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1 **A novel electrochemical immunosensor for ultrasensitive detection of tumor**
2 **necrosis factor α based on polystyrene - PAMAM dendritic polymer blend**
3 **nanofibers**

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15
16 **Abstract:**

17 A simple, rapid, and highly sensitive label-free immunosensor based on polystyrene (PS)-polyamidoamine
18 (PAMAM) dendritic polymer nanofibers (NFs) for detection of tumor necrosis factor- α (TNF- α) cytokine
19 was developed. The choice of PAMAM was due to the possibility of designing the surface of NFs to create
20 a novel interface with the high control ability of the density of amine groups at the surface by which capture
21 antibody (anti-TNF- α) could be covalently attached to the surface of NFs through glutaraldehyde (GA)
22 coupling chemistry. Evaluation of the electrode surface using cyclic voltammetry (CV) proved the electrode
23 manufacturing stages were successful by decreasing the peak current values due to the blockage of electron
24 transfer by the biomolecule layer on the surface of NFs, as well as the ability to precisely control the current
25 peak by changing the diameter of the NFs and the concentration of dendrimer. Electrochemical impedance
26 spectroscopy (EIS) was used to test the detection capability of the immunosensors and compare the role of
27 the amount of PAMAM presented on the surface of NFs on their analytical performance. Increasing the
28 concentration of dendrimer greatly increased the sensitivity of immunosensors due to the higher

29 stabilization of the antibody on the nanofiber surface. The immunosensor had a very high affinity for TNF-
30 α cytokines, with a linear response ranging from 10 to 200 pg/mL and a low limit of detection (LOD) of
31 669 fg/mL. Interference results with Interleukin-10 (IL-10) cytokine showed that the immunosensor was
32 extremely specific and may be utilized to detect cytokines in clinical diagnosis.

33 **Keywords:** Electrospun nanofibers, Polystyrene (PS), Polyamidoamine (PAMAM), Electrochemical
34 impedance spectroscopy (EIS), immunosensor, tumor necrosis factor- α (TNF- α)

35

36 **1. Introduction**

37 Tumor Necrosis Factor-alpha (TNF- α) is a pro-inflammatory cytokine produced by lymphocytes and
38 macrophages that has been implicated in the stimulation of the acute phase response, anticancer activity,
39 and immune system regulation [1,2]. According to recent researches, TNF- α levels in a patient's blood
40 plasma and saliva are linked to heart failure (HF), and it can be used as a specific biomarker in detecting
41 the first signs of future cardiovascular events [3,4]. Since the concentration of TNF- α in healthy human
42 serum is less than 10 pg/mL, and in the serum of patients with severe autoimmune diseases is between 10-
43 300 pg/mL depending on the severity of the disease, accurate estimating its concentration in human fluids
44 is a significant challenge and important to early diagnosis of disease [4,5]. Traditional methods such as
45 ELISA for TNF- α detection have always disadvantages including long assay times, large sample volumes
46 for analysis, the inability to assess in real time, and the necessity for a specialist. [6,7]. Electrochemical
47 immunosensors have received much interest in recent years as an alternative to conventional approaches in
48 TNF- α detection because of their high sensitivity, easy instrumentation, accuracy, and quick response [8-
49 10].

50 In this work, based on two novel perspectives a highly sensitive electrochemical immunosensor for TNF- α
51 cytokine detection was developed. First, the usage of the NFs structure in immunosensor construction with
52 the goal of providing a diagnostic platform with a large surface area to immobilize TNF- α antibodies in

53 such a way that they have maximal TNF- α cytokine accessibility to improve immunosensor sensitivity. The
54 use of NFs in the production of electrochemical immunosensors might lead to the development of a new
55 generation of miniaturized immunosensors that can answer key issues of previous works such as increased
56 sensitivity, simplicity, and rapid detection. Second, fiber surface engineering in terms of the kind and
57 quantity of functional groups suited for covalent immobilization of TNF- α antibody with a specific distance
58 to build a protein-friendly interface with high stability so that the analyte may be detected in the smallest
59 amount feasible. PAMAM dendrimers are globular, highly branched polymeric macromolecules with a
60 polyvalent core (ethylene diamine (EDA)), internal repeating branched units (amidoamine), and high
61 terminal active surface groups [11,12]. Dendrimers provide the possibility of immobilizing bioreceptors for
62 the design of immunosensors due to their unique structural and chemical features, such as multiple
63 functional groups, hydrophilicity, high mechanical and chemical stability, and similar size to biomolecules
64 [13,14]. Accordingly, great emphasis has been placed on the use of PAMAM in the production of extremely
65 sensitive electrochemical immunosensors in order to speed up the electron transfer process, enhance
66 antibody immobilization efficiency, and improve electrochemical responses and signal amplification [15–
67 17]. A previous study on the modification of the ELISA kit surface using PAMAM for TNF- α cytokine
68 detection showed increased sensitivity and the detection limit through better orientation of the antibodies
69 compared to the commercially ELISA kit [18]. In this work, PS-PAMAM dendritic polymer NFs were
70 produced for the first time, with doped PS and PAMAM dendritic polymer with different concentrations,
71 then allowing covalent interaction with the Fc region of the TNF antibody after amine group activation. As
72 a result, the impact of increasing PAMAM concentration on its presence on the fiber surface and TNF- α
73 detection was investigated. Electrochemical impedance was utilized in the final step to detect changes in
74 electron transport properties after the development of antibody–antigen immune complexes on the surface
75 of NFs mats deposited on a gold electrode. The immunosensors' repeatability, reproducibility, and
76 selectivity were all examined.

77 2. Materials and methods

78 2.1. Chemicals and Reagents

79 In the supplementary information, the reagents, instruments, and characterizations parts were described in
80 detail.

81 2.2. PS and PS-PAMAM nanofibrous mats preparation

82 PS granules were dissolved in N,N,-dimethylformamide (DMF) solvent at room temperature (RT) under
83 magnetic stirring overnight to prepare a 20 wt. % PS solution for electrospinning. The preparation of PS-
84 PAMAM NFs mats with different mass ratios of 85-15, 75-25, and 65-35 was conducted in two step process.
85 The precise quantities of PS and PAMAM dendritic polymer were dissolved separately in DMF solvent for
86 each mass ratio. The overnight stirring was conducted at RT to obtain clear solutions. The PAMAM
87 dendritic polymer solution was gently added to the PS solution in the second step, and the mixture was
88 stirred for 4 h to obtain a PS-PAMAM 20 wt. % solution. After this step, PS-PAMAM solutions with
89 different mass ratios were used immediately for electrospinning. The solutions were loaded into a 1ml
90 syringe supplied with a laser-cut needle (22 gages) and fed through a polytetrafluoroethylene (PTFE) tube
91 from the syringe to the nozzle using the syringe pump (electrospinning instrument, Spraybase®, Ireland).
92 In order to obtain NFs with a bead-less morphology with uniformity in diameter, various parameters such
93 as voltage, injection rate and distance from the nozzle to the collector were changed. The electrospinning
94 parameters were 27 kV for the applied voltage, rate 0.25 ml/h and the tip-to-collector distance 20 cm.

