This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Abstract

Well-defined water-soluble block copolymers poly(ethylene glycol)-b-poly(N-(2-hydroxypropyl)methacrylamide-co-N-methacryloylglycylglycine) (PEG-b-P(HPMA-co-MAGG)) with different composition and narrow polydispersity were synthesized by reversible addition-fragmentation transfer (RAFT) polymerization. The in vivo blood clearance and biodistribution of copolymers with different compositions were studied in normal BALB/c mice. The results showed that the electronegative copolymers were rapidly eliminated from blood and tended to accumulate in liver and spleen. However, the copolymers with neutral or small amount of negative charges showed prolonged circulation time and no specific organ uptake. Combined with the quantitative analysis of in vitro hepatocytes uptake, we conclude that this was due to the balance between (i) the electrostatic repulsion between copolymer and the cell membrane and (ii) the tendency of macrophage-like cells to capture the negative charged copolymers. This work also revealed the significant roles of PEG chain length, negative charge and molecular weight for the copolymers as anticancer drug carriers with prolonged circulation and optimal biodistribution.
PEG-\textit{b}-PHPMA block copolymers with precisely controlled composition were synthesized and showed good biodistribution pattern and long circulation time.
The synthesis of poly(ethylene glycol)-b-poly(N-(2-hydroxypropyl) methacrylamide) block copolymers with well-defined structure and its influence on in vivo circulation and biodistribution

Qingsong Yu,ab Chengyan Dong,‡c Jiajing Zhang,ab Jiyun Shi,abc Bing Jiac, Fan Wang,*c and Zhihua Gan,*ab

Well-defined water-soluble block copolymers poly(ethylene glycol)-b-poly(N-(2-hydroxypropyl) methacrylamide-co-N-methacryloylglycylglycine) (PEG-b-PHPMA-co-MAGG) with different composition and narrow polydispersity were synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization. The in vivo blood clearance and biodistribution of copolymers with different compositions were studied in normal BALB/c mice. The results showed that the electronegative copolymers were rapidly eliminated from blood and tended to accumulate in liver and spleen. However, the copolymers with neutral or small amount of negative charges showed prolonged circulation time and no non-specific organ uptake. Combined with the quantitative analysis of in vitro hepatocytes uptake, we conclude that this was due to the balance between (i) the electrostatic repulsion between copolymer and the cell membrane and (ii) the tendency of macrophage-like cells to capture the negative charged copolymers. This work also revealed the significant roles of PEG chain length, negative charge and molecular weight for the copolymers as anticancer drug carriers with prolonged circulation time and optimal biodistribution.

1. Introduction

Historically, the administration of anticancer therapeutic agents has been limited by multiple factors, primary among these being poor solubility, low stability and rapid clearance. The consequence is short circulation half-life and low efficacy, making frequent administration necessary. Additionally, there can be significant side effects in non-diseased tissues due to the non-selective adsorption of therapeutic agent.1,2 In order to achieve the site-specific drug delivery, macromolecular drug carriers have been investigated such as synthetic polymers,3-5 albumin,6 micelles,7 nanoparticles8,9 and liposomes.10,11 These drug carriers can selectively accumulate in tumor regions due to the enhanced permeation and retention (EPR) effect12 and long circulating characteristics.13

Polymer-drug conjugates have been widely studied for three decades as a potential solution to overcome the limitations of current chemotherapy.3,14 The typical polymers include poly(ethylene glycol) (PEG),15 poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA),16 dextran,17,18 and polyglutamate19 etc. PEG has been widely studied due to its non-toxic, non-immunogenic characteristics, and especially the approval by the Food and Drug Administration (FDA) for clinical application. The use of PEG for nanoparticle surface functionalization has led to very favorable results such as the resistance to non-specific adsorption of proteins and the prolonged circulation time.20-22 Although the PEG-drug conjugates have been considered as the potential long-circulating drug delivery systems for systemic drug administration, the application is restrained by the limited number of reactive sites, low drug loading capacity15 as well as the accelerated blood clearance (ABC) by the immune system.23 PHPMA has also been explored as another water-soluble, inert, non-immunogenic and uncharged polymer which can be well functionalized by the copolymerization with its derivatives. It is anticipated that the integration of PEG and PHPMA may bring about a recombinant of their advantages such as better “stealth” properties, longer circulation time, lower normal organ uptake and better functionalization. Therefore, the copolymer with PEG and PHPMA components is of great potentials not only for the demonstration of the relationship between structure and biofunctions, but also for the clinical application due to the FDA approval of both components.
However, traditionally PHPMA is synthesized by free radical polymerization which leads to uncontrollable molecular weight and broad distribution, while PEG is usually synthesized by anionic ring-opening polymerization. It is unable to synthesize the block copolymer of PEG and PHPMA via traditional method. Fortunately, with the development of modern living radical polymerization techniques such as reversible addition-fragmentation chain transfer (RAFT) polymerization, the synthesis of PHPMA with well-controlled molecular weight and narrow distribution (PDI<1.1) is possible. This technique also make the preparation of PEG-b-PHPMA block copolymer available.

