



Universiteit
Leiden
The Netherlands

Hydrogel based drug carriers for controlled release of hydrophobic drugs and proteins

Ke, P.

Citation

Ke, P. (2011, September 14). *Hydrogel based drug carriers for controlled release of hydrophobic drugs and proteins*. Retrieved from <https://hdl.handle.net/1887/17833>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/17833>

Note: To cite this publication please use the final published version (if applicable).

Chapter 2

Cyclodextrin–dextran based *in situ* hydrogel formation: a carrier for hydrophobic drugs

Ke Peng^a, Itsuro Tomatsu^a, Alexander V. Korobko^b and Alexander Kros^a

^aLeiden Institute of Chemistry, Leiden University, 2300 RA Leiden, The Netherlands

^bNano Structured Materials, TU Delft, Julianalaan 136, 2628 BL Delft, The Netherlands

Published in: Soft Matter, **2010**, 6, 85–87

Abstract

A rapid *in situ* hydrogel forming system composed of thiol functionalized β -cyclodextrin and maleimide functionalized dextran has been prepared and the *in vitro* release profile of the hydrophobic drug all-*trans* retinoic acid was studied.

Introduction

For many years, great efforts have been made to develop hydrogels for biomedical applications because of their excellent biocompatibilities and the similarity between their highly hydrated three-dimensional polymeric networks and hydrated body tissues.¹ Among these applications, hydrogel based drug delivery systems are of interest, since their physiochemical characteristics renders them suitable for targeted drug delivery, i.e. for extension of circulation time, and reduced toxicity and side effects.² In particular, hydrogels that can be formed *in situ* under physiological conditions have recently received much attention as promising drug carriers.³ These gels can, in principle, be applied *in vivo* by direct injection with minimal surgical wounds. However, the typical hydrophilic nature of these gels prevents their use as efficient drug carriers for hydrophobic drugs because of rapid initial release of these drugs. This is due to the lack of adequate interactions between the hydrophobic drug and the hydrophilic polymer backbone of the hydrogel.⁴ Furthermore hydrophobic interactions among lipophilic drugs can cause formation of large drug aggregates during drug loading process, which leads to side effects or even toxicity.⁵ Herein, we describe a rapid *in situ* forming hydrogel system composed of cyclodextrin and dextran, which is able to bind and release hydrophobic drugs in a sustained manner.

β -Cyclodextrin (β -CD) is a cyclic oligosaccharide composed of seven glucopyranose units with a truncated cone shape with a hydrophobic cavity and hydrophilic surface. This particular structure allows β -CD to accommodate a wide variety of hydrophobic molecules including lipophilic drugs into its cavity to form an inclusion complex. The complex formation results in improved solubility, better stability and enhanced bioavailability of the hydrophobic drug, which makes β -CD an attractive tool in drug delivery systems.⁶ Moreover the chemical structure of β -CD allows it to be used as a multifunctional cross linking reagent after chemical modifications.⁷

Dextran is a highly hydrophilic and biocompatible natural polysaccharide consisting mainly of linear chains of α -1,6 linked glucopyranose residues, which has been widely used for therapeutic purpose.⁸ A number of dextran based hydrogels have been reported⁹ and used in biotechnological applications including drug delivery of hydrophilic drugs.¹⁰

