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Sol-gel processing of actin to obtain homogeneous glasses at low temperatures

Gérard Prulière and Pierre Douzou

Unité de Recherche INSERM 310–INRA, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

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The lethal effects of freezing on cells are currently attributed to the crystallization of extracellular water which leaves concentrated solutions of salts, macromolecules and so forth in the extracellular space. This concentrated fluid establishes a strong osmotic gradient which draws water from the cells. Thus, a cell surrounded by ice can survive only if means can be found for reducing the osmotically driven outflow of cellular water. This is usually attempted through vitrification of the extracellular space, but may also be attained through suitable modifications of cellular plasms. Starting from microscopic observations on early rabbit embryos and related cryotolerance, we investigated purified actin solutions under similar conditions, and found that sol-gel processing could result in the formation of homogeneous glass, and through drying, give rise to monolithic solids, glasses and composites. The first process may be at least partially responsible for the induced cryotolerance of cells, while the second may be representative of new and useful biomaterials.

1. Introduction

One of the major problems in cryotechnics applied to living systems is the formation of ice crystals. Due to their size, they disturb the fine structure of cells and are responsible for both artefacts in microscopic observations and lethal effects in cryopreservation assays.

The best guarantee for avoiding such unwanted effects would be to obtain vitreous ice and in fact amorphous monolithic assemblies of macromolecules preserving the native organization.

It is known that the cell supramolecular structure defined as the cytoskeleton is based on the assembly of protein molecules, principally actin, which regulates the consistency of the cytoplasm by interacting with several proteic factors of the actin-binding protein family [1]. The three-dimensional structure formed is normally porous and the pores are filled with water, globular proteins and other particulate solutes. Such water-containing solutions or gels are susceptible to collapse and aggregate when the water is removed or when they crack during freezing as the result of large stresses due to the heterogeneity of the pores.

Advances in achieving homogeneity of the pores, thus minimizing stress caused by differential drying and freezing, could be made through the processing that brought a revolutionary breakthrough in the making of homogeneous glass and ceramics by optimizing gelation, aging, and drying to produce large-scale, fully dried, monolithic gels rapidly and routinely [2–5]. We tried to apply such 'sol-gel processing' to a number of biological macromolecules (especially actin) to form homogeneous solutions that, in addition to gels, give highly microporous materials able to produce glasses.

Correspondence address: G. Prulière, Unité de Recherche INSERM 310–INRA, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France.

In a previous work, it was shown that 1,2-propanediol strengthens the association between polymeric actin and α-actinin, a protein which cross-links microfilaments [6], a much stronger gel being obtained under these conditions. In the present work, we applied the sol-gel procedure and found that actin/α-actinin solutions in the presence of propanediol were able to give amorphous biomaterials.

2. Materials and methods

2.1. Sol-gel processing

Rabbit skeletal actin (purified as described previously [11]) was polymerized in the presence of 40 mM KCl and 1 mM $MgCl_2$ after the addition of chicken gizzard α-actinin (purified according to ref. 6) and organic solvents. Polymerization and gelation had reached completion after 3 h at room temperature. Final concentrations were 125 μM for actin (5 mg/ml) and 5 μM for α-actinin (1 mg/ml). For experiments carried out in the presence of organic solvents, 1,2-propanediol or glycerol was added at 15 and 30%, respectively. For final solvent concentrations, 6% glycerol must be taken into account in each case, since α-actinin contained 50% for preservation at −20°C and was added without dialysis.

2.2. Drying or freezing

For drying experiments, 100 μl of each sample were poured onto a glass slide. The drying was assayed for several days under a vacuum of 10^{-3} mmHg at 4°C. For freezing, 50 μl of each sample were poured onto a 0.2 mm thin glass cover slip. Cooling of the solution was initiated by contact of the cover slip with the surface of liquid nitrogen and solidification occurred within 30 s. The frozen solutions considered as apparently vitrified remained translucent under cooling, no white and opaque crystallization being visible.

