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Article

Covalent Capture of a Collagen Mimetic Peptide with an Integrin-Binding Motif

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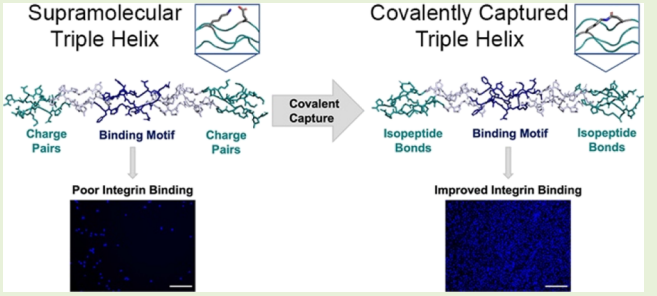
ABSTRACT: Collagen mimetic peptides (CMPs) are an excellent model to study the structural and biological properties of the extracellular matrix (ECM) due to ease of synthesis and variability in sequence. To ensure that synthetic materials accurately mimic the structure and function of natural collagen in the ECM, it is necessary to conserve the triple helix. However, CMP folding is subject to equilibrium, and frequently peptides exist in solution as both monomer and triple helix. Additionally, the stability of CMPs is highly dependent on peptide length and amino acid composition, leading to suboptimal performance. Here, we report the utility of covalent capture, a method to (a) direct the folding of a supramolecular triple helix and (b) form isopeptide bonds between the helix strands, in the design of an integrin-binding peptide with a GFOGER motif. Covalent capture effectively locked the triple helix and yielded a peptide with high thermal stability and a rapid folding rate. Compared to supramolecular triple helices bearing the same GFOGER-binding site, cell adhesion was substantially increased. *In vitro* assays using EDTA/Mg^{2+} and an anti-$\alpha2\beta1$ antibody demonstrated the preservation of the high specificity of the binding event. This covalently captured integrin-binding peptide provides a template for the future design of bioactive ECM mimics, which can overcome limitations of supramolecular approaches for potential drug and biomaterial designs.



INTRODUCTION

The extracellular matrix (ECM) serves varied roles, ranging from providing the structural support in tissues to influencing the biochemical signaling of cells.[1] Defects in the structure of the ECM can cause changes in tissue function and ultimately disease.[2−4] Cell adhesion to the ECM influences cell activity such as migration, proliferation, and differentiation, and this adhesion is often mediated through cell surface receptors such as integrins.[5,6] Collagen proteins are a major component of the ECM and contain numerous motifs to facilitate integrin binding. There are four known collagen-binding integrins: $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, and $\alpha11\beta1$, which display different binding affinities for different types of collagen.[7−9] All of the collagen-binding integrins associate with collagen through the interaction of a divalent metal ion-dependent (MIDAS) motif within the α(I) domain of the α subunit.[10−13] One well-studied integrin-binding motif is GFOGER, a hexapeptide sequence located in the $\alpha1$ chain of collagen I that is bound by the $\alpha2\beta1$integrin.[12,14,15] Mimicking this cell−collagen interaction is interesting from a biomaterial standpoint for modeling the ECM.[16−18]

The tertiary structure of collagen is important to its function within the ECM, especially cell adhesion.[19] The structure of collagen is a right-handed triple helix, which is composed of three poly-proline type II strands.[20−22] The primary sequence of collagen is typically characterized by a $(Xaa\text{-}Yaa\text{-}Gly)_n$ triplet repeat, in which Xaa is most frequently (2S)-proline, and Yaa is most frequently (2S,4R)-hydroxyproline.[20,23] The side chains of the amino acids in the Xaa and Yaa positions are oriented on the outside surface of the helix and are available for intra-helix interactions, as well as interactions with collagen receptors.[24,25]

The interaction of GFOGER with the $\alpha2\beta1$ integrin has previously been reported to be dependent on the presence of the triple helical structure of collagen.[26] Cell adhesion studies using natural collagen I indicated that the denatured and unfolded protein did not bind cells.[26] Further investigation using collagen mimetic peptides (CMPs) demonstrated that by covalently tethering the peptides together, cell adhesion to the peptide was generally improved.[27−29] Khew and Tong designed a peptide template utilizing glutamic acid residues as linkers to synthesize branched, covalently linked, peptides with a GFOGER motif. *In vitro* assays demonstrated that the templated and supramolecular peptides that had a triple helical structure promoted cell adhesion. However, none of the tested peptides (templated or supramolecular) performed as well as natural collagen.[27,30] Yamazaki and co-workers synthesized

