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Biochips: non-conventional strategies for biosensing elements immobilization

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TABLE OF CONTENT

1. ABSTRACT

The present article draws a general picture of non-conventional methods for biomolecules immobilization. The technologies presented are based either on original solid supports or on innovative immobilization processes. Polydimethylsiloxane elastomer will be presented as a popular immobilization support within the biochip developer community. Electro-addressing of biomolecules at the surface of conducting biochips will appear to be an interesting alternative to immobilization processes based on surface functionalization. Finally, bead-assisted biomolecules immobilization will be presented as an open field of research for biochip developments.

2. INTRODUCTION

The development of biochips for the detection of nucleic acids, proteins or enzyme–substrate interactions has benefited from increasing interest over the last decade since those tools were found to provide researchers and diagnostic companies with rapid sample screenings (1, 2). Nevertheless, one of the main handicaps to the development of these systems appeared to be the lack of generic immobilization procedures of the biologically active compounds, resulting in the obligation to adapt the coating protocols to keep the immobilized biological molecules active. The early immobilization procedures on silicon and glass supports based on silane technology, still in use for protein and DNA immobilization (2-5), are now

in competition with: (i) the grafting on soft lithography material such as elastomeric polymer, (ii) electrodeposition of biomolecules on conducting surfaces, (iii) micro-contact printing and (iv) beads arraying techniques and particle lithography.

The present review will focus on the latest developments and strategies in the field of the biomolecules immobilization for biochips developments. All the methods considered as standard such as photolithography, self assembled monolayers deposition and glass surface functionalization will not be treated in the present paper since numerous reviews were already published on those subjects (6-9). The presented methods will be the up-to-date and innovative technologies developed for on-chip immobilization of biomolecules.

3. BIOMOLECULES IMMOBILISATION ON PDMS BIOCHIPS

3.1. PDMS properties

PDMS (polydimethylsiloxane) is a silicone elastomer widely used for biomedical applications like breast implants, ocular lenses, dentistry (10) and micro- or bio-engineering (11). Its chemical and physical properties are making the polymer very attractive since it is chemically inert, permeable to gases and few solvents, optically transparent, cheap, not toxic and easy to handle in standard laboratory conditions (12, 13). Facing the increasing interest in microarrays and microfluidic development (14), PDMS appears more and more to be a powerful material compared to silicon, glass and others immobilization supports (15). However, its applications remain limited because of its very hydrophobic behavior (contact angle with water is 117°) (16) and its bio-fouling tendency leading to non specific adsorption of proteins (17). In order to overcome these problems, several groups currently try to chemically modify PDMS surfaces or turn at their advantage the unique PDMS properties. Based on these surface modification studies, several biochip applications were described. PDMS polymerization is characterized by a hydrosilylation reaction between silane and vinyl residues in the presence of a platinum based catalyst (18) following the reaction:

$$R_3SiH + R'_3SiCH_2{=}CH_2 \rightarrow R_3SiCH_2CH_2SiR'_3$$

Once polymerized, PDMS reveals a very hydrophobic surface due to methyl groups beard by the Si atoms of the polymer chain, and this lack of functional groups restrains covalent immobilization of biomolecules. Oxygen plasma treatment is to date the most widespread technique used to convert the methyl groups into hydroxyl ones rendering the surface reactive and hydrophilic (19), but UV-light irradiation (at 184 nanometers and 254 nanometers) (20) or ozone production using a corona discharge have also been used (21). Nevertheless, this modification is only temporary since un-polymerized buried chain could migrate to the surface, leading to the recovery of the PDMS hydrophobic property (22). However, a very recent study shown that hydrophobic PDMS recovery could be avoided through washing of the polymerized PDMS with permeable solvents in order to remove the buried un-polymerized chains (23).

3.2. PDMS modifications

3.2.1. Ozone treatment for PDMS surface functionalization

Well established silanisation reaction is commonly used for glass or silicon functionalization (24) and involves Si-OH silanol groups and silanes residues. PDMS oxidation could create silanol groups and Diaz-Quijada (25) used this property to functionalize PDMS surfaces. They succeeded in oxidizing PDMS with ozone only, without the need of any photolysis. This process allowed a homogeneous surface oxidation avoiding at the same time surface alteration of PDMS. Indeed, it has been shown that UV light, corona discharge and oxygen plasma exposure produce microstructures and crack patterns on PDMS surface. In the Diaz-Quijada study, silanization of the oxidized PDMS with (3-aminopropyl)triethoxysilane (APTES) was performed to immobilize single stranded oligonucleotide modified at the 5'-end with an activated carboxylic acid group.

3.2.2. Plasma oxidation for PDMS surface functionalization

Jang and co-workers (26) developed an electrochemical immunoassay based on PDMS channels functionalization. In order to activate the inert surface, they exposed PDMS to an air plasma treatment, thus creating silanol groups at the PDMS-air interface. The silanol groups were then reacted with 7-octenyltri(chloro)silane to generate a vinyl residues monolayer (Figure 1). An oxidative solution converted the vinyl in carboxylate functions and finally a traditional carbodiimide based coupling chemistry was used to chemically graft an amine containing biotin residue. This procedure allows then the grafting of a far-off biotin on the PDMS surface leading to a better recognition by the streptavidin. Finally, avidin was incubated in the channels allowing subsequent grafting of biotinylated mouse type G immunoglobulins (IgG). Eight minutes were necessary to achieve the sampling, reaction with alkaline phosphatase labeled anti-mouse IgG and washing steps, and to achieve a detection limit of 0.02 femtomole. However, one has to take in account the number of steps, the time (190 min) and the numerous chemicals needed to achieve such PDMS surface functionalization.

3.2.3. PDMS oxidation by hydrogen peroxide

In a different approach, Sui and co-workers (27) developed an improved and convenient way to oxidize PDMS using an acidic hydrogen peroxide solution trough microfluidic channels. Following the oxidizing step, PDMS was silanized either by a PEG-silane derivative for non-specific protein adsorption or by an amino-silane derivative. The amino group was then activated by thiophosgen to obtain isothiocyanate-grafted PDMS microchannels (Figure 2), which were subjected to attachment reactions with different amino containing biomolecules such as tripeptide RGD, single stranded DNA and PSCA (Protein Sequence Comparative Analysis) proteins. The PDMS modification was shown to be stable

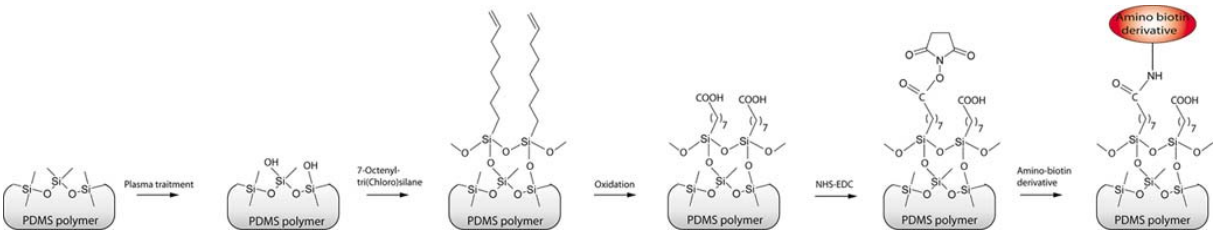

Figure 1. Schematic representation of the procedure for PDMS biochip surface functionalization with aminobiotin derivatives.

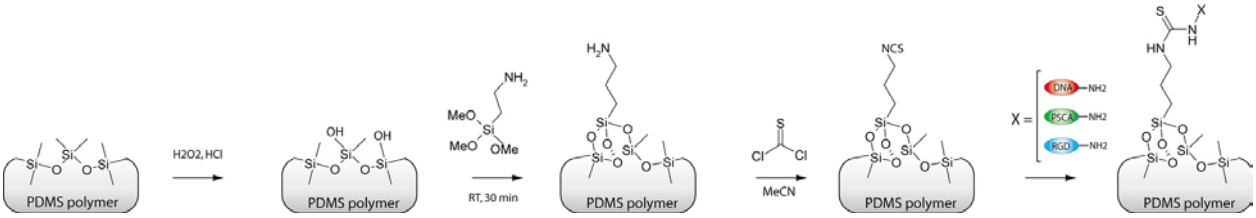

Figure 2. PDMS activation with thiophosgen for RGD peptide, DNA and PSCA protein immobilization.

