Journal of Pharmaceutical Sciences xxx (2016) 1-7

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Nanotechnology

Spontaneous Self-Assembly of Polymeric Nanoparticles in Aqueous Media: New Insights From Microfluidics, *In Situ* Size Measurements, and Individual Particle Tracking

Xue Li [1, 2], Giuseppina Salzano [1], Jiwen Zhang [2, *], Ruxandra Gref [1, *]

[1] *Institut des Sciences Moléculaires d'Orsay, Université Paris-Saclay, Université Paris-Sud, UMR CNRS 8214, 91400 Orsay, France*
[2] *Center for Drug Delivery Systems, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China*

ARTICLE INFO

Article history:
Received 12 April 2016
Revised 12 September 2016
Accepted 22 September 2016

Keywords:
cyclodextrins
nanoparticles
particle size
distribution
self-assembly

ABSTRACT

Supramolecular cyclodextrin-based nanoparticles (CD-NPs) mediated by host-guest interactions have gained increased popularity because of their "green" and simple preparation procedure, as well as their versatility in terms of inclusion of active molecules. Herein, we showed that original CD-NPs of around 100 nm are spontaneously formed in water, by mixing 2 aqueous solutions of (1) a CD polymer and (2) dextran grafted with benzophenone moieties. For the first time, CD-NPs were instantaneously produced in a microfluidic interaction chamber by mixing 2 aqueous solutions of neutral polymers, in the absence of organic solvents. Whatever the mixing conditions, CD-NPs with narrow size distributions were immediately formed upon contact of the 2 polymeric solutions. *In situ* size measurements showed that the CD-NPs were spontaneously formed. Nanoparticle tracking analysis was used to individually follow the CD-NPs in their Brownian motions, to gain insights on their size distribution, concentration, and stability on extreme dilution. Nanoparticle tracking analysis allowed to establish that despite their non-covalent nature, and the CD-NPs were remarkably stable in terms of concentration and size distribution, even on extreme dilution (concentrations as low as 100 ng/mL).

Introduction

Supramolecular nanostructures mediated by host-guest interactions based on cyclodextrins (CDs) have gained increased popularity for their potential biomedical applications, particularly in drug delivery.[1-3] Drug loadings were dramatically improved when including CDs in the nanoparticles (NPs), due to complex formation between CDs and drugs.[4] As an example, the loading of prednisolone in poly(alkyl cyanoacrylate) NPs was improved 129-fold when incorporated in CDs.[5]

More recently, CD-containing NPs (CD-NPs) were prepared by a mild, solvent-free method, at room temperature and without using surfactants.[6] CD-NPs were spontaneously formed by mixing 2 aqueous solutions of (1) dextran (Dex) grafted with alkyl side chains (Dex-C_{12}) and (2) a highly soluble CD polymer (poly-CD). The alkyl side chains of Dex-C_{12} formed inclusion complexes with the CDs in poly-CD, leaving most of the CDs free for the inclusion of other molecules of interest, such as anticancer drugs, contrast agents, nitric oxide donors, and cosmetic ingredients.[1,3] Based on a similar "lock and key" concept, the first small interfering RNA delivery system in humans using CDs was developed.[7] The self-assembly of cationic poly-CDs with anionic nucleic acids gave rise to the formation of NPs to which poly(ethylene glycol) and targeting agents could be associated.[7,8]

Benzophenone (Bz) was the most studied active ingredient in the NPs made of poly-CD and Dex-C_{12}.[6,9-14] Attractive features of the Bz-loaded NPs were their one step solvent-free preparation method, the small sizes and the possibilities to be freeze-dried and reconstituted in aqueous solutions.[6,14] Bz was incorporated in the polymeric solution and it was retained in the NPs as inclusion complexes with the CDs.[13] However, because of the competition between Bz and the alkyl chains for the CD cavities, Bz loadings reached at best 2.9 wt% and there was a significant amount of free Bz in the NP suspensions.[13]

