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Synthesis and Biomedical Applications of Functional Poly(α-hydroxy acids) via Ring-Opening Polymerization of *O*-Carboxyanhydrides

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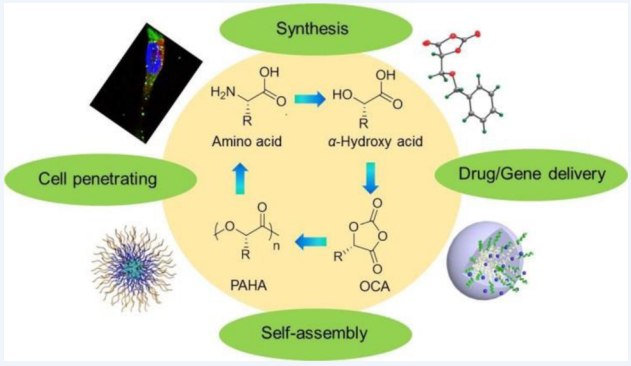
CONSPECTUS: Poly(α-hydroxy acids) (PAHAs) are a class of biodegradable and biocompatible polymers that are widely used in numerous applications. One drawback of these conventional polymers, however, is their lack of side-chain functionalities, which makes it difficult to conjugate active moieties to PAHA or to fine-tune the physical and chemical properties of PAHA-derived materials through side-chain modifications. Thus, extensive efforts have been devoted to the development of methodology that allows facile preparation of PAHAs with controlled molecular weights and a variety of functionalities for widespread utilities. However, it is highly challenging to introduce functional groups into conventional PAHAs derived from ring-opening polymerization (ROP) of lactides and glycolides to yield functional PAHAs with favorable properties, such as tunable hydrophilicity/hydrophobicity, facile postpolymerization modification, and well-defined physicochemical properties.



Amino acids are excellent resources for functional polymers because of their low cost, availability, and structural as well as stereochemical diversity. Nevertheless, the synthesis of functional PAHAs using amino acids as building blocks has been rarely reported because of the difficulty of preparing large-scale monomers and poor yields during the synthesis. The synthesis of functionalized PAHAs from *O*-carboxyanhydrides (OCAs), a class of five-membered cyclic anhydrides derived from amino acids, has proven to be one of the most promising strategies and has thus attracted tremendous interest recently. In this Account, we highlight the recent progress in our group on the synthesis of functional PAHAs via ROP of OCAs and their self-assembly and biomedical applications. New synthetic methodologies that allow the facile preparation of PAHAs with controlled molecular weights and various functionalities through ROP of OCAs are reviewed and evaluated. The in vivo stability, side-chain functionalities, and/or trigger responsiveness of several functional PAHAs are evaluated. Their biomedical applications in drug and gene delivery are also discussed. The ready availability of starting materials from renewable resources and the facile postmodification strategies such as azide−alkyne cycloaddition and the thiol−yne "click" reaction have enabled the production of a multitude of PAHAs with controlled molecular weights, narrow polydispersity, high terminal group fidelities, and structural diversities that are amenable for self-assembly and bioapplications. We anticipate that this new generation of PAHAs and their self-assembled nanosystems as biomaterials will open up exciting new opportunities and have widespread utilities for biological applications.

1. INTRODUCTION

Poly(α-hydroxy acids) (PAHAs) are a class of biodegradable and biocompatible polymers that have been widely used for a variety of biomedical and pharmaceutical applications such as restorable sutures and implants, drug delivery, and tissue engineering.[1−4] Well-known examples include poly(lactide) (PLA), poly(glycolide) (PGA), and poly(lactide-*co*-glycolide) (PLGA). They are readily available from inexpensive, renewable resources through ring-opening polymerization (ROP) of lactide (LA), glycolide (GA), and a mixture of LA and GA, respectively.[5] One disadvantage of these conventional PAHAs, however, is their lack of side-chain functionalities, which makes it difficult to conjugate active moieties to PAHAs or to tune the physicochemical properties of PAHA-derived materials through side-chain modifications. Thus, numerous efforts have been devoted to the development of methodology that allows facile preparation of PAHAs with a variety of functionalities for widespread utilities.[6−8] In this Account, we highlight the recent progress on the synthesis of functional PAHAs using well-controlled ROP of 1,3-dioxolane-2,4-diones (also called *O*-carboxyanhydrides (OCAs)) and the self-assembly of the

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corresponding materials into nanostructures for drug and gene delivery applications.

2. PROGRESS IN SYNTHESIZING FUNCTIONAL PAHAS

PAHAs are usually prepared through two synthetic routes. One is direct polycondensation of α-hydroxy acids.[9] The molecular weights (MWs) of the resulting PAHAs are generally low as a result of the cyclization side reaction, which hinders the growth of the polymer chain. Furthermore, long reaction times and high temperatures are usually required for the polymer synthesis, leading to various undesired side reactions. The other approach is ROP of cyclic polymerizable monomers derived from α-hydroxy acids. This methodology allows the synthesis of PAHAs with high MW, low polydispersity, and well-controlled molecular and structural characteristics under mild conditions. There have been numerous reports concerning the controlled synthesis of PAHAs through ROP of cyclic ester monomers, including GA, LA, caprolactone, dioxanone, and orthoester.[10−12] A variety of catalytic systems, such as metal complexes, organic catalysts, and enzymes, have been developed to mediate controlled ROP of α-hydroxy acid-derived cyclic monomers.[13−15] In biomedical applications for drug delivery, gene delivery, and surgical implantation, modulation of the polymer molecular weight, tacticity, polydispersity, and degradability are also highly demanded in response to specific utilities. Therefore, additional efforts have been devoted to the synthesis of various topologically different polymers with star, brush, cyclic, or cross-linked structures to further tailor their hydrophilicity/hydrophobicity and degradability.[16−18] While the physical properties of PAHAs can possibly be tuned through copolymerization,[19] the lack of side-chain functionality remains a major factor limiting the widespread applications of these materials in new fields.

Although highly challenging, the introduction of functional groups into polymers derived from ROP can yield functional PAHAs with favorable properties. In the past several decades, there have been few reports on the introduction of functionalities onto the side chains of PLA by the design and polymerization of lactide-like monomers. For example, the Hennink group[7] reported the synthesis of hydroxyl-containing LA analogues from *O*-benzylserine (Ser(Bn)). The Hillmyer group[20] reported the polymerization of bifunctional lactide derivatives to prepare PLA derivatives with enhanced mechanical properties. The Baker group synthesized glycolides with oligo(ethylene oxide) monomethyl ether-functionalized side chains.[21]

3. SYNTHESIS OF FUNCTIONAL PAHAS FROM RENEWABLE RESOURCES

Considering the structural similarity between α-hydroxy acids and amino acids and the availability of a wide range of terminal group functionalities on amino acids, much effort has been made to develop new monomers and prepare PAHAs with side-chain functionalities via ROP of the corresponding monomers. One such example is the readily available 1,3-dioxane-2,4-diones, so-called *O*-carboxyanhydrides (OCAs), a class of five-membered ring compounds derived from amino acids that have proved to be promising candidates for the synthesis of functionalized PAHAs in a highly controlled manner (Figure 1).[22−31]

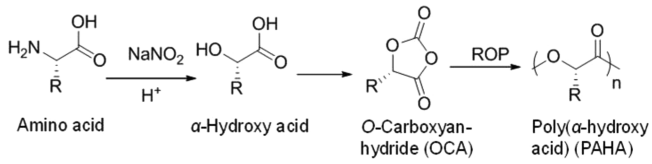

Figure 1. Synthesis of PAHAs via controlled ROP of OCAs.

