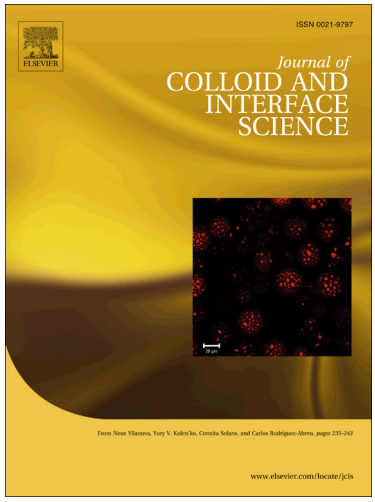
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C.A. Busatto, H. Labie, V. Lapeyre, R. Auzely-Velty, A. Perro, N. Casis, J. Luna, D.A. Estenoz, V. Ravaine

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Oil-in-microgel strategy for enzymatic-triggered release of hydrophobic drugs

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ABSTRACT

Polymer microgels have received considerable attention due to their great potential in the biomedical field as drug delivery systems. Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan composed of N-acetyl-D-glucosamine and D-glucuronic acid. This polymer is biodegradable, nontoxic, and can be chemically modified. In this work, a co-flow microfluidic strategy for the preparation of biodegradable HA microgels encapsulating hydrophobic drugs is presented. The approach relies on: (i) generation of a primary oil-in-water (O/W) nanoemulsion by the ultrasonication method, (ii) formation of a double oil-in-water-in-oil emulsion (O/W/O) using microfluidics, and (iii) cross-linking of microgels by photopolymerization of HA precursors modified with methacrylate groups (HA-MA) present in the aqueous phase of the droplets. The procedure is used for the encapsulation and controlled release of progesterone. Degradability and encapsulation/release studies in PBS buffer at 37 °C in presence of different concentrations of hyaluronidase are performed. It is demonstrated that enzymatic degradation can be used to trigger the release of progesterone from microgels. This method provides precise control of the release system and can be applied for the encapsulation and controlled release of different types of hydrophobic drugs.

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KEYWORDS

Drug delivery, Microgels, Biodegradable polymers, Hyaluronic acid, Hydrophobic drug, Oil-in-water-in-oil emulsion, Progesterone

INTRODUCTION

Among different types of drug delivery systems, polymeric hydrogels hold great promise thanks to their hydrophilic three-dimensional network which offers large water-retaining property and excellent biocompatibility. Moreover, particles made of hydrogels, namely microgels or nanogels, exhibit good colloidal stability and are injectable. All these lightly cross-linked polymers are porous and can be loaded with drugs. Stimuli-responsive hydrogels are fascinating materials because they swell or shrink in response to an external stimulus such as temperature, pH, irradiation or even the presence of a biomolecule in solution [1-4]. In this case, drug release can be additionally controlled by stimuli [5]. Microgels also offer opportunities as surface coatings when adhered to biomaterials or surgical implants to reduce infection and inflammation and to improve biocompatibility, particularly when containing antimicrobial and anti-inflammatory drugs.[6-8] Therefore, responsive hydrogels allow a fine tuning of drug release rate with tailored properties.

For most therapeutic applications, the drug delivery system needs to be readily biodegradable in order to avoid accumulation-related adverse effects. Biodegradability of the matrix can also be used as the trigger to control the release of the drug. HA is a naturally occurring glycosaminoglycan composed of N-acetyl-D-glucosamine and D-glucuronic acid, which exhibits biocompatible and biodegradable properties. This linear polysaccharide is ubiquitous in all tissues, as a major component of the extracellular matrix (ECM) in animal tissues[9]. Advantageously, chemical modification of HA is relatively easy[10, 11]. Being non-toxic and non- immunogenic, it has been widely used as a building block to design cross-linked architectures with a potential application in tissue regeneration [12, 13] and drug delivery[14] or cell growth [15]. Enzymatic degradation of HA occurs *in vivo* via hyaluronidases (Hase),

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which are present in both intra- and extracellular space[16]. Therefore, HA-based microgels are expected to deliver their payload after injection in the body by progressive enzymatic erosion of the matrix. In general, members of HA signaling pathway (receptors such as CD44 and CD168[17, 18], Hase[19, 20]) are also overexpressed in a variety of carcinomas. HA has been used in drug delivery formulations for the targeted delivery of chemotherapy drugs and other anticancer compounds to tumor cells through interaction with cell-surface HA receptors[21]. Furthermore, Hase-responsive containers appear as emerging technologies to deliver anticancer drugs since the high concentration of Hase in the tumor cells can increase the release efficiency[22-26].

In spite of the great interest for HA-based hydrogel materials, these systems are generally limited to water-soluble drugs, most often peptides and proteins[27-30], whereas many drugs are hydrophobic, such as anticancer drugs. The encapsulation of a hydrophobic drug has been reported using different strategies of functionalization of the micro- or nanogels: 1) via the incorporation of a hydrophobic core in a hydrogel shell [31] or hydrophobic domains in the hydrogel matrix[32, 33][36], and 2) via the introduction of cyclodextrin (CD) moieties, that can bind hydrophobic molecules through inclusion complexes[34]. However, the loading efficiency is often poor. Alternately, hydrophobic drugs can be solubilized in non-polar solvent that can further be dispersed in a hydrogel matrix. This latter solution offers the advantage of incorporating high payloads of drugs in a dissolved form. The concept has been explored recently using alginate matrices[35, 36] and thermoresponsive poly(*N*-isopropylacrylamide) (pNIPAM) ones [37, 38], where burst release could be achieved by collapsing the hydrogel upon heating.

In this paper, we focus on the preparation of a biocompatible system that could be used *in vivo* to deliver a hydrophobic drug upon Hase degradation (Figure 1). We demonstrate the concept using progesterone (PGR), a lipophilic steroid hormone with a low molecular weight.

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This drug requires long-time controlled delivery system to induce estrus and ovulation in production animals[39, 40]. Several progesterone delivery systems for estrus synchronization have been reported but they are based on non-biodegradable polymers[41]. HA microgels would provide a controlled release of the hormone after subcutaneous administration. Once the microgel is degraded and the oil droplets are dissolved, progesterone could be absorbed to systemic circulation and could reach sites of action thanks to its extensively binding to plasma proteins, primarily albumin.

