Complexation Hydrogels as Oral Delivery Vehicles for Insulin-Transferrin Conjugates

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Abstract: Protein bioconjugation is being currently investigated as a strategy to improve oral absorption of proteins. By conjugating proteins of therapeutic interest, such as insulin, to macromolecular PEG chains or other proteins, such as transferrin (Tf), the enzymatic stability of the protein drug and its transport characteristics across the intestinal epithelium may be improved. Complexation graft copolymers of poly(methacrylic acid-g-ethylene glycol), have also shown to be excellent carriers for oral protein delivery. In this work we demonstrated the use of complexation hydrogels as delivery vehicles for insulin-transferrin bioconjugates.

Introduction: Complexation graft copolymers of poly(methacrylic acid-g-ethylene glycol), designated as P(MAA-g-EG), have been shown to be effective in oral delivery of insulin. Their hydrogen bonding complexation/decomplexation characteristics render these responsive hydrogels able to protect the insulin in the harsh, acidic environment of the stomach before releasing the bioactive agent in the small intestine. Further, these network structures can inhibit the activity of Ca^{2+} dependent proteolytic enzymes [1], and increase the residence time of the drug in the small intestine by mucoadhesion. Oral administration of insulin entrapped in polymer microparticles resulted in high bioavailability of the drug in diabetic rats [2].

One of the other effective strategies for enhancing bioavailability of proteins exploits the receptor-mediated endocytotic pathway used by the cells for selective and efficient uptake of specific macromolecules required for various cell processes. By coupling proteins and peptides to ligands that can recognize specific receptors on the epithelial cells, transcellular delivery of these macromolecular biopharmaceuticals may be achieved [3]. Since only those molecules that are conjugated to the ligands are transcytosed, this process eliminates the potential side effects associated with the unspecific transport via the paracellular pathway. Researchers have used insulin-transferrin conjugates for enhancing the oral bioavailability of insulin [4]. Transferrin is a naturally occurring protein involved in the uptake of iron by the cells that binds specific receptors on the epithelial cells and is endocytosed.

We are investigating the use of the complexation hydrogel as a delivery vehicle for the insulin conjugates. Since the insulin conjugates thus released from the polymer microparticles will have better enzymatic resistance and enhanced permeability across the epithelium, high bioavailability of the drug could be achieved. In this work we investigated the loading and release profiles of transferrin and insulin-transferrin conjugates from P(MAA-g-EG) microparticles.

Experimental:
Polymer Synthesis: Polymer microparticles of 150-210 μm size rage were prepared by free radical UV polymerization as described elsewhere [5]. The initial monomer feed ratio of MAA:EG was 1:1. Tetraethylene glycol dimethacrylate (TEGDMA) was added as a crosslinker at 0.75 mol% of the total monomer. 1-Hydroxylcyclohexyl phenyl ketone (Irgacure-184) was used as the free-radical initiator and added in the amount of 0.1 wt% of the monomer mixture.

Conjugate Synthesis and Analysis: The conjugates were synthesized by coupling the proteins via succinimidyl 3-(2-pyridyldithio)propionate (SPDP), an amine reactive heterobifunctional crosslinker [4, 6]. Briefly, The N-terminal amino groups of bovine insulin (2 mg/ml) were protected by reaction with dimethylmaleic anhydride (DMMA) at a controlled pH of 6.8-7.0. Following this, the reaction products were dialyzed overnight (MWCO 3500) to remove the unreacted DMMA. The dialyzed protein was then reacted with 1 mg SPDP dissolved in minimum quantity of dimethylformamide for 2 hr. Insulin-PDP thus prepared was then dialyzed overnight and reacted with human holo transferrin-PDP complex (Tf-PDP) prepared by a similar procedure. The PDP:protein ratio was measured spectrophotometrically by measuring absorbance at 343 nm after reaction with 25 mM dithiothreitol (DTT) solution [6]. The conjugate was purified by size exclusion chromatography by elution on a Sephacryl-S200 column and protein modifications were confirmed by mass spectroscopy. The insulin: transferrin ratio in the conjugates was also measured to determine the number of insulin molecules coupled to a single transferrin molecule.

Protein Loading and Release Studies: The proteins were loaded into the polymer microparticles by equilibrium partitioning from a concentrated protein solution at pH 6.8 [7, 8]. Briefly 140 mg of polymer particles were soaked in protein solution for 6 hr. The microparticles were then collapsed by addition of 20 ml of 0.1 M HCl, filtered through 0.45 µm pore size membrane, and freeze dried for 24 hrs. The release studies were performed in a USP II dissolution apparatus. 10 mg of the protein loaded particles were placed in 50 ml pH 2.0 buffer solution. 50 µl samples were withdrawn at different time points and analyzed by reversed phase HPLC. After 1 hr, the pH was increased to 7.4 by addition of NaOH solution. The samples were withdrawn and analyzed for 2 hrs. The fractional release of the protein from the formulations, defined here as the ratio of the amount released at any time (Mt) to the total amount released at the end of release experiment (M_∞) was calculated.

Results: Mass spectroscopy analysis of the prepared conjugates confirmed both the N-terminal primary amine blocking and PDP attachment to the lysine primary amine at β-29 position of insulin. Insulin conjugation to Tf was also confirmed by mass spectroscopy. The loading and release were performed initially for Tf as a preliminary step towards the development of conjugate loaded formulation since the Tf molecule is significantly larger (hydrodynamic radius, R_h, of 40Å) as compared to insulin (R_h=20Å). Hence the diffusion of Tf in and out of the polymer network may be significantly hindered. The release profile of Tf from P(MAA-g-EG) microparticles is shown in Fig. 1. The loading efficiency in these studies, based on the initial protein present in the loading solution was 54.6± 4.7% and the release efficiency based on the loaded amount was 64.4± 6.7%. The loading and release profiles of the conjugates from the polymer formulation was also studied in this work.

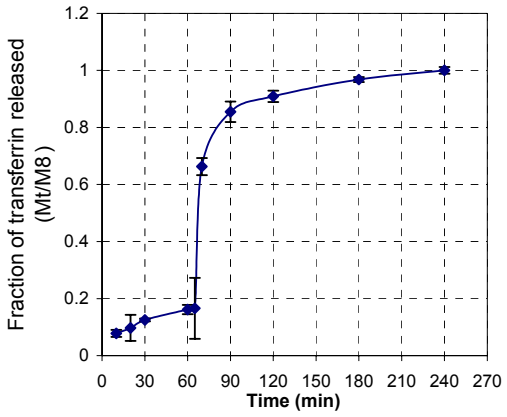

Fig.1. Transferrin release from P(MAA-g-EG) microparticles, pH was increased to 7.0 by addition of NaOH solution at 60 min. Release efficiency of transferrin was 64.4± 6.7%. Error bars represent one standard deviation (n=3).

Discussion: Complexation hydrogels when used as delivery vehicles for protein conjugates such as insulin-transferrin conjugates may provide a novel and effective strategy for oral delivery of therapeutic proteins. Since the proteins in the conjugated form, delivered at the site of absorption by the hydrogel carriers, will be able to traverse the intestinal barrier without getting degraded inside the epithelial cells this may result in increased bioavailability of the therapeutic protein. In this work we have studied the conjugation reaction of insulin and transferrin by mass spectroscopy and also investigated the loading and release profiles of the conjugated macromolecules from complexation hydrogels. Understanding the loading and release behavior of the conjugate is critical to developing a delivery system for the insulin-transferrin conjugates.

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