Two-Component In situ Forming Supramolecular Hydrogels as Advanced Biomaterials in Vitreous Body Surgery[a]

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Polymeric β-CD and poly{(2-acrylamido-2-methyl-1-propanesulfonic acid sodium salt)-*co*-[6-(acrylamido)-*N*-adamantylhexaneamide]} are synthesized to build in situ forming hydrogels based on host/guest interactions, so called physical hydrogels. The use of these hydrogels as a potential vitreous body substitute is discussed and recommended. Potential changes in cell morphology and cell vitality of the retinal ganglion cell line RGC-5 are determined. DSC experiments with artificial membrane structures are performed. The analyses show that β-CD overrides the harmful effects of the highly toxic adamantyl-modified polymer. Although the final hydrogel is considered to be biocompatible, the application as a biomaterial has to be reconsidered.

1. Introduction

Recently, hydrogels have received much attention because of their numerous advantages in biomedical applications, since their soft nature and high water content minimize mechanical irritation to tissues.[1] In general, hydrogels are clear and tend to be biocompatible. A variety of polymers have properties that closely resemble those of soft tissues and they are used to replace soft tissues including skin, cartilage, tendon, and lens.[2] Furthermore, the replacement of the vitreous body seems to be an especially interesting problem. After the removal of the vitreous body during vitrectomy, the volume has to be restored by an adequate substitute.[3] Since the native vitreous body represents a hydrogel with high water content (≈98%),[4] the most rational substitute would also be a hydrogel. Despite presenting an acceptable biocompatibility, several reviews about previous work with preformed artificial polymeric hydrogels as vitreous substitutes have reported complications upon injection into the eye.[5] The main problem with such preformed gels is that they shear thin and fragment as a result of shear stress during injection through a small-gauge needle, a fact that causes some loss of the elasticity and leads to a more fluid-like character. In situ forming hydrogels could be injected by a small-gauge instrument in liquid form and undergo gelation within the vitreous cavity (in situ, lat. in the place) without fragmentation. In this

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context, we recently described the idea of an in situ forming thermoresponsive hydrogel as a vitreous body replacement.[6]

Hydrogels based on host/guest interactions with various β-cyclodextrin (β-CD) derivates have gained increasing interest in recent years, since the interactions with suitable guests like adamantyl-modified polymers represent an ideal system to prepare physical hydrogels.[7–9] β-CD is well known to form inclusion complexes with various organic molecules of appropriate size and hydrophobic segments.[10–12] In this context, we recently described host/guest interactions and supramolecular complexes in aqueous solution.[13,14]

In this work, we present the interesting concept of in situ forming hydrogels based on host/guest interactions.

2. Experimental Section

The syntheses of polymer **1**, **2**, and of the hydrogel **3** were conducted according to literature[7] (see the Supporting Information).

2.1. Viscosity Measurements

Viscosities were measured with a Thermo scientific Haake Mars viscosimeter by plate/plate configuration using a MP35 plate and a PP35Ti plate.

2.2. Cell Culture and Treatment

The immortalized rat RGC-5 cells were grown in growth medium [Dulbecco's modified Eagle medium (DMEM) containing 10% newborn calf serum, 10^{-3} M l-glutamine, 200 μg mL^{-1} streptomycine, and 200 U ml^{-1} penicillin, all from Biochrom AG, Berlin, Germany] in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were cultured in 75 cm^2 culture flasks (NUNC, Wiesbaden, Germany) until they formed a confluent cell layer. For passaging, cells were rinsed twice with phosphate-buffered saline (PBS) and incubated with 0.25% trypsine (Biochrom AG, Berlin, Germany) in PBS to promote detachment from the flask. For experiments, cells were allowed to reach about 80% confluence. Medium was subsequently changed to serum-free medium (SFM, growth medium without newborn calf serum) with or without **2**, **1**, or their mixture. Here, a solution of 20 mg mL^{-1} of **2** in serum free medium was mixed overnight with 9.6 mg mL^{-1} of **1** to allow the hydrogel to form. These ratios match the numeric molecular amount of cyclodextrin cavities and adamantane groups to be inserted. This step was necessary to avoid free adamantyl groups or cyclodextrin cavities which could manipulate the biocompatibility assessment. Cells incubated with serum free medium alone served as a control.

