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Actin Cytoskeleton-Disrupting and Magnetic Field-Responsive Multivalent Supramolecular Assemblies for Efficient Cancer Therapy

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Cite This: ACS Ap	ppl. Mater. Interfaces 2020, 12,	13709–13717	Read Online	
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ABSTRACT: Actin cytoskeleton disruption is a promising and intriguing anticancer strategy, but their efficiency is frequently compromised by severe side effects of the actin cytoskeleton-disrupting agents. In this study, we constructed the biocompatible actin cytoskeleton-targeting multivalent supramolecular assemblies that specifically target and disrupt the tumor actin cytoskeleton for cancer therapy. The assemblies were composed of β -cyclodextrin-grafted hyaluronic acid (HACD) and iron oxide magnetic nanoparticles (MNPs) grafted by an actin-binding peptide (ABP) and adamantane (Ada)-modified polylysine. Owing to the multivalent binding between cyclodextrin and Ada, HACD, and peptide-grafted MNPs (MNP-ABP-Ada) could self-assemble to form MNP-ABP-Ada \subset HACD nanofibers in a geomagnetism-dependent manner. Furthermore, the presence of ABP rendered the assemblies to efficiently target the actin cytoskeleton. Interestingly, with the acid of a low-frequency alternating magnetic field (200 Hz), the actin cytoskeleton-targeting nanofibers could induce severe actin disruption, leading to a remarkable cell cycle arrest and drastic cell death of tumor cells both in vitro and in vivo,



but showed no obvious toxicity to normal cells. The actin cytoskeleton-targeting/disrupting supramolecular assembly implies an excellent strategy for realizing efficient cancer therapy.

KEYWORDS: supramolecular assembly, cyclodextrin, magnetic nanoparticle, actin cytoskeleton, cancer therapy

INTRODUCTION

Actin cytoskeleton, one of the main cytoskeletal components, is critical for numerous cell processes in tumor tissues, for example, survival, proliferation, and metastasis.¹⁻⁴ Therefore, targeted disruption of actin cytoskeleton is a promising strategy for high-efficient treatment of cancerous diseases, especially those resistant to common chemotherapeutic drugs.^{5,6} However, many of the agents targeting the actin cytoskeleton (e.g., phalloidin, cytochalasin B, and palytoxin) are toxic not only to tumor cells but also to normal cells, leading to severe side effects on our human bodies.⁷⁻¹⁰ One strategy that may facilitate the development of safe, effective actin-cytoskeletondisrupting therapies is the use of biofunctional assemblies. In particular, supramolecular assemblies that show reversible activity and respond to multiple stimuli have been explored for controlled and targeted delivery of drugs and genes and for preventing metastasis of tumor cells.¹¹⁻¹³ Unfortunately, there have been no reports to date on the use of actin-cytoskeletondisrupting supramolecular assemblies for cancer therapy.

Cyclodextrins are star molecules with good biocompatibility and strong carrying ability, having a wide application in a biomedical area.^{14–17} For example, native and modified cyclodextrins have been developed to carry poorly hydrophilic agents for targeted drug delivery and to remove hazardous metabolic products (e.g., cholesterol and bile acid).^{18–20} Especially, cyclodextrin-based supramolecular assemblies have been proven to be useful tools for delivering chemotherapeutic drugs or disrupting specific organelles and biomacromolecules for tumor therapy.^{21–27} Furthermore, we found that cyclo-dextrin-based supramolecular assemblies could confine tumor cells and consequently suppressing tumor invasion and metastasis.^{28,29} However, it remains a great challenge of cyclodextrin assemblies to simultaneously realize CAK therapy—that is, confining tumor cells at the primary site, arresting their growth, and killing them in situ to overcome the shortfalls of traditional therapies.³⁰

Development of cancer therapies targeting tumor cells and specific cellular components (especially cytoskeletons and organelles) is becoming a hot topic.^{31–36} Hyaluronic acid (HA) and specific polypeptides are widely used biomedical targeting molecules. On the one hand, because HA receptors are overexpressed on the surface of cancer cells rather than normal cells,^{31,32} HA could specifically mediate tumor-cell targeting. On the other hand, abundant polypeptides, especially mitochondrion-targeting peptides (MitPs) and

Received: January 30, 2020 Accepted: March 2, 2020 Published: March 2, 2020







tubulin-targeting peptides (e.g., plugH), actin-targeting peptides (ABPs), have been proven to specifically target intracellular organelles or tubulin cytoskeletons, leading to dysfunction of their targets with the aid of photodynamic or cross-linking agents.^{33–36} However, there is no report to construct multiplex supramolecular assemblies based on these targeting molecules in combination and to develop their biological applications, as far as we know.

