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Poster Abstracts

I. Polymeric Materials for Drug Delivery

PROTECTING SUBSTRATES FROM ENZYMATIC CLEAVAGE: HYDROGELS OF LOW MOLECULAR WEIGHT GELATORS DO THE TRICK

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Summary

An enzymatically cleavable low molecular weight gelator (LMWG)–drug conjugate is described that is capable of gelating water at concentrations as low as 0.45 mM (=0.03 wt.%). By comparing the enzymatic cleavage kinetics of the LMWG–drug conjugate with those of a nongelating substrate, it was shown that although the enzyme (α -chymotrypsin) is still functional in the gel, molecules present within the gel fibers are protected from enzymatic cleavage.

Introduction

Hydrogels of low molecular weight gelators (LMWGs) [1] are an attractive complement or even alternative for pharmaceutically interesting polymeric gel systems [2] as they possess properties unattainable by polymeric gelators [3]. The most important properties being a very rapid response to external stimuli, an inherent thermoreversibility owing to the noncovalent nature of the aggregation process, and a low molecular weight of the gelator, facilitating a fast clearance from the body after triggering the gel-to-sol transition. As we want to use LMWG systems in pharmaceutical applications, we require responsive and biocompatible systems, especially when envisioning their use for the *triggered* release of a pharmaceutical. Enzymatically cleavable systems are especially attractive triggerable systems, as they allow release of a pharmaceutical in very specific locations (e.g., tumors or designated areas of the GI tract). Enzymatically cleavable LMWG–drug conjugates (Fig. 1) could result in gels of which the molecules are protected against enzymatic cleavage as a result of their unavailability to the enzyme because they are incorporated in the fibrous aggregates. Upon triggering the gel-to-sol transition (e.g., via a pH or temperature change: Δ) the fibrous aggregates fall apart and the individual molecules can be cleaved by the enzyme. Here, we present the first enzymatically cleavable LMWG–drug conjugate system and prove that incorporation of a substrate into the gel fibers does indeed protect it from enzymatic cleavage.

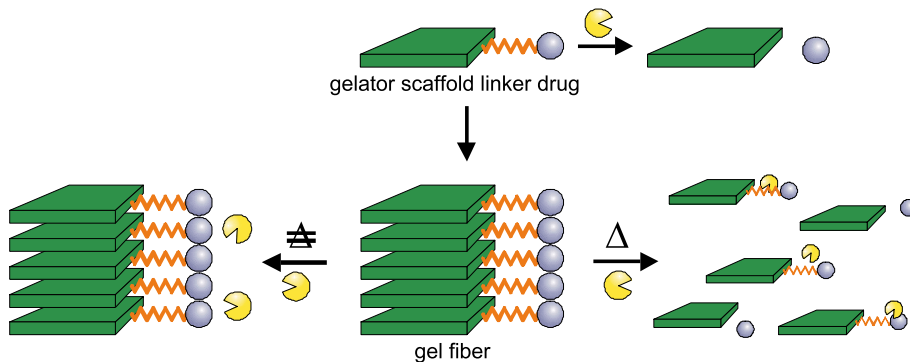


Fig. 1. Schematic representation of the enzymatic cleavage of a gelator–drug conjugate and the influence of aggregation on enzymatic cleavage.

Results and discussion

We designed and synthesized compound **1**, containing like many of our other LMWGs a cyclohexane core to which different moieties are linked via amide bonds [4]. The L-phenylalanylamidoquinoline [5] moiety was introduced to allow enzymatic cleavage to take place, using α -chymotrypsin capable of cleaving amide bonds at the C=O terminus of aromatic amino acids. The thus released fluorogenic "model drug" 6-aminoquinoline (6-AQ) allows easy monitoring of the cleavage kinetics [5]. Although gels of LMWG **1** can be easily switched into solutions and back into gels, either by temperature or pH changes, neither of these methods is very suitable for the inclusion of α -chymotrypsin in gels of **1**, as α -chymotrypsin does not respond well to large changes in either temperature or pH. A solution was found by using a mixed solvent system. Gelator **1** was dissolved in a small amount of DMSO, whereas α -chymotrypsin was dissolved in a buffer solution (Tris–HCl, 0.1 M, pH 7.75). The rapid addition of the aqueous α -chymotrypsin solution to the DMSO solution of **1** resulted in the instantaneous formation of a clear, homogeneous gel that could be used for fluorescence experiments (cryo-TEM measurements showed no differences in the gel fiber structure in this mixed solvent system when compared to the pure aqueous system). Compound **2** was synthesized in order to have a nongelating substrate, allowing us to evaluate the effect of gelation on enzymatic cleavage.