2.3. Phase Contrast Microscopy

To determine morphological changes, RGC-5 cells were seeded at a density of 4500 cells per well on a 12–well plate and were allowed to attach and grow for 15 h in growth medium. After cell treatment (see cell culture and treatment), microscopy analysis was carried out after 24 h incubation using a Leica DM IRB phase contrast microscope and Leica IM50 V1.20 software.

2.4. Cell Vitality Assay

Cellular metabolic activity was determined by using the MTS assay (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega, Wisconsin, USA) according to the manufacturer's instructions.[15] RGC-5 cells were seeded onto a 96-well microplate (Corning, Kaiserslautern, Germany) at a density of 2250 cells per well in SFM and allowed to attach for 15 h. Cells were treated with hydrogel components (see cell culture and treatment) and the microplate was analyzed by a Tecan GENios microplate reader. For the determination of the lethal concentration (LC_{50}), a range of 0.1 to 100 mg mL^{-1} of **2** was examined after 24 h as described.

2.5. Differential Scanning Calorimetry

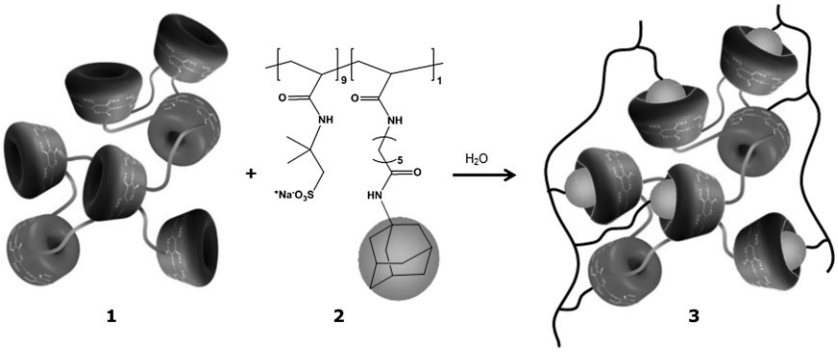
Dipalmitoylphosphatidylcholine (DPPC) was dissolved in chloroform/methanol (1:1, v/v) and dried under a stream of nitrogen at 50 °C for 30 min. Traces of solvent were removed by keeping the vial under vacuum at 50 °C overnight. The hydration of lipid films was achieved by adding a pre-heated HEPES buffer (pH = 7.0). The vesicle suspension was kept for 30 min at 50 °C in a water bath and vortexed for 30 s at regular intervals to obtain multilamellar vesicles (MLVs). DSC experiments were performed using a PC controlled Setaram Micro DSC III differential scanning calorimeter. One of the two chambers was filled with the sample; the other was filled with the corresponding HEPES buffer solution. The volume of the vesicle suspension was 600 μL. A scan rate of 30 °C h^{-1} was used in the temperature range between 10 and 60 °C. Phase transition temperatures were taken at the maximum of the transition peak.

3. Results

In this paper, we explored a physical hydrogel (**3**) based on polymeric β-CD (**1**) as host and poly{(2-acrylamido-2-methyl-1-propanesulfonic acid sodium salt)-*co*-[6-(acrylamido)-*N*-adamantylhexaneamide]} (**2**) as a hydrophobic guest molecule (Scheme 1).

Mixed in water, the adamantyl part of the polymer **2** interacts with the hydrophobic cavity of β-CD (**1**) and forms a host/guest complex (**3**) leading to a hydrogel. Figure 1 shows the drastic increase of the viscosity by mixing the host and guest polymer in aqueous solution. Considering the single components (**1** and **2**) their rheological behavior with $\eta < 0.1$ Pa s (at a shear rate from 0.5 to 100 s^{-1} and a concentration of 100 mg mL^{-1}) differs dramatically from the mixture (**3**) with $\eta = 900$ Pa s at a shear rate of ca. 0.5 s^{-1} and a concentration of 100 mg mL^{-1}.

