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COMMUNICATION

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We report a new crosslinked polymersome with pH-responsive swelling properties through acidic hydrolysis of hydrophobic contents from the amphiphilic polymer chains. The unique stability at physiological condition and large swelling capability under low pH condition give this polymersome promising potential for anticancer drug delivery.

Delivery of anticancer drugs in a carrier could minimize damage to healthy tissues, prolong drug circulation time, and selectively accumulate drugs in tumors through enhanced permeability and retention (EPR) effect.[1] Various carrier systems such as liposomes,[2] quantum dots,[3] and self-assembled nanoparticles or vesicles,[4] have been reported in recent years. Amphiphilic polymers containing hydrophobic and hydrophilic segments can be readily self assembled into nano-sized vesicles in aqueous solution. As one type of important self-assembled vesicles, polymersomes with stabilized structures have special capability in encapsulating guest molecules into their empty core domains and thus possess great potential for drug delivery.[5] As compared to liposomes, polymersomes are reported to have better mechanical strength, colloidal stability and lower drug leakage thus are emerging as superior alternatives to liposomes.[6] At the same time, all drug carrier systems, no matter if they are liposomes or polymersomes, are always associated with a practical challenge, i.e., inferior *in vivo* stability.[7] Self assembled particles were reported to be easily eliminated from circulation due to *in vivo* disintegration.[8] Hence, an intensified covalent or noncovalent bonding that can resist the physiological destabilisation forces, is highly desired for carriers aiming at sustained *in vivo* drug delivery. In recent years, several studies have incorporated cross-linkable properties to the carriers for improving stabilities.[9] The crosslinking could occur in either the hydrophilic shell, the hydrophobic core, or the core-shell interface, and various methods including photo irradiation or chemical reactions can be applied for the crosslinking.[10]

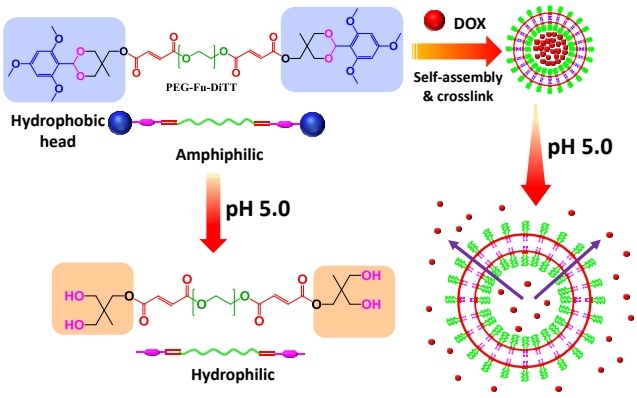

Fig. 1 Schematic demonstration of the pH responsive expansile PEG-Fu-DiTT polymersome system.

Multiple stimuli, e.g., pH, temperature, light, enzyme and oxidation/reduction, were studied for triggering drug release from nanoparticles in the past decades.[11] To utilize these stimuli, a large variety of responsive bonds, such as acetal, orthoester and disulfide-linkers, were designed and incorporated into the nanoparticle.[12] Upon the application of stimuli, the responsive bonds break or change properties and lead to the disassembly or expansion of the particles, resulting in the release of encapsulated drugs.[13] Among these triggers, pH-responsive property is one of the most convenient and frequently selected characteristics in designing delivery system for tumor targeted drug delivery due to the lower pH profile in cancerous tissues and in lysosomes after cellular endocytosis.[13] In this study, we report a new crosslinked polymersome system with pH-sensitive drug release capability. The key design feature for these polymersomes lies in the hydrophobic to hydrophilic transformation in response to low pH condition with subsequent size swelling. The polymer chain is synthesized to have both hydrophobic and hydrophilic segments thus can be readily self-assembled into polymersomes and crosslinked to prevent disintegration. However, the protecting group is cleaved upon