95 After obtaining uniform and bead-less NFs, they were electrospun on the surface of gold electrode that was
96 placed on a collector for 1min. Before electrospinning, the gold electrodes were placed in acetone for 15min
97 and then in ethanol in an ultrasonic bath for 15min for cleaning. At each stage and at the end, the surface
98 of the gold electrodes was rinsed thoroughly with ultrapure water. They were dried with a gentle stream of
99 nitrogen and then placed in an UV/ozone ProCleaner™ for 30min to eliminate any possible organic
100 contamination.

101 2.3. GA-PS and GA-PS-PAMAM nanofibrous mats preparation

102 GA coupling agent was used to activate PAMAM dendritic polymer amine groups on the surface of NFs to
103 allow covalent bonding with amino groups in the Fc region of anti-TNF- α . Briefly, gold electrodes
104 containing PS and PS-PAMAM NFs were exposed to saturated GA vapors in the desiccator for 2h.

105 *2.4. Antibodies and Antigens standard solutions preparation*

106 The anti-TNF- α and AgTNF- α have been reconstituted in PBS buffer (10 mM, pH 7.4) and then aliquoted
107 at 20 μ L according to the protocol provided by the supplier to get a stock solution with a final concentration
108 of 500 μ g/mL and 100 μ g/mL, respectively. The aliquots were stored at -20°C until use. The anti-TNF- α
109 solution was diluted at 10 μ g/mL in PBS (10 mM, pH 7.4). The standard solutions containing of AgTNF- α
110 were prepared at five different concentrations (10, 25, 50, 100, and 200 pg/mL) by dissolving the appropriate
111 amount of the stock solution in PBS (10 mM, pH 7.4) and stored at 4°C. All the standard solutions were
112 prepared by weighing.

113 *2.5. immunosensor preparation*

114 Immunosensor construction was done by pipetting 50 μ l of anti-TNF- α (10 μ g/mL) on the surface of NFs
115 and then incubating for 1h at 4°C. After this step, the electrode surface was gently washed three times with
116 PBS (10 mM, pH 7.4) to eliminate unbound antibodies from the NFs 's surface. Finally, blocking treatment
117 for reducing non-specific binding and blocking remaining active sites was done by pipetting 100 μ l of BSA
118 1% solution on the NFs and incubating for 1h at 4°C.

119 *2.7 Electrochemical measurements*

120 For the detection of AgTNF- α , the NFs modified gold electrode was subjected to successive immobilization
121 with standard solutions (50 μ l) containing different concentrations of AgTNF- α prepared as described in the
122 section 2.4 for 30min at RT ($20 \pm 1^\circ\text{C}$) in the electrochemical cell, followed by PBS washing to remove
123 unbounded AgTNF- α cytokine. At each AgTNF- α concentration, an EIS response was then recorded by
124 filling the cell with 5ml of 5mM of $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ in PBS buffer at pH 7.4. After each EIS

125 measurement, the electrolyte solution in the cell was removed and the cell were washed with PBS solution
126 to remove all adsorbed electrolyte solution from surface of NFs and prevent denaturation of anti-TNF- α .

127 **3. Results and discussion**

128 *3.1. Characterization of nanofibrous mats*

129 *3.1.1. FTIR Spectroscopy*

130 The ATR-FTIR spectra of PS and PS-PAMAM NFs with different concentrations are presented in Fig. 1.
131 The bands at 3080-3024, 2920, and 2846 cm^{-1} associated with the aromatic C-H stretching vibration
132 absorption and the existence of CH_2 , respectively were found in the spectrum of pure PS NFs. Also, the
133 peaks at 1939, 1869, 1801, and 1741 cm^{-1} were representative to overtone aromatic. The C=C stretching
134 vibration absorption were seen at 1600 cm^{-1} and bending vibrations of CH_2 were observed at 1492, and
135 1451 cm^{-1} . The absorption peaks at 755 and 696 cm^{-1} associated with C-H out-of-plane bending vibration
136 absorption were also seen. In comparison to pure PS, the amine and amide peaks of PAMAM emerged in
137 the spectra of NFs containing dendrimer. The spectrum of PS- PAMAM NFs with different mass ratio along
138 with characteristic PS peaks exhibited a new peak at 3268 cm^{-1} correspond to N-H stretching vibration. The
139 C=O of amide I band, N-H bending and C-N stretch of amide II band, and C-N stretch of amide III bands
140 appeared at peaks of 1645, 1551, and 1372 cm^{-1} , respectively. In addition, the peaks at 1155 and 1122 cm^{-1}
141 ¹ that belong to the C-N stretch of amine were found. The peak at 2810 cm^{-1} due to the stretching bands of
142 C-H close to NH group was observed. The intensity of the mentioned peaks increases as dendrimer
143 concentrations increase, confirming the existence of more dendrimer on the PS-PAMAM NFs surface.

