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Two-stage enzyme mediated drug release from LMWG hydrogels

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An enzymatically cleavable low molecular weight gelator–(model) drug conjugate system can be employed to effect a two-step enzyme mediated drug release, demonstrating the potential of LMWG systems for the development of drug delivery devices.

Introduction

One of the central problems in drug delivery today is striking the balance between toxicity and therapeutic effect of pharmaceuticals. By limiting the delivery to specific target sites, possible toxic effects at non-target sites can be avoided and the efficiency of the drug is increased. For this reason, smart drug delivery systems have been a major focus of pharmaceutical and materials research.[1] Two-stage drug delivery systems are particularly attractive as they allow an even higher degree of selectivity, with release depending on the consecutive action of two trigger mechanisms. One of the most interesting stimuli that can be employed for drug release is the action of enzymes, which allows the release of pharmaceuticals in very specific locations. For instance, drug release in tumors as a result of the enzymatic action of tumor-associated proteases (*e.g.*, plasmin),[2] or in designated areas of the GI tract under the influence of digestive enzymes has been reported.[3]

In our search for smart, responsive drug delivery systems,[4] that can be used as alternatives to polymer gel-based systems we investigated two-step drug delivery hydrogels based on low molecular weight gelator (LMWG)-drug conjugates.[5] Hydrogels of LMWGs[6,7] can be made responsive to a variety of stimuli to which they can respond by a fast gel to solution phase transition. Hence, gels of LMWGs can have rapid response times (in the order of a few seconds), not attainable by conventionally studied polymeric systems.[8] Here we present an enzymatically cleavable LMWG–(model) drug conjugate system (Fig. 1) and demonstrate that incorporation into the gel fibers protects molecules from enzymatic cleavage. Upon applying a stimulus (*e.g.*, *via* a pH or temperature change, as in Fig. 1) these gel fibers dissociate into individual molecules that can be cleaved by the enzyme, resulting in a two-step release mechanism for drugs.

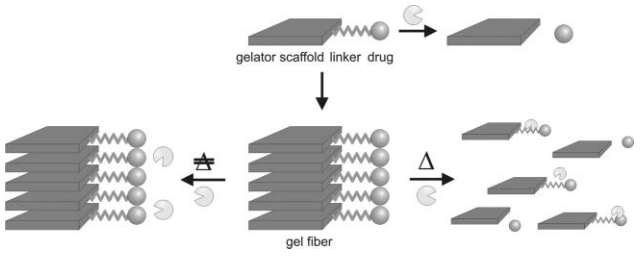
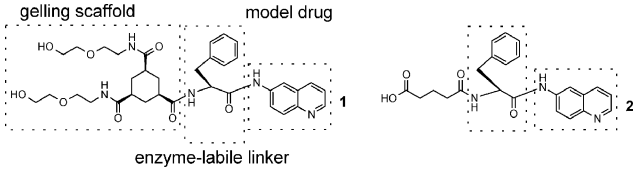


Fig. 1 Schematic representation of the enzymatic cleavage of a gelator–drug conjugate and the influence of aggregation on enzymatic cleavage; Δ = trigger for gel-to-sol transition (*e.g.*, temperature, pH).

Results and discussion

Recently we reported the excellent gelation properties of a novel class of cyclohexane trisamide-based hydrogelators with a modular architecture and demonstrated that, through the introduction of pH sensitive moieties onto the gelator scaffold, the inherently thermoreversible gels could be made responsive to changes in pH as well.[4b] For the two-step release system described here, use was made of the cyclohexane trisamide scaffold to which an L-phenylalanyl–amidoquinoline (L-Phe–AQ) moiety as well as two ethylene glycol chains were connected (Scheme 1). The L-Phe–AQ moiety can be enzymatically cleaved by α-chymotrypsin (α-chy), resulting in the release of the fluorogenic "model drug" 6-aminoquinoline (6-AQ).[9] The two ethylene glycol chains were introduced to give the overall structure a minimum degree of water solubility, which improves the gelation properties.



Scheme 1 Enzymatically cleavable LMWG **1** and non-gelator **2**.

