GLYCOSAMINOGLYCAN-BASED MATERIALS AS AN ENGINEERED BIOCOMPATIBLE CELLULAR MATRIX

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Abstract

Disclosed herein is a cross-linked polymeric system comprising thiolated hyaluronic acid (HA), thiolated chondroitin sulfate (CS), and functionalized polyethylene glycol (PEG), wherein said functionalized PEG cross-links thiolated HA and thiolated CS. Methods of fabrication and utilization of the same are also claimed. This polymeric system may be used as an engineered biocompatible cellular matrix for 3D cell culture, tissue engineering and regenerative medicine applications.
Figure 2
Figure 3
Figure 9A

Figure 9B

Figure 9
Harvest hMSCs from 2D and gently mix with the hydrogel precursor solution

Transfer to pre-cut syringe tip molds

Incubate in the culture hood for crosslinking

Plate cell-laden hydrogels

Culture

hMSCs in 2D plate

Analysis: viability
Actin staining

Figure 14
Figure 17
Cross-linker: 4 arm PEG

\[ \text{PEG} = \left( \text{CH}_2 - \text{CH}_2 - \text{O} \right)_n \]
FIG. 27A

FIG. 27B

FIG. 27C

Figure 27
FIG. 29A

Normalized fluorescence lifetime fold changes

PEG3400
PEGDA3400

Time (min)

FIG. 29B

Normalized fluorescence lifetime fold changes

Time (min)

Figure 29
Figure 30
GLYCOSAMINOGLYCAN-BASED MATERIALS AS AN ENGINEERED BIOMATERIAL CELLULAR MATRIX

CROSS REFERENCE


TECHNICAL FIELD

[0002] The present disclosure generally relates to engineered biocompatible cellular matrices, and in particular to polymeric systems that may be used as engineered biocompatible cellular matrices for 3D cell culture, tissue engineering and regenerative medicine applications.

BACKGROUND

[0003] This section introduces aspects that may help facilitate a better understanding of the disclosure. Accordingly, these statements are to be read in this light and are not to be understood as admissions about what is or is not prior art.

[0004] A biomaterial-based three-dimensional (3D) cell microenvironment (niche) is often designed to mimic a natural extracellular matrix (ECM) and support cell growth, differentiation, and organization to form regenerated tissue [1]. Hydrogels composed of polymer networks swollen in water provide an attractive biomaterial platform to provide ECM-mimicking and cell-interactive properties [2]. The regenerative efficacy is greatly dependent on precise control of material properties and optimization of cell-material interactions. For example, mesenchymal stem cells (MSCs) can sense the differences in mechanical environments such as stiffness and initiate the mechanotransduction pathways, which ultimately affect cell fate and distinct lineage differentiation [3, 4].

[0005] Glycosaminoglycans (GAGs) constitute an important part of the ECM. GAGs may be divided into hyaluronic acid (HA) and sulfated GAGs including chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin sulfate, and heparan sulfate (HS). HA is not linked to proteins, while sulfated GAGs in nature are covalently linked to proteins. HA is an immunoneutral polysaccharide that includes alternating disaccharide units of [β(1,4)-D-glucuronic acid-β(1,3)-N-acetyl-D-glucosamine] linkages [5] and is widely distributed throughout the body, especially in the synovia of joints, the corpus vitreum of the eyes, and the dermis of the skin. HA is predominantly localized to the extracellular and pericellular matrix. Functionally, HA contributes to the elastoviscosity of fluid connective tissues including synovial fluid and vitreous humor, and it modulates hydration and transport of water through tissues [6]. HA also plays an important role in many biological processes including facilitating wound healing [7], regulating cell adhesion and proliferation [8], cell motility, angiogenesis, cellular signaling, and matrix organization [9]. The enzymatic degradation of HA results from the action of three types of enzymes: hyaluronidase, β-D-glucuronidase, and β-N-acetylhexosaminidase [10]. Inherently biocompatible and biodegradable [5], HA has been used clinically for more than thirty years [5, 11, 12]. For example, it has been approved by the US Food and Drug Administration for the treatment of osteoarthritis in humans since 1997 [13]. Therefore, hyaluronic acid (HA) has attracted significant research interests to develop hydrogels because it offers both a biomimetic microenvironment found in natural ECM and the availability of carboxylic acids and hydroxyl groups necessary for modification [5].

[0006] Chondroitin sulfate (CS), a polysaccharide composed of alternating units of β-1, 4-linked glucuronic acid and β-1, 3-N-acetylgalactosamine, is sulfated on either the 4 or 6 position of the galactosamine residues. CS is the major glycosaminoglycan (GAG) in the ECM of all vertebrates and is found in bone, cartilage, and connective tissues [14]. GAGs in normal human meniscal tissues include 60% chondroitin-6-sulfate and 10-20% chondroitin-4-sulfate [15]. CS is an appealing material in tissue regeneration due to its biological implications in supporting cellular activities in the ECM [16, 17], as well as anti-inflammatory and wound healing properties [4, 18, 19].

[0007] However, hydrogels derived from either HA or CS suffer from limited mechanical properties and uncontrollable degradation rates, which necessitates the need to combine GAGs with other polymers [20, 21]. Here, we developed a novel GAG-based hydrogel system as a tunable cell niche through combination of functional polysaccharides and a cross-linker. GAG hydrogels may be used for tissue engineering due in part to their ability to efficiently encapsulate cells. Mechanical and structural properties, and cell-material interactions may be manipulated by modification of the crosslinking density and structures of polysaccharides and a cross-linker. In some cases, cross-linked polymers or gels may have a high, tissue-like water content, which may allow nutrient and waste transport.

SUMMARY

[0008] This disclosure provides a composition comprising thiolated hyaluronic acid (HA), thiolated chondroitin sulfate (CS) and functionalized polyethylene glycol (PEG) derivative, wherein said PEG derivative cross-links thiolated HA and thiolated CS.

[0009] In some preferred embodiment, the aforementioned functionalized PEG derivative contains a plurality of activated vinyl groups.

[0010] In some embodiment the aforementioned composition has a constant molecular ratio of the total thiol group of HA and CS vs. the activated vinyl group. In some preferred embodiment, the molecular ratio of the total thiol groups vs. the activated vinyl group is about 1.07.

[0011] In some preferred embodiment, the aforementioned activated vinyl group is selected from the group consisting of poly (ethylene glycol) diacrylate (PEGDA), poly (ethylene glycol) Divinyl Sulfone (PEGVS), and 4-arm poly (ethylene glycol) vinyl Sulfone (4PEGVS).

[0012] In some preferred embodiment the aforementioned thiolated HA further comprising a plurality of amino groups (—NH2)) selected from the following formula I-IV:
wherein R₁, R₂, R₃, R₄, and R₅ may include any one of or a combination of halogenated, dihalides, amines, thiols, carboxylic acids, aldehydes, ketones, active hydrogen sites on aromatic ring, dienes, azide isothiocyanates, isocyanates, acyl azides, N-hydroxysuccinimide (NHS) esters, sulfo-NHS, sulfonate chloride, epoxides, carbonates, aryl halides, imidoesters, carbodiimides (e.g. N, N'-dicyclohexylcarbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)), alkylphosphate compounds, anhydrides, fluoroaryl esters, hydroxymethyl phosphonates, guanidino groups, isoosacetyl derivatives, maleimides, aziridines, acryloyl derivatives, arylation agents, diazido-derivatives, vinylsulfone, phenylthioethers, cisplatins, diazoacetates, carbonyl dichlorides, oxiranes, N, N'-disuccinimidyl carbonates, N-hydroxysuccinimimidyl chloroformates, alkyl halogens, hydrazines, alkynes, and phosphorus-bound chloride.

This disclosure further provides an osteochondral regenerative engineering composite comprising poly (lactide-co-glycolide) (PLGA) grafted to aforementioned composition via —NH₂ group to form a bone mimetic.

In some preferred embodiment, the aforementioned PLGA is made of lactic acid/glycolic acid at a ratio of about 85:15.

In some preferred embodiment, the molecular weight and length of aforementioned PEG derivatives are adjustable to modify the composite modular storage and loss value.