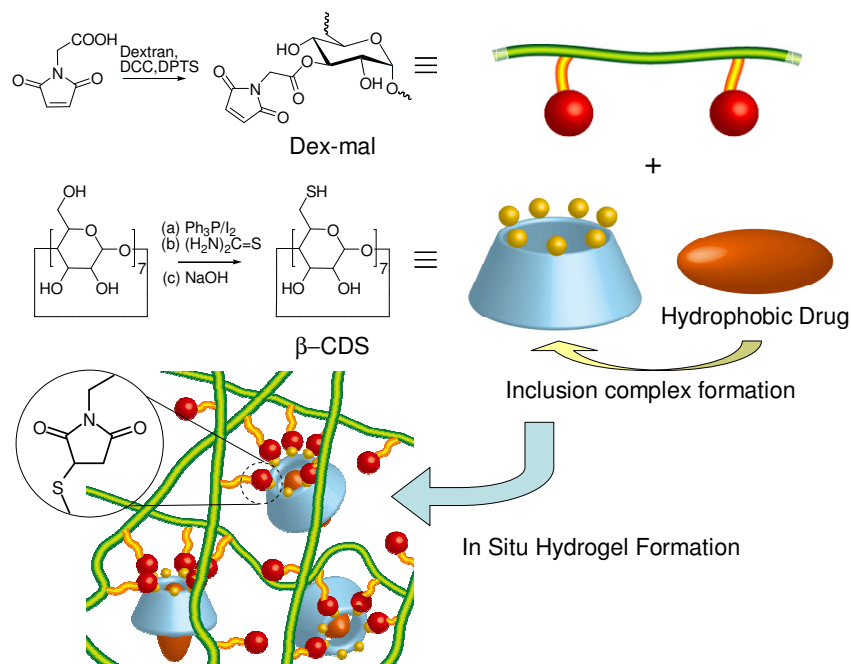


Fig. 1 Schematic representation of the *in situ* hydrogel forming system in which β -CDS plays two roles: a cross-linker of dextran back bones and a host for a hydrophobic drug. Inclusion complex formation can increase the affinity of the hydrogel for hydrophobic drugs and can avoid the aggregation of drugs.

Combining these potential materials, we designed an *in situ* hydrogel forming system employing maleimide functionalized dextran (Dex-mal) and thiol functionalized β -cyclodextrin (β -CDS), where Dex-mal acts as the backbone and β -CDS plays two essential roles as a cross-linker and as a host for the hydrophobic drug simultaneously (Fig. 1). Hydrogels were formed by mixing Dex-mal and β -CDS solutions *via* multiple Michael additions. The obtained hydrogels were characterized by rheometry and scanning electron microscopy.

To demonstrate the constant release of a hydrophobic drug from the gel over a longer period of time, all-*trans* retinoic acid (RA) was chosen as a model drug. RA has a strong *in vivo* bioactivity and plays an important role in development of animals. Major effects of RA on tissue re-growth and bone formation and patterning can easily be quantified and phenotype-based assays have been used for studying the effect of RA *in vivo* such as in zebrafish and chicken embryos.¹¹ So far, RA-soaked ion exchange resin were implanted using time consuming microsurgery to study the effect of long-term local doses of RA.¹²

However these microscopic delivery systems lack control over the total amount, the release rate and time of RA, which makes these methods unsuitable for a more extensive analytical study on the biological functions of RA.

Results and discussion

Maleimide functionalized dextrans were obtained by a conventional *N,N'*-dicyclohexylcarbodiimide mediated esterification of the hydroxyl groups of dextran with *N*-maleoylamino acid. *N*-maleoylamino acid was prepared by a synthesis procedure as previously reported.¹³ The obtained Dex-mal was characterized by ¹H NMR; signals at δ 6.9 and 4.4 from maleimide were clearly observed in addition to the signals attributed to dextran. The conjugation of *N*-maleoylamino acid to dextran was further confirmed with the peaks of the glucose unit at δ 5.3 and 5.1 those showed shifts after the esterification. The degree of substitution (DS; the number of substitutes per 100 anhydroglucose units) was determined based on the integrals of signals corresponding to maleimide at δ 6.9 and the dextran glucosidic protons at δ 3.2–4.0, 5.1, and 5.3. A series of Dex-mals with different DS of 10, 17 and 26 were obtained by varying the feeding ratio and the reaction