144 *3.1.2. Morphological Studies*

145 In order to evaluate NFs in terms of diameter, uniformity, and the effect of PAMAM dendritic polymer on
146 PS morphology, FE-SEM micrographs were prepared. Fig. 2a and b demonstrate FE-SEM micrographs of
147 PS NFs at two magnifications, with the average diameter of 985 ± 303 nm. Fig. 2c and d show micrographs
148 of PS-PAMAM NFs (65-35), which have significantly reduced fiber diameters compared to pure PS. This

149 decrease in diameter is due to a decrease in solution viscosity, increasing the conductivity of electrospinning
150 solution and surface charge density of ejected jet with increasing dendrimer ratio due to low molecular
151 weight and the cationic characteristic of PAMAM dendritic polymer, respectively, whereby the polymer jet
152 is subjected to more elongation force in a constant electric field. This increase in elongation is also evident
153 in the morphology of the NFs so that the porosity of the PS nanofiber surface was disappeared and the
154 surface became smoother. Table 1 shows the results of the electrical conductivity of electrospinning
155 solutions. According to the results, the electrical conductivity of the PS solution was 0.4 $\mu\text{S}/\text{cm}$, while it
156 reached 24.0 $\mu\text{S}/\text{cm}$ in the sample 65-35. The diameter of PS-PAMAM NFs with ratios of 85-15, 75-25,
157 and 65-35 were 350 ± 126 nm, 319 ± 86 nm, and 295 ± 94 nm, respectively. Significant results can be
158 summarized based on the morphology of the resulting NFs. (1) Modification with PAMAM has
159 substantially decreased the diameter and increased the accessible surface area of the resultant NFs compared
160 to pure PS, allowing more anti-TNF- α to be immobilized on the surface and increasing the immunosensor's
161 sensitivity. (2) The uniformity of the diameter, whether in a single fiber or in the average diameter of the
162 fibers, as well as the bead-less morphology, contribute to immunosensor reproducibility. (3) The porous
163 structure of the NFs mats allows the desired analyte to access even the immobilized bioreceptor in the lower
164 layers of the fibers, and provides no penetration resistance. (4) The morphology of PS NFs has not changed
165 after doping with PAMAM dendritic polymer, which is a benefit for the creation of reproducible
166 immunosensors.

167 3.1.3. *Water contact angle*

168 The images of the water droplets on the pure PS and PS-PAMAM NFs (65-35) on the surface of the gold
169 electrode are inserted in Fig. 2. Pure PS NFs showed a contact angle of $147.2\pm 1.1^\circ$, which is due to the lack
170 of functional groups in their structure and their hydrophobic nature. The contact angles of samples 85-15,
171 75-25, and 65-35, have been greatly reduced to $48.2\pm 1.2^\circ$, $31.8\pm 1.3^\circ$, and $15.4\pm 1.2^\circ$, respectively.
172 According to the results, the presence of PAMAM in the structure of NFs on the one hand reduces the
173 diameter of fibers and increases their surface area and, on the other hand, increases more hydrophilic amine

174 groups on the surface of PS NFs, both of which play an effective role in NFs surface properties. As a result
175 of the high hydrophobicity of PS and absence of functional groups on its surface, there is a lack of
176 homogeneous distribution of anti-TNF- α on the fiber surface, as well as covalent bonding with the NFs'
177 surface. In PS-PAMAM samples, increasing the concentration of the PAMAM provides increased
178 hydrophilicity and higher functional groups to the anti-TNF- α on a more uniform immobilization at the
179 fiber surface. Regardless of the chemical structure and morphology of NFs and only based on the shape of
180 the water droplet on the NFs surface in Fig. 2, unlike the PS sample, the droplet in the PS-PAMAM (65-
181 35) spread well on the surface. While the procedures of immobilizing anti-TNF- α on the surface of the
182 fibers were accomplished by pipetting a specific quantity of antibody onto the fibers' surface, the
183 hydrophilic properties of PS-PAMAM fibers allow for more uniform antibody immobilization. However,
184 immobilization of anti-TNF- α on the surface of PS NFs confined to the droplet's position. To examine the
185 surface properties of NFs following treatment with GA and anti-TNF- α immobilization, the contact angle
186 of the NFs was measured (Table 1). The contact angle increased after applying PS-PAMAM NFs with GA
187 owing to the interaction of dendrimers amine groups on the surface of NFs with GA and the formation of
188 Schiff bases. The contact angle of sample 65-35 rose from $15.4 \pm 1.2^\circ$ to $35.9 \pm 4.7^\circ$ after treatment with GA,
189 indicating that GA forms covalent bonds with amine groups. However, the contact angle of PS NFs did not
190 change after GA treatment due to the lack of amine groups at their surface. The contact angle fell to
191 $16.4 \pm 2.5^\circ$ after immobilization of anti-TNF- α on sample 65-35, indicating that antibodies were
192 immobilized at the fiber surface. This trend has been true for samples 85-15 and 75-25. The slight decrease
193 in the contact angle observed in the PS sample is related to the grafting of the anti-TNF- α on the surface of
194 the NFs.

195 *3.2. Immunodetection and Interference of TNF- α cytokines*

196 The Cyclic voltammograms (CVs) were performed in 10 mM PBS (pH 7.4) with 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as
197 the redox probe from -0.2 to $+0.6$ V and the scan rate of 60 mV/s to provide more specific information
198 about electrodes containing NFs and to analyze electrode behavior changes throughout stepwise

199 immunosensor fabrication. As shown in Fig. 3a, the PS-PAMAM NFs with different concentrations exhibit
200 a couple of reversible redox peaks. As the concentration of PAMAM increases, the peak current increases,
201 which is related to the increase in effective surface area with decreasing fiber diameter as well as the effect
202 of dendrimer on electron transfer. The disappearance of the oxidation and reduction peaks of the redox
203 couple in the pure PS NFs is related to their non-conducting properties, large diameter, and high
204 hydrophobicity. How PAMAM improves electron transfer in NFs is better understood by imagining a
205 network of it within a 3D nanofiber and on its surface that some of them face the working electrode and the
206 others facing the solution. This indicates that from a certain concentration onwards the amount of PAMAM
207 reaches a critical level to form a network within the NFs and on their surface, which increases the electron
208 transfer. This network is well-formed in sample 75-25, and with increased dendrimer concentration and
209 decreasing fiber diameter (sample 65-35), more PAMAM will be present on the surface of the fibers,
210 resulting in enhanced electron transport. Surface modification of the glassy carbon electrode with PAMAM
211 produced similar outcomes in terms of increased electric flux, improved conductive capacity, noise
212 reduction, and faster electron transfer processes on the electrode's surface [19].

213 The redox current of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ after electrospinning of NFs (65-35) on the electrode surface (129.3 μA)
214 has increased compared to the bare gold electrode (116.7 μA) due to the increase in high specific surface
215 area and improved charge-transport by the PAMAM (Fig. 3b). After the immobilization of the anti-TNF- α
216 and blocking with BSA, the redox current (111.2 μA) has decreased due to the formation of a layer of
217 protein molecules on the surface of the fibers that insulate the electrode and prevent electron transmission
218 between electrode and electrolyte. This confirms the ability of the substrate to immobilize anti-TNF- α .
219 Antibody activity on the surface of NFs was assessed by incubating 50 μl AgTNF- α (10 pg/mL) on the
220 electrode surface for 30 min. The decrease in redox current (102.7 μA) and increase in peak-to-peak
221 separation in voltammogram confirm the immunosensor is highly sensitive to TNF- α cytokine through the
222 formation of immune complexes that could hinder electron transfer between the electrode and the
223 electrolyte as insulating biomolecules.