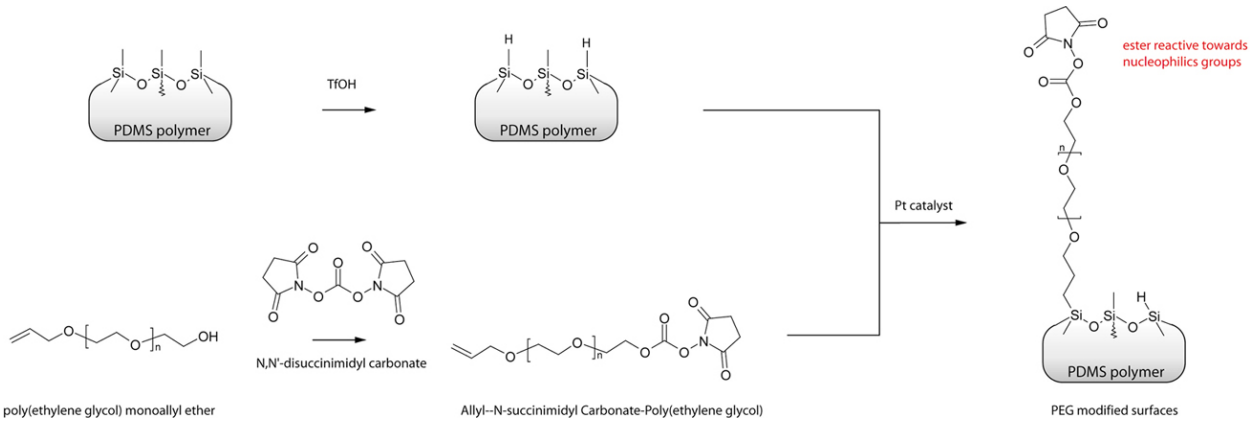

Figure 3. Overview of the strategy for PDMS biochip surface functionalization with PEG derivatives.

in time with no hydrophobic recovery and was successfully used for immunoassays, DNA hybridization and cell immobilization. This method has the particularity of avoiding radiation exposure or energetic beam commonly used to modify PDMS surface.

3.2.4. Lipid bilayer formation

Yang and co-workers (28) developed a heterogeneous microfluidic immunoassay based on solid supported bilayer containing dinitrophenyl (DNP)-conjugated lipids. The lipid bilayer was deposited on previously oxidized PDMS surface using oxygen plasma treatment and the lipid bilayer was created by vesicle fusion method. A linear array of channels was deposited and fluorescently labeled antibodies directed against DNP were flowed, giving a binding constant of 1.8microM for the antibody toward the immobilized antigen. This approach, taking advantage of the lateral diffusion properties of lipids, allows movements of the immobilized probe using the lipid bilayers properties. This method is particularly interesting for proteins which could suffer of unfolding when deposited using adsorption or covalent bonding on solid support.

3.2.5. Ester terminated silicones surfaces

Recently, a generic route has been proposed by Chen and co-workers (29) to functionalize PDMS containing hydrosilane residues (Figure 3). First, hydrosilane residues were created on polymerized PDMS surfaces using strong acidic conditions (triflic acid) in the presence of (MeOSiO)n. In parallel, alpha-Allyl-N-succinimidyl carbonate-poly(ethylene glycol) molecules were synthesized and reacted with the hydrosilane PDMS in the presence of little amount of Karstedt's Pt catalyst. Thus, covalent grafting was created between PDMS and PEG modified molecule which contained a free N-hydroxy-succinimide (NHS) group. The procedure allowed then the immobilization of PEG chains acting as a brush with its NHS groups pointing to the solution. Various biomolecules such as peptides for cell culture or proteins (mucin, heparin, lysozyme, human serum albumin and epidermal growth factor) were successfully immobilized on this PEG modified PDMS material.

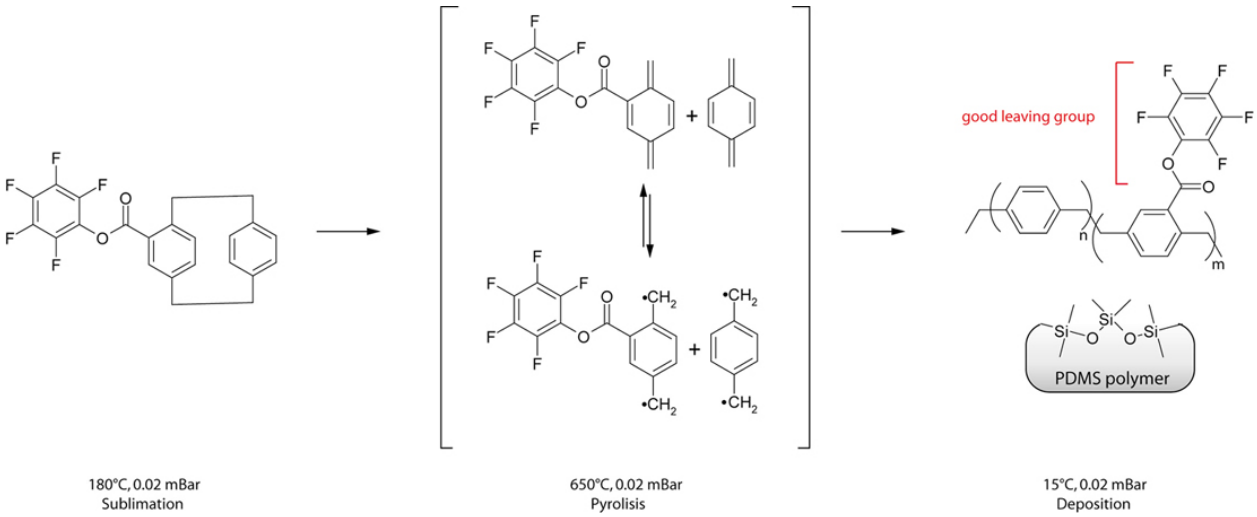

Figure 4. Chemical vapour deposition (CVP) of poly(p-xylylene carboxylic acid pentafluorophenolester-co-p-xylylene) on PDMS biochip surface.

3.2.6. Chemical vapour deposition enable

Lahann and co-workers (30, 31) used chemical vapor deposition (CVD) to deposit thin layers (90 nanometers to 150 nanometers) of poly(*p*-xylylene carboxylic acid pentafluorophenolester-*co*-*p*-xylylene) (PPX-PPF) on PDMS surfaces (Figure 4). First, PPF was sublimated at 180°C at reduce pressure and was then pyrolyzed at 600°C leading, with customized conditions, to the formation of p-quinodimethane. Afterwards, these reactive species were brought in a cold chamber (15°C) to polymerize homogeneously on PDMS surfaces. This technique has the advantage of being performed at room temperature without the need of additional catalyst, solvent or initiator, and to lead to a nanometer size PDMS modified layer. Furthermore, the polymer layer is insoluble in aqueous solutions or organic solvents. Using this strategy, the authors were able to coat PDMS-(PPX-PPF) with an amino-biotin derivative via an amide bond formation, followed by an incubation step with a streptavidin containing solution. As streptavidin can bind four biotins residues, two were believed to be linked with the functionalized surface, leaving two binding sites for biomolecules assembly. The authors validated their approach through cells adhesion on immobilized integrins for the development of on-chip cell-based bioassays.

3.2.7. Photo-linking

Brooks and co-workers (32) reported the use of a carbene generating photolinker to pattern biotin derivatives on various surfaces, including PDMS. A solution containing a biotin derivative bearing a (trifluoromethyl)diazarine function was deposited on a flat PDMS surface and carbene formation was generated using UV light exposure (254 nanometers). A particular feature of this experiment is the use of a dynamic confocal apparatus to create customized pattern of biotin by simply moving and focusing the UV light beam on un-activated PDMS surfaces. The authors didn't investigate the chemical modification involved in the biotin grafting onto PDMS substrate but proposed that the carbene function was inserted in C-H and C=C bonds of the hydrophobic chains of the polymer. This procedure created a homogeneous layer of biotin (6 nanometers thick) subsequently incubated with streptavidin. The system was efficient for the detection of biotinylated antibodies or fluorescent probes.

3.2.8. Photo-grafting

Using a photografting procedure, Wang and co-workers (33) investigated an efficient procedure to functionalize PDMS surfaces. Benzophenone used as a photoinitiator, was adsorbed and diffused in PDMS. These benzophenone groups were then used to graft polyacrylic acid (PAA) under UV-light exposition. The use of an exposure mask led to the achievement of localized grafting. After 25 minutes of UV-light illumination, the polymeric layer created (about 150 nanometers above the PDMS surface) allowed conventional carbodiimide amide bond formation with biomolecules containing free amino groups. As biochip applications, the authors performed on chip cells culture on immobilized ethylenediamine layers and immunoassays using anti-GFP antibodies covalently attached on PAA modified PDMS. Interestingly, no clean-room facilities were required for the achievement of the present PDMS surface patterning and the formation of the interpenetrating polymeric layer leads to a spatial resolution of 5 micron.

