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Graphical abstract
HIGHLIGHTS

1. Polymer coated nylon membranes as bioreceptor surfaces.
2. Carboxylated porous surface for protein immobilization.
3. High level of biosensing performance for dengue virus detection.
A novel approach for application of nylon membranes in the biosensing domain

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Abstract

In this paper we report the polymer-coated microporous nylon membranes and their application as platforms for protein immobilization and subsequent detection of the dengue virus (DV) in blood serum. Protein recognition experiments were performed with enzyme-linked immunosorbent assay (ELISA). The polymers used for coatings were synthesized by free-radical polymerization reaction between methyl methacrylate (MMA) and methacrylic acid (MMA) in different concentrations. The MAA monomer has carefully been chosen to generate polymers with pendant carboxyl (–COOH) groups which also exist on polymer surfaces. A high degree of control over surface-exposed –COOH groups has been achieved through variation of monomers concentration in polymerization reaction. The general aspect of this work relies on the dengue antibody (Ab) immobilization on surface –COOH groups via physical attachment or covalent immobilization. Prior to Ab immobilization and ELISA experiment, polymer-coated nylon samples were analyzed in detail for their physical properties by atomic force microscopy (AFM), scanning electron microscopy (SEM) and water-in-air contact angle (WCA) measurements. Membranes were further analyzed by Fourier transform infra-red spectroscopy (FTIR) in order to establish the relationship between wettability, porosity and surface roughness with chemical composition and concentration of –COOH groups on the coating’s surface. Optimized coatings have shown high sensitivity towards
dengue Ab molecules, revealing fundamental aspect of polymer-protein interfaces as a function of surface –COOH groups’ concentration.

**Key words:** Biosensor; nylon membrane; polymer coating; surface properties; protein immobilization.

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1. Introduction

The dengue virus (DV) is a mosquito-borne infection that threatens more than 100 countries in tropical/subtropical regions and presents one of the most dangerous viral diseases with the statistics of 50-100 million infected cases every year [1, 2]. In that perspective, the development of highly-sensitive biosensors is of crucial importance for early detection and successful treatment of dengue-affected patients. Todays’ methods for DV detection mostly rely on enzyme-linked immunoassays (ELISA), which is still the most practically applied assay in the clinical practice. Since 1971, when the ELISA method has been invented, it was continually employed for many different applications, namely: (i) detection of microorganisms; (ii) determination of allergens in food industry; and (iii) detection of viruses and cancer biomarkers [3, 4]. Apart from the world-wide use of ELISA, many drawbacks have frequently been reported such as tedious preparation procedures, time-consuming technical steps, relatively uncertain results and, most importantly, the lack of desirable reactive functionalities on the surfaces of ELISA substrates [5-7]. In that sense, significant research efforts have been dedicated to the improvement of this method in order to achieve higher levels of analytical selectivity and sensitivity. In particular, novel systems such as ultramicro ELISA, fluorophore linked
immunosorbent assay (FLISA), dot-ELISA, capsid ELISA, dipstick ELISA and enzyme linked immunospotting (ELISPOT) have been developed for DV detection [1, 8-12].

Detection by biorecognition in ELISA is a heterogeneous process that relies on interfacial phenomena. Various polymeric materials have been used for immobilization of biomolecules and subsequent virus detection (including dengue) in biosensor devices. These are namely: polystyrene (PS), polydimethylsiloxane (PDMS), polyethyleneterephthalate (PET), polycarbonate (PC), cyclic olefin copolymer (COC) and polymethylmethacrylate (PMMA) [6, 13, 14].

