Title:
Effects of cell-adhesive ligand presentation on pentapeptide supramolecular assembly and gelation: Simulations and Experiments

Running heading:
Cell-adhesive ligand impact on peptide assembly and gelation

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TITLE

Abstract
The extracellular matrix (ECM) is a complex, hierarchical material containing structural and bioactive components. This complexity makes decoupling the effects of biomechanical properties and cell-matrix interactions difficult, especially when studying cellular processes in a 3D environment. Matrix mechanics and cell adhesion are both known regulators of specific cellular processes such as stem cell proliferation and differentiation. However, more information is required about how such variables impact various neural lineages that could, upon transplantation, therapeutically improve neural function after central nervous system (CNS) injury or disease. Rapidly Assembling Pentapeptides for Injectable Delivery (RAPID) hydrogels are one biomaterial approach to meet these goals, consisting of a family of peptide sequences that assemble into physical hydrogels in physiological media. In this study, we studied our previously reported supramolecularly-assembling RAPID hydrogels functionalized with the ECM-derived cell adhesive peptide ligands RGD, IKVAV, and YIGSR. Using molecular dynamics simulations and experimental rheology we demonstrated that these integrin-binding ligands at physiological concentrations (3-12 mM) did not impact assembly of the KYFIL peptide system. In simulations, molecular measures of assembly such as hydrogen bonding and pi-pi interactions appeared unaffected by cell-adhesion sequence or concentration. Visualizations of clustering and analysis of solvent accessible surface area (SASA) indicated that the integrin-binding domains remained exposed. KYFIL or AYFIL hydrogels containing 3 mM of integrin-binding domains resulted in mechanical properties consistent with their non-functionalized equivalents. This strategy of doping RAPID gels with cell-adhesion sequences allows for the precise tuning of peptide ligand concentration, independent of the rheological properties. The controllability of the RAPID hydrogel system provides an opportunity to investigate the effect of integrin-binding interactions on encapsulated neural cells to discern how hydrogel microenvironment impacts growth, maturation, or differentiation.

1. Introduction

Hydrogels are water-swollen polymer networks capable of mimicking biochemical and biophysical attributes of the native extracellular matrix (ECM) [Murphy, and Lampe, 2015; Russell, and Lampe, 2016; Brown, and Anseth, 2017] . Due to their tailorable properties, engineered hydrogels have a wide range of therapeutic applications including cell-based therapies [Marquardt et al., 2020; Zhong et al., 2010; Payne et al., 2019] . Hydrogels require crosslinking for assembly, which can be mediated by either covalent or physical interactions. In addition to naturally-occurring physical hydrogels like gelatin, collagen, and alginate, synthetic and natural polymers have been grafted with moieties that physically interact to create robust hydrogels [Mann et al., 2017; Dånmark et al., 2016; Ding et al., 2018; Wong Po Foo et al., 2009; Clarke et al., 2017; Rodell et al., 2013; Sumey et al., 2023]. Another physical gelation mechanism builds on supramolecular assembly of small peptide molecules, in some cases only 2-5 amino acids long, that create fiber networks held together by physical interactions such as hydrogen bonding and pi-pi stacking [Tang et al., 2019a; Schneider et al., 2002; Hartgerink et al., 2001; Jayawarna et al., 2006; Smith et al., 2008]. Frequently, these physical interactions endow the bulk hydrogel with shear-thinning and self-healing abilities, thereby enabling injection and cell delivery. With their robust and controllable physical properties, peptide gels

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make excellent biomaterial candidates for cellular instruction if they can be modified to support direct cellular interactions.

While hydrogels are useful for mimicking aspects of the ECM and are conducive to cell growth, understanding the factors that influence specific processes in central nervous system (CNS) cell types remains elusive. Some mimics of the CNS extracellular matrix (ECM) support physiologically relevant development of cell morphologies and interactions [Larsen, and Yong, 2004; Banerjee et al., 2009; Veiga et al., 2011; Lee et al., 2012; Diao et al., 2015; Meco et al., 2020]. Both specific cell-matrix interactions as well as mechanical properties of the microenvironment influence the differentiation and proliferation of lineage-restricted progenitor cells, such as oligodendrocyte precursor cells (OPCs) [Asmani et al., 2013] [Urbanski et al., 2016; Russell, and Lampe, 2017]. Thus, in order to properly investigate cell-matrix interactions of neural cells and their surrounding microenvironment independent of the influence of differential biomechanical cues, it is important to decouple the synergistic effects of biomechanical properties and integrin-binding on cell fate. We have previously shown our hydrogel system, rapidly assembling pentapeptides for injectable delivery (RAPID), to be an excellent candidate for encapsulating neural cells with easily tunable rheological properties [Tang et al., 2019a; Tang et al., 2019b]. The modularity of this fiber-forming peptide system would permit the study of integrin-mediated cell-matrix interactions of encapsulated cells independently of hydrogel stiffness.

