COMPOSITE CELLULOSE FIBRE FOR AFFINITY CHROMATOGRAPHY APPLICATION

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Affinity chromatographic supports are nowadays one of the most frequently used and expensive consumable materials for protein purification at the laboratory and industrial scales. The introduction of cost-effective materials is an important issue to address in order to spread the usefulness of this technology.
Cotton fibres are a highly available natural material with excellent mechanical and structural properties, which can be used for this purpose. Nevertheless, fibre insolubility and low chemical reactivity are the major drawbacks preventing the use of this material for protein chromatography.
In this work, a composite prepared from polymethacrylate/cellulose fibres was used for the preparation of chromatographic materials containing immobilised proteins as adsorptive ligands. A green fluorescent protein and a protein A-similar ligand, containing cysteine in the terminal tags, were immobilised onto the fibres by epoxy-thiol chemistry. Buffer salt, pH, reaction time and pre-swelling procedure were optimised. The materials were characterised by fluorescence and electron microscopy, in addition to the binding capacity of the adsorbent materials.

Keywords: affinity chromatography, protein A, immunoglobulin purification, cellulose fibres

INTRODUCTION

Affinity chromatography is the preferred method for purifying proteins and other biomacromolecules at the laboratory and industrial scales; especially for proteins present in low concentrations in complex mixtures. Its specificity and selectivity allow simplifying the purification protocols to minimum steps, increasing the product yield and reducing the processing time.

Affinity chromatography using protein ligands is one of the first chromatographic methods to be described for protein purification.[1] However, the very high cost limits their application to a few industrial processes. One of the most often industrially used affinity chromatographic supports are Protein A matrixes. This chromatographic material allows purifying polyclonal and monoclonal antibodies for different applications in the biological/biomedical fields, and it is the preferred generic ligand for affinity purification of antibodies and molecules tagged with an antibody Fc-region.[2] Protein A is a 42-kDa protein, which contains four antibody binding sites (one per domain); however, only two of them can simultaneously bind the target protein as a consequence of the steric effect. Therefore, a short recombinant protein A called *Avipure*, of 14 kDa, containing only two domains, has been used as chromatographic ligand.[3]

Chromatographic supports involve two main building blocks, the chromatographic matrix and the adsorptive ligand. Matrix supports based on agarose beads are the gold standard for this technique; however, new supports are emerging, such as monolith columns,[4] adsorptive membranes[5,6] and adsorptive fibres.[7,8] Fibre-based materials show excellent physicochemical properties and offer some process advantages, such as mechanical robustness, high swelling

capacity, good mechanical strength and convenient usage.[9]

The introduction of cost-effective chromatographic materials, especially protein A based adsorbents, is a critical issue to address in order to enhance the growth of this technology through the use of disposable and highly specific columns for laboratory and industrial applications. Also, small devices with different affinity adsorptive properties are emerging;[10,11] in addition to the commercial micro and spin columns. However, one of the problems in the development of these modules is the compatibility of the container and the adsorptive (functional) material, in addition to the issues related to the hydrodynamic properties. Self-contained adsorptive materials are potentially superior to beaded ones to overcome these drawbacks. Porous monoliths and fibre materials, instead of the traditional chromatographic beads, could improve microdevice functionality.[12] In addition to that, industrial downstream processing also requires novel materials with improved hydrodynamic properties and adsorption capabilities.

Cellulose fibres have been used to prepare adsorptive protein materials without changing their physical morphology.[13] This new composite material has been modified with small ligands with enhanced ion-exchange adsorptive capabilities; while keeping the tensile strength, mechanical and morphological shape of the natural fibres.[7] However, the immobilisation of large ligands, such as proteins, in the inner structure of cellulose fibres, maintaining their physicochemical properties is still a challenge.

In this work, the immobilisation of protein ligands, containing a His-Cys tag, in the inner structure of composite cellulose fibres has been investigated. The protocol was optimised based on the fluorescence properties of a green fluorescent protein (GFP). The immobilisation procedure was applied to the immobilisation of the *AviPure* ligand, and the resultant chromatographic matrix was tested for human immunoglobulin adsorption.

