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Star-branched amphiphilic PLA-b-PDMAEMA copolymers for co-delivery of miR-21 inhibitor and doxorubicin to treat glioma

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ABSTRACT

The combined treatment of chemotherapeutant and microRNA (miR) has been proven to be a viable strategy for enhancing chemosensitivity due to its synergistic effect for tumor therapy. However, the co-delivery of drugs and genes remains a major challenge as they lack efficient co-delivery carriers. In this study, three amphiphilic star-branched copolymers comprising polylactic acid (PLA) and polydimethylaminoethyl methacrylate (PDMAEMA) with AB_3, $(AB_3)_2$,and $(AB_3)_3$ molecular architectures were synthesized respectively by a combination of ring-opening polymerization, atom transfer radical polymerization, and click chemistry via an "arm-first" approach. The star copolymers possessed a low critical micelle concentration (CMC) and formed nano-sized micelles with positive surface charges in water as well as exhibiting a much lower cytotoxicity than PEI 25 kDa. Nevertheless, their gene transfection efficiency and tumor inhibition ability showed a remarkable dependence on their molecular architecture. The $(AB_3)_3$ architecture micelle copolymer exhibited the highest transfection efficiency, about 2.5 times higher than PEI. In addition, after co-delivering DOX and miR-21 inhibitor (miR-21i) into LN229 glioma cells, the micelles could mediate escaping miR-21i from lysosome degradation and the release of DOX to the nucleus, which significantly decreased the miR-21 expression. Moreover, co-delivery of DOX and miR-21i surprisingly exhibited an anti-proliferative efficiency compared with DOX or the miR-21i treatment alone. These results demonstrated that amphiphilic star-branched copolymers are highly promising for their combinatorial delivery of genes and hydrophobic therapeutants.

1. Introduction

Accumulating evidences indicate 'one target strategy' remains suboptimal in cancer chemotherapy [1]. Recent clinical trials using combined administration of multiple-target chemotherapeutants provide promising results in glioblastomas, lung cancers and breast cancers [2,3]. Moreover, RNA-based drug development, including RNAi based strategy and miRNA-based strategy are still in its infancy. Many efforts have been devoted to siRNA-based combination therapy [4]. However, the intrinsic drawbacks of siRNA methodology, such as the off-target effects and elicitation of the interferon response, greatly hamper its therapeutic use [5].

MiRNAs are a class of short, non-coding RNAs with post-transcriptional regulatory functions [6]. "One shot, multiple targets" signature of miRNA allow it to be exempt from the problem of siRNA therapy, which might more closely resemble the action of the so-called "dirty drugs" used in the clinic today, thereby, make miRNA a better tool for gene therapy [7]. However, miRNA-based delivery still has another balk that makes it difficult to delivery therapeutic miRNA and chemo-drugs into tumor cells while keep surrounding normal cell away from inappropriate therapy [8]. It is expected the chemotherapeutic drug and miRNA should be simultaneously delivered to the same tumor cell after *in vivo* administration and, ideally, be distributed in the cells at their corresponding points to maximize intracellular cooperation [9]. In spite of the progress in liposomal [10] and silica-based [11] cationic

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nanoparticles, more attention is focused on polymer supports due to the easy synthesis of polymers, their strong stability, and their biodegradability [12].

The amphiphilic star copolymer, as a result of its unique structure and distinct physical properties, serves as a good support for genes and medication. Star polymers are three-dimensional globular or branched polymers containing multiple arms connected to a central core [13]. As compared with the linear polymers, star polymers have very high molecular weights but still possess a solubility and viscosity similar to that of linear or branched polymers of relatively low molecular weights [14]. The core cross-linked amphiphilic star copolymer has a lower critical micelle concentration (CMC), which avoids the problem of being disassemble as a result of dilution in linear amphiphilic copolymers. As the inner core of a star polymer is degradable, the star polymer micelles will release medicines through degradation when they reach the focus of the infection. Compared with dendrimers, star polymers enjoy the advantages of facile synthesis, flexible compositions, and tunable sizes (10–100 nm), which allow them to be able to carry more "cargo" within one molecule [15]. Up to now, star polymers are widely applied in the delivery of chemotherapy and genes [16,17]. However, there is no report on the simultaneous support of genes and medicine, nor is there a report on the synergic effects caused by the star polymer structure.

