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Biomimetic Hydrogels Incorporating Polymeric Cell-Adhesive Peptide to Promote the 3D Assembly of Tumoroids

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Abstract

Towards the goal of establishing physiologically relevant *in vitro* tumor models, we synthesized and characterized a biomimetic hydrogel using thiolated hyaluronic acid (HA-SH) and an acrylated copolymer carrying multiple copies of cell adhesive peptide (PolyRGD-AC). PolyRGD-AC was derived from a random copolymer of *tert*-butyl methacrylate (*t*BMA) and oligomeric (ethylene glycol) methacrylate (OEGMA), synthesized *via* atom transfer radical polymerization (ATRP). Acid hydrolysis of *tert*-butyl moieties revealed the carboxylates, through which acrylate groups were installed. Partial modification of the acrylate groups with a cysteine-containing RGD peptide generated PolyRGD-AC. When PolyRGD-AC was mixed with HA-SH under physiological conditions, a macroscopic hydrogel with an average elastic modulus of 630 Pa was produced. LNCaP prostate cancer cells encapsulated in HA-PolyRGD gels as dispersed single cells formed multicellular tumoroids by day 4 and reached an average diameter of ~95 μ m by day 28. Cells in these structures were viable, formed cell-cell contacts through E-cadherin (E-CAD) and displayed cortical organization of F-actin. Compared to the control gels prepared using PolyRDG, multivalent presentation of the RGD signal in the HA matrix increased cellular metabolism, promoted the development of larger tumoroids and enhanced the expression of E-CAD and integrins. Overall, hydrogels with multivalently immobilized RGD is a promising 3D culture platform for dissecting principles of tumorigenesis and for screening anticancer drugs.

Keywords

Hyaluronic Acid; Cell-Adhesive Peptide; Multivalent; Hydrogels; Prostate Cancer; Tumoroids

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Supporting information: qPCR primer information, ¹H NMR of HA-SH, GPC traces for P(OEGMA-*r*-*t*BMA), peptide synthesis and characterization by HPLC and ESI-MS, rheological characterization of PolyRDG gels and a representative cryoSEM image of HA-PolyRGD gels.

1. Introduction

Cancer progression and metastasis depend on the dynamic and intricate interactions between cancer cells and their surrounding microenvironment in a three-dimensional (3D) context.¹ For mechanistic investigations and for drug screening purposes, traditional 2D monolayer culture platforms have been progressively replaced by 3D culture systems that more realistically recapitulate the *in vivo* conditions and allow for a better understanding of cell-cell communication and cell-matrix interactions in a physiologically relevant manner.²⁻⁶ Owing to their structural similarities to natural extracellular matrices (ECM),⁷⁻⁹ synthetic or semi-synthetic hydrogels have been widely used for the assembly of multicellular tumor spheroids.¹⁰⁻¹³

We¹⁴⁻¹⁷ and others¹⁸⁻²⁰ have demonstrated the applicability of hyaluronic acid (HA)-based hydrogels for the engineering of physiologically relevant tumor models. As a major component of the natural ECM in various tissues and tissue fluids, HA can interact with cell surface receptors (e.g. CD44 and RHAMM) and HA-binding proteins to mediate cell adhesion, migration, and proliferation. Moreover, elevated HA is found in tumor tissues (75~80% in prostate tissue) as tumor-associated stroma produces HA.²¹ Additionally, HA degrading enzyme, hyaluronidase (HAase), secreted by tumor cells, can promote tumor progression, facilitate cancer cell invasion and foster tumor angiogenesis. High levels of tumor-associated HA and tumor-derived HAase can also protect cancer cells against immune surveillance and chemotherapeutic drugs.²²⁻²³ These unique properties, combined with its susceptibility to chemical modification, render HA an ideal macromolecule for the construction of hydrogel-derived 3D tumor models.

In addition to HA, cancer cells interact with integrin binding proteins in the tumor microenvironment to modulate cancer progression and metastasis.²⁴⁻²⁵ Interestingly, blockage of such interaction led to the restoration of normal tissue structure.²⁶ For in-depth mechanistic investigations, the engineered tumor microenvironment should present biological signals to foster integrin engagement with the resident cancer cells. This can be accomplished by introducing cell adhesive proteins to HA hydrogels via chemical and physical means.²⁷⁻²⁸ While these methods are straightforward to apply, the use of matrix constituents for biofunctionalization has disadvantages associated with purification, processing, reproducibility, denaturation and immunogenicity. To exert a greater control over material properties, short synthetic peptides have been used for matrix functionalization.²⁹ While these short peptides have proven efficacious in promoting cell adhesion and growth factor binding initially, they do not recapitulate the multivalent nature of the natural protein, thereby lacking the specificity, and tunability needed for the regulation of highly integrated biological processes.