Thus, we report the preparation of oil-in-microgel containers, made of fully biocompatible ingredients, encapsulating PGR. The materials are prepared by a two-step method which allows a fine and robust control of the structural parameters: the drug is incorporated in an oil-in-water nanoemulsion, that is further incorporated within monodispersed HA microgels prepared by microfluidic approach. The enzymatic degradability of the microgels and their ability to release the PGR-loaded oil nanodroplets upon enzymatic degradation is finally presented.

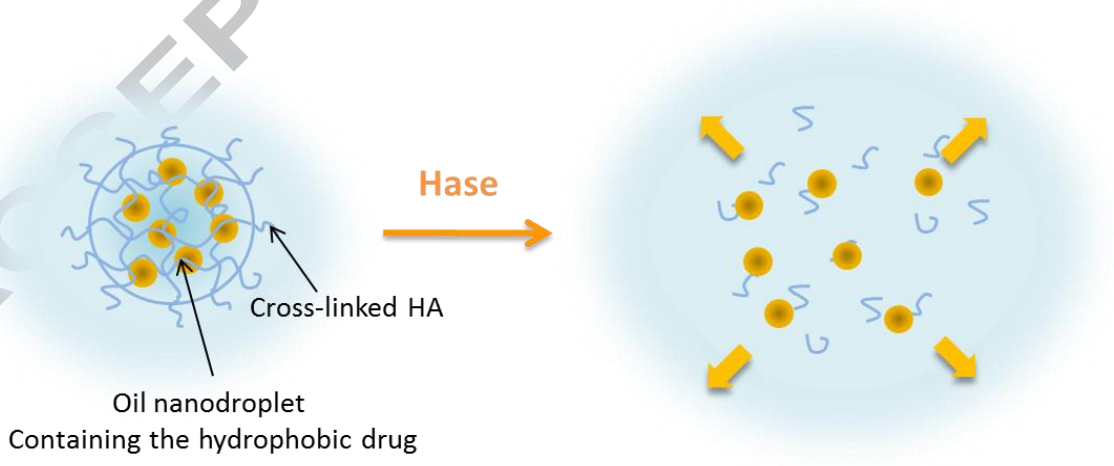


Fig. 1. Concept of the oil-in-microgel system, encapsulating a hydrophobic drug. The HA matrix degrades upon addition of Hase and releases the oil nanodroplets.

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EXPERIMENTAL

Materials

All the reagents were purchased from Sigma-Aldrich unless otherwise noted. Methacrylic anhydride (AMA), N,N-dimethylformamide (DMF), ethanol, n-hexadecane, octadecyltrichlorosilane (OTS), chloroform, toluene, soybean oil, sunflower oil, sorbitan monooleate (Span 80), polysorbate 80 (Tween 80), hyaluronidase from bovine testes (type I-S), N,N'-methylenebisacrylamide (BIS), sodium caseinate (SC), 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propan-1-one (Irgacure 2959), 2,2'-azobis(2-methylpropionamidine) dihydrochloride, Nile Red (NR), progesterone (PGR) and phosphate buffered saline (PBS) were used without any further purification. NIPAM was recrystallized from n-hexane. Hyaluronic acid (M_w = 60 000 g mol^{-1}) was purchased from Lifecore (USA).

Synthesis of methacrylated hyaluronic acid macromer (HA-MA)

The synthesis of hyaluronic acid modified with methacrylates is fully reported by Hachet *et al.*[42] In brief, 1 g of hyaluronic acid (2 wt. %) was solubilized for 4 hours at room temperature in ultrapure water. Then, DMF was added dropwise to the solution (volume ratio 3:2 water–DMF) and the mixture was cooled down to 4 °C. The reaction medium was cooled after addition of DMF to avoid a temperature increase upon addition of the anhydride in the hydro-alcoholic medium that could promote side reactions (hydrolysis of esters formed and hydrolysis of the anhydride). Then, AMA (1 molar equivalent with respect to the moles of the repeating unit of HA) was added dropwise and the pH was maintained between 8 and 9 for 4 hours by the addition of 0.5 M NaOH. The reaction was run overnight and then, 0.5 M NaCl was added to the mixture. The polymer was precipitated by the addition of ethanol (with a water–EtOH volume ratio of 2:3). After the removal of the supernatant, the precipitate was

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successively washed with mixtures of water–EtOH using volume ratios of 3:7, 1:4 and 1:9. The final precipitate was dissolved in ultrapure water and further dialyzed against ultrapure water by difiltration (ultramembrane Amicon YM10). The purified macromer was recovered by freeze-drying and characterized by ^{1}H-NMR analysis.

^{1}H-NMR spectra of HA-MA in D_2O (C = 8 mg mL^{-1}) were recorded at 80 °C with 128 scans using a Bruker 400 MHz spectrometer. The temperature was set to 80 °C in order to shift the water peak towards lower chemical shifts thus avoiding its overlapping with those of anomeric protons. The degree of methacrylation was determined by digital integration of the methacrylate proton signal at 5.8 ppm or 6.2 ppm relative to the anomeric proton signal of HA at 4.5 ppm and 4.7 ppm.

Preparation and characterization of primary emulsion

The aqueous phase of the emulsions consisted of 2 mL of aqueous phase containing the surfactant and the oil phase consisted of hexadecane or sunflower oil (20 wt%). The surfactant was Tween 80 or SC with different compositions. The oil phase was often replaced by a solution of Nile Red in hexadecane (3.1 x 10^{-4} M) to provide visual inspection of an encapsulated hydrophobic specie. The emulsions were prepared by ultrasonication for 20 min (with 1 min ON and 1 min OFF), with the output level set at 6, using a probe sonicator (Branson sonifier 250, frequency =20 kHz, operating with an output of 90% of 200 W and a Duty Cycle of 90%). The solution was cooled on an ice water bath throughout the sonication process. The diameter of oil droplets and the emulsion stability was studied using the dynamic light scattering technique with a Zetasizer NanoZS Malvern Instruments apparatus operating with a HeNe laser at 173 °.

