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Comparison of in vitro and in vivo protein release from hydrogel systems
Comparison of In Vitro and In Vivo Protein Release from Hydrogel Systems

Stefanie Wöhl-Bruhn, Muhammad Badar, Andreas Bertz, Brigitte Tiersch, Joachim Koetz, Henning Menzel, Peter P. Mueller, Heike Bunjes

1Technische Universität Braunschweig, Institute of Pharmaceutical Technology, Mendelssohnstraße 1, 38106 Braunschweig, Germany
2Helmholtz Centre for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany
3Technische Universität Braunschweig, Institute of Technical Chemistry, Hans-Sommer-Straße 10, 38106 Braunschweig, Germany
4Universität Potsdam, Institute of Chemistry, Karl-Liebknecht Straße 24-25, 14476 Potsdam, Germany

†corresponding author (Technische Universität Braunschweig, Institute of Pharmaceutical Technology, Mendelssohnstr.1, 38106 Braunschweig, Germany, phone: +49 531 391 5657, fax: +49 531 391 8108, e-mail: heike.bunjes@tu-braunschweig.de)
Abstract

Hydrogel systems based on hydroxyethyl starch polyethylene glycol methacrylate (HES-P(EG)\textsubscript{6}MA) or hydroxyethyl starch methacrylate (HES-MA) were used to assess the protein release behavior. Here, we analyzed the \textit{in vitro} release of FITC-anti-human antibodies incorporated in either HES-P(EG)\textsubscript{6}MA or HES-MA hydrogel delivery systems in PBS or human serum. In addition, hydrogel disks and microparticles prepared from the two polymers were subcutaneously implanted in BALB/c mice. The \textit{in vivo} release of FITC-IgG was non-invasively monitored by an \textit{in vivo} imaging system (IVIS 200) over a time period of up to 3 months. The imaging system allowed to assess individual animals over time, therefore only a small number of animals was required to obtain high quality data. The reduction in fluorescence intensity at the site of administration was compared to \textit{in vitro} release profiles. These investigations demonstrated a sustained release from HES-MA hydrogel disks compared to rapidly degrading HES-P(EG)\textsubscript{6}MA disks and microparticles. The sustained release from HES-MA disks could be further optimized by using increased polymer concentrations. Human serum as \textit{in vitro} release medium reflected better the \textit{in vivo} conditions than PBS, suggesting that the presence of organic substances like proteins or lipids may play a significant role for the release kinetics.

\textbf{Keywords:} In vivo imaging system (IVIS), hydrogel disks, hydrogel microparticles, release, \textit{in vivo} - \textit{in vitro} correlation, hydroxyethyl starch (HES)
1.1 Introduction

Hydrogel drug delivery systems are an attractive delivery system for the administration of therapeutically relevant biomacromolecules such as antibodies and other proteins. With their high water content hydrogels form a protein-protecting environment with good tissue compatibility and provide the possibility to influence the protein release [1-2]. Formation of hydrogels from aqueous prepolymer solutions can be achieved by various methods, e.g., crosslinking by radical polymerization [3]. In this context, the derivatization of water-soluble polymers with polymerizable groups has been studied widely [4-6]. Biodegradable natural substances like gelatin, pectin or dextran are often used as a polymeric backbone [1]. Recently, polymers based on modified hydroxyethyl starch (HES) have been used as matrix for drug delivery systems [7-8]. HES (MW 130,000) has been used as a plasma expander already since the 1970s and is considered as highly biocompatible material [9]. The synthesized polymers can be processed by UV-induced crosslinking into bulk hydrogels and hydrogel microparticles which were characterized in detail previously [7-8, 10-11]. Polymers were produced with various degree of substitution (DS) leading to variable network densities and consequently different release kinetics. In contrast to HES-based polymers derivatized with hydroxyethyl methacrylate (HES-HEMA) which show a limited water solubility [8], better properties were obtained for the new HES-based derivatives. Linkage of the methacrylate groups via polyethylene glycol spacers with 6 or 10 ethylenoxide units (HES-P(EG)$_6$MA, HES-P(EG)$_{10}$MA) increased the water solubility of the prepolymers. A better water solubility was also observed upon direct linkage of the methacrylate groups to the HES backbone (HES-MA) which resulted in a polymer with higher hydrolytic stability. All new polymers led to hydrogel systems with improved release profiles in particular showing a reduced burst release [10].

