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Preparation and In Vivo Imaging of Lucifer Yellow Tagged Hydrogels

Lena Möller,[1] *Andreas Krause,*[1] *Ivonne Bartsch,*[2] *Andreas Kirschning,*[1] *Frank Witte,*[2] *Gerald Dräger**[1]

Summary: The synthesis of new Lucifer Yellow labeled hyaluronic acid- and alginate hydrogels that are covalently cross-linked is reported. Biocompatibility and biodegradation of the fluorescent tagged hydrogel is investigated using an *in vivo* fluorescence imaging system.

Keywords: alginate; biocompatibility; hydrogel; hyaluronic acid; *in vivo* imaging; lucifer yellow

Introduction

Hydrogels are widely used as matrix scaffolds in the field of tissue engineering.[1,2,3] Especially *in situ* generated gels show various advantages: a) the cells can be added before the gelation occurs enabling a homogenous cell distribution within the gel, and b) the shape of the gel can be adjusted individually according to the mould used. Furthermore, a fast *in situ* gelation process allows to generate hydrogels *in vivo*, prepared by injecting both components simultaneously and punctual at the area of defect.[4]

We disclose a gel system, composed of hydrazide-modified alginate and hyaluronic aldehyde providing these desired features. Its properties for cell entrapment and tissue formation as well as the physical-rheological behavior will be reported in due course.[5]

In this work, the practicability of our gels for medical application regarding a fast, easy and reproducible preparation and implantation was evaluated. The biocompatibility of the prepared hydrogels was investigated *in vivo*. In order to simultaneously analyze the degradation process, the hydrogels were tagged with the dye Lucifer Yellow. This staining enables the visualization of the gel in living mice using the *in vivo* fluorescence imaging system Maestro™ (CRi/a multispectral acquisition and analysis system).[6] It allows the *in vivo* imaging of small laboratory animals as well as repetitive measurements within the same laboratory animal over a long period of time. This leads to a reduced animal consumption according to the EU guideline (3Rs: Replacement, Reduction and Refinement). In addition the usage of a fluorescent dye such as Lucifer Yellow is possible and the disturbing background autofluorescence can be subtracted.

Materials and Methods

General Remarks

Unless otherwise stated, all chemicals and solvents were purchased in "per analysis quality" from *Sigma-Aldrich*, Steinheim, Germany or *Acros Organics*, Nidderau, Germany and used as received. All dialysis steps were performed using Visking membrane tubes (regenerated cellulose, 0.025 mm membrane thickness, 28.6 mm diameter, obtained from *Roth*, Karlsruhe, Germany) with a molecular weight cut-off of 14.000 g/mol. All modified polymers were exhaustively dialyzed for 3 to 5 days against distilled

[1] Gottfried Wilhelm Leibniz University Hannover, Centre of Biomolecular Drug Research (BMWZ)/ Institute of Organic Chemistry, Schneiderberg 1b, D-30167 Hannover, Germany
Fax (+49) 511 762-3011;
E-mail:draeger@oci.uni-hannover.de
[2] CrossBIT, Center for Biocompatibility and Implant-Immunology, Hannover Medical School, Feodor-Lynen-Straße 31, D-30625 Hannover, Germany

water. Lyophilization was performed with a *Christ* Alpha 2-4 (Christ, Osterode, Germany) freeze dryer. Absorption of UV-light was measured with a *Shimadzu* UV-1601PC spectrophotometer (Kyoto, Japan). The preparation of uniform hydrogels was performed in CultureWell Silicone Gaskests (*BioCat*, Heidelberg, Germany).

Synthesis of Alginate Hydrazide

The functionalization of polysaccharides with the hydrazide functionality has been described in the literature. Commonly, the hydrazide is either directly attached to the polymer-backbone[7,8] or *via* adipic dihydrazide.[9,10,11] In contrast to these methods, we developed a direct route based on standard carbodiimide-based chemistry which allows to transform the carboxylic acid moieties of alginate and hyaluronic acid into the corresponding hydrazides.[5] Thus, alginic acid sodium salt (Alg, very low viscosity, *ABCR*, Karlsruhe, Germany) gave the hydrazide derivative (Alg-Hyd) **4** with a degree of functionalization of about 35%. Finally Ald-Hyd **4** was purified by thorough dialysis against distilled water for 4 days under repeated water change. After lyophilization alginate hydrazide was collected in colorless cotton like form.

