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Enhancing Biopolymer Hydrogel Functionality through Interpenetrating Networks

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Abstract

Traditional hydrogels are strong candidates for biomedical applications but suffer from drawbacks such as weak mechanics, static properties, and an inability to fully replicate aspects of the cellular microenvironment. These challenges can be addressed through the incorporation of second networks to form interpenetrating polymer network (IPN) hydrogels. The objective of this review is to establish clear trends on the enhanced functionality achieved by incorporating secondary networks into traditional, biopolymer-based hydrogels. These include mechanical reinforcement, 'smart' systems that respond to external stimuli, and the ability to tune cell–material interactions. Through attention to network structure and chemistry, IPN hydrogels may advance to meet challenging criteria for a wide range of biomedical fields.

Addressing the Need for Enhanced Properties in Biopolymer Hydrogels

Hydrogels (see Glossary) are water-swollen polymer networks that have demonstrated great utility in biomedical applications due to their tunable properties and ability to recapitulate aspects of native tissues. In addition to synthetic polymers for hydrogel formation, biopolymers derived from tissues [e.g., hyaluronic acid (HA), chondroitin sulfate, collagen, gelatin] or from natural materials (e.g., chitosan, alginate, cellulose) have gained attention in hydrogel design. Biopolymers are linked together to form hydrogels, either by leveraging their native intermolecular interactions or through chemical modifications that permit crosslinking. An advantage to using biopolymers for hydrogel formation is that many biopolymers exhibit inherent properties such as bioactivity, degradability, and biocompatibility. For example, HA is a non-sulfated glycosaminoglycan that exhibits unique viscoelastic behavior and plays an important role in regulating cell adhesion and tissue morphogenesis through specific chemical receptors.

Despite their promise, biopolymers also suffer potential drawbacks such as generally weaker mechanical properties, as well as wider distributions in molecular weights, undefined chemical compositions, and possible immune responses depending on how the biopolymer is sourced. For example, while hydrogels comprised of decellularized extracellular matrix (ECM) or **Matrigel®** are quite promising due to their native fiber arrangement and

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bioactivity, they often exhibit weak mechanical properties, heterogeneous structures, and there is limited control over the presentation of physicochemical signals.

Biopolymer chemical modifications and the introduction of various hydrogel crosslinking chemistries have expanded upon the library of attainable hydrogel properties from biopolymers; however, they may still fail to achieve all of the requisite properties for many biomedical applications [1]. Polymer blends and composite hydrogel formulations have enabled a wider range of hydrogel physical properties [2]; yet, these materials can often negatively impact encapsulated cells, undergo phase separation, and result in the deterioration of hydrogel properties over time.

To further improve upon the attainable and desirable properties of biopolymer hydrogels, there is growing interest in the design and incorporation of interpenetrating secondary networks. Interpenetrating polymer network (IPN) hydrogels are formed by the combination of independent, yet interdigitating polymer networks at the molecular level. IPN hydrogels differ from conventional composite hydrogels as IPN networks cannot be separated from one another without breaking crosslinks (Box 1). IPN hydrogels may be preferred over polymer blends due to their improved mechanical strength, efficient drug loading capacity, and controlled swelling behavior. Various physical and covalent chemistries can be leveraged for the crosslinking of individual networks within IPN hydrogels, with crosslinking occurring either simultaneously or in a sequential manner (Box 2). IPN hydrogels are generally characterized by their structure (topography, porosity, swelling), mechanical properties (tensile/compressive modulus, toughness, fracture energy), and bio-functionality (degradation, *in vitro* and *in vivo* biocompatibility).

By virtue of secondary networks, IPN hydrogels can readily achieve tough mechanical properties, smart responsiveness, and the ability to closely recapitulate complex cell–material interactions for applications in drug delivery, tissue engineering, ***in vitro*** **disease modeling**, and biofabrication (Figure 1, Key Figure). Synthetic polymers have also been widely used within IPN hydrogels; yet the objective of this review is to highlight the fabrication and use of IPN hydrogels that both consist of at least one biopolymer network and have demonstrated favorable *in vitro* and/or *in vivo* biomedical performance. Semi-IPN hydrogels, formed by the molecular interdigitation of a secondary polymer rather than a network, can also enhance the functionality of hydrogels (Box 3); however, the scope of this review has been restricted primarily to IPN hydrogels. Herein, we will introduce examples of IPN hydrogels where the design has improved their performance over single network (SN) biopolymer hydrogels. Throughout the review, we will rely on the terms ‘primary network’ and ‘secondary network’ as the standard naming convention for IPN hydrogels, although these terms are often interchangeable.

Mechanical Reinforcement through Interpenetrating Networks

The lack of mechanical strength in hydrated SN biopolymer hydrogels can be attributed to heterogeneous distributions of crosslinking sites, varying molecular weights between crosslinks, and the lack of stress dissipation mechanisms to prevent crack propagation [3,4]. Although increasing the polymer concentration or the crosslinking density can improve the

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mechanical properties of SN biopolymer hydrogels, these approaches can negatively impact cellular behavior by inhibiting nutrient transport or adversely affecting cell **mechanotransduction**. Thus, new strategies are needed.

Designing Tough IPN Hydrogels

To address these issues with the mechanical properties of SN hydrogels, the Gong laboratory first reported on the fabrication of double network (DN) hydrogels, a special class of IPN hydrogels formed by sequential polymerization of each network [3,5,6]. These hydrogels exhibit exceptionally high toughness under load because the failure of the primary, sacrificial network protects the secondary network from fracture via energy dissipation. Specifically, the rigid (primary) network may break into smaller clusters that further act as physical crosslinkers for the ductile, loosely crosslinked (secondary) network, allowing stress dissipation from the failure site into the surrounding area (Figure 2A).

Primary networks formed by free-radical polymerization often lack **self-recovery**, as rupture of these sacrificial networks causes permanent damage and reduced mechanics of the hydrogel under further loading [7]. To address this, **self-healing** primary networks have been introduced that allow reversible damage to DN hydrogels under deformation, enhancing their **fatigue resistance**. For example, self-healing catechol modified HA/methacrylated HA (MeHA) DN hydrogels have been fabricated via mussel-inspired reversible Fe^{+3}-catechol interactions [8].

Incorporation of dynamic covalent bonds such as acylhydrazone can also endow DN hydrogels with self-healing capabilities [9]. This approach allows DN hydrogels to recover their initial mechanical properties even after multiple loading and unloading cycles.

Alginate, an anionic biopolymer comprised of mannuronic and guluronic acid repeat units, has been widely adopted to form the primary network in DN hydrogels due to its tunable crosslinking density, high swelling capacity, and erosion under physiological conditions. Polyacrylamide (PAAm), owing to its elasticity and ease of fabrication via polymerization of acrylamide (AAm) in the presence of crosslinker and a photoinitiator, has gained tremendous attention as a synthetic secondary network in DN hydrogels. Sun and colleagues [10] reported on a highly stretchable and tough alginate/PAAm DN hydrogel that could sustain strains up to 20 times the hydrogel's original length, which is much greater than that of the SN alginate hydrogels. The hydrogel also demonstrated only a mild fibrotic encapsulation when implanted subcutaneously into a murine model [11]. Different multivalent cations used for ionic crosslinking of the alginate network also affect the mechanical strength and biological stability of the alginate/PAAm DN hydrogel [12]. For instance, Zn^{2+} crosslinked alginate/PAAm DN hydrogels exhibited remarkable antibacterial properties and accelerated wound closure in a rat model [13]. Since the development of these first alginate-based DN hydrogels, several similar studies have been reported on DN hydrogels formed by other biopolymers (e.g., agar, collagen, albumin) with PAAm as the secondary network [14–16].

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DN Hydrogels from Purely Biopolymer Components

In an effort to avoid the use of synthetic polymers such as PAAm in DN hydrogels, there has been a growing interest in the use of multiple biopolymer networks to form DN hydrogels. Some examples of these mechanically strong DN hydrogels include but are not limited to chitosan/gelatin methacryloyl (GelMA) DNs for cartilage repair [17], fibrin/HA/laminin DNs for neural and vascular tissue engineering [18], and MeHA/silk fibroin DNs for soft tissue engineering [19]. In all cases, the mechanical properties were enhanced with the DN hydrogel design when compared with SN hydrogels.

DNs have also been utilized to mechanically reinforce biological tissues through the sequential, *in situ* synthesis of an interpenetrating secondary network. For example, bovine osteochondral explants were soaked in phosphorylcholine-based monomer solution and crosslinked to form a seminatural-semisynthetic IPN hydrogel [20]. Owing to the physical entanglement of networks and cartilage interstitial fluid support, these IPN hydrogels exhibited excellent wear resistance [21]. Bilayered DN hydrogels for osteochondral tissue repair have also shown promise due to their ability to promote both cartilage matrix deposition and osteogenesis [22,23].

