Synthesis and Analysis of Semitelechelic Poly [N-(2-Hydroxypropyl)Methacrylamide]

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ABSTRACT

Protein therapy is reviewed including its main challenges and solutions being studied with an emphasis on polymer-protein conjugates. Semitelechelic[1] N-(2-hydroxypropyl) methacrylamide [ST-p(HPMA)] polymers ending in carboxyl groups were synthesized by a free radical polymerization using the chain transfer agent 3-mercaptopropionic acid and the initiator N,N'-azobisisobutylonitrile. The molecular weights of the polymers were low (≤ 8000 Da) and the polydispersity was very small (≤ 1.2). Esterification of the carboxyl groups with *p*-nitrophenol (ONp) was successful as determined by UV/Vis spectroscopy. The number of carboxyl groups found by esterification was compared to the number found by titration of the polymer with NaOH. Purification problems and incongruencies between the spectroscopy and titration data did not allow conclusive determination of the amount of carboxyl groups per chain. Conjugation of the esterified polymer to the amino groups of chymotrypsin was unsuccessful as determined by SEC. It was possible that hydrolysis of theONp groups was faster than aminolysis. This was supported by the conjugation with tyrosinamide. Only 9% of the ONp groups were conjugated to tyrosinamide under aqueous conditions whereas 27% of the ONp groups were conjugated to tyrosinamide under non-aqueous conditions.

[1] a semitelechelic polymer contains only one functional group at the chain's end

INTRODUCTION

My second rotation under the Higuchi Fellowship was in the labs of Professor Kopecek. The project entailed the free-radical polymerization of HPMA to yield semitelechelic[**] N-(2-hydroxypropyl)methacrylamide [ST-p(HPMA)] polymer chains ending in a carboxyl group and their attachment to chymotrypsin. This report will briefly review polymer modification of proteins, present the experimental methods, and review and discuss the results.

Peptides, enzymes, hormones, and other proteins have an enormous therapeutic potential as thrombolytics, immunomodulators, growth factors, chemotherapeutics, and cardiovascular drugs. Protein therapy also has several problems inhibiting the actualization of their great potential.[1-3]

An immune response to foreign proteins is one of the greatest challenges to overcome. Initially, the proteins used were not of human origin. Repeated use lead to an immunogenic response (formation of antibodies) and an antigenic response (reaction to specific antibodies). The development of large scale production of human-derived proteins and recombinant DNA technology has significantly reduced the immunogenicity and antigenicity of the proteins. Despite this, the proteins are still recognized as foreign to the body and are rapidly cleared by the reticuloendothelial system, kidney, spleen, or liver. The rate of clearance depends on the ionic charge, the size of the molecule and the presence of cellular receptors.[1-3]

Besides being cleared from the body, proteins can also be metabolized by peptidases resulting in their biological activity being rapidly lost. They are generally not stable for extended times and many have poor solubility in aqueous systems.[1-3]

[**] a semitelechelic polymer contains only one functional group at the chain's end

Possible solutions to these problems include sustained release technologies such as liposomes and microspheres, and chemical modification of the protein's surface. Liposomes and microspheres have immune and clearance problems similar to those of proteins.[1-3] Modification technology can be applied to both the protein and to their carriers mentioned in this report. Surface modification shows great promise and was the goal of this work.

Surface modification can greatly change the recognition of foreign entities. Many surface modifications have been explored including, succinylation, acylation, guanidation, and deamination. Polymer conjugation of proteins has also been explored. Polymers used as conjugates include dextran, albumin, DL-poly (amino acids), polyvinylpyrrolidone, poly (ethylene glycol) [PEG], styrene-maleic anhydride copolymers, divinylether-maleic acid copolymers, and poly-N-(2-hydroxypropyl)- methacrylamide [p-HPMA]. The main goals of polymer conjugation are a stable linkage, increased half-life, low immunogenicity, high biological activity, and high water solubility.[1-3]

PEG is one of the most extensively used polymer conjugates due to a combination of several factors. PEG is an uncharged, hydrophilic, linear polymer, available in a range of molecular weights. Its uncharged properties allow PEG large flexibility in bonding to substrates. PEG is non-immunogenic and has little toxicity when given orally or parentally. PEG also has FDA approval for use in drugs, foods, and cosmetics.

