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Engineering of pH-sensitive, cross-linked micelles for drug delivery

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ABSTRACT: Drug delivery systems have been more interested in two decades because of their enormous advantages over free drugs. Especially, stimuli-responsive carrier systems such as, pH-sensitive, enzyme sensitive, temperature-sensitive, or ultrasound sensitive particles, have been the most attractive drug delivery strategy. For that purpose, I designed pH-sensitive cross-linked micelles that could be used for cancer therapy. Micelles nanoparticles were formed with triblock copolymers for this system containing of a hydrophilic PEG block, a central functionalized block, and a hydrophobic block to establish stable nano sized micelle particles. The hydrophobic block can provide room for hydrophobic drugs with the physical hydrophobic drug-hydrophobic core interaction and form stable, spherical 25-35 nm nanoparticles in the presence of the aqua system. In order to control the release rate of hydrophobic drugs (fluorescence hydrophobic dye Nile Red (NR) is preferred for the hydrophobic model drug), a pH-sensitive layer at the middle block of the micelles that embraces the hydrophobic block was constructed. Shell cross-linked (SCL) micelles are obtained upon introducing the glutaraldehyde as a cross-linker that able to react with NH_2 functional groups of the middle block of the triblock copolymer. The formed cross-link shell contains acid-labile hydrazone linkages that are cleavable in response to acidic conditions such as cellular uptake mechanism, endocytosis, and bone lacuna not in physiologic pH.

KEYWORDS: Micelles; pH-sensitive; drug delivery; nile red, cabazitaxel.

1. INTRODUCTION

In the past three decades, great efforts have been performed to enhance the delivery efficiency of anticancer therapeutics in tumor tissue. Nanomedicine tools are the major interests for drug formulations in nanoparticles. Most of these trials based on the synthesis of self-assembled amphiphilic block copolymer into core-shell micelles to develop nanocarriers, which can enhance and improve the delivery of hydrophobic drugs [1-10]. These polymeric nanocarriers have explicit huge benefits for the controlled release of the hydrophobic anticancer agents due to their ability to reduce rapid renal clearance from the blood, improvement the solubility of the hydrophobic drugs, and prolonged circulation time [11-15]. In addition, polymeric micelles with stability in blood circulation are promising to passive targeting by improving the enhanced permeability and retention (EPR) effect that led to high accumulation in the solid tumor. However, most of polymeric micelles have low stability once introduced in blood stream resulting in nonspecific release at undesirable healthy tissues [16-19]. Thus, micelles should have high stability in blood, secure the drug to reach target site, and facilitate the release of encapsulated drugs within target cells. Enhancement of the structure stability of the micelles are achieved by shell or core cross-linking to retain in thermodynamically frozen state [20-26]. Whereas, inside the targeting tissue, the cross-linker should be cleaved, and encapsulated chemotherapeutic agent is controlled-release because the cross-link can act as a barrier. Environmental pH differences between regular pH of healthy tissues and diseased tissues (acidic tumor micro-environment) or cellular compartments like endosomes (pH 5.0) and lysosomes (pH 4.0-4.5) is one of the key factors in designing of the cross-linked micelles [27-29].

In recent years, substantial studies about pH-sensitive cross-linked nanoparticles have been reported that antitumor drugs release as a function of pH [30, 31]. Most of the systems are relied on nondegradable polymer with limited biocompatibility [20-26].

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The aim of this study was to design pH-sensitive micellar nanoparticles that could load hydrophobic drugs, which would be used for antitumoral therapy in the following studies. With this perspective, this study reports that the synthesis of cross-linking micelles with a pH sensitive hydrazone linkage to achieve a powerful, biocompatible, and unique micelles that can release Nile Red (NR) as a model drug in response to acidic endosomal pH.

Micelle nanoparticles were formed with tri-block copolymers consisting poly (ethylene glycol) (PEG), a derivative of poly (acrylic acid) (PAA), and poly (methyl methacrylate) (PMMA) approved by FDA in the United States. Thus, the component of triblock copolymers is able to construct biocompatible micelles for nanomedicine uses. In addition, each block of the triblock copolymer has unique properties for the micelle formulation. For example, PEG, is one of the most investigated biocompatible polymers because of its high flexibility, high water solubility, low protein adsorption, low cell adhesion and to enhance EPR effect [32-35]. The middle block was Boc-protected PAA was hydrolyzed in TFA and leading to gain NH_2 groups, which could be reacted with glutaraldehyde to form pH-sensitive hydrazone linkages. Lastly, the core block was formed by hydrophobic PMMA, which was mediated to trigger micellization process in aqua environment and encapsulated hydrophobic drug in the core. Moreover, the cross-linked micelles are gained two functional vital advantages, such as increasing the micellar stability against the destabilizing condition and protection from extracellular release in physiologic environment (pH 7.4) over non-crosslinked or other polymeric nanocarriers. After particles are internalized by the cells, the cleavage of the hydrazone cross-linker is promoted by an acidic endosomal pH, which intracellularly triggers release of the loaded drugs.

In this study, I showed that synthesis of triblock amphiphilic copolymers, which were conjugated to each other's with a sequential "click" reactions and formation of hydrophobic drug encapsulated micelle nanoparticles in aqua system. Furthermore, the synthesis of cross-linked micelles (CLMs) and the cross-linking effect on micellar strength was also investigated at endosomal and physiologic pH values.

