In Vitro and In Vivo Evaluation of a Multifunctional Hyaluronic acid Based Hydrogel System for Local Application on the Retina

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Summary: Conventional treatment of retinal detachment with laser and/or triamcinolon acetonide (TAAC) does not prevent loss of vision in all patients. Therefore, the development of degradable hydrogel patches covering retinal breaks was envisioned as alternative. Stable hydrogels could be formed by crosslinking hyaluronic acid with 1,2,3,4-diepoxybutane. Triamcinolone was diffusible in the gels. The hydrogels were slowly degrading, and mass loss during hydrolytic degradation was observed starting after three weeks. The sterilized gels showed excellent intraocular biocompatibility *in vivo* in rabbit eyes when applied as a patch on the retina. The good retinal adherence of the patch and absence of cellular growth and proliferation in and around the gel indicated the suitability as a material for a retinal patch to prevent cell-migration and proliferation after a retinal break and for local drug application.

Keywords: degradable; hyaluronic acid polymer; network; retina

Introduction

The volume of the vitreous body decreases with age. In some cases, the volume reduction of the vitreous body leads to disruptions in the retina because of the adhesion between the vitreous body and the retina.[1–5] Through the tears in the retina, liquid can penetrate into the subretinal area and detach photoreceptors from the retina pigment epithel cells, leading to blindness when untreated. Classical intervention include fixation of the retina by laser pulses or cryotherapy after pars plana vitrectomy, or administration of triamcinolone (TAAC) for the treatment of inflammatory processes.[6] For both treatments some patients are non-responders, who suffer from a partial or full loss of vision. Therefore, we envisioned a multifunctional[7] hydrogel network combining the required mechanical properties with biofunctionality and biodegradability. The hydrogel has to attach to the retina preferably by strong physical interactions and should allow functional regeneration of the retina.[8] Such a patch could potentially be used as a drug delivery system additionally, which would require to introduce this function without negatively affecting the other capabilities.

The need of multifunctional biomaterials in modern therapies is large, e.g. in the field of intravascular, urogenital, ophthalmic, and orthopaedic applications.[9–10] The combination of different functions is a major challenge in designing the material.[11–12] Polymer networks provide a versatile architecture for multifunctional materials. Covalently crosslinked polymer networks offer control over mechanical properties during the degradation.[13] In case of thermoplastic

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multiblock copolymers, the degradation rate can be adjusted independently from the mechanical properties, which are dominated by the weight fraction of hard segments. Variation of the hard segment enabled linear mass loss.[11–14] In this proceeding covalently crosslinked polymer networks based on hyaluronic acid (HA) have been prepared and investigated. The requirements for the polymer networks were: I) the patch must be injectable; II) strong adhesion to the retina; III) no degradation *in vivo* within six weeks; IV) no induction of cellular proliferation; V) no inflammatory processes caused by the patch.

HA is a component of the vitreous body and carries a large number of OH-groups which enable strong physical interactions through hydrogen bonds.[15–18] Pure HA forms viscous solutions in concentrations above 2 wt.-% in water or aq. buffer, but does not form sticky gels. The free hydroxyl and carboxylic acid groups of HA can be used for the formation of polymer hydrogel networks by crosslinking.[19] A suitable option is the reaction with diepoxides, which readily react with alcohols under basic conditions to form not easily hydrolizable ether bonds, whereby unreacted epoxide groups can be easily removed. The formation of the network can be performed outside the body, the gel purified and sterilized, and implanted on demand by folding of the patch and injecting with an injector system (see below). Such a procedure furthermore allows the incorporation of TAAC into networks of HA during the synthesis or after the synthesis by swelling or precipitation on the surface. The release time can potentially be varied by the amount of incorporated drug as well as the thickness of the patch.

In the following, the synthesis and sterilization of the gels, TAAC diffusion in the gel, and biological performance of the patch in a rabbit model are discussed.

