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Hydrogels that listen to cells: a review of cell-responsive strategies in biomaterial design for tissue regeneration

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The past decade has seen a decided move from static and passive biomaterials to biodegradable, dynamic, and stimuli responsive materials in the laboratory and the clinic. Recent advances towards the rational design of synthetic cell-responsive hydrogels—biomaterials that respond locally to cells or tissues without the input of an artificial stimulus—have provided new strategies and insights on the use of artificial environments for tissue engineering and regenerative medicine. These materials can often approximate responsive functions of a cell's complex natural extracellular environment, and must respond to the small and specific stimuli provided within the vicinity of a cell or tissue. In the current literature, there are three main cell-based stimuli that can be harnessed to create responsive hydrogels: (1) enzymes (2) mechanical force and (3) metabolites/small molecules. Degradable bonds, dynamic covalent bonds, and non-covalent or supramolecular interactions are used to provide responsive architectures that enable features ranging from cell selective infiltration to control of stem-cell differentiation. The growing ability to spatio-temporally control the behavior of cells and tissue with rationally designed responsive materials has the ability to allow control and autonomy to future generations of materials for tissue regeneration, in addition to providing understanding and mimicry of the dynamic and complex cellular niche.

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Huey Wen Ooi obtained her Bachelor in Biotechnology and PhD in Polymer Chemistry at the University of Queensland. Her PhD was on the study of reversible-deactivation radical polymerization techniques and click chemistries to form precisely controlled hydrogels under the supervision of Prof. Andrew Whittaker. Following this, she worked on developing thermoresponsive microparticles for protein separation in the groups of Prof. Matthias Franzreb and Prof. Christopher Barner-Kowollik (Karlsruhe Institute of Technology). At present, she works within the Complex Tissue Regeneration (CTR) department at the MERLN Institute for Technology-Inspired Regenerative Medicine, Maastricht University, the Netherlands. Her work focuses on the development of dynamic hydrogel systems towards bioinks for 3D printing.

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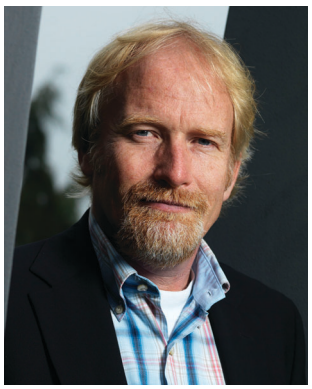
Shahzad Hafeez received his Bachelor of Science (BSc) from the University of Engineering and Technology, Lahore, Pakistan. In 2013, he moved to Germany where he studied a Master's program "Advanced Materials and Processes" from Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany, and carried out a collaborative Master's thesis between Julius Wolff Institute, Charite, Berlin (Dr Evi Lippens, Dr Taimoor H. Qazi) and Institute of Biomaterials, Friedrich-Alexander-Universität (Prof. Aldo. R. Boccaccini). In his thesis work, he developed void forming composite hydrogels for bone tissue engineering. Since December 2016, he has been a PhD student within the CTR department at the MERLN Institute, Maastricht University. In his PhD, he is investigating supramolecular hydrogels for corneal tissue engineering applications.

1. Introduction

A cell, and its niche, represents an instructive symphony of complex interactions, feedback loops, and signals aimed at controlling cellular behavior and function in real time. The cell's extracellular matrix (ECM), provides the cell a physical environment, a setting, to exist and communicate within. Made predominantly of proteoglycans and fibrous assemblies of proteins, the ECM provides a multitude of functions, including mechanical and structural support, spatio-temporal growth factor presentation, traction and movement, and the ability to remodel in response to a cell or external stimuli.[1,2] Made from a few key components, the responsiveness, instructiveness, and variability of the ECM (with respect to different tissues and organs) has long been a source of emulation. The recapitulation, control, and amelioration of ECM–cell interactions all remain significant targets for rationally designed systems.

Regenerative medicine aims to create therapies to replace or regenerate cells, tissues, and organs towards the restoration of impaired function resulting from genetic defects, disease, trauma, or aging; tissue engineering focuses a bit more to apply materials, cell biology, fabrication, and bioengineering strategies to control the growth or regrowth of tissues from basic cellular units, often progenitor cells.[3,4] In both regenerative medicine and tissue engineering, there is a large need to develop controllable materials that recapitulate functions of the natural ECM, including cell signaling, delivery of bioactives at controlled rates, and tunable and responsive mechanical properties, yet can still allow for regeneration and growth of the nascent tissue. Viewing Nature as a source of inspiration, the materials design within these fields focuses on mimicking features of a cell's natural environment.

Various three-dimensional (3D) scaffolds that share aspects of the native ECM, including electrospun meshes,[5–7] patterned surfaces,[8,9] and 3D printed scaffolds have been investigated;[10–12]



C. A. van Blitterswijk

Clemens van Blitterswijk is a University professor, director of the MERLN Institute, and chair of the CTR Department at Maastricht University. Before this, he had positions as Scientific Director and full professor at University of Twente and as associate professor at Leiden University. He obtained his PhD in 1985 from Leiden University, the Netherlands and graduated as a cell biologist from the same university in 1982. He has received a number of prestigious international awards including the George Winter award of the European Society for Biomaterials (ESB), the Career Achievement Award of the Tissue Engineering and Regenerative Medicine International Society (TERMIS), the 2015 Huibregtsen prize, and has been elected as member of the KNAW (Royal Academy of Arts and Sciences). He has co-founded multiple successful biomedical companies including CellCoTec Ltd, Progentix B.V., and Materiomics B.V. Recently, he founded RegMed XB, a public–private partnership, which aims to be the largest cross-border regenerative medicine consortium in Europe.

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M. B. Baker

Matthew Baker received his BS in Chemistry at Clemson University, after which he spent a short time translating academic research into the market with Tetramer Technologies. He obtained his PhD in 2012 in Physical Organic Chemistry under the guidance of Dr Ronald K. Castellano at the University of Florida, studying atypical small molecule organogelators and developing structure/property relationships toward the one-pot multifunctionalization of small symmetric synthons. He then moved to Eindhoven University of Technology to design and characterize water soluble supramolecular polymers and materials under guidance of Prof. E. W. Meijer. In 2015, he joined the MERLN institute to focus on developing polymeric and supramolecular materials for tissue engineering. His research interests include the synthesis and characterization of dynamic materials to mimic the cellular environment for biofabrication strategies, and to influence cellular behavior.

however, hydrogels remain one of the most promising ECM replacements. Hydrogels are made out of a 3D network of crosslinked hydrophilic macromolecules, which possess the ability to immobilize high amounts of water (*ca.* 90 to >99%). The natural ECM, itself, is a hydrogel. Many of the individual components of the ECM can form hydrogels (*e.g.* collagen, elastin). Synthetic hydrogels are a natural choice for the creation of artificial environments around cells.

The utilization of hydrogels for biomedical applications started approximately 60 years ago, with the invention of contact lenses from crosslinked poly(hydroxyethyl methacrylate) (PHEMA) networks.[13] Henceforth, hydrogels have played an increasingly important role in the design of biomaterials for drug delivery,[14–16] tissue engineering,[15,17–20] and regenerative medicine.[17,21] Although a wide array of hydrogels have been investigated, ranging from purely natural to purely synthetic polymers, each hydrogel holds its own advantages and limitations. For example, naturally derived hydrogels often provide desirable performance, but are difficult to standardize and modify; synthetic hydrogels are highly customizable, yet can be difficult to impart complex mechanical properties and the correct bioactivity. Consequently, the search for hydrogel systems that can best provide cells a designed and controllable alternative microenvironment remains an active area of research.

With significant progress in the field of polymer chemistry, synthetic chemistry, and supramolecular chemistry, the capacity to design and tailor polymer architecture and hydrogel networks has advanced.[22–24] No longer confined to synthesizing covalent and static networks from conventional radical crosslinking methods, current reports of instructive, stimuli responsive, and biodegradable hydrogels bring closer the realization of complex and dynamic systems that mimic ECM functions. Aligned with these efforts, focus has been shifted to exploring different chemistries to form dynamic hydrogels. Properties like cell-mediated remodeling, cellular adhesion, growth factor release, strain-stiffening, and viscoelasticity are being introduced through the incorporation of cleavable bonds, reversible bonds, supramolecular bonds/polymers, self-assembly, or flexible polymer backbones. Several excellent reviews on this current trend have come out in the last few years.[25–30]

Dynamic hydrogel architectures have found success as responsive materials to large external stimuli, yet they also have the potential to produce responsive materials that act biomimetically upon cues from a cell.[31] For example, the ability of a material to release growth factors in response to enzymes from a nearby cell can be viewed as a significant advance over a material relying entirely on external light irradiation. Consequently, current efforts to sense and respond to cells have quickly gained steam. As we obtain greater skill in rational design and more insight into cellular behavior, we are on the cusp of a new biomaterials revolution. Fully autonomous hydrogels that can sense, respond, and influence cells aim to become truly smart biomaterials of the future.

This review will delineate and focus attention to the growing trend of cell responsiveness within the hydrogel and biomaterials community. We have chosen to focus on systems designed for tissue engineering and regenerative medicine; however, significant advances from other fields (*e.g.* drug delivery) will also be presented with an eye towards their applications in regenerative medicine. First, we will quickly introduce the main behaviors of a cell (inputs) that have been utilized to engineer such responsive hydrogels. Secondly, we will briefly introduce the prevalent chemistries used in the construction of such materials. In the main body of this review, successful examples will be broken down into three sections, namely enzyme responsive, mechanical responsive, and metabolite/small-molecule responsive hydrogels (Fig. 1). In conclusion, we will look forward at the potential of cell responsive hydrogels and entertain the idea that dynamic and responsive synthetic environments can bring us close to the creation of an artificial ECM.

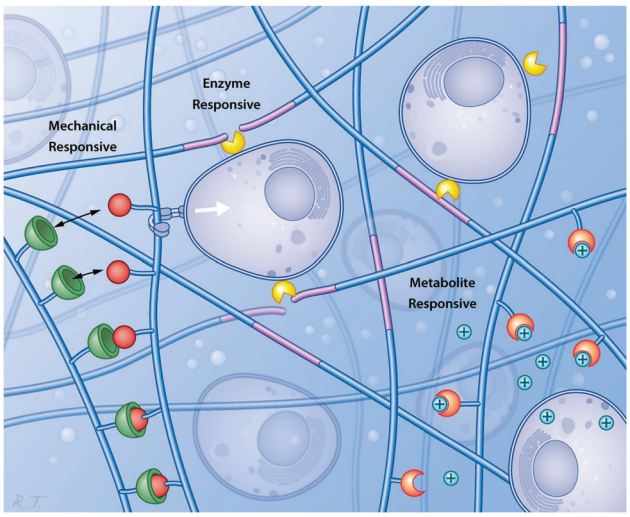

Fig. 1 Shown schematically are the three main strategies for the creation of cell-responsive materials. Enzymatic catalysis (top center) of hydrogel breakdown has been extensively used in tissue engineering to allow matrix remodelability. By incorporating enzyme cleavable units into the hydrogel structure (*e.g.* MMP cleavage sites, depicted in purple), cells are able to infiltrate and migrate through the material *via* the action of enzymes (depicted in yellow) cleaving the hydrogel network (blue fibers). Most recently, mechanically responsive hydrogels (bottom left) have been developed to allow for response based on a cell's traction forces. Shown schematically, a cell transfers traction forces to the matrix *via* integrins (light blue), which can remodel or rearrange dynamic interactions in the hydrogel, illustrated as the binding between a green cup and a red ball. Still in their infancy, such materials heavily rely on dynamic covalent and supramolecular interactions. Metabolite and small molecule responsive (bottom right) hydrogels represent a broad class of materials that sense and respond to small molecules, proteins, and metabolic by-products around a cell or tissue. As an example, depicted are pH sensitive receptors (orange) that become charged (light blue spheres). Such metabolite responsive materials often rely on highly specific non-covalent interactions, and are largely biohybrid type materials.

