Polymethacrylate coated electrospun PHB fibers: An exquisite outlook for fabrication of paper-based biosensors

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Abstract

Electrospun polyhydroxybutyrate (PHB) fibers were dip-coated by polymethyl methacrylate-co-methacrylic acid, poly(MMA-co-MAA), which was synthesized in different molar ratios of the monomers via free-radical polymerization. Fabricated platform was employed for immobilization of the dengue antibody and subsequent detection of dengue enveloped virus in enzyme-linked immunosorbent assay (ELISA). There is a major advantage for combination of electrospun fibers and copolymers. Fiber structure of electrospun PHB provides large specific surface area available for biomolecular interaction. In addition, polymer coated parts of the platform inherited the permanent presence of surface carboxyl (−COOH) groups from MAA segments of the copolymer which can be effectively used for covalent and physical protein immobilization. By tuning the concentration of MAA monomers in polymerization reaction the concentration of surface −COOH groups can be carefully controlled. Therefore two different techniques have been used for immobilization of the dengue antibody aimed for dengue detection: physical attachment of dengue antibodies to the surface and covalent immobilization of antibodies through carbodiimide chemistry. In that perspective, several different characterization techniques were employed to investigate the new polymeric fiber platform such as scanning electron microscopy (SEM), atomic force microscopy (AFM), water contact angle (WCA) measurement and UV–vis titration. Regardless of the immobilization techniques, substantially higher signal intensity was recorded from developed platform in comparison to the conventional ELISA assay.

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1. Introduction

There is a continuous demand for inexpensive and sensitive/ selective analytical devices which are reliable, portable, rapid and capable of high-throughput detection in the area of biosensing. To date, many different complex biosensors of the most advanced categories have been developed (Lin et al., 2010; Nie et al., 2014; Xiangjie et al., 2014). When it comes to the actual clinical practice, however, minor percentages of them are playing a vital role in routine diagnostic procedure. This simple and straightforward fact has to lead and encourage researchers to dedicate efforts to the more practical solutions for production of new generations of analytical platforms. Enzyme-linked immunosorbassay (ELISA) is perhaps the most well-known and widely applied assay for virus detection. Nevertheless, even to date, patients might enter to the acute phase of the illness due to the frequently reported serious drawbacks of this very conventional assay. Some of the major shortages of ELISA assay can be listed as: time consuming and laborious procedure, inconsistency of the detection signal, errors in reproducing the results and large detection range required for relatively more accurate diagnosis (Alkon et al., 2002; Hosseini et al., 2014b). Therefore, the necessity of an additional intermediary that can enhance the performance of ELISA, leading to the timely detection and subsequently better surveillance, is highly desirable.