In some preferred embodiment, the aforementioned PEG derivatives have molecular weight ranging from about 700 Da to about 8000 Da.

This disclosure further discloses a method of making a composition of cross-linked HA, CS and PEG, the method comprising the steps of:

- preparing thiolated HA;
- preparing thiolated CS;
- preparing PEG derivative containing a plurality of activated functionalities;
- mixing said HA, CS, and PEG derivative in an aqueous medium to crosslink HA, CS;
- wherein the activated functionality is selected from the group consisting of alkoxysulfonyl, arylation sulfonyl, heteroaryl sulfonyl, maleimido, ether NHS esters, and sulfo-NHS;
- wherein the structures of the cross-linker comprise any one of or a combination of linear, dendrimer-like, star-shaped, hyper-branched, combed, brushed, cross-linked architectures, fibers, microspheres, and nanoparticles.

In some preferred embodiment, the aforementioned activated functionalities of PEG comprises any one of or a combination of isothiocyanate, isourea, amide, sulfoamide, secondary amine, sulfonyamide, shift-base, secondary amine-methyl, carbamate, aryl amine, amidine, amide, phosphoramidate, guanidine, substituted imidocarbonate, thioether, 4-amin derivative of cytosome, aryl thiocarbonyl, sulfonyl, benzotriazole, ester, carbamate, hydrazide, dizzo, triazoles, iodinated compound, carbohydrates, amino acid esters bond, cycloalkene, oxime triazole, and triazolone.

In some preferred embodiment, the aforementioned engineered composite further comprising peptides selected from the group consisting of arginine-glycine-aspartate (RGD), fibronectin, laminin, and fibronogen, wherein said peptides comprising amine-functionalized laminin, carboxyl functionalized laminin, amine functionalized RGD, and thiolated RGD. These peptides can be conjugated with HA, CS, or functionalized PEG (e.g., PEGDA, PEGDS, 4PEGVS) through thiol-ene click reactions and esterification.

In some preferred embodiment, the aforementioned composition further comprising cells selected from the group consisting of mesenchymal stem cells, osteoblasts, chondrocytes, adipocytes, fibroblasts, hepatocytes, enterocytes, urothelial cells, blood cells, skin cells, endothelial cells, nerve cells, sex cells, cancer cells and combination thereof.

In some preferred embodiment, the aforementioned composition further comprising small molecules as therapeutic agents.

In some preferred embodiment, the aforementioned composition further comprising at least one growth factor.

These and other features, aspects and advantages of the present invention will become better understood with reference to the following figures, associated descriptions and claims.

**BRIEF DESCRIPTION OF THE FIGURES**

**FIG. 1** shows the formation of GAG-based polymeric systems via covalent or non-covalent interactions.

**FIG. 2** shows functionalized monomeric polymeric hyaluronate represented by formula I, II, III, IV.

**FIG. 3** shows examples of covalent chemistries for crosslinking functionalized HA with a cross-linker.

**FIG. 4** shows schematic and structural representations showing cross-linker structures.

**FIG. 5** shows an example of non-covalent chemistries for crosslinking functionalized HA with a cross-linker.
[0035] FIG. 6 shows a schematic illustration of design of bio-inspired composite hydrogels as an engineered cell niche via thiol-ene click chemistry. Modulation of hydrogel properties enables regulation of cellular behavior.

[0036] FIG. 7 shows a biominetic Hydrogel Formation. Carboxyl groups in HA or CS were reacted with cystamine dihydrochloride by activation with EDC and NHS. Reduction of cystamine by DTT resulted in free thiol groups in the HA or CS backbone. Crosslinking of HA-SH and CS—SH mixtures by conjugate addition using PEGDA.

[0037] FIG. 8. FIG. (8A) H NMR spectrum confirms successful synthesis of HA-conjugated cystamines. Compared to the spectrum of HA (the N-acetyl methyl proton of HA were at δ 1.95), new resonances appeared at δ 2.53, 2.54, 2.77 and 2.90 corresponding to methylenes (—CH₂CH₂—). FIG. (8B) H NMR spectrum confirms successful synthesis of CS-conjugated cystamines. Compared to the spectrum of CS (the N-acetyl methyl proton of CS were at δ 1.95), new resonances appeared at δ 2.25, 2.55, 2.77 and 2.90 corresponding to methylenes (—CH₂CH₂—).

[0038] FIG. 9. FIG. (9A) H NMR spectrum confirms successful synthesis of HA-SH. FIG. (9B) H NMR spectrum confirms successful synthesis of CS—SH.

[0039] FIG. 10. FIG. (10A) The inversion of a hydrogel. FIG. (10B) Hydrogels in cell culture.

[0040] FIG. 11 shows Cryo SEM images of FIG. (11A), FIG. (11D) HCP 700, FIG. (11B), FIG. (11E) HCP 3400, and FIG. (11C), FIG. (11F) HCP8000 hydrogels.

[0041] FIG. 12 shows tunable HCP hydrogel properties. FIG. (12A) Gelation time. FIG. (12B) Swelling ratio. FIG. (12C) FTTC-dextran (70k) release from hydrogels as a function of time at pH 7.4, 37°C. Demonstrating the potential to achieve tunable release profiles of macromolecules.

[0042] FIG. 13 shows tunable mechanical properties. FIG. (13A) Strain sweeps of hydrogel HCP7000L. The linear viscoelastic limit was determined with respect to strain. Storage modulus (G') was determined from 0.01 to 10% strain. FIG. (13B) Frequency sweeps of hydrogel HCP7000. The linear modulus plateau (G') with respect to frequency was determined. FIG. (13C) Time evolution of hydrogel HCP7000L, HCP3400L, HCP8000L with time at 37°C. FIG. (13D) The rheological properties for hydrogels. Increasing the chain length of cross-linker (PEGDA) results in a stiffer gel.

[0043] FIG. 14 shows 3D encapsulation procedures.

[0044] FIG. 15. Live/Dead assay of hMSCs encapsulated in hydrogels after 1 day of culture (FIG. 15A, FIG. 15B) HCP7000 (FIG. 15B, FIG. 15F) HCP3400 (FIG. 15C, FIG. 15E) HCP8000 hydrogels.

[0045] FIG. 16. Live/Dead assay of hMSCs encapsulated in HCP3400 hydrogels after (FIG. 16A, FIG. 16C) 1 day of culture (FIG. 16D, FIG. 16D) 5 days of culture.

[0046] FIG. 17 shows actin staining of hMSCs encapsulated in hydrogels after 1 day of culture. Actin, red; nucleus, blue. FIG. (17A) HCP7000 (FIG. 17B) HCP3400 (FIG. 17C) HCP8000 hydrogels.

[0047] FIG. 18 shows stained hydrogels exhibiting increased focal adhesion kinase phosphorylation of hMSCs as evidenced from FLJM. FAK is an early mechanosensor that responds to changes in substrate stiffness.

[0048] FIG. 19. FIG. (19A) SEM image of dehydrated hydrogel HCP8000, 200 μm scale. Cell deposited matrix visible on surface. (FIG. 19B) SEM image of dehydrated hydrogel HCP8000 showing cell deposited matrix on surface, 50 μm scale. (FIG. 19C) SEM image of dehydrated hydrogel HCP8000 showing a porous structure appropriate for cell migration, 50 μm scale.

[0049] FIG. 20. Comparison of PEGVS and PEGDA. FIG. (20A) Time evolution of hydrogel HCPVS3400 with time at 37°C. The gelation time was 6.2 min. FIG. (20B) Storage modulus at the equilibrium stage. Due to more reactive vinyl sulfone moiety, PEGVS demonstrates significantly higher storage modulus compared to PEGDA.

[0050] FIG. 21. In vitro study. FIG. (21A) Live/Dead assay of primary chondrocytes encapsulated in HCPVS3400 hydrogels after 21 day of culture. FIG. (21B) Primary chondrocytes were isolated freshly from pig. At P2 stage, cells were encapsulated in the hydrogel and cultured for 21 days with DMEM supplemented with 20% FBS. Cells were labeled with actin (red) and aggrecan (green). Nuclei were counterstained with DAPI (blue).

