# Ultrafast in situ forming poly(ethylene glycol)-poly(amido amine) hydrogels with tunable drug release properties via controllable degradation rates

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# Ultrafast *in situ* forming poly(ethylene glycol)-poly(amido amine) hydrogels with tunable drug release properties via controllable degradation rates

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**Abstract**

Fast *in situ* forming, chemically crosslinked hydrogels were prepared by the amidation reaction between N-succinimidyl ester end groups of multi-armed poly(ethylene glycol) (PEG) and amino surface groups of poly(amido amine) (PAMAM) dendrimer generation 2.0. To control the properties of the PEG/PAMAM hydrogels, PEGs were used with different arm numbers (4 or 8) as well as different linkers (amide or ester) between the PEG arms and their terminal N-succinimidyl ester groups. Oscillatory rheology measurements showed that the hydrogels form within seconds after mixing the PEG and PAMAM precursor solutions. The storage moduli increased with crosslink density and reached values up to 2.3 kPa for hydrogels based on 4-armed PEG. Gravimetrical degradation experiments demonstrated that hydrogels with ester linkages between PEG and PAMAM degrade within 2 days,

whereas amide-linked hydrogels were stable for several months. The release of two different model drugs (fluorescein isothiocyanate-dextran with molecular weights of $4 \cdot 10^3$ and $2 \cdot 10^6$ g/mol, FITC-DEX4K and FITC-DEX2000K, respectively) from amide-linked hydrogels was characterized by an initial burst followed by diffusion-controlled release, of which the rate depended on the size of the drug. In contrast, the release of FITC-DEX2000K from ester-containing hydrogels was governed mainly by degradation of the hydrogels and could be modulated via the ratio between ester and amide linkages. *In vitro* cytotoxicity experiments indicated that the PEG/PAMAM hydrogels are non-toxic to mouse fibroblasts. These *in situ* forming PEG/PAMAM hydrogels can be tuned with a broad range of mechanical, degradation and release properties and therefore hold promise as a platform for the delivery of therapeutic agents.

**Keywords**

Hydrogel; *in situ* formation; poly(ethylene glycol); poly(amido amine) dendrimer; tunable degradation; controlled drug delivery.

## Introduction

Hydrogels are three-dimensional polymer networks whose properties resemble those of natural soft tissues due to their high water content [1-3]. They generally exhibit excellent biocompatibility and are accordingly widely investigated for use in biomedical applications such as tissue engineering and systems for the controlled delivery of therapeutic agents. In biomedical applications the use of *in situ* forming, injectable hydrogels is preferred over pre-formed hydrogels as it omits the need for surgical procedures [4]. Furthermore, the initial flowing nature of the precursor solution allows for proper shape adaptation and therapeutic agents can be incorporated in the hydrogel by simple mixing with the precursor polymer solution. Compared to injectable physically crosslinked hydrogels, chemically crosslinked hydrogels usually have better mechanical properties and are more resistant to degradation and dissolution. Injectable chemically crosslinked hydrogels have been mostly prepared by

photocrosslinking of (meth)acrylate functionalized polymers using UV light [5,6] or by covalent crosslinking between polymers with complementary functional groups [7,8].

Recently, dendrimers have attracted increasing attention in the biomedical field for the preparation of hydrogels thanks to their uniformity combined with the control of their size, architecture, density, and surface groups [9]. In addition, the high degree of functionality of the dendrimer allows formation of hydrogel crosslinks as well as the simultaneous use of end groups for advanced functions, such as the attachment of drugs. Much of the research to date involves crosslinking between poly(amido amine) (PAMAM) dendrimers and another polymer such as poly(methacrylic acid) [10], poly(vinyl alcohol) [11], or poly(ethylene glycol) (PEG). For example, Holden et al. reported on PEG/PAMAM hydrogels for the delivery of antiglaucoma drugs [12]. PEG/PAMAM conjugates were prepared by reaction of the 4-nitrophenyl chloroformate (NPC) group of heterobifunctional NPC-PEG-acrylate with part of the surface amino groups of PAMAM. UV irradiation of the PEG/PAMAM conjugates in PBS resulted in a photocrosslinked hydrogel. The hydrophobic antiglaucoma drug brimonidine was loaded in the hydrogel by dissolving the drug in the PEG/PAMAM conjugate solution. The solubility of brimonidine increased by 75 % compared to PBS, which was attributed to encapsulation of the drug inside the hydrophobic core of PAMAM. Compared to eye drop formulations of brimonidine in PBS, drug-loaded PEG/PAMAM hydrogels resulted in a significantly higher brimonidine uptake in human corneal epithelial cells and increased transport across bovine corneas. PEG/PAMAM hydrogels have also been prepared by covalent crosslinking between complementary functional end groups. Wang et al. synthesized *in situ* forming PEG/PAMAM hydrogels via the aza-Michael addition reaction between amino groups of PAMAM and acrylate groups of PEG diacrylate [13]. The dendrimer was acetylated to varying degrees using acetic anhydride, thereby giving control over the number of available amino groups. By adjusting the dendrimer surface acetylation degree and dendrimer concentration, hydrogel properties such as the gelation time could be controlled. The hydrogels were shown to be cytocompatible and supported cell adhesion and proliferation. A hydrogel loaded with the anticancer drug 5-fluorouracil efficiently inhibited tumor growth after intratumoral injection in mouse xenografts.

Other chemical methods for the preparation of PEG/PAMAM hydrogels include disulfide bond formation [14], click chemistry [15] and amine-epoxide chemistry [16]. In most of the PEG/PAMAM hydrogels, linear PEGs were employed as crosslinking agents. However, star-shaped PEGs offer various advantages over linear PEGs, such as increased solubility in aqueous media and a higher concentration of functional end groups [17]. These properties are highly desirable with regard to *in situ* hydrogel formation as they may result in faster gelation. Furthermore, control over hydrogel degradation is an important item that has yet received little attention regarding PEG/PAMAM hydrogels. This prompted us to explore the preparation of *in situ* forming PEG/PAMAM hydrogels by reacting PAMAM dendrimers with multi-armed PEG polymers containing either a hydrolysable ester group or a stable amide group near each PEG chain end. We show that by varying the PEG arm number (either 4 or 8) and the functional group (either amide or ester) near the PEG chain end, important hydrogel properties like swelling, degradation and the release of model drugs can be controlled.

## Materials and methods

### Materials

8-Armed poly(ethylene glycol) with succinamide N-succinimidyl ester end groups and a total molecular weight of 20 kg/mol (8-PEG-A) was obtained from Creative PEGWorks (Chapel Hill, USA). 8-Armed poly(ethylene glycol) with succinate N-succinimidyl ester end groups and a total molecular weight of 20 kg/mol (8-PEG-E) as well as 4-armed poly(ethylene glycol) with glutaramide N-succinimidyl ester end groups and a total molecular weight of 10 kg/mol (4-PEG-A) were ordered from Jenkem (Plano, USA). Poly(amido amine) dendrimer generation 2.0 (PAMAM, 20 wt. % solution in methanol), 2,4,6-trinitrobenzenesulfonic acid (TNBS) and fluorescein isothiocyanate-dextran (average molecular weight 4 and 2000 kg/mol, FITC-DEX4K and FITC-DEX2000K respectively) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). All chemicals were used as received, except for the PAMAM solution, which was dried to the air before use to evaporate the methanol.

Cells and extract controls were chosen in accordance with International Standard Organization (ISO) 10993-5 guidelines (biological evaluation of medical devices, part 5: tests for *in vitro* cytotoxicity).

Murine fibroblast L929 cells (ECACC 85011425) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) high glucose supplemented with 5 % fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco) and 1 % penicillin/streptomycin (Sigma) and cultured at 37 °C and 5 % $CO_2$.

Negative control films (RM-C high density polyethylene, noted C-) were purchased from Hatano Research Institute (Ochiai 729-5, Hadanoshi, Kanagawa 257, Japan).