For the biocompatibility assessment of the single components and the final physical hydrogel, experiments



Scheme 1. The formation of physical hydrogels (**3**) from polymeric β-CD (**1**) and adamantyl polymer (**2**).

with both living cells and artificial membranes seem necessary, since the adamantyl-modified polymer strands are expected to interfere with membrane structures because of their highly hydrophobic regions. The most sensitive layer in contact with a vitreal endotamponade is the nervous tissue of the retina, foremost the retinal ganglion cell layer. Thus, all in vitro experiments were made on the immortalized retinal ganglion cell line RGC-5.[16] To further investigate effects of hydrophobic gel components on membranes, phospholipid vesicles of DPPC served as models for cell membrane structures and the interactions were elucidated using the sensitive DSC technique.

3.1. Effect of Hydrogel Components on RGC-5 Cell Morphology

By means of microscopic analysis, possible influences exerted by single gel components and the physical hydrogel on the RGC-5 cell morphology were investigated. For this purpose, phase contrast micrographs were taken 24 h after incubation with the hydrogel components and examined for morphologic changes. Untreated control cells show the typical ganglion cell morphology with flat cells with characteristic short processes (Figure 2A). Only a few rounded detached cells were visible. The presence of **2** led to a complete cell lysis of cellular structures (Figure 2C). No cells were visible after 24 h of incubation with the adamantyl polymer. After incubation with **1**, the morphology of the RGC-5 cells was slightly affected (Figure 2D). The cell bodies became rounder and showed shortened processes, several cells detached. The combination of 20 mg mL^{-1} of **2** and 9.6 mg mL^{-1} of **1** did not affect the RGC-5 morphology (Figure 2B). Cells presented a typical healthy appearance compared to control cells with sharp short processes and a flattened cell body.

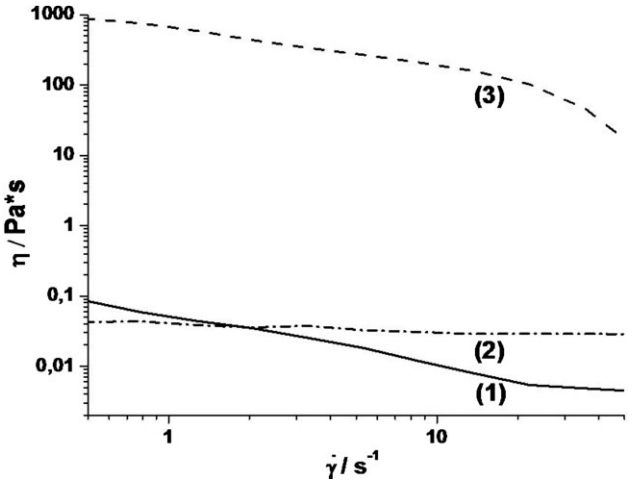


Figure 1. Viscosity of an aqueous solution **3** (100 mg mL^{-1}) as well as aqueous solutions of the single components **1** and **2** (100 mg mL^{-1}) as a function of the shear rate ($T = 20$ °C, pH = 7).

3.2. Effect of Hydrogel Components on RGC-5 Cell Vitality

The morphological observations showed clearly reduced effects on the cellular morphologies when both hydrogel components were applied in the form of a physical hydrogel. These results were quantified and further supported by a cell vitality assay. RGC-5 cells were incubated with the adamantyl polymer (**2**) and β-CD polymer (**1**) alone and in combination (Figure 3B). Pure **2** led to entire cell death, as after 24 h, the cell vitality was reduced to 2.1 ± 2.5%. Incubation with **1** notably reduced the cell vitality to 84.1 ± 2.3%. Within 24 h, the combination of **1** and **2** did not significantly affect cellular vitality compared to untreated control cells (98.3 ± 5.6%).

By determination of the cell vitality, a LC_{50} value (lethal concentration 50) for the adamantyl polymer (**2**) was assessed over a wide concentration range of 0.01 to 100 mg mL^{-1} (Figure 3A). The LC_{50} value was determined to be 3.2 ± 0.38 mg mL^{-1}.

