Squaramide-based interpenetrated networks for load-bearing applications

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Chapter 1

Introduction

1.1. The concept and design of 3D cell culture models *in vitro*

In the 1980s, Mina Bissell demonstrated the significance of the culture environment on cellular behaviour paving the way for *in vitro* 3D cell culture.[1,2] In this study she investigated casein production in mouse mammary epithelial cells when cultured in an extracellular matrix (ECM) derived from Engelbreth-Holm-Swarm (EHS) tumor cells that resembles the *in vivo* basement membrane. Distinct morphological differences between cells cultured on tissue culture plastic and in the EHS matrix were observed, with the latter exhibiting the formation of structures that comprise the secretory alveoli. Since this seminal finding, 3D cell models continue to show their utility in closing the gap with the *in vivo* condition in relation to 2D culture.[3,4] 3D cellular models better replicate characteristics of the natural tissue environment that can have an effect on cell morphology, proliferation, and differentiation.[3,4] Today, 3D cell culture is being explored broadly in several areas that can impact healthcare including tissue regeneration, therapeutic applications, pharmaceutical research, and diseases modeling.[5,6]

Hydrogels are gel-like materials that are composed of insoluble macromolecular chains that absorb and retain significant quantities (> 90%) of water or biological fluid, offering a versatile platform for various biomedical applications.[7] The hydrogel materials used for 3D cell culture can be natural or synthetic.[8] Natural hydrogels are obtained from animals and plant sources, such as Matrigel, collagen, hyaluronic acid, cellulose, or alginate. A commonly used matrix is Matrigel (EHS matrix) that forms hydrogels between 22-37 °C. It mainly consists of laminin (~60%), collagen IV (~30%), entactin (~8%) and the heparin sulfate proteoglycan perlecan (~2-3%).[9,10] Moreover, it possesses intrinsic soluble factors (e.g., chemokines, growth factors, and integrin ligands) that target transmembrane proteins involved in important cell signaling pathways.[7,11] Recent applications of this material in cell culture involve 3D (stem) cell expansion and organoid assembly.[12] However, it is a costly material that shows inconsistency between lots. Additionally, the incapability to further tune the low stiffness of Matrigel makes it unsuitable for some applications along with decoupling of various cues.[13] Synthetic hydrogels demonstrate the potential to overcome these challenges as they offer the potential to control and customize the cell microenvironment. They can be prepared from a range of synthetic polymers and combined with natural polymers providing handles to modulate their chemical and physical properties. This section is expanded on in 1.14.

1.2. The Extracellular matrix (ECM) of cell niche

The extracellular matrix of all tissues consists of an acellular fibrous protein network. This natural material not only physically support cells, but also guides their behaviour. A range of insoluble proteins (e.g., collagens, fibronectin, heparan sulfate), growth factors (e.g., transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF)), and other secreted proteins (e.g., proteolytic enzymes, protease inhibitors) can be found within this material.[2,14] The precise composition and presentation (e.g., density, stiffness, topography, fiber size and orientation, chemical/physical properties) of the ECM is specific to a given tissue, and adapts constantly to its environment.[15,16]

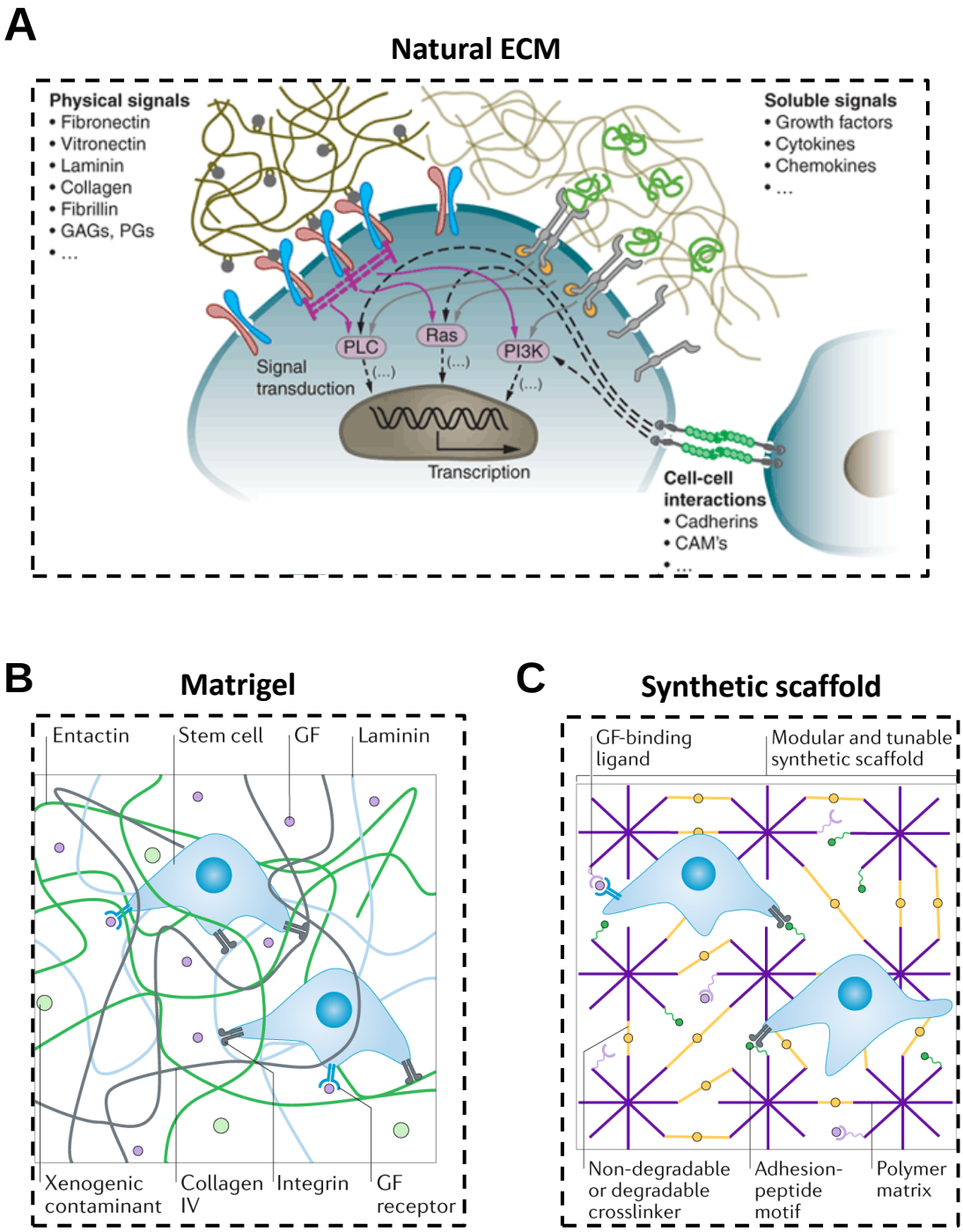


Figure 1.1. Dynamic interactions between cells and different ECMs (natural ECM[17], Matrigel and synthetic scaffold[9]) which is regulated by the complex and mutual molecular interactions occurring between cells and their surrounding environment. Images adapted from references.

