MNE 11 (2021) 100085

Contents lists available at ScienceDirect

Micro and Nano Engineering

journal homepage: www.sciencedirect.com/journal/micro-and-nano-engineering

Review paper

Methods for immobilizing receptors in microfluidic devices: A review

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ARTICLE INFO

Keywords:
Microfluidics
Surface chemistry
Microdevices
Functionalization
Receptors

ABSTRACT

In this review article, we discuss state-of-the-art methods for immobilizing functional receptors in microfluidic devices. Strategies used to immobilize receptors in such devices are essential for the development of specific, sensitive (bio)chemical assays that can be used for a wide range of applications. In the first section, we review the principles and the chemistry of immobilization techniques that are the most commonly used in microfluidics. We afterward describe immobilization methods on static surfaces from microchannel surfaces to electrode surfaces with a particular attention to opportunities offered by hydrogel surfaces. Finally, we discuss immobilization methods on mobile surfaces with an emphasis on both magnetic and non-magnetic microbeads, and finally, we highlight recent developments of new types of mobile supports.

1. Introduction

Over the past 30 years, the field of microfluidics has demonstrated great opportunities in many areas of research [1]. Biology, biochemistry, chemical analysis, clinical chemistry, environmental sciences, and physics benefit from the advantages offered by microfluidics [2–5]. Microfluidics provides the possibility to perform experiments at a small scale, with minute amounts of samples and reagents, and faster than at a larger scale [3–6]. Experiments and biochemical analysis done using microfluidics can be parallelized, less expensive, and may feature better performance than when done at the macroscale [3,4,7]. However, working at the microscale has an impact on the physics of the fluidic system [7]. At such a scale, dominating parameters are different and vary more rapidly than in macrosystems [6,7]. Gravitation forces are reduced at the expense of surface tension and capillary forces [3]. Inertial effects most often can be neglected [7]. Laminar flows dominate [8], and mass transport depends on the convection, dispersion [3] and diffusion inside liquids [4,7,8]. To this end, special considerations must be taken into account to study and optimize the transport of analytes and to perform biochemical reactions inside microfluidic chips [5,8,9]. Numerous applications in microfluidics require receptors immobilized on a specific surface, and analytes flowing in the vicinity of these immobilized receptors [8]. Receptors can be diverse in nature and include oligonucleotides, antigens, antibodies, enzymes, peptides, small chemical entities, etc. Different strategies of immobilization can be employed in microfluidics depending on (i) the nature and properties of receptors, (ii) the type of surface, static or mobile, on which the immobilization is performed, and (iii) the method of detection used.

This review focuses on the different methods of immobilization of receptors in microfluidic devices. Specifically, we review the principles and chemistry of immobilization techniques that are most commonly employed in the field of microfluidics. Furthermore, we focus on immobilization strategies on static surfaces and mobile surfaces and highlight well-established methods as well as recent research examples. Additionally, we decided to review this topic from a micro-engineering standpoint and provide examples of recent research developments impacting biomedicine, bioanalysis, and the life sciences.

2. Principles and chemistry overview of immobilization techniques

The choice of the type of surface for immobilizing receptors of interest depends on the application that is covered and the microfabrication strategy of the devices [10]. The immobilization of receptors is influenced by several factors, such as the chemical and physical properties of the surface, the method of immobilization and chemical conditions (e.g. buffer or solution used, pH, temperature), properties of the molecules to attach, and the desired position, orientation and density of the receptors over the surface. All these factors are important to consider because they can strongly impact the sensitivity and limit of detection (LOD) of the resulting device [10,11]. Receptors need to be functional not only immediately after their immobilization but must also

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https://doi.org/10.1016/j.mne.2021.100085
Received 10 December 2020; Received in revised form 6 April 2021; Accepted 10 April 2021
Available online 19 April 2021

have a shelf lifetime commensurate with the storage duration of the devices. In this section, we present an overview of techniques used to immobilize (bio)molecules in microfluidic structures and the underlying chemistry.

2.1. 2D versus 3D surface immobilization

Methods of immobilization significantly depend on the surface geometry (Fig. 1), which can be either planar (2D) or three-dimensional (3D). 2D surfaces used for immobilization include microfluidic channels made from different substrates, or surfaces coated with for example gold, platinum, or graphene (Fig. 1a-c). 2D surfaces are the simplest support on which receptors can be immobilized, but these surfaces have limited capacity in terms of density and amounts of molecules that can be immobilized. Traditional inorganic materials for fabricating microfluidic devices are silicon and glass. Such substrates have similar surface chemistry and have therefore common methods for immobilizing receptors. A broadly used method of immobilization employs silanization of the surface and receptors anchoring to functional groups [10,11]. Polymer substrates such as polydimethylsiloxane (PDMS), polycarbonate (PC), polystyrene (PS), polymethyl methacrylate (PMMA), and cyclic olefin copolymer (COC) are broadly used in microfluidic fabrication [10–12]. However, these types of surfaces lack functional chemical moieties for the direct attachment of biological receptors and binding sites. These surfaces therefore need to be chemically modified to create functional groups before immobilizing receptors. Metallic films offer another option for immobilizing receptors on a 2D surface, and in the case of Au, Pd and Pt, these films can be functionalized with self-assembled monolayers. Extended details on immobilization on 2D surfaces are given in Section 3.

The immobilization of receptors on 3D surfaces is illustrated in Fig. 1 (d-f). 3D structures can be functionalized with receptors after they have been formed on a substrate or after assembly of all components of a device. Pre-assembly fabrication techniques are performed by patterning microstructures, such as microposts, or by integrating porous membranes. Post-assembly fabrication techniques can be implemented using paper or nitrocellulose as a substrate, various polymers and beads. Paper and nitrocellulose are especially used to develop point-of-care diagnostic devices for low-resource setting countries because they enable low cost and simple-to-use devices, absorption-based immobilization of receptors, and direct visualization of signals [13]. Immobilization on polymers, such as hydrogels, can rely on copolymerization, covalent linking, and electrostatic capture. Beads carrying receptors can be suspended in a solution or localized and packed using specific microstructures. In either case, the receptor immobilization strategy depends on the surface composition of the beads. For silica beads, immobilization strategies can be similar to the ones employed for glass and silicon surfaces. For agarose beads, diverse techniques of immobilization have been developed, such as copolymerization with proteins, graft polymerization, and oxidative activation of functional groups [11]. Recently, graphene sheets suspended in a solution have been employed to immobilize receptors in microfluidic devices [14–16]. Compared to 2D surfaces, 3D surfaces have many advantages. 3D surfaces have a higher surface-to-volume ratio than 2D surfaces, which leads to more sites for immobilizing receptors. Moreover, the diffusion length between reactants is reduced for receptors immobilized on 3D surfaces. This can result in assays having higher sensitivity and being faster. More details about 3D surfaces are given in Section 4.

2.2. Covalent versus non-covalent immobilization methods

Non-covalent immobilization is presented in Fig. 2a-b, and it includes physisorption (Fig. 2a) and immobilization based on bioaffinity (Fig. 2b) [10,11]. Specifically, physisorption is realized for physical

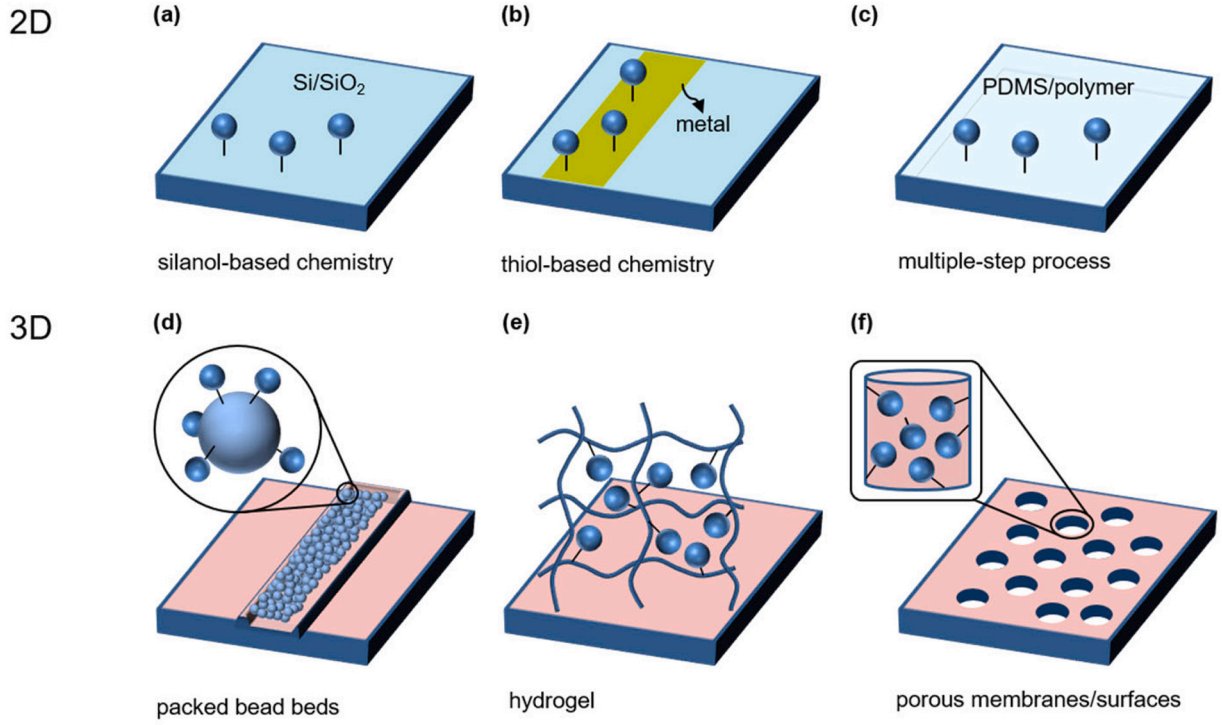

Fig. 1. Immobilization strategies on 2D surfaces and 3D geometries for commonly-used receptors in microfluidics such as proteins. (a) Immobilization on silicon or glass, (b) metal, and (c) PDMS or polymer, respectively. (d) Immobilization on beads packed into a 3D structure. Bead materials include polystyrene, silica agarose, and ferromagnetic materials. (e) Immobilization into a hydrogel. Commonly used hydrogels include PEGDA, polyacrylamide gel, chitosan, and agarose. (f) Immobilization on a porous membrane. Porous membranes can be fabricated for example using polymer monoliths (e.g. ethylene dimethacrylate, acrylamide, 2-hydroxyethyl methacrylate) and porous silicon. Alternatively, porous membranes such as polycarbonate, nitrocellulose, polyvinylidene fluoride, and paper are also commonly used.

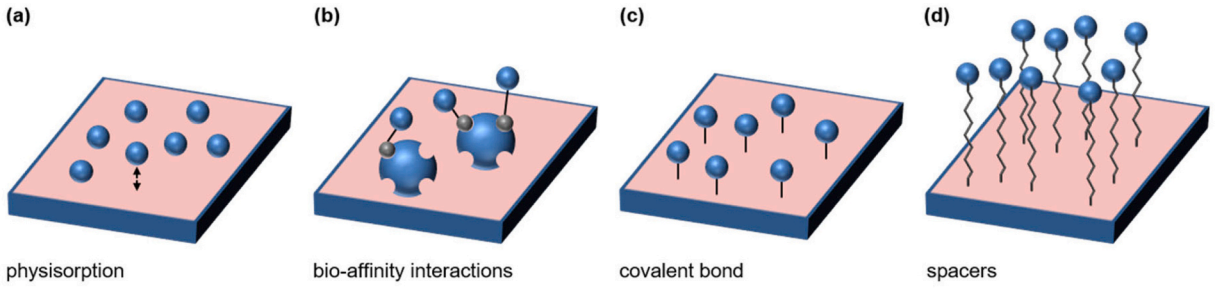

Fig. 2. Illustrations of physical, chemical, and biochemical methods for immobilizing receptors on surfaces. (a-b) Non-covalent immobilization methods based on (a) physisorption involving intermolecular forces, and (b) bio-affinity interactions between a ligand and a receptor. (c) Immobilization based on covalent bonding. (d) Covalent bonding with spacers between the surface and the immobilized receptors.

encapsulation and entrapment of receptors without involving complex chemistry or reagents. The method is environment-dependent and the intermolecular forces created are weak (electrostatic, hydrophobic, van der Waals, hydrogen bonds). Bioaffinity is based on particular binding phenomena that can be found in nature. It is specific, provides orientational control, minimizes loss of proteins, and allows good steric access to binding partners from solution. This type of attachment is often combined with other immobilization strategies (e.g. physisorption, covalent bonding). It is possible as well to reverse the immobilization process by detaching the receptors using chemicals, or changing the pH or temperature. The most employed partners based on bioaffinity interaction are avidin/biotin and streptavidin/biotin [10–12]. This ligand/receptor system has a very high affinity, is fast, and can mediate the immobilization of numerous proteins without affecting their functionality. One example of receptor immobilization by means of bioaffinity is the binding of protein A or G to the constant Fc region of antibody Immunoglobulin G (IgG). Another common technique is to use a spacer to link proteins to a planar surface in conjunction with protein A or G. Bioaffinity interaction is involved as well in ligand capture, where the C- or N-terminus of proteins is modified with an oligohistidine (His) segment to chelate a metal ion and a second chelating agent (e.g. NTA). Finally, to perform DNA hybridization, DNA and proteins are frequently covalently bonded or attached using a biotin-streptavidin linkage. In particular, aptamers, which are synthetic oligonucleotides, are increasingly used for biosensing applications [11,17]. These promising ligands offer many advantages, such as an excellent binding affinity for analytes, a simple chemical modification using linker molecules and dyes, and high-density functionalization of surfaces [17,18].

Covalent immobilization is frequently used for the non-reversible immobilization of receptors for assays performed in microfluidic chips (Fig. 2c) and it should be preferred to physisorption especially in cases where a continuous flow rate and associated shear forces require stable, irreversible immobilization of biomolecules, which cannot always be achieved by physisorption. Such a covalent immobilization approach is usually performed after fabrication of the microfluidic chips and before using them for a specific assay. For protein immobilization, surfaces are commonly modified with reactive groups that can react with amino acid residues present around proteins. Active functional groups that did not react should be blocked or made inactive using a blocking solution such as bovine serum albumin (BSA) in PBS. Covalent immobilization ensures non-reversible attachment of receptors to surfaces but leads to a random orientation of the receptors, which can impact their biological activity and ability to bind analytes from solution. Covalent immobilization can also take time in particular when expensive and low concentration solutions of receptors must be used during the attachment step. Various covalent conjugation chemistries are presented in Table 1. Finally, non-covalent and covalent immobilization can be combined using a spacer between a surface and receptors, especially when proteins need to be immobilized (Fig. 2d). In-depth description of each method can be found in the extensive reviews by Kim and Herr [11] and Welch *et al.* [12].

Table 1
Summary of different types of covalent immobilization chemistries and their characteristics, based on chemistries described in [11,19,20].

Covalent conjugation types	Characteristics
Amine—glutaraldehyde—amine	- Amino groups are the most prevalent covalent binding sites
Amine—NHS (N-hydroxysuccinimide)	- NHS ester is frequently employed to create covalent linking - NHS ester reacts with amines on proteins to form stable amide bonds
Amine/sulfhydryl—epoxide	- Formation of covalent bonds between epoxide and primary amines at alkaline ph - Or formation of covalent bonds between epoxide and sulfhydryl groups (-sh) in a physiological ph range
Amine—isothiocyanate	- Formation of stable linkage with primary amine groups
Amine—azlactone	- Formation of amide bonds between azlactone and nucleophiles
Amine—p-nitrophenyl ester p-nitrophenyl	- Reaction between ester and amines at basic ph (7-9).
Amine—tyrosinase (TR)—tyrosine	- Based on biocatalysis
Carboxylate—1-ethyl-3-(3-dimethylamonipropyl) carbodiimide—amine	- Edc is a carbodiimide commonly employed - Carboxylates and amines bond with amide linkage
Carboxylate—EDC+NHS—amine NHS	- Formation of NHS esters with carbodiimide, NHS, and carboxylates
Sulfhydryl—maleimide	- Maleimide is commonly employed to crosslink sulfhydryl groups
Reactive hydrogen—benzophenone	- Benzophenone is extremely reactive under uv light - Coupling of benzophenone with a protein using reactive hydrogen
Thiol group	- Reaction between thiol groups and maleimide or iodoacetyl present on modified surfaces
Click chemistry	- Azide and alkyne react by 1,3-dipolar cycloaddition - Formation of an oriented system
Carbohydrate groups	- Glycosylate of antibodies at the fc region
Spytag/spycatcher system	- Covalent isopeptide bond between the lysines of a spycatcher protein and an aspartate residue of a spytag
Covalent anchoring	- Commonly employed to form enzyme complexes - Covalent link between a substrate to a linker located in the center of the enzyme complexes

2.3. Random versus directed immobilization

Random immobilization refers to the deposition of receptors without control of the orientation of the receptors on a surface. In contrast, directed immobilization refers to the attachment of receptors with orientational control, usually resulting in the active site of the receptor looking away from the surface and being more sterically accessible to species from solution. Directed immobilization can also generate surfaces exhibiting higher density of receptors if the receptors have a non-symmetric conformation. Both types of deposition can employ covalent or non-covalent chemistries. Random immobilization can be achieved using chemistries described in Section 2.2 and that are either non-covalent (ionic bonds, physisorption) or covalent (amine-reactive surfaces, carboxy-reactive surfaces, photoactive surface) as shown in Table 2 [10].

Directed immobilization of receptors necessitates modified receptors

Table 2
Random immobilization of proteins using covalent attachments, adapted from [10].