We propose here an original approach to prepare Bz-containing CD-NPs, avoiding the presence of free Bz in the suspensions. Bz was grafted to Dex, replacing the alkyl side chains. The interaction of this novel conjugate (Dex-Bz) with poly-CD, leading to CD-NP formation, was investigated by a set of complementary novel

* *Correspondence to*: Ruxandra Gref (Telephone: +33-1-69158234; Fax: +33-1-69156777) and Jiwen Zhang (Telephone: +86-21-20231980; Fax: +86-21-20231980).
E-mail addresses: jwzhang@simm.ac.cn (J. Zhang) and ruxandra.gref@u-psud.fr (R. Gref).

http://dx.doi.org/10.1016/j.xphs.2016.09.024
0022-3549/

characterization techniques. For the first time, CD-NPs were instantaneously produced in a microfluidic interaction chamber by mixing 2 aqueous solutions of neutral polymers, in the absence of any organic solvent.

Interestingly, an *in situ* method was developed to study the CD-NP formation directly in the preparation vessel. Nanoparticle tracking analysis (NTA) was used to individually follow the NPs in their Brownian motion to gain insight into their size distribution, concentration, and stability upon extreme dilution.

Experimental

Materials

Dex (40,000 g/mol) was purchased from Pharmacia (Uppsala, Sweden). β-CD was kindly provided by Roquette (Vecquemont, France). 1-Adamantyl chloride and 4-benzoylbenzoic acid were obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France). MilliQ water was purified by reverse osmosis (Millipore®, Billerica, MA) and filtered using a 0.22-μm membrane (Millipore). All organic solvents were analytical grade and obtained from Thermo Fisher (Illkirch, France).

Synthesis of Modified Dextran Polymers

To synthesize Dex bearing Bz side units (Dex-Bz), 4.0 g of Dex was solubilized in 100 mL of anhydrous dimethylformamide containing 1.0 g of lithium chloride. Then, 0.46 g of 4-benzoylbenzoic acid and 31 μL of pyridine were added to the Dex solution. The reaction was carried out at 80°C for 3 h. The obtained Dex-Bz conjugate was isolated by precipitation in isopropyl alcohol. Then, Dex-Bz conjugate was further solubilized in distilled water, extensively purified by dialysis to remove unreacted Bz and finally freeze-dried overnight.

Dex bearing adamantane (Ad) side units (Dex-Ad) was obtained in a similar manner, by reacting Dex with 1-adamantanecarbonyl chloride as previously reported.[11,15] Briefly, 1.0 g of lithium chloride was dissolved in anhydrous N,N-Dimethylformamide by heating at 80°C. To this mixture, 4.0 g of Dex was added, followed by 0.5 g of 4-(dimethylamino)pyridine, 0.42 g of 1-adamantanecarbonyl chloride, and 30 μL of pyridine. The mixture was stirred for 3 h at 80°C followed by 15 h at room temperature. The polymer was then isolated by precipitation in 2-propanol. It was purified by dialysis and finally freeze-dried.

Dex-Ad and Dex-Bz were characterized by ^{1}H-NMR spectroscopy in D_2O using a Varian VXR-600 at 400 MHz and their average molar mass was determined by size exclusion chromatography (SEC) using pullulan standards. SEC studies were conducted using multiangle light scattering and refractive index detectors (SEC/multiangle light scattering/refractive index; Wyatt Technology, Santa Barbara, CA) with TSK6000PW/TSK2500PW columns, and ammonium acetate-acetic acid pH 4.5 as the eluent at a flow rate of 0.5 mL/min, using dn/dc of Dex = 0.147 mL/g. After Bz grafting, the average molar mass was shifted from 37,700 ± 400 (native Dex) to 43,400 ± 4300 g/mol (Dex-Bz), indicating the successful grafting reaction. ^{1}H NMR enabled to determine the substitution yield, showing that 5% of the Dex glucose units were effectively grafted with Bz or Ad.