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Cell-ECM interactions are frequently governed by adhesive peptide sequences like RGD, IKVAV, and YIGSR. These sequences have been successfully immobilized in synthetic 3D hydrogels to bind integrins on the cell surface and modulate cell behavior [Weber et al., 2007; Tibbitt, and Anseth, 2009; Miller et al., 2010; Madl et al., 2016]. These adhesive sequences have specific impacts on neural phenotype in 3D hydrogels, where their presence may be an important determining factor. RGD is derived from fibronectin, but is also found in collagen type I, fibronectin, laminin, and other matrix proteins [Thompson et al., 1991]. In hydrogels, RGD increases neurite outgrowth from both dorsal root ganglia neurons [Lampe et al., 2013] and neural stem cells (NSCs) [Fischer et al., 2007]. The laminin-derived adhesion sequence IKVAV significantly enhances regeneration of neural tissue in a rat brain surgery model [Ty et al., 2013]. The integrin receptor ligand YIGSR, found in the laminin β chain, promotes neuronal cell adhesion [Yu et al., 1999; Weber et al., 2007], neurite sprouting, and regeneration [Cui et al., 2016]. Collectively, these integrin-binding sequences have proven beneficial for neural cell growth and development and therefore should be considered when designing a biomaterial system for the CNS.

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Incorporating such adhesive peptides into a self-assembled hydrogel system may be advantageous. Some peptide-based biomaterials including RADA and IKVAV-modified PAs specifically support neural cell growth and differentiation in vitro and in vivo [Ylä-Outinen et al., 2014; Liedmann et al., 2012; Gelain et al., 2007; Silva et al., 2004; Tysseling et al., 2010]. Furthermore, the supramolecular and dynamic nature of peptide amphiphiles (PAs) incorporating IKVAV, such as previously noted peptide amphiphiles, further enhances neuronal branching and maturation [Álvarez et al., 2023]. However, there is a potential tradeoff between new functionality and assembly/gelation: adding any additional motifs to a peptide hydrogel has the potential to disrupt physical crosslinking, especially with particularly small peptide assemblers.

Furthermore, the sequence intended for cell-binding must be accessible to integrin interactions, rather than buried in a physical crosslink. In addition to experiments probing hydrogel mechanics, molecular dynamics can help characterize assembly behavior and adhesion motif availability by monitoring the early stages of molecular assembly, providing a map of molecular interactions responsible for gelation as well as ligand accessibility to solvent within clusters of peptides .

Leveraging recent advancements in computing power, simulation of soft materials systems has become a useful tool to synergistically inform, direct, and supplement *in vitro* biomaterial design and development [Smith, and Hall, 2001; Gartner, and Jayaraman, 2019]. While various methods of simulations aid in the design of soft materials, molecular dynamics (MD), has proven to be effective for studying peptide design and engineering as a means for linking molecular driving forces to self-assembled structure and macroscopic properties. [Frederix et al., 2015; Tang et al., 2019a; V. Alegre-Requena et al., 2019; Prhashanna et al., 2019; Wang et al., 2020; Hilderbrand et al., 2021]. Using MD, the molecular interactions controlling peptide self-assembly can be probed such as hydrogen bonding and pi-pi stacking, thus informing new peptide designs and their impact on hierarchical structures spanning multiple length scales (e.g., peptide oligomerization and hydrogel assembly) [Condon, and Jayaraman, 2018; Li et al., 2014a; A. Taylor et al., 2022; A. Taylor et al., 2020; Mansbach, and Ferguson, 2017]. Frederix et al. used simulations and experiments to explore the behavior of short (3 amino acid) peptide sequences, connecting course-grained MD predictions of assembly to experimental observations of gelation [Frederix et al., 2015]. Previously, we used atomistic MD to characterize the assembly of several different RAPID sequences into hydrogels, demonstrating excellent agreement between simulation and experiment regarding propensity for β-sheet secondary structure, peptide clustering, hydrogel formation, and hydrogel stiffness [Tang et al., 2019a]. Overall, atomistic MD simulations provide an opportunity to probe the effects of incorporating cell-adhesive sequences into small peptides molecules on the early stages of peptide clustering and fiber assembly, thus facilitating the molecular design of neural tissue-mimetic biomaterials.

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Inspired by our previous computational and experimental efforts examining RAPID hydrogel assembly, in this study we investigate the assembly of RAPID peptides functionalized with RGD, IKVAV, and YIGSR integrin-binding peptide sequences at various concentrations. Through a combined computational/experimental approach, we relate the intra- and inter-molecular interactions of each amino acid in the system to peptide assembly, cell-adhesive sequence availability, and hydrogel formation. We present a hydrogel system that can be facilely prepared and ultimately used to direct cell fate. Through atomistic MD, we examine the intra- and inter-molecular interactions of RAPID peptides governing the efficacy of hydrogel assembly and cell adhesive sequence presentation *in vitro*. Our results indicate the potential of RAPID hydrogels to provide suitable integrin-binding and mechanical cues to create a microenvironment for directing cell fate.

