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ARTICLE

Alginate-based Hydrogels Functionalised at the Nanoscale using Layer-by-Layer Assembly for Cartilage Repair

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Injuries to articular cartilage are frequently difficult to repair, in part because of the poor regenerative capacity of this tissue. To date, no successful system for complete regeneration of the most challenging cartilage defects has been demonstrated. The aim of this work was to develop functionalised hydrogels at nanoscale by Layer-by-Layer (LbL) assembly to promote cartilage healing. Hydrogels, based on sodium alginate (NaAlg) and gelatin (G), were prepared by an external gelation method consisting in $CaCl_2$ diffusion and genipin addition for G crosslinking. Successively, hydrogels were coated with G to obtain a positive charge on the surface; then functionalised by LbL assembly to create 16 nanolayers, based on (poly (styrene sulfonate)/poly(allyl amine) (PSS/PAH), including a specific peptide sequence (CTATVHL) and transforming growth factors β1 (TGF-β1). Physico-chemical properties were evaluated by XPS, ATR-FTIR and rheological analyses while *in vitro* cytocompatibility was studied using bovine articular chondrocytes (BAC). XPS spectra showed N_{1s} and S_{2p} peak, indicating PAH and PSS have been introduced with success. ATR-FTIR indicated the specific PAH and PSS absorption peaks. Finally, the biomolecules incorporation influenced positively with the processes of BAC adhesion and proliferation, and glycosamynoglycans secretion. The functionalised alginate-based hydrogels described here are ideally suited to chondral regeneration in terms of their integrity, stability, and cytocompatibility.

Introduction

Articular cartilage is well known to have poor intrinsic capacity for self-repair and is therefore extremely difficult to regenerate following injury, especially after the onset of degenerative joint disease. Up to now, clinical treatments, i.e. abrasion arthroplasty, chondral shaving, subchondral drilling, micro-fracturing, mosaicplasty, and prosthetic joint replacement have been largely employed for patients suffering from full thickness articular cartilage lesions [1, 2]. However, although giving some symptomatic relief these treatments present some disadvantages, such as a lack of donor tissue availability, donor site morbidity in mosaicplasty, and formation of an inappropriate cartilage (fibrocartilage) in subchondral drilling which usually degenerates within a few years. Eventually, prosthetic joint replacement may be needed to restore joint mobility but these have limited durability and can loosen leading to implant failure [3].

Alternatively, tissue engineering (TE) represents an emerging strategy to substitute and replace the patient-painful treatments. Among the TE constituents, hydrogels are suitable for cartilage regeneration for their distinctive biocompatibility, ability to include chemical biocues, and intrinsic hydrated structure [4, 5]. Different biomaterials have been proposed as matrices for cell supporting. Natural-based polymers represent a suitable choice, due to their similarities with extracellular matrix (ECM), chemical adaptability and biological behaviour [6, 7]. Furthermore, they have excellent flexibility to be shaped to desired forms through different casting and moulding methods [8]. One of the largely used natural polymer in the preparation of scaffolds for cartilage repair is the sodium alginate (NaAlg) which is an anionic and hydrophilic polysaccharide, derived from bacteria and brown seaweed [7]. Alginates are a family of linear binary copolymers, consisting of (1-4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. Chemical structure may differ largely between the species of algae and the period of the year when they are harvested [9]. According to the implant site, hydrogels are exposed to different pH conditions, which influence the degradation rate and swelling behaviour as well as the mechanical properties. Alginate is highly influenced by the molecular weight, in terms of the long-term stability (i.e. degradation rate) and performance (i.e. mechanical properties) [10]. NaAlg gels for TE field can be subdivided into physical and covalent hydrogels, according to their mechanism of gelation. Several approaches have been used for the alginate-based hydrogel preparation, such as thermal gelation, ionic interaction, “click” reaction and free radical

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polymerization [11]. In the last decade, ionic cross-linked alginate hydrogels have been developed using different setting parameters and different ions, such as Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{2+} or Sr^{2+}. Particularly, Ca^{2+} is the largely divalent cations utilised for the ionic alginate crosslink, where $CaCl_2$ is its most commonly used source [12]. However, the gelation occurs quickly because of the high $CaCl_2$ solubility in aqueous solution. Therefore, for obtaining tuneable gelation rate, different methods can be used. Recently, Hunt and Kaklamani proposed to control the gelation rate externally using porous micro-cellulose sheets, as boundary sheets, impregnated previously in a calcium chloride solution that were separated by a layer of aqueous NaAlg solution [13, 14].

However, alginate did not show to have good response after seeding mammalian cells. Therefore, the addition of cell adhesion biomaterials, such as collagen, gelatin have been shown to improve the alginate biocompatibility with this type of cells [15]. Gelatin is a very cheap material with an increased interest in tissue engineering [16, 17]. Because gelatin may be quickly degraded *in vivo*, a crosslinking method is required in order to improve its stability and mechanical strength. Genipin, the aglycone of geniposide (an iridoid glucoside derived from the fruits of *Genipa americana* and *Gardenia jasminoides Ellis*) has been largely used for the crosslinking of amino-containing materials, such as gelatin and collagen [18]. Genipin has been used as a crosslinking agent of different composite films and scaffolds for Guided Bone Regeneration (GBR) and microcapsules for drug delivery [19, 20].

