A Virus-Mimicking pH-Responsive Acetalated Dextran-Based Membrane-Active Polymeric Nanoparticle for Intracellular Delivery of Antitumor Therapeutics

Saowanee Wannasarit, Shiqi Wang,* Patrícia Figueiredo, Claudia Trujillo, Francesca Eburnea, Lorena Simón-Gracia, Alexandra Correia, Yaping Ding, Tambet Teesalu, Dongfei Liu, Ruedeekorn Wiwattanapatapee, Hélder A. Santos,* and Wei Li*

Achieving cellular internalization and endosomal escape remains a major challenge for many antitumor therapeutics, especially macromolecular drugs. Viral drug carriers are reported for efficient intracellular delivery, but with limited choices of payloads. In this study, a novel polymeric nanoparticle (ADMAP) is developed, resembling the structure and functional features of a virus. ADMAP is synthesized by grafting endosomolytic poly(lauryl methacrylate-co-methacrylic acid) on acetalated dextran. The endosomolytic polymer mimics the capsid protein for endosomal escape, and acetalated dextran resembles the viral core for accommodating payloads. After polymer synthesis, the subsequent controlled nanoprecipitation on a microfluidic device yields uniform nanoparticles with high encapsulation efficiency. At late endosomal pH (5.0), the ADMAP particles successfully destabilize endosomal membranes and release the drug payloads synergistically, resulting in a greater therapeutic efficacy compared with free anticancer drugs. Further conjugation of a tumor-penetrating peptide enhances the antitumor efficacy toward 3D spheroids and finally leads to spheroid disintegration. The unique structure along with the synergistic endosomal escape and drug release make ADMAP nanoparticles favorable for the intracellular delivery of antitumor therapeutics.

1. Introduction

Cytoplasmic delivery of therapeutics is critical for achieving satisfying efficacy.^[1,2] The precise control over the drug release on a cellular level can maximize the available pharmacologically

active molecule concentration.^[3,4] However, most membrane-impermeable drugs, including oligonucleotides and proteins, rely on endocytosis for cellular internalization.^[5–7] After endocytosis, the internalized payloads are typically entrapped in endosomes and finally processed to lysosomes where they may become degraded by enzymes.^[8,9] Therefore, endosomal escape before lysosomal degradation is critical for successful delivery of these payloads.

Viruses are nanosized infectious particles, composed of compactly packaged genomes surrounded by protective capsid protein assemblies.^[10] The capsid proteins not only protect the inner genome from exogenous degradation, but also undergo conformational changes and become endosomolytic once activated by acidic endosomal environments.^[11,12] This enables the viruses to escape endosomes within minutes and be released into cytoplasm, followed by nuclear transport.^[13,14] Because

of the naturally evolved, elaborate and hierarchical structure, viruses can efficiently infect cells and replicate their genomes within host cells.

Inspired by the hierarchical structure of viruses, there are some artificial virus-mimicking particles developed for

S. Wannasarit, Dr. S. Wang, P. Figueiredo, C. Trujillo, F. Eburnea, A. Correia, Dr. Y. Ding, Dr. D. Liu, Prof. H. A. Santos, Dr. W. Li
Drug Research Program
Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
FI-00014 Helsinki, Finland
E-mail: shiqi.wang@helsinki.fi; helder.santos@helsinki.fi; wei.li@helsinki.fi
S. Wannasarit, Prof. R. Wiwattanapatapee
Department of Pharmaceutical Technology
Faculty of Pharmaceutical Sciences
Prince of Songkla University
90110 Hat Yai, Thailand

Dr. L. Simón-Gracia, Prof. T. Teesalu
Laboratory of Cancer Biology
Institute of Biomedicine
Centre of Excellence for Translational Medicine
University of Tartu
50411 Tartu, Estonia
Dr. D. Liu, Prof. H. A. Santos
Helsinki Institute of Life Science (HiLIFE)
University of Helsinki
FI-00014 Helsinki, Finland

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intracellular delivery of drugs, genes and proteins, such as self-assembled capsid proteins, modifying capsid proteins on nanoparticles (NPs) and multicomponent polymeric assemblies.^[15–19] Whatever the components of the virus-mimicking particles, the key requirements for fulfilling the functions include biocompatibility, cell binding/internalization and endosomal escape.^[20]

Compared with viral protein derived delivery systems, polymeric intracellular delivery materials are more versatile, capable of incorporating different payloads and ease to modify with target moieties.^[21,22] A common approach is to use cationic polymers, such as polyethylenimine (PEI) and polyamidoamine (PAMAM), with buffering capacity and subsequent osmotic swelling of endosomes, to induce “proton-sponge” effects.^[23] Along with membrane destabilization by these positively charged polymers, endosome can burst and release its contents.^[24] Despite the high efficiency, these polymers also have high cytotoxicity profile, because of the nonspecific binding to cell membranes and possible cellular apoptosis triggered by complete endosomal rupture.^[25] Other polymers induced endosomal escape by membrane fusion or pore formation, which are considered as less cytotoxic compared with endosome rupture.^[26] With temporarily or permanently enhanced membrane permeability, payloads can be released from endosomes without significant leakage of endosomal contents. For example, Hoffman and co-workers and Leroux and co-workers reported poly(acrylic acid) derivatives with short hydrophobic pendant groups, which destabilized lipid membranes at endosomal pH, but not physiological pH for gene and protein delivery.^[27–33] Recent modifications of these poly(acrylic acid) derivatives led to endosomolytic polymers capable of antigens and immunostimulators to enhance cancer immunotherapy.^[34,35] Chen et al. also did systematical studies about anionic pseudopeptides derivatives with hydrophobic amino acids or different structures for intracellular drug delivery.^[36–38]

Herein we report a virus-mimicking pH-responsive endosomolytic polymer-based NP, namely, poly(lauryl methacrylate-co-methacrylic acid)-grafted acetalated dextran (ADMAP, which stands for acetalated dextran (AcDX)-based membrane-active polymer). We chose acetalated dextran mimicking the virus-like core composed of compactly packed genome. AcDX can accommodate various drug payloads, ranging from hydrophobic anti-tumor drug (paclitaxel) to antigen proteins (ovalbumin), with advantageous loading efficiency.^[39,40] Moreover, the acetal group is susceptible to hydrolysis at acidic pH, which can trigger drug release.^[41] The poly(lauryl methacrylate-co-methacrylic acid) grafts mimic capsid proteins on the viral surface and enable endosomal escape at acidic pH. Compared with reported virus-mimicking particles, the ADMAP NPs designed here do not contain biosynthesized viral components, which require delicate purification and may have immunogenicity and safety concerns.^[15,42] Moreover, the anionic pH-dependent endosomolytic polymer grafts remain nonlytic to cell membranes at physiological pH, thus minimizing the nonspecific binding to cell membranes and potential cytotoxicity.^[25,26] Although the nanoparticle does not have the function of replication and tropism as real viruses, the hierarchical structure, cell internalization and endosomal escape, controlled drug delivery, and the subsequent induction of cancer cell apoptosis make it resemble a nonenveloped oncolytic virus.

We have developed a simple method for preparing ADMAP polymer by conjugating a reversible addition–fragmentation chain transfer (RAFT) agent on AcDX, following by a controlled polymerization of lauryl methacrylate and methacrylic acid. After polymer synthesis, uniform ADMAP NPs were fabricated by nanoprecipitation on a microfluidic device with antitumor therapeutics encapsulated. The biocompatibility and endosomal escape properties of ADMAP NPs was evaluated using three colon cancer cell lines. Finally, the pH-dependent drug-loading and release behavior was quantified in vitro, and the cytotoxicity of drug-loaded ADMAP NPs were studied on both 2D cultured cells and on 3D tumor spheroids, after the conjugation of a tumor-penetrating peptide (TT1).

2. Results and Discussions

2.1. Membrane-Active Polymer Optimization

According to literature, once the endosomes gradually become acidic, viral capsid proteins undergo conformational changes and form hydrophobic domains for membrane insertion and finally allow the endosomal escape.^[11,14] To achieve similar properties, polymeric mimics should incorporate hydrophobic moieties to hydrophilic polymer backbones, such as poly(acrylic acid) derivatives with short alkyl pendant groups,^[27–30,43,44] and anionic pseudopeptide derivatives with hydrophobic alkyl anchors.^[38] However, there is no systematical investigation about the effects of alkyl length and grafting degree on membrane permeability. Therefore, we first designed two series of polymers by copolymerization of methacrylic acid (MAA) and hydrophobic methacrylate monomers with different alkyl chain lengths at different ratios. We chose butyl methacrylate (BMA, 4 carbons), lauryl methacrylate (LMA, 12 carbons) and stearyl methacrylate (SMA, 19 carbons) as methacrylate representatives with short, middle and long carbon chains to investigate the effects of hydrophobic chain length, while keeping the ratio of MAA and methacrylate the same (140:10). The other series include the copolymerization of MAA and LMA at different ratios (140:0, 140:10, and 140:40). As shown in **Figure 1a**, polymerization was performed at 70 °C with the presence of a reversible addition–fragmentation chain transfer agent. The RAFT agent was chosen due to its capability of controlling living radical polymerization and good tolerance toward acidic monomers like MAA. After polymerization, the polymers were characterized by nuclear magnetic resonance (NMR) spectroscopy. **Figure 1b** shows an identical ¹H-NMR of the polymer product (LMA10). The peaks from 7.4 to 7.9 ppm (zoomed in in **Figure 1b**) were attributed to the 5 protons on the benzyl ring of RAFT agent, whose areas were normalized as 5. The integration of the MAA peak (12.4 ppm, –COOH group) and the LMA peak (3.8 ppm, –CH₂ adjacent to the ester bond) was used for calculation of the real MAA and LMA incorporated in the final polymer. The results of other polymers (BMA10, SMA10, LMA0, and LMA40) were calculated in the same way and summarized in Table S1 (Supporting Information). For all polymers, the experimental degree of polymerization (DP) was almost the same as the DP designed, which suggested near quantitative conversion.