Preparation of HA-MA microgels

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A co-flowing microfluidic device was used. The system consisted of two coaxial capillaries connected with a T-junction (Fig S1). The inner fused silica tube [inside diameter (ID) 75 µm; outside diameter (OD) 148 µm; Polymicro technologies] associated with adjusted tubing sleeves (UpChurch Scientigic, 1/16 ID 180 µm) was inserted inside the T-junction (UpChurch Scientific, 1/16) along it main axis. At the exit of the T-junction, the inner capillary is introduced in the outer silica tube (ID, 250 µm; OD 356 µm; Polymicro technologies). The T-junction allowed the injection of the liquid dispersed phase into the continuous phase. The surface of the outer capillary was modified in order to increase its hydrophobicity. To this effect, 3 mL of a dilute OTS solution in dodecane (1 wt%) was passed through the silica capillary at the flow rate of 6 mL/ h. Then, the capillary was rinsed with a mixture of 5 mL of toluene and 5 mL of chloroform.

Soybean oil containing 2 wt% Span 80 was used as continuous phase. Separately, 3 wt% HA-MA macromer was dissolved in saline water (0.2 M NaCl) in the presence of 0.2 wt% photoinitiator (Irgacure 2959) and eventually BIS (2 wt%) and mixed with an equal volume of emulsion. Alternately, 10 wt% of NIPAM and 0.2 wt% of BIS were dissolved in saline water (0.2 M NaCl) in the presence of 0.08 wt% photoinitiator (2,2'-azobis(2-methylpropionamidine) dihydrochloride) mixed with an equal volume of emulsion. The resulted solution was used as dispersed phase in the microfluidic device. The dispersed and continuous phases were injected into the device using a syringe pump and the flow rates were 3 µL min^{-1} and 6 µL min^{-1}, respectively. Polymerization was carried out by placing the device under a UV lamp (Hamamatsu LC8, L9588-06). The UV irradiation (λ=300-450 nm, centered at 365 µm) was directed to an unprotected window on the outer silica tube (20 mm). The lamp was used at 100% of its power, and equipped with a filter allowing the transmittance of wavelengths between 300 and 400 nm. The microgels were separated from the continuous

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phase by centrifugation at 800 rpm for 10 min at room temperature and washed two times in hexane and then two times in ultrapure water to remove the oil.

Enzymatic degradation with hyaluronidase

The biodegradability of the microgels by hyaluronidase was investigated using a Leica optical microscope (Leica DMI600 B) with a coupled camera. The microgels were observed in an imaging chamber and micropipette technique was used to hold the microgels by suction. The microgels were incubated at 37 °C in PBS buffer (pH = 7.4) containing 300 U hyaluronidase, type I-S (Hase) and 1 wt% Tween 80.

Encapsulation and release of progesterone from microgel particles

A solution of progesterone in sunflower oil (0.06 M) was used to prepare the primary emulsion. The hormone release from the microgels was investigated by enzymatic degradation with hyaluronidase. Microgels collected from 10 min-microfluidic experiments were incubated at 37 °C, together with different concentrations of Hase in 150 µL PBS buffer (pH = 7.4) containing 1 wt% Tween 80. Aliquots of 20 µL of the solution were taken at different intervals of time and mixed with 480 µL of ethanol. Progesterone concentration was analyzed by HPLC (Merck-Hitachi LaChrom D7000 HPLC system) equipped with a L-7250 autosampler, a L7100 high pressure pump and a L-7455 diode array detector. A mixture of methanol/water (95:5) was used as the mobile phase at a flow rate of 1 $mL.min^{-1}$. Oven temperature and detection wavelength of the assay were 25 °C and 254 nm, respectively.

Results and discussion

Microgel preparation

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The preparation of microgels encapsulating oil nanodroplets requires several steps, which are summarized in Fig. 2. Firstly, an oil-in-water nanoemulsion is produced. This step is performed separately to allow a fine control of the volume fraction of oil and of the drop size distribution. Then HA water-soluble hydrogel precursors are added in the continuous phase of the nanoemulsion. This primary oil-in-water emulsion, noted O_1/W, is further emulsified using a microfluidic device to yield a double oil-in-water-in-oil emulsion, noted $O_1/W/O_2$. This method allows a good reproducibility of the drop size and of the number of O_1 nanodroplets per drop [43]. The resulting monodisperse aqueous drops are then irradiated by UV to photopolymerize the precursor and form the gel phase entrapping the O_1 nanodroplets. The preparation is collected in a vial. Water is finally added to provoke phase separation between the outer oil phase and water. The microgels remain dispersed in the aqueous phase. For the sake of comparison, poly(*N*-isopropylacrylamide) (pNIPAM), a synthetic polymer, will be used instead of the HA. It will be obtained by radical polymerization of a water-soluble monomer, NIPAM, and *N*,*N'*-methylenebisacrylamide, BIS.