3.3. Effect of Hydrogel Component 2 on Lipid Vesicles

To obtain a more detailed insight into the underlying mechanisms of the toxic effects of the polymeric components of the physical hydrogels to cells, experiments using the DSC technique were performed. Here, the interactions between hydrophobic parts of the adamantyl-functionalized polymer strands with artificial membranes composed of the DPPC were elucidated. DSC was used to study changes in the specific phase transitions of the phospholipid vesicles

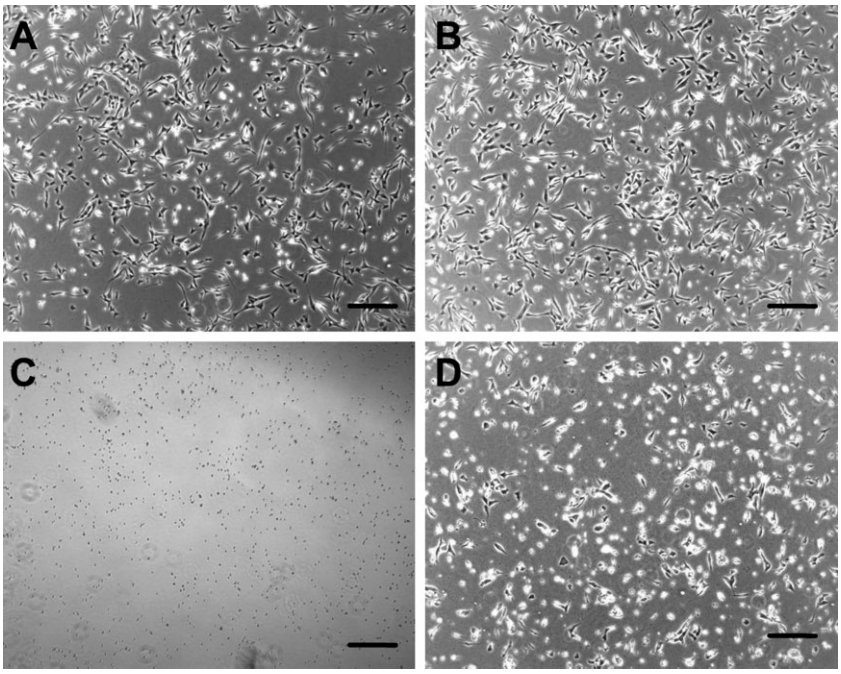


Figure 2. Cell morphology of RGC-5 cell line after incubation with physical hydrogels monitored over 24 h. Cells were incubated with A) serum-free medium (control), B) **2** (20 mg mL^{-1}) + **1** (9.6 mg mL^{-1}), C) **2** (20 mg mL^{-1}) and D) **1** (9.6 mg mL^{-1}). Phase contrast micrographs were taken after 24 h. Magnification 10×, scale bars represent 200 μm.

in the presence of the adamantyl polymer (**2**). These changes served as very sensitive parameters for the constitution of the lipid vesicles.

Pure DPPC MLVs were characterized by a strong pre-transition peak at 36.5 °C and a sharp peak for the main transition at 42.2 °C. The addition of **2** caused the disappearance of the pre-transition peak and a slight shift of the main transition peak to 41.8 °C (Figure 3C).

4. Discussion

In this paper, we highlight an innovative concept to overcome obstacles in vitreal surgery. Occupying the largest part of the eye, the transparent vitreous body plays an important role in the maintenance of vision.[17] We assessed the biocompatibility of a hydrogel following a new concept to bypass the common problems with liquid artificial vitreous body replacements (LAVRs) concerning fragmentation and cytotoxic effects.

The concept presented in this study involves hydrogels based on host/guest interactions of an adamantyl-substituted polymer (**2**) and the hydrophobic cavities of a corresponding polymeric β-CD (**1**) strand. The advantage is to instill the two single components separately into the ocular cavity in their liquid form without fragmentation with the final gelling in situ.

