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Squaramide-based interpenetrated networks for load-bearing applications

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Chapter 6

Summary and outlook

6.1 The importance of supramolecular-based hydrogels

Supramolecular hydrogels have emerged as advanced artificial platforms for *in vitro* engineering of the cellular microenvironment within living systems. Despite the advantages offered by non-covalent dynamic interactions, which confer dynamic and reversible properties to the material, their application in stiff and load-bearing tissue engineering has been limited. In this work, I systematically modulated the design of supramolecular monomers based on squaramides, covalent polymers, and their mixtures to tune the mechanical characteristics of the resulting hydrogels to direct cell behavior for mimicking the extracellular matrix (ECM). Hence, the information presented in this thesis contributes to the expansion of current fundamental knowledge regarding the assembly of squaramide-based supramolecular hydrogels and their *in vitro* studies, which serve as a basis for exploring functional supramolecular biomaterials. This chapter summarizes the main discoveries within this thesis, their implications for the field, and provides an outlook for advancing research on the respective topics.

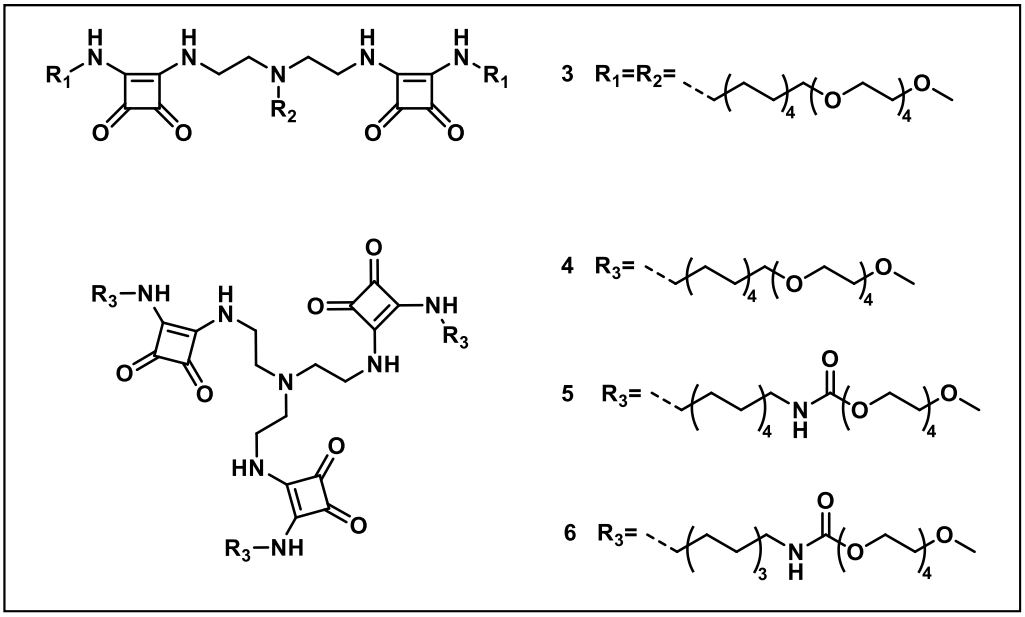

Figure 1. Chemical structures of squaramide-based gelators developed/used in this thesis. **3**, **4** and **5** were used in **Chapter 2**. **6** (**C8-SQ**) was used in **Chapter 3**. **5** and **6** are parental squaramide-based gelators that are commonly used for modifications and applications.

In **Chapter 2**, a small library of tripodal squaramide monomers was examined in an aqueous environment with respect to their self-assembly behavior and thermoresponsive properties. The tripodal squaramide-based hydrogelator as earlier reported by our group yields fibrous hydrogel at the critical gelation concentration, showing the importance of hydrophobic and hydrogen bonding interactions for supramolecular hydrogel formation. To gain a deeper view of the balance of hydrophilicity and hydrophobicity, and hydrogen bonds on gel formation, a family of tripodal squaramide-based monomers were designed and synthesized in moderate yields (Figure 1), and their self-assembly properties were investigated and compared with the corresponding parent tripodal squaramide-based hydrogelator. The exchange of the carbamates with the ether linkages resulted in a reduction in hydrogel stiffness, whereas the reduction of the number of squaramide units and

