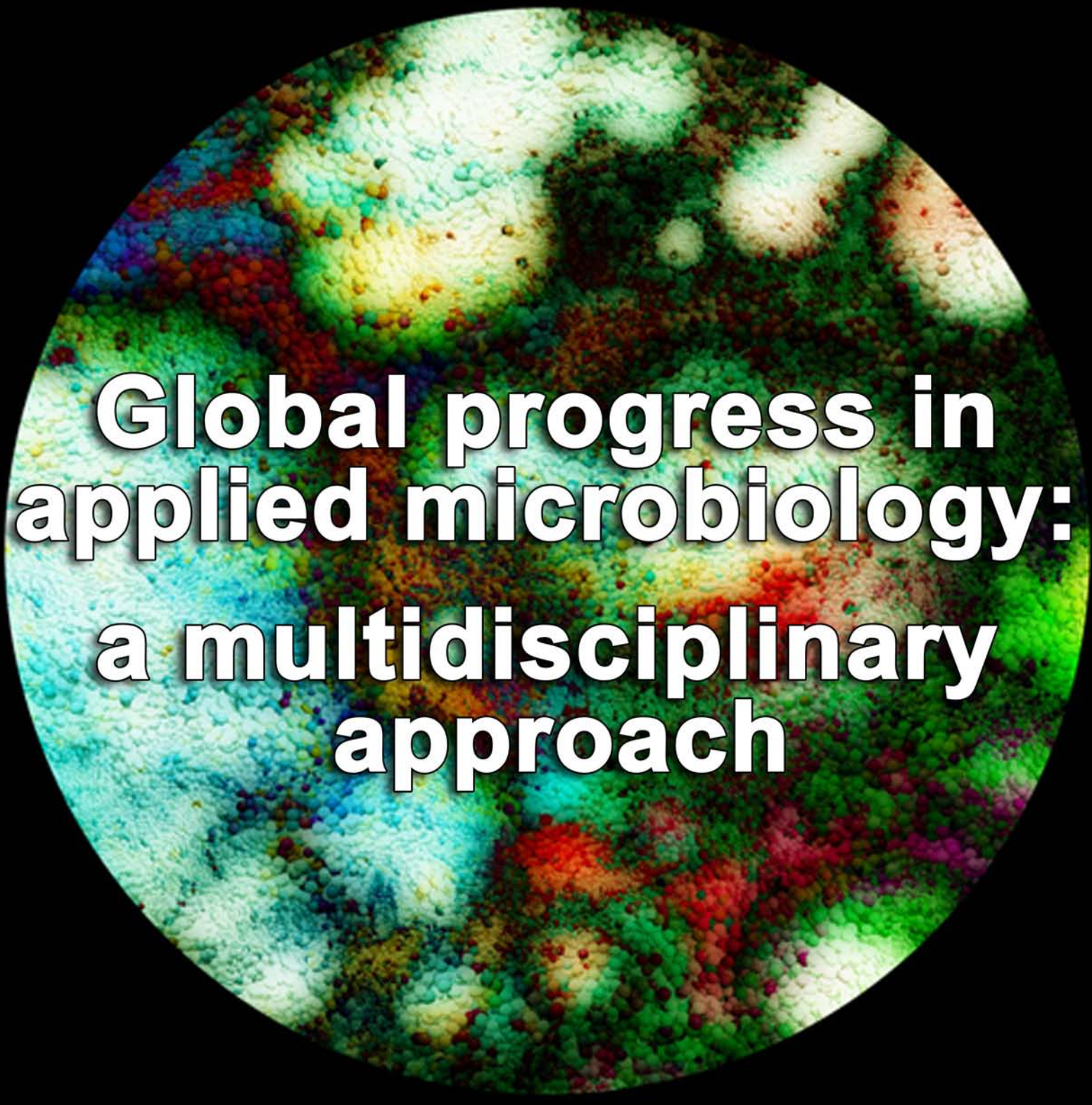

Global progress in applied microbiology: a multidisciplinary approach

A. Méndez-Vilas, Ed.

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Biofunctionalization of nylon nanofibers to be used in immunobiosensor for biological warfare agents detecting

