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1 **An impedimetric immunosensor based on PAMAM decorated
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3 cytokine**

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14

15 **Abstract:**

16 Cytokine storms are known as the uncontrolled overproduction of inflammatory cytokines that can be
17 produced by a variety of viral or non-infectious disorders and inflict significant damage to many organs.
18 Interleukin-10 (IL-10) is an anti-inflammatory cytokine, and rapid detection of its levels in serum and saliva
19 is important for many diseases, including severe COVID-19 patients. In this study, Polystyrene (PS) fibers
20 were electrospun over a gold electrode and modified by air plasma to allow their further decoration with
21 polyamidoamine (PAMAM) dendritic polymer for providing many active sites on the fiber surface. The
22 fabricated three-dimensional (3-D) architecture was employed as a platform in an impedimetric
23 immunosensor for the quantitative detection of interleukin-10 cytokine (AgIL-10). Scanning electron
24 microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), contact angle measurements,
25 fluorescence microscopy, UV-vis spectroscopy, and electrochemical methods including cyclic
26 voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to characterized the
27 proposed electrospun fiber-based platform and electrochemical immunosensor. The PAMAM properties
28 increased not only the amperometric response to the ferro/ferri cyanide redox probe, of the modified gold

29 electrode but also the active surface area available for covalently binding of anti-IL-10 capture antibody,
30 resulting in the sensitive detection of AgIL-10 in the concentration range of (1-50 pg/mL) in phosphate
31 buffer saline (PBS) with a limit of detection (LOD) of 1 pg/mL. The immunosensor's performance in
32 detecting AgIL-10 in artificial saliva (AS) as a complex medium was likewise satisfactory. This
33 immunosensor provides a new opportunity for clinical immunoassays thanks to its great
34 sensitivity, selectivity, and stability.

35 **Keywords:** Immunosensor, Fibers, Plasma, Polystyrene, Polyamidoamine (PAMAM), Interleukin-10

36 1. Introduction

37 Cytokine storms are defined as excessively high and exaggerated cytokine production originating from a
38 variety of viral and non-infectious disorders, causing severe inflammatory responses and organ damage. [1]
39 Interleukin-10 (IL-10) is an anti-inflammatory cytokine produced by T helper 2 cells that plays an important
40 role in immunoregulation and inflammation management. [2,3] IL-10 has been considered as a viable
41 therapeutic and diagnostic tool for a variety of diseases, including inflammatory autoimmune diseases. [4,5]
42 Recent studies with coronavirus (COVID-19) patients' non-survivors have reported elevated concentrations
43 of interleukin-6 (IL-6) and IL-10 cytokines compared with survivors owing to hyperinflammation [6–8].
44 The findings from 102 COVID-19 patients reveal that the median level of IL-10 cytokine in their serum,
45 (5.37 pg/mL) was 37% higher than the median level in healthy serum (3.91 pg/mL) [1]. Salivary IL-10
46 cytokine levels in COVID-19 and post-COVID-19 patients have been reported to be 10.01 ± 2.05 pg/mL
47 and 9.08 ± 1.17 pg/mL, respectively. These findings suggest that elevated IL-10 concentrations could be
48 utilized to predict the severity of COVID-19 [9].

49 The enzyme-linked immunosorbent assay (ELISA) is regularly used as a standard laboratory test for
50 cytokine detection. Despite its great sensitivity and precision, this approach is expensive, time-consuming,
51 and requires the analysis of professional specialists. [10,11] Hence, more accessible, highly sensitive, and
52 selective diagnostic methods for the detection of inflammatory cytokines are urgently needed to enable

53 early therapeutic intervention and improve patient quality of life. [12] Electrochemical immunosensors, as
54 opposed to these suggested techniques, have attracted a lot of interest because of their significant features,
55 such as ease of use, swiftness, portability, time and cost effectiveness, and excellent sensitivity. [13] These
56 immunological responses triggered by particular probe-target complexes are characterized by legible
57 current signals. The signal amplification strategy is the most widespread and effective way for increasing
58 the sensitivity of electrochemical immunosensors. In this regard, several approaches can be followed to
59 improve the electron transport efficiency and surface area of the substrate while keeping the immunological
60 molecule's activity. [14] Multiple nanomaterials have been employed in the creation of electrochemical
61 immunosensors and improve their performance as a result of the quick development of nanomaterial
62 breakthroughs due to their numerous benefits, including high stability, strong electrocatalytic activity, and
63 ease of modification. [15–17]

64 The ability to electrospun fibers (ESFs) mats with a high surface area, mechanical flexibility, good surface
65 functionalization, and interconnected porosity, which helps in the stabilization of higher receptors with
66 better contact with the surrounding medium and access to the target analyte, underpins their use in the
67 production of 3-D interfaces for electrochemical nano-biosensors. [18–21] Among the various fiber-
68 forming technologies, electrospinning is known as an efficient, low-cost, simple, and versatile tool which
69 uses electrostatic forces to generate polymer nanofibers (NFs) of different morphologies and diameters
70 from solutions, suspensions, or melts[20]. Normally, in the electrospinning synthesis of NFs, the solution
71 is extruded at a constant feed rate through the capillary to the tip of the spinneret, resulting in a pendant
72 liquid drop due to surface tension. When an electric field is applied, electrostatic repulsion of surface
73 charges of the same sign on the polymer droplet occurs, causing it to deform into a cone shape (known as
74 a Taylor cone). By increasing the electric field and the dominance of electric force over surface tension, a
75 charged polymer jet emerges from the cone, undergoing stretching, elongation, solvent evaporation, and
76 solidification in the distance between the nozzle and the collector. [20,22,23]

77 Our major objective in this project was to create a new ESF-based transducer for the detection of AgIL-10
78 by directly electrospinning PS ESFs on the surface of a gold electrode. PS was chosen as a polymer for its
79 high mechanical characteristics, great shape stability in aqueous media without swelling, and low cost. [24]
80 However, PS alone cannot offer an ideal interface because it lacks the surface-active groups and sufficient
81 hydrophilicity needed for biological recognition elements' covalent attachment. [19] For this reason, plasma
82 treatment, a process that alters the surface of polymers, increase their surface energy, and their
83 hydrophilicity while maintaining their advantageous bulk features intact [25,26], was utilized to produce
84 carboxyl groups on the PS fiber surface before decorating the fibers with the second generation (G2) of
85 PAMAM dendritic polymer. PAMAM dendrimers with huge peripheral groups have been employed as
86 branching polymeric macromolecules in the construction of different biosensors to readily interact with a
87 large variety of bioreceptors, boosting their sensitivity, and improving their detection limit. [27,28] Grafting
88 highly branched PAMAM polymer onto the fiber's surface can overcome the drawbacks of PS fibers, such
89 as their hydrophobicity and lack of functional groups. Furthermore, the synergistic properties of electrospun
90 fibers and highly branched polymers to create immunosensors with high capture antibody immobilization
91 and increased sensitivity would be beneficial.

92 In a previous report, our group employed electrospinning to create a composite nanofiber from PS and
93 PAMAM dendritic polymers in a one-step process by blending their solutions and using it as a 3-D interface
94 to detect TNF-alpha cytokines. [29] As novelty, the benefit of decorating PAMAM on the PS fiber surface
95 avails higher accessibility to PAMAM amine groups due to their larger loading on the fiber surface at
96 considerably lower PAMAM concentrations. To create a strong covalent bond between the anti-IL-10 and
97 the fiber surface, glutaraldehyde coupling chemistry was used to create a bond between the PAMAM
98 dendritic polymer groups and the amine groups in the Fc region of the anti-IL-10. Schematic 1 depicts all
99 stages of the immunosensor fabrication. Cyclic voltammetry (CV) and electrochemical impedance
100 spectroscopy (EIS) were used to characterize the electrochemical characteristics of the developed ESFs-
101 based immunosensor at each stage and AgIL-10 impedimetric detection, respectively.

102 Schematic.1.

103 **2. Experimental**

104 *2.1. Chemicals and reagents*

105 The reagents, instruments, and characterizations sections were thoroughly discussed in the supplementary
106 information.

