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Thin Peptide Hydrogel Membranes Suitable as Scaffolds for Engineering Layered Biostructures

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Abstract

A short tetramer peptide, Ac-IVKC, spontaneously formed a hydrogel in water. Disulfide bonds were introduced via hydrogen peroxide (H_2O_2)-assisted oxidation, resulting in $(\text{Ac-IVKC})_2$ dimers. The extent of disulfide bond formation and gel stiffness increased with the amount of H_2O_2 used and 100% dimerization was achieved with 0.2% H_2O_2 . The resultant gel achieved an elastic modulus of ~ 0.9 MPa, which to our knowledge, has not been reported for peptide-based hydrogels. The enhanced mechanical property enabled the fabrication of thin and transparent membranes. The hydrogel could also be handled with forceps at mm thickness, greatly increasing its ease of physical manipulation. Excess H_2O_2 was removed and the membrane was then infused with cell culture media. Various cells, including primary human corneal stromal and epithelial cells, were seeded onto the hydrogel membrane and demonstrated to remain viable. Depending on the intended application, specific cell combination or membrane stacking order could be used to engineer layered biostructures.

Statement of significance

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A short tetramer peptide – Ac-IVKC – spontaneously formed a hydrogel in water and disulfide bonds were introduced via hydrogen peroxide (H_2O_2)-assisted oxidation. The extent of disulfide-bond formation and gel stiffness were modulated by the amount of H_2O_2 . At maximum disulfide-bond formation, the hydrogel achieved an elastic modulus of ~ 0.9 MPa, which to our knowledge, has not been reported for peptide-based hydrogels. The enhanced

mechanical property enabled the fabrication of thin transparent membranes that can be physically manipulated at mm thickness. The gels also supported 3D cell growth, including primary human corneal stromal and epithelial cells. Depending on the intended application, specific combination of cells or individual membrane stacking order could be used to engineer layered biostructures.

Keywords: Ultrashort peptide hydrogel, disulfide bonds, tissue engineering, scaffolds, layered biostructures, membranes

1. Introduction

Pioneering reports demonstrating the potential of tissue engineering appeared in the 1980s [1]; and by early 1990s, its principles had been formally conceptualized [2]. Essentially, the premise of tissue engineering is that a collection of cells, when grown in an appropriate extracellular matrix (ECM)-like environment and subjected to relevant biochemical and biophysical cues, can be used to repair or reconstruct a tissue or organ as part of regenerative medicine. As such, much research efforts have focused on the design of the scaffolding materials that can structurally support three dimensional (3D) cell growth [3].

One approach to achieve 3D cell growth is to first culture cells in 2D close to confluency. The cellular monolayer can then be removed from the 2D surface by enzymatic digestion, mechanical scrapping [4, 5] or more elegantly, by lowering the ambient temperature below the lower critical solution temperature of a surface-coated thermosensitive polymer, such as poly(*N*-isopropylacrylamide) [6]. Often, the cell sheet should be preserved in its entirety. The cell sheet can next be stacked or arranged as desired to yield higher-ordered structures. This technology, termed “cell sheet engineering” [7], has been used in various applications, such as dermal [4, 8], vascular [5], cardiac [9], hepatic [10], oesophageal [11], urothelial [12] as well as dental [13].

Another approach is to use scaffolds that can offer greater structural support for cell growth. Such scaffolds can be in the form of membranes obtained directly from an animal or human origin. Examples include matrigel (a basement membrane secreted by murine sarcoma cells) [14], reconstituted rat cardiac ECM [15], decellularized porcine small intestinal submucosa [16] and human amniotic membrane [17]. Alternatively, scaffolds can be fabricated by using hydrogels [18].

