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Publication Date	09 Oct 2023
Article Type	Full Research Paper
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STUDY OF THE REUSABILITY AND STABILITY OF NYLON NANOFIBRES AS AN ANTIBODY IMMOBILISATION SURFACE

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Abstract

In the case of a biological threat, early, rapid and specific detection is critical. In addition, ease of handling, use in the field and low-cost production are important considerations. Immunological devices are able to respond to these needs. In the design of these immunological devices, surface antibody immobilisation is crucial. Nylon nanofibres have been described as a very good option because they allow an increase in the surface-to-volume ratio, leading to an increase in immunocapture efficiency. In this paper, we want to deepen the study of other key points, such as the reuse and stability of these nanofibres, in order to assess their profitability. On the one hand, the re-use of nanofibres has been studied using different stripping treatments based on different pH on the nylon nanofibres with well-oriented antibodies anchored by protein A/G. Our study shows that stripping with glycine buffer, pH 2.5, allows nanofibres to be reused as long as protein A/G is previously anchored, leaving both nanofibre and protein A/G unchanged. On the other hand, we investigated the stability of nylon nanofibres. To achieve this, we analysed any loss of immunocapture ability of well-oriented antibodies anchored both to the nylon nanofibres and to a specialised surface with high protein binding capacity. This immunocapture system maintained its immunocapture ability unchanged for a longer time than a planar specialised surface. In conclusion, nylon nanofibres seem to be a very good choice as an antibody immobilisation surface, offering not only higher immunocapture efficiency, but also more cost efficiency as they are reusable and stable.

Keywords

biothreat; biosensor, nanofibre, nylon, immunodetection.

Introduction

Biological threat involves a wide range of risks that attack not only the human population, but also at livestock and crops [1], causing effects on both human health (mortality/morbidity/incapacity) and the economy (crop failures, livestock deaths, investments in health and safety) [2]. For this reason, early, rapid and specific detection of a biological threat becomes a very important objective in order to be able to react as early as possible. Many efforts have been made in this direction. When designing a new sensor device, not only the rapid and specific identification has to be taken into account, but also the ease of handling, on-site use and low cost production.

In this regard, immunodetection appears to be a very good option [3]. The specificity of antigen-antibody binding and 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]. Thus, immobilization surface of the device is one of the key points in the development of new sensors.

Nylon has been used as an immobilisation surface in numerous applications, such as the immobilisation of enzymes and microorganisms [5, 6] and the immobilisation of antibodies in enzyme immunoassays [7]. Thus, nylon nanofibre has been used as an immobilisation surface in biosensors [8]. In this sense, efficiency studies of nanofibres manufactured by electrospinning have been carried out in our laboratory, determining the optimal nanofibre thickness in terms of stability and biofunctionalisation [9]. Our results showed that the nylon nanofibre surface provides advantages over the planar nylon surface in terms of increased immunocapture efficiency, as the higher surface area/volume ratio in the nanofibre allows for a greater amount of immobilised antibody in the same designed space [10, 11]. In addition, some studies demonstrate the suitability of electrospun nylon nanofibres for the development of Fabry-Pérot (FP)-based optical biosensors [12, 13]. On the other hand, for the selection of these nanofibres as immobilisation surfaces in biosensors, it seems necessary to study those characteristics of the immobilisation surface that contribute to their lower cost.

In this sense, this paper not only investigates the reuse of nylon nanofibres, but also whether this immobilisation surface provides a longer life for the immunocapture system. These characteristics are key points to choose a more cost effective and environmentally friendly immobilisation surface.

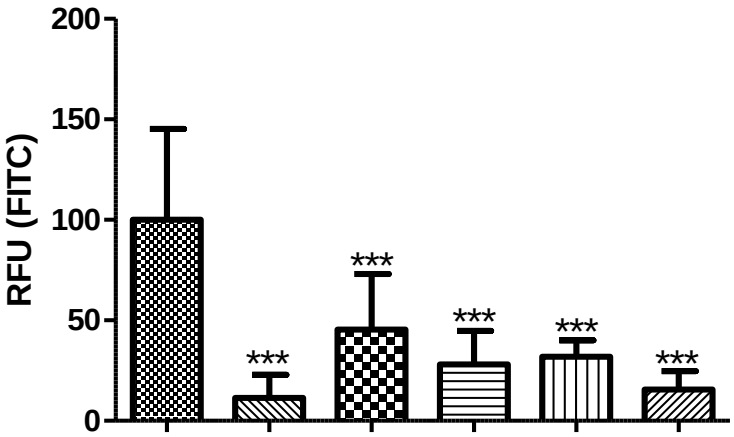
Results and Discussion

Nanofibre reusing study

Commercial Ag/Ac elution buffer pH 6.6 with high salinity was able to remove almost all the antibody fixed by these nanofibres through A/G protein (88.6 % compared to the total antibody fixed in the reference group (group 1), Figure 1, group 1 versus group 2), as indicated by the FITC-fluorescence associated with the antibody (index explained in Experimental section).

