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PAPER

Tailoring uptake and release of ATP by dendritic glycopolymer/PNIPAAm hydrogel hybrids: first approaches towards multicompartment release systems†‡

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A multicompartment release system is described which combines the advantages of dendritic architectures and hydrogels to enhance the desired delivery features in complex biological compartments. Here, a hydrogel hosts dendritic glycopolymers as nanocontainers and a delivery system for drug molecules. The dendritic glycopolymer used consists of a hyperbranched poly(ethylene imine) with a maltose shell and acts as a host for the guest molecule adenosine triphosphate disodium salt hydrate (ATP). The ATP uptake and release from the dendritic host have been elucidated in detail with dependence on the dendritic glycostructure and pH. The complex interactions within the three components ATP, dendritic glycopolymer and hydrogel have been evaluated and could be fine-tuned. A selective release at pH 5.4–7.4 only of ATP from the multicompartment release system ATP@dendritic glycopolymer@hydrogel has been achieved when a boronic acid containing hydrogel was used which allowed chemical binding between the maltose units from the dendritic glycopolymer and the boronic acid (BA) units in the hydrogel. However, when using a hydrogel without BA units, simultaneous release of ATP and the dendritic glycopolymer scaffold from the ATP@dendritic glycopolymer@hydrogel multicompartment release system is observed in the pH range 2–7.4. This multicompartment release system can be applied in complex biological environments with changing pH values and has potential in biomedical applications and sensory devices.

Introduction

Hyperbranched polymers and dendrimers have been established as highly interesting macromolecular architectures with unique features.[1] One promising application of these dendritic architectures is their use as delivery systems and polymeric therapeutics and diagnostics in biomedical applications.[2] The various prerequisites of their successful use in this field are derived from their multifunctional and highly branched structure leading to specific complexing and release features. Recent efforts are directed towards the development of multicompartment delivery systems where dendritic architectures are integrated into liposomes[3] and vesicles[4] or self-assembled into higher hierarchical and stable aggregates.[1c,5] These complex structures fulfil better the manifold requirements of a delivery system in a complex biological environment as there are: high complexation capacity for the drug as well as retarded release, tailored delivery of the drug and of course high biocompatibility and protection against biological attacks. In this context, recent developments focus on the formation of hydrogels loaded with (non-)covalently linked dendritic architectures.[6–14] This allows in a better way to tailor the drug delivery *e.g.* from injectable hydrogels[12] or from hydrogel particles taken up in cell lines.[13]

Stimulated by these recent developments of multicompartment delivery systems and the increasing popularity of hydrogels in the field of diagnostics,[15,16] separation technology,[15,16] or delivery of small and large macromolecules,[17–19] we report on the first steps towards a multicompartment release system unifying the advantages of a hydrogel as a storage matrix for drug-binding/releasing moieties and of dendritic glycopolymers[20] as delivery systems for drugs. Our idea is presented in Scheme 1: dendritic glycopolymers which can be loaded with a drug are integrated as specific drug-binding/releasing moieties into a hydrogel

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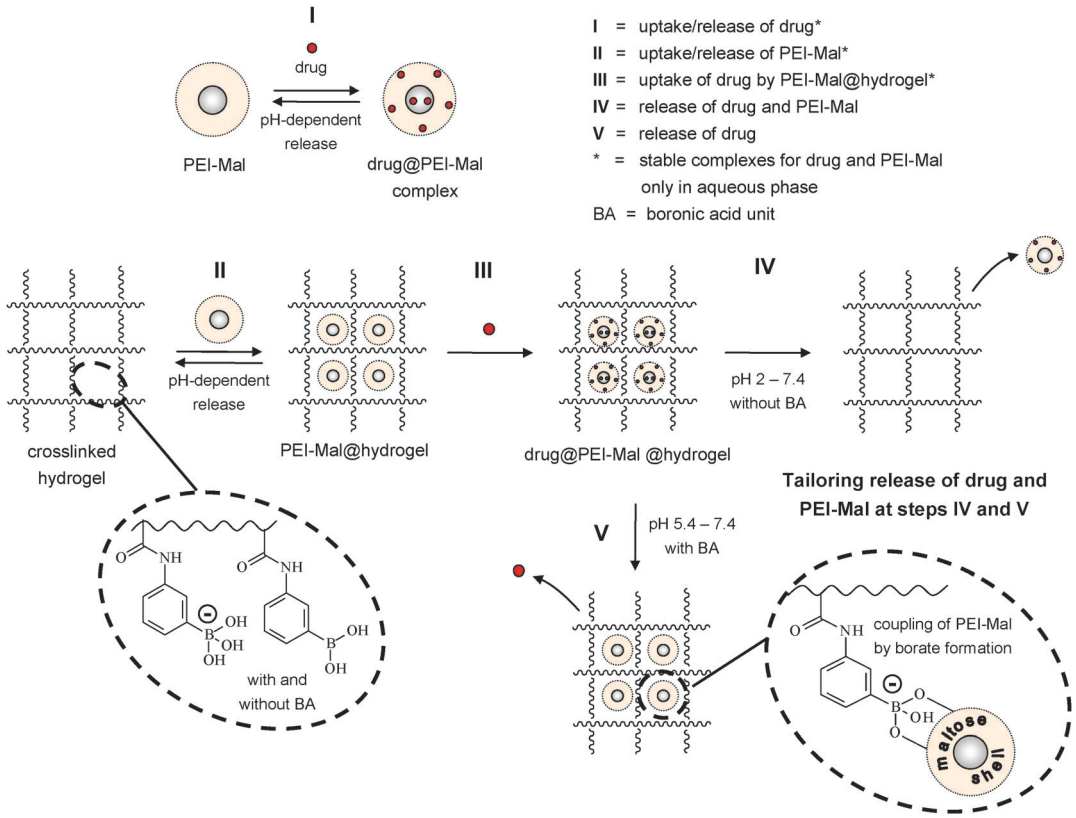


Scheme 1 Multicompartment release drug@glycopolymer@hydrogel system tailored by non-covalently driven interactions between the drug and dendritic glycopolymer PEI-Mal (Scheme 2) and non-covalently and covalently driven interactions between dendritic glycopolymer PEI-Mal and hydrogel.

leading to a multicompartment release system. With our concept we aim at preventing the undesired destruction of the drug@dendritic glycopolymer complex by incorporation into a hydrogel and at the same time introducing targeting capabilities for the drug delivery by having a controlled pH-dependent release of the individual drug (indicated by step V in Scheme 1) or the drug@dendritic glycopolymer complex (step IV in Scheme 1) from the hydrogel matrix. In the latter case, the released drug@dendritic glycopolymer complex itself then can act as a carrier system *e.g.* allowing cell uptake. In order to realize the described system and to allow for the described controlled simultaneous or stepwise release, one has to understand and fine-tune the interactions firstly, between the drug and dendritic scaffold, and secondly, between the drug@dendritic glycopolymer complex and the hydrogel. Therefore, the following components were selected for studying the underlying principles of interactions as outlined in steps I–V presented in Scheme 1: adenosine triphosphate disodium salt hydrate (ATP) as a model drug molecule, maltose-modified poly(ethylene imine) (PEI–Mal) as a binding/delivery system and as a nanocontainer for the drug within the hydrogel, and poly-*N*-isopropylacrylamide (PNIPAAm) as the main component for the hydrogel.

The key step of our concept is the uptake and binding of PEI–Mal in the hydrogel. For step IV, simultaneous release of the drug@dendritic glycopolymer complex, non-covalent interactions between the PEI–Mal and the hydrogel should be realized to allow a rather fast release in a defined pH range. A system according to step V, allowing for the controlled release of the drug only at the targeting site, is realized more likely by covalent bonding of PEI–Mal within the hydrogel. We assume that long-term stability of PEI–Mal in the hydrogel in a specific pH range between 5.4 and 9 will be realized by the presence of boronic acid units[21] in the polymeric network allowing for covalent binding *via* borate formation between vicinal diol containing maltose units from PEI–Mal macromolecules and boronic acid containing hydrogels.

The use of dendritic glycopolymers as a nanocontainer and nanocarrier in this proof of principle study was inspired by two facts: first, dendritic glycopolymers have been established as a (highly) biocompatible nanocarrier for drugs, but also in polymeric therapeutics and diagnostics.[20,22–26] Our studies have shown that dendritic glycopolymer PEI–Mal can be used as a nanocarrier for enhancing ATP molecules uptake in cells[22] and as polymeric cross-linked nanocontainers for the storage of different phosphate-containing drugs in aqueous solution.[27] The second reason relates to the potential interactions between the dendritic glycopolymers and the hydrogel: a high stability of borate bonds has been described between boronic acid-containing polymers and glucose molecules at pH 7.4.[28] This stability of borate bonds to sugar units at pH 7.4 was a good prerequisite for our study and thus, we planned to use the borate formation between PEI–Mal macromolecules and boronic acid containing hydrogels for establishing different pH-dependent release of ATP and PEI–Mal from the hydrogel.

Moreover, the use of acid-containing PNIPAAm hydrogels was based on the following facts: acid-containing PNIPAAm hydrogels[29] possess the desired anionic charge necessary to

allow the uptake of the cationic PEI–Mal macromolecules described in step II of Scheme 1. Additionally, these hydrogels guarantee the required porosity during the different uptake steps II and III in the aqueous phase (Scheme 1) in the desired temperature range since the incorporation of hydrophilic acrylic acid units into the PNIPAAm hydrogel guarantees a good degree of swelling up to 40 °C and higher as known from the literature.[30] Thus, the focus of this study is only directed towards the pH-dependent release of ATP molecules and PEI–Mal macromolecules from that hydrogel at room temperature.