3.3. Immobilisation of biomolecules on un-modified PDMS surfaces

Considering the highly hydrophobic properties of PDMS, some biomolecules strongly adsorb on it by their hydrophobic domains. Taking advantages of this property, Etheshola and co-workers (34) have built the first heterogeneous immunoassay using bare PDMS surfaces coated with anti-sheep type M immunoglobulins (IgM) antibodies. Non-specific adsorption problem following the immobilization step was overcome by using an appropriate solution containing BSA (Bovine serum albumin), casein

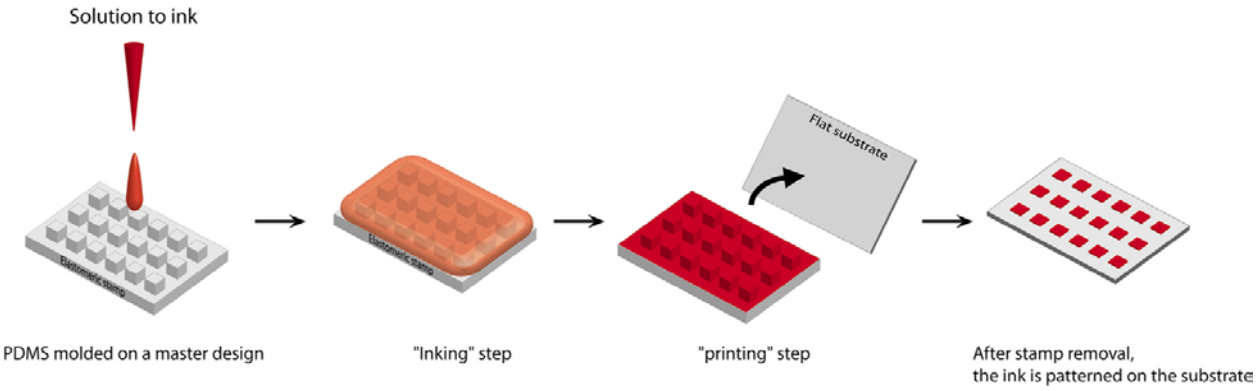

Figure 5. The four main steps for micro-contact printing preparation of biochips.

and Tween20. The sensitivity of the assay reached 425 femtomole of IgM in the PDMS modified channels. Using a similar approach, Linder and Verpoorte (35) used biotin-conjugated protein coating as the first layer for PDMS microchannels surface treatment. Neutravidin solution was then flowed through the system and reacted with the immobilized biotin to create a uniform layer which was functionalized with a second biotinylated antibody, thus creating a three layer sandwich surface. However, when an antigen solution was added to the system, some unwanted binding with the first antibody was detected, leading to interferences between the analyte and the coating. More recently, Cesaro-Tadic (36) used a microfluidic based immunoassay for the detection of tumor necrosis factor alpha (TNF-alpha) by simply coating the capture antibodies on a PDMS surface. The target protein was injected into the chip followed by fluorescently labeled antibodies and the detection limit reached was 0.02 femtomole.

3.3.1. Microcontact printing (microCP)

Considering the particular properties of PDMS, Whitesides group introduced in the early 1990's an original way to pattern biomolecules, using PDMS as a soft stamp (37, 38). This soft lithographical technique, called micro-contact printing (microCP), is simple, inexpensive and offers multiple possibilities to transfer monomolecular layers onto surfaces. Indeed, PDMS is the most widely used polymer for this application because it can be easily molded on a 3D master in classical laboratory conditions. Once peeled off, an "ink" containing the desired molecules is applied and incubated to the elastomeric stamp. The inked stamp is then subsequently brought into contact (i.e. printed) with a flat surface, enabling the transfer of the biomolecules to the biochip surface (39) (Figure 5).One of the main advantages of this technique is to pattern homogeneously large or small surface areas with biomolecules, particularly proteins, with very good spatial resolution (39). Unfortunately, microCP is also known to lead to proteins drying, and one have to consider that only proteins remaining active in a dry state, such as some antibodies (39) or resistant proteins, can be used with this method.

In the field of the well established DNA biochip, Thibault and co-workers (40) used this technology to adsorb oligonucleotides on a hydrophobic PDMS surfaces using microCP. They demonstrated the efficient DNA transfer from a PDMS stamp to flat substrate leading in an increase of the detection signal when compared to traditional pin spotted slides. MicroCP seems then to be a very promising procedure to address biomolecules but some limitations exist. Inking process tends to create protein diffusion at few micro-meter or nano-meter scale and one has to take in account some stamp deformation during the process using low scale 3D features (40). Another phenomenon pointed out is the siloxane contamination (41, 42) of the surface during the printing step. Indeed, it is well known that PDMS exhibits a dynamic behavior related to un-polymerized buried PDMS chain migration from the bulk to the surface (19) and, very recently, Asberg and co-workers (42) were able to assess the conformational states of different printed biomolecules (a synthetic peptide, calmodulin and the horseradish peroxidase).

3.3.2. Affinity contact printing (alphaCP)

An original way to use microCP is to functionalize the PDMS stamps with antibodies or other affinity proteins. Thus, immersing the stamp in a complex solution enables the selective extraction of antigens and their patterning on a dedicated surface. Bernard and co-workers (43) were the first to develop this technique by chemically grafting proteins on a previously activated PDMS stamp with a hetero-bifunctional crosslinker (oxygen plasma oxidation followed by silanization). In this particular case, stamps modified with NgCAM (a protein responsible for neural cell adhesion) specific antibodies were used to extract the protein from a culture medium and print it to generate a cell growth pattern.

More recently, Renault and co-workers (44) demonstrated the possibility to use alphaCP to create proteins microarray with resolution of the immobilized protein patterns of few micrometers. The authors also introduced the concept of microfluidics networks (microFN), exhibiting high resolution pattern of 3 x 3 micron. This technique allows an easy reproduction of the target array from the initial α-stamp which represents the critical step.

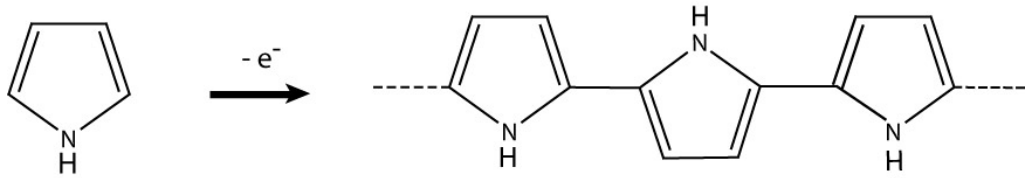

Figure 6. Polymerization of pyrrole through electro-oxidation process.

3.3.3. Patterning functionalized lipid-bilayer membranes

Supported lipid bilayers are useful to study dynamic processes between proteins and lipids and Hovis and co-workers (45) introduced two methods for patterning fluid lipid bilayers based on microCP. In the first one, named blotting, a lipid bilayer is created on a flat surface by the vesicle fusion method and used to ink a PDMS stamp. The second one, called stamping, implies lipid bilayer formation directly on the hydrophobic surface of the PDMS stamp. The modified stamp is then bring into contact with a glass slide, creating a lipid bilayer pattern.

Kung and co-workers (46) created complex surfaces containing both lipid bilayers and protein layers. Using microCP of proteins with a particular pattern on a glass slide, the authors were able to fill the areas leaved unmodified with lipid bilayers. The resolution of the patterned features between proteins and lipids can be scaled down to one micrometer depending of the stamp pattern resolution but one of the main advantages of this technique is to restrict the natural tendency of lipids to diffuse, through the use of protein layers as barriers.

With the same approach, Philips and Cheng (47) used phosphatidylcholine membranes containing receptors specific of cholera toxin to create a heterogeneous immunoassay on oxidized PDMS surface. Vesicles carrying the receptor were fused on the polymer surface and the so designed biochip succeeded in the detection of 210 picoM of cholera toxin. These results showed how lipid deposition can be an efficient way to functionalize PDMS surfaces since proteins with appropriate domains can be inserted inside the bilayer and used for biochip fabrication (48).

4. BIOMOLECULES IMMOBILISATION THROUGH ON-BIOCHIP ELECTROCHEMISTRY

4.1. Conductive electro-polymers

The electro-addressing of biomolecules is a modern objective to create new kinds of immobilization strategy for biochips. This wish responds to the ever-increasing demand of new efficient technologies for biochip development. The electro-chemical addressing of biomolecules intends to take advantage of direct, specific and spatially controlled way of immobilization, thus reducing time and cost of biochip production. Compared to other classical immobilization methods which necessitate a chain of chemical reactions and/or further consecutive technical steps, the electro-addressing of biomolecules can be considered as a direct method. Fundamentally, the method requires the application of a difference of potential, using a basic electrochemical setup, to achieve the localized fixation of the biomolecules, i.e. the "addressing". The technical advantage seek is to avoid the use of mechanical apparatus such as solution deposition systems. Nevertheless, the biochips useful for electro-addressing will have to be composed of either a matrix of electrodes or at least a conductive material.

Historically, biomolecules immobilization via electro-addressing is considered as a recent technology which emerged with the conductive electro-polymer researches. Innovations in the field started in 1979 (49), with the demonstration and the study of the electro-polymerization of pyrrole at a platinum electrode surface. Polypyrrole films prepared under controlled electrochemical environment were shown to be interesting materials with improved conductivity, strong adhesion to the metal surface and good stability under electrochemical conditions. The conducting property of these polymers is due to the strong electron mobility related to the regular conjugation of C=C bonds inside the polymer structure (Figure 6). Years after years, numerous electrochemical techniques such as cyclic voltammetry, potential step experiments and coulometry were used to investigate the electrodeposition of polypyrrole films from acidic, neutral and basic aqueous or organic solutions (50).

