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Multifunctional Albumin Nanoparticles As Combination Drug Carriers for Intra-Tumoral Chemotherapy

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Abstract

Current cancer therapies are challenged by weakly soluble drugs and by drug combinations that exhibit non-uniform biodistribution and poor bioavailability. In this study we have presented a new platform of advanced healthcare materials based on albumin nanoparticles (ANPs) engineered as tumor penetrating, delivery vehicles of combinatorially applied factors to solid tumors. These materials were designed to overcome three sequential key barriers: tissue level transport across solid tumor matrix; uptake kinetics into individual cancer cells; therapeutic resistance to single chemotherapeutic drugs. The ANPs were designed to penetrate deeper into solid tumor matrices using collagenase decoration and evaluated using a three-dimensional multicellular melanoma tumor spheroid model. Collagenase modified ANPs exhibited 1–2 orders of magnitude greater tumor penetration than unmodified ANPs into the spheroid mass after 96 hours, and showed preferential uptake into individual cancer cells for smaller sized ANPs (<100 nm). For enhanced efficacy, collagenase coated ANPs were modified with two therapeutic agents, curcumin and riluzole, with complementary mechanisms of action for combined cell cycle arrest and apoptosis in melanoma. The collagenase coated, drug loaded nanoparticles induced significantly more cell death within 3-D tumor models than the unmodified, dual drug loaded ANP particles and the kinetics of cytotoxicity was further influenced by the ANP size. Thus, multifunctional nanoparticles can be imbued with complementary size and protease activity features that allow them to penetrate solid tumors and deliver combinatorial therapeutic payload with enhanced cancer cytotoxicity but minimal collateral damage of healthy primary cells.

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1. Introduction

Dense tumor extracellular matrices (ECM) remain a significant barrier limiting diffusive transport and penetration of drug delivery agents.^[1, 2] Limited penetration can lead to heterogeneous drug distribution and concentration gradients within a tumor, further complicating a treatment regimen by promoting drug resistance in cancer cells exposed to low concentrations of therapeutics.^[3, 4] In addition, prolonged treatment of tumors with single therapeutic agents has been universally shown to contribute toward drug resistance.^[5]

Despite advances in drug discovery, many chemotherapeutic compounds fail to elicit response in solid tumors due to poor solubility,^[6, 7] limited tumor penetration,^[3, 8] and susceptibility toward drug resistance.^[9–13] Drug delivery vehicles have attempted to overcome some these challenges by increasing drug solubility,^[14, 15] controlling drug pharmacokinetics and tissue distribution,^[16, 17] or prolonging drug stability.^[18, 19] Solvent-based delivery approaches are able to improve drug solubility but pose significant risk of toxicity and result in uncontrollable systemic distribution limiting the overall effectiveness of potent drugs.^[20–24] The development of nanoscale drug delivery materials, such as nanoparticles, liposomes and micelles, has resulted in greater control over the release and stability of therapeutic cargo, and has led to safer and more effective treatment strategies.^[25, 26] However, solid tumor penetration and therapeutic resistance remain significant challenges for chemotherapeutics delivered by nanoscale materials.^[3, 27, 28]

Advanced nanomaterials have the multifunctional potential to overcome the varied barriers that hinder drug delivery into solid tumors.^[29] Previous work has shown that modifying the permeability of the tumor ECM can facilitate deeper nanoparticle penetration, while combinatorial drug delivery approaches that utilize multiple therapeutic compounds can further reduce the likelihood of drug resistance. Pre-treatment of tumors with protease enzymes that degrade ECM components, for example, can improve the degree of nanoparticle penetration and distribution within the tumor environment.^[30] Combinatorial therapies that capitalize on the synergistic effects of two or more agents acting on separate tumor-promoting pathways have been described using several drug delivery materials.^[31, 32] However, drug delivery materials that combine these two capabilities into a single, multifunctional platform have yet to be advanced.

Here, we report on the design of albumin-based nanoparticles that can readily integrate both active tumor penetration and multi-drug delivery within a single structure while enabling size-based tunability of cellular uptake. We hypothesized that the modification of albumin nanoparticles (ANPs) with ECM degrading proteases would increase the degree of nanoparticle penetration into a solid tumor model, which could be further tuned by controlling nanoparticle size (Figure 1). As carriers of a novel therapeutic cocktail of two complementary drugs, *riluzole* (an inhibitory regulator of glutamate receptors) and *curcumin* (an herbal derivative with chemotherapeutic and anti-inflammatory effects based on suppression of the Nuclear Factor- κ B Activation Pathway), the protease modified ANPs markedly enhanced drug functionality in terms of specific cancer cell killing within solid melanoma tumor models *in vitro*.

