JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 559–565

A new process for cell microencapsulation and other biomaterial applications: Thermal gelation and chemical cross-linking in 'tandem'

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The very rapid gelation of a cell- or biomolecule-containing solution is at the basis of most processes employed in microencapsulation. Adequately quick ('instantaneous') gelation kinetics are provided by a number of phenomena based on physical association.

On the other hand, physical gels are inherently reversible structures, which can be solubilized or disrupted in response to often poorly controllable phenomena in the environment of application, such as dilution, changes in temperature, ion strength and composition, pH, or other physical or chemical parameters.

Chemically cross-linked hydrogels would have therefore significant advantages in terms of stability and end-properties; however, the time required for chemical reactions to produce a chemically cross-linked material is in a more general case hardly compatible with microencapsulation processes.

In a recent study of our laboratory we have proposed a new approach for providing both quick gelation kinetics and good stability, by simply combining the rapid kinetics of a physical hardening phenomenon with a slower chemical curing; the former process is thus responsible of the morphogenesis of the material, while the latter develops its end-properties.

1. Introduction

1.1. Microencapsulation and related machinery

The encapsulation of active principles, such as living cells or biomolecules, aims primarily to provide the means for protecting them from a possibly aggressive (bio)environment.

Specifically for cells, the encapsulating material is rather a membrane or a capsular matrix [1] that allows nutrients, wastes, and therapeutic products to permeate and diffuse freely, but acts as a barrier to dangerous components, such as the mediators of the immune system of a host organism.

In this area, considerable advantages are presented by constructs featuring sub-mm dimensions, as opposed to bigger artefacts: for instance, microencapsulation devices are easier to implant, more resistant to breakage, produce less cell necrosis due to diffusion limitation, and often cause less non-specific fibrotic overgrowth [2, 3]. These devices are generally produced in form of spherical beads or hollow capsules, a variety of different materials, mostly polymeric hydrogels, have been employed for their preparation [3].

We wish to focus our attention on the techniques used for processing the encapsulating materials; in general, they must satisfy strict requirements in terms of toxicity and formation kinetics. For example, the materials must be produced in the presence of cells; therefore non-toxic precursors must be mixed together with cells and then harden without harming them through chemical (toxic by-products) or physical action (heat production, mechanical or osmotic damage). In addition, the transition from solution to solid matrix must be rapid, for preserving the same dispersed state of cells as in solution, and also allowing for a possibly continuous and up-scalable processing.

Physical gelation processes, such as polyelectrolyte complexation or thermally-induced gelation, have traditionally been preferred for this purpose, while, with a few exceptions [4, 5] the slow kinetics and the toxicity problems of chemical curing reactions have generally hindered their application in microencapsulation.

Among physically gelling materials, calcium alginate, due to its easy and cell-friendly processing (calcium-induced ionotropic gelation) and its natural

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origin, has since decades represented the standard hydrogel material for cell encapsulation and dedicated machineries have been developed for its processing [6–10], with the aim to provide in a reproducible and up-scalable fashion objects with very narrow dispersity in size. In most cases they are generated from 'monodisperse' droplets of a water solution of sodium alginate, which instantaneously harden when exposed to an aqueous environment containing calcium or other multivalent cations. The methods used for droplets generation vary from case to case; we have focused our work on to the jet break-up technique, where a laminar flow of a solution is converted in a succession of identical droplets by the action of an ultrasound vibrating nozzle.

1.2. The concept of a 'tandem' process

A feature shared by most processes for the formation of physical gels, including calcium alginate-based materials, is the substantial overlap between morphogenesis of the material and development of its end-properties (mechanical and transport properties). The 'instantaneous' and initially interfacial complexation of calcium by alginate chains—more specifically by their G sequences—determines both sol-gel transition and a substantial part of the final elastic modulus.

We have developed an alternative concept of 'tandem' process, where these two actions are ideally decoupled into completely separate kinetic domains: a rapid sol-gel process followed by a change in mechanical (and transport) properties based on other transitions.

