

Hybrid hydrogels for biomedical applications

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Abstract

The use of hydrogels in biomedical applications dates back multiple decades, and the engineering potential of these materials continues to grow with discoveries in chemistry and biology. The approaches have led to increasing complex hydrogels that incorporate both synthetic and natural polymers and functional domains for tunable release kinetics, mediated cell response, and ultimately use in clinical and research applications in biomedical practice. This review focuses on recent advances in hybrid hydrogels that incorporate nano/microstructures, their synthesis, and applications in biomedical research. Examples discussed include the implementation of click reactions, photopatterning, and 3D printing for the facile production of these hybrid hydrogels, the use of biological molecules and motifs to promote a desired cellular outcome, and the tailoring of kinetic and transport behavior through hybrid-hydrogel engineering to achieve desired biomedical outcomes. Recent progress in the field has established promising approaches for the development of biologically relevant hybrid hydrogel materials with potential applications in drug discovery, drug/gene delivery, and regenerative medicine.

Key words: Biomedical application, drug/gene delivery, hybrid hydrogel, microstructure, nanostructure, regenerative medicine

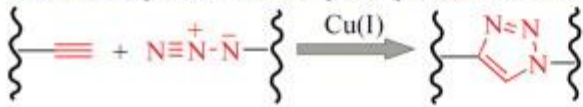
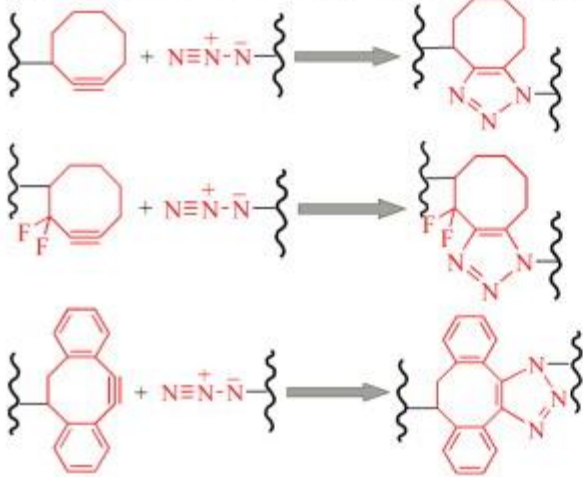
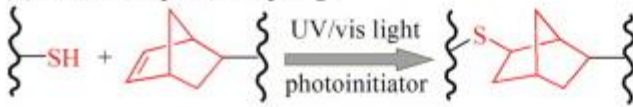
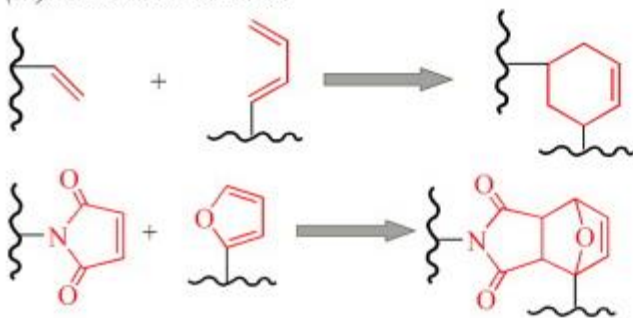
Introduction

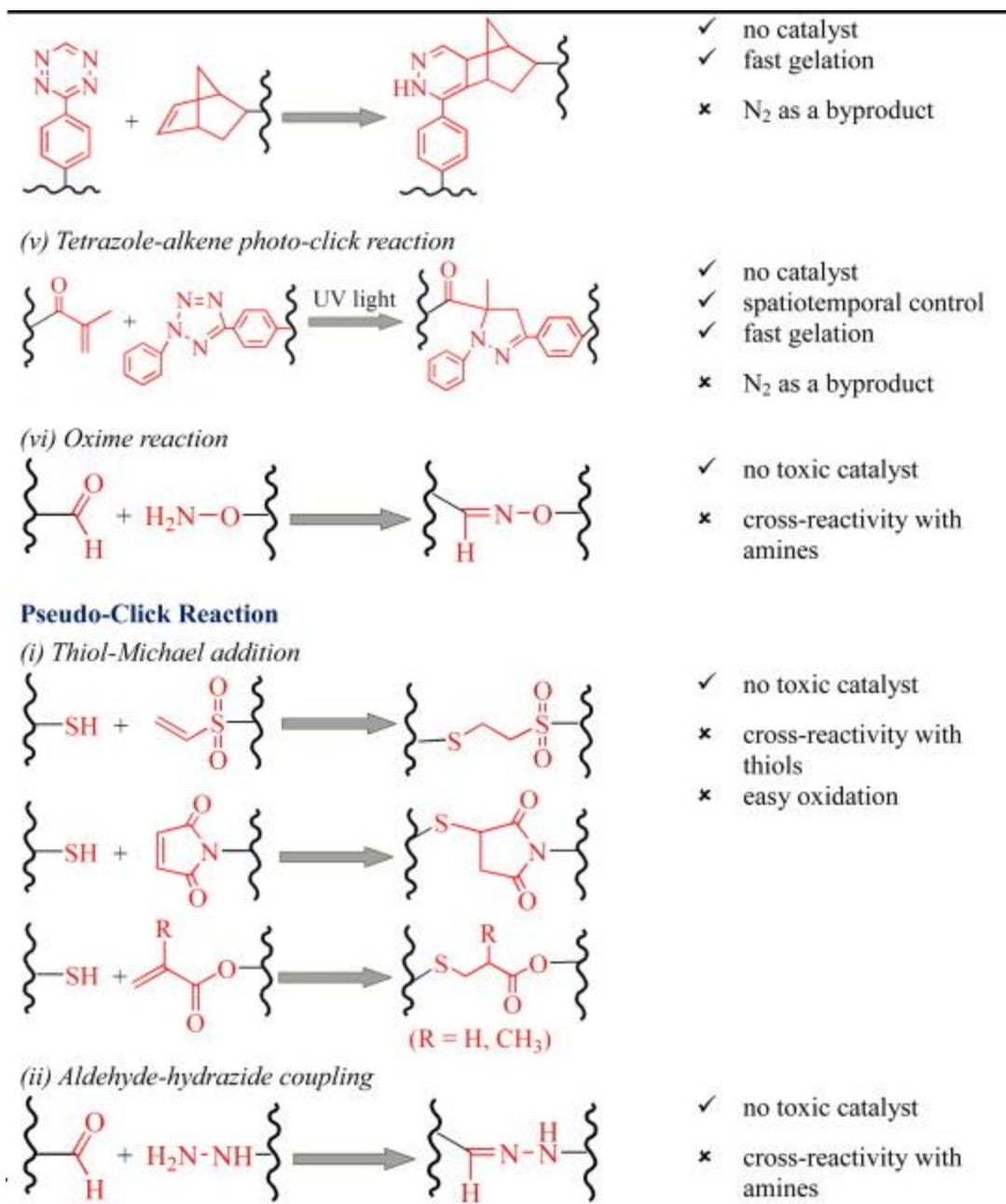
In their simplest form, hydrogels are hydrophilic polymer networks, that can absorb, swell, and retain large amounts of aqueous fluid [1]. Their high water content and permeability, as well as their tunable viscoelasticity and structural similarity to the extracellular matrix, make hydrogels inherently well suited for biological applications. These key properties make them attractive for biomedical use as pioneered by Wichterle and Lim in 1960, and continued today as platforms for drug delivery and tissue engineering [2]. Increased versatility and function of the materials has been enabled by chemical advances that allow incorporation of molecules that can direct cell activity and/or be released with temporal control, and also for inclusion of carriers such as microstructured domains and nanoparticles in the hydrogel network.