2. Methods

2.1. Reagents

Molecular biology grade ethanol (99.5%), HPLC grade methanol (99.8%), HPLC grade water with 0.1% trifluoroacetic acid (TFA) and HPLC grade acetonitrile (ACN) with 0.1% TFA were purchased from Sigma-Aldrich and used without further purification. Collagenase from *Clostridium histolyticum* was also purchased from Sigma-Aldrich (St. Louis, MO, USA). Curcumin was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Riluzole hydrochloride was purchased from Tocris Bioscience (Ellisville, MO, USA).

2.2. Cell Culture

WM239A human melanoma cells were provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). C8161(+) and C8161(−) human melanoma cells were from Dr. Mary J.C. Hendrix (Children's Memorial Research Center, Chicago, IL). AR7119 immortalized human melanocytes were provided by Dr. Dorothy Bennett (St. George's, University of London, UK).^[33] RPMI-1640 media was purchased from American Type Culture Collection (ATCC, Manassas, VA). Fetal bovine serum (FBS), trypsin-EDTA, streptomycin, and penicillin were purchased from Invitrogen (Carlsbad, CA, USA). Cells were routinely maintained in RPMI-1640 media, supplemented with 10% FBS, penicillin (100 µg ml^{−1}) and streptomycin (100 µg ml^{−1}) at 37 °C, in humidified atmosphere of 5% CO₂.

2.3. Albumin Nanoparticle Fabrication

Albumin nanoparticles were fabricated as described previously (Figure 1A).^[34] Albumin derived from human serum (HSA, fraction V, purity 96–99%, 65 kDa) and glutaraldehyde (8% solution) were obtained from Sigma-Aldrich and used without further purification. 2% (w/v) solution of HSA was dissolved in 10 mM NaCl to prepare 200 nm ANPs, and 2 mM NaCl to prepare 100 nm ANPs. The pH of HSA solution was adjusted to 8.50 ± 0.05 with 0.1 M NaOH. Under continuous stirring at 700 rpm at room temperature, 2 mL ethanol was added at 1.5 mL min^{−1} to the HSA solution (500 µL) with a syringe pump (Harvard Apparatus PHD 2000, Holliston, MA). 2.34 µL glutaraldehyde was added to ANPs suspension as a cross-linker following ethanol addition. The particles were left for crosslinking overnight under stirring at 700 rpm at room temperature.

2.4. Albumin Nanoparticle Purification

ANPs were purified by three rounds of centrifugation for 8 min at 4 °C (Beckman Coulter, Avanti J-E Centrifuge). 16 100× *g* was set for 200 nm ANPs, while 48 400× *g* was set for 100 nm ANPs. Resuspension of the pellets to original volume with PBS was done following each round of centrifugation. Resuspension was performed in an ultrasonication bath (Fisher Scientific, FS60).

2.5. Physical Characterization of Albumin Nanoparticles

ANPs were characterized using DLS and SEM. Particle size distributions and polydispersity indices were determined using a zetasizer (Zen 3690, Malvern, Worcestershire, UK). Samples were diluted 40 times with deionized water for *z*-average size and polydispersity

measurements of three sequential sample scans (3 runs scan⁻¹, 60 s scan⁻¹) at a 90° scattering angle and 37°C. Three separate batches of particles were measured and averaged. ANP autofluorescence was measured using a spectrofluorometer (SpexFluoroMax-3, Horiba Jobin Yvon, Edison, NJ, USA).

2.6. Preparation of Collagenase-Coated Nanoparticles

2% (w/v) solution of HSA and 0.1% w/v collagenase were dissolved in 10 mM NaCl to prepare 200 nm ANPs, and 2 mM NaCl to prepare 100 nm ANPs. Similar with the methods for preparing ANPs, the pH of HSA collagenase solution was adjusted to 8.50 ± 0.05 with 0.1 M NaOH. Under continuous stirring at 700 rpm at room temperature, 2 mL ethanol was added at 1.5 mL min^{-1} to 500 μL HSA solution with a syringe. 2.34 μL glutaraldehyde was added and the particles were left to cross link overnight under stirring at 700 rpm and room temperature. Using the same methods with ANPs, collagenase-coated nanoparticles were purified and characterized. Following centrifugation, ANPs pellets were resuspended to 1/10 of original volume with PBS for introduction to the tumor spheroid model.

2.7. Collagenase Activity Assay

The amount of collagenase conjugated on ANPs was measured by Rapid Collagenase Assay Kit with Type I Collagen Substrate (Chondrex, Redmond WA). Series dilutions of Reference recombinant Human Collagenase MMP-8 were used to build up standard curves. 100 μL of large or small collagenase coated ANPs in PBS were activated by 10 μL 0.1% w/v trypsin solution at 35°C for 15 min. 200 μL 0.05% w/v FITC-collagen was mixed with activated collagenase-ANP samples and reference standards, followed by 120 min incubation at 35°C. The collagenase-degradation products were then extracted and centrifuged at 10,000 rpm for 10 min. Supernatants of samples were transferred into a black 96-well plate and fluorescence intensities measured at $\lambda(\text{em}) = 520 \text{ nm}$ and $\lambda(\text{ex}) = 490 \text{ nm}$ (Detector Module 168, Beckman Instruments, San Ramon, CA). The amounts of collagenase on ANPs were quantified by comparing the fluorescence intensities of samples with reference standards.