2.3. Electron microscopy

Actin and α-actinin were mixed at the same molar ratio (α-actinin/actin = 1:25) as that for freezing and drying experiments, however, the respective final concentrations were 3.12 and 0.125 μM (for fig. 1a and b), 6.25 and 0.25 μM (fig. 1c and d). The solutions were set on formvar-coated grids before negative staining with 1% uranyl acetate.

3. Results

3.1. Freezing and drying of actin solutions

The stability of polymeric actin on removal of water was assayed by submitting the protein solutions to freezing or drying. Under drying, aggregation of proteins was observed, leading to the rapid elimination of water. The analysis of dehydrated solutions under modulation contrast microscopy confirmed the presence of large aggregates of proteins (results not shown). These results suggest that the concentration of actin used was not sufficient to stabilize all of the water and that a phase transition occurred prior to complete drying of the solution. Similar conclusions were drawn from freezing assay, since the appearance of white crystals accompanied the solidification of actin solutions. In order to overcome the disruptive effect of the phase transition, drying and freezing experiments were carried out in the presence of 30% glycerol or 15% propanediol. White and opaque crystallization was observed when actin solutions were frozen in the presence of both cryoprotectants. In each case, the dehydration was completed after several days under vacuum. However, the films obtained differed in some aspects. While dehydrated actin solutions containing glycerol were totally opaque such as those obtained in the absence of solvent, dehydration of actin solutions containing propanediol led to the formation of a translucent and very homogeneous film. The observation of these films under light microscopy did not reflect any collapse of actin filaments into compact masses (not shown).

3.2. Vitrification of actin solutions in the presence of a cross-linker

Successful vitrification was achieved when actin solutions were frozen in the presence of both

α-actinin and propanediol. The interaction between both proteins in the presence of solvent resulted in the formation of a strong gel which remained stable under freezing. The solid obtained appeared translucent without formation of any crystals compared to actin solutions containing only α-actinin or propanediol which, when frozen, led to the formation of white crystals.

The drying of actin gels performed in the presence of α-actinin and propanediol also determined the formation of very homogeneous solids. Similar experiments were carried out where propanediol