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exposure to lower pH of ~5, transforming the polymersomes into fully hydrophilic particles similar to hydrogels. As such, water penetrates to dissolve the hydrophilic segments, which in turn causes swelling of the hydrogel particle and releasing of the encapsulant in the core. Advantages of this expansile polymersome system includes: 1) capable of encapsulating hydrophilic drugs such as doxorubicin (DOX); 2) good extracellular stability through internally crosslinking the polymersomes at the core-shell interface; 3) potent drug release upon exposure to the acidic environment in endosomes/lysosomes after endocytosis; 4) average size around 100 nm thus utilizing the EPR effect for selective accumulation in tumors.

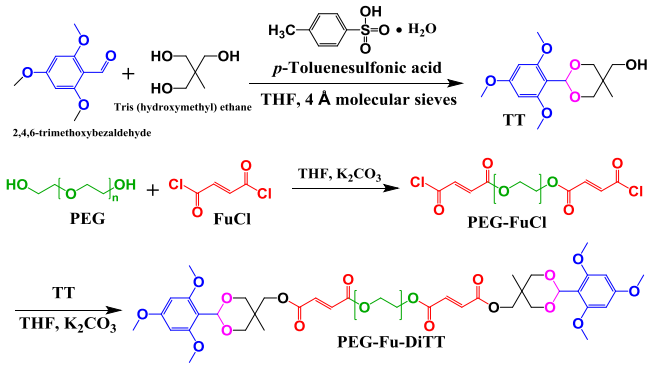

Fig. 2 Synthesis route of crosslinkable PEG-Fu-DiTT polymer using TT monomer and PEG chains.

The hollow polymersomes were prepared by self-assembly of poly(ethylene glycol)-Fumarate-Di-2,4,6-trimethoxybezylidene-1,1,1-tris(hydroxymethyl) ethane (PEG-Fu-DiTT) in 0.3% polyvinyl Alcohol (PVA) aqueous solution. The amphiphilic PEG-Fu-DiTT polymer was synthesized by linking hydrophilic PEG chain with hydrophobic 2,4,6-trimethoxybezylidene-1,1,1-tris(hydroxymethyl) ethane (TT) chains using fumarate chloride (see Supporting Information for details). Specifically, TT monomers were synthesized from 1,1,1-tris(hydroxymethyl)ethane and 2,4,6-trimethoxybenzaldehyde in tetrahydrofuran (THF) and purified using pH 8.0 Tris buffer (Fig. 2). The chemical shifts determined using ^{1}H NMR showed peaks corresponding to all functional groups in the monomer (Fig. S1), consistent with previous reports.[14] Crosslinkable PEG-Fu-DiTT polymer was synthesized by linking the terminal hydroxyl groups on PEG and TT using fumaryl chloride. PEG600 purchased from Sigma has an average M_n of 540 g mol^{-1}. After reaction, the purified PEG-Fu-DiTT polymer was determined to have M_n = 1380 g mol^{-1}, M_w = 1710 g mol^{-1} and PDI = 1.24 by GPC (spell out) using universal calibration method (Table S1). This indicates that PEG and TT molecules were successfully coupled using fumaryl chloride. Proton NMR was further conducted and the chemical shifts in ^{1}H NMR spectra could be well assigned to the functional groups inside the PEG-Fu-DiTT polymer, confirming successful polymer synthesis (Fig. S2).