An attractive intermediary between short peptides and intact proteins is a polymer/peptide conjugate consisting of a hydrophilic, protein-resistant polymer backbone and repetitive functional sequences identified from the integrin binding proteins. Such hybrid conjugates can elicit highly coordinated and dynamic interactions with the targeted cells,³⁰⁻³² driving specific cell phenotypes essential for the growth and phenotypic retention of cancer cells. Finally, the hybrid copolymers combine the unique features associated with synthetic

polymers and short peptides to exhibit enhanced biological functions and improved enzymatic stability. Stable linking of peptide signals in HA matrices can be achieved if a chemically addressable functional group is introduced to the hybrid copolymer. Overall, the hybrid copolymers can be engineered to mimic the natural proteins in terms of their molecular architectures, dynamic responsiveness and cell-instructive properties, with the added attributes of tunability and processibility provided by the synthetic polymer constituents.

Here, synthetic strategies were developed for the preparation of peptide/polymer conjugates that can be covalently integrated in a HA matrix to promote the 3D assembly of prostate cancer (PCa) tumoroids from dispersed LNCaP cells, originally isolated from a lymph node metastasis of a prostate cancer patient³³ (Figure 1). Specifically, atom transfer radical polymerization (ATRP) of *tert*-butyl methacrylate (*t*BMA) and oligomeric ethylene glycol methacrylate (OEGMA), followed by acid hydrolysis produced hydrophilic copolymers with protein-repellent OEG side chains and chemically addressable carboxylate groups. Modification of the copolymer with 2-hydroxyethyl acrylate installed reactive acrylates (AC), through which bioactive peptides, with a basic sequence of GRGDSP, were introduced (Figure 2). The resultant peptide-conjugated, chemically crosslinkable copolymer (PolyRGD-AC) was mixed with thiolated HA (HA-SH) to form a macroscopic hydrogel under physiological conditions. The HA-PolyRGD gels were characterized chemically, mechanically and morphologically. The synthetic matrix was used for the establishment of multicellular tumoroids and the effects of PolyRGD on cell growth, spheroid expansion, and gene/protein expression were systematically investigated. Overall, the bioactive, peptide-functionalized hydrogels are attractive 3D culture platforms for dissecting principles of tumorigenesis and for testing of new anticancer drugs.

2. Materials and Method

2.1. Chemicals and Reagents

Oligo (ethylene glycol) methyl ether methacrylate (OEGMA, 300 g/mol), *tert*-butyl methacrylate (*t*BMA, 98%), methyl-2-bromopropionate (MBP, 98%), copper (I) chloride (CuCl, 99.999%), 2,2'-bipyridine (bpy, 99%), 4-dimethylaminopyridine (DMAP, 98%), di-*tert*-butyl dicarbonate [(Boc)₂O, 98%] and bovine testicular hyaluronidase (HAase, Type VI-S, 30,000 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO). HA (sodium salt, *M_w* 450 KDa) was a gift from Genzyme Corp. (Cambridge, MA). Trifluoroacetic acid (TFA), tetrahydrofuran (THF), dichloromethane (DCM), dimethylformamide (DMF), hexane and ethyl ether were purchased from Thermo Fisher Scientific (Waltham, MA) and were used as received. Monodisperse polystyrene (PS) standards were purchased from Polymer Source Inc. (Dorval, Quebec, Canada). Monomers, *t*BMA and OEGMA in THF, were passed through a neutral Al₂O₃ column to remove the inhibitors. The OEGMA/THF eluate was concentrated on a rotary evaporator, and dried under reduced pressure. PEGylated (methoxy-PEG5000-SH) gold nanoparticles (PEG-AuNPs) were purchased from Cytodiagnosics (Burlington, ON). Bovine serum albumin (BSA, Jackson Immuno Research), primary anti-integrin β1 (mouse-derived monoclonal 12G10; Abcam, Cambridge, MA), primary anti-rabbit E-CAD (H-108; Santa Cruz Biotechnology, Dallas, TX),

secondary antibodies, including goat anti-mouse IgG Alexa Fluor® 488 and goat anti-rabbit Alexa Fluor® 647 (Life Technologies, Grand Island, NY), DAPI (Millipore, Billerica, MA) and Alexa Fluor® 568 phalloidin (Life Technologies) were used as received. All other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Water was deionized and filtered through a Barnstead NANOpure Diamond water purification system.

2.2. Synthesis and Characterization of PolyRGD-AC (Figure 2)

2.2.1. Instrumentation—¹H NMR spectra were recorded on a Bruker AV600 spectrometer using CDCl₃, DMSO-*d*₆ or D₂O as solvents. Gel permeation chromatography (GPC) was carried out using a Waters GPC (Milford, MA) equipped with two Waters Styragel (HR1 and HR4) columns, a Waters 2695 auto-sample pump and a refractive index (RI) detector (Waters 410). THF was used as the eluent at a flow rate of 1.0 mL/min, and calibration was performed with polystyrene standards.

