LETTERS

Multi-membrane hydrogels

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Polysaccharide-based hydrogels are useful for numerous applications, from food[1] and cosmetic processing to drug delivery and tissue engineering[2,3]. The formation of hydrogels from polyelectrolyte solutions is complex, involving a variety of molecular interactions. The physical gelation of polysaccharides can be achieved by balancing solvophobic and solvophilic interactions[4]. Polymer chain reorganization can be obtained by solvent exchange, one of the processing routes forming a simple hydrogel assembly. Nevertheless, many studies on hydrogel formation are empirical with a limited understanding of the mechanisms involved, delaying the processing of more complex structures. Here we use a multi-step interrupted gelation process in controlled physico-chemical conditions to generate complex hydrogels with multi-membrane 'onion-like' architectures. Our approach greatly simplifies the processing of gels with complex shapes and a multi-membrane organization. In contrast with existing assemblies described in the literature, our method allows the formation of free 'inter-membrane' spaces well suited for cell or drug introduction. These architectures, potentially useful in biomedical applications, open interesting perspectives by taking advantage of tailor-made three-dimensional multi-membrane tubular or spherical structures.

Here we report on the processing of multi-membrane structured materials (Fig. 1) based on physical hydrogels of amphiphilic polymers obtained without external cross-linker. We have applied the approach to chitosan and alginate, but it could be generalized to numerous amphiphilic polyelectrolyte polymers. We mainly focused our work on chitosan, well known to be biodegradable[5], bioactive[6] and biocompatible[7]. Chitosan is an amphiphilic natural co-polymer in which the relative proportion of acetylated and de-acetylated residues plays an important role in the balance between hydrophilic and hydrophobic interactions[8–10]. In addition, chitosan is haemostatic[11], fungi- and bacterio-static[12,] and is known to be important in cell proliferation and tissue regeneration[13,14]. For the processing of physical hydrogels made only of chitosan and a solvent, we previously proposed methods based on a simple hydrogel assembly[4,15]. Knowledge of the gelation mechanism, including physico-chemical events involved during gelation and the molecular organization at various levels, was essential to understand the formation of our multi-membrane 'onion-like' structures. This gelation process is applicable to a variety of other amphiphilic polymers, especially natural polyelectrolytes such as alginates or hyaluronic acid.

An alcohol gel is easily formed from a chitosan solution in a 50/50 water/1,2-propanediol mixture by water evaporation at 55 °C. This is not limited to propanediol and has been performed with several other alcohols[15]. A key parameter for the gelation is to keep the initial polymer concentration above the critical concentration for chain entanglements. When water is almost fully removed, a gel is formed. Neutralization in a sodium hydroxide solution and subsequent washings in water yield a physical hydrogel that contains only water (over 95 wt%) and chitosan in the free amine form. Although evaporation induces a decrease in the charge density of the polymer chains, when gelation is fully achieved almost 40% NH_3^+ groups are still present[15] and have to be neutralized to favour inter-chain interactions allowing the formation of a stable hydrogel. Indeed, an alcohol gel in contact with water before neutralization rapidly converts into a hydro-alcoholic solution. Therefore we investigated the neutralization step using different concentrations of aqueous solutions of NaOH.

Figure 2a illustrates the macroscopic shrinkage of the hydrogel during neutralization and reveals that an increase in the concentration

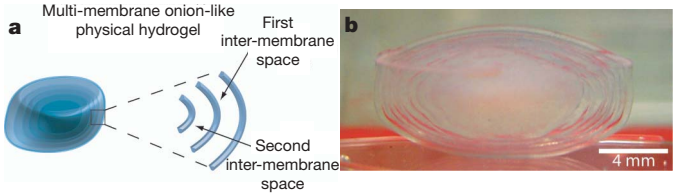

Figure 1 | Multi-membrane hydrogels. a, Schematic diagram of the multi-membrane onion-like structures; **b**, multi-membrane biomaterial with 'onion-like' structure based on chitosan.

