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# Native protein-initiated ATRP: A viable and potentially superior alternative to PEGylation for stabilizing biologics

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**Abstract**

Comparison of *in vitro* serum stability and enzyme activity retention for PEGylated chymotrypsin and structurally different, biocompatible vinyl polymer grafts of chymotrypsin was performed. These polymer grafts were synthesized by atom transfer radical polymerization (ATRP) initiated by chymotrypsin covalently modified with 2-bromoisobutyric acid, the ATRP initiator. The maximum number of ATRP initiators attached to chymotrypsin was adjusted to be as close as possible to the maximum number of polyethylene glycol chains attached to chymotrypsin for better comparison and then polymerizations were conducted. In mouse serum, native and PEGylated chymotrypsin deactivated within 24 h, whereas chymotrypsin-graft-poly(*N*-2-hydroxypropylmethacrylamide) retained >50% of its catalytic activity even after 5 days of incubation. In human serum, PEGylated chymotrypsin deactivated within 4 days of incubation, whereas native chymotrypsin and chymotrypsin-graft-poly(*N*-2-hydroxypropylmethacrylamide) and chymotrypsin-graft-poly(2-methacryloyloxyethyl phosphorylcholine) retained >25% catalytic activity after 5 days of incubation. Biocompatible vinyl polymer grafts of chymotrypsin synthesized by protein-initiated ATRP had higher catalytic activity retention and molecular weights and lower polydispersity than PEGylated chymotrypsin. In summary, studying the effects of structures of conjugated polymers on the stability and activity retention of modified proteins can lead to identification of a polymer–protein conjugate having superior pharmacological properties than conventionally PEGylated protein. Also, since vinyl monomers that form biocompatible polymers are easily polymerizable by ATRP, protein-initiated ATRP can become a viable and potentially superior alternative to PEGylation for stabilizing biologics.


*Keywords:* Protein; ATRP; Polymer conjugate; Stability; PEGylation

## 1. Introduction

Covalent attachment of polyethylene glycol (PEG) to therapeutic proteins and peptides, or "PEGylation" of proteins and peptides, can increase their bioavailability and reduce the antigenicity [1–7]. A handful of FDA-approved PEGylated biologics are in use currently to treat cancer, hepatitis C, anemia and diabetes. But PEGylation still suffers from problems. It produces diverse mixture of conjugates with variable potencies [8,9]. PEGylation leads to drastic drops in the protein's catalytic activity [10–12]. PEGylated proteins are difficult to purify and isolate in high yields [13–15]. Because of such drawbacks in PEGylation, only a few new PEGylated therapeutic proteins and peptides among the many known enter clinical trials. Researchers have tried to address the heterogeneity issues in PEGylated proteins by genetically engineering proteins containing unique lysine or cysteine residues which serve as specific PEGylation sites [16,17]. However, such uniquely reactive and genetically engineered proteins cannot always preserve the original biological activity of native proteins [18,19]. Site-specific PEGylation has also been conducted using proteins containing unnatural amino acids. For example, Shultz and co-workers [20] expressed various proteins in which amino acids with azide, alkenyl,

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iodo and keto functional groups were incorporated site specifically via genetic engineering and PEGylation was conducted at these unnatural but reactive sites.

Researchers have also tried to address problems in PEGylation by developing protein-initiated polymerization methods that allow better control over the molecular weight ($M_w$) of growing polymers and simpler purification of conjugates. Both native and genetically engineered proteins have been used in these *in situ* polymerizations. We and other workers [21–24] have covalently modified proteins with initiators of controlled radical polymerization, such as atom transfer radical polymerization (ATRP) or reversible addition fragmentation chain transfer (RAFT) polymerizations, and polymerized vinyl monomers to synthesize catalytically active protein–polymer bioconjugates. Recently a refinement in these strategies was reported by first modifying a trifunctional amino acid with ATRP initiator and then incorporating it into a polypeptide chain at a desired location. Polymerization of vinyl monomers was then initiated from this desired site in the peptide [25].

Growing polymers from a surface of a material is classified as a "grafting from" approach as it grafts polymer chains on that surface. Analogously, polymer–protein conjugates synthesized by protein-initiated polymerizations could be termed as polymer–protein grafts (PPGs). Vinyl monomers that form PPGs are structurally and functionally different from PEG, and therefore PPGs are quite different from PEGylated proteins. This could be advantageous in improving serum stability of protein beyond that which is achievable by conventional PEGylation by carefully selecting the structure of the grafted polymer. To our knowledge, such an effort has not been reported in the literature.

To date, PEGylation has dominated the bioconjugation field for stabilizing therapeutic proteins. However, reports in the literature suggest that biocompatible vinyl polymers have also displayed properties that could be an improvement over PEG in therapeutic protein stabilization. For example, Enada et al. [26] reported modification of L-asparaginase with comb-shaped PEG derivatives by multipoint attachment through maleic anhydride groups present on a polymer chain. These conjugates held up to 85% enzyme activity, as opposed to 11% for PEGylated asparaginase, and showed complete loss of immunogenicity [26]. Miyamoto et al. [27] compared the stability of papain–poly(2-methacryloyloxyethyl phosphorylcholine) (poly(MPC)) and papain–PEG conjugates. Papain–poly(MPC) had higher stability than papain–PEG at 40 °C in buffer [27]. Oupick and Ulbrich [28] synthesized chymotrypsin-poly(*N*-2-hydroxypropylmethacrylamide) (poly (HPMA)) conjugates with improved proteolytic stability and reduced immunogenicity. In this work we have applied protein-initiated ATRP to synthesize grafts of high $M_w$ poly (MPC), poly (HPMA) and poly(monomethoxy-polyethyleneglycol-methacrylate) (poly (MPEGMA)) with the model enzyme chymotrypsin (CT); which are difficult to synthesize (in high $M_w$ ranges) using conventional conjugation techniques. We have compared the *in vitro* serum stability of biocompatible vinyl polymer grafts of CT with the stability of PEGylated CT and demonstrated that structural variations in the polymer attached to protein can achieve serum stabilization of modified protein beyond that which is achievable with conventional PEGylation. Also, since vinyl monomers that form biocompatible polymers are easily polymerizable by ATRP, protein-initiated ATRP can become a viable and potentially superior alternative to PEGylation for stabilizing biologics.