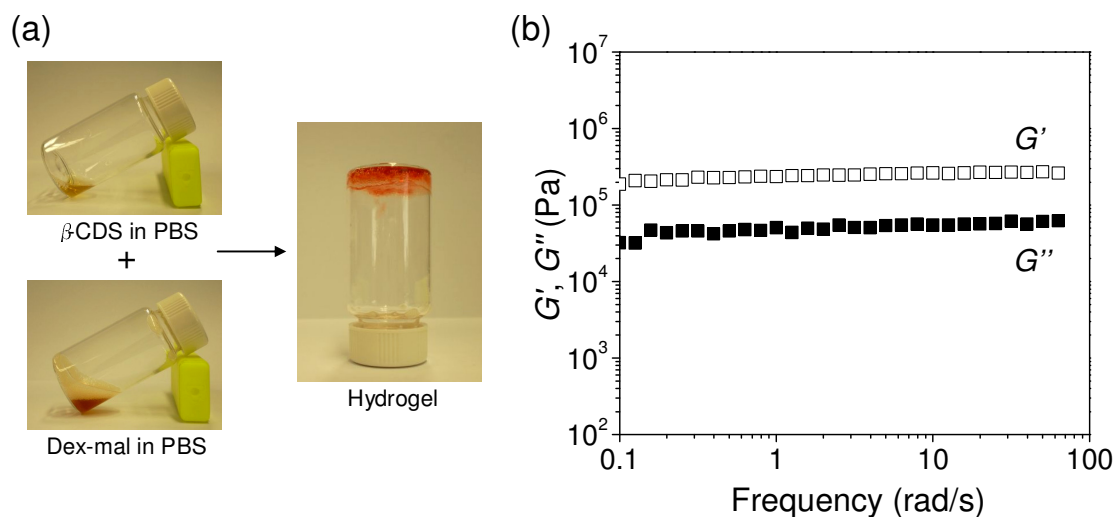


Fig. 2 (a) Photographs of *in situ* hydrogel formation. Hydrogel was formed immediately after mixing solutions of β -CDS (24 mg in 400 mL PBS) and Dex-mal (DS =17, 180 mg in 800 mL PBS). (b) Frequency dependency of storage moduli (G') and loss moduli (G'') of the obtained hydrogel.

time. All the primary hydroxyl groups of β -CD at the 6 positions were changed to thiol groups by the reported procedure.¹⁴ In order to improve the water solubility, the obtained thiolated β -CD was deprotonated by sodium hydroxide, forming the sodium salt of per-6-thio- β -cyclodextrin (β -CDS).

As shown in Fig. 2 (a), hydrogels were formed *via* Michael addition between maleimide and thiol groups by mixing solutions of β -CDS and Dex-mal in phosphate buffered saline (PBS). Upon the addition of β -CDS, a solution of 15 wt% Dex-mal (DS = 17) turned into a gel within 20 s. The gelation time was shown to depend on the polymer concentration and the DS. (DS = 10, gelation time = 30 s; DS = 27, gelation time = 15 s).

The obtained hydrogels were characterized with rheology measurements and scanning electron microscopy (SEM). Dependences of storage (G') and loss (G'') moduli on angular frequency were used to characterize the viscoelastic behavior, where G' and G'' reflect the elastic and viscous properties of the sample, respectively. It was confirmed that individual solutions of β -CDS and Dex-mal showed a dominant viscous property whereas the mixed solution showed a dominant elastic property. In Fig. 2 (b), G' and G'' were plotted as a function of angular frequency for a mixed sample of 15 wt% Dex-mal