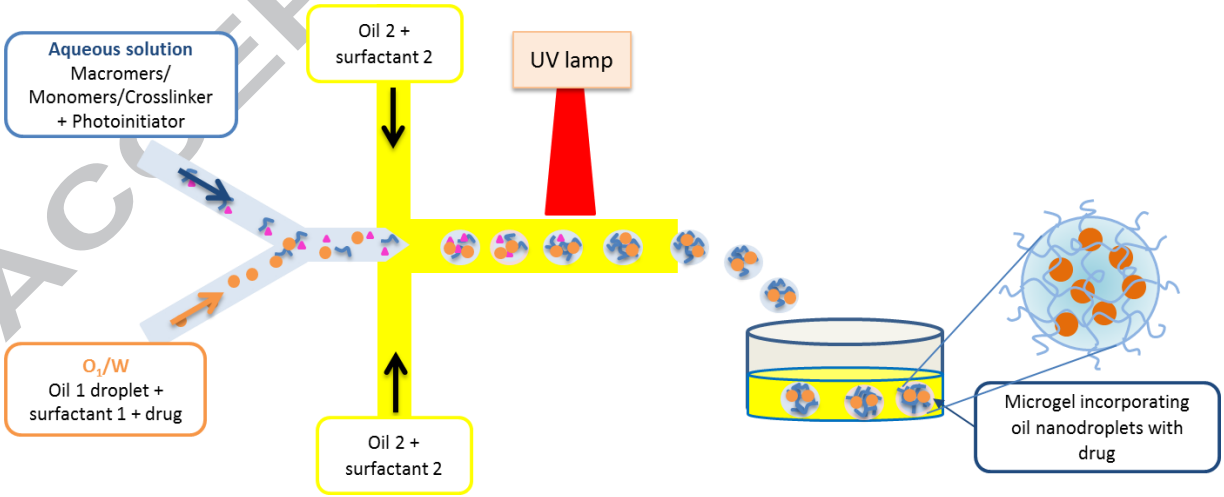


Fig. 2. Conceptual representation of the multistep process to prepare oil-in-microgels using the microfluidic approach.

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The inner oil drops (from primary emulsion) should be smaller than the double emulsion drops. In the present study, we chose to use a primary nanoemulsion, in order to promote the rapid diffusion of the droplets after matrix degradation and, eventually, to take advantage of their ability to locally deliver their payload. Therefore, attention was paid to generate the smallest drops as possible. A high energy emulsification method was used to produce the primary organic/water (O/W) nanoemulsion [44]. For simplicity, ultrasonication was chosen. However, it could be replaced by high pressure homogenization if fragile molecules should be used[30]. A first set of nanoemulsions was produced using hexadecane as a dispersed phase (10% vol) and Tween 80 as a water-soluble surfactant (5 wt% in water). Immediately after preparation, the average diameter of the hexadecane droplets in aqueous solution was 140 nm, with a polydispersity index close to 0.22 (Fig. 3). The droplet size increased and reached a plateau of 180 nm after 1 day. At the same time, the polydispersity index decreased from 0.22 to 0.1. This trend suggested that the emulsion was subjected to Oswald ripening, *i.e.* the diffusion of O_1 from small drops to larger ones due to the Laplace pressure difference. Over time, the size distribution becomes narrower and the diffusion slows down. After one day, the size remains constant. It should be noted that the HA precursor was added in the continuous phase after emulsification of the O_1/W nanoemulsion, and not prior to the nanoemulsion preparation. There was two reasons for that: firstly, the presence of HA-MA increases the viscosity, which has an impact on the size of drops; secondly, HA molecules are fragile and can depolymerize under ultrasonication[45], [30].

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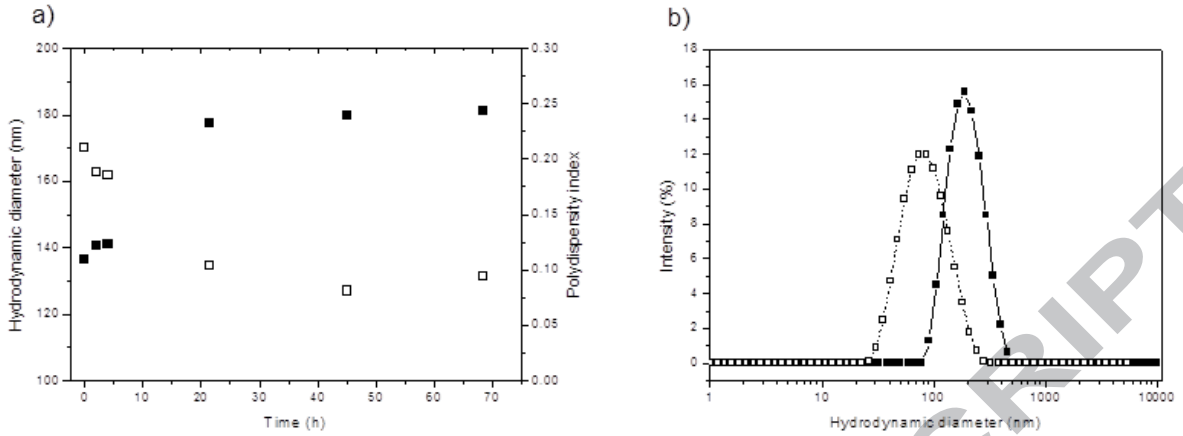


Fig. 3. Characteristics of the O_1/W emulsion with O_1=hexadecane, W=aqueous solution of Tween 80 (5 wt%): a) Kinetic evolution of the drop average diameter and of the polydispersity index; b) Size distribution just after emulsion preparation and after 24 h of ageing.

The conditions to prepare HA microgels were tested separately, in the absence of O_1/W nanoemulsion. HA chains modified with methacrylates were chosen as HA precursors. These moieties have been widely studied to yield photopolymerizable hydrogels, which can self-crosslink by radical polymerization of the methacrylate groups[14, 42]. The grafting density of methacrylates can be easily tuned during the synthesis step. It allows a fine control of the cross-linking density and thus of the elastic modulus of the gels. In our case, a HA-MA precursor having 10% mol. of methacrylates was synthesized. Its concentration was 15 g/L in the continuous aqueous phase of the nanoemulsion. A photoinitiator was also introduced in the solution. Irgacure 2959 was selected for its known biocompatibility with cells[46]. The aqueous phase containing HA-MA was introduced in the microfluidic device using a syringe pump and emulsified in the continuous oil phase, soybean oil containing Span 80. The flow rates were adjusted to 3 µL min^{-1} and 6 µL min^{-1}, respectively. After irradiation, the drops were collected in a vial. In the absence of photopolymerizable precursor or of photoinitiator, the drops were unstable and phase separation occurred. In the presence of photoinitiator

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together with HA-MA, the drops were stable, indicating the change in their mechanical properties due to the success of the cross-linking step. The resulting particles did not exhibit any aggregation and sunk in the vial. They were monodispersed and had a slug shape (Fig. 4a). The shape indicated that the drops were produced in a squeezing regime, *i.e.* a regime where the drops are compressed by the capillary [47]. As cross-linking took place during the progression of the drops in the capillary, the shape of the compressed liquid drops was translated to the resulting elastic hydrogel particles. The size of the drops was controlled by the flow rate, the viscosity of the continuous phase and the interfacial tension, which could be altered by the composition of the system. A similar shape was also obtained with a pNIPAM matrix, where HA-MA was replaced by a mixture of NIPAM (10 wt%) and BIS (0.2 wt%) (results not shown). It means that changing the viscosity ratio between the dispersed and the continuous phase did not alter significantly the shape of the drops. Note that the NIPAM solution has a viscosity close to that of water, whereas a solution of HA-MA at 15g /L has a viscosity of 15 times higher. Furthermore, as shown below, the addition of a second surfactant present in the nanoemulsion did not alter the shape, although it changed drastically the interfacial tension[48].