2.2.2. Preparation of random copolymer of OEGMA and tBMA [P(OEGMA-*r*-tBMA)]—MBP (10 μL, 0.088 mmol), OEGMA (5.28 g, 17.6 mmol), tBMA (3.75 g, 26.4 mmol), CuCl (8.8mg, 0.088 mmol) and bpy (27.6 mg, 0.176 mmol) were successively added to a 50 mL Schlenk flask containing 6 mL of ethanol. Thereafter, the reaction mixture was purged with argon for 10 min and the flask was sealed. The solution was stirred at 35 °C for 48 h and the polymerization was subsequently quenched by exposure to the ambient air. The reaction mixture was diluted with ethanol and passed through a neutral Al₂O₃ column to remove the copper catalyst. The solution was concentrated and precipitated dropwise into cold hexane three times. The resulting viscous solid, P(OEGMA-*r*-tBMA), was dried under vacuum at room temperature for 48 h. Yield: 41%; ¹H NMR (CDCl₃, δ, Figure 3A): 0.9-1.1 ppm (e, -CH₃), ~1.8 ppm (d, -CH₂-), ~1.4 ppm (h, -C(CH₃)₃), ~3.7 ppm (f, -CH₂OCH₃), ~3.4 ppm (g, -OCH₃); GPC: *M*_n: 35,910 g/mol, *M*_w/*M*_n: 1.43.

2.2.3. Synthesis of random copolymer of OEGMA and methacrylic acid [P(OEGMA-*r*-MAA)]—Briefly, TFA (1.5 mL, 20.4 mmol) mixed with 2 mL of DCM was added to a stirred solution of P(OEGMA-*r*-tBMA) (2.0 g, 0.056 mmol) in 10 mL of DCM at 0 °C. The reaction was allowed to proceed at room temperature for 72 h. After the excess solvent was removed by a rotary evaporator, the polymer was precipitated into a mixture of cold hexane and ethyl ether (10/1, v/v) three times. The product, P(OEGMA-*r*-MAA), was collected and dried in vacuum at room temperature for 24 h as a white powder. Yield: 91%; ¹H NMR (DMSO-*d*₆, δ, Figure 3B): ~12.4 ppm (i, -COOH).

2.2.4. Modification of P(OEGMA-*r*-MAA) with hydroxyethyl acrylate (HEA) [P(OEGMA-*r*-(MMA-g-HEA))]—HEA (0.9 g, 7.8 mmol), (Boc)₂O (0.68 g, 3.12 mmol) and DMAP (0.019 g, 0.156 mmol) were successively added to a solution of [P(OEGMA-*r*-MAA)] (0.4 g, 1.56 mmol of MAA) in 5 mL DMF. The reaction was allowed to proceed for 24 h at room temperature. The mixture was diluted with DI water and was subsequently purified by dialysis against DI water (MWCO 10K) for 72 h, followed by freeze-drying to yield a white solid. Yield: 89%; ¹H NMR (DMSO-*d*₆, δ, Figure 3C): 5.8-6.3 ppm (j, k, l, -CH=CH₂).

2.2.5. Synthesis of RGD containing, thiol-reactive copolymer (PolyRGD-AC)—

P(OEGMA-*t*-(MMA-*g*-HEA)) (62.3 mg) was dissolved in 4 mL DI water and the solution pH was adjusted to ~7.8 using 1 mM NaOH. Separately, a cysteine-containing peptide with a sequence of CGGWGRGDSPG (RGD-SH, 14.6 mg), synthesized and purified following standard solid phase peptide synthesis protocol (see supporting information), was dissolved in 0.5 mL pH-adjusted DI water at ~7.8. The peptide solution was then added dropwise to the polymer solution and the mixture was stirred at ambient temperature for 3 h. The conjugate was purified through dialysis against DI water (MWCO 10K) for 24 h before lyophilization, yielding the white solid product (PolyRGD-AC). PolyRDG-AC was prepared following the same procedure using scrambled peptide, CGGWGRD~~G~~SPG (RDG-SH). Yield: 93%; ¹H NMR (DMSO-*d*₆, δ , Figure 3D): 6.7-8.8 ppm (-CO-NH-, peptide backbone), ~10.8 ppm (-NH-, indole of the tryptophan residue),

2.3. Hydrogel Synthesis and Characterization

2.3.1 Hydrogel synthesis—Thiolated HA (HA-SH) was synthesized via a carbodiimide-mediated coupling reaction with 3,3'-dithiobis-propanoic dihydrazide (DTP) in an aqueous media at pH 4.75, followed by reduction with 1,4-dithiothreitol, according to reported methods.³⁴ HA-SH was obtained at ~75% yield, with a 33% thiol incorporation based on ¹H NMR (Figure S1). To prepare the hybrid hydrogels, HA-SH and PolyRGD-AC were separately dissolved in PBS at a concentration of 20 mg/mL, and the solution pH was adjusted to ~7.8 using NaOH. Gelation was initiated by mixing the above solutions at a thiol/acrylate molar ratio of 1/1, with the final RGD concentration maintained at 2 mM. The mixture was incubated at 37 °C for 3 h to complete the gelation.

2.3.2 Mechanical properties—The viscoelastic properties of the hydrogels were evaluated using a controlled stress rheometer (AR-G2, TA Instruments, New Castle, DE) with a 20-mm parallel plate geometry. The aqueous mixture was carefully loaded on the bottom plate immediately after the gel components were mixed. Time sweep experiments were carried out at a strain of 1% and a frequency of 1 Hz and the frequency sweep was conducted at 1% strain from 0.01 Hz to 10 Hz. All measurements were performed at 37 °C with a gap size of 100 μ m in triplicate and the average storage (*G'*) and loss (*G''*) moduli are reported.