Various physical and chemical cross-linking strategies have been implemented for fabrication of hydrogels. Physical hydrogels are formed by using a variety of environmental triggers (temperature, pH, ionic strength) and physicochemical interactions (stereocomplexation, charge condensation, hydrophobic interactions or supramolecular chemistry) [3]. They can be formed under mild conditions but are usually weak and possess poor long-term stability [4]. Conversely, chemical hydrogels (e.g., photopolymerized [5] or enzymatically cross-linked [6]) are generally characterized by better stability and enhanced mechanical properties (e.g., enhanced hydrogel stiffness). Advances in modern polymer chemical transformations have enabled the design of sophisticated material systems with a broad range of applications. In particular, “click reactions” (e.g., thiol-maleimide Michael addition, thiol-norbornene click reaction) have been especially valuable because they are orthogonal to many naturally occurring chemical functionalities, have few byproducts, and the resulting thioether succinimide linkage can be tuned to show reversible and dynamic properties [7,8]. They have particular utility for creating hydrogels

with high biocompatibility, tunable viscoelasticity, and most importantly the ability to carry, protect, and release cargo to the surrounding tissue in a responsive fashion (e.g., triggered by biological substrates like glutathione for glutathione-mediated cleavage of maleimide-thiol adducts, or triggered by a change in pH) [9]. For example, copper(I)-catalyzed azide-alkene cycloaddition (CuAAC) based hyaluronic acid hydrogels were used as cell scaffolds and drug reservoirs [10]. Furthermore, the potentially toxic catalysts used for copper-catalyzed reactions can be minimized with copper-free click chemistries such as those employed in radical mediated thiol-ene/yne chemistry [11], azide-alkyne cycloaddition [12], tetrazole-alkene photo-click chemistry [13], the Diels-Alder reaction [14], and the oxime reaction [15], as presented in **Table 1** (adapted from Jiang et. al.) [16].

Table 1: Summary of click chemistry strategies employed to form hydrogels (adapted from Jiang et. al. 2014) [16].
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Click Reactions	Features
Cu(I) Catalyzed Azide-Alkyne Cycloaddition (CuAAC) 	✓ bioorthogonality ✗ toxic copper catalyst
Copper-Free Click Reaction <i>(i) Strain-promoted azide-alkyne cycloaddition (SPAAC)</i> 	✓ no catalyst ✓ bioorthogonality ✗ difficult synthesis of cyclooctynes
<i>(ii) Thiol-ene photocoupling</i> 	✓ spatiotemporal control ✓ fast gelation ✗ potential toxicity from photoinitiators and radicals ✗ cross-reactivity with thiols
<i>(iii) Diels-Alder reaction</i> 	✓ no catalyst ✓ accelerated by H₂O ✓ thermoreversible ✗ slow gelation
<i>(iv) Inverse electron demand Diels-Alder reaction</i>	



While hydrogels as a class of materials possess key properties that make them suitable for biomedical applications (e.g., drug delivery and tissue engineering) [17,18], their mechanical strength, release/degradation kinetics, and the bioactivities of incorporated therapeutic molecules must still be optimized. Traditional synthetic hydrogels exhibit significant heterogeneity and

network defects (e.g., chain ends, entanglements, and phase-separated regions), which dramatically affect mechanical properties, and alter the diffusion rates and biological activity of active molecules [17]. Therefore, hybrid hydrogels have been developed to address and ameliorate the issues to improve existing formulations as well as to expand the range of applications from medicinal (e.g., spatially and temporally controlled drug release profiles [19,20]) to optoelectronics and magnetic materials (e.g., high-tech applications such as fabrication of organic electronic devices with excellent charge transport and related properties [21,22]). Hybrid hydrogels are composed of chemically, functionally, and morphologically distinct building blocks, which can include biologically active proteins, peptides, or nano/microstructures, interconnected via physical or chemical means. Proteins and peptides that are incorporated into networks are generally reacted with synthetic polymers via polymerization or conjugation (click chemistry) strategies to yield hybrid hydrogels compatible for *in vitro* (cell differentiation, proliferation and migration studies) and *in vivo* (drug delivery, tissue engineering, and wound healing) applications [23]. Nano/microstructures can be encapsulated within the hydrogels via physical or chemical encapsulation to yield improvement in mechanical reinforcement as well as for controlled cargo delivery [24] or for sequestration of growth factors from the surrounding milieu [25]. Furthermore, the heterogeneity of hybrid hydrogels can improve cell adhesion, organization, and cell-cell interactions to create tissue constructs with enhanced mechanical integrity, electroactivity, and improved cellular organization [26,27]. This review will focus on recent developments in the production of nano/microstructured hydrogels and their potential use in biomedical applications.

Hybrid hydrogels incorporating nanostructures

Recently, nanoparticles have gained increased attention for potential applications in biomedical fields owing to their small size, surface functionality, stability, and drug loading capacity. Different approaches can be implemented to develop nanoparticle-hydrogel composites with varying nanoparticle types and varying bulk hydrogel frameworks, either through noncovalent or covalent immobilization strategies [28]. The homogenous distribution of nanoparticles within hydrogels is achieved by five main approaches: (a) hydrogel formation directly in a suspension of nanoparticles; (b) physically embedding of the nanoparticles into a hydrogel matrix after gelation (typically achieved in three steps: a preformed hydrogel is placed in aprotic solvent to release water; the shrunken gel is placed in nanoparticle suspended aqueous solution; and the hydrogel is washed with water to remove weakly surface-adsorbed nanoparticles); (c) reactive nanoparticle formation within a preformed gel; (d) nanoparticle-mediated crosslinking to form hydrogels; and (e) gel formation by crosslinking a mixture of nanoparticles, polymers, and distinct gelator molecules. These various methods are presented in **Figure 1** [28].

