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Recent advances in surface functionalization techniques on polymethacrylate materials for optical biosensor applications

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Biosensor chips for immune-based assay systems have been investigated for their application in early diagnostics. The development of such systems strongly depends on the effective protein immobilization on polymer substrates. In order to achieve this complex heterogeneous interaction the polymer surface must be functionalized with chemical groups that are reactive towards proteins in a way that surface functional groups (such as carboxyl, –COOH; amine, $–NH_2$; and hydroxyl, –OH) chemically or physically anchor the proteins to the polymer platform. Since the proteins are very sensitive towards their environment and can easily lose their activity when brought in close proximity to the solid surface, effective surface functionalization and high level of control over surface chemistry present the most important steps in the fabrication of biosensors. This paper reviews recent developments in surface functionalization and preparation of polymethacrylates for protein immobilization. Due to their versatility and cost effectiveness, this particular group of plastic polymers is widely used both in research and in industry.

Introduction

Biosensors, "biochip" or "lab-on-chip" devices are important to various fields such as food safety, medical diagnostics, environmental regulation, and many others.[1–11] The major principle behind such devices is that the signal is generated by the recognition of an analyte by a bioactive compound and the

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generated signal is further analyzed by different transduction methods such as: electrochemical biosensing, fluorescence-based detection, cyclic voltammetry, electrochemical impedance spectroscopy, surface plasmon resonance (SPR), potentiometric signal transduction, gravimetric, thermal and other analytical techniques.[12–15] Recent advances in biosensor technology also include quartz crystal microbalance (QCM)[16] and Fourier transform infra-red attenuated reflection (FTIR-ATR) based sensors that allow determination of spectroscopic signatures of the interacting molecules.[17] In most cases the bioactive compounds are proteins such as antigens, antibodies, DNA, cell-binding proteins, *etc.* One of the major challenges is the preservation of protein activity in order to achieve high sensitivity and accuracy of biosensors.[18] In view of the current analytical challenges smaller molecules called "aptamers" have been used in order to overcome the limitations of conventional antigens/antibodies.[16] The use of smaller molecules such as protein sequences, can possibly offer better stability and less complicated analytical procedures. Whichever approach is used, there is still a strong demand for high accuracy, selectivity, lower detection limits and robustness of biosensors.[15]

In recent years, polymer supports to which bioactive compounds have been immobilized for subsequent analyte recognition and signal generation have shown high potential in sensitivity improvement and ultimately miniaturization of bioanalytical devices.[19] The ideal biosensor should possess high sensitivity, high accuracy (detectability) and selectivity, cost effectiveness of manufacture protocol and mobility.[20] One of the widely known applications of such polymer-based devices is a technique called enzyme-linked immune-sorbent assay (ELISA).[21] ELISA is currently used in many applications such as determination of serum antibody concentrations, food allergens, concentrations of certain types of drugs, as well as detection of cytokines and cancer biomarkers.[13] Possibly the most well-known clinical application of ELISA tests world-wide is the detection of HIV virus infection in human blood.[13] The basic principle of this method relies on antibody (protein) immobilization on polymeric surfaces and subsequent antigen detection in the blood samples from infected humans.

One of the most significant challenges of manufacturing viable lab-on-chip biosensors is the correct choice of the solid surface and the development of appropriate surface chemistry, using a diverse range of proteins; the proteins immobilized on the solid surface must maintain their integrity, native conformation, and biological function/activity.[21] Protein attachment must be closely controlled with respect to chemical selectivity; the functional groups of proteins that are directly immobilized to the functional groups of the polymer surface must be defined in detail.[22] There are many parameters that would influence protein activity when placed in direct contact with the surface. For that reason it is desirable to control the regio-selectivity of the reaction (protein immobilization) as the protein orientation could vary thus compromising the effectiveness of immobilization methods.[23] Possibly the most common surface functionalization of polymers is depicted in Scheme 1: the first step in successful protein immobilization is the formation of chemical functionalities such as hydroxyl (–OH), amine ($–NH_2$) or carboxyl (–COOH) groups. Note that there are several other functional groups that can be generated on the surface such as carbonyl, thiol, aldehyde, phosphate, silane, and hydroxyl.[23] In any case, surface modification must be carried out with great care. For example, if initial surface functionalization does not generate enough surface reactive groups, the compound (protein) can lose activity when placed in direct contact with the hydrophobic polymer surface (Scheme. 1A). On the other hand, too many functional groups can cause steric repulsion between proteins which can also cause protein deactivation (Scheme. 1B). For these reasons, polymer functionalization must be completed with a high degree of control over the surface properties upon potential chemical and physical treatments.[23]

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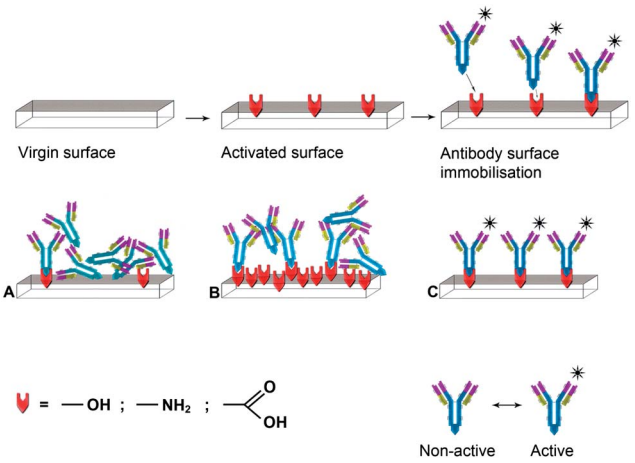