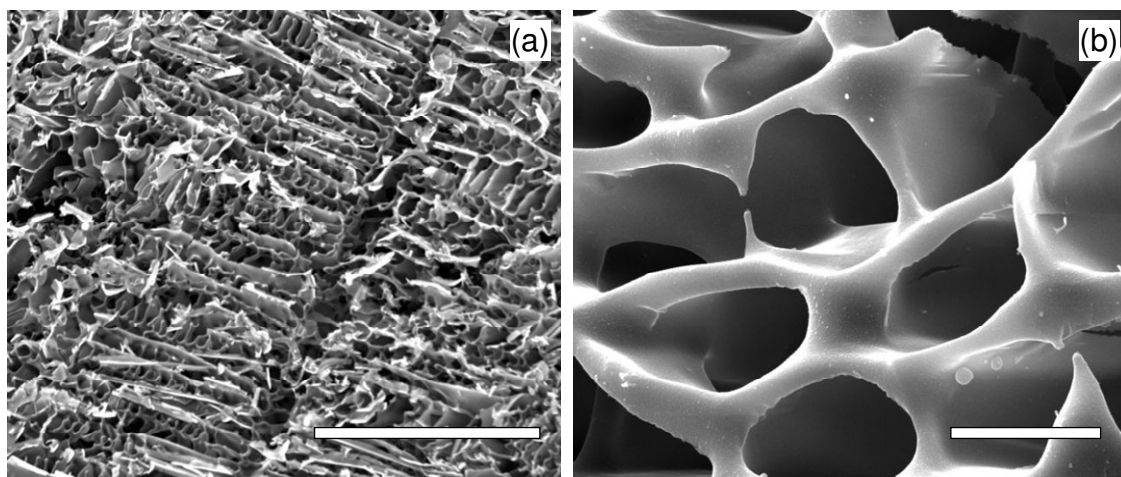


Fig. 3 SEM images of the freeze-dried hydrogel prepared from Dex-mal (15 wt%) and β -CDS solutions; with lower and higher magnifications. Scale bars in the images (a) and (b) represent 100 and 5 μ m, respectively.

(DS = 17) and β -CDS solutions. The mixed sample exhibited the characteristic viscoelastic behavior of gel, the values of G' varied little with angular frequency, and G' was significantly larger than the G'' over all measured frequencies indicating an elastic solid behavior.

The structures of the obtained hydrogels were studied with SEM on freeze-dried hydrogel samples. As shown in Fig. 3, the hydrogel had a macroporous network with a relatively regular structure indicating a highly hydrated structure and a homogeneous reaction during the gelation, respectively. In contrast only dense amorphous solids were observed for the freeze-dried individual solution of β -CDS or Dex-mal.

To examine the release characteristics of a hydrophobic drug from the hydrogel, all-*trans* retinoic acid (RA) carrying hydrogels were prepared. It is noteworthy that addition of RA did not alter the gelation time and also RA did not react with the hydrogel components. The released amount of RA per day from the hydrogel was determined by HPLC over a period of two weeks.¹⁵ As shown in Fig. 4, the release rate of RA became constant in a few days (20 nmol day^{-1}), which was controlled by the solubility of RA in PBS.¹⁶ And the release was sustained: ca. 270 nmol of the loaded RA was released in two weeks. It is important to note that the release profile did not show an initial rapid release phase (burst

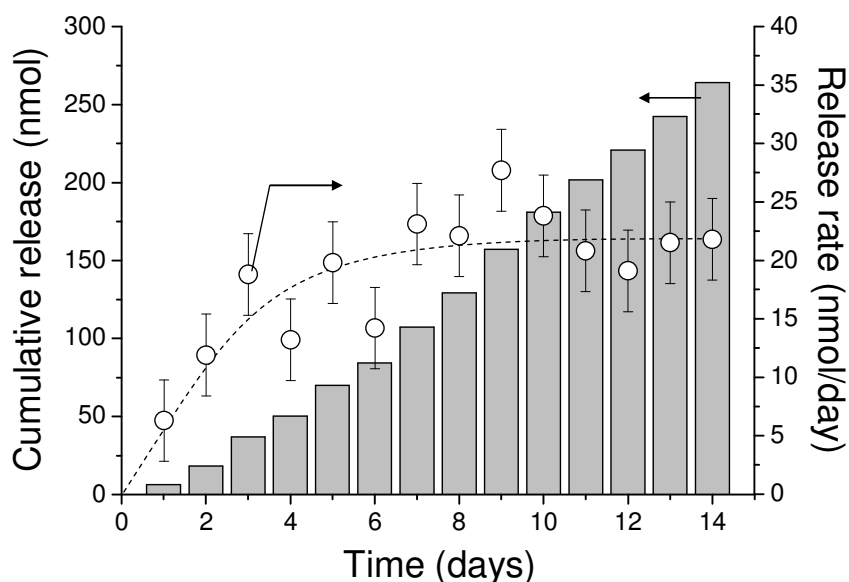


Fig. 4 Release profile of RA from 400 mL of the cyclodextrin–dextran based hydrogel carrying $3.3 \mu\text{mol}$ RA. The cumulative release (grey bars) and released amounts per day (circle) are shown.

effect), which has been commonly observed in other hydrogel systems.^{4,17} In this hydrogel, the β -CDS acts not only as a cross-linker but also as a host for RA,¹⁸ which increases the affinity for the hydrophobic drug, thus resulting in a constant release from the hydrogel without showing a burst effect. In addition, by forming the inclusion complex with β -CDS, aggregation of hydrophobic drugs during the drug loading process is prevented, thus evading the problems caused by a high local concentration of the drugs.

