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Hyaluronic acid-based hydrogels to study cancer cell behaviors

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Abstract

Hyaluronic acid (HA) is a natural polysaccharide and a key component of the extracellular matrix (ECM) in many tissues. Therefore, HA-based biomaterials are extensively utilized to create three dimensional ECM mimics to study cell behaviors *in vitro*. Specifically, derivatives of HA have been commonly used to fabricate hydrogels with controllable properties. In this review, we discuss the various chemistries employed to fabricate HA-based hydrogels as a tunable matrix to mimic the cancer microenvironment and subsequently study cancer cell behaviors *in vitro*. These include Michael-addition reactions, photo-crosslinking, carbodiimide chemistry, and Diels-Alder chemistry. The utility of these HA-based hydrogels to examine cancer cell behaviors such as proliferation, migration, and invasion *in vitro* in various types of cancer are highlighted. Overall, such hydrogels provide a biomimetic material-based platform to probe cell-matrix interactions in cancer cells *in vitro* and study the mechanisms associated with cancer progression.

Keywords: hyaluronic acid, hydrogels, crosslinking, extracellular matrix, cancer

1. Introduction

Hyaluronic acid (HA), also known as hyaluronan, is a naturally occurring linear polysaccharide made up of repeat units of N-acetyl-D-glucosamine and D-glucuronic acid. The monosaccharide units are linked via alternating β-1,4- or β-1,3-glycosidic bonds.[1–3] HA is the main component of the extracellular matrix (ECM) in many tissues. The HA chain is flexible and has various structural properties in distinct tissues that can affect HA signaling functions.[4] In addition, superior physiochemical characteristics of HA such as high water-binding capacity, biocompatibility, biodegradability, and nontoxicity make this polysaccharide an attractive candidate for a variety of biomedical applications.[5,6] Accordingly, HA-based biomaterial scaffolds, have been employed for applications in tissue engineering, and as *in vitro* cell culture models to study cell-matrix interactions in variety of contexts, including cancer.[7–9]

Several studies have highlighted the effects of HA on cancer cell behaviors mediated via HA binding to cell surface receptors (i.e., CD44 and the receptor for hyaluronan-mediated motility RHAMM).[10–12] Also, different types of cancer cells are known to overexpress the aforementioned cell surface receptors.[13,14] HA binding to these receptors is known to regulate various cancer cell behaviors including cellular proliferation, migration, and invasion.[15–17] Higher content of HA in the ECM is known to promote tumor progression, subsequently leading to a reduced life expectancy in patients.[18,19] Given these observations, in recent years, HA-based biomaterial scaffolds, particularly, hydrogels have been extensively explored as tunable tissue mimics to probe cell-matrix interactions, in the context of cancer.[20]

HA has several reactive functional groups, which offer the possibility of obtaining a variety of HA-based derivatives that can employed to form HA hydrogels. Fabrication of HA-based hydrogels has been performed through a wide range of chemical modifications generally targeting

the reactive hydroxyl and carboxyl chemical groups of the polysaccharide.[21,22] Modifying the HA chains allows fabrication of HA-based hydrogels with various crosslinking chemistries with tunable properties.[23] In addition, these chemistries provide the ability to tailor the hydrogel properties to closely match with the properties of the tissue of interest, thereby providing a biomimetic culture system to probe cancer cell behaviors *in vitro*.[23,24]

This review focusses on various chemistries used to fabricate HA-based hydrogels from various HA-derivatives and how they have been employed as tissue mimics to study cancer cell behaviors *in vitro*. Commonly employed Michael-addition reactions, photo-crosslinking, Carbodiimide crosslinking, Diels-Alder, as well as other less commonly employed chemistries are discussed. Each of these chemistries employ specific HA-derivatives. The utility of these HA-based hydrogels to examine cancer cell behaviors such as proliferation, migration, as well as invasion *in vitro* in various types of cancer are reviewed. Overall, such hydrogels provide a controllable platform to probe cell-matrix interactions in cancer cells *in vitro* and study the mechanisms associated with cancer progression.

2. Chemistries to fabricate HA-based hydrogels to study cancer cell behaviors *in vitro*

HA has several chemical groups that can be modified for fabricating hydrogels. Specifically, the hydroxyl and carboxyl groups of HA can be chemically modified and subsequently used as target sites for hydrogel fabrication with desired physiochemical properties. Methacrylated, acrylated and thiolated HA are some of the common derivatives of HA that have been employed to fabricate HA-based hydrogels. Although these HA derivatives form hydrogels with significantly different properties, fabricated HA-based hydrogels maintain biocompatibility and biodegradability of native HA.[23–25]

2.1 Michael addition reactions

One of the commonly employed chemistries to synthesize HA-based hydrogels is the Michael type addition reaction. The facile and orthogonal nature of this reaction combined with the yield of a highly specific product under mild conditions, is particularly beneficial for biological systems.[26] The Michael addition reaction, generally characterized as the reaction of an enolate-type nucleophile to an α,β unsaturated carbonyl group in the presence of a base, yields highly selective products under environmentally friendly and mild reaction conditions.[27] The α,β-unsaturated compound undergoing Michael-type addition is termed as the Michael acceptor, the nucleophile is termed as the Michael donor, and the product is termed as the Michael adduct. More specifically, this reaction is a conjugate addition in which the Michael adduct is formed from a negatively charged enolate intermediate resulting from the nucleophilic attack on the β carbon of an α,β-unsaturated carbonyl group. This thermodynamically favored condition generates C−C, C−O, C−N, C−S, and other C−X bonds within the Michael-type addition reactions. Based on the final formed bonds, the Michael-type addition reactions include the carbon-, oxa-, aza-, and the thiol-Michael reactions.[24,26,28] HA-based hydrogels are typically crosslinked by thiol-Michael reaction and can be divided into two main groups.

In the *first* group, the nucleophile (the donor) is a crosslinker with a thiol end group. In this group, the crosslinker acts as a nucleophilic agent and reacts with unsaturated carbonyl group of HA derivatives. Typically, methacrylated HA, or acrylated HA have been used as the Michael acceptor in the thiol-Michael addition reaction. In the *second* group of thiol-Michael reactions, the nucleophile (the donor) role is played by the HA derivative, instead of a cross-linker. Thiolated HA derivatives are commonly employed for this purpose. Accordingly, in this type, the Michael acceptor has unsaturated carbonyl groups which reacts with thiol groups on HA chains. The

acceptor could be an unsaturated carbonyl group (e.g., acrylated HA) or carbon-carbon double bond. In addition, other polymers such as thiolated Gelatin could be used to fabricate the hydrogel network by utilizing a Michael acceptor crosslinker such as poly(ethylene glycol) diacrylate (PEGDA). In all these cases, reactive SH group is part of the polymer (i.e., thiolated HA) structure. Below we discuss three HA derivatives commonly used in Michael addition reactions (i.e., Methacrylated HA, acrylated HA, and thiolated HA (Figure 1A)).

2.1.1. Methacrylated HA

Methacrylated HA is a commonly employed HA derivative to fabricate HA hydrogels via the thiol-Michael addition reaction (Table 1). HA is typically reacted with Methacrylic anhydride or Glycidyl methacrylate; the primary and secondary hydroxyl groups on HA will then be chemically altered with methacrylate groups via an esterification reaction. Thus, the unsaturated carbonyl double bond in methacrylate group potentially can react with the other double bond of same chemical group on the other HA chain. Typically, Dithiothreitol (DTT) is employed as a crosslinker. These HA-based hydrogels have been largely used to study the behavior of brain cancer (i.e., glioblastoma multiforme) and brain metastatic breast cancer cells *in vitro*, given the abundance of HA in the brain ECM.[29] For example, Ananthanarayanan et al., employed HA hydrogels fabricated using methacrylated HA to investigate glioblastoma cell behavior as a function of HA hydrogel stiffness and ligand (RGD peptide) density. Their results showed that glioma cell spreading had a positive correlation with RGD peptide density and hydrogel stiffness. Moreover, they reported that higher stiffness supported enhanced cell proliferation and cell migration speed.[30] These hydrogels have also been employed as a three dimensional (3D) culture model to study various cell-matrix interactions such as CD44 or integrin-based adhesion and their impact on glioblastoma cell motility.[16,31] Likewise, Nakod et al., showed that HA hydrogels are a

suitable environment for long-term culture of glioblastoma cells (U87, and patient derived D456 cells) encapsulated in hydrogels. Encapsulated U87 and D456 cells formed cell spheroids over time and these HA hydrogels also supported maintenance of the stem-like phenotype in glioblastoma cells. Interestingly, in this system, incorporating of the RGD peptide did not significantly impact glioblastoma spheroid sizes grown in HA-based hydrogels.[32]

