The effect of amidic moieties on polysaccharides: evaluation of the physico chemical and biological properties of amidic carboxymethylcellulose (CMCA) in the form of linear polymer and hydrogel

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A new derivative of carboxymethylcellulose was synthesised by converting some of the carboxylic groups into amidic moieties (CMCA). The synthetic procedure allowed for a good control of the degree of substitution. The new polysaccharide was characterised in terms of physico-chemical properties and biocompatibility. It was also used for the realisation of a hydrogel, and its physico-chemical behaviour, such as water uptake and rheological parameters, were evaluated. The new CMCA hydrogel showed physico-chemical properties closer to those of Hyaluronan (Hyal) rather than native carboxymethylcellulose (CMC). Hydrogel cytotoxicity and biological activity were then comparatively investigated on osteoblast-like cells, taking into consideration the possible application of the hydrogel as a cell scaffold in orthopaedic tissue engineering. Finally, the effectiveness of cellular scaffolds for β-pancreatic islet cells was also studied.

Introduction

Cellulose, a long chain polymer with repeating units of D-glucose, shows several reaction sites, permitting the realisation of several derivatives characterised by the presence of new functional groups. As a carbohydrate, the chemistry of cellulose is primarily the chemistry of alcohols. Consequently, it forms many of the common derivatives of alcohols, such as esters, ethers, *etc*. These derivatives form the basis for most of the industrial technology of cellulose in use today. The most common cellulose derivatives are hydroxypropylmethylcellulose (HPMC),[1-3] ethylcellulose (EC),[4-6] and carboxymethylcellulose (CMC).[7,8] CMC appears in several applications in the food industry[9,10] and in environmental care,[11] supplied in large quantities at a very low cost. These characteristics make the CMC polysaccharide useful as surfactant[12] and as an aggregating agent. Thanks to its properties, CMC guarantees a good stabilising action.[13] CMC is also used as stabiliser for coal-water slurries in order to prevent the formation of excessive sediment and guarantee the maintenance of an optimal viscosity value.[14]

In the biomedical field, CMC, as for other cellulose derivatives, is principally used as an excipient for the preparation of a matrix system used in sustained release formulations.[15-17] Neverthless, once implanted, it induces an inflammatory reaction and is not quickly degraded *in vivo*. In fact, not existing specific enzymes for its degradation, only aspecific lyases are able to break cellulose chains. In order to improve its biological properties, CMC was modified by introducing a new functional group. Fifty percent of the carboxylic groups of the polysaccharide chain were converted into an amido group and a new polysaccharide was obtained (amidic carboxymethylcellulose – CMCA) (Fig. 1). The amidic moieties were introduced in order to increase the hydrophilicity of the material. A greater water upta guarantees a better environment for cellular growth a reduces the inflammatory action of the material. The n polysaccharide was characterised by infrared analysis, pote tiometric titration and viscosimetric measurements. Gene cytotoxicity of the polysaccharide was evaluated on fib blasts. The CMCA linear polysaccharide was then cross-link and the obtained hydrogel was characterised by swelling a rheological measurements. The parameters of the CM hydrogel were compared with those of the corresponding H and the CMC hydrogels. By taking into consideration differ clinical applications, the hydrogel was characterised in ter of cytotoxicity, inflammatory reaction and bioactivity osteoblast-like cells. Finally, hydrogel biological activity v also investigated as scaffold for β-pancreatic cells.

Experimental

Materials

The sodium salt of carboxymethylcellulose (CMC, visco 402 mPa s in 2% w/v aqueous solution at 25 °C, with carboxymethylation degree of 0.9 ± 0.1 per monosacchar unit, M_w = 100 000) was supplied by Hercules Italia S.p (Italy). The polysaccharide was used as received. The chem composition was confirmed by FT-IR spectroscopy. All

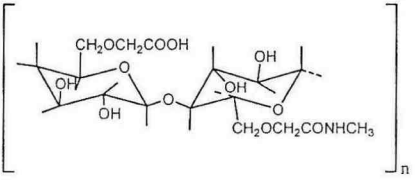

Fig. 1 Disaccharidic unit of CMCA polysaccharide.

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other reagents were purchased from Fluka Chemie AG (Switzerland) and used without further purification.