Conclusions

In conclusion, *in situ* hydrogel forming system composed of thiol functionalized β -cyclodextrin (β -CDS) and maleimide functionalized dextran (Dex-mal) was prepared and investigated the *in vitro* release of the hydrophobic drug all-*trans* retinoic acid (RA). Dex-mal with different degrees of substitution were synthesized and used for hydrogel formation. By mixing aqueous solutions of β -CDS and Dex-mal, hydrogels were formed *via* Michael addition within 30 s. The obtained hydrogel behaved as an elastic solid and showed to have a regular network structure. The *in vitro* release of RA did not show an initial burst effect making it an interesting drug carrier to study the effect of local doses of RA on development. This *in situ* forming hydrogel system will be further studied as an injectable drug delivery system with RA *in vivo* using phenotype-based assays in the near future. Furthermore, in principal it can also be applied to deliver any other hydrophobic drug that can form an inclusion complex with β -CDS such as doxorubicin,^{19a} diltiazem^{19b} and ketoprofen.^{19c}

Experimental

Materials.

Maleic anhydride, thiourea, *N,N'*-dicyclohexylcarbodiimide (DCC), all-*trans* retinoic acid (RA) and *p*-toluene sulfonic acid monohydrate were obtained from Fluka. Triphenylphosphine and 4-dimethylaminopyridine were obtained from Aldrich. β -cyclodextrin hydrate and iodine were obtained from Acros. All these chemicals were used as received. 4-(Dimethylamino)pyridinium 4-toluenesulfonate (DPTS) was synthesized from 4-(dimethylamino)-pyridine and *p*-toluenesulfonic acid monohydrate and recrystallized from toluene.^{9g} Dextran (Mw = 10, 000, Pharmacia Fine Chemicals,

Sweden) was dried in the vacuum oven for several days prior to use. *N,N'*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were dehydrated with molecular sieves. Water used in all experiments was purified through deionization and filtration with a Millipore purification apparatus.

Synthesis of maleimide functionalized dextran (Dex-mal).

Dex-mal was prepared through DCC mediated esterification of the hydroxyl group of the dextran with *N*-maleoylamino acid, which was obtained through a procedure reported previously.¹³ Typically, *N*-maleoylamino acids (0.96 g, 6.2 mmol) was dissolved in 30 mL DMSO followed by addition of DPTS (0.29 g, 0.9 mmol) and DCC (1.92 g, 9.3 mmol). Dextran (1.84 g, 10.3 mmol anhydroglucose (AHG) units) was dissolved in 10 mL DMSO and added to the reaction mixture slowly. After stirring for 24 h at room temperature, the formed *N,N'*-dicyclohexylurea salt was removed by filtration, and the crude product was obtained by precipitation in cold ethanol. The precipitate was collected by filtration and washed with ethanol and then dissolved in water and purified by ultrafiltration (MWCO 3500). The product was recovered by freeze drying; yield: 1.48 g (80 %). ¹H NMR (400 MHz, D₂O): δ 3.2-4.0 (m, dextran glucosidic protons), 4.4 (s, maleimide), 4.9 (s, dextran anomeric proton), 6.9 (s, maleimide). The degree of substitution (DS) of dextran is defined as the number of maleimide groups per 100 AHG units. The DS of Dex-mal was calculated from the ¹H NMR spectra based on the protons of the maleimides (δ 6.9) and the glucosidic protons of dextran (δ 3.2 - 4.0, 5.1 and 5.3). In the case mentioned above, DS was determined to be 17. Using a different feeding ratio of *N*-maleoylamino acids to dextran and different reaction time, a series of Dex-mal with different DS were obtained; Dex-mal (DS = 26) was prepared by changing feed ratio from 0.6 to 0.9 and Dex-mal (DS = 10) was prepared by shorting the reaction time to 12 hours.

