eXPRESS Polymer Letters Vol.10, No.2 (2016) 96–110
Available online at www.expresspolymlett.com
DOI: 10.3144/expresspolymlett.2016.11

Electrochemical impedance and spectroscopy study of the EDC/NHS activation of the carboxyl groups on poly(ε-caprolactone)/poly(m-anthranilic acid) nanofibers

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Received 2 Juy 2015; accepted in revised form 2 September 2015

Abstract. Electrochemical impedance spectroscopy (EIS) and spectroscopy was applied to investigate the surface activation of carboxyl group (–COOH) containing nanofibers by the reaction of 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxyl succinimide (NHS) in different concentrations. Poly(ε-caprolactone)/poly(m-anthranilic acid) (PCL/P3ANA) nanofibers were fabricated by electrospinning and were activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC/NHS. The surface activation was investigated by Attenuated Total Reflectance Fourier transform infrared spectroscopy (FTIR-ATR) and activation yield was estimated. Albumin was immobilized after surface activation and the amount of covalently immobilized protein was determined by bicinchoninic acid (BCA) assay. Morphology and composition of albumin immobilized nanofibers were characterized by Scanning Electron Microscopy/Energy-Dispersive X-ray Spectroscopy (SEM/EDX) and Atomic force microscope (AFM). EIS measurements indicated that nanofibers become resistant after albumin immobilization. The obtained data revealed that the highest amount of albumin bound to nanofibers activated with 50/50 mM of EDC/NHS which was found to be the optimum concentration for the activation of PCL/P3ANA nanofibers.

Keywords: *nanomaterials, covalent immobilization, electrochemical impedance spectroscopy (EIS), poly(m-anthranilic acid)*

1. Introduction

Using nanostructured materials as supports for biomolecule immobilization have recently attract great interest considering their extremely high surface area to volume ratio [1]. Among these nanostructured materials, electrospun nanofibers stand out as the better alternative for immobilization of biomolecules due to the higher loading capacity and porosity compared to polymer films [2, 3].

Polymeric nanofibers allow further optimization of properties such as electrical conductivity [4] to meet different requirements in various applications. In the case of conductive polymer containing nanofibers, modification of a surface with a biomolecule can be detected by electrochemical impedance spectroscopy (EIS) [5, 6]. There other methods such as X-ray photoelectron spectroscopy (XPS), Thermal gravimetric analysis (TGA), Scanning electron microscopy (SEM) etc. can be used to investigate the surface modification [7], however EIS is a very sensitive method for the analysis of the interfacial properties such as resistance and capacitance of a surface by using very small amplitude sinusoidal voltage signals without significantly disturbing the properties being measured. Therefore, EIS is a sensitive, easy and cost effective method which allows

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reuse of samples after EIS measurements. Poly(m-anthranilic acid) (P3ANA) is a very convenient conductive polymer for immobilization of biomolecules covalently due to the presence of carboxylic acid group –COOH on the main polyaniline backbone [8].

Covalent immobilization of the biomolecules by using reactive groups has been recognized as an attractive strategy since it provides the control of the immobilization of biological probes on the surface [9]. Covalent immobilization of biomolecules onto a –COOH group containing surface consists of preparation of a succinimidyl ester (–COOSuc)-terminated surface and its reaction with an amino ($–NH_2$) group on the biomolecule. This reaction is referred as surface 'activation' which is conducted by reacting a surface bearing carboxyl end groups with N-hydroxysuccinimide (NHS), in the presence of carbodiimide such as 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) [10]. EDC/NHS activation of carboxylic acids has been widely applied to various kinds of substrates of polymers [11], silicon [9], nanotubes [12] or nanoparticles [13, 14]. In these studies, the concentrations of EDC and NHS strongly vary in a wide range (from M to the mM range) from one study to another [15–17]. Voicu *et al*. [15], used 0.1 M NHS and 0.4 M EDC in order to activate silicon surface. In another study, equal amounts of EDC and NHS (100 mM) were used for activation of carboxylic acid terminated self-assembled monolayers [18]. Zander *et al*. [19] activated the surface of plasma-treated PCL nanofibers by using approximately 25 mM EDC and 43 mM NHS. Electrospun collagen or gelatin nanofibers were activated by 30 mM EDC and 6 mM of NHS [20]. Based on the EDC/NHS concentrations used in the literature, for the activation of carboxyl groups of PCL/P3ANA nanofibers, it was decided to use equal amounts of EDC/NHS (EDC/NHS = 5/5 mM and EDC/NHS = 50/50 mM), excess amount of EDC over NHS (EDC/NHS = 5/0.5 mM) and excess amount of NHS over EDC (EDC/NHS = 0.5/5 mM). These concentrations were selected since very large concentrations of EDC or NHS result the formation of the byproducts at the surface which can prevent the formation of –COOSuc surface and affect the success of the surface activation. In the case of EDC and NHS concentrations are very low, then the surface activation reaction remains incomplete [7]. Although the EDC/NHS activation has been widely used, the details of EDC/NHS activation of carboxyl groups of a semiconducting nanofiber and of the following amidation of NHS-ester for protein immobilization have not been reported. This study aims to investigate the optimum EDC/NHS concentration for surface activation of PCL/P3ANA nanofibers by spectroscopy and electrochemical impedance spectroscopy.

Poly(ε-caprolactone)/poly(m-anthranilic acid) nanofibers were fabricated by electrospinning. Figure 1 represents the chemical structure of PCL/P3ANA and shows the interaction between partial positive charges of amine groups in P3ANA [21, 22] and partially negative charges in PCL backbone [23]. The activation of carboxyl groups on the surface of PCL/P3ANA nanofibers were performed with different concentrations of EDC and NHS. Due to its low price and availability in large amounts, albumin was selected as model protein and covalently immobilized to activated nanofibers. The success of activation procedure and the amount of immobilized albumin were investigated by FTIR-ATR and the surface activation yield was estimated. The amount of covalently immobilized protein was determined by bicinchoninic acid assay. Morphology and composition of nanofibers before and after albumin immobilization were analyzed by AFM, (SEM)/(EDX). EIS analysis and equivalent circuit modeling was performed to observe the immobilized albumin depending on the EDC/NHS concentrations used in activation. The nanofibers become resistive due to albumin immobilization. The obtained data revealed that electrochemical impedance spectroscopy can be used for investigation of surface modification with biomolecule immobilization by the determina-

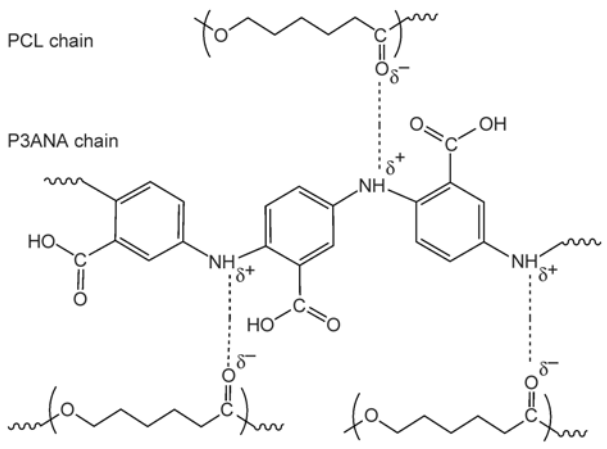

Figure 1. Schematic representation of the chemical structure of PCL/P3ANA

tion and quantification of the optimum and effective concentration for the activation of carboxyl group bearing polymeric nanofibers.