Figure 1. a) The synthetic scheme of alkyl-grafted poly(methacrylic acid) by RAFT polymerization. b) The ¹H-NMR spectrum of LMA10. The peaks from 7.0 to 8.2 ppm in the enlarged image are designated to the RAFT agent. c) The pH-dependent relative hemolysis of human RBCs in the presence of BMA10, LMA10, and SMA10 polymers at 100 μg mL⁻¹. d) The concentration-dependent relative hemolysis at pH 5.0 in the presence of BMA10, LMA10, and SMA10 polymers. e) The pH-dependent relative hemolysis of human RBCs in the presence of LMA0, LMA10, and LMA40 polymers at 100 μg mL⁻¹. f) The concentration-dependent relative hemolysis at pH 5.0 in the presence of LMA0, LMA10, and LMA40 polymers. c–f) The results were normalized by positive controls (the hemolysis of RBCs treated by H₂O). Data are presented as the mean ± s.d. (n = 3).

The interaction between polymers synthesized above and plasma membrane was evaluated by hemolysis assay, which is a well-established methodology for membrane interaction study.^[37,45,46] If polymers cause significant defects in the membrane of red blood cells (RBC), hemoglobin is released from cells and diffuses into buffer. For screening less toxic endosomolytic materials, it is preferable to have hemolysis only at acidic endosomal pH, but not at physiological pH, in order to induce endosomal escape, but still remain inert to cell membranes in physiological conditions.

As shown in Figure 1c, we screened all the membrane-active polymers synthesized above over a broad pH range (4.5–7.4), which represents the gradually acidifying environment during endocytosis. Both LMA10 and SMA10 displayed significant hemolytic activity at pH 5.0 (late endosomal pH), but negligible hemolysis at pH 7.4 (physiological pH). This is due to the pH-dependent protonation of the polymers. At pH 7.4, the MAA residues are deprotonated, and thus, negatively charged. The negative charge and the hydrophilicity of the polymers hindered their interaction with cell membranes due to electronic repulsion. Once acidified at pH 5.0, the MAA residues became partially protonated, and thus, more hydrophobic. The increased hydrophobicity enhanced the interaction with

RBC membranes, which caused significant hemolysis. As for BMA10, the optimal hemolysis pH is 4.5 instead of 5.0. Such low pH (similar to lysosomal pH) may lead to lysosomal degradation of payloads before the cytosolic release.

The hemolytic ability of BMA10, LMA10, and SMA10 at late endosomal pH (5.0) and physiological pH (7.4) was evaluated as a function of polymer concentration. As shown in Figure 1d, LMA10 showed superior hemolytic activity at pH 5.0 than the other two polymers at lower concentration ranges. Even at 10 μg mL⁻¹ (0.67 × 10⁻⁶ M), the hemolysis reached almost 80%, which is as efficient as a natural cell penetrating peptide melittin.^[47,48] Further increase in the concentration led to a decrease in hemolysis, probably due to the self-aggregation of polymers at higher concentrations. This means the available free polymer for destabilizing cell membranes decreased despite the higher overall concentration. A preliminary study of the critical micelle concentration (CMC) of LMA10 by dynamic light scattering (DLS) showed that polymer started to form micelle when the concentration was higher than 216 μg mL⁻¹. This correlated well with the decrease in hemolysis from 100 to 1000 μg mL⁻¹ (Figure S1, Supporting Information).

The effects of LMA grafting density on hemolytic activity were also assessed on a pH-dependent and concentration-dependent

manner (Figure 1e,f). Without hydrophobic LMA moieties, PMAA displayed only a moderate hemolysis at pH 4.5, similar to BMA10. Both LMA10 and LMA40 showed the highest hemolysis at pH 5.0, but LMA10 was more hemolytic at lower concentrations. Thus, we decided to choose LMA10 as the optimal structure for pH-responsive membrane-permeation and use it for the further studies.

2.2. ADMAP Polymer Synthesis and Characterization

ADMAP polymer was synthesized from dextran, an FDA approved polysaccharide. As shown in Figure 2a, dextran first reacted with 2-methoxypropene to form AcDX, and then modified by the RAFT agent, to conjugate LMA10 via a “grafting from” process. The acetalation of dextran switched the solubility from water soluble to organic solvent soluble, thus facilitating the loading of hydrophobic drugs. In addition, the attached acetal groups are susceptible to hydrolysis in an acidic environment, which allows the drug release and escape from endosomes by LMA10.

The synthesis and structure of ADMAP was confirmed by NMR spectra shown in Figure 2b. The representative peaks from AcDX, RAFT agent and polymerized MAA and LMA, were all presented. The peaks from 4.6 to 5.6 ppm were attributed to the anomeric proton of AcDX, while the small peaks from 7.5 to 8.0 ppm were attributed to the benzyl ring of RAFT agent. The characteristic peaks at 12.4 ppm (–COOH from MAA), 3.8 ppm (–CH₂ from LMA, partially overlapping with the glucose ring protons from AcDX) and 0.8–2 ppm (–CH₃ and –CH₂

from MAA and LMA, partially overlapping with the –CH₃ from AcDX) suggested the successful grafting of MAA and LMA. The NMR spectra of intermediates and their peak assignments are shown in Figures S2–S4 (Supporting Information).

Fourier Transform infrared spectroscopy (FTIR) was also used to confirm the structure of ADMAP (Figure 2c). After acetalation, the strong broad band from 3000 to 3600 cm^{−1} (O–H stretch from dextran glucose unit) significantly reduced, while a new band from 2850 to 3050 cm^{−1} (C–H stretch from acetal groups) appeared. After the grafting of MAA and LMA, the strong carbonyl characteristic band (1680 to 1760 cm^{−1}) in ADMAP spectrum indicated the successful polymerization.

2.3. ADMAP NP Preparation, Characterization, and Endosomal Escape Capability Test

The ADMAP NPs were prepared by nanoprecipitation on a glass-capillary microfluidic device (Figure 3a). ADMAP polymer was dissolved in ethanol as inner fluid, while the antisolvent (H₂O, pH 7.4 in this case) was used as outer fluid. As the polymer solution rapidly mixed with outer fluid, the supersaturation initiated the nanoprecipitation. DLS results (Figure S5, Supporting Information) showed that ADMAP has a size and polydispersity index (PDI) of 231 nm and 0.16, respectively. Transmission electron microscope (TEM) images (Figure 3b,c) further confirm the size and the uniformity of ADMAP particles. Most particles were spherical with diameter of ≈200 nm, which correlates well with the DLS results.

Figure 2. a) The synthetic scheme of ADMAP. b) The ¹H-NMR spectrum of ADMAP with proton designations. # Represents the peak of NMR solvent DMSO-d₆. * Represents the peak of residual water. c) The FTIR spectra of Dextran, AcDX, and ADMAP.

Figure 3. a) The schematic illustration of ADMAP NP preparation on a glass-capillary microfluidic device. b, c) TEM images of ADMAP NPs. Scale bars are b) 2 μm and c) 200 nm. d, e) The pH- and concentration-dependent relative hemolysis of ADMAP NPs. The results were normalized by the positive controls (the hemolysis of RBCs treated by H_2O). Data are presented as the mean $\pm$ s.d. ($n = 3$). f–h) The confocal microscopy images of CT-26 cells, showing the intracellular translocation of calcein. The cells were treated with f) calcein only (0.2 mg mL^{-1}), g) calcein with AcDX nanoparticles ($50 \mu\text{g mL}^{-1}$), and h) calcein with ADMAP ($50 \mu\text{g mL}^{-1}$) for 1 h. The images were acquired at 3 h after the 1 h incubation with the particles and calcein. Scale bars = 50 μm . i) Zoomed-in confocal images of ADMAP NP treated CT-26 cells show the calcein release from endosomes. Scale bar = 10 μm . j) Flow cytometry results of ADMAP NPs uptake and the inhibition of uptake by low temperature, sodium azide and ammonium chloride. Data are presented as the mean $\pm$ s.d. ($n = 3$).

