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Review

Advanced polymers for molecular recognition and sensing at the interface☆

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Abstract

In the few last years, the need of reliable, fast and inexpensive methods for selective analysis of specific substances in complex mixtures has grown exponentially. In particular, the detection of biomolecules, such as oligonucleotides, proteins, peptides and carbohydrates is of outstanding importance in gene expression, drug design and medicine studies. To these purposes, molecular recognition on microarray-configured devices is one of the most promising tools. This technology uses a number of different substrates such as glass, silicon, alumina or gold-coated slides. The use of polymers is a very effective way to tailor surface properties introducing functional groups able to bind biomolecules and prevent denaturation and non-specific binding. Furthermore, advanced polymers, thanks to their particular physico-chemical properties, can be used to improve selectivity and sensitivity during assays. This review will provide very recent examples of polymer-mediated molecular recognition between guest molecules in solution and host molecules located at the solid phase.

Keywords: Polymers; Molecular recognition; Surfaces; Coatings; Poly(acrylic acid); Poly(dimethylacrylamide); Conjugated polymers

Contents

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1. Introduction

In the fields of medical, environmental and chemical sciences there is an increasing need for the selective identification of specific molecules in complex mixtures of related substances. The development of techniques based on molecular recognition events that allow measurements of the concentration of certain compounds in complex mixtures is of great interest.

An example of a method based on this principle is given by the so called microarray technology [1]. This technique has attracted tremendous interest among biologists as it allows one to quickly and reliably analyze in parallel a large number of biomolecules such as nucleic acids [2,3], proteins [4,5] carbohydrates [6] and peptides [7,8]. The huge amount of information provided by microarray experiments facilitates the identification of genes or pathways for new drug targets, disease biomarkers [9], the prediction of individual drug responsiveness and the personalisation of therapeutic strategies [10,11]. In any array technology, different biopolymers, mainly proteins or DNA, are arranged in a predetermined configuration on a substrate. When the arrays are exposed to a population of analytes they will exhibit a pattern indicative of the presence of the various components separated spatially. Binding patterns of nucleic acids and/or proteins can then be detected by using a variety of suitable target labels and quantified. Currently, two dimensional arrays of macromolecules are made either by depositing small aliquots on flat surfaces under conditions which allow the macromolecules to bind the surface, or by synthesizing macromolecules on the surface using light-activated reactions. Probe arrayed substrates can be made from a variety of materials. Although solid flat plates, e.g. glass slides, silicon and gold substrates are suitable and are preferably employed, some microporous membranes, such as nitrocellulose or nylon membranes, may be used. An optically transparent substrate, such as glass or clear polystyrene, is convenient for detection modalities using fluorescence as it allows transmission of light through the microspots. However, reflective optical methods, allow the use of opaque substrates such as three-dimensional hydrogels or alumina, that are, in some cases, more favourable due to their higher binding capacity.

The chemistry used for the immobilization of probe molecules on the substrate plays a significant role in the success of any experiment. This is particularly true with protein arrays. Unlike DNA, proteins tend to bind to surfaces in a non-specific manner and, in doing so, sometimes lose their biological activity [12,13]. Therefore, the attributes for a substrate used to immobilize proteins are different from those for a DNA microarray in that surfaces for proteins must provide functionalities that are capable of interacting with protein capture agents without affecting their conformation. A further requirement of a good coating is its ability to resist non-specific protein binding to areas where no protein capture agents have been deposited. Selective binding of proteins to coated surfaces with antibodies or specific biomolecules is crucial in a number of areas, such as proteomics, diagnostics and disease monitoring, medical and biological research and in drug screening. Despite the high number of recent successful applications of such surfaces, many problems still remain to be solved [14,15]. The biggest challenges are (i) development of suitable protein-binders, (ii) preparation of supports able to prevent non-specific binding on surface avoiding false-positive detection, (iii) synthesis of protein denaturation-resistant coatings, and (iv) development of surfaces characterized by a high biomolecule loading capacity. This is of outstanding importance because, for example, the abundance of some proteins in animal plasma is very low (also lower than 10^{-12} g/ml) and their detection is very problematic.

The most common methods of immobilization relying on the formation of monolayers, either by protein adsorption on positive-charged poly-L-lysine or by covalent binding on silanized surfaces with suitable functionalities (i.e. epoxides, aldehydes, activated esters or maleimides), do not overcome these problems.

Background binding of proteins, carbohydrates, cell lysates to glass or other substrates employed in biosensing at interfaces is one of the major problem of this technology. The use of polymeric materials for modifying/functionalizing substrates is one of the most effective ways for tailoring molecular recognition events at surfaces in biosensing.

This review focuses, in no way exhaustively, on the development of materials and polymers with application in molecular recognition and on their use in sensing, with a particular attention to methods that are suitable for application in the microarray field. The aim of this article is to provide examples, taken from recent literature, of how polymer properties can be tailored to optimize the selective binding between guest molecules in solution and host molecules located at the solid phase and to optimize photonic or electronic detection of analyzed substances.

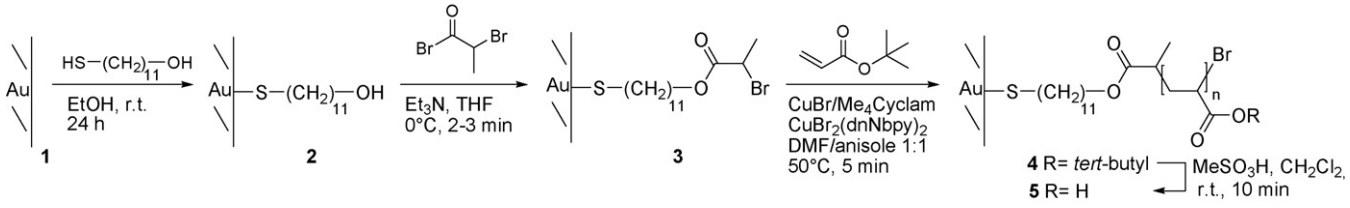

Fig. 1. Synthesis of PAA brush **5** through ATRP polymerization [20].

2. Use of polymers for selective binding of biomolecules to surfaces

2.1. Poly(acrylic acid) (PAA) brush coatings

Poly(acrylic acid) (PAA) is a very interesting substrate to anchor biomolecules through amide bond formation between its carboxylic functions and amino-modified oligonucleotides or protein amines. In particular, PAA brushes showed high protein-binding capacity. Brushes are polymeric chains bound by one end to a surface with a density of grafting points high enough to allow the extension of the polymeric chains from the surface [16,17]. This particular configuration, characterized by a high density of reactive groups together with a good swellability in appropriate solvents without necessity of crosslinks between chains, seems one of the most promising approaches to enhance the biorecognition on gene and protein chips.

2.1.1. PAA brushes via atom transfer radical polymerization (ATRP)

Recently, PAA brushes were obtained on gold-coated silicon wafers through atom transfer radical polymerization (ATRP) [18,19]. A gold-coated surface **1** was treated with mercaptoundecanol in order to prepare a self-assembly monolayer **2** which was subsequently reacted with α-bromopropionyl bromide affording a dense initiator monolayer **3** (Fig. 1). Immersion of initiator-coated surface in a mixture of *tert*-butyl acrylate, Cu(I)-1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane (Me_4Cyclam) and Cu(II)-4,4′-dinonyl-2,2′-bipyridyl (dnNbpy) in DMF/anisole 1:1 at 50 °C gave the corresponding polymer **4**. PAA brush **5** was obtained through *tert*-butyl ester hydrolysis with methanesulfonic acid in dichloromethane [20] (Fig. 1). PAA brushes were also synthesized *via* ATRP on flat silicon substrates by using an initiator-modified surface and *tert*-butyl acrylate as monomer. Interestingly, in order to avoid undesired side-reactions, ester cleavage through reagentless pyrolysis at 190–200 °C for 30 min was proposed [21].

2.1.2. Metal-ion affinity protein binding to PAA polymer brushes

With the aim of using metal-ion affinity interaction for binding proteins [22], polymer **5** was further derivatized in order to introduce a nitrilotriacetic acid (NTA) ligand [23]. Activation of carboxylic acids of **5** with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS) to give **6** was followed by treatment with aminobutyl NTA and subsequently with $CuSO_4$ providing, after derivatization with proteins, PAA-NTA Cu^{2+} complex **7** (Fig. 2).