In this study, the amphiphilic hyper branched star macromolecules of the AB_3, $(AB_3)_2$, and $(AB_3)_3$ architecture by using PLA as a hydrophobic branch and PDMAEMA as a hydrophilic core were synthesized. The aim of this work was to investigate the feasibility of using the star branched micelles as a drug and gene co-carrier treating the glioma as shown in Scheme 1. Thus, miRNA condensation ability and physiochemical properties of star copolymers, including size and zeta potential, were characterized. Meanwhile, the cytotoxicity of star branched copolymers and gene transfection efficiency were evaluated. The endosome escape ability of DOX-loaded and miR-21i-binded polyplex was also measured. Futhermore, the suppressive effects of co-delivery of DOX and miR-21i using these nanoparticles were investigated via colony formation and flow cytometry assays in LN229 glioma cells *in vitro* as well as in subcutaneous nude mouse models.

2. Materials and methods

2.1. Materials

3-Butyn-1-ol, 2-bromo isobutyryl bromide, CuBr, sodium azide, 2,2′-Bipyridine, and 2-(N,N-dimethylaminoethyl) methacrylate (DMAEMA, 97%) were purchased from Alfa Aesar. Stannous octoate, and 3-Bromo-2,2-bis(bromomethyl) propanol were purchased from Sigma–Aldrich. Trimesoyl chloride and 1,4,7,10,10-hexamethyl triethylenetetramine were supported by J&K. All other chemicals were of reagent grade and were used as received. Doxorubicin hydrochloride was also purchased from Sigma–Aldrich. Human glioma cell lines LN229 was obtained from the China Academia Sinica cell repository (Shanghai, China). The LysoTracker Blue DND-22 was obtained from Molecular Probes (Invitrogen). The 2′-O-methyl (2′-O-Me) miR-21 inhibitor (sequences: miR-21i: 5′-GTC CAC TCT TGT CCT CAA TG-3′, scrambled sequences: 5′-AAG GCA AGC UGA CCC UGA AGU-3′) and the FITC-labeled miR-21 inhibitor were chemically synthesized by Shanghai Gene Pharma (Shanghai, China). They were then dissolved in diethylpyrocarbonate (DEPC) water and frozen at −20 °C.

2.2. Synthesis and characterization of amphiphilic star-branched copolymers

2.2.1. Synthesis of pentaerythriol triazide-terminated PLA (PLA-$(N_3)_3$)

2.2.1.1. Synthesis of pentaery thrioltriazide. 3-Bromo-2,2-bis(bromomethyl) propanol (5.0 g,15.4 mmol) and sodium azide (6.0 g, 92.3 mmol) were added into 30 mL of DMF. The solution was flushed with dry N_2 and stirred at 70 °C for three (3) days. Afterwards, the reaction mixture was filtered, and the solvent was removed under reduced pressure using a rotary evaporator. The crude product was dissolved in 50 mL of double distilled water, and then extracted with 100 mL of dichloromethane twice. The solution was dried over $CaCl_2$ and filtered, and pentaerythriol triazide was obtained after removing dichloromethane by rotary evaporation. The structure was determined by ^{1}H NMR and ^{13}C-HMR (Varian 500 MHz spectrometer, USA) (Supplementary information, Figs. S1 and S2).

2.2.1.2. Synthesis of PLA-$(N_3)_3$ by ROP. First, 6.0 g of lactide were added to a reaction tube. Next, a solution of stannous octoate and pentaerythriol triazide (mole ratio, monomer and initiator to catalyst, M/I/C = 500/1/1) in dry chloroform was added. The solvent was removed in vacuo, and the tube was sealed and immersed in a silicone oil bath at 130 °C. At the end of polymerization (12 h), the product was dissolved in a small amount of chloroform and precipitated in an excess of methanol. The purification was repeated three (3) times, and the final product was dried in a vacuum oven at 40 °C for 48 h. Then, the weight-average molecular weight (Mw) and polydispersity index (PDI) were determined by gel permeation chromatography (GPC). The elution solvent was tetrahydrofuran (THF), and the flow rate was 1 mL/min (30 °C). Calibration was based on polystyrene standards. The number average molecular weight (Mn) and the chemical structure were confirmed by ^{1}H NMR. ^{1}H NMR spectra were recorded in $CDCl_3$ with a Varian 500 MHz spectrometer instrument.