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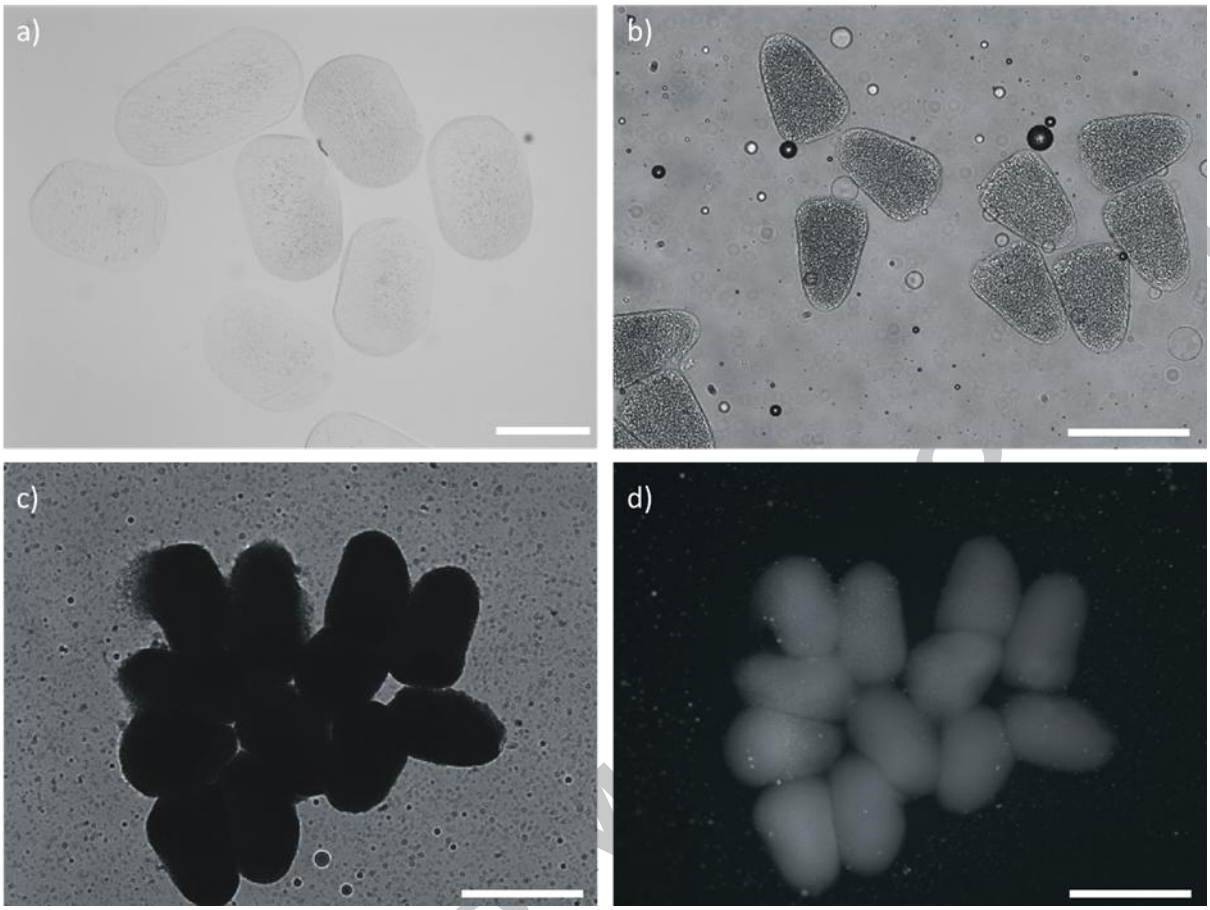


Fig. 4. a) Bright-field micrograph of HA-MA microgels (without the O_1/W nanoemulsion); b) Bright-field micrograph of pNIPAM microgels containing the O_1/W nanoemulsion stabilized with 5% wt. Tween 80 ; c) Bright-field and d) Fluorescence micrograph of pNIPAM microgels containing the Nile Red loaded O_1/W nanoemulsion stabilized with 1 wt. % sodium caseinate. Scale bar is 400 µm.

The aqueous phase was then replaced by the O_1/W nanoemulsion, containing the polymerizable precursors. The nanoemulsion was aged for 1 day before use. The encapsulation of oil nanodrops was set up with the pNIPAM system. Composite microgels were successfully obtained, as proved by their heterogeneous internal structure (Fig 4b). The microgels adopt a bullet-like shape induced by a modification the droplets wettability on the outer capillary wall.[49] It should be noted that the inner nanodrops could not be resolved by microscopy due to their size below 200 nm. In order to visualize the encapsulation process,

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Nile red was incorporated into the oil phase prior to emulsification. However, the fluorescence could not be observed for these first trials. Photobleaching due to UV irradiation was suspected but it was ruled out by a control experiment, where the oil solution containing Nile Red was irradiated in similar conditions. It was hypothesized that the dye could be transported from the inner oil phase O_1 to the outer oil O_2 phase, during the preparation process. This could be achieved by diffusion mediated by the micelles and/or by permeation through the surfactant film of the inner droplets located at the surface of the large water drop[50]. To address this issue, we limited the amount of excess surfactant and decreased the surfactant concentration in the primary emulsion down to 2 and 1 wt%. Slight improvement could be achieved, which confirmed our hypothesis. However, the fluorescence remained very low. Therefore, the low molecular weight surfactant was replaced by a high molecular weight one, while respecting the criteria of biocompatibility. It is indeed expected that the presence of large molecules at the interfaces reduces the dye diffusion, either by changing the interface permeability or by decreasing the number of micelles, which could transport the dye. Sodium caseinate was chosen to replace Tween 80. This time, the inner nanodroplets were slightly bigger (350 nm) and remained stable over time. After polymerization of the pNIPAM in the aqueous drops, fluorescent microgels were successfully obtained (Fig. 4c and d).

