Thermo-responsive methylcellulose hydrogels as temporary substrate for cell sheet biofabrication

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Abstract Methylcellulose (MC), a water-soluble polymer derived from cellulose, was investigated as a possible temporary substrate having thermo-responsive properties favorable for cell culturing. MC-based hydrogels were prepared by a dispersion technique, mixing MC powder (2, 4, 6, 8, 10, 12 % w/v) with selected salts (sodium sulphate, Na_2SO_4), sodium phosphate, calcium chloride, or phosphate buffered saline, to evaluate the influence of different compositions on the thermo-responsive behavior. The inversion test was used to determine the gelation temperatures of the different hydrogel compositions; thermo-mechanical properties and thermo-reversibility of the MC hydrogels were investigated by rheological analysis. Gelation temperatures and rheological behavior depended on the MC concentration and type and concentration of salt used in hydrogel preparation. In vitro cytotoxicity tests, performed using L929 mouse fibroblasts, showed no toxic release from all the tested hydrogels. Among the investigated compositions, the hydrogel composed of 8 % w/v MC with 0.05 M Na_2SO_4 had a thermo-reversibility temperature at 37 °C. For that reason, this formulation was thus considered to verify the possibility of inducing in vitro spontaneous detachment of cells previously seeded on the hydrogel surface. A continuous cell layer (cell sheet) was allowed to grow and then detached from the hydrogel surface without the use of enzymes, thanks to the thermo-responsive behavior of the MC hydrogel. Immunofluorescence observation confirmed that the detached cell sheet was composed of closely interacting cells.

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1 Introduction

Regenerative medicine approaches considered either cell delivery or biodegradable scaffolds to support regeneration of specific tissues. Since a number of problems arise with both these approaches, in recent years there has been extensive research into smart substrates enabling spontaneous cell harvesting in response to an environmental stimulus [1–4]. This novel approach to tissue engineering entails in vitro culturing cells and developing tissues on a temporary material sub-strate, thus eliminating the use of biodegradable scaffolds and the possible problems related to their in vivo degradation (e.g. fast degradation rate, release of toxic products, undesired inflammatory response) [5–7]. Cell-sheet engineering is one novel scaffold-free approach, suited for the regeneration of cell-dense tissues such as corneal [8], cardiac [1, 9, 10], and 3D tissues [11]. In addition, thermo-reversible polymers can be used as delivery systems that can be injected into the body as a solution and form a gel immediately after injection (i.e. upon heating), thus allowing the transplantation of a cell support engineered system in a minimally invasive manner. Compared to cells delivery systems based on the use of microcapsules, cells sheets have the advantage of being

This is a post-peer-review, pre-copyedit version of an article published Journal of Materials Sciences. Materials in Medicine, 2016, 27:95, 95-108. The final authenticated version is available online at: http://dx.doi.org/10.1007%2Fs10856-016-5703-8

already organized, and to avoid the in vivo typical degradation rate of the microcapsules.

The use of thermo-responsive poly(N-isopropylacrylamide) (PIPAAm) covalently immobilized on tissue culture polystyrene (TCPS) dishes or multiwells enables intact cell sheets to be harvested by simple temperature change, thus avoiding the use of proteolytic enzymes [12–14]. PIPAAm is a non-ionic polymer that undergoes phase separation in water when heated above a critical temperature, the so-called lower critical solution temperature (LCST). This phenomenon is closely connected to the hydrophobicity of the isopropyl side groups of the polymer, and enables the surface of PIPAAm to change its cell adhesive properties as a function of temperature [15]. Nevertheless PIPAAm cannot be used alone as adequate cell delivery system, because it needs to be covalently bonded to a substrate. For this reason, PIPAAm has been associated with other macromolecules to produce thermo-reversible systems that in solution can switch from a liquid to a gel [16, 17].

Due to the relatively complex and time-consuming process for preparation of cells sheets by using PIPAAm, many efforts have been made to extend the range of thermo-responsive culture surfaces with thermo-responsive hydrogels for cell-sheet production [18, 19] and as vehicle for cell/drug delivery [18]. Recently, a new class of smart polymers based on cellulose has been investigated for harvesting live cell sheets [18–21]. Cellulose, in fact, is the most abundant polysaccharide on earth and numerous new derivatives can be developed, easily produced and modified. Cellulose is not soluble in water but, by introducing hydrophilic moieties, cellulose derivatives can become water soluble and these aqueous solutions exhibit reverse thermo-gelation (gelation at elevated temperatures).

Among the investigated cellulose derivatives, methylcellulose (MC), a viscosity-enhancing polymer, is widely used as a thickener in the food and paint industries. It is recognized as an acceptable food additive by the U.S. Food and Drug Administration. Its chemical structure is characterized by the presence of both hydrophobic methoxy (–CH_3O) and hydrophilic hydroxy (–OH) groups. MC-based polymers are capable of self-structuring upon temperature variation, exhibiting thermo-reversible sol–gel transition with a specific LCST [22, 23]; water becomes a poorer solvent with increasing temperature, and polymer–polymer interactions become dominant at higher temperatures, resulting in a gel. Thus, MC-based hydrogels have interesting possible applications in the bio- and nano-engineering fields, not only for the fabrication of cell sheet, but also, for example, as cell delivery system [24, 25].

Salts in general have a greater affinity for water than do polymer molecules, leading to a removal of hydration water from the polymer, and thus dehydrating it. The presence of ionic compound in the MC aqueous solution can affect the LCST; in particular, ions in solution are defined as salting-out or salting-in, if they lower or raise the LCST, respectively [26, 27], meaning that water is forced out of the polymer structure or forced in [27]. In particular, the ability of a salt to salt-out a polymer gen-erally follows the salts' order in the lyotropic series [26]. Cations follow the order $Li^+ > Na^+ > K^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$, and the more common anions follow the order $CNS^- < I^- < Br^- < NO^{3-} < Cl^- <$ tartrate $< SO_4{}^{2-} <$ -$PO_4{}^{3-}$ [26]. The salting-out effect depends mostly on the anion.

The aim of our work is to exploit the possibility of fabricating cell sheets by means of an easy and fast pro-cess. Compared to other works on MC, we want to deeper investigate the role of different salts to improve the preparation process, to better control the transition temperature and the stability of the thermo-responsive hydrogel. A systematic study of the thermal properties of MC solutions of varying salt and polymer concentrations was undertaken, and from this the optimal compositions of MC-aqueous solutions that form stable gels at the experimentally-viable temperature of 37 °C were determined. In particular, rheological analysis was employed to determine variations of complex viscosity (η^*), conservative shear modulus (G') and viscous shear modulus (G'') of hydrogels after temperature increase. The thermo-reversible features of MC-based hydrogels were also investigated, by the inversion test and rheological characterization. Selected prepared MC-based hydrogels were tested preliminarily in vitro to investigate cytotoxicity on mouse fibroblasts. Finally, it was shown that cells cultured directly onto the MC hydrogel surface can be spontaneously induced to detach, thanks to the hydrogel thermo-reversibility. This means that cells are not detached as single units, but as a continuous monolayer of tightly-interconnected cells (cell sheet). Histological analysis confirmed this hypothesis.

