Assessment of block and random copolymer overlayers on polymer optical fibers towards protein detection through electrostatic interaction

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ABSTRACT

A simple fiber optic based scheme for the selective detection of proteins, based on surface electrostatic interactions, is presented. The implementation of this method is conducted by using a modified polymer optical fiber's (POF) surface and thin overlayers of properly designed sensitive copolymer materials with predesigned molecular characteristics. Block *poly(styrene*-b-*2vinylpyridine)* (PS-b-P2VP) and random *poly(styrene-r-2vinylpyridine)* (PS-r-P2VP) copolymers of the same monomers and similar molecular weights, were modified and used as sensing materials. This configuration proved to be efficient concerning the fast detection of charged proteins, and also the efficient discrimination of differently charged proteins such as lysozyme (LYS) and bovine serum albumin (BSA). Results on the sensing performance of block and random copolymers are also discussed drawing conclusion on their efficiency given their considerable different fabrication cost.

Keywords: Block copolymers, Random copolymers, Optical Biosensors, Proteins, Fiber sensors

INTRODUCTION

It has been identified lately an immense need for biosensing technologies capable for application in a cost efficient way in real applications in pharmaceutical, food and chemical industry. Further to the technological platform- optical, electrical, MEMS etc [1-REF riziotis]- other components of those biosensing platforms lie entirely on the availability of suitable sensitive materials that can have customizable properties. The selection, characterization and composition of such functional materials suitable for bio-sensing applications is therefore intensely needed and thus several studies have been reported. More specifically, polymer materials exhibiting extensively customizable properties together with biocompatibility characteristics are in the forefront of this research. Furthermore the special class of block and random copolymers[1-4] has been identified as strong candidate for such applications drawing research interest on the study of their morphology, chemical synthesis and physicochemical properties. A major sector of bioassays associated with the ability to trace biomolecules such as proteins, since proteins play a key role in cellular processes and diseases diagnosis, makes the need of detection very important, in biological and biochemical research, biotechnology, food analysis and clinical diagnostics[5]. So far protein detection is based mostly on expensive and complex spectroscopic techniques, such as surface plasmon resonance (SPR)[6] for label-free detection, bragg grating based optical sensing [ref2 pgrs], fluorescence detection which enables the identification of specific protein modifications, nanoscale biosensors that use aptamers as molecular recognition[7] and sensitive surface-enhanced Raman scattering (SERS)-based biosensors using optical fibers for label-free macromolecule detections[8]. Despite the intense research in the field little has been achieved in terms of applications of those technologies in actual applications mainly due to cost, complexity, difficulty of use and especially due to lack of standardization.

Although certain detection techniques could provide highly sensitive detection (< 0.2 μg/mL) of a target analyte, there is still a growing need for rapid, simple and low cost detection methods that could be vital to certain applications in chemical or food industry. Several studies have proved the high functionality and adaptability of the polymer optical fiber (POF) platform for bio-detection, while there is also a growing

research towards the sensitivity enhancement by using resonant type devices based on single or few moded POFs. Recently De Nazare et al. evaluated a series of optical fiber taper sensors to achieve the best tapering characteristics, which will provide an increased sensitivity[9]. Beres et al. used U-shaped chemically treated POF with immobilized antibodies to detect target cells, indicating the POF biosensor as a potential device to detect cells in aqueous medium[10].

However, in this work we propose and follow an alternative simpler approach regarding the protein detection without using recognition elements, as already mentioned in our previous studies[11-13]. The efficiency of this method relies, firstly, on the adsorption of the proteins on specific sensing materials and secondly on the interaction of the enhanced evanescent field (EF) with the sensing materials. The enhancement and optimization of sensitivity in this work relies exclusively on the choice of polymer sensing materials while retaining the low cost multimode POF as the sensing photonic platform. Parameters such as the adsorption of proteins by the active materials and the chemical modification of the polymer substrate surface are very important during the detection process, thus many investigations are devoted to studying the adsorption mechanism of proteins from multi-component systems on different surfaces[14] and the procedure of proper chemical treatment of such polymer surfaces[15]. *The results drawn on polymer materials choice could be afterwards applied also in other resonant like photonics platforms for serving more demanding applications with the associated corresponding coast of integration complexity [ref spie flat fiber]*

EXPERIMENTAL

Materials.