Surface functional groups	Protein functional groups	Product
NHS ester	H_2NR	Amide
Aldehyde	H_2NR	Imine
Isothiocyanate	H_2NR	Thiourea
Epoxide	H_2NR	Aminoalcohol
Amine	$HO(O)CCH_2R$	Amide

or the functionalization of surfaces with ligands targeting specific domains of the receptors. Proteins, and more specifically fusion proteins, can be immobilized on surfaces modified with nickel nitrilotriacetic acid using a tandem tagging method. Non-covalent attachments are additionally available for surfaces that can be modified with biotin, antibody-binding proteins (protein A and G), and DNA-modified surfaces for DDI [10,21–23] which is an efficient affinity-based method for immobilizing proteins on a solid surface. Fig. 3a illustrates DDI, based on the seminal work from Niemeyer *et al.*, where DNA-streptavidin are covalently coupled with biotinylated proteins to form "preconjugates" [22,23]. The formed preconjugates hybridize by base-pairing to the complementary biotinylated capture-DNA attached to a streptavidin-coated surface. Such an immobilization is highly specific and can be reversed [22]. In the case of covalent attachment, derivatization of a protein with a unique chemical group at a defined position is performed first, followed by a chemoselective reaction with a complementary group on a solid support [12]. Furthermore, the methods employed for the directed-immobilization of antibodies typically involve amine, carboxyl, thiol, and carbohydrate moieties attachment (Fig. 3b) [12]. Thiol-reactive surfaces can be reacted with cysteines present on the shell of proteins to achieve specific coupling and immobilization [12]. One powerful technique for immobilizing proteins on a surface is to use a

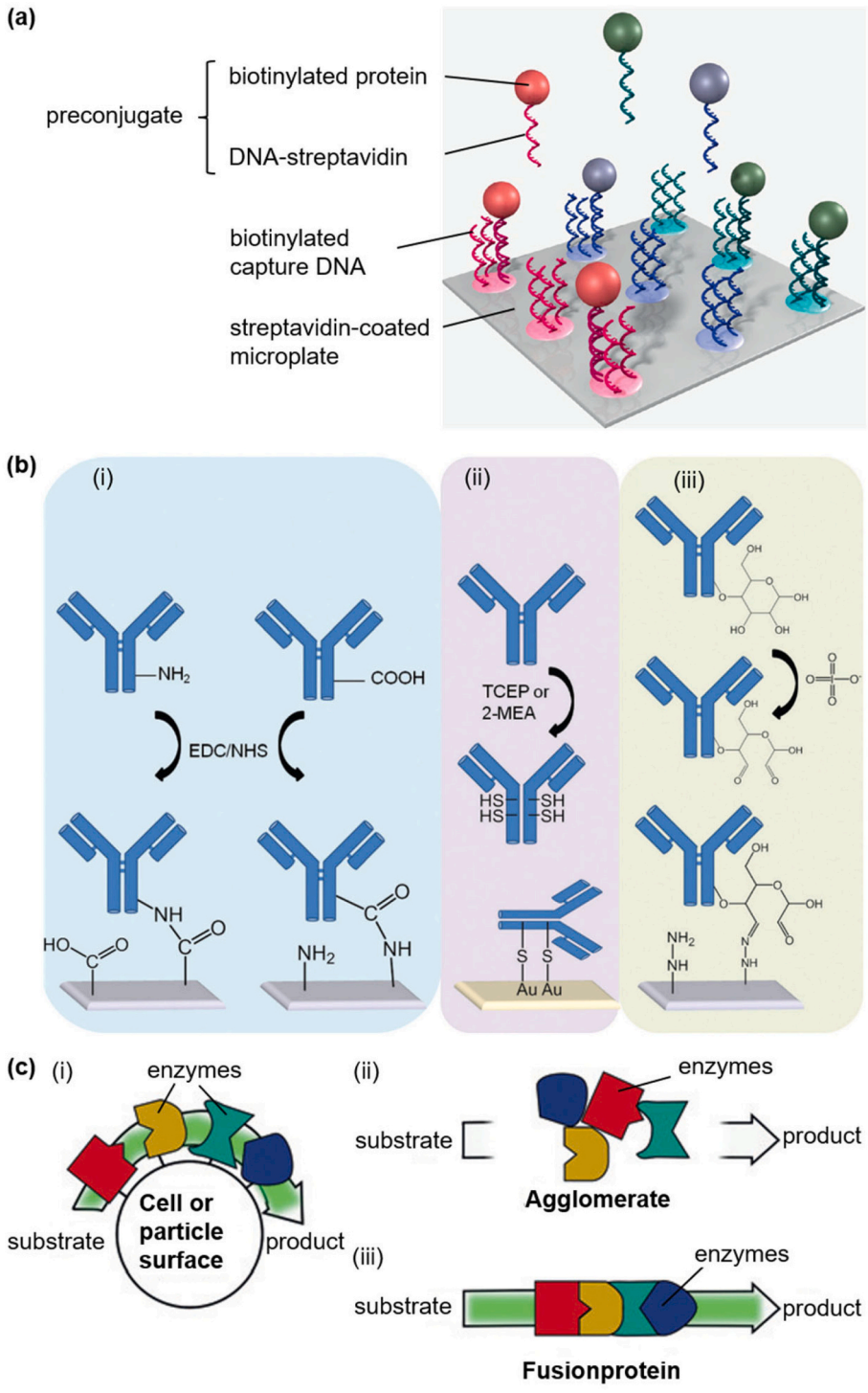

Fig. 3. Directed immobilization method. (a) DDI based on the hybridization of a preconjugate, composed of DNA-streptavidin, with a biotinylated protein, on a streptavidin-coated microplate modified with complementary biotinylated capture DNAs. (b) Antibody-directed immobilization based on (i) the binding of functionalized antibodies with carboxyl and amine groups on a surface functionalized with amine and carboxyl groups using EDC/NHS coupling, (ii) the binding of reduced disulfides from antibodies onto a gold surface using TCEP or 2-MEA, and (iii) periodate oxidation of carbohydrates of the Fc region of antibodies on a hydrazide surface chemistry. (c) Enzyme cascades transforming a substrate into a product based on four enzymes, (i) directly immobilized on cells or particle surfaces, (ii) non-covalently and (iii) covalently attached together. (a) Adapted from [23], (b) adapted from [12], and (c) adapted from [20].

native chemical ligation method [10]. Covalent immobilization can be performed as well by Staudinger ligation using azide groups and phosphines containing an ester or thioester to form an iminophosphorane intermediate, the nitrogen atom of which is nucleophilically attacked by a five-membered ring [10]. Such an attack results in the formation of an aminophosphonium salt. Cycloaddition reactions can be realized using "click chemistry". An example is the formation of a 1,2,3-triazole as a result of a Huisgen 1,3-dipolar cycloaddition of an azide and an alkyne [10]. Hodneland *et al.* developed a method to capture a protein via the intermediate of an enzyme that binds irreversibly a substrate present on a surface [10,24]. Directed immobilization of enzymes is based on the binding of a serine residue located in the active site of a cutinase, attached to a protein of interest, on a surface functionalized with phosphonate groups [24]. Finally, Fig. 3c presents immobilized enzymes during cascade reactions transforming a substrate into a product [20]. In this process, enzymes are immobilized either in a directed way on the surface of cells or particles, or attached non-covalently or covalently together.

3. Immobilization of receptors on a static surface

In this section, we focus on methods for immobilizing receptors on static surfaces. In particular, we will describe examples where immobilization is directly performed on the surfaces of microfluidic structures. Next, we will discuss immobilization strategies on metallic surfaces with an emphasis on gold electrode surfaces and sensor surfaces used for applications such as SPR and SERS. Finally, we will cover immobilization on 3D surfaces such as hydrogel surfaces.

3.1. Microfluidic channels

Immobilization of receptors in microfluidics is commonly performed directly on the surface of microfluidic structures [11], such as microchannels. Microchannels are simple structures on which receptors are typically functionalized with a single layer of receptors. Microchannels can provide large areas for immobilization and a direct contact between analytes flowing inside a microchannel and immobilized receptors [11]. The substrate to create microchannels can be made from different materials such as polymers, glass, silicon and silicon alloys.

3.1.1. Polymer surfaces

Polymers, such as PDMS, PC, PMMA, PS, and COC, represent the most commonly used materials for fabricating microfluidic devices for research and large scale applications such as in-vitro diagnostics and bioanalysis [25]. These polymers can be covered with proteins, are optically transparent, easy to prototype or make in large series, do not necessitate cleanroom facilities, and are economical to produce [1,26]. We therefore describe below methods for modifying the surface of these polymers.

3.1.1.1. Polydimethylsiloxane (PDMS). PDMS is an elastomeric material

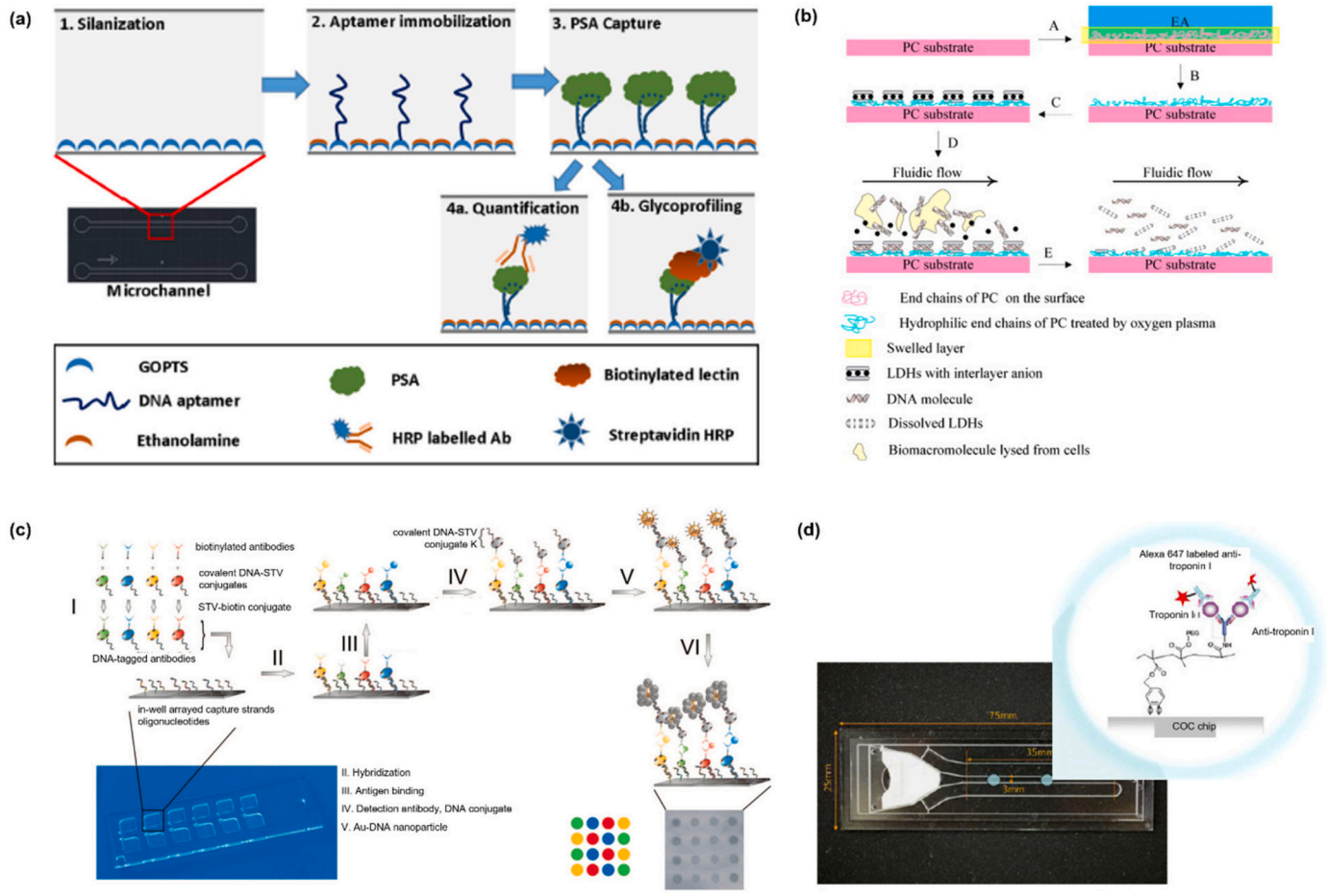

Fig. 4. Immobilization of receptors on polymeric surfaces of microfluidic chips. (a) Description of the implementation of a DNA aptamer-based sandwich immunoassay for prostate cancer diagnostic on the functionalized surface of a PDMS microfluidic device. (b) Immobilization process of layered double hydroxides on the PC surface of a microfluidic device for extracting DNA sequences from solutions. (c) Illustration of a multiplexed immunoassay performed in a PMMA microfluidic device and based on DNA-directed immobilization. (d) Functionalized COC microfluidic chip device for the detection of cTnI using a sandwich immunoassay format. (a) Adapted from [41], (b) adapted from [48], (c) adapted from [53], and (d) adapted from [66].

[27], which is relatively chemically inert and can conform to hard and soft substrates to create closed microfluidic devices [28,29]. PDMS has limited compatibility with inorganic solvents, [18] and is permeable to gas [30]. PDMS is hydrophobic [26,27,31] but it can readily be made hydrophilic [30–32]: plasma treatments can oxidize a PDMS surface for subsequent covalent immobilization of receptors [33,34] using silanol groups [35–38], or epoxy groups [39]. Plasma treatment has also been combined with graft polymerization, layer-by-layer assembly, metal coating and a sol-gel method to modify the surface of PDMS [38,40]. Fig. 4a illustrates the functionalization of a PDMS layer with aptamers for detecting biomarkers relating to cancer prostate using a sandwich-based immunoassay [41]. After silanization of the microfluidic surface, a solution of DNA aptamer, followed by a blocking solution of ethanolamine, was flowed over the PDMS surface using active pumping. This alternative system to standard antibody-based immunoassays is sensitive and here reached a LOD of 0.5 ng mL^{-1}. Alternatively, Roy *et al.* developed a microfluidic competition assay via equilibrium binding of analytes in solutions to detect fluorescently labeled insulin [42]. Biotinylated IgGs receptors were covalently immobilized using avidin coating a PDMS microchannel. Taylor *et al.* [43] and Shamansky *et al.* [44] used as well covalently immobilized proteins to capture biotinylated vesicles. To study enzymes in a PDMS microfluidic chip, Mao *et al.* immobilized them using streptavidin-conjugated alkaline phosphatase linked to a biotinylated phospholipid bilayer deposited onto PDMS microchannels [45]. A series of enzymatic reactions were then performed in the microfluidic chip and showed potential for multistep chemical synthesis at the microscale. The group of Bi *et al.* studied strategies for immobilizing unstable enzymes in PDMS channels and showed that the best protocol was to modify the PDMS surface with absorption of an alumina (Al_2O_3) xerogel [46]. Recently, Schneider *et al.* took advantage of DDI to immobilized proteins and cells on PDMS surfaces [47].

3.1.1.2. Polycarbonate (PC). Protein immobilization strategies on PC usually rely on intermolecular forces. Fig. 4b presents the immobilization of "inorganic layer double hydroxides" (LDHs) on the PC surface of a microfluidic device used for the specific extraction (capture and release) of DNA molecules from lysed cells [48]. The surface of the PC was modified using a solution of tetrahydrofuran ethyl acetate, which extended the end of the PC chains. The surface was afterward treated with oxygen plasma to improve the adhesion between LDHs and the PC. LDHs were immobilized on the modified surface by entanglement from the ends of the PC chains and DNA molecules from lysed cells were encapsulated by ion-exchange reactions between the LDH layers. The authors demonstrated that the encapsulated DNA could be released by flowing an acidic solution in the microfluidic system. The developed system improved the extraction efficiency of specific DNA present at low concentration in a sample solution. Moreover, in the work of Xu *et al.*, PC was photoactivated for the purification of DNA sequences produced using dye-terminator sequencing [49]. DNA sequences were immobilized on the PC surface before gel electrophoresis sorting. To this end, the PC surface of the microchannels was exposed to UV light, which produced carboxylate groups on the surface. The DNA sequences were in solution in an immobilization buffer composed of ethanol and triethylene glycol (TEG) and bound to the carboxylate groups of the PC surface. The DNA can be eluted from the surface using water for subsequent reading of the sequence. With such an approach, the authors reached a DNA sequencing accuracy of ~98.9%, which is similar to conventional systems.

3.1.1.3. Polymethyl methacrylate (PMMA), polystyrene (PS), and cyclic olefin copolymer (COC). PMMA, PS and COC surfaces lack functional groups and require a surface treatment using oxygen plasma or strong bases/oxidizers to introduce functional groups [11,50,51]. In 2003, Liu *et al.* tested different immobilization procedures for attaching three oligo probes on four different types of polymer surfaces (PS, PC, PMMA, and polypropylene) [52]. The procedures tested employed Surmodics™, cetyltrimethylammonium bromide (CTAB)-based, and Reacti-Bind™, with the least effective procedure being the Reacti-Bind™ one. The CTAB-based procedure gave fair results and showed that the concentration of CTAB had a significant effect on oligo immobilization efficiency. Amine-modified oligo probes provided better results than unmodified oligo probes. The most effective procedure in terms of spot quality and hybridization sensitivity was the Surmodics™ procedure.

Recently, DDI showed great potential for performing multiplexed assays in PMMA microfluidic chips (Fig. 4c) [53]. In this work, the functionalization of the PMMA chip was performed in several steps. First, specific DNA strands were inkjet-spotted. Capture DNA strands covalently attached by photocrosslinking to the PMMA surface and the surface was functionalized after sealing the chip with different antibody-DNA conjugates prepared using biotin-streptavidin conjugation. After capturing antigens, the detection of antibody-DNA conjugates was performed using DNA-streptavidin conjugates and Au-DNA nanoparticle complexes, which allowed silver deposition where antigens had been captured. This technique offers adaptable building blocks for creating protein microarrays, can use small volumes of samples, is inexpensive, and features a short processing time. Furthermore, other methods were developed for immobilizing antibodies or enzymes on PMMA. Su *et al.* improved the sensitivity of IgG detection 8 fold compared to conventional quartz crystal microbalance by simple adsorption of BSA on PMMA micropillar surfaces [54]. There, protein immobilization and protein-protein interaction were monitored and measured in real-time. For enzyme immobilization on PMMA surfaces, physical adsorption is commonly used [55]. The review by Hajba *et al.* gives an overview of recent enzyme immobilization strategies developed especially for microflow reactors for biocatalysis, bioconversion, and bioanalytical purposes [56].

PS shows as well good properties for the investigation of a wide range of analytes including hydrophobic analytes [57]. Microfluidic chips in PS offer advantages, such as non-toxicity and very good mechanical properties for developing diagnostic platforms [58–60]. However, PS is a hydrophobic material and a surface modification is necessary if receptors need to be covalently attached to this surface. Surface modification of PS can be performed using graft polymerization or ion beam, plasma or UV/ozone treatments [58–60]. Immobilization of receptors on PS surfaces is realized by covalent attachment [58,59]. Darain *et al.* took advantage of PS microfluidic chips to develop an immunoassay for the detection of myoglobin [58]. Antibodies were immobilized on a PS surface using UV/ozone, silanization, and a cross-linking treatment. Based on fluorescence detection of analytes, they reached a LOD (16 ng mL^{-1}) lower than reported clinical cut-off values. Ryu *et al.*, also immobilized capture antibodies by deposition on the PS surface of a microfluidic device to detect cardiac markers such as creatinine kinase-MB and myoglobin [61]. PS microfluidic chips are also employed for studying enzymes. Hu *et al.* developed a new method to immobilize enzymes for realizing microreactors in PS microfluidic structures [62]. To this end, they modified specific regions of the PS surface using UV light, which allowed the formation of carboxyl groups. These regions were treated with ethylenediamine to form amino groups. The treated PS surface was incubated overnight with glutaraldehyde, which reacted with the amino groups. A solution containing the enzymes was introduced in the microfluidic chip after a washing step using a buffer. The immobilization of the enzymes on the PS surface results from the reaction between the enzymes and the functional groups present on the PS. Such a protocol allowed to cover 96% of the PS surface with immobilized enzymes. PS surfaces showed better results for immobilizing cells than PDMS [57]. Dusseiller *et al.* developed a microwell platform in PS that can also be transferred into a microfluidic device for studying cells. They modified the surface of the PS surface using a graft-copolymer method [63].

The last polymer discussed here on which molecules can be

immobilized is COC. Microfluidic chips made from COC have been used for DNA immobilization [64], microarrays [65], and immunoassays [66]. This material presents excellent physicochemical features such as low density, resistance to many solvents, high transparency, low autofluorescence, and high flowability [66]. Nevertheless, it is difficult to modify the surface of COC, which is a hydrophobic material made of pure hydrocarbons without native groups for covalent functionalization [67,68]. In addition, the immobilization process is uncontrollable and physical adsorption methods often lack reproducibility in terms of spatial density of adsorbed biomolecules [11]. Alteration of COC can be done by photografting [64,66,69] ozonolysis [66,70] or oxygen plasma treatment [50,66,71,72]. In the latest case, the oxidation of the non-reactive COC surface produces hydroxyl functional groups. Such a process enhances the immobilization of NH_2-terminated silanol compounds, such as aminopropyl tri-ethoxysilane (APTES), (3-aminopropyl) dimethylethoxysilane, and (3-aminopropyl)trimethoxysilane [66]. A linker molecule is employed afterward to attach receptors. Another technique consists in the coating of silica on the surface of COC using a plasma jet and a precursor (tetraethylorthosilicate) [73]. Such a method described in detailed by Bourg *et al.* results in a homogeneous coating of the COC surface with silanol groups, which permits immobilizing proteins on the polymer surface. Fig. 4d presents a COC surface functionalized with biomolecules for detecting cardiac troponin I (cTnI). Antibodies are immobilized on the COC surface using a solution of amphiphilic polymers (poly(DMA-r-mPEGMA-r-NAS) and poly(BMA-r-mPEGMA-r- NAS)) at room temperature. Such a solution of polymer contains activated secondary carboxylic acids that have a higher reactivity and less steric hindrance than tertiary carboxylic acids. The polymer-coated COC surface was transferred into a specific cTnI antibody solution for several incubation steps. The advantage of this technique is the simple formation of pre-activated polymer layers on the COC surface and the selective protein immobilization. Using this method, a LOD of 10 ng mL^{-1} was reached using fluorescence-based detection.

