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Method for preparation of planar alginate hydrogels by external gelling using an aerosol of gelling solution

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ABSTRACT

Preparation of planar alginate hydrogels by external gelling requires slow rate of exposure of alginate solution to gelling ions to control gelling process and hydrogel properties. We tackled this issue by exposing solution of sodium alginate to solution of $CaCl_2$ applied as aerosol at exposure rate of 7.5 mg cm^{-2} s^{-1}. Gelling conditions varied with respect to concentrations of sodium alginate (1–3 wt.%) and $CaCl_2$ (0.5–4 wt.%), exposure time (2.5–40 min), the 2nd gelling step in the presence of barium ions, and the storage step. Dimensional stability and Young's modulus values were the principal determined quantities to examine the correlation between hydrogel properties and gelling protocol. The content of calcium ions in hydrogel after gelling by $CaCl_2$ aerosol reveals that the maximum binding capacity of calcium ions by alginate chains was reached. Obtained data suggest that an unusual gelling mechanism related to exposure of sodium alginate to aerosol of gelling solution does not need to be considered since the properties of planar alginate hydrogels follow the trends relevant to general knowledge about alginate hydrogels.

1. Introduction

Sodium alginate is one of the most important polysaccharides used for preparation of hydrogels (Draget, 2000). Its unique gelling capability in the presence of divalent ions makes this polysaccharide irreplaceable in biomedical (Lee & Mooney, 2012; Smidsrød & Skjåk-Bræk, 1990), biotechnological (Nedović, Willaert, Hofman, & Anné, 2004), food (Brownlee, Seal, Wilcox, Dettar, & Pearson, 2009) and other applications. The gelling mechanism by divalent ions has been described in detail (Donati & Paoletti, 2009; Fang et al., 2007; Grant, Morris, Rees, Smith, & Thom, 1973; Mørch, Donati, Strand, & Skjåk-Bræk, 2006; Smidsrød, 1974; Stokke et al., 2000). The hydrogel properties are typically controlled by alginate chemical microstructure (determined by α-L-guluronic and (G) and β-D-mannuronic (M) acid monomer units) and molecular weight, type of gelling ions and gelling conditions (Donati & Paoletti, 2009; Draget et al., 2001; Liu et al., 2002; Mørch et al., 2006; Mørch, Donati, Strand, & Skjåk-Bræk, 2007; Skjåk-Bræk, Grasdalen, & Smidsrød, 1989; Smidsrød, 1974; Strand, Mørch, Espevik, & Skjåk-Bræk, 2003).

The way of gelling with respect to the mode of introducing the gelling ions is an additional parameter influencing the properties of alginate hydrogels (Liu et al., 2002; Quong, Neufeld, Skjåk-Bræk, & Poncelet, 1998). When the alginate solution is exposed to the solution of gelling ions, alginate hydrogel is formed upon diffusion of ions. This is the external gelling mode. The second mode called internal gelling is based on mixing the insoluble source of gelling ions with alginate solution followed by releasing the gelling ions most typically by lowering the pH value after addition of organic acids or slowly hydrolyzing lactones (Draget, Østgaard, & Smidsrød, 1990; Hoesli et al., 2011; Mørch et al., 2006; Quong et al., 1998).

Both internal and external gelling methods exhibit specific characteristics. A distinct advantage of internally gelled alginate hydrogels is the flexibility with respect to the hydrogel shape determined by the shape of mold in which the hydrogel is formed (Kuo & Ma, 2001). The spherical shape can also be attained when gelling proceeds in droplets of alginate solution emulsified in the oil phase (Hoesli et al., 2011; Poncelet et al., 1992). However, the internal gelling is associated with (i) typically long gelling times in the range of several hours (Kuo & Ma, 2007; Mørch et al., 2006), although some protocols in case of alginate bead production show the option for using several minutes (Hoesli et al., 2011; Poncelet et al., 1992), (ii) the necessity to lower the pH value, which may influence the viability of immobilized cells (Hoesli et al., 2011), and (iii) decreased transparency due to residual particles of insoluble form of crosslinking ions (Wang, Liu, Gao, Liu, & Tong, 2008). The

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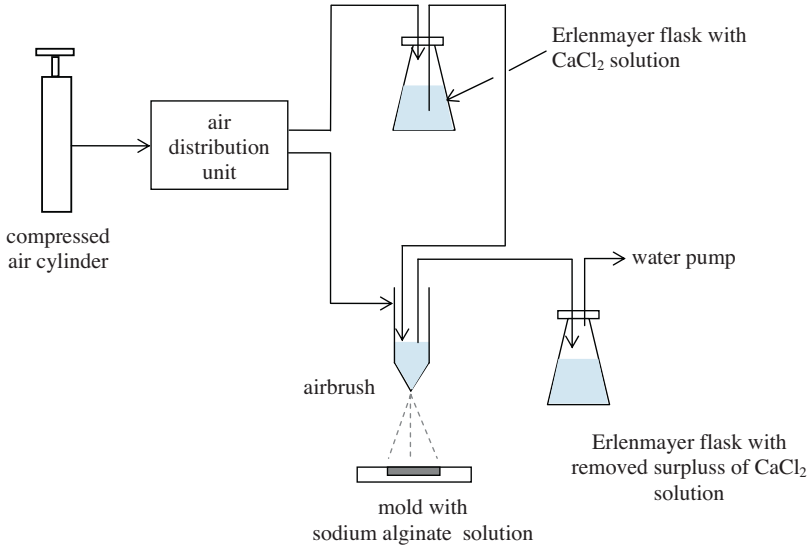

Fig. 1. Experimental setup for formation of planar alginate hydrogels by external gelling method.

advantages of externally gelled hydrogels are mainly represented by short gelling times (in the range of minutes) and physiological gelling conditions. This gelling process is frequently used for immobilization of various bioactive substances in spherical alginate beads (Qi et al., 2008; Tuch et al., 2009) and/or microcapsules (de Vos, Faas, Strand, & Calafiore, 2006; Wang et al., 1997). The spherical shape is the most common one obtained by external gelling process. Yet, some applications require the alginate hydrogels of planar (or other) shapes. For example, planar geometry was used for sustained multi-responsive drug delivery in a case of rheumatoid arthritis (Shi, Zhang, Qi, & Cao, 2012), wound dressing treating the burn injuries (Meng et al., 2010) and tissue engineering (Hwang et al., 2010; Rowley, Sun, Goldman, & Mooney, 2002). In case when the external gelling method is intended to be used for preparation of planar alginate hydrogels, the gelling process cannot be directly derived from the conditions used for formation of spherical alginate beads, since direct exposure of alginate solution to solution of gelling ions results in the hydrogel of an uncontrolled shape.

The identification of proper conditions for formation of externally gelled planar (or cylindrical) alginate hydrogels appears to be significant and several strategies have been described in the literature. The principal one is based on a slow exposure of alginate solution to gelling ions using a dialysis membrane separating alginate and gelling solutions (Junter & Vinet, 2009; Prang et al., 2006; Sriamornsak & Kennedy, 2006; Wong, Siegrist, Wang, & Hunziker, 2001), or a slow release of gelling ions from, for example, agarose hydrogel (Franzesi, Ni, Ling, & Khademhosseini, 2006) and the Kimwipe tissue (Willenberg, Hamazaki, Meng, Terada, & Batich, 2006). Other principles use alginate solution of high viscosity that is resistant to shape distortion after immersion to solution of gelling ions (Inukai & Masakatsu, 1999), or expose dry alginate films to the gelling ions by dipping (Aslani & Kennedy, 1996; Julian, Radebaugh, & Wisniewski, 1988), using aerosol (Cathell & Schauer, 2007) or vacuum to draw gelling solution through the film (Jejurikar, Lawrie, Martin, & Grøndahl, 2011).

