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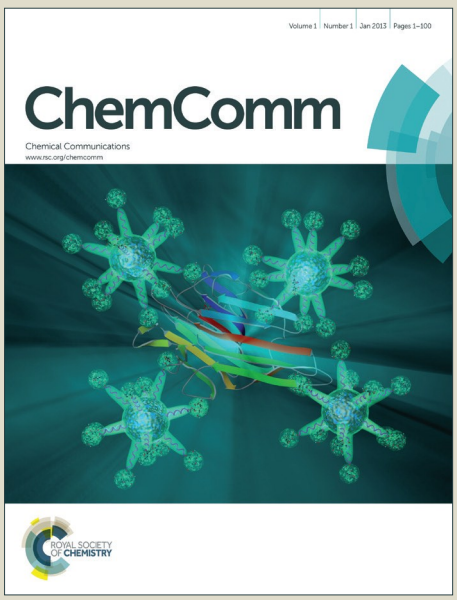

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Highly Robust Hydrogels via a Fast, Simple and Cytocompatible Dual Crosslinking-based Process

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A highly robust hydrogel device made from a single biopolymer formulation is reported. Owing to the presence of covalent and non-covalent crosslinks, these engineered systems were able to (i) sustain a compressive strength of *ca.* 20 MPa, (ii) quickly recover upon unloading, and (iii) encapsulate cells with high viability rates.

Hydrogels, as highly hydrated 3D polymeric networks, are soft and compatible with the majority of living tissues, highlighting their potential use as analogues of the native extracellular matrix.[1] However, their high water content along with intrinsic structural inhomogeneity and lack of efficient energy dissipation mechanisms often yields hydrogels with a poor mechanical performance. Recognizing the aforementioned drawback, several researchers have been focusing their work on the design of innovative and distinctive hydrogel microstructures with exceptional mechanical properties,[2] as the case of the double-network (DN) methodology, which is quickly becoming an essential tool in material science field.[3] Beneath their outstanding mechanical behavior is the specific combination of two crosslinked networks with contrasting properties, namely, a highly crosslinked brittle matrix and a loosely crosslinked ductile network. While the brittle network contributes to an increase on the elastic modulus, the ductile structure is responsible for the increase on strain.[3b] In fact, under optimized conditions, most of the DN gels have water contents higher than 90 wt% and are stiff, with an elastic modulus of 0.1-1.0 MPa, strong, as proved by a failure compressive stress of 20-60 MPa, and tough, possessing a tearing fracture energy of 100-4400 J m^{-2}.[3] This excellent mechanical performance is comparable to and even exceeds some soft load-bearing tissues. For instance, cartilage typically exhibits a compressive strength of *ca.* 35.7 MPa, while the tendon possesses a tensile strength of *ca.* 23.65-78.52 MPa.[4] Traditionally, DN hydrogels are covalently crosslinked networks produced by a two-step sequential free radical polymerization process.[3] This methodology presents some drawbacks: (i) is time-consuming, as it involves a swelling/diffusion step which can take several days; (ii) has limited reproducibility, due to the difficulty of controlling the exact molar ratio of both structures; (iii) yields hydrogels with low recoverability, as its high toughness depends on the irreversible damage of the brittle network; and (iv) require harsh processing conditions, hampering their use as cell-laden devices.[3a,5]

Herein, strong and cell-laden chitosan (CHI) hydrogels were fabricated from a single polymeric precursor formulation and under physiological conditions. CHI is of particular interest for biomedical purposes as it exhibits a plethora of desirable physicochemical and biological properties including biocompatibility, biodegradability, adhesiveness and versatility in both modification and fabrication.[6] These systems were produced through a DN-based methodology combining chemical and physical crosslinking mechanisms. Contrarily to chemical crosslinks, physical crosslinks are able to reversible associate/dissociate in response to an applied load, resulting in hydrogels with high toughness as well as with the ability to recover their mechanical properties upon bond reformation.[7] Although this combination of strong and weak crosslinks was already applied in other hydrogel devices,[5a,5e] this is the first time, that both polymer networks are composed of the same macromolecule, in this case CHI, differing only on the molecular weight and the crosslinking mechanism employed. Additionally, with the present technology, both polymer components are mixed together in a single hydrogel precursor solution, which solves the aforementioned drawbacks of the current DN strategies with the additional advantage of enabling cells encapsulation with high viability rates.