EXPERIMENTAL

Material and methods

AviPure protein was prepared as described by Kangwa[3] and green fluorescent protein containing a $(His\text{-}Cys)_2$ tag, called GFP-Cys, was prepared according to Kikot.[14] Glycidyl methacrylate (GMA), dimethyl acrylamide (DMA), Fluorescein isothiocyanate Isomer I (FITC), tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma Chemical Co® (USA). Phosphate and citrate salts were purchased from Anedra (Argentina). Thermo-treated Antitetanic gamma globulin (IgG) (165 mg/mL; GammaTet® 250 UI) was purchased from Gador S.A. (Argentina). All other employed chemicals were of analytical grade (RA-ACS) and were purchased from local suppliers. Deionised distilled water was used for all the experiments.

Phosphate buffered saline (10 mM, pH 7.4 (PBS)) was prepared by dissolving NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, and KH_2PO_4 0.24 g/L in distilled water; the pH was adjusted and the volume was brought to 1 L.

For IgG fluorescence labelling, FITC was linked to IgG following the product instructions with some modification, as described below. IgG protein was purified by a desalting column (PD-10, GE Healthcare) to remove the excipients. FITC solution in DMSO (1 mg/mL) was prepared fresh and diluted in an alkaline buffer for coupling procedures immediately before use. The protein solution was diluted in 0.1 M sodium carbonate buffer pH 9 (final protein concentration: 2 mg/mL). A volume ratio of 20:1 (IgG:FITC) was used. The mixture was incubated overnight in darkness at 5 °C. The reaction was stopped with NH_4Cl 50 mM final concentration during 2 h. IgG-FITC was purified by a PD10 column and stored in PBS at 5 °C in the dark until use.

Composite preparation

Cellulose cotton was provided by TN & Platex, S.A., Buenos Aires, Argentina. Fibres were pretreated with an alkaline solution, as described in Singh.[15] After exhaustive washing, they were dried and used for further functionalization. Composite fibres were prepared by radiation-induced grafting polymerisation. Briefly, 0.5 g of dried pretreated fibres was enclosed with 50 mL of nitrogen-purged grafting solution containing 3.2% (v/v) GMA and 7.6% (v/v) DMA in 1:1 v/v ethanol/water. Samples were irradiated in a ^{60}Co gamma source at the Semi-Industrial Irradiation Plant (PISI) – CNEA (National Atomic Energy Commission of Argentina), Ezeiza Atomic Center (CAE, Ezeiza, Buenos Aires, Argentina). A dose rate of 1 kGy/h and 10 kGy doses at room temperature (RT) were used. After irradiation, the epoxy-grafted fibres were washed with 50% ethanol (v/v), water and ethanol and then dried at 50 °C.[12] A small piece of wet fibres (in the order of 50 mg) was exactly weighed and dried overnight at 40 °C in an oven. The dried fibres were weighed again after their temperature went down to RT. The percent of moisture was calculated.

The fibres were rinsed with PBS containing 0.05% Tween 20 (PBST) prior to immobilisation. In the case of a pre-swelling step, the fibres were incubated in DMSO in a 50 mL tube.

The partial sulfonation protocol was performed by incubating 250 mg of epoxy cellulose fibres (grafting degree 70%) in DMSO containing 15 mg of sodium sulfite (Na_2SO_3); the mixture was incubated at 40 °C overnight under gentle shaking. The fibres were deeply washed with distilled water and dried in an oven at 37 °C overnight.

Composite fibres (210 mg) was soaked in 7 mL of 2 M sodium citrate buffer (pH 8.6) and 1.5 mL of glycerol (10% final concentration). TCEP of 0.1 mM final concentration and EDTA solution of 1 mM final concentration were added. Seven mL of 5 mg/mL *AviPure* solution was added. The mixture was incubated for 72 h at 5 °C with gentle shaking. After incubation, the supernatant was carefully removed, and fibres were washed with PBST (10 mL) 3 times and with PBS (10 mL) 3 times and drained gently with a pipette. For storage at 5 °C, 1% benzyl alcohol in 0.1 M sodium acetate, pH 5.2, was used as preserving solution.

