

## PAPER



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## Hybrid alginate–protein cryogel beads: efficient and sustainable bio-based materials to purify immunoglobulin G antibodies†

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Antibodies present in mammal's serum are of high relevance for therapeutic purposes, particularly in passive immunization and in the treatment of some chronic diseases. However, their widespread use is still compromised by the requirement of several process steps for their purification and the difficulty in keeping antibodies stable to guarantee their therapeutic efficiency. These challenges significantly contribute to the current high cost of biopharmaceuticals, namely antibodies such as immunoglobulin G (IgG). Accordingly, the development of effective and sustainable purification strategies for antibodies and other biopharmaceuticals is in critical demand to decrease economic, environmental and health burdens. Herein, bio-based and low-cost hybrid alginate–protein cryogel beads were prepared, characterized, and applied as novel adsorbent materials for the purification of IgG from human serum. It is shown that hybrid materials are more efficient than the respective alginate beads since the presence of proteins increases the material selectivity for IgG, which is due to the specific interactions occurring between the target antibody and amino acid residues in the hybrid materials. Several operating conditions, such as pH, adsorption time and serum concentration, were optimized to improve the recovery yield and purity of IgG. Adsorption isotherms were determined to infer the adsorption mechanism of IgG onto the cryogel beads and to determine their adsorption capacity (175 mg of IgG per g of cryogel beads). Under the optimized conditions, IgG can be recovered from the hybrid materials using buffered aqueous solutions, with a purity of 80% and a recovery yield of 91%. The stability and integrity of the antibody are retained after the desorption step. Finally, the regeneration and reuse of the cryogel beads were evaluated, with no losses in the IgG adsorption performance and antibody stability. Although significant efforts have been put on the development of novel affinity ligands to replace the standard chromatographic methods to purify IgG, this work demonstrates the potential of bio-based and low-cost hybrid materials as promising alternatives, in which proteins can be used to improve the material selectivity.

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†Electronic supplementary information (ESI) available: Detailed information of the Langmuir and Freundlich isotherms for IgG adsorption on Alg-SSF hybrid cryogel beads; the macroscopic aspect of restructured Alg-SSF hybrid cryogel beads; detailed information about the yield and purity values of IgG. See DOI: 10.1039/c9gc04449c

## Introduction

Significant advances in therapeutic applications have been achieved in the past few decades with the use of antibodies or immunoglobulins (Igs) as biopharmaceuticals.<sup>1</sup> Antibodies are glycoproteins present in blood serum and other body fluids of vertebrates.<sup>1,2</sup> Their function is to identify and eliminate pathogens, such as viruses, bacteria or fungi, through a recognition mechanism of high specificity.<sup>3</sup> The growing interest in immunoglobulin G (IgG) is the main result of the expanding number of biomedical applications in which this plasma-derived protein can be used, *e.g.* to prevent infections in patients with immune deficiencies by providing passive immune protection, in the treatment of cancer, inflammatory and autoimmune diseases, as well as in immunodiagnosis.<sup>4–7</sup>

In most of these applications, IgG with high quality and purity is required. Chromatographic techniques, such as ion exchange, gel permeation and affinity chromatography, are commonly used in the purification of IgG, but still require a number of additional steps for the efficient removal of impurities.<sup>8–12</sup> Although high purity levels are obtained, these techniques lead to low yields and render antibodies as high-cost products, thus limiting their widespread and recurrent use. Furthermore, by being a protein, IgG is a labile macromolecule. Accordingly, there is a crucial need for developing cost-effective methods to purify and recover IgG from the original complex matrices without compromising its stability and biological activity.

In addition to the use of liquid–liquid systems, particularly aqueous two-phase systems that have been largely explored for such a purpose,<sup>1</sup> solid-phase extractions applying materials as novel adsorbents or chromatographic supports have been investigated. Dextran-coated iron oxide magnetic particles modified with ligand 22/8 (a protein A mimetic ligand) were used to purify IgG from Chinese hamster ovary (CHO) cell cultures, with a final IgG purity of 95% in the eluted fraction; however, from the 56% of the total protein bound to the support, recoveries of 5 and 16% of the total protein at pH values of 3 and 11, respectively, were obtained.<sup>13</sup> Applied to the same type of matrix, the capture of IgG with magnetic nanoparticles coated with protein A and starch led to 69% of IgG recovery.<sup>14</sup> In addition to these works focused on the recovery of monoclonal antibodies from culture cells,<sup>13,14</sup> nano-poly(ethylene glycol dimethacrylate-2-methacryloylamidohistidine) nanoparticles were used to purify IgG from human plasma, yielding an IgG purity of 94%; however, the recovery yield was not provided. Affitin D1Sso7d-DM (anti-IgG) was used to prepare affinity/magnetic adsorbents, and also for the purification of IgG from human plasma.<sup>15</sup> Under the optimised conditions, the recovery of IgG was above 95% with a purity of 81%.<sup>15</sup>

Based on the possibility of using solid-phase extraction to purify IgG, we focused on the development of bio-based and low-cost materials, namely hybrid cryogel beads composed of polysaccharides (alginate) and protein fibers. In this work, we avoided the material functionalization with specific and high cost ligands, thus contributing to the development of more sustainable purification technologies.

