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(54) Title: SELF-HEALING ANTI-MICROBIAL PEPTIDE HYDROGELATORS

(57) Abstract: A hydrogelling peptide having the sequence X1-WTW–Link–WTW-X2 (eg. SWTW–Link– WTWK.) X1 and X2 are independently selected from any amino acid, for example Leucine (L), Isoleucine (I), Valine (V), Alanine (A), Methionine (M), Phenylalanine (F), Tryptophan (W), Proline (P), Glycine (G), Serine (S), Asparagine (N), Glutamine (Q), Threonine (T), Cysteine (C), Tyrosine (Y), Aspartic acid (D), Glutamic acid (E), Lysine (K), Arginine (R) or Histidine (H), where Link is a peptide motif selected to provide the hydrogelling peptide with hydrogelling properties. Link can be any number (e.g. 4-8) amino acid residues, for example, Link is a four amino acid residue of the following formula -aa1-GN-aa2- where aa1 and aa2 are independently selected from E, K, V or Q, (e.g. X1-WTWQGNVWTW-X2). Also the resultant hydrogels and methods of controlling mechanical properties (for example stiffness) of hydrogels and controlling basal-out: apical-out generation of organoids.

SELF-HEALING ANTI-MICROBIAL PEPTIDE HYDROGELATORS

Technical Field

The invention relates to peptide hydrogelators which mimic natural extracellular matrices while having other desirable properties such as the uniformity of starting material, ease of synthesis, biodegradability, low cytotoxicity, characteristics of yield-stress fluids, and self-healing behavior.

Background

Natural extracellular matrices are composed of a meshwork of multiple proteins with an interconnected and hierarchical structure that are ideal for guiding tissue assembly. These are predominantly Matrigel or collagen materials. While natural hydrogel materials are the gold standard for 3D cell culture, these materials are derived from animals and are hampered by poor uniformity and batch-to-batch variability. The in vitro use of these matrices also gives rise to concerns of immunogenicity. Thus, the discovery of synthetic alternatives remains a principal goal for cell biologists and tissue engineers. In pursuit of this, hydrogels comprised of self-assembling synthetic peptides have attracted broad interest due to the uniformity of starting material, ease of synthesis, biodegradability, and low cytotoxicity. These synthetic hydrogelators have been designed to form entangled networks of peptide nanofibers that mimic the structural characteristics of native matrices, including mesh size, pore size, and nanofiber architecture.

One approach to forming new peptide hydrogelators is via functionalization of ultra-short hydrophobic peptides (2-5 residues), with large N-terminal capping groups that favor self-assembly through pi-stacking interactions.

Another approach is through peptide amphiphiles, which are another class of gelators typically formed from longer peptide (16 residues) with alternating charged amino acids that self-assemble through electrostatic interactions.

Still further alternative approaches involve rational design of peptide sequences that imitate naturally occurring secondary protein structures, such as the alpha helix or beta sheet. However, despite extensive research into supramolecular peptide assembly, the discovery of

new hydrogelators is most often driven by serendipity or permutation of pre-existing gelator sequences.

There exists a need for new forms of peptide hydrogelator which can conveniently mimic native extracellular matrices.

It is an object of the present invention to overcome or ameliorate at least one of the disadvantages of the prior art, or to provide a useful alternative.

Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

Summary of the Invention

According to a first aspect, the invention provides a peptide having a sequence SWTWQGNVWTWK.

In another aspect, the invention also provides peptides which have a sequence selected from the group consisting of:
Sub1-SWTWQGNVWTWK-Sub2,
Sub1-SWTWQGNVWTWK and
SWTWQGNVWTWK-Sub2
wherein Sub1 and Sub2 are independently variable peptide substituents.

The peptide may, for example comprise the laminin motif at a terminal position-Ile-Lys-Val-Ala-Val (IKVAV), i.e., SWTWQGNVWTWKIKVAV Other adhesion sequences could be added at the terminal positions, for example other laminin derived sequences (YIGSR), adhesive fibronectin (RGD) type peptides such as GRGDSC, and other adhesive peptides of the GFOGER type.

The terminal modifications need not be restricted to amino acid sequences, but can include other types of species such as small molecules; proteins; enzymes; carbohydrates (for example polysaccharides); nucleic acids; synthetic polymers; natural polymers; metal nanoparticles; metal oxide nanoparticles; lipid nanoparticles; quantum dots; and combinations thereof.

The invention also provides a hydrogel comprising a peptide according to any one of the preceding aspects and water. The peptide may be present in an amount of at least 0.1% w/v, or it may be present in an amount of at least 3% w/v. Typically, the concentration range for cell culture applications is around 0.5% w/v. In some embodiments, the peptide is present in an amount up to 5.0% or even up to 10.0% by weight. The balance is generally water but other agents (for example pH adjusters, tonicity adjusters, colourants, bioactive agents) may all be added depending on the intended purpose of the hydrogel. The hydrogel may contain other components depending upon the intended purpose, for example, it may contain one or more of small molecules, proteins, enzymes, carbohydrates (such as polysaccharides), hydrogel forming polymers (such as poly(ethylene glycol) for example.

The hydrogel may comprise a peptide having the sequence SWTWQGNVWTWK and a peptide having the sequence SWTWQGNVWTWKIKVAV. These may be present in any desired total amount with respect to the water and they may be present in any desired ratio. For example, the ratio of SWTWQGNVWTWK: SWTWQGNVWTWKIKVAV maybe from 1:10 to 10:1 and the total amount of peptides in the hydrogel maybe at least 0.1% w/v, or at least 3% w/v from 0.1% w/v – 1% w/v, or 1% w/v to 3% w/v for example.

In some embodiments, the hydrogel of the present invention may be antibacterial, for example, with antibacterial activity against gram positive and gram negative strains. For example, the hydrogel exhibits at least about a 4 log reduction in *S. aureus* and a 3 log reduction in *E. coli*, that is at least a 99.99% reduction in growth for *S. aureus* and 99.75% reduction in growth for *E. coli*.

In some embodiments, the hydrogel of the present invention may be self-healing, that is the material can reform a gel after being damaged. For example, at ambient temperature and pressure, after exposure to 5% strain for 5 minutes, the hydrogel (1% w/v peptide) reforms immediately upon cessation of applied strain and returns to its initial stiffness within one hour.

In some embodiments, the hydrogel of the present invention may have low yield point, which for the hydrogel (1% w/v peptide) occurs at a strain of 0.3%. The yield point of the gel may be varied using standard techniques to be lower (0.1% yield point) or much higher (100% yield point.

In some embodiments, the hydrogel of the present invention exhibits stress relaxation which mimics the stress relaxation of natural matrices, for example, a hydrogel of the present

invention comprising 1% w/v peptide had a stress relaxation half-time of between 40-90 s, for example 50-75s. Matrices that are not stress-relaxing do not possess a stress-relaxation half-time.

In another aspect, the invention provides a method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising adjusting the concentration of a peptide of sequence SWTWQGNVWTWK.

In another aspect, the invention provides a method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising adjusting the concentration of a peptide of sequence SWTWQGNVWTWK and a peptide of sequence SWTWQGNVWTWKIKVAV.

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In another aspect, the invention provides a method of controlling the mechanical properties (for example stiffness) of a hydrogel of the present invention by altering the nature and concentration of counterions (e.g., trifluoroacetate, acetate, chloro etc.) present in the gel.

In yet another aspect, the invention provides a method of controlling the mechanical properties of hydrogel networks by adding the hydrogelling peptides of the present invention (such as SWTWQGNVWTWK) to polymer forming natural hydrogels like alginate, gellan gum, fucoidan, hyaluronic acid, and silk for example

Also broadly contemplated are hydrogelling peptides having the sequence SWTW–Link–WTWK, where link may be for example a peptide motif selected to provide the hydrogelling peptide with hydrogelling properties. The peptide motif may have any number of amino acid residues, for example, 4, 5, 6, 7, 8 or more amino acid residues.

In some embodiments, Link is a four amino acid residue of the following formula -aa1-GN-aa2- where aa1 and aa2 are independently selected from E, K, V or Q.

Similarly, also broadly contemplated are hydrogelling peptides of the sequence Sub1-SWTW-Link- WTWK-Sub2, where Link Sub1 and Sub2 are as disclosed above, with the proviso that Link is not a 4 amino acid residue comprising EGNK, ENGK or EpNK.

Also contemplated are peptides which have modifications at the first and last positions, that is peptides of the structure X_1-WTWQGNVWTW-X_2, where X1 and X_2 may be independently selected from any amino acid, for example Leucine (L), Isoleucine (I), Valine (V), Alanine (A), Methionine (M), Phenylalanine (F), Tryptophan (W), Proline (P), Glycine (G), Serine (S), Asparagine (N), Glutamine (Q), Threonine (T), Cysteine (C), Tyrosine (Y), Aspartic acid (D), Glutamic acid (E), Lysine (K)m Arginine (R) or Histidine (H).

Another aspect involves appending a peptide motif corresponding to the hydrogelling peptides of the present invention (such as those having sequence SWTWQGNVWTWK) to hydrogel forming polymers, like poly(ethylene glycol) and hyaluronic acid, to control the polymer hydrogel mechanical properties and cell bioactivity. The peptide motif can be added as a complete unit (e.g. by covalently bonding a hydrogelling peptide of the present invention to a conventional hydrogel forming polymer) or can be built upon the hydrogel forming polymer (e.g. by addition of single amino acids to a conventional hydrogel forming polymer to form species which comprise both a hydrogelling peptide of the present invention and a conventional hydrogel forming polymer) .

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

Description of the Drawings

Figure 1 shows coarse-grain molecular dynamics simulations to identify Trpzip sequences prone to nanofibril aggregation. (A) Cartoon representation of the tryptophan zipper folded in a beta hairpin conformation, with interlocking tryptophan residues depicted. (B) Schematic demonstrating the role of lysine as an aggregation gatekeeper in original Trpzip1 and Trpzip2, and the effect of successful aggregation gatekeeping on the nanofiber-forming ability of the peptides. (C) Results of 100 μs coarse-grain simulations run for Trpzip variants with serine, proline, alanine, or valine in the place of the eighth lysine residue present in the original Trpzip1

sequence. (D) Comparison of 100 μs coarse grain simulations run for the original Trpzip1 and Trpzip-V variant. Center boxes show the largest cluster of peptides extracted from each simulation and aligned on the same axis for visual comparison of size, length, and fibril morphology of peptide aggregates.

Figure 2 shows synthesis, optimization, and characterization of Trpzip hydrogels. (A) Chemical structures of Trpzip1 and Trpzip1-QV, indicating changes in amino acid sequence. The photograph depicts Trpzip1 and Trpzip1-QV peptides dissolved in pure water at 1 mg/mL after 24 h at 37ºC. (B) Circular dichroism spectra of Trpzip1 (dashed line) and Trpzip-QV (solid line) across a temperature range of 0-60 ºC. (C) Transmission electron micrographs of Trpzip-QV nanofibers. Scale is 200 nm (left) and 50 nm (right). (D) Cryo-TEM of Trpzip-QV hydrogels (2% w/v in DMEM, pH 7) at 30 s and 24 h post gelation at 37ºC. Scale is 50 nm (left) and 100 nm (right). (E) Small angle neutron scattering profiles of Trpzip-QV hydrogels in deuterated DMEM at 1% w/v (far left), 3% w/v (center left), separate data fitting to the lamellae model fit and the power law fit (center right) and the combined data fitting against experimental scattering of 1% w/v Trpzip-QV hydrogels (far right). (F) Scanning electron micrographs of Trpzip-QV hydrogels (3% w/v, DMEM) at pH 14 and pH 7. Scale bars are 100 μm, 5 μm, 200 μm and 10 μm, from left to right. (G) Schematic of proposed self-assembly mechanism of Trpzip-QV peptide monomers into hydrogels. -V variant. Center boxes show the largest cluster of peptides extracted from each simulation and aligned on the same axis for visual comparison of size, length, and fibril morphology of peptide aggregates.

Figure 3 shows mechanical characterization of Trpzip hydrogels. (A) Oscillatory time sweeps of Trpzip-QV hydrogels of varied concentration represented in percent weight per volume (% w/v). (B) Oscillatory time weeps of Trpzip-QV hydrogels (1% w/v, DMEM, pH 7) at 20 and 37ºC (light and dark blue, respectively). (C) A strain sweep of a Trpzip-QV hydrogel (1% w/v, DMEM, pH 7). Dotted pink line indicates yield-point. (D) A strain rate sweep of Trpzip-QV hydrogels (1% w/v, DMEM, pH 7). (E) Thixotropic test on Trpzip-QV hydrogels (3% w/v, DMEM, pH 7) involving exposure to 5% shear strain for 5 minutes, followed by reduction of shear strain to 1% for 2 h, repeated three times in succession. (F) Stress-relaxation profiles of Trpzip-QV hydrogels (1% w/v, DMEM, pH 7), Matrigel and polyethylene-glycol dimethacrylate (PEG-DM) hydrogels. Data are shown as mean±s.d. (shaded area) from $n = 3$ independently prepared gels. (G) Relaxation half-time ($\tau_{1/2}$) of Trpzip-QV hydrogels (1% w/v, DMEM, pH 7) and Matrigel, ($n = 3$; P = 0.0014). (H) Effect of IKVAV peptide (10% w/w of total peptide concentration) on bulk Trpzip-QV hydrogel stiffness ($n = 3$; P = 0.0004).

Figure 4 shows Trpzip-QV hydrogels support cell growth, syringe extrusion, biofabrication and show antimicrobial properties. (A) Confocal microscopy image of human fibroblast cells stained with Calcein AM and ethidium homodimer in Trpzip-QV hydrogels (1% w/v, DMEM,

pH 7) after 5 days in culture. Scale is 100 µm. (B) Quantified percentage of cell viability of fibroblasts cultured in Trpzip-QV hydrogels compared to culture on glass. (C) Confocal microscopy image of fibroblasts cultured in Fmoc-GFF hydrogels (1% w/v) and Trpzip-QV hydrogels (1% w/v) after seven days, without the use of RGD binding ligands. The scale bar is 50 µm. (D) Quantification of cell area (left) and cell aspect ratio (right) for fibroblasts cultured in Fmoc-GFF hydrogels and Trpzip-QV hydrogels for seven days. Error bars show mean±s.d. (E) Confocal microscopy image of HFFs cultured in Matrigel and Trpzip-QV gels (no RGD) for 7 days. The scale bar is 100 µm. (F) Deposition of endogenous collagen I by fibroblast cells after fourteen days in culture in Trpzip-QV hydrogels without RGD ligands. The scale bar is 100 µm. (G) Viability of cells encapsulated in Trpzip-QV hydrogels and then exposed to shear compared to cells experiencing no shear seeded on glass (2D) or within Trpzip-QV gels (3D). Error bars show mean±s.d. Scale is 100 µm. (H) Bioprinted constructs of cells in a Trpzip-QV hydrogel support bath, either as droplets (left panel) or lines (right panel) Scale is 500 µm in optical images and 200 µm in immunofluorescence images. The measured average diameter of printed cellular droplets (n = 8) or lines (n = 4). Error bars show mean±s.d. (I) Antibacterial activity of Trpzip-QV hydrogels against both a gram positive (*S. aureus*) and a gram negative (*E. coli*) strain, assessed using a bacterial growth inhibition assay. Photographs depict agar plates used to count colony forming units (CFUs) per mL of each bacterial strain after incubation with Trpzip-QV gels for 24 h at 37ºC (n = 3).

Figure 5 shows the development of adult-stem cell derived intestinal organoids in Trpzip-QV hydrogels compared to Matrigel. (A) Schematic describing experimental design to evaluate organoid development in Trpzip-QV hydrogels compared to in Matrigel. (B) Representative images of organoid morphology after 7 days in Matrigel and Trpzip-QV gels across three participant organoid lines. The scale is 100 µm (top panel); 50 µm (bottom panel). (C) Circularity of organoids following 7 days of culture in either Matrigel or Trpzip-QV gels across three participant organoid lines. (D) Filamentous actin stain to highlight microvilli localization on the exterior of organoids grown in Trpzip-QV gels (scale is 100 µm). Inset shows microvilli from cross-section view of organoid (scale is 50 µm). (E) Forskolin-induced swelling (FIS) assay in organoids cultured in Matrigel, Trpzip-QV gels and in suspension. (F) Principal component analysis (PCA) of protein expression profiles of organoids cultured in Matrigel and Trpzip-QV gels. (**G**) Volcano plot indicating significantly differentially expressed proteins. Dashed horizontal line shows p-value cut-off and vertical lines indicate up/down-regulated proteins (**H**) Ingenuity pathway analysis of top enriched canonical signalling pathways of organoids grown in Trpzip-QV gels vs Matrigel. (**I**) Representative images of organoids grown in either Trpzip-QV gels or Matrigel marked for differentiated cell types. Paneth cells, goblet cells and enteroendocrine cells are marked by LYZ, MUC2 and CHGA, respectively. Scale is

100 µm. (**J**) Frequency of mature cell type differentiation in Trpzip-QV gels (blue) and Matrigel (red). Data points represent the percentage of organoids expressing each marker as assessed by antibody staining across three participant organoid lines with $n \geq 10$ organoids per marker. Error bars show mean±s.d.

Figure 6 shows a simple inversion test to determine if a hydrogel is formed. In this case, a hydrogel formed from SWTWQGNVWTWV with a colouring agent to highlight the visual appearance of the hydrogel which is formed.

Figure 7 shows the characterisation of human small intestinal organoid polarity quantified for percentage of basal-out or apical-out in commercially available basement membrane matrix Matrigel, Trpzip-QV gel (0.5 wt%), and Trpzip-QV gel (0.5 wt%) supplemented with 50% laminin protein.

Figure 8 shows a scheme for mixing SWTWQGNVWTWV with photopolymerizable hydrogels like poly(ethylene glycol) dimethacrylate to create a hybrid material with different mechanical properties than either hydrogel forming species alone.

Figure 9 shows a scheme for mixing SWTWQGNVWTWV with anionic polymers, like alginate, gellan gum, silk, or hyaluronic acid, where electrostatics enhances gelation to create a hybrid material with different mechanical properties than either hydrogel forming species alone.

Figure 10 shows a scheme for covalently attaching SWTWQGNVWTWVC to polymer macromers to create hydrogels with polymer and nanofibre content.

Description

The present inventors have found that by exploiting the relatively unexplored tryptophan zipper ('Trpzip') motif, a new and useful hydrogelator can be prepared. The Trpzip motif has been described by Cochran et al (Cochran, A. G., Skelton, N. J. & Starovasnik, M. A. Tryptophan zippers: Stable, monomeric β-hairpins. *Proc Natl Acad Sci U S A* **98**, 5578–5583 (2001) and is characterized by four cross-strand tryptophan residues that interlock via the indole rings, folding the peptide into a beta hairpin conformation. As a result of this highly stabilizing 'zipper' effect, beta hairpins can be formed from Trpzip peptides that are as short as twelve amino acids. Trpzip sequences have been shown to assemble into nanofibers over the course of several weeks, however, the Trpzip peptide motif has not yet been used to form hydrogels.

The Trpzip peptides of the present invention undergo hierarchical assembly within minutes, first forming long nanofibers, followed by assembly into microscale domains with periodic

architecture. The resulting hydrogel showed thermoresponsive gelation with tunable modulus, self-healing, and stress-relaxing characteristics, cell viability and spreading even without cell adhesion motifs, and antimicrobial activity. Adding a pendant cell adhesion motif enabled culture of adult stem cell and induced pluripotent stem cell derived intestinal organoids. The low yield-strain of the material facilitates rapid fluidization under shear, providing a simple mechanism to retrieve embedded cells and tissue, and to disperse cell-laden Trpzip gels via syringe towards cell delivery and bioprinting applications.

There are many forms of hydrogelators in the prior art. However, the present invention represents the first instance of the specific peptide sequence as a hydrogelator.

Similar sequences are included in an earlier patent describing use as a model for studying folding in US20030175799A1 "Hairpin peptides with a novel structural motif and methods relating thereto". However, these differ from the present invention in terms of the motif between the terminal Trpzip moieties. The US 20030175799A1 provides no suggestion that the compounds disclosed therein are useful hydrogelling agents or have any inherent useful hydrogelation properties.

