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On the intracellular release mechanism of hydrophobic cargo and its relation to the biodegradation behavior of mesoporous silica nanocarriers

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ABSTRACT

The intracellular release mechanism of hydrophobic molecules from surface-functionalized mesoporous silica nanoparticles was studied in relation to the biodegradation behavior of the nanocarrier, with the purpose of determining the dominant release mechanism for the studied drug delivery system. To be able to follow the real-time intracellular release, a hydrophobic fluorescent dye was used as model drug molecule. The *in vitro* release of the dye was investigated under varying conditions in terms of pH, polarity, protein and lipid content, presence of hydrophobic structures and ultimately, in live cancer cells. Results of investigating the drug delivery system show that the degradation and drug release mechanisms display a clear interdependency in simple aqueous solvents. In pure aqueous media, the cargo release was primarily dependent on the degradation of the nanocarrier, while in complex media, mimicking intracellular conditions, the physicochemical properties of the cargo molecule itself and its interaction with the carrier and/or surrounding media were found to be the main release-governing factors. Since the material degradation was retarded upon loading with hydrophobic guest molecules, the cargo could be efficiently delivered into live cancer cells and released intracellularly without pronounced premature release under extracellular conditions. From a rational design point of view, pinpointing the interdependency between these two processes can be of paramount importance considering future applications and fundamental understanding of the drug delivery system.

1. Introduction

Drug delivery systems (DDS) aim to protect drug molecules from degradation, minimize harmful side-effects and increase the accumulation of the drug at the disease site (Saad, 2008). An ideal drug delivery carrier would further biodegrade and be excreted within a reasonable time course after the drug has been released, with no risk of cellular or organ accumulation. Among all different kinds of materials studied for their suitability as DDS, inorganic materials have during the last two decades gained increased attention, among others, due to their superior mechanical and chemical stability and flexible design strategies (Arruebo, 2012; Sekhon and Kamboj, 2010a, 2010b). Inorganic porous matrices can readily serve as protective reservoirs for a high load of poorly soluble drugs (Saad, 2008), the release of which can be controlled (Arruebo, 2012; Coll et al., 2013; He and Shi, 2011) employing the versatility of organic functionalization to produce hybrid materials (Sekhon and Kamboj, 2010a). Especially silica (SiO_2) has already quite long been studied as DDS due to its high available porosity and biocompatibility (Barbé et al., 2004), and synthetic advances have pushed especially mesoporous silica nanoparticles (MSN) to the forefront of drug delivery nanosystems during the 2000s (Desai et al., 2014; Simovic et al., 2011; Zhang and Rosenholm, 2015; Zhang et al., 2012). Amorphous silica is known to dissolve in aqueous solvents in a pH-dependent manner (Iler, 1979), thus rendering the material biodegradable and essentially biocompatible; being classified by the FDA as "generally regarded as safe" (GRAS) (Coll et al., 2013; Jaganathan and Godin, 2012; Yu et al., 2011). Quite surprisingly, mesoporous silica has in many previous publications been regarded as inert (non-degrading in the physiological environment), supported by observations where silica concentrations considerably above the water solubility limit of silica have been used. Regrettably, this naturally leads to erroneous predictions regarding their biobehavior, *i.e.* under actual application conditions. Various sophisticated release mechanisms have been built into the carrier system (Alberti et al., 2015; Ambrogio et al., 2011; Colilla et al., 2013; Park et al., 2009; Vallet-Regí, 2012; Yang et al., 2012; Zhang et al., 2014), from which the release is triggered by changes in pH, temperature, magnetic field or redox potential; or by exposure to light, ions, enzymes and other bio-stimuli. These triggers often rely on capping agents that keep the pores closed, but the translation to *in vivo* conditions becomes cumbersome when considering the fact that the silica matrix itself