Hydrogels are water swollen, 3D structures consisting of an interwoven, porous network of fibers that allow the percolation of cells, nutrients and oxygen. Hydrogels

therefore provide a microenvironment that closely mimics the ECM. A large spectrum of naturally-derived (gelatin [19], chitosan [20], hyaluronic acid [21] and collagen [22, 23]) and synthetic polymers (poly(vinyl alcohol)-acrylamide [24], poly(lactic-*co*-glycolic acid) [25], poly(ϵ -caprolactone) [26], poly(glycolic acid) [27], poly(*N*-isopropylacrylamide) [28] and poly(ethylene glycol) [21]) have been investigated alone or in combination to form hydrogels for tissue engineering. However, the use of naturally-derived scaffolds is laden with concerns over chemical heterogeneity, susceptibility to batch variation and the risk of immunogenicity as well as pathogen transmission. On the other hand, complicated and lengthy synthesis or the requirement for toxic reagents may ultimately limit the biomedical applicability and economic feasibility of industrial production of synthetic scaffolds.

Peptide-derived hydrogels based on naturally-occurring amino acids (AA) are thus attractive as scaffold materials. They are biocompatible and can be facilely batch-synthesised with precise control over its sequence and polydispersity. The various families of peptide hydrogels being investigated have been reviewed extensively elsewhere [29-31]. Our group, for instance, focuses on ultrashort peptides whose sequences consist of three to seven AA [32, 33]. These AA are arranged in a gradient of hydrophobicity which causes them to self-assemble spontaneously into physical hydrogels. The synthesis of peptides that are short in length is advantageous from a cost perspective. Recently, we have further improved the hydrogel in terms of its functionality and mechanical properties by introducing cysteine-mediated disulfide bonds into the nanotopography of the hydrogels [34]. Bioconjugation with the RGD motif can now also be easily performed [34]. This family of peptide hydrogels has since been demonstrated to be useful as a carrier for anti-cancer drugs [35], anti-microbial agents [36] or as a dressing to promote the healing of burn [37] and incision [38] wounds. We now report on a particular cysteine-containing peptide, Ac-IVKC, whose stiffness increased tremendously after oxidative disulfide bond formation. This enhancement in mechanical

property enabled the preparation thin and transparent peptide hydrogel membranes. For the first time in our laboratory, these hydrogels can be physically manipulated and cut to mm thickness. This, in turn, permitted their use to build up thin, cell-loaded, layered biostructures.

2. Materials and methods

2.1 Extent of disulfide bond formation by ultra performance liquid chromatography (UPLC)

Peptide Ac-Ile-Val-Lys-Cys (Ac-IVKC) was synthesised by American Peptide Company (CA, USA) with 95.6% purity (Figure S1 in supplementary information). To study the extent of disulfide bond formation, Ac-IVKC was first dissolved in PBS (pH 7.2, Invitrogen, Singapore) containing varying amount of hydrogen peroxide (H_2O_2) (Merck Millipore, Singapore), followed by incubation at room temperature for 24 hours. Samples were then injected into an Aquity UPLC (Waters, USA) fitted with an UV detector and a single-quadrupole mass spectrometer.

2.2 Oscillation rheology

Gel discs were formed by transferring 200 μL of peptide solution (dissolved in PBS containing varying amount of H_2O_2) into a custom-made hollow ring cast with an internal diameter of 0.8 cm. Gelation occurred at room temperature over 24 hours before the gel discs were loaded onto an ARES-G2 (TA Instruments, USA) for rheological measurements. Frequency-sweep studies were performed at angular frequency, $\omega=0-100$ rad/s and strain, $\gamma=0.1\%$. The gel stiffness was represented by plotting elastic modulus, G' against ω ($n=3-4$).

2.3 Transmission electron microscopy (TEM)

10 μL of a diluted Ac-IVKC gel sample (10 mg/mL) was dripped onto glow-discharged formvar-carbon-coated 200 mesh copper grids (Electron Microscopy Sciences).

After one min of incubation, grids were rinsed with distilled water and contrasted with 4% uranyl acetate. Grids were then analysed with a TEM (JEM1010, JEOL) operating at 80kV equipped with a bottom-mounted SIA model 12C high resolution full-frame CCD camera (16 bit, 4K).

