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A Hydrogel-Based Tumor Model for the Evaluation of Nanoparticle-Based Cancer Therapeutics

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Abstract

Three-dimensional (3D) tissue-engineered tumor models have the potential to bridge the gap between monolayer cultures and patient-derived xenografts for the testing of nanoparticle (NP)-based cancer therapeutics. In this study, a hydrogel-derived prostate cancer (PCa) model was developed for the *in vitro* evaluation of doxorubicin (Dox)-loaded polymer NPs (Dox-NPs). The hydrogels were synthesized using chemically modified hyaluronic acid (HA) carrying acrylate groups (HA-AC) or reactive thiols (HA-SH). The crosslinked hydrogel networks exhibited an estimated pore size of 70–100 nm, similar to the spacing of the extracellular matrices (ECM) surrounding tumor tissues. LNCaP PCa cells entrapped in the HA matrices formed distinct tumor-like multicellular aggregates with an average diameter of 50 μ m after 7 days of culture. Compared to cells grown on two-dimensional (2D) tissue culture plates, cells from the engineered tumoroids expressed significantly higher levels of multidrug resistance (MDR) proteins, including multidrug resistance protein 1 (MRP1) and lung resistance-related protein (LRP), both at the mRNA and the protein levels. Separately, Dox-NPs with an average diameter of 54 ± 1 nm were prepared from amphiphilic block copolymers based on poly(ethylene glycol) (PEG) and poly(ϵ -caprolactone) (PCL) bearing pendant cyclic ketals. Dox-NPs were able to diffuse through the hydrogel matrices, penetrate into the tumoroid and be internalized by LNCaP PCa cells through caveolae-mediated endocytosis and macropinocytosis pathways. Compared to 2D cultures, LNCaP PCa cells cultured as multicellular aggregates in HA hydrogel were more resistant to Dox and Dox-NPs treatments. Moreover, the NP-based Dox formulation could bypass the drug efflux function of MRP1, thereby partially reversing the resistance to free Dox in 3D cultures. Overall, the engineered tumor model has the potential to provide predictable results on the efficacy of NP-based cancer therapeutics.

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Keywords

Hyaluronic acid; hydrogel; 3D tumor model; nanoparticles; cancer therapeutics; drug resistance

1. Introduction

Over the past few decades, a concerted effort has been made to develop nanotechnology-based cancer diagnosis and therapy, with the premise of potentially prolonging patients' life expectancy, at the same time reducing treatment-related side effects [1, 2]. Encapsulating anticancer drugs in polymeric nanoparticles (NPs) are proposed, and in some cases have been shown, to improve the pharmacological properties of drugs [3]. The NP-based drug delivery systems also have the potential to increase the therapeutic efficacy through the enhanced permeability and retention (EPR) effect or the active targeting strategies [4]. To date, the promise of nanomedicine in patients has not been fully realized owing to the numerous biological barriers to the delivery of cancer therapeutics [5, 6].

Currently, preliminary screening of NP-based cancer therapeutics is usually performed on two-dimensional (2D), monolayer cell culture systems. While straightforward and easy to perform, 2D cultures identify promising drug formulations that do not translate similarly *in vivo* in animal models or in patients [7], and many limitations associated with NP formulations are not discovered until a later stage of product development. The inconsistency in therapeutic outcomes can be attributed, in part, to the inability of monolayer cultures to accurately account for the extracellular barriers [8]. While NPs delivered to a monolayer cell culture typically reach cells without any physical restriction, the diffusion of NPs administrated *in vivo* would be hindered by the complex, tumor-associated extracellular matrix (ECM) [8, 9]. The 3D organization of a tumor mass also fundamentally alters the diffusion profile for drugs, both through the cell-cell contacts and cell-matrix interactions [8].

In addition to altered cell organizations and extracellular environments, 2D monolayer cultures promote cells to adopt a non-natural phenotype, thereby influencing cellular responses to the delivered drugs [8]. Whereas cells in 2D cultures are exposed to a uniform environment with sufficient oxygen and nutrients, cells in the solid tumor tissues are exposed to gradients of critical chemical and biological signals [10]. Such a unique microenvironment can exert both stimulatory and inhibitory effects on tumor progression [10]. Moreover, tumor cells from cancer patients are frequently found to be resistant to a broad spectrum of chemotherapeutic drugs without previous exposure to those cytotoxic agents [11-13]. The intrinsic drug resistance can be attributed, in part to the overexpression of the multidrug resistance (MDR) proteins by tumor cells [12-14]. The tumor microenvironments, namely hypoxic conditions [12, 15], low nutrients supply [12] and low pH [16], all have been suggested to upregulate the expression of MDR proteins through specific cellular signaling pathways. Obviously, these essential environmental conditions cannot be recapitulated in traditional 2D monolayer cultures.

To overcome the limitations associated with traditional 2D monolayer cultures, various 3D culture systems aiming to recreate the tightly controlled molecular and mechanical microenvironment typical of tumors *in vivo* have been developed and characterized [17]. These systems may bridge the gap between 2D experiments and animal studies, providing physiologically relevant platforms for optimizing the drug formulations prior to the *in vivo* assessment [8]. Both natural (e.g. type I collagen [18-20] and basement membrane extract [21, 22]) and synthetic materials (e.g. poly(ϵ -caprolactone) (PCL) [23], poly(lactic-co-glycolic acid) (PLGA) [24] and poly(ethylene glycol) (PEG) [25]) have been used as the

scaffolding materials for the engineering of 3D tumor models. While natural materials derived from animal tissues are chemically ill-defined and suffer from batch-to-batch variations, most synthetic polymers are mechanically inappropriate and physiologically irrelevant [17]. These drawbacks limit their utility as artificial matrices for the construction of physiologically relevant tumor models.