2. Experimental

2.1. Materials

Poly(ε-caprolactone) (440744) (M_w = 80 000 g/mol), 3-aminobenzoic acid (m-anthranilic acid) (127671), albumin (M_w ~ 66 kDa) (A2153), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic acid (MES, low moisture content, ≥99) and the bicinchoninic acid (BCA) Protein Quantitation Kit were purchased from Sigma Aldrich (St Louis, MO, USA). Phosphate buffer saline (PBS) was obtained from Gibco (Carlsbad, CA). Dimethyl formamide (DMF Analytical Grade) (103034) and Tetrahydrofurane (THF Analytical Grade) (109731) were supplied from Merck (Darmstadt, Germany). Poly(m-anthranilic acid) (P3ANA) was synthesized according to our previous study [8]. Briefly, 3-aminobenzoic acid (0.043 mol) has been dissolved in 90 mL of 0.5 M NaOH. Equal mole of ammonium persulfate in water (30 mL) has been added drop wise to this solution and the mixture was stirred at room temperature for 24 h. The precipitated polymer has been collected by filtration, washed with 1.2 M HCl and water until the filtrate became colourless and dried in vacuum at 60 °C for two days. This polymer has been recovered in 70% yield as a brown powder. All of these chemicals were analytical grade and used as received.

2.2. Fabrication of PCL/P3ANA nanofibers by electrospinning

0.125 g of poly(m-anthranilic acid) were dissolved in 5 mL THF and DMF mixture (1/1 v/v) containing 0.5 g poly(ε-caprolactone). The obtained polymer blend was stirred for 3 h at room temperature to obtain homogeneous solution for optimum electrospinning conditions.

Electrospinning solution was placed into a syringe needle (outer diameter of 0.7 mm) which was connected to a high-voltage direct current (DC) power supply (ES 30 Model Gamma High Voltage Inc., Florida, USA). PCL/P3ANA solution was horizontally electrospun onto aluminum foil at 15 kV driving voltage with a collection distance of 15 cm. The flow rate of the solution was 1 mL/h and controlled by a syringe pump (NE-500 Model, New Era Pump Systems, Inc. New York, USA). For reproducible electrochemical measurements, nanofibers were also collected onto ITO-PET (NV Innovative Sputtering Technology, Zulte, Belgium, PET 175 lm, Coating ITO-60) substrate with dimensions of 0.5×2.5 mm, for 5 min.

2.3. Surface activation and covalent immobilization of albumin onto the PCL/P3ANA nanofiber mats

In order to activate the –COOH groups available on the surface of the nanofiber mat, the appropriate concentrations (5/0.5, 0.5/5, 5/5 and 50/50 mM) of EDC and NHS solutions were prepared with cold water. In order to investigate the influence of EDC and NHS concentrations during activation of carboxyl –COOH groups in nanofibers, PCL/P3ANA nanofiber mats on ITO-PET substrates were treated by shaken gently with 5 mL of the EDC/NHS solutions for 2 h at room temperature [24]. Activated mats were then washed twice with water. Activated mats were treated with 1 mL of albumin dissolved in MES buffer (pH 5.7) with the concentration of 2 mg/mL. Nanofiber mats were incubated with albumin for 2 h at +4 °C, being shaken gently. Finally, mats were taken out, washed twice again with water and dried for characterization. Initial albumin solutions as well as the residual solutions of each consecutive washing step were collected for further characterization.

2.4. Spectroscopic and morphological quantification of protein amount on the nanofiber mats

The activation of carboxylic acid groups on PCL/P3ANA nanofibers and the gradual change in the amount of immobilized protein depending on the EDC and NHS concentrations in the activation procedure was determined by FTIR-ATR spectrophotometer (Spectrum One, with a universal ATR attachment with a diamond and a ZnSe crystal) (Perkin Elmer, Massachusetts, USA), SEM/EDX (QUANTA 400 F) (FEI, Oregon, USA), AFM (Nanosurf Easy-Scan2) and BCA protein assay. The surface activation yield were estimated by calculation of the FTIR peak ratios of the formed succinimidyl ester and O–H stretching of carboxylic acid group by using Beer-Lambert law. All FTIR-ATR spectra were collected with 12 scans in the 600–4000 cm^{-1} spectral region at 4 cm^{-1} resolution. SEM/EDX analyses were performed before and after albumin immobi-

lization, with 10 kV accelerating voltage after nanofiber mats were coated with gold by Ion Sputter Metal Coating Device (MCM-100 Model). The average nanofiber diameters were determined using Image J by randomly measuring the diameters of 20 individual fibers shown in SEM images with 1 μm magnification. The influence of EDC and NHS concentrations on the structure and composition of the albumin immobilized PCL/P3ANA nanofibers were analyzed by detection of elemental concentrations for nitrogen and oxygen with EDX. The distribution of immobilized albumin on the surface of nanofiber mats was examined by EDX-mapping. The amount of immobilized albumin on the activated nanofiber mats was determined by BCA protein assay by subjecting the initial and residual solutions from albumin immobilization. As a control, albumin immobilization was carried out on non-activated PCL/P3ANA nanofibers under the same experimental conditions. The total protein content in each sample was calculated by comparing the means of absorption values with a standard curve of bovine serum dilutions (between 0 and 2 mg/mL). The samples were incubated with BCA working solution at 37 °C for 30 minutes and the absorbance was followed at 562 nm by UV-Vis spectrophotometer (Lambda 45) (Perkin Elmer, Massachusetts, USA) [25]. AFM images of PCL/P3ANA and albumin immobilized nanofibers after activation with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC and NHS, were taken in non-contact mode using Al coated high resonance frequency silicon tips (Nanosensors NCRL tips, 40 μm width, 225 μm length). Root-mean-square (RMS) roughness values of nanofibers were calculated *via* Easy Scan 2 Software™ (version 3.0.2.4) by selecting raw data.

2.5. Electrochemical impedance spectroscopic measurements of albumin immobilized nanofibers

The effect of the concentrations of the EDC and NHS reactants on the activation reaction and on the amount of immobilized protein was investigated by Electrochemical Impedance Spectroscopy (EIS). EIS measurements were performed in PBS (0.1 M, pH 7.4) using potentiostat 2263 Electrochemical Analyser (Princeton Applied Research, Tennessee, USA) with frequency range between 0.01 Hz and 100 kHz and AC voltage of 10 mV. Three-electrode system, consisting of nanofiber mats after albumin immobilization as working electrode, platinum wire as counter electrode, and silver wire as pseudo reference electrode, was used. All measurements were repeated three times for confirmation. The measured impedance spectra were analyzed in terms of electrical equivalent circuits using the analysis program ZSimpWin V.3.10 (Princeton Applied Research, Tennessee, USA).

