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Surface modification of chitosan for selective surface–protein interaction

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Abstract

This study evaluates the chromatographic performance of a support obtained by the reaction of a variety of ligands with di-epoxide (1,4 butanediol diglycidyl ether) activated chitosan beads. Chitosan beads 400–625 microns (μm) in diameter and with a solid contents of 4.5% were selected for this study. The activation step was optimized with respect to time, temperature, and starting epoxide concentration. Epoxide activity in the range of 600–1200 μmoles of epoxide per gram of chitosan was obtained. Epoxide activated beads were then reacted with synthetic ligands to yield a support for use in bioseparations. Ligand modified chitosan beads were observed to selectively bind human serum albumin (HSA) over human fibrinogen and human immunoglobulin. Purity of the products, obtained from a single purification step, as judged by electrophoretic analysis was estimated to be greater than 95%.

Keywords: Chitosan; Chemical modification; Albumin binding

1. Introduction

A natural polymer that is receiving increased attention because of its inherent biocompatibility is chitin and its derivative chitosan. Chitin and chitosan, ingredients in the shells of crustaceans, are natural polymers that exist widely in nature. Due to their good biological activity, biocompatibility and biodegradability, chitosan and its derivatives have attracted attention as potential applicants in the field of biomedical polymers (Hirano, Tanaka, Hasekawa, Tobetto, & Nisioka, 1985; Muzzarelli, Ilari, & Tomasetti, 1994; Denuziere, Ferrier, Damour, & Domard, 1998). Chitosan [β (l–4)-2 amino-2-deoxy-D-glucose] is a natural polyaminosaccharide derived from N-deacetylation of chitin [β (l–4)-2 acetamide-2-deoxy-D-glucose]. Chitin is generally insoluble in water, while chitosan is readily soluble in various acidic solvents. Depending on the degree of deacetylation, chitosan is a copolymer of *N*-acetyl-D-glucosamine and glucosamine units, with the glucosamine units predominating. The pH dependent solubility of chitosan provides a convenient mechanism for processing under mild conditions and the high charge density of chitosan in solution allows for chitosan to form insoluble ionic complexes with a wide variety of water soluble polyanionic species (Hirano & Noishiki, 1985; Hirano, Zhang, Nakagawa, & Miyata, 2000), thus allowing for facile substitutions.

Considerable interest has grown in the modification of chitosan to impart new and improved properties; namely surface properties and protein binding properties. A variety of methods have been employed to alter the chemical and biological properties of chitosan by taking advantage of the reactive amine group on the polymer. Chitosan and modified chitosan may find applications in biomedical applications, including biomaterials for use in tissue engineering, and drug delivery systems, and in bioseparations applications, including nano- and macro-scale separations, and classical affinity and non-affinity-based chromatographic systems. Previously chitosan has been modified

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with synthetic polymers in an attempt to improve the mechanical properties and generate hybrid materials that could manifest desirable properties (Shigeno, Kondo, & Takemoto, 1982; Blair, Guthrie, Law, & Turkington, 1987; Lagos & Reyes, 1988; Singh & Roy, 1994; Amiji, 1997; Hirano et al., 2000). To alter the chemical properties of the chitosan surface, films of chitosan containing either polyethylene glycol (PEG) or polyvinyl alcohol (PVA) were prepared by mixing PEG or PVA with a solution of chitosan acetate (Chandy & Sharma, 1991, 1992) or wheat germ agglutinin were prepared. In an attempt to improve the biological properties of chitosan, it was modified with heparin (Jdezuki et al., 1975), sulfated (Hirano et al., 1985; Gamzazade & Nasibov, 1997), N-acylated (Lee, Ga, & Park, 1995), modified using acid–chloride and acid anhydrides (Tangpasuthadol, Pongchaisirikul, & Hoven, 2003), and with wheat germ agglutinin (Wang, Kao, & Hseih, 2003).

Our *research goal* is to design, develop and synthesize chitosan-based surfaces with tailored chemistries to control the biological response at material surfaces. We are interested in understanding and optimizing the structure–property correlations between chemical modification of the surface and the resultant biological activity; namely protein adhesion. In our approach for the chemical modification of chitosan, we will use bifunctional epoxide (1,4 butanediol diglycidyl ether) for the introduction of reactive oxirane groups and for the stabilization of the hydrogel beads by simultaneous cross-linking. The use of bifunctional epoxides allows for the attachment of molecules or adsorptive groups to the matrix via a four-carbon long hydrophilic or amphiphilic spacer arm. In this paper, chitosan beads with a diameter of 600–800 μm were first reacted with 1,4 butanediol diglycidyl ether and subsequently reacted with a variety of ligands. In this particular study we will chemically modify chitosan such that modified chitosan can selectively, and specifically interact with albumin over other plasma proteins.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Vanson Chemical Company (Redmond, Washington) and was used without further purification. Lyophilized, 95% pure human IgG (I-4506), lyophilized human serum (S-2257), rabbit antiserum against mouse IgG's (M-8645), affinity purified goat-anti-mouse (whole molecule) IgG's conjugated to horseradish peroxidase (A-4416) and fluorescein isothiocyanate (FITC) labeled hIgG (F-9636) were purchased from Sigma Co. (St. Louis, MO). Immulon II microtiter plates were purchased from Fisher Scientific (Itasca, IL). *O*-Phenylenediamine-2HCl tablets were purchased from Abbott Laboratories (Chicago, IL). Dithiothreitol and β-mercaptoethanol were obtained from Fluka (Ronkonkoma, NY). Immunoaffinity separations were performed with Pharmacia C-10 columns (15 cm × 1 cm), a Masterflex peristaltic pump, a Knauer spectrophotometer, and a Rainin data acquisition system was used to monitor chromatography. Columns were kept at 4 °C with a Lauda Super RMT water cooler. 8–16% Tris–glycine gels were purchased from Novex (San Diego, CA) and gel electrophoresis was carried out with a X-Cell II Novex unit. The solid contents of the beads were measured by thermogravitametric analysis (TGA) and TGA analyses were performed by Dr. John Belot (Chemistry, University of Nebraska, Lincoln).

2.2. Methods

2.2.1. Preparation of chitosan beads

Chitosan was dissolved in 1–5% wt acetic acid to provide a chitosan concentration of between 0.5 and 5 wt% by stirring at room temperature. The dissolved chitosan was atomized into a 0.1 N aqueous sodium hydroxide solution to form porous chitosan beads in a hydrogel form. The chitosan beads were removed from the NaOH solution by decantation and filtration. The beads were washed multiple times with distilled water to obtain beads at neutral pH. The solid contents of the beads were determined by lyophilization and thermogravimetric analysis. The beads were fractionated into various ranges as listed: fraction-1 (200–400 μm), fraction-2 (400–625 μm), and fraction-3 (>625 μm) using standard screens. The beads were stored hydrated at 4 °C.