2.3.3 Swelling ratio and sol fraction—Freshly made gel disks were incubated in PBS (pH 7.4) at 37 °C for 24 h to extract soluble HA-SH and PolyRGD-AC that were physically entrapped in the matrix. Individual disks were then thoroughly washed with DI water, dehydrated using graded ethanol solutions and dried at 37 °C overnight. After the dry weight (*W_d*) was recorded, samples were rehydrated in PBS for 24 h and the wet weight (*W_w*) was measured. The swelling ratio (SW) and sol fraction (SF) were calculated as $SW = W_w / W_d$ and $SF(\%) = (1 - W_d / W_s) * 100$, where *W_s* represents the initial solid mass used in gel preparation. The measurements were performed in triplicate.

2.3.4 Degradation—Hydrogel disks were immersed in PBS with or without HAase (5 U/mL) at 37 °C. At a predetermined time, the degradation solution was collected and stored at -20 °C for further analysis, while the degradation medium was replenished with PBS or

HAase-containing PBS. The amount of HA degraded (W_1) at each time point was quantified by the carbazole assay.³⁵ HA content (wt%) in the remaining hydrogels at each time point was calculated as $(W_0 - W_1)/W_0 \times 100$, where W_0 represents the initial solid mass of HA in each sample. Three independent measurements were averaged for each sample.

2.3.5 Pore-size—A probe retention method was employed to measure the average pore size of the hydrogels using PEG-AuNPs with an average diameter of 35, 50, 70 and 100 nm, following our previously reported procedures.¹⁵ Briefly, PolyRGD-AC containing dispersed PEG-AuNPs was mixed with HA-SH to produce nanoparticle-containing hydrogels. The hydrogel samples were then equilibrated in PBS at ambient temperature for 48 h. The amount of PEG-AuNPs released into the supernatant was determined using UV-Vis spectrophotometer by monitoring the size-dependent absorbance at 518, 524, 530 and 548 nm for 35, 50, 70 and 100 nm PEG-AuNPs, respectively. Particle retention by the hydrogel, determined by comparing the concentration of PEG-AuNPs initially loaded in the hydrogel with that in the pooled supernatant, was plotted as a function of particle diameter. Hydrogel pore size was estimated from the retention plot based on the transition from a low to a high percent retention.

2.4. Cell Culture and Biological Characterization

2.4.1. Cell maintenance and 3D culture—LNCaP prostate cancer cells were maintained in a RPMI-1640 medium (ATCC, Manassas, VA) supplemented 5% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (P/S) at 37 °C under a 5% CO₂ atmosphere. Media was changed every 2 days and cells were passaged using 0.25% (w/v) trypsin containing ethylenediaminetetraacetic acid (EDTA-4Na). For 3D culture, LNCaP cells were dispersed in HA-SH (2 wt%) before polyRGD-AC (2 wt%) was added and the solution pH was maintained at 7.8. The mixture, with a cell loading density of 1×10^6 cell/mL, was transferred to a wet cell culture insert in a 24-well plate and was incubated at 37 °C for 1 h before the addition of cell culture media around and on top of the insert. Media was changed every 2 days and the cell/gel constructs were imaged using an Eclipse Ti-E microscope (Nikon, Tokyo, Japan).

2.4.2. Live/Dead staining—On day 1, 7, 14, 28, the cell/gel constructs were carefully rinsed with PBS and incubated in PBS containing calcein-AM (1:1000, v:v) and ethidium homodimer-1 (1:500, v:v) for 20 min. After another PBS wash, constructs were examined under a confocal laser scanning microscope (CLSM, Zeiss LSM 710) and images were collected as maximum intensity projection of ~300 μ m thick z-stacks. The size and size distribution of the LNCaP tumoroids grown in the hydrogels were quantified using ImageJ software (NIH, Bethesda, MD) based on 8-9 different CLSM images. The spheroid size distribution at different time points was created respectively using a histogram plot with a 5 μ m bin for the spheroid diameters measured. The resulting histogram was fitted with a Gaussian distribution using OriginPro Software.

2.4.3. PrestoBlue assay—LNCaP cells were cultured in HA-PolyRGD gels under conditions described above. At predetermined time points, medium was replaced with 10% (v/v) PrestoBlue®-fresh medium mixture. After 3-h incubation, 100 μ L of the medium

inside the inset was aliquoted to a 96-well plate and the fluorescence intensity was monitored using a Perkin-Elmer microplate reader (Ex: 550 nm; Em: 590 nm). Analysis was performed in triplicate for each condition.

2.4.4. Immunofluorescence—After 28 days of culture, the cell-laden hydrogel constructs were transferred to an 8-well Lab-Tek™ II chambered cover glass, immersed in 4% (v/v) paraformaldehyde (PFA) solution at room temperature for 30 min and washed twice with PBS (1×). Cells were permeabilized with 0.1% Triton X-100 in PBS for 45 min, then blocked in 3% BSA for 30 min. After wash, the primary antibody solution, including anti-integrin β_1 and anti-E-CAD (1:100 dilution in PBS containing 0.1% Triton X-100 and 3% BSA), was introduced and samples were incubated at room temperature for 3 h. The secondary antibody solution, Alexa Fluor® 488-conjugated goat anti-mouse IgG (1:200 dilution) or Alexa Fluor® 647-conjugated goat anti-rabbit IgG (1:200 dilution), mixed with Alexa Fluor® 568-labeled phalloidin (1:500 dilution), was introduced and the constructs were incubated at room temperature for additional 1.5 h. Finally, cell nuclei were counter stained with DAPI (1:1000 dilution) for 10 min. After a copious wash with PBST (0.05% Tween-20 in 1× PBS), constructs were inspected using a Zeiss LSM 710 confocal microscope and images were presented as a maximum intensity projection of 200-300 μm thick z-stacks.