The main reason for PEG's use is its success. PEG modification has resulted in increased circulating life, decreased immunogenicity and/or antigenicity, increased resistance to proteolysis, and enhanced solubility and stability. Several properties of PEG could account for the effects including PEG's low interfacial free energy with water, unique solution properties, molecular

conformation in aqueous solutions, hydrophilicity, high surface mobility, and steric stabilization effects. Size exclusion chromatography (SEC) shows PEG to have a larger hydrated radius than p-HPMA polymers of the same molecular weight. It is thought that the hydrated polymer strands provide a mobile surface which sterically hinders receptors and enzymes from approaching the protein, thereby reducing recognition and degradation. The increase in molecular weight would also reduce clearance by glomerular filtration in the kidney.[1-5]

Although PEG has yielded much success, other polymers have the potential as good protein conjugates. P-HPMA is a good candidate and was used in this work. Like PEG, p-HPMA is an uncharged, hydrophilic, linear polymer. SEC indicates that poly-HMPA chains form smaller, more tightly bound conformations than PEG in water; this suggests that larger p-HPMA chains would be needed to match PEG's effect.

The activity of conjugated proteins varies from being more active to inactive, but is generally lower than that of the native proteins. It is thought that the greatly increased circulation life more than makes up for decreases in activity; regardless, greater activity is wanted. It has been found that the activity of PEG-enzyme conjugates was dependent on the method and extent of conjugation.[6] Polymers with multiple functional groups can cause "cross-linking" connections between proteins and decrease the effectiveness of the polymer "shield." Harsh coupling conditions can also lead to decreased activity.

A single point attachment of polymers under gentle reaction conditions has become an important objective for protein conjugation. This would eliminate the possibility of crosslinking and most likely results in a more extended polymer conformation providing better shielding. Kamei and Kopecek recently reported the synthesis of ST-p(HPMA) chains ending in a single amino groups

which they coupled to polymer nanospheres.[7] This work was concerned with the synthesis of ST-p(HPMA) chains ending in a carboxyl group. The carboxyl group was to be activated by esterification and the polymer conjugated to chymotrypsin's amine groups.

Free radical polymerization of a semitelechelic polymer is difficult and purification of a telechelic[***]/semitelechelic mixture is even more challenging. Takei et. al. reported obtaining semitelechelic chains ending in carboxyl groups using the chain transfer agent 3-mercaptopropionic acid (MPA).[8] Synthesis of ST-p(HPMA) was accomplished by free radical polymerization of the monomer HPMA with the chain transfer agent MPA. A possible mechanism is seen in Figure 1. The reactions begin with the initiator, N,N'-azobisisobutylonitrile (AIBN), breaking into two radicals. A chain terminal semitelechelic polymer can be obtained if the chain transfer of the radical occurs next. Propagation allows the polymer to grow until chain transfer or another form of termination occurs.

METHODS

Polymerization

The ST-p(HMPA) polymer was made by a chain transfer free radical reaction as described above. Two polymerization techniques were used. The first was a precipitation polymerization in acetone for polymer 1. This was compa3Tj,.5se8uzatioel

Figure 1. An ideal reaction mechanism to produce ST-p(HPMA).

Molecular weight determination

The molecular weight was estimated by SEC. A Superose 12 column was calibrated with p-(HPMA) fractions characterized by laser light scattering. Detection of the eluting sample was performed using a refractive index detector and UV detector set at 254 nm.

Analysis

The carboxyl group was "activated" by polymeranalogous esterification with *p*-nitrophenol. The ST-p(HPMA) was reacted with *p*-nitrophenol and dicyclohexylcarbodiimide at -10°C for 3 hours and stored overnight at 4°C. Qualitative determination of esterification was done by UV/Vis spectrophotometry. The polymer was first dissolved in dilute HCl and the absorbance of bound ONp was measured at $\lambda = 272$ nm, $\varepsilon = 10{,}000$ L/(mol cm) in water. NaOH was then added to hydrolyze the ONp groups and the absorbance of free ONp^- was measured at $\lambda = 400$ nm, $\varepsilon = 18{,}000$ L/(mol cm) in water. Carboxyl and ONp content were also analyzed by titration with NaOH

Protein conjugation

Binding of the esterified polymer to chymotrypsin was attempted. The reaction occurred in PBS buffer (pH=7) at 4°C overnight. The subsequent day, the pH was raised to about 9 with $Na_2B_4O_7$ and stirred for several more hours and the reaction was ended by adding 1-amino-2-propanol. The solution was partially purified on a PD-10 column to remove low molecular weight compounds and analyzed by SEC. ination/Attachzed[/Bototo]/BBox [7237095601 312 108.439] >>BDCBT/

The esterfied polymer was attached to tyrosinamide in an aqueous solution using the same procedure as for chymotrypsin and in DMSO at room temperature following the same procedure. Detection of polymer/tyrosinamide conjugates was accomplished by UV/Vis spectroscopy.