This section describes the fabrication process of the copolymers and presents their main properties. Initially, for the production of the block copolymer PS-b-P2VP the well known and extensively described technique of anionic polymerization[16-18] was used. More specifically, firstly styrene was polymerized at -78ºC in THF using n-BuLi as initiator. Then 2-vinylpyridine was distilled inside the reaction mixture and allowed to react for 30 minutes. Finally, the active chain ends were deactivated using methanol and the synthesized copolymer was precipitated in hexane and allowed to dry in vacuum. The corresponding random copolymer PS-r-P2VP was synthesized by radical copolymerization using 2-vinylpyridine and styrene as the monomers and AIBN as the polymerization initiator in dioxane. The mixture was allowed to polymerize at 60ºC for 24hrs. The resulting copolymer was also precipitated in hexane and allowed to dry in a vacuum oven. The molecular weight, the molecular weight distribution and the composition of the polymers used in this study were derived by using ^{1}H-NMR spectroscopy and size exclusion chromatography (SEC). In table 1 the molecular characteristics of the copolymers used are shown, while in Fig. 1 the chemical structure and the molecular architecture of both copolymers are presented. Additional information on the structure of the block and random PS-P2VP copolymers were gathered by using attenuated total reflectance Fourier transformed infrared spectroscopy (ATR-IR) (Fig. 2). The peaks observed at the region between 1600cm^{-1} and 1550cm^{-1}, as well as the one observed at 1434cm^{-1} are associated with vibration modes of the pyridine ring[19].

Table 1. Molecular characteristics of the copolymers used.

Sample	M_w x 10^3 (by SEC/NMR)	M_w/M_n (by SEC)	Composition (by ^{1}H NMR)
PS-b-P2VP	7.04	1.01	44 wt % PS
PS-r-P2VP	4.53	2.14	47 wt % PS

Both block copolymer PS-b-P2VP and random copolymer PS-r-P2VP were dissolved in THF (Aldrich) in order to prepare polymer solutions of concentration ca. 50mg/mL. Solutions of bovine serum albumin (BSA

from Aldrich) and hen egg white lysozyme (LYS also from Aldrich) of various concentrations were prepared using deionized water and phosphate buffer saline solution as solvents.

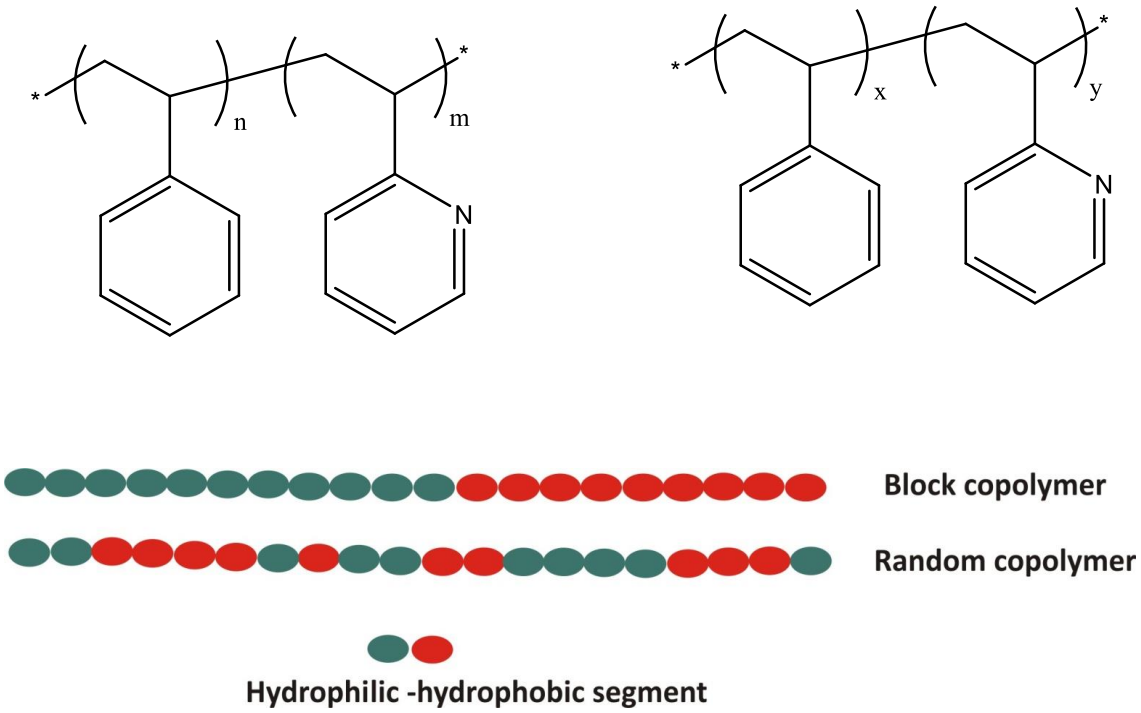

FIGURE 1 Structural formulas (top) of the CPs used and schematic representation of block and random copolymers segment sequences (bottom).