224 Electrodes containing sample 65-35 NFs with 4 different deposition durations of 1, 2, 3, and 5 min were
225 studied using SEM and CV to discover the ideal electrospinning time and to evaluate the influence of
226 deposit time on the redox current of $[\text{Fe}(\text{CN})_6]^{3-/4-}$. Increasing the electrospinning duration induces more
227 NFs to deposit, resulting in a thicker layer mat, as seen in Fig. S 1 (SI). The remarkable point in the images
228 is the uniform diameter and the beaded-free structure of the NFs mats in different samples. This reaffirms
229 the developed immunosensor is reproducible. The CV results show a decreasing trend in redox current with
230 increasing electrospinning time (Fig. S 2). It is related to increasing the thickness of the NFs mats and
231 decreasing the porosity between the NFs, which prevents penetration of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ to the electrode
232 surface. The 1 min electrospinning was selected as the ideal time due to the following reasons. (1) having
233 the highest current intensity in comparison to others, (2) supplying the appropriate and adequate quantity
234 of NFs on the electrode surface, and (3) having the best adherence to the electrode surface and suitable
235 stability throughout immobilization, blocking, and washing processes. Despite the role of the PAMAM in
236 the good adhesion of NFs to the electrode surface, increasing the electrospinning duration leads the NFs to
237 be attached only with their bottom layer and not in touch with the electrode surface. As a result, 1 min of
238 electrospinning was chosen as the optimal time, and all immunosensors were made using this time.

239 The EIS was utilized to quantitatively analyze the performance and sensitivity of the developed
240 immunosensor in detecting TNF- α cytokine and confirming the formation of immune complexes between
241 anti-TNF- α and AgTNF- α on the NFs surface. The acquired Nyquist impedance plots for developed
242 immunosensors are shown in Fig.4, where the charge transfer resistance at the electrode surface (R_{ct}) is
243 proportional to the semi-circle diameter. The first semi-circle relates to anti-TNF- α immobilization on the
244 surface of NFs at 4 °C for 1h. After this step, the immunosensors were evaluated to detect AgTNF- α in the
245 concentration range of 10-200 pg/ml. The Nyquist plot of an immunosensor based on pure PS NFs is shown
246 in Fig. 4a. Because of the lack of functional groups on the surface of pure PS NFs, it is not feasible to
247 immobilize antibodies uniformly and covalently on these NFs. Only the anti-TNF- α was physically attached
248 to the NFs' surface, leading to antibody accumulation, denaturation, and desorption at different sensing

249 stages. The immunosensor did not work and the semi-circles did not increase due to a low amount of
250 immobilized anti-TNF- α and saturation of them on the surface of PS NFs. The Nyquist plots of PS-PAMAM
251 NFs with different mass ratios 85-15, 75-25, and 65-35 are shown in Figures 4b-d. The semi-circles
252 gradually increased with increasing the AgTNF- α content in all PS-PAMAM NFs samples, indicating
253 AgTNF- α biorecognition with anti-TNF- α on the surface of NFs. By comparing the semi-circle and linear
254 part of the impedance spectra of the immunosensors, a smaller semi-circle with better diffusion of
255 $[\text{Fe}(\text{CN})_6]^{3-/4-}$ toward the surface of the electrode is observed with increasing PAMAM dendritic polymer
256 concentration. This is due to decreased diameter and increased hydrophilicity, and the presence of PAMAM
257 on the NFs surface, which enhances charge transfer for $[\text{Fe}(\text{CN})_6]^{3-/4-}$. This is in line with the findings of
258 the CV of NFs with different PAMAM concentrations. After each step, the electrochemical parameters
259 were calculated by fitting the Nyquist plot with Randles equivalent circuit, which provided the best fit for
260 the impedance parameters (Fig.4 inset). It consists of electrolyte resistance (R_s) in series with a parallel
261 combination of constant phase element Q_{CPE} and charge transfer resistance (R_{ct}). The calibration curve of
262 developed immunosensors was drawn based on $(R_{(\text{antibodies})} - R_{(\text{antigens})})/R_{(\text{antibodies})}$ obtained from the equivalent
263 circuit against the logarithmic value of the AgTNF- α concentration (Fig. 4e). The calibration graph of PS-
264 PAMAM NFs-based immunosensors showed a linear correlation in ranges between 10 - 200 pg/mL.
265 Increasing the concentration of PAMAM has increased the slope of the graph and the higher detection
266 sensitivity of the immunosensor due to the higher loading of antibodies on the surface of NFs. In comparison
267 to the other samples, PS-PAMAM with the concentration of 65-35 has the highest slope, with a linear
268 regression equation of $\Delta R/R = 0.379 \ln [\text{AgTNF-}\alpha] - 0.461$ and a correlation coefficient of $R^2 = 0.987$,
269 indicating that this immunosensor has a higher sensitivity to antigen changes due to the higher presence of
270 PAMAM and immobilization of antibodies on the surface of NFs.

271 The LOD of immunosensor was 669 fg/mL based on $3.S/m$, where S is the residual standard deviation and
272 m is the slope of the regression line. The trend is similar in samples 85-15 and 75-25, but they have a lower
273 slope than samples 65-35: $\Delta R/R = 0.210 \ln [\text{AgTNF-}\alpha] - 0.132$ for sample 85-15 and $\Delta R/R = 0.2680 \ln$

274 [AgTNF- α]-0.235 for sample 75-25. Table S1 compares the detection range and LOD of the developed
275 immunosensor to those of previous electrochemical TNF- α immunosensors.

276 Table S1 compares the prepared immunosensor to previously reported literature in detail. The
277 immunosensor has a wide linear detection range with the lowest LOD than previous electrochemical TNF-
278 α cytokine immunosensors. This is rooted in the high surface-to-volume ratio of NFs and allows antibodies
279 to be covalently immobilized with a significantly higher density on the fiber surface, a high accessibility
280 due to the porous structure. The electrospun NFs-based immunosensor produced in this study provided a
281 flexible interface with a low-cost and simple production method, allowing the development of a new
282 generation of immunosensors for insertion in a point of care testing.