Alginic acid and its derivatives are important hydrophilic biopolymers that can be extracted from certain species of brown seaweeds (*Phaeophyceae*).<sup>16–18</sup> They are highly relevant biopolymers for various applications due to their relevant features, such as biodegradability, biocompatibility, non-toxicity and low cost.<sup>19,20</sup> They can be processed in various formats, such as beads, fibres, films, capsules, blends with other natural and synthetic polymers, *etc.*<sup>20–23</sup> Furthermore, alginic acid allows the formation of water insoluble calcium alginate beads able to encapsulate therapeutics and/or other macromolecular agents,<sup>24–28</sup> and with high efficiency to adsorb metal ions, dyes or proteins.<sup>29–35</sup> Aiming at improving their physicochemical properties and selective adsorption features,

cryogel beads can be prepared by combining polysaccharides and protein fibrils,<sup>36</sup> resulting in hybrid materials. Protein fibers are aggregated proteins that have attracted considerable interest in the preparation of functional biomaterials, including foams, composites, films and gels.<sup>29,37</sup> These biomaterials are suitable for various biomedical applications, such as biosensors, drug delivery systems, tissue regeneration, cell encapsulation, wound dressing, among others.<sup>29,37</sup> Amongst proteins able to produce protein fibers, spider silk fibres (SSF) are a natural light weight protein with excellent physicochemical and mechanical properties.<sup>38</sup> SSF are of low cost, and display biocompatibility, biodegradability, non-toxicity and cell adhesion properties, making them useful in various biomedical applications.<sup>38</sup> Based on these properties, alginate and protein fibril (hybrid) cryogel beads can be considered promising adsorbent materials, although they were never investigated in the purification of proteins or antibodies such as IgG.

Given the novelty of the described materials, in this work, hybrid alginate–protein cryogel beads were first prepared and characterized by Fourier transform-infrared spectroscopy (FT-IR), Powder X-ray diffraction (PXRD), Thermo Gravimetric Analysis (TGA) and Scanning Electron Microscopy (SEM), followed by their application in the purification of IgG from human serum samples. Several operational conditions, such as pH, adsorption time and serum concentration, were optimized in order to improve the IgG recovery yield and purity. The purity and stability of IgG after extraction from human serum were evaluated and compared with commercial/pure IgG by Size Exclusion High-Performance Liquid Chromatography (SE-HPLC) and Circular Dichroism (CD) spectroscopy. The adsorption isotherms of IgG were additionally determined to infer the molecular-level mechanisms ruling the IgG adsorption in the prepared hybrid cryogels. Despite being low-cost and bio-based materials, the recovery of IgG from the cryogel beads and their reuse were finally addressed, with no significant losses in the adsorption performance and purity of IgG, contributing towards the development of efficient and sustainable separation/purification techniques of current high cost biopharmaceuticals.

## Materials and methods

### Materials, chemicals and biologics

Alginic acid (sodium salt) was extracted from the brown seaweed *Sargassum tenerrimum*, which was collected from the west coast of India (Okha, 22°28'N, 69°05'E), following a previously reported method.<sup>39</sup> 50 g of washed *Sargassum tenerrimum* was treated with 5% HCl (1 : 10 w/v) for 12 h, followed by filtration and washing of the residue with water to remove the adhered acid. The washed seaweed residue was treated with 5% Na<sub>2</sub>CO<sub>3</sub> at room temperature for 12 h, followed by filtration. Isopropyl alcohol (1 : 3 v/v) was added to the filtrate to precipitate sodium alginate, followed by vacuum drying to

obtain the polysaccharide, with a yield of  $25 \pm 2$  wt% with respect to the seaweed used.

AR grade isopropyl alcohol and calcium chloride ( $\text{CaCl}_2$ ) were purchased from Sisco Research Laboratories, Mumbai, India. Sodium alginate, cholinium bicarbonate (80% in water), sodium sulphate ( $\text{Na}_2\text{SO}_4$ ), dipotassium phosphate ( $\text{K}_2\text{HPO}_4$ ), monopotassium phosphate ( $\text{KH}_2\text{PO}_4$ ), IgG and human serum were purchased from Sigma Aldrich, USA. Tetrabutylammonium hydroxide (TBAH, 40 wt% in  $\text{H}_2\text{O}$ ) was purchased from Moly Chem, Mumbai, India. The water used was double distilled, passed by a reverse osmosis system and further treated with a Milli-Q + 185 water purification apparatus.

Spider silk protein fibres (SSF) were prepared by a previously reported method.<sup>38</sup> SSF were collected from naturally accumulated locations in the laboratory building of CSIR-CSMCRI, Bhavnagar ( $21.7600^\circ$  N,  $72.1500^\circ$  E). Collected SSF were washed with water several times to remove dust prior to their degumming and tetrabutyl ammonium hydroxide (TBAH, 40% aqueous solution) pre-treatment. To obtain TBAH treated SSF, 5 mg of degummed SSF was added to a vial containing 1.0 g TBAH (40 wt% in  $\text{H}_2\text{O}$ ) followed by stirring at room temperature until the complete dispersion of SSF in TBAH. The SSF in the dispersed solution were regenerated by adding excess isopropyl alcohol (IPA) followed by drying.