1.3. The relevance of mechanics on cell behaviour in disease

Cells continuously adapt the stiffness of their environment *in vivo* to accommodate external forces, otherwise known as mechanical homeostasis.[18] However, this balance can be disrupted in disease states where changes in matrix deposition, removal, and restructuring occur lead to aberrant cell behaviors. For instance, tumor formation can be promoted by mechanical cues from cancerous tissues that trigger biochemical signaling cascades stimulating cell cycle progression and motility.[19] In blood vessels, low and/or high shear stresses can activate or inhibit angiogenic signaling pathways during embryonic development. For adult blood vessels that generally encounter large shear forces,

low shear forces in curved and branched regions trigger abnormal signaling and the start of diseases such as artherosclerosis.[20] Thus, alteration of mechanical homeostasis can lead to disease progression and understanding how cells interact with their environment mechanically, or the study of mechanobiology is critical to understand disease and its treatment.

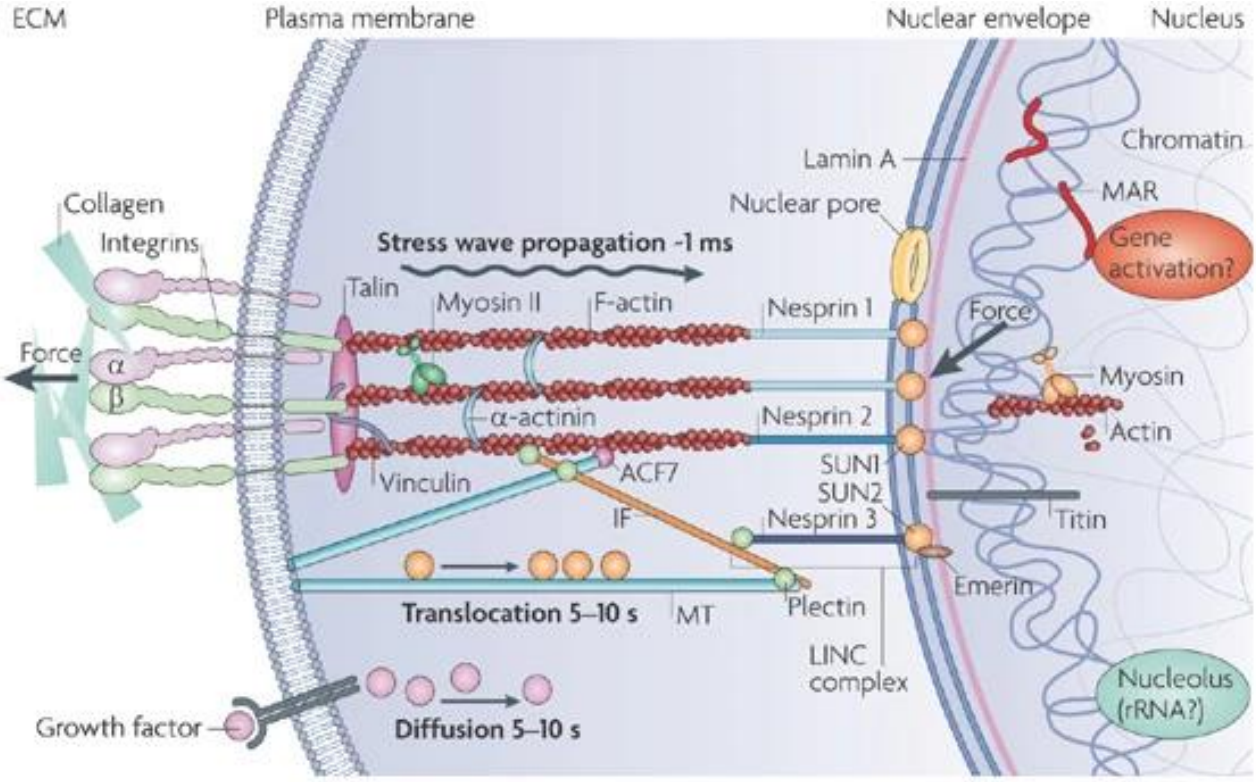


Figure 1.2. The transfer of external mechanical signals into the cell nucleus.[21] When an external force is applied on integrins on the ECM, it is initially concentrated at focal adhesions and transmitted to filamentous (F)-actin. This F-actin is bundled by α-actinin and tensioned by myosin II, creating prestress in the cell. The force could be transferred through multiple pathways within the cell due to the close connection with microtubules (MTs) and intermediate filaments (IFs). MTs and IFs are connected with Nesprin 1-3 that could further transmit force signal to SUN1 and SUN2 on the nuclear. The entire process can occur about 1ms. The force directed into the nuclear scaffold can impact gene activation within milliseconds of surface deformation. In contrast, it needs several seconds for growth factors to transfer the signal into the nuclear and cause changes. Image adapted from reference.

1.4. Mechanotransduction governs cell-matrix crosstalk

Mechanotransduction is the conversion of mechanical forces cells sense from their microenvironment extracellularly into intracellular biochemical signals.[21] Transmembrane mechanosensitive receptors or integrins provide a link from the ECM to the cytoskeleton to facilitate this process.[21,22] When integrins are subjected to force, a series of structural modifications are triggered translating the mechanical stimulus into a chemical signal that impacts gene expression within the nucleus. [21] Such as the tracts of disulfide bonds are involved in the conformational changes of the protein through bond opening and closing.[23] They can interact with a diverse ECM proteins (e.g., collagen, fibronectin) enabling cellular sensing and response to external mechanical stimuli, and thus are behind numerous cellular activities including cell motility, proliferation, and differentiation.[21,22]

Besides sensing the physical properties of their surroundings, cells also exert mechanical forces on their environment.[24] Cells generate force through cell-matrix adhesions and actin-myosin interactions. When the matrix is soft, the adhesion to the matrix is low with little focal adhesions and reduced actin fiber alignment. Whereas when the matrix is stiffened, cell adhesion to it increases

recruiting vinculin to the focal adhesions and actin fiber bundling generate higher traction forces.[25] Cells have the ability to enhance the rigidity of their matrix up to 15 µm radially from themselves by the mechanical strain they impose on their matrix to adjust the surrounding environments.[26] Bundling and alignment of the cell's actin filaments then occurs permitting the cell to exert more force forming a positive feedback loop. This further remodels the ECM and its mechanics with lasting changes, even after the cells have been removed.[27-31] This two-way communication between cells and ECM mechanics is referred to as mechanoreciprocity.[28]

