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Strong, Self-Healing Gelatin Hydrogels Cross-Linked by Double Dynamic Covalent Chemistry

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Abstract: Hydrogels constructed from natural sources received increased attention and applied in biomedical fields. They are protein or polysaccharide cross-linked scaffolds that display water retention and are able to recognize host cargos. Their excellent biocompatibility does not always combine with high mechanical strength (up to 136 kPa) and thermostability, making them less useful in biomedical applications. This paper reports biocompatible gelatin hydrogels, double cross-linked *via* imine and Diels-Alder (DA) dynamic covalent frameworks. They showed integrated advantages of adjustable and durable mechanical strength, good thermal stability, biocompatibility for promoting cell growth and reasonable degradable rate. These hydrogels possess remarkable self-healing property, acid/alkali resistance at 65 °C and good integrity in organic solvents at 130 °C, holding great potentials for biomedical applications in the areas such as cartilage regeneration, articular reconstruction or soft robotics.

Hydrogels are fibrillar functional soft materials with high surface area, great responsiveness and valuable storage and delivery abilities for biological targets. Thanks to their large content of water inside their three-dimensional (3D) polymeric networks and their good integrity under physiological conditions, hydrogels have been widely applied in biomedical fields, including wound dressing,^[1] biosensors,^[2] controlled drug release,^[3] etc. Recent developments in areas of soft robotics,^[4] tissue engineering and regenerative medicine,^[5] show that biocompatibility is one of the key features for hydrogel application. In this respect, low toxic natural polymers, such as collagen, chitosan, silk and cellulose,^[6] have become better options to synthetic polymers.

Gelatin is a natural protein, derived from collagen hydrolysis, which shows good biocompatibility, proper biodegradability, non-antigenicity and comparatively low costs.^[7] As a typical thermo-reversible gel, pure gelatin transforms into gel state upon cooling ($t < 30$ °C) and becomes a type of popular raw material for soft robotic applications. For example, entirely degradable gelatin bio-gels have been applied for electronic skin-*E-skin* and gelatin methacrylate hydrogels present strain-sensitive conductivity.^[8] However, most of these protein-based hydrogels still lack desirable mechanical tunability and stability, which may bring obstacles for practical applications. In order to address this issue, various chemical modifications or cross-linking methods have been used to improve the gelatin hydrogels.^[9]

Compared to strong covalent connections, reversible covalent bonds can not only strengthen the cross-linking of the polymeric networks, but also provide the dynamic rearrangement of their backbones, resulting in self-healing, stimuli-responsive and bio-interactive features of the hydrogels.^[10] Typical dynamic covalent chemistry includes imine, acyl-hydrazone, boronic ester, disulfide bonds and Diels-Alder (DA) reactions.^[11] Imine reaction is one of the most commonly used for its mild conditions, fast equilibration rate and good biocompatibility.^[12] Biomaterials cross-linked by imine bonds have been frequently reported with properties of self-healing, pH responsiveness and found wide applications in anti-bacterial, wound healing, adhesive dressing, 3D cell-printing studies.^[13] However, imine-based materials may not survive to acidic conditions from either metabolic or extreme skin surface environments.^[14] Diels-Alder (DA) reaction is a cycloaddition reaction with "click" characteristics, which shows reversibility at high temperature of around 110 °C.^[15]

For these reasons, in the current work, we combine imine and DA reversible covalent bonds to design and synthesize double cross-linked gelatin hydrogels with integrated multiple functions: tough yet biocompatible, thermostable yet degradable, durable yet self-healing, acid/alkali resistance and organic solvents tolerance. The presence of double imine/DA connections further increase the cross-linking of such double network DN, hydrogels, meanwhile, introduced good thermostability, adjustable mechanical strength (up to 136 kPa), acid/alkali resistance at 65 °C and good integrity in DMF and DMSO solvents at 130 °C. The originality of this work is not based on reversible crosslinking points involved in hydrogel structures, or on the use of gelatin as natural sources of material, but rather on the very interesting chemical and mechanical properties of the resulting materials. Moreover, the biocompatibility of the DN hydrogels was confirmed by MTT assays and confocal laser scanning microscopy, indicating great potentials for applications in various biomedical related or extreme E-skin conditions.