Narkhede et al., utilized HA hydrogels fabricated via thiol-Michael addition reaction to evaluate the behavior of brain metastatic breast cancer cells (i.e., MDA-MB-231Br cells) *in vitro*. They found that MDA-MB-231Br cells exhibited differential response on HA hydrogels with varying stiffnesses (Figure 1B, 1C, and 1D). In particular, cell adhesion, cell spreading, cell proliferation and cell migration significantly increased with hydrogel stiffness when cells were cultured on top of these hydrogels.[33] In a subsequent study, Narkhede et al., utilized this system and developed an *in vitro* model of dormancy for brain metastatic breast cancer cells, specifically demonstrating that brain metastatic breast cancer cells show a dormant phenotype when they are cultured on soft (~ 0.4 kPa) HA hydrogel, whereas they show a proliferative phenotype on stiff HA (~ 4.5 kPa) hydrogels.[34] This system was also subsequently employed to study microenvironmental regulation of dormancy and proliferation in brain metastatic breast cancer cell clusters *in vitro*.[35] In sum, these studies demonstrate the utility of HA hydrogels fabricated using methacrylated HA to study cell-matrix interactions *in vitro* in both, primary and secondary brain cancers.

2.1.2. Acrylated HA

Acrylated HA is another HA derivative commonly used to fabricate HA hydrogels via the thiol-Michael addition reaction, however, few studies have utilized hydrogels prepared using this derivative to study cancer cell behaviors *in vitro* (Table 1). For example, Shen et al., employed acrylated HA to fabricate hydrogels to study HT1080 fibrosarcoma cell proliferation and invasion.

They studied the impact of matrix stiffness (soft, medium, and stiff HA hydrogels) on HT1080 cell fate in different oxygen levels (atmospheric, hypoxic and severely hypoxic) by producing hydrogels with different concentration of matrix metalloproteinase (MMP) sensitive peptide crosslinkers. HT1080 cells adapted better to hypoxia with no correlation noted with matrix stiffness. In addition, their results showed that endothelial sprouting and invasion were affected by varying stiffness and oxygen tension in the presence of HT1080 cells. Increasing endothelial sprouting and invasion for all hydrogels was reported under hypoxia conditions and was accompanied by vascular endothelial growth factor (VEGF) as well as angiopoietin-1 (ANG-1) upregulation. However, under atmospheric condition, a negative correlation was observed between endothelial sprouting and invasion with matrix stiffness.[36]

2.1.3. Thiolated HA

Thiolated HA have been commonly employed to form hydrogels to study cancer cell behaviors *in vitro* (Table 1). For instance, Xiao et al., employed thiolated HA to investigate the role of the ECM in drug resistance in glioblastoma cells. Herein, thiolated HA was utilized in conjunction with Maleimide-terminated 4-arm polyethylene glycol (PEG) (one arm of which was conjugated with the RGD peptide), and PEG thiol.[37,38] Thiolated HA has also been frequently combined with thiolated Gelatin to further mimic the tumor microenvironment.[39] In these systems, thiol reactive PEGDA has been used as a crosslinker. By similar chemistry, thiolated Gelatin can be crosslinked to thiolated HA via the two acrylate end groups of PEGDA. Additionally, using PEGDA as a crosslinker has been shown to provide hydrogels with well-defined properties including defined gelation times.[40,41] Herein, in addition to thiolated HA, the crosslinker (i.e., PEGDA) also plays the acceptor role. This type of Michael addition reaction has been employed to create 3D culture systems that mimic the cancer cells ECM, maintains high cell viability, and mostly brain, prostate,

and colon cancer cells behaviors such as morphology and proliferation have been examined.[42,43] For instance, Leite et al., used this hydrogel system to encapsulate human glioblastoma cell lines (U-87MG, SNB-19 and UP-007). The hydrogel showed high viability for all the cell lines after one week and also supported cell proliferation.[44] Hydrogels fabricated using thiolated HA have also been employed to investigate cancer cell migration, invasion and study the impact of wide range of drugs such as Temozolomide, Vincristine, Marimastat, Cisplatin, Carmustin, Docetaxel, and Cabazitaxel.[41,43,45–47] HA based hydrogels have also been fabricated by utilizing thiolated and acrylated HA. This strategy has been thus far utilized to fabricate 3D culture models with distinct designs, such as multilayer HA-based hydrogels with controllable gelation timing, with a goal of designing novel drug delivery systems[48] or for drug screening applications.[49]

Finally, thiolated HA can be crosslinked via self-gelation. Specifically, thiolated HA can be crosslinked via disulfide crosslinking to fabricate HA hydrogels. Although this reaction is not categorized as Michael addition reaction, this strategy has been used in wide range of applications, including studying cancer cell behaviors *in vitro*.[50–52] For example, Rao et al., fabricated a composite hydrogel by mixing various types of collagen with different concentrations of thiolated HA to study the effect of increasing HA concentration on the morphology and migration of patient derived OSU-2 glioblastoma cells *in vitro*. They found that tumor cells exhibited spindle-like morphology at lower concentration of HA. Additionally, cell migration had an inverse relation with HA concentration, with increasing HA concentration eventually impeding cell migration .[52] Overall, the various HA derivatives provide opportunities to fabricate HA-based hydrogels with controllable properties via the Michael-addition reaction to probe cancer cell behaviors *in vitro*.

2.2. Photo-crosslinking

Photo-crosslinking allows fabrication of hydrogels with consistent mechanical and physical properties, with high reproducibility.[53,54] To fabricated photo-crosslinkable HA hydrogels, the HA chemical structure needs to be modified to support photo-crosslinking by a water-soluble photoinitiator. To obtain photo-crosslinkable HA, HA is methacrylated by reacting it with Methacrylic anhydride or glycidyl methacrylate as mentioned previously. Photoinitiators such as Irgacure 2959 or Lithium Acylphosphinate (LAP) are then added to the prepolymer solution, a free radical reaction will then be initiated by UV irradiation (Figure 2A). Propagation step of this reaction occurs between the methacrylate groups on different chains. Then, the termination step includes termination by creating covalent bond between two propagating HA chains or between one propagating HA chain and a second radical.[23,55]

Photo-crosslinkable HA based hydrogels provide several advantages such as quick gelation times and have been typically employed to encapsulate the cancer cells inside the hydrogel or to seed them on top of the hydrogel. For instance, Xu et al., fabricated a copolymeric HA-based hydrogel to study the effect of degree of methacrylation on the behavior of MCF-7 breast cancer cells. Poly (N_{ε}-acryloyl L-lysine)/HA hydrogel was synthesized with three different mole percentage of grafted glycidyl methacrylate. As a cationic polymer, poly L-lysine (PLL) promotes cell adhesion to the hydrogel via electrostatic interactions. Therefore, all copolymeric HA-based hydrogels showed higher cell adhesion when compared with tissue culture polystyrene (TCPS). As expected, higher degree of methacrylation lead to higher values of elastic and loss modulus. Moreover, presence of PLL decreased the swelling ratio of copolymeric hydrogels compared to the HA hydrogel. Encapsulated MCF-7 cancer cells in these hydrogels exhibited higher migration and invasion abilities, and expressed higher levels of pro-angiogenic growth factors.[56]