2.8. Cellular uptake of collagenase coated nanoparticles

C8161(+) cells were seeded onto 48-well microtiter plates at a density of 10^5 cells per well, and incubated at 37°C for 24 hours. To quantify amount of nanoparticle uptake, the cells were treated with collagenase coated large and small ANPs for various times up to 24 hours. The cells were then trypsinized, washed, and fixed prior to being analyzed via fluorescent flow cytometry (FACScalibur; BD Biosciences, San Jose, CA) using the autofluorescence of the ANPs to determine uptake. Data was processed and analyzed using FlowJo. Data was collected for 10,000 cells.

2.9. Multicellular Tumor Model Spheroid Formation

Melanoma cells (C8161+) and human foreskin-derived fibroblast cells (HFFs) were grown in monolayer cultures prior to spheroid formation. To produce spheroids, both types of cells were trypsinized, centrifuged and mixed in 1:1 ratio in 200 ml complete media (RPMI media with 10% FBS and antibiotics). 20 μL of cell suspensions were aspirated and applied on a plate cover to form small droplets. Cell droplets were kept in an incubator at 37°C for 4

days. Spheroids that expanded to a diameter of 300–400 μm , were transferred to a 24-well agar plate and allowed to incubate for 2 additional days before processing.

2.10. Nanoparticle Penetration in Tumor Model Spheroids

The spheroids were aspirated with a pipette and transferred to a new 24-well agar plate. The medium was removed by aspiration, and spheroids were washed with PBS (pH 7.4) before further processing. The spheroids were treated with base ANPs, dual drug loaded ANPs, base collagenase-coated nanoparticles and dual drug loaded collagenase-coated nanoparticles with varying concentrations of 0% v/v, 5% v/v, 10% v/v and 25% v/v in culture medium for 72 h and 96h.

For the analysis of spheroids penetration by nanoparticles, spheroids were fixed, Hoechst stained and then processed in two ways: 1) cross sectioning, 2) direct confocal imaging. Spheroids were transferred to a cryomold, covered in optimal cutting temperature compound (O.C.T, Tissue-Tek, Torrance, CA), flash frozen, and processed by cutting 25 μm - thick sections using a cryostat (Shandon Cryotome E, Thermo Scientific, Waltham, MA). Fluorescent images of spheroid sections were obtained with a confocal laser microscope (Leica Microsystems Inc., Bannockburn, IL) and analyzed by a series of Matlab algorithms (Supplementary Figure 1). Two fluorescence modes were imaged: the green autofluorescence of the albumin shells and the blue nuclear Hoechst stain. The two channels were converted into binary masks, and any holes in the images were filled. The number of pixels per mask was then calculated and used to create a densitometric ratio of albumin fluorescence to cellular fluorescence. This was used as a measure of ANP penetration, as the greater the area of the albumin fluorescence is associated with a greater percentage of nanoparticles within the spheroids.

2.11. Drug Loading of Albumin Nanoparticles

Curcumin was dissolved in ethanol to yield a final concentration of 1 mg mL^{-1} and was dissolved at room temperature for 30 min. The curcumin solution was added drop-wise into a suspension of ANPs to yield final concentrations of 10 μM , 20 μM , 30 μM , 40 μM , 50 μM , 80 μM , 100 μM and 140 μM .

Riluzole was dissolved in ethanol to give a final concentration of 10 mM. Riluzole solution was then added into a suspension of ANPs to yield final concentrations of 50 μM (0.135% w/v), 60 μM (0.162% w/v), 70 μM (0.189% w/v), 80 μM (0.216% w/v), 90 μM (0.243% w/v), and 100 μM (0.270% w/v).

For combination drug loading, the curcumin solution was added drop-wise into ANPs suspension to yield final concentrations of 0.1% w/v, while the riluzole solution was added to yield final concentrations of 50 μM (0.135% w/v), 60 μM (0.162% w/v), 70 μM (0.189% w/v), 80 μM (0.216% w/v), 90 μM (0.243% w/v), and 100 μM (0.270% w/v). In all instances, the drug mixtures were adsorbed onto the nanoparticles by agitation for 3 hours at room temperature.

Drug loaded ANPs were purified by one round of centrifugation for 8 minutes at 4 $^{\circ}\text{C}$ (16 100 $\times$ g for 200 nm ANPs, while 48 400 $\times$ g for 100 nm ANPs). To characterize the drug

loaded ANPs, the pellets were resuspended to original volume with PBS following the centrifugation. To test the drug loaded ANPs in physiological conditions, pellets were resuspended to 1/10 of original volume. Resuspension was performed as described before.