The natural gelatin possesses chemical reactivity *via* different side groups. In the current work, we decided to increase the number of amino groups for further imine cross-linking on gelatin backbone *via* the amidation reaction with diethylenetriamine.

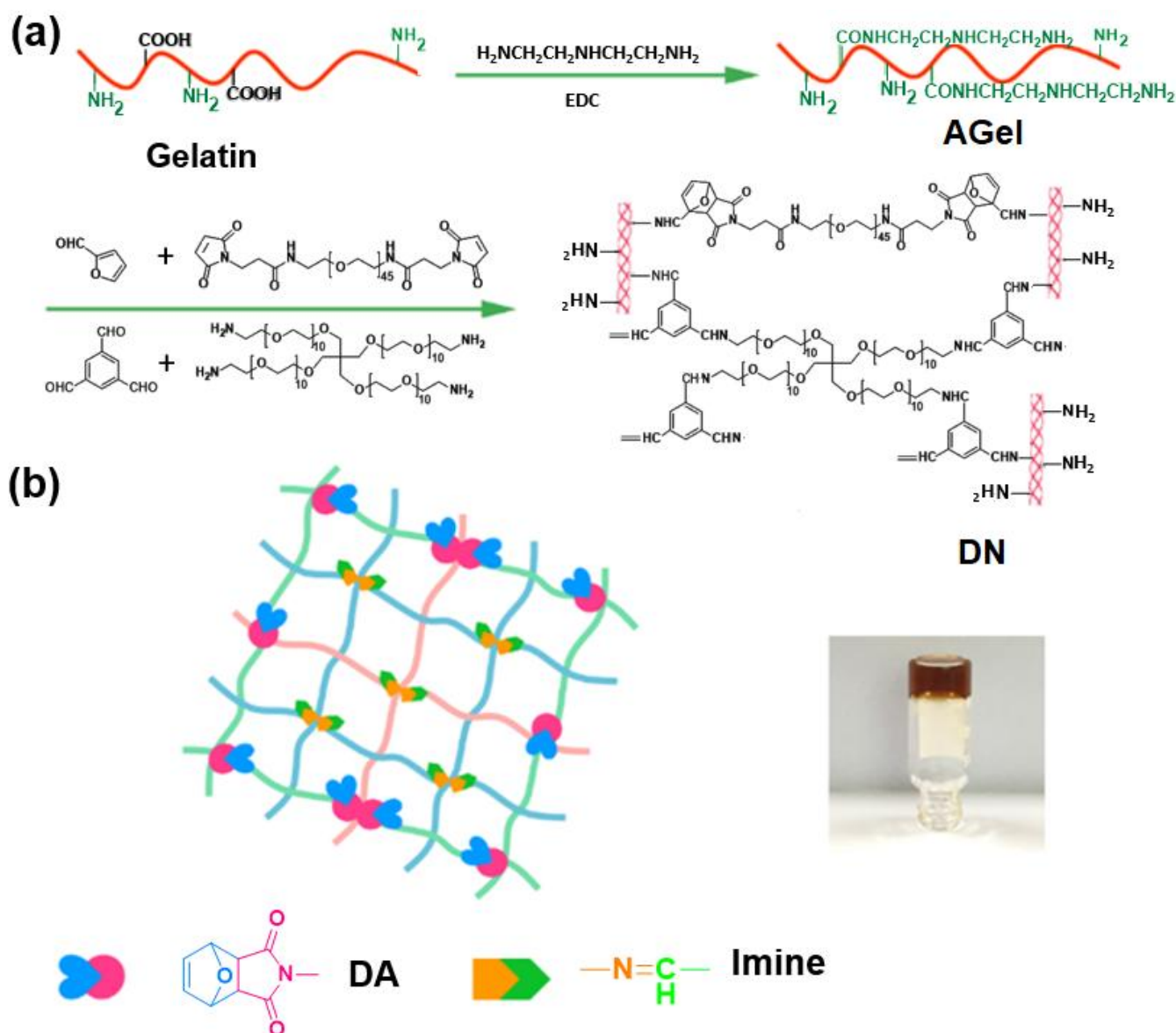


Figure 1. a) Synthesis of amino functionalized-gelatin, **AGel** and of the hydrogels **DN1-3** via synergistic imine/DA reversible reactions; b) schematic illustration of the double cross-linked dynamic network and the picture of the resulted hydrogel.