2. RESULTS AND DISCUSSION

In here, pH-sensitive cross-linked micelle nanoparticles were developed. Each segment of triblock copolymer was synthesized and coupled to each other via sequential "click" reactions. First, commercially available mono hydroxyl end functional PEG was reacted with 4-pentynoic acid to give alkyne functional PEG block (alkyne-PEG) (*1*) for a subsequent azide-alkyne "click" reaction. The conjugation efficiency was > 95% according to ^{1}H NMR. Second, poly(*tert*-butyl acrylate) (P*t*BA) (*3*) as a precursor of cross-linkable block was synthesized by atom transfer radical polymerization (ATRP) aiming 25 repeating *tert*-butyl groups which could easily be hydrolyzed into acid groups that can be functionalized for shell cross-linked after self-assembly of block copolymer into micelles to protect burst release of the drug. It was aimed to have two sequential "click" reactions. Thus, the middle block (Anthracene poly(*tert*-butyl acrylate)-Br, (Anth-P*t*BA-Br)) was produced as double side functionalities with bromine transferred to azide for azide-alkyne "click" reaction and DA "click" reaction, respectively. Moreover, poly (methyl methacrylate) (PMMA) (*8*) as hydrophobic block was synthesized using protected maleimide functional initiator via ATRP. The numbers of MMA units were calculated by following the ratio of total methyl protons of MMA to the initiator protons as 55 units from the ^{1}H NMR spectra. The numbers of MMA units are enough to self-assembly of triblock polymers to form efficiently micelles.

After well-characterization of each block, they were conjugated to each other with two different sequential "click" reactions as seen in Figure 1. First, alkyne-PEG and Ant-P*t*BA-Azide were coupled to each other with CuBr/PMDETA at room temperature by following copper catalyst azide-alkyne cycloaddition (CuCAAC) reaction (*9*). Successful coupling was confirmed with appearance of triazole ring proton in ^{1}H NMR spectrum which also confirmed anthracene groups still exist as polymer end groups for following DA "click" reaction. Moreover, this reaction was monitored with Fourier transform infrared spectroscopy (FTIR) that did not exhibit any azide/alkyne peak, which shows solely existence of block copolymer (Anth-P*t*BA-*b*-PEG) without any precursor. After perfectly characterized Anth-P*t*BA-*b*-PEG copolymer, DA coupling reaction was set up at 110 ^{o}C in toluene for 48 h. Based on the ^{1}H NMR analysis aromatic protons of anthracene in the range of 7.4-8.6 ppm were totally disappeared, whereas bridgehead protons of cycloadduct were formed at 4.7 ppm and the cycloadduct related methylene protons were also seen in the region of 5.2-5.6 ppm. All these results showed that PEG-*b*-P*t*BA-*b*-PMMA copolymer (*10*) was successfully acquired. Further, gel permeation chromatography (GPC) system was also preferred to evaluate molecular weight distribution and molecular weight of individual blocks and copolymers, as well. After each click reaction, the molecular weights of related block copolymers were shift to lower elution volume, which indicates molecular weight increase (Figure 2). Narrow polydispersity of the curves without a tail indicate that block copolymers were obtained without any impurities such us their precursors.

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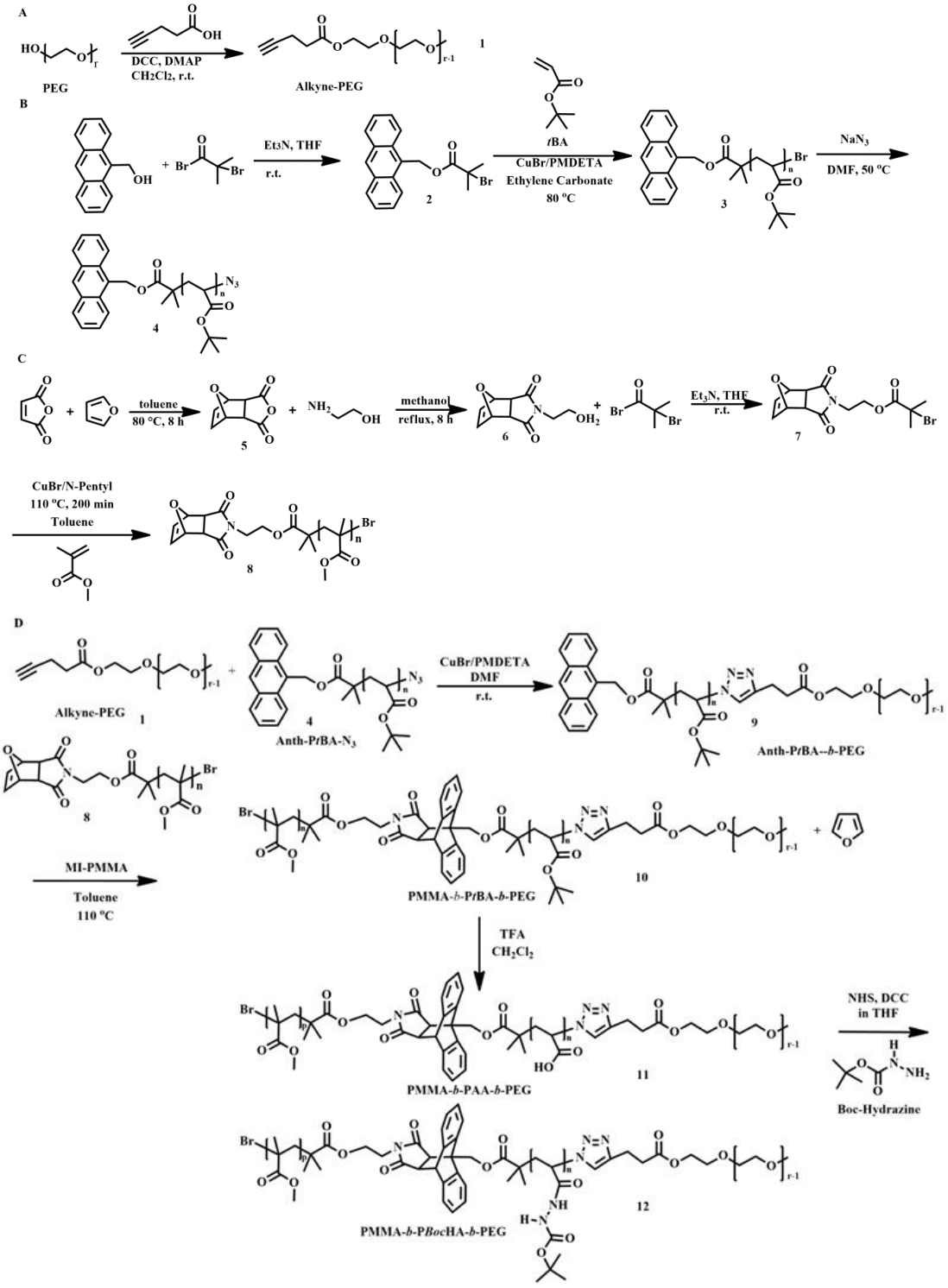