This study delved into the biocompatibility of polymers with adamantyl residues, linked to the adamantyl polymer (**2**) backbone by flexible alkyl spacers. Adamantane is a tricyclodecane cage-shaped compound that has been shown to be highly valuable to improve the physical properties when inserted in the backbones of many polymers.[18] The adamantyl moieties at the end of flexible alkyl spacers on the polymer strands are expected to interfere with hydrophobic cell membranes because of their highly hydrophobic character.

In this paper, the adamantyl-functionalized polymer (**2**) was revealed to have obvious cytotoxic impacts on a cultured retinal ganglion cell line (RGC-5). The devastating observed effects on the cultured cell monolayers in this study are comparable to the effects of detergents, e.g., sodium dodecylsulfate (SDS), on cell membranes. The phase contrast photographs did not only show detached or deformed cells, but indeed a rapid ongoing cell lysis. We attributed the underlying cytotoxic effects to the interactions of the hydrophobic adamantyl groups with the lipids of cell membranes. This seems evident, since the chemical structure shows the adamantyl present at the end of flexible alkyl spacers of varying length.

The most striking observation of the presented results is the fact that the clearly high toxicity of the adamantyl polymer is completely attenuated by masking the adamantyl groups with an equimolar number of cyclodextrin cavities. Applied to cell layers alone, **2** caused a complete cell death. When mixed with equimolar amounts of cyclodextrin cavities (**1**) to form a physical hydrogel (**3**), the detrimental effects on both cell morphology and vitality were nearly completely reversed. In this constellation, the harmful adamantyl groups were completely embedded in the hydrophobic β-CD cavities and were unable to reach the cell membrane.

This explanation holds for the observations that neither the cellular morphology nor the cell vitality is affected when both components were applied as a preformed physical hydrogel. The combination of both components in a preformed physical hydrogel distinctly enhanced the biocompatibility reaching control levels, and completely overrode the cytotoxic effects of the single components.

The cyclodextrin strand alone only showed mild cytotoxic effects in morphology and vitality experiments.