The modified amino-gelatin, **AGel** lost the gel state and appears as fluid at r.t. and 15 wt % (Figure S1). Two bands for amide group at 1635 and 1540 cm^{-1} were observed in FTIR spectra of gelatin and **AGel**, while the peak intensities of **AGel** were obviously not useful to prove the successful attachment of diethylenetriamine (Figure S2a). Thereafter, stoichiometric point method was adopted to measure the degree of the amino substitution. As a result, the amino content of the **AGels** with glue strength ~ 100 g Bloom and ~ 240 g Bloom were named as **DN1** and **DN2**, respectively and were determined to be 1.027 mmol/g and 0.958 mmol/g , respectively, with no significant differences observed between these two types. The Bloom number indicates the firmness or softness of a specific type of gelatin and is mainly related to its viscosity.

To construct the DN hydrogels through both imine and DA reactions were used: a) a 25 mM ethanolic solution of 1 eq. of 1,3,5-benzenetricarboxaldehyde and 0.6 eq. of 4-arm-amino-

terminated-poly ethyleneglycol-PEG2000 were first mixed, so after the equilibration 0.6 eq. of aldehyde remains unreacted; then the mixture was added to amino gelatin probes resulting in the construction of the first dynamic imine framework. B) the rest of the amino groups in functionalized gelatin was further reacted with increasing amounts of 2-furaldehyde and subsequently, stoichiometric amounts of MAL-PEG-MAL (2 : 1, mol : mol) were added to construct the second dynamic framework within hydrogels **DN1-1** to **DN1-3** and **DN2-1** to **DN2-3** with increasing DA connections (Figure 1a,b, Table S1). The imine formation between 4-arm PEG and 2-furaldehyde it is less favorable as subsequent cross linking between the two networks via transamination is substantially reduced in the presence of excess of amino groups on gelatin backbones.

FTIR spectroscopy was also used to emphasize the structural changes of the prepared DN hydrogels, after the double cross-linking steps, (Figure S2b). In **DN1-3**, the vibration

band of C=C at 1463 and 1466 cm^{-1} corresponding to the furan and maleimide groups, decrease reminiscent with the DA reaction between 2-furaldehyde and MAL-PEG-MAL. Moreover, the vibration band of aldehyde at 1692 cm^{-1} was almost disappearing after cross-linking with **AGel**, indicating the high conversions toward the imine-bond formation.

Then, compressive tests were performed to evaluate the mechanical strength and compressibility of the **DN** hydrogels (Figure 2). With the increasing amounts of DA constituents (2-furaldehyde and MAL-PEG-MAL) compressive modulus of the hydrogels increased from 52.91 kPa to 136 kPa.

Among these, **DN2-3** showed the largest compressive strength of 136 kPa and a breaking strain of 65%, indicating that the dynamic double cross-linked networks were soft and easy to extend. Thereafter, a strain of 5-10% was used to determine the compressive modulus. For the **AGel** with both glue strengths, the same trend was observed: higher contents of DAs led to higher compressive moduli from 12 ± 4.5 to 53 ± 9 kPa. Meanwhile, the obtained compressive moduli were higher for **DN1** series with ~ 100 g Bloom than **DN2** series. Subsequently, the **DN1-3** was selected to characterize the shape recovery and anti-fatigue properties with loading and unloading processes. Moreover, the **DN1-3** was subjected to compression loading-unloading tests under 40% strain. After 20 or 50 consecutive tests, the compressive strength of the hydrogels restored to 91% and 79.9% of its original strength, respectively, without any rupture, demonstrating good fatigue resistance and self-recovery ability (Figure 2c-d). As shown in Figure 2e, after removing the external force, the shape of **DN1-3** is restored immediately.