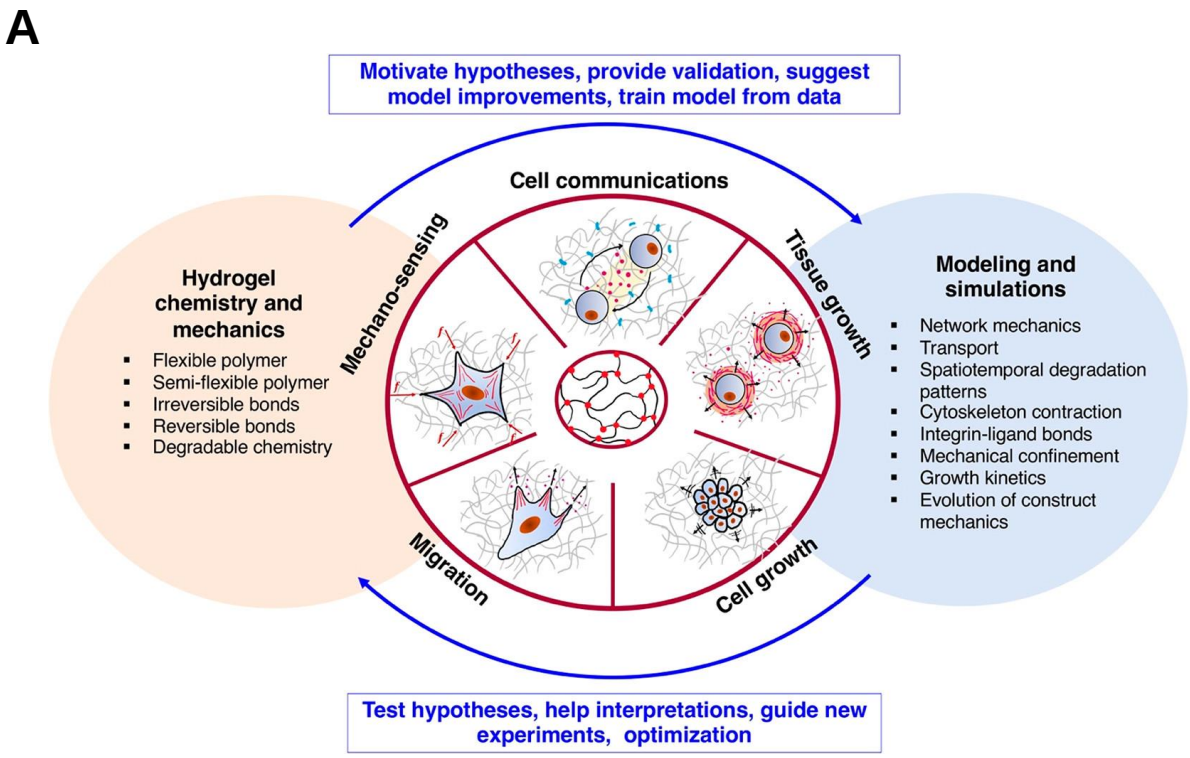
The elastic modulus or stiffness of the matrix was believed to be the most important mechanical property in mechanobiology over several decades. However, recent studies show that more complex mechanical characteristics such as strain stiffening, viscoelasticity and poroelasticity can majorly influence numerous cell behaviours that are critical in development and disease.[32] In the next sections, I will examine these various mechanical properties in detail.

1.4.1. Stiffness

The stiffness of a hydrogel is its resistance to deformation under an applied force and is described by the elastic modulus (G') or Young's modulus (E).[33,34] Differences in tissue stiffness of various tissues in the human body range from very soft (e.g., brain, several Pa - 1 kPa) to very stiff (e.g., bone, 10 kPa - 15 MPa).[35] Since the recognition that substrate stiffness influences cell behaviour over two decades ago, the amount of research on this topic continues to grow as this mechanical parameter has been implicated in numerous developmental (e.g., embryogenesis) and disease (e.g., tumor metastases) processes.[36-39] On a scan of hydrogel stiffnesses (1-100 kPa) developed by Chen and co-workers, fibroblasts were demonstrated to elongate on a stiffer hydrogel and align in the direction of the exerted force on a more stiff hydrogel.[40] The impact of stiffness on cell behaviour can be further seen from the work of Weaver and co-workers that demonstrated breast cancer cells (Basal-like and Her2 tumor subtypes) derived from stiff matrix (~5 kPa)were more invasive and resistant to chemotherapy (paclitaxel) to cells (Luminal A and B subtypes) grown on a soft matrix (400 Pa), the stiffness of the matrix affected gene activation implicated in cancer cell signaling and motility (TGFβ, YAP), providing a potential mechanism for how matrix stiffness can promote cancer progression.[41-45] These studies point that the ECM affects cancer cell behavior and highlight an opening for new therapies that target it to prevent or slow tumor growth.

1.4.2. Nonlinear elasticity

Hydrogels that show a non-linear response to applied mechanical stress or strain are called strain stiffening or non-linear elastic. This phenomenon is typically associated with biopolymer networks such as fibrin or collagen. Beyond a particular threshold the materials' stiffness increase, arising from the non-linear relationship between force and extension in individual filaments that resist stretching.[46-50] Their elastic behavior arises due to the ability to resist bending and stretching of their semi-flexible filaments that can be described by two theoretical frameworks. Bending of the filaments results in a non-affine type of deformation that is enthalpic,[47] whereas their stretching is affine and can be described as an entropic spring with a uniform distribution of strain and alignment in response to the strain direction.[46-51] Strain-stiffening can drive the transmission of mechanical stimuli to organize cells over considerable distances, typically on the order of hundreds of microns[47,52] The critical stress or deviation from linearity for several biopolymers is typically less than 20 Pa[47,52,53] Once this critical stress is exceeded, cells are limited to perceiving and modifying the material within its linear elastic region.[47] Kouwer and co-workers demonstrated this strain-stiffening property can exist in hydrogels consisting



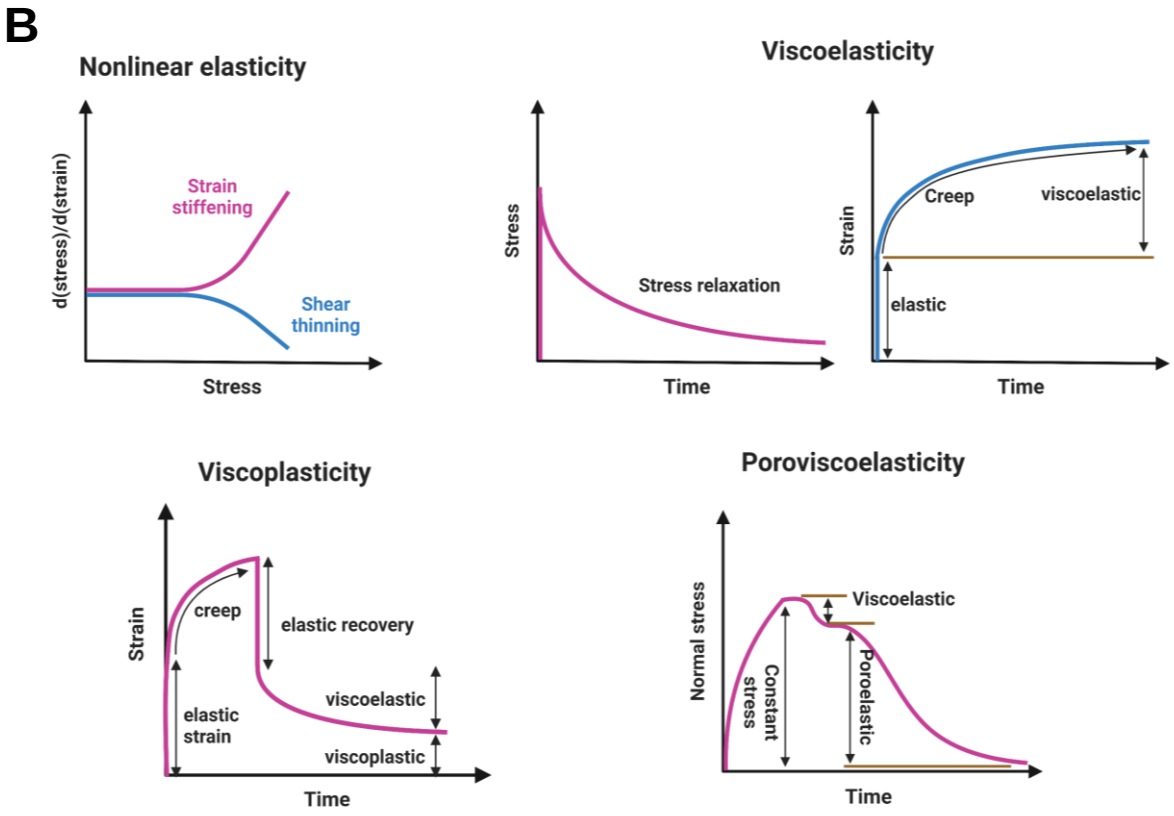


Figure 1.3. (A) Summary of the key interactions between cells and hydrogels upon cell encapsulation.[59] (B) Mechanical properties of (bio)materials can be assessed using a range of rheological tests, picture made in Biorender.