Our approach introduces the method for formation of planar alginate hydrogels by external gelling, where sodium alginate solution is exposed to the aerosol of gelling solution. The advantage of this method is in the possibility to tightly control the gelling conditions and to elucidate their effect on hydrogel properties. Planar hydrogels with the thickness of around 1 mm were prepared in this work, but the method is flexible in this respect. The sensitivity to process parameters (i.e., concentration of components and exposure time) is investigated, including the conditions for the additional gelling by barium ions. Hydrogels are characterized with respect to dimensional stability (syneresis and swelling) and Young's modulus in compression mode to demonstrate that hydrogel characteristics are comparable to those obtained by a more conventional internal gelling technique used for preparation of planar alginate hydrogels. These results are accompanied with determination of concentration of gelling ions in hydrogels and spatial distribution of alginate chains. This work demonstrates that proposed method for preparation of alginate hydrogels by external gelling can be applied for any situation where the planar geometry is required.

2. Experimental part

2.1. Materials

Sodium alginates with high content of G-monomer units Protanal LF 10/60, Protanal LF 200 S, Protanal LFR 5/60 and sodium alginates with low content of G-monomer units Protanal LF 10/60 LS, Protanal HF 120 RBS were purchased from FMC Biopolymer (Norway). Other chemicals were of analytical grade and they were purchased as indicated: $CaCl_2{\cdot}2H_2O$, $BaCl_2{\cdot}2H_2O$ and HNO_3 (CentralChem, Slovakia), NaCl and NaN_3 (Penta Chemicals, Czech Republic), NaOH (AFT Bratislava, Slovakia), acetic acid (≥99%, Mikrochem, Slovakia). $CaCO_3$ (≥99.999+%) and D-(+)-glucono-δ-lactone (GDL, ≥99.5%) were purchased from Sigma–Aldrich, USA. Fluorescein diamine isomer I (99%) was obtained from Acros Organics (Belgium). Distilled water (Separate Water Supply 2004, GFL, Germany) was used for preparation of solutions.

2.2. Preparation of planar alginate hydrogels by external gelling method

The solution of Protanal LF 10/60 of selected concentration (1–3 wt.%) and the $CaCl_2$ solution of concentration between 0.5 and 4 wt.% were prepared in 0.9 wt.% NaCl. The pH values of these solutions were set by concentrated acetic acid and sodium hydroxide solutions to the range between 7.0 and 7.5 and solutions were filtered through 0.22 μm filters. The setup used for formation of alginate hydrogels by external gelling method is shown in Fig. 1. The glass mold of dimensions (in mm) $19 \times 19 \times 1$ was filled with sodium alginate solution (0.3805 ± 0.0005 g) and placed

40 cm under the tip of nozzle of the airbrush AB-200 (Rich®, Japan). The airbrush was connected to the compressed air cylinder via polyurethane tubing (I.D. 4 mm), precision pressure regulator operated at the pressure of ~80 mBar and *in-house* made air-distribution unit (all components purchased from Festo, Germany). The $CaCl_2$ solution was pushed from Erlenmeyer flask to airbrush via tubing (I.D. 6 mm) and needle (I.D. 1.2 mm) inserted to the bottom of the airbrush reservoir. The aerosol of gelling $CaCl_2$ solution was leaving the nozzle at the flow rate V_1 of 1500 ± 60 mg min^{-1} reaching the surface of sodium alginate solution in the mold with the surface flow rate V_2 of 7.5 ± 0.5 mg min^{-1} cm^{-2}. Both flow rates were determined gravimetrically. To keep V_1 and V_2 values constant throughout the operation, the constant level of the gelling solution in the airbrush reservoir is required. This was achieved by using a higher flow rate of $CaCl_2$ solution than V_1 and an additional line to remove the surplus of gelling solution from the airbrush reservoir. This line consisted of the needle (I.D. 2 mm) positioned in the air-brush reservoir above the needle with $CaCl_2$ inlet line, and tubing (I.D. 6 mm) connected to Erlenmeyer flask attached to water pump. These conditions assured uniform exposure of alginate solution in the mold to aerosol of gelling $CaCl_2$ solution. The exposure time and $CaCl_2$ concentration provide a broad range of theoretical molar ratio, ν_{theor}, expressed as moles of calcium ions to moles of uronic acid residues, varied from 0.39:1 to 6.31:1. After gelling the hydrogels were immersed to the 2nd gelling solution (40 ml) composed of either 15 mM $BaCl_2$, 0.2 M NaCl or 1 mM $BaCl_2$, 50 mM $CaCl_2$, 0.2 M NaCl for a defined time between 10 and 60 min. At ν_{theor} of 0.39:1, hydrogels were let to equilibrate for 10 min after exposure to aerosol before removal from the mold as they were too soft for further manipulation. Hydrogels were stored for at least 24 h at 6 °C in storage solution (40 ml) containing 5 mM $CaCl_2$, 0.9 wt.% NaCl, 100 ppm NaN_3.

2.3. Preparation of planar alginate hydrogels by internal gelling method

Internally gelled hydrogel made of Protanal LF 10/60 was prepared following the published protocols (Draget et al., 1990; Holtan, 2006). In this work, 2 wt.% sodium alginate, 20 mM $CaCO_3$ and 60 mM GDL in 0.9 wt.% NaCl solution were used. The gelling solution was prepared by mixing of 4.5 ml of 2.2 wt.% sodium alginate solution in 0.9 wt.% NaCl with 10 mg of $CaCO_3$ dispersed in 0.25 ml of 0.9 wt.% NaCl solution by ultrasound PS04000 (Notus-Powersonics, Slovakia) for 3 min (resulting pH is ~9.3). Subsequently, 0.25 ml of 1.2 M GDL solution in 0.9 wt.% NaCl was quickly added to this mixture that decreased the pH to about 6.5. Immediately after GDL addition, the mixture was loaded between two glass plates of dimensions (in mm) $20 \times 50 \times 1$, secured with an adhesive tape with the thickness adjusted with a glass spacer. Hydrogel was formed for 24 h at 6 °C followed by the 2nd gelling step in 1 mM $BaCl_2$, 50 mM $CaCl_2$, 0.2 M NaCl for 20 min and equilibration for at least 24 h at 6 °C in storage solution (40 ml) containing 5 mM $CaCl_2$, 0.9 wt.% NaCl, 100 ppm NaN_3.

2.4. Preparation of alginate beads by external gelling method

Alginate beads were prepared for testing the effect of selection of sodium alginate on the weight change values using 3 wt.% (Protanal LF 10/60, Protanal LF 200 S, Protanal LFR 5/60, Protanal LF 10/60 LS and Protanal HF 120 RBS) and 1 wt.% (Protanal LF 10/60, Protanal LF 10/60 LS) sodium alginate solutions prepared in 0.9 wt.% NaCl. Sodium alginate solution was pressed from the syringe via needle (I.D. 1 mm) into 100 ml of gelling solution of 50 mM $CaCl_2$ dissolved in 0.9 wt.% NaCl. Gelling time for alginate beads of diameter of around 4 mm was 72 h.