3.1. Organocatalyzed ROP of OCAs

The first example of using L-lactic acid *O*-carboxyanhydride (L-Lac-OCA) as a readily available and highly polymerizable monomer to prepare homo- and copolymers of lactic acid was investigated by Kricheldorf in the 1980s.[22] Great improvement was achieved by the Bourissou group[23] with organocatalytic ROP of Lac-OCA to prepare PLA with controlled MW and narrow polydispersity under relatively mild polymerization conditions. Compared with ROP of lactide, the ROP of Lac-OCA was considered to be thermodynamically more favorable by computational prediction because of the release of a carbon dioxide molecule during the polymerization process of OCAs. This prediction was later confirmed by experimental results, as the ROP of Lac-OCA proceeded much faster than that of lactide under mild conditions (the polymerization finished typically within a few minutes at 25 °C with Lac-OCA as opposed to a few days at 35 °C with lactide).[23] Further computational mechanistic study carried out by the Bourissou group[24] revealed that base activation of the alcohol through multiple hydrogen bonding is energetically more favorable than nucleophilic activation of the monomers in dimethylaminopyridine (DMAP)-promoted ROP of Lac-OCA and that DMAP may serve as a bifunctional catalyst through its basic nitrogen center and an acidic *o*-hydrogen atom in this process.[24] Following the initial efforts on the synthesis and polymerization of Lac-OCA, the same group reported the synthesis and polymerization of L-Glu-OCA, an OCA derived from benzyl-protected L-glutamic acid.[25,26] Well-controlled homopolymerization of L-Glu-OCA was obtained in the presence of DMAP at room temperature within 5 min at a monomer-to-initiator (M/I) ratio of 50 with the expected MW and narrow polydispersity (M_n = 6300 g mol^{-1}, PDI = 1.18). Block and copolymerizations with L-Lac-OCA were also successful in rendering copolymers with pendant carboxylic acid groups under similar conditions.[25]

PAHA functionalized with pendant carboxylic acid groups can also be synthesized starting from L-malic acid.[27,28] The Dove group[27] recently reported the synthesis of (*S*)-5-[(benzyloxycarbonyl)methyl]-1,3-dioxolane-2,4-dione (L-Mal-OCA) derived from β-benzyl α-L-malate. ROP of L-Mal-OCA was carried out with neopentanol initiator and DMAP as a catalyst at 25 °C in dichloromethane (DCM) solution. The evidence suggested that the DMAP-catalyzed ROP of L-Mal-OCA was highly controllable, but some polymer impurities were found in the ^{1}H NMR and MALDI-TOF MS spectra. A range of para-substituted pyridines were then applied, and 4-methoxypyridine was found to enable the controlled ROP of L-Mal-OCA and minimize transesterification side reactions. Hydrophilic poly(L-malic acid) was then obtained by hydrogenolysis of the benzyl ester side groups of the resulting polymers.[27]

Our group recently reported the design and synthesis of *O*-benzyl-L-serine carboxyanhydride (Ser(Bn)-OCA) derived from serine. The synthesis is highly efficient, and the monmomer can be prepared on a gram scale in an overall yield of 50% (Figure 2A).[29] ROP of Ser(Bn)-OCA in the presence of DMAP as the

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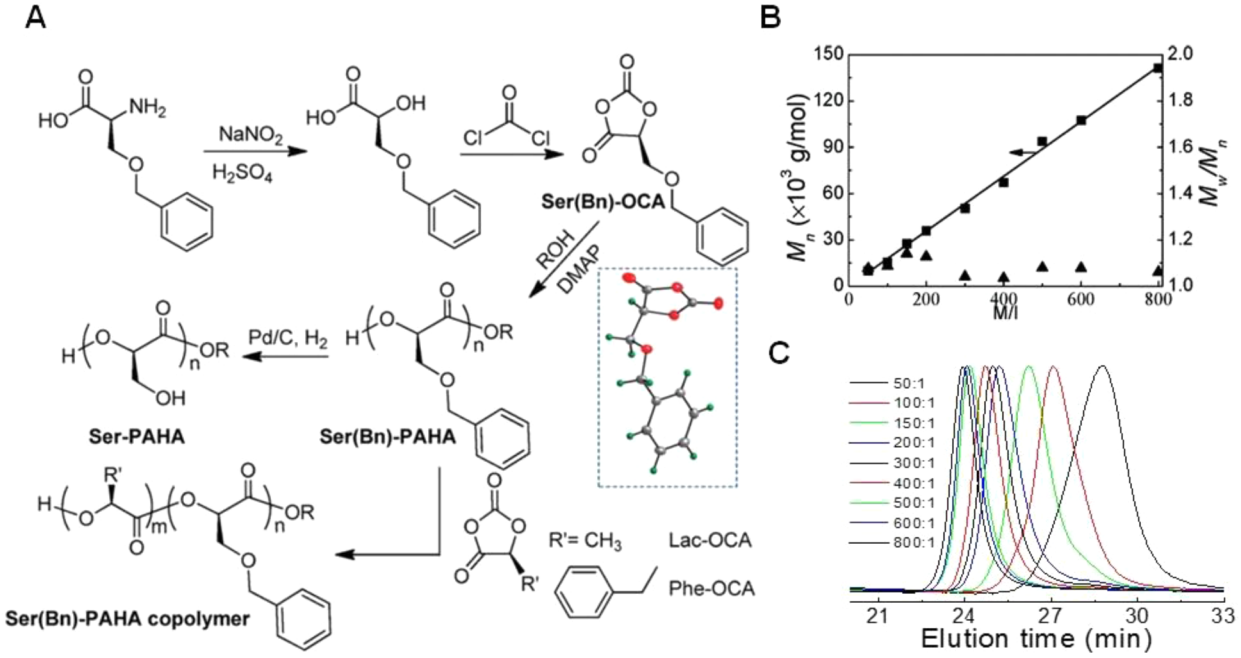

Figure 2. (A) Synthesis and polymerization of Ser(Bn)-OCA. The molecular structure of Ser(Bn)-OCA obtained by X-ray diffraction is shown in the dotted frame. (B) Plots of M_n (■) and M_w/M_n (▲) of Ser(Bn)-PAHA vs M/I for polymerization with DMAP as the catalyst and IB as the initiator in DCM ($[IB]_0$ = $[DMAP]_0$ = 0.001 M) at room temperature. (C) Overlay of GPC curves for DMAP-catalyzed, IB-initiated polymerization of Ser(Bn)-OCA at various M/I ratios. Adapted from ref 29. Copyright 2012 American Chemical Society.