In this work, we propose an alginate-based hydrogels functionalised by Layer-by-Layer (LbL) assembly in order to incorporate bioactive molecules as an efficient approach for improving the chondrocytes adhesion and proliferation. This is because the top surface of hydrogels is the first contact region with chondrocytes suspensions [21, 22]. Recently the potential of different peptides has been investigated. As example, EPLQLKM peptide (E7) was covalently conjugated onto poly(ε-caprolactone) electrospun meshes and, then, implanted into a cartilage defect site of rat knee joints with endogenous Mesenchymal stem cells (MSCs). The authors found that, after 1 week only, E7 peptide sequence showed high specific affinity to MSCs and enhanced MSCs recruitment *in vivo* [23]. In another work TATVHL peptide was grafted to polyethylene oxide/chitin/chitosan scaffolds and the biologically properties were evaluated using bovine knee chondrocytes. The results demonstrated that TATVHL peptide improved the chondrocytes proliferation and the glycosaminoglycans (GAGs) secretion [24]. Furthermore, growth factors from the transforming growth factor-β (TGF-β) superfamily demonstrated to have a great potential in cartilage repair. Particularly TGF-β1 stimulated chondrocyte activity and decreased the catabolic activity of Interleukin-1 (IL-1) [25], as well as encouraged the chondrogenesis of bone marrow-derived MSCs [26].

In this study, we describe the application of an alternative method called layer-by-layer assembly, to create on the hydrogel surface a multilayered structure incorporating TATVHL peptide sequence and TGF-β1 in order to control at nanoscale the chondrocyte biological response. LbL method, based on the alternating exposure of positively and negatively solutions of charged polymers called polyelectrolytes (PEs), is inexpensive and environmentally-friendly. Particularly, given the versatility of the LbL assembly method, the development of polyelectrolyte multilayer coating on hydrogels is currently emerging as a useful tool to functionalise hydrogel surface for various biomedical applications. Sakaguchi et al. first reported the LbL deposition of PEMs on synthetic poly(vinyl alcohol) hydrogels to control their coagulation properties [27]. Recently, biomimetic stratified structures have been created by spray-deposition where PEMs were alternated with alginate gel layers containing cells to mimic multilayered 3D structures found in various tissues such as skin [28]. On the other hand, Mehrotra et al. have modified agarose hydrogels using synthetic polyelectrolytes built in a LbL manner to control the release of the model protein lysozyme from hydrogels [29].

While LbL is highly attractive as a method to functionalise materials that are otherwise unable to stimulate specific biological processes, there is no report to date on the effect of LbL deposition on the microstructure, physico-chemical, mechanical and biological properties of a polysaccharide hydrogel for cartilage regeneration. In this work, an investigation of different hydrogel compositions was reported in order to select the most appropriate scaffold for functionalisation. Physico-chemical and mechanical properties were investigated using attenuated total reflectance and Fourier transform infrared spectroscopy (ATR-FTIR), swelling tests, and rheology measurements. Then, to confirm the incorporation of biomolecules by LbL, the hydrogels were characterised using X-ray Photon Spectroscopy (XPS) and further ATR-FTIR. Finally, the functionalised hydrogels were studied *in vitro* by seeding them with bovine articular chondrocytes (BAC) to assess the influence of the combination of the two bioactive molecules on BAC biological response. This work has important implications for the development of new implantable constructs for the unmet clinical need of regenerative osteochondral repair.

Experimental

Materials

Alginic acid sodium salt from brown algae (NaAlg; Low viscosity, CAS No: 9005-383), gelatin from porcine skin (Type A powder; CAS No: 9000-70-8), genipin (CAS No: 6902-77-8), calcium chloride (CAS No: 10043-52-4), sodium chloride (CAS No: 764714-5), poly(sodium4-styrenesulfonate) (CAS No: 25704-18-1), ethylenediamine (CAS No: 107-15-3), ε-Maleimidocaproic acid (CAS No: 55750-53-3), N-Hydroxysuccinimide (CAS No: 6066-82-6) and N-(3 Dimethylaminopropyl) - N-ethylcarbodiimide hydrochloride (CAS No: 25952-53-8) were purchased from Sigma Aldrich, UK. Poly (allylamine hydrochloride) (CAS No: 71550-12-4) was purchased from Alfa Aesar Company, UK). CTATVHL peptide sequence was synthesized (purity more than 95% by analytical HPLC) and supplied by Biomatik, Taiwan. Rat TGF-β1 was purchased from Stratech Scientific Ltd, UK. The cysteine (C) aminoacid has been added to the TATVHL peptide sequence to facilitate the PAH conjugation.

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Methods

Alginate-based hydrogel preparation

Four different solutions were prepared by dissolving NaAlg and G powders in deionised water at 50 °C: 3% w/v NaAlg, 5% w/v NaAlg, 3% w/v NaAlg -G blend (80:20 weight ratio) and 5% w/v NaAlg -G blend (80:20 weight ratio) coded as A3%, A5%, AG3% and AG5% respectively.

G-crosslinking was obtained by adding GP in a concentration of 2% (w/w) calculated with respect to the gelatin content. All the solutions were stirred for 30 min at 50 °C, then sonicated in ultrasound bath for 15 min. Filter papers (Whatman) were soaked in 5M $CaCl_2$ solution for 5 min at ambient temperature and deposed on the bottom of plastic Petri dishes (5 cm diameter). Then 20 ml of each solution was casted in different Petri dishes and covered with another $CaCl_2$-embeded filter paper. For ensuring the complete gelation by $CaCl_2$ diffusion, the Petri dishes were stored at +4 °C for 2 h. The obtained gel samples were removed from the casting mould and cut in small circles (diameter: 8 mm; thickness: 1 mm) for further characterisation.

Gelatin coating

Gelatin coating was casted on the hydrogel top surface in order to obtain the first charged layer for the following LbL functionalisation. Samples coating was achieved by immersions in 3% w/v gelatin for 15 min, then dried for 15 min (procedure repeated two times. Then hydrogels were immersed in 2.5% w/w GP solution for 24 h at room temperature.