Herein, we report the synthesis of actin-cytoskeletontargeting/disrupting supramolecular assemblies that respond to magnetic fields. The assemblies are composed of biocompatible β -cyclodextrin-grafted hyaluronic acid (HACD) and iron oxide (Fe₃O₄) magnetic nanoparticles (MNPs) grafted by ABP and adamantane (Ada)-modified polylysine (Scheme 1, Figure S1). Owing to the specific binding ability of HA to the tumor cell surface and of ABP to the actin cytoskeleton, and to the presence of MNPs responding to magnetic fields, the HACD-bearing magnetic assemblies exhibited magnetic field-directed aggregation behavior, and could bind and disrupt the actin cytoskeleton of tumor cells, thereby arresting the cell cycle and killing the cells in situ with the aid of an alternating magnetic field (AMF). Moreover, the formed nanofibers could also confine the tumor cells at the primary sites, resulting in remarkable suppression of tumor metastasis. This is the first report of the use of biocompatible supramolecular assemblies for disruption of the tumor actin cytoskeleton. Our findings may facilitate the development of new stimuli-responsive supramolecular biomaterials with greatly improved therapeutic efficacy.

EXPERIMENTAL SECTION

Materials. Aminopropyltriethoxysilane (ATPES) and glutaraldehyde were purchased from Sigma (USA). All other reagents were purchased from Aladdin (China). HACD was synthesized by an 1ethyl-3-[3-dimethylaminopropyl]carbodiimide/N-hydroxysulfosuccinimide—mediated reaction between HA sodium and mono-6-deoxyl-6-ethylenediamino- β -CD, according to the literature method.³⁷

Synthesis of FITC-Labeled ABP and Ada-Modified Polylysine. FITC-labeled ABP (FITC-ACP-MGVADLIKKFESISKEEC, MW = 2,530)³⁸ was synthesized using the solid-phase method by Beijing Protein Innovation (China). The obtained peptide was characterized by high-performance liquid chromatography (LC3000, Constant Innovation, China) and mass spectrometry (LC-MS2010, Shimadzu, Japan).

For the synthesis of Ada-modified polylysine, 5 mg of Ada-COOH was dissolved in 100 mL of PBS (50 mM, pH = 7.4), and then 10 mg of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide and 10.5 of mg *N*-hydroxysulfosuccinimide were added. After the solution was stirred

for 30 min at room temperature, 50 mg of polylysine (MW \approx 5,000) was added, and stirring was continued at 4 °C for 12 h. The resulting Ada-modified polylysine was isolated by dialysis (MW cutoff = 2000–5000) in distilled H₂O (dH₂O) and then lyophilized in a vacuum freeze drver.

Synthesis of MNPs. The MNPs used in this study were prepared by the co-precipitation method. Briefly, 100 mL of 0.3 M FeCl₂ in dH₂O was mixed with 100 mL of 0.6 M FeCl₃ in dH₂O. Then, 100 mL of 0.3 M NaOH was slowly added to the mixture, which was then heated at 80 °C for 30 min. The precipitated MNPs were obtained by filtration, washed with dH₂O until the pH of the wash solution reached 7.0, and then dried at room temperature. The morphology of the MNPs was characterized by transmission electron microscopy (TEM, Tecnai G² F-20, FEI, USA), and their crystal structure and composition were determined by X-ray diffraction analysis (D/max-2500, Rigaku, Japan).

Synthesis and Characterization of the MNP-ABP-Ada and MNP-ABP-Ada CHACD Assemblies. For preparation of MNP-ABP-Ada, 50 mg of MNPs were suspended in 50 mL of ethanol and then 2.5 mL of ATPES were added. After the mixture was stirred at 80 $^\circ\mathrm{C}$ for 2 h, the products were collected by centrifugation and washed three times with ethanol and twice with distilled water. The resulting silanized MNPs with free amino groups (MNP-NH₂) were suspended in 40 mL of 8% glutaraldehyde (prepared in PBS, pH 7.4). The suspension was magnetically stirred at room temperature for 6 h and then centrifuged to pellet MNP-NH-glutaraldehyde. The pellet was washed three times with PBS and then suspended in 30 mL of the same buffer. ABP (5 mg) and 5 mg of Ada-modified polylysine were dissolved in 1 mL of DMSO and then added to the suspension, which was stirred at 4 °C for 24 h. The suspension was centrifuged, and the pellet was washed twice with dH2O and lyophilized in a vacuum freeze dryer to afford the MNP-ABP-Ada assemblies. Equal volumes of the MNP-ABP-Ada assemblies (100 mg/L) and HACD (100 mg/ L) were mixed and sonicated for 5 min to generate the MNP-ABP-AdaCHACD assemblies. The MNP-ABP-Ada and MitP-MNP-Ada⊂HACD assemblies were characterized by TEM (Tecnai G² F-20. FEI, USA) and confocal microscopy (FV1000, Olympus, Japan).