3. Results and discussion

3.1. FTIR-ATR characterization of activated PCL/P3ANA nanofibers

FTIR-ATR spectra of PCL/P3ANA nanofibers were obtained after activation procedure in order to reveal the influence of NHS and EDC concentrations (Figure 2). P3ANA in the PCL/P3ANA nanofibers displays the peaks at 2615, 1690, 1580 and 1510 cm^{-1} which were attributed to O–H stretching, C=O

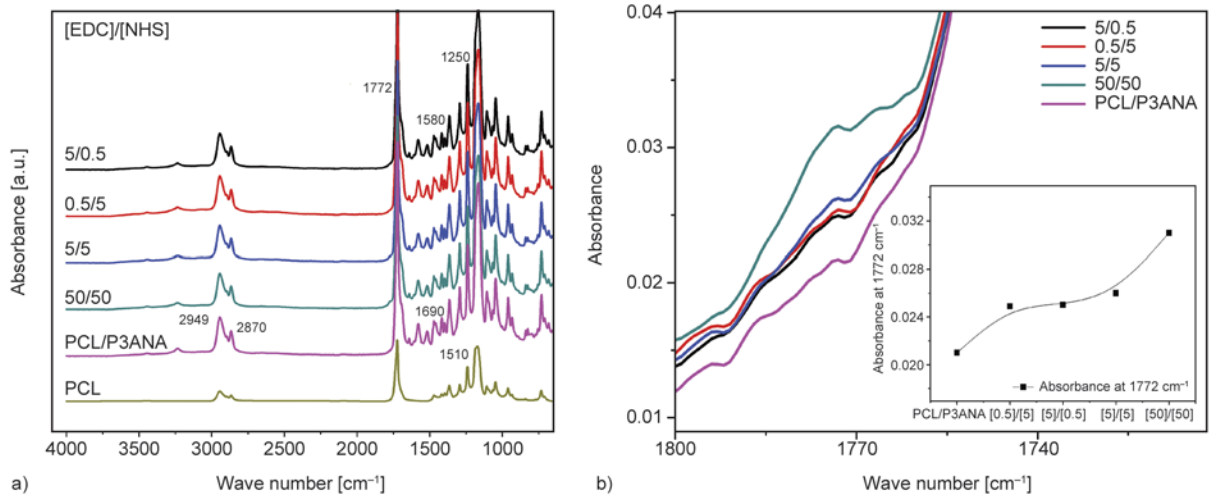

Figure 2. FTIR-ATR spectra of PCL, PCL/P3ANA and PCL/P3ANA nanofibers activated with EDC/NHS mixtures of various concentrations (a), succinimidyl ester absorbance (b) and the increase in absorbance (b inlet) after activation

stretching, C=C stretching and N–H stretching, respectively. The peaks at 1250 and 1070 cm^{-1} were attributed to C–N stretching [8, 26]. The carboxyl groups on P3ANA provide active sites for albumin binding through forming succinimidyl ester (–COOSuc) by reacting with NHS, in the presence of EDC [7]. The attachment of a succinimidyl ester termination to PCL/P3ANA nanofibers can be evidenced by the triplet in the spectral range of the C=O stretching vibrations. The activation can be observed in some spectra by a shoulder or an asymmetry of the 1772 cm^{-1} peak ascribed to the contribution of the C=O stretching mode [9]. The Figure 2b shows the C=O stretching vibrations of PCL/P3ANA nanofibers activated with different concentrations of EDC and NHS. When comparing the succinimidyl ester absorbance at 1772 cm^{-1} shown in Figure 2b (inlet), at low concentrations (0.5 mM or 5 mM) of EDC and NHS, the activation reaction was incomplete. Compare to lower concentrations the activation was more successful when EDC = NHS = 50 mM, since the peak absorbance at 1772 cm^{-1} was increased when the activation was performed by 50/50 mM of EDC/NHS (Figure 2b inlet).

The surface activation yield (Figure 3) was estimated by calculation of FTIR peak ratios of the formed succinimidyl ester and O–H stretching of carboxylic acid group by using Beer-Lambert law. The relative percentages of surface species can be derived from their corresponding absorption bands by means of the Beer-Lambert law ($A = \varepsilon bc$), where A is the absorbance, ε the extinction coefficient, c the concentration and b is the length of the sample layer which was the same for all nanofiber mats. For calculation, the peaks at 1772 and 2615 cm^{-1} were used for succinimidyl ester and O–H stretching of carboxylic acid, respectively. Due to high absorbance of peak at 1772 cm^{-1},absorbance of peak at 2615 cm^{-1} was measured in an enlarged spectrum of 2000–2750 cm^{-1} region.To estimate the reaction yield, peak height was measured to represent the absorbance (A) and the linear relationship among the individual extinction coefficients of succinimidyl ester and acid groups was 1:2 [14]. Activation yield for each PCL/P3ANA nanofibers activated with different amounts of EDC and NHS, was calculated by Equation (1):

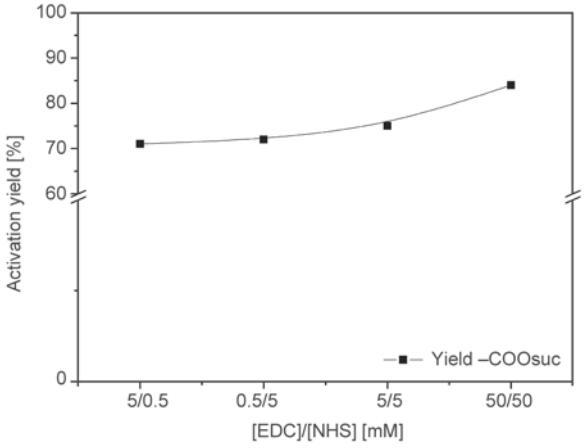

Figure 3. Surface activation yields of carboxylic acid groups on PCL/P3ANA nanofibers depending on the EDC/NHS concentrations used

The activation yield of carboxylic acid groups on PCL/P3ANA nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC and NHS was 71, 72, 73 and 82%.

Albumin immobilization was performed on the PCL/P3ANA nanofibers after activation with different concentrations of EDC and NHS (Figure 4). When compared to the PCL/P3ANA nanofibers which were not treated with albumin solution and nanofibers activated with lower concentration (0.5 and 5 mg) of EDC and NHS, there were no significant changes on FTIR spectra. On the contrary, after albumin immobilization onto nanofibers activated with 50/50 mM of EDC/NHS a broad peak was observed

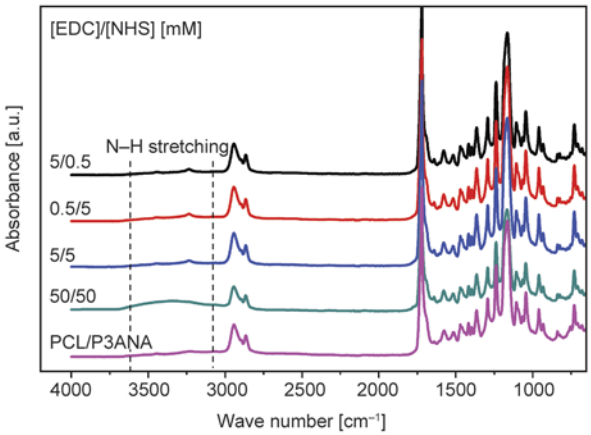

Figure 4. FTIR-ATR spectra of albumin immoblized PCL/P3ANA nanofibers activated with EDC/NHS mixtures of various concentrations

$$yield = \frac{A_{1772/\varepsilon_{\text{succinimidyl ester}}}}{A_{1772/\varepsilon_{\text{succinimidyl ester}}} + A_{2615/\varepsilon_{\text{carboxylic acid}}}} \cdot 100\% \quad (1)$$

between 3000 and 3600 cm^{-1}. The absorption in this region can be influenced by the contribution of water but in proteins the N–H groups are generally in greater numbers than O–H groups [27]. Also other albumin immobilized nanofibers showed no absorption at this region. The broad peak observed between 3000 and 3600 cm^{-1} region of FTIR spectrum of nanofibers activated with 50/50 mM of EDC/NHS was attributed to the N–H stretching vibration of albumin [28]. FTIR data (Figure 4) showed that PCL/P3ANA nanofibers were activated successfully only when [EDC] = [NHS] = 50 mM, therefore covalent binding of albumin was achieved on this nanofiber mat.