Conjugation of CTATVHL peptide to PAH

CTATVHL peptide was conjugated to PAH following the same protocol used recently by the authors [30]. 0.15 g of EMCA was mixed with 100 ml of PAH solution (0.2 g PAH, 2.37 g EDC and 1.47 g NHS) and incubated at room temperature for 2 h. Gel filtration was performed in order to remove non-reacted reactants and additional by-products. CTATVHL peptide was grafted to PAH-g-EMAC via reaction between the thiol groups of cysteine (C) and the maleimide groups (5 ml of PAH-g-EMAC solution with 100 µg of CTATVHL peptide). The obtained solution was then incubated at 4 °C for 24 h and coded as PAH-g-CTATVHL.

Layer-by-Layer functionalisation

The assembly of nano-scale multilayers was performed at room temperature. The polyelectrolytes were dissolved in solutions in 0.1 M NaCl with a pH of around 4.5 (5 mg/mL). The ζ-potentials of the polyelectrolyte solutions was measured by laser Doppler electrophoresis (Zetasizer Nano, Malvern instrument, USA). The coated hydrogels were dipped firstly in PSS solution (5 mL) for 15 min. Then, they were washed in water containing 0.1 M NaCl at pH 4.5 for 5 min. The, the samples were finally soaked in PAH solution (5 mL) for 15 min followed with water washing step using the same parameters described before. This dipping process was repeated for 4 cycles for creating 8 layers. Then the LbL assembly procedure was repeated for other 4 cycles (8 layers) but using the PAH-g-CTATVHL embedded with TGF-β1 (20 µg/ml) as cationic solution. Finally, after the obtainment of 16 layers, the hydrogels were washed with distilled water for 10 min, left to dry under hood and stored in the fridge at 4 °C.

Characterization methods

Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR (Nicolet iS10) was used to analyse the chemical composition of the sample surface over a range of 4000-550 cm^{-1}, with a resolution of 4 cm^{-1}. Before ATR-FTIR test, samples were frozen at -20 °C for 12 h and then freeze-dried at -20 °C for 24 h.

Swelling test

Dried samples (after freeze-drying) were firstly weighted (w_d). Then samples were soaked in Phosphate Buffer Solution (PBS) to measure the swelling behaviour in 24 hours at 37 °C. Samples were weighted after each time point (w_w). Swelling degree percentage (S_w%) of the gels was calculated using the following equation:

$$S_w\% = (w_w - w_d)/w_d \times 100 \qquad \text{Eq. (1)}$$

The test was performed in triplicate and results were reported as mean ± standard deviation.

Colorimetric Test (Orange II)

Orange II was selected, among commonly used dyes, to predict the amount of amine groups available on coating film for subsequent Lbl functionalisation. In the colorimetric test, 4 mg of Orange II dye (500 µM) was dissolved in 18 ml of aqueous acidic solution (pH 3 adjusted with 1 M HCl). The samples were immersed in this solution for 30 min at 37°C and then intensively rinsed 3 times using an acidic solution (pH 3) to remove unbound dye. Once air-dried, the coloured gels were soaked for 1 hour in alkaline solution (pH 12 adjusted with a 1 M NaOH solution). The absorbance of the solutions was measured by Ultraviolet-Visible (UV-Vis) spectrometry (Lambda 2S Perkin Elmer) at 484 nm.

Rheology analysis

The hydrogels were analysed for rheology (Physica MCR 301, Anton Paar) and the results were examined by Rheoplus/32 software. For frequency-sweep tests, gels (8 mm diameter and 1 mm thick) were soaked in PBS at 37 °C for 30 minutes. The frequency sweep was done by varying the angular frequency from 1 to 150 rad s^{-1} at 1 % strain. For this purpose a 7.99 mm measuring system was used. The angular frequency for measurements was set at 1 rad s^{-1}. The storage modulus (G'), loss modulus (G'') of the samples were recorded as a function of frequency.

X-Ray Photoelectron Spectroscopy (XPS)

XPS spectra were acquired on Theta Probe (Thermo Scientific, UK), equipped with a microfocused AlKa X-ray source (1486.6 eV), operated with a 400 µm spot size. Process parameters were: 200 eV pass energy, 1 eV step size of and of 50 ms dwell time in not angle-resolved lens mode. At least 3 single area were evaluated on each membrane surface.

In vitro biomolecules release

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LbL-functionalised -loaded hydrogels (samples (ϕ~ 6 mm diameter discs, thickness ~ 1 mm) were immersed in glass vials containing 5 mL of Phosphate Buffered Solution (PBS; pH 7.4, Sigma-Aldrich). The medium was withdrawn at different time point for the measurement and replaced with fresh buffer.

UV-Vis spectrophotometer (Lambda 2S Perkin Elmer) was used to determine the biomolecules release at 280 nm [31, 32]. Six replicates were measured, and the results were averaged with standard deviation. The initial content of the biomolecules drug was measured by UV-Vis after dissolving the LbL-functionalised hydrogels in acetic acid solution. As control, the same initial content of CTATVHL and TGF-β1 was mixed in the alginate-based solution before the gelation with $CaCl_2$ in order to obtain biomolecules-loaded hydrogels as control.

Cell tests

Preliminary to the seeding of cells, samples (ϕ~ 6 mm diameter discs, thickness ~ 1 mm) were sterilised using UV light for 4 hours in 48-well plates and rinsed five times with PBS. Bovine Articular Chondrocytes were isolated following a protocol described previously [33]. BACs were grown in Dulbecco's modified Eagle's medium (DMEM; high glucose) (Sigma-Aldrich), containing 10 mM Hepes buffer (Sigma-Aldrich), l-alanyl-l-glutamine (Sigma-Aldrich), non-essential aminoacids (Sigma-Aldrich) 10,000 units ml^{-1} penicillin, 10,000 µg ml^{-1} streptomycin (Sigma-Aldrich), 10% foetal calf serum (Biosera) and 10 ng ml^{-1} basic fibroblast growth factor (bFGF) (PeproTech). The medium was replaced two-three twice per week. For all experiments we used cells from up to two passages. A number of $6x10^5$ cells were seeded onto each scaffold in 1 ml DMEM. The medium was refreshed every 2-3 days. For this cell test, the culture medium was the expansion medium without bFGF but enriched with 1 mg ml^{-1} of insulin (Sigma-Aldrich) and 1 mg ml^{-1} of ascorbic acid) (Sigma-Aldrich).