2. Cell outputs

The chemical makeup and manner in which the multicomponent ECM is assembled directs cellular behavior. Reciprocally, the responsive and weak interactions between the ECM components create a dynamic environment that can respond to and

influence the activities of a nearby cell.[32] For example, the spatio-temporal control of growth factors is a major role of the ECM and regulates many *in vivo* processes. The ECM can sequester transforming growth factor-β (TGFβ) in an inactive form, and release its active form in response to cellular traction forces during tissue remodeling.[33] Additionally, laminin cleavage *via* matrix-metalloproteases (MMPs) has been shown to release fragments capable of regulating stem cell differentiation.[34] Numerous ECM components are released or presented in response to cellular cues, such as mechanical force or protease activity.[35]

When designing materials aimed at mimicking such responsive functions, it is crucial to understand what cues are both produced by a living cell and have been shown to trigger responsive materials. Shown schematically in Fig. 1, three main cell outputs have been employed as stimuli in responsive hydrogels: (1) enzymes, (2) mechanical force, and (3) metabolites/small molecules.

2.1 Enzymes

The fundamental role of enzymes as catalysts is to increase the rate of biochemical reactions. Enzymes are ubiquitous in living systems,[36] have high specificity for chemical transformations, and maintain high activity under physiologic conditions, making them ideal triggers for the chemical design of responsive hydrogel systems. Furthermore, cells highly rely on enzymes for the *in vivo* regulated remodeling of the natural ECM, a process crucial for the maintenance of normal biological functions.[2] Differences in enzyme expression is associated with many events including stem-cell differentiation, tissue repair, and pathological disorders. Altered expression level of specific enzymes can be used to catalyze a reaction in hydrogels for controlled release or degradation at desired sites or under certain events.[37,38]

2.2 Mechanical force

In the last decade, extensive research has been carried out to investigate the influence of mechanical forces generated between cells and their environment. Researchers now have developed understanding of the forces generated by cells in tissue remodeling and cellular functions ranging from cell adhesion, migration, proliferation, differentiation and morphogenesis.[39–42] Generally, cells first sense the matrix and pull to deform the matrix. These contractile forces are generated by actomyosin contraction and are transmitted to the ECM *via* focal adhesions—an integrin containing multi-protein complex connecting the cytoskeleton to the ECM. Cellular traction forces are in the range of piconewtons to nanonewtons and occur at the nanometer to micrometer length scale,[43] yet the mechanical feedback a cell receives from its environment can activate specific signaling pathways or alter gene expression in a surprisingly quick fashion.[44] There are numerous reviews on the complex interplay between a cell's mechanical sensing framework and its ECM environment.[37,45–48] Only in the past few years has this understanding been translated to materials that can also alter their properties in response to the small mechanical forces generated by a cell.

2.3 Metabolites and small molecules

Metabolites and small molecules produced (or consumed) by a cell also offer the possibility to be used as a trigger for cell-responsive systems. Metabolism (a set of chemical reactions taking place within a cell) is very important for a cell to regulate pathways and maintain critical concentrations of important building blocks (and by-products) within and outside of a cell. Any up and down regulation of these pathways leads to a change in the extracellular environment. For example, changes in the reductive power[49] and pH around cancerous cells,[50] higher glucose levels in diabetic patients,[51] and higher levels of reactive oxygen species under stress[52] have all been successfully utilized to create responsive hydrogel systems. In natural systems, cell metabolites are extensively used in signaling pathways, can initiate very specific interactions, and can maintain integrity and activity over long ranges. However, the difficulty and challenge lies in translating the sensitivity, specificity, and amplification, of these sometimes small differences to synthetic hydrogel systems.

3. Chemistries developed

The range of chemical structures and systems available to engineer cell-responsive hydrogels is a limited, but growing, set. Such chemistry must be reversible (or degradable), both stable and responsive under physiologic conditions, synthetically accessible, and ultimately, cytocompatible. Although all the systems described rely on water soluble macromolecules, it is the incorporation of dynamic chemical units that provide responsive function. To highlight the characteristics of available systems, employed approaches can be categorized into five main chemical strategies. Shown schematically in Fig. 2, cell-responsive materials mainly rely on pH responsiveness, enzyme catalyzed bond breakage, dynamic covalent chemistry, supramolecular interactions, and protein or DNA engineering. One should note that often, more than one category is implemented in more complex systems; the illustrative examples herein have been classified according to the main strategy employed.

3.1 pH responsive

The earliest hydrogel architectures to allow for responsive biomaterials were pH responsive matrices. Such pH responsive hydrogels normally contain amines (pK_a 8–10) or acids (pK_a 4–6) that are protonated/deprotonated to form charges within the hydrogel network. This change in protonation state introduces swelling/deswelling due to changes in the ionic character of the network. In a classic example, poly(acrylic acid) hydrogels swell at higher pHs *via* formation of the ionic carboxylate. General acid or base catalyzed hydrolysis of polymer backbones can also be utilized for the creation of pH responsive materials.

Such pH responsive approaches have been extensively studied for drug delivery,[53] but often lack the sensitivity or specificity needed for fine control in more complex applications. To overcome this limitation, often pH responsive hydrogels are coupled with enzymes that catalyze the conversion of a signal into an

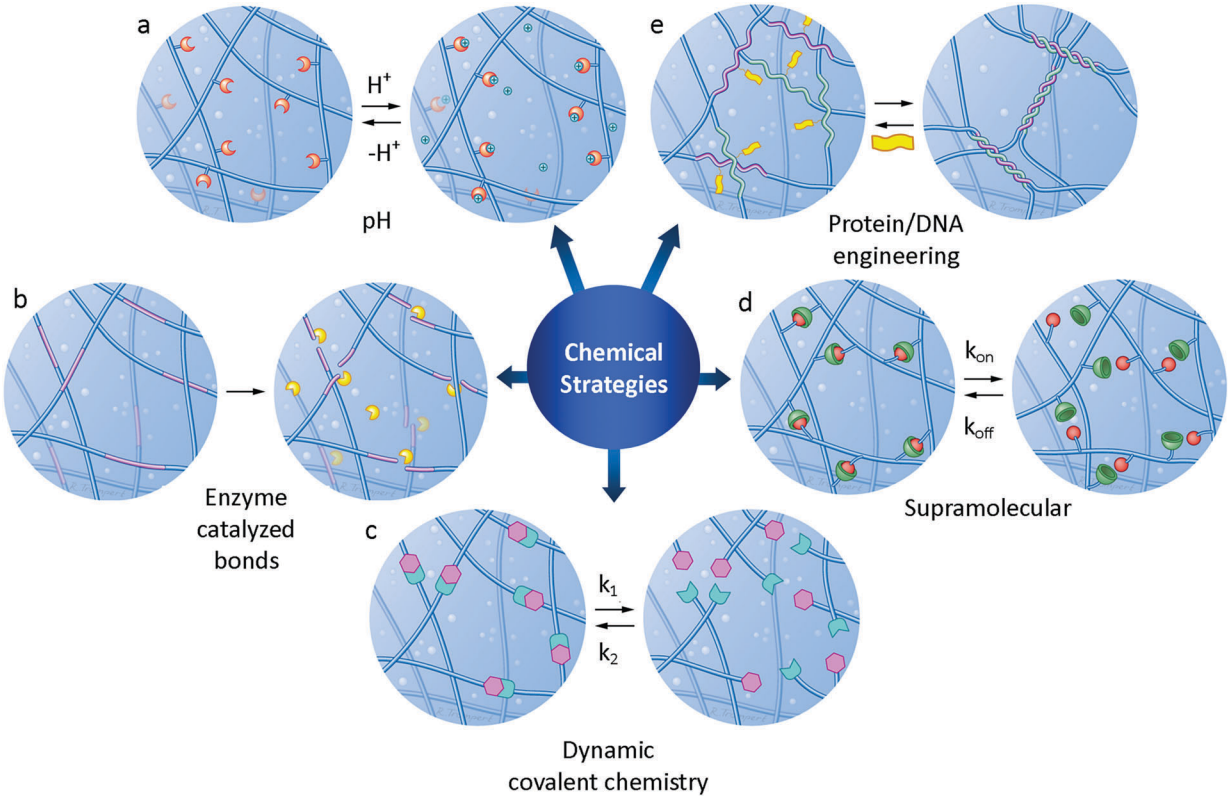

Fig. 2 Chemical strategies commonly utilized for cell-responsive hydrogels. (a) pH (redox) sensitive units (orange) can change protonation (oxidation) state, often becoming charged (blue positive charges), leading to changes in binding constants or the osmotic pressure within the hydrogel; *e.g.* amine or acid protonation, disulphide reduction (b) enzyme (yellow) catalyzed irreversible bond breakage of specific peptide sites (purple) in the hydrogel network (or precursor); *e.g.* MMP specific cleavage, enzymatic cleavage of supramolecular hydrogelator precursor (c) dynamic covalent chemistry whereby two units (green and pink) can dynamically, and reversibly, form covalent bonds depending on their reaction rates k_1 and k_2 and equilibrium constant (K_{eq}); *e.g.* hydrazone exchange/hydrolysis and disulphide formation, (d) supramolecular chemistry shown as the reversible binding and unbinding of a red ball and a green cup based on the binding constants k_{on}, k_{off}, and equilibrium (K_{eq}); *e.g.* polymer aggregation, host–guest interactions, H-bonding units, and protein-small molecule binding, and (e) protein/DNA engineering as shown by two engineered biomolecules in the network (light green and purple) reversibly bind and undergo conformational changes in response to the presence of a signal (yellow); *e.g.* incorporation of shape changing proteins (calmodulin, adenylate kinase) and RNA aptamer binding.

acidic (or basic) product and lowering (or rising) the local pH. An example is the glucose/glucose oxidase (Glu/GOx) system that produces gluconic acid. This type of pH responsive hydrogel has been used extensively for glucose responsive and insulin releasing hydrogel systems.[54]

3.2 Enzyme catalyzed bond breakage

One of the most visible cell-responsive strategies in tissue engineering has been enzyme catalyzed lysis of bonds. In such a strategy, specific peptide sequences, or enzymatically cleavable bonds (*e.g.* phosphates, esters) are engineered into the hydrogel network or into hydrogel precursors. These systems are fairly stable in the absence of enzyme activity, but readily degraded, or activated, in the presence of a cell secreting the correct enzyme. Various enzymes including MMPs, elastase, alkaline phosphatase (ALP), esterases, thrombin, plasmin, and cathepsin K have already been utilized to create functionally responsive hydrogels.[55] One will notice an absence of enzyme catalyzed bond formation in this area of responsive hydrogels.