#### Preparation and characterization of hybrid alginate–protein cryogel beads

Alginate (Alg) hydrogel beads were prepared following a previously reported extrusion dripping method.<sup>30</sup> Briefly, 0.5 g of sodium alginate was dissolved in 25 mL Milli-Q water. This solution was added dropwise to a  $\text{CaCl}_2$  (2 wt%) aqueous solution under continuous stirring (300 rpm) at  $30^\circ\text{C}$ , using a syringe with a 22 gauge/0.72 mm diameter needle. After 15 min of contact, the nearly round shaped and colourless alginate hydrogel beads obtained were filtered, followed by lyophilization (Fig. 1a, Alg cryogel beads). The excess of  $\text{CaCl}_2$  adhered on the surface of the beads was removed by washing with Milli-Q water for 30 min at room temperature. For the preparation of alginate–protein composite-based cryogel beads, 40 mg of SSF were added to 10 g of a sodium alginate (2 wt%) aqueous solution and dispersed by ultra-sonication for 15 min. The alginate–protein composite was then added dropwise at room temperature using a syringe with a 22 gauge/0.72 mm diameter needle to a  $\text{CaCl}_2$  (2 wt%) aqueous solution (under continuous stirring at 300 rpm and at  $30^\circ\text{C}$ ). The nearly round shaped alginate–protein beads were filtered and lyophilized to obtain the hybrid composite cryogel beads (Fig. 1b, Alg-SSF cryogel beads).

Both the alginate and hybrid alginate–protein cryogel beads were characterized by spectroscopic, microscopy and thermogravimetric techniques. Fourier transform-infrared spectroscopy (FT-IR) spectra of the degummed SSF and cryogel beads were recorded on a PerkinElmer FT-IR instrument (Spectrum GX, GSA) using KBr discs. The powder X-ray diffraction (PXRD) spectra of cryogel beads were obtained on a

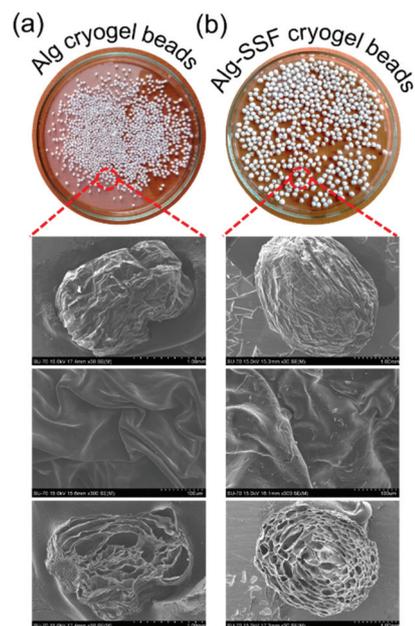


Fig. 1 Optical and SEM images of the prepared (a) Alg and (b) Alg-SSF hybrid cryogel beads; the surface and inner core morphologies are shown.

Philips X'Pert MPD system using  $\text{Cu-K}\alpha$  radiation ( $\lambda = 0.15405$  nm) with a  $2\theta$  range from  $2^\circ$  to  $80^\circ$  at a scan rate of  $0.1^\circ\text{ s}^{-1}$ . Thermogravimetric analysis (TGA) of SSF and cryogel beads was carried out on a NETZSCH TG 209F1 Libra TGA209F1D-0105-L instrument using a temperature programmer from room temperature to  $500^\circ\text{C}$  at a heating rate of  $10^\circ\text{C min}^{-1}$  under a nitrogen gas atmosphere. Scanning electron microscopy (SEM) was carried out with a Hitachi SU-70 scanning electron microscope operating at 15 kV.

#### Adsorption and desorption studies of IgG from human serum and material reuse

A screening of the different cryogel beads (Alg and Alg-SSF) for the purification of IgG from human serum was firstly carried out by the evaluation of several operational conditions, namely pH, adsorption time, and serum dilution. Experiments were carried out at room temperature ( $25 \pm 1^\circ\text{C}$ ) using 5 mg of cryogel beads in contact with 500  $\mu\text{L}$  of human serum diluted 25 times at pH values ranging from 4.0 to 9.0. These pH values were chosen based on the IgG pI, which ranges between 6.3–9.0.<sup>31,32</sup> The concentration of human IgG was evaluated by diluting the serum from 5 to 50 times in glycine buffer, pH 5.0. Under the optimal conditions of pH and serum concentration, the adsorption time was evaluated from 30 to 180 min. The cryogel beads with the adsorbed IgG and other proteins were collected after centrifugation (300 rpm, 5 min) and the supernatant was analysed for the quantification of IgG and other proteins by SE-HPLC – conditions given below.

After the successful adsorption of IgG onto the cryogel beads, the recovery of IgG was performed by applying two desorption steps. In the first desorption step, cryogel beads with

the adsorbed IgG and other proteins were equilibrated with an aqueous carbonate buffer solution (pH 11.0). In this desorption step, mainly human serum and other protein impurities were removed, with no significant removal of IgG. In the second desorption step, the cryogel beads, now mainly containing adsorbed IgG since the remaining proteins were preferentially removed in the first desorption step, were incubated with a phosphate buffer solution (pH 7.0) for 90 min at room temperature. The first desorption step is required to remove the non-target proteins, by taking advantage of the electrostatic interactions occurring between the material and proteins, since the second type of approach will lead to the desorption of most proteins with low selectivity. These steps were optimized in terms of pH and the time of incubation needed.

After the recovery of IgG from the hybrid cryogel beads (Alg-SSF), these materials were tested for regeneration and new adsorption assays. The procedure for the preparation of regenerated hybrid cryogel beads is similar to the protocol that was used for the preparation of hybrid cryogel beads from fresh materials. To this end, 2 wt% of composites in aqueous solution were prepared from the regenerated materials followed by ultra-sonication for 15 min. The composites were then added dropwise to a stirred CaCl<sub>2</sub> (2 wt%) aqueous solution at room temperature. The regenerated round shaped hybrid cryogel beads were washed with Milli-Q water and filtered off, followed by lyophilisation (Fig. S1, ESI†).