To investigate whether ADMAP NPs maintained the pH-dependent membrane-lytic activity, we repeated the hemolysis assay as a function of pH and NP concentration. As shown in Figure 3d, the pH-dependent hemolysis was identical compared

with LMA10, which demonstrates the highest hemolytic activity at pH 5.0, but negligible hemolysis at pH 7.4. The concentration-dependent hemolysis (Figure 3e) was slightly different from LMA10. When the concentration was lower than $50 \mu\text{g mL}^{-1}$,

the hemolysis was less than 20%, less hemolytic than free LMA10. This may be attributed to the lower mass percentage of membrane-active polymer grafts within the final ADMAP formulation. However, when the concentration reached $50 \mu\text{g mL}^{-1}$ and beyond, the hemolysis increased to 68–78%, which was comparable to free LMA10. Unlike free LMA10, the membrane active polymer grafts on the surface of ADMAP NPs cannot form micelles. Therefore, the decreased hemolysis at higher concentrations was not observed on ADMAP NPs.

After the verification of pH-dependent membrane-lytic activity on RBCs, ADMAP NPs were applied on mammalian cells, to investigate their endosomal escape. The NPs were incubated with CT26 colon carcinoma cells for 1 h, along with a membrane-impermeable dye calcein, which is taken up by endocytosis. After the 1 h incubation, free NPs and calcein were removed by washing and the cells were incubated for additional 3 h. Without endosomal escape reagents, calcein would be entrapped in endosomes/lysosomes. The confocal images of cells incubated with calcein only are shown in Figure 3f. The green channel (calcein) showed bright spotty fluorescence which colocalized with lysotracker in the red channel. Following incubation with ADMAP NPs, however, most cells showed evenly distributed green fluorescence (Figure 3h,i) within cells, which means calcein escaped from endosomes/lysosomes and diffused into cytoplasm. This means ADMAP NPs destabilized the membrane of endosomes upon acidification, which facilitated the release of calcein. In contrast, cells incubated with calcein and AcDX NPs (Figure 3g) showed spotty green fluorescence similar to control group, which confirmed that the endosomal escape is due to the pH-dependent membrane-lytic moieties of ADMAP, and not to the AcDX part. The endosomal escape capability of ADMAP was further tested on two other colon carcinoma cell lines, HT-29 and Caco-2, using the same calcein assay, as described above. Similar diffused green staining was observed on both cell lines (Figure S6, Supporting Information), which proved the universal endosomal escape capability of ADMAP NPs.

To further investigate the particle uptake and the endosomal escape mechanism, we used several endocytosis inhibitors, including low temperature (4°C), sodium azide, and ammonium chloride. The ADMAP NPs were labeled with Alexa-633, and incubated with cells pretreated with different inhibitors for 1 h. The flow cytometry results shown in Figure 3j indicate that the treatment with low temperature (4°C) and sodium azide (an ATPase inhibitor)^[49,50] significantly reduced the ADMAP uptake by 61%, which means the particle uptake was mainly energy-dependent. Notably, the endosomal acidification inhibitor ammonium chloride^[51] also inhibited 57% of uptake, comparable to energy-depletion inhibitors. This suggests that the acidic endosomal environment is critical for ADMAP NP uptake. Without endosomal acidification, the ADMAP lost the endosomolytic capability. In this case, ADMAP cannot translocate into cytoplasm, and finally get exocytosed.

2.4. ADMAP NP Drug Loading, Release, and In Vitro Therapeutic Efficacy

The drug loading of ADMAP NP was performed on the same microfluidic nanoprecipitation platform shown in Figure 3a.

Asiatic acid (AA), a pentacyclic triterpenoid, abundantly found in *Centella asiatica*, has anticancer effects on various types of malignant cells, such as colon (SW480 and HT-29), lung (A549), liver (HepG2), and ovarian (SKOV3 and OVCAR-3) cancer cells.^[52,53] It had also been used as a starting material for the development of new semisynthetic anticancer derivatives, which possess an anticancer activity against multiple cancer cell lines as reported in the previous literatures.^[54] In addition, AA was found to be a promising natural chemosensitizer for increasing the sensitivity of multidrug-resistant cancer cells (A549/DDP lung cancer cells).^[55] Further studies found that the anticancer mechanism of AA is related to the inhibition of phosphoinositide-3-kinase (PI3K)/protein kinase B(AKT)/mTOR signaling pathway, which regulates the proliferation, migration and apoptosis in colorectal carcinoma cells.^[52] The inhibition of such signaling pathways requires the intracellular delivery of AA to cytoplasm and mitochondria. Because of these attractive anticancer profiles and the mechanism, we chose AA as a model drug payload in this study. The encapsulation efficiency and loading degree were $80.7 \pm 5.5\%$ (w/w) and $3.9 \pm 0.3\%$ (w/w), respectively. After loading, the size of the particles showed a slight increase to 268 nm, but the PDI remained low (0.15, size distribution histogram shown in Figure S5, Supporting Information). TEM images shown in Figure 4a,b indicated that the drug loaded particles (AA@ADMAP) were uniform.

To investigate the pH-responsive properties of the ADMAP, the release profiles of the drug were studied at pH 5.0 and pH 7.4, mimicking the intracellular (late endosomal pH) and extracellular conditions, respectively. As shown in Figure 4c,d, free AA showed burst release at both pH conditions. At pH 7.4 almost 50% of the free AA was released in 15 min and complete drug release was observed at 24 h. In contrast, the release of AA@ADMAP exhibited only a slight release ($\approx 20\%$) at pH 7.4, which means the polymer matrix can protect the release of the payload in the physiological pH over 24 h. At pH 5.0, AA@ADMAP achieved almost 50% release after 2 h, and finally reached similar amount of release (86%) after 24 h compared with free AA. Overall, the results from the drug release study demonstrate the pH-responsive properties of ADMAP carrier, which can be degradable and release of the drug in acidic conditions.

To evaluate the cytotoxicity of ADMAP NPs, CT-26 cells were exposed to the different concentrations of the ADMAP NPs, ranging from 50 to $1000 \mu\text{g mL}^{-1}$ for 6 and 24 h, and the cell viability was assessed by CellTiter-Glo luminescence assay. As shown in Figure 4e, the percentage of viable cells are over 90% within the entire concentration range for both time points, suggesting that these ADMAP particles were noncytotoxic. The cytotoxicity on another two human colorectal cancer cell lines (HT-29 and Caco-2) also suggested that ADMAP NPs have negligible cytotoxicity even at a concentration as high as $1000 \mu\text{g mL}^{-1}$ (Figure S7, Supporting Information).

Then, we used drug-loaded ADMAP particles to investigate their anticancer activity in CT-26 tumor cells (Figure 4f,g). Compared with free AA, AA@ADMAP showed better therapeutic efficacy at the same concentration. After 6 h incubation, the AA@ADMAP at the concentration of $12.5 \mu\text{g mL}^{-1}$ showed a decrease in cell viability. Higher efficacy was observed after 24 h of incubation. At $25 \mu\text{g mL}^{-1}$ of the drug, the AA@ADMAP can greatly

Figure 4. a,b) TEM images of AA@ADMAP NPs. Scale bars are a) 2 μm and b) 200 nm. c,d) The cumulative release of free AA or AA-encapsulated in ADMAP at pH 5.0 and pH 7.4 buffers. Data are presented as the mean $\pm$ s.d. ($n = 3$). e) The cytotoxicity of ADMAP on CT-26 cells after 6 and 24 h incubation. f,g) The therapeutic efficacy of free AA in 1% Poloxamer 407 (P407) and AA@ADMAP on CT-26 cells after 6 and 24 h incubation. e–g) The results were normalized to the untreated controls. Data are presented as the mean $\pm$ s.d. ($n = 5$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

reduce the cell viability of the CT-26 tumor cells to 40% compared with the free drug-treated group, which did not exhibit the cytotoxicity effect. With higher concentration of the drug (50 $\mu\text{g mL}^{-1}$), the cell viability further reduced to 20% in AA@ADMAP, while the cell viability in the free AA-treated group was about three times (60%) higher. The improved anticancer potency of AA@ADMAP was due to the synergistic effect of successful endosomal escape and pH-triggered drug release.