The copolymerization of HPMA with other functional monomers can provide flexible chemical structures that allow for the conjugation of various moieties such as targeting molecules, anti-cancer drugs, fluorophores as well as ligand for radionuclide. N-methacryloylglycylglycine (MAGG) and N-methacryloylglycylphenylalanlyl-leucyl-glycine (MAGFLG) are commonly used comonomers for the further modification of PHPMA. MAGG can be used to conjugate anti-cancer drugs to evaluate the metabolic pathway of polymer-drug conjugates before the drug was released. However, there is still several problems exist in the study of copolymerization of HPMA and MAGG. i.e. Whether MAGG hinder the living polymerization process or can the composition be precisely controlled? Does the introduction of MAGG content have influence on the solution properties of the prepared copolymers? And whether the resultant chemical change impacts their biological functions?

It has been reported that the molecular weight (Mn), and thus hydrodynamic volume (Vh) play critical roles in pharmacokinetics (PKs) and in vivo biodistribution for water-soluble polymer drug conjugates. Once the hydrodynamic diameter (Dh) of a polymer exceeds the renal threshold of 5nm, the filtration rate of macromolecules decreases with increasing Dh, as long as other factors, such as molecular conformation and chemistry, remain constant. Thus the blood circulation time increases with increasing Mn as a result of decreased filtration by the kidneys.

In addition to the Mw, the surface charge of drug nanocarriers has been reported to be a very important factor in determining the efficiency and mechanism of cellular uptake, as well as the in vivo fate. However, the optimum surface charges and charge densities were reported differently for different nanocarriers. For PHPMA copolymers, the in vivo biodistribution and renal elimination rate are dependent on both molecular weight and charge. The neutral copolymers were found to accumulate in lung, liver and spleen, while no specific organ uptake except kidney can be found for negative copolymers. The copolymers with higher molecular weight show higher uptake for all non-renal tissue (heart, lung, spleen, liver, and muscle).

The aim of this work is to investigate the relationship between chemical composition and in vivo biofunctions for polymers as drug delivery carriers. For this purpose, the synthesis of PEG-b-P(HPMA-co-MAGG) block copolymers with well-controlled molecular weight and composition by RAFT polymerization was studied. The solution properties of the copolymers were characterized by dynamic light scattering (DLS) and capillary electrophoresis (CE). The cellular experiments and animal tests were performed to demonstrate the effects of molecular weight and negative charges of copolymers on in vitro hepatocytes uptake, PKs as well as in vivo biodistribution in BALB/c normal mice. The results of this work will provide an theoretical evidence for the design and synthesis of efficient polymer conjugates with long circulation time and optimal biodistribution for anticancer drug delivery.

2. Experimental section

2.1 Materials

N-(2-hydroxypropyl)methacrylamide (HPMA, reagent grade, Polysciences Inc), 4,4′-Azobis(cyanopentanoic acid) (V501, >98%, Fluka), tyrosine (Tyr, >98%, Alfa Aesar) and 4-(N,N-dimethylamino)pyridine (DMAP, 99%, Alfa Aesar), Iodogen (Sigma, St. Louis, MO), Na125I (Beijing Atom High Tech, Beijing, China) and PD MiniTrap column (GE Healthcare, Buckinghamshire, UK) were used as received without further purification. Water (HPLC grade) was obtained with a Milli-Q system (Millipore, Bedford, MA). Sodium cholate was purchased from Sigma. Dichloromethane and acetonitrile (HPLC grade) were obtained from Acros Organics. The other chemicals (reagent grade) in this work were purchased from Aldrich and used without purification. PEG macromolecular chain transfer agent (PEG-CTA), N-methacryloylamino propyl fluorescein thiourea (MA-FITC) and N-methacryloylglycylglycine (MAGG) were synthesized according to the literature procedure. The NCTC-1469 murine hepatocytes were obtained from the American Type Culture Collection (ATCC; Rockville, MD).

2.2 Synthesis of methacryloyl tyrosyl methyl ester (MA-Tyr)

Tyrosyl methyl ester hydrochloride (2.31 g, 10.0 mmol) and triethylamine (2.22 g, 22.0 mmol) were added into dichloromethane (30mL). The solution was kept in an ice bath for 30 min. A solution of distilled methacryloyl chloride (1.04 g, 10.0 mmol) in dichloromethane (DCM) (10 mL) was added slowly to the above mixture. After the addition of methacryloyl chloride, the resultant mixture was stirred at room temperature overnight. The DCM solution was successively washed with hydrochloric acid (100 mL, 1 M), saturated sodium bicarbonate solution (100 mL) and water, dried over magnesium sulfate and then evaporated to dryness. Yield: 2.24 g (85%) pale yellow oil. 1H NMR (400MHz, Acetone-d6): δ [ppm] 7.08 (d, 2H), 6.95 (d, 2H), 5.67 (s, 1H), 5.35 (s, 1H), 4.88 (dd, 1H), 3.75 (s, 3H), 3.10 (m, 2H), 1.93 (s, 3H).