Experimental Part

Hydrogel films of crosslinked HA (Figure 1a) were prepared by adding 2 ml 0.2 M aq. NaOH to 100 mg HA. The mixture was stored at 5 °C over night. Afterwards different amounts of diepoxybutane (DEB) (4.2–188 mmol) and 1.5–3.0 mg (40–80 μmol) $NaBH_4$ were added. The mixture was stirred at 50 °C for 2 h to crosslink (Figure 1b).[20] After this time, the sticky product was placed between two silanized glass plates separated by a 1 mm thick Teflon spacer by a spattle. After three days at room temperature a stable film was formed. Each side of the film was washed three to five times with distilled water. In later biological experiments (see below), no effect of potentially too high pH or remaining epoxides were found. After washing procedure one side of the crosslinked HA film was covered by a 0.05 mm thick Polyimide foil for a better handling in further use before the glass plate was placed on the top again.

The thermal properties were characterized by DSC in the range form −100 to + 150 °C. The water uptake was characterized by placing crosslinked HA films into water, aqueous solutions with different amounts of NaCl, and intra-occular solution (Acri.Pur®) at room temperature and into Acri.Pur® at 35 °C. The *in vitro* mass loss of crosslinked HA was characterized by storing a gel in (30 ml of) a liquid extract of vitreous body at 37 °C.

First experiments to test the capability of the crosslinked HA film as drug delivery system diffusion experiments were performed. Therefore a disc of crosslinked HA film was locating between two parts of an Ussing chamber. One chamber was filled with a saturated solution of TAAC in Acri.Pur® (0.14 mg/ml); the other chamber was filled with pure Acri.Pur® in the beginning. The experiment was performed at 37 °C. The UV extinction of TAAC at 240 nm was measured of both liquids in the chambers for 14 days.

After standard steam sterilization, the biocompatibility was tested in an *in vitro* perfusion cell system (Minucells&Minutissue™, Bad Abbach, Germany). In three chambers pieces of crosslinked HA films were placed on explanted retinal epithelial

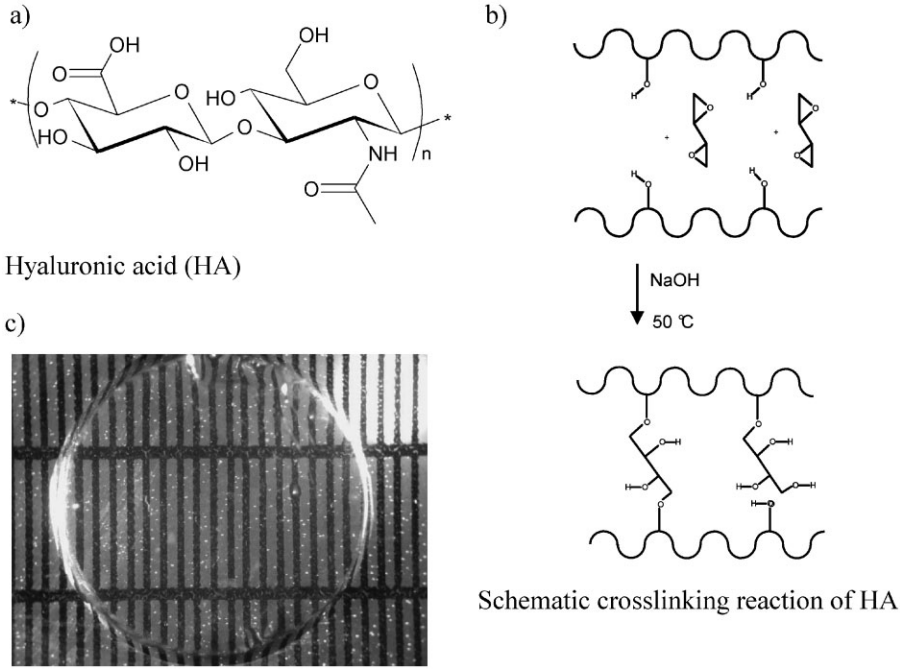

Figure 1.
a) Chemical structure of hyaluronic acid; b) Schematic representation of network formation: HA reacts with diepoxybutane under basic conditions; c) crosslinked HA film (1 mm thick, stained with phenolred).