Presto Blue assay. After 3 and 7 days of cell culture, the medium was removed and the sample were transferred to new 24-well plates; after addition of 10 % PrestoBlue solution (5 mg/mL in DMEM; Fisher Scientific), the multiwell plates were kept in incubation for 1 h at 37 °C. After the supernatant removal, the solution (now dark blue) was transferred in 96-well plates (0.2 mL) and quantified spectrophotometrically at 560 nm (Leica DM2500).

PicoGreen assay. PicoGreen® dsDNA reagent (Invitrogen, USA) was used to calculate the cell number for each sample in order to make a correct normalisation of the fluorescence values. After each culturing period, for having cell lysis, samples were washed with PBS and then incubated at 37 °C for 4 h. Finally the samples were frozen at -80 °C overnight in 1 mL ultra pure water. The fluorescence was analysed at 485 nm excitation wavelength and 528 nm emission wavelength. The mean ± standard deviation were calculated for five tests.

Dimethylmethylene blue assay for glycosaminoglycan (GAG) quantification. Samples were removed from the culture media after 1 month, freeze-dried and, finally, digested to permit the separation of the new formed-ECM from the gels. The digestion solution consisted of the addition of papain type III (0.05%, Sigma-Aldrich) and N-acetyl cysteíne (0.096%, Sigma-Aldrich) to 50 ml of digestion buffer (0.2 mM of phosphate buffer with 1 mM EDTA at pH 7). Hydrogels were incubated into 1.5-ml screw-cap Eppendorf tubes with 0.6 ml of the digestion solution at 60 °C for 12 hours (at least overnight). Then, samples were centrifuged for 10 min at 13,000 rpm. Supernatant was collected and frozen at -20 °C. FOR GAG assay dimethylmethylene blue (DM) solution was prepared by dissolving 16 mg DM in 0.9 L of bidistilled water containing 32.73 g NaCl and 3.04 g glycine and stirred for 120 minutes (protected from the light with Al foil). Then the pH was titrated to 3.0 with addition of HCl and reached the final volume of 1 L. The prepared solution was kept at ambient temperature and protected from the light. As control, chondroitin sulphate (CS, Sigma-Aldrich) solution was prepared by dissolving 5 mg in 1 ml of water and stored in the fridge. Then this stock solution was diluted (from 0 to 50 µg/ml) for the obtainment of the standard curve. In a 96 multiwell plate 20 µl of distilled water was added for the blank. 20 µl of sample supernatants (diluted with distilled water if necessary) and CS solution were added in triplicate, Finally, 0.25 ml of DM solution was added in each well, and then, analysed spectrophotometrically at 525 nm (Leica DM2500). As a further control, hydrogels without cells were utilised.

Statistical analysis

Tests were performed at least in triplicate for each sample. The results were represented as mean ± standard deviation. Analyse-it v2.22 software has been used for determining the statistical analysis., considering Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA) for the statistical differences between the groups. Statistical significance was declared as significant at (*) at $p< 0.05$ and very significant (**) at $p< 0.001$.

Results and discussion

The aim of the work was to develop a polymeric hydrogel functionalised firstly with a gelatin coating and then by Layer-by-Layer assembly at the nanoscale, incorporating the CTATVHL peptide sequence and the TGF-β1 growth factor for cartilage repair. Preliminary samples were prepared with different concentration of sodium alginate and gelatin (crosslinked with the natural biocompatible GP crosslinker) that has been demonstrated in literature to facilitate cell adhesion and proliferation mimicking ECM [15]. The hydrogel gelation was achieved by using Ca^{2+} ion crosslinking technique. Four different compositions were prepared and evaluated in order to find the optimal physico-chemical properties combination for the following LbL functionalisation and *in vitro* cell tests.

Physico-chemical characterisation of NaAlg and NaAlg/G hydrogels

ATR-FTIR analysis. Freeze-dried samples were analysed by ATR-FTIR for studying the chemical compositions of the four different samples. Figure 1(A) shows the ATR-FTIR spectra of the obtained hydrogels in a region ranging from 2000 to 550 cm^{-1}. The characteristic absorption bands at 1411 and 1593 cm^{-1} of NaAlg samples can be attributed to COO^- symmetric and asymmetric stretching bonds **[34]**. Furthermore, the peaks at 1310 cm^{-1}, 1088 cm^{-1} and 946 cm^{-1} (C–O stretching), and 1031 cm^{-1} (C-O–C symmetric stretching) correspond to NaAlg structure. G spectrum is characterised by peaks at around 1630 cm^{-1} and 1554 cm^{-1} that correspond to Amide I and II

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(C=O stretching and N-H bending vibration). Moreover, the typical peaks (from 1220 cm^{-1} to 1040 cm^{-1}) of the amino chains were detected **[35]**. Considering the ATR-FTIR of the blend sample (based on 80% sodium alginate and 20% gelatin), it was observed that carboxyl group band (1593 cm^{-1}) in sodium alginate shifted at higher wavenumbers for AG3% and AG5%.

Meanwhile, the intensities of bands at 1593 cm^{-1} and 1411 cm^{-1} of sodium alginate only progressively reduced with a higher content of gelatin. Finally, gelatin compared with NaAlg/G blend showed that the characteristic peaks at 1637 cm^{-1} and 1554 cm^{-1} (Amide I and II) moved to lower values of wavenumbers. All these shifts implied strong intermolecular interactions, such as electrostatic attractions and hydrogen-bonding created between gelatin and sodium alginate chains **[36]**.