This optimized composition was finally applied to HA-MA based microgels. When the nanoemulsion was mixed with HA-MA macromers and further photopolymerized, oil-in-microgels were obtained but the size of the inner droplets was larger than their initial size, meaning that the stability of the inner emulsion was not guaranteed (Fig S2). The shape of the microgels was also different from the pNIPAM ones. The anisotropic bullet shape was partially lost. The microgels looked more swollen, probably due to a lower cross-linking ratio. Increasing the substitution degree of methacrylates up to 20% mol. did not give significant

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changes. Therefore, a second cross-linker, BIS, was added to the HA-MA macromer. This strategy allowed obtaining monodisperse bullet structures, with relatively homogeneously distributed fluorescence (Fig. 5). The inner drops were not resolved optically, indicating that they remained close to their initial size.

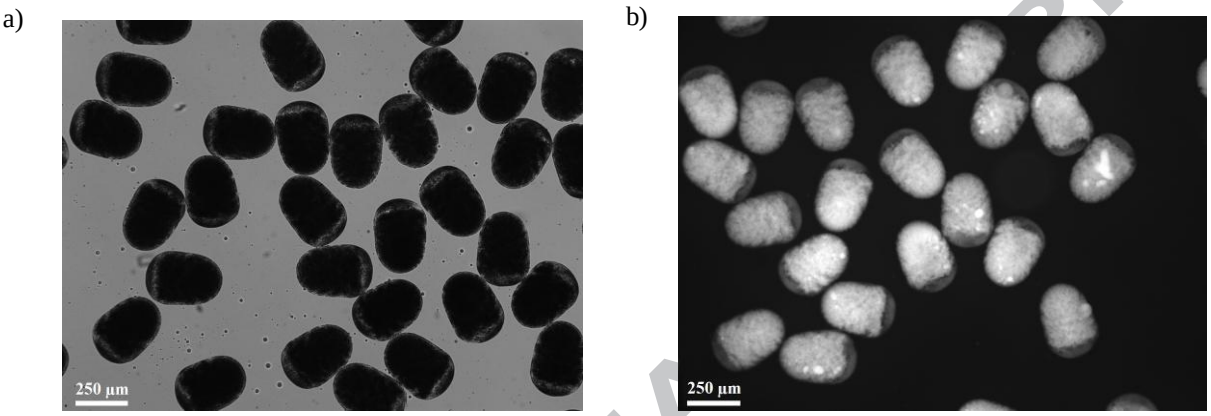


Fig.5. HA-MA microgels prepared with BIS as crosslinking agent (1% wt.) containing the Nile Red loaded O_1/W nanoemulsion stabilized with 1 wt. % sodium caseinate a) Bright-field images; b) Fluorescence images. Scale bar is 250 µm.

Degradation and release studies

The biodegradability of HA-MA microgels prepared with BIS as crosslinker and Nile Red as encapsulated compound was investigated by enzymatic degradation with hyaluronidase (Fig. 6). The microgels were incubated at 37 °C in PBS buffer (pH = 7.4) containing 1 wt% Tween 80 in presence of 300 UI/mL hyaluronidase.

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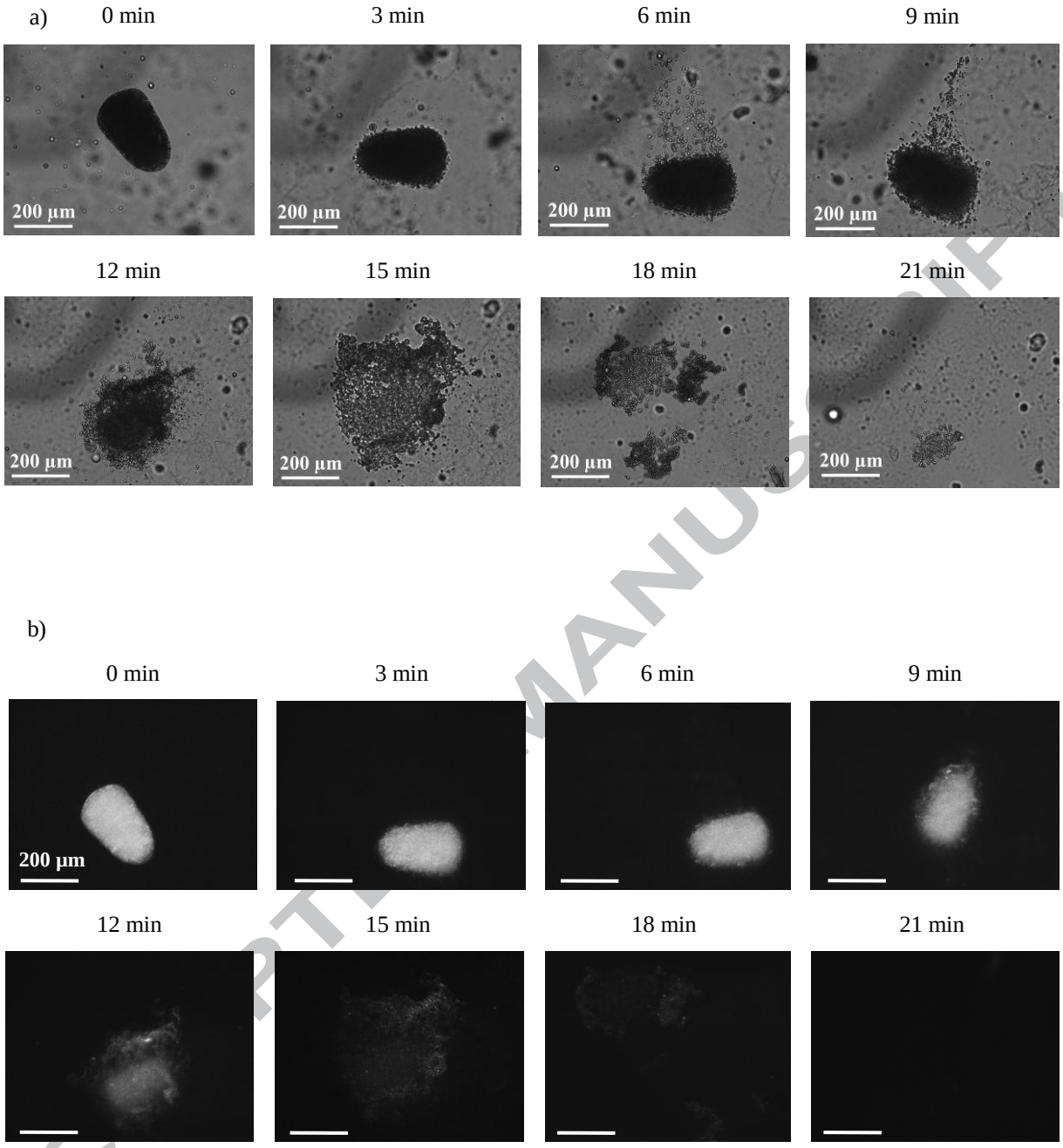