By combining a quick physical gelation with a slower chemical cross-linking (Fig. 1) it is therefore possible, in principle, to obtain a solid material within the very short time scale typical of physical processes. It naturally follows that such material can be processed by employing machineries used for physical gels (e.g. calcium alginate), which are generally based on quick solidification kinetics.

On the other hand, after an appropriate incubation time, the material is rendered irreversible and mechanically stronger by covalent cross-linking.

Poly(ethylene glycol)-poly(propylene glycol) triblock copolymers, known as Pluronics or Poloxamers when linear, as Tetronics or Poloxamines when 4-armed, show a rich phase behaviour in water, which, in dependence on polymer composition and concentration, can also present a reverse gelation transition in water solution [11].

Since long, it has been demonstrated this transition to be due to the formation of a colloidal 'crystal' with cubic symmetry composed of spherical micelles [12, 13], is almost athermic [14] and, within an appropriate range of concentrations and polymer compositions, can be caused to happen virtually at any physiologically acceptable temperature (Fig. 2). These features and the

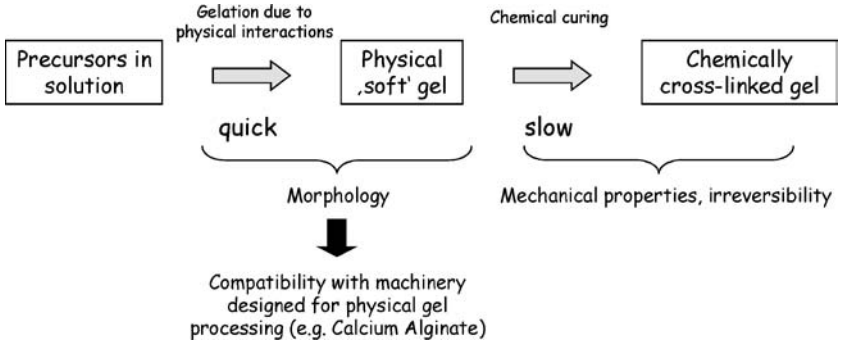

Figure 1 Schematic view of a 'tandem' process. The physical gelation determines a quick hardening process; the material is then cured with a slower kinetics.

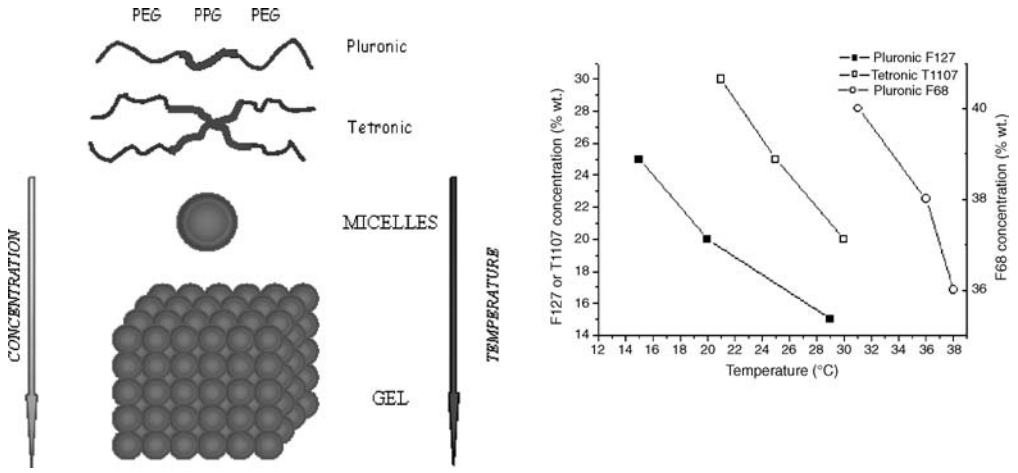

Figure 2 Above: pictorial representation of Pluronic and Tetronic blocky structures (PPG: poly(propylene glycol), PEG: poly(ethylene glycol)). Left: graphical summary of the colloidal 'crystallization' (gelation) of Pluronic micelles. Right: gel temperatures as a function of concentration for three Pluronic or Tetronic systems (F127: MW = 12,600 g/mol, 70% wt. PEG; F68: MW = 8,400, 80% wt. PEG; T1107: MW = 15,000, 70% wt. PEG).