In the last two decades, polymer fibers and membranes had undergone through significant progress in the field of biomaterials engineering and biotechnology (Chen et al., 2009; Hong et al.,...
2013; Lu et al., 2014; Tang et al., 2014). Having applications in areas such as tissue engineering, controlled drug release, wound dressings, molecular separation, preservation of bioactive agents and biosensors have drawn a great deal of importance in fiber developments (Luo et al., 2010; Zhang et al., 2005). Among existing fabrication techniques, electrospinning remains the most popular and preferred method for fabrication of polymer fibers. Electrospinning has shown major advantages over other techniques as it is a simple and versatile method that can be used for a wide range of polymer solutions (Ma et al., 2006). The laboratory set up can be customized in a relatively low price and the produced fibers can be controlled in diameter range (micro/nanofibers) depending on the size of the needle (Chantasirichot and Ishihara, 2012; Cipitria et al., 2011). Electrospun fibers have proven great potentials in biosensing domain due to the high interconnectivity, porosity, micro/nanointerstitial space and high surface area available for biomolecular interaction (Ma et al., 2006). On the other hand, presence of effective functional groups such as carboxyl (−COOH), amine (−NH₂), hydroxyl (−OH) and sulfhydryl (−SH) is essential when covalent immobilization is aimed (Hosseini et al., 2014a,b). Therein, a suitable biosensor material would be credited not only for a large available surface area but also for bearing desirable functionalities. In response to the mentioned factual requirements for a well-designed bioreceptor surface, combination of electrospun fiber and functionalized polymer may offer a protein-friendly platform with high chance of bimolecular interaction associated with binding stability. This paper presents the fabrication of electrospun poly-hydroxybutyrate (PHB) fibers by widely applied electrospinning method. In the second step, different compositions of polymethyl methacrylate-co-methacrylic acid, poly(MMA-co-MAA), were synthesized via free radical polymerization. Dip-coating of electrospun fibers in poly(MMA-co-MAA) solution creates a unique biosensing platform at which the high surface area is originated from the structure of PHB fibers and surface –COOH functional groups are inherited from MAA segments of the copolymer (Hosseini et al., 2014a). In immobilization domain, if one aspect matters more than presence of desirable functionalities, it would be the optimum concentration and proper distribution of them on the surface. An insufficient number of surface functional groups may result in deactivation of the immobilized proteins as biomolecules, in general, could be very sensitive toward solid phases (Hosseini et al., 2014d). On the other hand “too many” surface functionalities might result in an overly functionalized surface which again deactivates proteins due to the steric repulsion (Hosseini et al., 2014c,d). Therefore to assess and optimize versatile surfaces of polymer coated electrospun fibers with variety of –COOH concentrations, different molar ratios of the monomers (MMA/MAA) have been used in polymerization reaction. Designed platforms (uncoated and dip-coated PHB fibers) were employed for detection of the dengue enveloped virus through physical and covalent immobilization in ELISA method. Dengue fever (DF) is a mosquito transmitted viral disease which is mainly widespread in tropical and subtropical regions (Bessoff et al., 2008). However, the risk of this dangerous infection can become worldwide by people who contracted the infection while traveling abroad (Shu et al., 2003). Large reactive surface area that goes hand in hand with stable binding for antibody conjugation, provide a biomolecule-compatible environment that can lead to an adaptive technique for fabrication of new generation of paper-based diagnostic devices with higher performance.

2. Materials and methods

2.1. Chemicals and reagents

Methyl methacrylate (MMA), methacrylic acid (MAA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), polyhydroxybutyrate (PHB), bovine serum albumin (BSA), monosodium phosphate (NaH₂PO₄), chloroform (CHCl₃), Tween 20 and disodium hydrogen phosphate (Na₂HPO₄) were purchased from Sigma, US. MMA monomer was purified by distillation technique prior to polymerization reaction. Other materials were used as received. Tetrahydrofuran (THF, solvent in free-radical polymerization and dip-coating), dimethylformamide (DMF) and phosphate buffer saline (PBS) were purchased from Thermo Fisher Scientific, US. Azobisobutyronitrile (AIBN, polymerization initiator), was purchased from Friedemann Schmidt Chemical, Germany.

2.2. Fabrication of electrospun PHB fibers

PHB fiber membrane were fabricated by electrospinning method. Briefly, 20 ml PHB solution (10 wt%) in chloroform/DMF (9:1) was ejected out of a 20 G needle (inner diameter=0.9 mm) at a speed of 3 ml/h. 10 kV voltage was loaded between the needle and an aluminum plate (40 cm x 40 cm). The distance between needle and aluminum plate was 18 cm (Scheme 1a). Polymer solution was drawn into fibers and deposited on the aluminum plate to form PHB fiber membrane. The electrospun fiber was then peeled off from the aluminum plate. The white-colored and paper-like PHB membranes with thickness of ~500 μm were cut into the circle shape pieces with dimension of 6 mm (Scheme 1, center) for further experiments.

2.3. Poly(MMA-CO-MAA) synthesis and processing

Four different compositions of poly(MMA-co-MAA) were prepared by free-radical polymerization reaction in THF using AIBN as an initiator. The abbreviations of the copolymers were used to identify the initial molar ratio of the monomers. For instance, Poly(MMA-co-MAA-9:1) corresponds to 90% of MMA and 10% of MAA in reaction mixture. Further copolymer compositions are as follows: poly(MMA-co-MAA-7:3) and poly(MMA-co-MAA-5:5). For the ease of discussion, further in the text, mentioned copolymer compositions are named as follows: comp.(9:1), comp.(7:3) and comp.(5:5). Pure PMMA (when MMA is the only monomer involved in polymerization reaction) was also synthesized under the same reaction conditions and used as control in all experiments. A two-neck round-bottom flask was fitted with a condenser and sealed inlet, used for reactants feed. The set up was charged with pre-calculated amount of MMA in 50 ml of THF under stirring condition for 5 min. A mixture of second monomer (MMA) and initiator (AIBN, 0.164 g) was gradually added to the reaction mixture. Polymerization has been carried out for 8 h at 90 °C and was stopped by adding reaction mixture into 1000 ml of distilled water. Immediate white color precipitation was observed confirming the formation of copolymer compositions. Mentioned precipitation was filtered and thoroughly washed with distilled water and dried in vacuum oven at 40 °C (Hosseini et al., 2014a).

