MICROFLUIDIC-BASED PREPARATION OF HYALURONIC ACID MICROGELS FOR PROGESTERONE DELIVERY

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SUMMARY

Progesterone delivery systems have been proposed for regulating the estrous cycle in cattle by incorporating progesterone into biodegradable and non-biodegradable polymeric matrices. In recent years, 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 that can be cleaved by hyaluronidase present in vivo. The objective of this work is to prepare biodegradable HA microgels by a co-flow microfluidic strategy for the encapsulation and controlled release of progesterone. The approach relies on (i) the generation of a primary organic/water (O/W) nanoemulsion, (ii) microfluidic emulsification of the primary emulsion, and (iii) photopolymerization of HA precursors with polymerizable methacrylate groups (HA-MA) present in the aqueous phase of the droplets. The potential use for progesterone delivery applications was evaluated through degradability and encapsulation/release studies in presence of hyaluronidase.

Keywords: progesterone, hyaluronic acid, drug delivery.

INTRODUCTION

Controlled delivery systems of progesterone have been proposed for regulating the estrous cycle in cattle by incorporating the hormone into biodegradable and non-biodegradable polymeric matrices.¹² Polymer microgels have received considerable attention in recent years due to their great potential in the biomedical field as drug delivery systems. Microgels are crosslinked hydrogel particles which present high water content and adjustable chemical and mechanical properties. In addition, these soft particles are of great interest for their stimuli-responsiveness. Indeed, their swelling properties are ruled by the balance between the inner and outer osmotic pressures in the gel phase. Thus, any unbalance between the osmotic pressures induces swelling or shrinking. Such modification can be triggered by external
parameters acting on the solubility of the polymer network, its charge density or its cross-linking density. The modulation of the swelling state is accompanied by a modification of the hydrogel physical properties such as its weight (water content), optical properties (e.g. refractive index), mechanical properties (elasticity), or even porosity. The latter is of huge interest in drug delivery applications, since microgels can entrap a molecule and release it at a rate depending on its diffusion through the network. This rate depends not only on the size of the network, but also on the mesh size of the gel and can be modulated by the application of an external stimulus. To be used as drug delivery systems, microgels should fulfill both biocompatibility and biodegradability criteria. In other words, the matrix must be safe, not generate excessive immune responses, possess acceptable biocompatibility, be nontoxic, and be eliminated in a controlled manner. Biodegradability can serve two main purposes: the hydrogel matrix can be degraded after the particle has delivered its payload, thus controlling the excretion pathway and particle disintegration can also be the mechanism to trigger drug release. In drug formulation it is important to control the particle size and size distribution in order to achieve an optimal pharmokinetic effect. The polydispersity in size and the lack of process repeatability resulting from the conventional preparation methods may produce undesirable variation in the rate of microparticle degradation, the stability of the drug and the release rate and drug dosage. Several methods have been developed to control the size of the particles and thus to control the profiles of drug release. Among them, microfluidic technologies offer low-cost and easy-to-use platforms for the fabrication of highly monodisperse drug-loaded polymer particles.

Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan composed of N-acetyl-D-glucosamine and D-glucuronic acid. This linear polysaccharide is ubiquitous in all tissues, as a major component of the extracellular matrix (ECM) in animal tissues. It has important structural and biological functions, including cell proliferation, differentiation, morphogenesis, inflammation and wound healing. HA is abundant in nature, renewable and nontoxic, and can be chemically modified. In addition, HA can be cleaved by hyaluronidase present in vivo. To be designed as a crosslinked network, HA can be modified by crosslinkable groups such as
glutaraldehyde (ADH), divinylsulfone (DVS), thiol or methacrylate functional groups.\textsuperscript{10,11,12}

The objective of this work is to prepare biodegradable hyaluronic acid (HA) microgels by a microfluidic strategy for the encapsulation and controlled release of progesterone. The approach relies on (i) the generation of a primary organic/water (O/W) nanoemulsion by the ultrasonication method, (ii) microfluidic (MF) emulsification of the primary emulsion, and (iii) photopolymerization of the monomer present in the aqueous phase of the droplets.