We will show (a) the ATP binding and release capabilities of PEI–Mal depending on PEI–Mal structure and pH; (b) design of the hydrogel and the integration of the PEI–Mal and the ATP@PEI–Mal complexes within the hydrogel depending on the hydrogel chemical structure; and (c) pH dependent ATP release from the ATP@PEI–Mal@hydrogel multicompartment systems on varying the hydrogel structure. Ultrafiltration, UV-Vis-measurements, dynamic light scattering, zeta potential measurement, (cryo)scanning electron microscopy and dynamic scanning calorimetry will be applied to characterize steps I–V in Scheme 1.

Experimental section

Materials

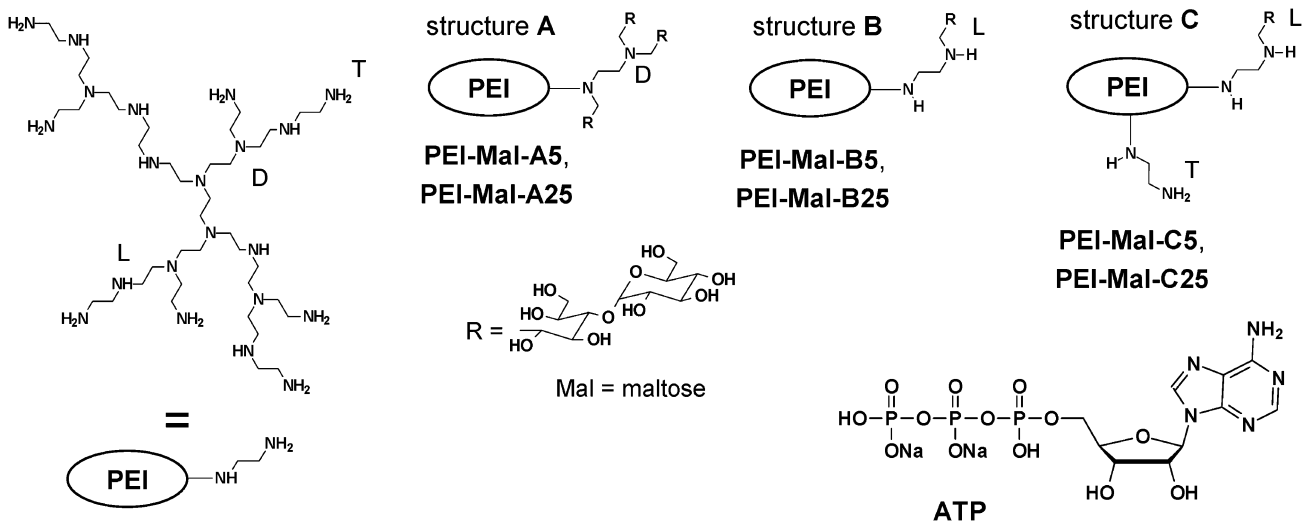
Adenosine triphosphate disodium salt hydrate (ATP), acrylic acid (AA), fluorescein 5(6)-isothiocyanate (FITC), *N*-isopropylacrylamide (NIPAAm), 4-(4-isothiocyanatophenylazo)-*N*,*N*-dimethylaniline (DABITC), *N*,*N*-methylenebis(acrylamide) (BIS), 1,4-piperazine-1,4-bis(ethanesulfonic acid) (PIPES), phosphoric and acetic acids, sodium acetate, sodium chloride, and sodium phosphate mono- and dibasic were purchased from Sigma-Aldrich (Munich, Germany). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was purchased from Merck (Darmstadt, Germany). Compositions of the used buffer solutions are presented in the ESI.‡ PEI–Mal is an abbreviation for hyperbranched poly(ethylene imine) modified with the disaccharide maltose. Maltose monohydrate was used as purchased from Fluka. Hyperbranched poly(ethylene imine) (PEI as general abbreviation; Lupasol G100 with M_w = 5000 g mol^{-1} and Lupasol WF with M_w = 25 000 g mol^{-1}) was received from BASF SE (Ludwigshafen, Germany). The synthesis and characterization of the resulting PEI–Mal structures have been carried out as described previously.[22] The various structures A–C of PEI–Mal and the definition of the abbreviations PEI–Mal-A5, PEI–Mal-A25, PEI–Mal-B5, PEI–Mal-B25, PEI–Mal-C5 and PEI–Mal-C25 are shown in Scheme 2. Syntheses of acrylamidophenylboronic acid (AAPBA), fluorescein-modified PEI–Mal-B25 (fl–PEI–Mal-B25) and DABITC-modified PEI–Mal-B25 (d–PEI–Mal-B25) are presented in the ESI.‡

Equipment

All UV-Vis measurements were carried out using 3 ml Plastibrand plastic cuvettes (Y198.1; Carl-Roth GmbH, Germany) in a Varian Cary 100 Spectrophotometer (Varian, Inc., USA). ATP was detected at a wavelength of 258 nm, d–PEI–Mal-B25: 421 nm, and fl–PEI–Mal-B25: 498 nm. For the ultrafiltration procedures solvent-resistant stirred cell XFUF07601 (Millipore Corp., USA) with ultrafiltration membranes of poly(ethylene sulfone) with the nominal molecular weight limit (NMWL) of 5000 Da (PBCC07610, Millipore Corp., USA) at a rotation speed of 50 rpm under 5 atm pressure of nitrogen was used. Lyophilisation procedures were carried out using a Christ Alpha 1–2 LD plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). In all the experiments the ultrapure MQ water was obtained with a Milli-Q Reference purification system (Millipore Corp., USA).

Synthesis of various hydrogels

NIPAAm was purified by recrystallization from *n*-hexane (70 °C, 2.5 ml of hexane per 1 g of NIPAAm). TEMED and



Scheme 2 Structures of PEI with different maltose architectures A–C, generally mentioned as PEI–Mal, and ATP molecules used for complexation and uptake into hydrogel. Abbreviations: T = terminal unit; L = linear unit; D = dendritic unit. PEI–Mal-A5–PEI–Mal-C5 possess PEI Lupasol G100 (M_w = 5000 g mol^{-1}) as the core. PEI–Mal-A25–PEI–Mal-C25 possess PEI Lupasol WF (M_w = 25 000 g mol^{-1}) as the core.

BIS were used without further purification. Dry nitrogen gas was bubbled through a 100 ml aqueous solution, composed of total 5 wt% of NIPAAm, BIS and ionic monomers, for 15 min. After that 5 ml of aqueous 0.02 g ml^{-1} ammonium persulfate solution and 200 μl of TEMED were added as the initiator and accelerator to the monomer solution, respectively. The reaction mixture was stirred vigorously for 30 s and allowed to polymerize at room temperature for 19 h on covered Petri dishes. After the polymerization hydrogels were removed from the dishes and washed for at least 72 h in water to extract unreacted compounds. For the preparation of "bulk" samples (**A**, **AB3** and **AB5**; Table 2), after purification/extraction with water swollen hydrogels were cut into pieces with a mass of around 1 g which were used for the different experiments (Fig. 6 and 8; S2–S5, ESI‡). Additionally, further pretreatment of the bulk hydrogel was carried out to transfer them into a kind of microgel defined as mechanically crushed hydrogels μ**A**, μ**AB3** and μ**AB5** (experimental description for Fig. 7 and 9, S2, S3, S6 and S7 (ESI‡), and Table 3). Washed and dried hydrogels were frozen in liquid nitrogen and crushed in the analytical mill for 10 min with the subsequent lyophilization procedure to obtain them in a dry state.

Determination of degree of swelling

(I) Experiments using bulk hydrogel **A**: swollen hydrogel samples with a mass of ~1 g were weighed and dried at 40 °C under vacuum for a minimum of two days. Then samples were weighed again. (II) Experiments using mechanically crushed hydrogels μ**A**, μ**AB3** and μ**AB5**: 15 mg of dry hydrogels μ**A**, μ**AB3** and μ**AB5** were weighed in a 2 ml Eppendorf tube and mixed with 2 ml of MQ water. The tube was allowed to swell to equilibrium for 72 h, after that it was centrifuged at 6000 rpm for 5 min to remove non-absorbed water. Then the tube was weighed once again to get the mass of the swollen gel (W_t). The weight degree of swelling (DS) is defined as the mass of absorbed water per mass of dried copolymer network (W_d):

$$DS = (W_t - W_d)/W_d$$

Uptake of ATP by several PEI–Mal macromolecules (PEI–Mal-A5, PEI–Mal-A25, PEI–Mal-B5, PEI–Mal-B25, PEI–Mal-C5 and PEI–Mal-C25): preparation of ATP@PEI–Mal complexes

(I) ATP and the various PEI–Mal macromolecules were mixed together in the required complexation ratios in 100 ml of 10 mM phosphate buffer solution at pH 7.4 (complexation ratios are presented in Table S1, ESI‡, and Fig. 3) and stirred for 24 h at room temperature. Then the complexes were separated by ultrafiltration *via* a 5 kDa PES membrane. Purified complexes were freeze-dried and the amount of the hosted ATP was investigated by UV-Vis spectroscopy. (II) To determine the kinetics of uptake a special experiment was carried out undertaking ultrafiltration of 10 ml of the complexation solution followed by the determination of non-complexed ATP molecules in the filtrate by UV-Vis spectroscopy (Fig. 1). (III) Complex capacity and complex efficiency were calculated using the following equation:

$$\text{Complex capacity} = (\text{amount of ATP in PEI–Mal}/\text{initial amount of ATP in the system}) \times 100\%$$

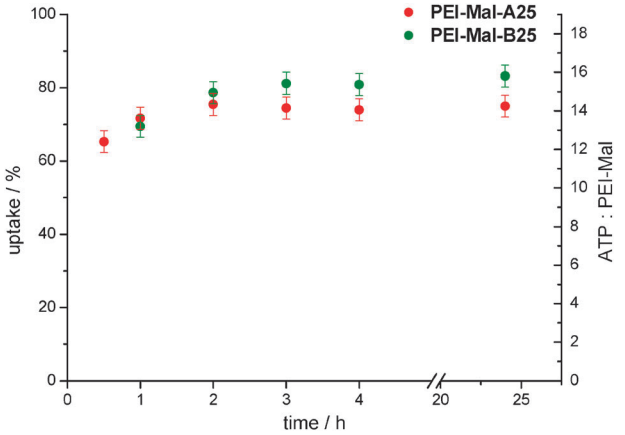


Fig. 1 Time-dependent complexation of PEI–Mal-A25 and PEI–Mal-B25 with 20 ATP molecules in 10 mM phosphate buffer solution at pH 7.4.