We are interested in the *in vitro* engineering of 3D models of prostate cancer (PCa) using hyaluronic acid (HA)-based hydrogel systems [17, 26, 27]. Produced by PCa cells and the stromal cells, HA is found to be enriched in the tumor-associated stroma [28, 29]. In addition to providing the structural support to the tumor tissues [17], HA interacts with its cell surface receptors, such as CD44 or RHAMM [26], to alter cell adhesion, migration and proliferation [26]. HA's biodegradation by hyaluronidase (HAase) helps the cancer cells to escape from the primary tumor mass [26], and degraded HA fragments promote angiogenesis to allow further tumor expansion [30]. Finally, HA protects the tumor tissues to evade the immune surveillance [28]. Using HA derivatives carrying orthogonal functionalities, we have developed HA-based hydrogel systems that not only support the tumoroid formation from PCa cells [27], but also simulate the reciprocal interactions with the tumor-associated stroma [17]. The resultant tumoroids closely mimic the morphology of the native tumor tissues and exhibit strong angiogenic potentials [17]. The engineered tumor models have also been used successfully to test the efficacy of anti-cancer drugs [27].

Herein, we report the physical and biological characterization of the hydrogel-based PCa model, and explore the utility of the engineered model in the evaluation of an anti-cancer drug, doxorubicin (Dox), both in its free form and encapsulated in polymeric NPs. To this end, HA-based hydrogels were prepared using HA derivatives carrying complementary reactive groups via a Michael-type addition reaction. The resultant hydrogel matrices were used for the 3D culture of LNCaP PCa cells *in vitro*. Separately, Dox was formulated into NPs derived from an amphiphilic block copolymer. The goal of this investigation was to repurpose the nanoformulations readily available in our laboratories rather than to design polymeric nanocarriers. Using the engineered tumor model, the transport properties of the NPs and the apoptotic properties of the nanoformulation were evaluated.

2. Materials and methods

2.1. Chemicals and reagents

HA (500 kDa, sodium salt) was generously donated by Genzyme Corporation (Cambridge, MA). All chemicals necessary for the synthesis of HA derivatives and the amphiphilic block copolymers were purchased from Sigma-Aldrich (St. Louis, MO). Doxorubicin hydrochloride (Dox-HCl) was obtained from BIOTANG Inc. (Waltham, MA). Nile red (NR), bovine testicular HAase (30,000 U/mg), bovine serum albumin (BSA), sucrose, chlorpromazine hydrochloride, methyl- β -cyclodextrin (M- β -CD), genistein, colchicine and quercetin were purchased from Sigma-Aldrich (St. Louis, MO). Cell Titer-Blue® cell viability assay was obtained from Promega (Madison, WI). PEGylated (methoxy-PEG5000-SH) gold nanoparticles (PEG-AuNPs) were purchased from Cytodiagnostics (Burlington, ON). Cell Death Detection ELISA^{PLUS} was obtained from Roche Applied Science (Mannheim, Germany). Mouse anti-multidrug resistance protein 1 (MRP1) mAb (MRPm6) was purchased from Kamiya Biomedical Company (Seattle, WA). Mouse anti-lung resistance-related protein (LRP) mAb (LRP 1014) and goat anti-mouse IgG-FITC were obtained from Santa Cruz Biotechnology (Dallas, TX). All cell culture reagents were purchased from Life Technologies (Grand Island, NY). All other reagents were used as received unless otherwise noted.

2.2. Particle preparation and drug/dye encapsulation

Amphiphilic block copolymers consisting of hydrophilic PEG and hydrophobic polyester bearing pendent cyclic ketals [PEG-*b*-P(CL-*ran*-TSU)] were synthesized by ring-opening co-polymerization of ϵ -caprolactone (CL) and 1,4,8-trioxaspiro-(4,6)-9-undecanone (TSU), using α -hydroxyl, ω -methoxy PEG as the initiator and stannous octoate as the catalyst [31]. At a 20 wt% TSU in feed, the copolymer (abbreviated as ECT2) was found to contain 14 mol% TSU in the hydrophobic segment. ECT2 with a number average molecular weight (M_n) of 40.6 KDa was used to formulate Dox-loaded NPs. Prior to drug encapsulation, Dox-HCl was neutralized to generate Dox, following reported procedures [32]. NPs were prepared using a nanoprecipitation method. Briefly, an acetone/DMSO (1:1, v/v) solution of ECT2 (10 mg/ml, 1ml) was slowly added to a stirred (900 rpm) aqueous phase (5 ml DI water). The mixture was allowed to stabilize for 2 h under constant agitation at room temperature to obtain blank-NPs. Dox- or NR-loaded NPs were prepared using an acetone/DMSO (1:1, v/v) solution of ECT2 (10 mg/ml, 1ml) containing 2 mg/ml Dox or 0.1 mg/ml NR. Centrifugation (4,000 rpm for 10 min) was applied to all NP suspensions to remove large polymer aggregates. The supernatant containing NPs was collected, and the free drug or dye and the solvent were removed by filtration using Amicon regenerated cellulose centrifugal filters (MWCO=30kDa, EMD Millipore). The collected NPs were thoroughly washed with DI water or phosphate buffered saline (PBS, pH 7.4) three times by centrifugation using the centrifugal filters. The resultant formulations were immediately subjected to physical and biological analyses.

2.3. Characterization of NPs

The hydrodynamic diameters of NPs were measured using a Zetasizer nanoZS (Malvern Instruments, Westborough, MA) by dynamic light scattering (DLS) at a concentration of 0.5 mg/ml in PBS. Transmission electron microscopy (TEM) was used to examine the particle size and morphology. TEM samples were prepared by applying a drop of NP suspension (3 μ l) directly onto a carbon-coated copper TEM grid. Samples were allowed to dry under ambient condition prior to imaging using a Tecnai G2 12 Twin TEM (FEI Company, Hillsboro, OR). The mean diameter of the NPs was estimated using ImageJ based on 50 counts of the particles from the TEM image.

2.4. Drug/dye loading and release

Aliquots (1 ml each) of the Dox-NPs and NR-NPs suspension in DI water were collected and lyophilized. The dried powder was weighed accurately before being dissolved in DMSO (1 ml). The fluorescence intensity of the drug or dye was determined using a plate reader (DTX880 Multimode Detector, Beckman Coulter, Fullerton, CA) at 485 nm (excitation) / 595 nm (emission) for Dox and at 535 nm (excitation) / 595 nm (emission) for NR. The concentration of the drug or dye was then derived from a series of standards in DMSO at Dox concentrations of 3.125-25 μ g/ml, and NR concentrations of 6.25-100 ng/ml. The drug-loading content (LC) was defined as the amount of drug (μ g) loaded per milligram of Dox-NPs. The drug encapsulation efficiency (EE, as percentage of the total) was calculated by dividing the amount of Dox loaded into the NPs with the amount of Dox initially added during the nanoprecipitation process. All measurements were carried out in triplicate, and the results were indicated as the mean $\pm$ SD.