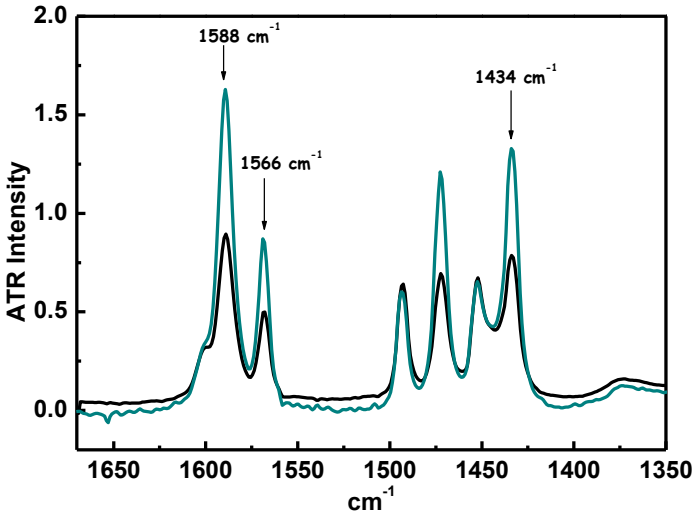

FIGURE 2 ATR-FTIR spectra of PS-b-P2VP (black line) and PS-r-P2VP (blue line) copolymers

Sensors' Development

The functionalization of the polymer fiber active region is achieved, firstly, by removing the jacket and the fluorinated polymer cladding [Fig. 3a], thus exposing the fiber core as sensing zone, followed by proper chemical treatment of the PMMA surface. This generates an area with improved bio-contact properties and, in parallel, gives some additional properties that influence the procedure in which sensing materials are coated. The optical fiber was permanently bended, with an angle of curvature approximately 180◦ [Fig. 3b], in order to enhance the penetration depth of evanescent wave and hence, the sensitivity of the probe. This procedure was conducted by using a heat gun, which exhibited a bend loss of around 3 dB, while the fluorinated polymer cladding of a 5cm effective probe length was removed with a 30% solution of acetone in deionized water, allowing thus the ester groups of the PMMA to be exposed. Figures. 3(c), (d) show a detailed view of the fiber surface topography of the PMMA probe.

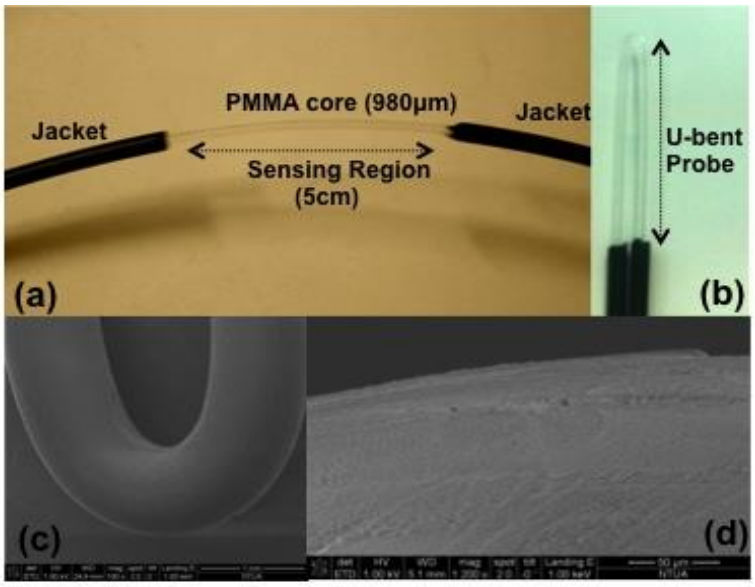

FIGURE 3 (a) POF after removing the jacket and cladding, (b) the U-bent sensing probe and (c), (d) the corresponding scanning electron microscope (SEM) images of the fiber surface.

By having a clean PMMA fiber core, the second step was to investigate how the modification of the surface properties, like hydrophilicity and surface charge, affect the protein adsorption. In order to chemically modify the exposed active region of the fiber two different methods for the evaluation of the random copolymer were followed while for the block copolymer, as regarding the responsivity measurements, only the optimum methodology was chosen to present. The reason is that the main goal of this work is to compare the random and block copolymers by following the most functional chemical treatment of the fiber surface. However in Figure 4 a schematic representation of the chemical functionalization for the case of the block copolymer is presented in order to clarify the two different methodologies.

In the first method, the active region of the fiber was immersed into isopropanol and in sodium hydroxide solution 0.1M consecutively. This process resulted in creating partially negative charges onto the PMMA surface due to the formation of carboxylic groups ($-COO^-$, due to partial hydrolysis of PMMA material), while the hydrophobic nature of the surface was slightly modified. In the first case after the deposition of the PS-b-P2VP polymer, a thin film was created where the P2VP block formed a layer in the inner part and PS block was the outer part of the film, resulting in an overall hydrophobic surface. Finally the active coated region of the fiber was immersed in HCl 1M solution and washed several times with deionized water. The polymer blocks were redistributed and the pyridine ring of the 2VP block was protonated and transferred to the outer material surface. In the case of the random copolymer PS-r-P2VP, the random placement of styrene and 2-vinylpyridine segments within the macromolecular chain is expected to modify the arrangement of each

monomeric unit in the outer surface of the overlayer, compared to the case of the block copolymer. In order to obtain representative results five different fibers were modified for each case with exactly the same procedure.