In spite of the fact that the authors do not report any recognition of biomolecules by this surface, PAA-NTA brush **7** seems to be a promising tool to this aim. A high complexing ability toward proteins such as lysozyme, myoglobin, bovine serum albumine (BSA) and anti-IgG was reported with a binding capacity of 3.3, 7.7, 5.8 and 9.6 μg/cm^2, respectively. Furthermore, PAA-NTA

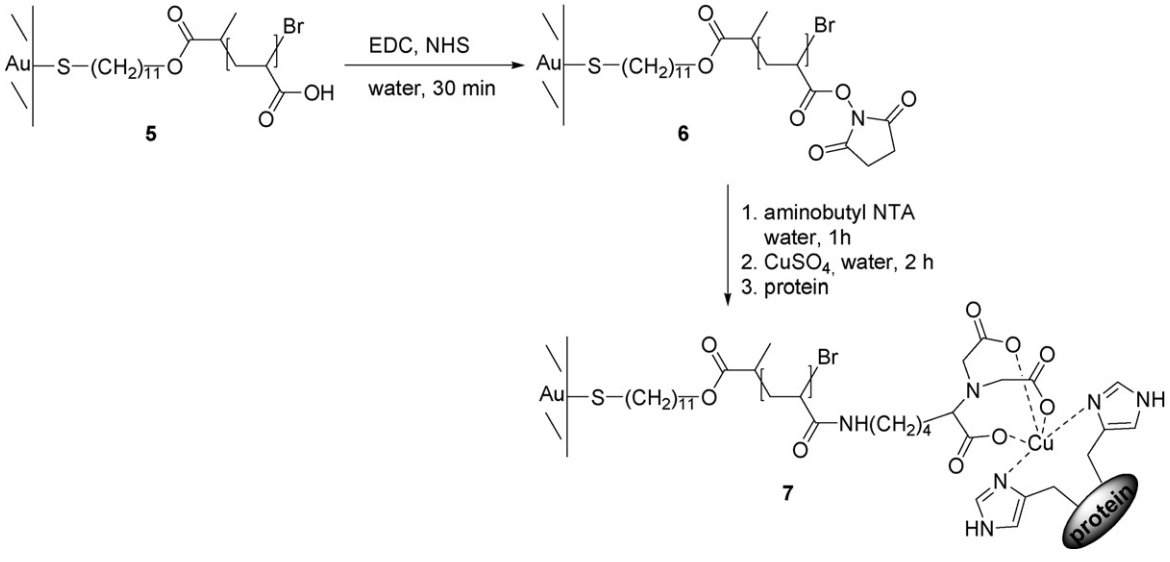

Fig. 2. Protein immobilization onto nitriloacetic acid-modified PAA brushes through metal affinity interaction [23].

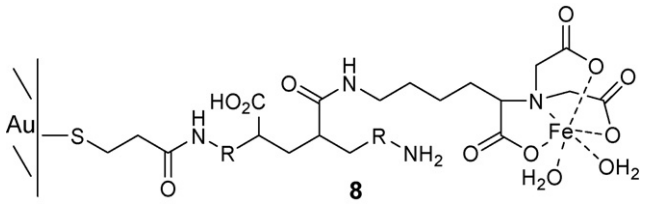

Fig. 3. Structure of a MALDI plate coated with PAA-NTA Fe^{3+} **8** useful in phosphopeptides detection [24].

brushes **7** evidenced a good robustness allowing several cycles of protein binding without any decreasing of protein loading in each cycle.

The combination of metal-ion affinity binding and PAA-NTA Fe^{3+} **8** (Fig. 3) was recently used for surface modification of matrix assisted laser desorbtion/ionization (MALDI) gold plates [24].

It is well known that six-coordinated metal ions, such as Fe (III), bound to tetradentate ligands, such NTA, have a strong affinity with phosphoryl groups present on serine, threonine and tyrosine residues in phosphopeptides resulting from tryptic digest of phosphorylated proteins [25]. Protein phosphorylation plays an essential role for a number of cellular functions such as gene expression and membrane transport [26], therefore identification of phosphorylation sites is crucial to understand many biological processes. Mass spectroscopy, in particular MALDI, proved to be one the most useful technique for identifying such sites, nevertheless low ionization efficiencies of phosphopeptides and low degree of phosphorylation make this identification a challenging task. However, derivatization of MALDI plates with PAA-NTA Fe^{3+} **8** provided interesting results because the immobilized complex allows a preferential adsorbtion of phosphorylated peptides resulting in mass spectra dominated by peaks corresponding to phosphopeptides. This surface allowed detection of all the three phosphorylated fragments (one monophosphorylated and two tetraphosphorylated) obtained from tryptic digestion of β-casein, while conventional MALDI gave a signal only for the monophosphopeptide. In addition, the intensity of the peaks was higher (6.5- and 4-fold for tetraphosphorylated peptides and 2-fold for monophosphopeptide) than any signal assigned to nonphosphorylated fragment. Utilization of modified plates also allowed a great decrease of signals due to nonphosphorylated peptides in ovalbumin digest analysis. The signals assigned to phosphopeptides obtained from digestion were up to 13-times higher than signals of nonphosphorylated fragments. Noteworthy, the results obtained by means of MALDI plates derivatized with PAA-NTA Fe^{3+} **8** were substantially better than the results previously reported using MALDI plates covered with PAA grafted with polyethylenimine or complexed directly with Fe(III) without NTA [27].

2.2. PAA coating of polydimethylsiloxane (PDMS) microdevices

Poly(acrylic acid) was used also in combination with poly(dimethylsiloxane) (PDMS). PDMS is one the most utilized polymers for biosensors and lab-on-chip fabrication thanks to its favourable properties such as low cost, ease of fabrication, durability, low Young's modulus, biological compatibility and optical transparency. Despite these advantages, PDMS shows significant drawbacks for most bioanalytical purposes. The high hydrophobicity of PDMS is the biggest limitation in biological applications because this property increases non-specific adsorption of biomacromolecules, reduces cell adhesion and causes difficulties in surface wetting and channel filling in microdevices. In addition, native PDMS lacks functional groups useful to immobilize biomolecules in DNA assays or immunoassays.

2.2.1. Graft polymerization of PAA onto PDMS surface

A number of approaches have been utilized to tailor the properties of PDMS surface [28]. Among them PDMS grafting with PAA seems to be the most promising methodology. Two strategies are currently adopted to this aim, both concerning an acrylic acid radical polymerization triggered by ultraviolet radiation (UV) onto or just beneath the PDMS surface.

One-step process: in the first method, graft polymerization of acrylic acid onto PDMS surface was achieved using an aqueous solution of monomers, $NaIO_4$ as oxygen scavenger and benzyl alcohol as chain-transfer agent (CTA) in order to decrease solution viscosity thus increasing polymerization efficiency [29,30]. This method was successfully employed by Li and Albritton in microfluidic devices fabrication [31].

Two-step process: in 2004 the same group reported a second PDMS modification methodology based on a two-step strategy involving (i) an implantation of benzophenone (a polymerization photoinitiator) onto or just beneath PDMS surface favoured by acetone-induced PDMS swelling leading to photoinitiator diffusion in PDMS pads and (ii) UV irradiation of PDMS-benzophenone in an aqueous solution of monomer, $NaIO_4$ and benzyl alcohol giving the formation of polymer brushes [32]. This method gave a higher polymerization rate than the previous one, providing an efficient surface grafting polymerization in only 5 min of exposure to UV light rather than in 4 h as reported in one-step process [31].

2.2.2. Photografting of PAA on PDMS for surface patterning

Using the latter strategy, PDMS surface was micropatterned by photografting through an iron oxide photomask with 5- and 10-μm PAA circles and squares [33] (Fig. 4).

Atomic force microscopy (AFM) analysis revealed a maximal height of the PAA graft of 150 nm above PDMS surface with a spatial resolution of 5 μm while the depth of grafting in the inner PDMS core was determined through 6-aminofluorescein incubation. This positively charged, low molecular weight fluorophore is able to diffuse into the PDMS core and to establish electrostatic interaction with negatively charged PAA carboxylates. Scanning of the surface by fluorescence microscopy showed the interpenetrating polymer network PAA-PDMS as brilliant fluorescent up to a 50 μm depth into the material core, while intervening regions were nonfluorescent. Also worthy of mention is the ease of fabrication of this covalent surface modification which can be done in a standard laboratory,

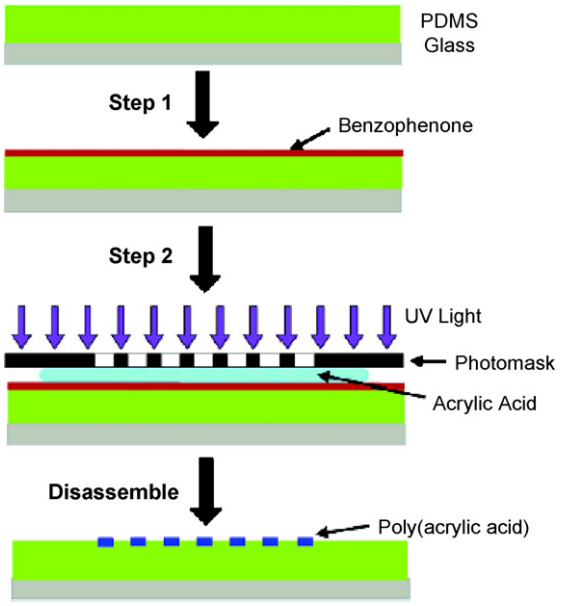

Fig. 4. Schematic representation of UV-mediated micropatterning of PDMS with PAA. From [33] with permission.

without the need for clean-room facilities and using benchtop procedures.

The PDMS substrate coated with grafted PAA was used in heterogeneous antibodies immunoassays, where an anti-GFP antibody able to bind the enhanced green fluorescence protein (EGFP) was immobilized onto the surface. The binding capacity of this surface was enhanced through the covalent attachment of protein A, a bacterial product which binds selectively four anti-GFP molecules. The results demonstrated that in the latter case the fluorescence obtained upon hybridization was 3-fold higher than that provided by the surface without protein A.