283 The selectivity of the PS-PAMAM NFs-based immunosensor was also considered as a significant property
284 of immunosensors. For this, all of the procedures used for AgTNF- α were repeated for cytokines IL-10
285 (Fig. 4f). According to the results, the developed immunosensor had higher selectivity and sensitivity to
286 AgTNF- α compared to IL-10.

287 **4. Conclusion**

288 Nanostructured transducers along with the ideal surface chemistry design can lead to the development of
289 highly sensitive immunosensors. In this work, a PS-PAMAM NFs-based immunosensor was developed
290 that provided the ideal protein-friendly platform with a high surface area and a well-designed receptor
291 surface for the immobilization of anti-TNF- α . The role of PAMAM dendritic polymer and its presence on
292 the fiber surface at three different concentrations were investigated and sample 65-35 had the highest
293 sensitivity and linear response for AgTNF- α due to the higher presence of dendrimer on the surface. This
294 highly sensitive immunosensor, along with the easy and fast preparation method, provided conditions for
295 the anti-TNF- α to be covalently immobilized on the fiber surface without losing their activity to detect the
296 AgTNF- α with high sensitivity. This could be promising for the development of a point of care and flexible
297 immunosensor with maximum analyte detection ability.

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376 Figure Captions

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378 Schematic.1. The schematic of the stepwise preparation process of NFs based EIS immunosensor.

379 Fig. 1. ATR-FTIR spectra of (a) pure PS, (b) PS-PAMAM-(85-15), (c) PS-PAMAM-(75-25), and (d) PS-
380 PAMAM-(65-35) NFs.

381 Fig. 2. FE-SEM images of (a) and (b) pure PS, (c) and (d) PS-PAMAM-(65-35) NFs, and (e) diameter
382 distribution histograms of NFs. Insets in Fig.2 are the images of water droplets on the surface of electrospun
383 NFs on a gold electrode.

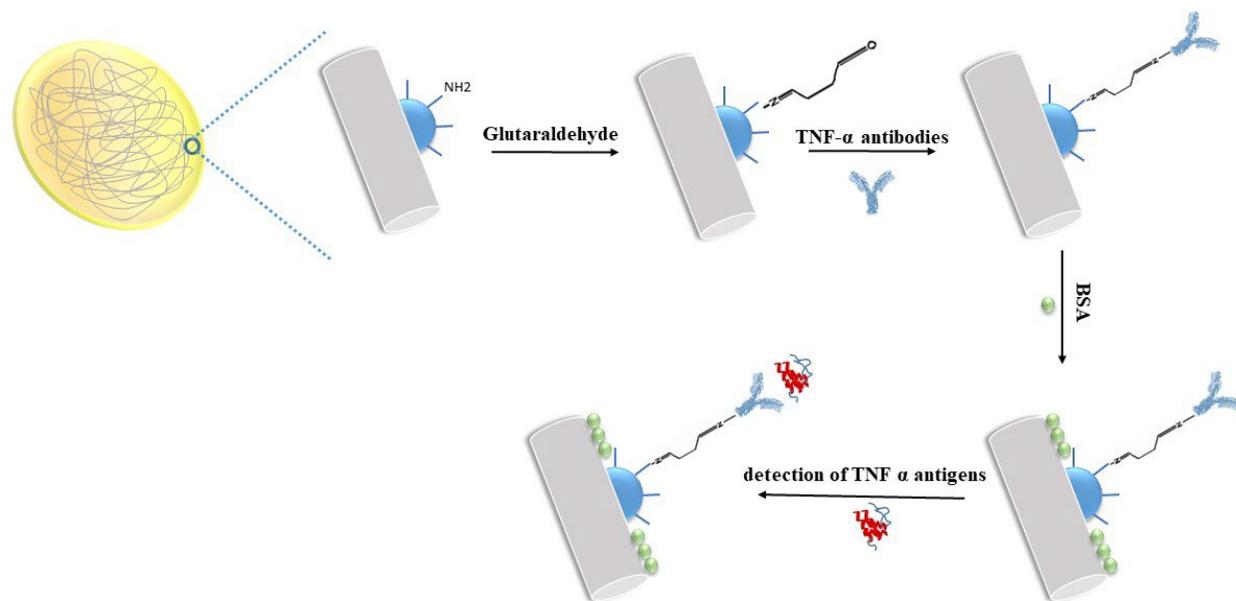
384 Fig. 3. CVs of (a) pure PS and PS-PAMAM NFs with different concentrations, (b) bare gold, gold electrode
385 containing PS-PAMAM-(65-35) NFs, anti-TNF- α -GA-PS-PAMAM-(65-35) NFs, and AgTNF- α -anti-
386 TNF- α - GA-PS-PAMAM-(65-35) in a 10 mM PBS (pH 7.2) containing 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (scan rate
387 = 60 mV/s).

388 Fig. 4. Nyquist plots of impedance spectra obtained in 10 mM PBS (pH 7.4) solution containing 5mM
389 $[\text{Fe}(\text{CN})_6]^{3-/4-}$ for gold electrodes containing (a) pure PS, (b) PS-PAMAM-(85-15), (c) PS-PAMAM-(75-
390 25), and (d) PS-PAMAM-(65-35) NFs for AgTNF- α detection ranging from 10-200 pg/mL. EIS:
391 polarization potential of 0.2 V vs Ref, 1 Hz–100 kHz frequency range, and a sinus amplitude of 10 mV.
392 Inset was the equivalent circuit model used to fit the impedance data. (e) calibration curves of the developed
393 immunosensors for the detection of AgTNF- α cytokine. (f) Selectivity histograms of the developed
394 immunosensors for the detection of Ag-TNF- α and IL-10 cytokine.

395 Table. 1. The characteristics of electrospinning solutions conductivity, diameter of NFs, and water contact
396 angle of NFs on the surface of the gold electrode (a) before GA treatments, (b) after GA treatments, and (c)
397 after anti-TNF- α immobilization.