hydrophilic and hydrophobic properties resulted in the ability to form gel phase materials. The observed effects on gelation likely result from the reduced degrees of polymerization of the squaramide monomers, with a reduction in the number of the squaramide synthons affecting their entanglement. Furthermore, hydrogels **3**-**5** exhibited lower critical solution temperature (LCST) behavior, opening the door for developing thermoresponsive hydrogels. Specifically, hydrogels **4** and **5** maintained their original shapes but experienced shrinkage, ultimately transforming into highly oriented materials upon heating to 130°C and subsequent cooling to room temperature. Collectively, these findings not only underscore the essential structural features guiding the gelation and thermoresponsiveness of tripodal squaramide-based monomers in water but also suggest their potential adaptability to structural modifications for future endeavors aimed at their transformation into functional supramolecular biomaterials.

Based on these results, it is obvious that the design of supramolecular hydrogelators necessitates careful consideration of several factors.[1] Foremost among these is the hydrophilic-hydrophobic balance, serving as the main force driving the self-assembly process . Additionally, the characteristics of hydrogen bonds, encompassing their quantity, positioning, and types, exert considerable influence on intramolecular and intermolecular forces. Other factors, such as π-π stacking, also affect the dynamics of the system.

Our group previously functionalized tripodal squaramide-based monomers with 1,2-dithiolanes (DT) to trigger photocrosslinking within the networks.[2] This modification facilitated the precise control of material mechanics in supramolecular hydrogels by establishing dynamic covalent bonds among the fibers to increase the stiffness of the ECMs. Inspired by these results, I demonstrated the potential to modulate the mechanical properties of supramolecular hydrogels by coupling phenol groups (Phe) onto the squaramide-based monomer (**SQ-Phe**) derived from **Chapter 2** for *in situ* covalent photopolymerization by visible light stiffening the hydrogel while modulating its complex mechanical characteristics (e.g., strain-stiffen, stress relaxation, viscoplasticity) (figure 2A-C). Strikingly, co-assembly of a small amount (up to 5 mol%) of the reactive **SQ-Phe** monomer with the native squaramide-based monomer (**C8-SQ**) resulted in a dramatic increase in the stiffness of the hydrogels. By tuning the concentration of the hydrogel or the quantity of **SQ-Phe**, this filamentous supramolecular hydrogel displayed strain-stiffening, strain-enhanced stress relaxation, and viscoplasticity properties like biopolymers (e.g., actin, fibrin) of the ECM that are used for developing organ and tissue models. Moreover, the supramolecular hydrogel affected the growth of individual 3T3 cells, fostered the formation of anisotropic spheroids under a 3D environment without additional bioactive cues (e.g., RGD), in which their morphology could be modulated by the strategic adjustment of crosslinker concentration and fiber density (figure 2D). Additionally, the neuronal stem cells could be 3D differentiated into nerve cells within these supramolecular hydrogels in a soft environment. Overall, this study demonstrates the broad applicability of supramolecular hydrogel for diverse 3D cell cultures where anisotropy of the substrate is desired, with the potential to guide stem cells to differentiate for tissue engineering and disease modeling applications.