2.3. PAA coating on microporous alumina support

Poly(acrylic acid) was used in combination with protonated poly(allylamine) (PAH) in alumina coating to generate a membrane-based protein microarray. The use of microporous alumina supports resulted in a 500-fold increase in surface area respect to two classical 2-D supports [34] allowing a decrease in detection limit of up to 2 orders of magnitude. Additionally, binding kinetics of biomolecules could be accelerated by the flow of analyte solution through alumina small pores [35]. The PAA coating of alumina is appealing due to its ability to suppress non-specific protein adsorption [36] thus avoiding surface blocking with BSA which sometimes masks the access to binding sites of the immobilized probe [37]. Moreover, the acidic functions of PAA can be transformed into activated (i.e. succinimidyl) esters and reacted with amines allowing the covalent binding of biomolecules.

Bruening and co-workers [38] prepared a microporous alumina support coated with a multilayer of PAA/poly(allylamine) polyelectrolytes. PAA acidic functions **9** were thus readily converted into their respective succinimidyl esters **10** using EDC and NHS and then reacted with anti-IgG obtaining **11** (Fig. 5).

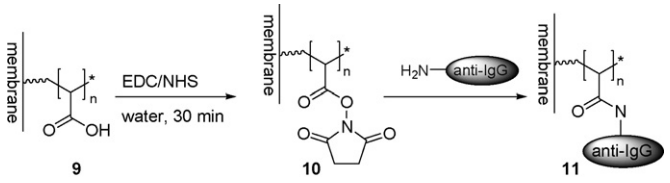

Fig. 5. Immobilization of anti-IgG onto PAA coated microporous alumina [38].

A flow of Cy5 labelled IgG solution through the alumina pores coated with polymer **11** at various flow-rates allowed quantitative antibody analysis with a detection limit of 0.02 ng/ml without need for blocking with BSA. This limit was 100- and 500-fold greater than that achieved in the same assay accomplished either by shaking the membrane in contact with the solution or in a static mode. Furthermore, in order to assess the capacity of the PAA/PAH coated alumina to enhance signal to noise ratio in the analysis of a complex mixture of proteins, the binding of three different IgGs dissolved in 10% fetal bovine serum to their corresponding anti-IgGs immobilized was investigated. Interestingly no cross-reactions were observed and the background signal was very low.

2.4. Poly N,N-dimethylacrylamide (PDMA) coatings

Poly *N*,*N*-dimethylacrylamide (PDMA) is a well known polymer in capillary electrophoresis. It has been introduced by Madabhushi et al. [39] for DNA sequencing and rapidly gained high popularity for its self-coating properties. This polymer physically adsorbs on a silica capillary wall thus eliminating electroosmotic flow (EOF) [40,41]. The dynamic coating is very rapid (few minutes) and do not require special pre-treatments. In electrophoresis, the coating is challenged by a number of factors (shear forces, competition by urea, detergents and proteins). Therefore, strategies to form irreversible polymer films on a capillary wall that involve polymer physisorption followed by its covalent attachment to the surface have been explored. Some of the strategies exploited in capillary electrophoresis to coat the silica surface in an irreversible manner were also applied to microarray substrates.

2.4.1. Poly(dimethylacrylamide-silane) copolymer coatings

A new family of polydimethylacrylamide-silane copolymers, that rapidly adsorb on the wall from ultra-diluted aqueous solutions, was synthesized and used to form a capillary coatings [42]. This copolymer, copoly(DMA-MAPS) **12**, was synthesized by a free radical chain polymerization of *N*,*N*-dimethylacrylamide (DMA) **13** and [3-(methacryloyl-oxy) propyl]trimethoxysilane (MAPS) **14** in THF and in presence of 2,2′-azo-bis(isobutyronitrile) (AIBN) (Fig. 6).

Upon thermal treatment, hydroxy silyl groups on **15**, obtained through silylethers hydrolysis of **12** and pendant from the backbone, condense with the surface silanols **16** and covalently bind the copolymer to the capillary wall giving **17** [43] (Fig. 7).

In this system, physisorption of polymer chains to the surface is essential to bring surface and polymer silane groups in close proximity thus facilitating the formation of covalent bonds. The

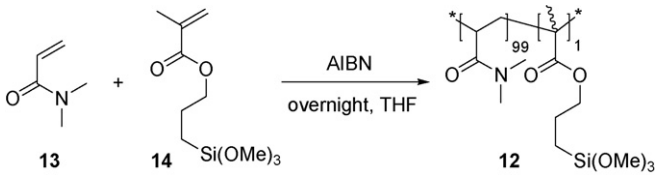

Fig. 6. Synthesis of copoly(DMA-MAPS) **12** using radical chain polymerization [42].

adsorbtive properties of polydimethylacrilamide onto glass and the stability of the bond obtained by the reaction between silyl groups of **15** and glass surface silanols **16** were subsequently combined with the use of functionalities extending in solution and able to covalently bind biomolecules as modified DNA molecules, peptides or proteins. The *ter*-copolymer obtained by this approach, named copoly(DMA-NAS-MAPS) **18** is the result of an optimized composition of DMA **13**, MAPS **14** and *N*-acryloyloxysuccinimide (NAS) **19** [44] (Fig. 8), and was first reported to covalently attach biomolecules on glass slides for the preparation of low-density DNA microarrays [45].

The innovative aspect in this approach relies in the fact that the coating procedure provides a fast and inexpensive method of producing hydrophilic functional surfaces bearing active ester. The polymer self-adsorbs onto the glass surface very quickly, simply by immersing glass slides in a diluted aqueous solution of the polymer and without time-consuming glass pre-treatments. Notably, in order to best exploit at best the poly *N*,*N*-dimethylacrylamide self-adsorbing properties, the molecular weight of the functional copolymers cannot be lower than 120,000 Da.

DNA microarrays produced by physisorption of copoly (DMA-NAS-MAPS) **18** provided a maximal attachment density of 0.9×10^{13} molecules/cm^2, one order of magnitude superior to that obtained with the copolymer (DMA-NAS) [46].

The copoly(DMA-NAS-MAPS) **18** coated slide performance was also investigated as a polymeric surface for protein microarrays in the assessment of rheumatoid factor (RF) in human serum samples [47]. The results demonstrated that the proteins immobilized on the polymeric surface maintain a native conformation and are easily accessible for molecular recognition, providing a detection limit of 54 amol/spot; moreover, after the assay, the slides exhibited a very low background which is an important feature directly affecting protein microarrays sensitivity. The polymeric surface was also tested as a peptide microarray support in an epitope mapping study on human chromogranin [48]. This study suggested that although the copoly(DMA-NAS-MAPS) **18** slides bind the capture molecule in a random conformation, the aqueous micro-environment created by the polymeric coating provided a good accessibility of the ligand without need for a spacer between the probe and the surface as required by most small molecule microarray supports.

2.4.2. *Copoly(DMA-NAS-MAPS) coating of nanospheres*

The versatility of the copoly(DMA-NAS-MAPS) **18** coating was also exploited to functionalize substrates different from the common microscope glass slides. Glass nanobeads (330 nm) were coated by copoly(DMA-NAS-MAPS) **18** by simply dispersing the nanospheres in a dilute aqueous polymer solution. Coated nanobeads, bearing proteins, were entrapped in a PDMS substrate (**C** in Fig. 9) in confined regions leading to a strong enhancement of the protein deposited on the surface [49]. The analytical performance of the novel arrayed surface was compared to that obtained, either with copoly(DMA-NAS-MAPS) **18** coated glass slides (**A** in Fig. 9) or with latex microbeads based systems (**B** in Fig. 9).

Evidence was obtained that the new material exhibits interesting characteristics in term of protein immobilization and accessibility. The immobilized protein, used as antigen, enabled the specific binding of a large amount of polyclonal antibodies. This system combines the advantages of the highly efficient protein immobilization of the copoly(DMA-NAS-MAPS) **18** with the enhanced surface of the glass beads. Chemiluminescent detection of anti-rabbit-IgG was obtained through peroxidase labelled antibodies in the 5 μg/l to 10 mg/l range. Application of the developed system to real samples was achieved for the

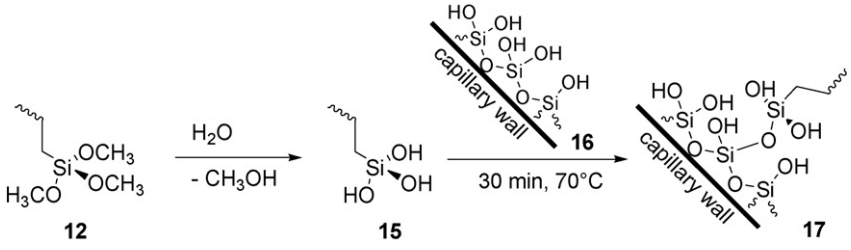

Fig. 7. Schematic representation of copoly(DMA-MAPS) **12** capillary wall coating [43].

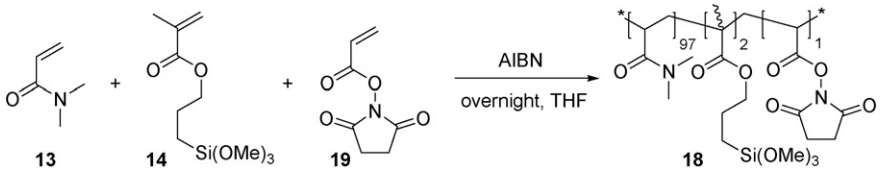

Fig. 8. Synthesis of copoly(DMA-NAS-MAPS) **18** using radical chain polymerization [44].