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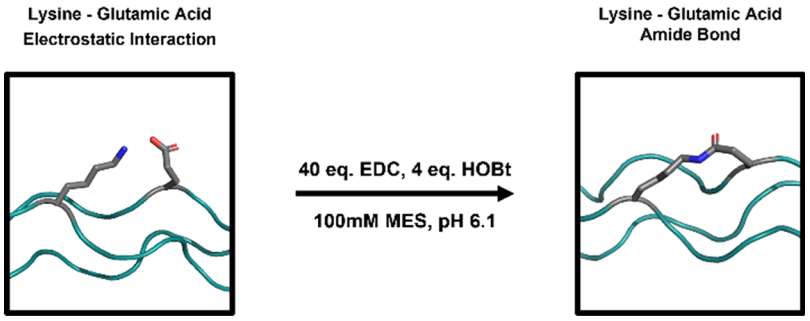

Figure 1. Covalent capture of lysine−glutamic acid interactions within the triple helix.

collagen peptides with a GFOGER mimic using an on-resin synthetic strategy. Regioselective cysteine chemistry in tandem with a 9-fluorenylmethoxycarbonyl (Fmoc)-based synthesis strategy was used to synthesize staggered trimers with disulfide bonds intended to promote fiber formation. These collagen mimetic polymers were able to bind integrins in a fashion similar to natural collagen I; however, the material properties of the fibrous mimic may not be suitable for all applications in biomaterial design.[28] Reyes and García synthesized a CMP with a GFOGER motif and disulfide bonds, which demonstrated cell adhesion comparable to that of natural collagen I at coating concentrations as low as 10 μg/mL.[29] However, cysteine-based cross-linking schemes can leave some cysteine residues as free thiols upon assembly into a triple helix, which can promote peptide oligomerization and other undesirable side reactions.[31]

Using CMPs with integrin-binding sequences, such as GFOGER, for the coating and functionalization of biomaterials, offers a method to accurately mimic the structure and function of natural collagen. The García laboratory has extensively studied the use of their GFOGER CMP to coat implants of materials such as titanium and polycaprolactone through passive adsorption.[32,33] *In vivo*, these materials enhanced osteoblastic differentiation and bone defect repair.[34,35] A number of laboratories have utilized GFOGER mimetic peptides with cysteine residues for incorporation into polyethylene glycol hydrogels via Michael addition chemistry.[16,17,34] These hydrogels were good scaffolds for the survival, proliferation, and differentiation of mesenchymal stem cells.[16,17,36] The Kiick laboratory has developed vesicles by conjugating elastin-like peptides to CMPs with a GFOGER sequence. The vesicles were used to thermally control delivery of fluorescein to collagen-containing matrices.[37,38] CMP amphiphiles, containing the GFOGER motif, have demonstrated the ability to form nanofibers that were able to promote cell adhesion.[39] However, several of these strategies either use monomeric GFOGER peptides and do not guarantee the presence of the triple helical structure in the material which can lead to poor or non-specific integrin binding, or rely on cysteine disulfide bonds, which could be problematic for covalently linking the peptide to a gel material. A highly stable collagen mimic which can present the GFOGER sequence exclusively in its integrin-specific triple helical fold would be a highly desirable improvement for many biomaterial designs. In this study, we aim to develop a covalently stabilized CMP that contains a GFOGER mimic that (1) requires no complex protection strategies on resin, (2) leaves no highly reactive functional groups, (3) is a freely soluble molecular species, and (4) supports specific integrin-mediated cell adhesion at low concentrations.

Utilizing CMPs to mimic the ECM is desirable due to the high degree of control of sequence and thereby structure.[40,41] Strategies for supramolecular assembly of CMPs can include the use of charge pairs, cation-pi, and amide-pi interactions.[42−44] The Hartgerink group and others have previously demonstrated the ability to direct the assembly of CMPs using only supramolecular interactions.[42,45−52] However, supramolecular assembly can be slow, and the thermal stabilities of the CMPs are often lower than what is biologically relevant.

There are a number of possible strategies for the covalent tethering, and therefore stabilization, of collagen peptides, such as disulfide bond formation, the use of organic templates, and oxime ligation.[53−59] As previously mentioned, several of these methods have been utilized to model integrin binding to the ECM, and while they result in a covalently tethered system, the chemistries employed do not promote supramolecular assembly and detract from the flexibility of the material design.[28−30,60] Covalent capture, as a method for peptide stabilization, is useful because it allows supramolecular assembly prior to bond formation, which can be used to set the composition and register of the triple helix.[61,62] Covalent capture of CMPs utilizes supramolecular charge pair interactions first for assembly and then for proximity-induced amide bond formation between the strands of the triple helix.[63] Figure 1 illustrates this reaction between strands of the triple helix. Recently, the Farndale laboratory has used this approach to determine the register of type I collagen and mimic discoidin domain receptor interactions with collagen.[64] The covalent capture reaction is performed in aqueous folding conditions with the water-soluble reagents: 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt). These reagents activate side-chain carboxylic acids to drive amide bond formation. Establishing covalent bonds between the strands of the triple helix can dramatically decrease the folding time and increase the thermal stability.[63] Importantly, covalent capture of lysine (K) and glutamic acid (E) residues does not distort the backbone structure of the triple helix (as verified by X-ray crystallography), and therefore, covalent capture of CMPs should provide an idealized mimic of the triple helical structure of natural collagen.[65]