2.4. Coating procedure

Polymer coatings were prepared on fabricated PHB fibers by simple and straightforward method of dip-coating (Scheme 1b). PHB fibers (substrates) were coated by immersing fiber sheets into the polymer solutions (5%) by using THF as solvent. Coated fibers of different copolymer compositions have been taken out after 3 s
of dipping and dried in ambient temperature.

2.5. Morphology analysis by scanning electron microscopy (SEM) and atomic force microscopy (AFM)

Surface morphology of polymer coated and uncoated PHB fibers was analyzed by SEM equipped with a field emission gun (FESEM, JEOL, JSM7600F) operated at an accelerating voltage of 0.5 KV in secondary electron mode. Samples were mounted on a double-sided conductive carbon tape and coated with platinum, avoiding the surface charging. Frontal view and cross-section images of the samples were carefully analyzed in order to detect the traces of the coating on the PHB fibers. Surface topology of the polymer coated PHB fibers was recorded by AFM (Ambios, Q scope) in non-contact mode. Mean roughness (Ra), root mean-square roughness (Rq) and total roughness (Rt) were recorded for all of the coated samples.

2.6. Water-in-air contact angle measurement

Water contact angle (WCA) measurement was carried out by depositing droplets of distilled water on the coated surfaces of the PHB fibers. WCA experiment was done by sessile drop method in room temperature. The instrument used for these measurements was Dataphysics contact angle system (OCA). Measurement was performed after 1 min of depositing droplets of distilled water (0.3 μl) on the coated surfaces (3 samples of each composition). The average contact angle was calculated from measurements performed for five separate droplets of distilled water, one on the center and four on the corners of the samples. Standard deviation was negligible in plotting the data as a very small spreading (± 2°) was observed for almost all of the measurements (n = 15).

2.7. UV–vis titration and determination of surface –COOH groups on polymer coated fibers

The qualitative measurement of surface –COOH can be performed by spectroscopic UV–vis toluidine blue (TB) titration which is a pH dependent adsorption/desorption technique. As it can be seen from Scheme 1d, the structure of poly(MMA-co-MAA) contains carboxyl (–COOH) groups which are generated from MAA polymer segments. By immersing the polymer coated PHB fibers into the TB solution (5 mM TB in 0.1 mM NaOH, 2 h at ambient environment) positively charged TB ions interact with surface –COOH groups which were dissociated in alkaline condition (Djordjevic et al., 2010; Sano et al., 1993). Samples were taken out
and rinsed with 0.1 mM NaOH solution in order to eliminate the non-complexed TB dyes. By soaking samples in 5 ml of 50% acetic acid for 20 min, TB desorbed into the acidic medium. The absorbance of released TB dyes in acetic acid solution was measured by a UV–vis (Varian, Cary 1) spectrophotometer at 635 nm. Measured concentration of TB dye corresponds to the concentration of available –COOH groups on the coated samples. Recorded spectroscopic data was then converted to surface concentration of –COOH expressed in μM/mm² (n=5).

2.8. Dengue antibody immobilization on polymer coated PHB fibers

Polymer coated PHB fibers of all compositions were cut into the circle shapes with the diameter of 6 mm which can be perfectly fit at the bottom of the ELISA 96-well plates (SPL, life science, China). Dengue Ab immobilization was performed by two different techniques: (1) physical attachment of antibody (Ab) to the coated surfaces (Scheme 1e); and (2) covalent immobilization of Ab through carbodiimide chemistry (Scheme 1f). In the case of covalent immobilization, samples (n=12) were treated in EDC/NHS solution (0.155 g of EDC and 0.115 g of NHS in 200 ml of PBS) for 1 h prior to immobilization. After carbodiimide treatment, samples were thoroughly washed in PBS before being used in ELISA. As control, conventional ELISA in 96 well-plate has been conducted as well.