Improving DN Hydrogel Fabrication Processes

Despite the high water content of DN hydrogels, they exhibit synergistically higher toughness and **tensile strengths** as compared with their SN components. However, there are challenges to the traditional fabrication of DN hydrogels. First, swelling/diffusion of the second monomer/macromer into the first network lacks reproducibility and scalability, as it is often challenging to control the exact molar ratio and distribution of the monomer/macromer. Second, crosslinkers, photoinitiators, and processing conditions employed may be toxic to encapsulated cells. And third, multistep processes make it difficult to fabricate DN hydrogels into unique shapes.

To address these limitations, simple, one-pot synthesis methods have now been developed, often relying on thermoreversible sol–gel transitions [14] or simultaneous crosslinking through **orthogonal** chemistries (Box 2). DN hydrogels can also be processed into more complex shapes through the employment of molds or *in situ* crosslinking chemistries [24,25]. To avoid nonphysiological crosslinking conditions (toxic crosslinkers, high temperature, low pH), enzymatic crosslinking [26,27] and self-assembling peptides [28–30] have also gained tremendous attention for the formation of DNs. Additionally, metal-coordination complexes [31], hydrophobic associations [32], ionic interactions [33], and supramolecular bonding [34] have been explored for the formation of DN hydrogels.

Tough, Composite DN Hydrogels

Techniques have also been developed to convert composite hydrogels into extremely tough DN hydrogels. For instance, chitosan chains within chitosan/PAAm composite gels form dense microcrystalline structures or undergo chain entanglement when soaked in alkaline or saline solutions, respectively [35]. This one-step tailoring of chitosan physical networks provides excellent load-bearing and self-recovery capacity to the hydrogel (Figure 2B). Another study utilized a similar gelation and soaking protocol to fabricate tough chitosan/

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gelatin DN hydrogels [36]. Chitosan is an antibacterial, alkaline polysaccharide obtained by deacetylation of chitin, while gelatin is obtained by thermal denaturation of collagen; these biopolymers possess abundant hydroxyl (−OH) and primary amine ($-NH_2$) groups, respectively, that can be utilized for crosslinking, chemical functionalization, or conjugation of bioactive ligands.

Unlike traditional DN hydrogels, wherein energy dissipation is mainly borne by the primary network, these chitosan/gelatin 'conjoined' network hydrogels consist of intertwined networks with similar or equal energy dissipation mechanisms (Figure 2C). When these hydrogels are subjected to load, bonds within both networks break randomly to absorb energy, thereby resulting in high initial modulus. Upon continued deformation, the networks can withstand large strains due to their reduced crosslinking density. This addresses an important challenge within the field by increasing hydrogel stiffness and toughness simultaneously.

Tough, Adhesive DN Hydrogels

With regard to biomedical applications, hydrogels often suffer from poor adhesion to surrounding tissues, posing a challenge for their clinical translation. To overcome this limitation, surface mineralization techniques that promote bonding to bone at the hydrogel–tissue interface *in vivo* have been utilized [37,38]. The adhesion of IPN hydrogels can also be achieved without the need for surface modification by increasing the ability of the hydrogel to transfer stress through interfacial bonds [39,40]. A common approach involves the use of an interpenetrating, bridging polymer such as chitosan, collagen, or gelatin containing a positively charged primary amine to form bonds with the negatively charged surface of tissues, while also being compliant with their dynamic movement (Figure 2D). This strategy has been widely utilized to design biocompatible tough adhesives, where adhesion is greatly improved over SN hydrogel counterparts [41–43]. By contrast, antiadhesive properties are important for applications that involve frequent removal and replacement of hydrogels, such as in wound dressings. This has been addressed by the addition of a nonadhesive poly[3-dimethyl-(methacryloyloxyethyl) ammonium propane sulfonate] (PDMAPS) secondary network to chitosan/PDMAPS/poly(hydroxyethyl acrylate) (PHEA) multinetwork hydrogel [44].

Smart Responsive Interpenetrating Network Hydrogels

Although many hydrogels are static in their properties, the additional network within IPN hydrogels permits further design flexibility to the fabrication of smart responsive hydrogels where spatiotemporal properties can be engineered in the presence of extrinsic signals. These signals may include both physical (temperature, light, shear/strain) or chemical (pH, ionic strength, redox, enzymes) stimuli to modulate hydrogel properties. Although SN hydrogels can be designed to be responsive, the secondary networks within IPN hydrogels permit more modular control over individual network mesh sizes or selective network degradation to control hydrogel features (e.g., drug delivery) (Figure 3A) [45]. Examples of smart IPN hydrogels are shown later, categorized based on the stimuli used to alter hydrogel properties.

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Temperature-Responsive IPN Hydrogels

Poly(N-isopropylacrylamide) (PNIPAAm), a polymer with a phase transition temperature that can be designed to be close to physiological conditions, has been an attractive choice for temperature-responsive hydrogels. PNIPAAm hydrogels alone are incapable of sustaining long-term release of cargo due to their rapid collapse at temperatures above 32°C; however, the incorporation of secondary networks can increase the sequestration or allow the chemical conjugation of desired molecules within IPN hydrogels [46]. For instance, heparin was incorporated into thermoresponsive star poly(ethylene glycol) (PEG)-heparin/(PNIPAAm) IPN hydrogels, allowing growth factors such as vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2 (BMP-2) to be reversibly bound to the hydrogel [47]. Importantly, the thermal crosslinking of PNIPAAm in these hydrogels resulted in decreased mesh sizes and volumetric swelling ratios, further modulating long-term growth factor release (Figure 3B).

Glucose-Responsive IPN Hydrogels

Mechanical properties and hydrogel stability are important features in many biomedical applications. Smart IPN hydrogels can be designed to exhibit stimuli-responsive behavior without incurring substantial losses in mechanical integrity. For instance, silk fibroin-based IPN hydrogels were recently developed as smart microneedles capable of on-demand insulin release in response to local glucose concentrations [48]. Owing to the incorporation of silk fibroin, these microneedles demonstrated enhanced mechanical strength and remained intact for longer times after skin insertion when compared with SN hydrogels.

pH-Responsive IPN Hydrogels

Among various stimuli, pH responsiveness has gained special attention since it can be readily adapted to different tissues (e.g., tumor microenvironment, gastric fluid [49], colon [50]), where the design of the IPN hydrogel allows localized release of a bioactive payload based on the pH of the target tissue. For example, in the case of chronic wound dressings, hydrogels must possess high swelling capacity to absorb the secreted exudates, which has been achieved through chemically crosslinked PEG diacrylate (PEGDA) and acrylic acid networks within alginate hydrogels with pH modulation [51]. As compared with SN hydrogels, these IPN hydrogels supported increased ingrowth of keratinocytes in a 3D human skin model of chronic wound [51]. It has also been shown that high levels of reactive oxygen species (ROS) at wound sites can adversely affect healing. To this end, alginate/Rhodamine-B acrylate (RhB-Ac)-based IPN hydrogels were designed to undergo structural collapse in the presence of local ROS, thereby allowing controlled release of an encapsulated drug [52].

Beyond drug delivery, pH-sensitive IPN hydrogels have also been adapted to exhibit dynamic, **shape memory** properties that may be leveraged to create actuators or biosensors [53,54]. For instance, in case of agar/carboxymethyl chitosan (CMC) DN hydrogels, CMC exhibits metal–ligand chelation and pH-dependent solubility, important for shape-memory properties. Such IPN hydrogels become ideal candidates for shape memory applications as one network (e.g., agar) can be used to memorize the original shape, while the stimuli-

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responsive network (e.g., CMC) can be leveraged for reversible gel–sol transition, enabling shape recovery [54].

Magnetically Responsive IPN Hydrogels

In addition to local pH, IPN hydrogels have been designed to respond to exogenous triggers such as magnetic fields. Fe_3O_4 nanoparticles encapsulated in alginate/PAAm DN hydrogels [55] exhibited local magnetic induction heating in the presence of an external magnetic field, allowing for accelerated drug release due to increased diffusivity. By tuning the magnetic field strength or the composition of these magnetic hydrogels, noninvasive, tissue-specific heating behavior was achieved. Additionally, these magnetic DN hydrogels demonstrated high toughness and **ion-resistant stability**, two major drawbacks that limit the use of magnetic SN hydrogels.

Enzyme-Responsive IPN Hydrogels

Several IPN hydrogels have also been designed to undergo gradual degradation upon exposure to **proteases** (thermolysin, trypsin [27], papain [26]), such as those found in many tissues. For example, the utilization of matrix metalloproteinase (MMP)-degradable crosslinkers endows IPN hydrogels with enzyme-responsive behavior [56], including for *in vivo* imaging in **hyperthermia** cancer therapies with the incorporation of iron oxide nanoparticles [57]. The presence of two networks within IPN hydrogels allows selective, partial, or complete degradation of networks, toward controlled drug delivery. For example, Li and coworkers reported an IPN hydrogel in which the first network formed by self-assembly of DNA could be selectively digested by nucleases, while the second network formed by guest–host interactions between phenylalaninefunctionalized carboxymethyl cellulose and cucurbit[8]uril could be selectively cleaved by cellulase [34].