Peptide Design Strategy. The supramolecular integrin-binding peptide was designed considering three variables: (1) residues used for charge pair interactions, (2) location of the integrin-binding site, and (3) peptide length. In determining the sequence, the goal was to design a peptide that had residues in the correct geometry for facile covalent capture to

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preserve the structure of the triple helix. When determining the peptide length, we considered the benefits additional triplet repeats offer in regards to triple helix stability. We also considered potential future applications of peptides such as these and desired to design the smallest peptide both for synthetic ease and to minimize disruption to properties of the material they would be integrated with. K-E axial interactions were chosen because the isopeptide bonds form more quickly than K−D bonds in covalent capture and generally provide a more stable triple helix after covalent capture.[65] The GFOGER-binding site was situated in the center of the peptide, away from the locations of covalent capture, so that the reaction did not disrupt the binding site as the E residue in GFOGER is essential for binding. Several peptides with an increasing number of POG triplets were synthesized and evaluated since canonical POG triplets contribute to the triple helical structure and help stabilize the folded peptide.

EXPERIMENTAL DETAILS

Peptide Synthesis. PKGEOG$(POG)_2$FOGERG-$(POG)_2$PKGEOG (GFOGERsupra) was synthesized on an Aapptec Focus XC automatic peptide synthesizer. $(POG)_{10}$, GFOGERG, PKGEOGFOGERGPKGEOG (shGFOGER), PKGEOGPOGFO-GERGPOGPKGEOG (medGFOGER), and $(POG)_3$PKGEOG-$(POG)_3$ (KGE) were synthesized manually. All peptides were synthesized using a standard fluorenylmethyloxycarbonyl (Fmoc) protecting strategy on a Rink Amide MBHA low loading (0.36 mmol/gram) resin to yield a peptide with an amidated C-terminus. Deprotection was performed using a 25% (v/v) solution of piperidine in dimethylformamide. Coupling was performed using the desired amino acid, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*] pyridinium 3-oxid hexafluorophosphate (HATU), and *n*,*n*-diisopropylethylamine (DIEA), in a 1 resin: 4 amino acid: 4 HATU: 6 DIEA ratio. The N-terminus was acetylated using acetic anhydride and DIEA in dichloromethane. Peptides were cleaved in excess trifluoroacetic acid (TFA) using ultrapure H_2O, triisopropylsilane, anisole, and ethanedithiol (EDT) as scavengers. The TFA was evaporated under nitrogen, and the crude peptide was triturated using excess ice cold diethyl ether. The crude peptide was centrifuged and washed with ether twice.

Peptide Purification. Crude peptide was dissolved in ultrapure H_2O to achieve a concentration of ∼22 mg/mL and filtered through a 0.2 μm filter. The peptides were purified using reverse phase high-performance liquid chromatography (HPLC) on a Waters Prep 150 LC system with a Waters Atlantis T3 C18 column. The mobile phase was a gradient of ultrapure H_2O and acetonitrile with 0.05% TFA. The detailed methods for HPLC purification are provided in the Supporting Information. Acetonitrile was removed via rotary evaporation before the samples were frozen and then lyophilized. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was used to confirm the mass of the peptide. A Waters ACQUITY ultra performance liquid chromatography (UPLC) system was used to confirm the purity of the peptide. Peptides were stored at −20 °C.

Sample Preparation. 3 mM samples were prepared by dissolving peptide powder in 100 mM 2-(N-Morpholino)ethanesulfonic acid (MES) buffer (pH 6.1) to maintain neutral pH. The sample was heated at 85 °C for 15 min and then cooled to room temperature and stored at 5 °C.