Scheme 1 Bio-activation strategy for production of polymer detection platforms in biosensor devices. Top: polymer surface modification by introduction of reactive functional groups and subsequent antibody surface immobilization. Middle: effect of the functionalized polymer surface on protein bioactivity: (A) protein denaturation (or deactivation) due to the low surface concentration of functional groups which causes the proteins to "fall" on the polymer surface; (B) reduced antibody immobilisation and protein deactivation due to over-functionalization of the polymer surface; and (C) optimal surface concentration of functional groups and formation of a detection platform with active immobilized antibody molecules.[23]

Another novel strategy to design the reusable functionalized platform for protein immobilization is a reversible protein interaction on the biosensor surface. Such systems have been developed on silicon nanowire field-effect transistors (SiNW-FETs) as promising devices with ultra-sensitivity, multiple use and label-free selective capabilities.[24] In such cases, the existence of surface functional groups prior to protein immobilization is highly desirable. In a recent study by X. Duan *et al.* the SiNW-FETs were used for quantification of kinetics of protein interactions by translation of the analyte–surface interaction into an electrical signal for high sensitivity measurements.[25] The authors have used amine-functionalized SiNWs with a 2.9 nm polyethylene glycol (PEG) spacer in order to effectively immobilize biotin for investigation of biotin/streptavidin interaction.[25] Whether the biosensor detection is performed on polymeric surfaces (chips) or other types of devices (nanowires) the existence of the surface functional groups still presents an important feature of biosensors. In any case, one should be aware of the analytical challenges in establishing the exact conformation of proteins on the surfaces used in biosensor applications.[26] The existing way to analyze the effectiveness of biosensors is only through direct verification of protein activity once the immobilization has been performed, regardless of the type of detection and signal transduction applied in biosensor development.[13,27]

It is clear that the polymer surface functional groups play important roles in protein immobilization. Polymeric materials offer such possibility as they can be processed and designed by means of synthetic procedures and controlled surface chemistry. The most common polymeric materials for biosensor devices are polystyrene (PS), polydimethylsiloxane (PDMS), polyethylene terephthalate (PET), polycarbonate (PC), cyclic olefin copolymer (COC) and poly(methyl methacrylate) (PMMA).[28,29] Table. 1 shows the advantages and disadvantages, together with some recent applications of these substrates. The major advantages of polymeric materials are their low cost, processability on the mass scale and the possibility to integrate microelectronics and microfluidic technology onto the surface of designed chips.[30–33] In comparison to polymer systems for developing fluorescence-based diagnostic devices, PMMA has many advantageous properties such as low intrinsic fluorescence, transparency and versatility in fabrication.[34]

Microfluidic devices based on plastic materials show great promise due to their versatility, the small amount of sample required for analysis and the possibility of high throughput screening.[19] Miniature devices called Biological Micro-Electro-Mechanical Systems (BioMEMS) have shown important biomedical applications as high sensitivity diagnostic devices.[35–37] BioMEMS can be defined as "devices or systems, constructed using techniques inspired from micro/nano-scale fabrication, that are used for processing, delivery, manipulation, analysis, or construction of biological and chemical entities".[38–41] Many BioMEMS systems are based on PMMA materials and other polyacrylate systems and in most cases surface modifications are required since the surface of PMMA is not reactive and hydrophobic.[40]

The surface of the PMMA can be modified in different ways (both chemically and physically) while maintaining both transparency and original mechanical properties. Although several approaches are available to modify the PMMA surface for protein detection, the major strategy is to chemically functionalize the PMMA plastic by surface treatment and introduction of hydroxyl (–OH) carboxyl (–COOH), or amine ($–NH_2$) reactive groups,[23] as depicted in Scheme 2. Those functionalities can be further reacted in order to immobilize (both physically and chemically) proteins in biochips and microfluidic devices produced from PMMA.[29] In this article, we will discuss some recent advances in the design and surface modification of polyacrylate materials (mainly PMMA) for biosensor applications.

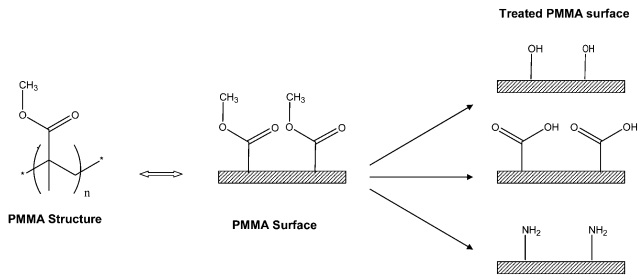
The discussion is divided into three general parts, reviewing strategies for the polymer surface treatments such as: (i) wet chemical treatment; (ii) plasma treatment; and (iii) radiation and photo-grafting. Fabrication techniques for biochip integration and micro-fluidic channel production for BioMEMS are excluded from the present discussion. For protein immobilization strategies that are not covered in the current review, readers are referred to the excellent review papers published previously.[19,22,23]

