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Tailoring the stealth properties of biocompatible polysaccharide nanocontainers

Biao Kang [a], Patricia Okwieka [a, b], Susanne Schöttler [a], Oliver Seifert [c], Roland E. Kontermann [c], Klaus Pfizenmaier [c], Anna Musyanovych [a], Ralf Meyer [b], Mustafa Diken [d, e], Ugur Sahin [d, e], Volker Mailänder [a, b], Frederik R. Wurm [a, *], Katharina Landfester [a]

[a] Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany
[b] Department of Hematology, Medical Oncology, and Pneumology, University Medical, Center Mainz, Langenbeckstr. 1, 55131 Mainz, Germany
[c] Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
[d] Biontech AG, An der Goldgrube 12, 55131 Mainz, Germany
[e] TRON – Translational Oncology at the University Medical Center of Johannes Gutenberg University Mainz, Langenbeckstr 1, 55131 Mainz, Germany

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ABSTRACT

Fundamental development of a biocompatible and degradable nanocarrier platform based on hydroxyethyl starch (HES) is reported. HES is a derivative of starch and possesses both high biocompatibility and improved stability against enzymatic degradation; it is used to prepare nanocapsules via the polyaddition reaction at the interface of water nanodroplets dispersed in an organic miniemulsion. The synthesized hollow nanocapsules can be loaded with hydrophilic guests in its aqueous core, tuned in size, chemically functionalized in various pathways, and show high shelf life stability. The surface of the HES nanocapsules is further functionalized with poly(ethylene glycol) via different chemistries, which substantially enhanced blood half-life time. Importantly, methods for precise and reliable quantification of the degree of functionalization are also introduced, which enable the precise control of the chemistry on the capsules' surface. The stealth properties of these capsules is studied both *in-vitro* and *in-vivo*. The functionalized nanocapsules serve as a modular platform for specific cell targeting, as they show no unspecific up-taken by different cell types and show very long circulating time in blood (up to 72 h).
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1. Introduction

As a rapid growing interdisciplinary research field, nanomedicine has extended the number of possible therapeutic methods, including nanobiosensors [1], nanovaccination [2,3], nanoparticle related imaging [4], nanodrug carriers [5,6], etc. Among these many potential applications, nanocarriers for targeted drug delivery are a major research field due to promising therapeutic effects, e.g. specific cell targeting, protecting the drug simultaneously on its way to the desired site, and the controlled release of the delivered cargo [7,8]. Of utmost importance for targeted delivery and release are the functional properties of the nanocarrier itself: unspecific cell-uptake has to be prevented, and the carrier needs to exhibit long blood circulation half-life in order to achieve enrichment and selective, targeted activity at the diseased tissue. High drug loading capacity is needed to maximize therapeutic effect. In addition, the nanocontainer should be biodegradable, but only subsequent to uptake and release of the bioactive principle at the target site. Finally, of equal important is the precise quantification of the chemistry of nanocarrier synthesis, to allow systematic investigation and understanding of the factors, which influence the performance of the system. Many studies have so far addressed the question of suitable targets and provide a large toolbox of targeting structures [9–21]. In contrast, information on the development of new synthesis mechanisms for the nanocarrier platform itself is sparse. The performance of the whole targeting system is often hindered by properties of the nanocarrier itself [22–25], which often possesses one or more of the setbacks like low stability, cytotoxicity, no precise

* Corresponding author.
E-mail address: wurm@mpip-mainz.mpg.de (F.R. Wurm).

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quantification (of functional groups, drug loading, etc.), unspecific interaction with healthy cells, and short plasma half life time.

In this study, miniemulsion was chosen as the preparation method, due to its outstanding performance to generate nanoobjects with tunable sizes, various surface functionalization possibilities, and efficient encapsulation of a payload [26]. Nanoparticles with diameters between 50 nm and 500 nm have been successfully prepared by miniemulsion, through different reaction mechanisms, including radical polymerization [27], polycondensation [28], oxidative polymerization [29], and click chemistry [30].