The *in vitro* release of Dox and NR was analyzed under sink conditions. Briefly, freshly formulated NPs dispersed in serum containing cell culture medium were incubated at 37 °C. At predetermined time points, the release medium was separated from the NPs using centrifugal filters as described above. NPs were re-dispersed in fresh cell culture medium to continue the release study. The collected filtrates containing the released Dox or NR at each time point were lyophilized, and the resultant solid was re-dissolved in DMSO (undissolved

salt was removed by centrifugation). Subsequently, the fluorescence intensity of the Dox and NR was measured using the plate reader, and the concentration was derived based on the standard curve as described above. Three repeats were performed for each time point, and the cumulative release profile was calculated by dividing the amount of drug or dye released in one specific measurement time by the total mass initially loaded.

2.5. Hydrogel preparation

Acrylated HA (HA-AC) and thiolated HA (HA-SH) were synthesized following previously reported procedures [33, 34]. Briefly, HA-AC was synthesized by reacting adipic acid dihydrazide-modified HA (HA-ADH, 35% ADH incorporation determined by ^1H NMR [35]) with N-acryloxysuccinimide (NHS-AC) in DI water. The product was obtained as a lyophilized solid (yield: 70%). Sulfhydryl groups were incorporated in HA via the reaction with a disulfide-containing dihydrazide compound, followed by reduction with dithiothreitol (DTT). The dry solid HA-SH was obtained after lyophilization (yield: 80%). The HA derivatives were stored at -20°C prior to use. ^1H NMR analyses revealed a degree of modification of 9% and 45% for HA-AC and HA-SH, respectively [17]. The respective weight average molecular weights, as determined by GPC, were 345 kDa and 314 kDa. HA hydrogels were prepared by mixing equal volume of HA-AC and HA-SH, dissolved in PBS at a concentration of 20 mg/ml. The gelation process started 17 min after mixing and was completed after 6 h incubation at 37°C [17].

2.6. Analysis of hydrogel pore size

The pore size of the hydrogel was determined by a probe retention method [36] using a series of PEG-AuNPs with mean hydrodynamic diameters of 35, 50, 70 and 100 nm, with the respective Au core of 5, 20, 40 and 70 nm. The concentration of PEG-AuNPs in the stock solution, C_0 (mg/ml), was determined by UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA) at a wavelength of 518, 524, 530 and 548 nm for 35, 50, 70 and 100 nm PEG-AuNPs, respectively. An aliquot (V_0 ml) of PEG-AuNPs stock solution was spun down by centrifugation and the pellet was re-dispersed in HA-AC solution in PBS (20 mg/ml, 75 μl). The HA-AC solution containing the disperse PEG-AuNPs was subsequently mixed with the HA-SH solution in PBS (20 mg/ml, 75 μl). The mixture was incubated at 37°C for 6 h. The hydrogels were then immersed in fresh PBS solution at room temperature for two days under constant shaking. The supernatant was collected and the hydrogel was washed with PBS. The concentration of released PEG-AuNPs (C_1 , mg/ml) in the combined supernatant and the washing solution (total volume V_1) was determined by UV-Vis spectrophotometer. The retention of PEG-AuNPs in the hydrogel was quantified as follows: $((C_0 \times V_0 - C_1 \times V_1) / C_0 \times V_0) \times 100$.

2.7. Analysis of NP diffusion in HA hydrogels

HA hydrogels were prepared as described above. The precursor mixture was added to the channel ($17 \times 3.8 \times 0.4 \text{ mm}^3$) connected by two cylindrical reservoirs at each end on a channel slide (ibidi, Verona, WI). After 6 h incubation at 37°C , one of the reservoirs was filled with PBS containing NR-NPs (1 mg/ml, 70 μl) and the other with PBS. The device was equilibrated at 37°C for 18 h. Control experiments using free NR (1 $\mu\text{g/ml}$ in PBS) were performed as described above. Fluorescence images (16-bit) were taken with a PCO-Tech Pixelfly CCD camera after being magnified 5 times using a lab-built fluorescence microscope. A 1W 470 nm LED was used to excite the NR using an epifluorescence design, and the fluorescent light was filtered through a 570 nm long-pass filter. ImageJ was used to construct a $0.8 \text{ mm} \times 2.5 \text{ mm}$ image from a row scan of four images, and to generate intensity line profiles. The background intensity was subtracted from all line profiles prior to curve fitting (Igor Pro, Wavemetrics). The concentration profile for NR-NPs was fitted to

the following one-dimensional model to obtain the diffusion coefficient in the HA hydrogel [37]:

$$C(x, t) \propto \operatorname{erfc}\left(\frac{x}{2\sqrt{D_{eff}t}}\right)$$

Where erfc is the complementary error function, x is the diffusion distance (μm), t is time (s), and $C(x, t)$ is the measured concentration (intensity) as a function of time (t) at a known distance (x). The measurement was carried out with three independent repeats.

2.8. Cell culture

LNCaP prostate cancer cells were maintained in Corning (Lowell, MA) tissue culture flasks (75 cm^2) at 37°C in 5% (v/v) CO_2 in a RPMI-1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin G sodium and 100 $\mu\text{g/mL}$ streptomycin sulfate in 0.085% (v/v) saline (P/S). The medium was changed every other day, and cells were routinely passaged using 0.25% (w/v) trypsin containing ethylenediaminetetraacetic acid (EDTA*4Na). Hydrogel culture of LNCaP prostate cancer cells were performed following previously reported procedures [17]. Briefly, cell culture inserts were pre-wet with PBS before being placed into a 24-well culture plate. HA-SH (100 μl , 20 mg/ml) was added to the HA-AC solution (100 μl , 20 mg/ml) containing dispersed LNCaP cells (0.2 million). After thorough mixing, the cell suspension was transferred into the cell culture insert and was incubated for 45 min at 37°C before the medium was added around the insert. The resultant cell-gel construct had a cylindrical geometry of 1.8 mm in height and 12 mm in diameter. The cell culture medium was changed every day for each culture.