2.3 Synthesis of PEG-b-P(HPMA-co-MAGG) and PEG-b-P(MAGG) block copolymers

A given amount of PEG-CTA, HPMA, MAGG, MA-Tyr, MA-FITC and V501 (CTA/initiator=4/1) in DMSO was added into a
schlenk tube, degassed via three freeze–thaw cycles. The polymerization was conducted at 70 °C for 48 h. Then the polymerization was halted by immediate exposure to air and cooling with liquid nitrogen. The reaction solution was precipitated into acetone for three times to remove unreacted monomer and PEG. The final product was obtained as pink solid after drying in vacuum. Yield: 70–90 %. The chemical structure of the copolymers was analyzed by 1H NMR. The number-average molecular weight (Mn) and polydispersity indices (Mw/Mn) were measured by a SHIMADZU LC-10Avp aqueous size exclusion chromatography (SEC) equipped with SD-806HQ column in 0.1 mol/L NaNO₃ at a flow rate of 1.0 mL/min and at 35 °C. The fluorescence spectra of all copolymers were acquired on a Hitachi F4500 apparatus.

2.4 Capillary electrophoresis
All electrophoretic experiments were performed on a P/ACE model 2050 (Beckman, Fullerton, CA, USA) mounted with a bare fused-silica capillary of 47 cm (40 cm to detector)×50 μm id, from Yongnian Optical Fiber Factory (Hebei, China). Prior to every injection, the capillary was successively flushed with 0.1 M NaOH, water, and running buffer (10 mM phosphate buffer, pH=7.4) for 3 min each. Samples were introduced by pressure injection at 0.5 psi (1 psi = 6894.76 Pa) for 2 s and separated at +15 kV. The separated bands were detected by UV absorption at 254 nm through a slit aperture of 100×200 μm. The data were acquired at 5 Hz and processed with the P/ACE workstation. DMSO was used as the Electroosmotic flow (EOF) marker. Unless otherwise noted, the running buffer was renewed every run.

2.5 125I labeling of PEG-b-P(MAGG) and PEG-b-P(HPMA-co-MAGG)
The diblock copolymers were labeled at room temperature and ambient pressure with 125I using the Iodogen method as previously reported. Briefly, 100 μg of P2M40 and 37 MBq Na125I in 0.2 M pH 7.4 phosphate buffer were added to a vial coated with 20 μg Iodogen. The molar ratio of Na125I to tyrosine residues is 10–120 in the feeding reaction mixture. Generally, all the tyrosine residues can be labeled under this labeling condition. The resultant labeled copolymer was purified by a PD MiniTrap G-25 column (28-9180-07-07, GE Healthcare) equilibrated with phosphate buffer (0.2 M, pH7.4) to remove unreacted radiiodine. The radioactive fractions of 125I-P2M40 were collected and passed through a 0.2 μm syringe filter for further in vivo experiments. A similar procedure was used for the rest of the copolymers. The quality control was carried out with Instant Thin-Layer Chromatography (ITLC) method using Gelman Sciences silica-gel paper strips and acetone as eluent. By this method, free 125I migrated to the solvent front while 125I labeled polymer remained at the origin.

2.6 Solution stability
The solution stability of all 125I radiotracers was determined in saline at a final concentration of 2 mCi/mL. The samples were analyzed by ITLC at 12, 24 and 48 h.

2.7 Cell lines and cell culture
NCTC 1469 (ATCC CCL-9.1) murine normal hepatocytes were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with penicillin, streptomycin and 10 % heat inactivated horse serum. The concentration of L-Glutamine was adjusted to 4mM. Cells were maintained at 37 °C in a humidified atmosphere of 5 % carbon dioxide and 95 % air. All tissue culture media were obtained from Gibco Life Technologies, Inc. (Grand Island, NY). Cells were used 2 days after being plated unless otherwise stated.

2.8 Animal studies
All the 125I-labeled copolymers were purified using a PD MiniTrap G-25 column before animal experiments. The PD MiniTrap G-25 column was washed with 6 mL of PBS and was activated with 2 mL of 1 % BSA before purification. After that PD MiniTrap G-25 column was loaded with radiotracer (~100μL) and then washed with 4 mL of PBS, the 0.5 mL between 0.6 and 1.1 mL of eluent was collected. We prepared doses for animal studies by dissolving the purified radiotracer in 0.9 % saline to give a concentration of 100 μCi/mL for biodistribution studies and 2.5 mCi/mL for imaging. Each animal was injected with 0.1 mL of radiotracer solution (10 μCi/mouse). All animal experiments were performed in accordance with guidelines of Peking University Health Science Center Animal Care and Use Committee.