2.5. Size-exclusion chromatography

Molecular weights of sodium alginates were determined by aqueous-phase size-exclusion chromatography adopting the conditions used in our laboratories for characterization of anionic polymers using 0.1 M Na_2HPO_4 and 200 ppm of NaN_3 as an eluent and set of Suprema columns (Beuermann, Buback, Hesse, & Lacík, 2006). A combination of a multi-angle laser light scattering detector PSS SLD 7000 (PSS, Germany) and refractive index detector Waters 2414 DRI (Waters Corporation, MA, USA) was used to obtain the absolute values of molecular weight. The values of refractive index increment dn/dc were determined by differential refractometer BP-2000-V (Phoenix Precision Instrument Comp., PA, USA) in eluent: dn/dc for pullulan (Polymer Laboratories Ltd., UK) of molecular weight 113 kg mol^{-1} (an isorefractive standard) was 0.149 mL g^{-1} and dn/dc for sodium alginate was 0.146 mL g^{-1}, both at the wavelength of 633 nm.

2.6. Young's modulus

Young's modulus was determined employing a Texture Analyzer TA-XT2i (Stable Micro Systems, UK) equipped with a force transducer of a resolution of 1 mN and a Texture Expert software version 1.16 used for data acquisition and evaluation. The measurements were performed at room temperature on alginate hydrogel cylinders of diameter 4 mm, cut with the cork bore, in a compression mode using a vertically moving mobile probe of diameter 3.87 mm at a constant speed of 0.1 mm s^{-1}. The cylinders were positioned on the wetted sandpaper (800 grit) to prevent slipping during compression. The final deformation was set to 97%. Young's modulus was evaluated from the stress-strain curves using the approach described recently, which is suited for characterization of soft hydrogel samples (Krupa, Nedelčev, Račko, & Lacík, 2010). The arithmetic averages and standard deviations of mean were obtained for at least six replicates using the Peirce's criterion (Ross, 2003) for excluding the outliers.

2.7. Dimensional stability

Dimensional stability was determined gravimetrically and expressed as the weight change of hydrogel (in %) with respect to the initial weight of sodium alginate solution using Eq. (1):

$$weight\ \ change = 100 \cdot \frac{m - m_0}{m_0} \quad (1)$$

where m_0 is the initial weight of sodium alginate solution and m is the weight of hydrogel at a particular preparation step. Then, the *weight change* < 0 and the *weight change* > 0 correspond to syneresis and swelling, respectively. The excess liquid was gently wiped by tissue prior to weighing of hydrogels. For planar hydrogels, the arithmetic averages and standard deviations of mean were determined from three individual samples. The data for beads characterize the weight change determined for 10 beads.

2.8. Confocal laser scanning microscopy

A confocal laser scanning microscopy (CLSM) was employed to determine the spatial distribution of alginate chains in hydrogels. Sodium alginate Protanal LF 10/60 was labeled by fluorescein diamine (about one fluorescein diamine molecule per 600 uronic acid units) following the protocol described by Strand et al. (2003). The LSM 510 META on Axiovert 200 inverted microscope (both Zeiss, Germany) was used with the 477 nm line of Ar:ion laser for excitation and the emission was detected with a long-pass LP 505 nm emission filter. The confocal hydrogel image was obtained (Lacík & Chorvát, 2009) by scanning in the *XZ* plane (depth-profile)

with spatial sampling of 1.76 μm/pixel (*X* axis) and of 5 μm/pixel (*Z* axis). An A-plan 10/0.25 objective with confocal pinhole opening of 1 Airy unit and 16× line-averaging was used.

2.9. Content of gelling ions in alginate hydrogels

The inductively coupled plasma optical emission spectrometry, ICP-OES (Jobin-Yvon 70 Plus, France), at Ar plasma power of 1.0 kW, was employed with wavelengths of 317.933 nm and 455.403 nm for calcium and barium determination, respectively. Sodium content was analyzed by flame atomic absorption spectrometry (Perkin Elmer 4100, MA, USA) with flame of C_2H_2–air mixture and wavelength of 589.0 nm. In order to suppress ionization of the analyte in the flame, Cs was added to analyzed solution with final Cs concentration of 1 g l^{-1}. Prior to the ICP-OES analysis, hydrogels were rinsed in ultrapure water, then immersed to 100 ml of water for 3 h with water replacement every 30 min, stored in water overnight and freeze-dried. The residual water content, determined by thermogravimetry (TGA/SDTA 851^e, Mettler Toledo, Switzerland) was 5.1 ± 2.5 wt.%, which was taken into account in evaluation of the content of ions. Freeze-dried samples were placed to the PTFE vessel of a pressure assisted decomposition device ZA-1 (JZD Zahnašovice, Czech Republic), 5 mL of HNO_3 was added, vessel was closed and heated for 4 h at 160 °C. After cooling to the ambient temperature, the solution was quantitatively transferred to 25 ml volumetric flask, filled by re-distilled water and subjected to analysis. The content of sodium ions was found to be at the level of expected values after gelling (in average around 20 mol.% to total content of uronic acid units) that proved effective in washing of non-bound salts from hydrogels. Samples were analyzed in duplicates with less than 5% difference in obtained values. The calcium and barium content is reported as the molar ratio, ν_{exp}, expressing the number of moles of respective ion to the total number of moles of uronic acid residues in hydrogel sample.

3. Results and discussion

3.1. Repeatability in preparation of planar alginate hydrogels

Fig. 2 reveals the level of repeatability in preparation of alginate hydrogels by external gelling method introduced in this work. This is demonstrated by weight change and Young's modulus values determined for hydrogels prepared using 2 wt.% sodium alginate solution exposed for 20 min to aerosol of 2.5 wt.% $CaCl_2$ solution. Alginate hydrogels were prepared under identical conditions in the time span of approximately one year (numbers 1 through 4) and repeated experiments prepared on the same day from the same alginate and $CaCl_2$ solutions (small letters *a* through *c*). The weight change values are shown for different preparation steps: after hydrogel formation and after the 2nd gelling and storage steps. The negligible weight change after hydrogel formation compared to the weight of initial sodium alginate solution below 1% was observed (syneresis for samples 1, 3 and 4, and no change for sample 2). The 2nd gelling step increases the level of syneresis to about 5%, which is maintained after the storage step. The Young's modulus values are reported for hydrogels obtained after the storage step. They are between 50 and 90 kPa with the average value 70 kPa and standard deviation 15 kPa. This indicates that a scatter in data may be associated with Young's modulus values due to a soft nature of samples. Fig. 2 thus demonstrates that preparation of planar alginate hydrogels by protocol proposed in this work is satisfactorily repeatable and allows for evaluation of the effects of preparation conditions on properties of resulting hydrogels.