2.9. Expression of multidrug resistance proteins

LNCaP cells were cultured on 2D or in 3D as described above. At day 7, cells on 2D plastic surfaces were trypsinized and collected. Cells cultured in HA hydrogels were harvested by degrading the HA matrices using HAase (5KU/ml) at 37°C for 4 h. RNA extraction, reverse transcription and quantitative polymerase chain reaction (qPCR) analysis using ABI Prism 7300 Sequence Detection System (AB Applied Biosystems, Foster City, CA) were performed as described previously [36]. Primer sequences for specific genes were as follows [17, 38]: MRP1 (XM_003281512.2): forward (5'-GTC GGA ACA AGT CGT GCC TG-3'), reverse (5'-CAA AGC CTC CAC CTC CTC A-3'); LRP (XM_004615945.1): forward (5'-TGG CTG AGG TGG AGG TGA AG-3'), reverse (5'-ATC GGT GAT GAG GGT TGA TTT C-3'); β -actin (NM_001101.3, the housekeeping gene): forward (5'-GGA CTT CGA GCA AGA GAT GG-3'), reverse (5'-AGC ACT GTG TTG GCG TAC AG-3'). The relative gene expression was calculated using the equation below [36]:

$$\text{ratio} = \frac{E_{\text{target}} \Delta C_{\text{target}}(\text{control-sample})}{E_{\text{ref}} \Delta C_{\text{ref}}(\text{control-sample})}$$

where E_{target} and E_{ref} are the real time PCR efficiencies of the target and the reference genes, respectively. ΔC_{target} is the difference in the threshold cycle (Ct) of the target genes between the control and the test samples. ΔC_{ref} is the difference in the Ct value of the control versus the test samples with reference to β -actin.

To analyze the expression MDR proteins at the protein level, tumoroids in 3D hydrogel cultures were collected by the enzymatic degradation using HAase as described above. Single cell suspension was obtained by treating the tumoroids with trypsin. Cells on the

monolayer after 7 days of culture were treated with the same concentration of HAase as used in 3D, and then were collected by trypsinization. Single cell suspensions from both 2D and 3D cultures were plated onto the 8-well chamber slides at the same density (5×10^4 cells per well). Immunocytochemistry staining for MRP1, LRP and cell nucleus were performed 24h after seeding, following previously reported procedures [17]. Mouse anti-MRP1 mAb (1:4 in 3% BSA) and mouse anti-LRP mAb (1:20 in 3% BSA) were used as the primary antibodies for the MRP1 and LRP, respectively. Goat anti-mouse IgG-FITC (1:400 in 3% BSA) was used as the second antibody. Cell nucleus was stained with Draq5 (1:1000 in PBS). Samples were imaged using the Zeiss510 NLO confocal microscope.

2.10. Cellular uptake of Dox and Dox-NPs

LNCaP cells were grown on 8 well chamber slides as 2D monolayer or in HA hydrogels as 3D tumoroids, following the procedures described above. Cells on 2D and in 3D were incubated with free Dox (50 $\mu\text{g/ml}$) or Dox-NPs (containing 50 $\mu\text{g/ml}$ equivalent Dox) for 2 h at 37 °C. After extensive wash with PBS, cells or cell-gel constructs were inspected using Zeiss510 NLO confocal microscope to detect the internalized Dox or Dox-NPs (excitation wavelength at 488 nm with a band pass filter of 565-615 nm). Because of the short duration of the uptake study, tumoroids close to cell/gel constructs were imaged. Z-stack images were collected at a 3- μm interval to observe the localization of Dox-NPs in the tumoroid.

2.11. Endocytic mechanism

LNCaP cells seeded in a 96 well plate at a density of 6.4×10^3 per well were incubated for 24 h. After the medium was removed, cells were incubated at 37 °C for 30 min with one of the following compounds dissolved in cell culture medium: 0.45 M hypertonic sucrose and 10 $\mu\text{g/ml}$ chlorpromazine hydrochloride (both are inhibitors of clathrin-mediated endocytosis); 5 mM m- β -CD and 50 $\mu\text{g/ml}$ genistein (both are inhibitors of caveolae-mediated endocytosis); 8 $\mu\text{g/ml}$ colchicine (an inhibitor of macropinocytosis) and 6 $\mu\text{g/ml}$ quercetin (an inhibitor of caveolae- and clathrin-independent endocytosis), followed by a 2h incubation with NR-NPs (0.5 mg/ml). Subsequently, 2D cultures were thoroughly washed with PBS, and the internalized NR was recovered by DMSO extraction and quantified using the plate reader. For 3D samples, LNCaP cells were cultured in HA-based hydrogel for 7 days before the treatment. After the inhibitor treatment, cell-gel constructs were exposed to NR-NPs for 24 h at 37 °C. Constructs were then degraded by using HAase and the collected cells were subjected to DMSO extraction. The mean fluorescence intensity of cells cultured on 2D and in 3D without any inhibitor treatment served as the control and was considered to be 100%. The fluorescence intensity of cells treated with various inhibitors was compared with the control group to obtain the percentage of cellular uptake.

2.12. Drug treatment and apoptosis assay

Cells cultured on 2D in a 12-well plate and in 3D in HA hydrogels as stated above were allowed to proliferate for 24 h and 7 days respectively, to reach a similar cell number of 2.5×10^5 [17]. Fresh medium containing free Dox or Dox-NPs at various concentrations were then added to the respective cultures and the drug treatment was allowed to proceed for 24 h. Subsequently, cells were collected by trypsinization or HAase degradation for 2D and 3D respectively. Cell apoptosis analysis was performed using Cell Death Detection ELISA^{PLUS} according to manufacturer's instructions. Colorimetric detection by absorbance was carried out using the plate reader at 405 nm.

2.13. Statistical analysis

All quantitative measurements were performed on three replicate samples. The results were reported as mean $\pm$ standard deviation. Statistical significance was evaluated by analysis of

variance (one-way ANOVA), followed by Tukey's post-hoc test. A P-value of <0.05 was considered to be statistically different.