Compound **1** indeed proved capable of gelating water with a critical gelator concentration (CGC, *i.e.*, the lowest concentration at which gelation occurs) at room temperature as low as 0.45 mM (= 0.03 wt%), making it a so-called super hydrogelator.[10] All gels up to 0.75 wt% are highly transparent, indicative of gelator aggregates with small diameters. This was confirmed by cryo-transmission electron microscopy experiments, which showed that hydrogels of LMWG **1** consist of a dense network of unbranched, very long, relatively straight tubular fibers (Fig. 2). Remarkably, all fibers were found to have identical diameters of *ca.* 4.2 nm, corresponding to the length of two molecules. Such monodispersity is rare and indicates that fibers are formed under thermodynamic, rather than kinetic, control.[11]

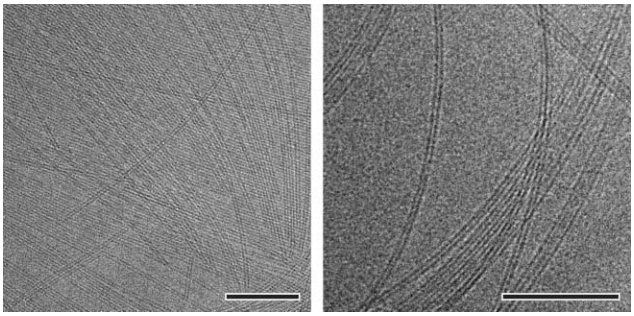


Fig. 2 Cryo-TEM images of a hydrogel of **1** (0.25 wt% = 4.77 mM). Note the uniform fiber thickness (bars correspond to 100 nm).

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Gel formation by **1** is completely thermoreversible, with gel–sol transition temperatures[4b] ranging from 52 °C for a 0.03 wt% gel, to 114 °C for a 0.55 wt% gel. Furthermore, owing to the presence of the basic quinoline moiety, gels of **1** can be reversibly switched to solutions by decreasing the pH (between pH 3–5, depending on the concentration of the LMWG). Several cycles of temperature- or pH-induced dissolution-gelation can be carried out without any loss in gelation capability, demonstrating the excellent responsiveness, as well as the robustness, of our system.

The first step in demonstrating this two-step drug release system is to prove that incorporation into gel fibers protects molecules from enzymatic cleavage. To this end, enzyme kinetics for the cleavage of LMWG **1** and non-gelating model compound **2**[9] were studied by means of fluorescence spectroscopy. In order to prevent the diffusion of the enzyme from being rate limiting, α-chy needs to be included homogeneously into gels of **1**, rather than being injected into or added as a solution on top of a gel. As α-chy is sensitive to large changes in temperature[12] and the experiments necessitated the use of buffered solutions (pH 7.75), enzyme incorporation *via* temperature or pH induced dissolution–gelation was not possible. A solution was found in the use of mixed solvent systems. The rapid addition of a buffer solution of α-chy to a solution of LMWG **1** in a small amount of DMSO resulted in the instantaneous formation of a clear, homogeneous gel suitable for fluorescence experiments (buffer : DMSO = 9 : 1).

The initial rates of hydrolysis (V_0) of the non-gelating substrate **2** as a function of the concentration (S) are accurately described using standard enzyme kinetic models (Fig. 3). The maximum initial rate V_{max} (22.4 μmol min^{-1}) and Michaelis constant K_m (4.8 mM) were calculated by means of an Eadie–Hofstee plot.

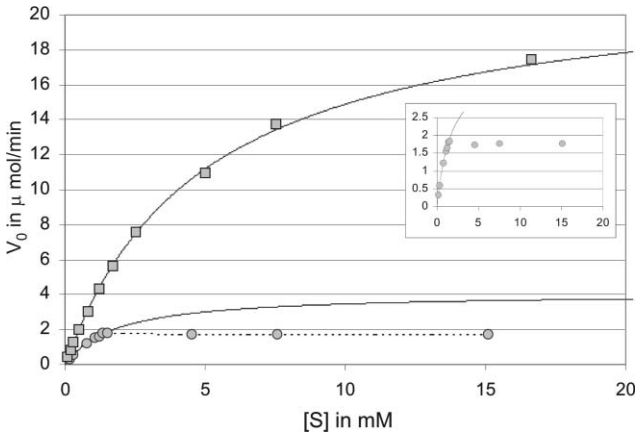


Fig. 3 Initial rate of hydrolysis of LMWG **1** (●) and model substrate **2** (■) as a function of the substrate concentration. Solid lines: theoretical curves based on the calculated V_{max} and K_m values. Dashed line: experimental curve. Inset: enlarged view of data points and theoretical curve for LMWG 1. Conditions: 25 °C, [α-chy] = 40 μM, buffer (tris–HCl, 0.1 M, pH 7.75) : DMSO = 9 : 1.

