unit with aldehyde groups, which is usually achieved by reaction with sodium periodate (Kim et al., 2017). In this way, cellulose-derivatives with high content of free amino groups can be easily prepared. The adsorption behavior of water soluble cellulose derivatives, such as cellulose-4-[N-methylammonium]butyrate chloride (CMABC) with a DS = 0.86, on cellulose substrates was studied as a function of electrolyte concentration and pH of the polymer solution, using the QCM-D technique, with the aim to determine the mass of deposited cationic cellulose (Mohan et al., 2013b). CMABC was shown to be irreversibly adsorbed onto cellulose surfaces at high ionic strengths (25–100 mM NaCl) at pH 7 (Δf_3: 15 to-17 Hz). When the ionic strengths were decreased from 1 to 10 mM, smaller amounts of CMABC (Δf_3: 2 to −12 Hz) were deposited on cellulose surfaces, as revealed by the frequency changes using QCM-D. Rising of the surface with water (pH: 7 to 8), lead to an increase in adsorption. Notably, no adsorption was detected at lower pH values (3 and 5). The morphology of CMABC coated surfaces was changed from fibrilar to a particle like structure as shown by AFM (Mohan et al.,

2013b). The authors successfully adsorbed cationic TMC (DS = 0.64) on cellulose thin films (regenerated from TMSC) as followed by SPR technique. The latter showed irreversible adsorption of TMC, which was further confirmed by ζ-potential measurements. This resulted in a positive ζ-potential value of +40 mV for the TMC-modified surface, compared to the pure cellulose film, ζ-potential ranging from −13 to-17 mV at pH: 4.7–7.2 (Niegelhell et al., 2018; Ristic et al., 2014).

Crystalline nanoribbons of cellulose oligomer derivatives with primary amino groups were synthetized via a cellodextrin phosphorylase-catalyzed single-step reaction, employing α-D-glucose l-phosphate monomers and 2-aminoethyl-β-D-glucoside primers (Nohara et al., 2017). The primary amino groups attached on the nanoribbon surfaces induce a positive charge (ζ-potential of the measured sample before adsorption measurements was determined as +24 mV), which effectively attracts negatively charged proteins, but not the positively charged ones, such as cytochrome *c*, lysozyme, and trypsin (with isoelectric points of 10.1 (Lvovj et al., 1995), 10.5 (Koutsopoulos et al., 2007), and 11 (Lvovj et al., 1995), respectively. Moreover, the amino nanoribbons showed no apparent cytotoxicity towards cultured cells (Nohara et al., 2017).

2.1.2. Surface modification with anionic components

Similar to the attachment of cationic polymers, several works can be found in the literature involving the modification of the cellulose surface with anionic components. For example, the CMC molecule is negatively charged in aqueous solution, has a pKa value of ~4.5, have been often used for the modification of cellulose thin films with anionic moieties. The resulted CMC-modified films have been used to prevent nonspecific protein binding (Nordgren et al., 2009). CMC adsorption occurs in a manner similar to that of Chi. The slope of the ΔD-Δf profiles of CMC is comparable to that of Chi (Fig. 1), and adsorption is highly irreversible, with rather small amounts of desorption observed, as indicated by a small loop in the ΔD-Δf diagram at the end of the process. While the poor adsorption of chitosan with positive charges on the cellulose surface can be explained by electrostatic interactions, in the case of CMC adsorption, with negative charges on cellulose substrates, the authors suggested a different mechanism responsible for its irreversible adsorption. Thus, it is suggested that both the adsorbent site of CMC and the base substrate (cellulose) have high affinity/selectivity due to structural and chemical similarities. The existence of such selective interaction between CMC and cellulose has been addressed in numerous cases (Kargl et al., 2012; Laine et al, 2000, 2002; Zemljic et al., 2008). However, to date, there is no direct evidence in the literature to confirm such selective CMC-cellulose interactions. A reasonable explanation for these interactions can be attributed to the hydrogen bonding interactions between CMC unsubstituted glucopyranosides and cellulose glucopyranosides (Laine et al, 2000, 2002). It can be predicted that such an interaction is only feasible when the charges on the CMC are minimized. This can be achieved either by increasing the electrolyte concentration (Liu et al., 2011) or by lowering the pH of the CMC solution (Kargl et al., 2012). Consequently, CMC molecules can be irreversibly deposited on cellulose surfaces. Recently, a new evidence was provided for the specific adsorption of CMC on cellulose supports at low pH and under salt-free conditions, compared to non-cellulosic supports. This specific adsorption is again based on the fact that there is a selective CMC-cellulose interaction due to the structural similarity between the CMC polyelectrolyte and the cellulose surface (Kargl et al., 2012; Orelma et al., 2011). It is worth noting that the CMC-coated surfaces were found to be particularly stable under different rinsing conditions. This high stability of CMC molecules, which can be conjugated with any kind of functional molecules, recommends their easy anchoring on cellulose surfaces.

Unlike cellulose, xylans carry a variety of different side groups, such as residues of O-methylglucuronic acid, O-acetyl residues, even ramose and galacturonic acid, or α-L-arabinofuranose, providing a rather complex polysaccharide structure. Xylan was precipitated on four different cellulose samples, including white and unbleached kraft pastes (SKPB and SKPUB), a bleached sulfur paste and viscose fibers (Miletzky et al., 2015).. Deposition of xylan on cellulose samples led to increased xylan content, where the affinity for xylan depended on the type of cellulose and the deposited xylan content decreased from bleached softwood kraft pulp (SKPB) to unbleached softwood kraft pulp (SKPUB), bleached sulfur pulp (SPB). Interestingly, no adsorption/precipitation of xylan was detected on the viscose fibers. Ahola et al. (2008) investigated the dynamic adsorption of anionic CMC and neutral xyloglucan molecules (0.1 g/L) on the swollen surfaces (in 1 mM $NaHCO_3$/1 mM NaCl) of cellulose nanofibrils using QCM-D and SPR techniques (Ahola et al., 2008). It was observed that the adsorption of xyloglucan occurs by a diffusion effect in the film, through the nanofibrils, which means that the nanofibril/polymer layer becomes weaker and softer upon adsorption and also increases the water adsorption capacity of the film. Although CMC adsorption induced a film dispersion effect, it was not irreversibly adsorbed on the nanofibril surfaces.

2.2. Chemical methods for the cellulose functionalization

2.2.1. Cross-linked cellulose microspheres/nanospheres

Crosslinked cellulose microspheres/nanospheres play an important role in various applications due to their unique properties, for example the large area to volume ratio, considerable flexibility for various analyzes and data acquisition compared to native cellulose. Cellulose microspheres were prepared using epichlorohydrin as a crosslinking agent, using a mixture of aqueous NaOH/urea as a solvent for the cotton pulp (Fig. 2). These microspheres were used to immobilize polymyxin B, where they were preactivated with 1,4-butanediol diglycidyl ether and NaOH solution (Cao et al., 2016). However, the resulting material was also tested for adsorption of endotoxin, a constituent of the outer cell wall of Gram-negative bacteria, also called lipopolysaccharide (LPS), and adsorption was studied in both physiological saline and human plasma.

The maximum adsorption capacity observed in this study was 3605 EU/g in physiological saline e (Cao et al., 2016), which increased the possibility of using these microspheres for blood purification applications. Another approach in the preparation of cellulose nanospheres uses a mixture of lithium chloride (LiCl) and N, N-dimethylacetamide (DMAc) as the solvent for cellulose, followed by emulsification using a vortex mixer in silicone oil. In the final step, the emulsion in ethanol (a non-solvent) containing a polysorbate-type nonionic surfactant (Tween 20) is forced through a filter with a specific pore size, by nitrogen gas under pressure, resulting in nanospheres of perfectly spherical cellulose (Carrick et al., 2014).

Recently, an interesting approach was reported, using a mixture of bacterial cellulose (BC) and chitosan for the preparation of hybrid hydrogel beads. Both polysaccharides were dissolved in ionic liquids, such as 1-ethyl-3-methylimidazole acetate, followed by the reconstitution step with distilled water (Kim et al., 2017). They have been used as supports for the immobilization of Candida rugosa lipase, both by physical adsorption and covalent crosslinking. BC-chitosan hydrogel beads were found to be more effective for lipase immobilization compared to hydrogel beads obtained from microcrystalline cellulose (MCC) or chitosan alone. The amount of protein adsorbed on BC-Chi beads was almost 4 times higher than the amount adsorbed on MCC-Chi hydrogels, while lipase on BC-Chi beads exhibited twice the catalytic activity. Spectacularly, the half-life at 60 °C of the crosslinked enzyme on BC-Chi hydrogel beads was more than 20 times longer than that of free lipase (Kim et al., 2017).

Fig. 3 shows scanning electron microscopy (SEM) images of lipase-coated cellulose-chitosan beads. It is remarkable that the BC chitosan beads retain their spherical shape after freeze-drying. Characteristics such as: surface morphology and size remain unchanged by the lipase crosslinking process. The MCC-chitosan beads have a more pronounced microporous structure than the BC-chitosan beads. Despite the fact that

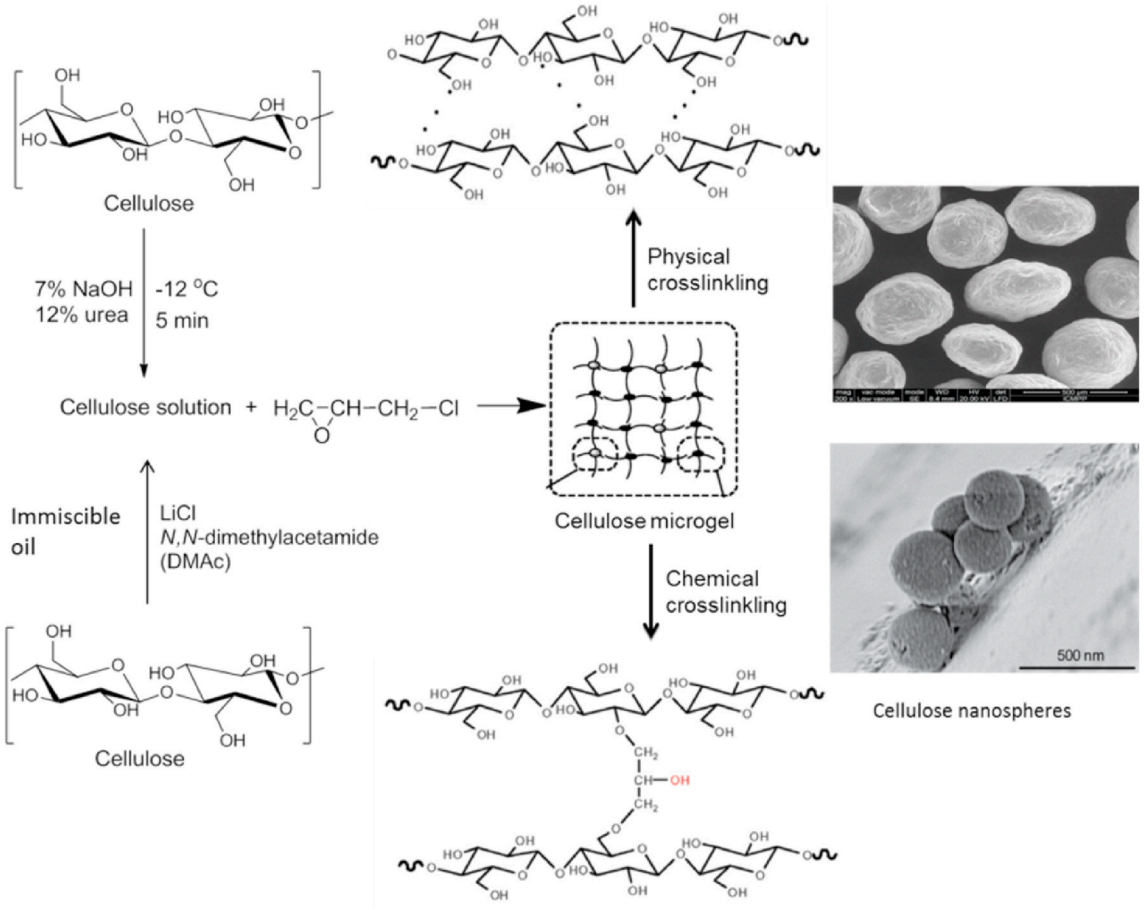

Fig. 2. Chemical route for the preparation of cellulose microspheres. Reproduced from (Cao et al., 2016) with permission from Elsevier.