When the immunocapture system was reconstituted after the stripping procedure, only 45 % of the total bound antibody was found compared to the total antibody fixed in the reference group (Figure 1, group 1 versus group 3), suggesting that antibody binding was altered by this buffer. In this sense, the same results were obtained in the reconstituted immunocapture system, whether BSA alone or antibody plus BSA were reapplied after the stripping process (28.1 % and 31.9 % compared to the total antibody fixed in the reference group, respectively, Figure 1, groups 4 and 5 versus group 1). In addition, bare nanofibres (nanofibres that underwent the stripping process without first binding the immunocapture system) were damaged in the same way, to the extent that they were unable to bind the immunocapture system (15.5 % compared to the total antibody fixed in the reference group) (Figure 1, group 6 versus group 1).

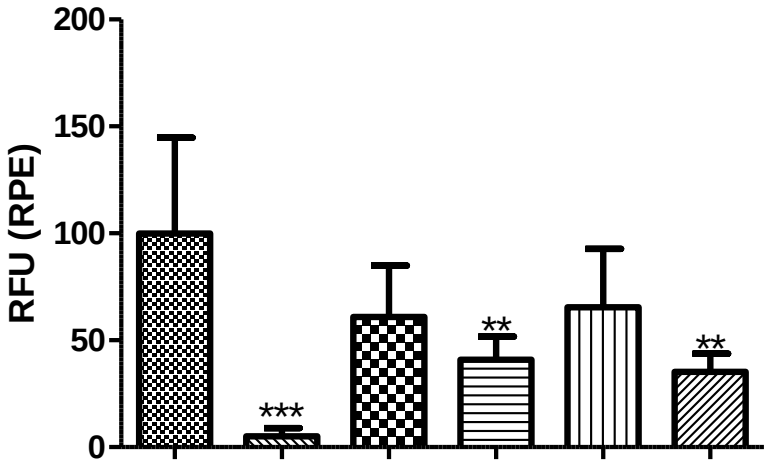
Therefore, it seems that Commercial Ag/Ac elution buffer pH 6.6 with high salt content damages the nylon nanofibre by altering its immunocapture ability.



	Groups 1	2	3	4	5	6
Original immunocapture system						
Protein A/G	+	+	+	+	+	-
Antibody	+	+	+	+	-	-
BSA	+	+	+	-	-	-
Stripping with high salinity pH 6,6	**-**	**+**	**+**	**+**	**+**	**+**
Reconstructed immunocapture system						
Protein A/G	-	-	+	-	-	+
Antibody	-	-	+	-	+	+
BSA	-	-	+	+	+	+
Percentage from original system						
Mean	100	11.4	45.4	28.1	31.9	15.5
SEM	18.4	5.1	13.9	7.4	3.7	4.1

Figure 1: FITC fluorescence due to anti-BSA antibody. Data are expressed as a percentage of the antibody fluorescence of the original immunocapture system (group 1, reference group), n = 5-6. Stripping treatment with commercial Ag/Ac elution buffer pH 6.6 was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman-Keuls test. Difference from original immunocapture system fluorescence (group 1, reference group): ***$p < 0.001$.

Regarding BSA immunocapture, the results showed some unspecific BSA binding to the altered nanofibres after stripping treatment, as the bound antibody was lower than the immunocaptured BSA (45.4 % of bound antibody and 60.9 % of BSA immunocaptured, both compared to the values obtained with the initial immunocaptured system (reference group)) (Figures 1 and 2, group 1 versus group 3). Similar results were obtained in the reconstituted immunocapture system when BSA alone or antibody plus BSA were administered after stripping. While 28.1 % and 31.9 % of bound antibody was found respectively, 40.9 % and 65.5% of immunocaptured BSA was detected (Figures 1 and 2, groups 4 and 5 versus group 1). Furthermore, after buffer treatment, the bare nanofibre was only able to bind 15.5 % of the total antibody initially bound (reference group), whereas 35.3 % of the BSA was immunocaptured compared to that bound in the initial system (reference group) (Figure 1 and 2, group 6 versus. group 1).