## 2. Experimental

### 2.1. Materials

CT (from bovine pancreas, 3× crystallized), MPEGMA ($M_w$ 2000 Da), human male serum, mouse serum, *N*-succinyl-Ala-Ala-Pro-Phe-para-nitroanilide, 2-bromoisobutyryl bromide, copper (I) bromide, 2,2′-dipyridyl, etc. were obtained from Sigma–Aldrich. *N*-2-Hydroxypropylmethacrylamide (HPMA) was obtained from Polysciences. 2-methacryloyloxyethyl phosphorylcholine (MPC) monomer was obtained from Vertellus. Monomethoxy poly(ethylene glycol) succinimydyl succinate (MPEG-SS) of $M_w$ 5–35 kDa was obtained from LaySan Bio and Jenkem USA.

### 2.2. Instrumentation

Molecular weight characterization of PPGs synthesized by protein-initiated ATRP was performed at Polyanalytik, ON, Canada. Size-exclusion chromatography coupled with detectors for refractive index, viscosity, right angle and low angle light scattering was performed. Three columns, PAA 202.5, PAA 203, and PAA 204, were used in series on a Viscotek TDA chromatograph. The mobile phase was 0.1 M $NaNO_3$ and the flow rate was 0.75 ml $min^{-1}$. Ultraviolet–visible spectroscopy was performed using a Perkin-Elmer Lambda 2 spectrophotometer. Magic angle laser desorption ionization-time of flight (MALDI-TOF) spectrometry was performed at the Center for Molecular Analysis, Carnegie Mellon University. Briefly, a PerSeptive Biosystems' Voyager elite MALDI-TOF spectrometer was used to determine the molecular weights of native, initiator-attached and PEGylated proteins. Acceleration voltage was set at 20 kV in a linear mode. Protein solution (0.5–1.0 mg $ml^{-1}$) was mixed with an equal volume of matrix (0.5 ml of water, 0.5 ml of acetonitrile, 2 μl of trifluoroacetic acid and 8 mg of 4-hydroxy-3,5-dimethoxy-cinnamic acid) and 2 μl of the resulting mixture was spotted on the plate target. Spectra were recorded after solvent evaporation.

### 2.3. Serum stability and activity retention study of CT

Native, PEGylated or vinyl polymer-grafted CT (protein concentration 0.1 mg $ml^{-1}$) was incubated at 37 °C in mouse or human serum (obtained from Sigma). At different time intervals, serum aliquots (0.1 ml) were mixed with 1.0 ml

Tris–HCl buffer (0.1 M, pH 8.0). Substrate solution of *N*-succinyl-Ala-Ala-Pro-Phe-para-nitroanilide (0.1 ml in DMSO, 10 mg ml$^{-1}$) was added and mixed well. Enzymatic hydrolysis of substrate was monitored by recording the increase in the absorbance at 412 nm for 15 min. The background hydrolysis of the substrate by serum was negligible. Residual enzyme activity was determined from the slopes of the graph for increase in absorbance at 412 nm vs. time. Initial activity of native, PEGylated and vinyl polymer-grafted CT in buffer was determined using the same procedure described above except that the enzyme solutions (0.1 mg ml$^{-1}$) were made in Tris–HCl buffer (0.1 M, pH 8.0).

### 2.4. Synthesis of CT–2-bromoisobutyramide (protein initiator)

The ATRP initiator, 2-bromoisobutyric acid, was conjugated to CT according to our earlier reported procedure [23]. Briefly; 1 g CT was dissolved in 100 ml of phosphate buffer (100 mM, pH 8.0). The protein solution was continuously stirred at 800 rpm using a magnetic stir bar. To this, a predetermined amount of 2-bromoisobutyryl bromide dissolved in 2–5 ml dichloromethane was added slowly. The reaction mixture was stirred for 30–60 min while keeping the pH at 8.0 by intermittently adding small amounts of concentrated sodium hydroxide solution. After the completion of the reaction, the pH was adjusted to 6.0 and the reaction mixture was centrifuged to remove unreacted and precipitated 2-bromoisobutyric acid. The protein initiator was purified by centrifugal ultrafiltration using ultrafiltration tubes of 5 kDa molecular weight cut-off. Yields as determined from protein recovered (bicinchoninic acid assay) ranged from 70% to 80%. The average number of ATRP initiators conjugated per protein was determined by MALDI-TOF spectrometry.

### 2.5. Synthesis of PPGs by protein-initiated ATRP

Protein-initiator (100 mg) was dissolved in phosphate buffer (100 ml, 0.1 M, pH 6.0) and the solution was placed in a round-bottomed flask equipped with a nitrogen gas inlet and outlet. MPEGMA or other monomer (1 g) was added and the reaction mixture was purged with nitrogen gas for 10 min at room temperature. Polymerization was initiated by adding copper (I) bromide (2 mg) and 2,2′-dipyridyl (5 mg) and allowed to proceed for 60 min at 40 °C (25 °C for MPC). PPGs were purified by first swirling the reaction mixture in 5 g of silica gel to adsorb the copper salts and then subjecting the copper-free solution to centrifugal ultrafiltration using ultrafiltration tubes of 30 kDa $M_w$ cut-off. Yields as determined from protein recovered (bicinchoninic acid assay) ranged from 60% to 70%.

