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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 constraint of 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 cargos. 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 materials selectivity for IgG, which is due to specific interactions occurring between the target antibody and amino acids 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). At 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 is kept after the desorption step. Finally, the regeneration and reuse of the cryogel beads was evaluated, with no losses on the IgG adsorption performance and antibody stability. Although significant efforts have been placed 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 materials selectivity.

Introduction

Significant advances in therapeutic applications have been achieved in the past decades with the use of antibodies or immunoglobulins (Igs) as biopharmaceuticals. Antibodies are glycoproteins present in blood serum and other body fluids of vertebrates. Their function is to identify and eliminate pathogens, such as viruses, bacteria or fungi, through a recognition mechanism of high specificity. The growing interest in immunoglobulin G (IgG) is a 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. 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 requiring a number of additional steps for the efficient removal of impurities. Albeit 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 on 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 stems, particularly aqueous two-phase systems that have been largely explored for such a purpose, 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. 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. In addition to these works focused on the recovery of monoclonal antibodies from culture cells, nano-polyethylene...
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 D15so7d-DM (anti-IgG) was used to prepare affinity/magnetic adsorbents, also for the purification of IgG from human plasma. 15 At the optimised conditions, the recovery of IgG was above 95% with a purity of 81%. 15 Based on the possibility of using solid-phase extractions to purify IgG, we focused on the development of bio-based and low-cost materials, namely hybrid cryogel beads composed of polysaccharides (alginate) and proteins fibrils. In this work, we avoided the materials 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). They are highly relevant biopolymers for various applications due to their relevant features, such as biodegradability, biocompatibility, non-toxicity and low-cost. 19-20 They can be processed in various formats, such as beads, fibres, films, capsules, blends with other natural and synthetic polymers, etc. 20-23 Furthermore, alginic acid allows the formation of water insoluble calcium alginate beads able to encapsulate therapeutics and/or other macromolecular agents, 24-28 and with high efficiency to adsorb metal ions, dyes or proteins. 29-35 Aiming at improving their physicochemical properties and selective adsorption features, cryogel beads can be prepared by combining polysaccharides and protein fibrils, resulting in hybrid materials. Protein fibrils are aggregated proteins that have attracted considerable interest in the preparation of functional biomaterials, including foams, composites, films and gels. 29-37 These biomaterials are suitable for various biomedical applications, such as biosensors, drug delivery systems, tissue regeneration, cell encapsulation, wound dressing, among others. 29,37 Amongst proteins able to produce protein fibrils, spider silk fibres (SSF) are a natural lightweight protein with excellent physicochemical and mechanical properties. 38 SSF are of low cost, and display biocompatibility, biodegradability, non-toxicity and cell adhesion properties, making them useful in various biomedical applications. 38 Based on these properties, alginate and protein fibrils (hybrid) cryogel beads can be considered promising adsorbent materials, although never investigated in the purification of proteins or antibodies such as IgG.

Given the novelty of the described materials, in this work, the 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 Microscopic (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 of low-cost and bio-based materials, the recovery of IgG from the cryogel beads and their reuse was finally addressed, with no significant losses on 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 °C 28 `N, 69 °C 05` E), following a previously reported method. 39 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% Na2CO3 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 dry to obtain the polysaccharide, with a yield of 25 ± 2 wt% with respect to the seaweed used.