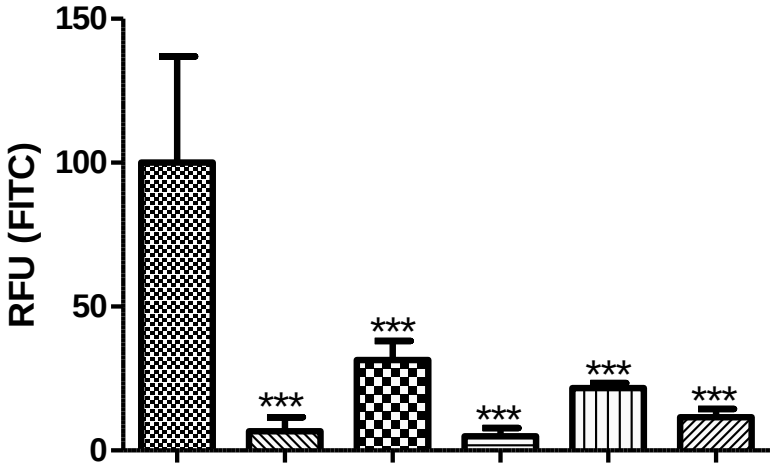
Groups	1	2	3	4	5	6
Original immunocapture system						
Protein A/G	+	+	+	+	+	-
Antibody	+	+	+	+	-	-
BSA	+	+	+	-	-	-
Stripping with high salinity pH6,6	**-**	**+**	**+**	**+**	**+**	**+**
Rebuilt immunocapture system						
Protein A/G	-	-	+	-	-	+
Antibody	-	-	+	-	+	+
BSA	-	-	+	+	+	+
Percentage from original immunocapture system						
Mean	100	5.1	60.9	40.9	65.5	35.3
SEM	25.8	1.9	10.8	4.9	13.6	4.2

Figure 2: RPE fluorescence due to immunocaptured BSA. Data are expressed as a percentage of the immunocaptured BSA by the complete immunocapture system (group 1, reference group), n = 5-6. Stripping treatment with commercial Ag/Ac elution buffer pH 6.6 was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman-Keuls test. Difference from original immunocapture system fluorescence (group 1, reference group): ***$p < 0.001$, **$p < 0.01$.

The pH11 ammonium hydroxide buffer gave similar results to the commercial pH 6.6 high salt Ag/Ac elution buffer (Figure 3). Thus, although this treatment was able to remove almost all of the antibody captured by these nanofibres via the A/G protein (93.4 % compared to the total antibody bound in the reference group, Figure 3, group 1 versus group 2), it interfered with the reconstituted immunocapture system to such an extent that only 31.5% of captured antibody was detected in the reconstruction process, compared to the initial bound antibody (Figure 3, group 1 versus group 3). When bare nanofibre was treated with this ammonium buffer, almost no antibody was bound (only 11.6 % compared to the total antibody fixed in the reference group; Figure 3, group 6 versus group 1).

As with the commercial pH 6.6 high salt Ag/Ac elution buffer, non-specific binding of BSA was observed, as more immunocaptured BSA was detected than bound antibody (31.5 % bound antibody and 50.3 % immunocaptured BSA, both compared to the values obtained with the reference immunocapture system) (Figures 3 and 4, group 1 versus group 3). In this line, in the reconstituted immunocapture system with BSA only or antibody only plus BSA, reapplied again after stripping process, while 4.9 % and 21.6 % of bound antibody was found respectively, 21.6 % and 53.1% of immunocaptured BSA was detected (Figures 3 and 4, groups 4 and 5 versus group 1). Furthermore, after buffer treatment, the bare nanofibres were able to bind only 11.6 % of the total antibody and immunocaptured 36 % of the BSA, compared to the reference group (Figures 3 and 4, group 6 versus group 1).

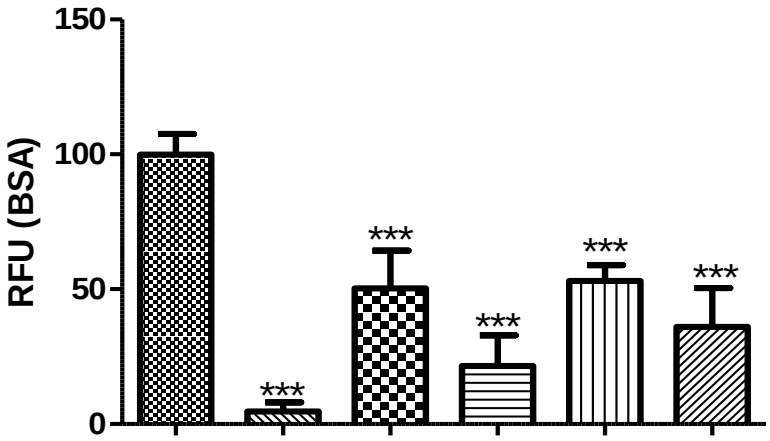
Thus, both ammonium hydroxide and commercial elution buffer had a detrimental effect on the nylon nanofibres, so neither of these well-known solutions should be used as stripping buffers with these nanofibres.