Self-assembled PEG-Fu-DiTT polymersomes were readily prepared using THF and 0.3% polyvinyl alcohol (PVA) solution. Dynamic light scattering (DLS) measurements showed average diameters of 101.6 ± 3.6 nm, PDI of 0.46 ± 0.03 and zeta potential of -2.4 ± 1.5 mV (Table S2). Polymers incorporated with fumarate segments are biocompatible and can be easily crosslinked through photo or chemical crosslinking. Widely studied examples include poly (propylene fumarate) (PPF), polycaprolactone fumarate (PCLF), oligo (poly (ethylene glycol) fumarate) (OPF), poly(propylene fumarate)-*co*-poly(L-lactic acid) (PPF-*co*-PLLA) and poly(propylene fumarate)-*co*-polyhedral oligomeric silsesquioxane (PPF-*co*-POSS), which are readily crosslinked and widely investigated for tissue engineering and drug delivery applications.[15] After chemical crosslinking using ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) solution, an obvious shrink in the polymersomes size was detected with average diameters decreased to 96.3 ± 2.4 nm (Table S2). The size distribution of crosslinked PEG-Fu-DiTT polymersomes was presented in Fig. 3A.

In addition to DLS measurement, the size and morphology of the crosslinked polymersomes were observed using transmission electron microscopy (TEM). As seen in Fig. 3B, morphological images observed by TEM demonstrated a distribution of polymersomes within the range of 50-100 nm, consistent with the sizes determined from DLS. Moreover, the TEM images demonstrated a void configuration for PEG-Fu-DiTT (Fig. 3C), similar to the polymersome or hollow nanosphere structures reported in previous studies.[16] The dark edge represents the shell of the polymersome whereas the central white region indicates the hollow core of the polymersome. The larger sized particles observed under TEM are mainly caused by the collapse of large polymersomes during the drying process, which is a normal phenomenon for polymersomes.[14] A detailed schematic demonstration of different conformations of polymersomes created by the drying process including void core collapse, overlap and edge overlap of multiple polymersomes was also presented (Fig. S3) After crosslinking, a slight change in the surface zeta potential was determined with average values of -2.8 ± 1.1 mV for crosslinked polymersomes (Table S2).

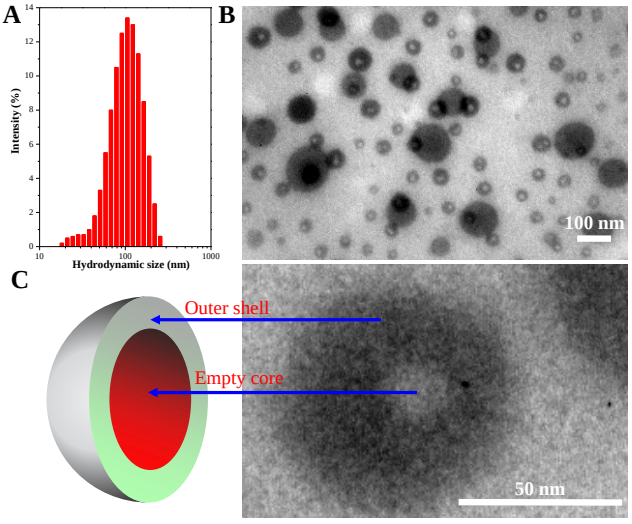

Fig. 3 (A) Hydrodynamic size distribution, (B) TEM images, and (C) core-shell structures of self-assembled PEG-Fu-DiTT polymersomes.

Stability test of PEG-Fu-DiTT polymersomes upon exposure to proteins, surfactants and salt ions were evaluated. Results showed that crosslinked polymersome mainly kept a stable size in 10% FBS, 5 mM SDS and 0.9% NaCl solution (Fig. 4). However, for uncrosslinked polymersomes, a large portion was detected to easily disassemble and form a population of particles with size ~ 10 nm upon environmental changes (Fig. 4C-D). These results indicate that polymersome gained enhanced stability after crosslinking of inside chains, which is consistent to several previous studies that also reported an enhanced stability of polymersomes or nanoparticles after crosslinking.[17] These crosslinked PEG-Fu-DiTT polymersomes are potentially useful for *in vivo* drug delivery.