One can refer to the review by Henares *et al.* for a deeper overview of the different developments in microfluidic immunosensing chips [74]. There, a particular focus is given to surface modification, antibody immobilization, detection, and analyte sensing on polymer chips. Moreover, new polymers (parylene C and fluorinated polymers) used in microfluidics have been functionalized using plasma treatment to

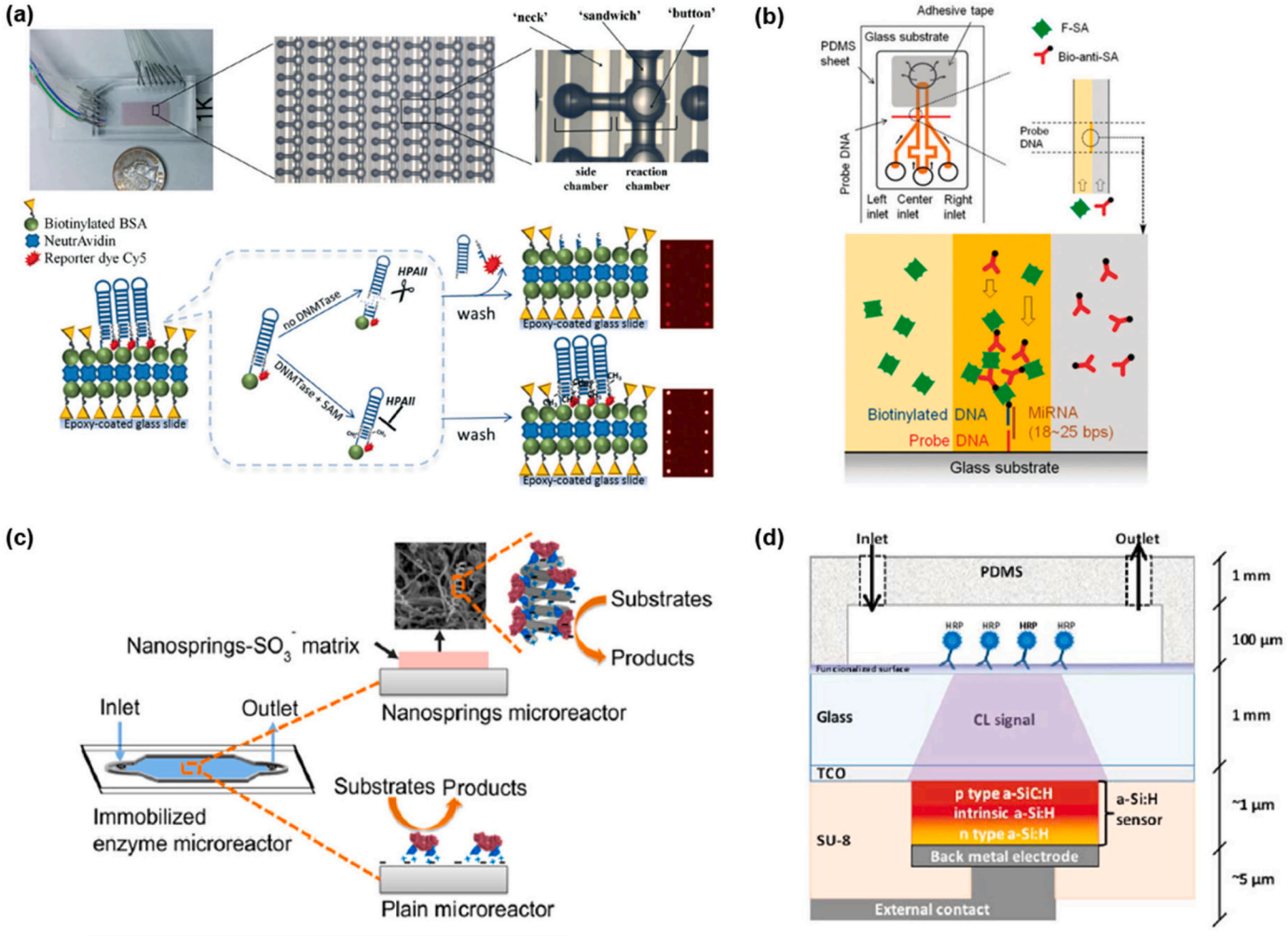

Fig. 5. Examples of receptors immobilized on the glass surfaces of microfluidic devices. (a) Microfluidic-based fluorometric assay for studying DNA methylation in vitro. The most upper right inset presents a reaction unit with a flowing liquid (black), control layers (white), and that is composed of two chambers controlled by a neck, sandwich, and button microvalves, respectively. The methylation assay was performed on an epoxy-coated glass surface using a biotinylated hairpin-shaped DNA probe substrate immobilized through biotin-avidin interaction. (b) Illustration of a laminar flow-assisted dendritic amplification device. The glass substrate is functionalized with DNA probes and a probe-micro RNA-biotinylated DNA sandwich initiates the formation of a streptavidin-biotin dendrimer complex. (c) Microfluidic enzyme microreactor with silica nanosprings grown on the surface of a functionalized glass surface to enhance enzyme immobilization. (d) Illustration of a microfluidic chip for chemiluminescence detection of immobilized bio-specific probes on a functionalized glass surface. (a) Adapted from [86], (b) adapted from [18], (c) adapted from [87], and (d) adapted from [32].

immobilize proteins [75] and a fluorescent alkyne [76].

3.1.2. Glass surfaces

Immobilization on glass surfaces is a technique mainly used in optical sensing applications [77]. Glass is transparent with a low autofluorescence, robust, chemically homogeneous, and stable [18,78–80,81]. It can be manufactured on very large scales and with very low surface roughness. Glass is compatible with solvents, acids, and slightly alkaline solutions; it is possible to control its surface properties, such as polarity and wettability using various coating and surface functionalization methods [82,40,83]. Despite these advantages, glass is brittle and expensive to microstructure [18,40]. To immobilize receptors on glass surfaces, several chemistries are available to create covalent linking between silanol groups on the glass surface and receptors in solution [11,82,40,84,85]. Almost all chemical modification processes are based on the easily accessible and highly modular chemistry of functionalized organosilanes [10]. Fig. 5 shows different glass-based microfluidic chips used for various applications and involving different methods of functionalization. Fig. 5a presents a microfluidic platform for DNA methylation assays, in vitro, and for high-throughput screening with the possibility to perform thousands of methylation reactions in nanoliter reaction volumes [86]. In this system, DNA probes were immobilized on an epoxy-coated glass slide surface through biotin/neutravidin interaction by flowing a biotinylated DNA solution inside the chip. Another immobilization method developed to detect microRNA by sandwich hybridization is the functionalization of an aminated glass slide with aminated DNA probes by incubating a glass surface into a glutaraldehyde solution (Fig. 5b) [18]. A LOD of 0.5 pM using only 0.5 µL of sample solution was reached with this technique. For enzymes, a method using silica nanospring was developed by Valikhani *et al.* (Fig. 5c) and consists of coating borosilicate microchannels with silica nanosprings using a modified vapor-liquid-solid deposition process [87]. In addition to being a well-defined material, silica nanosprings present a large surface area with many silanol groups, which enables anchoring genetically-modified enzymes via a "silica-binding module". This method enhanced the activity of immobilized enzymes by 4.5 fold, and up to 10 fold when the nanospring surface was modified with sulfonate groups. This system is a promising approach for improving microreactors used for biocatalysis applications. Fig. 5d shows a microfluidic chip having an integrated amorphous-Si:H (a-Si:H) photodiode for chemiluminescence bioassays [32]. There, the glass surface was cleaned with a "piranha" solution and functionalized with APTES and glutaraldehyde to introduce aldehyde groups, which react with amino groups from antibodies in an alkaline solution. Probes were deposited afterward on the glass slides and aligned with the a-Si:H photodiode to detect horseradish peroxidase by measuring a photocurrent at the attomole level with a sensitivity of 6.4. 10^4 A mol^{-1}. Very recently, Schneider *et al.* developed a microreactor fabricated with glass and PDMS structures to study the communication between cells using a DDI approach [88].

Miniaturization of microfluidic devices to the nanometer scale can extend the capabilities of biosensors in terms of sensitivity, time to result and volume of sample required. In fact, by decreasing the dimensions of channels below the micrometer lengthscale, diffusion paths of molecules can be reduced, the surface to volume ratio of channels can be increased, and less volumes of samples can be employed [89–91]. Shirai *et al.* demonstrated a new process to pattern antibodies in a nanochannel and achieved a limit of detection as little as 3 zeptomoles, which is 5 orders of magnitude lower compared to a standard microfluidic immunoassay format. Specifically, these authors patterned antibodies on an APTES-modified glass surface by crosslinking the amino groups of APTES molecules with those of antibodies using glutaraldehyde [92]. Nanofluidic devices were also used in combination with protein microarrays to achieve real-time multiplexed biosensing and kinetic monitoring [93]. Putallaz *et al.* integrated a nanofluidic sensor into a fully-automated device for POC testing and managed to decrease the time to reach binding equilibrium to minutes and achieved a high sensitivity [94]. This patented technology is being used in a commercial product by the company Abionic, for example.

3.1.3. Silicon-based surfaces

Silicon is the workhorse substrate of the semiconductor industry [95,96]. It allows fabrication of microfluidic structures with nanometer precision and having very large surface-to-volume ratios [97]. An oxide can be naturally or artificially grown on a Si surface [98,99] to immobilize proteins, and cells, [100,101]. Its main drawbacks are its opacity, cost, and incompatibility with electrokinetic methods [11,96,102]. For example, a microfluidic cantilever device was successfully implemented to immobilize and detect bacteria and measure their susceptibility to antibodies (Fig. 6a) [103]. The cantilever (32 µm wide and 600 µm long) was fabricated using silicon nitride (Si_3N_4) and microchannels (32 µm by 3 µm) were embedded on it. The interior of the cantilever was functionalized with specific receptors and target bacteria were trapped inside the 50 pL-volume channel. The immobilization strategy consisted of two steps: ethanolamine interaction with an oxidized silicon nitride surface after DMSA treatment to form free accessible amine groups and binding to monoclonal antibodies using NHS/EDC chemistry. The detection was performed in-situ, in real-time and it was possible to detect a single bacteria per µL of sample [103]. In 2002, Yakovleva *et al.* studied the immobilization efficiency of antibodies (polyclonal anti-atrazine) according to the surface treatment of a Si surface to perform enzyme-based immunoassays [104]. Two different immobilization protocols were tested. A common step for both methods was to use APTES, 3-glycidoxypropyltrimethoxysilane (GOPSA), linear polyethylenimine (LPEI), and branched polyethylenimine (BPEI). The results showed an unstable coating when the immobilization of antibodies was performed by adsorption on a silica surface modified with LPEI and by covalent linkage to physically adsorbed BPEI. A stable coating of antibodies on three functionalized silica surfaces was instead obtained (APTES-GA, LPEI-GA, and GOPS-BPEI-GA) using glutaraldehyde (GA). The best long-term stability was obtained for the LPEI-GA functionalized Si surface. Fig. 6b illustrates the immobilization of proteins on such a Si substrate [100]. A 4-layer microfluidic biosensor comprising a SiO_2 microring resonator with a Si core layer was functionalized with IgG antibodies. Immobilization steps consisted in a surface modification with APTES, glutaraldehyde, and piranha solution, followed by the functionalization of the ring with an antibody solution flowing inside the chip using active pumping, incubation, rinsing steps, and blocking with a BSA solution. Quantitative detection was label-free, real-time and the LOD (0.5 µg ml^{-1}) was 14 times lower using this method than the reported state-of-the-art on microring resonators. Lee *et al.* studied the binding kinetics of protein-ligand interactions at the nanoliter scale on Si substrates [105]. They patterned a hydrophobic and inert fluorocarbon layer, which prevents non-specific binding, on a silicon nitride substrate. A streptavidin solution reacted covalently with aldehyde chemical linkers on the silicon nitride surface and fluorescein isothiocyanate-BSA-biotins presented in solution were captured. This approach allows the study of protein-protein interactions, the characterization of receptor-ligand interactions in functional genomics, proteomics, and drug discovery at the microscale and nanoliter level. Another technique of immobilization on Si consists in using a lipid bilayer [101]. The illustration in Fig. 6c is a side view of a microfluidic device coated with a lipid bilayer containing receptors to detect cholera toxin b [101]. A silver surface was covered with a silica shell to enhance the rupture of vesicles from solution. Phospholipid vesicles containing the cholera receptor (GM1) were injected into the microfluidic channels and ruptured on the silica-coated nanohole array to form the supported lipid bilayer. Cholera toxin b was introduced and bound to the oligosaccharide portion of the receptors. This set-up allows a high-throughput, label-free, real-time multi-channel SPR biosensing with the possibility to individually functionalize each channel with a wide range of receptors, reaching in this case a LOD of 8.4 nM.

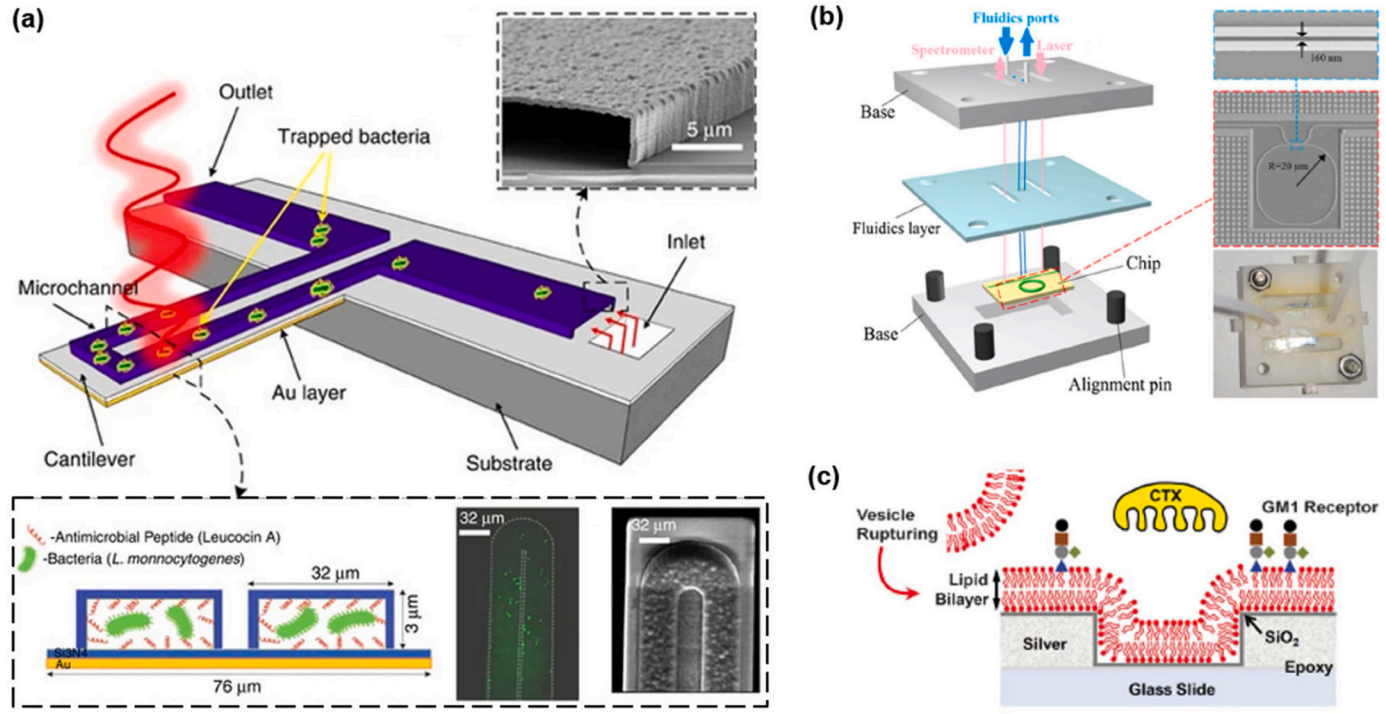

Fig. 6. Immobilization of receptors on silicon-based substrates. (a) Cantilever with an embedded microfluidic channel having surfaces functionalized with receptors for the selective capture of bacteria passing through the channel. (b) Illustration of a microfluidic biosensor composed of four layers: a top and bottom base, a fluidic layer, and a chip containing a SiO_2 microring resonator functionalized with IgG antibodies. (c) Illustration of one unit of a microfluidic device composed of 50 parallel microchannels coated with a liquid bilayer containing ganglioside (GM1) receptors for detecting cholera toxin b. (a) Adapted from [103], (b) adapted from [100], and (c) adapted from [101].

3.1.4. *Paper surfaces*

Paper is a popular material in the microfluidic community interested in conceiving POCDs, which are low cost and easy to use [13,106–109]. Despite being opaque and leading to a lower LOD compared to other transparent substrates, paper offers many advantages [13,18,110]. Paper is cheaper than polymers or Si, easy to fabricate, flexible, lightweight, disposable, and biocompatible [111]. In addition, a liquid wicking paper can be passively transported by capillary action to a detection zone or to zones containing immobilized reagents [11,13,111,112], which avoids external, bulky, or expensive peripherals. Microchannels on paper can be patterned using photoresist, wax, local oxidative plasma treatment, or cutting methods [113–117]. Screen-printed electrodes can be added as well to the detection zones to perform electrochemical assays [13,113–115]. Paper is macroscopically

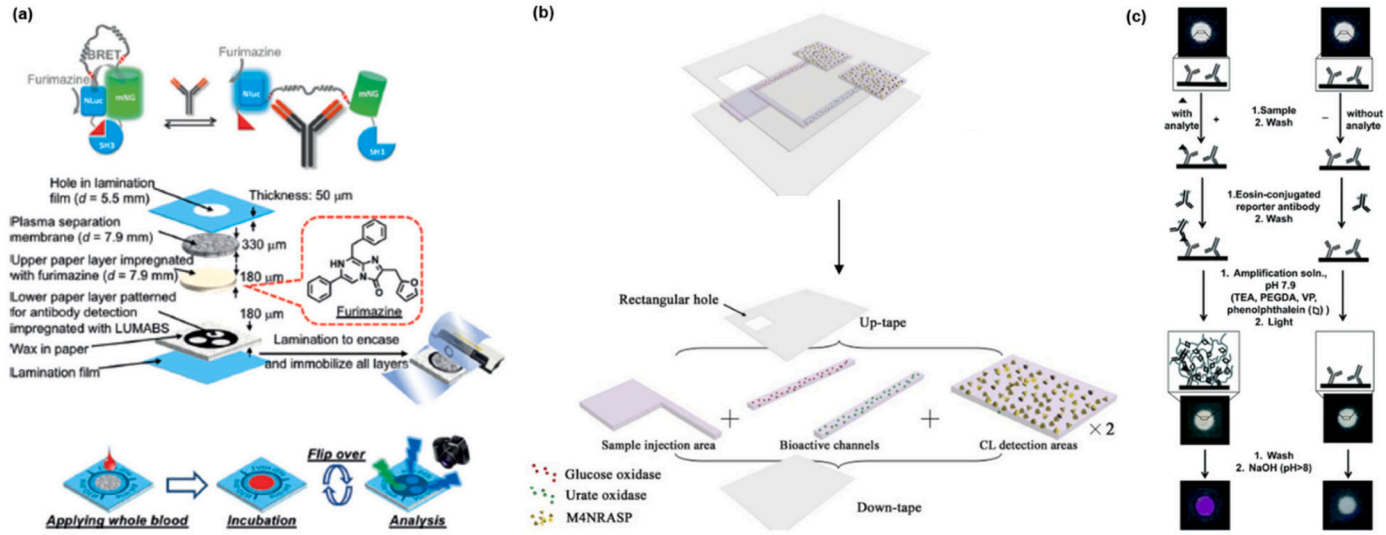

Fig. 7. Immobilization of receptors on paper-based microfluidic devices. (a) Illustration of an antibody detection device using a BRET-switching fluorescence measurement. BRET-based antibodies were integrated into the different layers of paper constituting the sensor protein. (b) Illustration of the fabrication of a microfluidic paper-based chemiluminescence device for the detection of glucose and uric acid using oxidase enzyme reactions. (c) Paper-based immunoassay and polymerization-based amplification to detect PfHRP2 protein using a colorimetric detection. (a) Adapted from [110], (b) adapted from [121], and (c) adapted from [123].

planar but it is a porous material with microscopic 3D pores made of a cellulose membrane [11,107]. Therefore, paper offers a high surface-to-volume ratio, which can improve reaction kinetics and accumulation of signal reporting molecules [118].