2 Materials and methods

2.1 Materials

Methylcellulose (MC, Methocel A4M, $\eta = 4000$ mPa × s for a 2 % w/v aqueous solution at 20 °C) was kindly supplied by The Dow Chemical Company. All basic chemicals were from Sigma-Aldrich unless stated otherwise.

2.2 Preparation of the thermo-reversible methylcellulose-based hydrogels

Aqueous solutions of different concentrations of MC (Table 1) were prepared with the addition of different concentrations of selected salts (sodium sulphate, Na_2SO_4,

sodium phosphate, Na_3PO_4, calcium chloride, $CaCl_2$) or phosphate buffered saline (PBS, containing NaCl 137 mM, KCl 2.7 mM, phosphate buffer 10 mM) to the MC solutions (Table 1). The MC hydrogels tested will be referred to by acronyms, in which the first number indicates the MC con-centration (% w/v, Table 1), the letters indicate the salt (SO, PO, Cl, PBS) and the last number indicates the concentration of salt or PBS (mol/l for salts, g/l for PBS, Table 1).

Preparation of the MC hydrogels comprised three main steps, summarized in Fig. 1: (1) preparation of the saline solution at the concentration given in Table 1; (2) addition of MC to the saline solution at the concentration given in Table 1; (3) hydration of the MC powder (i.e. sol phase).

Step 1: preparation of saline solution.	Saline solutions were prepared by mixing the appropriate quantity of salt or PBS tablet with distilled water (Table 1) at 55 °C under magnetic stirring a Falcon tube
Step 2: mixing.	The MC powder was first added to the saline solution at 55 °C under magnetic stirring to distribute particles throughout the solution. The MC suspension was poured into tissue-culture polystyrene Petri dishes
Step 3: hydration of MC suspension.	To allow complete hydration of the MC powder, after the mixing step the suspension was cooled to 30–35 °C, depending on the MC hydrogel composition. The MC powder began to hydrate and the viscosity of the solution increased. The prepared MC solutions (Table 2) were then stored at 4 °C overnight, resulting in a transparent hydrogels in the sol phase

2.3 Gelation test

The physical gelation of MC hydrogels was determined visually, using an inversion method reported in the literature [27]. Briefly, aliquots (10 ml each, $n = 3$) of MC hydrogels (Table 2) were placed in Falcon tubes (15 ml), and heated to 40 °C in a standard bath. The temperature was then decreased to 20 °C, at approximately 0.5 °C/min. At 37 and 20 °C, the Falcon tube was inclined by 90° and any flow of the MC solution was noted. At each temperature, the solutions/gels were allowed to equilibrate for 1 h. The gelation criterion was defined as the temperature at which the clear solution did not flow upon inversion of the Falcon tube [27].

2.4 Rheological characterization

Rheological characterization of MC hydrogels was performed with a rotational rheometer (AR-1500ex, TA Instruments, USA), using flat plate geometry (diame-ter = 2 cm, working gap = 1 mm). A home-made isola-tion chamber in polymethyl methacrylate (Plasting srl, Segrate, MI, Italy) was designed and attached to the rheometer to prevent dehydration of the hydrogels during the test. Tests were performed using five specimens for each MC hydrogel composition tested. To investigate the rheological properties of the MC hydrogels, dynamic vis-cosity (η^*), shear storage modulus (G') and shear viscous modulus (G'') were recorded over the temperature range 5–50 ° C, with a temperature ramp of 5 ° C/min. The oscillation frequency during the temperature ramp was maintained at 1 Hz. Thermo-reversibility characteristics of the MC hydrogels were studied, with a first run increasing the temperature from 4 to 40 °C, and a second run decreasing the temperature to 4 °C (temperature ramp = 10 °C/min, oscillation frequency = 1 Hz).

2.5 In vitro cytotoxicity study

In vitro cytotoxicity of the MC hydrogel extracts was assessed on murine fibroblasts (L929, ECACC No. 85011425). The hydrogels with the highest salt concentration (Table 3) were prepared under laminar hood, using saline solutions that had been previously sterilized by filtration (Minisart 20 nm, Sartorius Stedim Biotech). The prepared hydrogels were sterilized by UV light exposure

Table 1 Salt and MC concentrations used for the preparation of the MC hydrogels

Salt	Salt concentration	Salt abbreviation
Na_2SO_4	0.02 M–0.05 M–0.07 M–0.1 M–0.2 M	SO
Na_2PO_4	0.1 M–0.2 M–0.3 M–0.4 M	PO
$CaCl_2$	0.05 M–0.1 M–0.2 M	Cl
PBS	5 g/l–10 g/l–50 g/l	PBS
MC concentration (% w/v)	2–4–6–8–10–12	

The different salts investigated for the preparation of MC hydrogels are reported together with salt concentration selected and the salt abbreviation used for their identification. The different concentration of MC considered for the preparation of the hydrogels are here reported