Confocal Microscopy of Intracellular Supramolecular Aggregates. The A549 cells (purchased from Cell Resource Center, China Academy of Medical Science Beijing, China) were precultured for 24 h and then incubated with MNP-ABP-Ada (50 mg/L) or MNP-ABP-Ada \subset HACD (50 mg/L of MNP-ABP-Ada plus 50 mg/L of HACD) for a further 24 h. The cells were then washed with PBS and fixed with 4% formaldehyde for 1 h. The fixed cells were treated with 0.5% Triton X-100 for 10 min, stained with rhodamine B-tagged phalloidin (100 nM) and 4',6-diamidino-2-phenylindole (5 mg/L), washed three times with PBS, and observed by confocal microscopy (Olympus, FV1000, Japan).

Cell Cycle and EdU Incorporation Assays. A549 cells were precultured for 24 h and then treated with MNP-ABP-Ada (50 mg/L) or MNP-ABP-Ada CHACD (50 mg/L of MNP-ABP-Ada plus 50 mg/



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Figure 1. Self-assembly of MNP-ABP-Ada and HACD into supramolecular nanofibers. (a) TEM images of irregularly distributed MNP-ABP-Ada assemblies in the visual field; (b,c) TEM images of MNP-ABP-AdaCHACD assemblies ([MNP-ABP-Ada] = [HACD] = 100 mg/L); and (d) confocal microscopy images showing the formation of the MNP-ABP-AdaCHACD assemblies over time.

L of HACD) for a further 24 h, fixed overnight with 95% ethanol, and then suspended in 50 mM citrate buffer (pH = 7.4). The fixed cells were treated with RNase (0.5 mg/mL, Sigma, USA) at 37 °C for 1 h and then stained with propidium iodide (PI) (20 mg/L) for flow cytometry analysis (FACS Calibur, BD, USA). Incorporation of EdU by the treated cells was measured with a BeyoClick EdU-555 kit (Beyotime, China).

In Vitro Cell Viability Assay. A549 cells were treated with MNP-ABP-Ada (50 mg/L) or MNP-ABP-Ada⊂HACD (50 mg/L of MNP-ABP-Ada plus 50 mg/L of HACD) in the absence or presence of an AMF (200 Hz, 10 A, 10 V) for 24 h. The growth of the treated cells was analyzed by means of a Cell Counting Kit-8 (Dojindo, Japan). Apoptosis of the treated cells was detected with an FITC-Annexin V/ PI assay kit (Sungene, China).

In Vitro Tumor Cell Invasion and Migration Assay. The A549-Luc2-tdT-2 cells were purchased from the Cell Resource Center, China Academy of Medical Science (Beijing, China) and were cultured in McCoy's 5A medium supplemented with 10% FBS (Hyclone, Australia) in a CO₂ incubator at 37 °C for 48 h. The cells were then suspended in FBS-free McCoy's 5A medium at 5×10^5 cells/mL. Matrigel (Corning, USA) was diluted in coating buffer (0.01 M Tris, 0.7% NaCl, pH 8.0) to a final concentration of 300 μ g/ mL and then added to 24-well invasion chambers, which were incubated at 37 °C for 2 h for gelation. Cell suspensions (400 μ L) containing MNP-ABP-Ada (50 mg/L) or MNP-ABP-AdaCHACD (50 mg/L of MNP-ABP-Ada plus 50 mg/L of HACD) were added to the Matrigel in the invasion chambers and cultured for 12 h. The cells that invaded the Matrigel were observed by a confocal microscope. The fluorescence intensity of the cells at different heights in the Matrigel was quantified by means of Image J software.

In Vivo Tumor Metastasis Model. The animal experiments were approved by the Animal Care and Use Committee at Nankai University. In vivo tumor metastasis was evaluated in 4T1-bearing nude mice. For this purpose, 200 μ L of the 4T1 cell suspension (1 × 10⁸ cells/mL, prepared in saline) was subcutaneously inoculated into the rear legs of 4-week-old female BALB/c nude mice (Huafukang, China). After 3 days, MNP-ABP-Ada (50 mg/kg) or MNP-ABP-Ada⊂HACD (50 mg/kg of MNP-ABP-Ada plus 50 mg/kg of HACD) assemblies were then intravenously injected into the mice repeatedly every 3 days. At 12 days after the first intravenous injection, the lungs of the mice were stained with India ink. The ink could fill the normal alveoli and result in black-staining of healthy lung tissues, but could not enter into the metastatic sites, which may therefore exhibit white spots.³⁹ The stained lungs were sampled and photographed. The areas of metastasis in the lungs were quantified using ImageJ software.