Methods

Synthesis of the amidic derivative of carboxymethylcellulose (CMCA). The carboxymethylcellulose sodium salt was dissolved in doubly distilled water in order to obtain a 1% solution. The aqueous solution was subjected to sodium–hydrogen ionic exchange using DOWEX monosphere 650C resin and then added to a 5% tetrabutylammonium hydroxide (TBAOH) solution until a pH of 8–9 was reached. The solution was then lyophilised to obtain the tetrabutylammonium salt of the polysaccharide. Afterwards, the TBA-CMC salt was dissolved in *N,N'*-dimethylformamide (DMF) with stirring and under a nitrogen flow. The solution was kept at 4 °C before adding the activating agent 2-chloro-*N*-methylpyridinium iodide (CMP-J) the amount of which depended on the number of carboxylate groups to be activated, in this case 50% of the carboxylic groups of the polysaccharide chain. The stoichiometric amount, plus 10% of the methylamine solution was then added, together with two to three drops of triethylamine, which acted as a catalyst. The reaction was maintained for 3 h. The amidic polysaccharide was precipitated by addition of EtOH 95% (2 : 1 v/v), and leaving the mixture overnight at 4 °C. The precipitate was dissolved in water and dialysed against water and 0.1 M NaCl until no more byproducts were found in the washing solutions (Fig. 1).

Synthesis of CMCA-based hydrogel. The amidic polysaccharide in the Na^+ form was exchanged (DOWEX resin) to the TBA^+ form, and the cross-linking reaction was conducted as previously reported.[18] Briefly, the TBA salt was dissolved in DMF with stirring and under a nitrogen flow. The solution was kept at 0 °C before adding the activating agent, CMP-J. An excess amount of CMP-J was added with respect to the free carboxylate groups in order to guarantee complete cross-linking. 1,3-Diaminopropane (1,3-DAP) was used as cross-linking agent. The reaction was catalysed by a small amount of triethylamine as hydrogen iodide captor. The reaction was left at room temperature for 3–4 h. The hydrogel formed was then washed alternately with doubly distilled water and ethanol until no more solvents or secondary products were found in the washing solution, as shown by UV absorption measurements.

The ninhydrin assay was performed to check for the presence of unreacted NH_2 groups in the 1,3-DAP used as a cross-linker.[19] The same procedure was used to synthesise Hyal and the CMC-based hydrogels with a cross-linking degree of 100% (all the carboxylic groups of the polysaccharide chain were involved in the cross-linking reaction).[20,21]

Physico-chemical characterisation

FTIR-ATR analysis. ATR spectra of the samples in dry form were recorded on a Bio-rad FTS6000 apparatus between 4000 and 750 cm^{-1} following the classic procedure.[22] An MCT (Mercury-Cadmium-Tellurium) detector was used, and the apparatus was purged with nitrogen. As previously, 50 scans at a resolution of 1.0 cm^{-1} were averaged. The frequency scale was internally calibrated with a helium–neon reference laser to an accuracy of 0.01 cm^{-1}. A deconvolution method was applied to the 1800–1500 cm^{-1} spectral region, which is the most representative for this system.

Potentiometric titration. Potentiometric titrations were performed in order to evaluate the percentage of amidation present in the polymer, and in a second step, to check the cross-linking degree of the hydrogel. The same procedure was used for both the polysaccharide and the hydrogel. Only the experimental conditions were modified.

Polysaccharide titration: the sodium salt of amidic carboxymethylcellulose was converted to the acidic form by an exchange resin (DOWEX 650 monosphere) and then dissolved in a thermostatic glass cell at 25 °C, at a constant ionic strength of 0.1 M NaCl with a known amount of 0.1 M HCl. The titration was performed with 0.1 M NaOH and back-titration with 0.1 M HCl. The default conditions for the experiment were as follows: a stabilisation time of 360 min for the initial system and a delay time of 10 min between each addition of titrant.

Hydrogel titration: the dried hydrogel was finely dispersed in a thermostatic glass cell at 25 °C, at a constant ionic strength of 0.1 M NaCl with a known amount of 0.1 M HCl. The titration was then performed with 0.1 M NaOH and back-titration with 0.1 M HCl. The default conditions for the experiment were as follows: a stabilisation time of 24 h for the initial system and a delay time of 30 min between each addition of titrant.

The titration data were collected by a Crison MicropH-2002 apparatus, equipped with a combined electrode (mod. 6.0204.000), together with an automatic Crison microburette (mod. 3031), connected to a PC.

Viscosity. The viscosity of 10% solutions of CMCA, CMC and Hyal was evaluated on a strain-controlled AR2000 Rheometer (TA-Instruments, Leatherhead, United Kingdom) equipped with a concentric cylinder system at a controlled temperature of 25 °C.

