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Ultrasonically Assisted Preparation of Polysaccharide Microcontainers for Hydrophobic Drugs

T.N. Borodina[1,*], R.A. Akasov[2,†], T.V. Bukreeva[1,‡], E.A. Markvicheva[2,§]

[1] *Shubnikov Institute of Crystallography RAS, 59, Leninskiy Prospect, 119333 Moscow, Russia*
[2] *Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS, 16/10, Miklukho-Maklaya Str., 117997 Moscow, Russia*

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Stable polysaccharide microcontainers are fabricated by ultrasonically assisted procedure. Ultrasound induces formation of permanent microcontainer shell due to interaction between chitosan and xanthan gum. The obtained system has a core-shell structure with high loading capacity for hydrophobic molecules. The permanent polymer shell thickness of 7-10 nm allows to maintain the microcontainer stability for more than 4 months. The microcontainers in a wide size range of 350-7500 nm were obtained by changing an overall emulsion viscosity. Uptake of the microcontainers by mouse melanoma M3 cells was studied by flow cytometry and confocal microcscopy.

Keywords: Microcontainers, Ultrasonication, Core-shell structure, Hydrophobic Drug.

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1. INTRODUCTION

Although a lot of physicochemical methods are used to encapsulate lipophilic bioactive substances, a number of encapsulation techniques increases continuously because of constant growth of poor water soluble drug formations and necessity to create stable delivery system for them. In most cases, water-immiscible components are encapsulated by emulsification method which requires additional stabilization of a system by various surfactants, surface-active polymers, proteins, polysaccharides or their mixtures. Up-to-date to produce emulsions, different techniques, such as high pressure homogenization, microfluidization, membrane contractor technique, and ultrasonication have been developed [1].

Suslick was the first who proposed a sonochemical technique to prepare protein microspheres from bovine serum albumin, human serum albumin, hemoglobin and myoglobin using high-intensity ultrasound [2]. Since his first report, various research groups used the proposed approach to fabricate various microspheres/microcontainers [3]. So far, they focused on microsphere fabrication utilizing various proteins or polymers which contain protein moieties.

We propose a new class of microcontainers based on chitosan and xanthan gum prepared in one-step procedure applying a low-frequency ultrasound. Both polymers belong to natural polysaccharides which are widely used for fabrication of different types of microcapsules. The main advantage of natural polymers is their biocompatibility and biodegradability performance, without producing systemic toxicity upon drug administration [4]. Chitosan is widely used in numerous applications, ranging from pharmaceutical and cosmetic products to water treatment and plant protection [5]. Xanthan gum has occupied an important place in food industry as well as in pharmaceutical, cosmetic and industrial applications [6].

An advantage of proposed microcontainers over earlier ones is their polysaccharide shell which allows to stabilizes the containers within a broad pH range.

2. MATERIALS AND METHODS

Chitosan (20-300 cP), xanthan gum (from Xanthomonas campestris), soybean oil, Nile Red were supplied from Sigma-Aldrich. Phosphate buffer (pH 7.4) was from PanEko, Russia.

In our study mouse melanoma cell line M3 was cultured in DMEM medium (PanEko, Russia) supplemented with 10% fetal bovine serum (HyClone) at 37 C in a 5% CO_2 humidified atmosphere. The medium was replaced every 3-4 days after previous cell detachment using 0.02 % (w/v) trypsin solution.

2.1 Microcontainer preparation

Equal volumes of chitosan and xanthan gum solutions (0.25 w%, pH 2) were mixed, then layered with soybean oil and exposed to high-intensity ultrasound using a 7-mm diameter titanium sonotrode (56 W cm-2, 20 kHz, 5 min). The synthesized microcontainers were separated from the reaction mixture by centrifugation (6.708 × g, 5 min) and washed three times with water using centrifugation/resuspension technique. The containers loaded with Nile Red were prepared following the same procedure but Nile Red was added to soybean oil.

2.2 Flow Cytometry Analysis of Microcontainer Uptake by Cells

A BD FACSCalibur fluorescent-activated flow cytometer and the BD CellQuest software were used to

* tatiana_borodina@hotmail.com
† zoolcat@yandex.ru
‡ bukreeva@ns.crys.ras.ru
§ lemarkv@hotmail.com

perform flow cytometry analysis.

To study entrapment of red–loaded microcontainers by cells, they were incubated with murine melanoma M3 cells in DMEM supplemented with 10% fetal bovine serum for 30 min and 2 hs. Then non-entrapped microcontainers were removed by washing three times with phosphate buffer (pH 7.4), and the cells were detached by trypsinization. After centrifugation (1000 rpm, 5 min), they were resuspended in a 0.4% (w/v) trypan blue in Hank's Balanced Salt Solution (HBSS) to quench extracellular fluorescence, thus enabling determination of the fraction which was actually internalized. The treated samples were subsequently washed twice, and then analysed by flow cytometry with at least 10,000 cells being measured in each sample.

