Dynameric Collagen Self-Healing Membranes with High Mechanical Strength for Effective Cell Growth Applications

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Biomaterials

Dynameric Collagen Self-healing Membranes with High Mechanical Strength for Effective Cell Growth Applications

Yan Zhang[+],[a] Ye Zhang[+],[a] Han Cui,[a] Mihail Barboiu,*[b] and Jinghua Chen*[a]

Abstract: The fabrication of biocompatible adaptive materials with high stiffness and self-healing properties for medical applications is a challenging endeavor. Collagen is a major extracellular matrix component acting as a substrate for cell adhesion and migration. Dynamers are constitutional polymers whose monomeric components are linked through reversible bonds, able to modify their constitution through reversible exchange of their components. In the current work, we demonstrate that the rational combination of collagen and dynameric networks connected with reversible covalent imine bonds is a very important and previously unreported strategy to provide biocompatible membranes with self-healing ability and excellent mechanical strength. The key challenge in the construction of such membranes is the required adaptive interaction between collagen chains and the dynamic cross-linkers, preventing the formation of defects. For example, by varying structure and molecular lengths of the dynamers, the tensile strength of the dynameric membranes reach over 80 MPa, more than 400% higher than that observed for the reference collagen membrane, and the highest value for break strain found, was 19%. The self-healing properties were observed when reconnecting two membrane pieces or even from crushed status of the membranes. Moreover, both MTT assay and confocal laser scanning microscopy method demonstrated the good biocompatibility of the collagen membranes, leaving more than 90% viability for NIH 3T3 cells after 24 h co-culture.

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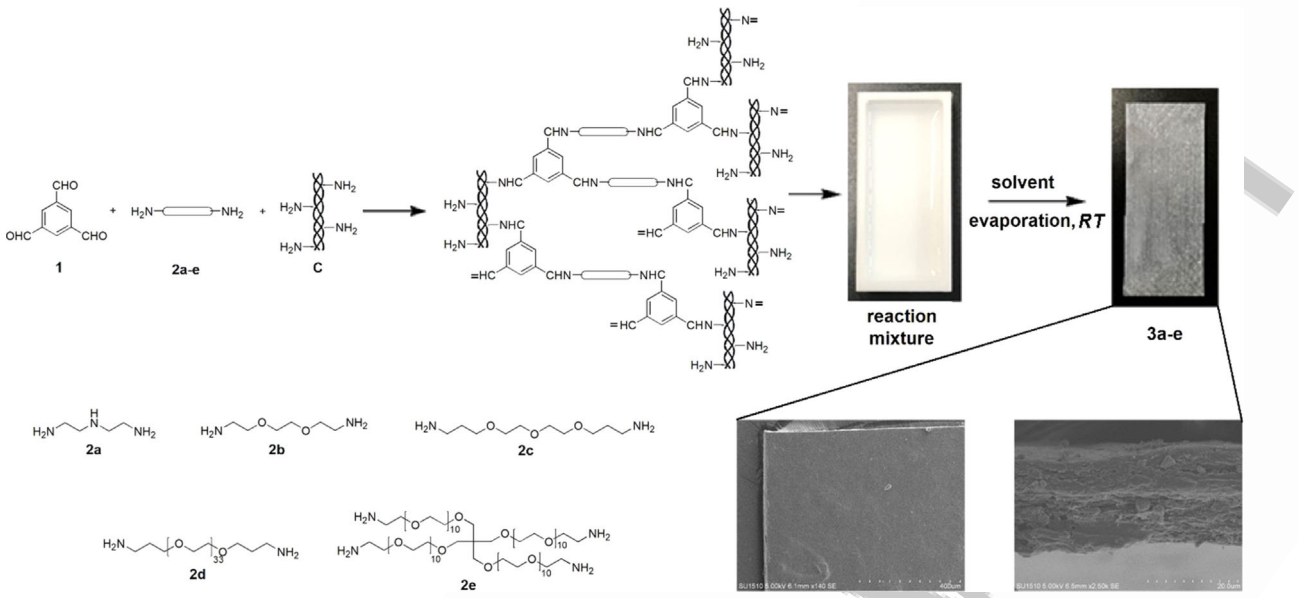
Constitutional dynamic chemistry (CDC) represent an evolutional approach to produce chemical diversity, implementing dynamic molecular/supramolecular interfaces between reversible interacting constituents.[1] CDC has been spreading into different fields, including materials,[2,3] catalysis,[4,5] drug discovery or[6,7] sensing,[8] among others.