2. Methods and Materials

Simulation Setup of KYFIL Peptide System

We conducted simulations of both unmodified (control) KYFIL as well as systems containing modified KYFIL (Figure 1). Using the molecular editing software *Avogadro 1.2.0* [Hanwell et

al., 2012] , we generated protein databank (.pdb) files of the LYS-TYR-PHE-ILE-LEU (KYFIL) peptide as well as the following peptides modified with known integrin-binding motifs: ARG-GLY-ASP-LYS-TYR-PHE-ILE-LEU (RGDKYFIL), ILE-LYS-VAL-ALA-VAL-LYS-TYR-PHE-ILE-LEU (IKVAVKYFIL), and TYR-ILE-GLY-SER-ARG-LYS-TYR-PHE-ILE-LEU (YIGSRKYFIL). The C-terminus of all four peptide sequences were amidated using *Avogadro 1.2.0*. We used the web-based CHARMM-GUI built-in "PDB Reader" to generate corresponding protein structure files (.psf) for these peptides using a CHARMM36m forcefield [Jo et al., 2008; Jo et al., 2014; Park et al., 2023]. The individual peptide .pdb and .psf files can be found in Supplemental Information. Next, we used *Visual Molecular Dynamics 1.9.3* (VMD) along with an in-house Tcl script, available in the SI, to add and arrange components of the initial peptide system [Humphrey et al., 1996]. For the unmodified KYFIL sequence, we used the corresponding .pdb and .psf files for KYFIL to generate a cubic array of 64 KYFIL peptides (4 x 4 x 4). Each peptide was positioned 32 Å away from other peptides, and 5 Å from the edge of the water box, as measured from the peptide center of mass, and oriented randomly as to not introduce any artificial bias in the starting configuration. This spacing ensured that solvating the system with explicit TIP3P water using VMD's built-in *solvate* plugin followed by neutralizing and ionizing using VMD's built-in *ionize* plugin to add a physiological NaCl concentration (~150 mM), yielded a peptide concentration in the system of ~45 mM (~3 wt%), similar to concentrations used to form gels experimentally [Tang et al., 2019a]. The initial configuration for each system contained between ~180,000 and ~240,000 atoms within an approximately cubic box of water measuring 130 Å x 130 Å x 130 Å for all systems. For each modified KYFIL peptide (RGDKYFIL, IKVAVKYFIL, and YIGSRKYFIL), we generated three unique systems containing 64 peptide systems, having either 4, 8, or 16 modified peptides substituted in place of unmodified KYFIL peptides. To distribute the RGD-, IKVAV-, and YIGSR-modified peptides throughout the entire system, we divided the cube into four sections of 16 peptides. A random number generator was then used to determine which unmodified KYFIL peptide(s) in each section would be replaced with either an RGDKYFIL, IKVAVKYFIL, or YIGSRKYFIL peptide. These modified KYFIL systems were made to achieve physiologically-relevant adhesion sequence concentrations of ~3 mM, ~6 mM, and ~12 mM [Jung et al., 2011]. A more detailed description of this process can be found in the SI containing the full in-house script.

Performing a Simulation on a Peptide System

We used *Nanoscale Molecular Dynamics 2.14* (NAMD) to simulate the peptide behavior in these systems for 100 ns at constant number of moles, temperature, and pressure (NPT) using periodic boundary conditions to mimic benchtop conditions [Phillips et al., 2020]. Before each simulation, the system was first energy minimized for 10,000 steps. The temperature and pressure of the system were set to 300 K and 1 atm, respectively, via Langevin dynamics of all non-hydrogen atoms and a hybrid Nose-Hoover Langevin piston. The full configuration file can be found in SI. The systems were then simulated for 100 ns with a 2 fs time step and configurations were stored every 500 time steps. We quantified and visualized peptide self-assembly over the simulation trajectory by using information on atom position, energy, and bond angles and lengths provided by in binary files (.dcd) and NAMD standard output files (.out). Visualizations of each system are available in SI Figures 1-3. Energy profiles in each simulation confirmed that the potential energy reached a plateau within the first 60 ns for each trajectory and that systems have reached equilibrium (SI Figure 8).

Clustering Analysis
We performed clustering analysis to quantify peptide proximity to other peptides to gauge the extent to which they assemble. A pair of peptides was defined to be part of the same "cluster" if the distance between the two peptide's centers of mass fell below a specified cutoff value of 15 Å. The center of mass distance cutoff was determined by first analyzing two different peptide systems (the unmodified KYFIL system and the 16 peptide IKVAVKYFIL system) at 14 Å, 15 Å, and 16 Å with all three values yielding qualitatively similar clustering results (SI Figure 4). Using an in-house Tcl script, we parsed through every combination of a peptide's position in relation to the other 63 peptides to generate a list of peptide pairs, with data analyzed every 12.5 ns in each system. The number of unique groupings was counted to determine the number of clusters at each time step in the analysis. Block averaging of the final 40 ns was conducted to provide relative comparisons between systems (SI Figure 5).

