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Evaluation of human interferon adsorption performance of Cibacron Blue F3GA attached cryogels and interferon purification by using FPLC system

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ABSTRACT

In this study, we have focused our attention on preparing supermacroporous cryogels as a potential dye-affinity adsorbent for interferon purification. For this purpose, 2-hydroxyethyl methacrylate (HEMA) and Cibacron Blue F3GA (CB) were selected as main monomer and dye–ligand. Cibacron Blue F3GA attached supermacroporous poly(2-hydroxyethyl methacrylate) [poly(HEMA)/CB] cryogels were prepared and characterized by swelling test, scanning electron microscopy, elemental analysis, and FTIR. After that, the effecting factors such as pH, concentration, interaction time, and ionic strength on the interferon separation were evaluated. The maximum adsorption capacity of poly(HEMA)/CB cryogels was obtained as 38.2 mg/g at pH 6.0. Fast protein liquid chromatography (FPLC) system was used for interferon purification from human gingival fibroblast extract. The chromatography parameters, capacity and selectivity factors, resolution and theoretical plate number were found as 7.79, 9.62, 4.23 and 554, respectively. Although some decreases in total protein content, from 320 μg to 18 μg, and interferon activity, from 2.6×10^3 IU to 2.2×10^3 IU, were determined, specific antiviral activity increased from 7.19 IU/μg to 122.2 IU/μg. The purified interferon samples have 97.6% purity determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After repeated ten adsorption–desorption cycles, no significant decrease was determined in adsorption capacity of cryogel. In result, poly(HEMA)/CB cryogels have an application potential for rapid, cheap and specific purification of interferon.

1. Introduction

Over the last decades, there has been increasing interest and innovations in the field of biotechnology, biomedicine and applied biochemistry including research and development of vital biochemicals and biopharmaceutical compounds [1]. In proportion to the ascending scientific treatment, extraordinary development and expanding use of biomolecules, especially proteins, in the medical, food, drug and cosmetic industries, commercial values of treated biomolecules also increased [2]. Many of the biomolecules must be separated and purified before use and it is urgently needed to meet requirements for high purity and yield. Because of some difficulties and limitations, numerous separation procedures mainly based on the physicochemical and biological properties of the interested molecule such as antibodies, cytokines, enzymes, therapeutic DNA, and plasmids have been under investigation [3]. Among the numerous studied techniques, adsorption based separation and purification methodologies are widely investigated phenomenon having its origin in several different disciplines of science [4].

Cryogels are considered as one of the new types of polymeric hydrogels with a considerable potential use in biotechnology [5]. Beside the general advantages, i.e. high chemical and physical stability, easy protein adsorption and elution capability, low-cost and reusability, of mostly used polymeric supports, cryogels are very good alternatives for protein purification with additional advantages such as large pores, short diffusion path, and low pressure drop [6]. For example, short diffusion path allow optimal utilization of the immobilized ligand on the pore walls [7]. Osmotic stability can be also listed as one of the main advantages of cryogels [8]. They are gel matrices formed in moderately frozen solutions of monomeric or polymeric precursors and typically have interconnected macropores (or supermacropores), allowing unhindered diffusion of solutes of practically any size such as nano- and even microparticles [9]. Cryogels can be used as a monolithic form as well as in a membrane form to combine the advantage of membrane chromatography, in terms of high flow-rates and high productivity [5]. They allow high flow-rates enabling the processing of large volumes within short process times for both adsorption and elution processes. They are cheap materials and they can be used as

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disposable avoiding cross-contamination between batches [10]. Hence, it is possible to use cryogels in chromatographic separation of biological nanoparticles (plasmids, proteins, viruses, cell organelles) and even all of cells (*Escherichia coli*) from natural sources [11].

In affinity chromatography, numerous molecules, including enzymes, coenzymes, cofactors, antibodies, amino acids, oligopeptides, proteins, nucleic acids, and oligonucleotides may be used as bioligands for designing the novel and specific adsorbents [12]. Because of their restrictions and precautions in the adsorption and elution studies, difficulties in retention of their original biological activity, storage stability, their needing to extensive purification processes, high cost of production and complexity in the immobilization of these bioaffinity ligands, the numbers of the attempts and interest in developing biomimetic ligand are getting more. The synthetic dye–ligands have been considered as important alternatives for affinity chromatography; hence, dye-affinity chromatography is an effective and widely used method for the purification of biomolecules [13]. Dye–ligands are commercially available, inexpensive, and can be easily immobilized, especially on solid matrices bearing hydroxyl groups and able to bind most types of proteins in a remarkably specific manner [14]. It should be especially pointed out that, although dyes are all synthetic in nature, they are still classified as affinity ligands because they can interact with the active sites of many proteins by mimicking the structure of the substrates, cofactors, or binding agents for those proteins. Therefore, a number of textile dyes, also known as reactive dyes, have been used for protein purification [15]. Dye–ligands can exhibit selectivity resulting from the cumulative effects of multiple weak binding such as electrostatic, hydrophobic, hydrogen binding and van der Waals interactions with fast kinetics; in spite of having low binding constants (10^{-4} to 10^{-6} M^{-1}) [16,17].

Interferons are biologically active and very important proteins for the immune system of mammalian species. They can be classified as a member of large group of glycoproteins known as cytokines and were named as "interferon" because of their ability to interfere with viral proliferation [18]. Interferons are most rapidly synthesized by peripheral blood leukocytes, lymphoblastoid and myeloblastoid cell lines on somatic cells in response to an appropriate stimulus in the presence of pathogens such as viruses, bacteria, or parasites, or other antigens or tumor cells [19]. Then, they are secreted into the surrounding medium, bind to receptor on target cells and induce transcription of some genes and these results in an anti-viral state in the target cells [20]. Although they modulate specific cellular functions in some cases, the main function of these proteins in the mammalian bodies is preventing viral replication in newly infected cells as gain a resistance to infection especially in the first line of defense against viral infections [21]. Beside the uses of interferons in treatments of kidney cancer, multiple myeloma, carsinoid, lymphoma and leukemia, especially recombinant human interferon-α, that comprises a family of extracellular signaling proteins with antiviral, antiproliferating and immunomodulatory activities, is especially used for the treatment of AIDS-related Kaposi's sarcoma, hairy cell leukemia, and chronic hepatitis B and C [22]. Therapeutic value of interferons against certain types of tumors such as brain tumors and malignant melanomas caused both increasing interest in these proteins [23] and more focusing on investigations aimed to obtain treated and purified interferons [24]. The purification of human interferons from various sources has been attempted by a variety of methods including metal-chelation, precipitation, cation or anion-exchange, gel filtration, hydrophobic and immunoaffinity chromatography over many years and some protocols have been proposed that yield homogenous protein [25–29].