107 *2.2. Immunosensor preparation*

108 *2.2.1. Preparation of PS ESFs*

109 PS granules were dissolved in DMF and stirred overnight to make a 25 wt.% electrospinning solution. A
110 vertical electrospinning setup (Spraybase®, Ireland) was used for producing ESFs, which included a
111 polytetrafluoroethylene (PTFE) tube to transfer the polymer solution from the pump to the nozzle. The
112 electrospinning parameters, including voltage, feed rates, and nozzle to collector distance, were set at 27
113 kV, 0.25 mL/h, and 20 cm, respectively, with these parameters having been previously optimized. [19] To
114 achieve the highest level of similarity between the electrodes in terms of the thickness and amount of ESFs
115 deposited, fibers were electrospun on gold electrodes for 1 min each by placing each on a specific location
116 of aluminum foil. Before electrospinning, the gold electrodes were cleaned in an ultrasonic bath for 15 min
117 in an acetone solution and then in an ethanol solution and were rinsed with ultrapure water at each stage
118 and at the end. Then they were dried with a mild stream of nitrogen (N_2) and then placed in a UV-ozone
119 ProCleanerTM for 30 min to eliminate any possible organic contamination. This process results in a gold
120 electrode with a hydrophilic feature, which increases not only fiber adherence to the electrode surface but
121 also electrode wettability and electron transfer. Then the electrodes containing ESFs were stored at room
122 temperature (RT) for subsequent use.

123 *2.2.2. Air plasma treatment*

124 Gold electrodes modified with ESFs were treated with a frequency/low pressure (13.56 MHz, 50 W) plasma
125 generator (Pico PCCE, Diener Electronic, Ebhausen, Germany) to produce carboxylic acid groups on the
126 surface of the PS ESFs. By the time the electrodes were placed in the center of the chamber, the pressure
127 within the chamber was reduced to 0.2 mbar by pumping to remove the air. The chamber was then filled
128 with filtered air for 1 min before plasma was applied at a pressure of 0.2 mbar for 10 s. To find the optimal
129 plasma time, five different plasma timings (10 s, 20 s, 30 s, 60 s, and 120 s) were tested.

130 *2.2.3. PAMAM decoration of PS ESFs*

131 The plasma-generated carboxylic acid groups were activated by carbodiimide chemistry to decorate the
132 surface of the fibers with PAMAM. Gold electrodes containing PS-plasma (10 s) treated ESFs were dipped
133 in a 2 mL solution of EDC (0.4 M)/NHS (0.1 M) mixture in deionized water for 1 h. The electrodes were
134 immediately immersed in an aqueous PAMAM solution for 4 h, rinsed with deionized water, and then dried
135 at RT. To determine the optimal dendrimer concentration, three PAMAM solution concentrations of 0.5, 1,
136 and 2 wt.% were investigated.

137 *2.2.4. Covalent immobilization of antibodies and blocking steps*

138 The PAMAM amine groups on the PS ESFs surface were activated to form a covalent bond with the Fc
139 region of anti-IL-10 using glutaraldehyde as a coupling agent. For this purpose, the electrodes were put in
140 a desiccator containing glutaraldehyde saturated vapor for overnight. Then, 20 µL of anti-IL-10 (10 µg/mL)
141 was kept in contact with the ESF-modified electrodes for 1 h at 4 °C. After that, the immunosensor was
142 carefully rinsed with PBS (10 mM, pH 7.4) and the blocking treatment was done by incubating it in 40 µL
143 of BSA 2% in PBS solution (10 mM, pH 7.4) for 1 h at 4 °C to reduce non-specific binding and deactivate
144 the unbounded sites on the ESFs surface.

145 *2.3. Detection of AgIL-10 in PBS buffer and in artificial saliva (AS)*

146 The ESFs based immunosensor was immobilized in the electrochemical cell. The immunosensor was
147 incubated in 40 µL of different concentrations of AgIL-10 standard solution in PBS or AS for 30 min at

148 RT, followed by PBS washing to eliminate unbound AgIL-10 cytokine. The electrochemical cell was then
149 filled with PBS (10 mM, pH 7.4) containing 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] at each detection to record
150 an EIS response. The electrolyte solution in the cell was withdrawn and the cell was washed with PBS
151 solution after each EIS measurement.

152 *2.4. Characterizations*

153 Instrumentation and procedures used for the physicochemical characterization are presented in the
154 supplementary information.

155 *2.5. Electrochemical measurements*

156 Instrumentation and procedures used for the electrochemical measurements are presented in the
157 supplementary information.

158 **3. Results and discussion**

159 *3.1. Choosing the optimal plasma time*

160 *3.1.1. Morphological analysis*

161 Plasma-induced chemical changes are typically accompanied by morphological changes. It should be
162 highlighted that extremely harsh surface treatment results in uneven surface etching, which has a significant
163 influence on the immobilization and performance of bio-interface. The surface morphology influences the
164 activity of immobilized biomolecules as well as the biophysical and chemical aspects of the biointerface,
165 therefore picking the optimal time for plasma is crucial to minimize undesirable ion-assisted chemical
166 etching and damage of PS fibers. [30] Five different times (10 s, 20 s, 30 s, 60 s, and 120 s) were tested to
167 find the best plasma time for causing the least amount of damage to the PS ESFs surface and generating the
168 most functional groups. The SEM images of ESFs are shown in Fig. S1. PS ESFs produced from
169 electrospinning solution with a concentration of 20 wt.% had a bead-on-strings morphology, therefore a PS

170 solution with concentration of 25 wt.% was chosen to enhance polymer chain entanglement and produce
171 fibers with a uniform morphology and reproducible characteristics (Fig. S1A).

172 According to the SEM images (Fig.S1 B-D), there are no signs of damage to the PS ESFs for 10 s, 20 s,
173 and 30 s of plasma, but after that, the ESFs diameter begins to decrease (Fig. S1E and F). The mean diameter
174 of pure PS ESFs was 1805 ± 336 nm, while the diameter after 60 s of plasma decreased to 1271 ± 253 nm.
175 The ESFs structure was destroyed and the fibers were charred after 120 s of plasma. Consequently, using
176 plasma to reduce fiber diameter for some specific purposes may be deemed a technique. Because PS ESFs
177 were utilized as an interface in this study, their strength was especially crucial during the washing and
178 immobilization processes. As a result, preserving their strength was prioritized, and SEM evaluation led to
179 the selection of 10 s for plasma.

180 *3.1.2. wettability analysis*

181 Contact angle measurements were conducted to assess the involvement of plasma treatment in the creation
182 of functional groups on the surface of PS ESFs and their hydrophilic characteristics. According to the results
183 (Fig. S2), the contact angle of the PS ESFs after 10 s of plasma was 0° , while the contact angle of pure PS
184 ESFs was $145^\circ \pm 2.5^\circ$. The formation of COOH, OH, C=O, and C–O, leads to the contact angle decreasing.
185 [31] The production of singly and doubly bonded C–O functionality is based on the removal of hydrogen
186 from tertiary C–H bonds or benzene rings, followed by oxygen substitution in free radical sites. [32]

187 A duration of 10 s was chosen as the optimal plasma time since it reduced the contact angle to 0° and
188 caused the least amount of damage to the ESFs. It should be mentioned that fiber hydrophilicity is a key
189 feature for the homogenous immobilization of antibodies on the fiber surface, as well as improved electron
190 transfer between the electrolyte and the electrode.

191 *3.2. Evaluation of fiber-based immunosensor*

192 *3.2.1. FE-SEM characterization*

193 FE-SEM microscopic analysis of the fibers' surface before and after the plasma indicated that a plasma time
194 of 10 s was appropriate since there were no signs of fracture or cracking on the surface of the fibers (Fig.
195 1A-B). The significant difference between before and after the plasma is the rise in nanoporosity size on
196 the surface of the fibers, which increases surface area and enhances potential interaction with the
197 environment [33]. The fibers' morphology changed to peach skin after being decorated with PAMAM (Fig.
198 1C). The average diameter of PS-plasma 10 s-PAMAM 2 wt.% ESFs was 1565 ± 290 nm (Fig. 1D).

199 Fig. 1.

200 *3.2.2. fluorescence microscopy*

201 Fluorescence microscopy of PS ESFs revealed that plasma-modified PS fibers had autofluorescence (4.26 ± 1) at 488 nm wavelength excitation as compared to pure PS (Fig. 2A-B). A high level of autofluorescence
202 has been proven in thermoplastic polymers, including PS, particularly between the wavelengths of 300 -
203 400 nm. A recent study has shown that thermal and solvent treatment increases the levels of
204 autofluorescence of PS plate and changes its wavelength to longer wavelengths. [34] Here, the PS ESFs
205 have no autofluorescence at 488 nm wavelength excitation, whereas autofluorescence and spots of different
206 sizes are observed on the PS-plasma 10 s ESFs. The autofluorescence of PS fibers has been attributed to
207 excimers resulting from polymer chain reorganization, which have longer wavelengths than the monomer
208 emission peak. It should be noted that due to the high interest in the use of ESFs in various fields,
209 particularly optical immunosensors, and the proximity of the fluorophores wavelength used for antibody
210 conjugation to the plasma treated PS wavelength, serious consideration should be given to their
211 autofluorescence after plasma to avoid false positive results. [34] Fig. 2C-E shows images of fibers after
212 being decorated with various concentrations of PAMAM. Because PAMAM has fluorescence properties at
213 488 nm excitation [19], the fluorescence increase in PS-plasma 10 s-PAMAM ESFs could be noticed when
214 PAMAM concentration increases. The quantity of grafted PAMAM at the fiber surface is larger in the PS-
215 plasma 10s-PAMAM 2 wt.% sample than in the other samples. The average fluorescence intensity for each
216 of the mentioned ESFs was obtained by preparing 5 distinct samples and assessing 5 different points of

each sample. Fig. 2F shows the fluorescence intensities of different ESFs samples, according to which the fluorescence intensities for the 0.5, 1, and 2 wt.% dendrimer samples were 30.5 ± 4.4 , 47.2 ± 6.7 , and 60.8 ± 5.5 , respectively. Because of its higher fluorescence properties, PS-plasma 10 s-PAMAM 2 wt.% was chosen as the best sample, and all immunosensors were prepared with this concentration.