Microscopy analysis

Electron microscopy images were captured with a Siemens Elmiskop I electron microscope (LANAIS-MIE, CONICET). For sample preparation, the fibres were socked in a fixative solution (4% (w/v) paraformaldehyde, 0.25% (v/v) glutaraldehyde in 0.1 M phosphate buffer of pH 7.4 at 4 °C). After 4 h, the material was washed with distilled water. Afterwards, the sample was postfixed for 1 h in OsO_4 (1%, v/v), contrasted with uranyl acetate (1%, v/v), dehydrated and embedded in Durcupan (Fluka Chemie AG). Ultrathin sections were cut from cross-sectional areas and mounted onto grids.

Fluorescence microscopy quantification was performed by a Cytation™ 5 Cell Imaging Multi-Mode Reader. Fluorescence intensity was used as a detection mode. The endpoint method was established using two filter sets to quantify the RFU: FITC filter 469/10 and 525/10 nm, and others to determine auto-fluorescence (Excitation 485/20 nm; Emission 528/20 nm). Spectral scanning: DAPI and GFP were applied as imaging filter cubes. Fluorescence and brightfield were used as imaging mode; single colour (2.5x and 10x objectives) and montage (2.5x objective) were implemented as an imaging method with a 16-bit Sony CCD camera in grayscale. Fibres (approximately 30 mg wet weight) were washed with PBS, PBST and finally were incubated overnight with 1 mL of 0.5 mg/mL IgG-FITC and 2 mg/mL BSA in PBST with gentle agitation at RT. After incubation, the fibres were washed many times under stirring with PSBT and PBS. They were drained (or squeezed) and then placed on a 96-well plate containing 50 µL of PBS. The 96-well plate was preserved in darkness until the fluorescence measurement. The fluorescence determination was performed immediately after the incubation treatment with the labelled protein.

Adsorptive studies

The binding capacity was determined by measuring fluorescence by the Cytation™ 5 Cell Imaging Multi-Mode Reader, as mentioned previously. One mL of 0.5 mg/mL IgG-FITC solution was incubated overnight with *AviPure* fibres (approximately 100 mg wet weight). Before and after incubation, fluorescence was measured in the supernatant.

The *AviPure* fibres were packed in home-made polypropylene columns (internal diameter: 6.2 mm; length: 33 mm; volume: 1 mL). Chromatographic experiments and dynamic binding capacity (DBC) determination were performed using an ÄKTA Prime Plus System, running on Unicorn 4.10 software. The column was equilibrated with PBS; the IgG sample was injected into the buffer PBS, and 0.1 M citrate buffer, pH 3, was used for elution. DBC was performed using the IgG solution (2 mg/mL) in PBS, and an acetone pulse response experiment was performed to confirm the column packing and determine the dead volume of the system.

RESULTS AND DISCUSSION

Cellulose is the most abundant organic polymer on Earth, composed by glucose moieties linearly linked by glycosidic β(1-4) bonds. Also, the polymer has a massive network of weak interactions, which prevents cellulose dissolution in most solvents, with a few exceptions, such as ionic liquids.[16] The insoluble microfibrils have high tensile strength as a result of this intricate network of hydrogen bonds, occurring within or between neighbouring long polymeric chains, holding them tightly linked together side-by-side.

The use of cellulose fibres as protein adsorptive materials can be supported by their properties of tensile strength, low non-specific protein adsorption, and chemical neutrality. Also, fibres have at least two-dimensional units in the micron range, which can maintain good adsorptive properties as a consequence of a micron-scale diffusion pathway; even lower than those of the chromatographic beads currently used in protein chromatography.

The treatment with alkaline solutions allows partial and temporary hydrophilization of the cellulose fibre. This swelling will drastically increase their adsorption capabilities; however, a permanent hydrophilization required for this application, without destroying the fibre structure, is a challenging task to achieve.