In the second method, in order to thoroughly remove any remaining polar segments, due to the treatment of the fiber with acetone, the active region of the fiber was immersed into cyclohexane. (Fig. 4B). Afterwards the fiber was removed from the solvent and immediately dried under a nitrogen flow. This treatment also helped in slightly increasing the hydrophilicity of the PMMA fiber. Then, exactly the same procedure was followed as already described, in the first method. The fiber was dip coated into the PS-b-P2VP polymer solution, however this time the PS block is expected to form the inner layer and the P2VP block the outer layer resulting in the formation of a hydrophilic outer surface. Accordingly, the fiber was immersed into HCl and washed with water resulting in the protonation of the pyridine ring.

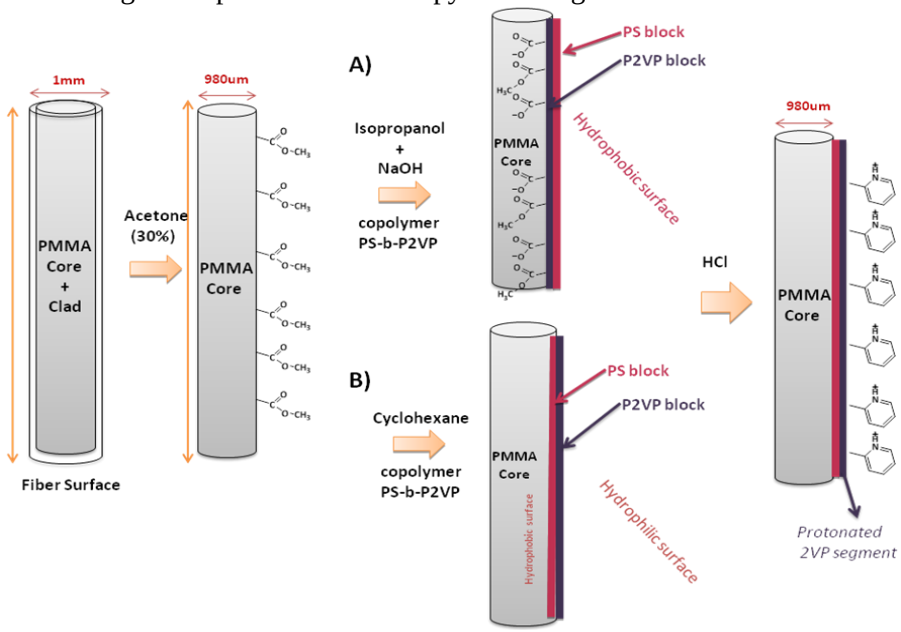

FIGURE 4 Schematic representation of fiber's surface sensitization following two alternative approaches.

The above study allowed us to optimize the way in which we can intervene on the fiber surface topography and comprehend how the chemical functionalization process influences the wettability of the surfaces/overlayers and hence the final protein adsorption. The aforementioned overlayer preparation methods were evaluated taking into account the final responsiveness of the sensor at different protein concentrations. The deposition of polymeric thin films onto PMMA fiber tips was achieved by using the commonly used dip coating technique, which allowed the quick (within 6 min) and stable (over a month) formation of a layer using low-complexity and inexpensive infrastructure. Comparing the ATR-FTIR spectra [Fig. 5] of the sensor probe before and after the deposition of the sensing material, it was observed that absorption peaks corresponding to the PS-P2VP copolymers immerged, while the main peaks of PMMA at 1157, 1398 and 885 cm^{-1} were essentially absent. This result indicated the presence of copolymer coating layer on the PMMA fiber surface and proved the efficient deposition of the sensing material onto the PMMA fiber surface.

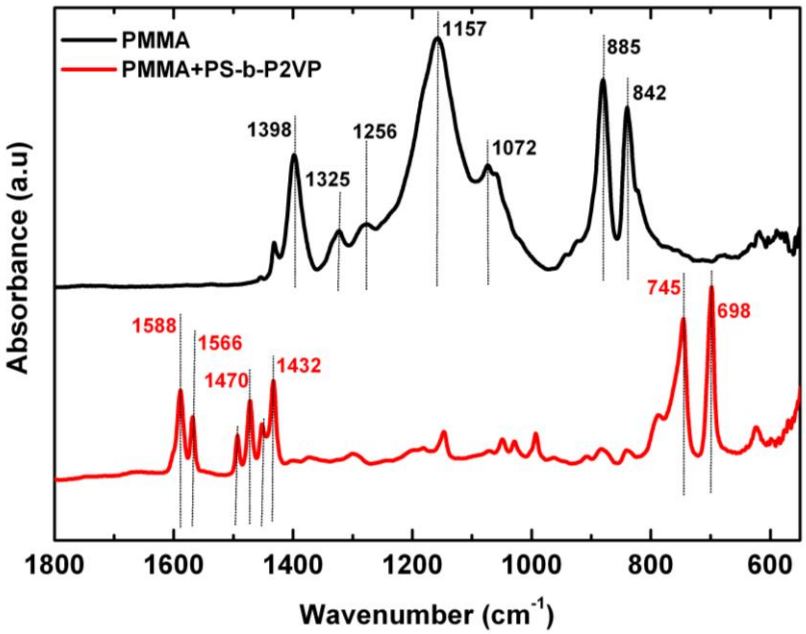

FIGURE 5 ATR-FTIR spectra of the PMMA core of the sensing probe before and after the deposition of the block copolymer material.