3. Results

3.1. Formulation and characterization of drug-loaded NPs

The amphiphilic block copolymer, ECT2 (M_n : 40.6 kg/mol, PDI: 1.57) containing 14 mol% TSU in the hydrophobic block, was synthesized following a previously reported procedure [31], and used to formulate the NPs. Dox or a fluorescent hydrophobic dye, NR, was loaded into the NPs by a nanoprecipitation method without using of any surfactants. Particle size analysis by DLS (Figure 1A) showed that Dox-NPs and NR-NPs were similar in size (Dox-NPs: 54 ± 1 nm; NR-NPs: 50 ± 7 nm) and exhibited an overlapping size distribution. Inspection of Dox-NPs by TEM revealed the presence of spherical objects with an estimated diameter of 40 ± 5 nm (Figure 1B). The TEM results are in good agreement with DLS analyses, considering the fact that the TEM image was acquired on dry samples under vacuum whereas the DLS profile was obtained in an aqueous solution. The formulated NPs did not dissociate upon dilution, nor did they aggregate during prolonged incubation in both PBS and serum containing cell culture medium (Figure S1). Cytotoxicity evaluations on blank NPs confirmed that the NPs were not toxic to LNCaP cells under experimental conditions employed in the study (Figure S2).

Dox was effectively entrapped in the NPs with a loading content and encapsulation efficiency of 75.6 ± 3.5 μ g/mg and 39.3 ± 0.9 %, respectively. Comparable values were obtained from our previous studies using this type of amphiphilic copolymer as the drug carrier [39]. *In vitro* release was evaluated by incubating Dox-NPs in serum-containing cell culture medium under sink conditions at 37 °C for up to 7 days (Figure 1C). From day 0 to day 3, Dox was released at a cumulative rate of 15.9 ± 0.3 wt% per day. Thereafter, Dox was released at a slower rate (8.4 ± 0.2 wt% per day) until day 7 when the experiments were terminated. By day 7, a total of 81.2 ± 0.8 wt% of Dox initially loaded was released. Separately, the loading content and encapsulation efficiency for NR were quantified as 3.2 ± 0.1 μ g/mg and 32.3 ± 0.4 %, respectively. Less than 0.4 wt% of NR was released at day 1 and 12.1 ± 2.4 wt% NR was detected in serum containing media by day 7 (Figure 1C). The limited release of NR is attributed to the hydrophobicity of the dye [40]. The high retention of the hydrophobic fluorescent probe by NPs demonstrates the feasibility to use NR to track the location of NPs without undesirable dye leaking concerns.

3.2. NP diffusion in HA hydrogels

In our study, the HA hydrogel was developed to mimic the tumor-associated ECM, which is the first barrier to the NP-based cancer therapeutics [37]. We speculate that the diffusion of the ECT2-derived NPs through the hydrogels would be strongly dependent on the average pore size of the hydrogels, not the NP-matrix interaction, considering the fact that the NPs are PEGylated. To analyze the hydrogel microstructure, PEG-modified gold nanoparticles (PEG-AuNPs) ranging from 35 to 100 nm were employed as probes. PEG-AuNPs were encapsulated in the HA hydrogels *in situ* and the relative retention of PEG-AuNPs reflects the average pore size of the hydrogel matrix. For 35 nm PEG-AuNPs, approximately 27 ± 4 % of initially loaded particles was retained by the hydrogel after two days of incubation (Figure 2A). As the size of PEG-AuNPs increased, the average retention capacity increased accordingly, reaching a plateau value of 77 ± 2 % for 70 nm PEG-AuNPs. Further increase in particle size didn't result in any significant increase in the average retention for 100 nm PEG-AuNPs (79 ± 7 %). When the size of the PEG-AuNPs is smaller than the average pore size of the hydrogel, the probe can diffuse out of the hydrogel without significant restriction, consequently a relatively low overall retention was observed. When the average pore size of

the hydrogel became comparable to the size of PEG-AuNPs, high retention was observed. Large PEG-AuNPs entrapped in the hydrogel could not diffuse out due to the steric hindrance imposed by the hydrogel matrix. Our probe retention experiments suggest that the HA hydrogel contains water-filled pores of approximately 70-100 nm. The estimated pore size is in agreement with the results observed by cryogenic scanning electron microscopy (CryoSEM) as reported in our previous study [17].

We next evaluated the diffusive properties of the polymeric NPs in HA hydrogels (Figure 2B). NR was entrapped in NPs as the fluorescent probe. The NR-NPs suspension was placed in contact with one end of the hydrogel-filled channel. Infiltration of NR-NPs into the HA matrices was visualized using a fluorescence microscope after NPs were equilibrated with the HA hydrogel for 18 h. NR-NPs were found to distribute across the hydrogel-filled channel in a gradient fashion, reaching the interior of the channel at least 1000 μm away from the source reservoir in 18 h (Figure 2B(a)). By fitting the intensity profile of the NR-NPs (Figure 2B(b)) to a one-dimensional diffusion model [37], the diffusion coefficient (D_1) of NR-NPs was calculated as $4.04 \pm 0.03 \mu\text{m}^2/\text{s}$. As a control experiment, free NR solution (1 $\mu\text{g}/\text{ml}$) was prepared and the diffusion study was performed similarly as that for NR-NPs. As expected, no fluorescence signal was detected for the free NR within the hydrogel. This can be attributed to the fact that the fluorescence of the free dye was quenched in water [41]. Therefore, the fluorescent signal detected is from NR molecules entrapped in the NPs, not from free NR release in the medium. The diffusion coefficient of NR-NPs in water (D_0) was

calculated as $9.21 \mu\text{m}^2/\text{s}$ using the Stokes-Einstein equation: $D = \frac{k_B T}{6\pi\eta r}$, where k_B is the Boltzmann's constant, the viscosity (η) of water at 295.6 K is $9.4 \times 10^{-4} \text{ Pa}\cdot\text{s}$ [37], and the hydrodynamic radius (r) of NR-NPs is 25 nm as measured by DLS. The lower D_1 value, in comparison to D_0 , indicates the ability of the HA matrix to hinder the NP diffusion [37, 42].