2.9. Sandwich ELISA method

Among different protocols for performing ELISA such as direct, indirect, sandwich and competitive, sandwich ELISA is the most specific and reliable method for biomolecular recognition. In present case, by using a sandwich ELISA technique, risk of non-specific binding was minimized and reasonably accurate detection signals were resulted. Each well of the ELISA well-plate was charged with 100 μl of capture Ab, Goat IgG anti DV (SC-325014, Santa Cruz, US) which was diluted (1:300) in coating buffer (0.85 g of NaCl, 0.14 g of Na₂HPO₄ and 0.02 g of NaH₂PO₄ in 100 ml of PBS, pH=7.4). Incubation was carried on for 2 h at 37 °C. Washing step was performed with 200 μl per well of washing buffer (0.05% Tween 20 in PBS, pH=7.4) at room temperature. ELISA kits of both, empty and including polymer coated PHB fibers were washed for 3 times (each time 5 min) by using shaker with shaking speed of 1000 rpm. The exact same washing procedure was performed between each two steps of the ELISA. In order to achieve high selectivity and to avoid the non-specific bindings, blocking procedure was conducted by adding 100 μl of blocking buffer (1 g of BSA in 100 ml of washing buffer, pH=7.4) to each well (37 °C for 1 h). Dengue enveloped virus (virus propagation procedure can be found in Supplementary section) was diluted in coating buffer by serial dilution. Variety of virus concentrations have been used depending on the purpose of conducted assays. Each well was charged with 100 μl of the virus solution and incubation was carried out overnight in 4 °C. The concentration range of the virus used in the assay has been selected accordingly: calibration curves were plotted by running the assay in the virus concentration range between 3.5 × 10⁻⁸ p.f.u/ml and 3.5 × 10⁸ p.f.u/ml; detection range study was performed by conducting the assay in the concentration range of 3.5 × 10⁻¹⁰–3.5 × 10⁻⁶ p.f.u/ml. Sandwich ELISA was performed on all the platforms via physical and covalent immobilizations in order to compare the efficiency of developed surfaces in DV detection on selected concentration of 3.5 × 10⁸ p.f.u/ml. Primary Ab solution was prepared (1:200) by diluting mouse IgG2a anti DV (ab155863, Abcam. US) in diluting buffer (0.4 g BSA, 4 ml PBS buffer and 120 μl of Trintonx-100 in 36 ml of distilled water, pH=7.4). Each well has received 100 μl of Ab solution and ELISA kits were placed in incubator for 2 h at 37 °C. For accuracy of the judgment, samples were taken out from the well-plate, washed and placed in the new ELISA kit before proceeding with further experiments. The incubation of 30 min was conducted in 37 °C by adding 100 μl of anti-mouse IgG2a alkaline phosphatase (ab97242, Abcam. US) as secondary Ab which was diluted in diluting buffer (1:500). Eventually wells were thoroughly washed (as it was described) and filled with 100 μl of mixed substrate (Alkaline phosphatase blue microwell substrate components A and B). The reaction was stopped after 10–15 min by adding 50 μl of alkaline phosphatase stop solution (AS585, Sigma. US) and signal intensity was recorded by using Bio-Rad (model 680) at the wavelength of 570 nm. Negative controls were calculated as a result of the assay which was conducted in the absence of antigen (n=12). Samples that showed the optical density (OD) greater than twice of the mean value of negative controls (cut-off value) were considered as positives (Alcon et al., 2002). Detection results were plotted by subtraction of the cut-off values from obtained data. It is noteworthy that the chance of cross-reactivity in this assay was less than 2%. Limit of detection (LoD) was determined from the average standard deviation and the slope of the calibration curves by previously reported method (Shrivastava and Gupta, 2011). Total number of 16 readings for positive samples with predetermined DV concentration was used in ELISA for each platform. In the case of negative controls (non-infected samples) 10 replicates have been tested in sandwich ELISA. Non-specific bindings at intentional negative controls resulted in background signal that was quantitatively compared to the actual results in order to determine sensitivity and specificity of the proposed methodology (Linares et al., 2013). Accuracy of the method was calculated by using a well-known method that involves true positives and true negatives in comparison to the total number of the samples.