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The use of biological warfare agents involves a growing threat to society**.** Thus, the most countries have been forced to increase resources in research for their detection and identification. One of the critical points in the effective fight against these agents is the development of devices that allow their detection and early identification. The best choice is the immunobiosensors easy to use on-site. However, how the antibody is attached to the biosensor surface, in terms of density and orientation, will determine the diagnosis capability of the device. In this study, both a functional nanofiber able to increase the surface / volume relation, and a chemically-similar planar membrane were used as support for the immobilization of antibodies. Different antibody immobilization systems were carried out to biofunctionalize both surfaces: passive adsorption, covalent bond by glutaraldehyde and well-oriented immobilization by protein A/G. Our results showed that nanofibers in combination with protein A/G were a very effective immunocapture system for being used in a biosensor.

Keywords immunobiosensor; nanofibers; biofunctionalization; biological agent warfare.

1. Introduction

Biological warfare agents involve a wide range of risk since they can be deliberately directed not only at human population but also at livestock and crops [1], causing effects both on human health (mortality/morbidity/incapacity) and on the economy (failed harvest, death of livestock, health and safety investment) [2]. Therefore, much effort has been devoted to the research, design and development of new technologies for their detection and identification. New low-cost sensor devices that allow their detection and early identification are very significant in the effective fight against these agents. The need to carry out analytical determinations with specific devices, easy to use and low cost has led to the development of immunobiosensors [3]. The specificity of antigen-antibody binding is used in a multitude of biosensors like biological warfare-agent detection equipment. However, how the antibody is attached to the biosensor surface, in terms of density, orientation and stability, will determine the diagnosis capability of the device [4]. In this line, both the properties of the anchoring surface and the antibody immobilization method are crucial in the effectiveness of immunocapture system. The use of nanofibers manufactured by electrospinning is currently in expansion for the sensors design and development due to their high surface-to-volume ratio and nanofiber diameter customization [5]. In the present work, authors carried out a study of different antibody immobilization methods using eletrospinning nanofibers as anchoring surfaces and conventional surface for the comparison of their effectiveness.

2. Aim

We have carried out a study of different antibody immobilization methods using different surfaces.

3. Material and methods

3.1 Materials

The selected surfaces used in this study were two chemically-similar surfaces, made of nylon, which is widely used to retain biomolecules such as proteins. The first one was a planar surface, the commercial Zprobe (Bio-Rad Laboratories, S.A). The second one was specifically manufactured by authors to increase the areal surface of the substrate. It was made by electrospinning and the composition of the ultra-thin nanofibers (NFs) was

polyamide 6, PA 6, purchased from BASF (Ultramid® B24 N 03). Solvents and additives was purchased form Aldrich (Spain).

Bovine serum albumin (BSA, from Sigma Aldrich) labelled with R-phycoerytrin was selected as toxin surrogate. Fluorescein (FITC)-labelled sheep polyclonal antibody (Thermo Fisher Scientific Inc.) against BSA were immobilized onto the selected surfaces. The blocking buffer used was a phosphate buffered saline (PBS) with casein (Pierce).

3.2 Nanofiber manufacture

The electrospinning solutions were prepared by solving 6 wt.%. of PA6 in Acetic Acid: Formic Acid, 2:1, and the subsequent addition of 5 wt.%. of Pyridine. The electrospinning setup consists in a needle free equipment form Nanospider^{TM} 500 from El Marco (Fig. 1a). The polymeric solution was place in the tank, where the roller spinning electrode is immersed. The roller spinning electrode consists in four metallic wires. The electrospinning process (Fig 1b), consists in the jet formation from a solution when the applied voltage overcomes the solution surface tension, causing a cone-shaped deformation of the solution drops. During the jet flight movement from the spinning electrode to the ground electrode, the fibers were stretched by the extremely high electrostatic forces. NFs were collected in solid stated, solvents are evaporated during NFs flight, in the ground electrode covered by an antistatic substrate and located in the top of the electrospinning apparatus. The overall NF formation process occurs in a sort period with stretching rates up to 10^5 s^{-1} [6].