Membrane substrates, from the family of polymeric materials, could facilitate analyte-surface interaction purely by their porous nature and desirable surface chemical compositions [15]. The modified ELISA methods have previously been developed on porous filter-membranes as an alternative to the conventional assay [4]. Membranes are materials that can provide a large specific surface area for biomolecular immobilization due to their highly porous structures [16, 17]. Microporous polyamide membrane (nylon) is an engineering plastic that offers narrow pore size distribution and good mechanical rigidity [18]. Nylon is a material of choice in many biomedical applications because of its outstanding properties, such as lightweight, low production costs, strength and durability [19-22]. Despite the superior material characteristics, nylon lacks desirable surface-reactive functionalities, such as amine (–NH₂), hydroxyl (–OH) and carboxyl (–COOH) groups, that would facilitate immobilization of proteins. The importance of surface functional groups and different “functionalization” techniques for polymer membranes have been described many times in the literature [23-25]. However, most of those surfaces are prone to “aging effect” which often results in unstable surface chemistries and consequent inefficient performance in bio-activation [2, 4]. For those reasons, a development of novel,
robust polymeric platforms with high degree of control over surface functional groups is essential.

In this article, we introduce a novel approach for fabrication of a cost-effective and flexible class of bioreceptor platforms which can be used for enhanced DV detection. Polymethyl methacrylate-co-methacrylic acid (poly(MMA-co-MAA)), synthesized by free-radical polymerization, has been used for coating a commercially available nylon membrane. A direct control of the concentration of surface –COOH groups has been achieved by variation of initial reactant concentration, namely methacrylic acid (MAA) monomer. Pre-determined polymer compositions were expected to influence the performance of polymer-coated membranes in regards to the protein attachment. Such combination creates a unique structure that offers considerably high surface area for biomolecular immobilization. Analyte-substrate interaction was performed via two different techniques: (1) physical adsorption; and (2) covalent immobilization with the aid of carbodiimide chemistry (Scheme 1). Results in this paper describe the method for production of flexible and functional platforms with controllable interface between Ab molecules and biosensor substrates. Apart from the new methodology for production of highly sensitive and cost-effective ELISA tests, here we also reveal some important fundamental processes that occur between functional polymer surfaces and Ab molecules in general.

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), bovine serum albumin (BSA), monosodium phosphate (NaH₂PO₄), Tween 20 and disodium hydrogen phosphate (Na₂HPO₄) were purchased
from Sigma, US. Purification of MMA monomer was performed by distillation method before free-radical polymerization reaction. Other materials were used as received. Tetrahydrofuran (THF), solvent for synthesis and coating procedures, and phosphate buffer saline (PBS) were purchased from Thermo Fisher Scientific, US. Azobisisobutyronitrile (AIBN), initiator for free-radical polymerization, was purchased from Friedemann Schmidt Chemical, Germany. Nylon transfer membranes (MAGNA, nylon transfer membrane, pore size 0.22 μm) were purchased from Osmonics and used as the substrate for polymer coatings and subsequent protein immobilization.

2.2 Poly(MMA-co-MAA) synthesis and processing

Four different compositions of the poly(MMA-co-MAA) have been polymerized (Scheme 1a) by free-radical polymerization reaction based on the previously reported method: pure PMMA; poly(MMA-co-MAA-9:1); poly (MMA-co-MAA-7:3); and poly (MMA-co-MAA-5:5) [2, 7, 26, 27]. For the ease of discussion, copolymer compositions are presented further in text as follows: comp.(9:1); comp.(7:3); and comp.(5:5). Numbers in brackets indicate the ratio (MMA/MAA) of monomers in polymerization reaction. The synthesis reaction has been carried out for 6 hours in THF by using AIBN as an initiator at 90°C. The washing procedure of the precipitated polymers was conducted by using distilled water to eliminate the excess of unreacted monomers. Polymer compositions were freeze-dried and stored in refrigerator.

2.3 Coating Procedure

Polymer coatings were prepared on nylon membranes by dip-coating procedure (Scheme 1b). Cleaned nylon membranes (substrates) have been coated by immersing membrane sheets inside
the polymer solutions (5% in THF). Polymer-coated nylon membranes were taken out after 5s of
the contact with polymer solution and dried at ambient temperature.

