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Sequentially Moldable and Bondable 4D Hydrogels Compatible with Cell Encapsulation

Mariana B. Oliveira*, Henrique X. S. Bastos, and João F. Mano*

Department of Chemistry, CICECO – Aveiro Institute of Materials. University of Aveiro. 3810-193 Aveiro, Portugal

Abstract

Hydrogels have captivated the attention of several research and industry segments, including bioengineering, tissue engineering, implantable/wearable sensors and actuators, bioactive agent delivery, food processing, and industrial processes optimization. A common limitation of these systems is their fixed shape. The concept of hydrogel moldability is often assigned to the injectability potential of liquid precursors, and this feature is often lost right after hydrogel formation. Hydrogel modulation is a recent trend that advocates the importance of designing materials with shape fitting ability targeting on-demand responses or defect filling purposes. Here, we present a compliant and cell encapsulation-compatible hydrogel prepared from unmodified natural origin polymers with the ability to undergo extreme sequential shape alterations with high recovery of its mechanical properties. Different fragments of these hydrogels could be bonded together in spatiotemporally-controlled shape- and formulation-morphing structures. This material is prepared with affordable off-the-shelf polysaccharides of natural origin using a mild and safe processing strategy, based solely on polyelectrolyte complexation followed by an innovative partial coacervate compaction and dehydration step. These unique hydrogels hold potential for multifield industrial and healthcare applications. In particular, they may find application as defect filling agents or highly compliant wound healing patches for cargo release and/or cell delivery for tissue regeneration and cell-based therapies.

Keywords

Cell encapsulation; 4D hydrogels; moldable hydrogels; bondable hydrogels

Introduction

Shape-morphing materials are added-value assets for multiple applications, and have been reported as self-folding robots,¹ on-demand drug delivery systems², and stimulus-responsive textile devices.³ Four-dimensional (4D) hydrogels are highly hydrated materials able to transform their shape, composition or physical properties after fabrication, and are useful as customized drug delivery systems, controllable actuators or cell-encapsulating devices.⁴ The modulation of hydrogels' shape is often related to on-demand induction of reversible phenomena, often triggered by stimuli-responsive swelling variations^{5–7} or

*Corresponding Author: mboliveira@ua.pt, jmano@ua.pt.

molecular re-arrangements in response to exposure to temperature,^{8–10} light¹¹ and conductivity¹² cycles. Such “on/off” materials are often limited in the number of shapes and configurations they may acquire because of associated binary reversible molecular configurations.

Moldable hydrogels can acquire a versatile array of shapes through the application of mechanical stress. The value of moldable materials was showcased for industrial setups through the application of safe shear-thinning and rapidly self-healing dispersions of silica nanoparticles in cellulose, targeting the cleaning of food processing pipelines and as fire retardants.¹³ Recently, nanocomposite adhesive hydrogels made of a mussel-inspired polymer and dispersed nanosilicates were suggested as fit-to-shape sealants, introducing moldable hydrogels as functional materials for the healthcare field.¹⁴

Functional hydrogels with moldable features may play a vital role in circumventing current drawbacks associated with defect-filling injectable materials. Therapeutic injectable hydrogels are usually formed by (i) the use of liquid polymeric precursors that react upon the addition of crosslinking agents and/or exposure to external stimuli (e.g. light irradiation, temperature), or (ii) the injection of chemically-modified polymeric chains that spontaneously gellify inside the defect site.^{15–16} Defect-filling hydrogels usually (i) show lack of homogeneity of ionic or irradiation-driven crosslinking mechanisms in large 3D defects due to ionic or light penetration impairment, leading to uncontrolled degradation phenomena and uneven cell-sensing physical and chemical microenvironments,^{17–20} (ii) feature poor delivery/cell retention inside defects associated with too slow or too fast crosslinking kinetics of liquid polymeric precursors (limiting material and cell retention efficiency),¹⁷ and (iii) often require the chemical modification of raw polymeric materials to achieve self-reacting properties, resulting in time-consuming, possibly low-yield and costly processes.²¹