METHODOLOGY

Materials

All the reagents were purchased from Sigma-Aldrich unless otherwise noted. Methacrylic anhydride (AMA), N,N-dimethylformamide (DMF), ethanol, hexane, 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), progesterone and phosphate buffered saline (PBS) were used without any further purification. Hyaluronic acid ($M_w = 60000$ 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 in the literature.\textsuperscript{13,14} In brief, 1 g of hyaluronic acid (2 wt\%) was solubilized overnight at room temperature in ultrapure water. Then, DMF was added dropwise to the solution (ratio 3/2 water–DMF) and the mixture was cooled down to 4 °C. Then, methacrylic anhydride (AMA) (1 molar equivalents 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 (v/v) ratio of 2/3). After the removal of the supernatant, the precipitate was successively washed with mixtures of water–EtOH, v/v of 3/7, 1/4 and 1/9. The final precipitate was dissolved in ultrapure water and further dialyzed against ultrapure water for a period of 3 days. Finally, the purified macromer was recovered by freeze-drying. The macromer was characterized by $^1$H-NMR analysis. $^1$H-NMR spectra of HA-MA derivatives in D$_2$O at a concentration of 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 (DM) 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 1 wt% SC and the oil phase consisted of hexadecane or sunflower oil 20 wt%. 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. 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

A co-flowing microfluidic device was used for microgels preparation involving fused silica capillaries with an inner diameter of 75 µm and an outer diameter of 250 µm. For surface modification, 3 mL of OTS solution (1 wt%) in dodecane was passed through the outer capillary at a 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 2 wt% BIS and 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 microfluidic device using a syringe pump and the flow rates were 3 µl/min and 6 µl/min, respectively. Polymerization was carried out by placing the device under a UV lamp (Hamamatsu LC8, $\lambda = 350-385$ nm). The microgels were separated from the continuous phase by centrifugation at 800 rpm for 10 min at room temperature and washed first with hexane and then with 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 a micropipette was used to hold the microgels. 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 with UV detection by diode array. 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

The degree of methacrylation determined by $^1$H-NMR was 10%. The $^1$H-NMR spectra is depicted in Fig. 1.

Microgels containing oil nanodroplets were prepared by the microfluidic generation of organic/aqueous/organic (O/W/O) double emulsions and subsequent photopolymerization of the monomer in the aqueous phase of the droplets. In order to visualize the encapsulation process, nile red was incorporated into the oil phase prior to emulsification at a concentration of 3.1 x 10$^{-4}$ M. Average diameter and polydispersity index of the hexadecane oil droplets in aqueous solution were 355 nm and 0.33, respectively. Optical images of microgels are shown in Fig. 2.

![Figure 1. $^1$H-NMR spectra of HA-MA derivatives.](image)
As shown in the Fig. 2, microgels are monodisperse in size and morphology and they can encapsulate the emulsion. They exhibit a slug shape originating from the co-flow technique, where the drops are deformed by the walls of the capillary. This means that the microgels adopt the shape of the drops during the photopolymerization step and do not relax their shape after being released from the capillary. Some phase separation can also be observed during microfluidic preparation of microgels (Fig 2). However, this phenomenon was not investigated in details as it was not visible with further compositions. 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. As shown in Fig. 3, the average diameter of the sunflower oil droplets in aqueous solution was 54 nm and the polydispersity index was close to 0.37. The size of the oil droplets and polydispersity remained constant over time. Optical images of the microgels containing this emulsion are shown in Fig. 4. The image suggests that the individual oil droplets were successfully encapsulated, retained, and uniformly distributed within the hydrogel network of the particles.
Figure 3. Characteristics of the W/O emulsion: a) evolution of the average diameter; b) evolution of the polydispersity index.

Figure 4. Microscopy images in transmission of HA-MA microgels loaded with progesterone.

**Enzymatic degradation with hyaluronidase**

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. 5). 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.
As shown in the Fig. 5, slow enzymatic degradation of the matrix occurs in a period of 21 min approximately. The slug shape of the microgel was progressively lost (Fig 5a). Simultaneously, small drops appear at the surface of the microgel, which rapidly diffuse away. More droplets are visible when the slug shape vanishes. This scenario is very similar to the dissolution of aspirin effervescent tablets, except that the gaz bubbles are replaced by the tiny oil droplets..

**Progesterone release in microgels**

The potential of these microgels to be used as drug delivery systems was assessed. Progesterone was incorporated in the oil phase during the microfluidic preparation of the HA-MA microgels. Fig. 6 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. Progesterone release was not observed in the control test carried out in absence of enzyme in the period studied.

Figure 6. Photographs of progesterone release experiments from HA-MA microgels in presence of hyaluronidase.

The degradation-responsive release of progesterone was analyzed by HPLC (Fig. 7). 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 progesterone 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.
CONCLUSIONS

Microfluidics provides an effective route to the preparation of monodisperse degradable microgels based on hyaluronic acid for the encapsulation and controlled release of progesterone. In particular, the reported method provides the ability to controllably encapsulate submicrometer-size non-polar droplets in the interior of hydrophilic microgel particles. The enzymatic degradation of the microgels was studied and it was demonstrated that it can be used to trigger the release of progesterone from microgels.

BIBLIOGRAPHY