2.9 Blood clearance and biodistribution experiments
Seven BALB/c normal mice were used as one group for the blood clearance experiment of one copolymer radiotracer. The 125I-labeled copolymer (10 μCi in 0.1 mL 0.9% saline) was administered intravenously to each mouse. Blood was harvested from orbital sinus at 1, 3, 5, 7, 10, 15, 20, 30, 60, 90, 120, 180, 240, 480, 1440 and 2880 min postinjection (p.i.), and the radioactivity was measured using a γ-counter (Wallac 1470-002, Perkin-Elmer, Finland). The uptakes of radiotracer in blood were calculated as the percentage of the injected dose per gram of blood mass (%ID/g).

For biodistribution studies, 16 BALB/c normal mice were randomly divided into four groups. The 125I-labeled copolymer (10 μCi in 0.1 mL 0.9% saline) was administered intravenously to each mouse. Time-dependent biodistribution studies were carried out by sacrificing mice at 1, 4, 24, and 48 h postinjection. Blood, heart, liver, spleen, kidney, stomach, intestine, muscle, and bone were harvested and measured for radioactivity in a gamma counter (Wallac 1470-002, Perkin-Elmer, Finland). The organ uptake was calculated as a percentage of injected dose per gram of wet tissue mass (%ID/g).

This journal is © The Royal Society of Chemistry 2014 Polym. Chem., 2014, 00, 1-3 | 3
2.10 Scintigraphic imaging
The imaging study was performed using three BALB/c normal mice. Each animal was administered with 500 μCi of 125I labeled copolymers in 0.2 mL saline. Animals were anesthetized and placed supine on a three-head-camera (GE Healthcare, Millennium VG SPECT) equipped with a parallel-hole, low energy, and high-resolution collimator. Anterior images were acquired at 24 h postinjection and stored digitally in a 128×128 matrix. The acquisition count limits were set at 200 K. After completion of the imaging study, animals were sacrificed by cervical dislocation.

2.11 Isolation of murine liver cells
The karat parenchymal and non-parenchymal cells were isolated from adult mouse livers (8–14 weeks of age) after the administration of fluorescent copolymers (20 mg/kg) according to the method described in the literature. Parenchymal cells were separated from non-parenchymal cells by centrifugation at a relative centrifugal force of 50 g for three times. The isolated parenchymal cells were incubated with ASGR1 Antibody (8D7) PE (Santa Cruz Biotechnology, Inc.), MCAM Antibody (P1H12) PE(Santa Cruz Biotechnology, Inc.) and anti-mouse F4/80 Antigen PE BM8 (eBioscience, Inc.) were used to stain the Liver Sinusoidal Endothelial Cells (LSEC) and Kupffer Cells (KC) in non-parenchymal portion respectively. Both the parenchymal cells and non-parenchymal cells isolated at 24 h after the injection were stained by the Goat anti Rabbit IgG1 PE (GAR IgG1 PE) and used as the isotype control. All these cells were analyzed by Accuri C6 flow cytometer (Becton & Dickinson). Cells stained with FITC or PE were used to manually compensate the PE and FITC channels. Compensation levels were set at 1.6 % PE from FITC subtraction, 20 % FITC from PE subtraction. Data analysis was performed using FCS Express 4 Flow Research edition (De Novo Software).

2.12 Cellular uptake of polymers with different amount of negative charge
For the analysis of cellular uptake by flow cytometry, NCTC-1469 cells were plated in 24-well plates (7.5 ×10^4 per well) two days prior to the experiment. Cells were treated with 200 μL of polymer solutions in FBS-free media or assay buffer. Cells were incubated for 12 h at 37 °C/5% CO2, washed subsequently thrice with ice-cold PBS, trypsinized and centrifuged. The cell pellet was resuspended in 400 μL PBS with 1 % bovine serum albumin, split in two aliquots and analyzed using flow cytometry. Each data point was performed in triplicate. The mean fluorescence intensity was analyzed using Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter). Data were acquired in linear mode and visualized in logarithmic mode. Approximately 10,000 digital list mode events were collected and the data gated on forward and side scatter parameters to exclude debris and dead cells. Cells without labeled polymers were used as the negative control for autofluorescence. Data analysis was performed using WinMDI 2.9 software.

2.13 Confocal laser scanning microscopy
Polymer uptake studies of NCTC-1469 cells were performed using a FluoView Confocal Laser Scanning Microscopes-FV1000 (Olympus, Tokyo, Japan) equipped with a 100×Olympus UPlanApo oil immersion objective (NA, 1.35). One day prior to each study, 5–10 ×10^4 cells were seeded on 35 mm glass-bottom dishes (Nest Biotechnology Co., Ltd., Shanghai, China). The fresh media (2 mL) containing micromolar concentrations of polymer conjugates (20 μM FITC equivalent) was added and then incubated for 12 h. Before observation, the incubation media was replaced with fresh media. The untreated cells were observed to reduce background fluorescence.

2.14 Statistical analysis
The biodistribution data and blood clearance curve are reported as an average plus the standard variation. Comparison between two different radiotracers was also made using the one-way ANOVA test (GraphPad Prism 5.0, San Diego, CA). The level of significance was set at p=0.05.