Wet chemical treatment and creation of polymer surface functional groups

Wet chemical treatment methods of PMMA surfaces in biosensor applications can be generally divided into four approaches: (i) acid or alkaline treatments; (ii) amine functionalization; (iii) glutaraldehyde treatment of aminated

Table 1 Development of polymeric substrates for diagnostic biosensor applications

Polymer	Advantages	Disadvantages	Recent applications
PMMA	Low intrinsic fluorescence High optical transparency Low cost High scratch resistance Versatility in fabrication Impact resistance	Limited compatibility with organic solvents	A microfluidic biosensor with interdigitated ultramicroelectrode (IDUA) arrays for electrochemical quantification of specific nucleic acid sequences[42]
PS	High optical transparency Chemical inertness Commercially available in different colours Low cost Standard cell-culture material	Limited compatibility with organic solvents Ineffective barrier to oxygen and water vapor	High refractive index coating for a label-free long period grating (LPG) biosensor[43]
PC	Thermal molding Durability and high strength High optical transparency Commercially available	Low scratch resistance	A microfluidic electrochemical biosensor chip for micro flow-injection amperometric determination of glucose[44]
PET	Recycled material Semi-transparent Moisture barrier Semi-crystalline Impact-resistant Light weight and flexible	Discoloration over the period of degradation	Silver-nanoparticles-on-plastic (PET): a localized surface plasmon resonance (LSPR) biosensor[45]
COC	Good performance in microfabrication High optical transparency High moisture resistance Low dielectric constant High heat resistance	Relatively expensive Limited data available in the literature	On-chip DNA, LSPR detection from a gold nano-ring coated substrate integrated with a COC based microfluidic device[46]
PDMS	Elastomeric material Good performance in microfabrication Non-toxic	Gas-permeable Limited compatibility with organic solvents Degree of elasticity is temperature dependent	PDMS/paper/glass hybrid microfluidic biochip for one-step multiplexed pathogen detection[47]



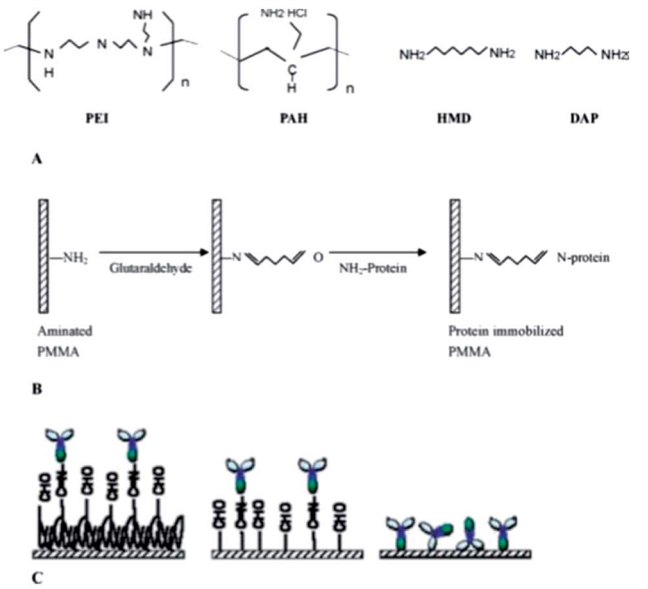
Scheme 2 Functionalization of PMMA and expected outcome with reactive surface groups: (top) hydroxyl; (middle) carboxyl; and (bottom) amine functional groups on the treated PMMA surface.

surfaces; and (iv) siloxane treatment by the sol–gel technique. The expected result of all wet chemical treatments is a polymer surface with reactive functional groups for further protein immobilization.[48–51] Possibly the major advantage of this classical approach is that the method does not require specialized equipment. Another important advantage is that the reagent could penetrate porous three-dimensional substrates important for functionalization of microfluidic devices.[23,52–54]

Base and acid hydrolysis have been used to generate carboxylic acid groups on PMMA with concentrated sodium hydroxide and sulfuric acid.[55,56] In particular it has been reported that 16 h treatment in 10 M sodium hydroxide at 40 °C resulted in 0.66 nmol cm^{-2} carboxylic acids on PMMA.[23] Other reports also describe the chemical conversion of PMMA methyl ester side chains by various chemical agents.[23,57,58] For example, Y. Liu *et al.* have used base treatment in order to produce PMMA microfluidic chips coupled with an electrochemical detection system for detection of trace levels of α-fetoprotein hepatocellular carcinoma biomarker (AFP) by immobilizing the AFP antibody on the PMMA microfluidic channels.[21] The microchips were first treated with 1 M NaOH solution at 55 °C for 30 min in order to produce –OH surface groups and the chips were subsequently immersed in poly(ethyleneimine) (PEI) solution (0.2%, pH = 7.0) at room temperature for 1 h to derive $–NH_2$ for

further protein attachment (surface characterization of PMMA following surface treatment has not been reported).[21]