Water uptake measurements. The water uptake (W.U.) was determined for each sample, and calculated by the following formula:

$$\text{W.U.} = [(W_s - W_d)/W_d] \times 100$$

W_s and W_d are the weight of the swollen and dried hydrogels respectively. In practice, known amounts of the dried samples were deposited on a propylene cell strainer with a nylon pore mesh of 100 μm and immersed several times at 25 °C in 50 mL of a buffer solution (pH 2: 6.5 mL 0.2 M HCl mixed with 25 mL 0.2 M KCl pH 7.4: PBS (phosphate buffer solution); pH 9: 50 mL 0.1 M TRIS with 5.7 mL 0.1 M HCl). They were then placed on a piece of dry filter paper to remove the excess water. The filter containing the swollen hydrogels were weighed to calculate the water up-take and the swelling kinetics.

Rheological characterisation. The rheological properties of the hydrogels were evaluated on a strain-controlled AR 200

Rheometer (TA-Instruments, Leatherhead, United Kingdom) at a controlled temperature of 25 °C. The geometry was plate–plate (PP 20 cell).

Small-amplitude oscillatory shear experiments were performed to measure the time-dependent response of the samples. The frequency range was 0.1–10 Hz. In particular, $G'(\omega)$ (shear storage modulus) and $G''(\omega)$ (shear loss modulus) were evaluated. G' gives information about the elasticity or the energy stored in the material during deformation, whereas G'' describes the viscous character or the energy dissipated as heat.

Strain sweep tests at a fixed oscillation frequency (consisting in monitoring the viscoelastic properties while logarithmically varying the strain amplitude γ_0) were performed on the materials to determine the strain amplitude at which linear viscoelasticity is valid.

Biocompatibility. *Cytotoxicity on fibroblasts.* The test was performed with mouse fibroblasts (cell line 3T3). Fibroblasts 3T3 were maintained in culture in Dulbecco's modified eagle's medium (DMEM), supplemented with 10% Fetal Calf Serum (FCS), 1.2% L-glutamine and 1% penicillin–streptomycin (Sigma, Germany) in polystyrene flasks. The fibroblast cultures were incubated at 37 °C in an atmosphere of 5% CO_2 until cells were harvested with the aid of trypsin and suspended in fresh medium.

The cytotoxicity of CMCA polysaccharide solution towards mouse fibroblasts 3T3 was evaluated. Briefly, 3T3 cells (4000 cells mL^{-1}) were suspended in DMEM containing 10% fetal calf serum and placed on the bottom of each well of a 24-well plate. The plate was then incubated in a humidified atmosphere containing 5% CO_2 at 37 °C until the cells reached confluence (24 h). Afterwards, 2 mL of sterilised (by filtration) solution was added to each fibroblast monolayer and incubated at 37 °C for 24 h. The cells were fixed with glutaraldehyde, stained with trypan blue, and counted by direct observation with an optical microscope (BX40, Olympus).

Cytotoxicity and bioactivity on osteoblasts. Freeze-dried samples of of carboxymethylcellulose (CMC), carboxymethylcellulose amide (CMCA) and Hyal were weighed (15 mg each) and sterilised with ethylene oxide. Human osteoblast-like cells (MG63) were cultivated in DMEM with 10% added FCS, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin solution, and incubated at 37 °C in a humidified 95% air + 5% CO_2 atmosphere. Cells were released before confluence with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA, counted (Coulter Counter Z1, Beckman Coulter Inc., Miami, FL, USA), and used at a concentration of 2×10^4 cells mL^{-1} for the experiment.

Six sterile samples of each group were placed in the same number of wells of two 24-well plates. The material samples were reconstituted with 0.5 mL of cell culture medium (DMEM additioned with 10% FCS). The same quantity of medium alone was put in empty wells for negative control (CTR). After 24 h, to permit the hydrogel to stabilise, a suspension of 100 μl of osteoblasts at the concentration of 0.5×10^5 cells mL^{-1} was seeded in every well. After 1 h, 950 μl of DMEM was added to each well. Plates were incubated in standard conditions, changing the medium every two days. the end of experimental time, the supernatant was collec from the wells and centrifuged to remove particulates, if a Aliquots were dispensed in Eppendorf tubes for storage −70 °C and assayed for Interleukin-6 (IL-6, Human I Immunoassay kit, Biosource International, CA, USA), Tun Necrosis Factor α (TNF-α, Human TNF-α Immunoass R&D Systems, MN, USA), and C-terminal Propeptide Type I Collagen (CICP, Metra CICP EIA kit, Quidel, C USA). Lactate dehydrogenase release (LDH, Sigma Kin method kit, St. Louis, MO, USA), and Bone Alkal Phosphatase (BALP, Metra BAP EIA kit, Quidel, CA, US were tested on supernatants immediately after collection. T measured concentrations were normalised by cell numb Finally, the Cell Proliferation Reagent WST-1 test performed to assess cell proliferation and viability: 100 μl WST1 solution and 900 μl of medium (final dilution: 1 : were added to the cell monolayer, and the multi-w plates were incubated at 37 °C for an additional 4 Supernatants were quantified spectrophotometrically 450 nm with a reference wavelength of 640 nm and the resu have been reported as optical density (OD).