According to increase in demand of interferon market, there is a necessity to develop simple, fast and effective techniques for the purification of them. Hence, the design and application of novel affinity sorbent for the adsorption of recombinant human interferon-α (rHuIFN-α) from aqueous solution and the purification from human gingival fibroblast were studied and reported here. Firstly, poly(HEMA) cryogels were synthesized by cryopolymerization of HEMA monomer. Then, Cibacron Blue F3GA was covalently attached on them through substitution reaction between triazine groups of the dye molecules and hydroxyl groups of HEMA. After that, Cibacron Blue F3GA attached poly(HEMA) [poly(HEMA)/CB] cryogels were characterized using swelling test, FTIR, elemental analysis, and SEM. After the characterization step, system parameters such as adsorption rate, pH, rHuIFN-α concentration and ionic strength were investigated to evaluate their effects on the rHuIFN-α adsorption performances of dye-affinity cryogels. The reusability of the cryogels was also tested. Finally, poly(HEMA)/CB cryogels were used for interferon purification from natural source, human gingival fibroblast.

2. Experimental

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA), N,N′-methylene bisacrylamide (MBAAm) and ammonium persulfate (APS) were obtained from Sigma (St. Louis, USA). N,N,N,N′′-tetramethylene ethylenediamine (TEMED) was purchased from Fluka A.G. (Buchs, Switzerland). Recombinant human interferon-α (rHuIFN-α) (freeze-dried powder; 99% pure by RP-HPLC) was supplied from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). Cibacron Blue F3GA was purchased from Polyscience (Warrington, USA) and used as received. All other chemicals were of reagent grade and purchased from Merck A.G. (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion-exchange packed-bed system.

2.2. Preparation of poly(2-hydroxyethyl methacrylate) [poly(HEMA)] cryogels

A typical preparation procedure is as follows: HEMA monomer (0.5 ml) and crosslinker N,N′-methylene bisacrylamide (100 mg) were dissolved in 10.0 ml deionized water and; then, nitrogen gas was passed through the solutions under the vacuum (100 mmHg) for removal of dissolved oxygen gases for 5 min. The total monomer concentration is 6.0%. The cryogels were then produced by free radical polymerization initiated by TEMED (120 μl) and APS (10 mg). After adding APS (1% of the total monomers, w/v) the solution was cooled in an ice bath for 2–3 min. TEMED (1% of the total monomers, w/v) was added and the reaction mixture was stirred for 1 min. Then, the reaction mixture was divided and poured into sealed glass tubes (i.d. 5 mm × 20 mm). The polymerization solutions in the glass tubes were frozen at −12 °C for 24 h and then thawed at room temperature. After that, cryogels were washed with water and ethanol three times; then, stored in buffer containing 0.02% sodium azide at 4 °C until use.

2.3. Cibacron Blue F3GA attachment onto poly(HEMA) cryogels

Dye attached cryogels were obtained by immobilization of dye–ligand onto poly(HEMA) cryogels [30]. Immobilization studies were performed in the shaking water bath for controlling the reaction temperature. Cibacron Blue F3GA was dissolved in 50 ml of water (dye concentration: 5 mg/ml). Then, this aqueous dye solution was transferred to 50 ml of distilled water containing

poly(HEMA) cryogels. After that, 5.0 g of NaOH was added to the mixture. The medium was heated at 80 °C in a sealed reactor and stirred in a shaking bath at 125 rpm for 4 h. Immobilization was followed by observing depletion of absorbance of dye solution at 630 nm, maximum wavelength of Cibacron Blue F3GA. In order to remove the non-specifically attached dye molecules, an extensive cleaning procedure was applied by using deionized water, ethanol and water:ethanol mixtures as solvents. At the last stage, cryogels were washed with deionized water and stored in a solution containing 0.02% sodium azide to prohibit microbial contamination at 4 °C.

2.4. Characterization of poly(HEMA) cryogels

Swelling ratio of the cryogel was determined in distilled water. The experiment was conducted as follows: initially cryogel sample was washed until washing solution was clear. Then, it was dried until reaching to constant mass, the mass of dried sample was weighed (±0.0001 g). This dried sample was being placed into swelling medium at room temperature for 4 h. Then, the mass of swollen sample was recorded. The swelling ratio was calculated by using following expression (Eq. (1)):

$$\text{Swelling ratio } \% = \left[\frac{W_s - W_0}{W_0}\right] \times 100 \quad (1)$$

where W_0 and W_s are the weights of cryogel (g) before and after swelling, respectively.

The morphology of a cross section of the dried cryogel was investigated by scanning electron microscope. Cryogel samples were dried at room temperature for 7 days, then, coated with gold–palladium (40:60) and examined using a JEOL JSM 5600 (Tokyo, Japan) scanning electron microscope.

The characteristic functional groups of dye and cryogels were analyzed by using a Fourier transform infrared spectrophotometer (FTIR, 8000 Series, Shimadzu, Tokyo, Japan). The samples were prepared by mixing 2 mg of dried/crushed cryogels and 98 mg of powdered KBr (IR Grade, Merck, Germany) and pressed into a pellet form. The FTIR spectrum was then recorded.