2.4 Quantification of H₂O₂ removal from gel

200 μ L of peptide solution (dissolved in PBS containing 0.2% H₂O₂) was transferred into a 48-well plate and allowed to gel overnight at room temperature. After which, excess H₂O₂ not consumed by disulfide bond formation was removed by layering 500 μ L of PBS over the gel. At appropriate time points, the entire supernatant was aspirated for testing and replaced with fresh PBS. The amount of H₂O₂ in the supernatants was quantified with a peroxide assay kit (Pierce PeroXOquant, Thermo Fisher Scientific, Singapore). The amount of residual H₂O₂ in the gel, at the end of purification, was similarly quantified. Therefore, the sum of H₂O₂ present in the gel and supernatants represented the starting amount of H₂O₂ (100%) before purification. The cumulative amount of H₂O₂ removed was plotted as a function of time.

2.5 Casting of thin Ac-IVKC membrane

Peptide solutions were prepared and enclosed between two flat glass plates separated by spacers of known thickness. The ends of the glass plates were sealed with parafilm to prevent excessive evaporation. After incubation at room temperature for 24 hours, the glass plates were carefully peeled apart to yield thin transparent membranes of Ac-IVKC hydrogel.

2.6 Cell culture and viability

Thin membranes of Ac-IVKC hydrogel were formed as above and cut into 1×1 cm squares using a surgical blade. The membranes were then transferred with forceps into 8-chamber Lab-Tek wells (Nunc, NY, USA). To prepare for cell culture, excess H₂O₂ was removed from the gel using a dialysis-inspired method [34].

Mouse corneal endothelial cells [39] and human dermal fibroblasts (Lonza, Singapore) were cultured in DMEM (Invitrogen) completed with 1% penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen) and maintained at 37°C and 5% CO₂. During cell seeding, 0.5 mL of cell suspension was deposited onto the purified gel membrane, which was returned to the incubator to allow for attachment and proliferation. 0.5 µL of calcein-AM (Invitrogen) was then added to the wells to identify live cells and images were obtained with an inverted confocal microscope (LSM5 DUO, Carl Zeiss, Germany).

2.7 Sequential seeding of human corneal cells and live cell imaging

Human corneal stromal fibroblasts (HCSF) were isolated from the corneas of cadaveric donors (SightLife, WA, USA) as previously described [40]. Briefly, the Descemet's membrane/endothelial layer was carefully peeled away and any remnant epithelial layer was scraped off. Stromal buttons were then washed with serum free F99 medium (1:1 Ham's F12 and M199, Thermo Fisher Scientific) and digested in collagenase IV (2mg/ml) overnight. Stromal keratocytes released from the stromal button were washed with PBS, and plated in growth factor-reduced matrigel (Corning Inc., NY, USA, diluted 1:50 with DMEM/12 serum free medium from Life Technologies, USA) coated plates. The cells were cultured with F99 medium, supplemented with 5% FBS, 20 ng/ml ascorbic acid (Sigma-Aldrich), 1×insulin-transferrin-selenium (Lonza, Singapore), 1×antibiotic/antimycotic (Thermo Fisher Scientific) and 10 ng/ml of fibroblast growth factor-2 (FGF-2, Merck

Millipore). Corneal stromal keratocytes were transformed into corneal stromal fibroblasts in serum-supplemented medium, which was refreshed every alternate day.

Human corneal epithelial cells (HCEC) were commercially obtained (Merck Millipore, SCCE016) and cultured on tissue culture polystyrene (TCPS) surfaces in serum-free EpiGRO ocular epithelia complete media kit (Millipore, Darmstadt, Germany) as per manufacturer's recommendations.