Hydrogen Bond Quantification
To quantify the number of hydrogen bonds at each time point over the course of the 100 ns simulation, we used the built-in VMD hydrogen bond calculator. VMD's hydrogen bond calculator quantifies hydrogen bonds by determining the distance and angle between hydrogen, oxygen, and nitrogen atoms that are capable of hydrogen bonding for each peptide in the system and returning a list of hydrogen bonds found every 50 frames. In this work, we examined peptide-peptide hydrogens and used the VMD hydrogen bond calculator's geometric criteria, which defines hydrogen bonds as a bond between a hydrogen and an atom (the donor, D) and another atom (the acceptor, A) such that the distance D-A is less than or equal to 3 Å and the angle D-H-A is less than or equal to 20^{o} [McDonald, and Thornton, 1994; Weinhold, and Klein, 2012]. Hydrogen bond data was decimated using the Python package SciPy's *decimate* function, using a decimate value of 5 [Virtanen et al., 2020]. The data was further smoothed by applying a moving average algorithm using a window size of 21 for each respective system. Additionally, we performed a block averaging analysis on this decimated data to understand overall trends after equilibration (SI Figure 6). The analysis was conducted by taking the average and standard deviation from the aforementioned decimated list of hydrogen bonds for the last 40 ns in each system.

Pi-Pi Interaction Quantification
We used the VMD plug-in RIP-MD to quantify pi-pi interactions in each peptide system every nanosecond [Contreras-Riquelme et al., 2018]. RIP-MD measures the distance between the geometric centers of aromatic rings of each peptide in the system. If the distance between the two centers of mass of aromatic rings of different peptides was less than 6 Å in any one of three configurations (T, sandwich, or parallel-displaced), then this was counted as a pi-pi interaction. In this work, we only examine pi-pi interactions between peptides and exclude other pi-pi interactions such as pi-cation interactions. Additionally, we performed a block averaging analysis on this data to understand overall trends after equilibration (SI Figure 7). The analysis was conducted by taking the average and standard deviation from the number of pi-pi interactions for the last 40 ns in each system.

Relative Solvent Accessible Surface Area (relSASA) Quantification
We quantify solvent accessible surface areas (SASA) to examine the accessibility of cell adhesion motifs. Higher SASA values would suggest a greater amount of amino acid

accessibility to cells in experiments. To quantify the amount of solvent that is accessible to each amino acid in the system after 100 ns, we used an in-house script that leveraged the built-in solvent accessible surface area (SASA) tool in VMD with a water probe radius of 1.4 Å. Relative SASA (relSASA) for each amino acid was then calculated by normalizing each SASA value to the maximum empirical SASA value found in literature [Tien et al., 2013].

Peptide Synthesis

All peptides were synthesized with an amidated C-terminus. Amidated KYFIL was purchased from Genscript (>98% purity) and used as provided. All integrin-binding motif peptides were synthesized by solid-phase chemistry on a Gyros Protein Technologies Tribute (for rheology experiments). From a TentaGel R Rink Amide Resin, peptides were synthesized in the C-terminal to N-terminal direction using standard Fmoc techniques as previously described [Tang et al., 2019a]. Solvents and Fmoc (fluorenylmethoxycarbonyl) protected amino acids were purchased from Gyros Protein Technologies. After all amino acids were coupled, the resin was washed three times with dichoromethane (DCM). Cleavage of the peptides from the resin was accomplished by shaking the resin with 10 mL of an acid cocktail containing trifluoroacetic acid (TFA)/triisopropylsilane/H_2O (95:2.5:2.5 v/v/v) for 2 h at room temperature. The solution containing deprotected cleaved peptide was collected, and the peptide precipitated by the addition of cold diethyl ether followed by two washes with cold ether after centrifugation. Peptides were dried overnight, redissolved in deionized water, and purified by dialysis in semipermeable cellulose ester membranes with a molecular weight cutoff of 100-500 Da (Spectra/Por, Spectrum Laboratories Inc., Rancho Dominguez, CA). The dialysis buffer (deionized water) was changed every 12 h for 2 days. All peptides were lyophilized and stored at 4 °C until use.

Hydrogel Formation and Rheological Properties

Lyophilized peptides were dissolved in 1X phosphate-buffered saline (PBS) to a final concentration of 1.5 wt.%, inclusive of the specified concentration of modified KYFIL. To evaluate the viscoelastic properties of the hydrogel forming peptides, 25 μL aliquots of the hydrogel were pipetted into 5 mm molds on glass cover slips. Gels were removed from the molds and rheology was performed 10 minutes after induction of gelation (Anton Par, P25S 25 mm parallel steel plates) with a gap height of 250 μm. Storage (G') and Loss (G") moduli were measured as a function of strain (%) ranging from 0.01 to 100% with a constant frequency of 10 rad/s. Frequency sweeps were performed at angular frequencies ranging from 1 to 100 rad/s at 0.1% strain. For recovery experiments, a step-time procedure was utilized with a series of applied oscillatory strains. Initially, samples were subjected to 0.01% strain for 100 s followed immediately by a 500% strain for 50 s, and cycled 5 times. All steps were performed at a fixed oscillation frequency of 10 rad/s.