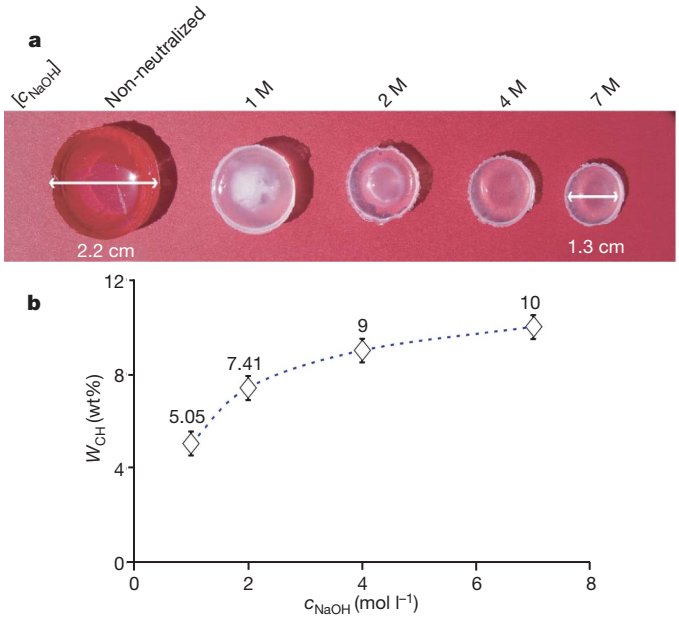

Figure 2 | Parameters influencing the polymer mass fraction of physical gels based on chitosan. a, Variation of hydrogel shrinkage during neutralization as a function of the concentration of sodium hydroxide. The initial polymer concentration in the non-neutralized alcohol gel is constant and close to 4.5wt% in each case. **b**, Evolution of the chitosan mass fraction in the gel (W_{CH}) at different steps of the hydrogel neutralization as a function of the NaOH concentration in the neutralization bath. Error bars represent ±1 standard deviation.

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of the neutralizing agent favours the gel depletion. Chitosan mass fractions in the hydrogels (W_{CH}) after neutralization for an initial alcohol gel with $W_{CH} \approx 4.5$ wt% are shown in Fig. 2b. The neutralization of NH_3^+ sites into NH_2 led to the disappearance of ionic repulsions between polymer chains so that physical cross-links corresponding to hydrogen bonding, hydrophobic interactions and crystallite formation were favoured. Volume changes in gels can also be driven by inter-chain interactions[16,17] and by pH variations for synthetic polyelectrolyte gels[18,19].

As previously discussed, this result can be interpreted by considering different contributions such as the change in the electrostatic potential during the neutralization inducing the formation of physical crosslinks, and ionic strength effects. The concentration of the neutralization agent (C_{NaOH}) also determines the kinetics of neutralization. At high C_{NaOH}, chains are rapidly and completely neutralized, and their condensation is maximum, leading to gels with a high physical cross-linking density. In contrast, for low C_{NaOH}, water diffusion within the alcohol gel must be considered, because it contributes to disturb hydrophobic interactions, influencing the final density of physical cross-links and thus W_{CH} of the neutralized hydrogel. Moreover, the presence of salts in a non-neutralized polyelectrolyte gel induces a screening of the electrostatic repulsions between polymer chains and should therefore favour physical junctions inside the gel. Additionally, the salting-out effect in amphiphilic polymer gels[20] could be considered. This effect corresponds to a displacement of water in the gel to form strongly hydrated ions (small electrolytes in solution), thus contributing to dehydrate polymer chains and increase hydrophobic inter-chain interactions, and increasing the density of cross-linking in the neutralized hydrogel. Nevertheless, the neutralization of an alcohol gel in a solution containing 1 M NaOH and 5 M NaCl only results in a slight increase (0.91%) in W_{CH} compared with the value obtained for an alcohol gel neutralized in the same conditions without NaCl. To summarize, different salt effects occur during neutralization but their contributions to the shrinkage of the gel are small compared with pure neutralization effects. The neutralization step seems complex, with several different phenomena acting simultaneously.