Synthesis of Poly-β-CD Polymers (Poly-CD)

Poly-CD was prepared by poly-condensation of β-CD with epichlorohydrin (EP) under alkaline conditions as previously described.[6,14] Briefly, 100 g of anhydrous β-CD was dissolved in 160 mL sodium hydroxide, 33% wt/wt solution under magnetic stirring overnight. Then EP (molar ratio β-CD/EP = 10) was rapidly added to the solution heated at 30°C. The reaction was stopped in the proximity of the gelation point by adding acetone. The obtained aqueous phase was then heated at 50°C overnight. The reaction mixture was neutralized with hydrochloric acid (6 N) and ultrafiltered using membranes with a cut-off of 100,000 g/mol. The β-CD polymer was finally recovered by freeze-drying.

The β-CD content was 77% (wt/wt) as determined by ^{1}H-NMR spectroscopy analysis. Polymer average molar mass was 2.1×10^5 g/mol according to SEC measurements using pullulan standards.

Nanoparticle Preparation

The NPs of poly-CD and Dex-Bz polymers, namely poly-CD/Dex-Bz NPs, were prepared by a microfluidic technique (NanoAssemblr™ Benchtop Instrument; Precision NanoSystems, Ltd., Vancouver, BC). The mixing chamber (Fig. 1) has channels of around 100 μm. Aqueous solutions of poly-CD (5 μg/mL to 1 mg/mL) and Dex-Bz (5 μg/mL to 1 mg/mL) were obtained by dissolving the 2 polymers in MilliQ water at room temperature for 12 h. Then, the poly-CD and Dex-Bz solutions were mixed in the microfluidic mixing chamber at different volume ratios at a flow rate of 1 mL/min. The start waste and end waste indicated in the NanoAssemblr™ software were set at 0.25 and 0.05 mL, respectively. These volumes correspond to the non-steady state regime of NP formation. The NPs formed in these conditions were not further recovered for characterization purposes.

As a control, poly-CD/Dex-Bz NPs were prepared by mixing the 2 polymer solutions using a pipette at room temperature using the same experimental conditions (volume ratios and polymer concentrations).

Nanoparticle Characterization

Size Distribution and Stability

The mean diameter and the size distribution of poly-CD/Dex-Bz NPs were determined at 25°C by dynamic light scattering (DLS) at 90° angle using Zetasizer ZS 90 Instrument (Malvern Instruments Ltd., Orsay, France). Experiments were performed in triplicate. DLS enabled studying the stability of the NPs during storage at room temperature.

In Situ *Kinetic Study of NP Formation*

The kinetics of NP formations were studied *in situ* by means of a Cordouan Vasco Flex particle analyzer (Cordouan Technologies Ltd., Pessac, France) equipped with an *in situ* head detector. Samples of 100 μL of Dex-Bz solution (0.5 mg/mL) were added to 1 mL of poly-CD solution (0.5 mg/mL) every 2 min until a 1:1 volume ratio between Dex-Bz and poly-CD was reached. Size analysis of the forming NPs were performed immediately after each addition of the Dex-Bz solution to the poly-CD one.

Vasco Flex allows non-intrusive measurements of NP size distribution in indicative size range of 0.5 nm to 10 μm, and a concentration range of 10^{-5} to 5%-10% (vol/vol). The *in situ* head detects the scattered light at an angle of 170°. Size distributions were determined using the NanoQ 2.5 software with Pade-Laplace, Cumulant, and SBL inversion algorithms.

Individual Particle Tracking and NP Concentration Measurements by NanoSight

The NP concentration and stability upon dilution were investigated by NanoSight analysis (NanoSight LM10 Instrument; Malvern Instruments Ltd.), combining a conventional optical microscope with a laser to illuminate the NPs in Brownian motion in a defined volume.[16] Of main interest here, NPs could be visualized one by one,