2.4 Morphology analysis by Atomic force microscopy (AFM) and scanning electron
microscopy (SEM)

Surface topography of the samples was recorded by AFM (Ambios, Q scope) in contact mode. Mean roughness (Ra) and the root mean-square roughness (Rq) for all of the coated samples were measured. Surface morphology of coated and uncoated nylon membranes was analyzed by SEM equipped with a field emission gun (FESEM, JEOL, JSM7600F), which was operated at an accelerating voltage of 0.5 kV. The samples were mounted on a double-sided conductive tape and coated with platinum, retaining the surface from charging. Frontal view and cross-section images of the samples were recorded in the secondary electron mode. Pore size distribution of the coated and uncoated membranes has been calculated for 500±5 pores (taken from frontal view SEM images) for each composition category. It is notable that standard deviations were negligible for this pore size measurement as a very small variation has been observed.

2.5 Water-in-air contact angle measurement

Water contact angle (WCA) measurements were carried out by depositing distilled water drops on the coated surfaces in room temperature by sessile drop method. The apparatus used for WCA analysis was Dataphysics Contact Angle System (OCA) instrument. Measurement was performed in 1 minute after depositing droplets of water (0.1µL) on the surfaces. The average result from five separate drops (on the center and four corners of the surface) was calculated (n=5) for 3 different samples. Standard deviations were found to be negligible as a minor error (±2°) resulted from the experiment.
2.6 Polymer analysis by Fourier-transform infrared spectroscopy (FTIR)

The coated samples of different compositions have been examined by FTIR spectroscopy operated in attenuated total reflectance (ATR) mode with 1 cm\(^{-1}\) resolution in the transmission mode and the wavenumber region between 400 and 4000 cm\(^{-1}\). The exhibited spectra were taken with a Perkin–Elmer model 1600 series FTIR-ATR spectrometer. For each spectrum, a circular shape of coated sample of 6 mm dimension was placed on specimen holder of the spectrophotometer. Presented spectra were result of 4 scans at the speed of 1 scan per 4s.

2.7 Dengue Ab immobilization on poly(MMA-co-MAA) coatings

Nylon membranes (coated and uncoated) were carefully cut into the round shapes with a diameter of 6 mm in order to fit into the ELISA 96-well plates (SPL, life science, Korea). Immobilization of the dengue Ab was performed via two different techniques: (1) physical adsorption of Ab molecules on the surfaces of the coated samples; and (2) covalent immobilization of Ab through carbodiimide chemistry (Scheme 1c). In the case of covalent immobilization, samples have been incubated in EDC/NHS solution (0.155 g of EDC and 0.115 g of NHS in 200 ml of PBS) for 1 hour prior to Ab immobilization. Samples were thoroughly washed with PBS prior to ELISA assay. Conventional ELISA on 96-well plate was conducted as control.