3.2. Determination of immobilized protein amount by BCA assay

In order to determine the amount of covalently bound albumin onto nanofibers, the amount of bound protein on both non-activated and activated nanofibers was determined by BCA protein assay. After each reaction step, the nanofiber mats were taken out and washed with PBS to remove any residual albumin on the nanofiber mats. They were then reintroduced into a fresh reaction medium, and the albumin amount was detected. Figure 5 shows the amount of covalently bound albumin onto nanofibers after each immobilization step. 2 mg/mL of albumin was incubated with non-activated PCL/P3ANA nanofibers (control) and PCL/P3ANA nanofibers which were activated by EDC/NHS at different concentrations. After incubation with albumin, albumin amount of the remaining solutions (cyan) were decreased. The difference between albumin amounts of the initial solutions (2 mg/mL) and solution obtained after binding step indicates that some of the protein is attached onto nanofibers. The highest amount of albumin (~1.85 mg) was attached onto nanofibers activated with 50/50 mM of EDC/NHS. In control or other samples activated with lower concentrations of EDC and NHS, the residual albumin amounts in the solutions obtained after binding step were similar to each other. Two washing steps were applied in order to remove physically absorbed protein. The amounts of washed albumin from non-activated nanofibers or nanofibers activated with lower concentrations of EDC/NHS were higher compared to amount of albumin washed from PCL/PANA nanofibers activated by 50/50 mM of EDC/NHS. This result indicates that the attachment was mostly physical on the nanofibers activated with lower concentrations of EDC/NHS. On the other hand, there was only 0.012 mg protein in the solution remained from PCL/PANA nanofibers activated by EDC/NHS at 50/50 mM concentration after two washing steps. In correlation with the FTIR-ATR data, albumin immobilization onto PCL/P3ANA nanofibers activated with 50/50 mM of EDC/NHS was achieved with covalent binding, since the attached albumin cannot be removed with washing steps. The amount of albumin bound covalently (purple) was the highest (~1.78 mg/mL), when nanofibers were activated with 50/50 mM of EDC/NHS.

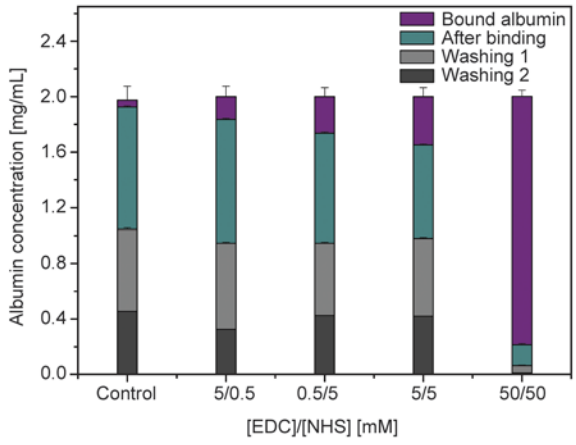

Figure 5. Amount of initial and residual albumin after each immobilization step

3.3. Morphological and elemental composition analyses of activated PCL/P3ANA nanofibers

The morphology (Figure 6, 7) and elemental composition (Figure 8, Table 1) of albumin immobilized PCL/P3ANA nanofibers activated by different concentrations of EDC/NHS were investigated. The average diameter of albumin immobilized PCL/P3ANA nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC/NHS were determined as 86±19, 75±16, 87±21 and 96±19 nm, respectively. The average fiber diameter of PCL/P3ANA before activation was determined as 89±16 nm. There is no significant change in diameter upon the binding of albumin onto PCL/P3ANA nanofibers activated with different concentration of EDC and NHS. AFM images of PCL/P3ANA and albumin immobilized nanofibers were represented in Figure 7. The surface of PCL/P3ANA nanofibers retained its topography after covalent immobilization of albumin indicating the EDC and/or NHS were not assembled or remained

on the surface after washing steps [29]. PCL/P3ANA nanofibers have RMS roughness of 150.3 nm. The surface roughness was slightly increased after albumin immobilization. The RMS roughness values of albumin immobilized nanofibers which were pre-activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of

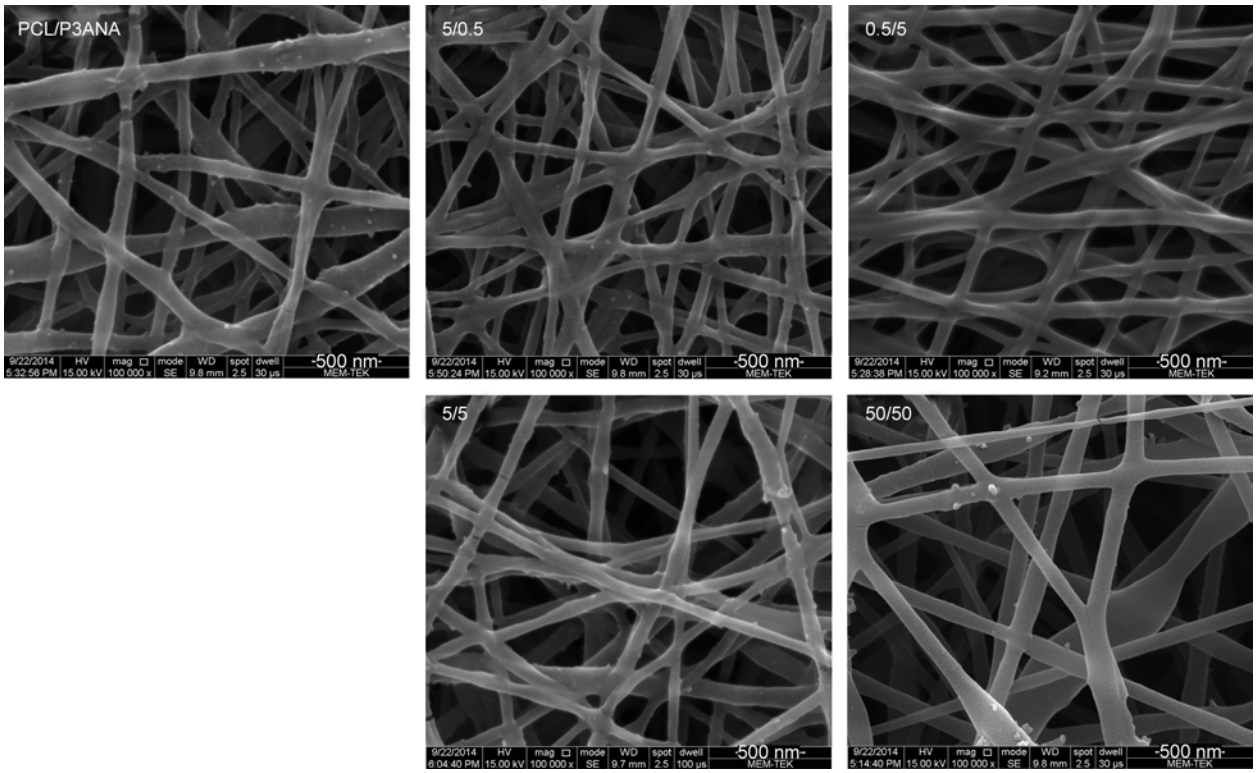

Figure 6. SEM images of PCL/P3ANA nanofibers and albumin immobilized PCL/P3ANA nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC/NHS

