Prolonged Blood Circulation in Rats of Nanospheres Surface-Modified with Semitelechelic Poly[N-(2-Hydroxypropyl)methacrylamide]

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Semitelechelic poly[N-(2-hydroxypropyl)methacrylamide] (ST-PHPMA) containing one amino end-group and differing in molecular weight were synthesized by radical polymerization in the presence of 2-aminoethanethiol (AET) as chain transfer agent. These polymers were covalently attached via amide bonds to the surface of nanospheres based on a copolymer of methyl methacrylate, maleic anhydride, and methacrylic acid. When compared to unmodified nanospheres, those with the surface modified with ST-PHPMA possessed a decreased protein (albumin, IgG, fibrinogen) adsorption in vitro, an increased intravascular half-life as well as a decreased accumulation in the liver after intravenous administration into rats. The higher the molecular weight of the ST-PHPMA, the more pronounced the changes in these properties. The results obtained methodically demonstrated that covalently attached ST-PHPMA chains are efficient in decreasing the biocorrection of negatively charged (hydrophilic) polymer surfaces.

KEY WORDS: semitelechelic poly[N-(2-hydroxypropyl)methacrylamide]; nanospheres; surface modification; prolonged blood circulation; avoidance of RES.

INTRODUCTION

Nanoparticles are spherical polymer particles with a wide use in biomedical applications (1), for example as carriers of anticancer agents (2). Following administration into the bloodstream, nanoparticles are rapidly cleared by the reticuloendothelial system (RES), typically due to phagocytosis by macrophages in the liver and spleen (3). Numerous attempts have been made to modify the biocorrection of nano- and microspheres (4-8), and liposomes (9) by the RES by changing their surface structure. The most frequently used procedure is the adsorptive or covalent attachment of poly(ethylene oxide) (PEO) chains to nanoparticle surfaces. It is well established that PEO chains possess protein repulsion properties (10). This phenomenon is probably due to PEO's low interfacial free energy with water, hydrophilicity, high surface mobility and steric stabilization effects (11).

However, other polymers have been successfully used to decrease biocorrection. For example, albumin was used to modify L-asparaginase (12) and N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers to modify trypsin (13), chymotrypsin (13), and acetylcholinesterase (14). In the latter case a dramatic increase in the acetylcholinesterase survival in the bloodstream of mice and in the thermostability was observed when the enzyme was attached to an HPMA copolymer. However, the chemistry used did not permit one-point attachment of the polymer chains to the surface of the protein. Multiple attachment points resulted from the reaction of HPMA copolymers containing side-chains terminated in reactive ester groups and amino groups of the enzyme (13,14).

To be able to test the hypothesis that poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) attached to nanophasic surfaces at one of their chain termini will decrease their biocorrection in the RES, semitelechelic PHPMA (ST-PHPMA) containing one terminal amino group but differing in molecular weight were synthesized, and covalently attached to the surface of nanospheres based on a copolymer of methyl methacrylate, maleic anhydride, and methacrylic acid. The modified nanospheres were characterized by physicochemical methods, by protein (albumin, IgG, fibrinogen) adsorption, and by the evaluation of their intravascular half-life, and accumulation in the liver and the spleen after intravenous administration to rats.

MATERIALS AND METHODS

HPMA was prepared as described previously (15). Methyl methacrylate (MMA) and methacrylic acid (MAA) were distilled under reduced pressure. 2,2'-azobisobutyronitrile (AIBN), 4,4'-azobis-4-cyanovaleric acid (ACVA) were recrystallized, and 2-aminoethanethiol (AET) was purified by sublimation. Other reagents and bovine proteins were commercially obtained and used without further purification.

Synthesis and Characterization of ST-PHPMA

ST-PHPMA (Scheme 1) was prepared by solution polymerization of HPMA in methanol with AET and AIBN as a chain-transfer agent and an initiator, respectively, at 50°C for 24 h. The polymers were precipitated by pouring the reaction mixture into an excess of diethyl ether, and purified by repeated reprecipitation from methanol solutions into diethyl ether. Their weight-average (Mw) and number-average (Mn)

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\text{Scheme 1. Structure of ST-PHPMA.}
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molecular weights were estimated by size exclusion chromatography (FPLC Pharmacia system) on a Superose 12 column connected to a differential refractometer using Tris buffer (0.05 M 2-amino-2-hydroxyethyl-1,3-propanediol + 0.5 M NaCl, pH = 8) as eluent. The column was calibrated using PHPMA fractions of known molecular weight (determined by static light scattering). The Mn of the polymers was also calculated from the content of amino end-groups as determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS) (16). The compositions of monomer mixtures and the characterization of polymers are summarized in Table I.