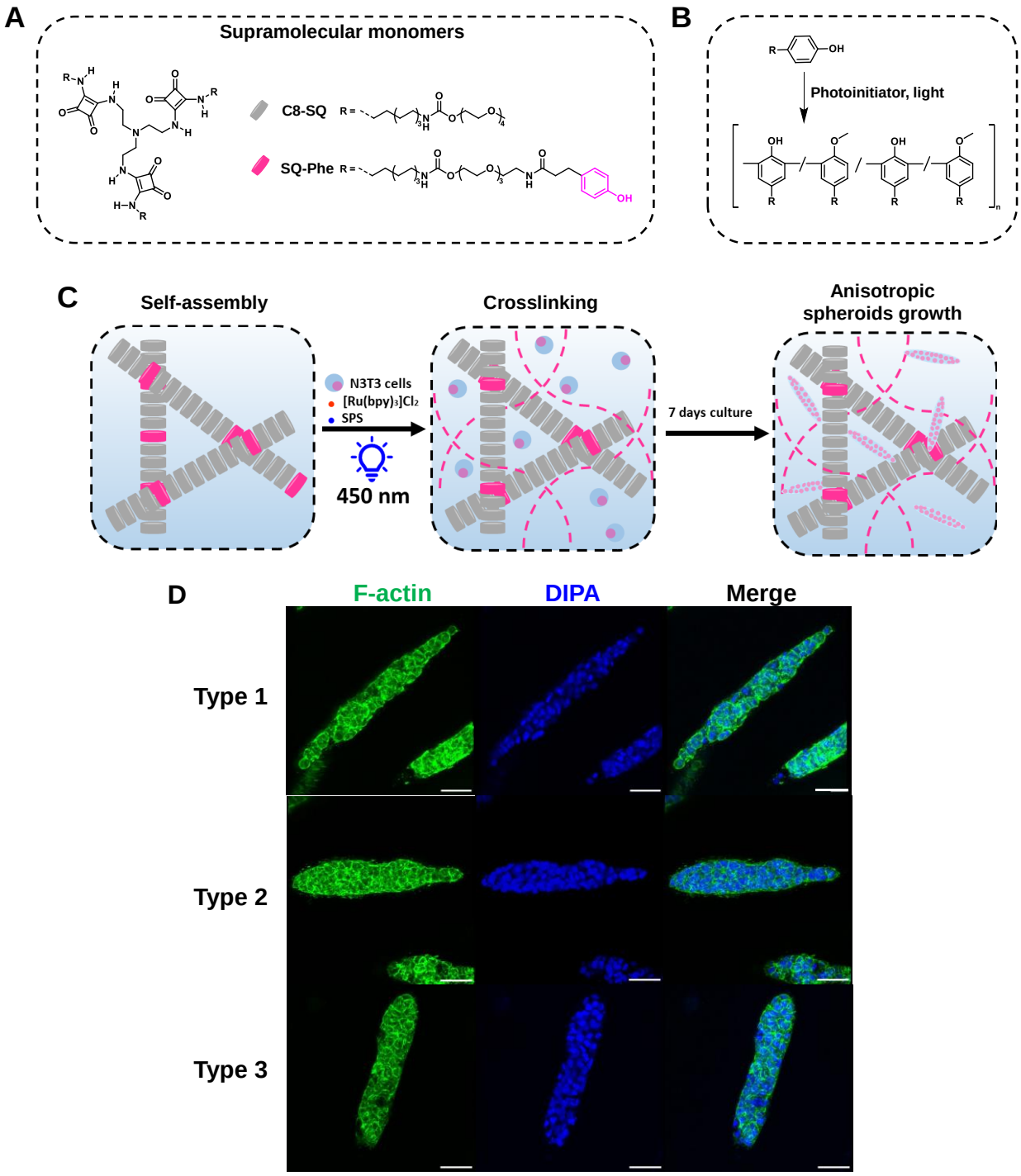

Figure 2. (A) Supramolecular monomer (**C8-SQ**, **SQ-Phe**) components are used to prepare hydrogels. (B) Phenol photopolymerization for crosslinking supramolecular nanofilaments. (C) The visible light-induced photopolymerized supramolecular networks for 3D cell culture promote anisotropic spheroid growth. (D) Representative confocal images of three major morphological growth patterns of NIH 3T3 fibroblast spheroids stained with F-actin and DAPI in tripodal squaramide-based hydrogels with different amounts of **SQ-Phe** after 10 days culture. Scale bar: *100 μm*.

While the reinforcement of supramolecular hydrogel's mechanical properties can be achieved through nanoscale fiber crosslinking that can mimic certain features of the ECM, significant challenges

still exist in the application of filamentous supramolecular polymers as stiff tissues especially where loading is necessary. **Chapter 3** disclosed a rapid ring-opening photopolymerization of DT and norbornene (NB) to prepare a covalent network through chain-growth polymerization simultaneously, that crosslinks nanoscale supramolecular filaments, yielding a tough double network hydrogel that can sustain dynamic compression in 3D cell culture. These hydrogels show cartilage-like mechanical characteristics, namely their biomimetic complex mechanical properties, stress response and relaxation under compression that can be tuned according to component ratios and concentrations using light. The distinctive mechanical properties are connected to their inhomogeneous network architectures, generated through the polymerization of monomers, resulting in the concurrent formation and reinforcement of poly(disulfide)s and poly(disulfide-norbornene)s networks that are achieved through UV exposure at the same wavelength. Notably, the potential of this double network for 3D culture was exploited through the encapsulation of human primary articular chondrocytes (hPACs) where the cells show high cell viability. Moreover, the cyclic compressive loading was applied on hPACs-laden hydrogels in 3D, resulting in increased deposition of sulfated- glycosaminoglycans after daily loading for 14 days. Notably, exploiting the photoreactive macromonomers, I mechanically pattern the materials at cell-relevant length scales to engineer spatially distinct mechanical domains, stiff and soft, to mimic developmental and disease conditions encountered in cartilage with superimposed compressive loading. The enclosed covalent network fortification strategy combined with bioorthogonal photopolymerization provides a base to tune the mechanics of filamentous supramolecular biomaterials, opening avenues to expand their 3D cell culture application where cyclic mechanical loading is involved. Compared with **Chapter 3**, this work introduced dynamic covalent bonds into **DN** hydrogels that provide the potential for degradability in the designing of stiff hydrogels.