Shear-thinning hydrogels were suggested as injectable and printable cell carriers able to provide improved cell retention efficacy.^{22–24} Reported strategies that enable one-step injection and shape fixation of cell-laden hydrogels inside tissue defects rely on the use of specifically designed chemically-modified self-assembling materials.²² The injection of hydrogels with finely controlled crosslinking kinetics was also reported as a plausible strategy to circumvent low cell fixation rates in defect sites. However, shape fixation of these structures was only achieved through a two-step tandem crosslinking strategy, based on UV irradiation after injection.^{22,24} Importantly, the adequacy of shear-thinning cell-laden hydrogels to be sequentially modulated into different self-standing shapes outside the scope of defect filling purposes remains, to the best of our knowledge, unexplored. Hydrogels with on-demand spatiotemporally controlled 3D composition may be designed to tailor e.g., biological, mechanical or electrical response overtime. This may be achieved by simply bonding or mixing hydrogel pieces containing different cell types, chemical compositions or loaded drugs on different time points. The possibility of handling moldable material to direct different sequential shapes may also dictate the development of safe “play dough”-like materials with potential application on food and cosmetic industries.

Experimental Methods

1 Materials

Medium molecular weight chitosan (MMW-CHT; ref.448877; 75% degree of deacetylation calculated by ^1H NMR – Figure S1 - Bruker DRX 300 Avance at 300.13 MHz; CHT was dissolved in a solution containing 98% D_2O and 2% DCl (v/v)), alginic acid sodium salt (ref. A3249), glacial acetic acid and phosphate buffer saline (PBS) were purchased from Sigma-Aldrich, and used as received. Sodium chloride (NaCl) was purchased from LabChem, and sodium hydroxide (NaOH) from AkzoNobel. Deuterium oxide and deuterium chloride (20% in deuterium oxide) were purchased from Acros. Amicon® Ultra 4 mL centrifuge tubes with Ultracell 100 membrane (100 kDa cutoff), were purchased from Merck Millipore. Mouse fibroblast cell lines L929 (ref. 85011425) were purchased from Sigma-Aldrich, and human adipose-derived mesenchymal stem cells (hASCs; ref. ATCC® PCS-500-011™) from ATCC. Cell culture media Dubelcco's Modified Eagle Medium (DMEM) was purchased from Sigma-Aldrich (ref. D2902). Minimum Essential Medium Eagle-Alpha Modification (αMEM ; ref. 12000014), fetal bovine serum (FBS; ref. 10270106), Alamar Blue reagent (ref. DAL1025), Calcein AM (ref. C3099) and propidium iodide (ref. P1304MP), TrypLe™ Express Enzyme (ref. 12605010) and penicillin/streptomycin solution (ref. 15140122) were acquired from ThermoScientific.

2 Hydrogel Synthesis

A solution of CHT was prepared using MMW chitosan dissolved at 0.5 mg mL^{-1} in a solution of 1% (v/v) glacial acetic acid (Sigma) in distilled water. A 0.5 mg mL^{-1} ALG solution was prepared by dissolving sodium alginate in distilled water. Both solutions were prepared overnight at room temperature, with an agitation of 300 rpm. After total dissolution of the polymers, sodium chloride was added to the solutions. The two polymers were mixed in the presence of distinct amounts of salt (NaCl) in the polymeric solutions: (i) absence of salt, here described as hypotonic condition - 0 M; (ii) isotonic salt concentration - 0.15 M; and (iii) hypertonic condition - 0.5 M NaCl . The solutions were stirred until total NaCl dissolution. The pH of all solutions was adjusted to 4 using 2 M NaOH . The analysis of the ^1H NMR spectrum of CHT showed an acetylation degree of approximately 25%. The molar equivalence of ALG and CHT was calculated, so equivalent charges of both polymers could be determined. Considering the average molecular weight of CHT monomers for a deacetylation degree of 75%, a neutral net charge between both polymers is obtained in a mass proportion of approximately 1ALG:1.1CHT. The molecular weight of each monomer of chitosan was considered 179.17 g/mol for deacetylated monomers, and 221.2 g/mol for the N-acetylglucosamine groups. The molar mass was calculated considering the determined deacetylation degree of 75%. The average molecular weight of sodium ALG monomers was considered 216.1 g/mol, Mass proportions of ALG and CHT of 0.8:1, 1:1, 1:1.2 and 1:1.4 were studied (here, addressed as 0.8C, 1C, 1.2C and 1.4C). To prepare CHT:ALG coacervates, a beaker containing a CHT solution (pH 4) was heated at 37°C and stirred at 600 rpm. An ALG solution (pH 4) was then added to the CHT, and the mixed solution was stirred for 10 minutes. Aliquots of polyelectrolyte complexes at pH 4 were then collected for analysis (ζ -potential measurements). The pH of the remaining solution was then adjusted to 7 using 2 M NaOH , while the coacervate solution was stirred at 600 rpm. The coacervates