Fig. 2.

3.2.3. FTIR analysis

ATR-FTIR analysis were done in order to more accurately evaluate the chemical composition of PS ESFs during the surface modification and immunosensor fabrication processes. The bands at $3080\text{-}3024\text{ cm}^{-1}$, 2920 cm^{-1} , and 2846 cm^{-1} in the PS spectra are attributable to aromatic C–H stretching vibration absorption and the presence of CH_2 , respectively. The peaks at 1939 cm^{-1} , 1869 cm^{-1} , 1801 cm^{-1} , and 1741 cm^{-1} are associated with overtone aromatic. The C=C stretching vibration absorption is seen at 1600 cm^{-1} , while CH_2 bending vibrations are seen at 1492 cm^{-1} and 1451 cm^{-1} (Fig. 3Aa). [19] A comparison of spectra of PS and PS-plasma 10 s shows two new peaks at 3454 cm^{-1} and 1726 cm^{-1} , which correspond to OH and C=O from carboxylic acids, respectively (Fig. 3Ab). Fig. S3. depicts a schematic representation of the likely PS structure following plasma treatment. [31] According to previous study, the major mechanism for plasma treatment is an oxidative assault on the benzene ring followed by ring-opening to create aldehyde/carboxylate species. Oxidation of PS to a higher oxidation state and carbonyl/carboxyl species formation has been confirmed by IR-visible sum frequency generation (SFG) spectra. [35] The spectrum from PS-plasma 10 s-PAMAM 2 wt.% has amide bands of PAMAM vNH at 3293 cm^{-1} , vCO (amide I band) at 1652 cm^{-1} , δNH and vCN (amide II band) at 1553 cm^{-1} . [29] It should be noted that the peak of carboxylic acid is decreased due to covalent bonding with PAMAM. Also, PS peaks are still present in the spectrum (Fig. 3Ac).

Previous studies have noted the N–H stretching vibration peak in the PAMAM dendrimer at 3293 cm^{-1} , which increased with increasing dendrimer concentration. [19,36] In the spectra of PS-plasma 10 s-

242 PAMAM 2 wt.%, a broad peak in the range of 3100 cm^{-1} to 3600 cm^{-1} is observed, which consists of two
243 OH peaks in the region of 3454 cm^{-1} formed by plasma modification and the amide bands vNH of PAMAM
244 at 3293 cm^{-1} , which are overlapped. When the two spectra in Fig. 3B are compared, the tip of the peak in
245 the PS-plasma 10 s-PAMAM 2 wt.% spectra is placed near the N–H stretching vibration peak, confirming
246 the presence of this peak of PAMAM in the spectrum.

247 The spectrum of PS-plasma 10 s-PAMAM 2 wt.%-glutaraldehyde before and after anti-IL-10 ($10\text{ }\mu\text{g/mL}$)
248 immobilization is shown in Fig. 3Ca and b, respectively. Fig. 3Ca shows the characteristic peak at 1715 cm^{-1}
249 for glutaraldehyde and also the peaks at 1662 cm^{-1} and 1552 cm^{-1} of the amide bands of PAMAM in
250 the spectrum. After anti-IL-10 antibody immobilization, the glutaraldehyde peak at 1715 cm^{-1} decreased
251 significantly, indicating a covalent bond between the antibody and glutaraldehyde (Fig.3 Cb). [37,38]
252 However, it should be noted that due to the peak overlap of PAMAM and anti-IL-10 amine groups, it is not
253 possible to distinguish between them.

254 *3.2.4. UV-vis analysis*

255 Fig. 3D shows the UV-visible absorption spectrum of the supernatant after washing PS-plasma 10 s-
256 PAMAM-glutaraldehyde with washing buffer, PBS solution (10 mM , pH 7.4) for 5 min. An absorption
257 peak is observed at 235 nm , which is connected to the carbon – carbon double bond $\pi - \pi^*$ transition of
258 aldehyde groups. [39,40] According to the findings, the amount of unreacted glutaraldehyde in the
259 supernatant is inversely linked to the concentration of PAMAM, so the PS-plasma 10 s-PAMAM 2 wt.%-
260 glutaraldehyde supernatant has the lowest amount of glutaraldehyde adsorption. This shows that increasing
261 the PAMAM concentration while also increasing the number of amine groups on the fiber surface induces
262 more glutaraldehyde molecules to establish stronger covalent bonds with them.

263 Fig. 3.

264 *3.2.5. Electrochemical characterization*

The ESFs modified-gold electrode in stepwise fabrication process of ESFs based immunosensor was evaluated by CV measurement in PBS (10 mM, pH 7.4) containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-4-}$ because of any alterations in the bio-interface physically hinder the electrochemical behavior of the redox probe. As shown in Fig. 4A, the bare gold electrode presents well-defined anodic/cathodic peaks of $[\text{Fe}(\text{CN})_6]^{3-4-}$ and a reversible voltammogram with peak-to peak separation (ΔE_P) of ~ 182.5 mV, with a peak maximum intensity ($I_{pa} = 115.6 \mu\text{A}$). After electrospinning of the PS fibers on the electrode, the oxidation and redox peaks of the redox probe almost disappeared ($I_{pa} = 14.6 \mu\text{A}$). Electron transfer from the electrolyte to the gold electrode surface happens as a result of tunneling through the porosity of the fibrous mats. The lack of conductivity and high hydrophobicity of the PS ESFs block the diffusion of redox probe to the electrode surface. Plasma modification for 10 s significantly increased the amperometric response of the electrode containing PS ESFs ($I_{pa} = 113.5 \mu\text{A}$). The formation of oxygen-containing groups after plasma is the main factor contributing to the increasing mobility of the redox probe towards the electrode. [41] Modification of the fiber surface with PAMAM increased oxidation/reduction current ($I_{pa} = 135.1 \mu\text{A}$) and decreased ΔE_P from ~ 210.5 to ~ 186.5 mV, which indicates the successful attachment of PAMAM at the PS fiber surface [29]. The pKa of the peripheral functional groups ($-\text{NH}_3^+$, pKa ~ 9.52) gave the PAMAM a protonated interface in aqueous media at neutral pH. [42] Positively charged PAMAM accumulates the ferro/ferricyanide redox probe on its surface because of its electrostatic interaction. The electron-rich PAMAM surface amine groups and the electron-deficient interior create an electric field that accelerates electron transfer on the electrode's surface. [43] Fig. 4B depicts the role of PAMAM in increasing current response; when dendrimer concentration rose from 0.5 to 2 wt.% due to increased dendrimer presence at the fiber surface, the current increased from ($I_{pa} = 121.7 \mu\text{A}$) to ($I_{pa} = 135.1 \mu\text{A}$). The results were compatible with the fluorescence images in section 3.2.2. Due to improved electron transfer and a larger presence of amine groups on the surface, the concentration of 2 wt.% was determined as the best concentration for immunosensor preparation.