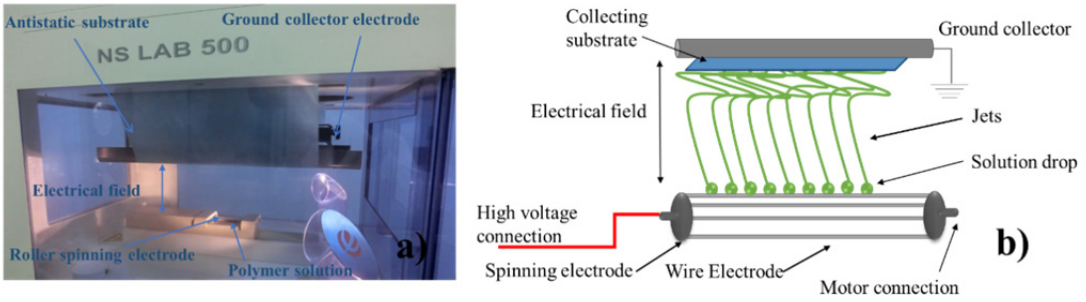

Fig 1. Needle free electrospinning setup Nanospider ^{TM} (a) and electrospinning process and NFs formation scheme (b).

PA 6 nanofibres were electrospun into samples of 25 mm^2 on one side polished, boron doped, silicon wafer pieces to avoid substrates roughness interference in the nanofibers mesh formation. The silicon wafers were located in the middle of the deposition area on the antistatic substrate. The electrospinning parameters were 65 kV as applied voltage and 170 mm as the distance between roller electrode and the ground one. The ambient conditions were 19 ± 2 ºC and 40 ± 5 % RH and the collecting time was 40 min, in order to achieve the suitable NF layer thickness

3.3 Biofunctionalisation methods

The immunocapture protocol used in this study consisted in a fluorescein-labelled antibody immobilized on the surface. After blocking step with PBS-casein, RPE-labelled BSA was immunocapture by the anchored antibodies. Slices of 5x5 mm of each kind of membrane were put into the multi-well black plate, the immunocapture protocol was carried out there and fluorescence (from both antibody and BSA) measured. Three biofunctionalisation methods were used as described below.

- Passive adsorption: Antibody (10 µL 100 µg/mL) was incubated on each surface in phosphate buffer saline (PBS 1x) over night (o/n).
- Well-oriented immobilization by the intermediate protein A/G: Protein A/G (10 µL 100 µg/mL) was incubated on each surface in PBS 1x o/n. Then, antibody was incubated for 1 hour.
- Covalent bond: Surfaces were treated with glutaraldehyde. Then, antibody (10 µL 100 µg/mL) was incubated on each surface in PBS 1x o/n. It is important to notice that this method was combined with both passive adsorption and Protein A/G.

3.4 Studies

3.4.1 First study: comparison of antibody immobilization method

Fluorescence from both antibody and BSA was measured by a microplate fluorescence reader (SpectraMaxGemini XPS, Molecular Devices). FITC measurement allows us to determine the retained antibody density. RPE measurement allows us to calculate immunocaptured BSA. The ratio RPE-BSA / FITC-immobilized antibody allows us to know how the effective the immunocaptured system is. Data from each

surface (nanofiber or Z probe) were represented and statistically analysed separately. Results were expressed as a percentage of its respective "passive adsorption without glutraldehyde" and statistically compared by One Way ANOVA followed by Tukey's Multiple Comparison test.

3.4.2 Second study: analysis of saturation surfaces.

Different antibody concentrations were assayed in both surfaces (10 µL of both 50 and 100 µg/mL: 500 ng and 1 µg respectively) using the following immobilization methods: passive adsorption without glutaraldehyde, considered as control group, and the selected method according to the results obtained in the first study.