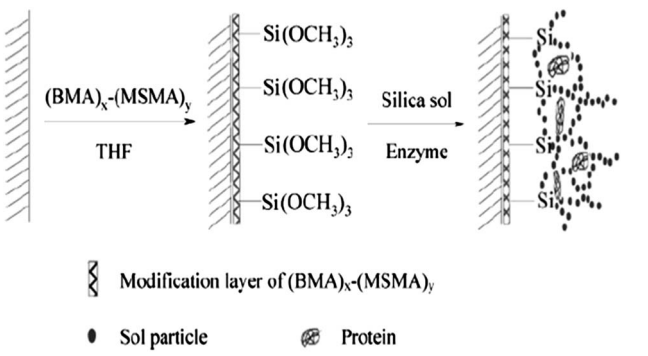
Amine-terminated molecules of different sizes and structures are often used as intermediate compounds between the PMMA surface and the actual proteins. These molecules are usually called "spacers" and they provide a useful way to preserve the activity of immobilized proteins (Scheme 3). The chain length and the structural morphology of amine-terminated linkers are important parameters to consider for potential protein immobilization on biochips or BioMEMS devices. In order to examine the effect of amine structure on protein activation and detection sensitivity of microfluidic immunoassay chips, Y. Bai *et al.* have developed a microfluidic immunoassay ELISA device produced from PMMA (140 μm wide, 125 μm deep, and 1.5 cm long) by using a computer numerical control (CNC) machine. A surface wet treatment method was introduced using different amine-bearing polymers: PEI, poly(allylamine hydrochloride) (PAH), hexamethylenediamine (HMD), and 1,3-diaminopropane (DAP), in order to enhance antibody binding on the PMMA surface (Scheme 3).[31] For PEI or PAH treatment, PMMA plates were first treated in 1 N NaOH and then immersed in a PEI or PAH solution (0.2%, pH 7) at room temperature for 1 h. For, HMD and DAP treatment, PMMA plates were immersed directly in polymer solutions without prior NaOH treatment.



Scheme 3 Schematics of antibody binding on different PMMA surfaces treated with different amine-bearing chemicals: (A) chemical structures of PEI, PAH, HMD, and DAP amine spacers; (B) steps in PMMA surface treatment and subsequent antibody binding: amination, cross-linking with glutaraldehyde and covalent binding of antibody; (C) illustration of the spatial effects of different surface modifications on antibody binding on the PMMA surface. Left: PEI or PAH coated PMMA surface provides a larger spacer length to allow the antibodies to stay away from the solid surface. Middle: the HMD or DAP treated surface provides active binding sites on the PMMA surface. Right: passive adsorption of antibodies on the PMMA that could result in disorientation and activity loss of the antibody upon its binding on the surface. Reprinted with permission from ref. 31, Y. Bai *et al.*, *Langmuir*, **22**, 2006, 9458. © 2006, American Chemical Society.

Aminated PMMA plates were subsequently treated with glutaraldehyde for antibody binding as depicted in Scheme 3. By treating the PMMA surface of the micro-channel on the microfluidic device with a longer amine spacer such as PEI, antibodies can be bound to the micro-channel surface with the antibody activity preserved. As compared to surfaces treated with small amine spacer molecules (HMD and DAP, Scheme 3), both PEI and PAH were able to significantly increase the distance between antibody and surface, and thus afforded the antibody a sufficient spacial freedom. The larger spacer also allowed more antibodies to bind on the surface thus increasing the sensitivity of the potential diagnostic device: PEI-treated PMMA micro-channels gave higher fluorescence signals with an improved signal/noise ratio and performed better for ELISA applications.[31] However, use of spacers in general may present a more complicated approach for direct binding of proteins thus requiring specialized expertise.[27]

The precise surface patterning of proteins is critical in the development of micro analytical biosensor systems. The major goal is to create a selective biochip device that enables high-affinity binding to the analytes of interest.[59] Sol–gel technology, a technique considered to be a relatively mild route for the immobilization of proteins, has been used to generate protein microstructures on solid polymer or glass supports.[60] In this particular process the biomolecules are entrapped in the swelling gel network instead of being chemically attached to the surface functional groups.[60] In terms of protein stability, it has been reported that sol–gel entrapment provides improved resistance of biomolecules towards chemical and thermal denaturation.[61] Due to this reason there is a promising future for utilization of the sol–gel technique in the production of robust biosensor devices (Scheme 4). However, there are two important factors that surface scientists must consider in terms of sol–gel protein immobilization onto the solid PMMA surface: (1) in the case of relatively hydrophobic plastic surfaces (such as PMMA), direct contact with proteins might cause protein denaturation; and (2) because there is no intermolecular interaction between the gel matrix and the PMMA substrate, it is



Scheme 4 Process summary of the functionalized PMMA with silane groups originating from the $(BMA)_x$–$(MSMA)_y$ copolymer coating as a surface modification layer. The copolymer coating of the PMMA substrate is followed by sol–gel protein immobilization. Reprinted with permission from ref. 41, H. Qu *et al.*, *Anal. Chem.*, **76**, 2004, 6426. © 2004, American Chemical Society.