Photo-crosslinkable HA has also been combined with other photo-crosslinkable moieties to generate composite hydrogels and subsequently study the impact of HA incorporation on cancer cell behaviors, including morphology, proliferation, and invasion (Table 2). For instance, HA has been routinely combined with Gelatin methacrylate (GelMA), which can also be similarly photo-crosslinked.[57–61] To fabricate GelMA, Gelatin chains are modified by an esterification reaction with Methacrylic anhydride and methacrylate groups are added to the Gelatin backbone. But in this case, the modification occurs at both hydroxyl and primary amine group on the Gelatin chain.[62,63] Chen et al., employed hydrogels with similar composition and same concentration of methacrylated HA but with different molecular weights to study the effect of chain length of HA crosslinked with GelMA network on GBM39 brain cancer cell invasion. Their results showed a negative correlation between invasion and matrix-immobilized HA molecular weight.[64] Likewise, the impact of HA concentration on the invasion of U251MG brain cancer cells has also been investigated. In this study, the authors reported significantly lower mean invasion distance for hydrogels incorporating HA compared to hydrogels lacking matrix immobilized HA (Figure 2B, 2C).[65] In a subsequent investigation, Chen et al., observed a negative correlation between invasion of glioblastoma cell lines (U87 & U87^{VIII}) and presence of matrix-bound HA. However, they found invasion of both cell lines in all hydrogels (with or without HA) significantly increased under hypoxia compared to normoxia.[66] The impact of incorporating HA in GelMA or 4 arm-PEG hydrogels on proliferation of glioblastoma cells has also been examined. In this study, methacrylated HA was incorporated into GelMA or 4 arm-PEG hydrogels via photo-crosslinking at similar concentrations. Interestingly, the presence of HA minimally impacted proliferation in U87 cells in PEG hydrogels, while U87 cells showed significantly higher proliferation in the absence of HA in GelMA hydrogels. Further, HA incorporation also promoted clustering of cells

in both hydrogels tested.[67] Finally, photo-crosslinking has also been employed in multiple stages to create dynamically stiffened HA hydrogels. This is particularly attractive as dynamic changes occurring in the tumor microenvironment *in vivo* can be studied using such hydrogels *in vitro*. For example, these hydrogels have been employed to study the impact of mechanical cues on the behavior of mammary epithelial cells.[68]

A combination of Michael type reaction and photo-crosslinking has also been employed to fabricate HA-based hydrogels. Dual crosslinking of HA-based hydrogels provides a 3D culture system with tunable elastic modulus.[45,47] For example, Sivakumar et al., utilized this method to fabricate HA-based hydrogels with varying stiffnesses. The hydrogel stiffness was tuned by crosslinker manipulation, particularly by utilizing linear PEGDA and 4-arm PEGDA, thereby providing a wide range of matrix stiffness to study the impact of matrix stiffness on glioma cell proliferation.[47] In addition, by incorporating a modified PEGDA with a cleavable disulfide bond, the hydrogel elastic modulus was dynamically tuned through the addition of N-acetyl-l-cysteine which cleaves disulfide bonds.[47] In sum, photo-crosslinking is an attractive strategy to fabricate HA-based hydrogels with well-defined properties, including dynamic tuning of hydrogel properties, to study cancer cell behaviors *in vitro*.

2.3. Carbodiimide chemistry

Carbodiimide chemistry has been commonly used to make HA-based hydrogels (Table 3). As dehydration agents, carbodiimide compounds tend to react with carboxylic acid groups and alter the functional group to produce an unstable intermediate, which is very reactive with primary amine functional groups.[70–72] Specifically, Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) has been used to couple carboxylic acid group containing component (i.e., HA) with an amine containing component (e.g., adipic dihydrazide) (Figure 3A). Because adipic dihydrazide is

amine terminated at both ends, they have been employed as crosslinkers to form HA hydrogels.[73,74] Compared to Michael addition reaction or photocrosslinking, modification of HA chains is not needed for carbodiimide chemistry. Also, this method of crosslinking allows fabrication of cytocompatible HA-based hydrogels for a variety of biomedical applications, including studying cancer cell behaviors *in vitro*.

HA hydrogels fabricated by this method were employed by Jin et al., to test the invasiveness of highly malignant glioma cells *in vitro*. Herein, the formation of cell colonies (diameter > 150 μm) was considered as a quantitative indicator of invasiveness. Out of the four cell lines tested (U87MG, U251MG, U343MG-A, and U373MG), cell lines that expressed hyaluronidase (HAase) (U251MG and U343MG-A) exhibited greater invasion compared to cell lines that were deficient in HAase (U87MG and U373MG) suggesting that HAase expressing glioma cells can degrade the HA hydrogel fabricated via the EDC chemistry and produce more colonies.[75] Similarly, these HA hydrogels were utilized by David et al., to correlate invasion of various cancer cells with HA-binding sites (CD44 expression) and ability to release HAse/HA. The results showed a negative correlation between CD44 expression and cancer cell invasion. In addition, less secretion of HA was correlated with a higher number of colonies.[76] Conversely, cell lines that secreted more HAse produced significantly higher number of colonies in the HA hydrogels consistent with observations by Jin et al., (Figure 3B).[75] A similar HA-based system fabricated via EDC chemistry, was utilized to examine the impact of collagen types (type I, III, IV, V) on cancer cell invasiveness *in vitro*. This study also used the number of cell colonies ((diameter < 150 μm) and (diameter > 150 μm)) as an indicator to evaluate the invasive potential of cancer cells. Based on morphometry and number of colonies, collagen types I and III were shown to better support invasion compared to other types with type III collagen having a stronger effect compared to type I in three of the four

cell lines tested. These hydrogels were also used to study the role of stromal cell-derived factor-1α and basic fibroblast growth factor in cancer cell invasion.[77]

In addition to measuring cancer cell invasion, HA hydrogels fabricated via the EDC chemistry have also been used for testing drug response of cancer cells *in vitro*. For instance, David et al., employed HA hydrogels as a desirable 3D model to investigate impact of 5-Fluorouracil (5-FU) and Doxorubicin as anticancer drugs. The results were also compared with traditional 2D models. Testing a wide range of anticancer drug concentration in both models demonstrated that cancer cell lines tested (SA87, NCI-H460 and H460M) were more responsive to drugs in the 2D model than in HA hydrogels. Moreover, adding soluble HA to the 2D system showed the presence of HA increased drug resistance especially in lower doses of 5-FU for SA87 and NCI-H460 cell lines. Overall, these results showed the utility of HA hydrogels to study drug responsiveness in cancer cells *in vitro*.[78]

2.4. Diels-Alder and other chemistries

The Diels-Alder reaction is a very selective cycloaddition that occurs between a diene and a dienophile and is accelerated in the presence of water.[79] This approach is an attractive method to fabricate HA hydrogels because this method is not time consuming and is achievable in a simple one-step reaction. In addition, this aqueous-based orthogonal crosslinking chemistry that is free of additives has lower potential toxicity in comparison with photo-crosslinking that normally requires a coupling agent, or a photoinitiator.[80–82] To reach these goals, HA chains are typically modified by reacting with 5-methylfurfurylamine to functionalize furan chemical groups on HA chains which replace the carboxyl groups. This strategy provides a crosslinking reaction between HA-furan and a crosslinker with maleimide end groups (i.e., Bis(maleimide)-PEG or maleimide functionalized peptides) via the Diels-Alder chemistry (Figure 4A).[83]

Functionalizing HA hydrogels with motifs comprising diolefin also provides a tailorable platform to incorporate different types of ligands using Diels-Alder chemistry.[83,84] To this end, HA-furan hydrogels were employed by Fisher et al., to investigate the impact of a MMP cleavable peptide crosslinker and the RGD ligand on invasion of breast cancer cell lines *in vitro*. Maleimide end groups of both MMP cleavable peptide and the adhesive ligand were added to the HA backbone by a single step Diels-Alder reaction. Out of four tested cell lines, only MDA-MB-231 breast cancer cells infiltrated the hydrogels and all the other cell lines remained on the hydrogel surface. Invasion was shown to be inversely correlated with crosslinker density and was not impacted with varying ligand density. However, the hydrogel with highest ligand density had significantly more number of cells in comparison with control samples without the RGD ligand, demonstrating that increased ligand density supported cell proliferation (Table 4).[9]

Similar approach was utilized by Baker et al., to immobilize various bioactive ligands to a bifunctional HA-based hydrogel with independently controlled mechanical and chemical properties. To decouple the chemical from mechanical properties, HA hydrogels were functionalized with both methyl furan as well as aldehyde motifs as active sites for conjugating ligands and crosslinking, respectively. The probable competition for the same furan groups on the HA backbone in reversible Diels-Alder reaction (retro Diels-Alder) would be between the bioactive ligands and the crosslinker comprising diolefin motif (such as bis (maleimide)-PEG). To address this concern, aldehyde motifs were added to HA backbone to provide a new avenue to chemically crosslink the hydrogel using oxime chemistry. By screening several formulations, the authors found that hydrogels with an elastic modulus of ~0.6 kPa and incorporating IKVAV peptide optimally supported culture of breast cancer spheroids. In addition, cells in this formulation

exhibited higher expression of multidrug resistance protein MDR1, demonstrating the utility of such systems for drug screening applications.[85]

Conjugating a cyclic structure such as phenol to HA backbone also provides opportunities to fabricate enzymatically crosslinked HA hydrogels (Figure 4A). In this chemistry, horseradish peroxidase (HRP) serves as the crosslinker and hydrogen peroxide as the catalyst control the gelation rate and crosslinking density, respectively.[86,87] HA-Phenol (HA-Ph) hydrogel was fabricated by Tan et al., and these hydrogels enabled enrichment of breast cancer stem cells facilitated via HA-CD44 interactions (CD44 has been reported to be a marker for cancer stem cells [88,89]). They found that adhesion of breast cancer cells (MCF-7, HCC1937 and MDA-MB-231 cells expressing low to high levels of CD44, respectively) to the hydrogel decreased with increasing crosslinking density of hydrogel. Also, their results demonstrated that breast cancer cell line adhesion to HA-Ph hydrogels positively correlated with CD44 expression.[90] Similarly, hydroxyphenyl derivative of HA modified with RGD can also be enzymatically crosslinked,[91] however, this has not been employed to study cell-matrix interactions in cancer cells *in vitro*.