The simple peptide (SWTWQGNVWTWK) of the present invention can be dissolved in water and will spontaneously assemble into a hierarchically structured nanofibre network. The resultant material is antimicrobial and will kill bacteria (due to the tryptophan content) making it amenable as an antimicrobial coating or bandage. Dispersing live cells into the gel maintains viability for prolonged culture even without a specific adhesion sequence.

Importantly, the simple peptide (SWTWQGNVWTWK) of the present invention forms a hydrogel whereas the original peptides of Cochran (SWTWEGNKWTWK) did not: See for example Figure 2 – the original peptides of Cochran in solution remained fluid and flowed to the bottom of a container on inversion. In contrast, the peptides of the present invention formed a gel of sufficient rigidity that on inversion of a container, the resultant hydrogel remained at the upper end of the inverted container. This demonstrates that the hydrogelling capabilities of the present invention arise not solely from the Trpzip moieties, but as a consequence also of the structure between the Trpzip moieties. Before the present invention, it was not recognized that modification of this central region could result in molecules with hydrogelling properties.

However, adding distal short peptide sequences (for example, up to 10 or less peptides such does not compromise gel forming ability making the material very flexible to add complementary functionality.

Suspension of organoids into the gel allows long term growth, with unique advantages in terms of differentiation. This suggests scope as a better alternative to the widely used Matrigel material for organoids.

The mechanical characteristics of the gel allow dissolution with brief agitation. In this way a complex cell culture can be harvested from the material easily rather than requiring enzymatic digestions as currently needed with other materials. The yielding characteristics and self-healing provide scope for the material to be used as an injectable for delivery of sensitive biological macromolecules or live cells.

The peptide system of the present invention has several unique, favourable and unexpected attributes. These include low yield stress for simple and fast harvest of complex cell cultures, self-healing capabilities for cyclic use, cytoprotection for cell injection and biofabrication, and antimicrobial properties.

This peptide shows unique properties with many favorable attributes which has potential for the following markets:

- Cell culture reagent for organoid culture (Matrigel and/or collagen replacement)
- Reagent for cell stabilisation for syringe delivery, e.g., CAR T-Cell, MSC, pancreatic islets, miscellaneous stem cell treatments
- Void filler biomaterial for short-term orthopedic, dental, etc.
- Antibacterial hydrogel for broad medical and biotechnology use
- Shear thinning fluid for extrusion and 3D printing
- Yield-stress support matrix for 3D bioprinting

The peptides of the present invention may be prepared readily by any known method, including the most common method of solid phase peptide synthesis (where the starting amino acid is tethered to a support and each desired amino acid is added sequentially via a series of reactions forming amide bonds). Peptides can readily be produced by commercial suppliers having amino acid sequences made to order.

The initial steps towards the present invention were commenced using computational screening to identify self-assembling tryptophan zipper variants.

As mentioned above, the Trpzip peptide motif has enabled synthesis of the shortest reported beta hairpins to date (Fig. 1A). Originally designed for studying the thermodynamics of protein folding, minor changes in Trpzip peptide sequences have been shown to drastically alter aggregation and nanofiber formation (Fig. 1A). To test the hypothesis that the positively charged lysine residue in the eighth position may acted as an 'aggregation gatekeeper' by

preventing peptide monomer association through repulsive forces the present inventors ran coarse-grain MD simulations of Trpzip variants with small uncharged amino acids in place of lysine (Fig. 1B).

One hundred microsecond simulations were run for Trpzip1 (SWTWEGNKWTWK) peptide variants (Fig. 1B) and the moments of inertia for the largest cluster of peptides in the last frame of the simulations were calculated and compared (Fig. 1C). Comparing all variants, it was found that the valine substitution (SWTWEGNVWTWK) herein named Trpzip-V, showed the largest difference between aggregation modes compared to the original sequence (Fig. 1D). Both moments of inertia calculations and backmapping to all-atom resolution data indicated Trpzip1 formed spherical aggregates while Trpzip-V formed fibrillar aggregates.

The inventors then synthesized Trpzip-V (SWTWEGNVWTWK to experimentally assess its potential for self-assembly under physiological conditions. It was observed that Trpzip-V was able to form a gel under both acidic and basic pH conditions (Fig. S2) but precipitated out of solution at neutral pH. The loss in solubility at neutral pH was believed to be due to the peptide's isoelectric point of 6.97.

A further variant was designed with an uncharged glutamine residue to replace the negatively charged glutamic acid (Trpzip-QV SWTWQGNVWTWK; Fig. 2A). This new variant has an overall charge of +1 at pH 7 to enable solubility at neutral pH. After overnight incubation at 37ºC in pure water, Trpzip-QV (0.1% w/v) formed a self-supporting hydrogel while Trpzip1 remained liquid (Fig. 2A). Circular dichroism (CD) spectroscopy confirmed Trpzip-QV still folds into a beta hairpin, evidenced by the positive band at 228 nm, which is indicative of tryptophan's indole rings packing into a hairpin conformation (Fig. 2B). Additionally, Trpzip-QV showed a higher level of aggregation after 24 hours compared to Trpzip1, as revealed by a lower CD signal at 228 nm, which is correlated with the reorganization of the peptide hairpin into fibrillar aggregates.

Transmission electron microscopy (TEM) of the formed aggregates revealed an entangled network of fibrillar nanostructures, approximately 4.12 ± 1.03 nm in diameter (Fig. 2C; Fig. S4). To visualize the nanostructures under hydrated conditions, Trpzip-QV gels were imaged over 24 hours using cryo-TEM, demonstrating gradual assembly and elongation of nanofibers (Fig. 2D; Fig. S6). Additionally, Fourier transform infrared (FTIR) spectra showed prominent peaks in the Amide I (1600–1800 cm^{-1}) and Amide II (1500–1600 cm^{-1}) regions (Fig. S5), suggesting gelation was driven by beta sheet aggregation of the Trpzip-QV monomers.

To further probe the self-assembly pathway of Trpzip-QV hydrogels, small angle neutron scattering (SANS) was performed. The data was fitted initially with the shape-independent power-law model, which revealed the slope in the high Q region was –2 slope (Fig. 2E; far

left). This suggested the formation of lamellae or disc-shaped particles on the order of 1-10 nm. The slope in the low Q region was found to be –3.7, indicating the formation of large mass fractal-like aggregates exceeding 500 nm in size. These large fractal aggregates continue to increase in size over the course of seven days, as indicated by the decrease in slope in the low Q region. This was further confirmed by ultra-small angle neutron scattering (USANS) measurements (Fig. S7). Data fitting to a lamellae model indicates the size of the lamellae particles is approximately 2.2 nm. At higher hydrogel concentrations (3% w/v), a decrease in intensity in the high Q region indicates the assembly of larger fractal aggregates (Fig. 2E; center left). The scattering profile of Trpzip-QV gels fit well with a combination of the lamellae model in the high Q region and the power law fit in the low Q region, as further confirmation of the presence of both nanometer-scale lamellae particles and micrometer-scale fractal aggregates.

Scanning electron microscopy was carried out to explore the microscale assembly. Gelation at basic pH produced a highly homogenous network of lamellae stacks, with fibrillar nanostructures on the order of a few nanometers comprising this meshwork (Fig. 2F; Fig. S8). Upon pH balancing to neutral pH, this highly organized, discrete honeycomb network is disrupted, and the resulting gel network is reformed through entanglement of more irregularly shaped nanofibers.

Based on the above, it appears that Trpzip-QV self-assembles into hydrogels via the following mechanism: Peptide monomers fold into a beta hairpin conformation and assemble into a disc/ellipsoidal-shaped particle, as suggested by SANS. These particles stack via peptide backbone interactions to create nanofibers, which align to create a meshwork of lamellae stacks which give rise to the macro-scale order (Fig. 2G). Thus, the peptides of the present invention form Hydrogel assemblies with multiscale hierarchical order.

In-situ oscillatory parallel plate rheometry was carried out on gels at various peptide concentrations to evaluate the mechanical properties of Trpzip-QV hydrogels. The stiffness of Trpzip-QV hydrogels increased with peptide content, with *G'* values ranging from 1–50 kPa and all gels reaching an equilibrium storage modulus after approximately 12 hours (Fig. 3A). Trpzip-QV gels display temperature-dependent gelation behavior, with a ten-fold higher stiffness at 37ºC compared to 20ºC (Fig. 3B). Surprisingly, the yield point of Trpzip-QV gels occurs at a strain of only 0.3% (Fig. 3C). This is consistent with our observations of rapid fluidization of the gel under moderate force. The Trpzip-QV gel also shows shear-thinning behavior (Fig. 3D), with viscosity decreasing linearly in proportion to shear rate, suggesting it may be an ideal biomaterial for extrusion and biofabrication. A thixotropic test was carried out on Trpzip-QV gels to determine how fast they self-heal and recover after shear. After exposure

to 5% strain for 5 minutes, the hydrogels rapidly re-crosslink, returning to the initial stiffness within an hour (Fig. 3E).

One of the important aspects of natural materials is their ability to show non-linear viscoelastic properties like stress-relaxation, essential in distributing cellular forces generated during tissue expansion and morphogenesis. In order to assess whether Trpzip-QV gels possess a similar stress relaxation profile to natural materials, constant shear deformation measurements (Fig. 3F) were obtained and the stress relaxation half-time of Trpzip-QV gels (1% w/v) was compared to the natural matrix Matrigel and polyethylene glycol dimethacrylate (PEG-DM) hydrogels. Unsurprisingly, PEG-DM exhibited little stress relaxation due to its covalently crosslinked network; however due to their dynamic nature, Trpzip-QV gels show a similar stress-relaxation profile to Matrigel. The stress relaxation half-time of Trpzip-QV gels was found to be lower (58.5 ± 0.4 s) compared to Matrigel (87.1 ± 0.76 s) ($P = 0.0014$) (Fig. 3G), but within range of other natural matrices.

Another important aspect of natural materials is the presence of cell adhesion motifs. A laminin-derived sequence Ile-Lys-Val-Ala-Val (IKVAV) adhesion motif integrated from 100% Trpzip-QV-IKVAV to X mM Trpzip-QV-IKVAV in Trpzip-QV showed no discernible differences in physical properties. This suggests that inclusion of distal molecules or objects does not disrupt the core assembly mechanisms. Adding a peptide with a different salt formulation (Trpzip formulated in TFA salt; Trpzip-QV-IKVAV formulated as formate salt) was introduced into the Trpzip-QV hydrogels. Integrating Trpzip-QV–IKVAV (10% w/w) into a Trpzip-QV gel resulted in ~10-fold decrease in stiffness compared to Trpzip-QV gels with no adhesive ligands (86.7% decrease; $P = 0.0004$) (Fig. 3H), comparable to the stiffness of natural matrices like Matrigel. This suggests that pendant peptides can be used to tune the structural and mechanical properties as desired if formulated with different counterions.

Thus, the above clearly shows that Trpzip-QV hydrogels can have pendant molecules/objects without disrupting the assembly process, with potential for tunable mechanics using molecules of variable electrostatics (e.g., a different counterion in the added material), along with a conserved assembly mechanism, self-healing and stress relaxation.

The ability of Trpzip-QV hydrogels to support mammalian cell growth was investigated. The low yield point of Trpzip-QV gels enables cells to be easily resuspended after adjustment to neutral pH, circumventing the need for large pH switches common to peptide-based hydrogels. Human fetal fibroblasts (HFFs) were encapsulated in Trpzip-QV hydrogels and their viability after five days was observed to be to be comparable to cells grown on tissue culture plastic (Fig. 4A-B). Interestingly, cells exhibited spreading and elongation in Trpzip-QV hydrogels in the absence of exogenous ECM proteins or adhesive peptide motifs. To explore this further,

HFFs were encapsulated in both Trpzip-QV gels and Fmoc-GFF gels, another bioinert peptide hydrogel (Fig. 4C). Cells grown in Trpzip-QV and Fmoc-GFF gels were initially similar in size at day 1 (P = 0.0008), however cells in Trpzip-QV gels doubled in area after 7 days compared to those grown in Fmoc-GFF gels (Fig 4D) (23.7% increase; P = 0.0002). Cellular aspect ratio also increased a greater amount for cells grown in Trpzip-QV and Fmoc-GFF gels by day 7 (Trpzip-QV: 1.9 ± 0.8; Fmoc-GFF: 1.6 ± 0.6; P = <0.0001; Fig. S10). Strikingly, cells in Trpzip-QV gels show similar morphology to cells cultured in Matrigel after 7 days of culture (Fig. 4E; Fig. S11). Through immunofluorescence imaging, the deposition of collagen I throughout Trpzip-QV gels was observed, suggesting endogenous deposition of ECM is occurring (Fig. 4F; Fig. S12). Taken together, these data indicate Trpzip -QV hydrogels foster robust cell attachment, spreading and elongation without the need for cell adhesion cues. Other synthetic peptide-based hydrogels of which the present inventors are aware lack this inherent bio-adhesivity, suggesting Trpzip-QV gels may prove an optimal 3D cell culture material.

Given Trpzip-QV gels display properties of yield-stress fluids – shear-thinning, fluidization under shear, and self-healing – the present inventors assessed its utility as a 3D bioprinting support medium. High-density fibroblast cell inks were printed into various droplet and line constructs within a support bath of Trpzip-QV gel (Fig. 4H; Fig. S13). Dot diameters averaged 650 ± 80 µm (*n*=8), and line diameters averaging 350 ± 50 µm (*n*=4; 14% from theoretical line width). Immunofluorescent imaging of the printed constructs show they consist of tightly packed cells (Fig. 4H), suggesting that the yield point is high enough for use as a bioprinting support medium. The low yield point and self-healing properties also indicate the potential for both injectable cell delivery and extrusion bioprinting of cell-laden inks. During cell injection procedures, mechanical membrane damage results in significant loss of viability at clinically relevant injection rates. Trpzip-QV gels may be able to shield cells from the damaging mechanical forces experienced during flow and so to assess this, HFFs encapsulated in Trpzip-QV gels were extruded through a syringe needle at high shear (Fig. 4G). After 24 hours, the sheared cell viability was comparable to cells grown on glass and non-extruded cells encapsulated in Trpzip-QV gels.

For some applications, stabilizing the Trpzip network to withstand mechanical forces and increase toughness is desirable. To expand the mechanical characteristics, Trpzip-QV with a terminal cysteine was appended to the ends of a maleimide terminated poly(ethylene glycol) (PEG) macromer through Michael-type addition reactions. Addition of the PEG-Trpzip-QV to trpzip solutions increased gelation time, stiffness, yield point and flowpoint (Figure 10). Similarly, trpzip-QV was blended with anionic polysaccharides alginate and gellan gum, and with the anionic protein silk, which also increased gelation time, stiffness and yielding properties (Figure 9). Finally, Trpzip-QV was blended with polyethylene glycol dimethacrylate,

where photopolymerization led to a mixed network that shows mechanical properties of both PEG and trpzip, providing an approach where viscoelastic characteristics may be introduced to predominantly elastic hydrogels (Figure 8).

The amino acid tryptophan has been shown to possess antibacterial activity through its ability to permeabilise bacterial membranes, thereby causing bacterial cell death. It is hypothesized that the high tryptophan content in Trpzip-QV peptides may confer some antibacterial activity. To test the hypothesis that the high tryptophan content in Trpzip-QV peptides may confer some antibacterial activity, sterilised Trpzip-QV hydrogels were challenged with both a Gram-positive and Gram-negative strain of bacteria (*Staphylococcus aureus* and *Escherichia coli*, respectively) and the antimicrobial activity after 24 hours was assessed using a bacterial growth inhibition assay (Fig. 4I; Fig. S14). A marked reduction in bacterial growth was observed for both strains, with Trpzip-QV hydrogels showing particularly high activity against *S. aureus* (99.99% reduction in growth for *S. aureus*, 99.75% reduction in growth for *E. coli*). The dual activity of Trpzip-QV gels against both Gram-positive and Gram-negative strains suggest they are promising therapeutic candidates against polymicrobial infections. While there are examples of antimicrobial hydrogels by virtue of backbone properties, the present invention is the first example of an extrudable self-healing hydrogel with antimicrobial properties. Trpzip-QV hydrogels are antimicrobial and support mammalian cell growth which make them an exciting candidate material for *in vivo* applications.

Organoid culture relies heavily on Matrigel as an exogenous extracellular matrix support material, despite being of murine origin and issues with batch variability. Trpzip-QV gels, having similar porosity and stress-relaxing characteristics to Matrigel, could conceivably serve as a synthetic, minimally supportive matrix alternative for organoid growth, without many of the attendant drawbacks of Matrigel. Human intestinal organoids—derived from adult stem cells and induced pluripotent stem cells (iPSCs)—were selected to test this hypothesis as their morphogenesis and tissue patterning pathway has been extensively studied. Recent work has demonstrated the importance of laminin protein in promoting the growth of organoids in synthetic cultures, and thus a Trpzip-QV variant with the laminin-derived IKVAV peptide at the N-terminus was used for further investigation.

The inventors passaged and seeded intestinal organoids from three participants with cystic fibrosis in Trpzip-QV gels with 10% Trpzip-QV-IKVAV and cultured them for seven days before comparing them alongside organoids grown in Matrigel (Fig. 5A). A striking difference in morphology was observed between organoids grown in Matrigel and Trpzip-QV hydrogels (Fig. 5B). As expected, organoids grown in Matrigel were not cystic but rather compact and with projections and budding, with a central lumen. Despite some evidence of a central lumen in organoids grown in Trpzip-QV hydrogels (Fig. S15B), they displayed a uniform and spherical

morphology. Morphometric analysis revealed an average circularity value of 0.421 in Matrigel-grown organoids and 0.807 in Trpzip-QV -grown organoids, across the three participant lines (Fig. 5C).

In contrast to Matrigel-grown organoids, Trpzip-QV -grown organoids possessed an apical out polarity, as evidenced by a layer of filamentous actin and the apical tight-junction ZO-1 protein on the organoid exterior, along with the visualization of microvilli of brush border cells (Fig. 5D; Fig. S15A). The apical-out polarity of organoids grown in Trpzip-QV gels was confirmed with a forskolin-induced swelling assay on organoids grown in Matrigel and organoids grown in Trpzip-QV gel for 7 days, with apical-out suspension organoids as a control (Fig. 5E). As the organoids were derived from patients with the homozygous DF508-CFTR mutation, organoids with a basal-out polarity are expected to swell upon correction and activation of the CFTR chloride ion channel. Upon treatment, organoids grown in Matrigel swelled and increased in size within one hour as expected (Fig. S16). In contrast, organoids grown either in suspension or within the Trpzip-QV gel showed negligible changes in size, due to their apical-out polarity change. To assess whether polarity reversal of other organoid models occurs in Trpzip-QV gels, iPSC-derived intestinal organoids in Trpzip-QV gels were also cultured for 7 days. Like the adult stem cell derived organoids, immunofluorescence imaging showed a polarity change to apical-out in iPSC-derived organoids (Fig. S17). Thus, it can be seen that Trpzip -QV hydrogels induce polarity reversal in human intestinal organoids.

A global proteomics analysis on the lysate collected from Matrigel and Trpzip-QV hydrogel cultured organoids. It was observed that the Matrigel and Trpzip-QV cultured organoids clustered separately in PCA plots (Fig. 5F). While 70% of expressed proteins were common between organoids grown in Matrigel and Trpzip-QV gels, analysis of the top differentially expressed proteins (Fig. 5G) showed that organoids grown in Trpzip-QV gels had upregulation of laminin subunit proteins (LAMB3 and LAMA3), as well as secreted glycoproteins involved in intestinal homeostasis (MUC13, AOC1), hormones or hormone-interacting proteins (INS, HSD17B2), and numerous proteins involved in intestinal metabolic processes (AKR1C2, NMES1, CYP2S1 and CES2). Interestingly, organoids grown in Trpzip-QV gels also showed lower stress signaling than those grown in Matrigel (Fig. 5H). Additionally, several actin cytoskeleton remodeling pathways were significantly upregulated in Trpzip-QV -grown organoids (Rho family GTPases, actin nucleation, actin-based motility by Rho pathways), along with several hormone regulation pathways (GNRH signaling, oxytocin signaling, cholecystokinin/gastrin-mediated signaling and insulin receptor signaling) (Fig. 5H; Fig. S18). Together, these results suggest that culture in Trpzip-QV hydrogels impact key features of endogenous intestinal morphogenesis.