Thin Ac-IVKC hydrogels were prepared as above in 4-chamber Lab-Tek II chamber slides (Nunc). Prior to cell seeding, HCSF and HCEC were treated, respectively, with the green or deep red CellTracker CMFDA dye (Molecular Probes, Singapore). On day 0, HCSF were seeded at 8×10^5 cells/well and allowed to percolate through the gel. On day 2, culture medium was changed to EpiGRO medium for equilibration before HCEC was seeded onto the same gel at 8.5×10^5 cells/well. Cells were allowed to attach for another 24 hours before live-cell imaging.

For live-cell imaging, the chamber walls were removed and the gel was sliced into thin cross-sectional pieces. These pieces were then oriented, laid down on ProLong Gold Antifade Mountant with DAPI (Molecular Probes) and viewed under a fluorescent microscope (Olympus IX81, Singapore).

2.8 Polymerase chain reaction (PCR)

Total RNA was extracted using RNeasy kit (Qiagen, Singapore) with DNase digestion according to the manufacturer's instructions and then reverse transcribed using Superscript III first strand synthesis kit (Thermo Fisher Scientific). The resulting cDNA was used for quantitative PCR with gene-specific primer pairs (Table S1). PCR was performed with the FastStart Universal SYBR green master mix (Roche Life Sciences, Singapore) using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Singapore). Gene expression data was

calculated according to the Bio-Rad CFX manager software program. All expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression level.

2.9 Light transmittance study

Ac-IVKC gels were filled into a quartz cuvette with a path length of 1 mm and allowed to gel overnight. Light transmittance study was then performed with a U-2800 spectrophotometer (Hitachi, Japan) across the entire visible light spectrum of 400-750 nm.

2.10 Statistical analysis

T-testing was performed where $p < 0.05$ was denoted by * and accepted to be statistically significant.

3. Results and discussion

We now describe a short tetramer peptide hydrogel – Ac-IVKC – whose mechanical stiffness increased by more than two orders after H_2O_2 -assisted oxidative disulfide bond formation. We first determined the minimum peptide concentration required to form a self-supporting gel within 24 hours at room temperature. Using PBS as solvent, a transparent gel capable of supporting its own weight in an inverted vial was achieved at 20 mg/mL. However, gels at this concentration were weak and gave inconsistent rheological data. Therefore, 25 mg/mL was tested and shown to reproducibly form Ac-IVKC gels. PBS was also the preferred solvent as water alone gave only a viscous liquid at 25 mg/mL.

To achieve simultaneous self-assembly and dimerization of Ac-IVKC gels, H_2O_2 was added to PBS and used directly to dissolve the peptide powder. The extent of disulfide bond formation can then be modulated by the amount of added H_2O_2 , as shown by UPLC analysis. Figure 1a shows that Ac-IVKC remained mostly as a monomer and was <4% dimerized in

the absence of H_2O_2 . Therefore, there was already a small extent of disulfide bond formation due to air-oxidation. Ac-IVKC, however, became ~100% dimerized in the presence of 0.2% H_2O_2 . The identities of the respective peaks were confirmed with mass spectrometry (Figure S2).

The stiffness of the gel, as reflected by its elastic modulus (G'), varied with the extent of disulfide bond formation. In the absence of H_2O_2 , Ac-IVKC gels achieved G' values of 3-5 kPa (Figure 1b). The resultant gel also appeared more viscous than solid-like, as shown in Figure 1c. Upon the addition of H_2O_2 , stiffness increased significantly and a G' of ~0.9 MPa was achieved with 0.2% H_2O_2 . Increasing the amount of H_2O_2 to 1%, however, did not increase gel stiffness any further, consistent with the earlier observation that Ac-IVKC was already completely dimerized with 0.2% H_2O_2 . To our knowledge, such a high G' value has not been reported for other peptide-based hydrogel systems. Dimerized Ac-IVKC gels appeared more solid-like and featured sharp, well-defined edges (Figure 1d-f).