Fig. 6. Enzymatic degradation of a single HA-MA microgel with time: a) bright-field, b) fluorescence micrographs. Scale bar is 200 µm.

As shown in the Fig. 6, slow enzymatic degradation of the matrix occurs in a period of 21 min approximately. The bullet-like shape of the microgel was progressively lost (Fig 6a). Simultaneously, small drops appear at the surface of the microgel, which rapidly diffuse away. More droplets are visible when the bullet-like shape vanishes. This scenario is very

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similar to the dissolution of aspirin effervescent tablets, except that the gas bubbles are replaced by the tiny oil droplets.

Application to progesterone encapsulation and release

In order to use biocompatible compounds for the hormone encapsulation, hexadecane was replaced with sunflower oil containing 0.06 M of progesterone during the preparation of the primary emulsion. The average hydrodynamic diameter of the sunflower oil droplets in aqueous solution was about 60 nm, with a PDI of 0.3. Therefore, the distribution was broader than the one with hexadecane. Droplets as large as 200 nm were also present in the distribution. Optical images of the HA-MA microgels containing this emulsion are shown in Fig. S3. The image suggests that the individual oil droplets were successfully encapsulated, retained, and uniformly distributed within the hydrogel network of the particles.

The potential of these microgels to be used as drug delivery systems was assessed. Fig. 7 shows pictures taken at different times during the release assay in presence of different concentrations of hyaluronidase. The release experiments were this time performed on a collection of microgels. Since the microgels were filled with oil droplets, their density was lower than that of the aqueous phase and they floated at the top of the suspension. The subnatant was initially clear. Upon HA degradation, the subnatant became cloudy, indicating that the oil droplets invaded this part of the solution. Thanks to their small size, they could diffuse in the whole volume. For microgels exposed to a concentration of 300 UI/mL, the degradation process begins to be macroscopically visible after 3 hours of testing, whereas for microgels exposed to a concentration of 100 UI/mL, this situation is visible after 8 hours. As expected, the release of oil nanodroplets was not observed in the control test carried out in absence of enzyme in the period studied.

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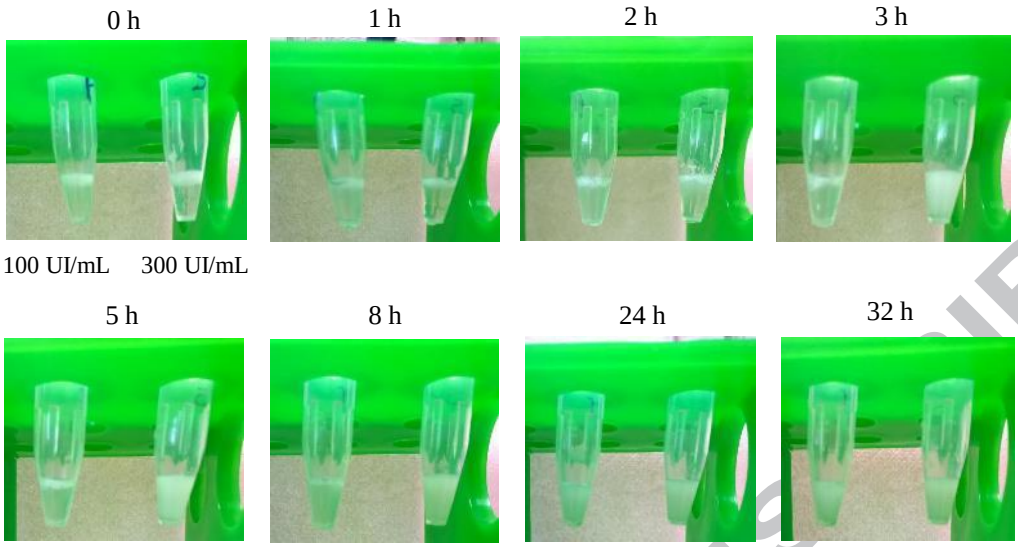


Fig. 7. Photographs of release experiments of PGR-encapsulating oil nanodroplets from HA-MA microgels in presence of hyaluronidase. Each snapshot presents a solution with microgels containing 100 UI/mL of Hase (left vial) and 300 UI/mL of Hase (right vial). At initial time, the microgels are located near the air-water interface, where a turbid area is visible. Over time, the vial on the right becomes progressively turbid in the whole volume of the solution, indicating a progressive diffusion of the nanodroplets of oil.