2.4.5. Real-time quantitative polymerase chain reaction (qPCR)—Following 28 days of 3D culture, constructs were snap-frozen in a dry ice/isopropanol slurry and were stored at $-80\text{ }^{\circ}\text{C}$ for further analysis. The frozen samples ($n=3$ to 4 per sample) were crushed with a pestle and the total RNA was extracted using Trizol reagent (ThermoFisher, Waltham, MA). The QuantiTect reverse transcription kit (Qiagen, Valencia, CA) was utilized to convert mRNA to cDNA. Template cDNA (4 ng) was then combined with primers (400 nM) and Power SYBR green master mix (Invitrogen, Carlsbad, CA). PCR was performed in an ABI 7300 real-time sequence detection system (Applied Biosystems, Foster City, CA). All primers were obtained from Integrated DNA Technologies (Coralville, IA). The target primer sequences are summarized in Table S1 and the expression of each gene was compared to GAPDH using a modified Ct method, which accounted for the different efficiency of primers.³⁶

2.5. Statistical Analysis

All quantitative measurements were performed on at least three repeats. The data were expressed as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were carried out using student's t-test. Differences were considered statistically significant when p values are <0.05 .

3. Results and Discussion

3.1. Synthesis and characterization of PolyRGD-AC

A thiol-reactive copolymer carrying multiple copies of cell adhesive peptide, PolyRGD-AC (Figure 2), was synthesized and characterized. First, a random copolymer of OEGMA and β BMA was prepared by ATRP using MBP as the initiator, CuCl as the catalyst and bpy as

the ligand. The ^1H NMR spectrum shows characteristic chemical shifts from both monomers and a comparison of peak integration for $-\text{C}(\text{CH}_3)_3$ (h, Figure 3A) and $-\text{OCH}_3$ (g, Figure 3A) revealed a copolymer composition of $\text{P}(\text{OEGMA}_{65}\text{-}r\text{-BMA}_{115})$. The number-average molecular weight (M_n) of 35,910 g/mol and a polydispersity index (M_w/M_n) of 1.43 were measured by GPC (Figure S2). Subsequent TFA treatment led to the hydrolysis of the *tert*-butyl groups, producing hydrophilic copolymer, $\text{P}(\text{OEGMA}\text{-}r\text{-MMA})$, with protein-repellent OEG side chains and chemically addressable carboxylate groups. The complete disappearance of methyl protons in the *tert*-butyl groups at 1.4 ppm ($-\text{C}(\text{CH}_3)_3$, h, Figure 3A) and the appearance of the carboxylic proton at ~ 12.4 ppm ($-\text{COOH}$, i, Figure 3B) confirmed the complete deprotection. Esterification reaction of $\text{P}(\text{OEGMA}\text{-}r\text{-MMA})$ with HEA led to the installation of thiol reactive acrylates, as evidenced by the addition of vinyl protons at 5.8–6.3 ppm (j, k, l, $-\text{CH}=\text{CH}_2$, Figure 3C). A comparison of peak integration for these protons with that for the methyl protons (g, $-\text{OCH}_3$) in the OEGMA repeats revealed that approximately 20 ± 1 mol% of the MMA repeats were acrylated. Finally, a cysteine-containing cell adhesive peptide, synthesized and purified following standard procedures (Figure S3), was conjugated to copolymer $\text{P}(\text{OEGMA}\text{-}r\text{-}(\text{MMA}\text{-}g\text{-HEA}))$ via Michael Addition through the dangling double bonds. The reduction of the peak integration for the vinyl protons relative to the methyl protons in OEGMA for the parent copolymer (Figure 3D) indicates that approximately 5 ± 1 mol% MMA repeats in PolyRGD-AC are RGD-conjugated, while 15 ± 1 mol% MMA repeats were acrylated. On average, each PolyRGD-AC carries approximately 6–7 RGD peptides and 17–18 double bonds.

3.2. Hydrogel synthesis and characterization

Hybrid hydrogels were prepared via a Michael-type addition reaction between HA-SH and polyRGD-AC under physiological conditions at a thiol/acrylate ratio of 1/1. The hydrogels exhibited an average swelling ratio of 22.8 ± 0.3 and a sol fraction of $29.2 \pm 2.1\%$. The gelation kinetics and viscoelastic properties were characterized by oscillatory rheology. Time sweep experiments (Figure 4A) revealed that the gel point was reached ~ 13 min after HA-SH and polyRGD-AC were mixed, as evidenced by the crossover of storage modulus (G') and loss modulus (G''). Thereafter, G' value continued to increase, reaching a plateau modulus of 630 ± 62 Pa 2 h post mixing. The storage modulus (Figure 4B) was insensitive to frequency from 0.01 to 10 Hz, and the corresponding $\tan(\delta)$ (G''/G') value was 0.005, indicating the elastic nature of the HA-PolyRGD hydrogels. In order to assess the effect of RGD on the growth and aggregation of LNCaP cells, scrambled negative control polyRDG-AC was prepared via the same synthetic pathway using RDG-SH. Control gels prepared using PolyRDG-AC exhibit similar viscoelastic properties as those made using PolyRGD-AC (Figure S4). Overall, the hybrid hydrogels exhibit viscoelastic properties comparable to those measured for bone marrow, lymph and brain tissues,^{37–38} through which PCa frequently metastasizes.