### 2.6. Conventional PEGylation

CT (100 mg) was dissolved in borate buffer (20 ml, 0.1 M, pH 8.5) and amine-reactive PEG (MPEG-SS of $M_w$ 5–35 kDa, 2.0 g) was added. The reaction mixture was stirred for 30 min at room temperature and PEGylated CT was purified by centrifugal ultrafiltration using ultrafiltration tubes of 30 kDa $M_w$ cut-off. Yield as determined from protein recovered (bicinchoninic acid assay) ranged from 60% to 70%.

## 3. Results and discussion

As mentioned earlier, PEGylation of native proteins typically results in formation of a mixture of conjugates containing a different number of PEG chains attached to the protein. Therefore, the activity or stability results obtained are representative of the whole mixture of conjugates unless they are separated from each other. Also, conjugates containing the same number PEG chains per protein make up a mixture of positional isomers that could have activity significantly different from each other [8]. Despite this tremendous heterogeneity in conjugates, PEGylation of native proteins has proved to be successful in producing effective biopharmaceuticals such as PEGASYS®, Oncaspar®, and Adagen®. Although native proteins cannot be modified with polymers in a controllable manner, uniquely "PEGylable" and genetically engineered proteins do not necessarily give conjugates with desired activity and stability [18,19]. Also, such de novo versions are not common for all proteins and peptides that have therapeutic activities. We therefore focused our efforts on comparing protein-initiated ATRP with PEGylation using native proteins only. In order to do this comparison as fairly as possible, we first conducted conventional PEGylation of a well-characterized model enzyme, CT, and determined the number of PEG chains covalently attached per molecule of native CT. We then modified native CT with 2-bromoisobutyric acid, the ATRP initiator, to closely match the maximum number of PEG chains found in PEGylated CT and conducted ATRP of vinyl monomers that form biocompatible polymers.

### 3.1. Synthesis and characterization of PEGylated CT

PEGylation of CT was performed using the amine-reactive PEG reagents of $M_w$ 5, 20, 30, and 35 kDa, as described earlier (Fig. 1). MALDI-TOF characterization of CT PEGylated using 5 kDa PEG showed attachment of 1–2 PEG chains per protein molecule and presence of significant amount of unmodified CT in the conjugate (Fig. 2). MALDI-TOF spectra of CT PEGylated with 20–35 kDa PEG showed trace amounts of the modified CT and the presence of >90% unmodified CT (data not shown). Therefore in this work we used CT PEGylated with 5 kDa PEG only for serum stability and activity retention studies. We did not attempt to separate free enzyme from PEGylated CT as its serum stability was expected to be much less that that of the major fraction of modified enzymes in the conjugate. Native and PEGylated CT were also characterized by size-exclusion chromatography.

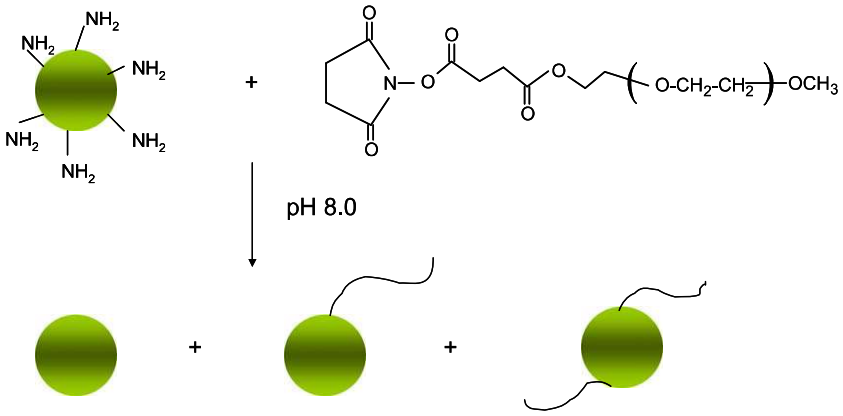


Fig. 1. Schematic representation of PEGylation of CT and the composition of resulting conjugate.

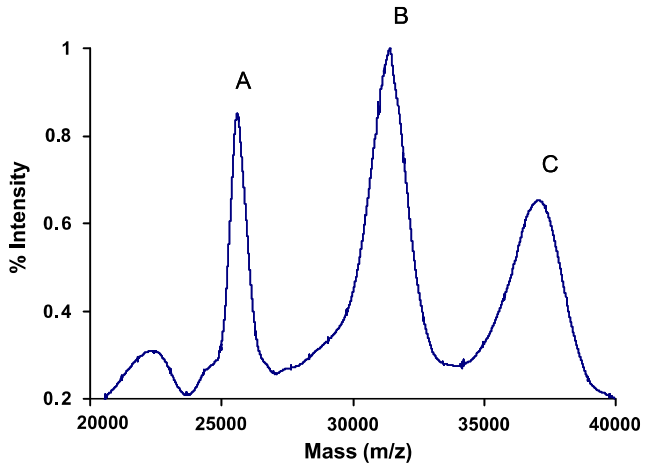


Fig. 2. MALDI-TOF spectrum of CT PEGylated with PEG of 5 kDa $M_w$. (A) Unmodified CT remained in the conjugate obtained after centrifugal ultrafiltration. (B) CT conjugated with 1 PEG chain. (C) CT conjugated with 2 PEG chains.