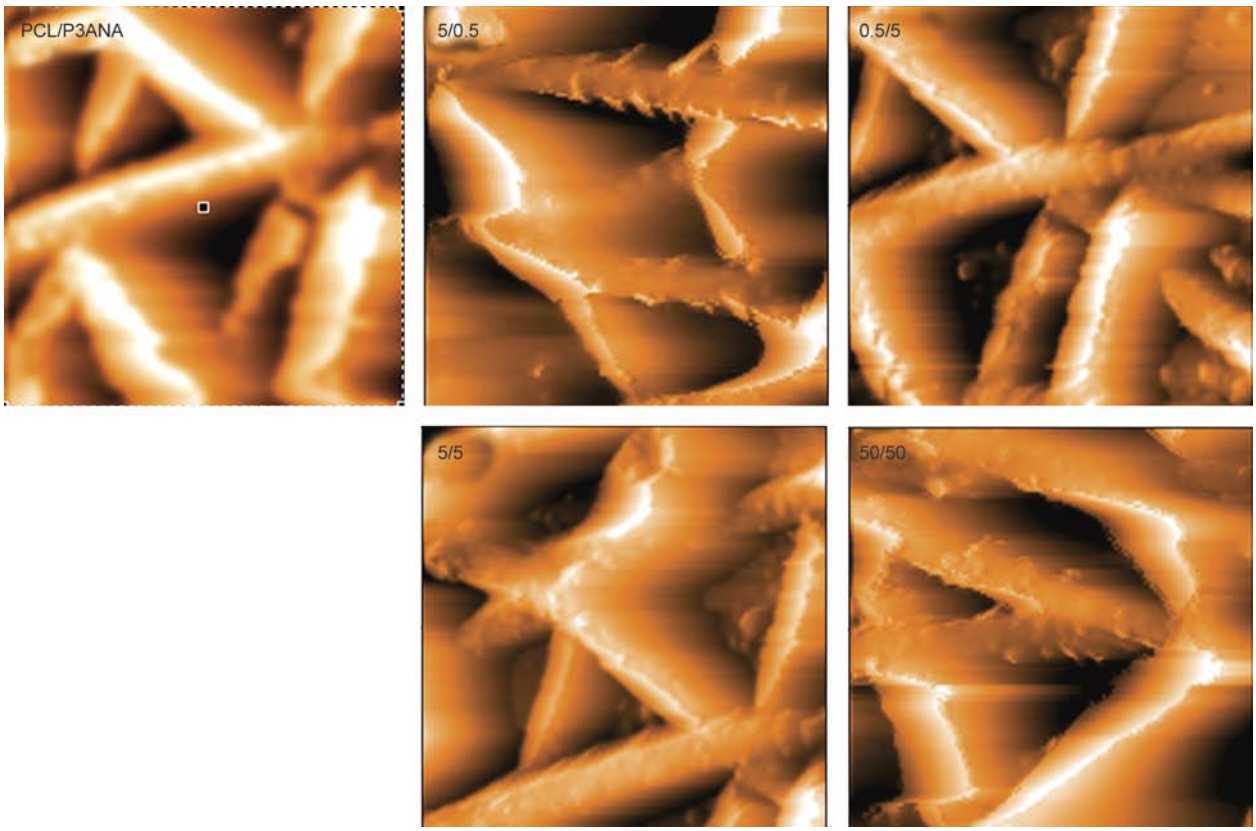

Figure 7. Topography AFM images (6 μm×6 μm) of PCL/P3ANA nanofibers and albumin immobilized PCL/P3ANA nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC/NHS

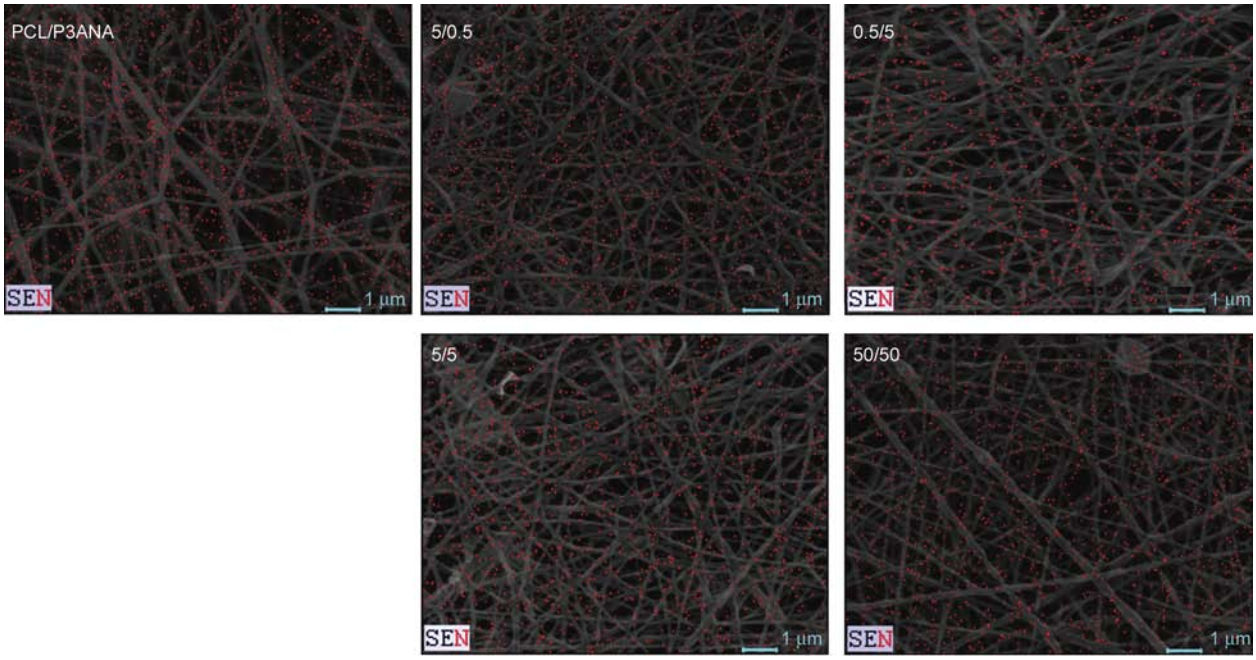

Figure 8. EDX-mapping of nitrogen (red) atoms on the surface of PCL/P3ANA nanofibers and albumin immobilized PCL/P3ANA nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC/NHS

Table 1. Elemental concentrations for nitrogen and oxygen atoms in PCL/P3ANA nanofibers and albumin immobilized nanofibers after activation with EDC/NHS

Samples [EDC]/[NHS] [mM]	Elements [wt%]	
	N	O
PCL/P3ANA	8.51	91.49
[5]/[0.5]	8.80	91.20
[0.5]/[5]	8.48	91.52
[5]/[5]	8.91	91.09
[50]/[50]	10.97	89.03

EDC and NHS, were 176.2, 193.2, 208.9 and 226.69 nm, respectively. This increase in roughness indicates the immobilization on albumin on the surface of activated PCL/P3ANA nanofibers [6, 30]. In correlation with FTIR-ATR, EDX and BCA, AFM images also indicates that the highest amount of albumin bound to nanofibers activated with 50/50 mM of EDC/NHS.