The behavior observed for LMWG **1** is in total contrast to that of **2**. At low concentrations of **1** the values of V_0 increase as a function of the substrate concentration ($V_{max} = 4.1$ μmol min^{-1}, $K_m = 1.8$ mM).[13] However, V_0 levels off abruptly at a concentration of *ca.* 1.5 mM (inset of Fig. 3), which corresponds to the CGC in this solvent mixture.[14] Since the fraction of gelator molecules in excess of the CGC is incorporated in the gel fibers, the CGC is also the maximum concentration of gelator in solution. As the value for V_0 no longer increases once the concentration of LMWG **1** has reached the CGC, this strongly indicates that only the molecules in solution are cleaved, and molecules that are incorporated into the gel fibers are protected from enzymatic cleavage. The inclusion of the α-chy in the gel did not lead to an inhibition of its activity, as was shown in a reference experiment using a gel sample of LMWG **1** containing substrate **2** (both present in 7.54 mM). The observed value for V_0 (13.7 μmol min^{-1}) is well above the maximum observed value for the pure gel samples of **1** (=1.8 μmol min^{-1}; see Fig. 3) and close to the value observed for an equimolar solution sample of **2** (=13.8 μmol min^{-1}; see Fig. 3), demonstrating that α-chy included in the gel matrix retains an activity level similar to that in solution.

As more gel fibers of **1** disassociate at higher temperatures, this should result in an increased release of 6-AQ. In order to demonstrate that the rate of enzymatic cleavage is controllable by altering the gel–sol equilibrium, hydrolysis experiments were carried out over the temperature range 25–45 °C. Studying the formation of 6-AQ in solutions of **2**, demonstrates that the increase in the rate of hydrolysis (V) as a result of the temperature-induced increase in enzymatic activity only corresponds to *ca.* 100% over a 20 °C interval (Fig. 4, lower data points). Conversely, in the gel sample of LMWG **1**, V increased by nearly 1200% over a 20 °C interval (upper data points), confirming that increasing the temperature results in an augmented release of 6-AQ as a result of the two-step mechanism of gel fiber dissolution followed by enzymatic cleavage.

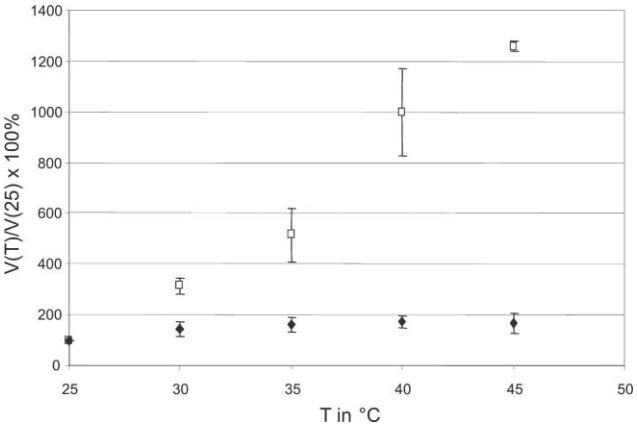


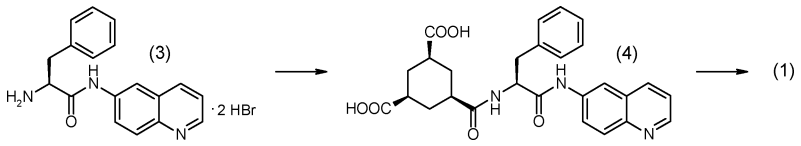
Fig. 4 Temperature dependent increase in enzymatic hydrolysis (V) with respect to $V(25)$, in 10 mM gels of **1** (□) and 10 mM solutions of **2** (◆). Error bars: standard deviation over three independent measurements. For experimental conditions see Fig. 3.

