Polymersomes

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Concurrent Block Copolymer Polymersome Stabilization and Bilayer Permeabilization by Stimuli-Regulated "Traceless" Crosslinking**

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Abstract: *The fabrication of block copolymer (BCP) vesicles (polymersomes) exhibiting synchronized covalent crosslinking and bilayer permeabilization remains a considerable challenge as crosslinking typically leads to compromised membrane permeability. Herein it is demonstrated how to solve this dilemma by employing a stimuli-triggered crosslinking strategy with amphiphilic BCPs containing photolabile carbamate-caged primary amines. Upon self-assembling into polymersomes, light-triggered self-immolative decaging reactions release primary amine moieties and extensive amidation reactions then occur due to suppressed amine pK_a within hydrophobic milieu. This leads to serendipitous vesicle crosslinking and the process is associated with bilayer hydrophobicity-to-hydrophilicity transition and membrane permeabilization.*

Learning from the structures and functions of complex biological systems such as cells and viral capsids has continuously inspired the creation of artificial self-assembled nanostructures,[1] with lipid vesicles (liposomes) and block copolymer (BCP) vesicles (polymersomes) being the most representative examples.[2] Both polymersomes and liposomes consist of an aqueous interior enclosed by a hydrophobic bilayer membrane. They have been increasingly used to construct drug delivery nanocarriers,[3] nanoreactors,[4] and artificial organelles.[2e,5] However, compared to liposomes, the more robust polymersomes are subjected to severe membrane permeability issues, that is, they are almost impermeable to small organic molecules, ions, and even water.[6] Previously, a few approaches have been developed to enhance polymersome permeability, including membrane incorporation of channel proteins,[7] construction of stimuli-sensitive polymersomes (e.g., pH, CO_2, sugar, light irradiation),[8] co-assembly of oppositely charged BCPs,[9] self-assembly of helical rod–coil BCPs,[4b] and post-modification of vesicle membranes.[10]

The use of above strategies involves the introduction of external additives and complex procedures, or results in structural disintegration. On the other hand, the stability of polymersomes is another important issue and they tend to be disrupted upon large dilution, high shear forces, or subjected to complex biological milieu.[11] Conventional chemical crosslinking approaches have been used to solve the stability issue, but nonetheless, usually led to further compromised bilayer permeability towards both hydrophilic and hydrophobic substances.[12] Previously, a two-step procedure has been applied with stimuli-responsive BCP polymersomes to partially solve the stability/permeability dilemma, that is, crosslinking at first for enhanced stability and subsequent permeability control by external stimuli (pH, temperature, and light).[13] However, the crosslinking and permeability tuning processes are independently conducted and the decoupled nature renders this approach less satisfactory at optimizing both microstructural stability and permeability.

Thus, the fabrication of polymersomes exhibiting synchronized covalent crosslinking and bilayer permeabilization remains a considerable challenge. Presumably, the design of such an optimized polymersome system requires unique crosslinking chemistries and extensive functional group transformation during crosslinking. Here we demonstrate a new strategy to solve this dilemma by employing a light-regulated "traceless" crosslinking strategy (Scheme 1). In brief, we designed amphiphilic BCPs with the hydrophobic block containing photolabile carbamate-caged primary amine moieties. Upon self-assembling into polymersomes, UV-triggered self-immolative decaging releases primary amine moieties, prominent amidation reactions then occur and this leads to prominent vesicle crosslinking instead of vesicle-to-unimer transition, which we initially expected to occur. Most importantly, the crosslinking process is associated with bilayer hydrophobicity-to-hydrophilicity transition. We further demonstrate light-tunable co-release of both hydrophilic and hydrophobic molecules encapsulated within polymersomes and light-switchable enzymatic biocatalysis.