2.2.2. Diepoxide activation

The bead activation procedure detailed elsewhere was adapted [Roy et al., 1994]. Briefly, the beads were solvent exchanged in pure ethanol and in isopropanol, and incubated with molar excess of 1,4 butanediol diglycidyl ether in isopropanol at room temperature for 24 h. Upon the completion of the reaction step the ether solution was removed and the beads were washed copiously with isopropanol (IP) and stored in IP at 4 °C till further derivatization. At the end of each reaction step a small aliquot of the beads were washed and saved for determination of total epoxide content by sodium thiosulfate and total sulfur analysis.

2.2.3. Determination of epoxide groups

The amount of pendant epoxide groups were determined by sodium thiosulfate titration method described elsewhere (Scoble & Scopes, 1996). In brief, 1 ml of decanted activated beads was mixed with 3 ml of 1 M sodium thiosulfate solution. The mixture was rotated for 2 h at room temperature to release the reactive vinyl groups. Supernatant was titrated with 0.1 N HCl to a pH of 7.0 and the volume of HCl used was recorded. The amount of HCl acid added was converted into equivalent μmoles of epoxide groups and reported.

As an alternate method, the titrated beads were washed with PBS buffer at pH 7.0 and reacted with a 1 M solution of mercaptoethanol for 24 h. Upon completion of the reaction step, the beads were washed with water and dried via lyophilization and submitted to the Research and Soil Test laboratories at the University of Minnesota for percent sulfur (% S) analysis. Analysis was performed on a Leco SC-132 sulfur system from Leco Corporation, St. Joseph, Michigan. Typically, 20 mg of dried beads was placed in a sample container and 50 mg of vanadium pentoxide was added on top of the sample. Samples were then heated to 2600 °F and an infrared detector quantified the vapors of sulfur dioxide.

2.2.4. Ligand modification of epoxy activated beads

Typically, epoxide modified beads were washed with water and incubated with an aqueous solution of ligand (L_i) for 10–16 h at room temperature to yield the pseudo-bioaffinity matrix. The list of ligands employed in this study are listed in Table 1. Upon completion of the activation step, the beads were sequentially washed with distilled water, 0.1 M HCl and distilled water and stored at 4 °C until further use. The epoxide activity was determined by the titration method using sodium thiosulfate before and after the activation step to measure the degree of conversion (Sundberg & Porath, 1974). Chemical structures and their relative concentrations per mole of chitosan were determined by FTIR-analysis (Chemistry department, UNL-Lincoln).

2.2.5. Purification of HSA from mock-serum and pure component solution

A protein mixture containing 25% (w/w) human albumin, 15% (w/w) human γ-globulin (γIgG) and 7.5% (w/w) human fibrinogen in PBS was prepared and will be referred to as the mock serum. In a typical experiment, 1 ml of ligand-derivatized beads was incubated with 3 ml of mock serum for 24 h at 4 °C by placing on a end-to-end rotator. Upon completion of the coupling step, the supernatant was pipetted off and saved for further analyses. The beads were washed thoroughly with three sequential washes of 20 ml loading buffer per wash step till the absorbance 280 nm of the wash fraction. The bound proteins were eluted batch-wise by the addition of 3 ml of a 10 mM phosphate, 0.5 M NaCl, pH 7.0 buffer. All the fractions were saved for analyses by ELISA analysis and gel electrophoresis.

2.2.6. Determination of hIgG, hfib, and HSA by ELISA

The concentrations of hIgG in various fractions were determined by the ELISA procedure outlined elsewhere (Subramanian, Van Cott, Milbrath, & Velander, 1994). Briefly, the plates were coated with rabbit anti-human IgG and the bound hIgG was detected by HRP-conjugated rabbit anti-human IgG. The bound chromophore was detected at 490 nm. The concentrations of hfib in various fractions were determined by the ELISA procedure outlined elsewhere (Subramanian, Butler, Gwazdauskas, Lubon, & Velander, 1997). Briefly, the plates were coated with rabbit anti-human fibrinogen and the bound hfib was detected by HRP-conjugated rabbit anti-human fibrinogen. The bound chromophore was detected at 490 nm. The concentrations of HSA in various fractions were determined by the ELISA procedure outlined elsewhere (Tateno, Yoshizane, Saito, & Yoshizato, 2004). Briefly, the plates were coated with goat anti-human HSA and the bound HSA was detected by HRP-conjugated rabbit anti-human HSA. The bound chromophore was detected at 490 nm.

2.2.7. Gel electrophoresis

The purity of the recovered albumin was analyzed by SDS–PAGE gel electrophoresis under non-denaturing conditions and denaturing conditions (Laemmli, 1970). In brief, all fractions were diluted to a yield a total protein of 2 μg per well. Samples were mixed with non-reducing buffer at a ratio of 1:1 and were heated to 95 °C for 5 min in a water bath. Proteins were analyzed on 8–16% gradient gel and visualized by Gelcode reagent. Western blots were run according to standard procedures and the blots were probed with HRP-conjugated anti-human albumin and the bands were visualized with DAB substrate.

2.2.8. Molecular simulations

HyperChem 7.0 was used to perform the molecular simulations. Templates, comprised of the structure of the chitosan, chemical ligands and the spacer arm, were drawn in 2-dimensions using Hyperchem 7.0, a 2D structure-sketching tool. Chemical structures were then converted into a reasonable 3D model using optimization routines provided in the software. These structures were then imported into molecular modeling software and either manual-or computer-assisted procedures were followed to obtain electrostatic potential, hydration energies, and other parameters.

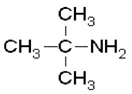
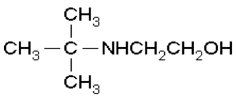
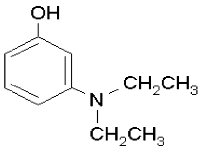
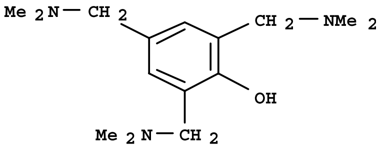
2.2.9. FTIR and environmental spectroscopy

Underivatized, epoxy-derivatized, and ligand-derivatized beads were washed with distilled water and lyophilized to dryness. The dried samples were supplied to the spectroscopy facility with the University of Nebraska, Lincoln. These results were analyzed by a NICOLET 20SX FT-IR using transmission sampling techniques by KBr pellets. Hydrated beads were viewed under a environmental scanning electron microscope with the Laser facility at the department of electrical engineering at the University of Nebraska, Lincoln.