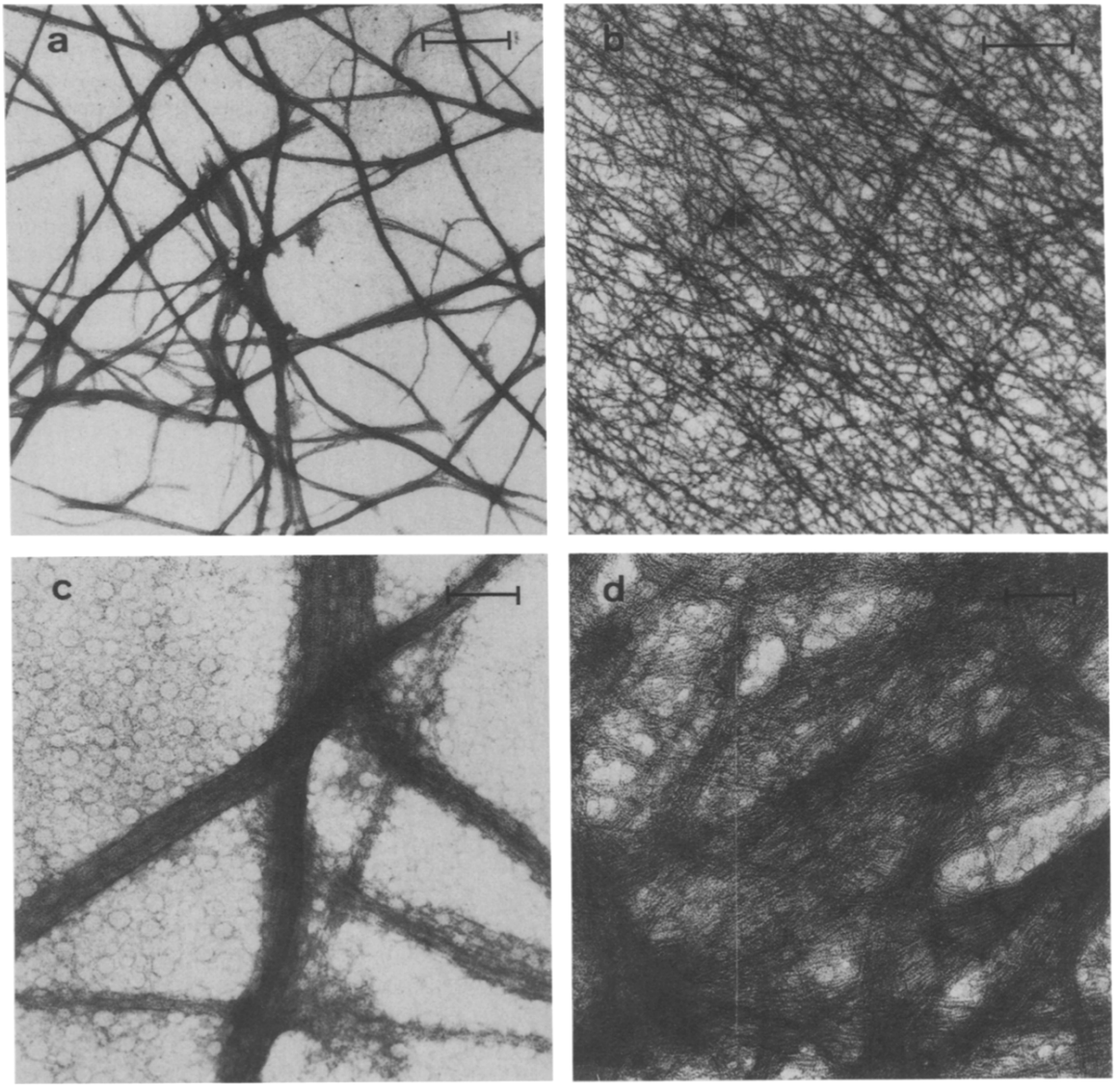

Fig. 1. Electron micrographs of F-actin/α-actinin solutions in the absence (a, c) or presence (b, d) of 1,2-propanediol. Scale bars: 1 μm (a, b) and 100 nm (c, d).

was replaced by 30% glycerol. In this case, the formation of a fairly homogeneous solid with actin/α-actinin solutions was observed in no case at all, suggesting a specific effect for propanediol.

We compared the structures of gels formed by actin and α-actinin in the absence or presence of propanediol using electron microscopy after negative staining of proteins (fig. 1). It appears that propanediol not only increases the proportion of α-actinin bound to actin [6] but also induces the formation of a very homogeneous three-dimensional network at low (fig. 1b) and high (fig. 1d) protein concentrations. In comparison, the formation of characteristic bundles where filaments are associated side-by-side was visualized when proteins interacted in the absence of propanediol (fig. 1a, c). The transparence obtained in freezing and drying assays may arise from the great regularity in size and spacing of cross-linked actin filaments observed under solvent conditions. Finally, preliminary investigations made on monomeric actin solutions treated under similar conditions of medium and temperature have provided evidence showing that it could be transformed into homogeneous and vitreous solids, a result which will be briefly discussed in the context of the cryotolerance of some types of cells.

4. Discussion

The present results clearly show that formation of amorphous solids from solutions of purified actin requires the simultaneous presence of selected amounts of α-actinin and propanediol. α-Actinin is a well established gelling agent of actin microfilaments through a mechanism of physical (noncovalent) cross-linking [1] but does not promote vitrification on its own. The generation of cracks during freezing or drying of the actin/α-actinin gels is probably due to stresses that behave as functions of both the heterogeneity of the actin network and the rate of freezing or dehydration of the pore liquid. In those cases, large differential freezing or evaporation induces large stresses irrespective of the rate of these operations. Propanediol (15% v/v) increases gelation of actin/α-actinin mixtures as the result of a significant increase in cross-linking capacities of α-actinin and stabilizes the materials into homogeneous networks which are transformed in solid gels upon freezing or drying as the result of uniform stress distribution suppressing cracks in the samples.