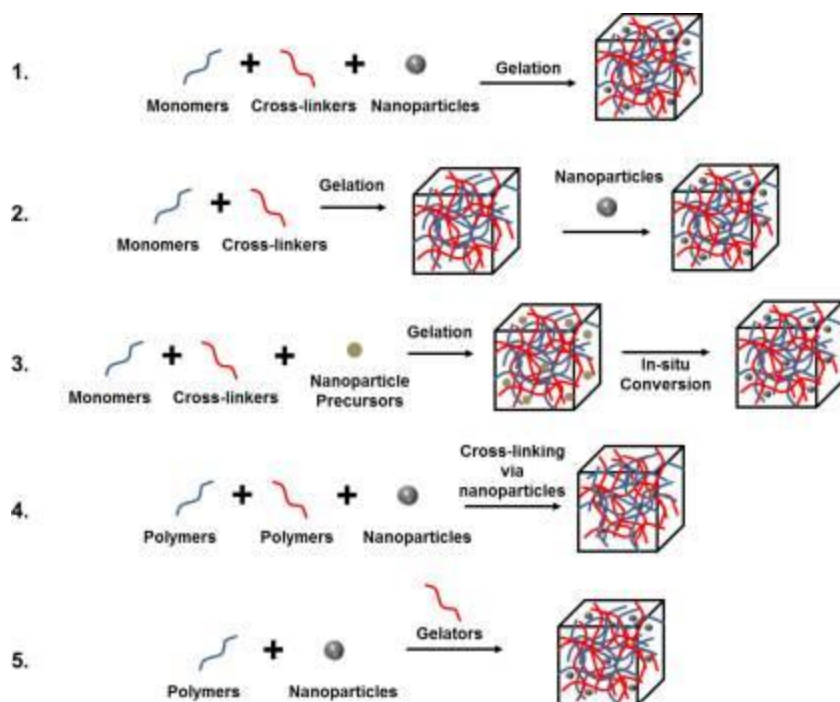


Figure1. Five main approaches that have been used to obtain hydrogel-nanoparticle conjugates with uniform distribution: (1) hydrogel formation directly in a suspension of nanoparticles; (2) physically embedding the nanoparticles into hydrogel matrix after gelation; (3) reactive nanoparticle formation within a preformed gel; (4) nanoparticle-mediated crosslinking to form hydrogels; and (5) gel formation by crosslinking a mixture of nanoparticles, polymers, and distinct gelator molecules. Adapted from Thoniyot et. al. [28]. (Reproduced with permission from John Wiley and Sons, Copyright 2015.)

Many types of nanoparticle-containing hydrogel networks have been aimed at enhancing the mechanical properties of the hydrogel, such as the incorporation of silica nanoparticles for improved mechanical stiffness, bioactivity of therapeutic molecules, and tissue stickiness as compared to unmodified hydrogels [29,30]. A 10-fold increase in stiffness of collagen-based hydrogels was realized following mixing with nitro-dopamine modified oleic acid coated iron oxide nanoparticles working as crosslinker epicenters that connect collagen chains on the surface of nanoparticles [31]. Incorporation of carbon nanotubes into methacrylated gelatin hydrogels led to a 3-fold increase in the hydrogel tensile modulus [26]. Addition of gold nanoparticles into poly(N-isopropylacrylamide) (PNIPAM) hydrogels enhanced shear modulus by 6-fold [32].

Conventional hydrogels have enabled controlled release of a variety of water-soluble therapeutic molecules; however, entrapment of hydrophobic therapeutic molecules has been limited [33]. These poorly soluble therapeutic agents have thus been encapsulated in nanoparticles such as micelles and liposomes to achieve controlled and sustained delivery. The direct loading of the hydrophobic drug dexamethasone acetate (DMSA) into poly-2-hydroxyethyl methacrylate (pHEMA)-based hydrogels for ocular drug delivery was reported [34]. Direct loading of DMSA in the hydrogel supported delivery for only three days, whereas DMSA-loaded poly(ethylene glycol)-poly(ϵ -caprolactone) (PEG-PCL) micelles, physically incorporated into the hydrogels, resulted in sustained release for up to 30 days –depending on the drug loading level. Erythromycin (a hydrophobic antibiotic drug) was encapsulated in Pluronic F-127 diacrylate macromer micelles, and hydrogels were developed by photopolymerization under a low-intensity UV light that sustained drug release for > 2 days [35]. A pH-responsive hydrogel containing poly(methyl methacrylate) (PMMA) nanoparticles, crosslinked with methacrylic acid grafted with poly(ethylene glycol) tethers, was developed for delivery of hydrophobic drugs [36]. A similar approach was used to develop a nanocomposite hydrogel for curcumin delivery that comprised gelatin and poly(3-hydroxybutyrate) (PHB) polymeric nanoparticles [37]. Uniform core-shell microparticles encapsulating cisplatin and paclitaxel were fabricated using coaxial electrohydrodynamic atomization technique and subsequently were embedded into an injectable hydrogel composed of a mixture of alginate-aldehyde and branched polyethyleneimine (PEI-25k). [38]. Controlled paclitaxel and cisplatin release for 45 days was evident with synergistic combination anticancer effects on MDA-MB-231 breast cancer cells.

Stimuli-responsive hybrid hydrogels, such as those desired for chemotherapeutic applications [39], have motivated the development of chemical and biochemical approaches for

hydrogel degradation and nanoparticle/drug release in response to specific stimuli. Liposome cross-linked hybrid hydrogels were developed by using the Michael-type addition of thiolated PEG-polymers with maleimide-linked liposomes, creating a hydrogel matrix that was capable of degradation in response to thiol-containing environments (i.e., GSH) (**Figure 3**) [40]. Liposomes encapsulating doxorubicin were prepared using maleimide-functionalized DSPE-PEG that cross-linked with alkyl- or aryl-thiol-functionalized four-arm PEG to form hybrid hydrogels. Controlled doxorubicin (DOX) release in PBS was observed with 25% drug release in 6 days, whereas addition of 10 mM GSH increased the cumulative release to 70% for the arylthiol-containing PEG-SH hybrid hydrogel. Furthermore, cytochrome c (CytC) also was encapsulated in the polymer network, enabling simultaneous release of DOX and CytC with differential release profiles driven by degradation-mediated release and Fickian diffusion, respectively. A bioactive nanocomposite hydrogel composed of hyaluronic acid and self-assembled pamidronate-magnesium nanoparticles (which acted as crosslinker for hyaluronic acid resulting in hybrid hydrogel formation within 30 sec) was developed for the localized elution and on-demand simultaneous release of bioactive ions (Mg^{2+}) and a small drug (dexamethasone phosphate) following application in bone defects [41]. Osteogenic differentiation of the hydrogel-encapsulated human mesenchymal stem cells (hMSCs) and subsequent activation of alkaline phosphatase was promoted by the released magnesium ions. The activated alkaline phosphatase then catalyzed dephosphorylation (activation) of dexamethasone phosphate (a prodrug of dexamethasone) and expedited the drug release for the promotion of osteogenesis to enhance bone regeneration in the hydrogel implantation site. Biodegradable polycarbonate-based ABA triblock copolymers (synthesized via organocatalyzed ring-opening polymerization) were formulated into chemically cross-linked hydrogels by strain-promoted alkyne-azide cycloaddition [42]. Doxorubicin loaded catechol-functionalized

polycarbonate micelles were encapsulated in the hydrogels to sustain the release of doxorubicin for one week (the cumulative drug release after 6 h in pH 7.4 media was 16 ± 1 % and 79 ± 3 % for micelles in hydrogels and free micelles, respectively). A pH-dependent doxorubicin release profile (i.e., higher release at pH 5.0 than at pH 7.4 due to doxorubicin's higher solubility at lower pH) was evident with a corresponding reduction in MDA-MB-231 cell viability ($< 25\%$). The significant alteration in MDA-MB-231 viability was attributable to accelerated drug release in the endolysosomes of cancer cells, ultimately enabling increased doxorubicin activity. These stimulus responsive behavior suggests the potential for such chemically degradable hybrid hydrogels as a potential platform for controlled and/or targeted delivery of multiple cargoes [41,43,44].