In conclusion, a simple model system based on hydrogels from a LMWG–(model)drug conjugate has successfully been used to demonstrate the concept of two-stage enzyme mediated drug release. It was shown that LMWG–(model) drug molecules are protected from enzymatic cleavage by incorporation into the gel fibers, although the enzyme included in the gel is still fully functional. By raising the temperature of the LMWG system, the gel–sol equilibrium is altered and more gelator becomes available for enzymatic cleavage, resulting in a dramatic increase in the rate of release of the model 'drug'. The results reported here demonstrate the potential of LMWG gels for controlled drug release systems and we plan to use such systems for the development of drug delivery devices.

Experimental

Materials

α-Chymotrypsin (α-CT) from bovine pancreas (MW 40.0 kDa) was purchased from Sigma and used without further purification. α-CT was Type II Sigma preparation, 3 times crystallized, dialyzed, and lyophilized. All other chemicals were purchased from Aldrich or Fluka and used without further purification. Doubly-distilled, deoxygenated water was used for all gelation tests.



Scheme 2 Synthesis of LMWG **1**.

Characterization

NMR experiments were performed using a Varian Gemini NMR spectrometer operating at 200 MHz, or a Varian VXR NMR spectrometer operating at 300 MHz. All spectra were recorded in DMSO-d6 unless stated otherwise. MS-spectra were measured on a JEOL JMS-600H or a Science API 3000 mass spectrometer. All gel-to-sol transition temperatures (T_{gs}) were determined using the "dropping ball" method,[15] which consists in carefully placing a stainless steel ball (65 mg, 2.5 mm in diameter) on top of a gel that had been prepared 16 h earlier in 2 mL glass vials and subsequently placing these vials in a heating block where the gels can be monitored by means of a CCD camera. The temperature of the heating block is increased by 5 °C h^{-1} and the T_{gs} is defined as the temperature at which the steel ball reaches the bottom of the vial. pH values (pH_{gs}) were determined by dissolving a weighed amount of the gelator in acidic water (HCl). Subsequently, small volumes of a slightly basic solution were added until the onset of gelation could be observed (either a thickening of the solution or the appearance of small "gel flakes"), upon which the pH of the solution was measured. The total volume of the gelator solution was then used to calculate the exact gelator concentration. The pH measurements were carried out with a WTW inoLab pH Level 1 meter equipped with a Hamilton minitrode that was calibrated before use. Cryo-transmission electron microscopy (CryoTEM): A few microliters of suspension were deposited on a bare 700 mesh copper grid. After blotting away the excess of liquid the grids were plunged quickly in liquid ethane. Frozen-hydrated specimens were mounted in a cryo-holder (Gatan, model 626) and observed in a Philips CM 120 electron microscope, operating at 120 KV. Micrographs were recorded under low-dose conditions on a slow-scan CCD camera (Gatan, model 794). Enzyme kinetics studies were conducted using 1 mL samples that were prepared by rapid addition of enzyme stock solution (900 μL, α-chy in 0.1 M Tris–HCl buffer, pH 7.75) to a DMSO solution of **1**, **2** or **1** + **2**. Enzyme and substrate solutions were prepared immediately before their use in experiments. The enzyme concentration after mixing with the DMSO was 40 μM. Substrate concentrations ranged between 0.09 and 15.49 mM. No cleavage of substrates **1** and **2** was observed in the absence of α-chy ($t = 5000$ sec). Fluorescence measurements were carried out on a Sim-Aminco SPF-500C spectrofluorometer equipped with a thermostated cell holder controlled at 25, 30, 35, 40, or 45 ± 0.1 °C. Excitation and emission wavelengths used to monitor the appearance of 6-AQ were 400 and 550 nm, respectively.

Synthesis

Compounds **2** and **3** were synthesized according to a literature procedure.[9] The synthesis of compounds **4** and **1** is depicted in Scheme 2.