The average pore size of the hydrogels was estimated based on the relative retention of the encapsulated PEG-AuNPs of varying diameter. As shown in Figure 5A, approximately $32 \pm 8\%$ of 35-nm PEG-AuNPs initially loaded were retained in the hydrogel after 48 h of incubation. As the particle size increased, a higher retention was observed. Further increase in particle size beyond 70 nm resulted in little particle release. As the particle size

approached the average pore size of the hydrogel network, particle diffusion through the matrix and into the supernatant became significantly restricted. Because the particles are pegylated, minimal particle-matrix interaction is anticipated. In summary, our particle retention experiment revealed that the average pore size of the HA-PolyRGD hydrogels was 50-100 nm. Characterization of the synthetic hydrogel by cryogenic scanning electron microscopy (cryo-SEM, Figure S5) further confirmed the presence of interconnected nanopores in the matrix.

For the 3D growth of LNCaP cells and the progressive expansion of the multicellular tumoroids, ideally, the synthetic matrix should be degradable by cell-secreted enzymes, at the same time, maintaining structural integrity to enable long term culture. Hydrogel degradation was monitored indirectly by following the release of HA from the gel disks under physiological conditions with or without HAase (Figure 5B). The HAase concentration employed in the degradation study is significantly higher than that in various tissues and in cell cultures.^{21, 23} Hydrogels treated with 5 U/mL of HAase were gradually degraded and completely disappeared by day 18. Initially from day 1 to day 5, HA was released at a rate of ~5% per day. A rapid loss of HA was observed from day 5 to day 12, likely due to enhanced accessibility of HAase to the exposed HA chains as a result of the reduction in network connectivity. By day 14, HA was essentially depleted from the hydrogel. Therefore, the synthetic hydrogels can be readily degraded by HAase via the glycosidic bonds in HA. In the absence of HAase, less than 30% HA was lost during 18 days of incubation. The percent gel mass remaining after 18 days of incubation without HAase was measured gravimetrically as $80.9 \pm 4.5\%$. Note that HA accounts for 39 wt% of the polymer mass in the hydrogel and that the hydrogel exhibit a sol fraction of 29%. Thus, within the time frame of the experiment, enzyme-free hydrolytic degradation via the side chain ester bonds is minimal. It is worth mentioning that LNCaP cells are known to secrete elevated levels of esterases, and these cell-secreted enzymes have been used as drug targets for prostate cancer.^{23, 39} Overall, esterase and/or HAase-mediated hydrogel degradation can facilitate the rapid growth of LNCaP in 3D. Alteration of the order of D and G residues in PolyRDG-AC is not expected to change the network mesh size and degradation as compared to the PolyRGD gels.

3.3. Characterization of LNCaP tumoroids

3.3.1. Cell viability, metabolic activity and tumoroid expansion—Cell-laden gel constructs were prepared by mixing HA-SH and PolyRGD-AC with dispersed LNCaP cells. To ascertain the role of integrin-binding in tumor growth without altering the gel structure or the stiffness, control samples were prepared using PolyRDG-AC in place of PolyRGD-AC. Live/dead staining (Figure 6) revealed minimal cell death during the initial encapsulation and throughout the 28 days of culture, confirming the cytocompatibility of the hydrogel formulations. LNCaP cells were initially entrapped in the hydrogels homogeneously in a single cell state. Viable multicellular aggregates greater than 40 μm were detected on day 7 and the size of individual structures increased over time. By day 28, abundant large (> 80 μm) tumoroids were found in both types of gels. The tumoroid structures were intact even after 28 days in culture. While the distribution of single cells in the hydrogel was uniform on day 1, on day 7, 14 and 28, the size of the tumoroids in any randomly selected area was

heterogeneous. This is probably due the heterogeneous nature of the growth pattern of LNCaP cells. In large tumoroids with an average diameter greater than 100 μm , the center region was weakly fluorescent owing to insufficient dye penetration throughout the structures.