Release of ATP from ATP@PEI–Mal-B25 complexes

A defined amount of the dry ATP@PEI–Mal-B25 complex was dissolved in the appropriate buffer or water solution (conditions are given in Table S2, ESI‡). To determine the amount of released ATP from complexes after a defined time period, 10 ml of the complex solution were separated and ultrafiltrated. The absorbance of the filtrate was investigated by UV-Vis spectroscopy.

Uptake of PEI–Mal-B25 by anionic hydrogels

(I) Experiments using bulk hydrogels **A**, **AB3** and **AB5**: swollen hydrogel samples with a mass of ~1 g were dried at 40 °C under vacuum for a minimum of two days and then mixed with 25 ml of 1 mg ml^{-1} fl–PEI–Mal-B25 water solution adjusted with NaOH or HCl to the desired pH value. Every hour for the first day and then once a day 3 ml of the solution were repeatedly taken out and measured by UV-Vis spectroscopy. Each experiment was carried out in triplicates (Fig. 6). (II) Experiments using mechanically crushed hydrogels μ**A**, μ**AB3** and μ**AB5**: 240 mg of dry hydrogel were added to 174 ml of 1 mg ml^{-1} water solution of d–PEI–Mal-B25 and stirred overnight. Then excess of the solution was removed by centrifugation at 10 000 rpm for 10 min. The resulting supernatant was investigated by UV-Vis spectroscopy to determine the amount of hosted d–PEI–Mal-B25 (100% uptake for d–PEI–Mal-B25 was usually observed; Table S6, ESI‡). The swollen d–PEI–Mal-B25@hydrogel complex was dried by lyophilization overnight (Fig. S3, ESI‡).

Release of PEI–Mal-B25 from the PEI–Mal-B25@hydrogel complexes

(I) Experiments using bulk hydrogels **A**, **AB3** and **AB5**: fl–PEI–Mal-B25 was taken up into the hydrogel as described

above. Then, swollen fl–PEI–Mal-B25@hydrogel complexes were separated from the solutions and carefully dried with a piece of tissue and immersed into 25 ml of the solution at pH 7.4, 11.0, or MQ water. Every hour for the first day and then once a day 3 ml of the solution were repeatedly taken out and measured by UV-Vis spectroscopy. Each experiment was carried out in triplicates (Fig. S4, ESI‡). (II) Experiments using mechanically crushed hydrogels μ**A**, μ**AB3** and μ**AB5**: d–PEI–Mal-B25 was taken up into the hydrogel as described above. 10.6 mg of the dry d–PEI–Mal-B25@hydrogel complex were immersed in 8 ml of the buffer solution at pH 2.0, 5.4 and 7.4 also containing 154 mM NaCl. Every hour for the first day and then once a day 1 ml of the solution was exchanged with the fresh buffer and investigated by UV-Vis spectroscopy (Fig. 7).

Uptake and release of ATP by PEI–Mal-B25@hydrogel complexes to realize the ATP@PEI–Mal-B25@hydrogel multicompartment release system. (I) Experiments using bulk hydrogel **A**: (a) for uptake of the ATP_{46}@fl–PEI–Mal-B25 complex into the hydrogel swollen hydrogel samples with a mass of ~1 g were dried at 40 °C under vacuum for a minimum of two days and then mixed with 25 ml of 0.12 mg ml^{-1} ATP_{46}@fl–PEI–Mal-B25 water solution. (b) For uptake of ATP into the fl–PEI–Mal-B25@hydrogel complex swollen fl–PEI–Mal-B25@hydrogel samples (prepared as described above) with a mass of ~1 g were dried at 40 °C under vacuum for a minimum of two days and then mixed with 25 ml of 0.05 mg ml^{-1} ATP water solution. Every hour for the first day and then once a day 3 ml of the solution were repeatedly taken out and measured by UV-Vis spectroscopy. Each experiment was carried out in triplicates (experimental description for Fig. 8). (II) Experiments using mechanically crushed hydrogels μ**A** and μ**AB3**: A defined amount of the d–PEI–Mal-B25@hydrogel complex was added to 0.5 mg ml^{-1} ATP water solution and stirred at 4 °C for 24 h (ratios are presented in Table S3, ESI‡). Then hydrogels were centrifuged (10 min, 10 000 rpm) and lyophilized. The amount of the hosted ATP was investigated by UV-Vis spectroscopy of the supernatant solution. Release experiments from the mechanically crushed hydrogels μ**A** and μ**AB3** were carried out as described for the release of PEI–Mal-B25. Namely, 10.6 mg of the dry ATP@d–PEI–Mal-B25@hydrogel complex were immersed in 8 ml of the buffer solution at pH 2.0, 5.4 and 7.4 also containing 154 mM NaCl. Every hour for the first day and then once a day 1 ml of the solution was exchanged with the fresh buffer and investigated by UV-Vis spectroscopy (experimental description for Fig. 9 and S7, ESI‡).

Experiments of differential scanning calorimetry analysis (DSC) for hydrogels μ**A**, μ**AB3** and μ**AB5** are presented in ESI‡ (Fig. S6).

Experiments of scanning electron microscopy (SEM) and cryo-SEM for hydrogels **A**, **AB3** and μ**A** are presented in ESI‡ (Fig. S2).

Details of the experiments on dynamic light scattering (Fig. 2) and zeta potential measurements (Fig. S1 and Fig. S4, ESI‡) of ATP@PEI–Mal complexes are presented in ESI.‡

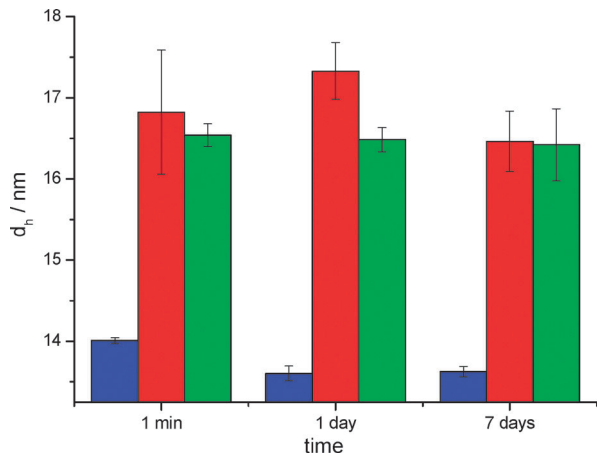


Fig. 2 Results of DLS measurements for ATP and PEI–Mal-B25 128 : 1 mixture in water (blue); ATP@PEI–Mal-B25 46 : 1 complex in 10 mM phosphate buffer solution (pH 7.4 + 154 mM NaCl, red); ATP@PEI–Mal-B25 46 : 1 complex in 10 mM acetate buffer solution (pH 5.4 + 154 mM NaCl, green) (concentration of PEI–Mal-B25 in all samples 0.5 mg ml^{-1}).

Results and discussion

The realization of a dendritic glycopolymer/hydrogel multicompartment release system for ATP, as presented in Scheme 1, requires first the investigation of the formation and stability of ATP@PEI–Mal complexes at various pH including their solution and charge properties. Attention was directed to pre-select the corresponding PEI–Mal structure where the ATP@PEI–Mal complexes exhibit variations in surface charge and isoelectric point using different complex ratios of ATP molecules and PEI–Mal. The knowledge about the right balance of covalent or non-covalent interactions within the multicompartment release system of ATP@PEI–Mal@hydrogel is the deciding parameter which allows us to tailor the release of ATP and PEI–Mal macromolecules[22] integrated in the hydrogel. For that purpose various PEI–Mal structures were tested as potential carrier macromolecules for ATP molecules. The structures A–C of PEI–Mal and their molecular properties are shown in Scheme 2 and Table 1. Briefly, structure A is characterized by a dense maltose shell (PEI–Mal-A5 with PEI M_w = 5000 g mol^{-1} and PEI–Mal-A25 with PEI M_w = 25 000 g mol^{-1}) with the prevalent conversion of nearly all primary amino groups (T units) and secondary amino groups (L units) into tertiary amino groups (D units) by reaction of PEI with maltose. Structure B has a more loose maltose shell comprised mostly of L units (monosubstitution) and only few D units (disubstitution) as peripheral groups. In other words, the periphery of structure B is dominated mostly by secondary amino functions carrying one maltose unit (PEI–Mal-B5 with PEI M_w = 5000 g mol^{-1} and PEI–Mal-B25 with PEI M_w = 25 000 g mol^{-1}). Structure C is mainly characterized by a mixture of T (~50%) and L (~50%) units as peripheral groups (PEI–Mal-C5 with PEI M_w = 5000 g mol^{-1} and PEI–Mal-C25 with PEI M_w = 25 000 g mol^{-1}) and still contains a significant amount of unsubstituted primary amino functions.