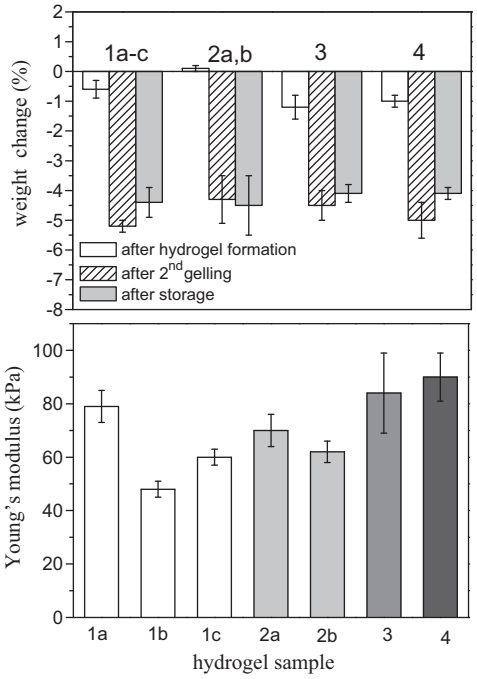

Fig. 2. Weight change determined during different stages of hydrogel preparation and Young's modulus after storage step for planar alginate hydrogels prepared by external gelling method using 2 wt.% sodium alginate solution exposed to aerosol of 2.5 wt.% $CaCl_2$ solution for 20 min. Numbers (1 through 4) and letters (a through c) denote experiments done on different days in span of one year and repeated samples prepared on the same day, respectively.

3.2. Effect of gelling conditions on properties of planar alginate hydrogels

Table 1 summarizes experimental conditions used for preparation of externally gelled planar alginate hydrogels. These conditions approximately follow those typically used for preparation of alginate hydrogels by external gelling in terms of concentration of chemicals and ν_{theor} (Jejurikar et al., 2011; Mørch et al., 2006, 2012; Quong et al., 1998). The varied parameters are sodium alginate and $CaCl_2$ concentrations, time of exposure to aerosol of $CaCl_2$ solution, simultaneously varied time of gelling and $CaCl_2$ concentration while keeping the same value of ν_{theor}, and the conditions for the 2nd gelling step. All hydrogels were equilibrated in the storage solution. The properties of resulting planar alginate hydrogels were evaluated in terms of dimensional stability and Young's modulus shown in Figs. 3 and 4. These data are supported by ν_{exp} determined after exposure to aerosol of gelling $CaCl_2$ solution (Table 1) and after the 2nd gelling and storage steps (Table 2).

3.2.1. Effect of sodium alginate concentration

The increase in sodium alginate concentration from 1 to 3 wt.% (Fig. 3a) is accompanied with an increase in Young's modulus from 40 to 180 kPa for final hydrogels (after storage). Both the range of Young's modulus values and their increase with alginate concentration is a typical result for alginate hydrogels corresponding to higher number of junctions in alginate network (Donati & Paoletti, 2009; Junter & Vinet, 2009; Martinsen, Skjåk-Bræk, & Smidsrød, 1989; Ouwerx, Velings, Mestdagh, & Axelos, 1998). Fig. 3a also shows a trend of decreased syneresis upon increasing the alginate concentration. This is seen for all steps of hydrogel formation, where hydrogels for 1 and 2 wt.% exhibit slight syneresis after gelling by aerosol that increases after the 2nd gelling and storage steps. The hydrogel prepared at 3 wt.% of sodium alginate exhibits even slight swelling after formation with the negligible weight

Table 1
Experimental conditions used for preparation of externally gelled planar alginate hydrogels: concentration of sodium alginate solutions, concentration of $CaCl_2$ in aerosol, exposure time of sodium alginate solution to $CaCl_2$ aerosol, theoretical, ν_{theor}, and experimental, ν_{exp}, ratio of Ca^{2+} ions to total uronic acid units after gelling step by aerosol, and type and time used for application of the 2nd gelling step.

Type of experiment	Sodium alginate (wt.%)	$CaCl_2$ (wt.%)	Exposure time (min)	ν_{theor} (mol/mol)	ν_{exp}[c] (mol/mol)	Type of 2nd gelling solution	Time of 2nd gelling (min)
Concentration of sodium alginate	1	2.5	20	6.31:1	*ND*	[a]	20
	2	2.5	20	3.16:1	*ND*	[a]	20
	3	2.5	20	2.08:1	*ND*	[a]	20
Concentration of $CaCl_2$	2	0.5	12.4	0.39:1	0.27:1	[a]	20
	2	1.5	12.4	1.16:1	0.41:1	[a]	20
	2	3.0	12.4	2.31:1	0.42:1	[a]	20
	2	4.0	12.4	3.08:1	0.42:1	[a]	20
Gelling time	2	2.5	2.6	0.39:1	0.41:1	[a]	20
	2	2.5	7.7	1.16:1	0.43:1	[a]	20
	2	2.5	15.3	2.31:1	0.42:1	[a]	20
	2	2.5	20.5	3.08:1	0.42:1	[a]	20
Concentration of $CaCl_2$ and gelling time	2	0.5	40.1	1.16:1	0.39:1	[a]	20
	2	1.5	13.4	1.16:1	0.41:1	[a]	20
	2	3.0	6.7	1.16:1	0.41:1	[a]	20
	2	4.0	4.4	1.16:1	0.42:1	[a]	20
The 2nd gelling step	2	2.5	20	3.23:1	*ND*	–	–
	2	2.5	20	3.23:1	0.42:1	[b]	10
	2	2.5	20	3.23:1	0.43:1	[a]	10
	2	2.5	20	3.23:1	*ND*	[a]	20
	2	2.5	20	3.23:1	*ND*	[a]	40
	2	2.5	20	3.23:1	*ND*	[a]	60

ND: not determined.
[a] 1 mM $BaCl_2$, 50 mM $CaCl_2$, and 0.2 M NaCl.
[b] 15 mM $BaCl_2$ and 0.2 M NaCl.
[c] Determined by ICP-OES.

change after the 2nd gelling and storage steps. Although the trend of decreased syneresis with increased alginate concentration was reported by others, for example (Nunamaker, Otto, & Kipke, 2011; Quong et al., 1998), a minor syneresis observed in our experiments is considered as puzzling, since formation of alginate hydrogels is usually accompanied by a more significant syneresis. We deal separately with this question in Section 3.3.

3.2.2. *Effect of $CaCl_2$ concentration in aerosol*

Fig. 3b shows dependence of weight change (at different preparation steps) and Young's modulus (after gelling and storage steps) on concentration of $CaCl_2$ in aerosol for 2 wt.% of alginate and 12.4 min of gelling time (Table 1) with ν_{theor} between 0.39:1 and 3.08:1. At the lowest $CaCl_2$ concentration of 0.5 wt.%, hydrogels show a slight swelling, which was decreased but interestingly not suppressed by the 2nd gelling and storage steps unlike in case of higher $CaCl_2$ concentrations. It was expected that after the storage step the saturation conditions with respect to binding of divalent ions to available binding sites of sodium alginate will be reached and all hydrogels in this set of experiments would exhibit similar properties. This was not the case and upon increasing the $CaCl_2$ concentration to and beyond 1.5 wt.% $CaCl_2$, a clear trend of increased syneresis after the 2nd gelling and storage steps is seen along with increased dimensional stability after the gelling step by aerosol. An increased syneresis after the 2nd gelling step is connected with a higher affinity of barium than calcium ions to alginate chains and increased number of gelling junctions between barium ions and alginate MM junctions (Donati & Paoletti, 2009; Mørch et al., 2006; Smidsrød, 1974). During the storage step hydrogels equilibrate in the presence of calcium and sodium ions contained in the storage solution. These results demonstrate that the concentration of gelling ions in aerosol influences the properties of alginate hydrogels after further hydrogel stabilization and equilibration. It is hard to suggest the origin of this phenomenon from available data. Yet it should be noted that the range of weight change for all samples in Fig. 3b is very narrow, i.e., only between 10% of swelling and 5% of syneresis. The sensitivity of dimensional stability to preparation conditions in this set of experiments is not reflected in the Young's modulus values. Although there is slight variation in Young's modulus values between about 60 and 80 kPa after the gelling step by aerosol, this fully disappears after the 2nd gelling and storage steps and Young's modulus values reach the common value of 95 kPa regardless the initial $CaCl_2$ concentration in aerosol. Hence, these hydrogels may not be considered as significantly different in spite of slight but systematic increase in syneresis with increased $CaCl_2$ concentration.