The first immobilization procedure experimented for biosensing was realized through the electro-polymerization of pyrrole, simply used to entrapped biomolecules during the polymerization process (Figure 7-a). The technique used the ability of pyrrole and/or its derivatives to form insoluble films attached on the electrode surface. Thus, Umana and Waller (51) reported a protein-modified glassy carbon electrode based biosensor, using glucose oxidase entrapped in a polypyrrole film available for electrochemical detection of glucose. At this point two major classes of electro-addressing immobilization using electropolymers have to be distinguished: (i) the entrapment of biomolecules during the electro-polymerization process which was shown to be efficient with high molecular weight molecules, (ii) the co-polymerization of single unit monomers together with monomer units functionalized biomolecules, which seems to be more efficient with small biomolecules such as oligonucleotides and peptides (Figure 7b).

4.1.1. On-chip electro-entrapment of biomolecules

On the model of the entrapment, oxidase enzymes were immobilized by electro-polymerization into conductive polymers films of polyaniline, polyindole, polypyrrole and poly(o-phenyldiamine) (52). The kinetic

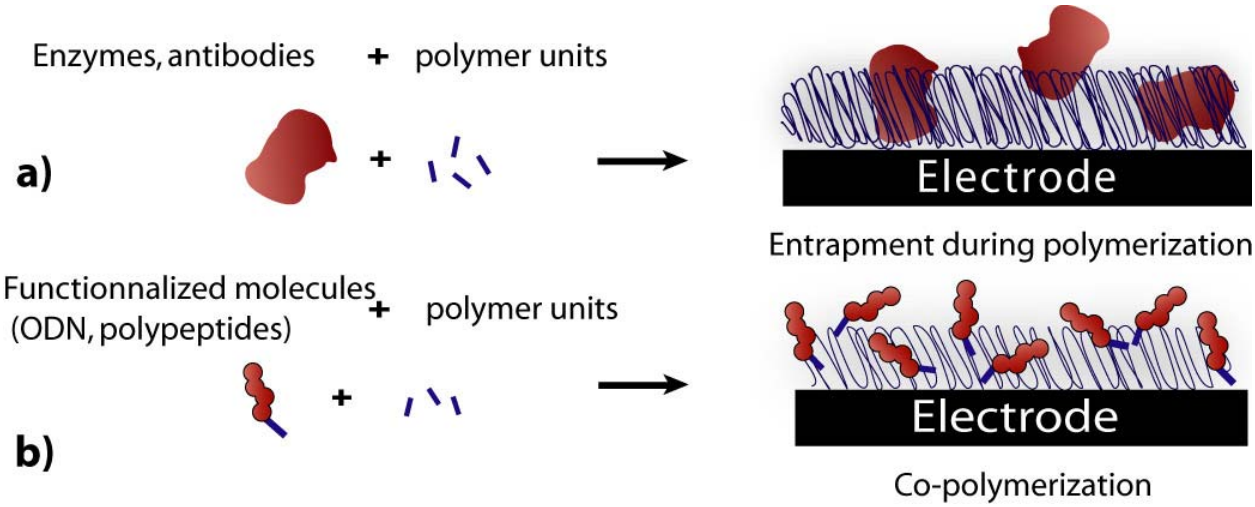

Figure 7. a) Immobilization via entrapment of biomolecules during the electro-polymerization process at the electrode surface. b) Immobilization via co-electro-polymerization of monomer units and monomer-functionalized biomolecules at the electrode surface.

and the behavior of the entrapped enzyme toward temperature, solvent and pH were studied. All the bio-films tested evidenced a clear decrease of the current generated in the presence of electro-active interfering species. This behavior was attributed to the permselective effect of the polymer. Other enzyme types were successfully immobilized using this approach, extending the usefulness of the technology to a wide variety of molecules. For example, Bekir Yildiz and co-workers (53) have shown that the electro-addressing of invertase through the electro-polymerization of poly(pyrrole)/PMMA-*co*-PMMT (polymethyl methacrylate-*co*-polymethyl thienyl methacrylate) matrices was possible. Results shown that the enzyme was kept active, and that the biosensor offered great measurement stability over time (40 measures in 1 day). In a similar way, Vedrine and co-workers (54) immobilized a tyrosynase in an electrogenerated polythiophene film at the surface of a glassy carbon electrode. It was then shown that the electro-polymerized film provided an optimal response of the biosensor. The amperometric enzyme sensor generated was used to detect toxic mono- and di-phenols but also herbicides through the inhibition of the tyrosinase activity. In addition to immobilized enzyme, the electro-polymerization entrapment technique was described to generate immunosensors.

A one-step entrapment of type G immunoglobulins (IgG) during the polymerization of pyrrole was shown to generate a sensitive immunosensor at the surface of a glassy carbon electrode biochip (55). Following the immobilization, impedance measurements were used to provide a labelless method to detect antibodies/antigens interactions. Recently, Shi and co-workers (56) also entrapped antibodies in polypyrrole films during electro-polymerization, leading to a specific electro-addressing on interdigitated micro-electrodes array. Antigens in sera samples were detected through cyclic-voltammetry experiments in $Fe(CN)_6^{3-/4-}$. This chip based immunoassay offered high sensitivity and good specificity against antigens for testing in human sera samples.

4.1.2. On-chip co-polymerization of biomolecules

The second class of electro-polymer immobilization is based on the use of biomolecules functionalized with monomer and co-electro-polymerized with free monomer (Figure 7b). This co-polymerization procedure was mainly used to immobilize small molecular-weigh biomolecules.

In this way, Garnier and co-workers (57) have built a peptide functionalized conductive polymer layer. Monomers were prepared on a pyrrole basis, allowing the co-electro-polymerization. The sensing layers formed permitted the selective recognition of the immobilized peptides by the carboxypeptidase A. Using the same co-polymerization addressing approach, biotin derivative was electro-addressed in polydicarbazole film at the surface of a glassy carbon electrode (58). A complex of avidin-polyphenol oxidase was used to immobilize the enzyme which enabled the detection of L- and D-norepinephrines. Microarrays composed of 50 micron gold electrodes were used as support for oligonucleotides/pyrrole electro-addressing, via the co-electro-polymerization of single pyrrole molecules together with oligonucleotides-5'-pyrryole modified. The oligonucleotides biochips realized were successfully tested for the genotyping of hepatitis C virus in blood samples (59). The immobilization via direct electro-addressing on electrode microarray was also demonstrated for the achievement of a nucleic acid biochip. The system was used to detect nucleic acid gene mutation (60). Peptide biochips were developed based on this pyrrole co-polymerization technology. Immobilized peptides on micro-electrode arrays were then available for the immunodetection of specific antibodies (61).

Interesting applications of the pyrrole co-polymerization procedure were developed on surface plasmon resonance imaging gold substrates. The co-

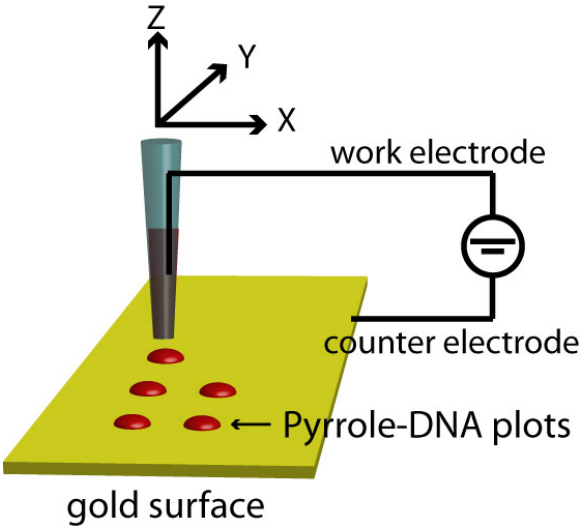

Figure 8. Arraying using the electro-spotting method onto gold plated SPRi biochip.

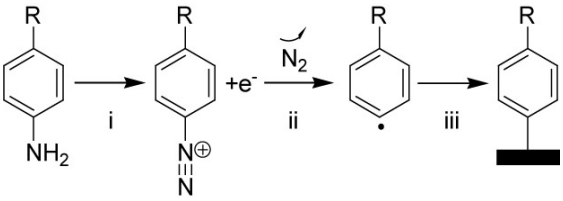

Figure 9. Reaction sequence for the electro-grafting of aryl-diazonium molecules.

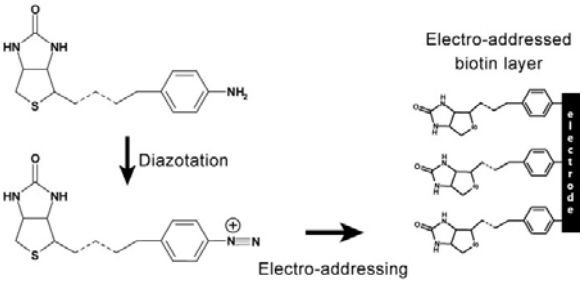

Figure 10. Strategy for creating biotin layer covalently attached to an electrode surface

polymerization of biomolecule-pyrrole adducts and pyrrole units was used to generate spots through an electro-spotting procedure (Figure 8) (62). This strategy was shown to be successful for the direct monitoring of DNA interactions through SPRi measurement (63). The same method was extended to the co-polymerization of pyrrole and pyrrole-modified proteins in order to address antibodies at surface of the gold chip. This approach enabled the real time observation of interactions between antibody and antigens (64). Further sophisticated immobilization strategies were studied for protein electro-addressing on screen-printed microarray, involving different reactive conjugates. Therefore, HIV-1 P24 capsid protein modified with pyrrole monomers was immobilized through co-polymerization (65). Various pyrrole-protein complexes were tested for electro-addressing, notably protein fixed on (maleic-anhydride-*alt*-methyl vinyl ether). This latter was shown to provide the most accessible immobilized protein.