Furthermore, the structures A–C of PEI–Mal are characterized by an increasing cationic charge density explicitly shown for PEI–Mal-A5–PEI–Mal-C5[23] and by an increasing isoelectric

Table 1 Molecular properties of the applied PEI–Mal structures

PEI–Mal	DF[a] of Mal (%)	M_w[b]/g mol^{-1}	IEP[c]
PEI–Mal-A25	90	75 400	8.0
PEI–Mal-B25	40	35 600	9.1
PEI–Mal-C25	20	21 200	9.4
PEI–Mal-A5	89	28 200	8.8[d]
PEI–Mal-B5	42	13 600	9.5[d]
PEI–Mal-C5	21	8100	9.8[d]

[a] DF = degree of functionalisation; further details described in ref. 22. [b] M_w = molecular weight determined by elemental analysis; further details described in ref. 22. [c] IEP = isoelectric point. [d] Determined in ref. 23.

point from structure A to structure C (Table 1). On the other hand, ATP is an anionic molecule with pK_a 4.68 and 7.60.[31] Therefore, two types of interactions between ATP and PEI–Mal can be considered in their complex formation. One is driven by electrostatic forces between the cationic dendritic PEI scaffold of the PEI–Mal macromolecule and phosphate groups of ATP. This type of interaction is widely discussed in the literature.[22,32–34] The second type of interactions are H-bonds between hydroxy and amine groups of ATP and the maltose shell in structures A–C. Formation of H-bonds between (A) ATP[35] and various sugars and (B) liposomes[36] and sugars was previously described in the literature. Therefore, the parameters and conditions which define uptake and release of ATP in PEI–Mal structures A–C had to be determined.

Uptake and release of ATP molecules by PEI–Mal

In this study a combination of ultrafiltration and UV-Vis measurement for determining the non-complexed ATP was used to investigate uptake and release properties of PEI–Mal towards ATP, a method which has been established previously for PEI–Mal carrier systems.[37] For this purpose two types of membranes were tested for the ultrafiltration step: regenerated cellulose and poly(ethylene sulfone) (PES). In the first case strong interactions of ATP with regenerated cellulose were found and no reproducible results have been achieved. In contrast to this, PES membranes were able to pass ATP molecules without hampering the quantification of the ATP uptake and release by PEI–Mal. The details of ATP uptake and release quantification are given in the experimental part.

In the first complexation series the complexation saturation of PEI–Mal macromolecules (PEI–Mal-A25 and PEI–Mal-B25) was determined to define the complexation time for uptake of ATP molecules. Fig. 1 presents the time-dependent complexation of PEI–Mal-A25 and PEI–Mal-B25 with 20 ATP molecules. After 15 min more than 60% of the ATP molecules are complexed with PEI–Mal-A25 and PEI–Mal-B25, while after more than 3h complexation for both PEI–Mal structures with ATP reached 75 to 80%.

In a second complexation series the time-dependent stability of ATP@PEI–Mal structures was tested at various pH values. While complexes of structures A and B are stable over a long time period at various pH values (Fig. 2), complexes with structure C tend to precipitate. Thus, one can state that structures PEI–Mal-C5 and PEI–Mal-C25 are not suited to form stable complexes with excess ATP in comparison to structures A and B, probably because all charges are fully compensated which leads to aggregation.

In the following, only PEI–Mal-A5, PEI–Mal-B5, PEI–Mal-A25 and PEI–Mal-B25 were used to determine the complexation capacity towards ATP molecules, and a complexation time of 24 hours was selected for the achievement of highly saturated ATP@PEI–Mal complexes (Fig. 1). The following complexation ratios of excess ATP to PEI–Mal were applied to obtain an overview of the complexation capacity of structures A and B: 32 : 1 and 128 : 1 for PEI–Mal-A5; 7 : 1, 30 : 1 and 121 : 1 for PEI–Mal-B5; 21 : 1, 36 : 1 and 146 : 1 for PEI–Mal-A25; 1 : 1, 8 : 1, 18 : 1, 32 : 1, 64 : 1 and 128 : 1 for PEI–Mal-B25. The results of complexation capacity of PEI–Mal with structures A and B are presented in Fig. 3. For the smaller PEI–Mal-A5 and PEI–Mal-B5, the highest amount of bound ATP molecules is about 20. Thus, for a 5K PEI core there is no dependency of ATP complexation on the maltose shell density. The same structure-independent behavior was also observed for the ATP complexation of maltose- and maltoheptaose-modified PEI by using isothermal titration calorimetry (ITC).[22] In contrast to this behaviour, ATP complexation by the larger PEI–Mal-A25 and PEI–Mal-B25 is dependant on the maltose shell density. The maximal amount of complexed ATP obtained is ~40 for PEI–Mal-A25 and ~50 for PEI–Mal-B25. This correlates well with the results of very sensitive ITC experiments for maltotriose-modified PEI complexing 40 and 60 ATP molecules for structures A and C, respectively.[22] One can conclude that the used approach "ultrafiltration followed by UV-Vis measurement and determination of uncomplexed ATP" is suited to determine reproducibly the number of ATP molecules complexed by PEI–Mal.

PEI–Mal-B25 was selected as the most promising carrier system for our multicompartment release PEI–Mal@hydrogel system since a larger variation of the cationic surface charge (Fig. S1, ESI‡) was expected upon the addition of ATP to the PEI–Mal@hydrogel system. For this purpose, the change of cationic surface charge of purified ATP@PEI–Mal-B25 complexes at various complexation ratios (5 : 1, 14 : 1, 30 : 1 and 46 : 1 with excess ATP; presented in Fig. 4) was evaluated

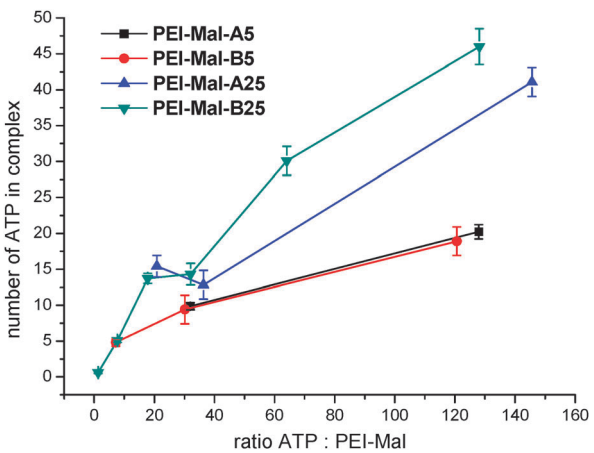


Fig. 3 Complexation capacities of PEI–Mal A and B structures (PEI–Mal-A5, PEI–Mal-B5, PEI–Mal-A25 and PEI–Mal-B25) towards ATP in 10 mM phosphate buffer solution at pH 7.4.

by zeta potential measurements. The assumption that the PEI core charge is only partially shielded in PEI–Mal-B25 leading to remaining cationic surface charge was confirmed by the increase of the number of ATP molecules complexed by PEI–Mal-B25. Additionally, the isoelectric point (IEP) of each complex decreased (from IEP $\approx$ 9.3 for pure PEI–Mal-B25 to IEP $\approx$ 5.1 for the ATP_{46}@PEI–Mal-B25 complex) when the number of ATP molecules was increased in the complexes. Additionally the 46 : 1 complex reveals a high stability, measuring particles with a defined diameter ($\sim$17 nm at pH 5.4 and 7.4 with and without 154 mM NaCl, Fig. 2) over several days, although the surface charge is nearly zero/weakly anionic (Fig. 4) under these experimental conditions. This remarkable stability of the ATP_{46}@PEI–Mal-B25 complex is astonishing since usually a high tendency of aggregation/precipitation of polyelectrolyte complexes with nearly neutral surface charge is observed.

In the following, the stability of isolated ATP@PEI–Mal with PEI–Mal-B25 was investigated at various pH. The results of the complex stability for 15 : 1 and 46 : 1 ATP@PEI–Mal-B25 complexes are presented in Fig. 5. Generally, the release of ATP from complexes strongly depends on the pH of the solution and on the ratio of ATP : PEI–Mal-B25 in the complex. For example, while in pure water the ATP_{15}@PEI–Mal-B25 complex remains virtually unchanged after one week, addition of NaCl results in the rapid release of 50% of the hosted ATP molecules from the complex. This fast release of 50% of ATP in the presence of NaCl indicates that the ATP molecules are complexed in different locations within the dendritic PEI–Mal-B25 macromolecule. While strong electrostatic interactions in the dendritic PEI core of PEI–Mal-B25 are expected to bind one part of the ATP molecules tightly, weaker electrostatic interactions and H-bonds are involved in binding some ATP molecules in the outer shell and/or at the interface of the PEI scaffold and maltose shell of PEI–Mal-B25. The weak H-bonds instantly collapse in the presence of NaCl leading to a fast release of about 50% of the loaded ATP. Furthermore, for both complexes studied the lowest stability was observed at pH 2.0 and pH 7.4 and the highest stability was found at pH 5.4 (Fig. 5).

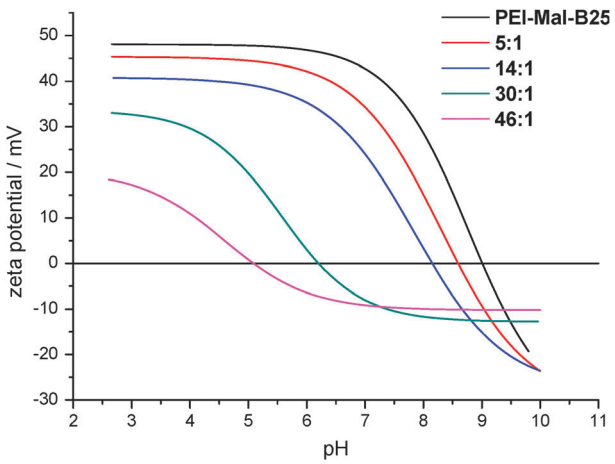


Fig. 4 pH-dependency of zeta potential for various ATP_x@PEI–Mal-B25 complexes (x = 5, 14, 30, 46).