Light-Responsive IPN Hydrogels

Light-responsive IPN hydrogels are another option that have been engineered for remote, noninvasive release of therapeutics via morphological network changes (i.e., degradation or deformation) [58]. Near-infrared (NIR) light-responsive hydrogels were formed by incorporating polypyrrole (PPy) nanoparticles within an agarose/alginate DN system [59]. On demand release of a model drug was achieved by local melting of the agarose network facilitated by PPy nanoparticles and light exposure, while the nonthermal responsive secondary network retained the shape integrity of the hydrogel.

Mechanically Responsive IPN Hydrogels

The ability to fill irregular defects and administer cargo in a minimally invasive manner has made strain-responsive or injectable hydrogels promising vehicles for the delivery of cells, microspheres, and nanogels loaded with growth factors or bioactive drugs [60–62]. Upon injection, cell retention and survival *in vivo* constitute major challenges that can be effectively improved using an IPN approach [63–65]. Secondary networks incorporated into injectable IPN hydrogels can achieve clinically relevant mechanical properties, requisite crosslinking kinetics and stability required for injection [66], or allow favorable rheological properties [45] when compared with SN hydrogels alone.

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Another general strategy that has been adopted for injectable IPN hydrogels is to engineer *in situ* network formation [67]. An initially **shear-thinning**, self-healing network may be formed *ex vivo* to homogeneously encapsulate and protect cells during injection [68]. Then, postinjection, a second network can be crosslinked under physiological conditions to reduce the rate of network degradation, to enhance cargo retention *in vivo*, and/or to adhere with the surrounding tissue [69]. For instance, a supramolecular guest–host network between adamantane (Ad)- and β-cyclodextrin (CD)-modified HA can impart shear-thinning, self-healing properties to a hydrogel, while a secondary network that forms can provide increased mechanical integrity (Figure 3C) [70]. To this end, different crosslinking chemistries (physical or dynamic covalent) and additives (cellulose nanofibrils [71] or Laponite® [72–74]) have also been studied to tune rheological properties. This approach has been leveraged to render strain-responsive IPN hydrogels amenable to **extrusion bioprinting** [72,75]. For example, PEGDA-alginate-nanoclay inks containing $CaSO_4$ solution have been printed into various shapes and then exposed to UV light to allow stabilization of the PEGDA network (Figure 3D).

Multistimuli-Responsive IPN Hydrogels

Most ‘smart’ hydrogels reported thus far can only respond to a single environmental stimulus and it remains a challenge to develop multifunctional hydrogels with the ability to respond to multiple stimuli. To achieve this, IPN hydrogels can be fabricated with multiple ‘smart’ networks, wherein each network is capable of responding to a different stimulus. For example, IPN hydrogels could be endowed with pH and NIR responsiveness through a Fe^{3+}-catechol-modified poly(glycerol sebacate)-co-PEG network, while also rendered with injectable, self-healing characteristics through a ureido-pyrimidinone-modified gelatin network [76] (Figure 3B). Similarly, other dual responsive systems based on temperature/strain [77] and temperature/pH [78,79] have been developed to offer additional control over the delivery of therapeutic drugs and proteins.

Interpenetrating Networks for Studying and Modeling Cell–Material Interactions

In addition to enhanced mechanical properties and stimuli-responsive behavior, the inclusion of secondary networks within IPN hydrogels can enhance their use in studies of cell–material interactions. Hydrogel properties such as stiffness, **viscoelasticity**, porosity, topography, and ligand presentation can greatly influence cell behaviors such as adhesion, proliferation, migration, and differentiation [80]. However, many of these aforementioned properties are coupled together and cannot be readily tuned in an independent manner using SN hydrogels. IPN hydrogels, however, are excellent tools to decouple the effects of certain factors on cell behavior (Figure 4A) [81]. For instance, the stiffness of IPN hydrogels may be adjusted by increasing the polymer concentration or crosslinking density of the first network, while the presentation of ECM ligands or ECM composition may be independently tuned via the second network [82]. The below examples highlight where IPN hydrogels have been leveraged to improve studies of cell–hydrogel interactions.

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Controlling Cell Adhesion and Topography with IPN Hydrogels

The integration of cell-adhesion peptides (e.g., **RGD peptide sequences**) within synthetic SN hydrogels is widely adopted to permit cell adhesion; however, unlike biopolymers, these peptides can only interact with a limited set of integrins and their inclusion fails to mimic the native topography of ECM. To address this, biopolymer IPNs have been used to incorporate cell-adhesion ligands or to impart protease sensitivity to hydrogels, while conserving tunable mechanics. Examples of this include fibrin/alginate [83] and gelatin/PEG [84], where the fibrin or gelatin was used to facilitate adhesion and degradation, while the alginate or PEG was used to control mechanics. As another example, electrically conductive poly(thiophene-3-acetic acid) (PTAA) SN hydrogels have been previously prepared for cardiac tissue engineering, but normally undergo rapid hydrolysis and lack innate cell-adhesion sites. To improve the hydrogel properties, methacrylated aminated gelatin (MAAG) secondary networks were incorporated in PTAA hydrogels to introduce cell adhesion and hydrogel stability towards facilitating the cardiomyogenic differentiation of brown adipose-derived stem cells [85].

With regards to hydrogel topography, collagen is an important structural protein and an excellent choice of biopolymer to recreate the hierarchical architecture of the native ECM. In comparison with many traditional SN hydrogels that are nonfibrillar, interpenetrating networks of collagen with alginate [86], HA [87], or chitosan [88] can effectively capture aspects of the fibrous organization of native tissues, while affording an additional network through which hydrogel properties can be modulated independent of the collagen. In addition to collagen, self-assembling peptides can also be used within IPN hydrogels to mimic native ECM topography.

IPN Hydrogels to Improve Hydrogel Stability

Secondary networks can also be utilized to impart enhanced hydrogel stability to support long-term cell culture and characterization [18,89,90]. For example, traditional **immunostaining** methods to study cell–material interactions often require substantial washing of hydrogels, which may cause physically crosslinked hydrogels to fail. To address this challenge, secondary networks may be utilized to improve hydrogel integrity. Towards this, cell-laden DNA supramolecular hydrogels were immersed into AAm monomer to form a secondary PAAm network, increasing the stability of the hydrogel without compromising embedded cell position or morphology [91]. This technique can be extended to other weak SN hydrogels for cell immobilization or imaging. IPN hydrogels are also expanding upon capabilities for bioimaging by harnessing unique features of the secondary network, such as with the photoluminescent property of sericin within sericin/alginate IPN hydrogels that has been exploited for *in vivo* bioimaging [92].

Investigating Mechanobiology with IPN Hydrogels

While elastic hydrogels are known to influence cell behavior through the generation and transmission of **traction forces** via focal adhesions, increased attention is being focused on the roles of viscoelasticity [93,94] and **viscoplasticity** [95] on cellular mechanotransduction. IPN hydrogels have been designed with tunable dissipation properties independent of their elastic moduli to better recapitulate and elucidate the roles of these mechanical properties on

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cellular outcomes. For example, reconstituted basement membrane matrix and alginate were formed into IPN hydrogels that exhibited differential viscoplasticity independent of stiffness, allowing the roles of ECM viscoplasticity on cancer cell migration to be elucidated (Figure 4B). While cancer cell migration is often attributed to protease-dependent processes, studies with IPN hydrogels revealed protease-independent migration via plastic deformation and opening of micron-scale pores by the protrusive forces of **lamellipodia** [95].

Cell volume confinement is another mechanosensing mechanism for cells in 3D environments, where elastic stresses in some nanoporous SN hydrogels may restrict ECM formation and affect embedded cell function. Stress-relaxing properties can be included through secondary networks to facilitate hydrogel remodeling by cells or deposited matrix and to support differential cell behavior [96,97]. For example, incorporation of a stress-relaxing supramolecular network within a covalently crosslinked HA hydrogel favored mechanosensing, spreading, and subsequent differentiation of encapsulated human mesenchymal stem cells (hMSCs) (Figure 4B) [98].