Dynamic constitutional materials show practical multi-stimuli (i.e. light, temperature and pH)[9] or inherited self-healing properties,[10] for biomedical,[11] drug delivery,[12] tissue engineering[13] and recyclable plastics[14] applications. Essentially, such features are achieved through supramolecular interactions or dynamic covalent reactions, in which the component connections can be reversibly tuned. Most of the existing dynamic covalent materials are made of synthetic components, such as poly(dimethylsiloxane),[15] methacrylate,[16] polyethylene glycol,[17] and so on. Their biocompatibility is comparatively low to other biodynamers mostly based on chitosan,[18] which may induce inflammation responses when biomedicine related applications are concerned.

Among natural polymers, such as chitosan, gelatin, silk and cellulose, collagen is a main composite in extracellular matrix and contributes as the most abundant protein in mammals.[19a] With its good biocompatibility, high cell affinity and low antigenicity,[19b] collagen has been widely used for biomedical applications, including guided bone regeneration, corneal reconstruction,[20] wound dressing,[21] therapeutic contact lenses,[22] etc. However, pure collagen membranes usually lack desirable mechanical properties, which may bring obstacles for clinical uses. For instance, when barrier membranes are applied for prevention of postsurgical peritoneal adhesion,[23] mechanical stiffness is important to keep the material integrated during all operational procedures. Thus, different approaches have been developed to improve the properties of collagen membranes, for example, addition of cross-linkers such as oxidized polysaccharides[22] or β-cyclodextrin,[24] or mixing with other polymers,[25] including chitosan or cellulose. Despite all these efforts, the reported tensile stress values of the existing collagen membranes were mostly limited to a few MPa, lower than some other membranes from synthetic sources.

Amino-carbonyl/imine reversible chemistry is one of the most frequently adopted approach for building dynamic covalent materials under mild exchange conditions close to physiological environments.[26] Meanwhile, pH can be used to externally switch between condensed imine and hydrolyzed states, which in turn control the polymerization states.[27,28]

In the current work, we combined the dynamic polymers -dynamers- with collagen to provide biocompatible mem-



Scheme 1. Synthesis and preparation of the dynameric collagen membranes **3a**–**e** from collagen, **C** and dynameric frameworks of trialdehyde core centers **1** and polyamine connectors **2a**–**e**. Surface (**3e**) and cross-section (**3a**) images of the membranes have been obtained via scanning electronic microscpopy (SEM).

branes with good self-healing properties and strong mechanical stiffness for effective cell-growth applications. Upon air-drying method is aimed to be as simple and broadly applicable as possible to obtain thin and compact layer of collagen membranes. This study leads to a greater fundamental understanding of how swelling, self-healing, mechanical strength, degradation, thermostability and cell toxicity can be optimized at the nanoscale to optimize mechanical stiffness of newly designed collagen membranes that hold great potentials for various biomedical applications. The relatively straightforward quantification of mechanical strength of dynameric collagen, relevant to subsequently construct membranes in larger scale based on such probes, is a very important and previously unreported strategy.