**Hydrogel preparation**

Hydrogel precursor solutions were prepared by separately dissolving PEG and PAMAM in $NaH_2PO_4/Na_2HPO_4$ buffer (0.2 M, pH 8.0) at concentrations of 12.5 w/v % and 4 w/v % respectively, with N-succinimidyl ester and amino end groups present in equimolar amounts. The hydrogel precursor solutions were mixed in a cylindrically shaped rubber mold at room temperature, resulting in the immediate formation of a hydrogel. The amidation was allowed to proceed for 24 h at room temperature to allow for an optimal hydrogel formation. Selected hydrogels were prepared in phosphate buffered saline (PBS, pH 7.4) at 37 °C.

**Hydrogel properties**

*Rheology*

Oscillatory rheology experiments were performed to determine the kinetics of gel formation and the mechanical properties of the chemically crosslinked hydrogels. A solution (0.4 ml) of PEG in $NaH_2PO_4/Na_2HPO_4$ buffer was applied to a TA Instruments AR1000 rheometer and the storage modulus G' and the loss modulus G'' of samples were monitored as a function of time at 20 °C. After 2 min, 0.1 ml of PAMAM in $NaH_2PO_4/Na_2HPO_4$ buffer was mixed with the PEG solution *in situ* and G' and G'' were monitored for another 30 min. Experiments were performed using a parallel plate geometry (diameter 20 mm, gap 1 mm) utilizing a strain of 1 % and a frequency of 1 Hz.

*Mesh size*

The mesh size ξ in freshly prepared hydrogels was approximated using equation (1):

$$\xi = \varphi_p^{-\frac{1}{3}} \times l \times (n \times C_\infty)^{\frac{1}{2}} \qquad (1)$$

where $\varphi_p$ is the polymer volume fraction in the swollen state, l is the average bond length, n is the average number of bonds between crosslinks and $C_\infty$ the Flory characteristic ratio [8,18]. Because of the small contribution of PAMAM (7 %) to the network mass, the $C_\infty$ value for PEG (3.8) was used. The PAMAM dendrimer and the core moieties of the multi-armed PEGs were considered as the crosslink points; n was therefore assumed to be equal to the number of bonds in the individual PEG arms (~180) whereas the average bond length in PEG ($1.5 \cdot 10^{-10}$ m) was used for l.

*TNBS assay*

TNBS was used to determine the remaining number of amino groups in the hydrogels after 24 h of reaction [19]. To this aim, an 8-PEG-E/PAMAM hydrogel was prepared as described above and subsequently immersed in $H_2O$ until the hydrogel was fully degraded and dissolved. After lyophilization, 1.5 mM TNBS in 0.2 M sodium borate (pH 9.0) was added. After 1 h, the absorption at 420 nm was measured using a BMG Labtech Clariostar plate reader. A calibration curve was constructed using PAMAM. Control measurements without TNBS confirmed that none of the samples absorbed at 420 nm.

*Hydrogel swelling and hydrogel degradation/dissolution*

The swelling and the stability of the hydrogels were investigated by a gravimetrical procedure. Freshly prepared hydrogel samples were dried in air and their initial weight $W_0$ was determined. For determination of the degree of swelling, dry networks were immersed in water until equilibrium was

reached. Excess water was carefully removed with tissue paper and the swollen hydrogel weight ($W_s$) was measured. The degree of swelling was calculated from equation (2):

$$\text{degree of swelling} = \left(\frac{W_S - W_0}{W_0}\right) \times 100\ \% \qquad (2)$$

For the degradation experiments, hydrogels were immersed in PBS at 37 °C. Periodically, samples were taken out and dried in air overnight to yield the dry weight ($W_D$).

The relative polymer mass during degradation was calculated from equation (3):

$$\text{relative polymer mass} = \left(\frac{W_D}{W_0}\right) \times 100\ \% \qquad (3)$$

*Release of model compounds*

The model compound FITC-DEX (molecular weight 4 or 2000 kg/mol) was loaded in the hydrogels by dissolving it in the PEG solution prior to hydrogel preparation as described above. The initial FITC-DEX concentration was 1 wt. % relative to the polymer mass. The FITC-DEX loaded hydrogels were placed in 3 ml of PBS and the vials were kept at 37 °C under static conditions. At specific time points, the entire release medium was removed and replaced with 3 ml fresh PBS. The concentration of FITC-DEX in the release samples was determined by reading the absorbance at 492 nm using a BMG Labtech Clariostar plate reader. A calibration curve was constructed using FITC-DEX solutions of known concentrations.

*Evaluation of cytotoxicity of hydrogels via direct cell contact method*

The direct cell contact method was used to assess the cytotoxicity of PEG/PAMAM hydrogels [20]. L929 fibroblasts cells were seeded at $8 \cdot 10^4$ cells per well in a 96-well plate and allowed to proliferate for 2 days. Cylindrically shaped 4-PEG-A/PAMAM hydrogels (diameter 6 mm, height 1 mm) were immersed in ethanol for 2 h, washed 3 times with PBS and kept overnight in PBS. The gels were subsequently dried in a laminar flow hood for 1 day. The hydrogels were pre-swollen in complete growth cell culture medium and placed carefully on the cell layer, followed by the addition of 100 µl of

growth medium. The cytotoxicity after 24 h was determined by the lactate dehydrogenase activity assay (LDH assay, Pierce) and the CellTiter-Glo luminescence assay kit (Promega) according to the manufacturer's instructions. Briefly, for the LDH assay, equal amounts of cell supernatant and LDH reaction mixture were mixed and incubated for 30 minutes at room temperature. Subsequently, the absorbance was read at 490 nm using a BMG Labtech Clariostar plate reader. Lysis buffer and cell culture medium were used as positive and negative control, respectively. For the CellTiter-Glo luminescence assay, CellTiter-Glo reagent was added in each well with a volume equal to the volume initially present in each cell culture well. After 10 min incubation at room temperature, luminescence was recorded on a BMG Labtech Clariostar. The luminescent signal is proportional to the amount of ATP present in cell medium after cell lysis and hence to the number of cells in the well.

*Evaluation of hydrogel cytotoxicity via extraction method*

Material decontamination step

4-PEG-A/PAMAM hydrogels were immersed in ethanol (70 % v/v) for 30 min, washed 3 times with PBS and pre-swollen in DMEM without serum. Reference C- material was prepared under the same conditions as the hydrogels. All materials were prepared in triplicate.

Extract preparation

The 4-PEG-A/PAMAM hydrogels and control material (denoted C-) were extracted according to ISO 10993-12 in a ratio of 0.1 g material per ml extraction vehicle (DMEM without serum) in chemically inert closed containers by using aseptic techniques. Extraction was done at 37 °C for 72 h under stirring. The hydrogel extracts were then diluted in culture medium to achieve final concentrations of 100, 75, 50, 25 and 10 %.

Test on L929 cells

L929 fibroblasts were seeded at $1 \cdot 10^4$ cells per well (96-well plate) and allowed to adhere overnight. The medium was then removed and replaced by 0.1 ml of liquid extracts or growth medium only. As a positive control (denoted C+), phenol (Sigma) having final concentrations of 0.64 and 0.064 % m/v were used. The cytotoxicity after 24 h was determined by the LDH assay as described above.

The percentage of cytotoxicity was calculated from equation (4):

$$\text{cytotoxicity (\%)} = \left(\frac{\text{(sample LDH activity) - (LDH-)}}{\text{(LDH+) - (LDH-)}}\right) \times 100 \qquad (4)$$

Where LDH- represents spontaneous LDH release control (water-treated) and LDH+ represents maximum LDH release control activity obtained after cell lysis.

*Evaluation of the cytotoxicity of NHS*

L929 fibroblasts were seeded at $1 \cdot 10^4$ cells per well (96-well plate) and allowed to attach overnight. N-hydroxysuccinimide (NHS) solution was prepared extemporaneously in sterile PBS at a concentration of 46 mg/ml. After filtration through a 0.22 µm membrane, dilutions were made using cell growth medium and applied on the cell monolayer. After 24 h, cell viability was assessed using the CellTiter-Glo luminescence assay kit as described above.