Spatial Patterning of IPN Hydrogels

The ample functional groups (e.g., hydroxyl, carboxyl) present along the backbone of biopolymers can also be used for chemical derivatization. Biopolymer-based IPN hydrogels can thus be easily patterned with cell-instructive cues (e.g., viscoelasticity, stiffness, cell-signaling ligands) towards achieving dynamic, spatiotemporal cell behaviors. However, since conjugation of signaling ligands may compromise or alter the innate bioactivity of some biopolymer networks [99], secondary networks can be employed to enable hydrogel patterning while conserving the bioactivity of the primary network. As an example, norbornene-modified HA as a secondary network facilitated the orthogonal **photopatterning** of hydrogel constructs with thiolated molecules via click reaction [75]. In another study, IPN hydrogels comprised of photocrosslinkable MeHA and **Puramatrix®** were designed to study the influence of stiffness on neurite growth and proliferation, including when patterned for spatial control over mechanical properties (Figure 4C) [100]. In addition to adding new material, photo-degradation techniques can also be used to spatially soften IPN hydrogels via degradation of a target network [101].

Biofabrication of IPN Hydrogels

IPN hydrogels also allow for improved processing of hydrogels in microfluidics [102], bioprinting [72], and high-throughput screening devices [103]. For example, IPN hydrogels comprising agarose and gelatin were fabricated to create perfusable microvasculature-on-a-chip devices with well-controlled mechanics to model the disruption of endothelial barrier function during hemato-logical and inflammatory diseases (Figure 4D) [102]. Agarose, due to its minimal swelling, enabled high resolution pattern transfer, whereas gelatin allowed multilayer assembly, which together resulted in mechanical properties similar to native tissue.

The inclusion of secondary biopolymers into IPN hydrogels can render nonviscous hydrogel inks amenable to extrusion bioprinting [104], permit secondary crosslinking to increase stability of 3D constructs postprinting [70,75], or be used as sacrificial templates to

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introduce perfusable channels or voids within a printed construct [105]. With the development of **embedded printing** techniques, IPN hydrogels have also been printed into granular support materials [106]. A major limitation for 3D printing of SN hydrogels is the achieved print resolution; however, an alternative printing strategy was recently developed using IPN hydrogels. Specifically, primary networks (e.g., MeHA, GelMA, alginate) that are polyanionic are first printed and then subsequently submerged in a solution of polycationic chitosan to form semi-IPN hydrogels. When formed, these semi-IPN hydrogels undergo syneresis via charge complexation, resulting in the controlled shrinkage of the printed hydrogels to achieve enhanced print resolution [107].

Optical projection lithography and other 3D printing strategies have also been leveraged to construct IPN hydrogels with high resolution, complex structures [108]. To achieve this, solutions containing alginate and AAm are first projected with digital UV light patterns to enable covalent crosslinking of AAm and build a 3D structure. The printed construct is then submerged in a calcium bath to allow ionic crosslinking of alginate, thereby forming an IPN hydrogel [108].

Concluding Remarks and Future Perspectives

The use of biopolymers has grown for a wide range of biomedical applications, particularly as there are a number of advantages to the incorporation of biopolymers into biomaterials over purely synthetic materials. Biopolymers harness innate features due to their origin, such as desirable cell interactions, degradability, intermolecular interactions, and physical structures. Despite many examples where biopolymers are used as SN hydrogels, there are advances being made by incorporating biopolymers into IPN hydrogels. This review summarized a number of important areas of advancement where biopolymer IPNs have achieved desired mechanics, responsiveness to stimuli, and cell–material interactions.

From the perspective of clinical translation, recent IPN hydrogel systems with favorable *in vivo* application are summarized (Table 1). Owing to their excellent load-bearing capacity, IPN hydrogels have been most widely studied for musculoskeletal tissues. However, they can be adapted for other target tissues such as lung, gut, cardiac, and neural with the same precision. The tunability of IPNs may also allow application in new biomedical areas, such as the growing field of **immunomodulatory** biomaterials. IPN hydrogels inherently necessitate more components than SN hydrogels, which may pose a regulatory challenge for future clinical translation. However, it is expected that continued advancements in material commercialization and the use of biopolymers will support the future translation and implementation of IPN hydrogels in clinical settings.

In addition to translatable tissue engineering and regenerative medicine approaches, IPN hydrogel-based multifunctional systems are strong candidates for fields such as mechanobiology, organ-on-a-chip technology, biosensing, drug screening, and development (see Outstanding Questions). For example, IPN hydrogels have been used as model platforms to investigate the role of ECM mechanics on transfection for gene delivery [82] and transport of extracellular vesicles as paracrine signals [109]. Further, IPN hydrogels have been employed to model *in vitro* tumor microenvironments to explore the independent

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effects of tumor ECM composition and mechanics on cancer cell malignancy or invasion, tumorigenicity, and drug resistance [81,110–112]. Remote field (magnetic, electrical, optical, and acoustic) enabled technologies that have been promising for directed spatial assembly of tissue engineering components (e.g., controlling matrix fiber alignment to guide cell behavior) may also be implemented in combination with IPN hydrogels to further their utility [113].

There are still advances to be made with respect to IPN hydrogel mechanical properties and structure. For instance, there is an emerging interest in further reinforcement of IPN hydrogels using nanofillers (i.e., Laponite® [114], calcium polyphosphate [115], cellulose nanocrystals [116], or inorganic polyhedral oligomeric silsesquioxane [7]). As the aggregation of nanofillers negatively impacts reinforcement, future strategies must address the uniform distribution of reinforcing nanomaterials. To that end, a few recent studies have utilized micro/macroscale structures as sacrificial networks within DN hydrogels [117]. For instance, macro-DN hydrogels formed by a gellan gum microgel reinforced gelatin network [118] exhibited higher strength and a higher osteogenic behavior of MC3T3-E1 preosteoblasts than molecular scale bulk gellan gum/gelatin DN hydrogels [119]. Furthermore, the ability to mimic features such as anisotropy and tension-compression nonlinearity, important for load-bearing tissues, remains a challenge to the field. Incorporation of 3D bioprinted [120] or woven fibers [121] into IPN hydrogels can provide control over internal architecture and mechanical properties at multiple length scales and future studies could focus on tuning the fiber arrangement to mimic the native organization of collagen fibers within load-bearing tissues.

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Highlights

The extracellular matrix (ECM) is a complex assembly of biopolymers, the organization and composition of which combine to provide structural, mechanical, and biochemical signals to cells. Although single network hydrogels recapitulate features of the ECM, further advancements are needed to expand their functionality for many applications.

Incorporation of secondary networks into biopolymer hydrogels imparts mechanical reinforcement, the ability to respond to stimuli, and increased mimicry of the ECM. These interpenetrating polymer network hydrogels are promising for tissue engineering, drug delivery, and *in vitro* disease models for drug discovery and screening.

Addition of a second network makes conventional hydrogels amenable to many emerging biofabrication techniques geared towards achieving hierarchical architectures and personalized medicine.

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Glossary

Embedded printing: approach by which material is extruded through a nozzle via pressure and deposited in a supporting hydrogel to stabilize printed structures.

Extrusion bioprinting: approach by which material is extruded through a nozzle via pressure and deposited layer-by-layer to create a 3D construct.

Fatigue resistance: ability of a material to sustain cyclic loading without fracture or failure through structural damage or crack propagation.

Hydrogel: 3D crosslinked hydrophilic polymer network that swells in an aqueous environment.

Hyperthermia: therapy in which tissues are exposed to heat (at temperatures higher than normal body temperature), commonly practiced with radiation or chemotherapy.

Immunomodulatory: ability to control (activate or suppress) and modify (proversus anti-inflammatory) the host immune system response.

Immunostaining: standard techniques that employ dye-tagged antibodies to detect and visualize the presence of various molecules.

***In vitro* disease models**: an approach to mimic cell and tissue structures *in vitro* to gain insights into mechanisms of human disease or to develop and screen the efficacy of new therapeutics.

Ion-resistant stability: ability to retain original structure and/or mechanical properties even after exposure to an ionic solution, typically physiological fluids for biomedical applications.

Lamellipodia: cytoskeletal branched actin protein filaments that provide force for membrane protrusion and form the leading edge of migrating cells.

Matrigel®: basement membrane rich in ECM proteins such as laminin, collagen IV, and heparin sulfate proteoglycans secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma and used to form hydrogels for cell culture.

Mechanotransduction: biological mechanism by which cells convert mechanical stresses to biochemical signaling.

Optical projection lithography: microfabrication approach based on light-induced crosslinking to create high resolution patterns or constructs.

Orthogonal: (referring to) independent reactions that occur concurrently without influencing the properties of each other.

Photopatterning: decoration or chemical functionalization of substrates with ligands, crosslinkers, or peptides in a controlled pattern (using a photomask) through optical exposure.

Protease: enzyme that can cleave/degrade proteins or peptides.

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Puramatrix®: self-assembling peptide hydrogel comprised of amino acids with alternate hydrophilic and hydrophobic side chains.

RGD peptide sequence: arginine-glycine-aspartic acid peptide sequence identified in adhesive proteins that can promote cell adhesion through integrin binding.