necessary to develop a routine, simple, and well-defined surface functionalization of PMMA and modification protocols in order to improve the biomolecule immobilization entrapped within the gel system.[41,61,62] For these reasons, gel systems with entrapped active proteins must be appropriately anchored to the solid surface through created surface functional groups such as silane functionalities for silicon gel anchoring. H. Qu *et al.* have designed a graft copolymer system in order to introduce the silane functional groups onto the plastic PMMA surface (Scheme 4).[41] For that purpose the authors reacted butyl methacrylate (BMA) and methylacryloxy-propyl-trimethoxysilicane (MSMA) in a well-defined free-radical polymerization reaction by using azobisisobutyronitrile (AIBN) as a free-radical initiator. The concentration of pending silane functional groups from the resulting $(BMA)_x$–$(MSMA)_y$ copolymer was controlled by simple variation of the initial BMA–MSMA molar ratio (x/y) in free-radical polymerization. The control over the copolymer composition provides an optimal concentration of pendant silane groups (Scheme 4). The schematic view of $(BMA)_x$–$(MSMA)_y$ modification of the PMMA surface and subsequent immobilization of encapsulated proteins within silica gel is presented in Scheme 4. With this technique a physical immobilization of proteins can be achieved onto the hydrophobic PMMA micro-channels with the bioactivity preserved as far as possible. Due to a relatively large microstructured surface area and hydrophilic, cross-linked silica gel morphology, the encapsulation of proteins in this matrix has a strong potential for high biomolecule loading and an increase of detection sensitivity through preserving bioactivity in the microfluidic device.[41]

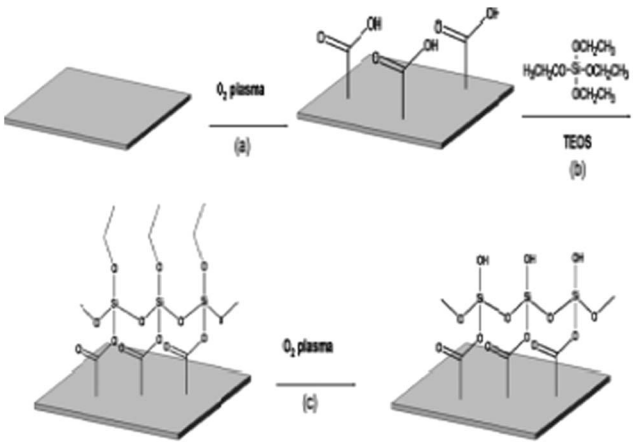
However cost-effective and simple the wet chemical treatment might appear, there are considerable disadvantages of the method. One of the major concerns is that the wet chemical methods could often be non-specific, generating undesirable irregular surface etching.[23] Furthermore, acid or alkaline treatments can produce a range of oxygen-containing functional groups on the plastic surface. In particular the chemical (wet-treated) PMMA ester conversion of side chains will strongly depend on the side chain orientation.[5] Another important aspect is the stability of chemically modified surfaces. For example, siloxane linkages (described in the previous paragraph) can be hydrolyzed in alkaline solutions or at elevated temperatures.[63] There is an environmental concern about generation of toxic wastes when wet chemical treatments are applied on an industrial scale. For the above mentioned reasons, the efforts in scientific research have also been and still are focused on alternative methods for surface modifications through plasma and radiation treatments. The relevant findings in plasma treatments of polymethacrylate surfaces are described further in the text.

Plasma surface treatment of PMMA platforms for protein immobilization

Possibly one of the major advantages of plasma treatments of plastics (PMMA) over wet chemical methods is that the plasma treatment can provide modification of the top nanometer layer with much less roughening and surface degradation.[64] Another important advantage is that plasma treatment does not involve toxic and corrosive solvents and therefore can be utilized on the industrial scale without major concerns about the environmental impact.[65–71] There are several types of plasma functionalization of plastic surfaces and the imparted functionalities can be varied by selection of precursor gases in plasma treatments and the operating parameters (precursor flow rate, pressure inside the plasma chamber, power and time of the sample exposure).[72,73] Many different gases have been used in plasma treatments such as: Ar, O_2, N_2, CO_2, NH_3, as well as vapors from H_2O and various liquid amines.[73–79] The major idea remains the same as for the wet chemical surface treatments: generation of reactive functional groups for further protein immobilization (amine, $-NH_2$, carboxyl, –COOH and hydroxyl, –OH). Functional groups such as aldehyde or epoxide can also be achieved through "plasma polymerization" for subsequent coupling of proteins with "gentle" methods that would retain protein function.[27]

Plasma polymerization methods offer many advantages such as: single-step dry surface functionalization, fabrication of thin functional coatings on materials of different shapes and sizes, and uniformity of the coated layers.[80] There are many biological applications of plasma polymerization including fabrication of both bio-reactive and non-bio-reactive coatings for microfluidics, cell culture substrates, regenerative medicine and tissue engineering.[80,81] Another important aspect is that plasma treatment changes the surface from the hydrophobic to the hydrophilic state, a very important feature for protein stability on the plastic surface.[23]