Synthesis of thiol functionalized β -cyclodextrin.

Per-6-thio- β -cyclodextrin sodium salt (β -CDS) was prepared following a reported procedure.¹⁴ Triphenylphosphine (20.20 g, 77.1 mmol) was dissolved in 80 mL dry DMF, I₂ (20.30 g, 79.7 mmol) was added to this solution carefully with vigorous stirring. Then vacuum oven dried β -cyclodextrin hydrate (5.40 g, 4.7 mmol) was added to this dark brown solution and stirred overnight at 70 °C under N₂ atmosphere. DMF was removed

under reduced pressure and pH of the resulted solution was adjusted to 9–10 by adding 3 M sodium methoxide in methanol. The solution was allowed to cool down to room temperature and was kept at room temperature for 30 min. Then the reaction mixture was poured into 400 mL methanol to form a precipitate. The precipitate was filtered, washed with methanol and further purified by Soxhlet extraction with methanol. The product per-6-iodo- β -cyclodextrin was dried in a vacuum oven for 1 week; yield 5.37 g (60%). ^1H NMR (400 MHz, DMSO- d_6) δ 3.2–3.4 (m, 21H), 3.5–3.7 (m, 14H), 3.8 (d, 7H), 4.9 (d, 7H), 5.9 (d, 7H), 6.05 (d, 7H).

Per-6-iodo- β -cyclodextrin (0.97 g, 0.5 mmol) was dissolved in 10 mL dry DMF and then thiourea (0.30 g, 4.0 mmol) was added to this solution slowly. The resulted mixture was stirred overnight at 70 °C under N_2 atmosphere. Then DMF was removed under reduced pressure and yellow oil was obtained. 50 mL water and 0.26 g sodium hydroxide were added. The mixture was heated to a gentle reflux under N_2 atmosphere for 1 hour. After the suspension was acidified with 2 M HCl (2 mL), the resulted precipitate was collected by filtration and washed thoroughly with distilled water. The product was dried in a vacuum oven for 2 days; yield 0.56 g (58 %). ^1H NMR (400 MHz, DMSO- d_6) δ 2.1 (t, 7H), 2.7 (m, 7H), 3.2 (br m, 7H), 3.4–3.7 (m, 28H), 4.9 (d, 7H), 5.83 (s, 7H), 5.95 (d, 7H). Obtained per-6-thio- β -cyclodextrin (0.10 g) was suspended in 10 mL H_2O and one equivalent of sodium hydroxide (0.23 g) to the thiols was added. After stirring for 30 min, excess NaOH was neutralized with 1 M HCl by monitoring the pH of the solution. Water soluble per-thio- β -cyclodextrin sodium salt was recovered by freeze-drying with a quantitative yield.

Hydrogel Formation.

Hydrogels were obtained by mixing solutions of Dex-mal and β -CDS. Typical procedure was following: 200 μL Dex-mal (60 mg) and 200 μL β -CDS (8 mg) in phosphate buffered saline (PBS) solutions were mixed by vortexing. The gelation time was determined by the vial tilting method. When there was no flow of the sample within 5 seconds, it was regarded as a gel. In order to keep the ratio of cross-linkable groups at 1:1, 10% excess thiols to maleimide groups were used due to the fact that thiol groups may be oxidized or form disulfide bonds. Due to the high abundance of thiol groups on β -CDS, it gives amber solution and it turns into red after the reaction with Dex-mal.

Characterizations of the Hydrogel.

Sample for visco-elastic measurements was prepared by mixing two solutions of Dex-mal (0.18 g in 800 μ L PBS) and β -CDS (24.0 mg in 400 μ L PBS). A small sample of the hydrogel to be analyzed was manually applied to a rheometer (Ares AR-G2 TA) with plate-plate geometry (40 mm in diameter) and a 400 microns gap distance. Prior to the measurements, the strain sweep tests were performed on the sample to determine the maximum limit of the linear viscoelastic regime. Data acquisition started when steady state was reached, as indicated by normal forces. Frequency sweeps were done between 0.1 and 100 Hz in the linear response regime at 25 $^{\circ}$ C.