3.3 Dynamic covalent chemistry

Dynamic covalent chemistry (DCvC)[56] has played a significant role in the current trend towards dynamic hydrogels. Sometimes classified as covalent adaptable networks, hydrogels based on DCvC are characterized by the breaking and reforming of covalent bonds based on their equilibrium (K_{eq}) and rate constants (k_{off} and k_{on}). These networks generally maintain the number of linkages or crosslinks based on the K_{eq} value, and the time of the bond is generally governed by k_{off}.[57] Several DCvC systems are biocompatible and have already been used to form hydrogels, including boronic acids,[58] thioesters,[59] hydrazones,[60,61] imines,[62,63] and disulfides.[64,65] However, hydrazone/imine and disulfide systems have recently come to the forefront of use. Hydrazones remain dynamic under physiologic conditions, imines become more dynamic with drops in pH, while disulfides are responsive to local changes in sulfide concentration or reductive power of the environment. Most DCvC systems are tunable *via* physical organic chemistry principles, and the adaptation of existing systems for use under physiologic conditions remains an active area of research.

3.4 Supramolecular

Supramolecular assemblies and non-covalent interactions have also been a rich source for the creation of dynamic and responsive biomaterials.[26] Nature, itself, relies heavily on the use of supramolecular assemblies. In a truly biomimetic fashion, the engineering of hydrogels designed from specific, directional, tunable, and reversible non-covalent interactions have enabled the creation of modular platforms with tunable physical, chemical, and biological information.[66,67] Supramolecular hydrogels[68] owe their properties to the binding, association, or assembly of discrete units. The assbembly of these units is again governed by an equilibrium constant (K_{eq}), which is simply a ratio of association (k_a) and disassociation (k_d) kinetics. Numerous supramolecular systems are capable of creating hydrogels including host/guest interactions,[69,70] directional hydrogen bonding,[71,72] peptide amphiphiles,[73–75] metal–ligand coordination,[76] and small molecule gelators.[77] In theory, such systems built on weak and non-covalent interactions are well situated to quickly respond to changes in the environment or secreted factors. Although prolific as materials to drive tissue formation,[75] supramolecular materials are currently under-represented in the area of cell-responsive hydrogel systems. Progress in rational design, characterization, and control over the sometimes sensitive supramolecular interactions remain both a hurdle, and a promising research area.

3.5 Protein/DNA engineering

When creating bioresponsive systems, utilizing nature's own building blocks is a pragmatic approach. Numerous responsive enzymes and protein/protein complexes are known, expressible, modifiable, and purifiable from recombinant protein expression. Integrating such functional or responsive protein units within the network of a hydrogel allows a biosynthetic hybrid approach to materials that can sense, respond, and influence cellular behavior.[78–80] In addition, DNA origami and DNA aptamer technologies allow for the formation of spatially controlled materials and high specificity to the binding of specific analytes.[81,82] While such biohybrid approaches are less scalable and more expensive for the production of hydrogels, their specificity and fine control can justify the high cost.

4. Enzyme responsive systems

Extensive research has developed enzyme-responsive hydrogels for applications in drug delivery,[83,84] imaging,[85] diagnostics,[86] tissue engineering,[87] and regenerative medicine.[21] The design of enzyme-responsive hydrogels that enable cell and/or tissue specific responses is currently based on two types of mechanisms: (1) hydrogel networks with enzyme cleavable crosslinks or tethers; (2) supramolecular assemblies generated from enzymatic conversion of a non-self-assembling precursor. These mechanisms mimic the breakdown and the formation, respectively, of ECM; however, enzymatically based systems to regulate both have not yet been developed.

4.1 Proteolytically-degradable hydrogels

Proteolytic sensitive hydrogels, engineered with enzyme sensitive crosslinks of customized peptide sequences, directly enable modulation of hydrogel degradation based on cell-secreted enzymes (shown schematically in Fig. 3a). When compared to their static and non-degradable counterparts, cells can spread, migrate, and proliferate more freely within these protease sensitive hydrogels.[88,89] Cellular infiltration, vascularization, tissue remodeling, and enhanced differentiation capacity of stem cells have all been observed in these biomimetic hydrogel systems.

One of the earliest works to utilize enzymatically degradable gels, from the 1980s, was based on crosslinked poly(2-hydroxyethyl-L-glutamine) (PHEG, a poly(α-amino acid)). This hydrogel showed significant degradation during post-implantation inflammatory response, which was attributed to the presence of proteolytic enzymes.[90] Spurred from such initial studies, the field of

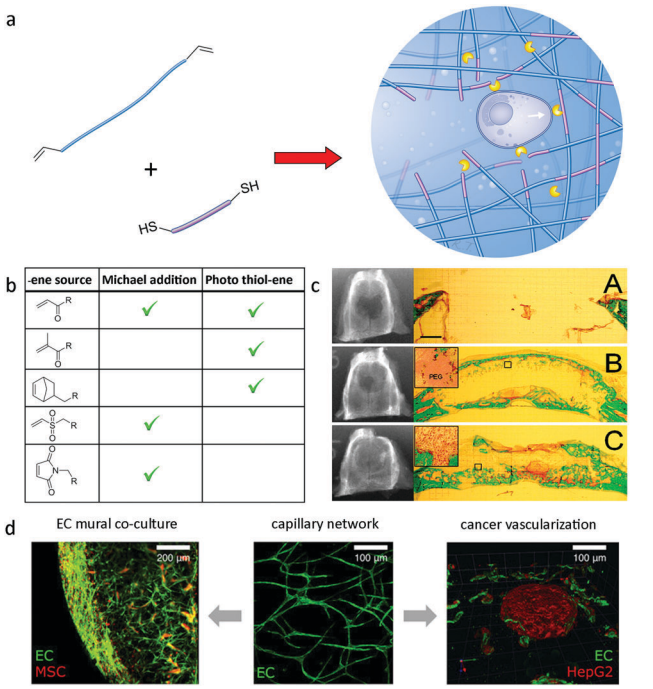

Fig. 3 (a) Water soluble polymers (blue) containing activated double bonds can be crosslinked with thiol terminated peptide sequences (purple) to form enzyme degradable hydrogels. A cell can remodel and migrate through these hydrogel networks *via* proteolytic enzymes (yellow) cutting covalently attached peptides (purple) within the network. (b) Polymer chains (*e.g.* multi-arm PEG) are commonly functionalized with (meth)acrylates, norbornene, vinyl sulfone, and maleimides. Advantages of MMP-degradable hydrogels are shown in (c) rat calvarial defects 4 weeks after implantation bone formation was observed within gels, which were (A) not susceptible to MMP degradation, (B) with moderate degradation by MMP, and (C) highly susceptible to MMP degradation. Assessed by radiography (left) and histology (right), gels with highly degradable MMP networks showed significantly higher bone formation than gels with less or no MMP-sensitive sites due to cellular infiltration and remodelling. (d) MMP-cleavable starPEG–heparin hydrogels provides a platform for extensive *in vitro* studies of heterocellular cell–cell interactions between endothelial cells and mural cells (left), as well as heterotypic cell–cell contacts *via* a tumor angiogenesis model (right). (c) was reprinted from ref. 99. Copyright (2003) National Academy of Sciences. (d) was reproduced with permission.[103] Nature Publishing Group 2014.

proteolytically-degradable hydrogels has become widely studied in the context of cell-responsive biomaterials, as shown by several thorough reviews specifically on this topic.[91–93]

More recent approaches have relied heavily on the use of cross-links sensitive to MMPs, a large family of proteases which effect natural breakdown of the ECM during processes such as tissue resorption and remodeling.[94] Although there is often overlap between MMPs and their substrates, MMPs can show substrate specificity,[95] and the rate of degradation between MMP cleavable sites can vary significantly.[96] Depending on the application in sight, careful choice of peptide substrates can be matched with enzymes present in targeted sites, events, or cell types.

Pioneering this approach of MMP degradable gels for tissue engineering was a 4-arm poly(ethylene glycol) (PEG) system with terminal vinyl sulfones to enable Michael-type additions for functionalization with cysteine containing biomolecules. By crosslinking the system with MMP cleavable peptides, and functionalization with thiol-containing adhesive RGD sequences, hydrogels were created where cells could attach, degrade, and migrate through.[97] By incorporating growth factors like vascularization endothelial growth factor (VEGF) or bone morphogenic protein (BMP-2), these systems showed tissue formation with vascularization[98] and bone regeneration,[99] respectively, in *in vivo* models, non-degradable crosslinked hydrogels were free of cellular infiltration (Fig. 3c). The modularity and tailorability of this straightforward PEG–vinylsulfone system has effected the creation of highly customizable cell culture environments. For example, recently a stiffness-switching hydrogel was designed to replace Matrigel in intestinal organoid expansion[100]—a previous bottleneck for organoid technologies. Of note, the most successful hydrogel was not the enzyme-responsive gel tested (collagen I sequence); a hydrolyzable ester linkage with slowed degradation provided the relevant timescale for this specific application.

Due to the synthetic accessibility of alkenes and the common presence of thiols in biomacromolecules, thiol–ene reactions like the Michael addition and photo-activated thiol–ene, are natural choices for hydrogel formation with biomolecules. Some of the commonly used alkenes for Michael addition and photo-initiated thiol–ene to form hydrogels in a biological environment are presented in Fig. 3b. It should be noted that methacrylates can undergo Michael addition under appropriate conditions, yet these conditions are usually not cell compatible.[101]

PEG–maleimide has shown some significant improvements in crosslinking speed and efficiency over other systems (including vinyl sulfone).[102] Multi-component PEG–maleimide hydrogels containing RGD, MMP degradable crosslinks, VEGF, and hepatocyte growth factor (HGF), have been shown to increase angiogenesis in a rat infarcted myocardium.[37] Furthermore, injectable microgels based on a similar architecture have shown increased vascularization in the dorsum of mice.[38] Microgel slurries have emerged as a convenient work-around to the lack of injectability for covalently crosslinked hydrogels.