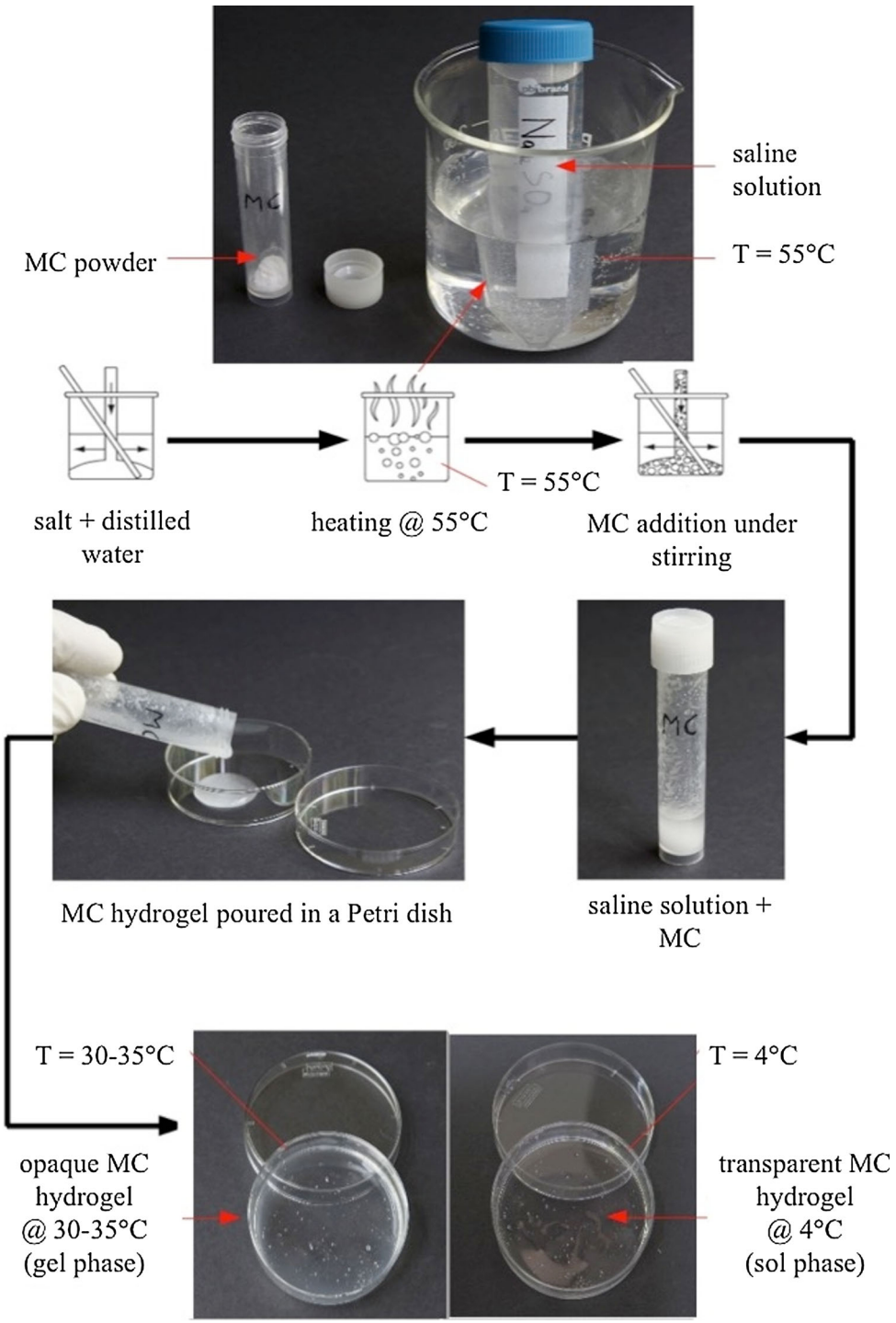

Fig. 1 Scheme showing preparation of MC thermo-reversible hydrogels, starting from saline solution and MC powder. Step 1: preparation of the saline solution at the concentration given in Table 1; step 2: addition of MC to the saline solution at the concentration given in Table 1; step 3: hydration of the MC powder. The saline solution was pre-heated to 55 °C before adding the MC powder (step 1); an opaque solution containing MC was then formed, and gently and uniformly distributed over the surface of a Petri dish (step 2). After 24 h hydration at 30–35 °C and storage at 4 °C, the gel became transparent (step 3). The gel returned opaque when heating again up to 37 °C for 30 min

(t = 30 min). L929 fibroblasts were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10 % fetal bovine serum (Sigma) and 1 % penicillin/streptomycin at 37 °C, 5 % CO_2. When cells reached 80–90 % confluence, they were detached with trypsin–EDTA solution, harvested, and used for the experiments. Two hundred microliters of the hydrogels (Table 3) were placed in the wells (n = 3) of a 24 TCPS multiwell plate (CellStar, VWR-PBI International) and submerged for 1 week with 2 ml of complete medium. The surnatants were then collected and used to cultivate cells (cell density = 1 × 10^6 cells/cm^2, cell suspension = 500 µl/well) for 24, 48, or 72 h. At each time-point, cell viability was evaluated by the (3-(4,5-

Table 2 Prepared MC hydrogels; on the row the salt concentration and the PBS concentration are reported, on the columns the MC concentration

Salt concentration	MC concentration (% w/v)					
	2	4	6	8	10	12
0.02 M					Na_2SO_4	Na_2SO_4
0.05 M				Na_2SO_4	Na_2SO_4	Na_2SO_4
					$CaCl_2$	$CaCl_2$
0.07 M				Na_2SO_4		Na_2SO_4
0.1 M		Na_2SO_4	$CaCl_2$	Na_2SO_4	Na_2SO_4	Na_2SO_4
				$CaCl_2$	$CaCl_2$	$CaCl_2$
0.2 M		Na_2SO_4	$CaCl_2$	$CaCl_2$	Na_2SO_4	$CaCl_2$
					$CaCl_2$	
5 g/l		PBS		PBS	PBS	PBS
10 g/l	PBS	PBS		PBS	PBS	PBS
50 g/l	PBS	PBS		PBS	PBS	

In each cell, the salts or PBS blended to the MC solution are reported

Table 3 MC hydrogels investigated in the in vitro cytotoxicity test

	Salt	Salt concentration	MC concentration
4SO0.2	Na_2SO_4	0.2 M	4 % w/v
4Cl0.2	$CaCl_2$	0.2 M	
4PBS50	PBS	50 g/l	

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric metabolic assay (MTT, Sigma). Briefly, 50 µl of MTT solution (3 mg/ml in PBS) were added to each well and incubated for 4 h in the dark. The surnatants were then removed; formazan crystals were dissolved in 100 µl of dimethyl sulphoxide (DMSO), and 50 µl were spotted into a 96 TCPS multiwell plate. Sample optical density (O.D.) was evaluated by spectrophotometer (SpectraCount, Packard Bell) at 570 nm. Cells cultivated with fresh DMEM were used as positive control, and their O.D. was taken as 100 % viability. Cell morphology was also evaluated 72 h after seeding, by light microscopy (Leica AF6500, Leica Instruments). Experiments were performed in triplicate.

2.6 Feasibility of in vitro cell-sheet biofabrication

NIH-3T3 mouse embryo fibroblasts (CRL1658, American Type Culture Collection, Manassas, VA) and NIH-3T3 mouse embryo fibroblasts transduced with third-generation lentivirus, using the pCCLsin.PPT.hPGK.eGFP.pre vector transfer construct, were used to verify the suitability of the MC hydrogel as a temporary substrate for the preparation of cell sheets. Since the MC hydrogel is not fully transparent at 37 °C (the cell culture temperature), cells expressing GFP were used, to facilitate daily checking of cell adhesion, spread, and morphology. Cells were expanded in flasks, using DMEM added with 10 % FBS, and detached with trypsin–EDTA when 80–90 % confluence was reached.

Two hundred microliters of MC hydrogel (8 % w/v MC and 0.05 M Na_2SO_4) were used to coat each well of a 24 multiwell plate (TCPS, CellStar). The plate was heated for 2 h at 37 °C, 95 % humidity, and coated with 20 µl of Type I collagen (BD, 2 mg/ml) prior to cell seeding. Cells were seeded at high concentration (1×10^6 cells/cm^2) onto the MC hydrogel surface and cultivated for 72 h until 100 % confluence. Cell viability was checked every 24 h by the MTT assay; cells cultivated on TCPS alone at the same density were taken as control. Viability was expressed as % vs. control. After 72 h, the 24 well TCPS was cooled to 4 °C for 30 min to allow gel–liquid transition of the hydrogel. The detached cell sheets were collected using a 25 ml pipette and washed three times with PBS. Cell adhesion, spread, and detachment were checked visually by fluorescence microscopy (Leica DM5500 B, Leica Microsystems, IL, USA).

Lastly, the capability of the detached cell sheets to adhere onto a new well was tested. A portion of cell sheet approximately 1 cm in diameter, detached from a 24 multiwell plate, was seeded into a new polystyrene Petri dish (3.5 cm diameter, Sigma) without medium for 2 h at 37 °C. A few drops of medium were gently spotted directly onto the cell sheet to avoid dehydration. 5 ml of fresh medium (DMEM) were then added and, after 72 h, the dish were visually checked by fluorescent microscopy (Leica DM5500 B).