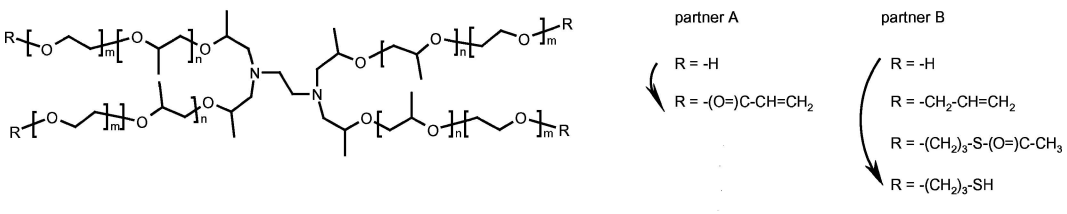

Figure 3 Structure of a Tetronic polymer (left) and brief description of the path for the synthesis of the two reaction partners in Michael-type addition cross-linking (right). Tetronic acrylate was prepared by direct conversion of terminal alcohols into acrylic esters. In the conversion into thiols, on the other hand, the alcohols were first transformed into allyl ethers and then into thioacetic esters (radical addition of thioacetic acid onto the double bonds); the last groups were *in situ* hydrolyzed to thiols.

well-known biocompatibility of poly(ethylene glycol) materials make therefore of this gelation an ideal candidate as the first gelation step in a 'tandem' scheme.

Pluronic and Tetronic structures, besides the thermal gelation properties, offer also the possibility of a functionalization at their termini with reactive groups, which can be conveniently used for the second step of covalent cross-linking.

In our previous studies, Michael-type addition of thiols onto electron-poor olefins [15], such as acrylates, has been extensively employed for the synthesis of PEG-based cross-linked hydrogels, even in presence of cells [16]. Due to its favourable properties—biocompatibility, negligible interference with biomolecules' reactivity and easy preparation of the reaction partners—we have employed Michael-type addition also as the curing reaction in a 'tandem' scheme. 'Tandem' gels have therefore been prepared by mixing two mutually reactive partners based first on the linear Pluronic structure [17], then on the tetra-armed Tetronics one [18]; in the latter case the nucleophilic reaction partners (thiols) were generated *in situ*, in order to avoid their uncontrolled dimerization to disulfides upon storage (Fig. 3).

2. Materials and methods

2.1. Synthesis of precursors

Tetronic T 1107 (MW = 15000, 70% wt. PEG) was kindly supplied by BASF (BASF AG, Ludwigshafen, Germany). Tetronic derivatives in form of tetra-acrylate (T1107 ACR) and tetra-thioacetate (T1107 TAC) were synthesized and purified according to a previously reported procedure [18].

2.2. Preparation of the Tetronic reacting solution

1 g of solid T1107 TAC was dispersed in 3.0 ml of a 0.2 M NaOH solution and the mixture was left in an ice bath (0 °C) for 90 min under stirring. The cold polymer solution was buffered with 1.6 ml of 0.2 M HCl and with 40 μl of 0.2 M acetic acid. 1 g of solid T1107ACR was added and dissolved under stirring at 0 °C for 75 min.

2.3. Encapsulation process

2.3.1. Machinery

A Inotech IE-50R encapsulator (Inotech Encapsulation AG, Dottikon, Switzerland) was employed for the production of beads or capsules.

Cold (5 °C) polymer solutions were pushed through a capillary to an ultrasound-vibrating nozzle nozzle. The laminar jet was forced to break-up into equally sized droplets by the nozzle vibration at controlled frequency and amplitude. The chain of falling droplets gelled immediately in a stirred hardening bath.

A circulating cooling system was added to refrigerate the capillary and the nozzle at roughly 1–3 °C, in order to provide temperature control and prevent undesired gelation before the jet break-up.

When the encapsulator was used for the production of capsules, an core material was used for producing, by the use of a double nozzle, a laminar coaxial flow.

2.3.2. Production of beads

A nozzle with an internal diameter of 200 μm was used for the preparation of hydrogel beads.