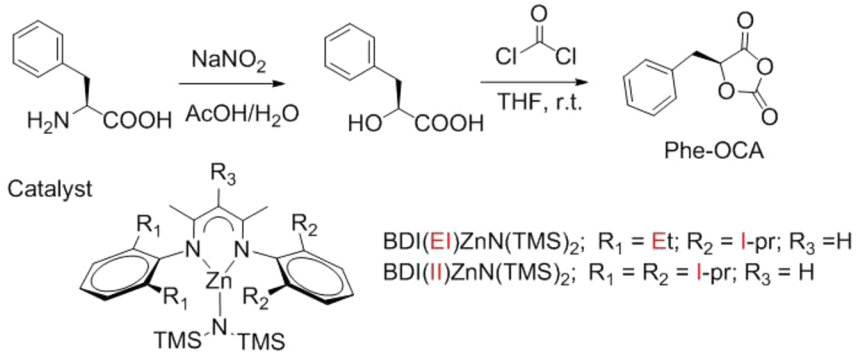

Figure 3. Synthesis and polymerization of Phe-OCA, and the structure of the BDI(m)ZnN(TMS)$_2$ catalysts (m = EI, II).

catalyst and isobutanol (IB) as the initiator afforded Ser-PAHA, a pendant hydroxyl group-functionalized PAHA with remarkable water solubility.[23−26] The polymerization process was well-controlled over a broad range of M/I ratios varying from 50 to 800. The obtained number-average molecular weight (M_n) values of Ser(Bn)-PAHA were in excellent agreement with the calculated M_n values, and the molecular weight distributions (MWDs) of Ser(Bn)-PAHA were very narrow (around 1.05−1.15) (Figure 2B). The results were further confirmed by the monomodal, symmetrical MWD curves obtained by gel-permeation chromatography (GPC) analysis of Ser(Bn)-PAHA (Figure 2C). After the polymerization, the hydrophobic benzyl ether protecting groups were removed by hydrogenolysis of Ser(Bn)-PAHA in the presence of the Degussa-type Pd/C catalyst, affording highly water-soluble Ser-PAHA.

3.2. Metal-Catalyzed ROP of OCAs

Previous studies regarding the polymerizations of OCAs have been entirely based on the use of organocatalysts.[23−30] We turned our attention to the BDI-Zn catalyst, an active-metal catalyst developed by Coates and co-workers.[32] We synthesized BDI(II)ZnN(TMS)$_2$ and BDI(EI)ZnN(TMS)$_2$, two Coates catalysts with slightly different sterically hindered β-diimine (BDI) ligands,[32,33] and studied their mediated ROPs of the OCA derived from L-phenylalanine (Phe-OCA) in the presence of 1-pyrenemethanol (PyrOH) as the initiator (Figure 3). The alkoxide BDI(II)ZnOPyr was presumably generated in situ, initiated the ROP of Phe-OCA, and controlled the chain propagation. Excellently controlled polymerization of Phe-OCA with complete monomer conversion was observed. The obtained MWs were nearly identical to the expected MWs with very narrow MWDs at M/I ratios ranging from 50 to 300. Compared with BDI(II)ZnN(TMS)$_2$, BDI(EI)ZnN(TMS)$_2$ gave even better controlled polymerization of Phe-OCA. The MW of the obtained Phe-PAHA polymer (45.8×10^3 g/mol) was in excellent agreement with the calculated MW (44.9×10^3 g/mol) and the MWD was narrow (1.12), substantiating the precise control over OCA polymerization by fine-tuning of the catalyst.

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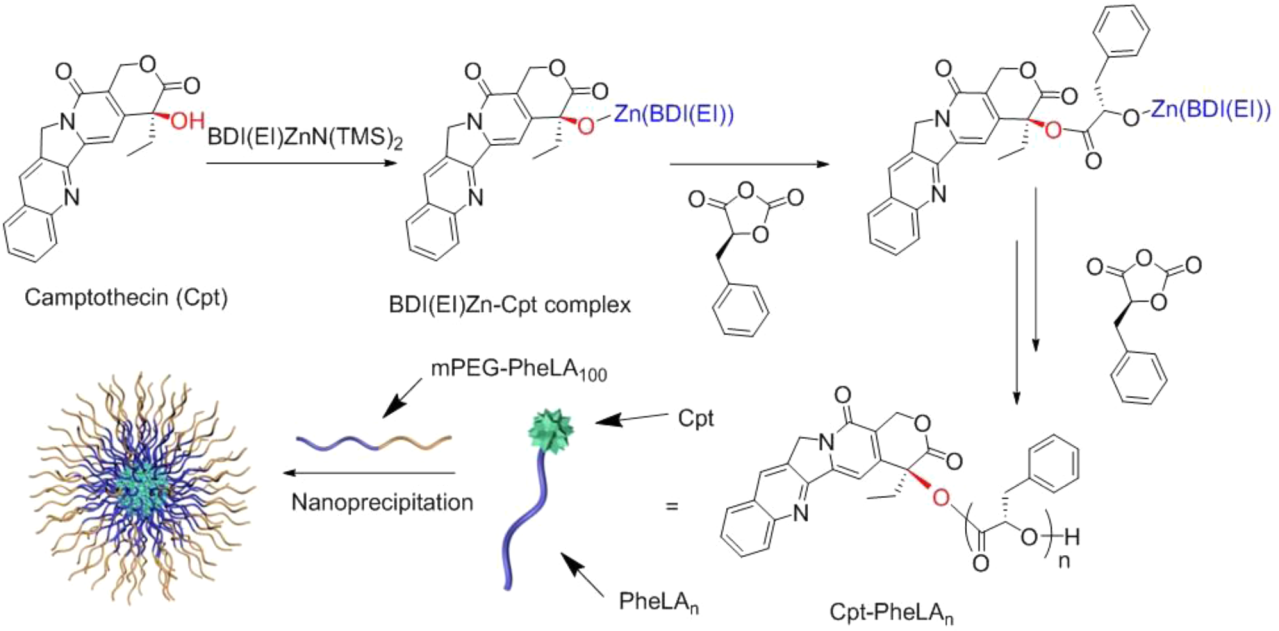

Figure 4. Schematic illustration of BDI(EI)ZnN(TMS)$_2$/CPT-mediated ROP of Phe-OCA followed by formulation of the CPT−PheLA$_n$ nanoconjugates (NCs) (n = the Phe-OCA/CPT feed ratio) using nanoprecipitation.