We used this mechanism of neutralization to generate multi-membrane onion-like architectures. As discussed above, the modification of the balance between hydrophobic and hydrophilic interactions induces a contraction of the neutralized gel (Fig. 3a–c),

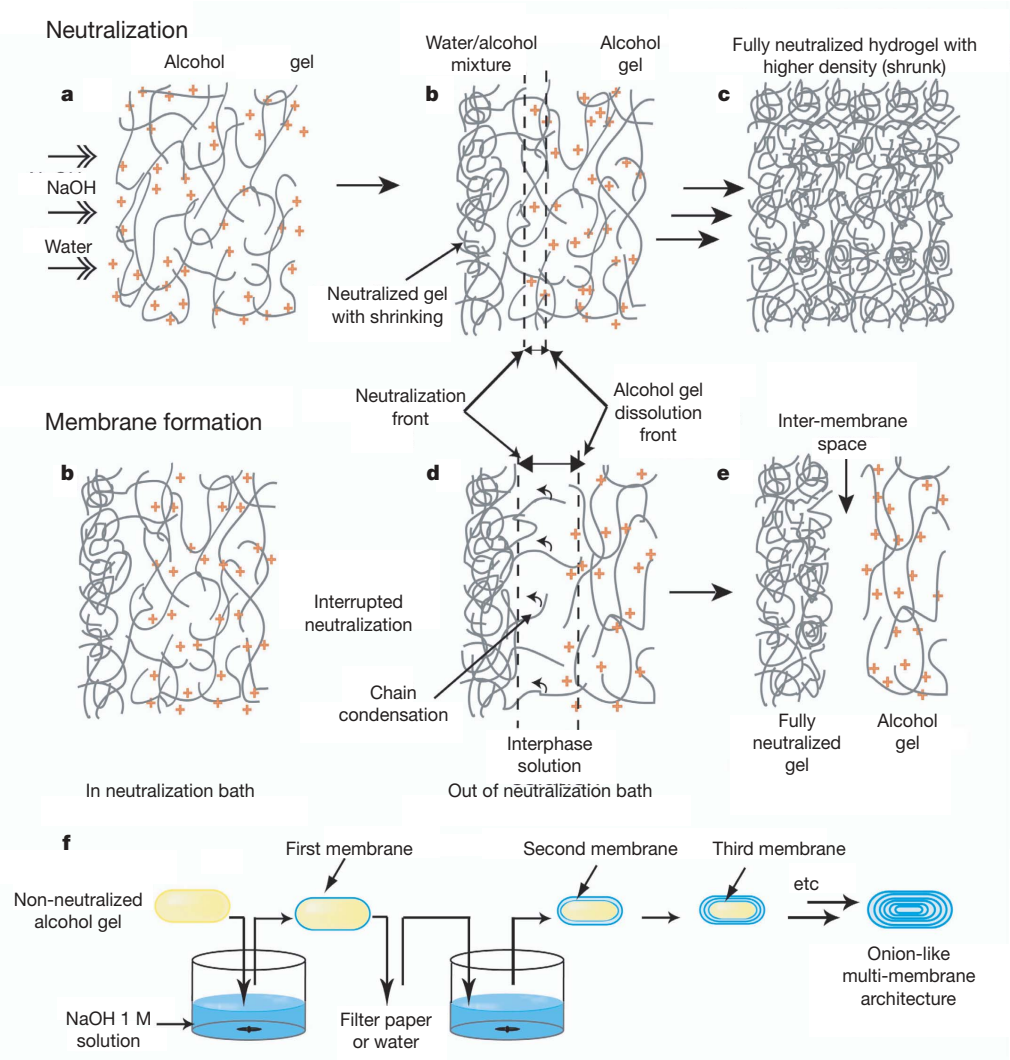

Figure 3 | Schematic representation of neutralization of a polyelectrolyte alcohol gel and derived methodology for building a multi-membrane structure. a, Non-neutralized alcohol gel introduced into the neutralization bath. **b**, Chain condensation and shrinkage of the alcohol gel with the disappearance of ionic repulsions during the neutralization step. **c**, Fully neutralized shrunk hydrogel after a classical neutralization step. **d**, Formation of the interphase solution and collapse of the polymer chains onto the neutralized shrunk gel during the interruption of the neutralization step. **e**, Inter-membrane space formation after complete condensation of the residual polymer chains in the interphase solution. At the end of the process, the multi-membrane system was thoroughly washed in distilled water to eliminate the excess of NaOH, the salts formed during neutralization and the alcohol. **f**, Overview of the multi-step neutralization process.