2.7 Histological analysis

After detachment, cell sheets made of cells expressing GFP were fixed for 30 min at room temperature with 4 % phosphate buffered formaldehyde, embedded in Kilik

(Sigma) tissue-freezing compound, and stored at −80 °C. Samples were cryosectioned at 10 μm and slices were seeded for 30 min onto charged glass slides (SuperFrost, Menzel-Glaser, Germany); samples were then stained with phalloidin to visualize F-actins (Molecular Probes Inc, OR, USA, 1:150 in PBS) in order to evaluate cell distribution, concentration, and morphology in the cell sheets.

Moreover, cell sheets made of cells non-expressing GFP were stained with an anti-N-cadherin (Abcam, ab98952, 1:400 in PBS containing 2 % goat serum and 1 % bovine serum albumin) and anti-Collagen type II (Abcam, ab3092, 1:500 in PBS containing 2 % goat serum and 1 % bovine serum albumin) antibody prior to be co-stained with phalloidin and 4′,6-diamidino-2-phenylindole (DAPI, Sigma, 1:150 in PBS) to visualize actin filaments and nuclei. Cells were incubated overnight at 4 °C with pri-mary antibodies and then washed carefully three times with PBS prior to be co-stained with an appropriate secondary antibody (FITC-conjugated, 1:500 in PBS). Images were collected by fluorescence microscopy (Leica DM5500 B, Leica Microsystems, IL, USA) at various magnifications.

2.8 Statistical analysis

Experiments were performed by using at least three replicates for each assay. Data were expressed as mean ± standard deviation, and compared statistically by means of the two-sample *t* test (significance level = 0.05; Origin® Pro v. 8.5 software) or by one-way ANOVA (significance level = 0.05 and Tukey means comparison; Origin® Pro v. 8.5 software).

3 Results

3.1 Gelation test

All test compositions of MC blended with saline solutions changed from a clear solution at a lower temperature (sol phase) to an opaque gel (gel phase) at a higher temperature ($T \geq 37$ °C). As a representative example, the 4SO0.2 hydrogel appeared as a clear solution (sol phase, Supple-mentary Data 1a) at low temperature ($T = 4$ °C), then when gelation occurred, an opaque gel is formed at 37 °C (gel phase, Supplementary Data 1b, c).

The inversion test, run for all test compositions (Table 2), showed the influence of MC and salt concen-trations on the gelation temperature (Fig. 2). In general, hydrogels containing high MC concentrations showed a slower flow rate, indicating higher viscosity. Conversely, hydrogels with low MC concentrations showed lower viscosity, thus flowing faster. Further, at the same concentration of MC, different gelation temperatures could be achieved by varying the salt (Fig. 2a–d). In particular, comparing 4SO0.1 and 4PBS10, the gelation temperature was higher for the hydrogel prepared with PBS. For the 8 % w/v MC concentration, no correlation with the type of salt was found. Considering the highest MC concentration, PBS and Na_2SO_4 showed the same low gelation tempera-ture ($T = 20$ °C); conversely, the gel prepared with $CaCl_2$ maintained a higher gelation temperature ($T = 37$ °C). For all the salts investigated (Fig. 2b, c) and for PBS (Fig. 2d), the gelation temperature decreased to below 37 °C on increasing the salt concentration.

In addition, the critical salt concentration, corresponding to a gelation temperature of 37 °C, was not the same for all the salts investigated. This is due to the different ionic interaction between the salt ion and MC. As an example, for 10SOx and 12SOx, the critical concentration was 0.05 M, while for 12Clx it was 0.1 M. Moreover, for PBS, the critical concentration was 10 g/l for 2PBSx and for 8PBSx, while for 10PBSx and 12PBSx it was 5 g/l.

3.2 Rheological characterization

Figure 3a, b shows curves of the storage shear modulus (G') and the loss shear modulus (G'') obtained from tem-perature sweep rheological analysis, for representative compositions with gelation temperature close to 37 °C at the inversion test. The gelation kinetics of the MC hydro-gels investigated differed widely, depending on their composition, as is clear from the G' and G'' values. For all the MC solutions selected, at low temperatures (approxi-mately in the range 5–10 °C), G' was lower than G'' (Fig. 3a, b) due to the viscous/liquid-like behavior of the MC solution, i.e. the sol phase. On increasing the tem-perature, G' first showed a decrease, reaching a minimum, after which it rapidly increased at the sol/gel transition point, as a soft elastic gel was formed. In addition, on increasing the MC concentration, values of G' increased. Further, on increasing the salt concentration at constant MC concentration, G' increased, and the gelation temper-ature shifted slightly towards lower temperatures ($T < 37$ °C), confirming the inversion test results. When the storage modulus value exceeded the one of the loss modulus, gel is assumed to be formed. The crossover temperature is generally higher than the one detected in the inversion test because of the test protocol, because the inversion test is a very qualitative analysis instead of the rheological one that gives as output a quantitative value detectable by G' and G'' trend. In particular, in the rheo-logical tests it was not always possible to detect the tran-sition temperature in the test condition used in this work, because of the high viscosity of the MC solution due to the high MC concentration (i.e., 10 and 12 % w/v). In fact, in