289 The CV responses of the PS-plasma 10 s-PAMAM 2 wt.%-glutaraldehyde ESFs electrode at different scan
290 rates (10 to 100 mVs⁻¹) are shown in Fig. S4A. Increasing the scan rate, which in turn reduces the
291 penetration layer, increases the current peak and ΔE_p. Fig. S4B shows the square root of the scan rate versus
292 anodic and cathodic peak currents [$I_{pa}(\mu A) = 14.8739 v^{1/2}[(mV/s)^{1/2}] + 9.2634. R^2 = 0.99$] and
293 [$I_{pc}(\mu A) = -14.7623 v^{1/2}[(mV/s)^{1/2}] - 12.4408. R^2 = 0.99$], respectively. Fig. S4C shows the square
294 root of the scan rate versus anodic and cathodic peak potentials, [$E_{pa}(V) = 0.0083 v^{1/2}[(mV/s)^{1/2}] +$
295 $0.2549. R^2 = 0.99$] and, [$E_{pc}(V) = -0.0087 v^{1/2}[(mV/s)^{1/2}] + 0.1888. R^2 = 0.99$], respectively. Peak
296 currents and peak potentials of developed ESFs-based electrodes have a linear relationship with the square
297 root of the scan rate (square of regression coefficient of 0.99), demonstrating that the electron transfer
298 process in developed immunosensor is mostly based on a diffusion-controlled process. [44]

299 The electro-active surface area provided by the ESFs developed immunosensor was determined to be 0.149
300 cm² by the Randles–Sevcik equation $I_p = 268600AD^{1/2}n^{3/2}Cv^{1/2}$ in which I_p is the redox peak current
301 (A), A is electroactive surface area (cm²), D is the diffusion coefficient (D = 7.60×10^{-6} , at 25 °C), n is
302 the number of electrons transferred in the redox reaction (n = 1), C is the concentration of the reactant (5
303 mM Fe(CN)₆^{3-/4-}) (mol/cm³), and v is the scan rate (V/s). [45,46] For bare gold electrode, the electro-active
304 surface area of 0.127 cm² was measured, indicating the role of fibers in 17.3% increasing the available
305 surface-active area.

306 To evaluate ESFs based electrode's capability as an effective bio-interface, 40 μL of different concentrations
307 of anti-IL-10 were immobilized on the surface of the PS-plasma 10 s-PAMAM 2 wt.%-glutaraldehyde ESFs
308 electrode at 4 °C for 1 h and the electrode characterized with CV measurement in PBS (10 mM, pH 7.4)
309 containing 5 mM [Fe(CN)₆]^{3-/4-} with a scan rate of 60 mV s⁻¹ (Fig. 4C). A drop in redox current was seen
310 following the immobilization of the antibody on the fiber surface due to the insulating characteristics of
311 antibodies and this was a valid indicator of successful grafting on the fiber surface. Increasing the antibody

312 concentration further reduced the anodic peak intensity, confirming the higher immobilized density of the
313 antibody at the extraordinarily high available surface area provided by the fibers.

314 The activity of the anti-IL-10 on the surface of the fibers and the performance of the immunosensor before
315 and after antigen incubation were also examined with CV in PBS (10 mM, pH 7.4) containing 5 mM
316 $[\text{Fe}(\text{CN})_6]^{3-/-}$ at the final stage of immunosensor preparation (Fig. 4D). The current of $[\text{Fe}(\text{CN})_6]^{3-/-}$ on
317 PS-plasma 10 s-PAMAM 2 wt.% ESFs electrode was ($I_{\text{pa}} = 130.4 \mu\text{A}$). After incubating 10 pg/mL of
318 AgIL-10 on the electrode surface, the redox current ($I_{\text{pa}} = 112.8 \mu\text{A}$) were lower than the PS-plasma 10
319 s-PAMAM 2 wt.%-anti-IL-10-BSA current ($I_{\text{pa}} = 124.0 \mu\text{A}$). This decrease is due to immune complexes
320 formed by Anti-IL-10 and Ag-IL-10, which could obstruct electron transfer to the electrode surface as an
321 insulating layer and confirms the developed ESFs immunosensor's high affinity for Ag-IL-10 cytokine.

322 The results of the developed immunosensor's stability after 20 consecutive cycles at a scan rate of 60 mVs^{-1}
323 are shown in Fig. S4D. The covalent bond between anti-IL-10 and glutaraldehyde, as well as the strong
324 fiber adhesion to the electrode surface, resulted in constant redox peaks and peak potential separation, with
325 relative standard deviations (RSDs) of 0.68 % and 0.87 % for I_{pa} and I_{pc} , respectively. [47]

326 *3.3. Electrochemical detection of AgIL-10 in PBS*

327 EIS was employed as a sensitive approach to assess antibody-antigen interactions at the immunosensor
328 interface and to quantitatively determine AgIL-10 in PBS and AS. The presented EIS results in the Nyquist
329 diagram for AgIL-10 detection from 1 pg/mL to 50 pg/mL in PBS (10 mM, pH 7.4) are shown in Fig. 4E,
330 where the semicircle at higher frequencies refers to the electron transfer process and the linear section at
331 low frequencies belongs to the diffusion process. The gray semicircle corresponds to the immobilization of
332 10 $\mu\text{g/mL}$ of anti-IL-10 on the surface of the PS-plasma 10 s-PAMAM 2 wt.%-glutaraldehyde ESFs
333 electrode for 1 h at 4 °C and shows low R_{ct} , which is consistent with the CV results and demonstrates the
334 role of PAMAM in fast electron transfer and easily accessible redox probe to the electrode surface. As
335 expected, after immobilization of each concentration of the AgIL-10 for 30 min at RT (20 ± 1 °C), the

336 diameter of semicircles increased owing to immunorecognition of AgIL-10 by surface-fixed antibodies that
337 can hinder redox probe charge transfer as an electrical contact barrier. Furthermore, the results show that
338 the proposed immunosensor has a high affinity for AgIL-10 with rising R_{ct} , due to the increased antigen
339 steric hindrance on the electroactive interface for redox probe and restriction of the Faradaic reaction. [48]
340 The electrochemical parameters were derived after each detection by fitting the Nyquist plots with the
341 Randle's equivalent circuit, where R_s corresponds to the ohmic electrolyte resistance, Q_{CPE} to constant
342 phase element, R_{ct} to charge transfer resistance, and \mathcal{W} to Warburg impedance (inset of Fig. 4E). The
343 calibration diagram of immunosensor obtained from the impedance fitting data based on $\Delta R/R$
344 , $(R_{antigen} - R_{antibody})/R_{antibody}$, versus different concentrations of AgIL-10 (1, 5, 10, 20, 35, and 50
345 pg/mL) is shown in Fig. 4F. The immunosensor showed two linear correlation in the ranges from 1 pg/mL
346 to 10 pg/mL with a linear regression equation of $\Delta R/R = (0.03188 \pm 0.0018)[IL - 10] +$
347 0.21685 (± 0.01) and a correlation coefficient of $R^2 = 0.99$. The second range of 10 pg/mL to 50 pg/mL
348 was similarly adjusted to a linear regression equation of $\Delta R/R = (0.00838 \pm 0.0008)[IL - 10] +$
349 0.45164 (± 0.01), with $R^2 = 0.98$. The smaller change in redox current at higher Ag-IL-10 concentrations,
350 which led the calibration curve to be separated into two segments, is due to anti-IL-10 antibody saturation
351 and a reduction of free antibodies available on the surface of the electrospun fibers to form an
352 immunocomplex. The immunosensor has a limit of detection (LOD) of 1 pg/mL based on $3S/m$, where S
353 represents the residual standard deviation and m denotes the slope of the regression line.

354 The inter- and intra-assay precisions of the results obtained using the designed immunosensor were studied.
355 The inter-assay reproducibility of ESFs immunosensor was performed using five independent electrode
356 prepared in the same manner for assessing samples containing 10 pg/mL of AgIL-10. While for the intra-
357 assay, the same designed immunosensor was used five times to detect 10 pg/ml of AgIL-10. The RSD of
358 inter- and intra-assay was around 3.18% and 0.79%, respectively. This result indicated that the developed
359 ESFs immunosensor's precision, reproducibility, and repeatability were desirable.

360 Table 1 compares the analytical performance of the ESFs-based immunosensor to that of other
361 immunosensors for the detection of IL-10 that have been reported to date. To our knowledge, this is the
362 first study to use ESFs as an interface to detect IL-10. The increased surface area provided by the fibers
363 permitted a significantly higher number of antibodies on the surface (100 µg/mL) and provided a
364 comparable detection range and extremely low detection limit to previous studies.

365 Fig. 4.

366 Table 1.

367 *3.4. Interference study*

368 To evaluate the specificity of the immunosensor, an aliquot of 20 µL of anti-IL-10 (10 µg/mL) was fixed
369 on the ESF modified-gold electrode at 4 °C for 1 h and 40 µL of TNF-alpha antigen with different
370 concentrations (1, 5, 10, and 20 pg/mL) was incubated with the ESF modified-gold electrode at RT for 30
371 min. The results of the interference studies in Fig. 5A show that the developed immunosensor is selective
372 for AgIL-10.

