Communication

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Facile Functionalization of Polyesters through Thiol-yne Chemistry for the Design of Degradable, Cell-Penetrating and Gene Delivery Dual-Functional Agents

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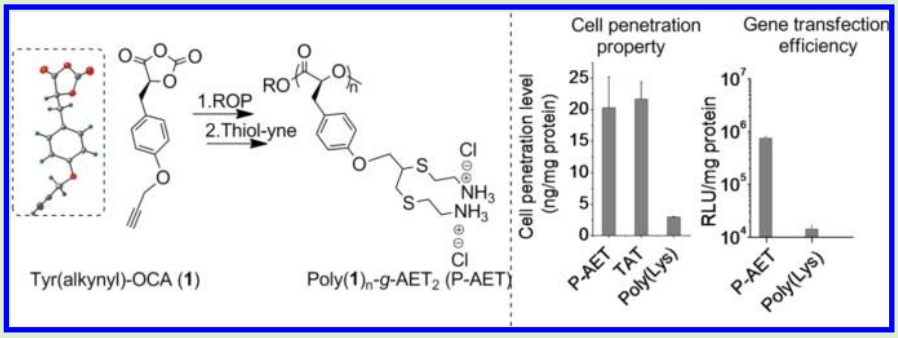
Supporting Information



ABSTRACT: Synthesis of polyesters bearing pendant amine groups with controlled molecular weights and narrow molecular weight distributions was achieved through ring-opening polymerization of 5-(4-(prop-2-yn-1-yloxy)benzyl)-1,3-dioxolane-2,4-dione, an *O*-carboxyanhydride derived from tyrosine, followed by thiol-yne "click" photochemistry with 2-aminoethanethiol hydrochloride. This class of biodegradable polymers displayed excellent cell penetration and gene delivery properties with low toxicities.

Polypeptides are the first set of materials used as nonviral gene delivery vectors. Well-known examples include poly-L-lysine (PLL)[1−3] and poly-L-arginine (PLR).[4−8] These cationic polypeptides can bind and condense anionic DNA through electrostatic interactions, forming nanoscale polymer/DNA complexes, termed polyplexes, and promoting intracellular delivery of the nucleic acids that are otherwise impermeable to cell membranes.[3,9−16] When used in gene delivery, however, polypeptides often suffer from low transfection efficiencies,[17,18] which is attributed in part to their low membrane activities. Limited cellular internalization and poor endosomal release of polyplexes are usually observed in synthetic polypeptide-mediated gene delivery. In addition, polypeptide-based materials are subject to slow degradation, resulting in prolonged retention when applied in vivo, which in turn gives rise to appreciable toxicity associated with their high molecular weights (MWs).[19−22] A polycation with faster degradation profiles and high cell penetration and transfection efficiency can potentially be a less toxic alternative to polypeptide in nonviral gene delivery.

Polyesters are a class of well-known biodegradable materials and have been extensively used in controlled release and drug delivery.[23−27] Amino-functionalized polyesters are potentially excellent candidates for nonviral gene delivery due to their biocompatibility and biodegradability. To the best of our knowledge, aminated polyesters used as gene delivery vehicles are still rare, and most of the cationic polyesters are prepared through polycondensation, which affords polymers with poorly controlled MWs and broad molecular weight distributions (MWDs).[28−33] Controlled synthesis of aminated polyesters for gene delivery applications has been attempted.[34−37] Synthesis of side-chain functionalized polyesters via *O*-carboxyanhydrides (OCAs) were recently reported;[38−41] ring-opening polymerizations (ROPs) of OCAs in the presence of 4-dimethylaminopyridine (DMAP) allowed the preparation of polyesters bearing protein-like side chains with controlled MWs and narrow MWDs. Taking advantage of the OCA ROP strategy, we report here the design and synthesis of side-chain aminated poly(α-hydroxy acids) (PAHAs) by integrating ROP of 5-(4-