The amounts of Cibacron Blue F3GA incorporation on the cryogels were obtained using an elemental analysis instrument (Leco, CHNS-932, USA) by considering the sulfur stoichiometry. Cryogel sample (1.0 mg) was placed on the aluminum sample holder of the instrument and weighed (with the maximum error range of ±0.0001). The amounts of carbon (C), hydrogen (H), nitrogen (N) and sulfur (S) were measured after burning. Differently, amount of oxygen was calculated from the difference between the sample weight and the total weight of other elements.

2.5. Adsorption studies of recombinant human interferon-α

Affinity adsorption of recombinant human interferon-α (rHuIFN-α) onto poly(HEMA)/CB cryogel was studied at different conditions enabled varying different parameters such as initial protein concentration, pH, contact time and ionic strength of the medium in order to observe the effect of these parameters on the adsorption process (some data given as supplementary information). rHuIFN-α adsorption was studied at various pH values, either in acetate buffer (0.1 M, pH 4.0–6.0) or in phosphate buffer (0.1 M, pH 7.0–8.0). Initial rHuIFN-α concentration was varied between 0.1 mg/ml and 2.0 mg/ml. The rHuIFN-α adsorption was followed by monitoring the decrease in rHuIFN-α concentration of the solutions by UV absorbance at 280 nm using UV spectrophotometer (Shimadzu 1601, Tokyo, Japan).

At the beginning of this step, rHuIFN-α buffer solution without cryogels was stirred until observing transparency (about 5 min) at a stirring rate of 30 rpm. Then, 0.5 ml of sample was taken from this solution to determine the initial concentration. The cryogels were added to solutions, then, rHuIFN-α adsorption experiments were conducted at room temperature with a stirring rate of 30 rpm for 2 h. At the end of this period, the rHuIFN-α adsorbed cryogels were removed from the solution and washed with the same buffer three times. Amount of adsorbed rHuIFN-α was determined using the concentration differences depending on absorbance taken from sample solution before and after adsorption processes.

2.6. Desorption and repeated use

Adsorbed proteins were desorbed using buffer solutions (pH 4.0) containing 1.0 M NaCl. The adsorbed rHuIFN-α was placed in the elution medium and continuously stirred at 25 °C with a stirring rate of 30 rpm for 1 h. The desorption ratio was calculated from the amount of protein adsorbed on the cryogel and the final protein concentration in the desorption medium. In order to test the reusability of poly(HEMA)/CB cryogel, rHuIFN-α adsorption–desorption cycles were repeated 10 times using the same cryogels. poly(HEMA)/CB cryogels were washed with 20 mM NaOH solution after each adsorption–desorption cycle to regenerate and remove residuals.

2.7. Interferon purification from natural source

Interferon purification from natural source, human gingival fibroblast, was performed in this part. For this purpose, interferon samples extracted from human gingival fibroblast cell culture were used. The cell culture was gift from Hacettepe University, Faculty of Science, Department of Biology. The preparation procedure was summarized as follows [31]. Inflammation-free human gingival tissue was obtained from biopsies of the attached gingiva of premolar teeth of healthy people, with informed consent based on an appropriate protocol, reviewed and approved by the Institutional Review Board. The explants were stored overnight at 4 °C and washed thereafter twice in Hank's balanced salt solution (HBSS) supplemented with 5 ml of bicarbonate and antibiotic additive [penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin (2.5 mg/ml); Sigma, St. Louis, USA] and cooled to 4 °C. The explants were cut into small pieces of 1–2 mm^3 and then washed twice with HBSS. The explants were then transferred into tissue flasks (25 cm^2) and were incubated with culture medium containing 10% fetal calf serum in RPMI-1640 containing 2.3 mM glutamine, 117.2 μg/ml streptomycin and 117.2 IU/ml penicillin in a humidified 5% CO_2 in air at 37 °C. The culture medium was replaced after 5–7 days and subsequently twice a week. When the cells became confluent, they were passaged. All experiments were performed using cells between the third and the eighth passages. Human gingival fibroblast cells grown in cell culture medium were added to 100 ml of 2 M NaCl solution in order to rupture the cell-walls at room temperature for 2 h. Then, the obtained mixture was centrifuged at 4000 rpm for 10 min to remove the cellular residues. The supernatant was extracted and dialyzed through the Servapor® dialyze tube (diameter: 29 mm: mesh(pore) size: 12,000 Da, Serva Electrophoresis GmbH, Heideiberg, Germany) for 24 h. After that, sodium azide (0.02%) was added to dialyzed sample and stored at +4 °C until use. Interferon purification performance of poly(HEMA)/CB cryogel, separately prepared cryogel applying same procedure given above (i.d. 1.0 cm × 5.0 cm), from natural source was tested with AKTA-FPLC (Amersham Bioscience, Uppsala, Sweden) system equipped with a UV detection system. Fast protein liquid chromatography (FPLC) system includes M-925 mixer, P-920 pump, UPC-900 monitor, INV-907 injection valve and Frac920 fraction collector. Separation was carried out at GE Healthcare column (10/10, 19-5001-01) filled with synthesized poly(HEMA)/CB cryogel. FPLC mobile phases A and B were prepared using 20 mM

phosphate buffer (pH 6.0) and 20 mM acetate buffer (pH 4.0) containing 1 M NaCl, respectively. The chromatographic separation was performed using a linear gradient at 0.5 ml/min flow rate. After a 6 min starting period with 100% mobile phase A, a linear gradient started from 0% B to 100% B in 1 min, continued with 7 min 100% eluent B and finished last 8 min 100% buffer A. All buffers and protein solutions were filtered before use. In this step, we applied firstly standard interferon solution in different concentration in order to determine the retention time. Then, the sample (2 ml) from the extract was applied to the column. Absorbance was monitored at 280 nm and the separation was performed at room temperature. Potassium bromide was chosen as the void marker because of its known density, 1.21 g/ml. Capacity factor (k') and separation factor (α) were calculated as:

$$k' = \frac{t_R - t_o}{t_o}, \qquad \alpha = \frac{k_{2'}}{k_{1'}} \tag{2}$$

where t_R is the retention time of the protein and t_o is the retention time of the void marker (KBr), $k_{2'}$ is capacity factor for fibroblast interferon and $k_{1'}$ is capacity factor for other contents. The resolution (R_s) and theoretical plate numbers (N) were calculated using the following equations:

$$N = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2 \tag{3}$$

$$R_s = \frac{2(t_{R,2} - t_{R,1})}{w_2 + w_1} \tag{4}$$

where $w_{0.5}$ is the peak height at the corresponding peak height fraction, $t_{R,1}$ and $t_{R,2}$ are the retention times of two adjacent peaks, w_1 and w_2 are the widths of the two adjacent peaks at the baseline.