2.5. Linear TT1-Conjugated ADMAP NP for 3D Tumor Spheroid Penetration and Treatment

Since AA@ADMAP NPs showed advantageous therapeutic efficacy on 2D tumor cells, we decided to further test them on a 3D tumor spheroid model, which better mimics the in vivo solid tumor conditions.^[56–58] Tumor penetrating linear TT1 peptide (sequence: AKRGARSTA) targets an aberrantly expressed receptor (p32) on the surface of CT-26 colon carcinoma cells.^[59,60] After binding with p32, the TT1 peptide is proteolytically cleaved, C-terminally exposing the RGAR C-end Rule (CendR) motif, capable of binding tissue penetration receptor NRP-1.^[61–63] The NRP-1 binding activates the subsequent transcytosis pathway, which facilitates the particle transportation inside tumor spheroids.^[60]

TT1 was conjugated onto the ADMAP NPs via a two-step reaction, as shown in Figure 5a. First, the ADMAP or AA@

ADMAP was conjugated with a crosslinker molecule 3-(2-pyridylthio)propionyl hydrazide (PDPH) via a 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling reaction. Next, a fluorescent label (Alexa-633 hydrazide) was conjugated using the same EDC chemistry. The PDPH-conjugated ADMAP was incubated with fluorescein-labeled cysteine-modified TT1 peptide, to allow for the reduction of 2-pyridylthio group on PDPH. The final TT1 conjugated ADMAP (ADMAP-TT1) was characterized by DLS and TEM (Figure 5b). DLS results showed an average size of 317 nm and PDI of 0.2 (Figure S5, Supporting Information).

The ADMAP-TT1 particles were first incubated with 2D CT-26 cells to verify their cellular uptake. After 1 h treatment at 50 $\mu\text{g mL}^{-1}$, the flow cytometry showed significant cellular uptake (Figure S8, Supporting Information). Then ADMAP and ADMAP-TT1 particles were incubated with CT-26 spheroids for 1 and 3 h, respectively, to follow the penetration. The spheroids were fixed, stained by DAPI, and imaged by confocal microscopy directly using Z-stacking. As shown in Figure S9 (Supporting Information), both ADMAP and ADMAP-TT1 particles were found attached to the spheroid surface after 1 h incubation. Over time, the ADMAP-TT1 particles showed increased spheroid binding and penetration, and the green fluorescence from TT1 peptides colocalized well with the red fluorescence from ADMAP particles, suggesting that the peptide remained bound to the particles.

Figure 5. a) The scheme of ADMAP-TT1 conjugation. The ADMAP particles were first activated EDC/NHS, followed by PDPH and Alexa-633 labeling. The ADMAP-PDPH particles were further incubated with cysteine-terminated, fluorescein-labeled TT1 peptides. b) TEM images of ADMAP-TT1 NPs. Scale bar = 500 nm. c–g) The confocal microscopy images of CT-26 spheroid cryosections. The spheroid was treated without particles c), with ADMAP NPs d) and f), or with ADMAP-TT1 NPs e) and g) at $50 \mu\text{g mL}^{-1}$. The treatment lasted for 1 h in (d) and (e), and 3 h in (f) and (g). Scale bars = 200 μm . h) The zoomed-in confocal image of 3 h ADMAP-TT1 treated spheroid cryosections. The white arrows show the ADMAP-TT1 NPs penetrated inside the spheroid. Scale bar = 50 μm . i, j) The flow cytometry results of spheroids treated with or without different particles for 1 and 3 h. Orange bars represent the positive event percentage and green bars represent the mean fluorescence intensity. Data are presented as the mean $\pm$ s.d. (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001.

In order to study the penetration of the NPs into the spheroids, we next analyzed the cryosections of the spheroids. As shown in Figure 5c–g, the spheroids ($\approx 500 \mu\text{m}$ in diameter) appeared compact and solid, with uniform blue staining of cell

nuclei by DAPI. Similar to the 3D-projection images shown in Figure S9 (Supporting Information), both ADMAP and ADMAP-TT1 showed strong fluorescence on the surface of spheroids after 1 h incubation. With increasing incubation time,

the ADMAP-TT1 particles showed more intense fluorescence inside the spheroid, whereas ADMAP without TT1 remained confined on the spheroid surface (Figure 5f,g). In the enlarged image of ADMAP-TT1 after 3 h treatment (Figure 5h), there was red color within tumor spheroids at a depth of more than ten layers of cells, which demonstrates the deep penetration of the NPs inside the 3D-spheroid. Notably, the green color in Figure 5g,h only appeared on the surface of the spheroid, which suggests that only the surface cells were associated with TT1. Therefore, we deduced that TT1 peptide was cleaved from ADMAP-TT1 NPs after interaction with the surface cells, while ADMAP managed to penetrate inside the spheroids.

The fluorescence of ADMAP and ADMAP-TT1 NPs were also quantified by flow cytometry after dissociating the spheroids using trypsin. As shown in Figure 5i,j, ADMAP-TT1 showed higher positive events number and mean fluorescence intensity than ADMAP. The positive events percentage of ADMAP-TT1 almost doubled after 3 h incubation. Despite the $\approx 64.0\%$ positive events of ADMAP (analyzed by the Alexa-633 channel) after 3 h incubation, the positive events of TT1 (analyzed by the fluorescein channel) was only 9.7%, indicating that the some TT1 peptides got cleaved from the NP during transcytosis and penetration which is consistent with confocal results shown above. After cleavage, the CendR fragment binds to NRP-1 and it triggers the penetration, not only of the peptide attached to the NP, but also the NPs that are near the CendR peptide, even if they are not attached to the peptide.^[61] This so-called “by-stander” effect enables the transcytosis of ADMAP-TT1 NPs no matter the peptide is cleaved or not, which may explain such significant tumor penetration.

Based on the significant cell uptake and spheroid penetration, we proceeded with AA@ADMAP-TT1 NPs and characterized their antitumor efficacy on the 3D spheroids. The release behavior of AA@ADMAP-TT1 was similar to AA@ADMAP (Figure 6a). More than 40% of the drug was released after 2 h at pH 5.0, and almost 100% release was achieved after 24 h due to the complete hydrolysis of AcDX. At pH 7.4, however, the particles remained stable during the duration of the study with only a small burst release at the first hour.

When incubated with 3D spheroids, AA@ADMAP-TT1 showed greater therapeutic efficacy than both free AA (in 1% P407) and AA@ADMAP, in terms of spheroid size, volume and cell viability. The optical images of spheroids with or without treatment for 5 days are shown in Figure 6b. The control group without treatment showed a constant size (550 μm) during the studied period, while the spheroids that received treatments decreased steadily by time from 550 to 472, 445, and 423 μm for free AA, AA@ADMAP and AA@ADMAP-TT1, respectively. The spheroids receiving AA@ADMAP-TT1 decreased in diameter from day 1 to day 5, and cells disintegrated from the spheroid's surface. Instead of an integral sphere with a smooth surface and a solid core seen at the beginning of the treatment, the AA@ADMAP-TT1 treated spheroids became irregular with rough surfaces and individual cells scattered around eventually. This suggested that AA@ADMAP-TT1 NPs not only killed the cells on the spheroid surface, but also managed to disintegrate the spheroids over time. The corresponding volumes of the spheroids plotted in Figure 6c further proved that the treatment with AA@ADMAP-TT1 NPs reduced the spheroid

volume to 64% after 1 day of treatment, and eventually to 45% at the end of the treatment, whereas the volume in the other groups remained above 50% within the studied range.

The quantification of the cell viability within the spheroids after treatment was done using trypsin to disassemble the spheroids and analyzed the metabolic activity of remaining tumor cells (Figure 6d). After 1 day incubation, the viability of the AA@ADMAP-TT1 NP-treated group decreased significantly to 69%, and further decreased to 40% and 11% after 3 days and 5 day treatment, respectively. The free AA-treated group only showed a modest reduction in the viability: 80% on day 1 and to 54% on day 5. Although the AA release within the spheroid was not visible, the higher cell-killing potency along with the 3D-spheroid disintegration indicated that the TT1 peptide conjugated on ADMAP may have facilitated the tumor penetration and enabled drug delivery both to the cells' surface and deep inside the spheroids after transcytosis. Thus, the TT1 functionalization improved the ability of AA@ADMAP to lyse 3D spheroids. This observation in combination with known tumor tropism of TT1-guided nanoparticles (REFs) suggests the potential of AA@ADMAP-TT1 NPs for in vivo antitumor therapy in the future.

3. Conclusions

A pH-responsive, virus-mimicking polymer ADMAP was synthesized by grafting endosomolytic polymers from AcDX by RAFT polymerization, and subsequently fabricated into drug-loaded, uniform NPs on a glass-capillary microfluidic device. The ADMAP NPs maintained the endosomolytic activity, thus enabling the cytoplasmic delivery of endocytosed payloads. After the encapsulation of an anticancer therapeutic (AA) as a model drug, the AA@ADMAP showed greater therapeutic efficacy than free anticancer drugs in vitro. Functionalization with a tumor-penetrating peptide TT1 enhanced the therapeutic efficacy of the NPs on 3D tumor spheroids, leading to both cell-killing effect and spheroid disintegration, probably by penetrating deep inside the spheroids. The simple preparation, pH-responsive drug release, successful endosomal escape property and the compatibility with affinity targeting of this nanocomposite makes it promising for antitumor therapeutics delivery.