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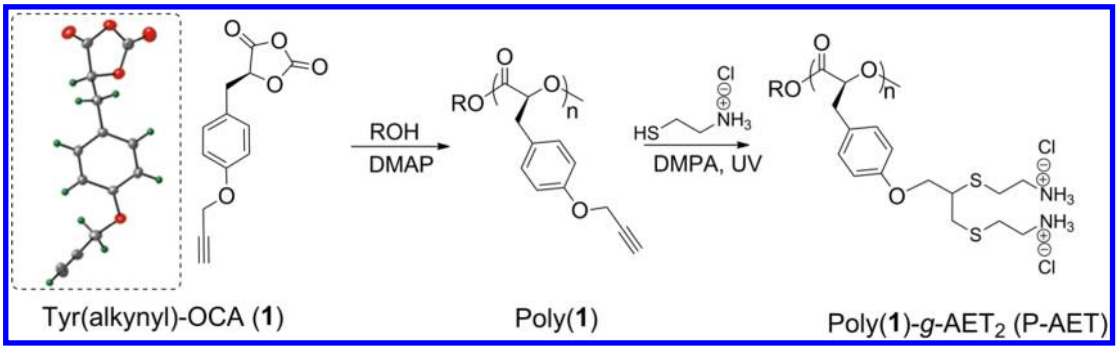
Scheme 1. Synthesis and Functionalization of Poly(1) to Form Poly(1)-*g*-AET$_2$



(prop-2-yn-1-yloxy)benzyl)-1,3-dioxolane-2,4-dione (Tyr-(alkynyl)-OCA or **1**, Scheme 1) with thiol-yne "click" photochemistry with 2-aminoethanethiol hydrochloride (AET); the latter reaction was recently developed and has found widespread applications in materials functionalization and conjugation.[42−65] Poly(**1**)-*g*-AET$_2$ (Scheme 1), a PAHA bearing pendant amine group, was found to display excellent cell penetration and gene delivery properties.

To synthesize these materials, we first attempted to use a well-controlled polymerization method to prepare the parental PAHAs that are amenable for side group amination (Scheme 1). OCA monomer **1** was synthesized through the ether-

Table 1. ROP of 1 by Using DMAP as the Catalyst[a]

entry	M/I	$M_{n,cal}$[b] ($\times 10^{-3}$ g/mol)	$M_{n,GPC}$[c] ($\times 10^{-3}$ g/mol)	M_w/M_n[c]
1	30	6.1	5.8	1.15
2	50	10.3	11.1	1.12
3	100	20.5	21.0	1.02
4	150	30.5	32.0	1.04
5	200	40.6	40.5	1.03
6	300	60.8	63.3	1.04
7	400	80.8	78.8	1.04
8	600	121.2	116.2	1.07

[a]All reactions were performed in a glovebox at room temperature using pyrenebutanol as the initiator. The conversion of monomer was monitored by analyzing the OCA anhydride band (1810 cm^{-1}) using FTIR. [b]Calculated from monomer to initiator ratio with 100% monomer conversation. [c]Determined by GPC.

ification of commercially available Boc-$_L$-Tyr-OH (Boc = *tert*-butoxycarbonyl) followed by introduction of an alkyne group by using propargyl bromide, deprotection of the Boc group, and cyclization of the resulting α-hydroxyl acid (Scheme S1). The crude OCA was purified by silica gel column chromatography with dichloromethane (CH_2Cl_2) as eluent,[66] and then recrystallized with hexane/CH_2Cl_2 to afford **1** in crystalline form. The molecular structure of **1** was verified by NMR, FTIR, and X-ray (Figure S1, S2, S3 and S9).