A stable jet break-up was obtained by using an oscillation frequency of 800 Hz and a flow rate of 5 ml/min. The droplets were hardened in 200 ml of a bath of 10 mM phosphate buffered saline (PBS) at pH 7.4, stirred at 37 °C and added of Pluronic F127 (10% wt.) and of Tween 20 (0.1% wt.) (Sigma, Buchs, Switzerland), (10 mM PBS pH 7.4 was prepared from 0.2 g KCl, 0.2 g KH_2PO_4, 2.14 g $Na_2HPO_4 \cdot 7H_2O$ and 8 g NaCl dissolved in 1 L of distilled water).

During the process the hardening bath was covered by a layer of n-hexane (approximately 2 ml/dm^2). When beads were stable enough for manipulation and hexane was completely evaporated (45 min), the bath mixture was transferred in a glass bottle and there incubated for additional 12 h. Spherically shaped beads with an average diameter of 600 μm were finally obtained.

2.3.3. Production of capsules

A concentric double nozzle with an external diameter of 400 μm and an internal core diameter of 300 μm was used.

A solution made of 5 ml of 10 mM PBS pH 7.4 with 10% vol. of Fetal Bovinum Serum (FBS) (Gibco BRL/Life Technologies AG, Basel, Switzerland) and 2% wt. of dextran MW 2,000,000 (Fluka, Buchs, Switzerland) was used as core liquid, extruded at a flow rate of 3 ml/min. The Tetronic reacting solution was used as the shell-forming liquid, extruded at a flow rate of 14 ml/min. The nozzle vibration frequency was lowered to 500 ÷ 550 Hz; the bath composition and the hardening procedure was the same as in the production of beads.

Spherically shaped capsules, external diameter 900 μm with a core diameter of 200 μm, were finally obtained.

2.3.4. CHO cells preparation and encapsulation

Chinese hamster ovary cells (CHO-K1, American Type Culture Collection (ATCC; Rockville, MD), CCL-121) were cultured in 75 cm^2 tissue culture flask containing 10 ml of CHO culture medium. CHO cell culture medium was prepared from FMX-8 Medium (Cell Culture Technologies GmbH, Zurich, Switzerland) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories GmbH, Linz, Austria) and 1% penicillin-streptomycin solution (Sigma, Buchs, Switzerland).

Cells were harvested before reaching confluence using 3 ml trypsin solution (Gibco BRL/Life Technologies AG, Basel, Switzerland), re-suspended in PBS-washing buffer to have a cell concentration of 20×10^6 cell per ml.

10 ml of core solution, composed by PBS 10 mM pH 7.4, 2% wt. of dextran MW 2,000,000 and 10% vol of FBS, was filtered with a 0.2 μm filter and mixed with 500 μl of cell suspension, to have a final cell concentration of approximately 2×10^6 cell per ml.

The suspension was then transferred to a 20 cc syringe which was then connected to an encapsulator located under a sterile hood.

The hardening bath (200 ml of Pluronic F127 10% wt. in 10 mM PBS pH 7.4.) was previously filtered with a 0.2 μm filter before the warming up at 37 °C and the addition of n-hexane.

The capsules were produced according to the procedure described above.

After 45 min of hardening the capsules were filtrated, re-suspended in CHO culture medium and incubated under normal tissue culture conditions.

2.4. Moulding

0.5 g of low melting point agarose (Tgel = 29–33 °C for 1.5% wt. solution, purchased from Fluka) was dissolved in 50 ml of PBS 10 mM pH d 7.4 (~1% wt. agarose solution) heating the suspension at boiling point in a microwave oven for a minute. The hot agarose solution was cast in a mould constituted by a cylindrical plastic container displaying a silicone tube in the centre; the tube was fitted with and supported by a sterilized metallic needle; needle, tube and cylinder are all coaxial. After cooling the solution to room temperature, the silicone tube was removed, leaving a hollow cylindrical crown around the needle, which was filled with the cold Tetronic reacting solution. After incubation at 37 °C for 12 h, the agarose gel and the needle were gently removed from the tandem gel, which preserved the original shape of the silicon tube.