Reagents can be stored or immobilized directly on paper devices [13,119] using simple immobilization strategies, such as the adsorption from solution of biomolecules based on a combination of intermolecular forces (Van der Waals and electrostatic interactions [120]). In Fig. 7a, a fully integrated paper-based microfluidic device for LUMABS detection of antibodies based on a novel type of bioluminescence resonance energy transfer (BRET) is presented [110]. The device consists in multiple layers of paper that are vertically arranged. One layer contains a substrate and another a BRET-switching protein. Reagents were simply immobilized by direct adsorption. The device was flipped and detection was performed using a digital camera after the formation of the antibody-LUMABS complex. Multi-target assays were achieved on a single device with good LOD in the nanomolar range. Protein enzymes can in addition be immobilized in paper microfluidic devices by adsorption. Fig. 7b illustrates a microfluidic paper-based chemiluminescence biosensor developed to measure glucose and uric acid simultaneously [121]. The device was composed of one sample injection area, two bioactive channels, and two cathodoluminescence detection areas. Components were assembled between two water-impermeable single-sided adhesive tapes. Microchannels and the detection zone were functionalized by adsorption of reagents from solution. The device showed high sensitivity and selectivity in a short time (LOD of 0.14 mmol L^{-1} for glucose and 0.52 mmol L^{-1} for uric acid) using only a small volume of sample.

A chemical modification can be performed to take advantage of the abundance of hydroxyl groups, which occur in the cellulose and wood pulp materials of paper [119,120]. Such a modification provides functional groups for covalent immobilization of receptors, and provides therefore a strong binding between the receptor and the surface. Fu *et al.* evaluated the modification efficiency of various biofunctionalization methods for biosensing and compared five surface chemistries for protein immobilization on paper [120]. Potassium periodate (KIO_4)-modified cellulose paper gave the best performance with a 53% increase in the signal output and a 59% decrease in the background noise of colorimetric ELISA. Glavan *et al.* silanized paper with decyl trichlorosilane to immobilize antigens and antibodies on the surface of different microwells of a paper microfluidic device to perform, by folding of the device, an electrochemical sandwich ELISA for the detection of malaria and IgG [122]. Fig. 7c describes the different steps of the functionalization of a paper immunoassay and polymerization-based assay using an eosin-conjugated reporter antibody [123]. There, aldehyde groups were used in a hydrophilic test zone of oxidized chromatography paper to immobilize covalently monoclonal antibodies. Detection was colorimetric-based and very fast upon the addition of NaOH.

Paper has been combined as well with other materials to create hybrid microfluidic systems [118,124,125]. Zuo *et al.* combined a glass slides having PDMS channels with a paper chromatography bottom surface for the immobilization of aptamer probes [118]. Adsorption and storage of aptamer-graphene oxide complexes in the paper avoided complicated surface treatment and enabled simultaneous detection of two infectious pathogens in a simple and fast manner (10 min). A hybrid system from a paper/PMMA microfluidic microplate to diagnose infectious diseases (IgG and HBV in human serum) based on colorimetric ELISA was additionally developed [126]. There, reagents flew through the micro-porous paper, which avoided complicated surface modifications and multiple reagents pipetting. The formation of antigen/antibody complexes was fast, the LOD was good (1.6 ng mL^{-1} for IgG and 1.3 ng mL^{-1} for HBsAg), and the results were comparable to commercial ELISA kits using specialized equipment.

Overall, the excellent flow, binding properties of paper and nitrocellulose, and the numerous options for reagent integration on these substrates supported the widespread use of these materials in products involving lateral flow assays [127,128], and commercialized by companies such as Abbott Laboratories, F. Hoffmann-La Roche, Siemens AG, Thermo Fisher Scientific Inc., QIAGEN N.V., Abcam plc, bioMérieux SA.

The emerging field of "open microfluidics" created possibilities for designing microfluidic chips and performing bioassays using several substrates [129]. Open microfluidics enables the access to every location on the surface of the microfluidic device and allows to perform localized chemistry and bio-analysis. A review from 2012 highlights available methods to perform localized chemistry in open microfluidics [130].

3.2. Metallic surfaces

Immobilization of receptors can be performed as well on metallic surfaces. Metallic surfaces can be patterned directly on microfluidic structures for example to define areas for local chemical surface modification, for obtaining optical properties needed for specific biosensing principles [131,132], for heat detection or heat generation [133,134], or to create electrodes for liquid control [135,136], flow monitoring [137,138], or electrochemical reactions [139,140]. The immobilization of receptors on metals therefore allows applications for optical biosensing, electrochemical sensing, and molecular diagnostics [141–143], and takes advantage of the outstanding electrical conductivity of metals to perform surface-enhanced Raman spectroscopy (SERS) or surface plasmon resonance (SPR) detection [144–146]. We first review the immobilization of receptors on electrodes before addressing the functionalization of metallic surfaces for optical biosensing. Electrodes are often fabricated in gold, palladium, or platinum and new types of electrodes made from graphene or carbon are increasingly used [147–149]. Receptors on gold and palladium can be immobilized using thiol-based chemistry [150,151], which conveniently relies on self-assembly of monolayers from solution.

3.2.1. Gold electrode surfaces

Gold electrode surfaces are frequently used to immobilize receptors DNA, antibodies, and enzymes in microfluidic biosensing systems. Immobilization of receptors is only one part of a challenge and it is necessary to find the correct surface chemistry to passivate the electrode surface properly to avoid non-specific binding that could compromise detecting specific analytes from a complex sample [152]. The immobilization of proteins on a gold surface is presented in Fig. 8a. As an example, the protocol steps for the detection of PSA in a low-cost, label-free, and multiplexed biosensor are detailed in the Fig. [141]. Functionalization was performed in-situ on the gold electrode without the need for a lengthy (overnight) functionalization process or complex fabrication method. Electrodes were blocked with a PEG-thiol solution and modified with thiolated biotin. Streptavidin was deposited on the biotin layer and capture biotinylated antibodies were added before performing the immunoassay. With this protocol, Parra-Cabrera *et al.* demonstrated a more specific detection of PSA than currently available diagnostic tests [141]. Alternatively, Wang *et al.* developed a highly sensitive and specific system for pathogen antigen detection based on a glass-coated with gold and SERS nanoparticle clusters as labels [153]. They used nanoyeast single-chain variable fragments (NYscFv) that are stable in solution, inexpensive, and target-specific instead of using traditional monoclonal antibodies. The gold surface of the system was functionalized as follows: channels were cleaned using a piranha solution and functionalized with a solution of biotin-PEG-thiol. Streptavidin attached to the biotin layer on the gold surface and mediated the deposition of biotinylated anti-HA antibodies from solution. The nanoyeast-scFv was then bound to the anti-HA antibody and this functionalized gold surface was incubated with the antigen solution and corresponding SERS labels to perform the immunoassay.

Nucleic acids offer great potential in biosensing owing to their relative ease of integration with electronics components, their selectivity to bind complementary probes, and the numerous options for

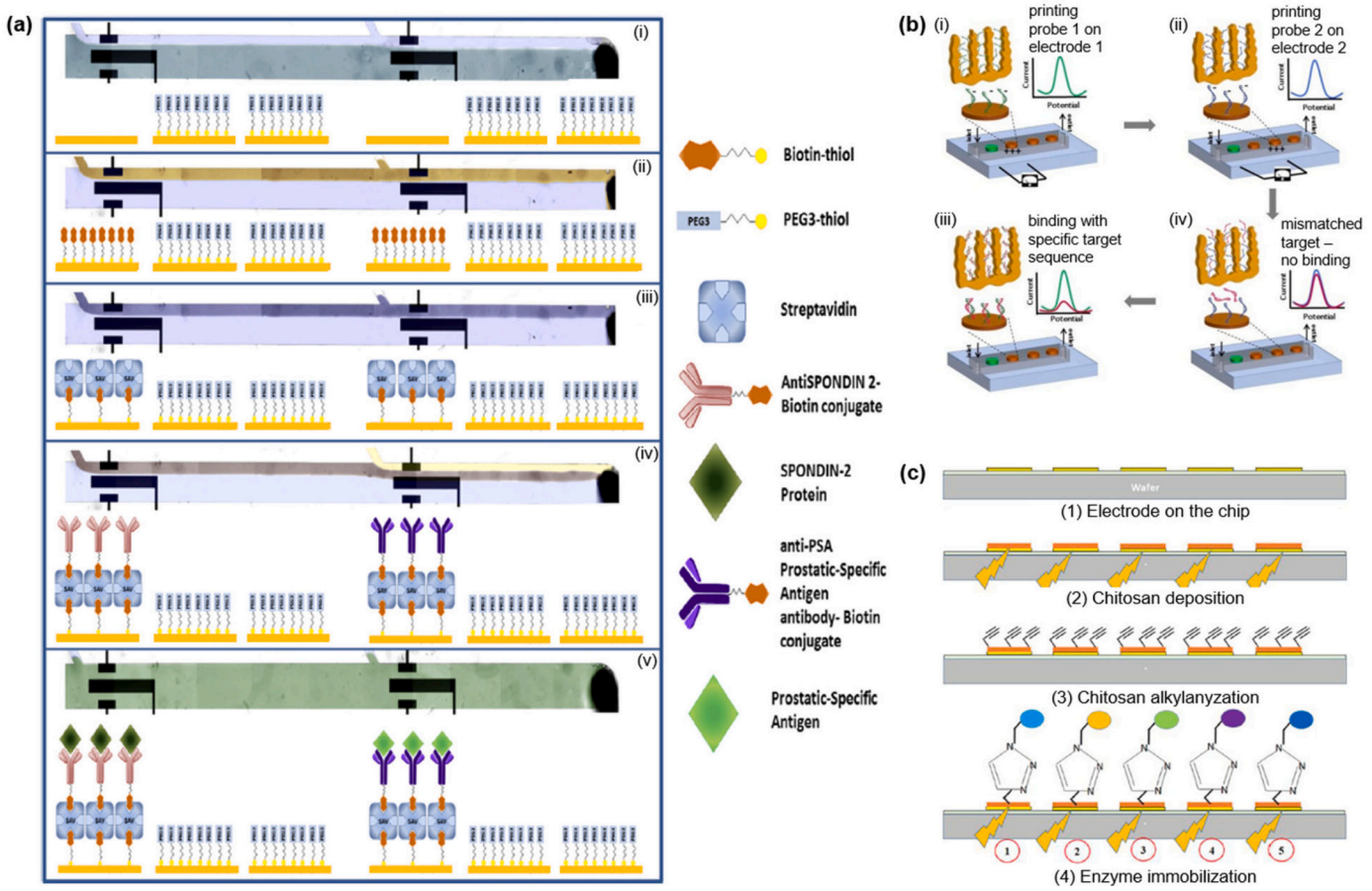

Fig. 8. Immobilization of receptors on a gold surface electrode. (a) Protocol for multiple detection of prostate cancer biomarkers. (i-ii) Specific electrodes were modified with a thiolated biotin, while other electrodes were blocked using a PEG-thiol solution. (iii-iv) Streptavidin deposition before the addition of biotinylated antibodies and (v) detection of prostate cancer biomarkers. (b) Illustration of an electrically guided DNA immobilization on nanoporous gold electrodes. (i-ii) Two DNA probes are patterned on electrodes 1 and 2. (iii-iv) Change in signal current when the complementary probes bind to the specific target in solution and negative control showing no change in current in the absence of binding. (c) Multi-functional enzyme immobilization in a fabricated device using "electro click chemistry" and individual electrode activation. (a) Adapted from [141], (b) adapted from [142], and (c) adapted from [143].

designing them with arbitrary sequences, spacers, linkers, and signal reporting moieties [142]. Fig. 8b shows the concept of electrically-guided DNA grafting. The authors studied the impact of electrode nanostructures on electrically-guided DNA grafting for the multiplexed detection of nucleic acids [142]. Electrodes were localized in a micro-channel and fabricated using an electro-grafting method. Electro-grafting is a versatile solution for nanostructuring electrodes and improving the overall performances of the sensor. The electrode diameter was reduced, thereby allowing a higher hybridization efficiency and reduction of the incubation time to immobilize DNA probes into the porous electrodes (< 10 min). DNA probes were then patterned on the nanoporous gold electrodes. Different probes were patterned on different electrodes (for multiplexed detection of breast cancer markers) and immobilization was performed using a passive incubation.

Enzymes can as well be immobilized on gold electrodes. Fig. 8c describes the immobilization of enzymes on the bottom surface of a microchannel patterned with gold electrodes [143]. The functionalization of the gold electrode was performed by electrodeposition of a thin film of chitosan on top of the electrode. Chitosan is a biopolymer material that can be applied to assemble biological molecules on conductive surfaces. The chitosan layer was modified with alkaline groups, and enzymes were immobilized on the chitosan using an electro-click chemistry process. Such a method is useful to immobilize enzyme, especially for developing multi-functional biosensors because it allows temporal and spatial control of enzyme immobilization.

3.2.2. *Metallic surfaces for plasmonic applications*

SPR is a label-free [154,155], real-time [155], rapid [101], mass- and temperature-sensitive technique [155] based on the propagation of electromagnetic waves on an interface between a metallic film and a dielectric medium [101]. SPR is used to detect molecular interactions occurring in proximity to a surface made of noble metals (gold or silver) by measuring the refractive index changes caused by the binding of biomolecules on the surface of the metal without the need for fluorescent or optical labels [101,155]. A variant of SPR is surface plasmon resonance imaging (SPRi) for which a charge-coupled device (CCD) camera is used for reflection detection and surface imaging [155–158]. Nanoplasmonic biosensors extend this concept and offer a robust sensing platform with direct coupling of the perpendicularly incident light, which allows direct detection of pathogens without alignment requirements [159].

Proteins and DNA are common immobilized receptors in the field of plasmonic-based biosensing. Proteins, such as IgG, are often immobilized to perform SPR and nanoplasmonic detection of an antigen. Fig. 9a describes the fabrication steps of an optofluidic nanoplasmonic gold sensor functionalized with antibodies for the detection of RNA viruses [160]. The device was composed of a suspended nanohole array, the surface of which was functionalized with IgG antibodies for detecting virus membrane proteins. Detection and recognition of either small enveloped RNA viruses (vesicular stomatitis virus and pseudo-typed Ebola) or large enveloped DNA viruses (vaccinia virus) within a dynamic range spanning three orders of magnitude was demonstrated.

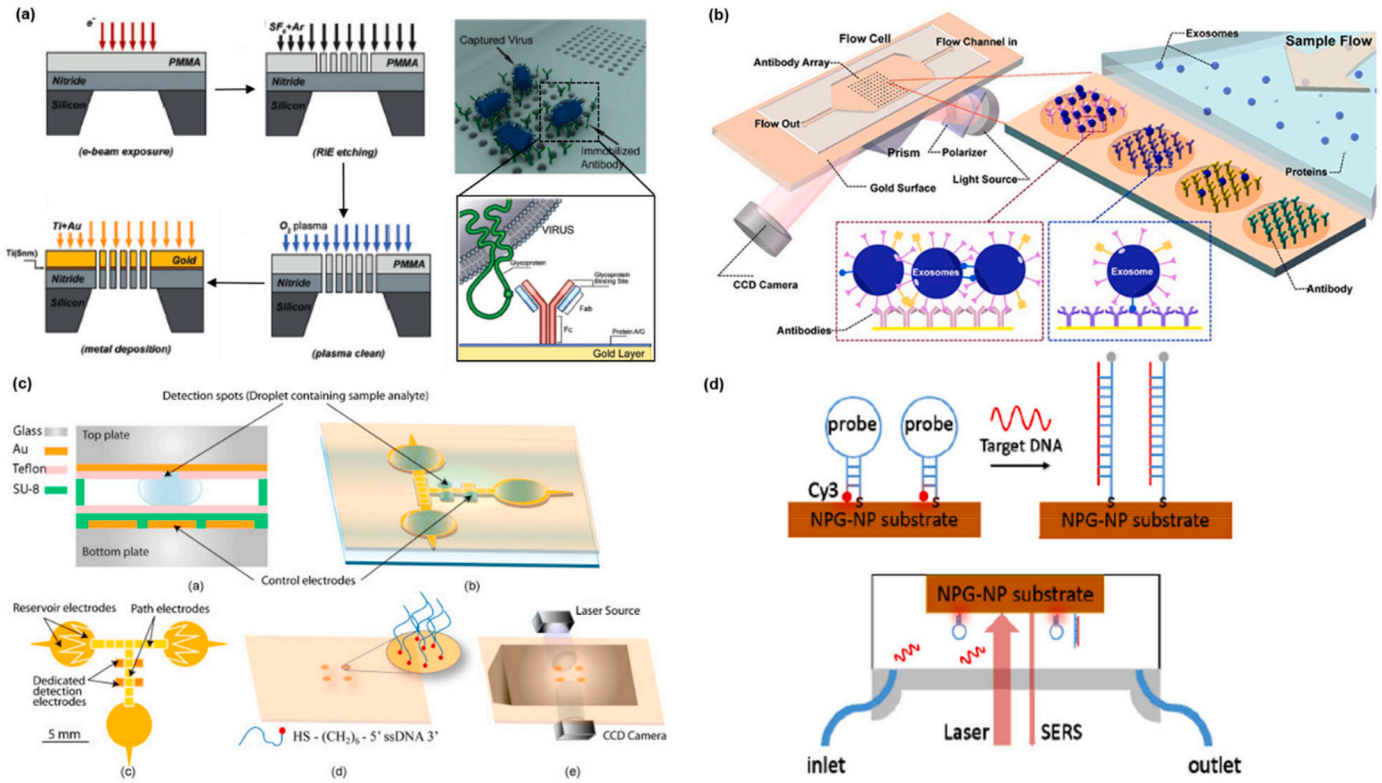

Fig. 9. Immobilization of receptors for specific SPR and nanoplasmonic applications. (a) Fabrication process and rendering of an optofluidic nanoplasmonic gold sensor functionalization for the immuno-recognition of RNA viruses. (b) SPR microfluidic biosensor system comprising capture antibodies on the surface of gold surfaces for the detection of exosomes. (c) Presentation of a micro-array SPR using an EWOD microfluidic chip for immobilizing DNA probes on gold surfaces. (d) Scheme of a nanoplasmonic sensor based on the immobilization of molecular sentinels on nanoporous gold nanoparticles within a microfluidic device. (a) Adapted from [160], (b) adapted from [161], (c) adapted from [164], and (d) adapted from [165].

Detection was based on resonance transmission and showed a high signal to noise ratio. Fig. 9b illustrates a SPR microfluidic biosensor based on the functionalization of gold surfaces by antibodies for the detection of exosomes using a prism coupling and a CCD detector [161]. Gold was coated on the glass surface of the microfluidic chip. Antibodies were printed (protocol involving incubation steps, a blocking step, and rinsing steps) and immobilized on a gold-coated glass sensor chip before injection of exosomes by active pumping. Moreover, Lee *et al.* developed a surface-sensitive optical technique for detecting two-dimensional spatial phase variation caused by rabbit immunoglobulin G absorption on an anti-rabbit IgG film by SPR using a self-assembled monolayer technique to immobilize anti-rabbit IgGs [162]. This assay was highly sensitive (LOD of 0.67 nM) and highly specific. Proteins can alternatively be physically adsorbed based on electrostatic and hydrophobic interactions, which avoids disturbances caused by chemical modifications [163]. This is the method adopted by Zhang *et al.* for their portable SPR biosensor immunoassay device, which reached a LOD of 25 mg L^{-1} without surface modification.