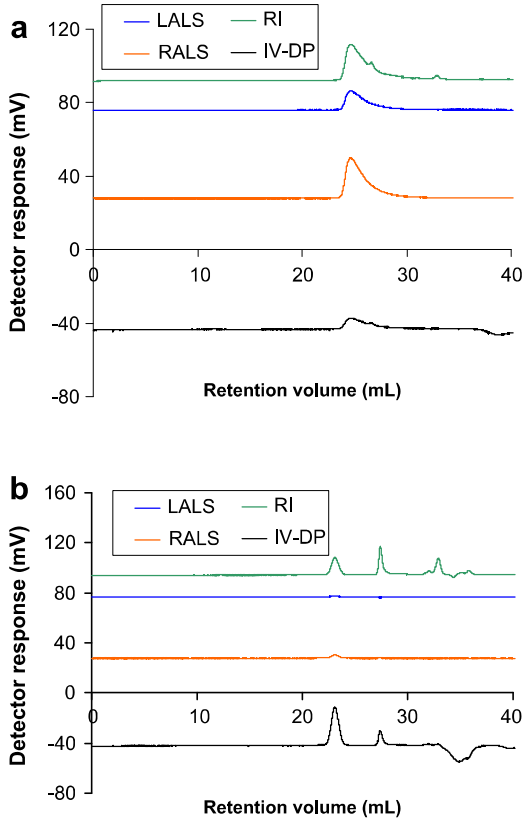


Fig. 3. (a) Size exclusion chromatogram of native CT using a tetra-detector analyzer (TDA) from Viscotek. LALS = Low angle light scattering signal, RALS = Right angle light scattering signal, RI = Refractive index signal, IV-DP = Viscometer signal. Retention volume = 24.97; $M_w$ = 28,120 Da; $M_n$ = 26,263; $M_w$ / $M_n$ = 1.07. Hydrodynamic radius = 2.2 nm. (b) Size exclusion chromatogram of PEGylated CT using a tetra-detector analyzer (TDA) from Viscotek. LALS = Low angle light scattering signal, RALS = Right angle light scattering signal, RI = Refractive index signal, IV-DP = Viscometer signal. Retention volumes = 23.4, 27.5, 33.03. Molecular weight estimation could not be performed satisfactorily because of very low light scattering signal.

Native CT elutes as a single peak but with some tailing due to hydrophobic interactions with the column material (Fig. 3a). The refractive index signal in the chromatogram of PEGylated CT shows three peaks (native, mono, and diPEGylated CT), just like its MALDI-TOF spectrum (Fig. 3b). We note here that, due to polydispersity in the PEG reagents used, molecular ion peaks for mono- and diPEGylated peaks do not occur exactly at differences in multiples of 5 kDa.

### 3.2. Synthesis of CT–2-bromoisobutyramide (protein initiator)

Chymotrypsin contains 13 lysine residues and thus theoretically it is possible to conjugate or grow (via ATRP) 13 polymer chains per enzyme molecule. Previously we have shown that it is possible to control the average number of 2-bromoisobutyric acid conjugated to lysine residues in CT by adjusting the molar ratio of CT to 2-bromoisobutyryl bromide in Schotten Baumann acylation reactions between the two. Through a series of experiments that determined the number of ATRP initiators conjugated to CT as a function of increasing molar ratios of CT:2-

bromoisobutyryl bromide, we were successful in conjugating 1–3 molecules 2-bromoisobutyric acid per molecule of CT (Table 1, Fig. 4). A 1:20 molar ratio of CT:2-bromoisobutyryl bromide achieved this modification, which was close to the desired conjugation of 1–2 ATRP initiators per CT. MALDI-TOF spectrum of this protein initiator is also free of native CT (Fig. 5). Residual enzyme activity in this protein initiator was >90%.

We are aware that the protein initiator described above can lead to PPGs in which some or all initiator sites will be consumed to grow polymers of the same or different $M_w$s. However, as shown in the following sections, we have confirmed the presence of single peaks in the chromatograms and relatively narrow polydispersity indices for the PPGs evaluated in this work against PEGylated CT, which clearly shows multiple peaks in its chromatogram. Unfortunately, we were unable to ionize and run PPGs on the MLADI-TOF spectrometer and therefore at present we do not have any direct data to show the exact number of vinyl polymer chains grown per CT. However, since a maximum of three vinyl polymer chains can be grown from the protein initiator used here, we believe that the PPGs synthesized can be compared with the PEGylated CT described above.

Table 1
Effect of varying molar ratios of CT:2-bromoisobutylbromide on number of ATRP initiators conjugated to CT

| CT:2-bromoisobutyryl bromide | Number of ATRP initiators conjugated to CT[1] |
|---|---|
| 1:20 | 1–3 |
| 1:43 | 4–7 |
| 1:85 | 7–10 |

[1] Determined by MALDI-TOF spectrometry. Calculation of a number of conjugated ATRP initiators detailed in the legend for Fig. 5.

### 3.3. Synthesis of PPGs

An important advantage of protein-initiated ATRP is that one can grow high $M_w$ polymers from protein surfaces since the "living" center of the polymer is carried away from the bulk of the protein as the polymer chain grows. This is in stark contrast with conventional conjugation, which suffers from too many steric hindrances between the high $M_w$ polymer and the protein for effective conjugation (poor yields of conjugates were achieved when PEGylation was conducted using high $M_w$ PEG reagents). We therefore used protein initiator:monomer ratios of 1:100–1:2000 to synthesize high $M_w$, biocompatible vinyl polymer grafts of CT and compared these with PEGylated CT for initial activity retention and serum stability. In our previous paper we reported synthesis of PPGs by protein-initiated ATRP using a tenfold molar excess of Cu(I)Br and 2,2′-dipyridyl over the protein initiator [23]. The removal of such a large quantity of copper requires time-consuming procedures, such as passing the reaction mixture through a silica gel column and eluting the column with a large amount of water. In this work we used an ~1:1 molar ratio of Cu(I)Br: initiator and conducted polymerizations for 1 h. The onset of polymerization was indicated by an increase in the solution viscosity as soon as the catalysts were added. Within 5 min, the reaction mixture had turned from colorless to faint brown. After polymerization, the