2.7.1. Assaying biological activity of purified rHuIFN-α

The antiviral activity of rHuIFN-α was determined in vitro by protection of human amnion WISH cells against VSV-induced cytopathic effects as described by traditional method [20,32]. In the procedure, virus-induced cytopathic effects were determined and the concentration was expressed as the inverse dilution that provided 50% protection of cells from viral induced cytopathic effects. The rHuIFN-α concentrations in IU/ml were derived from the human rHuIFN-α standard reference. For these purpose, 5.0×10^4 cells were seeded into each well of well plates and incubated with rHuIFN-α samples at 37 °C for 18 h. After incubation, the cells were challenged with VSV and the plates were consecutively incubated at 37 °C for 18 h. The measurements were carried out thrice and mean values were reported. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was also used to evaluate the purity of the collected samples. The three samples, taken from fibroblast extract, before (i) and after (ii) adsorption, and collected from bound region of FPLC chromatogram (iii), were subjected to gradient SDS–PAGE. During SDS–PAGE analysis, the fractions were assayed by using 10% separating gel (9 cm × 7.5 cm), which were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid–methanol–deionized water (1:5:5, v/v/v) to visualize the bands and destained in ethanol–acetic acid–water (1:4:6, v/v/v). PageRuler™ Prestained Protein Ladder, 3-color ladder with 10 proteins covering a molecular weight range from 10 to 170 kDa, was used as standard for monitoring protein migration on SDS–PAGE gel.

3. Results and discussion

3.1. Synthesis and characterization of poly(HEMA) cryogels

Supermacroporous poly(HEMA) cryogels were synthesized by cryopolymerization in the frozen state of 2-hydroxyethyl methacrylate monomer. Functional groups (—OH) on the cryogels allow modification such as Cibacron Blue F3GA immobilization. Internal structure of the cryogel was monitored using scanning electron microscope (SEM) and given in Fig. 1. As shown in the figure, poly(HEMA) cryogels have large supermacropores (up to 200 μm) and wide flow channels having thin polymeric walls. Interconnected flow channels and supermacropores provide easy diffusion of interferon molecules into the structure and effective interaction with dye molecules. As a result of the convective flow of the mobile phase through the pores, the mass transfer resistance is practically negligible. Also, in the chromatographic applications, it is possible to work at high flow rate since very low back pressure drop properties of these cryogels. It should be mentioned, here, the pore size of the cryogel is much larger than the size of the protein molecules, 5.53 nm × 6.59 nm × 12.15 nm [24]. This property allows them to pass and diffuse easily through the pores. Equilibrium swelling ratio of poly(HEMA) cryogels was found to be 7.14 g H_2O/g dry gel, which also confirms the supermacroporosity of the cryogel.

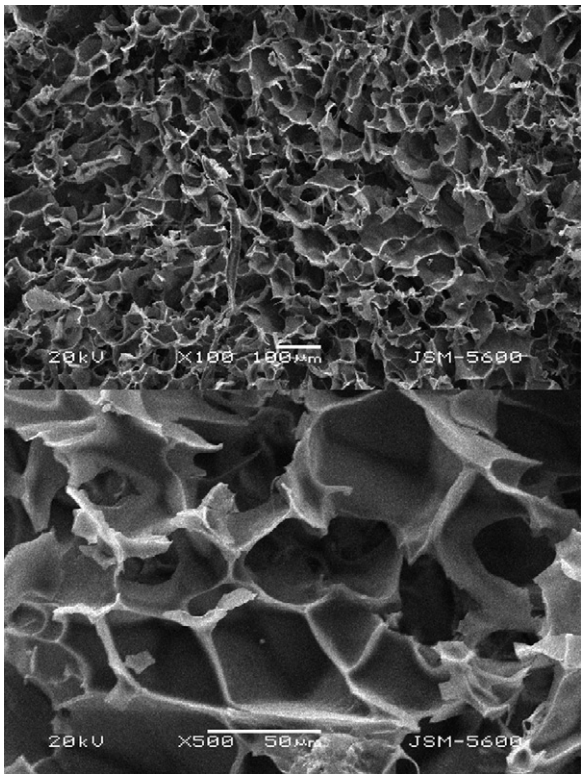

Fig. 1. SEM micrographs of poly(HEMA) cryogels.

3.2. Cibacron Blue F3GA immobilization onto poly(HEMA) cryogel

Cibacron Blue F3GA is a monochlorotriazine dye (Fig. 2a), and it contains three sulfonic acid groups and four primary and secondary amino groups. In the preparation of dye attached poly(HEMA) cryogel, Cibacron Blue F3GA was covalently bound onto poly(HEMA) cryogels (Fig. 2b) via nucleophilic substitution reaction between hydroxyl groups of the cryogel and triazine ring of the dye (Fig. 2c). Elemental analysis was performed in order to determine the amount of dye immobilized onto poly(HEMA) cryogels. According to the result, amount of immobilized dye was found to be 74.7 μmol/g cryogel, respectively.