In Vivo Tumor Damaging Assay. The ability of the assemblies to damage tumor cells was evaluated in S180 tumor-bearing mice. S180 tumor cells (100 μ L, suspended in saline, 10⁷ cells/mL) were subcutaneously injected at the hind leg into 4-week-old female BALB/ c mice (Huafukang, China). After 7 days, 15 mice were randomly divided by three groups (5 mice per group), that is, the control group (received PBS), the MNP-ABP-Ada group, and MNP-ABP-Ada + HACD group. For the MNP-ABP-Ada and MNP-ABP-Ada + HACD groups, MNP-ABP-Ada (50 mg/kg) or MNP-ABP-AdaCHACD (50 mg/kg of MNP-ABP-Ada plus 50 mg/kg of HACD) assemblies were then intravenously injected via tail veins into the mice repeatedly every 3 days. After exposure to an AMF (200 Hz, 10 A, 10 V, 30 min per day) for 12 days of treatment, the tumors were excised and weighed. The tumor samples were fixed with a 4% formaldehyde solution, embedded in paraffin, and sectioned. The sections were stained by means of a TUNEL assay kit and observed with a fluorescence microscope (DM3000, Leica, Germany), and the number of TUNEL-positive cells was calculated. At least 20 fields were quantified.

Statistical Analysis. Each experiment was performed with three replicates, and values are given as means \pm standard deviations. Differences between groups were compared by means of one-way ANOVA (p < 0.05). All statistical tests were performed using SPSS Software (ver. 20, IBM, USA).

RESULTS AND DISCUSSION

Construction of the Magnetic-Field-Responsive MNP-ABP-Ada CHACD Assemblies. To prepare ABP-modified MNPs, we first synthesized iron oxide (Fe_3O_4) nanoparticles by using a co-precipitation method.⁴⁰ TEM and X-ray diffraction analysis demonstrated that the obtained MNPs were cube-like with a diameter of 10-20 nm (Figure S2a). Xray diffraction analysis also indicated that they were pure Fe₃O₄ (Figure S2b). Next, MNPs were silanized with ATPES to install surface -NH₂ groups, which were then covalently decorated with fluorescein isothiocyanate (FITC)-labeled ABP (FITC-ACP-MGVADLIKKFESISKEEC) (Figure S3)³⁸ and Ada-modified polylysine (MW = \sim 5,000, which facilitates multivalent binding of Ada to β -cyclodextrin and uptake of the nanoparticles) with mediation by glutaraldehyde (Figure S1). The resulting MNP-ABP-Ada assemblies had the desired abilities to bind to actin and interact with β -cyclodextrin. Like the initial MNPs, the MNP-ABP-Ada particles were cube-like (as indicated by TEM), but they tended to form morecondensed nanoclusters (Figure 1a). Higher-magnification TEM images confirmed that the surface of the MNPs was decorated with ABP-Ada (Figure S4).

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Figure 2. Disruption of the actin cables by the MNP-ABP-Ada \subset HACD assemblies without AMF (–AMF, a) or with AMF (+AMF, b). "Rho B-Pha" indicates rhodamine B-tagged phalloidin. Noting that while MNP-ABP-Ada has no obvious impact on the formation of actin cables, the MNP-ABP-Ada \subset HACD assemblies induce partial aggregation of actin cables (indicated by the yellow arrow), and AMF in combination with the assemblies severely disrupt the cables (indicated by the white arrows).



Figure 3. Actin cytoskeleton targeting and cell cycle arrest by the MNP-ABP-Ada \subset HACD assemblies in A549 tumor cells. (a) Confocal microscopy images showing intracellular distributions of the MNP-ABP-Ada and MNP-ABP-Ada \subset HACD assemblies. The A549 tumor cells were treated with MNP-ABP-Ada (50 mg/L) or MNP-ABP-Ada \subset HACD (50 mg/L of MNP-ABP-Ada plus 50 mg/L HACD) for 24 h, stained with Rho B-Pha (for actin staining), and observed by confocal microscopy. DAPI = 4,6-diamidino-2-phenylindole. (b) FACS of the cell cycle in cells treated with MNP-ABP-Ada or MNP-ABP-Ada \subset HACD. (c) 5-Ethynyl-2-deoxyuridine (EdU)-incorporation assays. (d) Cell growth evaluated by means of a cell proliferation assay. (e) Cell death evaluated by PI staining. * Indicates significant between-group difference (P < 0.05). Error bars indicate \pm standard derivations.