formed at pH 7 were then left to sediment in the bottom of the beaker for approximately 1 hour, and 75% of the total solution volume was discarded. A volume of 5 mL of the concentrated CHT:ALG coacervates (pH 7) was poured into Amicon Ultra-4 Centrifugal filter units (Millipore), with a filter membrane with a cutoff of 100 kDa. The tubes were then centrifuged at 500 *g* for 10 minutes. The supernatant was then removed from the upper part of the tube and (i) for physical-chemical characterization studies, the concentrated coacervates were disturbed using a 200 μ L micropipette (“sham” procedure) or (ii) for cell studies, a volume of 50 μ L of cell suspension was added to the hydrogels and mixed using a micropipette. The tubes were then centrifuged again at 300 *g* – the same speed used to compact cell pellets in standard animal cell culture – for 5 minutes. A spatula was used to retrieve the remaining hydrogel from the bottom of the filter to clean Petri dishes.

3 Hydrogel characterization

a Zeta (ζ)-potential characterization—CHI and ALG solutions (pH 4) were prepared in 1% acetic acid or distilled water, respectively, at 0.5 mg mL⁻¹ with different NaCl concentrations: 0, 0.15, and 0.5 M. The prepared coacervates at pH 4 and pH 7 were collected and ζ -potential values were determined using a Nano-ZS equipment and DTS1070 folded capillary cells from Malvern (United Kingdom) at room temperature. All measurements were performed in triplicate, in three independent experiments.

b Water content and uptake—After the preparation of the hydrogels, excess water was removed from the hydrogels using a cellulose filter and the wet weight (W_w) was measured. After freezing at -80°C and freeze-drying for at least 24h, dry weight (D_w) was measured and the water content was calculated. The water content percentage of the processed hydrogel was calculated using equation (S1).

$$\text{Water content (\%)} = \frac{W_w - D_w}{D_w} \times 100 \quad (\text{equation S1})$$

c Recovery assessment: rheology studies—The recovery ability of the hydrogels was quantitatively assessed by a dynamic oscillatory rheology assay. The rheological measurements were collected using a Kinexus Pro+ Rheometer at room temperature, using a stainless steel parallel plate geometry. The hydrogels were first strained from 0.1% to 500% until failure at a frequency of 1 rad s⁻¹. To assess sequential recovery of the materials, a 0.5% strain was applied for two minutes, followed by a 500% strain, also for two minutes. This cycle was repeated four times. Storage modulus (G') and loss modulus (G'') were recorded and analyzed.

d Chemical characterization: Fourier Transform (FT)-Raman—The FT-Raman spectra of CHT and ALG powders, as well as of their air-dried hydrogels, were measured to study the interactions of both polymers. The samples were air dried during 48 hours at room temperature. The spectra of all 1.4C samples (0 M, 0.15 M and 0.5 M NaCl) were obtained using a FT-Raman Bruker RFS/100S equipment (Laser: Nd-YAG; wavelength: 1064 nm; Laser power: 350 mW; number of scans: 1000; resolutions: 4 cm⁻¹).