*Statistics*

Statistical analysis of the effects of the number of PEG arms and the functional group near the PEG chain end on the hydrogel degradation and drug release was performed using one-way analysis of variance (ANOVA) at various time points. Post hoc comparisons of the means of individual groups were performed using Tukey's honestly significant difference (HSD) test. Statistical significance was defined as $p < 0.05$.

## Results and Discussion

### Preparation of chemically crosslinked PEG/PAMAM hydrogels

Being the most investigated class of dendrimers, PAMAM dendrimers have been frequently applied for biomedical applications, including targeted drug delivery [21], gene vectorization [22] and imaging [23]. We explored the preparation of hydrogels based on PAMAM dendrimer generation 2.0, which possesses 16 amino surface groups. N-succinimidyl ester-terminated 4- or 8-armed PEG was used to crosslink the dendrimer molecules via an amidation reaction, which has been employed previously for hydrogel formation thanks to its fast kinetics under mild conditions [24,25].

To be able to tune various properties of the PEG/PAMAM hydrogels, including the storage modulus and drug release behavior, PEGs were selected with different arm numbers as well as different linkers between the PEG arms and their terminal N-succinimidyl ester groups (Figure 1). The use of succinamide N-succinimidyl ester or glutaramide N-succinimidyl ester end groups leads to two amide groups between PAMAM and PEG (Figure 2), whereas succinate N-succinimidyl ester end groups yield one ester and one amide group. Generally, the hydrolytically labile ester linker allows for fast degradation, whereas the stable amide linker may provide hydrogels that are degrading much slower [26,27]. To vary the crosslink density, we used PEGs with 4 or 8 arms with a constant molecular weight of 2.5 kg/mol per arm.

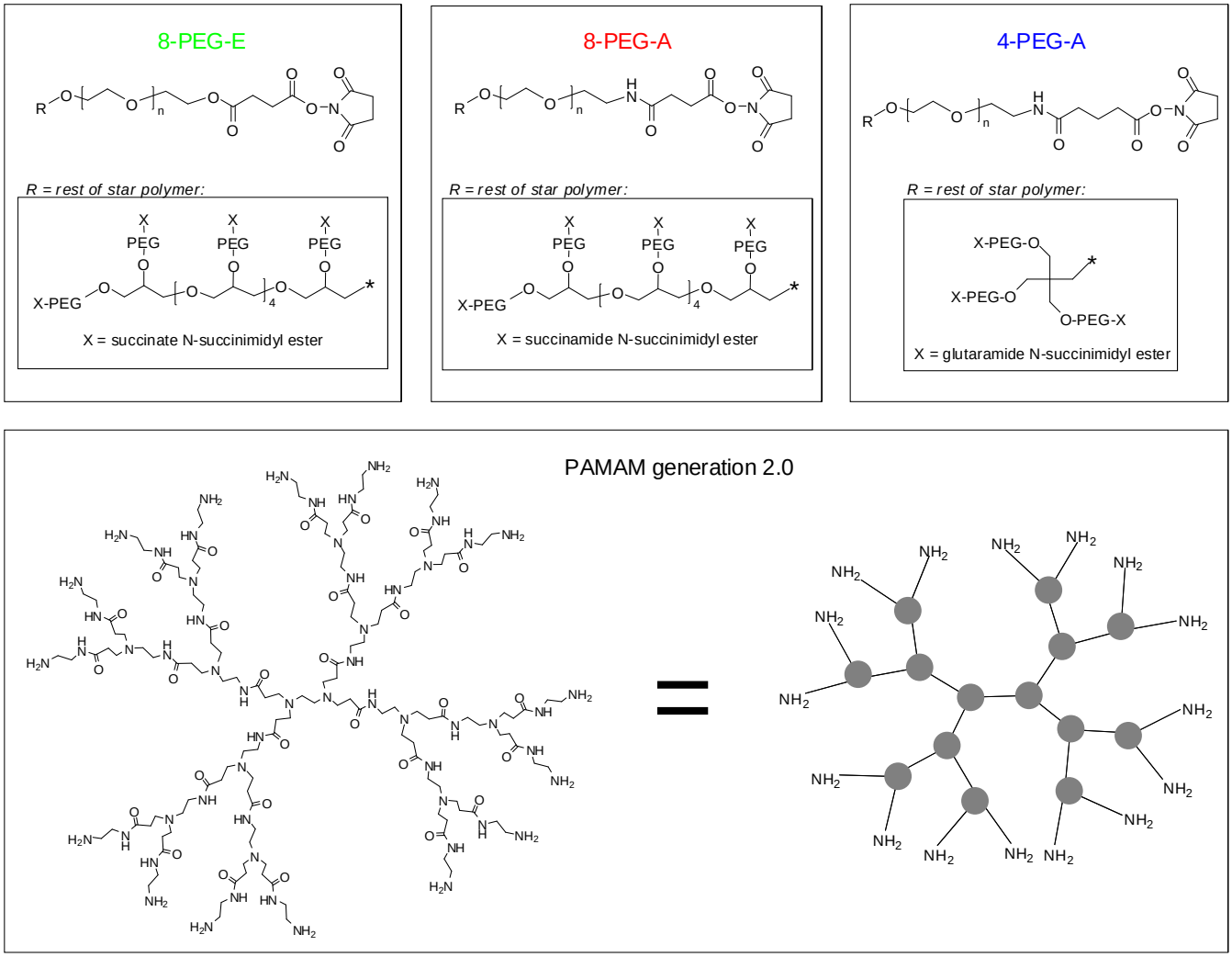


**Figure 1.** Structure of the PEG and PAMAM hydrogel precursors used in this study.

Different combinations of precursor solutions of multi-armed PEG and PAMAM in $NaH_2PO4/Na_2HPO_4$ buffer (pH 8.0) were applied to prepare hydrogels via a crosslinking amidation reaction between the terminal N-succinimidyl ester and amino groups. In this study a pH of 8 was used as a compromise between the contradicting requirements of a high pH value to obtain a high concentration of reactive, unprotonated amino groups and a low pH value to limit the competing hydrolysis of the NHS ester. An idealized structure of a 4-PEG-A/PAMAM hydrogel network is presented in Figure 2. Although all hydrogels formed *in situ* after mixing the precursor solutions, immediately yielding clear, transparent hydrogels, the crosslinking reaction was continued for 24 h to facilitate an optimal hydrogel formation.