3.3. Characterization of engineered tumoroids

In our previous investigation [17], we characterized PCa tumoroids grown from LNCaP cells in HA hydrogels, in terms of cell morphology, cell-cell adhesion, and the expression of pro-angiogenic factors. Herein, the expression of two important multidrug resistance proteins, MRP1 and LRP were first evaluated at the mRNA level by qPCR analysis. Our results showed that the hydrogel culture resulted in a significant upregulation of MRP1 and LRP expression (Figure 3A). Specifically, cells in 3D expressed 3.2 ± 1.2 and 1.4 ± 0.1 times more MRP1 and LRP than those cultured on 2D at day 7 ($p < 0.05$, Figure 3A). To further corroborate our finding, cellular production of multidrug resistance proteins was assessed by immunocytochemical staining (Figure 3B and 3C). LNCaP cells with a history of 7-day 2D or 3D culture were trypsinized and re-plated on 2D for direct comparison purposes. Twenty four hours post plating, cells with a 2D culture history appeared larger and their cytoplasm was more elongated, whereas those with a 3D culture history appeared smaller and were prone to aggregate. The difference in cell morphology implies that cells with a history of 3D culture may still maintain their 3D phenotype after being re-plated on 2D plastic surface. For both MRP1 and LRP, only a faint background staining was detected for cells cultured on 2D. Contrarily, cells with a history of 3D hydrogel culture exhibited robust MRP1 and LRP staining (Figure 3B and 3C), indicating a significant overexpression of these two multidrug resistance proteins in cells grown under 3D conditions. Overall, the trend obtained from immunostaining is consistent with the qPCR results.

3.4. Evaluation of Dox-NPs using the engineered tumor models

Dox's intrinsic fluorescence enables the direct monitoring of the intracellular transport of the NPs via confocal microscope. Free Dox and Dox-NPs were found to localize in different intracellular compartments after being exposed to cells cultured on 2D for 2 h. The short

exposure time was chosen to avoid complications introduced by Dox released from NPs at a longer incubation time. While a stronger fluorescent signal was found in the cell nuclei in the case of free Dox (Figure 4A), Dox-NPs were excluded from the nuclei and distributed predominantly in the cytoplasm (Figure 4B). These results confirm that Dox-NPs can be readily internalized by LNCaP cells and that limited Dox release from NPs occurred within the time frame of the experiment, given the fact that Dox signal in the cell nuclei was undetectable (Figure 4B). Interestingly, under 3D culture conditions, free Dox localized predominantly in the cytoplasm of individual cells in the tumoroids (Figure 4C and Figure S3A), rather than in the cell nuclei as observed for free Dox in 2D cultures (Figure 4A). On the other hand, Dox-NPs were found to be distributed mainly in the cytoplasm of 3D tumoroids (Figure 4D and Figure S3B). The faint pinkish background staining surrounding the tumoroid in Figure 4D could be a result of the Dox-NPs being retained by the HA matrix. To ascertain that Dox-NPs were indeed internalized by cells in 3D and not simply adhered to the surface of the spheroids, Z-stack slides of the confocal image at a 3- μ m interval were examined. Our results (Figure 5) confirm the homogeneous penetration of Dox-NPs through the entire tumoroid.

Our cellular uptake studies show that Dox-NPs can be readily internalized by LNCaP cells. Next, we investigated the endocytic pathways involved in the cellular uptake of NPs, employing specific endocytic inhibitors (Figure 6). NR-NPs were used in these studies in place of Dox-NPs to eliminate possible cytotoxic effects induced by the released Dox. Under the 2D conditions when treated with colchicine, a macropinocytosis inhibitor, cellular uptake of NR-NPs was reduced by $32 \pm 4\%$ ($p < 0.05$) relative to the untreated controls. Treatment on 2D monolayer cultures with other inhibitors did not significantly alter the degree of NP uptake. As shown in Figure 6, the inhibitory effects of M- β -CD, genistein (both inhibit caveolae-mediated endocytosis) or colchicine on 3D cultures was evidenced by $34 \pm 1\%$, $27 \pm 2\%$ and $23 \pm 2\%$ ($p < 0.05$) reduction of NP uptake, respectively. These results suggest that cells cultured on 2D internalize NPs predominantly via the macropinocytosis mechanism, whereas those in 3D employ both caveolae-mediated endocytosis and macropinocytosis mechanisms in NP uptake.

Following the cellular uptake studies, we evaluated the toxicity of free Dox and Dox-NPs on 2D and in 3D using a standard cell apoptosis assay. As shown in Figure 7, the drug induced a dose-dependent toxicity to the cultured cells, both on 2D and in 3D. The apoptosis experiments conducted on 2D cultures revealed that as the Dox concentration increased, both for free Dox and Dox-NPs, the respective apoptosis levels increased accordingly. A similar plateau level was reached (50.3 ± 4.6 and 52.2 ± 4.2 , fold change compared to untreated control) when the Dox concentration was higher than 10 and 20 μ M for free Dox and Dox-NPs, respectively. The half maximal inhibitory concentration (IC_{50}) for free Dox and Dox-NPs on 2D was calculated as 5.0 ± 0.1 μ M and 11.0 ± 0.8 μ M, respectively. The apoptosis experiments conducted on 3D cultures showed a similar trend of dose dependence, although the plateau values of apoptosis were significantly lower as compared to the 2D cultures. Compared to cells cultured in 3D without any drug treatment, the apoptosis level for cells receiving the treatment of free Dox and Dox-NPs was 26.8 ± 1.9 and 25.0 ± 1.2 fold higher, respectively. The calculated IC_{50} value for free Dox and Dox-NPs in 3D cultures is 15.0 ± 0.4 and 12.3 ± 0.8 μ M, respectively.

4. Discussion

The hydrogel-derived tumor model reported herein simulates the native tumor tissue and its associated microenvironment. First, the HA hydrogel is viscoelastic and exhibits characteristic pore sizes similar to that found in the native tumor tissue, thereby serving as an artificial tumor-associated matrix. Second, cancer cells cultured in the hydrogel matrix

organize into tumor-like structures and overexpress MDR proteins. Finally, the potential interactions between the aggregating cancer cells and the surrounding HA matrix, via the cell surface HA receptors such as CD44 and RHAMM, may recapitulate the early stage of tissue metastasis [26]. The engineered tumor model was used as a predictive platform for assessing therapeutic efficiencies of Dox-NPs.