AR grade isopropyl alcohol and calcium chloride (CaCl2) were purchased from Sisco Research Laboratories, Mumbai, India. Sodium alginate, cholinium bicarbonate (80% in water), sodium sulphate (Na2SO4), dipotassium phosphate (K2HPO4), monopotassium phosphate (KH2PO4), IgG and human serum were purchased from Sigma Aldrich, USA. Tetrabutylammonium hydroxide (TBAB, 40 wt%) 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. 38 SSF were collected from naturally accumulated locations in the laboratory building of CSIR-CSMCRI, Bhavnagar (21.7600° N, 72.1500° E). Collected SSF were washed with water for several times to remove dust prior to their degumming and tetrabutyl ammonium hydroxide (TBAB, 40% aqueous solution) pre-treatment. To obtain TBAB treated SSF, 5 mg of degummed SSF was added to a vial containing 1.0 g TBAB (40 wt% in H2O) followed by stirring at room temperature until the complete dispersion of SSF in TBAB. 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. 30 Briefly, 0.5 g of sodium alginate was dissolved in 25 mL milli-Q water. This solution was added dropwise to a CaCl2 (2 wt%) aqueous solution under continuous stirring (300 rpm) at 30°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 CaCl2 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 fibrils 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 CaCl2 (2 wt%) aqueous solution (under continuous stirring at 300 rpm and at 30°C). The nearly round shaped algin-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, microscopic and thermogravimetric techniques. Fourier transform-infrared spectroscopy (FT-IR) spectra of degummed SSF and cryogel beads were recorded on a Perkin-Elmer FT-IR instrument (Spectrum GX, GSA) using KBr disc. The powder X-ray diffraction (PXRD) spectra of cryogel beads were carried out on a Philips X’Pert MPD system using Cu-Kα radiation (λ = 0.15405 nm) with 2θ range from 2° to 80° at a scan rate of 0.1° s⁻¹. Thermo gravimetric analysis (TGA) of SSF and cryogel beads were carried out on a NETZSCH TG 209F1 Libra TGA209F1D-0105-L instrument using a temperature programmer from room temperature to 500°C at a heating rate of 10°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 materials 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 ± 1) °C using 5 mg of cryogel beads in contact with 500 µL 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. The concentration of human IgG was evaluated by diluting the serum from 5 to 50 times in glycine buffer, pH 5.0. At 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 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 are 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 first the non-target proteins, by taking advantage of 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 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 w% 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 CaCl₂ (2 w%) 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 x 300 mm). The mobile phase was prepared with 100 mM sodium phosphate buffer pH 7.0 containing NaCl 0.3 M and run isocratically with a flow rate of 0.5 mL min⁻¹. 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_iG) and IgG recovery yield (Y_iG) were determined by the following equations,

\[ P_{iG} = \frac{C_{iG} \times 100}{C_{total}} \]  
\[ Y_{iG} = \frac{C_{iG} \times 100}{C_{total}} \]

where \( C_{iG} \) is the concentration of IgG adsorbed on the material, \( C_{total} \) is the total concentration of all proteins (IgG and other proteins) adsorbed on the material and \( C_{iG} \) 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 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⁻¹ and band width 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 Na₂SO₄ 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 reveal 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 fibrils (Alg-SSF) in the hybrid cryogel beads. The IR stretching band of amide-I (C=O stretching vibration) and amide-II (C-N stretching vibration) appear in the range of 1620-1700 cm⁻¹ and 1500-1580 cm⁻¹, respectively. After the preparation of cryogel beads with CaCl₂ aqueous solution, amide-I and amide-II stretching vibration bands merge, with a broad stretching vibration in the range of 1500-1700 cm⁻¹, confirming the existence of protein fibrils in the hybrid cryogel beads.

The crystalline behaviour of protein fibrils and cryogel beads with and without protein fibrils was additionally addressed by PXRD, whose results are depicted in Fig. 2b. SSF exhibit a sharp peak at 2θ = 24°, indicative of the crystalline nature of the protein fibres due to
their β-sheet. 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 fibrils.

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

The thermogravimetric analysis of sodium alginate powder, protein fibrils 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°C, alginate beads start to degrade at ~206°C, and Alg-SSF materials start to degrade at ~241°C.

Figure 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-SFF beads).

Overall, hybrid cryogel beads incorporating protein fibrils 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, 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. 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 (pKa = 3.5) and, 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 pKa of 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 ~13 min), the target IgG (retention time ~14.8 min), and the high-abundant human serum albumin (retention time ca. 15.8 min). Being the most abundant protein in serum (~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 protein fibrils. 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 materials 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. In the case of the 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. Furthermore, it was shown that calcium alginate beads tend to display oblate shape at lower pH values and spherical shape at higher pH values. Accordingly, these differences in the materials 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 yield and purity of 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, the studied Alg-SSF hybrid cryogel beads reduce the required adsorption time by half.

