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Biocompatible long-circulating star carboxybetaine polymers

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Abstract:

Polyethylene glycol (PEG) is considered as the most effective material to prolong the circulation time of nanoparticles through reducing non-specific protein adsorption in blood. However, it is recognized that PEG decomposes in most physiological solutions; and an anti-PEG antibody has been dectected in some normal blood donors as a response to injection with PEGylated polymer particles. Zwitterionic polymers are potential alternatives to PEG for biomedical applications due to their super resistance to non-specific protein adsorption. Thus, finding one polymer processing long circulation time and resisting immune response is of great importance. Here, we prepared four star carboxybetaine polymers of different molecular weights via atom transfer radical polymerization (ATRP) from a *β*-cyclodextrin (*β*-CD) initiator for investigating the biocompatibility of carboxybetaine polymer, a typical zwitterionic polymer. The circulation half-life of the largest star polymer (123 kDa) in mice was prolonged to 40 h in vivo, with no appreciable damage or inflammation observed in major organ tissues. Furthermore, the circulation time of repeat injections showed similar results to the first injection, with no obvious increase of antibody amount occurred in blood. The internalization of the star carboxybetaine polymers by macrophage cell was a relatively slow process. The cell viability in presence of star carboxybetaine polymers is up to 2 mg/mL. The hemolytic activity of the star carboxybetaine polymers at 5 mg/mL was almost undetectable. I*n vitr*o results prove a key prediction of excellent biocompatibility in vivo. All the results suggest that the carboxybetaine polymer, perhaps most of the zwitterionic ones, might be a good alternative to PEG in the development of drug

delivery system.

Key words: star carboxybetaine polymers; long-circulating; repeated injection;

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1. Introduction

Non-ionic polyethylene glycol (PEG)[1] and oligo(ethylene glycol) (OEG)[2], which exhibit both hydrophilic and amphiphilic characteristics, are the most commonly used stealth materials. However, it has been recognized that PEG and OEG decompose in the presence of oxygen and transition metal ions found in most biochemically relevant solutions for long-term application.[3] Furthermore, PEG antibodies occur in animals and even humans, which may lead to strongly reduced bio-availability of a PEGylated drug or even cause undesirable pathological side effects.[4, 5] A possible interaction of PEG with biological macromolecules suggests a possible mechanism of PEG protection might be involved due to the amphiphilic nature of PEG.[6, 7] Unlike amphiphilic PEG, zwitterionic polymers, such as poly(2-methacryloyloxyethyl phosphorylcholine) (pMPC)[8-10], poly(sulfobetaine methacrylate) (pSBMA)[11-13], poly(carboxybetaine methacrylate) (pCBMA)[14-16] and simply mixed-charge materials, etc[17-19] are super-hydrophilic via electrostatic interaction with water and have been recognized as effective nonfouling materials which can resist protein adsorption and cell attachment. Polyzwitterionic materials might be a good alternative to PEGs to achieve excellent stability in blood stream. Thus, it is important to illustrate the biocompatibility of zwitterionic polymers both *in vivo* and *in vitro*, especially long circulation time and no immune response of zwitterionic polymers in vivo. Meanwhile, more and more experimental results showed the advantages of zwitterionic polymer in biomedical applications.[20, 21] Jiang and coworkers reported good retention of hydrophilic drug and long blood circulating characteristics of pCBMA-modified liposomes without cholesterol shine a light on the superior biocompatibility.[22] Moreover, they also prepared poly(lactic-co-glycolic acid) (PLGA)-b-pCBMA block copolymers (PLGA-PCB), and found that their

self-assembled nanoparticles were remarkably stable without any cryoprotectant additives after freeze-drying.[14] Recent results reported by Wooley and coworkers indicated a better shielding efficiency provided by PEG than PCB polymers to the immunotoxic nanoparticles.[23] In fact, the biocompatibility of the materials forming nano-drug vehicles (NDVs) plays a decisive role in nano-drug delivery. NDVs administered by the parenteral route could spread to most organs and be uptaken by them, mainly by reticulo-endothelial system. Moreover, NDVs will be repeatedly injected, which might cause immunoresponse-related clearance. Previous reports have shown that anti-PEG antibody was dectected in some normal blood donors (22%-25%) as a response to injection with PEGylated polymer particles. Rapid clearance of PEGylated polymer particles has been observed.[21] Meanwhile, some recent studies have reported that nanoparticles may generate potential harm to the body. Such informational shortages become a big hindrance to expand the biomedical applications of related polymers, especially in nano-drug delivery. It is desired to get systematic information on long circulation time and no immune response of zwitterionic polymers.

To investigate the biocompatibility of zwitterionic polymers, four RhB-labeled star polymers of different chain lengths were synthesized via atom transfer radical polymerization (ATRP) from a β-CD initiator (Scheme 1) as reliable nanoparticle models due to their well-defined, chemically stable molecular entities with moderate flexibility in structure and surface functionality.[24] In addition, it has been shown that at equivalent areas per molecule, a star polymer will be superior to resist non-specific protein adsorption than linear grafts, which have the potential to provide the pharmacokinetic advantages of delivery systems and increase the possibility of drug targeting. Moreover, their diameters are relatively fixed and stable since branches are covalently bonded.[25] In this work, both *in vitro* behaviors, such as cell uptake, cytotoxic and hemolytic activity and *in vivo* behaviors, including blood circulation, serum-biochemistry and histopathological responses of the star zwitterionic polymers were investigated systematically. The objective of the study was to determine whether there was little potential toxicity on cell and tissue level, and whether there was long

circulation time and no immune response upon repeated injections of zwitterionic polymer. The results of the biocompatibility of zwitterionic polymers are rather encouraging. The circulation half-life of the largest star polymer (123 kDa) in mice was prolonged to 40 h, with little change between the first and the next two doses. The results illustrate that zwitterionic polymers could be excellent alternatives to PEG for drug delivery system.

2. Experimental Methods

2.1 Materials

β-Cyclodextrin (*β*-CD, 98%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 98%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%), 2-hydroxyethyl methacrylate (HEMA, 98%), 2-bromoisobutyryl bromide (BIBB, 98%), 4-dimethylaminopyridine (DMAP, 99%), copper(I) bromide (CuBr, 99%), *N,N′,N′,N″,N″*-pentamethyldiethylenetriamine (99%), triethylamine (99.5%), rhodamine B (95%), polypropylene oxide 1000 (PPO 1 K), polyethylene glycol 2000 (PEG 2 K), sodium nitrate ($NaNO_3$) and all the organic solvents were purchased from Aladdin-reagent (Shanghai). *β*-propiolactone (98%) and dicyclohexylcarbodiimide (DCC, 99%) were purchased from TCI (Shanghai). Fibrinogen from bovine was purchased from Sigma-Aldrich. *β*-CD was vacuum-dried at 100 °C overnight before use. Carboxybetaine methacrylate (CBMA) was prepared following the procedures reported previously.[16]

2.2 Characterization

All ^{1}H NMR spectra were recorded in $CDCl_3$ or D_2O using a Bruker ADVANCE2B/400MHz instrument at room temperature. Gel permeation chromatography (GPC) measurements were conducted on a Waters GPC system equipped with Waters Ultrahydrogel columns and a Waters refractive index detector. The molecular weight and molecular weight distributions were calibrated against polyethylene glycol standards with 0.2 mol/L $NaNO_3$ aqueous solutions as eluent at a

flow rate of 0.5 mL/min, and the column temperature was 40 °C. The averages diameters of the star polymers were measured by Zetasizer Nano-ZS (Malvern Instruments), the scattering angle was kept at 173° and the temperature was 37 °C.