Swelling tests. The swelling behaviour is fundamental for evaluating the application of hydrogels in tissue engineering field, due to the fact that gel properties are correlated with the swelling degree (i.e. flexibility, degradation rate, mechanical stiffness). Figure 1(B) show the swelling degree (S_w%) of the prepared hydrogels at different time points after 1h, 2h, 4h, 6h and 24h. Trends of all samples were similar and swelling degree increased quickly in the first 6 h and then reached a plateau value. Blend samples showed a lower swelling degree (1967±65 for AG3% and 1656±53 forAG5% respectively) due to the gelatin crosslinking with genipin that provided more stability. As described recently, swelling phenomenon is due to the network polymeric relaxation when an osmotic pressure is present **[37]**. Hydrogel swelling takes place in aqueous solution in about 6 hours since the crosslinking bonds forces are equalled by the osmotic pressure, and the gel network structure remains stable. As consequence of this equilibrium between these forces, no more water is absorbed from the samples. Therefore, when samples are placed in PBS at physiological pH, the sodium ions present in the buffer medium start an ion exchange with the calcium ions that are present in the poly-mannuronate sequences (bonded to the COO^- groups). Consequently, there is an increase of the electrostatic repulsion among COO^- negatively charged groups which finally enhance the swelling of the hydrogel due to the relaxation of the polymeric chain. In the later stage of swelling process, the Ca^{2+} ions, bonded to the COO^- groups of the polyguluronate units, create the tight "egg-box" structure, taking place to a further ion-exchange with the PBS sodium ions **[37]**.

Rheological tests. The storage modulus (G') and the loss modulus (G") are important material properties for cartilage substitutes, because they measure the combined elastic and viscous effects in dynamic shearing. The mechanical parameters of the sodium alginate-based hydrogels were influenced by the frequency value, that are presented in Figure 2(A) and (B). The sample were preconditioned in PBS after incubation for 30 minutes. The accurate examination of the results indicated that specific concentrations of sodium alginate and gelatin were able to generate an interpenetrating polymer network in the hydrogels. The G' values of all tested hydrogels were higher than G'' (around a magnitude order) over all the range frequency considered in the experiment. This indicated a prevalence of an elastic performance respect with the viscous one, that is typical of gel behaviour **[38]**. Sample AG5% presented the higher values of loss and storage modulus (234±17 kPa and 188±23 kPa at 1 rad/s respectively). Moreover, its G'' measured at lower frequencies was higher than G' thus indicating a "liquid like" behaviour of this hydrogels, in contrast to a "solid-like behaviour" for higher frequency deformation.

In general, it was obvious that both the G' and G'' values slowly increased for higher deformation frequencies. G'' denoted the gel stiffness and was measured to be >1000 Pa.

Actually, cartilage responds to shearing forces by both stretching and deformation of the solid matrix, so that in pure shear, the tissue is likely to deform with no change in volume, no pressure gradient and no fluid flow through the matrix [39]. In our work the rheological analysis showed that alginate-based hydrogels showed a similar behavior, corroborated by the fact that by comparing the rheological performance of human cartilage and that of the proposed hydrogels, they appeared to be similar. The obtained values for storage and loss modulus are fairly good when compared to other hydrogels frequently described in the literature for cartilage regenerative approaches. As example, comparable behaviours have been discussed for self-assembling hydrogels composed of ascorbyl palmitate and peptides **[38]**, which were studied as promising injectable drug-delivery system, mimicking the joint lubricants, for the osteoarthritis treatment. In this work, Strehin et al. showed that the hydrogel adhesive nature can be controlled by changing the damping factor, that consists into the loss and storage modulus ratio **[40]**. Finally, human cartilage exhibits slightly higher modulus values (monotonically increase from 0.4 to 2.5 MPa with the increase of the angular frequency). This difference is expected to be compensated by ECM deposition during the formation of a cartilaginous structure [41].

AG5% was selected as optimal composition for the second step of the work due to the favourable physical and mechanical properties.

Physico-chemical characterisation of LbL-functionalised hydrogels

Orange II test and laser Doppler electrophoresis. Colorimetric Orange II test was behaved in order to verify the presence of ε-amino groups present in deposited gelatin coating in order to ensure the chemical bound with polyelectrolyte of the first layer of LbL functionalisation. Figure 3 shows the evidence of presence of amino groups in samples with gelatin coating (0.452±0.047) compared to the control of AG5% that showed a small amount of amine group content due to the presence of gelatin in the blend (0.089±0.075).

Furthermore, the gelatin coating showed a ζ-potential of +12.2 mV, that represents a fundamental charge in order to initiate the electrostatic LbL assembly after immersion in the polyelectrolytes solutions solution (ζ-potential of +14.8 mV for pure PAH, while PSS solution was negatively charged with ζ-potential of -18.5 mV).

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The gelatin coating was estimated to have a thickness of ~1-2 µm. This range was measured by casting the same amount of gelatin on a glass coverslip (with the same size of the hydrogel sample), dried overnight and analysed by SEM (data not shown).

Chemical analyses by ATR-FTIR and XPS

LbL deposition was applied onto the hydrogel top surface in order to incorporate the peptide previously conjugated with the amino group of PAH for improving the chondrocyte behaviour. Multilayered assembly was composed by alternating PSS and PAH without CTATVHL peptide and TGF-β1 until the eight layer followed by PSS and PAH_biomol for the remaining 8 layers. In order to characterise the polyelectrolytes multilayer absorption on the hydrogel surface, ATR-FTIR analysis was performed (Figure 4). The coated AG5% sample showed the strong bands at 1637 cm^{-1} and 1554 cm^{-1}, attributed to amide carbonyl (C=O and C–N stretching vibration) of the gelatin layer, while the peak at 1031 cm^{-1} (CO–C stretching) peculiar of alginate structure decreased the intensity due to the G peaks overlap. After LbL assembly, the spectra presented the presence of the characteristic PAH and PSS peaks that increased the intensity with the increase of the number of layers.