373 *3.5. Electrochemical detection of AgIL-10 in AS*

374 The function of the immunosensor for AgIL-10 detection in AS as a complex medium and natural saliva
375 simulator was assessed by EIS. The EIS concepts and immunosensor fabrication procedures were the same
376 as mentioned before. Initially, the likelihood of any unspecific absorption by AS was examined by
377 incubating 40 µL of it on the surface of the ESFs electrode (without AgIL-10). Fig. 5B shows the 3 times
378 incubation of AS on the ESFs immunosensor. After the first incubation of AS, an increase in R_{ct} was
379 observed. This impact was detected in all AS measures in this investigation and is thus considered a
380 constant. The matrix effect, also known as unspecific adsorption, has been discovered in prior
381 investigations. [49] Re-incubation of AS (second and third semicircles) did not result in increased
382 impedance or undesired absorption. It should be emphasized that the immunosensor surface was thoroughly
383 washed with PBS between each incubation step. Fig. 5C shows the Nyquist plot for the detection of AgIL-

384 10 in AS. After 3 times incubation of AS and maintaining a consistent R_{ct} , the AS containing various
385 concentrations of AgIL-10 (10, 25, 50, and 100 pg/mL) was applied to the fiber surface for 30 min at RT
386 each time. The detection of AgIL-10 in AS as a complex medium is highlighted by increasing the Nyquist
387 plot semicircle each time. Furthermore, the EIS signal displayed a linear relationship with IL-10
388 concentrations ranging from 10-100 pg/mL and a LOD of 4,63 pg/mL, with a regression equation of
389 $\Delta R/R = (0.00483 \pm 0.0002)[IL - 10] + 0.00382 (\pm 0.008)$ and a correlation coefficient of $R^2 = 0.96$
390 (Fig. 5D).

391 Fig. 5.

392 **4. Conclusion**

393 In this study, PAMAM polymer was used to decorate the air plasma-modified PS electrospun fibers' surface,
394 resulting in the development of a 3-D open-porous interface and an impedimetric immunosensor with very
395 high sensitivity to the IL-10 cytokine. The formation of carboxylic acid functional groups on fiber surfaces,
396 a rise in hydrophilicity, and the creation of autofluorescence characteristics in PS fibers were all induced
397 by 10 s of air plasma. Increased PAMAM concentration on the fiber surface causes more glutaraldehyde
398 molecules to form covalent bonds with them. Anti-IL-10 capture antibody was successfully immobilized
399 on the PS fiber surface by glutaraldehyde coupling chemistry on the amine functional groups of PAMAM.
400 The immunosensor showed ameliorated stability due to covalent bonding between PAMAM amine groups
401 and anti-IL-10 using a glutaraldehyde coupling agent. Modification of the fiber surface with PAMAM
402 increased oxidation/reduction current and promoted antibody immobilization by increasing surface area.
403 The PS-plasma-PAMAM 2 wt.% ESFs-based immunosensor is a new 3-D framework with great sensitivity
404 and selectivity within a wide linear range of AgIL-10 detection in both PBS and AS. The proposed ESFs
405 based interface is a promising material that can be employed in the clinical detection of cytokines or as a
406 substrate for immunosensors development.

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620 **Figure Captions**

621 Schematic.1. The schematic depicts the step-by-step preparation procedure for the developed
622 immunosensor.

623 Fig. 1. FE-SEM images of A) pure PS, B) PS after 10 s plasma treatment, C) PS-plasma 10 s-PAMAM 2
624 wt.% ESFs, and D) the diameter distribution of PS-plasma 10 s-PAMAM 2 wt.% ESFs.

625 Fig. 2. Fluorescence images of A) pure PS, B) PS-plasma 10 s, C) PS-plasma 10 s-PAMAM 0.5 wt.%, D)
626 PS-plasma 10 s-PAMAM 1 wt.%, E) PS-plasma 10 s-PAMAM 2 wt.%, and F) quantification of fluorescent
627 intensity of different samples (a) pure PS, (b) PS-plasma 10 s, (c) PS-plasma 10 s-PAMAM 0.5 wt.%, (d)
628 PS-plasma 10 s-PAMAM 1 wt.%, and (e) PS-plasma 10 s-PAMAM 2 wt.%.

629 Fig. 3. A) ATR-FTIR spectra of (a) pure PS, (b) PS-plasma 10 s, and (c) PS-plasma 10 s-PAMAM 2 wt.%
630 ESFs, B) zoom at 3000-4000 cm⁻¹, C) (a) PS-plasma 10 s-PAMAM 2 wt.%-glutaraldehyde and (b) PS-
631 plasma 10 s-PAMAM-2 wt.%-glutaraldehyde-anti-IL-10, and D) UV-vis spectra of unreacted
632 glutaraldehyde in (a) PS-plasma 10 s-PAMAM 0.5 wt.%-glutaraldehyde, (b) PS-plasma 10 s-PAMAM 1
633 wt.%-glutaraldehyde, (c) PS-plasma 10 s-PAMAM 2 wt.%-glutaraldehyde, and (d) pure PS.

634 Fig. 4. A) CVs of different stages of immunosensor preparation, B) CV of PS-plasma10 s-PAMAM with
635 different concentrations electrode, C) effect of different immobilized anti-IL-10 concentrations (10, 20, 40,
636 50, and 100 µg/mL) on reducing the redox current, D) evaluation of anti-IL-10 activity (10 µg/mL) on the
637 fiber surface, E) Nyquist plots of PS-plasma10 s-PAMAM 2 wt.%-glutaraldehyde-anti-IL-10 modified gold

638 electrode of AgIL-10 detection ranging from 1 to 50 pg/mL, and F) calibration curves of the ESFs based
639 immunosensors for the detection of AgIL-10.

640 Fig. 5. A) Selectivity resualts of the developed immunosensors, B) Nyquist plots of three times incubation
641 of AS (without AgIL-10) on PS-plasma 10 s-PAMAM 2 wt.% ESFs modified gold electrode, C) Nyquist
642 plots of the immunosensor for AgIL-10 detection in AS, and D) detection calibration curves of the
643 immunosensor toward AgIL-10 in AS.

644 Table 1. Comparison of analytical characteristics of other immunosensors reported so far for IL-10
645 detection with the presented ESFs based immunosensor.

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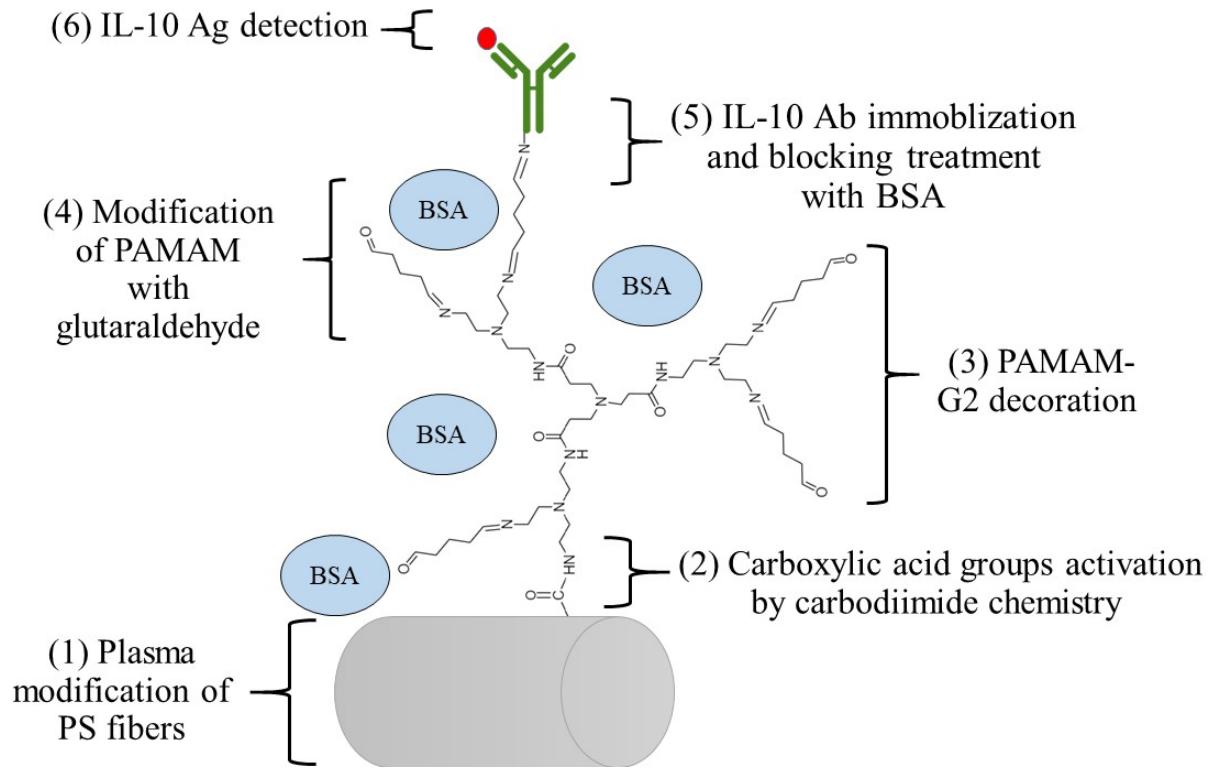
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 657 immunosensor.