Both density of the immobilized labelled antibody and immunocaptured labelled BSA were obtained (see 3.4.1). For each immobilization method, results were expressed as a percentage of the value obtained from the corresponding antibody 50 µg/mL group (nanofibers or Zprobe). T-student tests were carried out to compare the results from the antibody 100 µg/mL group obtained from nanofibers and Zprobe.

4. Results

4.1 Morphological characterization of the nanofibers (NFs)

The morphology of PA6 electrospun NFs layer was examined using a Zeiss Ultra 55 Field Emission Scanning Electron Microscope (FESEM) to verify the beds defects absence, irregularities or small droplets from the preparation process, as well as, the homogeneity of the NFs deposition before the immobilization of the antibody-antigen. The average diameter of the NFs was calculated using Image-J image analysis program and the number of measurements used to estimate the average diameter of the NFs and their distribution were near 100 measurements at different areas of the FESEM images, (Fig 2). The average diameter of PA 6 nanofibers was 23 nm and its standard deviation ± 5.8 nm. The very ultrathin nature of the PA 6 electrospun nanofibers is due to the addition of the 5 wt.% of pyridine as has been demonstrated in other polyamide 4,6 systems [7]. Pyridine was eliminated by evaporation during the electrospinning manufacturing process, avoiding their presence in the final substrate before their functionalization.

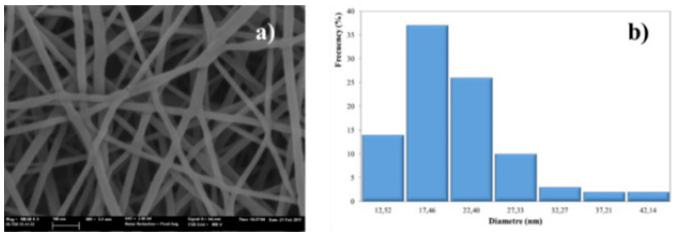

Fig. 2. FESEM images of PA 6 with 5 wt.% of Pyridine electrospinning porous layer (a) and nanofiber diameter distribution as obtained from Image-J software images analysis (b).

4.2 First study: Comparison of Antibody Immobilization methods

No statistical differences were observed between the assayed immobilization methods when nanofibers were used. However, it can be seen a slight and opposite effect with glutaraldehyde: an increasing tendency was observed when passive adsorption was combined with glutaraldehyde while a decreasing tendency was seen with protein A/G in combination with glutaraldehyde. This trend resulted to be statistically significant when the planar surface Z probe was used (Fig. 3a). Regarding the capability of immunocapture BSA, protein A/G without glutaraldehyde achieved the best results in both surfaces (Fig 3b), resulting to be the statistically more effective immunocapture system (Fig.4).

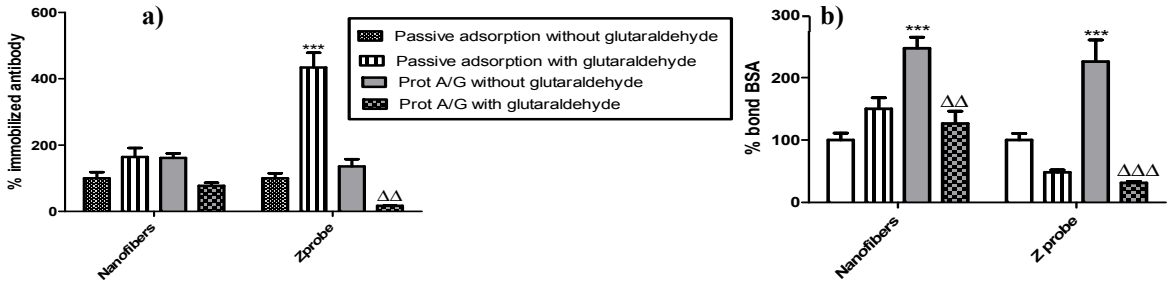

Fig. 3. a) Density of immobilized antibody. b) Immunocaptured BSA. Results showed as a percentage of its respective "passive adsorption without glutaraldehyde" group. *** P<0,001 differences from its respective "passive adsorption without glutaraldehyde" ; ΔΔΔ p > 0,01; ΔΔ p< 0,01 differences from protein A/G (Prot A/G) without glutaraldehyde (One Way ANOVA, followed by Tukey's Multiple Comparison test, carried out