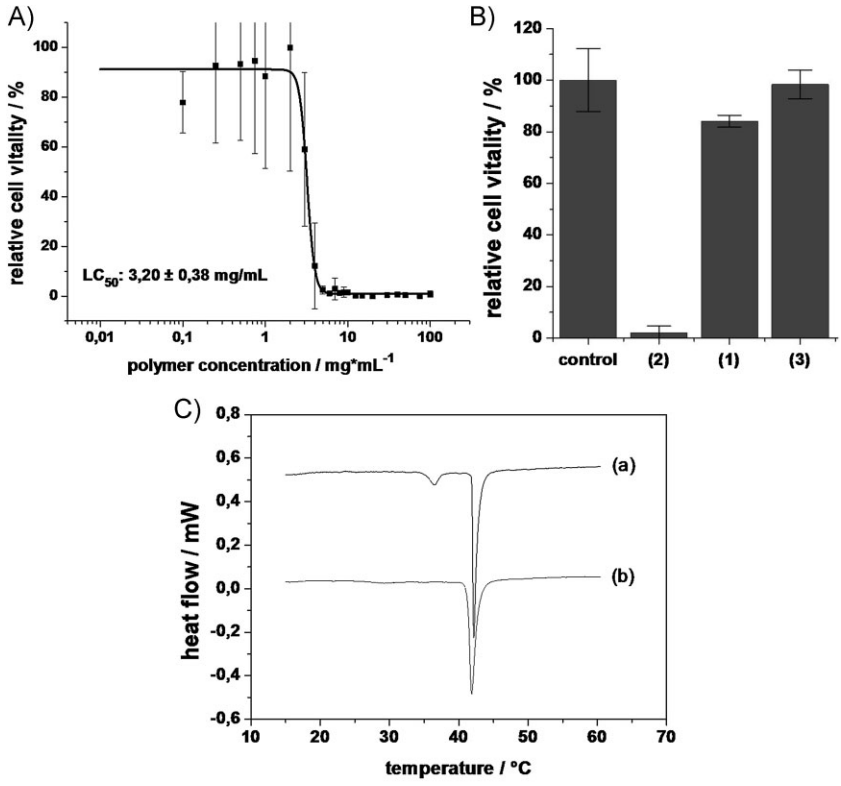


Figure 3. A) Determination of the lethal concentration 50 (LC_{50}) value of **2** on RGC-5 cell line. To determine the LC_{50} value for the polymer **2** on cultured RGC-5 cells, cells were cultured with concentrations from 0.1 to 100 mg mL^{-1} for 24 h. Obtained values for cell vitality were normalized to untreated control cells. Values are expressed as percentage of control. Data show mean of three experiments with different passages (mean ± SD). B) Cell vitality of the RGC-5 cell line after incubation with the physical hydrogel and its components. Metabolic activity was measured with MTS assay after 24 h of direct contact with **2** (20 mg mL^{-1}), **1** (CD, 9.6 mg mL^{-1}), and the final physical hydrogel (**3**). Values of relative cell vitality are expressed as a percentage of the control. Data shows mean of three experiments with different passages (mean ± SD). C) Differential scanning calorimetry scans of DPPC lipid vesicles with **2**. The influence of polymer **2** on MLVs at a concentration of 20 mg mL^{-1} on the phase transition behavior was determined over a temperature range of 15 to 60 °C. For better visual inspection, the graphs were vertically shifted.

These results are consistent with the fact that the incubation of cell membranes with different CDs is a common method to modify the cholesterol content of cell membranes because of the presence of relatively hydrophobic cavities.[19] Here, a loss of cell vitality is often observed.

Consistent with these results, the adamantyl polymer (**2**) clearly interacted with DPPC MLV model membranes and influenced their transition state temperatures and enthalpies. Vesicles composed of DPPC were used as artificial model membranes.[20] Model membranes composed of well-defined lipids can exist in different phases.[21] The gel-state exists at low temperatures, where the occurring hydrophobic interactions between the acyl chains are maximized. Heating of this phase results in a progressive increase in the mobility of the phosphate head group and leads to the pre-transition. As the temperature is raised above T_m, the main transition temperature where the lipid chains melt, a fluid liquid-crystalline phase is formed. When external substances are inserted, the lateral organization of the lipid vesicles specifically changes. These delicate changes in a sample undergoing an endothermic or exothermic transition served as sensitive markers for the inclusion of polymer molecules in this study, and were measured by DSC.[22]

The same explanation as mentioned for the impacts on cell morphology and vitality holds for the high impacts of the tested polymer strands on the transition states of the lipid vesicles in DSC-based experiments. The pre-transition can serve as a very sensitive marker for contaminations in pure lipid layers.[23] In the experiments, the pre-transition completely disappeared in the presence of **2**. These results imply a massive interaction with the model membranes. The main transition temperature, where the lipid layer melts, was slightly shifted. Taken together, **2** interferes with both cell membranes and model membranes in a distinct way. These results show that the underlying toxic mechanisms may be the destruction of the lipid bilayer of the cell membranes.

In spite of the results for the absence of toxic impacts of the final hydrogel, the concept of physical hydrogels based on host/guest interactions for the use as LAVR in the current combination has to be reconsidered. The application design plans to administer the single cyclodextrin and adamantyl-linked strands to finally gel in situ within the eye bulbous. Here, the toxic properties of unbound polymer could have dramatic and rapid effects on the surrounding tissues including the retina, the lens, and the ciliary body. Furthermore, the concept of physical bonds includes the risk of long-term effects. Since the polymer system is based on the formation of complexes between the hydrophobic cavities of the cyclodextrin (host) and the lipophilic adamantyl groups (guest), the *law of mass action* has to be considered. According to this law, depending on both the association and dissociation constants for the complex formation, chemical equilibrium is a dynamic process and free

components will be present at every point of time and exert harmful effects on the eye interior.

In conclusion, this study provides new insights into the in vitro biocompatibility and usefulness of an innovative concept for liquid artificial vitreous body replacements. These results may support the way to the development of new functional polymeric biomaterials in the future. Indeed, the development of physical hydrogels with non-toxic components and different host–guest systems could be convenient as liquid artificial vitreous body replacements in the future.

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