To determine the release of incorporated proteins or antibodies from drug delivery systems in vitro release studies are commonly performed. The released amount can be measured in the release medium which typically consists of buffered salt solutions. Due to a limited comparability to in vivo conditions such studies may, however, not be highly predictive of the in vivo release behavior. For investigations of the in vivo release from subcutaneously (s.c.) administered delivery systems various methods can be used. In a conventional setup, for example, several animals are sacrificed at each time point and the residual drug content of the remaining delivery system is analytically determined [12]. Other methods detect the released drug amount in blood samples, e.g., by HPLC [13] or radioactivity measurements of the labeled drug [14]. Recently, in vivo imaging techniques have become available that are able to track the in vivo behavior of fluorescently labeled substances and can be used for biodegradation and biodistribution studies [15-16]. In comparison to conventional in vivo studies, these imaging systems are advantageous because individual animals can be monitored over an extended period of time. This leads to an improved quality of data and additionally a much smaller number of animals is required for such studies. In order to identify in vitro release conditions leading to results that reflect the in vivo behavior as closely as possible comparative studies need to be carried out. Finally this may allow establishing an in vitro-in vivo correlation [17]. A good correlation of in vitro studies and results gained from computer modeling of pharmacokinetic data to in vivo experiments was shown for protein-loaded tristearin implants [18]. Other studies revealed a faster release of incorporated substances from implantable system in vivo than in vitro [19-20].
The present study aimed at comparing the release from HES-P(EG)$_8$MA and HES-MA hydrogel disks and microparticles in vitro and in vivo. While hydrogel disks are supposed to have a higher sustained release potential due to their smaller surface-to-volume ratio, hydrogel microparticles have the advantage of being applicable by simple injection. Hydrogel systems prepared from these starch-based polymers (Fig. 1) showed a long term in vitro release of FITC-labeled substances (FITC-dextran, FITC-IgG) of up to one year in phosphate buffer at 37 °C [10]. To gain information about the comparability of these data with those obtained under in vivo conditions, the release of FITC-IgG from implanted hydrogel disks and subcutaneously injected microparticles was studied in mice. The decrease of fluorescence intensity of the incorporated FITC-IgG was compared to in vitro release data obtained by a half-change method using phosphate buffered saline (PBS) or human blood serum as release media.

2 Materials and Methods

2.1 Materials

Anti-human IgG (whole-molecule)-FITC antibody produced in goat (FITC-IgG, 12.7 mg/mL) was purchased from Sigma Aldrich (Germany). FITC-IgG solution was dialyzed by Slide-A-Lyzer® Mini Dialysis Units 10,000 (Pierce, Rockford, USA) against PBS to remove sodium azide. Irgacure®2959 was purchased from Ciba Specialty Chemicals (Basel, Switzerland). Polyethylene glycol with an average molecular weight of 12,000 (PEG 12,000) was purchased from Fluka (Steinheim, Germany). Converted human serum and antibiotic/antimycotic solution (consisting of 100 U/mL penicillin R-Na, 100 µg/mL streptomycin-sulfate and 0.25 µg/mL amphotericin B) were obtained from PAA (Cölbe, Germany). Phosphate buffered saline (PBS, pH 7.4) consisted of 8.0 g NaCl, 0.2 g KCl, 1.44 g Na$_2$HPO$_4$ and 0.24 g KH$_2$PO$_4$ ad 1000 ml MilliQ water (Arium 611, Sartorius, Göttingen, Germany).

2.2 Polymers

Both polymers were based on hydroxyethyl starch 130,000. HES-P(EG)$_8$MA (hydroxyethyl starch-polyethylene glycol methacrylate) was synthesized and characterized according to the procedure of van Dijk-Wolthuis et al. [21] as described previously by our group [10]. HES to polyethylene glycol methacrylate-imidazole carbamate (P(EG)$_8$MACI) to DMAP was used in a concentration ratio of 1 : 0.1 : 0.1. After a 3 h reaction, a polymer with a degree of substitution (DS) of 0.026 was obtained. Hydroxyethyl starch-methacrylate (HESMA, DS 0.083) was synthesized within 20 h using HES : GMA : DMAP in a ratio of 1 : 0.5 : 0.25. The DS was determined by $^1$H-NMR in D$_2$O [10].
2.3 Preparation of Hydrogel Disks