Thus, if actin polarization could be governed by the assemblies with the aid of the geomagnetic field, we prepared supramolecular assemblies consisting of MNP-ABP-Ada non-covalently cross-linked with HACD. Although the MNP-ABP-Ada assemblies were freely distributed in the visual field (Figure 1a), when MNP-ABP-Ada and HACD were combined, they self-assembled into nanofibrous supramolecular assemblies that were 50–200 nm in diameter and hundreds of micrometers in length (Figure 1b,c).

To confirm that these assemblies responded to the geomagnetic field (~0.05 mT), we monitored their formation in real-time by means of confocal microscopy. Initially, the MNP-ABP-Ada and HACD interacted with each other to form small aggregates with lengths of $10-50 \ \mu m$ (Figure 1d, 2 min). Over the course of 10 min, the aggregates elongated and adhered to one another along the direction of the geomagnetic field lines, eventually forming long nanofibers ($100-200 \ \mu m$; Figure 1d, 5–10 min). MNP-ABP-Ada and HACD could also respond to an artificial magnetic field ($1.8 \ mT$), rapidly forming nanofibers (within 2 min) along the direction of the magnetic field (Figure S5).

Strong Actin-Binding and Actin Cytoskeleton-Disrupting Activity of the Assemblies. Owing to the presence of ABP, we hypothesized that the assemblies may have an actin-binding ability. When incubated in the actin polymerization buffer, actin could self-assemble into long actin cytoskeleton cables (stained by rhodamine B-tagged phalloidin, Figure 2a, control). When co-incubated with the preformed actin cables, both MNP-ABP-Ada and the MNP-ABP-Ada \subset HACD assemblies could bind the cables (Figure 2a). As compared to MNP-ABP-Ada, the assemblies led to obvious swelling of the cables (indicated by the yellow arrow, Figure 2a), indicating that the assemblies might induce partial actin aggregation. Especially, with the aid of AMF, the assemblies, rather than MNP-ABP-Ada, caused severe disruption of the actin cables (indicated by the white arrows, Figure 2b). Therefore, the assemblies could target and severely impair the actin cytoskeleton, especially under the treatment of AMF. The increased actin cytoskeleton-disrupting efficiency of the assemblies as compared to MNP-ABP-Ada may be attributed to the enhancement of multivalent binding of the NPs to actin cytoskeleton.

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Figure 4. In vitro and in vivo antitumor activity of MNP-ABP-Ada \subset HACD assemblies in combination with an AMF. (a) Apoptosis assay of A549 tumor cells treated with MNP-ABP-Ada (50 mg/L) or MNP-ABP-Ada \subset HACD (50 mg/L plus 50 mg/L) assemblies in the absence of an AMF (-AMF) or in the presence of an AMF (+AMF). (b) Disruption of the actin cytoskeleton by treatment with the MNP-ABP-Ada \subset HACD assemblies and an AMF. The white arrows indicate a disrupted actin cytoskeleton. (c) NMR images of S180-tumor-bearing mice 24 h after injection with MNP-ABP-Ada or MNP-ABP-Ada \subset HACD. The dotted circles indicate the tumor sites, and the blue arrow indicates assemblies accumulated at the tumor site. (d) Images of representative tumors removed from mice treated with the assemblies and an AMF. Five tumor-burden mice were used for each group. (e) Weights of tumors removed from mice treated with the assemblies and an AMF. (f) Time-dependent change of tumor volumes of the mice treated by MNP-ABP-Ada or MNP-ABP-Ada \subset HACD with the aid of AMF. (g) Confocal images of TUNEL kit-stained tumor slices. (h) Quantification of the TUNEL-positive cells. * Indicates significant between-group differences (P < 0.05). Error bars indicate \pm standard derivations.