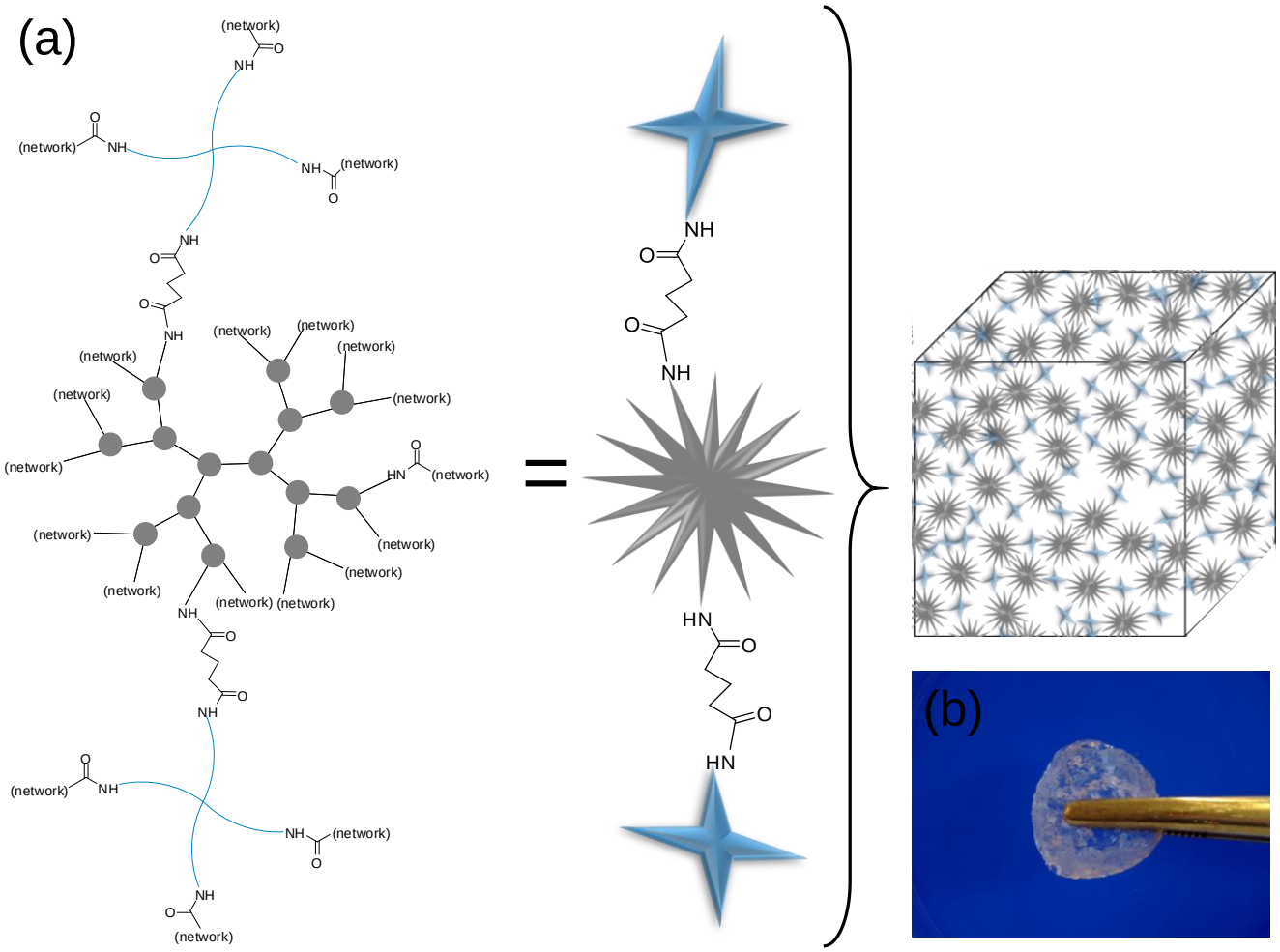


**Figure 2.** (a) Idealized structure of a 4-PEG-A/PAMAM hydrogel network. PEG chains are represented by blue lines and stars, PAMAM dendrimers are represented by grey stars. (b) Photograph of a swollen 4-PEG-A/PAMAM hydrogel.

The degree of swelling was determined by immersing dry networks in water and measuring the swollen hydrogel weight. Although it was observed that the networks swelled rapidly, they were kept in water for 24 h to guarantee that equilibrium swelling was reached. The 8-PEG-E/PAMAM hydrogel was excluded from swelling experiments because important hydrolytic degradation was observed at short timescales for this network (vide infra). After 24 h immersion in water, 4-PEG-A/PAMAM and 8-PEG-A/PAMAM hydrogels exhibited a degree of swelling of 945 (± 7) % and 1282 (± 79) %, respectively (n = 3, values between accolades correspond to standard deviations). For hydrogels with equimolar

amounts of PEG and PAMAM endgroups, the number of 4-armed PEG polymers ($M_n$ = 10 kg/mol) in 4-PEG-A/PAMAM hydrogels is twice as high compared to the number of 8-armed PEG polymers ($M_n$ = 20 kg/mol) in 8-PEG-A/PAMAM hydrogels. Since each star polymer contains one central branching point, acting as a crosslink, 4-PEG-A/PAMAM networks possess a higher number of crosslinks than 8-PEG-A/PAMAM networks. The 4-PEG-A/PAMAM networks are therefore more resistant to expansion, which explains their lower degree of swelling. Despite their relatively high equilibrium water content, the 8-PEG-A/PAMAM and 4-PEG-A/PAMAM hydrogels could easily be manipulated without visibly damaging them.

The TNBS assay was used to determine the amount of free amino groups after network formation for 8-PEG-E/PAMAM hydrogels [19]. To make any remaining amino groups accessible for reaction with TNBS, the hydrogels were placed in water to induce full hydrogel degradation via hydrolysis of the ester linkages. It was found that only 1% of the free amino groups present in the initial PAMAM solution remained after network formation, which confirms that the amidation reaction between the amino groups of PAMAM and the N-succinimidyl ester end groups of PEG occurs very efficiently. The remaining end groups can be employed to post-functionalize the hydrogel with specific chemical or biological cues.

**Rheology**

The gel formation kinetics and mechanical properties of the PEG/PAMAM hydrogels were investigated by oscillatory rheology. Figure 3 shows that separate 4-PEG-A and 8-PEG-A solutions exhibit a very low storage modulus (G' < 1 Pa). Upon addition of the PAMAM solution, G' quickly increases for both formulations, demonstrating that hydrogels are formed *in situ*. For the 4-PEG-A/PAMAM hydrogel, the storage modulus reaches a value of 1 kPa within 3 minutes, after which G' levels off to attain a value of 2.3 kPa after 30 min. The 8-PEG-A/PAMAM hydrogel also forms very quickly, but develops a lower stiffness (G' = 0.7 kPa) than the 4-PEG-A/PAMAM hydrogel due to fewer branching points in the

network. Following the addition of PAMAM solution, the loss modulus (G'', Figure S1) was inferior to G' during the entire rheological experiment for both hydrogel formulations. After 30 min the 8-PEG-A/PAMAM hydrogel displayed a higher damping factor (tan $\delta$ = G''/G') compared to the 4-PEG-A/PAMAM hydrogel (0.04 vs. 0.01), demonstrating a less elastic (i.e. gel-like) behavior in accordance with the lower crosslink density. Whereas the rheological experiments were performed at 20 °C using $NaH_2PO_4/Na_2HPO_4$ buffer (pH 8.0), additional experiments on the benchtop, in which separate polymer solutions in PBS at 37 °C were mixed into a rubber mold, demonstrated that the hydrogels also formed within seconds under physiological conditions. The gelation times are faster than those of previously reported dendrimer-based PEG [28], PEG/PAMAM [13] and PEG-polyglycerol [29] hydrogels where linear PEGs were employed as crosslinking agents. The rapid gelation of the 4-PEG-A/PAMAM and 8-PEG-A/PAMAM hydrogels is likely due to the high number of end groups on both PEG and PAMAM in combination with the efficient amidation reaction. This hypothesis is supported by the observation that mixing linear bifunctional PEG with PAMAM, which was performed as a control experiment, did not yield hydrogels. The results show that hydrogels with good mechanical properties can be obtained quickly by simple mixing of the precursor solutions in PBS. The gel stiffness can be tuned, similar to the equilibrium swelling, by varying the crosslink density.