e Cell encapsulation and characterization—An immortalized mouse fibroblast cell line (L929) was grown in 150 cm² tissue culture flasks using DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C until approximately 80% confluence. Cell culture medium was replaced every 3 days. Adipose-derived mesenchymal stem cells (hASCs) were grown using the same protocol, using α MEM, supplemented with 10% FBS and 1% penicillin-streptomycin, as cell culture medium. The L929 cell line is well established for the characterization of biomaterials cytocompatibility (ISO Standard 10993-5). ASCs have shown the potential to be used in cell therapy and differentiated into distinct cell lineages, including the osteogenic, chondrogenic and adipogenic ones, targeting the development of disease models or tissue regeneration products. As such, their study allows envisioning the applications of biomaterials as supports for stem cell delivery and differentiation. After 80-90% confluence, cells were washed with 1x calcium- and magnesium-free PBS, and enzymatically detached from the culture flasks using 0.05% TrypleExpress solution for 5 to 10 minutes, incubated at 37°C. The cells were then centrifuged at 300 *g* for 5 minutes, and the pellets were re-suspended in 50 μ L of the respective cell culture medium, at cell densities of 1×10^6 cells/50 μ L and 2×10^5 cells/50 μ L for L929 and ASCs, respectively. These cell concentrates were added to the hydrogel precursors and mixed vigorously with the help of a 100 μ L micropipette, right after the first centrifugation cycle of hydrogel preparation, i.e. after the removal of the remaining liquid following the first centrifugation step. The cell/hydrogel mixture was then centrifuged for 5 minutes in sterile Amicon[®] tubes (100 kDa cutoff) for 5 minutes at 300 *g*. The cell-laden hydrogels were then removed from the filter membrane to suspension 48 well plates using a sterile spatula. All procedures were performed under sterile conditions, inside a vertical laminar flow chamber. Cells were cultured at 37°C, in a 5% CO₂ humidified environment, for 7 days. Cell culture was fully replenished every 2 days.

Cellular viability and metabolic activity was assessed after 1, 4 and 7 days of cell culture, using a LIVE/DEAD cell staining assay and alamarBlue[®] test, respectively, accordingly to manufacturers' instructions. Briefly, for the LIVE/DEAD assay, hydrogel samples were incubated in 500 μ L of 1x PBS with 0.5 μ L propidium iodide (PI) and 1 μ L of calcein-AM for 20 minutes. The samples were then visualized in a fluorescence microscope (Axio Imager 2, Zeiss), using the 488 nm and 610 nm filters. Viable cells were stained in green, because of the entrance of calcein-AM in their cytoplasm, the further cleavage of the calcein-AM bond by live cell esterases. Dead cells were stained in red due to the linkage of PI to cell nucleus caused by membrane disruption. Cell metabolic activity was assessed overtime using the alamarBlue[®] assay. Cell-laden hydrogels were incubated in cell culture medium with 10% (v/v) of alamarBlue[®] reagent. Hydrogels were incubated at 37°C, 5% CO₂ for 16 hours. Cell culture medium was then retrieved from the well plates to a new sterile 96 well plate, and the conversion of resazurin into resorufin through reagent reduction by viable cells was monitored by absorbance quantification at 570 nm, normalized by absorbance at 600 nm, accordingly to manufacturer's instructions.

4 Statistical Analysis

For zeta potential, all experiments were performed at least in triplicate in three independent experiments ($n = 3$) and results are presented as mean $\pm$ standard deviation. For rheology

tests and water content assessment, samples were analyzed at least in triplicate. For biological analysis, at least five hydrogels were used to perform each analysis. Statistical analysis was performed with the GraphPad 6.0 software, using the one-way analysis of variance test with Bonferroni post hoc multiple comparison test; differences were considered statistically significant for a $p < 0.05$.

Results and Discussion

The synthesis of sequentially moldable, rapidly recovering, shape self-standing, fit-to-shape, bondable and biocompatible hydrogels is described using two non-modified affordable and abundant natural polysaccharides: chitosan (CHT) and alginate (ALG). A schematic representation of the preparation of these hydrogels can be found in Scheme 1, and a characterization of their precursors' ζ -potential, hydrogel's aspect and water content/uptake properties in Figure 1a-c. Their ability to be molded into different shapes is showcased in Figure 2a-d,f. Oscillatory rheology characterization showed that structural loss at shear deformations of 500% was rapidly recovered by the hydrogels (up to 43% shear storage modulus (G') recovery after the first complete structural destruction cycle, and around 100% on three subsequent cycles) – Figure 2e. High structural recovery imparted the hydrogels with a “play dough”-like behavior. While hydrogels could be deformed using low stress values, their ability to rapidly recover their viscoelastic solid features allowed fixing sequential shapes (Figure 2a). Moreover, by applying a shear movement to two distinct pieces of hydrogel, those could be bonded together, and their shape could be altered several times (Video 1). This peculiar characteristic may be particularly valuable in the biomedical field to control materials' 3D configuration and spatial distribution overtime. Besides the ability to acquire macrostructural shapes (Figure 2a), the hydrogels also showed the ability to fill pre-determined hollow shapes with accuracy upon manually-applied deformation (Figure 3c,d). The highly hydrated state of these hydrogels – with 95% to 97% water content (Figure 1c) – makes them interesting materials for applications that demand high water retention, such as wound patches^{25,26} or fire retardants (as suggested before for systems showing similar rheological properties¹³). Cells from a rat fibroblast cell line and clinically relevant human mesenchymal stem cells derived from the adipose tissue (hASCs) showed high viability rates while cultured in the hydrogels for 7 days.