The impact of disulfide bond formation on the increase in gel stiffness is interesting because each Ac-IVKC monomer had only one thiol group and thus, could maximally form dimers via a disulfide bond. However, it is important to understand that these disulfide bonds could be formed intra- or inter-peptide fibers, which extended throughout 3D space. This established a network which essentially “locked” the fibers in place and presumably resulted in the enhanced mechanical properties.

To understand the relationship between self-assembly, disulfide bond formation and mechanical property, dimerized Ac-IVKC gels [25 mg/mL (0.05 M) in PBS containing 0.2% H_2O_2 (0.06 M)] were prepared and subsequently freeze-dried. Water lacking or containing the reducing agent, dithiothreitol (DTT, 0.07 M), was then added to the lyophilized gels and vortex-mixed before being re-cast to form gels. The volume used for reconstitution was equal to the volume of water lost during lyophilization. In both cases, gels were re-formed within

24 hours after reconstitution. A representative image of a gel reconstituted in water can be seen in Figure S3a.

Gels reconstituted in water alone were less stiff compared to before lyophilization (Figure S3b). This suggests the importance of starting the initial phase of gel formation with monomeric Ac-IVKC – during which, self-assembly of the monomeric Ac-IVKC occurred concurrently with disulfide bond formation. Once formed, dimerized gels reconstituted in water did not self-assemble in the same fashion and could not recover its original stiffness. As expected, reducing the dimerized gels with DTT also produced gels that were less stiff compared to before lyophilization. Interestingly, dimerized gels reconstituted in DTT were still stiffer than gels formed without disulfide bonds in the first place (0% H_2O_2) and dimerized gels reconstituted in water. This again suggests that the self-assembly process is sensitive to the starting state of the peptide (i.e., monomeric or dimeric Ac-IVKC). Consequently, the assembly process can take on a different trajectory, eventually producing gels with different mechanical properties. Taken together, this highlights the importance of beginning the process of self-assembly and disulfide bond formation with monomeric Ac-IVKC, in order to achieve gels with high stiffness.

TEM revealed that the nanotopography of Ac-IVKC gels prepared with 0.2% H_2O_2 consisted of an interwoven meshwork of fibers (Figure 1g), with diameters mostly between 10-20 nm (Figure 1h).

The enhanced mechanical property enabled the fabrication of thin hydrogel membranes using Ac-IVKC in the presence of 0.2% H_2O_2 . This was previously not possible as gels were too fragile to be physically handled at mm thickness. To prepare thin membranes, the peptide solution was enclosed within two flat glass plates separated by a 1 mm-thick spacer. As observed in Figures 2a-b, the gel membrane was clear and >95% transparent to the entire visible light spectrum (400-750 nm). It is thus suitable for

applications requiring optical clarity. Upon carefully peeling apart the glass plates, the membrane still had sufficient mechanical integrity to be picked up with a pair of forceps (Figure 2c). This greatly facilitates the physical manipulation of the membrane and opens up new avenues in terms of possible biomedical applications, especially in cases where it is important for the scaffold to be thin and strong at the same time. A caveat, however, is that the membrane is generally brittle at the same time (due to its high stiffness) and probably less suitable for applications requiring high levels of membrane flexibility.

To make the hydrogel membrane compatible with cells, excess H_2O_2 had to be removed. This was achieved by soaking the membrane in PBS and periodically refreshing the external PBS sink after it had accumulated H_2O_2 leached from the membrane. Doing so, it was determined that ~96% and 99% of H_2O_2 was removed after 8 and 24 hours respectively (Figure 3).

