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]s (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 have clearly demonstrated that covalently attached ST-PHPMA chains are efficient in decreasing the biorecognition 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 blood stream, 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 biorecognition 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 biorecognition. 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).

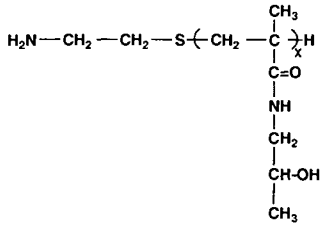
To be able to test the hypothesis that poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA) attached to nanosphere surfaces at one of their chain termini will decrease their biorecognition in the RES, semitelechelic PHPMA (ST-PHPMA) containing one terminal amino group but differing in molecular weight were 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′-azobisisobutyronitrile (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)



Scheme 1. Structure of ST-PHPMA.

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Abbreviations: AET, 2-aminoethanethiol; ACVA, 4,4′-azobis-4-cyanovaleric acid; AIBN, 2,2′-azobisisobutyronitrile; AP, 1-amino-2-propanol; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HPMA, N-(2-hydroxypropyl)methacrylamide; IgG, immunoglobulin G; MA, maleic anhydride; MAA, methacrylic acid; MMA, methyl methacrylate; PEO, poly(ethylene oxide); PHPMA, poly[N-(2-hydroxypropyl)methacrylamide]; ST, semitelechelic; linear macromolecule possessing a functional group at one end of the molecule; ST-PHPMA, semitelechelic poly[N-(2-hydroxypropyl)methacrylamide] terminated in one amino group; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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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 [^{14}C]-maleic anhydride (MA) and MAA 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 elutriation using an ultracentrifuge (Sorvall RC2-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 ^{14}C-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 ECONOFLUOR, 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 μl 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-aminoethanethiol (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[a] of HPMA and Characteristics of the Resulting Polymers

Lot	N-ST	ST-1	ST-2[b]	ST-3
HPMA (mg)	300	2,400	2,400	2,400
AET (mg)	0	40	800	800
AIBN (mg)	14	110	110	110
MeOH (ml)	2.5	20	20	20
Mw_{FPLC} = A	55,300	18,800	9,300	6,300
Mn_{FPLC} = B	31,400	12,500	8,000	5,000
A/B	1.76	1.50	1.16	1.25
Mn_{TNBS} = C	∞	13,400	7,200	4,600
B/C	0.00	0.94	1.10	1.10

[a] Polymerized 24 h at 50°C.

[b] ST-2 PHPMA was further purified by dialysis using a membrane tubing (cut-off Mw: 1000) after purification by repeated precipitation.

The number average molecular weight of the polymers synthesized was determined by two methods, namely size exclusion chromatography and determination of amino end-groups using 2,4,6-trinitrobenzene sulfonic acid. The results are in very good agreement, indeed. The differences in the molecular weights of samples ST-2 and ST-3 are most probably due to the lost of the low molecular weight fraction during dialysis.

It appears that radical polymerization in the presence of a potent chain transfer agent is a suitable general method for the synthesis of semitelechelic polymers. However, two limitations should be mentioned. First, it is important to use an excess of the chain transfer agent to ensure that it reacts predominantly with primary radicals. Polymerization in the presence of smaller amounts of AET (the composition of ST-1 in Table I appears to be on the borderline) does not result in the synthesis of semitelechelic polymers (results not shown). Consequently, only low molecular weight polymers can be produced by this method. Second, it is important to work in a homogeneous solution. Attempts to produce semitelechelic PHPMA by radical precipitation polymerization of HPMA in the presence of large amounts of AET were not successful (results not shown). One possible explanation might be the precipitation of short growing chains before the chain transfer reaction occurred, their coalescence with the precipitated inactive polymer, followed by diffusion controlled chain growth.

Synthesis and Surface-modification of Nanospheres

Monodisperse nanospheres were synthesized by emulsifier-free emulsion copolymerization of methyl methacrylate (MMA), maleic anhydride (MA), and methacrylic acid (MAA). The conditions used are listed in Table II. Cold and hot nanospheres were synthesized in parallel. It was assumed that the content of surface COOH groups (41.8 μeq/g) was identical. The polydispersity as calculated from quasielastic light scattering data was very narrow (1.03).