Hydrogels were synthesized by an innovative simple and mild process, depicted in Scheme 1. Polyelectrolyte complexation of CHT and ALG was promoted at pH 4 (37°C), under stirring (600 rpm). At pH 4, CHT is below its pKa (~6.5),²⁷ showing positively charged amine groups. ALG is above its pKa (~3.5),²⁸ and carboxylic groups are negatively charged. The formation of polyelectrolyte complexes, widely reported for this polyelectrolyte pair, 29–33 was driven mainly by electrostatic interactions, and occurred upon mixing of the polymeric solutions. CHT/ALG polyelectrolyte complexes (PECs) were prepared with distinct polyelectrolyte ratios. The calculation of theoretical charge ratios was based on CHT degree of deacetylation – calculated from ¹H NMR data (Figure S1, Supporting Information), monomer mass of each polyelectrolyte, and the pre-assumption that at pH 4 all deacetylated amine and carboxyl groups will be charged. The detailed description of charge ratios calculation can be found on Supporting Information. After PEC formation, the pH of the dispersed coacervates was gradually raised to 7. At this pH value, CHT is above its pKa,

and positively charged amines that did not react with alginate in the first complexation step at pH 4 are expected to be, at least partially, neutralized. Layer-by-layer free-standing CHT/ALG films showed low thickness and water uptake variations at pH values in the range of 7 to 9, probably indicating that the unprotonation of amine groups can occur at those pH values without the perturbation of CHT/ALG electrostatic assembly.³⁴ The increase from pH 4 to pH 7 drove the formation of larger CHT/ALG aggregates able to sediment within 30 minutes to 1 hour at rest (Figure S2, Supporting Information). The remaining solutions were then decanted down to 25% of their initial volume, and the concentrated coacervates were then filtered and compacted through a 100 kDa membrane at 500 *g*. After this step, a cell suspension was mixed with the remaining compacted coacervates, and a partial dehydration process was carried out by another centrifugation at 300 *g*. For samples prepared without cells, a “sham” protocol, consisting of pipetting the compacted coacervates, was developed to emulate the introduction of a cellular suspension. A 300 *g* centrifugation speed was chosen because it is commonly used for pellet retrieval on animal cell culture protocols. Formed cell-laden (or cell-free) hydrogels were retrieved from the membrane using a sterile spatula.

The zeta potential of the formed ALG/CHT complexes was characterized after polyelectrolyte complexation at pH 4, and after pH raising to 7. Solutions of 0.5 mg mL⁻¹ ALG and CHT, initially used to form coacervates at pH 4, were first characterized in the presence of 0, 0.15 and 0.5 M NaCl. ALG solutions showed negatively charged ζ -potential values (-40 to -20 mV), correlating with charged carboxylic groups, in accordance to previously reported results.³⁴ CHT showed positively charged ζ -potential (+20 to +40 mV), also in accordance with previously reported data,²⁹ corroborating the presence of unprotonated amines at pH 4. Modular ζ -potential values were lower for solutions prepared with increasing concentrations of NaCl, which can probably be associated with the ion screening effect.³⁵ CHT and ALG showed similar modular ζ -potential values at pH 4, which was previously theorized to lead efficient CHT/ALG coacervate formation.³⁵ Stoichiometric ratios higher than the 1:1 net charge of both polymers (equivalent to ~1C mass ratio) led, in general, to gradually higher ζ -potential values, indicating a predominantly positively charged environment at the surface of the formed coacervates (Figure 1a). Only for the 0.8C condition, with 0 M and 0.15 M NaCl, polymer complexes showed negative surface charge. After transition to pH 7, all coacervates tended to negative ζ -potential values. This occurred after the neutralization of unreacted amine groups of CHT, indicating that at this stage PECs are negatively charged on their surface with the remaining unreacted alginate carboxylic groups (still charged at pH 7, above ALG pKa).