Chapter 4 demonstrated a visible light-induced photopolymerization of phenol groups (**Phe**) to simultaneously prepare double networks (**DN**s) by using the filamentous supramolecular polymer (with 5% mol **SQ-Phe**) and poly(ethylene glycol) network (functionalized with **Phe** group) for 3D culture with cyclic compressive loading. These networks maintained the filamentous supramolecular structure but overcame the mechanical fragility of the supramolecular materials. Importantly, these hydrogels show biomimetic complex mechanical properties, such as a cartilage-like stress response, and relaxation under compression that can be tuned according to component ratios and concentrations. The incorporation of filamentous supramolecular structures was observed to expedite stress relaxation in both shear and axial direction, even in cases where the stiffness was elevated. These **DN** hydrogels can be used for human primary articular chondrocytes culture in 3D and cyclic compressive loading of these gels for 2 days showed enhanced deposition of sulfated-glycosaminoglycans (s-GAGs) compared to the free swelling condition. These **DN** hydrogels demonstrate potential for multiple applications in 3D cell cultures across diverse tissue types requiring compressive loading, opening avenues to map the essential chemical parameters in the next-generation hydrogels for advanced 3D *in vitro* modeling and targeted regenerative therapies, especially osteoarthritis (OA).

Stimuli-responsive hydrogels provide possibilities to control hydrogel properties in response to external stimuli, which has been applied in many aspects such as controlled drug delivery, sensing materials, and tissue engineering.[3] **Chapter 5** disclosed a stimulus-responsive **DN** hydrogels with

mechanical properties that mimic the compressive properties of cartilage and the potential to provide localized heating that can be used for various *in vitro* and *in vivo* aims, including therapy. In this work, I used the chemistry and **DN** material from **Chapter 4** and incorporated the 15 nm gold nanoparticles to create a dual-network hydrogel with enhanced mechanical properties and additional functions. The uncrosslinked hydrogels were injectable and the injected shape could stay stable in PBS after crosslinking. Additionally, the addition of gold nanoparticles enhanced the energy dissipation ability of the hydrogels as well as introduced tunable conductivity and photothermal properties when irradiated with 532 nm. The hydrogel showed good cytocompatibility and the photo-thermal effect successfully applied in the MDA-MB-231-B1 3D cancer model. The phototherapy effect is positively correlated with AuNPs concentration and light dosage, and the position could be precisely controlled by the laser, providing the potential applications of squaramide-based hydrogels for photothermal therapy involving cancerous tissues and valuable insights for studying complex diseases.

6.2 Future Perspective

The modification of tripodal squaramide-based gelators may appearstraightforward, yet it involves several considerations to achieve the desired self-assembly. Several factors come into play, such as the hydrophilic-hydrophobic balance of the monomer that can play a dominating role. As we can see, both DT and Phe functionalized squaramide-based monomers exhibit limited solubility under physiological conditions. While co-self-assembly can mitigate this limitation permitting the dissolution of the functionalized monomers for co-assembly to attain the maximum stiffness of the supramolecular material. Another critical factor is the crosslinking conditions, including light type and intensity, crosslinking speed, and photoinitiator quantities, all of which may affect cell behaviour. Consequently, implementing a mild crosslinking method becomes essential for subsequent biological studies.

