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Cyclodextrin/dextran based drug carriers for a controlled release of hydrophobic drugs in zebrafish embryos†

Ke Peng,^a Chao Cui,^b Itsuro Tomatsu,^a Fabiola Porta,^a Annemarie H. Meijer,^b Herman P. Spaink^b and Alexander Kros^{*a}

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Hydrogel-based drug carriers have been developed from biocompatible materials, cyclodextrin, dextran and poly(ethylene glycol) and were used in zebrafish embryos. Maleimide modified dextrans (Dex-mal) were functionalized with cyclodextrins and crosslinked to form a hydrogel using either per-6-thio- β -cyclodextrin (PSCD) or a combination of mono-6-thio- β -cyclodextrin (MSCD) and di-thiolated poly(ethylene glycol) (DSPEG). Using all-*trans* retinoic acid (RA) as a model hydrophobic drug, a sustained release from these cyclodextrin modified hydrogels was observed *in vitro* without an initial burst. This is because the cyclodextrin moiety in these hydrogels acts as a binding site for the RA. Furthermore, the nanosized hydrogel particles were injected into early stage zebrafish embryos in order to test *in vivo* release of RA and biocompatibility. We found the gel particles prepared from Dex-mal, MSCD and DSPEG were suitable for use in zebrafish embryos and it showed the release of RA in the embryos occurs in a controlled manner.

Introduction

Hydrogel-based drug delivery systems are of interest due to their attractive characteristics, which can lead to targeting delivery, extension of circulation time, and reduction of toxicity and side effects.¹ Particularly, hydrogels that can be formed *in situ* under physiological conditions have recently been paid much attention as promising drug carriers.²

To advance applications of hydrogels in the biomedical field, careful design of the hydrogel materials is critically important.^{3–10} For instance, applications of current hydrogels as drug delivery tools are limited by their hydrophilic nature, which prevents their use as drug delivery systems for hydrophobic drugs because of a rapid drug release in the initial phase. This is due to the lack of adequate interactions between the hydrophobic drug and the hydrophilic polymer network.^{11–13} Furthermore, because of the hydrophobic interactions, large drug aggregates may be formed during the drug loading process, which can result in a high local concentration and cause side effects or even toxicity.¹⁴ However, around 40% of bioactive new chemical entities identified in combinatorial screening programs are poorly water soluble.¹⁵ Therefore, a hydrogel system with the ability to load and release hydrophobic drugs without the aforementioned problems is desired.

Recently, we have reported an *in situ* forming hydrogel system using per-6-thio- β -cyclodextrin (PSCD) as a binding site for the hydrophobic drugs and a crosslinker simultaneously (Fig. 1).¹⁶

^aLeiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. E-mail: a.kros@chem.leidenuniv.nl; Fax: +31 71 527 4397; Tel: +31 71 527 4234

^bInstitute of Biology, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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β -Cyclodextrin (β -CD) is a cyclic oligosaccharide composed of seven glucopyranose units with a structure that can accommodate a wide variety of hydrophobic molecules including lipophilic drugs into the cavity to form an inclusion complex.¹⁷ The inclusion results in improved solubility, better stability and enhanced bioavailability of the hydrophobic drugs, which make cyclodextrins highly attractive in drug delivery systems.^{18–20} As a result of β -CD functionalization, our hydrogel showed an increase of the affinity to the hydrophobic drug, and a well controlled *in vitro* release profile of a model hydrophobic drug, all-*trans* retinoic acid (RA), was observed without initial burst effect (see ESI†).