Bourissou reported previously that DMAP mediated controlled ROP of Lac-OCA, an OCA derived from alanine.[40] Following the same strategy, we attempted to use DMAP as the catalyst for the ROP of **1**. At monomer/initiator (M/I) ratios ranging from 30 to 600, the MWs of poly(**1**) were found to agree excellently with the theoretical MWs (Figure 1a, Table 1). The M_n values of poly(**1**) at M/I ratios of 30 and 600 were 5.8×10^3 and 116.2×10^3 g/mol, respectively, both of which were very close to the expected MWs (Table 1). Polymerizations at other M/I ratios between 30 and 600 also gave poly(**1**)s with expected MWs (Table 1). The MWDs of poly(**1**)s were fairly narrow, ranging from 1.02 to 1.15 (Table 1). Analysis of the poly(**1**)s obtained at various M/I ratios by gel permeation chromatography (GPC) showed monomodal distributions (Figure 1b). The MWs of poly(**1**)s was in linear correlation with the conversions of **1** (Figure 1c), suggesting that the polymer chains were propagated through living chain ends. To demonstrate that the polymerization chain ends were still active after the polymerization completed, we monitored the MW changes of poly(**1**)s with sequentially added monomers. The first block of poly(**1**) had a monnomodal GPC curve with a M_n value of 20.5×10^3 g/mol and MWD of 1.06 at a **1**:pyrenebutanol:DMAP molar ratio of 100:1:1. After addition of the second portion of 100 equiv of **1** or *O*-benzyl-$_L$-serine carboxyanhydrides (Ser(Bn)-OCA), monomodal GPC curves were obtained and the MWs of the final polymers were in excellent agreement with the theoretical values (Figure S5, Supporting Information). The plot of Ln($[M]_0/[M]$) versus polymerization time revealed that the polymerization was in first order correlation with the concentration of **1** (Figure 1d). These experiments demonstrated that DMAP facilitated well-controlled, living ROP of **1** and allowed for preparation of poly(**1**)s containing alkynyl pendant functional groups amenable for "click" type of conjugation.

We next modified the side chain alkynyl funcitonal groups of poly(**1**)s through the thiol-yne "click" reaction (Scheme 1).[67,68] Poly(**1**)$_{50}$, poly(**1**) prepared at the M/I ratio of 50, was allowed to react with AET in solvent mixture of dimethylformamide (DMF) and H_2O (9:2, v/v) with the molar ratio of thiol/alkyne controlled at 8 and 2,2-dimethoxy-2-phenylacetophenone being used as the photoinitiaor at UV intensity of 20 mW/cm^2. The reaction was followed by monitoring the methylene proton peak adjacent to the alkyne group (peak e, Figures 2 and S6). The conversion of the thiol-yne reaction was over 98% for a 40-min reaction (Figure S7). The alkyne group of the poly(**1**)$_{50}$ reacted readily with an AET molecule to form a vinyl sulfide, followed by a subsequent thiol-ene reaction of the vinyl sulfide with the second AET molecule to yield the 1,2-disubstituted adduct of the alkyne.[67,69,70] The water-insoluble poly(**1**) was changed to an amine-containing, water-soluble poly(**1**)-*g*-AET$_2$ via the one-step thiol-yne reaction. The thiol-yne chemistry is highly efficient and reproducible, does not generate side products, and does not involve harsh conditions or require the use of metal catalysts, making thiol-yne chemistry a powerful postmodification method.

Poly(**1**)$_{50}$-*g*-AET$_2$ (P-AET) possesses cationic amine groups that are essential for DNA condensation and hydrophobic side chains connecting the amine groups to the polymer backbone. Because polymers with a long hydrophobic side chain

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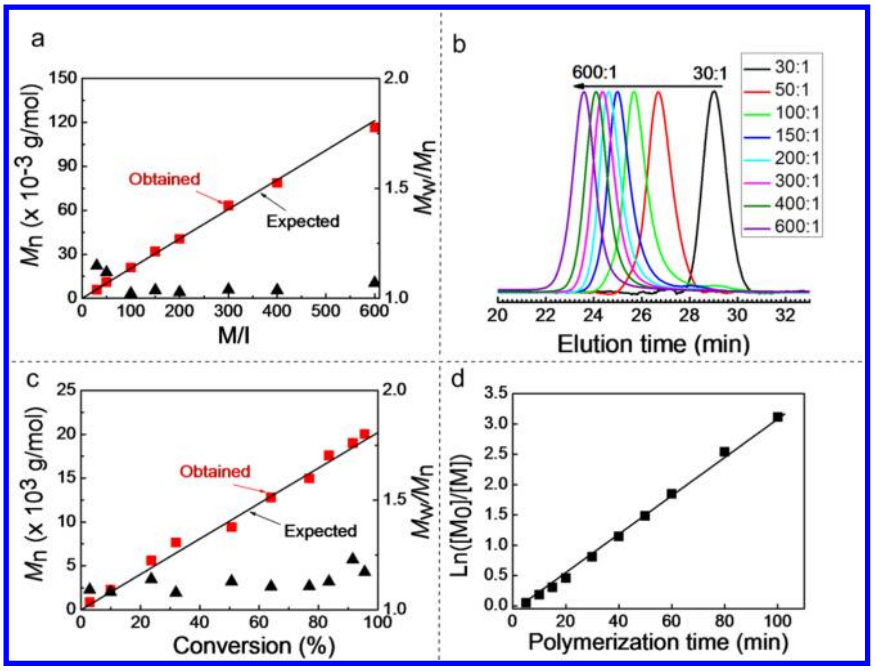