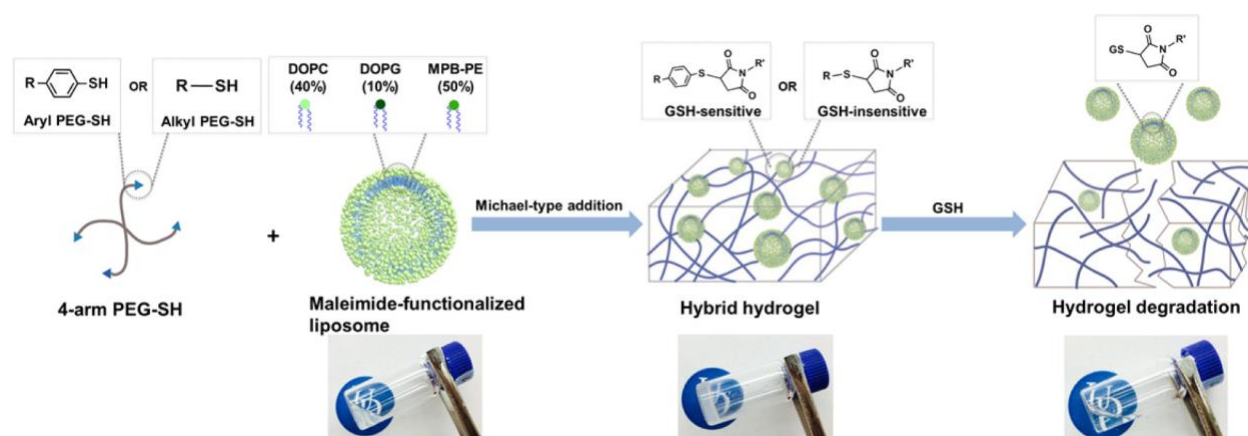


Figure 2. Schematic representation of the formation and degradation of the liposome cross-linked hybrid hydrogels. Adapted from Liang and Kiick [40]. (Reproduced with permission from the American Chemical Society, Copyright 2016.)

Hydrogels incorporating nanoscale structures also have been studied for tuning cell behavior and responses through optimization of the mechanical properties or an enhancement in the stability of hydrogels [45]. Although the mechanical properties of hydrogel can be controlled by the density and chemistry of cross-links, highly cross-linked 3D hydrogels may limit cell proliferation, migration and morphogenesis. Therefore, nanoparticles including carbon-based nanostructures, dendrimers, and ceramic nanoparticles in hydrogels can be used for the

development of biomimetic 3D environments enabling reconstruction of the complexity of native tissue for potential regenerative medicine and drug discovery applications [46,47]. Carbon-based nanostructures can be used to reinforce the mechanical properties of hydrogels [48]. Furthermore, the hybrid hydrogels can enhance cell proliferation [49,50] and differentiation [48] mediated by an increase in scaffold mechanical properties to match the properties of biological tissues such as bone, muscle and brain. Carbon-based nanocomposite hydrogels can also be used to engineer electrically conductive tissues such as muscles and cardiac tissues [26,48]. A 0.5 mg/ml carbon nanotube-gelatin methacrylate (CNT-GelMA) hydrogel improved compressive modulus by 3-fold when compared to 5% GelMA while facilitating cell survival and spreading [48]. Furthermore, the CNT-GelMA's higher biocompatibility and modulation of cell morphology for hMSCs is expected to promote hMSC differentiation to skeletal muscular, osteoblastic, and neural lineages dependent on their stiffness [48]. An enhancement in cell proliferation was achieved by using primary amine-terminated polyamidoamine (PAMAM) dendrimer crosslinked collagen scaffolds [50]. The stability of naturally-derived proteins like collagen also plays a vital role in cell proliferation. A significantly higher cell proliferation rate was achieved for the dendrimer-collagen scaffolds (225%) as compared to the collagen scaffold alone (150%), attributable to the increased biostability of collagen nanofibers with dendrimers. **Table 2** summarizes some of the examples of these hybrid hydrogels.

Table 2. Hybrid hydrogels encapsulating nanoparticles for tuning cell behavior and responses.

Hydrogel component	Nanoparticle component	Cell type	Outcome	Reference
Methacrylated gelatin	Gold nanoparticle	Adipose derived stem cells	Promoted osteogenesis	[51]

Methacrylated gelatin	Graphene oxide	Cardiac cells (H9c2, HUVEC)	Controlled VEGF gene delivery to the cells	[52]
Collagen	Functionalized bioactive glass nanoparticles	Mesenchymal stem cells	Improved stability to enzymatic degradation and shrinkage behavior of cells	[53]
Collagen/Polyvinyl alcohol	Nano hydroxyapatite	Osteoblastic cells (MC3T3)	Enhanced cell adhesion, proliferation, and differentiation	[54]
Oligo-poly(ethylene glycol) fumarate	Graphene oxide	Cardiomyocytes	Enhanced cell electrical signaling after implantation into myocardial infarct	[55]

Hybrid hydrogels incorporating microstructures

The addition of micron-sized particles or domains in a hydrogel network allows for better structural integrity and ultimately can serve as a method for directing cell behavior. It is well known that cells are sensitive to material properties such as elasticity, modulus, and pore size; therefore, taking advantage of these cell-modulating material properties is of great interest to develop materials that promote cell growth, proliferation, signaling, expression, and migration [56,57].

Despite the many outstanding properties of hydrogels for drug release and cell culture, they are typically isotropic materials that can only undergo uniform volumetric expansion and contraction in response to stimuli [58], in distinct contrast to the properties of ECM and various human tissues, which incorporate a vast array of microstructures such as spheres, tubules, and fibrils. Inspired by microscale structural motifs that occur naturally such as those mentioned, hybrid hydrogels that incorporate micron-sized domains have been developed to sustain cellular

growth, promote microstructure-mediated cell migration, and release bioactive agents in a controlled fashion.