While notable progress has been made in enhancing supramolecular hydrogels' compressive properties, the current achievements fall short of meeting the properties of native cartilage tissues (Figure 3). They require further refinement to reach levels comparable to native cartilage, which can withstand stress on the order of megapascals and exhibit recoverability. While the supramolecular materials are toughened by the disclosed approaches in this thesis, they still exhibit softening in response to loading over time. This limits their use for long-term applications and will need to be addressed in future designs. Furthermore, our present materials are primarily characterized under compressive forces, and investigations into stretching properties remain relatively scarce in the supramolecular hydrogel field. Therefore, a meticulous molecular structure design, careful selection of appropriate chemical bonds for network formation, and consideration of polymer types and ratios become paramount in developing stretchable hydrogels, which is pivotal for creating smart materials with versatile applications.

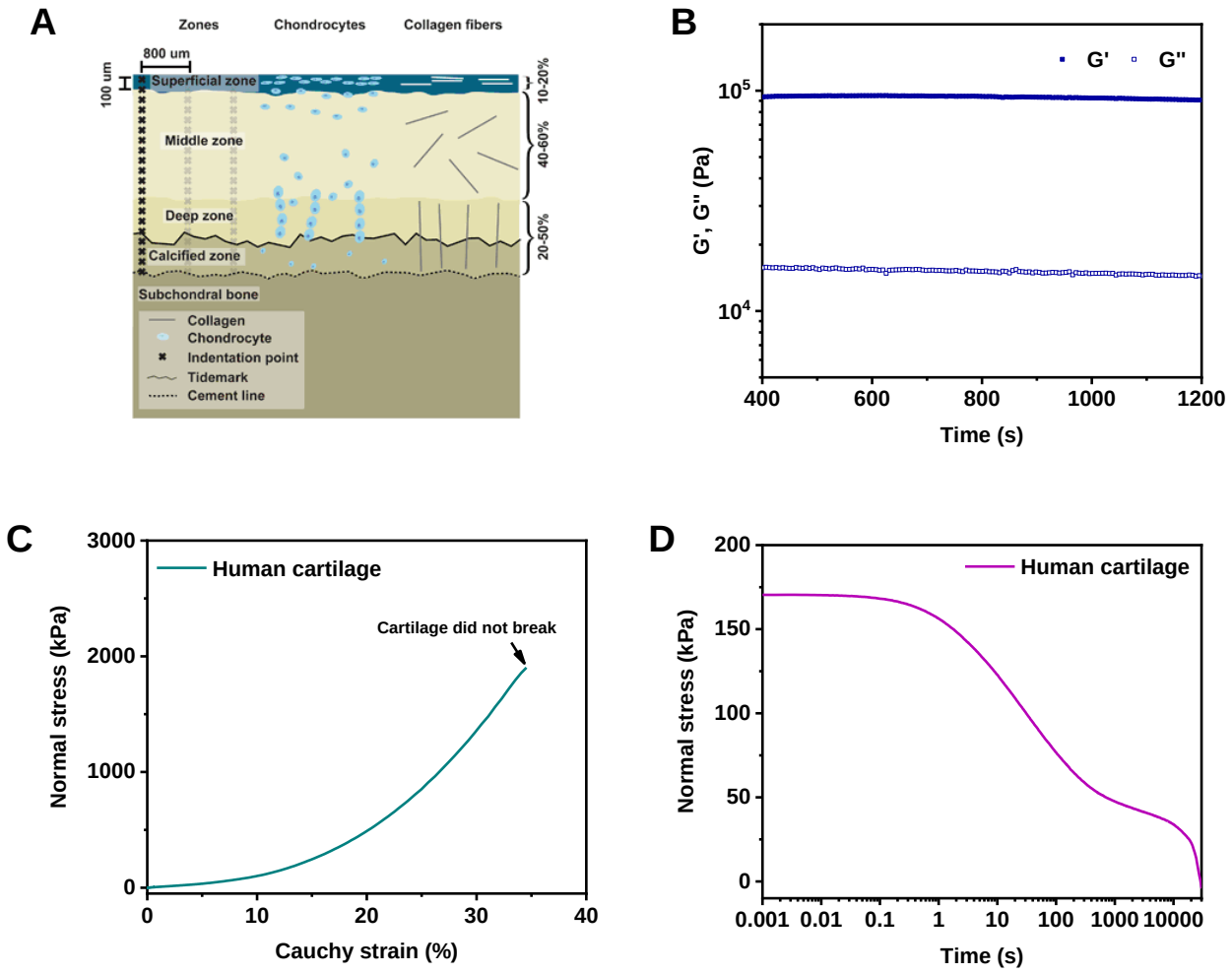

Figure 3. (A) Cross section of human knee joint including four zones: superficial zone (10–20%), middle zone (40–60%), deep zone (20–50%) and calcified zone.[4] (B) Time sweeps measurements of human cartilage at RT. (C) Uniaxial stress-strain curves of human cartilage under a consistent speed of 10 µm/s, the cartilage did not break at the end of the measurement. (D) Poroviscorelaxation curves of human cartilage under 15% consistent normal strain.