Electro-polymerized films sensing layers were then evidenced as really versatile and powerful tools which could be used for numerous analytical systems such as optical or electrochemical, immuno- and enzyme-based sensors. However their involvement for biochip mass arraying and detection are not yet fully exploited.

4.2. Diazonium salt based electro-addressing

The biomolecules electro-addressing based on the diazonium electro-reduction is a recent technology, taking advantage of the electrochemical grafting possibilities of aryl-diazonium residues. Figure 9 presents the reaction sequence involved: (i) an aryl-amine residue is diazotated in presence of $NaNO_2$ and HCl giving an aryl-diazonium, (ii) this latter is electro-reduced to an aryl-radical which (iii) reacts with the electrode material surface. The radical generated attacks the surface and forms a C-X bond; where X is the electrode material, namely Au, Cu, C (carbon or diamond) or Si (66-74).

The use of this reactions was first described on carbon material surface (75, 76), and was applied to the indirect covalent immobilization of an enzyme. A glassy carbon electrode surface was functionalized by the electro-reduction of 4-acetic-phenyl-diazonium, leading to a 4-phenyl-acetic layer. Then a chemical covalent grafting of the glucose oxidase was performed to generate a glucose sensitive electrode. Diazotation reaction on an aniline derivative was also used to create a biotin layer, electro-addressed at a screen-printed carbon electrode surface (Figure 10) (77). An aniline-biotin residue was diazotated and subsequently electro-grafted on the electrode surface, providing a covalent anchoring point for streptavidin.

Recently, our team has developed a real one-step electro-addressing immobilization strategy using aryl-diazonium modified biomolecules (78). The overview of this electro-addressing strategy is presented in figure 11. The initial model developed was an immunobiochip. Immunoglobulins were first functionalized with an aniline derivative through carbodiimide activation (Figure 11-i) leading to immunoglobulin-anilines adducts. The aryl-amine present on the biomolecule could then be transform in diazonium via a diazotation in the presence of HCl and $NaNO_2$ (Figure 11-ii). Afterwards, the diazonium bearing immunoglobulins were shown to be successfully electro-addressed on specific electrodes of a screen-printed carbon microarray. The immuno-biochip obtained using this novel approach enabled the specific detection of anti-rabbit IgG with a detection limit of 50 femtomole of protein. The immobilization strategy via diazonium-protein electro-addressing was also shown to provide an excellent spatial specificity of the immobilization on the screen-printed carbon microarrays.

This method was successfully extended to the detection of rheumatoid factor (RF, which is a group of

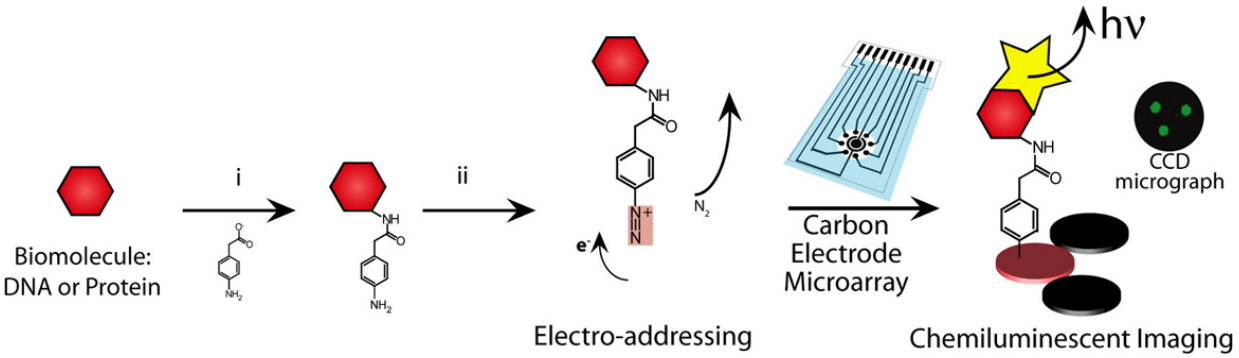

Figure 11. Biomolecules electro-addressing strategy onto screen-printed electrochemical biochips.

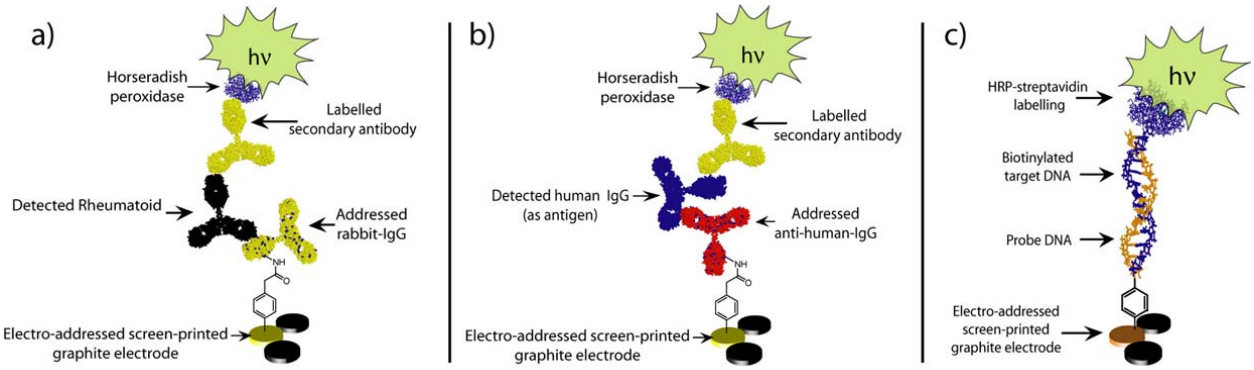

Figure 12. Schematic representations of different biochip models using the electro-addressed immobilization of (a) an immunoglobulin antigen, (b) an active anti-human antibody and (c) a probe DNA sequence.

antibodies) in human sera samples (79). A capture type immunoassay was then realized (Figure 12-a) where rabbit IgG were electro-addressed on the carbon electrodes of a screen-printed microarray and were recognized by RF antibodies present in the serum samples. It was also demonstrated that electro-grafted immunoglobulins could be used as recognition element and were then keeping their fully binding ability (Figure 12-b). The functionalization protocol and the electro-addressing technique were pushed forward in order to perform oligonucleotides grafting. A 20^{mer} sequence from a "hot spot" of the exon 8 of the p53 tumor suppressor gene was functionalized with 4-aminobenzylamine, electro-addressed and used as stationary phase probe sequence for hybridization testing of biotinylated target sequence (Figure 12-c). As for the protein biochips, the system was shown to provide a strong specific signal, only due the specific recognition of immobilized biomolecules.

This biomolecule electro-addressing technology appears to be promising, taking advantage of simple, direct and covalent immobilization of biomolecules. Up to now, this technology was demonstrated on carbon material surfaces, but may be extended to a wide variety of conducting materials, such as iron (67, 71), platinum, cobalt, nickel, zinc, copper (70), gold (80, 81), indium thin oxide (82) and silicon (68).

5. BIOMOLECULES IMMOBILIZATION THROUGH BEADS

Micro- and nano-beads have become a major tool in analytical chemistry sciences. Indeed, micrometer size beads were developed with a very large range of properties: magnetic and/or fluorescent beads, having different surface functional groups for coupling chemistry or having different physicochemical properties.

Biomolecule immobilization on beads, when compared to a flat surface, presents the advantage of generating higher specific surfaces. Those textured surfaces obtained were shown to improve the sensitivity of the developed assays (83). Three different systems could be distinguished according to the organization of the beads: bead arrays, composed of highly organized beads; bead biochips, using beads as immobilization support without precise positioning of each bead; and the beads in microfluidic networks, characterized by a possible displacement of the beads.