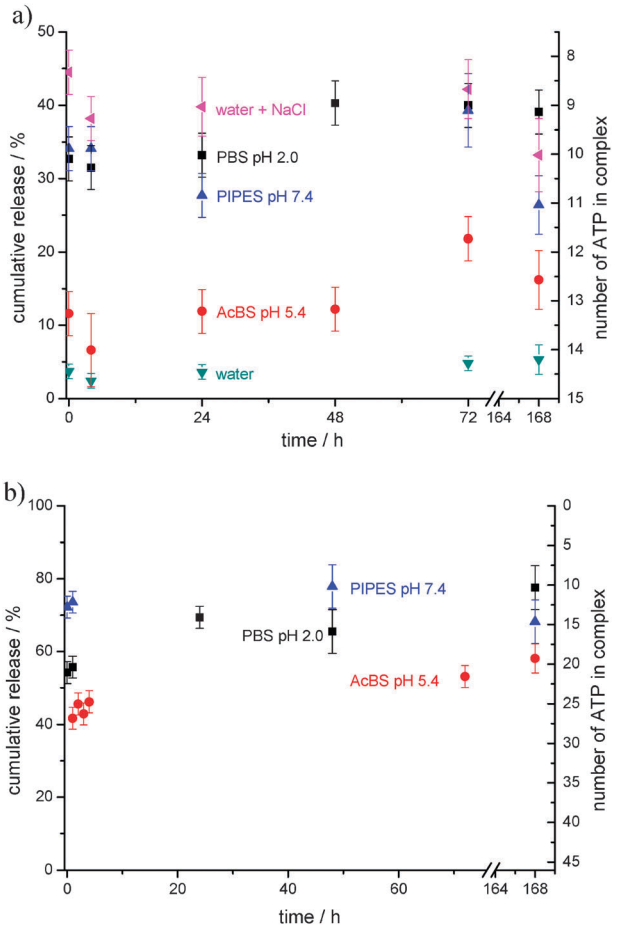


Fig. 5 Stability of 15 : 1 (a) and 45 : 1 (b) ATP@PEI–Mal-B25 complexes in various media. All buffer salines contained 154 mM NaCl (further details in ESI‡).

The pH-dependent stability of the obtained ATP@PEI–Mal-B25 complexes can be explained by considering various factors: (A) non-covalent interactions (electrostatic and H-bond interactions) between ATP and PEI–Mal-B25 and (B) some hydrolysis of ATP catalysed by PEI–Mal. Looking at the interactions between ATP and acyclic and cyclic polyamines, amine-catalysed hydrolysis of ATP was extensively studied.[33,34] The stability of *polyamine–ATP* complexes increases when the number of protonated amine groups is increased in those complexes. In contrast to this, stability of *polyamine–ATP* complexes decreases at very low pH value ($\sim$2) due to increasing amount of protonated ATP molecules as a result of an increasing rate of ATP hydrolysis.[33] Furthermore, incubation of ATP with an aza-containing macrocycle resulted in the decrease of pK_{a1} of ATP from 6.75 down to 3.5[33] and acyclic polyamines induced a larger rate of ATP hydrolysis in comparison to cyclic polyamines.[34]

Having these finding in mind it is possible to partially explain the ATP complexation behaviour of PEI–Mal-B25 at different pH values. At pH 2 PEI–Mal-B25 bears a strong positive charge at which most of the amino groups in PEI–Mal-B25 are protonated. So it is reasonable to expect the highest conversion of ATP into ADP and AMP at pH 2 compared to pH 5.4 and 7.4. This facilitates the release of ATP

molecules from the complexes at pH 2 (Fig. 5) and thus 40% and 60% release after one week was found for the release of ATP from the 15 : 1 and 46 : 1 complexes, respectively. At pH 5.4, the optimal balance of non-covalent binding of ATP molecules by positively charged PEI–Mal-B25[22] is found resulting in the lowest degree of release of ATP at both complexation ratios (Fig. 5). Under these conditions we can also expect the lowest degree of hydrolysis of ATP by PEI–Mal-B25. Thus, PEI–Mal-B25 can be considered as a rigid dendritic scaffold with a globular shape[38] showing ideal complexation of ATP molecules in the respective cavities at pH 5.4 which is nearly not influenced by external parameters especially at complexation ratio 15 : 1. At pH 7.4, PEI–Mal-B25 has the lowest cationic surface charge in the dendritic PEI scaffold which explains the higher release of ATP molecules from scaffold due to lower electrostatic binding (Fig. 5). Additionally, the PIPES (piperazine-1,4-bisethanesulfonic acid) buffer, which is used to reach pH 7.4, may interact with ATP and this can further weaken the non-covalent interactions between ATP molecules and PEI–Mal-B25.

Finally, the stability of the ATP complexes depends significantly on the ATP : PEI–Mal-B25 complexation ratios. In the same buffer solutions, in all cases, the 15 : 1 complexes release not more than 40% of ATP, while the 46 : 1 complexes loose 60–80% of complexed ATP after one week (Fig. 5). Since one can assume for both complex ratios that about the same amount of ATP is bound electrostatically by the core, the 46 : 1 complex should contain much more ATP molecules in the sugar shell than the 15 : 1 complex. As discussed above, the H-bonds towards ATP in the maltose shell are less strong, and thus, a higher percentage of ATP is released from the 46 : 1 complexes.

Having verified that a large amount of ATP can be complexed by PEI–Mal-B25 and stable complexes can be achieved under suitable conditions, in the next step the uptake and release of the cationic PEI–Mal-B25 in a suited anionic hydrogel had to be studied.

Uptake and release study of PEI–Mal-B25 by anionic hydrogels

Hybrid systems combining properties of dendritic polymers and hydrogels for potential use in drug delivery[39,40] and tissue engineering[41] have been discussed previously. Dendrimer@hydrogel materials have been prepared either by (1) uptake of the dendritic macromolecule by the hydrogel from the solution driven by ionic forces,[6,7] (2) incorporation of the dendritic macromolecule by physical entrapment during the synthesis of the hydrogel,[8] (3) chemical crosslinking of the dendritic macromolecule with other macromonomers to form the desired hydrogel network,[9–13] or (4) self-assembly of dendritic macromolecules resulting in the formation of gel-like materials.[14]

The approach used in this study to establish a potential multirelease PEI–Mal@hydrogel system is adapted from previous work of Kabanov *et al.*[6] We prefer to use electrostatic interactions as the main driving forces for the desired uptake of cationic PEI–Mal-B25 into an anionic hydrogel and we aim for a long-term binding of dendritic macromolecules in those hydrogels in a specific pH range. For this purpose three different anionic hydrogels were synthesised based on a procedure described by Kuckling *et al.*[42] The chemical composition of the three anionic hydrogels **A**, **AB3** and **AB5** are presented in Table 2. All anionic hydrogels **A**, **AB3** and **AB5** were mainly composed of the monomers *N*-isopropylacrylamide (NIPAAM), acrylic acid (AA) and *N*,*N*-methylenebis(acrylamide) (BIS). **AB3** and **AB5** possess additionally 3 and 5 mol% of acrylamidophenylboronic acid (AAPBA) as a complexing ligand. The incorporation of AAPBA in the hydrogel should allow the covalent binding of PEI–Mal-B25 to the hydrogel *via* borate formation between maltose and AAPBA (Scheme 1). The anionic NIPAAM hydrogel with varying acid comonomer contents is a material with well-known swelling and shrinking properties which has been successfully applied as bulk material and in microstructured layer technology.[29]

The results of pH-dependent uptake of PEI–Mal-B25 in 1 g of shrunken hydrogel **A** (further details in experimental part) are presented in Fig. 6a. PEI–Mal-B25 was labeled with FITC (fl–PEI–Mal-B25) for the uptake study. The amount of fl–PEI–Mal-B25 incorporated into the anionic hydrogel **A** strongly increases from pH 5 to pH 9. However, at pH 11, the lowest amount of fl–PEI–Mal-B25 was determined in hydrogel **A** (Fig. 6a). With increasing pH from acidic to basic (~pH 9) more and more acid groups of AA are deprotonated in hydrogel **A** which leads to a highly negatively charged material. This allows the increasing uptake of PEI–Mal-B25 although the surface charge of PEI–Mal-B25 is continuously decreased from pH 2 to pH 9 (Fig. 1S; Table S5, ESI‡). At pH 11, both PEI–Mal-B25 and hydrogel **A** are characterized by an anionic charge which leads to nearly zero uptake of PEI–Mal-B25 due to electrostatic repulsion.