Covalent Capture. GFOGERsupra and KGE were covalently captured to yield GFOGERcc and KGEcc, respectively. 3 mM samples of GFOGERsupra was prepared, as described above. A 3 mM sample of peptide is equivalent to 1 mM triple helix, which for this peptide contains 6 mM isopeptide bonds. A 600 mM solution of EDC and a 36 mM solution of HOBt were prepared in 100 mM MES (pH 6.1). The final reaction contained 1 mM peptide (2 mM isopeptide bond), 8 mM HOBt, and 80 mM EDC in MES. Thus, the final reaction ratio of the isopeptide bond/EDC/HOBt was 1:40:4, respectively. A 3 mM sample of KGE was prepared, as described above. For this peptide, a 3 mM sample is equivalent to 1 mM triple helix, which is equal to 3 mM isopeptide bonds. Therefore, to have a ratio of isopeptide bond to EDC to HOBt of 1:40:4, the final reaction conditions were 1 mM KGE peptide, 40 mM EDC, and 4 mM HOBt. Both reactions were quenched after 4 days using an equivalent volume of 1 M hydroxylamine (NH_2OH). After 24 h, the NH_2OH was neutralized with an equivalent volume of 1 M HCl. Reaction completion was monitored using mass spectrometry.

Purification and Sample Preparation of Covalently Captured Peptide. Neutralized samples were centrifuged using a Pierce protein concentrator with a 3K MWCO to concentrate the covalently captured trimer and remove the small molecules left in the reaction mixture. The covalently captured helix was then purified using HPLC. Acetonitrile was removed using rotary evaporation, and samples were lyophilized and stored at −20 °C.

Circular Dichroism. Samples were prepared, as described previously, and then further diluted to a concentration of 0.3 mM in ultrapure H_2O. A wavelength scan was performed from 180 to 250 nm. Thermal denaturation studies were then performed by monitoring the molar residual ellipticity (MRE) value at the previously determined maximum wavelength as the sample was heated from 5 to 85 °C at a rate of 10 °C per hour. For refolding experiments, the sample equilibrated at 85 °C for 30 min before being cooled from 85 to 5 °C at a rate of 10 °C per hour. All experiments were performed on a JASCO J-810 spectropolarimeter with a Peltier temperature-controlled stage. The derivatives of the melting and refolding curves were generated using the Savitzky−Golay algorithm. The minimum of the derivative provides melting and refolding temperatures.

Mass Spectrometry. Samples were prepared using α-Cyano-4-hydroxycinnamic acid as the matrix and were run on a Bruker Autoflex Speed MALDI ToF. GFOGERcc and KGEcc were also examined using electrospray mass spectrometry on an Agilent 1290 Infinity II system with an Agilent Pursuit 5 diphenyl column due to difficulty with the signal for the high-molecular-weight peptides.

Cell Culture. Human fibrosarcoma cells HT1080 (ATCC, Manassas, VA) were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% fetal bovine serum and 1% penicillin−streptomycin at 37 °C and 5% CO_2. Cells were passaged every 2−3 days using standard techniques.

Cell Adhesion Assay. Nunc MaxiSorp 96-well plates were coated with 100 μL of rat tail collagen I, bovine serum albumin (BSA), or synthetic peptide at the desired concentration in Hank's Balanced Salt Solution (HBSS, Corning) overnight at room temperature. This 1× HBSS contains 0.977 g/L $MgSO_4$ (anhydrous). The well coating was removed and allowed to air dry for 1 h. All wells were blocked with 1% BSA and rinsed twice with HBSS. HT1080 cells were detached from culture plates with 0.25% trypsin-0.5 mM EDTA solution. After neutralization with EMEM, the cells were recovered using centrifugation. The pellet was resuspended in serum-free EMEM to a concentration of 1×10^6 cells/mL. This EMEM formulation contains 0.09767 g/mL $MgSO_4$ (anhydrous). 100 μL of the cell suspension (100,000 cells) was added to each well for 1 h under sterile conditions at room temperature in air. The media was removed, and non-adherent cells were washed away with HBSS. Formalin was added for 10 min to fix the cells. The wells were washed again with HBSS before staining with 4′,6-diamidino-2-phenylindole (DAPI). After 30 min, HBSS was added to each well, and the solution was removed. Finally, HBSS was added to each well before imaging. For each well, three images were captured and evaluated. Images of the cells were taken on an EVOS fluorescence microscope and processed using ImageJ. Cell adhesion assays with GFOGERcc concentrations 50 μg/mL or higher were repeated four times; assays with GFOGERcc concentrations lower than 50 μg/mL were repeated twice. Cell adhesion assays with GFOGERsupra concentrations 100 μg/mL or lower were repeated six times; higher concentrations were repeated 10 times.

Additional cell adhesion assays were performed with the addition of EDTA/$MgCl_2$. Cells were incubated with a treatment of 4 mM

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Table 1. Peptide Sequences and Corresponding Melting Temperatures[a]

peptide	sequence	T_M (°C)
shGFOGER	PKGEOGFOGERGPKGEOG	NT
medGFOGER	PKGEOGPOGFOGERGPOGPKGEOG	15.5
GFOGERsupra	PKGEOGPOGPOGFOGERGPOGPOGPKGEOG	36.5
GFOGERcc	PKGEOGPOGPOGFOGERGPOGPOGPKGEOG	69.0
$(POG)_{10}$	POGPOGPOGPOGPOGPOGPOGPOGPOGPOG	65.0
GFOGERG	GFOGERG	NT
KGEcc	POGPOGPOGPKGEOGPOGPOGPOG	60.5

[a]NT indicates no transition.