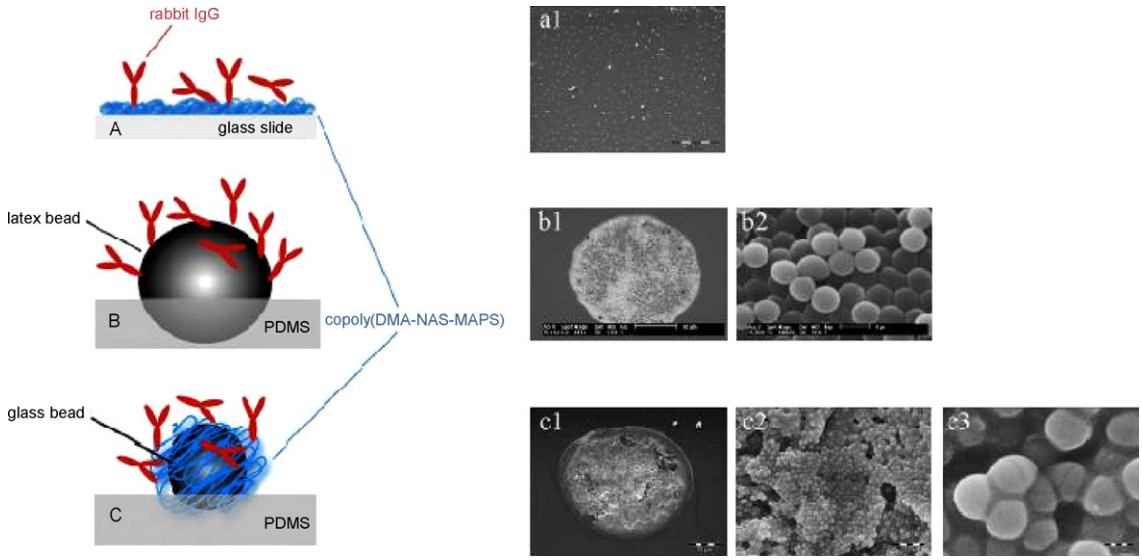

Fig. 9. (Left) graphical representation of the three microarray systems: (A) glass slide coated with **18**; (B) latex microbeads in a PDMS matrix; (C) latex microbeads coated with polymer **18** in a PDMS matrix. (Right) scanning electron microscopy images (15 kV) of the three systems. From Ref. [49] with permission.

detection of rheumatoid factor through a capture assay. Interesting results were obtained, with a RF detection over the 5.3–485 IU/ml range and without measurable matrix effect or non-specific signal. Such novel nanosized structures could help increasing the performance of analytical microsystems.

2.4.3. Copoly(DMA-NAS-MAPS) coating of silicon microcantilevers

Recently, copoly(DMA-NAS-MAPS) **18** was used to functionalize silicon microcantilevers by dip-coating. A microcantilever is a device that can act as a physical, chemical or biological sensor by detecting changes in cantilever bending or vibrational frequency [50]. The polymer coating microstructure was thoroughly investigated by analyzing the resonance frequency values of bare and coated microcantilevers, by scanning electron microscopy (SEM) and scanning force microscopy (SFM) imaging, by SFM tip-scratch tests and X-ray reflectometry (XRR) experiments. The results of the tests indicate that the polymeric coating is 2.5 nm thick. The coating surface seems to be nanostructured, displaying nanoblobs, which are from few up to 20 nm wide and, on average, 1.6 nm high. The diameter of the biggest nanoblobs is of the same order of magnitude of the gyration radius of the copolymer chains, suggesting that nanoblobs may identify individual macromolecules [51]. The article discusses also the successful coating of microcantilevers to generate functional layer for making microcantilever-based

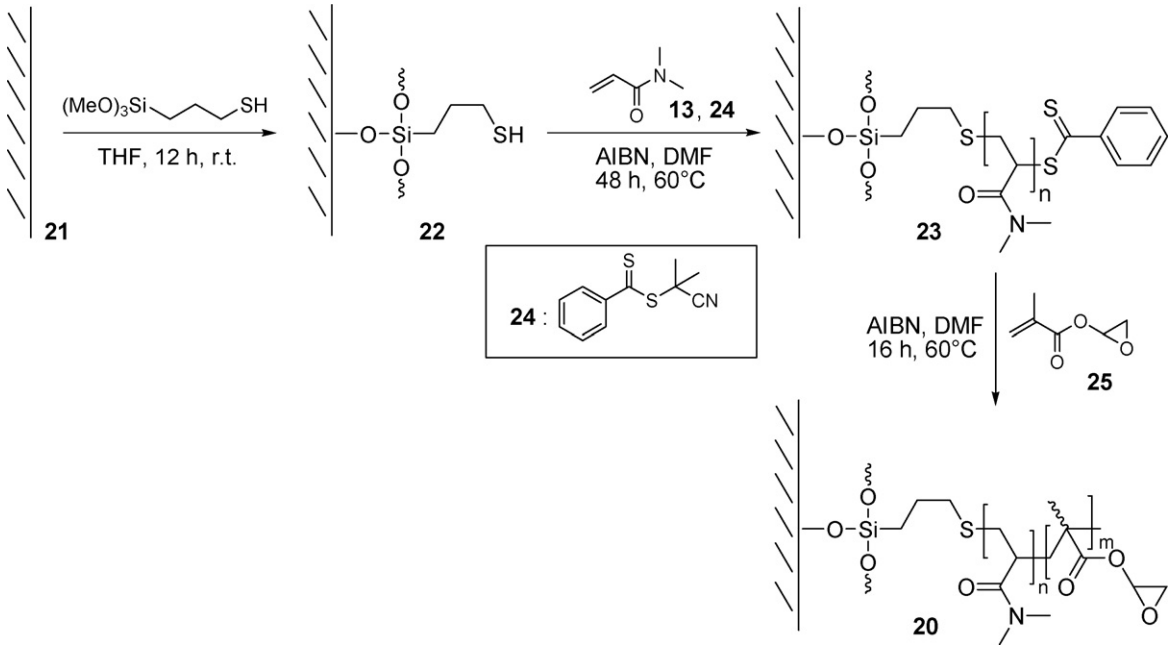

Fig. 10. Preparation of glass slides coated with a brush block copolymer **20** using RAFT polymerization [56].

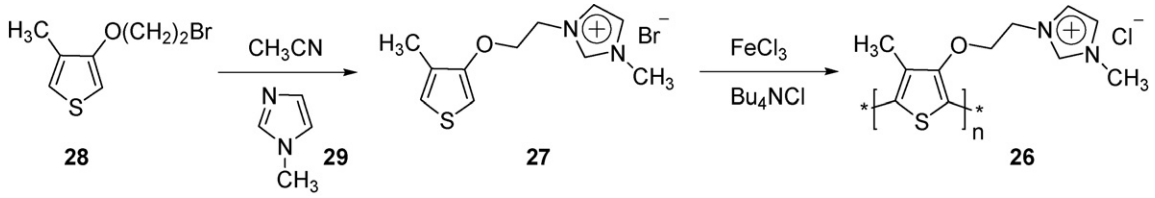

Fig. 11. Synthesis of imidazolium-substituted poly(thiophene) **26** [59].

(bio)sensors which pose unique challenges due to the microscopic dimensions of the microcantilevers.

2.4.4. PDMA brush copolymer coatings

PDMA was also used to obtain brush copolymers by means of reversible addition-fragmentation chain-transfer (RAFT) polymerization [52], a controlled/living polymerization method used to synthesize polymers with controlled architectures and low polydispersities [53] through the use of thiocarbonylthio compounds acting as chain-transfer molecules. This mechanism allows a polymeric chains growth starting from a suitable initiator bound to the surface leading to formation of chains covalently linked by one end to the surface. Polymer brushes are attractive in solid phase molecular recognition since their 3-D structure enhances hybridization efficiency by avoiding the problem of probe accessibility due to hindrance [54,55].

Recently, block copolymer brushes **20** on glass slides **21** were obtained. Derivatization of **21** with (3-mercaptopropyl)trimethoxysilane gave **22** which was reacted in a first step with DMA **13** in presence of cyanoisopropyl dithiobenzoate **24** as CTA giving brush **23**. Polymerization with glycidyl methacrylate (GMA) **25** gave block brushes **20** (Fig. 10) [56].

Epoxy functions were used to bind amino-modified oligonucleotides and a grafting density of 3×10^{12} molecules/cm^2 was assessed. Additionally it was calculated that, on average, every chain bears ~20 probes, which corresponds to about one oligonucleotide every five monomer residues.

3. Use of polymers for sensing of biomolecules

3.1. Devices based on cationic polymers

3.1.1. Poly(thiophene)s

Conjugated polymers are very interesting emerging tools for recognition of biomolecules [57]. This research field emerged with the pioneering studies of Leclerc and co-workers on the electrostatic interaction between a cationic conjugated polymer and DNA. In 2002 Leclerc's group demonstrated that the optical properties of a water-soluble imidazolium-substituted poly(thiphene) **26** [58,59] depended on its conformational modification and changed when the polymer electrostatically interacted with single- or double-stranded DNA. This makes it useful in the detection of oligonucleotides without any labelling of the probe or target. Polymer **26** was easily obtained by means of an oxidative polymerization in chloroform, in the presence of $FeCl_3$ as the oxidizing agent, starting from monomer **27** synthesized by a reaction between 3-(2-bromoethoxy)-4-methylthiophene **28** and *N*-methylimidazole **29** (Fig. 11).