Hydrogel disks were produced by dissolving HES-P(EG)₆MA with a degree of substitution (DS) of 0.026 and HES-MA with a DS of 0.083 in a PBS solution of 0.05 wt% photoinitiator Irgacure® 2959 and 2 % FITC-IgG solution (containing 25.4 µg FITC-IgG) adjusting a polymer concentration of 20 wt% or 30 wt%. 20 µl of the mixture were dropped on a piece of parafilm and exposed to UV light for 40 min (366 nm, model NU-8 KL, Benda, Wiesloch, Germany) for crosslinking. Each disk contained 25.4 µg FITC-IgG. Hydrogel disks without FITC-IgG were prepared as controls in the animal experiments.

2.4 Preparation of Hydrogel Microparticles

HES-P(EG)₆MA and HES-MA microparticles were prepared according to the following general procedure by a water-in-water emulsion process [8, 10]: aqueous solutions (PBS) of HES-P(EG)₆MA or HES-MA (2 wt%, 9 g), the photoinitiator Irgacure® 2959 (0.1 wt%, 0.9 g) and FITC-IgG solution (65 µl containing 825.5 µg FITC-IgG) were filled in a 20 mL glass vial in the mentioned order. An aqueous solution (PBS) of PEG 12,000 (30 wt%, 6.0 g) was added. After cooling at 0 °C over 10 min, the system was vigorously mixed with a vortex-Genie mixer (Bender & Hobein AG, Zurich, Switzerland) for 1 min leading to a water-in-water emulsion. After exposure to UV light (Nu-8 KL, Benda, Wiesloch, Germany) at 366 nm for 30 min the microparticle suspension was washed five times with demineralized water to remove adherent PEG from the surface of the microparticles. After decanting off the main fraction of the last washing water the remaining highly concentrated microparticle suspension (about 1.1 g containing ~ 0.75 mg FITC-IgG / g microparticle suspension) was used for further studies.

2.5 Characterization of Hydrogel Microparticles

To determine the encapsulation efficiency for the hydrogel microparticles the amount of FITC-IgG in the supernatants of the five washing steps (including the aqueous PEG solution that was used in the preparation step) was analyzed spectrophotometrically with a fluorescence micro-plate reader (Tecan Austria GmbH, Grödig, Austria). The encapsulation efficiency was calculated from the cumulative loss of the labeled substance into the PEG phase and the supernatants and the total initially incorporated amount.

Furthermore the hydrogel microparticles were investigated by laser diffraction (Beckman Coulter LS 13320, Fullerton, USA) for their particle size distribution. A sample of about 10 mg microparticle suspension was diluted in 2 mL MilliQ water (Arium 611, Sartorius, Göttingen, Germany) and, in the case of HES-MA microparticles, sonicated for 15 min before the measurement. The resulting suspension was diluted to the final concentration with demineralized water up to approximately 125 mL within the measurement system. The particle size was calculated from the average of six measurements (each 90 s). The evaluation model applied on the samples was based on the Mie theory [22] with a refractive index of 1.33 for water and 1.4 for the sample. The volume distributions were calculated and the results are given as d10, d50 and d90 values.

Fluorescence microscopy (Axio Observer A.1, Carl Zeiss AG, Jena, Germany), brightfield microscopy (DMLM photo microscope, Leica Microsystems, Wetzlar, Germany) and cryo-SEM imaging (Hitachi S-4800, Hitachi, Japan) were used to characterize the morphology of hydrogel microparticles. For cryo-SEM images microparticles were rapidly frozen in melting nitrogen and fractured in the cryo chamber at -180 °C. After etching for 45 sec at -98 °C the samples were sputtered with a thin platinum layer.
2.6 In Vitro Release Studies