Experimental Detection Setup

The evaluation performance of the active materials was conducted using large core PMMA polymer optical fibers demonstrating also the potential of low cost implementation of refractometric based biosensors, taking advantage of such novel sensing materials. The optical platform consists of a U-bend multimode polymer optical fiber (POF) (ESKA GH-4001P, Mitsubishi-Rayon Co.), with an overall fiber diameter of 1 mm, and a core diameter of 980μm. The core of the POF is polymethylmethacrylate (PMMA, n_{core}=1.492), while the cladding is fluorinated polymer (n_{clad}=1.417). The light source used is a LED operating at 650 nm with maximum output power of 1 mW. The power meter used in the current work is a Newport model 2832-C Dual Channel equipped with detectors model 818-UV. The experimental set-up used for the experiments is similar to our previous relative works[11-13].

Sensing Mechanism

Generally, every molecular interaction is determined by a combination of the basic physical forces like hydrophobic interactions[19], electrostatic interactions[20], hydrogen interactions and van der Waals forces[21]. In this case we take advantage of the electrostatic interactions, which are generated due to different charges between the sensing material and the detectable protein. The sensing mechanism is based on the interaction between the evanescent field and the copolymer, which becomes stronger due to increased losses of propagation light after the bending of the active area of the POF. The detection method relies on successful adsorption of the proteins on the sensing materials, which is accomplished due to strong electrostatic attractive forces generated between the protein molecules and the block or random copolymer material. This procedure increases the thickness of the deposited layer and causes variations in the refractive index at the outer material interface, leading to significant changes in the output guided wave light.

RESULTS AND DISCUSSION

ATR-FTIR analysis and responsivity measurements were performed in order to evaluate the aforementioned copolymers. Both PS-b-P2VP and PS-r-P2VP materials revealed that BSA, which is negatively charged at neutral pH, was adsorbed on positively charged top-layered material surfaces with a fast initial rate and large adsorbed amount, due to the complimentary charge characteristics of the substrate and the positive charge of the sensing materials. The opposite observation was made for lysozyme which is positively charged and hardly adsorbed onto the positively charged fiber surface, due to electrostatic repulsion. In particular, the ATR-FTIR analysis of the sensor probe [Fig. 6] after the adsorption procedure showed the presence of bands associated with adsorbed BSA (amide bond frequencies at 1655, 1537 and 1403 cm^{-1}), while the absence of LYS peaks proved the low adsorption of the particular protein by the copolymer materials. These results indicate the efficient adsorption of the BSA onto the copolymer coating overlayer and prove the detection capability and selectivity of the proposed fiber sensor towards specific charged proteins.

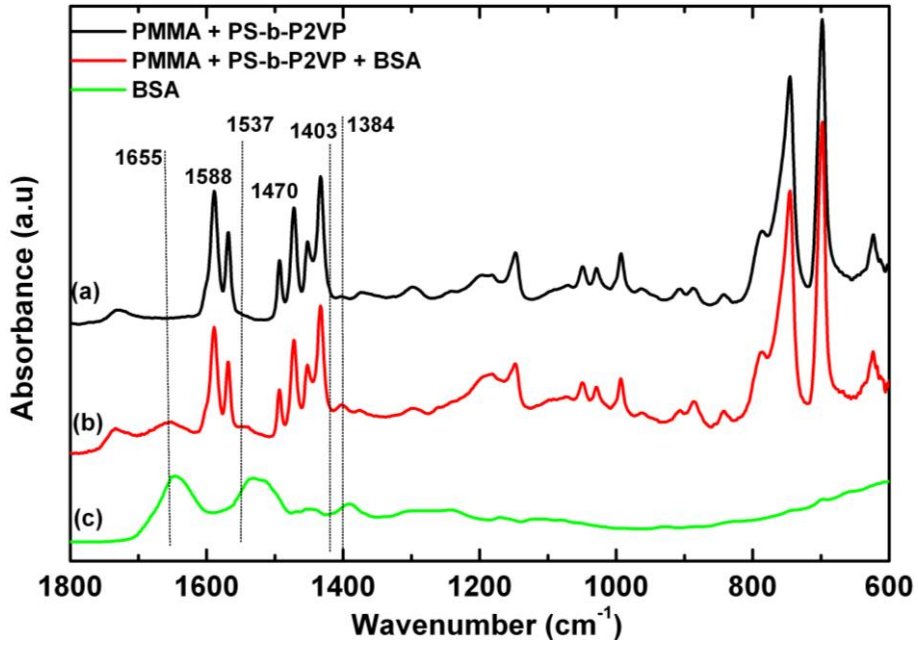

FIGURE 6 ATR-FTIR spectra of the sensing probe with the block copolymer coating overlayer before (black line) and after (red line) BSA adsorption, and the ATR-FTIR spectrum of BSA (green line). Peaks at ca. 1655, 1537 and 1384 cm^{-1} in spectrum (b) are associated with the presence of adsorbed BSA.