Immunofluorescence analysis was performed to understand the differentiated state of these intestinal organoids. Organoids grown in both Matrigel and Trpzip-QV gels were examined for the presence of intestinal epithelial patterning and polarization, as well as markers of fully differentiated intestinal cell types including Paneth cells, goblet cells and enteroendocrine cells. Immunofluorescence analysis revealed the expression of the intestinal epithelial marker CDX2 and the tight junction marker ZO-1 in 100 % of organoids, irrespective of participant origin or matrix condition, confirming Trpzip-QV gels support intestinal lineage differentiation and proper epithelial polarization (Fig. 5I). The percentage of organoids expressing the markers MUC2, CHGA and LYZ was quantified to assess abundance of the differentiated goblet cells, enteroendocrine cells and Paneth cells, respectively (Fig. 5J). Matrigel-grown and Trpzip-QV -grown organoids express all markers with a similar frequency, although variability between participant lines was evident. In addition, there was a small increase in enteroendocrine cell ($CHGA^+$) frequency in organoids grown in Trpzip-QV gels. As enteroendocrine cells are specialized cells in the intestine that secrete gastrointestinal hormones, an increase in their frequency in Trpzip-QV hydrogels corresponds to the observation of multiple upregulated hormones signaling pathways in the proteomics analysis.

The surrounding matrix in organoid culture can play an important role in regulating functional activity. Most protocols use Matrigel as the encapsulate; however, the use of alternative biomaterials has been of great interest in recent years. Early iterations of synthetic matrices were often PEG-based, with incorporation of MMP-degradable peptides to enable organoid morphogenesis contingent on matrix degradation but have also included polysaccharide and other full-length protein-based hydrogels. It has recently been shown how a fully synthetic hydrogel with both covalent and physical crosslinks enabled comparable organogenesis to Matrigel by virtue of matrix stress-relaxation. Trpzip-QV gels show strong potential as a fully synthetic organoid matrix, emulating both the stress-relaxing nature and dynamic hierarchical assembly observed in natural materials.

Materials that foster apical-out polarity would benefit a range of studies, since this is the side that natively interacts with the external environment. Most techniques for accessing the apical surface involve invasively penetrating the organoid barrier through either microinjection, shearing or mechanical disruption of organoids, or dissociation of 3D organoids to be reseeded as 2D monolayers on Transwell plates. Co et al. only recently reported the first non-destructive 3D method of generating apical-out organoids through suspension culture. However, this approach precludes studies of the epithelial microenvironment, an aspect that can be tuned using Trpzip-QV hydrogels.

Initial experiments have suggested that the hydrogels of the present invention allow controlled tuning of organoid polarity by the addition of laminin and/or certain peptides. Tuning of polarity allows the formation of both apical-out organoids (with the Trpzips of the present invention alone) and basal out organoids upon the addition of laminin protein. In this way different organoid structures can be generated with tuneable morphology starting from the Trpzip material, suggesting broad versatility of the Trpzip motif as a general matrix for tissue and organoid engineering. Figure 7 shows the characterisation of human small intestinal organoid polarity quantified for percentage of basal-out or apical-out in commercially available basement membrane matrix Matrigel, Trpzip-QV gel (0.5 wt%), and Trpzip-QV gel (0.5 wt%) supplemented with 50% laminin protein, clearly demonstrating the control of organ polarity by hydrogel composition.

The present invention demonstrates a peptide hydrogelator based on the tryptophan zipper motif that self-assembles into a nano- and micro-structured material with unique mechanical and biological properties. Trpzip-QV hydrogels are easily formed without the need for rigid temperature control, in contrast to the requirements for Matrigel gelation. The tunable modulus and low yield stress provides the first example of a material where viscoelasticity can be varied to direct functional biological outcomes followed by quick harvest through simple agitation. This will prove highly beneficial for molecular characterization, which usually requires invasive enzyme-mediated dissolution of the surrounding matrix. Similarly, the low yield stress and self-healing properties provide a means for syringe extrusion, where fluidization of the hierarchical material protects the cells from shear, towards applications in cell delivery and in biofabrication. Considering how these hydrogels are simultaneously bactericidal and bioactive to mammalian cells, there is broad scope for using Trpzip-QV hydrogels *in vitro* as well as *in vivo* as a therapeutic biomaterial.

As mentioned above, the Trpzip compounds of Cochran are not hydrogelling, indicating the criticality of the central moiety in achieving such a result. The present inventors have also conducted preliminary studies of a hydrogel of the present invention having a terminal modification to one of the Trpzip arms, that is changing SWTWSQGNVWTWK to SWTWQGNVWTWV. This compound was found to exhibit hydrogelling properties in a simple inversion test, i.e., a sample of the peptide dissolved in water was allowed to reach equilibrium. The container was inverted, and it was observed that the solution was sufficiently solid to remain in the container.

Claims

1. A hydrogelling peptide having the sequence SWTW–Link–WTWK, where Link is a peptide motif selected to provide the hydrogelling peptide with hydrogelling properties.

2. A hydrogelling peptide according to claim 1 wherein Link is a peptide motif having any number of amino acid residues.

3. A hydrogelling peptide according to claim 2 wherein the peptide motif has 4, 5, 6, 7 or 8 amino acid residues.

4 A hydrogelling peptide according to claim 1 where Link is a four amino acid residue of the following formula -aa1-GN-aa2- where aa1 and aa2 are independently selected from E, K, V or Q.

5. A hydrogelling peptide having the sequence X1-WTW–Link–WTW-X2, wherein Link is a peptide motif selected to provide the hydrogelling peptide with hydrogelling properties and where X1 and X2 are independently selected from any amino acid, for example Leucine (L), Isoleucine (I), Valine (V), Alanine (A), Methionine (M), Phenylalanine (F), Tryptophan (W), Proline (P), Glycine (G), Serine (S), Asparagine (N), Glutamine (Q), Threonine (T), Cysteine (C), Tyrosine (Y), Aspartic acid (D), Glutamic acid (E), Lysine (K), Arginine (R) or Histidine (H).

6. A hydrogelling peptide according to claim 5 wherein Link is a peptide motif having any number of amino acid residues.

7. A hydrogelling peptide according to claim 6 wherein the peptide motif has 4, 5, 6, 7 or 8 amino acid residues.

8 A hydrogelling peptide according to claim 5 where Link is a four amino acid residue of the following formula -aa1-GN-aa2- where aa1 and aa2 are independently selected from E, K, V or Q.

9. A hydrogelling peptide having the sequence SWTWQGNVWTWK.

10. A hydrogelling peptide of the structure X1-WTWQGNVWTW-X2, where X1 and X2 are independently selected from any amino acid, for example Leucine (L), Isoleucine (I), Valine (V), Alanine (A), Methionine (M), Phenylalanine (F), Tryptophan (W), Proline (P), Glycine (G), Serine (S), Asparagine (N), Glutamine (Q), Threonine (T), Cysteine (C), Tyrosine (Y), Aspartic acid (D), Glutamic acid (E), Lysine (K), Arginine (R) or Histidine (H).

11. A hydrogelling peptide having the sequence selected from the group consisting of:
Sub1- SWTWQGNVWTWK-Sub2,
Sub1-SWTWQGNVWTWK and
SWTWQGNVWTWK-Sub2
wherein Sub1 and Sub2 are independently variable substituents.

12. A hydrogelling peptide according to claim 11 wherein Sub1 and/or Sub2 is a peptide substituent which contains the laminin motif -Ile-Lys-Val-Ala-Val (IKVAV).

13. A hydrogelling peptide according to claim 11 wherein Sub1 and/or Sub2 substituent is a peptide substituent which contains motif selected from one or more of YIGSR, GRGDSC or GFOGER.

14. A hydrogelling peptide according to claim 11 wherein Sub1 and/or Sub2 is selected from one or more of a small molecule; a protein; an enzyme; a carbohydrate (for example a polysaccharide); a nucleic acid; a synthetic polymer; a natural polymer; a metal nanoparticle; a metal oxide nanoparticle; a lipid nanoparticle, a quantum dot, or a combination thereof.

15. A hydrogelling peptide having the sequence Sub1-SWTW–Link–WTWK-Sub2, where Link is a peptide motif selected to provide the hydrogelling peptide with hydrogelling properties and Sub1 and Sub2 are independently variable substituents.

16. A hydrogelling peptide according to claim 15 wherein Link is a peptide motif having any number of amino acid residues.

17. A hydrogelling peptide according to claim 16 wherein the peptide motif has 4, 5, 6, 7 or 8 amino acid residues.

18 A hydrogelling peptide according to claim 15 where Link is a four amino acid residue of the following formula -aa1-GN-aa2- where aa1 and aa2 are independently selected from E, K, V or Q.

19. A hydrogelling peptide according to any one of claims 15 to 18 wherein Sub1 and/or Sub2 is a peptide substituent which contains the laminin motif -Ile-Lys-Val-Ala-Val (IKVAV).

20. A hydrogelling peptide according to any one of claims 15 to18 wherein Sub1 and/or Sub2 substituent is a peptide substituent which contains motif selected from one or more of YIGSR, GRGDSC or GFOGER.

21. A hydrogelling peptide according to any one of claims 15 to18 wherein Sub1 and/or Sub2 is selected from one or more of a small molecule; a protein; an enzyme; a carbohydrate (for example a polysaccharide); a nucleic acid; a synthetic polymer; a natural polymer; a metal nanoparticle; a metal oxide nanoparticle; a lipid nanoparticle, a quantum dot, or a combination thereof.

22. A hydrogelling peptide according to any one of the preceding claims with the proviso that Link is not a 4 amino acid residue comprising EGNK, ENGK or EpNK.

23. A hydrogel comprising a peptide according to any one of the preceding claims and water.

24. A hydrogel according to claim 23 wherein the peptide is present in an amount of 0.1% w/v – 10% w/v.

25. A hydrogel according to claim 23 or 24 wherein the peptide is present in an amount of at least 0.5% w/v to 3% w/v.

26. A hydrogel according to any one of claims 23 to 25 comprising one or more of a small molecule, a protein, an enzyme, a carbohydrate (for example a polysaccharide), a hydrogel forming polymer.

27. A hydrogel according to any one of claims 23-26 comprising a peptide having the sequence SWTWQGNVWTWK and a peptide having the sequence SWTWQGNVWTWKIKVAV.

28. A hydrogel according to any one of claims 23-27 which is antibacterial.

29. A hydrogel according to any one of claims 23-28 which is self-healing.

30. A hydrogel according to any one of claims 23-29 which has low yield stress.

31. A method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising adjusting the concentration of a peptide of sequence SWTWQGNVWTWK.

32. A method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising adjusting the concentration of a peptide of sequence SWTWQGNVWTWK and a peptide of sequence SWTWQGNVWTWKIKVAV.

33. A method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising adjusting the pH > 7 of a peptide of sequence SWTWQGNVWTWK.

34. A method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising adjusting the pH > 7 of a peptide of sequence SWTWQGNVWTWK and a peptide of sequence SWTWQGNVWTWKIKVAV.

35. A method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising a peptide of sequence SWTWQGNVWTWK and a synthetic hydrogel forming polymer.

36. A method of controlling the mechanical properties (for example stiffness) of a hydrogel comprising a peptide of sequence SWTWQGNVWTWK and a natural hydrogel.

37. A method of generating an organoid mix having a predetermined ratio of basal-out: apical-out organoids, the method comprising the step of selecting an organoid forming hydrogel having a predetermined ratio of Trpzip:laminin

38. The method according to claim 36 wherein organoid forming hydrogel is predominantly Trpzip and the organoids formed are predominantly apical out.

39. The method according to claim 36 wherein the organoid forming hydrogel comprises Trpzip and laminin in an amount such that the organoids formed are predominantly Basal out.

FIGURE 1

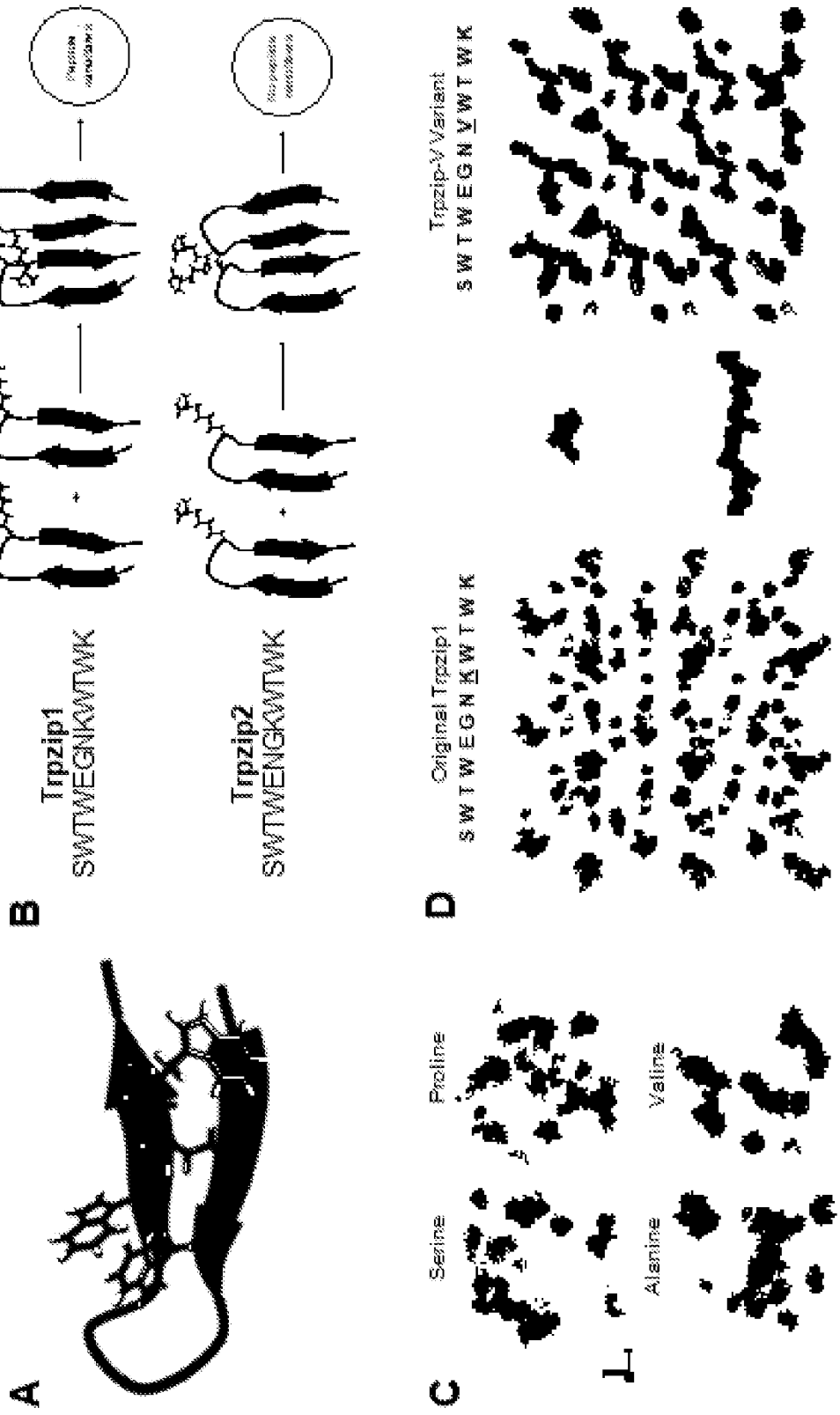

A
B
Trpzip1
SWTWEGNKWTWK
Trpzip2
SWTWENGKWTWK
C
Serine
Proline
Alanine
Valine
D
Original Trpzip1
S W T W E G N K W T W K
Trpzip-V-Variant
S W T W E G N V W T W K

FIGURE 2

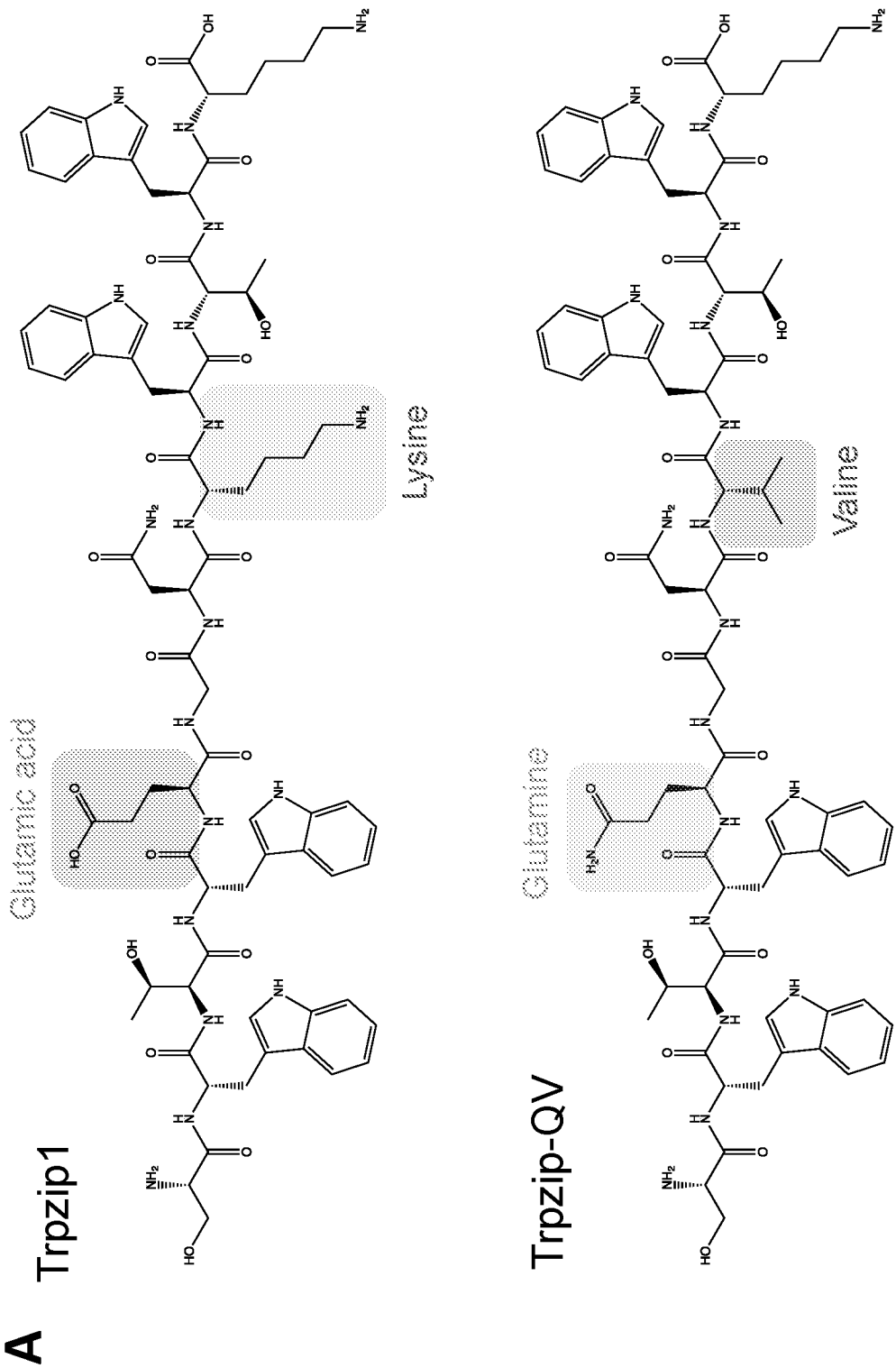


FIGURE 2 (CONT)