Synthesis of Lucifer Yellow Tagged Hyaluronic Acid Aldehyde

Hyaluronic acid aldehyde (HyA-Ald) **1** was synthesized from hyaluronic acid sodium salt (HyA, *Streptococcus equi*, $M_w = 1.63 \times 10^6$ Da, *Sigma-Aldrich*, Steinheim, Germany). With Alg-Hyd **4** the aldehyde is used to form spontaneously jellying hydrogels for applications.[5] In addition, the fluorescent dye Lucifer Yellow was coupled to HyA-Ald **1** in order to monitor the degradation process *in vivo* by optical imaging.

The oxidation process was performed by addition of sodium periodate to an aqueous solution of HyA, following a procedure related to the protocol of Bulpitt *et al.* or Jia *et al.* [9] After 12 hours, HyA-Ald **1** was obtained with a degree of oxidation of about 100% as judged by ^{1}H-gel NMR spectroscopy.[5] The oxidation led to cleavage of the C_2-C_3 diol in the monomeric D-Glucoronic acid unit, present in HyA.

Coupling of Lucifer Yellow to the polymer was achieved by hydrazone formation between the aldehyde group of the oxidized polymer and the hydrazide moiety in Lucifer Yellow (Figure 1). Thus, HyA-Ald **1** (20 mg, 100.23 μmol aldehyde, 1.0 equiv.) was dissolved in phosphate buffer (1.33 mL, pH= 6.5, 0.1 M) at 50 °C. Lucifer Yellow (6.88 mg, 15.03 μmol, 0.15 equiv.) was added and the reaction mixture was stirred for another 6 hours at the same temperature. Because hydrazones easily undergo hydrolysis, the newly formed linkage was selectively reduced by treatment with sodium cyanoborohydride (0.95 mg, 15.03 μmol, 0.15 equiv.) for 12 hours at 4 °C yielding polymer **3**. The reaction mixture was dialyzed against distilled water and the dialysate was investigated using UV-spectroscopy in order to determine the amount of dye released into the dialysate. This procedure was repeated for polymer-dye conjugates **2** which had not been subjected to reductive conditions. Finally, the functionalized polymers were lyophilized.

Hydrogel Formation

Hydrogels were prepared according to Ossipov *et al.* [7] and Hudson *et al.* [12] Therefore alginate hydrazide **4** and hyaluronic acid aldehyde **1** were synthesized, purified and lyophilized as reported above. In a general procedure both polymeric components were dissolved in $H_2O_{dest.}$ At 40 °C until a clear polymer solution was obtained. Typically, 1% aqueous solutions were prepared and mixed well for 30 sec forming stable transparent gels within minutes.[5] Lucifer Yellow tagged hydrogels were obtained from hyaluronic acid derivative **3** and alginate hydrazide **4**. Although about 15% of the aldehyde functionalities of polymer **3** are blocked by the dye, the remaining aldehydes are still sufficiently functionalized to produce stable gels. Regarding the *in situ* hydrogelation process, the basic principles of the covalent cross linking is schematically depicted in Figure 2.