Figure 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 at the optimized conditions and after two desorption steps.

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 at 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, being given in Fig. 4. Adsorption isotherms are fundamental to characterize the interactions occurring between the materials 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 (R²), the experimental equilibrium data were best fitted by the Langmuir model, with a Langmuir’s equilibrium constant (kL), which describes the strength of interactions between proteins and the materials surface, of 3.3. The poorer performance of the Freundlich model on describing the experimental data reveals that the formation of IgG multilayers, potentiated by IgG-IgG interactions, do not occur. According to the Langmuir isotherm, the maximum adsorption capacity of the hybrid material (qₑₓ) 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. According to this work, the cooperative Langmuir–Freundlich model correlated better than the Langmuir model, whereas a qₑₓ Langmuir–Freundlich parameter of 99.1 mg.g⁻¹ 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 that these differences on 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 are well fitted using the Langmuir model, with a maximum capacity of 17 mg.g⁻¹, whilst data obtained with commercial protein A immobilized on iron oxide magnetic particles are better fitted using the Langmuir model, with a maximum capacity of 109 mg.L⁻¹, being the Langmuir model not 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 materials type, pH, adsorption time and human serum dilution, 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, 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 steps process, the purity of IgG in solution was ~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%. However, this type of affinity chromatography requires biological ligands of high cost and low stability, being responsible for the IgG current high cost. 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. IgG purity may be further increased by improving the materials selectivity, using e.g. other waste proteins to produce hybrid beads, or by applying an additional step, such as ultrafiltration, to the recovered supernatant comprising IgG.

Albeit no hybrid protein-alginate cryogel beads have been investigated up to date, some works can be found in the literature resorting to the use of polysaccharides beads or nanoparticles to purify IgG. Jain and Gupta investigated the purification of IgG from goat serum by alginate beads charged with Cu(II). At 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. studied the separation of IgG from human plasma using chitosan nanoparticles. After the purification step, the adsorption ratio of IgG was of 63 mg.g⁻¹ with a desorption ratio >95% of the adsorbed IgG. The purity and yield were however not given in this study. 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, were obtained. At the same type of matrix, the capture of IgG with magnetic nanoparticles coated with protein A and starch allowed 69% of IgG recovery. The best results to adsorb IgG from human plasma reported up 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. Affinity D15so7D-DM (anti-IgG) was used to prepare affinity/magnetic adsorbents for the purification of IgG from human plasma. At the optimised conditions, the recovery of IgG was above 95% with a purity of 81%. 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⁻¹. 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 was 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 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. 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, it is noticed the presence of peaks at lower retention times, ~13.5 min, 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. Comparing 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, further requiring additional polishing steps, such as hydrophobic interaction, gel-filtration or ion-exchange. In this work, IgG is purified with the complete removal of protein aggregates.

Figure 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 at optimized conditions.

The secondary structure of purified IgG was evaluated by circular dichroism (CD). 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, at 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 β-plated sheets (β-helices) have negative bands at ~218 nm and positive bands at ~195 nm, whereas disordered proteins have very low ellipticity above 210 nm and negative bands near 196 nm. At ~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 β-sheet
structure), occurred. 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 ~80% and ~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 decrease of the IgG final cost.

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

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

**Conclusions**

Bio-based hybrid alginate-protein cryogel beads were successfully prepared, characterized and used to purify high-cost proteins, namely immunoglobulin G from human serum. The introduction of proteins in the materials allows to improve 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 at the best identified conditions, the desorption of IgG from the hybrid cryogel beads was addressed, allowing to obtain IgG with a purity of 80% and a recovery yield of the adsorbed protein of 91%. The stability and integrity of the recovered IgG was 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 on their separation performance and on 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 investigation in the separation of other high-value products.

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