3. Results and discussion
3.1 Synthesis of copolymers with well-defined structure

Scheme 1. Synthetic routes of MA-Tyr and PEG-b-P(HPMA-co-MAGG) copolymers.
Scheme 1 shows the synthetic routes of tyrosine residue contained monomer (MA-Tyr) and the copolymers composed of PEG, HPMA, MAGG, MA-Tyr and MA-FITC. The MA-Tyr and MA-FITC were synthesized for radiolabeling and fluorescent labeling, respectively. Although the copolymers with different compositions were designed, the amount of tyrosine and FITC residue was kept constant in copolymers.

Figure 1 shows the SEC results of PEG-CTA and copolymers. For the sake of clarity, the theoretical molecular weights were used in the nomenclature of the copolymers, e.g. P2M40 stands for PEG\textsubscript{2k}-b-PMAGG\textsubscript{40k}, P5H30M5 stands for PEG\textsubscript{5k}-b-P(HPMA\textsubscript{30k}-co-MAGG\textsubscript{5k}). Compared to the elution time of PEG\textsubscript{5k}-CTA, the shorter elution time of copolymers P5M35, P5H30M5 and P5H80 indicated the successful synthesis of copolymers from macro-CTA via RAFT polymerization. The unimodal peaks and very narrow polydispersity indicated that the RAFT copolymerization of HPMA and MAGG in the presence of PEG-CTA is a well-controlled process.

Figure 2 shows the \textsuperscript{1}H NMR spectra of monomer and copolymers. The appearance of peaks at 3.9 ppm, 3.6 ppm and 3.0 ppm from MAGG, PEG and HPMA respectively clearly proved the successful synthesis of copolymers. The successful introduction of tyrosine can be confirmed by the appearance of the characteristic peaks at 6.5-7.5 ppm. The contents of HPMA, MAGG and tyrosine were calculated from the integral ratio of the characteristic peaks at 3.9 ppm (MAGG), 3.6 ppm (PEG), 3.0 ppm (HPMA) and 6.8 ppm (tyrosine).

Figure 3 shows the aggregation behavior of copolymers with different compositions in water and PBS. In aqueous solution, length and the content of carboxyl groups in our copolymers were precisely controlled during polymerization process by changing the molar ratio of HPMA to MAGG monomers in feed or by changing the PEG block length.

### 3.2 Hydrodynamic diameter of copolymers

Based on the nature of living polymerization, a series of copolymers P2M40, P2H30M5, P5H20M15, P5H30M15, P15H25, H40, P5H50 and P5H80 with well-defined composition were synthesized to demonstrate the influence of composition on the solution behavior and biological functions. To evaluate the solution properties of these copolymers, these copolymers were tested by DLS and CE respectively.

Figure 3 shows the aggregation behavior of copolymers with different compositions in water and PBS. In aqueous solution,
Table 1. Characterization of PEG-b-P(HPMA-co-MAGG) copolymers.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>P</th>
<th>H</th>
<th>M</th>
<th>Tyr</th>
<th>FITC</th>
<th>Mw</th>
<th>PDI</th>
<th>Dₜ(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2M40</td>
<td>2,000</td>
<td>0</td>
<td>45,000</td>
<td>6.0</td>
<td>2.0</td>
<td>47,000</td>
<td>1.10</td>
<td>8.38±0.63</td>
</tr>
<tr>
<td>P2H20M5</td>
<td>2,000</td>
<td>29,500</td>
<td>4,100</td>
<td>6.2</td>
<td>2.1</td>
<td>35,600</td>
<td>1.11</td>
<td>10.36±1.15</td>
</tr>
<tr>
<td>P5H20M15</td>
<td>5,000</td>
<td>17,900</td>
<td>21,600</td>
<td>6.1</td>
<td>1.9</td>
<td>44,500</td>
<td>1.13</td>
<td>11.69±0.77</td>
</tr>
<tr>
<td>P5H30M5</td>
<td>5,000</td>
<td>28,700</td>
<td>3,900</td>
<td>5.9</td>
<td>2.2</td>
<td>37,600</td>
<td>1.06</td>
<td>12.51±1.08</td>
</tr>
<tr>
<td>P5H50</td>
<td>5,000</td>
<td>54,000</td>
<td>0</td>
<td>5.9</td>
<td>2.0</td>
<td>59,000</td>
<td>1.08</td>
<td>8.64±0.37</td>
</tr>
<tr>
<td>P5H80</td>
<td>5,000</td>
<td>86,000</td>
<td>0</td>
<td>5.8</td>
<td>2.0</td>
<td>91,000</td>
<td>1.13</td>
<td>10.68±0.95</td>
</tr>
<tr>
<td>P15H25</td>
<td>15,000</td>
<td>23,000</td>
<td>0</td>
<td>6.3</td>
<td>1.9</td>
<td>38,000</td>
<td>1.07</td>
<td>11.54±1.31</td>
</tr>
<tr>
<td>P5M35</td>
<td>5,000</td>
<td>0</td>
<td>34,000</td>
<td>5.9</td>
<td>2.1</td>
<td>39,000</td>
<td>1.08</td>
<td>11.25±0.78</td>
</tr>
<tr>
<td>H40</td>
<td>0</td>
<td>38,000</td>
<td>0</td>
<td>6.0</td>
<td>2.1</td>
<td>38,000</td>
<td>1.08</td>
<td>10.23±0.94</td>
</tr>
<tr>
<td>P5H50 W/O Tyr</td>
<td>5,000</td>
<td>50,000</td>
<td>0</td>
<td>6.0</td>
<td>2.0</td>
<td>55,000</td>
<td>1.08</td>
<td>8.58±0.67</td>
</tr>
</tbody>
</table>