but to form separate gel membranes, an interphase that was less concentrated in polymer had to be generated between the neutralized and the alcohol gels (Fig. 3d). Control of the degree of entanglement across this interphase was essential to control the formation of separate membranes in the final structure (Fig. 3e). This, in turn, was determined by the kinetics of several phenomena occurring near the neutralization front, such as water diffusion within the alcohol gel. Indeed, going back to the gelation process, the sol–gel transition occurred after a complete water evaporation of a water/alcohol solution of chitosan. This transition is reversible after rehydration of the alcohol gel. As a result, during neutralization, if the water diffusion kinetics was fast enough in comparison with the neutralization kinetics, a thick interfacial solution could be formed in which the polymer hydration and mobility were high enough to allow disentanglement of the polymer chains and then their condensation onto the neutralized gel (Fig. 3d to e). In contrast, when the kinetics of neutralization was faster, a continuous entangled network existed between the alcohol and neutralized gels (Fig. 3c), without any possibility of forming a membrane and therefore a multi-membrane organization. The formation of inter-membrane spaces could be promoted by slowing down the neutralization simply by removing the gel from the neutralization bath or by washing with water. As discussed above, this resulted in (i) the formation of a water/alcohol solution between the neutralized and the alcohol gels, (ii) the disentanglement of chains located within the interphase, and (iii) condensation and contraction onto the neutralized gel.

The sequence including the neutralization and interruption steps could be repeated several times (Fig. 3f), for example 20 times, for a macroscopic gel of a few cubic centimetres, to build a multi-membrane onion-like structure composed of non-adherent and then fully independent gel membranes. The membranes were progressively formed from the periphery of the initial alcohol gel inwards, so it was possible to structure the gels fully into a succession of membranes, or to combine a continuous gel in the core (Fig. 1b)

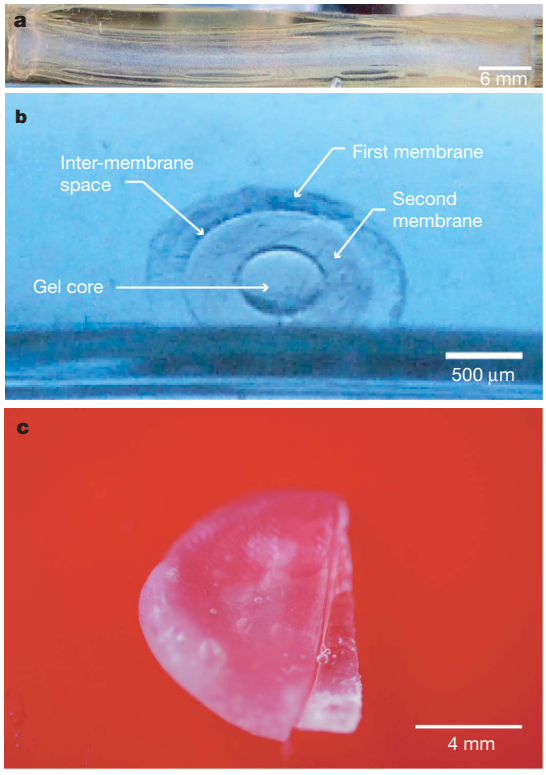

Figure 4 | Versatility of the multi-membrane architecture process. **a**, Multi-membrane tubular architecture, resembling a blood vessel. **b**, Microscopic multi-membrane capsule. **c**, Macroscopic multi-membrane architecture based on alginate hydrogel.