Immobilization of DNA on metal surfaces for specific SPR and nanoplasmonic applications is presented in Fig. 9c-d. Fig. 9c shows an electrowetting-on dielectric digital (EWOD) microfluidic device involving SPR detection of DNA hybridization [164]. EWOD microfluidics is a widely employed technology, which manipulates a liquid by dividing it into discrete, independently controllable droplets that are sandwiched between two hydrophobic surfaces [164]. The method is flexible and easy to use. EWOD allows forming robust, reproducible arrays of DNA sequences tethered to a gold-coated surface, which is an essential requirement for SPR. Droplets containing DNA probes are transported between parallel plates for covalently attaching DNA probes to self-assembled monolayers present on gold electrodes. Immobilization was performed dynamically in-chip with or without an electric field, which enhanced DNA hybridization efficiency [164]. Fig. 9d illustrates a nanoplasmonic sensor based on the immobilization of molecular "sentinels" on nanoporous gold nanoparticles, which were arrayed in microfluidic device [165]. DNA probes were attached to the gold surface by the intermediate of a thiol group at the 5' end using an incubation step. SERS detection was very fast (< 10 min). Other groups developed protocols to immobilize DNA on gold surfaces for specific SPR detection [166–168]. Zhou *et al.* reported in 2013 a gravity-induced flow injection system to perform on-chip DNA hybridization [166]. To this end, a solution of SH-DNA flew through the unmodified gold surface sensing area. The SH-DNA immobilization on the surface and hybridization with complementary DNA strands was measured using SPR in real-time. Sonntag *et al.* achieved DNA immobilization on-chip by inverse microcontact printing in order to implement a microfluidic device for the detection of label-free DNA hybridization and protein-protein interaction using SPR [167]. Very recently, Chuang *et al.* developed a SPR array based on gold-capped nanowires and a microfluidic platform for detecting amplified DNA products without labeling or complex sample processing [168]. This new and highly sensitive method allowed a specific hybridization of target molecules with immobilized probes on gold nanostructures. PCR products were detected using a low number of amplification cycles due to the high specificity between DNA probes and DNA analyte molecules.

Finally, interesting reviews on SPR biosensing are available for a more complete overview of this field of research. Wasburn *et al.* reviewed the advancements of waveguide-based optical sensors, from the fabrication of such sensors to biosensing applications [169]. The review by Spackova *et al.* [170] covers the fundamentals of optical affinity biosensors based on plasmonic application and details the functional coating of sensor surfaces.

3.2.3. Other types of conductive surfaces

During the last few years, new types of conductive surfaces have been developed for microfluidics. Such surfaces have been patterned in carbon [154,155], indium tin oxide [159,171], and graphene [172,173]. Consequently, new methods of immobilization of receptors and biomolecules, and detection strategies have been developed. Electrodes doped with carbon nanotubes (CNTs) have promising characteristics for electrochemical sensing [28,174]. They present interesting electrochemical and catalytic properties [159,175], a very high surface area [174,176] high electrical conductivity [174,175], excellent electron transfer rates [154,155,176], and exciting biosensing characteristics [159,175]. These types of electrodes are widely used as sensors in various electroanalysis and biosensing applications [154,174,176]. Fig. 10a represents the fabrication of a novel microfluidic nano-biochip based on the functionalization of CNTs for the detection of cholesterol [154]. There, CNTs showed excellent sensing performance with good reproducibility, selectivity, high sensitivity, low detection potential, and fast response. The CNTs were fabricated using a composite made of nickel oxide nanoparticles (nNiO) and multiwalled carbon nanotubes (MWCNTs). The surface of the CNTs was functionalized with a bienzyme (cholesterol oxidase and cholesterol esterase), and the CNTs were integrated with PDMS microchannels. Amide bonds between the nNiO-MWCNT and the NH_2 groups of the bienzyme formed with carboxyl groups present in the nNiO-MWCNT. Wisitsoraat *et al.* developed another system for detecting cholesterol using a PDMS/glass chip and functionalized CNTs nanotube electrodes [177]. CNTs were functionalized in flow with cholesterol oxidase. Detection was performed in real-time using chronoamperometry. This method was fast, sensitive, and had low cross-sensitivity between the different analytes. Fig. 10b presents a microfluidic separation and sensing device fabricated for monitoring neurotransmitters in human plasma based on functionalized carbon electrodes [155]. In this example, the electrodes were functionalized with bi-functional probe molecules. The surface of the electrode acted as a mediator between the electrons and interacted selectively with the target NTs. The redox mediator was covalently immobilized using an amide bond between NH_2 groups of the mediators and the carboxyl groups of pTBA. This biocompatible system demonstrated specific catalytic properties, good stability, and superior performance than conventional systems owing to the formation of CeN and CSe moieties in the carbon matrix. It was possible to detect 6 neurotransmitters in human plasma after the optimization of several parameters.

Oxide-based electrodes have furthermore been developed and functionalized for sensing applications [159,171,178,179]. Fig. 10c presents the functionalization of an electrode surface for the detection of salmonella Typhimurium by protein conjugation and where anti-

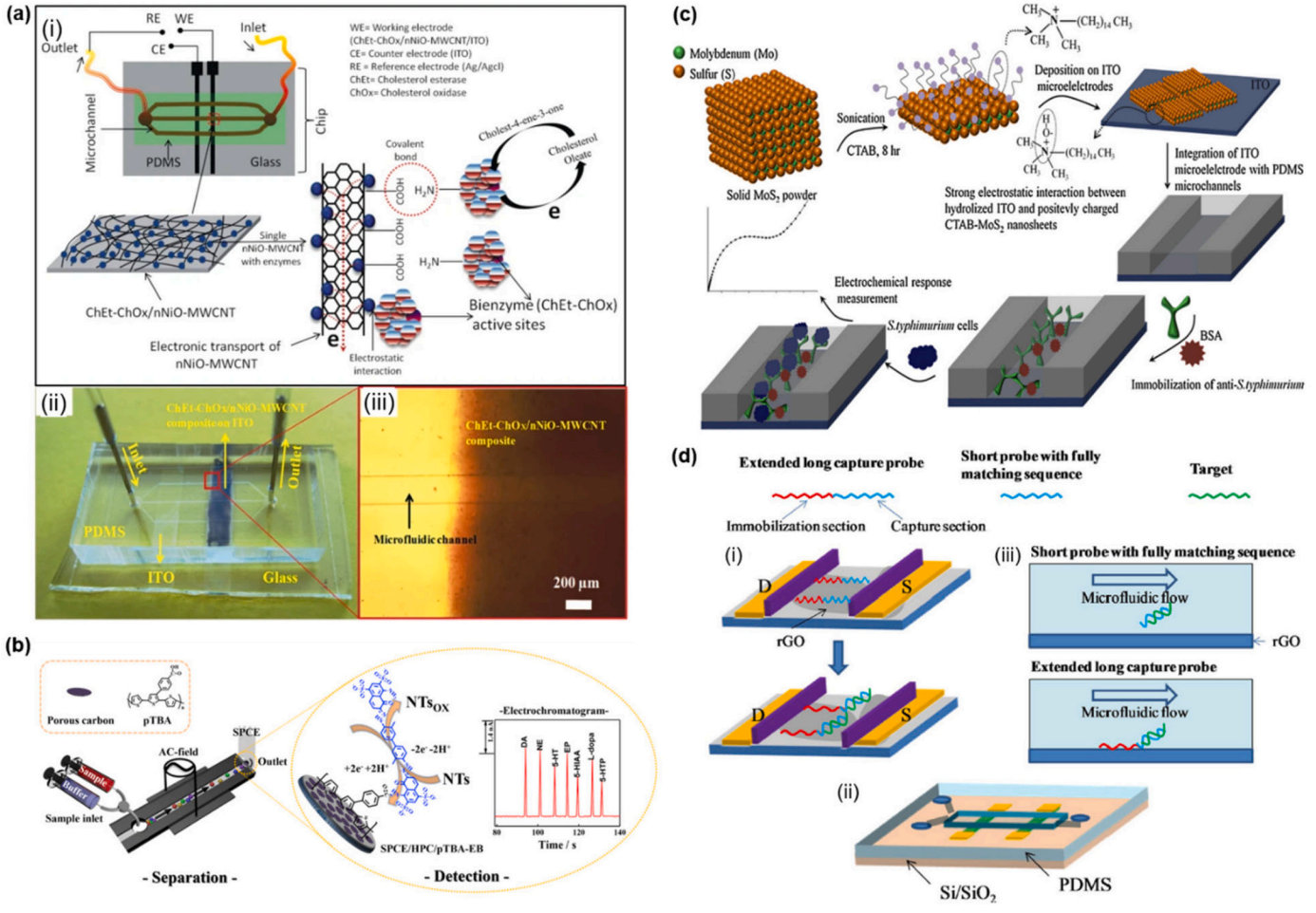

Fig. 10. Immobilization of receptors on various types of electrode surfaces. (a) (i) Illustration and (ii) photograph of a microfluidic biochip for the detection of cholesterol based on the functionalization of carbon nanotubes. (iii) Enlarged view of the biochip. (b) Illustration of an electrochemical microfluidic separation and sensing device to monitor neurotransmitters in human plasma using a functionalized carbon electrode. (c) Illustration of an immunosensor for Salmonella typhimurium based on the conjugation of proteins to indium tin oxide electrodes. (d) Illustration of an rGO transistor for influenza virus gene detection with (i) long capture probe immobilized on an rGO surface, (ii) integration of the rGO transistor in a PDMS microfluidic chip, and (iii) short probes with complementary sequences being flushed away in contrast to extended long capture probes, which remain immobilized on the rGO. (a) Adapted from [154], (b) adapted from [155], (c) adapted from [159], and (d) adapted from [172].

salmonella Typhimurium antibodies were immobilized onto the surface of a MoS_2-NS electrode [159]. Molybdenum disulfide (MoS_2) nanosheets were synthesized from bulk MoS_2 using cetyltrimethyl ammonium bromide (CTAB) assisted exfoliation, which results in positive charges on CTAB-MoS_2-NS. CTAB-MoS_2-NS was deposited onto a patterned hydrolyzed indium tin oxide (ITO) microelectrode by electrostatic interaction. The microelectrode was directly integrated with the PDMS chip. Detection was based on electrochemical impedance spectroscopy and showed high performances in terms of sensitivity and selectivity. Kaur *et al.* modified a platinum electrode with nickel oxide thin-film deposition using a sputtering technique to monitor cholesterol [171]. The surface of the nickel oxide was functionalized with cholesterol oxidases, which served as bioreceptors for cholesterol. Electrochemical amperometry detection showed that the system was sensitive (LOD of 0.1 mM) over a wide range of concentrations.

Finally, the use of graphene to fabricate conductive surfaces for biosensing generates a lot of interest [172]. Graphene is composed of a unique layer of atoms with an extended surface for immobilization of receptors, and chemical changes on its surface can easily be detected by electrical measurements [172,173]. Key challenges for using graphene for biosensing are functionalizing it with DNA in a stable manner and preserving graphene conductivity without reducing its sensitivity performance [172]. Immobilization on graphene is usually performed by covalent bonding [172,180]. Most of the DNA graphene transistors already developed are based on dip-and-dry methods [172,180]. There are so far few studies on the impact of receptor immobilization strategies on the performances of graphene transistors exposed to solutions containing analytes [172]. Fig. 10d describes the extension mechanism of long capture probe immobilization on a reduced graphene oxide transistor of a microfluidic chip for the detection of influenza virus [172]. The microfluidic channels were patterned in PDMS and the graphene transistor was fabricated on a Si/SiO_2 substrate. The PDMS layer and the transistor were bonded afterward. Several techniques of immobilization of DNA capture probes were tested: the immobilization of extended long and short capture probe based on π-π stacking, and a covalent immobilization based on a linker (pyrenebutyric acid). The immobilization was performed by incubating the transistor channel overnight in solution. For the π-π stacking method, the solution was a buffer containing the DNA, and for the linker solution-based method the solution contained the DNA, the linker solution and dimethylformamide. Both methods were characterized and compared after a rinsing step. The best immobilization in terms of sensitivity and stability was obtained for the π-π stacking method and using extended long capture probes. In the work of Qin *et al.*, CNTs and reduced graphene oxide (rGO) nanosheets were combined with gold nanowire arrays to implement a glucose biosensor [173]. Glucose oxidase was covalently immobilized on CNTs nanoelectrodes. The flow was driven by gravity forces inside a microfluidic setup. rGO nanosheets in combination with gold nanowires array showed better performances than glucose oxidase-CNT-gold nanowire arrays for glucose sensing and achieved a linear range of detection with a good LOD.

3.3. Hydrogel surfaces

Hydrogels are more and more employed in biosensing [181,182], POCDs [183], and tissue engineering [181]. These polymers are very popular for immobilizing receptors because they present numerous advantages. For example, their mechanical properties can be tailored [181]. They are hydrophilic, permeable to solvent molecules and analytes, and have excellent optical properties. They are resistant against non-specific deposition of proteins, biologically and chemically inert unless they are functionalized with specific groups for immobilizing receptors. They can be deposited or grafted on surfaces to yield a film of well-defined thickness thereby offering numerous sites for attaching receptors [182,181]. Hydrogels can also be photopatterned on a substrate or inside sealed microfluidic structures [184,185]. Functionalization with biomolecules (e.g. proteins, oligonucleotides, glycans) or non-biological material (e.g., nanoparticles, crosslinkers) is straightforward and well-documented [182,186]. Chemical modification of hydrogels for the specific immobilization of receptors can employ covalent bonds or photochemistry [186,187].

Immobilization of protein receptors on a hydrogel is often performed using photopolymerization [187–189]. Lin *et al.* photopatterned a 3D polyacrylamide gel inside a glass microfluidic device to serve as an immobilization matrix to perform a barcode sandwich immunoassay [188]. Proteins were patterned quickly with a good spatial resolution. Target antigens (hepatitis C virus) were then driven by electrophoresis to the capture sites present into the hydrogel [188]. Kucukal *et al.* immobilized various reagents in the hydrogel of a microfluidic chip to monitor the coagulation of blood using a dielectric sensor [190]. The upper part of the microchannels was patterned with a PEG using UV photo-crosslinking. The PEG layer was functionalized with receptors (either an anti- or pro-coagulant). Such a hydrogel did not interfere with the blood coagulation process and was resistant against unspecific platelet and protein. More recently, Kalme *et al.* developed a point-of-care microfluidic immunoassay for the multiplexed detection of fertility markers using hydrogel as a sensing surface [191]. The hydrogel was synthesized from a pre-polymer mixture by UV photopolymerization in a separate device. Acrylate groups on the PEG diacrylate photocrosslinked with the acryl moieties on the conjugated capture antibodies by photopolymerization reaction. Covalently-bound antibodies were present in the hydrogel, which allowed the capture of analytes by diffusion into the hydrogel. These hydrogels were trapped in a microfluidic device by taking advantage of their flexibility. The hydrogels presented low non-specific binding, which improved signal-to-background ratios and the overall sensitivity of the assay. Detection was fluorescence-based and results were accurate and precise. Polyacrylamide gels and electrostatic adsorption can also be used for protein immobilization [186]. In Fig. 11a, a polyacrylamide gel was functionalized with streptavidin-biotin-anionic moieties to allow the immobilization of proteins by electrostatic adsorption [186]. Different size of proteins was immobilized into the hydrogel and detected using fluorescently-labeled antibodies. Another strategy for immobilizing protein on hydrogels is the use of benzophenone, which facilitates covalent binding of proteins into the hydrogel via photocrosslinking [192]. Fig. 11b, depicts such a modified hydrogel for performing a fluorescence-based multiplexed immunoassay.

Hydrogels have been combined with graphene to immobilize proteins in microfluidic devices [182]. The combination of both materials leads to excellent signal transduction properties. rGO is a conductive material with pores and functional groups such as carboxylic groups on its surface [182,193]. rGO is commonly modified by patterning a self-assembled monolayer of L-cysteine amino acid for the fabrication of electrochemical or plasmonic biosensors [182]. Cysteine enables stable thin film formation on gold surfaces by adsorption of thiols groups from the amino acid residue. Fig. 11c presents a dual-modality microfluidic biosensor for quantifying cardiac myoglobin in human serum [182]. Mesoporous cysteine-graphene hydrogels were prepared and integrated into a microfluidic device. Cysteine-rGO hydrogel and the microfluidic channel were prepared separately. Au electrodes were dipped into a hydrogel solution for adsorption of the thiolated hydrogel on the Au surface. Using an oxygen plasma treatment, microfluidic channels were bound to a glass substrate covered with the Au electrodes, cysteine-rGO hydrogel, and a reference electrode. A solution containing cardiac myoglobin antibodies (cMAbs) was injected into the chip to functionalize the hydrogel using an EDC–NHS coupling chemistry followed by a blocking step using a solution of BSA. This system demonstrated excellent performance with a high surface reactivity, fast electron transfer, good reaction kinetics, and real-time detection capability.

Nucleic acids have also been successfully immobilized on hydrogel surfaces. In Fig. 11d, miRNA probes were immobilized in a hydrogel using projection lithography to incorporate probes composed of two

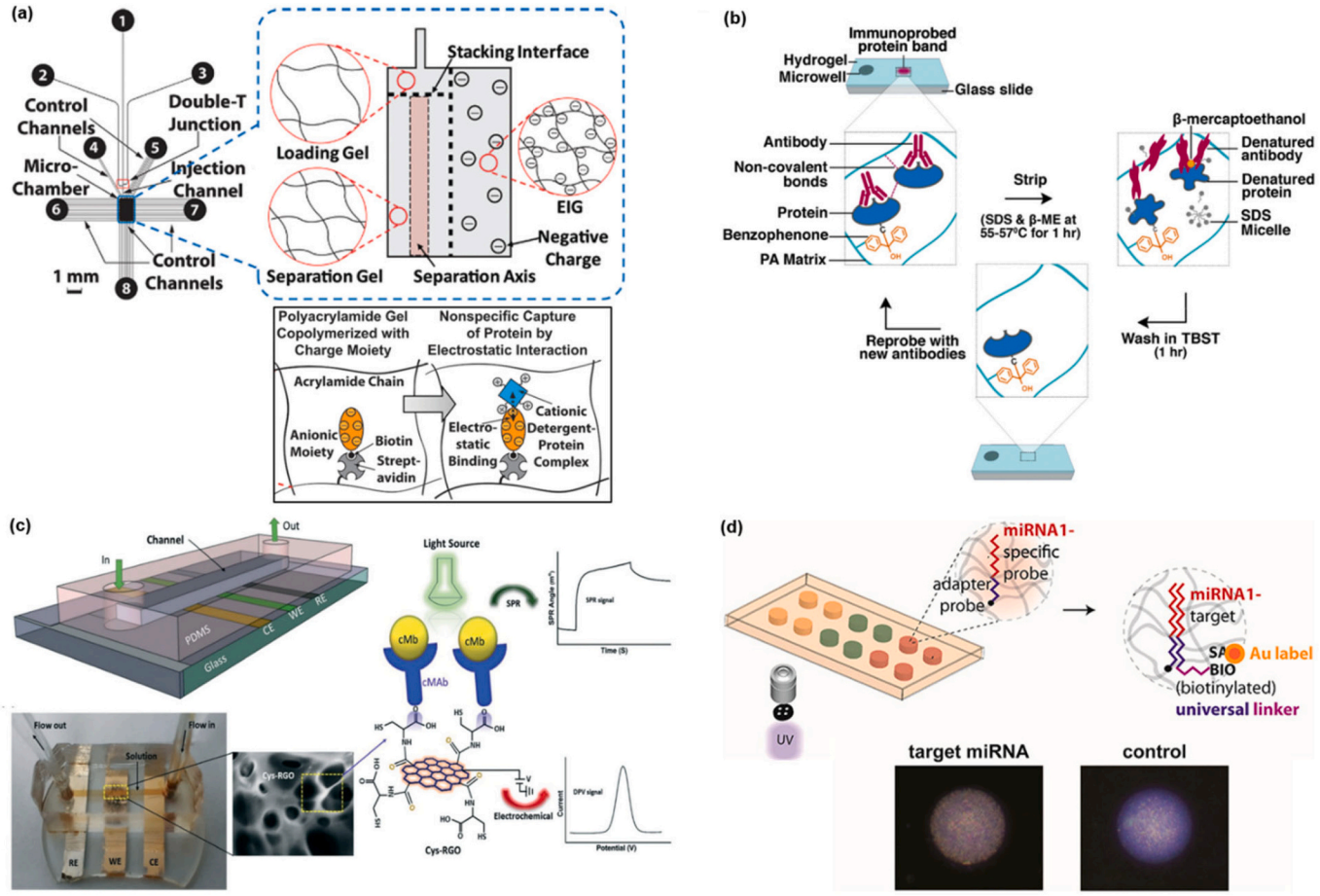

Fig. 11. Immobilization of receptors on a hydrogel. (a) Protein immobilization on an electrostatic polyacrylamide gel matrix for microfluidic Western blotting. (b) Protein-antibody complex immobilized on a hydrogel using benzophenone and description of the potential loss of receptor function during steps for stripping captured proteins. (c) Microfluidic chip biosensor fabricated using a mesoporous nanostructured cysteine-graphene hydrogel for detecting human cardiac myoglobin. (d) Illustration of a microfluidic chip integrating functionalized hydrogels for the multiplex detection of miRNA. (a) Adapted from [186], (b) adapted from [192], (c) adapted from [182], and (d) adapted from [183].

domains (miRNA-specific domain and universal domain) [183]. The probe-target was functionalized with a biotinylated universal linker using a T4 DNA ligase. The probe-target complex was labeled using a streptavidin-conjugated gold nanoparticle to perform signal amplification by gold deposition. This platform offers multiplexing opportunities and a low LOD (260 fM). Recently, Gao *et al.* developed a microfluidic device for on-site analysis of DNA adenine methyltransferase (Dam) activity [181]. They used DNA tetrahedra-based hydrogel to trap glucose-producing enzymes. Enzymes were encapsulated non-covalently into a DNA hydrogel, and enzymatic substrates were modified separately on papers. The enzyme and their substrates were combined afterward onto a commercial glucose test strip for the sensitive evaluation of Dam activity. A large amount of enzyme could be entrapped into the hydrogel with high sensitivity and selectivity (LOD of 0.001 $U.mL^{-1}$), which is superior to previously reported biosensors.