Generally, the PMMA surface is considered to be hydrophobic in terms of protein–surface interaction. The reported water-in-air contact angle values for PMMA plastic are in the range of 80°–90°.[34] For example, in antibody/antigen immobilization techniques for diagnostic microfluidic devices, the interaction of antibodies with hydrophobic surfaces may cause protein denaturation leading to poor protein activity.[19] Making a surface protein-compatible is the necessary step in any type of surface modification including plasma treatment. Plasma treatment creates the desired charged groups and increases the "overall surface energy", which in turn also increases hydrophilicity and wettability.[40] Through the generation of polar functional groups on the polymer surface, hydrogen or covalent bonds may be formed across the interface thus strengthening the bonds between the two contacting surfaces.[27] Y. N. Tennico *et al.* reported an improved bonding method *via* surface modification of plastic and glass materials. The PMMA surface was treated in oxygen plasma in order to produce –COOH groups on the polymer surface as illustrated in Scheme 5.[82] Immediately following the plasma treatment, the surface was silanized by wet chemical treatment (10% tetraethyl orthiosilicate TEOS solution in 60 : 40 (v/v) isopropyl alcohol–water mixtures). The silane-functionalized surface was activated for the second time with oxygen plasma under the same experimental conditions as the first plasma treatment. Although the authors used this technique for physical bonding (gluing) of polymer surfaces for



Scheme 5 Schematic representation of the PMMA surface modification using oxygen plasma and TEOS. Secondary oxygen plasma treatment has been performed in order to activate the PMMA surface for physical bonding (gluing) of polymer surfaces and fabrication of micro-channels in micro-fluidic devices. Reprinted with permission from ref. 82, Y. H. Tennico *et al.*, *Sens. Actuators, B*, **143**, 2010, 799. © 2009, Elsevier.

fabrication of microfluidic channels, it is important to note that the reported functionalization method could also be used for protein immobilization through either covalent binding (*via* –COOH groups) or physical encapsulation *via* the sol–gel technique (Scheme 5). One of the major concerns in plasma treatments of polymers (such as PMMA) is surface ageing. Plasma-generated functional groups are generally not stable with time, as the high-energy plasma-treated polymer surface tends to "relax" to its untreated state. Reports indicate that oxygen-treated PMMA results in more hydrophilic –COOH groups so that aging can affect the induced hydrophilicity thus compromising the performance in terms of the protein activity immobilized on the treated PMMA surface.[23,72]

Generally, the ageing effect of plasma-treated polymers is dependent on different storage conditions such as humidity, temperature, *etc.* The major processes responsible for surface ageing are: (i) re-orientation of the polar groups on the surface; and (ii) mobility of small polymer fragments into the polymer bulk. In either case, the result of aging is usually the loss of surface activity and decrease in surface energy. A. Vesel and M. Mozetic have observed the ageing effect on PMMA surface after oxygen plasma treatment. The ageing experiment was performed in order to compare different plasma treatment times and storage conditions: the surface was characterized by contact angle experiments, atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS) for the PMMA samples stored in water and air.[83] Oxygen-plasma treated PMMA samples were also stored in different temperatures in order to establish the temperature effect on ageing. The authors reported that increased treatment time induces higher crystallinity at the surface thus decreasing the rate of aging significantly. High crystallinity induces limited surface mobility and therefore prevents the surface re-organization after treatment. As expected, the highest aging effect was observed for the samples stored in water and in elevated temperatures.[83,84] Note that further protein immobilization onto the plasma-treated PMMA surface also causes alteration in the surface morphology (measured with AFM; Fig. 1).[84]

As we mentioned at the beginning of this section, different precursors in plasma treatments can be used for PMMA surface functionalization. For example, ammonia and nitrogen plasmas have been used to impart amine groups to the polymer surface, while inert gases can be used to introduce radical sites on the polymer surfaces thus introducing the reactive sites for subsequent graft polymerization.[85] For instance, polymers can be pre-treated with Ar in order to activate the surface for further graft polymerization of acrylic acid (AA) thus producing a polymer coating with surface –COOH groups.[86] E. T. Kang *et al.* pre-treated PTFE (Teflon™) with Ar plasma in order to graft polymerize AA on the PTFE surface. In a process called radio frequency glow discharge (RFGD), plasma is used to produce reactive species from vaporized monomers and thus induce a subsequent graft polymerization.[87] One of the most important advantages of RFGD treatment is that it can be used to modify surfaces of porous structures without compromising the bulk properties of the material.[73] Apart from RFGD there are other technical approaches in ionized-gas/plasma treatments such as the Low Pressure Chemical Vapor Deposition (LPCVD) process where the materials (precursors) are deposited under low pressure conditions. The result is usually a more uniform layer of material; however, a high temperature is required so this fabrication process is not suitable for some thermally unstable precursors. Contrary to LPCVD, the Plasma Enhanced Chemical Vapor Deposition (PECVD) process allows reactions in plasma chamber at lower temperatures. In this technique the gases/vapors (precursors) are ionized by plasma within the evacuated chamber and subsequently deposited onto the polymer substrate. The major drawback of PECVD is that the films (coatings) produced by this process could result in a questionable quality.[39]

One of the considerable disadvantages of plasma treatments is reorganization of the surface and the ageing effect. Apart from that, the vacuum technology such as plasma treatment requires a close control over many parameters involved in optimization, which might cause serious complications when

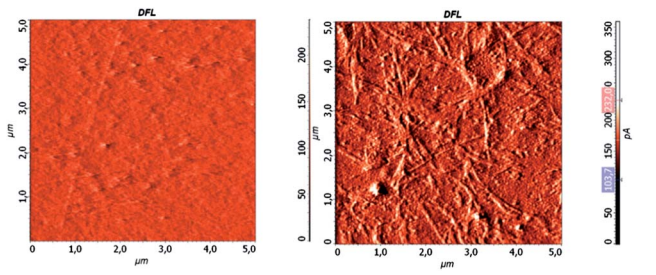


Fig. 1 AFM investigation of the protein immobilization effect on the surface morphology of PMMA. Left: virgin PMMA surface. Right: PMMA with immobilized streptavidin through RFGD plasma-generated functional groups on the treated PMMA surface. Reprinted with permission from ref. 84, A. Vesel *et al.*, *Vacuum*, **86**, 2012, 773. © 2012, Elsevier.