Based on our previous findings, this study deals with the *in vivo* testing of the hydrogel-based drug carriers. For *in vivo* testing, we have chosen to use zebrafish as an animal model, since it can be a cost-effective bridge between cell-based assays and mammalian models in the drug screening technology in the future.^{21,22} In particular, zebrafish embryos are attractive for the following reasons. First, they are very sensitive to foreign compounds. Second, the optical transparency of the embryos makes it possible to examine their early development. Third, the zebrafish embryo is easily accessible to experimental manipulations. Due

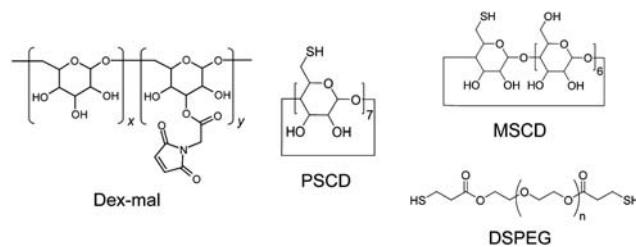


Fig. 1 Structures of the chemicals used in this work. Dex-mal is used as a backbone. PSCD plays two roles: a binding site for the hydrophobic drugs and a crosslinker, simultaneously. These functions are undertaken by MSCD and DSPEG separately in the other system.

to these advantages, the early development of the zebrafish embryos is well documented.²³ For example, the bioactivity of RA has been investigated, which can result in serious developmental defects.^{24–26} In addition, zebrafish embryos are frequently used to analyze the toxicity of compounds,²⁷ thus we can also evaluate the biocompatibility of our systems.

To use the hydrogel as an injectable drug delivery system in small animals (the size of the zebrafish embryo is about 1 mm), the size of the gel has to be smaller than the tip size of the needle used for the injection and the viscosity should not be high. Therefore, we decided to reformulate the former macroscopic hydrogels to nanosized hydrogel particles, which renders these hydrogels injectable.^{28–34} For this we made a moderate change in the procedure to synthesize a hydrogel-based drug carrier with nanometre scale dimension.

In this contribution, we present the fabrication and characterization of the newly developed hydrogel-based drug delivery system that can be used in zebrafish embryos. Using this assay, we found that one of our candidates for *in vivo* testing (*i.e.* the one composed of Dex-mal and PSCD) was not suitable for the zebrafish embryos; most of the embryos did not survive after the injection. However, the hydrogels composed of Dex-mal, mono-6-thio- β -cyclodextrin (MSCD) and di-thiolated poly(ethylene glycol) (DSPEG) showed excellent biocompatibility. Furthermore, the latter system showed well controlled release of the model hydrophobic drug in the zebrafish assay. Based on these results, we conclude that the nanogel particles composed of Dex-mal, MSCD and DSPEG will be useful to establish a new drug screening assay using the early stage zebrafish embryos.²²