The statistical evaluation of data was performed using software package SPSS/PC+ Statistics® 10.1 (SPSS In Chicago, IL, USA). The experiment was repeated three tin and the results presented are the mean of the three valu Data are reported as mean ± standard deviations (SD) a significance level of $p < 0.05$. After having verified norr distribution and homogeneity of variance, a one-way ANO was performed for comparison between groups. Fina Scheffé's *post hoc* multiple comparison tests were perform to detect significant differences between groups.

Evaluation of growth of β-pancreatic cells on hydrog Pancreatic islets were prepared by the method reported Weir *et al.*[23] Energy metabolism and morphology of pancrea islet cells were monitored by both Autofluorescence Mic Spectroscopy (AMS) and Multicolour Imaging Au fluorescence Microscopy (MIAM) techniques immediat after the recruitment and at following times after encapsulation in hydrogels.

Sample preparation. 1.5–2.5 mg mL^{-1} of collagenase w dissolved in RPMI 1640 medium without calf serum. T medium was utilised to remove pancreas, in accordance w standardised surgical procedures. Each pancreas was placed a 50 mL falcon tube on ice. The pancreas-containing tul were then placed into a water bath at 37 °C for 15–25 min. the end of incubation, 20 mL of medium with newborn c serum was added to each falcon tube on ice. After further application of 20 mL of medium, the tubes w shaken for 5–10 s to disperse the tissue. The collagen was removed by washing the islets three to four times w 25–35 mL of medium containing 10% newborn calf serum.

For the gradient separation procedure, the pellet w resuspended in 10 mL Histopaque 1.077 and vortexed ur the suspension was homogeneous. 10 mL medium with serum was carefully overlaid on the Histopaque to maintain sharp interface. The tubes were then spun for 20 min in

refrigerated centrifuge (10 °C) at 2400 rpm (900*g*). After centrifugation, the islets were collected from the interface and placed in 50 mL conical tubes. The islets were then washed three times to remove the Histopaque and resuspended in 20–30 mL of media with 10% FCS. Pancreatic islet cells were seeded for culturing in standard conditions or in a hydrogel enivironment.

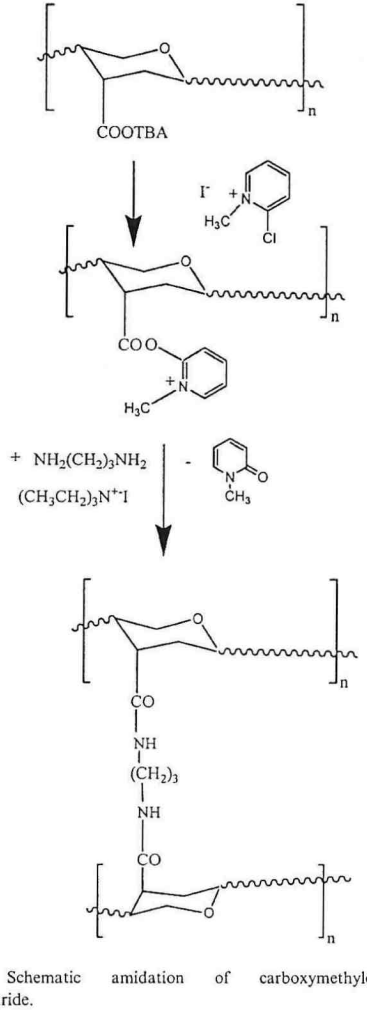
Hydrogel preparation. 5 mg of polysaccharide hydrogels (Hyal and CMCA and CMC hydrogels) were placed in a well with 1 mL of 70% ethanol for sterilisation. About 40 min later, the ethanol was removed and the hydrogels were dried at room temperature. Then, 1 mL of RPMI 1640 medium was added to the well to swel the hydrogel. After 24 h, pancreatic islet cells (1×10^4) were seeded in the hydrogel.