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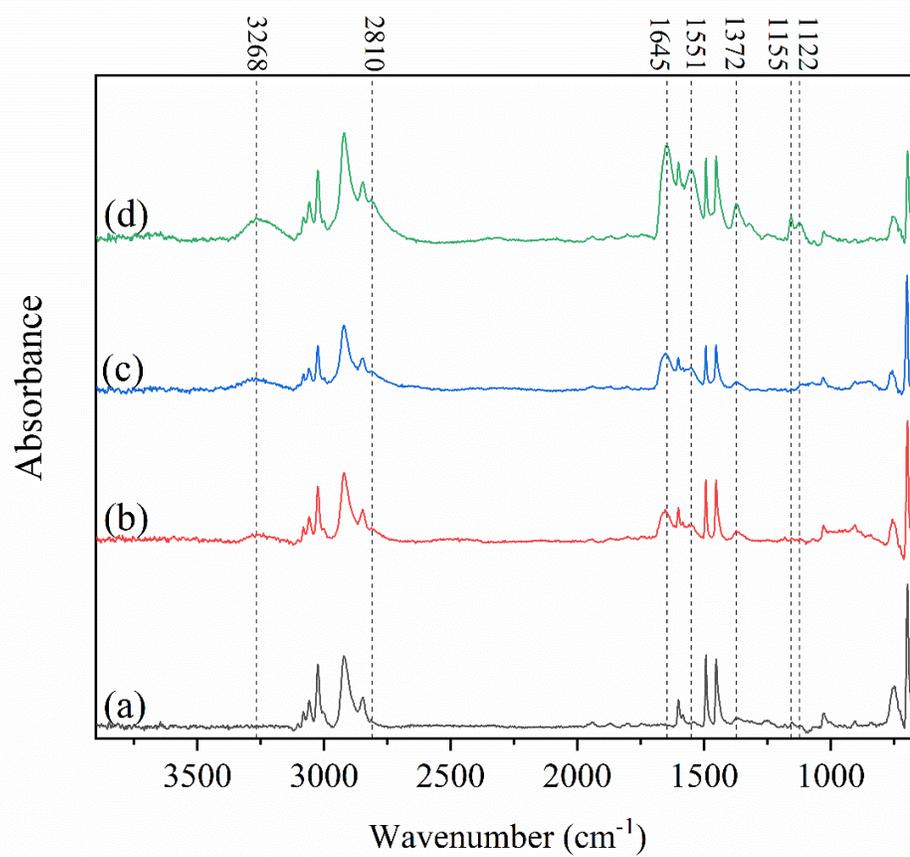


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Schematic.1.