[0051] FIG. 22. Mechanical properties of HA/CS/4 arm PEG (HC4PVS). FIG. (22A) Time evolution of hydrogel HC4PVS20000 with time at 37°C. The gelation time was 12 seconds. FIG. (22B) Storage modulus at the equilibrium stage of hydrogel HC4PVS20000 at day 1 and day 40.

[0052] FIG. 23. FIG. (23A) Live/Dead assay (FIG. 23B) Actin staining of hMSCs encapsulated in HC4PVS20000 hydrogels after 21 days of culture. Actin, red; nucleus, blue.


[0054] FIG. 25. SEM images of FIG. (25A), FIG. (25C) PLGA 3D scaffolds. FIG. (25B), FIG. (25D) PLGA 3D scaffold-hydrogel integration.

[0055] FIG. 26. Raman spectra of the HA-SH, CS—SH, PEGDA700, and hyphosphilized HCP700 hydrogel at 0 min and 60 min.

[0056] FIG. 27. Physical characterization of hydrogels: FIG. (27A) Plateau of G'. FIG. (27B) Gelation times determined by the vial tilting method in PBS at 37°C. FIG. (27C) Time sweep showing storage (G') and loss (G'') with increasing MW of crosslinker and high thiol degrees of substitution (i.e., HCP700, HCP3400 and HCP8000 hydrogels).

[0057] FIG. 28. Dynamic FAK phosphorylation dependent on hydrogel stiffness. FIG. (28A) Schematic illustration of real time measurement of FAK phosphorylation in hMSCs laden hydrogels. FIG. (28B) Quantitative analysis showed average fluorescence lifetime for hydrogels (n=26-50) with different plasmid G. FIG. (28C) Quantitative analysis shows EAKSOR average lifetime in hMSC-laden HCP7000 (n=20) and HCP700 hydrogels (n=50). At the same as total polymer percentage and MW of PEGDA, HCP 700L use low thiol substitution degree of HA-SH and CS—SH and HCP700 utilized high thiol substitution degree of HA-SH and CS—SH.

[0058] FIG. 29. FIG. (29A) Stiffened hydrogels exhibiting increased FAK phosphorylation of hMSCs encapsulated in 3400 hydrogels (n=3). FIG. (29B) FAKSOR average lifetime in hMSC-laden HCP3400 hydrogels remain constant over 30 minutes right after G' reached plateau (n=5).

[0059] FIG. 30. Quantitative Real Time PCR for (Bone morphogenetic protein 2) (BMP2), alkaline phosphatase (ALP), collagen (Col1), and osteonectin (ON) expression in the hydrogels with PEG700DA or PEGDA34000 as a crosslinker after 5 days in growth medium.
FIG. 31. hMSCs encapsulated in hydrogels after 3 day culture with RGD concentration of (FIG. 31A) 0.25 mM, (FIG. 31B) 0.5 mM and (FIG. 31C) 1 mM. Scale bar: 10 μm.

**DETAILED DESCRIPTION**

**0060** For the purposes of promoting an understanding of the principles of the present disclosure, reference will now be made to the embodiments illustrated in the drawings, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of this disclosure is thereby intended.

**0062** While the concepts of the present disclosure are illustrated and described in detail in the figures and the description herein, results in the figures and their description are to be considered as exemplary and not restrictive in character. It is understood that only the illustrative embodiments are shown and described and that all changes and modifications that come within the spirit of the disclosure are desired to be protected.

**0063** Unless defined otherwise, the scientific and technological nomenclature have the same meaning as commonly understood by a person in the ordinary skill in the art pertaining to this disclosure.

**0064** As disclosed herein, in at least one embodiment, a GAG-based polymeric system can be prepared by following two strategies: (i) chemical covalent bonding and (ii) physically entrapped and/or entangled (non-covalent) strategies as shown in FIG. 1.

**0065** In at least one embodiment, GAG polysaccharides can be modified in predictable synthetic routes to control the properties of the resulting materials, including modifications leading to hydrophobicity and biological activities. In part, the present disclosure provides for a composition comprising at least one monomeric unit of HA functionalized by at least one functional group moiety. Chemical modifications of HA can be targeted to three functional groups: the glucuronic acid carboxylic acid, the primary and secondary hydroxyl groups, and the N-acetyl group (following deamination). In some embodiments, compositions of HA in the present disclosure are provided that may be represented by Formula I, II, III and IV (FIG. 2).

**0066** Carboxylates in a HA backbone can be modified by carbodiimide-mediated reactions, esterification, and amidation. Hydroxyls in a HA backbone can be modified by etherification, divinylsulfone crosslinking, esterification, and bisepoxide crosslinking. Additionally, converting diols to aldehydes can be achieved through periodate oxidation of HA. Finally, decyclization of the N-acetyl group of HA recovers an amino group which can then react with an acid using the same modification.

**0067** The functional groups R₁, R₂, R₃, R₄, and R₅ may include any one of or a combination of haloacetates, dihydroxyls, amides, thiols, carboxylic acids, aldehydes, ketones, active hydrogen sites on aromatic ring, dienes, azide isothiocyanates, isocyanates, acyl azides, N-hydroxy succinimide (NHS) esters, sulfo-NHS, sulfonyl chloride, epoxides, carbonates, aryl halides, imidoesters, carbodimides (e.g. N, N′-diacylhexylcarbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)), alkylphosphate compounds, anhydrides, fluoroephyl esters, hydroxymethyl phosphines, guanidine groups, imidoacetyl derivatives, maleimides, aziridines, acryloyl derivatives, arylating agents, disulfide derivatives, vinylsulfone, phenylthioester, cisplatin, diazoacetate, carbonyl dimidazole, oxiranes, N,N′-disuccinimidyl carbones, N-hydroxysuccinimidyl chloroformates, alkyl halogens, hydrazines, alkynes, and phosphorus-bound chloride.

**0068** The functionalized polysaccharides may include the combination of HA and CS. HA may be used in an amount ranging from 0.1% to 99% by weight. HA has an average molecular weight in the range of 10-100 Kilo Daltons, preferably 1000-5000 Kilo Daltons. Sulfated GAGs may be used in an amount ranging from 0.1% to 99% by weight.

**0069** Cross-linkers can be both synthetic polymers and natural polymers. The natural polymers may include any one of or a combination of fibrin, collagen, matrigel, elastin, elastin-like peptides, albumin, natural poly (amino acids) (e.g. cyanophycin, poly (lysine), and poly (γ-glutamic acid)), polysaccharides (e.g. chitosan, dextran, chondroitin sulfate, agarose, alginate, methylcellulose, and heparin), α-cyclodextrin (CD), β-CD, γ-CD, and blends thereof.

**0070** Synthetic polymers may include any one of or a combination of poly (aliphatic ester) (e.g. poly(lactide) (PLA)), poly(ε-caprolactone) (PCL), poly(glycolic acid) (PGA), poly(lactico-glycolic acid) (PLGA), poly(trimethylene carbonate) (PTMC), polydioxanone (PDS), poly(ortho ester), polyanhydrides, poly(anhydride-co-imide), poly(anhydride-esters), polyurethanes (e.g. degradals), poly (amides), poly(esteramides), poly(orthoesters), poly(dioxanones), poly(�acetals), poly(β-ketals), poly(carbonates), poly (orthocarbonates), poly(esterbutoxynates), poly(esterbutyrates), poly(esteramide values), poly(alkylene succinates), poly(malic acid), poly(amino acids), poly(vinylpyrrolidone), poly(esterbutoxyethyl), poly(glycerol sebacate), poly(ethylene imine), poly(acrylic acid)(PAA), poly (N,N-diolethylaminocarbonyl methacrylate) (PDEAMMA), polyethylene glycol (PEG), poly(propylene oxide) (PPO), PEO-b-PPO block copolymers (e.g. pluronic or poloxamers, and tertronic), poly(vinyl alcohol) (PVA), poly(N-isopropylacrylamide) (PNNPAM), poly(N,N-diolethylcarbamate) (PDEAAm), poly (oxazolines) (e.g. poly(2-methoxyoxazoline and poly(2-ethyl-2-oxazoline)), oligo(poly(ethylene glycol) fumrates), poly(propylene fumurate), poly(alkyl cyanoacrylates), poly(acrylic amide), synthetic poly(amino acids) (e.g. poly (L-glutamic acid) (L-PLA), poly(esteramide), poly(phosphazenes), poly(orthoesters), and blends thereof.