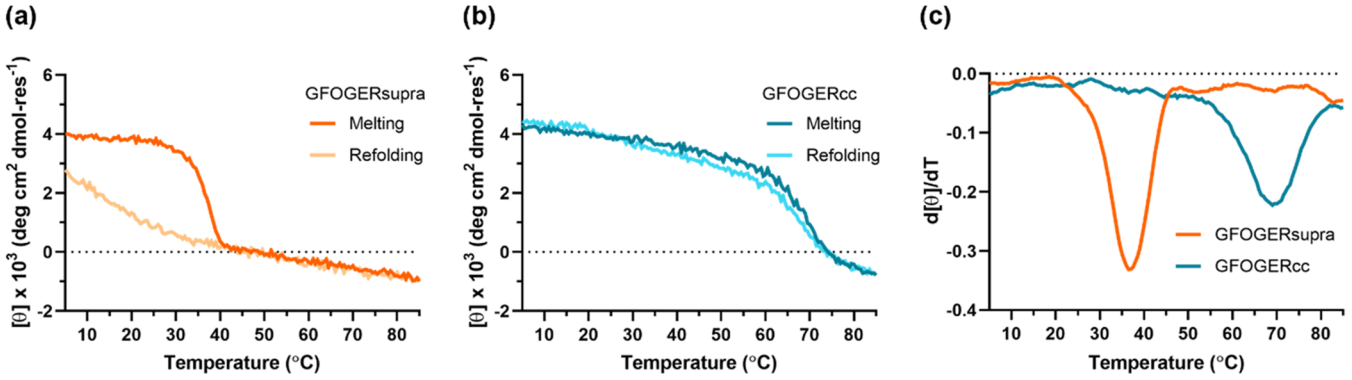

Figure 2. Circular dichroism of GFOGERsupra and GFOGERcc. The signal was monitored at 225 nm for all experiments. (a) Melting and refolding experiments for GFOGERsupra. (b) Melting and refolding experiments for GFOGERcc. (c) Derivatives of the melting curves of GFOGERsupra and GFOGERcc used to determine melting temperatures.

EDTA, 4 mM EDTA + 8 mM $MgCl_2$, or 4 mM $MgCl_2$ for 20 min prior to the addition to the pretreated well plates. The inhibition assay was performed twice.

Antibody Inhibition Assay. Nunc MaxiSorp 96-well plates were coated with collagen I or GFOGERcc. Cells were passaged, as previously described, and incubated with 100 μg/mL anti-integrin $\alpha2\beta1$ antibody (MAB1998Z, Chemicon) for 20 min prior to the addition to the coated well plate. The cell adhesion assay was carried out, as described above. The antibody was purchased from Sigma-Aldrich. The antibody inhibition assay was performed twice.

Statistical Analysis. All statistical analysis was performed using GraphPad Prism software. One-way ANOVA tests with Tukey's multiple comparison test were performed for comparison of the mean of each treatment group. The detailed results of the statistical analysis are available in the Supporting Information.

RESULTS AND DISCUSSION

Peptides with the GFOGER-binding motif were synthesized with varying numbers of POG triplet spacers. The goal was to synthesize a peptide with the smallest number of POG spacers possible. However, in order to drive proximity-induced amide bond formation by covalent capture, it was necessary to have a folded triple helix. The shortest peptide (shGFOGER)- with no POG triplets did not fold into a triple helix and was therefore not suitable for our application (Figure S12). Peptide medGFOGER had one POG triplet on each side of the binding site and did fold into a triple helix. However, the melting temperature of medGFOGER was lower than room temperature, making covalent capture challenging (Figure S11). The final peptide synthesized has two POG triplets on each side of the binding site, totaling 30 amino acids. This peptide (GFOGERsupra) folded into a stable triple helix, which is easy to work with at room temperature and was therefore chosen for further experiments.

Several control peptides were synthesized to evaluate important variables including the presence of the binding sequence, triple helical structure, and the presence of isopeptide bonds. A short seven amino acid peptide with the sequence GFOGERG was synthesized as a control for the presence of the integrin-binding site. $(POG)_{10}$ was synthesized as a control for the presence of the triple helix. KGEcc was used as a control for the presence of isopeptide bonds. All peptides were synthesized with an amidated C-terminus and an acetylated N-terminus to avoid destabilizing charge repulsion. After purification with HPLC, the synthesis and purity of each peptide were confirmed using mass spectrometry and UPLC. These data are available in the Supporting Information.