Uptake of fl–PEI–Mal-B25 in 1 g of shrunken anionic boronic acid containing hydrogel **AB3** at pH 7.5 and 9.6 is presented in Fig. 6b. For hydrogel **A**, the highest hosted amount of fl–PEI–Mal-B25 was found at pH 9, and similarly, the maximum amount of fl–PEI–Mal-B25 was hosted by the boronic acid anionic hydrogel **AB3** at both pH values, 7.5 and 9.6 (Fig. 6b) after 200 h under the given experimental conditions. This is not surprising, since the maximal coupling of sugar to boronic acid will preferably occur at pH 7 to pH 10. However, comparing the uptake kinetics, fl–PEI–Mal-B25 is incorporated much faster by **A** as compared to **AB3**. Additionally, the hosted amount of PEI–Mal in **AB3** is impressively lower in comparison to that in **A** in the mentioned pH range (Fig. 6). The anionic hydrogel **AB5** with further increased amount of boronic acid groups showed a further reduced uptake of fl–PEI–Mal-B25 (data not shown) compared to hydrogel **AB3**. The significant lowering of PEI–Mal-B25 uptake in the boronic acid containing hydrogels is the result of various parameters, but one can assume that the degree of

Table 2 Compositions of the synthesized hydrogels

Monomer	Hydrogel **A**/mol%	**AB3**/mol%	**AB5**/mol%
NIPAAM	93	90	88
AA	4	4	4
BIS	3	3	3
AAPBA	—	3	5

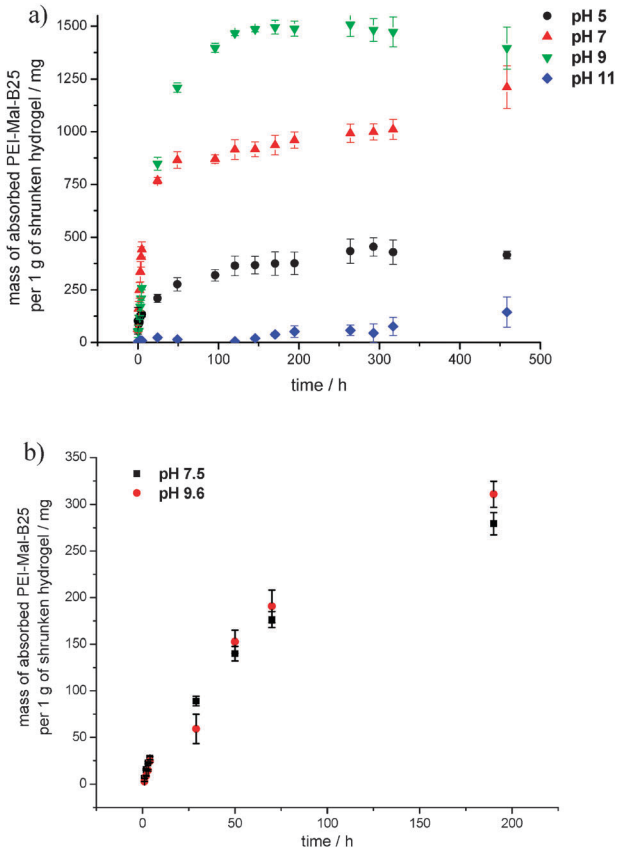


Fig. 6 Uptake of fl–PEI–Mal-B25 at different pH values by hydrogels **A** (a) and **AB3** (b).

swelling may be one deciding factor (further details later). In general, the uptake of PEI–Mal-B25 by the various anionic hydrogels is slow and especially, it takes extremely long for **AB3** and **AB5** to reach the uptake saturation.

Thus, for the development of an effective multicompartment release system, different hydrogel characteristics are needed which provide faster uptake of PEI–Mal-B25. Furthermore, also the release of PEI–Mal-B25 from the anionic hydrogels **A** and **AB3** should not explicitly be hampered by the material properties of bulk hydrogels. In order to achieve this, swollen and liquid N_2-treated hydrogels **A** and **AB3** were mechanically crushed in the analytical mill to obtain a flocculent powder of anionic hydrogels **A** and **AB3** consisting of microparticles of 100–1000 μm (named in the following as μ**A** and μ**AB3**; cryo-SEM of μ**A** and SEM of **A** and **AB3** are presented in Fig. S2, ESI‡). Using μ**A**, a reproducibly incorporated amount of fl–PEI–Mal-B25 and faster saturation of the fl–PEI–Mal-B25 uptake were identified (Fig. S3, ESI‡) which fulfilled our above-mentioned hydrogel characteristic requirements for their further use in the multicompartment release system. Thus, in the following, all studies were carried out using the mechanically crushed μ-hydrogel if not mentioned otherwise.

Fig. 7 summarizes the release properties of PEI–Mal-B25, labeled with DABITC (DABITC as opposed to FITC is stable under acidic solutions), from anionic hydrogels μ**A** and μ**AB3** at pH 2.0, 5.4 and 7.4. For this release study DABITC-modified PEI–Mal-B25 (d–PEI–Mal-B25) was quantitatively hosted in μ**A** and μ**AB3** after 24 h (Table S6, ESI‡), then the isolated d–PEI–Mal-B25@hydrogel complexes were lyophilized followed by the determination of d–PEI–Mal-B25 in the hydrogel complex using UV-Vis measurement. A defined amount (10.6 mg) of the d–PEI–Mal-B25@hydrogel complex was dissolved in the corresponding buffer solution (8 ml) at pH 2.0, 5.4 and 7.4, all containing 154 mM of NaCl. Especially, the release of d–PEI–Mal-B25 from hydrogel μ**A** complexes (Fig. 7a) can be generally characterized by two phases: a fast and a slow one mainly tailored by the localization of d–PEI–Mal-B25 in the hydrogel. The part of d–PEI–Mal-B25 adsorbed more on the surface of the hydrogel microparticles is released rapidly, while the part of d–PEI–Mal-B25 preferably adsorbed in the interior of the hydrogel particles is released very slowly or not at all (Fig. 7a). Furthermore, at pH 7.4 about twice as much d–PEI–Mal-B25 is released from hydrogel μ**A** than at pH 2. A similar increased release is seen when the pH was dropped from 7.4 to 5.4. The observed high amount of retained d–PEI–Mal-B25 within the hydrogel at pH 2 can be explained by combination of electrostatic interaction and physical cross-linking. In line with this release observation of d–PEI–Mal-B25 from hydrogel μ**A**, a similar, but slightly slower, release percentage of fluorescein-labeled PEI–Mal-B25 from hydrogel **A** (using 1 g of shrunken hydrogel for uptake

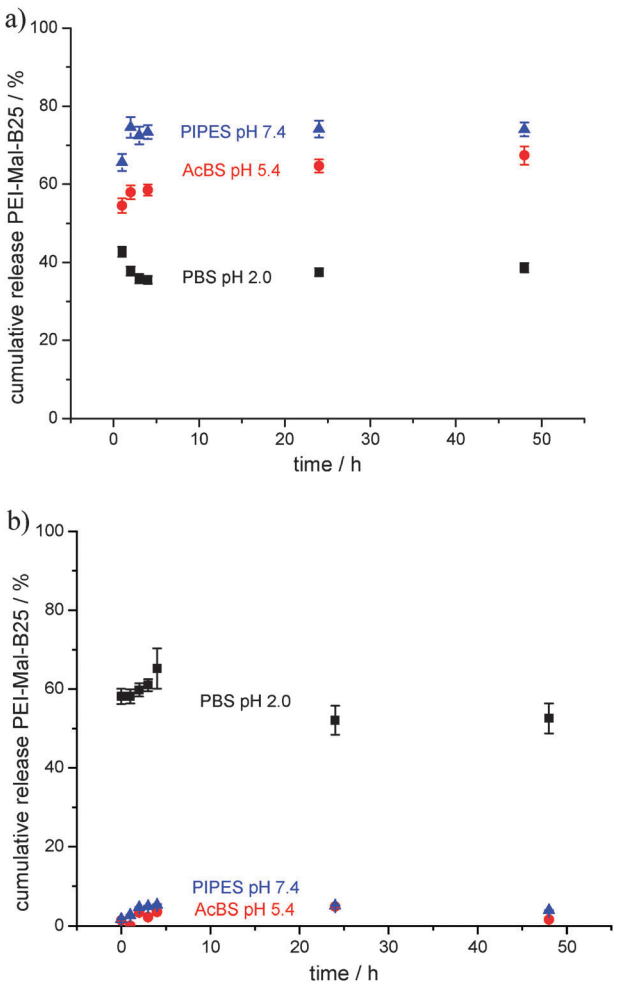


Fig. 7 Release of PEI-Mal-B25 at different pH values from hydrogels μ**A** (a) and μ**AB3** (b) (further details for buffer salines in ESI‡).

and release of fl–PEI–Mal-B25) was determined (Fig. S4, ESI‡). The size and shape of hydrogel **A** and mechanically crushed hydrogel μ**A** may play a crucial role in the duration of the burst release: hydrogel **A** has been studied as large pieces on a scale of 1 cm (Fig. S2a, ESI‡) whereas the powder-like hydrogel μ**A** contains 100–1000 μm sized particles (Fig. S2c and d, ESI‡). Thus, for a defined multicompartment release system reproducible bulk characteristics of the anionic hydrogel should be attained to be able to manipulate reliably the cumulative release of ATP or PEI–Mal-B25 from the ATP@PEI–Mal@hydrogel system.

Before discussing the release results of d–PEI–Mal-B25 from hydrogel μ**AB3**, a few words are directed towards the ability of areneboronic acid moieties to form covalent bonds with vicinal diol-containing molecules which is widely used in the development of various sugar-sensitive[28,43–45] or protein delivery[46,47] devices. Numerous authors mentioned the importance of pH in the formation of a stable covalent bond in this reaction, but indicated also its stability at pH 7.4.[28] Indeed, being formed from the charged state under basic or neutral pH the resulting borate complexes are stable under physiological conditions, whereas formation of the maltose–boronic acid complex from the uncharged state does not usually lead to success due to high susceptibility to hydrolysis.[48,49] It was remarkable to observe that d–PEI–Mal-B25@μ**AB3** complexes, prepared in MQ water, possessed the same stability at pH 7.4 (Fig. 7b) as found for the same complexes (data not shown) prepared in borate buffer at pH 9.6. It should be noted that d–PEI–Mal-B25@hydrogel μ**AB3** complex formation was only carried out in MQ water in this study to avoid non-desirable influence of buffer salts adsorbed by the hydrogel and to support the stability of borate complex under physiological conditions (pH 7.4 PBS and 154 mM NaCl).