EDX analyses were performed to confirm the presence of the albumin onto nanofibers. The structure and composition of the albumin immobilized nanofibers were analyzed by detection of elemental concentrations for nitrogen (N) with EDX [31, 32]. Also, the distribution of immobilized albumin on the surface was investigated by EDX-mapping of PCL/P3ANA nanofiber mats (Figure 8). P3ANA has N atoms in its backbone, as well as albumin. The distribution of nitrogen atoms on PCL/P3ANA nanofiber mat seems to be random. It can be explained by the porous structure of nanofiber mat which affects the penetration depth of electrons during EDX analysis [33]. It is known that depending on the polymer chemical nature and porosity of mat, a loss of X-rays can be occurred [34]. Also, P3ANA and PCL blended in a weight ratio of 25% w/w between two polymers. Since both PCL and P3ANA contains oxygen atoms on their backbones, the amount of O atoms [wt%] was 91.49 while N atom amounts [wt%] was 8.81 on the surface of PCL/P3ANA nanofibers. The amount of N [wt%] on the surface of PCL/P3ANA and of the nanofiber mats activated with 5/0.5, 0.5/5 and 5/05 mM of EDC/NHS were approximately the same. However, when nanofibers were activated with increasing (50/50 mM) amounts of EDC and NHS, the amount of N [wt%] on the surface increased (10.97 wt%) which indicated that the N atoms were introduced to the structure of nanofibers through covalent binding of the albumin. The compositions of included atoms (nitrogen and oxygen) in the structure are given in Table 1. EDX-mapping images of albumin immobilized nanofibers showed the distribution of the immobilized albumin (Figure 8). EDX-mapping images indicate that the distribution of the albumin on the surface of nanofibers is affected by the distribution of activated functional groups –COOH of P3ANA. SEM/EDX results have been complementary with FTIR-ATR, BCA protein assay and able to verify the covalent

immobilization of albumin onto PCL/P3ANA nanofibers activated with higher and equal concentrations of EDC and NHS.

3.4. Electrochemical impedance spectroscopy and equivalent circuit modeling

Electrochemical impedance spectroscopy (EIS) measurements were performed on albumin immobilized PCL/P3ANA nanofibers on ITO-PET in order to understand the influence of NHS and EDC concentrations on the amount of bound albumin. The EIS data provide information about the nature of electrochemical process occurring at the electrode/electrolyte interface [35]. EIS data were obtained on PCL/P3ANA and on albumin immobilized nanofibers which were activated with different concentrations of EDC and NHS. A significant difference in the impedance spectra was observed depending on the covalent immobilization of albumin onto nanofibers and the concentrations of the EDC and NHS used in activation. In Nyquist plot (Figure 9), albumin immobilized PCL/P3ANA nanofibers exhibited a semicircle while PCL/P3ANA nanofibers showed a linear behaviour. The diameter of the semicircle of Nyquist plot represents the charge-transfer resistance (R_{ct}) [8] and linear line with high-slope indicates capacitive behavior [36]. Nanofibers became resistive after treated with albumin and the charge-transfer resistance of the albumin immobilized nanofibers was increased. PCL/P3ANA nanofibers activated with 50/50 mM of EDC/NHS had the largest diameter of the semicircle of the Nyquist plot indicated that the highest amount of albumin bound onto these nanofibers [37]. This result was in correlation with the data obtained from FTIR-ATR, SEM/EDX and BCA.

The measured impedance spectra were analyzed in terms of electrical equivalent circuits to evaluate the kinetics of the systems using the analysis program ZSimpWin. The circuits for PCL/P3ANA nanofibers and on albumin immobilized nanofibers which were activated with different concentrations of EDC and NHS, which describe the physical properties of the system and provide a good fit to the measured data with a reasonable number of circuit elements, were chosen. The calculated and measured data were fitted well together with the chosen equivalent circuits (Figure 9). The impedance spectra for nanofibers (PCL/P3ANA) described by the equivalent circuit of $R(CR)(QR)W$ (Figure 10) in short hand. For albumin immobilized PCL/P3ANA, the equivalent circuit of $R(CR)(CR)(QR)W$ were selected since there are additional electrical components (resistance and capacitance) arising from immobilized albumin. Table 2 represents the fitting parameters for the equivalent circuit elements by modeling of the impedance spectra.

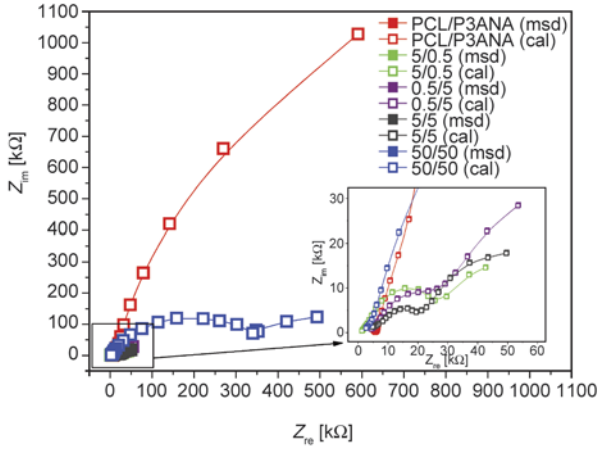

Figure 9. Measured (msd) and calculated (cal) Nyquist plots of PCL/P3ANA and albumin immobilized PCL/P3ANA nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC/NHS