Covalent capture of GFOGERsupra and KGE were performed using a ratio of 1 equiv isopeptide bond/40 equiv EDC/4 equiv HOBt. Analysis with mass spectrometry showed that the major species was a trimer with six water loss, indicating reaction completion and amide bond formation. The covalently captured trimer was purified using HPLC prior to further analysis. The covalently captured triple helix was freely soluble in aqueous buffers.

Circular dichroism (CD) was used to analyze the structure and the thermal stability of the peptides; Table 1 and Figures S10–S15 display these data. Peptides with polyproline type II (PPII) secondary structure display a characteristic peak at 225 nm in CD. As expected for the control peptides, at 5 °C, $(POG)_{10}$ and KGEcc displayed a peak at 225 nm, indicating the presence of a PPII structure. For control peptide GFOGERG at 5 °C, there was no signal at 225 nm, indicating that the peptide did not fold into a PPII twist, which is expected due to the short length of the peptide. Both GFOGERsupra and GFOGERcc formed stable PPII structures at 5 °C. Thermal denaturation experiments were performed to determine the stability of the peptide structures by monitoring

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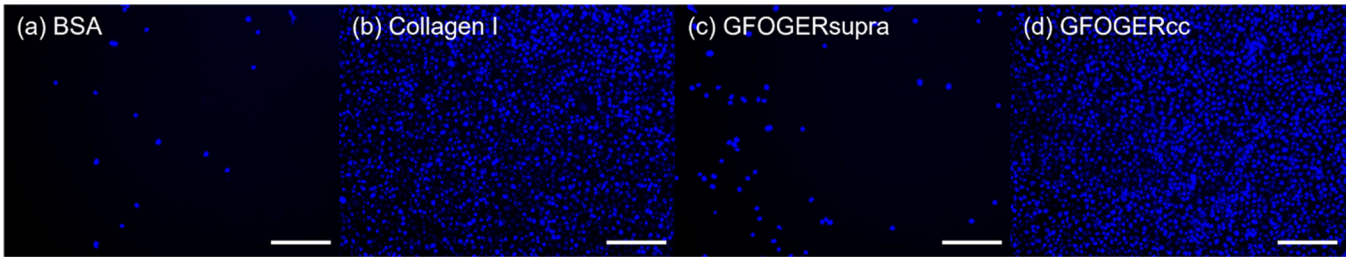

Figure 3. Cell adhesion of HT1080 cells to control proteins and GFOGER peptides. (a) BSA coating concentration is 10 mg/mL. (b) Collagen I, (c) GFOGERsupra, and (d) GFOGERcc coating concentrations are 50 μg/mL. Scale bar represents 100 μm.

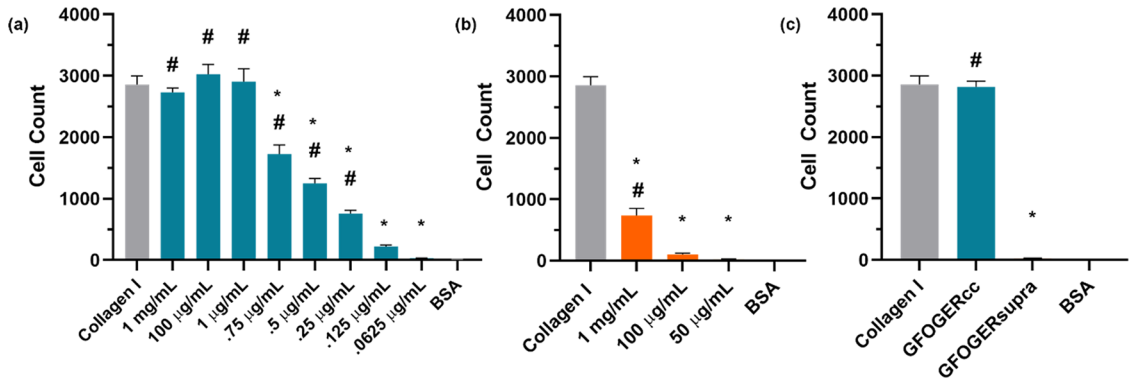

Figure 4. Quantification of cell adhesion. For all experiments, the coating concentration for collagen I is 50 μg/mL and 10 mg/mL for BSA. * Indicates treatment groups statistically significant from collagen I, # indicates treatment groups statistically significant from BSA ($p < 0.05$). (a) Cell adhesion to GFOGERcc. (b) Cell adhesion to GFOGERsupra. (c) Cell adhesion to GFOGERcc and GFOGERsupra at a coating concentration of 50 μg/mL. Data are shown as mean with SEM.