Synthesis and Characterization of the Collagen Membranes. Collagen (C) is known for its triple helix structure with three polypeptide strands binding together, where extra amino groups can be found in multiple Lysines for further dynamic modifications.[29] In the current study, various dynameric frameworks cross-linkers were selected to connect the collagen through reversible imine bonds. The cross-linkers themselves were also dynamic polymers synthesized through imine formation reaction, starting from 1,3,5-benzenetricarboxaldehyde (**1**) core-centers and multi-amino-connectors of different lengths and hydrophilicity (Scheme 1), including short chain diethylenetriamine (**2a**), extended chains with 2,2′-(ethylenedioxy)bis(ethylamine) (**2b**) and 4,7,10-trioxa-1,13-tridecanediamine (**2c**), also polyethylene glycol (PEG) based polymers, such as polyethylene glycol bis(3-aminopropyl) (**2d**, $M_n \approx 1500$) and amino-terminated 4-armed-polyethylene glycol (**2e**). Trialdehyde **1** was chosen for its high cross-linking ability, whereas the water solubility of the resulted dynamers is relying on the addition of amines **2a**–**e**. The ratio between the trialdehyde and amines were set as 1:1 (for **2a**–**d**) or 1:0.6 (for **2e**), where one third of the aldehyde groups was left for further reaction with amino groups of collagen. The resulted dynameric cross-linkers were firstly dissolved in ethanol to make the stock solutions of 50 mM, which were subsequently mixed with collagen water solution (10 mM) and transferred to Teflon molds for natural evaporation of solvent. After air-drying for 2–3 days at RT, thin layers of dynameric collagen membranes were obtained (Scheme 1).

Fourier transform infrared (FT-IR) spectroscopy was used to analyze the structural changes of the materials. As shown in Figure S1, the vibrational band at 1692 nm of aldehyde groups (**1**) almost disappeared after cross-linking with collagen and formed the membranes, showing high conversions of the imine formation process. Moreover, the surface morphology of collagen membranes was observed by using scanning electron microscopy (SEM), in which dense and nonporous structures were identified (Scheme 1). From the cross-section images under SEM, the thickness of the membranes was determined to be around 20 μm thinner than the collagen membranes prepared from other methods.[23,30]

Tailoring the Mechanical Strength of the Collagen Membranes. The mechanical stiffness of the collagen membranes was measured by placing the samples (3 cm × 7 cm) to tensile and compression testers, with a 50 N crosshead at a speed of 5 mm min^{-1} at RT (Figure S2). The typical stress–strain curves for all membranes have been illustrated in Figure 1. The membrane composed of pure collagen show the smallest tensile stress (21.9 MPa) and strain at break (2.4%). The cross-linking with various dynamers improve its mechanical properties. Among the membranes with shorter dynamer cross-linkers, such as **3a** (39.1 MPa, 3.0%), **3b** (41.7 MPa, 4.8%) and **3c** (65.1 MPa, 9.9%), both the values of tensile stress (up to