Much like nanoparticles, micron-sized particles can be covalently or non-covalently incorporated in a hydrogel network to yield increased mechanical strength, release bioactive molecules, and promote a variety of intended cellular responses. The mechanical properties of a hydrogel upon addition of graphene derivatives (GDs) in a matrix of peptide nanofibers was investigated for a set of hydrogels made from three different gelling β -sheet-forming, self-assembling peptides and five GDs with different surface chemistries to investigate the molecular interaction between the matrix and the filler [59]. By varying the physicochemical properties of the peptides and the surface chemistry of the GDs, the effect of coupled electrostatic and hydrophobic interactions and the subsequent outcome of those interactions on the modulus was determined [59]. When both the long-range electrostatic interactions and hydrophobic interactions are attractive, there is an increase in G' , and when the interactions are in competition it is the relative strength of each contribution that determines the result on G' [59].

Overall, the release of bioactive molecules from hybrid-hydrogels can occur through one or more of three mechanisms: (1) burst release associated with the dissolution of absorbed drug on the hydrogel surface; (2) release due to drug diffusion through the hydrogel matrix and/or through the micro-particle; and (3) release due to degradation of the hydrogel [60]. These mechanisms for release of bioactive molecules offer handles that can be modulated to influence transport; accordingly, polymer network and particle composition can be manipulated to control mesh size and rates of chemical degradation, and bio-responsive domains can be incorporated to enable cell-responsive behavior [61,62]. For example, upon inclusion of vancomycin hydrochloride HMPC (Van-HMPC) nanoparticles in a chitosan/glycerophosphate (Ch/Gp)

hydrogel, there was a notable effect of extending the release of Van, attributed to a diffusional mechanism of transport [60]. In this case, the release of the drug is slowed by having to diffuse through both the microparticle and the hydrogel network. In another application, the release of an active drug species was tailored through generation of a carbohydrate based injectable hydrogel containing anionically-functionalized microgels (acrylic acid-functionalized poly(NIPAM)) [63]. In this system, the release of the drug bupivacaine was not only controlled through diffusive contributions, such as the cross-linking density of the hydrogel, but also through the partitioning affinity of the drug between the bulk and microgel phases, which allowed the initial burst release to be minimized.

However, the ability to tune the release from and into micro-domains is constrained due to geometry. With increased particle diameter, diffusion is limited as spheres possess a lower surface area to volume ratio [64]. In order to account for this shortcoming, particles of different geometries can be made to enable increased surface area and a correspondingly increased range in potential drug release rates. One such example is the potential of using spheroid particles generated by squeezing chitosan droplets between two superamphiphobic surfaces, followed by UV-crosslinking, to increase the release rate of encapsulated BSA. BSA was encapsulated in the particles and faster release was observed from spheroids than from spheres, shown in **Figure 3** [64].

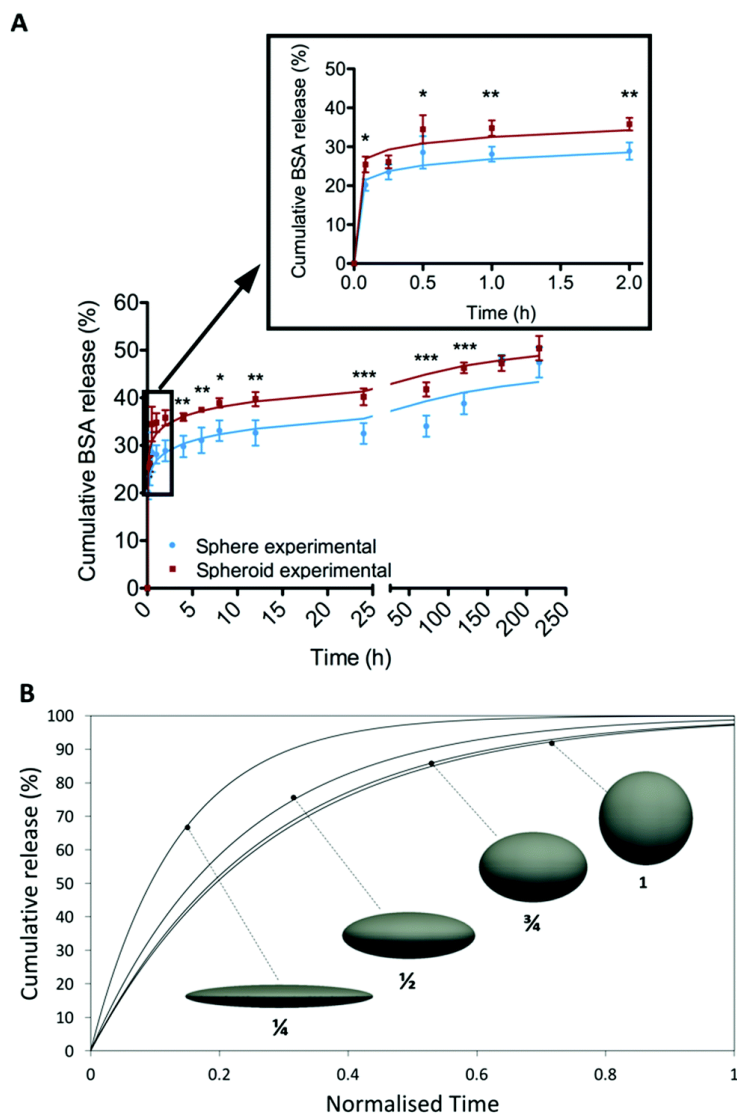


Figure 3: A) Cumulative BSA release from 2% M-CHT spheres (blue circles) and spheroids (62% deformation) (red squares), up to 216 hours, with a zoomed-in view for the initial 2 hours ($n = 3$). Values plotted are the mean $\pm$ error for 3 samples. Both experimental and curve fits using the Korsmeyer-Peppas equation are presented in the figure (lines with the same color of the corresponding symbols). Statistical significance of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) are represented for time-points when applicable. (B) Numerical modelling studies for the release of a compound from spherical versus spheroidal particles with varying particle height. Variation in height of spheroidal particles ($3/4$, $1/2$, and $1/4$) is presented relative to spherical particles, corresponding to 1. Adapted from Bjørge et.al [64]. (Reproduced with permission from the Royal Society of Chemistry, Copyright 2018.)

The benefits of anisotropic microparticles, such as spheroids, ellipsoids, rods, and disks, also have been shown to extend to hemodynamic settings, with both theoretical modeling and experimental studies indicating better margination, wall interaction, and adhesion [65]. The

enhancement of both diffusion and cell viability (demonstrated by the incorporation of spheroid particles) [64], as well as the potential hemodynamic transport benefits arising from anisotropic particles, suggests the potential of employing more complex and anisotropic particles in hybrid hydrogels. Non-circular micropatterned regions in hybrid hydrogels have also been shown to increase the absorption of toxins, thus increasing the detoxification efficacy of these materials [66]. Through hemolytic assays, it was shown that star shaped channels in a poly(ethylene glycol) diacrylate (PEGDA)/red blood cell membrane-coated nanoparticles (RBC-NPs) hybrid hydrogel resulted in relatively higher detoxification efficacy compared to circular channels, potentially due to the increased surface perimeter, multiple surface planes, and planar area [66].