2.3 Synthesis of *β*-CD-Based Macroinitiator (CD-BIBB)

β-CD-based macroinitiator was prepared following the procedures reported previously.[24] *β*-CD (4 mmol, vacuum-dried at 100 °C overnight before use) was dissolved in 35 mL of anhydrous 1-methyl-2-pyrrolidione (NMP) at room temperature with stirring and then it was cooled to 0 °C. BIBB (25 mmol) dissolved in anhydrous NMP (15 mL) was added dropwise to the *β*-CD solution with stirring. The reaction mixture was allowed to warm up to ambient temperature and stirred overnight. The final reaction mixture was precipitated with 400 mL of diethyl ether. The white powder precipitate was collected by filtration. The crude product was purified by suspending it in 200 mL of deionized water at room temperature overnight. The purified *β*-CD-based macroinitiator was filtered, and dried by lyophilization with a yield of 48%. [26]

2.4 Synthesis of Fluorescent Monomer (RhB-HEMA)

Typically, a solution of dicyclohexylcarbodiimide (20 mmol) and 4-dimethylaminopyridine (2 mmol) in 160 mL of methylene dichloride was added dropwise to a solution of rhodamine B (10 mmol) and 2-hydroxyethyl methacrylate (20 mmol) in 40 mL of methylene dichloride (Scheme 2). The reaction mixture was stirred overnight. After removing insoluble salts by suction filtration, the filtrate was concentrated and further purified by chromatography (silica gel, 10% isopropanol/ethyl acetate). After removing all the solvents by rotate evaporation, RhB-HEMA was obtained as a solid (2.7 g, yield: 45%) ^{1}H NMR ($CDCl_3$, 400 MHz): 8.31, 7.83, 7.76, 7.32, 7.09, 6.93 and 6.81 (m, 10 H, Aromatic protons), 6.60 (s, 1H), 6.53 (s, 1H), 4.18 (m, 2H), 4.18 (m, 2H), 3.65 (q, 8H), 1.35 (t, 12 H) (Figure 1a).

2.5 Synthesis of Star Carboxybetaine Polymers

A typical polymerization procedure of the CD-g-CBMA star polymers (CD-CBMA2) at [CBMA]/[CD-BIBB] ratio of 300 was carried out as follows: CBMA (2.3 mmol), RhB-HEMA (11 μmol), CD-BIBB (7.5 μmol), and 3 mL of methanol/water (9:1, v/v) was deoxygenated with nitrogen for 30 min before adding Cu(I)Br (0.045 mmol) and PMDETA (0.054 mmol) under nitrogen protection. The reaction mixture was maintained under nitrogen purge for the duration of the polymerization. After 12 h, the reaction mixture was exposed to air to stop polymerization. The polymer was dialyzed against methanol for 3 days using a 3500-MW cutoff membrane and against water for another 3 days. The star polymer was lyophilized to afford a rose pink solid. The molecular weight was determined by GPC.

2.6 Evaluation the Stability of Star Polymers in Protein Solution

The stability of the star polymers against fetal bovine serum (FBS) solution in PBS (pH 7.4, 0.15 M) was investigated using dynamic light scattering (DLS). 1 mL of star polymer (10 mg/mL) prepared in PBS was mixed with 1 mL of fetal bovine serum (FBS) solution (100%) at 37 °C. At 0.5 h, 1 h and 2 h, the size of the star polymer was measured. PPO 1 kDa and PEG 2 kDa were selected as the positive and negative controls.

2.7 *In Vitro* Cell Uptake

To study the cellular uptake of the star carboxybetaine polymers by fluorescence microscopy and flow cytometry, RAW 264.7 cells were seeded in 24-well plates supplemented with Dulbecco's modified eagle's medium (DMEM) and 10% fetal bovine serum (FBS) under 5% CO_2 at 37 °C for 24 h. Culture medium was removed and 1.0 mL of RhB-HEMA labeled polymer (0.5 mg/mL) or equivalent RhB-HEMA was added into each well. After 12 h incubation, the cells were rinsed 3 times with PBS and placed in 0.2 mL of DMEM for live cell imaging. Thereafter, the cells were rinsed 3 times with PBS and treated with trypsin. The cells were then suspended in PBS and analyzed immediately using a flow cytometer (BD FACSEALIBUR™).

2.8 Hemolysis Assay

The hemolysis assay was performed according to a previous report.[27] Fresh blood was collected from healthy male ICR mice. Red blood cells (RBCs) were separated by centrifugation of whole blood diluted in sterile phosphate-buffered saline (PBS) at 1500 rpm for 10 min. The plasma supernatant was removed and the erythrocytes were resuspended in sterile PBS. The cells were washed three times with sterile PBS solution, and then resuspended in PBS to get a 2% w/v RBCs suspension. Star carboxybetaine polymer solutions of different molecular weight were also prepared in sterile PBS. A 0.5 mL of polymer solution prepared in PBS was added to 0.5 mL of 2% w/v RBCs suspension to make the final polymer concentration at 5 mg/mL and it was incubated for 6 h at 37 °C. After incubation, the mixture was centrifuged and the supernatant was transferred to a 96-well plate. Release of hemoglobin was determined by spectrophotometric analysis of the supernatant at 575 nm. Complete hemolysis was attained using double distilled water as positive control and PBS as negative control.

2.9 *In Vitro* Cytotoxic Assay

The cytotoxic effect of star carboxybetaine polymers was determined by using MTT assay. RAW 264.7 or HUVEC cells were seeded (1×10^4 cell/well) in 96-well plates supplemented with Dulbecco's modified eagle medium (DMEM) and 10% fetal bovine serum (FBS) under 5% CO_2 at 37 °C for 24 h. For each well, cells were washed with PBS and incubated with 200 μL full medium containing varied concentration of star carboxybetaine polymers for 24 h. Cells were washed with PBS to remove star carboxybetaine polymers. Then, 100 μL of a stock solution containing 1 mg/mL of MTT in medium was added and incubated for another 4 h. Then, MTT-containing medium was replaced with 150 μL of DMSO to dissolve the formed crystals at 37 °C for 10 min. The absorbance at a wavelength of 570 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 570 nm with control wells.