**0071** A cross-linker may include a homo- or heterofunctional modifier of the following formula: A-cross-linker-Z

**0072** In some embodiment, one of A and Z is a moiety selected from the group consisting of hydroxyls, thiols, aminos, alkyls, alkenyls, alkoxy sulfonate, aroyl sulfonate, heterocycles, azides, maleimido, propargyl, haloacetate, dihydroxide, amines, carboxylic acids, aldehydes, ketones, active hydrogen sites on aromatic ring, dienes, azide isothiocyanates, isocyanates, acyl azides, ethynyls, N-hydroxy succinimide (NHS) esters, sulfo-NHS, sulfonyl chloride, epoxides, carbonates, aryl halides, imidoesters, carbodimides (e.g. N, N′-diacylhexylcarbodiimide (DCC) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)), alkylphosphate compounds, anhydrides, fluoroephyl esters, hydroxymethyl phosphines, guanidine groups, imidoacetyl derivatives, maleimides, aziridines, acryloyl derivatives, arylating agents, disulfide derivatives, vinylsulfone, phenylthioester, cisplatin, diazoacetate, carbonyl dimidazole, oxiranes, N,N′-disuccinimidyl carbonate, N-hydroxysuccin-
imidoyl chloroformate, alkyl halogens, hydrazine, maleimide, alkyne, and phosphorus-bound chlorine.

[0073] Structures of a cross-linker may be any one of or a combination of linear, dendrimers-like, star-shaped, hyperbranched, combed, brushed, cross-linked architectures, fibers, microspheres, and nanoparticles. Examples are shown in FIG. 4. The number of arms in star-shaped polymers may be two or more. Fibers may comprise any one of or a combination of polymer fibers, carbon fibers, and ceramic fibers. Microspheres and nanoparticles may comprise any one of or a combination of polymer micro/nanospheres, iron oxide, silica, gold, and mesoporous silica nanoparticles.

[0074] A cross-linker with homo- or hetero-functional groups can be prepared by addition or chain growth polymerizations, coordination polymerizations, and condensation or step growth polymerizations. Addition or chain growth polymerizations include free radical polymerization, controlled living radical polymerization (e.g., atom transfer radical polymerization (ATRP), reversible addition fragmentation transfer (RAFT) polymerization, and nitroxide-mediated radical polymerization (NMP)), cationic polymerizations, anionic polymerizations and the like.

[0075] The conjugation of functionalized HA and crosslinkers can be achieved by any one of or the combination of the following reactions: carboxylate-mediated reactions, esterification, amidation, aldehyde and ketone reactions, active hydrogen reactions, photochemical reaction, azide-alkyne cycloaddition (CuAAC), copper-free azide-alkyne huisgen cycloaddition or strain-promoted azide-alkyne cycloaddition (SPACC), thiol-based click reaction (thiol-yne, thiol-ene, thiol-isocyanate, thiol-Michael addition), Diels-Alder reactions, tetrazole cycloaddition, nitrile oxide cycloaddition, oxime/hydrazine formation, enzymatic crosslinking, and coordination chemistry, and ligand exchange reactions. Examples are shown in FIG. 3.

[0076] The conjugated linkages may include any one of or a combination of isothioura, isourea, amide, sulfonamide, secondary amine, sulfonamide, shift-base, secondary aminomethyl, carbamate, aryl amine, amidine, amide, phosphoramidate, guanidine, substituted imidocarbonate, thiocarboxylic, aryl thiocarboxylic, disulfide, sulfonate, thiosulfonate, ester, ether, carbamate, hydrazone, diazo, triazoles, iodinated compound, carbohydrates, amino acid esters bond, cycloalkene, oxime triazole, and triazolone.

[0077] Physical interactions may include any one of or a combination of hydrophobic interactions, hydrophobic interactions, hydrogen bonding, electrostatic interactions, and van der Waals interactions. Examples are shown in FIG. 5.

[0078] The GAG-based system can be used as a cellular matrix for 3D cell culture and tissue engineering as well as a delivery vehicle for therapeutic agents including but not limited to cells, growth factors, and small molecules.

[0079] Cells that are encapsulated within the Engineered composite may comprise any one of the combination of, but not limited to, mesenchymal stem cells, osteoblast, chondrocytes, adipocyte, fibroblast, hepatocyte, enterocyte, urothelial cells, blood cells, skin cells, endothelial cells, nerve cells, sex cells, and cancer cells.

**EXAMPLES**

Example 1: HA/CS/Poly(Ethylene Glycol) Diacrylate Composite Hydrogel as hMSCs Niches

[0080] We developed a novel biomimetic hydrogel system as a tunable stem cell niche through the combination of thiolated HA (HA-SH) and thiolated CS (CS-SH) cross-linked with poly(ethylene glycol) diacrylate (PEGDA) as shown in FIG. 6. The combination of HA and CS offers a cell-friendly microenvironment found in native tissues, whereas the selection of PEG is based on its established biocompatibility and chemical versatility. The efficient control of various hydrogel properties is demonstrated by simply varying the molecular weight of PEG. A thiol-ene click reaction was selected due to its high efficiency in aqueous media, reliability, lack of by-products, and substantial degradation of the polysaccharide backbone, and without the use of any metal catalysts[22]. To our knowledge, this is the first report that tricopolymer of HA, CS, and PEG in the forms of natural/synthetic composite hydrogels were fabricated through thiol-ene click chemistry. We showed the efficient control of various hydrogel properties (e.g., swelling, modulus, and gelation time) by simply varying the PEG molecular weight (MW). We also demonstrated that the composite hydrogels could support 3D encapsulation of human MSCs (hMSCs) with high viability as well as tunable cell-hydrogel interactions by varying the properties of hydrogels. The ability of fine-tuning hydrogel properties paves the way for further optimization of cellular responses to promote in situ tissue regeneration.

[0081] Materials and Methods

[0082] Hyaluronic acid sodium salt (HA) with molecular weight (MW) of 2-3 million Daltons and chondroitin sulfate sodium salt (CS) with average MW 10-30k Daltons were purchased from Carbosynth Limited (Berkshire, UK). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and polyethylene glycol diacrylate (PEGDA) with MW of 700, 3400, and 8000 were purchased from Alfa Aesar (Ward Hill, Mass.). PEGDA700 was passed through a short column containing basic alumina to remove the inhibitor before use. PEGDA3400 and PEGDA8000 were precipitated in diethyl ether twice to remove the inhibitor before use. PEG divinylsulfone (MW 3500 Daltons) and 4 arm PEG vinylsulfone (MW 20000 Daltons) were purchased from JenKem Technology USA Inc. N-hydroxysuccinimide (NHS), cystamine dihydrochloride, 5, 5'-dithiobis (2-nitrobenzoic acid) (Ellman’s Reagent), DL-Dithiothreitol (DTT), and 2-(N-morpholino) ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, Mo.). 10x Phosphate buffer saline (PBS) was purchased from Fisher Bioreagents (Pittsburgh, Pa.). Penicillin-Streptomycin (Pen-Strep), ethanol hydrate-1 (ethOH); and calcein AM were purchased from Life Technologies (Carlsbad, Calif.).