Radiation technology is an important industrial process for polymer treatment.[17] This technology is gaining novel industrial applications as it is environmentally sustainable. Under this premise,

a novel method to prepare a composite material based on cellulose fibres using radiation technology has been described.[13]

The radiation-induced grafting polymerisation technique was demonstrated as able to induce a bulk or surface modification of cellulose fibres.[18,19] Bulk modification can be achieved without destroying the macroscopic structure of cellulose fibres, if the irradiation is performed after specific sample preparation. Cellulose fibres should be alkaline treated and should be kept wet to avoid rebuilding of the H-bond network. Swelled fibres were mixed with the monomer solution for the radiation-induced polymerisation process. The amount of free monomer was adjusted to yield poly(glycidyl methacrylate) (polyGMA) grafting in the order of fifty percent of the initial fibre weight, ensuring that a high amount of reactive sites will be available in the fibre structure. Later on, using a similar approach, chemical grafting was also performed on the alkaline-treated cotton fibres for preparing the chromatographic materials.[15] Composite fibres containing different ionic ligands, such as ion-exchange fibres, were successfully prepared. They showed very high protein adsorption capacity when they were loaded in standard chromatographic columns.[7] Also, protein adsorptive fibrous systems have low-pressure drop and high biomass tolerance.[20]

The strong electrostatic charge of ion-exchange ligands improves the swelling character of the composite fibres, this statement being especially true for sulfonic ligands, helping to interfere with the H-bonding network and therefore with the compact cellulose arrangement. However, organic molecules and protein-based ligands could be more difficult to introduce into the cellulose matrix.

The composite material contains a high amount of reactive epoxy rings, thus, it does not require additional chemical reagents for matrix activation. Besides, it is stable in water at neutral pH and can be stored in a dry condition. However, the polyGMA fraction of the composite is also a water-insoluble material.

Proteins have many chemical functionalities to be used for linkage to epoxy rings. However, the thiol-epoxy reaction offers chemical link robustness and the advantages of click chemistry to immobilise the protein-based ligands (Fig. 1A).

The immobilisation of protein-based ligands also has additional issues. Proteins cannot be exposed to high-temperature reactions and organic solvent media. Also, the insolubility of the composite material in aqueous media is a significant drawback to perform the immobilisation reaction of water-soluble ligands, such as proteins.

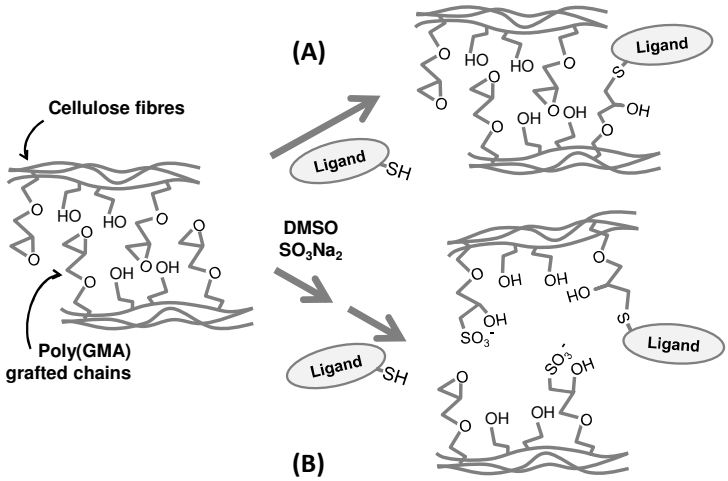

Figure 1: Scheme of immobilisation reactions onto composite fibres

Affinity chromatography matrices for immunoglobulin purification are prepared with Protein A and Protein A-like ligands. Recently, a short protein A-like ligand called *AviPure*, based on the B domains of the native protein, has been described. This protein, with a molecular weight of approximately 14 kDa, contains two repeats of the B domain and two (His-Cys) sequences at the C-terminal.[3] The two free thiols of Cys allow a standard immobilisation reaction by the epoxy-thiol chemistry. This reaction is one of the most preferred chemistries as a consequence of the high

stability of the thioether linkage. Besides, this chemical coupling is a cost-effective reaction under mild conditions (pH 7 and RT) due to the fact that no additional coupling reagents are required. One of the few drawbacks of this chemistry is the slow reaction kinetics, which is in the range of hours or days.

In order to study this coupling reaction and its optimisation on the composite fibres, a fluorescent protein GFP-Cys, containing the $(His\text{-}Cys)_2$ tag, was used as a model protein. The straightforward determination of this protein by fluorescence measurements allows developing a fast method to improve the immobilisation protocols. The GFP-Cys was previously described and purified by IMAC chromatography.[20] This protein can be detected and quantified by fluorescence at low concentrations in different protein solutions.