4. Experimental Section

Materials—Polymer Synthesis: Dextran (40 000 Da), pyridinium-*p*-toluenesulfonate (PPTS), 2-methoxypropene, triethylamine (TEA), cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPADB), *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 2,2'-azobis(2-methylpropionitrile) (AIBN) solution (0.2 M in toluene), methacrylic acid (MAA), lauryl methacrylate (LMA), butyl methacrylate (BMA), stearyl methacrylate (SMA), ethyl acetate, dichloromethane (DCM), dimethyl acetamide (DMAc), anhydrous dimethyl sulfoxide (DMSO), 2-propanol, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Fluor 633 and 3-(2-pyridyldithio)propionyl hydrazide (PDPH) were purchased from ThermoFisher Scientific (Waltham, MA, USA). All materials were used as received.

Cell Studies: LysoTracker red and 4',6'-diamidino-2-phenylindole (DAPI) was purchased from ThermoFisher Scientific (Waltham, MA, USA).

Figure 6. a) The cumulative release of AA from AA@ADMAP-TT1 NPs at pH 5.0 and 7.4. Data are presented as the mean $\pm$ s.d. ($n = 3$). b) The optical microscopy images of representative 3D-spheroid cells with and without treatments in five consecutive days. The CT-26 tumor spheroids are incubated with cell culture medium only (control), 1% P407, 50 $\mu\text{g mL}^{-1}$ of AA in 1% P407, AA@ADMAP (1 mg mL^{-1} , drug contents equivalent to 50 $\mu\text{g mL}^{-1}$ of AA), and AA@ADMAP-TT1 (1 mg mL^{-1} , drug contents equivalent to 50 $\mu\text{g mL}^{-1}$ of AA). Scale bars = 500 μm . c) The size of the spheroids measured from the microscopy images. The volume data were calculated and normalized to the untreated control at day 0 ($n = 3$). d) The cell viability of the spheroids quantified by CellTiter-Glo assay after dissociation using trypsin. The results were normalized to the untreated controls. Data are presented as the mean $\pm$ s.d. ($n = 5$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Fluorescein (FAM) labeled linear TT1 peptide (sequence: FAM-Cys-Ahx-AKRGARSTA-NH₂; Cys = cysteine; Ahx = amino hexanoic acid) was purchased from TAG Copenhagen (Denmark). 10 $\times$ Hank's Balanced Salt Solution (HBSS), Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Life Technologies Gibco, USA. 96-well plates and 75 cm^2 cell culture flasks were purchased from Corning Inc. (NY, USA). CellTiter-Glo assay was purchased from Promega Corporation, USA. 2-(4-(2-Hydroxyethyl)pipezarin-1-yl) ethanesulfonic acid (HEPES), asiatic acid and calcein were purchased from Sigma-Aldrich (St. Louis, MO, USA). 10 $\times$ Phosphate buffered saline (PBS), nonessential amino acids (NEEA), L-glutamine 200 $\times 10^{-3}$ M, penicillin (100 IU mL^{-1}), streptomycin (100 mg mL^{-1}), and trypsin (2.5%) were purchased from HyClone, GE Healthcare Lifesciences (Logan, UT, USA). Triton X-100 was purchased from Merck Millipore (Darmstadt, Germany). NanoShuttle-PL magnetic nanoparticles for 3D spheroid

preparation were purchased from Greiner Bio-One International GmbH (Frickhausen, Germany). Red blood cells were purchased from Finnish Red Cross (Veripalvelu).

Synthesis of Membrane-Active Polymer: RAFT agent CPADB (10 mg , 0.036 mmol) was weighed in round-bottom flask and dissolved in 2-propanol (5 mL). Two monomers, MAA (425 μL , 5 mmol) and methacrylate (either BMA, LMA or SMA at a specific ratio shown in Table S1 in the Supporting Information) were added directly into this solution, followed by AIBN solution (54 μL , 0.2 m in toluene). The flask was sealed and purged with nitrogen for 20 min, and then immersed in an oil bath at 70 $^{\circ}\text{C}$ for 16 h. The polymer was purified by precipitation in cold diethyl ether, and then isolated by centrifugation at 3214 $\times g$ for 5 min. The precipitant was dissolved in methanol, and then dropped into diethyl ether to precipitate again. The precipitation process was repeated three times, and the final polymers were dried in a vacuum

oven, yielding pink solid products. The salt form of the polymers for hemolysis study were obtained by dissolving the pink solid in 1×10^{-3} M sodium bicarbonate solution, following by overnight dialysis (MWCO 3.5 kDa) against Milli-Q water to remove excessive sodium bicarbonate. The final solution after dialysis was freeze-dried, yielding light pink fine powders.

The polymer structure was verified by ^1H -NMR spectroscopy on an Avance III 400 MHz NMR spectrometer (Bruker, Switzerland), and the spectra were analyzed by MestReNova. The chemical shift was calibrated by NMR solvent ($\text{DMSO}-d_6$) peak. The integral of the peaks (7.4–7.9 ppm) of CPADB was normalized as 5, corresponding to the five protons on benzyl rings. Then the integration of the MAA peak (12.4 ppm, $-\text{COOH}$ group) and the RMA peak (3.8 ppm, $-\text{CH}_2$ adjacent to the ester bond) were obtained and the amount of MAA and RMA incorporated in the final polymer was calculated. The calculated results were shown in Table S1 (Supporting Information).

Synthesis of ADMAP: To prepare ADMAP, acetalated dextran (AcDX) was first prepared, as reported elsewhere.^[41] Briefly, dextran (1.0 g) was dissolved in 10 mL anhydrous DMSO. PPTS (15.6 mg) and 2-methoxypropene (3.4 mL) were added and the reaction was allowed for 4 h under the argon atmosphere before quenching by TEA (1 mL). The polymer product was obtained by precipitation in Milli-Q water (100 mL). After centrifugation (15 min, $16\,110 \times g$), the pellet was redissolved in ethanol (20 mL), and precipitated in water again. The purification process was repeated twice, and the residual water was removed by freeze-drying for 48 h. The final AcDX polymer was characterized by FTIR (Figure 2c) and ^1H -NMR (Figure S3, Supporting Information) on a Bruker Vertex 70 spectrometer to confirm the structure before proceeding to the next step.

The AcDX macroRAFT was synthesized, following the previous literature.^[64] AcDX (100 mg) was dissolved in DCM (1.6 mL), and then CPADB (10 mg) was added and stirred until dissolved. DCC (20 mg) and DMAP (1 mg) were separately dissolved in additional 0.2 mL DCM. DCC solution was added to the polymer solution, followed by DMAP solution. The reaction proceeded overnight at room temperature. The reaction mixture was dialyzed against acetone for 2 days using dialysis bag (MWCO 3.5 kDa), and the solvent was replaced twice a day. After dialysis, the pink polymer solution was dried by rotary evaporator to obtain the AcDX macroRAFT, which was characterized by ^1H NMR spectroscopy (Figure S4, Supporting Information). By the peak integration of benzyl rings (five protons) from 7.4 to 7.9 ppm and the anomeric proton of AcDX from 4.7 to 5.2 ppm, it is possible to deduce the amount of RAFT agent on AcDX. On average, there are approximately 3.6 RAFT agent per AcDX chain.

The ADMAP polymer was obtained by RAFT polymerization of MAA and LMA using AcDX macroRAFT agent ($[\text{RAFT agent}]/[\text{AIBN}]/[\text{MAA}]/[\text{LMA}] = 1:0.3:140:10$). The AcDX macroRAFT (550 mg, equivalent to 0.1 mmol RAFT agent) was dissolved in DMAc and IPA mixture solution (2.5 mL, 1:10 v/v). After AcDX macroRAFT completely dissolved, MAA (1.21 mL, 14.3 mmol) and LMA (0.3 mL, 1.02 mmol) were added, followed by the addition of AIBN solution (0.15 mL, 0.2 M in toluene, 30.6 μmol). The flask was sealed and purged with nitrogen for 40 min, and then immersed in an oil bath at 70°C for 16 h. The ADMAP polymer was purified by precipitation in cold diethyl ether and separated by centrifugation at $3214 \times g$ for 5 min. The collected pellet was redissolved in methanol and precipitated again in diethyl ether. The precipitation was repeated three times to removed unreacted monomers. The final polymer pellet was dried in a vacuum oven at 40°C for 1 day. The ADMAP was subsequently characterized by NMR and FTIR.

Fabrication of the Glass-Capillary Microfluidic Device: A coflow microfluidic nanoprecipitation device was fabricated by assembling borosilicate glass capillaries on a glass slide.^[40] Cylindrical capillary (inner and outer diameter of 580 and 1000 μm ; World Precision Instruments Inc., USA) was tapered using a micropipette puller (P-97, Sutter Instrument Co., USA), and then further enlarged to a diameter of 100 μm . This tapered capillary was inserted into another larger cylindrical capillary with an inner diameter of 1100 μm (Vitrocom, USA). A transparent epoxy resin (5 Minute Epoxi, Devcon) was used to seal the

capillaries when required. Two miscible liquids were injected separately into the microfluidic device in the same direction through polyethylene tubes attached to syringes at constant flow rates. The flow rate of the different liquids was controlled by pumps (PHD 2000, Harvard Apparatus, USA).