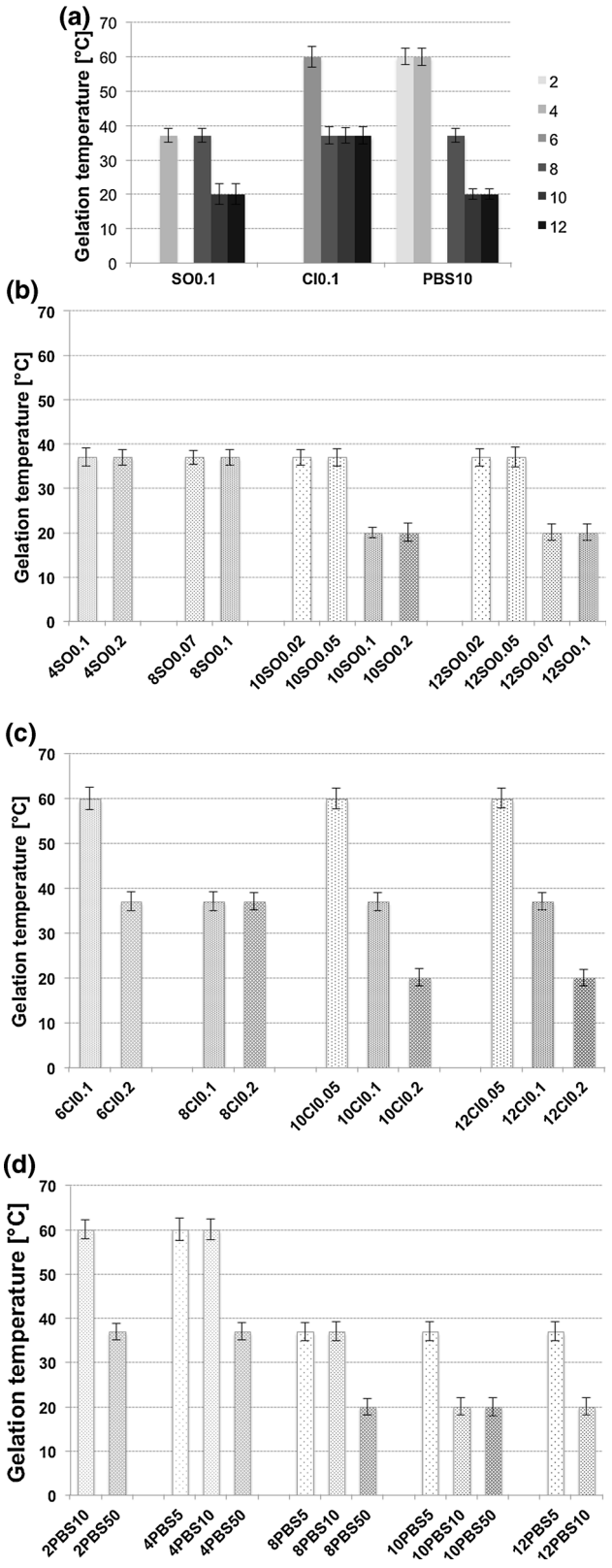

Fig. 2 Gelation temperature of MC blended with salt: **a** effect of MC concentration when blended with 0.1 M Na_2SO_4 or $CaCl_2$, or 10 g/l PBS; **b** effect of Na_2SO_4 or **c** $CaCl_2$ concentrations; **d** effect of PBS concentration

the literature, only MC hydrogels with a concentration of 2–4 % w/v have been characterized by rheometer.

In the heating and cooling tests (Fig. 3c–e), for all the investigated solutions hysteresis occurred. In particular, greater hysteresis was detected when the MC concentration increased, for MC blended with salts (Fig. 3c [e.g. 12SO0.02, 8SO0.1], Fig. 3d [e.g. 10Cl0.2], Fig. 3e [e.g. 12PBS5]). he marked hysteresis detected for some MC compositions might suggest that the solution did not achieve thermal equilibrium in the gelled state, and that the dissociation occurring at the MC gel–sol transition (during the cooling run) was not an exact reversal of the association process (during the heating run) [28].

3.3 In vitro cytotoxicity study

The MTT assay showed that, at each time-point considered (Fig. 4a, left panel), no cytotoxic effect was caused by the release of toxic compounds from the tested MC hydrogels. Cell viability was in the 96–99 % range for all time-points, which is comparable to control values, with no statistically-significant differences ($P > 0.05$) between controls and eluates. Further, MTT results were supported by light microscopy observations (Fig. 4b, right panel). Cells cultivated for 72 h in contact with the MC hydrogel eluates showed morphology, spread, and density comparable to cells seeded in fresh medium, confirming that no toxic compounds were released into the culture medium.

3.4 Feasibility of in vitro cell-sheet biofabrication

Once the optimal hydrogel composition (i.e. 8 % w/v MC and 0.05 M Na_2SO_4) had been selected, the possibility of fabricating a cell sheet detachable from the surface of the MC hydrogel was tested (as schematized in Fig. 5a). Although cell adhesion took about 10–12 h, after 48 h cultivation, spread, morphology, and density were comparable between polystyrene (Fig. 5b, upper panel) and hydrogel (Fig. 5b, lower panel) seeded cells. The results of the MTT assay for up to 72 h showed no statistically-significant difference between cells seeded onto the MC hydrogel and controls (i.e. TCPS), suggesting that no toxic compounds were released from the hydrogel towards cells in direct contact with it (Fig. 5c), and confirming the results reported above for indirect cytotoxicity. The possibility of inducing spontaneous detachment of the cells by lowering the temperature to 4 °C was subsequently checked (Fig. 5d). The 24 mutilwell TCPS plates con-taining cells cultivated on the hydrogel were thus placed in a refrigerated room (4 °C) and detachment of the cell monolayer (i.e., the cell sheet) was verified every 5 min. Full cell-sheet detachment was achieved after about

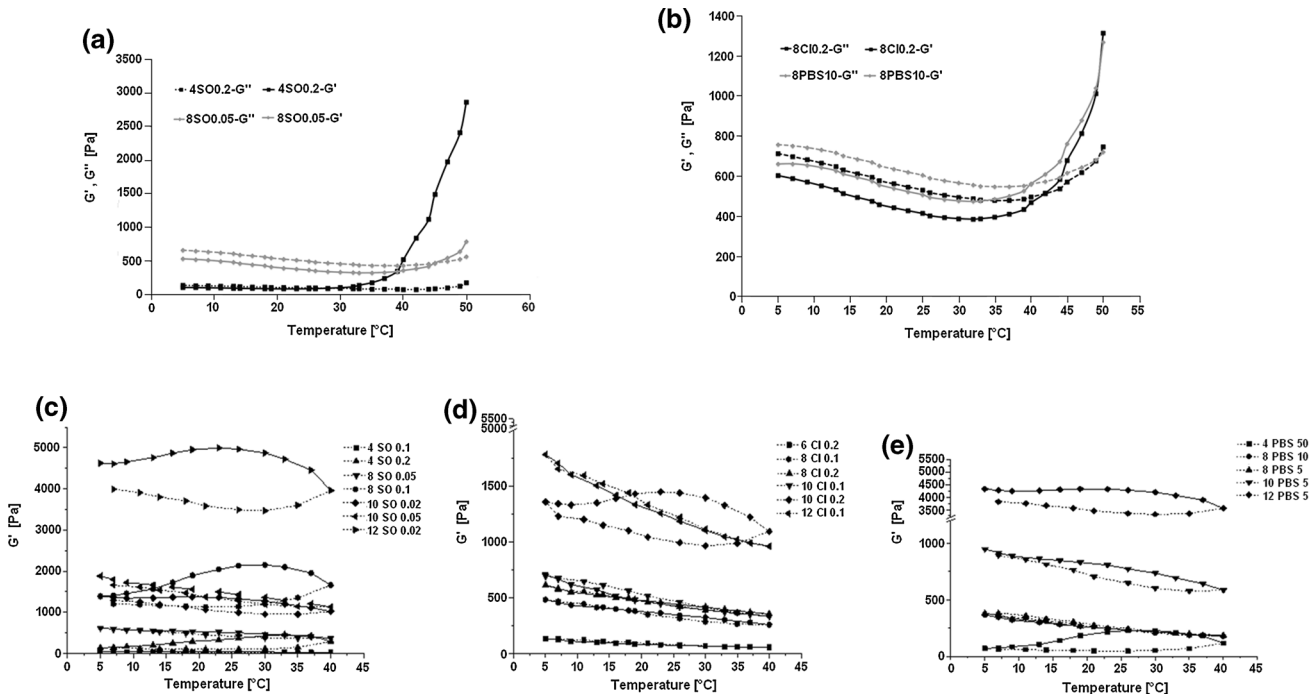

Fig. 3 Shear storage modulus (G') and shear loss modulus (G'') versus temperature for the solutions prepared varying MC and salt concentration: **a** blended with 4SO0.2 or 8SO0.05 and **b** blended with 8Cl0.2 or 8PBS10. *Curves* show the average trend of G' ad G'' obtained from rheological data collected at 0.5 °C temperature intervals, at a frequency of 1 Hz. Temperature dependence of the shear storage modulus (G') for solutions prepared varying either MC or salt concentrations: **c** MC blended with Na_2SO_4; **d** MC blended with $CaCl_2$; **e** MC blended with PBS. *Straight lines* show the average trend of G' during heating to 40 °C; *dashed lines* show trend during cooling to 5 °C