Hydroxyethyl starch (HES) is a starch derivative, whose hydroxyl groups at the C2 or C6 positions of each glucose unit are partially functionalized with a hydroxyethyl group. It is approved for clinical application, where in most cases it is used as a volume expander for the treatment of a hypovolemic shock [31]. Due to the presence of hydroxyethyl groups, the enzymatic degradation of HES is much slower compared to native starch, while the biocompatibility is rather unchanged [32]. Furthermore, it has been shown that, when HES is attached on the surface of micelles, i.e. "HESylation", it shows similar properties as membrane-grafted poly(ethylene glycol) (PEG), i.e. "PEGylation", with respect to prolonged blood circulation times or reduced unspecific cell uptake, often called "stealth effect" [33,34].

In the present study, we investigated the combination of both strategies: First, HES nanocapsules have been prepared by interfacial polyaddition in an inverse (water-in-oil) miniemulsion with diameters ranging from 150 to 250 nm. Then, the surface of these polysaccharide nanocapsules was further functionalized with PEG via different chemistries: either the coupling of PEG was conducted within the organic phase directly after the polyaddition, or the bare HES-nanocapsules were transferred into an aqueous dispersion and subsequently PEGylated. Both strategies were compared with respect to toxicity, stability in long time storage, protein adsorption, cell uptake *in vitro*, and plasma half life time *in vivo*. In addition, a detailed investigation of every step in the reactions was carried out to allow a precise quantification of functional groups and added PEG chains on the nanocontainers.

2. Experiment part

2.1. Materials

Different hydroxyethyl starch samples were donated by Fresenius Kabi. 2,4-Toluene diisocyanate (TDI) and cyclohexane (>99.9%) were purchased from Sigma Aldrich. The isocyanate-PEG and amine terminated PEG (PEG molecular weight of 2000 g mol^{-1}, 44 ethylene oxide units) was purchased from Nanocs Inc., USA. The maleimide-PEG (PEG molecular weight of 2000 g mol^{-1}, in average 44 ethylene oxide units) was purchased from Sigma Aldrich. The PBS buffer (pH 7.4, without Ca^{2+} and Mg^{2+}) was purchased from Gibco, Germany. Boric buffer (1 M, pH 9.5) was prepared by dissolving boric acid (B6768 Sigma) in water, and adjusted to the desired pH by sodium hydroxide solution. IR dye 800CW carboxylate was bought from Li-COR. Oil soluble surfactant poly((ethylene-*co*-butylene)-*b*-(ethylene oxide)), P(E/B-b-EO), consisting of a poly(ethylene-*co*-butylene) block (M_w = 3700 g mol^{-1}) and a poly(ethylene oxide) block (M_w = 3600 g mol^{-1}) was synthesized starting from ω-hydroxypoly-(ethylene-*co*-butylene), which was dissolved in toluene after addition of ethylene oxide under anionic polymerization conditions [35]. The anionic surfactant sodium dodecylsulfate (SDS) was purchased from Fluka. The fluorescent dye sulforhodamine 101 (SR101) (M = 606.71 g mol^{-1}) was purchased from BioChemica, Aldrich. Dimethylsulfoxide (<50 ppm water content) was purchased from Acros Organics. Demineralized MilliQ water was used for the synthesis of nanocapsules. All the other chemicals were purchased from Sigma Aldrich and used as received.

2.2. Methods

2.2.1. Dynamic light scattering (DLS)

The average size of the capsules is measured by DLS using a PSS Nicomp Particle Sizer 380. The capsule dispersion is diluted by MilliQ water to a solid content of 0.1%. The scattered light is detected at 90°, while the temperature is maintained at 25 °C.

2.2.2. Electron microscopy

Scanning electron microscopy (SEM) measurements are carried out by using a LEO (Zeiss) 1530 Gemini device (Oberkochen, Germany). The samples are diluted to a solid content of 0.01% with MilliQ water, then 15 μl of the diluted sample is placed on the silica wafer and dried at room temperature overnight. Transmission electron microscopy (TEM) measurements are carried out by using Jeol 1400 device at 80 kV accelerating voltage. The samples are diluted in the same way as for SEM sample preparation. Then 2 μl of the diluted sample is placed on a copper grid, which is covered by a 5 nm thick carbon film.

2.2.3. Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectrums of functionalized capsules are measured at 300 MHz on a Bruker Avance 300 Spectrometer, by using deuterated water as solvent.