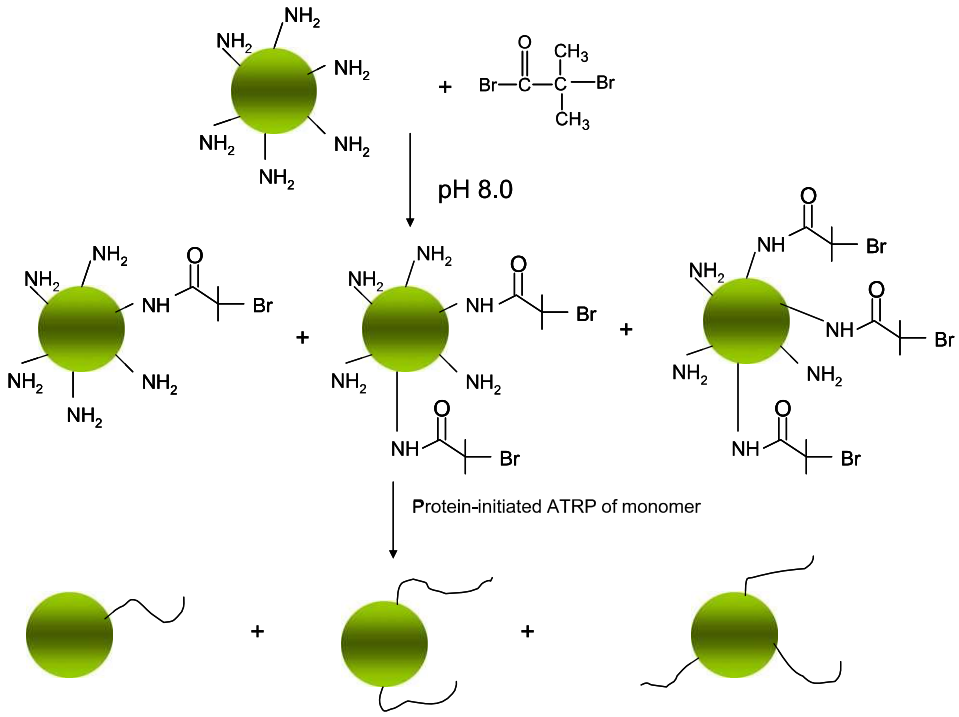


Fig. 4. Schematic representation of synthesis of protein initiator and protein-initiated ATRP to synthesize biocompatible, vinyl polymer grafts of CT.

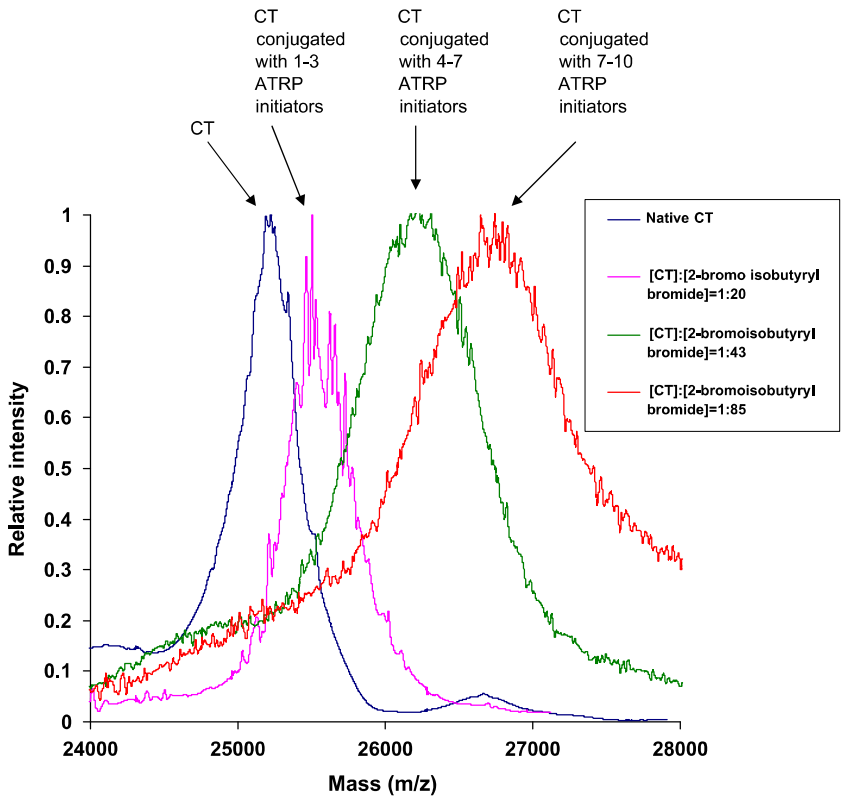


Fig. 5. MALDI-TOF spectra of CT and CT-initiators synthesized using increasing molar ratios of [CT]:[2-bromoisobutyryl bromide] in acylation reactions. Number of initiators conjugated to CT were calculated from the difference in the mass of molecular ion peak for CT ($m/z = 22230$) and for CT modified with different molar ratios of 2-bromoisobutyryl bromide. In case of CT modified with 1:20 ratio, the molecular ion peak exhibited splitting which was not in the multiples of 150 Da (molecular weight of conjugated ATRP initiator moiety). Increased mass covered under the topmost peak area ranged from 25410 to 25730. Based on this upper and lower limit, the number of ATRP initiators conjugated to CT modified with 1:20 molar ratio was assigned to range from 1 to 3. Similar calculations for remaining protein initiators were performed. The ranges for number of conjugated ATRP initiators given in Table 1 represent results obtained when a typical ratio of the reagents is used in different experiments.

faint brown solution was added to silica gel and stirred for 1 min to adsorb any copper salts and obtain a colorless solution, which was ultrafiltered for 15–20 min to remove unreacted monomers. This simplicity of synthesis allowed us to obtain a 60–70% yield of purified PPGs. We have not pursued kinetic studies for ATRP in this work and chose a polymerization time of 1 h based on reports in the literature. Armes et al. [29,30] studied the kinetics for ATRP of MPEGMA and MPC in water and showed that major conversion of monomers takes place within first 30 min of polymerization.