Another approach to fabricate 3D HA-based hydrogels employs two distinct HA derivatives. Specifically, in this approach, crosslinking occurs by hydrazone bond formation between aldehyde-carrying HA (HAALD) and adipic acid dihydrazide–derived HA (HAADH) (Figure 4A).[12,92,93] HA-based hydrogels fabricated via hydrazone crosslinking have considerable advantages such as their simple *in-situ* gelation process, pH-responsiveness, as well as self-healing ability.[93] For instance, Gurski et al., fabricated HA-based hydrogels by hydrazone crosslinking method and demonstrated the ability of this system to study anti-cancer drug efficacy of Camptothecin, Docetaxel and Rapamycin on C4-2B bone metastatic prostate cancer cell line. The hydrogel also supported high cell viability, and tumor cell cluster formation similar in morphology

to that observed *in vivo* (Figure 4B, C, D, E).[94] In addition, to compensate for the high degradation rate of the hydrazone bond in the hydrogel, photo-crosslinking process was used (dual crosslinking) to improve the mechanical properties and stability of the hydrogel.[92] These hydrogels mimicked the topographical and mechanical properties of the breast tumor microenvironment and supported enhanced migration and invasion of MCF-7 breast cancer cells. Overall, the chemistries discussed herein, provide exciting avenues to create HA hydrogel-based tumor microenvironment mimics to study cancer progression *in vitro*.

3. Conclusions and Perspectives

In conclusion, a variety of chemistries have been employed to develop HA based hydrogels as tissue mimics to study cancer cell-matrix interactions *in vitro* as well as response to drug treatment in several types of cancer, including brain, breast, and prostate cancer. Michael-addition reaction and photo-crosslinking have been more commonly used compared to other chemistries such as EDC chemistry. Choosing a particular crosslinking chemistry is one of the key aspects of creating hydrogel-based tissue mimics. Factors such as cytocompatibility, biodegradability, mechanical properties, gelation time, and byproducts of the crosslinking reaction can help in choosing the appropriate chemistry or a combination of chemistries if needed. For example, for studies that require cancer cell encapsulation, gelation time is one of the key factors to successfully fabricate cell-laden 3D HA hydrogels. The choice of chemistry is also critical for three dimensional bioprinting, a technology that has now been increasingly employed for creating *in vitro* culture models. HA derivatives discussed herein, such as methacrylated HA, and thiolated HA, have been successfully employed for 3D bioprinting of HA-based hydrogels.[69,96] For example, Maloney et al., recently employed thiolated HA in combination with methacrylated collagen as a bioink to print tumor organoids for drug screening applications.[69] The utility of these HA derivatives to

create 3D bioprinted models of the tumor microenvironment is expected to increase in the coming years.

In addition to the chemical crosslinking techniques discussed herein, physical crosslinking has also been used to fabricate HA-based hydrogels. Physical crosslinking offers advantages such as simple preparation techniques that include adjusting parameters like temperature or pH compared to chemical crosslinking. In addition, hydrogels prepared via physical crosslinking do not typically contain residual or unreacted agents such as initiators or catalysts. However, precisely controlling the molecular interactions can be challenging. Physically crosslinked HA-based hydrogels can formed via various methods, including, utilization of block copolymers of PEG and cationic poly(2-aminoethyl methacrylate) (ionic interactions),[97] thiolated oligo DNA modified HA (DNA hybridization),[98] HA-benzoyl cysteine derivative (π–π stacking interactions),[99] adamantane-modified and β-cyclodextrin-modified HA (guest-host interactions),[100,101] as well as collagen binding peptide grafted HA to form Collagen-HA hydrogels (molecular recognition).[102] However, to the best of our knowledge, these approaches have not been employed to study cancer cell behaviors *in vitro* but should provide avenues to do so in the future.

Developing controllable HA hydrogel environments also enables study of various cues influencing cancer cell fate *in vitro*. In addition, the choice of specific chemistries allows decoupling of hydrogel properties governing cancer cell behaviors. Thus, the impact of various cues of the tumor microenvironment, such as biophysical cues, and biochemical cues, on the phenotype of cancer cells *in vitro* can be studied utilizing HA based hydrogels. In addition, these hydrogels provide the ability to probe HA-CD44/RHAMM interactions, not typically afforded by synthetic hydrogels. In the future, the various chemistries discussed herein, are expected to provide opportunities to create increasingly complex tissue mimics closely mimicking several aspects of the tissue of

interest. These tissue mimics can subsequently be utilized to probe cancer cell phenotype, study mechanisms of cancer progression, and response to treatment with the potential to identify therapeutic targets for intervention in various types of cancers.

Conflict of Interest

The authors declare no conflict of interest.

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References

1 T. C. Laurent, U. B. Laurent and J. R. E. Fraser, *Immunol. Cell Biol.*, 1996, **74**, a1–a7.

2 J. Necas, L. Bartosikova, P. Brauner and J. Kolar, *Vet. Med. (Praha).*, 2008, **53**, 397–411.

3 A. Fallacara, E. Baldini, S. Manfredini and S. Vertuani, *Polymers (Basel).*, 2018, **10**, 701.

4 M. Hemshekhar, R. M. Thushara, S. Chandranayaka, L. S. Sherman, K. Kemparaju and K. S. Girish, *Int. J. Biol. Macromol.*, 2016, **86**, 917–928.

5 J. Martin, S. Garcia, A. Panitch and S. Calve, *Acta Biomater.*, 2019, **84**, 169–179.

6 T. Walimbe, A. Panitch, P. M. Sivasankar and W. Lafayette, *J. Voice*, 2017, **31**, 416–423.

7 L. Lapcik, S. De Smedt, J. Demeester and P. Chabrecek, *Chem. Rev.*, 1998, **98**, 2664–2683.

8 M. Sepantafar, R. Maheronnaghsh, H. Mohammadi, F. Radmanesh, M. M. Hasani-sadrabadi and M. Ebrahimi, *Trends Biotechnol.*, 2017, **35**, 1074–1087.

9 S. A. Fisher, P. N. Anandakumaran, S. C. Owen and M. S. Shoichet, *Adv. Funct. Mater.*, 2015, **25**, 7163–7172.

10 K. J. Williams, I. J. Stratford, N. Tirelli and M. Mehibel, *Mol. Pharm.*, 2019, **16**, 2481–2493.

11 J. Min, S. Jintaek, I. Richard, S. Nho, Y. Hwan, P. Upadhyaya and F. Kassie, *Mol. Carcinog.*, 2019, **58**, 321–333.

12 L. A. Gurski, X. Xu, L. N. Labrada, N. T. Nguyen, L. Xiao, K. L. Van, X. Jia and M. C. Farach-carson, *PLoS One*, 2012, **7**, Article e50075.

13 Z. Cai, H. Zhang, Y. Wei and F. Cong, *Biomacromolecules*, 2017, **18**, 1677–1696.

14 K. J. Wolf and S. Kumar, *Biomater. Sci. Eng.*, 2019, **5**, 3753–3765.

15 R. Golshani, L. Lopez, V. Estrella, M. Kramer, N. Iida and V. B. Lokeshwar, *Cancer Res.*, 2008, **68**, 483–492.

16 Y. Kim and S. Kumar, *Mol. Cancer Res.*, 2014, **12**, 1416–1430.

17 S. Misra, V. C. Hascall, R. R. Markwald and S. Ghatak, *Front. Immunol.*, 2015, **6**, Article 201.

18 M. Il and J. Correia, *Carbohydr. Polym.*, 2016, **150**, 139–148.

19 R. Tammi, J. Parkkinen, M. Tammi, U. Ågren, R. Johansson, P. Hirvikoski and M. Eskelinen, *Am. J. Pathol.*, 2000, **156**, 529–536.