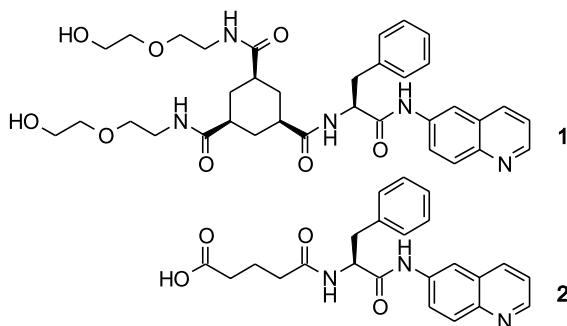
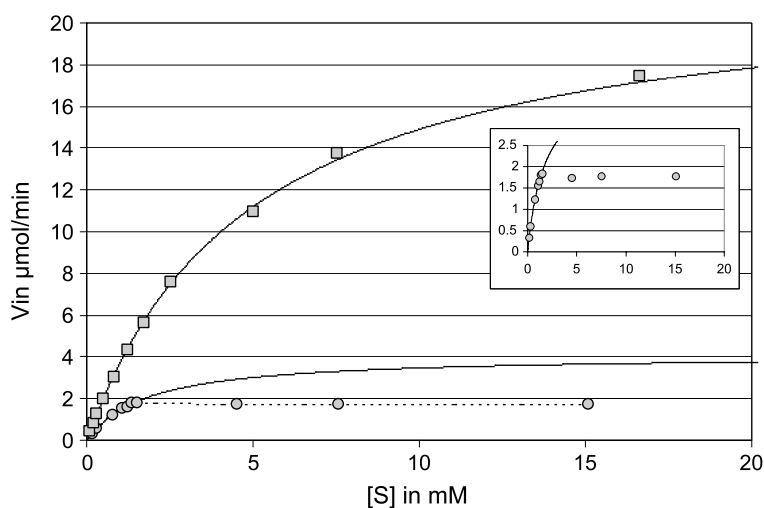


Chart 1. Molecular structures of LMWG **1** and nongelator **2**.

In order to investigate the kinetics of enzymatically catalyzed cleavage of LMWG **1** as well as model substrate **2**, experiments were carried out using different concentrations of substrates (i.e., **1** or **2**). The initial velocity of hydrolysis of each experiment was then plotted as a function of the substrate concentration (S), resulting in the points plotted in graphic 1. From the points belonging to each substrate the V_{\max} (limiting or maximum enzyme velocity) and K_m (Michaelis constant) can be determined, using either an Eadie–Hofstee or Lineweaver–Burke plot, both plots giving identical results for V_{\max} and K_m with R^2 values of >0.995 . Using the values thus obtained for substrate **2** ($V_{\max}=22.3 \mu\text{mol}/\text{min}$, $K_m=4.9 \text{ mM}$), it was possible to plot the theoretical curve, which accurately followed the experimentally determined points. For LMWG **1**, however, the experimentally determined points (circles in graphic 1) clearly did not follow the trend common for enzyme substrates. Although the values for V_{\max} did increase with increasing substrate concentrations, they abruptly leveled

off at a concentration of ca. 1.5 mM (see also inset of Graphic 1). Using only the experimentally obtained points at concentrations below 1.5 mM, values were calculated for V_{\max} and K_m (4.1 $\mu\text{mol}/\text{min}$ and 1.8 mM, respectively), which again allowed a theoretical curve to be drawn. It is clear that the experimentally determined points for **1** deviate from the theoretical curve at concentrations above 1.5 mM. As a result of its gelating properties LMWG **1** has an imposed V_{\max} of 1.8 $\mu\text{mol}/\text{min}$, rather than the expected 4.1 $\mu\text{mol}/\text{min}$. Interestingly, the concentration of 1.5 mM corresponds to the critical gelation concentration (CGC) of **1** in this particular solvent mixture. In gels of LMWGs, the concentration of gelator in solution no longer increases once the total concentration of gelator is above the CGC (i.e., all gelator in excess of the CGC will be in the gel fibers).