3. Results and discussion

Chitosan beads with a nominal diameter between 425 and 600 μm, and with a solid contents of 4.25% were selected for this study. The availability of the amino group on the chitosan backbone for facile substitutions enables the synthesis of modified chitosan with different chemical and

Table 1

Ligand #	Ligand structure	Chitosan amine N-terminal ligand carbon	
		No. of units	Max. distance, Angstrom
1	$CH_3(CH_2)_3NH_2$	18	17.66
2	 $CH_3-\overset{CH_3}{\underset{CH_3}{C}}-NH_2$	16	15.98
3	$CH_3(CH_2)_6CH_2NH_2$	22	23.28
4	 $CH_3-\overset{CH_3}{\underset{CH_3}{C}}-NHCH_2CH_2OH$	20	19.71
5		18	19.88
6		18	22.51

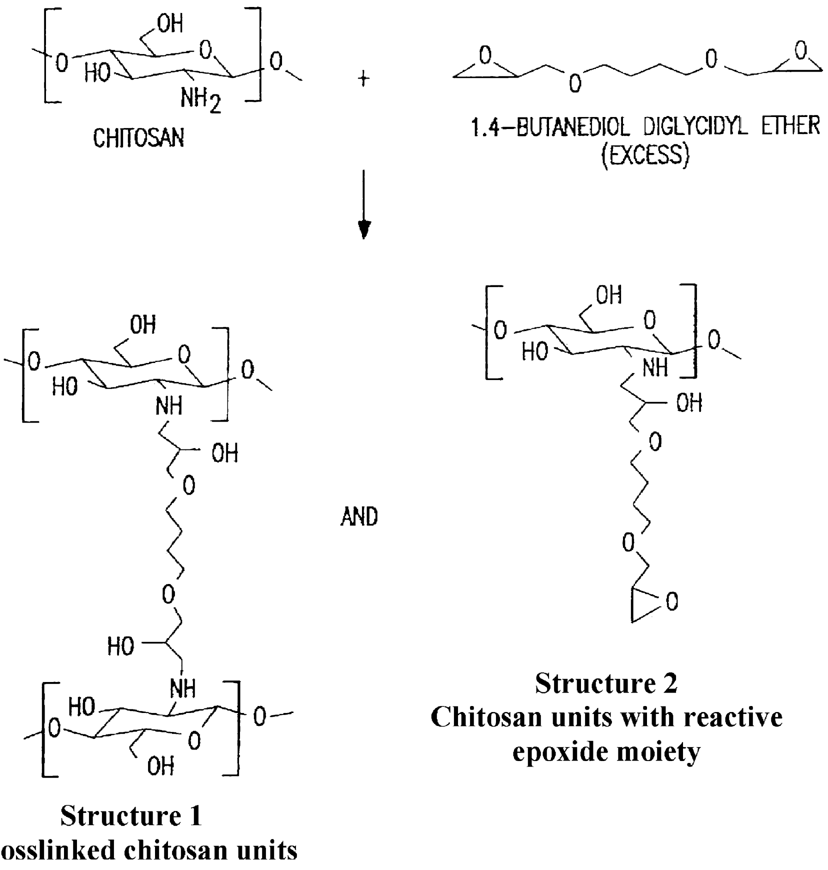


Fig. 1. Schematic for the bead-activation strategy with 1,4 butanediol diglycidyl ether. Chitosan-based hydrogel beads were first neutralized with 0.05 N NaOH, washed with deionized water and dehydrated with isopropanol (Roy et al., 1998). Solid line indicates the chitosan backbone. Surfaces were incubated with a known stoichiometric excess of 1,4 butanediol diglycidyl ether (BUDGE).

biological properties. We have studied the modification of the amino group on chitosan with a bifunctional epoxide, 1,4 butanediol diglycidyl ether. As represented in Fig. 1, the reaction yields two end products: cross-linked chitosan (structure 1) and chitosan plus a spacer (structure 2) with a reactive epoxide terminal group (Roy, Todd, Glasser, & Number, 1998). This latter structure is subsequently available for nucleophilic substitution reactions, to yield modified chitosan with varying chemical and biological properties. The reaction strategy proposed ensures that structure 1 will always be obtained thus allowing for cross-linking of the base matrix to confer mechanical stability.

3.1. Epoxide activation and ligand modification

The dependence of the extent of derivatization (μmoles epoxide per gram of chitosan) on the amount of percent epoxide in the reaction mixture is shown in Fig. 2. As expected, the amount of reactive epoxide groups increased with an increase in the amount of diepoxide used in the activation process. A control incubation of the beads at the same pH, but with epoxide omitted, showed no oxirane vinyl groups. All the beads were examined under a microscope before and after the activation process to record any gross change in morphology and representative scans are presented in Fig. 3. As seen from the micrographs the sur-

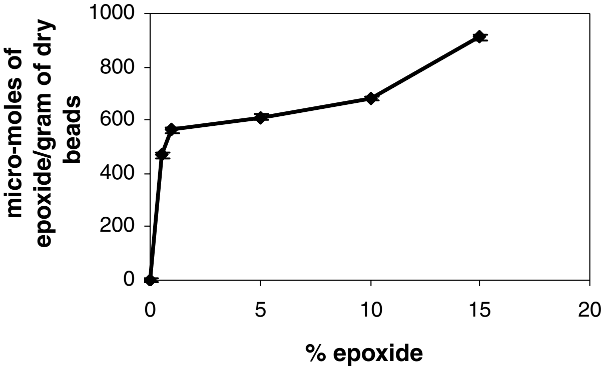


Fig. 2. Effect of percent BUDGE in the reaction mixture on the epoxide content in the dry beads. The experiment was carried on at room temperature (73 °F) for 24 h and the epoxide content of the beads (μmoles of epoxide per gram of dry beads) was determined by sodium thiosulfate titration.

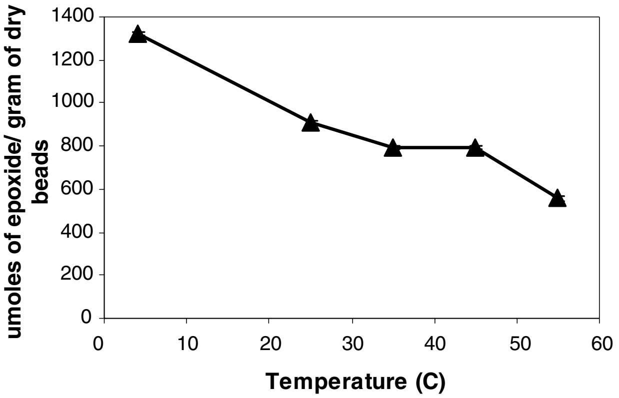


Fig. 4. Effect of the temperature of the reaction on the epoxide content of the beads. A known amount of chitosan beads was incubated with 15-molar excess of BUDGE for 24 h at different temperatures (4, 25, 35, 45, and 55 °C). The epoxide activity found out by sodium thiosulfate titration.