Original immunocapture system						
Protein A/G	+	+	+	+	+	-
Antibody	+	+	+	+	-	-
BSA	+	+	+	-	-	-
Stripping buffer ammonium pH 11	**-**	**+**	**+**	**+**	**+**	**+**
Rebuilt immunocapture system						
Protein A/G	-	-	+	-	-	+
Antibody	-	-	+	-	+	+
BSA	-	-	+	+	+	+

Percentage from original immunocapture system						
Mean	100	6.6	31.5	4.9	21.6	11.6
SEM	13.9	2.2	2.9	1.3	0.9	1.3

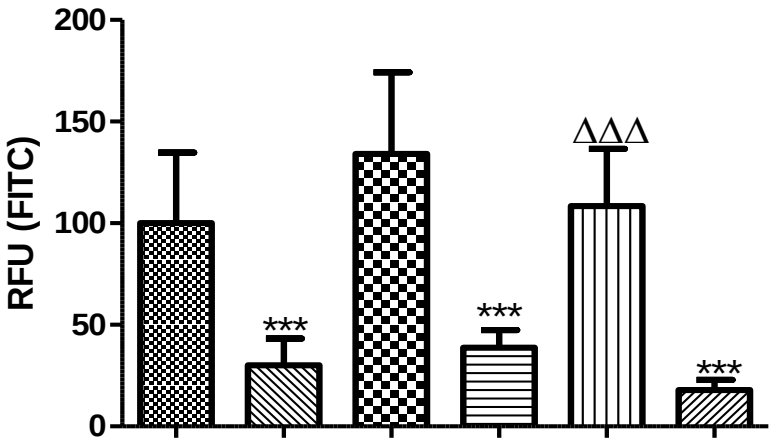
Figure 3: FITC fluorescence due to anti-BSA antibody. Data are expressed as a percentage of the antibody fluorescence of the original immunocapture system (group 1, reference group), n = 5-6. Stripping treatment with pH 11 ammonium hydroxide buffer was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman-Keuls test. Difference from original immunocapture system fluorescence (group 1, reference group): ***$p < 0.001$.



Groups	1	2	3	4	5	6
Original immunocapture system						
Protein A/G	+	+	+	+	+	-
Antibody	+	+	+	+	-	-
BSA	+	+	+	-	-	-
Stripping buffer ammonium pH 11	**-**	**+**	**+**	**+**	**+**	**+**
Reconstructed immunocapture system						
Protein A/G	-	-	+	-	-	+
Antibody	-	-	+	-	+	+
BSA	-	-	+	+	+	+
Percentage from original immunocapture system						
Mean	100	4.7	50.3	21.6	53.1	36
SEM	4.3	1.5	6.3	5.6	3.4	6.4

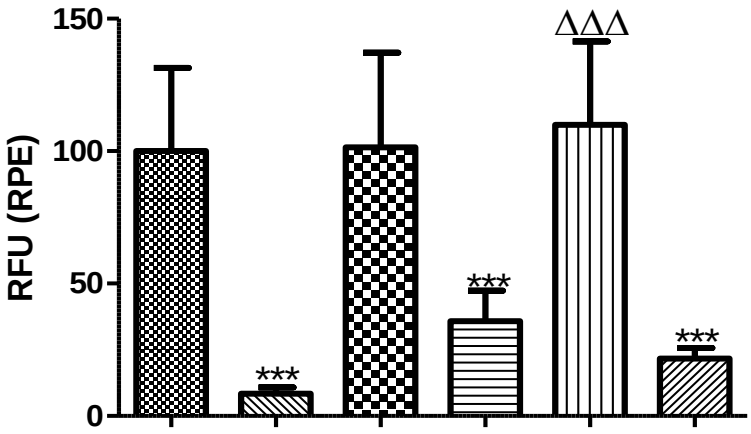
Figure 4: RPE fluorescence due to immunocaptured BSA. Data are expressed as a percentage of the immunocaptured BSA by the complete immunocapture system (group 1, reference group), n = 5-6. Stripping treatment with pH 11 ammonium hydroxide buffer was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman-Keuls test. Difference from original immunocapture system fluorescence (group 1, reference group): *** $p < 0.001$.

In contrast, buffer containing glycine pH 2.5 was able to remove the 70 % of the total fixed antibody (Figure 5, group 1 versus group 2) but, when the immunocapture system was rebuilt again after stripping procedure, the amount of bound antibody was the same as the total antibody binding before stripping (Figure 5, group 1 versus group 3). It was also consistent with the BSA immunocapture results, as the reconstituted immunocapture system was able to bind the same amount of BSA as the immunocapture system before stripping (reference group) (Figure 6, group 1 versus group 3).