of grafted polyisocyanopeptide (PIC)-polymers.[54-56] These polymers can access parallel intramolecular hydrogen bonds along the backbone similar to proteins and form a semi-flexible fiber network. The polymers can undergo considerable deformation before reaching their maximum elongation and

resistance. Recently, Webber and co-workers incorporated dynamic boronate ester cross-links into flexible, non-filamentous networks to develop strain-stiffening hydrogels that could be adjusted by the composite concentration, pH and temperature).[57] Interestingly, in these hydrogels the critical stress exceeds the known range for most biological filaments (20 Pa).[58]

1.4.3. Viscoelasticity and plasticity

Viscoelasticity describes the ability of hydrogels to exhibit both viscous and elastic behavior under mechanical deformation while maintaining their structural integrity. The motion of entangled polymer chains, breaking of weak crosslinks or movement of fluid influences this property. [25] This property is found across tissues (e.g., skin, tendons, and muscles) and is related to the underlying collagen networks.[60] In synthetic hydrogels, this property is influenced by factors such as crosslinking density, polymer composition, polymer chain length and architecture and solvent conditions.[61] This property can be described by the Maxwell-Wiechert model that encompasses multiple parallel Maxwell elements to represent both elastic and viscous features.[62] Mooney and co-workers showed that physically crosslinked alginate hydrogels display rapid stress relaxation that influences mesenchymal stem cells (MSCs) spreading and osteogenic differentiation without the need for matrix degradation in 3D cell culture.[63] Anseth and co-workers further showed that myoblast cells spread rapidly on stress-relaxing polyethylene glycol (PEG) hydrogels crosslinked with hydrazone bonds.[64,65] These investigations indicate that stress relaxation is an key mechanical characteristic of the natural ECM that is needed in hydrogels to steer cell responses.

Viscoplasticity refers to the irreversible deformation of a material under applied stress, which is often accompanied by yielding or flow.[66] During rheometer measurements, the level of plasticity is determined by calculating the ratio between the permanent strain observed after stress removal and the total strain encountered during stress application in a creep and recovery test.[66,67] As viscoelastic substances undergo a time-dependent rise in strain when subjected to a constant stress, also known as creep, the plasticity of viscoelastic materials varies with time.[68,69] Studies indicate that viscoplasticity may affect cell behaviour in disease processes with cancer cells using this property to overcome the structural barriers imposed by the ECM to infiltrate tissues. In the disease process, the cells execute force-mediated remodeling and matrix metalloproteinase (MMP)-mediated degradation[70] resulting in realignment of matrix fibers and plastic remodeling.[66] This changes to the matrix in this process remain even after the cells are removed demonstrating a plastic response.

1.4.4. Poro(visco)elasticity

Poroelasticity refers to the elastic behavior of hydrated materials that arises from pore trapped water.[71] When the hydrogel matrix is compressed, the entrapped water is compelled to flow through the pores, leading to a decrease in water pressure due to friction with the matrix walls. This decrease in water pressure generates stress in response to compression. As water moves through the pores on compression, the water pressure decreases due to the frictional forces between the water molecules and the walls of the pores. This decrease in water pressure generates stress in response to compression. Features of the material that influence this property include porosity, pore size distribution, amount of water and mechanics.[72,73] Deguchi and co-workers studied the poroelasticity of a cellulose-based hydrogel dissolved in LiCl (8 wt%) or DMAc (92 wt%).[74] When subjected to gradual compression (0.05 mm/min), water was released at 70% strain without rupturing. In comparison, rapid compression (1.0 mm/min) resulted in a stiffer hydrogel with a steeper initial slope. At 25% strain, the hydrogel ruptured due to more rapid rate of water flow during fast compression. When polymer

materials that show both viscous and elastic properties are examined under compressive stress, they can show a poroviscoelastic response to compressive stress.[75] Monitoring of the stress relaxation process of viscoelastic materials in compression can yield this information.[76] Viscoelastic relaxation is first observed due to polymer chain and dynamic bond rearrangement, and then the poroelastic response arising from the gel microstructure and porosity.[76] Poroviscoelastic relaxation is observed in both biological tissues (e.g., cartilage) and synthetic materials (e.g., hydrogels), opening a new parameter to control and understand its influence on cell behaviour for application in areas such as tissue engineering and biomechanical studies.

1.4.5. Bioactive cue deposition in synthetic materials

While the ECM architecture and mechanics are critical to guide cell responses, engagement of a range of cell surface receptors is also critical.[77-79] Cell-adhesive proteins, such as selectins, laminin, and cadherins, bind to integrin receptors on the cell surface to promote cell adhesion and migration.[80] Growth factors and cytokines, such as TGF-β, regulate cell behavior, including proliferation and differentiation.[79] Proteoglycans, such as heparan sulfate proteoglycans, sequester and present growth factors, modulating their activity.[81] On the surface of synthetic materials, Ikada and co-workers found that a polymer film exhibiting a water contact angle ranging from 60° to 80° (moderately hydrophilic) in its dried state exhibited a higher preference for fibronectin adsorption and facilitated better growth of fibroblast cells, in comparison to films with contact angles ranging from 20° to 50° (highly hydrophilic).[82] Kawakami and co-workers incorporated phenol groups on to carboxymethylcellulose derivatives (CNC-Ph hydrogels) to obtain a hydrogel with high crosslinking speed and crosslinker density, showing that more L929 fibroblasts could adhere and grow on the hydrogel surface containing 15.4 phenol groups per 100 repeat units of uronic acid (with a 97% adhesion rate) compared to the gel with only 8.4 phenol groups (with a 62% adhesion rate). This cell behaviour was ascribed to the increased hydrophobicity of the gel resulting in the enhanced binding of cell-adhesive proteins (e.g., fibronectin, vitronectin).[83] Arinzeh and co-workers discovered that human mesenchymal stem cells (hMSCs) showed enhanced osteogenic differentiation when placed on a hydrophobic tyrosine-derived polyarylates and polycarbonates.[84] Overall, these studies point out the importance of hydrogel properties on the binding of proteins secret.