Self-healing: ability to recover the original state and properties upon releasing an applied stress or strain.

Self-recovery: ability to regain original mechanical properties after sustaining multiple loading and unloading cycles.

Shape memory: ability of a material to return to its original shape from a deformed state induced by external stimuli.

Shear-thinning: ability to exhibit a reduction in viscosity and to flow under shear stress or deformation.

Tensile strength: stress that a material can withstand under tension (stretching)without breaking.

Traction forces: force applied by cells to a substrate (i.e., extracellular matrix) that allows spreading and migration.

Viscoelasticity: ability of a material to exhibit time-dependent deformational behavior via both elastic (slow stress-relaxation) and viscous (fast stress-relaxation) properties.

Viscoplasticity: ability of a material to exhibit rate-dependent deformational behavior.

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Outstanding Questions

With the expansion of attainable hydrogel properties via the formation of IPNs, what additional empirical or computational methodologies may be employed to rapidly screen IPN hydrogel designs?

In what settings are multicomponent IPNs (greater than two networks) more advantageous than IPNs comprising just two networks to justify their increased complexity?

Can ‘smart’ IPN hydrogels be designed to achieve sequential delivery of multiple bioactive factors, for both the *in vitro* and *in vivo* engineering of tissue repair and tissue structures?

Can multilevel patterning and other biofabrication technologies be developed to leverage the dissimilar networks within IPN hydrogels?

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Box 1.

Defining Terminology around Interpenetrating Polymer Networks

IPN hydrogels are formed by the combination of two or more topologically interlocking crosslinked polymer chains. The resulting two or more polymer networks within these IPN hydrogels are mutually independent yet held together by internetwork entanglement. This approach increases the functionality of hydrogels when compared with single network (SN) hydrogel designs. IPN hydrogels comprising more than two distinct interpenetrating networks may be referred to as multicomponent network hydrogels [122,123].

Double network (DN) hydrogels are a special class of IPN hydrogels that are characterized by: (i) two networks that are interpenetrating yet independent, with asymmetric and contrasting properties; and (ii) the molar concentration of the secondary network being 20–30 times higher than that of the primary network. The primary network with high stiffness (i.e., brittle) acts as a sacrificial network, whereas the secondary network is ductile and can sustain large deformations, resulting in synergistically high strength and toughness. Ionic-covalent entanglement hydrogels are DN hydrogels formed by an ionically crosslinked rigid network and a covalently crosslinked elastic network, which allows self-healing of the primary network upon rupture [124,125].

In contrast to IPN hydrogels, hybrid or composite hydrogels are SN hydrogels that include nano- or microstructures, which are often attached physically or via chemical conjugation strategies. Some of these hydrogels have demonstrated noteworthy improvements in mechanical properties, which are often additive of the individual components; however, they often require large amounts of reinforcing agents to achieve desirable mechanical properties. Commonly employed fillers (e.g., Laponite®, gold nanorods, hydroxyapatite, carbon nanotubes) may aggregate over time and undergo irreversible failure, which limits their self-healing ability.

Another hydrogel design that has been implemented to tailor hydrogel properties includes ‘dual crosslinked’ [126,127] or ‘bicomponent network’ [128] hydrogels. These are SN hydrogels formed by two or more chemically different polymers that are crosslinked together using single (e.g., purely covalent) or multiple types (e.g., both covalent and physical) of crosslinking. Although these hydrogel systems combine the desired properties of various polymers, they are typically unable to achieve the mechanical properties or cell interactions that are possible with IPN hydrogels.

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Box 2.

Synthesizing Interpenetrating Network Hydrogels

A wide range of physical or covalent crosslinking methods have been employed towards the formation of biopolymer IPNs (Figure I), with synthesis occurring primarily through either sequential crosslinking or simultaneous crosslinking.

The sequential crosslinking of IPN hydrogels entails a multistep process. For biopolymer networks, the primary network is first formed by the crosslinking of biopolymers through physical or chemical interactions, which is then immersed in a solution of a monomer or functional macromer that then diffuses throughout the primary network. The secondary network is then formed by the polymerization/reaction of the monomer/macromer. This is the most commonly adopted technique to fabricate IPN hydrogels, such that the two polymer networks interpenetrate each other. Poor swelling capacity of a primary neutral network can hinder the diffusion of the monomer/macromer into the primary network. This problem can be circumvented by a novel molecular stent approach [129], wherein a linear, strong polyelectrolyte (e.g., glycosaminoglycans such as chondroitin sulfate or HA) is incorporated into the primary network, generating a higher osmotic pressure difference and a higher swelling ratio. The molecular stent method allows simultaneous incorporation of biopolymers without any chemical modifications. Additionally, these molecular stents can undergo degradation in lieu of the networks, conserving the structural integrity of the entire IPN hydrogel for a longer period of time.

By contrast, simultaneous crosslinking involves the direct mixing of monomers/functional macromers (e.g., biopolymers) that correspond to the networks with simultaneous crosslinking via independent routes to form an IPN hydrogel [130]. This allows control over the ratios of the two networks and is far less time consuming since diffusion is not needed, providing a facile, one-pot fabrication route to IPN hydrogel formation. However, this approach may lead to entanglement of the macromers before crosslinking or undesired chemical interactions between the networks, which can negatively impact their mechanical performance or functionality. Hence, IPN hydrogels fabricated via simultaneous crosslinking require that the kinetics of polymerization/crosslinking of the two polymer networks are manipulated to promote interpenetration and avoid phase separation. DN hydrogels formed via sequential crosslinking have demonstrated superior mechanical properties and cell-adhesion characteristics when compared with those fabricated through simultaneous crosslinking [131].

More recently, some studies have utilized a combination of the two techniques for IPN fabrication, wherein the macromers are mixed simultaneously but crosslinked in a sequential manner. It is also important to note that the sequence of network synthesis can influence the properties and functionality of the IPN hydrogel.

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Box 3.

Semi-Interpenetrating Polymer Network Hydrogel

Semi-IPN hydrogels are formed by the incorporation of a secondary polymer within a crosslinked SN hydrogel. Semi-IPNs can be fabricated similarly to IPN hydrogels, where the second polymer may be added via diffusion into an already crosslinked primary network or by selective crosslinking of a primary polymer (or polymerization of a monomer) with itself in the presence of a secondary polymer (that itself is never crosslinked into a network or tethered to the primary network). Semi-IPNs are similar to polymer blends, as the constituent secondary polymer can be separated from the primary crosslinked network without breaking any chemical bonds.

Since the mechanical strength of semi-IPN hydrogels is inferior to IPNs, they are not widely used for load-bearing applications or where mechanical properties drive the hydrogel functionality. However, these hydrogels have gained attention for the sustained delivery of antitumor therapeutics [132] or recombinant proteins [133]. For example, SN alginate hydrogels could be endowed with temperature responsiveness by incorporating elastin-like polypeptides (ELPs) [134], making them attractive for cell encapsulation and delivery. Biocompatible semi-IPN hydrogels capable of multistimuli response behavior have also been developed for controlled and sustained release of drugs, proteins, and growth factors [135]. The incorporation of hydrophobic payloads into hydrophilic hydrogels is often challenging; therefore, SN hydrogels comprising secondary, emulsifying polymers such as PVA have also been employed to achieve uniform encapsulation of hydrophobic drugs [136].

There are numerous examples where semi-IPNs have included biopolymers to control cell behaviors. For example, the incorporation of secondary biopolymers such as HA [137], or Matrigel® [138] within SN hydrogels allowed higher cell spreading and migration. Highly porous and interconnected Matrigel®-collagen semi-IPNs have also enhanced porcine valve interstitial cell viability and improved the response to *in vitro* cyclic stretching [139]. Using soft lithography techniques, photo-crosslinkable collagen/HA semi-IPN hydrogels could be patterned into cell laden microchannels and microstructures useful for microscale tissue engineering [140].