cells. In one chamber only retinal epithel cells were placed. Through all chambers a steady flow of 40 μl/min from the upper side and lower side was created. DMEM plus 15 vol.-% porcine serum, HEPES-buffer, and 1 wt.-% Penecillin/Streptomycin was used as medium at 37 °C. After four days the retinal epithel cells of all chambers were characterized macroscopically and fixed by paraformaldehyde to characterize the tissue in histological aspects.

In vivo tests were performed in rabbits. The animal study was approved by the local ethical committee (Government of Oberbayern; approval number 55.2-1-54-2531-48-09). Therefore, the HA films were cut in pieces (size 2 × 3 mm, 1 mm thickness) and stained with brilliant blue (Brillant Peel™) by adding a drop of a solution of brilliant blue on the film (Figure 1c) before loading the injector system (VISCOJECT™). The patches were implanted into one eye each at a total number of 10 rabbits (chinchilla bastard, female, 6–9 month old, general anesthetic by i. m. injection of Ketamin [35 mg/kg] and Rompun [2 mg/kg]) after pars plana vitrectomy, in three of them after intentional retinal break, in another three of them after additional lensectomy. The gel-patch was injected into the eye with the help of the injector system through a 2.5 mm sclerotomy and placed on the retina or on a retinal break under physiological NaCl-solution or air. After an observation period of 2 weeks or 6 weeks respectively, the animals were sacrificed by T61; the treated eyes were enucleated and processed for histology (light microscopy).

Result and Discussion

HA can be crosslinked by 1,2,3,4-diepoxybutane (DEB) with good results (Figure 1b). In case of 80 wt.-% (1.6 ml, 16.7 mmol) DEB and 2.8 mg (7.4 mmol) $NaBH_4$ the best gels for the later use have been formed, therefore this composition

was used for all further experiments. HA does not show any transition temperature in DSC measurements in the range from – 100 - +150 °C, while crosslinked HA exhibited a glass transition temperature at 59 °C (with a broad transition from ca. 50-70 °C).

The water uptake of crosslinked HA was in a range 43600 wt.-%. The uptake of water in solutions with different amounts of NaCl was quite different and also resulted in an obvious volume change, so swelling can be adjusted by placing the gel in solutions with different concentrations of NaCl. In case of physiological NaCl-solution, the gel took up 21800 wt.-% saline only. The process of uptake and release due to different salt concentration in the surrounding medium was reversible.

The crosslinked HA gel is stable in Acri.Pur® for more than six weeks at room temperature and at 37 °C (Figure 2). The degradation of a crosslinked HA film took place faster in an extract of the vitreous body of a pig at 37 °C. The mechanism of degradation of crosslinked HA gels in the extract of vitreous body is not yet fully understood and needs further investigation. For many materials, degradation mechanisms *in vivo*, which involve not only hydrolysis, but also enzymatic degradation, direct interaction with cells, and mechanical stress, are not well understood and hardly predictable.[11] A full study with defined model systems *in vitro* and diverse methods employed *in vivo* is needed to achieve a much better understanding of degradation *in vivo* in order to develop the basis for a knowledge-based design of novel materials.

Hydrogels can be applied as drug delivery systems.[21] The diffusion of TAAC, which is relevant in the treatment of retinal breaks, through crosslinked HA films was therefore investigated as basic requirement for potential additional drug release from the gels. The UV extinction at 240 nm of the liquid in both chambers of an Ussing chamber was determined over 14 days (Figure 3) to determine at what time point a homogeneous distribution was reached. A significant diffusion of TAAC through the crosslinked HA film was observed within the first 24 h. After 120 h the equilibrium had been reached. The slight increase in absolute values of UV extinction after 120 h might be caused by HA film gel degradation products.