The release of the incorporated FITC-IgG was investigated in vitro by the half-change method. Hydrogel disks (accurately weighed) were placed in 500 µl release medium (PBS containing 0.02 % sodium azide or converted human serum containing 0.02 % sodium azide and 1 % antibiotic/antimycotic solution) in 2 mL micro tubes (Sarstedt, Nuembrecht, Germany). Hydrogel microparticles (~ 30 mg, accurately weighed) were dispersed in 1 mL release medium. All samples were incubated in a shaking water bath at 37 °C. At defined time points the samples were slightly shaken and, in case of microparticles, centrifuged for 1 min at 16,060 x g (Biofuge pico, Heraeus, Hanau, Germany). 50 % of the supernatants were removed and replaced by tempered (37 °C) fresh release medium. The supernatants were analyzed for the fluorescence label with a fluorescence micro-plate reader (Tecan Austria GmbH, Grödig, Austria) for FITC-IgG. All samples were incubated and analyzed in triplicate; data given in the graphs correspond to the mean values ± standard deviation. For tracking a possible loss of fluorescence, FITC-IgG in PBS or human serum was used as control. The vials were incubated and analyzed under the same conditions as the samples. No loss of fluorescence intensity was detected within the measurement interval. In order to determine the 100 % release values of hydrogel cylinders the release of FITC-IgG in PBS was monitored for the HES-P(EG)_6MA disks until a plateau in the release curves was reached. HES--MA cylinders which did not release 100 % of FITC-IgG under the experimental conditions applied were assumed to contain the same concentration of IgG as released from the HES-P(EG)_6MA disks. The 100 % values for the HES-P(EG)_6MA microparticles were determined in analogy to the disks. The cumulative released FITC-IgG values over the time are given in relation to these 100 % values assumed for the corresponding systems. Unfortunately the 100 % values for the HES-MA microparticles could not be determined since these particles did not completely release their protein load within the tested period of time and a completely analogous behavior to HES-P(EG)_6MA microparticles in the preparation process cannot simply be assumed. The release data for HES-MA microparticles are not shown for this reason. In spite of the addition of antimicrobial agents the samples studied in serum as release medium tended to show signs of contamination after one month of study under the non-sterile conditions of the release experiment. For this reason the amount of released FITC-IgG was considered for the calculation until day 15 only. Since all hydrogel samples contained equal amounts of FITC-IgG the 100 % release values for the studies in serum were adopted from the release studies in PBS. For better comparability of in vitro and in vivo measurements, the single supernatant fractions collected in the half-change test were additionally imaged and analyzed for their fluorescence intensity in the IVIS system (chapter 2.7). The values obtained by the two fluorescence methods were in good agreement for all tested samples (data not shown).

2.7 In Vivo Release Studies

In vivo studies conformed to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals [23] and were performed with the approval of the local Institutional Animal Care and Use Committee (permission nr. 33.42502/07-10.05). Mice were kept in individually aerated cages and fed with a cholesterol-free standard diet and water ad libitum. Male BALB/c mice, approximately 4 months of age, were anesthetized with ketamin (10 mg/kg) and xylazine (4 mg/kg). After shaving, 20 µl hydrogel disks (20 wt% or 30 wt% polymer concentration) containing FITC-IgG were placed in subcutaneous pockets at the left and right side of the tail base, respectively. The pockets were closed with simple interrupted sutures. Over a period of up to 3 months after implantation of the disks, the mice were scanned in predetermined intervals in an IVIS® In Vivo Imaging System 200 (Xenogen). A
second group of mice received s.c. injections of hydrogel microparticle suspensions (50 µl) containing a comparable amount of FITC-IgG in the lower dorsal region between the hind limbs. These mice were scanned over a time period of 10 days. To confirm the general traceability of the fluorescent label 20 µl FITC-IgG solution (25.4 µg FITC-IgG in PBS) were injected s.c. on the dorsal side of the neck of three mice. As a negative control, blank polymer disks were implanted on the dorsal side of the neck of three mice. Scanning was performed under ketamin anesthesia using the GFP filter (488 nm / 509 nm) and low fluorescence level string for 1 sec. exposure. The fluorescence intensity of each sample was calculated from the fluorescence signal (p/sec/cm²/sr) using the Living Image® software Version 2.6 (Xenogen). The fluorescence intensity was corrected by using GFP background filters to eliminate tissue autofluorescence. To ensure reproducibility all tests were performed in three mice each. The results given in the graphs correspond to the mean values ± standard deviation. After the final imaging time point, the mice were euthanized. In selected cases the implantation site was inspected afterwards.