Particularly, Figure 4 shows for PSS: H_2O stretching vibration (3700-3000 cm^{-1}), aromatic =C-H stretching vibrations (3100 cm^{-1}), alkyl C-H stretching vibrations (2920 cm^{-1}), aromatic C-H bending vibrations (1810 and 1925 cm^{-1}), aromatic –C=C– stretching vibrations (1600, 1500, 1450 and 1410 cm^{-1}) and -SO_3^- symmetric (1040 and 1005 cm^{-1}) and asymmetric (1190 and 1130 cm^{-1}) stretching vibrations.

For PAH, it was noticed: N-H stretching vibration (3360 cm^{-1}), alkyl C-H stretching vibrations (2920 cm^{-1}), N-H asymmetric and symmetric scissoring vibrations (1580 and 1490 cm^{-1}), and N-H asymmetric stretching vibrations (1330 cm^{-1}).

XPS analysis was conducted as a further analysis for the detection of the gelatin coating and LbL assembly multilayer. In Figure 5(B) the peak of nitrogen at 400 eV showed the presence of gelatin (with a consequent decrease of calcium and chlorine peaks of alginate) compared with the uncoated sample (Figure 5(A)). Figure 5(C) shows the spectra of the hydrogels with LbL deposition. The peak N_{1s} at 400 eV and the peak S_{2s} at 168 eV confirmed that two polyelectrolytes were successfully deposed on the surface and the multilayer was made. Finally, a quantification of the bonded CTATVHL peptide to the hydrogel was performed using FITC- CTATVHL peptide (Figure 6). The measured amount of grafted peptides was 74.3% ± 5.2 % (calculated as a percentage of the peptide amount added for synthesising the PAH-g-CTATVHL).

In vitro release of CTATVHL peptide and TGF-β1 in PBS at different interval times was shown in Figure 7. UV-Vis spectrophotometry was used to measure the initial content of the biomolecules added in the multilayered coating. The content was quantified as around 48.6 ± 7.5 µg/cm^2. The LbL-functionalised hydrogels were characterised by a tri-phasic release profile: (1) linear pattern with 10 % of the biomolecules released within 18-24 hours, (2) constant controlled and linear release (75-80 %), and (3) linear zero-order release after 14 days. On the other hand, for the hydrogels containing the absorbed CTATVHL/TGF-β1, a very fast burst release (~80-90% during the initial 12 hours of incubation) was observed.

Biological characterisation of LbL-functionalised hydrogels

The adhesion and proliferation of bovine articular cartilage on the non-treated and LbL-functionalised hydrogels were studied, focusing particularly on the single influence of the CTATVHL peptide and TGF-β1 and in their combination on the cell behaviour. In this study BAC viability has been investigated using the PrestoBlue assay after 1, 3 and 7 days (Figure 8(A)). All the data were normalised for the number of cells measured using the PicoGreen test described before. After 1 day PrestoBlue assay showed that the LbL functionalised samples with the incorporation of the peptide sequence demonstrated a good adhesion (379 ± 46 RFU for CTATVHL peptide, 388 ± 32 RFU for TGF-β1 and 398 ± 28 RFU for their combination), comparable with the results obtained for the tissue culture plate (409 ± 48 RFU) and the G-coated hydrogels (394 ± 42 RFU), but higher respect with the LbL-functionalised hydrogels without biomolecules incorporation (259 ± 39 RFU).

Viability test at long-term showed that cell viability increased along the period of cell culture. Particularly, the simultaneous presence of the biomolecules in the multilayer enhanced significantly the cell viability (1159 ± 78 RFU) compared to their un-functionalised counterparts (718 ± 58 RFU for AG5%) as observed after 7 days of incubation, showing also higher values respect with the TCP control (955± 79 RFU). Interestingly, the presence of CTATVHL peptide or TGF-β1 only in the multilayered coating showed to be biocompatible but with lower values (933 ± 88 RFU for CTATVHL peptide and 918 ± 78 RFU for TGF-β1), indicating the interesting potential of their co-adsorption.

This results indicated an effect similar to the one reported by other authors, that studied the influence of the combination of different peptides in cell response **[42, 43]**. The good cytocompatibility of the functionalised hydrogels was further demonstrated by optical microscopy (Figure 9). After 7 days of incubation, BAC cells showed a good adhesion on the sample top surface, grafted with the peptide sequence, presenting a spreading behaviour (Figure 9(C)).

Finally, preliminary tests on the detection of GAGs was quantified by the DM assay after 28 days of BAC seeding on the hydrogels (Figure 8(B)). Significantly higher GAG production was detected in all the samples including the CTATVHL peptide in comparison with the samples presenting TGF-β1 only. This result seems to be in accordance with previous works, that showed TATVHL peptide influenced significantly the secretion of glycosaminoglycans [24].

Analysing and understanding the impact of the nanocoating is fundamental for improving the ECM production *in vivo* **[44]**. Therefore, the relevant characteristics, including ie. hydrogel surface and texture, regulate the cell adhesion, proliferation, phenotype maintenance and ECM formation **[45]**.