FTIR spectra of Cibacron Blue F3GA, poly(HEMA) and poly(HEMA)/CB cryogels are given in Fig. 3. From the FTIR spectrum of Cibacron Blue F3GA, it can be observed that the

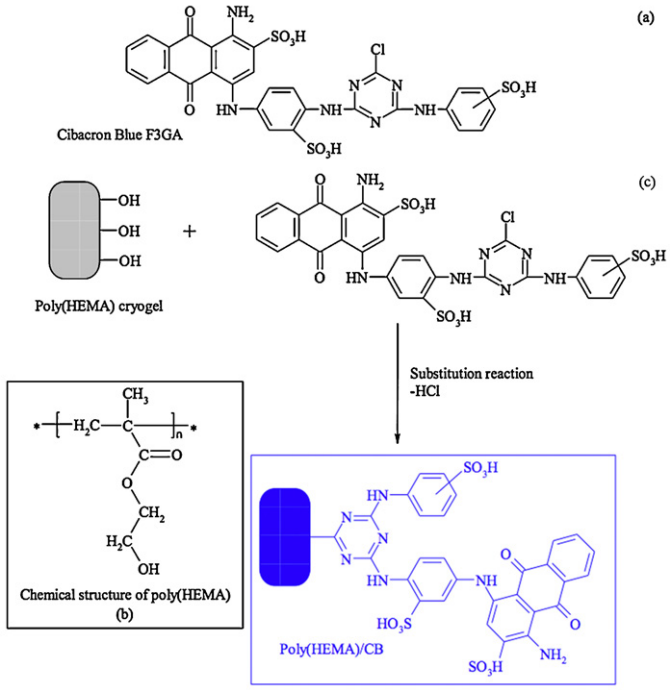

Fig. 2. Molecular structure of (a) Cibacron Blue F3GA, (b) poly(HEMA) cryogel and (c) chemical representation of Cibacron Blue F3GA immobilization onto poly(HEMA) cryogel.

band at 1088 cm^{-1} belongs to the C—Cl stretching. At the lower wavenumber such as 1042 cm^{-1} and 1025 cm^{-1}, S—O stretching vibration band is also seen in the spectrum. In addition, it is possible to see the C—Cl stretching vibrations of the dye at different wavenumbers in the range of 500–900 cm^{-1}. The N—H stretching vibration bands of this dye appear at 3854 cm^{-1} frequency range as multiple bands [13,14]. FTIR spectrum of poly(HEMA) cryogel presents that the C=O band is observed at 1729 cm^{-1} as a sharp peak. The peaks at 1263 cm^{-1} and 1159 cm^{-1} characterize the C—O (ester) stretching vibrations. In addition, the bands originated from hydroxyl groups stretching vibrations were determined at 3522 cm^{-1} as a broad band. Poly(HEMA)/CB cryogels have additional bands according to dye immobilization. The bands at 1321 cm^{-1} and 1299 cm^{-1} were stemmed from sulfonic acid groups of the dye molecule. In addition, the bands at 859 cm^{-1} and 879 cm^{-1} were originated from wagging vibration of primary and secondary amine groups of dye molecules. Hence, the FTIR spectra confirm that Cibacron Blue F3GA was successfully attached onto poly(HEMA) cryogels.

3.3. rHuIFN-α adsorption studies from aqueous solutions

3.3.1. Effect of initial concentration on rHuIFN-α adsorption

In order to investigate the effect of initial rHuIFN-α concentration on the adsorption behaviors of both plain and poly(HEMA)/CB cryogels, they were interacted with rHuIFN-α solutions in different concentration range of 0.1–2.0 mg/ml. Adsorption capacities of the cryogels were given as a function of the initial rHuIFN-α concentration in Fig. 4. As expected, rHuIFN-α adsorption onto poly(HEMA)/CB cryogels significantly increased with initial concentration up to 1 mg/ml. This increase indicates the high affinity between the rHuIFN-α molecules and dye-affinity cryogel. After this high affinity region, the adsorption capacity became nearly constant and reached a plateau value, 38.2 mg/g, which represented saturation of the active binding sites that are available and accessible for rHuIFN-α on these cryogels. Increasing tendency seems to be nearly linear until 0.5 mg/ml and, then, started to decline between 0.5 and 1.0 mg/ml. It is pointed out that one of the main requirements in dye-affinity chromatography is high selectivity and specificity of the affinity adsorbent for the target molecule [12]. The non-specific interactions between the support, i.e. plain poly(HEMA) cryogels, and the interested molecules, i.e. the rHuIFN-α, should be minimum in order to consider the interaction as specific.

As also seen in the figure, negligible amount of rHuIFN-α was adsorbed non-specifically on the plain poly(HEMA) cryogels, which was 0.48 mg/g. Since the plain cryogels have no reactive functional groups to interact with rHuIFN-α molecules, the obtained adsorption may be only due to diffusion of rHuIFN-α through wide flow-channels and macropores and weak interactions between rHuIFN-α molecules and the plain cryogel. Cibacron Blue F3GA immobilization significantly increased the rHuIFN-α binding specificity and capacity of the poly(HEMA) cryogels (up to 38.2 mg/g). This can explain that the strong binding of the dye–ligands to the proteins may have resulted from a cooperative effects of different mechanisms such as hydrophobic, hydrogen bonding, van der Waals and electrostatic interactions caused by the aromatic structures and functional groups on the dye molecules and the amino acid residues on surface of the protein molecules [33].

3.3.2. pH dependency of rHuIFN-α adsorption

The pH value of the medium can be considered as an important parameter which generally affects the protein–ligand interaction, because of the total charge, solubility, chemical stability and spatial arrangement, a function of primary, secondary, tertiary and quaternary structure of proteins, are strongly depend on it. The effect of pH on the rHuIFN-α adsorption was investigated in the pH range of 3.0–8.0 at 1.0 mg/ml of initial rHuIFN-α concentration (Fig. 5). In all investigated cases, the maximum adsorption capacity was obtained at pH 6.0 that is near to the isoelectric point, p*I* is 5.9, of rHuIFN-α. At pH values lower and higher than pH 6.0, the adsorbed amount of rHuIFN-α onto poly(HEMA)/CB cryogels significantly decreased. The pH value of surrounding medium has direct effect on determining the dominant interaction between dye–ligand and protein molecules. The changes in pH values effect on the ionization states of several groups on both the Cibacron Blue F3GA (i.e., sulfonic acid and amino) and surface residues of the rHuIFN-α. Therefore, the decrease in the rHuIFN-α adsorption capacity in more acidic and more alkaline pH regions can be attributed to electrostatic repulsion effects between the oppositely charged groups [30]. It has been shown that proteins have no net charge at their isoelectric points; therefore, the maximum adsorption capacity is usually observed at the isoelectric point [15]. Fig. 5 also shows that rHuIFN-α adsorption onto plain poly(HEMA) cryogels was non-specific and do not depend on pH value.