Water content of the hydrogels was calculated by weighting the hydrogels after preparation, and then after complete drying by lyophilization. In general, water content average values of all formulations ranged from 95 to 97%, and different processing conditions did not lead to statistically significant differences in hydrogels' water content (Figure 1c-e). Immersion in distilled water for 24 hours at room temperature did not change significantly the water content of hydrogels prepared with 0 M or 0.5 M NaCl; nonetheless, it led to an increase in such values for 0.15 M NaCl samples, which reached up to 99% of water content for the 0.8C condition. In the isotonic phosphate buffer saline (PBS) solution, water content also varied mainly for 0 M NaCl samples that, in general, lost water. In general, water and PBS

uptake results seem to be mainly influenced by salt osmotic gradients created by NaCl (used during the preparation of the hydrogels), and water or PBS ionic strength. For 0.15 M samples, hydrogels' water content after processing and after 24 hours immersion decreased gradually with increasing relative proportion of CHT used to prepare polymeric coacervates. Since 0.15 M NaCl and PBS have the similar ionic strengths (150 mM and 162 mM, respectively), we hypothesize that water uptake by hydrogels prepared with 0.15 M NaCl will be directed mainly by molecular mobility and hydrophilicity-driven mechanisms, instead of osmotic pressure phenomena. Indeed, in samples with higher modular ζ -potential, the presence of unreacted highly hydrophilic alginate – possibly attributed to a low complexation efficiency driven by a poor charge compensation – are a plausible explanation for the higher water uptake observed in both 0.8C and 1C conditions (Figure 1c-e). In general, all hydrogels showed high degrees of hydration (>94%) either after preparation, immersion in water or in a physiological-like saline solution, showing their versatile application as highly hydrated water-retaining systems for both salt-free applications or salt-dependent applications (e.g. as industrial cleaning agents or cell encapsulation systems, respectively).

CHT/ALG hydrogels showed the ability to withstand different shapes induced by handling, with a behavior resembling “play-dough” (Figure 2a-d). Different shapes could be repeatedly induced using the same hydrogel (Figure 2e, Video 1 and Video 2). Such molded hydrogels showed stable morphology after immersion in cell culture medium. A quantitative assessment of such compliant and shape-fixing ability was assessed by rheology tests using 1.4C 0.15 M hydrogels. To assess an effective strain value to assure hydrogels' structural destruction, the hydrogels were exposed to increasing shear strains (from 0 to 500%). A steep decrease in shear modulus (G') from 649 Pa to ~160 Pa was observed after the first cycle of deformation at around 100% shear strain (Figure S3, Supporting Information). A complete loss of the structural properties of the hydrogel was observed at 252% strain, when the G'/G'' crossover was observed. The hydrogels also showed the ability to flow with the application of shear stress, exhibiting a shear-thinning response (Figure S4, Supporting Information). Two minutes sequential cycles of 0.5% and 500% shear deformations were performed to assess the structural recovery ability of the hydrogels (Figure 2e). After exposure to 500% shear strains, the materials were able to recover to the solid state ($G' > G''$) immediately after applied stress relaxation (Figure 2e), in a partial structural recovery behavior.^{13, 36} After the first cycle, hydrogels showed an average 43.4 ± 2 % recovery rate of their initially measured G' at room temperature. Three additional cycles were performed to check hydrogels' ability to recover from sequential cycles of complete destruction of their solid structure. In those cycles, the hydrogels could recover their structure with G' recovery efficiencies of approximately 100%, as compared to the G' value measured in the previous deformation cycle (Figure 2e and Table S1, Supporting Information). The partial recovery of hydrogels' storage modulus after the first application of a 500% shear strain, followed by a complete recovery upon subsequent destructive strains suggests that a primary structure of the hydrogels is destroyed on the first cycle of deformation, which is unable to recover under the experimental conditions used in this study. The ductile and compliant behavior of these hydrogels is showcased in Video 3. A similar rapid recovery of high shear deformations was observed for 1.4C 0 M hydrogels, indicating the independence this property from the

presence of salt during hydrogels preparation (Figure S5 and Table S1, Supporting Information).