### 3.4. Characterization of PPGs

We used the analytical services from Polyanalytik to characterize the PPGs. Data in Table 2 show results from absolute $M_w$ estimations performed on size-exclusion column chromatography coupled with a tetra-detector system comprising detectors for refractive index, viscosity, right angle and low angle light scattering. As stated previously, this method of characterization does not reveal whether all the initiator sites on the protein are utilized in polymer synthesis. However, it does give information on the polydispersity and absolute $M_w$ of the entire PPGs. Monomers MPC and MPEGMA undergo efficient and rapid ATRP in aqueous solvents [29,30]. This allowed us to synthesize grafts between CT and high $M_w$ polymers (100–450 kDa). However, ATRP of HPMA is not very efficient in the presence of commonly used ATRP ligands such as 2,2′-dipyridyl and therefore it resulted in CT-graft-poly(HPMA) with rather lower $M_w$ (56.5 kDa) [31]. Chromatograms of all PPGs show single peaks with fairly low polydispersity indices (1.2–1.7) considering the 1–3 initiator sites on the CT and the high $M_w$ of the PPGs (Fig. 6a–c). It is interesting to note that chromatograms for PPGs that could potentially contain different numbers of polymer chains attached per protein show the presence of single peaks, and a chromatogram for PEGylated CT which certainly contains 1 or 2 PEG chains attached per protein shows the presence of multiple peaks. The hydrodynamic radii of all the PPGs were small (3–13 nm), which suggests the absence of large aggregate formation (Table 2).

### 3.5. Initial activity retention of native CT, PEGylated CT, and vinyl polymer-grafted CTs in buffer

Initial activity retention data in buffer alone is shown in Fig. 7. PEGylated CT had much reduced catalytic activity

Table 2
Molecular weight characterization data for PPGs

| PPG | $M_w$ (Da)[1] | $M_n$ (Da)[1] | $M_w/M_n$[1] | $R_h$[1] (nm) |
|---|---|---|---|---|
| CT | 28,100 | 26,300 | 1.06 | 2.2 |
| CT-graft-poly(HPMA) | 56,500 | 40,200 | 1.40 | 3.5 |
| CT-graft-poly(MPEGMA) | 146,800 | 116,400 | 1.26 | 6.4 |
| CT-graft-poly(MPC) | 497,500 | 283,500 | 1.75 | 13 |

$M_w$ = Weight average molecular weight, $M_n$ = Number average molecular weight, $R_h$ = Hydrodynamic radius.
Monomer:initiator ratios for HPMA ($M_w$ = 143), MPEGMA ($M_w$ = 2000), and MPC ($M_w$ = 282) were 1850, 130, and 950, respectively, assuming three initiators conjugated to CT (100 mg protein: 1000 mg monomer were used in feed). For 100% monomer conversions, theoretical $M_w$ of conjugates range from 260,000 to 780,000. As seen from the data, only MPC monomer shows potential for efficient polymerization by protein-initiated ATRP under the conditions used. In our previous report <17% monomer conversion was also observed after 16 h of polymerization [23]. In the present work, polymerization was stopped after 1 h as there was no significant benefit of increased polymerization time on monomer conversion under the reaction conditions used.
[1] Determined by size exclusion chromatography coupled with a tetra detector analyzer.

(25% of CT) than what we reported in our previous paper (70–80% of CT) [23]. (The PEG reagent used in this work is from a different source than the one from Nektar Therapeutics that was used in the earlier study. Nektar has discontinued catalog sale of its proprietary PEG reagents and therefore these reagents were not available at the time this work was performed.) But more interestingly, the catalytic activity of CT-graft-poly(HPMA) was almost equal to that of native CT. The catalytic activity of CT-graft-poly(MPC) was also slightly higher than that of PEGylated CT. Retention of high levels of catalytic activity despite grafting of high $M_w$ polymer on protein is a unique feature of protein-initiated ATRP that we have noted here. The following sections show that the ability to synthesize high $M_w$ PPGs with high biological activity can also have pharmacological advantages over PEGylated conjugates.

### 3.6. Mouse serum stability of native CT, PEGylated CT, and vinyl polymer-grafted CTs

To assess pharmacological benefits (if any) of structurally different PPGs over conventional PEGylation we compared the *in vitro* mouse serum stabilities of CT, PEGylated CT and vinyl polymer-grafted CTs. Fig. 8 shows a comparison of the initial catalytic activities of the three in mouse serum at 0.1 mg enzyme $ml^{-1}$ concentration. Only CT-graft-poly(HPMA) was found to retain substantial enzymatic activity in mouse serum. These results indicate that biocompatible vinyl polymer structures other than PEG can exert superior stabilization effects on modified protein in pharmacologically relevant conditions. Relative activity retentions were monitored for 5 days of incubation at 37 °C in mouse serum. Fig. 9 shows that native and all polymer-attached CTs except CT-graft-poly(HPMA) deactivated within 1 day. CT-graft-poly(HPMA) retained >50% of its catalytic activity for all 5 days of the study. Mouse serum itself did not exhibit any significant CT activity. Both free and polymer-attached enzyme present in PEGylated CT deactivated rapidly; therefore there was no ambiguity in interpretation of the results obtained in the mouse serum stability study.