In the following, evaluation of the toxicity of the gel in an *in vitro* system, the surgical technique for implantation and closure of retinal tears, and the full *in vivo* study are described, which have been performed as comprehensive biofunctionality study of the gels.

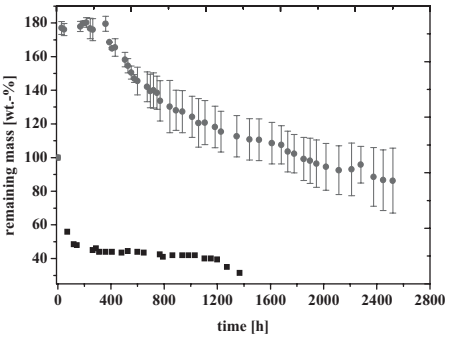

Figure 2.
Remaining mass of swollen crosslinked HA films in Acri.Pur® at 35 °C (dots) and remaining mass of swollen crosslinked HA film in an extract of the vitreous body of a pig (squares) at 37 °C.

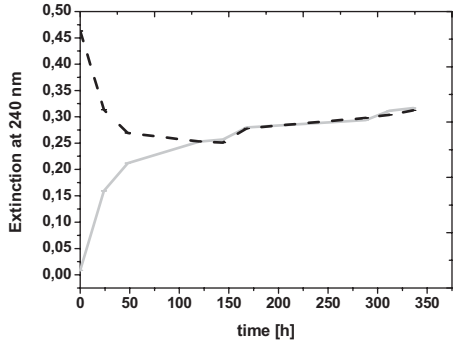

Figure 3.
Diffusion of TAAC through a 1 mm thick crosslinked HA film separating the cells of an Ussing chamber at 37 °C (--: extinctions of chamber one with sat. TAAC solution in the beginning; ——: extinctions of chamber two with pure Acri.Pur® in the beginning).

Basic evaluation of potential toxic effects of the gels on relevant cells was performed in an *in vitro* perfusion culture experiment by contacting an explanted retina with a gel for four days and comparing the results with retinal tissue cultured without gel-patch. In histological aspects no damage of the tissue treated with the patch was observed in the tissue perfusion culture.

Surgical Technique: Implantation of the Gel Patch in Rabbit Eyes with the Help of An Injector System

The first *in vivo* tests were surgical tests. The adhesion of the hydrogel patch on the retina was tested. After pars plana vitrectomy (Figure 4a), the gel-patch could be injected into the vitreous cavity with the injector system (Figure 1b-da) and showed spontaneous adhesion to the retina (Figure 4e and f).

In a second *in vivo* test, a retina break could be sealed with the patch of crosslinked HA (Figure 5a-c). However, exact placement of the patch on the retina or a retinal break was difficult in cases, in which a strong flow rate of the intraocular infusion was necessary for stabilisation of the eye. So in three eyes extended retinal breaks developed intraoperatively. The mechanical fragility of the patch limits the applicable size of the patch.

Clinical Findings, Biocompatibility in Rabbit Eyes

In case of all *in vivo* tests the biocompatibility of the hydrogel was excellent in all eyes without any inflammatory reaction. The gels remained solid and optically clear and showed adherence to the retinal tissue (Figure 6a-d). In the three eyes with extended retinal breaks complete retinal detachment developed within two weeks.

Histological Findings

Histology (paraffin embedded 4 μm-sections, HE-staining) did not show any morpho-logical alterations of the retina or the surrounding ocular tissue caused by the implant. Na scare tissue can be found. There was no cellular reaction or cell proliferation in the area around the gel or cellular migration and proliferation within the gel (Figure 7a). The gel remained clear and free of cells in all cases and was attached to retinal tissue, also in the cases with retinal detachment (Figure 7b). Crosslinked HA films enabled the organism to regenerate the tissue without forming scar tissue.