2.5. Live/dead cytotoxicity tests

Human foreskin fibroblasts (HFF) were cultured on tissue culture polystyrene (TCPS) in 96-multiwell culture dishes until confluence was achieved. The Tetronic reacting solution was then added until complete coverage of the cell layer and incubated for 5 min until a physical gel layer was achieved. Then 100 μL of cell culture medium were added to each well. The cells were incubated for an additional 24 h and then stained (without removing the gel layer) with fluorescein diacetate and ethidium bromide (Fluka, Buchs, Switzerland) to determine the number of live and dead cells, respectively. Cell counting was accomplished by monitoring the resulting fluorescence.

2.6. Swelling measurements

A 1 mL syringe was filled with cold Tetronic reacting solution and incubated at 37 °C for 15 h. A cylinder-shaped gel was then extracted from the syringe and cut in uniform 0.3 ml portions. Each portion was incubated in 10 mM PBS pH 7.4 at a temperature of 37, 20 and 5 °C respectively. The time behaviour of the gel swelling was monitored and expressed through the ratio $[w(t) - w(0)]/w(0)$, where $w(t)$ is the gel weight at time t and $w(0)$ is the gel weight before the incubation.

2.7. Mechanical tests

A ZWICK Z100 tensile testing machine was used to perform tensile stress measurements on tandem gel tubes produced with the method described above. Pieces of gel tube having inner diameter 2 mm and outer diameter of 3 mm where connected to the machine so that the initial length of the sample was 10 mm at a minimal normal force of 0.01 N. The applied axial load and the corresponding deformation were then measured up to material fracture.

3. Results and discussion

3.1. Development of a 'tandem' system and its application in microencapsulation

In the frame of a 'tandem' process, we have combined in polymer structures of known biocompatibility (Pluronics and Tetronics) a physical gelation mechanism (colloidal 'crystallization') with a biocompatible chemical cross-linking (Michael-type addition). As a preliminary step for the application, the compatibility of the products of this 'tandem' process with microencapsulation processes was to be characterized in terms of gelation kinetics and effectiveness, mechanical and transport properties and cell compatibility.

Under appropriate conditions of concentration (30% wt.) and temperature differences (5 → 37 °C), water solutions of Michael-type reactive Tetronics (T1107) could provide both a rapid gelation kinetics (<0.1 s) and a slower chemical cross-linking. The chemical curing considerably hardens the material and can therefore be followed by shear rheometry; this has shown that the cross-linking proceeds in the physical gel phase at a rate and with a final yield comparable to those observed for cross-linking from solution (Fig. 4) [17, 18].

The elastic modulus (G') of the 'tandem' gels, considerably higher than that of similar cross-linked gels from solutions or of physical gels, could be easily modulated by varying the functionality of the reactive

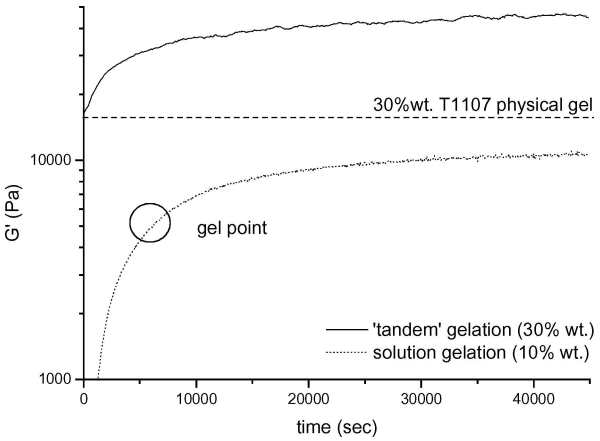

Figure 4 Time behaviour of the elastic (G') shear modulus in the gelation of a 10% wt. and 30% wt. water solutions of Michael-type reactive Tetronic T1107, at constant temperature of 37 °C. At low concentration (10% wt) the solution does not present a physical gelation and the elastic modulus increases only by the chemical cross-linking mechanism. At higher concentration (30% wt) a fast physical gelation can occur increasing the elastic modulus immediately. Then cross-linking proceeds in the physical gel phase at a rate and with a final yield comparable to those observed at 10% wt.