The degradation-responsive release of PGR was analyzed quantitatively by HPLC (Fig. 8). The microgels exposed to a concentration of 300 UI/mL released their load within 1 day whereas those exposed to a concentration of 100 UI/mL released their load within more than 2 days. In both cases, a PGR release of about 80% was observed. Additional quantitative analyses were performed in order to evaluate the influence of the compound transport into the continuous oil phase during the preparation of microgels. It was estimated that the compound loss by transfer was about 6%, whereas additional losses may occur during washing and redispersion steps. These small losses could explain why the plateau did not reach 100%. This meant that the progesterone was quasi-quantitatively released in the outer medium. Interestingly, this result also indicated that the drug was not degraded by the UV irradiation

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during particle synthesis. Therefore, it could be concluded that the enzymatic degradation could lead to a full release of the entrapped species.

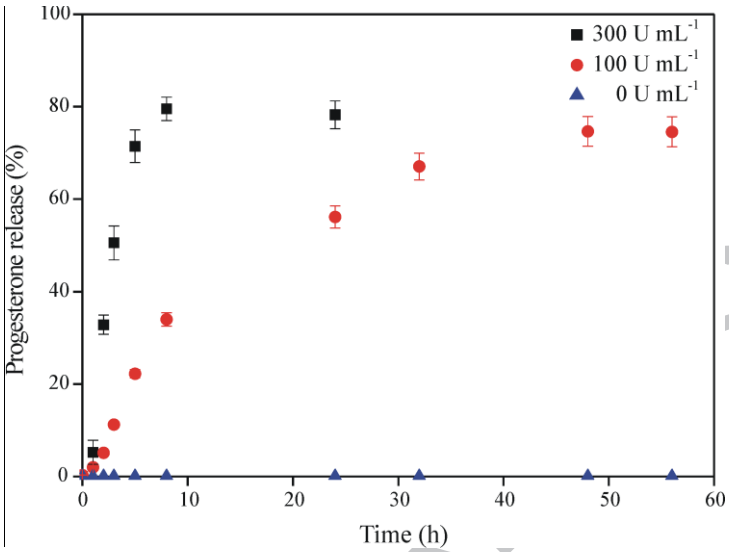


Fig. 8. Effect of enzymatic degradation on progesterone release from HA-MA microgels (T=37 °C).

CONCLUSIONS

In this work, we provided an effective route to the preparation of monodisperse degradable microgels based on hyaluronic acid, which encapsulate and release hydrophobic drugs upon enzymatic degradation. Whereas hydrogel particles are highly sought-after as highly hydrated biocompatible drug delivery systems, their limitation often lies in their poor capability to encapsulate hydrophobic drugs. The incorporation of submicrometer-sized non-polar droplets in the interior of hydrophilic microgel particles provides an efficient route of encapsulation, which is achieved using a double oil-in-water-in-oil emulsion template, where the aqueous phase is further converted into the gel phase. The overall process is precisely controlled by microfluidics, which offers a tailoring process to create controlled double emulsion template, and allows online photopolymerization. Up to now, very few examples have reported the

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entrapment of oil droplets into hydrogel particles [35-38], and the concept has never been applied to a biodegradable matrix for controlled release application. Here, the enzymatic degradation of the microgels is used to trigger the release of progesterone-loaded oil nanodroplets. In particular, it is demonstrated that hyaluronidase, which is present *in vivo*, can promote the release with a concentration-dependent kinetics. The droplets can then diffuse freely in the body to transport the drug.

This concept of oil-in-microgel material is general and can be applied to a broad range of hydrophobic drugs. Depending on the application, the required kinetics of release and the delivered amount will be different. Those parameters will be easily tuned upon an appropriate choice of the injection site – because the amount of Hase varies in the body- but also upon appropriate structure of the microgel. Further work will be required to demonstrate the impact of the structure on the release kinetics. The microfluidic process can be easily adapted to tune the shape of the microgels and yield spherical microgels instead of bullet-like ones by changing the position and width of the channels, using a flow-focusing technology for example. In general, the synthesis procedure can be optimized in order to produce particles with pre-specified physico-chemical and structural characteristics (size of microgels and drops, drop concentration, crosslinking degree of HA-MA, chemical composition) to achieve a desired drug release profile. Besides, the drug is released in the solubilized form, in oil nanodroplets. Conjugation of the droplets may also be required to further promote site-specific targeting. In future works, the preparation of microgels with tunable properties will be studied and other hydrophobic drugs will be assayed.

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Supporting information

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Fig. S1. Co-flowing microfluidic device used for the preparation of the microgels; Fig. S2 HA-MA microgels prepared using HA-MA with a methacrylation degree of 20 % without crosslinking agent; Fig. S3. Bright-field micrograph of HA-MA microgels entrapping nanodroplets with progesterone.

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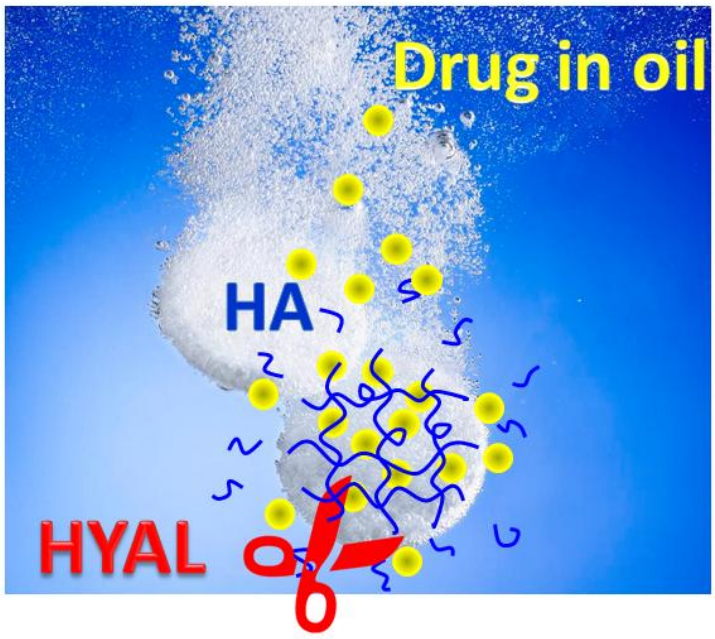
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