Michael-type MMP cleavable hydrogels have also proven utility as a laboratory platform to study tissue formation and the study of cell–cell interactions. For example, a MMP-cleavable biohybrid starPEG/heparin hydrogel system has been optimized to support the morphogenesis of endothelial cells.[103] Due to its susceptibility for remodelling, the hydrogel platform enabled *in vitro* studies of heterocellular cell–cell interactions between endothelial cells and other cells (Fig. 3d: left), as well as heterotypic cell–cell contacts using a tumor angiogenesis model (Fig. 3d: right).

Michael-type additions typically require a slightly alkaline pH to occur (pK_a of the Michael donor), which precludes the use of some base sensitive molecules.[104] Photo-initiated thiol–ene conjugations can overcome this limitation. Photo-initiated thiol–ene occurs quickly and in high yield at neutral pH, which can be beneficial for *in situ* gelation. Furthermore, spatio-temporal control (*via* light) of the biomolecule conjugation and gelation has resulted in several novel materials applications in biofabrication. Recent advances include complex patterns within hydrogels[105–107] and the development of photo-crosslinkable thiol–ene hyaluronic acid[108] and polyglycidol[109] based bioinks for 3D printing of cell–hydrogel mixtures.

An excellent example of photo-initiated thiol–ene formation is the thiol–norbornene photopolymerizable 4-arm PEG hydrogels, crosslinked with an MMP degradable sequence (KCGPQG↓IWGQCK).[110] When human mesenchymal stem cells (hMSCs) were encapsulated in such hydrogels, significant increases in cell spreading were observed. Furthermore the osteogenic, adipogenic, and chondrogenic differentiation capacity of hMSCs increased with increasing MMP-degradability of the networks, showing a clear link between differentiation capacity and the ability of a cell to remodel the synthetic ECM.

Not only for degradation, enzymes can also be used to trigger localized release through tethering of active molecules on the crosslinker. For example, norbornene-functionalized PEG hydrogels were photo-crosslinked with MMP releasable pro-angiogenic sequences. In this design, pro-angiogenic peptides ($SPARC_{113}$ and $SPARC_{118}$) were end-capped with IPES↓LRAG sequences, which are sensitive to MMP cleavage. These hydrogels successfully promoted angiogenesis when injected subcutaneously in mice in an entirely degradation dependent manner.[111] However, compared to other pro-angiogenic protein delivery hydrogel systems, the release profile of these enzyme degradable systems were much shorter and would still need further optimization.

Applying such strategies to synthetically-modified naturally-derived polymers, MMP-cleavable hyaluronic acid (HA) hydrogels that shut-down or allowed cell mediated remodeling have also been created. By functionalizing HA with both maleimides and methacrylates, thiol–ene crosslinking *via* an MMP-cleavable peptide (GCRDVPMS↓MRGGDRCG) could (or could not) be followed by a secondary non-degradable photo-initiated crosslinking of the methacrylates.[112] In these hydrogels, osteogenic/adipogenic differentiation of hMSCs was observed to be directed mainly by degradation-mediated cellular traction. Permissive (remodelable, without secondary crosslinking) matrices gave rise to osteogenesis, while non-permissive (non-remodelable, with secondary crosslinking) matrices led to adipogenesis. Interestingly, when a stem cell's environment was switched from permissive to non-permissive *via* delayed secondary crosslinking,

the cell also switched from an osteogenic to adipogenic phenotype. The osteogenic cell morphology remained, yet an adipogenic phenotype dominated. Recently, this same group utilized the efficiency of thiol–ene photochemistry to print fibroblast-laden HA modified with norbornene groups.[108] To highlight the versatility of the 3D printing technique, they created complex hydrogel structures comprising of MMP-degradable and non-degradable regions, and observed cell spreading throughout the printed hydrogel when a degradable linker was used.

Moving towards complexity and autonomous systems, the versatility of MMP-cleavable hydrogels has been shown in the rational design of a negative feedback loop to inhibit MMP activity.[113] By loading an MMP cleavable hydrogel network with a physically-associated MMP inhibitor (TIMP-3), hydrogels were designed to buffer the local MMP activity. A significant decrease in MMP activity was observed after delivery of these injectable hydrogels to an infarcted myocardium (MMP overexpressed region) in a pig model. Controlled delivery of MMP inhibitors is always a challenge, due to dose limiting side effects; however, this hydrogel system is a significant example of how cell-responsive hydrogel systems can be designed for feedback loops to autonomously control pathological levels of enzyme expression.

MMP is not the only enzyme trigger used for hydrogels in tissue engineering and regenerative medicine. Hydrogels cross-linked with cathepsin K sensitive peptide sequences have been shown to specifically degrade in the presence of osteoclasts,[114] while elastase sensitive Ala–Ala repeat segments incorporated in PEG hydrogels have been shown to allow both *in vivo* degradation and cellular infiltration in mice[115] and controlled protein delivery.[116,117] Thrombin, a key enzyme of blood coagulation cascade, has been used to trigger anti-coagulant release to prevent clotting. A modular feedback-loop PEG hydrogel system with thrombin sensitive peptide sequences has been developed to prevent clotting through the release heparin on demand,[118] or respond to and reverse clot formation *via* the release of tissue plasminogen activator.[119] Both of these thrombin mediated hydrogels have been shown to successfully suppress critical points in the blood coagulation cascade.

The highlighted examples throughout this section only represent some of the more commonly used enzymes and some examples of their complimentary peptide. More exhaustive lists of enzymes/peptides can be found elsewhere.[36,91,95,96,120]

4.2 Enzymatic assembly of nanostructures

Enzymes can not only be used to breakdown hydrogels, but also to induce hydrogel formation *via* self-assembly of low-molecular weight hydrogelators. This enzymatically triggered build-up of fibrous nanostructures can mimic the enzymatic remodeling and production of the ECM. The enzyme triggered dynamic assembly, and in the future disassembly, is potentially a promising strategy to mimic the behavior of natural fibers in the cellular environment.

Most enzyme responsive supramolecular hydrogelators are peptide based and are converted from an inactive (non-self-assembling) to an active (self-assembling) form *via* hydrolysis or condensation of peptide precursors (Fig. 4a).[77] Generally, a

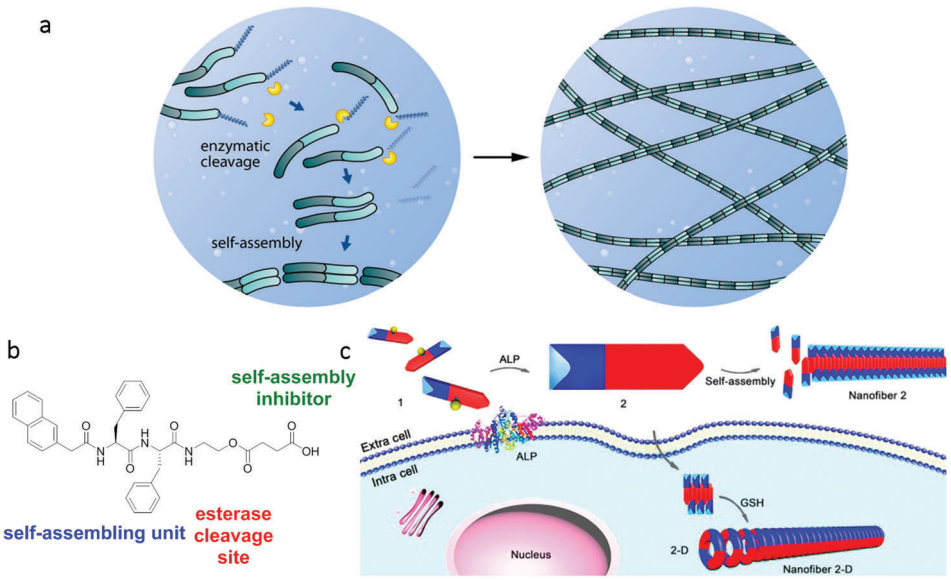

Fig. 4 (a) General schematic of an enzyme-triggered self-assembly mechanism. Peptide based precursors (shaded green units) are equipped with a tail (blue), that prevents self-assembly. Upon specific enzyme (yellow) cleavage, the tail is removed and the active hydrogelator is formed, assembling into a fibrous network (right). Often these hydrogelators can self-assemble into several nanostructures. (b) A Nap-C(O)-FF based unit possesses all of the characteristic design elements. A self-assembling peptide with a hydrophobic moiety (naphthalene) to drive self-assembly and a dipeptide unit (Phe–Phe) as acceptor/donor of hydrogen bonds, an enzyme cleavable bond (esteraste, ester), that can remove a self-assembly inhibitor (deprotonated carboxylic acid).[121] (c) Self-assembly of a cyano-6-aminobenzothazole (CBT) based precursor 1 into nanofiber 2 was catalyzed by extracellular ALP removal of a phosphate that prevented self-assembly. After cellular uptake of nanofiber 2, reduction of a protected thiol by glutathione (GSH) in the intracellular environment leads to intermolecular disulfide formation (hydrogelator 2) and the formation of the second nanofiber (2D). (c) was reprinted with permission.[126] Copyright (2016) American Chemical Society.

known self-assembling unit is conjugated to an enzymatically labile group that inhibits self-assembly. Upon cleavage of this inhibitor, hydrogelators then freely self-assemble to form nanostructures, mainly *via* hydrophobic interactions, hydrogen bonding, electrostatics, and π–π interactions. Through design of the peptide sequences and dictation of the self-assembly pathways, the morphology of the self-assembled structures can be controlled and used to direct cellular behavior.[30] A majority of the work reported to-date on enzymatically triggered hydrogelation has not yet been applied to tissue regeneration, but has focused on cell death and entrapment for cancer therapy.[77] Despite this, these systems carry interesting design principles, and their application towards tissue regeneration is overdue.

An illustrative example of enzyme triggered nanostucture assembly can be seen in systems based off naphtyl–Phe–Phe self-assembling units. Towards creating enzyme sensitive self-assembling units, a monomer (shown in Fig. 4b) was designed with three functional parts: a dipeptide capable of forming a hydrogen-bonding motif, a napthyl group to provide a hydrophobic driving-force to self-assembly, and an enzymatically cleavable pendant ester of butyric diacid, which provides electrostatic repulsion to prevent self-assembly.[121] Upon exposure to esterases within HeLa cells, the anionic butyric acid is removed, and the resultant hydrogelators self-assemble to form intracellular nanofibers, leading to cell death.

Other self-assembling systems have also been created with specific MMP cleavable tails. A peptide–lipid conjugate precursor (palmitoyl-GGGHGPLGLARK-$CONH_2$) has been successfully designed with an MMP-7 cleavage site that removed a highly charged Arg-Lys sequence. The supramolecular hydrogelator self-assembles intracellularly into nanofibers upon enzymatic cleavage, leading to death of cancer cells.[122] Importantly, this supramolecular system showed high cytotoxicity to five different cancer lines (HeLa, MIAPaCaII, SKBR3, MCF-7, A431), yet low cytotoxicity to normal cells (microvascular endothelial cells and pancreatic epithelial cells), even in co-culture situations.