Tetronics and thus the final cross-link density. Finally, it was shown that the 'tandem' gels exhibited a size-dependent permeability; the recorded cut-off, in the range of 3–6 nm, is ideal for the exclusion of high MW macromolecules, such as those acting as immune system mediators (immunoglobulins, complement).

The 'tandem' process performed in the presence of living cells showed negligible toxicity; the gelation of reactive Tetronic solutions on the top of monolayers of cells (Human Foreskin Fibroblasts) preserved quantitatively the cell viability (Fig. 5).

The 'tandem' system was finally applied to the production of spherical beads and hollow (liquid core) capsules, employing encapsulators based on the jet break-up technology and originally designed for calcium alginate processing [18, 19]. In this technique, the solution of interest is converted by a ultrasound vibrating nozzle to monodisperse liquid droplets; falling by gravity in a hardening bath, they are there transformed into hard spheres. Through a precise modulation of the viscosity of the precursors solution [18] and the surface tension of the bath [19], 'tandem' spherical beads with controlled diameter were finally obtained (Fig. 6(A)). In the jet break-up technique, it is possible to employ engineered nozzle geometries; for example, double nozzles, whose concentric structure allows for the production of core-shell structures, such as liquid-core capsules if the gelling material is present only in the shell. The 'tandem' process could be applied also for the production of these capsules, possibly containing viable cellular material in the internal cavity (Fig. 6(B)).

Figure 5 Live/dead cytotoxicity assay for human foreskin fibroblasts (HFF) covered with a layer of Tetronic tandem gel. *Left.* Cells were cultured on tissue culture polystyrene (TCPS) until confluence was achieved. The Tetronic reacting solution was then added until complete coverage of the cell layer. After the instantaneous physical gelation, a fresh warm culture medium was added and cells were cultured in this environment for an additional 24 hr. *Right.* Cell counting was accomplished by staining with fluorescein diacetate and ethidium bromide (without removing the gel layer) and monitoring the resulting green/red fluorescence. The negligible cytotoxicity of the tandem process was demonstrated by the high number of green (alive) spread cells compared with the low number of red-stained nuclei (dead cells).

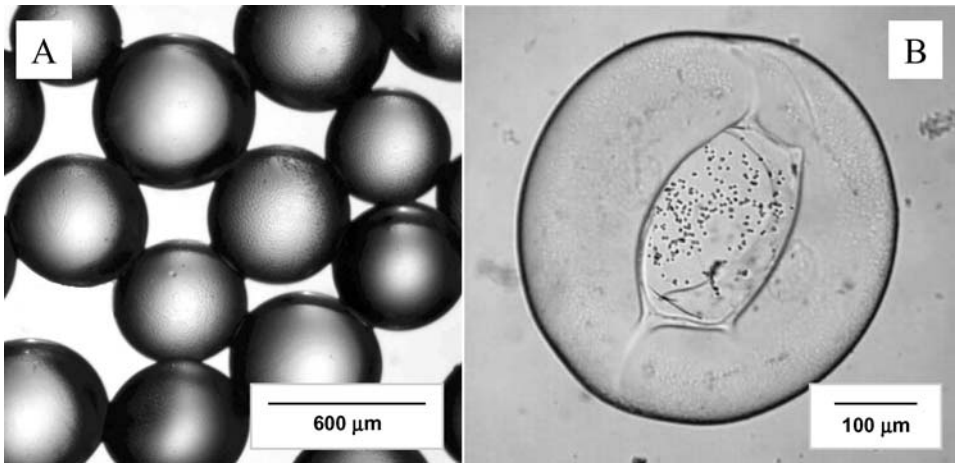

Figure 6 (A) Collection of regularly shaped Tetronic gel micro-beads (diameter 500 ÷ 600 μm) photographed after removal of the suspending liquid bath. (B) Tetronic gel capsule (average shell diameter 900 μm) loaded with viable CHO cells. The capsules are produced employing the double nozzle technique so that the gel material is present only in the shell and cells are confined in the liquid core of the particle.