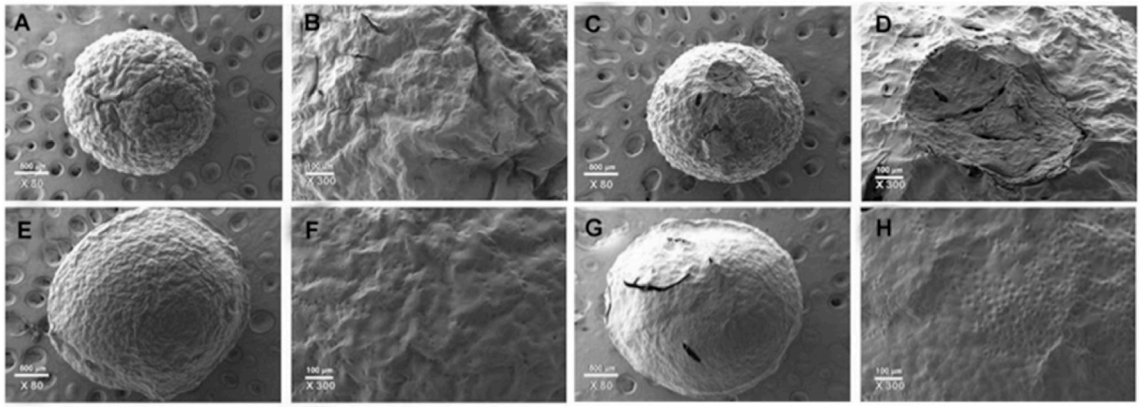

Fig. 3. Morphology of cellulose-chitosan hydrogel beads. Scanning electron microscopy pictures of lipase-coated cellulose-chitosan beads (A, C, E, G) along with their surfaces (B, D, F, H) are shown. (A, B) images of BC-chitosan hydrogel beads with adsorbed lipases, and (C, D) cross-linked lipase. (E, F) Images of MCC-chitosan hydrogel beads with adsorbed lipases, and (G, H) cross-linked lipases. Reproduced from (Kim et al., 2017) with permission from Springer.

the microporous structures of the MCC-chitosan beads appear to be more advantageous for protein adsorption, the amount of adsorbed protein on the BC-chitosan beads was surprisingly much higher. This suggests that the higher enzymatic adsorption capacity of BC-chitosan beads could be the result of higher chemical interactions between BC and lipase. A different approach for the preparation of cationically modified cellulose (SC) superporous beads was reported by Wang et al. (2007) using calcium carbonate granules as porogenic agent by emulsification in a water-oil mixture, followed by thermal regeneration (Wang et al., 2007; Wang and Sun, 2007). Cellulose previously functionalized with glycidyl methacrylate was cationized by diethylaminoethyl grafting (DEAE) followed by testing the electrosatic adsorption of BSA. At the same time, double crosslinking was performed to increase the mechanical strength of the particles. Electron microscophs of the SC beads revealed the presence of several micrometric “craters” on the surface of the beads. These “craters” led to higher water content, but also higher porosity. The two beads were then modified with a diethylaminoethyl group to prepare anion exchangers. The experimental results regarding the dynamic adsorption of bovine serum albumin (BSA) showed that the diffusivity of the BSA pores in the DEAE-SC bead was two to three times higher than in

the DEAE-MCC bead.

2.2.2. Oxidized cellulose

Under the generic term *'oxidized cellulose'*, there is a wide range of products containing carboxyl, aldehyde and/or ketone groups in the anhydroglycoside unit. In particular, it is possible to introduce mainly the carboxyl group by oxidation on the C_6 unit of the anhydroglucose unit, while oxidation taking place on the OH groups attached to C_2 and/or C_3 results in the introduction of the aldehyde groups, simultaneously with the cleavage of the C_2–C_3 bond. For the oxidation of C_6 in the cellulose unit (Vuoriluoto et al., 2016), the TEMPO-mediated protocol is largely used, as one of the most selective methods (Baron et al, 2019a, 2019b; Madalina Elena Culica et al., 2019; Tarbuk et al., 2020; Toshikj et al., 2019), while the sodium (meta) periodate reaction with cellulose selectively provides dialdehyde cellulose (Kim et al., 2017). Recently we reported a combination of the two oxidation protocols that can produce a tricarboxyl cellulose derivative (Coseri et al., 2015) with plenty of carboxyl groups in the structural unit, rendering the product water soluble (Fig. 4(Fig. 5).

Oxidation of the primary OH groups of the anhydroglucose unit in cellulose is an affordable technique for the introduction of carboxyl groups, but it has been successfully used as a pretreatment step for the preparation of nanocellulose (Coseri, 2017). It requires the presence of a stable nitroxyl radical, such as 2,2,6,6-tetramethyl piperdine *N*-oxy, which mediates the overall process, in the presence of sodium hypochlorite and sodium bromide, at pH 10 (Isogai and Kato, 1998; Okita et al., 2010; Saito and Isogai, 2004). Alternatively, other nitroxyl radicals, such as those derived from *N*-hydroxyphthalimide (NHPI) (Coseri, 2007, 2008, 2009a, 2009b), have been satisfactorily applied to selectively oxidize C_6 in cellulose to carboxyl groups (Biliuta et al., 2017; 2011; Coseri et al., 2018; 2013; Coseri and Biliuta, 2012; M.E. Culica et al., 2019). TEMPO-oxidation reaction leads to easier disintegration, yielding very thin fibrils with a diameter of 3–5 nm (Saito et al., 2007), which also have specific properties that are modified compared to the starting cellulose, as they have a high negative charges on the surface.

Dialdehyde cellulose (DAC), resulting from the oxidation reaction of cellulose with sodium periodate, under acidic conditions (pH = 3), at temperatures close to room temperature, can be prepared with varying degrees of oxidation (OD), mainly by changing the reaction time (Kim et al., 2000). To shorten the reaction time, other parameters are changed, such as reaction temperature, but also metal salts could be used, as an activator for cellulose (Sirvio et al., 2011). DAC, due to the presence of two aldehyde groups per anhydroglucose (AGU) unit in its structure, but also due to its natural origin as well as its low toxicity, aroused an increased interest as it was perceived as a much more convenient alternative crosslinking agent to replace glutaraldehyde (GA), which is now widely used, but highly toxic (Kim et al., 2017; Woo et al., 2012). DAC films obtained from the dissolution of DAC in dimethylformamide (DMF) and deposited on a glassy carbon electrode (GCE) have been successfully used to immobilize antibodies for the detection of human IgG. The obtained electrochemical immunosensor is capable of detecting hIgG with good specificity, stability and reproducibility in the range of 0.5–45 ng/mL, while the detection limit is 0.3 ng/mL (Kim et al., 2017).

A DAC sample with an aldehyde group content of 5.15 mmol/g has been shown to be effective for BSA binding by Schiff base formation (Zhang et al., 2018). Delighted by the large amount of immobilized BSA, the authors introduced the idea that the use of DAC could be more effective than the traditional Sevag method, in cases of deproteinization and protein recovery (Zhang et al., 2018). Another strategy using DAC was the coupling of its aldehyde groups with the chitosan amino groups by forming the Schiff base, followed by the reduction of the imine groups using sodium borohydride (Kim et al., 2017). At pH values of 5–5.5, crosslinked chitosan with DAC has a remarkable adsorption capacity of BSA, as an effect of the high content of unreacted amino groups during the Schiff base formation reaction between DAC and chitosan. Furthermore, it is worth noting that the cytotoxicity of DAC crosslinked chitosan is absent, as observed in a 21-day long-term stability test. Therefore, crosslinked chitosan DAC could be successfully recommended as a biomaterial in protein immobilization applications, drug delivery, but also other applications in the biomedical field in general (Kim et al., 2017). Another grafting reaction performed on the CHO groups in DAC was reported by Du and Dan (2018), who modified the DAC (e.g., macroporous cellulose monolith, MCM), by grafting pentaethylenehexamine (Du and Dan, 2018). In this way, the newly introduced amino groups reacted successively with glutaraldehyde and iminodiacetic acid (IDA), to prepare carboxyl-terminated cellulose monoliths, which were, subsequently complexed with Cu^{2+} ions to obtain Cu (II) –IDA-MCM. This system is expected to be very effective for the selective binding of glycoproteins, such as Concanavalin A (Con A), by a reversible mechanism.

2.2.3. Carboxymethyl cellulose

Bacterial cellulose is synthesized by some bacteria such as *Acetobacter xylinum* and is used as a superior cellulose source because it is obtained with a high degree of purity and uniformity and has possessing remarkable physical and mechanical properties. These properties have recommended BC for use in many biomedical applications, including dressings (Gershlak et al., 2017), prostheses for blood vessels (Czaja et al., 2007), tissue regeneration (Jonas and Farah, 1998) but also in the composition and construction of a variety of other biomedical devices (Czaja et al., 2007; Svensson et al., 2005). A classic example of BC derivatization is the conversion of the primary OH groups from the anhydroglycoside unit to carboxymethyl (CBC) by reaction with sodium

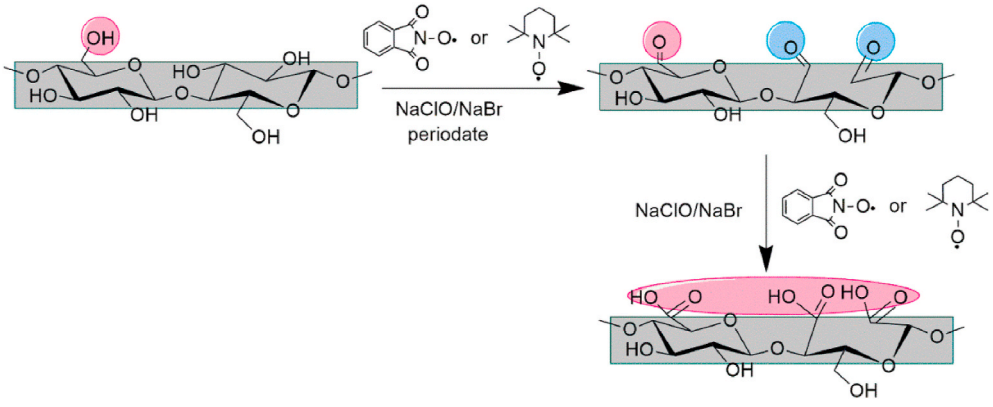

Fig. 4. The fully oxidization of the three OH groups in the presence of sodium hypochlorite (NaClO), sodium bromide, TEMPO and/or phthalimide-N-oxy (PINO) radical, emphasizing the two stages of the conversion. Reproduced from (Coseri et al., 2015) with permission from Royal Society of Chemistry.