Another interesting property observed in the hydrogels was their ability to be bonded, either after (1) hydrogel breakage or (2) by using hydrogels initially synthesized as independent pieces (Figure 2f). This bonding process was dependent on the application of a shear deformation in the interface of both hydrogel pieces, using shear deformation, in a similar manner to the one applied to re-define hydrogels' shape. After this process, the shape of the new hydrogel formed by different aggregated single pieces can be modulated sequentially into a multitude of shapes (Figure 2f). Tandem deformation steps allow bonding and deforming different hydrogel pieces into different shapes and 3D-controlled complex materials. This property may be useful to establish complex cell cultivation strategies, in which the composition and temporary relative position of each hydrogel piece can be controlled and altered overtime. This may find application on the design of 4D on-demand changing hydrogel compositions, including compartmentalized spatiotemporally-controlled hydrogels made from bond hydrogel fragments that may contain different cargos, including bioactive agents or cell types.

FT-Raman spectroscopy was used to assess the type of chemical interactions present in ALG/CHT hydrogels. The condition 1.4C 0.15 M - relevant for potential cell encapsulation strategies due to its isotonic character - was characterized, along with ALG and CHT dry powders, and a physical dry powder mixture at the same polyelectrolyte/polyelectrolyte ratio (Figure S6, Supporting Information). The characteristic peaks assigned to the hydrogels analysis can be found in Table S2 (Supporting Information).^{37–39} The polyelectrolyte complexation occurring between CHT charged amines and ALG charged carboxylic groups was visible in several peaks. Characteristic peaks of ALG corresponding to carboxylic groups (COO^- ; 1656 cm^{-1}),⁴⁰ as well as amine I detected in CHT (here, 1597 cm^{-1})⁴¹ were lost or severely decreased in hydrogels. Also, the decrease in the peak 2885 cm^{-1} , corresponding to the asymmetric stretching of CHT's $-\text{CH}_2$ group,⁴¹ suggests that uncharged groups as $-\text{OH}$ (linked to $-\text{CH}_2$) present in both polyelectrolytes, may have a role in coacervate and hydrogels' formation by dipole-dipole interactions.³⁹ Also, the peak at 1086 cm^{-1} detected in CHT, attributed to the asymmetric stretching of C-O-C bonds (in literature, at $1093\text{--}1099\text{ cm}^{-1}$ for chitosan,⁴¹ and 1150 cm^{-1} for alginate³⁹), is lost in hydrogels, indicating the role of other uncharged groups in the stability of the hydrogels. A plausible theory for the formation of the hydrogels is the establishment of PECs by electrostatic bonds occurring in the first phase of complexation (with charged polymers) at pH 4 followed by, upon pH raising to 7, a possible mixture of electrostatic bonds (using possible remaining charged CHT amines) and, mainly, hydrogen bonds, hydrophobic interactions, among other van der Waals interactions driving the gathering and stabilization of individual PECs. Due to their weak nature, van der Waals interactions have been reported to show a reversible behavior after breakage, allowing materials' rapid partial healing,^{36,42} which here imparts the materials with easy and reversible moldability.

The compatibility of the 0.15 M hydrogels with cellular encapsulation was tested with two cell types: (i) L929 rat fibroblast cell line and (ii) adipose-derived stem cells (hASCs). Both cell types were viable after 7 days of cell culture (Figure 3a) and dispersed throughout the

whole hydrogel structure (Figure 3b). Moreover, cells contained in hydrogels that went through re-shaping using pre-defined hollow shapes (Figure 3c,d) were also viable and did not show any decrease in cell viability. The alamarBlue® test corroborated cellular viability inside the hydrogels (Figure 3e). For L929 cells, an increase in the reduced alamarBlue® reagent was observed overtime (Figure 3e), which was consistent with the increasing live cells density observed from day 1 to day 7 of cell culture (Figure 3a). For ASCs, however, no significant increase in cell metabolic activity could be observed overtime (Figure 3e). These results are consistent with results previously obtained for alginate hydrogels crosslinked with calcium ions.⁴³ In alginate beads, L929 cells show the ability to proliferate, even in the absence of any cell-recognition domains. ASCs, on the other hand, have shown the ability to remain viable for long *in vitro* culture times while encapsulated in those hydrogels, without any visible signs of cellular proliferation.⁴⁴ Two-dimensional membranes prepared using CHT and ALG, using the layer-by-layer methodology, showed the inability to promote effective cell adhesion, namely with ASCs cells,^{45,46} suggesting that the chemical environment provided by the electrostatic assembly of these two polyelectrolytes does not favor spontaneous cell attachment and proliferation of most cell types. The use of PECs formed at acidic pH to prepare cell-loaded hydrogels was previously reported for chitosan-chondroitin sulfate hydrogels⁴⁷; hASCs showed high viability and differentiation potential into the chondrogenic lineage while encapsulated in platelet lysate-loaded hydrogels. However, the mechanical compliance and self-recovery properties of these structures was not mentioned, and future studies must be performed to characterize the mechanical behavior of hydrogels prepared with distinct polyelectrolyte pairs. Irradiation-crosslinked hydrogels containing embedded coacervates pioneered the use of these structures as spatially-controlled delivery systems with the ability to locally modulate cell and tissue response.⁴⁸ The high cell viability observed in CHT/ALG hydrogels allows envisioning their future as cell differentiation-triggering materials, with 3D spatially-controlled delivery or presentation of biomolecules.