Recently, the ability of enzyme to induce self-assembly to enable both cell and organelle specific targeting was shown by conjugating triphenyl phosphinium (TPP), a redox modulator capable of targeting cancer mitochondria, to an enzyme inducible tetra-peptide precursor capped with a fluorophore (4-nitro-2,1,3-benzoxadiazole, NBD).[123] The tetra-peptide precursor, ((NBD)-FFYpK(TTP)) undergoes dephosphorylation selectively in the environment of cancer cells (upregulated ectophosphatases). The conjugate then self-assembles, is endocytosed by cancer cells, escapes from lysosomes, and the TPP specifically targets the mitochondria.

Enzyme responsive self-assembling systems have also been employed to image local enzymatic activity. Direct attachment of fluorophores to enzymatically self-assembling systems[85] and co-assembly strategies[124] have allowed the imaging of organelles within cells and enhanced theranostic treatments, respectively. Imaging of fluorescent nanostructures in breast cancer cells (MDA-MB-468) has even been shown *via* a bond-breaking bond-forming condensation to create a fluorescent monomer upon enzymatic cleavage (among other stimuli).[125]

In an advanced enzyme-responsive self-assembly system, Liang and co-workers designed a small-molecule hydrogelator that can change its self-assembled nanostructure, depending on the cellular environment (Fig. 4c).[126] In the extracellular matrix, the 2-cyano-6-aminobenzothazole (CBT)-based precursor (C(SEt)EY(H_2PO_3)FFG-CBT) undergoes dephosphorylation, in response to ALP, forming a hydrogelator that self-assembled into nanofibers in the extracellular environment. The fibers underwent endocytosis where intracellular glutathiones (GSH) reduce the disulfide bonds of the protected cysteine residue (Cys(SEt)), allowing intermolecular cyclic dimers of the hydrogelator to form. This new molecular structure led to the formation of a different morphology of nanofibers. The environmentally specific self-assembly was validated through *in vitro* studies with HeLa cells and characterization of the structures and compounds formed extra- and intracellularly when ALP or GSH was introduced; however, the effects of this differential self-assembly system on the cells has not yet been fully reported.

While the majority of research on self-assembling structures have focused on homochiral molecules, in particular L-amino acids, there has also been increasing efforts to use D-amino acids and mixtures.[127] In the molecular design of enzyme responsive supramolecular hydrogelators, simple changes in chirality can control the interactions of the self-assembled system with target cells. For example, molecules with more D-amino acid substitution were found to be more toxic than their corresponding enantiomer potentially due to their resistance towards proteolysis.[128] The accumulation of nanoscale networks in the pericellular space hindered regular cellular activities, such as migration and adhesion, and led to cell apoptosis. Based on these findings, nanoscale hydrogel networks have been developed as inhibitors of cancer cells;[129] however, inhibition of migration and adhesion can also be effectively used to control tissue formation. In future designs of chiral self-assembling structures for biomaterials, it is clear that the chirality of each amino acid is essential to control the morphology, degradation, and cell signaling behavior of the resultant nanostructures.[130]

4.3 Prospects

Several other enzyme responsive systems present concepts that hold potential for future design of biomaterials. For example, Roberts *et al.* have developed a dynamic PEG-grafted surface with elastase sensitive dialanine protected RGD peptides.[131] The protected-RGD surfaces were shown to have lower, while the elastase deprotected-RGD surface had higher amounts of cell spreading and adhesion. Therefore, when MSCs were cultured on these surfaces, they showed a change from a growth state (low adhesion) to a differentiating state (high adhesion) upon elastase addition. Although this system was not shown to be cell-responsive (exogenous delivery of elastase), the use of enzyme triggers to control the phenotype of progenitor cells paves the way to develop cell culture environments that can control phenotypic changes in cells and tissue formation.

In living systems, enzymatic processes are often part of a feedback network. Mimicking such feedback loops, a hydrogel system with both enzymatically cleavable crosslinks and

hydrolytically activated crosslinks (thioester/maleimide) has been designed.[132] In-depth studies on the reaction kinetics have led to fine control of gel transitions (gel–gel or gel–liquid–gel) based on time and enzyme concentration. The incorporation of such a design in the development of hydrogels will allow progress towards more complex materials, which have pre-programmable responses to environmental biochemical triggers.

Despite advances in using enzymes to create cell-responsive hydrogel systems, there are some limitations. A noticeable drawback is the current lack of reversibility. Hydrogel degradation, self-assembly, or phase changes are currently a one-way street, limiting some applications that may benefit from more reversible, controllable, and long-lasting systems. Studies, such as the enzymatically cleavable and hydrolytically activated crosslinking system (*vide supra*), show clues to the utility and behavior of systems that both degrade and rebuild; however, currently these two phenomena are triggered with different mechanisms.

While enzymes are famous as highly selective catalysts, most of the cleavable peptide sequences incorporated in these hydrogels are degradable by more than one type of protease. In addition, limited studies have been conducted to tease out the changing biophysical properties of enzyme degradable hydrogels, which play an important role in stem cell fate.[39,40] There is little control over the homogeneity of these hydrogels when cells are encapsulated, making the physical characteristics of the networks difficult to predict or measure. These systems have seen utility and good proof-of-concept in well-controlled environments, yet testing in more complex *in vivo* systems is their next hurdle, especially with respect to self-assembling systems. Studies of *in vivo* implantation of enzyme-responsive hydrogels has shown promising cellular infiltration,[99] vascularization,[37,133] and neobone formation in bone defects,[99,134] yet efficient translation towards clinically relevant tissue regeneration requires more work in terms of upscaling, large animal model proofs, and clinical trial feasibility.

5. Mechanically responsive systems

One of the emerging areas of cell–biomaterial interactions is the interplay between the mechanical properties of a material and the mechanosensing framework of a cell. Mechanobiology is quickly becoming an active field,[135] and mechanically instructive and responsive biomaterials are advancing in a concerted fashion. In the past decade, it has become a central pillar of biomaterials that a hydrogel's stiffness can directly influence cellular behavior[136] and affect the differentiation of stem cells into different lineages.[39,137] At the most basic level, neuronal cells perform best in soft matrices, while osteoblasts perform best in hard matrices; the differentiation of progenitors is most efficient in a matrix with elasticity comparable to the mature cell's natural niche.

Natural ECM components exhibit non-linear rheological properties including strain-stiffening,[138] stress-relaxation and viscoelasticity,[139] due largely to non-covalent and reversible interactions, allowing for the matrix to respond to the presence of mechanical forces from cellular adhesion. Such materials display frequency (time) and magnitude (force) dependent relationships between the storage (G') and loss (G'') moduli of the materials. Efforts to mimic these behaviors in synthetic systems are uncovering elements of rational design of such properties. Nicely compared, the differences in mechanical properties of peptide based natural and synthetic hydrogels have been topics of a comprehensive review.[140] In general, mechanically responsive networks have the potential to display different mechanical information over time, in response to force, and even allow disruption and reformation of bonds during cell and tissue ingrowth.

5.1 Strain-stiffening

Strain-stiffening is a common feature of natural physically associating hydrogels,[141] allowing for cellular traction and communication even in very soft matrices.[142] Unfortunately, biomimetic strain stiffening has been difficult to engineer in synthetic hydrogels. A breakthrough in this area was introduced in 2013 with ethylene glycol functionalized poly(isocyanides) (PICs, Fig. 5a) that were able to recreate the magnitude and response of many ECM strain-stiffening properties. Akin to collagen bundling, this biomimicry was experimentally attributed to the supramolecular bundling of the PIC polymers into a nanofibrous network.[143,144] The synthetic modularity of the system allowed engineering of the mechanical properties, including the critical stress point (the strain at which the hydrogels exhibit stiffening).[145]

Control over the differentiation of 3D encapsulated stem cells was shown *via* changes in the strain stiffening behavior of the biomimetic PIC hydrogels.[41] Within a series of soft (0.2–0.4 kPa) PIC gels, moving the critical stress point from 8 Pa to 20 Pa induced a switch in hMSC differentiation from adipogenesis to osteogenesis (cultured in a 1 : 1 osteogenic:adipogenic culture medium). These gels all exhibit similar elasticity at rest, yet when the cells exert mechanical force on the adhesion sites (covalently attached GRGDS), the materials respond differently. To further advance this study, the authors were able to find a correlation between DCAMKL1 expression (a microtubule-associated protein) and the onset of the strain stiffening. This observation supported DCAMKL1/RUNX2 as a potentially important mechanotransduction switch in these 3D strain-stiffening matrices.

More recently, a self-assembly, covalent fixation, and covalent crosslinking strategy to design strain-stiffening hydrogels was introduced.[146] Utilizing bis-urea bola-amphiphiles with internal acetylenes, covalent fixing of the self-assembled flexible fibers, followed by azide–alkyne crosslinking, led to strain stiffening hydrogels. Pre- and post-crosslinking both showed bundled fibers, and control of concentration led to control of stiffening onset. These observations give promising insights in the rational design of biomimetic strain-stiffening materials. Cell-based studies with the bis-urea materials have not been performed, yet will prove difficult due to their non-transparent nature (poly(acetylene) backbone).

5.2 Viscoelasticity

Viscoelastic materials exhibit both elastic and viscous properties that vary with the timescale of the deformation applied.