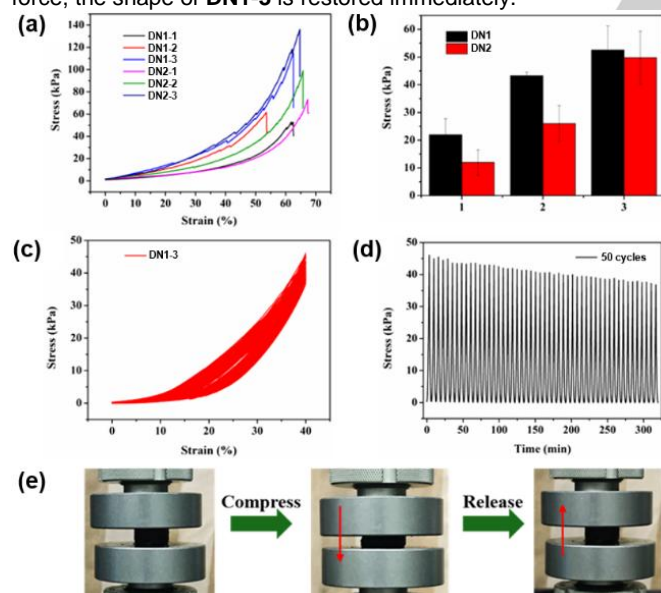


Figure 2. a) The compression strain-stress curve of the **DN** hydrogels; b) the compressive modulus; c) Loading-unloading continuous compression tests and d) the stress recovery property of hydrogel **DN1-3** at 40% strain for 50 cycles; e) photograph of the loading-unloading processes for compression tests.

Then, **DN1-3** was selected to carry out the macroscopic self-healing experiments. The hydrogels were made into round- or cylindrical-shapes and cut into different pieces. After contacting the pieces for 30 min at 37 $^{\circ}\text{C}$, **DN1-3** not only bore its own weight (Figure 3a), but also kept as a whole piece when being bent for nearly 90 $^{\circ}$, showing good toughness and recombining

ability (Figure 3b). Moreover, the hydrogel did not break off from the cutting point when being stretched, indicating its ability to withstand considerable tension after healing (Figure 3c).