1.5. Synthetic hydrogels as ECM mimicking materials

Synthetic polymer hydrogels provide access to materials where precise control of their physical and chemical characteristic can be achieved. They can be synthesized from a range of synthetic polymers and combined with natural materials. [85,86] These synthetic polymers can be further subdivided into those that are covalent or supramolecular and will be discussed in detail below. Moreover, numerous possibilities abound when the multiple polymer networks when their own properties are combined and will also follow. This burgeoning research area that aims to emulate the ECM of various tissues and has provided a wealth of information on how cells behave in the context of a dynamic and static interfaces.[85,87,88]

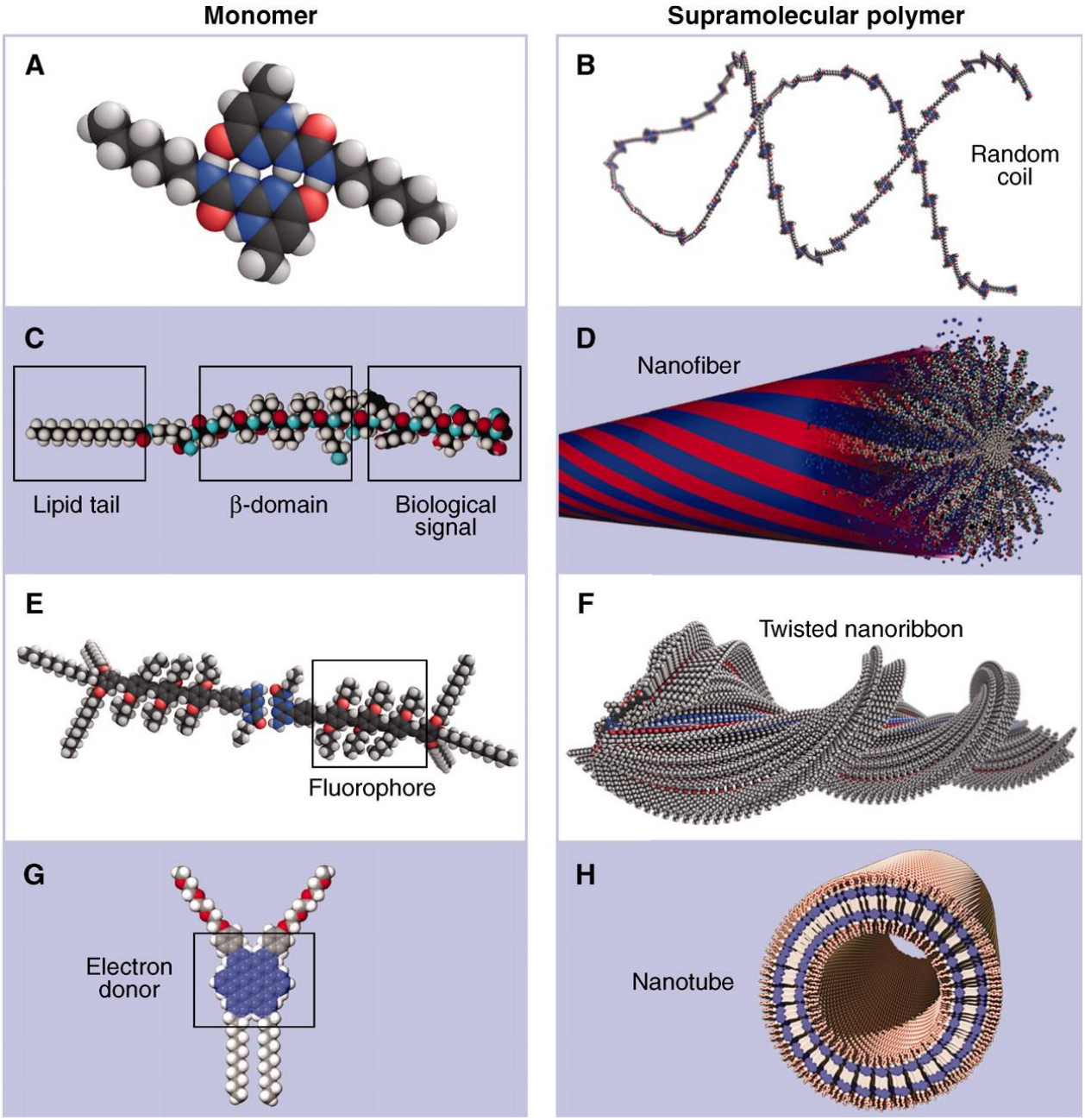


Figure 1.4. Four examples of monomers that can form supramolecular polymers. **A** and **B** The first example consists of a hydrogen bonding ureidopyrimidinone unit (left) that leads to a random-coil structure on polymerization (right). **C** and **D** The second set shows a peptide amphiphile (left) consisting of three segments: a biological cue, a β sheets forming domain, and a hydrophobic domain. The resulting supramolecular polymer (right) adopts a cylindrical structure with twisted β sheets. **E** and **F** A fluorophore oligo(phenylene vinylene) (left) modified with hydrophobic alkyl moieties and a chirality center forms stable dimers through quadruple hydrogen bonds at each monomer end leading to a chiral spiraled ribbon (right). **G** and **F** A hexabenzocoronene core (left) serving as an electron donor and adorned with phenyl triethylene glycol and dodecyl chains. The resulting shape of this supramolecular polymer is a nanotube (right), with thickness of the tube wall dictated by the dimensions of the individual building blocks.[89] Images adapted from references.

1.5.1. Supramolecular polymers

Supramolecular polymers are macromolecules held together by reversible, non-covalent interactions, exhibiting dynamic properties and functionalities with applications in diverse fields.[90] Supramolecular polymers mainly consist of two types based on the structures that are formed: random-coil supramolecular polymers without internal order that form random-coils and those that are shape-persistent nanostructure with a high degree of internal order.[89] The second type polymer is

different from covalent polymers and could achieve mechanical or biological functionalities depending on the monomers used.[89] Supramolecular polymerization can be described by one of three mechanisms: isodesmic (σ=1), cooperative (σ>1), and anti-cooperative (σ<1).[91] Random-coils usually polymerize according to an isodesmic polymerization while the ordered structures that polymerize result from an cooperative or anticooperative polymerization. The monomers that show cooperative polymerization include macrocycles, peptides and C3 symmetric monomers. Benzene-1,3,5-tricarboxamide (BTA) derivatives represent one of the most studied C3 symmetric cores in supramolecular chemistry,[92] with monomers prepared from them self-assembling into supramolecular polymers with a high level of cooperativity through π-π interactions and hydrogen bonding.[92-99] Moreover, when a critical concentration is surpassed, gel phase materials can be obtained.[94-99] Meijer and coworkers showed that supramolecular polymer hydrogels based on the 2-ureido-4[1H]-pyrimidinone (UPy) unit demonstrate mechanical properties relevant for biomedical applications. Dankers and coworkers designed a customizable single-component supramolecular transient network in water with unique nonlinear structure by modifying PEG with quadruple hydrogen bonding motifs that is shielded by a hydrophobic alkyl chain, which could incorporation of active proteins during gelation process and gradually releases the protein through the dissolution and stability of both the protein and polymer components after being implanted into the living organisms.[100] Sijbesma and co-workers demonstrated that diacetylene bis-urea bolaamphiphiles after light irradiation can show strain stiffening and self-healing attributes attributed to its semi-flexible fibers and dynamic properties.[101] Stupp and coworkers developed two different peptide amphiphiles that could self-assemble into supramolecular hydrogels with different stiffness, which could be applied to studied the influence in neuronal development.[102] These studies point out that supramolecular polymers can show relevant mechanical properties for biological application, but also the need for methods to modulate their mechanics as these non-covalent materials are often weak in mechanical character.