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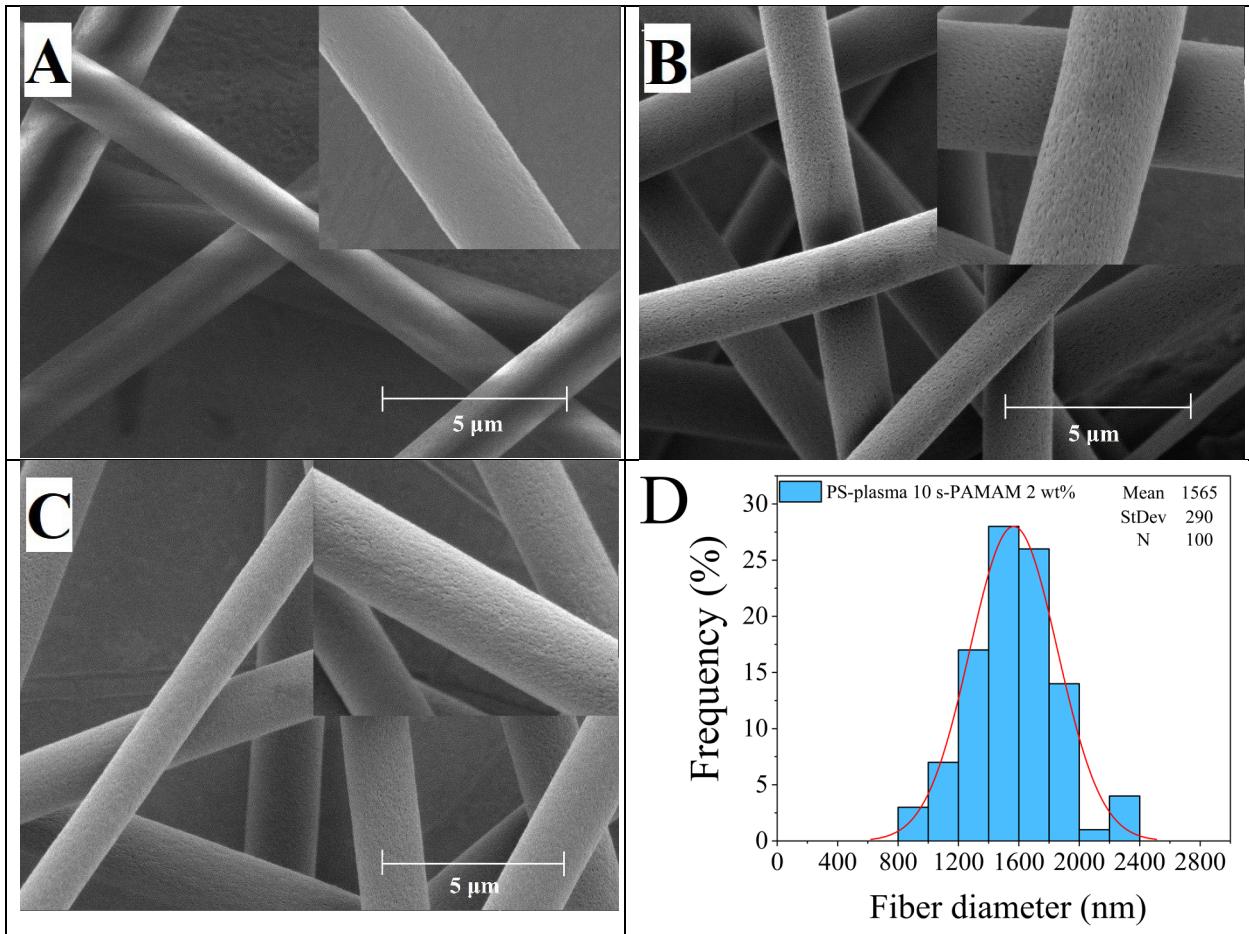
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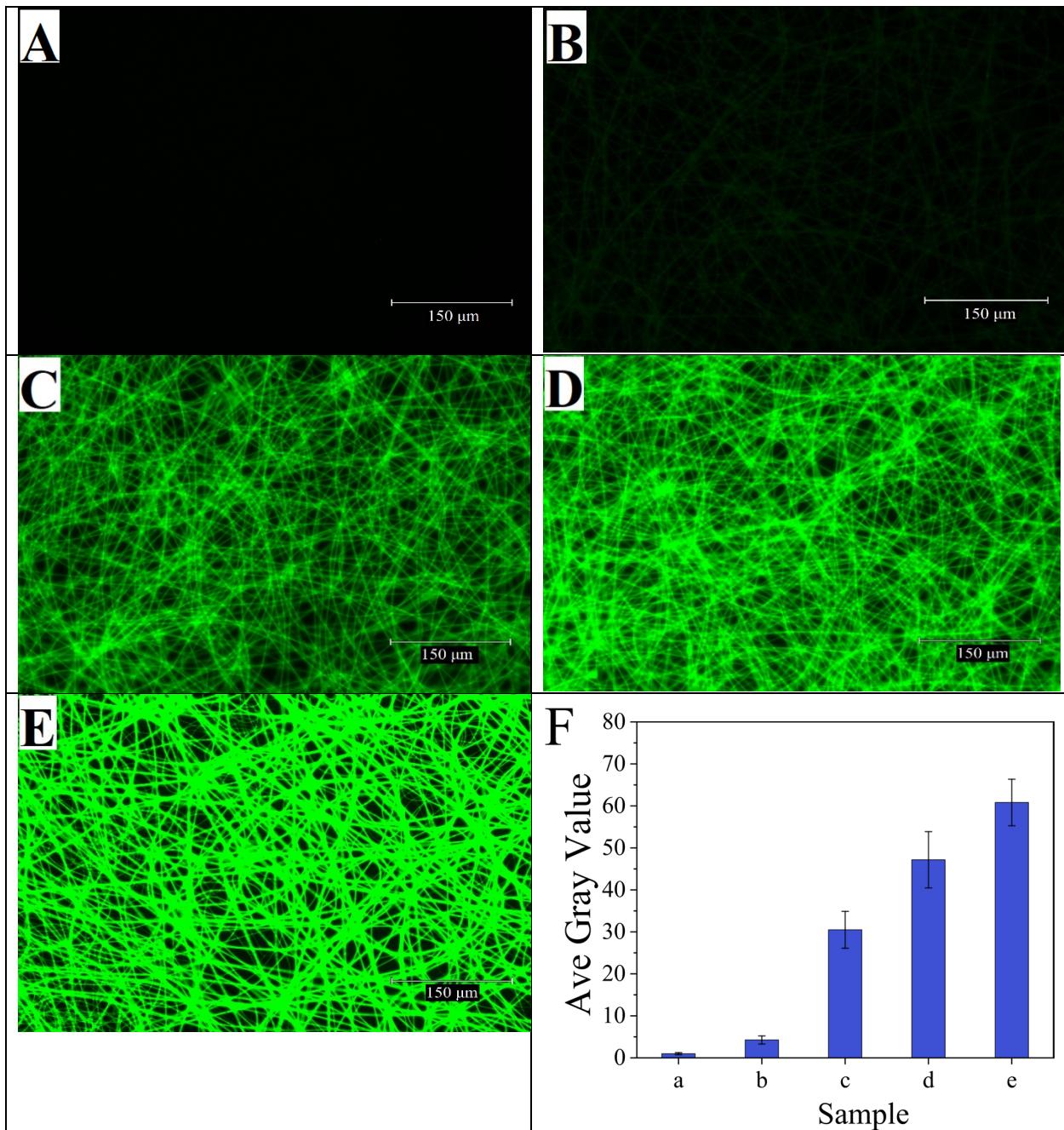
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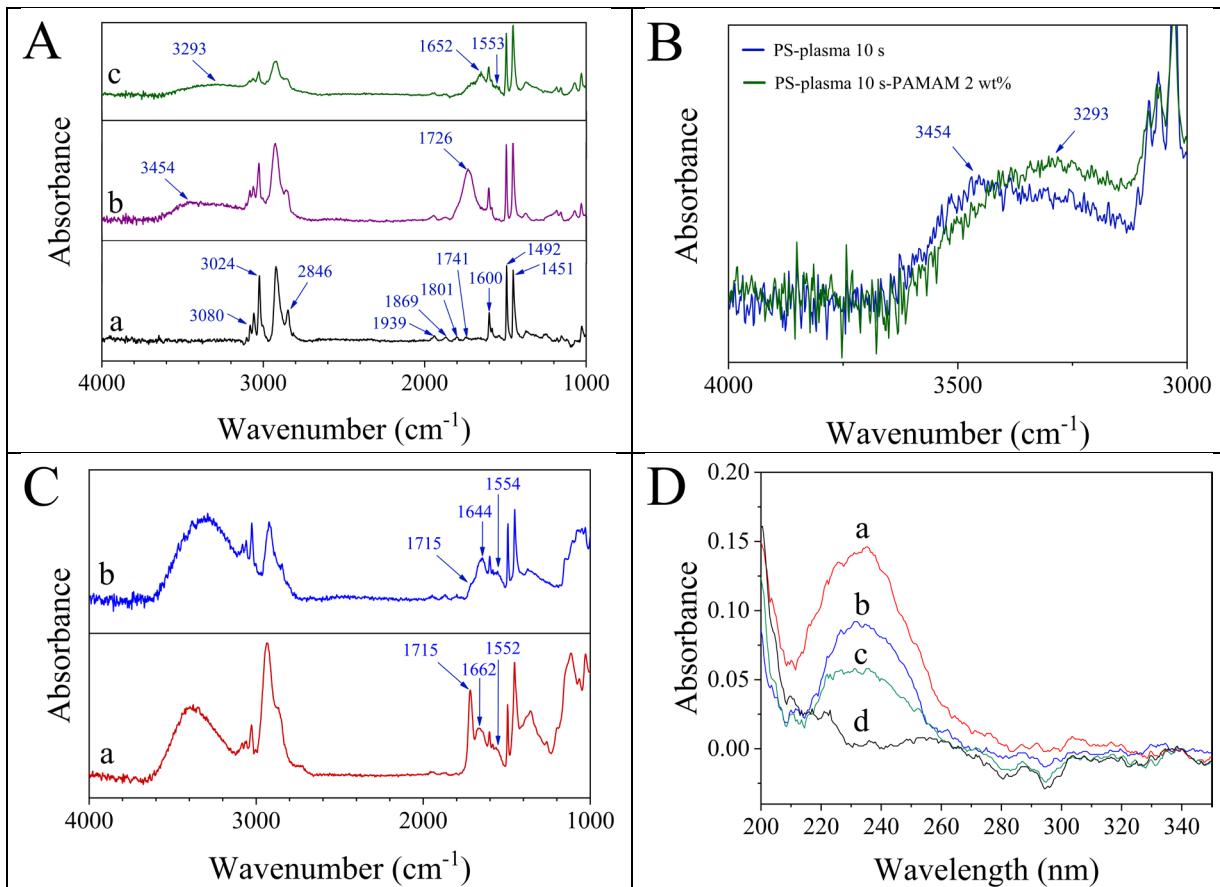
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676 Fig. 2. Fluorescence images of A) pure PS, B) PS-plasma 10 s, C) PS-plasma 10 s-PAMAM 0.5 wt.%, D)
 677 PS-plasma 10 s-PAMAM 1 wt.%, E) PS-plasma 10 s-PAMAM 2 wt.%, and F) quantification of
 678 fluorescent intensity of different samples (a) pure PS, (b) PS-plasma 10 s, (c) PS-plasma 10 s-PAMAM
 679 0.5 wt.%, (d) PS-plasma 10 s-PAMAM 1 wt.%, and (e) PS-plasma 10 s-PAMAM 2 wt.%.