P represents the molecular weight of PEG; H and M represent the sum of the molecular weight of HPMA and MAGG, respectively. Tyr represents the molar ratio of tyrosine residue in P(HPMA-co-MAGG) block. FITC represents the molar ratio of FITC residue in P(HPMA-co-MAGG) block. Mw is the molecular weight of the copolymers. All of the above data were calculated from ¹H NMR in deuterium oxide. The polydispersity index was determined by SEC in 0.1M NaNO₃ (aq). The hydrodynamic diameter of the block copolymers was determined by DLS measurement in 10 mM phosphate buffer (pH=7.4). Represents P5H50 copolymer without tyrosine residue.

The copolymers containing more MAGG showed larger $D_h$. For example, $D_h$ of P2M40 was 150 nm, $D_h$ of P5H20M15 was 30 nm, while $D_h$ of P5H80 was only 10 nm. These results suggested that the copolymers with more MAGG components are aggregated in water. However, in PBS (pH 7.4, 10 mM), all copolymers showed $D_h$ around 10 nm, suggesting that no aggregation occurred for all copolymers in PBS. In this case, the copolymers with higher molecular weight of PEG or PHPMA showed larger $D_h$ values. Furthermore, from the $D_h$ values of P5H50 (9.29 nm) and P5H50 W/O Tyr (9.16 nm), we can conclude that the introduction of a small amount of tyrosine and FITC shows no obvious influence on the chain conformation of copolymers in PBS.

The above phenomenon could be attributed to the different hydrophilicity of carboxyl group of MAGG in aqueous solution and PBS. It has been reported that the carboxyl groups only in buffer solution were ionized and shielded by the counterions which prevent the formation of aggregates. The increased hydrophilicity and chain length of P5H80 and P15H25 copolymers also led to their larger size in PBS. Therefore, the size difference of copolymers in water and buffer solution remind us not to ignore the possible aggregation when the copolymers are injected into body, which may lead to the different PKs and biodistribution.

3.3 Charges of copolymers with different compositions

Due to the negative charge of carboxylic group of MAGG, the change of MAGG content should bring about the alteration of the net charge of PEG-b-P(HPMA-co-MAGG) copolymers. According to the report that the micelles with different surface charges could be characterized by using agarose gel electrophoresis, the net charge of copolymer was determined qualitatively by capillary electrophoresis (CE) in this work. The zeta potentials of these copolymers were also determined by zetasizer(Figure S1). These results showed similar tendency as the CE results in Figure 4.
Figure 4 represents the electrophoretic mobility of PEG-b-P(HPMA-co-MAGG) copolymers with different amount of MAGG. The peak time of DMSO, PEG5k, P5H35, P5H30M5, P5H20M15, P5H15M20, P5H5M30, P5M35, P2M40 and P2MAA40 was 2.0, 2.0, 2.2, 2.7, 2.8, 3.0, 3.2, 4.1, 4.3 and 4.5 min, respectively. DMSO was used as the electroosmotic flow (EOF) marker (neutral). The PEG-b-poly(methacrylic acid)40k (P2MAA40) with the highest electronegativity was used as the negative control. Due to the forward voltage applied in the test, those copolymers with elution time longer than DMSO carried negative charge. The longer the elution time, the more negative the net charge.

According to the CE results in Figure 4, it can be found from these diagrams that PEG and PHPMA carried neutral charge at pH 7.4. With increasing more MAGG contents, the net charge of copolymers became more negative accordingly. Therefore the introduction of PMAGG brings negative charge to the copolymers. We can also conclude that the net charge of these copolymers can be precisely controlled by altering the molar ratio of comonomers in feed.

Based on the above results, the PEG-b-P(HPMA-co-MAGG) copolymers with well-controlled chemical composition and negative charges were successfully synthesized by RAFT copolymerization. For the further investigation on the influence of chemical composition and negative charge on the biofunctions of copolymer as anticancer drug conjugates, the PEG-b-P(HPMA-co-MAGG) block copolymers were labeled with radionuclide $^{125}$I and characterized in vivo for blood clearance and biodistribution in normal mice. The radiolabeling efficiency for all copolymers was greater than 90 %, and the radiochemical purity (RCP) (for example $^{125}$I labeled P5H30M5) after PD MiniTrap column purification was higher than 99 % (Figure S2). For all radiolabeled copolymers, it was found that their RCP values were still above 90 % after purification for 48 h, and still high enough after 72 h, around 80 %. These results revealed the stability of radionuclide labeled copolymers in saline solution, which guaranteed the further in vivo tests.