The authors used fluorescence spectroscopy to detect the binding between ss-DNA and its complementary strand. They measured as few as 3×10^6 molecules of complementary ss-DNA in a liquid volume of 200 μl (2×10^{-14} M). In addition to the high sensitivity they could also observe high selectivity. The oligonucleotide probe electrostatically bounded to the polymer, selectively recognized its fully complementary strand in mixtures containing strands with one or two mismatches.

In 2005 Leclerc and co-workers used this strategy on DNA-chips [60]. The system consisted of the cationic polymer **26** as fluorophore, ss-DNA and a peptide nucleic acid (PNA) [61] tethered onto a solid surface as the capture strand.

PNA is a non-ionic DNA analogue in which a sugar-phosphate backbone is replaced by neutral pseudo-peptide chain that links nucleobases through a carboxymethylene bridge. A PNA strand, being uncharged, does not bind to the cationic polymer **26**. However, after hybridization with a complementary ss-DNA, PNA/DNA duplex acquires negative charges and interacts with the polymer. This hybridization event is then translated into a fluorescence signal (Fig. 12). The authors observed a sensitivity of 2.5×10^{-13} mole of oligonucleotide in a volume of 20 μl.

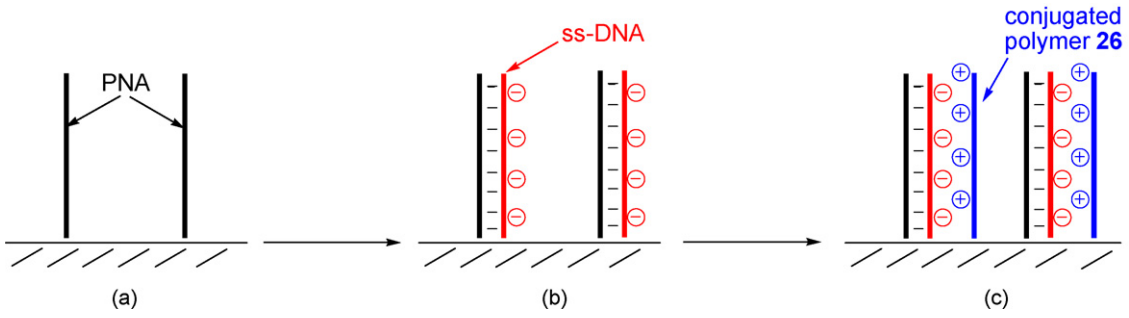

Fig. 12. Schematic representation of detection system with polymer **26**. (a) PNA tethered to a glass slide surface. (b) Hybridization with a complementary ss-DNA. (c) Electrostatic interaction between negatively charged DNA and fluorescent-cationic polymer [60].

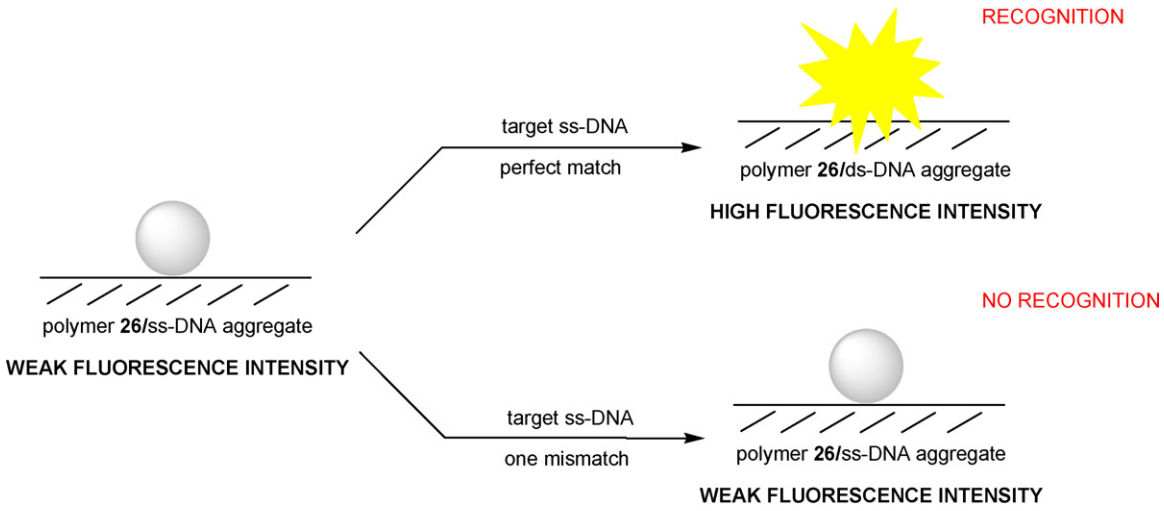

Fig. 13. Schematic representation of biochip platform based on a polymer **26**/ss-DNA micelles (on the left side). When the capture probe is hybridized with a complementary ss-DNA it shows a high fluorescence enhancement (top right); interaction with target ss-DNA having one mismatch shows a very weak fluorescence (bottom right) [62].

Conjugated polymer **26** was used to build a biochip able to detect as few as 300 ss-DNA molecules, directly and specifically, even in the presence of large amounts of ss-DNA strands with one mismatch [62]. The novel biochip was composed of an aggregate of polymer **26** and a Cy3-labelled ss-DNA tethered on a glass surface as the capture probe. The hybridization with mismatched targets gave rise only to a signal of weak fluorescence intensity whereas the hybridization with a complementary ss-DNA strand caused a conformational change of polythiophene **26**, from planar to helical [59], with a strong emission of fluorescence (Fig. 13).

In addition to the usual fluorescence resonance energy transfer (FRET) phenomenon, the fluorescence enhancement observed upon hybridization was ascribed to a fast and efficient energy transfer mechanism among neighbouring polymer chains favoured by the helical conformation. This process was called either "superlighting" or "fluorescence chain reaction" (FCR) [63,64]. Using this biochip platform a detection limit of 5×10^{-16} M was reached, a value not too far from that achieved with the same detection system in an homogeneous medium [65]. Furthermore, the polymeric transducer **26** was successfully combined with an aptamer ligand (i.e. a synthetic oligonucleotide with characteristic 3-D structure and specific binding sequence) in order to detect with high specificity human α-thrombin in the attomole range (6.1×10^{-11} M) at room temperature and in less than one hour [66].

Ferrocene-functionalized cationic polythiophene was also used for the electrochemical detection of DNA. Polymer **30** was easily prepared by reacting bromo functionalized thiophene **31** with the commercially available ferrocene amine **32** leading to formation of the corresponding ferrocene-labelled monomer **33** which was subsequently polymerized in presence of $FeCl_3$ (Fig. 14) [67].

This electrochemical detection method involved, in addition to polymer **30**, a PNA tethered onto a gold surface as the capture probe and a single strand DNA fragment. The neutral PNA can interact with the cationic polymer only after the hybridization with a ss-DNA allowing transduction into an electrical signal (Fig. 15, path a). The detection limits reported for this sensor are in the order of 5×10^{-10} M with a good selectivity.

A similar strategy was used for the indirect detection of human α-thrombin in solutions of unknown concentration [68]. Excess of specific human α-thrombin aptamer was added to a human α-thrombin solution. After aptamer/protein complex formation, S1 nuclease enzyme was added to the solution in order to specifically hydrolyze the free ss-DNA aptamer. Subsequent protein denaturation released the aptamer from the aptamer/protein complex which was electrochemically detected by the PNA capture probe grafted onto a gold electrode (Fig. 15, path b). With this device a detection limit of 7.5×10^{-10} M was achieved.

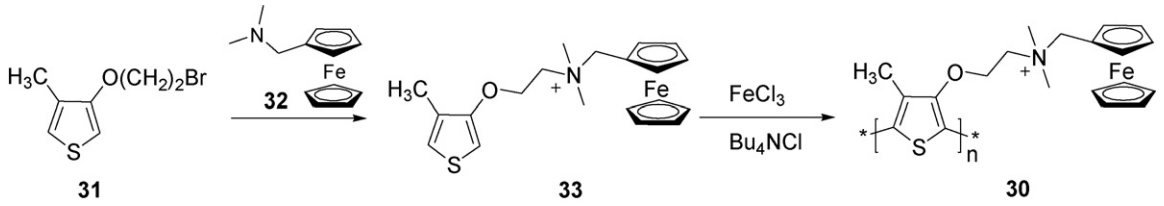

Fig. 14. Synthesis of ferrocene-labelled polythiophene **30** [67].