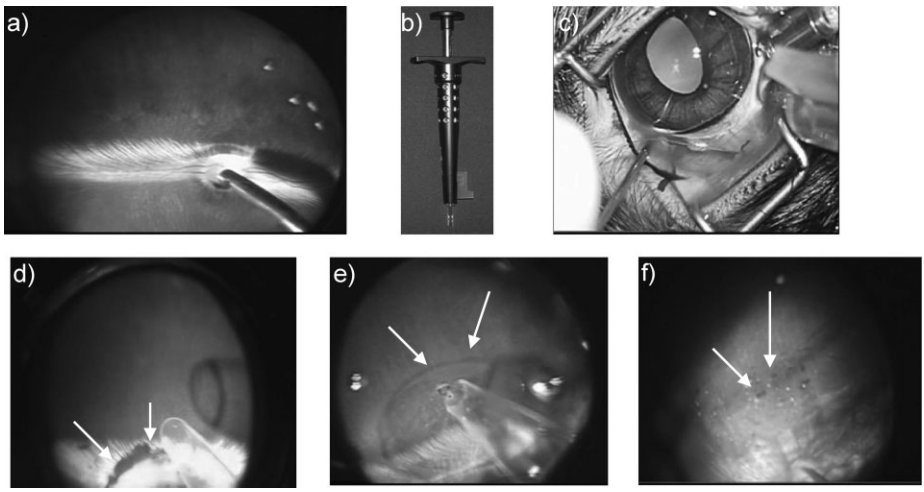

Figure 4.
a) Pars plana vitrectomy; b) injector system; c) preparation of the eye before the injection of the patch; d) locating of the injector system in the vitreous cavity; e) injection of the patch (below the arrows) into the vitreous cavity with the injector system; f) spontaneous adhesion of the patch to the retina (below the arrows).

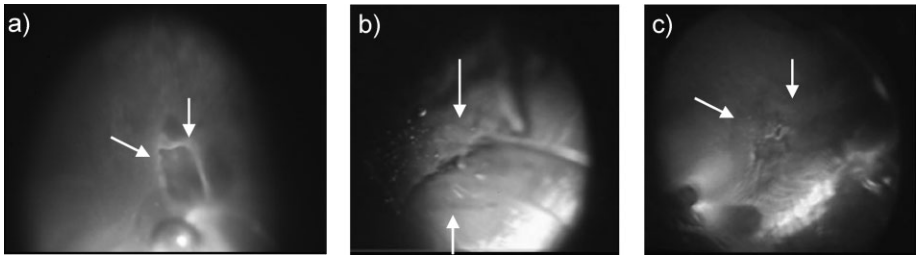

Figure 5.
The patch (stained blue by brilliant blue) is injected through the injector (a) and placed over the retinal break (b, c) a) Retina break (marked by arrows); b) injection of the hydrogel patch (between the arrows) through the injector to cover the retina break; c) sealed retinal break with a hydrogel patch (between the arrows).

Conclusion

HA could be crosslinked by 1,2,3,4-Dipeoxybutan to give flexible, solid hydrogels. TAAC was shown to diffuse through the gel. Both HA and TAAC are components of FDA approved products for the use in ophthalmology. The crosslinked hyaluronic acid hydrogel showed excellent intraocular biocompatibility *in vivo* in rabbit eyes, but were somewhat delicate to handle in injection through a minimized incision. The good retinal adherence of the patch and absence of cellular proliferation in the