#### Quantification of IgG and other proteins using SE-HPLC and evaluation of the IgG stability

Human IgG and other proteins in human serum and supernatant samples (collected after adsorption and desorption studies) were analysed and quantified by SE-HPLC (Chromaster, VWR Hitachi), equipped with a size exclusion column (Shodex Protein KW-802.5; 8 mm × 300 mm). The mobile phase was prepared with 100 mM sodium phosphate buffer pH 7.0 containing 0.3 M NaCl and run isocratically with a flow rate of 0.5 mL min<sup>-1</sup>. The column oven and autosampler temperatures were kept at 40 °C and 10 °C, respectively. The sample injection volume was 25 μL and the wavelength was set at 280 nm using a DAD detector. Commercial pure IgG obtained from human serum was used for the calibration curve.

The purity of IgG ( $P_{\text{IgG}}$ , %) and IgG recovery yield ( $Y_{\text{IgG}}$ , %) were determined by the following equations,

$$P_{\text{IgG}}(\%) = \frac{C_{\text{IgG}}}{C_{\text{Total}}} \times 100 \quad (1)$$

$$Y_{\text{IgG}}(\%) = \frac{C_{\text{IgG}}}{C_{\text{IgGi}}} \times 100 \quad (2)$$

where  $C_{\text{IgG}}$  is the concentration of IgG adsorbed on the material,  $C_{\text{total}}$  is the total concentration of all proteins (IgG and other proteins) adsorbed on the material and  $C_{\text{IgGi}}$  is the initial concentration of IgG in human serum, taking into account all dilutions carried out.

Commercial IgG and recovered IgG from the cryogel beads were characterized by SE-HPLC and CD spectroscopy. SE-HPLC

was performed as described above. CD spectra of the standard and recovered IgG were recorded using a Jasco J-815 CD spectrometer from 190 to 300 nm, at a scanning speed of 10 nm min<sup>-1</sup> and bandwidth of 1 nm. All spectra were acquired with a 0.1 cm path length quartz cuvette cell at 25 °C. All solutions were prepared in stock solutions of 5 mM Na<sub>2</sub>SO<sub>4</sub> in 10 mM of potassium phosphate buffer, pH 7.0.

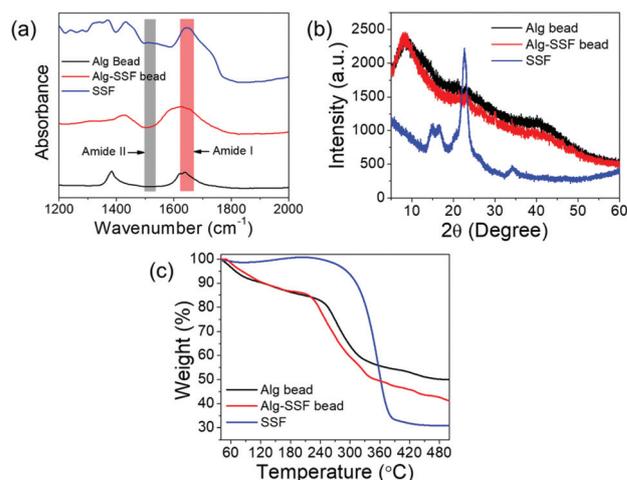
## Results and discussion

### Preparation and characterization of cryogel beads

This work aims to identify bio-based materials to be used in the purification of biopharmaceuticals, namely hybrid alginate–protein cryogel beads for the purification of IgG from human serum. Before the evaluation of their separation performance, and due to their novelty, cryogel beads were chemically and morphologically characterized by FT-IR, PXRD, TGA and SEM. Their macroscopic aspect and morphological characterization are shown in Fig. 1.

SEM images show considerable differences in the surface and inner core morphology of the alginate beads and hybrid alginate–protein cryogel beads. Alginate beads show a smooth and irregular surface whereas hybrid cryogel beads show a moderately rough and regular surface incorporating protein fibres. Furthermore, the inner core of the hybrid cryogel beads reveals a well-ordered morphology, in contrast to the unordered inner core morphology of alginate beads.

FT-IR spectra of protein fibrils, alginate beads and hybrid alginate–protein cryogel beads are given in Fig. 2a. The characteristic IR band in the amide region indicates the presence of protein fibers (Alg-SSF) in the hybrid cryogel beads. The IR stretching bands of amide-I (C=O stretching vibration) and amide-II (C–N stretching vibration) appear in the range of 1620–1700 cm<sup>-1</sup> and 1500–1580 cm<sup>-1</sup>, respectively. After the preparation of cryogel beads with CaCl<sub>2</sub> aqueous solution,



**Fig. 2** (a) FT-IR spectra, (b) PXRD spectra and (c) TGA of spider silk fibres (SSF), alginate beads (Alg beads) and alginate–protein hybrid cryogel beads (Alg-SSF beads).

amide-I and amide-II stretching vibration bands merge, with a broad stretching vibration in the range of 1500–1700  $\text{cm}^{-1}$ , confirming the existence of protein fibers in the hybrid cryogel beads.

