Squaramide-based supramolecular materials for 3D cell culture applications

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CHAPTER 6

Summary and perspectives

Over the past decades, synthetic hydrogels developed through physical or chemical strategy, have been widely explored as artificial platforms *in vitro* for engineering the cellular microenvironment. Due to the dynamic nature of the non-covalent interactions, supramolecular hydrogels with their unique, reversible and adaptable properties are particularly interesting for tissue engineering. Moreover, supramolecular hydrogels with hierarchical features could be achieved through structural design of the monomer or by controlling the self-assembly process, that possibly mimic the real structure feature of the ECM *in vivo*. Although numerous peptide-based supramolecular hydrogels have been used as smart biomaterials for 3D cell culture, the development of novel small-molecular gelators that are cost-effective are still largely in demand and allow large-scale synthesis.

One of the major challenges for supramolecular materials is their relatively weak mechanical strength and stiffness that limits their application as materials that mimic load bearing tissues. On the other hand, to push the limits of synthetic materials in replicating the dynamic features of native tissues to drive complex biological processes remains an inspiring and unsolved challenge in the field. In this thesis, I develop squaramide-based supramolecular hydrogels and explore their application towards engineering the cell microenvironment. Moreover, these hydrogel materials can be outfitted with biological cues. For example, cell-adhesive cues (e.g., RGD peptide) can be chemically incorporated prior to monomer co-assembly and distributed throughout the network, or by spatial and temporal modification by UV irradiation. Importantly, a wide range of hydrogel stiffnesses to guide cell behavior 3D can also be achieved by simply modifying the monomer with the photo-activatable groups. Further incorporating the new type of photo-induced polymeric hydrogel into supramolecular hydrogel network influences the formation of double network hydrogels with the improved mechanical stiffness and strength. The preliminary studies of 3D cell culture applications reveal their potential in biomedical fields.

In **chapter 2**, a novel family of self-assembled supramolecular hydrogels containing flexible TREN core, three squaramide synthons, hydrophobic and hydrophilic domains was developed and explored. Oscillatory rheology studies demonstrated gel formation under physiological conditions and also their self-recovering properties. Cryo-TEM showed the formation of a hydrogel network that consists of entangled fibrils with a few nanometers in width and micrometers in length. SAXS measurements in the solution phase further indicated the

formation of high-aspect-ratio one-dimensional nanofibers that have 1 monomer/nm along the fibrillar axes. Further insight into the self-assembly process at the molecular scale, spectroscopic measurements (UV-Vis, fluorescence, and Fourier Transform Infrared (FTIR)) showed the importance of hydrophobic and hydrogen bonding interactions for supramolecular hydrogel formation. Moreover, the simplicity of 3D cell encapsulation and release, and its cytocompatible response for a range of cell types, demonstrated its applicability for a broad range of targets in 3D cell culture. In this study, I showed that the squaramide motif can be used to build new supramolecular hydrogel materials in an efficient, economical and biocompatible manner, opening the door to act as synthetic platforms to mimic the cell microenvironment. The importance of tripodal core, squaramide motif, and hydrophobic domain were demonstrated to be critical for gel formation in water. The hydrophilic PEG chain length also plays a crucial role in determining the solubility of the gelator by affecting the hydrophilic/hydrophobic balance. However, oxygen atoms from PEG chains may also interfere with the designed hydrogen bonding units when the longer spacers are used through their folding. Further investigation through 2D NMR and energy minimization studies are needed to understand the role of PEG chains in the self-assembly process. On the other hand, due to its structural flexibility, the end group of the supramolecular monomer could be easily modified with bioactive cues (e.g., RGD) or other biomolecular units to further improve the properties of materials and widen their application scope for tissue engineering.

Chapter 3 shows the potential to tune the mechanical properties of covalent polymer hydrogels by functionalizing light active 1,2-Dithiolane (DT) into the supramolecular monomer from **chapter 2** and triggering the photoreaction with UV irradiation. First, the supramolecular hydrogel was prepared by co-assembly of the monomers without and with the 1,2-Dithiolane (DT) unit. Next, wide range of hydrogel stiffness (from 10 Pa to 10 kPa) could be obtained by varying the molar concentration of DT group and also the light source parameters (e.g., irradiation time, light intensity) by oscillatory rheology. UV photoirradiation did not alter the aggregate morphology at the nanoscale as confirmed by cryo-TEM and SAXS studies. Moreover, bioactive cues (RGD peptide) could be introduced into the hydrogel network through co-assembly of peptide-modified squaramide-based supramolecular monomers. On the other hand, the RGD peptide was functionalized with the DT motif and could be incorporated into the hydrogel network by photo-patterning using a photomask or direct laser writing.

The encapsulation of several cell lines (e.g., NIH 3T3, C2C12 and Hs578t) within the supramolecular hydrogel showed a respond with high viability both pre- and post-UV irradiation. The difference in C2C12 morphology and Hs578T cell migration within the hydrogels with respect to UV light irradiation indicated the potential to spatially and temporally control the cell microenvironment in 3D. These studies demonstrated the potential of using the 1,2-dithiolane unit to introduce dynamic cross-links into supramolecular polymer materials under user-defined conditions with UV light, providing a foothold to create hydrogel platforms that can be spatiotemporally modified enabling mimicry of complex ECM processes.