20 M. Y. Kwon, C. Wang, J. H. Galarraga, E. Puré, L. Han and J. A. Burdick, *Biomaterials*, 2019, **222**, 119451.

21 M. N. Collins and C. Birkinshaw, *Carbohydr. Polym.*, 2013, **92**, 1262–1279.

22 A. Cadete and M. Jose Alonso, *Nanomedicine*, 2016, **11**, 1–17.

23 C. E. Schanté, G. Zuber, C. Herlin and T. F. Vandamme, *Carbohydr. Polym.*, 2011, **85**, 469–489.

24 S. Khunmanee, Y. Jeong and H. Park, *J. Tissue Eng.*, 2017, **8**, 1–16.

25 S. K. Seidlits, Z. Z. Khaing, R. R. Petersen, J. D. Nickels, J. E. Vanscoy, J. B. Shear and C. E. Schmidt, *Biomaterials*, 2010, **31**, 3930–3940.

26 D. P. Nair, M. Podgórski, S. Chatani, T. Gong, W. Xi, C. R. Fenoli and C. N. Bowman, *Chem. Mater.*, 2014, **26**, 724–744.

27 B. D. Mather, K. Viswanathan, K. M. Miller and T. E. Long, *Prog. Polym. Sci.*, 2006, **31**, 487–531.

28 C. F. Nising and S. Brase, *Chem. Soc. Rev.*, 2008, **37**, 1218–1228.

29 S. S. Rao, J. J. Lannutti, M. S. Viapiano, A. Sarkar and J. O. Winter, *Tissue Eng. Part B*, 2014, **20**, 314–327.

30 B. Ananthanarayanan, Y. Kim and S. Kumar, *Biomaterials*, 2011, **32**, 7913–7923.

31 A. D. Rape and S. Kumar, *Biomaterials*, 2014, **35**, 8846–8853.

32 P. S. Nakod, Y. Kim and S. S. Rao, *Biotechnol. Bioeng.*, 2019, **117**, 511–522.

33 A. A. Narkhede, J. H. Crenshaw, R. M. Manning and S. S. Rao, *J. Biomed. Mater. Res.*, 2018, **106**, 1832–1841.

34 A. A. Narkhede, J. H. Crenshaw, D. K. Crossman, L. A. Shevde and S. S. Rao, *Acta Biomater.*, 2020, **107**, 65–77.

35 R. V. Kondapaneni and S. S. Rao, *Biomater. Sci.*, 2020, **8**, 6637–6646.

36 Y. Shen, H. E. Abaci, Y. Krupski, L. Weng, J. A. Burdick and S. Gerecht, *Biomater. Sci.*, 2014, **2**, 655–665.

37 W. Xiao, S. Wang, R. Zhang, A. Sohrabi and Q. Yu, *Matrix Biol.*, 2019, **85**–**86**, 128–146.

38 W. Xiao, R. Zhang, A. Sohrabi, A. Ehsanipour, S. Sun, J. Liang, C. M. Walthers, L. Ta, D. A. Nathanson and S. K. Seidlits, *Cancer Res.*, 2018, **78**, 1358–1370.

39 J. M. Heffernan, D. J. Overstreet, L. D. Le, B. L. Vernon and R. W. Sirianni, *Ann. Biomed. Eng.*, 2015, **43**, 1965–1977.

40 K. Ghosh, X. Z. Shu, R. Mou, J. Lombardi, G. D. Prestwich, M. H. Rafailovich and R. A. F. Clark, *Mol. Pharm.*, 2005, **11**, 2040–2050.

41 B. J. Engel, P. E. Constantinou, L. K. Sablatura, N. J. Doty, D. D. Carson, M. C. Farach-carson, D. A. Harrington and T. I. Zarembinski, *Adv. Healthc. Mater.*, 2015, **4**, 1664–1674.

42 E. L. S. Fong, M. Martinez, J. Yang, A. G. Mikos, N. M. Navone, D. A. Harrington and M. C. Farach-carson, *Mol. Pharm.*, 2014, **11**, 2040–2050.

43 Y. Liu, X. Z. Shu and G. D. Prestwich, *Tissue Eng.*, 2007, **13**, 1091–1101.

44 D. M. Leite, B. Zvar, M. Gumbleton and G. J. Pilkington, *FASEB J.*, 2020, **34**, 1710–1727.

45 S. Soker and N. Carolina, *Biotechnol. Bioeng.*, 2020, **113**, 2020–2032.

46 C. Jiguet, N. Baeza-kallee and E. Denicolaï, *Exp. Cell Res.*, 2014, **321**, 99–108.

47 H. Sivakumar, R. Strowd and A. Skardal, *Gels*, 2017, **3**, 1–18.

48 X. Xu, L. A. Gurski, C. Zhang, D. A. Harrington, M. C. Farach-carson and X. Jia, *Biomaterials*, 2012, **33**, 9049–9060.

49 X. Xu, C. R. Sabanayagam, D. A. Harrington, M. C. Farach-carson and X. Jia, *Biomaterials*, 2014, **35**,

3319–3330.

50 C. Fu, H. Li, N. Li, X. Miao, M. Xie, W. Du and L. Zhang, *Carbohydr. Polym.*, 2015, **128**, 163–170.

51 K. Xu, H. Yao, D. Fan, L. Zhou and S. Wei, *Carbohydr. Polym.*, 2021, **254**, 117286.

52 S. S. Rao, J. Dejesus, A. R. Short, J. J. Otero, A. Sarkar and J. O. Winter, *ACS Appl. Mater. Interfaces*, 2013, **5**, 9276–9284.

53 S. Pedron, E. Becka and B. A. Harley, *Adv. Mater.*, 2014, **27**, 1567–1572.

54 M. Tang, Q. Xie, R. C. Gimple, Z. Zhong, T. Tam, J. Tian, R. L. Kidwell, Q. Wu, B. C. Prager, Z. Qiu, A. Yu, Z. Zhu, P. Mesci, H. Jing, J. Schimelman and P. Wang, *Cell Res.*, 2020, **30**, 833–853.

55 K. Yue, G. Trujillo-de Santiago, M. M. Alvarez, A. Tamayol, N. Annabi and A. Khademhosseini, *Biomaterials*, 2015, **73**, 254–271.

56 W. Xu, J. Qian, Y. Zhang, A. Suo, N. Cui, J. Wang, Y. Yao and H. Wang, *Acta Biomater.*, 2016, **33**, 131–141.

57 M. T. Ngo and B. A. Harley, *Adv. Healthc. Mater.*, 2017, **6**, 1700687.

58 S. Pedron, J. S. Hanselman, M. A. Schroeder, J. N. Sarkaria and B. A. C. Harley, *Adv. Healthc. Mater.*, 2017, **6**, 1700529.

59 S. Pedron, G. L. Wolter, J. E. Chen, S. E. Laken, J. N. Sarkaria and B. A. C. Harley, *Biomaterials*, 2019, **219**, Article No: 119371.

60 S. Pedron, H. Polishetty, A. M. Pritchard, B. P. Mahadik, C. R. Woese and G. Biology, *MRS Commun.*, 2017, **7**, 442–449.

61 N. K. Narayanan, B. I. N. Duan, J. T. Butcher, A. Mazumder and B. A. Narayanan, *In Vivo (Brooklyn).*, 2014, **28**, 67–74.

62 M. Alonso-nocelo, M. Theresa and K. H. Vining, *Biomed. Mater.*, 2017, **12**, 025009.

63 J. Vasudevan, C. T. Lim and J. G. Fernandez, *Adv. Funct. Mater.*, 2020, **30**, 2005383.

64 J. E. Chen, S. Pedron, P. Shyu, Y. Hu, J. N. Sarkaria and B. A. C. Harley, *Front. Mater.*, 2018, **5**, Article 39.

65 J. E. Chen, S. Pedron and B. A. C. Harley, *Macromol. Biosci.*, 2017, **17**, 1700018.

66 B. Harley and H. R. Gaskins, *Biomater. Sci.*, 2018, **6**, 854–862.

67 S. Pedron, E. Becka and B. A. C. Harley, *Biomaterials*, 2013, **34**, 7408–7417.

68 M. G. Ondeck, A. Kumar, J. K. Placone, C. M. Plunkett, B. F. Matte, K. C. Wong, L. Fattet, J. Yang and A. J. Engler, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 3502–3507.