Intracellular Targeting and Disruption of the Actin Cytoskeleton by the Supramolecular Assemblies. The actin-binding abilities of the MNP-ABP-Ada and MNP-ABP-AdaCHACD assemblies were evaluated in A549 tumor cells. Because MNP-ABP-Ada and HACD spontaneously form micrometer-large nanofibers that are difficult to be internalized by cells, their mixture was vigorously vortexed to temporarily disrupt the nanofibers and then immediately added into the culture media. After being incubated with the mixture, the cells were stained with the actin probe rhodamine B-phalloidin (Rho B-Pha). Confocal microscopy clearly demonstrated that MNP-ABP-Ada and HACD entered into the cells and reassembled to form MNP-ABP-Ada⊂HACD nanofibers (as indicated by the fluorescence of FITC-ABP, Figure 3a). Moreover, the nanofibers were co-localized with the actin cytoskeleton (Figure 3a), confirming that MNP-ABP-Ada and HACD could be internalized and could again form nanofibers

to target the actin cytoskeleton. Quantification assay of Rho B-Pha/FITC co-localization further showed that the Pearson's correlation coefficient of the MNP-ABP-AdaCHACD group was higher than that of the MNP-ABP-Ada group (0.831 vs 0.656, Table S1), indicating that the nanofibers had a higher actin cytoskeleton-targeting ability than MNP-ABP-Ada. Interestingly, unlike the control cells and the MNP-ABP-Ada-treated cells, the MNP-ABP-Ada⊂HACD-treated cells exhibited partial disruption of the actin cytoskeleton, as indicated by the aggregation of the actin cables in some regions (Figure 3a, white arrows). Moreover, free ABP, a competitive agent of the assemblies, remarkably inhibited the assemblies to target the intracellular actin cytoskeleton (Figure S6). These results implied that the supramolecular assemblies targeted actin cytoskeleton and affected actin dynamics, which was attributed to the actin cytoskeleton-targeting ability of ABP.

Cell Cycle Arrest and Growth Inhibition Caused by the MNP-ABP-AdaCHACD Assemblies. The actin cytoskeleton is a key regulator of the cell cycle, and its dysfunction can severely disrupt the cycle.⁴¹ Because the MNP-ABP-AdaCHACD assemblies disrupted the actin cytoskeleton, we evaluated their effect on the cell cycle by means of a flow cytometry-based assay. The assay showed that the assemblies increased the percentage of G1-phase cells (from 51% in the control to 81%) and decreased the percentage of S-phase cells (from 29.19 to 12.19%) (Figure 3b), implying that the assemblies induced cell cycle arrest at the G1 phase. Furthermore, EdU-incorporation analysis, which measures DNA synthesis in the S-phase cells owing to the incorporation of EdU (an analogue of thymidine) into replicating DNA and following the introduction of a Biotin group via the Cu(I)catalyzed click reaction,^{42,43} which indicated that the assemblies decreased the percentage of EdU-containing cells from 28 to 4.5%, confirming that DNA synthesis was severely suppressed (Figure 3c). This observation is consistent with reports that disruption of the actin cytoskeleton may arrest cell cycle progression at the G1/S phase and reduce 5-bromo-2-deoxyuridine incorporation.^{44,45} In addition, a cell proliferation assay showed that the MNP-ABP-AdaCHACD assemblies decreased cell viability to a greater extent than the MNP-ABP-Ada assemblies (54 vs 15%, Figure 3d) did. This decrease was not attributed to cell death because both the MNP-ABP-Ada and MNP-ABP-AdaCHACD assemblies caused comparable levels of cell death ($\sim 20\%$, Figure 3e).

Severe Tumor Damage Induced by the MNP-ABP-Ada_CHACD Assemblies with AMF. Because the actin cytoskeleton is important for cell survival,^{46–48} we speculated that the partial disruption of actin caused by treatment with the MNP-ABP-AdaCHACD assemblies might lead not only to cell cycle arrest but also to cell death. However, an apoptosis assay showed that both the MNP-ABP-Ada and the MNP-ABP-Ada⊂HACD assemblies induced apoptosis in only ~30% of the cells (Figure 4a). Therefore, we investigated whether an AMF could enhance the ability of the assemblies to kill tumor cells. Exposure to an AMF had no impact on untreated control cells, with both the AMF-untreated and AMF-treated control cells showing a low percent of apoptosis (7.3-7.4%), caused by long-term culturing (48 h) of the cells) (Figure 4a). In contrast, the field remarkably enhanced apoptosis of cells treated with the MNP-ABP-Ada or MNP-ABP-Ada⊂HACD assemblies (Figure 4a). Strikingly, in the presence of an AMF, treatment with the MNP-ABP-AdaCHACD assemblies led to the death of almost 100% of the tumor cells (~94% apoptotic cells and ~5% necrotic cells); in contrast, the MNP-ABP-Ada assemblies killed only ~55% of the cells under the same conditions (Figure 4a). However, neither of the two types of assemblies decreased the viability of noncancerous cells (e.g., NIH3T3 and 293T), even in the presence of an AMF (Figure S7). The difference in toxicity of MNP-ABP-Ada or MNP-ABP-Ada⊂HACD to cancerous and noncancerous cells may be attributed to the distinct uptake of nanoparticles or nanoassemblies between these two kinds of cells (Figure S8).⁴⁹