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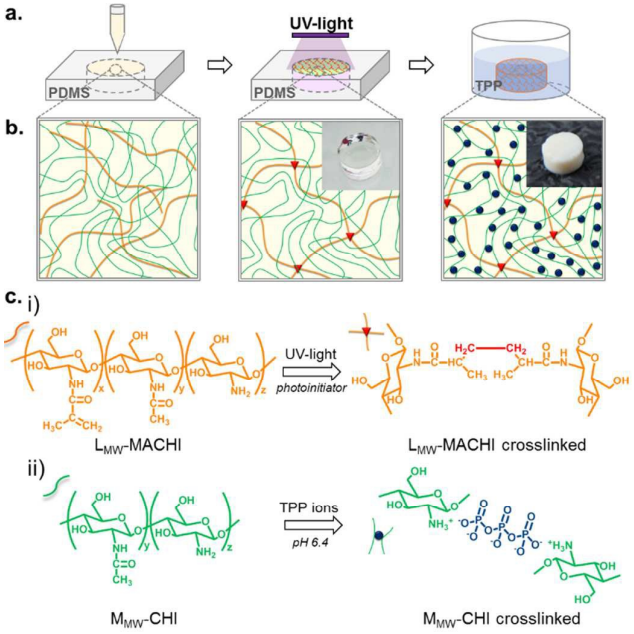

Scheme 1. (a) CHI DN hydrogels fabrication process. (b) Schematic representation of the hydrogel structure after each crosslinking step as well as their appearance. (c) Chemical structures of the L_{MW}-MACHI (i) and M_{MW}-CHI (ii) polymers as well as their respective crosslinked form.

To fabricate strong DN hydrogels, two CHI derivatives, namely low molecular weight methacrylamide CHI (L_{MW}-MACHI) and medium molecular weight CHI (M_{MW}-CHI) were mixed with a photoinitiator (I2959) (ESI; Section S3) and a weak base, β-glycerolphosphate. Afterwards, a dual-crosslinking process was performed by (i) UV-light exposure of the methacrylic groups on L_{MW}-MACHI forming a covalently crosslinked network (Scheme 1c i) and (ii) immersing the resultant hydrogel in a solution containing negatively-charged tripolyphosphate (TPP) ions to ionically crosslink M_{MW}-CHI through their positively-charged amine groups (Scheme 1c ii). Both crosslinking times were kept in the minimum value which allowed the attaining of strong hydrogels without ultimately compromise cell viability (ESI; Sections S4 and S5). Then, the resultant hydrogels had a homogeneous composition and morphology (ESI; Section S6). It is worth to notice that the described strategy is simple, relatively fast and occurs at physiological conditions.