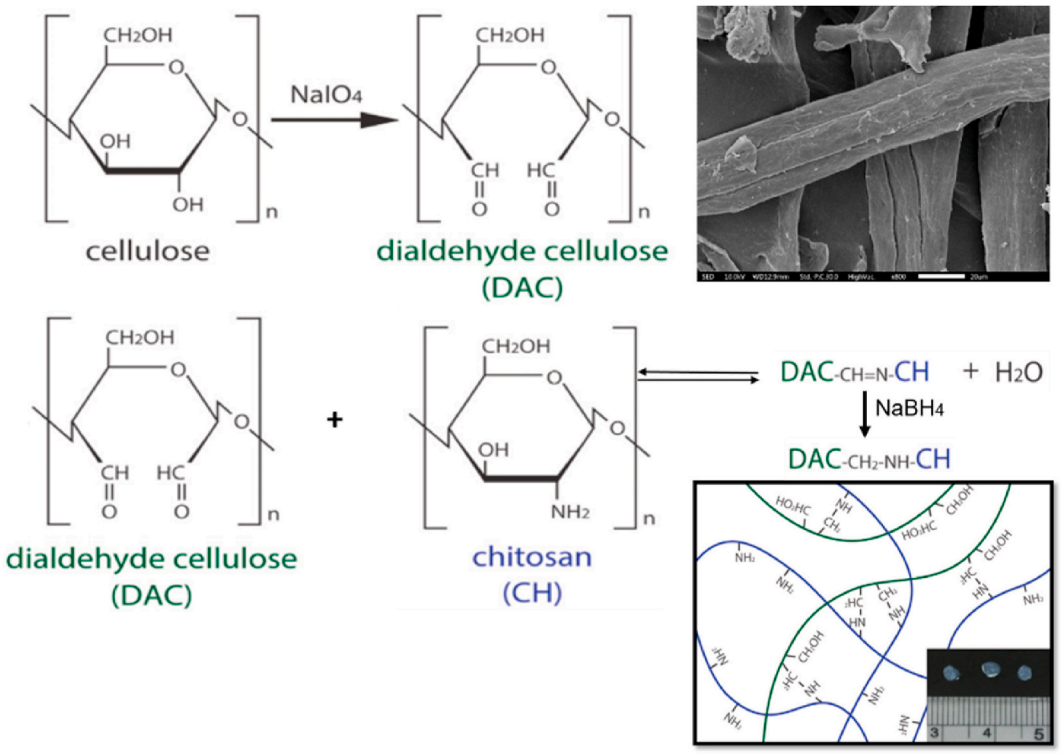

Fig. 5. Preparation of DAC from the reaction of cellulose with sodium periodate, SEM picture of fibrous DAC, the interaction between DAC and chitosan, and the possible interaction occurred between DAC and chitosan. Reproduced from (Kim et al., 2017) with permission from Elsevier.

chloroacetate (Fig. 6), with the resulting derivative having a special affinity for adsorption of bovine serum albumin (Lin et al., 2015).

The amount of adsorbed BSA is highly dependent on the degree of substitution (DS) of the synthesized CBC. For example, the sample with the highest degree of substitution (carboxymethyl cellulose for 36 h) has a maximum adsorption capacity of up to 0.217 mg BSA/mL. Not only the chemical structure of CBC and the degree of substitution are the factors that determine protein adsorption, but also the electrostatic interactions that occur between CBC and model protein. In the case of BSA, the carboxymethyl sample was adsorbed at a pH below its isoelectric (PI) point and no adosrption occurred at a higher pH above the PI value (Lin et al., 2015).

2.2.4. *Phosphorylated cellulose*

Another common route to functionalize cellulose in the preparation of protein adsorption is phosphorylation. In a recent study, two types of cellulose, namely bacterial cellulose and plant cellulose (PC), were tested to synthesize their corresponding phosphorylated derivatives, with different degrees of substitution that can be adjusted by varying the amounts of urea and phosphoric acid used in the reaction (Fig. 7) (Oshima et al., 2011).

The phosphorylated cellulose products were tested for lysozyme adsorption and it was found that lysozyme adsorption depends on the phosphorylation. Moreover, adsorption also depends on the type of cellulose, for example, phosphorylated bacterial cellulose has a much higher adsorption capacity than plant cellulose at the same degree of substitution. This behavior was explained by the specific surface area of phosphorylated bacterial cellulose, which is much larger than that of phosphorylated cellulose, leading to a greater number of potential sites for protein binding. Lysozyme is adsorbed on phosphorylated BC by electrostatic interaction, at pH values lower than its isoelectric point.

2.2.5. *Quaternary ammonium cellulose*

The same two cellulose sources used for phosphorylation (BC and PC) were used for the preparation of quaternary ammonium cellulose using (3-chloro-2-hydroxypropyl)-trimethylammonium chloride, with the reaction taking place in ethylene diamine (EDA) or triethylene tetramine (TETA) (Fig. 8) (Niide et al., 2010).

The adsorption capacity of quaternary ammonium cellulose derivatives such as bacterial cellulose (QABC) and cellulose plants (QAPC) was studied in detail on a wide range of molecules, including hemoglobin and myoglobin under basic conditions, and the results are shown in Table 1.

It can be seen from Table 1 that the adsorption of proteins, onto quaternary cellulose derivatives occurred in much larger quantities compared to unmodified cellulose. Again, quaternary ammonium derivatives of bacterial cellulose have an impressive adsorption capacity compared to derivatives from plant cellulose (almost 20 times higher in myoglobin adsorption and about 25 times higher hemoglobin adsorption). An unconventional approach for the preparation of a cellulosic oligomer with amino groups in the structure (2-aminoethyl-β-D-glicoside) using an enzymatic pathway was proposed by Nohara et al. (2017). The oligomer obtained was used as a model compound for studying and understanding the physical interaction between its functional groups of different types of proteins, such as BSA, IgG, lysozyme, transferrin and trypsin (Nohara et al., 2017). In the work of Lombardo et al. (2017)

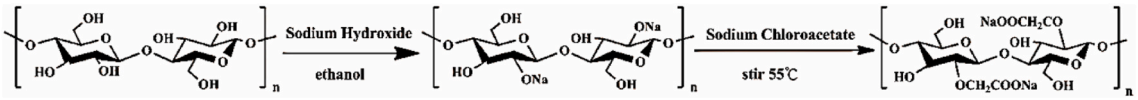

Fig. 6. Synthesis of carboxymethyl cellulose from bacterial cellulose. Reproduced from (Lin et al., 2015) with permission from Elsevier.

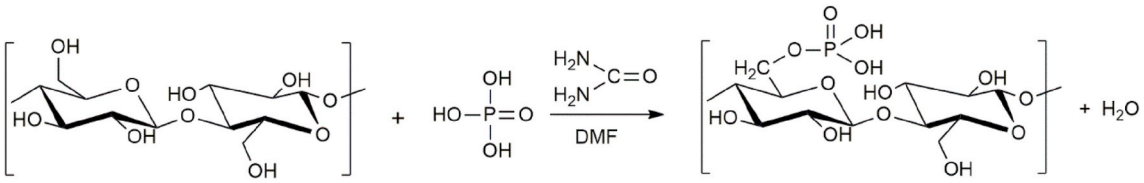

Fig. 7. Reaction scheme for the preparation of phosphorylated cellulose. Reproduced from (Oshima et al., 2011) with permission from Elsevier.

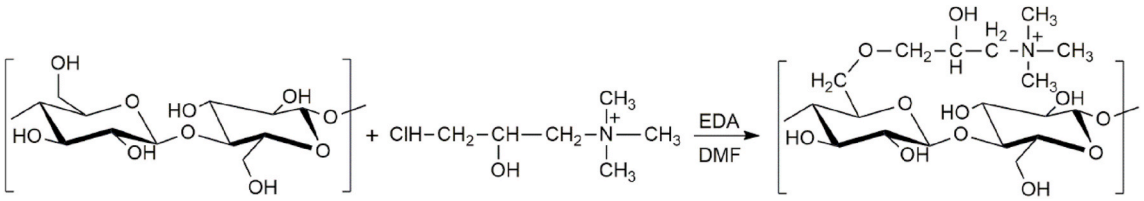

Fig. 8. Synthetic route for the quaternary ammonium cellulose preparation. Reproduced from (Niide et al., 2010) with permission from J-STAGE.

Table 1
Adsorption of different proteins on various types of cellulose, under common conditions (adsorbent: 20 mg, volume = 15 cm^3) (Niide et al., 2010).

Adsorbent	Material	Hemoglobin	Myoglobin
	M.W. (A.W.)	64,500	17,200
	Initial Concentration ($\mu mol/dm^3$)	6.20	23.3
QABC		88.0×10^{-3}	423×10^{-3}
QAPC	q ($mmol/g^1$)	3.88×10^{-3}	22.8×10^{-3}
BC		0.68×10^{-3}	9.32×10^{-3}
PC		0.53×10^{-3}	6.87×10^{-3}

cellulose nanocrystals were grafted with pyridinium ions, obtaining different degrees of positive charge, in the range DS = 0.19–0.91 to obtain an in-depth perspective on the adsorption properties of individual amino acids, as well as other molecules, such as BSA.

2.3. Cellulose membranes-surface conjugation

Mechanical treatment is used intensively in the laboratory, but also in large companies in practice, for the disintegration of cellulose to individualize cellulosic fibers, for the preparation of microfibrillated cellulose (MFC) and nano-sized cellulose components. Even though the process ensures the individualization of cellulosic components, there are a number of disadvantages. For example the process itself is expensive and it requires very high energy consumption, with relatively low efficiency (Qing et al., 2013). Given these disadvantages, a new approach has recently been considered that could be a viable alternative to the disintegration of cellulose fibrils. This new approach considers the chemical treatment of cellulose, which can break down the myriad of hydrogen bonds present in cellulose nanostructures, resulting in nano cellulosic forms such as: Cellulose nanofibers (CNF) or Nanocrystalline cellulose (NCC), also known as Cellulose nanocrystals. CNC) (Khalil et al., 2012; Yousefi et al., 2013). Fig. 9 schematically shows the distribution of cellulose, starting from the level of a tree, up to the molecular level. Depending on the methods used to individualize cellulose fibers down to the microscopic level, several types of nanocelluloses can be obtained, such as: CNC, CNF, bacterial nanocellulose (BNCC) and hairy nanocellulose (HNC) (Sheikhi et al., 2019; Tavakolian et al., 2020).