To determine whether the killing activity of the assemblies was associated with the disruption of the actin cytoskeleton, we observed the intracellular distribution of the cytoskeleton upon treatment with the assemblies in the presence of an AMF. Whereas the control cells showed normal actin cables and patches, cells treated with the MNP-ABP-Ada or MNP-ABP-Ada CHACD assemblies had obviously disrupted actin

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cytoskeletons (Figure 4b, white arrows). Remarkably, the disruption was more severe in the MNP-ABP-AdaCHACDtreated cells than in the MNP-ABP-Ada-treated cells; in fact, the cytoskeleton in the MNP-ABP-Ada⊂HACD-treated cells was totally collapsed (Figure 4b). Moreover, neither bare MNPs nor MNP-MitPCHACD assemblies (which target mitochondria) disrupted the actin cytoskeleton (Figure S9) or had a strong impact on cell viability (Figure S10) exhibited by the MNP-ABP-Ada⊂HACD assemblies. Notably, under the conditions used for AMF treatment, no obvious temperature increase was observed (Figure S11). This is attributed to the application of low-frequency AMF (200 Hz), which may avoid AMF-induced overheating of the nanoparticle-enriched sites, and therefore prevent AMF-induced damage of normal tissues in the body. The result ruled out the possibility that a magnetocaloric effect caused the observed cell death. These results confirm that actin disruption, which further induced cell cycle arrest and apoptosis, contributed to the ability of the MNP-ABP-AdaCHACD assemblies to kill tumor cells with the aid of a low-frequency AMF.

The in vivo anticancer activity of the supramolecular assemblies in combination with an AMF was evaluated in S180-tumor-bearing mice. NMR imaging 24 h after injection of the assemblies revealed that they accumulated mainly at the tumor site but were also present in the lung and the liver (Figure 4c). Consistently, inductively coupled plasma mass spectrometry revealed that the tumor tissues had much higher contents of iron than the representative organs, including lungs, livers, spleens, hearts, and kidneys (Figure S12). These results indicated that the assemblies had good tumor-targeting ability. In contrast, the MNP-ABP-Ada assemblies showed low distribution at the tumor sites.

After exposure to AMF for 12 days, the MNP-ABP-Ada⊂HACD assemblies showed much stronger antitumor activity than the MNP-ABP-Ada assemblies, almost completely eliminating the tumors; tumor weight in the treated group was approximately 5 and 11% of tumor weight in the control group and MNP-ABP-Ada-treated group, respectively (Figure 4d,e). Consistently, the MNP-ABP-AdaCHACD-treated group maintained extremely low tumor volumes during the whole period, while the control and MNP-ABP-Ada-treated groups exhibited increased tumor volumes with the increase in culture time (Figure 4f). A TdT-mediated dUTP nick-end labeling (TUNEL) assay further revealed that the MNP-ABP-AdaCHACD assemblies killed a much higher percentage of tumor cells (>95%) than the MNP-ABP-Ada assemblies (<50%) (Figure 4g,h) did. Meanwhile, the MNP-ABP-AdaCHACD assemblies, similar to MNP-ABP-Ada, had no obvious impact on body weights of the mice (Figure S13), indicating good biocompatibility of the assemblies. Taken together, these results show that the combination of the assemblies and an AMF had strong antitumor activity both in vitro and in vivo, and had no obvious toxicity to the bodies.

Confinement of Tumor Cells and Suppression of Metastasis by the MNP-ABP-Ada HACD Assemblies. Because the MNP-ABP-Ada HACD assemblies formed nanofibers in response to the geomagnetic field and because HACD can target tumor cells, we further hypothesized that the MNP-ABP-Ada HACD assemblies would exhibit tumor cellconfining activity like that shown by the MNP-MitPCHACD nanofibers (where MitP = mitochondrion-targeting peptide) reported previously.²⁴ We were pleased to find that the MNP-ABP-AdaCHACD assemblies specifically bound to lung