3.3. Enzyme-Catalyzed ROP of OCAs

In addition to ROP with organo- and metal catalysts, enzyme-catalyzed ROP of OCAs provided another route to obtain functional PAHAs and has the potential to avoid harsh reaction conditions and minimize the use of toxic reagents.[30] As a representative example, Bourissou and co-workers[30] reported the ROP of Lac-OCA in the presence of lipase PS or Novozym 435 at 80 °C. In sharp contrast to the ROP of lactide, these two enzymes showed similar catalytic efficacies toward Lac-OCA, and both of them afforded PLA with relatively high MWs and narrow polydispersities within a few hours. The MW of the obtained PLA could be further tuned by varying the lipase loading. As such, Lac-OCA demonstrated its high potential for use in preparing PLA in a controlled manner with biocatalysts under mild conditions.

4. BIOMEDICAL APPLICATIONS OF FUNCTIONAL PAHAS

Over the last several decades, nanomedicine has grown explosively for cancer diagnosis and treatment. Among a variety of polymeric nanomedicines, PAHA-based nanoparticles (NPs) have attracted much attention.[34−36] Compared with traditional widely used PAHAs such as PLA, PLGA, and PCL, introducing functional groups into the polymer backbone can largely tune the physicochemical properties, including hydrophobicity/hydrophilicity, crystallinity, and degradation kinetics,[13−15] making the materials more suitable for biomedical applications. Functionalized PAHAs can be accessed by two approaches: homopolymerization of functionalized monomers[20,21] and copolymerization with other cyclic esters.[19] Because of their ready availability, structure modulability, and high polymerizability, OCAs are promising monomers for the preparation of PAHAs with tailored architectures and accessible functionalities. This section presents several PAHAs obtained from ROP of OCAs and their applications for drug and gene delivery.

4.1. In Vivo Stable Anticancer Drug−PAHA Nanoconjugates

PLA is one of the most widely used PAHAs for the preparation of polymeric NPs because of its excellent biocompatibility and easy synthesis. We recently developed a strategy to apply ROP to the formulation of drug−PLA NPs with precisely defined compositions and well-controlled physicochemical properties.[36−38] The method allows for the quantitative incorporation of therapeutic agents bearing hydroxyl groups, e.g., paclitaxel (Ptxl), camptothecin (CPT), and doxorubicin (Doxo), to the end of PLA via ester bonds.[36−38] The drug loading of the obtained drug−PLA NPs can be fine-tuned by modulating the chain length of the parent drug−PLA conjugate. As such, it is necessary to synthesize drug−PLA conjugates with very short PLA chains in order to obtain high drug loadings. However, the use of very short PLA chains usually decreases the interchain interactions within the drug−PLA conjugates and hence affects the colloidal stability of the resulting drug−PLA NPs. A similar issue is observed when the surfaces of NPs are modified with poly(ethylene glycol) (PEG) through coprecipitation of the mPEG−PLA and drug−PLA conjugates.[36] The decreased hydrophobic interactions between the PLA polymer chains lead to NPs with reduced stability, increasing the tendency for particle disassembly and aggregation upon postinjection dilution due to depletion of the PEG shell. To address these issues and formulate in vivo-applicable NPs with the desired colloidal stability as well as high drug loading, one solution is to enhance the interchain interactions by increasing the hydrophobicity of the PLA polymer chains. Thus, Phe-OCA was chosen to prepare a PLA derivative bearing hydrophobic phenyl side chains and further used to formulate in vivo-stable anticancer CPT−PAHA NPs (Figure 4).[39]

BDI(EI)ZnN(TMS)$_2$ was reacted with CPT to form BDI(EI)Zn-CPT alkoxide in situ, which then catalyzed the ROP of Phe-OCA in a controlled manner. No unconjugated drug was found after completion of the polymerization, indicating 100% drug incorporation efficiency. Via nano-

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precipitation, the CPT−poly(Phe-OCA) conjugates (termed CPT-PheLA) were able to self-assemble into nanoconjugates (NCs) with sub-100 nm size and narrow size distributions (Figure 5A). Adding a mixture of mPEG-PheLA$_{100}$ (an amphiphilic copolymer with a PheLA segment of 21 kDa and an mPEG segment of 5 kDa) and CPT-PheLA$_{25}$ to vigorously stirred nanopure water resulted in NCs with a core−shell nanostructure with hydrophobic CPT-PheLA$_{25}$ as the core and PEG as the shell. The stability differences between PEGylated PheLA NCs and PLA NCs were then compared. Since PheLA affords stronger interchain hydrophobic interactions, the corresponding PheLA NCs were expected to be more stable and have a lower critical micelle concentration (CMC). The results showed that the CMC of mPEG-PheLA$_{100}$ NCs (0.022 mg/mL) was 50% lower than that of mPEG-PLA$_{100}$ NCs (0.045 mg/mL) (Figure 5B). Upon 100-fold dilution, the particle size of the mPEG-PLA NCs increased by 30% from 110 to 143 nm, while the size of the mPEG-PheLA NCs increased by only 10% from 108.8 to 119.9 nm. Thus, PEGylated PheLA NCs could still maintain their stable nanostructures upon dilution, while PEGylated PLA NCs may have complete disassembly.

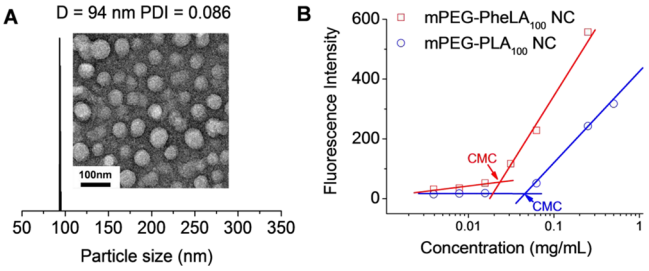

Figure 5. (A) DLS analysis and (inset) TEM image (negative stained, scale bar = 100 nm) of CPT-PheLA$_{100}$ NCs in water. (B) CMC determination of mPEG-PheLA$_{100}$ and mPEG-LA$_{100}$ from plots of the intensity of Nile Red versus the concentrations of mPEG-PheLA$_{100}$ and mPEG-LA$_{100}$, respectively. The CMCs were determined by taking the midpoints in the plots. Adapted from ref 39. Copyright 2013 American Chemical Society.