3. Results and discussion

Morphology of the uncoated and polymer coated PHB fibers were recorded by SEM (Fig. 1). A very clear confirmation of the successful coating can be observed by simple comparison between images of uncoated (Fig. 1a) and polymer coated PHB fibers (Fig. 1b). Fig. 1c shows the highly porous structure of coated fibers (coated and uncoated) was successful coating can be observed by simple comparison between images of pure PHB fibers and coated PHB fibers (Fig. 2a and b). Obvious capillary features with continuous and smooth structures were detected on coated PHB fibers with comp.(9:1). Those features are clearly recognizable from the polymer coated segments that occupy voids between the fibers (Fig. 2b). Complete AFM analyses of the uncoated and coated fibers by different compositions are displayed in Supplementary section (Fig. 2S). Fig. 2 (bottom, table) presents more detailed information obtained from AFM experiment. It can be seen that total roughness increased by introducing PMMA coating to the PHB fibers' structure. This is quite expected as polymethacrylate coated regions of the sample act as enhancer of the surface roughness. However, entangled poly (MMA-co-MAA) among PHB fibers leads the surface to a
completely different character. Considerably lower surface roughness detected for PHB fibers coated by comp.(9:1) and comp. (7:3) is factually the result of –COOH functional groups generated from MAA segments. In that sense, it can be concluded that surface roughness is the function of surface –COOH concentration. Surprisingly, PHB fibers coated with comp.(5:5) have shown the highest roughness among all the samples. The explanation of such a different behavior is that this composition is a gel-like material in its nature. According to our previous observations, this particular compound readily swells in water due to the dominant presence of structural –COOH groups (Hosseini et al., 2014a). Wetability of the samples has been analyzed by WCA measurement and results are shown in Fig. 3a. The average angle of ~116° was measured for uncoated PHB fibers which is in close

<table>
<thead>
<tr>
<th>Composition</th>
<th>Mean roughness (nm)</th>
<th>Root mean-square roughness (nm)</th>
<th>Total roughness (nm)</th>
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<td>412</td>
<td>507</td>
<td>1979</td>
</tr>
<tr>
<td>PMMA</td>
<td>423</td>
<td>509</td>
<td>2421</td>
</tr>
<tr>
<td>Comp.(9:1)</td>
<td>137</td>
<td>167</td>
<td>713</td>
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<tr>
<td>Comp.(7:3)</td>
<td>137</td>
<td>168</td>
<td>757</td>
</tr>
<tr>
<td>Comp.(5:5)</td>
<td>456</td>
<td>575</td>
<td>2433</td>
</tr>
</tbody>
</table>

Fig. 1. Representative morphology analysis of PHB fibers by FESEM: (a) PHB electrospun fibers; (b) and (c) copolymer coated PHB fiber; (d) cross-section image of the copolymer coated PHB fibers.

Fig. 2. Topology analysis of the uncoated and coated electrospun fibers, (a) PHB fibers and (b) coated PHB fibers by comp.(9:1) as a representative (bottom): surface characteristics of the uncoated and coated PHB fibers by different compositions of poly(MMA-co-MAA).
agreement with previously reported results (Sombatmankhong et al., 2007). PMMA coated electrospun PHB fibers has become extremely hydrophobic with the contact angle of almost 126° due to the PMMA coverage (Fig. 3b). Although PHB fibers are known as hydrophobic materials in their nature, there still would be a chance of water penetration because of the large scaled interstitial spaces between fibers. Polymer coatings however, occupy the voids between fibers and impose an even more hydrophobic behavior. Measured WCA gradually decreases as the number of MAA segment increases (from comp.(9:1) to comp.(5:5), Figs. 3a and 3S). Naturally hydrophilic surface –COOH groups generated from poly(MMA-co-MAA) convert the extreme hydrophobicity to more moderate levels. As expected, the minimum WCA was measured for PHB fibers coated with comp.(5:5) as this composition carries the highest concentration of surface –COOH groups (Fig. 3c). Data obtained from WCA measurements associated with AFM results are in very close agreement with the commonly known fact which indicates the following: for hydrophobic surfaces WCA increases with surface roughness and for hydrophilic surfaces, in contrast, WCA decreases with surface roughness (Ma et al., 2006).