Figure 1 displays the compressive mechanical behavior of the resultant hydrogels. L_{MW}-MACHI single network broke into small fragments upon reaching a strain value of *ca.* 60%, as easily observed by comparing Figure 1A and 1B. In contrast, the CHI DN hydrogels did not fracture after a strain of *ca.* 90% (Figure 1C and D). In fact, under optimized conditions, CHI DN structures exhibited an outstanding compressive strength of *ca.* 19.481 MPa, which is several orders of magnitude higher than those of their precursors: 0.094 MPa and 0.100 MPa for L_{MW}-MACHI and M_{MW}-CHI, respectively (ESI; Movie S1 and S2, and Figure 1E). This synergistic effect on their mechanical properties highlights their DN-based character, contrarily to interpenetrating networks (IPN), whose mechanical behavior results from the linear combination of their individual networks properties.[8] To assess the influence of each structure on the DN hydrogel mechanical properties, different amounts of each polymer were mixed together, keeping the total polymer amount fixed. By observing Figure 1F, it can be noticed that hydrogels with lower amounts of M_{MW}-CHI polymer, namely 0 and 25%, fractured at strains lower than 60%, however, for the same strain value, these same hydrogels exhibited higher strengths, compared to hydrogels with 50, 75 and 100% M_{MW}-CHI content, indicating that the L_{MW}-MACHI network can significantly improve the mechanical strength of the CHI DN hydrogels. The initial compressive modulus and fracture stress of each hydrogel were summarized on Figure 1G. By increasing the amount of M_{MW}-CHI from 25% to 50% of the total polymer mass, the strength increases drastically from *ca.* 0.1 MPa to *ca.* 19.5 MPa. On the other side, the elastic modulus decreased considerably from *ca.* 44.3 kPa to 4.7 kPa for an increase of the M_{MW}-CHI from 50% to 75% of the total polymer mass. An optimal balance of the mechanical properties was obtained for 1:1 ratio of both structures, yielding CHI DN hydrogels with an improved mechanical performance. Interestingly, these properties are even higher than other DN hydrogels composed of natural-origin polymers.[9] Taking into account all of these results, it is suggested that the L_{MW}-MACHI network is

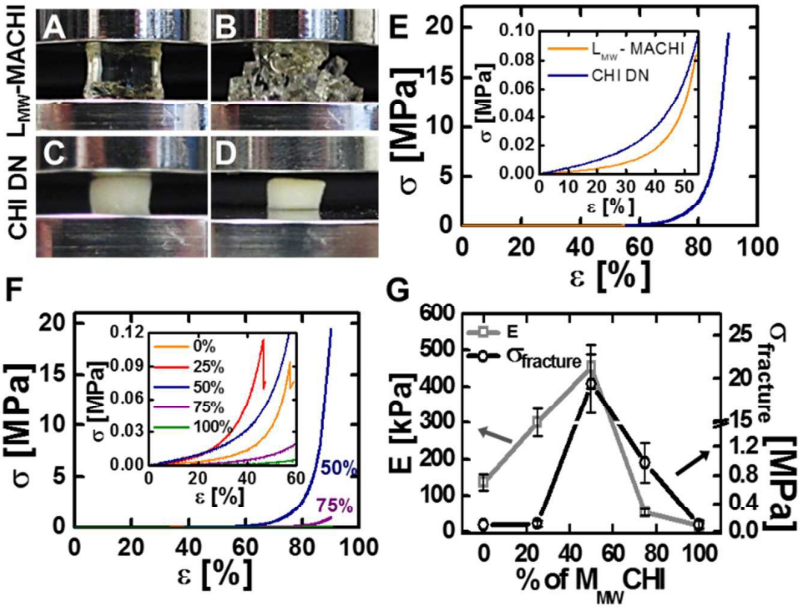

Figure 1. Photographs showing the L_{MW}-MACHI (A and B) and CHI DN (C and D) hydrogels before (A and C), and after (B and D) a ε = 90 %, respectively. (E) Representative compressive stress-strain curve of L_{MW}-MACHI and CHI DN hydrogels. (F) Compressive curves obtained by keeping the total polymer mass constant and changing the ratio of both structures. The percentage values are in relation with the M_{MW}-CHI polymer. (G) Compressive modulus and fracture stress as a function of % of M_{MW}-CHI in the hydrogel