Figure 1. Synthesis of tri-block copolymer PEG-*b*-PAH-*b*-PMMA.

Finally, to obtain desired triblock copolymer (*11*), *tert*-butyl groups of P*t*BA were effectively hydrolyzed by using trifluoroacetic acid (TFA). To monitor the success of reaction ^{1}H NMR was preferred as indicator of entire reduction on methyl protons of *tert*-butyl groups at 1.43 ppm for PEG-*b*-P*t*BA-*b*-PMMA copolymers. Furthermore, acid groups of obtained PEG-*b*-PAA-*b*-PMMA were reacted with *Boc* protected hydrazine (*tert-butyl* carbazate) (*12*) and efficient functionalization was confirmed by regain of *tert*-butyl protons of *Boc* at 1.43 ppm. This reaction was followed by hydrolysis of *Boc* groups using TFA to be able to obtain acyl hydrazide functionality (*13*), which can react with glutaraldehyde to form acid sensitive hydrazone linkage. In this case reaction efficiency was calculates as 80% based on ^{1}H NMR spectrum. The characteristics of all block copolymers and their precursors were summarized in the Table 1.

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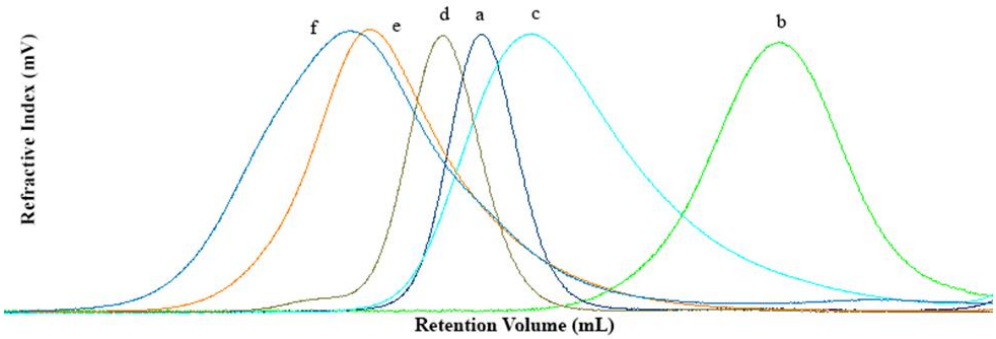

Figure 2. GPC traces of precursors and tri-block copolymer determined based on PMMA standard.

Table 1. Characteristics of PEG-b-PAH-b-PMMA, and its precursors.

GPC Name	Code	Conv.[d] (%)	# of Repeating Units[e]	M_{nNMR}[e] (g/mol)	M_{nGPC}[f] (g/mol)	M_w/M_n[f]
a	Alkyne-PEG_{5000}[a]	93	113	5,100	9,400	1.02
b	Anth-P*t*BA-Br[b]	10	25	3,625	3,347	1.20
c	MI-PMMA[c]	15	52	5,565	4,543	1.43
d	PEG-*b*-P*t*BA-Anth			8,725	12,231	1.05
e	PEG-*b*-P*t*BA-*b*-PMMA			14,290	14,850	1.22
f	PEG-*b*-PAA-*b*-PMMA			12,815	14,225	1.22
-	PEG-*b*-PBocHA-*b*-PMMA			14,960	8,206	1.35
-	PEG-*b*-PAH-*b*-PMMA			13,290	-	-

[a] Synthesized by esterification using DCC/DMAP in CH_2Cl_2 at room temperature.
[b] [M]/[I]/[CuBr]/[PMDETA]=100/1/1/1, T=80°C, Time: 240 min.
[c] [M]/[I]/[CuBr]/[PMDETA]=50/1/1/1, T=40°C, Time: 205 min.
[d] Determined by gravimetrically.
[e] Calculated by ^{1}H NMR Spectra.
[f] Determined with GPC based on PMMA standard, THF as an eluent at 35 °C.