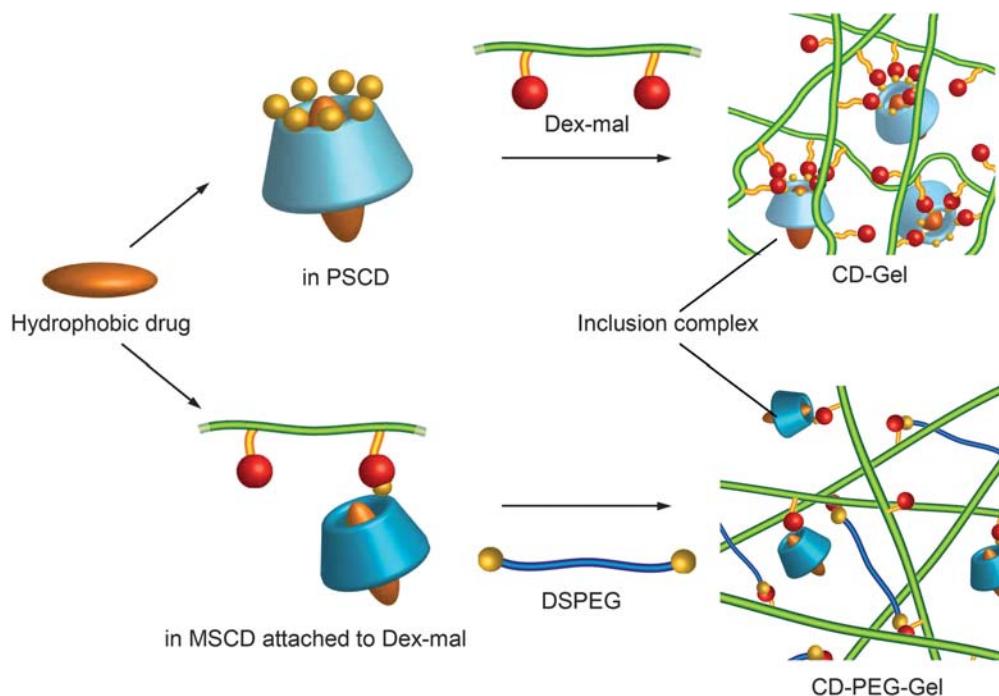
Results and discussion

Hydrogel formation

Maleimide modified dextran (Dex-mal) can be crosslinked to form a hydrogel by applying multi-thiol compounds as we previously showed with per-6-thio- β -cyclodextrin (PSCD). Similar hydrogels can also be obtained by using di-thiolated poly(ethylene glycol) (DSPEG). In these systems, macroscopic hydrogels were formed *in situ* via Michael addition between maleimide and thiol groups rapidly.³⁵

Since these hydrogel can be used as drug carriers, we investigated the *in vitro* release profiles from the obtained macroscopic gels. In the case of PEG-Gel (Dex-mal was crosslinked with DSPEG without CD moiety), about 250 nmol of RA were released in only 4 days (see ESI†). The rapid release profile of RA from the PEG-Gel is similar to many examples of reported hydrogels, which are generally biphasic with an initial rapid release phase (burst effect) and followed by a slower phase.^{11,12,36} In the case of the CD-PEG-Gel (Scheme 1), after an initial delay, a sustained and constant release started, in which about 250 nmol of RA were released in 20 days. Although the gel is composed of the same components except the grafted CDs, the CD-PEG-Gel shows a well controlled and sustained release. A similar controlled release (250 nmol of RA were released in 13 days) was observed with CD-Gel (Dex-mal was crosslinked with PSCD), in which PSCD acted as a binding site and crosslinker simultaneously¹⁶ (see ESI†).

These observations clearly show that the CD moiety acts as a binding site for the hydrophobic drug and plays an essential role to control the release of the hydrophobic drug. These



Scheme 1 Schematic representation of the drug loading processes. The first step: the hydrophobic drug (retinoic acid) form an inclusion complex either with per-6-thio- β -cyclodextrin (PSCD) or maleimide functionalized dextran (Dex-mal) partially modified with mono-6-thio- β -cyclodextrin (MSCD). The second step: hydrogels were formed *in situ* via Michael addition between maleimide and thiol groups. In the resulting gel, the inclusion complexes of hydrophobic drugs and cyclodextrins are conjugated to the polymer network.

findings motivated us to further study these CD functionalized gels *in vivo* as potential drug carriers in more detail.

Preparations and characterizations of the injectable hydrogels

To use a hydrogel as an injectable drug delivery system in the zebrafish embryos, there are two major physical limitations: (i) the viscosity of the solution should not be high; and (ii) the size of the gel has to be smaller than the tip size of the needle used for the injection. Therefore, we decided to synthesize nanometre scale, cyclodextrin-functionalized drug carriers based on the macroscopic hydrogels.

By changing the concentrations of the polymer solutions, the hydrogel particles can be formed due to the fact that the highly reactive combination of maleimide and thiol is employed in this system.^{37,38} We have tested several concentrations of Dex-mal and found that 3 wt% Dex-mal solution showed fluidic behavior even after mixing with DSPEG (see ESI†) although 5 wt% Dex-mal solution exhibited a highly viscous gel-like behavior after that. The compositions of the injectable hydrogels used for the *in vivo* studies in zebrafish embryos are summarized in Table 1.

The resulting injectable hydrogels were characterized with ¹H NMR and scanning electron microscopy (SEM). NMR data (Fig. 2) showed that the maleimide groups of Dex-mal were almost completely consumed within 15 min in the case of CD-PEG-Gel. However, when PSCD was used, only *ca.* 14% of the maleimide groups of Dex-mal were consumed in 15 min and about 60% of the maleimide groups still remained even after 1 day. This difference is presumably because of the steric hindrance of multi-thiol compounds preventing to reach high conversion of the crosslinking reaction.