These results can be correlated with data published by Martinsen et al. (1989). In a similar range of $CaCl_2$ concentrations

Table 2
Molar ratio of gelling Ca^{2+} and Ba^{2+} ions to uronic acid residues in alginate hydrogels after formation, after the 2nd gelling step and after the storage step. Hydrogels were prepared according to experimental conditions presented in Table 1 for studying the effect of the 2nd gelling step.

Solution used for the 2nd gelling	Divalent ion:uronic acid (mol/mol)				
	After hydrogel formation	After 2nd gelling		After storage	
	Ca^{2+}	Ca^{2+}	Ba^{2+}	Ca^{2+}	Ba^{2+}
Without the 2nd gelling	0.42	–	–	0.36	–
1 mM $BaCl_2$, 50 mM $CaCl_2$, 0.2 M NaCl		0.42	0.014	0.32	0.011
15 mM $BaCl_2$, 0.2 M NaCl		0.31	0.13	0.26	0.084

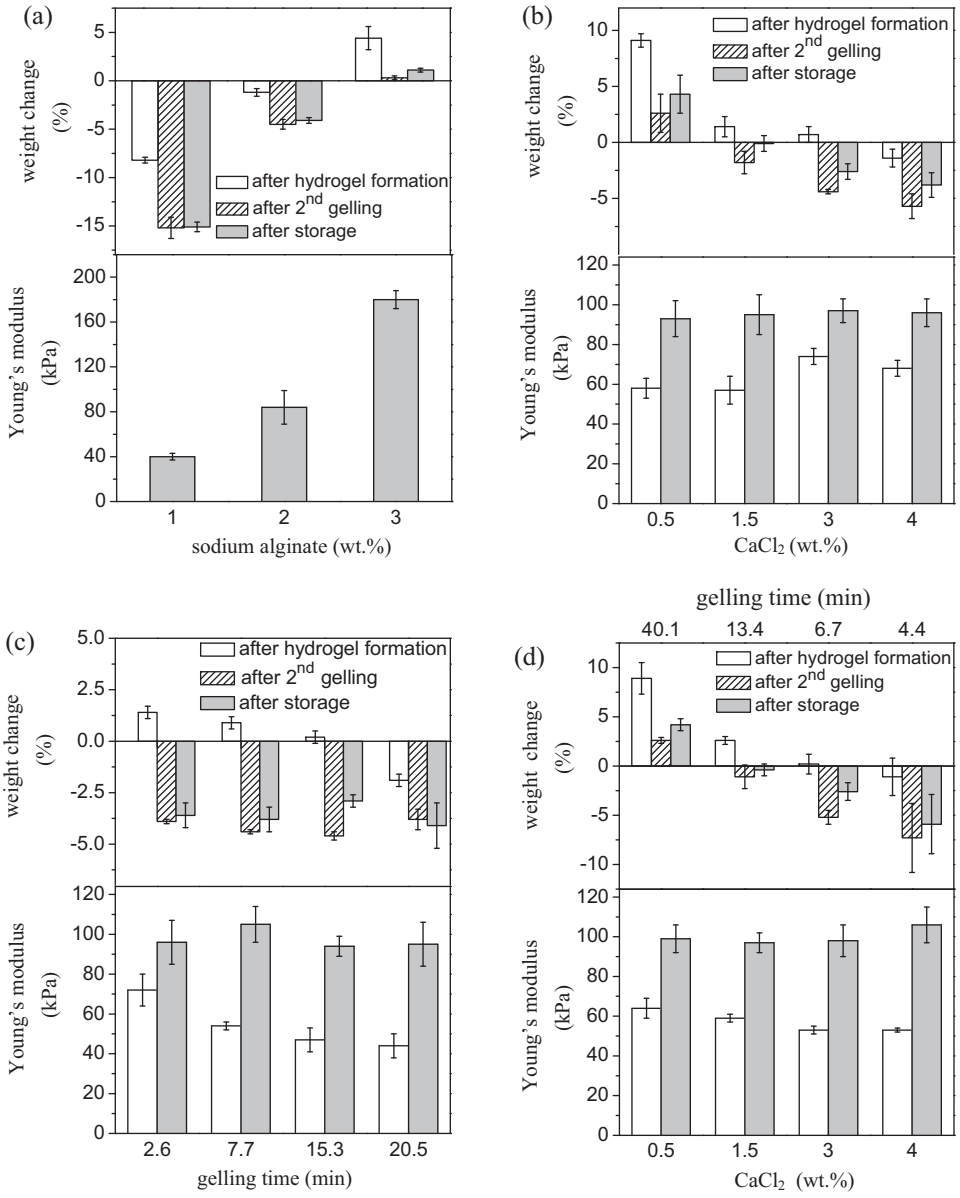

Fig. 3. Effect of gelling conditions on weight change and Young's modulus of alginate hydrogels prepared by external gelling: (a) concentration of sodium alginate, (b) concentration of $CaCl_2$ in aerosol, (c) gelling time, and (d) mutual effect of $CaCl_2$ concentration and gelling time keeping constant ν_{theor} of 1.16:1. Resulting alginate hydrogels were analyzed at various steps of preparation: after gelling by aerosol, after the 2nd gelling step (20 min in solution consisting of 1 mM $BaCl_2$, 50 mM $CaCl_2$, 0.2 M NaCl) and after storage. Further details on gelling conditions are given in Table 1.

(although exposing the alginate solution to gelling ions in significantly different mode, i.e., by $CaCl_2$ solution vs. aerosol), only a minor dependence of volume changes and strength on concentration of gelling ions was observed due to saturating the binding sites. The ν_{exp} values given in Table 1 imply that in this work the maximum binding capacity of calcium ions by alginate chains is attained during the gelling step for $CaCl_2$ concentrations equal and above 1.5 wt.%. These values are constant at 0.41:1 regardless of significantly different ν_{theor} values. The ν_{exp} value is by about 30% lower only for the lowest $CaCl_2$ concentration of 0.5 wt.%, which may be associated with higher swelling than for other hydrogels in this series. The ν_{exp} value of 0.41:1 refers to the ratio of moles of calcium per moles of uronic acid residues, which is around 0.6:1 in terms of G units (based on G-content 65–75% provided by manufacturer) capable of interactions with calcium ions. In correlation with recent work by Fang et al. (2007), who employed isothermal titration calorimetry to follow gelling of sodium alginate by calcium ions in dilute solutions of acetate buffer, these values indicate that planar hydrogels are prepared under the conditions providing the laterally associated egg-box multimer structure. It can be pointed out that ν_{exp} values determined in our work correspond to those obtained by others at various levels of ν_{theor} values (Jejurikar et al., 2011; Mørch et al., 2012; Quong et al., 1998).