2.3 Characterization of the microcontainers

Fourier Transform Infrared (FTIR) spectroscopy measurements were carried out with a Bruker Hyperion 2000 IR spectrometer. Confocal Laser Scanning Microscopy (CLSM) images were obtained using a Leica TCS SP confocal scanning system (Leica, Germany). Scanning electron microscopy (SEM) measurements were performed using a Gemini Leo 1550 instrument at an operation voltage of 3 keV. Z-potential, size and polydispersity index (PDI) of the containers were measured by DLS using the Zeta Sizer Nano (Malvern Instruments, UK).

3. RESULTS AND DISCUSSION

As known, chitosan and xanthan gum can form polyelectrolyte complex due to an ionic interaction. Prevention of a complex formation is an important factor for the microcontainer formation by the proposed technique. To avoid electrostatic interaction between the polymers, we adjusted pH value of their solutions to the acidic one. Since both polymers possess functional groups, such as amino, hydroxyl and carboxyl ones, we suggested that they could be cross-linked by direct interaction between their polymer chains. This assumption is based on a theory that acoustic cavitation arises during an ultrasonication process and induces production of highly reactive free radicals, especially $OH^{\cdot}$ and $H^{\cdot}$ [2]. These radicals could diffuse to a solution and react with a solvent or solutes, initiating radical chemical processes. As a result, polymer moieties located at an interface between a dispersed phase and a dispersion medium could be cross-linked. It leads to formation of a stable structure at the surface of emulsion droplets, and subsequently formation of a microcontainer permanent shell.

We investigated an influence of ultrasound on the polymer mixture by FTIR spectroscopy. Comparison of the initial polymer spectra and the spectrum of the polymer mixture extracted from the containers revealed various changes. A mechanism of container shell formation was explained by influence of the free radicals generated at sonication process on polymer molecules leading to their cross-linking earlier [2]. We suggested that in the case of chitosan/xanthan gum microcontainers, ultrasound shell formation is a multi-step procedure. The first step consists of hydrogen bridges formations (between the polar groups –COOH, $–NH_2$, -OH and –COOH) which maintain a primary structure of the microcontainer shell. The second step involves formation of ester binding and amide linkage network which contribute to preparation of the containers with permanent core-shell structure.

We examined an influence of a sonicated solution volume on the size of the obtained microcontainers. As seen in Figure 1A, 1B, it was possible to get the microcontainers in a wide size range of 350 nm - 7500 nm by varying an oil/water ratio (from 1:300 to 1:1.5 accordingly).

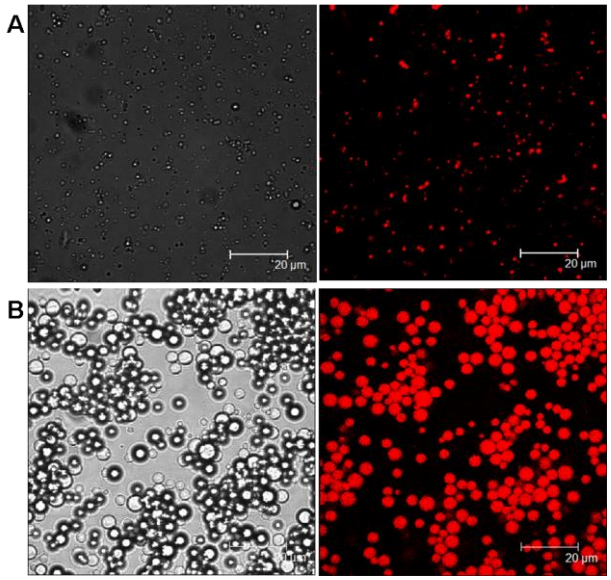

Fig. 1 – The confocal images of the microcontainers differed in size: 350 nm (A) and 7.5 µm (B).

A dispersed phase/dispersion medium ratio has an influence on the microcontainer size due to changing an overall emulsion viscosity [7]. An oil volume fraction increase led to an overall emulsion viscosity enhancement and, as a result, to the increase of the microcontainer size.

To visualize microcontainers, a hydrophobic fluorescence dye Nile Red was used. Figure 1A and 1B show two microcontainer fractions (namely, small MC1 and big MC2 fractions) recorded in transmission and fluorescence mode. The presence of fluorescence from Nile Red demonstrates an uniform distribution of a liquid core within the microcontainers.

The microcontainers were additionally monitored by cryo SEM. Figure 2 demonstrates the microcontainers with different size. The results were in a good agreement with confocal investigations.