Rather than the fabrication and subsequent incorporation of microparticles in hydrogels, a variety of other approaches have been used to produce hydrogels that include micron-sized domains such as liquid-liquid phase separation, emulsion stabilization, and photopatterning [67]. While all of these methods can yield desirable microstructure with various levels of control, the exploitation of phase separation has particular benefits, owing to the one-step fabrication, tunability, and specificity of the behavior [62]. These methods have been employed to produce microstructured resilin-like polypeptide (RLP)-PEG hydrogels in which liquid-liquid phase separation (LLPS) of the acrylate-functionalized RLP and PEG, combined with UV-crosslinking, is used to generate RLP-rich hydrogel microdomains that are dispersed in a PEG hydrogel matrix [62]. The RLP-PEG hydrogels support high cell viability as well as direct cell localization around RLP-rich domains. The domain size can be easily tuned by selection of the timepoint at which the phase-separating solutions are crosslinked after mixing, and selection of LLPS conditions can afford bicontinuous networks when crosslinking occurs during the initial stages of spinodal decomposition. **Figure 4** below [62] demonstrates the tunability of the domain size of the

microsphere structures with time and acrylate functionality. While this method is easily employed *in situ*, and offers tunability in terms of the size of the domains, the range of structures is limited.

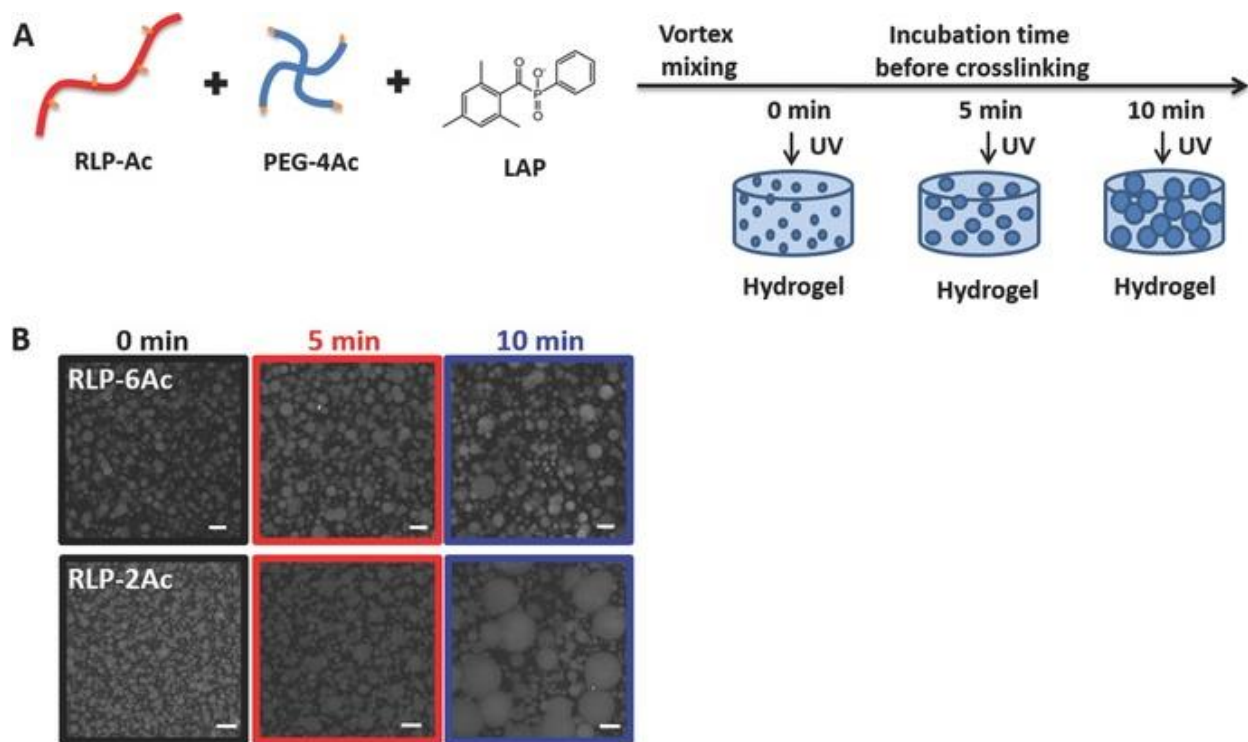


Figure 4: A) Schematic of hydrogel formation and microstructure development. B) Autofluorescence images of photo-crosslinked 10wt% 50/50 RLP-6Ac/PEG-4Ac and 10 wt% 50/50 RLP-2Ac/PEG-4Ac hydrogels. Microscale RLP-rich domains grow in diameter when gel precursors were incubated at room temperature for 0, 5, or 10 min prior to photo-crosslinking. Adapted from Lau et. al. [62]. (Reproduced with permission from John Wiley and Sons, Copyright 2018)

In order to develop hydrogels with detailed and specific microstructures, methods such as photopatterning and 3D printing have been used. However, techniques such as these can be labor-intensive and resolution-limited, which restricts production efficiency and fabrication flexibility [68]. In order to address these shortcomings, more rapid and equally accurate techniques are being explored, such as laser-mediated, UV photo-patterning [69]. Such photopatterning of gelatin hydrogels with riboflavin-5'phosphate as a non-toxic UV-photosensitizer has permitted more rapid

generation of micropatterned substrates with low variability and spatial resolution commensurate with that of traditional photolithographic and micromolding methods [68]. These approaches could be used in conjunction with casting and crosslinking of polymeric materials to generate hybrid-hydrogels with finely tuned domains. Furthermore, micropatterned gels could be stacked in order to generate multilayered heterogeneous materials with embedded high-resolution microchannels that allow for perfusion [70]. By use of silicon carbide as an adhesive to facilitate strong binding ($0.39 \pm 0.03\text{kPa}$) between micropatterned alginate and collagen hybrid hydrogel films, the result is a hydrogel with hollow microchannels ($150\text{ }\mu\text{m}$ - 1 mm) that allows for high cell viability ($90.61 \pm 3.28\%$) when tested over a 7 day period [70].

Biomedical applications of hybrid hydrogels

Hybrid hydrogels offer expanded opportunities in biomedical applications by enabling modulation of microscale hydrogel properties (suitable for cell adhesion, migration, and proliferation) as well as the inclusion and tailoring of drug or gene delivery features (suitable for microenvironment-sensitive and targeted therapy) (**Figure 5**). They have been employed as therapeutic interventions in a variety of conditions including wound healing [71,72], osteogenesis [51,73], cancers [39,74], myocardial infarction [75], Parkinson's disease [76], and infections [77,78] along with the development of biodevices and biosensors or contact lenses [79].