3. Results and Discussion

3.1 Computational analysis of peptide assembly incorporating adhesion motifs

We previously described a family of shear-thinning peptide hydrogels conducive to cell survival and injection based on the peptide sequences KYFIL [Tang et al., 2019a; Tang et al., 2019b]. While the presence of RGD, IKVAV, and YIGSR adhesion sequences cell adhesion sequences can improve cell viability and stem cell differentiation of synthetic biomaterials [LeBaron, and Athanasiou, 2000][Patel et al., 2019], in our system, the presence of these cell-adhesive

sequences may also impact assembly of KYFIL and resulting physical properties. Thus, we used atomistic molecular dynamics simulations to investigate the impact of both the presence and concentration of these three different integrin-binding, cell adhesion sequences on the assembly-governing molecular interactions of 10 different KYFIL self-assembling peptide systems (Figure 1, Table 1). A pure KYFIL system with 64 KYFIL peptides served as a control for all doped systems. The total peptide concentration in each system remained constant at ~ 30 mg/ml (~ 3 wt% or 45 mM).

We performed atomistic molecular dynamics for 100 ns atomistic simulations of each KYFIL system. Using VMD we visualized the trajectories of peptides in each system (SI Figures 1-3). Snapshots of the simulation trajectory in the modified 4 RGDKYFIL peptide system demonstrate dynamic peptide clustering (Figure 2). In this system, we readily observed that the RGD sequence remains accessible and is not buried internally to the clusters it forms. Similarly, the RGD integrin-binding motif appears to remain accessible in each of the simulated peptide systems at other RGD concentrations (SI Figure 1). By inspection, the IKVAV (SI Figure 2) and YIGSR (SI Figure 3) sequences appear to be well distributed through their respective systems at each concentration as well. For all systems, the peptides interacted in a dynamic manner, while leading to an increase in peptide assembly over time that tended to stabilize around 60 ns. The trajectories of each system were then analyzed to examine the ability of modified KYFIL peptides to form gels.

We first investigated the propensity for the various peptides to group, or "cluster", together as a means to study assembly. We previously showed that the assembly propensity of KYFIL peptides from MD simulations correlates with peptide gelation experimentally [Tang et al., 2019a]. In this study, we screened our peptide systems for clustering by determining the distance between the centers of mass of each of the peptides in the system. If the centers of mass of two peptides were closer than 15 Å, we considered them to be clustered and plotted the number of clusters as a function of time, peptide sequence, and concentration of modified peptides. We note that using 14 and 16 Å as a cut-off distance produced similar results for both a pure and modified KYFIL system (SI Figure 4). Over time, the number of peptide clusters rapidly decreased over the first 40 ns (and thus the number of peptides per cluster increased), suggesting a positive correlation between time and the number of peptide molecules assembled into supramolecular clusters (Figure 3). Overall, the little difference in cluster formation of the modified KYFIL systems compared to the pure KYFIL system suggests minimal impact of adhesion ligands on gelation *in vitro* at these concentrations. To confirm this observation, we performed a block average analysis on the clustering behavior after 60 ns (SI Figure 5), using a limited set of data points. When compared to KYFIL, there does not appear to be any notable (> 1 standard deviations) differences when compared to all modified KYFIL systems. However, there is perhaps a trend toward a lower degree of clustering (greater # of clusters) in with increasing concentrations in the RGDKYFIL and YIGSRKYFIL peptide systems.

While clustering suggests the propensity of peptide assembly and hydrogel formation, we can also use simulations to analyze the type and quantity of molecular interactions and thereby provide mechanistic insight into the assembly process. Interactions like hydrogen bonding and pi-pi stacking play a key role in the formation and stabilization of peptide secondary structure formation, and therefore we quantify them here to determine the role of the modified KYFIL

sequences on assembly [Bordo, and Argos, 1994; Wang et al., 2016; Gray et al., 2022]. Over the first ~40 ns of each simulation, the number of hydrogen bonds in each of our systems increases in a nearly linear manner, and then this increase slows and approximately plateaus after ~60 ns in all systems except the 4 RGDKYFIL, 16 RGDKYFIL, and 16 IKVAVKYFIL systems (Figure 4). This suggests that each system reaches equilibrium around 60 ns into the trajectory. Near the end of the simulation the amount of hydrogen bonding varies little from system to system, although most modified systems appear to have slightly greater H-bonding than the pure KYFIL system. We performed a block average analysis on the hydrogen bonding behavior after 60 ns. There are no quantifiable differences between any peptide system. This suggests that the existence of modified KYFIL sequences in the system does not impact hydrogen bonding capability.

