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Acta Biomaterialia 5 (2009) 560–569
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

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

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Received 6 May 2008; received in revised form 22 July 2008; accepted 12 August 2008
Available online 31 August 2008

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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doi:10.1016/j.actbio.2008.08.010

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

ID	Title	Pages
2596	Native protein-initiated ATRP: A viable and potentially superior alternative to PEGylation for stabilizing biologics	10