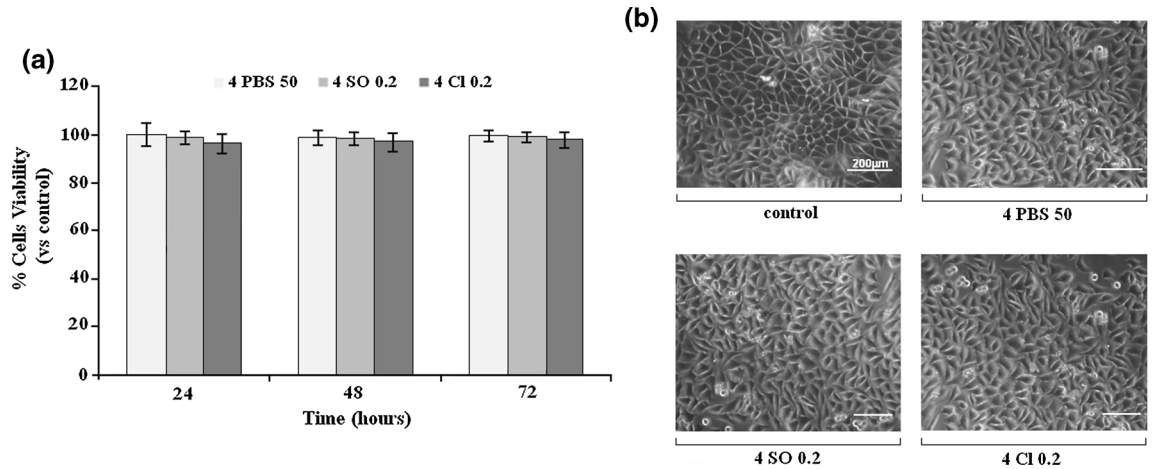

Fig. 4 In vitro cytotoxicity evaluation results. (**a**, *left panel*) At 24, 48 and 72 h after seeding in contact with hydrogel eluates; at each time-point, viability values were comparable between control and tested samples; no statistically-significant differences were found ($P > 0.05$); (**b**, *right panel*) optical microscopy images of L929 cells 72 h after seeding in contact with hydrogel eluates; cell morphology, spread, and density were comparable, confirming cell viability assay results. *Bars* represent means and standard deviations; *bar scale* 200 µm

20–30 min: the cell sheet began to detach from the well border (5–10 min); the centre of the cell sheet then also detached from the hydrogel substrate (15–20 min). Finally, the detached cell sheet was collected in a 25 ml pipette and dropped into a well of a fresh TCPS plate.

3.5 Detached cell-sheet analysis

Phalloidin staining on the fully detached cell sheet (Fig. 5e, left panel) confirmed that the cell sheets were composed of a layer of tightly-interconnected cells, comparable with a

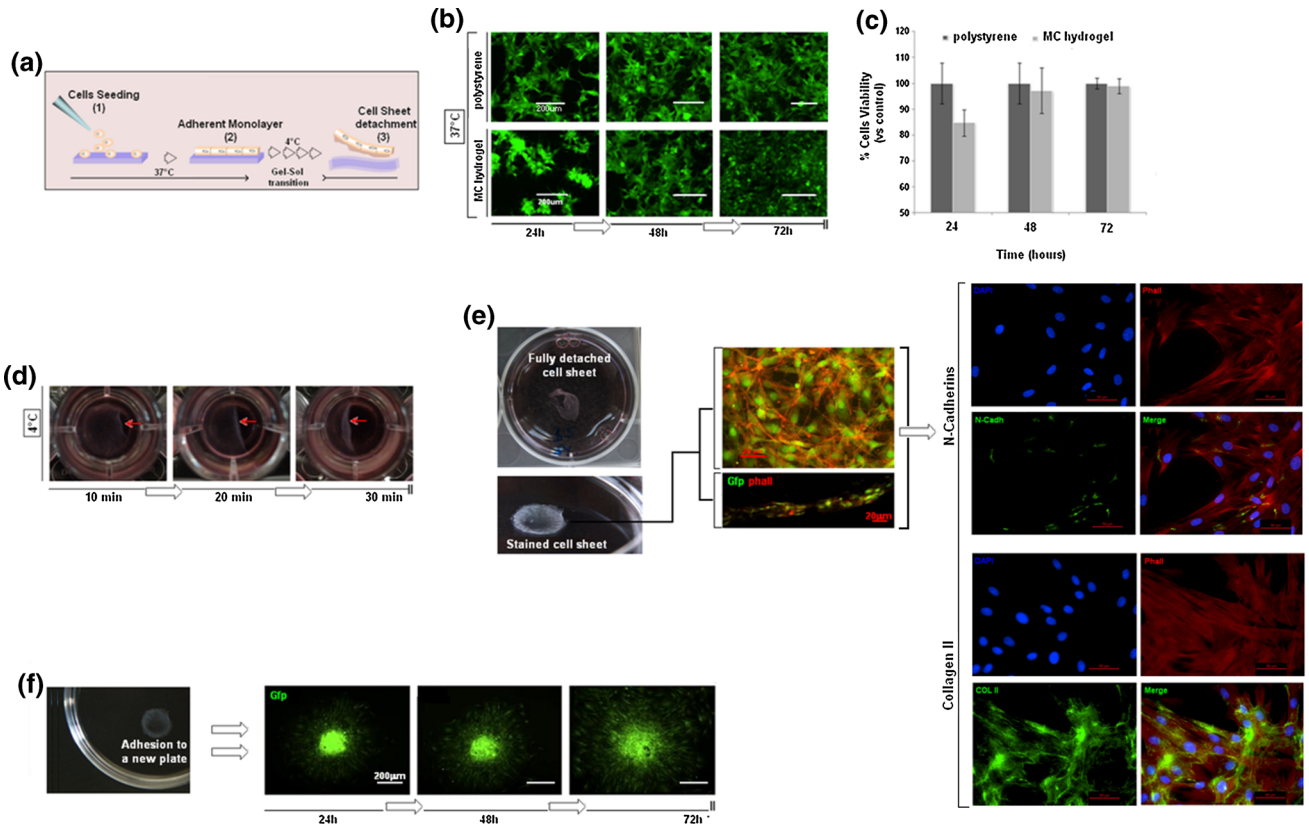

Fig. 5 **a** Schematic representation of the 8SO0.05 hydrogel thermo-reversibility in cell-sheet preparation and detachment; **b** cell adhesion onto polystyrene surface (*upper panel*) and 37 °C pre-heated hydrogel surface (*lower panel*) up to 72 h, spreading was achieved after 48 h, and 100 % confluence after 72 h; **c** cell viability results, no statistically difference values were detected ($P > 0.05$, *bars* represent means and standard deviations); **d** cell-sheet detachment was promoted by lowering the temperature to 4 °C: detachment from the well was observed after about 10 min and went on until complete detachment after about 30 min; **e** immuno-fluorescence images confirmed that the detached cell sheet was composed of high-density tightly-interconnected cells (F-actins in *red*, stained with phalloidin, magnification 20×) and that cells were connected to each others by N-cadherins (N-cadh, in *green*, *right panel*, *upper board*, magnification 40×) with ECM collagen II still present (COL II, in *green*, *right panel*, *lower board*, magnification 40×); **f** the detached cell sheet adhered to a new polystyrene surface (**f**, *left panel*) and cell population was determined after 24, 48 and 72 h by immuno-fluorescence observation (**f**, *right panel*) (Color figure online)