1.5.2. Squaramide-based supramolecular polymers and materials

The squaramide is a minimalistic ditopic hydrogen bonding synthon that consists of a cyclobutenedione ring with two N-H donor hydrogen bonding groups and two C=O acceptors. These groups positioned in an opposing manner on the ring, primes them to interact with another squaramide moiety as needed to facilitate the supramolecular monomer polymerization.[103] The squaramide unit has been widely used in molecular recognition, chemical catalysis, medicinal chemistry and self-assembly.[103,104] Our group first introduced squaramide units onto flexible amphiphilic monomers that polymerize into rigid filaments microns in length and nanometers in width.[105] Hydrogen bonds and hydrophobic effects drive the interaction of the monomer units when self-assembled in water. By systematically modifying the hydrophilic or hydrophobic domains present in the bolaamphiphiles, a transition from spherical aggregates to filamentous structures was induced.[105] Furthermore, co-assembly of a fluorescent monomer led to assessment of the biodistribution behaviour and potential for sequestration of the various nanoparticles by stabilin-2, a hepatic receptor in developing zebrafish embryos.[106] This investigation aimed to explore the impact of the morphology and dimensions of the supramolecular polymer nanoparticle on these biological mechanisms. Recently, our group reported a tripodal squaramide-based monomer that forms filamentous polymers and hydrogel materials above a critical concentration. The squaramide synthon was integrated into the hydrophobic domain of this tripodal amphiphile bearing a tris(2-aminoethyl)amine (TREN) core with peripheral tetra(ethylene glycol) chains. A series of

supramolecular monomers were designed by varying the aliphatic chain length, ranging from 6-12 methylene units. However, it was observed that only the monomers with 8 and 10 methylene units could form hydrogel materials.[107] Furthermore, our group also introduced 1,2-dithiolane (DT) onto the tripodal squaramide-based monomer to modulate the mechanics of the materials through the formation of dynamic covalent crosslinks in a light-mediated manner and an RGD peptide to guide cell adhesion and migration. This modified hydrogel exhibited strain stiffening, viscoelasticity, and plasticity, resembling the properties of biopolymer networks in the ECM and demonstrated the possibilities to tune the gel properties during cell culture.[108]

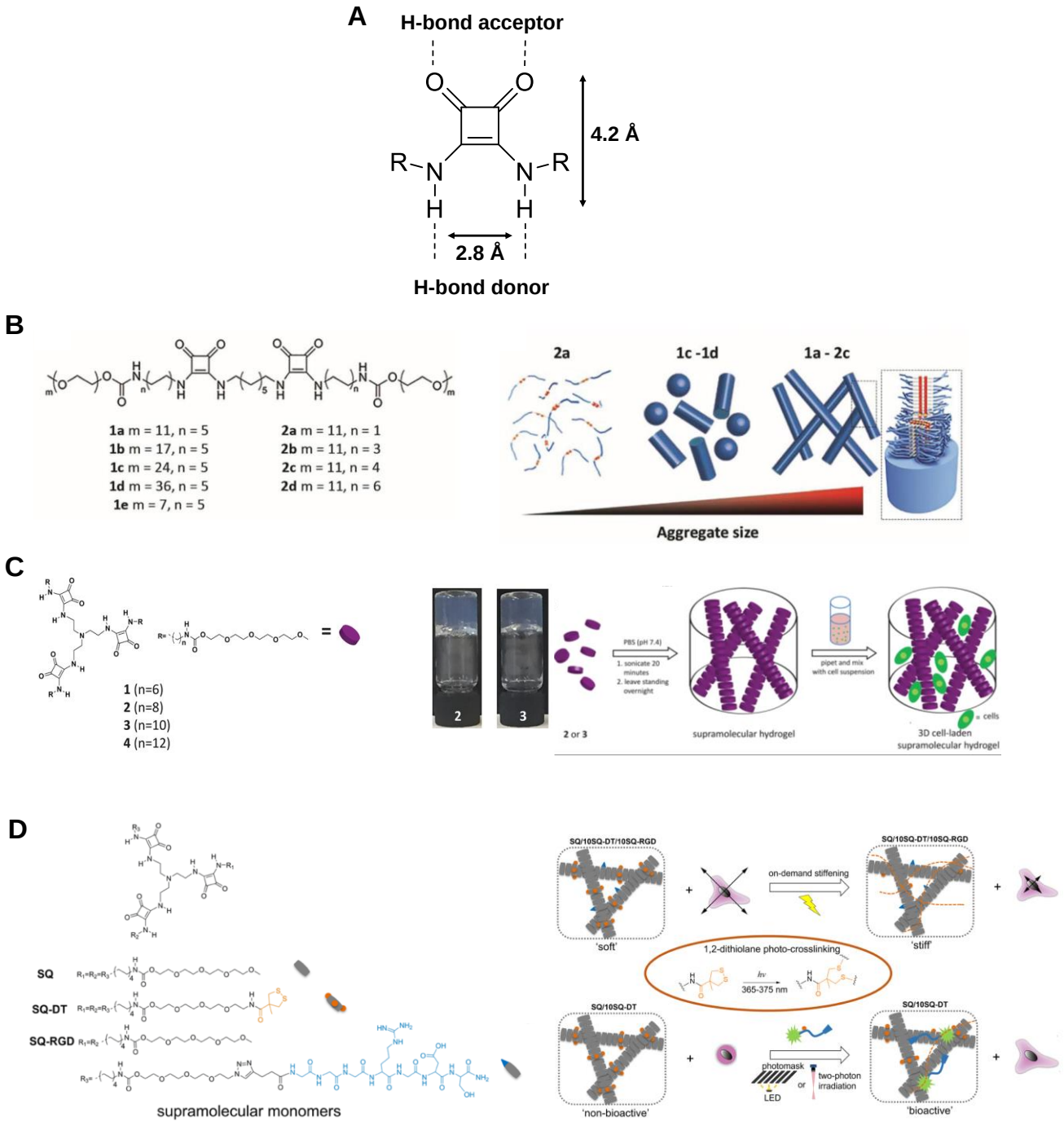


Figure 1.5. (A) Molecular structure of the squaramide.[109] (B) Chemical structures of squaramide-based bolaamphiphiles and a scheme of the observed structures of the supramolecular polymer in water as the ratio of hydrophilic to hydrophobic components was adjusted.[106] (C) Chemical structures of tripodal squaramide-based hydrogelators as the hydrophobic length was adjusted changed and a scheme of gelation process and cell culture study.[107] (D) Chemical structures of DT and RGD modified tripodal squaramide-based monomer, a scheme depicting mechanical modulation, and the controlled

arrangement over space and time of the biologically active RGD peptide (fluorescein)GK(DT)GGGRGDS within the hydrogel through DT cross-linking..[108] Images adapted from references.