Accordingly, the sensor was tested in successively diluted BSA and LYS solutions with different protein concentrations in order to determine the responsivity of the sensor and the detection limit, which in the case of biomolecular sensing, is the minimum amount of analyte that the sensor can accurately quantify[16]. Successive response measurements over time [Fig. 7] showed excellent repeatability in the case of the buffer and distilled water, while the detection limit was found to be 0.5 mg/ml. The experiments using the buffer solution were performed in order to simulate the human fluids at least in acidity (neutral pH) and salinity. Fig. 8 shows the response of the sensor in BSA and LYS, using as sensing material the PS-b-P2VP block copolymer and Fig. 9(a), (b) show the responsivity of the corresponding random copolymer PS-r-P2VP coated on fibers, which were subject previously to different chemical treatments, as described in the experimental part. One of the major issues is the optimization of the sensor design to improve the detection limit, working for example with fibers such as taper POF in U-bend scheme as it has been shown recently[9]. Nonetheless, this detection scheme proved to be suitable for easy, fast and low cost biosensing applications.

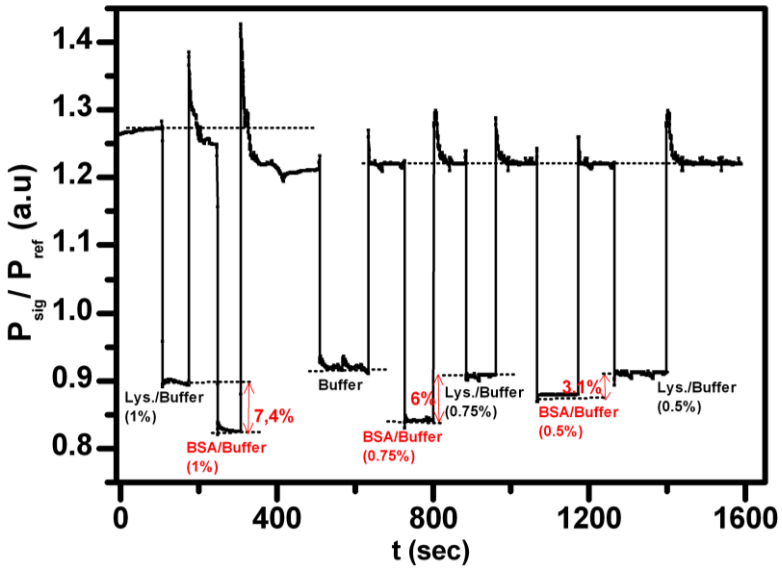

FIGURE 7 The experimental process indicates the relative response of the POF sensor over time in different concentrations of BSA and LYS and the fully reversible behavior of the sensor.The bottom dotted lines show the maximum reduction of the optical signal (P_{sig}) while the upper dotted lines show the reference baseline before the immersion of the POF in the protein solutions. The time needed in order the signal to return in the same reference value is about of a few seconds, while the response time was instantaneous.

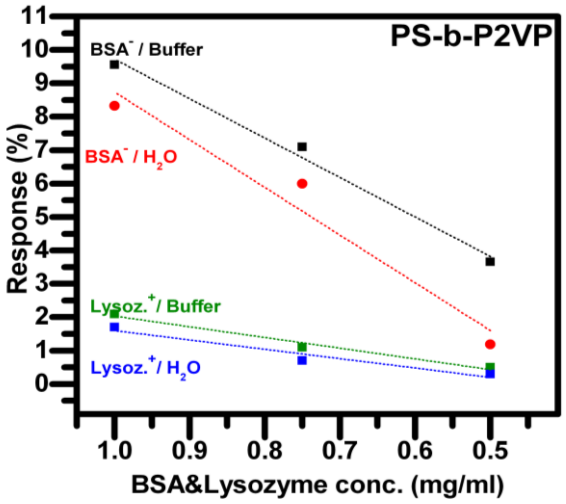

FIGURE 8 The absolute response of the block copolymer in different protein concentrations using isopropanol in the chemical treatment.