2.8 Statistical Analysis

The results from the release studies were analyzed statistically for selected time points. In order to demonstrate the significance of differences, first an F-test was applied to two independent groups of values (n = 3). Considering its result, an independent two-sided t-test for either equal or unequal variances was carried out subsequently (p<0.05). Significance (*) and non significance (o) are marked in the figures.

3 Results and Discussion

3.1 Characterization of Hydrogel Microparticles

Hydrogel microparticles with incorporated FITC-IgG were tested for their encapsulation efficiency, particle size distribution and morphology after incorporation of FITC-IgG. In Table 1 the encapsulation efficiency is given as percentage of the total amount of IgG used in the preparation process. In accordance with previous studies [10] encapsulation efficiencies of at least 94 % were achieved with both polymers. The results of the particle size distribution studies of the microparticles from HES-P(EG)₆MA and HES-MA polymer are presented as d₁₀, d₅₀, and d₉₀ values (Table 1). HES-P(EG)₆MA microparticles displayed a median particle size of 11 µm with a narrow size distribution in contrast to HES-MA microparticles for which a d₅₀ value of 51 µm and a rather broad size distribution was determined.

<table>
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<th>Table 1</th>
<th>Particle size distribution of hydrogel microparticles from HES-P(EG)₆MA and HES-MA.</th>
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<td>Polymer</td>
<td>Encapsulation Efficiency</td>
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<td></td>
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</tr>
<tr>
<td>HES-P(EG)₆MA</td>
<td>94 %</td>
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<tr>
<td>HES-MA</td>
<td>96 %</td>
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We hypothesized that this increase in size may be due to agglomeration of smaller particles. Indeed sonication of the microparticle suspension resulted in lower size values for d₅₀ (11 µm) and d₉₀ (38 µm) thus confirming this assumption. In addition microscopic studies of
HES-P(EG)$_6$MA and HES-MA hydrogel microparticles revealed randomly distributed conglomerates of HES-MA microparticles in contrast to homogeneously distributed individual particles of HES-P(EG)$_6$MA (Fig. 2). High resolution cryo-SEM imaging indicated a porous structure of the entire HES-P(EG)$_6$MA particles. In contrast the tested HES-MA microparticles had a more compact network with smaller pore sizes and were surrounded by a dense capsule. This capsule may be more hydrophobic due to the high polymer content which may explain the extensive agglomeration of these hydrogel microparticles.

![Fig. 2 Brightfield microscopy (A, D), fluorescence microscopy (B, E) and cryo-SEM images (C, F) of HES-P(EG)$_6$MA (A, B, C) and HES-MA (D, E, F) hydrogel microparticles loaded with FITC-IgG.](image)

### 3.2 Release from Hydrogel Disks

Hydrogel disks were produced with both starch-based polymers (HES-P(EG)$_6$MA, HES-MA) in two concentrations (20 wt% or 30 wt%) to obtain drug delivery systems with different release properties influenced by the network density. The \textit{in vitro} release was investigated in the conventional release medium (PBS) and in addition in human serum in order to generate \textit{in vivo} like conditions. Fig. 3A depicts the release of FITC-IgG from 20 wt% and 30 wt% HES-P(EG)$_6$MA and HES-MA hydrogel disks in PBS. After 10 days 70 - 75 % of the incorporated IgG was released from HES-P(EG)$_6$MA disks. Only small differences (without statistical significance) between the lower and higher polymer concentration were observed. Release from HES-MA disks was generally slower than from HES-P(EG)$_6$MA hydrogel disks. For HES-MA disks the release was significantly faster when 20 % hydrogels having a lower network density were used compared to 30 % hydrogels. The release kinetics changed when human serum was used as release medium (Fig. 3B). The release from HES-P(EG)$_6$MA hydrogels was accelerated with more than 90 % IgG being released from 20 wt% and 30 wt% hydrogel disks within 10 days. In contrast, the release from HES-MA hydrogel disks was even slower in serum than in PBS but again with a faster release from 20 wt% hydrogels than from the 30 wt% disks (Fig. 3B). Significant differences of the released FITC-IgG between 20 wt% and 30 wt% HES-P(EG)$_6$MA hydrogel disks were obtained in both release media. These findings may be explained by variations in the chemical structure of the two gel forming polymers associated with different degradation properties of the corresponding hydrogels: HES-P(EG)$_6$MA which contains a carbonate ester linkage is degraded more rapidly by hydrolysis than HES-MA in which the methacrylate group is linked by an ester.
bond to the hydroxyethyl starch backbone. An alternative way of degradation of these polymers may be the cleavage of the starch backbone by enzymes [10, 24]. The cause for the slower release from HES-MA hydrogel disks in human serum compared to PBS is not clear yet. Tentatively, it may be related to a kind of plugging of the already smaller pores by components of human serum.