The composite material was incubated with GFP-Cys under the following standard immobilisation conditions: 10 mM PBS pH 7.4, 5 °C, overnight incubation (previously developed in our laboratory). Quantification of GFP helped determine the immobilisation yield on the composite fibres by direct fluorescence detection on the material, and indirectly, by measuring the fluorescence in the reaction solution at the initial and final stages. The amount of GFP-Cys immobilised under these reaction conditions was negligible (data not shown). This result could be the consequence of the poor swelling properties of the composite material under the reaction conditions (aqueous environment, neutral pH and RT) used for ligand immobilisation. In order to overcome this drawback, several organic solvents were studied, as a pretreatment step, in order to improve the swelling of the composite material. Several polar solvents, such as alcohols (methanol, ethanol and isopropanol), acetone, dioxane, ethyl acetate, acetonitrile, DMF and DMSO were tested. The last two solvents showed some swelling effect upon naked-eye observation; and out of the two, DMSO was identified as the best swelling solvent for the composite fibres. However, proteins cannot be used with a high proportion of this solvent because it induces protein denaturation. Therefore, composite fibres were pre-swelled in the solvent, the excess of the solvent was removed, and then the fibres were incubated with an aqueous solution of protein-ligand under standard conditions to perform the immobilisation reaction. The preswelled fibre/aqueous solution ratio was selected, keeping the final solvent remaining in the reaction medium lower than 20%. The data in Table 1 describe the immobilisation yield, shown as mg GFP/mg fibre, using the selected pre-swelling solvents and following the procedure described in Figure 1A, using the standard immobilisation protocol (10 mM PBS, pH 7.4, 5 °C, overnight). The protocol using DMSO as a pre-swelling solvent was able to increase the reaction yield 20-fold, in comparison with the procedure without the pre-swelling step (water condition).

Considering that a low percentage of solvent is supported in the immobilisation reaction, some additives were added to the reaction buffer in order to preserve the cellulose swelled after solvent removal. The general strategy was to interfere with the restoration of the cellulose hydrogen bond network. In this way, the addition of the following reagents was proposed: glycerol, saccharides and cosmotropic salts, such as sulfate and phosphate anions.

Table 1
Immobilisation yields of GFP-Cys onto composite fibres using different pre-swelling solvents

Pre-swelling solvents[a]	Immobilisation yield (RFU/g fibre)[b]
Water	168 ± 20
Acetone	418 ± 92
DMF	139 ± 11
DMSO	2022 ± 53

[a]After solvent treatment, fibres were removed and incubated in 10 mM PBS (pH 7.4, 5 °C, overnight); [b]Immobilised GFP was quantified by direct fluorescence of the material (RFU: relative fluorescent units)

Table 2 lists the experimental conditions and reaction yield of GFP-Cys immobilisation per fibre weight, measured by direct fluorescence. Similar results were found by an indirect method (fluorescence difference of reaction media at the final and initial stages). From all the studied conditions, the citrate buffer reaches the highest yield, followed by the glucose and trehalose

conditions. Considering that the citrate salt has buffer properties, it was selected for further optimisation. The immobilisation reaction using citrate was compared with that involving phosphate buffer at two different concentrations and two different pH values. All the conditions were assessed using the DMSO pre-swelling treatment (Table 3).

Table 2
Immobilisation reaction conditions using GFP-Cys as protein-ligand

Condition[a]	Concentration	Immobilisation yield (RFU/g fibre)[b]	Immobilisation yield (mg GFP/g fibre)[c]
PBS pH 7.3	0.01 M	1218 ± 225	4.13
Glycerol	25 %v/v	731 ± 186	na
Phosphate buffer pH 7	1.5 M	888 ± 40	na
Na_2SO_4	1 M	Precipitate	na
Citrate buffer pH 8.4	1 M	1551 ± 324	5.40
Glucose	10 %p/v	1324 ± 10	4.96
Trehalose	20 %p/v	1357 ± 16	5.10
Lactose	14 %p/v	1136 ± 35	4.07
Sacarose	20 %p/v	1287 ± 82	4.33

[a] Fibre samples were pre-swelled in DMSO prior to incubation with GFP-Cys in the selected buffer; [b] Calculated as total RFU difference of the supernatant prior to and after incubation per fibre amount; [c] Calculated by direct fluorescence of fibres