**Fig. 1.**

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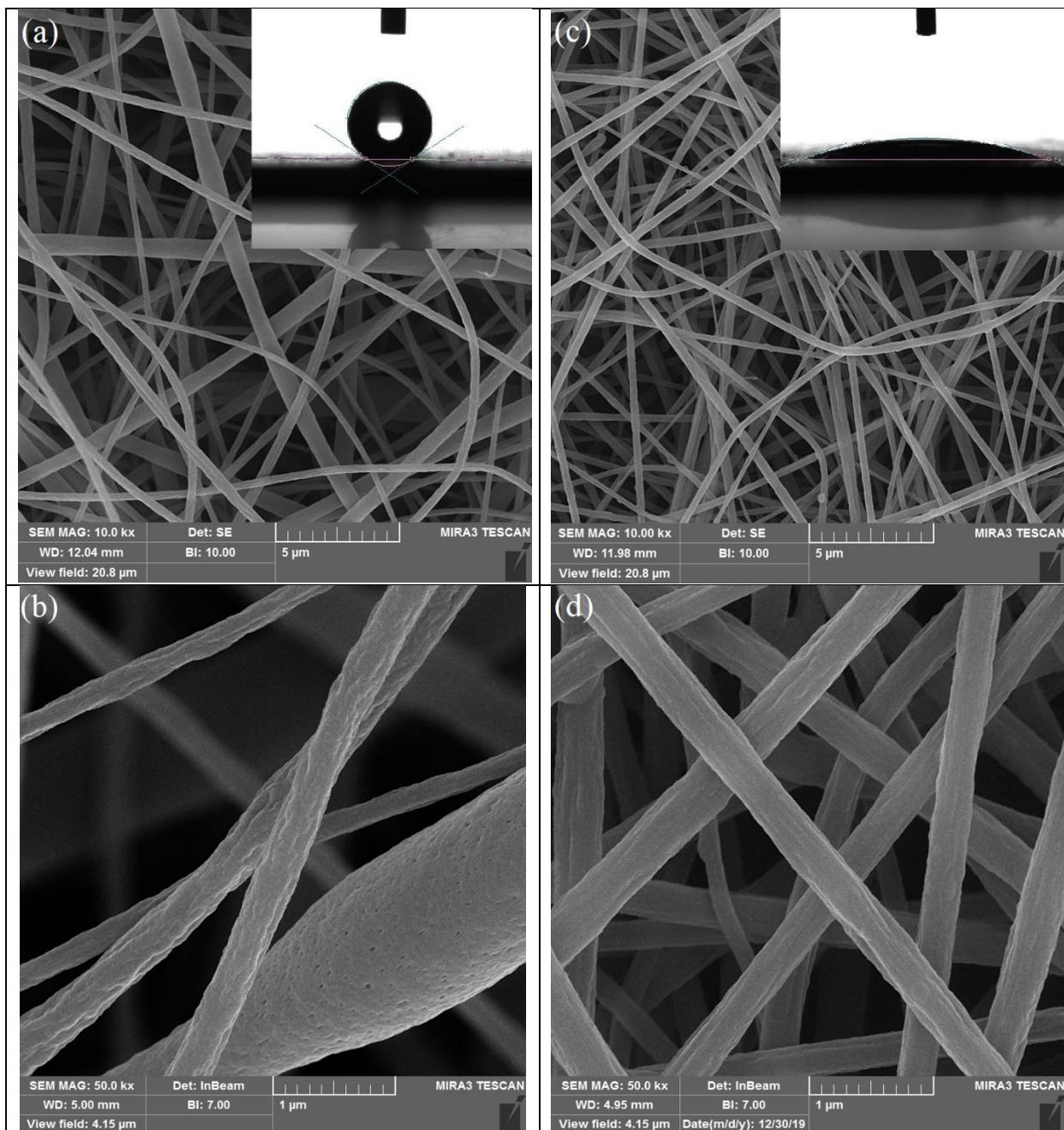
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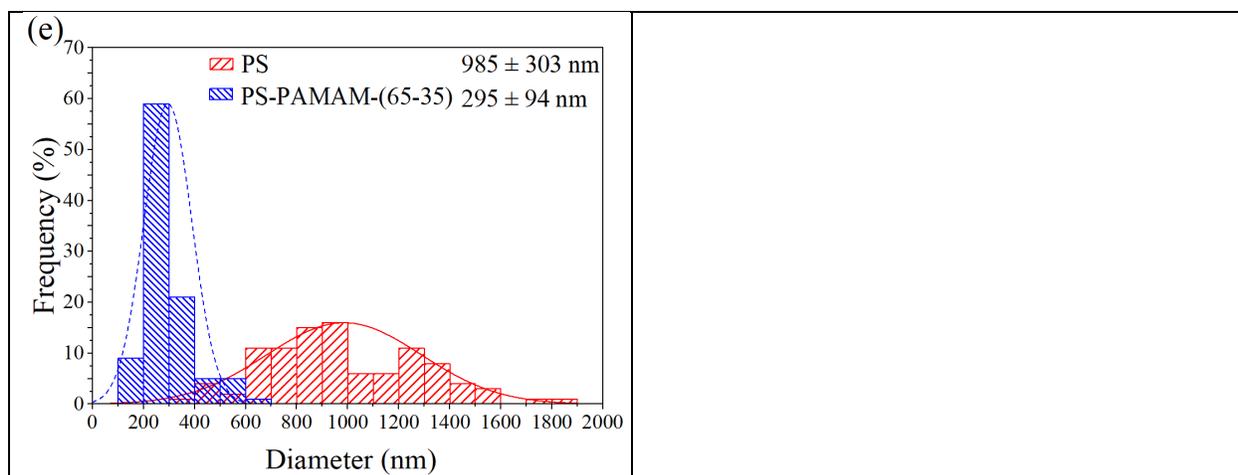
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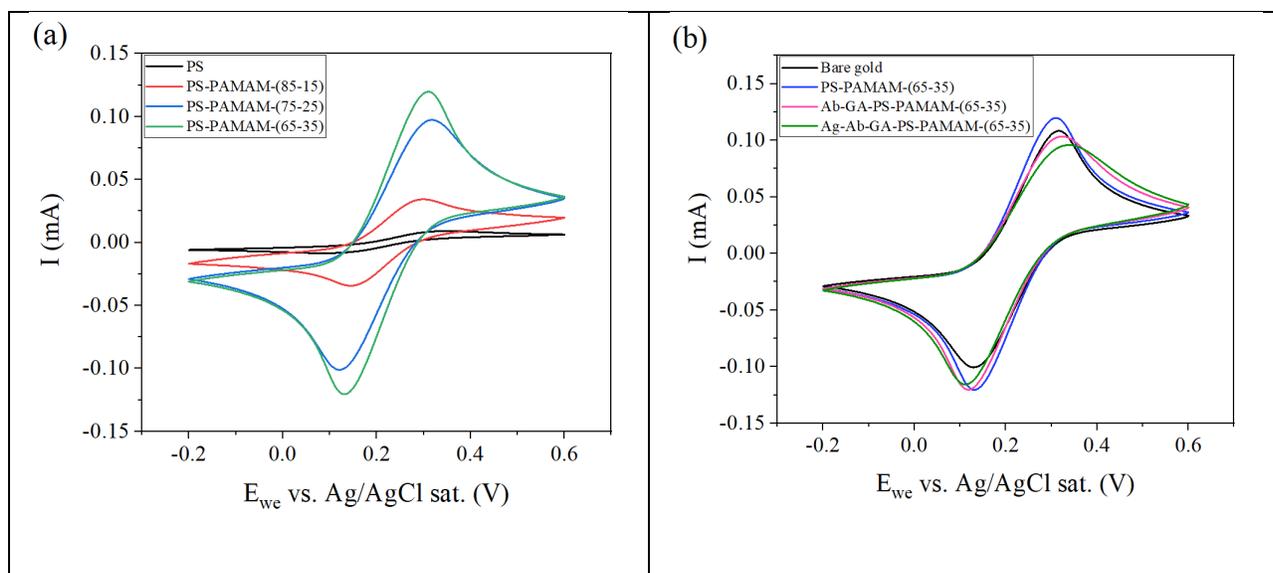
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Fig. 2.

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435**Table 1.**

Samples	Diameter (nm)	Conductivity ($\mu\text{S}/\text{cm}$)	Water contact angle		
			before GA	after GA	after TNF- α Ab
PS	985 \pm 303	0.4	147.2 \pm 1.1 $^\circ$	146.9 \pm 1.8 $^\circ$	132.4 \pm 2.5 $^\circ$
PS-PAMAM-(85-15)	350 \pm 126	11.5	48.2 \pm 1.2 $^\circ$	55.9 \pm 5.0 $^\circ$	31.4 \pm 2.3 $^\circ$
PS-PAMAM-(75-25)	319 \pm 86	18.2	31.8 \pm 1.3 $^\circ$	42.8 \pm 4.8 $^\circ$	28.9 \pm 3.3 $^\circ$
PS-PAMAM-(65-35)	295 \pm 94	24.0	15.4 \pm 1.2 $^\circ$	35.9 \pm 4.7 $^\circ$	16.4 \pm 2.5 $^\circ$

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Fig. 3.