3.2.3. *Effect of exposure time*

Fig. 3c depicts dependence of alginate hydrogel properties on time of gelling, i.e., the exposure time of sodium alginate solution to aerosol of $CaCl_2$ solution. Now the ν_{theor} values between 0.39:1 and 3.08:1 in the gelling step were achieved by varying the gelling time between 2.6 and 20.5 min for 2 wt.% alginate solution and 2.5 wt.% $CaCl_2$ concentration. Interestingly, unlike the situation in Fig. 3b, the gelling time only slightly affects the weight change values, which change from ~2% swelling to ~2% syneresis on the given time scale. The ν_{exp} values show that the maximum binding capacity is attained for all times of gelling used in this set of experiments. A higher Young's modulus of 70 kPa obtained after gelling for the

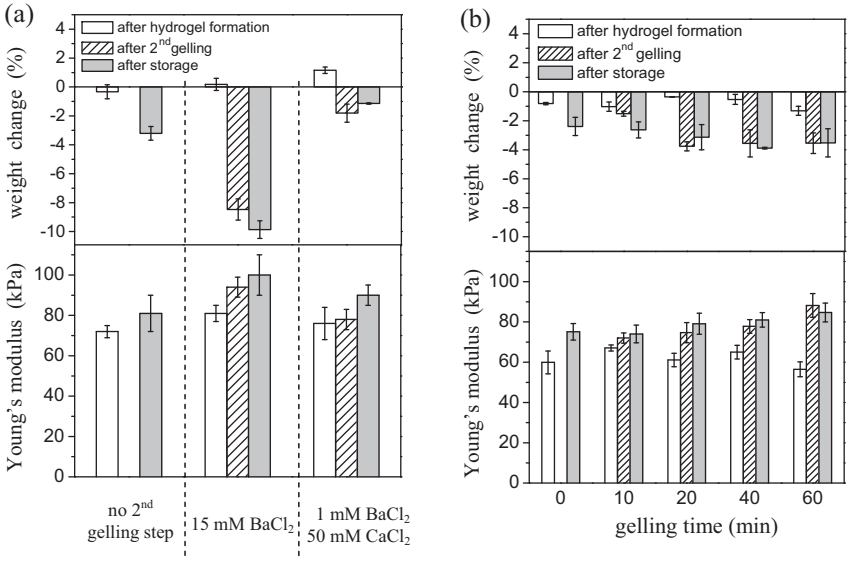

Fig. 4. Weight change and Young's modulus values for alginate hydrogels at different preparation steps in estimating the influence of the 2nd gelling step: (a) effect of composition of solution used in the 2nd gelling step with 10 min gelling time, (b) effect of gelling time for the 2nd gelling solution consisting of 1 mM $BaCl_2$ and 50 mM $CaCl_2$. Further experimental conditions are provided in Table 1.

shortest time (2.6 min) compared to the constant value of ~50 kPa for all three longer times of gelling is difficult to be understood and is proposed to be ascribed to a lower accuracy in determination of Young's modulus (Fig. 2). Of interest is the independence of both weight change and Young's modulus values on time of gelling after the 2nd gelling and storage steps. A slight impact of $CaCl_2$ concentration on dimensional stability shown in Fig. 3b is not coming into play when changing time at constant $CaCl_2$ concentration and the same ν_{theor} values (Fig. 3c).

3.2.4. *Effect of $CaCl_2$ concentration in aerosol and exposure time at constant ν_{theor}*

Fig. 3d shows data obtained for varied both time and $CaCl_2$ concentrations in such a way that ν_{theor} is maintained constant at the value of 1.16:1 (for 2 wt.% alginate solution). This again leads to identical ν_{exp} values of ~0.41:1. The weight change values shift from swelling to syneresis region upon increasing the $CaCl_2$ concentration and, in parallel, decreasing time. This closely follows the situation seen for variation in $CaCl_2$ concentration in Fig. 3b. The Young's modulus values are insufficiently sensitive to account for the differences in the weight change values. This finding points out that the way of delivery of gelling ions in terms of either time of exposure or $CaCl_2$ concentration matters and one cannot replace either of them without the slight impact on hydrogel properties. The origin of this observation is presently not understood. Any discussion based on available data along the line of the systematic effect of $CaCl_2$ concentration on the level of inhomogeneity (Skjåk-Bræk et al., 1989) of the alginate hydrogel appears redundant especially in light of CLSM measurements presented further below in Section 3.4.

3.2.5. *Effect of the 2nd gelling step*

Data in Fig. 3 show importance of the 2nd gelling step on dimensional stability and Young's modulus of alginate hydrogels, where hydrogels were exposed to solution consisting of 1 mM $BaCl_2$ and 50 mM $CaCl_2$ in 0.2 M NaCl for 20 min. Generally, this treatment leads to increased hydrogel stability demonstrated by a more pronounced syneresis and higher mechanical stability (Mørch et al., 2007). In order to further elucidate the effect of the 2nd gelling step for stabilization of planar alginate hydrogels, the effect of gelling time as well as the composition of gelling solution was investigated in a moderate number of experiments (Table 1) using 2 wt.% alginate solution and 20 min exposure time of 2.5 wt.% $CaCl_2$ in aerosol.

Fig. 4a shows the effect of composition of the gelling solution on weight change and Young's modulus for either solution of 15 mM $BaCl_2$ or solution of 1 mM $BaCl_2$ and 50 mM $CaCl_2$, both prepared in the presence of 0.2 M NaCl, with the gelling time of 10 min. Data are shown for samples after gelling step, after applying the 2nd gelling step and after storage. The final alginate hydrogel prepared in the absence of the 2nd gelling step shows minor syneresis of 3%. On the other hand, the gelling step in case of high barium content of 15 mM increases syneresis to 9%, which is maintained during the storage. A lower barium content in the gelling solution (1 mM $BaCl_2$ and 50 mM $CaCl_2$) results in syneresis of only 2% that again does not change upon further stabilization in the storage solution. The 2nd gelling and storage steps increase Young's modulus that is obviously more pronounced for higher barium content in the 2nd gelling solution. Nevertheless, the composition of the 2nd gelling solution, even the absence of the 2nd gelling step, does not lead to strong effect on Young's modulus since the difference between the lowest and highest values is only about 30%.

Table 2 provides the information on the content of gelling calcium and barium ions in different steps of preparation of alginate hydrogels with properties shown in Fig. 4a. The experimental ratio of calcium ions to uronic acid residues of 0.42:1 was found after hydrogel formation by exposure to aerosol of $CaCl_2$ solution. The equilibrium concentration of calcium ions in hydrogel after storage in the absence of the 2nd gelling step is slightly decreased to 0.36:1 (around 0.5:1 with respect to the G-content) likely due to the presence of non-gelling sodium ions in the storage solution along with the low concentration of calcium ions. The 2nd gelling step using solution containing 1 mM $BaCl_2$ and 50 mM $CaCl_2$ resulted in shifting the Ba/Ca molar ratio from 1/50 in gelling solution to 1/30 in alginate hydrogel. The occupation of binding sites by barium and its increased level in hydrogel does not lower the calcium concentration. Hence, in this case, barium ions seem to occupy predominantly MM junctions (to which calcium does not have a strong affinity) and do not replace calcium ions from GG and MG junctions (Donati & Paoletti, 2009; Mørch et al., 2006; Smidsrød, 1974). The molar ratio Ba/Ca equal to 1/30 is maintained also in hydrogel after the storage step although the absolute concentrations of both ions are decreased during the equilibration in the storage solution.