Micelles were prepared from the amphiphilic triblock copolymer using dialysis method. PEG-*b*-PAH-*b*-PMMA amphiphilic block copolymer was dissolved in THF and drop by drop the triblock polymer solution was added to Millipore water under sonication as illustrated in Figure 3. Afterwards, the polymer mixture in water was placed in a dialysis membrane (MWCO 1 KDa) to dialyze against water, which allowed self-assembly of amphiphilic block copolymer. Micelles formation was monitored by dynamic light scattering (DLS) measurements showing that these empty non-shell cross-linked micelles had average hydrodynamic size around 25 nm. Furthermore, NR probe was examined to determine whether or not the triblock amphiphilic copolymer formed micelles by encapsulating the probe in the core of micelles in aqua environment. As can be seen in Figure 4, the fluorescence intensity of NR in water or at low micelle concentrations results in relatively low intensity. However, with increasing micelle concentration, the dye settles in hydrophobic core, which leads to dramatically increase on the fluorescence intensity. The solutions were excited at 550 nm and where the fluorescence emission reads at 620 nm demonstrate noticeable increase in emission intensity as increasing concentration of the micelles. At the low concentrations, the weak emission intensity indicates that NR is in the water. On the other hand, the intensity is rapidly increased at high concentration levels. The observed inflection point corresponds to a critical micelle concentration (CMC) which calculated from the intersection of two lines, base line, and the tangent line of the rapid rising curve. Based on this intersection the CMC value was found about 13.9 µg/mL.

NR was preferred as the model drug and loaded into the polymeric micelles with dialysis method. The model drug encapsulation efficiency was above 80% with 8% drug loading content ratio as the NR feeding amount was 10%. After NR encapsulation, the size of NR loaded non-shell cross-linked micelle did not significantly change; it was around 25 nm again (Figure 5a). The critical concern about non-shell cross-linked micelles is lack of the stability of the micelle in aqua environment at low concentration even the micelles have low CMC value. This leads to dissociation of the micelles and releasing of the therapeutic reagent before reaching to targeted part. SCL micelles were obtained upon introducing of the glutaraldehyde as a cross-linker that able to react with NH_2 functional groups of the middle block of the triblock copolymer. The formed cross-

link shell contains acid labile hydrazone linkages that are cleavable in response to acidic conditions. The obtained pH label SCL micelles were then characterized by DLS and *in vitro* drug release test. The average hydrodynamic sizes of SCL micelles with and without NR were slightly increased to 33.53 and 28.22 nm, respectively (Figure 5a). Further, the TEM image of non-shell cross-linked particles were seen in Figure 5b, which is closely agreement with DSL results. The size of the particles were around 25-50 nm ranges. Larger particles were more likely aggregated particle forms.

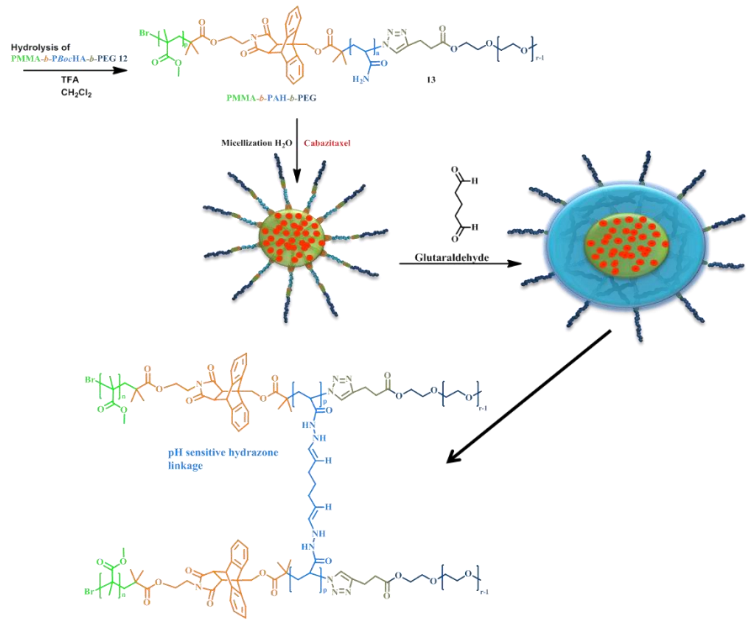

Figure 3. Illustration of micellization and cross-linking with CTX loading.

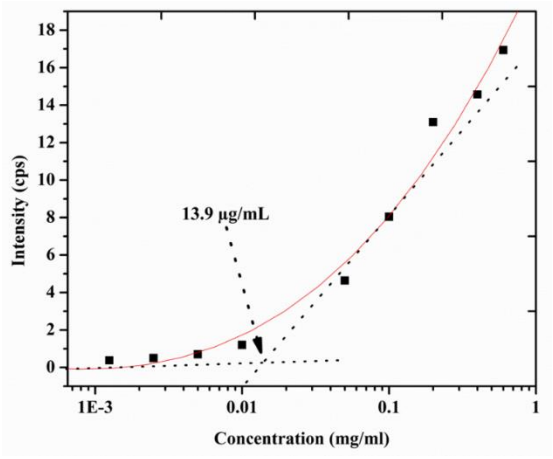

Figure 4. Calculation of CMC of the tri-block polymer.

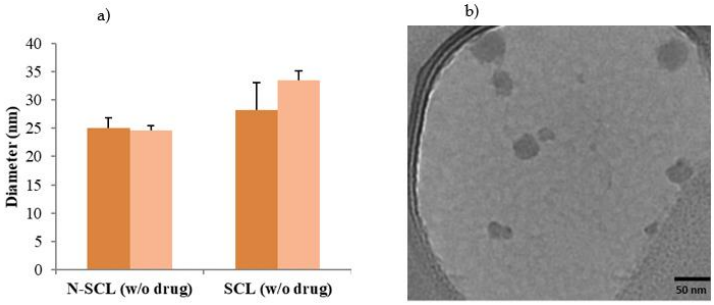

Figure 5. a) Micelle Sizes w/o Nile Red (model drug encapsulated) b) TEM image of the non-shell cross-linked micelles, scale bar indicates 50 nm.