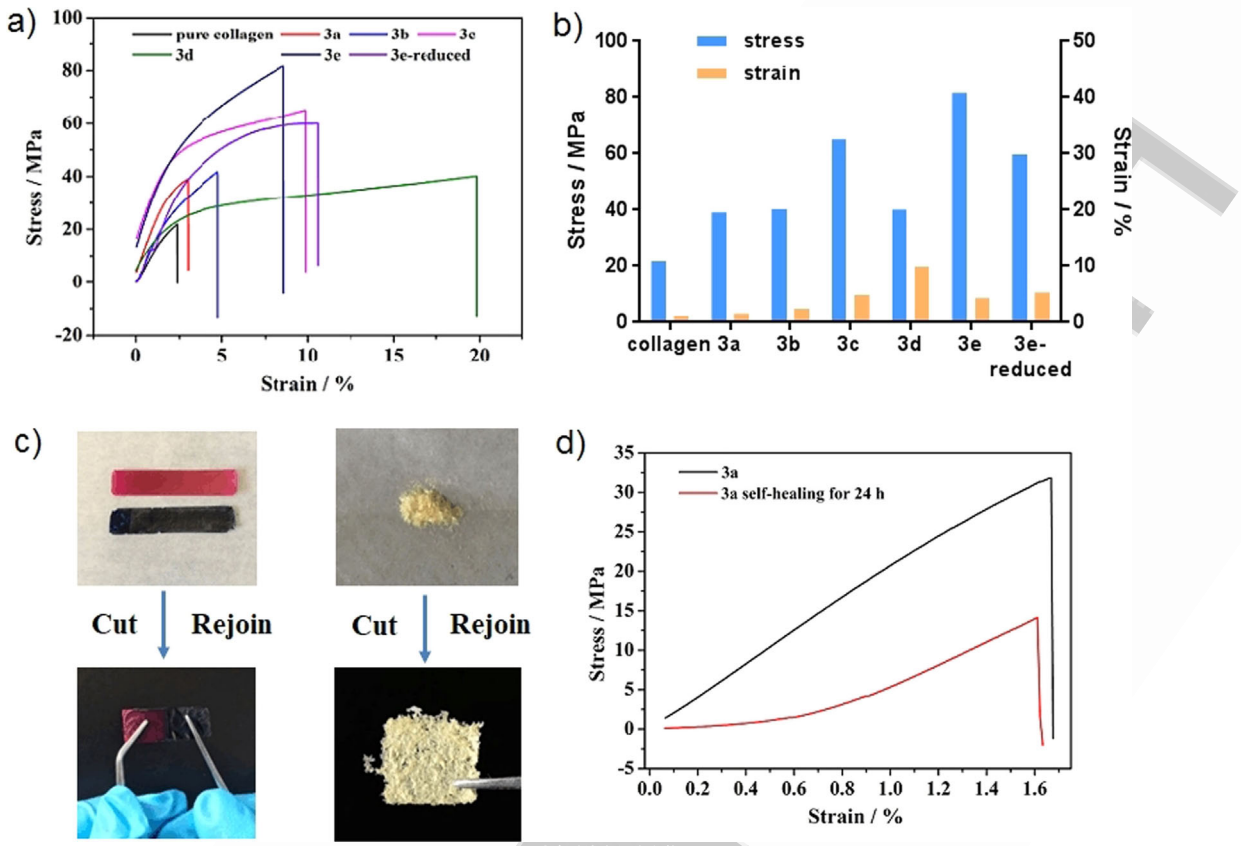


Figure 1. a) Tensile stress-strain curves collagen **C**, dynameric collagen **3a**–**e** membranes and reduced **3e** membrane. b) The highest tensile stress and break strain for each membrane. c) Cutting and rejoining of the self-healing membranes. d) Stress-strain curves of the original and the re-healed membrane **3a**.

175%) and the break strain (up to 400%) were increased with the length of the amine connectors. This phenomenon can be attributed to the combined effects of dynamically lubricant between the collagen structures and the higher water content from the longer chain dynamers. For the membranes containing polymeric PEG components (**3d**,**e**), their mechanical strengths were even higher. For example, the highest tensile stress (81.7 MPa, +400%) was observed from the membrane that linked with **3e**, much higher than most of the other collagen based membranes,[31] accompanied with a break strain of 8.6% (+400%), probably owing to the amine-terminated four-arm linker, leaving more spots for multiple cross-linking opportunities by imine bonds. On the other hand, the highest break strain was almost 10 times bigger for the membrane **3d** (19.8%) than the reference membrane **C**. This can be explained by the longest linker chain between neighboring amines, giving the highest flexibility for the resulted dynameric collagen membrane. However, due to the decreased cross-linking sites, its tensile strength (40.2 MPa) was a bit lower than that of **3e**.

Moreover, to examine the dynameric imine bonding effect during the membrane formation, the imine synthesized from trialdehyde **1** and amine **2e** was reduced with $NaBH_4$ first and further applied to physically cross-linked collagen. The resulted collagen membrane in which reduced polyamine dynamers would not bind to collagen, showed comparatively lower tensile strength, with the maximum value around 75% of that from membrane **3e**, indicating the importance of inserting reversibly imine bonds between collagen and dynamers during the membrane preparation process. Furthermore, the highest tensile stress and break strain for each collagen membrane have been summarized in Figure 1b, from which a clear trend of the influences of linker length and dynamer non-bonding effects on the mechanical properties of the membranes can be observed.