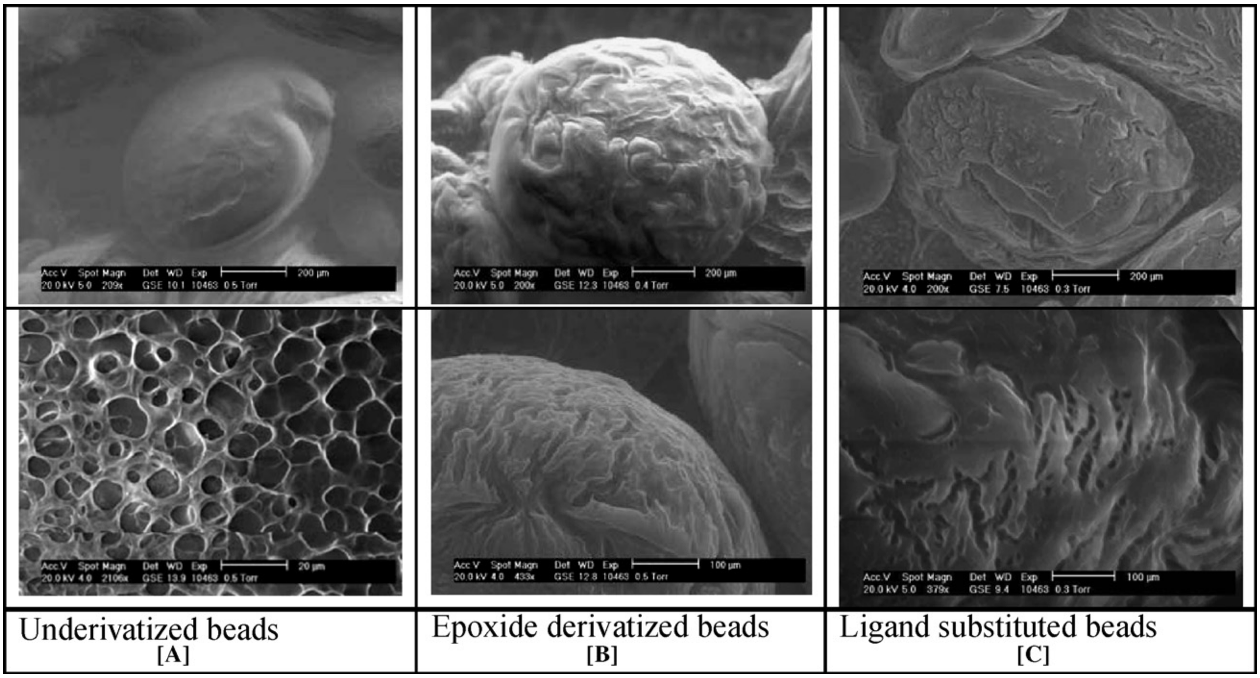


Fig. 3. The environmental microscopy scans of derivatized chitosan hydrogel beads. (A) plain chitosan bead; (B) BUDGE-derivatized bead; and (C) BUDGE-derivatized bead end-capped with ligand 6 (see Table 1).

face smoothness and morphology seem to be impacted by the activation step.

Based on our optimization experiment, all further experiments were carried out with a 15:1 molar ratio of diepoxide to primary amino groups. Fig. 4 shows the impact of temperature on the μmoles of epoxide groups per gram of chitosan. With an increase in temperature a decrease in the number of μmoles of epoxide (structure 2, Fig. 1) was observed. An epoxide content of 600 μmoles of epoxide per gram of chitosan was obtained at 55 °C and an epoxide content of 1400 μmoles of epoxide per gram of chitosan was obtained at 4 °C. Our stoichiometric-based calculations indicate that with an increase in temperature the reaction conditions favor the formation of cross-linked chitosan (structure 1). It is to be noted that thiosulfate base titrations quantify the terminal epoxide (structure 2). Thus at higher temperatures the chitosan-based matrix is more cross-linked when compared to reactions at lower temperatures thus yielding a lower percentage of terminal reactive moieties. We have studied the impact of the time of the reaction on the μmoles of epoxide groups per gram of support. No significant difference was observed between different reaction times and an average epoxide content of 1200 ± 200 μmoles of epoxide per gram of support was obtained for all reaction times studied.

Based on our optimization studies, a semi-preparative scale derivatization was carried out. A liter of beads were treated as described earlier and reacted with a 15-molar excess of diepoxide and the activation was carried out at 4 °C for 24 h. An epoxide activity of 1280 ± 10 μmole epoxide per gram of dry chitosan and 2250 ± 225 μmole epoxide per gram of dry chitosan was estimated by sodium thiosulfate titration and total sulfur analysis, respectively. The disparity in the values