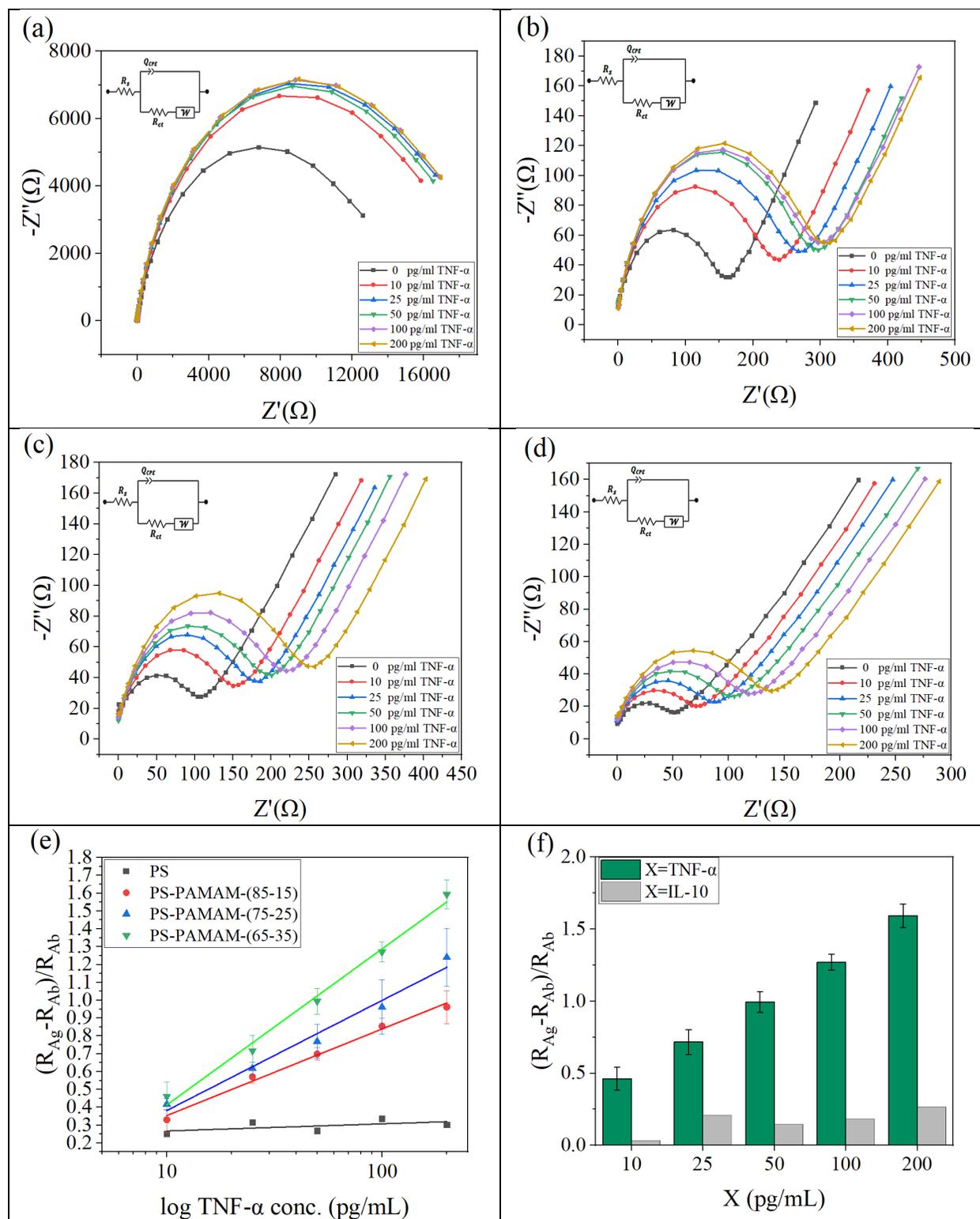


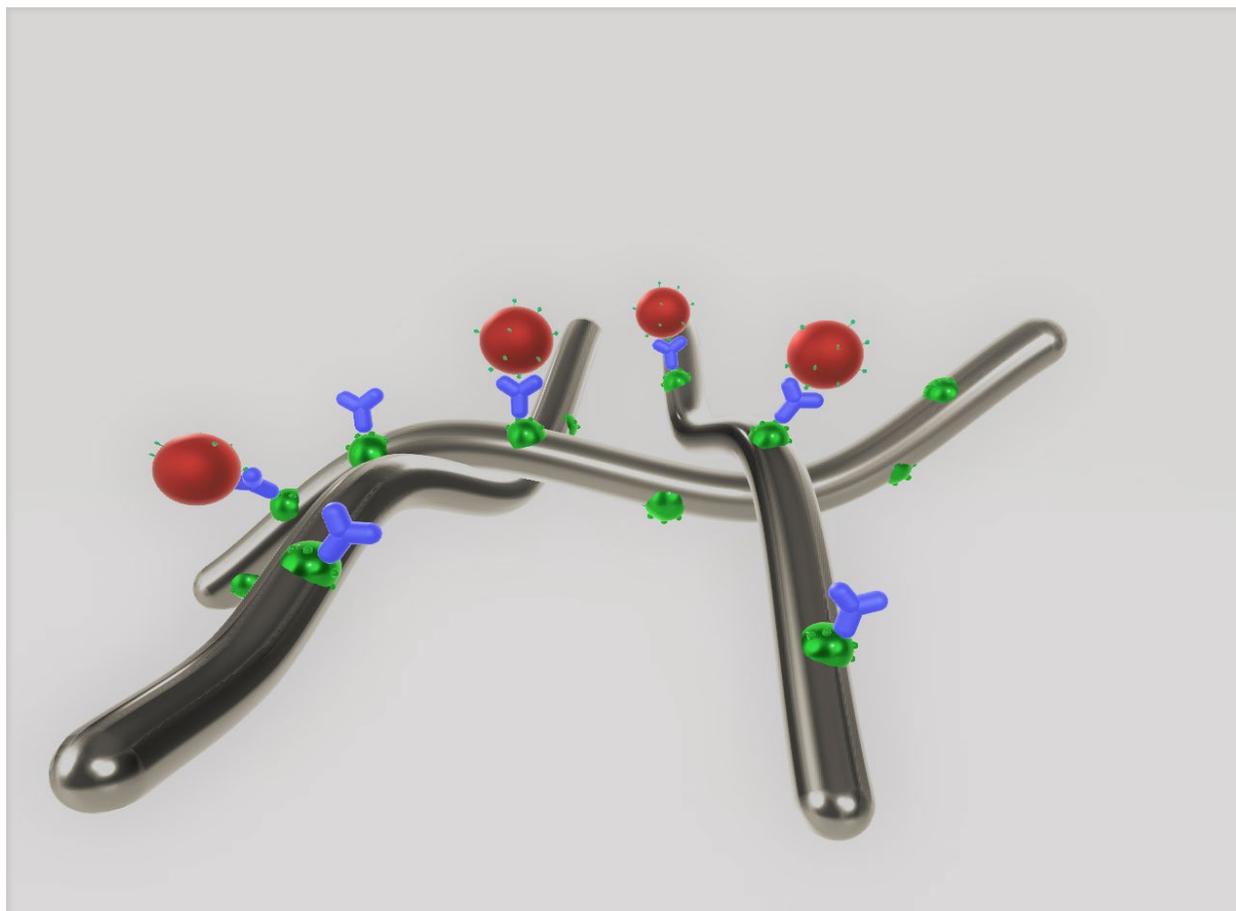
Fig. 4.

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Graphical abstract