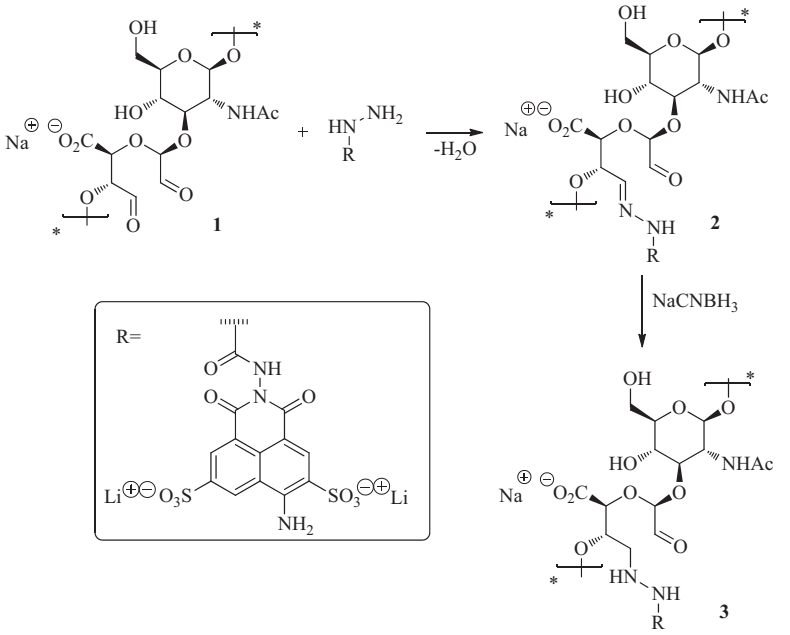

Figure 1.
Functionalization of hyaluronic acid with the dye Lucifer Yellow followed by reduction of the resulting hydrazone with sodium cyanoborohydride.

Preparation of the Hydrogels for the Implantation in Mice

All *in vivo* investigated hydrogel samples were freshly prepared before implantation. Therefore alginate acyl hydrazide **4** (2.0 mg, 10.53 μmol) and Lucifer Yellow tagged hyaluronic aldehyde **3** (4.0 mg) were dissolved in 100 μL distilled water each. 25 μL of both polymer solutions were mixed and filled in silicone gaskets with a diameter size of 6 mm. After 10 min of gelation the samples were directly implanted subcutaneously.

Animals

All animal experimental procedures have been approved by the local governmental animal care committee LAVES (Land-

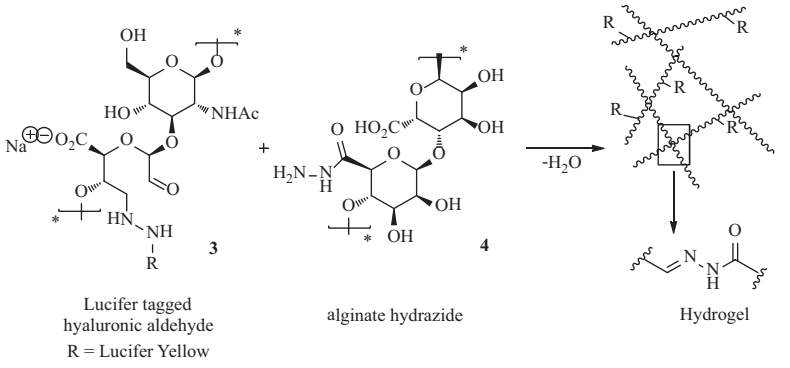

Figure 2.
Preparation of Lucifer Yellow tagged hyaluronic-alginate hydrogels.

esamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany, Approval No. 33-42502-04-10/0248).

In total, two female hairless mice (Crl:SKH1-*hr*) were used for that preliminary *in vivo* study. The Crl:SKH1-*hr* mice are hairless but immunocompetent, so they are capable of carrying out an immune response. The average weight was approximately 26 g. After implantation, each mouse was housed individually and received a standard diet of water and Altromin 1324 *ad libitum*. Rooms of animal husbandry were illuminated by artificial light 14 hours a day starting at 7 am.

Implantation Procedure

Animals were anesthetized by an intraperitoneal injection of xylazine 2% and ketamine 10%. The area of implantation was cleaned according to surgical guidelines. Subcutaneous pockets were created by blunt dissection with a forceps. The implants were placed in these pockets using a spoon spatula (Figure 3). The skin was closed with resorbable surgical suture material (Vicryl, Ethicon, *Johnson & Johnson GmbH*, Germany).

Multispectral Acquisition and Analysis System

The *in vivo* fluorescence imaging system Maestro™ from CRi (Minnesota, USA) is a special camera and software system that enables the imaging of whole small rodents. It provides users with a patented method of removing autofluorescence emitted from images of skin and other tissues.[6] According to the used fluorophore appropriate filter sets for excitation and emission light can be employed. The excitation wavelength of Lucifer Yellow was about 425 nm. The emission maximum was about 528 nm, so we used the blue filter set performing the *in vivo* measurements.

Figure 3.
Implantation of the Lucifer Yellow tagged hydrogel subcutaneously in an immunocompetent hairless mouse using a spoon spatula.

In Vivo Measurements

Animals were anaesthetized by intraperitoneal injection of xylazine 2% and ketamine 10%. In one mouse *in vivo* imaging was conducted at days 0 (immediately after implantation), 1, 2, 3, 7, 14, 21, 35, 43, 49, and 56. The other mouse served as a long-term trial and was additionally measured at days 63 and 125.