2.10 Blood circulation

All animal experiments were performed according to the guidelines established by the Institute for Experimental Animals of Zhejiang University. Healthy male ICR mice (18-22 g) were purchased from the animal center of Zhejiang Academy of Medical Sciences. The room was maintained at 20 ± 2 °C and 60 ± 10% relative humidity, with a 12 h light–dark cycle. Mice were fed on water and sterilized food. Prior to treatment, mice were kept fasted overnight. 1 mg of RhB-HEMA labeled polymer in 0.2 mL of PBS was injected via tail vein, and the control group was given only PBS injection. At 2 min, 0.5 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h and 96 h, blood samples (50−100 μl) were collected from orbit. The plasma was separated from the blood by centrifuging at a speed of 4,000 rpm for 5 min. Then, the plasma was diluted with methanol and centrifuged to remove the insoluble solid. The solutions were measured for fluorescent emission at 605 nm with the excitation at 556 nm using a microplate reader (SpectraMax M2e) and the corresponding RhB-HEMA concentration was calculated according to an established standard curve.

2.11 Serum-biochemistry evaluation and histopathological examination

For serum-biochemistry evaluation, 1 mg of various RhB-HEMA labeled polymer in 0.2 mL of PBS or 0.2 mL of PBS (control group) were injected via tail vein every two days. After three injections, blood samples were withdrawn from the posterior vena cava under ether anesthesia. HITACHI 7020 automatic analyzer (Hitachi, Tokyo, Japan) was used to measure the total protein (TP), albumin (Alb), globulin (Glo), total bilirubin (TB), bilirubin direct (BD), bilirubin indirect (BI), glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), alkaline phosphatase (AP), creatinine (Cr), usea nitrogen (UN) and lactate dehydrogenase (LDH). Then, mice were dissected for histopathological examination. A small piece of heart, lung, liver, spleen and kidney were fixed by using 10% formalin and then embedded into paraffin. The section was stained with hematoxylin–eosin (HE) and examined by light microscopy. The data were expressed

as mean ± standard deviation (n = 5).

3. Results and Discussion

3.1 Synthesis and Characterization of Star Carboxybetaine Polymer

A two-step reaction was performed to prepare the desired star carboxybetaine polymer (Scheme 3). First, the β-CD-based macroinitiator was prepared via the reaction of hydroxyl groups on the outside surface of β-CD with 2-bromoisobutyrl bromide.[26] From ^{1}H NMR spectroscopy(Figure 1b), a new peak located at δ = 1.9 ppm (***a,*** C(Br)-CH_3) is observed, which indicates the macroinitiator has been successfully synthesized. The signals located at broad chemical shifts in the region of 3.5 - 4.1 ppm are mainly associated with the inner methylidyne and methylene protons between the oxygen and carbon moieties. The peak located at δ = 4.6 ppm is assigned to the inner methylidyne protons between the oxygen moieties (***c***, O-CH-O). From the area ratio of peak ***a*** to peak ***c***, the degree of substitution of the hydroxyl groups on the CD is determined to be about 6.0 (Figure 1b). The star carboxybetaine polymer (CD-CBMA) was subsequently synthesized via ATRP of CBMA from β-CD-based macroinitiator, purified by dialysis against methanol and water to remove unreacted monomers and catalyst (Scheme 3). The CD-CBMAs with different molecular weights were synthesized by varying the feed ratio of monomer to initiator. Table 1 summarizes the GPC results of CD-CBMAs, the number average-molecular weight (Mn) of CD-CBMA increases from 14 kDa to 123 kDa with the increase of feed ratio and the polydispersity index (PDI) of these four CD-CBMAs varies from 1.28 to 1.51. These slightly high PDI might be caused by the complex star shape.

To evaluate the cell uptake *in vitro* and the pharmacokinetics *in vivo*, CD-CBMA were labeled with fluorescent monomer RhB-HEMA via ATRP. The GPC measurements proved the successful removal of physically adsorbed fluorescent monomer from the RhB-labeled CD-CBMA (Figure 2).

3.2 Blood circulation time

To evaluate the *in vivo* stealth property, CD-CBMAs labeled with RhB-HEMA were used for circulation kinetic studies. At various time points, blood was collected

to estimate the amount of CD-CBMA remained in bloodstream. Elimination half-life ($t_{1/2}$) and areas under % dose/g versus time curve from 0 to infinity ($AUC_{0\rightarrow\infty}$) were calculated using two-compartment model and the data are listed in Table 2. As expected, it is found that both $t_{1/2}$ and $AUC_{0\rightarrow\infty}$ values increase when the molecular weight of these polymers increases from 12 kDa to 123 kDa (Figure 3). The $t_{1/2}$ of CD-CBMA increases dramatically when the molecular weight increases from 12 kDa to 40 kDa. However, further increasing the molecular weight causes a slow increase of $t_{1/2}$. This clear change in $t_{1/2}$ indicates that the threshold MW to avoid rapid renal elimination of CD-CBMA is around 40 kDa, where the hydrodynamic size of CD-CBMA2 is 6.8 nm by DLS, and is a little higher than the pore size of the glomerular membrane of kidney (about 3–5 nm).[28] The $t_{1/2}$ for CD-CBMA2 (40 kDa), CD-CBMA3 (58 kDa) and CD-CBMA4 (123 kDa) is 26 h, 36 h and 39 h, respectively. This is a relatively long blood circulating characteristics *in vivo,* especially, molecules at the diameter of the serum protein level (less than 20 nm diameter). This value is comparable to the $t_{1/2}$ of CD-PEGMA300 at similar molecular weight (data not shown). CD-PEGMA300 is poly[poly(ethylene glycol) methyl ether methacrylate] via ATRP from the β-CD initiator for CD-CBMA to make head to head comparison. Jiang and coworkers reported that the circulation half-life of optimized linear pCBMA modified liposome was about 8 h[19], which also suggests the pCBMA could improve the circulation time.

Furthermore, the circulation time of repeat injections exhibit similar results to the first injection, implying no immune response occurred (Figure 4). This may be due to the excellent nonfouling properties (<0.3 ng/cm^2 adsorbed proteins) of zwitterionic polymer on flat surfaces[15] or nanoparticle surfaces[29].

3.3 Serum-biochemistry Evaluation and Histopathological Examination

A number of serum-biochemical and histopathological parameters were also compared after three repeat injections of CD-CBMA3 and PBS in order to evaluate *in vivo* biocompatibility. Obvious change in such parameters would presumably reflect the occurrence of any abnormality or toxicity in the body. All parameters in the

serum-biochemical evaluation, including total protein (TP), total bilirubin (TB), glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), alkaline phosphatase (AP), creatinine (Cr), usea nitrogen (UN) and lactate dehydrogenase (LDH) were compared. Among them, TB, AP, GPT and GOT levels were attributed to putative functioning of liver; Cr, UN, LDH were attributed to putative functioning of kidney. No significant differences between CD-CBMA3 treated mice and PBS treated mice were found (Tables 3). TP, Alb and Glo levels also showed no obvious increase of antibody expression in blood. Moreover, there is few anti-pCBMA antibody observed through a sandwich method detecting the mouse IgG on pCBMA coated gold surface (data not shown). Furthermore, the histopathological evaluation of major organs and tissues including heart, liver, spleen, lung and kidney showed no significant difference with respect to the control (Figure 5), indicating the major organs of mice are still in healthy condition after received three repeated injections of CD-CBMA3. In short, all the results show no clear difference between mice receiving three repeat injections of CD-CBMA3 and the control mice.