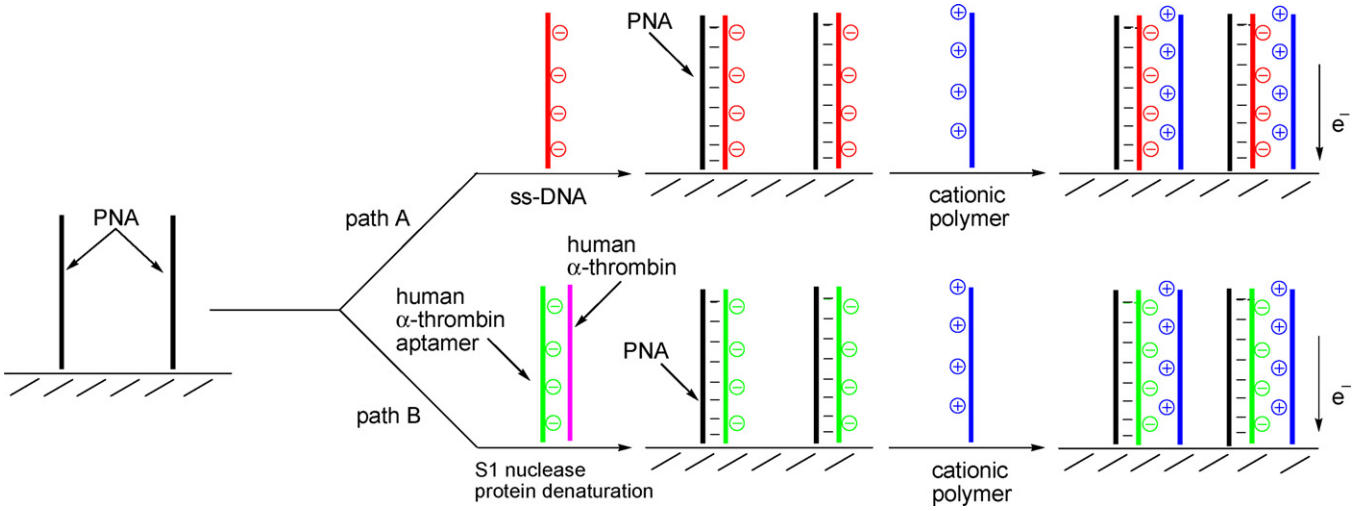

Fig. 15. Schematic representation of electrochemical detection mediated by cationic polymer **30**. Path A: recognition between grafted PNA and complementary ss-DNA allows signal transduction after interaction with electroactive cationic polymer **30** [67]. Path B: indirect detection of α-thrombin through complex formation with the corresponding aptamer followed by denaturation, subsequent PNA capture and signal transduction by means of polymer **30** [68].

3.1.2. *Fluorene-benzothiadiazole copolymers*

Liu and Bazan [69] detected DNA by means of a system comprised of (i) a PNA tethered to a surface, (ii) a complementary ss-DNA and a (iii) cationic fluorene containing conjugated copolymer.

Surface-bound PNA probe **34** was prepared starting from a glass slide **35** which was derivatized with a solution of aminopropyltrimethoxysilane. Amino-modified surface **36** was reacted with 1,4-phenylenediisothiocyanate affording **37** to which amino-modified PNA oligomer was immobilized (Fig. 16).

The copolymer, poly[9,9′-bis(6″-*N*,*N*,*N*-trimethylammonium)hexyl)fluorene-*co*-*alt*-4,7-(2,1,3-benzothiadiazole) dibromide] (PFBT) [70] **38**, was obtained by reaction between polymer **39** and trimethylamine. Polymer **39** was, in turn, synthesized using a Suzuki coupling [71] starting from 2,7-bis[9,9′-bis(6″-bromo-hexyl)-fluorenyl]-4,4,5,5-tetramethyl-[1.3.2]dioxaborolane **40** and 4,7-dibromo-2,1,3-benzothiadiazole **41** (Fig. 17).

This PFBT based DNA-sensing system exploits the FRET mechanism, also known as Förster Resonance Energy Transfer [72]. In FRET, a long-range dipole–dipole energy transfer occurs between a donor and an acceptor chromophore. The transfer strongly depends on the distance between them. The strong distance-dependence makes FRET useful for sensing because events that change the distance between the two chromophores can be detected as a variation of the acceptor emission intensity. Starting from their previous works concerning both RNA [73] and DNA-detection [74,75] the authors reported a method for DNA-detection on a microarray platform in which they used a PNA tethered on a glass surface **34**, a ss-DNA labelled with Cy5 dye and PFBT **38** (Fig. 18).

The hybridization of the non-ionic PNA probe on the surface with a complementary Cy5 label ss-DNA introduces negatively

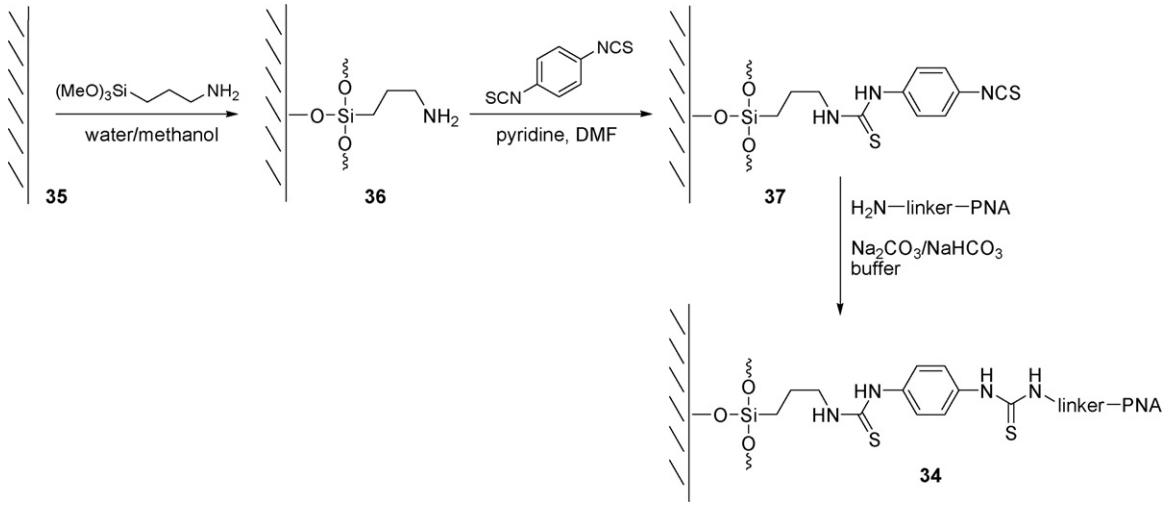

Fig. 16. Preparation of surface-bound PNA probe [69].

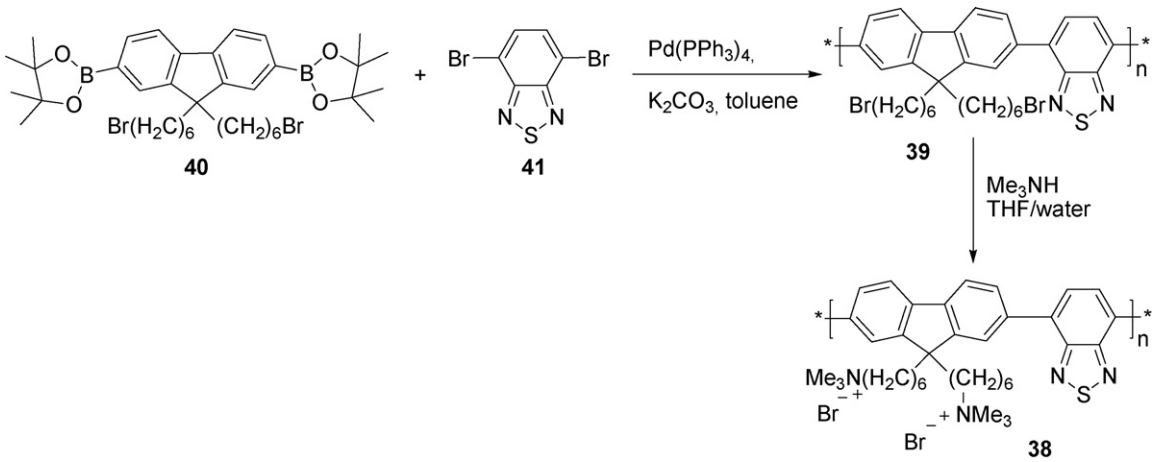

Fig. 17. Synthesis of poly[9,9′-bis(6″-*N*,*N*,*N*-trimethylammonium)hexyl)fluorene-*co*-*alt*-4,7-(2,1,3-benzothiadiazole)dibromide] (PFBT) **38** using Suzuki coupling and subsequent ammonium salt formation [70].

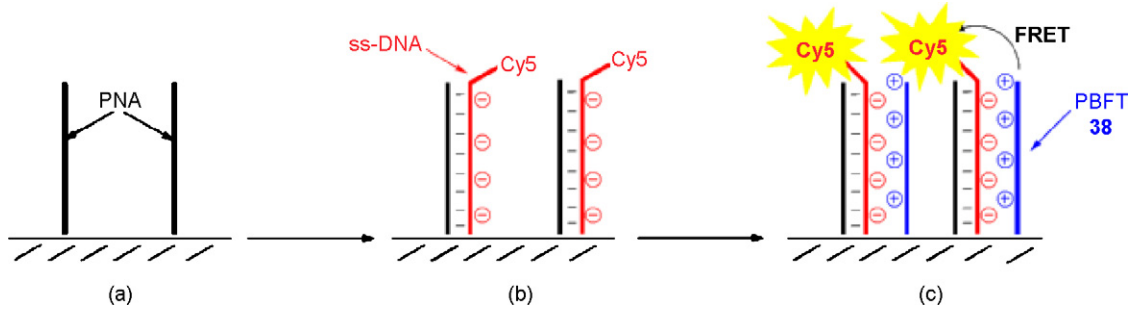

Fig. 18. Schematic representation of detection system based on PBFT **38** (a) PNA bound to the surface. (b) Hybridization between PNA and Cy5 labelled ss-DNA. (c) Interaction with cationic PFBT **38** and FRET detection [70].

charges on surface which can interact with positive-charged polymer **38**. The distance between the acceptor (Cy5) and the donor (PFBT) allows an efficient FRET. More than 1 order of magnitude amplification of the dye emission intensity was observed by the authors in the hybridization with complementary ss-DNA with respect to the non-complementary one.