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Conclusions

This study demonstrated the utility of LbL assembly to functionalise hydrogels to promote chondral regeneration. The main advantages of this environmentally-friendly technology that have been demonstrated here are that potent biomolecules may be incorporated without loss of functionality to improve the regenerative potential of a device or scaffold biomaterial. The presence of CTATVHL peptide sequence and the growth factor TGF-β1 on the alginate-based hydrogel maintained chondrocyte viability while promoting the secretion of glycosaminoglycans. This study is the first report of LbL being employed successfully to promote cartilage tissue regeneration, and it opens up new opportunities to enhance and tailor biofunctionality in regenerative medical devices.

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Figure 1. (**A**) ATR-FTIR spectra (2000–550 cm^{-1} range) of pure alginate and gelatin, and A3%, AG3%, A5%, AG5% hydrogels. (**B**) Water uptake of A3%, AG3%, A5%, AG5% hydrogels. n= 3.

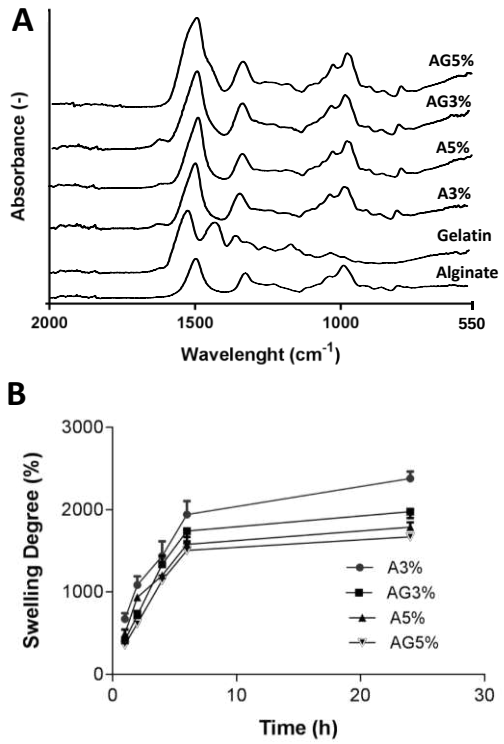


Figure 2. (**A**) Storage and (**B**) Loss Modulus of A3%, AG3%, A5%, AG5% hydrogels measured by conducting frequency sweep test (from 1 to 150 rad/s at 1% strain). n= 3.

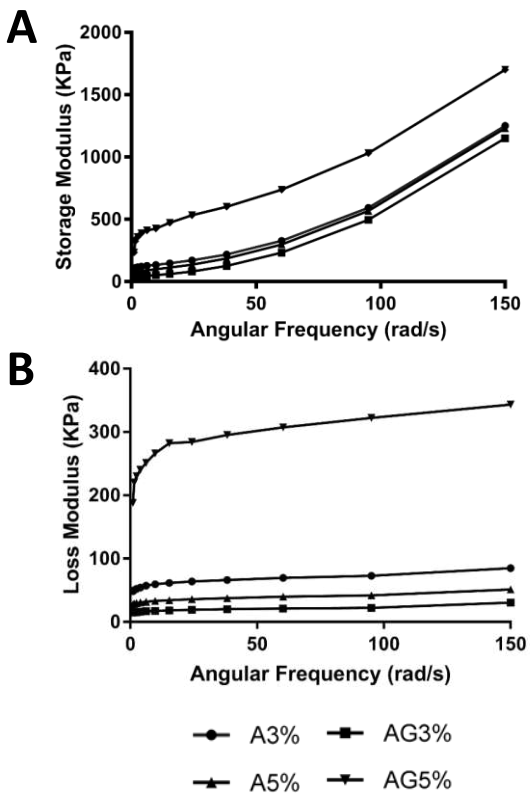


Figure 3. Absorbance values measured by Orange test of the amino groups present on the surface hydrogels before and after G coating. n= 3.

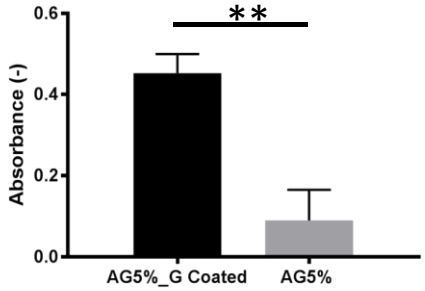


Figure 4. ATR-FTIR spectra of the AG5% hydrogels after G coating and Layer-by-Layer assembly. Resolution 4 cm^{-1}.

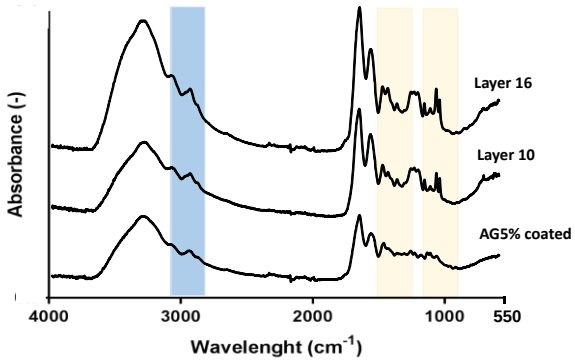
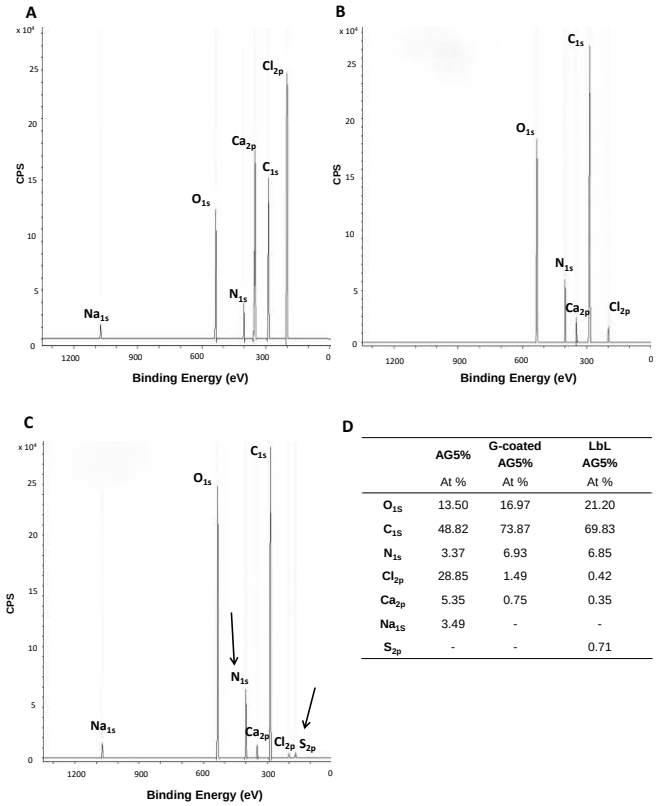