RESULTS AND DISCUSSION

The calibration of the Superose 12 column with HPMA standards is shown in Figure 2. The standards had been prepared by fractionating a polydisperse sample of p-HPMA. The molecular weight of the standards had been characterized by laser light scattering. A straight line described the data very well, $R^2 = 0.995$.

The polymerization information and analysis of the ST- p(HPMA) polymers are presented in Table 1. The polydispersity of the polymers is very low for a free radical polymerization. The largest value of 1.2 is well below that expected. This suggests that termination/chain transfer occurred at a specific polymer chain length. More studies are needed to make any conclusions about the polydispersity.

All of the molecular weights are below 10 kD. Free radical polymerizations characteristically produce large molecular weight polymers. The low molecular weight supports that chain transfer of the free radical occurred.

Polymers 1, 2, and 4 had similar amounts of HPMA monomer and MPA. The precipitation polymerization of polymer 1 produced the largest molecular weight polymer. The difference was not great and more studies are necessary to determine any correlation. Polymers 2 and 4 had the same reaction conditions. Polymer 4 has a slightly lower molecular weight that is probably due to

Figure 2. Calibration curve of Superose 12 column with p-HPMA standards.

more efficient precipitation of lower molecular weight chains in the acetone:ether mixture. Polymer 3 was synthesized with three times as much MPA as the other polymers. This was expected to produce shorter polymers since the growing polymer would be apt to collide with MPA quicker if it were in greater concentration. Precipitation problems occurred after polymerization of polymer 3. The polymer was precipitated into acetone as were polymers 1 and 2. Very little polymer precipitated although the solution was very viscous. Many lower molecular weight polymer chains were most likely soluble in the acetone and were lost. The molecular weight determination of the recovered polymer is therefore artificially high and does not accurately represent the true molecular weight.

Esterification of carboxyl groups and ONp determination had a twofold purpose. First, the esterified polymer is reactive and should easily couple to amine groups. Second, detection of ONp groups can be accomplished with small amounts of the polymer by UV/Vis spectroscopy. Direct detection of carboxyl groups requires larger amounts of the sample that we initially wished to avoid.

A semitelechelic polymer has one reactive group per polymer chain. It was hoped that the esterification of the carboxyl groups would be complete and that the ONp content of the polymers would equal the original carboxyl content of the polymer. The ONp fractions presented in Table 1 are the ratios of ONp groups detected divided by the number of polymer chains calculated based on the number average molecular weight. The amounts of ONp detected before and after hydrolysis are very similar. The agreement indicates that the ONp was covalently bound and not associated to the polymer. If the ONp had been associated to the polymer, no significant amount of bound ONp should have been detected.

The amount of ONp detected by the UV/Vis data in Table 1 is subject to question. In the esterification, the by product dicyclohexylurea (DCU) is produced and was very difficult to remove. A large amount of contamination would affect all determinations using the mass of the sample. The ONp content reported most likely represents the extent of purification. Thus, the values probably do not represent the actual ONp nor the original carboxyl content.

Table 1. Polymerization and analysis data of ST-p(HPMA).

	Polymer 1	**Polymer 2**	**Polymer 3**	**Polymer 4**
Polymerization method / Solvent	Precipitation / acetone	Solution / MeOH	Solution / MeOH	Solution / MeOH
HPMA (mg)	2000	2000	2000	2000
MPA (mg)	200	200	600	200
AIBN (mg)	96	92	92	92
Acetone (mL)	17.6	-	-	-
MeOH (mL)	-	16.7	16.6	16.7
Analysis results				
Mw (kDa)	8.0	7.2	(< 5.4)	6.7
Mn (kDa)	6.8	6.5	(< 5.1)	5.9
Z	1.2	1.1	(> 1.05)	1.1