2.8 Sandwich colorimetric ELISA

Sandwich colorimetric ELISA was performed in order to assess the potential performance of polymer-coated nylon substrates as bioreceptor platforms for detection of DV. Capture Ab (Goat
IgG anti DV 2 (D-15): SC-325014) was diluted in coating buffer (0.85g of NaCl, 0.14g of Na₂HPO₄ and 0.02g of NaH₂PO₄ in 100ml of PBS) in the ratio of 1:300, and added (100µl) to each well. The well-plate was incubated for 2h at 37°C. Well plate was thoroughly washed with 200µl of washing buffer (0.05% Tween 20 in PBS) per well at room temperature. Washing steps were performed 3 times (each time 5 minutes) by using a mechanical shaker with the speed of 1000 rpm. After washing procedure, each well was charged with 100µl of blocking buffer (1g of BSA in 100ml of washing buffer) in order to block the non-specific binding sites and achieve higher selectivity of the detection. Blocking step was performed in the incubator at 37°C for 1h. Dengue enveloped virus (virus propagation in mosquito cells and titration were performed based on the previously reported method [2, 7, 27, 28]) was diluted (serial dilution) in coating buffer and different concentrations of the virus have been chosen based on the application. For plotting the calibration curves the concentration range between 3.5×10² p.f.u/ml to 3.5×10⁻² p.f.u/ml was used. The quantitative detection study was performed in a wide concentration range of virus (3.5×10⁶ p.f.u/ml -3.5×10⁻² p.f.u/ml). The representative concentration of DV for performance comparison of the developed platforms was chosen to be 3.5×10² p.f.u/ml as this particular concentration has resulted in the highest detection signal (will be discussed further in the text). Each well of ELISA kit was charged with 100µl of virus solution and incubated overnight at 4°C. As the next step, wells were incubated with 100µl of diluted (1:200) primary Ab (mouse IgG2a anti DV, ab155863, Abcam US) in diluting buffer (0.4g BSA, 4ml PBS buffer and 120µl of Tritonx-100 in 36 ml of distilled water). Wells were washed after 2h of incubation at 37°C. Secondary Ab solution was diluted (1:500) by adding anti-mouse IgG2a alkaline phosphatase (ab97242, Abcam US) to diluting buffer. Each well of ELISA kit has received 100µl of secondary Ab solution and well plates were placed inside the incubator for 30 minutes at 37°C.
Wells then, were washed (based on the described method) and charged with 100µl of mixed substrate (Alkaline phosphatase blue micro well substrate components A and B). After 10-15 minutes, reaction was stopped by adding 50µl of alkaline phosphatase as stopping buffer (A585, Sigma) and signal intensity were recorded by using Bio-Rad microplate reader (model 680) at the wavelength of 570nm. Negative controls (n=8) have been measured in the absence of DV. It is important to note that presented results in this study have been plotted by excluding the background signals and negative control (except in the cases where those cut-off values are shown in reported figures). Nevertheless, cut-off values have also been plotted and added to detection graph for the ease of discussion. Sensitivity and specificity of the proposed methodology were investigated by running the assay with total number of 56 samples via sandwich ELISA. Positive samples (n=40) were measured with the DV, while the negative samples (n=16) were totally non-infected. Accuracy of the method (%) was calculated as the sum of the true positives and true negatives divided by the total number of samples [29]. Limit of detection (LoD) for each surface was calculated by analyzing slope of the related calibration curves and average standard deviations [30].

3. Results and discussion

3.1 Physical properties of polymer-coated nylon membranes

The morphology of coated membranes was analyzed with SEM and representative images are shown in Fig. 1. The porous structure remained unchanged upon dip-coating and subsequent drying for all the analyzed coatings, regardless of the polymer composition (Fig. 1, a and b). The cross-sectional view recorded by SEM reveals a polymer presence distributed over entire membrane volume, which is obvious in comparison to the uncoated nylon control (inserts, Fig. 1,
c and d). The pore size distribution has been calculated from SEM images and the largest average pore size was recorded within PMMA-coated membranes (Fig. 2). Such behavior could be a consequence of close packing between 2 hydrophobic polymers (nylon and PMMA) where the presence of hard PMMA has caused the shrinkage of nylon struts within porous structure. A quite opposite result was obtained from poly(MMA-co-MAA)-coated membranes that contain – COOH functional groups. The results in Fig. 2 show an obvious systematic decrease in pore sizes with an increased concentration of MAA segments in polymer chains.

The surface topography of both pure and coated nylon membranes was analyzed by AFM and the results are presented in Fig. 3. AFM images show a gradually increased roughness in copolymer-coated membranes which reached the peak point at comp.(5:5). This macromolecular structure showed significantly rougher surface in comparison to other two poly(MMA-co-MAA) compositions (Fig. 3, a-c). In particular, comp.(5:5) coatings showed signs of continuous and sharp fibers and deeper pits consistent with quantitative experimental results (Fig. 3, bottom). Apart from comp.(5:5)-coated membrane, the total roughness decreased significantly with the introduction of MAA into polymer composition. The coatings of comp.(9:1) and (7:3) have resulted in smoother surfaces, unlike the polymer coating with highest concentration of surface – COOH groups (comp.(5:5)). In our previous work we have observed that the comp.(5:5) is a gel-like material in its nature and is much softer than other analyzed compositions [2, 7, 26, 27]. This specific material integrity directly corresponds to macromolecular composition, thus causing an obvious change in both surface morphology and pore size distribution.