Figure 1. (a) Plot of the values of M_n (■) and M_w/M_n (▲) of poly(**1**)s versus the M/I ratios of the ROP of **1** with pyrenebutanol as the initiator and DMAP as the catalyst ($[DMAP]_0 = [pyrenebutanol]_0 = 1$ mM). (b) Overlay of the GPC light scattering curves of poly(**1**)s at various M/I ratios. (c) Plot of the values of M_n (■) and M_w/M_n (▲) of poly(**1**)s versus the conversion of **1** ($[M]_0 = 0.1$ M, $[pyrenebutanol]_0 = [DMAP]_0 = 1$ mM). (d) Plot of $Ln([M]_0/[M])$ versus the ROP time of **1** ($[M]_0 = 0.1$ M, $[DMAP]_0 = [pyrenebutanol]_0 = 0.001$ M).

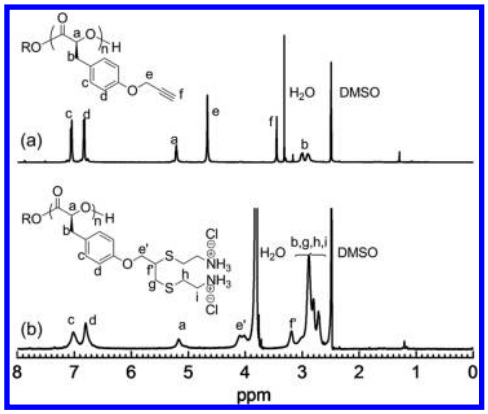

Figure 2. ^{1}H NMR spectra of poly(**1**)$_{50}$ (a) and poly(**1**)$_{50}$-*g*-AET$_2$ (b) in DMSO-d_6.

terminated with a cationic group are often found to have membrane activity,[71,72] we were motivated to explore the cell penetration property of P-AET. P-AET was labeled with rhodamine, and its cell penetration efficiency was evaluated in HeLa cells. As shown in Figure 3a, the rhodamine-labeled P-AET showed comparable cell uptake efficiency to nona-Arg (Arg9) and HIV-TAT, two well-known cell penetration peptides,[73] but outperformed PLL and PLR and got internalized to cells 3−5 times faster than these two extensively used cationic polypeptide gene delivery vectors. The uptake level increased with incubation time and the feeding concentration (Figure 3b,c). These experiments demonstrated that P-AET was able to efficiently penetrate mammalian cell membranes.

Upon identifying the cell penetration properties of the P-AET, we evaluated whether it could facilitate the intracellular