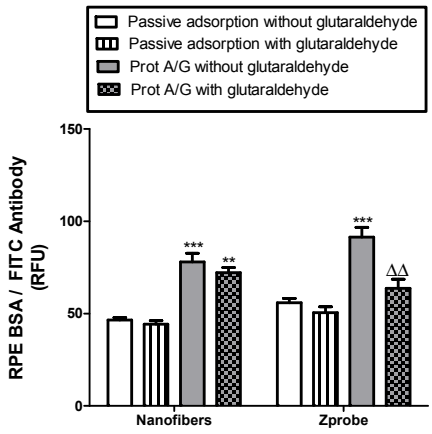

Fig. 4. Data from each surface (nanofiber or Z probe) were statistically analysed separately by One Way ANOVA, followed by Tukey's Multiple Comparison test. *** P<0,001 differences from its respective "passive adsorption without glutaraldehyde" ΔΔΔ p > 0,01; ΔΔ p< 0,01 differences from its respective "Prot A/G without glutaraldehyde".

4.3. Second study: saturation surface study

Increasing antibody concentration led to a higher density of immobilized antibody in nanofibers than in the Zprobe planar surface (fig. 5a). This effect was more striking, and statistically significant, when antibody retention was carried out with protein A/G (fig. 5b).

According to these results, even though both surfaces (nanofibers and Zprobe) tendered to inmuno-capture more BSA with the highest antibody concentration, this increase was only statistically significant with the nanofibers in both surfaces. When compared the BSA immunocapture capability of both biofunctionalised surfaces using the highest antibody concentration (100 µg/mL), nanofibers captured more BSA, being further striking with these nanofibers combined with protein A/G (Fig 6a and 6b).

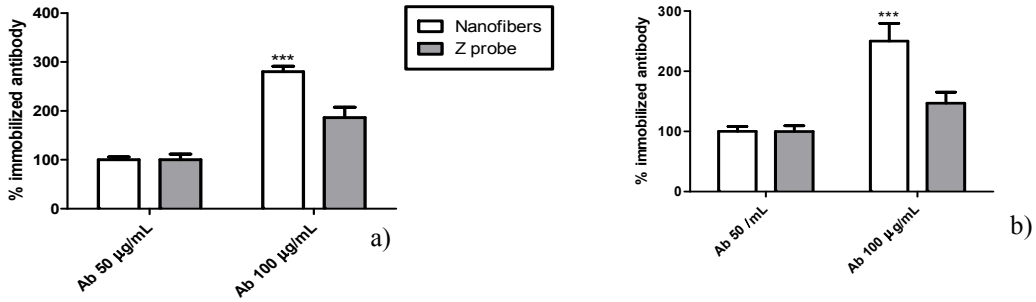

Fig. 5. Antibody immobilization methods: a) Passive adsorption, b) Protein A/G. Results were expressed as the percentage of the value obtained from the corresponding antibody (Ab) 50 µg/mL group (nanofibers or Zprobe). T-student tests were carried out to compare the results from the antibody (Ab) 100 µg group obtained from nanofibers and Zprobe: *** P<0,001 difference from Zprobe Antibody 100 µg/mL