Amphiphilic BCPs with varying hydrophobic block lengths, PEO_{45}-*b*-$PNBOC_{30}$ and PEO_{45}-*b*-$PNBOC_{54}$, were synthesized by reversible addition–fragmentation chain transfer polymerization of 2-nitrobenzyloxycarbonylaminoethyl methacrylate (NBOC) using poly(ethylene oxide) (PEO)-based macroRAFT agent (Scheme S1 and Table S1 in the Supporting Information). 2-Nitrobenzyl functionalities typically exhibit photolabile characteristics,[8e,f,14] and UV irradiation transforms NBOC into 2-aminoethyl methacrylate

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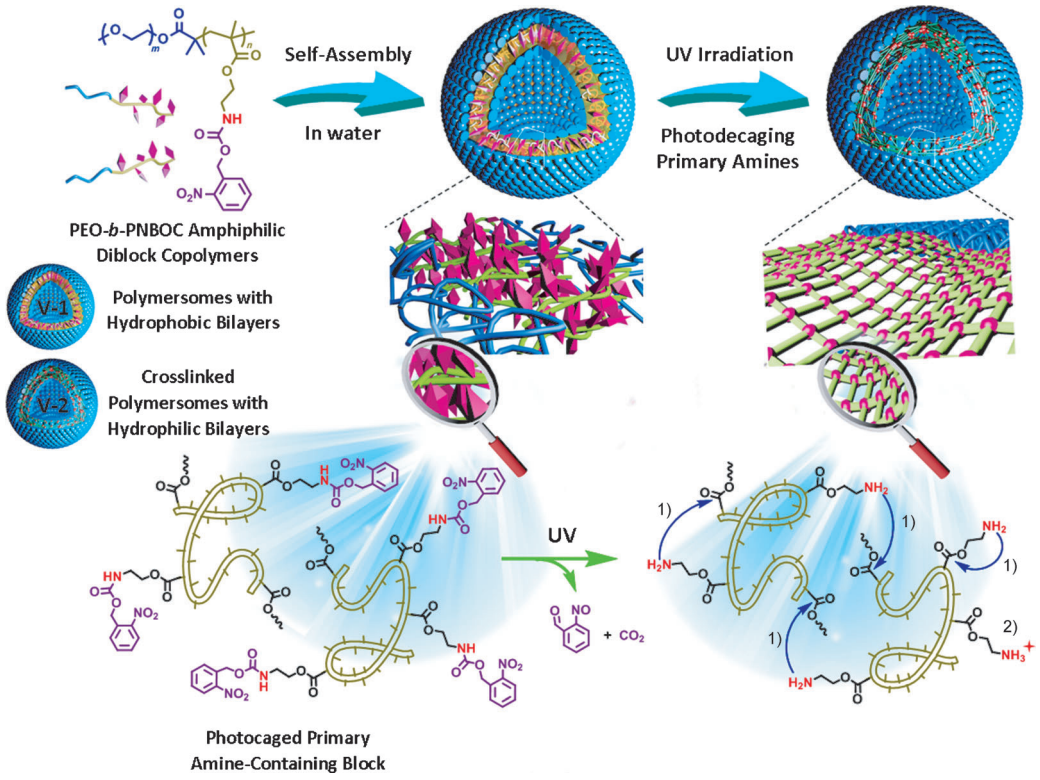

Scheme 1. Design of BCP vesicles exhibiting concurrent phototriggered "traceless" crosslinking and vesicle membrane permeabilization. PEO-*b*-PNBOC amphiphilic BCPs self-assemble into polymersomes with the hydrophobic bilayer containing carbamate-caged primary amine moieties. UV irradiation triggers decaging reactions and the release of primary amine functionalities, prominent amidation reaction then occurs because of a suppressed amine pK_a within the hydrophobic vesicle membrane, resulting in vesicle crosslinking instead of vesicle-to-unimer disassembly. 1) Enhanced amidation within the hydrophobic microenvironment. 2) Unreactive primary amines because of protonation.

(AEMA), accompanied with the release of 2-nitrobenzaldehyde and CO_2 (Scheme 1).[15]

The block copolymer self-assembly was triggered by adding water into PEO_{45}-*b*-$PNBOC_{30}$ solution in 1,4-dioxane at 25 °C. Transmission electron microscopy (TEM) observation confirms the formation of polymeric vesicles with a diameter of about 400 nm and a membrane thickness of about 23.5 nm (Figure 1 a). They should possess hydrophobic PNBOC bilayer membranes stabilized with both inner and outer PEO coronas (Scheme 1). The vesicular microstructure is also verified by scanning electron microscopy (SEM) and atomic force microscopy (AFM) observations (Figure 1 c and e).