It is well accepted that the transport of nano-therapeutics to the poorly perfused regions of tumor tissues is dominated by diffusion [43]. The rate of diffusion and the penetration depth depend on the size of the drug carrier and the interactions between the carrier and the interstitial matrices [43]. Although NPs in the range of 70-200 nm are optimal for tumor passive targeting through the EPR effect [4], NPs of ~100 nm have been shown to diffuse poorly through the interstitial space in the tumor tissue [44]. As a result, NPs accumulate around tumor blood vessels and do not penetrate into the tumor parenchyma [44]. On the other hand, NPs smaller than 50 nm exhibited efficient transport capability in both the 3D multicellular tumor spheroids model [43] and tumor xenografts *in vivo* [45, 46]. In this study, NPs formulated from PEG-*b*-P(CL-*ran*-TSU) were used as the drug carrier. By controlling the molecular weight of our copolymer and fine-tuning the nanoprecipitation parameters [31, 47], NPs with an average diameter of ~50 nm were obtained. Particles in this size range are expected to diffuse passively through the interstitial matrices in tumor tissues. The presence of PEG on the NP surface prevented the undesirable particle-matrix and particle-cell interactions [48].

The HA hydrogels, prepared from HA-AC and HA-SH, predominantly via a Michael type addition reaction, had an average elastic modulus of 234 ± 30 Pa [17], comparable to that of the native lymph tissue through which PCa frequently metastasizes [17]. Although HA-SH can potentially react with biological entities to form S-S linkages, the free thiols on cell surfaces are of low abundance and the disulfide formation is much slower compared to the thiol-acrylate reaction [17]. Overall, our cell encapsulation protocol does not negatively impact the cellular functions [49, 50]. Probe retention study using PEG-AuNPs revealed that the average pore size of the hydrogel was approximately 70-100 nm, comparable to the spacing (20-130 nm) found in tumor ECMs [51]. As revealed from the diffusion study (Figure 2B), the HA-based matrix mimics the ECM barrier in the tumor tissues, and its presence reduced NP diffusivity by over 2 folds. Considering the similarity in pore size between the HA hydrogel and the interstitial matrices in tumor tissues, we suggest that the transport properties of NPs obtained from our HA hydrogel model could be translated to *in vivo* settings.

Drug resistance remains an obstacle to effective chemotherapy of solid tumors [52], and has been attributed to the overexpression of the MDR proteins by tumor cells [12, 14]. The expression of two well-documented MDR proteins was investigated in this study. MRP1, a member of the superfamily of ATP-binding cassette (ABC) transporters, is well known for its function as a plasma membrane drug-efflux pump. MRP1 can extrude drugs from the cell and thereby decrease the intracellular drug accumulation, which ultimately cause the failure of chemotherapy [53, 54]. LRP, a major vault protein that is located in both cytoplasm and nuclear membrane, is also widely overexpressed in multidrug resistant tumor cells [55]. LRP may mediate MDR by the compartmentalization of drugs away from intracellular drug targets [55]. Specifically for Dox, it has been reported that LRP plays an important role in the transport of Dox from the cell nucleus (the active target of Dox) to the cytoplasm and thereby decreases the drug efficacy [56]. Our results show that the 3D hydrogel-based cell culture platform gives rise to cellular overexpression of MRP1 and LRP, possibly as a result of the competition for essential nutrients in cell aggregates [12]. The overexpression of MDR proteins has been suggested to be related to the native tumor microenvironment, as well as the tumor development process *in vivo* [13]. In agreement with our observations,

Sanchez and colleagues found that the primary cultures of PCa cells derived from patients expressed significantly higher levels of MRP1 and LRP than PCa cell lines (i.e. LNCaP, PC3 and DU145 cell lines) cultured on 2D [13]. Our hydrogel-based 3D tumor model closely simulates the *in vivo* tumor growth condition, and the elevated expression of MDR proteins suggested a MDR cell phenotype. Therefore, the drug response from cells cultured in 3D should be more physiologically relevant than that from cells cultured on 2D monolayer.

Interestingly, Dox was found to localize in the nucleus of cells cultured on 2D (Figure 4A), but was restricted to the cytoplasm of cells cultured in 3D (Figure 4C). A possible explanation is that Dox in the nucleus was transported and sequestered to the cytoplasm by LRP [56, 57]. In agreement with this observation, a decreased nuclear to cytoplasmic drug ratio was also reported in MDR cells overexpressing LRP [58, 59]. The cellular uptake study also revealed that Dox-NPs can penetrate the entire tumor spheroid and localize in the cytoplasm of individual cells (Figure 4D and Figure 5). The engineered tumoroids are likely to have loose structures, consisting of aggregating cells and interstitial matrix components. The HA immunostaining results (Figure S4) revealed the presence of HA in individual tumoroids. Such matrix-rich intercellular space within the spheroids may serve as a permissive pathway to facilitate the transport of NPs to the inner region of the tumoroids. These results are in line with previous reports confirming the NP penetration through the ECM between tumor cells to reach the inner region of the multicellular aggregates [43, 45, 60]. Particularly, Kim et al. have shown that polymeric micelles with an average diameter of 37 nm can penetrate into the core of 400 μ m human cervical tumor spheroids in 30 min [43]. In this study, the diffusion of NPs through the entire tumoroids is not surprising considering the relatively small size ($\sim$ 50 μ m) of the multicellular aggregates of approximately 20-25 cells per spheroid.

The HA-based tumor model is designed to mimic the *in vivo* tumor microenvironment to provide predictive results on the efficacy of NP-based cancer therapeutics. Our apoptosis assay revealed that the IC₅₀ value for Dox-NPs was higher than that for the free drug tested under 2D culture conditions. This is expected because there is a time delay for Dox-NPs owing to the slow release of the drug from the polymeric NPs as compared to the immediate exposure of the free drug to the cultured cells [39]. The higher IC₅₀ value and the lower plateau level of apoptotic cell death observed in 3D cultures treated with free Dox, as compared to that observed for 2D cultures, can be partially attributed to the aforementioned overexpressed MRP1 and LRP. Because Dox targets rapidly dividing cells and cells in 3D are less proliferative, the drug potency might be diminished in 3D cultures [17, 23]. This phenomenon cannot be explained by the limited drug access due to diffusion restrictions in the cell-gel construct, considering the fast diffusivity of the small molecule drug and the small size of the multicellular aggregates [14].