The recorded response of the nylon membrane to water interaction from WCA experiment is displayed in Fig. 4. Apparent high wettability of both nylon and PMMA-coated membranes is a consequence of water penetration through the membrane over the time of WCA experiment. This
is expected as the nylon membranes, used in our experiments, are generally produced for protein filtering through the diffusion process. The WCA measurements were impossible to record (supplementary video is available) due to a relatively high porosity. This behavior was also followed by PMMA-coated membranes with even larger pores (as presented in Fig. 2). Unlike PMMA, poly(MMA-co-MAA) coatings stopped the diffusion so WCA was conveniently measureable with a reasonably small standard deviations (negligible in plotting). As presented in Fig. 4, WCA measured for poly(MMA-co-MAA)-coated membranes, has gradually decreased by increasing the concentration of MAA monomers in free-radical polymerization [7, 26, 27]. The result confirms reasonable expectations that the hydrophilic nature of surface –COOH groups would certainly result in higher hydrophilicity of the platforms [2, 26]. Logically, the lowest contact angle was measured on the surface of the coated membrane with comp.(5:5) [7]. Apart from the water diffusion characteristics, a significant alterations in surface roughness, average pore size, wettability and surface concentration of –COOH groups are expected to have a strong influence on interaction with Ab molecules.

3.2 Chemical structure of polymethacrylate coatings: surface –COOH groups

FTIR spectra obtained for all poly(MMA-co-MAA) compositions, pure PMMA coating and uncoated nylon membrane are presented in Fig. 5. Amide groups that exist in nylon structure can be recognized in FTIR spectra from peaks located at 1660 and 1541 cm⁻¹, which correspond to the amide I and II bands respectively [31]. Detected wavelengths of the dominant bands in the range of 2800-3500 cm⁻¹ originate from the combination of different types of C–H stretching vibrations [32]. The appearance of the peak at 1729 cm⁻¹, which is attributed to the carbonyl (O=C=O) groups, has shown the major difference between spectra resulted from copolymer compositions analysis [33]. The intensity of the marked peak (1729 cm⁻¹) increased and the peak
became broader as the concentration of the MAA segments in copolymer compositions increased. This can be considered as a strong evidence for systematic replacement of –COOCH₃ with –COOH groups, generated from MMA and MAA respectively. Furthermore, the broad peak located at 3500 cm⁻¹ (attributed to –OH stretching vibration) is present in FTIR spectra, recorded for all polymer-coated samples, except for PMMA [34, 35]. Such –OH functional groups are formed as a result of MAA monomers in polymerization reaction which include –COOH functional groups. For reminder, concentration of –COOH functional groups has been controlled by changing the concentration of monomers in free-radical polymerization reaction.

3.3 Dengue virus detection in sandwich ELISA

Different concentrations of the DV were used in sandwich ELISA method performed on coated and uncoated surfaces in order to calculate the analytical calibration curves for the assay (Fig. 6). The optical density (OD) was measured at specific wavelength against the wide concentration range (Log scale) at which the coated membranes have displayed consistently higher OD in comparison to ELISA control. Furthermore, calibration curves obtained from the membranes are more reliable as the square correlation coefficients (R²) are significantly higher in comparison to the calibration curve from a conventional ELISA method. The best linear fit was obtained from comp.(5:5) with R² = 0.9967. This particular composition also resulted in the highest degree of hydrophilicity and the highest surface roughness, along with the smallest pore sizes as described in previous section. The detection range of the developed surfaces in comparison to a conventional ELISA was assessed and the results are presented in Fig. 7. Developed platforms have resulted in substantially higher OD values in comparison to ELISA control, which has shown the lowest signal intensity in all of the virus concentrations. It should be noted that higher signal intensity was obtained in the middle range of virus concentrations, in particular at the
value of $3.5 \times 10^2$ p.f.u/ml. This concentration has been used in further assessment of Ab immobilization on polymer-coated membranes.