In order to compare the signal intensity at the different measurement time points, the original exposure time derived from one sample was kept constant during repeated measurements.

Histology

Animals were anaesthetized by intraperitoneal injection of xylazine 2% and ketamine 10% and sacrificed via decapitation. Explanted tissues were immersion fixed in 4% neutral buffered formalin for 24 hours. Then, one half of each sample was embedded in paraffin and cut at 7 μm using a motorized microtome for histological examination after staining with (H&E) following routine procedures. The other half of the sample was stored at −80 °C and was cut using a cryostat for fluorescence detection.

Results and Discussion

Synthesis and Evaluation of Lucifer Yellow Tagged Hyaluronic Acid Aldehyde 3

Lucifer Yellow was coupled to hyaluronic acid in order to investigate the polymer/hydrogel degradation *in vivo*. If dye and polymer are covalently linked to each

other, the disappearance of the dye stands in direct correlation to the degradation of the polymer. In order to remove unbound Lucifer Yellow, the reduced and the non-reduced Lucifer Yellow hyaluronic acid conjugates **2** and **3** were dialyzed and the dialysates were investigated with UV spectroscopy at 430 nm, which is the absorption maximum of Lucifer Yellow. The obtained results are listed in Figure 4. The filled squares display the dialysate absorption of the reduced polymer-dye **3** over time, while the non-filled squares show the dialysate absorption for the non-reduced adduct **2**. The arrows show the timepoints when the dialysis water was renewed. Within the first 24 h the Lucifer Yellow concentration in reached a plateau and therefore water was exchanged once a day immediately after UV-measurement. It could be demonstrated, that without adding the reducing agent unbounded Lucifer Yellow could still be detected in the dialysate even one week after dialysis with several changes of water. This indicates that the hydrazone moiety is not stable under dialysis conditions likely due to hydrolysis. In contrast to these observations, reduced hydrazone cross-linked polymer **3** released no Lucifer Yellow from the polymer after 120 h of dialysis (Figure 4). Since Lucifer Yellow could not be detected in the supernatants afterwards, it can be stated that unbound or remaining hydrazone-linked Lucifer Yellow had been removed completely during dialysis. Finally, hydrogels were successfully prepared upon mixing the polymeric solutions of alginate acyl hydrazide **4** and Lucifer Yellow tagged hyaluronic acid aldehyde **3**. After 10 min. of gelation, the gels were directly implanted subcutaneously in the left and right shoulder region of two mice. The viscosity of all gels was adequate for subcutaneous implantation procedures.

The obtained results correlate well to the work from Hudson *et al.*, who prepared Amphotericin B loaded dextran cellulose hydrogels using a similar hydrazone cross linking technique.[12] Amphotericin B was bound to the polymeric network *via* hydrolysable imine bonds, comparable to the Lucifer Yellow ligation described above. It was shown that non reduced imine linkages led to a continuous drug release over eight days. Consequently, the reduction step is essential in order to achieve non hydrolysable Lucifer Yellow polymer linkages.

In Vivo Measurements

The signal intensity of the implanted Lucifer Yellow tagged hydrogel was reduced to 37% of its initial intensity after one day indicating a fast hydrolysis of

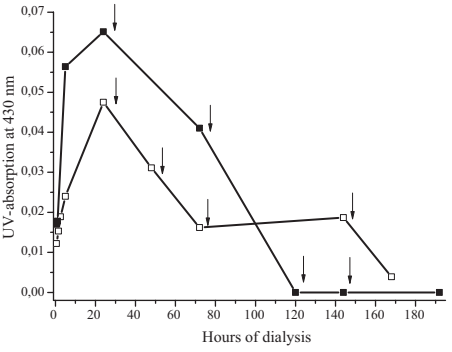

Figure 4.
UV-absorption at 430 nm of the dialysate of reaction mixtures **3** and **2**, measured at different times of dialysis; ■: reduced Lucifer Yellow hyaluronic acid conjugate **3**, □: non-reduced Lucifer Yellow hyaluronic acid conjugate **2**; ↓: point of time when dialysate was exchanged.