The crystalline behaviour of protein fibers and cryogel beads with and without protein fibers was additionally addressed by PXRD, whose results are depicted in Fig. 2b. SSF exhibit a sharp peak at  $2\theta = 24^\circ$ , indicative of the crystalline nature of the protein fibres due to their  $\beta$ -sheet.<sup>38,40</sup> Interestingly, after the preparation of the hybrid cryogel beads, this peak becomes broader and less intense, revealing the presence of interactions occurring between alginate and protein fibers.

The thermogravimetric analysis of sodium alginate powder, protein fibers and hybrid cryogel beads was additionally performed (Fig. 2c), with all thermograms showing a single step thermal degradation. Protein fibrils start to degrade at 290  $^\circ\text{C}$ , alginate beads start to degrade at  $\sim 206^\circ\text{C}$ , and Alg-SSF materials start to degrade at  $\sim 241^\circ\text{C}$ . Overall, hybrid cryogel beads incorporating protein fibers show an enhanced thermal stability in comparison to alginate beads.

### Adsorption and purification of IgG from human serum

After the materials preparation and characterization, the alginate and hybrid cryogel beads were evaluated for the selective adsorption and purification of IgG from human serum. The effects of pH, the concentration of human serum and contact time were addressed to optimize the IgG recovery yield and purity. The adsorption of IgG onto the two types of beads was initially investigated using human serum (diluted 25 times) at different pH values (from 4.0 to 9.0) and a fixed contact time (60 min). The results obtained are summarized in Fig. 3 and in Table S1 in the ESI.† Both the alginate beads with no protein and the hybrid Alg-SSF materials show a maximum adsorption of IgG from human serum at pH 5.0, with 70–74% of recovery yield (adsorbed IgG), and where the hybrid material presents a slightly better performance to adsorb IgG. According to the literature, the isoelectric point of polyclonal IgG is between 6.3–9.0.<sup>31,32</sup> Thus, at pH 5.0, IgG is positively charged, and electrostatic interactions can occur with the negatively charged beads. Alginic acid has negatively charged groups at neutral and acidic pH due to the speciation of carboxyl groups ( $\text{p}K_{\text{a}} = 3.5$ ),<sup>42,45</sup> thus capable of interacting by electrostatic interactions with IgG. At pH 4.0 IgG is also positively charged; nevertheless, this pH is close to the  $\text{p}K_{\text{a}}$  of the carboxyl groups of alginic acid, and therefore lower recovery yields in terms of IgG adsorption occur at pH 4.0. These results highlight the relevance of electrostatic interactions between IgG and the beads, although other interactions such as hydrogen bonding and hydrophobic interactions cannot be discarded. Based on this set of results, the pH value of 5.0 was selected for the subsequent studies.

The SE-HPLC chromatogram of diluted human serum aqueous solution (SHS), shown in Fig. 3b (black line), is characterized by three main peaks, corresponding to protein aggregates (retention time of  $\sim 13$  min), the target IgG (reten-

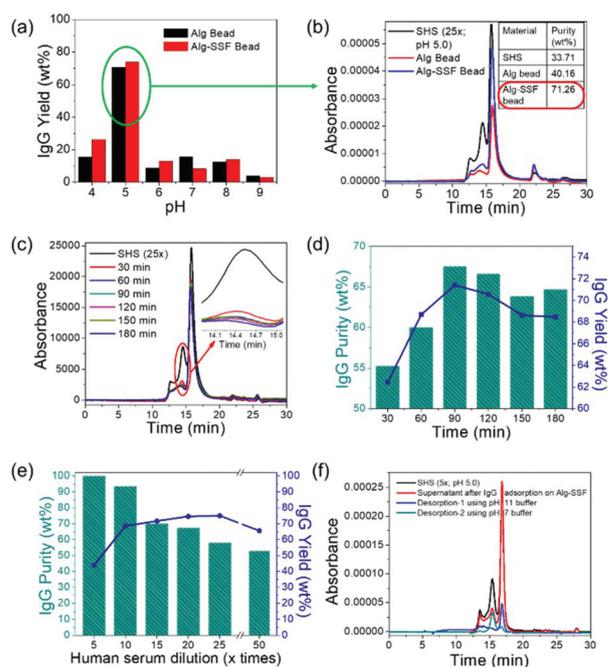


Fig. 3 Optimization of several operational conditions to improve the IgG purity and recovery yield: (a) optimization of pH from 4.0–9.0; (b) SE-HPLC chromatograms of the supernatant after separation of IgG at pH 5.0; (c) SE-HPLC chromatograms of the supernatant after adsorption of IgG at variable contact times; (d) IgG purity and yield at variable contact times; (e) IgG purity and yield at several serum dilutions; and (f) SE-HPLC chromatograms of human serum, supernatant after adsorption of IgG using Alg-SSF hybrid cryogel beads under the optimized conditions and after two desorption steps.

tion time  $\sim 14.8$  min), and the high-abundant human serum albumin (retention time *ca.* 15.8 min). Being the most abundant protein in serum ( $\sim 66\%$ ), the adsorption of human serum albumin (HSA) onto the beads largely affects the purity of the adsorbed IgG.