[0083] Synthesis of HA-SH and CS-SH

[0084] Carboxyl groups in HA and CS were functionalized to thiol groups by a simple two-step reaction scheme which is depicted in FIG. 7. In a typical procedure, HA (1 g, 2.5 mmol) was dissolved in 100 mL MES buffer (0.1 M MES, pH 6.0) in a 45° C oil bath and allowed to dissolve overnight. EDC (2.4 g, 12.5 mmol) and NHS (3.9 g, 34 mmol) were added and allowed to react for 2 h. Then cystamine dihydrochloride (5.65 g, 25 mmol) was added to the mixture and allowed to react overnight while stirring. The reaction mixture was exhaustively dialyzed (MWCO of
12-14,000) against distilled 0.1M NaCl for 60 h, 25% ethanol for 12 h and distilled (DI) water for 12 h. After dialysis, the product, HA-conjugated cystamine (HS-S—S—NH₂), was lyophilized and kept at in −20°C. HA-conjugated cystamine (0.25 g) was dissolved in PBS with a concentration of 5 mg/mL. Then DTT (0.75 g, 4.8 mmol) was added to the flask and pH was adjusted to 7.4 with 1 M NaOH. NaCl was added to produce a 5% w/v solution after 20 h and the modified HA was precipitated in 10-fold ethanol three times. The precipitation was dissolved in D₂O at a concentration of approximately 5 mg/mL and the purified product was freeze-dried and kept at −80°C freezer.

[0085] In a typical procedure, CS (1 g, 2 mmol) was dissolved in 40 mL MESS buffer (0.1M MESS, pH 6.0). EDC (3.086 g, 16.2 mmol) and NHS (2.280 g, 19.8 mmol) were added to the flask and allowed to react for 2 h. Following the 2 h activation step, the pH was raised to 7.2 using 1 M NaOH. Cystamine dihydrochloride (4.460 g, 20 mmol) was subsequently added to the solution and allowed to react overnight. The CS-conjugated cystamine (CS—S—S—NH₂) was exhaustively dialyzed (MWCO of 12-14,000) against distilled 0.1M NaCl for 60 h, 25% ethanol for 12 h and DI water for 12 h and then was lyophilized. The CS—S—S—NH₂ was reduced using DTT as the same procedure of reduction of HA. The structure of functionalized-HA or CS were confirmed 1H NMR spectroscopy (D₂O, Bruker ARX 400 MHz) and the degree of thiolation was also confirmed with an Ellman’s test using L-cysteine as the standard.

[0086] Characterization of HA-SH and CS—SH

[0087] The structures of HA and CS-conjugated cystamines were confirmed by proton nuclear magnetic resonance (1H NMR) spectroscopy, with the degree of modification (DS) estimated from integration of methylene protons relative to the N-acetyl methyl protons in FIG. 8. By varying the molar ratios of HA/EDC/NHS, variable DS values (4.1–43.1%) were obtained (Table 1), which shows the effects of EDC concentration on degree of substitution (DS) of —NH₂. For example, molar ratios of 1:5:13.5 and 1:10:13.5 yielded HA-conjugated cystamines with DS of 27% and 43.1%, respectively. Therefore, CS/EDC/NHS molar ratio of 1:10:13.5 was selected for CS modification.

Subsequently, reduction of HA and CS-conjugated cystamines by dithiothreitol (DTT) resulted in free thiol groups. The two-pot reaction allows for efficient control of DTT reduction reactions and minimizes the oxidation of thiols resulting in the disulfide formation and the insolubility of HA or CS. DS of both amine and thiol groups were controlled by simply adapting the pH of reaction mixture or the amount of DTT. The results of DS determined by the Ellman’s assay and 1H NMR were consistent and listed in Table 2, which shows the effects of DTT concentration and pH on DS of —SH.

TABLE 2

The effects of DTT concentration and pH on DS of —SH.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Reaction condition</th>
<th>DS % of —SH</th>
<th>Ellman's assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-SH</td>
<td>8-fold DTT</td>
<td>38.5</td>
<td>5.2</td>
</tr>
<tr>
<td>HA-SH</td>
<td>15-fold DTT</td>
<td>18.0</td>
<td>15.3</td>
</tr>
<tr>
<td>CS-SH</td>
<td>PBS buffer</td>
<td>38.8</td>
<td>34.1</td>
</tr>
<tr>
<td>CS-SH</td>
<td>pH = 7.2</td>
<td>25.5</td>
<td>23.5</td>
</tr>
<tr>
<td>CS-SH</td>
<td>pH = 8</td>
<td>18.5</td>
<td>17.7</td>
</tr>
</tbody>
</table>

[0088] 1H NMR spectra (FIG. 9) showed the decrease of proton signals from the cystamines disulfide pendant. Peaks at d is attributed to proton signals of methylene (—CH₂CH₂NH₃). Peaks at a, b, a' and b' correspond to resonances of the two side chain methylene (—CH₂—CH₂—SH). By altering the pH of reaction mixture, the thiol group DS of CS—SH could be controlled from 17.7% to 36.0% (Table 2). The pH at 7.2 was selected to offer reactive thiolate and avoid the possible degradation of thiolated HA. By altering the amount of DTT, the thiol group DS of HA-SH with 5.2% and 15.3% respectively were obtained (Table 2). For the remaining studies, HA-SH with a thiol group DS of 5.3% was employed because lower substituted HA hydrogels have been shown to display higher cellular activity [23]. CS—SH with a thiol group DS of 23.5% was employed. Moreover, the residual —NH₂ groups allow for additional functionalization of the hydrogels if desired.

[0089] The Formation of Hydrogels Using Thiol-Ene Chemistry

[0090] Crosslinking of HA-SH and CS—SH mixtures was achieved using PEGDA as shown in FIG. 6. To study the effects of chain length of cross-linker, the MW of PEGDA was altered to distribute either 700 Da, 3400 Da, or 8000 Da within hydrogel while the molar ratio of thiol groups to acrylate groups was kept at 1.07 and other conditions were fixed (Table 3, which shows the composition of HCP hydrogels). Hydrogels were formed by simple mixing of HA-SH, CS—SH, and PEGDA in PBS at 37°C. Specifically, 1% HA-SH (199 µL) and 5% CS—SH (217 µL) were mixed in the microcentrifuge tube. Either 5% PEGDA 700 (26.8 µL), 20% PEGDA 3400 (33.8 µL), or 33% PEGDA 8000 (46 µL), was added to the microcentrifuge tube to create HA/CS/PEGDA 700 (HCP700), HA/CS/PEGDA 3400 (HCP3400), and HA/CS/PEGDA8000 (HCP8000) respectively.

TABLE 3

The composition of hydrogels

<table>
<thead>
<tr>
<th>Hydrogel Type</th>
<th>m/m HA-SH</th>
<th>CS-SH</th>
<th>SH=ene</th>
<th>MW of PEGDA</th>
<th>Polymer percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCP700</td>
<td>1.5:1</td>
<td>1.07</td>
<td>5.4 ± 0.2</td>
<td>25.8 ± 1.8</td>
<td>700 3.2</td>
</tr>
<tr>
<td>HCP3400</td>
<td>1.5:1</td>
<td>1.07</td>
<td>5.4 ± 0.2</td>
<td>23.8 ± 1.8</td>
<td>3400 4.4</td>
</tr>
<tr>
<td>HCP8000</td>
<td>1.5:1</td>
<td>1.07</td>
<td>5.4 ± 0.2</td>
<td>23.8 ± 1.8</td>
<td>8000 6.2</td>
</tr>
<tr>
<td>HCP1000</td>
<td>1.5:1</td>
<td>1.07</td>
<td>11.2 ± 0.9</td>
<td>32.9 ± 1.9</td>
<td>1000 3.2</td>
</tr>
<tr>
<td>Hydrogel Type</td>
<td>mm HA:SeH</td>
<td>CS:StHy</td>
<td>DS of HA</td>
<td>DS of SeH</td>
<td>MW of PEGDA (Da)</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>---------</td>
<td>---------</td>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td>HCP3400</td>
<td>1.5:1</td>
<td>1.07</td>
<td>11.2 ± 0.9</td>
<td>32.5 ± 1.9</td>
<td>3400</td>
</tr>
<tr>
<td>HCP8000</td>
<td>1.5:1</td>
<td>1.07</td>
<td>11.2 ± 0.9</td>
<td>32.5 ± 1.9</td>
<td>8000</td>
</tr>
</tbody>
</table>

[0091] Cryo Scanning Electron Microscope (Cryo-SEM)

[0092] Hydrogel samples were imaged using an FEI NOVA nanoSEM field emission scanning electron microscope (FEI Company, Hillsboro, Oreg.) using ET (Everhart-Thornley) detector or the high-resolution throughput lens (TLID) detector operating at 5 kV acceleration voltage, spot 3, 5.0 nm working distance and 30 mm aperture.