Pi-pi interactions can also drive peptide assembly in biological systems [Wang et al., 2016; C. Edwards-Gayle, and W. Hamley, 2017; Gray et al., 2022]. Because of the aromatic groups present in each KYFIL system, it is pertinent to investigate not only the impact hydrogen bonding has on gelation, but also the impact of pi-pi interactions. Similar to hydrogen bonding, there appears to be a linear increase in the number of pi-pi interactions in each system until 50 ns (Figure 5). After 50 ns, the number of pi-pi interactions seems to plateau and remain relatively constant for the remainder of the simulation. Interestingly, the trend in pi-pi interactions for all modified KYFIL peptide systems over all 100 ns appears to follow the behavior of the pure KYFIL system much more closely than the trends seen with hydrogen bonding behavior. According to a block averaging analysis of the pi-pi interactions of each system from 60-100 ns, the systems have incredibly similar numbers of pi-pi interactions after their plateaus, all well withing one standard deviaion (SI Figure 7). However, there is a potential trend toward higher concentrations of integrin-binding-motif-modified KYFIL leading to fewer pi-pi interactions. We also note that despite YIGSRKYFIL having more capability to pi interact with other aromatic groups in the system (referring to the additional tyrosine on the N-terminus unique to this peptide), it does not appear to have additional pi-pi interactions in comparison to the other peptide simulations. Moreover, the presence of 30 or more pi-pi interactions in each system conveys the fact that pi-pi interactions are important contributors in KYFIL peptide hydrogel assembly.

Solvent accessible surface area (SASA) is an important metric for gauging accessibility of each amino acid in the system. Because we are doping KYFIL with adhesion-binding amino acid sequences, these sequences should be exposed to solvent *in situ* to avail them for interaction with cells *in vitro* to mediate adhesion to KYFIL-based hydrogels. In pure KYFIL systems, lysine is typically the most exposed and phenylalanine and tyrosine are the most buried (Figure 6). Phenylalanine and tyrosine are the only amino acids in our systems with the capability of pi-pi interaction. Given the presence of pi-pi interactions in all peptide systems, these amino acids may be buried (low SASA) because of these interactions. In all modified KYFIL systems, the N-terminal residues, and indeed the entire N-terminal integrin-binding portion, of each peptide consistently had the highest relSASA value. These data suggest that the cell adhesion sequences are likely to be available for cellular binding when presented in KYFIL-based hydrogels. The results of these simulations are particular interesting in the case of YIGSRKYFIL (Figure 6) since the tyrosine that belongs to the cell-adhesion motif YIGSR is one of, if not the most exposed amino acid in the YIGSRKYFIL systems despite YIGSRKYFIL having more capability

to pi interact with other aromatic groups in the system, indicating it does not take part in these interactions.

Together, the simulations suggest that cell adhesion motifs can be attached to the N-terminus of KYFIL without appreciably interfering with KYFIL molecular interactions important for assembly and gelation. The number of hydrogen bonds in the modified systems appears to be slightly greater than the pure KYFIL system (albeit not significantly so) which could support an association of XXX-KYFIL dopants with each other, rather than a broad distribution. However, the pi-pi interactions of YIGSRKYFIL would be expected to be higher if the modified KYFIL dopants were phase-segregating, and such an effect is not observed. It is possible that the YIGSR sequence is subject to intramolecular interactions via pi-pi interactions with the bound KYFIL sequence, although our simulation experiments do not indicate such unavailability. Visualizations of each system at snapshots throughout the simulation trajectory also provide no evidence of integrin-binding-motif segregation. The SASA data suggest that these sequences will be presented on the solvent-accessible periphery of these assemblies and therefore be available for interactions with cells. Encouraged by these findings, we proceeded to study these sequences experimentally, first using rheology to gauge the effects of the modified KYFIL sequences on hydrogel mechanics prior to cell studies.

3.2 Hydrogel rheological characterization

One advantage of these dynamic peptide hydrogels is their injectability, that is, their ability to shear-thin and self-heal due to their viscoelastic behavior, with a modulus that can be tuned in the regime of healthy brain tissue [Tang et al., 2019a]. Here, we evaluated the mechanical properties of 1.5 wt. % hydrogels, and found that the incorporation of RGD, IKVAV, and YIGSR-modified peptides did not alter the storage modulus of RAPID hydrogels. We tested the impact on both KYFIL and AYFIL gelation, as we previously established that these two sequences yield hydrogels with different mechanics, and therefore could approximate the stiffness of different biological tissues. In both cases, hydrogels were formed by mixing 22 mM of AYFIL or KYFIL peptides with 2 mM of the same peptide modified with the respective cell-adhesive ligands. This ligand concentration was based off published multifactorial experiments to optimize endothelial cell growth as a function of individual cell-adhesive ligand incorporation, and demonstrated that there was significant cell growth for cells seeded on hydrogels with cell-adhesive ligand concentrations from 1.5 mM to 6 mM [Jung et al., 2011]. For all KYFIL and KYFIL-functionalized samples, the storage modulus (G') was found to be ~8.5 kPa (Figure 6) versus ~3 kPa for AYFIL hydrogels (Figure 7) at 10 rad/s. These stiffnesses are consistent with previously published results of RAPID hydrogel systems [Tang et al., 2019a; Tang et al., 2019b], where it was reported that 1.5 wt % KYFIL hydrogels had a storage modulus ranging from 8-9 kPa, and 1.5 wt % AYFIL hydrogels had a stiffness of 3.2 kPa. This suggests we can reliably tune the mechanical properties of the hydrogel while maintaining a constant ligand concentration.