monolayer tissue (Fig. 5e, right panel). Accordingly, it was easy to collect the detached cell sheet with a 25 ml pipette and transfer it onto a new plate, without causing the cell sheet to disrupt. As a further confirmation of the tight cell-to-cell interactions, the presence of N-cadherins [29] was verified in the correspondence of cells interconnection sites (Fig. 5e, right panel, upper board images). Moreover, by avoiding the use of enzymes such as trypsin, it may be thought that the natural extracellular matrix was also still present on the detached cell sheet; the performed collagen Type II staining showed in Fig. 5e (right panel, lower board images) seems to confirm this sentence even if other proofs would be required for further confirmation. As final assay, the collected cell sheet (about 1 cm in diameter) was dropped into a new TCPS plate, to test cell-sheet adhesion (Fig. 5f, left panel). It was then possible to observe cells from the cell sheet colonizing the new surface (i.e. the well bottom) after 24 h, while after 48–72 h it was very interesting to note that almost all the plate surface was colonized by cells (Fig. 5f, right panel). Expression of GFP by the cells fully confirmed that these cells had originated from the cell sheet.

4 Discussion

Good results in terms of thermo-reversible behavior at 37 °C (for possible use in the formation and subsequent detachment of a cell sheet) have been reported using, as substrate, MC hydrogels with PBS, in particular 8PBS10 and 12PBS5 [20, 26, 30, 31]. The present study investigated the possibility of producing smart MC hydrogels as an alternative to those produced with PBS, described in literature [19, 30]. The thermo-responsive hydrogels can be used for cell sheet fabrication, but they could also be investigated as vehicle for cell delivery taking advantage of

switch from sol to gel. To select the hydrogel composition that would give the optimal results, hydrogels were prepared with Na_2SO_4 and $CaCl_2$ salts or with PBS, varying the MC concentration (2, 4, 6, 8, 10 and 12 % w/v).

The qualitative gelation test (i.e. inversion test) showed that the temperature at which gelation begins could be varied by changing either the MC concentration or the formulation of the saline solution, as others have previously reported [19, 26, 31]. The obtained results were confirmed by the rheological characterization that exhibited that only some MC hydrogel compositions hold a transition temperature closed to 37 °C, that would appear to be optimal for possible use as smart hydrogel in regenerative medicine applications.

The MC based hydrogels prepared in our work exhibited reversible thermo-responsive properties. Phase transition was confirmed by both physical and rheological characterizations. Interestingly, it was immediately clear that temperature determined variations in the MC hydrogel's mechanical properties. When samples were heated, mechanical parameters increased, confirming that the polymer structure became increasingly ordered and compact. Conversely, immediately after cooling, hydrogels showed a decrease in the mechanical parameters, indicating that the different MC-based materials were losing their compact structure as they recovered the sol phase. These properties are clearly related with the chemical structure of MC, which is characterized by the presence of both hydrophobic and hydrophilic groups. Methoxyl groups (–CH_3) comprise the hydrophobic regions, while hydroxyl groups (–OH) comprise the hydrophilic regions [31]. At low temperatures (<10 °C) hydrophilic interactions between –OH groups and solvent predominate, thus the MC molecules are hydrated and the polymer structure is held together by simple entanglements [31–33]. As the temperature increases, hydrogels absorb energy and grad-ually lose their hydration water. Polymer–polymer inter-actions take place between –CH_3 groups, forming a gel–network structure.

At rheological characterization, no plateau was reached on increasing the temperature, until the marked decrease in mechanical parameters that have been reported [31, 32]. These results may be due to the fact that the MC hydrogels here investigated did not achieve formation of a complete 3D network, owing to the low test temperature limit (i.e. T = 50 °C) [26]. Further, the rheological characterization showed that the hydrogel composition (i.e. concentrations of MC and salt) strongly influenced the hydrogels' mechanical properties [19, 26, 31]. As the concentrations of MC and of salt increased, the mechanical parameters also increased. This was an expected result, because on adding additional MC, the final polymer will be composed of a greater number of hydrophobic regions, which will form a more structured and compact gel–network. On increasing the salt concentration, water molecules are forced to locate around them, reducing the intermolecular hydrogen-bond formation between water molecules and the hydroxyl groups of MC [26, 29, 33–35].

Not only the concentration, but also the type of salt greatly influenced the hydrogels' mechanical properties [33]. According to the lyotropic series, with the same MC and salt concentrations, more water molecules were removed from MC hydrogels when Na_2SO_4 was used than with $CaCl_2$, evidencing the effect of the salts on the LCST value.

On evaluating the G' and η^* values obtained during the cooling phase, it was noted that they were higher than the respective values during the heating phase. In contrast to the sharp increase of both parameters during the heating process in the temperature range 30–40 ° C, the gradual decrease of the two parameters with temperature during cooling showed a marked deviation from the heating curve. This clearly indicates that the thermally-induced hydrophobic dissociation is not an exact reversal of the hydrophobic association occurring during the heating pro-cess. The hysteresis between the heating and cooling phases may be due to the existence of some associated aggregates or weak connections that have not completely disassociated [28, 35]. Differences may also be due to different dynamics being involved in forming and in breaking the chain-to-chain interactions, related to the different dynamics involved in absorbing and releasing water. Only below 7 ° C did the values again become comparable.

Another factor that may affect the breakdown of the polymeric structure is the length of time during which the system has equilibrated at the highest temperature before cooling. In this study, the cooling process began immediately after the heating process was completed. However, if the sample is allowed to equilibrate for some time before cooling starts, the equilibration period should have an effect on the hydrogel's behavior during the cooling phase. It could be expected that the hydrogel would have higher mechanical properties because of the more perfect network that had formed, compared to a freshly-formed gel at the same temperature; in consequence, a different trend would be recorded. The above results show that the temperature at which gelation begins, therefore the mechanical proprieties of a hydrogel, can be varied by changing the concentration of MC and the formulation of the aqueous solvent. In addition, the composition of a MC hydrogel can easily be manipulated to obtain specific physico-chemical characteristics for different uses.

In vitro cytotoxicity tests were performed on extracts of complete medium that had been in contact with 4SO0.2, 4Cl0.2, and 4PBS50 MC hydrogels for 7 days, in order to

rule out the release of toxic compounds. Cells viability was verified after 24, 48 and 72 h as required by international ISO 10993-5:2009 standards for biomedical devices in vitro cytotoxicity evaluation. The MC hydrogels were selected for the in vitro test because they contained the highest content of salt among the test hydrogels. Therefore, any possible cytotoxic effect would be more evident for these hydrogels than for those with lower salt concentra-tions. The preliminary in vitro biological characterization results showed the selected MC hydrogels not to be cyto-toxic to mouse fibroblasts. The viability of cells cultured in medium that had been in contact with the test hydrogels was comparable to that of cells cultured in fresh medium. These results suggest that, in 7 days of direct hydrogel-medium contact, no toxic products had been released into the medium. Since the hydrogels containing the highest salt concentrations were tested, these findings may be extended to the other formulations, with a lower salt concentration. Besides, MC itself cannot be considered to be a source of toxicity, since the selected material is a commercial MC powder in widespread use, including the food industry, and its cytocompatibility has been clearly established [27].