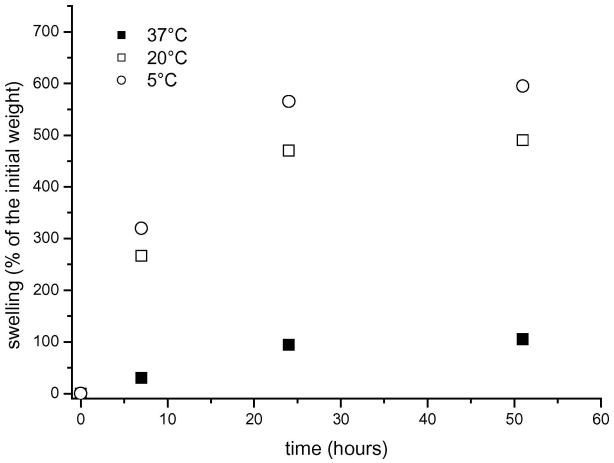

Figure 7 Time dependence of swelling for Tetronic tandem gels incubated under 10 mM PBS pH 7.4 at different temperatures. At 37 °C the micellar cubic structure of the physical gelation may be preserved after the chemical crosslinking, resulting in a decreased internal osmotic pressure and a subsequent limited swelling effect. Below the critical micellar temperature the micellar structure is lost and the swelling behaviour is more similar to a common PEG gel.

3.2. Other biomaterial applications of the 'tandem' gelation

Differently from hydrogels obtained through chemical cross-linking of low molecular weight compounds or polymers in solution, the 'tandem' gels are characterized by limited swelling in water.

This is likely to ascribe to the fact that the precursor of the cross-linked gel is a gel itself, which consists of colloidal objects (polymeric micelles); the reduction in mobility of these components during chemical cross-linking is therefore negligible, resulting in a moderate increase in the internal osmotic pressure.

As a proof of it, it is easy to see that the swelling extent of 'tandem' gels strongly depends on temperature; in particular, the swelling, and therefore the internal osmotic pressure after cross-linking, is maximal at temperatures where the precursors would be in form of isolated, freely moving micelles (Fig. 7), that is where one has the highest reduction in mobility. Analogous PEG hydrogels, where the precursors are even more mobile macromolecules in solution, at the same weight concentration and similar cross-linking density show even higher swelling (even above 1000%).

The low swelling of the 'tandem' gels when exposed to an external water environment is specifically advantageous, because it provides a minimal osmotic shock to the cells exposed to the gelation process. On the other hand, it can be also conveniently employed for the production of artefacts where dimensional stability *in situ* and *in vivo* is an issue.

We have considered the preparation of hydrogel nerve guides, potentially useful for peripheral nerve regeneration, as an example of this concept. The most commonly used materials for this purpose are either non-porous, non-degradable elastomers (such as silicones), or porous and hydrolytically degradable polyesters (of the poly(lactic or glycolic acid) family), which are largely advantageous in that they do not require the implant to removed. Despite advantages in biocompatibility and transport properties, hydrogels have seldom been used for such applications and to our knowledge never in a degradable formulation [20, 21]. We believe one of the main reasons for this lack of use to be the poor dimensional control of hydrogels upon implantation, which is problematic for suturing and for the restriction in the tube lumen, and is combined to often unsatisfactory mechanical properties.

On the other hand, 'tandem' gels present both low swelling in a water-rich environment and interesting values of elastic modulus (up to 100 kPa), while the presence of hydrolytically labile ester groups ensures the degradability of the whole constructs [18].