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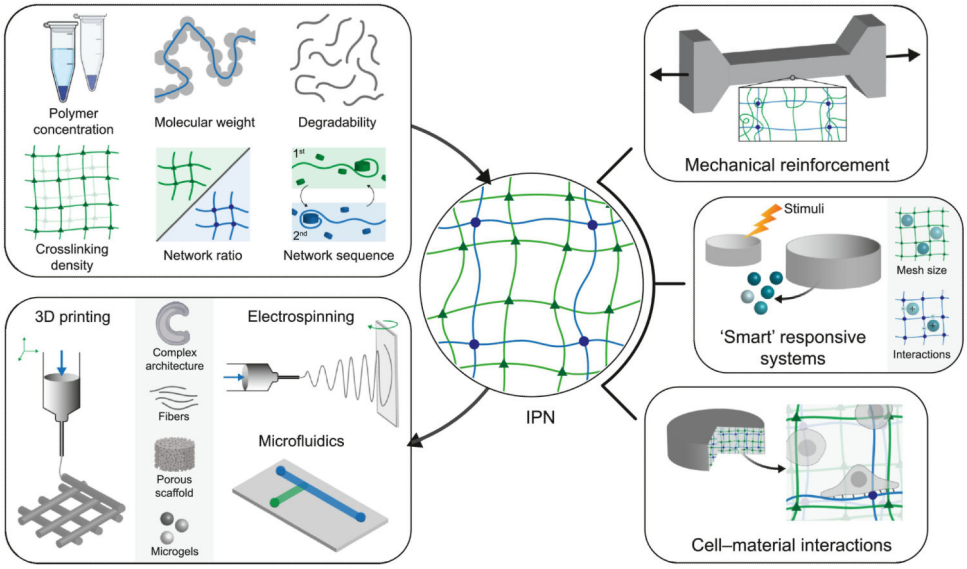

Figure 1. Key Figure. Overview of Interpenetrating Polymer Network (IPN) Hydrogels Employed for Applications in Tissue Engineering, Regenerative Medicine, Drug Delivery, and *In Vitro* Disease Models

In comparison with conventional single network hydrogels, the incorporation of secondary networks to form IPN hydrogels (two or more independent, yet entangled networks shown as green and blue) often results in advantageous properties such as enhanced mechanical strength, the ability to respond to stimuli, and mimicry of the native cellular microenvironment. The tuning of polymer components (concentration, charge, molecular weight, degradability, cell-adhesion ligand density) and control over network parameters (crosslinking density, network ratio, and sequence of formation) allow for the synthesis of functional IPN hydrogels with diverse properties. IPN hydrogels can be processed into various shapes and architectures as cell-laden or acellular structures using a variety of techniques (i.e., 3D printing, electrospinning, microfluidics, and lithography).

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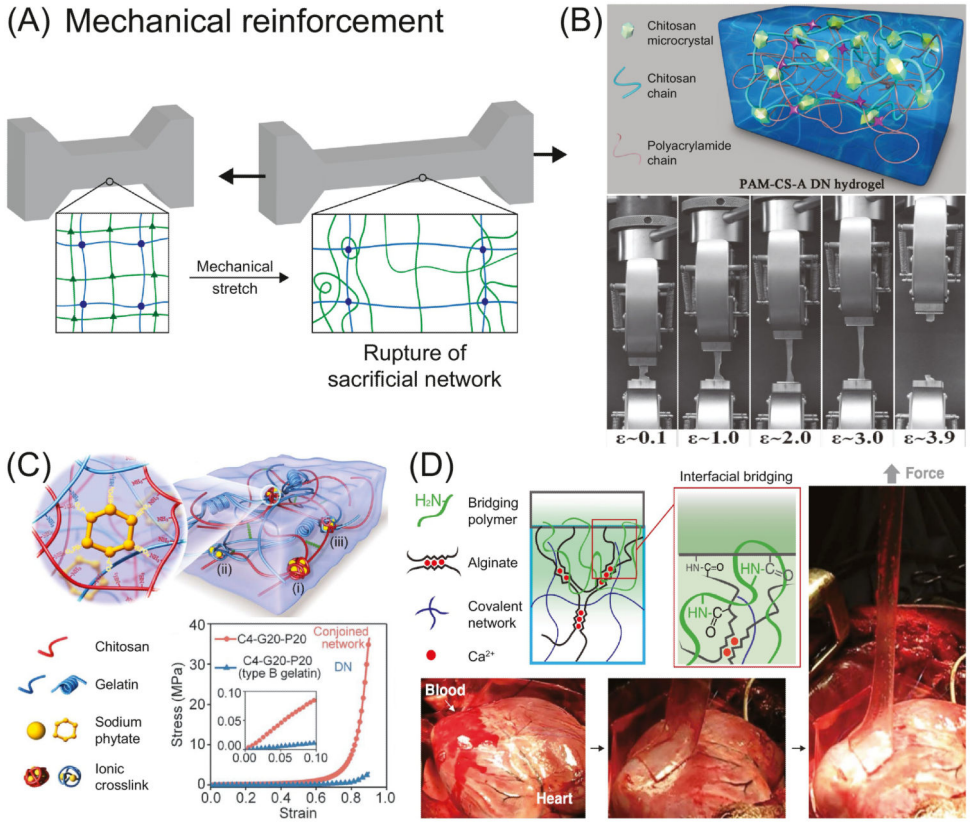

Figure 2.

Mechanical Reinforcement of Hydrogels through an Interpenetrating Secondary Network. (A) Schematic representation of energy dissipation facilitated by rupture of a sacrificial primary network under tensile loading. (B) Investigation of the load-bearing capacity and crack resistance of a notched double network (DN) hydrogel via tensile stretching. The hydrogel was comprised of a chitosan/polyacrylamide DN soaked under alkaline conditions to yield a chitosan microcrystalline network (PAM-CS-A DN). Adapted, with permission, from [35] (C) Chitosan/gelatin/phytate (C-G-P) formed an interpenetrating polymer network (IPN) hydrogel (i.e., conjoined network) through noncovalent interactions. Graphical representation of tensile stress-strain curve for DN and conjoined network hydrogel. Adapted, with permission, from [36] (D) Design of an alginate/polyacrylamide (PAAm) DN tissue adhesive consisting of a dissipative matrix (made of ionically and covalently crosslinked networks) and adhesive surface through a bridging polymer with primary amine groups. *In vivo* hydrogel adhesion performance on a beating porcine heart with exposed blood. Adapted, with permission, from [41].

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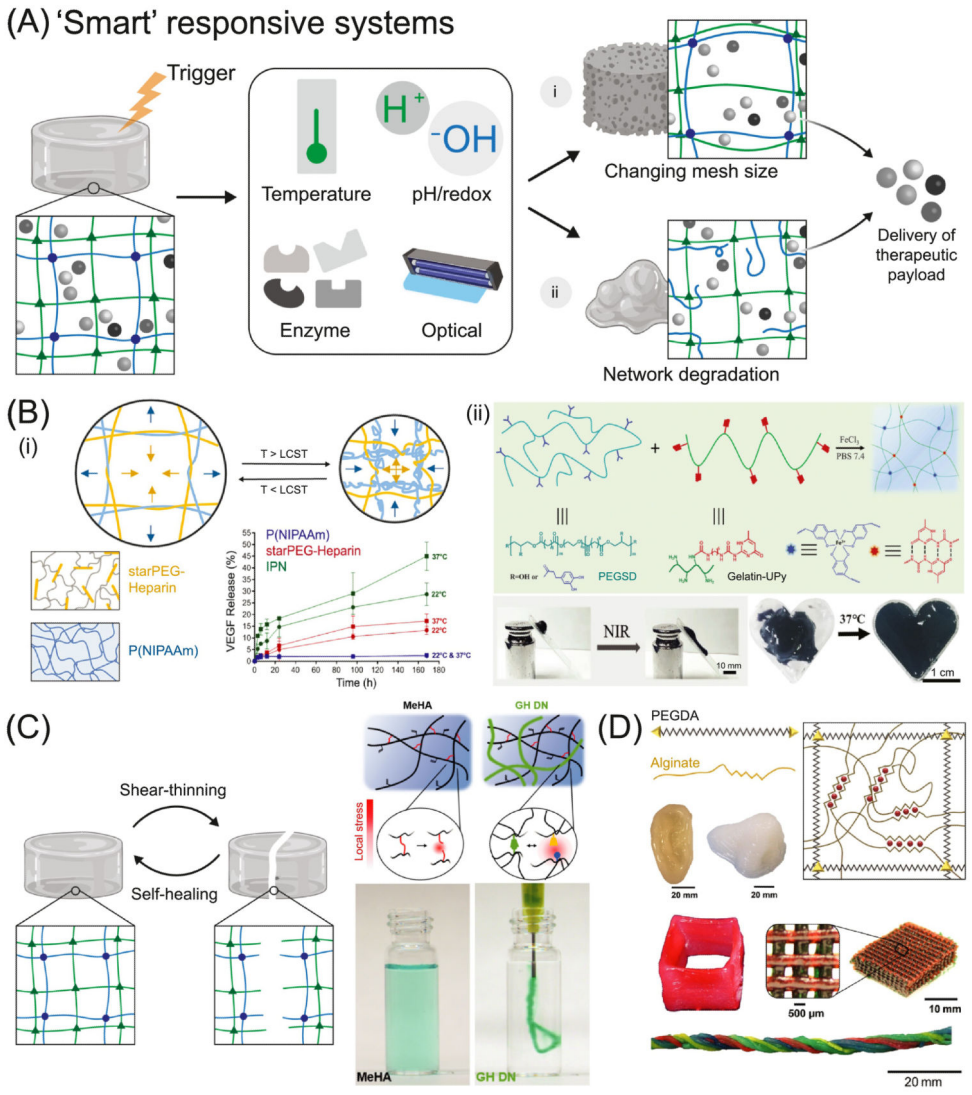

Figure 3.