Photolabile carbamate-caged primary amine moieties are located within hydrophobic membrane bilayers of polymersomes. Upon UV irradiation, the photocleavage of 2-nitrobenzyl functionalities and generation of 2-nitrosobenzaldehyde can be verified by time-dependent UV/Vis absorption spectra (Figure S1a). Upon irradiation of a mixture of fluorescamine (FA) and BCP in THF, fluorescence intensities at about 475 nm increase linearly with irradiation duration and then level off after about 30 minutes (Figure S2). This confirms the fresh generation of primary amines, which undergo fast reaction with FA. Comparable results were also obtained for PEO_{45}-*b*-$PNBOC_{30}$ vesicle dispersion in aqueous media (Figure S1e).

Dynamic laser light scattering (LLS) analysis revealed that before irradiation, the vesicle dispersion (pH 7.4, 25 °C) exhibits an intensity-averaged hydrodynamic diameter, $\langle D_h \rangle$, of about 462 nm and size polydispersity (μ_2/Γ^2) of 0.245 (Table S1 and Figure 2 b). Upon irradiation, the scattered light intensity only exhibits modest initial decrease and then levels off; meanwhile, $\langle D_h \rangle$ increases from 460 to 500 nm, suggesting partial bilayer swelling. Apparently, the vesicular dispersion shows almost no visual changes after irradiation (Figure 2 a, (1) and (2) in the inset). If the dispersions were subjected to a sixfold dilution with 1,4-dioxane, the nonirradiated one turned transparent, whereas the irradiated vesicular dispersion still exhibited a bluish tinge and no aggregate disassembly can be discerned (Figure 2 a, (3) and (4) in the inset).

TEM and SEM of the UV-irradiated vesicular dispersion further confirmed the retaining of hollow nanostructures (Figure 1 b and d). Furthermore, after irradiation the hollow sphere morphology is more explicit and stereoscopic (Figure 1 b). A collapsed and bowl-shaped morphology of the UV-irradiated vesicles is observed by SEM (Figure 1 d). The AFM height image also revealed spherical nanoparticles with an average height of 134 nm (Figure 1 g), which is much larger than that of nonirradiated vesicles (about 16 nm in the height profile, Figure 1 e). These results further verify that during irradiation, crosslinking reactions occur and vesicles are getting more rigid and endowed with improved mechanical stability.

UV irradiation will transform the PNBOC block into a PAEMA block. The PAEMA homopolymer is water-soluble and possesses a pK_a of 7.6. The homopolymer is chemically stable in acidic or neutral aqueous media.[15–16] Below pH 9,10, PAEMA in aqueous solution will not undergo degradation or crosslinking reactions even the extent of amine protonation is $< 10\,\%$. When the vesicle dispersion was UV irradiated at pH 7.4, we initially expected vesicle disassembly. However, all the above results indicated that vesicle crosslinking has occurred; we then speculate that this