Further investigations revealed that the dynameric frameworks **1·2a**–**e** can form nanoparticles in water, with average diameter ranging from 100–200 nm (Figure S4), as determined by dynamic light scattering (DLS). The comparatively small size of the dynamic particles can provide higher exposure of free aldehyde groups, leading to multivalent effects while interacting with the amino groups in collagen. This can explain the enhanced mechanical strength of this type of cross-linked collagen membranes than the ones made from other methods.

Self-Healing Ability of the Collagen Membranes. The reversibility of the amino-carbonyl/imine reaction enabled the self-healing properties of the membranes at RT. The membrane **3a** was first fabricated into rod-shaped samples (50 mm × 10 mm) and cut into two pieces, which was stained with rhodamine B and methylene blue, respectively, for better visualization effect. Afterwards, the two membrane pieces were put together with slight overlapping, with the presence of a few drops of deion-

ized water. As can be seen in Figure 1a, the two pieces were rejoined after 2 h at RT. Meanwhile, the collagen membrane was cut into a large number of very small pieces, and paved in a Teflon mold with the addition of a few drops of water. After 6 h at RT, one whole self-standing piece of membrane was obtained (Figure 1c), demonstrating again the strong self-healing and recovery ability of the dynamic covalent membranes. Moreover, a more precise analysis was carried out by cutting membrane **3a** into two pieces, the tensile stress was measured after re-healing the two parts, while comparing to the value of the original membrane. Figure 1d illustrates that the tensile strain of **3a** was maintained for the re-built membrane, but the mechanical stress was decreased to less than half of the original number, with resulted in a calculated healing efficiency of 44.4%. This can be explained by the shorter membrane size and stiffer texture after the overlapping of rejoining process. The dry membrane surface also increased the difficulty of the imine exchange reaction under this specific conditions as previously observed for chitosan membranes.[18a]

Swelling, Thermostability and Degradation of the Collagen Membranes. The swelling behavior is an important issue related to the cross-linking degree of the membranes and impacting on their performances in an aqueous environment. Therefore, all the freeze-dried collagen membranes were placed in phosphate buffered saline (PBS) solution at 37 °C, and the weight was measured after determined time intervals. Figure 2a illustrates the change of swelling ratios with time for the prepared membranes. It was clear that the membrane with pure collagen gave the highest swelling ratios due to the lack of cross-linking process. All the other membranes provided lower values, among which, the swelling ratio of membrane **3c** was comparatively lower than the others, probably owing to the good solubility and short length of the dynamic cross-linker contained in the structure. Additionally, the pure collagen membrane was gradually dissolved in water, making difficult to measure the weight after 2 h. These results also demonstrated that the physical stability of the membranes was greatly enhanced after the cross-linking procedure. Appropriate degradation rate is crucial for biomedical membranes. Figure 2b showed the in vitro degradation profile of the collagen membranes in PBS (pH 7.4) at 37 °C. Generally, fast degradation rate was detected for collagen membranes, **C** in the first 5 days, with up to 80% mass loss. The presence of cross-linking slowed down the degradation process, especially for the PEG-based membranes **2a** and **2d**,**e**, probably coming from the tight dynameric frameworks connections, leading to a higher retention of the membrane masses, up to 23% after 45 days.

The thermal stability of the collagen membranes were subsequently evaluated. From the thermogravimetric analysis (TGA) two significant weight losses were observed (Figure S5a): one from 50 to 150 °C, indicating the removal of water from the matrixes; and the other from 250–450 °C, representing thorough decomposition of the materials, with the collagen helix structure completely disintegrated into a random coil polypeptide. The differential thermal gravity (DTG) result was consistent with the TGA profile (Figure S5b). Meanwhile,