Groups	1	2	3	4	5	6
Original immunocapture system						
Protein A/G	+	+	+	+	+	-
Antibody	+	+	+	+	-	-
BSA	+	+	+	-	-	-
Stripping with buffer glycine pH 2,5	**-**	**+**	**+**	**+**	**+**	**+**
Rebuilt immunocapture system						
Protein A/G	-	-	+	-	-	+
Antibody	-	-	+	-	+	+
BSA	-	-	+	+	+	+
Percentage from original immunocapture system						
Mean	100	30	134	38.7	108.4	17.9
SEM	11	5.9	18	3.9	12.6	2.2

Figure 5: FITC fluorescence due to anti-BSA antibody. Data are expressed as a percentage of the antibody fluorescence of the original immunocapture system (group 1, reference group), n = 5-6. Stripping treatment with pH 2.5 glycine buffer was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman-Keuls test. Difference from original immunocapture system fluorescence (group 1, reference group): *** $p < 0.001$. Difference from bare nanofiber suffering stripping treatment and, then, constructed the immunocaptured system (group 6): Δ Δ Δ $p < 0.001$.



Groups	1	2	3	4	5	6
Original immunocapture system						
Protein A/G	+	+	+	+	+	-
Antibody	+	+	+	+	-	-
BSA	+	+	+	-	-	-
Stripping with buffer glycine pH 2,5	**-**	**+**	**+**	**+**	**+**	**+**
Reconstructed immunocapture system						
Protein A/G	-	-	+	-	-	+
Antibody	-	-	+	-	+	+
BSA	-	-	+	+	+	+

Percentage from original immunocapture system						
Mean	100	8.5	101.4	35.9	110	21.69
SEM	14.1	1.1	16	5.12	14.01	1.8

Figure 6: RPE fluorescence due to immunocaptured BSA. Data are expressed as a percentage of the immunocaptured BSA by the complete immunocapture system (group 1, reference group, n = 5-6. Stripping treatment with pH 2.5 glycine buffer was performed in all groups except group 1, which was used as the reference in the statistical analysis. One-way ANOVA followed by Newman-Keuls test. Difference from original immunocapture system fluorescence (group 1, reference group): ***$p < 0.001$. Difference from bare nanofiber suffering stripping treatment and, then, constructed the immunocaptured system (group 6): ΔΔΔ$p<0.001$.

After glycine pH 2.5 treatment, when BSA was administered alone, only 35.9% of immunocaptured BSA was detected, the same percentage of antibody detected after this stripping treatment (Figures 5 and 6, group 4 versus group 1). When antibody was re-administered after this treatment, the antibody was again fully bound (108.4 % of bound antibody compared to the reference group, Figure 5, group 5 versus group 1), and the BSA was immunocaptured in the same way (110 % compared to that bound by immunocapture system (reference group), Figure 6, group 5 versus group 1), suggesting that only antibody, but not protein A/G, was eluted from the nanofibres.

In contrast to the previous treatments, no unspecific BSA binding was found with nanofibres treated with glycine pH 2.5, as both antibody and immunocaptured BSA showed the same percentage values compared to the reference group.

Another interesting finding was that although glycine buffer pH 2.5 damaged the bare nanofibres by rendering it unable to bind to the immunocapture system (it was only able to bind 17.9 % of the total antibody compared to reference group) (Figure 5, group 6 versus group 1), when protein A/G was anchored prior to treatment with glycine buffer pH 2.5, it showed the same rates of antibody immobilisation and BSA immunocaptured as the nanofibres before undergoing the stripping procedure (108.4 % and 110 % respectively) (Figures 5 and 6, group 5 versus group 1). This suggests that the A/G protein protects the nanofibres from damage by the pH 2.5 glycine buffer.

Thus, the effect of pH on protein A/G was found to be very significant. A strong acid (pH 2.5) caused protein A/G to dissociate from the antibody, but not from the nylon nanofibre. A higher pH level, such as ammonium buffer pH 11, caused the A/G protein to dissociate and/or not to anchor to the nylon nanofibre. The same results were obtained with the commercial pH 6.6 Ag/Ac elution buffer, which operates under near-neutral conditions but has a high salt content.

The structures of protein A/G and nylon and their interactions may explain all these results.

On the one hand, protein A/G binds to the constant fraction (Fc) of the antibody by hydrophobic interactions through binding sites inside of its three-dimension structure [14, 15]. The polar side chains are located outside of the protein molecule, allowing the protein to form a hydrogen bond with nylon.

On the other hand, nylon is a polyamide that contains amide groups and free amine groups at the ends of its polymer chains, as well as carboxyl groups. These amide and amine groups provide excellent hydrogen bonding sites [16, 17].