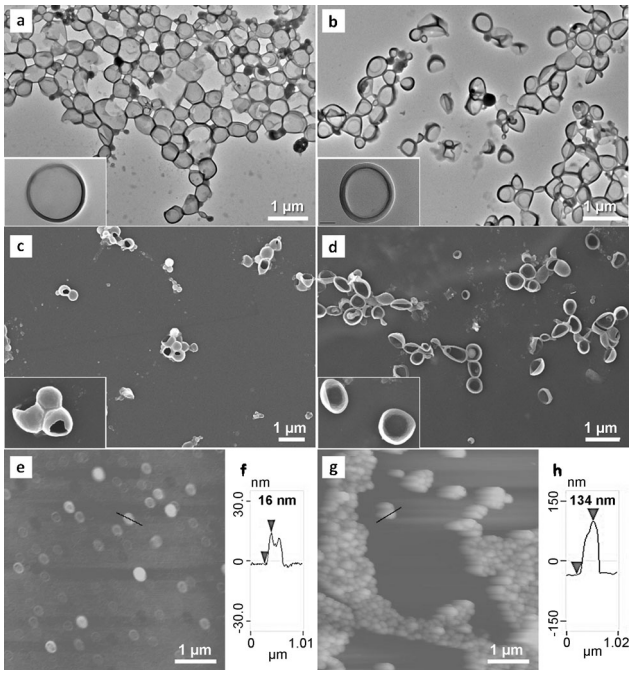

Figure 1. Microscopic characterization of polymersomes before and after UV irradiation. a,b) TEM images (the scale bars in the insets are 100 nm), c,d) SEM images, and e–h) AFM height images of PEO_{45}-*b*-$PNBOC_{30}$ polymersomes (a,c,d) before and (b,d,g) after UV irradiation; the insets show enlarged individual polymersomes. f,h) Cross-sectional profiles.

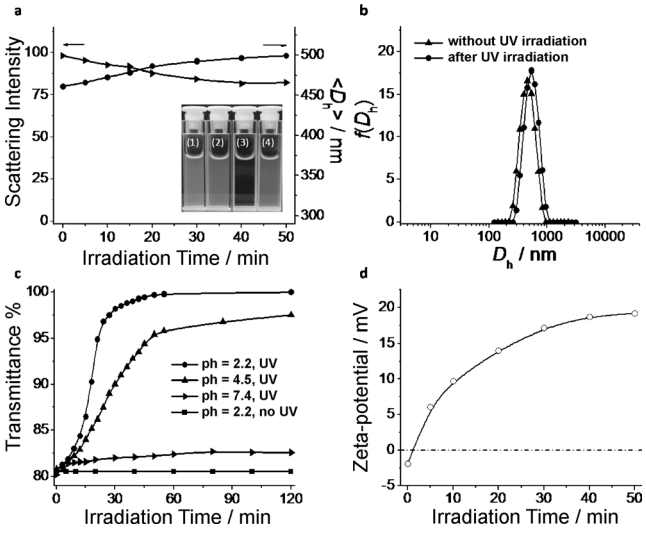

Figure 2. Characterization of polymersomes during UV irradiation. a) Irradiation duration-dependent evolution of (▶) scattered light intensity and (●) $\langle D_h \rangle$ of PEO_{45}-*b*-$PNBOC_{30}$ vesicles. The insets in (a) show photos of vesicular dispersions: 1 and 3) before and 2 and 4) after UV irradiation and then subjected to six-fold dilution with (1 and 2) water and (3 and 4) 1,4-dioxane. b) D_h distribution of vesicles before and after UV irradiation. c) Irradiation duration dependence of optical transmittance at 700 nm recorded for vesicles in buffer media at pH 2.2, 4.5, and 7.4; also included is the control sample at pH 2.2 but without UV irradiation. d) Irradiation duration dependence of the zeta potential of the vesicle dispersion at pH 7.

process should be related to amine-involved amidation reactions (Scheme 1).[17a] The extent of crosslinking should be affected by solution pH because the protonated amine will not undergo amidation reactions.[18] UV irradiation was then conducted for vesicle dispersions at varying condition (pH 7.4, 4.5, and 2.2).

For the nonirradiated dispersion, optical transmittance (700 nm) remains to be constant at 80.5 %. Upon irradiation at pH 7.4, optical transmittance only exhibits a modest increase to 82.6 % (Figure 2 c) and this verifies vesicular crosslinking. When irradiated at pH 4.5, where most newly generated amines should be protonated, the optical transmittance can increase to 96 %, implying incomplete vesicle disintegration. Only when irradiated at pH 2.2, the optical transmittance can reach 100 %, achieving the "initially" designed vesicle-to-unimer transition (Figure S3). pH-dependent crosslinking suggests that the crosslinking involves intermolecular amidation reactions of UV-decaged primary amines (Scheme 1). In addition, PEO_{45}-*b*-$PNBOCA_{15}$ was also synthesized as a control, which contains methacrylamide instead of a methacrylate functionality (Scheme S2 and Table S1). In this case, after irradiation primary amines are also released but micellar disintegration occurs because of the lack of possible amidation reactions (Figure S4).