Trpzip1
SWTWEGNKWTWK

Trpzip-QV
SWTWQGNVWTWK

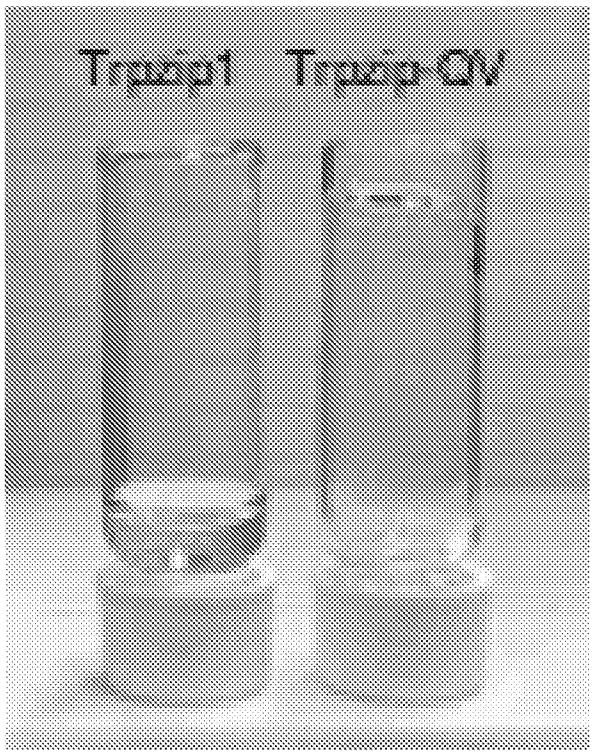
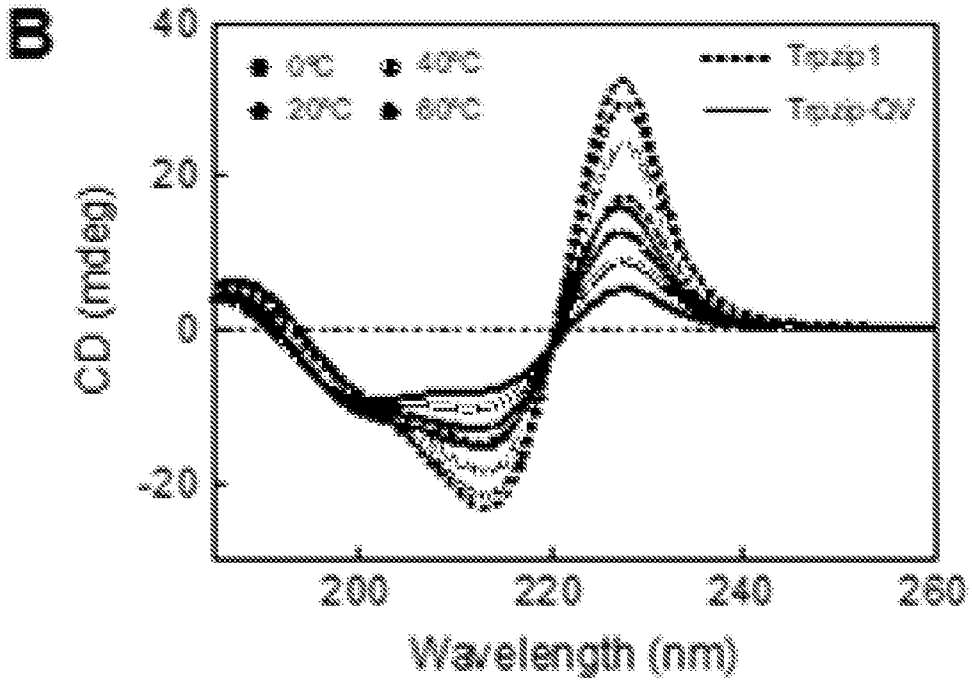


FIGURE 2 (CONT)



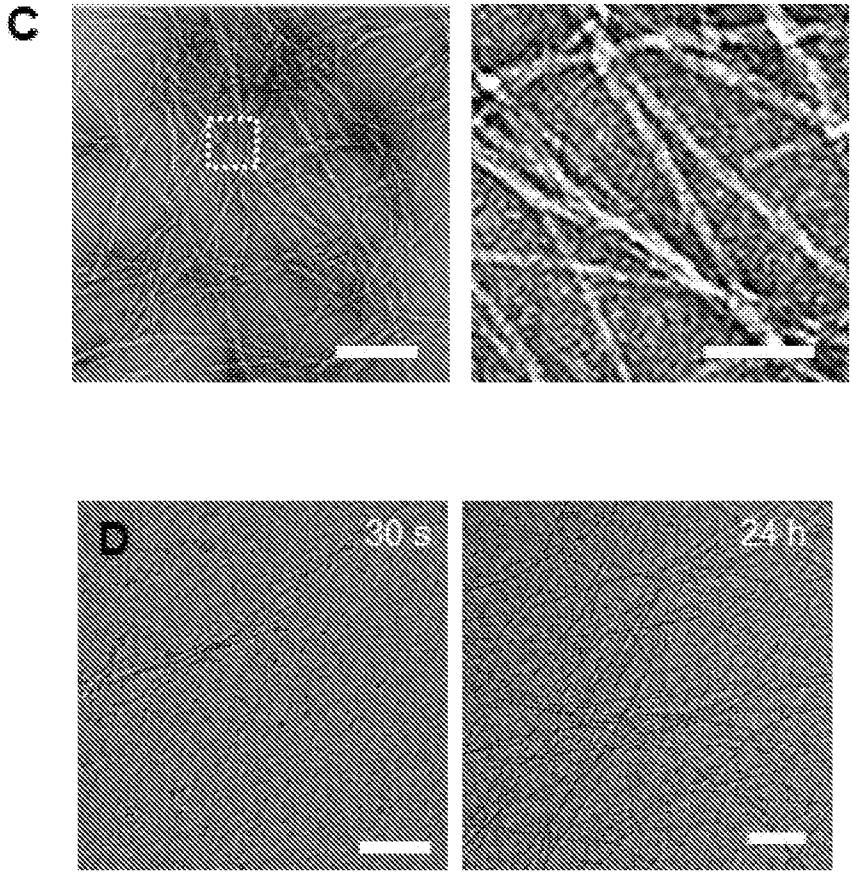

C
D
30 s
24 h

FIGURE 2 (CONT)

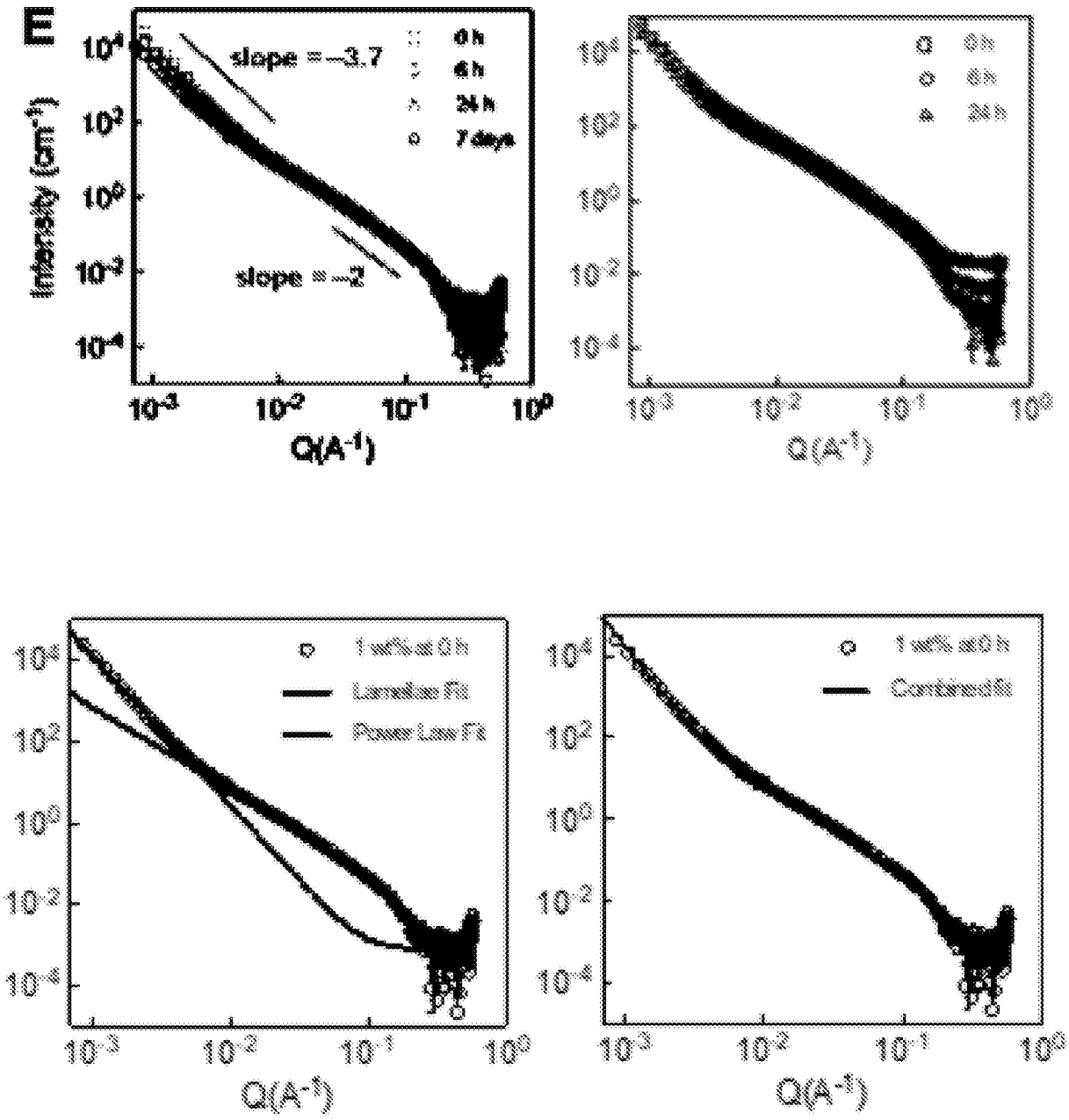


FIGURE 2 (CONT)

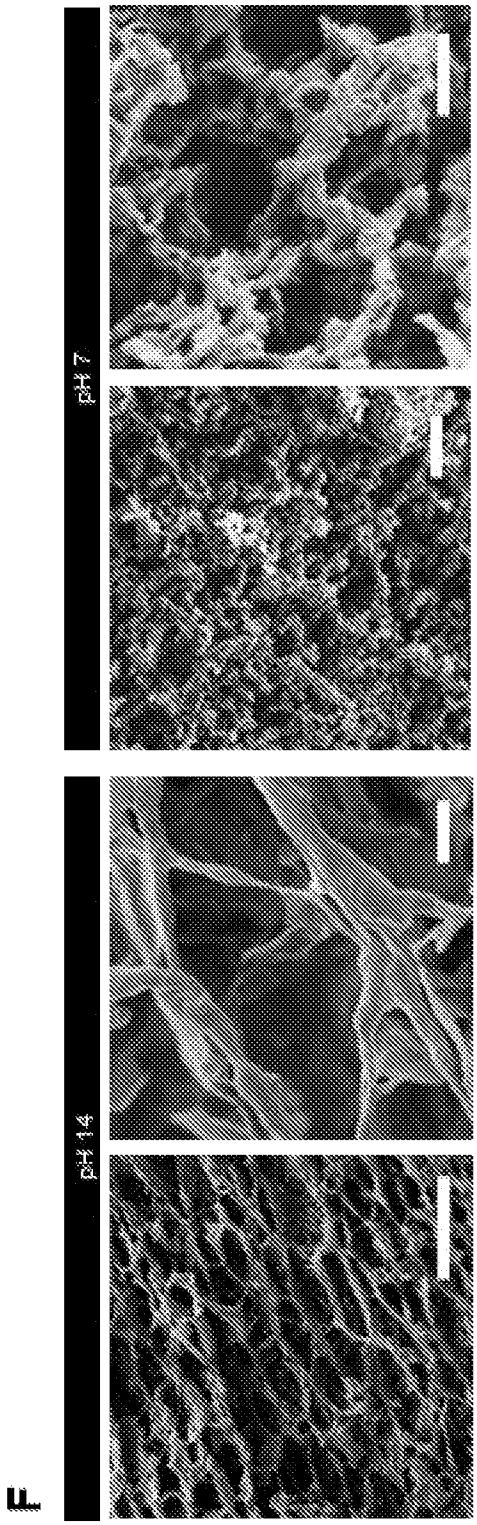


FIGURE 2 (CONT)

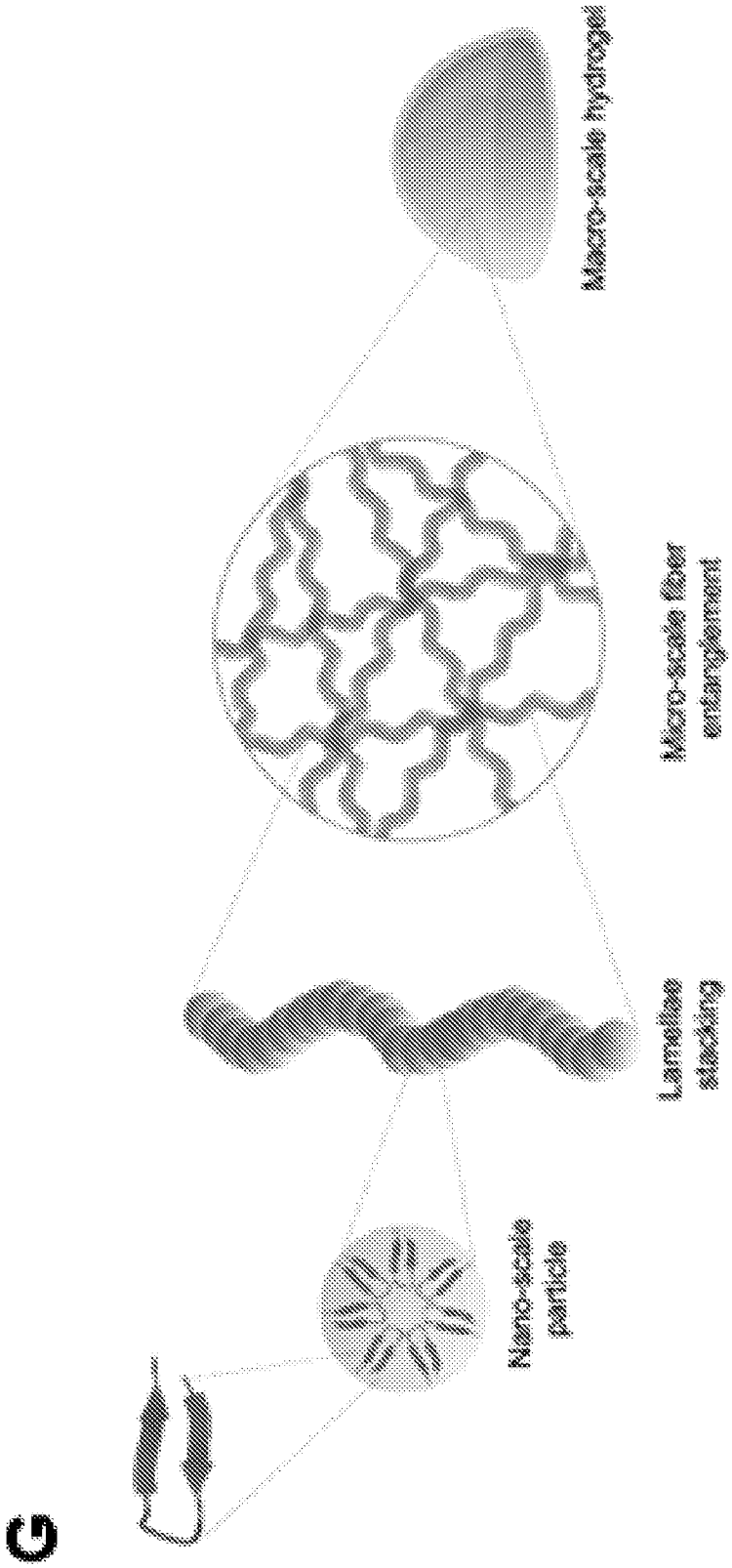


FIGURE 3

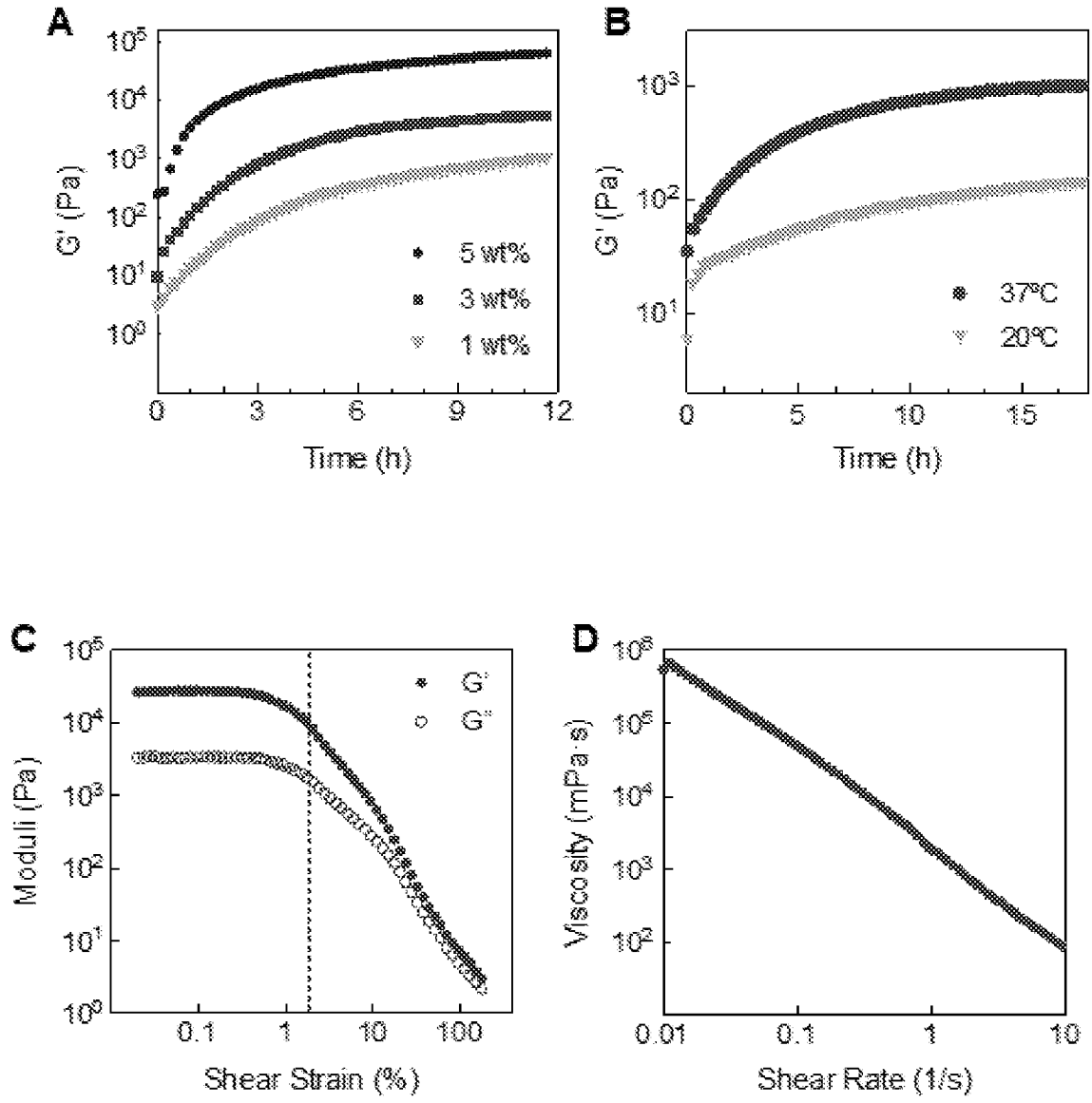

A
G' (Pa)
Time (h)
5 wt%
3 wt%
1 wt%
B
G' (Pa)
Time (h)
37°C
20°C
C
Moduli (Pa)
Shear Strain (%)
G'
G"
D
Viscosity (mPa·s)
Shear Rate (1/s)

FIGURE 3 (CONT)