As seen in Figure 6, for all micellar systems, the zeta potential of the micelles was neutral, which was enough to maintain their stability in physiological system. Furthermore, NR release profile of polymeric micelles as a response of pH was investigated using a dialysis cassette (MWCO 3500 Da) immersed in phosphate buffer solutions adjusted pH 7.4 and 5.0 within 32 h by comparing with non-shell-cross-linked micelles at physiological pH within same time frame. Since the micelles internalize into cells via endocytosis and the pH drops from neutral to the pH 5.0, this releasing experiment is necessary to investigate the pH response of cross-linked shell, which will directly affect the control release of drug. The concentration of triblock copolymer was much higher than CMC value. The release of physically entrapped NR model drug was significantly arrested in SCL micelles at pH 7.4 with minimal burst release, as compared to non-shell cross-linked micelles under the same conditions. Within 24 h, non-shell cross-linked micelles released almost 50% of loaded NR, while only less than 10% NR was leaked from SCL micelles at pH 7.4, 37 ºC. Moreover, the released percent of NR from SCL micelles under acidic conditions reached to 37% that was more than pH 7.4 due to the pH label possession of the cross-link shell. This release profiles indicated that cross-linked shell led to a noticeable decrease of NR release rate under normal physiologic conditions for the first 24 h but in acidic pH the loaded drug swiftly released from the core of the micelles comparing to pH 7.4 as seen in **Figure 7**. The cross-linked shell layer could behave like a diffusion barrier for drug release. Thus, cross-linked micelles exhibited a higher retention time of the drug.

After completing model drug loaded micelle and release characterization, we encapsulated a real cancer therapeutic agent, Cabazitaxel (CTX) and similarly analyzed CTX loaded micelle system as described in previous section. The loading efficiency of CTX was around 55% with 10% loading content rate. The size of CTX loaded SCL micelle was around 30.05 nm with similar dimension range of previous micellar systems (data not shown).

3. CONCLUSION

All in all, the results indicate that the amphiphilic triblock copolymer, PMMA-*b*-PAH-*b*-PEG successfully synthesized by sequential "click" reactions. Under the aqua condition, the triblock copolymers could form micelles, which can encapsulate hydrophobic molecules such as NR and CTX and having a pH-sensitive shell in the middle of the particles when introducing glutaraldehyde. The formed pH-sensitive hydrazone linkage could control the release rate of the encapsulated NR, model drug as a function of pH-level of the environment and time. Thus, the findings of the study extensively fit with the hypothesis. This pH-sensitive micelle formulation with "smart" nanoparticles phenomena could be used in cancer treatment. This study should be carried out for further research of *in vitro* and *in vivo* application studies in related to cancer model in the future.

4. MATERIALS AND METHODS

4.1. Materials

Methyl methacrylate (MMA, 99 %, Aldrich), tertiary-butylacrylate (*t*BA, 99%, Aldrich) and Styrene (St, 99%, Aldrich) were passed from a column containing basic alumina. Triethyl amine (TEA, 99.5%, Fluka), 2-bromoisobutyryl bromide (97%, Aldrich), propargyl alcohol (99%, Aldrich), 2,2′-bipyridine (99%, Aldrich), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl, Aldrich), 2-(N-morpholino)ethanesulfonic acid (MES, Aldrich), 4-dimethylaminopyridine (DMAP, Aldrich), CuBr (98%, Acros), CuCl (98%, Acros), Sodium azide (97%, Carlo-erba), ethylene glycol (95%, Aldrich), Poly(ethylene glycol) monomethylether (PEG, M_n: 5000 g/mol), resazurin sodium salt (Aldrich) were used as received. Dichloromethane (99.8%, J.T. Baker) was dried with P_2O_5. *N, N, N′, N″, N‴* – Pentametyldiethylenetriamine (PMDETA, 99%, Aldrich) was distilled before used as a ligand. Sodium azide (NaN_3) and trifluoroacetic acid (TFA, 99%) were purchased from Acros Chemicals (New Jersey, NJ). Cabazitaxel (CTX) was a gift from Sanofi-Aventis US LLC.

4.2. Characterization

^{1}H NMR spectra of 5–10 % (w/w) solutions in $CDCl_3$ with $Si(CH_3)_4$ as an internal standard were recorded at room temperature at 400 MHz or 500 MHz, respectively, on a Varian Mercury system (Palo Alto, CA). GPC measurements were acquired with an Autosampler system of Viscotek GPCmax involving of Waters refractive index detector and a pump. The molecular weight distribution and molecular weight of the final individual polymers and copolymers were analyzed based on the elution volume on an Styragel HR 4E column calibrated with a series of PMMA standards (PolyAnalitik Inc, Canada) using THF solvent system at

a flow rate of 1 ml/min, at 35 °C. The obtained GPC data were analyzed using Viscotek OmniSEC Omni-01 software. Further, a Perkin-Elmer FT-IR Spectrum 4100 was used to take FT-IR spectra.