69 E. Maloney, C. Clark, H. Sivakumar, K. Yoo, J. Aleman, S. A. P. Rajan, S. Forsythe, A. Mazzocchi, A. W. Laxton, S. B. Tatter, R. E. Strowd, K. I. Votanopoulos and A. Skadral, *Micromachines*, 2020, **11**, 208.

70 K. Tomihata and Y. Ikada, *J. Biomed. Mater. Res.*, 1997, **37**, 243–251.

71 M. Madaghiele, A. Maffezzoli, L. Ambrosio and L. Nicolais, *Polymer (Guildf).*, 2005, **46**, 11206–11212.

72 P. Bulpitt and D. Aeschlimann, *J. Biomed. Mater. Res.*, 1999, **47**, 152–169.

73 L. Zhang, K. Li, W. Xiao, L. Zheng, Y. Xiao, H. Fan and X. Zhang, *Carbohydr. Polym.*, 2011, **84**, 118–125.

74 C. Hsieh, Y. Huang, M. Sheu and H. Ho, *Int. J. Biol. Macromol.*, 2014, **64**, 45–52.

75 S. Jin, Y. Jeong, S. Jung, H. Ryu, Y. Jin and I.-Y. Kim, *J. Korean Neurosurg. Soc.*, 2009, **46**, 472–478.

76 L. David, V. Dulong, D. Le Cerf, C. Chauzy, V. Norris, B. Delpech, M. Lamacz and J.-P. Vannier, *Matrix Biol.*, 2004, **23**, 183–193.

77 L. David, V. Dulong, B. Coquerel, D. Le Cerf, L. Cazin, M. Lamacz and J.-P. Vannier, *Cell Prolif.*, 2008, **41**, 348–364.

78 L. David, V. Dulong, D. Le Cref, L. Cazin, M. Lamacz and J.-P. Vannier, *Acta Biomater.*, 2008, **4**, 256–263.

79 S. Otto and J. B. F. N. Engberts, *Org. Biomol. Chem.*, 2003, **1**, 2809–2820.

80 X. Z. Shu, Y. Liu, Y. Luo, M. C. Roberts and G. D. Prestwich, *Biomacromolecules*, 2002, **3**, 1304–1311.

81 C. B. Highley, G. D. Prestwich and J. A. Burdick, *Curr. Opin. Biotechnol.*, 2016, **40**, 35–40.

82 H. Yao, J. Wang and S. Mi, *Polymers (Basel).*, 2018, **10**, 1–27.

83 C. M. Nimmo, S. C. Owen and M. S. Shoichet, *Biomacromolecules*, 2011, **12**, 824–830.

84 S. C. Owen, S. A. Fisher, R. Y. Tam, C. M. Nimmo and M. S. Shoichet, *Langmuir*, 2013, **29**, 7393–7400.

85 A. E. G. Baker, R. Y. Tam and M. S. Shoichet, *Biomacromolecules*, 2017, **18**, 4373–4384.

86 M. Kurisawa, E. Chung, Y. Yang, J. Gao and H. Uyama, *Chem. Commun.*, 2005, **41**, 4312–4314.

87 F. Lee, J. E. Chung and M. Kurisawa, *Soft Matter*, 2008, **4**, 880–887.

88 I. Morath, T. N. Hartmann and V. Orian-rousseau, *Int. J. Biochem. Cell Biol.*, 2016, **81**, 166–173.

89 W. Li, H. Ma, J. Zhang, L. Zhu, C. Wang and Y. Yang, *Sci. Rep.*, 2017, **7**, Article number: 13856.

90 S. Tan, A. Yamashita, S. J. Gao and M. Kurisawa, *Acta Biomater.*, 2019, **94**, 320–329.

91 K. Zaviskova, D. Tukmachev, J. Dubisova, I. Vackova, A. Hejcl, J. Bystronova, M. Pravda, I. Scigalkova, R. Sulakova, V. Velebny, L. Wolfova and S. Kubinova, *J. Biomed. Res. Part A*, 2018, **106**, 1129–1140.

92 A. Suo, W. Xu, Y. Wang, T. Sun, L. Ji and J. Qian, *Carbohydr. Polym.*, 2019, **211**, 336–348.

93 J. Lou, R. Stowers, S. Nam, Y. Xia and O. Chaudhuri, *Biomaterials*, 2017, **154**, 213–222.

94 L. A. Gurski, A. K. Jha, C. Zhang, X. Jia and M. C. Farach-carson, *Biomaterials*, 2009, **30**, 6076–6085.

95 S. J. Florczyk, K. Wang, S. Jana, D. L. Wood, S. K. Sytsma, J. G. Sham, F. M. Kievit and M. Zhang, *Biomaterials*, 2013, **34**, 10143–10150.

96 T. B. Ngo, B. S. Spearman, N. Hlavac and C. E. Schmidt, *ACS Biomater. Sci. Eng.*, 2020, **6**, 6819–6830.

97 D. Cross, X. Jiang, W. Ji, W. Han and C. Wang, *Macromol. Biosci.*, 2015, **15**, 668–681.

98 S. Fujita, S. Hara, A. Hosono, S. Sugihara, H. Uematsu and S.-I. Suye, *Adv. Polym. Technol.*, 2020, **2020**, 1–7.

99 F. S. Palumbo, G. Pitarresi, A. Albanese, F. Calascibetta and G. Giammona, *Acta Biomater.*, 2010, **6**, 195–204.

100 C. B. Rodell, A. L. Kaminski and J. A. Burdick, *Biomacromolecules*, 2013, **14**, 4125–4134.

101 J. E. Mealy, C. B. Rodell and J. A. Burdick, *J. Mater. Chem. B*, 2015, **3**, 8010–8019.

102 S. Federico, U. Nöchel, C. Löwenberg, A. Lendlein and A. T. Neffe, *Acta Biomater.*, 2016, **38**, 1–10.

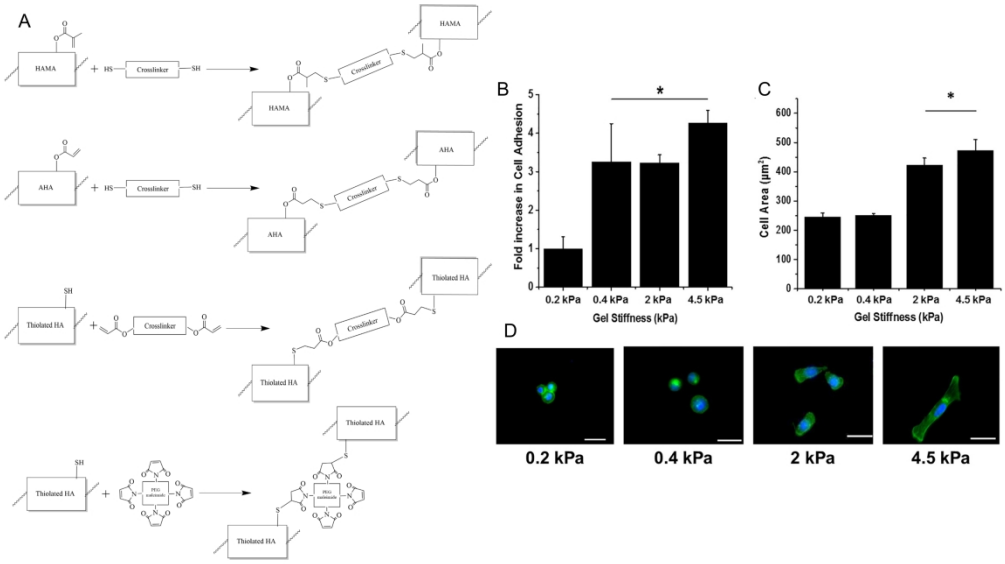

Figure 1: Michael type addition reactions to fabricate HA based hydrogels. (A) Schematic of the Michael type addition reactions employing methacrylated HA (HAMA), acrylated HA (AHA) and thiolated HA. HA hydrogels fabricated using methacrylated HA were employed to demonstrate that cell adhesion (B) and spreading (C) of brain metastatic breast cancer cells (MDA-MB-231Br) increases with HA hydrogel stiffness (*p < 0.01 compared to 0.2 kPa condition). (D) F-actin staining of cells on HA hydrogels with varying stiffness (Green = F-actin, Blue = DAPI, Scale bar = 50 μm). B, C, and D taken from 33 and reprinted with permission of Wiley Online Library (John Wiley and Sons).