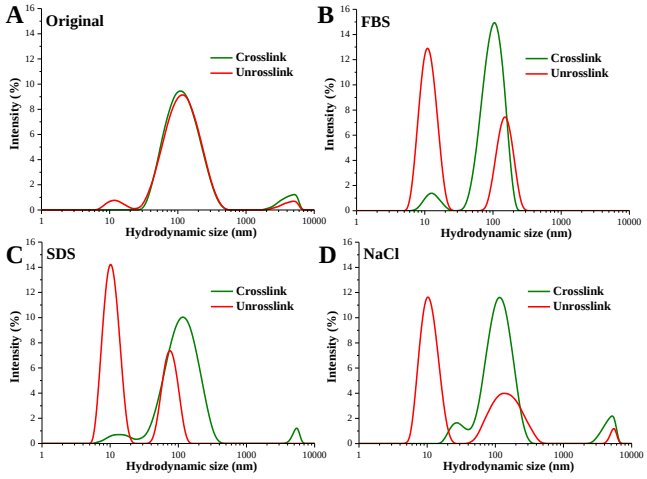

Fig. 4 Stabilities of crosslinked and uncrosslinked PEG-Fu-DiTT polymersome in the presence of proteins, surfactants and salt ions. Hydrodynamic size changes were evaluated by DLS for (A) original polymersomes and polymersomes in (B) 10% FBS, (C) 5 mM SDS and (D) 0.9% NaCl solution.

The size change of crosslinked PEG-Fu-DiTT polymersomes in response to acidic pH was followed by DLS measurements (Fig. 5). The results demonstrated little changes in the size of crosslinked polymersomes over 48 h at pH 7.4 conditions (Fig. 5A). This data indicates a strong stability for crosslinked PEG-Fu-DiTT polymersomes under neutral environments. However, under acidic condition, crosslinked polymersomes swelled severely as indicated by the appearance of sub-micrometer/micrometer-sized particle peak after 2 h incubation in pH 5.0 solution under otherwise the same conditions (Fig. 5B). With further increase of incubation time to 8 h, the majority of crosslinked polymersomes swelled continuously with the highest particle size peak shifted to 200 nm, as seen in Fig. 5B. In particular, a small portion of the polymersomes was detected to swell into micrometer level in sizes. After 8 h, no further changes in polymersome size were detected. This swelling effect was caused by the acetal hydrolysis of TT monomer under low pH conditions. According to several previous studies, half of the TT molecules were hydrolyzed within 5 to 8 hours in response to pH 5.0 condition.[14] Thus, the swelling effect detected in this study was consistent with the hydrolysis rates of TT molecules.

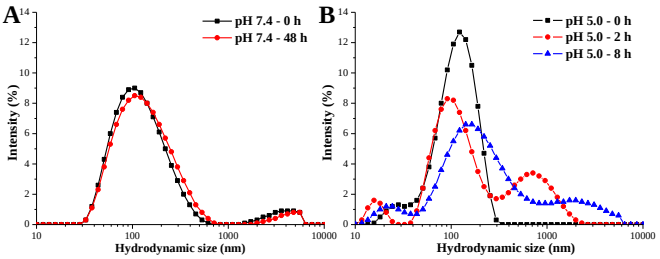

Fig. 5 DLS measurements of size changes for crosslinked PEG-Fu-DiTT polymersomes under (A) pH 7.4 and (B) pH 5.0 conditions.

DOX is one of the most widely used anticancer drugs for the treatment of malignant tumors by inhibiting nucleic acid synthesis. Here, DOX was readily encapsulated into the polymersomes during the self-assembly process. DOX-loaded uncrosslinked and crosslinked polymersomes were characterized to have hydrodynamic sizes of 113.9 ± 4.6 nm and 103.5 ± 5.3 nm, respectively. Compared with empty polymersomes without DOX loading, there is an increase in size due to drug encapsulation (Table S2). Zeta potential measurements detected values of -1.3 ± 0.5 and -1.3 ± 0.3 for uncrosslinked and crosslinked DOX loaded polymersomes, implying negative surface charges (Table S2). DLC and DLE of DOX in the crosslinked polymersomes were calculated to be 6.8% and 52.2%, respectively. These results indicate a successful loading of DOX into the polymersomes with a slightly enlarged size and negative surface charge.