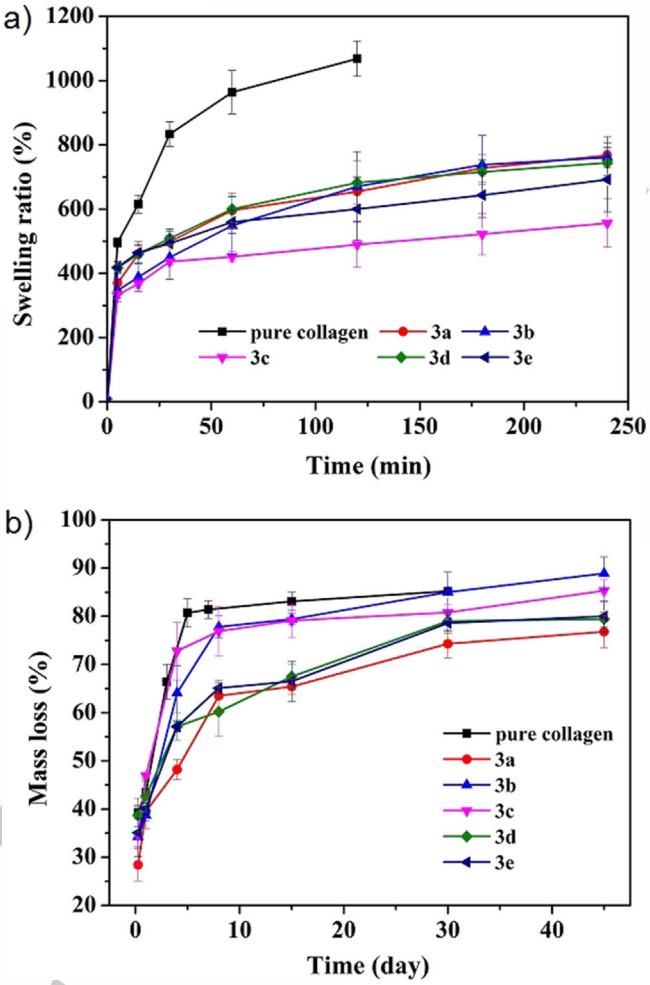


Figure 2. a) Swelling kinetics and b) degradation profile of the collagen, **C** and dynameric collagen **3a**–**e** membranes from PBS (pH 7.4) at 37 °C.

an extra valley at 320 °C was detected for the two membranes containing PEG, indicating the decomposition of the PEG part. Furthermore, in the differential scanning calorimetry (DSC) analysis (Figure S5c), the collagen membrane **C** showed a denaturation temperature at 107.3 °C, whereas the values for the cross-linked collagen membranes **3a**,**b** were slightly higher, up to 115.4 °C.

Biocompatibility of the Dynameric Collagen Membranes. The MTT assay was used to study the cytotoxicity of the dynameric collagen membranes. NIH 3T3 cells were treated with gradient-diluted membrane leachates, 100, 50, 25 and 12.5% of the original concentration, respectively (Figure 3a). All the leachates from pure collagen increased the cell viability to some extent, which is consistent with the good biocompatibility of collagen itself. After cross-linking, some membrane leachates, for example, **3c** and **3e** with lower concentrations exhibited an enhancement of the cell viability, whereas an slight decrease at above 95% after 24 h was observed for all the cross-linked membranes with 100% concentration.■■ok?■■ Therefore, the potential toxicity of the possible dynameric frameworks cross-linking did not obviously affect the cell viability in the current case. All the dynamic collagen membranes