We anticipated that the increased stability of the PEGylated PheLA NCs would affect their biological performance. Thus, the pharmacokinetics and in vivo biodistribution of ^{64}Cu-labeled mPEG-PheLA NCs and mPEG-PLA NCs were compared (Figure 6A). The results showed that the clearance of mPEG-PheLA NCs from the blood was much slower than that of mPEG-LA NCs, with an almost 2 times higher plasma concentration of mPEG-PheLA NCs left in the first 5 h post injection (Figure 6B). To monitor their in vivo biodistributions, ^{64}Cu-DOTA-PheLA NCs and ^{64}Cu-DOTA-PLA NCs (50 mg/

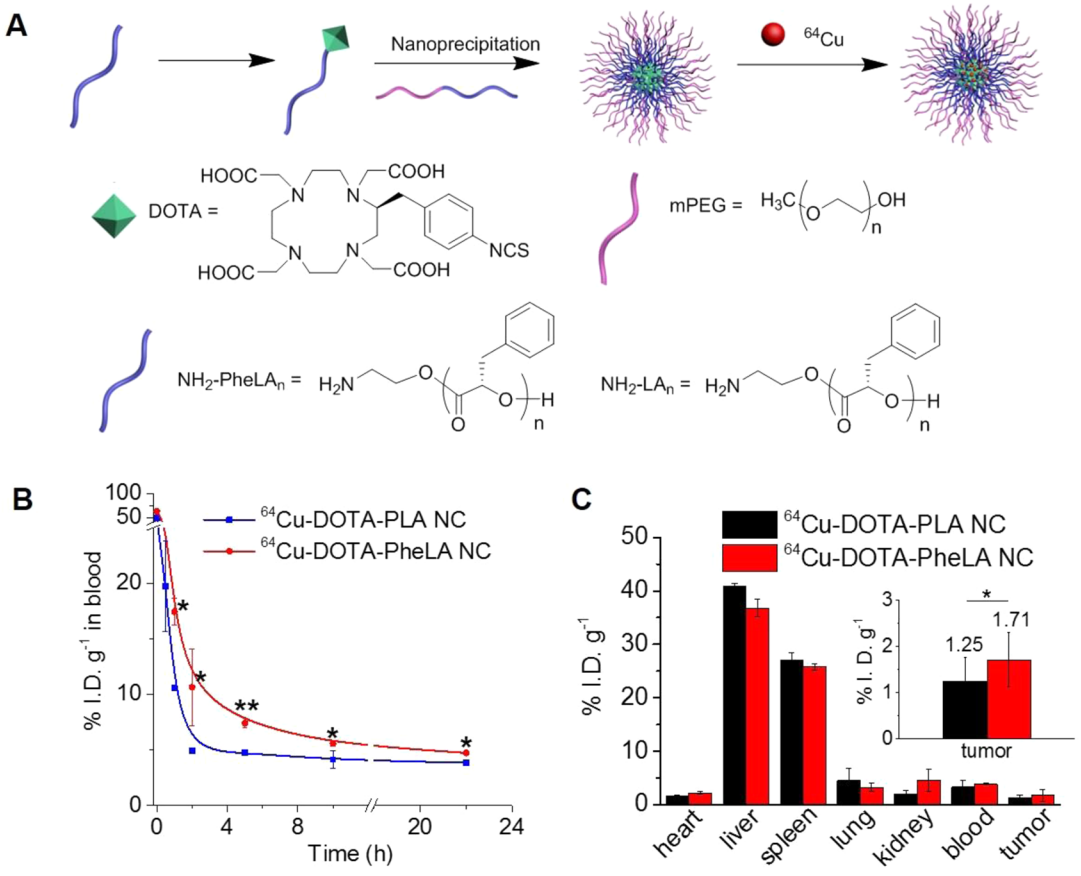

Figure 6. (A) Synthesis and formulation of ^{64}Cu-labeled PEGylated PheLA (^{64}Cu-DOTA-PheLA) NCs and PLA (^{64}Cu-DOTA-PLA) NCs. (B) Pharmacokinetics of ^{64}Cu-DOTA-PheLA NCs and ^{64}Cu-DOTA-PLA NCs in athymic nude mice (n = 3). Statistical significance was assessed by a two-sample unpaired Student's t-test; $0.01 < p \leq 0.05$ and $p \leq 0.01$ are considered statistically significant and highly significant differences and are denoted as * and **, respectively. (C) Biodistributions of ^{64}Cu-DOTA-PheLA NCs and ^{64}Cu-DOTA-PLA NCs in MCF-7 breast cancer bearing athymic nude mice (n = 3). All of the organ distributions are presented as percentages of injected dose per gram of tissue (% I.D. g^{-1}). Adapted from ref 39. Copyright 2013 American Chemical Society.

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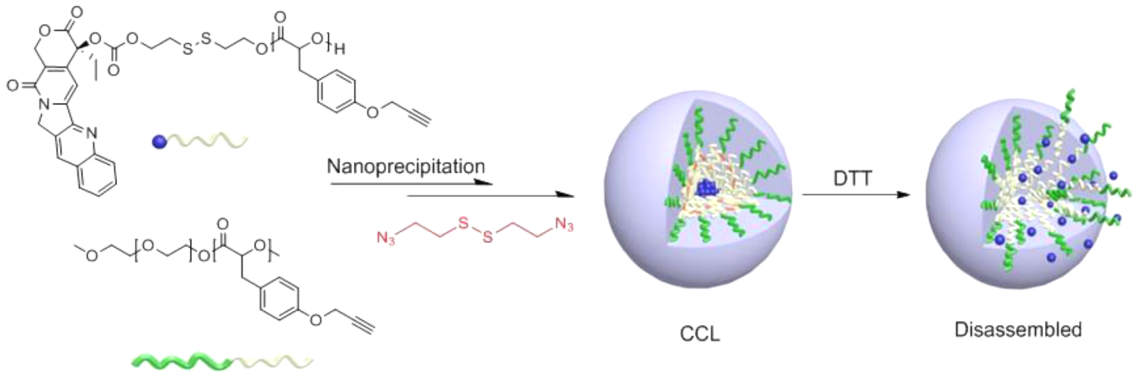

Figure 7. Preparation of core-cross-linked (CCL) micelles by co-nanoprecipitation of CPT-S-S-poly(Tyrosine(alkynyl)-OCA) and poly(Tyrosine-(alkynyl)-OCA). Adapted from ref 44. Copyright 2013 American Chemical Society.