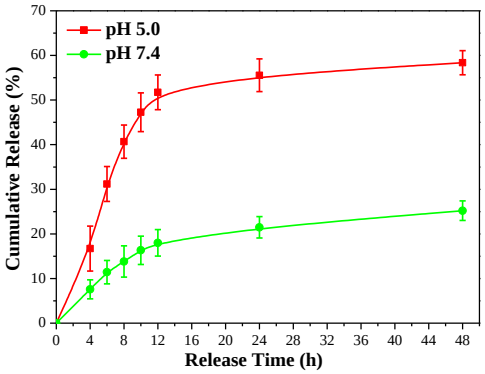

Fig. 6 Release profile of DOX from crosslinked PEG-Fu-DiTT polymersomes at pH 7.4 and pH 5.0 conditions.

Release of DOX from polymersomes under physiological (pH 7.4) or acidic conditions (pH 5.0, mimicking acidic conditions in lysosomes) was investigated. As can be seen in Fig. 6, a substantial higher DOX content was detected in acidic release medium (pH 5.0) than that of physiological condition (pH 7.4) over a period of 48 h. DOX-loaded polymersome (DOX-PS) demonstrated good stabilities in physiological conditions, and DOX amount of 21.5 ± 2.4% and 25.2 ± 2.2% were released after incubation in release medium for 24 h and 48 h, respectively. However, under acidic medium, DOX release was much faster and the release amount increased to 55.6 ± 3.7% and 58.4 ± 2.7% at 24 h and 48 h, respectively. This rapid and robust DOX release is largely due to the polymersome expansion caused by hydrophobic to hydrophilic transformation of PEG-Fu-

DiTT polymer chains through acetal hydrolysis, consistent with the size swelling trend shown above (Fig. 5). Real physiological conditions in cells are complicated with multiple enzymes that could help digest and hydrolyze the polymersomes in addition to pH change, which may result in even faster release of DOX. Therefore, these pH-sensitive degradable polymersomes based on PEG-Fu-DiTT polymers are able to efficiently load and rapidly release DOX triggered by low pH environment in endo/lysosomes, which renders them particularly appealing for the delivery of hydrophilic anticancer drugs.