with a multi-membrane region at the periphery. Moreover, depending on the initial shape of the alcohol gel, which was easy to mould, various geometries of multi-membranes such as spherical, ovoid, cubic or tubular shapes (Fig. 4a) were possible, with a broad range of sizes (Fig. 4b). We varied the conditions of membrane formation by varying the initial polymer concentration in the alcohol gel, and the concentration and the nature of the neutralizing agent. Chitosan membrane formation occurred with NaOH ($pK_a = 14$), ethanolamine ($pK_a = 9.51$) or ethylamine ($pK_a = 10.81$) in specific conditions but was not observed with aqueous ammonia ($pK_a = 9.25$), for any concentration of the base ranging from 1 to 7 M and polymer concentration of the (1,2-propanediol) alcohol gel ranging from 2 to 6 wt%. These neutralization experiments led to gels with different polymer fractions ranging from 4.7 to 12.8 wt% (Supplementary Table 1) with an initial alcohol gel of 4.5 wt%, but with no direct relationship with the capacity to form membranes.

In contrast, the viscosity of the base/water/alcohol mixture was found to be closely related to the membrane formation (Supplementary Fig. 5). The viscosity of bases in water/1,2-propanediol mixtures was significantly larger than in water (Supplementary Fig. 5b and Supplementary Table 1), except in the case of aqueous ammonia for which the increase was very weak. For NaOH and NH_4OH, this trend was measured either as a function of the concentration of the base in a 50/50 mixture (by weight) (Supplementary Fig. 5a), or as a function of the 1,2-propanediol fraction at a fixed base concentration (Supplementary Fig. 5b). Consequently, the higher viscosity of NaOH/water/1,2-propanediol solutions resulted in lower diffusion kinetics of the base through the alcohol gel and thus slower neutralization kinetics, promoting the hydration of the alcohol gel and the formation of membranes. At a microscopic scale, the high viscosity of NaOH/water/1,2-propanediol mixtures could be related to specific interactions of 1,2-propanediol with the ionic species ('structure making effects'), as reviewed elsewhere[21]. Similar bridging effects have previously been described between polymers and ionic species[22]. Finally, the role of the alcohol viscosity was confirmed when 1,2-propanediol was replaced by glycerol, a more viscous solvent. In the latter case, multi-membrane processing became possible even with aqueous ammonia.

We also studied the membrane formation conditions, showing that chitosan membranes were only obtained for low initial polymer concentration in the alcohol gel (C_{pi}) and low NaOH concentrations (Supplementary Fig. 5c). At high concentrations of neutralization agent, the neutralizing flux entering the alcohol gel was high and the resulting neutralization kinetics fast. At high C_{pi}, the entangled network (in the alcohol gel) was dense and the disentanglement kinetics too slow, compared with the neutralization kinetics, to form membranes. We observed that C_{pi} appeared to have a weak influence on the membrane thickness, whereas the latter increased with the concentration of NaOH and the root square of the neutralization time (Supplementary Figs 6 and 7). This offers a way to control the width of the membranes and again illustrates the strong influence of the diffusion mechanism on the membrane formation. It showed that the increase of C_{pi} only affects the polymer chain mobility and their disentanglement, but not the NaOH diffusion within the gel, essentially governed by the concentration and viscosity of the neutralization bath.

The concept involved in the multi-step assembly process could also be applied to other natural polymers of polyanionic nature. We checked this with alginate hydrogels. Alcohol alginate gels were easily obtained by evaporation of water/1,2-propanediol mixtures, as in the case of chitosan alcohol gels, and multi-membrane architectures were generated by interrupted complex hydrogel assembly in $CaCl_2$ baths (Fig. 4c). The kinetics of hydrogel assembly played a dominant role, as the membrane architecture could only form for low concentrations in $CaCl_2$.

To validate the usefulness of our systems as biomaterials, we carried out chondrocyte culture within multi-membrane onion-like

hydrogels of chitosan (Supplementary Fig. 8). Cell aggregates were observed in several inter-membrane spaces, showing that cells can be introduced and cultured within these new systems for tissue engineering. Details of a biological study of rabbit chondrocytes cultured in our systems will be published elsewhere.