3.4 *In Vitro* Biocompatibility Analysis of Star Zwtterionic Polymer

The biocompatibility of star zwtterionic polymers was analyzed including plasma compatibility, cell internalization, hemolysis and cell viability. The aims of this study were to investigate *in vitro* behavior to support the unique *in vivo* results.

3.4.1 Non-specific Interaction between Star Polymer and Plasma

Non-specific protein adsorption on the blood-contact materials is considered as the first step in foreign-body reaction. High concentration of FBS is one of the most challenging conditions to prevent non-specific protein adsorption on nano drug carriers. The change of particle size measured by DLS provides direct information of hemocompatibility of drug carrier because the adsorption-caused activation or denaturation of serum proteins could cause aggregation, thereby causing the increase of particle size.[29] As shown in Figure 6, the size of 5 mg/mL PPO 1 kDa in FBS increased to micrometer level after 1 h, which was attributed to the hydrophobic

interaction between PPO and protein in FBS. And there was no large particle detected by DLS after 5 mg/mL CD-CBMA or PEG 2 kDa mixed with FBS. The mixture of CD-CBMA with FBS exhibited almost the same hydrodynamic size as PEG 2 kDa with FBS. No aggregation as PPO 1 kDa was found in either CD-CBMA or PEG 2 kDa in FBS. This excellent stability of coexistence of CD-CBMA with FBS indicates very weak non-specific interaction between CD-CBMA with proteins in FBS.

3.4.2 Interaction between Star Polymer and Cell Membrane

To evaluate the biocompatibility of CD-CBMAs at cell level, RBC hemolysis assay and macrophage cell uptake were performed. Red blood cell lysis is a widely used method to study polymer−membrane interaction. The observed hemolysis of RBCs in water solutions at 37 °C were used as positive controls, respectively. Figure 7 shows that all CD-CBMAs exhibit an undetectable level of hemolytic activity which is comparable with the negative control PEG, while hydrophobic PPO 1 kDa exhibited about 20% hemolytic activity. These phenomenon indicate that the excellent nonfouling nature of zwitterionic polymers could avoid disrupting red blood cell membranes, and CD-CBMAs could be a promising candidate for *in vivo* application, such as systemic drug delivery, through using the hydrophobic cavity of CD to wrap drug molecules and modifying the targeting group with the carboxyl group of CBMA to increase cell uptake.[30]

Furthermore, cell uptake of star polymer CD-CBMA3 (RhB-HEMA labeled) and RhB-HEMA were investigated by fluorescence microscopy and flow cytometry using macrophage RAW 264.7 cell, which is a specialized phagocytic cell that attacks foreign substances, infectious microbes and cancer cells through destruction and ingestion. Even after 12 h incubation with CD-CBMA3, only very weak fluorescence was observed in the cells, whereas strong fluorescence was observed in all cells after 12 h incubation with RhB-HEMA at an equivalent concentration of RhB (Figure 8). This result was further confirmed by flow cytometry method quantitatively (Figure 9). After 12 h incubation, the intensity of red fluorescence of RhB from the cells cultured with RhB-HEMA was about 40 times higher than the value obtained from those

cultured with RhB-HEMA labeled CD-CBMA3, under condition that an equivalent concentration of RhB was used. These results indicate that the interaction between CD-CBMA and the cell membrane of macrophage cell is very weak due to the superhydrophilic and nonfouling nature of the zwitterionic groups, which is consistent with the result of hemolysis assay.

3.4.3 Cell Viability Assay

It is very important to evaluate the potential toxicity of polymer for drug delivery applications. RAW 264.7 and HUVEC cells were used to investigate the cytotoxicity *in vitro*. The cytotoxicity to RAW 264.7 and HUVEC cells in culture medium after 24 h incubation with CD-CBMAs was determined using MTT assay. Macrophage cells and endothelial cells were also used because one of the potential uses of these polymers is intravenous drug delivery systems. As shown in Figure 10, even at a concentration up to 2 mg/mL, CD-CBMAs show no obvious cytotoxicity to these two types of cells (> 90% cell viability). The excellent biocompatibility is greatly attributed to the low interaction between polymer and cell membranes, even when cells are growing.

4. Conclusion

In this study, star carboxybetaine polymers consisting of a β-CD core and six poly(carboxybetaine) arms were prepared via ATRP from β-CD initiator. *In vivo* experiments showed that this polymer has long-circulation time in mice, even after repeated injections; no appreciable damage or inflammation of major organ tissues, and no obvious increase of antibody occurred in blood. *In vitro* experiments showed that this polymer has reduced internalization, no obvious cytotoxity and undetectable hemolytic activity, which are consistent with the *in vivo* results. Thus, it is believed that the star carboxybetaine polymer shows great potential for drug delivery systems.