### 3.3.1 Physical attachment of Dengue antibody

As described in section 2.7, coated membranes of different compositions have been cut in round shapes with diameter of 6 mm in order to fit inside the ELISA 96 well-plates. A conventional ELISA has also been conducted in order to carefully assess the performance of the proposed platforms for protein immobilization. In particular, we monitored the Ab activity upon surface attachment and subsequent DV detection signal, generated from the coated nylon membranes. As depicted in Fig. 8, there is no significant difference in detection signal obtained from the uncoated nylon and the membranes coated with PMMA. This result was somehow expected as both surfaces showed approximately the same porous structures and wetting characteristics, obtained from pore size distribution calculations and WCA analysis (Fig. 2 and Fig. 4). Proteins initially attach to microporous structure of membranes by means of physical interaction, mainly via three fundamental forces: (1) hydrophobic interactions; (2) hydrogen bonding; and (2) ionic attraction [36]. In particular, the influence of hydrophobic interactions on protein binding to solid substrates has been frequently reported in literature [36]. In case of microporous substrates, the capillary forces can stabilize the layer of hydration around the nylon membranes; therefore, opportunity for hydrophobic interactions can be limited [37]. Although uncoated nylon has indicated relatively high signal intensity, the negative signal related to the nylon membrane (and PMMA-coated) was also in the highest level when compared to copolymer-coated membranes (Fig. 9). This experimental error was most likely introduced by the large pore sizes that allowed the diffusion of Ab solution (Fig. 2). The water diffusion made penetration of Ab molecules possible inside the porous structure and entrapped primary Ab molecules could not have been
eliminated by washing steps (section 2.8). Generally, this phenomenon is most likely to cause the false signal by coupling with labeled secondary Ab in sandwich ELISA method [38]. For that reason, the detection signals from uncoated and PMMA-coated nylon samples are insignificant as the negative controls are too high.

The surface of comp.(5:5) has resulted in the most efficient protein immobilization judging from the recorded detection signals and cut-off values (Fig. 9). This composition contains the maximum number of surface –COOH groups available for protein immobilization. Carboxylated surfaces are known to effectively trap proteins through hydrogen bonding which is a dominant interfacial force rather than hydrophobic interaction [36]. As the concentration of surface –COOH groups increases, hydrogen bonding between the protein molecules and the carboxylated surface would be favored interaction. The results obtained from ELISA experiment, performed on the polymer-coated membranes, should also be discussed in terms of physical morphology of the samples. The surface of comp.(5:5) has shown the highest degree of roughness in AFM experiment in comparison to other copolymer structures (Fig. 3, bottom). Generally, it is expected that higher roughness and higher surface area should lead towards more efficient protein attachment. In any case, the negative control signals should be subtracted as “noise” from the experimental signals (Fig. 9 contains original data along with cut-off values). If this methodology is adopted in practice, the obtained signals from pure and PMMA-coated nylon samples would generate OD that is comparable to clinical ELISA for DV detection (Fig. 9, insert). This is not the case for poly(MMA-co-MAA) coatings which generated the signal intensities 3 times higher even after subtraction of cut-off values. Detection performance of the assay has significantly increased when the –COOH groups have been introduced, although other parameters such as surface morphology, porosity and contact angle should not be neglected.
3.3.2 Covalent attachment of Dengue antibody