Autofluorescence techniques. Cell autofluorescence was analysed by an inverted epifluorescence microscope (Nikon Eclipse TE 2000 E), an oil-immersion CF-UV fluor 100× objective (N.A. 1.3) and 365 nm excitation. The fluorescence signal was detected by a diode-array spectrophotometer and a Hires III digital CCD camera (DTA, Italy). Fluorescence images were digitalised directly in the CCD controller with 14-bit dynamics and transferred to a storage computer with a digital interface. The size of the field detected by the 100× objective was approximately 69 × 69 mm (spatial calibration of 0.13 mm per pixel), as determined by imaging 6 mm fluorescent microspheres (Molecular Probes). For each sample, three 40 nm wide (full width at half maximum) spectral bands, with peaks close to 450, 550 and 650 nm (450 FS 40-25, 550 FS 40-25 and 650 FS 40-25 respectively, Andover, Salem, NH) which were used to sequentially acquire three fluorescence images in blue, green and red, with integration times of about 5 s. Monochrome images were then combined in a single multicolour image using the RGB technique. The multicolour images were obtained by the Image Combine Channels algorithm of Corel PHOTO-PAINT v. 6.0 software (Corel Corporation, Ottawa, Canada), after the identification of the three greyscale images (acquired at 650, 550 and 450 nm respectively) with the RGB components.

Results and discussion

Synthesis, physico-chemical characterisation and cytotoxicity evaluation of the amidic carboxymethylcellulose polymer

Polymer amidation (Scheme 1) was carried out with the CMC polymer in DMF solution at 4 °C in the form of TBA^+ CMC^- salt under a nitrogen flow, in order to guarantee an anhydrous environment since the polymer in the TBA form is a highly hygroscopic substance and a high humidity could alter the synthetic process. The temperature control was necessary because the CMP-J induces the formation of interchain ester bonds at a higher temperature, favouring the formation of physical hydrogel. This phenomenon led to a heterogeneous mixture and hindered the amidation process. CMP-J was added in a stoichiometric amount depending on the number of carboxylate groups to be activated. Activation of the carboxylate groups proceeded rapidly by nucleophilic attack of the carboxylate ion upon CMPJ and the liberation of



Scheme 1 Schematic amidation of carboxymethylcellulc polysaccharide.

TBA^+ Cl^-. Afterwards, the intermediate underwent nucle philic attack by the alkyl amine (methylamine) on t carboxylic group, with the elimination of 1-methyl-2-pyridor The reaction was catalysed by a small amount of trieth amine, as a hydrogen iodide captor.[18]

An infrared analysis was performed in order to check t presence of the new functional group in the polymer. As sho in the IR spectrum in Fig. 2, the presence of amidic groups w confirmed by the presence of a new band at 1645cm^{-1} due the amidic C=O stretch, which was absent in the nati polysaccharide.

The percentage of amidation was evaluated by potenti metric titration. Two steps were evident in the potentiomet curve. The calculation, performed using a previously report procedure,[20] showed that the amidation degree was 40 ± 3

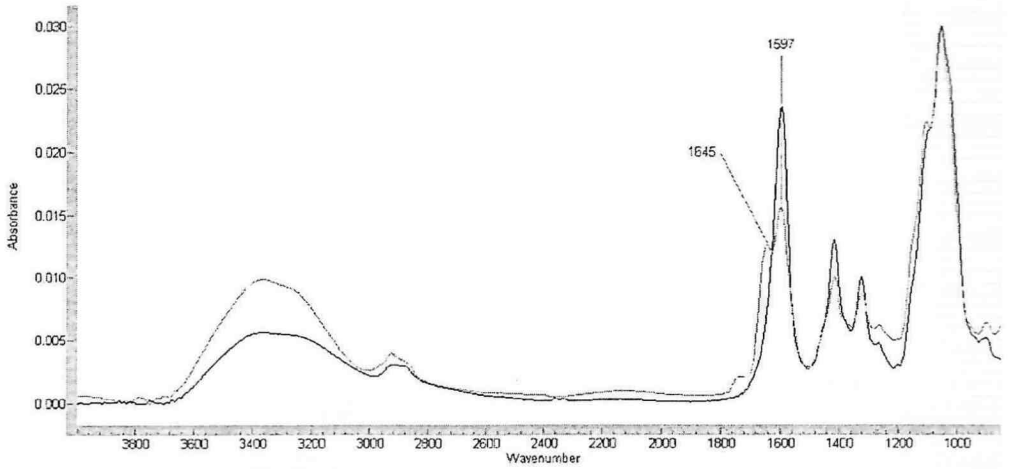


Fig. 2 IR spectrum of CMC (black) and amidic CMCA (grey). The presence of a new band at 1645 cm^{-1} confirms the formation of the functional group (the amidic moiety).

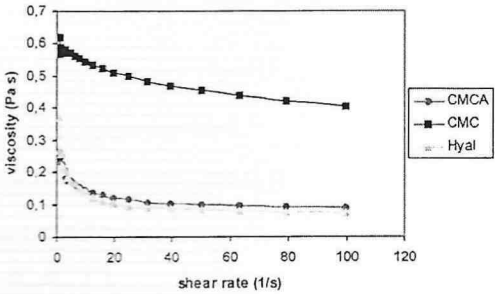


Fig. 3 Viscosity curves of 10% aqueous solutions of CMC (black), Hyal (light grey) and CMCA (dark grey).