2.12. Quantification of Drug Loading

The concentration of drugs loaded on ANPs were determined by high-performance liquid chromatography (HPLC, Beckman Coulter Model 166, Brea, CA) equipped with a Luna C18 column (3 μ m, 100 $\times$ 4.6 mm i.d., Phenomenex, Torrance, CA). The drug loaded ANPs were frozen at -80°C overnight and lyophilized for 48 hours. The powders were resuspended in ethanol to original volume, and subsequently assayed by HPLC to determine curcumin and riluzole concentrations. For chromatographic elution, the flow rate was 0.9 ml min^{-1} over the entire separation. A step gradient was utilized for elution in which the mobile phase consisted initially of 30% ACN and 65% water containing 0.1% TFA for the first 7 min, followed by 75% ACN and 25% water containing 0.1% TFA for the next 9 min. Curcumin was detected at 420 nm, while riluzole was detected at 255 nm (Detector Module 168, Beckman Instruments, San Ramon, CA). Serial dilutions of curcumin and riluzole standards were used to construct a calibration curve, with the linear portion utilized for measurements.

2.13. Cell Viability Assay

The metabolic activity of viable cells was assessed by CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Before drug treatment, 2-D melanoma cells were cultured on 96-well polystyrene tissue culture plates at a density of 5000 cells per well for overnight incubation at 37 $^{\circ}\text{C}$, while 3-D spheroids were directly transferred to 96-well culture plate. Wells were treated with different drug regimen (single drugs or single drug loaded nanoparticles or combination free drug or combination drug loaded nanoparticles) of varying concentrations starting from 1 μM to 50 μM . At 0 h, 24 h and 96 h time points, MTS reagent (20 μL) was added to each well of the assay plate containing 10% v/v test samples in culture medium (100 μL). The plates were incubated for 2 h at 37 $^{\circ}\text{C}$ in a 5% CO_2 atmosphere, after which the absorbance was measured at 490 nm using a plate reader. Background absorbance was corrected by preparing a set of control wells without cells containing the same volumes of culture medium, test sample, and MTS reagent. The measurements were carried out in triplicate. Cell viability was quantified by the relative number of live cells compared to a PBS negative control.

2.14. Cell Apoptosis Assay

Melanoma cell lines were cultured on a 48 well microtiter plate at a density of 100,000 cells per well. After an overnight incubation at 37 $^{\circ}\text{C}$, cells were treated with free drugs and incubated for 48 hours. Cells were then trypsinized, washed and fixed in 70% ethanol. DNA was extracted and the cells were stained with propidium iodide (Invitrogen, Carlsbad, CA, USA) prior to being analyzed via flow cytometry (FACScalibur; BD Biosciences, San Jose, CA). Data was processed and analyzed using FlowJo. Data was collected for 10,000 cells.

2.15. Drug Release

The release of drugs from ANPs was monitored using dialysis. The dialysis tubes (100 kDa, Spectrum Labs) were immersed in 15% ethanol for 30 min prior to use and then rinsed with phosphate buffered saline (PBS) solution. 1 mL of base ANPs, drug loaded ANPs and the native drug solutions were dialyzed against 200 mL of PBS (pH 7.4) with 10% ethanol. At each time point, 50 μ L of ANPs suspension was withdrawn from the tubes, and the PBS was replaced with fresh solution. The samples withdrawn at each time points were lyophilized and re-suspended with ethanol. The levels of curcumin and riluzole in each sample were measured using HPLC as described above.

2.16. In vitro Cellular Uptake of Drug Loaded ANPs

C8161(+) cells were seeded onto 8-well Lab-Tek slides (Thermo Scientific, Waltham, MA) at a density of 10^5 cells/well, and incubated at 37°C for 24 h. To quantify the relative degree of drug uptake, C8161(+) cells were treated with free drugs, base ANPs and drug loaded ANPs for 6 h. The cells were fixed, washed, and stained with Hoechst prior to imaging. Auto-fluorescence from albumin and curcumin in the ANPs was captured along with the Hoechst fluorescence by a confocal laser microscope (Leica Microsystems Inc., Bannockburn, IL). Images were processed and quantified using ImageJ.

3. Results

3.1. Characterization of Nanoparticles

Albumin nanoparticles (ANPs) were formed through the solvent-induced controlled coacervation of albumin. By varying the amount of sodium chloride in the HSA solution prior to coacervation, the size of the final nanoparticles could be controlled. Two sets of ANPs were generated with hydrodynamic diameter $\sim$ 100 nm (small) and $\sim$ 200 nm (large), as confirmed using dynamic light scattering (DLS). Scanning electron microscopy (SEM) of the ANPs revealed spherical, monodispersed populations of discrete nanoparticles (Figure 2A and 2B), corroborating the results seen with DLS. Both sets of ANPs exhibited low polydispersity and negative zeta potentials in phosphate-buffered saline (PBS, pH 7.4) (Figure 2G). The yields of human serum albumin (HSA) in producing small and large ANPs were approximately 65% and 50% respectively.