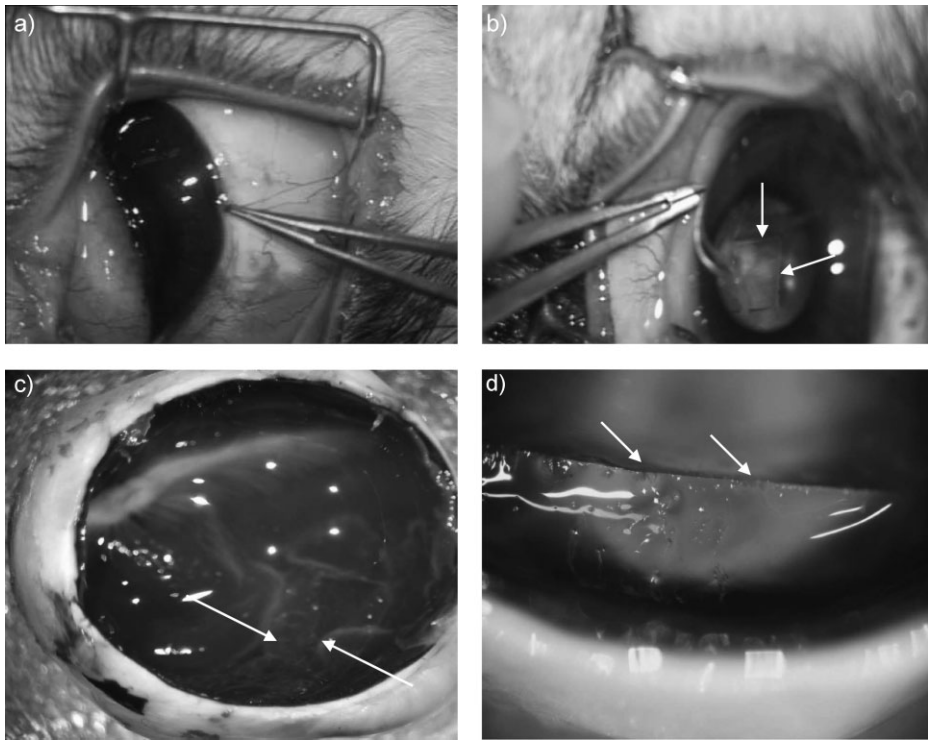

Figure 6.
a) Rabbit eye six weeks after implantation of the hydrogel patch, no irritation can be observed; b) view through the pupil on the retina, the patch (between the arrows) is located on the retina; c) eye explantation after two weeks with the hydrogel patch (between the arrows) on the retina; d) vertical cut through the eye with the patch (below the arrows) on the retina and on pigment epithel.

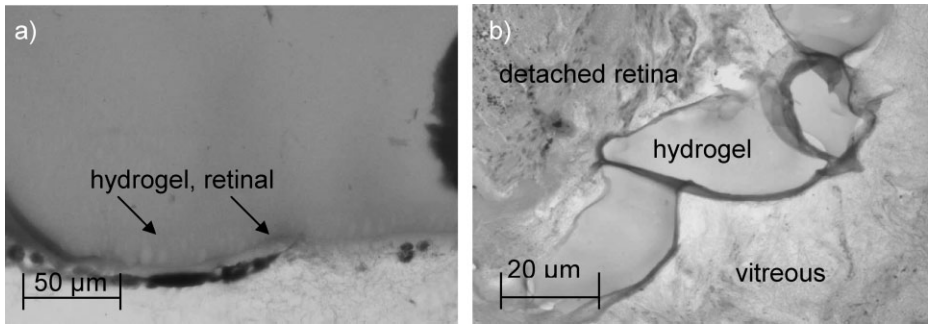

Figure 7.
Histological results after explantation: a) no cellular reaction can be observed; b) gel remained attached to the retina even in case of retina detachment. (paraffin-embeded, 4 µm transversal section through the eye-cup: area of the gel-patch, retina and vitreous; HE-staining).

gel and around the gel indicated the suitability as a retinal patch for immediate sealing, reduction of cell-migration and proliferative vitreoretinopathy (PVR) after a retinal break and for local drug delivery.

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