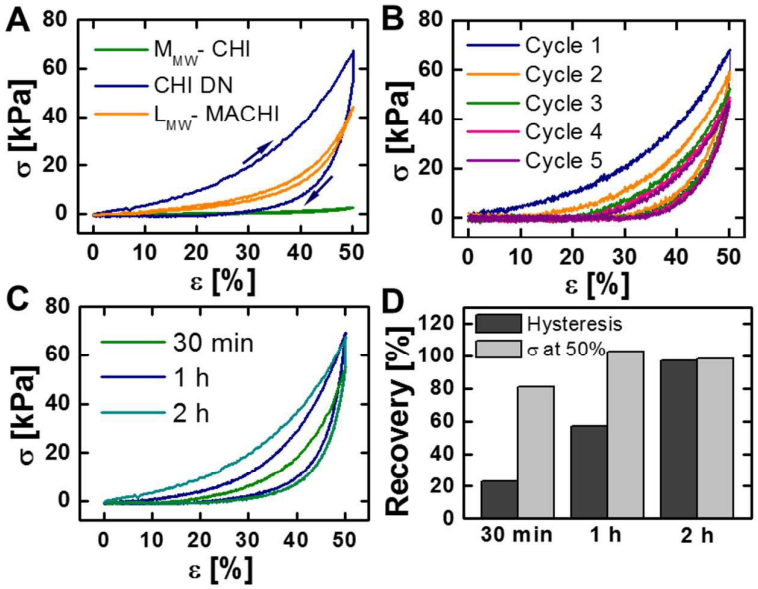

Figure 2. A) Representative load/unload compressive stress-strain curve of CHI DN, L_{MW}-MACHI and M_{MW}-CHI hydrogels up to ε = 50 %. (B) Five successive loading/unloading cycles of CHI DN hydrogels. (C) 2nd loading cycle after the CHI DN sample rested for 30 min, 1h and 2h after a 1st compression cycle. (D) Recovery [%] of CHI DN hydrogels in function of the time of recuperation at room temperature.

responsible for providing the hydrogel strength and the M_{MW}-CHI the high strain values, contributing to the simultaneous elastic and tough behavior of CHI DN hydrogels.

Moreover, the effective energy dissipation of the CHI DN hydrogel was proved by the presence of a pronounced hysteresis in the loading and unloading curves, being directly related with the toughness of the material (Figure 2). Indeed, the energy dissipated during a first cycle of compression was much higher for the CHI DN hydrogels than for hydrogels of the two extreme compositions (*ca.* 10 times for L_{MW}-MACHI and 55 for M_{MW}-CHI) for the same strain value (Figure 2A and ESI; Section S7). Then, CHI DN hydrogels were subjected to five successive compression cycles with a maximum strain of 50%, as shown on Figure 2B. The results suggest a decrease on the hysteresis and compressive strength values, being more pronounced from the first to the second cycle since the curves of the subsequent cycles almost overlapped. In order to assess if the initial mechanical properties could be recovered, the samples were allowed to rest after a first compression cycle at mild conditions. Figure 2C suggests different recovery rates depending on the time of recuperation. Nevertheless, these hydrogels were able to recover almost completely their initial mechanical properties if the second loading is delayed by 2 hours (Figure 2D), which is of outmost importance for repairing structural soft tissues that are continuously subjected to stresses. Based on previous works,[5a,5e,10] the high level of hysteresis with low permanent deformation is possibly the result of combining strong and weak crosslinks. In a single covalently or ionically crosslinked network, the polymer chains in direct contact with the applied load sustain it until one of the chains break or an ionic bond is disrupted, respectively. Afterwards, the crack is able to run throughout the hydrogel, resulting in low toughness as the fracture occurs in a localized injured area. Contrarily, in DN hydrogels containing strong and weak crosslinks, the damage zone is much larger, allowing for the accumulation of more damage before the macroscopic crack propagation. In this case, the weak ionic bonds are first disrupted and, as the stress increases, the neighbor ionic bonds are also dissociated in order to cooperatively sustain the load. Consequently, the spread of the stress and the damage zones are larger. In short, the L_{MW}-MACHI is responsible for preserving the initial hydrogel shape once the ionic crosslinks are broken, while the M_{MW}-CHI dissipate the energy over a wider region and protects the L_{MW}-MACHI structure from fracturing. If the load is released, the M_{MW}-CHI hydrogel structure is able to recover after the bond reformation, explaining the low permanent deformation. Although CHI polymer was already used to produce other strong hydrogels devices,[11] these mechanical properties were never observed in hydrogels only made of the same macromolecule, as far as our research goes.