The purity of IgG in the original serum sample is *ca.* 34%. After the solid-phase extraction with alginate beads (Alg beads) and with hybrid alginate–protein cryogels (Alg-SSF beads) there is an improvement in the IgG purity from 34% to 40% and 71%, respectively (Fig. 3b). The SE-HPLC chromatograms of the supernatant (Fig. 3b) at pH 5.0 reveal a more selective adsorption of IgG from human serum onto the hybrid alginate–protein beads over the alginate beads with no proteins. In particular, with hybrid materials there is a significant decrease of the peak corresponding to IgG whereas a slight decrease of the peak corresponding to HSA occurs. Based on these results, it is clear that incorporation of SSF is advantageous to improve the material selectivity for IgG. SSF is a polypeptide consisting of proteins possessing high quantities of glycine and alanine amino acids, and lower amounts of other amino acids such as glutamine, serine, leucine, valine, proline, tyrosine, and arginine. On the other hand, it is known that antibodies such as IgG interact with antigens through specific binding sites. For instance, for a particular antigen rhodopsin, it was observed that IgG interacts with the alanine

residue, and that interactions with the glycine residue do not occur.<sup>46,47</sup> In the case of Alg-SSF, apart from the interactions triggered by the alginate as discussed above and where the pH plays a major role, the amino acids and eventually the alanine residue present in SSF may be responsible for the higher selectivity for IgG afforded by the hybrid material. With the exception of pH 7 and 9, the IgG adsorption on Alg beads is lower when compared to the hybrid materials, reinforcing the possibility of hydrogen-bonding and hydrophobic interactions occurring between the target antibody and proteins present in the material. It has been reported that at lower pH values alginate beads shrink, while at higher pH they tend to swell.<sup>48</sup> Furthermore, it was shown that calcium alginate beads tend to display an oblate shape at lower pH values and a spherical shape at higher pH values.<sup>49</sup> Accordingly, these differences in the material morphology may be also contributing to the different IgG adsorption behaviour at different pH values and cannot be discarded.

After proving that the Alg-SSF hybrid cryogel beads are selective materials for IgG, the contact time was optimized at pH 5.0. The SE-HPLC chromatograms and IgG yield and purity data after adsorption of IgG onto Alg-SSF are shown in Fig. 3c and d. Both the yield and purity of the adsorbed IgG onto the hybrid cryogel beads increase up to 90 min of equilibrium, after which both parameters decrease due to the desorption of IgG and further adsorption of HSA (Fig. 3d). Overall, this set of results reveals that the highest yield and purity of IgG with Alg-SSF hybrid cryogel beads occur at pH 5.0 and 90 min of contact. Comparing the IgG equilibrium time with other alginate-based materials, *e.g.* cibacron blue F3GA immobilized onto epoxide chitosan/alginate,<sup>42</sup> the studied Alg-SSF hybrid cryogel beads reduce the required adsorption time by half.

The effect of the human serum dilution on the adsorption of IgG onto Alg-SSF hybrid cryogel beads at the previously optimized parameters is shown in Fig. 3e. The IgG yield (adsorption onto the hybrid materials) slightly increases with the increased dilution of human serum, whereas its purity decreases. Remarkably, the 5 times diluted human serum aqueous solution leads to the highest purity (94%) of adsorbed IgG. Although with a small compromise in the yield (64%), since IgG is a high-cost product used for therapeutic purposes, the 5 times serum dilution is in our opinion the best condition to purify IgG using the prepared hybrid Alg-SSF cryogel beads under the previously optimized conditions.

Aiming at better understanding the superior performance of the hybrid materials to selectively adsorb IgG, adsorption isotherms using commercial/pure IgG were determined, as given in Fig. 4. Adsorption isotherms are fundamental to characterize the interactions occurring between the material surface and the target protein. The isotherm parameters were determined by non-linear fitting of the Langmuir and Freundlich models to the experimental data. The modelling and determined parameters are given in the ESI.†

Considering the correlation coefficient ( $R^2$ ), the experimental equilibrium data were best fitted with the Langmuir model, with a Langmuir's equilibrium constant ( $k_d$ ), which

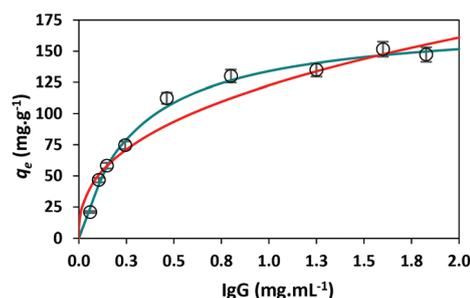


Fig. 4 Langmuir (green line) and Freundlich (red line) model isotherms for the IgG adsorption onto Alg-SSF hybrid cryogel beads. The open symbols correspond to experimental data and solid lines correspond to the fitting (nonlinear regression) of the experimental data.

describes the strength of the interactions between proteins and the material surface,<sup>33,41</sup> of 3.3. The poorer performance of the Freundlich model in describing the experimental data reveals that the formation of IgG multilayers, potentiated by IgG–IgG interactions, does not occur. According to the Langmuir isotherm, the maximum adsorption capacity of the hybrid material ( $q_m$ ) is 175 mg of IgG per g of Alg-SSF cryogel beads. A different behaviour was observed for the adsorption of human IgG on cibacron blue F3GA immobilized onto epoxide chitosan/alginate.<sup>42</sup> According to this work, the cooperative Langmuir–Freundlich model correlated better than the Langmuir model, whereas a  $q_m$  Langmuir–Freundlich parameter of 99.1 mg g<sup>-1</sup> was obtained, which is lower than the one obtained in the current work. Although both materials are based on an alginate matrix, it must be pointed out that these differences in IgG adsorption capacity are probably associated with the different modifications over the alginate surface, such as functional groups, surface area and ligand loading, which in our case is improved by the preparation of hybrid materials incorporating proteins. In the literature, adsorption isotherms of human IgG using protein A immobilized on agarose<sup>43</sup> are well fitted using the Langmuir model, with a maximum capacity of 17 mg g<sup>-1</sup>, whilst the data obtained with commercial protein A immobilized on iron oxide magnetic particles<sup>44</sup> are better fitted by the Freundlich model with a maximum capacity of 109 mg L<sup>-1</sup>, the Langmuir model not being adequate for this material. Overall, the hybrid alginate–protein cryogel beads proposed in this work show a higher adsorption capacity than those previously reported, which is highly advantageous when designing cost-effective and sustainable purification platforms for high-cost products.