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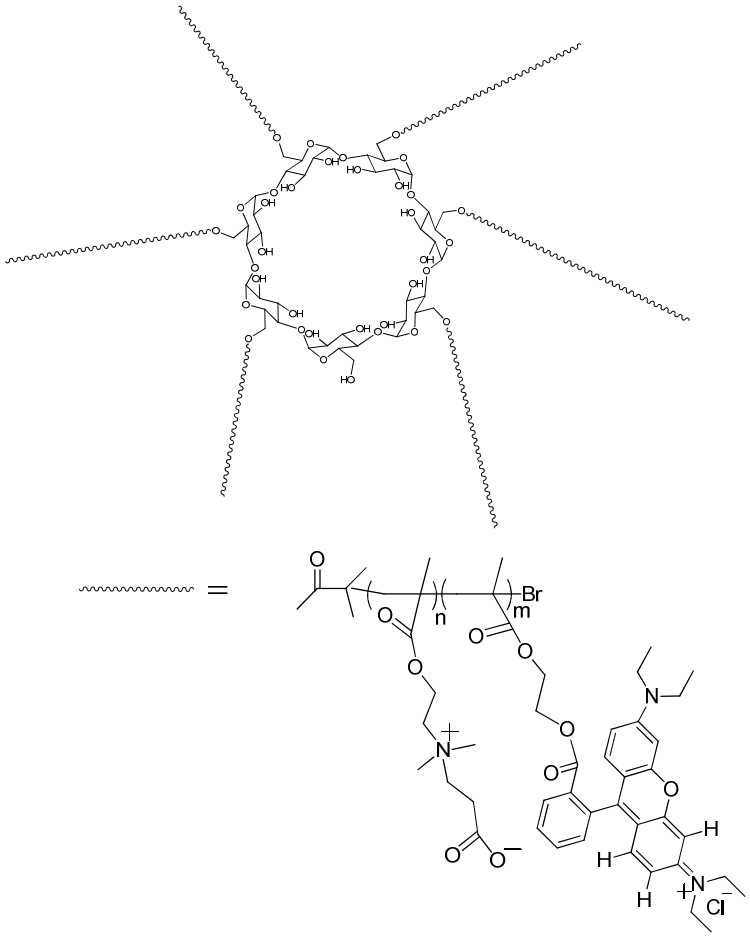
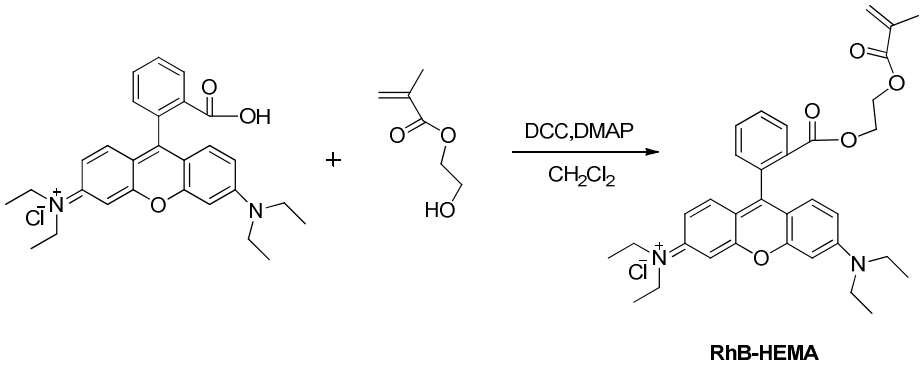
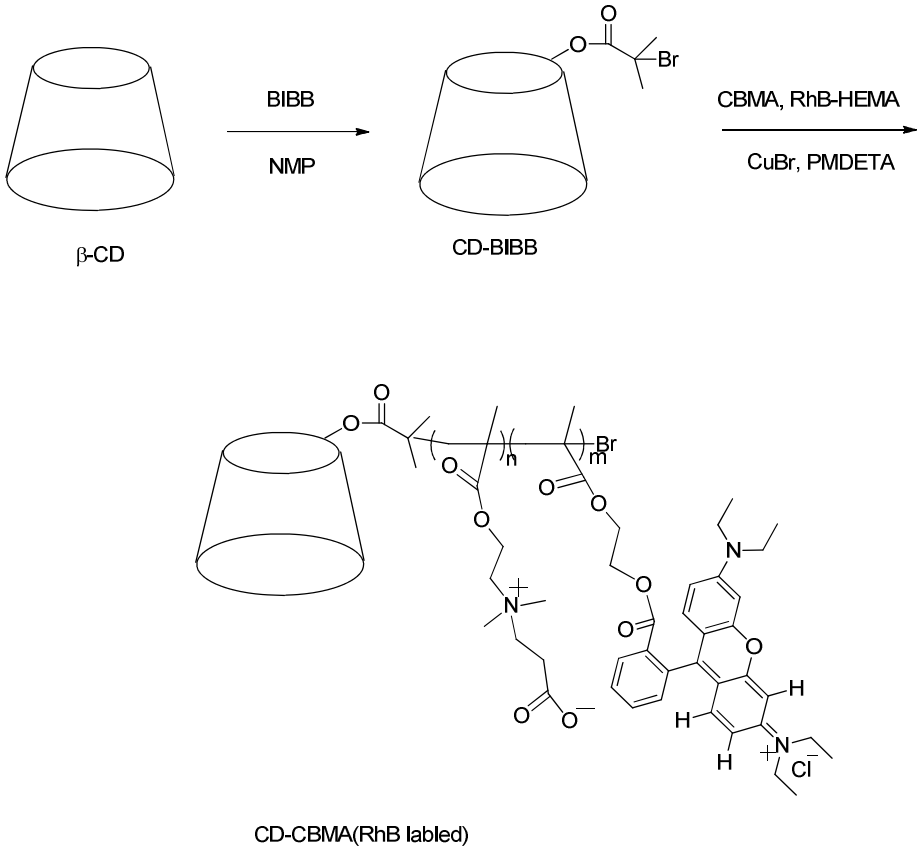


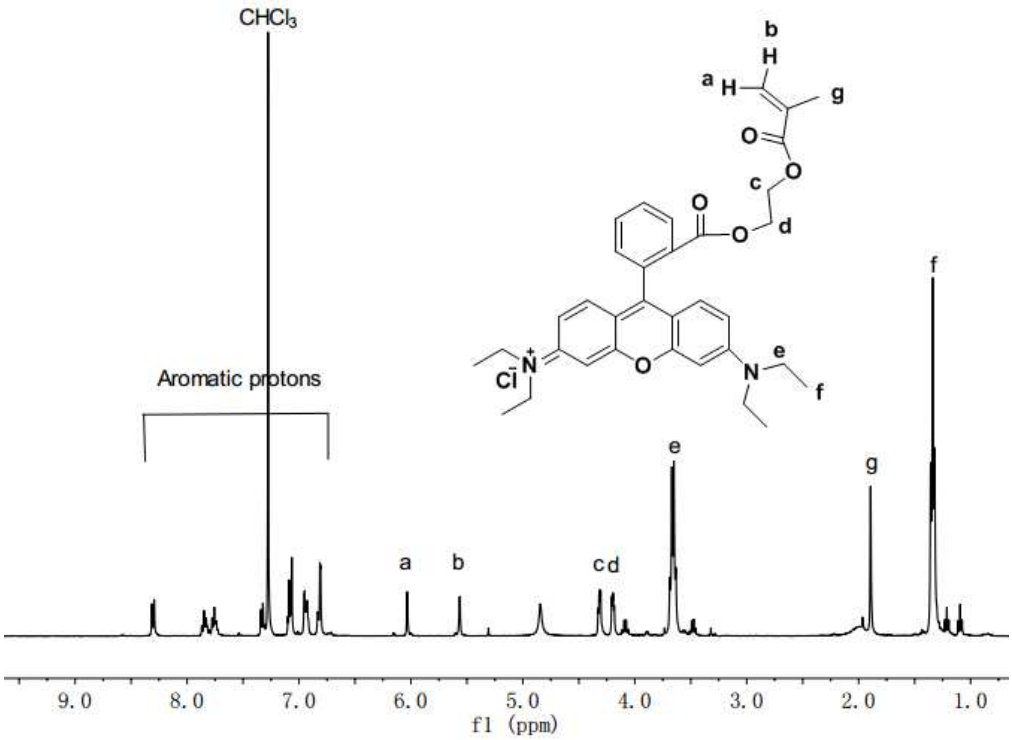
Scheme 1. Chemical structure of star carboxybetaine polymer.