To further prepare the membranes for cell culture, completed growth medium was used instead of PBS during the penultimate exchange. This served to infuse the membranes with nutrients and also to regulate the pH. For visual effects, phenol red was either present or absent in the growth media to colour-code the membranes. As an example, nine dimerized Ac-IVKC hydrogel membranes were stacked on top of one another to create a layered structure (Figure 2d). Here, the membranes were simply stacked upon one another. For future applications, tissue glue can be used to secure the membranes in place, prior to the cells producing enough ECM to further bond the structure together. The pink tinge observed in the phenol red-stained membranes further confirmed the ideal pH condition for cell growth. Depending on the specific application, cells can be seeded into the individual layers before layering. Here, mouse corneal endothelial cells (Figure 2e) were seeded into membranes stained with phenol red, while human dermal fibroblasts (Figure 2f) were seeded into

membranes without. As evidenced by the positive calcein staining, both populations of cells remained viable within the membranes.

Alternatively, cells can be seeded sequentially onto a single gel membrane to mimic a layered biostructure. As an example, human corneal stromal fibroblasts (HCSF) were first seeded into the gel, followed by human corneal epithelial cells (HCEC) (schematic in Figure 4a). This sequence is reminiscent of the two outermost layers of the human cornea, which account for the bulk of its thickness. To discriminate between the two populations, cells were pre-stained with different cell-tracking dyes prior to seeding. For illustration, cells were seeded individually on separate membranes in an initial experiment and observed top-down using a confocal microscope. As can be seen, HCEC were pre-stained red (Figure 4b) while HCSF were pre-stained green (Figure 4c). The successful staining of the cells also indicated that both cell populations remained viable within the gel. Next, the layering of cell populations was visualized by seeding the two cell populations sequentially on a single membrane, as aforementioned. The membrane was then sliced into cross-sectional strips using a scalpel and oriented accordingly onto slides for microscopic examination. Such a side-on or cross-sectional view revealed that the epithelial cells were successfully layered above the stromal cells (Figure 4d-e). Cells were also seen to percolate into the depth of the porous gel, consistent with observations reported previously [34].

PCR was performed on both HCEC and HCSF to further investigate the effects of Ac-IVKC gel culture on selected gene expression. Expression of HCEC markers such as cytokeratin 18 (CK18) [41], gap junction protein CX43 [42] and ocular surface marker PAX6 [43] were found to be higher in cells cultured on Ac-IVKC gel, compared to the control surface (Figure 4f). Transcriptional factor p63, however, was two-fold lower. p63 has been reported to be a marker for corneal epithelial progenitor cells and limbal stem cells [44]. Its expression was also reported to be lower in mature adult cells [45]. Hence, this gene

expression profile collectively suggests that HCEC cultured on Ac-IVKC gels may be of a more matured phenotype compared to control.

For HCSF, expression levels of ALDH3A1, lumican and decorin were analysed. ALDH3A1 is a major soluble protein reported to be constitutively expressed in the corneas of human and other species [46]. Lumican and decorin belong to a class of keratan sulfate-containing proteoglycans, which plays a part in maintaining corneal transparency [47]. HCSF cultured on Ac-IVKC showed lower decorin and lumican expressions compared to the matrigel control, while expression of ALDH3A1 was higher (Figure 4g). Studies had reported that during wound healing, keratocytes activated by FGF-2 and TGF- β 1 decreased their levels of keratocan and lumican expression [48]. Furthermore, an increased expression of ALDH3A1 was correlated with proliferation by stem and cancer cells [49]. In this light, this expression profile suggests that the HCSF may be in a more proliferative state compared to the control.

The dimerized Ac-IVKC membrane described here may thus be useful in tissue engineering to build layered biostructures. Since this technology is easily scalable, it is especially applicable in cases where 3D bioprinting is either not feasible [50] or uneconomical as the cost increases exponentially with the scale of printing [51].