As shown in Fig. 3, the mixture of Dex-mal, MSCD and DSPEG formed particles with a dimension of ≤ 100 nm. However, in the mixture of Dex-mal and PSCD, we found fewer

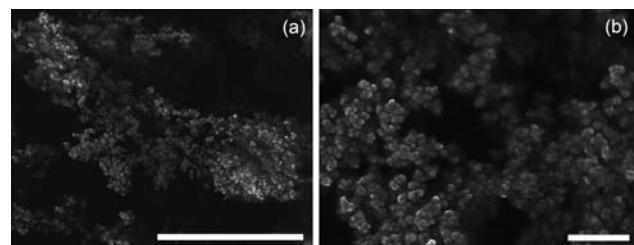


Fig. 3 Scanning electron microscopy images of the CD-PEG-Gel particles prepared from Dex-mal, MSCD and DSPEG in pure water with lower and higher magnifications. Scale bars in (a) and (b) represent 5 μ m and 500 nm, respectively.

particles even 1 day of reaction (see ESI†). We suppose that the low reactivity of the combination of Dex-mal and PSCD causes less efficient nanogel formation than the other combination crosslinked with DSPEG.

Biocompatibility of the injectable hydrogels

In order to examine the biocompatibility, the drug carriers were injected without any drug in zebrafish embryos at the one cell stage. Injections of the CD-PEG-Gel did not cause death of the embryos, which is contrasting with the injection of the CD-Gel prepared from Dex-mal and PSCD. When we used the CD-Gel, most of the embryos did not survive after the injection presumably because of unreacted thiol and maleimide groups in the system and/or may be because of higher salt concentration of the system which was required to increase the solubility of PSCD. This result showed that the biocompatibility of the CD-PEG-Gel particles composed of Dex-mal, MSCD and DSPEG was high enough to be used in zebrafish embryos that are very sensitive to foreign compounds.

As shown in Fig. 4, embryos injected with the CD-PEG-gel developed in the same way as the control experiments at 30 hours post fertilization (hpf); no severe phenotypic abnormality was observed and only less than 8% of the embryos ($n = 89$) showed mild phenotypes (such as an enlarged heart cavity or curved tail). This observation is comparable to the control experiment (the injections of Danieau buffer containing 2% DMSO); 7% of embryos showed mild phenotypes at 30 hpf ($n = 102$). Therefore, it was concluded that the drug carrier is biocompatible and does not affect the embryo development.

In vitro drug release from the CD-PEG-Gel particles

The *in vitro* release behavior of a hydrophobic drug from the obtained CD-PEG-Gel particles was examined by using all-*trans* retinoic acid (RA) as a model compound. The RA-loaded

Table 1 Composition of the injectable hydrogels

Sample	CD	Dex-mal (wt%)	DSPEG (wt%)
CD-Gel	3 mM (PSCD)	3	None
CD-PEG-Gel	3 mM (MSCD)	3	2

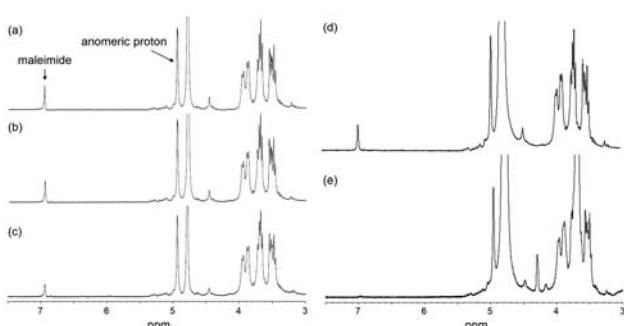


Fig. 2 Comparisons of ¹H NMR spectra of Dex-mal before (a), 15 min after (b), and 1 day after (c) addition of PSCD (left); and Dex-mal before (d) and 15 min after (e) adding DSPEG (right). The ratios between maleimide/anomeric protons are 0.29 (a), 0.25 (b) and 0.17 (c).