The same authors reported a label-free detection system in which intrinsic fluorescence properties of polymer **38** were used (Fig. 19).

The overall selectivity of the system is based on the ability of the capture PNA to discriminate complementary and non-complementary DNA strands. It was possible to demonstrate that PBFT emission in a system PNA/complementary DNA was 5-fold greater than the corresponding emission with non-complementary DNA. In addition, the label-free detection of 10^{10} molecules of ss-DNA complementary to the surface-bound PNA was demonstrated with a substantial simplification over the DNA sensing methods currently in use.

3.2. Devices based on non-charged polymers

3.2.1. Poly(pyrrole)s

Another way to achieve a label-free DNA sensing is represented by electrochemical assays based on non-charged

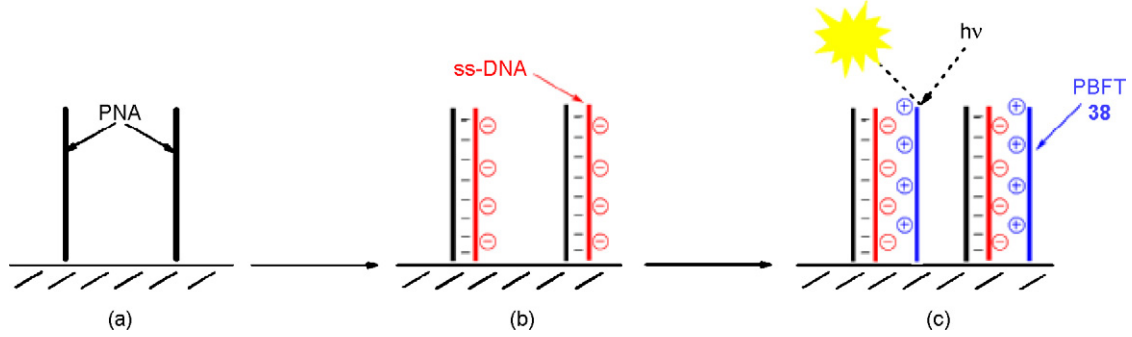

Fig. 19. Schematic representation of label-free detection system based on **38**. (a) Surface-bound PNA. (b) Hybridization with complementary ss-DNA. (c) Electrostatic interaction between ss-DNA and PBFT and polymer fluorescence emission [70].

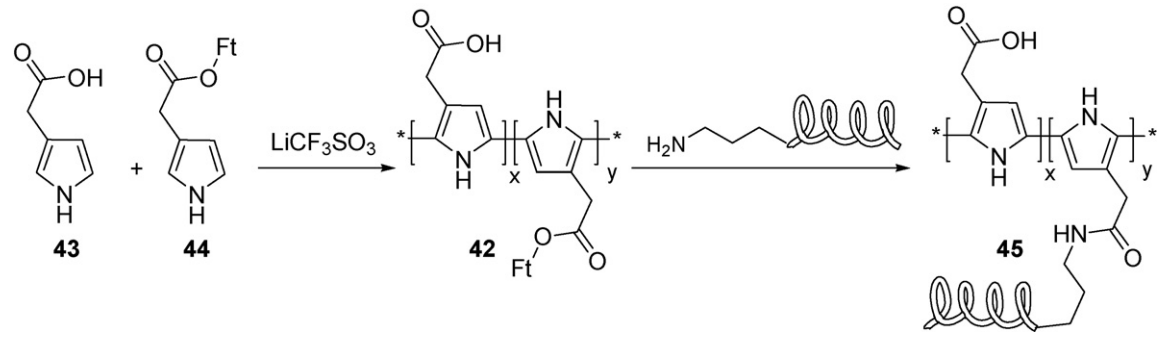

Fig. 20. Synthesis of bioconjugated copolymer **45** by means of electropolymerization and subsequent amino-modified ss-DNA coupling [76].

conjugated polymers. This detection method relies on the changes in conjugated polymers electrochemical characteristics after hybridization with complementary ss-DNA. Thanks to their simple electropolymerization, pyrrole-based polymers and copolymers are widely used in this field. Garnier et al. designed a novel pyrrole-based copolymer **42** [76] obtained from electropolymerization of 3-carboxymethyl pyrrole [77] **43** and the corresponding phthaloyl (Ft)-ester **44** and subsequent coupling with an amino-modified ss-DNA to obtain bioconjugated polymer **45** (Fig. 20).

Very recently, the bioconjugated copolymer **45** was synthesized on a plastic-based chip patterned with eight gold electrodes ($\phi = 100\ \mu m$) and used for DNA-detection [78]. When the surface of this device was contacted with a DNA strand non-complementary to the immobilized probe no modification of its electrochemical response was observed, whereas hybridization with complementary ss-DNA strand caused a decrease of the oxidation wave due to a new organization of the polymer structure. As a consequence of the electrochemical oxidation, polymer **45** shifts from a polyaromatic scaffold, less sensitive to the steric hindrance of pyrrole substituents, to a planar polyquinoid structure strongly affected by the substituent bulkiness [79].

Interestingly, this electrochemical device allows detection of complementary DNA with a detection sensitivity of 10^{-12} M, a value very close to the sensitivity of present fluorescence-based sensors. This high sensitivity was achieved using conventional electrochemical equipment. Its high efficiency was considered to be related to various cooperative amplification factors in the electrochemical response of the conjugated polymer based sensor.

3.2.2. Poly(p-phenyleneethynylene)

Recently, Kim and co-workers reported the synthesis of a conjugated poly(*p*-phenyleneethynylene) (PPE) **46** bearing a carboxylic acid function suitable for a bioconjugation with DNA strands, and alternating hydrophilic and hydrophobic chains for Langmuir–Blodgett [80] film fabrication [81]. This polymer was synthesized by Sonogashira coupling [82] between a *p*-diethynylbenzene bearing two hydrophilic triethylene glycol side chains **47** and a *p*-diiodobenzene substituted with two hydrophobic ethyl heptanoate **48**, followed by ethyl ester hydrolysis of **49** (Fig. 21).

Polymer **46** was transferred onto a glass slide and carboxylic groups were activated by means of EDC/*N*-hydroxysulfosuccinimide (sulfo-NHS) and then coupled with an amino-modified ss-DNA. Hybridization tests were performed using a complementary and non-complementary ss-DNA labelled with hexachlorofluorescein (HEX). Successful hybridization with HEX-labelled complementary ss-DNA places the chromophore at a correct distance leading to an efficient FRET with PPE. The authors proved that the interaction with non-complementary ss-DNA sequence does not give rise to energy transfer by FRET mechanism demonstrating the good selectivity of the system. Interestingly, a significant fluorescence emis-

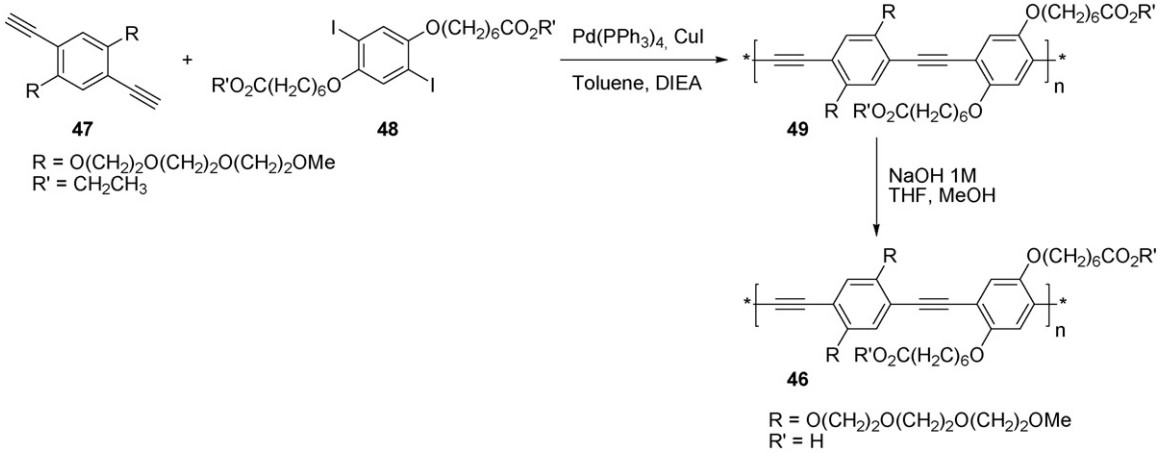

Fig. 21. Synthesis of poly(*p*-phenyleneethynylene) (PPE) **46** using Pd-catalyzed Sonogashira coupling in the key step [81].