CNCs are the crystalline component of cellulose, with dimensions up to 10 nm wide and 100–200 nm long. They are obtained by various methods aimed at removing the amorphous regions of cellulose fibrils, most often by acidic or enzymatic hydrolysis. Due to their composition, 100% crystallinity, CNCs exhibit amazing mechanical properties, such as a tensile strength of 7500 MPa and Young's modulus values between 100 and 140 GPa (Tang et al., 2017). Moreover, CNCs have exceptionally high specific surface area, between 150 and 250 m^2/g and lower thermal expansion coefficients, remarkable properties required of materials for use in tissue engineering and drug delivery applications

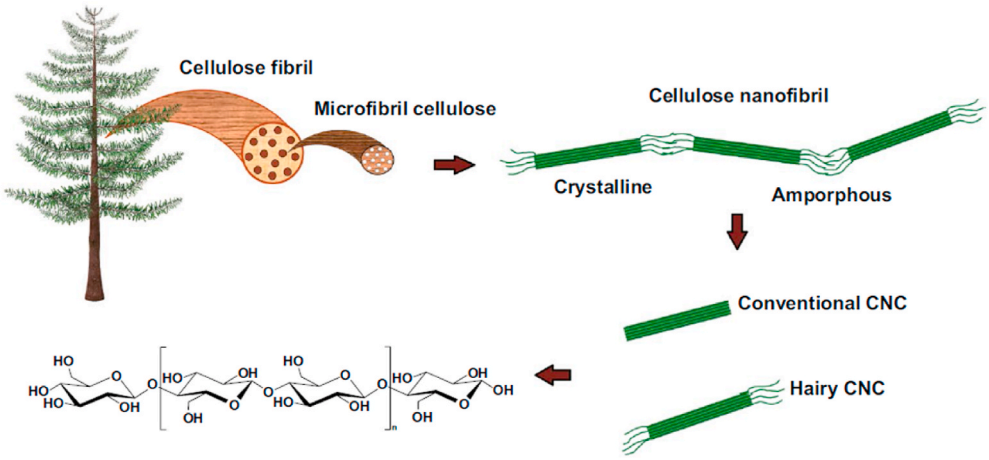

Fig. 9. Schematic representation of cellulose structures from resources to molecular level. Reproduced from (Tavakolian et al., 2020) with permission from Springer.

(Markstedt et al., 2015). Unlike CNC, CNF structure contains both crystalline and amorphous regions. CNF can be obtained mostly by harsh and long mechanical treatments of cellulose suspensions. It differs from CNC by larger dimensions, diameters up to 100 nm and fibril length of a several micrometers (Habibi et al., 2010). BNCC is a special kind of nanocellulose, produced by some types of bacteria, like *Gluconacetobacter xylinus*. The main advantage in its preparation is the absence of a mechanical process since the bacteria directly produce ribbon-like nanofibrils. BNCC exhibits larger fibrils width than CNF but similar length (Conley et al., 2017). Compared to CNF, BNCC has higher crystallinity, purity, and superior mechanical properties (Rol et al., 2019). In recent years, researchers have implemented a new class of nanocellulose in which only chemical treatments are applied to cellulose for the complete removal of amorphous parts and preserve the crystalline structure. This new class of nanocellulose has been called "*hairy*" nanocellulose, a name that actually suggests the existence of additional amorphous chains ("*wires*") protruding from both ends of the crystals, providing very good steric stability. The preferred chemical treatment for obtaining this type of nanocellulose is oxidation, a process that leads to solubilization and cleavage of the amorphous areas. Different types of HNCs can be prepared with neutral (Yang et al., 2015), negative (Yang et al., 2013), and positive charge content (Yang and Ven, 2016), which greatly expands the range of applications. Generally, HNC have similar sizes compared to conventional CNCs but the former have larger amounts of charged groups, which are introduced mainly in the amorphous areas during the preparation. It should be noted that the charged groups attached to the crystalline ends have very good accessibility compared to the crystalline part (Tavakolian et al., 2019) which contributes to higher reactivity of HNC. Due to the functional groups attached to the ends, HNC is an ideal substrate suitable for further chemical functionalization, reactions that are required in potential applications, including those as carriers of biological bodies such as drugs, proteins, antibacterial agents or bioactive elements (Yang et al., 2012). One such example is a microporous regenerated cellulose membrane immobilized with copper (II) ions. The method described by Wu et al. (2003) was used for the adsorption of various proteins, including BSA, lysozyme and ϒ-globulin (Wu et al., 2003).

3. Methods for the protein's adsorption monitoring

Protein adsorption on cellulose-based substrates can be monitored by two categories of methods: i) *in-situ - used* for real-time investigations of the protein adsorption process and ii) *ad mortem* methods used to explore the kinetics of protein adsorption after the process is complete. The first category includes surface sensitive methods, such as QCM-D (Crosson and Rossi, 2013; Salmain et al., 2012), and SPR (Dong et al., 2008; Jyoung et al., 2006). Other techniques, such as in-situ infrared and visible ellipsometry, a non-destructive technique, allow the study of protein adsorption on various polymers in aqueous solutions under various external stimuli, such as pH and temperature (Kroning et al., 2015).

In the second category, we can mention techniques such as fluorescence (Engström et al., 2006; Guo et al., 2014), chemiluminescence (Jain et al., 2004; Wu et al., 2013) lateral flow (López et al., 2013) and electrochemistry (Zhang et al., 2014). An interesting finding was recently proposed employing zeta potential measurements performed during adsorption using the streaming potential (Payerl et al., 2017).

3.1. Proteins attachment-the most studied ones

3.1.1. Immunoglobulins

Immunoglobulins, are proteins that exert excellent antibody activity and are produced by the terminal cells of B-cell differentiation, also known as plasma cells. Under the generic name of immunoglobulins, there are 5 major classes or isotypes: Immunoglobulin G (IgG) is the one that confers most of the immunity against invasive pathogens, based on antibodies, as it is the only antibody that can penetrate the placenta and provide passive immunity to the fetus, Immunoglobulin A (IgA), is mainly present in mucosal areas such as the intestinal tract or the respiratory and urogenital tracts, and is also found in saliva, tears and breast milk. Its role is to prevent colonization by pathogens. Immunoglobulin M (IgM), found on the surface of B cells (monomer) and in a secreted form (pentamer), which possesses a high avidity, immunoglobulin D (IgD), which acts as an antigen receptor on B cells, capable to activate basophils and mast cells to produce antimicrobial factors, and immunoglobulin E (IgE), which binds to allergens and activates histamine release from mast cells and basophils, is involved in allergies and is also a protective factor against parasitic worms. They play a decisive role in humoral immunity. Among immunoglobulins, IgG (Fig. 10) with a molecular weight of 150 kDa has the highest presence in the blood (73%). IgG is also detected in plasma and external secretions and is expressed on the B cell membrane. In normal human plasma, the concentration of hIgG is between 6.6 and 14.5 mg/mL, representing the key component of the system. immune (Beek and Hellstern, 1998). By measuring its concentration in the human body, valuable information can be obtained about the immune status of the individual to specific pathogens (Shiang et al., 2011).

3.1.1.1. Different strategies for the hIgG immobilization on cellulose based supports. Adsorption of human immunoglobulinG (hIgG) onto cellulose nanofibrils (CNF) was monitored using QCM-D (Zhang et al., 2013). Bioactive films prepared from cellulose nanofibrils (CNF) via conjugation with a short chain peptide molecule, acetylated-HWRGWVA, were reported to manifest a specific affinity towards hIgG (Zhuo Liu et al., 2011; Naik et al., 2011; Yang et al, 2009, 2010). To achieve the conjugation reaction, a hydrophilic copolymer, poly (2-aminoethyl methacrylate-*co*-2-hydroxyethylmethacrylate) (polyhydrochloride (AMA-*co*-HEMA)) was introduced by polymerization as a spacer and support for peptide immobilization, using an initiator previously coupled to the CNF. The authors evaluated two methods of grafting peptides: (I) the first method was to support the CNF suspension on QCM sensors, followed by conjugation of the peptides, peptide-CNF (I), and II) the peptide is mixed initially with the aqueous

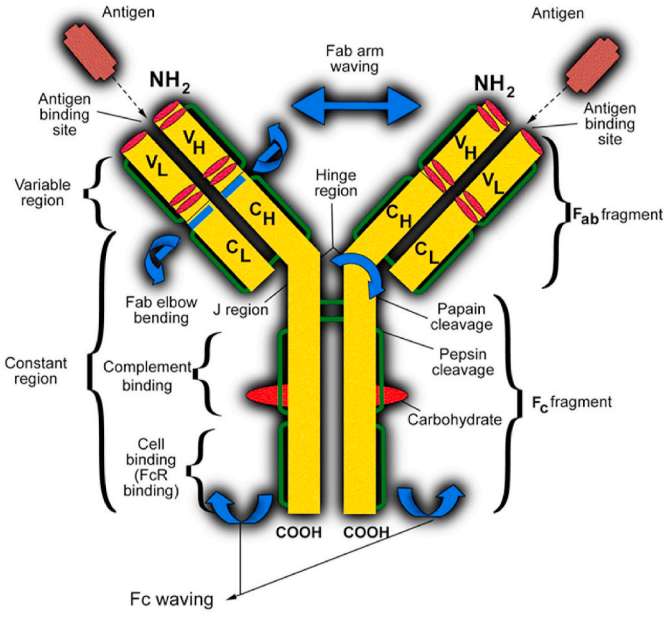

Fig. 10. Schematic representation of an immunoglobulin G molecule. CH represents constant region of heavy chain; CL, constant region of light chain; VH, variable region of heavy chain; and VL, variable region of light (Beek and Hellstern, 1998) chain. The size of this protein was reported as 14.5 × 8.5 × 4 nm, with a MW of ∼ 160 kDa (Lee et al., 2013).

suspensions of CNF and then spin coated on the QCM sensor, peptide-CNF (II) (Zhang et al., 2013).

It can be seen from Fig. 11, that the amount of immobilized hIgG is almost negligible in the case of the untreated CNF film, whereas, after activation by the two proposed routes, the amount of hIgG reached 16.4 ng/cm^2 for the peptide-CNF (I) films, and 17.8 ng/cm^2 for the peptide-CNF (II) films respectively. These values are higher than those reported by Orelma et al. (2011) for the cellulose films modified with CMC and chitosan (Table 2) (Orelma et al., 2011).

A recent and interesting study presents the manufacturing of an analytical device based on origami paper (3D-soPAD) which has a sliding band that has a large storage capacity of antibodies on a single sheet of paper and aims to increase the reliability and easiness of operation, with lower sample consumption (Chen et al., 2019). The construction of this device is simple and versatile and flexible due to its reduced size. HIgG was used as a model to test the 3D-soPAD device with detection limit of only 0.01 ng/mL and a response time of only 7 min. This device can store the test for longer periods without losing its effectiveness: 75 days at 4 °C, 30 days at 25 °C, and 2 days at 40 °C, when stabilizers (sucrose and D-(+)-trehalose) are added. The detection principle is shown in Fig. 12.

To demonstrate the applicability of the 3D-soPAD platform in clinical trials, horseradish peroxidase (HRP) – conjugated detection antibodies deposited on gold nanoparticles (AuNPs) were used to analyze protein A from human synovial fluid infected with *Staphylococcus aureus* collected during surgery. Five samples of human synovial fluid were tested, and the AuNPs had the role of signal amplification to obtain unequivocal test results (Fig. 12A), To induce a color change with the HRP-conjugated antibody, a 3,3 ′, 5,5′-tetramethylbenzidine (TMB) reagent was used. In this way, two out of five samples were identified as cultured negative, P1 and P2, while the other three were found infected with *Staphylococcus aureus*. To reduce the differences during detection caused by a variety of synovial fluids, with different viscosities and thus different ability to interfere with the matrix, the blank index (B/R) – (B/R) was adopted. As a result, a large standard deviation in the triplicate measurements of the infected samples can be seen from Fig. 12B. The presented results show that the test is able to discriminate the variation between the test (B/R) – blank values (B/R) of health controls and clinical infected samples, denoting that this proposed device could potentially be implemented as a reliable diagnostic kit for personal medical use and on-site analysis in an elementary and economic way.