3.2. Matrix Protease Modified ANPs

Tumor penetrating ANPs were formulated by decorating the surface of the particles with the matrix protease, collagenase. Small collagenase-embedded ANPs (cANPs) were approximately 10 nm larger in size than unmodified small ANPs and with only slightly larger polydispersity (Figure 2G). Large cANPs were similar in both size and polydispersity to unmodified large ANPs. SEM images revealed greater surface texture on both sets of cANP (Figure 2C and 2D). Large cANPs presented two fold higher levels of collagenase compared to the smaller cANPs (Figure 2H). Small and large cANPs were subsequently loaded with both curcumin and riluzole as before (Figure 2E and 2F). Loading of the drugs led to increase in size and polydispersity for both small and large cANPs (Figure 2G).

Unmodified and collagenase modified ANPs were evaluated in a 2-D cell culture to determine the effect of the ANP size and collagenase coating on uptake and association at the single cancer cell level. Notably, the small ANPs showed greater cellular uptake than the large ANPs, supporting the design of differentially sized ANPs to tune the uptake kinetics. The cANPs showed a higher cellular uptake than the uncoated ANPs as measured by flow cytometry (Figure 2I). Confocal microscopy showed internalization of both the nanoparticles and free drugs in solution after 6 hours (Supplementary Figures 7A–7F).

3.3. Penetration of cANPs in a 3-D Multicellular Spheroid Model

The collagenase-modified ANPs were further evaluated in a 3-D cell multicellular spheroid model consisting of C8161+ melanoma cells co-cultured with human foreskin-derived fibroblasts for tumor penetration. The penetration of cANPs into the spheroids was visualized using the cANP autofluorescence (Supplementary Figure 2).

Spheroids were exposed to small and large ANPs with and without collagenase for 96 hours. The imaging studies revealed that both in presence and absence of collagenase treatment, the size of the nanoparticles influenced their penetration significantly. After 96 hours, a majority of the undecorated particles remained on the periphery of the tumor (Figure 3A and 3B). Collagenase decoration improved tumor penetration for both the small and large particles (Figure 3C and 3D). The large cANPs showed greater regional accumulation around the periphery of the spheroid (Figure 3C), while small cANPs achieved penetration into the center of the spheroid (Figure 3D).

Images of cANP penetration were quantified and compared to those of ANPs (Figure 3E). Based on total area of the spheroid imaged, small cANPs penetrated approximately 6% of the spheroid compared to 0.6% for small ANPs 96 h. Large cANPs penetrated 27% of the spheroid compared to 0.1% for large ANPs in 96 h. The greatest penetration was exhibited by large cANPs, consistent with their greater collagenase activity.

3.4. ANP Drug Loading and Release Kinetics

Curcumin and riluzole were first adsorbed separately at various concentrations onto the surface of fully formed ANPs of both sizes. The particles were then characterized with DLS to determine the effect of drug loading on particle size and polydispersity (Supplementary Figure 3). For both small and large ANPs, the concentration of curcumin added to the formulations was fixed at 80 μM and the amount of riluzole varied. Across the concentrations tested, there was no change in the binding efficiency of riluzole or curcumin as quantified using HPLC, and curcumin adsorption had little effect on the binding efficacy to Riluzole (Supplementary Figure 4A, 4B and 4C). Riluzole and curcumin bound with efficiencies of ~25% and ~60%, respectively, for both the large and small ANPs (Supplementary Figure 4C), which is similar to the loading efficiencies for each drug individually (data not shown). Both drugs could be released over a time scale of approximately a day from the ANPs (Supplementary Figure 5).

3.5. In vitro Cytotoxicity of Curcumin, Riluzole and Combination Treatments

The therapeutic effects of curcumin and riluzole were evaluated on malignant versus healthy cells using C8161+ human melanoma cells and AR7119 immortalized human melanocytes, respectively. Both cell lines were exposed to curcumin (Supplementary Figure 6C and 6D), riluzole (Supplementary Figure 6A and 6B), or cocktails of both compounds for 96 h (Supplementary Figure 6E and 6F). C8161+ cells were more sensitive towards curcumin at concentrations above 10 μ M than AR7119 cells. Riluzole treatment showed greater dose-dependent cytotoxicity in the C8161+ cells than AR7119 cells. Cell viability assays revealed that while 40 μ M riluzole inhibited the proliferation of melanoma cells, cytotoxicity was also induced in healthy melanocytes (~30% reduction of proliferation). To test the effect of combining two therapeutics, the concentration of riluzole was fixed at 10 μ M and curcumin concentration was varied. From these studies, the combination of both drugs in solution was chosen as 10 μ M riluzole and 40 μ M curcumin. This combination of riluzole and curcumin in solution had high cytotoxicity effects against the C8161+ melanoma cells with minimal adverse effect on the viability of the AR7119 melanocytes (Figure 4A and 4B). These concentrations were used to determine the apoptotic effect of the drugs in solution. After staining with propidium iodide, apoptotic, G1/G0, and G2 cell populations could be detected based on characteristic fluorescence patterns^[35]. After 48 hours, the dual drug treatment resulted in more apoptotic and G2 phase cells than either individual therapy (Figure 4C).