2.2.2. Synthesis of monocapped PDMAEMA with an alkynyl group (PDMAEMA-C≡CH)

2.2.2.1. Synthesis of 3-Butyn-1-ol-2-bromoisobutyrate (BOBiB) ATRP initiator. To begin, 1.0 mL (13.2 mmol) of 3-Butyn-1-ol and 1.84 mL (13.2 mmol) of triethylamine (TEA) were dissolved in 15 mL of dichloromethane. The flask was cooled in a water/ice bath. Next, 1.8 mL (14.5 mmol, 1.1 equiv) of 2-bromoiso-butyryl bromide (BiBB) was diluted with 10 mL dichloromethane and added drop-wise to the solution under nitrogen while stirring. The reaction was left to proceed for approximately 24 h. The reaction products were filtered and extracted with H_2O. The organic phase was dried over $CaCl_2$ and filtered, and the solvent was removed by rotary evaporation. The product was purified by silica gel chromatography with dichloromethane as an eluant. The structure was determined by ^{1}H NMR (Supplementary information, Fig. S3).

2.2.2.2. Synthesis of PDMAEMA-C≡CH by ATRP. Initiator BOBiB (83 mg, 0.38 mmol) was dissolved in DMAEMA (3.0 g, 19 mmol) in a glass tube equipped with a Rotaflo at ambient temperature. The catalyst CuBr (54 mg, 0.38 mmol) and ligand 1,1,4,7,10,10-Hexamethyl triethylenetetramine (1.0 g, 0.45 mmol) were added, and the mixture was degassed by three (3) vacuum/nitrogen cycles. Polymerization was carried out at 60 °C. The product was then dissolved in THF and recovered by precipitation in cold heptane. After drying to constant mass, conversion was determined by gravimetry. The copper catalyst was removed by passing a solution of the copolymer in THF through a column of basic alumina, before characterization. The molecular weight (Mn) of the polymer was calculated by comparing the integrals of the alkynyl protons and the peaks of the PDMAEMA backbone. The Mw and PDI were determined by GPC. THF was used as the eluant at a flow rate of 1 mL/min (30 °C).

2.2.3. Synthesis of star-branched PLA-b-PDMAEMA copolymers

PLA-$PDMAEMA_3$ was synthesized by direct click chemistry of PLA-N_3 and PDMAEMA-C≡CH, while (PLA-$PDMAEMA_3)_2$ and (PLA-$PDMAEMA_3)_3$ were prepared via first-coupled PLA-N_3 onto terephthaloyl chloride and trimesoyl chloride respectively, and then clicked with PDMAEMA-C≡CH (Scheme 2).

Choosing (PLA-$PDMAEMA_3)_3$ as an example (Scheme 2), 2.0 g PLA-N_3 and TEA (1.1 equiv.) was dissolved in 10 mL dry THF, and the solution was cooled in an ice/water bath. Then, 27 mg (0.1 mmol) of trimesoyl chloride was dissolved in 10 mL of THF and was added dropwise to the solution under nitrogen. The reaction mixture was continually stirred at 0 °C for 3 h and then left stirring at room temperature overnight. The white precipitate was filtered, and the filtrate was concentrated before precipitated in methanol. The crude product was redissolved in dichloromethane and precipitated in methanol three (3) times. The star PLA-$(N_3)_3$ polymer (sPLA-$(N_3)_3$) was obtained after being dried at 40 °C for 48 h in a vacuum oven. The average number of arms was calculated through ^{1}H NMR by comparing the integrals of the aromatic protons and the appropriate peaks related to the PLA backbone. The Mw and PDI of sPLA-$(N_3)_3$ were measured by GPC.

For the click coupling reaction, 1.0 g (0.051 mmol) of star PLA-$(N_3)_3$ was reacted with an excess of PDMAEMA-C≡CH (1.2 × based on NMR determined functionality) in the presence of CuBr (21.5 mg, 0.15 mmol) and 80 mg (0.51 mmol) of 2,2-dipyridyl in 10 mL of THF. After stirring at 35 °C for 12 h under nitrogen, the reaction mixture was diluted with THF and then passed through an alumina column to remove excess copper. The reaction products were concentrated before precipitation into hexanes and diethylether (V/V = 3). The precipitate collected was dissolved in THF and then submitted to dialysis against water (Cut = 10 kDa) for 48 h to remove excess PDMAEMA-C≡CH. The star-branched copolymers were obtained after freeze-drying. The synthesis was confirmed by the ^{1}H NMR measurement, and the arm number ratio of PLA to PDMAEMA was calculated from the integration ratios of the PLA proton peak and PDMAEMA proton peak.

2.3. Determination of critical micelle concentration

The critical micelle concentration (CMC) was determined by a spectrofluorophotometer (F24500, Hitachi, Japan) with pyrene as a hydrophobic probe. The