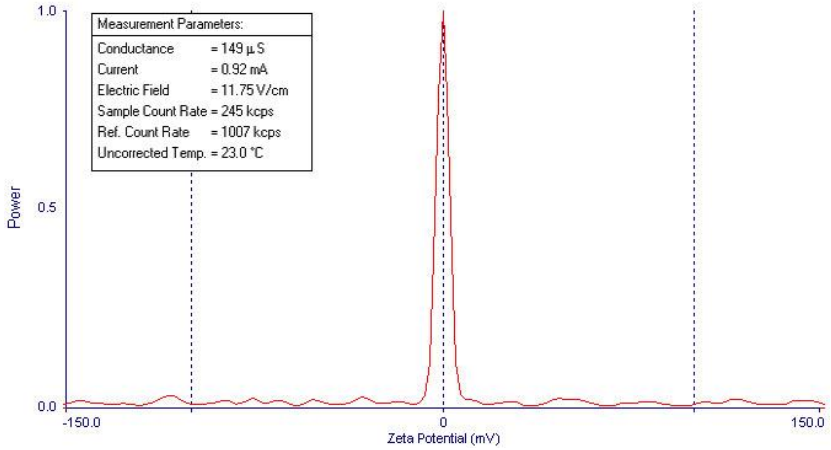

Figure 6. Evidence of zero zeta potential.

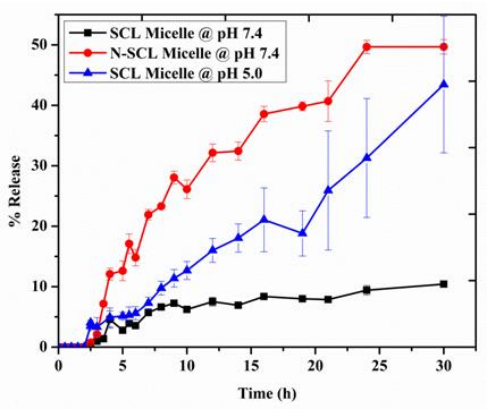

Figure 7. NR release profile from non-cross-linked and cross-linked micelles in the solution pH 7.4 and pH 5.0.

4.3. Tri-block copolymer synthesis

4.3.1. Synthesis of alkyne functional PEG (1)

PEG (M_n:5000 g/mol, 3.0 g, 6 x 10^{-4} mol) was dissolved in 25 mL of dichloromethane. After that, DMAP (0.073 g, 6 x 10^{-4} mol) and 4-Pentynoic acid (0.088 g, 9.0 x 10^{-4} mol) were successively added to the PEG solution. After the reaction mixed well for 5 min at room temperature, a solution of N,N'-Dicyclohexylcarbodiimide (0.135 g, 6.6 x 10^{-4} mol) in 15 mL of dichloromethane was added to the solution and stirred overnight at room temperature. After filtration of the precipitated salt in the reaction, the solution was concentrated with a rotary evaporator, and the solution containing alkyne-PEG was precipitated in diethyl ether three times and filtered after each precipitation.

4.3.2. Synthesis of 9-anthyrylmethyl 2-bromo-2-methyl propanoate (2)

9-anthyrylmethyl 2-bromo-2-methyl propanoate was synthesized by the direction of the following literatures [36]. DMAP (0.350 g, 2.88 mmol) and 9-Anthracene methanol (3.00 g, 14.36 mmol) were dissolved in 100 mL of dichloromethane, and triethylamine (1.2 mL, 17.2 mmol) was introduced to the solution. The reaction mixture temperature was set to 0 °C within an ice-bath. After that, 2-bromo isobutyryl bromide (1.82 g, 7.89 mmol) was added drop-by-drop within half an hour to this solution, then stirred for 15 min in the ice-bath. The reaction was then kept for overnight at the room temperature. The ammonium salt was removed by a paper filtration and the reaction solvent was evaporated by a rotary evaporator. The obtained residue was extracted with dichloromethane/water that saturated with sodium bicarbonate. The aqueous phase re-extracted with dichloromethane, and then combined all organic phases were dried over sodium sulfate. Afterwards, the solution was concentrated, and the crude product was purified by column chromatography

over silica gel eluting with hexane/EtOAc (10:1) to give **5** as yellow solid. ^{1}H NMR ($CDCl_3$, δ) 8.51 (s, 1H, ArH of anthracene), 8.33 (d, J = 8.7 Hz, 2H, ArH of anthracene), 8.03 (d, J = 8.2 Hz, 2H, ArH of anthracene), 7.60-7.45 (m, 4H, ArH of anthracene), 6.21 (s, 2H, CH_2-anthracene), 1.86 (s, 6H, $C(CH_3)_2$-Br).

4.3.3. Synthesis of anthracene functional poly(t-butyl acrylate) (3)

Anthracene functional initiator (0.122, 3.41 x 10^{-4} mol), PMDETA (71.2 µL, 3.41 x 10^{-4} mol), , *t*-BA as a monomer (5 mL, 3.41 x 10^{-2} mol), CuBr (48.9 mg, 3.41 x 10^{-4} mol), and ethylene carbonate (10% w/w, 0.46 g) were kept in a round-bottom flask under the argon atmosphere. The gas inside the mixture was removed by three freeze–pump–thaw cycles and kept under the argon at 80 °C for 50 min. End of the reaction period, THF was added to the reaction mixture to terminate it. To remove the copper complex, the reaction solution in THF was passed through a neutral alumina column. After collecting all reaction products and materials in THF, the excess THF was removed by rotary evaporation. To purify and get the polymer, the mixture was precipitated in cold water/ methanol (20/80 v/v). After decantation, the polymer was dissolved in dichloromethane, extracted against water. The water phase was re-extracted with dichloromethane. At the end, organic phases were collected and was dried within sodium sulfate. Finally, the solvent was evaporated to obtain Anth-P*t*BA polymer.