For newly generated amines within hydrophobic vesicle bilayers, we propose that the effective pK_a will decrease because of the local hydrophobic milieu, thus favoring subsequent amidation reactions (Scheme 1). In addition, FT-IR, X-ray photoelectron spectroscopy (XPS), and ^{1}H NMR spectroscopy of PEO_{45}-*b*-$PNBOC_{30}$ vesicles (lyophilized sample after dialysis) further corroborated UV-triggered carbamate decaging and crosslinking reactions (Figure S1b-d; see the Supporting Information for details). In particular, XPS core-level N1s spectra show that after UV irradiation, 10 % of the primary amines remain in the protonated state whereas the other 90 % form amide linkages (Figure S1c). The presence of residual protonated amines correlates with the fact that the zeta potential of the vesicular dispersion increases to +18 mV upon UV irradiation (Figure 2 d). We tentatively termed the stimuli-triggered crosslinking approach as a "traceless" one since the starting materials cannot be exactly deduced simply based on the chemical structure of the cross-linked nanostructures (Scheme 1). The proposed "traceless" crosslinking chemistry can also be applied to large compound vesicles self-assembled from PEO_{45}-*b*-$PNBOC_{54}$ (Figure S5), demonstrating the generality of this approach.

The vesicular crosslinking process is accompanied with the generation of residual protonated amines and a large amount of amide bonds at the price of more hydrophobic ester linkages (Scheme 1). We speculate that during crosslinking hydrophobic vesicle bilayers will be subjected to hydrophobic-to-hydrophilic transitions. This was further confirmed for Nile-red-loaded PEO_{45}-*b*-$PNBOC_{30}$ vesicles (Figure S6). Since hydrophobic and hydrophilic molecules can be co-loaded into hydrophobic bilayers and aqueous interiors of vesicles, after photoirradiation a vesicle dispersion should be capable of achieving co-release of two types of molecules, while maintaining the structural integrity of vesicles because

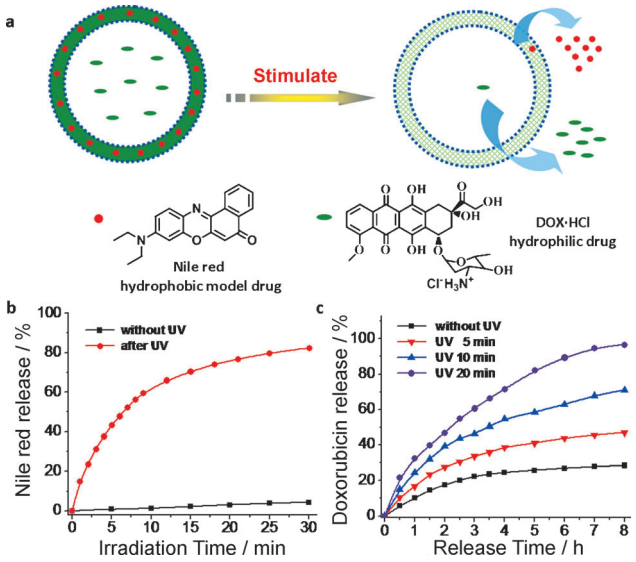

Figure 3. Polymersome nanocarriers for light-regulated co-release of both hydrophobic and hydrophilic drugs. a) Schematic illustration of vesicles co-loaded with Nile red and Dox·HCl; upon UV irradiation, the hydrophobic bilayer is crosslinked and concomitantly rendered hydrophilic and permeable, leading to the drug co-release. b) Release profiles of loaded Nile red from the vesicle bilayer without and with UV irradiation. c) Release profiles of encapsulated Dox·HCl from the aqueous interior of vesicles without and with UV irradiation.