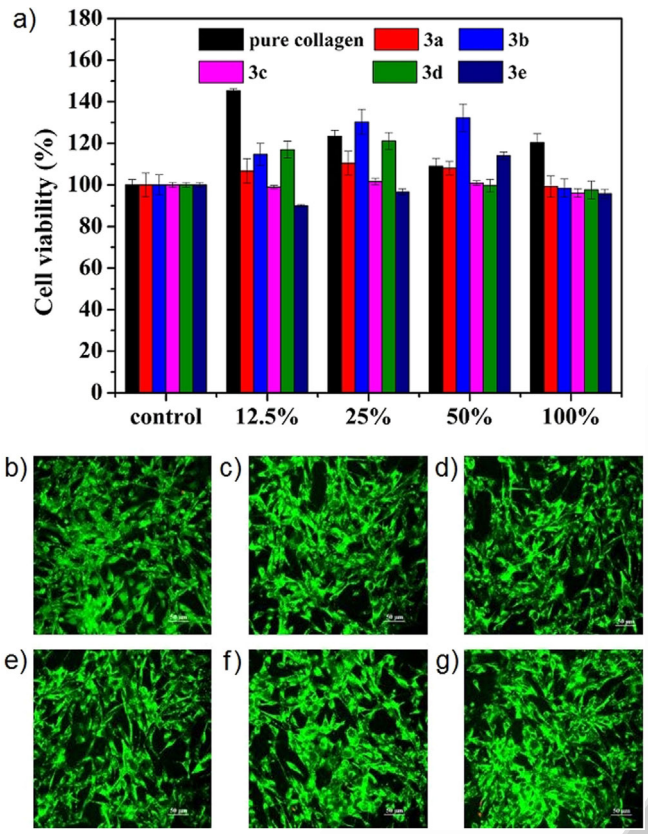


Figure 3. a) Cytotoxicity of the collagen membranes on NIH 3T3 cells for 24 h ($n=6$). Confocal laser scanning microscopy (CLSM) images of NIH 3T3 cells cultured on b) collagen membrane, **C** and dynameric collagen membranes: c) **3a**; d) **3b**; e) **3c**; f) **3d** and g) **3e** for 3 days in vitro, cells were stained green with Calcein-AM and red with Propidium Iodide (PI). Scale bar: 50 μm.

demonstrated enough■■ok?■■ biocompatibility for further biomedical applications.

Besides the membrane leachates, the direct contact tests were also employed to investigate the cytotoxicity of the dynameric membranes. In this experiment, NIH 3T3 cell suspensions were placed directly on the membrane surfaces and incubated for 72 h. All membranes exhibited appreciable stability during the cultivation period. Afterwards, the cells were stained with calcein acetoxymethyl ester (Calcein-AM, green-fluorescence) and propidium iodide (PI, red-fluorescence) for the observation of live and dead cells, respectively. By using confocal laser scanning microscopy (CLSM), cell numbers and morphologies can be clearly observed. As shown in Figure 3b–g, all the samples of dynameric membranes exhibited similar fluorescence for the live cells, whereas their spindle-shaped morphology showed no difference than the reference sample containing collagen membrane. Very few dead cells (red spots) were observed, demonstrating again the negligible cytotoxicity of the dynameric collagen membranes.

In summary, we have developed a series of dynameric collagen membranes connected through dynamic framework linkers, leading to excellent mechanical strength, self-healing properties and good biocompatibility. Nevertheless, the novel membranes were notably robust, showing self-healing ability even from a crushed status. Meanwhile, by varying the structural composition and length of the dynamic cross-linkers, the stiffness and flexibility of the membranes can be tuned accordingly, in which the tensile strength of the membranes can reach over 80 MPa, more than 400% higher than that was observed for the reference collagen membrane and highly superior compared to the current state-of-the-art biomaterial sourced membranes. Moreover, the implantation of natural collagen as the starting material also endowed the membrane with good biocompatibility, resulting in more than 90% viability for NIH 3T3 cells, which can also be directly observed from confocal laser scanning microscopy after live–dead cell staining. The current work provides a new strategy for the design and preparation of biocompatible membranes with excellent properties, which can further lead to broad applications in biomedical fields, such as wound healing, prevention of peritoneal adhesions or tissue regeneration, among others.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cell growth · collagen · dynamers · dynamic constitutional chemistry · self-healing

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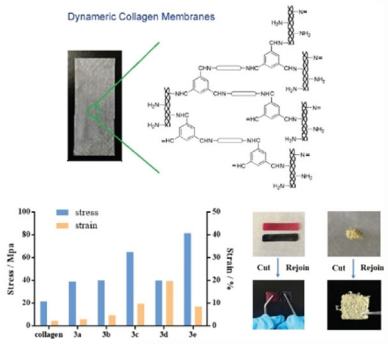
Self-healing technology: Dynameric collagen membranes with high mechanical strength, biocompatibility and self-healing properties have been prepared, showing a great potential for biomedical applications.



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