4.3.4. Synthesis of azide functional anthracene functional poly(tert-butyl acrylate)(4)

20 equivalents of NaN_3 (0.393 g, 6 x 10^{-3} mol) and Anth-P*t*BA (883 mg, 3 x 10^{-4} mol) were dissolved in DMF and stirred at 50 °C for overnight. After that the polymer was dissolved in dichloromethane, extracted against water and the water phase was extracted with dichloromethane again. Sodium sulfate was added to the collected organic phase to remove water residue. Lastly, the solvent was evaporated to give anth-P*t*BA-N_3.

4.3.5. Synthesis of 2-bromo-2-methyl-propionic acid 2-(3,5-dioxo-10-oxa-4 azatricyclo [5.2.1.02,6]dec-8-en-4-yl) ethyl ester (7)

The initiator, having protected-maleimide group was synthesized by following literature [37]. Maleic anhydride (10.0 g, 0.1 mol) was dissolved in 30 mL of toluene and the mixture was heated up to 80 °C. Following furan (11.3 mL, 0.15 mol) addition with a syringe, the solution was stirred for overnight. The mixture was then cooled down to room temperature. After white solids were precipitated, they were collected by filtration by following two times wash with 20 mL of diethyl ether afforded as white needless. ^{1}H NMR ($CDCl_3$, δ) 6.57 (s, 2H, CH=CH, bridge protons), 5.45 (s, 2H, -CHO, bridge-head protons), 3.17 (s, 2H, CH-CH, bridge protons).

The adduct 5 (10.0 g, 60.0 mmol) was suspended in 150 mL of methanol and the mixture temperature was cooled down to 0 °C with an ice-bath. 3.6 mL of ethanolamine in 30 mL of methanol was added drop-by-drop for 15 min to the reaction mixture, and then the resulting solution was stirred for 5 min at 0 °C and stirred additional 30 min at ambient temperature. The end solution was refluxed for 8 h. After cooling the mixture to room temperature, methanol was removed with a rotary evaporator, and the residue was dissolved in 150 mL of dichloromethane and washed three times with 100 mL of water. The organic layer was collected, dried over sodium sulfate, and filtered. The chloroform was removed again with a rotary evaporator and a white-color solid product was obtained and further purified by flash chromatography eluting with ethyl acetate to get the final product (5) as a white solid. ^{1}H NMR ($CDCl_3$, δ) 6.47 (s, 2H, CH=CH, bridge protons), 5.23 (s, 2H, -CHO, bridge-head protons), 3.72-3.66 (m, 4H, NCH_2CH_2OH), 2.84 (s, 2H, CH-CH, bridge protons).

Et_3N (1.44 mL, 10.54 mmol) and adduct 6 (2.0 g, 9.55 mmol) in 120 mL of THF were inserted into a a round-bottom flask. The reaction temperature was cooled down to 0 °C, and a solution of 2-bromo isobutyryl bromide (2.34 g, 10.0 mmol) in 15 mL of THF was introduced drop-by-drop for half an hour to the mixture. Afterwards, the mixture was stirred for 4 h in an ice-bath and then kept and stirred at room temperature for overnight. The ammonium salt was separated by filtering and excess THF was evaporated with a rotary evaporator. The final residue was a pale-yellow and further purified by column chromatography over silica gel eluting with ethyl acetate/hexane (1:4) to get a white solid compound. ^{1}H NMR ($CDCl_3$, δ) 6.47 (s, 2H, CH=CH, bridge protons), 5.22 (s, 2H, -CHO, bridge-head protons), 4.28 (t, J = 5.2 Hz, 2H, NCH_2CH_2OC=O), 3.77 (t, J =5.2 Hz, 2H, NCH_2CH_2OC=O), 2.83 (s, 2H, CH-CH, bridge protons), 1.85 (s, 6H, $C(CH_3)_2$-Br).

4.3.6. Synthesis of protected maleimide functional poly (MMA) (8)

As a monomer MMA (1.5 mL, 1.4 x 10^{-2} mol), protected maleimide functional initiator (7) (83.3, 2.23 x 10^{-4} mmol), N-(n-Pentyl)-2-pyridylmethanimine (86.5µL, 4.68 x 10^{-4} mol), CuBr (33.5 mg, 2.33 x 10^{-4} mol), and

2.5 mL of toluene were placed in a Schlenk tube under argon. The reaction temperature was set at 40 °C in an oil bath and stirred for 205 min. End of the time, the reaction was terminated with THF and passed through an alumina column to remove CuBr and salts. The polymer was precipitated in a 10-fold excess volume of cold methanol. At the end, the solid product was collected with centrifugation at 1000 rpm for 5 min.

4 3.7. Synthesis of diblock (PEG-b-PtBA-An) (9)

In a Schlenk tube, azide functional anthracene terminated P*t*BA (1.5 eq, An-P*t*BA-N_3; 2.36 x 10^{-4} mol) was dissolved in 10 mL of DMF. Then, PEG_{5000}-Alkyne (788 mg, 0.016 mmol), CuBr (0.035 g, 2.36 x 10^{-4} mol), and PMDETA (35 µL, 2.36 x 10^{-4} mol) were added to the solution. To remove gas from the reaction, 3 times freeze-pump-thaw cycles were carried out, then the reaction was stirred at ambient temperature for 12h. End of the time, the reaction was terminated with THF and passed through an alumina column to eliminate CuBr and salts. THF solvent was removed under reduced pressure. The mixture was first precipitated in diethylether and then collected with centrifugation at 1000 rpm for 10 min.