Of particular interest are hydrogels that incorporate biologically inspired molecules and delivery methods. A collagen mimetic peptide (CMP)-polyplex mediated delivery of platelet-derived growth factor-BB (PDGF-BB) genes from collagen gels improved PDGF-BB expression and promoted a diverse range of cellular processes associated with wound healing, including

proliferation, extracellular matrix production, and chemotaxis [72]. An injectable form of heat shock protein 27 (HSP27, molecular chaperones that protect heart muscle from ischemic injury) fused with TAT peptide and contained in a hybrid hydrogel system composed of poly(lactic-co-glycolic acid) (PLGA) microsphere and alginate hydrogel sustained the release of HSP27 for two weeks *in vitro* based on the pore size [75]. Intramyocardial injection of this hybrid hydrogel into a murine myocardial infarction model substantially suppressed apoptosis (3-fold decrease) in the infarction site, and improved the ejection fraction (2-fold increase), end-systolic volume (3-fold decrease), and maximum pressure development (1.3-fold increase) in the heart compared to PBS treatment [75]. A hydrolytically degradable hydrogel composed of PEG-b-poly(lactide)-b-dimethacrylate (PEG- b-PLA-b-DM)-containing, siRNA nanoparticles (siRNA/NPs) targeting Wwp1 (i.e., WWdomain-containing E3 ubiquitin protein ligase 1) was tested for sustained and localized delivery to bone fractures. These structures showed accelerated healing and increased bone formation in a murine mid-diaphyseal femur fracture model [80]. In another example, a phenylacrylamide nanoparticle hybrid hydrogel was prepared and abiotic hydrogel nanoparticles were engineered to bind to and modulate the activity of a diverse array of phospholipase A2 (PLA₂) and three-finger toxin (3FTX) isoforms found in Elapidae snake venoms, thus inhibiting the dermonecrotic activity of the venom in a dose-dependent manner [81], with potential to limit local tissue damage following a snake bite.

The development of biohybrid drug carriers to deliver various cargo to targeted sites is an area of research that could overcome limitations of synthetic nanoparticles such as suboptimal distribution, innate toxicity, and limited transport through biological barriers [82]. A noncovalent-bonded hybrid hydrogel was assembled by utilizing cucurbit[7]uril as a supramolecular linker to “stick” superparamagnetic γ -Fe₂O₃ nanoparticles onto the polymer backbone of catechol-

functionalized chitosan [83]. The γ -Fe₂O₃ nanoparticles displayed vibrational movement (to promote chemotherapy release) and heat generation under an alternating magnetic field supporting both thermo- and chemotherapy modalities *in vitro* and *in vivo* in xenograft models bearing HeLa cells. An advanced hybrid hydrogel system comprised of RBC membrane wrapped PLGA polymeric nanoparticles loaded in acrylamide gel was developed for the antivirulence treatment of local bacterial infection [77]. In a methicillin resistant *Staphylococcus aureus* (MRSA) subcutaneous mouse model, treatment with the nanosponge-hybrid hydrogel markedly reduced MRSA skin lesion development, attributed to the retention of pore forming toxins within the hydrogels and their subsequent neutralization [77]. No involvement of antibiotics in this unique treatment broadens the treatment area to antibiotic resistant bacterial infections and also rules out the possibility of the development of any new bacterial resistance.

However, hybrid-hydrogels are not limited in their biomedical application to only clinical implementation. Hybrid-hydrogels could address the need for *in vitro* systems that more closely represent physiological microenvironments for applications such as drug-screening and disease models or disease detection [84,85]. A versatile hydrogel containing DNA-capped gold nanoparticles for simultaneous and sensitive imaging of intracellular multiplex miRNAs was developed using a toe-hold strand-displacement reactions and hairpin-locked, DNAzyme-assisted miRNA recycling for dual signal amplification [86]. A wide linear range, low limit of detection, and good selectivity for simultaneous detection of multiple miRNAs was exhibited by the hybrid hydrogels. Given the abundance of intracellular cancer-related miRNAs in different cancer types, these structures pose enormous potential in accurate and sensitive differentiation of cancer cells. In order to study the delivery of soluble molecules to target cells in their native environment, *in vitro* systems that utilize hydrogels with tunable chemical, physical, and mechanical properties

that match native tissues can be used to determine cellular response [87]. In order to probe the nuanced relationship between the delivery of platelet derived growth factor-AA (PDGF-AA) and oligodendrocyte precursor cell (OPC) fate, a PEG-based hydrogel with encapsulated PLGA microparticles containing (PDGF-AA) was used to simulate seven different release schemes and study the effect of each on OPCs cultured in the hybrid hydrogel [87]. The results of these experiments support the hypothesis that burst release followed by withdrawal of PDGF-AA results in survival, proliferation, and differentiation of OCPs in the hybrid hydrogel and that OPC fate is dependent on release kinetics, rather than the amount of PDGF-AA delivered [87].

Table 3 summarizes studies involving a range of potential medical applications of hybrid hydrogels; these materials could also be used to as *in vitro* models of physiological microenvironments for applications such as drug screening [84,85].