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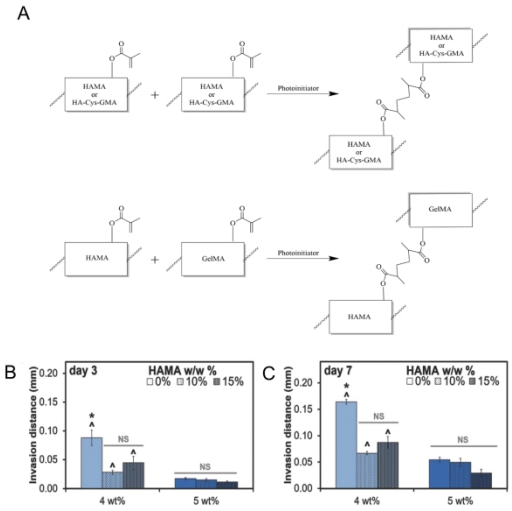

Figure 2: Photo-crosslinking to fabricate HA based hydrogels. (A) Schematic of the photo-crosslinking reactions employing methacrylated HA (HAMA) and/or methacrylated Gelatin (GelMA). HA based hydrogels fabricated via photo-crosslinking were employed to study invasion of encapsulated U251MG glioblastoma cells. Hydrogels were fabricated by using two different concentrations of GelMA (4 and 5 wt%) and HAMA was incorporated at 0, 10, 15 w/w% followed by utilization of LAP photoinitiator to fabricate HA-based hydrogels. Average invasion distance of U251MG cells at (B) day 3 and (C) day 7. Invasion distance was significantly increased in 4 wt% GelMA and invasion decreased with increasing HA in the matrix (^$p < 0.05$ compared to hydrogels with 5 wt% GelMA and similar HA content, *$p < 0.05$ compared to 10 and 15 w/w% HAMA). B, and C taken from 65 and reprinted with permission of Wiley Online Library (John Wiley and Sons).

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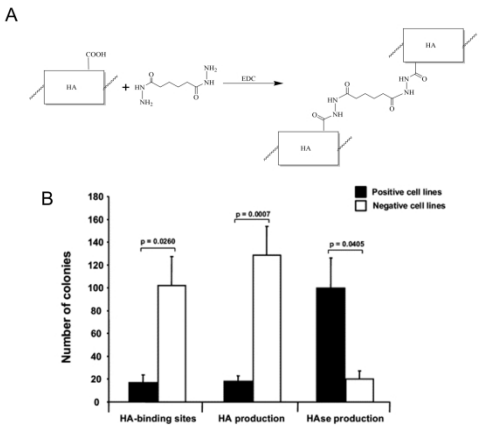

Figure 3: Carbodiimide chemistry to fabricate HA based hydrogels. (A) Schematic of the carbodiimide chemistry employing adipic dihydrazide. HA hydrogels fabricated via carbodiimide chemistry were employed to study invasion (measured via number of colonies) of various cancer cells grouped via positivity or negativity for HA/HAase production, and HA-binding sites (B). B taken from 76 and reprinted with permission of Elsevier.

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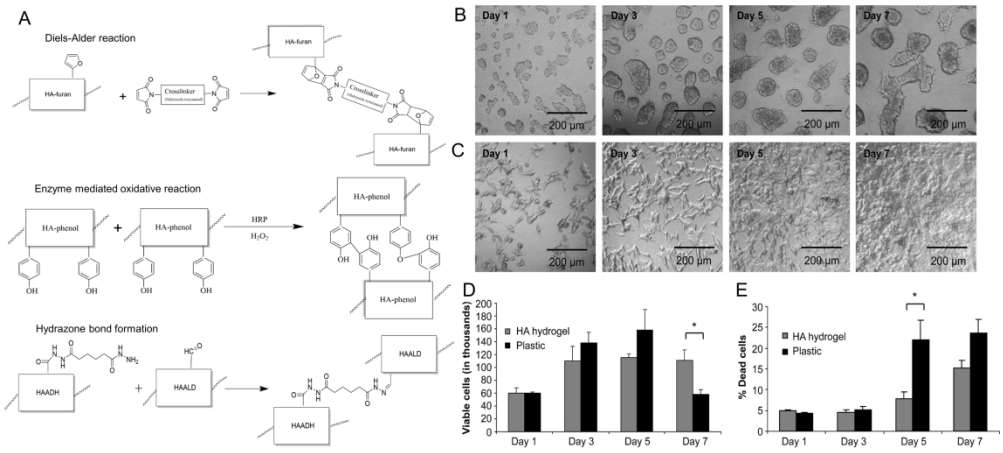

Figure 4: Diels-Alder and other chemistries to fabricate HA based hydrogels. (A) Schematic of Diels-Alder chemistry (employing HA-furan), enzyme mediated oxidative reaction (employing HA-phenol), and hydrazone bond formation (employing HA-aldehyde (HAALD) and HA-adipic dihydrazide (HAADH)) to fabricate HA based hydrogels. HA hydrogels fabricated using HAALD and HAADH were used to encapsulate bone metastatic prostate cancer cells (C4-2B) which formed clusters (B) similar to the morphologies observed in vivo compared to traditionally studied 2D TCPS (C). These hydrogels also supported high cell viability (*$p < 0.05$) (D, E). B, C, D, and E taken from 94 and reprinted with permission of Elsevier.

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Table 1: Summary of studies using HA based hydrogels fabricated by a Michael addition reaction to study cancer cell behaviors.

HA Modification	Other Component (s)	Crosslinking Method or Crosslinker(s)	Cancer Type	Cancer Cell Line	Cancer Cell Behavior(s) Examined	Reference
Methacrylated HA	NA	DTT	Brain cancer	U373-MG U87-MG	Cell morphology, spreading, adhesion, and invasion	[16]
Methacrylated HA	NA	DTT	Brain cancer	U373-MG U87-MG	Cell morphology, spreading, motility, and invasion	[30]
Methacrylated HA	NA	DTT	Brain cancer	U373-MG U87-MG	Cell adhesion and migration	[31]
Methacrylated HA	NA	DTT	Brain cancer	U87	Cell proliferation and apoptosis Gene expression (Nestin, SOX2, CD133)	[32]
Methacrylated HA	NA	DTT	Brain metastatic breast cancer	MDA-MB-231Br	Cell morphology, adhesion, spreading, proliferation, and migration	[33]
Methacrylated HA	NA	DTT	Brain metastatic breast cancer	MDA-MB-231Br	Cell proliferation and dormancy	[34]
Methacrylated HA	NA	DTT	Brain metastatic breast cancer	MDA-MB-231Br	Cell proliferation and dormancy	[35]
Acrylated HA	NA	MMP crosslinker (GCRDGPQGWGQDRCG)	Fibrosarcoma	HT1080	Cell proliferation, apoptosis, and invasion Gene expression (MMP-1, VEGF and ANG-1)	[36]
Thiolated HA	PEG maleimide-peptide 4-arm-PEG-SH	Self-gelation by crosslinking between thiol groups and 4-arm-PEG-SH	Brain cancer	K301 BM6 GS024 GS025	Cell adhesion and proliferation Drug resistance	[37]

Thiolated HA	PEG maleimide-peptide 4-arm-PEG-SH	Self-gelation by crosslinking between thiol groups and 4-arm-PEG-SH	Brain cancer	GBM39 HK301 HK423	Cell proliferation and apoptosis Drug resistance	[38]
Thiolated HA	Thiolated Gelatin	PEGDA	Prostate cancer	C4-2B	Cell morphology, viability, and growth	[41]
Thiolated HA	NA	PEGDA	Prostate cancer	MDA PCa 183 MDA PCa 118b	Cell viability and growth	[42]
Thiolated HA	Thiolated Gelatin	PEGDA	Breast cancer Ovarian cancer Colon cancer	MDA-MB-231, MDA-MB-468 SK-OV-3, OVCAR-3 CaCo-2, HCT-116	Cell proliferation	[43]
Thiolated HA	Thiolated Gelatin	PEGDA	Brain cancer	U118 U87R	Cell proliferation and invasion	[39]
Thiolated HA	Thiolated Gelatin	PEGDA	Brain cancer	U87MG SNB-19 UP-007	Cell viability and proliferation	[44]
Thiolated HA	Thiolated Gelatin	PEGDA 4-arm-PEG-Acrylate 8-arm-PEG-Acrylate Initiator: Irgacure 2959	Colon cancer	HCT-116	Cell spreading and migration Gene expression (ZO-1, β-Catenin, MMP-9, N-Cadherin, PCNA)	[45]
Thiolated HA	Thiolated Gelatin	PEGDA	Brain cancer	U87MG	Cell morphology and proliferation	[46]
Thiolated HA	Thiolated Gelatin	PEGDA 4-arm PEG linker Initiator: Irgacure 2959	Brain cancer	U373 A172S U87 U87 EGFRvIII	Cell proliferation	[47]
Acrylated HA Thiolated HA	NA	Self-gelation	Prostate cancer	LNCaP	Cell apoptosis Gene expression (MRP-1, LRP)	[49]
Acrylated HA Thiolated HA	NA	Self-gelation	Prostate cancer	LNCaP	Cell viability, morphology, and proliferation Gene expression (HYAL-1, VEGF, and IL-8)	[48]