4.3.8. Synthesis of triblock copolymer, PEG-b-PtBA-b-PMMA (10)

PEG-*b*-P*t*BA-An (877 mg, 11.63 x 10^{-5} mol) and 0.725 mg of Ma-PMMA (16.61 x 10^{-5} mol) were reacted in 20 ml of toluene at 110 °C for 48 hours via DA "click" reaction. The reaction was terminated, it was precipitated first in diethyl ether and then cold methanol. Precipitated copolymers were centrifuged to collect at 3000 rpm 5 min.

4.4. Formulation of acid sensitive micelles

4.4.1. Synthesis of PEG-b-PAA-b-PMMA (11)

PEG-*b*-P*t*BA-*b*-PMMA copolymer was dissolved in CH_2Cl_2, then 0.41 ml of trifluoroacetic acid (TFA) was mixed in ice-bath and kept for 30 minutes in it. It was kept at overnight in room temperature.

4.4.2. Boc-Hydrazine coupling to PEG-b-PAA-b-PMMA (12)

The PEG-b-PAA-b-SA triblock copolymers (100 mg, 9.34 x 10^{-6} mol) were dissolved in 20 mL of THF and 1.5 equivalent of NHS and DCC were introduced into the solution under Ar atmosphere and stirred for 1 h. 50 equivalent of Boc-Hydrazine was included to the mixture and kept more than 2 h at room temperature. To remove unreacted NHS of the tri-block copolymer, 100 equivalent excess of unreacted NHS was reacted with ethanol amine (4.21 x 10^{-3} mol, 506 µl) in 5 ml of THF overnight.

4.4.3. Hydrolysis of Boc protection from Boc-Hydrazine coupled PEG-b-PAA-b-PMMA (PMMA-b-PAH-b-PEG) (13)

The same strategy used in synthesis of PEG-b-PAA-b-PMMA was followed to hydrolysis of Boc groups.

4.4 4. Preparation of noncross-linked micelles

The PMMA-*b*-PAH-*b*-PEG triblock copolymers (5 mg) were dissolved in 5 mL of DMSO / THF mixture (1:9), placed in a semi-permeable dialysis membrane (Mw cut-off: 1000 Da), and dialyzed against Millipore water for 24 h. The water was changed in defined time intervals. The polymeric micelles obtained were then lyophilized.

4.4.5. Preparation of cross-linked micelles

The cross-linked micelles were obtained by the reaction between the amine groups in the middle block of tri-block copolymer and glutaraldehyde. 9.82 x 10^{-6} mol of NH_2 functional tri-block polymer was reacted with 4.91 x 10^{-6} mol of glutaraldehyde during micellization as in the step of preparation of noncrosslinked-micelles.

4.5. Encapsulation and release of model drug nile red (NR)

NR dye stock solution was dissolved and stirred overnight (4.8 mg of NR in 1.8 mL of THF and 0.2 mL of DMSO). From the stock solution taken 500 µL of NR and added into the tri-block copolymer solution (4 mg/ml in THF/DMSO; 9:1). The mixture was added into 2 ml of Millipore water by dropwise and stirred for 1 h. It was then dialyzed by a membrane with MWCO of 1 KDa for 12 h to obtain micelle solution. After the micelle solution was lyophilized, it was resuspended in 4 ml of PBS at pH 7.4 and divided two sample solutions, one of which was used for model drug release; the other one was used to determine drug loading content and efficiency calculations based on the equations 1 and 2. 3 mL of NR loaded micelle solution was

transferred into dialysis cassette (MWCO: 3500 Da) and placed the dialysis bag into 150 mL of release medium (the buffer solution pH 7.4) The release medium was stirred at 150 rpm at 37 ºC and at the predetermined time intervals triplicates of 100 µL of the micelle solution will be drawn and measured UV-Abs at 550 nm. Then, the sample solutions were returned to dialysis cassette and the buffer solution medium (300 mL) was replaced with an equal volume of the fresh the buffer solution medium preheated to 37ºC. The amount of released NR was determined by the decay in absorbance and fluorescence of the micelle solution in the cassette. The same model drug release strategy was applied on cross-linked micelles [38].

$$\text{Loading content (wt \%)} = \frac{Amount\ of\ the\ drug\ in\ the\ particles}{Total\ weight\ of\ the\ particles}\ x\ 100\ \% \quad \text{(Eq. 1)}$$

$$\text{Loading efficiency (\%)} = \frac{Amount\ of\ the\ drug\ in\ the\ particles}{Total\ amount\ of\ drug\ used\ in\ the\ preperation\ of\ particles}\ x\ 100\ \% \quad \text{(Eq. 2)}$$

4.6. Particle characterization with DLS and TEM

The prepared particles were dissolved in deionized water at a polymer concentration of 0.5 mg/mL. The size and zeta potential of each particles were measured using a 90Plus particle size analyzer with ZetaPALS capability (Brookhaven Instruments Corporation, NY, USA). Particle formulation were investigated in triplicate. To calculate size distribution of the particles the geometric mean of each measurement was calculated, and results are reported as the average of geometric mean with the standard error of the mean.

The shape and structure of the non-shell-cross-linked particles were investigated with the JEOL 3011 high resolution electron microscope. The particles were prepared in deionized water at a polymer concentration of 0.5 mg/mL. After 10 µl of the solution was placed on a 400-mesh grid (Ted Pella Inc., CA, USA), 25,000X magnification of the images were taken under an accelerating voltage of 300 kV.

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