Scanning electron microscopy (SEM) was used to study the structure of the hydrogel. It was conducted on a Nova NanoSEM (FEI) with an accelerating voltage of 10 kV and spot size of 3.5. Sample was prepared by freeze-drying from 400 μ L of the freshly made hydrogel. Fractured pieces were mounted onto an aluminum stub and coated with carbon before measurements.

Drug loading and release.

All-*trans* retinoic acid (RA) (1.0 mg, 3.3 μ mol) was dissolved in acetone (100 μ L) and then this solution was added to 200 μ L β -CDS (8.0 mg, 5.7 μ mol) PBS solution. Acetone was removed by vacuum evaporation. Upon the addition of 200 μ L Dex-mal (60.0 mg) in PBS to the mixture, the hydrogel was formed immediately. Before releasing experiment, the obtained hydrogel was aged overnight at room temperature. The RA carrying hydrogel was put into a dialysis tube (MWCO 1000) with 5 mL of PBS and the tube was placed in 100 mL PBS solution. The surrounding PBS was stirred at 50 rpm at room temperature and refreshed every 24 hours. The amount of RA released from the hydrogel was detected by the HPLC (Shimadzu) using UV detection at 345 nm equipped with an autoinjector (Shimadzu, SIL-10AD) and a dC18 column (Atlantis, Waters; 150 $\times$ 4.6 mm; 5 μ m particle size) with a 21 min linear gradient from 100% solvent A (methanol/acetonitrile/THF, 62:33:5, v/v/v) to 75% solvent A with 25% solvent B (acetic acid/H₂O, 2:98, v/v). For calibrations, a methanol solution of RA (5 mg/L) was used. In order to prevent decomposition of RA during the release experiment, the sample was kept in dark. It has also been confirmed that RA did not react with thiol functionalized cyclodextrin, with ¹H NMR in a DMSO solution.