The interaction between TB dyes and surface –COOH groups was utilized to conceptually investigate the concentration of this particular functionality on the polymer coated electrospun fibers. It is known that a positively charged blue colored dye (TB) forms a stable complex with –COO⁻ by the ratio of 1:1 via electrostatic interaction (Hosseini et al., 2014a; Ma et al., 2006). The adsorption/desorption of this salt (TB) which contains an aromatic cation and chloride anion is pH dependent (Fig. 3d). It can be observed from Fig. 3e that TB dyes have also been adsorbed on uncoated PHB fibers as well as PMMA coated fibers even though these samples do not contain any –COOH surface groups (Schemes 1c and d). This is due to the fact that overall electronegativity accumulated around oxygen atoms can attract the TB cations to some extent. Therefore, results presented in Fig. 3e should be interpreted only as a comparative study. It can be concluded from the plot in Fig. 3e that the gradual increase in TB adsorption (from comp.(9:1) to comp.(5:5) coated fibers) is a direct function of relative increase in MAA segments which can only be assigned to an increase in concentration of surface –COOH groups. Dramatically higher TB adsorption was recorded for PHB fibers coated by comp.(5:5) in comparison to other compositions. Such behavior is most likely a consequence of polymer swelling which allows diffusion of TB dyes into the bulk of the coatings as TB is known to form a complex with negatively charged compounds through diffusion process (Hosseini et al., 2014a; Ma et al. 2006). This particular composition, as it was mentioned in AFM discussion, has shown gel-like properties due to the abundance of –COOH groups in the structure (Hosseini et al., 2014a).

To compare the performance of different surfaces (coated and uncoated PHB fibers) in detecting target analyte, sandwich ELISA assay was conducted and results are summarized in Fig. 4a. In order to accomplish the comparison, conventional ELISA has also been performed (in polystyrene analytical kit) as control. Relative standard deviations were evaluated by conducting 16 successive experiments on the developed samples. Generated OD signal from either physical or covalent immobilizations, in general, was approximately in the range of 0.2–0.6 units of OD which is comparable to the previously reported values for dengue detection (Alcon et al., 2002; Shu et al., 2003; Xu et al., 2006).

In the case of physical immobilization, pure PHB fibers have shown considerably higher detection signal than conventional assay. Although results have been plotted by subtraction of the cut-off values, it was observed that PHB fibers have generated the wrong positive signal when the assay was performed in the absence of DV (Fig. 4a, inset). Such phenomenon has not been observed for coated PHB fibers, except for comp.(5:5) with described swelling integrity (original data before subtraction of the cut-off values are presented in Supplementary section, Fig. 4S). This is direct evidence showing that even high hydrophobicity of the PHB fibers cannot protect the substrate from the risk of penetration.
fibers coated with other two compositions, comp.(7:3) and comp. (5:5). These platforms offer more surface concentrated –COOH groups but not necessarily the optimum amount. These results can be interpreted referring to the well-known fact that overly functionalized surfaces would yield in lower immobilization rates due to the steric repulsion (Hosseini et al., 2014b,d).