Preparation of ADMAP and AA@ADMAP Nanoparticles: The nanoparticles without drug (ADMAP) and with drug (AA@ADMAP) were prepared by the coflow glass-capillary microfluidic device. The inner and outer fluids were ADMAP in ethanol (20 mg mL^{-1}) and Milli-Q water (adjusted to pH 7.4), respectively. The flow rate of the inner and the outer fluids was 2 and 20 mL h^{-1} , respectively. To produce AA@ADMAP, AA (1 mg mL^{-1}) was dissolved in the inner fluid, and the rest of the procedure was as the same as the method mentioned above. The produced particles were collected by centrifugation at $16\,110 \times g$ for 5 min and washed twice with Milli-Q water. The particle size, polydispersity index (PDI) and zeta-potential were measured using Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). The morphology of the different NPs was evaluated by transmission electron microscope (TEM; Tecnai F12, FEI Company, USA).

Conjugation of TT1 Peptide to AA@ADMAP: TT1 was chemically attached onto the surface of the AA@ADMAP using EDC/NHS chemistry together with the PDPH conjugation in water (pH 6.0). Briefly, 0.5 mg of NPs was dispersed in water (0.5 mL) containing 3.8 μL of EDC, 3.3 mg of NHS, and 0.5 mg of PDPH. The particle suspension was stirred for 2 h at room temperature to activate the carboxylic groups on the surface of the NPs, and further conjugated with PDPH. Afterward, the particles were centrifuged and washed with Milli-Q water twice to remove the unreacted compounds. To quantify the amount of the conjugated PDPH on the particle surface, the supernatant (100 μL) was taken and mixed with DTT ($10 \times 10^{-3}\text{ M}$, 5 μL) for 15 min and measured the UV-absorbance at 343 nm. The particles were redispersed again in 1 mL of TT1 peptide solution ($100\text{ }\mu\text{g mL}^{-1}$), and tip-sonicated for 10 s using a Vibra-Cell ultrasonic processor (Sonics, Sonics and Materials, Inc., USA). The particle suspension was stirred for 1 h at room temperature to allow for peptide conjugation. Finally, the final particles were washed with Milli-Q water twice to remove an excess peptide. To quantify the amount of the peptide conjugated on the surface of the particles, the fluorescence intensity (FI) before and after peptide-conjugation was detected at the excitation and emission wavelength of 485 and 520 nm, respectively, using Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Inc., NY, USA). The percentage of the peptide conjugation efficiency was $98\text{ }\mu\text{g peptide mg}^{-1}\text{ NPs}$, calculated according to the following equation

$$\% \text{ conjugation efficiency} = \frac{(\text{FI before peptide conjugation}) - (\text{FI after peptide conjugation})}{(\text{FI before peptide conjugation})} \times 100 \quad (1)$$

LMA10 CMC Measurement: The CMC of LMA10 polymer was measured by DLS. LMA10 aqueous solutions with different concentrations ranging from 5 to 500 mg mL^{-1} were prepared and measured by Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). Each sample was measured at 25°C for three times. The average derived count rate of 3 measurements was plotted against concentration and the linear fitting was performed by OriginPro 2018.

Hemolysis Assay: The pH responsive-cell membrane permeability properties of the membrane-active polymers (BMA10, LMA10, SMA10, LMA0, and LMA40) were investigated, using human blood hemolysis assay.^[37] Briefly, the blood (1 mL) at the cell density of 5×10^8 cells per mL was washed by $150 \times 10^{-3}\text{ M}$ sodium chloride solution and centrifuged at $1137 \times g$, 4 min for 3 times until the supernatant was almost clear. Each membrane-active copolymer (in salt form) was prepared at the concentration of $1\text{--}1000\text{ }\mu\text{g mL}^{-1}$ in Milli-Q water, and diluted in citric acid buffer solution (pH 4.5, 5.0, 5.5, and 6.0, respectively) or PBS 1X (pH 7.4). Then, the purified blood (4 μL , 2×10^6 cells) was added into each sample solution (200 μL) in triplicates and incubated with gentle shaking at 37°C for 1 h. After that, all samples were centrifuged at $1485 \times g$ for 4 min, and the supernatant (100 μL)

was transferred to the 96-well plate. The UV absorbance at 540 nm was recorded using microplate reader to identify the hemoglobin concentration. Blood containing buffer solution and Milli-Q water were used as negative control and positive control, respectively. The positive control is to lyse all red blood cells (RBCs) since the water causes RBC swollen and breaks the cells afterward. The positive control was defined as 100% hemolysis, and the RBC lysis of negative control is defined as 0%. The final hemolysis for a specific polymer sample was calculated as

$$\text{Relative Hemolysis (\%)} = \frac{\text{Abs(sample)} - \text{Abs(negative control)}}{\text{Abs(positive control)} - \text{Abs(negative control)}} \times 100\% \quad (2)$$

The hemolysis test on the ADMAP NPs at the concentrations of 10–1000 $\mu\text{g mL}^{-1}$ was later determined using the same method as described above.

Determination of Loading Degree (LD), Encapsulation Efficiency (EE), and In Vitro Drug Release Study—High-Performance Liquid Chromatography (HPLC): Agilent 1100 series HPLC system (Agilent Technologies, Germany) was used to analyze the AA compound loaded into NPs. A reversed phase column (C_{18}) with 5 μm particle size ($200 \times 4.6 \text{ mm}$, TSK-gel ODS-80Tm) was used as a stationary phase. The mobile phase was composed of Milli-Q water and acetonitrile/methanol (8% v/v) at the ratio of 23:73 v/v. The flow rate and injection volume were set at 1.0 mL min^{-1} and 20 μL , respectively. The wavelength of detection was 217 nm.

Determination of LD and EE: The loading degree (LD) for the drug loaded particles, expressed as [(weight of loaded drug/weight of drug loaded samples) $\times$ 100%], was determined by immersing them into methanol to dissolve the polymeric matrix and release all the drugs. The encapsulation efficiency (EE) was defined as the ratio of the actual and the starting amounts of drug encapsulated in the particles, expressed as [(actual amount of loaded drug/theoretical amount of loaded drug) $\times$ 100%]. The amount of drug was quantified by HPLC as stated above.

In Vitro Drug Release Study: The release profiles of AA from AA@ADMAP and AA@ADMAP-TT1 were studied and compared with free AA. AA-loaded particles were suspended in either PBS (pH 7.4) or HBSS-MES (pH 5.0) and incubated at 37 $^{\circ}\text{C}$ with shaking (60 rpm). Free AA was added into buffer solutions containing 1% (w/v) P407 due to the low solubility of AA. At selected time intervals (15 min, 30 min, 1, 2, 4, 6, and 24 h), 200 μL of the suspension was collected and replaced with the same volume of fresh and preheated medium. The suspension was then centrifuged at 16 110 $\times g$ for 5 min. The amount of AA in supernatants was quantified by HPLC as stated above. Triplicate samples were used for release study.

Cytotoxicity Studies: The in vitro cytotoxicity was performed using a commercial CellTiter-Glo luminescent cell viability assay (Promega Corp., WI, USA). CT-26 colon cancer cell lines were selected as a tumor cell model. The cells were seeded overnight on a 96-well plate at density of 1×10^4 cells per well in fresh DMEM medium (10% FBS). The medium was discarded and replaced with tested materials dispersed in cell culture medium (ADMAP NPs from 50 to 1000 $\mu\text{g mL}^{-1}$, free AA from 2.5 to 50 $\mu\text{g mL}^{-1}$ with 1% (w/v) P407, or AA@ADMAP at the concentrations based on the amount of AA encapsulated ADMAP). Triton X-100 solution (1% v/v in cell culture medium), and cell culture medium without any tested materials were used as negative and positive controls, respectively. Cells were incubated for 6 and 24 h in the incubator at 37 $^{\circ}\text{C}$ (5% CO_2 and 95% relative humidity). The cell viability was detected from the luminescent intensity, in which represent the amount of ATP produced by the viable cells. The assay was carried out using Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Inc., NY, USA). All samples were performed in six replicates.

Endosomal Release Study by Calcein Assay: CT-26 tumor cells (HT-29 and Caco-2 cells) were cultured in Lab-Tek chambered borosilicate cover glass (eight-chamber, ThermoFisher Scientific, USA) at a cell density of 15×10^3 cells per well in cell culture medium (DMEM) containing 10% FBS for 2 days. Calcein solution at the concentration of 0.2 mg mL^{-1} in DMEM with free FBS was prepared and used as a control. The

ADMAP and the AcDX-NPs at the concentration of 50 $\mu\text{g mL}^{-1}$ were dispersed in calcein solution containing DMEM. 200 μL of the samples were added into the well and incubated for 1 h. Then, the particles were gently removed and replaced with DMEM containing 10% FBS. After 3 h of incubation, the cells were incubated with LysoTracker red solution ($200 \times 10^{-9} \text{ M}$) prepared in DMEM for 10 min. Finally, the cells were imaged by a Leica TCS SP5 II HCS-A confocal microscope (Leica Microsystems, Wetzlar, Germany). The images acquired were processed by Fiji 1.51 software.