The nanospheres were modified by attachment of ST-PHPMA containing amino end-groups. By the reaction of the latter with surface carboxylic groups derived from hydrated MA (maleic acid), MAA, and ACVA, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as the coupling agent, PHPMA chains were attached to the surface via amide bonds, one per chain. As shown in Table III, the efficiency of binding was relatively high, although the efficiency tended to decline with increasing molecular weight of PHPMA. The relationship between the molecular weight of PHPMA and the hydrodynamic thickness of the coating layer (determined from the differences in the hydrodynamic volumes of control and modified nanospheres) is shown in Fig. 1. Illum et al. (22) determined the hydrodynamic volumes of polystyrene microspheres coated with poloxamers (block copolymers PEO-poly(propylene oxide)-PEO). Consequently, it was possible to compare the hydrodynamic thickness-molecular weight relationship for PEO and PHPMA. It appears that the thickness of the PEO layer is considerably larger based on the comparison of molecular weights than the PHPMA layer. However, if polymerization degrees (number of monomer units per chain) are compared, the difference is smaller (about three times). These results are in accordance with the fact that PEO has an extended conformation in water solutions (23), whereas PHPMA adopts a random coil conformation (24). The results are also in agreement with the observation that the elution volume of PHPMA chains in size exclusion chromatography on Superose beads in aqueous buffers is considerably higher when compared to a PEO chain of the same molecular weight (P. Kopečková, unpublished data).

Table II. Emulsifier-Free Emulsion Polymerization[a] of MMA, MA and MAA and Characteristics of the Resulting Nanospheres

	Cold nanospheres	Hot nanospheres
MMA (ml)	25	1
MA (mg)	100	4[b]
MAA (μl)	985	40
ACVA[c] (mg)	260	9
Water (ml)	300[d]	15[d]
Diameter[e] (nm)	180	207
$[COOH]_s$[f] (μeq/g)	41.8	N.D.[g]

[a] Polymerized 6 h at 70°C.
[b] ^{14}C-MA (50 μCi).
[c] Used as a stoichiometrical solution in 0.1 N NaOH.
[d] Total volume containing that of 0.1 N NaOH used as a solvent of ACVA.
[e] Determined by laser light scattering at 25°C.
[f] COOH concentration at the surface of nanospheres.
[g] Not determined.

Protein Adsorption

Unmodified and PHPMA surface modified nanospheres were incubated with solutions of bovine proteins (albumin, IgG, fibrinogen) and adsorption isotherms were determined (Fig. 2). A time period of 3 h was chosen to ensure that the adsorption equilibrium was reached. It is interesting to note that the adsorption isotherms for all three proteins studied show higher initial slopes on unmodified nanospheres than on modified ones, indicating a higher affinity of proteins for the unmodified surface.

The relationship between the thickness of the coating layer and the protein adsorption is shown on Fig. 3. The higher the thickness of the coating layer (and the molecular weight of PHPMA), the lower the amount of adsorbed protein. The mechanism of protein repulsion may be similar to the one discussed by Gombotz et al. (25) for long (folded) PEO chains. Inside the PHPMA coil, there may be a "loose" sharing of bound water molecules. The sharing of bound water creates an excluded volume from which proteins will be repelled. If a protein molecule attempts to compress the hydrated coil, it will take energy to remove the water molecules bound to PHPMA side-chains, while at the same time the PHPMA coil will loose entropy. It is evident that the released water molecules will be gaining entropy. However, the overall process will cause a rise in free energy and will not occur spontaneously. An osmotic driving force will "drive back" the released water molecules (25). However, there are other factors influencing the protein adsorption in the systems studied here. The modification of the surface with ST-PHPMA will change the surface charge since carboxylic groups are being converted into amide

Table III. Characteristics of Surface-Modified P(MMA-MA-MAA) Nanospheres

Modifier	Unmodified	AP	ST-1	ST-2	ST-3
Cold nanospheres					
Immobilization (μmol/g)					
Before wash	–	30.3	26.9	28.9	29.2
After wash[a]	–	30.2	26.7	28.9	29.2
Conversion of COOH[b] (%)	–	72.3	64.1	69.0	69.8
Occupied area[c] (A^2)	110#	152##	172	159	158
Diameter[d] (nm)	180.0	180.2	187.6	183.4	180.5
Hot nanospheres					
Immobilization (μmol/g)					
Before wash	–	30.4	26.6	27.8	29.7
After wash[a]	–	30.2	26.4	27.5	29.5
Occupied area[c] (A^2)	N.D.[d]	133##	152	146	136
Diameter[e] (nm)	207.0	206.8	213.8	210.2	207.7

[a] Rinsing three times with distilled water.
[b] Percentage of COOH conversion due to binding of ST-PHPMA.
[c] Occupied area per one COOH#, AP## or PHPMA moiety; calculated by dividing the surface area (based on the diameter of nanospheres) by the content of moiety. Surface area of cold and hot nanospheres are 27.8 and 24.2 m^2/g, respectively.
[d] Not determined.
[e] Determined by laser light scattering at 25°C.

groups. An indication of the importance of this factor is shown in an unusually high reduction of protein adsorption when 1-amino-2-propanol (AP) was used as the surface-modification agent. More experiments are needed, however, to explain these observations.