### 3.7. Human serum stability of native CT, PEGylated CT, and vinyl polymer-grafted CTs

The activity and stability retention results obtained in human serum were quite different from those observed in mouse serum. Fig. 10 shows a comparison of initial catalytic activities of CT, PEGylated CT and vinyl polymer-grafted CTs in human serum at 0.1 mg enzyme $ml^{-1}$ concentration. All the modified CTs, but not the native CT, showed a drastic reduction in initial retention of catalytic activity in human serum. However, upon incubation for 5 days at 37 °C, PEGylated CT deactivated on day 4, whereas native CT, CT-graft-poly(MPC) and CT-graft-poly(HPMA) retained 25–30% catalytic activity on days 4 and 5 (Fig. 11). We believe that the fraction of free enzyme present in PEGylated CT should still be active on days 4 and 5, but its concentration must be too low to show any significant activity in the assay performed. At present we do not have a plausible explanation for the higher stability of native CT in human serum. However, results obtained so far collectively show that it is possible to develop PPGs by protein-initiated ATRP having serum stability and activity retention higher than that of conventionally PEGylated proteins. Also, the differences in stability of modified proteins in mouse and human serum observed here re-emphasize the importance of selecting a polymer structure to stabilize a given protein in a given environment rather than relying on PEGylation alone. Another point to mention here is that *in vivo* stability study results for PPGs are likely to be significantly better than those for PEGylated proteins because the high $M_w$ of PPGs should keep them in circulation for a much longer time.

## 4. Summary and conclusion

*In vitro* mouse and human serum stability study results show that PPGs synthesized by protein-initiated ATRP can perform better than PEGylated proteins under pharmacologically relevant conditions. Protein-initiated ATRP has displayed several other advantageous features over conventional PEGylation, such as controllable modification of native proteins, synthesis of well-defined high $M_w$

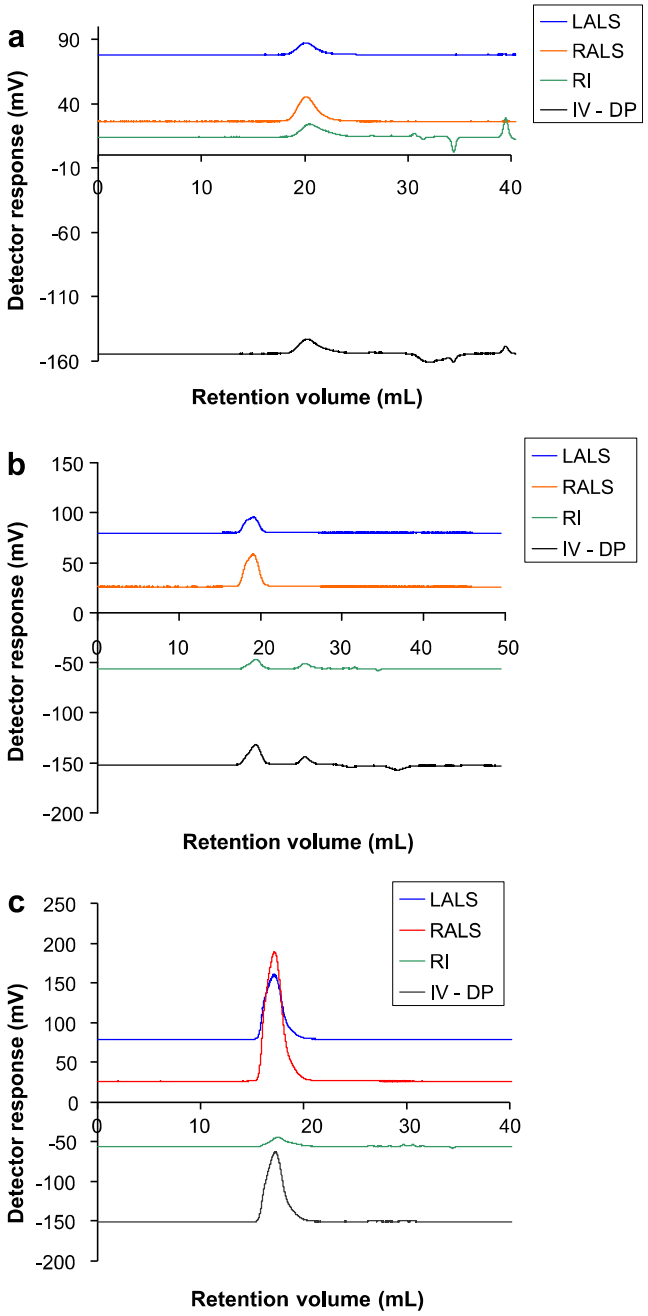


Fig. 6. (a) Size exclusion chromatogram of CT-graft-poly(HPMA) using a tetra-detector analyzer (TDA) from Viscotek. LALS = Low angle light scattering signal, RALS = Right angle light scattering signal, RI = Refractive index signal, IV-DP = Viscometer signal. Retention volume = 19.51; $M_w$ = 56,500 Da, $M_n$ = 40,200, $M_w$ / $M_n$ = 1.4. Hydrodynamic radius = 3.5 nm. (b) Size exclusion chromatogram of CT-graft-poly(MPEGMA) using a tetra-detector analyzer (TDA) from Viscotek. LALS = Low angle light scattering signal, RALS = Right angle light scattering signal, RI = Refractive index signal, IV-DP = Viscometer signal. Retention volume = 19.41; $M_w$ = 146,800 Da; $M_n$ = 116,400; $M_w$ / $M_n$ = 1.26. Hydrodynamic radius = 6.4 nm. (c) Size exclusion chromatogram of CT-graft-poly(MPC) using a tetra-detector analyzer (TDA) from Viscotek. LALS = Low angle light scattering signal, RALS = Right angle light scattering signal, RI = Refractive index signal, IV-DP = Viscometer signal. Retention volume = 17.50; $M_w$ = 497,500 Da; $M_n$ = 283,500; $M_w$ / $M_n$ = 1.75. Hydrodynamic radius = 13 nm.