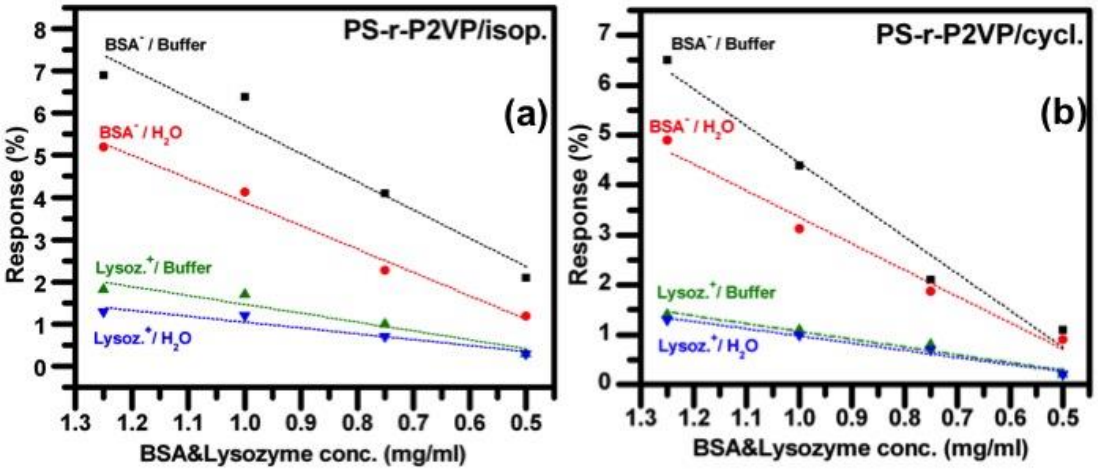

FIGURE 9 The absolute responses of the PS-r-P2VP material in similar protein concentrations using (a) isopropanol and (b) cyclohexane in the chemical treatment, in order to modify the surface topography and thus the protein adsorption

Particularly, the block copolymer PS-b-P2VP showed sensing capability up to 10% in a concentration range of BSA about 0.5-1% (wt/v) with an almost instantaneous response time, due to the electrostatic nature of the interaction described, while the response of the sensor in LYS did not exceed 2% [Fig. 8]. The corresponding random copolymer PS-r-P2VP showed comparable results (up to 7%) in detection of BSA as is shown in Fig. 9, while the response of the sensor in similar concentration of LYS was relatively low, indicating low levels of lysozyme adsorption from the copolymer material in both cases. The different chemical functionalization/treatment of the fiber surface seems to affect the responsivity of the sensor mainly at low concentrations of BSA, as it is shown in Fig. 9(a), (b), where the hydrophilic PMMA fiber proved to be more suitable, regarding the responsivity of the sensor, probably due to the more efficient deposition of the copolymer on the fiber surface. Moreover, the increased response in the case of the buffer solution gives an added value to the tested sensor, indicating the functionality of the sensor in simulated biological fluids.

From these observations it can be concluded that the electrostatic interactions, which govern the adsorption process, vary for the two investigated proteins. As a result a larger amount of BSA is adsorbed. The use of copolymer overlayers essentially induces a positively charged coated PMMA sensing region that could adsorb strongly negatively charged BSA. In contrast, as it was anticipated, positively charged lysozyme was adsorbed in small and almost undetectable amounts, demonstrating in this way an intrinsic electrostatic discrimination and selective adsorption mechanism. Generally, the control of protein adsorption is not easily feasible because it is necessary to know the physicochemical properties of the block copolymer films that are formed onto the fiber surface. However, it is clear that these differences in the sensor response can be attributed to electrostatic phenomena. Although, as stated by the results, this method is inherently limited in both sensitivity and effective range compared to the aforementioned complex techniques, there are advantages regarding the rapidness, simplicity and the inexpensive procedure of the proposed detection scheme.

CONCLUSIONS

In this paper a low cost optical biosensor is reported based on a polymer optical fiber capable to detect selectively specific proteins with negative charge, such as BSA. The proposed block and random copolymers of styrene and 2-vinylpyridine monomers have proven to be adequate as coating layers on the POF surface thus suitable to use as sensitive materials. The optimum response's dynamic range in various BSA concentrations for the PS-b-P2VP lie in the range of 0-10%, while PS-r-P2VP revealed comparable responses reaching the dynamic range of 0-7%. The chemical functionalization study of the surface sensor revealed different optical responsivity, allowing the determination of the optimum experimental

procedure, using such copolymers, concerning the detection of the studied proteins. Furthermore, the reversibility of the sensors was tested when returned in buffer and H_2O solutions with zero concentrations of proteins, after being cycled through a wide range of concentrations, verifying in this way the sensors capability and stable operation. The minimum detectable protein concentration was proved to be 0.5 mg/ml. Block copolymers often require laborious synthetic techniques for their production in contrast to random copolymers that are cheaper and easier in their production, providing thus a guide for the optimization of the tradeoff between cost and efficiency in sensors development. The deployed inexpensive and highly customizable POF platform, functionalized by suitable techniques with copolymers could lead to the development of customizable, rapid and inexpensive schemes for bio-detection. Further work is in progress in order to extend the performance characterization of block and random copolymers in other photonics platforms, like Bragg grating ring resonators etc, that exhibit resonant behavior of higher inherent sensitivity

ACKNOWLEDGEMENTS

This work was funded by the R&D Project WelCOM 09SYN-71-856 funded by National Strategic Reference Framework NSRF 2007-2013/Hellenic General Secretariat of Research and Technology GSRT and supported also by the NANOMACRO project which is implemented in the framework of the Operational Program "Education and Life-long Learning" (Action "ARISTEIA I") and it is co-funded by the European Union (European Social Fund) and by national funds.