Endocytosis Inhibition Study: CT-26 tumor cells (5×10^4 cells per well) were seeded in 48-well plates for 2 days at 37 $^{\circ}\text{C}$. The endocytosis inhibitors, including ammonium chloride $50 \times 10^{-3} \text{ M}$, and sodium azide $100 \times 10^{-3} \text{ M}$ were introduced to the cells for 1 h in DMEM (10% FBS). The low temperature group was incubated for 1 h at 4 $^{\circ}\text{C}$ condition. Then, the Alexa633-conjugated ADMAP (50 $\mu\text{g mL}^{-1}$) were added to the cells and incubated for 1 h. The cells incubated with Alexa633-conjugated ADMAP without any inhibitor pretreatment was used as positive control, while the cells without any particle exposure were used as negative control. After incubation, the particles were removed and the cells were washed twice with PBS before detachment by trypsin. The detached cells were centrifuged at $317 \times g$ and washed again with PBS before analysis on BD Accuri C6 Flow Cytometer. The mean fluorescence intensity of each sample (in triplicates) was calculated by BD Accuri C6 software and normalized according to the positive control.

3D Spheroid Culture and the Interaction with Particles: CT-26 3D tumor spheroids were prepared as reported elsewhere.^[65] Cells were seeded in a six-well plate at the concentration of 4×10^5 cells per well in DMEM, supplemented with 10% FBS, in a humidified chamber at 37 $^{\circ}\text{C}$ overnight. The NanoShuttle-PL magnetic nanoparticles (50 μL) were directly added into the cell culture medium and incubated for 8 h. To prepare the 3D spheroids, the cells were detached from the 6-well plate using trypsin- Ethylenediaminetetraacetic acid (EDTA), and seeded in the ultra-low attachment surface 96 well plate (Costar, Corning, Kennebunk ME, USA) at the density of 6×10^3 cells per well. After that, the plate was placed on the magnetic driver and incubated at 37 $^{\circ}\text{C}$ for 2 days to allow the cells, forming an intact spheroid. The ADMAP and ADMAP-TT1 were prepared in DMEM (10% FBS) at the concentration of 50 $\mu\text{g mL}^{-1}$ for 1 and 3 h at 37 $^{\circ}\text{C}$, and separately prepared for confocal imaging and flow cytometry analysis.

For confocal imaging, the spheroids were washed once with PBS and fixed in 4% paraformaldehyde (PFA, 100 μL) at 37 $^{\circ}\text{C}$ in the incubator. After 4 h, the cells were washed once with PBS and stained with DAPI overnight. The intact spheroids were imaged by a Leica TCS SP5 II HCS-A confocal microscope subsequently using Z-stack imaging. The final 3D image was reconstructed by Imaris 8.2. To further investigate the particle penetration, the spheroids were cryosectioned using Leica CM3050 S cryostat, and transferred to the glass slides and stored at 4 $^{\circ}\text{C}$, before imaging by a Leica TCS SP5 II HCS-A confocal microscope. The cryosectioned spheroid images were processed by Fiji 1.51 software.

For flow cytometry analysis, the spheroids were disassembled using 50 μL of trypsin-EDTA and incubated for 15 min at 37 $^{\circ}\text{C}$. The lysed cells were resuspended in 150 μL of PBS-EDTA and transferred to the V-shape 96-well plate (three spheroids combined in one well), centrifuged at $317 \times g$ for 5 min and discard the supernatant. The cells were washed once with PBS-EDTA (150 μL per well), and resuspended in 250 μL of PBS-EDTA for flow cytometry analysis on BD Accuri C6 Flow Cytometer. The spheroids without any particle treatment were used as negative control for events gating. The percentage of positive events and the mean fluorescence intensity of each sample (four replicates) were calculated by BD Accuri C6 software.

Therapeutic Efficacy Study on 3D Spheroid Models: The therapeutic efficacy of the free AA and AA@ADMAP was investigated using 3D spheroid model prepared by the same method as stated above. The spheroids were treated with 50 $\mu\text{g mL}^{-1}$ of free AA with 1% (w/v) P407, AA@ADMAP (1000 $\mu\text{g mL}^{-1}$, equivalent to 50 $\mu\text{g mL}^{-1}$ AA) and AA@ADMAP-TT1 (1000 $\mu\text{g mL}^{-1}$, equivalent to 50 $\mu\text{g mL}^{-1}$ AA) for 5 days and the medium containing sample solutions was replaced in every other day. On days 0, 1, 3, and 5, the spheroids were imaged using a

Leica DMi1 inverted optical microscope (Leica Microsystems, Wetzlar, Germany), and their diameters were measured by Nano Measurer 1.2 software. After imaging, the spheroids were washed with PBS once and incubated with 100 μ L of trypsin-EDTA for 15 min to disassemble the spheroids into cell suspension. The cell viability at each time point was quantified by CellTiter-Glo luminescence assay on a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific Inc., NY, USA). All samples were performed in five replicates.

Statistical Analysis: The experiments were performed in at least triplicates and the values are represented as mean $\pm$ standard deviation. The data were analyzed by one-way ANOVA by OriginPro 2018. The levels of significant differences were set at probabilities of * P < 0.05, ** P < 0.01, and *** P < 0.001.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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- [1] M. P. Stewart, R. Langer, K. F. Jensen, *Chem. Rev.* **2018**, *118*, 7409.
- [2] S. Mitragotri, P. A. Burke, R. Langer, *Nat. Rev. Drug Discovery* **2014**, *13*, 655.
- [3] R. Cheng, F. Meng, C. Deng, H. A. Klok, Z. Zhong, *Biomaterials* **2013**, *34*, 3647.
- [4] J. Vasir, V. Labhasetwar, *Adv. Drug Delivery Rev.* **2007**, *59*, 718.
- [5] E. Wagner, *Acc. Chem. Res.* **2012**, *45*, 1005.
- [6] F. Wang, Y. Wang, X. Zhang, W. Zhang, S. Guo, F. Jin, *J. Controlled Release* **2014**, *174*, 126.