Building 'Smart' Hydrogels through an Interpenetrating Polymer Network (IPN) Approach. (A) Schematic illustration of the release and delivery of a therapeutic payload (bioactive drugs, proteins, growth factors, nucleotides, cells, spheroids) via a stimuli-responsive IPN hydrogel. The release can be facilitated either via: (i) changes in the hydrogel mesh size, or (ii) through selective network degradation. (B) (i) Temperature-induced collapse of Poly(N-isopropylacrylamide) (PNIPAAm) [at temperature higher than lower critical solution temperature (LCST)] results in tighter physical entanglement between an IPN of PNIPAAm and heparin and increased release of vascular endothelial growth factor (VEGF) when compared with single network hydrogels. Adapted, with permission, from [47]. (ii) Schematic illustration of IPN hydrogel comprised of poly(glycerol sebacate)-co-poly(ethylene glycol)-g-catechol (PEGSD) and ureido-pyrimidinone (UPy) modified gelatin. Gel–sol transition of the IPN hydrogel occurs when irradiated with near infrared (NIR) (808 nm) or increase in temperature (37°C). Adapted, with permission, from [76]. (C) Shear-thinning and self-healing behavior of IPN hydrogels that permit extrusion through a syringe.

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Whereas single networks [methacrylated hyaluronic acid (MeHA)] disperse rapidly when injected into an aqueous environment, the double network [supramolecular adamantane (Ad)-HA and β-cyclodextrin (CD)-HA guest–host (GH) network and covalently crosslinked MeHA network) is stable due to the presence of reversible physical bonds. Adapted, with permission, from [70]. (D) Schematic representation of components [alginate and poly(ethylene glycol) diacrylate (PEGDA)] for a 3D printable double network hydrogel ink. The 3D printed hydrogel constructs in the shape of ear, nose, hollow cube, porous mesh, and twisted bundle. Adapted, with permission, from [72].

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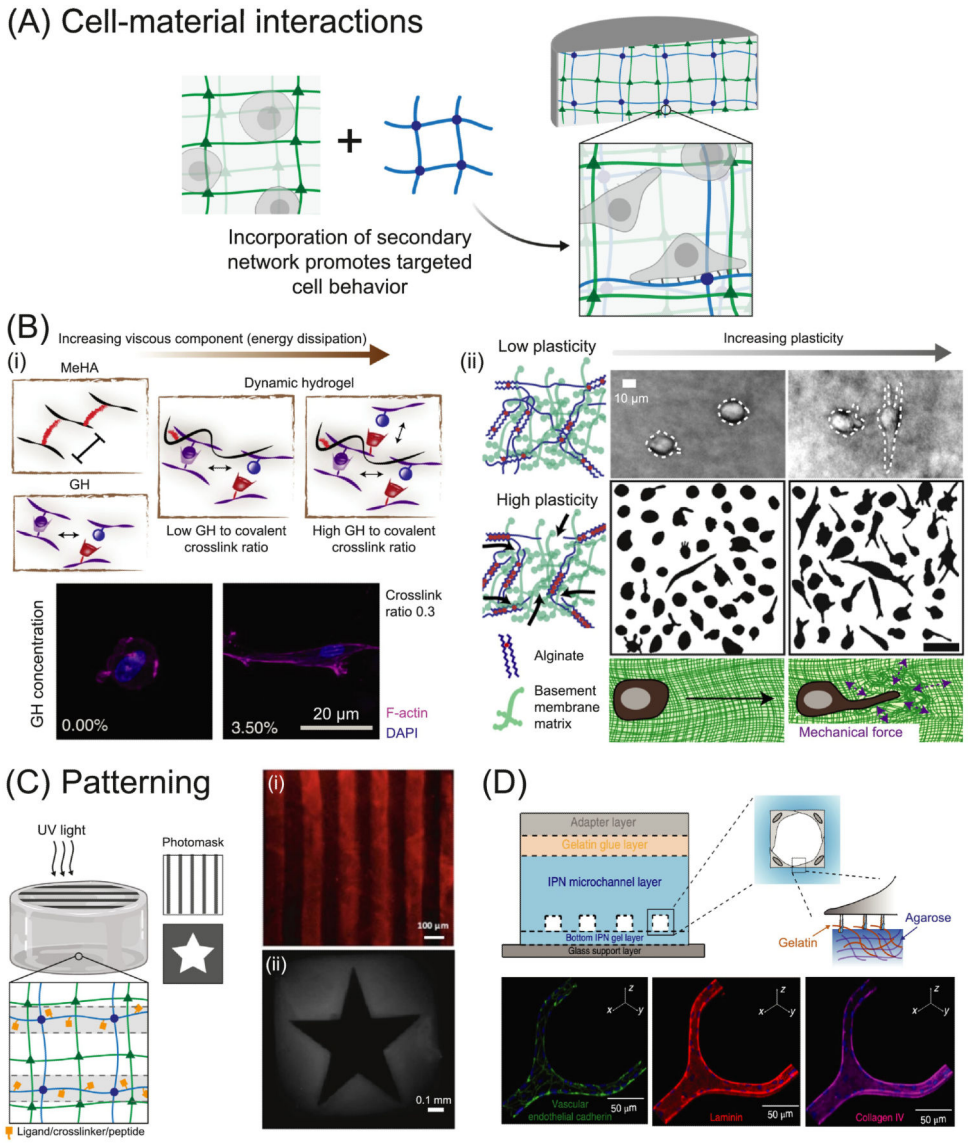

Figure 4.

Interpenetrating Network Hydrogels Designed to Tune and Study Cell–Material Interactions. (A) Schematic illustration of enhanced cell behavior within an interpenetrating polymer network (IPN) hydrogel. (B) (i) Schematic of dynamic, viscoelastic IPN hydrogel formed by the combination of guest–host (GH) interactions [between adamantine- and cyclodextrin-modified hyaluronic acid (HA)] and covalent crosslinking [methacrylated hyaluronic acid (MeHA)]. Cytoskeletal staining (i.e., actin) indicates increased cell spreading as a function of the concentration of the viscous GH network. Adapted, with permission, from [98] (ii) Design of viscoplastic IPN hydrogels consisting of alginate and reconstituted basement membrane matrix. Enhanced matrix plasticity promoted increased spreading and motility of MDA-MB-231 breast cancer cells, independent of protease activity (arrows indicate differences in molecular weight). Adapted, with permission, from [95]. (C) Schematic representation of photopatterning or photostiffening IPN hydrogels. (i) Hydrogel discs formed from hydrazide-aldehyde-modified HA and covalently crosslinked norbornene-

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modified HA were photopatterned with thiolated rhodamine. Adapted, with permission, from [75]. (ii) Photo-micropatterning of IPN (bright) and semi-IPN (dark) regions within glycidyl methacrylate-modified HA/self-assembling peptide (Puramatrix®). Adapted, with permission, from [100]. (D) Microvasculature-on-a-chip model fabricated using agarose/gelatin IPN hydrogel. Human umbilical vein endothelial cells seeded on the microfluidic device attach to gelatin and appropriately remodel the microenvironment, as seen *in vivo*, illustrated with confocal images after 14 days for cadherin and the basement membrane proteins laminin and collagen IV. Adapted, with permission, from [102].