the MRE value at 225 nm as the temperature increased from 5 to 85 °C. Figure 2 illustrates the notable differences in the melting temperatures and refolding abilities between the supramolecular and covalently captured peptides. GFOGERsupra displayed a cooperative transition showing the unfolding of the triple helix with a melting temperature of 36.5 °C. GFOGERcc had a much higher melting temperature: 69.0 °C. Covalent capture resulted in an over 30 °C increase in the melting temperature of the peptide. This demonstrates the utility of the covalent capture to raise the melting temperature of the triple helix, ensuring the presence of the tertiary structure at higher temperatures such as normal body temperature (37 °C) or higher temperatures that may be required during formulation of a biomaterial. This is especially useful because the thermal stability of natural human collagen I is near body temperature.[66] Next, the folding properties of the supramolecular and covalent assemblies were evaluated by cooling the peptides from 85 to 5 °C after thermal denaturation. The refolding curve for GFOGERcc is nearly superimposable with its unfolding curve. This lack of hysteresis suggests immediate refolding. In contrast, while GFOGERsupra did begin to refold, it was not fully refolded even after the full 8 h experiment. This slow folding rate is normal for CMPs and has been a major limitation of their application in many scenarios. Due to the covalent bonds between the individual strands of the triple helix, GFOGERcc has a dramatically higher thermal stability and folds more quickly than GFOGERsupra.

Cell adhesion assays were performed to test the ability of GFOGERsupra and GFOGERcc to promote $\alpha2\beta1$-mediated cell adhesion. HT1080 cells were chosen for the analysis of binding because $\alpha2\beta1$ is the only collagen-binding integrin present.[67,68] The desired material was used to coat the wells of a 96-well plate overnight, and after the wells were blocked to prevent non-specific binding, a cell suspension was added for 1 h. After the adhesion time, the cell nuclei were stained with DAPI and counted. See Figures 3 and 4 for cell images and quantification of adhesion.

Collagen I was chosen as a positive control as it is known to possess the GFOGER motif and bind the $\alpha2\beta1$ integrin.[12,14,15] BSA was used as a negative control to block the surface of the well. As expected, collagen I facilitated the adhesion of the HT1080 cells, and the well was highly coated with cells. BSA did not promote the cell adhesion, and very few cells adhered to the surface of the well. Next, the peptide controls were tested for cell adhesion properties. $(POG)_{10}$, which does not possess the bioactive motif but does fold into a triple helix, did not promote cell adhesion, indicating that the presence of the triple helix alone is not enough to drive $\alpha2\beta1$-mediated adhesion. KGEcc, which does not include the bioactive GFOGER sequence but folds into a triple helix stabilized with isopeptide bonds, also did not promote cell adhesion. Therefore, the isopeptide bonds formed through the covalent capture reaction are not involved in the cell adhesion mechanism. The cells also did not adhere to the final control peptide, GFOGERG, which did not fold into a triple helix. The lack of adhesion to this short peptide further supports the literature, which states that the triple helix of collagen is required for the GFOGER/$\alpha2\beta1$ interaction.[26] Images of cell adhesion (Figures S16–S21) and quantification of cell count (Figure S23) for all control peptides are available in the Supporting Information.

In contrast to these negative controls, HT1080 cells adhered to GFOGERcc-coated wells in high number. There was no

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significant difference between adhesion to GFOGERcc and the positive control collagen I (p = 0.98). A range of concentrations for coating solutions were used to evaluate the dose−response relationship of the cell adhesion to GFOGERcc. Adhesion remains excellent down to a concentration of 1 μg/mL (p < 0.05 for statistical comparison between collagen I and GFOGERcc treatments between 1 mg/mL and 1 μg/mL). Below this, cell counts begin to drop and when a coating concentration of 0.125 μg/mL is used, the cell binding is equivalent to the negative control BSA (p = 0.99), indicating that there is not enough peptide available on the surface of the well to promote the cell adhesion. Surprisingly, GFOGERsupra did not elicit significant cell attachment. Although CD experiments demonstrate that GFOGERsupra folds into a good triple helix, it is likely that at the low concentrations of the GFOGERsupra used for coating the plate for the cell adhesion assays, the folding equilibrium favors the monomer rather than the trimer. Alternatively, it may be that the peptide adsorption to cell culture plastic interferes with the rather fragile triple helical fold. In any case, this underlines the need for CMPs that are more robust than supramolecular assemblies.