of the "traceless" crosslinking (Figure 3a). The hydrophilic anticancer drug, doxorubicin hydrochloride (Dox·HCl), and the hydrophobic model drug, Nile red, were co-loaded into vesicles during self-assembly. Indeed, stimuli-triggered co-release of both drugs can be achieved by UV irradiation (Figure 3b,c); however the release mechanisms of the two model drugs are different. For Nile red, hydrophobic interactions between Nile red and the hydrophobic vesicle bilayer allows for efficient dye loading, and a UV-triggered bilayer hydrophobic-to-hydrophilic transition during crosslinking leads to immediate Nile red release, that is, the release is synchronized with the crosslinking and membrane permeabilization process. For Dox·HCl, the release is restrained after being loaded into the aqueous lumen because of the barrier effect of hydrophobic membranes (20% release within 8 h). Upon UV irradiation, the initially hydrophobic bilayer is crosslinked and hydrophilic network channels are generated through the bilayer membrane (Scheme 1). This allows for more facile passage of Dox·HCl. Indeed, upon irradiation for 5, 10, and 20 minutes, 40, 70, and 95% Dox·HCl can be released within 8 h (Figure 3c). The Dox release regulated by the UV irradiation duration also reveals that both vesicle crosslinking density and network hydrophilicity can be modulated in a proportional manner.

Since vesicle bilayer permeabilization can be achieved during irradiation-triggered crosslinking, we propose that vesicles encapsulating enzymes would serve as excellent candidates for enzymatic nanoreactors with switchable off/on activities. Triggered crosslinking will endow the system with superior structural stability and robustness, whereas the accompanied hydrophobicity-to-hydrophilicity transition will allow for the selective and specific diffusion of external substrates into the vesicular interior to actuate enzymatic reactions. In addition, vesicle bilayer permeabilization will also render possible the diffusion out of enzymatic reaction product. The use of enzyme-loaded vesicles as photoregulated nanoreactors was then explored by choosing alkaline phosphatase, ALP, as a prototypical enzyme of biological relevance (Figure S7).

Upon UV irradiation, the permeabilization of crosslinked vesicular bilayers allows for the diffusion of nonfluorescent water-soluble substrate (phosphate-caged fluorescein, *p*-FL[17b]) into the hydrophilic lumen, which are then subjected to ALP-catalyzed decaging reactions to afford highly fluorescent fluorescein (Figure S7b). To confirm that enzymatic activity switching was due to substrate diffusion control instead of enzyme release, we used dye-labeled dextran (TMR-dextran; molar weight (MW) of about 10 kDa) as a model macromolecule and fabricated dextran-loaded vesicles, dextran@vesicle. It was observed that upon irradiation, encapsulated TMR-dextran cannot diffuse out of vesicular nanoreactors (Figure S8). This suggests that the ALP enzyme, with a MW of 56 kDa, should also be retained within crosslinked vesicle interiors. In contrast, a nonirradiated vesicle dispersion does not exhibit appreciable enzymatic activities because of the bilayer barrier to the charged substrate (Figure S7c).

In conclusion, we developed a stimuli-regulated "traceless" crosslinking strategy to solve the dilemma of concurrent polymersome stabilization and bilayer membrane permeabilization, starting from amphiphilic block copolymers containing photolabile carbamate-caged primary amine moieties in the hydrophobic block. The "traceless" crosslinking process is accompanied with the generation of hydrophilic network channels within vesicle membranes and bilayer permeabilization, and this feature has been successfully used to achieve light-regulated co-release of both hydrophobic and hydrophilic substances and light-switchable biocatalysis of enzyme-entrapped vesicle nanoreactors. We expect that the reported strategy should also be feasible to other BCP hierarchical assemblies and even "top-down" fabricated nanostructures. Although UV irradiation was used as an external stimulus to trigger synchronized vesicle crosslinking and membrane permeability processes in the current work, other triggering motifs such as visible and infrared light and more biologically relevant stimuli (e.g., thiols, hydrogen peroxide, and enzymes) might also be exploited upon appropriate structural designing of block copolymers. Further work in this aspect is currently ongoing.

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