By taking advantage of these features, we have thus developed a method for moulding them in virtually any desired shape. In this method a cold precursor solution is introduced into a mould, which is prepared by solidifying another hydrogel material (agarose) around a model of the desired artefact. The use of water-rich materials as moulds can have a significant advantage for hydrogel processing, because the hydrogel precursors can spread well on the mould surfaces (very low contact angles). This reduces the pressure necessary for the application of solution in small size opening and assures a good reproduction of the contour. The filled

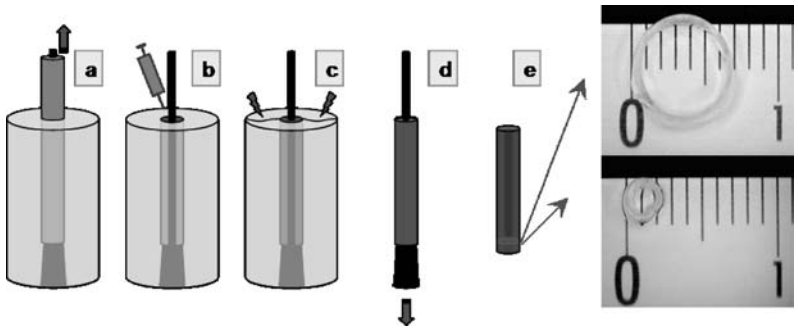

Figure 8 Schematic representation of 'tandem' gel moulding. A hot agarose-water solution (1% wt.) was gelled by cooling it at room temperature in a mould constituted by a cylindrical plastic container whose centre is occupied by a templating silicone tube that contains a sterilized metallic support. The silicone tube was removed (A) and the space between the agarose gel and the metallic support was filled with a cold (5 °C) Tetronic reacting solution (B). After incubation at 37 °C for 12 h the agarose gel (C) and the metallic support (D) were gently removed from the tandem gel, which templates its shape on that of the silicone tube (E). By varying the diameter of the silicone tube and the metallic support, it is possible to produce tubes with any desired diameter and thickness (right pictures).

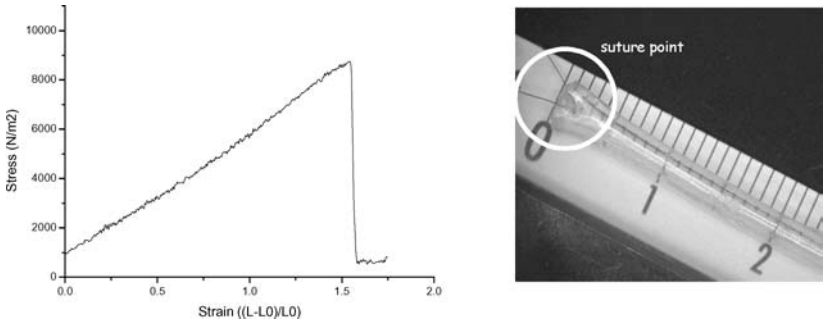

Figure 9 Left: stress-strain curve for the tensile deformation of a 'tandem' gel tube of initial length = 1 cm, i.d. = 2 mm, o.d. = 3 mm. The tube shows a completely elastic response until rupture occurs, at an ultimate strain of more than 150%. This compliance is particularly indicated for applications, such as nerve guides, where the implant is required to continuously extend under axial load. Right: picture of a 'tandem' gel tube with a suture point (polypropylene thread) at one of the extremities; the 'tandem' gels offer a good resistance to fracture propagation when tensile forces are applied to suture points.

mould is then brought at higher temperature, causing the rapid solidification of the 'tandem' gel, which later cross-links. If simple chemical cross-linking processes are used, the higher increase in osmotic pressure generally produces a swelling in the hydrogel, which takes water up from the mould and looses its original shape (Fig. 8).

Hydrogel tubes with controlled internal and external diameters and lengths were readily obtained with this method; they were shown to be elastic and compliant (Fig. 9, left), amenable to sutures (Fig. 9, right) and hydrolytically degradable, achieving complete dissolution in a period variable from 4 to 10 weeks.

4. Conclusions

Chemically cross-linked hydrogels can be prepared through hardening processes characterized by kinetics much quicker than that of chemical curing. This is possible when a secondary and rapid gelation transition is employed for the morphogenesis of the material.

With an appropriate choice of the gelation mechanisms and of the polymer structures, we have shown that this approach can be successfully adapted to applications in cell encapsulation or biomaterial design.

In particular, the 'tandem' gels based on Tetronic thermal gelation and Michael-type addition cross-linking have shown excellent biocompatibility, interesting mechanical and transport properties and very low swelling in water environment.

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Received 11 August
and accepted 4 November 2004