After confirming that HT1080 cells bind to GFOGERcc, several experiments were performed to confirm the mechanism and specificity of binding. The GFOGER-binding site of the $\alpha2\beta1$ integrin is located in the α(I) domain, and the binding is known to be metal ion dependent. The cell adhesion assays using standard conditions relied on the Mg^{2+} present in the buffer and culture media used for binding. Removing metal ions from the cell suspension using EDTA should abolish all $\alpha2\beta1$-mediated cell adhesion. Cells were incubated with either 4 mM EDTA, 4 mM $MgCl_2$, or 4 mM EDTA and 8 mM $MgCl_2$ prior to the addition to the coated wells. Treatment with only 4 mM $MgCl_2$ serves as a positive control for the metal ion-dependent cell adhesion. While adding excess salt slightly increases the adhesion, this is not significant (p > 0.05 for comparison to standard conditions). Incubating the cells with EDTA abolished the cell adhesion to both collagen I and GFOGERcc. Addition of EDTA with excess $MgCl_2$ to the adhesion media restored the cell adhesion to collagen I and GFOGERcc. This suggests that the removal of the metal ions by EDTA disrupted the metal ion-dependent binding site in the $\alpha2\beta1$ integrin. Binding to the peptide or protein presenting GFOGER was blocked, which demonstrates the expected Mg^{2+} dependency for cell adhesion (Figure 5).

Antibody inhibition assays provided further evidence for the mechanism of binding to GFOGERcc. Briefly, cells were incubated with an anti-integrin $\alpha2\beta1$ antibody for 20 min prior to adding to the coated well. Blocking the $\alpha2\beta1$ integrin prevented the cells from adhering to both collagen I and GFOGERcc. The combination of Mg^{2+} dependence and anti-$\alpha2\beta1$ blocking demonstrates that the covalently captured GFOGER peptide successfully mimics the binding mechanism of natural collagen for GFOGER.

CONCLUSIONS

Herein, we report a method for the synthesis of a stabilized CMP with an integrin-binding motif that effectively mimics cell adhesion. Charge pairs were incorporated into a CMP, which did not interfere with helix assembly but can subsequently be covalently captured through the formation of K−E isopeptide bonds. The GFOGER integrin-binding sequence was placed in the middle of the peptide, with an optimized number of flanking POG triplets, to promote the triple helical structure. The supramolecular peptide did not promote cell adhesion, despite having a GFOGER sequence. The supramolecular CMP was covalently captured, resulting in a dramatic increase in thermal stability and faster rate of refolding. Unlike the supramolecular CMP, the covalently captured peptide was able to strongly promote the cell adhesion equal to natural collagen I. Additionally, these peptides maintained good solubility in aqueous buffers and have no residual functional groups of high reactivity. Control experiments demonstrated that both the presence of GFOGER and the stability of the covalently captured triple helical template were needed for effective cell adhesion. Incubation with EDTA or anti-$\alpha2\beta1$ integrin antibody abolished cell adhesion, demonstrating that the binding of the HT1080 cells, for which the $\alpha2\beta1$ is the only collagen-binding integrin, to GFOGERcc is facilitated through the targeted receptor. Our approach in the design and stabilization of GFOGERcc serves as a template to model a wide range of collagen−cell interactions, including those which may require heterotrimeric sequences. Since the triple helix of the covalently captured bioactive peptides is locked into place, this method could be used for the functionalization of biomaterials for receptor binding or as a peptide-based drug.

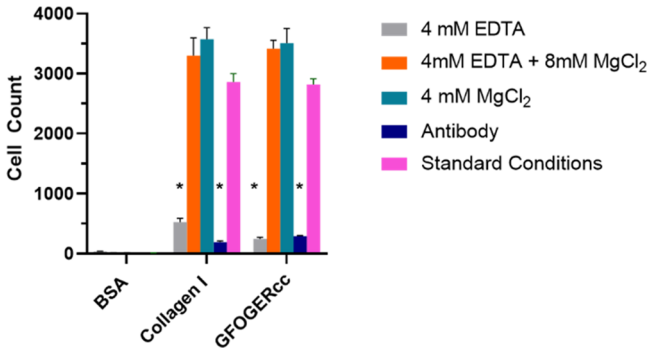

Figure 5. Specificity of HT1080 cell adhesion to GFOGERcc. Cell adhesion after incubation with EDTA and/or $MgCl_2$ or anti-$\alpha2\beta1$ antibody. Wells were coated with BSA at 10 mg/mL, collagen I at 50 μg/mL, and GFOGERcc at 50 μg/mL. Data are shown as mean with SEM. * Indicates treatment groups statistically significant from their respective standard conditions (p < 0.05).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.2c00155.

Full statistical analysis of cell adhesion assays; detailed characterization of peptides, including crude HPLC methods, mass spectra, and UPLC traces of pure peptides; CD of peptides; and additional cell adhesion images (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CMP, collagen mimetic peptide; CD, circular dichroism; NT, no transition

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