Fig. 3 In vitro release of FITC-IgG from HES-P(EG)<sub>6</sub>MA and HES-MA hydrogel disks containing 20 wt% or 30 wt% polymer in PBS (A) and human serum (B). n=3; statistically significant differences are marked with *, those without statistical significance with °.

To gain information about the comparability of conventional in vitro release studies with in vivo conditions, disks with FITC-labeled IgG were implanted subcutaneously into mice and the arising fluorescence was imaged in vivo. It was assumed that the reduction of fluorescence intensity at the implantation site over time largely reflects the release of FITC-IgG from the microparticles or hydrogel disk, respectively. By using FITC-IgG solution without polymers as control it could be confirmed that free FITC-IgG did not remain localized in the subcutaneous tissue at the injection site but was rapidly distributed such that no fluorescence signal could be detected after 24 h (data not shown). Secondly, we verified that unloaded HES-MA and HES-P(EG)<sub>6</sub>MA hydrogel microparticles injected in mice (Fig. 4A/B, neck) did not show any fluorescence signal. Therefore, the signals obtained within the long term experiments with FITC-IgG loaded disks were attributed to a sustained entrapment of FITC-IgG in the polymeric network. Fig. 4 shows the fluorescence images of two mice (A, B) scanned at defined time points. Independent of the polymer, fluorescence emission of hydrogel disks with higher concentration (30 wt%, Fig 4A, left side, Fig 4B, right side of the mouse) lasted longer compared to disks with 20 wt%. In line with the in vitro experiments, HES-P(EG)<sub>6</sub>MA hydrogel disks revealed a faster loss of fluorescence intensity than HES-MA disks. Quantitative analysis of diminishing fluorescence intensity with time using the Living Image® software confirmed that the fluorescence signals from 20 wt% HES-P(EG)<sub>6</sub>MA hydrogel disks had completely disappeared 5 days after implantation. In contrast 24 % retained fluorescence were detected for 20 wt% HES-MA hydrogels after the same time due to a significantly slower release of FITC-IgG (Fig. 5A).
Fig. 4 Time course of reduction in fluorescence intensity after implantation of FITC-IgG loaded HES-P(EG)$_6$MA hydrogel disks (left: 30 %, right: 20 %; time course from day 0 (d0) to day 12 (d12)) (A) and HES-MA hydrogel disks (left: 20 %, right: 30 %; time course from day 0 (d0) to day 91 (d91)) (B). As control the mice carried unloaded disks implanted into the neck which did not lead to any fluorescence signal. Studies were performed with three animals each, only one mouse of those is shown here for clarity.

Similarly, hydrogels with higher polymer concentration led to 11 % remaining fluorescence intensity of the incorporated FITC-IgG at day 5 in case of HES-P(EG)$_6$MA and 53 % for HES-MA (Fig. 5B). This could be explained by faster degradation of the HES-P(EG)$_6$MA hydrogel disks \textit{in vivo} probably caused by faster hydrolysis and enzymatic degradation. This assumption is strongly supported by studies by Cadée et al. [25] who found evidence of hydrolytic degradation of dextran-based hydrogels containing carbonate ester linkages. Hydrogels containing just an ester linkage showed no signs of degradation. Also in our study inspection of the implantation sites in the mice (after euthanasia) revealed strong degradation of HES-P(EG)$_6$MA disks, whereas HES-MA based systems remained almost unchanged. The finding that a higher polymer concentration was associated with a slower \textit{in vivo} release of FITC-IgG from both types of hydrogels used in this study is likely to reflect the network structure consisting of differently sized pores and meshes [24]. The release properties of these drug delivery systems can thus be adjusted by changes in polymer type and concentration depending on the desired release kinetics. For both hydrogel systems the \textit{in vitro} results obtained in the presence of human serum correlated much better with the \textit{in vivo} results than the \textit{in vitro} release in the absence of serum. Still, conventional \textit{in vitro} release studies performed in PBS led to a correct ranking of the sustained release potential of the different hydrogel systems despite being poorly similar with regard to the absolute release rate. From this point of view serum would be preferable but for long-term investigations with this medium the stability issue will have to be addressed.
3.3 Release from Hydrogel Microparticles