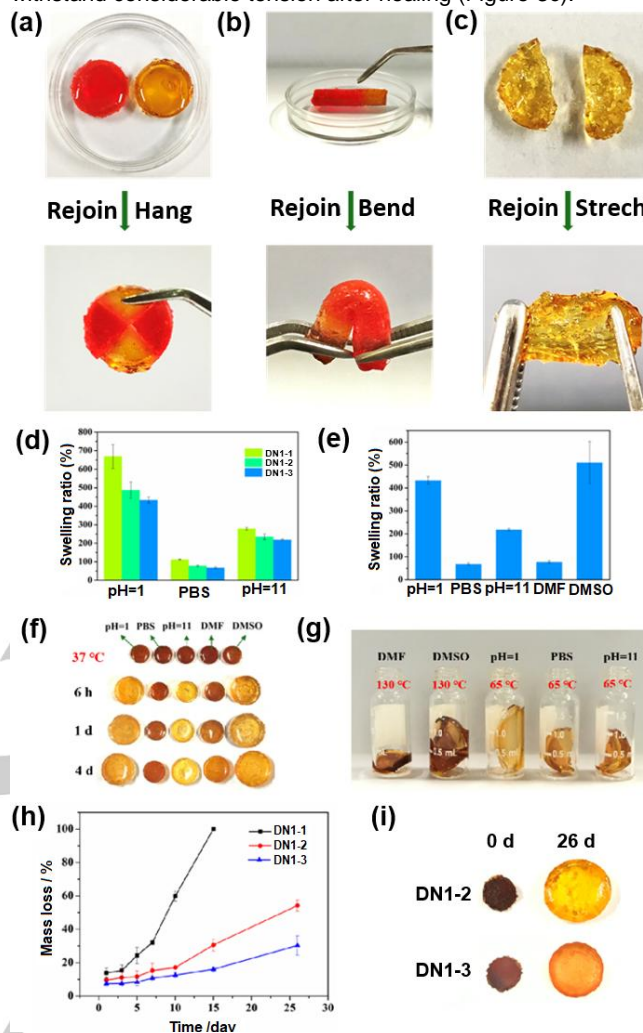


Figure 3. The self-healing behaviors of the hydrogel **DN1-3**: a) with manual hanging; b) with manual bending and c) with manual stretching of the healed hydrogel for 30 min. Some samples were stained with Rhodamine B for better visualization effect. Swelling ratio at equilibrium of hydrogels **DN1** d) in HCl (pH=1), PBS and NaOH (pH=11) solutions at 37 $^{\circ}\text{C}$; e) in HCl (pH=1), PBS, NaOH (pH=11), DMF and DMSO solutions; f) photographs showing the state of **DN1-3** in different solutions at 37 $^{\circ}\text{C}$ for 6 h, 1 d and 4 d, respectively; g) photographs of swelling in different solutions at high temperature for 6 h; h) degradation behavior of **DN1** hydrogel in PBS (pH=7.4) at 37 $^{\circ}\text{C}$; i) photographs of the initial freeze-dried hydrogels and hydrogels after degradation for 26 d.

The thermal stability of the **DN1** hydrogels were subsequently examined. From the thermogravimetric analysis (TGA), there were two significant weight losses (Figure S3a): the first one from 20 $^{\circ}\text{C}$ to 150 $^{\circ}\text{C}$ indicate the loss of water, while the second one extending from 200 $^{\circ}\text{C}$ to 450 $^{\circ}\text{C}$ is related to the decomposition of the cross-linking molecules and the amino-gelatin. In addition, the maximum degradation temperature (T_{max}) was examined for the **DN1** series, the gelatin and **AGel**. For **AGel**, the T_{max} increased from 305.5 $^{\circ}\text{C}$ to 321.4 $^{\circ}\text{C}$ (Figure S3b), indicating the increase of thermal stability. Owing to the double cross-linking effect of imine and DA bonds, the T_{max} of the hydrogel **DN1-3** increased to 402.5 $^{\circ}\text{C}$. Differential scanning

calorimetry (DSC) analysis show that two endothermic peaks at ~ 150 °C and 279 °C were detected for all samples; they relate to the volatilization of water and the content of triple helix in gelatin, respectively (Figure S3c). The last but not least, the second endothermic peak shifted from 278.7 °C to 285.5 °C for **AGel**, and further increased from 290.3 °C to 292.9 °C with the higher content of 2-furaldehyde and MAL-PEG-MAL, indicating that the amino-modification and the double cross-linking have a great impact on the helical structure of gelatin.

It is a great challenge for natural-sourced biomaterials to adapt to extreme environments either from inside the body or on the skin surfaces. Therefore, the hydrogels of **DN1** series containing various amounts of 2-furaldehyde and MAL-PEG-MAL were immersed in HCl (pH=1), PBS (pH=7.4) and NaOH (pH=11) solutions at 37 °C, and their swelling properties were evaluated. Decreasing swelling ratios (**DN1-1** > **DN1-2** > **DN1-3**) were observed with the increased amounts of 2-furaldehyde and MAL-PEG-MAL and higher cross-linking degrees (Figure 3d). The impacts of tested medium had a greater influence on the swelling behaviors of the hydrogels than the cross-linking degree of the structures (Figure 3e). For each individual hydrogel, its equilibrated swelling ratios in acidic medium was much higher than that of PBS and alkaline medium, which can be explained by the hydrolysis of imine bonds at low pH, resulting in collapse of the imine network. On the other hand, the equilibrated swelling ratios for all the **DN1** hydrogels were recorded as around 30% at the beginning, suggesting the immediate loss of water in PBS, and the values maintained lower than 100% after reaching equilibrium. Considering this feature, the hydrogel **DN1-3** was immersed in DMF and DMSO respectively at 37 °C in order to evaluate its solvent resistance. The results showed that the hydrogel displayed good anti-swelling property in PBS and DMF, with equilibrated swelling ratio even <100%. Comparatively higher swelling ratios in HCl solution (pH=1) and DMSO were recorded, but still showing a high structural integrity owing to the stable DA network. Figure 3f showed the photos taken during the swelling process of the

DN1-3 at 37 °C, which were in consistent with the recorded swelling curves (Figure S4).