[0093] The composite hydrogels exhibited interconnected porous structures with micron-sized pores as shown in SEM micrographs (FIG. 11). Generally, HCP700 hydrogels possessed larger pores than HCP3400 and HCP8000 hydrogels presumably due to lower cross-linking density of hydrogels.

[0094] Gelation Time

[0095] The time to form a gel (denoted as gelation time) is defined as the time when the gel, in an inverted state, shows no fluidity for 1 min[24] (FIG. 10). The experiment was performed in triplicate. The gelation times decreased with increasing molecular weight of PEGDA (p<0.05, n=3) (FIG. 12A). HCP700, HCP5400 and HCP8000 hydrogels exhibited a gelation time of -30 min, -18 min, and -16 min respectively.

[0096] Swelling Tests

[0097] For swelling tests, hydrogel samples (~0.35 mL) were prepared as described above. The hydrogels were freeze-dried and weighed (Ww). Subsequently, 1 mL of PBS was applied on top of the hydrogels and then the samples were incubated at 37°C for 24 h to reach the swelling equilibrium. The excess PBS was then aspirated away and the remaining saturated hydrogels were weighed (Ww). The experiments were performed in triplicate and the degree of swelling of the hydrogels was expressed as \((W_s - W_w)/W_w\) x 100%. All data are presented as mean±standard deviation. The swelling ratio is another important parameter for hydrogels, which is associated with hydrogel mechanical properties including strength and flexibility.

[0098] All the composite hydrogels were highly swollen in water resulting from the hydrophilicity of PEG, HA and CS molecules, and the maintenance of cross-linked HA/CS/PEG networks. As shown in FIG. 12B, the swelling ratio decreased significantly with the increase of MW of PEGDA from 300 Da to 3400 Da (p<0.05, n=3). Although not significant, a similar trend was also found while increasing MW of PEGDA from 3400 Da to 8000 Da.

[0099] Drug Release Study

[0100] Tricomponent hydrogels (350 μL) with different PEG molecular weights (700, 3400, and 8000) were prepared in a syringe with the end cut off. FITC-dextran (MW~70k, 20 μg) was added to each hydrogel precursor solution. The syringe was then placed in a 37°C incubator. The hydrogels were equilibrated for 2 h at 37°C. After the two hours the hydrogel was then dispersed into cell culture inserts (12 mm diameter with 3 μm pore size (Corning Incorporated, USA) in a 12 well plate. The hydrogels were then submerged with PBS and the well plate was placed into a 37°C water bath. At specified time intervals, 1 mL of the solution from individual wells was withdrawn and replaced with pre-heated water. The amounts of released FITC-dextran were determined by fluorescence measurements (excitation at 485 nm, emission at 528 nm).

[0101] FIG. 12C shows the percent cumulative release profiles of FITC-dextran from different composite hydrogels as a function of time at 37°C. After 58 hours, the FITC-dextran encapsulated in HCP700, HCP3400, and HCP8000 hydrogels resulted in about 84.3%, 77.6%, and 72.2% cumulative release of total FITC-dextran, respectively. The release of FITC-dextran from HCP hydrogels was mainly controlled by the diffusion. The release rate correlated well with the hydrogel pore size as a functional of PEGDA molecular weight. Increase of PEGDA chain length generates greater crosslinking density resulting in smaller pore size and slower release of FITC-dextran. This observation was consistent with our swelling ratio results.

[0102] Rheological Characterization

[0103] Rheological experiments were carried out with a new Discovery Series Hybrid Rheometer (DRH)-3 (TA) using parallel plate (20 mm diameter, 0°) in the oscillatory mode. Oscillatory time, frequency, and strain sweeps were performed at 37°C, and the storage (G') and loss (G'') moduli were recorded. 450 μL of gel precursor solution was mixed by vortexing at room temperature for 15 s before loading on to the rheometer. Hydrogels were cast between the lower plate (preheated at 37°C) and upper parallel plate. The 20 mm parallel plate geometry was set to a gap of 1000 μm. Each hydrogel sample was used for only one test. Strain sweeps and frequency sweeps were performed in duplicate and the data represents the average of the two tests. 1% strain and a frequency of 1 Hz were used for the time sweeps, with the same 20 mm parallel plate for 4800 S. Time sweep tests were performed in triplicate and the data represents the average of the three tests with corresponding standard deviation.

[0104] The composite hydrogels showed tunable rheological and mechanical properties by varying the PEGDA chain length. A strain sweep from 0.1% to 10% strain was conducted at a frequency of 1.0 Hz (chosen arbitrarily) on a formed gel. FIG. 13A showed constant storage modulus G' values as strain is varied up to 10% strain, indicating that strains in the range 0.1% to 10% was in the linear-viscoelastic regime (LVE)[25]. Consequently, a strain of 1% was selected for the subsequent frequency sweep tests. At low frequencies from 0.1 to 10 rad/s, G' does not change (FIG. 13B) indicating the solid-like nature of the gel. As a result, a frequency of 1 Hz was selected for future tests based on a favorable torque signal at that frequency. A time sweep was performed under the condition that the strain was small enough to be in the LVE and the frequency was a value under which the instrument provides a clear and favorable torque signal. Therefore, 1% strain, 1 Hz, and 37°C were
determined to be the appropriate conditions for the hydrogel time sweep tests. HA-CS/PEGDA formed a gel and reached stability in ~45 min (FIG. 13C). However, the hydrogels formed rapidly and the crossover of G' and G″ was not observed (G" was too low to measure for the extent of the tests). As noted in FIG. 13C, the gels have a solid-like viscoelastic behavior. Extensive rheology testing showed that by increasing the MW of the PEGDA, a significant increase in the hydrogel storage modulus (p<0.05. FIG. 13D) was observed, which is in good agreement with the conclusion derived from the SEM data. Accordingly, HCP700 hydrogels were the weakest with a G' value of 410±30 Pa, whereas HCP8000 hydrogels were the strongest with a G' value of 1810±106 Pa. Storage modulus of HCP3400 hydrogels was 1500±190 Pa, approximately triple that of HCP700 hydrogels.

[0105] 3D Cell Encapsulation and Culture

[0106] hMSCs were obtained from Lonza. Cells were cultured in growth medium containing DMEM (Life technologies, catalog #11885084) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 1% glutamine (Life technologies, catalog #25030081), and 1% penicillin/streptomycin (Life technologies, catalog #15140122) at 37° C with 5% CO2. For the cell encapsulation in the hydrogels, cell pellets were obtained by centrifugation, followed by a brief resuspension with hydrogel solution through pipetting up and down gently to ensure a homogeneous distribution. Then cell suspension with the density of 20,000 cells/25 μL was transferred to an inverted pre-cut 1 mL syringe and injected at 37° C for 45 min (FIG. 14). Subsequently, hydrogels were injected to 24 well tissue culture plate and incubated in growth medium. The medium was changed every other day thereafter.

[0107] Cell Viability: Live/Dead Staining

[0108] The viability of the cells encapsulated in hydrogels after 1 and 5 days' culture was investigated by the live/dead viability assay (Life technologies, catalog# L3224) according to the manufacturer’s instructions. Media were removed from all three samples, washed twice with sterile PBS and stained with calcein-AM and ethidium homodimer-1 solution. Samples in staining solution were incubated at room temperature for 30 min. Imaging was performed with a confocal microscope (Nikon A1R).

[0109] Mesenchymal stem cells (MSCs) offer a promising cell source for musculoskeletal regenerative engineering due to their ease availability, high expansion capacity, and multipotency. Thus, hMSCs were encapsulated in the composite hydrogels and characterized for cell viability and cellular responses. Confocal images using a live/dead assay demonstrated that all composite hydrogels supported progressive cellular growth with high viability (~95%) during cell culture (FIG. 15 and FIG. 16). Cells encapsulated within hydrogels displayed an overall spherical morphology, which is in line with previous literature reports for 3D hydrogel cell culture.[26]

[0110] Actin Immunofluorescence

[0111] Actin immunostaining was performed with the actin cytoskeleton and focal adhesion staining kit (Millipore). Briefly, cell/hydrogels were fixed in 4% paraformaldehyde and blocked with 1% BSA in PBS. Then samples were stained with TRITC-conjugated for phalloidin F-actin and with DAPI for nuclei. Imaging was performed with a confocal microscope (Nikon A1R).