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684 Fig. 3. A) ATR-FTIR spectra of (a) pure PS, (b) PS-plasma 10 s, and (c) PS-plasma 10 s-PAMAM 2
 685 wt.% ESFs, B) zoom at 3000-4000 cm⁻¹, C) (a) PS-plasma 10 s-PAMAM 2 wt.%-%glutaraldehyde and (b)
 686 PS-plasma 10 s-PAMAM-2 wt%-%glutaraldehyde-anti-IL-10, and D) UV-vis spectra of unreacted
 687 glutaraldehyde in (a) PS-plasma 10 s-PAMAM 0.5 wt%-%glutaraldehyde, (b) PS-plasma 10 s-PAMAM 1
 688 wt%-%glutaraldehyde, (c) PS-plasma 10 s-PAMAM 2 wt%-%glutaraldehyde, and (d) pure PS.

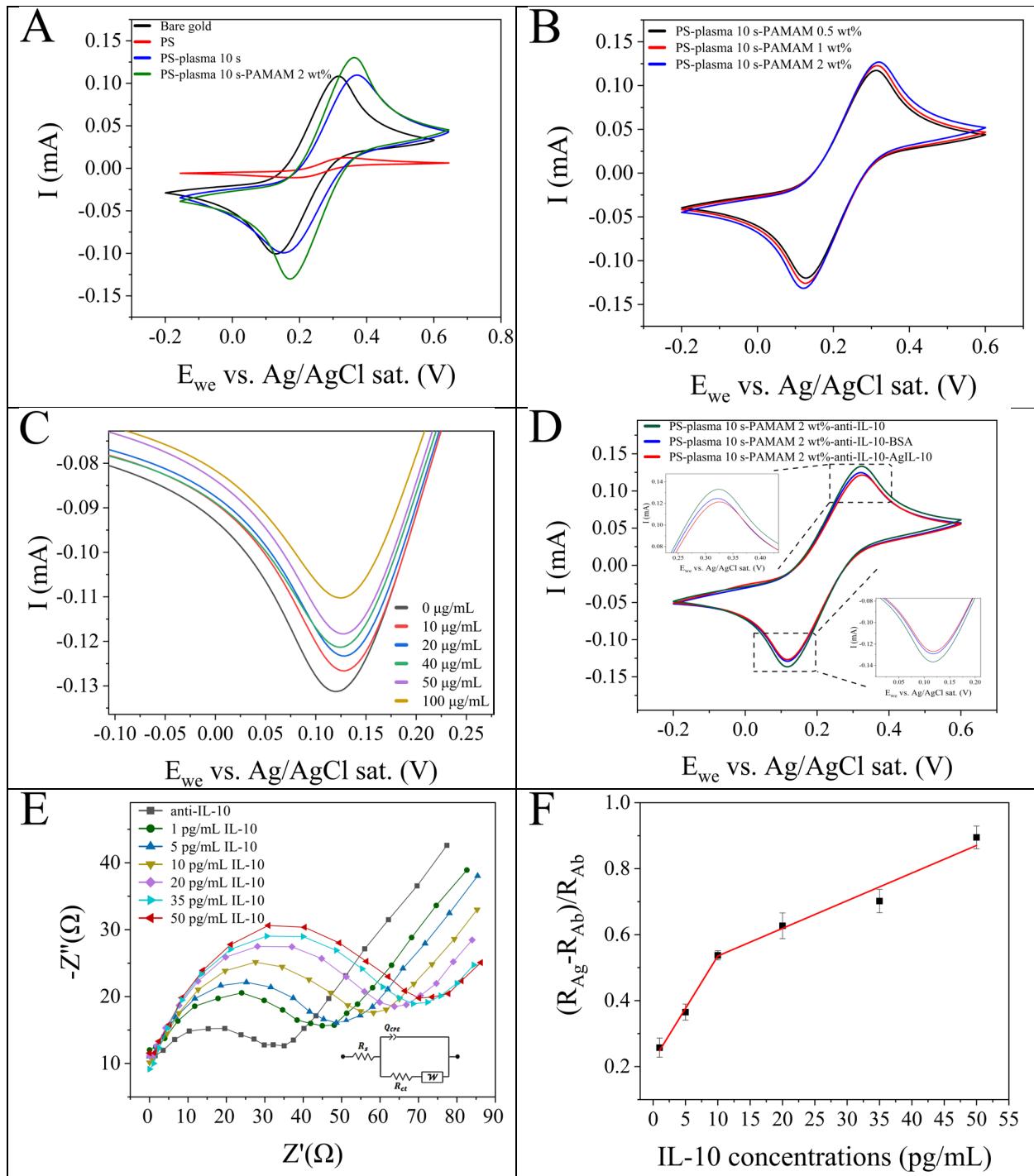
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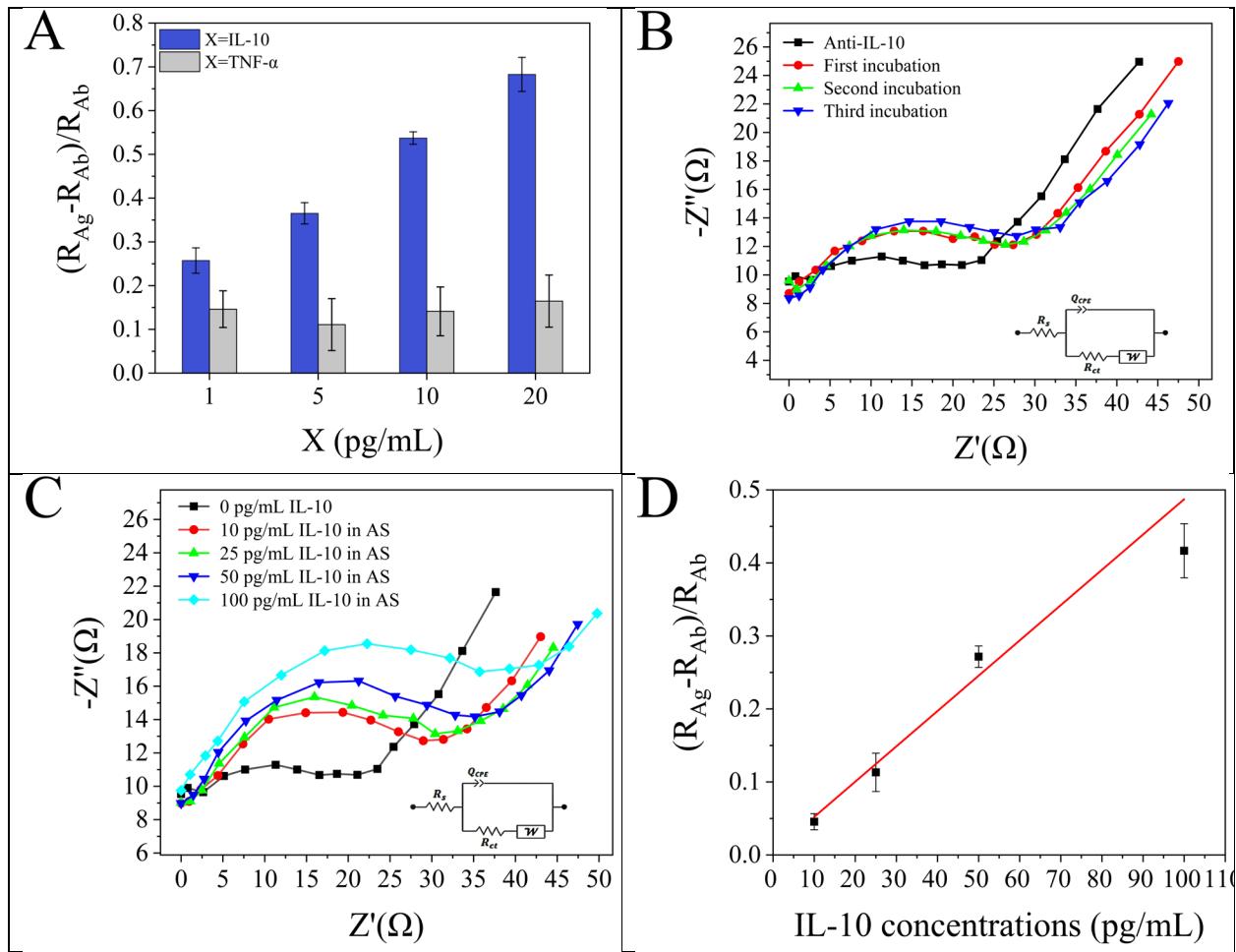
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Fig. 4. A) CVs of different stages of immunosensor preparation, B) CV of PS-plasma10 s-PAMAM with different concentrations electrode, C) effect of different immobilized anti-IL-10 concentrations (10, 20, 40, 50, and 100 µg/mL) on reducing the redox current, D) evaluation of anti-IL-10 activity (10 µg/mL) on the fiber surface, E) Nyquist plots of PS-plasma10 s-PAMAM 2 wt% glutaraldehyde-anti-IL-10 ESFs modified gold electrode of AgIL-10 detection ranging from 1 to 50 pg/mL, and F) calibration curves of the ESFs based immunosensors for the detection of AgIL-10.