References

- 1 (a) C. Wang, R. J. Stewart and J. Kopecek, *Nature*, **1999**, 397, 417; (b) W. E. Hennink and C. F. van Nostrum, *Adv. Drug Delivery Rev.*, **2002**, 54, 13; (c) M. P. Lutolf, J. L. Lauer-Fields, H. G. Schmoekel, A. T. Metters, F. E. Weber, G. B. Fields and J. A. Hubbell, *Proc. Natl. Acad. Sci. U. S. A.*, **2003**, 100, 5413; (d) N. A. Peppas, J. Z. Hilt, A. Khademhosseini and R. Langer, *Adv. Mater.*, **2006**, 18, 1345; (e) C. Hiemstra, W. Zhou, Z. Y. Zhong, M. Wouters and J. Feijen, *J. Am. Chem. Soc.*, **2007**, 129, 9918.
- 2 A. S. Hoffman, *Adv. Drug Delivery Rev.*, **2002**, 54, 3.
- 3 (a) L. Yu and J. D. Ding, *Chem. Soc. Rev.*, **2008**, 37, 1473; (b) T. R. Hoare and D. S. Kohane, *Polymer*, **2008**, 49, 1993.
- 4 M. C. Chen, H. W. Tsai, C. T. Liu, S. F. Peng, W. Y. Lai, S. J. Chen, Y. Chang and H. W. Sung, *Biomaterials*, **2009**, 30, 2102.
- 5 V. P. Torchilin, *Cell. Mol. Life Sci.*, **2004**, 61, 2549.
- 6 (a) M. E. Davis and M. E. Brewster, *Nat. Rev. Drug Discovery*, **2004**, 3, 1023; (b) K. Uekama, F. Hirayama and T. Irie, *Chem. Rev.*, **1998**, 98, 2045.
- 7 (a) L. Janus, G. Crini, V. El-Rezzi, M. Morcellet, A. Cambiaghi, G. Torri, A. Naggi and C. Vecchi, *React. Funct. Polym.*, **1999**, 42, 173–180; (b) J.-F. R. dos Santos, R. Couceiro, A. Concheiro, J.-J. Torres-Labandeira and C. Alvarez-Lorenzo, *Acta Biomater.*, **2008**, 4, 745–755.
- 8 R. Mehvar, *J. Controlled Release*, **2000**, 69, 1.
- 9 (a) S. J. de Jong, S. C. de Smedt, J. Demeester, C. F. van Nostrum, J. J. Kettenes-van den Bosch and W. E. Hennink, *J. Controlled Release*, **2001**, 72, 47; (b) S. H. Kim, C. Y. Won and C. C. Chu, *Carbohydr. Polym.*, **1999**, 40, 183; (c) W. N. E. van Dijk-Wolthuis, O. Franssen, H. Talsma, M. J. van Steenberg, J. J. Kettenes-van den Bosch and W. E. Hennink, *Macromolecules*, **1995**, 28, 6317; (d) S. H. Kim, C. Y. Won and C. C. Chu, *J. Biomed. Mater. Res.*, **1999**, 46, 160; (e) B. G. de Geest, W. van Camp, F. E. Du Prez, S. C. de Smedt, J. Demeester and W. E. Hennink, *Chem. Commun.*, **2008**, 190; (f) R. Jin, C. Hiemstra, Z. Y. Zhong and J. Feijen, *Biomaterials*, **2007**, 28, 2791; (g) C. Hiemstra, L. J. van der Aa, Z. Y. Zhong, P. J. Dijkstra and J. Feijen, *Macromolecules*, **2007**, 40, 1165; (h) S. G. Levesque and M. S. Shoichet, *Bioconjugate Chem.*, **2007**, 18, 874.
- 10 (a) L. Hovgaard and H. Brondsted, *J. Controlled Release*, **1995**, 36, 159; (b) S. H. Kim and C. C. Chu, *J. Biomater. Appl.*, **2000**, 15, 23; (c) W. E. Hennink, S. J. de Jong, G. W. Bos, T. H. J. Veldhuis and C. F. van Nostrum, *Int. J. Pharm.*, **2004**, 277, 99; (d) C. Hiemstra, Z. Y. Zhong, M. J. van Steenberg, W. E. Hennink and J. Feijen, *J. Controlled Release*, **2007**, 122, 71.
- 11 (a) S. A. Brittin, S. J. Duivestijn, W. Bitter, J. D. de Bruin, D. Champagne, E. Cuppen, G. Flik, R. Janssen, I. M. de Jong, R. de Kloet, A. Kros, A. H. Meijer, A. M. van der Sar, M. J. M. Schaaf, S. Schulte-Merker, H. P. Spaink, P. P. Tak, C. M. Vandenbroucke-Grauls, M. J. Vervoordeldonk, F. F. Vonk, F. Witte and M. K. Richardson, *Int. J. Dev. Biol.*, **2009**, 53, 835–850; (b) C. Tickle, B. Alberts, L. Wolpert and J. Lee, *Nature*, **1982**, 296, 564.

- 12 C. Tickle, J. Lee and G. Eichele, *Dev. Biol.*, **1985**, *109*, 82.
- 13 D. H. Rich, P. D. Gesellchen, A. Tong, A. Cheung and C. K. Buckner, *J. Med. Chem.*, **1975**, *18*, 1004.
- 14 M. T. Rojas, R. Koniger, J. F. Stoddart and A. E. Kaifer, *J. Am. Chem. Soc.*, **1995**, *117*, 336.
- 15 G. Eichele, C. Tickle and B. M. Alberts, *Anal. Biochem.*, **1984**, *142*, 542.
- 16 E. Z. Szuts and F. I. Harosi, *Arch. Biochem. Biophys.*, **1991**, *287*, 297.
- 17 Y. L. Zhang and C. C. Chu, *J. Biomed. Mater. Res.*, **2002**, *59*, 318.
- 18 P. Montassiera, D. Ducheneb and M.-C. Poelmana, *Int. J. Pharm.*, **1997**, *153*, 199.
- 19 (a) E. S. Gil, J. S. Li, H. N. Xiao and T. L. Lowe, *Biomacromolecules*, **2009**, *10*, 505; (b) K. Uekama, T. Horikawa, Y. Horiuchi and F. Hirayama, *J. Controlled Release*, **1993**, *25*, 99; (c) H. S. Woldum, K. L. Larsen and F. Madsen, *Drug Delivery*, **2008**, *15*, 69.