ImageJ analysis of the confocal images after live/dead staining revealed a gradual increase in tumoroid sizes for both PolyRGD and PolyRDG cultures. On average, tumoroids grown in PolyRGD gels were statistically ($p < 0.05$) larger than those grown in PolyRDG controls (Figure 7A). On day 7, tumoroids of varying sizes coexist with single cells, owing to the heterogeneous cell population and differential growth patterns. By day 28, multicellular tumoroids were predominant in both types of constructs. Consistent with this observation, size distribution histograms (Figure 7B-D) show a clear shift from smaller structures in PolyRDG gels to larger aggregates in PolyRGD gels on day 7, 14 and 28, respectively. Structures found in PolyRGD gels had a median size of $95 \pm 17 \mu\text{m}$, ~1.3 fold larger than those grown in PolyRDG gels on day 28. The formation and the expansion of multicellular tumoroids from cells singly dispersed in the covalent matrix during the initial encapsulation process can be attributed to the ability of LNCaP cells to remodel the synthetic matrix through secreted enzymes, including HAase and various esterases.⁴⁰⁻⁴¹ Our previous work⁴² describes a synthetic matrix that is purely HA-based, but is significantly softer (G' : ~234 Pa) and devoid of ester linkages between the HA backbone and the crosslinking points. LNCaP cells grown in this type of matrix reached an average diameter of 85 μm by day 7, and prolonged culture did not lead to further size expansion. Therefore, matrix parameters, such as stiffness, ligand presentation and stability/degradability collectively influence the tumoroid formation in 3D.

Because integrin binding stimulates cell proliferation, proper presentation of integrin-binding motifs in the synthetic matrix can promote tumoroid formation and expansion.⁴³ Cell proliferation was analyzed indirectly by PrestoBlue assay. Our results showed (Figure 8) a progressive increase in cellular metabolism for PolyRGD cultures from day 0 to day 10, followed by a moderate decrease during the last two weeks of culture. The data suggests that the metabolic activity of these cultures reached a steady state between days 10 and 14. Cells in PolyRDG gels were generally less metabolically active than those in the PolyRGD counterparts, most significantly from day 10 to day 28. A 2.5-fold increase in cell metabolism was seen over the first 10 days for PolyRGD cultures, whereas a significantly ($p < 0.05$) lower fold change (1.5 fold) was observed from the PolyRDG cultures. Therefore, multivalent incorporation of RGD signals in the hydrogel matrix significantly increased the metabolic activity of cells, suggesting that cells in these gels are significantly more proliferative. This data is consistent with the growth of the cells shown in Figure 6. The decrease in metabolic activity can be ascribed to the formation of large tumoroids after day 14, when cells in the central core exhibited different metabolic profiles and might present hypoxia. Meanwhile, due to the diffusion limit, inefficient mass transport also leads to metabolic waste accumulation inside the tumoroids.⁴⁴⁻⁴⁵

3.3.2. Cellular expression of E-CAD and Integrins—Tumorigenesis is characterized by progressive alterations of cellular machineries that give rise to uncontrolled cell growth.⁴⁶ Changes occurring in the tumor microenvironment further reinforce the abnormal growth

and facilitate the dissemination of the primary tumor to the distal organs. In response to external cues from the tumor microenvironment, cancer cells dynamically regulate E-cadherin-mediated cell-cell adhesions and integrin-mediated cell-matrix contacts to promote the invasive phenotype.⁴⁷⁻⁴⁹ Having confirmed the effects of polyRGD on cell growth and spheroid expansion, we next sought to understand how the engineered microenvironment modulates cellular expression of cell-cell and cell-ECM adhesions. Constructs were analyzed for expression of E-CAD and integrins after 28 days of culture. Our qPCR results (Figure 9A) showed that compared to the housekeeping gene (GAPDH), the mRNA levels of E-CAD, ITG α 5 and ITG β 1 were consistently lower in the control samples, but are significantly ($p < 0.05$) higher in the PolyRGD constructs. When normalized to the PolyRDG controls, the expression levels for E-CAD, ITG α 5 and ITG β 1 in PolyRGD constructs are 3.0 ± 0.3 , 3.3 ± 0.4 and 2.1 ± 0.4 fold higher, respectively. Confocal imaging (Figure 9B) of tumoroids growing in both types of gels revealed a rounded morphology with a clear cortical organization of F-actin delineating cell boundaries. No obvious differences in the organization and expression levels of F-actin were observed. On the other hand, intense E-CAD staining was only observed in PolyRGD samples. In this case, a colocalization of E-CAD and F-actin at the areas of cell-cell contacts is clearly revealed. Although both PolyRGD and PolyRDG constructs were stained positively for ITG β 1, the former staining is more intense. Because PolyRGD and polyRDG gels exhibit a similar substrate stiffness, the differences observed herein can be attributed to multivalent RGD presentation. Overall, multivalent presentation of RGD signals in the synthetic matrix did not compromise the ability of LNCaP cells to form multicellular spherical structures, but significantly enhanced cell-cell and cell-matrix adhesions.