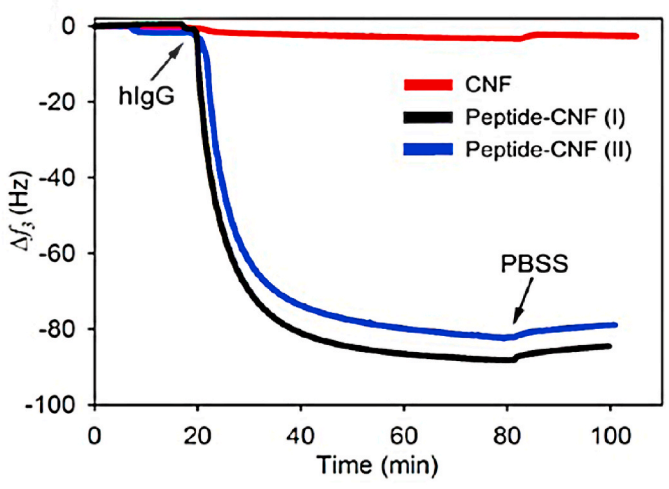

Fig. 11. Time-resolved QCM isotherms for adsorption and binding of hIgG on CNF, peptide-CNF (I), and peptide-CNF (II) surfaces. Injection of hIgG solution as well as rinsing with phosphate buffer saline (0.14 M NaCl) enriched with an additional 1 M NaCl (PBSS) buffer is indicated with arrows at the given times. Reproduced from (Zhang et al., 2013) with permission from American Chemical Society.

Table 2
Amount of immobilized hIgG on the different cellulosic supports.

No.	Cellulose type support	Amount of adsorbed hIgG (ng/cm^2)	Method of determination	Reference
1	Peptide-CNF (I)	16.4	QCM-D	Zhang et al. (2013)
2	Peptide-CNF (II)	17.8	QCM-D	Zhang et al. (2013)
3	Cellulose	2.3	SPR	Orelma et al. (2011)
4	Chitosan-modified cellulose	10.5	SPR	Orelma et al. (2011)
5	CMC-modified cellulose	5.8	SPR	Orelma et al. (2011)

In another very recent study, an ingenious strategy to introduce active channels on nanopaper and passivation towards non-specific hIgG was proposed (Fig. 13) (Rotaru et al., 2019).

Photolithography and inkjet printing techniques have been applied to the construction of two-dimensional fluid channels using a nanopaper produced from discolored birch kraft fibers as the support. This photolithographic approach used click-thiol-yne coupling, while for inkjet printing a polystyrene solution was used as the ink. Various protein blockers were developed using solutions with different concentrations of bovine serum albumin, fibrinogen or solutions of PDMAEMA (poly (2-(dimethylamino) ethyl methacrylate)) block-POEGMA (poly (oligo (ethylene glycol) methyl)) methacrylate ether)) copolymers (D33-EGMA-110 and D33-EGMA-137), which are allowed to adsorb on the surfaces of cellulose nanofibrils at pH 7.4. After adsorption, the cellulose surface was rinsed with phosphate buffer to remove weakly bound blocking agents., Then the adsorption of hIgG (0.1 g/L) on cellulose nanofibril surfaces was tested, and the process was monitored by SPR technique. Remarkably, due to the antifouling properties induced using protein blockers in the channels, the adsorption of hIgG on CNF decreased dramatically, up to 95%, as the POEGMA block copolymer was able to form a hydrated layer on the CNF surface. The efficient blocking of channels is corroborated with the adequate fluid flow and recommends the proposed cellulosic surface models as a high-performance platform for biosensitization. Unlike porous materials used in microfluids, the surface flow channels on the nanopaper have higher resolution, no leakage or lateral flow, instead showing higher sensitivity and much faster liquid transfer. In a related work, Vuoriluoto et al. (2016) chemically attached the anti-hIgG to the surfaces of TEMPO-CNF surfaces using EDC/NHS chemistry, Fig. 14 (Vuoriluoto et al., 2016). Chemical attachment of anti-hIgG (0.90 ± 0.09 mg/m^2) to the cellulose surface was monitored using QCM-D. Such surfaces were physically adsorbed with block and random copolymers such as poly (2-(dimethylamino)ethyl methacrylate) (PDMAEMA) or poly (oligo (ethylene glycol) methyl ether methacrylate) (POEGMA), to introduce non-fouling characteristics. Such anti-hIgG modified cellulose surfaces were used for quantitative detection of hIgG (1.28 ± 0.11 mg/m^2) using QCM-D. The authors also demonstrated that the bound IgG on the cellulose surfaces can be regenerated at lower pH (e.g. pH 2, see Figure below) and the anti-hIgG coupled cellulose surfaces can be reused with high selectivity.

3.1.2. Albumins

Albumins are natural proteins present in serum. Bovine serum albumin, due to its resemblance to human serum albumin (HSA) in structure and physicochemical properties (with more than 75.5% sequential similarity), is a commonly used marker to verify nonspecific binding (Akdogan et al., 2012). Albumins have pleiotropic tasks related to maintenance of plasma oncotic pressure, linking with endogenous and exogenous ligands such as fatty acids, hormones, toxic metabolites,

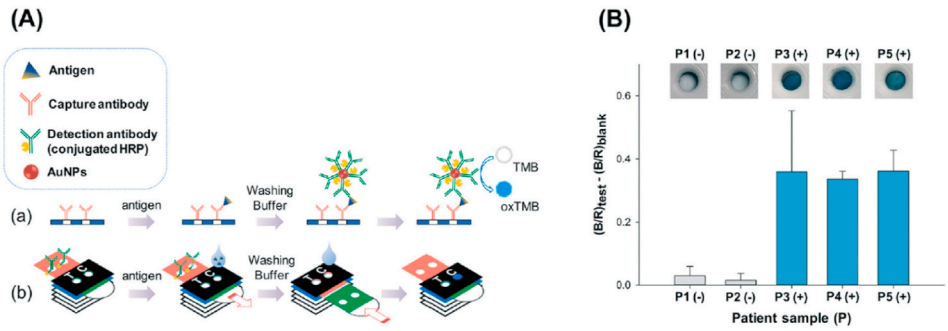

Fig. 12. (A) 3D-soPAD-ELISA test procedure for clinical *Staphylococcus aureus* infected human synovial fluid. (B) Clinical synovial fluid sample test results using the 3D-soPAD platform. The gray bars are the healthy controls and the blue bars are the infected human synovial fluid samples (n = 3). Reproduced from (Chen et al., 2019) with permission from Royal Society of Chemistry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

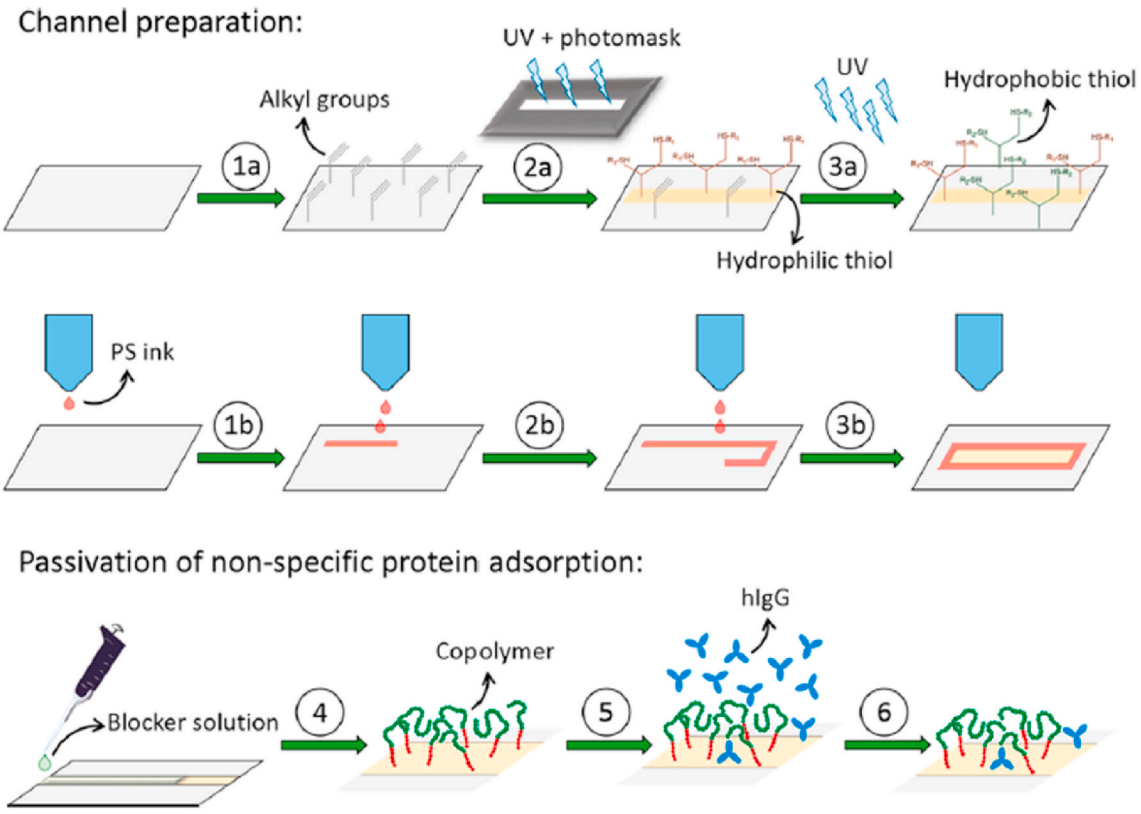

Fig. 13. Schematic illustration of the preparation of channels on nanopaper and passivation towards nonspecific hIgG adsorption. (1a) Esterification reaction using 4-pentynoic acid introduces thiol reactive alkyl groups on nanopaper. Thiol-yne reactions with (2a) hydrophilic and (3a) hydrophobic thiol-modified compounds fabricate the channel. Alternatively, (1b–3b) inkjet printing is employed for channel realization. In both cases (1a-3a) and (1b–3b), nonspecific protein passivation of the surface is achieved by (4) physical adsorption of blocker molecule (shown is the case of an adsorbing copolymer). This is demonstrated in (5) for hIgG adsorption, followed by (6) rinsing. Reproduced from (Solin et al., 2019) with permission from American Chemical Society.