Fate of Nanospheres in Vivo

The modified and unmodified P(MMA-MA-MAA) nanospheres were administered intravenously to rats. The surface modification of nanospheres with ST-PHPMA increased their intravascular half-life in a molecular weight dependent manner (Fig. 4). The higher the molecular weight of the PHPMA, the higher the intravascular half-life. For example, modification of the nanosphere's surface with PHPMA having Mn = 12,500 (ST-1) increased the intravascular half-life from 40 min (unmodified nanospheres) to 12 h. Concomitantly, the accumulation of the nanospheres in the liver 24 h after administration decreased from >80% (unmodified nanospheres) to <50% for ST-1 modified nanospheres (Fig. 5). The observed molecular weight dependence of the biorecognition seems to indicate the influence of the hydrodynamic thickness of the coating layer on the process of opsonization and capture by Kupffer cells of the liver and macrophages of the spleen. As mentioned above, the thickness of the coating layer (Fig. 1) was lower when compared

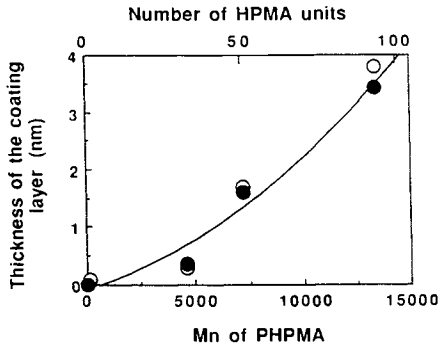

Fig. 1. Relationship between Mn of ST-PHPMA (as determined by the TNBS method) and hydrodynamic thickness of the coating layer at the surface of the hot (●) and cold (○) P(MMA-MA-MAA) nanospheres after surface modification with ST-PHPMA.

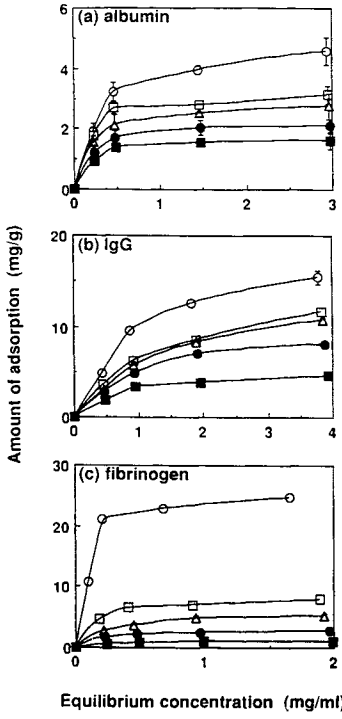

Fig. 2. Adsorption isotherms of (a) albumin, (b) IgG and (c) fibrinogen onto the surface-modified cold P(MMA-MA-MAA) nanospheres in 1/15 M saline at 25°C for 3 h. Surface modifier: (○) unmodified; (□) AP; (■) ST-PHPMA; (●) ST-2 PHPMA; (△) ST-3 PHPMA. Each point represents the mean ± SD (n = 3).

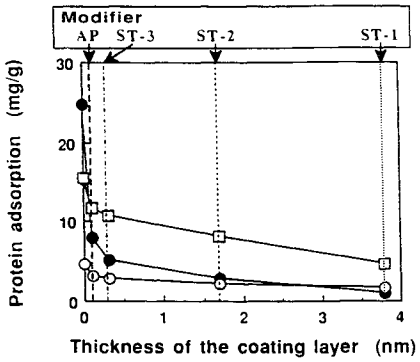

Fig. 3. Relationship between the hydrodynamic thickness of the coating layer and bovine protein adsorption onto cold P(MMA-MA-MAA) nanospheres. Adsorption conditions: initial concentrations of albumin (○), IgG (□) and fibrinogen (●) were 3, 4 and 2 mg/ml in 1/15 M saline, respectively; temperature, 25°C; time, 3 h. Each point represents the mean ± SD (n = 3).

to the effective thickness of PEO layers (22,26). In addition to the random coil conformation of PHPMA chains, their surface density (occupied area per molecule is around 150 Å^2; Table III) may play an important role. It was shown recently (27) that the biorecognition of PEO-modified polystyrene nanospheres decreased with increased surface density of PEO chains.

The data (Fig. 5) have shown that 24 h after i.v. administration, the unmodified and ST-PHPMA modified microspheres were present mainly in the blood stream or in the liver (>85% of the administered dose). On the other hand, nanospheres modified with AP have reached other compartments. Their accumulation in the blood and liver was less than 75% of the administered dose. It is well known that the biodistribution of nanospheres depends greatly on their surface structure. Spleen, lung, intestine, and bone marrow are the main compartments where accumulation of nanospheres was observed (2,7,8,27).