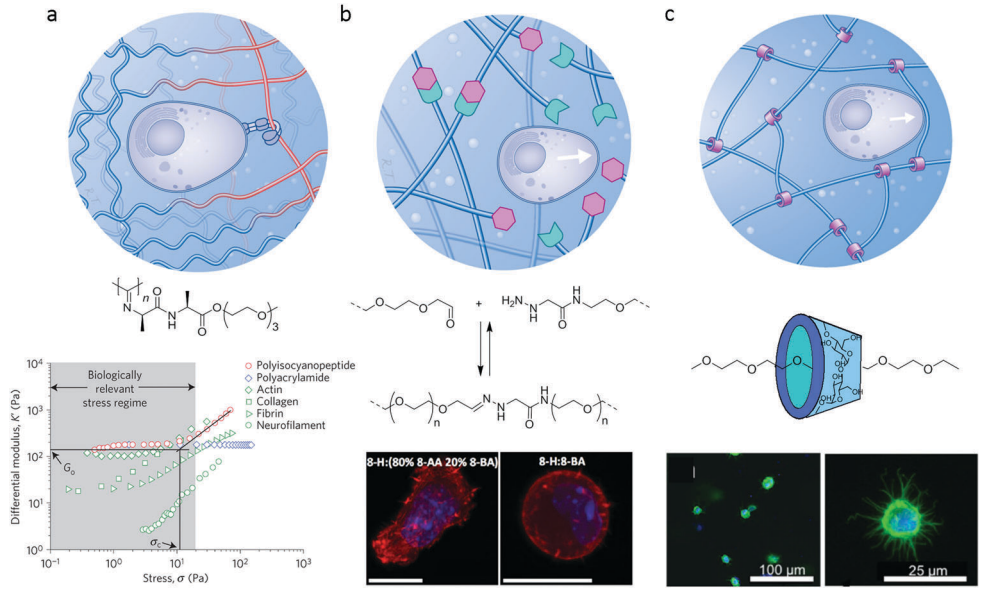

Fig. 5 Significant examples of hydrogels able to respond to the mechanical forces of a cell. (a) Strain-stiffening hydrogels, create a stiffer network (orange) upon applied strain from a cell, *via* its integrins (light blue hook), during the generation of traction forces. Poly(isocyanide) (PIC, structure shown) gels, allow for recapitulation of the strain-stiffening environment of the ECM. PIC gels showed stress stiffening behavior within the biologically relevant stress regime (see graph) in comparison to other hydrogel systems.[41] G_0 represents the equilibrium bulk stiffness and σ_c is the critical stress for the onset of stress stiffening of the polymer gel. The effect of varying these parameters through *e.g.* alteration in polymer chain length showed the ability to switch stem cell differentiation. (b) Viscoelasticity, as in dynamic covalent hydrazone based hydrogels, allows for facile cellular remodeling of the gel. Shown both schematically (above) and chemically (middle), these dynamically interchangeable bonds create materials with different mechanical information on different time scales, and greatly affects the ability of cells to grow protrusions into the material. The dynamism of the aliphatic vs aryl aldehyde on cell viability was studied *via* C2C12 myoblasts encapsulated in 8-H:(80% 8-AA,20% 8-BA) (comprised of 80% aliphatic aldehyde and 20% aryl aldehyde) showed actin filaments and extend lamellipodia and filopodia, while cells encapsulated in 8-H:8-BA (100% aryl aldehyde) remained round (bottom).[60] (c) Sliding hydrogels based on poly(rotaxanes) provide permanent, yet mobile crosslinks (above). Shown with α-cyclodextrin threaded PEG hydrogels (middle), this atypical architecture allows for the clustering of adhesive ligands and the remodeling of the network *via* forces from the cell. Again here, authors see enhanced propensity for differentiation in such dynamic hydrogel architectures. hMSCs in sliding hydrogels allowed formation of protrusions (bottom), which was attributed to their ability to rearrange crosslinks and ligands.[160] (a) was reproduced by permission from Macmillan Punishers Ltd: Nature[41] copyright year 2015. (b) was reproduced with permission[60] John Wiley and Sons 2013. (c) was reproduced with permission.[160] John Wiley and Sons 2016.

Many of the natural ECM components used for cell culture are viscoelastic and stress-relaxing (*e.g.* collagen, basement membrane, fibrin). This plasticity of the matrix allows for cellular remodeling of the material *via* cellular forces[139] (combination with enzymatic degradation). In attempts to recapitulate this dynamic nature, many of the synthetic systems rely on reversible and/or non-covalent interactions that have inherent timescales.[25,27,29]

The importance of viscosity on cell behavior was first observed within a series of 2D poly(acrylamide) hydrogels. An increase in the hydrogel's viscous behavior led to enhanced hMSCs differentiation potential to a number of lineages.[147] Later, in 3D alginate hydrogels, significant differences in cell spreading and focal adhesion formation were shown between elastic and viscoelastic 3D alginate hydrogels (covalent *vs.* ionic crosslinking) at a similar initial modulus.[148]

Recently it has been shown that the relaxation rate of viscoelastic hydrogels can directly influence MSC differentiation.[149] Utilizing a series of alginate hydrogels, from high molecular weight to low molecular weight with grafted PEG, stress relaxation of the gel was tuned independently of the stiffness, degradation, and adhesive ligand density (relaxation constants = 3300–70 s). Gels with a rapid rate of stress relaxation (low molecular weight alginate with grafted PEG) led to significantly higher proliferation, spreading, and osteogenic differentiation than gels with a low rate of stress relaxation (high molecular weight alginate). Clusters of adhesive ligands in the vicinity of cells were observed to a larger extent in the rapidly relaxing matrix; the force associated with this integrin-ligand clustering was previously shown to effect osteogenic differentation.[40] The rapidly relaxing alginate gel (~1 min) was the only matrix that allowed osteogenic differentiation and the formation of an interconnected, mineralized, and collagen I rich matrix—hallmarks of bone formation.

Viscoelasticity, and stress-relaxation, can also be engineered into hydrogels utilizing well-defined and reversible crosslinks within the material. While many dynamic covalent chemistries (DCvC) are triggered by external factors, such as pH, light, and temperature, the hydrazone exchange reaction maintains a dynamic equilibrium under physiological conditions and lends itself well to the formation of biomaterials. In an instructive example, Anseth and co-workers have developed a multi-arm PEG based hydrogel that is crosslinked *via* DCvC between hydrazones and aldehydes (Fig. 5b).[60] By tuning the nature of the aldehyde (aliphatic and aromatic) and the topology of the

gel (4-arm and 8-arm PEG), they report the ability to cover a wide range of elastic moduli (1.8–27 kPa) and relaxation time constants (10 s–approximately hours). Within this series of dynamic gels, C2C12 myoblasts were found to fuse and demonstrate myotube-like morphology only in the dynamic hydrogels (relaxation constant 91 s) (Fig. 5b). In the more static hydrogels (slower hydrazone exchange), the cells kept mostly a rounded morphology. Less than 30% of the cells grew external protrusions into the material after 10 days of culture.

Materials made from such well-defined chemical strategies can be optimized, correlated to, and rationally designed based on solution phase kinetics and equilibrium constants.[150] Furthermore, these dynamic materials have the ability to maintain a constant bulk behavior, while responding acutely to the local forces from a cell. Owing to the constant bulk properties, these hydrazone–PEG hydrogels have also enabled the recording and analysis of neurite outgrowth from embroyid bodies to be translated into cellular forces involved in the remodeling of the viscoelastic material.[151] Furthermore, recent results show that a hydrogel system based on the reversible hydrazone linkages was able to promote neural progenitor growth and have a marked increase in neural development in zebrafish when compared to a traditional alginate hydrogel.[62]

Numerous non-covalent and supramolecular hydrogels are capable of displaying viscoelastic behavior.[70,71,152–155] However, there are no known studies linking this viscoelasticity to cellular behavior. Cellular adhesion to supramolecular surfaces[156,157] and supramolecular fibers[75] has been well demonstrated, yet the correlation to viscoelasticity or timescale remains to be reported. The effect of viscoelasticity within self-assembled supramolecular matrices on 3D cell culture remains an open research question and will become crucial in upcoming designs of a fibrous synthetic ECM.

5.3 Mechanically interlocked systems

The mechanical remodeling of a network can also be accomplished with permanent, yet mobile, crosslinks within a hydrogel. Topologically interlocked networks like poly(rotaxanes)[158] have been extensively used in the toughening of materials due to their dynamic and stress-responsive network architectures.[159] Primed for a foray into biomaterials, a poly(rotaxane) sliding hydrogel with mobile crosslinks and adhesion ligands has recently been reported (Fig. 5c).[160] Based on an α-cyclodextrin threading PEG polymers, these hydrogels not only have enhanced toughness, but also the ability for cells to remodel the sliding crosslinks and/or adhesive ligands. Key advancements were both the ability to crosslink the poly(rotaxanes) in a cell-friendly manner and prevention of cyclodextrin crystallization. As seen above, MSCs showed increased ability for differentiation to a variety of lineages in this remodelable gel, when compared to a similarly constructed statically crosslinked hydrogel.

This initial study represents a promising area of research in the future as there is a wealth of information on the unique properties of topologically interlocked networks. One can quickly imagine adding shuttling functions (*e.g.* in relation to transport of oxygen and removal of cellular metabolite byproducts) and off/on switches within these materials for enhanced control over cellular behavior.

5.4 Mechanochemical bond

In the cell's environment, there are several versions of mechanically sensitive bonds that provide enhanced function and responsiveness in the presence of mechanical force.[45,161] Frequently termed "soft mechanochemistry,"[162] the mechanically responsive bonds in biological systems are often based on protein conformation changes. In an ideal bond, the lifetime and/or strength of the bond or interaction is constant regardless of the applied force on the bond. However, recent research has shown that many molecular interactions involved in cell adhesion and movement deviate from this ideal relationship.[163] Catch bonds are capable of increasing in strength and lifetime under applied force, while slip bonds decrease in persistence under force. An excellent example of these phenomena exist in cadherins; the presence of all three types of bonds allows for cadherins to withstand tensile force and tune the mechanical properties of adhesive junctions.[164]

Mechanochemistry within the realm of polymer chemistry has seen resurgence in recent years. Traditionally, these systems revolve around creating a chemical reaction (generally bond breakage) under applied force; however, recently inspired by molecular catch bonds, systems that strengthen with applied force are also being currently developed.[165] While these are covalently based systems, there is some evidence that supramolecular based hydrogels can undergo force mediated modulation of binding constants.[166] In addition, mechanophore research has recently allowed for the rational design of molecular sensors for mechanical force.[167,168] Currently, the mechanochemical systems developed have not been designed active on the cellular level of force generation, and only a few verified versions of synthetic catch bonds have been documented.[169] Yet, rational design and benchmarking[170] pave the way for innovation. Creating systems that are mechanochemically active to the forces of individual cells inherently will produce fragile materials that can create extreme difficulties in handling. It is likely that such systems may need an *in situ* activation step, or rely on reversible non-covalent interactions, to create functional hydrogel biomaterials.

6. Metabolite and small molecule responsive systems

While enzyme responsive hydrogels have proven utility for the construction of cell-responsive materials, these protein-based catalysts are not the only molecular stimuli in the environment of a cell. In the cellular niche, there are numerous pH or reductive changes, non-catalytic proteins and small molecule metabolites, and dissolved gases that provide functions ranging from structural support to inter-cellular signaling. Creating synthetic systems that are capable of responding to these highly varied metabolic products (or fuel) of a cell can prove difficult, but can allow significant advancements in

specificity, selectivity, and choice of triggers. Furthermore, these strategies can allow for reversibility of response, since many of these systems are based on binding or recognition events.

Within this section, one will find common use of biohybrid materials relying on synthetic integration of highly specific and bioengineered proteins and nucleic acids. Although fully synthetic materials are less prevalent, progress has been made *via* dynamic recognition events *e.g.* boronic acid sensing of glucose. Many of these metabolite/small molecule responsive systems have not yet found applications in tissue engineering or regenerative medicine; however, strategies able to respond to metabolic states or differentiation events in tissue formation, can start to be entertained.