Regarding the binding of the antibody to the A/G protein, it has been described that this occurs at a pH between 5 and 8 due to these hydrophobic interactions [14, 15]. Acidic pH value below 5, which causes protein A/G to separate from antibody, probably by imposing positive charges on amino acids responsible for this interaction, with pKa above 5, such as histidine, as described in Zarrineh et al. in protein A and Fc of antibody interaction [18]. Our results are consistent with this, since strong acidic pH, such as glycine buffer pH 2.5, caused protein A/G to dissociate from antibody.

Regarding the binding of the A/G protein to nylon, it was dissociated by basic pH conditions such as ammonium buffer pH 11. As the isoelectric point (pI) of protein A/G is 4.65, there is a higher percentage of acid groups such as aspartic and glutamic acids. These aminoacids have carboxylic acid groups in their side chains, which lose protons at pH values higher than their pKa and become negatively charged as a result. In addition, nylon is negatively charged at basic pH [19]. This is understandable as nylon is a polyamide that contains not only many amide groups and free amine groups at the ends of its polymeric chains, but also a large number of carboxyl groups, more than amine groups, which gives the nanofibre a negative charge in the basic pH range [16]. Therefore, basic pH levels, such as pH 11, but not strongly acidic pH levels such

as 2.5, could impart a negative charge to the carboxyl acid in both protein A/G and nylon nanofibres, preventing hydrogen bonding between them.

Acidic pH, such as pH 2.5, does not alter the binding of protein A/G to nylon. However, the bare nylon nanofibre was found to be altered by this treatment. This is understandable as polyamides, although containing both negative and positive centres, have amide and amine groups which are protonated at acidic pH [16]. When protein A/G is administered prior to this glycine pH 2.5 buffer, no effect is found, as these amide and amine groups will have previously formed hydrogen bonds with polar side chains outside of protein A/G [17].

In the case of the commercial Ag/Ac elution buffer pH 6.6 with high salt content, the high salt content, but not this pH value, may explain the results. These high salinity conditions create an environment of high ion concentration capable of interacting with any charge density group, disrupting the hydrophobic bonds between protein A/G and antibody, and the hydrogen bonds between protein A/G and nylon, as well as the bare nylon nanofibre.

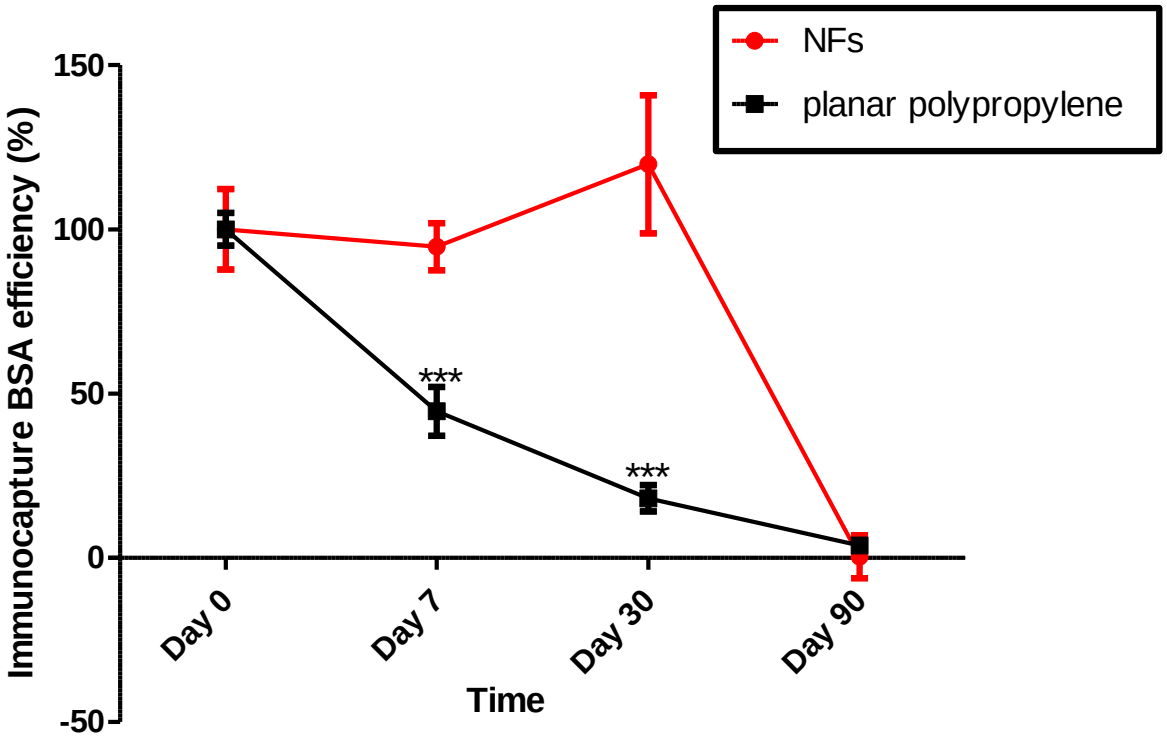
Stability study

As this system is designed to be used for the on-site detection of biological agents, we wanted to conduct this stability study using a potential biological warfare agent such as ricin.