- [7] J. Wang, Z. Lu, M. G. Wientjes, J. L.-S. Au, *AAPS J.* **2010**, *12*, 492.
- [8] S. Barua, S. Mitragotri, *Nano Today* **2014**, *9*, 223.
- [9] T. F. Martens, K. Remaut, J. Demeester, S. C. De Smedt, K. Braeckmans, *Nano Today* **2014**, *9*, 344.
- [10] H. R. Gelderblom, *Struct. Classif. Viruses* **1996**.
- [11] O. Meier, K. Boucke, S. V. Hammer, S. Keller, R. P. Stidwill, S. Hemmi, U. F. Greber, *J. Cell Biol.* **2002**, *158*, 1119.
- [12] T. Lagache, O. Danos, D. Holcman, *Biophys. J.* **2012**, *102*, 980.
- [13] P. Leopold, R. Crystal, *Adv. Drug Delivery Rev.* **2007**, *59*, 810.
- [14] O. Meier, U. F. Greber, *J. Gene Med.* **2003**, *5*, 451.
- [15] M. Elsabahy, K. L. Wooley, *J. Polym. Sci., Part A: Polym. Chem.* **2012**, *50*, 1869.
- [16] M. Somiya, S. Kuroda, *Adv. Drug Delivery Rev.* **2015**, *95*, 77.
- [17] M. J. Rohovie, M. Nagasawa, J. R. Swartz, *Bioeng. Transl. Med.* **2017**, *2*, 43.
- [18] Z. Su, Q. Wang, *Angew. Chem., Int. Ed.* **2010**, *49*, 10048.
- [19] Y. Zou, M. Zheng, W. Yang, F. Meng, K. Miyata, H. J. Kim, K. Kataoka, Z. Zhong, *Adv. Mater.* **2017**, *29*, 1703285.
- [20] E. Mastrobattista, M. A. E. M. van der Aa, W. E. Hennink, D. J. A. Crommelin, *Nat. Rev. Drug Discovery* **2006**, *5*, 115.
- [21] J. Nicolas, S. Mura, D. Brambilla, N. Mackiewicz, P. Couvreur, *Chem. Soc. Rev.* **2013**, *42*, 1147.
- [22] M. A. C. Stuart, W. T. S. Huck, J. Genzer, M. Müller, C. Ober, M. Stamm, G. B. Sukhorukov, I. Szleifer, V. V. Tsukruk, M. Urban, F. Winnik, S. Zauscher, I. Luzinov, S. Minko, *Nat. Mater.* **2010**, *9*, 101.
- [23] W. Gao, J. M. Chan, O. C. Farokhzad, *Mol. Pharmaceutics* **2010**, *7*, 1913.
- [24] L. M. P. Vermeulen, S. C. De Smedt, K. Remaut, K. Braeckmans, *Eur. J. Pharm. Biopharm.* **2018**, *129*, 184.
- [25] Z. Tan, Y. K. Dhande, T. M. Reineke, *Bioconjugate Chem.* **2017**, *28*, 2985.
- [26] W. B. Kauffman, T. Fuselier, J. He, W. C. Wimley, *Trends Biochem. Sci.* **2015**, *40*, 749.
- [27] N. Murthy, J. R. Robichaud, D. A. Tirrell, P. S. Stayton, A. S. Hoffman, *J. Controlled Release* **1999**, *61*, 137.
- [28] M.-A. Yessine, C. Meier, H.-U. Peterreit, J.-C. Leroux, *Eur. J. Pharm. Biopharm.* **2006**, *63*, 1.
- [29] M.-A. Yessine, M. Lafleur, C. Meier, H.-U. Peterreit, J.-C. Leroux, *Biochim. Biophys. Acta, Biomembr.* **2003**, *1613*, 28.
- [30] C. A. Lackey, N. Murthy, O. W. Press, D. A. Tirrell, A. S. Hoffman, P. S. Stayton, *Bioconjugate Chem.* **1999**, *10*, 401.
- [31] M. E. H. El-Sayed, A. S. Hoffman, P. S. Stayton, *Expert Opin. Biol. Ther.* **2005**, *5*, 23.
- [32] C. L. Duvall, A. J. Convertine, D. S. W. Benoit, A. S. Hoffman, P. S. Stayton, *Mol. Pharmaceutics* **2010**, *7*, 468.
- [33] S. Foster, C. L. Duvall, E. F. Crounover, A. S. Hoffman, P. S. Stayton, *Bioconjugate Chem.* **2010**, *21*, 2205.
- [34] D. Shae, K. W. Becker, P. Christov, D. S. Yun, A. K. R. Lytton-Jean, S. Sevimli, M. Ascano, M. Kelley, D. B. Johnson, J. M. Balko, J. T. Wilson, *Nat. Nanotechnol.* **2019**, *14*, 269.
- [35] M. E. Jacobson, L. Wang-Bishop, K. W. Becker, J. T. Wilson, *Biomater. Sci.* **2019**, *7*, 547.
- [36] S. Wang, R. Chen, *Chem. Mater.* **2017**, *29*, 5806.
- [37] R. Chen, S. Khormaei, M. E. Eccleston, N. K. H. Slater, *Biomaterials* **2009**, *30*, 1954.
- [38] S. Chen, S. Wang, M. Kopytynski, M. Bachelet, R. Chen, *ACS Appl. Mater. Interfaces* **2017**, *9*, 8021.
- [39] K. E. Broaders, J. A. Cohen, T. T. Beaudette, E. M. Bachelder, J. M. J. Frechet, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 5497.
- [40] D. Liu, S. Cito, Y. Zhang, C.-F. Wang, T. M. Sikanen, H. A. Santos, *Adv. Mater.* **2015**, *27*, 2298.
- [41] E. M. Bachelder, T. T. Beaudette, K. E. Broaders, J. Dashe, J. M. J. Fréchet, *J. Am. Chem. Soc.* **2008**, *130*, 10494.
- [42] A. Fu, R. Tang, J. Hardie, M. E. Farkas, V. M. Rotello, *Bioconjugate Chem.* **2014**, *25*, 1602.

- 1 [43] M. Yessine, *Adv. Drug Delivery Rev.* **2004**, 56, 999.
- 2 [44] D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, *Nat. Rev. Drug*
- 3 *Discovery* **2005**, 4, 581.
- 4 [45] B. C. Evans, C. E. Nelson, S. S. Yu, K. R. Beavers, A. J. Kim, H. Li,
- 5 H. M. Nelson, T. D. Giorgio, C. L. Duvall, *J. Visualized Exp.* **2013**,
- 6 <https://doi.org/10.3791/50166>.
- 7 [46] V. Bulmus, *J. Controlled Release* **2003**, 93, 105.
- 8 [47] M. T. Tosteson, S. J. Holmes, M. Razin, D. C. Tosteson, *J. Membr.*
- 9 *Biol.* **1985**, 87, 35.
- 10 [48] W. F. DeGrado, G. F. Musso, M. Lieber, E. T. Kaiser, F. J. Kézdy,
- 11 *Biophys. J.* **1982**, 37, 329.
- 12 [49] D. Vercauteren, R. E. Vandenbroucke, A. T. Jones, J. Rejman,
- 13 J. Demeester, S. C. De Smedt, N. N. Sanders, K. Braeckmans,
- 14 *Mol. Ther.* **2010**, 18, 561.
- 15 [50] S. Xiang, H. Tong, Q. Shi, J. C. Fernandes, T. Jin, K. Dai, X. Zhang,
- 16 *J. Controlled Release* **2012**, 158, 371.
- 17 [51] A. Sanchez, *J. Infect. Dis.* **2007**, 196, S251.
- 18 [52] M. F. Nagoor Meeran, S. N. Goyal, K. Suchal, C. Sharma, C. R. Patil,
- 19 S. K. Ojha, *Front. Pharmacol.* **2018**, 9, 892.
- 20 [53] R.-Z. Huang, G.-B. Liang, M.-S. Li, Y.-L. Fang, S.-F. Zhao,
- 21 M.-M. Zhou, Z.-X. Liao, J. Sun, H.-S. Wang, *MedChemComm* **2019**,
- 22 10, 584.
- 23 [54] J. A. R. Salvador, A. S. Leal, A. S. Valdeira, B. M. F. Gonçalves,
- 24 D. P. S. Alho, S. A. C. Figueiredo, S. M. Silvestre, V. I. S. Mendes,
- 25 *Eur. J. Med. Chem.* **2017**, 142, 95.
- 26 [55] Q. Cheng, M. Liao, H. Hu, H. Li, L. Wu, *Cell. Physiol. Biochem.* **2018**,
- 27 47, 279.
- 28 [56] J. Friedrich, C. Seidel, R. Ebner, L. A. Kunz-Schughart, *Nat. Protoc.*
- 29 **2009**, 4, 309.
- 30 [57] G. Mehta, A. Y. Hsiao, M. Ingram, G. D. Luker, S. Takayama,
- 31 *J. Controlled Release* **2012**, 164, 192.
- 32 [58] R. Sutherland, *Science* **1988**, 240, 177.
- 33 [59] H. Hunt, L. Simón-Gracia, A. Tobí, V. R. Kotamraju, S. Sharma,
- 34 M. Nigul, K. N. Sugahara, E. Ruoslahti, T. Teesalu, *J. Controlled*
- 35 *Release* **2017**, 260, 142.
- 36 [60] L. Paasonen, S. Sharma, G. B. Braun, V. R. Kotamraju,
- 37 T. D. Y. Chung, Z.-G. She, K. N. Sugahara, M. Yliperttula, B. Wu,
- 38 M. Pellicchia, E. Ruoslahti, T. Teesalu, *ChemBioChem* **2016**, 17, 570.
- 39 [61] E. Ruoslahti, *Adv. Drug Delivery Rev.* **2017**, 110-111, 3.
- 40 [62] L. Simón-Gracia, P. Scodeller, S. S. Fuentes, V. G. Vallejo, X. Ríos,
- 41 E. San Sebastián, V. Sidorenko, D. Di Silvio, M. Suck, F. De Lorenzi,
- 42 L. Y. Rizzo, S. von Stillfried, K. Kilk, T. Lammers, S. E. Moya,
- 43 T. Teesalu, *Oncotarget* **2018**, 9, 18682.
- 44 [63] P. Säälik, P. Lingasamy, K. Toome, I. Mastandrea, L. Rouso-Noori,
- 45 A. Tobí, L. Simón-Gracia, H. Hunt, P. Paiste, V. R. Kotamraju,
- 46 G. Bergers, T. Asser, T. Rätsep, E. Ruoslahti, R. Bjerkvig,
- 47 D. Friedmann-Morvinski, T. Teesalu, *J. Controlled Release* **2019**, 308,
- 48 109.
- 49 [64] H. T. T. Duong, F. Hughes, S. Sagnella, M. Kavallaris, A. Macmillan,
- 50 R. Whan, J. Hook, T. P. Davis, C. Boyer, *Mol. Pharmaceutics* **2012**, 9,
- 51 3046.
- 52 [65] P. Figueiredo, M. H. Sipponen, K. Lintinen, A. Correia, A. Kiriazis,
- 53 J. Yli-Kauhaluoma, M. Österberg, A. George, J. Hirvonen,
- 54 M. A. Kostainen, H. A. Santos, *Small* **2019**, 15, 1901427.

Q8