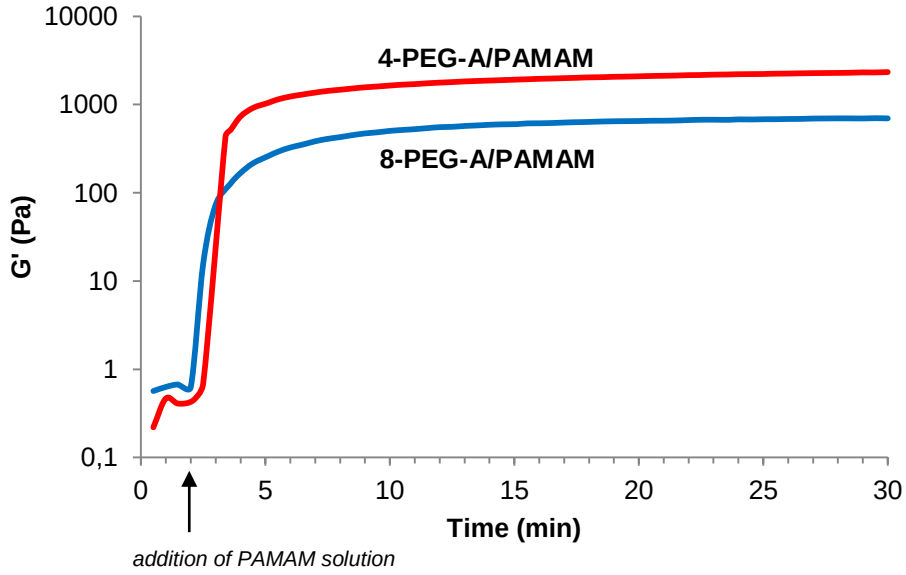


**Figure 3.** Storage modulus (G') as a function of time for 4-PEG-A/PAMAM (red) and 8-PEG-A/PAMAM (blue) hydrogels. During the first 2 minutes only PEG solution was present. At t = 2 min, PAMAM solution was added, immediately resulting in *in situ* hydrogel formation.

**Hydrogel degradation**

The *in vitro* degradation of the PEG/PAMAM hydrogels was investigated by a gravimetrical procedure, in which the polymer mass loss was followed up to 11 weeks (Figure 4). The 8-PEG-E/PAMAM hydrogels degrade rapidly due to hydrolysis of the labile ester linkage between the PEG and PAMAM chains. Consequently, PEG and PAMAM polymers as well as network fragments diffuse out of the hydrogel as a result of the concentration difference between the hydrogel and the surrounding medium and eventually, the system dissolves completely within 48 h. In contrast, the hydrolytically more stable amide linkage in 8-PEG-A/PAMAM networks affords hydrogels which degrade much slower, with 35 % of polymer mass loss after 11 weeks.

No degradation was observed for the 4-PEG-A/PAMAM hydrogel on the time scale of the experiments, which may be due to the synergistic effect of stable amide linkages in combination with a high crosslink density. Interestingly, a biphasic degradation profile can be obtained by using a 1:1 mixture of 8-PEG-A and 8-PEG-E during hydrogel preparation. In the resulting 8-PEG-AE/PAMAM hydrogels, where the

ratio of ester and amide linkages is 1:1 initially, the hydrolysis of esters is responsible for the rapid decrease in polymer mass during the first 5 days. As the network becomes progressively rich in amide linkages, the degradation rate levels off and a hydrogel remains that only loses 6 % polymer mass between day 22 and 57. The results show that by tailoring the precursor properties (i.e. PEG functionality and linker type between PEG and PAMAM) the degradation time can be adjusted from 2 days to several months. In comparison with previously reported degradable PEG/PAMAM hydrogels [13,14,30], the current system offers an unprecedented level of control over the *in vitro* degradation pattern. This study clearly demonstrates that the linker type (ester or amide) between two polymer constituents in a hydrogel has a pronounced effect on the degradation behavior, as shown previously for amide- or ester-linked PEG-PCL [26] and PEG-PLA [27] based hydrogels.

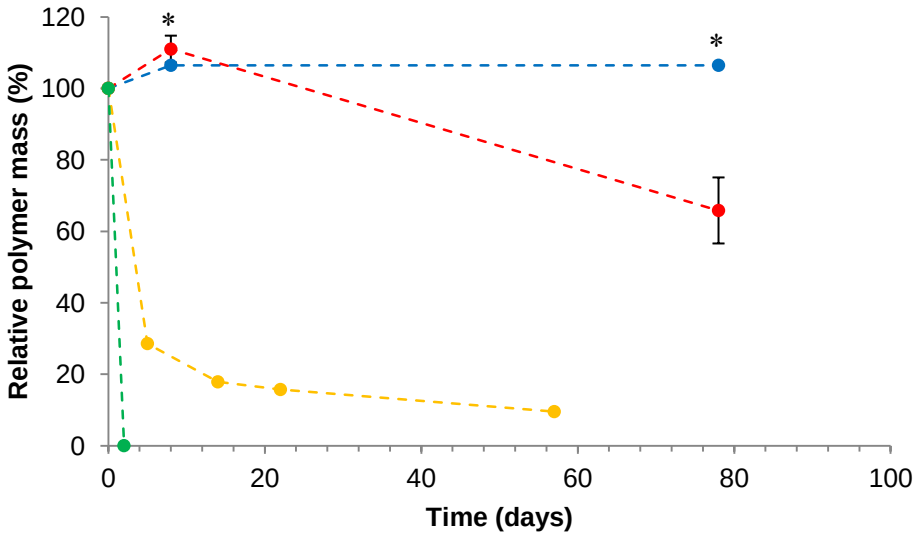


**Figure 4.** Polymer mass as a function of time for various PEG/PAMAM hydrogels in PBS at 37 °C. 4-PEG-A/PAMAM (blue), 8-PEG-A/PAMAM (red), 8-PEG-E/PAMAM (green), 8-PEG-AE/PAMAM (orange). Experiments with 4-PEG-A/PAMAM, 8-PEG-A/PAMAM and 8-PEG-E/PAMAM hydrogels were performed in triplicate. These data are expressed as mean ± standard deviation (error bars are smaller than the symbol size for 8-PEG-E/PAMAM and 4-PEG-A/PAMAM hydrogels). The data points corresponding to 8-PEG-AE/PAMAM hydrogels are based on one sample. The dashed lines are meant

to guide the eye. Significant differences were found at the indicated time points (*, $p < 0.05$, 4-PEG-A/PAMAM versus 8-PEG-A/PAMAM hydrogels).

**Release of model compounds**

FITC-DEX has been widely used as a model compound for therapeutic agents such as proteins and peptides to study the *in vitro* release kinetics of hydrogel drug delivery systems [31-33]. Two different FITC-DEX with molecular weights of 4 and 2000 kg/mol, spanning a size range including siRNA, small proteins, antibodies and plasmids [34], were used to investigate the drug release behavior of the PEG/PAMAM hydrogels. FITC-DEX4K and FITC-DEX2000K could be easily incorporated in the PEG/PAMAM hydrogels by dissolving them in the PEG solution prior to crosslinking. For the 8-PEG-A/PAMAM hydrogels an initial burst release is obtained (Figure 5) as typically observed during the release of hydrophilic compounds from hydrogels. A burst release allows the drug to rapidly reach an effective concentration, whereas subsequent sustained release allows the drug to maintain this concentration over time [15]. Following the burst release, the remaining FITC-DEX4K is released from the 8-PEG-A/PAMAM hydrogels in 7 d. Calculation of the mesh size (Equation 1) of 8-PEG-A/PAMAM hydrogels affords a value of approximately 10 nm, which is much larger than the hydrodynamic diameter of DEX-FITC4K (approximately 3 nm according to the manufacturer's specifications), suggesting diffusion controlled release of DEX-FITC4K from the 8-PEG-A/PAMAM hydrogels. The release of FITC-DEX2000K is significantly slower as the release increases only from 50 to 70 % in the 14 days following the initial burst (Figure 5). Since the hydrodynamic radius of FITC-DEX2000K (54 nm) is considerably larger than the network mesh size, the transport out of the hydrogel as a result of the concentration difference between the hydrogel and the surrounding medium is severely restricted by the polymer mesh. Even though amide bonds are quite stable under physiological conditions, it cannot be excluded that hydrolysis of a few amide linkages occurs on the timescale of the release experiments, also in view of the degradation behavior of the 8-PEG-A/PAMAM hydrogels at later timepoints (35 % polymer mass loss after 11 weeks, Figure 4). Although the polymer mass loss is

still negligible after 14 d, the scission of a limited number of chains between crosslinks may already result in a notably increased mesh size, facilitating the diffusion of FITC-DEX2000K through the polymer mesh. The release of FITC-DEX4K and FITC-DEX2000K from 4-PEG-A/PAMAM hydrogels (Figure S2) is similar to the release from 8-PEG-A/PAMAM hydrogels, in accordance with the similar initial mesh size (10 nm).