In vivo, environments exhibit dynamic complexity, characterized by varied human ECM compositions in different regions, each contributing to specific physiological functions. Nowadays, complex mechanical properties (e.g., strain-stiffening, viscoelasticity, viscoplasticity, poroelasticity) have been introduced into the hydrogel field (native and synthetic) to better understand specific cell behaviors. Therefore, in the future, it will also be crucial to delve into the intricate interplay between the complex mechanical properties of hydrogels and cellular behavior. Besides, integrating peptides onto the supramolecular monomer is imperative for enhancing biocompatibility and fostering a conducive cellular environment to promote beneficial cell functions (figure 4). This exploration aims to unveil increasingly sophisticated mechanical and biological characteristics that closely emulate diverse tissues. Identifying specific complex mechanical and biological properties influencing cellular behavior is imperative, and precision in orchestrating these properties is essential to sculpting a framework for controlling cell phenotypes and fostering the growth of organs or specific tissues.

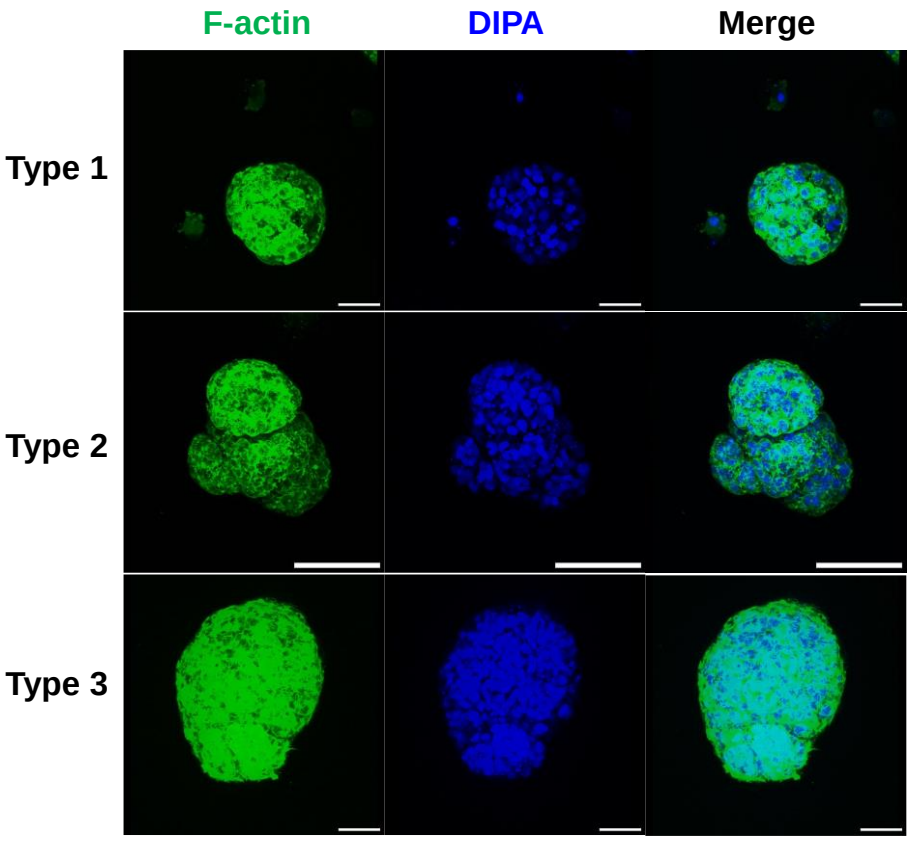

Figure 4. Representative confocal images of three major morphological growth patterns of NIH 3T3 fibroblast spheroids in tripodal squaramide-based hydrogels with bioactive cues (RGD) after 10 days of culture and stained with F-actin and DAPI. Scale bar: *100 μm*.

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