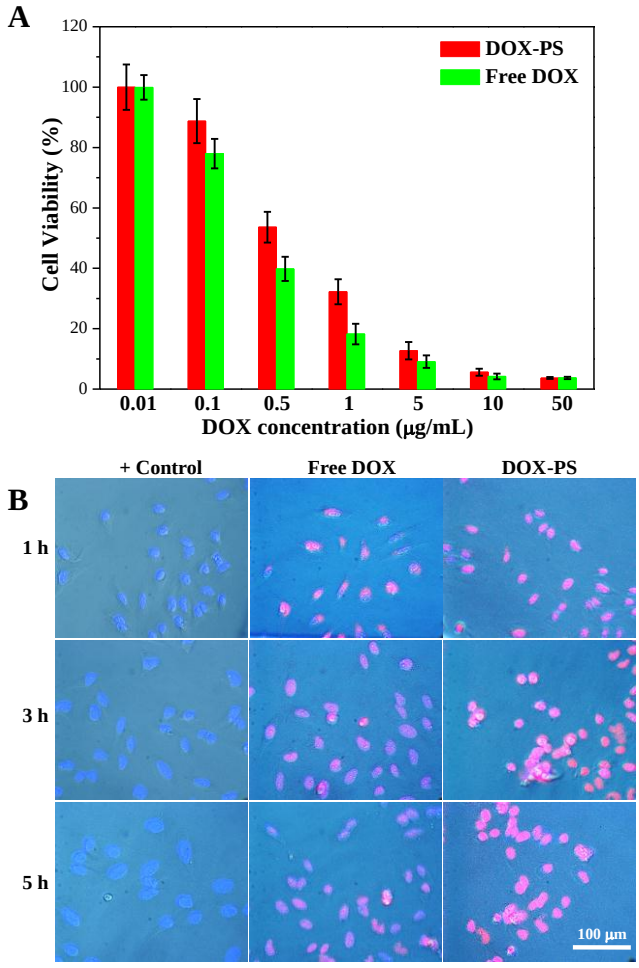

Fig. 7 (A) Cell viability under co-culture with varied concentrations of crosslinked DOX-PS or free DOX•HCl. (B) Cellular uptake of crosslinked DOX-PS or free DOX•HCl after 1, 3 and 5 h incubation at a DOX concentration of 10 μg mL^{-1}.

The cytotoxicity of crosslinked polymersomes before and after DOX loading were evaluated using HeLa cancer cells. Before loading of DOX•HCl, empty polymersomes showed good biocompatibility with HeLa cells (Fig. S4). However, after encapsulation of DOX•HCl into the polymersomes, an inhibition effect to cancer cells were detected at concentration > 0.1 μg mL^{-1}. As demonstrated in Fig. 7A, under the same concentration of DOX•HCl administration for 3 days, DOX-PS showed excellent killing effect close to the free drug. Free DOX as a positive control showed stronger cytotoxicity, which may be due to the fact that the DOX encapsulated in polymersomes requires more time to be released, while free DOX could kill cells immediately. Incomplete digestion of polymersomes in endosomes/lysosomes causing imperfect release of encapsulated DOX may also happen thus is another potential reason. Cellular uptake of DOX-PS particles were investigated through imaging of DOX fluorescence after 1, 3 and 5 hours of co-culture with HeLa cells. Positive control groups without drug administration showed no DOX fluorescence, as expected. Cells with free DOX•HCl demonstrated red fluorescence after 1 h incubation, with a slight increase in intensity at 3 and 5 h time points (Fig. 7B). HeLa cells with DOX-PS exhibited similar DOX fluorescence at 1 h, However, the fluorescence intensified remarkably at 3 and 5 h time points. Moreover, cells were observed to contract and detach from culture plates at 3 and 5 h with incubation of DOX-PS (Fig. S5-7). It is widely acknowledged that the cellular uptake of large particles are generally achieved by endocytosis.[18] After endocytosis, particles encounter an acidic environment in intracellular organelles, i.e., endosomes or lysosomes. This acidic environment is believed to cause rapid acetal hydrolysis of the PEG-Fu-DiTT polymersomes, which further result in immediate DOX release and robust cancer-killing effects, as evidenced by cell contraction and low cancer cell viability. After acetal hydrolysis and DOX release, the crosslinked polymersome left is similar to crosslinked nano-hydrogel with PEG chains as major components. PEG chains are biocompatible and biodegradable into non-toxic degradation products.

Conclusions

In summary, we have developed a new pH-responsive crosslinked nanosphere for hydrophilic anticancer drug delivery. The facile synthetic route, high loading capacity of drugs, physiological stability, and low pH triggered size swell and subsequent robust drug release render this pH-responsive polymersome a promising drug carrier for cancer chemotherapy.

Acknowledgements

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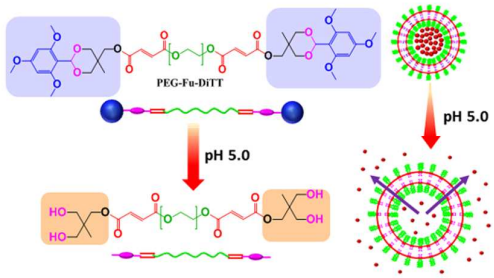

Shell crosslinked polymersome self-assembled by amphiphatic chains showed pH-responsive swelling through acidic cleavage of hydrophobic content.