3.4 Blood clearance

Figure 5 shows the plasma levels of copolymers over 48 h after a single injection of $^{125}$I labeled copolymers in normal BALB/c mice. It was found that the plasma level of copolymers at each time point p.i. was strongly dependent on molecular weight, PEG chain length and negative charge. The plasma concentration of P2M40 with highest electronegativity as shown in Figure 4 was 25 %ID/g at 1 min p.i. and decreased bi-exponentially to essentially undetectable levels after 30 min. On the contrary, those copolymers containing less negative charge had blood clearance rate much slower than that of P2M40, e.g. the plasma level of P5H30M5 and P5H80 was still above 5 %ID/g after 48 h.

Table 2 summarizes the PK parameters of PEG-b-P(HPMA-co-MAGG) copolymers. The PK data were determined using the Kinetica v4.2 program with a two-compartment model. The adjacent R-square ($R^2$) was larger than 0.99. The elimination half-lives $t_{1/2}(\beta)$ of the tested copolymers were ranked as follows: P5H80 > P5H30M5 > P5H50 ≈ P15H25 > H40 > P5H20M15 > P2M40 > P2H30M5. Generally, copolymers with higher molecular weight showed longer elimination time; at the same molecular weight level, the copolymers with small amount of negative charge possessed longer elimination time than the non-charged ones. The distribution half-lives $t_{1/2}(\alpha)$ were ranked as the following order: P5H30M5 > P5H80 > P15H25 > P5H50 ≈ H40 > P5H20M15 > P2H30M5 > P2M40. It took longer time for the copolymer with small amount of
negative charge to distribute into all tissues, which might be due to the electrostatic repulsion between polymer and cell membrane. The area under curve (AUC) values of the tested copolymers were ranked as follows: P5H30M5 > P5H80 > P15H25 > P5H50 > P5H20M15 > H40 > P2H30M5 > P2M40. P5H30M5 which carried small amount of negative charge and longer PEG block length possessed the largest AUC value which indicated the longest exposure time.

3.5 In vivo biodistribution

Figure 6(A) illustrated the retention of copolymers in different organs/tissues such as blood, heart, lung, liver, spleen, kidney, stomach, intestine, bone and muscle at 1, 4, 24 and 48 h after intravenous administration of radio-labeled copolymers. PEG and PHPMA themselves are loosely coiled macromolecules, and can only be slowly captured by fluid phase pinocytosis. Therefore, those copolymers contain PEG and PHPMA (e.g. P5H50 and P5H80) showed slow organ uptake. With the introduction of small amount of MAGG, the organ uptake was further reduced because of the electrostatic repulsion. However, when the PHPMA block of PEG-b-PHPMA copolymers was completely replaced by MAGG units, the results were totally different. The P2M40 was rapidly enriched in liver and spleen and consequently eliminated rapidly from plasma. Considering its negative charge with large amount, the enrichment of P2M40 might be due to the process called opsonization. The opsonin proteins tend to bind hydrophobic or charged polymers more than hydrophilic and neutral polymers to increase their recognizability to phagocytic cells, resulting in the acceleration of copolymers’ removal by liver and spleen. The P2M40 still showed obviously higher concentration in liver, spleen and kidney after 48 h, indicating the much slower body clearance rate of P2M40 with much negative charge compared to other copolymers.

The elimination of P5H80 with higher molecular weight was slower than its counterpart P5H50 with lower molecular weight. At the same molecular weight level, P15H25 have longer retention time than H40 due to the longer PEG length as well as larger size. These results were mainly due to the molecular weights and the hydrodynamic size which determine the possibility of copolymers to pass through the renal threshold. Therefore, from the viewpoint of efficient transportation of anticancer drug, the copolymers with slight negative charge such as P5H30M5 are the best candidates as drug conjugates with long circulation time and optimal biodistribution. Furthermore, compared with those PEG or HPMA modified Dex-g-PMAGGCONHTyr copolymers, PEG-b-PHPMA copolymers in this work show longer circulation time and better biodistribution patterns.