The upregulation of E-CAD and ITG β 1 is in line with previous reports on metastatic cancers. While the initial dispersal of cells from the primary tumor site is characterized by a decrease in cell-cell and cell-matrix interactions, this change is reversed and the expression of integrins and E-CAD is increased once the tumor has relocated.⁵⁰⁻⁵¹ At the target site, integrin-mediated cell adhesion to the matrix provides a survival signal to the cells,⁵² and E-CAD is required to form clusters necessary to develop tumoroids. For prostate cancer, bone metastasis is prevalent and represents a lethal prognosis, and both integrins and E-CAD are found to be upregulated in prostate cancer metastasis to bone.⁵³⁻⁵⁴ The multivalent RGD are engaged in integrin binding, which in turn, triggers cell-cell adhesion, and tumoroid formation.⁵⁵⁻⁵⁶ Collectively, the PolyRGD gels reported herein provide a biomimetic and cell-permissive environment that not only facilitate cell matrix interaction but also promote cell-cell adhesion, altogether contributing to the establishment of a more metastatic model. Integrins have been widely studied with regards to prostate cancer cellular proliferation and metastasis as potential targets for drug therapies due to their ubiquity among different cancer types. By introducing specific cell binding ligands to LNCaP cells then monitoring their transcriptional changes, it is possible to predict whether cells are becoming more metastatic or proliferative. Our future study is focused on the coculture of PCa and stromal cells in a biomimetic matrix, and the contributions of HA and PolyRGD to the establishment of complex and realistic tumor models will be systematically evaluated.

4. Conclusion

A thiol-reactive, protein-mimetic hybrid copolymer (PolyRGD-AC) with a multivalent presentation of cell adhesive motifs was synthesized and characterized. PolyRGD-AC was combined with HA-SH to establish a biomimetic matrix with an elastic modulus of 630 Pa, an average pore size of 50-100 nm and can be degraded by HAase. LNCaP prostate cancer cells cultured in PolyRGD gels are metabolically active and form multicellular tumoroids with an average diameter of 95 μm by day 28. Cells residing in the tumoroids establish cellular contacts through E-cadherin, interact with the matrix through integrin and develop cortical F-actin. Multivalent presentation of the RGD signals not only promoted the formation of larger tumoroids, but also enhanced cellular expression of E-CAD and integrin $\alpha 5/\beta 1$. PolyRGD-AC provides the added advantage of compositional versatility, enabled by its synthetic nature, and biological activity, through peptide installation. The hybrid HA-PolyRGD hydrogels present key signaling motifs in the tumor microenvironment to facilitate the encapsulation of LNCaP cells with high cell viability, at the same time, allowing cell expansion in 3D to form physiologically relevant tumor models. The engineered tumor model can be used as model to provide predictive results for optimizing the efficacy of cancer therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Fabrication of HA/PolyRGD hydrogels for the assembly of LNCaP prostate tumoroids.

Figure 2.

Synthesis of PolyRGD-AC by atom transfer radical copolymerization of OEGMA and tBMA, followed by side chain deprotection, partial esterification and peptide conjugation. The parent copolymer, P(OEGMA-*r*-tBMA), has a M_n of 35,910 g/mol and PolyRGD-AC contains approximately 6-7 peptides and 17-18 acrylates per chain.

Figure 3.
 ^1H NMR spectra of P(OEGMA-*r*-tBMA) (A), P(OEGMA-*r*-MAA) (B), P(OEGMA-*r*-(MAA-*g*-HEA)) (C) and PolyRGD-AC (D).

Figure 4.

Representative rheological time (A) and frequency sweep (B) measurements on HA/ PolyRGD hydrogels prepared using HA-SH (2 wt%) and PolyRGD-AC (2 wt%) at a thiol/ acrylate molar ratio of 1/1 (G' : ●; G'' : ○). Samples were loaded on the plate immediately after HA-SH and PolyRGD-AC were mixed.

Figure 5.

Characterization of hydrogel pore size and degradation by probe retention (A) and carbazole assay (B), respectively. (A): PEG-AuNPs of varying diameters were encapsulated in the hydrogels, and particle release was analyzed by UV-Vis (*: significant difference, $p < 0.05$). (B): Hydrogels were incubated in PBS at 37 °C with or without HAase and HA release was monitored by carbazole assay.

Figure 6. Representative confocal images of fluorescently stained LNCaP cells grown in HA/ PolyRGD gels at day 1, 7, 14 and 28. Cells cultured in HA/PolyRDG gels were included as the controls. Live and dead cells were stained Calcein AM (green) and ethidium homodimer-1 (red), respectively. Scale bar: 100 μm.

Figure 7.

The average diameter of LNCaP spheroids grown in PolyRGD and PolyRDG gels as a function of culture time (A). Characterization of 3D cultures in terms of tumoroid size distribution represented as histograms for day 7 (B), 14 (C) and 28 (D) cultures. (*: significantly higher than the corresponding control group, student's t-test, $p < 0.05$)

Figure 8.

Characterization of 3D cultures in terms of cellular metabolism. The metabolic activity was quantified by PrestoBlue assay as a function of culture time. The UV absorbance from each well was normalized to the initial day 0 level (*: significantly higher than the corresponding control group, $p < 0.05$).

Figure 9.

Characterization of E-CAD and ITG expression by LNCaP cells cultured in 3D hydrogels. (A) Quantification of gene expression by qPCR after 28 days of 3D culture. The error bars represent SEM. Expression of each gene was compared to GAPDH using a modified $\Delta\Delta C_t$ method which accounts for the different efficiency of primers (*: significantly different from PolyRDG cultures). (B): Representative confocal images, at low (10 $\times$) and high (40 $\times$) magnifications, of immunofluorescently stained LNCaP cells after 28 days of 3D culture.

Cell nuclei, ITG β 1, F-actin and E-CAD were stained blue, green, red and magenta, respectively.

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