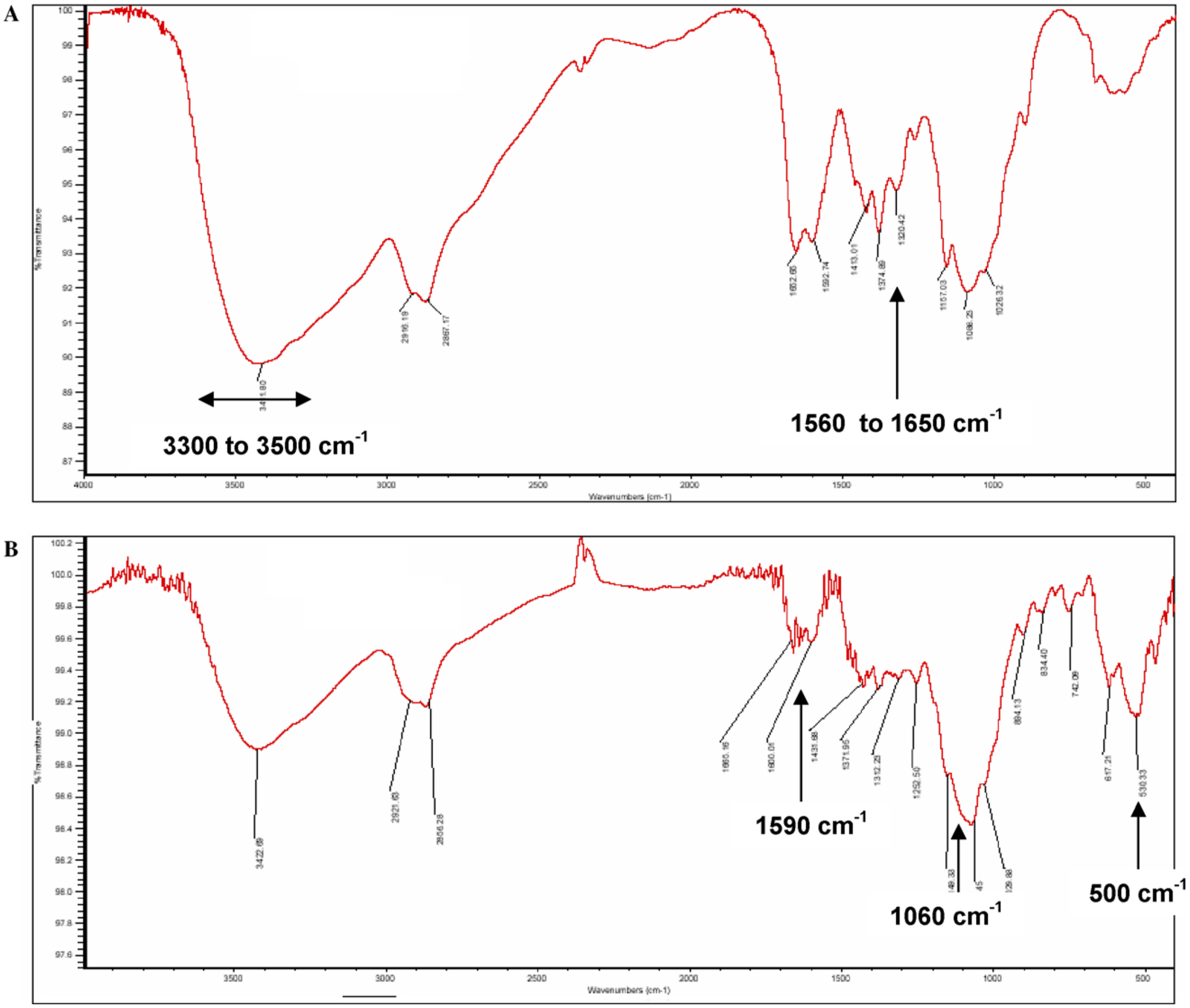


Fig. 5. IR spectra of unmodified chitosan and ligand modified chitosan. Percent transmittance (%*T*) is denoted on the *Y* axis and wavelengths (in cm^{-1}, range from 50 to 4000 cm^{-1}) are denoted on the *X* axis. Relevant wavelengths and regions are indicated with arrows. (A) FTIR of underivatized chitosan; (B) FTIR of BUDGE-derivatized chitosan; (C) FTIR of ligand-1 (L1) modified chitosan; (D) FTIR of ligand-2 (L2) modified chitosan; (E) FTIR of ligand-3 (L3) modified chitosan; (F) FTIR of ligand-4 (L4) modified chitosan; (G) FTIR of ligand-5 (L5) modified chitosan; and (H) FTIR of ligand-6 (L6) modified chitosan.

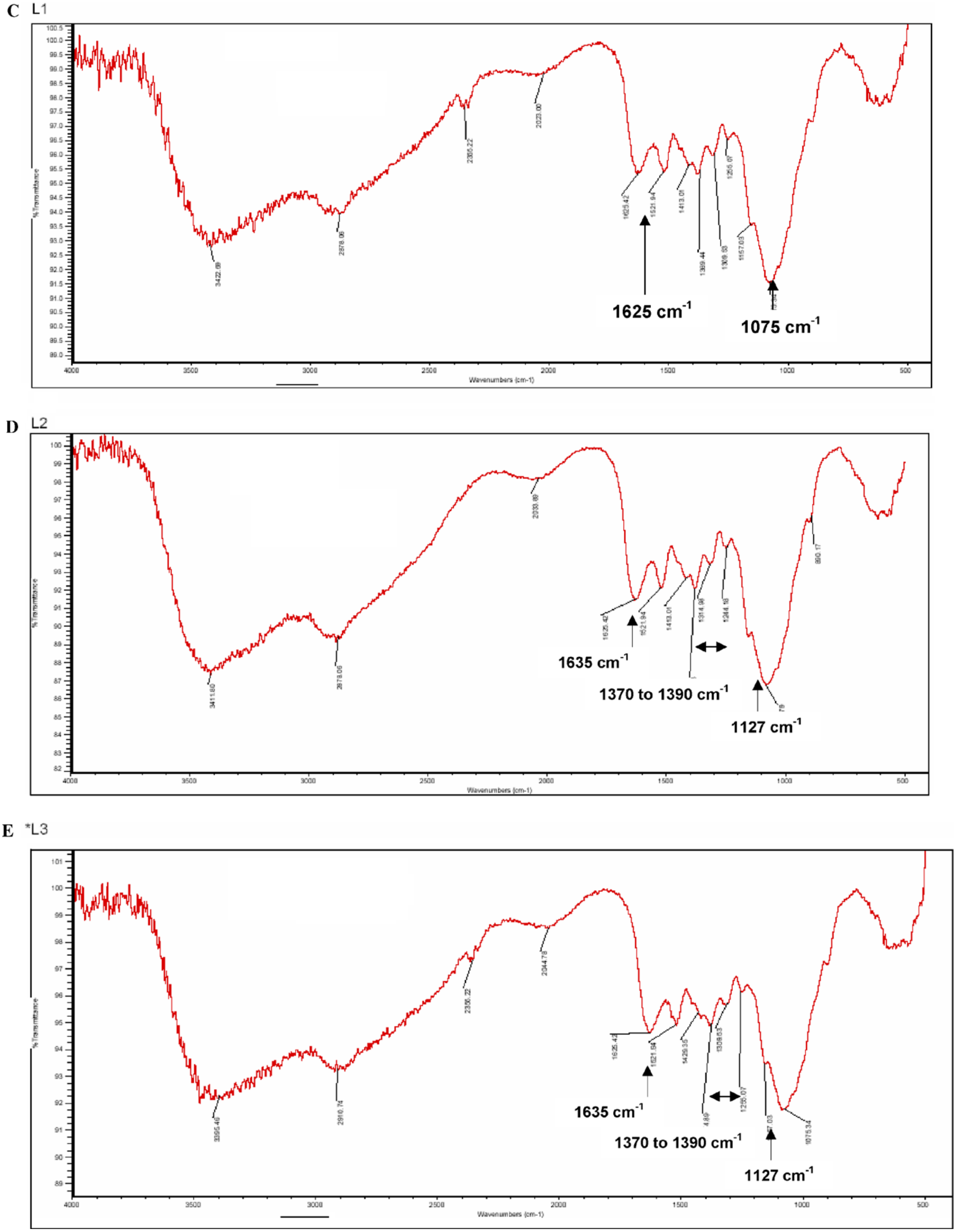


Fig. 5 (*continued*)