Moreover, two other interesting features about these hydrogels were also observed. First, contrarily to conventional DN hydrogels, these networks showed a de-swelling effect in water, PBS and culture medium (ESI; Figure S8). This behavior was already observed in other systems,[12] being ascribed to the release of water from the hydrogel structure either due to the presence of (1) hydrophobic methacrylic groups on L_{MW}-MACHI or (2) higher ions concentration in the enclosing medium. This property is relevant as swelling severely weakens hydrogels, compromising their behavior *in-vivo*.[11,13] Second, it was found that to create hydrogels with improved mechanical properties, it is necessary to combine two polymeric networks with

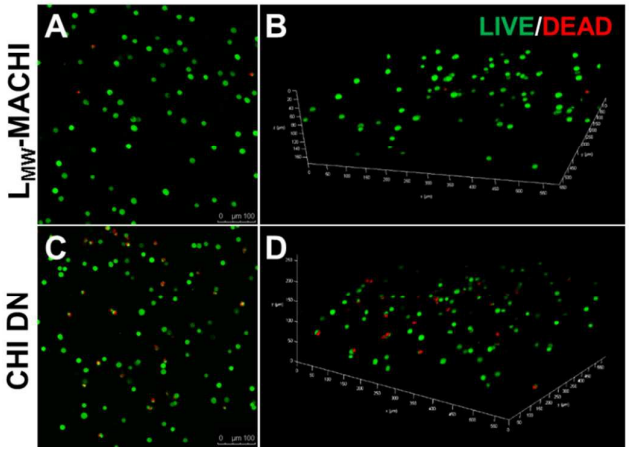

Figure 3. 2D and 3D LIVE/DEAD confocal images of L929 cells encapsulated within L_{MW}-MACHI (A and B) and on CHI DN hydrogels (C and D), respectively. Viable cells appear in green whereas dead cells are stained in red. Scale bar corresponds to 100 µm.

distinct features, in this case different molecular weights, and use two crosslinking processes. This fact was proved since the production of strong hydrogels was prevented by employing both crosslinking processes on the L_{MW}-MACHI network (ESI; Figure S9).

To further explore the biomedical or biological interest of the developed systems, L929 cells were mixed with the polymeric precursor solution to assess the ability of the hydrogels to encapsulate cells. Figure 3 shows that cells were uniformly distributed within the CHI DN hydrogels, exhibiting high viabilities rates (*ca.* 80%) after 24h of incubation at physiological conditions. These values are comparable with the ones obtained for the L_{MW}-MACHI hydrogels (*ca.* 95%) and for other DN hydrogels,[9a] proving the cytocompatible character of the developed strategy and emphasizing its potential to create cell-laden artificial substitutes of native soft-tissues.

This is the first report of single polysaccharide DN hydrogel with the ability to (i) withstand an impressive compressive stress in the same order of magnitude as the ones found in native load-bearing soft tissues, (ii) fast recover their mechanical properties upon unloading, and (iii) encapsulate cells with high viability rates. Such multifunctional devices could potentially be used for the repair of load-bearing soft tissues or as an encapsulation platform for a variety of biological applications, such as disease models for drug screening and therapies in a more realistic mechanical environment.

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Highly Robust Hydrogels via a Fast, Simple and Cytocompatible Dual Crosslinking-based Process

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Table of Contents

An extremely strong, tough and cell-laden chitosan-based hydrogel fabricated from a single polymeric precursor solution is reported.