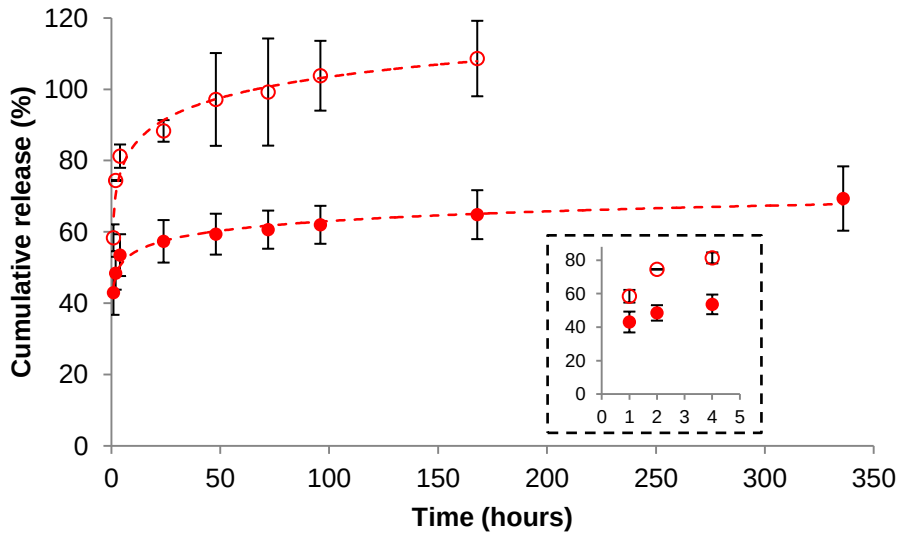


**Figure 5.** *In vitro* release of DEX-FITC4K (open circles) and DEX-FITC2000K (closed circles) from 8-PEG-A/PAMAM hydrogels. Data (n = 3) are expressed as mean ± standard deviation. The dashed lines are meant to guide the eye. The insert shows the cumulative release during the first 5 hours. Significant differences were found at all time points ($p < 0.05$).

Following these results, we investigated the possibility to modulate the release by changing the linker type between PEG and PAMAM. There is no significant difference ($p > 0.05$) among the different hydrogels for the initial release stage (between t = 0 and t = 4 h) of FITC-DEX2000K. At t = 24 h, the release of FITC-DEX2000K from 8-PEG-E/PAMAM hydrogels is complete and significantly faster than from the 8-PEG-A/PAMAM and 8-PEG-AE/PAMAM hydrogels ($p < 0.05$, Figure 6). Similar to the 8-PEG-A/PAMAM network, the estimated mesh size of the 8-PEG-E/PAMAM network is initially

10 nm, but this value increases quickly after the start of the release experiments due to hydrolytic degradation of the ester linkages, leading to increased swelling and complete dissolution of the hydrogel at day 2. The release from this hydrogel is likely to be governed by a combination of diffusion and degradation. The release of FITC-DEX2000K from 8-PEG-AE/PAMAM hydrogels (Figure 6) is more sustained than from 8-PEG-E/PAMAM hydrogels in accordance with the lower number of ester linkages (initial ratio of ester and amide linkages is 1:1) and slower degradation rate (Figure 4). The data show that the release of the model drug FITC-DEX2000K depends mainly on the degradation behavior of the hydrogels, which can be controlled via the linker between PEG and PAMAM.

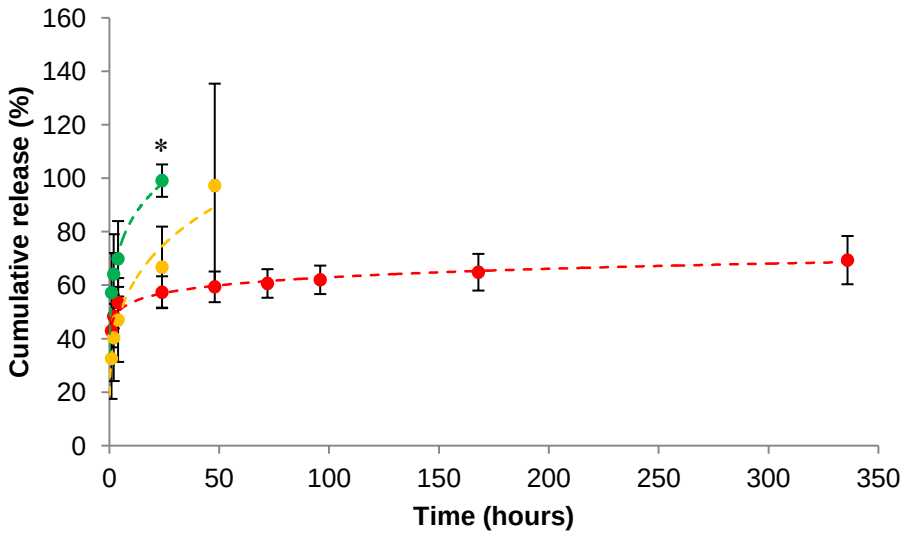


**Figure 6.** *In vitro* release of DEX-FITC2000K from 8-PEG-A/PAMAM (red), 8-PEG-AE/PAMAM (orange) and 8-PEG-E/PAMAM (green) hydrogels. Data (n = 3) are expressed as mean ± standard deviation. The dashed lines are meant to guide the eye. Significant differences were found at the indicated time point (*, $p < 0.05$, 8-PEG-A/PAMAM versus 8-PEG-E/PAMAM hydrogels and 8-PEG-AE/PAMAM versus 8-PEG-E/PAMAM hydrogels).

***In vitro* cytotoxicity of hydrogels**

The direct contact method and the extraction method were used to assess the cytotoxicity of 4-PEG-A/PAMAM hydrogels. Results concerning the direct contact method [20] are presented in Figure 7 and 8. Figure 7 shows the amount of lactate deshydrogenase (LDH), a cytosolic enzyme which is released upon cell membrane damage, in the cell culture medium after 24 h. As positive control, in a separate set of wells, lysis buffer was added to L929 cells to achieve maximum LDH activity. Cell culture medium only was used as negative control. It follows from Figure 7 that the 4-PEG-A/PAMAM hydrogels are non-toxic to L929 cells.

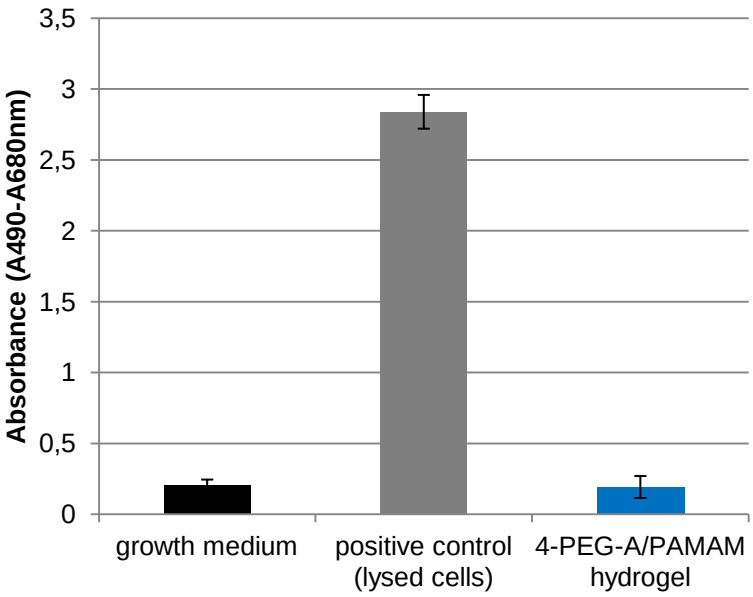


**Figure 7.** LDH cytotoxicity assay of fibroblasts grown in contact with 4-PEG-A/PAMAM hydrogels for 24 h. Data (n = 8) are expressed as mean ± standard deviation.