Given the time lag for Dox release from NPs, one would expect that the IC₅₀ value for Dox-NPs would be higher than that for the Dox under 3D culture conditions. Instead, similar IC₅₀ values were observed. While low molecular weight hydrophobic drugs diffuse readily through the cell membrane, the polymeric NPs investigated here were internalized by LNCaP cells predominantly through caveolae-mediated endocytosis and macropinocytosis. In previously reported studies, researchers have shown that nano-therapeutics entered cells through these endocytic pathways can bypass the drug efflux pump and reverse the MDR [61, 62]. In our study, the apoptosis assay was performed after 24 h drug treatment. Our NP uptake experiments showed the Dox-NPs were rapidly taken up within 2 h, during which minimal Dox was released from the NPs. Thus, cell death detected from 3D cultures was likely the result of intracellularly released Dox. Overall, NP formulation may avoid the drug efflux function of MRP1 and thereby the drug potency was enhanced. Of note, Because the

HA matrix can hinder NP diffusion, and Dox released from NPs may potentially be pumped out by MRP1, it is reasonable to expect Dox-NPs to exhibit a higher efficacy in treating matrix-free tumoroids. Although the NP formulation may potentially bypass the drug efflux function of MRP1, the plateau level of apoptotic cell death in 3D Dox-NPs condition is significantly lower than that from the 2D Dox-NPs conditions. The discrepancy can be mainly attributed to the drug resistance effect of LRP and the slow growth of LNCaP cells in 3D as mentioned above.

5. Conclusion

In this investigation, we evaluated the efficacy of NP-based cancer therapeutics using an engineered PCa tumor model. The 3D platform recapitulates characteristics of native tumor tissue both physically and biologically. The HA-based matrix not only supports the tumoroids formation from LNCaP PCa cells but also serves as a diffusion barrier for NPs. Cell cultured under the biomimetic conditions expressed a higher level of MDR proteins than those cultured on 2D. Dox-loaded NPs with an average size of 54 ± 1 nm can penetrate into the cell-gel construct, be taken-up by the resident cells and induce apoptotic responses. Moreover, cells cultured in 3D were more resistant to Dox treatment, in both soluble and NP-based forms, when compared to those grown under 2D conditions. The 3D tumor model provides an engineering advance that closely mimics the native carcinoma and can be used as an *in vitro* platform for the assessment of nanomedicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Characterization of Dox- and NR-loaded NPs. (A) DLS analysis (number-based size distribution) of Dox-NPs (black) and NR-NPs (gray). NPs were dispersed in PBS at a concentration of 0.5 mg/ml for DLS analysis. (B) Transmission electron micrograph of Dox-NPs dried on carbon-coated copper grid. Image was acquired without any staining. (C) *In vitro* release of Dox (filled) and NR (open) from NPs in serum-containing cell culture medium at 37 °C.

Fig. 2. Analysis of hydrogel pore size and NP diffusion in the hydrogels. (A) Analysis of hydrogel pore size by probe retention. PEG-AuNPs with an average diameter of 35, 50, 70 and 100 nm were used as the probe (*: significant difference, $p < 0.05$). (B) Analysis of NP diffusion in HA hydrogels. Hydrogels were equilibrated with NR-NPs for 18 h prior to the analysis. (a) Fluorescence image of HA hydrogel containing infiltrated NR-NPs. (b) Intensity profile of free NR and NR-NPs in HA hydrogel. The diffusion coefficient of NR-NPs in HA gels (D_1) was calculated as $4.04 \pm 0.03 \mu\text{m}^2/\text{s}$ using a one-dimensional diffusion model.

Fig. 3.

Characterization of MRP1 and LRP expression by LNCaP PCa cells cultured on 2D or in 3D HA gels. (A) Quantification of gene expression by qPCR. The expression was normalized to that of 2D at day 7. β -actin served as the normalization control (*, significant difference, $p < 0.05$). (B and C) Confocal images of immunocytochemically stained LNCaP PCa cells with a history of 7-day culture on 2D monolayer or in 3D HA hydrogels. Cell nuclei, MRP1 (B) and LRP (C) were stained blue, green and red respectively.

Fig. 4. Confocal images of LNCaP PCa cells, cultured on 2D or in 3D, exposed to free Dox (50 μg/ml) or Dox-NPs (0.67 mg/ml, corresponds to a Dox concentration of 50 μg/ml) for 2 h at 37 °C. On 2D, free Dox is primarily localized at the cell nucleus, while Dox-NPs are observed mainly in the cell cytoplasm. In 3D, both the free Dox and Dox-NPs are localized in the cytoplasm.

Fig. 5. Confocal images (Z-stack, 3 μm interval) of PCa tumoroids grown in the HA hydrogel and exposed to Dox-NPs (0.67 mg/ml) for 2 h. Dox-NPs penetrated the entire tumoroid.

Fig. 6.

NP uptake by LNCaP prostate cancer cells cultured on 2D or in 3D in the presence of hypertonic sucrose (0.45 M), chlorpromazine (10 μ g/ml), methyl- β -cyclodextrin (m- β -CD, 5 mM), genistein (50 μ g/ml), colchicine (8 μ g/ml) and quercetin (6 μ g/ml). NR-NPs (0.5 mg/ml) were used and the fluorescence intensity of NR in the non-inhibited cells was used as control. (# and *, significant difference compared to 2D or 3D non-inhibited controls, respectively, $p < 0.05$).

Fig. 7.
In vitro cytotoxicity of Dox and Dox-NPs in 2D and 3D cultures of LNCaP PCa cells. (A) Dose-dependent apoptosis induced by Dox and Dox-NPs. (B) Summary of the IC₅₀ values (μM) for Dox and Dox-NPs. (*, significant difference compared to the IC₅₀ value of 2D Dox condition, $p < 0.05$)