The nylon nanofibre allows the immunocapture capability of the system to remain absolutely intact for one month without the use of any preservative. In contrast, a polypropylene microplate specifically designed to optimise an Enzyme-Linked ImmunoSorbent Assay showed a decreasing immunocapture capability under these conditions, such that seven days after the immunocapture system was assembled,

only 44% of the ricin was immunocaptured compared to the initial measurement result (0 day), and 30 days after, only 18% was detected (Figure 7). Two-way ANOVA showed these differences to be statistically significant.

As described by Feng *et al* [21], hydrogen-bonded organic frameworks (HOFs) allow enzymes to diffuse into the pores, providing an additional layer of protection against denaturation factors. Since hydrogen bonds are formed between protein A/G and nylon, it is understandable that a three-dimensional nylon structure such as this nanofibre would provide more hydrogen bonds as attachment points than a planar surface, allowing the attached protein to be better protected.



	NF		Treated polystyrene	
	Mean	SEM	Mean	SEM
0 day	100	12,3	100	5,0
7th day	94.72	7,2	44,6	7,5
30th day	119,9	21,0	18,1	4,0
90th day	0,3	6,5	3,7	1,0

Figure 7: Fluorescence due to BSA immunocaptured by the immunocapture system immobilised on both NFs and a specialised polypropylene ELISA microplate over time, up to 90 days. Data are expressed as a percentage of fluorescence due BSA immunocaptured on day 0, n = 4. Two-way ANOVA. Difference between the immunocapture system in NFs and in a 96-microwell plate, for each time: *** p< 0,001.

Conclusion

In summary, nylon nanofibers with protein A/G are capable to be reused in a new immunocapture system, as long as stripping treatment is carried out with buffer glycine pH 2.5, since after this treatment protein A/G resulted to be separated from antibody but not from the nylon nanofibers, and no damage in its antibody binding capability was found.

It allows the system to be very cost-effective, not only because nanofiber can be used again but also protein A/G previously anchored. It reduces not only the cost but also the time needed to provide a new immunocapture ready to use. In addition, because the nylon nanofibre protects the immunocapture system better than a planar surface specialised for anchoring antibodies, it allows the immunocapture system to extend its shelf life.

Experimental

Chemicals

Polyamide 6 or nylon 6 (PA6) was made by electrospinning by Tecnalia Research & Innovation, the composition of the ultra-thin nylon nanofibres (NFs) was purchased from BASF (Ultramid® B24 N 03). The nanofiber manufacture was described in previous publications [10-13; 22-23]. NFs were presented in slice of 4x4 mm to be placed and assayed in 96-well microplate.

Nunc MaxiSorp® flat-bottom microplates were used in stability assay.

Ricin was obtained from Robert Koch Institute. Kit for FITC-labelled was purchase from BioRad (Spain) and peroxidase labelled by LYNX Rapid HRP Antibody Conjugation Kit (BioRad). Antibody anti-ricin was in house made in collaboration with National Center for Biotechnology (CNB) - CSIC. 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP, Ampliflu) was a fluorogenic substrate for horseradish peroxidase (HRP) (Sigma-Aldrich). 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.). The blocking buffer used was a phosphate buffered saline (PBS) with casein (Pierce).

Solvents and additives were purchased form Aldrich (Spain). Phosphate buffered saline (PBS) was purchased from Fisher Scientific.

Nanofiber reusing study

Immunocapture Protocol

The immunocapture protocol used was carried out for our laboratory and published in 2018, as mentioned in the Introduction section [10, 11], and it consisted in a well-

oriented antibody immobilization system by the intermediate protein A/G. Briefly, NFs were placed in microwells from a 96-well microplate, previously blocked with PBS-casein. In order to achieve a well-oriented antibody immobilization, protein A/G (10 µL 100 µg/mL in PBS) was added to each NFs surface and incubated over night at 4 ºC, followed by a blocking step with PBS-casein. Then, a fluorescein-labelled antibody against BSA was immobilized on the surface NFs containing protein A/G, after 1 hour incubation. Then, RPE-labelled BSA (10 µL 100 µg/mL in blocking buffer) was immunocaptured by the anchored antibodies, after one hour. Washing steps were carried out between each steps above in order to eliminate the non-linked reactive in excess. The fluorescence signals were measured using a Gemini XPS Microplate Reader (Molecular Device).