While application of cross-linkers is recommended even in contemporary commercial products, there still exist opponent viewpoints which take capability of such linkers under serious precaution (Sam et al., 2009; Wang et al., 2011). Perhaps one of the most applied cross-linkers for covalent binding is EDC. This carbodiimide agent (EDC) associated with NHS can activate the surface by converting –COOH groups to NHS-ester groups which are semi-stable and highly reactive compounds toward primary amines of proteins (Sam et al., 2009). As it can be seen from Fig. 4a, covalent immobilization, in general, yielded in lower detection signal than physical immobilization but higher than conventional ELISA. This trend was also recorded for coated PHB fibers with comp.(9:1) which offers available –COOH groups on the surface. Detection signal even significantly decreased when assay was performed with coated PHB fibers by other two compositions (comp.(7:3) and comp.(5:5)) which generously offer the abundance of surface –COOH groups. Sam et al. in an excellent research work revealed different possible functional groups that EDC/NHS treatment might generate instead of the expected functionality. For example, under particular circumstances, EDC/NHS reaction might result in formation of anhydride groups (instead of highly reactive NHS-ester groups) which are utterly unreactive towards proteins (Sam et al., 2009). EDC/NHS may also cause an early cross-linking inside the individual biomolecules which consequently makes the immobilization troublesome (Coad et al., 2013). A possibility of immobilization of –NH$_2$ groups, in close proximity to Ab active site, would also compromise the conditions of an immune complex. Apart from mentioned difficulties, even if desirable functionalities have been successfully achieved it is intolerably hard to reproduce them in the next lot of the experiments. Unlike covalent immobilization, physical attachment of the proteins to the surface was observed to be reliably reproducible. From all of the drawbacks which have been discussed, occurring one from many would be enough to make the obtained results questionable. For all the mentioned reasons, use of EDC/NHS as carbodiimide agents for covalent immobilization of the proteins must be taken under serious consideration.

A concentration range between $3.5 \times 10^{-10}$ and $3.5 \times 10^6$ p.f.u/ml has been chosen to assess the detection range of the developed platform in comparison to clinical ELISA (Fig. 4b). Selected platform for this experiment was coated PHB fibers with comp.(9:1) as this particular composition has shown the greatest detection performance among all. Results presented in Fig. 4b clarify that mentioned bioreceptor surface is adequate to be used in diagnostics as the signal received from this particular surface remains positive even in the lowest concentrations of the DV. In respect to cut-off values depicted for both methods, it can be observed that developed platform is successful in detection of DV in the concentration range that the conventional ELISA was found to be unreliable (detection signals at the cut-off point, Fig. 4b).

In order to evaluate the assay, sandwich ELISA was performed in polystyrene analytical kits, with and without developed platforms, in the concentration range between $3.5 \times 10^{-4}$ and $3.5 \times 10^5$ p.f.u/ml. Calibration curves were plotted and from the regression coefficients it can be concluded that the purposed platforms offer higher reliability (average $R^2=0.9861$) than actual conventional ELISA method ($R^2=0.9303$; Fig. 5S). The LoD values have been calculated for coated and uncoated electrosyn PHB fibers and results are shown in Table 1. All of the developed platforms have successfully detected DV in acceptable
concentration range which were reported for dengue penitents in the day 4–6 of the infection (Thomas et al., 2010). Coated electrospun fibers with comp.(9:1), in particular, have shown the capability of the detection in the lowest DV concentrations among all the examined platforms.

Calculated sensitivity and specificity of the proposed methodology are presented in Table 1. Total numbers of 130 samples have been examined to verify the potential of the assay in biological analysis. The precise calculations were determined and results are presented in Table 1 as well. Generally acceptable level of accuracy has resulted from coated surfaces in comparison to the conventional method.

In conclusion, developed method presented in this paper opens many ways for fabrication of cost-effective and highly sensitive platforms that can be used for detection of viruses not only limited to DV. Polymer coated DHB electrospun fibers have a great potential to be integrated into the microfluidic and/or paper-based bio-diagnostic devices after further optimization of analytical signals in presented DV concentration range. As our future direction, we are conducting research on production of new generation of installable analytical kits in separate parts at which polymer coated fiber segments are readily incorporated with the bottom part of the well-plate. Furthermore, in our laboratory, additional experiments are in progress in order to produce polymer/fiber blended material with tailored surface properties which make the coating procedure completely needless.

4. Conclusion

This paper presents processing of electrospun PHB fibers coated by poly(MMA-co-MAA) in different compositions which were synthesized via free-radical polymerization reaction. Developed surfaces have been successfully used for dengue virus diagnosis via physical and covalent immobilization through sandwich ELISA assay. In either case, use of polymer coated PHB fibers allowed the detection with higher sensitivity than conventional clinical method, ELISA. Proposed method in this paper opens many windows for production of cost-effective, portable, disposable and reliable diagnostic tools that can be used not only for detection of the dengue fever but many other neglected tropical diseases. Furthermore, such strategy can be applied for fabrication of new generation of analytical kits, microfluidic and paper-based bio-diagnostic devices with higher level of performance.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.02.034.