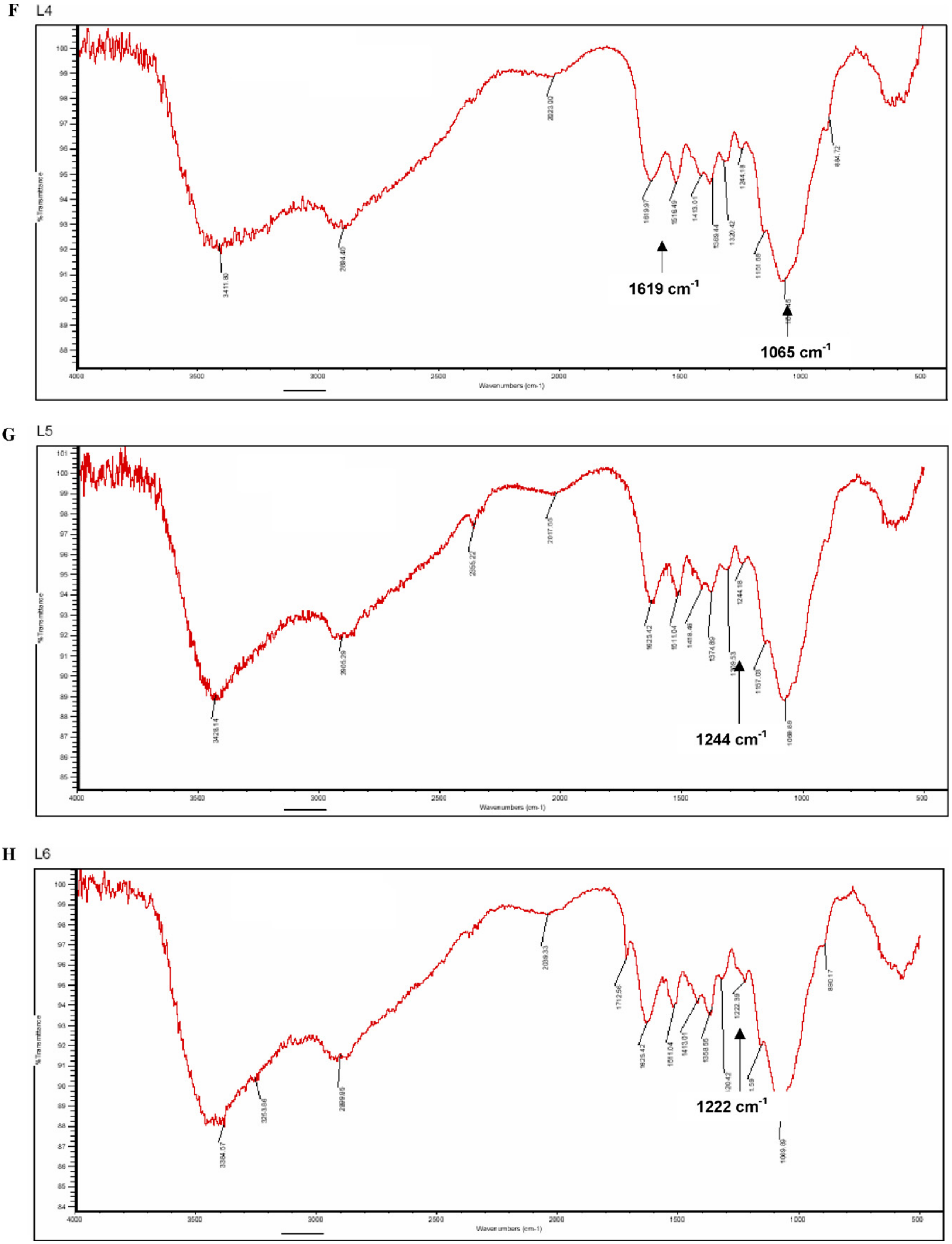


Fig. 5 (*continued*)

arises from the sensitivity of the assays and the sodium thiosulfate method has been reported to underestimate the epoxide activity. Hence, we have based our stoichiometric calculations on the value estimated by the total sulfur method.

3.2. FTIR analysis

The FTIR spectrum of the unmodified chitosan is shown in Fig. 5A. The signature of the chitosan molecule can be significantly recognized from the intensity of amine peak at wavelengths of 3300–3500 cm^{-1} and the strong N–H stretch at 1560–1650 cm^{-1}. The IR spectrum of the diepoxide modified chitosan is shown in Fig. 5B. After the modification of chitosan with 1,4 butanediol diglycidyl ether, the intensity of the characteristic amino peak at 3300–3500 cm^{-1} was found to decrease and a strong stretch at 1590 cm^{-1} was observed, signifying the modification of the primary amine group on chitosan with the di-epoxide. There is also an increase in the peak at 500 cm^{-1} which is due to C-H_2 stretch and the peak 1060 cm^{-1} is that from the C–O–H resulting from the opening of the oxirane moiety by the primary amine group. The stretch from 1000 to 1150 cm^{-1} represents the region of C–O signal resulting from the incorporation of terminal epoxide moiety (structure 2).

The diepoxide modified chitosan was further reacted with small synthetic ligands, listed in Table 1, to yield ligand modified chitosan. Figs. 5C–H represent the IR scans of ligand modified chitosan. A total of six ligands were used and the modifications were characterized by FTIR signals. Beads modified with *n*-butylamine (ligand 1) exhibited a strong N–H signal at 1625 cm^{-1} and strong C–O–H stretch at 1075 cm^{-1} and a representative scan is shown in Fig. 5C. Beads modified with tert-butylamine (ligand 2) and tert-octyl amine (ligand 3) exhibited a similar IR pattern to that of beads derivatized with ligand 1, giving a strong stretch at 1635 cm^{-1} and C–O–H stretch at 1127 cm^{-1}. There is also increase in the peak height in the region of 1370–1390 cm^{-1} signifying the presence of methyl groups and N–C stretch in the IR spectra obtained for beads derivatized with ligand 2 and ligand 3, respectively. Beads modified with tert-butyl amino-ethanol (ligand 4) exhibited a C–O–H stretch at 1065 cm^{-1} and N–H stretch