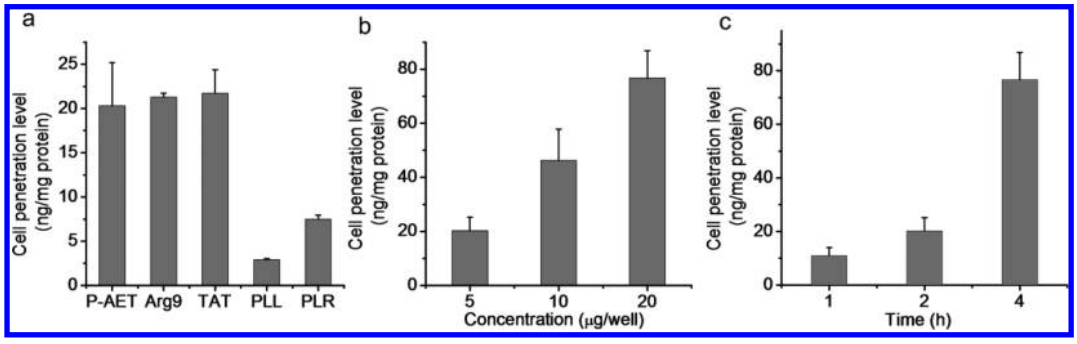

Figure 3. Cell uptake of rhodamine-labeled P-AET. (a) Uptake of rhodamine-labeled P-AET in HeLa cells. Cells in 96-well plates were treated with the polymer (5 μg/well) for 2 h. The uptake level was expressed as nanograms (ng) of P-AET per milligram (mg) of cellular protein ($n = 3$). TAMRA-labeled Arg9 (TAMRA = tetramethylrhodamine; Arg9 = nona-arginine) and HIV-TAT, rhoadmine-labeled PLL and PLR served as the controls. (b) Concentration-dependent uptake of rhoadmine-labeled P-AET in HeLa cells. Cells were treated with the polymer (5, 10, and 20 μg/well) for 2 h ($n = 3$). (c) Time-dependent uptake of rhoadmine-labeled P-AET in HeLa cells. Cells were treated with the polymer (5 μg/well) for 1, 2, and 4 h, respectively ($n = 3$).

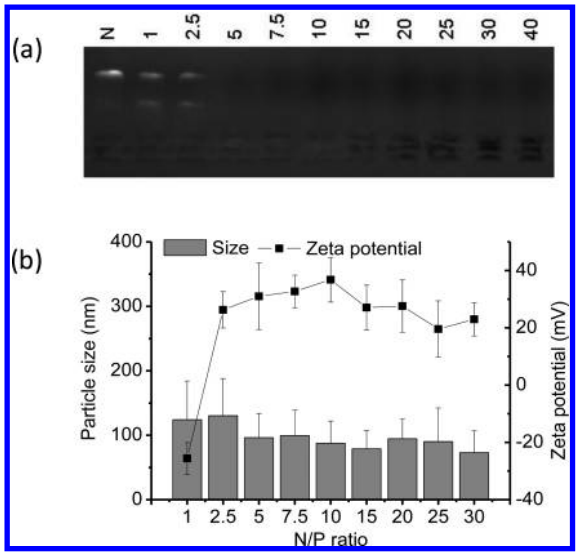

Figure 4. Characterization of the P-AET/pCMV-Luc plasmid DNA complexes. (a) Gel retardation assay for the analysis of condensation of pCMV-Luc plasmid DNA with P-AET. N refers to naked DNA; the numbers are the N/P ratios of P-AET/DNA. (b) The size and zeta potential of P-AET/pCMV-Luc plasmid DNA complexes at different N/P ratios as determined by DLS.

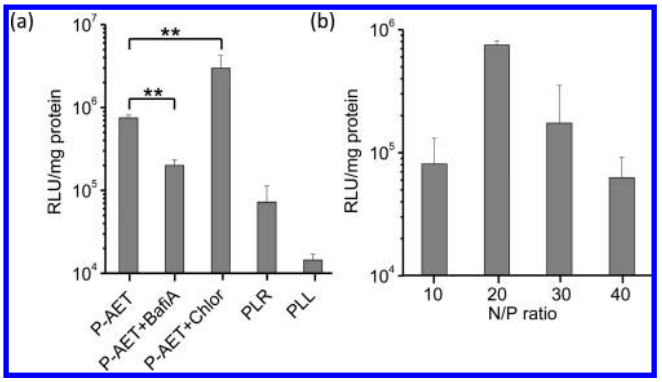

Figure 6. In vitro transfection of P-AET/pCMV-Luc plasmid DNA complexes in HeLa cells. (a) Transfection efficiencies of complexes (N/P ratio of 20) in the presence of bafilomycin A1 (BafiA) or chloroquine (Chlor). PLL and PLR served as controls. (b) Transfection efficiencies of complexes at various N/P ratios.