5.1. Bead arrays

Arraying beads in a highly ordered manner requires the development of a bead localization system. This technical difficulty has been overcome by different groups through the formation of physical traps as illustrated in figure 13. The beads are in those cases of micrometer

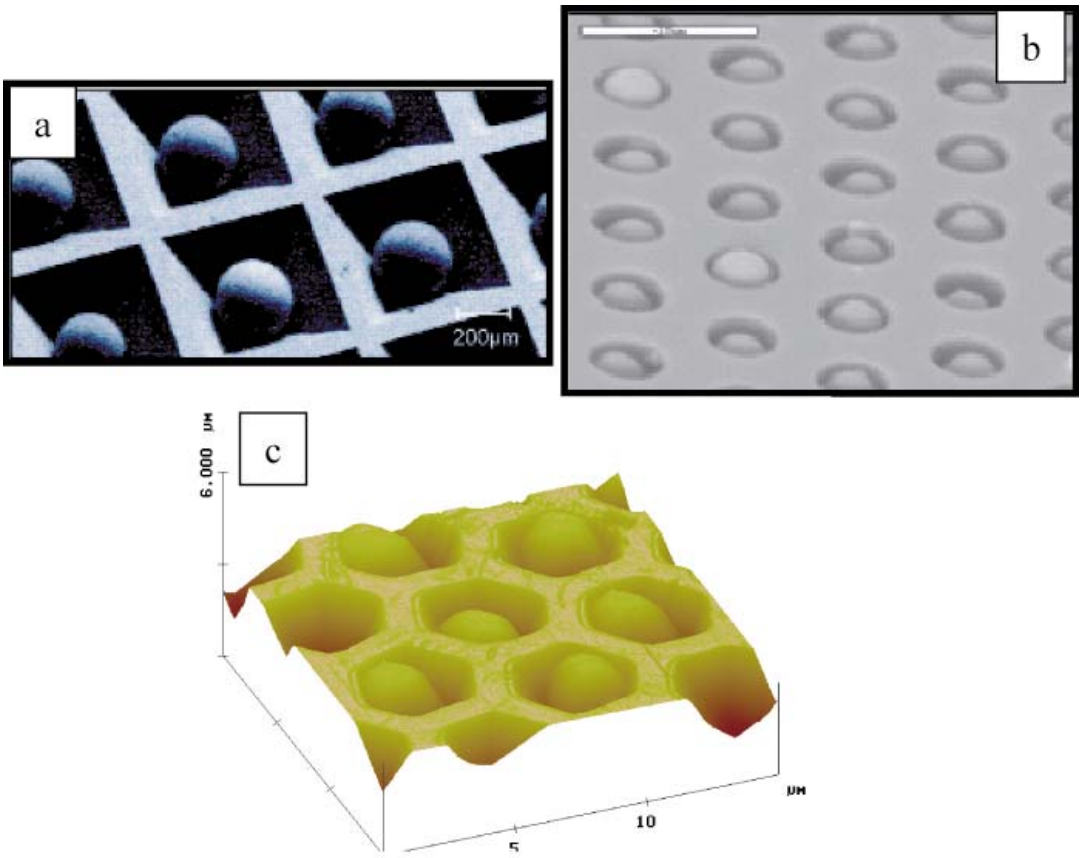

Figure 13. Scanning electron microscopy (SEM) images showing a: multiple well pits used to confine the sensor beads. b: etched fiber optic end filled with 3 micron beads. c: etched array housing 2 micron bead (from Illumina®).

size (2-5 micron). Such traps were obtained by chemical etching (hydrofluoric acid) of a silicon wafer (84), or imaging fiber optic bundles (85-87). A precise control of the basin size enabled a perfect matching of the beads in their traps. Moreover, as presented in figure 14-a, such traps could have a pyramidal shape and be open at both ends, leaving the solutions flowing from one side of the chip to the other, and then bringing the beads in a constant reagent flow. This last type of chip is therefore an interesting approach to obtain systems with high mass transfer and a high hybridization rate.

DNA and other biomolecules immobilization chemistries on the beads used in these systems are resumed in figure 14. Two main techniques were used, a direct immobilization through primary amino groups (Figure 14-a), or an affinity procedure using avidin modified beads and biotinylated biomolecules (Figure 14-b). In the second case, immobilization chemistry is required to graft avidin on the beads. When using agarose beads, the microspheres were purchased as terminated with aldehyde groups to which proteins could be linked via reductive amination (Figure 14-b). Another possibility is to use commercially available neutravidin coated beads (88). This affinity immobilization system enables, in the case of oligonucleotide immobilization, the achievement of perfectly orientated probes since the biotin is introduced, during the nucleic acid synthesis, only at one end of the sequence.

One major handicap of these bead arraying systems is that usually no physical addressing of the beads in a particular trap can be achieved; these ones are then randomly self organized in the traps. Currently, two methods co-exist to determine the position of the probe grafted beads on a self-assembled bead arrays. The first method requires fluorescent dye encoding of the microspheres, where each microsphere is labeled with a unique ratio of dyes in order to identify the attached probe sequence (89). The second method is based on the use of addressing oligonucleotide sequences co-immobilized on each bead with the sequence of interest. The microsphere positions were then determined by hybridization to a series of fluorescent complements (85, 90).

Both approaches present limitations, since on one hand the number of unique, distinguishable optical signatures that can be prepared with a fluorescent dye are limited, and on the other hand, decoding the array with the hybridization method could be time consuming and dependent on the addressed sequence quality. A solution to cope with this problem is to use beads with a larger diameter (230 micron) in order to permit their manipulation with a micro-manipulator (91). Each particular bead could

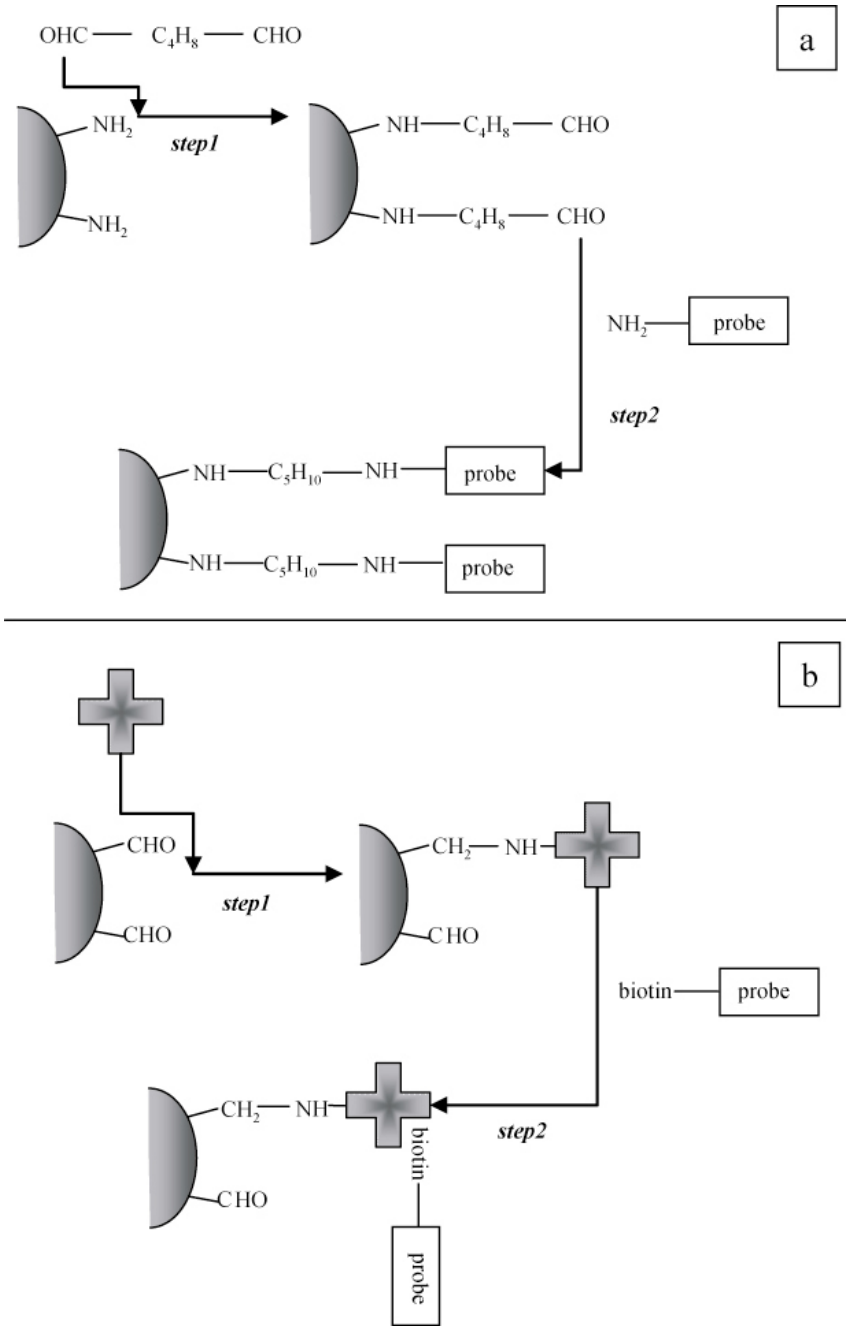

Figure 14. Probe immobilization onto a: amino modified beads, b: after the immobilization of avidin via reductive amination onto Sepharose beads (3 micron).

then be placed at a precise location of the etched array. Nevertheless, at the present time, only low density bead arrays (4*3 array) could be physically addressed simultaneously, since the procedure is found to be time consuming.

A last addressing system named LEAPS (for Light-controlled Electrokinetic Assembly of Particles near Surface), has been described by Seul and co-workers (88, 92) and appears to be more powerful. It is based on the concomitant use of (i) an in situ generated electric field, (ii) a computer generated illumination pattern and (iii) interfacial patterning. An electric field is generated at a silicon substrate surface patterned with etched traps. Beads present in this field will assemble into a cluster and are subjected to be retained in traps. The illumination of the silicon wafer enables the local modulation of the electrical properties of the semiconductor, leading to a possible addressing of the beads in particular traps.