3.3.3. Repeated use

One of the main problems in the affinity chromatography is high cost of both the supporting material and the required bioligand. Beside the use of biomimetic ligand such as metal ions, amino acids and reactive dye, the repeated usability of the support is considered as alternative and promising property for reducing process cost [34]. Repeated usability of any supporting material used in the protein adsorption improves economics of processes by means of decreasing the material cost. In order to determine the reusability of poly(HEMA)/CB cryogels, adsorption–desorption cycles were repeated 10 times using the same cryogels (Fig. 6). At the end of the 10th cycles, there was no noticeable loss in the adsorption capacity of the poly(HEMA)/CB cryogels and the capacity had only decreased by 4.3%. By these properties, poly(HEMA)/CB cryogel can be classified as an alternative adsorbent for rapid, easy and cost-effective separation of rHuIFN-α molecules.

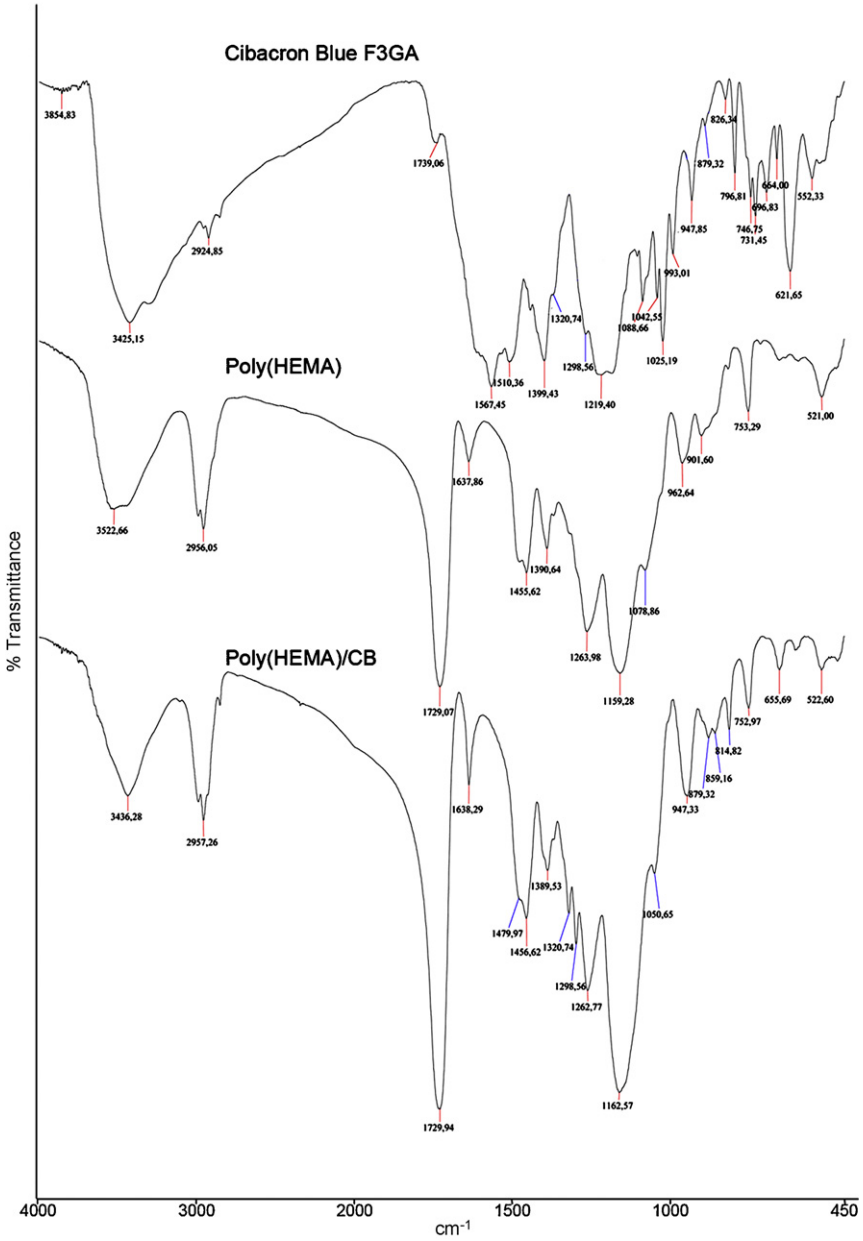

Fig. 3. FTIR spectra of Cibacron Blue F3GA, poly(HEMA) cryogel and poly(HEMA)/CB cryogel.

3.4. Interferon purification from natural source

For the determination of purification performance of poly(HEMA)/CB cryogels, interferon purification was performed from the human gingival fibroblast culture. Chromatograms were obtained by connecting of poly(HEMA)/CB cryogel to AKTA-FPLC system. Firstly, calibration chromatograms were taken by injecting of standard rHuIFN-α solution (50–1000 ppm) into FPLC system (Fig. 7a). As shown in the figure, excess rHuIFN-α pass through the column without any interaction. Bound rHuIFN-α molecules were recovered by the applying the gradient elution. The peak areas of eluted fragments were linearly increased with increasing concentration (Fig. 7b). In the light of the data obtained from the chromatograms of standard interferon solutions, the extract of human gingival fibroblast was injected into the FPLC system (Fig. 8).