3.6. Efficacy of Dual Drug Loaded ANPs In vitro

The efficacy of the dual drug-loaded nanoparticles was compared against the free drugs in solution using a 2-D monolayer of cultured cells. For equivalent levels of dual drug loading concentrations (10 μ M riluzole and 40 μ M curcumin), both large and small nanoparticles exhibited similar effectiveness by 96 hours against C8161+ melanoma cells (Supplementary Figure 7G). Comparative trends were similar for AR7119 melanocytes between free and ANP-bound formulations, though the drug treatment has a less pronounced effect on the melanocytes (Supplementary Figure 7H).

3.7. Efficacy of Matrix Protease-Modified, Combination Drug ANPs

Dual drug-loaded cANPs were evaluated in a 2-D cell culture model to determine whether the incorporation of collagenase altered the therapeutic efficacy of the riluzole and curcumin combination. Unloaded cANPs had little effect on cell proliferation for either the C8161+ (Figure 5A) or AR7119 cells (Figure 5B). Drug-loaded cANPs induced a similar degree of cytotoxicity when compared to both free drug and drug-loaded ANPs in C8161+ cells. This was also seen in AR7119 cells though cytotoxicity was significantly less than with the C8161+ cells.

The therapeutic efficacy of cANPs loaded with both curcumin and riluzole was next tested in a 3-D spheroid model. Spheroids were exposed to drug loaded ANPs with and without collagenase modification. After 24 h, drug-loaded small cANPs induced a significantly greater degree of cytotoxicity in the spheroids than unmodified drug-loaded ANPs (Figure 5C). By 96 hours, both the large and small drug-loaded cANPs had induced greater cytotoxicity in the spheroids as compared to ANPs unmodified with collagenase.

4. Discussion

The goal of this work was to develop an advanced health care material designed to integratively overcome three successive barriers for chemotherapy of cancerous tissues: intracellular chemo-resistance (through dual drug delivery); cancer cell uptake (through nanoparticle size and albumin chemistry); and multicellular tumor penetration (through enzymatic presentation) (Figure 1). We demonstrate that the modification of differentially sized albumin-derived nanoparticles (ANPs) with an ECM protease,^[36, 37] permits greater nanoparticle penetration into a solid tumor model and more effective delivery of a bound-therapeutic cocktail.

Clinically-used ANPs (*nab*-paclitaxel, Abraxane®, Abraxis BioScience Inc.)^[38, 39] have already been shown to sequester large amounts of therapeutic cargo,^[40, 41] improve the pharmacokinetics and circulation half-life of antitumor agents,^[42] reduce in vivo toxicity associated with solvent delivery systems^[21] and possibly enhance tumor uptake of therapeutics via albumin binding interactions.^[43–45] However, ANPs exhibit limited penetration into tumors and have not yet been reported to deliver a combination of synergistic chemotherapeutics simultaneously. Multicellular tumor spheroid models exhibit many characteristics of tumors, such as extracellular matrix generation and oxygen and nutrient gradients.^[46] Previously, work has shown that drug delivery to solid tumor masses is frequently challenging for nanoparticle-based systems, greatly limiting successful treatment.^[8] Here, we developed and characterized a novel multi-protein nanoparticle, consisting of human serum albumin doped with collagenase, and evaluated its ability to penetrate into a multicellular spheroid model of co-cultured human melanoma and fibroblast cells. Collagenase treated nanoparticles have shown increased penetration into tumor spheroid models and improved tumor accumulation in spheroid masses vs. unfunctionalized particles.^[36]

Two sizes of collagenase-modified ANPs (cANPs) were synthesized and loaded with both therapeutic compounds. The cANPs did not alter the binding of the drugs to ANPs or their release. Furthermore, cANPs retained the cytotoxic effects of curcumin and riluzole on the cancerous cells; their effect on the C8161+ melanoma cells was nearly identical to that of the ANPs loaded with drugs alone. As expected, ^[36] confocal imaging of cANP penetration into multicellular spheroids revealed strong size dependence for penetration and confirmed collagenase activity. While the distribution of smaller cANPs is more diffuse, suggesting cellular internalization, the larger cANPs exhibit pockets of density that appear localized between cells in the spheroid. Smaller sized cANPs, within the size limits of endocytosis,^[47] were more rapidly internalized by tumor cells after initially penetrating into the tumor. Large cANPs, decorated with higher levels of collagenase, were able to slowly degrade the collagen fibers and penetrate further into the tumor over time.