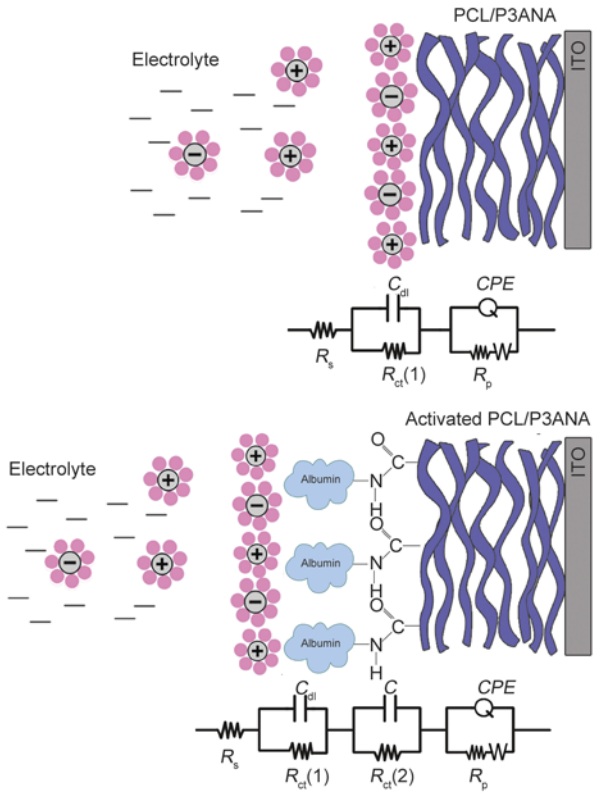

Figure 10. Equivalent circuits for the simulation of the EIS spectra of PCL/P3ANA (top) and albumin immobilized PCL/P3ANA nanofibers activated with EDC/NHS nanofibers (bottom)

Table 2. Fitting values for the equivalent circuit elements by the simulation of the impedance spectra

[EDC]/[NHS] [mM]	R_s [Ω]	C_{dl} [μF]	$R_{ct}(1)$ [Ω]	C [μF]	$R_{ct}(2)$ [kΩ]	Q(CPE) ·(10^{-7}) [$Ω^{-1}$]	R_p [kΩ]	W ·(10^{-5}) [$Ssec^5/cm^2$]	n	Chi-squared error ·(10^{-4})
PCL/P3ANA R(CR)(QR)W	2771	13.04	210	–	–	0.19	2.87	3.05	0.85	3.83
[5]/[0.5] R(CR)(CR)(QR)W	1108	0.08	1209	5.54	22.90	4.65	26.49	2.98	0.62	4.23
[0.5]/[5] R(CR)(CR)(QR)W	3403	0.09	1331	2.16	33.06	3.99	25.15	1.65	0.67	5.48
[5]/[5] R(CR)(CR)(QR)W	1143	0.08	4725	4.94	16.47	0.10	32.07	3.46	0.33	5.00
[50]/[50] R(CR)(CR)(QR)W	974	0.06	52 790	12.59	202.60	0.13	693.50	1.14	0.30	1.39

R_s, the first component of two circuits, represents the solution resistance of the electrolyte corresponds to the Ohmic resistance due to the presence of the electrolyte on the nanofiber and in solution [8] and of electrical contacts [38]. R_s values for PCL/P3ANA nanofibers, albumin immobilized nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC and NHS were 2771, 1108, 3403, 1143 and 974 Ω, respectively. The R_s values correspond to the behavior of the electrolyte, filling pores of nanofibers [39]. The second resistance in both circuits represents the charge transfer resistance (R_{ct}) between the solution and the surface of the PCL/P3ANA nanofibers or albumin immobilized nanofiber electrode surface. The charge transfer resistance $R_{ct}(1)$ of 210 Ω of PCL/P3ANA nanofibers increased to $R_{ct}(1)$ of 1209, 1331, 4725 and 52 790 Ω after albumin immobilization onto nanofibers activated with 5/0.5, 0.5/5, 5/5 and 50/50 mM of EDC/NHS, respectively. The increase in R_{ct} value is attributed to the formation of a new layer at the interface between solution and nanofibers [26]. First C in the circuits was attributed to double layer capacitance (C_{dl}) arise from alignment of solvated counter ions along nanofiber surface. The electron transfer through electrode occurs by overcoming activation barrier, charge transfer resistance and solution resistance [40]. Double layer capacitance (C_{dl}) along the surface of PCL/P3ANA nanofibers was decreased after albumin immobilization. The decrease in C_{dl} is attributed to increase in thickness of electronic double layer depending on the albumin immobilization [26]. For albumin immobilized nanofibers, additional circuit elements of capacitance (C) and resistance (R) are connected in parallel to first ones in order to describe the capacitance and the charge transfer resistance between immobilized albumin molecules and PCL/P3ANA nanofibers, respectively. When PCL/P3ANA nanofibers were activated with 50/50 mM of EDC/NHS, the capacitance (C) occurring at the interphase between immobilized albumin and nanofibers increased as well as the charge transfer resistance of $R_{ct}(2)$. The difference between capacitance values of albumin immobilized nanofibers can be explained by the different interfacial structures, assuming different structures or sizes [41]. PCL/P3ANA nanofibers are porous electrodes and they provide large surface areas which forms interfaces between electrodes and electrolytes, resulting in high capacitances. The immobilization of albumin onto the surface of nanofibers induces higher R_{ct} values [42, 43]. The increase in R_{ct} values can be explained by the formation of a new layer at the interface between solution and nanofibers was formed after attachment of albumin onto surface [26]. The different interfacial structure between protein layer and nanofibers can cause higher R_{ct} values. Also, EIS measurements were performed in PBS buffer at pH 7.4 where albumin become negatively charged [44]. In PBS buffer, the total positive charge concentration is greater than the total negative charge concentration [45]. After attachment of albumin on the surface, the negative charge on the surface of nanofibers was increased and this resulted in the alignment of positively charged solvated ions along the nanofiber surface and increase in R_{ct} values. Moreover, negatively charged ions can be repulsed by albumin on the surface and the transfer of positively charged and relatively big ions into positively charge nanofibers become challenging [24]. Q represents the constant phase element (CPE) which was applied in the equivalent circuit for the simulation of the impedance data, since CPE takes into account the non-homogeneity of the conductance [8] and the electrode [38]. The impedance of a non-ideal electrode is defined by the formula ($Z_{CPE} = T_{CPE}(jw)^{-n}$) where T_{CPE} and n are frequency-independent constants; w is the angular frequency [35],

n is a parameter describing the deviation from an ideal capacitor and arises from the slope of the log*Z* versus log*f* plot. The values for *n* vary from 0 to 1. *n* = 1 belongs to an ideal capacitor, while *n* = 0 and 0.5 denotes a resistance and Warburg behavior, respectively [46]. The n value of PCL/P3ANA nanofibers was 0.85 and it is significantly reduced up to 0.30 with albumin immobilization. The values for *n* = 0 indicates a resistance while *n* = 0.5 denotes Warburg behavior. *W* represents the Warburg impedance and it is attributed to the diffusion of counterions. Once the electron transfer begins, the electrode kinetics determined by Warburg impedance (*W*) due to the mass transport [40]. R_p represents the pore resistances of the nanofiber layer (Ohmic resistances of the electrolyte in the pores) [47]. R_p values for PCL/P3ANA nanofibers were increased after albumin immobilization. These differences in R_p can be explained by the fact that covalent immobilization of albumin by EDC/NHS activation forms a different nanofiber mat structure [48]. The highest value of 693.50 Ω of R_p was observed on nanofibers activated with 50/50 mM of EDC and NHS. AFM images indicated that surface roughness increased after albumin immobilization onto nanofibers activated with 50/50 mM of EDC and NHS, the increase in pore resistance value of PCL/P3ANA nanofibers (50/50 mM of EDC/NHS) can be related with the increase in surface roughness. EIS and equivalent circuit modeling indicated that 50/50 mM of EDC/NHS were the most effective concentration on the activation of carboxylic acid groups on PCL/P3ANA nanofiber.