In comparison to d–PEI–Mal-B25@hydrogel μ**A** complexes (Fig. 7a), an opposite release behaviour of d–PEI–Mal-B25 from hydrogel μ**AB3** was determined (Fig. 7b). There is nearly no release of d–PEI–Mal-B25 from μ**AB3** at pH 5.4 and 7.4, but a significant release of d–PEI–Mal-B25 (~60%) from μ**AB3** is seen at pH 2.0. The strong retention of d–PEI–Mal-B25 in hydrogel μ**AB3** at pH 5.4 and 7.4 can be explained by the high stability of the borate complex usually formed in the aqueous phase between maltose and phenylboronic acid having the characteristics of a covalent bond.[28] Moreover, it is possible that the basicity of d–PEI–Mal-B25 induces a microenvironment which locally shifts the pH around the boronic groups to higher pH values (pH 7.4) for stabilizing the interaction of boronic acid and hydroxyl groups of maltose units. This may explain the equally strong retention of d–PEI–Mal-B25 in hydrogel μ**AB3** at pH 5.4 and pH 7.4. On the other hand a reasonable explanation for the enhanced release of d–PEI–Mal-B25 at pH 2 can be the dissociation of the formed ester bond between arylboronic units and hydroxyl groups of maltose residues.

From our study we can conclude that the anionic hydrogel μ**AB3**, having boronic acid ligands, fulfils best the requirements for a sequential multi-release system as outlined in Scheme 1 under step V since retention and release of d–PEI–Mal-B25 can be fully controlled by the pH. This will allow in the first step for release of the drug from the dendritic scaffold and the hydrogel at the same time, and later on the dendritic scaffold can be reloaded with various drugs to realize again a drug@PEI–Mal@hydrogel multicompartment release system.

Degree of swelling (DS) and lower critical solution temperature (LCST) of anionic hydrogels and their complexes with PEI–Mal-B25

Before presenting the results of the uptake and release properties of ATP from the ATP@PEI–Mal@hydrogel multicompartment release system, the swelling behaviour of the hydrogels and the influence of physically and chemically incorporated PEI–Mal-B25 in hydrogels on the LCST behaviour of those hydrogels will be discussed.

As it was widely investigated,[50,51] the degree of swelling (DS) of ionic hydrogels depends on the pH of the solution. The same dependency was observed for hydrogel **A** as presented in Fig. S5 (ESI‡). In this context, hydrogel μ**A** shows the highest DS in the series of μ**A**, μ**AB3** and μ**AB5**, while a reduction of DS is observed when boronic acid units are incorporated in the backbone of the anionic hydrogels μ**AB3** and μ**AB5** (Table 3). Furthermore, an increasing amount of incorporated PEI–Mal-B25 in anionic hydrogel μ**A** reduces DS in comparison to that of pure hydrogel μ**A** in aqueous solution (Table 3).

As it is well known, the volume phase transition (VPT) in NIPAAm hydrogels is a direct consequence of the lower critical solution temperature (LCST 32–34 °C) behaviour of the linear polymer chain in water undergoing temperature-induced shape and structural changes.[52] Many parameters (salt, surfactant, balance of hydrophilicity/hydrophobicity, polymeric additives, drug) are responsible for lowering[28,30,53–55] or increasing[30] the LCST behaviour of NIPAAm hydrogels. For example, the simple presence of carbohydrate[56] in the hydrogel environment can lower LCST, but also the incorporation of cross-linkers[57] or more hydrophobic units.[28,52] Generally, most used additives (salt, polymer additives *etc.*) lower the LCST of NIPAAm hydrogels. In DSC studies the phase transition at LCST of NIPAAm hydrogels is observed by an endothermic peak related to the breaking of the hydrogen bonds of water and the polymer chain.

LCST data of anionic hydrogels μ**A**, μ**AB3** and μ**AB5** obtained from DSC measurements are presented in Table 3 and Fig. S6 (ESI‡). It is found that LCST of anionic and cross-linked

Table 3 Degree of swelling and LCST of the pure hydrogels (μ**A**, μ**AB3** and μ**AB5**), PEI–Mal-B25@μ**A** complexes of various composition (PEI–Mal-B25 concentration: (1) 7.5, (2) 13.8, (3) 24.2 wt%, g g^{-1} shrunken hydrogel) and PEI–Mal-B25@μ**AB3** complex (PEI–Mal-B25 concentration: 42 wt%, g g^{-1} shrunken hydrogel) in aqueous solution

	Degree of swelling	LCST(= T_{max}, heat)/°C
μ**A**	89	49.3 ± 0.3
PEI–Mal-B25@μ**A** (1)	47	45.6 ± 0.2
PEI–Mal-B25@μ**A** (2)	35	44.5 ± 0.3
PEI–Mal-B25@μ**A** (3)	24	42.9 ± 0
μ**AB3**	58	47.9 ± 0.6
PEI–Mal-B25@μ**AB3**	51	46.0 ± 1.3
μ**AB5**	49	46.2 ± 0.9

NIPAAm hydrogels is increased in comparison to the well-known LCST of about 32–34 °C for linear NIPAAm homopolymers. Furthermore, a gradual lowering of the LCST within the series μ**A**, μ**AB3** and μ**AB5** is observable from about 49 °C for μ**A** to about 48 °C for μ**AB3** and about 46 °C for μ**AB5** during the heating scan (Table 3). Keeping in mind the known parameters for tailoring LCST behaviour of hydrogels, incorporation of the hydrophilic monomer unit AA dominates the increase of LCST of the cross-linked NIPAAm/AA hydrogel in comparison to that of the linear NIPAAm homopolymer. Substitution of hydrophilic NIPAAm units by hydrophobic boronic acid units AAPBA in hydrogels μ**AB3** and μ**AB5** results in a very slight lowering of the LCST behaviour indicating no major difference in H-bonding behaviour towards water between hydrogel μ**A** and the boronic acid containing hydrogel.

LCST data of anionic hydrogels μ**A** and μ**AB3** with incorporated PEI–Mal-B25 obtained from DSC measurements are presented in Table 3 and Fig. S6 (ESI‡). With increasing amounts of PEI–Mal-B25 in the series from PEI–Mal-B25@μ**A-1** *via* PEI–Mal-B25@μ**A-2** to PEI–Mal-B25@μ**A-3** for anionic hydrogel μ**A** a gradual lowering of LCST of the μ**A** complexes determined in the heating scan can be stated (Table 3). Very surprisingly, there is nearly no effect on the LCST behaviour of μ**AB3** when PEI–Mal-B25 is chemically bound to boronic acid units in hydrogel μ**AB3**.

In summary, hydrogels retain their temperature-sensitivity and the differences in LCST of the various anionic hydrogels and PEI–Mal-B25/hydrogel complexes are weak with no evident correlation between DS and LCST.

Preparation and properties of ATP@PEI–Mal-B25@hydrogel

There are few studies that describe the uptake and release properties of hyperbranched polymer-based hydrogels.[12–13,58] In one special case, crosslinked hyperbranched poly(amine-ester)[12] was used as injectable hydrogel material for a locally applied multi-drug delivery system of an individual drug or combination of doxorubicin hydrochloride, 5-fluorouracil, and leucovorin calcium in cancer treatment. Depending on the hydrophilicity/hydrophobicity of incorporated drugs in hydrogels, different burst releases of the drugs were observed. Du *et al.*[58] and Vinogradov *et al.*[13] aimed for long-term release of triphosphate-containing nucleosides or their analogues by non-covalent complex formation with the hydrogel matrix. While in the first study[58] the chitosan-based hydrogel was characterized by rapid release of ATP, a hydrogel,[13] based on chemically integrated PEI, demonstrated much slower kinetics of ATP release. In both cases[13,58] the presence of two different types of interactions - weak H-bonds and strong ionic forces - between the drug and the matrix can be assumed, but with different contributions in the release of drug from the hydrogel matrix. Therefore, the ability of PEI–Mal-B25 to bind ATP by both types of forces should lead to a different kinetics of ATP release with dependence on the ATP/PEI–Mal-B25 ratio in the ATP@PEI–Mal-B25@hydrogel system in our study.

Two different approaches have been used to form the desired ATP@PEI–Mal-B25@hydrogel release system containing different amounts of ATP molecules (Scheme 1). The first one was based on freshly prepared ATP@PEI–Mal-B25 complexes followed by uptake by the anionic hydrogel in distilled water. The second approach started from PEI–Mal-B25@hydrogel complexes followed by the addition of different amounts of ATP in aqueous solution. In the first experiment using anionic hydrogel **A**, we evaluated the potential use of both approaches. The results are summarized in Fig. 8: nearly zero uptake of the ATP_{46}@PEI–Mal-B25 complex into the anionic hydrogel **A** over a period of 3 days was found while in the second case ATP (added in the ratio 128 : 1 ATP to PEI–Mal) was uptaken well by the PEI–Mal-B25@hydrogel **A** complex. The anionic hydrogel **A** can only take up directly positively charged ATP_x@PEI–Mal-B25 complexes with low numbers (x = 1–14) of ATP molecules (Fig. 4) whereas the ATP_{46}@PEI–Mal-B25 complex is negatively charged. Therefore, by the first approach the loading of the hydrogel with ATP molecules is limited. In contrast to that, the second approach allows time-dependent uptake of different amounts of ATP molecules in the PEI–Mal-B25@hydrogel **A** complex (Fig. 8). The amount of ATP incorporated into PEI–Mal-B25@hydrogel is tailored by the presence of residual cationic charge of PEI–Mal-B25 in the PEI–Mal-B25@hydrogel complex indicating preferably electrostatic interactions.