Table 2. The PK parameters of PEG-b-P(HPMA-co-MAGG) copolymers.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>$K_{fast}^{(a)}$ (min$^{-1}$)</th>
<th>$K_{slow}$ (min$^{-1}$)</th>
<th>Half-life (min)</th>
<th>AUC b) (%ID-min/g)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2M40</td>
<td>0.0032</td>
<td>0.1501</td>
<td>4.1</td>
<td>210.5</td>
<td>962.6</td>
</tr>
<tr>
<td>P2H30M5</td>
<td>0.0087</td>
<td>0.1553</td>
<td>7.5</td>
<td>79.6</td>
<td>8069.8</td>
</tr>
<tr>
<td>P5H20M15</td>
<td>0.0045</td>
<td>0.0824</td>
<td>8.4</td>
<td>153.0</td>
<td>11571.7</td>
</tr>
<tr>
<td>P5H30M5</td>
<td>0.0007</td>
<td>0.0345</td>
<td>26.1</td>
<td>913.7</td>
<td>24563.1</td>
</tr>
<tr>
<td>P5H50</td>
<td>0.0011</td>
<td>0.0523</td>
<td>13.2</td>
<td>618.5</td>
<td>12298.6</td>
</tr>
<tr>
<td>P5H80</td>
<td>0.0006</td>
<td>0.0298</td>
<td>23.2</td>
<td>1054.5</td>
<td>20952.6</td>
</tr>
<tr>
<td>P15H25</td>
<td>0.0011</td>
<td>0.0552</td>
<td>18.6</td>
<td>606.7</td>
<td>19555.9</td>
</tr>
<tr>
<td>H40</td>
<td>0.0017</td>
<td>0.0521</td>
<td>13.2</td>
<td>401.2</td>
<td>10048.1</td>
</tr>
</tbody>
</table>

$K_{fast}$ represents the faster rate constant and $K_{slow}$ represents the slower rate constant; AUC equals the area under the %ID/g of blood curve from zero to infinite time.
Figure 6. (A) Histograms of biodistribution data for $^{125}$I labeled copolymers with different charge and molecular weight in normal BALB/c mice at 1, 4, 24 and 48 h. The organ uptake was calculated as a percentage of Injected Dose per gram of tissue (%ID/g). Each data point represents an average of data in four animals. (B) Scintigraphic images of normal BALB/c mice administered with P2M40, P5H30M5, P5H80 and P2H30M5 copolymers.

Figure 7 represents the mean values of cell associated fluorescence from three parallel experiments calibrated by the fluorescence spectra. The flow cytometric histograms and the fluorescent spectra can be found in the supplementary data (Figure S5). It was found that the net charge of copolymers significantly affected their uptake by murine hepatocytes. The negative control P2MAA40 which carried the highest amount of negative charge showed the highest uptake level. With decreasing the negative charge, the cell uptake decreased accordingly. Exceptionally, the neutral copolymer P5H35 showed uptaking level higher than P5H30M5, P5H20M15 and P5H15M20, indicating that small amount of negative charge reduced the hepatocytes uptake. These quantitative results indicated again that small amount of negative charge is beneficial for preventing hepatocytes uptake due to the electrostatic repulsion, and therefore endow copolymer conjugates with long circulation time in blood for delivering drug efficiently to the cancer site.

This can also be confirmed by the confocal microscopic images, as shown in Figure 8. The copolymers with slightly negative charges (e.g., P5H30M5 and P5H20M15) showed the least and punctate distribution of the green fluorescence, while the copolymers with more negative charges such as P2M40 and P2MAA40 showed strong fluorescence distribution.

The above results revealed that the extension of circulation time for the copolymers with slightly negative charge may involve a balance between (i) the electrostatic repulsion between copolymer and the cell membrane and (ii) the tendency of macrophage-like cells to capture the negative charged copolymers.

4. Conclusion

The PEG-b-P(HPMA-co-MAGG) with well-defined molecular weight and composition were successfully synthesized by RAFT copolymerization. Based on the precisely controlled chemical structure, the influences of chemical composition and negative charge on blood clearance and in vivo biodistribution were studied. It was found that the increased molecular weight of copolymer and PEG chain length will lead to the prolonged circulation time and lower organ uptake. Meanwhile, slightly negative charge helps to avoid the nonspecific organ uptake. The quantitative analysis from in vitro hepatocytes uptake test revealed that the copolymers with small amount of negative charge are easier to escape from...
the hepatocytes. In summary, for PEG-b-P(HPMA-co-MAGG) copolymers for 4 h.

Figure 8. The confocal microscopic images of NCTC-1469 cells treated by copolymers with different negative charge: (a) P5H35, (b) P5H30M5, (c) P5H20M15, (d) P5H5M30, (e) P2M40 and (f) P2MAA40. Cells were incubated with 50 μg/ml copolymers for 4 h.

hepatocytes uptake due to the electrostatic repulsion, while too much negative charge makes the copolymer more accessible to the hepatocytes. In summary, for PEG-b-P(HPMA-co-MAGG) copolymers as potential efficient anticancer drug carriers, the introduction of small amount of negative charge is of great importance for the copolymer conjugates with prolonged circulation and optimal biodistribution.

Acknowledgements

This work was supported by the Outstanding Youth Fund (Grant no. 51025314), the National Natural Science Foundations of China (Grant no. 51390481, 50873109, 50830103, 30930030), the Knowledge Innovation Program of the Chinese Academy of Sciences (Grant No KJCX2-YW-H19), and 973 project (2011CB707703) from the Ministry of Science and Technology of China.

Notes and references

This journal is © The Royal Society of Chemistry 2014

Polym. Chem., 2014, 00, 1-3 | 11

Polymer Chemistry Accepted Manuscript

This journal is © The Royal Society of Chemistry 2014

Polymer Chemistry

Article

Polymer Chemistry

Page 13 of 13