Since DA reaction is thermally sensitive, it is necessary to reveal the influence of temperature on the stability of the DN hydrogels. In order to detect their responses to external stimuli, the **DN1-3** were severally immersed in hydrochloric acid solution (pH=1), PBS (pH=7.4) and sodium hydroxide solution (pH=11) and kept at 65 °C for 6 h, and in DMF and DMSO at 130 °C for 6 h because of their higher boiling points. When the temperature increased, the volume of the hydrogels decreases significantly, especially in HCl solution and DMSO (Figure 3g), indicating partial breakage of imine bonds in acidic environment and partial rupture of DA bonds at high temperature of 130 °C, respectively. However, the hydrogels still remained one whole piece during the processes, demonstrating good thermostability and integrity of the materials, which were still benefited from the double cross-linked structures. The cross-sectional morphology of freeze-dried hydrogels **DN1**, visualized by Scanning Electronic Microscopy-SEM, revealed dense structures, which can be related to the good mechanical and swelling properties of the hydrogels. The small holes in the cross section were probably caused by bubbles in the solution during the preparation process. To explain the impacts of DA constituents on the *in vitro* degradation rates of the hydrogels, the mass loss profiles of the **DN1** in PBS (pH=7.4) at 37 °C shown the slow degradation rates within the first 7 days (Figure 3h). On the 15 day, the **DN1-1** was completely degraded, while the **DN1-2** and **DN1-3** show remaining 69.4% and 83.9% mass residue, respectively. On 26 days, the hydrogels **DN1-2** and **DN1-3** still contained mass residues of 45.8% and 69.7%, respectively, and both maintained good shape integrity (Figure 3i). These results indicated that the increased contents of DA constituents and higher cross-linking slowed down the degradation process.

The MTT assay was used to evaluate the cytotoxicity of the DN hydrogels. NIH 3T3 cells were treated with gradient-diluted hydrogel leachates (100%, 50%, 25% and 12.5% of C_0). After 24 h of incubation, all the samples provided more than 100% cell viability (Figure 4a).