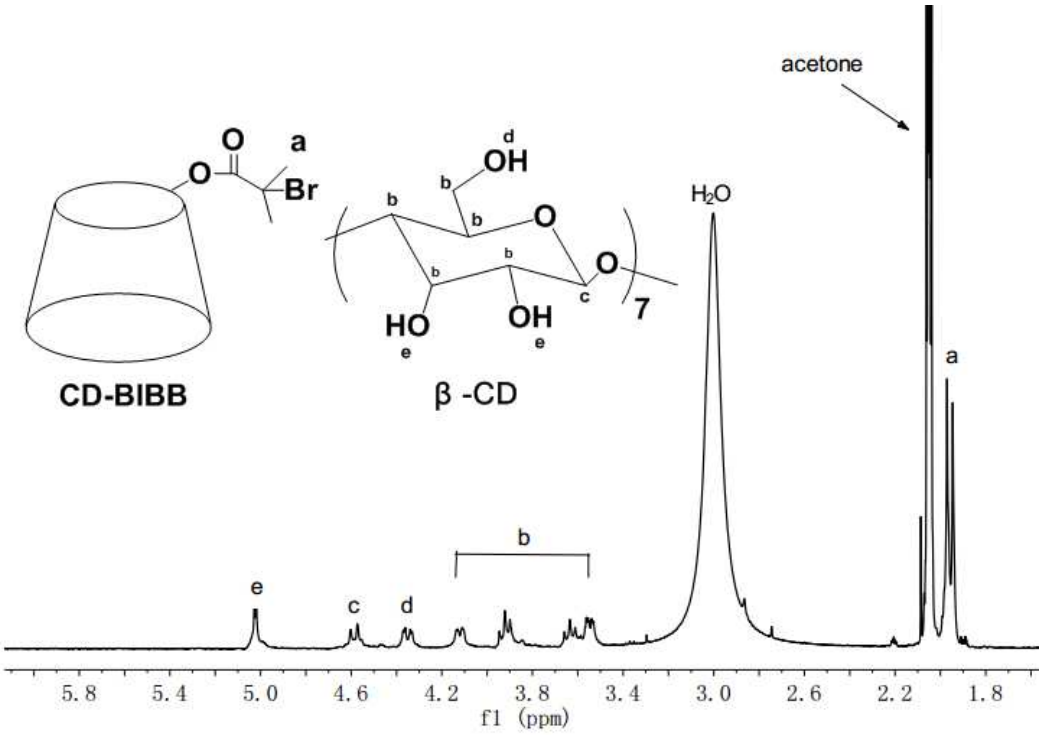
Scheme 2. Synthesis procedure of rhodamine B-based fluorescent monomer (RhB-HEMA).



Scheme 3. Synthesis procedure of macroinitiator CD-BIBB and RhB-labeled star polymer CD-CBMA.



(a)



(b)

Figure 1. ^{1}H NMR spectra of (a) RhB-HEMA in $CDCl_3$; (b) Macroinitiator CD-BIBB in acetone-D6.

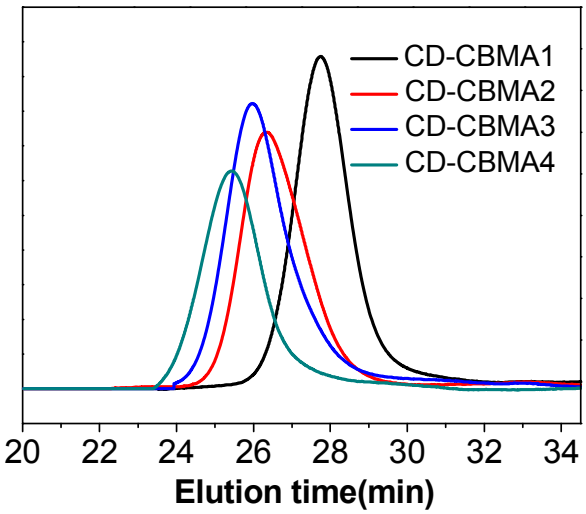


Figure 2. GPC traces of star polymer CD-CBMA.

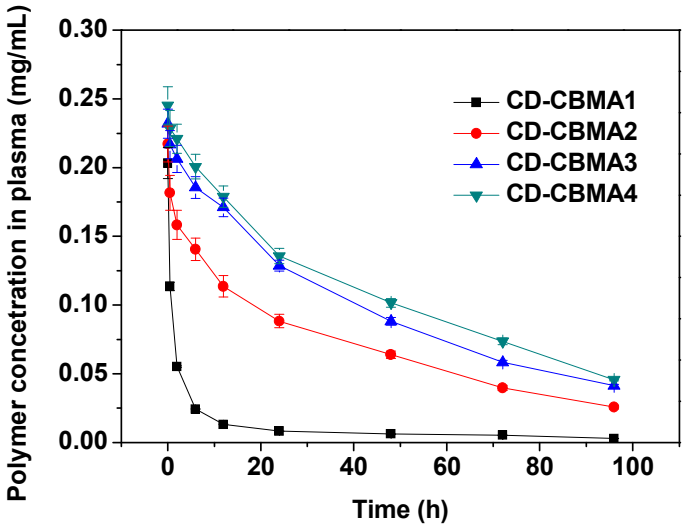


Figure 3. Polymer concentration in plasma with respect to time after intravenous administration (mean ± SD, n = 3).

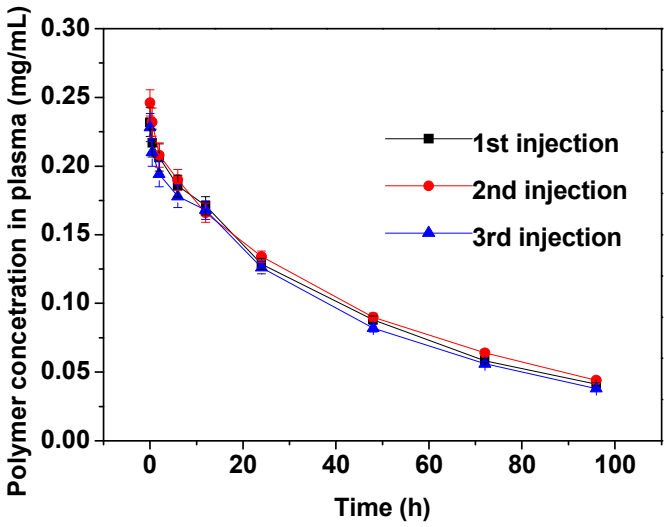


Figure 4. CD-CBMA3 concentration in plasma with respect to time after intravenous administration (mean ± SD, n = 3).