After the evaluation of all adsorption conditions, such as cryogel bead material type, pH, adsorption time and human serum dilution, the final desorption studies of IgG from the hybrid materials were carried out. Fig. 3f shows the SE-HPLC chromatograms of human serum, of the supernatant after adsorption of IgG using Alg-SSF hybrid cryogel beads, and of the aqueous solutions containing IgG after the two desorption steps. The initial purity of IgG in human serum was found to be 34%. During the first desorption step (blue chromatogram),

when the cryogel bead-IgG conjugate was incubated in water at pH 11.0, a main peak corresponding to HSA and two minor peaks corresponding to IgG and IgG aggregates were detected in the supernatant. This means that HSA is preferentially desorbed from the Alg-SSF. Although a small amount of IgG is also desorbed (25% of the total amount), the major fraction of IgG remains adsorbed on the surface of the beads. After the second desorption step, when the cryogel beads were incubated in water at pH 7.0, IgG is the main protein desorbed, indicating that the interactions between IgG and cryogel beads can be significantly reduced at pH 7.0. These two desorption steps were optimized and are needed to first remove the non-target proteins, followed by the recovery of IgG at more amenable pH values. After the two step process, the purity of IgG in solution is ~80%, with a desorbed yield of IgG from the beads of 91%. Although a promising IgG purity is reached in this work it should be remarked that affinity chromatography leads to values of purity higher than 98%.<sup>1</sup> However, this type of affinity chromatography requires biological ligands of high cost and low stability, being responsible for the IgG current high cost.<sup>1</sup> Even though lower purification values are obtained by us when compared to benchmark technologies, we are using low-cost and bio-based materials with no specific ligands. The IgG purity may be further increased by improving the material selectivity, using *e.g.* other waste proteins to produce hybrid beads, or by applying an additional step, such as ultrafiltration,<sup>47</sup> to the recovered supernatant comprising IgG.

Although no hybrid protein-alginate cryogel beads have been investigated to date, some works can be found in the literature resorting to the use of polysaccharide beads or nanoparticles to purify IgG. Jain and Gupta<sup>50</sup> investigated the purification of IgG from goat serum using alginate beads charged with Cu(II). Under the optimized conditions of copper content and in batch experiments the IgG recovery after the desorption step was 66.4% with a purification of 6.8 times. Kavaz *et al.*<sup>51</sup> studied the separation of IgG from human plasma using chitosan nanoparticles. After the purification step, the adsorption ratio of IgG was 63 mg g<sup>-1</sup> with a desorption ratio >95% of the adsorbed IgG. The purity and yield were however not given in this study.<sup>51</sup> Dextran-coated iron oxide magnetic particles modified with ligand 22/8 led to a final IgG purity of 95% in the eluted fraction; nevertheless, from 56% of the total protein bound to the support, a small recovery of 5 and 16% of the total protein at pH 3 and 11, respectively, was obtained.<sup>13</sup> Applied to the same type of matrix, the capture of IgG with magnetic nanoparticles coated with protein A and starch allowed 69% of IgG recovery.<sup>14</sup> The best results of adsorption of IgG from human plasma reported to date were achieved with nano-poly(ethylene glycol dimethacrylate-2-methacryloylamidohistidine) nanoparticles, yielding an IgG purity of 94% and a desorption rate of 97%, although the IgG recovery yield was not reported. Affitin D1Sso7d-DM (anti-IgG) was used to prepare affinity/magnetic adsorbents for the purification of IgG from human plasma. Under the optimised conditions, the recovery of IgG was above 95% with a purity of 81%.<sup>15</sup> Overall, promising results have been reported, but it should be

remarked that the materials used in this work are of low-cost and bio-based, while allowing to obtain IgG with a purity of ~80%, with a desorption yield of IgG from the beads of 91%, and a maximum adsorption capacity of IgG of 175 mg g<sup>-1</sup>. Furthermore, the current materials can be regenerated and reused, as shown below, thus contributing to the development of sustainable purification strategies.