Covalent immobilization of proteins is one of the most commonly applied methods for development of biosensors [39]. This is a heterogeneous chemical reaction between reactive sites that are present on both phases: solid phase (substrate) and aqueous medium (analyte). Surface functionalities are generally transformed to semi-stable and highly reactive groups that further react to covalently bind biomolecules, supposedly leading to higher detection signals and more robust detection protocols [40]. Covalent immobilization can be accomplished via cross-linkers of different sizes [40]. One of the most commonly used carbodiimide cross-linkers is EDC (associated with NHS) for variety of applications such as bio-recognition, drug delivery devices and tissue engineering templates. EDC/NHS method is normally used to activate carboxylated surfaces that further react with primary –NH2 groups of proteins; this reaction can also be conducted vice versa between aminated surfaces and pendant –COOH groups of proteins. In the present case, we made an attempt to convert available –COOH surface functional groups into semi-stable NHS-ester groups which are highly reactive toward proteins (Ab molecules in this case). EDC/NHS treated membranes, coated with comp.(5:5) have resulted in the highest detection signal intensity (as it can be observed from Fig.9) in comparison to the coated membranes with other polymer compositions. This particular composition has proven to be the most efficient one, not only for covalent immobilization but also for physical adsorption of Ab on the surface (discussed in section 3.3.1). The signal intensity, resulted from covalent immobilization of biomolecules to poly(MMA-co-MAA)-coated membranes, gradually increased (from 9:1 to 5:5; Fig. 9) with an increasing concentration of –COOH groups, generated from MAA co-polymer segments.
Despite the promising results from our experiments, there are several factors that have to be taken under serious consideration when dealing with heterogeneous chemical reactions mediated through carbodiimide chemistry. In some cases, the conversion of –COOH groups by EDC/NHS treatment can yield undesirable functionalities which would prevent further reaction with –NH₂ groups from Ab molecules. For example, in particular cases, EDC/NHS reaction can result in formation of anhydrides (instead of NHS-ester groups) which are unreactive towards amines [40]. Another phenomenon that could lead to protein deactivation is an early cross-linking inside the individual protein molecules [41]. Described uncertainties often contribute to low reproducibility and high standard deviations, which also have affected our experiments (note the comparatively high standard deviation values in Fig. 9). Due to the above mentioned obstacles and uncertainty of carbodiimide surface treatments, all potential biosensors must be thoroughly tested for protein activity upon immobilization. Considering experimental facts, physical adsorption of Ab molecules would be a recommended method due to the proven technical simplicity, cost effectiveness and highly reproducible analytical results [27].

The calculated values presented in Table 1, show sensitivity and specificity of investigated membrane platforms. A non-specific binding, resulted from the negative controls, produced background signal that was used to quantitatively determine sensitivity and specificity of developed analytical method [1]. A total number of 40 positive and 16 negative replicates from the total of 56 samples have been examined to assess the potential of each coated membrane group in biological analysis. Developed platforms, in general, have shown a good level of sensitivity. In particular, coated membranes with comp.(7:3) and comp.(5:5) have resulted in the highest sensitivity (100%). The level of specificity was observed to be very low for pure nylon membrane (6.25%). Similar result was obtained for PMMA-coated nylon (37.5%) and therefore
it can be concluded that those two substrates are not reliable platforms for the DV detection.

Unlike those two types of membranes, copolymer coatings resulted in increasingly higher specificity (up to 93.75% for comp.(5:5)-coated membranes). It is noteworthy that all the developed surfaces have shown significant level of stability as no sign of fragility or delamination was observed during the assay. Our results in Table 1 also demonstrate that coated nylon membranes with copolymer compositions, generally, increased the diagnostic accuracy of the conventional ELISA. Detailed analysis of the LoD was included in Table 1 as well.

Developed platforms (except uncoated nylon membrane) have proven to be capable of detecting significantly low concentrations of DV in blood serum. Specifically, the LoD for comp.(5:5) reached down to 117 units of the virus in the blood serum which is a promising results for early detection of DV [2, 27, 42].

In a parallel study, we have investigated the poly(MMA-co-MAA)-coated silicon wafers as biochip platforms [2]. In case of the flat silicon surfaces, comp.(9:1) resulted in the highest intensity of ELISA detection signal. This result is fundamentally different in the present case as far as the influences of both surface chemistry and micro-morphology are considered [2, 7, 26]. Performance comparison between these two platforms revealed an obvious influence of surface morphology and porosity on the activity of immobilized Ab molecules [27]. Nylon membrane is privileged with many practical advantages such as: (i) nylon membrane is an industrial material that is available for a reasonable price in comparison to relatively expensive silicon wafers; (ii) the flexibility and porosity of the membranes present further advantages which could enable the fabrication in variety of applications, shapes and sizes. Nonetheless, untreated commercial nylon membranes are not desirable materials for their applications as bioreceptor surfaces. Our methodology, however, provides a unique platform with a very special micro-morphology and
surface chemical profile, along with the vast specific surface area available for biorecognition.