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702 Fig. 5. A) Selectivity results of the developed immunosensors, B) Nyquist plots of three times incubation
 703 of AS (without AgIL-10) on PS-plasma 10 s-PAMAM 2 wt.% ESFs modified gold electrode, C)
 704 Nyquist plots of the immunosensor for AgIL-10 detection in AS, and D) detection calibration curves of the
 705 immunosensor toward AgIL-10 in AS.

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715 Table 1. Comparison of analytical characteristics of other immunosensors reported so far for IL-10 detection with the presented ESFs based
 716 immunosensor.

Immunosensors fundamentals	Development Method	Transduction technique	Linear Range	LOD	Ref.
Epoxy-functionalized mPDGs on a glass substrate	Immobilization	Fluorescence	0.25 µg/mL	-	[50]
HfO ₂ grown by ALD on silicon substrate	ALD	EIS	0.1–20 pg/mL	-	[5]
Diazonium modified gold microelectrodes	Soft lithography	EIS	1–15 pg/mL	-	[51]
Polypyrrole microwires (PPy-µWs)	Soft lithography	EIS	1–50 pg/mL	0.122 pg/mL	[52]
Antibody conjugated gold nanorods	Microfluidic patterning	LSPR	5–20 pg/mL	10.97 pg/mL	[53]
MHDA modified gold microelectrodes	Soft lithography	EIS	1–15 pg/mL	-	[54]
Diazonium modified gold microelectrodes	Soft lithography	EIS	1–15 pg/mL	0.3 pg/mL	[11]
Capture-end NPs modified PTFE membrane	Conjugation	SERS	0.01–100 pg/mL	0.1 pg/mL	[55]
Gold LSPR strip	Nanoimprinting lithography	LSPR	0.2–200 nM	-	[56]
Polypyrrole modified silicon nitride (Si ₃ N ₄)	Casting	EIS	1–10 pg/mL	0.347 pg/mL	[4]
Graphene interdigitated electrodes (IDEs)	Aerosol-jet-printed	EIS	0.1–2 ng/mL	46 pg/mL	[57]
One-dimensional photonic crystal (1DPC)	PIAD	BSW	250 pg/mL	110 pg/mL	[58]
PAMAM decorated plasma-treated electrospun PS fibers	Electrospinning	EIS	1–50 pg/mL	1 pg/mL	This work

717 LOD – limit of detection; mPDGs – magnetic poly (divinylbenzene-co-glycidyl methacrylate) colloidal particles; HfO₂ – hafnium oxide; ALD –
 718 atomic layer deposition; EIS – electrochemical impedance spectroscopy; LSPR – localized surface plasmon resonance; MHDA –
 719 mercaptohexadecanoic acid; NPs – nanoparticles; PTFE – polytetrafluoroethylene; SERS – surface-enhanced Raman spectroscopy; PIAD – plasma
 720 ion-assisted deposition technology; BSW – Bloch surface waves; PAMAM – polyamidoamine; PS – polystyrene.

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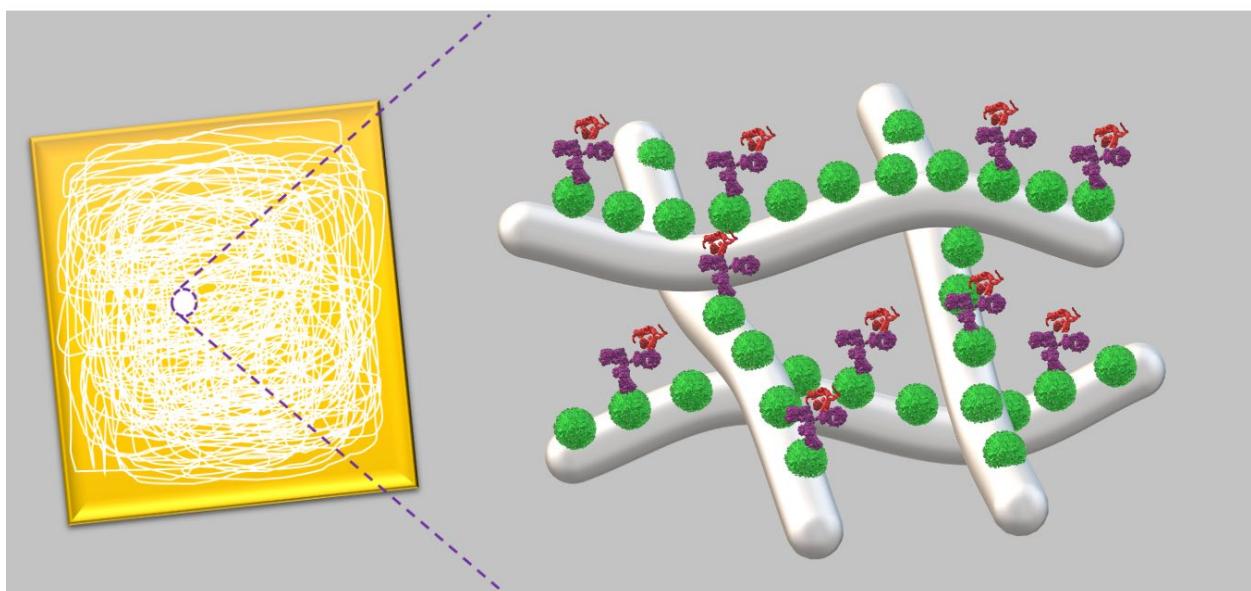
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