Table 3
Immobilisation yield of GFP-Cys onto composite fibres using different conditions

Buffer salt [a]	Concentration (M)	pH	Immobilisation yield (mg GFP/g fibre)
Phosphate	0.01	7.4	3.2 ± 0.3
Phosphate	0.01	8.4	3.1 ± 0.4
Phosphate	1	8.4	3.3 ± 0.3
Citrate	1	8.4	5.4 ± 0.3

[a] Fibre samples were pre-swelled in DMSO prior to incubation with GFP-Cys in the selected buffer

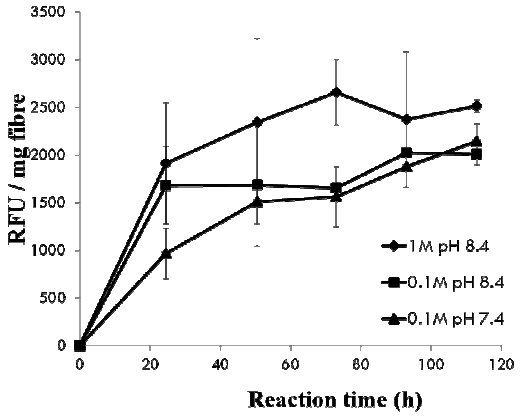

Figure 2: Progress of immobilisation reaction of Cys-tagged GFP protein onto composite fibres in citrate buffer

According to the experimental immobilisation yields, the phosphate buffer could not be as efficient as the citrate. The higher hydrodynamic radii and the H bonding capability of the citrate could be determinant for the better performance of this salt.

It is well known that the kinetic reaction of the epoxy group is relatively slow. Therefore, the immobilisation reaction was performed on a timescale of days. Figure 2 has shown the evolution of immobilised GFP fluorescence with the reaction time up to six days under different

citrate buffer conditions. GFP immobilisation was determined by direct fluorescence of the fibres. Some additives, such as 1 mM EDTA and 0.1 mM TCEP, were included into the buffer to avoid sulfur oxidation. According to Figure 2, the optimum incubation time was 72 h (3 days) and the reaction temperature of 5 °C, which will preserve the protein structure for long incubation periods.

In the following step, *AviPure* immobilisation reaction was performed on the composite fibres using some selected conditions (Table 3, entry 1 to 3). The success of the immobilisation reaction was determined by measuring a functional property, the binding capacity to IgG. This experiment was performed using FITC-IgG for the straightforward determination of IgG adsorption in the composite fibres (Table 5). The binding capacity was much higher in the case of the solvent pre-swelling treatment with DMSO.

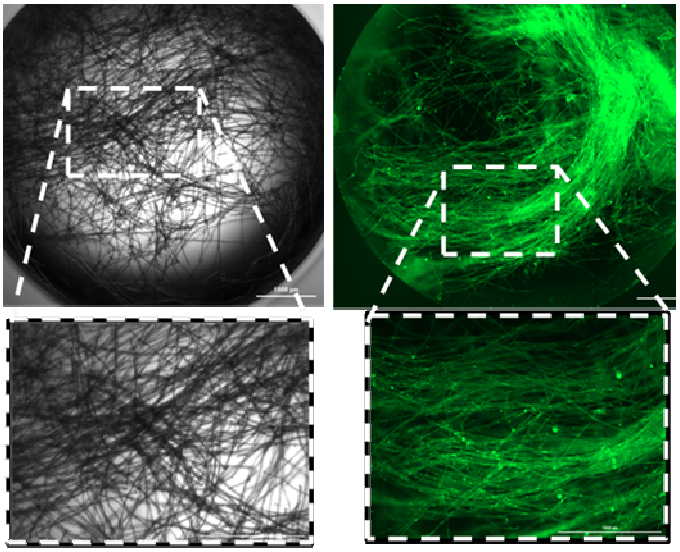

Figure 3: Fluorescence microscopy images (x10) under white field (left) and black field (right) of *AviPure* fibres

Table 4
Comparison of fluorescence emission of different fibres incubated with FITC-IgG