4. Conclusions

We have demonstrated that the tetrapeptide Ac-IVKC is able to spontaneously self-assemble in water into nanofibrous hydrogels and that chemical disulfide bonds could be introduced into the network via H₂O₂-assisted oxidation. The extent of disulfide bond formation and stiffness of the gel increased with the amount of H₂O₂ used and 100% dimerization was achieved with a concentration of 0.2% H₂O₂. The resultant gel achieved a G' value of ~0.9 MPa, which to our knowledge, has not been reported before for peptide-

based hydrogels. The enhanced mechanical property enabled the fabrication of thin membranes. Dimerized Ac-IVKC membranes were transparent, could be cut into slices using a scalpel and handled with forceps at mm thickness. This greatly increased its ease of physical manipulation. Excess H_2O_2 was removed from the membrane, which was subsequently infused with cell culture media. Various cells including primary human corneal stromal and epithelial cells were seeded onto the membranes and demonstrated to remain viable. Depending on the intended application, specific combination of cells or membrane stacking order could be used to engineer layered biostructures.

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Figure captions

Figure 1. Modulation of disulfide bond formation and mechanical stiffness of Ac-IVKC hydrogels with H_2O_2 . a) Ac-IVKC was dissolved in PBS containing various amount of H_2O_2 and incubated at room temperature for 24 hours before UPLC analysis. Ac-IVKC was <4% dimerized in the absence of H_2O_2 but became ~100% dimerized in the presence of 0.2% H_2O_2 . b) Stiffness increased tremendously after dimerization and a G' value of ~0.9 MPa was achieved with Ac-IVKC gels prepared with 0.2% H_2O_2 . Data was plotted as mean $\pm$ standard deviation of triplicates. c-f) Gross images of Ac-IVKC hydrogels formed in the presence of 0-0.2% H_2O_2 . g) Low (20k $\times$) and h) high (100k $\times$) magnification TEM images of diluted Ac-IVKC gels prepared with 0.2% H_2O_2 , revealing the dense fibrous network with fiber diameter ranging between 10-20 nm.

Figure 2. Harnessing the outstanding mechanical property of Ac-IVKC gels to construct layered biostructures. a) Thin transparent Ac-IVKC membrane dimerized with 0.2% H_2O_2 can be cast between two glass plates separated by a spacer of known thickness. b) Light transmittance study with Ac-IVKC gels (path length of 1 mm). c) The resulting gel with mm thickness can still be picked up with forceps. d) Layered biostructures can potentially be created with thin Ac-IVKC membranes. Shown here are nine Ac-IVKC membranes stacked together, with alternating membranes stained pink (infused with DMEM with or without phenol red) to increase visualization. Depending on application, relevant cell populations can be seeded onto the Ac-IVKC membranes. For illustration, e) mouse corneal endothelial cells were seeded onto the pink membranes while f) human dermal fibroblasts were seeded onto the colourless membranes. In both cases, cells were viable as evidenced by positive calcein staining. e-f) Each image consisted of a merger of several z-stacks.

Figure 3. Excess H_2O_2 was removed from Ac-IVKC gels using a dialysis-inspired method. Cumulatively, ~96% and 99% of H_2O_2 was removed after 8 and 24 hours, respectively. Data was plotted as mean $\pm$ standard deviation of triplicates.

Figure 4. a) Sequential seeding of HCSF (stained with a green live-cell tracker dye prior to seeding), followed by HCEC (red) on a single layer of thin Ac-IVKC hydrogel. b-c) Top-down views (merger of several z-stacks) of the respective cell populations where positive staining further confirmed the viability of cells in Ac-IVKC gel culture. d-e) Cross-sectional view showing that HCEC was successfully layered above the HCSF. Gene expression analysis of f) HCEC and g) HCSF cultured on Ac-IVKC or controls (tissue culture polystyrene, TCPS, or Matrigel) as determined by quantitative PCR. Genes analysed were tumor protein (p63), cytokeratin 18 (CK18), gap junction protein alpha 1 (CX43), paired box 6 (PAX6), aldehyde dehydrogenase 3 family member A1 (ALDH3A1), decorin (DCN) and lumican (LUM). All expression levels were normalized to GAPDH expression level and plotted as mean $\pm$ standard deviation of triplicates. T-test was performed where $p < 0.05$ was denoted by * and accepted to be statistically significant.

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Graphical abstract