metal ions, medicines, carrier for lipoproteins, small molecules and pharmaceuticals (Ha and Bhagavan, 2013). In recent years, albumins have played an exceptional role as typical model proteins in the study of novel drug delivery research (Hamidi and Zarei, 2009). However, albumins (BSA or HSA) are hardly adsorbed on cellulose (Orelma et al., 2011), due to the essentially amphiphilic nature of cellulose, the high degree of swelling of both, cellulose and BSA, and the lack of hydrophobic sites for BSA adsorption on cellulose (Pelton, 2009). Cellulose has been shaped in different forms to understand the adsorption or immobilization mechanism of BSA. To date, different physical and chemical methods have been developed to attach albumin to the cellulose surface. For example, Orelma et al. (2011) employed the CMC and Chi-modified thin films of cellulose for the adsorption of BSA. In this case, the introduction of cationic groups (in the case of Chi) or anionic groups (in the case of CMC) enhanced the adsorption of BSA close to its isoelectric point (pI = 5) compared to an unmodified cellulose film from 1.7 to 3.5 mg/m^2 as determined by using QCM-D and SPR techniques (Orelma et al., 2011). The same authors demonstrated the conjugation of BSA and antihuman IgG on TEMPO-oxidized thin solid surfaces of NFC using QCM-D. Conjugation of the proteins was achieved via EDC/NHS chemistry, resulted in the binding capacity of 9.8 mg/m^2 of BSA and 17.7 mg/m^2 of antihuman IgG on NFC (Orelma et al., 2012a). Orelma et al. (2014) also reported the modification of tubular bacterial nanocellulose (BC) with CMC by its introduction into a BC culture medium, resulting in the in-*situ* formation of a CMC–BC tubular network. In parallel, carboxylation of BC was performed via TEMPO–NaBr–NaClO oxidation (Orelma et al., 2014). These carboxylated BC surfaces were conjugated with anti-human serum albumin (anti-HAS, 84.7 ng/cm^2) affibody ligands via amide bonds using EDC/NHS. It was found that the specific binding of human serum albumin (HSA) was eight times higher

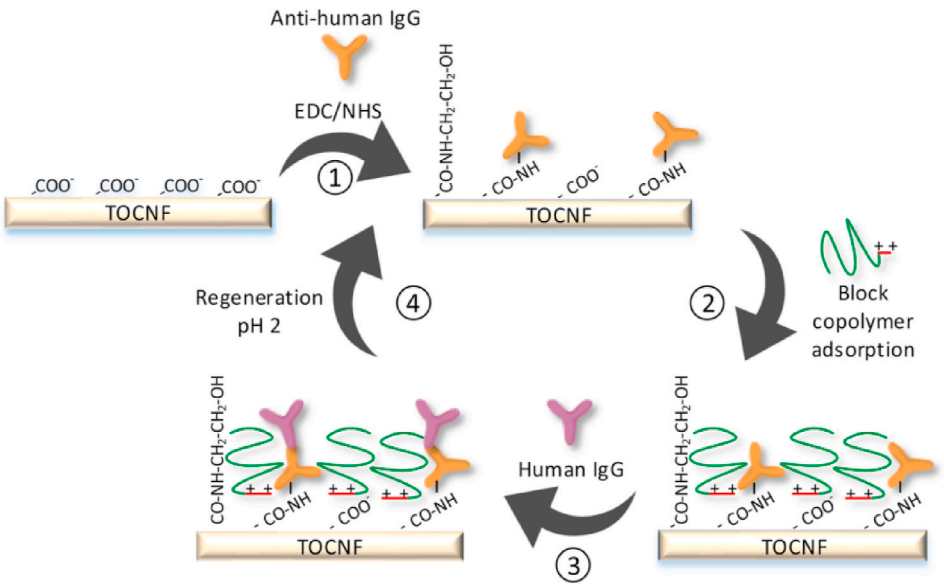

Fig. 14. Schematic illustration of (1) EDC/NHS Mediated Conjugation of Anti-human IgG on TOCNF and (2) Subsequent Blocking of Non-specific Human IgG Adsorption by Block Copolymers, (3) Detection of Human IgG by the Biointerface, and (4) Regeneration of the Biointerface for Reuse. Reproduced from (Vuoriluoto et al., 2016) with permission from American Chemical Society.

(81 ng/cm^2) when anti-HSA was conjugated to cellulose via EDC/NHS compared to direct adsorption of HSA (10 ng/cm^2) onto CMC modified BC surfaces. These specifically modified surfaces, equipped with the antibodies mentioned above, are expected to have greater potential for application in the filtration of blood proteins. It has been shown that cationic cellulose-modified cellulose and chitosan-derived thin films can be used to regulate BSA deposition by controlling the pH and ionic strength of protein solutions, or loading density with CMBAC or TMC used to modify the cellulose surfaces. CBAM modified surfaces allow control of BSA deposition in the range of 0.6–3.9 mg/m^2 (dry mass), as demonstrated by the SPR technique. In the case of cellulosic surfaces modified with TMC, the wet adsorbed mass of BSA ranges from 1.7 to 10.6 mg/m^2, amounts determined using QCM-D (Mohan et al, 2013a, 2014). Another interesting approach to immobilize BSA on cellulose membranes was proposed by Wei et al. (2011). In this approach, the cellulosic surfaces were first coated with a layer of polydopamine (PDA) by pH-induced polymerization (Wei et al., 2011). The PDA-functionalized cellulose membrane was subsequently conjugated with BSA (20 μg/cm^2) via Michael addition or Schiff base reactions by immersing the cellulose membrane in a BSA solution at physiological pH. In addition to thin films of solid cellulose, the use of porous cellulose membranes modified with various organic and inorganic components to improve albumin binding capacity, has also been reported. The work of Wu et al. (2003) highlighted the superior affinity of the porous cellulose membrane immobilized with different amounts of copper ions by various chelators and triazine dyes to bind BSA, which was performed at different concentrations of protein solutions and ionic strength (Wu et al., 2003). It was shown that cellulosic surfaces decorated with triazine dyes were superior for binding BSA via electrostatic interactions (Wu et al., 2003). Similarly, the surfaces of super-porous and micro-porous cellulose beads (SPCB and MPCB) equipped with cationic diethylaminoethyl groups were used for high-speed protein chromatography (Wang et al., 2007; Wang and Sun, 2007). This was demonstrated by adsorption of negatively charged BSA at different concentrations, between 0 and 1.2 mg/mL, at neutral pH. The binding efficiency of SPCB column has been shown to be 2 to 3 times higher as compared to MBCB, which was determined by batch adsorption experiments. In another study, it was shown that immobilization at physiological pH of BSA solutions with a concentration of 10 mg/mL can be adjusted by controlling the number of CMC layers adsorbed on thin cellulose films (Mohan et al., 2015a). While cellulose films adsorbed 17.7 mg/m^2 of BSA, cellulose films equipped with CMC showed different values of BSA adsorption depending on the pH, as follows: at pH 5, the adsorption is 15.9 mg/m^2, at pH 3: 12.4 mg/m^2 and 7 mg/m^2 at pH 2, respectively.

3.1.2.1. Other proteins. In addition to the above known proteins, another protein being studied by researchers is protein A, which is naturally present in the bacterial cell wall of *Staphylococcus aureus*. Adsorption of this protein is increasingly being studied because the functionalized surface of Protein A can be used to immobilize or target serum biomarkers in the blood, such as C-reactive protein (CRP) or anti-CRP. Zhang and Rojas (2016) immobilized protein A covalently on carboxylated or oxidized CNF surfaces using TEMPO, as well as by EDC/NHS coupling (Zhang and Rojas, 2016). Following these studies, TEMPO oxidized cellulose surfaces were found to be capable of adsorbing a higher amount of protein A compared to carboxylated cellulose surfaces (approximately 12.5 mg/m^2 compared to 2 mg/m^2). Despite these differences, approximately the same amount (approximately 3.3 mg/m^2) of anti-CRP attached to the protein-A ligand was identified. These functionalized surfaces were found to detect 0.2–0.7 mg/m^2 of CRP when incubated with the latter, at concentrations between 3 and 100 μg/mL. Since the methods developed for protein grafting often lead to longer reaction times, non-selective pH and non-physiological, a new two-step method was developed by Uth et al. (2014) wherein the protein was regioselectively coupled to TEMPO-oxidized CNC via an enzyme-mediated reaction (Uth et al., 2014). In this work, the primary OH groups of CNC were transformed to aldehyde groups that were subsequently coupled to the lysine-terminated oligopeptide motif by the Schiff base reaction, which was recognized under physiological conditions using enzymes such as sortase A transpeptidase. Using this enzyme, the authors demonstrated the possibility of targeted immobilization of various recombinant proteins at physiological pH for further functionalization with the protein of interest. In another work, two model proteins, such as alkaline phosphatase (AP) and anti-hydrocortisone antibody were chemically attached to functional NFC groups with amine, epoxy and carboxylic acid with different degrees of substitution using three approaches, (Arola et al., 2012). In this study, the conjugation methods for protein attachment were carefully chosen by considering all reactive groups

present on NFC (such as amination of epoxy groups, modification of amino groups, and activation of carboxylic acid activation by t EDC/NHS). The degree of conjugation of these two proteins varied significantly depending on to the type of functional groups present on the NFC surface. Protein-conjugated NFC was transformed into thin films of different thicknesses (by increasing the number of deposited layers) by the spin coating technique and used to analyze the specific activity of PA using QCM-D. It was found that a maximum of *ca.* 0.15 μg/cm^2 of AP and *ca.* 0.012 μg/cm^2 of anti-hydrocortisone antibody can be immobilized in the NFC matrices when the number of layers is increased. In this way, the authors developed an ingenious approach to control conjugation, the amount of incorporated protein, and at the same time, the preservation of protein activity. Yu et al. (2012) used divinyl sulfone chemistry (DVS) to conjugate glycoproteins such as Concanavalin A (Con A) to the backbone of cellulose (eg paper) (Yu et al., 2012). The Con A was specifically attached to the DVS activated cellulose substrate which was previously immobilized with various glycans, including maltose, mannose, glucose, amino-modified mannoside, etc. The authors demonstrated that the Con A could bind specifically and more to amino-modified mannoside than to other glycans (Du and Dan, 2018). As a reversible ligand, Con A was chelated with Cu (II)-IDA-MCM for the adsorption of glycoproteins. The reversible immobilization of Con A on Cu(II)-IDA-MCM was realized by Cu^{2+} ions bridging the Con A affinity ligand and held by strong chelation (metal-ion) interaction. In addition to their excellent structural stability and septicity, the MCM immobilized with Con A showed an adsorption capacity of 17.4 ± 0.6 mg/Ml when incubated with glucose oxidase, which can catalyze the oxidation of glucose to hydrogen peroxide and D-glucono-δ-lactone, demonstrating their potential for protein chromatography.