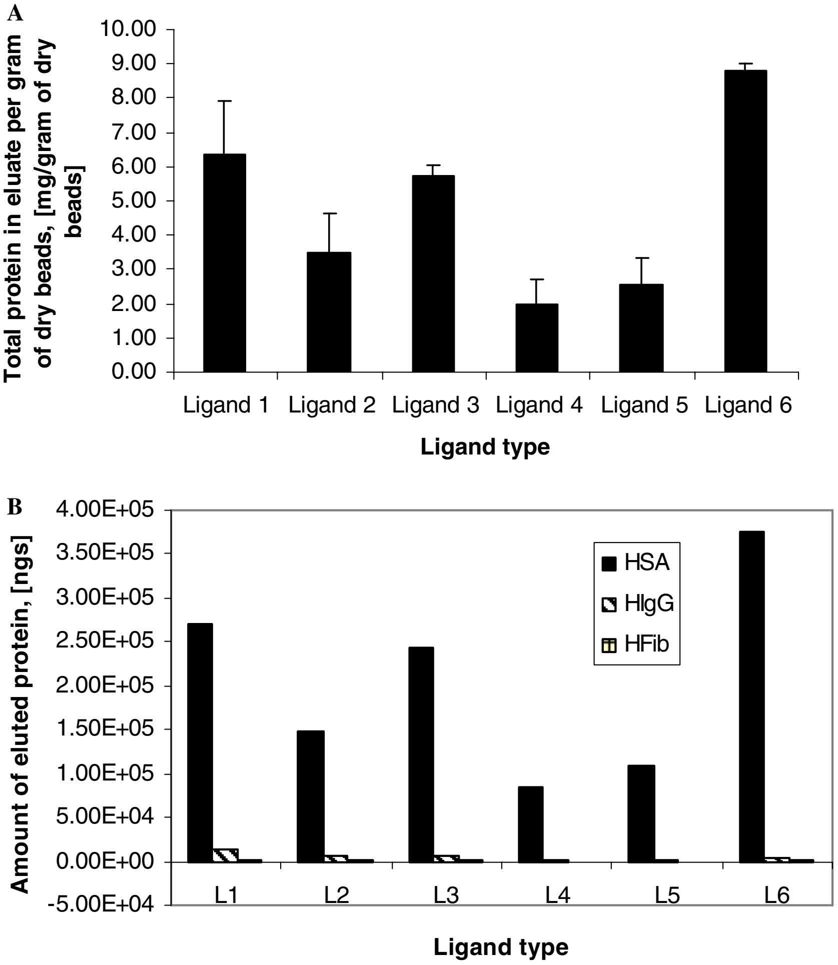


Fig. 6. (A) Total protein yields in the eluate fractions. One milliliter of ligand-derivatized beads was challenged with a 3-ml solution of mock serum with the following composition: 25% (w/w) human albumin, 15% (w/w) human γ-globulin (γIgG) and 7.5% (w/w) human fibrinogen in PBS. Upon washing, the bound protein was eluted with 0.5 M NaCl and the eluate fractions were assayed spectroscopically at 280 nm. (B) Protein concentrations in eluate fractions. The eluate fractions were subjected to specific ELISA and individual concentrations of HSA, hfib, and hIgG were determined.

at 1619 cm^{-1} and the representative stretch is shown in Fig. 5F. Beads derivatized with 3-diethyl aminophenol (ligand 5) exhibited a C–O–H stretch at 1070 cm^{-1} and a peak corresponding to the phenyl substitution at 1244 cm^{-1} and the corresponding IR spectra I shown in Fig. 5G. Beads derivatized with 2,4,6-tris (dimethylaminomethyl) phenol (ligand 6) exhibited a peak corresponding to the phenyl group substitution at 1222 cm^{-1} and additionally, an increase in the intensity of peaks in the region of 1320–1400 cm^{-1} signifying the presence of methyl groups and N–C stretch was also observed.

3.3. Protein binding experiments

Fig. 6A summarizes the total protein yields in the eluate fractions when mock human serum was used as feed to columns with various ligand modified beads. The total protein concentration in different fractions in each individual run was estimated by measuring the absorbance at 280 nm. The percent total recovery of protein was determined as a ratio of the total protein in the eluate fraction and fall through to the total protein in the feed sample. In runs using mock serum as a feed sample, total recoveries in the range of 82–90% were obtained. A majority of the protein was recorded in the fall through fraction thus allowing enrichment in the elution fraction. As indicated in Fig. 6A, beads derivatized with ligand-1, ligand-3, and ligand-6 exhibited higher levels of total protein eluted when challenged with the same of amount of total protein in the feed.

In order to better estimate the levels of hIgG, hfib, and HSA in the eluate fractions, the fractions were assayed for individual proteins by sensitive ELISA assays and the composition of the eluate fractions is presented in Fig. 6B. In all the fractions assayed, we observed the percentages of hfib ad hIgG to be lower than 0.5% and the HSA accounted for the remainder of the composition.

3.4. Gel electrophoresis

Fig. 7 shows a GELCODE™ reagent stained, SDS–PAGE gel of the mock serum (feed) and the eluate fractions from a typical separation run on ligand modified chitosan beads. Lane 1 shows a molecular weight ladder. Lane 2 shows an application of mock serum, feed that was used in all experiments. Lanes 3, 4, and 5 show an application of pure human immunoglobulin (hIgG, MW 155 kDa), human serum albumin (HSA, MW 56 kDa), and human fibrinogen (hfib, MW 400 kDa), respectively. The feed mock serum has three distinct protein bands corresponding to hfib, hIgG, and HSA. Lane 6 through 11 shows the elution (0.5 M NaCl) fractions obtained from the protein binding experiments on ligand modified chitosan beads. The eluate fractions gave a dominant band around 56 kDa similar to the pure HSA in Lane 4. In addition to the major HSA

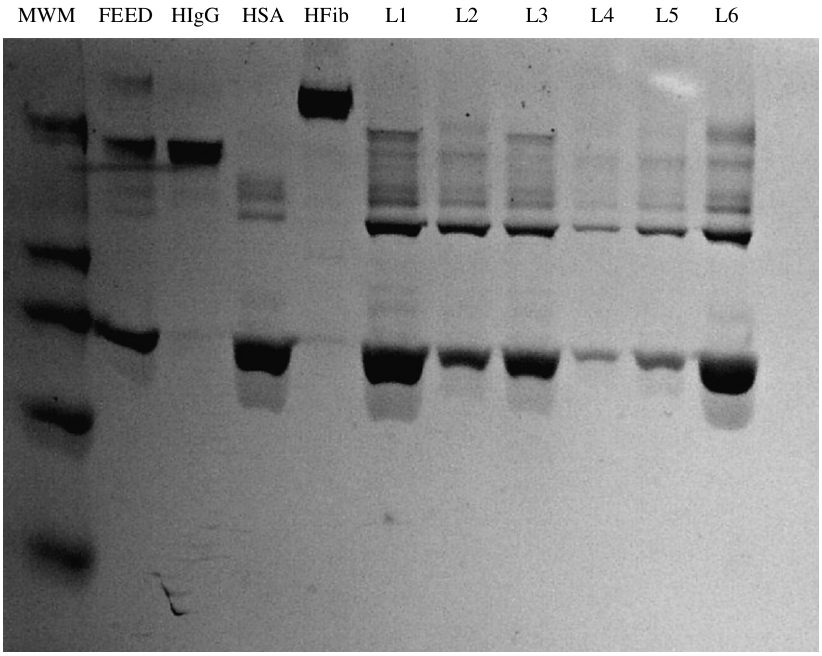


Fig. 7. SDS–PAGE gel electrophoresis of eluate fractions. Sodium dodecylsulfate (0.1%)–polyacrylamide gel (8–16% gradient) electrophoresis of HSA that was purified from mock serum using ligand modified chitosan. Lane 1 shows a molecular weight ladder. Lane 2 shows an application of mock serum, feed that was used in all experiments. Lanes 3, 4, and 5 show an application of pure human immunoglobulin (hIgG, MW 155 kDa), human serum albumin (HSA, MW 56 kDa), and human fibrinogen (hfib, MW 400 kDa), respectively. The feed mock serum has three distinct protein bands corresponding to hfib, hIgG, and HSA. Lanes 6 through 11 show the elution (0.5 M NaCl) fractions obtained from the protein binding experiments on ligand modified chitosan beads. Lane 6, ligand-1 modified beads; lane 7, ligand-2 modified beads; lane 8, ligand-3 modified beads; lane 9, ligand-4 modified beads; lane 10, ligand-5 modified beads; and lane 11, ligand-6 modified beads.