Abbreviations:

DTT: Dithiothreitol, PEGDA: Polyethylene glycol diacrylate, 4-arm-PEG-SH: Thiol-terminated 4arm-polyethylene glycol, MMP-1: Matrix metalloproteinase-1, MMP-9: Matrix metalloproteinase-9, VEGF: Vascular endothelial growth factor, ANG-1: Angiopoietin-1, ZO-1: Zonula occludens-1, β-Catenin: Beta catenin, N-Cadherin: Neural cadherin, PCNA: Proliferating cell nuclear antigen, MRP-1: Multidrug resistance-associated protein-1, LRP: Low density lipoprotein receptor-related protein, HYAL-1: Hyaluronidase-1, IL-8: Interleukin-8.

Table 2: Summary of studies using HA and HA containing hydrogels fabricated via photo-crosslinking to study cancer cell behaviors.

HA Modification	Other Component (s)	Photoinitiator	Cancer Type	Cancer Cell Line	Cancer Cell Behavior Examined	Reference
HA-Cys-GMA	N/A	Irgacure 2959	Breast cancer	MCF-7	Cell attachment, morphology, viability, proliferation, migration and invasion Growth factor expression (VEGF, bFGF and IL-8)	[56]
Methacrylated HA	GelMA	LAP	Brain cancer	GBM39	Cell invasion, metabolic activity	[64]
Methacrylated HA	GelMA	Irgacure 2959	Brain cancer	U87MG	Gene expression (VEGF, FN, HIF-1, MMP-2 and MMP-9)	[53]
Methacrylated HA	GelMA	LAP	Brain cancer	U87MG	Cell morphology, proliferation, and invasion	[57]
Methacrylated HA	GelMA 4-arm-PEG	Irgacure 2959	Brain cancer	U87MG	Cell morphology, viability and proliferation	[67]
Methacrylated HA	GelMA	LAP	Brain cancer	U251MG	Cell invasion, metabolic activity	[65]
Methacrylated HA	GelMA	Irgacure 2959	Brain cancer	GBM6 GBM10 GBM12	Gene expression (VEGF, FN, HIF-1, MMP-2 and MMP-9) Cell metabolic activity	[58]
Methacrylated HA	GelMA	LAP	Brain cancer	GBM U87 GBM U87 EGFR	Cell viability, metabolic activity, and invasion	[66]
Methacrylated HA	GelMA	LAP	Brain cancer	GBM6 GBM12	Cell proliferation and invasion	[59]
Thiolated HA	Methacrylated Collagen	Irgacure 2959	Liver cancer Colorectal cancer	HepG2 Caco2	Cell viability and proliferation	[69]

Abbreviations:

HA-Cys-GMA: Hyaluronic acid- Cystamin- Glycidyl methacrylate, GelMA: Gelatin Methacrylate, LAP: Lithium Acylphosphinate, PEG: Polyethylene Glycol, VEGF: Vascular endothelial growth factor, bFGF: Basic fibroblast growth factor, IL-8: Interleukin-8, FN: Fibronectin, HIF-1: Hypoxia-inducible factor-1, MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix metalloproteinase-9.

Table 3: Summary of studies using HA hydrogels fabricated via EDC chemistry to study cancer cell behaviors.

HA Modification	Additional Components Used	Cancer Type	Cell line	Crosslinking Method or Crosslinker(s)	Cancer cell behavior(s) examined	Reference
NA	NA	Brain cancer	U87 MG U251 MG U343MG-A U373MG	Adipic dihydrazide	Cell migration and invasion	[75]
NA	Collagen	Brain cancer, Gastric adenocarcinoma brain metastases Gastric adenocarcinoma hepatic metastases	CB191 SA87 SA87M1 SA87M2	Adipic dihydrazide	Cell adhesion and invasion Gene expression (MMP-2 and MMP-9)	[77]
NA	NA	Lung carcinoma Gastric adenocarcinoma brain metastases	NCI-H460M, NCI-H460 SA87	Adipic dihydrazide	Cell invasion and proliferation	[78]
NA	NA	Breast adenocarcinoma pleural metastases Prostate cancer, Hepatoma Lung carcinoma Brain cancer Gastric adenocarcinoma brain metastases Gastric adenocarcinoma hepatic metastases Osteosarcoma	CAL51 PC3 PLC/PRF/5 NCI-H460 NCI-H460M CB191 CB193 SA87 CAL51M PC3MH1 SA87M1 SA87M2 SaOs	Adipic dihydrazide	Cell invasion Gene expression (CD44)	[76]

Abbreviations:

MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix metalloproteinase-9.

Table 4: Summary of studies using HA hydrogels synthesized via Diels-Alder chemistry and other methods to study cancer cell behaviors.

HA Modification	Additional component(s)	Crosslinking Method or Crosslinker(s)	Cancer Type	Cell Line	Cancer Cell behaviors examined	Reference
HA-furan	GRGDS	Diels-Alder, Crosslinker: MMP cleavable peptide (GPQG↓IWGQ) (maleimide-terminated peptide)	Breast cancer	MDA-MB-231 MCF-7 SK-MEL-28 T-47D	Cell proliferation and invasion	[9]
HA-MF-dma HA-MF-Ald	Mal-IKVAV-L_6 peptide Mal-YIGSR-L_6 peptide RGD-L_6-Mal peptide	Diels-Alder, Crosslinker: Bis(maleimide)-PEG and bis(oxyamine)-PEG	Breast cancer	T47D	Cell morphology and proliferation	[85]
HA-Ph	NA	Enzyme mediated oxidative reaction Catalyst: HRP and H_2O_2	Breast cancer	4T1 MCF-7 HCC1937 MDA-MB-231	Cell attachment, viability, Gene expression (OCT-4, ALDH-1)	[90]
NA	Chitosan	Formation of polyelectrolyte complex through ionic bonding	Brain Cancer	U118 MG	Cell viability, growth, proliferation, and invasion Gene expression (Nestin, CD44, Musashi, GFAP, MMP-2, MMP-9, and TWIST-1)	[95]
AHA GHHA	NA	Dual crosslinking: Formation of hydrozane bonds and photo-crosslinking by Irgacure 2959	Breast Cancer	MCF-7	Cell viability, migration, and invasion Gene expression (VEGF, bFGF, IL-8)	[92]
HAALD HAADH	NA	Self-gelation by formation of hydrozane bonds	Prostate cancer	LNCaP C4 C4-2 C4-2B	Cell invasion Gene expression (RHAMM)	[12]

HAALD HAADH	NA	Self-gelation by formation of hydrozane bonds	Prostate cancer	C4-2B	Cell morphology, growth, viability, and apoptosis	[94]

Abbreviations:

HA-Ph: Hyaluronic Acid -Phenol (HA-Phenol), GRGDS: glycyl-arginyl-glycyl-aspartyl-serine, HA-MF-dma: Methyl furan/dimethoxy acetal substituted hyaluronan, HA-MF-Ald: Methylfuran/aldehyde-substituted hyaluronan, PEG: Polyethylene glycol, AHA: Hyaluronic Acid-derived polyvalent aldehyde (oxidized HA), GHHA: Hyaluronic Acid-derived polyvalent hydrazide (glycidyl methacrylated 3,3′-dithiobis(propionic hydrazide) functionalized HA), HAALD: Hyaluronic Acid aldehyde, HAADH: Hyaluronic Acid adipic dihydrazide, HRP: Horseradish peroxidase, OCT4: Octamer-binding transcription factor-4, ALDH-1: Aldehyde Dehydrogenase-1, GFAP: Glial fibrillary acidic protein, MMP-2: Matrix metalloproteinase-2, MMP-9: Matrix metalloproteinase-9, TWIST-1: Twist-related protein-1, VEGF: Vascular endothelial growth factor, bFGF: Basic fibroblast growth factor, IL-8: Interleukin-8, RHAMM: Receptor for hyaluronan-mediated motility.