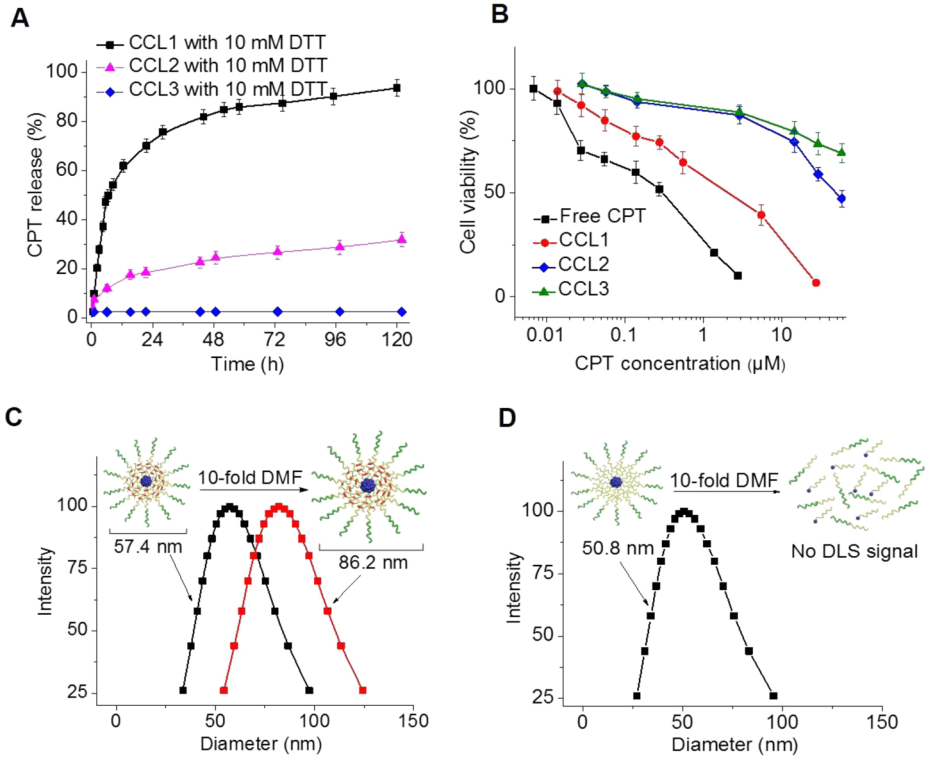

Figure 8. (A) In vitro release profiles of CPT from CCL1, CCL2, and CCL3 in phosphate-buffered saline (pH 7.4) at 37 °C (mean ± SD, n = 3). (B) Viability of MCF-7 breast cancer cells after treatment with free CPT or CCL micelles at various concentrations (CPT equivalent dose) for 48 h. (C, D) Distributions of hydrodynamic diameters of (C) CCLs and (D) UCLs in water before (black) and after (red) 10-fold dilution with DMF. Adapted from ref 44. Copyright 2013 American Chemical Society.

kg) were intravenously injected into MCF-7 human breast cancer bearing athymic nude mice. The tissues were harvested at 24 h post injection, and the ^{64}Cu radioactivities remaining in various organs including liver, heart, spleen, kidney, lung, and blood as well as in tumor tissues were measured and normalized against the total injected dose and the mass of tissue (%I.D. g^{-1}). The results demonstrated that the intratumoral accumulation of mPEG-PheLA NCs was 50% larger compared with that of mPEG-LA NCs (Figure 6C).

4.2. Core-Cross-Linked, Trigger-Responsive Drug–PAHA Micelles

In addition to in vivo stability, drug release kinetcis is another critical parameter when evaluating a drug delivery system. An ideal drug carrier should be able to control the release of the drug to minimize undesired systemic side effects. To meet this requirement, extensive efforts have been made to develop trigger-responsive polymeric micelles that can respond to a variety of internal or external triggers such as pH,[40] temperature,[41] glutathione (GSH),[42] and enzyme.[43] In view of the easy access of functional groups in PAHAs, this class of materials exhibits enormous potential for the preparation of smart, trigger-responsive micelles. We recently developed core-cross-linked (CCL) micelles that can undergo disassembly and concurrent drug release upon redox triggering.[44,45] CPT reacted with 2-hydroxyethyl disulfide to afford CPT-S-S-OH, which initiated ROP of 5-[4-(prop-2-yn-1-yloxy)benzyl]-1,3-dioxolane-2,4-dione (Tyrosine(alkynyl)-OCA) to yield CPT-S-S-poly(Tyrosine(alkynyl)-OCA) bearing a redox-responsive linker between the drug and poly(OCA) polymer. A mixture of CPT-S-S-poly(Tyrosine(alkynyl)-OCA)$_{20}$, mPEG-poly-

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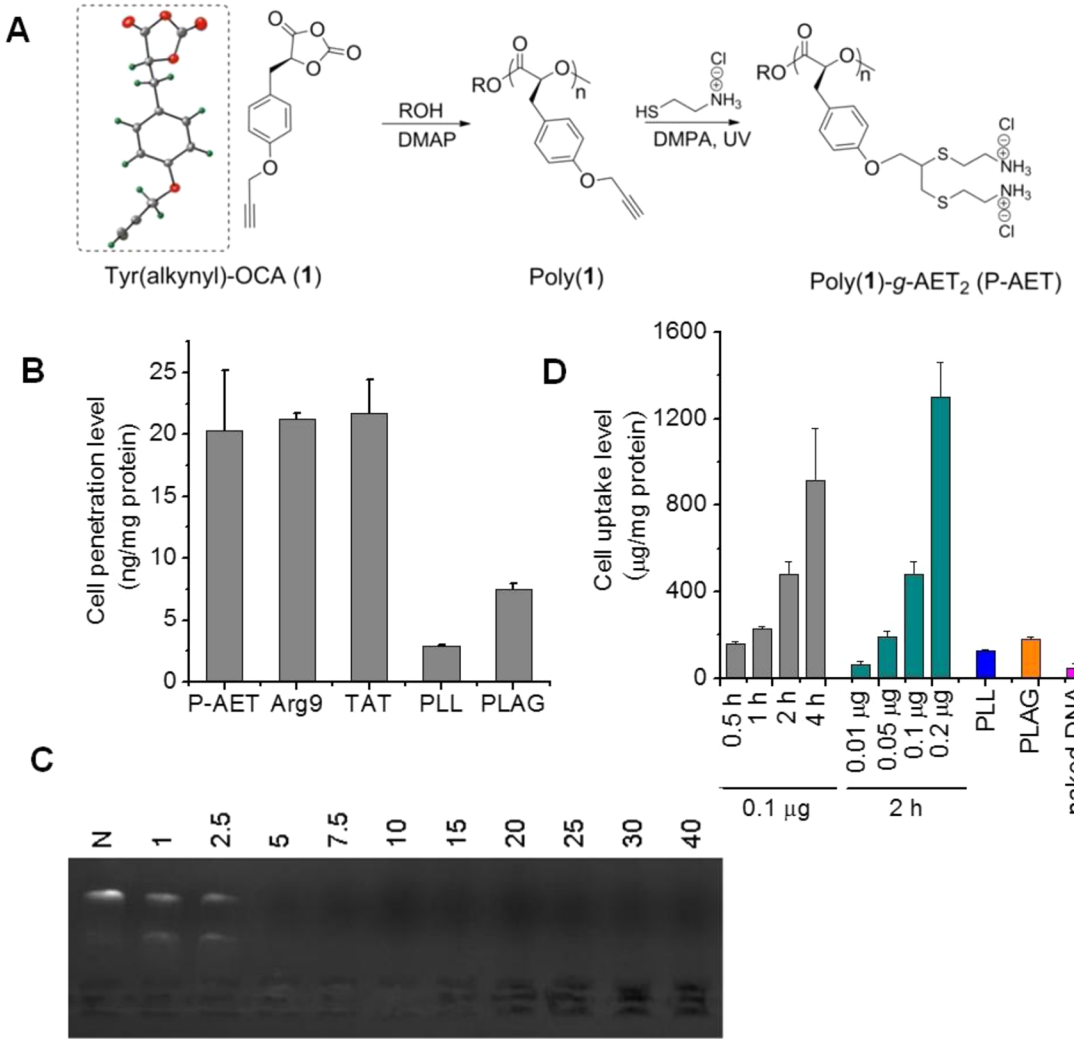