The activation of the carboxyl groups on the PCL/P3ANA nanofibers can be achieved in several steps. The first step is the addition of the OH group of the carboxylic acid across one of the double bonds of the carbodiimide reactant, forming an O-acylurea adduct. Then, the surface O-acylurea can be transformed into succinimidyl ester (–COOSuc) product with a nucleophilic attack by NHS. Then, this product reacts with a primary amine and yield a peptide coupling through an amide bond (Figure 11). FTIR-ATR (Figure 2) analyses of the PCL/P3ANA nanofibers which were treated with different concentrations of EDC and NHS showed that, 50/50 mM of EDC/NHS were the effective concentration to activate the nanofibers. Also, the success of covalent immobilization of the albumin is directly depending on the efficiency of the activation procedure. The subsequent analyses (FTIR-ATR, EDX, BCA and EIS) performed on albumin immobilized nanofibers indicated that the highest amount of albumin was bound onto nanofibers activated with 50/50 mM of EDC/NHS. Figure 12 represents the relationship between the surface activation and albumin immobilization. The concentrations of nitrogen (N) atoms at the surface and the charge transfer resistance of albumin immobilized nanofibers were increased as the activation of the nanofibers were achieved represented by the increase in the absorbance (1772 cm^{-1}) of the succinimidyl ester. During the activation pro-

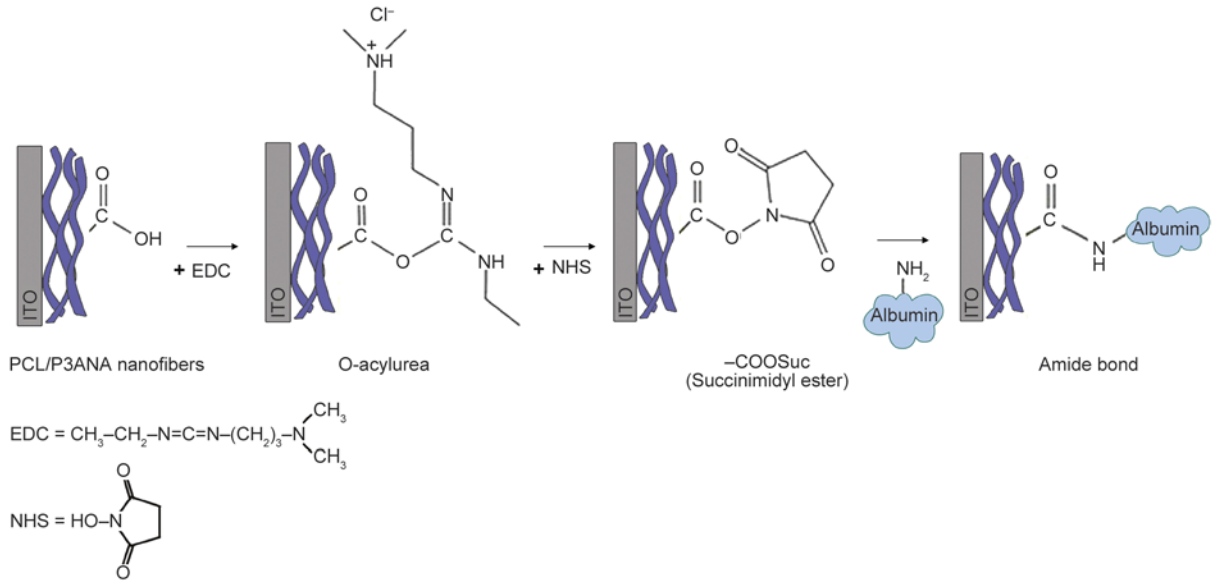

Figure 11. Schematic representation of covalent immobilization of protein through EDC/NHS activation

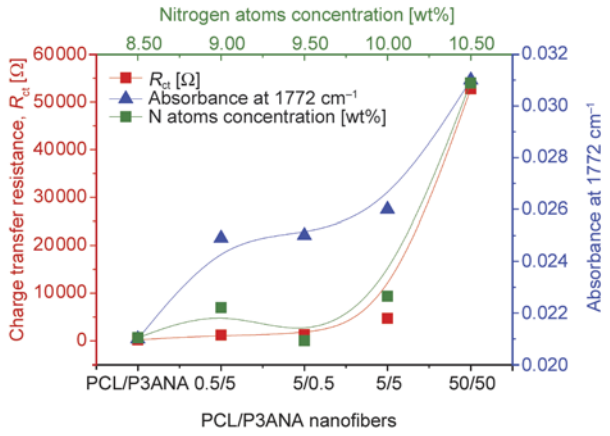

Figure 12. The relationship among charge transfer resitance (R_{ct}) from EIS, succinimidyl ester absorbance from FTIR-ATR and N atoms concentrations from EDX data of activated PCL/P3ANA nanofibers

cedure, the formation of the byproducts at the surface results a kinetic competition between different reactions [9]. In the case of EDC concentration is lower than NHS concentration (0.5/5 mM), the formation of O-acylurea become very slow resulting a slow succinimidyl ester formation. When EDC concentration is higher than NHS concentration (5/0.5 mM) O-acylurea formation become relatively fast however succinimidyl esters form very slow and byproducts of Anhydride and N-acylurea can be formed at the surface [49]. When NHS and EDC concentrations are equal to each other and both low (5/5 mM) then slow kinetics for whole reaction are expected. On contrary, when the concentrations of both EDC and NHS increased to 50 mM, great balance between formation of O-acylurea and transformation of it into succinimidyl ester product can be obtained.

4. Conclusions

The nanofibers of poly(ε-caprolactone)/poly(m-anthranilic acid) (PCL/P3ANA) nanofibers were fabricated by electrospinning. Carboxyl groups on the surface of PCL/P3ANA nanofibers were activated by EDC and NHS in different concentrations (5/0.5, 0.5/5, 5/5 and 50/50 mM). Albumin was covalently immobilized onto activated nanofibers. The surface activation was investigated by FTIR-ATR and activation yield was calculated. The amount of immobilized albumin was investigated by BCA assay and elemental analyses (EDX) of nanofibers. AFM images showed that surface roughness of the nanofibers was increased after albumin immobilization. The amount of bound albumin was changed depending on the activation of the carboxyl groups of P3ANA. Elemental analyses and EDX-mapping of nitrogen atoms showed that the distribution of the albumin on the surface of nanofibers is affected by the distribution of activated –COOH groups of P3ANA. Electrochemical Impedance Spectroscopy (EIS) analysis and equivalent circuit modeling was performed to observe the immobilized albumin depending on the EDC/NHS concentrations used in activation. The nanofibers become resistive due to albumin immobilization and the higher charge transfer resistance was observed for the nanofibers activated with 50/50 mM of EDC/NHS. This study showed that 50/50 mM of EDC/NHS was the most effective concentration for the activation of PCL/P3ANA nanofibers. This study indicated that Electrochemical Impedance Spectroscopic (EIS) analysis can be successfully applied for the determination of activation degree of covalent immobilization of proteins and enzymes onto polymeric substrates.

Acknowledgements

The authors express their thanks to Scientific and Technological Research Council of Turkey (TUBITAK) for the financial support on project 213M469.

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