In another recent study, amino and thiol groups were introduced on the surface of cellulose nanocrystals and the resulting materials were designed and used for the conjugation of transfer glycoprotein (TF) and iron oxide (Fe_3O_4) (NP) nanoparticles (Hazra et al., 2020). TF was succinylated under alkaline conditions to obtain TF, which was then conjugated to the amino functional groups of CNC (0.038 mg–0.028 mg TF/mg CNC) by amide binding using the EDC/NHS protocol. Newly developed CNCs with a protein-transferrin (TF) were used for targeted adsorption of circulating tumor cells (CTCs) in blood. The authors showed that CNC modified with TF effectively isolated CTCs from patients' blood, with a 85% of cell capture efficiency compared to the standard platform, even though the capture efficiency of CTCs varied with the concentration of TF conjugated to the CNCs, demonstrating its potential for use in early stage detection of metastasis in cancer. A simple and low-cost method to modify cellulose sheets under biocompatible conditions and to retain the bioactivity of the enzyme, namely ovalbumin (OVA), was developed Credou et al. (2013). The cellulose sheets were modified via diazonium-based chemistry, which involves a free-radical reaction, and led to the modification of the cellulose surface with various functional groups such as amine, carboxyl or thiol. Following this modification in water and at room temperature, conjugation of murine anti-OVA monoclonal proteins (or antibodies) was performed in different ways. For example, carboxyl-bearing cellulose was conjugated to OVA via EDC/NHS chemistry, amine-bearing cellulose was derivatized with N-hydroxysuccinimide ester (SATA) and converted into thiol-bearing cellulose, and thiol-bearing cellulose was reacted with maleimido-OVA. Despite the different strategies, more OVA was immobilized on the thiol-bearing (ca. 53%) cellulose surface than on the other two surfaces (-COOH: *ca.* 46% and –NH_2: *ca.* 39%) (Credou et al., 2013). Additionally, the authors modified bacterial nanocellulose (BNC) with cell-binding proteins such as fibronectin and collagen type I via an efficient conjugation method using cyano-4-dimethylaminopyridinium (CDAP) tetrafluoroborate as an intermediate catalyst, which formed a cyanate ester derivative after reaction with the hydroxyl groups of cellulose (Kuzmenko et al., 2013). The aforementioned proteins were directly conjugated to the CDAP-activated cellulose via the amine groups of the surface amino acids at pH values between 9 and 10, resulting in the formation of an isourea bond. More fibronectin was found to be immobilized on the cellulose surface than collagen type I, which was correlated with the higher molecular weight of fibronectin (~500 kDa) compared to collagen type I (139 kD). Zhu et al. (2018) successfully immobilized α-fetoprotein (AFP) as a model protein on the oxidized cellulose paper surface bearing dialdehyde groups via imine formation due to the reaction between aldehydes and amine groups of AFP (Zhu et al., 2018). Besides their good stability and reproducibility, the AFP immobilized cellulose substrates showed a detection limit of 0.04 ng/mL horseradish peroxidase (HRP), high sensitivity and reliability, which demonstrated a great opportunity for the development of the paper-based ELISA for clinical applications.

3.1.3. Enzymes

Enzymes belong to the class of proteins with high biological activity. In chemical industry, enzymes play an essential role, owning to their exceptional activity associated with high selectivity and specificity (Schmid et al., 2001). The fast development of bio oriented industries has enabled the production and commercialization of enzymes in many areas, including chemical, pharmaceuticals, food and beverages, plastics, cosmetics, and biofuels (Gennari et al., 2020). In the food industry, for example, enzymes are widely used to provide superior digestibility and nutritional value, in addition to improving other properties such as aroma, fragrance, and texture. From an industrial point of view, six main groups of enzymes can be distinguished: Hydrolases, oxidoreductases, transferases, isomerases, liases, and ligases. These classes of enzymes have special properties, such as high specificity and solubility, which make them suitable for industrial applications. In these applications, the enzymes are strong catalysts and do not interfere with reagents, chemical balance is not affected, enzymatic processes have low energy consumption. (Min and Yoo, 2014). The processes involving enzymes are extremely selective and, thus offer higher yields compared to conventional processes, side effects are almost completely eliminated, in addition, in most cases, the reactions occur under mild and friendly conditions, such as room temperature, pH-neutral or atmospheric pressure. By evaluating all these qualities and properties of enzymes, we can see that the use of enzymes leads to more sustainable industrial technologies, because they attenuate or completely avoid the use of organic solvents or other harmful chemicals (Rojanarata et al., 2018). However, there is a key issue in the use of enzymes as biocatalysts in biological processes that should be well documented, namely the problem of their immobilization to increase their activity and/or selectivity, as well as to improve their stability (Mohan et al., 2015b; Rodrigues et al., 2011). The main goal of enzyme immobilization is to improve the economy of biocatalytic processes. The process allows the reusability of the enzyme over an extended period while providing simplified procedures for separating the enzyme from the reaction products. With the immobilization of enzymes on various media, a wide range of parameters are affected, such as: improved solubility in organic solvents, higher pH tolerance, heat and functional stability (Barbosa et al., 2015; Guzik et al., 2014; Vaghari et al., 2016; Verma et al., 2014). A site-specific and high amount of green fluorescent protein (GFP) used as a model protein was reported based on the strong interactions between cohesion-dockerin and microcrystalline cellulose as a support binding a carbohydrate module (CBM) (Fig. 15A) (Li et al., 2016). The highest amount of charged GFP loaded was reported to be ~ 0.508 μmol/g cellulose. However, there are at least two issues to consider with this interesting approach, namely the loss of enzyme activity due to possible side interactions between the insufficiently rigidified enzyme chains, and the interference of the N-terminal dockerin domain.

A wide range of natural polymers are already used for the immobilization of enzymes, e.g. chitosan, alginate, agarose. Alginate and chitosan are two of the most commonly used carriers in enzyme

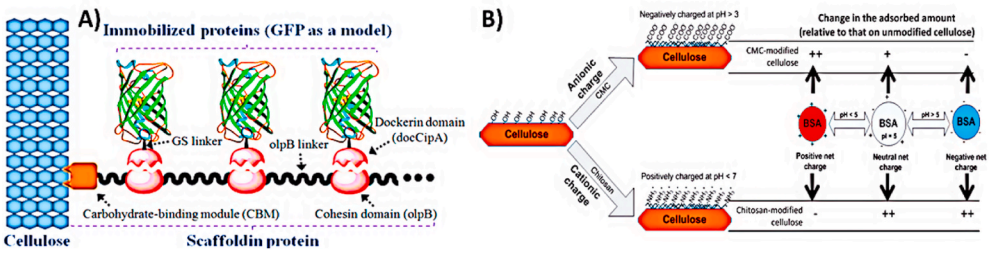

Fig. 15. Different strategies for the proteins immobilization: A) microcrystalline cellulose as a solid support to attach the carbohydrate-binding module (**CBM**), followed by the insertion of a short Gly-Ser (**GS**) linker between cohesion domains (***olpB***), serving as site-specific immobilization of dockerin (***docCipA***) for binding the green fluorescent protein (**GFP**) selected as an immobilization model. B) cellulose films primed with CMC and chitosan for the BSA adsorption. Reproduced from A) (Li et al., 2016) and B) (Orelma et al., 2011) with permission from American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

encapsulation due to their excellent chemical stability under pH and temperature conditions. There are still enough unresolved issues in this field, especially related to more rigorous control of the pore size of the formed capsules and poor mechanical properties (Dhiman et al., 2020; Mohapatra, 2020). Agarose, have some beneficial properties such as hydrophilic properties, large porosity, mechanical resistance, chemical and physical inertness, but its cost still constitute yet a major limitation for its use as immobilization support (Zucca et al., 2016).

3.1.4. Lysozyme (LY)

Chicken egg-white lysozyme (LY) is one of the most studied hydrolysis agents, both in fundamental research and practical applications, because of its lack of toxicity, low price, biocompatibility and well-defined chemical structure. LY is able to catalyze the hydrolysis of the β (1 → 4) linkages between N-acetylmuramic acid (NAM) and N– acetylglucosamine (NAG) in the peptidoglycan layer, the major component of Gram-positive bacterial cell wall (Pellegrini et al., 1997). Hen egg white lysozyme shows excellent antimicrobial activity against both Gram-positive and Gram-negative bacteria (Pellegrini et al., 1997) being found in many cells, tissues and secretions from many organisms (Torbeck and Prieur, 1979). Unfortunately, the stability of free LY is relatively low. To increase its stability, LY is immobilized on various solid substrates, while observing that the reactivity is kept within reasonable limits. LY is physically adsorbed or chemically conjugated to multiple colloidal nanoparticles for a wide range of purposes. Some examples include bactericide (Tavakolian et al., 2018), thermal (Ciolkowski et al., 2012) colloidal assembly (Yang et al., 2007), functional stabilizations (Bayazidi et al., 2018), or conformational analyses (Chakraborti et al., 2010). In this way, physically immobilized molecules are subjected to less agglomeration, proteolysis and harmful interactions with hydrophobic interfaces. On the other hand, irreversible and stronger covalent bond is one of the most largely utilized techniques for chemical immobilization of various macromolecules on solid supports. Lysozyme, has an isoelectric point of 10.7 (Croguennec et al., 2000) and is positively charged in neutral aqueous solutions. Moreover, LY plays a key role in the innate immune system of most animals, including human, and may be an ideal candidate for bacterial inhibition in the food industry (packaging and preservatives) and wound dressings, etc. Solutions of lysozyme and rectorite, were electrosprayed onto the negatively charged electrospun cellulose acetate nanofibrous mats prepared from 16% cellulose acetate solution in 2/1 (w/w) acetone/DMAc mixture (Li et al., 2014). Bacterial cellulose nanofibers were used for physical adsorption of LY, as recently reported (Bayazidi et al., 2018). The published results suggest that immobilization on BCNF does not affect the LY activity. More notably, the antimicrobial activity of LY against *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Yersinia entrocolitica, Aspergillus niger* and *Saccharomyces cerviseae* increased after immobilization. Cellulose nanocrystals carboxylated by Cu-catalyzed oxidation of cellulose fibrils by H_2O_2 at 60 °C and acidic pH served for LY immobilization via electrostatic interactions and covalent bonds in the study by van de Ven and Koshani (van de Ven and Koshani, 2020). Covalent immobilization of LY was possible by activating the carboxyl groups on the cellulose surface to react with the nucleophilic amino functionality present on the side chains of amino acids in lysozyme using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as a non-toxic triazine-based reagent (Fig. 16) (van de Ven and Koshani, 2020).

The adsorption isotherms showed a high loading capacity of the cellulose nanocrystals (∼ 240 mg/g), and fitting the data to the Langmuir model confirmed the monolayer coverage of lysozyme on their surface. The superior adsorption capacity and capability to form e amide bonds with amine-containing molecules with little adverse effects, in correlation with the high specific surface area of cellulose nanocrystals and their excellent colloidal stability and lack of cytotoxicity are desirable features to recommend carboxylate cellulose nanocrystals as ideal green nanocarriers for edible and in vivo applications. Liu et al. (2018) on the immobilization of LY on the surfaces of nonwoven regenerated cellulose combined with carboxylated carbon nanotubes (Liu et al., 2018). Immobilization of LY was done by using the EDC/NHS protocol, wherein the carboxyl groups of the carbon-nanotubes were chemically attached with the nucleophilic the amino groups of amino acids in LY. It was found that higher amounts of LY were immobilized on the carbon nanotube/cellulose surfaces than on pure cellulose itself. The LY immobilized substrate showed robust bioactivity and improved biocompatibility compared to bare substrates. In the work of Edwards et al. (2012) LY is conjugated to amino-glycine-modified cotton cellulose (nanocrystals) via an amide bond formed between the aspartate and glutamate residues of LY, which was esterified with 9-fluorenylmethoxycarbonyl-glycine (Fmoc-glycine) in DMF solvent (Edwards et al., 2012). For the conjugation of LY to modified cellulose, the well-established carbodiimide activation coupling protocol was employed, which resulted in the conjugation of lysozyme with 604 mg/g cotton nanocrystal. The conjugation of LY to the cotton nanocrystals showed excellent activity of 1500 U/mg cotton when measured using a Tag assay fluorescence to evaluate the antimicrobial activity against microbes such as Micrococcus

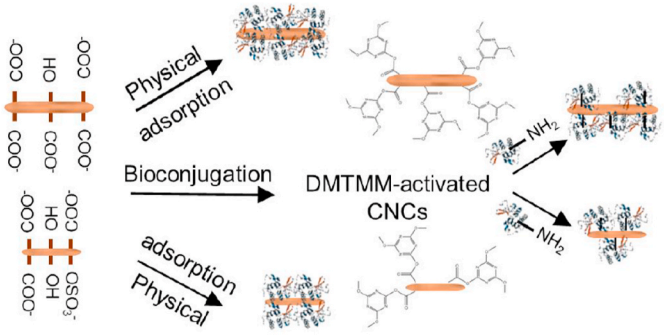

Fig. 16. Adsorption and bioconjugation dual mechanisms of LY onto carboxylated cellulose nanocrystals activated by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride. Reproduced from (van de Ven and Koshani, 2020) with permission from American Chemical Society.

lysodeikticus. The same author immobilized the LY on differently functionalized (carboxyl and amino) cotton cellulose (Edwards et al., 2011). The carboxyl functionalized cellulose was prepared by esterification with citric acid, while treatment with aminosilane such as (3-aminopropyl)-triethoxysilane (APTES) resulted in amino functionalized cellulose. Both functionalized surfaces were conjugated to LY via an amide bond using carbodiimide 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-p-toluene sulfonate in water. In this reaction, more LY was conjugated on carboxyl functionalized (1.4 mg/g) cellulose surfaces than on aminosilane-functionalized (0.54 mg/g) ones. Consequently, a higher activity of LY was determined for the carboxylate functionalized cotton than on aminosilanized cotton.