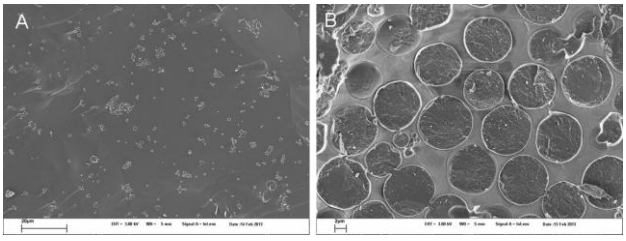

Fig. 2 – Scanning electron microscopy images of the microcontainers differed in size.

Figure 3 demonstrates an image of the microcontainer with broken shell where it is clearly seen that an inner container core is surrounded by an external polymer shell. This observation revealed a core-shell structure of the obtained microcontainers with the polymer shell thickness of 7-10 nm. The microcontainers were stable at storage for at least 4 months

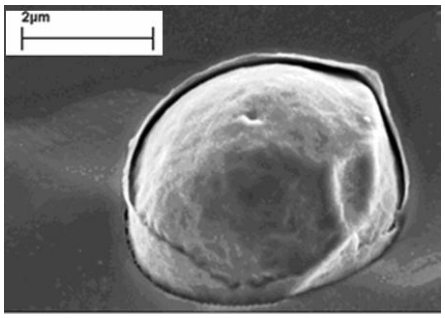

Fig. 3 – Micrograph of the microcontainer with a broken shell recorded by cryo scanning electron microscopy.

We performed a pilot experiment to investigate a behavior of the microcontainers towards M3 cells. The small (500 nm) and bigg (2 μm) microcontainer fractions were used to analyze their penetration into the cells. Figure 4 shows an evaluation of Nile Red-loaded microcontainers uptake by M3 cells after 30 min and 2.5 hours incubation. Results demonstrated that 30 min incubation was enough for the microcontainer internalization by M3 cells. The uptake rate revealed a time-dependent character with dramatic increase of the cellular uptake of the small microcontainer fraction.

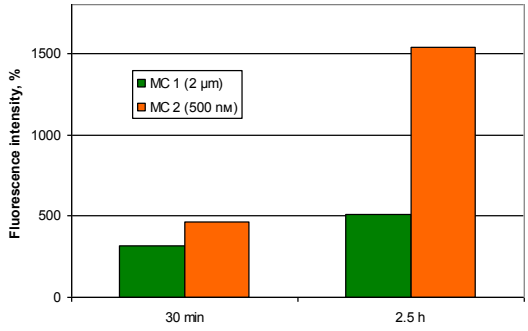

Fig. 4 – Uptake of the Nile Red-loaded microcontainer fractions MC1 (2 μm) and MC2 (500 nm) by mouse melanoma M3 cells after 30 min and 2.5 h incubation.

We also studied the microcontainer distribution within the cells by confocal microscopy. Figure 5 demonstrates that most of the microcontainers were accumulated within a cellular phospholipid bilayer but not within endosomes. It could be explained by a lipid-based nature of the microcontainers and their negative charge. Similar results were reported earlier [8] where targeted nanoparticles delivered lipophilic substances directly to a targeted cell membrane with subsequent trafficking via a lipid raft-dependent pathway.

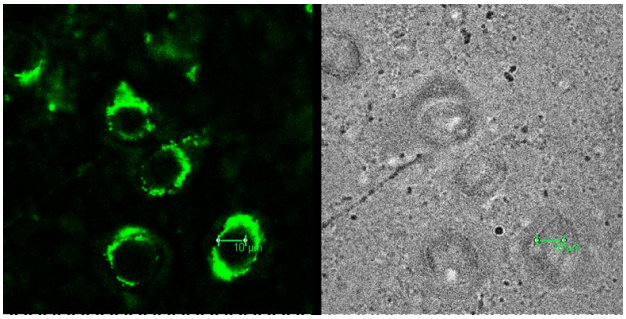

Fig. 5 – Confocal images of the microcontainer distribution within murine melanoma M3 cells.

4. CONSLUSIONS

A new class of the polysaccharide microcontainers for hydrophobic drug encapsulation was proposed. The microcontainers with a core-shell structure were ultrasonically obtained due to cross-linking between chitosan and xanthan gum. The microcontainer size could be easily adjusted from nano-scale to microscale by varying the sonicated solution volumes. Flow cytometry analysis revealed rather good microcontainer internalization by murine melanoma M3 cells. The Nile Red-loaded microcontainers were found to accumulate in the cellular membrane. The developed microcontainers could be promising for delivery of hydrophobic drugs, in particular lipophilic anticancer preparations.

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