Published data on the fate of poly(methyl [2-^{14}C]-methacrylate) nanospheres indicate their fast removal from the blood stream and accumulation predominantly in the liver (7,8). Their intravascular half-lives were considerably shorter even when compared to unmodified nanospheres synthesized in this study. The main reason might be the difference in the hydrophilicity of both microspheres. Whereas

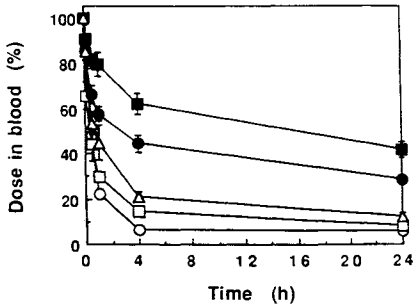

Fig. 4. Blood elimination in rats after intravenous administration of unmodified (○) and modified (□, ■, ●, △) [^{14}C]-P(MMA-MA-MAA) nanospheres. Modifier: AP (□), ST-1 PHPMA (■), ST-2 PHPMA (●), and ST-3 PHPMA (△). Each point represents the mean ± SD (n = 5).

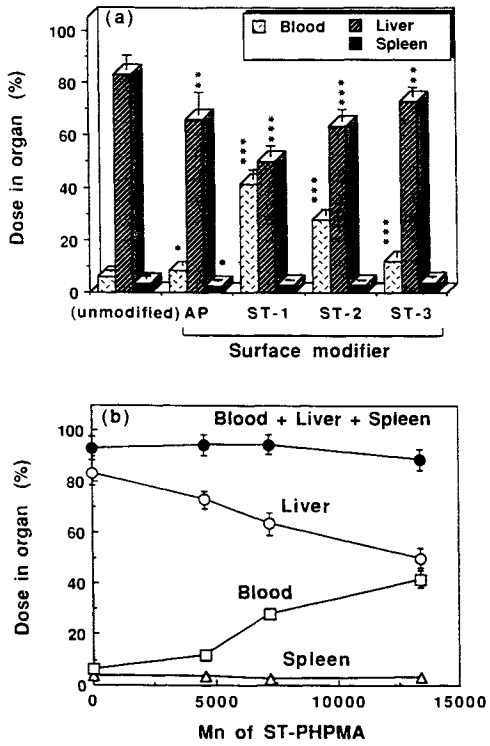

Fig. 5. Body distribution profiles for unmodified and modified [^{14}C]-P(MMA-MA-MAA) nanospheres in rats 24 h after intravenous injection (a); and the correlation with Mn of ST-PHPMA as surface modifier (b). Each point represents the mean ± SD (n = 5). Significantly different from the unmodified nanospheres by Student's *t*-test (*P < 0.05, **P < 0.01, ***P < 0.001).

the poly(methyl [2-^{14}C]methacrylate) nanospheres (7) were synthesized by γ-ray initiated polymerization of methyl [2-^{14}C]methacrylate, the nanospheres of this study were synthesized by copolymerization of methyl methacrylate, maleic anhydride, and methacrylic acid resulting in the presence of a relatively large amount (41.8 μeq/g) of ionized carboxylic groups at their surface under physiological conditions. The substantial and statistically significant changes of body distribution of these nanospheres after surface-modification with ST-PHPMA (Fig. 5b) demonstrate the potential of PHPMA to modify the biorecognition of relatively hydrophilic nanospheres whose structure prevents the use of adsorbable surfactants. The biocompatibility of HPMA homopolymer and copolymers (28) bodes well for future applications of PHPMA modified particles. For example, it was shown that unmodified poly(D,L-lactide-co-glycolide) nanospheres possess a short intravascular half-life after i.v. administration (29,30). Their modification (29) as well as the incorporation of targeting moieties are possibilities for future research.

CONCLUSIONS

1. Semitelechelic poly[N-(2-hydroxypropyl)methacrylamide] (ST-PHPMA) terminated in one amino end-group per macromolecule were synthesized by radical solution polymerization using 2-aminoethanethiol as chain transfer agent.
2. Nanospheres based on a copolymer of methyl methacry-

late, maleic anhydride, and methacrylic acid were synthesized by emulsifier-free emulsion polymerization. Their surface was modified by covalent attachment of ST-PHPMA of different molecular weights.

3. Modified nanospheres possessed a decreased biorecognition both in vitro and in vivo. When compared to unmodified nanospheres, the modified ones demonstrated a lower protein adsorption in vitro, and a longer intravascular half-life as well as a decreased accumulation in the liver after intravenous administration to rats.
4. The results clearly demonstrated the potential of poly[N-(2-hydroxypropyl)methacrylamide] as a surface modifier to achieve protein repulsion surfaces. It may be suitable in decreasing the biorecognition of moderately hydrophilic surfaces whose body distribution can not be changed by the adsorption of surfactants.

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