In contrast with systemic administration of collagenase, which may promote tumor metastasis,^[48, 49] our system confines the presentation of collagenase on the surface of the nanoparticles enabling strict control of ECM degradation. Passive tumor targeting of nanoparticles through the enhanced retention and permeation effect (EPR) combined with

active targeting approaches, such as the conjugation of cyclic RGD or other tumor-targeting moieties,^[34, 50] can further lead to controlled ECM degradation at tumor sites.

As drug delivery vehicles, ANPs have been shown to exhibit strong but reversible drug-binding properties.^[41] Albumin can simultaneously bind multiple, structurally dissimilar compounds at sites distributed across its surface.^[40, 51] We exploited this feature of albumin for the purpose of co-delivering two major therapeutic moieties, curcumin and riluzole, as proof-of-concept agents to cancer cells using ANPs. Curcumin, a poorly bioavailable herbal derivative,^[52] is known to have anti-cancer and anti-inflammatory properties.^[53–55] Furthermore, curcumin can act as a potent chemosensitizer, improving tumor cell sensitivity towards chemotherapy via downregulation of NF- κ B and NF- κ B-regulated gene products.^[53, 56] Riluzole is a noncompetitive glutamate release inhibitor that is currently approved for use in patients with amyotrophic lateral sclerosis (Lou Gehrig's disease). Recent work has indicated that neuronal glutamate receptor expression in melanocytes is highly oncogenic and present in human melanoma biopsies,^[57, 58] while disruption of this receptor with riluzole can trigger apoptosis in vitro and suppress the metabolic activity of human melanoma.^[59, 60] Together, riluzole and curcumin can act on two independent apoptotic pathways: inhibition of P13k/Akt^[60] and activation of NF- κ B mediated by caspase-8,^[61] respectively (Figure 1D). In our studies, both drugs were found to bind tightly to ANP substrates. The slower release of curcumin compared to riluzole from the ANPs may be due to its greater hydrophobicity.^[52, 62] Although it took 18–21 hours for 50% of the adsorbed drugs to be released from the ANPs in PBS, imaging studies using the inherent fluorescence of ANPs revealed that the nanoparticles were internalized into cells after 6 hours of incubation. Taken together with the evidence that both riluzole and curcumin maintained therapeutic efficacy against melanoma cells when formulated with ANPs in 2D cytotoxicity assays, nanoparticles likely facilitate cellular uptake of both drugs which is followed by release over a time scale appropriate for pharmacological activity. Similarly, other combinations of therapeutics could be delivered via this versatile nanoparticle platform material.

The multimodular nature of the albumin nanoparticles allowed us to explore the individual and combined roles of nanoparticle size, protease modification and dual drug presentation in the context of a tumor-equivalent, multicellular melanoma model using three-dimensional spheroids in vitro. Several interesting trends emerged from this study. First, the larger ANPs showed rapid penetration into tumor spheroids in a size-dependent manner, thus showing early increase in tumor cytotoxicity. Collagenase modification of these large ANPs did not further enhance the efficacy by 24 hours, suggesting that the size-governed transport rate of large ANPs was sufficient to elicit cytotoxicity early on. Because large ANPs also displayed higher levels of collagenase, these trends could not be easily decoupled. In contrast, the smaller ANPs showed a latent phase of cytotoxicity effects by 24 hours, which we attribute to increased cellular uptake at the spheroid margins, thus depleting the core of spheroids of ANPs. One of the intriguing trends observed was that at longer time points (96 h), these smaller ANPs effect higher levels of cytotoxicity, likely resulting from intracellular release of the dual drugs. When the intracellular activity of smaller ANPs was combined with the collagenase modification, the highest levels of cytotoxicity by 96 h were observed. We attribute this to cooperative effects of increased ANP intracellular uptake and enhanced

matrix transport, thus promoting both localized cytotoxicity due to dual drugs and spatially dispersed “reach” of the ANP carriers within the tumors. Thus, the ANP materials could be used as a versatile chemotherapy tool to modulate both the spatial and temporal aspects of cancer cell cytotoxicity within heterogeneous tumor matrix microenvironments.

5. Conclusions

A novel formulation of nanoparticles for targeted penetration of tumor tissues was advanced, based on human albumin-derived carriers of two complementary chemotherapeutic agents (curcumin, riluzole), and examined for enhanced cancer cell killing in a 3D melanoma tumor spheroid model. Collagenase decoration of ANPs improved both the ANPs penetration into a multicellular spheroid model and therapeutic outcomes. The proposed albumin nanoparticles thus integrate several functionalities: (1) multi-drug delivery, (2) active tumor penetration and (3) size-dependent single cell uptake. The synthesized nanoparticles could be a useful tool for further investigation of mechanisms of dual drug delivery into the core of a dense tumor mass, improving the efficacy of the associated therapeutic payload.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic summarizing the overall design of albumin nanoparticles as advanced healthcare materials for enhanced “intratumoral” chemotherapy