Multiple high-strain (100%) sweep cycles, with 30 s recovery periods, were performed to evaluate RAPID hydrogels' ability to self-heal following mechanical deformation. All cell-adhesive motif functionalized RAPID hydrogels had relatively similar recovery profiles compared to non-functionalized hydrogel controls (Figure 7I-L, Figure 8I-L). Following a 500% strain, KYFIL hydrogels repeatedly recovered gel behavior within 14 seconds. Within 1 minute,

the gel recovered ~70% of its initial *G′*. For AYFIL hydrogels, following a 500% strain, hydrogels recovered 82% of its initial *G′* within a minute. One potential difference amongst systems is that in the stress recovery experiments, RGDAYFIL and YIGSRAYFIL appear to recover from the high strain condition more slowly than pure AYFIL.

Sensitivity to ligand presence, or ligand type, may be dependent on stiffness of the hydrogel or the ability of cells to remodel it. AYFIL hydrogels are less stiff than KYFIL hydrogels and have a lower G'/G'' crossover point in strain sweeps. This indicates that encapsulated cells may be more able to push and pull on the matrix to rearrange the nanofibrous matrix and the integrin-binding sites. This increased mobility in supramolecular peptide hydrogels may be beneficial to cell development, but also may lead to more rapid hydrogel erosion/degradation [Álvarez et al., 2023]. In all cases, even after multiple high-strain cycles, the hydrogel rapidly and repeatedly recovers its mechanical strength—rendering these materials suitable for biomedical applications that require injection. These mechanical characteristics are well suited for uniform encapsulation of cells in 3D, *ex vivo*, and then injection via a minimally invasive technique.

4. Conclusions

Using atomistic molecular dynamics simulations, we established that incorporating cell-adhesive sequences into RAPID hydrogels up to 12 mM does not appreciably impact peptide assembly mechanisms. Quantification of peptide clustering demonstrated remarkable agreement across all our simulation systems and integrin-binding motif concentrations. Hydrogen bonding and pi-pi stacking are not negatively impacted by the presence of cell adhesion sequences, which may in fact increase inter-peptide interactions. This correlated well with the physical behavior of gelation, where adhesion sequences did not statistically change storage or loss moduli. Furthermore, the shear-thinning behavior of hydrogels was unchanged at concentrations of the integrin-binding motif relevant to cell encapsulation. IKVAV, RGD, and YIGSR functionalized hydrogels had similar material mechanics, and increased levels of cell metabolic activity compared to non-functionalized hydrogel controls. Collectively, our results demonstrate that we can append a number of different motifs without substantially impacting the assembly mechanics facilitated by the KYFIL (or AYFIL) sequence. Notably, for instance, RGD is particularly hydrophilic while IKVAV is substantially more hydrophobic, and both yield similar assembly as determined by both computational and physical experiments. The hydrogel system presented here allows for independent adjusting of the concentration of multiple cell-adhesive ligands without any changes to the mechanical properties of the hydrogel. Such a platform presents an opportunity to further explore the effect of peptide ligand presentation on a number of facets of cell behavior, laying the groundwork for future explorations of neural cell fate. The facile preparation of functionalized RAPID hydrogels should be widely applicable to other studies at understanding the effects of matrix-bound ligands involving 3D hydrogel encapsulation studies.

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6. Statement of Ethics

This work was conducted according to ethical practices for experimental design and analysis. All authors have contributed to the manuscript and approved submission. No animal or human data were used in this study.

7. Conflict of Interest Statement

The authors declare that they have no conflicts of interest to disclose.

8. Funding Sources

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9. Author contributions

KJL, ATT, ARK, CDA, and JDT conceived the experimental concepts and design. ATT, JDT, CEC, LJH, and PAT developed computational simulation and analysis methods. ATT conducted computational simulations and analysis. ARK and JDT conducted rheology experiments and performed data analysis. ATT, ARK, CDA, JDT, PAT, and KJL interpreted the data and wrote and edited the manuscript. KJL supervised the project.

10. Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author. Simulation data and code will be shared in a publicly available repository upon acceptance of this article.

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Captions:

Figure 1. Schematic of computational methods for MD system formation. For a pure KYFIL system, the C-terminus-amidated KYFIL peptide is replicated and placed into a 4 x 4 x 4 box with 32 Å spacing. This system is then solvated with explicit TIP3P water and ~150 mM of NaCl. For modified KYFIL systems, a similar 4 x 4 x 4 box is created with 4, 8, or 16 randomly located KYFIL peptides replaced with a corresponding modified KYFIL peptide. This system is then solvated with explicit TIP3P water and ~150 mM of NaCl. Each system is then run for 100 ns (water not shown for clarity) and subsequently analyzed. The example modified peptide system shows 60 KYFIL (grey) and 4 RGDKYFIL (red) peptides.

Figure 2. Modified KYFIL-based peptides assemble into supramolecular structures. Simulations in explicit solvent of KYFIL (grey) and RGDKYFIL (red) begin with 64 peptides randomly oriented on a 4 x 4 x 4 grid. These example snapshots are rendered from the simulation of the RGDKYFIL 4 Pep system. Simulations over the course of 100 ns display dynamic clustering of peptides, observable even after as little as 25 ns. RGD remains accessible and is not buried internally to the clusters.