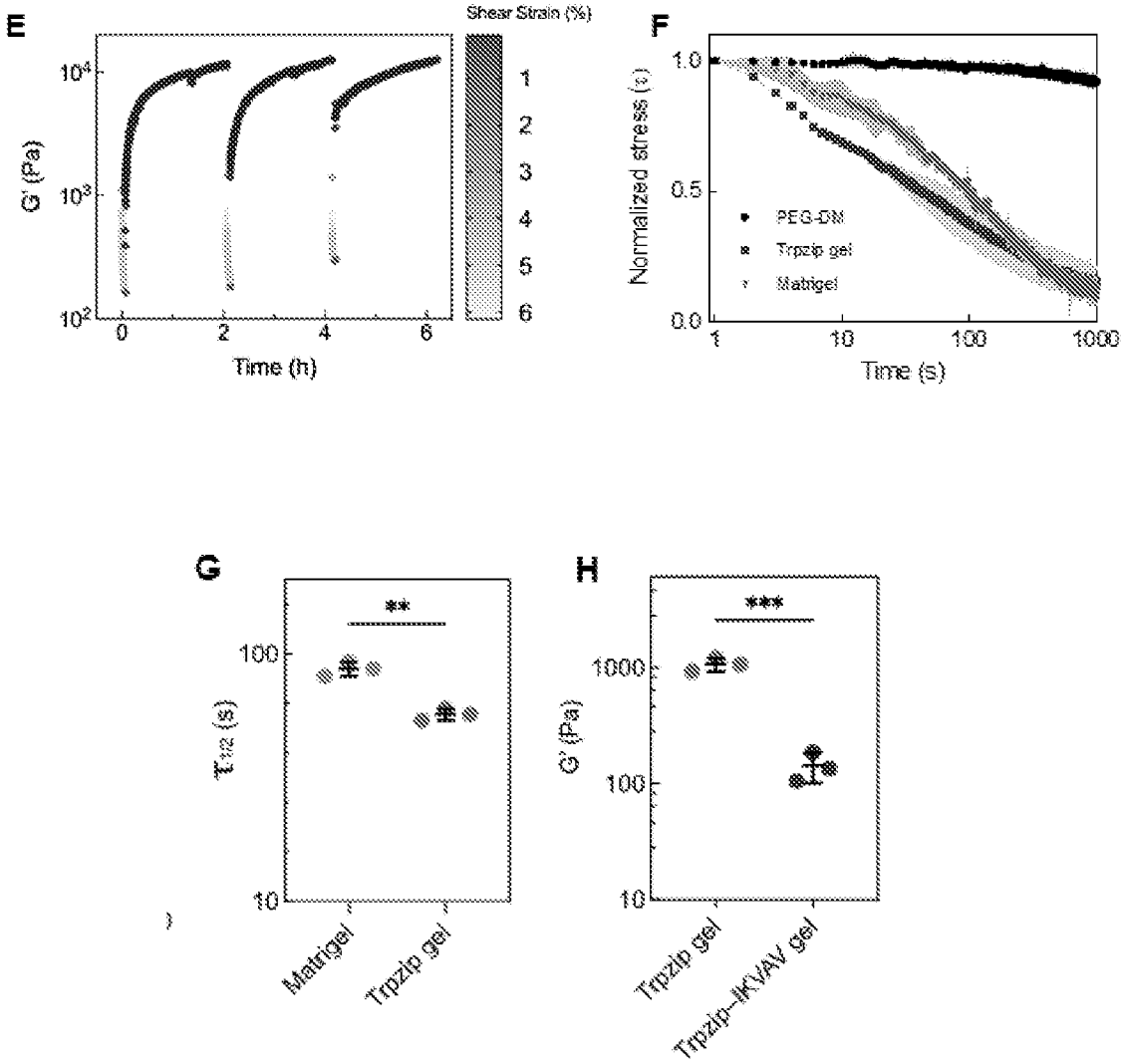


FIGURE 4

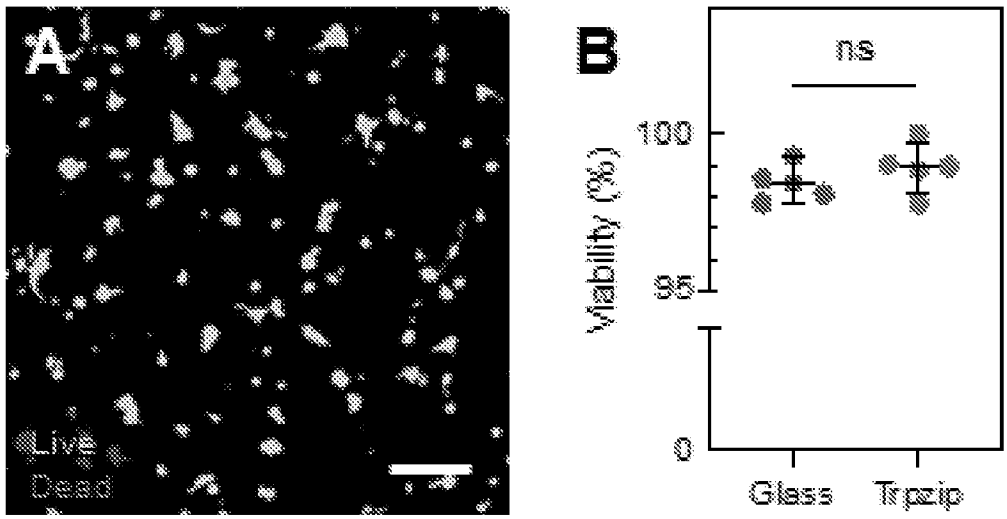


FIGURE 4(CONT)

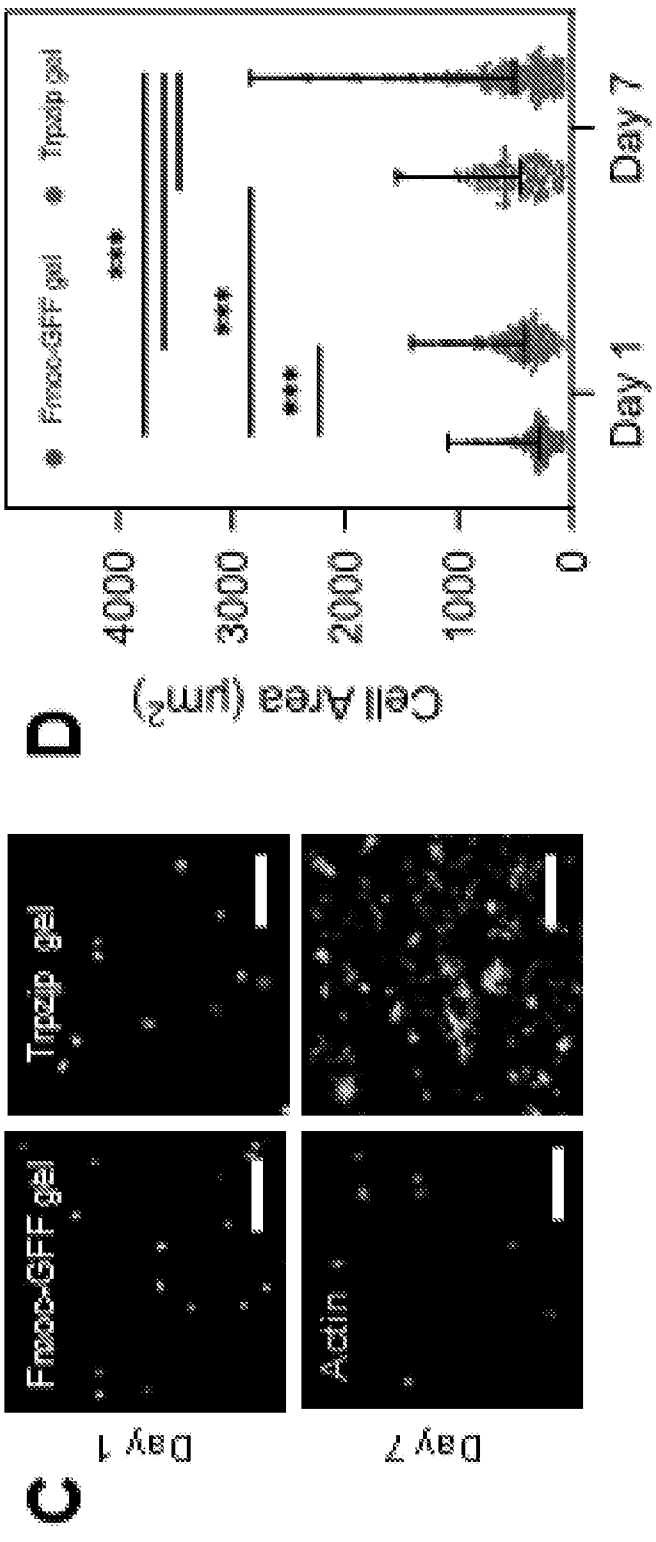


FIGURE 4 (CONT)

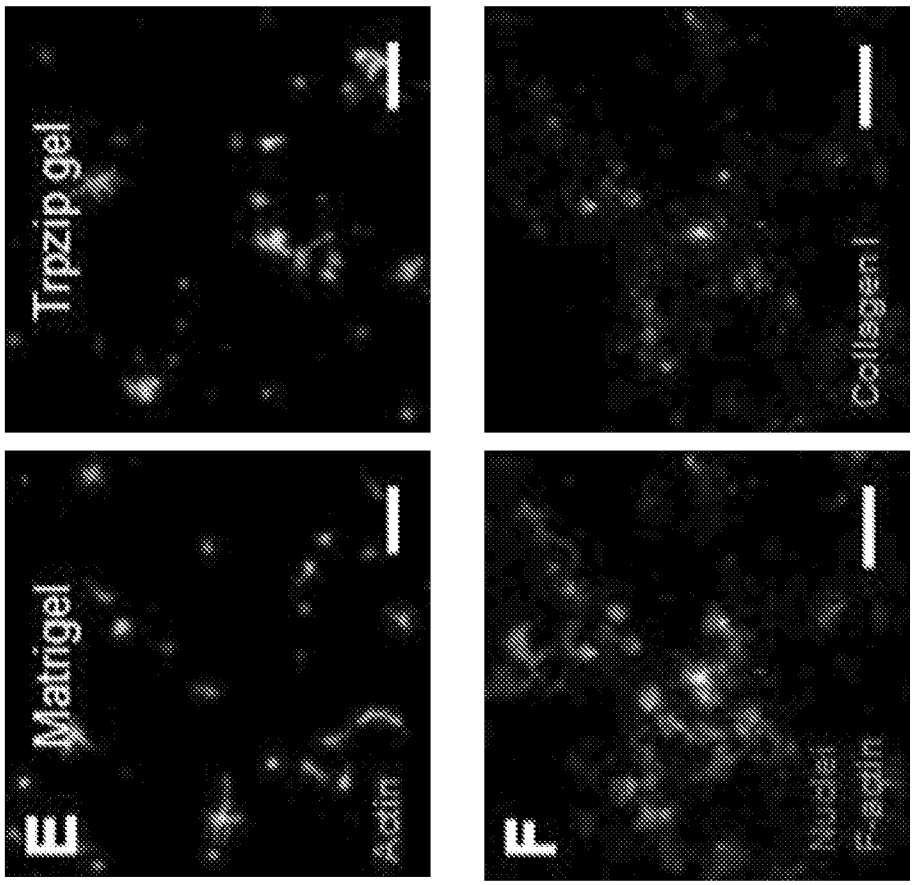


FIGURE 4(CONT)

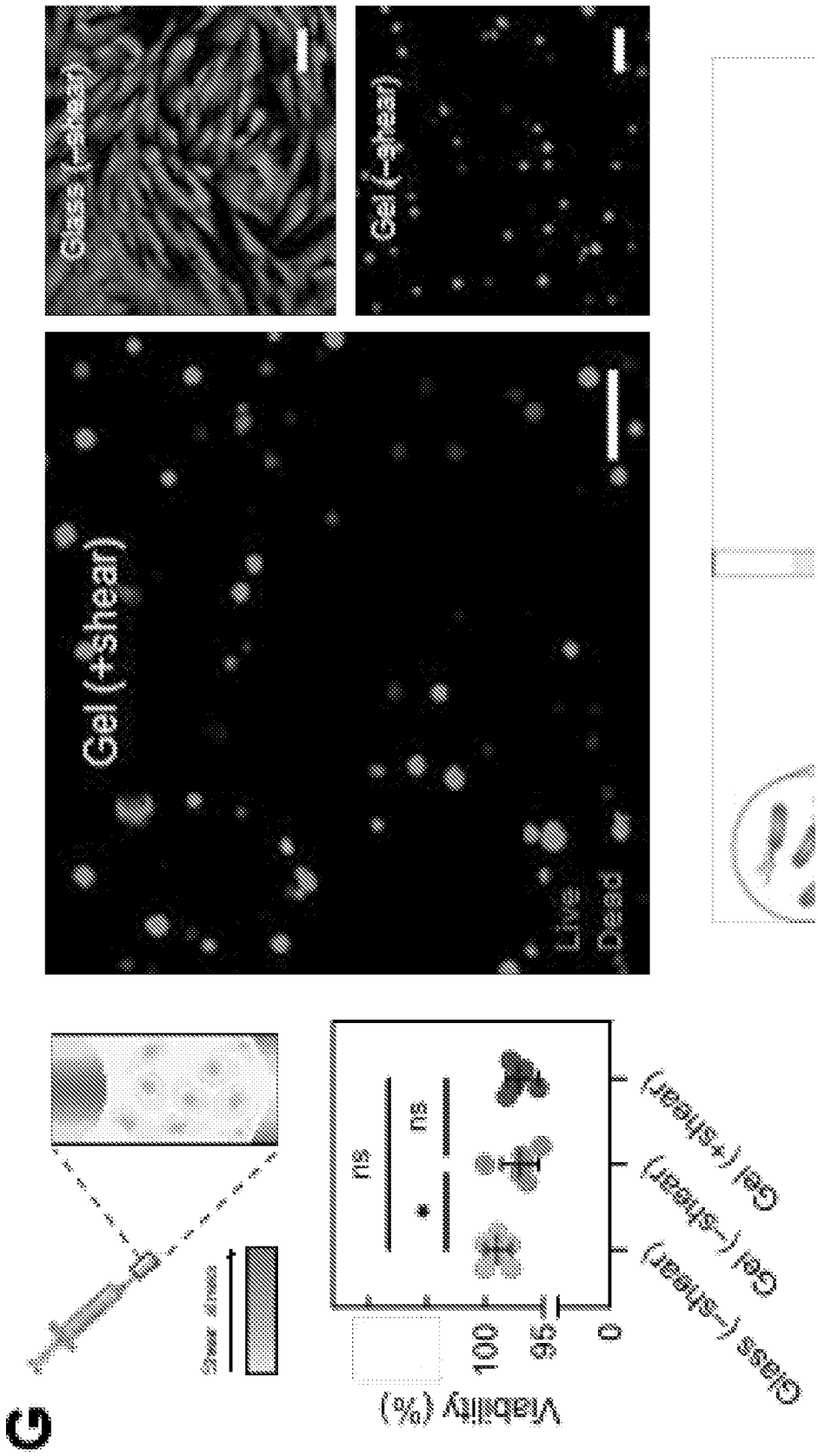


FIGURE 4(CONT)

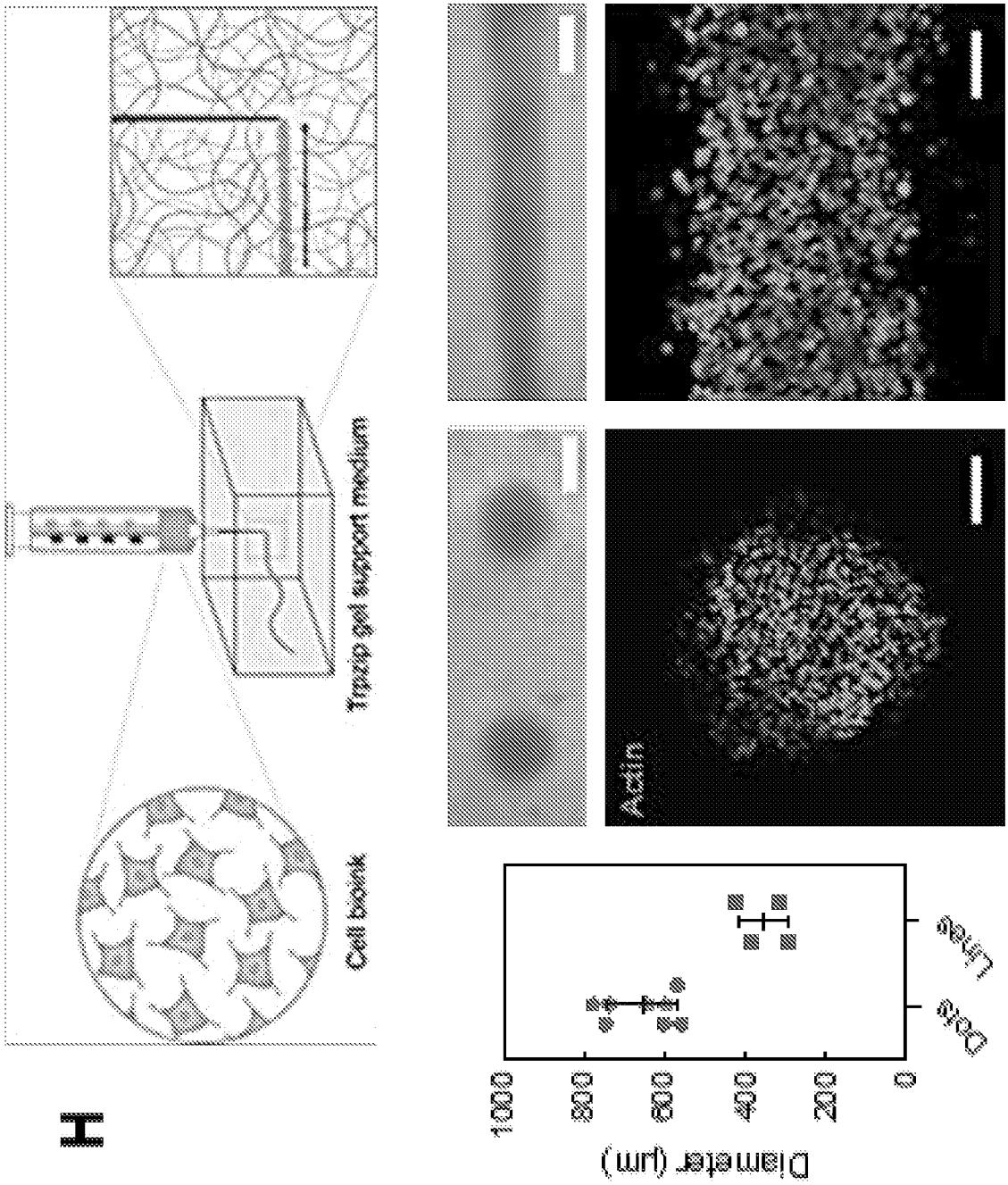
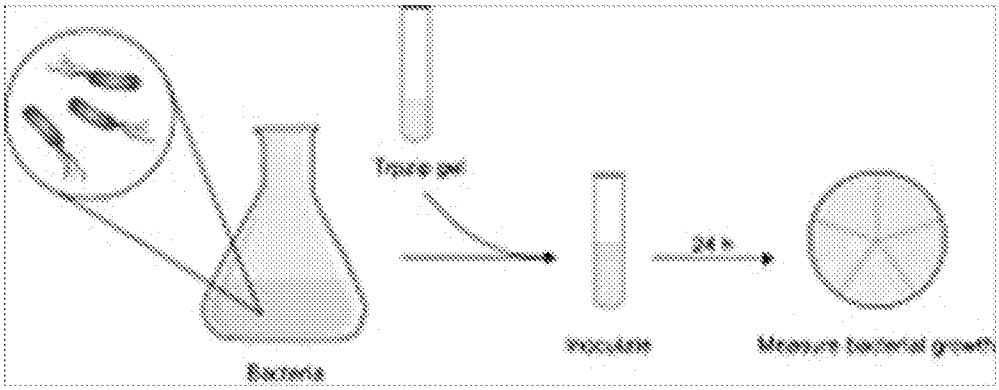
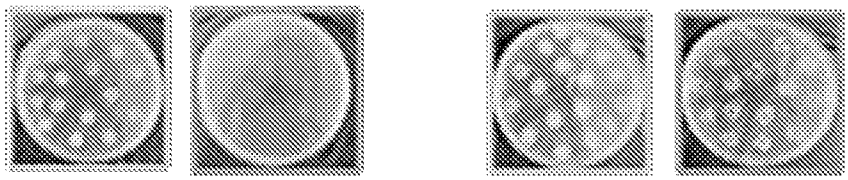


FIGURE 4(CONT)