**Synthesis and Surface Modification of Nanospheres**

MMA copolymer-based nanospheres were produced by an emulsifier-free emulsion copolymerization (17) of MMA with [14C]-maleic anhydride (MA) and MMA as ionic comonomers to introduce radioactivity and carboxylic groups for surface modification (Table II). Cold nanospheres of the same composition were synthesized in parallel. A solution (phosphate buffer, pH 5.7) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to a suspension of the nanospheres purified by centrifugation using an ultracentrifuge (Sorvall RC-2-B). The suspension was incubated for 4 h at 4°C, and a solution of ST-PHPMA in phosphate buffer, pH 5.7, was added. In one case ST-PHPMA was replaced with 1-amino-2-propanol (AP). The mixture was further incubated for 16 h at 4°C. Stoichiometric amounts of EDC and ST-PHPMA to that of surface carboxylic groups of the nanospheres (as determined by acid-base titration) were used. After the surface modification reaction, the nanospheres were purified by elutriation three times with purified water. The amount of immobilized PHPMA was estimated from the difference in PHPMA concentrations (determined by the TNBS method) in the supernatant before and after the surface modification reaction. Parallel experiments on the incubation of ST-PHPMA with poly(methyl methacrylate-co-methacrylic acid) nanospheres in the absence of coupling agent have shown negligible non-specific (non-covalent) adsorption of ST-PHPMA onto the surface of nanospheres. The diameters of the control and surface-modified nanospheres were evaluated by quasielastic laser light scattering using a Brookhaven Instruments apparatus equipped with an argon laser (vertically polarized, λ = 514.5 nm) at 25°C. The size of the particles (equivalent hydrodynamic radius) was calculated from the diffusion coefficient using the Stokes-Einstein equation (18). The nanospheres were characterized in Table III.

**Protein Adsorption**

An equal volume of the respective protein solution (in 2/15 M saline) was added to the suspension of nanospheres (solid content about 2.8% (w/w)) and left for 3 h at 25°C. The suspensions were centrifuged, and the protein concentration in the supernatant determined by UV spectrophotometry at 280 nm. The amount of protein adsorption was calculated from the difference in protein concentrations in the supernatant before and after exposure to the nanospheres.

**Fate of Nanospheres in Vivo After Intravenous Administration to Rats**

The suspensions of 14C-labeled nanospheres (1 ml containing about 40 mg nanospheres; 2 μCi) in physiological saline were injected intravenously into the femoral vein of Sprague-Dawley rats (200 g). Blood was serially withdrawn from the tail vein at time intervals indicated. After 24 h, the animals were sacrificed and the liver and spleen dissected after abdominal exsanguination. Blood and homogenized organs were weighed into glass vials and dissolved in solubilizer (PROTOSOL, DuPont). After addition of scintillation cocktail (BIOFLUOR and ECONOFUOR, DuPont) to each vial, the radioactivity of the samples was measured in a scintillation counter (Beckman LS1801). The percentages of the administered dose of nanoparticles in the blood, liver, and spleen were calculated using a standard (25 μg of nanosphere suspension), the total blood volume (6 ml/100 g-body weight (19)), and the organ weight.

**RESULTS AND DISCUSSION**

**Synthesis of Semitelechelic Poly[N-(2-hydroxypropyl)methacrylamide]**

The synthesis of semitelechelic polymers containing a reactive group at one end of the molecule is a prerequisite for their one point attachment to the surfaces of biomaterials or proteins. Unfortunately, it is not possible to synthesize semitelechelic polymers by radical polymerization of monomers such as HPMA using polymerization initiators containing reactive groups. The occurrence of two mechanisms of termination of growing polymer chains (disproportionation and recombination) results in products which are a mixture of semitelechelic and telechelic polymers making their purification very difficult (J. Kopeček, J. Strohalm, unpublished data).

A suitable route for the synthesis of semitelechelic polymers is radical solution polymerization in the presence of chain transfer agents. Thiol compounds have been shown to be effective at introducing functional groups to the ends of growing polymer chains and regulating the molecular weight via chain-transfer reactions (20,21). Using 2-aminoethanol (AET) Okano et al. (20) have synthesized semitelechelic poly(2-hydroxyethyl methacrylate). As shown in Table I, this method is suitable for the synthesis of semitelechelic PHPMA (ST-PHPMA) with different molecular weights.

**Table I. Solution Polymerization of HPMA and Characteristics of the Resulting Polymers**

<table>
<thead>
<tr>
<th>Lot</th>
<th>N-ST</th>
<th>ST-1</th>
<th>ST-2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ST-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPMA (mg)</td>
<td>300</td>
<td>2,400</td>
<td>2,400</td>
<td>2,400</td>
</tr>
<tr>
<td>AET (mg)</td>
<td>0</td>
<td>40</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>AIBN (mg)</td>
<td>14</td>
<td>110</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>MeOH (ml)</td>
<td>2.5</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mw&lt;sub&gt;FPLC = A&lt;/sub&gt;</td>
<td>55,300</td>
<td>18,800</td>
<td>9,300</td>
<td>6,300</td>
</tr>
<tr>
<td>Mn&lt;sub&gt;FPLC = B&lt;/sub&gt;</td>
<td>31,400</td>
<td>12,500</td>
<td>8,000</td>
<td>5,000</td>
</tr>
<tr>
<td>A/B</td>
<td>1.76</td>
<td>1.50</td>
<td>1.16</td>
<td>1.25</td>
</tr>
<tr>
<td>Mn&lt;sub&gt;TNBS = C&lt;/sub&gt;</td>
<td>∞</td>
<td>13,400</td>
<td>7,200</td>
<td>4,600</td>
</tr>
<tr>
<td>B/C</td>
<td>0.00</td>
<td>0.94</td>
<td>1.10</td>
<td>1.10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Polymerized 24 h at 50°C.
<sup>b</sup> ST-2 PHPMA was further purified by dialysis using a membrane tubing (cut-off Mw: 1000) after purification by repeated precipitation.