Figure 3. Clustering analysis of modified peptide systems incorporating integrin-binding ligands. The number of clusters for the 3 mM, 6 mM, and 12 mM **A)** RGDKYFIL, **B)** IKVAVKYFIL, and **C)** YIGSRKYFIL peptide systems from 0 ns to 100 ns. Over 100 ns, the number of clusters decreases (from 64 at t = 0) as a function of time in each system. There appears to be little difference between each of the respective systems, suggesting that the addition and concentration of adhesion sequences to the parent KYFIL system has little impact on clustering behavior in this concentration regime.

Figure 4. Hydrogen bonding quantification in simulations of modified peptide systems. The number of hydrogen bonds increases over time for the 3 mM, 6 mM, and 12 mM **A)** RGDKYFIL, **B)** IKVAVKYFIL, and **C)** YIGSRKYFIL systems over 100 ns. Over 100 ns, there appears to be a linear increase in the number of hydrogen bonds for each sample for the first ~60 ns with the hydrogen bonding activity having a less consistent trend there after. At the end of 100 ns, the amount of hydrogen bonding varies little as a function of the concentration of modified KYFIL peptides in the system. For all modified KYFIL systems hydrogen bonding is greater than in the pure KYFIL system after 100 ns. Hydrogen bond data shown has been decimated by a factor of 5 and smoothed using a moving average algorithm.

Figure 5. Quantification of pi-pi stacking in modified peptide systems. Pi-pi interactions of the 3 mM, 6 mM, and 12 mM A) RGDKYFIL B) IKVAVKYFIL, and C) YIGSRKYFIL systems increase over 100 ns. After 100 ns, there appears to be a similar number of pi-pi interactions for each of the modified peptide systems as well as the unmodified KYFIL control. The data was smoothed by applying a moving average algorithm using a window size of 5 for each respective system.

Figure 6. The relative solvent accessible surface area (relSASA) values for each modified peptide after a 100 ns MD simulation. SASA values have been normalized to each amino acid's respective molecular weight to account for differences in amino acid surface area. For all

samples, the N-terminus of each modified peptide consistently has the highest relSASA value, with well solvated amino acids throughout the integrin-binding ligands. This indicates that these cell adhesion sequences could be available for cellular binding in an experimental hydrogel. This is particularly notable as some of these amino acids are more hydrophobic or capable of pi-pi interactions, but they appear well solvated, whereas the KYFIL motif in the modified systems appears largely buried, apparently due to intermolecular peptide interactions. Based on the pure KYFIL control system, amino acids containing aromatic groups (Y, F) appear to be driving the supramolecular assembly.

Figure 7. Rheological properties of 1.5 wt. % KYFIL hydrogels at pH 7.4 (n = 3 for all samples). A - D) Angular frequency sweep of control KYFIL, RGDKYFIL, IKVAVKYFIL, and YIGSRKYFIL peptide sequences, respectively, at constant strain of 0.1%. G' is an order of magnitude greater than G" indicating hydrogelation has occurred. E - H) Strain sweep of gelling control KYFIL, RGDKYFIL, IKVAVKYFIL, and YIGSRKYFIL peptide sequences, respectively, at constant angular frequency of 10 rad/s. Above the critical strain at 5%, the material becomes progressively more fluid-like, where G' starts to increase and G" begins to decrease. I - L) Five step strain sweeps of 0.1 % (100 s) and 500 % strain (50 s), followed by a 100 s recovery period, were performed on control KYFIL, RGDKYFIL, IKVAVKYFIL, and YIGSRKYFIL hydrogel peptide sequences, respectively. The hydrogel recovered 80% of its initial G' within several seconds. The hydrogel repeatedly recovered its mechanical strength following multiple high strain cycles.

Figure 8. Rheological properties of 1.5 wt. % AYFIL hydrogels at pH 7.4 (n = 3 for all samples). A - D) Angular frequency sweep of control AYFIL, RGDAYFIL, IKVAVAYFIL, and YIGSRAYFIL peptide sequences, respectively, at constant strain of 0.1%. G' is an order of magnitude greaten than G" indicating hydrogelation has occurred. E - H) Strain sweep of gelling control AYFIL, RGDAYFIL, IKVAVAYFIL, and YIGSRAYFIL peptide sequences, respectively, at constant angular frequency of 10 rad/s. Above the critical strain at 5%, the material becomes progressively more fluid-like, where G' starts to increase and G" begins to decrease. I - L) Five step strain sweeps of 0.1 % (100 s) and 500 % strain (50 s), followed by a 100 s recovery period, were performed on control AYFIL, RGDAYFIL, IKVAVAYFIL, and YIGSRAYFIL hydrogel peptide sequences, respectively. The hydrogel recovered 80% of its initial G' within several seconds. The hydrogel repeatedly recovered its mechanical strength following multiple high strain cycles.