Titration of the polymer with NaOH to detect the number of carboxyl groups was attempted next. The NaOH titration standard was calibrated with potassium acid phthalate. The titration curve is shown in Figure 3. The titration curve looked good and the obtained result of 0.10 M agreed with the reported value of 0.0989 M. Figure 4 shows the titration of polymer 4. Again the titration curve looks very typical and has an unmistakable neutralization point. The amount of NaOH used to neutralize the polymer indicated that there were 3.5 acid groups per polymer chain. This was much higher than expected and it was thought that some low molecular weight MPA could be associated with the polymer chains and be responsible for the large acid content. The polymer was purified by precipitation and titrated. The second titration gave 3.0 acid groups per polymer chain which was still three times higher than expected.

The titrated samples were combined and subject to dialysis under ethanolic, acidic, basic and neutral conditions. The polymer was then freeze dried and titrated. It was expected that the dialysis would remove all lower molecular weight compounds (the molecular weight cutoff of dialysis tubing was 1000 Da). The titration revealed 2.4 acid groups per polymer chain. This result was closer to the expected value but it was still too high and opposed the data obtained from UV/Vis spectroscopy.

The esterified polymer 4 was also titrated with NaOH. Figure 5 presents the titration curve. The pH was initially low which indicates the presence of acid groups. (If the esterification was not complete, residual acid groups would be expected, but only small amounts.) The titration curve looks normal up to the neutralization point of the acid. Calculation revealed 2.1 neutralized acid groups per chain. It remained unclear whether the acidic groups were part of the polymer or due to impurities that had not been removed. At pH = 7, hydrolysis of the ONp groups should be minimal.

Figure 3. The calibration of NaOH with potassium acid phthalate.

Figure 4. The titration of polymer 4 with NaOH.

Figure 5. The titration of esterified polymer 4 with NaOH.

Table 2. The ONp content of polymer 4 determined by titration agrees with the UV/Vis determined amount.

Acid groups / chain of the dialyzed polymer (titration data)	2.4
Acid groups / chain of the esterified polymer (titration data)	2.1
Calculated ONp groups / chain[a]	0.3
Measured ONp groups / chain (UV/Vis data)	0.32

[a] The calculated ONp groups / chain was determined by subtracting the acid content of the unmodified polymer from the acid content of the esterified polymer.

This was confirmed by absence of yellow color until pH = 7.1, at which point a very faint yellow color (due to free ONp^-) could be detected. The titration curve took an unusual path to pH = 9.3. It appeared that some base was being consumed, presumably by the hydrolysis of the ONp group. This was supported by an increase in the yellow color with increasing pH.

Table 2 contains the data from the titrations of the dialyzed and esterified polymers and the data from the UV/Vis spectroscopy. The number of ONp groups / polymer chain was estimated using the titration data by subtracting the acid content of the esterified polymer from the acid content of the unmodified dialyzed polymer. The result of 0.3 agrees with the value obtained by UV/Vis spectroscopy.

An explanation of the high acid content remains unknown. Only two peaks eluted during the SEC analysis which were assumed to consist of the polymer and the buffer. The buffer peak contains all of the material too small to be resolved by the column. All lower molecular weight contaminants should have been removed during dialysis. The joining of two growing chains would have given an acid content of 2 per chain. A value above 2 was an unexpected result. Contamination by a neutral species would result in a calculated value being lower than the actual. Only a small acidic contaminant could raise the acid content of the polymer. Determination of the number of carboxyl groups might have been solved by a sulfur content analysis of the polymer since each acid group should be connected to the polymer by a sulfur, but time was insufficient.

Binding of the esterified polymer to chymotrypsin was attempted as described above. After termination of the reaction, the product was analyzed by SEC. A polymer bound to chymotrypsin would have a much larger size and elute earlier than an unbound polymer chain. No detectable

amount of bound polymers was detected. It was thought that the ONp groups were hydrolyzed before they could react with the amino groups on chymotrypsin.

Three conjugations reactions with *L*-tyrosinamide were performed to determine if elimination of water from the reaction increased binding to the amino groups. Table 3 contains a summary of the reaction conditions and the results. Two aqueous reactions with different amounts of Tyr yielded binding of only 9% of the ONp groups. The non-aqueous reaction had three times more binding than the aqueous reactions which tends

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