(A) Albumin nanoparticles (ANPs) fabricated by controlled coacervation can be engineered to overcome multiple barriers to chemotherapy. The synthesis process was modified to display an extracellular matrix protease (cANP). Drugs can be adsorbed onto the surface after fabrication to further enhance chemotherapeutic efficacy. (B) On a multi-cellular tumor level, collagenase coating of ANPs allows for rapid penetration through the dense extracellular matrix to allow for increased intratumoral drug availability. (C) ANP size can be modulated to engineer size-dependent uptake by individual cancer cells, thus triggering controlled intracellular drug release. Smaller ANPs are hypothesized to exhibit enhanced cellular uptake over the larger ANPs. (D) A novel dual-drug regimen of ANP-based presentation and release of complementary melanoma drugs, curcumin and riluzole, was implemented. These drugs would act on distinct but convergent pathways that promote apoptosis and inhibit cellular proliferation, thus enhancing cancer cell chemotherapy intratumorally.

Figure 2. Multimodal Design of Albumin Nanoparticles as Monodisperse Substrates for Size Modulation, Intracellular Uptake, Protease Modification, and Dual Drug Binding

Scanning electron microscopy (SEM) images of (A) small and (B) large ANPs reveal monodisperse, spherical nanoparticles. Collagenase modification altered the morphology of the (C) small and (D) large ANPs. This change in morphology was more evident after both drugs were loaded on the (E) small and (F) large collagenase ANPs. (G) ANPs were synthesized and characterized at two different sizes by modifying the concentration of NaCl used during synthesis. Both sizes exhibit narrow size distributions and negative zeta potentials in PBS. Hydrodynamic diameter, polydispersity and loading efficiency of ANP formulations with and without collagenase incorporation were characterized. (H) Large Collagenase ANPs carried 2 fold greater levels of collagenase compared to smaller C-ANPs. One unit of collagenolytic activity was defined as the cleavage of 1 mg of collagen substrate per minute. (I) Cellular uptake of collagenase conjugated and unmodified ANPs in C8161+ cell line was analyzed by flow cytometry for up to 24 h. The decrease in size and collagenase conjugation accelerates the uptake of nanoparticles (I). $\pm$ Values: standard deviation ($n=3$); Error bars represent standard deviation (SD) for $n=3$. *** $p < 0.005$ (t test).

Figure 3. Protease Modified ANPs Exhibit Increased Transport Into Melanoma Tumor Spheroid Model

Confocal microscopy images of multicellular spheroids stained with Hoechst (blue) treated with various nanoparticles (green) for 96 hours (A–D): (A) larger ANPs, (B) small ANPs, (C) large collagenase conjugated ANPs, and (D) small collagenase conjugated ANPs. Images indicate increased penetration of both collagenase modified ANPs compared to ANPs. (E) Large C-ANPs penetrate further into the spheroids than the small ones, while the smaller C-ANPs displayed deeper penetration by 96 hours. Error bars represent standard deviation for n=5. ** $p < 0.02$; *** $p < 0.005$ (t test).

Figure 4. Dual Drug Combination of Curcumin and Riluzole Elicits Increased Melanoma Cytotoxicity

In vitro cytotoxicity of riluzole, curcumin, and dual drugs on (A) C8161+ melanoma cells and (B) AR7119 melanocytes. (C) Apoptotic effects of 40 μ M curcumin, 10 μ M riluzole, and dual drugs (10 μ M riluzole + 40 μ M curcumin) on C8161+ melanoma cells after 48 hours of incubation were obtained by flow cytometry. The dual drug treatment resulted in more apoptotic and dividing (G2) cells than either single drug therapy. Error bars represent standard deviation for n=3.

Figure 5. Multimodal ANPs exhibit enhanced tumor cytotoxicity in a size, protease-modification, and dual drug-display dependent manner

A dual drug (DD) combination of 10 μ M riluzole and 40 μ M curcumin bound ANPs nanoformulations were tested within 2-D monolayer cultures and showed enhanced cytotoxicity of (A) C8161+ human melanoma cells compared to (B) AR7119 healthy human melanocytes. The cANPs showed no inherent cytotoxicity in either cell line. (C) The multimodal ANPs showed unique effects in the 3-D multicellular tumor spheroid model as a function of time and ANP size, collagenase modification, and dual drug treatment. ANP-based dual drug delivery showed enhanced efficacy over free drugs. The collagenase modification enhanced efficacy for both large and smaller ANPs but particularly for smaller ANPs. The smaller cANPs showed the most pronounced time dependent therapeutic effect. Overall, the ANPs can be designed as versatile chemotherapy tools to modulate matrix transport rates as well as intracellular uptake rates and effect short-term and longer-term tumor killing. Error bars represent standard deviation for $n=3$. * $p<0.05$; ** $p<0.02$ (t test).