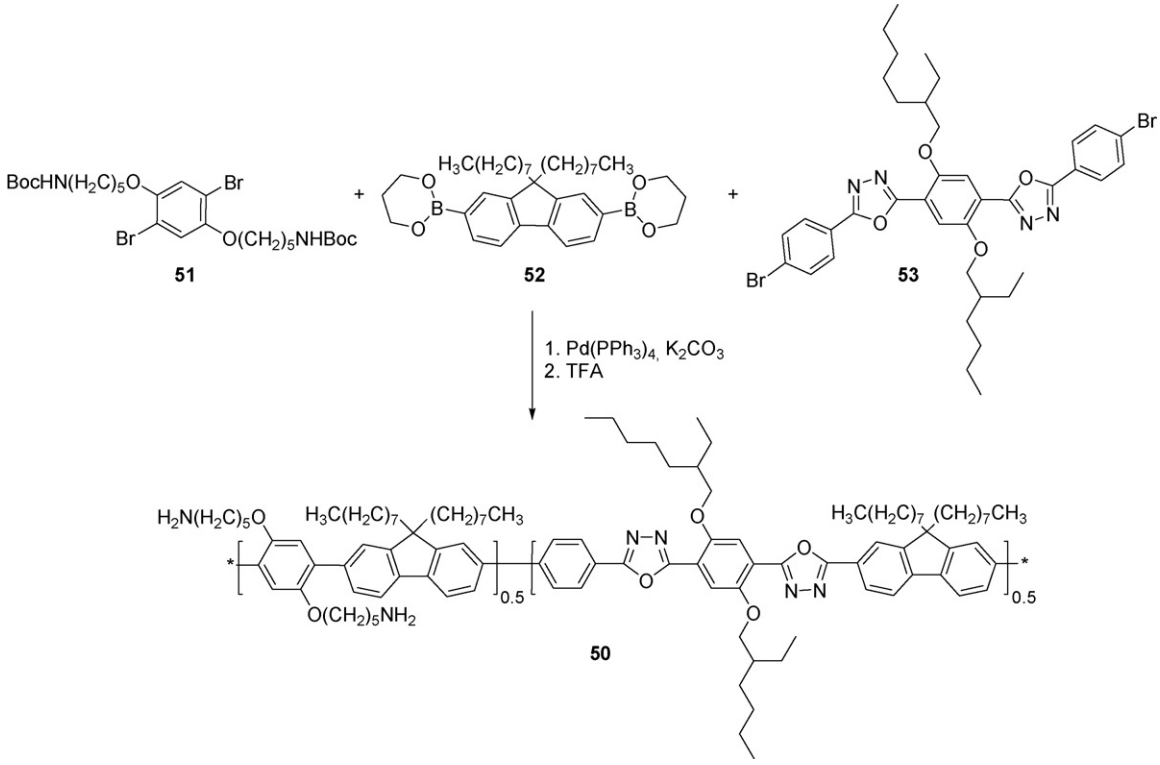

Fig. 22. Synthesis of conjugated polymer **50** through Suzuki coupling [84].

sion enhancement was observed upon hybridization between HEX-labelled ss-DNA and its complementary PPE-DNA bioconjugate immobilized on a solid phase, whereas the same experiment in solution did not provide any fluorescence emission enhancement. This behaviour was explained, according to the literature [83], taking into consideration the different intermolecular packing in solution or in solid-state for this kind of polymer.

3.2.3. *Oxadiazole-phenylene-fluorene copolymers*

In 2007 Kim's group [84] reported on a strategy to produce a signal-amplifying DNA microarray making use of a novel conjugate polymer combined with an on-chip synthesized DNA approach [85]. Poly(oxadiazole-*co*-phenylene-*co*-fluorene) **50** was obtained starting from monomers **51**, **52** and **53** by Pd-catalyzed Suzuki reaction and subsequent Boc deprotection with trifluoroacetic acid (Fig. 22).

Every monomer was tailored with the aim of conferring special properties to the final polymer **50**. The amine groups on **51** were introduced both to immobilize the polymer on the surface and link DNA strands directly synthesized on-chip. Monomer **52** contains a fluorene unit to confer to **50** good solubility in organic solvents and to ensure a good spectral overlap with the commonly used organic dyes in order to provide an efficient FRET. Monomer **53** comprises an electron-poor oxadiazole units to confer resistance against harsh conditions, such as long expo-

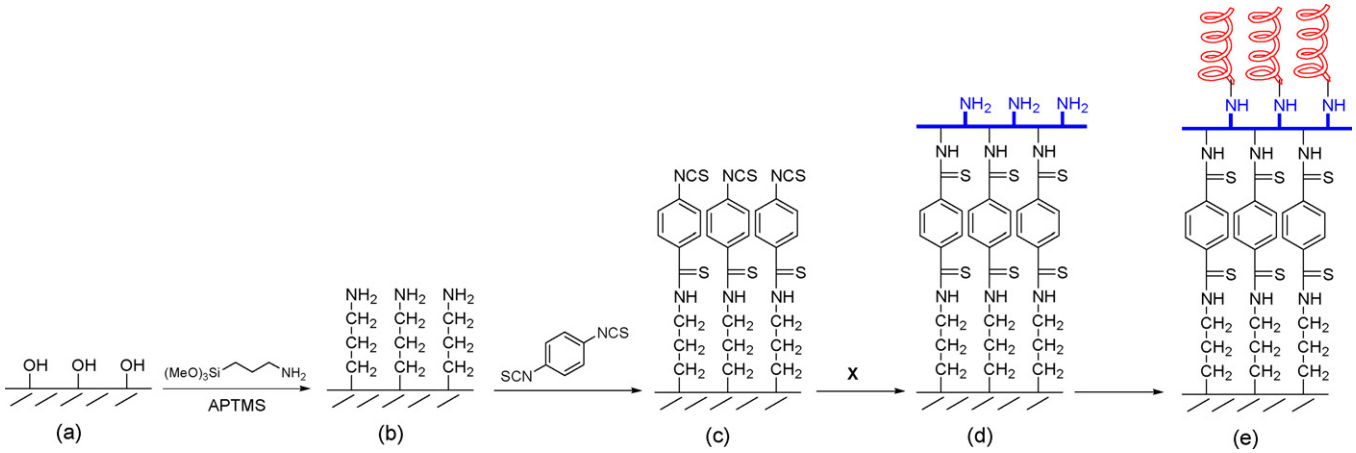

Fig. 23. Schematic representation of on-chip DNA synthesis on a glass slide polymer **50** coated. (a) Bare glass. (b) Amine group introduction. (c) Derivatization with 1,4-phenylenediisothiocyanate. (d) Polymer **50** immobilization. (e) On-chip DNA synthesis [84].

sure to UV light or to the strongly acidic environment needed for the on-chip DNA synthesis.

Glass slides were functionalized with primary amine groups by organosilanization with aminopropyltrimethoxysilane and subsequent reaction with 1,4-phenylenediisothiocyanate. The conjugate polymer **50** was linked to the surface through formation of a thioureic bond involving some of its amines. The remaining amino groups were used to synthesize ss-DNA using 5′-(4,4′-dimethoxytrityl) (DMT) nucleophosphoramidite monomers as building blocks, which were step-by-step easily deprotected with UV-induced decomposition of a photoacid generator (Fig. 23).

This on-chip synthesis provided a surface with an oligonucleotide density of 2.44 pmol cm^{-2} and a detection limit of 10^{-10} M. A good selectivity in discriminating sequence mismatches was demonstrated.

4. Conclusions

The examples reported in this review demonstrate the great potentiality of synthetic polymers in the field of biomacromolecule recognition. Polymers obtained with a number of different procedures were conveniently used as surface coatings. The possibility of modifying polymer characteristics by appropriate derivatization allowing the tailoring of surface properties and the introduction of functional groups useful to bind biomolecules. In addition polymers can be used to create a surface environment that prevents non-specific binding and protein denaturation.

Another interesting field of research in the context of biorecognition is the synthesis of polymers, with optical and electrochemical properties, suitable for highly sensitive and selective detection of biomacromolecules. The examples presented in this review give a general picture of the potentiality in high-throughput screening for drug discovery and diagnostic applications.

Nomenclature

AFM atomic force microscopy
AIBN 2,2′-azo-bis(isobutyronitrile)
ATRP atom transfer radical polymerization
BSA bovine serum albumine
CTA chain-transfer agent
DMA *N*,*N*-dimethylacrylamide
DMF dimethylformamide
dnNbpy Cu(II)-4,4′-dinonyl-2,2′
ds-DNA double strand DNA
EDC *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide
(E)GFP (enhanced) green fluorescent protein
EOF electroosmotic flow
FCR fluorescence chain reaction
FRET fluorescence resonance energy transfer
GMA glycidyl methacrylate
HEX hexachlorofluorescein
IgG immunoglobuline G
MALDI matrix assisted laser desorbtion/ionization
MAPS [3-(methacriloyl-oxy)propyl]trimethoxysilane
Me_4Cyclam Cu(I)-1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane
NAS *N*-acryloyloxysuccinimide
NHS *N*-hydroxysuccinimide
NTA nitriloacetic acid
PAA poly(acrylic acid)
PAH poly(allylamine)
PDMA poly(*N*,*N*-dimethylacrylamide)
PDMS poly(dimethylsiloxane)
PFBT poly[9,9′-bis(6″-*N*,*N*,*N*-trimethylammonium)hexyl)fluorene-*co*-*alt*-4,7-(2,1,3-benzothiadiazole) dibromide]
PNA peptide nucleic acid
PPE poly(*p*-phenyleneethynylene)
RAFT reversible addition-fragmentation chain-transfer
RF rheumatoid factor
ss-DNA single strand DNA
sulfo-NHS *N*-hydroxysulfosuccinimide
SEM scanning electron microscopy
SFM scanning force microscopy
THF tetrahydrofurane
XRR X-ray reflectometry

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