As seen in Fig. 8, fibroblast interferon molecules were successfully eluted from poly(HEMA)/CB cryogels. The retention times of standard and fibroblast interferon are similar. The calculated t_R, N, k', and R_s values are summarized in Table 1. According to the accurate quantitative measurement, R_s should be above 1.5. The resolution is a critical value when working with complex samples such as drug impurities and degradation products, or when the formulation is complex and excipients might interfere with the quantitative measurements. Therefore, it is an essential part of the system suitability measurement stage before the quantitative work of these types of samples [35]. R_s value for poly(HEMA)/CB cryogels is very high (4.23). Due to the result, we can say that this dye-affinity

Table 1
Chromatographic parameters for poly(HEMA)/CB cryogels.

	t_R	N	k'	α	R_s
Fibroblast interferon	10.64	554	7.79	–	–
Other content	2.19	30	0.81	9.62	4.23

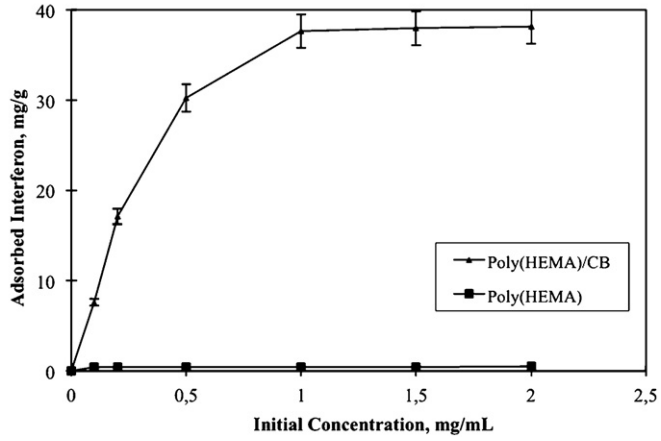

Fig. 4. Effect of initial concentration on the rHuIFN-α adsorption capacity of cryogels. pH: 6.0; *T*: 25 °C.

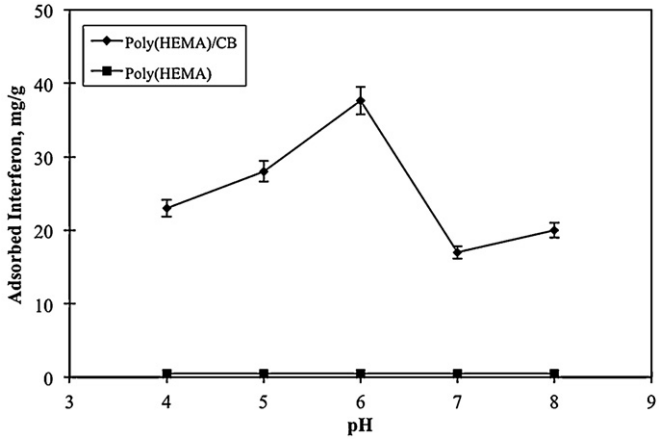

Fig. 5. Effect of pH on the rHuIFN-α adsorption capacity of cryogels. rHuIFN-α concentration: 1.0 mg/ml; *T*: 25 °C.

cryogel system is suitable for purification of interferon from even complex mixture such as fibroblast extract.

Separation factor, α, is a parameter shows that the separation selectivity and this value should be higher than 1.0 for a good resolution of two adjacent peaks in a chromatography system. Also, the separation factor, a marker for the separation performance of the column, was calculated as 9.62, respectively. The collected sample from bound region of the chromatogram was assayed for antiviral activity of the purified rHuIFN-α. Although some decreases in total protein content, from 320 μg to 18 μg, and interferon activity, from 2.6×10^3 IU to 2.2×10^3 IU, were determined, specific antiviral activity increased from 7.19 IU/μg to 122.2 IU/μg, which shows that the rHuIFN-α purification from crude extract was

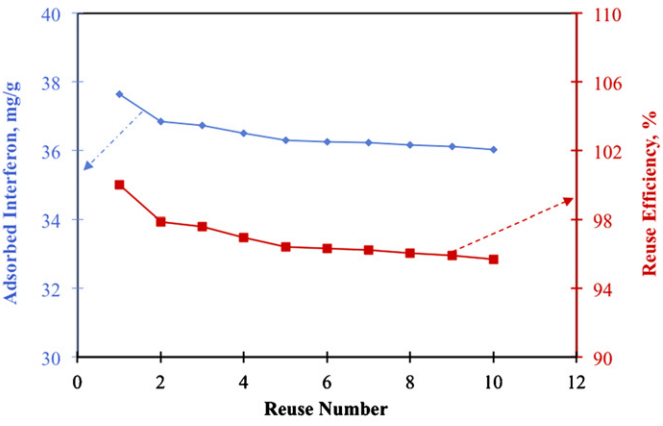

Fig. 6. Repeated use of cryogels. rHuIFN-α concentration: 1.0 mg/ml; pH: 6.0; *T*: 25 °C.