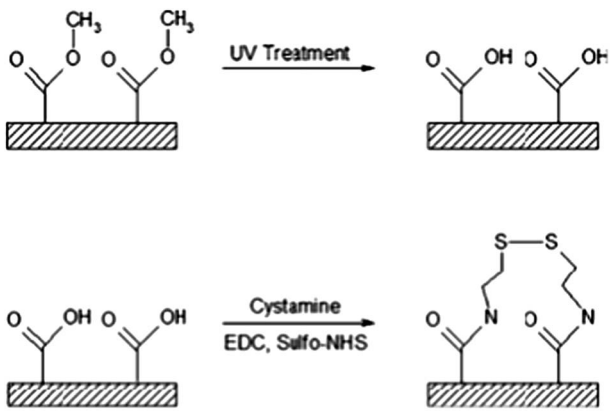
intended for the industrial scale. Other than that, plasma treatment results are sometimes difficult to repeat between the laboratories due to the complicated process requirements.[74] The most important parameters include reaction time, process temperature, chamber pressure, orientation of the reactor, distance between the substrate and electrode and the gas/precursor flow rate.[73] There is also a substantial risk of contamination due to the high sensitivity of the method so for that reason the plasma chamber should be cleaned thoroughly after each alteration of the reaction gas or the chemical precursor.[23]

UV treatment and polyacrylate photo-grafting

One of the accepted approaches in polymer surface activation is an exposure to UV light. The expected outcome of such treatment is generation of intermediate reactive sites. These semi-stable active sites can become functional groups in the second stage of the treatment depending on the gas chosen for surface treatment.[23,88–91] Generally, the exposure of PMMA to UV light should result in the formation of surface –COOH groups that are later utilized for protein immobilization *via* carbodiimide chemistry. S. R. Nugen *et al.* have developed a disposable electrochemical microfluidic biosensor based on the PMMA plastic. The strategy was to immobilize cystamine proteins onto the thiolated PMMA surface previously treated with UV light (Scheme 6).[92] The surface concentration of –COOH groups have been measured with toluidine blue assay and contact angle measurements.[92–94] The results have been compared for both UV and UV/ozone treatments and it has been found that the UV treatment (without ozone) causes much higher decrease in the contact angle in comparison to the virgin PMMA which is directly related to the generation of hydrophilic –COOH groups on the PMMA surface.[92]

In regards to the UV treatment of PMMA surfaces, the surface concentration of generated –COOH groups is strongly dependent on the exposure time. C. Situma *et al.* have performed surface modification of PMMA by UV treatment for subsequent fabrication of DNA microarrays. The treatment time ranged from 5 to 30 min. The surface concentration of –COOH groups was measured and the results show saturation within 15 min. Another important finding was that the surface density of immobilized protein was found to decrease after 20 min of UV photo-oxidation. Such behavior was attributed to the PMMA surface degradation at long exposure times.[95] According to the report, the main-chain scissions of methyl ester groups are the most likely result of high UV doses.[95] For those reasons, high exposure times are not desirable due to the high degradation rate which reduces the number of methyl ester groups converted to –COOH groups by UV photo-oxidation.[95] Another way of introducing –COOH functionalities on the polymer surface is UV-induced photo-grafting.[49] This reaction is important for the close control over the surface concentration of immobilized proteins and creation of protein gradients on the polymer surface. B. Li *et al.* described the method of graft polymerization of AA on pre-irradiated PS surface.[93,94] In their work, peroxides were generated on the substrate (PS) surface by UV pre-irradiation and they initiated graft polymerization of AA onto the surface upon second UV irradiation (Fig. 2). A PET mask was used to create the surface gradient of polyacrylic acid (PAA) as PET film can be used to cut-off UV light with a wavelength below 310 nm. The proteins were immobilized to PAA-grafted surfaces by the coupling reaction between $-NH_2$ groups of proteins and –COOH groups on grafted chains by carbodiimide chemistry (Fig. 2).[94]

Although, in this work the authors have created protein gradients in order to observe fundamental cell properties when attached on the bio-activated polymer surface, the reported method can be used for creation of biosensor devices with a high level of control and potential increase of sensitivity.[93,94] One of the major advantages of the photo-grafting process is that the surface modification can be carried out under mild conditions thus eliminating the need for high energy consumption, expensive equipment and excessive use of solvents.[96–103] The surface activation with proteins can be performed in only few steps such as: UV light irradiation, carbodiimide coupling and washing.[93,94] According to B. Li *et al.* the photo-activation method makes array fabrication simple and less time consuming and does not require extensive amounts of specialized reagents to produce robust linkages with high surface densities.[94] Another important characteristic is that the active functional groups are introduced with simultaneous polymerization. For that reason no additional activation process is required for covalent immobilization such as alkaline/acid treatment or plasma activation. Furthermore, there is an indication of lower detection limits reported for photo-grafted epoxy resin-based immunoassay devices.[95] However, UV treatments can affect the optical properties of the treated polymer. There is also a question of polymer degradation at the surface and creation of unstable functionalities that would affect the storage capacity due to the surface relaxation and ageing effect.