delivery of DNA. Plasmid DNA encoding luciferase (pCMV-Luc) was used as the model gene, and gel retardation assay was adopted to evaluate the ability of P-AET to condense DNA. As shown in Figure 4, P-AET could fully condense DNA at any N/P ratio at or above 5, evidenced by the DNA retardation assay in a 1% agarose gel. Dynamic light scattering (DLS) measurement further demonstrated that P-AET was able to form nanoscale complexes with DNA at N/P ratios above 5

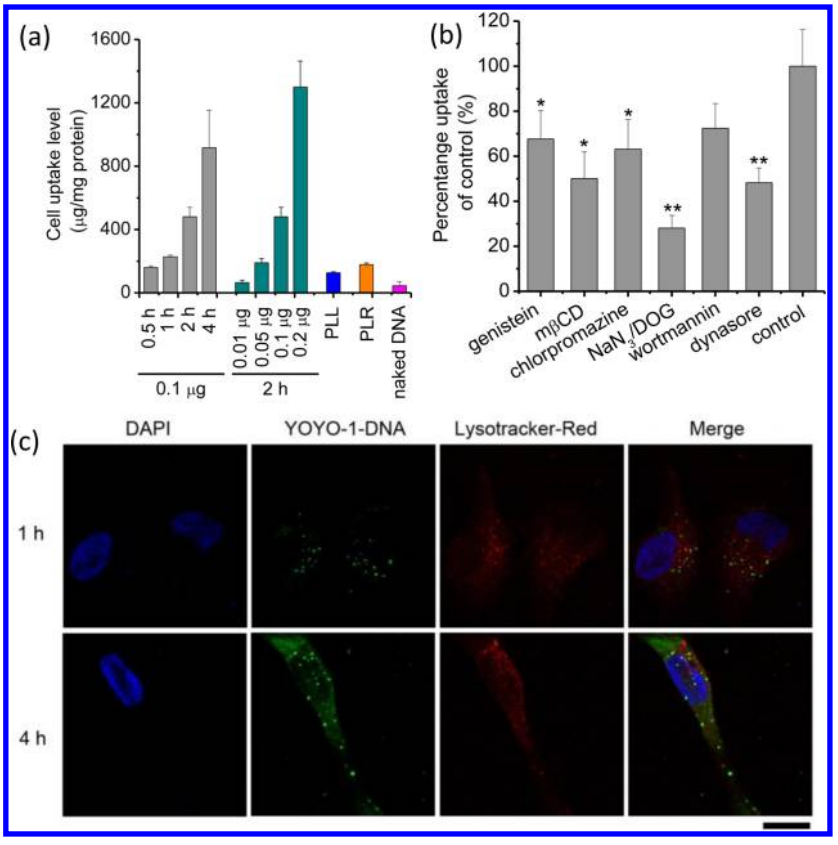

Figure 5. Cell uptake level and intracellular kinetics of P-AET/pCMV-Luc plasmid DNA complexes in HeLa cells (pCMV-Luc was labeled with YOYO-1). (a) Uptake of polyplex in HeLa cells at different concentrations with fixed incubation time (2 h) or with different incubation times at a fixed amount of DNA used for cell uptake (0.1 μg/well). PLL/DNA complexes, PLR/DNA complexes, and naked DNA served as the controls. (b) Elucidation of the cell uptake mechanisms of P-AET/DNA complexes by using various endocytic inhibitors. Results were expressed as the percentage of uptake level of control cells in the absence of the inhibitors. (c) 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. Bar = 20 μm.