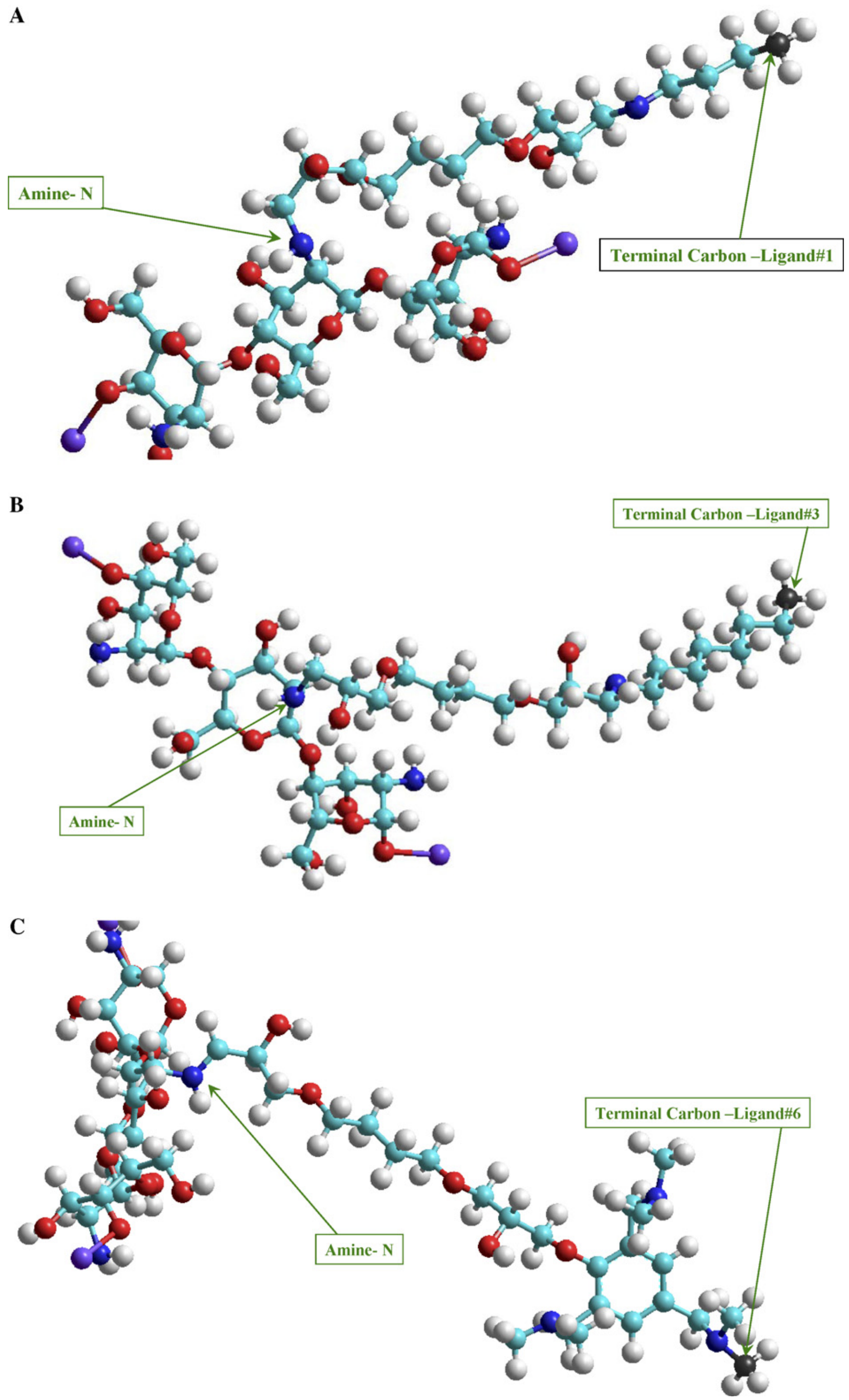


Fig. 8. Molecular simulations. HyperChem 7.0 was used to perform the molecular simulations. Templates, comprised of the structure of the chitosan, chemical ligands and the spacer arm, were drawn in 2-dimensions using Hyperchem 7.0, a 2D structure-sketching tool. Chemical structures were then converted into a reasonable 3D model using optimization routines provided in the software. The chitosan backbone comprised of three chitosan-units and the ligands were substituted to the amine group of the chitosan-molecule in the center. (A) Schematic of ligand-1 substitution; (B) schematic of ligand-3 substitution; and (C) schematic of ligand-6 substitution.

band at 56 kDa, several bands corresponding to higher molecular weights were observed. In order to ascertain the nature of these fragments, the samples were assayed by Western blot analysis (data not included). The fractions shown in lanes 2, 4, 6 through 11 gave a band around 56 kDa similar to the pure HSA along with several higher molecular weight bands. Thus, we conclude that the higher molecular weight bands correspond to multimers of HSA, which could have been present in the pure HSA that was purchased commercially and used without further purification. However, in a future study we also plan to further investigate the molecularity of this impurity.

3.5. Molecular modeling

Table 1 summarizes the parameters obtained from molecular simulations. The effect of substitutions on the inter-atomic distances is shown in Table 1. Figs. 8A–C depict molecular models of ligand 1, ligand 3, and ligand 6 substituted diepoxide modified chitosan, respectively. While detailed simulations involving multi-substitutions on the chitosan backbone are necessary to predict charge-based interactions, we have used single-point substitutions to gauge the atomic distances between the chitosan backbone and the ligand.

4. Conclusions

We have shown that matrices based on naturally occurring polysaccharides like chitosan can be designed to yield chromatographic supports or surfaces for use in bio-chromatography. Specifically, we have demonstrated the utility of these matrices in a pseudo-affinity mode. Also, our results show that these matrices were able to completely remove fibrinogen from the feed mixture. We believe the true leaps in throughput and productivity in bioprocessing processing will result when the merits of large bead technology will be merged with new and improved activation or ligand immobilization strategies.

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