Once MC hydrogels in vitro cytocompatibility was verified, the possibility of fabricating a cell sheet detach-able from the surface of the MC hydrogel was tested. Cells adhesion represented the first hurdle. In fact, cells seeded onto hydrogels, uncoated or coated with FBS or human gelatin, formed spherical agglomerates but did not adhere as single cells (data not shown). To overcome this draw-back, hydrogels were pre-coated with type I collagen. After this surface treatment, cells successfully attached onto the MC hydrogel as single units, avoiding the formation of spheroids. Then, also cells density was found to be a cru-cial parameter to promote adhesion. By seeding cells at a concentration of 1×10^6 cells/cm^2, 100 % confluence was reached after 72 h. However, when cells were seeded at lower concentrations (1×10^4 or 1×10^5 cells/cm^2), spherical agglomerates were observed even if the collagen coating had been applied (data not shown). Direct cells seeding onto collagen-coated hydrogel showed a difference in term of viability between cells seeded onto MC hydrogel and TCPS after 24 h that was not significant ($P > 0.05$). This result could be probably related to the effective number of cells adhered to the substrate as some cells floating were noticed in the wells filled with hydrogel while all the seeded cells were adherent into polystyrene wells. By seeding cells at a density of 1×10^6 cells/cm^2, after 72 h 100 % confluence was achieved and the temperature-dependent cell sheet detachment was assayed. The entire detachment process was achieved after about 20–30 min: the cell sheet began to detach from the well border (5–10 min) and then from the hydrogel substrate (15–20 min). This not homogeneous process is probably due to the greater cell density in the centre of the wells compared to the edges, caused by the cell suspension being dropped onto the centre of the MC hydrogel surface. However, after complete detachment of the cells sheet from the hydrogel, it was not difficult to handle it for further assays, confirming the feasibility of the entire process. As first evaluation of the detached cell sheet, phalloidin was used to study density and morphology of cells. The staining confirmed that the cell sheets were composed of a layer of tightly-interconnected cells, com-parable with a monolayer tissue. This finding strongly validates the hypothesis that the cells had detached from the MC hydrogel surface still strongly connected to one another as a continuous monolayer. Accordingly, it was easy to collect the detached cell sheet with a 25 ml pipette and transfer it onto a new plate, without causing the cell sheet to disrupt. This is due to the tight cell-to-cell inter-actions that confer strong mechanical resistance to the cell sheet as clearly demonstrated by the presence of N-cad-herins in the correspondence of most of the cells inter-connection regions [29]. Thus, it can be speculate that the cell sheet mechanical resistance during and after the detachment step is mostly due to the tight cell-to-cell interconnections. Moreover, it must be underlined that no enzymes (such as trypsin) were used to determine or drive the cell sheet detachment. Therefore, it was supposed that the cell ECM was still present also in the detached cell sheet. This hypothesis was firstly verified by the presence of collagen assayed after the separation from the hydrogel. Finally, as last assay, the collected cell sheet was dropped into a new TCPS plate, to test cell-sheet adhesion. For the adhesion step, the cell sheet was seeded onto a medium-free plate: if medium is present, the cell sheet tends to flow into the liquid instead of attaching to the plate surface. Thus, it was verified that it is necessary to promote a first “adhesion phase” prior to adding medium. This phase took about 2 h in an incubator, and thus a few drops (about 1 ml) of medium were added to avoid cell dehydration, without detaching the cell sheet from the TCPS surface. It was then possible to observe cells from the cell sheet colonizing the new surface (i.e. the TCPS well bottom). The entire process was daily observed up to 72 h during which the number of cells in the well increased. These cells expressed GFP, confirming that they were originated from the cell sheet. These data are very encouraging, suggesting that this technology could appropriately be applied to the biofabrication of implantable artificial proto-tissues. Indeed, the natural extracellular matrix could play a gluing role in adhesion of the cell sheet to damaged tissues. Moreover, the very promising data obtained concerning cell-sheet adhesion to a new TCPS plate, leads to the speculation that cell sheets might not only be useful to fill tissue-damaged regions, but also to promote regenerative

potency of the surrounding area, as a source of proliferative cells. Thanks to these findings, we can suppose that the recovered cell sheets could be suitable for two- and three-dimensional manipulation techniques and represents a promising tool with high investigative potential. In fact, in naive tissues, the parenchyma is mainly composed by tightly interconnected layers made of cell sheets [36]. As an example, kidney glomeruli are composed of podocytes layers and fenestrated endothelial cells interconnected via basement membrane to form a continuous three-dimensional tissue [37]. Comparable bilayers are also known for renal tubules, where epithelial cells and peritubular capillary form different monolayer cell sheets interconnected by basement membrane [37]. Therefore, by the perspective to develop new cellular in vitro model or a novel hybrid artificial organ with a three-dimensional structure similar to naive tissues, we were aimed to the development of an easy, reproducible and low-cost technology. We here demonstrate that this latter allows cell sheets biofabrica-tion, improving the most common technique based on single cells manipulation. To accomplish this, temperature-responsive MC-based hydrogel was very promising. Using the above-mentioned cell sheet detachment/recovery tech-nique, we successfully developed and managed an in vitro-biofabricated cell sheet composed by viable tightly interconnected cells still presenting their natural ECM.

However, it must be pointed out that this research is only a preliminary study, aimed at validating MC-hydro-gels as temporary substrate for the biofabrication of cell sheets. Any speculation concerning their regenerative potency will need to be validated in future work, with specific assays also involving in vivo studies.

5 Conclusions

Different salt concentrations were tested to investigate their influence on MC-hydrogel properties, in particular focusing on the thermo-reversible characteristic. It emerged that some of the prepared MC hydrogels not only possessed thermo-responsive properties but also reversible thermo-responsive properties. In particular the 8 % w/v MC and 0.05 M Na_2SO_4 hydrogel, showed a transition temperature around 37 °C and it maintained this transition temperature with a relatively small amount of salt. It also showed a lower hysteresis compared to the other MC and salt concentration, thus indicating a better stability. Moreover, during the in vitro cells assay this hydrogel showed the best handling and it was found to induce the spontaneous detachment of a single layer of confluent cells. These very encouraging results, including a preliminary in vitro cytotoxicity evaluation, suggest that selected hydrogels might be particular as temporary substrate for cell seeding and cell-sheet biofabrication.

Acknowledgments The authors would like to thank Prof. Antonia Follenzi for kindly providing transduced NIH GFP cells and Dr. Frances Cooper for language revision.

Funding A. Cochis was partially supported by PRIN 2010e2011 (PRIN 20102ZLNJ5_006), financed by the Ministry of Education, University and Research (M.I.U.R.), Rome, Italy.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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