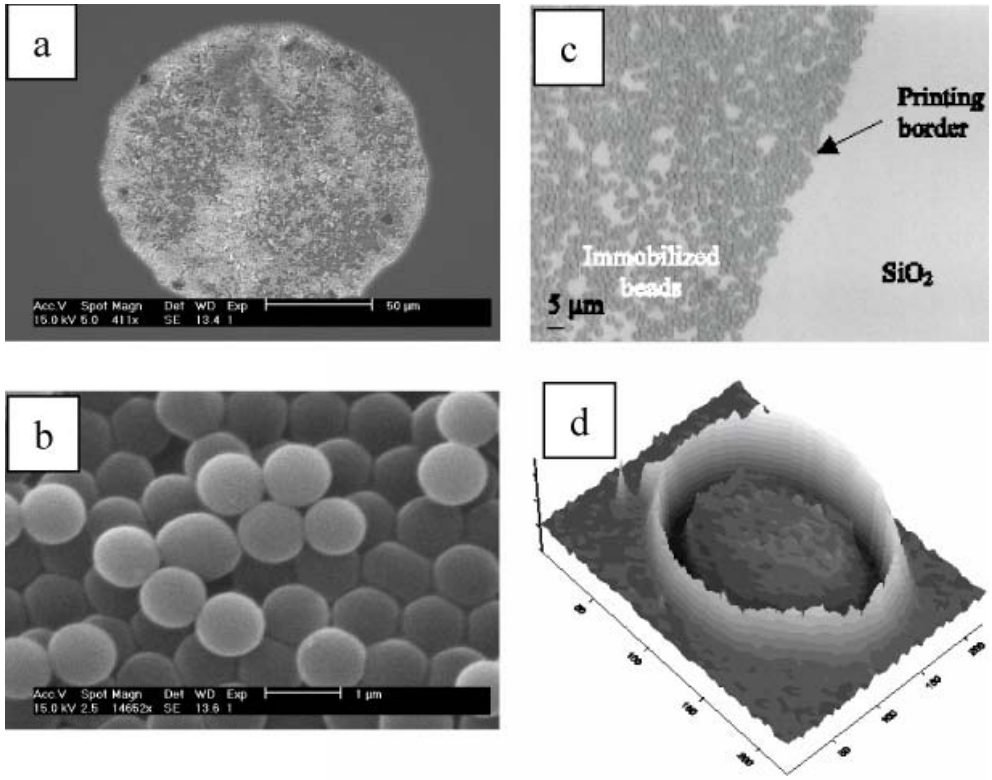

Figure 15. Scanning electron microscopy (SEM) images of a: a 150 micron diameter latex beads spot, b: a closer view of the latex beads arrangement within a spot c: immobilized streptavidine-coated beads (2.8 micron) on a biotin modified surface, d: 3D representation of the SEM image of a Sepharose bead trapped at the PDMS / air interface.

All those systems, based on beads arraying and using optical detection are characterized by a very low nucleic acid target detection limit with a lowest value at 1 zeptomole (86). The bead arrays are therefore interesting tools in terms of integration and miniaturization. Indeed, having sensing elements localized in a micron size sphere could lead to the achievement of highly integrated systems. Nevertheless, the integration degree appeared at the present time to be proportional to the technical difficulty degree.

5.2. Bead biochips

Bead biochips are characterized by a non-ordered relative position of each bead. The microspheres are in this case used to increase the specific surface of the support and to facilitate either the immobilization chemistry or the handling of the biomolecules. Two main categories of bead biochips could be distinguish associated to the use of the beads in an immobilized state or in a homogeneous phase.

5.2.1. Immobilized beads

Systems using immobilized beads bearing nucleic acid sequences are suffering from a lack of technical possibility to immobilize the beads at the surface of a solid support. Indeed, only a few examples are found in the literature which propose such analytical systems. The electron microscopy images presented in figure 15-a and 15-c illustrate two possibilities of arraying populations of identical beads at the surface of a biochip (93, 94, 96). Figure 15-c shows the organization of immobilized 2.8 micron sized beads at a biotin modified surface. The surface was previously modified by micro-contact printing with a biotinylated protein that reacted subsequently with streptavidine grafted beads to generate a self assembled and self sorted array. In a similar way, modifying the surface by microcontact printing with particular chemical functions (anhydride) immobilization of hydroxy- or amino-functionalized beads was enabled (94). Therefore, using beads bearing both the immobilization function (biotin or chemical) and the biomolecule of interest, led to the grafting of a high surface density of probes, generated by the surface enhancement obtained. Nevertheless, since these methods are using a chemical modification of the flat surface, the problem is then resumed to the classical addressed modification of a flat and homogeneous surface. The use of bead assisted biomolecule immobilization is in that case only useful to increase the specific immobilization area.

Another interesting bead immobilization technique is based on the entrapment of 1-100 micron sized microspheres at an elastomer (PDMS: polydimethyl siloxane)/air interface. Such a method enabled the immobilization of biomolecules bearing beads in an addressed manner (spotted) and with a high microsphere density, as could be seen in figure 15-b. Compared to the methods presented above, this system enables the use the bead assisted immobilization with a large scale of bead

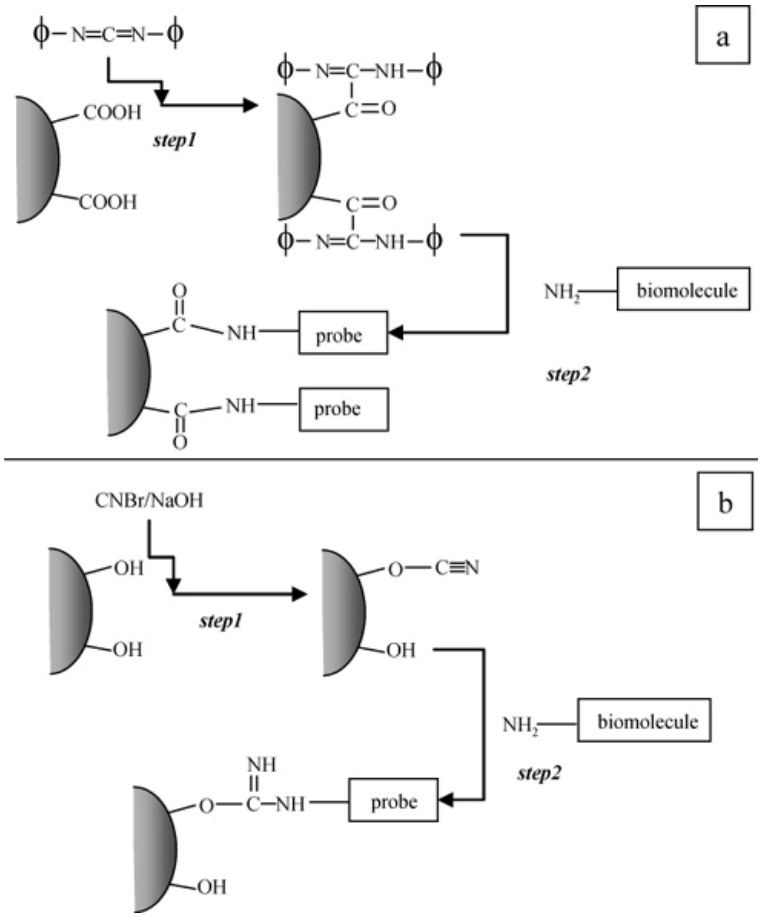

Figure 16. Biomolecule immobilization onto a: carboxylate modified latex beads (1 micron) via carbodiimide reaction and b: cyanogen bromide (CNBr) activated Sepharose beads (100 micron). (cyclic structure, e. g. dicyclohexylcarbodiimide).

coverage, since the bead surface chemistry is not used during bead immobilization.

Numerous analytical applications are described using this technique in particular for the study of point mutation in the codon 273 of the gene of the anti-cancer p53 protein (93) and the detection of anti-HIV capsid protein antibodies. In this example, 5'-amino-C6 probe sequences were immobilized via carbodiimide (dicyclohexylcarbodiimide) reaction onto carboxylate modified latex beads (1micron), prior to their immobilization (Figure 16-a). In another study, 5'-amino-C6 modified nucleic acid was immobilized onto cyanogen bromide activated Sepharose beads (Figure 16-b) (95). The 100 micron diameter beads were then subsequently transferred at the PDMS/air interface (Figure 15-d). Such porous polymeric beads have enabled a high enhancement of the specific surface since targets could be hybridized with the probes immobilized outside but also inside the Sepharose beads. Target detections (20mer sequences) with such systems were in the 0.1 picomole to 0.1 femtomole range.

Immobilized bead biochips are furthermore an interesting alternative to the dramatically complex bead arrays. Indeed, different populations of biomolecule-grafted beads could be easily immobilized at the surface of a solid support, leading to the achievement of easy to prepare, adjustable nucleic acid biochips.