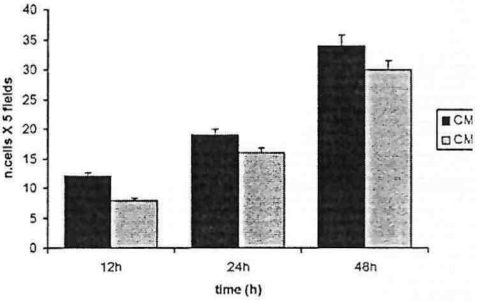


Fig. 4 Cell proliferation at 12, 24 and 48 h in the presence of C and CMCA solution

i.e. 40% of the carboxylic groups of the native polysaccharide were converted into amidic moieties.

The viscosity of a 10% aqueous solution of the modified carboxymethylcellulose (CMCA) was evaluated and compared with that of native CMC and Hyaluronan. The presence of amidic groups decreased the viscosity of CMC polysaccharide rendering the CMCA more similar, in terms of viscosity, to Hyaluronan (Fig. 3). Without shear stress, the native 10% solution of CMC showed a viscosity of 0.619 Pa s which decreased immediately to 0.403 Pa s at a shear rate of 100 s^{-1}. The CMCA solution showed a decrease with the shear rate as well. Moreover, at the beginning the viscosity of the CMCA solution was 0.260 Pa s, whereas with a shear rate of 100 s^{-1} it decreased to 0.087 Pa s. The Hyaluronan solution showed a greater decrease of the viscosity by increasing the shear rate. In fact, the viscosity decreased from 0.370 to 0.071 Pa s.

The cytotoxicity of the polysaccharide was evaluated following the NIH procedure, using 3T3 fibroblasts. No cytotoxic effect was revealed by either CMC or CMCA polymers, even though the CMCA solution behaved better than the native C one, showing a better growing of the cell *versus* time (Fig

Synthesis and physico-chemical characterisation of CMCA-based hydrogel

The hydrogel was obtained with a 100% degree of cross-link (all the carboxylic groups not involved in the amida reaction were involved in the cross-linking reaction, *i.e.* 40% the native carboxylic groups were modified into methylam groups, whereas the remaining 60% were involved in the cr linking reaction). The absence of free carboxylic groups confirmed by potentiometric titration. In fact, the titra curve showed only one step which was relative to the hydrogen ions added in excess.

The swelling behaviour of the CMCA-based hydrogel evaluated at three pH values (pH 2, pH 7.4 and pH 9) compared with 100% CMC and 100% Hyal hydrogels. CMCA and Hyal hydrogels showed a similar water uptak pH 2 and 7.4, which was much higher than that of native 1(

CMC at the same pH values. The higher water uptake of Hyal and CMCA hydrogels can be related to the presence of the highly hydrophilic amidic group in their chains, which is absent in the native CMC chain. In principle, no 100% hydrogels should have any dependence on the pH. In fact, the occupation of all the carboxylic groups by cross-links excludes them from the mechanism of ionisation–neutralisation which affects the water uptake mechanism. The CMC and CMCA hydrogels did not show a practical pH dependence. The behaviour of 100% Hyal, sensitive to basic pH, could be due to an incomplete cross-linking reaction (cross-linking degree 96 ± 2%) or to a degration of the hydrogel at pH 9 (Fig. 5).[21]

A rheological analysis was also performed. CMCA, Hyal and CMC-based hydrogels behaved as strong gels with the storage modulus greater than the loss modulus.[24] The presence of chemical cross-linking among the chains led to permanent stable networks. For all the polysaccharide-based hydrogels, both G' and G'' increased with increasing frequency even though the increase observed for CMCA hydrogel was the most consistent. In fact, for the CMCA hydrogel G' rose from 5×10^3 Pa to 1×10^4 Pa and G'' increased only from 2.5×10^3 to 3×10^3 Pa, whereas for the CMC hydrogel a very small increase was observed (4.9×10^3 Pa to 5.8×10^3 Pa). In comparison, G' of 100% Hyal rose from 1.8×10^3 Pa to 2.9×10^3 Pa. Therefore, the 100% CMCA-based hydrogel showed a harder consistency than the Hyal and CMC hydrogels (Fig. 6).