Condition	Fluorescence ratio [a] (IgG-FITC[b]/AutoFluorescence[c])	Error (%)
Cellulose fibre	0.44 ± 0.09	21
Cellulose fibre + IgG-FITC	0.35 ± 0.10	29
Alkaline-treated fibre + IgG-FITC	1.08 ± 0.34	32
Composite fibre + IgG-FITC	2.80 ± 0.57	20
Sulfonic fibre + IgG-FITC	2.84 ± 1.24	44
AviPure-fibre + IgG-FITC	81.73 ± 8.44	10

[a] n = 3; [b] FITC: Excitation 469/10 nm; Emission at 525+/-10 nm; [c] Polymer autofluorescence: Excitation 485/20 nm; Emission at 528+/-10 nm

Also, the buffer citrate condition was better than the use of the phosphate buffer, as it was previously determined for GFP-Cys, leading to a binding capacity of 15.5 ± 2.0 mg IgG/g fibre. Therefore, pre-swelling of the material with DMSO was the main factor that led to higher immobilisation yields. Image analysis of *AviPure* fibres was performed using a Cytation 5 fluorometer. Figure 3 shows a well (10x magnification) containing fibres under white light and fluorescence. Pictures of the lower panels present higher magnification of selected regions of the fibre mats. *AviPure* fibres were previously immersed into FITC-IgG and intensively washed with loading buffer. The green colour comes from the FITC-IgG, which was adsorbed onto *AviPure* fibres. The fluorescence threshold was set using unreacted composite fibres to subtract the

cellulose autofluorescence. Homogeneous green fluorescence through the materials can be appreciated. In Table 4, the specific and non-specific fluorescence of different cellulose fibres is compared. The specific fluorescence corresponds to FITC-IgG adsorption, and the non-specific fluorescence comes from the intrinsic fluorescence of the polymer. Previous to the fluorescence detection, the fibres were washed with loading buffer to remove the non-specific IgG adsorption to the matrix.

The second round of binding capacity optimisation was performed based on the induction of a permanent opened structure of the cellulose fibre, by improving the matrix swelling under aqueous conditions. Two alternative strategies were analysed, keeping the fibre structure unaltered: (a) the cellulose backbone acetylation using acetic anhydride onto alkaline treated cellulose fibres, and (b) partial hydrophilization of the composite fibre. No improvement in the swelling properties of the fibres and binding capacity was found using the first strategy. The second alternative was to perform partial epoxy add-on reactions, using a highly hydrophilic ligand. Chemical reactions with thioglycolic acid to introduce a carboxyl group and sodium sulfite to introduce a sulfonic acid were analysed (see Fig. 1B). Both of them showed some improvement in the binding capacity to IgG; however, thioglycolic acid treatment was discarded because the free unreactive reagent is quite challenging to remove from fibres. Meanwhile, *AviPure* fibres prepared from partially sulfonated fibres reached a binding capacity of 18.8 ± 0.9 mg IgG/g fibre (Table 5, entry 4).

Table 5
Binding capacity (BC) of *AviPure* composite fibre prepared under different buffer conditions*

#	Matrix	Pre-swelling/buffer condition	Buffer concentration (M)	pH	BC (mg IgG/g fibre)
1	Composite fibre	Water/Citrate	0.01	7.4	1.1
2	Composite fibre	DMSO/Phosphate	0.01	7.4	9.2 ± 3.0
3	Composite fibre	DMSO/Citrate	1	8.4	15.5 ± 2.0
4	Sulfonic composite fibre	DMSO/Citrate	1	8.4	18.8 ± 0.9

*BC was determined using IgG-FITC solution

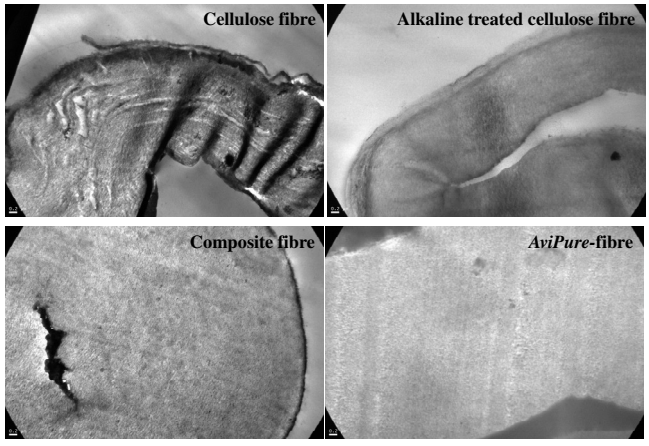

Figure 4: Cross-sectional TEM images of cellulose fibres

Another strategy was to perform two modification steps (two grafting processes) to obtain composite fibres, where the epoxy groups of the first round were converted into sulfonic (hydrophilic) groups and a second grafting process was applied to *AviPure* immobilisation according to the standard protocol. However, this strategy failed to reach higher binding capacities than those of the previous ones. An additional study on the fibre material was performed by