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with average diameters of 90−100 nm and zeta potentials of 20−30 mV. By labeling the DNA with YOYO-1 (one YOYO-1 molecule per 50 DNA base pairs),[74] we evaluated the uptake of P-AET/YOYO-1-DNA complexes in HeLa cells. As shown in Figure 5a, P-AET notably enhanced the cell uptake level of DNA, and the amount of P-AET internalized were in proportional to the incubation time or the amount of P-AET applied to the cells. P-AET outperformed PLL and PLR for gene delivery, which could be attributed to its substantially better cell penetration property. By performing the cell uptake study in the presence of various endocytic inhibitors, we probed into the mechanisms underlying the cellular internalization of P-AET/DNA complexes. NaN_3/deoxyglucose (DOG) completely blocked endocytosis by depleting the energy; chlorpromazine inhibited clathrin-mediated endocytosis (CME) by triggering the dissociation of the clathrin lattice; genisteine and methyl-β-cyclodextrin (mβCD) inhibited caveolae pathway by inhibiting tyrosine kinase and depleting cholesterol, respectively; dynasore inhibited both CME and caveolae by inhibiting dynamin; wortmannin inhibited macropinocytosis by inhibiting phosphatidyl inositol-3-phosphate.[75,76] As shown in Figure 5b, the cell uptake level of YOYO-1-DNA was inhibited by 70% in the presence of NaN_3/DOG, suggesting that majority of the complexes were internalized through endocytosis while the rest of the complexes entered the cells via energy-independent processes, such as physical binding and diffusion. The cell uptake was inhibited by genistein, mβCD, chlorpromazine, and dynasore, suggesting that the endocytosis was related to both caveolae- and clathrin-mediated pathways. Comparatively, wortmannin exerted unappreciable inhibitory effect, which indicated that the macropinocytosis pathway was not involved during the internalization of the P-AET/DNA complexes. Confocal laser scanning microscopy (CLSM) was further used to observe the intracellular distribution of complexes. Punctated spots of DNA (green fluorescence) were observed in the cytoplasm after 1-h incubation of the cells and the P-AET/DNA complexes, which correlated to the endocytosed complexes (Figure 5c). When the incubation time was increased to 4 h, green fluorescence spread throughout the cell, indicating extensive intracellular internalization of DNA. We also noted nuclear distribution of DNA after 4-h incubation, suggesting that internalized DNA could be transported to the nuclei and potentially facilitate gene transcription. Internalized DNA colocalized with LysoTracker-Red-stained endolysosomes after 1 h of incubation, which confirmed endosomal entrapment of the complexes and endocytosis uptake mechanism. However, partial separation of the green fluorescence from red fluorescence was noted after 4 h of incubation, suggesting that P-AET might be able to mediate effective DNA release from endosomes, one of the most critical barriers to effective gene transfection.

In light of the capability of P-AET to penetrate cell membranes efficiently and deliver DNA intracellularly, it led to effective gene transfection in HeLa cells at the optimal N/P ratio of 20, markedly outperforming PLR and PLL (Figure 6a,b). Chloroquine that buffers the pH of late endosomes/lysosomes[77,78] significantly enhanced the gene transfection efficiency of P-AET/DNA complexes (Figure 6a), suggesting that the P-AET/DNA complexes experienced endosomal entrapment during transfection. Bafilomycin A1, which inhibits proton transport into endosomes,[77] resulted in significantly reduced transfection efficiency when it was used in P-AET/DNA complex-mediated gene delivery (Figure 6a). Such outcome strongly suggested that P-AET was able to mediate endosomal escape of polyplexes via the "proton sponge effect",[77] which was likely attributed to the amine groups in the P-AET structure that buffered the protons in endosomes and thereby increased the osmotic pressure to ultimately cause the rupture of endosomes. MTT assay revealed much lower cytotoxicity of P-AET than PLL and PLR in HeLa cells following a 4-h incubation in the presence of the polymer and an additional 20-h incubation in the absence of the polymer (Figure S8), substantiating the usefulness of this class of biodegradable polymers in nonviral gene delivery without compromising cell viability and biological functions.

In conclusion, we presented a novel and facile strategy for the synthesis and functionalization of polyesters via well-controlled ROP of OCAs and subsequent side-chain modification via thiol-yne photochemistry. Using this strategy, we designed and developed poly(**1**)-*g*-AET_2, a polyester bearing pendant amine groups capable of both cell penetration and gene delivery. With the desired biodegradability, low toxicity, and simplicity of tuning the backbone and side-chain structures, this class of functional polyesters can potentially have broad biological and biomedical applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, including NMR spectra, FTIR, X-ray, and MTT results. This material is available free of charge via the Internet at http://pubs.acs.org

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Author Contributions

‖Equal contribution.

Notes

The authors declare no competing financial interest.

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