3.1.5. Streptavidin

Streptavidin is a protein with a molecular weight of 53 kDa, obtained from the bacterium *Streptomyces avidinii*. The most remarkable feature of streptavidin is its ability to bind 4 biotins, (also known as vitamin B7 or vitamin H) with an extraordinarily affinity; the dissociation constant (K_d) of the streptavidin-biotin complex is $\approx 10^{-14}$ mol/L (Green, 1927), one of the strongest binding found in nature, close in strength to a covalent bond. Streptavidin is used in bio-nanotechnology and molecular biology primarily, due to the recalcitrance of the streptavidin-biotin complex in organic solvents also denaturants (e.g. guanidinium chloride), as well as detergents, and proteolytic enzymes, at extreme temperature and pHs. The great affinity for biotin has led to streptavidin being used as a precursor for the attachment of sensitive biomolecules, the direct immobilization of which would result in a loss of activity (Rusmini et al., 2007). Moreover, numerous other bioreceptors and bioactive molecules containing biotin are commercially available, making streptavidin functionalization very tempting, as a universal platform for further attachment of specified bioreceptors. Fig. 17 shows two different strategies for streptavidin immobilization on cellulose-functionalized substrates. Route A) shows membranes made of cellulose modified with an adenosine receptor antagonist, a derivative of theophylline-oligo (ethylene glycol)alkene, (Theo 1) used for the immobilization of streptavidin (SA) using a fluorophore-labeled protein streptavidin and monitored by using bright field microscopy (Hierrezuelo et al., 2014). The authors assume that the entire amount of streptavidin was bound to the cellulosic support, as evidenced by the significant decrease in UV residual adsorption s compared to the original.

Route B) describes in the first step, the tetrazole decoration of cellulose, which can ligate maleimide-bearing molecules after irradiation. The arylation reaction of tetrazole acid chloride performed on the hydroxyl moieties of cellulose would generate the Cel-Tet intermediate. Cel-Tet surfaces were subsequently immersed in a solution of phosphate buffered saline (PBS) containing streptavidin functionalized with maleimide moieties, and irradiated at $\lambda = 320$ nm for 2 h, at room temperature (Tischer et al., 2014).

The authors envisioned that the reported immobilization protocol could be translated to the development of new bioactive papers and advanced microfluidic paper-based analytical devices (μPADs) necessary for the forthcoming progresses in medicine (Tischer et al., 2014).

3.1.6. Other enzymes

Proteolytic enzymes, such as chymotrypsin (CT), are important enzymes especially from an industrial point of view, known primarily for their ability to hydrolyze a wide range of proteins and peptides, to the terminal carboxyl group of the constituent amino acids. Due to its anti-inflammatory properties, CT is also widely used to cure digestive disorders, mela absorption, nutrient deficiency, and bloating. In the work of Bilal et al. (2020) the CT was conjugated with dialdehyde cellulose ether, via Schiff-base reaction. The dialdehyde cellulose was obtained by etherification with various alkyl bromides followed by oxidation with sodium periodate. The immobilized CT showed excellent reusability and storage capacity besides its higher activity under harsh physiochemical environments (e.g. pH and temperature) compared to the free CT. It was also shown that the CT retained its original activity, between 88 and 95% even after 10-times reuse, when stored up to 30 days at 4 °C and 25 °C, respectively (Bilal et al., 2020). Prikryl et al., 2012 reported a divinyl sulfone (DVS) chemistry protocol to conjugate CT to magnetic cellulose beads. Conjugation of the enzyme was carried out under different pH values (neutral to alkaline) and at different concentrations of CT (Prikryl et al., 2012). The immobilized CT showed specific activity that remained unaltered during storage, which is a key feature of the CT enzyme, because of its use in the design of protein digestion in proteomic studies. Researchers have also investigated the different immobilization strategies for the enzyme laccase on cellulose substrate (Rekuć et al., 2008; Zhai et al., 2019). Zhai et al. (2019) developed a new and highly efficient method to immobilize and retain the activity of enzymes such as laccase enzyme on bacterial cellulose. The latter was functionalized with polydopamine nanoparticles (size: 100–500 nm) at pH 9 followed by immobilization with laccase enzyme at different concentrations, temperature and pH. It was reported that the immobilized laccase (165 mg laccase per g nanocellulose), exhibited excellent enzyme activity, great thermal stability and operational stability (Zhai et al., 2019). Glucose

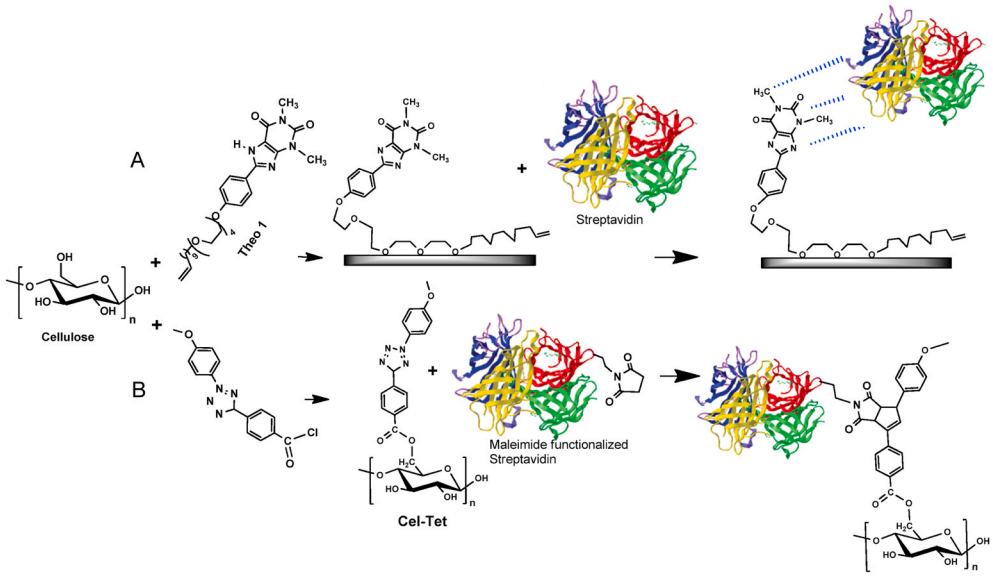

Fig. 17. Different strategies for the streptavidin immobilization on cellulosic substrates: A) cellulose functionalization using theophylline-oligo (ethylene glycol)alkene derivative, followed by the physical adsorption of streptavidin, and B) cellulose functionalization using 4-(2-(4-methoxyphenyl)-2H-tetrazol-5-yl)benzoic acid chloride, followed by the adsorption of streptavidin functionalized with maleimide moieties. Reproduced from (Hierrezuelo et al., 2014; Tischer et al., 2014) with permission from Elsevier and Wiley Online Library.

oxidase enzyme (GOx), which can catalyze the oxidation of glucose to hydrogen peroxide and D-glucono-δ-lactone, was conjugated to CNC (size: 100 nm length, 10 nm width) by Incani et al. (2013) prior to this step, and the anionic CNC was adsorbed electrostatically with polyethyleneimine and gold nanoparticles (Au NPs). The deposition of Au NPs latter on CNC was controlled by using PEI at different pH values, which was then functionalized with thiol using various carboxyl-based cross-linkers before the immobilization of GOx. The latter was conjugated to the CNC/PEI/Au NPs by EDC/NHS and further activation of the carboxylic acid moiety. It was reported that the amount of immobilized GOx on the CNC-based platform depends on the length of the linker used, for example longer chain thiol linkers (11 carbon chain) can immobilize about 20 mg of GOx per mg of cellulose, while the shorter ones linkers (from by 3 carbon chain) are able to immobilize greater values of GOx (more than 25 mg/mg of cellulose support) (Incani et al., 2013).

4. Conclusions and future perspectives

In recent years, we have all witnessed remarkable progress in the field of protein immobilization on new substrates of natural origin, with the cellulose-based matrix being considered a priority. In this field, there are great hopes for the manufacture of new, more stable and reliable sensors, new detection devices with higher sensitivity, shorter response time, smaller size and simple to use. In this perspective, the main concepts related to methodology, design and fine chemical synthesis are almost solved. However, there remain troubling issues to be addressed in terms of sensitivity, speed of analysis, and accuracy of results obtained in the absence of complex instrumentation or classical laboratory equipment. Current trends address the incorporation of various nanoparticles (NPs) into cellulose films, resulting in protein-assisted 2D assemblies. The wide variety of NPs can contribute significantly to the tremendous development of cellulose-based architectures with applications in drug administration, cell analysis and encapsulation. As 2D constructs could be converted into 3D architectures, they are expected to build robust, multi-component 3D cellulose scaffolds that include various nano entities. These new structural construct offer a special advantage over the individually taken component materials (polysaccharide and NP), namely a much higher degree of precision. An additional advantage of these architectures is the possibility of developing cellular network models, applicable, for example, in drug delivery, which may facilitate the use of these systems in analytical applications related to proteins and cellulose. Moreover, the use of activated or modified proteins through a click chemistry approach, could open new avenues for the engineering of different cellulose surfaces. With the drive to use low-cost and environmentally friendly materials, we believe that the requirements for cellulose-based biosensors will increase significantly. Therefore, it is expected that various future research will be stimulated to investigate the beneficial results of using cellulose-based biosensors for biodefense, food safety, environmental monitoring, healthcare, and biological and medical applications. Immobilization of bioactive molecules within the nano- or microstructure of cellulose brings new strategies to improve their analytical efficiency. Even though numerous cellulose-based biosensors have been developed for a wide range of laboratory-scale applications, only a few cellulose-based biosensors are commercially available until now. Therefore, further efforts are required in research innovation to develop automated, real-time, and continuous biosensor devices for healthcare and biomedical applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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