The multifunctionality of the CHT/ALG hydrogels reported here may benefit several biotechnology-related fields due to their safety and versatile mechanical properties. We envision particularly interesting future application in the design of tissue regeneration devices. In particular, their shear-thinning and cell-encapsulation compatible properties makes them potential candidates as high-retention systems for *in situ* cell delivery; their moldability may be useful to fill tissue defects with complex shapes, or to be used as fit-to-shape patches in topical dermal applications, including wounds. The ability to bond hydrogels' fragments into different positions may be useful in the preparation of spatiotemporally-controlled drug delivery systems, or co-culture devices, in which direct or indirect contact between different cell types may be altered overtime.

Conclusion

We present a fully polymeric non-covalent hydrogel with high compliance, rapid recovery ability, sequential shape-morphism and moldability, as well as bonding ability. For the first time, a hydrogel capable of withstanding cell encapsulation shows moldability, on-demand positioning of different fragments, and ability to change its composition over time. The synthesis method relying on the partial dehydration of coacervate compact structures allows

preparing highly hydrated hydrogels in salt-free conditions, as well as in conditions with different ionic strengths. We envision the application of this technique in the preparation of complex materials made of different ionic pairs of polyelectrolytes, rendering materials with distinct chemical and biological features. The combined properties of these environmentally friendly hydrogels allied to their safety makes them potential candidates as materials for the food processing industry, cosmetics developments, electronics and sensors, as well as tissue regeneration/drug delivery strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) ζ -potential measurement of CHT and ALG solutions (CHT, ALG) and coacervates produced with different molar charge ratios, right after preparation (pH 4) and after transition to pH 7, with distinct amounts of NaCl. (b) Pictures of hydrogels (0.15 M NaCl) right after preparation (scale bar = 5 mm). Water content of hydrogels after preparation, after 24 hours of immersion in distilled water and after 24 hours immersion in PBS, prepared with (c) 0 M NaCl, (d) 0.15 M NaCl and (e) 0.5 M NaCl. # indicates statistically significant difference between the water content values of the same hydrogel condition while immersed in different solutions ($p < 0.05$).

Figure 2.

(a) A piece of hydrogel is retrieved in shape #1 and sequentially molded into shape #2 and shape #3. (b) Mold used to achieve shape #3. (c) The samples are easily handled using tweezers after partial dehydration using an absorbent filter, and (d) can then be adapted to confined geometries, such as a pipette tip. (e) Oscillatory rheology tests applying sequential shear strains of 0.5% and 500%, for time periods of 2 minutes (1 rad s^{-1} , 4 destruction/recovery cycles). (f) Simultaneous molding and binding of three different hydrogel fragments into one single piece, with shape-morphing features.

Figure 3.

Calcein AM (green, live cells) and propidium iodide staining (red, dead cells) staining of (a) L929 fibroblast cell line encapsulated into 0.15 M 1.4C hydrogels after preparation (D0) and after 1, 4 and 7 days of incubation at 37°C, 5% CO₂ in a humidified saturated atmosphere (scale bar = 100 μm); (b) hASCs after 7 days of cell culture (image of a full hydrogel, with borders marked with dashed line); (c) after hydrogel re-shaping in a hollow mold with the shape of a U; (d) after hydrogel re-shaping in a hollow mold with the shape of the number 2. (e) alamarBlue[®] corrected absorbance values for both cell types while cultured in 0.15 M 1.4C hydrogels. ** indicates a statistically significant difference ($p < 0.01$).

Scheme 1.

Schematic representation of the steps performed to produce cell-laden moldable CHT/ALG hydrogels.