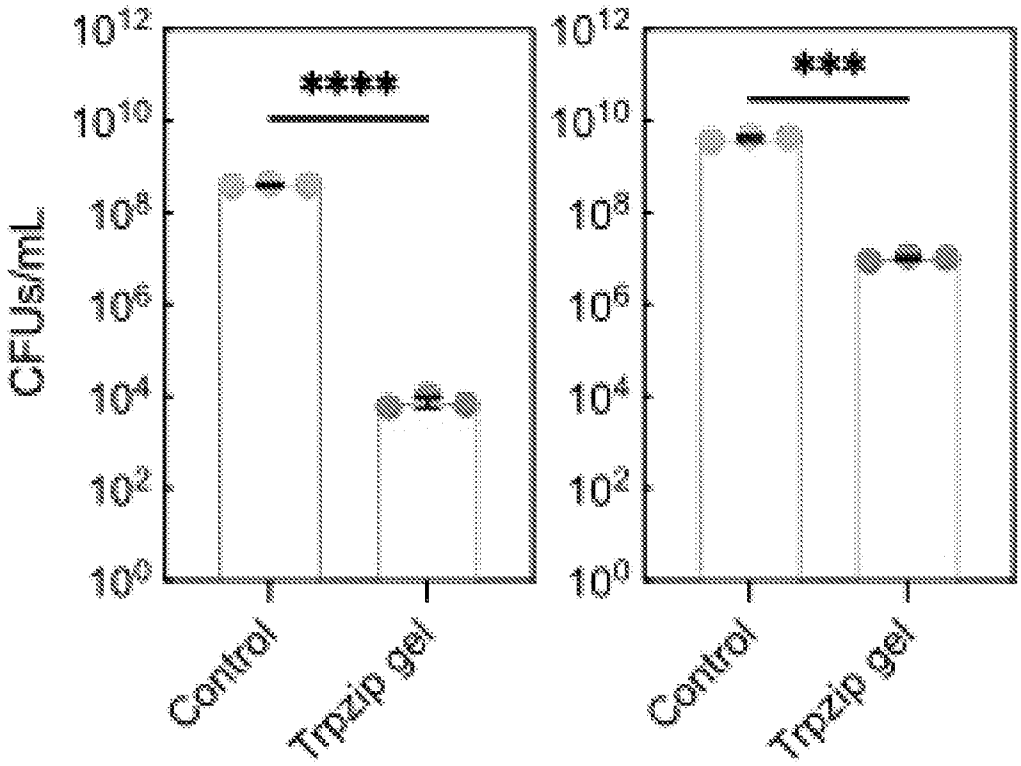


FIGURE 5

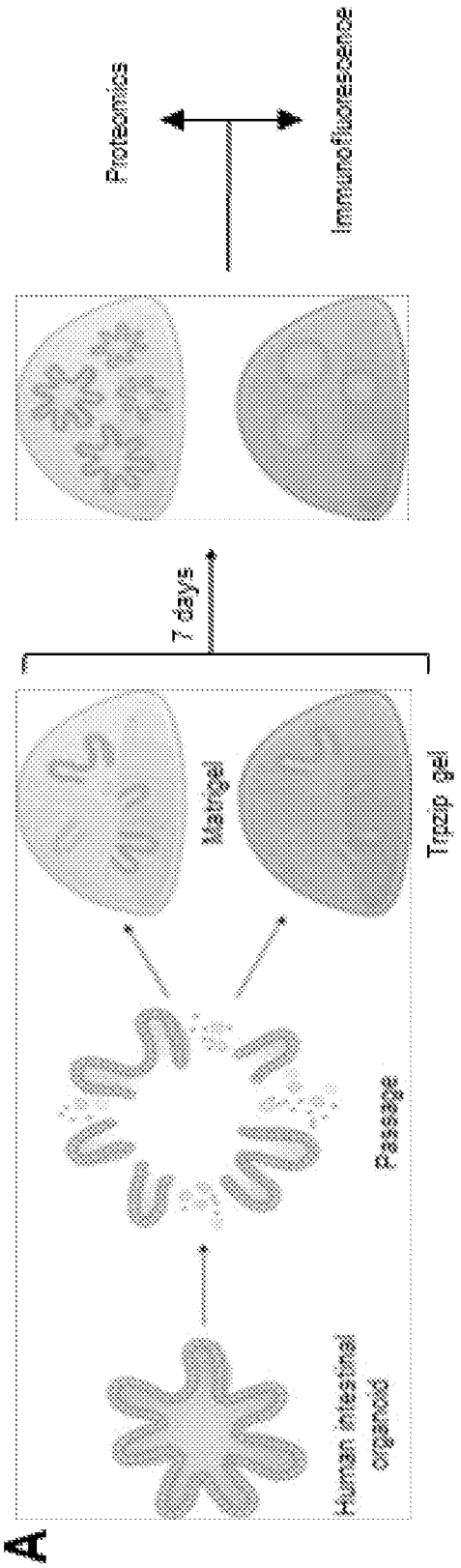

A
Human intestinal organoid
Passage
Matrigel
Trypsin gel
7 days
Proteomics
Immunofluorescence

FIGURE 5 (CONT)

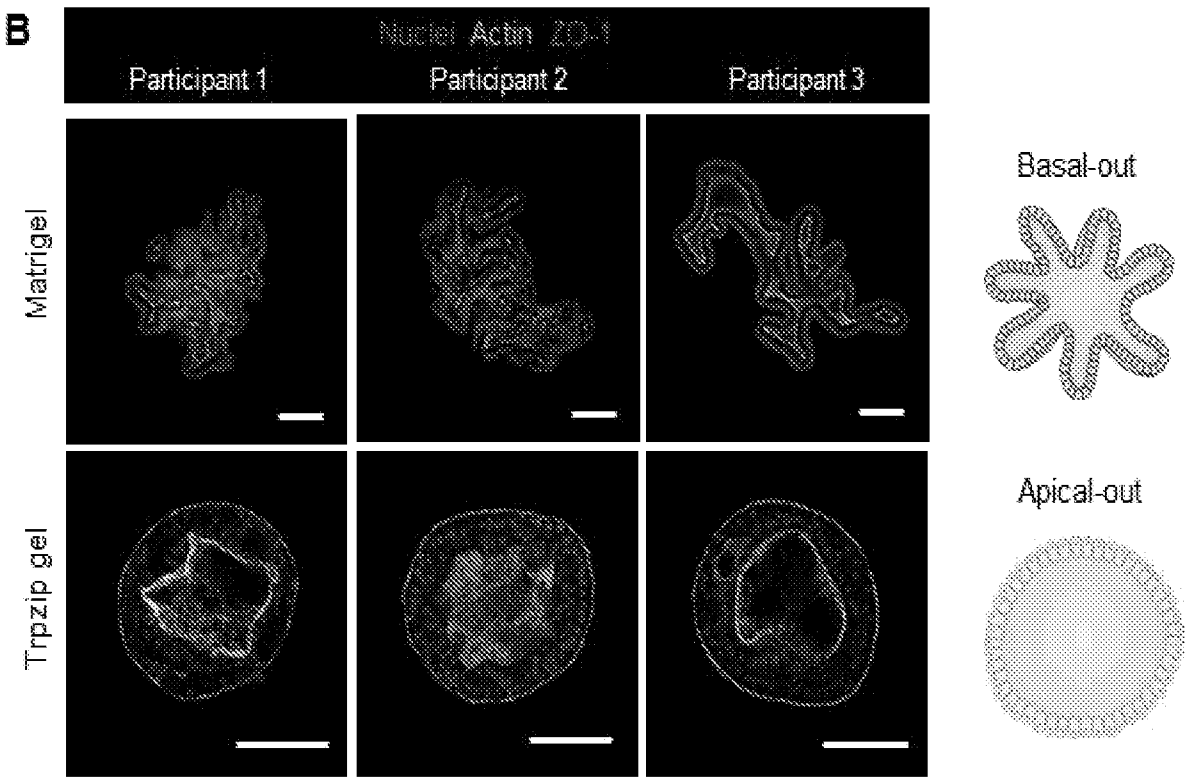


FIGURE 5 (CONT)

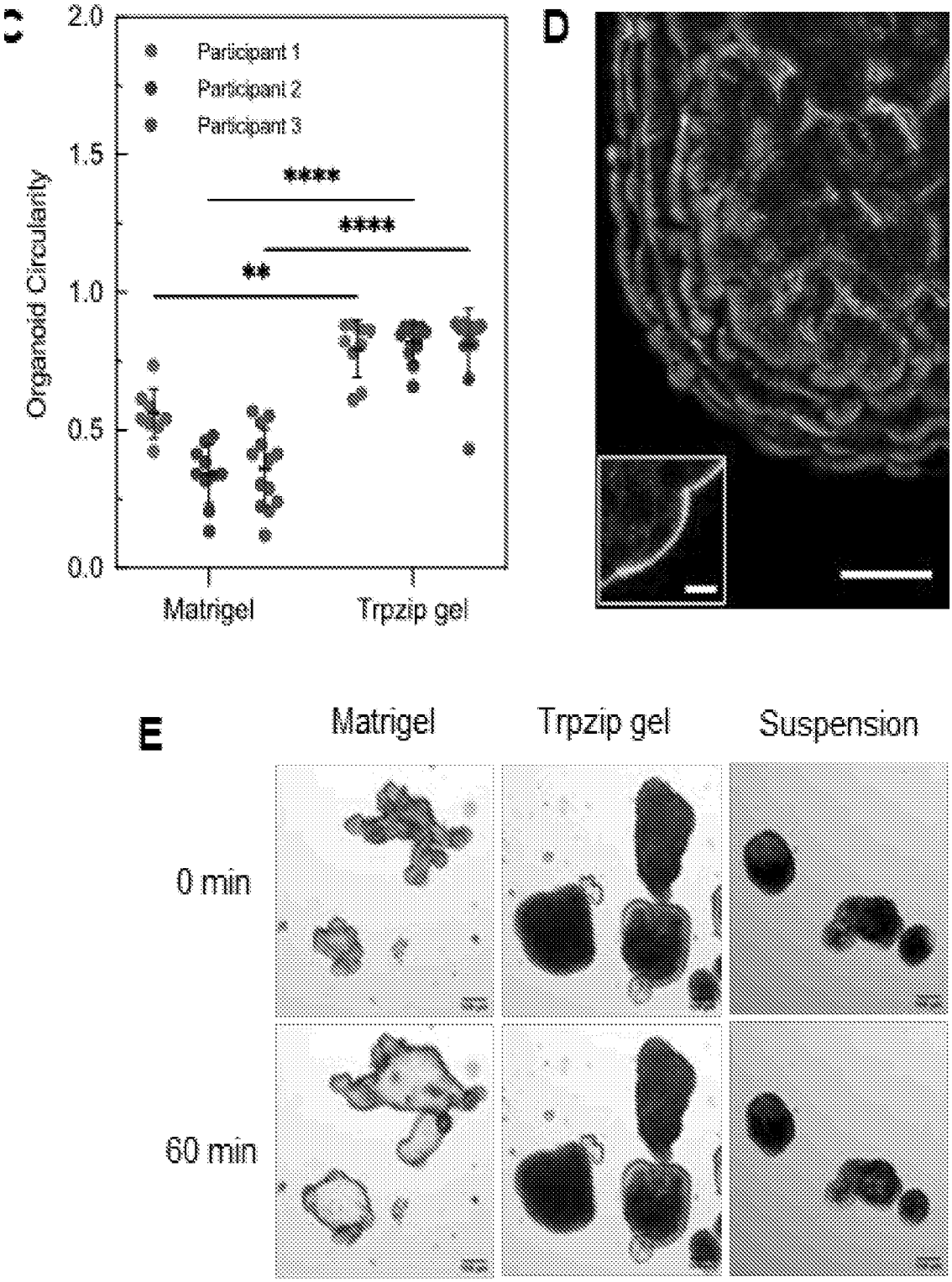


FIGURE 5 (CONT)

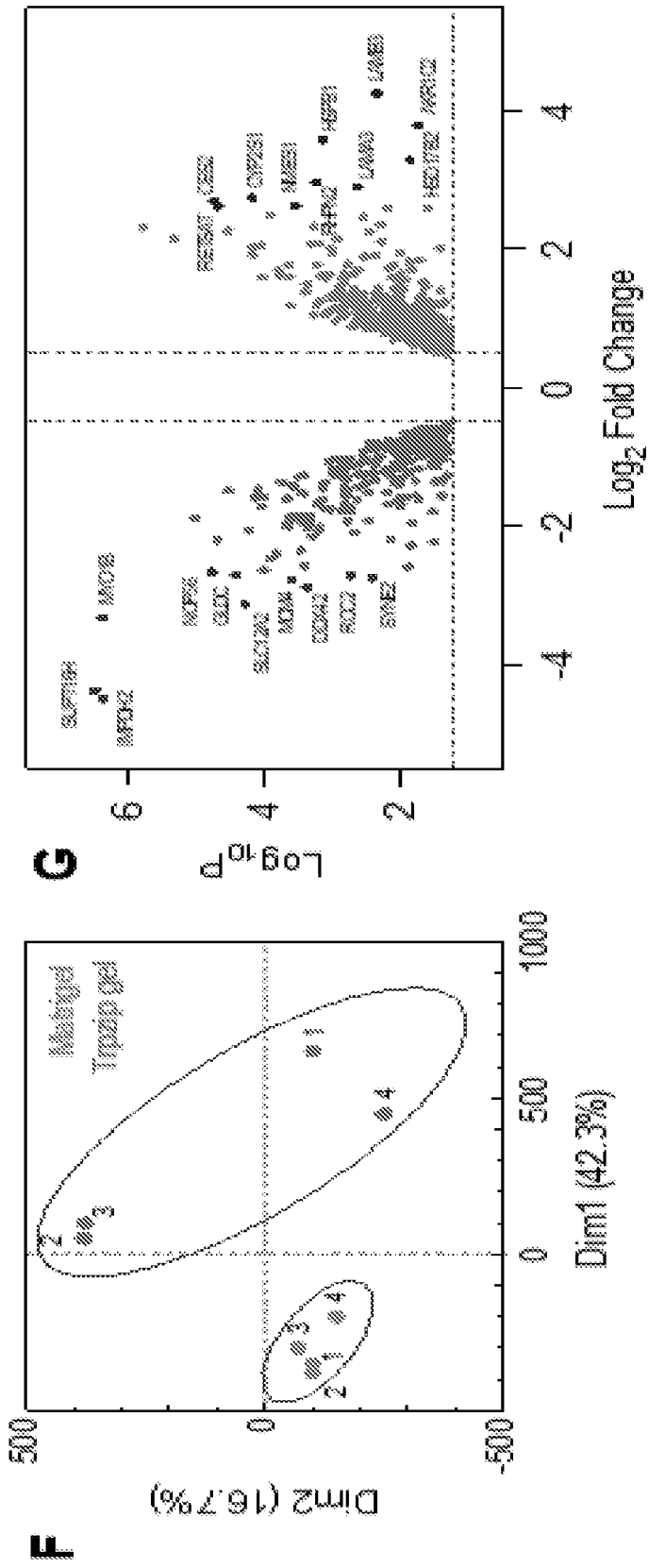


FIGURE 5 (CONT)

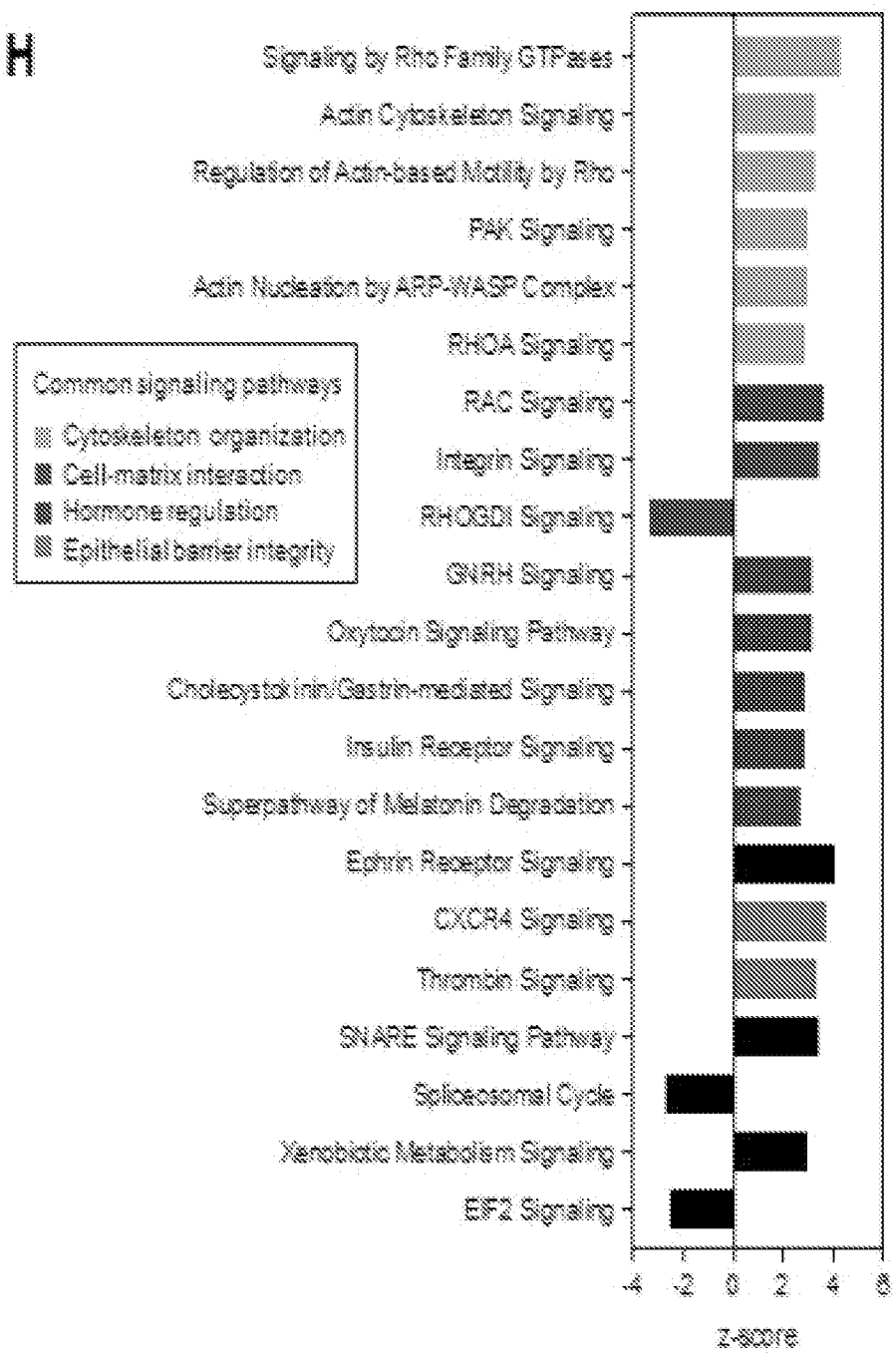


FIGURE 5 (CONT)