To compare the release from hydrogel disks with that from hydrogel microparticles HES-P(EG)$_6$MA and HES-MA microparticle suspensions with the same amount of FITC-IgG as in the studied hydrogel disks were prepared. The release from hydrogel microparticles was investigated in vitro and in vivo as described above. After 9 days 46% of FITC-IgG was released in phosphate buffered medium (PBS), a faster release of 72% was achieved in human serum (Fig. 6A). This was probably caused by faster hydrolytic and enzymatic degradation in serum as already discussed above for the hydrogel disks. Human serum also led to a higher initial release rate. After s.c. injection of HES-P(EG)$_6$MA microparticle suspension the images showed an almost complete loss of fluorescence signal after 9 days (Fig. 7). The remaining fluorescence intensity at day 9 was 2% (Fig. 6B). Additionally a much higher amount of FITC-IgG initially released was observed in vivo as compared to in vitro as determined by the half-change method in the shaking water bath using PBS or human serum. Direct comparisons of in vitro and in vivo data for all tested drug delivery system can be found in the supplementary data.
In vitro studies using HES-MA microparticles revealed a much slower release than HES-P(EG)$_6$MA microparticles [10]. The subcutaneous injection of HES-MA microparticle suspension, however, led to an inflammatory reaction with swelling at the injection site and with a purulent exudate as well as an unexpected loss of fluorescence intensity of over 90% already at day 2 (not shown). We assume that the rapid reduction in fluorescence intensity was induced by an adverse response against the microparticle suspension. Inflammation and tissue swelling may have shielded the fluorescence. Microscopic investigations of the exudate confirmed the presence of fluorescent particles. It is unlikely that the inflammatory reaction was due to the HES-MA polymer itself because the studies with the HES-MA hydrogel disks did not cause such effects. The reaction may rather be associated with the specific structure of the HES-MA microparticles which, in contrast to the HES-P(EG)$_6$MA microparticles, contained a dense capsule wall surrounding the single particles which formed large agglomerates (Fig. 2). The inflammatory reaction towards HES-MA microparticles is reminiscent of strong tissue reactions to nondegradable dex-MA microspheres in comparison to slowly degradable microspheres. These were observed independently of the particle size distribution [26]. This leads to the assumption that not the agglomeration of microparticles itself but the highly crosslinked polymeric capsule wall surrounding HES-MA microparticles may cause the inflammatory reaction due to its compact, non-porous and hydrophobic structure. Further studies will be required to fully understand the tissue reaction to HES-MA microparticles.

4 Conclusion

In vitro release studies performed with hydrogel drug delivery systems in buffered solutions are known to be hardly comparable with in vivo drug release. Therefore, the non-invasive in vivo imaging system (IVIS) was used for monitoring the in vivo release of FITC-IgG incorporated in HES-MA and HES-P(EG)$_6$MA based hydrogel disks and microparticles. The ability of the IVIS to assess individual animals allowed to work with the comparatively small number of animals while still obtaining high quality data. The release in vivo was slower from HES-MA compared to that from HES-P(EG)$_6$MA hydrogel disks. In addition, higher concentrated cylinders showed a more sustained release of FITC-IgG than cylinders with lower polymer concentration. In vitro release studies in PBS demonstrated a long-term release
from HES-P(EG)_xMA hydrogel microparticles. In contrast in vivo release lasted only for about one week. In vitro studies using human serum were generally in better agreement with in vivo data than studies performed in PBS but the samples were pruned to contamination over time. Consequently, the choice of the optimal release medium should be well considered. Trends concerning slower or faster release profile can be deduced from studies in PBS, but the time period of release was clearly shortened in human serum and under in vivo conditions. The optimization of HES-MA microparticles showing less aggregation tendency and a uniform porous structure may lead to formulations with a sustained in vivo release. Therefore, this study strongly supports the notion that for pharmaceutical drug release systems in vivo studies are essential to select relevant in vitro assay conditions and to validate the results.

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References