Scheme 6 Surface functionalization of PMMA with UV irradiation and subsequent carboxylic acid conjugation to cystamine using the carbodiimide (EDC/sulfo-NHS) immobilization protocol. Reprinted with permission from ref. 92, S. R. Nugen *et al.*, *Biosens. Bioelectron.*, **24**, 2009, 2428. © 2008, Elsevier.

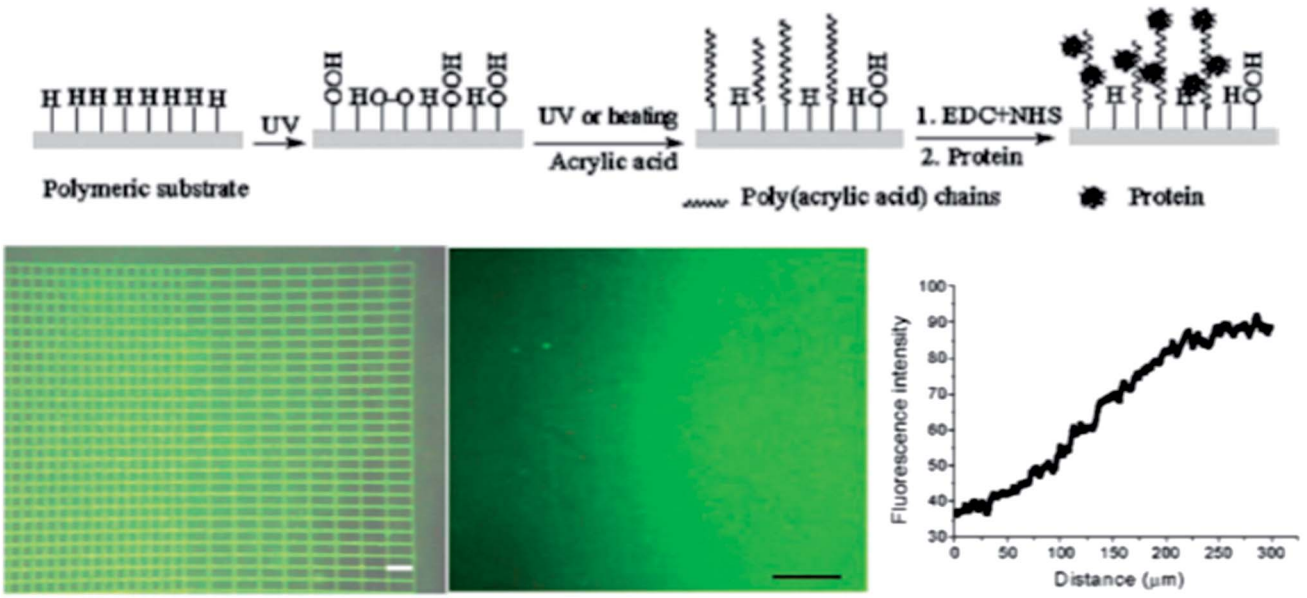


Fig. 2 Top: schematic illustration of the two-step UV irradiation method for grafting AA onto polymeric substrate surfaces followed by covalent carbodiimide (EDC/NHS) immobilization of protein to –COOH groups present on UV-grafted PAA molecular chains. Bottom (left): fluorescence microscopic image of micro-patterned PAA conjugated with fluorescent-labeled avidin. Bottom (middle and right): fluorescence microscopic image showing the density gradient of avidin on the PET surface (scale bars, 50 μm). Reprinted with permission from ref. 94, B. Li *et al.*, *Biomaterials*, **26**, 2005, 1487. © 2005, Elsevier.

Concluding remarks and future perspectives

Surface chemistry of the polymer substrate and protein immobilization techniques are the key issues in application of protein chips and biosensors. In ideal cases, the protein should retain the biological activity and should provide selective interaction with other proteins of interest such as the antigens/viruses present in the blood of affected patients. In that perspective, the development of efficient methods for polymer substrate surface modification is essential. Surfaces that contain highly controlled concentration of functional groups could provide the preservation of the native conformation of attached proteins: the most important requirement for development of biosensors. Furthermore, the optimum surface chemistry and reactivity towards protein macromolecules should lead to high sensitivity biosensor devices of crucial importance for early diagnosis. Polymeric materials such as polymethacrylates have shown great potential for creating high protein loading capability while their functionalized surface maintains active conformation of proteins. Advances in micro-fluidics have opened new opportunities for fabrication of single autonomous lab-on-chip systems; however, the fabrication of a fully functional and highly-sensitive chip still remains a challenge. Future research in this area will provide important answers and understanding of fundamental principles behind protein interaction with polymer surfaces, an important aspect that is still not fully understood. The success and future development of diagnostic devices, which would be translated from the research laboratories into clinics, is strongly dependent on the advances in polymer surface chemistry.

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