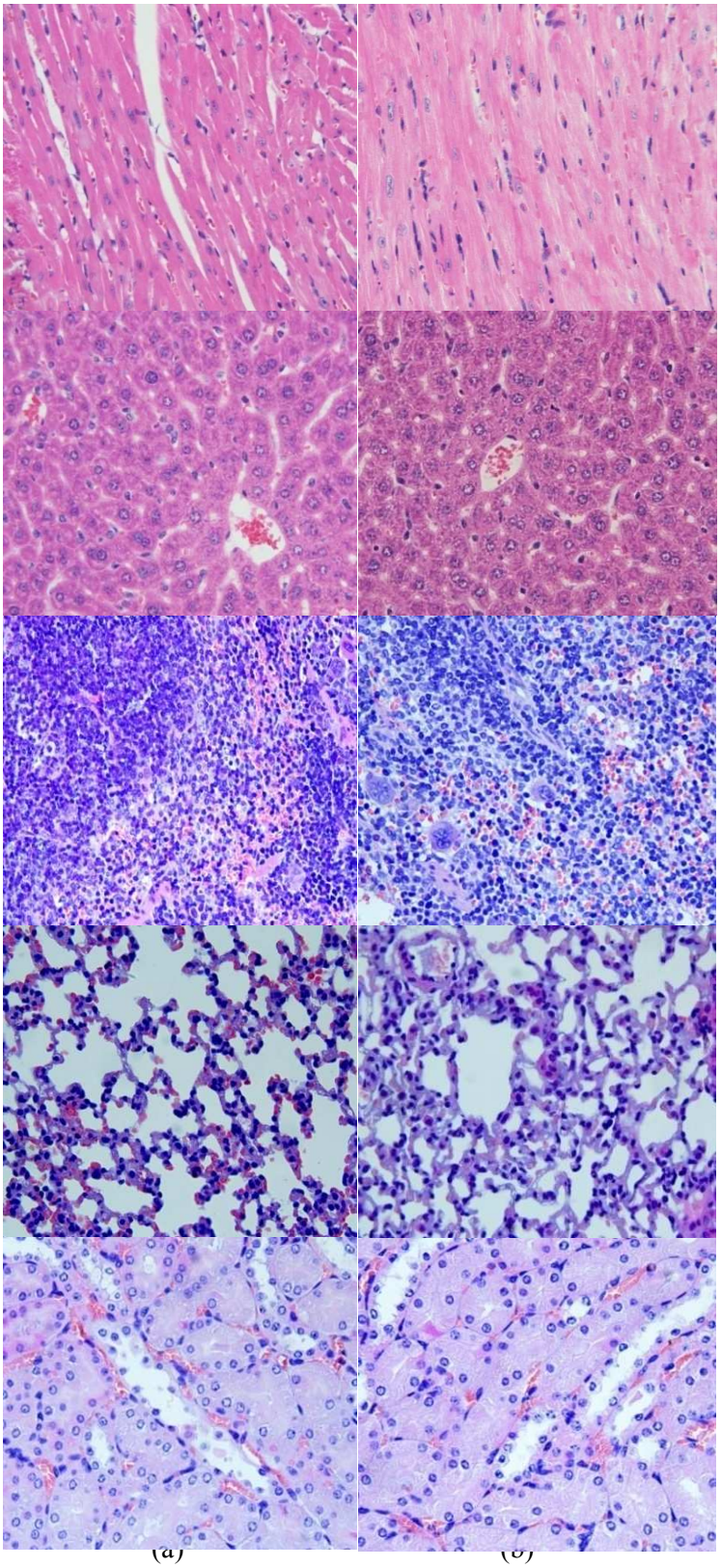


Figure 5. Histopathology of heart, liver, spleen, lung, kidney after (a) PBS and (b) CD-CBMA3 treatment.

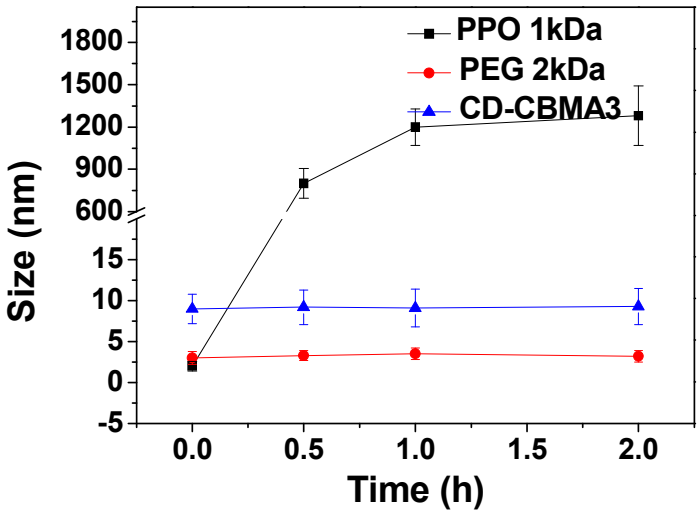


Figure 6. Size of CD-CBMA3, PPO 1 kDa and PEG 2 kDa versus time in FBS at 37 °C.

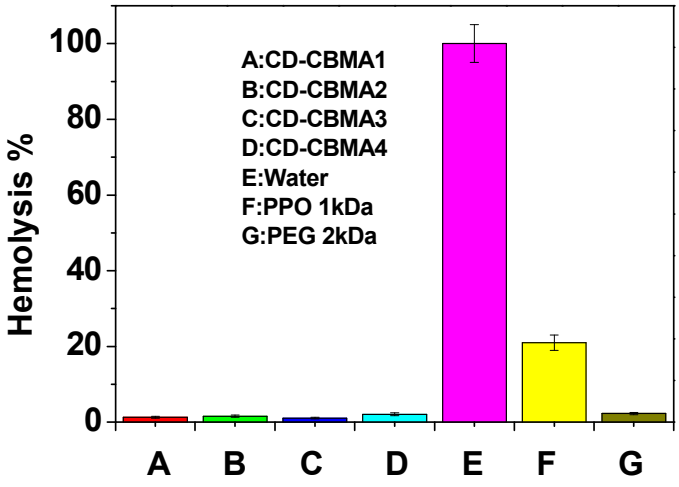
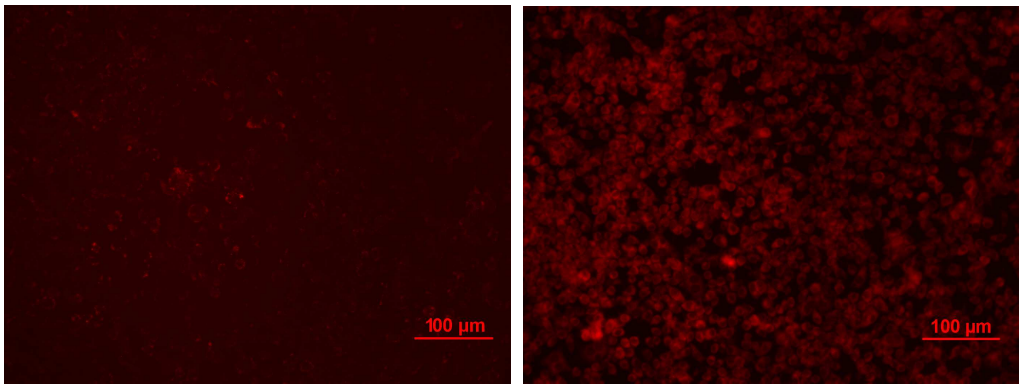
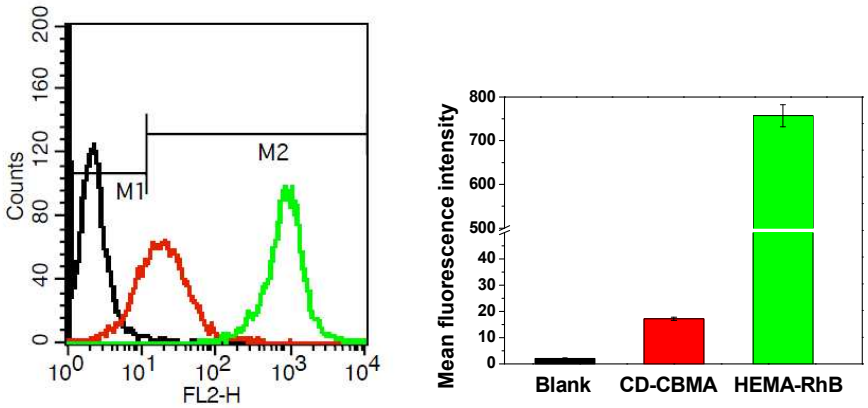


Figure 7. Hemolysis assay of star polymer CD-CBMA, PPO 1 kDa and PEG 2 kDa with different molecular weights (mean ± SD, n = 3).