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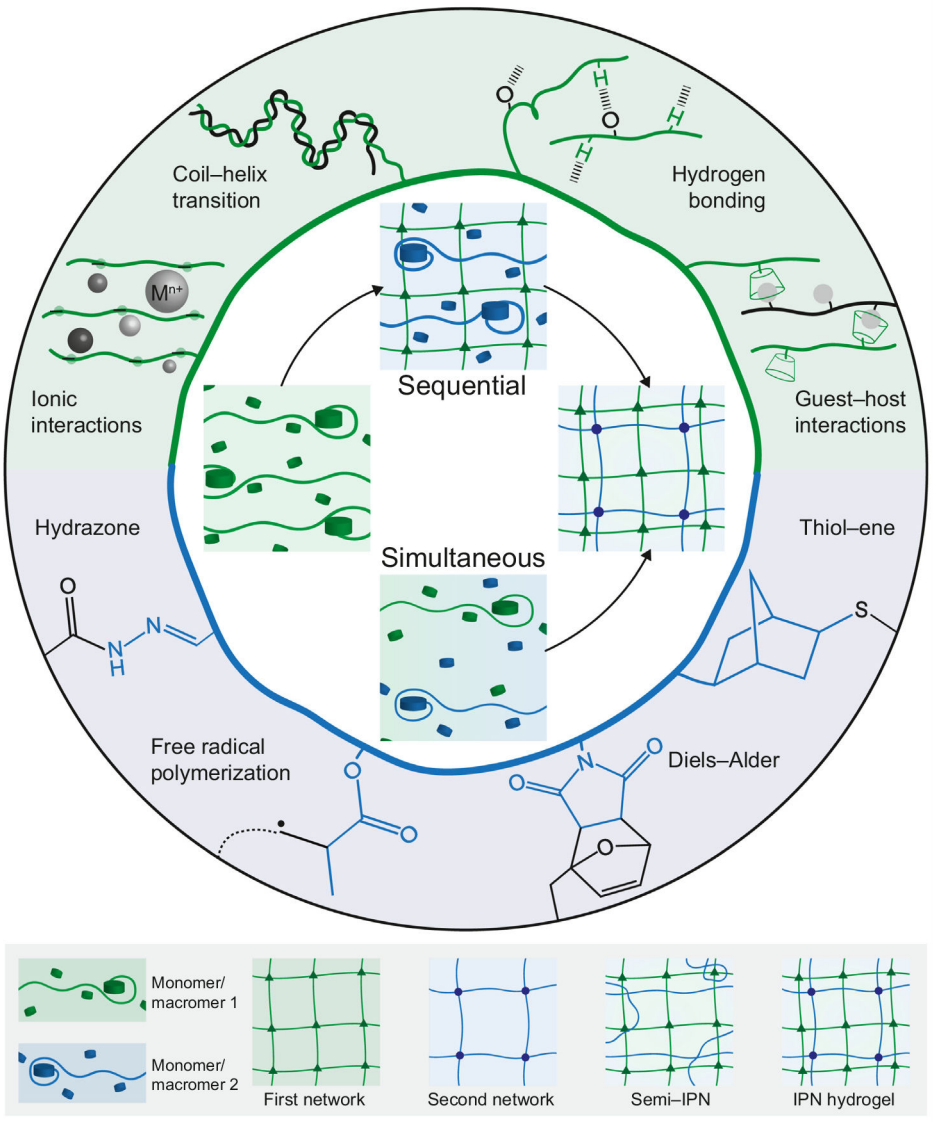

Figure I. Synthesis of Interpenetrating Polymer Network (IPN) Hydrogels.

IPN synthesis techniques can be broadly classified into sequential (swelling of first network in a secondary monomer/macromer) or simultaneous (orthogonal crosslinking of both first and second networks). The selection of biopolymer and type of crosslinking chemistry utilized for each network influences the interactions between networks within IPN hydrogels. Representative modes of physical crosslinking (top, green) include ionic crosslinking (e.g., alginate, chitosan), temperature-induced coil–helix transition (e.g., agar, gelatin), hydrogen bonding (e.g., cellulose, dextran), and hydrophobicity (e.g., guest–host-modified biopolymers). Chemicals such as glutaraldehyde, genipin, or carbodiimide-based crosslinking can be used for the direct crosslinking of biopolymers via primary amines on biopolymers such as gelatin, chitosan, or collagen. Biopolymers can also be chemically modified with functional groups for covalent crosslinking (bottom, blue), such as acylhydrazone, diol-boronic acid, Schiff-base, Michael addition, Diels-Alder, disulfide

linkage, azide-alkyne or thiol-ene/thiol-yne click chemistry, and free-radical polymerization (e.g., methacrylates, methacrylamides).

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Table 1.

Representative Examples of Crosslinking Chemistries Used for the Formation of IPN Hydrogels That Have Been Explored for *In Vivo* Applications

Chemistry	First network	Second network	*In vivo* model	*In vivo* response	Refs
Physical crosslinking of first network					
Ionic	Alginate	Poly(Y-glutamic acid) crosslinked by lysine	Full-thickness cartilage defect in the trochlear grooves of rabbit femurs	Achieved good integration between the neo-subchondral bone and the surrounding host bone	[23]
	Alginate	PAA	Implanted in a rabbit calvarial defect	New bone formation at the interface of host bone and hydrogels, suggesting improved osseointegration	[115]
	Chitosan	PEGDA	Full-thickness skin defect model in rats	Accelerated *in vivo* wound healing by reducing inflammation and increasing angiogenesis	[141]
Coil to helix transition	Agarose	4-arm PEG-NH2 and 4-arm PEG-NHS	Subcutaneous implantation into rats	Reduced foreign body reaction while achieving enhanced fibroblast proliferation	[25]
	Gellan gum	Poloxamer-heparin (PoH)	Dorsal subcutaneous implantation in athymic nude mice	BMSC seeded hydrogels exhibited high cell adhesion and infiltration while maintaining low inflammatory response	[142]
Hydrogen bonding	Chitosan	PVA	Rabbit lateral femoral condyle bone defect model	Significant increment in differentiation, osseointegration, and new bone growth	[38]
	Chitin	PVA	Full excision wound model in rats	Accelerated wound healing and tissue remodeling process by promoting epithelial cell regeneration and fibroblast migration while also exhibiting antibacterial activity	[58]
Metal-ligand coordination	Catechol-modified CSMA	CSMA	Mouse hemorrhaging liver model and full thickness mouse cutaneous wounds infected by bacteria	Rapidly formed a bleeding barrier upon *in situ* gelation and promoted wound healing, granulation tissue formation, and angiogenesis	[69]
	PGS-co-PEG-g-catechol	Ureido-pyrimidinone-modified gelatin	Full-thickness skin defect model in rats	Better wound closure and healing of skin incision than medical glue and surgical suture and significantly accelerated collagen deposition, granulation tissue formation, and vascularization	[76]
Self-assembly	Self-assembling peptides	Copolymerization of acrylamide, acrylate-terminated multi-armed PEG and MeHA	Full-thickness cartilage defects in rabbit model	High regeneration capacity along with formation of uniform and smooth cartilage that was well integrated with the surrounding tissue	[28]
	Pluronic F127-g-chitosan	Furan-modified gelatin with maleimide-functionalized PEG	Subcutaneous administration into nude mice	High retention and survival of cardiomyocytes embedded within the hydrogel	[65]
	Gelatin nanoparticles	Platelet-rich fibrin	Implanted in the back of nude mice	Able to withstand the pressure within rabbit sinus augmentation model and promoted angiogenesis	[60]
Covalent crosslinking of first network					
Hydrazone formation	3,3'-Dithiodipropionate hydrazide-modified	pLysAAm	Subcutaneous implantation into rats	Supported higher cell infiltration and maintained excellent *in vivo* biocompatibility	[143]

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Chemistry	First network	Second network	*In vivo* model	*In vivo* response	Refs
	HA and PEG dilevulinate				
Radical polymerization	Methacrylated aminated gelatin	PTAA	Subcutaneous implantation into rats	Reduced inflammatory response and high vascularization suggested *in vivo* biocompatibility	[85]
	CSMA	GelMA	Implanted into rat calvarial defects	Accelerated *in situ* bone regeneration by promoting bone tissue invasion and mineral apposition	[7]
	Cystamine or glycidyl methacrylate-modified HA	pLysAAm	Subcutaneously injected into mice	Effectively mimicked *in vivo* tumor ECM and maintained the malignant phenotype of MCF-7 cells	[110]
Schiff-base reaction	Oxidized dextran and amine-modified gelatin	4 arm PEG acrylate	Porcine disc degeneration model and subcutaneous implantation into a rat model	Supported long-term cell retention and survival in the rat intervertebral discs and promoted long-term regeneration and enhanced ECM deposition and reduced fibrotic response *in vivo*	[144]
	Glycol chitosan and dibenzaldehyde functionalized PEG	Alginate	Subcutaneous injection into mouse model	Slight inflammation and production of GAG at the site of implantation	[64]
	Glycol chitosan and dibenzaldehyde functionalized PEG	Alginate	Subcutaneous injection into nude mice	Exhibited negligible inflammatory response and promoted neovascularization as well as osteogenic differentiation	[89]
Thiol-ene click reaction	Thiolated HA and MeHA	Azido-modified collagen and dibenzocyclo-octyne (DBCO)-modified collagen	Rabbit corneal defect fillers	Reduced myofibroblastic activity in the wounded stroma, prevented corneal haze and scarring and did not cause epithelial hyperplasia	[130]

Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; CSMA, methacrylated chitosan; GAG, glycosaminoglycan; GelMA, gelatin methacryloyl; HA, hyaluronic acid; MeHA, methacrylated hyaluronic acid; NHS, N-hydroxysuccinimide; PAA, poly(acrylic acid); PAAm, poly(acrylamide); PEG, poly(ethylene glycol); PEGDA, poly(ethylene glycol) diacrylate; PGS, poly(glycerol sebacate); pLysAAm, poly(Nε-acryloyl-L-lysine); PTAA, poly(thiophene-3-acetic acid); PVA, poly(vinyl alcohol).