5.2.2. Homogeneous phase bead biochips: suspension array technology

Biomolecules immobilized beads could be used in a non-heterogeneous phase to perform hybridization assays. The probe molecules are in this case grafted onto non-immobilized beads. Such systems present the advantage of being heterogeneous regarding the immobilized probes and to enable therefore the separation between hybridized and non-hybridized target. Moreover, bead suspensions could be approximated to homogeneous solutions, which could be turned into heterogeneous through the application of physical forces (gravity, magnetism).

Numerous studies on bead biochips were therefore based on magnetic (97, 98), glass or silica (99-101), and polystyrene beads (102). The biomolecule immobilization chemistry on those beads could be very different, from the classical avidin/biotin affinity reaction (Figure 14-b) to the disulfide bridging onto thiol modified

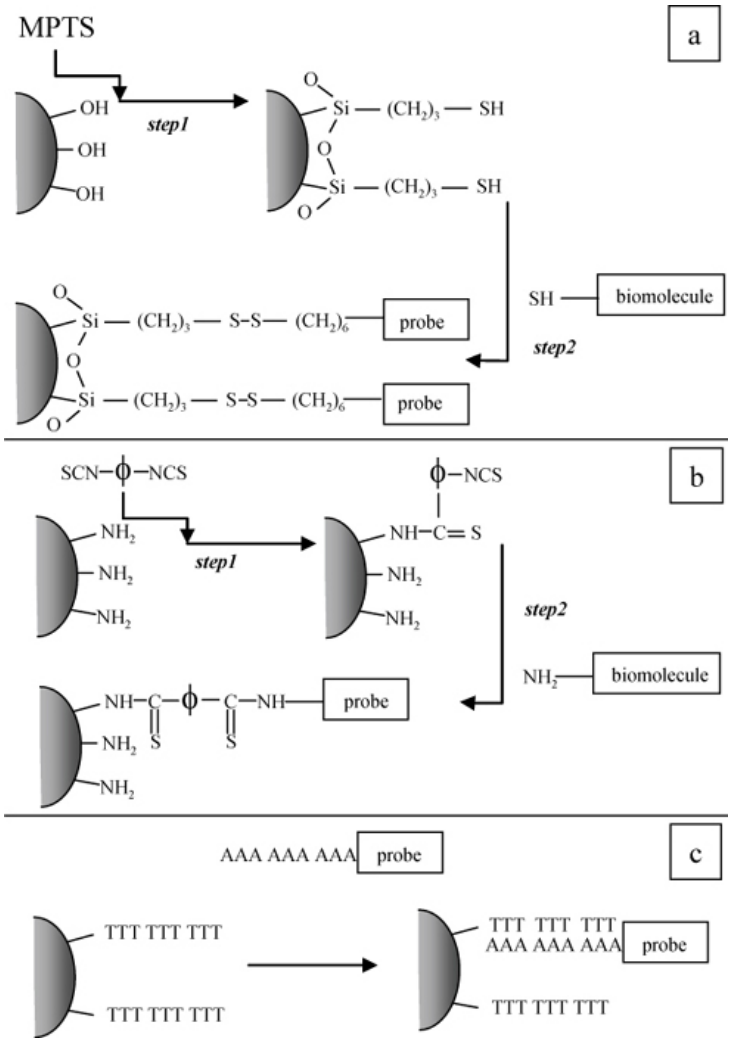

Figure 17. Biomolecule immobilization onto a: silica 60 nanometers particles, b: amino terminated 0.31 micron latex beads and c: poly(T) modified Dynabeads®. MPTS: 3-mercaptopropyltrimethoxysilane.

silica (100) (Figure 17-a), the thiocyanate reaction onto amino terminated latex beads (101) (Figure 17-b), and finally the hybridization based immobilization of poly(A) tagged probes onto poly(T) bearing magnetic beads (97) (Figure 17-c). A last possibility to obtain probes grafted beads is to directly synthesize oligonucleotide probes onto glass beads via phosphoramidite reaction (99). Hybridization onto those different homogeneous systems could be detected through a large range of detection methods such as fluorescence (100, 102, 103), fluorescence quenching (99) or stripping voltammetry (98). Interesting studies were also performed based on a suspension of beads in conjunction with flow cytometry measurements (104, 105). Flow cytometry, which was the standard methodology for cell population study during the last 20 years, has now begun to serve for in vitro microspheres analysis (106). Such systems were described as multiplex microsphere bead assays and were used to detect different nucleic acid sequences hybridized on beads having different properties (size, fluorescent label). Discriminating on one hand the bead type and on the other hand the hybridized sequence leads to a sensitive and high throughput technique with detection limits in the 10 femtomole range (105). Finally, as a prospective issue, flow cytometry is planned to have a potential throughput of nearly 300 thousand analyses per day, and to play an important role in genomics and proteomics (106).

5.3. Beads in micro-fluidic systems

Beads in microfluidic systems are an interesting evolution of the homogeneous phase beads biochips. Indeed, examples of microfluidic platforms in which biospecific molecules are immobilized on an internal channel surface are still rare (107). Bead based material is therefore a clearly viable alternative to introduce immobilized biological compounds in micro-channels. Microfluidic structures used to retain beads might then fulfill the dual purpose of holding back particles while allowing samples and reagents to be delivered. A first type of device is presented in figure 18 in which microspheres are confined either in a microchamber bordered by a "leaky" wall (Figure 18-a) (108) or by a pillar made wall

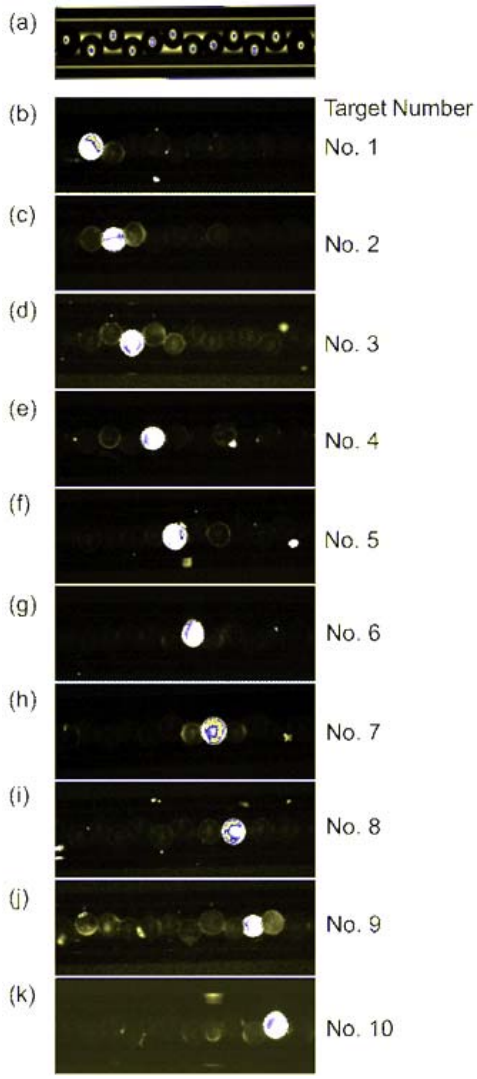

Figure18. a) Beads packing device working in a flow system, taking advantage of a "leaky" wall, b) A scanning electron microscopy image of a flow through reactor composed of pillar made walls used to pack microspheres.

(Figure 18-b) (109). Such systems were used to perform single nucleotide polymorphism (SNP) analysis of the codon 72 of the gene of the anti-cancer p53 protein.

In a similar way, magnetic beads supporting nucleic acid sequences could be retained in a microfluidic system by a magnet and act as supporting phase (110, 111). A good example is the study presented by Fan and co-workers (111) in which magnetic beads bearing different nucleic acid probes (up to eight) were magnetically packed in an eight channels microfluidic biochip. Hybridization was then taking place in a well adapted flow through format.

More organized systems were also described based on the alignment of beads bearing biomolecules in capillaries (112, 113). Such systems enabled the achievement of ordered beads as presented in figure 19-a. Different beads bearing different DNA probes could then be aligned in a capillary. In a typical experiment performed by Kambara and co-workers (113), 103 microns sized glass beads modified with DNA were arrayed in a capillary with an internal diameter of 150 microns. This bead handling required the development of bead alignment devices such as microchamber rotating cylinders or microvacuum tweezers, in order to manipulate and introduce the beads in the capillary in an ordered manner. The hybridization of the different probes immobilized on the different beads led to the achievement of the images presented in figure 19-b,k. Such systems therefore have a real potential in analytical development since beads with particular bio-specificity could be addressed and arrayed in a fluidic system which could be used to carry the different reagents and which could be read out optically.

6. CONCLUSION

The present review has proposed a selection of non-conventional methods to obtain biomolecules immobilization on biochip. As a matter of fact, each technology – i.e. PDMS, electrochemical and bead based immobilization – appeared to be linked to very special biochip designs. Indeed, these extremely particular methods, which were demonstrated as generic, were also highly dependant of (i) the use of a special polymer for the first one, (ii) the conductivity of the biochip support for the electro-immobilization and (iii) the possibility to handle bead for the last system. Thus, the current multiplication of coexisting powerful methods for biochip development leads to an interesting situation where one developed system could match one analytical need of the biomedical, safety, research or diagnostic field.

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