Figure 5. XPS Analysis. Survey spectra of (**A**) AG5%, (**B**) G-coated and (C) LbL functionalised hydrogels. (**D**) Table summarising the recorded atomic percentages (At%).



D

	AG5%	G-coated AG5%	LbL AG5%
	At %	At %	At %
O_{1S}	13.50	16.97	21.20
C_{1S}	48.82	73.87	69.83
N_{1s}	3.37	6.93	6.85
Cl_{2p}	28.85	1.49	0.42
Ca_{2p}	5.35	0.75	0.35
Na_{1S}	3.49	-	-
S_{2p}	-	-	0.71

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Figure 6. Fluorescence microscopy images of (**A**) AG5%, (**B**) LbL functionalised hydrogels without biomolecules addition, and (**C**) LbL functionalised hydrogels with the addition of FITC- CTATVHL peptide and TGF-β1. Bar= 100 µm.

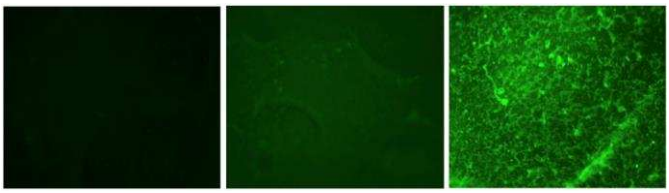


Figure 7. *In vitro* release kinetics of TGF-β1 and CTATVHL from hydrogels containing the biomolecules by physical absorption (TGF&CTATVHL) and by LbL functionalisation (LbL_ TGF&CTATVHL) (n= 6).

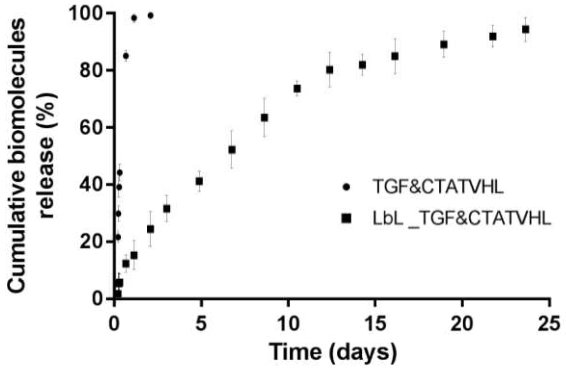


Figure 8. *In vitro* cell tests. (**A**) BAC metabolic activity (PrestoBlue® assay) after culturing for 1, 3 and 7 days. (**B**) GAG quantification using DB assay (* p<0.05 and ** p< 0.001).

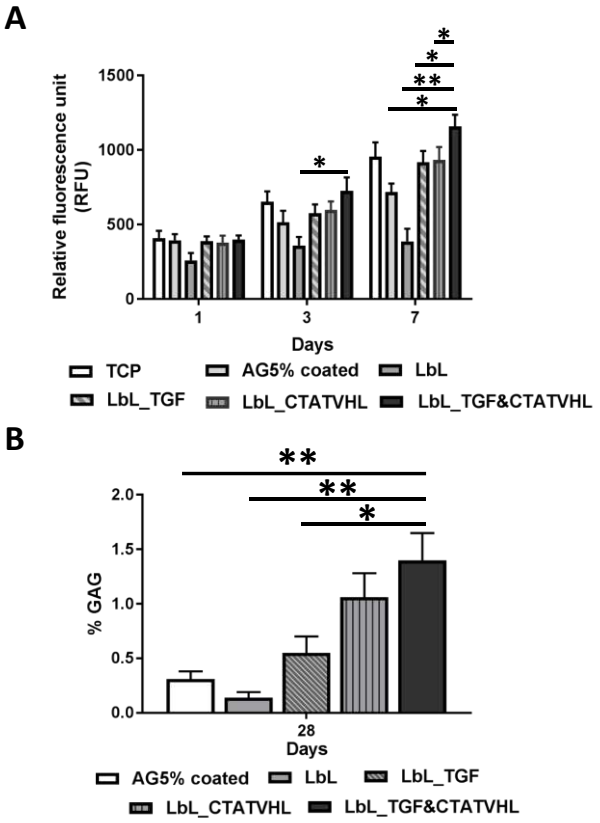
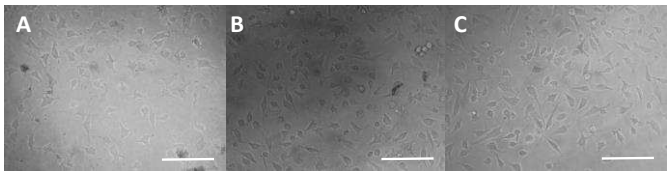


Figure 9. Optical microscopy images of (**A**) G-coated AG5% hydrogel, (**B**) LbL functionalised hydrogel with the addition of CTATVHL peptide, and (**C**) LbL functionalised hydrogel with the addition of CTATVHL peptide and TGF-β1. Bar= 100 µm.



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