Hydrogel cytotoxicity and bioactivity on osteoblast-like cells

Hydrogel cytotoxicity was assessed by osteoblast-like cells (MG63). The cell proliferation was evaluated using a colorimetric method which measures the cell viability on the basis of cellular mitochondrial metabolic activity (WST1). Other factors, such as LDH, IL-6, and TNF-α were taken into account and the results are reported in Table 1. A polystyrene (PS) dish was used as negative control (CTR) and the CMC-, Hyal- and CMCA-based hydrogels were tested and compared to one another.

The CMCA-based hydrogel appeared to be the optimal scaffold for cell proliferation. Starting from the same baseline condition, on day seven, proliferation of osteoblasts grown on hydrogel was not statistically different from cells grown on polystyrene (CTR).

LDH is a cytoplasmic enzyme present in all cells, rapidly released in cell culture supernatant when the membrane is

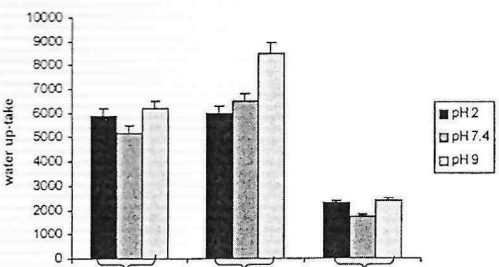


Fig. 5 Water uptake values of 100% CMC, 100% CMCA and 100% Hyal at pH 2, 7.4 and 9 at room temperature.

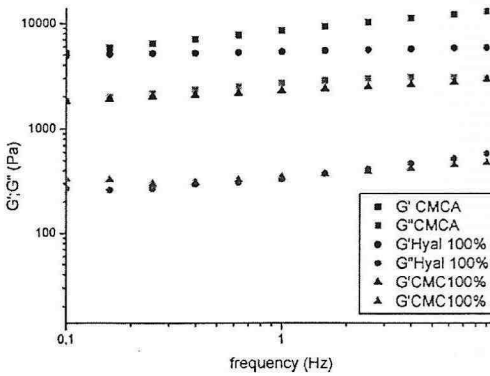


Fig. 6 Rheological behaviour of CMCA-, CMC- and Hya based 100% hydrogels determined at 25 °C in the frequency ran 0.1–10.00 Hz.

damaged. On day seven, the differences in LDH release we no longer evident. IL-6 is a cytokine favouring bone resorptio and plays an important role as the mediator of inflammation. IL-6 production in CMC was higher than CTR, CMCA ar Hyal. TNF-α also has a role in inflammation response ar apoptosis: it is reported to promote inflammatory ce infiltration by upregulating leukocyte adhesion molecules c endothelial cells, serve as a chemotactic agent for monocyt and activate phagocyte killing mechanisms.[26] It is therefo involved in differentiation of hematopoietic stem cell. TNF production showed no differences among the samples.

BALP and CICP production are established markers f osteoblast phenotype and activation.[27] The increase of BAL in the osteoblast culture is considered an early expression of more differentiated state of cells. Collagen type I, the maj organic macromolecule in the extracellular bone matrix, produced as a large procollagen molecule, which is converte to collagen by the release of N- and C-terminal propeptide After seven days, BALP in the CMC hydrogel was signi cantly lower than in all the other samples (CTR: $p < 0.00$ CMCA: $p < 0.05$; Hyal: $p < 0.01$). CICP production aft seven days was significantly higher in the CMCA ar Hyal hydrogels when compared to CTR groups ($p < 0.0$ and $p < 0.005$ respectively) (Fig. 7).

Evaluation of growth of β-pancreatic cells on the CMCA-base hydrogel

Cell viability and cell energy metabolism were monitored b autofluorescence microscopy over a period of 10 weeks.

Table 1 WST1, LDH, IL-6, and TNF-α production by osteoblast li cells (MG63) in contact with Hyal-, CMC- and CMCA-base hydrogels

MG63	Control	50% CMC	CMCA	50% HYA
WST1	0.67 ± 0.4	0.63 ± 0.26	0.81 ± 0.14	0.83 ± 0.
LDH	12.4 ± 1.9	18.0 ± 3.9	15.8 ± 5.2	21.4 ± 1.
IL-6	0.7 ± 0.06	8.23 ± 1.74	1.87 ± 0.27	3.2 ± 1.
TNF-α	8.15 ± 0.72	7.08 ± 1.1	6.7 ± 1.03	8.32 ± 1.