2.2.4. General protocol for the synthesis of HES nanocapsules

HES nanocapsules were prepared in a water-in-oil miniemulsion system similarly to the previously published protocols [38]. An aqueous solution of HES is emulsified with cyclohexane containing a block copolymer, P(E/B-*b*-EO) as surfactant. In a typical reaction, 100 mg of the stabilizer is dissolved in 9.5 g of cyclohexane at 60 °C. The water phase consists of 1 mg of SR101 (dye acting as a model for a low molecular weight drug), 10 mg of NaCl, 100 mg HES and 590 mg of PBS buffer. 7.5 g of the oil phase are mixed with the water phase and are magnetically stirred at 1000 rpm for 30 min, followed by ultrasonication at 70% amplitude for 3 min, with a pulse of 20 s on and 10 s off (cooling the mixture with an ice bath), using a Branson Sonifier W-450-Digital and a ½" tip. Then, 85 mg of TDI is added into 2 g of the oil phase, and added dropwise (over a period of 2 min) into the formulated miniemulsion. The interfacial polymerization is allowed to proceed at 25 °C for 24 h. HES molecules with different molecular weight and degree of substitution is used, with the detailed composition listed in (Table S1, Supporting information).

2.2.5. HES capsules with sizes lower than 200 nm

The reaction is conducted in the same manner as described above, however, an additional amount of oil phase is added after the sonication step, followed by an additional sonication step before the addition of the crosslinker TDI. The oil phase is prepared by dissolving 160 mg of P(E/B-*b*-EO) in 14.5 g of cyclohexane. The water phase is mixed with 7.5 g of the oil phase, magnetically stirred at 1000 rpm for 30 min, followed by ultrasonication under the same conditions as for the preparation of larger HES nanocapsules. After ultrasonication, 5 ml additional oil phase is introduced into the system, and magnetically stirred for the other 30 min at 1000 rpm, followed by an additional ultrasonication step. To start the polyaddition, TDI is added into 2 g of the oil phase, and added dropwise into the miniemulsion system. The interfacial polymerization is allowed to proceed at 25 °C for 24 h.

2.2.6. Redispersion of HES nanocapsules in water

1.5 g of the nanocapsule dispersion in cyclohexane is centrifuged at 4000 rpm for 30 min, and the precipitate is redispersed in 400 μl of fresh cyclohexane by pipetting up and down. Then, a 0.3 wt% SDS solution is prepared, and filtered (sterile, with 0.2 μm pore size). 5 ml of this SDS solution is gently shaken in a sonication bath (Bandelin Sonorex, type RK 52H), while the nanocapsule dispersion in cyclohexane is added slowly. Then the whole dispersion is magnetically stirred at 1000 rpm at room temperature overnight, in an open vial, to allow evaporation of cyclohexane. The obtained dispersion is ultrafiltrated using Amicon Centrifugal Filter (Ultra-0.5, Ultracel-100 Membrane, 100 kDa), in order to remove the excess of SDS and used for further measurements.

2.2.7. Quantification of primary amine groups at the surface of nanocapsules

The fluorescamine (FA) assay [36] is used to quantify the available primary amine groups at the surface of the nanocapsules. A stock solution of FA is prepared by mixing 2 mg of FA and 1.5 ml of anhydrous acetone. A standard working function is established from an aqueous hexylamine solution as reference (see Supporting information Fig. S4). For a typical quantification step, 25 μl of the nanocapsule dispersion in water (1 wt% solid content) are added into 725 μl of boric buffer (pH 9.5), followed by the addition of 200 μl of the FA stock solution. The mixture is vortexed (Heidolph REAX2000 at maximum speed) for 30 s, and the fluorescence intensity is measured by a Tecan plate reader at 25 °C by excitation at 410 nm and detecting the emission at 470 nm. All fluorescence measurements are repeated three times.

2.2.8. Functionalization of HES capsules with maleimide-PEG

12 mg of maleamide-PEG (MeO-PEG_{44}-Mal, with M_n = 2000 g mol^{-1}) are dissolved in 1 ml of MilliQ water. 5 ml of the nanocapsule dispersion in water (1 wt% solid content) are magnetically stirred, while the maleimide-PEG solution was added in one portion. The system is stirred at room temperature overnight, Millipore ultrafilter with a molecular cut off of 100 kDa is used to remove the free PEG chain after coupling.

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