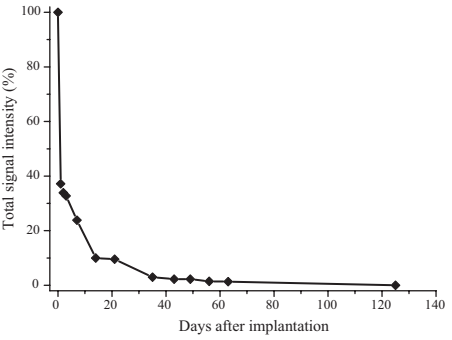

Figure 5.
Signal intensity of the implanted Lucifer Yellow tagged hydrogel is not detectable anymore after 125 days of implantation.

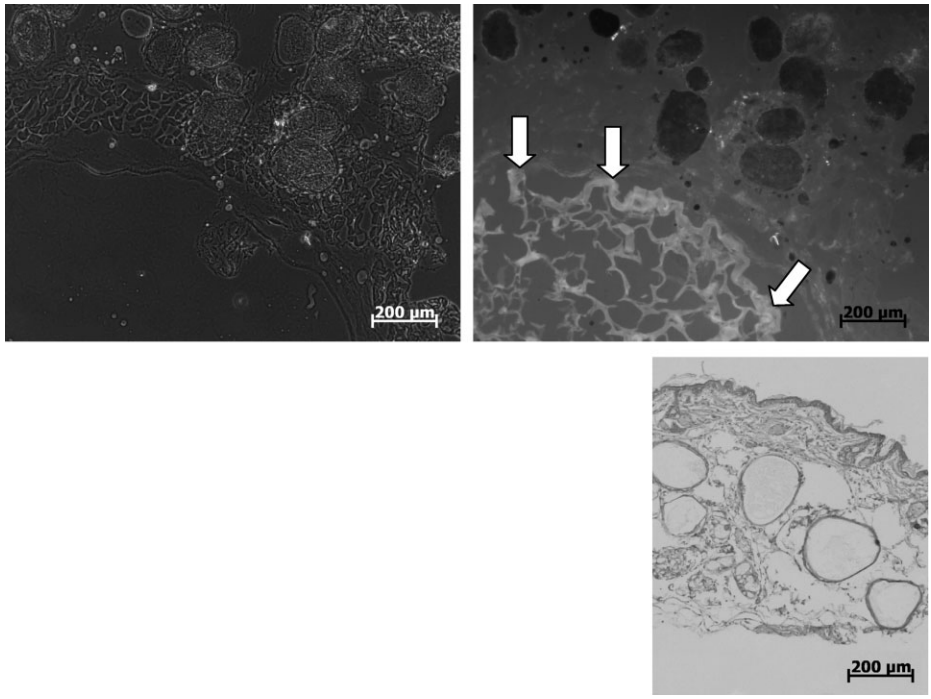

Figure 6.
upper left: Histologic cryo section (10 µm thick, phase contrast) of the mouse skin adjacent to the implanted Lucifer Yellow tagged hydrogel after 51 days of implantation. A thin fibrous capsule is ensheathing the hydrogel implant. upper right: Histologic cryo section of the mouse skin adjacent to the implanted Lucifer Yellow tagged hydrogel after 51 days of implantation. The fluorophore Lucifer Yellow can still be seen (white arrows). lower right: Histologic paraffin section (7 µm thick, hematoxylin-eosin staining) of the mouse skin adjacent to the implanted Lucifer Yellow tagged hydrogel after 125 days of implantation.

unbound Lucifer Yellow imine. It could be shown, that Lucifer Yellow was still in place inducing a measureable fluorescent signal at day 63. At day 125 no fluorescent signal could be detected any more (Figure 5).

Biocompatibility of Implanted Hydrogels

The implanted hydrogels did not evoke any clinical adverse effects in form of allergic or other immunological rejection in the hairless mice during the entire study interval. The missing inflammation could be approved by the histological sections. No foreign body reaction in the form of a fibrous capsule or the presence of inflammation cells as neutrophils or macrophages could be observed (Figure 6).

Conclusion

In conclusion we showed that Lucifer Yellow labeling of hyaluronic acid derived aldehydes yields materials reactive enough to undergo hydrogel formation with alginic acid hydrazide. The resulting hydrogels were implanted in immunocompetent hairless mice, could be monitored by *in vivo* imaging and showed promising biocompatibility.

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