To provide insight into the assembly of the monomers, **SQ**, **SQ-DT** and **SQ-RGD**, super-resolution microscopy experiments can be performed. The monomers can be labeled with specific dyes, e.g., sulfo-Cy3 and sulfo-Cy5, to visualize their distribution in the fibrillar aggregates at the nanoscale. To further explore the molecular picture behind the changes in mechanical stiffness before and after UV irradiation is still required, as it is unclear whether inter- or intra-fiber crosslinks take place. One of the challenges in understanding the self-assembly of the aggregates is the limited concentration of the 1,2-dithiolane-based monomer (e.g., maximum of 10 mol% of total concentration) that is used in the multicomponent self-assembled system. Therefore, a large quantity of monomers would be required to provide a sufficient signal to monitor the change in the 1,2-dithiolane before and after UV irradiation. Gaining molecular insight into their crosslinking will be pursued, for example, through solid-state NMR studies.

Moreover, the photochemical reaction used in this work can affect both cell viability, metabolism and genomes. During the photo-crosslinking process to create 3D scaffolds, the use of UV light and the generation of the free radicals could also interact with other cellular components (e.g., cell membranes, proteins, and DNA), that can alter cell viability, metabolism, and DNA integrity. Therefore, further analyzing cell metabolism and DNA damage within our photochemically crosslinked hydrogel materials is required. For example, genotoxicity can be explored by measuring the expression of γ-H2AX, an early detection marker of DNA damage.

In **chapter 4**, I further apply the 1,2-Dithiolane (DT) moiety to modify a covalent PEG polymer and use it as a latent thiol that can be reacted with the norbornene (NB) group on the second PEG polymer through a highly efficient and byproduct-free reaction using light in the presence of a photoinitiator. A new type

of branched polymeric hydrogel was formed through UV light induced reaction of DT-NB in one-pot. Oscillatory rheology results showed that hydrogel stiffness can be easily modified by varying the total monomer or photoinitiator concentration, DT/NB molar ratio and light source parameters (e.g., light irradiation time and light intensity). The combination of the oscillatory rheology and NMR studies demonstrated the DT moiety could yield a bifurcated cross-linker upon ring opening that can efficiently cross-link with itself and NB resulting in the formation of reversible and irreversible cross-linked hydrogels. Photo-patterning using a photomask in 2D or direct laser writing in 3D had been used to spatiotemporally introduce the RGD peptide into the hydrogel network. 3D cell encapsulation and a live/dead study were performed and indicate their cytocompatibility with NIH 3T3 cells opening the door to engineering the cell environment. Here, I disclosed the development of a covalent polymer scaffold that is synthetically accessible and economical for applications in the biomedical field. In the following works, many possibilities can follow these studies for 3D cell culture *in vitro*. One step can be to connect cell behavior with the mechanical stiffness or RGD peptide cues spatially or temporally through photo-activation. Furthermore, because of the existence of dynamic crosslinks in the one-pot reaction, their unique properties over other traditional covalent crosslinks (e.g., SH-NB reaction) on cell behavior could be explored. Therefore, it is critical to understand the following properties, for example, viscoelasticity and stress-relaxation because of their known influence on cell behaviour.

In **chapter 5**, I exploit the fabrication of a new type of supramolecular and covalent polymer double network hydrogel using a simple preparation strategy. The multicomponent tripodal squaramide based supramolecular hydrogels as described in **chapter 3** and the polymer hydrogels from **chapter 4** were chosen for the contribution of the double networks. The use of 1,2-dithiolane (DT) enables to generate the additional chemical junctions between the two networks. Oscillatory rheology showed the tunable mechanical stiffness and compressive property through varying the total supramolecular concentration, molar percent of dithiolane motif from supramolecular monomer, total polymer concentration and polymer architectures. Moreover, the self-recovering property was observed from the step-strain experiment from rheology due to the combination of the physical crosslinks and the dynamic disulfide crosslinks under photo-irradiation. SEM results showed the microscopic structures of the double network hydrogel, which was consistent with the rheology results. Other basic parameters for double

network hydrogel, for example, equilibrium water content (EWC) and swelling ratio, were also collected. Moreover, there was a high percentage of viable NIH 3T3 cells and primary chondrocyte cells after encapsulation within the various double network hydrogels. Additionally, the matrix produced from the primary chondrocytes during the 3D cell culture could be modulated by varying the RGD concentration and hydrogel stiffness. Here, the use of supramolecular and covalent polymer hydrogels that consist of entanglements and covalent cross-links gave rise to the unique properties obtained for the double network materials, for example, viscoelastic, self-recovering, and also improved mechanical stiffness and strength. As an outlook, these hybrid materials are demonstrating their potential for cartilage tissue engineering.

In the future, the application of dynamic mechanical loading on the 3D chondrocyte cell-laden double network hydrogels would be an important route to pursue. Furthermore, due to the viscoelastic character and self-recovery property of the supramolecular hydrogel, its application for extrusion-based 3D bioprinting can be explored; as UV curing would enable maintenance of the structural integrity after printing through double network formation. While still many parameters like printing settings, hydrogel compositions and concentration, cell source and cell density have to be taken into consideration to improve the cell viability during the printing process, the strategy used in this work could be further developed to fabricate the complex architecture of tissues and organs.