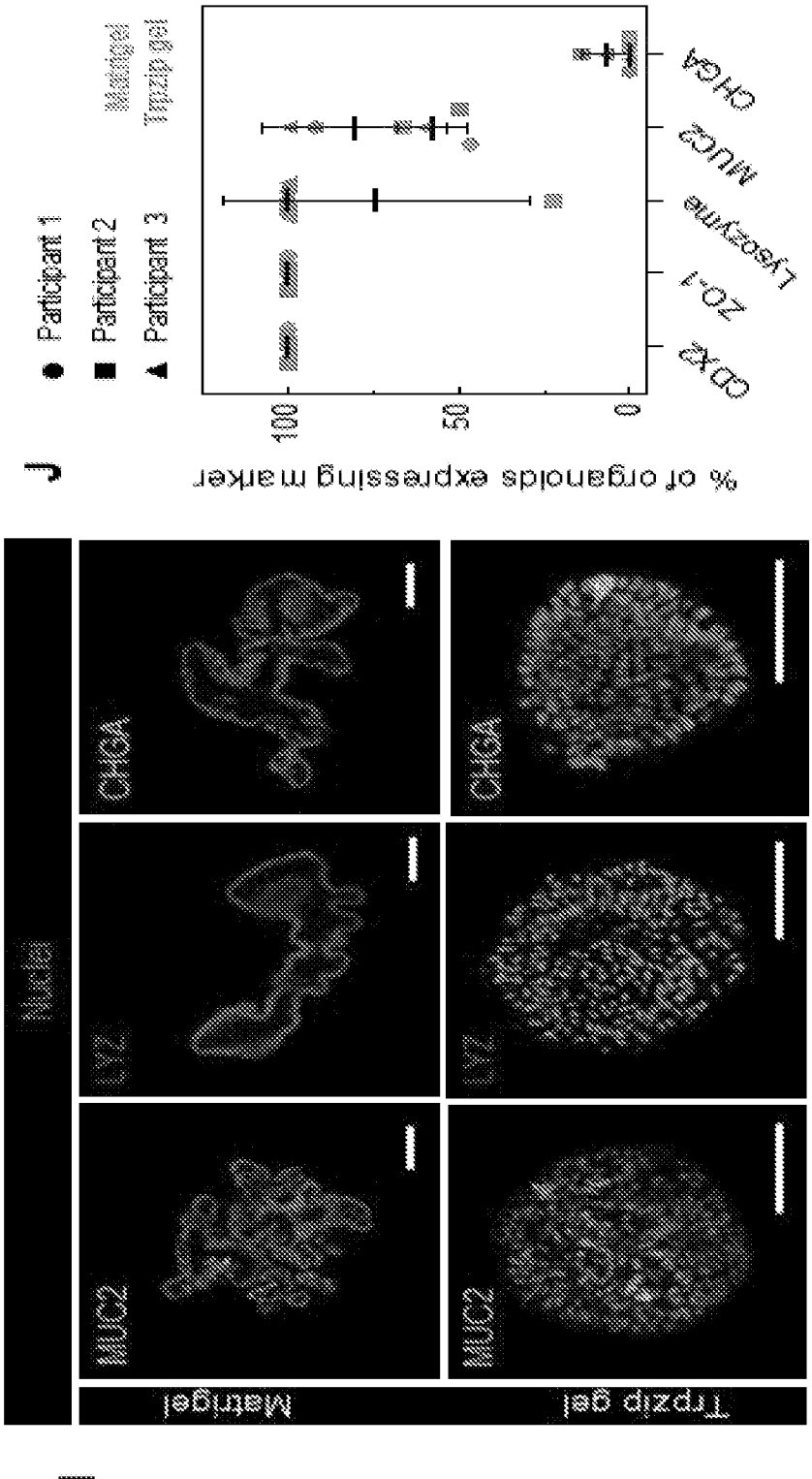


FIGURE 6

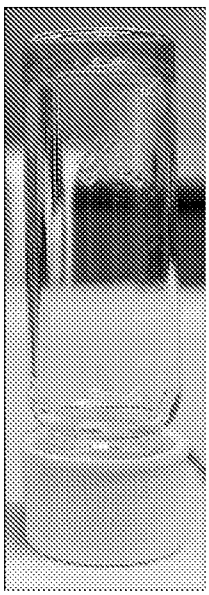


FIGURE 7

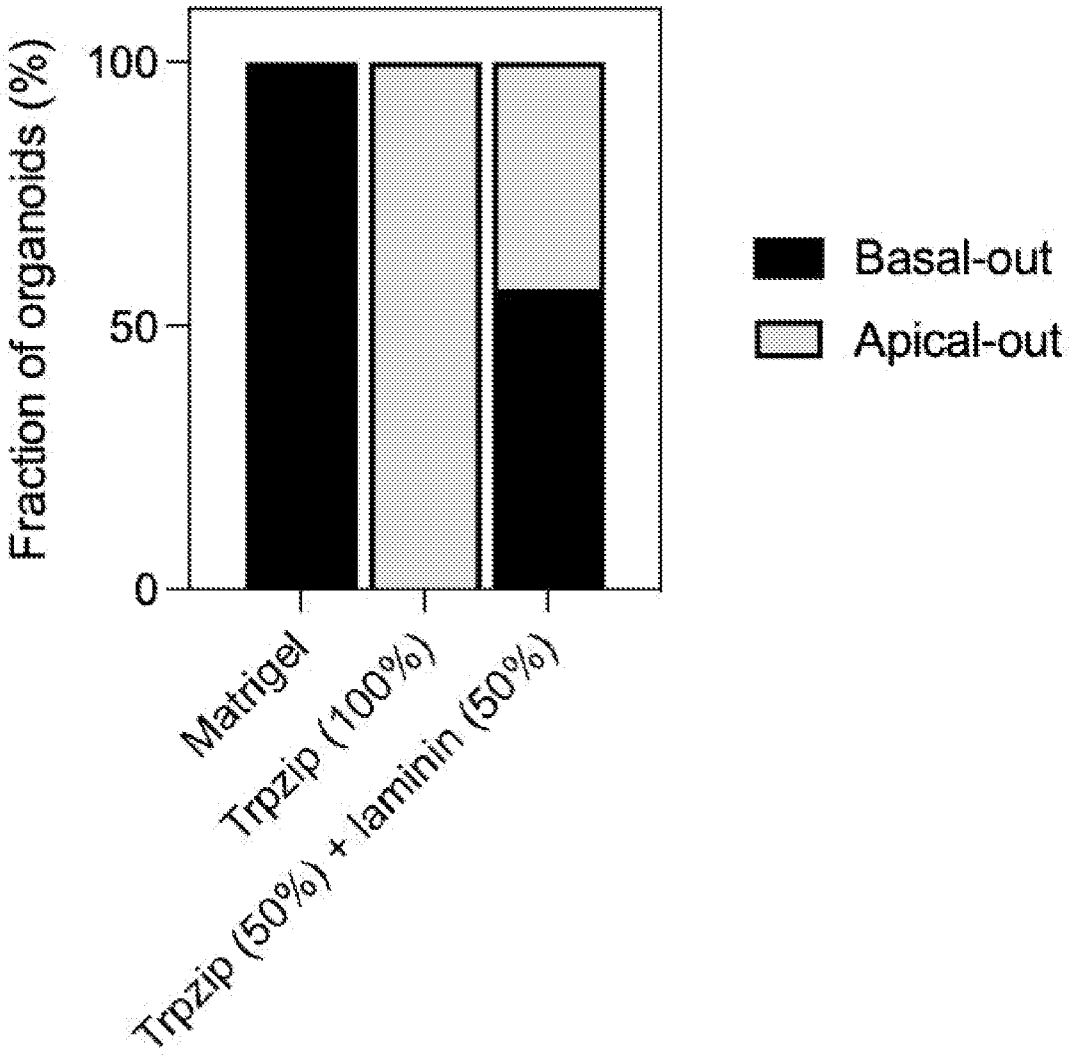

Fraction of organoids (%)
100
50
0
Matrigel
Trpzip (100%)
Trpzip (50%) + laminin (50%)
Basal-out
Apical-out

FIGURE 8

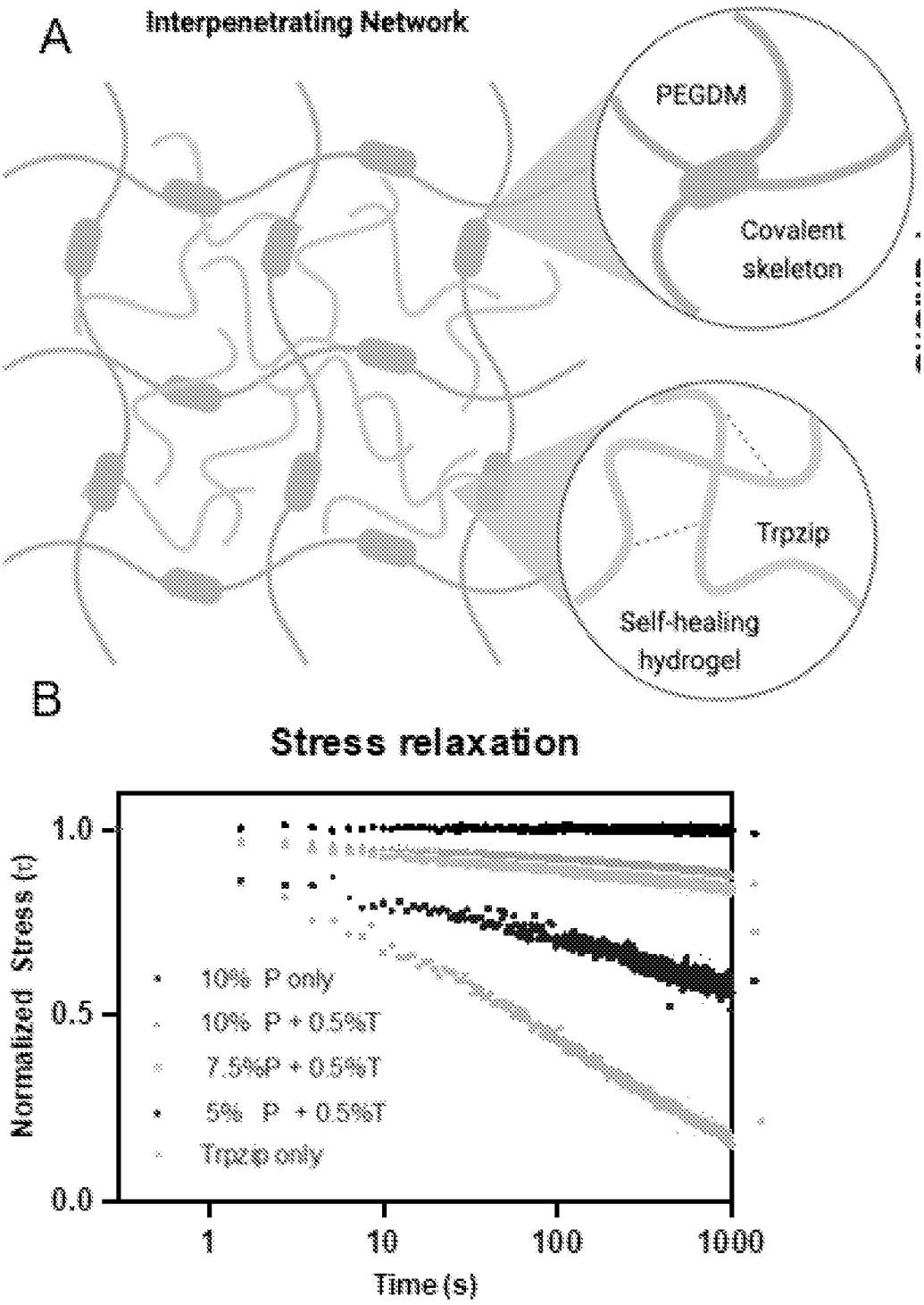

A
Interpenetrating Network
PEGDM
Covalent skeleton
Trpzip
Self-healing hydrogel
B
Stress relaxation
Normalized Stress (τ)
1.0
0.5
0.0
10% P only
10% P + 0.5%T
7.5%P + 0.5%T
5% P + 0.5%T
Trpzip only
1
10
100
1000
Time (s)

FIGURE 8(CONT)

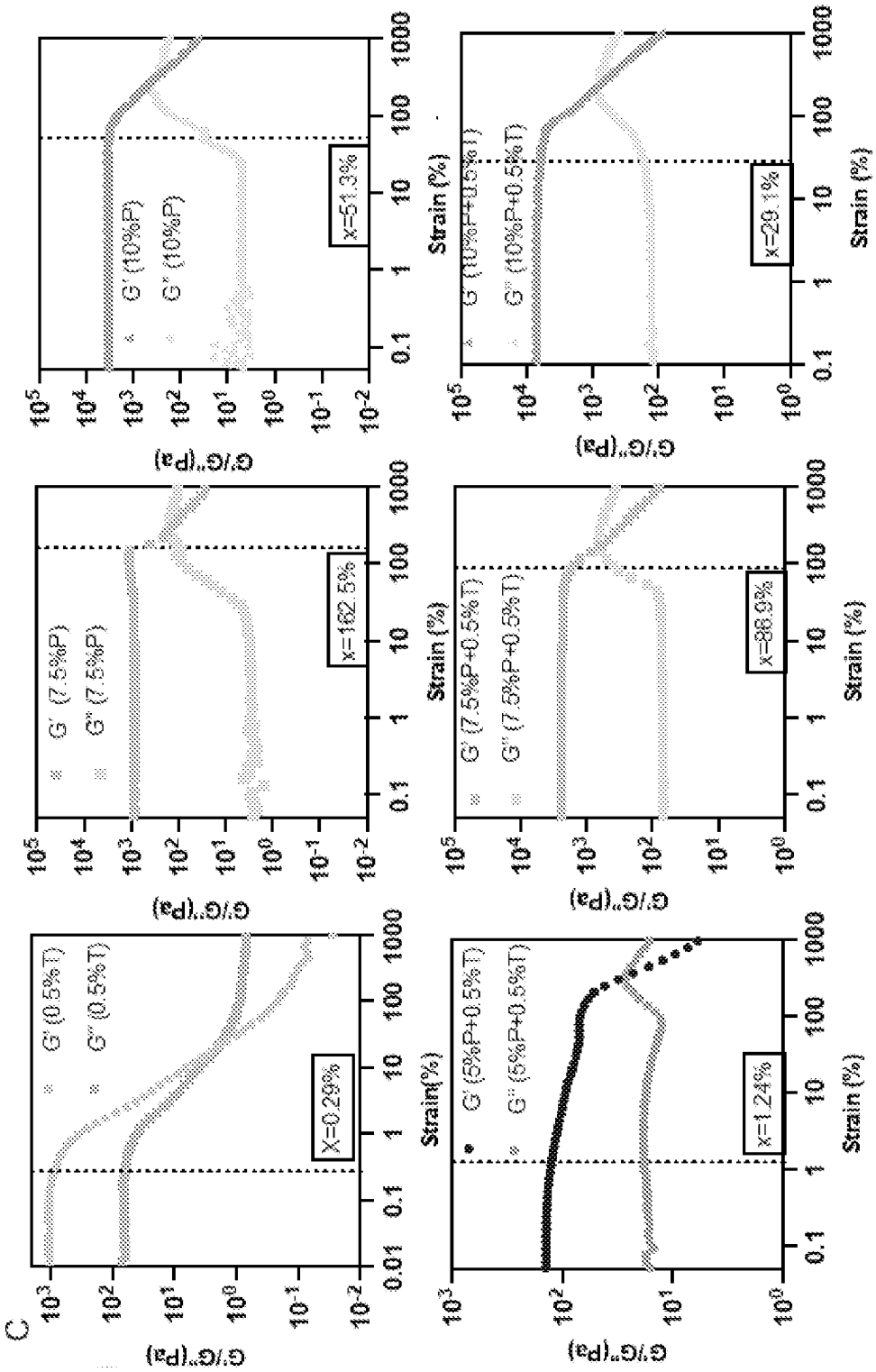


FIGURE 9

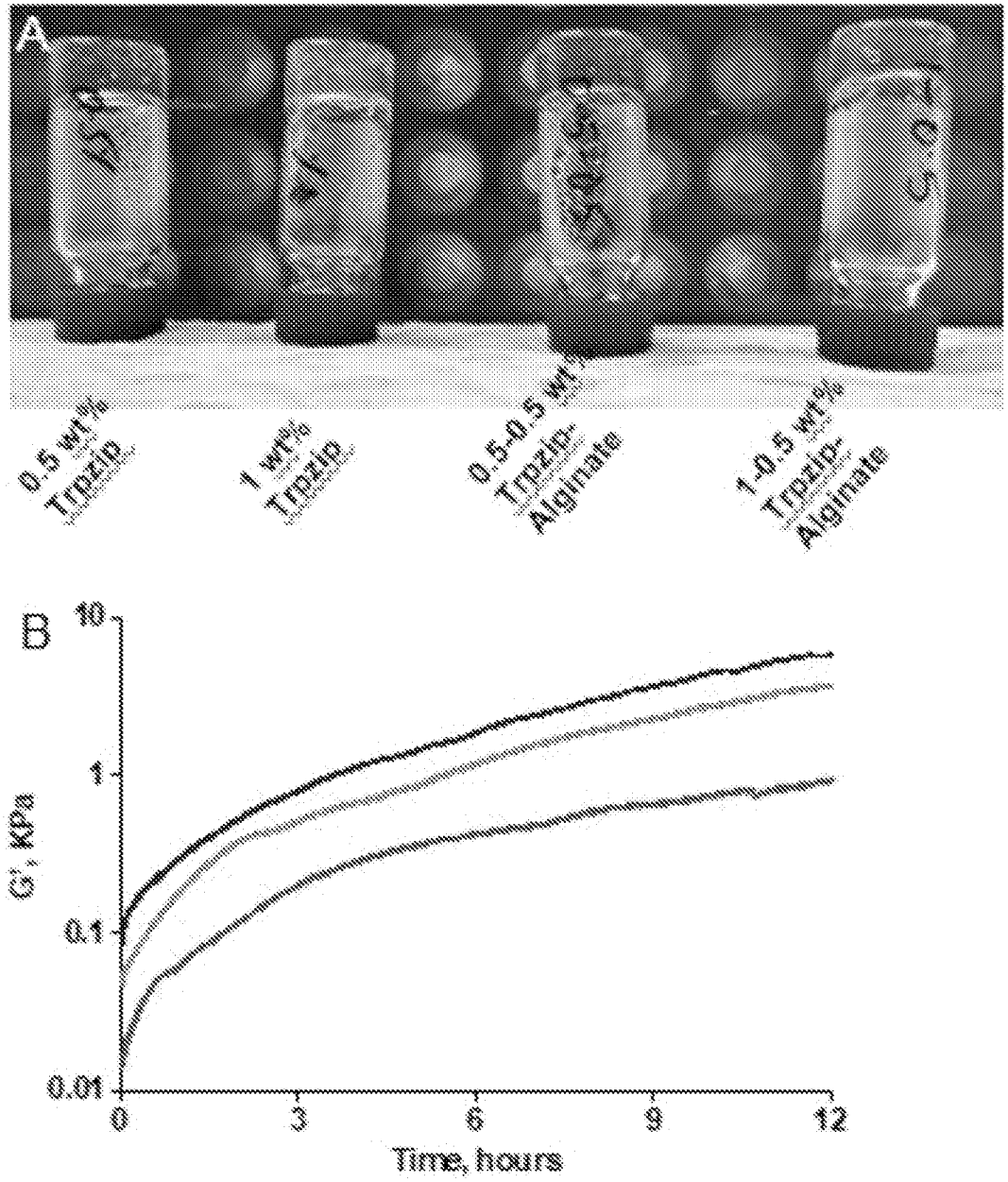

A
0.5 wt% Trazio
1 wt% Trazio
0.5-0.5 wt% Trazio-Alginate
1-0.5 wt% Trazio-Alginate
B
G', kPa
10
1
0.1
0.01
0
3
6
9
12
Time, hours

FIG 9 (CONT)