4. Immobilization of receptors on mobile surfaces

Immobilization of receptors on static surfaces is convenient because it leads to devices with integrated receptors and well-defined areas for reading signals. The drawback of this immobilization strategy is that potentially large volumes of samples will need to pass over the receptors in order to detect rare analytes. This can take a considerable time for assays where analytes are not amplified using PCR, for example. A careful design of the geometry of the microfluidic structure holding the receptors and good understanding of the transport/kinetic characteristics of the ligand-receptor binding reaction is necessary [8,194,195]. Mobile surfaces offer a remedy to these challenges by giving the possibility of "exploring" a sample by dispersing and regrouping particles functionalized with receptors in a sample for optimal capture of analytes and reading of a signal. Similarly to static surfaces, immobilization of receptors on mobile surfaces can lead to random orientation of receptors and/or their denaturation. A carrier for receptors should have a high surface-to-volume ratio, be compatible with surface treatments for receptor attachment, be stable mechanically and chemically, and should not induce non-specific binding with other carriers or microfluidic walls. Capture efficiency and capture capacity can be improved using many solutions that increase the surface-to-volume ratio and enhance the binding kinetics [196–198]. Microbeads present several and unique advantages compared to static supports, especially when they are immobilized and packed into specific microfluidic structures [199]. Such a support can offer a much higher surface-to-volume ratio than walls forming microchannels [196,197,199–202]. Microbeads are suitable for the analysis of complex biological samples [203], and multiplexing assays can be performed by using different surface coatings [198,201,204]. Analyte binding reactions on microbeads can lead to high sensitivity detection of analytes [202,204,205], fast binding kinetics with excellent mixing efficiency [196–198], and a reduction of the incubation time [205] and background noise. In addition, beads can be produced in large quantities at low-cost [201], from various types of materials [200]. A large variety of surface modifications and libraries of pre-functionalized beads are also commercially available

[204].

In the remaining parts of this section, we discuss the immobilization of receptors on mobile surfaces. The mobile surfaces can be microbeads or microparticles, nanoparticles, and can be magnetic or non-magnetic. In the last part, we present developments of an immobilization performed on suspended graphene, which is a promising, very recent alternative to using microbeads.

4.1. Magnetic mobile surfaces

Magnetic mobile surfaces are often used for biological assays in the form of magnetic beads [206–208], paramagnetic and superparamagnetic beads [206,209,210], or magnetic nanoparticles [211–213]. Magnetic beads can be coated with specific biomolecules such as nucleic acids, aptamer, or proteins and can be manipulated with external magnets to localize them in microfluidic devices [206,214,215]. Such localization using magnets can be reversible and used to expose the functionalized beads to reagents and samples.

4.1.1. Magnetic beads

Magnetic beads are very popular for bioseparation and bioassays performed at the macroscale and can also be used in microfluidics for immobilizing receptors in the context of experiments involving DNA hybridization, mRNA isolation, and immunoassays [215]. Examples of microfluidic devices taking advantage of the functionalization of magnetic beads to immobilize nucleic acids based on streptavidin attachment are described in Fig. 12. In Fig. 12a, aptamer-coated magnetic beads are used to measure the amount of glycated hemoglobin in a blood sample using a microfluidic chip [207]. All steps of the assay were integrated into a PDMS device composed of several reaction chambers and valves. Magnetic beads were coated with streptavidin to allow functionalization with aptamers. Beads were blocked with BSA to avoid nonspecific binding during the measurement process. Functionalized beads were added to blood to capture hemoglobin and glycated hemoglobin based on a "sandwich" configuration. Beads were immobilized in a specific area of the device using a magnet and an external magnetic field. Specific antibodies were added to bind the captured targets and magnetics beads were collected afterward. The measurements of hemoglobin and glycated hemoglobin were performed on-chip based on a chemiluminescence detection. This approach enabled high-sensitivity and high-specificity assays, the use of small volumes of samples, and a short assay time (~25 min). Fig. 12b presents a multi-channel PMMA microfluidic biosensor using paramagnetic beads for the quantification of DNA sequences [209]. Magnetic beads were coated with streptavidin and coupled with biotinylated DNA capture probes off-chip. Probe-tagged liposomes were entrapped off-chip with ferri/ferro hexacyanide as a redox marker. The prepared sample solution was introduced into the microfluidic chip using active pumping. DNA capture probes and liposome reporter probes formed a complex through DNA hybridization on the surface of the beads. External magnets were placed over the microchannels, which allowed capturing the beads in a microchannel. Such a capture facilitated washing steps and lysis of the liposomes using detergents. Detection was performed on microelectrodes downstream of the capture zone using amperometry. The system showed good signal

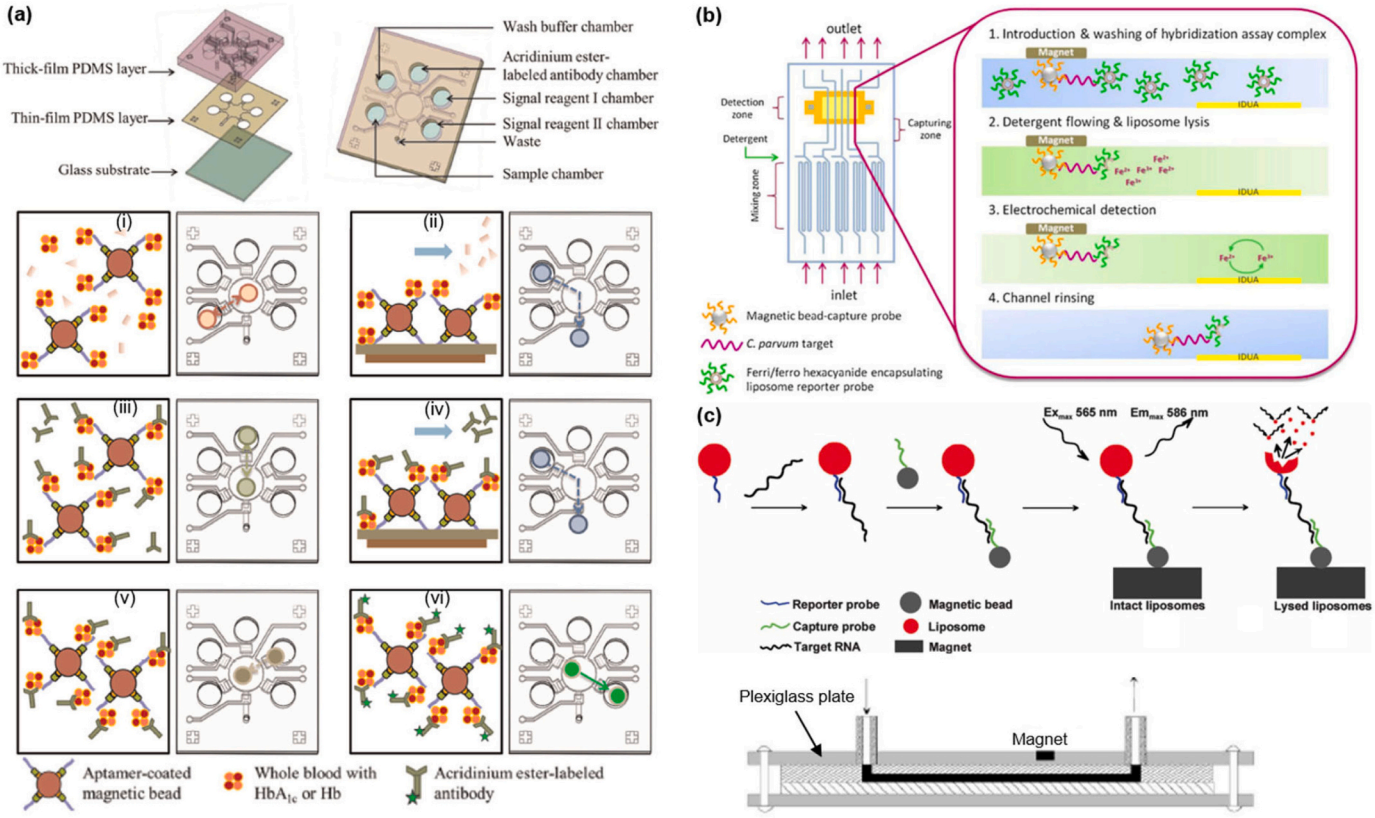

Fig. 12. Immobilization of nucleic acids on magnetic beads. (a) Exploded and top views of an integrated microfluidic device using aptamer-coated magnetic beads for measuring hemoglobin and glycated hemoglobin in blood samples. (i) Aptamer-coated beads are incubated with blood to accumulate specifically targets on the surface of the beads and (ii) to collect the beads using an external magnetic field. (iii) Labeled antibodies were transported and reacted with the captured targets before (iv) collection, (v) resuspension of the magnetic beads, and (vi) fluorescence detection. (b) Quantification of target DNA sequences inside a multi-channel microfluidic chip composed of interdigitated ultra-microelectrode arrays. The system used paramagnetic beads coated with DNA capture probes and liposomes tagged with DNA reporter probes to bind the target DNA sequences. (c) Principle of a microfluidic biosensor for the detection of pathogens. Superparamagnetic beads were used as substrates and functionalized with DNA capture probes to bind RNA analytes and liposomes. The lysis of liposomes on the beads provided a fluorescence reporting signal. (a) Adapted from [207], (b) adapted from [209], (c) adapted from [215].

linearity, robustness, a good LOD, and reliability compared to previously published work. A microfluidic biosensor for the detection of RNA from pathogens is presented in Fig. 12c [215]. There, superparamagnetic beads were incorporated into the system as mobile substrates and were manipulated using an external magnet. Capture probes were immobilized at their 5' end on the surface of the beads using biotin-streptavidin interaction. Once captured on the beads, RNA analytes were bound by liposomes functionalized with complementary strands and an optical signal triggered the release of reporting molecules from the liposomes. This elegant assay architecture permitted fast tests (15 min including incubation) with an excellent signal-to-noise ratio. Nguyen *et al.* developed recently a quick and precise droplet microfluidic platform to coat magnetic beads with quantum dots, which offer great possibilities for solid-phase assays involving DNA [216]. They took advantage of microdroplets as reaction vessels, which allowed better control of local reaction conditions. A high-density layer of quantum dots was coated on the surface of single magnetic beads even when the concentration of quantum dot was low. The conjugation between the beads and the quantum dots was enhanced through a confined environment and fast mixing. Magnetic beads and quantum dots were co-encapsulated into individual droplets. This system was then improved to achieve high-quality quantum dots-DNA conjugation as evaluated using FRET [217].

The immobilization of proteins on magnetic beads usually involves functionalizing beads with capture antibodies as illustrated by various immunoassays depicted in Fig. 13 [200,210,218–220]. Fig. 13a illustrates the detection principle of dengue virus in a microfluidic device using antibodies conjugated to magnetic beads [208]. All steps of the assay were performed on-chip. Antibodies were coated onto the magnetic beads and the functionalized beads were incubated in a solution containing target viruses. The beads were attracted by 14 planar microcoils for purification and washing steps, and pumped into a collection chamber for detection of the viruses. This device showed high specificity and sensitivity. An automated microfluidic platform employing a three-stage incubation step and paramagnetic beads was developed by Sasso *et al.* to perform multiplexed immunoassays (Fig. 13b) [200]. Antigens of interest were captured by incubating the antibody-coated microbeads with a sample in a continuous flow environment. The resulting bead-antibody-antigen-complexes were incubated with a biotinylated secondary antibody that bonded to the antigen, leading to the formation of a sandwich structure. Microbeads were then exposed to streptavidin-phycoerythrin conjugates to generate a fluorescence signal. An external magnet separated and actuated microbeads by pulling them from one reagent stream to the next one. This system proved to be robust, fast and automated, versatile, economic of sample, with a high sensitivity (LOD between 10 pg mL^{-1} and 1 ng mL^{-1}), and amenable to multiplexed detection of analytes. Fig. 13c presents another multiplex assay using magnetic beads [206]. Three different types of magnetics beads (Dynabeads™) were used for the assay. Magnetic beads were either coated with epoxy groups, streptavidin, or with protein A. All types of beads were suspended in a glycine solution. For

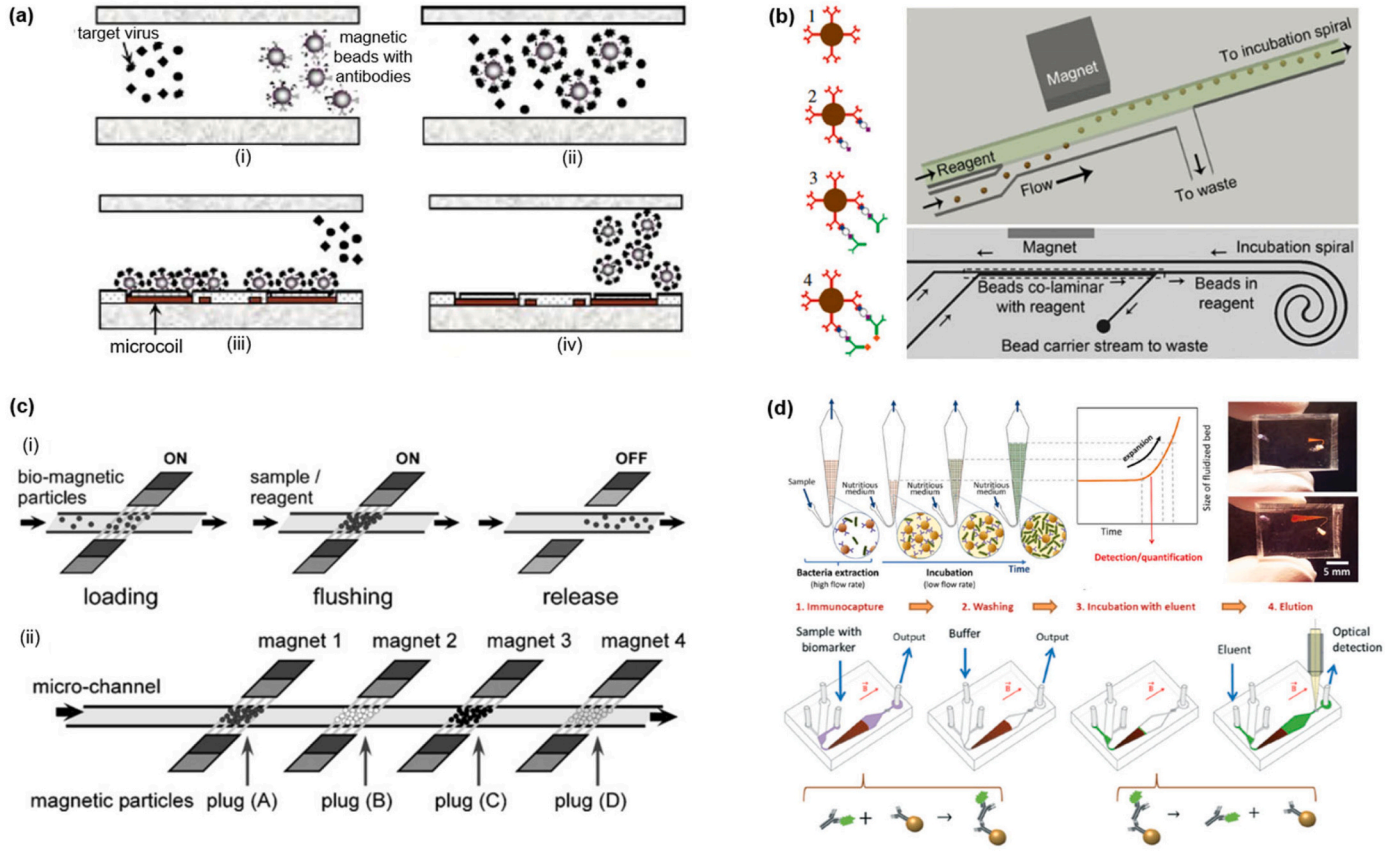

Fig. 13. Immobilization of proteins on magnetic beads. (a) Principle of a microfluidic device for virus detection using antibody-conjugated magnetic beads. (i-ii) Target viruses were captured on magnetic beads. (iii) Viruses were purified and magnetically enriched using planar microcoils (iv) before being released. (b) Automatized multiplexed immunoassay in a microfluidic chip using conjugated antigen-paramagnetic beads. An external magnet transferred the paramagnetic beads along the flow path, allowing the formation of an immunocomplex on the bead. (c) (i) Principle of a microfluidic magnetic bead-based assay. Magnetic beads were loaded and trapped by an external magnetic field. Solutions containing the analytes were flushed over the beads allowing binding of the analytes on the surface of the beads. The beads were released after detection by stopping the magnetic field. (ii) Simultaneous bioassays can be performed by coating the magnetic beads with different biomolecules and by controlling independently different magnets. (d) Microscale fluidized bed for capturing and detecting bacteria in a microfluidic chip using and external magnet and superparamagnetic beads functionalized with antibodies. (a) Adapted from [208], (b) adapted from [200], (c) adapted from [206], and (d) adapted from [196] and [197].

the epoxy coated beads, such a suspension in glycine resulted in a deactivation of the surface of the beads owing to the binding of amino groups of glycine to the epoxy groups on beads. These beads were then used to perform the negative control of the assay. A solution of fluorescently-labeled biotin and fluorescently-labeled IgG were used to perform the assay with the streptavidin-coated beads and the protein A-coated beads, respectively. Sets of magnets were placed along the microchannel to localize, trap and detect a small volume of each type of beads in a microfluidic chip to realize multiplexed assays (Fig. 13c, ii). Different samples containing either biotin or IgG were flushed in a continuous flow. Detection of biotin and IgG was based on fluorescence. Biotin bound to the streptavidin-coated beads, IgG to the proteins A-coated beads, and no binding occurred on the epoxy/glycine beads, as expected. Incubation times were reduced during the assay owing to a short diffusion distance between the analytes and the surface of the beads in such a configuration setup. This setup allowed a fast and easy way to realize multiplex assays. Moreover, Paratore *et al.* used isotachophoresis to accelerate surface-based immunoassays [221]. Target proteins were pre-concentrated and delivered to the surface of beads functionalized with specific antibodies using isotachophoresis. An enhanced green fluorescent protein was used as a model protein. The system showed a 1300-fold improvement in LOD compared to a standard immunoassay and the protein-antibody reaction was fast (6 min). Systems combining a giant magnetoimpedance-based platform and superparamagnetic beads have been investigated as well to detect proteins. Yang *et al.* developed a method for the determination of magnetic bead-labeled with C-reactive protein [222]. To this end, a gold film was deposited on a magnetoimpedance sensing element as a substrate. A sandwich immunoassay was then performed using antibody-antigen combinations and biotin-streptavidin interactions directly on the gold film surface via self-assembled layers. Finally, Wang *et al.* employed as well magnetoimpedance using labeled superparamagnetic Dynabeads™ to perform an immunoassay and detect alpha-fetoprotein [223]. There, the gold layer was modified with 11-mercaptoundecanoic acid for immobilizing alpha-fetoprotein monoclonal antibodies.