Figure 8 shows the amount of ATP, which is present in metabolically active cells, in the cell culture medium after 24 h. According to the ISO 10993-5 guidelines, a reduction in cell viability of more than 30 % is considered as a cytotoxic effect. Fibroblast viability in contact with 4-PEG-A/PAMAM hydrogels is 76 % with respect to the control (growth medium only), confirming that the 4-PEG-A/PAMAM hydrogels are non-toxic to L929 cells after 24 h of direct contact. The observed diminution of cell viability could be due to cell detachment when the hydrogels were removed from the wells.

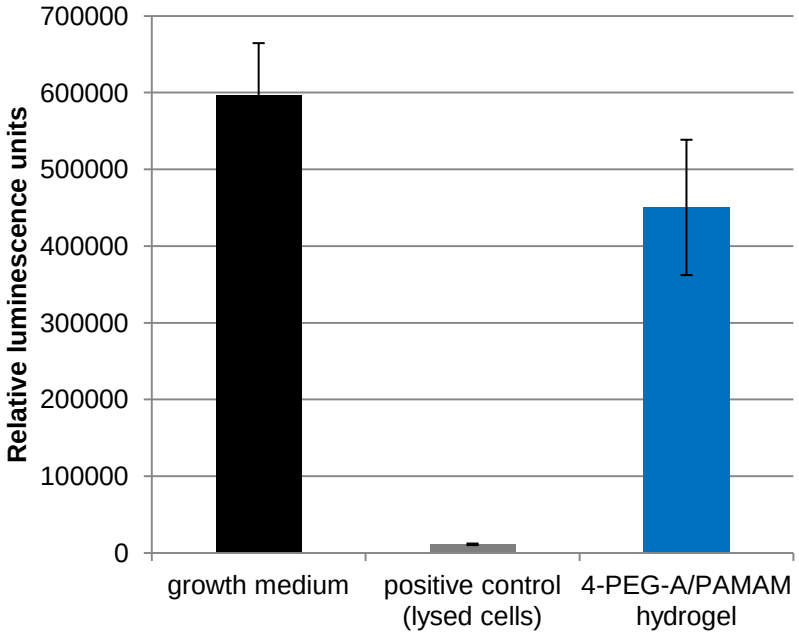


**Figure 8.** Viability of fibroblasts grown in contact with 4-PEG-A/PAMAM hydrogels for 24 h. Data (n = 8) are expressed as mean ± standard deviation.

The cytotoxicity of 4-PEG-A/PAMAM hydrogels was further evaluated using the extraction method. The 4-PEG-A/PAMAM hydrogel extracts show a very low toxicity towards fibroblasts after 24 h (Figure S3), comparable to the toxicity of the polyethylene extracts (negative control), corroborating the cytotoxicity results obtained with the direct cell method.

It should be noted that these *in vitro* results were obtained with pre-formed hydrogels. When it comes to future applications, our hydrogels are intended as *in situ* forming systems for the controlled delivery of therapeutic agents. With the currently used chemical end groups, NHS is released upon crosslinking (4.6 mg per ml hydrogel for quantitative end group conversion), which can be a concern for *in vivo* injectability. To evaluate the cytotoxicity of NHS, the viability of fibroblasts after treatment with NHS solutions for 24 h was determined using the lactate dehydrogenase activity assay (Figure S4). The results demonstrate that NHS shows some cytotoxicity towards fibroblasts at a concentration of 4.6

mg/ml. In this regard, the use of chemoselective, bioorthogonal cross-linking strategies, which do not interfere with biochemical processes or biomolecules, are very appealing. For example, the use of strain-promoted azide-alkyne cycloaddition (SPAAC) is attracting increasing interest [1]. The use of reactive azide and alkyne groups in biomedical applications also raises the issue of toxicity, but good biocompatibility was observed previously for networks crosslinked via copper-free click chemistry [35]. In our current contribution, which can be regarded as a proof-of-principle, we have chosen NHS-amine chemistry because of the availability and affordability of the polymers containing NHS or amine groups as well as the efficiency of the crosslinking reaction. The uniqueness of our approach resides in the possibility to control important hydrogel properties by varying the PEG arm number and the functional group near the PEG chain end. This concept will be combined with bioorthogonal crosslinking in a forthcoming publication.

## Conclusions

Chemically crosslinked hydrogels were prepared from multi-armed PEG star polymers bearing N-succinimidyl ester end groups and PAMAM dendrimers through an amidation reaction. Hydrogels formed quickly by simple mixing of PEG and PAMAM precursor solutions at low concentrations. The hydrogel equilibrium swelling and the mechanical properties could be tuned via the number of arms of the PEG precursor, whereas the degradation behavior could also be modulated via the ratio of ester and amide linkages between PEG and PAMAM. The degradation of the hydrogels, ranging from a few days to several months, had a clear effect on the release behavior. FITC-DEX2000K was released from the 8-PEG-A/PAMAM hydrogels during several weeks, whereas the release of FITC-DEX2000K from the 8-PEG-E/PAMAM and 8-PEG-AE/PAMAM hydrogels was much faster resulting from a combination of diffusion and degradation. The possibility to be formed *in situ* and their tunable mechanical, degradation and release properties make these PEG/PAMAM hydrogels appealing as controlled drug delivery systems.

**Acknowledgements**

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**Conflicts of interest**

Declarations of interest: none.

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*Supporting information for*

# Ultrafast *in situ* forming poly(ethylene glycol)-poly(amido amine) hydrogels with tunable drug release properties via controllable degradation rates

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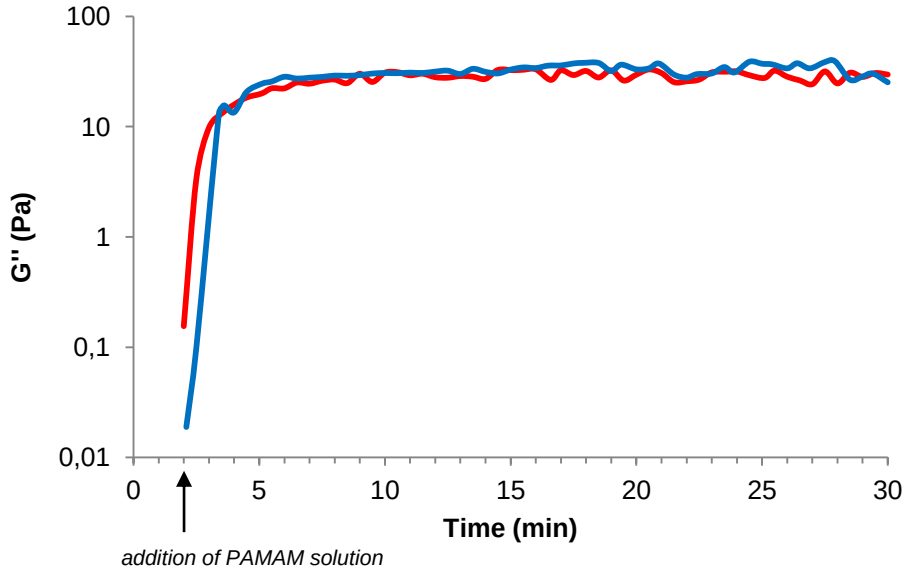


**Figure S1.** Loss modulus (G'') as a function of time for 4-PEG-A/PAMAM (red) and 8-PEG-A/PAMAM (blue) hydrogels. During the first 2 minutes only PEG solution was present and G'' values were below the detection limit of the rheometer. At t = 2 min, PAMAM solution was added, immediately resulting in *in situ* hydrogel formation.