Antibody anchored was measured as FITC-fluorescence (λ emission 490 nm and λ excitation 521 nm wavelengths) after its incubation and the subsequent wash, divided by its FITC-fluorescence obtained just before antibody incubation (system autofluorescence of the system).

BSA immunocaptured was measured as RPE-fluorescence (λ emission 495 nm and λ excitation 570 nm wavelengths) after its incubation and the subsequent wash, divided by its RPE-autofluorescence obtained just before antibody incubation.

Since a lot of handling is required, BSA was used as a toxin surrogate in this study by safety and economic reasons.

Stripping treatments

Since we wanted to evaluate the role played by pH, three different pH buffers from acid to basic were assayed. In this line, we used a Thermo Scientific™ Pierce™ Gentle Ag/Ab Elution Buffer, pH 6.6, a glycine buffer pH 2.5 containing glycine 200 mM in

PBS, and ammonium hydroxide pH 11 containing NH_4OH 1 N in PBS (both chemicals were purchased from Merck-Sigma-Aldrich).

The stripping protocol using any buffer was as follow:

Buffer stripping was added (200 µL *per* NF) and incubated at room temperature for 10 minutes, two times. Thrown away the stripping buffer from nanofibers, they were washed with PBS (adding it and incubated for 10 minutes, two times). The two stripping buffer steps were repeated and three 5 minutes-PBS wash steps took place after them (adapted from *abcam* stripping protocols [24]).

Reconstructing of the immunocapture system

Immunocapture system was rebuilt as describing above. However, in order to study how each treatment affects both immunocapture system and nanofibers, several groups were assayed:

Group 1: complete immunocapture system (protein A/G + antibody-FITC+BSA-RPE) without stripping treatment after it (group using as reference one).

Group 2: complete immunocapture system (protein A/G + antibody-FITC+BSA-RPE) with stripping treatment after it.

Group 3: complete immunocapture system (protein A/G + antibody-FITC+BSA-RPE), then stripping treatment and complete rebuilt immunocapture system (protein A/G + antibody-FITC+BSA-RPE) after it.

Group 4: immunocapture system without BSA-RPE, then stripping treatment and only BSA-RPE added after it.

Group 5: only protein A/G anchored to nanofiber, then stripping treatment and only antibody-FITC incubation and BSA-RPE added after it.

Group 6: only bare nanofiber suffering the stripping treatment and complete rebuilt immunocapture system (protein A/G + antibody-FITC+BSA-RPE) was added after it.

Anchored antibody-FITC and immunocaptured BSA-RPE fluorescences were measured as described above. Results are shown as percentage fluorescence from complete immunocapture system (group 1).

Data were statistically analyzed by two Two-way analysis of variance (ANOVA) using GraphPad Prims 5 Software.

Stability Study

Since less handling is required in this study, ricin is used as toxin instead of a surrogate as BSA.

Immunocapture System in Stability Study

The immunocapture system was similar as one described above. Briefly, NFs were placed in microwells from a 96-well microplate, previously blocked with PBS-casein. Protein A/G (10 µL 100 µg/mL in PBS) was added to each NFs surface and incubated over night at 4 ºC, followed by a blocking step with PBS-casein. The control planar surface group began in this step, incubating the protein A/G overnight and then, blocking with PBS-casein. Since only immunocapture capability was measured in this study, no-labelled *in house* antibody (10 µL 500 µg/mL) against ricin was incubated for one hour at room temperature. Then, biotin-labelled ricin (1 µL 1 mg/mL in blocking buffer) was immunocaptured by the anchored antibodies, after one hour incubation. Biotin-ricin was added to both NFs and microplate immunocapture systems at different times: 0 day (immediately after antibody anchoring; it is considered the reference value), 7 days, 30 days and 90 days after antibody anchoring, placing the immunocapture systems at room temperature and only covered by aluminum foil.

Washing steps were carried out between each steps above in order to eliminate the non-linked reactive in excess. Peroxidase-labelled streptavidin was added in order to detect immunocaptured ricin through biotin and streptavidin binding. A fluorescent peroxidase substrate (ADH, Ampliflu) was added and fluorescence measured (λ emission 530 nm and λ excitation 590 nm wavelengths). This value was divided by the fluorescence obtained just before biotin-labelled ricin incubation (system autofluorescence). Results are expressed as a percentage of ricin immunocaptured fluorescence from the initial immunocapture system (0 day).

Acknowledgements

We would like to thank the whole team at Nieves Murillo of TECNALIA - Industry and Transport Division for providing the nylon nanofibres.

Funding

These results belongs to OPTONANOSENS TEC2015-63838-C3-2-R, a coordinated project supported by MINECO / FEDER funds, and the project TEC2015-63838-C3-1-R-OPTONANOSENS (MINECO/FEDER),UE, and KK-2017/00089-μ4F from the Basque government.

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