The functionalization of magnetic beads can be employed as well for detecting cells [224]. Fig. 13d presents a microscale fluidized bed to capture and detect infectious bacteria in a microfluidic chip using an external magnet and superparamagnetic beads functionalized with antibodies [196,197]. Fluidized beds have several non-negligible advantages: they lead to a high mixing efficiency, and homogenous flow rates and temperatures during experiments and assays. In this work, beads were conjugated with antibodies, and an external magnetic field, collinear to the general direction of the flow, brought them within the chip and localized them in a specific chamber [196]. In addition to offering a high dynamic range of detection, sensitivity, and specificity, this system enhanced target capture efficiency and was equivalent to quantitative PCR. This system can operate at the living cell level rather than at the DNA level [196].

4.1.2. *Magnetic nanoparticles*

Nanoparticles are broadly used for bioanalysis and especially for rapid and ultra-sensitive detection methods where immunoassays need to be employed [211]. Nanoparticles can offer excellent optical, electrical, and magnetic properties combined to a high surface-to-volume ratio and larger diffusivity than larger particles [211]. Fig. 14 illustrates the immobilization of receptors on nanoparticles. Fig. 14a presents a colorimetric biosensor for the detection of Escherichia coli bacteria [212]. Magnetic nanoparticles were modified with capture antibodies against Escherichia coli and polystyrene microspheres were in parallel modified with detection antibodies. Functionalized magnetic nanoparticles and polystyrene microspheres were mixed in chip with catalase and a solution of bacteria. Magnetic nanoparticles, bacteria and polystyrene beads formed complexes that were localized in a separation chamber using external magnets. A solution of hydroxide peroxidase was added to react with the catalase, and a solution of gold nanoparticles and crosslinking agents were injected to perform the detection: Au nanoparticles aggregated, resulting in a change of color that was detected using a smartphone.

Fig. 14b illustrates a microfluidic device used to perform an immunoassay to detect immunoglobins E [211]. To this end, magnetic nanoparticles were entrapped into silica shells to form a so-called "magnetic core-shell nanoparticles". Antigens were grafted on the surfaces of these nanoparticles following a chemometric method using EDC, NHS and MOPS solutions. Target antibodies attached to the antigens present on the surface of the magnetic core-shell nanoparticles in solution. The resulting complexes were focused in a chamber surrounded by magnets. Detection antibodies were injected in the chamber to bind and reveal antibody-antigen complexes on the surface of the nanoparticles. This approach proved to be fast (20 min), sensitive (LOD of 1 ng mL^{-1}), and economical of sample (5 μL) and reagents.

Hybrid methods taking advantage of nanoparticles have also been developed. For example, Esmaeili, *et al.* implemented a system to enhance the capture and detection of DNA in microfluidics [225].

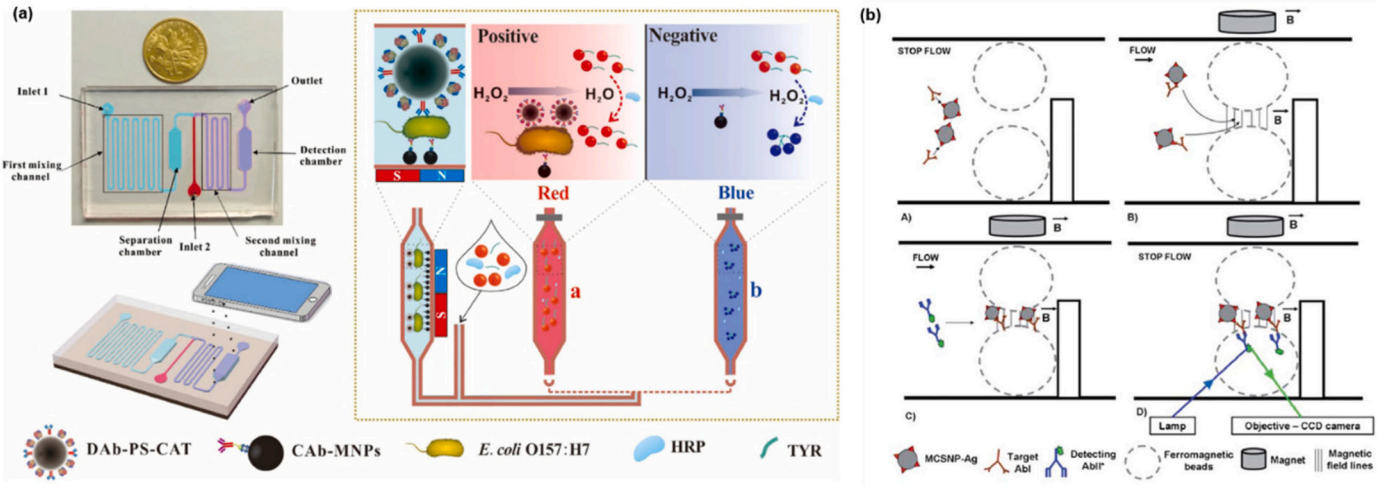

Fig. 14. Immobilization of receptors on the surface of nanoparticles. (a) Illustration of a colorimetric biosensor for detecting Escherichia coli using functionalized magnetic nanoparticles and functionalized polystyrene microspheres. (b) Illustration of an immunoassay using magnetic core-shell nanoparticles in a microfluidic chip. The nanoparticles are functionalized with antigens to capture specific antibodies before being focused in a microfluidic chamber using magnets. Detection of captured antibodies uses fluorescently-labelled antibodies. (a) Adapted from [212], and (b) adapted from [211].

Magnetic nanoparticles were functionalized with probe DNA sequences. Antibodies conjugated to complementary sequences of the probes formed a complex with the magnetic nanoparticles. Such a complex was introduced into a solution containing antigens and fluorescently-labeled detection antibodies, which resulted in the formation of immunocomplexes on the surface of the magnetic nanoparticles. The resulting solution was introduced in a microfluidic chip and the nanoparticles were captured for detection by an external magnet in a specific zone of the chip. This system was fast and sensitive (LOD of 0.7 nM) and had low steric hindrance and short time for reaching equilibrium.

4.2. Non-magnetic bead surfaces

Non-magnetic beads represent a simpler system compared to magnetic beads because these beads can be made of many materials (polystyrene [198,226], PMMA [205,227], agarose [199,203], silica [228], glass [229,202]), can have a lower density than magnetic beads and tend to sediment less, are not necessarily opaque, and do not require microfluidic devices having magnets. Positional control and functionalization of non-magnetic beads can be done in flow [230]. In this section, we review examples of microfluidic devices based on such beads and their surface functionalization with nucleic acids and proteins.

4.2.1. Immobilization of nucleic acids on non-magnetic beads

Nucleic acids can successfully be immobilized on polystyrene, PMMA, and silica beads, as illustrated in Fig. 15 using surface activation of the beads and streptavidin attachment. Specifically, Fig. 15a describes how to immobilize molecular beacons on the surface of polystyrene beads for the genotyping of single nucleotide polymorphisms [198]. Polystyrene microbeads were coated with streptavidin and biotin-BSA. The resulting coated beads were rinsed, functionalized with avidin, rinsed again and injected into one inlet of a microfluidic chip device. At the same time, biotinylated molecular beacons were injected into a second inlet of the device, and attached to the avidin-BSA-biotin-streptavidin beads in a first mixing chamber of the chip. Target DNA sequences were injected into a third inlet of the chip, hybridized to the molecular beacons attached on the beads, which resulted in the opening of the beacons and a fluorescence signal. The beads were trapped downstream on arrays of microposts for rapid detection of the fluorescence signal. Such a system demonstrated high performances and offered a great opportunity to create arrays of densely packed microbeads inside microfluidic devices. Kim *et al.* employed as well polystyrene beads coated with streptavidin and modified with DNA [230]. These authors studied the factors influencing the capture of DNA on such beads. The beads were introduced inside a microfluidic channel using a pressure-induced flow. Hybridization time was on the order of minutes and the system showed a good LOD [230]. Fig. 15b presents a microfluidic chip for the solid-phase detection of cell-free mitochondrial DNA (cfmDNA) [205]. PMMA beads were functionalized with streptavidin to immobilize biotinylated DNA capture probes. Microbeads were injected in the microfluidic chip and immobilized as a planar layer in a so-called flow cell section of the chip using a specific commercial solution. Controls were performed using different DNA strands (EUB338 and HPV72) and beads without immobilized DNA captured probes. A solution containing the cfmDNA strands was filled to the "reagent reservoirs" of the chip and then pumped to the "flow cell" of the chip. The cfmDNA hybridized to complementary sequences on the surface of the beads. The detection of cfmDNA was fluorescence-based and automatically performed after hybridization. Approximately 50% of the maximum of fluorescence signal was reached within 7 min and the LOD was improved by at least a factor of five. Moreover, multiplexing was successfully achieved with this rapid system. PMMA beads were employed as well by Caneira *et al.* to perform DNA hybridization [231]. These authors specifically used nanoporous PMMA chromatography beads to detect a specific 22-mer DNA sequence. Detection was performed using labeled quantum dots. No amplification procedure was required to perform this easy, rapid, and sensitive assay, which presented a hybridization efficiency above 95% and a hybridization dissociation constant below 1 nM. In Fig. 15c a sandwich molecular beacon assay using silica beads is implemented on a 1D chip [228]. The surface of the silica beads was activated with Na_2CO_3. After binding of biotinylated BSA by physical absorption, the beads were functionalized with streptavidin. Biotinylated DNA capture probes were immobilized on the surface of the beads. The beads were introduced into a pre-treated PDMS chip using an active pumping system. A solution containing target DNAs was then pumped in the chip, resulting in hybridization of target DNA to complementary sequences present on the surface of the beads. Molecular beacons were then flowed in the chip to partially hybridize with captured targets and generate a fluorescence readout. With this implementation, the fluorescence background was low, resulting in a high sensitivity of the assay (LOD of ~0.05 nM) and multiple targets can be detected without the need for laborious and time-consuming elution of captured target analytes.

4.2.2. Immobilization of proteins on non-magnetic beads

Non-magnetic beads can be used as substrates for immobilizing proteins as shown in Fig. 16 where immunoassays are performed on the surface of beads made from polystyrene, PMMA, and agarose, and using

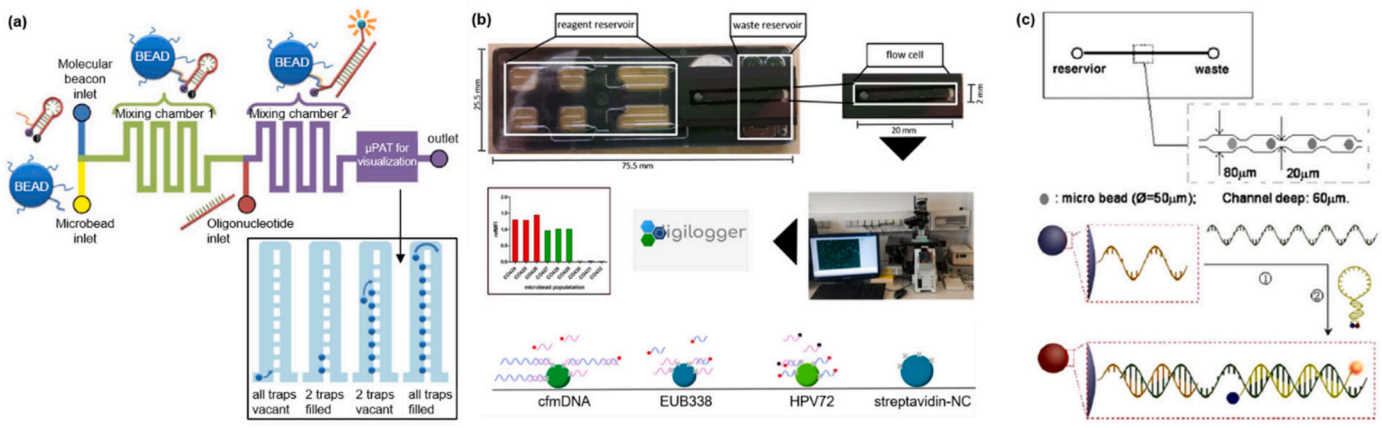

Fig. 15. Immobilization of DNA probes on microbeads. (a) Illustration of a microfluidic device for the genotyping of single nucleotide polymorphism using polystyrene beads. (b) Microfluidic chip platform for the solid-phase detection of biomarkers. Microbeads were immobilized in a flow cell section of a chip and detection was performed by hybridization with a complementary DNA sequence that was fluorescently labeled. (c) Implementation of a 1D chip for a "sandwich" assay using silica beads functionalized with DNA. (a) Adapted from [198], (b) adapted from [205], and (c) adapted from [228].

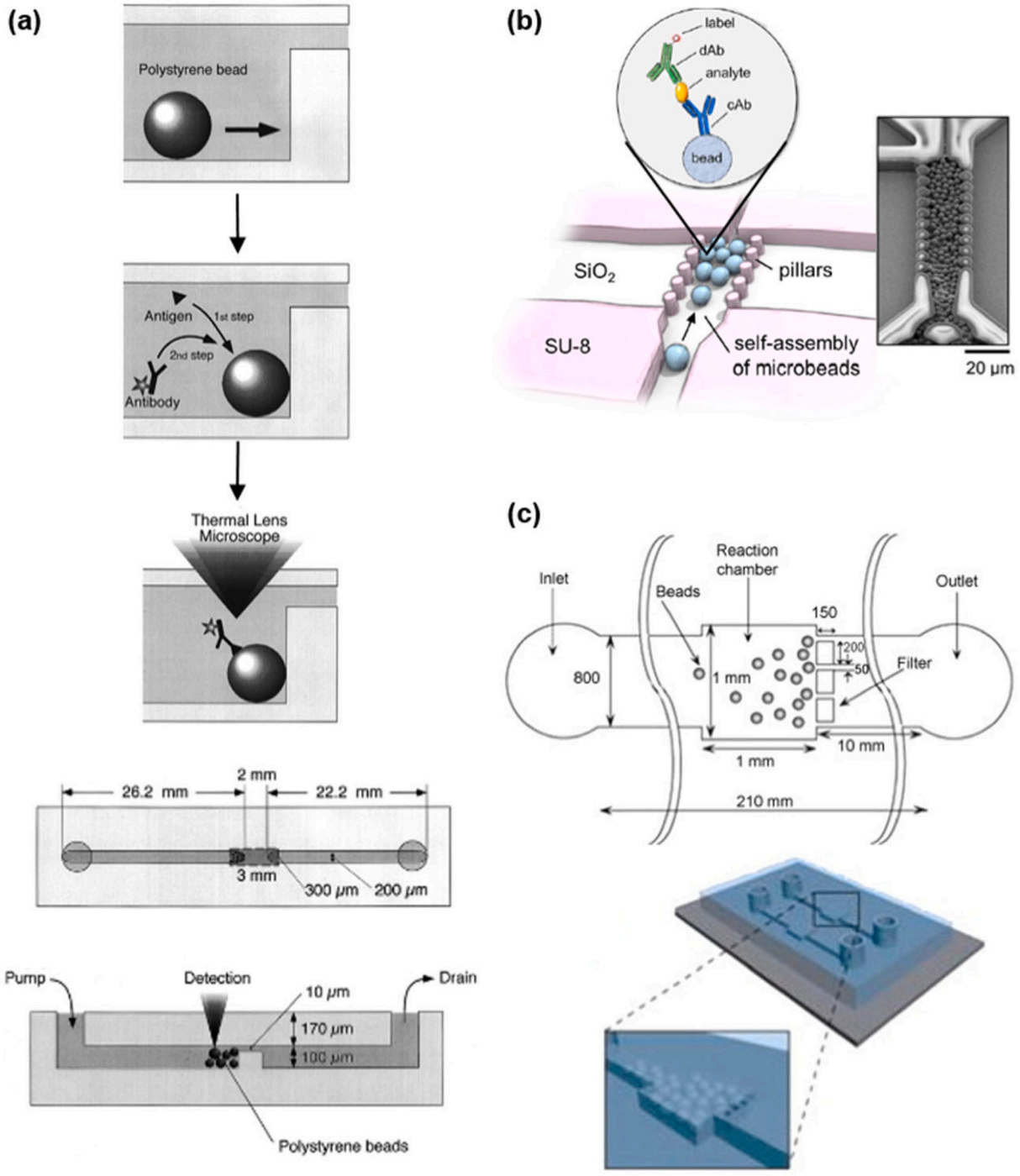

Fig. 16. Immunoassay on non-magnetic beads. (a) Illustration of the implementation of an immunosorbent assay inside a glass microfluidic chip. Polystyrene beads were introduced into a microchannel. Human s-IgA antigens were adsorbed on the surface of the beads, and detection antibodies labeled with colloidal gold bound to the attached s-IgA. The detection of colloidal gold was performed using a thermal lens microscope. (b) Illustration and SEM image of the trapping of PMMA microbeads functionalized with capture antibodies for an immunoassay in a "bead lane" and capillary-driven microfluidic chip. (c) Design of a PDMS microfluidic chip for packing agarose beads. Beads were functionalized with IgG capture antibodies, introduced into an inlet port, and trapped into a filter structure. The detection of IgG was performed using an enzymatic reaction based on alkaline phosphatase. (a) Adapted from [232], (b) adapted from [227], and (c) adapted from [199].

methods of adsorption, streptavidin attachment, or covalent bindings. Fig. 16a illustrates the implementation of an immunosorbent assay inside a glass microfluidic chip [232]. Polystyrene beads were used as mobile surfaces for the assay. The beads were introduced in a 100-μm-high microchannel and antigens (s-IgA) adsorbed on the beads. Detection antibodies labelled with colloidal gold formed a complex with s-IgA on the surface of the beads. Detection of the resulting complex used thermal lens microscopy, which led to fast, low cost and sensitive detection of analytes. Javammaed *et al.* employed as well polystyrene beads functionalized with antibodies to perform an immunoassay [226]. There, beads were functionalized with primary antibodies and introduced into a solution of analytes. The functionalized beads captured the analytes and the resulting solution was introduced into a microfluidic chip. Gold electrodes patterned on the surface of the chip were functionalized with secondary antibodies. The complex beads/analytes was captured by the secondary antibodies and formed a complex on the

surface of the electrode. Detection of the sandwich was based on impedance measurement between the electrodes and was performed in less than 1 h with a dynamic range of three orders of magnitude, and a LOD of 1 ng mL^{-1}.