ACS Applied Materials & Interfaces Research Article www.acsami.org MNP-ABP-Ada⊂HACD **(C)** ஆ (a) Control MNP-ARP-Ada 3.5 -Control 3 -MNP-ABP-Ada Relative fluorescend intensity 5 1.5 1 2.5 MNP-ABP-Ada⊂HACD Hoechst 33342 staining 1 0.5 50 µm 50 µm 50 µm 0 100 200 300 Invasion depth (µm) 0 400 (b) 0 µm 100 µm 200 µm 300 µm 400 µm MNP-ABP-Ada ⊂HACD (d) Control MNP-ABP-Ada Control 100 µm (**a**) % Area of metastasis 100 MNP-ABP-Ada 80 60 100 µm 40 20 MNP-ABP-Ada 0 MNP-ABP-Ada MNP-ABP-Ada CHACD Control 00 µm

Figure 5. Suppression of metastasis by the MNP-ABP-Ada \subset HACD assemblies. (a) Microscopy images of the MNP-ABP-Ada \subset HACD assemblies during incubation with A549-RFP tumor cells (stained by Hoechst 33342). The red arrow indicates that the nanofibrous assemblies intertwined to form a network that confined the tumor cells. (b) Confocal microscopy images of tumor cells treated with the MNP-ABP-Ada or MNP-ABP-Ada \subset HACD assemblies. (c) Quantification of the red fluorescent protein fluorescence from the A549-RFP cells in various layers of the Matrigel. (d) In vivo suppression of metastasis from the rear legs to the lungs in 4T1-bearing nude mice by the MNP-ABP-Ada \subset HACD assemblies, but not by the MNP-ABP-Ada assemblies. (e) Quantification of areas of metastasis in the lungs. * Indicates significant between-group difference (P < 0.05). Error bars indicate \pm standard derivations.

carcinoma cells (A549-RFP, i.e., A549 cells expressing the red fluorescence protein) but not to noncancerous cells (NIH3T3 and 293T) (Figure S14). Moreover, when incubated with A549-RFP cells, the assemblies intertwined to form a network that bound the cells, whereas the MNP-ABP-Ada assemblies failed to do so (Figure 5a). Similarly, unlike MNP-ABP-Ada, the MNP-ABP-Ada⊂HACD assemblies markedly inhibited invasion of the tumor cells into Matrigel (Figure 5b,c) in an in vitro assay, indicating that the assembly-intertwined network strongly confined the tumor cells and prevented their invasion. In addition, in a standard in vivo metastasis assay involving 4T1-bearing nude mice, the nanofibers suppressed metastasis from the primary tumor site (rear leg) to the lungs; no obvious metastatic areas were observed in the lungs treated with the MNP-ABP-Ada⊂HACD assemblies (Figure 5d,e). These results revealed that the assemblies not only killed most of the tumor cells but also prevented them from metastasis.

CONCLUSIONS

In this study, we realized high-efficient actin cytoskeleton disruption for cancer therapy using nanofibrous supramolecular assemblies. The assemblies were composed of HACD noncovalently cross-linked to MNPs modified with both ABP and Ada-modified polylysine. The assemblies selectively targeted and disrupted the actin cytoskeleton of tumor cells, leading to cell cycle arrest and activation of apoptosis, especially in the presence of an AMF. Moreover, the assembly could also form extracellular network to confine the tumor cells and to prevent them from metastasis. Our results indicate that supramolecular assembly-mediated actin cytoskeleton disruption has the potential application in efficient cancer therapy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.0c01762.

Synthetic routes of MNP-ABP-Ada and HACD; characterization of MNP; HPLC and MS analysis; magnified TEM image; rapid assembly of MNP-ABP-Ada⊂HACD; inhibition of ABP addition on targeting of MNP-ABP-Ada⊂HACD to actin cytoskeleton; cell death assay of NIH3T3 and 293T cells; different uptake contents of MNP-ABP-Ada and MNP-ABP-Ada⊂-HACD; effect of MNPs, MNP-MitP⊂HACD, and MNP-ABP-Ada⊂HACD on actin cytoskeleton distribution of the A549 tumor cells under AMF treatment and on cell death; iron contents in tumor tissues and representative organs; temperature change of MNP-ABP-Ada and MNP-ABP-Ada⊂HACD; body weight of the tumor-burdened mice; quantification of fluorescence intensity; and statistical analysis of co-localization (PDF)

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Author Contributions

Q.Y., B.Z., and Y.-H.L. synthesized and performed the chemical and biological experiments and characterization. All authors analyzed and discussed the results. Q.Y. and Y.-M.Z. wrote the main manuscript. Y.L. supervised the work and edited the manuscript. All authors reviewed the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Yanan Chen and Dr. Jie Liu for confocal microscopy. This work was funded by the National Natural Science Foundation of China (grant nos. 21432004, 31870139, 21472100, 21772099, and 91527301).

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