6.1 pH and redox

Hydrogels that are sensitive to pH changes[84] were among the first classes of responsive hydrogels designed to change in response to physiochemical factors,[171] while redox systems have traditionally been used intracellularly.[84] Both systems respond to local extra and intra-cellular environments and are popular with drug-delivery strategies. Easily accessible chemical motifs can impart pH and/or redox sensitivity; however, pH and redox changes nearby a cell are generally neither large, nor specific. For these reasons, pH and redox responsive systems are sometimes coupled with an enzyme for signal amplification (*i.e.* glucose responsive hydrogels, *vide infra*).

There exist two main strategies to design hydrogel pH responsive systems: the use of polymers with ionizable chemical groups that undergo conformational or solubility changes, and polymeric systems with acid-sensitive bonds. A major application of pH sensitive hydrogels includes the sensing and response of hydrogel networks to the acidic environment (due to accelerated glycolysis) around cancerous cells.[50] Poly(acrylic acid) (PAA, anionic polyelectrolyte) and poly(diethylaminoethyl methacrylate) (PDEAEMA, cationic polyelectrolyte) are the most commonly studied polymers with ionizable chemical groups, and can swell/deswell depending on pH changes, often releasing a drug.[14,172] As an example of bond sensitivity to pH, Schiff base hydrogel systems, including alginate–chitosan,[173] and low-molecular weight hydrogelator (LMWG) hydrogels,[174,175] have found application in cancer therapeutics and wound healing.[63] Self-assembled hydrogelators with doxorubicin (DOX) have been reported to form injectable systems that effectively and selectively deliver DOX to breast cancer in mice and significantly inhibit tumor growth.[173,175]

Moving towards redox-sensitive systems, disulfide crosslinked hydrogels, prone to rapid cleavage by glutathione, have recently been used in effective hydrogels for gene-therapy and tissue engineering. Reduction sensitive self-assembled DNA nanogels (held together by disulfide links between the DNA building blocks) have been shown to disassemble within a cell, and were capable of effectively delivering an anti-proliferation gene.[82] In addition, Varghese and co-workers designed a disulfide crosslinked PEG hydrogel that was shown to degrade in the presence of multiple cells without external stimulus. They observed that this degradation rate could be tuned *in vitro via* cell type, encapsulated cells density, and disulfide cross-link density. These PEG hydrogels were successfully used to deliver, and increase retention of, hMSCs into mouse dorsal muscle.[64] Various other redox responsive systems have been employed for drug delivery, but require the input of a user-defined stimulus.[84,176]

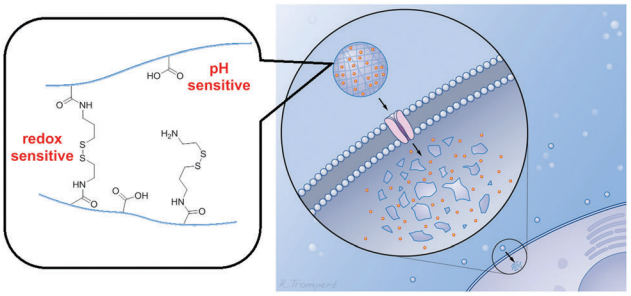

Fig. 6 Many pH and redox responsive systems are engineered for drug delivery across a cell membrane. Shown schematically are hydrogel microspheres, loaded with a drug or growth factor (orange spheres), being taken up by a cell and then releasing its cargo upon breakdown of the hydrogel network. Poly(methacrylic acid) (PMAA) hydrogels with disulfide crosslinks and with dual pH and redox sensitivity have been successfully created for delivery of doxyrubicin to cancer cells. These hydrogels swell at neutral pH and uptake DOX, degrade in cytosolic space due to presence of gluthatione (GSH), which reduces disulfide bonds and release DOX. Adapted with permission.[49] Copyright (2015) American Chemical Society.

pH and redox sensitive hydrogels can also be combined into one functional system. Poly(methacrylic acid) (PMMA) hydrogels of micrometer sized with dual pH and redox sensitivity have been reported by Kharlampieva and co-workers.[49] Again targeting DOX delivery to cancerous cells, the pH sensitivity facilitated DOX encapsulation, while the incorporation of disulfide crosslinks triggered rapid degradation of these hydrogels in the reducing environment of a cell's cytoplasm. The concept of this system is represented in Fig. 6 where DOX (red spheres) are encapsulated in the hydrogels at neutral pH and degraded in the cytosolic space by gluthathione. The authors have shown that these DOX containing mirco-hydrogels were capable of 90% cytotoxicity when incubated with HeLa cells for 48 h.[49] Such dual responsiveness has also been effectively translated to self-assembled cyclodextran/benzimidazole nanogels for controlled release.[177]

A newer concept to design responsive hydrogels is to use a cell or tissue's generation of reactive oxygen species (ROS). These species play important roles in cell signaling pathways and abundantly exist at sites of inflammation and tissue healing.[52] Several studies have been reported on 3D polymeric scaffolds, crosslinked with oligo(proline) ROS cleavable sites. These scaffolds showed enhanced degradation when incubated with ROS generating macrophages, as well as improved host cell infiltration and angiogenesis when implanted subcutaneously in mice.[178,179] This concept is transferrable to hydrogels for a general degradation mechanism during tissue growth and remodeling, while also providing a potential protective effect. ROS protective hydrogels have been shown to protect both pseudo-islets and hMSCs under high oxidative stress.[180]

6.2 Molecule specific

Molecule specific hydrogels commonly rely on a biohybrid approach. The specificity (and amplification) of biochemical recognition, and the ability of this recognition to provide a response, is difficult to recreate in fully synthetic systems. However, it is this same specificity that empowers molecule specific responsive systems greater potential in the complex biological environment. Some of the best known and most advanced molecule specific hydrogels have been created towards the treatment and control of diabetes. Glucose responsive and insulin releasing hydrogels hold promise to replace simple insulin injection, and such hydrogel systems have been one of the few to be approximated by fully synthetic systems.

Most hydrogels designed for glucose responsiveness, utilize enzymatic conversion of glucose to gluconic acid, in presence of glucose oxidase (GOx), to induce the swelling of pH responsive hydrogels for insulin release (Fig. 7a).[181] For example, glucose responsive chitosan (protonatable amine) microgels have been designed using this approach, and their glucose dependent insulin release showed *in vivo* efficacy in controlling glucose levels in diabetic mice.[54] The production of H_2O_2 from GOx (by oxidizing glucose) has also been utilized to engineer glucose responsive hydrogels.[182] Based on the sensitivity of ferrocene/cyclodextrin complexation to oxidation, redox-sensitive hydrogels have been created from ferrocene terminated pluronic and a polymer with cyclodextrin pendant groups. The hydrogels underwent a gel–sol transition upon exposure to glucose solutions.

While the previous examples are effective, these systems lack specificity to only glucose, due to the hydrogel response being secondary in nature. In order to increase glucose specificity, fully synthetic polymers containing phenyl boronic acid (PBA) have gained much attention (Fig. 7a).[58] Boronic acids are well known to form dynamic covalent bonds with diols, especially sugars, in aqueous media. Sakurai and co-workers were among the first to use such a system, where the creation of a charged PBA glucose complex swelled the hydrogel and allowed release of insulin.[183] More recently, work by the Anderson group has created a series of self-healing, tunable, shear-thining, injectable, and glucose responsive hydrogels by incorporating both the PBA and a competitive diol (hydrolyzed sugar) within the polymer network.[152,184] The glucose responsiveness (and insulin or IgG release) of these hydrogels were shown with hyperglycemia mimicking conditions, and these hydrogels showed a typical foreign body reaction upon implantation in mice, making them promising candidates for insulin delivery applications.

As seen by the glucose–PBA based hydrogels, systems based on the response to a binding event from a specific small molecule are possible in many fashions. Relying on a urate responsive protein–DNA complex, Weber and co-workers developed a hydrogel designed to provide a protective environment in patients with gout.[185] The hydrogel system was based on a urate repressor protein (HucR), conjugated to poly(acrylamide), and crosslinked *via* the addition of oligomeric hucO DNA ($[hucO]_n$). This system forms a crosslinking HucR·hucO complex that dissociates at elevated urate concentrations, resulting in dissolution of the hydrogel. By incorporating urate oxidase, the authors have shown that the hydrogel system responded protectively to uric acid pulses in a mice model.[186]

An exciting approach to design molecule specific dynamic hydrogels is by translating binding induced protein conformational

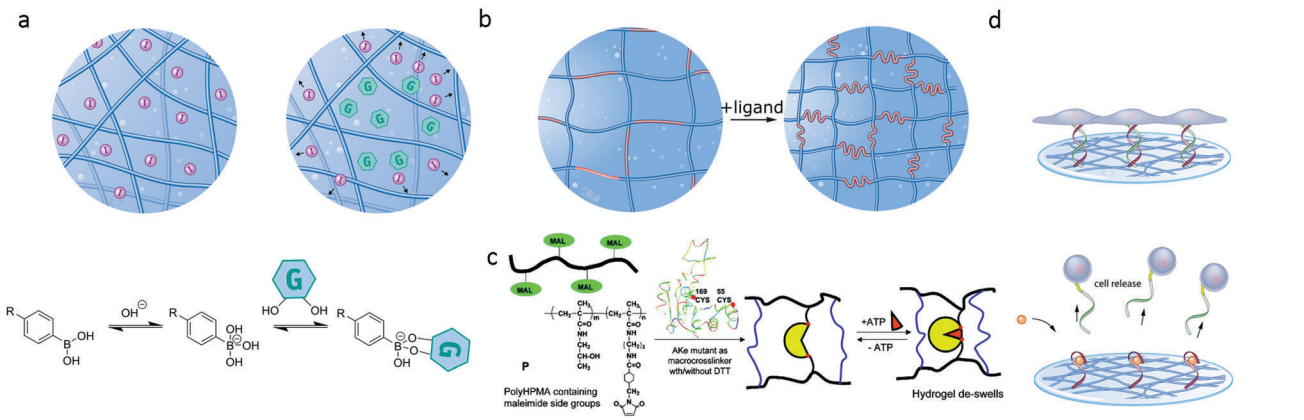

Fig. 7 Selected examples of molecule specific responsive hydrogels (a) phenyl boronic acid (PBA) hydrogels for insulin (purple spheres) delivery: PBA specifically recognizes diols such as glucose. A charged PBA–glucose (G hexagons) complex is formed swelling the hydrogel network and releasing insulin as a function of glucose concentration. Due to the dynamic nature of the PBA–glucose complex, such hydrogels are often quick to respond and can be made injectable (shear-thinning).[152] (b) Protein conformational changes (orange sections of hydrogel network) upon ligand binding can translate nanoscale motion into macroscale motion or materials properties. For example, calmodulin (CAM) conjugated PEG hydrogels showed volume changes upon binding to a trifluoroperazine (TFP) ligand.[189] CAM goes from its extended shape (in the presence of calcium ions (left)) to a collapsed conformation (right) upon binding to TFP ligand. (c) Adenylate kinase (another conformationally changing protein) hydrogels were made by crosslinking polyHPMA (with maleimide side groups) with a thiolated mutant of adenylate kinase (AKtm). These hydrogels undergo macroscopic motion when exposed to ATP. (d) DNA aptamer recognition for cell release: apatmers (purple) sensitive to ATP (orange) are initially hybridized to complementary strands (green) with adhesive RGD attached (yellow), creating a cell-adhesive environment (top). Upon introduction of ATP (bottom, exogenous or cell-secreted), aptamers release their complementary strand and bind to ATP, thus creating a non-adhesive environment.[193] Aptamer–target interactions can be designed for virtually any target, making this a highly promising, though costly, strategy. (c) was reprinted with permission.[191] Copyright (2008) American Chemical Society. (d) was adapted from ref. 193 under creative commons license (CC BY 3.0).