PMMA beads have been successfully functionalized with proteins and used in capillary microfluidics by Delamarche *et al.* [136,201,227]. Fig. 16b presents the filling of a 20-μm-wide specific structure ("bead lane") of a capillary-driven microfluidic chip with PMMA microbeads functionalized with antibodies for detecting the cardiac biomarker troponin I [227]. The antibodies on these 5 μm beads were immobilized using biotin-streptavidin attachment. The functionalized beads were drawn into the chip by capillarity where they packed in the bead lane in less than 30 s. This $\sim 3 \times 10^{-5}$ mm^3 bead lane was positioned across the flow path of the chip and can hold ~300 microbeads. Analytes were introduced into the chip and were captured by the functionalized beads. A ~1 μL of sample containing fluorescently-labeled detection antibodies was sufficient to perform an assay having a LOD of ~4 ng mL^{-1} in only 25 min. Pham *et al.* used the same concept of bead lanes and 5 μm fluorescent beads functionalized with capture antibodies to perform a sandwich immunoassay for detecting malaria antigens [201]. In this work, fluorescence from the beads was masked by electroless deposition of silver on the gold-labeled detection antibodies, which resulted in a LOD below 6 ng mL^{-1} in 20 min. Another example used streptavidin-coated PMMA microbeads with biotinylated alkaline phosphatase. Such functionalized beads were packed into a bead lane of a capillary-driven microfluidic chip to study the impact of the flow rate, set by a programmable hydraulic resistor array, on the enzymatic conversion of a specific substrate (ELF-97) [136].

Furthermore, proteins were immobilized on agarose beads using a streptavidin-coating method and in particular into superporous agarose beads using a covalent method. Such beads offer many advantages compared to other types of beads and are commonly used for affinity chromatography [199]. The homogeneous surface of other types of beads limits the capture of molecules by receptors to the outer surface of the beads. Superagarose beads in contrast have two varieties of pores: diffusion pores, and superpores, with the latest representing a considerable volume fraction of the beads [199]. The surface area available for immobilizing receptors is therefore considerable with such pores. Superpores allow an extensive fraction of the flow to pass inside each bead, which enhances mass transfer inside microchannels and the possibility to work with an increased flow rate. This last point is still valid with compactly packed beads, which enhances the performances of immunoassays performed in microfluidic chips [199]. Fig. 16c presents the design of a PDMS microfluidic chip in which superporous agarose beads were functionalized and trapped to detect IgG antibodies [199]. Superporous agarose beads were covalently conjugated to a protein A following a procedure described in detail in the paper. Briefly, beads were prepared using a double emulsification procedure and partially hydrolyzed, which produced aldehyde groups to immobilize protein A. Functionalized beads were then trapped inside a filter structure (400 μm high and made in SU-8) to perform an immunoassay. The different elements of the sandwich immunoassay (IgG antibodies, analytes, and detection antibodies conjugated to alkaline phosphatase) were sequentially introduced inside the microchannel. The immuno-complex formed on the surface of the beads and detection was based on an enzymatic reaction with alkaline phosphatase visible to the naked eye. A LOD of 100 pg mL^{-1} of goat IgG in PBS was achieved. Such a low LOD results from the access of the analytes to both outer and inner surfaces of the superporous agarose beads. Agarose beads were employed as well in the work of Jokerst *et al.* to detect a specific biomarker (carcinoembryonic antigens) inside a programmable microfluidic chip [203]. The agarose beads had a ~300 μm diameter and were introduced inside the device. The impact of the pore sizes of the beads (ranging from 45 to 620 nm) on the immobilization process was studied in detail. The best results in terms of rapidity, efficiency, detection, and non-specific immobilization, were obtained with the 140 nm-pore-size beads. In the setup by Thompson *et al.*, streptavidin-coated agarose beads were immobilized on a specific region of a microfluidic channel using hot embossing [204]. This approach is convenient, repeatable, and enables the localization of functionalized beads at specific locations of microfluidic devices. Quantum dots functionalized with fluorescently-labeled biotin were used to capture analytes in solution and to detect them on the surface of the beads. Based on this method, analytes were separated from complex biological samples. Detection of the resulting complex (bead, analyte, biotin/quantum-dot) was fluorescence-based and presented a low signal of background.

Finally, glass beads can be used as mentioned in the work of Lee *et al.* to perform immunoassays on the surface of beads for detecting various bacteria [229]. Glass beads were treated with aminosilanes and glutaraldehyde. Treated beads were functionalized with antibodies via covalent bonds between the carboxyl-terminated of the beads and the primary amines of the antibodies. Glass beads of different sizes and with different antibodies attached on their surfaces were sequentially packed into a microfluidic chamber, which allowed the simultaneous detection of multiple analytes inside the chip. Guan *et al.* functionalized glass beads with antibodies to detect *Escherichia coli* inside a microfluidic device [202]. To this end, antibodies were covalently attached to the surface of the glass beads. Beads were afterward introduced inside a microfluidic chamber to capture the bacteria present in flow. Detection of the bacteria was bioluminescence-based, fast, and showed high specificity and sensitivity.

4.3. Suspended graphene

Other types of surfaces, such as functionalized graphene, can be used as a suspended solid surface to immobilize receptors in microfluidic chips (Fig. 17). Graphene is a good alternative to bead functionalization due to its excellent mechanical strength and the advantages already mentioned in previous sections [233]. Graphene can be functionalized using surfactants and ionic liquids, which allows a better dispersion of such a material in aqueous or organic solution [14]. In particular, functionalized suspended graphene demonstrated good performances for enzymatic microreactors (Fig. 17a) [14]. Enzymes were immobilized on suspended graphene sheets for biocatalysis in microfluidics. To this end, immobilization of enzymes has been performed by adsorption from solution with the help of nanoparticles. Moreover, suspended graphene surfaces can also be functionalized with DNA aptamers by adsorption from solution (Fig. 17b). Due to the high quenching ability of graphene, the immobilization of a fluorescently-labeled DNA on the surface of such a material is particularly suited [15]. Fig. 17b shows a fluorescence sensor using graphene oxide as an energy acceptor for a system based on FRET [15]. The setup employed a microfluidic electrophoresis device integrating an optical sensor platform to detect the binding of aptamers/proteins. Graphene oxide was functionalized with fluorescently labeled ssDNA aptamers by non-covalent binding. Proteins were introduced afterward and bound to the aptamers present on the graphene surface, which affected the interaction between the aptamers and the graphene leading to the separation of the aptamer/protein complexes from the graphene surface and permitted their detection by fluorescence. This setup did not require sample pre-treatment or pre-labeling, and was highly sensitive with a low background noise due to the graphene oxide strong quenching ability. In Fig. 17c, graphene oxide was employed in a microfluidic device that combined a micromotor-based immunoassay and an electrochemical detection system to detect C-reactive protein [16]. Micromotors were fabricated in rGO and functionalized with streptavidin. EDC/NHS chemistry was used to activate the carboxyl-terminate of the graphene surfaces. The microreactors were incubated with a biotinylated antibodies solution and introduced afterward into a solution containing C-reactive protein analytes. Analytes were captured and detection antibodies bound directly in flow. Microreactors were magnetically controlled in the system, which allowed the capture of the immuno-complex (antibodies, C-reactive protein, detection antibodies).

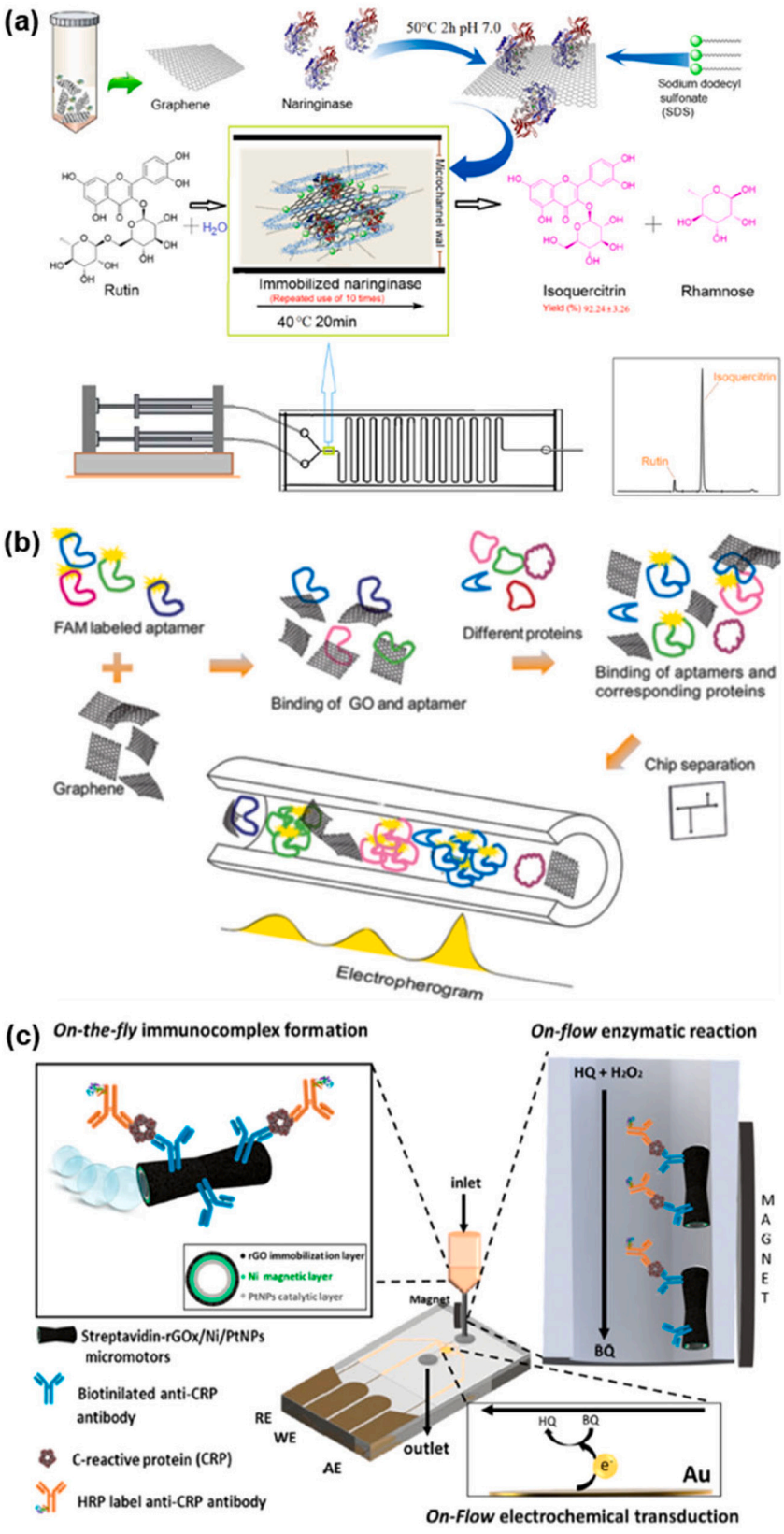

Fig. 17. Immobilization on a graphene surface. (a) Enzyme immobilization on mobile and suspended graphene sheets for microfluidic biocatalysis applications. (b) Illustration of a microfluidic chip electrophoresis system integrating a parallel optical sensor platform for the detection of label-free protein by binding of aptamer on a graphene surface. (c) Electrochemical microfluidic chip used to detect C-reactive protein using streptavidin-rGOx/Ni/PtNPs microreactors. (a) Adapted from [14], (b) adapted from [15], and (c) adapted from [16].

This automated system enabled an accurate and fast (8 min) determination of C-reactive protein using a low volume of sample (10 μL).

5. Patterning-based receptor immobilization

Throughout this review, we described methods for immobilizing receptors and biomolecules on various surfaces using different techniques. In this last section we will detail immobilization techniques from

a technological point of view. We will introduce available wet-based techniques for integrating linkers and biomolecules on functionalized surfaces and then focus on dry-processes for immobilizing receptors, such as plasma processing, plasma immersion ion implantation and laser processing.

5.1. Wet-based techniques for reagents integration

There are several methods available for the integration of reagents on modified surfaces using wet-based techniques. Two main approaches are available and can be classified as "contact printing" and "non-contact printing". Among contact printing, "pin printing" is a reliable, but slow technique, which relies on the surface tension of a liquid to transfer, and the wettability of that liquid on the substrate [234]. A high-throughput alternative technique is microstamping, which allows hundreds of spots to be printed in parallel. This technique is simple and inexpensive and often used to fabricate microarrays. In order to achieve a higher spot density, nano-tips and an atomic force microscope set-up can be used to precisely deposit a sample to the substrate. Non-contact printing techniques allow higher throughput and can reduce contamination. Examples of non-contact printing techniques are photochemistry-based printing, electro-printing, droplet dispensing, such as inkjet printing and electrospray deposition, and laser writing. These techniques are described in details in the review from Barbulovic-Nad *et al.* [234].

5.2. Plasma processing

Among dry processes, plasma is a key technology that is frequently used for surface modification. Plasma is formed by energizing atoms and molecules sufficiently to form charged species and radicals [235]. The generated ions can be accelerated towards a substrate to modify the physical, chemical and morphological properties of its surface. Plasma processing can be used to enhance protein adsorption on various surfaces. Research groups proposed various methods to change the properties of surfaces and control protein adsorption on these surfaces with spatial control [236]. Oehr [237] showed the general trend of having less protein adsorption on more hydrophilic surfaces. Holländer *et al.* were able to create acidic groups (sulfonic acid, hydrogel sulfates) on polyethylene, poly(propylene) and polyester surfaces using SO_2-containing plasmas. They also showed how to further process the acidic groups with diaminoethane in order to produce primary amines on the surface [238]. Holländer *et al.* also showed in another publication that low-pressure oxygen plasma can be used for activating polymer surfaces produced by additive manufacturing technologies [239]. Penache *et al.* introduced a low-cost method, which they refer to as "plasma printing", in order to selectively functionalize polymeric surfaces. They use dielectric barrier discharges at atmospheric pressure to change locally the surface chemical composition and the related physical-chemical properties of the surface [240]. Bayati *et al.* in 2009 proposed a method for high-density patterning of proteins on silicon substrates. They first patterned SiO_2 or Si_3N_4 on a silicon substrate followed by c-C_4F_8 plasma treatment to etch SiO_2 and Si_3N_4 while creating a hydrophobic fluorocarbon film on the silicon. They showed that proteins would adsorb mostly on the etched SiO_2 or Si_3N_4 areas [241]. In 2012 Malainou *et al.* showed a method for patterning protein microarrays on commercially-available glass slides. First a photoresist is patterned using photolithography on a glass slide to define the areas for protein immobilization. Then plasma processing is used to enhance adsorption of proteins on the photoresist pattern [242]. Rucker *et al.* used reactive ion etching to generate microroughness on various polymer substrates and found that antibodies attached preferentially on etched PMMA, Zeonex, but as well on non-etched regions on PC surfaces. This suggested that the introduction of certain RIE-induced functional groups may play a critical role in the immobilization of proteins [243]. In 2012, Tsougeni *et al.* introduced a method for selectively controlling surface wettability and protein adsorption on a PMMA surface. They used O_2 deep reactive ion etching to create rough PMMA surfaces and then employed C_4F_8 plasma deposition to obtain superhydrophobic areas. They showed that protein adsorption significantly increased in the rough hydrophilic areas and was nearly absent in the superhydrophobic ones [244]. Using a similar method, Tsougeni *et al.* also showed an increased cell attachment on rough PMMA and no cell attachment on hydrophobic patches [245]. By introducing a thermal annealing step, these authors showed direct covalent immobilization of proteins on nanotextured PMMA surfaces and demonstrated an increased immunoassay sensitivity for C-reactive protein and salmonella lipopolysaccharides compared to a smooth, untreated surface [246,247]. Rough nanotextured PMMA surfaces generated by plasma processing were also used for DNA purification in a microfluidic channel [248,249].

5.3. Plasma immersion ion implantation

Plasma immersion ion implantation (PIII) uses high voltage pulsed DC to accelerate ions and target them into the desired substrate. Due to the higher energy employed, PIII allows to modify the substrate deeper than previously possible, opening new possibilities for longer-lasting surface modification and covalent binding capacity [250]. Bilek *et al.* reviewed methods to immobilize biomolecules on plasma modified surfaces without using chemical linkers [251] and showed a method that can make any surface hydrophilic and, at the same time, immobilize covalently biomolecules using an energetic ion-assisted plasma process. Hydrophilic surfaces do not induce denaturation of immobilized proteins, but show a lower protein binding affinity. To keep the advantage of a hydrophilic surface and allow covalent binding, Bilek *et al.* created free radicals from a source, which can migrate to the surface and allow covalent binding of biomolecules on the surface [252,253]. In 2017 Kondyurin *et al.* published a one-step method for the surface functionalization of a conductive polymer (polypyrrole), by activating it using PIII. They demonstrated covalent binding of proteins to the activated surface and claim no loss in electrical conductivity [254]. One year later, Wakelin *et al.* showed that treating polyether ether ketone (a material used for orthopedic implants) with PIII, allowed for the immobilization of tropoelastin, which improved osteoblast interactions [255]. Kosobrodova *et al.* used PIII to modify the inner surface of a bundle of hollow fibers enclosed in a cassette. They achieved a 40 nm-deep carbonization of the capillary surfaces, which increased wettability of the capillaries and allowed protein immobilization by covalent bonding [256]. Plasma-based processes have enhanced our capabilities to functionalize surfaces and there is still space for fundamental research to understand and master this technology, for example, to characterize the vertical stacking of different chemical structures generated by the different penetration depth of various components of plasma. Holländer highlighted some of the main challenges and how chemical derivatization can be useful to expand our understanding of functionalized surfaces [257].

5.4. Laser surface treatment

Another technique used for modifying the physical and chemical properties of a surface is laser treatment. Shah *et al.* described the use of laser treatment to optimize bone formation and osseointegration of metal implants. In particular, laser technology gives the ability to tune precisely the surface roughness of the medical implant device and adjust the thickness of oxide layer by increasing locally the temperature and control the reaction with ambient oxygen. This affects the osteoconductive behavior and favors tissue bonding [258]. In 2009 Hook *et al.* used laser ablation to selectively remove PEG from a coated allylamine plasma polymer (ALAPP) surface and create ALAPP wells, where cells could be seeded and would grow only inside these wells. This setup was demonstrated for transfected cell microarray applications [259]. Daskalova *et al.* illustrated how to improve cellular attachment and orientation by laser microstructuring. In particular, they studied the

effects on the physical, chemical and morphological properties of the chitosan and its ceramic composite blends, when treated with femtosecond laser pulses and showed an improved cellular adhesion on the treated surfaces [260].

6. Conclusion

The field of microfluidics has been offering more and more opportunities to perform analysis and research in chemistry, biomedicine and environmental sciences during the last 30 years. Since the early days of microfluidics, the immobilization of receptors in microfluidic devices was and still remains a challenge. An efficient immobilization of receptors can ensure assays to be performed with good sensitivity and specificity, and it is important that receptors, which are typically fragile biomolecules, conserve their conformation and activity during the immobilization process. In addition, receptors in microfluidics are typically located in microstructures, which are sealed during operation. For this reason, receptor immobilization is not just a matter of surface chemistry, functionalization and cross-linking biomolecules from solution to a surface, but it also needs to be thought of in the broader context of device fabrication and packaging. Mobile surfaces based on beads, or more recent materials such as graphene sheets, provide options for bringing receptors inside microfluidics post-fabrication. Many materials for device fabrication and commercially-available linkers and chemicals for surface modification can be used to facilitate receptor immobilization. Receptor immobilization does not require deep expertise in chemistry or biochemistry but instead requires a workflow that must include early on the receptor immobilization strategy during designing a specific device and planning its fabrication. The options and combinations of components for devising assays have exploded during the last years, with an ever-increasing number of concepts for binding analytes involving natural and synthetic compounds. For this reason, we hope that this review provided a detailed survey of most recent methods, which can be used for immobilizing receptors on various surfaces and that experts on microtechnology and biosensing can take advantage of these methods for realizing robust, high-performance microfluidic devices.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Y. Temiz from IBM and G. Gygli from KIT for discussions and T. Scharnweber (KIT) and H. Riel (IBM) for their continuous support. This work received funding from the European Union's Horizon 2020 research and innovation program under the grant agreement No. [764476]. Further permissions related to the material shown in Fig. 11d should be directed to the ACS.

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