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REFERENCES

[1] V. Pruneri, C. Riziotis, P.G.R. Smith, A. Vasilakos, Fiber and integrated waveguide-based optical sensors, J. Sens. (2009) art. No. 171748, doi:10.1155/2009/171748

[2] Y. Wang, J. Xu, Y. Zhang, H. Yan, K. Liu, *Macromol. Biosci.* **2011**, 11, 1499–1504.

[3] M. W. M. Fijten, J. M. Kranenburg, H. M. L. Thijs, R. M. Paulus, B. M. V. Lankvelt, J. D. Hullu, M. Springintveld, D. J. G. Thielen, C. A. Tweedie, R. Hoogenboom, K. J. Van Vliet, U. S. Schubert, *Macromol.* **2007**, 40(16), 5879-5886.

[4] J. F. Kenney, *Polym. Eng. Sci.*, **1968**, 8(3)

[5] C. Simão, W. Khunsin, M. Salaün, M. Zelsmann, M. Morris, C. M. Sotomayor Torres, Nanotechnol. **2014,** 25 (17), 175703

[6] P. J. Scully, L. Betancor, J. Bolyo, S. Dzyadevych, J. M. Guisan, R. F. Lafuente, N. J. Renault, G. Kuncov´a, V. Matˇejec, B. O'Kennedy, O. Podrazky, K. Rose, L. Sasek, J. S. Young, *Meas. Sci. Technol.* **2007**, 18, 3177–3186.

[7] N. Cennamo, D. Massarotti, L. Conte, L. Zeni, *Sens.* **2011**, 11, 11752-11760.

[8] IJG Sparrow, PGR Smith, GD Emmerson, SP Watts, C Riziotis Planar Bragg grating sensors—Fabrication and applications: A review, Journal of Sensors 2009

[9] J. O. Lee, H. M. So, E. K. Jeon, H. Chang, K. Won, Y. H. Kim, *Anal. Bioanal. Chem.* **2008**, 390, 1023–1032.

[10] X. Yang, C. Gu, F. Qian, Y. Li, J. Z. Zhang, *Anal. Chem.* **2011**, 83, 5888–5894.

[11] F. V. B. D. Nazare, C. Beres, N. Correa, C. D. Souza, M. M. Werneck, M. Antonio, M. Lemos, *Elect. Engin.* doi: 10.1109/IMTC.2011.5944172.

[12] Beres, Fábio Vieira Batista de Nazaré, N. Corre, C. D. Souza, M. A. L. Miguel, M. M. Werneck, *Biosens. Bioelectron*. **2011**, 30, 328–332.

[13] L. Athanasekos, A. El Sachat, S. Pispas, C. Riziotis, *J. Polym. Sci., Part B: Polym. Phys.* **2014**, 52, 46-54.

[14] L. Athanasekos, N. Aspiotis, S. Pispas, and C. Riziotis, *Key Engineering Materials* **2013** 543, 385-388.

[15] A. El Sachat, A. Meristoudi, C. Markos, S. Pispas, C. Riziotis, **(2014)***,* *8983,* 89830I. doi:10.1117/12.2039529
[16] T. G. Vladkova, *Intern. Jour. Polym. Scien.* **2010**, 296094.
[17] F. Poncin-Epaillard, T. Vrlinic, D. Debarnot, M. Mozetic, A. Coudreuse, G. Legeay, B. El Moualij, W. Zorzi, *J. Funct. Biomater.* **2012**, 3, 528-543.
[18] C. Riziotis, K. Kalli, C. Markos, A. Posporis, C. Koutsides, A.S. Webb, C. Holmes, J. C. Gates, J.K. Sahu and P.G. R. Smith, "Flexible glass flat-fibre chips and femtosecond laser inscription as enabling technologies for photonic devices ", Proc. SPIE 8982, Optical Components and Materials XI, 89820G (March 7, 2014); doi:10.1117/12.2039643; http://dx.doi.org/10.1117/12.2039643
[19] D. Topouza, K. Orfanou, S. Pispas, *J. Polym. Sci. Part A: Polym. Chem.* **2004**, 42, 6230-6237.
[20] A. Meristoudi, S. Pispas, N. Vainos, *J. Polym. Sci. Part B: Polym. Phys.* **2008**, 46, 1515-1525.
[21] Antonietti, M.; Wenz, E.; Bronstein, L.; Seregina, M. *Adv. Mater*. **1995,** 5, 1000
[22] B. W. Matthews, *Encyclopedia of life Science, Nature Publishing Group Oregon, USA.* doi: 10.1038/npg.els.0002975.
[23] V. Z. Spassov, A. D. Karshikov, B. P. Atanasov, *Biochimica et Biophysica Acta.* **1989**, 999, 1-6.
[24] M. White, X. Fan, *Opt. Express.* **2008**, 16, 1020-1028.