Our adaptive technique presented in this article, creates numerous opportunities in fabrication of cost-effective and disposable biosensing platforms that can be integrated into flexible diagnostic devices.

4. Conclusion

Polymer-coated nylon membranes have been produced with different concentrations of surface carboxyl groups. The close control over surface chemical composition has been achieved by variation of initial monomer concentrations in the free-radical polymerization reaction. Dengue Ab molecules have been immobilized on coated membranes for ELISA experiment and the immobilization procedure has been conducted by two different methods: (1) physical adsorption; and (2) covalent immobilization of Ab via carbodiimide chemistry. In either case, our results showed significantly higher detection signals generated from nylon membranes coated with synthesized copolymer, in comparison to conventional dengue detection used in clinical practice. The experiments demonstrated high selectivity of Ab molecules towards surfaces with different concentrations of carboxyl groups and the effective surface attachment of biomolecules has been achieved while preserving the activity of the proteins to a large extent. Polypeptides in general have pendant carboxyl and amine groups that could be used for surface immobilization. In that perspective, nylon substrates coated with developed polyacrylate macromolecules could possibly be used for any type of protein immobilization. For that reason, our method can be considered as universal approach in development of biosensors for different types of viruses. Furthermore, flexible nylon membranes have promising future in production of disposable microfluidic biosensors with great potential for development on industrial scale.
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References


Scheme, Figures and Table:

**Scheme 1.** Polymer-coated nylon membranes for the dengue virus detection: (a) free-radical synthesis of poly(MMA-co-MAA) with AIBN initiator; (b) dip-coating of nylon membranes in the polymer solution; (c) dengue antibody attachment to polymer-coated membrane and colorimetric ELISA experiment with (left) EDC/NHS cross-linking, and (right) physical adsorption of dengue Ab molecules.

**Figure 1.** SEM images of polymer-coated membranes (a and b); cross-sections of the uncoated nylon membrane control (c); and representative poly(MMA-co-MMA) coated membrane (d); the magnification bar of 10µm also applies to inserts.

**Figure 2.** Pore size distribution calculated from optical microscopy analysis (measured for 500±5 pores from SEM images of different coated samples).

**Figure 3.** AFM images of poly(MMA-co-MMA) coated nylon membranes (top): (a) comp.(9:1), (b) comp.(7:3), and (c) comp.(5:5); (bottom) detailed AFM analysis for coated and uncoated samples.

**Figure 4.** Water-in-air contact angle measurements for coated and uncoated nylon membranes (a); representative images of the droplets on the coated surfaces with: (b) comp.(9:1); and (c) comp.(5:5).

**Figure 5.** FTIR spectra recorded for pure nylon membrane (a) and polymer-coated membranes: (b) PMMA; (c) comp.(9:1); (d) comp.(7:3); and (e) comp.(5:5).

**Figure 6.** Calibration curves obtained from colorimetric ELISA for dengue virus detection (experiments performed on polymer coated and uncoated membranes in comparison to conventional ELISA as control).

**Figure 7.** Results from the DV detection range experiments performed on different types of membranes and conventional ELISA on standard PS 96-well plates (cut-off values calculated as 2 x the average of negative control).

**Figure 8.** The dengue detection performance measured for selected DV concentration ($3.5 \times 10^2$ p.f.u/ml) ; (insert) clinical ELISA for DV detection with plotted cut-off value.

**Table 1.** Analytical data and limit of detection (LoD) of coated and uncoated nylon membranes; the total number of 56 replicates (40 positive and 16 negative) for each composition was examined in sandwich ELISA for DV detection.
Scheme 1.
Figure 1.
Figure 2.
### Figure 3.

<table>
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<tr>
<th>Composition</th>
<th>Mean roughness (nm)</th>
<th>Root mean-square roughness (nm)</th>
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<td>Comp.(5:5)</td>
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Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
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<th>Comp.(7:3)</th>
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