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Immobilization of protein-coated drug nanoparticles in nanofibrillar cellulose matrices—Enhanced stability and release

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ABSTRACT

Nanosizing is an advanced approach to overcome poor aqueous solubility of active pharmaceutical ingredients. One main problem in pharmaceutical nanotechnology is maintaining of the morphology of the nanometer sized particles during processing and storage to make sure the formulation behaves as originally planned. Here, a genetically engineered hydrophobin fusion protein, where the hydrophobin (HFBI) was coupled with two cellulose binding domains (CBDs), was employed in order to facilitate drug nanoparticle binding to nanofibrillar cellulose (NFC). The nanofibrillar matrix provides protection for the nanoparticles during the formulation process and storage. It was demonstrated that by enclosing the functionalized protein coated itraconazole nanoparticles to the external nanofibrillar cellulose matrix notably increased their storage stability. In a suspension with cellulose nanofibrils, nanoparticles around 100 nm could be stored for more than ten months when the specific cellulose binding domain was fused to the hydrophobin. Also freeze-dried particles in the cellulose nanofibrils matrix were preserved without major changes in their morphology. In addition, as a consequence of formation of the immobilized nanodispersion, dissolution rate of itraconazole was increased significantly, which also enhanced the in vivo performance of the drug.

1. Introduction

Nanoparticles have been long seen as a promising way to enhance the dissolution rates of poorly water-soluble drugs due to their larger surface area and increased saturation solubility. Stable storage without losing the benefits of small size and delivery of the nanosized drug particles to the gastrointestinal provide typically further challenges in addition to their synthesis [1]. Typically the stability of drug nanoparticles is enhanced by protecting them by a polymer matrix or a surfactant layer. Nevertheless, to overcome existing stability problems as well as poor bioavailability or biocompatibility challenges, new strategies and new materials must be explored. Surface functionalization of nanoparticles is one of the key questions in novel pharmaceutical applications. Recently bio-based composites have been in the focus for pharmaceutical applications, as they offer materials with versatile characteristics [2,3].

Hydrophobins are a group of surface active proteins secreted by filamentous fungi [4–6]. Hydrophobins have an ability to spontaneously self-assemble onto a hydrophobic–hydrophilic interface as monolayers, which changes the hydrophobicity of the surface. Also their adhesive behavior has been studied for utilization as an immobilizer or a linker. Based on hydropathy plots, hydrophobins have been divided into class I and class II. These classes also have significant differences in the solubility of the assembled structures they form [7]. Hydrophobins, especially of class II (e.g., HFBI, II and III), have an awe-inspiring potential for several applications in pharmacy due to possibility to produce fusion proteins. In our previous studies, hydrophobins HFBI and HFBII were used to form and coat drug nanoparticles [8]. There, the functionalization of the surfaces was demonstrated by utilizing HFBI fusion with the green fluorescent protein (GFP-HFBI).

In this study drug nanoparticles were coated with an HFBI that was genetically fused with two cellulose binding domains (CBD) to obtain drug nanoparticles that can bind to cellulose. Most of the cellulolytic enzymes include a catalytic domain (CD), interdomain linker, and a discrete cellulose-binding domain (CBD). CBD domains fold independently and have a major role in targeting of the enzyme to the cellulose, which makes them ideal tags for different applications with cellulose fibers [9]. The double CBD (DCBD) was expected to increase the affinity to cellulose in comparison with the single CBD [10]. Strong affinity between CBDs and cellulose can be used to bind HFBI-DCBD coated drug nanoparticles to the cellulose network, which will protect the particles during the storage and the formulation processes [11].

Cellulose and its derivatives have a long history in pharmaceutical technology, mainly as an excipient in oral solid dosage forms. Cellulose morphological hierarchy consist of glucose β(1→4) D-glucopyranose

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units packed into elementary fibrils with the cross-sectional dimensions of ca. 5–30 nm and over 1 μm lengths e.g. in wood plant cells. Recently, these nanoscale fibers, disintegrated from microfibrils by enzymatic hydrolysis combined with mechanical shearing and high-pressure homogenization, have been recognized as rheology modifiers [12] and aerogel formers with tunable networks [13–15]. Therefore, cellulose nanofibrils (NFC) could also be used to form macroscopic structures that can act as templates for nanoparticles in stabilization and drug delivery purposes. In this study, our aim was the utilization of NFC for bottom-up building of immobilization matrices for drug nanoparticles.

A design of a drug delivery system where the drug nanoparticles are protected, stored and released from the NFC matrix is described. The hydrophobic drug compound, itraconazole (ITR), was precipitated to form nanoparticles with either hydrophobins (HFBI or HFBII) or genetically modified fusion protein (HFBI-DCBD). NFC was evaluated as a long-term stabilizer of the itraconazole nanoparticles and we wanted to demonstrate that enclosing of the nanoparticles to an external nanofibrillar cellulose matrix enhance their processability. Finally, due to the retained small particle size, improved dissolution rate of the freeze-dried nanoparticles was maintained and the enhanced oral bioavailability of the drug was shown in animal studies.