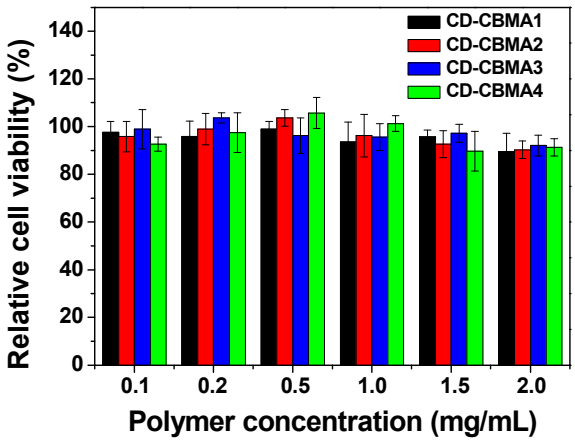
(a) (b)

Figure 8. Cell uptake of star polymer CD-CBMA3 (RhB-HEMA labeled) and RhB-HEMA followed by fluorescence microscopy using macrophage RAW 264.7 cells. (a) After 12 h incubation with CD-CBMA (exposure time is 3 s); (b) After 12 h incubation with RhB-HEMA (exposure time is 0.2 s).

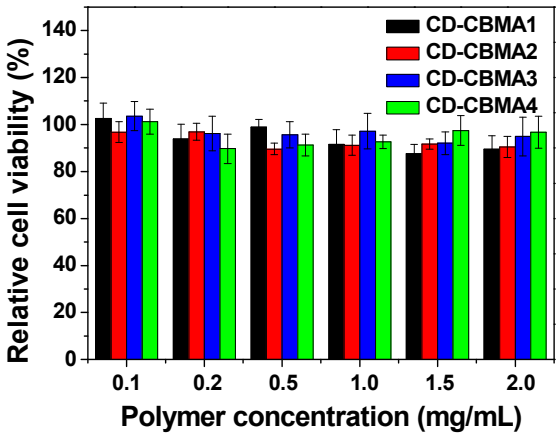


(a) (b)

Figure 9. (a) Flow cytometry results of RAW 264.7 cells treated by PBS (black), CD-CBMA (RhB-HEMA labeled) (red), free RhB-HEMA (green) at 37 °C for 12 h; (b) Mean fluorescence intensity located in the RAW 264.7 cells incubated in PBS, CD-CBMA(RhB-HEMA labeled) and free RhB-HEMA at 37 °C for 12 h. RhB-HEMA concentration was 2 μg/ml.



(a)



(b)

Figure 10. Cytotoxic of star polymer CD-CBMA at polymer concentrations ranging from 0.1 mg/mL to 2 mg/mL after 24 h incubation with cells. Relative cell viability are shown as mean ± SD, $n = 4$. (a) RAW 264.7 cells, (b) HUVEC cells.

Table 1. Feed molor ratio of monomer to initiator, number-average molecular weights (Mn), polydispersity index (PDI) and size for star polymer CD-CBMA.

Sample	[CBMA]:[CD-BIBB][a]	Mn (kDa)[b]	PDI[b]	Size (nm)[c]
CD-CBMA1	120	14	1.28	3.0 ± 0.4
CD-CBMA2	300	40	1.34	6.8 ± 0.8
CD-CBMA3	600	58	1.31	8.7 ± 2.6
CD-CBMA4	1200	123	1.51	11.9 ± 2.5

[a]Reaction molar ratios of CBMA monomer to CD-BIBB initiator used.

[b]Determined by GPC.

[c]Estimated by DLS.

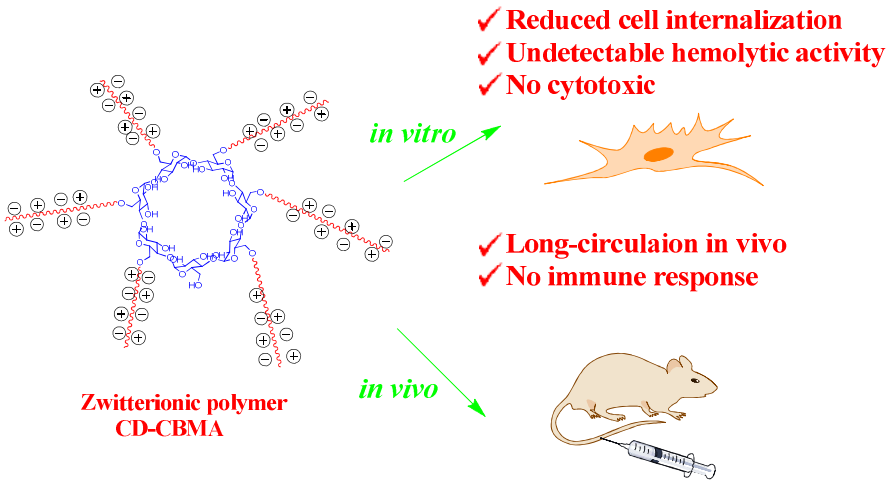
Table 2. $T_{1/2}$ and $AUC_{0\text{-}\infty}$ determined from pharmacokinetic analysis for the Evaluated Polymers.

Sample	$t_{1/2}$ (h)	$AUC_{0\rightarrow\infty}$ (mg/mL/h)
CD-CBMA1	0.92 ± 0.08	0.26 ± 0.04
CD-CBMA2	26.6 ± 2.3	6.97 ± 0.71
CD-CBMA3	36.0 ± 3.1	11.3 ± 1.7
CD-CBMA4	39.1 ± 3.7	13.0 ± 1.5

Table 3. Blood test parameters for mice treated with PBS and CD-CBMA (mean ± SD, *n* = 3).

Materials	PBS	CD-CBMA3
Total Protein(g/L)	50.9 ± 0.5	49.2 ± 0.6
Albumin (g/L)	32.6 ± 0.2	31.1 ± 0.3
Globulin (g/L)	18.3 ± 0.3	18.1 ± 0.3
Total Bilirubin(umol/L)	5.8 ± 0.2	5.0 ± 0.2
Bilirubin Direct(umol/L)	2.6 ± 0.2	2.0 ± 0.2
Bilirubin Indirect(umol/L)	3.2 ± 0.1	3.3 ± 0.2
Glutamic-Pyruvic Transaminase(U/L)	19 ± 4	24 ± 2
Glutamic-Oxaloacetic Transaminase(U/L)	75 ± 6	74 ± 8
Alkaline Phosphatase(U/L)	101 ± 8	97 ± 4
Creatinine(umol/L)	42.7 ± 1.8	36.7 ± 1.3
Usea Nitrogen(umol/L)	8.3 ± 0.4	8.8 ± 0.2
Lactate Dehydrogenase(U/L)	593 ± 60	618 ± 67

1 Graphical Abstrate



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