Figure 9. (A) Synthesis and functionalization of Poly(**1**) to form Poly(**1**)-*g*-AET$_2$. (B) Uptake of rhodamine-labeled P-AET in HeLa cells after incubation for 2 h. The uptake levels are expressed as nanograms of P-AET per milligram of cellular protein (n = 3). Tetramethylrhodamine-labeled Arg9 and HIV-TAT, rhodamine-labeled PLL, and PLAG served as the controls. (C) Gel retardation assay for analysis of the condensation of pCMV-Luc plasmid DNA with P-AET. N refers to naked DNA; the numbers are the N/P ratios of P-AET/DNA. (D) Uptake of Poly(**1**)/DNA polyplexes, PLL/DNA complexes, PLAG/DNA complexes, and naked DNA in HeLa cells at different concentrations (incubation time fixed at 2 h) or different incubation times (DNA amount fixed at 0.1 μg/well). Adapted from ref 31. Copyright 2012 American Chemical Society.

(Tyrosine(alkynyl)-OCA)$_{20}$, and diazide cross-linker in a molar ratio of 1:1:1.4 in DMF, copper chloride, and sodium ascorbate were added together into rapidly stirring deionized water to prepare CCL micelles via azide−alkyne click chemistry (Figure 7).

To analyze drug-release profiles upon redox triggering, we formulated three structurally different CCL micelles (CCL1 with disulfide linkers in both the conjugate and the core, CCL2 with disulfide linkers in the conjugate but not the core, and CCL3 with no disulfide linker in the conjugate or the core) and closely monitored CPT release from these micelles. We found that 81.7 ± 2.9% of the CPT was released from CCL1 within 24 h in the presence of 10 mM dithiothreitol (DTT) (Figure 8A) while no released CPT was detected in the absence of DTT, demonstrating the redox-triggered drug release. In comparison, the CPT release was much slower in CCL2, with 20% of the CPT being released under the same DTT treatment, presumably because the nondegradable, hydrophobic, cross-linked core made disulfide bonds inaccessible to DTT and further hampered the diffusion of released drug molecules. No CPT release after 4 days of DTT treatment was observed with the uncleavable CPT-poly(Tyrosine(alkynyl)-OCA) conjugate (Figure 8A). These distinct drug release kinetics greatly affected the anticancer efficiency of CCLs. In MCF-7 cells, CCL1 showed the highest cytotoxicity with an IC$_{50}$ value of 2.24 μM, which is presumably due to the structure disruption of the micelles and subsequent release of CPT upon intracellular redox triggering.

To further demonstrate the cross-linking of the hydrophobic core, we evaluated the diameter change of un-cross-linked (UCL) and CCL micelles after addition of DMF. CCL and UCL micelles showed nearly identical diameters in water (51 nm for UCL micelles and 57 nm for CCL micelles), as characterized by dynamic light scattering (DLS). Upon 10-fold dilution with DMF, the DLS signal of the UCL micelles disappeared, indicating complete disruption of the micellar structure (Figure 8D). In comparison, the structure of CCL micelles was well-maintained with only a slight increase in the particle diameter to 86 nm as a result of swelling of the core (Figure 8C). It was therefore substantiated that as a result of

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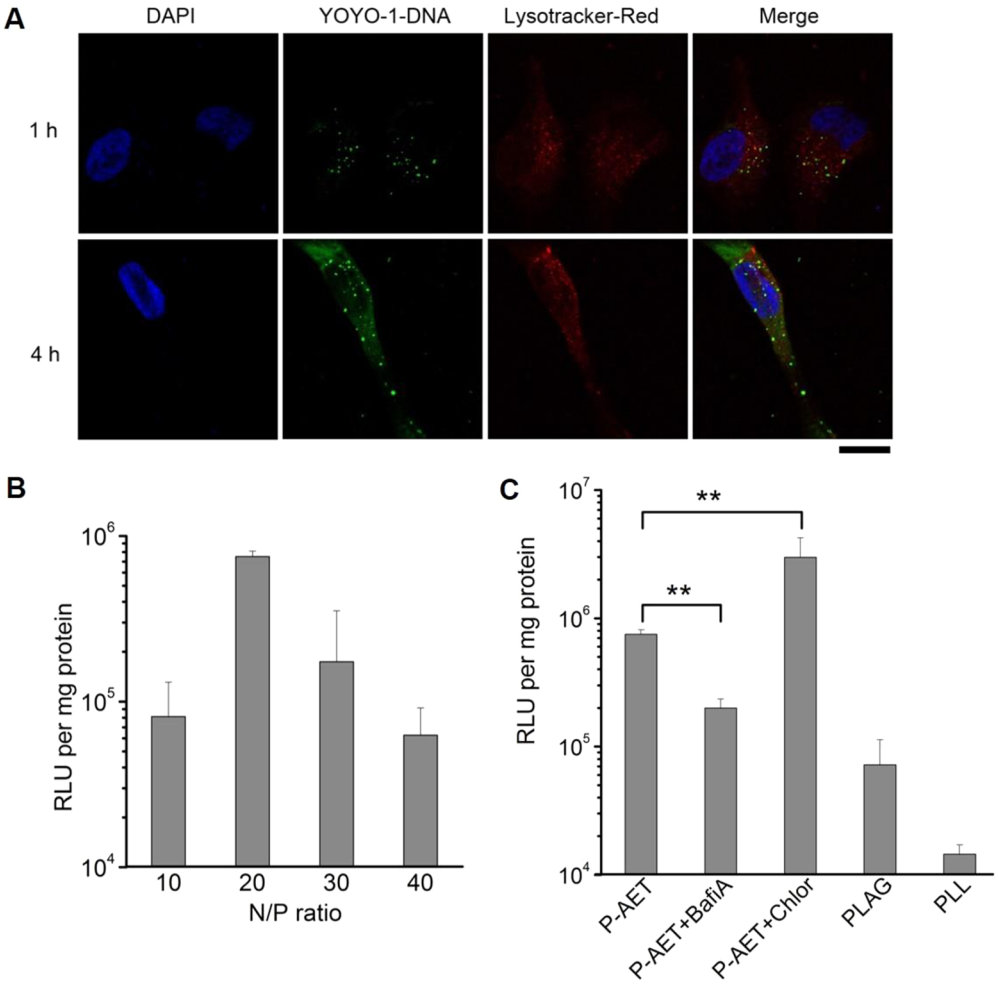

Figure 10. (A) CLSM images of HeLa cells following incubation with P-AET/pCMV-Luc plasmid DNA complexes for 1 or 4 h and subsequent staining with LysoTracker-Red. Scale bar = 20 μm. (B) In vitro transfection of P-AET/pCMV-Luc plasmid DNA complexes at various N/P ratios in HeLa cells. (C) Transfection efficiencies of complexes (N/P ratio = 20) in the presence of bafilomycin A1 (BafiA) or chloroquine (Chlor). PLL and PLAG served as controls. Adapted from ref 31. Copyright 2012 American Chemical Society.

the core cross-linking, CCL micelles afforded excellent stability against dilution and released the drug cargo upon redox-triggered cleavage of the disulfide bonds.