Table 3
Weight change for externally gelled alginate beads prepared using sodium alginates differing in molecular weight, M_w, and chemical composition in terms of guluronic acid monomer units (G-content). Hydrogels were prepared by adding sodium alginate solution droplets into 50 mM solution of $CaCl_2$ in 0.9% NaCl with gelling time of 72 h.

Sodium alginate	M_w (kg mol^{-1})[a]	G-content (mol.%)[b]	Concentration (wt.%)	Weight change (%)
Protanal LF 200 S	235.8	65–75	3	−24
Protanal LF 10/60	116.7	65–75	3	−5
Protanal LF 10/60	116.7	65–75	1	−11
Protanal LFR 5/60	38.4	65–75	3	35
Protanal HF 120 RBS	242.5	45–55	3	−43
Protanal LF 10/60 LS	109.1	35–45	3	−36
Protanal LF 10/60 LS	109.1	35–45	1	−46

[a] This work by size-exclusion chromatography.
[b] From manufacturer.

When 15 mM of $BaCl_2$ in the 2nd gelling step is used, the Ba/Ca ratio in hydrogel equals to 1/3 and the absolute concentration of calcium ions is decreased by 25% (a decrease in ratio of calcium ions to uronic acid residues from 0.41:1 to 0.31:1). This indicates that barium ions create new gelling junctions (MM) as well as substitute calcium ions from GG and MG gelling junctions. The equilibration of hydrogels during storage again leads to decreasing the absolute concentrations of gelling ions and maintaining the Ba/Ca ratio at around 1/3. The quantitative data presented in this work correspond to recent work by Mørch et al. (2012) related to in vitro and in vivo studies on binding and leakage of barium from alginate beads, where the preferential binding of barium to calcium ions in hydrogel compared to the gelling solution was reported.

Fig. 4b reveals the effect of time used for application of the 2nd gelling step for zero (absence of the 2nd gelling step), 10, 20, 40 and 60 min gelled by 1 mM $BaCl_2$ and 50 mM of $CaCl_2$. Note that the gelling times of 10 and 20 min demonstrate repeated experiments to those shown in Fig. 4b (for gelling components 1 mM $BaCl_2$ and 50 mM $CaCl_2$) and Fig. 3a (2 wt.% sodium alginate), where tolerable differences were observed in dimensional stability and Young's modulus values. The ν_{exp} values are not available for all samples in this data set but it is safe to assume, based on the rest of data in Table 1, that the gelling step by $CaCl_2$ aerosol leads to saturation conditions. The time of 10 min used for the 2nd gelling step gives only minor syneresis (~2%) and does not cause a major difference in weight change of hydrogel after storage (similar to hydrogel prepared in the absence of the 2nd gelling step). Only at higher gelling times ≥20 min, the 2nd gelling step leads to syneresis of ~4%. The increased gelling time for the 2nd gelling step contributes to a minor increase in Young's modulus after both the 2nd gelling and storage steps. This data suggests that lower barium content in the 2nd gelling solution does not significantly influence properties of planar alginate hydrogels prepared in this work. Consequently, higher barium content in the 2nd gelling solution is needed to effectively tune dimensional stability and mechanical properties of alginate hydrogels prepared in this work.

3.3. Dimensional stability of alginate beads

Our objective is to conclusively elucidate whether the low weight changes seen in our experiments depicted in Figs. 2 through 4 should be ascribed to the way of gelling, when alginate solution is exposed to the aerosol of gelling solution, or it simply reflects the applied gelling conditions and type of sodium alginate. A number of papers deal with syneresis of alginate hydrogels prepared by both internal (Draget et al., 1990, 2001; Kuo & Ma, 2001, 2007; Mørch, Holtan, Donati, Strand, & Skjåk-Bræk, 2008; Quong et al., 1998) and external (Chin, Khattak, Bhatia, & Roberts, 2008; Nunamaker et al., 2011; Quong et al., 1998) gelling. The syneresis values are usually much higher, in the range of several tens of percent, than those obtained in our work. On the other hand, the close-to zero syneresis was reported for both externally (Junter & Vinet, 2009) and internally (Draget et al., 1990) gelled alginate hydrogels depending on the gelling conditions. Since the level of weight change (syneresis and swelling) depends on molecular weight and chemical structure of sodium alginate as well as on the gelling conditions (time of gelling and composition of gelling and storing solutions) (Donati & Paoletti, 2009), we decided to perform a moderate screening and determine the level of weight change for hydrogels made of sodium alginates differing in molecular weight, chemical composition and concentration, which were prepared as hydrogel beads by external gelling.

The principal data are contained in Table 3. For high G-content alginates and at 3 wt.% of sodium alginate concentration, the weight change is pronounced toward either strong syneresis (24%) or strong swelling (35%) for the highest and the lowest molecular weight alginates, respectively. For the medium value of molecular weight, i.e. Protanal LF 10/60 used in this work for preparation of planar alginate hydrogels, the syneresis value is around 5%. This value corresponds with the weight change values determined for planar alginate hydrogels shown in Figs. 2 through 4. A more pronounced syneresis is associated with both the low G-content and the lower sodium alginate concentration. Also for the low G-content sodium alginates, a higher molecular weight and lower

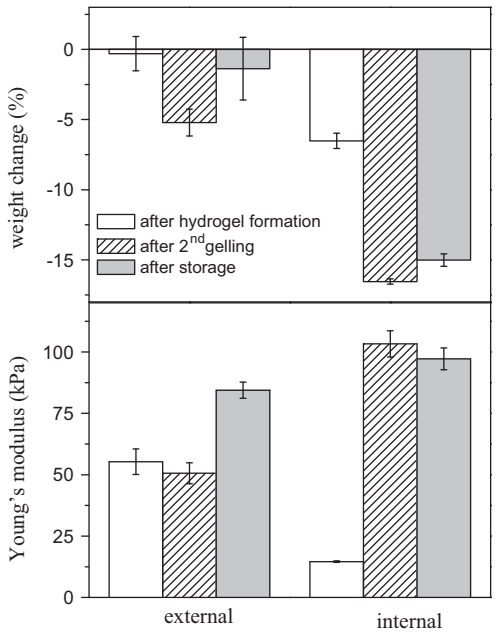

Fig. 5. Comparison between externally and internally gelled alginate hydrogels with weight changed determined after each step and Young's modulus for hydrogels after storage step. Conditions for externally gelled hydrogel: 2 wt.% sodium alginate, 2.5 wt.% $CaCl_2$, 2.5 min exposure time to aerosol. Conditions for internally gelled hydrogels: 2 wt.% sodium alginate, 20 mM $CaCO_3$, 60 mM GDL, 24 h, 6 °C. The 2nd gelling step and storage conditions were identical for both types of hydrogels: 20 min in 1 mM $BaCl_2$, 50 mM $BaCl_2$ and 0.2 M NaCl followed by storage for 24 h at 6 °C.