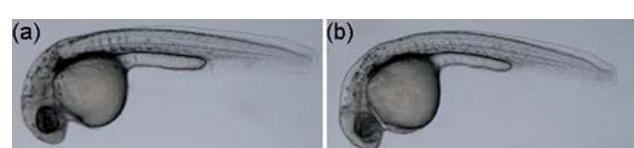


Fig. 4 Comparison of the results from control (a) and CD-PEG-Gel injection (b) without a drug at 30 hpf. Embryos are shown in lateral view, anterior to the left.

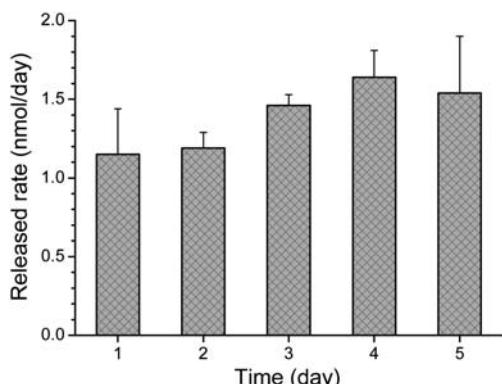


Fig. 5 *In vitro* release of RA from 700 μ L of the CD-PEG-Gel particles prepared from Dex-mal, MSCD and DSPEG, loaded amount of RA was 210 nmol.

CD-PEG-Gel was prepared as shown in Scheme 1 and the released amounts of RA from the particles were determined by HPLC every 24 hours.

As shown in Fig. 5, the CD-PEG-Gel particles showed a controlled release; the release rate of RA was about constant at 1.3 nmol per day. It is important to note that this nano scale gel particles showed a well controlled release profile of RA without an initial rapid release phase (burst effect), which is comparable to its macroscopic gel matrix form.³⁰

In vivo drug release from the CD-PEG-Gel particles

To examine if the RA release from the CD-PEG-Gel particles can be controlled *in vivo* in the same manner as *in vitro*, RA-loaded CD-PEG-Gel particles were injected in the embryos because it is known that the exposure of zebrafish embryos to certain amount of RA during embryogenesis causes severe phenotypic abnormalities.^{25,26} As shown in Fig. 6, due to the strongly controlled release of RA, 25% of the embryos showed normal development, 50% of the embryos showed mild phenotypes (microcephaly, microphthalmia, enlarged heart cavity and short or curved tail;

representative example is shown in Fig. 6b), and only 25% of the embryos showed severe malformations. This is contrasting with the results from the injections of the same volume of RA solution in the absence of the CD-PEG-Gel, in which only less than 10% of the embryos showed normal development, 30% showed mild phenotypes, and more than 60% of the embryos showed severe phenotypic abnormalities including yolk sac edema, cyclopia, and caudal dysplasia (representative example is shown in Fig. 6c). These observations indicated that the hydrophobic drug RA was released from the hydrogel system in a well controlled manner in the zebrafish embryo assay. As a result of the retention of RA inside the carrier and slow release during embryo development in a sustained manner without a burst effect, severe disruption of embryogenesis was largely prevented while RA release still caused mild developmental defects.

It should be noted here that the current drug delivery system can be prepared on site by mixing the solutions and directly injected into the living body with a capillary needle or syringe. This makes the system highly attractive to use not only in a high-throughput drug screening system with zebrafish embryos or other animal models but also in the future human clinical applications.

Conclusions

In conclusion, an *in situ* forming hydrogel-based drug delivery system was developed, which is composed of biocompatible materials, cyclodextrin, dextran and poly(ethylene glycol). Maleimide modified dextrans (Dex-mal) were functionalized with cyclodextrins and crosslinked to form a hydrogel using either per-6-thio- β -cyclodextrin (PSCD) or a combination of mono-6-thio- β -cyclodextrin (MSCD) and di-thiolated poly(ethylene glycol) (DSPEG). In these systems, cyclodextrin moiety acts as a binding site for a hydrophobic drug and regulates the release of the drug. Using all-*trans* retinoic acid (RA) as a model hydrophobic drug, a sustained release from these cyclodextrin modified hydrogel matrices was observed *in vitro* without an initial burst. By lowering the polymer concentrations, hydrogel-based particles on a scale of tens of nm were prepared as drug carrier to be used in zebrafish embryos. Although one of the gels prepared from Dex-mal and PSCD (CD-Gel) exhibited toxicity, the drug carrier prepared from Dex-mal, MSCD and DSPEG (CD-PEG-Gel) showed high biocompatibility. Furthermore, controlled release of RA from the CD-PEG-Gel particles was confirmed both *in vitro* and *in vivo*. As the hydrogel-based drug carrier showed the convenience and efficiency for the delivery of hydrophobic drug RA into the zebrafish embryos, it will be a highly attractive tool in high-throughput drug screening systems based on zebrafish.^{21,22} Moreover, in future the present system could be a promising candidate not only for zebrafish but also for human clinical applications of administrating hydrophobic drugs that can form an inclusion complex with cyclodextrins.