4.4. Degradable PAHAs toward Cell Penetration and Gene Delivery

Amino-functionalized PAHAs with the characteristics of biocompatibility and biodegradability have great potential for nonviral gene delivery applications. However, the use of aminated PAHAs as gene delivery vehicles has rarely been reported. The currently used cationic PAHAs are mainly synthesized by polycondensation. The molecular weights of the resulting PAHAs are usually poorly controlled, and the MWDs are generally broad.[46] Given the fact that ROP of OCAs affords functional PAHAs with controlled MWs and narrow MWDs, we proposed the synthesis of the side-chain-aminated PAHA Poly(**1**)-*g*-AET$_2$ via ROP of 5-(4-(prop-2-yn-1-yloxy)benzyl)-1,3-dioxolane-2,4-dione (Tyr(alkynyl)-OCA or **1**) followed by the thiol−yne click reaction with 2-aminoethanethiol hydrochloride (AET) (Figure 9A) and its use for nonviral gene delivery applications.[31]

Polymers with rich positive charge and long hydrophobic side chains are often reported to have cell penetration properties.[47] Thus, we hypothesized that Poly(**1**)$_{50}$-*g*-AET$_2$ (P-AET) would possess the desired cell penetration property because of its amine-terminated hydrophobic side chains. To demonstrate the hypothesis, the cellular uptake efficiency of P-AET was evaluated in HeLa cells. Nona-Arg (Arg9) and HIV-TAT, two well-known cell-penetrating peptides,[48] and poly(L-lysine) (PLL) and poly(L-arginine) (PLAG), two widely used cationic polypeptide-based gene delivery vectors, were used as controls. As shown in Figure 9B, P-AET exhibited performance comparable to those of Arg9 and HIV-TAT and better than those of PLL and PLAG by 3−5 times in terms of faster internalization (Figure 9B), demonstrating that P-AET can penetrate mammalian cell membranes efficiently. The DNA condensation ability of P-AET was then evaluated by a gel retardation assay with plasmid DNA encoding luciferase (pCMV-Luc) as the model gene. As shown in Figure 9C, P-AET was able to fully condense DNA at N/P ratios higher than 5, as confirmed by the restricted DNA migration in the agarose gel. With YOYO-1-labeled DNA, the cellular uptake level of P-AET/YOYO-1-DNA complexes in HeLa cells was quantified. The results showed that the cellular internalization of DNA was significantly enhanced by P-AET, and the amount of internalized YOYO-1-DNA was in proportion to the incubation time and P-AET addition (Figure 9D).

The cellular distribution of P-AET/YOYO-1-DNA complexes could also be observed by confocal laser scanning

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microscopy (CLSM). The colocalization of DNA (green fluorescence) in the cytoplasm with LysoTracker-Red-stained endolysosomes (red fluorescence) after treatment for 1 h indicated that the complexes were trapped in the endosomes after being endocytosed (Figure 10A). After incubation for 4 h, green fluorescence started to spread throughout the cell and some was colocalized with the nuclei, indicating that the internalized DNA could be transported into nuclei for gene transcription. Importantly, the green fluorescence and the red fluorescence were partially separated, indicating that P-AET could assist DNA in overcoming endosomal entrapment, one of major intracellular barriers against nonviral gene delivery. As a consequence of P-AET-mediated effective cell penetration and intracellular DNA delivery, the gene transfection efficiency of P-AET is significantly higher than those of PLAG and PLL at the optimal N/P ratio of 20 in HeLa cells (Figure 10B,C). It is noted that the gene transfection efficiency of P-AET/DNA complexes could be further improved by the addition of chloroquine (Figure 10C), presumably because chloroquine can buffer the pH of late endosomes/lysosomes and facilitate the escape of P-AET/DNA complexes from endosomal entrapment.[47] In contrast, the transfection efficiency of P-AET/DNA complexes was decreased when cells were treated with bafilomycin A1, which inhibits proton transport into endosomes (Figure 10C). Such observations strongly suggested that the primary amine groups in the P-AET structure play a vital role in buffering the protons in endosomes, thereby increasing the osmotic pressure, finally leading to endocytic vesicle disrupture and facilitating the escape of complexes from endosomal entrapment.

5. CONCLUSION

There has been fascinating progress in the PAHA field in the past decade. The ongoing advances in the synthesis and ROP of OCAs will generate a streamlined process for the facile synthesis of various functionalized PAHAs with well-defined physicochemical and biological properties. The widely available starting materials from naturally existing amino acid renewable resources and easy postmodification strategies such as azide–alkyne cycloaddition and thiol–yne click reaction have made it possible to prepare a handful of PAHAs with controlled MWs, narrow MWDs, high terminal group fidelities, and structural diversities. This new generation of PAHAs and their self-assembled nanosystems as biomaterials are opening up new exciting approaches for biological applications. PAHA-based, advanced nanosystems used for controlled release, tissue engineering, and drug and gene delivery applications are anticipated. Nevertheless, several important questions remain to be answered to fully demonstrate OCA polymerization as an attractive approach for the synthesis of PAHAs. First, the synthesis of OCAs should be further simplified. ROP of OCAs resembles to some extent the ROP of amino acid *N*-carboxyanhydrides (NCAs). The use of phosgene for monomer preparation is one of the biggest limitations of both NCA and OCA. To fully advance the synthesis of functional PAHAs via OCAs that can be as broadly used as PLA, the synthesis of OCAs must be further simplified using easy-to-handle, safer reagents. Second, only less than a dozen OCA monomers have been reported to date. An open question is how many more OCA monomers can be synthesized easily and whether they are easy to handle and polymerize in a controlled manner. Third, it is still unclear whether there are key applications or important biomedical or biological properties for PAHAs that are only accessible via OCA polymerization. We are aware of these existing challenges and see exciting opportunities in the field of ROP of OCAs and the derived PAHAs.

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Notes

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