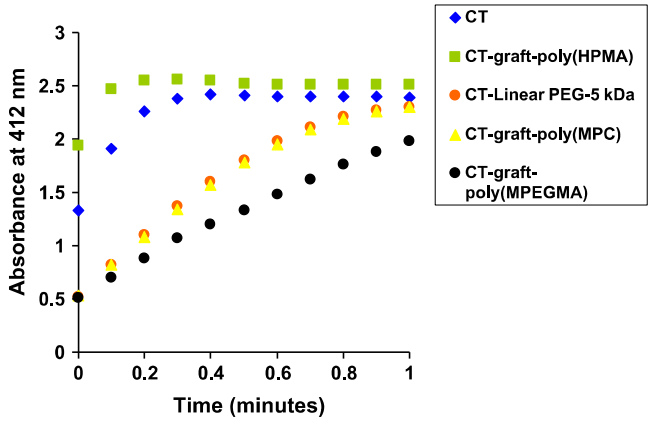


Fig. 7. Initial activity retention of native, PEGylated and vinyl polymer-grafted CTs in buffer at enzyme concentration 0.1 mg ml$^{-1}$.

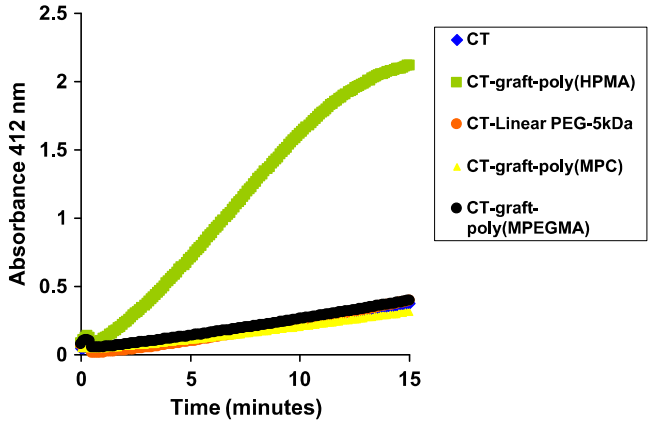


Fig. 8. Initial activity retention of native, PEGylated and vinyl polymer-grafted CTs in mouse serum at enzyme concentration 0.1 mg ml$^{-1}$.

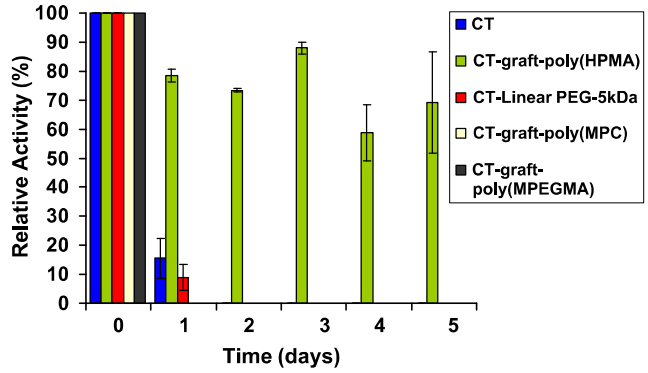


Fig. 9. Results of *in vitro* mouse serum stability study conducted in triplicate for native, PEGylated and vinyl polymer-grafted CTs at 37 °C at enzyme concentration 0.1 mg ml$^{-1}$.

conjugates with high residual activity retention, ease of purification and high yields of purified conjugates. Novel monomers can be designed and used in ATRP to give modified proteins unique properties that are not available to PEGylated proteins. These advantages over PEGylation make protein-initiated ATRP a promising and attractive

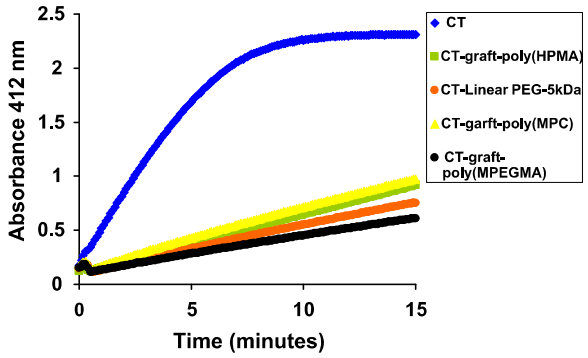


Fig. 10. Initial activity retention of native, PEGylated and vinyl polymer-grafted CTs in human serum at enzyme concentration 0.1 mg ml$^{-1}$.

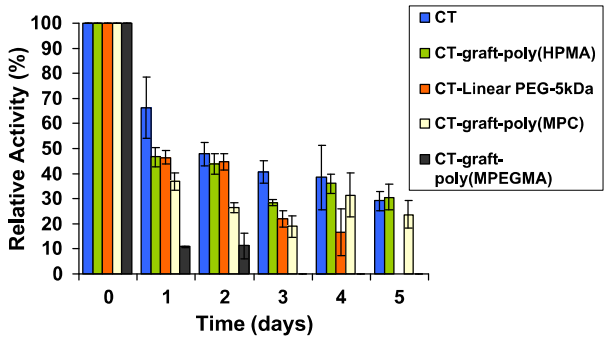


Fig. 11. Results of *in vitro* human serum stability study conducted in triplicate for native, PEGylated and vinyl polymer-grafted CTs at 37 °C at enzyme concentration 0.1 mg ml$^{-1}$.

alternative technique for further investigation to develop new, well-defined biological therapeutics or diagnostics. Also, vinyl biocompatible polymers used in this work are known to reduce the antigenicity of the protein modified. Work on demonstrating the compatibility of ATRP with various proteins and comparing the *in vitro* and *in vivo* serum stability of therapeutic proteins modified by PEGylation and by protein-initiated ATRP is currently in progress and will be reported in subsequent communications.

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