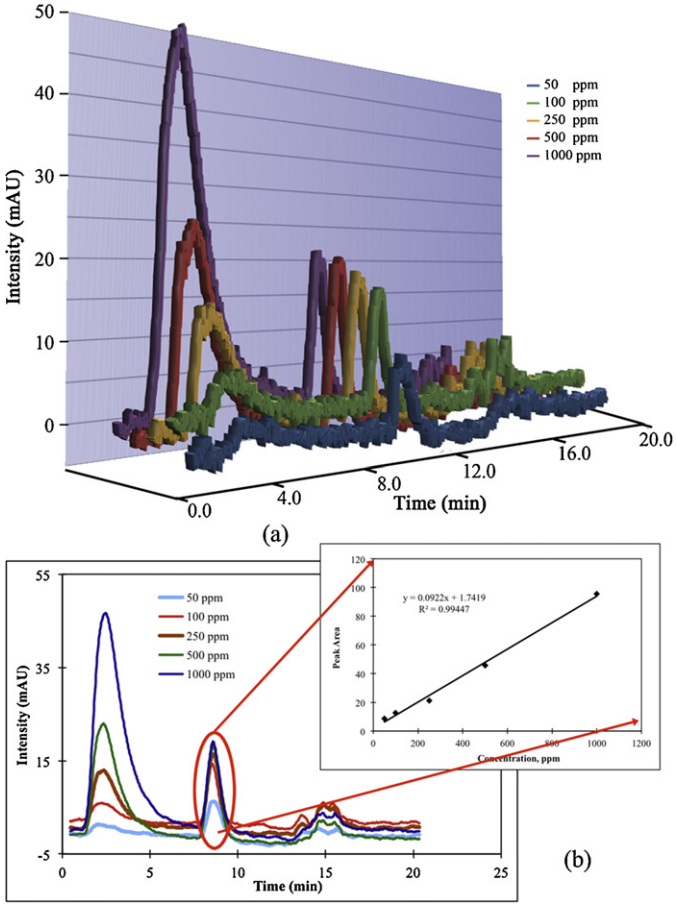

Fig. 7. The rHuIFN-α separation with poly(HEMA)/CB cryogels. (a) FPLC chromatograms of standard rHuIFN-α solutions. (b) The relation between initial concentration and eluted peak areas. Flow rate: 0.5 ml/min; binding buffer: 20 mM phosphate buffer (pH: 6.0); elution buffer: 20 mM acetate buffer (pH: 4.0) containing 1.0 M NaCl; wavelength: 280 nm.

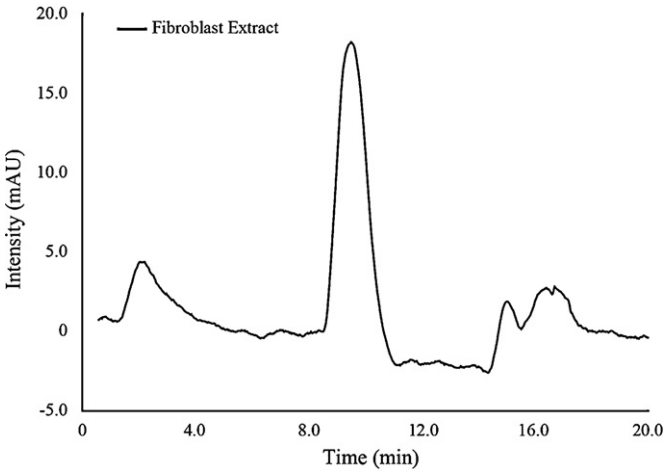

Fig. 8. AKTA-FPLC chromatogram of the interferon extracted from human gingival fibroblast. Flow rate: 0.5 ml/min; binding buffer: 20 mM phosphate buffer (pH: 6.0); elution buffer: 20 mM acetate buffer (pH: 4.0) containing 1.0 M NaCl; wavelength: 280 nm.

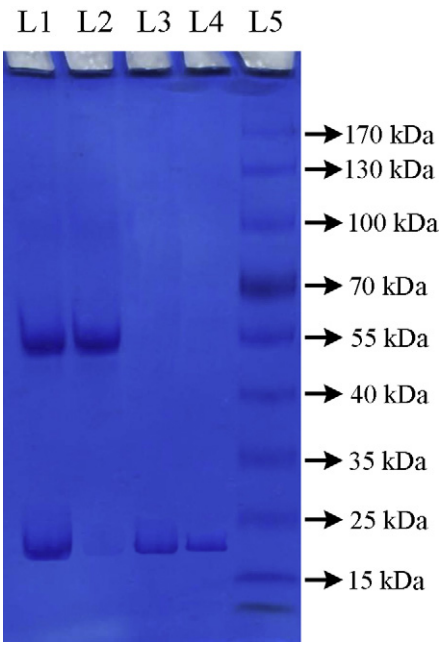

Fig. 9. SDS–PAGE analysis of collected fractions. Lane 1: fibroblast extract before adsorption; Lane 2: fibroblast extract after adsorption; Lane 3: eluted fraction; Lane 4: standard interferon solution; Lane 5: marker (PageRuler™ Prestained Protein Ladder in molecular range of 10–170 kDa). Separating gel concentration: 10%; staining solution: 0.25% (w/v) Coomassie Brillant R 250 in acetic acid–methanol–deionized water (1:5:5, v/v/v) and destaining solution: ethanol–acetic acid–water (1:4:6, v/v/v).

achieved with purification fold as 17.0-fold and recovery as 84.6%, respectively. The fractions collected from FPLC system were also assayed with SDS–PAGE (Fig. 9). As seen in the figure, the intensity of the band around 20 kDa due to interferon molecules having molecular weight approximately 19.5 kDa decreased after adsorption onto poly(HEMA)/CB cryogel (Lanes 1 and 2). Lane 3 contains the fraction eluted from FPLC column. As clearly seen, it only has a specific band for interferon molecules shown in Lane 4. The purity of eluted fraction was found as 97.6%. As conclusion, we can say that poly(HEMA)/CB cryogel has acceptable chromatographic performance for separation and purification of interferon from human gingival fibroblast extract.

4. Conclusion

Reactive dyes have been extensively used as biomimetic ligand, because, they are effective, rapid applicable, and ability to easy attachment onto support without multistep immobilization procedure [15,30]. Cryogels are very good alternatives for protein purification with their promising advantages and there is an increasing interest to use them as adsorbents for diverse applications including protein purification and biomedical therapy [36–39]. In this study, we have focused our attention on developing a dye affinity cryogel for affinity adsorption of interferon molecules from both artificial and natural source using cryogels. For this purpose, HEMA and CB were selected as monomer and biomimetic ligand, respectively. FPLC chromatograms were taken from both standard rHuIFN-α solutions and fibroblast extract at optimum adsorption condition that were determined with experiment performed from aqueous solutions. In the light of FPLC analysis, resolution (4.23) and separation (9.62) factors show that effective and selective separation of interferon molecules was achieved from human gingival fibroblast extract. The purity determined via SDS–PAGE assay of the eluted interferon fraction was found as 97.6%. The results presented in this study are very encouraging and show that poly(HEMA)/CB cryogels can be considered as a potential candidate with acceptable chromatographic performance for the affinity purification of interferon molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.036.

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