Thus, propanediol allows one to produce large-scale, frozen or dried monolithic gels rapidly and routinely. These homogeneous microporous networks withstand thermal and osmotic shocks. If produced in vivo, they should preserve the cellular ultrastructure and may at least be partially responsible for cryotolerance of some cells.

Propanediol can be compared to the drying control chemical agents (DCCA) used to optimize gelation, aging and drying of solutions of silicates and other materials involved in homogeneous glass, ceramics and composites that are now prepared at low temperatures through so-called sol-gel processing [1–4] – DCCAs make it possible for one to produce with 100% reliability a wide range of sizes and shapes of optically dried gel monoliths of silicon, barium, lithium, aluminium, sodium or titanium oxides.

Propanediol used in vivo as a cryoprotectant and in vitro as an additive in F-actin/α-actinin solutions could be termed a freezing control chemical agent (FCCA). Samples containing these ingredients can be compared to composites, that are particle reinforced, or fiber-whisker sol-gel matrices. Their cracking can again be controlled with glycerol, which also provides flexibility and durability of the dried samples [4]. This liquid gelling agent accelerates the gelation of low-viscosity sols to occur within seconds, and this freezes the materials into a homogeneous distribution. Sol-gel derived materials are then characterized by the very large (up to 70%) percentage of microporosity in the materials after drying. A wide range of physical properties and ultrastructural control can be achieved with materials of the same chemical composition by varying volume fraction, size distribution and connectivity of the microporosity [5].

At present, the mechanism of formation of the crack-free gels remains to be clarified and sol-gel processes for producing monolithic glasses, ceramics and composites are still in their infancy. Almost all experimental and theoretical work in

sol-gel science has been performed on silica, and polymeric molecules have only recently been used as reinforcement materials in composites [5]. It is expected that the impregnants of the future will be polymers with a high degree of order and anisotropy, and able to display physical cross-linking and determine reversible gel formation. Combination of inorganic, organic and polymeric compounds through sol-gel processing could lead to materials not obtainable by other methods. Composites with submicrometer dimensions (on the scale of 1–10 nm units), referred to as nanocomposites, have been obtained through use of polymer fibers [7].

Thus, sol-gel processing now traverses basic topics in polymer science which may be a source of inspiration for new composites, including biomaterials and more precisely fibrous and globular protein mixtures.

These proteins are believed to constitute the 'background' solution in the cytoplasm, and a mechanism of physical cross-linking establishing connectivity throughout fibrous polymeric systems can lead to gelation. Such networks are reproducible in vitro but may be quite different in vivo when they involve all their biological ingredients. Usually, these networks of purified proteins do not withstand freezing or drying, however, according to the present observations, new mixtures and sol-gel processing could be developed for obtaining monolithic solids. We are currently investigating such new biomaterials, or biocomposites, with raw material involving protein fibers and fillers as well as linkers and gelling control chemical agents.

On the other hand, the challenge that lies ahead is to devise experiments that enable one to determine whether and how such particular structures are formed in cells and to identify actin regulatory proteins and conditions able to enhance and to control gelation of actin/actin-binding protein complexes.

Preliminary results obtained with monomeric actin suggest that the procedure applied (essentially the addition of α-actinin and propanediol) can favor the formation of fairly homogeneous ultra-divided solutions such as those obtained with colloidal particles [8]. Such processes may at least partially explain the cryoprotective effect of propanediol on early rabbit embryos [9] in which it governs significant depolymerization of cortical actin [10]. According to such an assumption, a number of cellular microstructures might form homogeneous sols in which water could be osmotically inactive and then unable to flow across plasma membranes under freezing of extracellular spaces. These processes are currently under investigation.

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