CHex(AmPhe–6AQ)(COOH)$_2$ (4). To a solution of *cis*,*cis*-1,3,5-cyclohexanetricarboxylic acid (6.48 g; 30.0 mmol) and HOBT (2.55 g, 18.87 mmol) in DMSO (200 mL) was added CDI (1.62 g, 10.0 mmol). After stirring for 2 h at RT, **3** (4.51 g, 10.0 mmol) and Et_3N (4.04 g, 40.0 mmol) were added and stirring was continued overnight after which the solution was poured into H_2O (600 mL). The solid that was collected by filtration was dissolved in DMSO–H_2O–acetone and filtered, after which the acetone was slowly evaporated, resulting in the formation of a precipitate that was collected by filtration and subsequently dried to give pure **4** as a light orange solid. Yield: 2.95 g (6.03 mmol = 60.3%). ^{1}H NMR: δ 12.2 (bs, 2 H, COOH), 10.45 (s, 1 H, NH-Qui), 8.76 (d, 1H, $J = 3.4$ Hz, Qui-H), 8.35–8.26 (m, 3H, Qui-H + NH), 7.96 (d, 1H, $J = 9.0$ Hz, Qui-H), 7.77 (d, 1H, $J = 9.0$ Hz, Qui-H), 7.45 (m, 1H, Qui-H), 7.24 (m, 5H, ArH), 4.71 (m, 1 H, NHC*H*), 3.05 (m, 2H, CH_2Ar), 2.31 (bm, 3H, CHex), 2.03 (m, 2H, CHex), 1.75 (m, 1H, CHex), 1.23 (bm, 3H, CHex). ^{13}C NMR: δ 174.7, 173.1, 169.8, 148.1, 143.7, 136.6, 135.7, 134.5, 128.5, 128.2, 127.2, 127.0, 125.3, 122.3, 120.7, 114.2, 53.8, 40.8, 39.8, 37.2, 36.6, 30.1, 29.4. EI-MS m/z 490.1 $[M + H]^+$, calcd. for $C_{27}H_{27}N_3O_6$: 489.2.

CHex(AmPhe–6AQ)(AmEtOEtOH)$_2$ (1). A solution of compound **4** (2.80 g, 5.73 mmol), 2(-2-aminoethoxy)-1-ethanol (1.36 g, 12.94 mmol), and DMT-MM (3.58 g, 12.94 mmol) in MeOH (100 mL) and DMSO (150 mL) was stirred overnight at RT. After completion of the reaction H_2O (300 mL) was added and the resultant precipitate was filtered off, washed with H_2O (3 × 100 mL), and dried. The crude product was purified by column chromatography (SiO_2, CH_2Cl_2 : MeOH = 9 : 1–8 : 2) to give pure **1** as a light yellow solid. Yield: 1.60 g (2.41 mmol = 42.1%). ^{1}H NMR: δ 10.50 (s, 1H, NH), 8.80 (d, 1H, $J = 4.4$ Hz, ArH), 8.37 (s, 1H, ArH), 8.28 (m, 2H, ArH), 7.98 (d, 1H, $J = 9.2$ Hz, ArH), 7.82 (m, 3H, ArH), 7.49 (m, 1H, ArH), 7.35–7.17 (m, 5H, PhH), 4.74 (m, 1H, CH), 4.59 (m, 2H, OH), 3.5–3.35 (m, 12H, $CH_2OCH_2CH_2N$), 3.19 (d, 4H, $J = 5.5$ Hz, CH_2OH), 3.2–3.0 + 3.0–2.9 (m, 2H, CH_2Ph), 2.35–2.15 (m, 3H, CHex), 1.70 (m, 2H, CHex), 1.56 (m, 1H, CHex), 1.45–1.30 (m, 3H, CHex). ^{13}C NMR: δ 173.3, 173.2, 169.8, 148.0, 143.6, 136.6, 135.7, 134.5, 128.6, 128.1, 127.2, 127.0, 125.3, 122.3, 120.7, 114.1, 71.0, 68.0, 59.1, 53.7, 41.4, 41.1, 39.7, 37.4, 37.2, 36.5, 30.4. EI-MS m/z 664.2 $[M + H]^+$, 686.2 $(M + Na)^+$ calcd for $C_{35}H_{45}N_5O_8$: 663.3. Anal. calcd. for $C_{35}H_{45}N_5O_8 + H_2O$: C, 61.66; H, 6.95; N, 10.27; found: C, 61.6; H, 6.9; N, 10.3.

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