Experimental

Materials

Maleic anhydride, thiourea, *N,N'*-dicyclohexylcarbodiimide (DCC), *p*-toluenesulfonic acid monohydrate, 3-mercaptopropionic

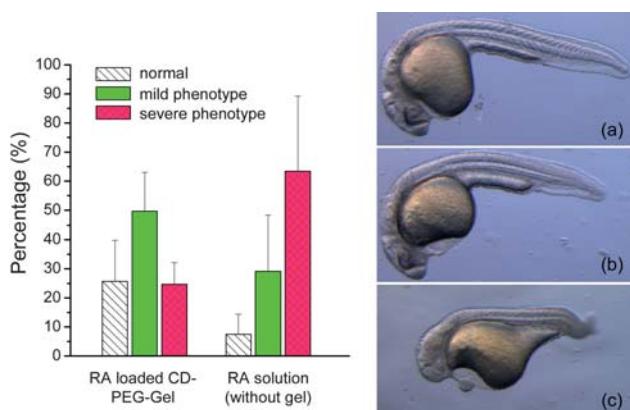


Fig. 6 Effects of RA injections on development of zebrafish embryos. Pictures show typical examples of normal development (a), with mild phenotype (b) and with severe phenotype (c). Embryos are shown in lateral view, anterior to the left. The 5 nL of the samples containing the same amount of RA were injected.

acid and all-*trans* retinoic acid were obtained from Fluka, dithiothreitol (DTT) and 4-dimethylaminopyridine (DMAP) were obtained from Aldrich, β -cyclodextrin and *p*-toluenesulfonyl chloride were obtained from Acros, glycine and poly(ethylene glycol) (PEG, M_w = 2000) were obtained from Merck. All these chemicals were used as received. 4-(Dimethylamino)pyridinium 4-toluenesulfonate (DPTS) was synthesized from DMAP and *p*-toluenesulfonic acid monohydrate and recrystallized from toluene. Dextran (M_w = 10 000, Pharmacia Fine Chemicals, Sweden) was dried in the vacuum oven for several days before use. Ethyl acetate, methanol, diethyl ether, toluene and dimethyl sulfoxide (DMSO) were previously dried with molecular sieves. Triethylamine (TEA) and absolute ethanol were obtained from Riedel-de Haen. Water used in all experiments was purified through deionization and filtration with a Millipore purification apparatus to the resistivity higher than 18.0 M Ω cm.

Per-6-thio- β -cyclodextrin (PSCD),³⁹ mono-6-thio- β -cyclodextrin (MSCD),⁴⁰ maleimide modified dextran (Dex-mal)^{16,41} and di-thiolated poly(ethylene glycol) (DSPEG)⁴² were prepared by following previously reported procedures. In this study, we used Dex-mal with 14 maleimide groups per 100 monomer units of dextran.

Preparation of the injectable hydrogels

Hydrogel-based injectable drug carriers were prepared from phosphate buffered saline (PBS) solutions of Dex-mal and thiol compounds. In the case of CD-Gel, to a solution of 1.25 mL Dex-mal (75.0 mg, 57 μ mol of maleimide groups), 1.25 mL PBS solution of PSCD (10.0 mg, 1 eq. of thiol groups to the maleimide groups of Dex-mal) was added by vortexing. In the case of CD-PEG-Gel; to a solution of 1.25 mL Dex-mal (75.0 mg), MSCD (8.4 mg, 0.1 eq. of thiol to the maleimide groups of Dex-mal) was added and the resulted solution was stirred for 5 min. After that a solution of 1.25 mL DSPEG (66.0 mg, 1 eq. of thiol to the maleimide groups of Dex-mal) in PBS solutions was added by vortexing.