2. Materials and methods

2.1. Materials

Itraconazole (purity 99.7%) used as a model compound in precipitation was purchased from Apotecnia (Murcia, Spain). Itraconazole used as reference in the in vivo experiments (98%) was from Sigma-Aldrich (St. Louis, Mo, USA). Hydroxy itraconazole (98%), Itraconazole-d5 (chemical purity 98%, isotopic purity 99%) and Hydroxy itraconazole-d5 (chemical purity 97%, isotopic purity 99%) were from Toronto Research Chemicals (Toronto, Canada). HFBI, HFBII [16] and HFBI-DCBD [17] were expressed and purified as described elsewhere. In the HFBI-DCBD fusion protein DCBD was genetically fused to HFBI by a short peptide linker. NFC used for stabilization of the nanoparticles was obtained from UPM-Kymmene Corporation as a dilute hydrogel (solids content 1.9%). The sample was prepared by mechanical disintegration of bleached birch pulp by ten passes through a M7115 Fluidizer (Microfluidics Corp.) essentially according to the previous reports [12]. D ± trehalose dihydrate (TRE) was used as a lyo- and cryoprotectant (Sigma-Aldrich, St. Louis, Mo, USA). All chemicals were of analytical grade obtained from standard sources and used without further purification.

Tetrahydrofuran (THF, p.a., Riedel-de Haën, Germany) was used as the solvent for ITR. Chromatographic separations were performed using acetonitrile, methanol and 2-propanol (all LC–MS chromasolv grade, ≥99.9%), tetrahydrofuran (chromasolv for HPLC, ≥99.9%), formic acid (puriss p.a. for mass spectroscopy ~98%) and ammonium formate (puriss p.a. for mass spectroscopy, ≥99%) were from Sigma-Aldrich (St. Louis, MO, USA). De-ionized H_2O was produced using a Milli-Q water purification system from Millipore (Milford, MA, USA).

2.2. Drug nanoparticle preparation and binding to cellulose

An anti-solvent precipitation technique was used to form nanoparticles of the hydrophobic drug, itraconazole, and the hydrophobins, as described previously. Tetrahydrofuran was used as the solvent for ITR. HFBI, HFBII or HFBI-DCBD was first dissolved in water (0.6 mg/ml). The solution was sonicated and placed on an ice bath. Before use, the ITR solution (12 mg/ml) was filtered with 0.2 μm PVDF syringe filters (PALL, Ann Arbor, MI, USA) to remove possible dust particles. Then, the ITR (0.25 ml) solution was rapidly added into the hydrophobin solution (5 ml). The receiving liquid was stirred vigorously with a magnetic stirrer and temperature of the solution was controlled by keeping the sample on ice. A white precipitate was observed as a turbid solution immediately after the ITR addition, indicating the formation of the nanoparticles. The solution was stirred for 20 min before binding to cellulose nanofibrils. NFC dispersion was used at a concentration of 8.4 mg/ml and was sonicated immediately prior to use. Expressed in terms of dry weight, ratio of ITR: HFB: NFC of 1:1:2 was used in all the experiments.

2.3. Removal of organic solvent

Regenerated cellulose tubular membrane MWCO 3500 (CelluSep, Spectrum Labs, San Antonio, USA) was used to remove residuals of the organic solvents from the nanoparticle suspensions. The dialysate was ultrapurified water and it was changed after 2 and 6 h during the total dialysis time of 14 h.

2.4. Freeze-drying of nanoparticles

For the removal of water, the suspensions were immediately placed into a freeze-drying chamber (Kinetics Thermal Systems Lyostar II, SP Industries Inc., Warminster, USA). Particle dispersions were dried on the freeze-dryer with or without cryo- or lyoprotectants. With D ± trehalose the ratio of drug:excipients was 1:1.25. The primary drying was performed at −40 °C for 3 h and −30 °C for 17 h followed by the secondary drying, where the temperature was increased from −25 °C to +40 °C using 5 °C steps. The temperature was kept 1 h in each step. Pressure was maintained at 100 mTor (20 Pa). Powders were analyzed immediately after drying by differential scanning calorimetry (DSC).

2.5. Differential scanning calorimetry experiments

Thermal behavior of the freeze-dried nanopowders was studied using a differential scanning calorimeter (Mettler-Toledo Inc., Switzerland). The samples were held at 25 °C for 5 min before heating. All the samples were heated from 25 to 250 °C and then cooled down to 25 °C. The heating rate was 10 °C min^{-1}, and cooling rate was 20 °C min^{-1}. The data was analyzed with STARe software (Mettler-Toledo Inc., Switzerland).

2.6. Storage stability study

The long-term stability tests of the nanoparticle powders were performed at specific climatic conditions, 40 °C/75% RH. Samples for dissolution were taken before and after 12 weeks storage. The nanosuspensions were stored at +4 °C for 10 months.

2.7. Dissolution studies

Dissolution tests were performed using a paddle type dissolution apparatus (Erweka DT-6, Germany) with a rotation speed of 150 rpm. Dissolution medium (400 ml) was 0.2% (w/V) NaCl-HCl pH 1.2 and temperature was maintained at +37 ± 0.5 °C. Powders were loaded inside gelatin capsules (size 0). Theoretical amount of ITR was 0.5 mg in each sample. Capsules were dropped to the bottom of the dissolution vessels. Samples (1 ml) were taken at various time points, and the extracted medium was replaced with fresh medium. The samples were centrifuged at 13,000 rpm for 10 min. The released amount of ITR was determined by a HPLC-UV spectrophotometer (Agilent 1100 series, Santa Clara, CA, USA) at a wavelength of 261 nm.

2.8. Morphology and size

Characterization of the particle size and morphology was done by transmission electron microscope (TEM) (FEI Tecnai F12, Philips Electron Optics, The Netherlands). Nanoparticle dispersions were dried or spread (freeze-dried powder) on formvar film-coated copper grids with mesh size 300 (Agar Scientific, Essex, UK).