1.5.3. Interpenetrated networks

Single network hydrogels (e.g., supramolecular and polymeric-based hydrogels) have been utilized widely in many biological applications. However, their weak and brittle character prevent them from mimicking load-bearing tissues. Several strategies have been explored to tackle these constraints including augmenting the monomer concentrations and cross-linking density.[110-112] To date, strategies based on semi-interpenetrating networks (IPNs) and double networks (DN), two common subclasses within this area, have also been employed to increase the mechanical properties of these gels bringing the needed mechanical characteristics to mimic these tissues in reach.[110,111] IPN hydrogels are individually cross-linked networks of multiple polymer chains that interpenetrate each other, providing access to properties such as enhanced thermal stability, mechanics, and chemical resistance.[113,114] Chaudhuri and co-workers developed an IPN hydrogel system consisting of hyaluronic acid (HA) and collagen I. The HA network chemically crosslinked through dynamic covalent bonds (hydrazine and aldehyde). This strategy aimed at replicating the viscoelastic and fibrillar properties of the ECM in native tissues. These IPN hydrogels showed distinguishable stress relaxation due to HA crosslinking within the network that can be modulated by varying the affinity of the crosslinkers, molecular weight or concentration of the HA network. This work showed that a faster stress relaxation process in the IPN hydrogels can permit cells to rearrange the fibers within the network, help to establish the focal adhesion sites, and accelerate their maturation.[115] Qin and co-workers developed a viscoelastic alginate-collagen hydrogel that shows both fast- and slow-relaxation supporting osteocyte viability within the hydrogel. They further discovered that a fast-relaxing environment could promote early cell spreading, while slow-relaxing hydrogels facilitate osteogenic differentiation, indicating a potential role in advancing 3D osteocyte models for bone mechanobiology research.[116] While IPN hydrogels do exhibit improved mechanical properties and retain the advantages of two polymer networks, they still fall short in terms of matching the toughness or loading capabilities found in certain native tissues (e.g., bone, cartilage), mainly due to the absence of crosslinking between the two networks. As a result, double network (DN) hydrogels have emerged as a response to overcome this limitation and enhance their suitability for biological applications.[117-119]

The DN hydrogel consists of a soft and ductile network (first network) as well as a rigid and brittle network (second network) that are covalently connected in between[117]. The soft and highly ductile is usually consists of long polymers that maintain the hydrogel integrity and tolerate high stress before break[120]. The second network serves as a “sacrificial network” that breaks first to dissipate energy when external stress is applied. Gong and coworkers[117,121,122] demonstrated the DN concept with the addition of poly(acrylamide) (PAAm) to a poly(2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) and their crosslinking leading to hydrogels with exceptional mechanical strength (elastic modulus: 0.1-1 MPa; fracture tensile stress: 1-10 MPa, strain: 1000-2000%; tearing fracture energy of 100-1000 J/m^2) and the ability to sustain compressive pressure (fracture compressive stress 20-60 MPa, strain 90-95%) with an extremely low coefficient of friction.[117] DN hydrogels integrate many extraordinary properties in one network compared with traditional single hydrogel network and IPN hydrogel.[120,123,124] However, many DN hydrogels exhibit irreversible damage under external loading and could not totally recover to their original state after releasing the stimulations. Therefore, many sacrificial frameworks such as

ionic bonds[125,126], hydrophobic interactions[127,128], or crystallites[129,130], have been widely used to enhance the recovery of the network after the loading.

Filamentous supramolecular polymers have been applied into DN hydrogels for their self-healing capabilities, energy dissipation, and viscoelastic properties[131-133]. Jiang and coworkers developed a supramolecular-polymer DN hydrogel, combining acrylic acid functionalized EFK peptides (AA-EFK) and covalent polymer networks (a mixture of acrylamide, 4-arm PEG-acrylate and methacrylated hyaluronic acid (HAMA)) to exhibit remarkable mechanical properties (Young's modulus: ~209 kPa, compression limit: >70%, toughness: ~47 kJ/m^3) and rapid recovery. The DN hydrogel outperformed the single network hydrogels showing enhanced cell (SW1353) viability, differentiation in 2D culture. Subsequently, the crosslinked gel was implanted into rabbit models with cartilage defect. Successful regeneration of cartilage was observed, with regenerated tissues displaying native cartilage-like ECM and well organized cell arrangement after 12 weeks of *in vivo* study.[134] Heise and co-workers presented a synthetic polypeptide-based DN hydrogel that consisted of alkyne-functionalized polypeptides and azide-functionalized PEG, that could crosslink together through copper-catalyzed alkyne-azide cycloaddition. The resulting hydrogels demonstrated improved mechanical strength (ultimate compressive strength ~820kPa, strain ~30%) compared to conventional peptide-based hydrogels tunable and good cell compatibility (>68%), thus suggesting their suitability as scaffolds for soft tissue engineering.[135] Cao and coworkers introduced two peptides, acrylic acid-Ile-Ile-Ile-Lys-C (I_3K-AA) and acrylic acid-Ile-Ile-Ile-Thr-C (I_3T-AA), into a polymer network composed of polyacrylamide (PAM) and 4-arm PEG-acrylate (Mw: 20 kDa). Although both peptides could effectively enhance the mechanical strength of DN hydrogels, I_3T-AA exhibited 3-5 times higher values in all mechanical properties (e.g., Young's modulus: 107.4 kPa; fracture stress: 0.48 MPa; toughness: 0.38 MJ/m^3) compared to I_3K-AA. It also showed a better recovery rate of the dissipated energy (I_3T-AA: 93%; I_3K-AA: 86.6%) after 20 stretching-relaxation cycles without fracture.[136] These studies provide valuable insights into manipulating supramolecular network architectures to enhance the mechanical strength and rapid recovery of DN hydrogels. While DN hydrogels have garnered interest within the realm of biology because of their appealing properties, the protocols to prepare them are often bioincompatible and they lack the fibrillar structure of the extracellular matrix.

1.5.4. Nanoparticle-reinforced double networks

The introduction of nanoparticles into synthetic polymer materials opens the door to simultaneously modulate hydrogel mechanics as well as introduce new properties (e.g., electrical, magnetic).[138] Silica nanoparticles (SiO_2) were introduced into DN gels composed of poly(2-acrylamido-2-methylpropanesulfonic acid) (PAMPS) and poly(acrylamide) (PAAm) of as macro-crosslinkers. The hydrogels exhibited high compressive toughness, with no fracturing observed even under loading conditions of up to 73 MPa and a strain exceeding 98%.[139] Beyond silica, the remarkable optical and plasmonic characteristics exhibited by gold nanoparticles (AuNPs) and nanorods (GNRs) render them highly attractive additives to tune the mechanics of synthetic polymer materials and develop photo-thermo responsive hydrogels.[140]. Khademhosseini and co-workers[141] used gelatin methacrylate (GelMA) coated GNRs to crosslink a GelMA hydrogel with photoinitiator under UV irraditation to develop conductive bioinks for culturing cardiac cell culture. They found that the incorporated GNRs improved the electrical resistance of the material and cell coupling resulting in synchronized contraction of the bio-printed constructs.[142] Moreover, the photo-thermal responsiveness of GNRs has also been examined in single network hydrogels, presenting opportunities for their utilization of the

materials in photothermal therapy[143] and imaging applications.[144] Chen and co-workers developed an injectable thermosensitive hydrogel composed of β-glycerophosphate-bound chitosan (CGP), dopamine-modified alginate (Alg-DA), and polydopamine-coated GNRs (GNR-PDA) which could be used for efficient photothermal therapy. The strong interactions between the polymer (chitosan and alginate) and polydopamine (PDA) on the nanoparticles help to stabilize and immobilize GNR-PDA within the hydrogel uniformly. Both *in vitro* and *in vivo* studies demonstrated good biocompatibility and efficient tumor ablation, showing promise for long-term, repeated photothermal therapy for tumors.[137] Overall, incorporating nanoparticles into the hydrogel network is a practical approach for both introducing new functionalities and enhancing mechanical properties.