TEM microscopy of cross-sections of cellulose fibres. Figure 4 shows TEM images (magnification of 20,000X) of cross-sections of fibres under different treatments: raw fibre; alkaline-treated fibre, composite fibre, and *AviPure* fibre. The microstructure of the polymer shifts from a concentric parallel ordered structure of cellulose to a final image of homogeneously distributed small dots included into the cellulose matrix.

Figure 5 displays higher magnification TEM pictures (magnification of 85,000X), showing the structure of the composite fibre and *AviPure* fibre in greater detail. The former one shows a random distribution of small dots, which can be assigned to the polyGMA, and the *AviPure* fibres show more significant randomly distributed spots, where the protein could be linked.

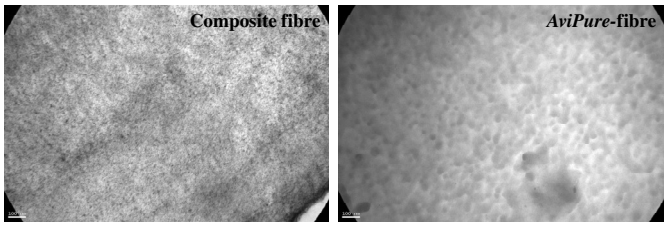

Figure 5: Cross-sectional TEM images of cellulose fibres at higher magnification

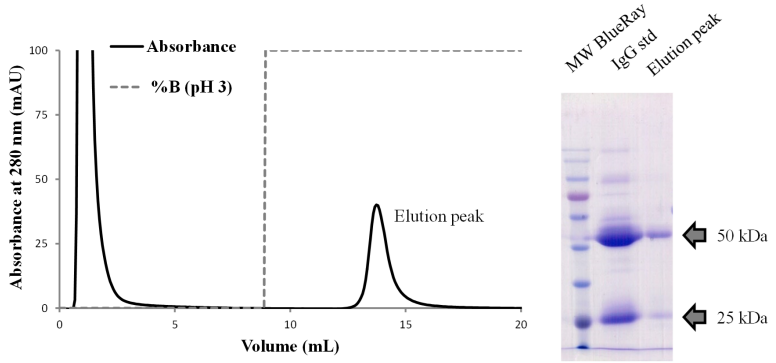

Figure 6: Chromatographic plot corresponding to the adsorption and desorption process of IgG sample

AviPure fibres, prepared according to Table 5 entry 4, were used for an adsorption experiment by packing a small chromatographic column with them. Column hydrodynamics was checked by pulse-response analysis, using acetone tracer. Figure 6 depicts the elution peak after injecting a one-mL sample of a commercial human IgG solution into a one-mL column of *AviPure* fibres. SDS-PAGE gel (12%) shows the proteins (light and heavy chains of IgG), corresponding to the elution peak. In the following experiment, the dynamic binding capacity (DBC10%) was determined, using a 2 mg/mL IgG solution. The DBC10% using a human IgG sample exhibited a fibre capacity of 10.3 mg/g fibre.

CONCLUSION

In this work, affinity adsorbents were prepared based on composite cellulose fibres and protein A-like ligand for immunoglobulin adsorption. A robust immobilisation protocol has been developed and optimised based on GFP-Cys protein and fluorescence techniques. Different strategies for protein-ligand immobilisation were quantitatively studied using fluorescence methods.

The microscopy analyses demonstrated that the *AviPure* fibre material was homogeneously modified at micro- and macroscopic levels. The immobilisation protocols were compared with IgG adsorption experiments under static and dynamic conditions.

It is envisioned that *AviPure* fibres could be applied as low-cost alternative materials to prepare disposable devices for immunoglobulin purification.

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