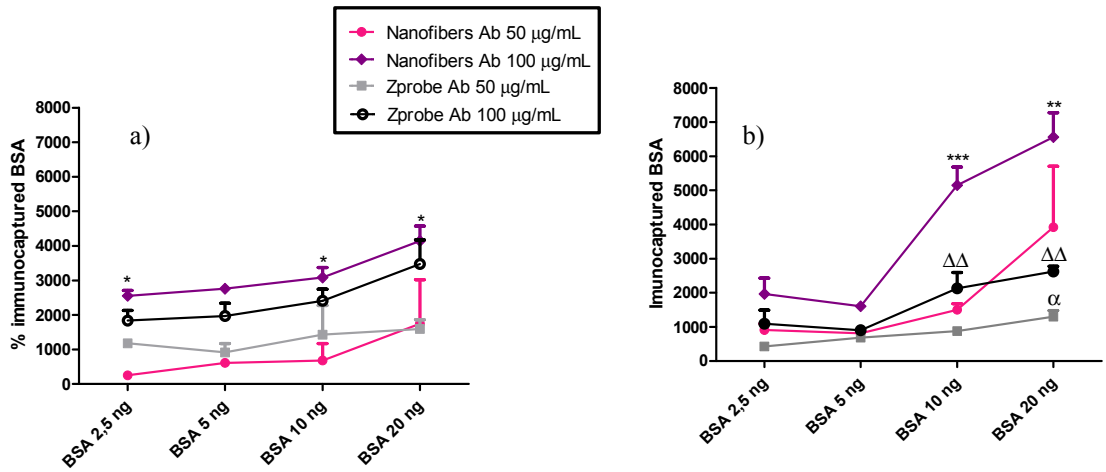

Fig 6. Measure of Immunocaptured BSA. Passive adsorption (a); Protein A/G (b). Two Way ANOVA with a Bonferroni post test * p< 0,05; ** p< 0,01; *** p>0,001 Difference from the corresponding surface using antibody 50 µg/mL. ΔΔ p<0,01. Difference from nanofibers 100 µg/mL. α p< 0,05. Difference from nanofibers 50 µg/mL.

5. Discussion and conclusions

Not only the biofunctionalised method but also the antibody-attached surface resulted to be crucial in the design of an effective immunobiosensor.

With regards to the surface, smaller nanofibers diameters, 23nm, provide higher surface area for the same sample volume and, hence, higher surface area to immobilize antibodies unoccupied for the immunocapture of a higher number of antigens, with the consequents sensibility increase. The very ultrathin nature of the PA 6 electrospun NFs is due to the addition of the 5 wt.% of pyridine. Many salts soluble in acetic acid or /and formic acid can be used to increase the conductivity of the solutions; nevertheless, pyridine increases the solution conductivity by its reaction with formic acid without affects to the surface tension and induces a very small increase of the solution viscosity. An additional advantage of the pyridine use is its elimination by evaporation during the electrospinning manufacturing process.

Regarding to the biofunctionalised method, even though the glutaraldehyde tends to retain more antibody in combination with passive adsorption, over all in Zprobe membrane, it proves to be the less effective system since it showed a worse BSA immunocapture capability. Since glutaraldehyde is very reactive with pH over 6, it could lead to change in the antigenic recognition of the antibodies [8]. Instead, protein A/G without glutaraldehyde was the most able to immunocapture BSA, in spite of not being the most able to retain antibody, in both nanofibers and Zprobe. It appears to be due to the well-orientation of the immobilized antibody, as demonstrated in previous works [9].

With regards to the behavior of both surfaces in anchoring antibodies, even though both surfaces tendered to inmuno-capture more BSA with the highest antibody concentration, this increase was only statistically significant with the NFs. When compared the BSA immunocapture capability of both biofunctionalised surfaces using the highest antibody concentration (100 µg/mL), NFs captured more BSA, being further striking with these NFs combined with protein A/G (Fig 4a and 4b). It seems that a saturation effect is happening in the planar surface and not in NFs. In this sense, the small nanofibers diameters provide a higher surface area for the same sample volume, allowing to immunocapture a higher number of antigens, with the consequents sensibility increase.

To sum up, nanofibers in combination with protein A/G were found to be the more effective system for being used in a biosensor since it was able to retain, in the same area than a planar surface, more amount of well-oriented antibody which results in a very effective immunocapture system.

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