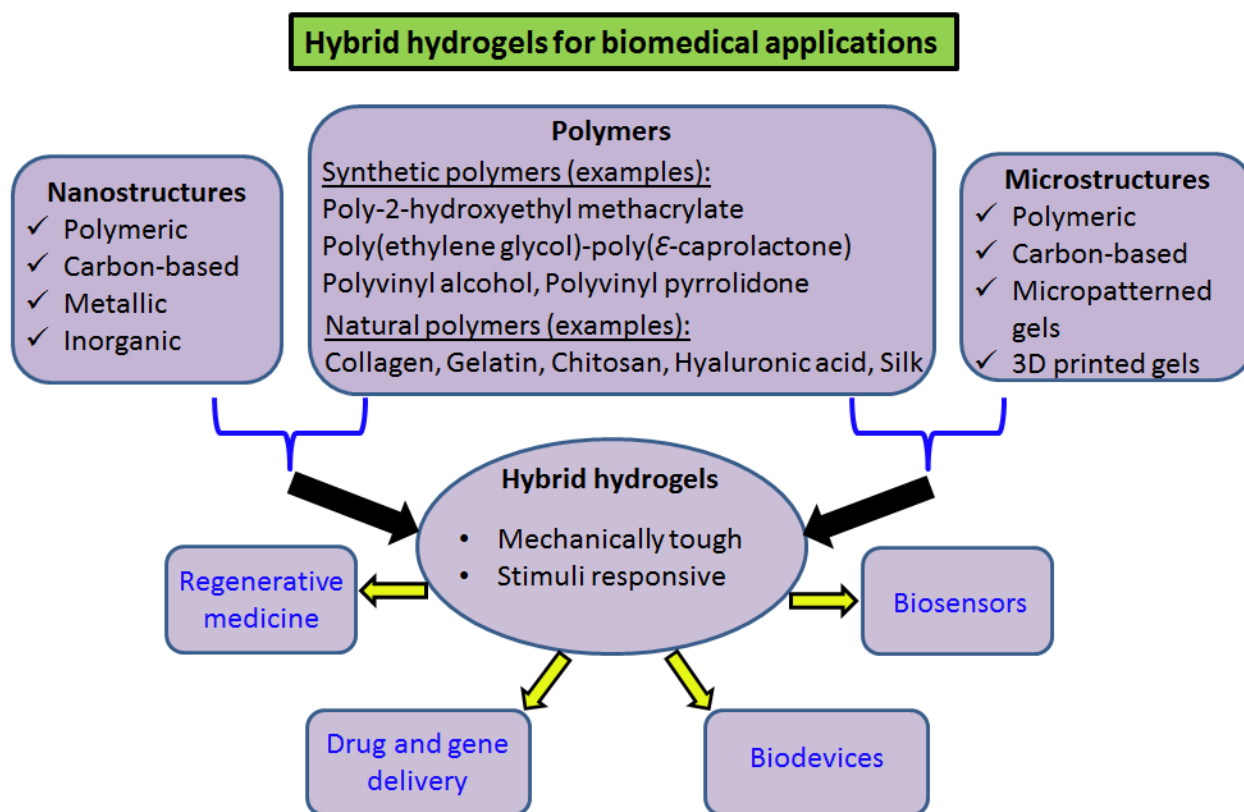


Figure 5: Examples of types of hybrid hydrogel compositions and select biomedical applications.

Table 3: Hybrid hydrogels and their reported biomedical applications

Hybrid hydrogel components	Nano/microstructure incorporation strategy	Nano/microstructure or drug release mechanism	Targeted disease	Potential outcome	Reference
Sodium alginate/PLGA microspheres loaded with HSP27 (heat shock protein 27) fused to TAT peptide	Physical incorporation of microspheres followed by alginate crosslinking with calcium sulfate	Pore size regulated HSP27 release from microspheres	Myocardial infarction	Suppression of apoptosis and improved ejection fraction, end-systolic volume and maximum pressure development in the heart	[75]
Sodium alginate/3D Ormocomp scaffold	Sodium alginate solution along with human adipose-derived stem cells (ASCs) were added to 3D scaffold and crosslinked using ionic cross-linking or RGD	The gel containing scaffolds are stable for eight weeks and ASCs produced dopamine	Parkinson's disease	Prevention of immune rejection of the non-autologous cells by the extremely small pore size of the alginate gel;	[76]

	based crosslinking method			higher dopamine secretion by hybrid hydrogel was evident as compared to conventional alginate hydrogel	
PECA [poly(ϵ -caprolactone)-acryloyl chloride]/COS-GMA (glycidylmethacrylated chitooligosaccharide)/NIPAm (N-isopropylacrylamide)/AAM (acrylamide)/Gold nanorods (GNR)/doxorubicin	Physical encapsulation of GNRs and doxorubicin in hydrogels developed by heat-initiated free radical polymerization using ammonium persulfate as a heat initiator	Temperature and pH dependent doxorubicin release profile; NIR laser irradiation of GNRs increased the temperature and thus improved doxorubicin release	Breast cancer	Significantly reduced post-operative tumor recurrence in <i>in vivo</i> mouse model	[74]
Acrylamide/RBC membrane coated PLGA nanoparticles (nanosponges)	Physical encapsulation of nanosponges before gelation	Pore forming α -toxin absorption by nanosponges	Methicillin resistant <i>Staphylococcus aureus</i> (MRSA) infection	Effective detoxification with marked reduction in MRSA skin lesion development in mouse models	[77]
Quaternized chitosan (HTCC)/silver nanoparticles/graphene oxide (GO)/voriconazole	Physical encapsulation of silver nanoparticles and GO in HTCC solution; electrostatic interactions between GO and HTCC resulted in hydrogel cross-linking	Voriconazole is loaded onto GO by π - π stacking interactions and released slowly	Fungal keratitis	Enhanced antibacterial properties <i>in vitro</i> and anti-fungal properties <i>in vivo</i> in fungal keratitis mouse model	[79]
Rapamycin-loaded unimolecular micelles/PLGA-PEG-PLGA	Physical dispersion in triblock gel	Diffusion of rapamycin from the unilamellar micelle dispersed triblock gel	Prevention of neointima-caused re(stenosis) after open surgery such as bypass surgery	Sustained rapamycin release for four months; inhibition of re(stenosis) by 80% even after three months as compared to no drug treatment	[88]
Carboxymethyl chitosan (CC)/Aldehyde	Physical encapsulation of VEGF loaded porous	Vancomycin linked to injectable hydrogel via the reversible Schiff's	Non-healing	Inhibited bacterial growth; accelerated vein	[78]

hyaluronic acid (AHA)/VEGF loaded porous PLGA microsphere/vancomycin	PLGA microspheres in CC/AHA hydrogel	base reaction is released by change in the pH from neutral to acidic in infected wounds; VEGF release was dependent on the pore size of PLGA microspheres	infected wounds	endothelial cell proliferation with reduced inflammation; promoted angiogenesis.	
Chondroitin sulfate (CS) and poly(ethylene glycol) (PEG)	FXIIIa –mediated crosslinking of CS-Mal grafted with MMP-Lys and PEG-Gln	The degree of CS grafting with MMP-Lys and stoichiometry of the hydrogel components dictated hydrogel properties, and the gel was degradable by chondroitinase and MMP to promote cell proliferation, migration, and viability.	Osteogenesis is	Tuned growth factor binding and release; generated a cell instructive matrix; promoted stem cell proliferation and osteogenic differentiation.	[73]

Conclusions and future perspectives

The incorporation of nano/microstructures in hydrogel formulations allows for the generation of hybrid hydrogels that can achieve a multitude of functionalities for applications in biological systems. Incorporation of particles and fabrication of domains not only allows for targeted drug therapy, tuned cellular response, and stimuli-responsive material behavior, but also allows for improved mechanical and physical properties. As the development of hybrid-hydrogels continues, the design of new systems will continue to be inspired by biological structures and motifs that closely resemble the environment for which they are intended. Hybrid hydrogels that are easily synthesized by one-step methods to achieve this level of architecture will be influential to the field, as well as those that can be precisely tuned in a modular fashion for the desired application. The potential to generate materials through facile methods that mimic the native environment of cells for a wide range of biomedical applications is germane to the field. As innovation and research progresses in the creation of these hybrid hydrogels, advances in chemical, biomedical, and materials engineering as well as chemistry, biology, and medicine is inevitable.

Acknowledgments

Related work in the authors' laboratories has been partially supported by grants from the National Institutes of Health (R01 AR067247A, RO1 DC011377A, and for instrument support: S10 OD016361, S10 RR027273, 1 P30 GM110758-01), the National Science Foundation (PFI-1700980, DMR 1609544), and the University of Delaware Research Foundation.

Conflict of interest

The authors declare no conflict of interest.

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Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

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