METHODS SUMMARY

Sample purification and characterization. We purified and characterized chitosan from squid pens (Mahtani Chitosan, batch 114) as described previously[4]. Its degree of acetylation was $2.5 \pm 0.1\%$, with a weight-average molecular weight $M_w = 550{,}000 \pm 25{,}000\ \mathrm{g\,mol^{-1}}$. Sodium alginate (System Bio-Industries, lot Satialgine SG800) had a mannuronic/guluronic ratio of 0.5.

Gelation. After purification, lyophilized chitosan or alginate were ground with a cryo-grinder, then dispersed in water. For chitosan, we added a stoichiometric amount of hydrochloric acid to neutralize the amino groups. After dissolution, we added an equivalent weight of 1,2-propanediol and stirred the mixture at room temperature for 1 h before evaporating the solution in a ventilated oven.

Chitosan multi-membrane structures. After collecting the gel from its mould, we placed it in 1 M NaOH for 5 min, then took it out of this medium for 3 min and carefully removed the excess NaOH from its surface with a filter paper. This sequence allowed the formation of both a first membrane and a first inter-membrane space, and could be repeated to produce several membranes and inter-membrane spaces. The 'onion' was then washed extensively in water to eliminate the alcohol and NaOH.

Alginate-based multi-membrane systems. After evaporation, the gel was at a concentration of 2%. We treated it alternately in a coagulation bath of 0.3 M $CaCl_2$, then in a water bath, for 5 min each.

Viscometry. We carried out viscometry at 25 °C with an automatic capillary viscometer (Viscologic TI1, SEMATech).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Purification and characterization of biopolymers. The initial chitosan produced from squid pens was purified as described previously[4]. Chitosan corresponds to the series of the linear copolymers of (1→4)-2-amino-2-deoxy-β-D-glucan (GlcN) and (1→4)-2-acetamido-2-deoxy-β-D-glucan (GlcNAc). The degree of acetylation (DA) is the molar fraction of acetyl units within the polymer chains and is below 70% for chitosan. The purified sample used in this work is a highly deacetylated chitosan (DA = 2.5 ± 0.25%) with a high weight-average molecular weight (M_w = 550,000 g mol^{-1}) and a low polydispersity index (I_p = 1.5 ± 0.1). The techniques of characterization were accurately described in previous works[4,9]. In the case of alginate, we used Satialgine SG800 with a mannuronic/guluronic ratio of 0.5.

Preparation of physical alcohol gels based on chitosan or alginate. Lyophilized chitosan or alginate was dispersed in water in the form of powder. In the case of chitosan, we added a stoichiometric amount of hydrochloric acid to achieve the exact protonation of the amino groups. After complete dissolution, we added an equivalent weight of 1,2-propanediol (50 wt%) and stirred the mixture for 1 h at room temperature. The solution was evaporated at 55 °C for 60 h in a ventilated oven.

Processing of the multilayer onion-like structure. For chitosan: after taking off the alcohol gel from the mould, the multilayer onion-like structure was formed by introducing the gel in 100 ml of a 1 M solution of NaOH for 5 min. We then took the gel out of the solution for 3 min and carefully removed the NaOH excess on the surface with a filter paper. We then placed the gel again in 100 ml of 1 M NaOH for 5 min, followed by another storage time on a filter paper for 3 min. When the required number of membranes was reached, it was necessary to neutralize the core of the gel for 12 h with 1 M NaOH. We then washed the capsule several times in de-ionized water to remove NaOH and alcohol fully.

For alginate: the multi-membrane system based on alginate was obtained from a 1,2-propanediol gel of alginate with a final polymer concentration close to 2% (w/w). Based on the same process, we treated the alcohol gel of alginate alternately by coagulation baths ($CaCl_2$ 0.3 M aqueous solution) and water baths of 5 min each.

Viscometry measurements. We made measurements three times at 25 °C with an automatic Ubbelohde capillary viscometer with an inner diameter of 0.79 mm.