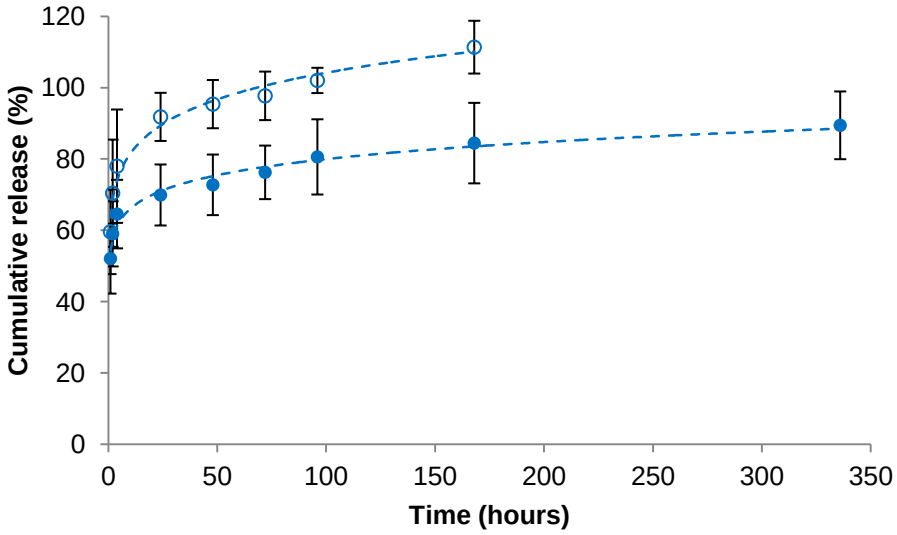


**Figure S2.** *In vitro* release of DEX-FITC4K (open circles) and DEX-FITC2000K (closed circles) from 4-PEG-A/PAMAM hydrogels. Data (n = 3) are expressed as mean ± standard deviation. The dashed lines are meant to guide the eye.

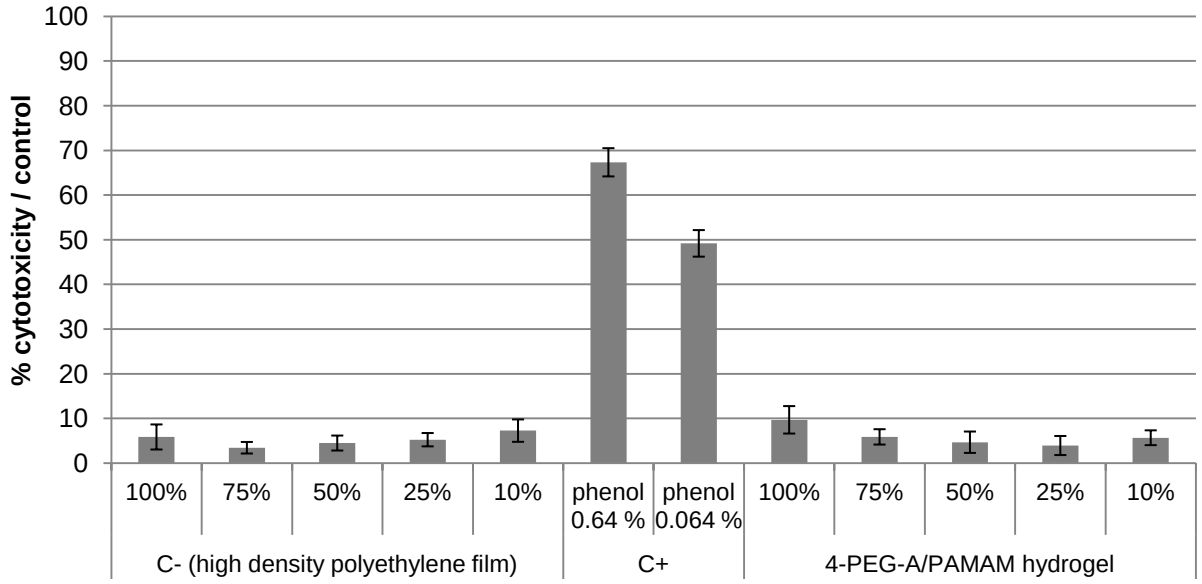


**Figure S3.** LDH cytotoxicity assay of fibroblasts grown in contact with extracts of 4-PEG-A/PAMAM hydrogels for 24 h. Data (n = 9) are expressed as mean ± standard deviation. Maximum LDH activity obtained after cell lysis was taken as 100 %.

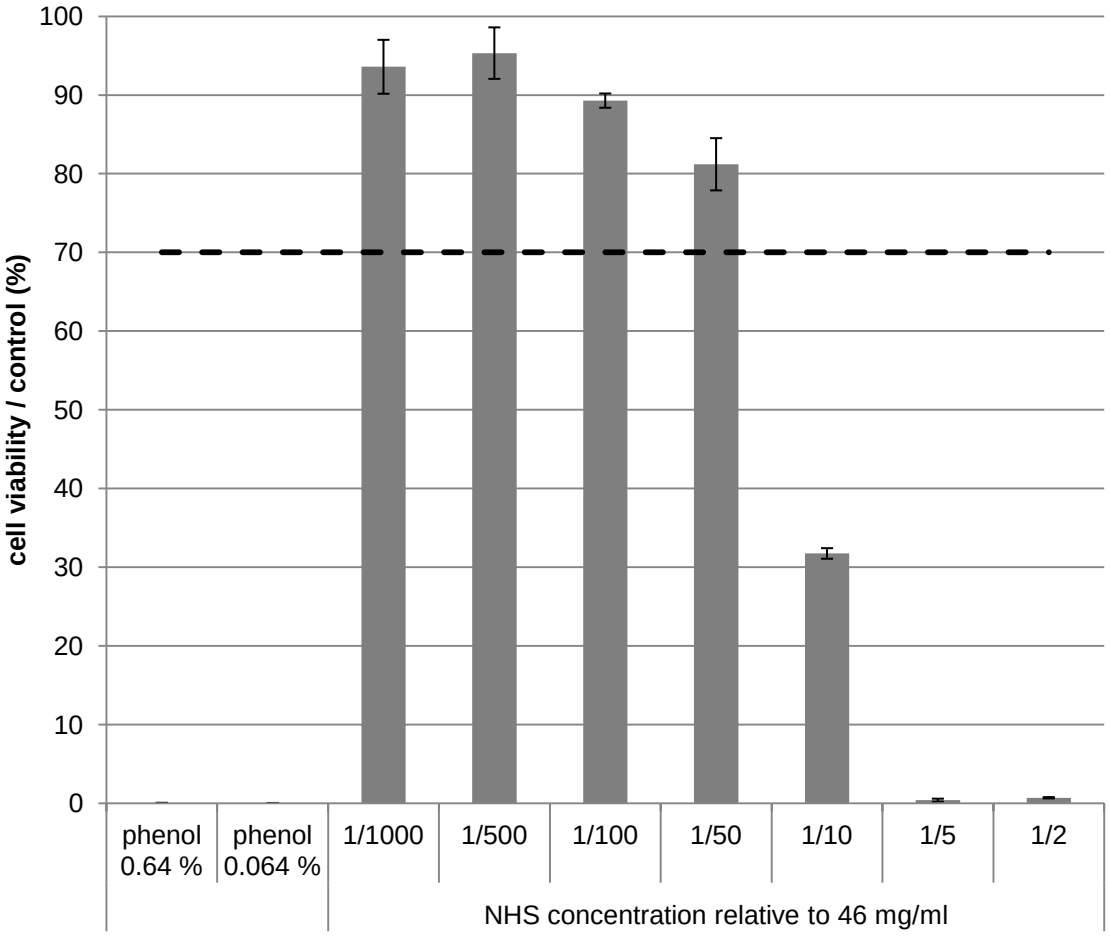


**Figure S4.** Viability of fibroblasts after treatment with NHS solutions for 24 h. Data (n = 3) are expressed as mean ± standard deviation. The viability of cells without any treatment (growth medium only) was taken as 100%. According to the ISO 10993-5 guidelines, a reduction in cell viability of more than 30 % is considered as a cytotoxic effect. Viabilities of fibroblasts after treatment with phenol solutions (positive controls) are lower than 0.1 %.