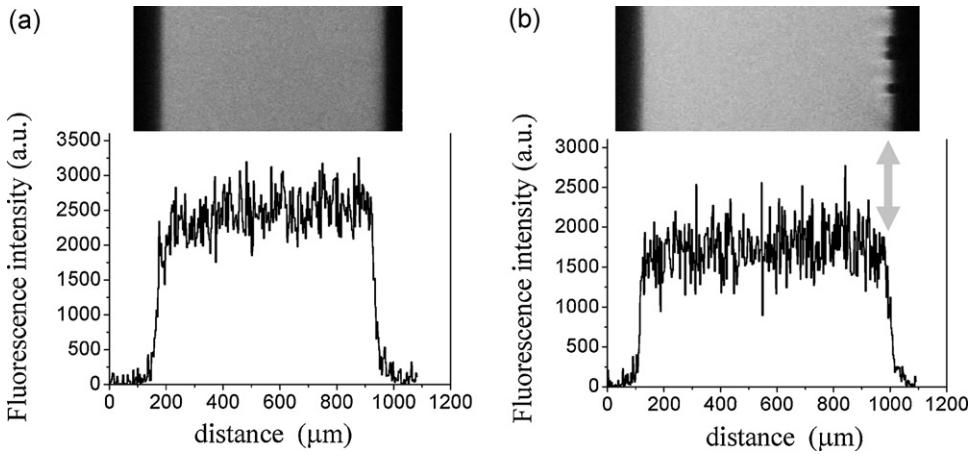

Fig. 6. Depth-profiles of fluorescently labeled alginate chains and associated fluorescence intensities determined by CLSM for internally (a) and externally (b) gelled alginate hydrogels. Images were rotated by 90° clockwise. An arrow for externally gelled hydrogel points at the surface of alginate solution exposed to the aerosol of gelling solution. Conditions for externally gelled hydrogel: 2 wt.% sodium alginate, 2.5 wt.% $CaCl_2$, 2.5 min exposure time to aerosol. Conditions for internally gelled hydrogels: 2 wt.% sodium alginate, 20 mM $CaCO_3$, 60 mM GDL, 24 h, 6 °C. The 2nd gelling step and storage conditions were identical for both types of hydrogels: 20 min in 1 mM $BaCl_2$, 50 mM $BaCl_2$ and 0.2 M NaCl followed by storage for 24 h at 6 °C.

alginate concentration result in a higher syneresis. These results follow the experience described in literature on effects of molecular weight, gelling conditions and chemical composition of sodium alginates on syneresis and, importantly, support our results on the level of syneresis for planar hydrogels prepared by external gelling. Hence, the low degree of syneresis observed in our experiments for planar alginate hydrogels should not be ascribed to using the aerosol of gelling solution but rather to selection of sodium alginate in terms of its molecular weight and chemical composition.

3.4. Comparison between externally and internally gelled planar alginate hydrogels

The aim of this manuscript is to demonstrate that alginate hydrogels prepared by employing the external gelling method with controlled exposure of alginate solution to aerosol of gelling solution are similar to those prepared by standard protocols, represented, for example, by the internal gelling method. Fig. 5 reveals the comparison of dimensional stability and Young's modulus for alginate hydrogels prepared using initial 2 wt.% sodium alginate solutions under the gelling conditions of similar ν_{theor} (0.39:1 and 0.19:1 for external and internal gelling, respectively). These hydrogels differ mainly in the first gelling step, i.e., $CaCl_2$ aerosol and release of gelling calcium ions from $CaCO_3$, while the 2nd gelling and storage steps are identical. After the gelling step, the internally gelled hydrogel exhibits syneresis of 6%, whereas no dimensional change was obtained for the externally gelled hydrogel. Since syneresis is highly sensitive to a number of parameters, it is difficult to formulate the generalized correlation between external vs. internal gelling and the weight change of hydrogel. For internally gelled hydrogel the higher syneresis after the gelling step is transferred to higher syneresis after the 2nd gelling and storage steps. The Young's modulus values differ significantly after the gelling step with higher value for externally (55 kPa) than internally (15 kPa) gelled alginate hydrogels, which may be related to lower value of ν_{theor} in the latter case. On the other hand, the 2nd gelling step strongly increases Young's modulus to 100 kPa for internally gelled hydrogel, while no major difference is introduced by the 2nd gelling step to externally gelled hydrogel (as was seen in Figs. 2 through 4 for low content of barium ions in the 2nd gelling solution). Young's modulus values become very close to each other (~90 kPa) after equilibration of both alginate hydrogels in the storage solution. Differences seen in Fig. 5 are interesting, nevertheless, their understanding is beyond the scope of this paper.

The final data concerns the spatial distribution of fluorescently labeled alginate chains in hydrogels, which dimensional stability and Young's modulus are characterized in Fig. 5. Fig. 6 reveals the homogeneous profile in alginate concentration for both internally and externally gelled hydrogels, which were determined by CLSM after the gelling step. While for internally gelled hydrogel the homogeneous distribution of alginate chains is expected (Draget et al., 1990), the absence of inhomogeneous spatial distribution of alginate chains for externally gelled hydrogel was surprising. The concentration of gelling ions supplied by $CaCl_2$ aerosol is very low of around 1 $\mu mol\,cm^{-2}\,s^{-1}$. Since the inhomogeneous spatial distribution of alginate chains is promoted by decreased rate of gelling (Skjåk-Bræk et al., 1989; Thu et al., 2000), a significantly higher alginate concentration was expected to be observed at the hydrogel surface exposed to the aerosol of gelling ions than in the hydrogel toward the bottom of the mold. Nevertheless, the inhomogeneous profile of alginate was not observed and, thus, the presence of non-gelling sodium ions in alginate solution and $CaCl_2$ aerosol appears to be the crucial factor responsible for a homogeneous spatial distribution of alginate chains (Fig. 6b). This agrees with classical work by Skjåk-Bræk et al. (1989) showing a homogeneous profile of alginate in externally gelled hydrogels in the presence of 0.2 M NaCl in gelling solution that is a comparable concentration to that used in our work (0.16 M NaCl). In addition, our preliminary data (manuscript in preparation) indicate that the inhomogeneous alginate profile can be obtained in the absence of non-gelling ions.

4. Conclusion

We designed a relatively simple setup for preparation of externally gelled planar alginate hydrogels with slow exposure of sodium alginate solution to gelling solution applied as aerosol. The results demonstrate that conventional and well-described principles in the field of alginate hydrogels dominate also the method for preparation of planar alginate hydrogels presented in this work. This method provides the opportunity for preparation of planar alginate hydrogels by external gelling with the applicability for immobilization of biological species. The principal sodium alginate used in this work was high G-content and medium molecular weight Protanal LF 10/60, for which the Young's modulus, dimensional stability and content of gelling ions were determined in a

broad range of gelling conditions. These conditions are obviously suitable for other types of sodium alginate, which are expected to provide a wider range of properties of alginate hydrogels. In addition to calcium gelling ions applied as an aerosol, the interesting data may be obtained, in terms of mechanical properties, chemical stability and dimensional stability, by combination of calcium and barium gelling ions that would also shorten the time and number of steps needed for preparation of stable hydrogels.

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