changes into macroscopic motion or reorganization (Fig. 7b). For example, calmodulin (CaM) is a protein with three distinct shapes: unstructured, extended (in presence of Ca^{2+}), and collapsed (in the presence of phenothiazine anti-psychotics). CaM was first successfully incorporated into star PEG hydrogels that were shown to undergo a significant volume decrease (up to 15%) upon binding of trifluoroperazine.[187] Incorporating a phenothiazine ligand into an acrylamide network containing CaM allowed for a triple-state responsive gel system that swelled and de-swelled as a function of calcium and phenothiazine concentrations.[188] These hydrogels were shown to allow active control of molecular transport across the gel and control fluid flow from a microfluidic device. CaM based hydrogels have even found application for tissue engineering *via* spatio-temporal control of growth factors delivery. Murphy *et al.* have designed growth-factor laden PEG–CaM–PEG acrylate based hydrogel microspheres, using two-phase suspension polymerization that showed a maximum volume change around 76%. The authors have shown that temporal release of VEGF and BMP-2 could be achieved by varying the timings of CaM's ligand trifluoperazine induced volume changes.[189] CaM is just one good example of molecular motion in protein engineering for hydrogel design, as touched upon briefly in a recent review.[190] Notably, systems like adenylate kinase-ATP responsive gels (as depicted in Fig. 7c) may see more application in the future, as they respond to a more relevant cell metabolite (adenosine triphosphate).[191]

Although the *a priori* engineering of target-responsive hydrogels remains difficult, directed evolution of DNA aptamers give a strategy to create systems that respond to a desired target molecule. Aptamer based hydrogels can be made responsive to specific analytes, for example, an excess of adenosine and thrombin.[192] Taking this design principle further, Qu and co-workers have recently created cell responsive hydrogels that control 2D cellular adhesion in response to ATP concentration (Fig. 7d). By conjugating ATP aptamers to an alginate hydrogel and hybridizing with RGD functionalized complementary DNA, a cell adhesive surface was formed. Exogenous delivery of ATP was shown to cause dehybridization of the DNA, rendering the surface non-adhesive and releasing cells. The system was demonstrated to be highly selective to ATP and even responsive to ATP signaling from cells in co-culture.[193] Such a strategy requires non-trivial aptamer generation, but can be applied to a wide variety of targets and incorporated in a wide variety of systems.

6.3 Gas sensitive

The responsiveness of hydrogels to and control of gaseous metabolites is a significant need in 3D scaffolds for tissue engineering. The difficulty in maintaining oxygen partial pressures within tissue engineered constructs and in 3D cell culture is well known, but is difficult to control or to quantify/image. Ultimately, re-vascularization is desired to enable the long term viability of an engineered tissue;[194,195] however, biomaterials that can sense and/or control the concentration of gaseous metabolites remain highly sought after. Biomaterials capable of delivering gases remain an active area of research,[196–198] and hydrogels capable of sensing gaseous metabolites are valuable research tools.

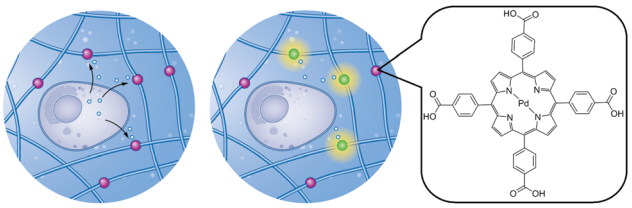

Fig. 8 Gas sensitive hydrogels have mainly been designed for imaging applications. Proper concentrations of gaseous metabolites and by-products within regenerating tissue is crucial for success, yet very difficult to measure. Incorporating gas sensitive fluorophores (purple sphere) in the hydrogel network allows the artificial environment to sense and provide this information. For example, oxygen responsive Pd-porphyrins (left) have been used to crosslink hydrogels for monitoring of oxygen concentration subcutaneously.[206]

There is currently only one strategy for materials that can loosely be defined as delivery of oxygen in response to local consumption. Perfluorocarbon (PFC) based materials, have high oxygen solubility (up to 35 times the aqueous environment) and can act as oxygen conduits when the local partial pressure drops. Hyaluronic acid hydrogels with a covalently attached polyfluorinated oxadiazole have been shown to significantly increase the viability of fibroblasts in both normoxic and hypoxic conditions.[199] Fluorinated methacrylamide chitosan hydrogels have also shown enhanced oxygen distribution, and higher fluorine content (*via* conjugation of longer fluorinated chains) facilitated both the highest cell proliferation and neuronal differentiation within neural progenitor cells.[200]

While no materials are currently able to truly respond to gaseous metabolites and influence a cell, responsive hydrogels that allow the imaging of gaseous metabolites still provide significant information to tissue engineering and regenerative medicine. For example, measuring the distribution of oxygen within a tissue engineered construct can lead to better 3D designs.[201] For biological use, oxygen responsive probes (usually organic dyes) are physically encapsulated in a hydrophobic polymer or solid.[202] Beautiful core–shell[203] and dual-responsive[204] (to O_2 and interleukin-6) hydrogels working on this principle have already found application for tissue engineering. Recent efforts to covalently incorporate porphyrin dyes into the hydrogel matrix, has opened up the potential for hydrogel networks with immobilized (non-leachable) dyes (as depicted in Fig. 8).[205,206] Huang *et al.* were even able to show the ability of a porphyrin crosslinked PEG hydrogel to perform well as an implantable oxygen sensing material,[206] showing the possibility for long-term oxygen distribution imaging of tissue engineered constructs *in vitro* and *in vivo*.

7. Outlook

As we move towards more sophisticated hydrogel architectures, the progress in stimuli responsive systems has the potential to move from large, user generated stimuli, to smaller, locally generated biological stimuli. The ability to engineer these

hydrogel systems to listen and respond to cell-based stimuli can pave the way to the creation of autonomous biomaterials to be used in tissue engineering, drug delivery, sensing, and cancer therapeutics, among other fields. With smart engineering and design, one can imagine the design of materials that can not only recapitulate the real-time response of the natural ECM, but also move towards multi-component[207] systems that provide non-natural or enhanced function,[208] controlling the growth, differentiation, and migration of cells towards the formation of regenerated tissues. For example, the design of bioresponsive hydrogels with complex logic gate functions has already been demonstrated,[209] and such logic gate architectures allow complex responses from complex inputs.

The toolbox of chemistries to use in the design of cell-responsive systems can be considered small, but more importantly, such chemistries are often poorly studied in the context of complex biological systems. For example, supramolecular biomaterials hold great promise to recreate and mimic the complex and dynamic extracellular environment.[26,66] Numerous biological systems revolve around the dynamics, specificity, responsiveness, and information richness of non-covalent interactions and self-assembly for proper function.[210] As we grow more sophisticated in our engineering of synthetic supramolecular systems, demonstration of control over molecular assembly pathways,[211,212] energetics,[213,214] consumption or dissipation of energy,[215–217] and the resultant dynamics,[218,219] clustering,[220,221] and function in a biological setting,[66,67,222] have all been demonstrated. Control of dynamics can enable the hydrogel to interact with different mechanosensing events, clustering can effect signal transduction or raft formation *via* superselective assembly, and control over pathways can enable different environmental structures given different inputs. Of particular interest, the creation of out-of-equilibrium and dissipative self-assembled hydrogels will allow new insights into the assembly of the natural ECM and the potential to mimic this dynamic environmental switching in biorelevant hydrogel systems.

As seen with CaM hydrogels (*vide supra*) molecular machines can generate a large output from a simple biological signal. Synthetic (supra)molecular machines, such as rotaxanes,[223] catennanes,[224] and molecular rotors[225] have all gained much recent attention due to recognition through the 2016 Nobel Prize. These systems have a long road towards functional materials in a complex environment, yet open up exciting new possibilities Directed shuttling of nutrients and growth factors in a spatio-temporal manner, and stimuli-generated mechanical forces, as already seen in rotaxane molecular muscles,[226] give scientists the potential to go above and beyond some of the complexities of natural ECM.

The use of dynamic covalent, protein/DNA engineered, and enzyme degradable gels stand to provide good benchmarks for fundamental development of hydrogel systems in the immediate future. While dynamic covalent systems can specificity, their synthetic accessibility and low cost (comparatively) render them excellent choices for the production of larger amounts of hydrogel for study. On the other hand, protein/DNA engineered hydrogels allow ultimate levels of control and biomimicry, but come at an extremely high price. Their replacement by purely synthetic systems seems inevitable; however, it must be noted that production of designed hydrogelators *via* protein expression shows promise for scalability.[227] Enzymatically responsive hydrogels are excellent at creating remodelable artificial environments, and are poised to remain a valuable platform. Such systems are in need of new enzyme responsive motifs and orthogonality between motifs to enable complex functions such as complex signal processing and remodeling *via* both degradation and creation.

A major driving force for the creation of hydrogels that can respond to cellular signals or events has been the biochemical and biophysical characterization of cellular behavior in space and time. Marker identification, unravelling of signaling pathways, (bio)chemical quantification, and high content and super-resolution microscopy techniques have all been paramount to measure the interplay between materials and cells. When moving towards more dynamic systems, reliable cellular readouts remain an ever present bottleneck to characterization.

Despite progress in spatio-temporal characterization of cells and tissues, we still have a long way to go to understand the different time scales that dominate cellular behavior and decision making. While general timescales are known (*e.g.* cell division normally takes 24 hours),[228] the heterogeneity of timescales within populations and in signaling pathways is less understood. Recent studies of time-dependant materials and their effect on tissue engineering reinforce this importance.[229,230] For example, the observation stem cells contain a ''memory'' for a few days for the stiffness of the substrate on which it has been previously cultured[231] clearly show the importance of timescales for signaling pathways, adhesion formation, differentiation, and homeostasis.

While it is the dream of many that a simple hydrogel architecture will provide an effective surrogate for the natural ECM or an instructive environment for tissue engineering, we tend to think that replacement of such a multi-functional environmental scaffold for cells can only be accomplished effectively by multi-component complex chemical systems. Complex, but not complicated. We support the idea that the combination of simple chemical systems to work together in a smart manner is the way forward for this, and potentially many other fields.[232]

Conflicts of interest

There are no conflicts to declare.

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