Using the second approach we addressed step II to step V outlined in Scheme 1. For that, the stability of the hosted ATP molecules in the ATP@PEI–Mal-B25@hydrogel multi-compartment release system was studied preparing systems with different ATP and d–PEI–Mal-B25 ratios (5 : 1, 15 : 1 and 30 : 1) in aqueous solution and only using hydrogels μ**A** and μ**AB3**. After a centrifugation and lyophilisation step, a defined amount of the dried ATP@d–PEI–Mal-B25@hydrogel multi-compartment release system was dissolved in buffer solution at pH 2.0, 5.4 and 7.4 to determine the time and pH dependent release of ATP from the multicompartment release system. The results are summarized in Fig. S7 (ESI‡; ATP@d–PEI–Mal-B25@hydrogel μ**A**) and in Fig. 9 for the multicompartment release system with hydrogel μ**AB3**.

The use of different ATP : PEI–Mal-B25 complexation ratios is stimulated by the possibility to generate different surface charges (Fig. 4), but the microenvironment in the PEI–Mal-B25@hydrogel complex may present different conditions

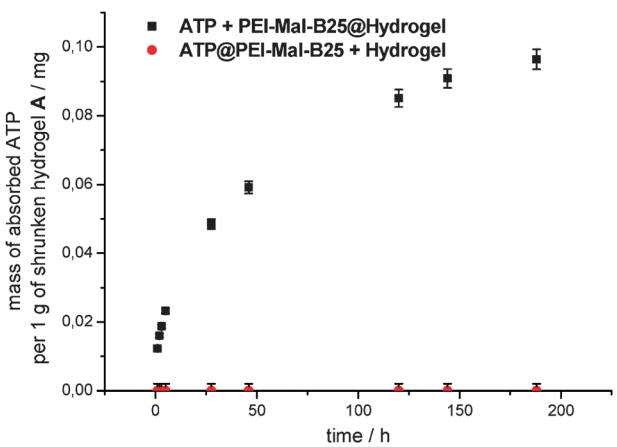


Fig. 8 Comparison of uptake capacity of ATP by the PEI–Mal–B25@hydrogel **A** complex and ATP@PEI–Mal-B25 by hydrogel **A** in aqueous solution.

for the complexation of ATP in the multicompartment system compared to the direct complexation of ATP in PEI–Mal-B25 in solution (Fig. 5).

For the multicompartment release system based on anionic hydrogel μ**A** (Fig. S7, ESI‡), a simultaneous release of ATP and d–PEI–Mal-B25 is observed at each pH value. Furthermore, there is nearly no differentiation between the different ATP : d–PEI–Mal-B25 complexation ratios at each pH value. Finally, one can conclude that there is no practical use of the ATP@PEI–Mal-B25@hydrogel system based on μ**A** since no control of the release can be achieved.

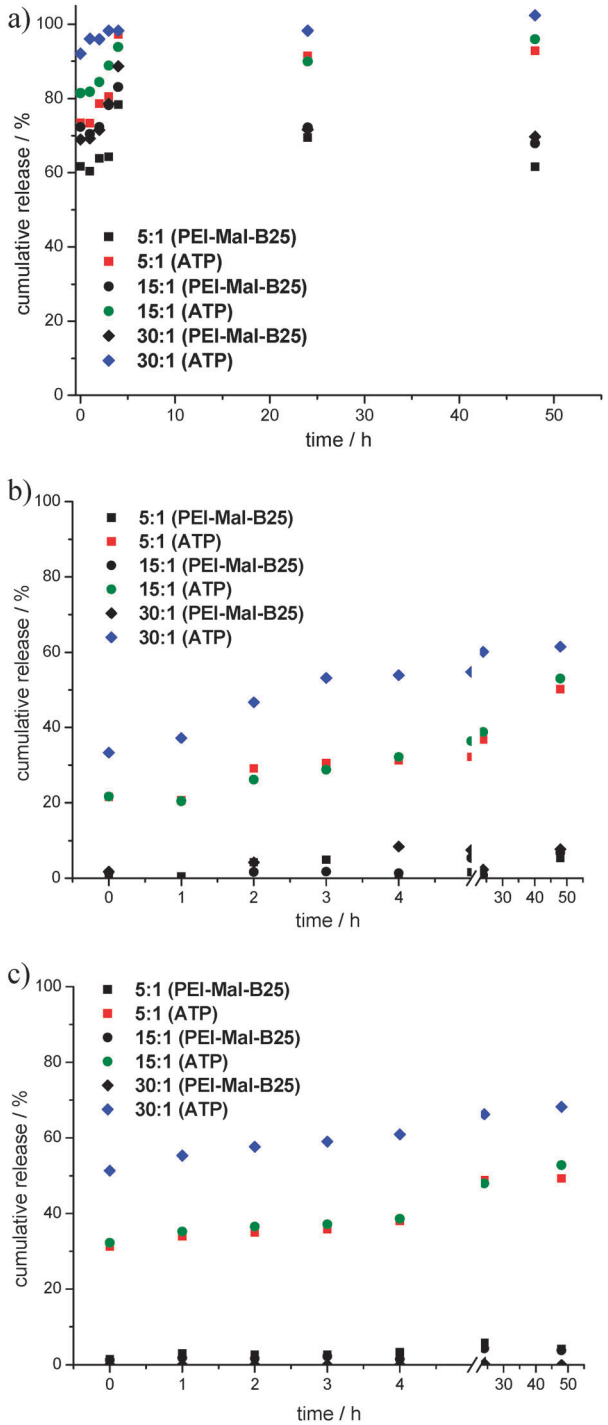


Fig. 9 Release of ATP from the ATP@PEI–Mal-B25@μ**AB3** hydrogel multicompartment release system with different ratios between excess ATP and B25 (5 : 1, 15 : 1 and 30 : 1) at pH 2.0 (a), 5.4 (b) and 7.4 (c) (further details for buffer salines in ESI‡).

In contrast to this, the boronic acid containing ATP@d–PEI–Mal-B25@hydrogel μ**AB3** multicompartment release system shows the desired selective release of ATP molecules at pH 5.4 and 7.4 (Fig. 9) due to the strong bonding of PEI–Mal-B25 by the boronic AAPBA unit in the anionic hydrogel μ**AB3** at those pH values. At pH 2, due to the preferred dissociation of d–PEI–Mal-B25 from the boronic acid unit, a rapid and simultaneously high release of d–PEI–Mal-B25 and ATP occurs. At pH 5.4 and 7.4, one can observe further the desired ratio-dependent release of ATP: the higher the ATP : d–PEI–Mal-B25 ratio, the faster is the ATP release. Therefore, the boronic acid containing ATP@PEI–Mal-B25@hydogel μ**AB3** multicompartment system offers the potential for sequential release of drug and drug carrier molecules tailored by the change of pH.

Conclusion

In this study we have successfully developed a hydrogel multicompartment and potential multirelease system in which pH-dependent sequential release of drug and dendritic carrier molecules from the hydrogel can be induced (Scheme 1, step V). Alternatively, a simultaneous release of drug molecules and nanocarriers from the hydrogel is also possible by adjusting the pH and hydrogel structure (Scheme 1, step IV). In detail, an anionic hydrogel PNIPAAm–AA, having in addition boronic acid binding sites, has the function of hosting cationic PEI–Mal macromolecules by mainly covalent interaction between the boronic acid units and the maltose units in the shell of the dendritic carrier moiety. The PEI–Mal macromolecules, themselves incorporated in anionic hydrogel, host anionic ATP molecules governed by non-covalent interaction. It was found that selective pH-dependent release of ATP from the multicompartment release system is possible assuring that the nanocarrier macromolecules will not simultaneously escape with the drug molecules from the hydrogel. This is achieved by the strong boronic acid–maltose complexes formed at pH 5.4 and 7.4 in the hydrogel. Switching to pH 2, the borate bonds are hydrolyzed leading to simultaneous release of PEI–Mal carrier loaded with ATP (Scheme 1). This is a highly interesting feature since it will allow for pH-controlled release of small biocompatible drug loaded nanocarriers into the blood stream which may be taken up by cells. On the other hand, PEI–Mal@hydrogel systems based on hydrogels without specific boronic acid binding units for the dendritic carrier do not allow for pH controlled drug delivery. In this case ATP and the dendritic scaffold are released quickly from the hydrogel over a broad pH range. It should be noted, however, that effective uptake of the drug loaded PEI–Mal into the gel needs also an optimization of the hydrogel structure with regard to porosity and high surface area for accessibility of the binding units. In our case, we were able to realize by milling a micropowder from our hydrogel which shows reasonable uptake characteristics and kinetics.

Essential for the success of our concept has been the design of the dendritic nanocarrier which in our case is hyperbranched poly(ethylene imine) (PEI) decorated to a different degree of modification by maltose units (PEI–Mal with structures A–C). We evaluated in detail the complexation capacities of these structures towards ATP. One has to note that the uptake of ATP alters the properties of PEI–Mal, *e.g.* the surface charge changes and thus, in the case of structure C immediate precipitation occurs during ATP uptake. Finally we were able to identify PEI–Mal-B25, core–shell structures with a 25 K PEI core having mainly mono-substitution on the terminal amino functions with maltose, as the ideal candidate for well-balanced interactions, firstly, because of excellent retention of PEI–Mal-B25 within the boronic acid modified hydrogel at pH 5.4 and 7.4 and secondly, because of very good uptake and release properties towards ATP. Two types of interactions are present between ATP and PEI–Mal: strong electrostatic ones between the positively charged PEI core and the anionic ATP and weaker hydrogen bonding through the maltose shell. Thus, ATP is bound to the PEI core much tighter than within the maltose shell. Depending on the amount of the hosted ATP and the pH, a two-step release can be realized for the drug and later at lower pH for the dendritic nanocarrier.

In summary, with our optimized drug@PEI–Mal@hydrogel hybrid system the concept of a multicompartment release system could be well demonstrated and it offers the possibility for the development of the next generation of drug delivery systems.

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