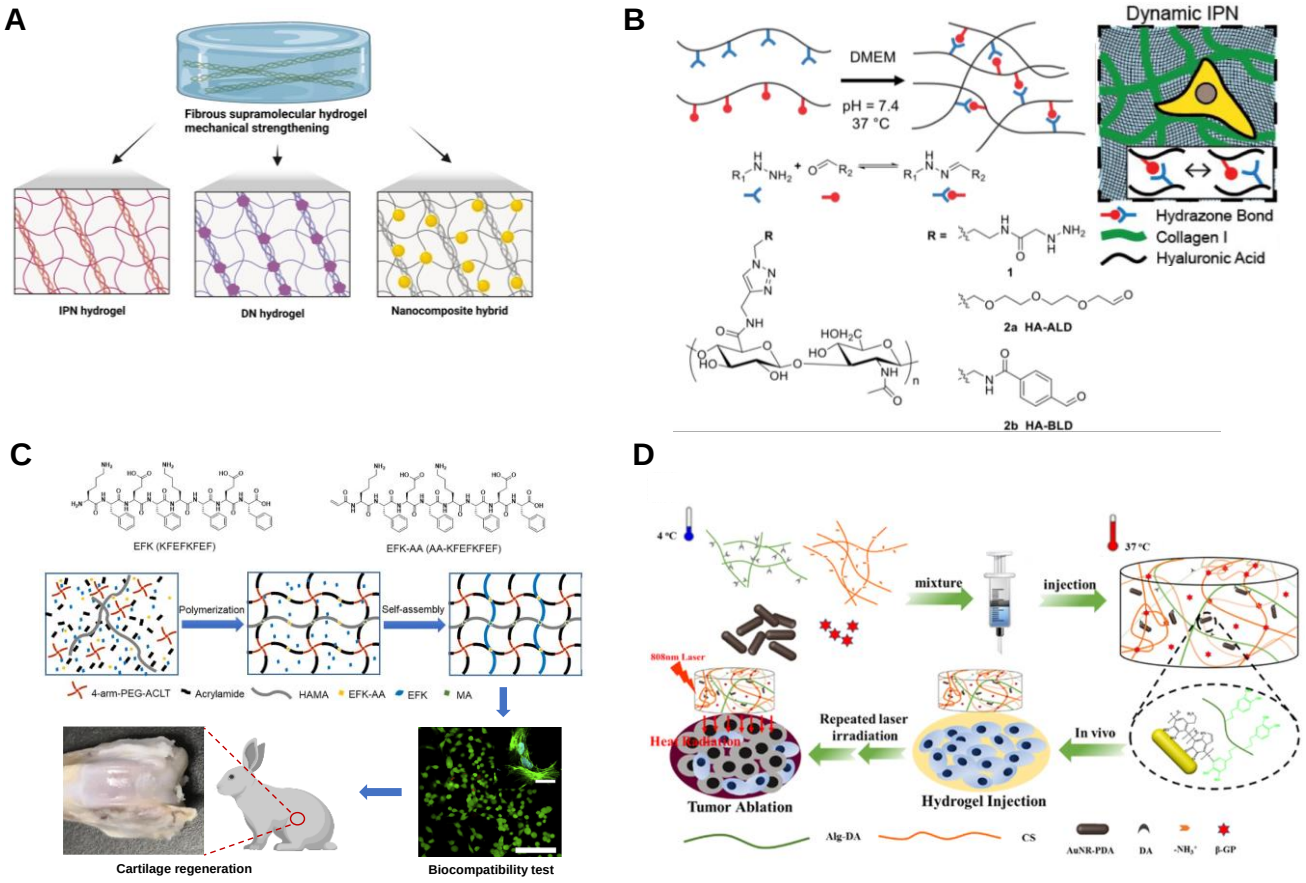


Figure 1.6. (A) Strategies to reinforce the mechanical characteristics of supramolecular hydrogels, figure made in biorender. (B) Molecular structures of hydrazine and aldehyde modified HA and their dynamic properties under physiological conditions and a scheme demonstrates that dynamic covalent interactions can regulate cell morphology in 3D cell culture.[115] (C) Molecular structures of EFK and EFK-AA peptides and their formation into a DN network, along with biocompatibility testing and cartilage regeneration in rabbits are presented.[134] (D) A GNRs-reinforced DN hydrogel that has potential applications in tumor ablation.[137] Images adapted from reference.

1.6. Aim and outline

This thesis first explores the squaramide-based supramolecular polymer properties by tuning the chemical structures, and further develop multiple synthetic hydrogels aiming at enhancing their mechanical strength and investigating their applications in 3D cell culture. A comprehensive range of chemical, biophysical, and biological studies are employed to analyze the impact of squaramide-based synthetic hydrogels on cell behavior.

Chapter 2 examines the relationship between the chemical structure of tripodal squaramide-based monomers and properties of the supramolecular polymer materials by tuning the hydrophilic-

hydrophobic ratio and hydrogen bonds within the monomers. Using a combination UV-Vis, cryo-TEM and oscillatory rheology experiments, it was demonstrated that the hydrophilic-hydrophobic ratio and hydrogen bonds can influence the thermal responsiveness of the polymers.

Chapter 3 discloses a mechanically enhanced DN hydrogel featuring a rapid ring-opening photopolymerization of DT and norbornene (NB) to prepare a DN hydrogel. The DN networks consist of supramolecular filaments and PEG polymers and show cartilage-like mechanical characteristics that can sustain dynamic compression in 3D cell culture. Furthermore, human primary articular chondrocytes (hPACs) were 3D seeded in these hydrogels and the composite could be applied for long-term loading to stimulate the production of cartilage ECM components (e.g. sGAGs).

Chapter 4 describes a novel DN hydrogel phenol-crosslinked hydrogel consisting of the filamentous squaramide-based supramolecular hydrogels from chapter 3 and modified 4-arm PEG network. The resulting hydrogel materials that are crosslinked by visible light show mechanical properties resembling cartilage and bone. Moreover, the ECM production by hPACs entrapped within this DN hydrogel highlights its applicability for cartilage-mimicking applications.

Chapter 5 discloses the use of gold nanoparticles (AuNPs) as crosslinkers in DN hydrogels that consists of a filamentous squaramide-based supramolecular polymers and a 4-arm PEG network. Crosslinking of these components results in improved compressive properties and enhanced energy dissipation levels of the DN hydrogels. Additionally, the incorporation of AuNPs provides the network with a photo-thermal effect that effectively eliminates MDA-MB-231-B1 cells (bone derived human breast cancer cells) showcasing its potential for bone cancer therapy.

Chapter 6 outlines the key findings of this thesis and explores potential avenues for future research of squaramide-based supramolecular materials that mimic the ECM for a wide variety of 3D cell culture aims.

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