### Characterization of the purified IgG

The purity and structural stability of IgG before and after purification with Alg-SSF hybrid cryogel beads were evaluated by SE-HPLC and CD spectroscopy. Although SE-HPLC was used to quantify IgG and other protein impurities, this technique was also used to evaluate the structural integrity of the purified IgG since the aggregation or degradation of proteins will affect the respective retention times. The SE-HPLC chromatograms of standard human IgG, standard human serum and purified IgG from human serum are shown in Fig. 5a. This technique is important to evaluate the formation of protein aggregates since aggregation reduces the content of the biologically active protein, which results in a lower therapeutic efficiency and may be fatal for patients who have a variety of diseases involving protein aggregation.<sup>52</sup> According to the results depicted in Fig. 5a, for both standard IgG and purified IgG, a peak corresponding to IgG at a retention time of ~15.6 min is obtained. Before the purification step, the presence of peaks at lower retention times, ~13.5 min, is noticed indicating undesired IgG aggregates and/or impurity protein aggregates present in human serum. However, after the purification step, no protein aggregates are observed, meaning that these are completely eliminated after the IgG purification with the hybrid materials. Compared to standard protocols for IgG purification, a high removal of protein impurities is obtained with Protein A or G affinity chromatography. Nonetheless, a significant amount of aggregates is still observed with these approaches,<sup>53</sup> further requiring additional polishing steps, such as hydrophobic interaction, gel-filtration or ion-exchange.<sup>54</sup> In this work, IgG is purified with the complete removal of protein aggregates.

The secondary structure of purified IgG was evaluated by circular dichroism (CD) analysis. The CD spectra of standard human IgG, human serum and purified IgG from human serum after the desorption steps using Alg-SSF hybrid cryogel beads,

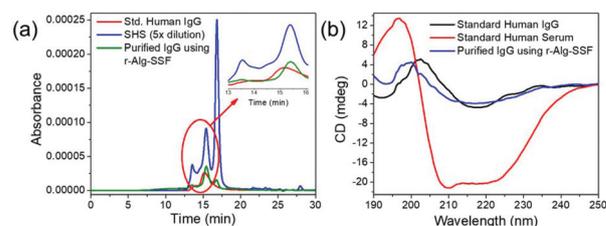


Fig. 5 (a) SE-HPLC chromatograms and (b) CD spectra of standard human IgG, human serum and purified IgG from human serum using Alg-SSF hybrid cryogel beads under the optimized conditions.

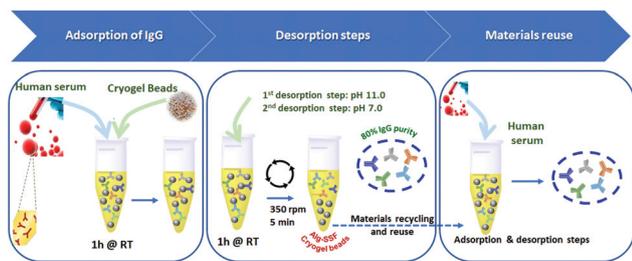


Fig. 6 Schematic representation of the use of the prepared hybrid cryogel beads to purify IgG and their regeneration and reuse.

under the optimized conditions, are presented in Fig. 5b. The CD spectrum of the purified IgG shows a single band (with a positive ellipticity), which is very similar to that shown by the standard IgG, in both the amplitude and magnitude in the range of 200–250 nm. Proteins with well-defined antiparallel  $\beta$ -plated sheets ( $\beta$ -helices) have negative bands at  $\sim$ 218 nm and positive bands at  $\sim$ 195 nm, whereas disordered proteins have very low ellipticity above 210 nm and negative bands near 196 nm.<sup>54</sup> At  $\sim$ 228 nm, small differences can be observed between the standard and purified IgG confirming that no conformational changes, associated with second tryptophan side chains (dominant  $\beta$ -sheet structure), occurred.<sup>55</sup> These results indicate that the purified IgG, obtained after the optimized purification process, maintains its native secondary structure. A different spectrum was obtained however for human serum since this is a complex sample containing different proteins.

### Recovery and reuse of hybrid cryogel beads

The sustainability of the IgG purification process was finally addressed by the regeneration and reuse of the hybrid cryogel beads. After the IgG purification and desorption steps, the recovered Alg-SSF cryogel bead materials were dissolved in aqueous media and restructured using an extrusion dripping method, as mentioned above. The restructured Alg-SSF beads were further used for the purification and desorption of IgG from human serum without compromising the IgG purity and yield, with similar values of  $\sim$ 80% and  $\sim$ 90% of purity and recovery yield obtained (Fig. S2a in the ESI†). The secondary structure of the purified IgG using the recovered Alg-SSF cryogel beads also remains unchanged, as shown in Fig. S2b in the ESI.† Overall, the prepared hybrid materials can be recovered and reused without compromising their separation performance and IgG stability, thus contributing to the process sustainability and the decrease of the IgG final cost.

A schematic representation showing the process sustainability comprising the material reuse to purify IgG from human serum is depicted in Fig. 6.

## Conclusions

Bio-based hybrid alginate–protein cryogel beads were successfully prepared, characterized and used to purify high-cost pro-

teins, namely immunoglobulin G from human serum. The introduction of proteins in the materials allows the improvement of the material selectivity for IgG and its purification. Several operating parameters, namely pH, contact time and serum dilution, were optimized to improve the IgG purity and recovery yield. After the adsorption of IgG under the best identified conditions, the desorption of IgG from the hybrid cryogel beads was addressed, obtaining IgG with a purity of 80% and a recovery yield of the adsorbed protein of 91%. The stability and integrity of the recovered IgG were confirmed by SE-HPLC and CD. The sustainability of the purification process was finally ascertained, by regenerating and reusing the hybrid materials, with no losses in their separation performance and in the IgG stability. Although several materials have been investigated and a wide range of affinity ligands are under continuous research to replace the high-cost standard methods to purify IgG, this work demonstrates the potential of bio-based and low-cost materials to purify this added-value protein, while paving the way for their use in the separation of other high-value products.

## Conflicts of interest

There are no conflicts to declare.

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