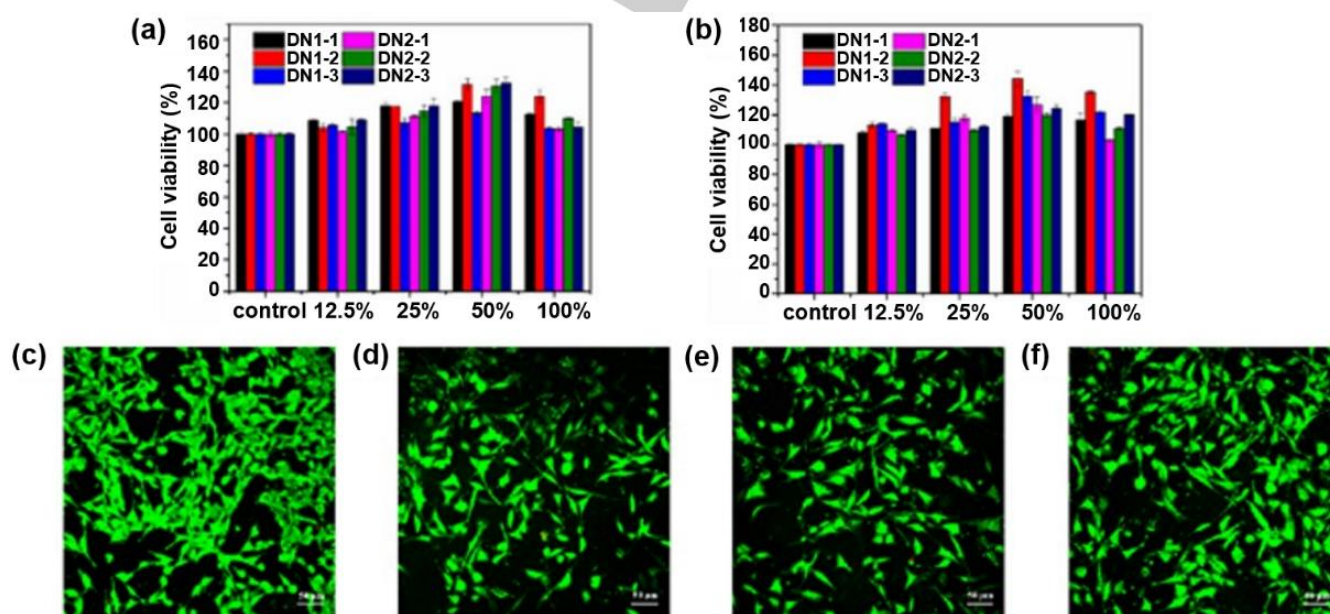


Figure 4. Cell viabilities of NIH 3T3 cells incubated with the the double cross-linked hydrogels, measured by the MTT assay for a) 24 h and b) 72 h. Confocal laser scanning microscopy (CLSM) images of NIH 3T3 cells cultured on the double cross-linked hydrogels: c) control, d) **DN1-1**, e) **DN1-2** and f) **DN1-3**.

After 72 h of incubation, the same trend was observed, and the cell survival rates were even higher than those at 24 h (Figure 4b), indicating continuous cell growth within the DN hydrogels that demonstrate excellent biocompatibility. Further, the DN hydrogels were put in direct contact with the NIH 3T3 cells and incubated for 72 h to investigate their cytotoxicity, while all the hydrogels maintained good integrity during the whole testing period. Thereafter, the cells were stained with Calcein-AM (green-fluorescence) and PI (red-fluorescence) for the observation of live and dead cells, respectively. As shown by using confocal laser scanning microscopy, all the tested hydrogels revealed similar fluorescence for the live cells with the spindle-shaped morphology similar to the reference sample in pure medium. Moreover, almost no dead cells (red spots) were observed, confirming the negligible cytotoxicity of the DN hydrogels (Figure 4c-f).

In conclusion, a chemically modified amino-enriched gelatin was used to prepare acid-, alkali- and solvent-resistant hydrogels via dynamic imine and Diels-Alder (DA) reversible reactions. The use of double dynamic covalent frameworks to cross link the protein backbones led to strong self-healing property of the hydrogels. The insertion of a secondary reversible DA bonds provided stability to the network structure and up to 3-fold improved the mechanical strength, leading to good fatigue-resistance and self-recovery capacities. After 50 consecutive compression loading and unloading tests, the compressive strength of the DN hydrogel was still restored to 79.9% of its original strength without rupture. Meanwhile, the DN hydrogel also exhibited good stability in response to pH and temperature changes, still maintained shape integrity in acidic/alkali solutions at 65 °C or in organic solvents at 130 °C. Importantly, they showed good biocompatibility, as confirmed by MTT assay and confocal laser scanning microscopy. With all these synergetic properties integrated in one type of materials, the present DN hydrogels hold great potentials in various fields of biomedical or soft robotic applications.

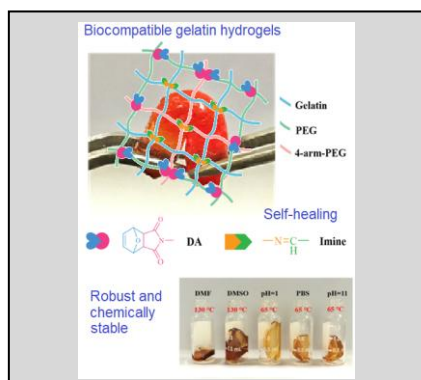
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Keywords: dynamic covalent chemistry • Diels-Alder (DA) reaction • gelatin • hydrogels • imine

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Double dynamic covalent gelatin hydrogels with integrated functions: tough mechanical strength (up to 136 kPa) yet biocompatible, thermostable yet degradable, durable yet self-healing, plus properties of acid/alkali resistance at 65 °C and organic solvent tolerance at 130 °C. These hydrogels are biocompatible revealing similar fluorescence for the live cells with the spindle-shaped morphology similar to the reference sample in pure medium.