As the value for V_{\max} no longer increases once the concentration of LMWG **1** has increased above the CGC, this suggests that only the molecules in solution and not those in the gel fibers can be cleaved by the enzyme. By performing an enzymatic cleavage experiment in which both LMWG **1** as well as nongelating substrate **2** were present, it was shown that the incorporation into a gel did not influence the functioning of the enzyme α -chymotrypsin.



Graphic 1. Initial velocity of hydrolysis of model substrate **2** (squares) and LMWG **1** (circles). Conditions: 25 °C, α -chymotrypsin: 40 μM , buffer solution: Tris-HCl, 0.1 M, pH 7.75. 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**.

Conclusions

In conclusion, we designed and synthesized the first example of an enzymatically cleavable LMWG-(model) drug conjugate capable of gelating water at very low concentrations. Furthermore, we proved that although the LMWG-drug conjugate can be cleaved by α -chymotrypsin, the molecules that are incorporated into the gel fibers are protected from enzymatic cleavage. We plan to use similar systems for the controlled release of pharmaceuticals.

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STUDY OF THE SURFACE MOBILITY OF PLGA MICROSPHERES USING HIGH-RESOLUTION TOPOGRAPHY MEASUREMENTS WITH THE ATOMIC FORCE MICROSCOPE

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Summary

An atomic force microscope (AFM) has been used to obtain high-resolution topography measurements of surface properties of poly(lactide-co-glycolide) (PLGA) microspheres. The effect of the tapping AFM mode on a low T_g polymer (T_g=37.5±0.2) was investigated and was shown to have no discernible effect on the surface stability. Real-time AFM measurements provides a novel means of observing moisture induced transformations to the porous structures of microspheres under controlled environmental conditions.

Introduction

Biodegradable polymeric microspheres have received much attention for the sustained release of bioactive macromolecules, such as peptides or proteins. The various microencapsulation techniques lead to a wide variety of microcapsule behaviours [1,2]. The understanding of the different features is necessary in order to control and to manufacture specific pattern releases. The scanning electron microscope (SEM) is commonly used to observe the external morphology of the microspheres. However, conventional SEM requires coating of the sample with a conductive layer and operates in vacuo, thus limiting the mesoscopic resolution and study of the dynamic stabilities of microspheres particles. In contrast, the AFM offers the possibility of assessing accurately the conformational aspect and stability of any microspheres without the need for any significant sample preparation. Surprisingly, only very limited amount of work has been undertaken to study the surface conformational arrangement [3,4]. The aim of this study was to use high-resolution topographical AFM to investigate the surface properties of PLGA microspheres and to measure in situ moisture-induced transformations of porous microspheres.

Experimental methods

Preparation of microspheres

The encapsulation procedure used in this study was the water-in-oil-in-water double emulsion evaporation technique [5]. The inner aqueous phase contained 90 µl of phosphate-buffered saline (PBS, pH 7.5) with 5 µl of poly-vinyl alcohol (PVA, 5% w/v) in water, which was injected into 950 µl of oil phase consisting of dichloromethane (DCM) with 5% w/v PLGA. This primary emulsion was homogenised at 22 000 rpm for 15 s (with an Ultra TURRAX IKAT 18 basic), and transferred into 40 ml of water containing poly-vinyl alcohol (PVA, 0.5% w/v), then stirred at 500 rpm for 2 h at room temperature (lab-egg IKA RW11 basic). The solvent was allowed to evaporate for the capsides to harden. The microspheres were then harvested by centrifugation at 41 000 rpm for 1 min, washed, snap-frozen in liquid nitrogen and finally lyophilised overnight.

Scanning Electron Microscope (SEM)

Lyophilised microspheres were sprinkled onto a carbon adhesive disc, which mounted on an aluminium stub. Samples were coated with a thin layer of gold (Edwards Sputter Coater S150B). The SEM was operated at 10 kV (JEOL JSM6310).

Topography measurement with the Atomic Force Microscope (AFM)

All AFM surface topography images were recorded in TappingMode™ operation (TM-AFM). The effect of RH on surface topography was investigated using a custom-built perfusion unit. The partial vapour pressure of water within the imaging chamber of AFM was controlled by varying a mixture of dry nitrogen gas with the same gas humidified to 100% under constant temperature 25 °C (±0.2 °C).