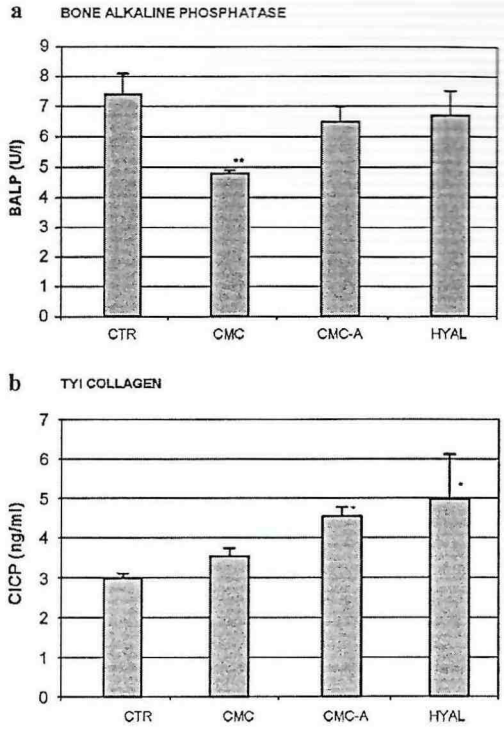


Fig. 7 (a) BALP and (b) CICP production in the presence of CTR, CMC, CMCA and Hyal hydrogels.

Hydrogels were suitable environments for cell culturing: cell viability and function were even better in hydrogels than under standard conditions (Fig. 8).

In fact, after 21 days small changes in energy metabolism were found in cells cultured in the standard, whereas there was no change in cells cultured in the hydrogels. The CMCA-based hydrogel appeared more stable than Hyal, which was partially degraded by cells (Fig. 9).

For culture times greater than 21 days, both autofluorescence spectroscopy and imaging showed an increase of oxidised flavin content in cells cultured in standard conditions, as well as in cells cultured in hydrogels.

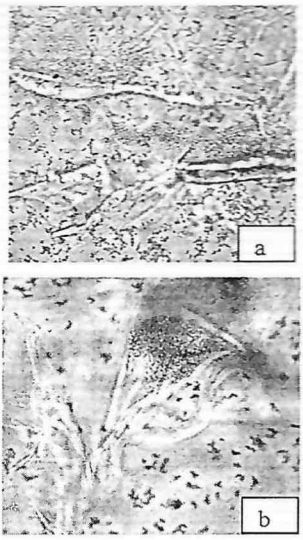


Fig. 9 Pancreatic islet cells after 14 days (transmitted light micr scopy – 20×): (a) CMCA hydrogel, (b) Hyal hydrogel.

Conclusion

A new semi-synthetic derivative of cellulose polysacchari was synthesised by introducing new amidic groups along th chain. The conversion of some of the carboxylic groups in amidic groups drastically modified the properties of th polysaccharide with respect to the native CMC. CMC has physico-chemical properties very similar to those Hyaluronan. The CMCA-based hydrogel performed bett than Hyal in terms of hardness, thus resulting in a bett support for cells and resistance towards enzymatic attacks. It well-known that the Hyaluronic acid hydrogel shows sever disadvantages as a cellular scaffold, undergoing very quic degradation by hyaluronidase and other enzymes. On the bas of these results, the CMCA polysaccharide can be considere to be a lower cost substitute for hyaluronic acid. As far orthopaedic purposes are concerned, these preliminary da demonstrate that the addition of amide to CMC significant ameliorated the biocompatibility of the material and the ce expression of inflammatory cytokines (as demonstrated by ce proliferation rate, LDH and IL-6 values). CMCA al

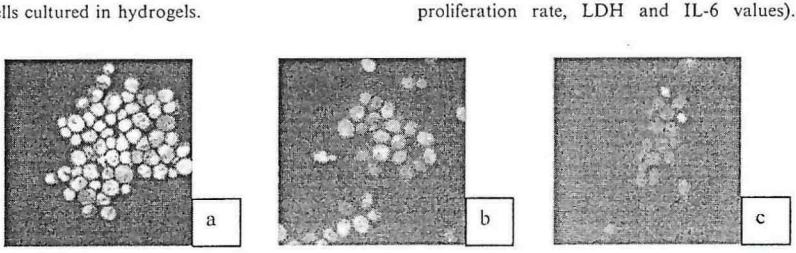


Fig. 8 Autofluorescence imaging of pancreatic islet cells after 21 days (150× magnification) using: (a) standard conditions (RPMI 1640 mediun (b) CMCA hydrogel, (c) Hyal hydrogel.

positively influenced osteoblast activity, having higher BALP and CICP production than CMC.

The results obtained with the pancreatic cells show that the CMCA-based hydrogel performed better than the Hyal-based hydrogel, since the former was still present after 70 days, whereas the latter was completely degraded after 21 days.

Furthermore, the easy synthetic procedure and modification of the polysaccharide chain allows us to foresee a wide application for other polysaccharides. Therefore, in conclusion, as ascertained by the results here reported, the introduction of amidic moieties along the polysaccharide chain drastically increases the biological performance of polysaccharides.

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