[0112] Interestingly, hMSCs responded to differences in the mechanical properties of hydrogels as evidenced from the changes in actin cytoskeleton. In specific, hMSCs encapsulated in HCP700 hydrogels showed less defined actin fibers as compared to those in HCP3400 and HCP8000 hydrogels (FIG. 17). Furthermore, cortical actin protrusions into the surrounding hydrogel matrix were only observed with hMSCs encapsulated in HCP3400 and HCP8000 hydrogels.

[0113] Focal Adhesion Kinase (FAK)

[0114] hMSC cells were incubated with a cell permeable focal adhesion kinase (FAK) phosphorylation biosensor and were washed gently with PBS (3×1 mL). After FAK biosensors were successfully delivered into hMSCs, they were encapsulated in HCP hydrogels. The dynamic monitoring of focal adhesion kinase (FAK) activity was conducted to examine cell-hydrogel interactions using fluorescence lifetime imaging microscopy (FLIM).[27]

[0115] The dynamic monitoring of FAK activity using FLIM confirmed a similar finding where stiffened hydrogels exhibited increased FAK phosphorylation of hMSCs (FIG. 18). It is important to note that cells in different tissues are tuned to the specific mechanical environments in which they reside.[28]

[0116] 3D Cell Adhesion and Culture

[0117] The prepared HCP hydrogels were freeze-dried, sterilized for 20 min under UV light, and pre-incubated in the growth medium containing DMEM (Life technologies, catalog #11885084) which was supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 1% glutamine (Life technologies, catalog #25030081), and 1% penicillin/streptomycin (Life technologies, catalog #15140122). hMSCs were seeded on the surface of the lyophilized hydrogel scaffolds with a density of 20,000 cells/scaffold and were pre-incubated at 37° C with 5% CO2 for 1 h. Subsequently, the lyophilized hydrogel scaffolds seeded with hMSCs were cultured in 500 μL growth medium at 37° C with 5% CO2 for 1 day.

[0118] SEM Characterization

[0119] Cell seeded hydrogel scaffolds were dehydrated sequentially with 50, 70, 80, 90, 95, and 100% ethanol for 10 minutes each and then were freeze dried. The samples were then cut to expose the cross-sectional area as well as the surface, and then sputter coated with platinum for 60 seconds. The hydrogels were examined on a scanning electron microscope (Nova NanoSEM) at 3.0 kV.

[0120] The lyophilized HCP hydrogel scaffolds were shown to have porous structures (FIG. 19A, C) and to be able to support cell growth on the surface of scaffolds (FIG. 19A, B). Pore structure was roughly 40 to 50 μm in diameter.

Example 2. HA/CS/Poly (Ethylene Glycol) Divinyl Sulfone (PEGVS) Composite Hydrogel as Primary Chondrocytes Niches

[0121] The Formation Hydrogels using Michael-type Addition Chemistry

[0122] In all experiments, the molar ratio —SH—ene concentration was held constant. Specifically, 1% HA-SH (192 μL), 5% CS—SH (199.2 μL), and poly (ethylene glycol) divinyl sulfone (PEGVS) 3400 (52.8 μL) were mixed at 37° C in the microcentrifuge tube to create HA/CS/PEGVS 3400 (HCP3400).
[0123] Rheological Characterization
[0124] Rheological experiments were carried out with a new Discovery Series Hybrid Rheometer (DHR)-3 (TA) using parallel plate (20 mm diameter, 0°) in the oscillatory mode. Oscillatory time, frequency, and strain sweeps were performed at 37° C, and the storage (G') and loss (G'') moduli were recorded. 450 µl of gel precursor solution was mixed by vortexing at room temperature for 15 s before loading on to the rheometer. Hydrogels were cast between the lower Peltier plate (preheated at 37° C) and upper parallel plate. The 20 mm parallel plate geometry was set to a gap of 1000 µm. Each hydrogel sample was used for only one test. Time sweep tests were performed under 1% strain, 1 Hz, and 37° C conditions (n=4). The data represents the average of the three tests with corresponding standard deviation.

[0125] Due to more reactive vinyl sulfone moiety, PEGVS demonstrates significantly shorter gelation time and higher storage modulus compared to PEGDA (FIG. 20).

[0126] Chondrocytes isolation
[0127] Chondrocyte cultures were prepared from pig articular cartilage[29]. Shavings of cartilage were removed from the outside of the articular cartilage, such that contamination with bone cells or other connective tissue cells could be avoided. The pig cartilage was finely chopped and the chondrocytes were released from their extracellular matrix by sequential digestion at 37° C with collagenase. Cells obtained from the collagenase digest were pooled and passed through a sterile 60 µm aperture nylon screen (Nitex) to remove any undigest chondrocyte fragments.

[0128] 3D Cell Encapsulation and Culture
[0129] Primary chondrocytes were cultured in growth medium containing DMEM (Life Technologies, catalog #11885084) supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals), and 1% penicillin/streptomycin (Life Technologies, catalog #15140122) at 37° C with 5% CO2. For the cell encapsulation in the hydrogels, cells pellets were obtained by centrifugation, followed by a brief resuspension with hydrogel solution through pipetting up and down gently to ensure a homogeneous distribution. Then cell suspension with the density of 20,000 cells/25 µl was transferred to an inverted pre-cut 1 ml syringe and incubated at 37° C for 45 min. (FIG. 14). Subsequently, hydrogels were injected to a 24 well tissue culture plate and incubated in growth medium. The medium was changed every other day thereafter.

[0130] Cell Viability and Immunofluorescence
[0131] Cell immunostaining was performed with the actin cytoskeleton and focal adhesion staining kit (Millipore). Briefly, cell/hydrogels were fixed in 4% paraformaldehyde and blocked with 1% BSA in PBS. Then samples were stained with TRITC-conjugated phalloidin for F-actin, with aggrecan antibody (H-300) (Santa Cruz Biotech) for aggrecan, and with DAPI for nuclei. Imaging was performed with a confocal microscope (Nikon A1R).

[0132] Confocal images using a live/dead assay demonstrated that all composite hydrogels supported cellular growth with high viability after a 21-day culture (FIG. 21A). Primary chondrocytes are easy to de-differentiate after passages. Here we show that our hydrogel can maintain chondrocytes morphological characteristics after 21 days indicated by the staining of aggrecan (FIG. 21B).

Example 3: HA/CS/4-Arm Poly (Ethylene Glycol) Divinyl Sulfone (4PEGVS) Composite Hydrogel as hMSC Niches

[0133] The Formation Hydrogels using Michael-type Addition Chemistry
[0134] In all experiments, the molar ratio —SH/ene-concentration was held constant. Specifically, 1% HA-SH (140 µL), 5% CS—SH (150 µL), and 25% 4-arm PEG) divinyl sulfone (4PEGVS) 20k (115 µL) were mixed in the 1.5 mL centrifuge tube to create HA/CS/4PEGVS 3400 (HCPV3400).

[0135] Rheological Characterization
[0136] Rheological experiments were carried out with a new Discovery Series Hybrid Rheometer (DHR)-3 (TA) using parallel plate (20 mm diameter, 0°) in the oscillatory mode. Oscillatory time, frequency, and strain sweeps were performed at 37° C, and the storage (G') and loss (G'') moduli were recorded. 450 µl of gel precursor solution was mixed by vortexing at room temperature for 15 s before loading on to the rheometer. Hydrogels were cast between the lower Peltier plate (preheated at 37° C) and upper parallel plate. The 20 mm parallel plate geometry was set to a gap of 1000 µm. Each hydrogel sample was used for only one test. Time sweep tests were performed under 1% strain, 1 Hz, and 37° C conditions (n=4). The data represents the average of the three tests with corresponding standard deviation.

[0137] Compared with 2-arm PEGVS, 4-arm PEGVS demonstrates significantly shorter gelation time and higher storage modulus compared to PEGDA (FIG. 22).