Characterizations of the injectable hydrogels

The obtained injectable hydrogels were characterized with NMR, scanning electron microscopy (SEM), and viscometry. For NMR measurements D₂O was used instead of PBS and the ratios between the materials were kept as we described above. The viscosities of the solutions were measured with the Haake CV 100 rheometer equipped with the Rotovisco RV20 and the Rheocontroller RC20 at 23 °C. The rheograms were obtained in the shear rate range up to 300 s⁻¹ and analyzed as Newtonian fluids. For the viscometry, the sample was prepared in a bigger scale: Dex-mal (30.0 mg) in 500 μ L PBS solution was reacted with MSCD (3.4 mg) and then mixed with DSPEG (26.4 mg) in 500 μ L PBS solution. SEM was conducted on a Nova NanoSEM (FEI) with an accelerating voltage of 10 kV and spot size of 3.5. The sample was prepared from the freshly made hydrogel in pure water, mounted onto an aluminium stub and coated with carbon before measurements.

In vitro drug release

The model hydrophobic drug, all-*trans* retinoic acid (RA) loaded CD-PEG-Gel particles were employed to test the release profile, which were prepared by using an RA containing stock solution instead of using PBS in the procedure described above. The stock solution was prepared by the following procedure: RA (0.9 mg, 3.0 μ mol) was dissolved in DMSO (200 μ L) and 9.8 mL of PBS were added. Typically, Dex-mal (75.0 mg) was dissolved into 1.25 mL of this RA solution and MSCD (8.4 mg) was added and vortexed for 5 min. After that, DSPEG (66.0 mg) in 1.25 mL of the RA solution was added and vortexed for 15 min.

The CD-PEG-Gel solution (0.7 mL) was then put into dialysis tubes (MWCO 1000) and the tubes were placed in 100 mL PBS solution. The surrounding PBS was stirred at 50 rpm at room temperature and refreshed every 24 hours. The amounts of RA released were detected by the HPLC (Shimadzu) equipped with an autoinjector (Shimadzu, SIL-10AD) using a dC18 (Atlantis, Waters) column (150 × 46 mm; 5 μ m particle size) with a 21 min linear gradient from 100% solvent A (CH₃OH/CH₃CN/THF, 62 : 33 : 5, v/v/v) to 75% solvent A with 25% solvent B (AcOH/H₂O, 2 : 98, v/v), UV absorbance detection was performed at 345 nm. For calibrations, 30 nM RA stock solution was employed. To prevent decomposition of RA during the release experiment, the samples were kept in dark.

In vivo testing of the CD-PEG-Gel particles

The *in vivo* testing was conducted by injections into the yolk of zebrafish embryos at the one cell stage. Zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (<http://ZFIN.org>). For each embryo, 5 nL of the samples were injected with a Femtojet microinjector (Eppendorf) and a micromanipulator with pulled microcapillary needles: (i) Danieu buffer containing 2% DMSO; (ii) the RA-free CD-PEG-Gel (Dex-mal 7.5 mg, MSCD 0.8 mg, DSPEG 6.6 mg in 250 μ L Danieu buffer containing 2% DMSO); (iii) 0.3 mM RA solution (in Danieu buffer containing 2% DMSO); and (iv) the CD-PEG-Gel loaded with 0.3 mM RA (Dex-mal 7.5 mg, MSCD 0.8 mg, DSPEG 6.6 mg in 250 μ L Danieu buffer containing 2% DMSO). Zebrafish embryos were maintained at 28 °C in egg water (60 μ g mL⁻¹ Instant Ocean sea salts) in the dark and developmental abnormalities beyond 30 hours post fertilization (hpf) were analyzed with a Leica MZ16FA stereo fluorescent microscope. The number of embryos injected (n) were in the range of 50–150 for each sample. The *in vivo* release experiments were repeated 3 times.

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