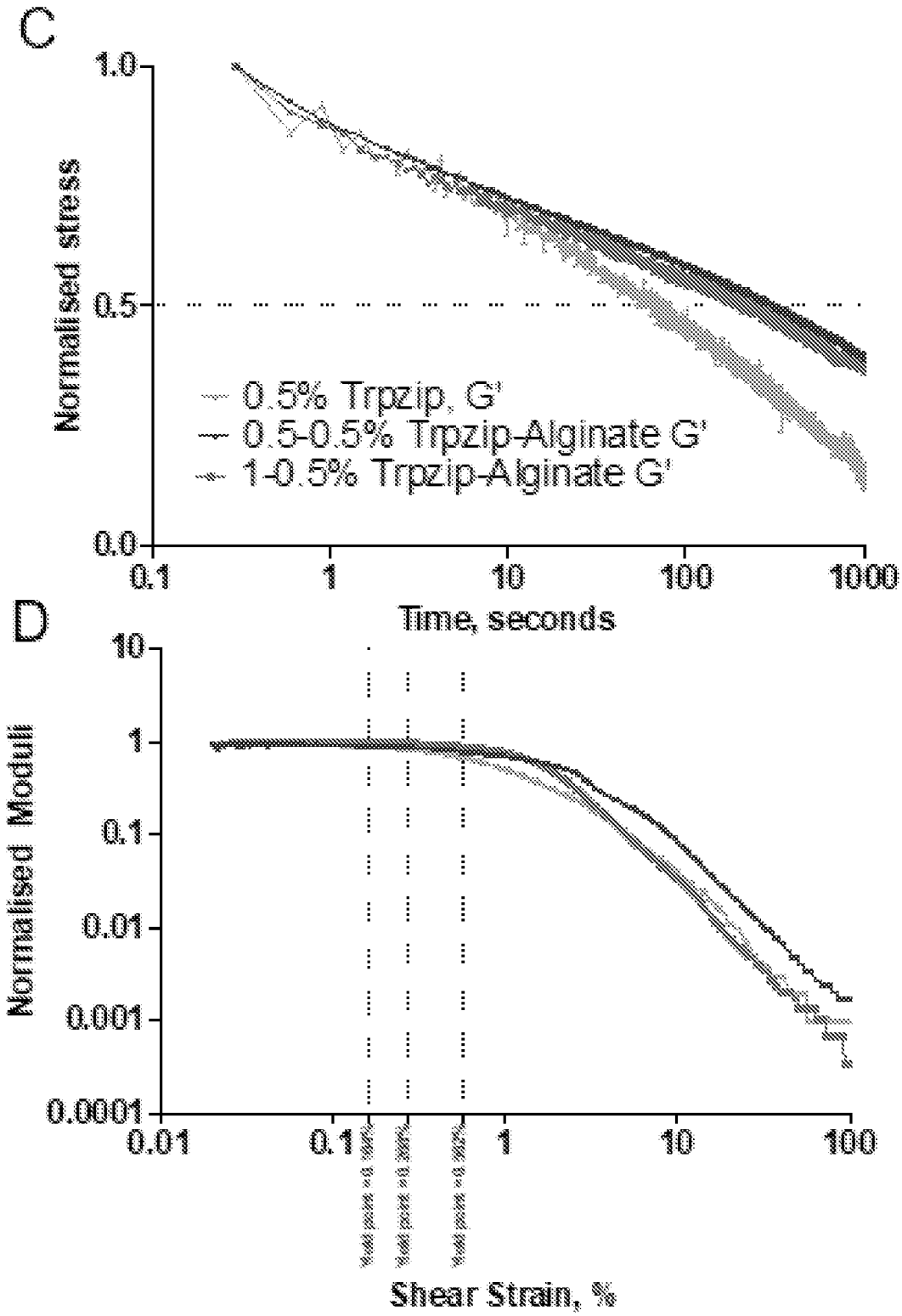

C
1.0
0.5
0.0
Normalised stress
0.5% Trpzip, G'
0.5-0.5% Trpzip-Alginate G'
1-0.5% Trpzip-Alginate G'
0.1
1
10
100
1000
Time, seconds
D
10
1
0.1
0.01
0.001
0.0001
Normalised Moduli
0.01
0.1
1
10
100
Shear Strain, %

FIG 9 (CONT)

E

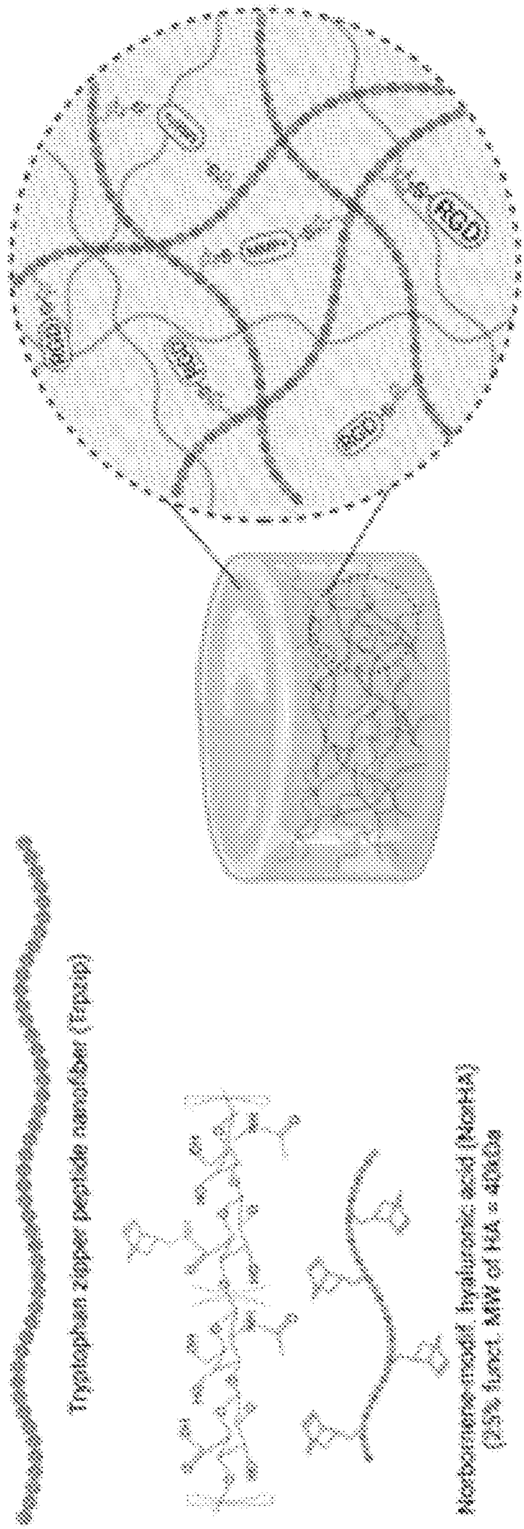


FIG 9 (CONT)

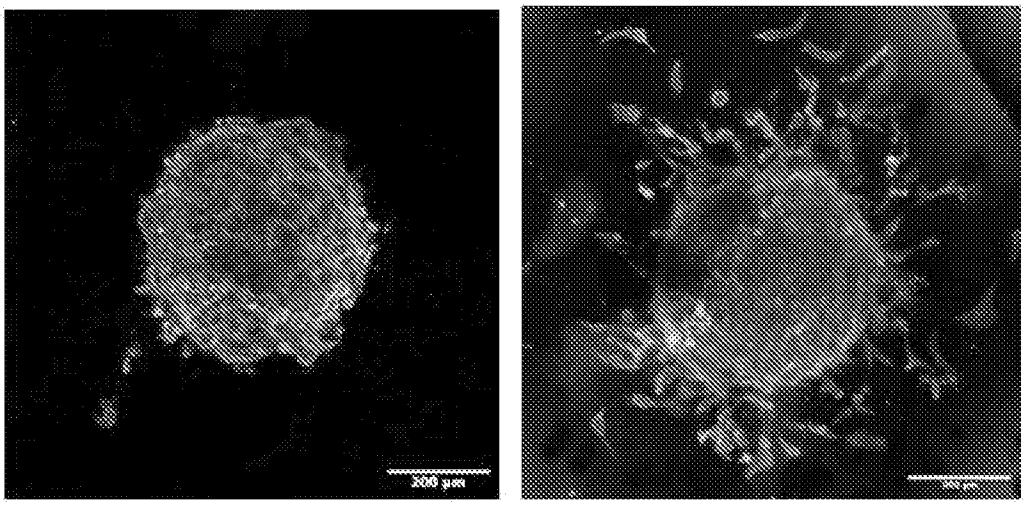


FIG 10

A

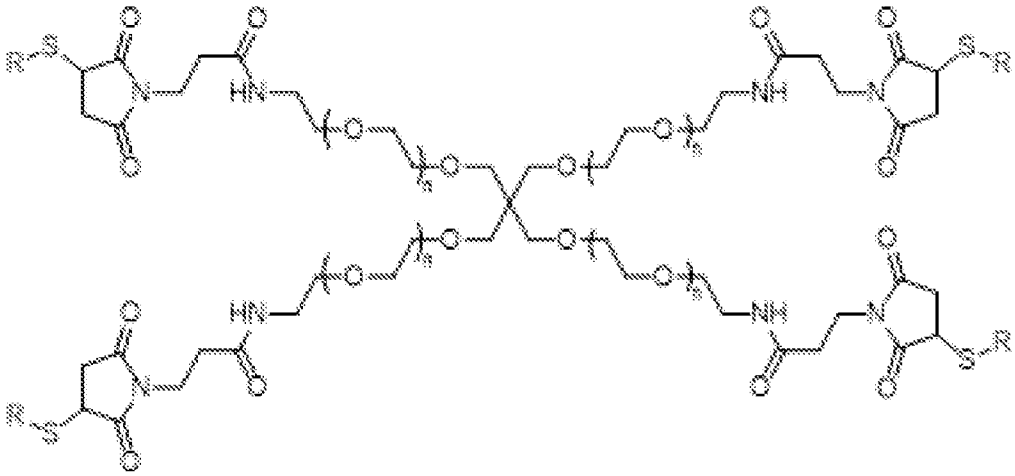


FIG 10(CONT)

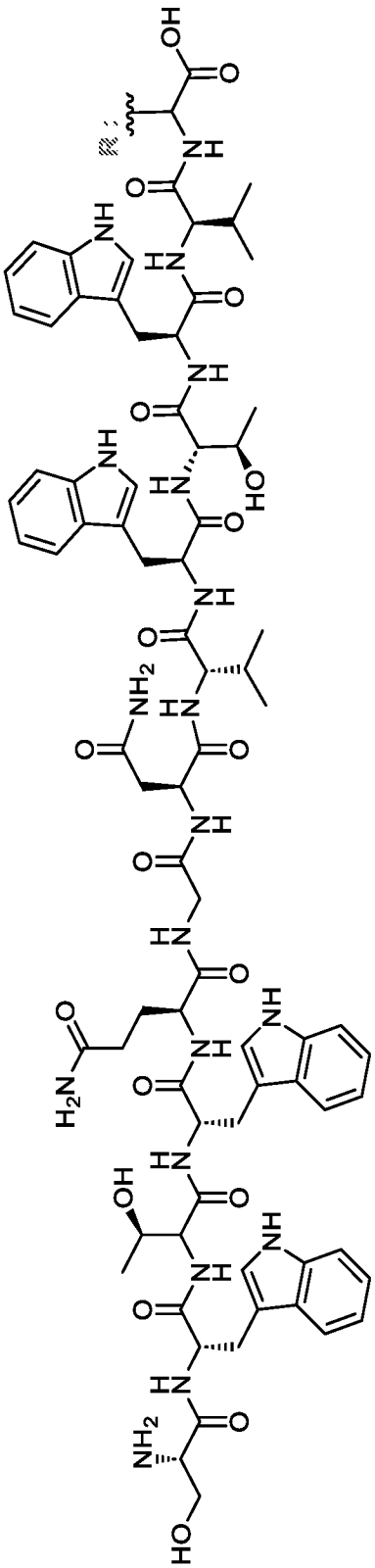


FIG 10(CONT)

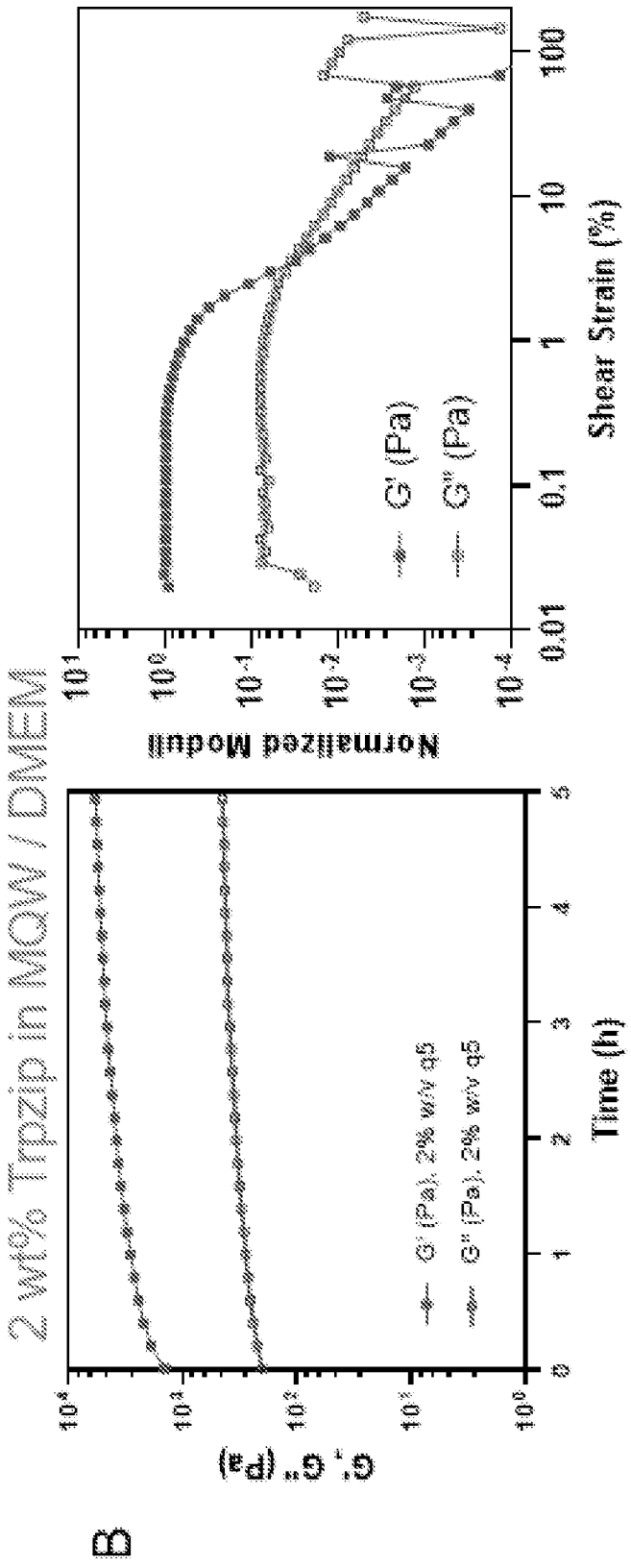
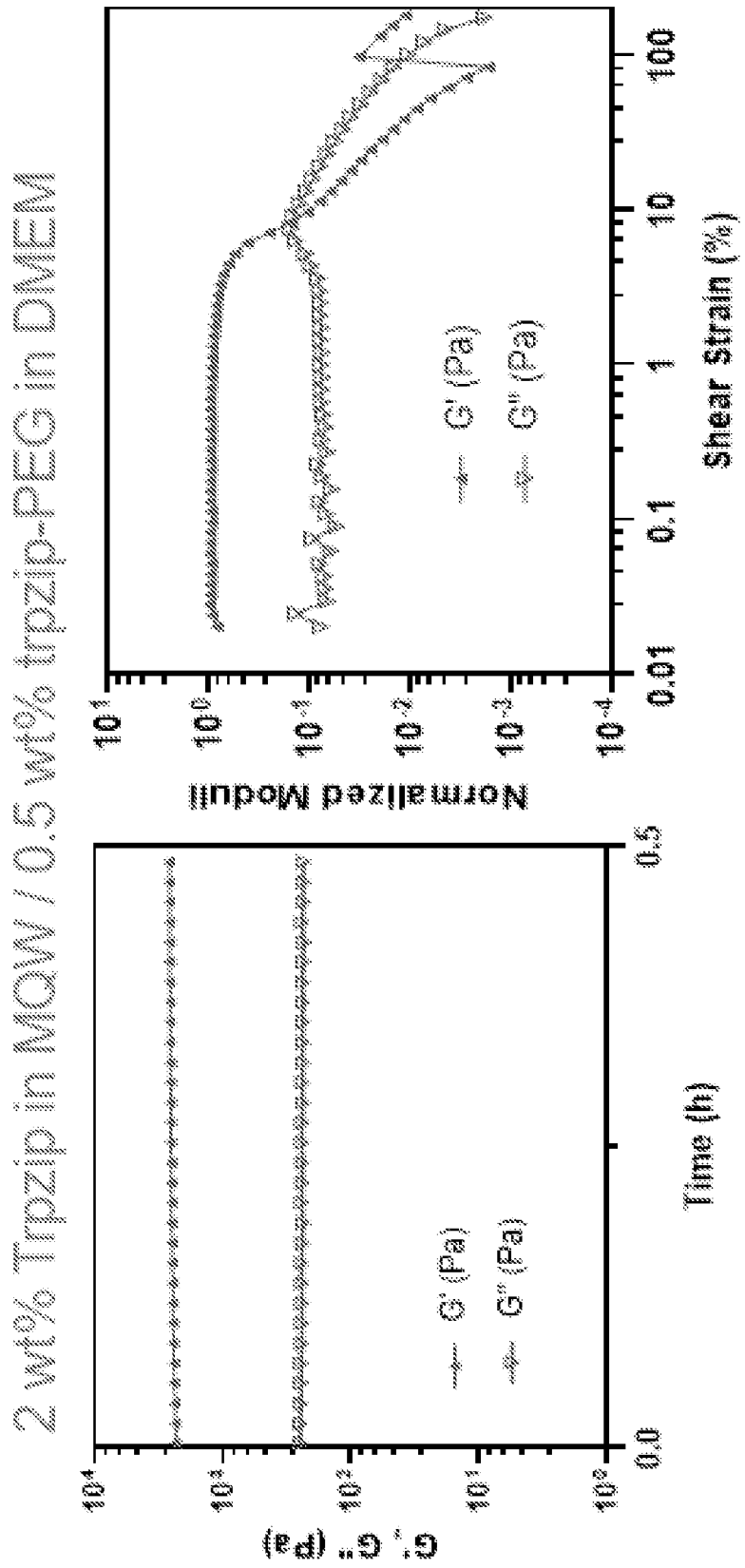

B
2 wt% Trpzip in MQW / DMEM
G', G'' (Pa)
Time (h)
G' (Pa), 2% w/v q5
G'' (Pa), 2% w/v q5
Normalized Moduli
Shear Strain (%)
G' (Pa)
G'' (Pa)

FIG 10(CONT)


2 wt% Trpzip in MQW / 0.5 wt% trpzip-PEG in DMEM
G', G" (Pa)
Time (h)
G' (Pa)
G" (Pa)
Normalized Moduli
Shear Strain (%)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2024/050729

A. CLASSIFICATION OF SUBJECT MATTER

C07K 7/08 (2006.01) A61L 27/22 (2006.01) A61L 27/52 (2006.01) C12N 5/071 (2010.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN sequence searches. Files: Registry, CaPlus. Sequences searched: WTW[EKVQ]GN[EKVQ]WTW; QGNV. STN Keyword search. File: CaPlus. Keywords: Hydrogel?; extracellular?; matri?; trpzip?; Tryptophan zipper. GenomeQuest sequence searches (GenePAST, Motif). Sequences searched: WTW[EKVQ]GN[EKVQ]WTW; QGNV. Databases: GQ- Pat GoldPlus protein, GQ-Pat Platinum protein, Protein Data Bank, Genpept (Translated Genbank), ENSEMBL protein, Swiss-Prot, RefSeq, Translated EMBL, Genpept (SARS-CoV-2 protein).

Applicant/Inventor search. Files: IP Australia internal databases, DOCDB, DWPI. Keywords: NEW SOUTH INNOVATIONS PTY LIMITED (and variations thereof); NGUYEN, Ashley Khanh Mai (and variations thereof); KILLIAN, Kristopher Alan (and variations thereof).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

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"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 September 2024	17 September 2024

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2024/050729**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2003/0175799 A1 (COCHRAN ET AL.,) 18 September 2003 See Table 1	1-39
A	Cochran, A. G. *et al.*, "Tryptophan Zippers: Stable, Monomeric β-Hairpins", PNAS. 2001, vol. 98(10), pages 5578-5583. See Table 1	1-39
A	Desai, R. *et al.*, "Mechano-Sensitive Peptide-Polymer Hydrogels", Abstracts of Papers, 245th ACS National Meeting & Exposition, New Orleans, LA, United States, April 7-11, 2013. American Chemical Society, Washington, D.C. See entire abstract	1-39
A	Fang, Y. *et al.*, "Biomaterial-Interrelated Bacterial Sweeper: Simplified Self-Assembled Octapeptides with Double-Layered Trp Zipper Induces Membrane Destabilization and Bacterial Apoptosis-Like Death", Small Methods. 2021, vol. 5: 2101304, pages 1-19. See abstract; Figure 1B	1-39
A	Chen, L. *et al.*, "Molecular Dynamics and Ion Mobility Spectrometry Study of Model ß-Hairpin Peptide, Trpzip1", The Journal of Physical Chemistry A. 2011, vol. 115, pages 4427-4435. See abstract and Table 1	1-39
P,X	Nguyen, A. K. *et al.*, "Hierarchical Assembly of Tryptophan Zipper Peptides into Stress-Relaxing Bioactive Hydrogels", Nature Communications. 23 October 2023, vol 14:6604, pages 1-13. See entire document	1-39

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2024/050729**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2003/0175799 A1	18 September 2003	US 2003175799 A1	18 Sep 2003
		US 6914123 B2	05 Jul 2005
		US 2005196810 A1	08 Sep 2005
		US 7229777 B2	12 Jun 2007

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.