Example 4: Bio-inspired Composite Hydrogels for Osteochondral Regenerative Engineering

[0142] Treatment of osteochondral defects encompassing injury or degeneration to both the articular cartilage as well as the underlying subchondral bone presents a significant medical challenge. Current treatment options including autografts and allografts suffer from limited availability and risk of immunogenicity, respectively. The long term goal of this work is to develop an integrated scaffold system for treatment of osteochondral defects via in situ regeneration of bone, cartilage and the bone-cartilage interface (FIG. 24). Hydrogels provide an attractive biomaterial platform for regeneration of cartilage. In the present disclosure, we have
developed a novel composite hydrogel that includes thiol-ated HA and CS cross-linked with PEG to mimic cartilage. The bone mimetic is based on PLGA/nano-hydroxyapatite composite scaffolds[30].

[0143] Here, integration between hydrogels and PLGA 3D scaffolds was achieved using a novel multifunctional HA. HA was chemically functionalized with both —SH and —NH₂ groups to form two functional arms: one arm to covalently bond to a cross-linker to form hydrogels and the second arm bonding to the PLGA 3D scaffold surface. This multifunctional HA provides a biologically active and mechanically functional bridge with scaffolds.

[0144] PLGA 3D Scaffold Preparation

[0145] (PLGA) 85:15 was purchased from Lakeshore Biomaterials. PLGA microspheres were obtained using the emulsion-solvent evaporation method. PLGA 3D scaffolds were fabricated by heat sintering at an optimized condition[31].

[0146] PLGA 3D Scaffold-Hydrogel Integration and SEM Characterization

[0147] The -SH, CS —SH and PEGDA solution (PIS) was placed onto PLGA 3D scaffold surface for 45 min at 37°C. PLGA 3D scaffold-hydrogel integration was lyophilized and then sputter coated with platinum for 60 seconds. The integration was examined on a scanning electron microscope (Nova NanoSEM) at 3.0 kV. SEM images of PLGA 3D scaffolds-hydrogel integration was shown in FIG. 25.

[0148] Statistical Analysis

[0149] Differences among groups were assessed by one-way ANOVA with Bonferroni post hoc correction to identify statistical differences among three treatments. A p-value of 0.05 was set as the criteria for statistical significance. Graphs are annotated where values are represented as *p<0.05.

[0150] Additional disclosure is found in Appendix-A, filed herewith, entirety of which is incorporated herein by reference into the present disclosure.

[0151] Those skilled in the art will recognize that numerous modifications can be made to the specific implementations described above. The implementations should not be limited to the particular limitations described. Other implementations may be possible. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.

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[0173] (22) Mergy J, Fournier A, Huchet E, Azély-Vely R. Modification of polysaccharides via thiol-ene chemis-


1. A composition comprising thiolated hyaluronic acid (HA), thiolated chondroitin sulfate (CS) and a functionalized polyethylene glycol (PEG) derivative, wherein said PEG derivative crosslinks thiolated HA and thiolated CS.

2. The composition of claim 1 wherein said functionalized PEG derivative contains a plurality of activated vinyl groups.

3. The composition of claim 1 wherein the molecular ratio of the total thiol group of HA and CS vs. said activated vinyl group is maintained as approximately 1.07.

4. The composition of claim 1 wherein said activated vinyl groups are selected from the group consisting of poly (ethylene glycol) diacrylate (PEGDA), poly (ethylene glycol) Divinyl Sulfone (PEGVS), and 4-arm poly (ethylene glycol) vinyl Sulfone (4PEGVS).

5. The composition of claim 1 wherein thiolated HA further comprising a plurality of amino groups (—NH₂) groups selected from the following formula I-IV:

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 Carbon O O
 |     |     |
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 R₁|     |     |
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---|-----|-----|
 R₂|     |     |
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 |     |     |
---|-----|-----|
 R₃|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₄|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₅|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₆|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₇|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₈|     |     |
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 |     |     |
---|-----|-----|
 R₉|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₁₀|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₆₀|     |     |
     |     |     |
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---|-----|-----|
 R₁₂|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₄₄|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₆₆|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₈₈|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₁₀₀|     |     |
     |     |     |
 |     |     |
---|-----|-----|
 R₁₂₀|     |     |
     |     |     |
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wherein the functional groups R₁, R₂, R₃, R₄, and R₅ comprise any one of or a combination of haloacetates, dihydroxides, amines, thiois, carboxylic acids, ald hydrates, ketones, active hydrogen sites on aromatic rings, dienes, nitro, isothiocyanates, isocyanates, acyl azides, NHS esters, sulfo-NHS, sulfonyl chloride, epoxides, carbonyls, aryl halides, imides, carboxamides, (e.g. DCC and EDC), alkylphosphate compounds, anhydrides, fluorophenol esters, hydroxymethyl phosphines, guanidino groups, iodoacetyl derivatives, maleimides, aziridines, acryloyl derivatives, arylating agents, disulfide derivatives, vinylsulfone, phenylthioester, cisplatin, diazocates, carbonyl dimidazoles, oxiranes, N, N'-disuccinimidyl carbonates, N-hydroxysuccinimidyl chloroformates, alkyl halogens, hydrazines, alkynes, and phosphorus-bound chlorine.

6. An osteochondral regenerative engineering composite comprising poly (lactide-co-glycolide) (PLGA) grafted to a composition via —NH₂ group to form a bone mimetic, wherein said composition comprising thiolated hyaluronic acid (HA), thiolated chondroitin sulfate (CS) and a functionalized polyethylene glycol (PEG) derivative, wherein said PEG derivative crosslinks thiolated HA and thiolated CS.

7. The osteochondral regenerative engineering composite of claim 6 wherein said PLGA is made of lactide/glycolide at a ratio of about 85:15.
8. The composition of claim 1 wherein the molecular weight and length of said PEG derivatives are adjustable to modify the composite modular storage and loss value.

9. The composition of claim 8, wherein the molecular weight of said PEG derivatives ranges from about 700 Da to about 8000 Da.

10. A method of making a composition of cross-linked HA, CS and PEG comprising the steps of: preparing thiolated HA;
   a. preparing thiolated CS;
   b. preparing PEG derivative containing a plurality of activated functionalities;
   c. mixing said HA, CS, and PEG derivative in an aqueous medium; and
   d. initiating cross-linking;
wherein said activated functionality is elected from the group consisting of alkoxyxysulfonate, arylsulfonate, heteroarylsulfonate, maleimido, ether NHS esters, sulfon-NHS,
wherein the structures of said PEG derivative is any one of or a combination of linear, dendrimer-like, star-shaped, hyper-branched, combed, brushed, cross-linked architectures, fibers, microspheres, and nanoparticles.

11. The method of claim 10, wherein the activated functionality of PEG comprises any one of or a combination of isothiourea, isourea, amide, sulfonamide, secondary amine, sulfonamide, shift-base, secondary amino-methyl, carbamate, aryl amine, amidine, amide, phosphoramidate, guanidine, substituted imidocarbonate, thioether, 4-amino derivative of cytosine, aryl thioether, disulfide, sulfonate, β-thiosulfonyle, ester, carbamate, hydrazide, diazo, triazoles, iodinated compound, carbohydrates, amino acid esters bond, cycloalkene, oxime triazole, and triazole.

12. The composition of claim 1 further comprising functionalized peptides selected from the group consisting of arginine-glycine-aspartate (RGD), fibronectin, laminin, and fibrinogen, wherein said peptides are functionalized by carboxyl, amine or thiol group, and conjugated with HA, CS, or functionalized PEG derivatives through thiol-ene click reactions and esterification.

13. The composition of claim 1 further comprising tissue engineering cells, wherein the cells are selected from the group consisting of mesenchymal stem cells, osteoblast, chondrocytes, adipocytes, fibroblast, hepatocytes, enterocytes, urothelial cells, blood cells, skin cells, endothelial cells, nerve cells, sex cells, cancer cells, and combination thereof.

14. The composition of claim 1 further comprising small molecules as therapeutic agents.

15. The composition of claim 1 further comprising at least one growth factor.