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dissolves rapidly in an environment where a large volume of water is present (higher than the solubility limit of silica in water at pH 7, 120 ppm) (Krauskopf, 1956) and that surface functions, attaching the capping agents to the particles, are prone to detach if the material itself dissolves even though the bonds inherently are stable. Even for simple diffusion-driven release, the mechanisms have generally been established for inert matrices (Higuchi, 1963), whereby the release mechanism would be distorted upon dissolution of the matrix, depending on the dissolution mechanism (surface or bulk erosion). Thus, these two events should be highly interconnected, and we here aim at establishing not only the drug release mechanism, but also its interdependency on the degradation rate of the carrier matrix. In general, water-soluble drugs that are incorporated into a porous matrix are mainly released by diffusion, whereas for the release of poorly water-soluble drugs, self-erosion of the matrix would be the more prominent mechanism (Costa and Sousa Lobo, 2001). An increasing number of studies with regard to dissolution mechanisms of pure siliceous MSN are becoming available (Braun et al., 2016; He et al., 2010; Lin et al., 2012; Yamada et al., 2012). Our own previous investigations (Li, 2010) suggest that the dissolution mechanism will further largely differ depending on surface functionalization (Cauda et al., 2010a, 2010b; Chen et al., 2012), type of drug and loading degree, thermal history, surfactant extraction method, likely also particle size when working on the nanoscale, and inherently, pH. Furthermore, core@shell designs employing silica coatings have indicated that the degradation mechanism for porous silica shells on the nanoscale obey different rules than silica nanoparticles (He et al., 2010, Chen et al., 2012). Accumulated data (Chen et al., 2012; He et al., 2010) points to a mechanism where the pore structure is initially enlarged, still preserving the particle morphology (bulk erosion). This would be an ideal scenario for a circulating DDS, as size, one of its key properties determining biodistribution and pharmacokinetics, remains unchanged (Li and Huang, 2008).

We have to date shown that our developed MSN platform can be functionalized for cancer cell specific internalization and is suitable for targeted delivery of hydrophobic compounds including commercially available anticancer drugs (Mamaeva et al., 2011; Niemelä et al., 2015; Senthilkumar et al., 2015; Wittig et al., 2014), for which the release of cargo has been mainly confined to the intracellular environment (Lu et al., 2007). *In vivo* we have shown that the carrier can be intravenously, locally and orally administered, accumulates in tumor tissue, lacks cytotoxic side-effects, is biodegradable and most likely eliminated through renal excretion after intravenous administration (Mamaeva et al., 2011). Furthermore, the drug-loaded particles have exhibited higher therapeutic efficacy than corresponding amounts of free drug (Desai et al., 2016; Mamaeva et al., 2011). In our abovementioned studies, we have been able to indirectly establish that no premature leakage of drug takes place and that the drug is primarily intracellularly released. Though this is the optimal scenario from an *in vivo* drug delivery point of view for intracellular drug targets, knowledge around fundamental mechanisms related to drug release is still lacking.

Therefore, we now seek to determine the decisive parameters for the drug release mechanism, which from a rational design point of view would be of paramount importance, considering both assessment and potential improvement of already existing MSN-based DDS; but also for development of novel designs and applications. Thus, we will hereby investigate the sought-for drug release mechanism by studying the MSN system, empty or loaded with hydrophobic cargo, in different environments under *in vitro* conditions; in one- and two-phase systems as well as by mimicking intracellular conditions in terms of pH and presence of proteins and lipids. Silica dissolution will be quantitatively determined by spectrophotometric means, and the drug release kinetics will be evaluated for the same MSN. These will be further correlated to establish the underlying mechanisms of cargo release for the rational design of MSN-based DDS. The intracellular uptake of MSN will finally be verified, along with the real-time drug release being studied by FRAP (fluorescence recovery after photobleaching) in live cells.

2. Materials and methods

2.1. Preparation of mesoporous silica nanoparticles

The preparation of amino-functionalized mesoporous silica nanoparticles (MSNs) was carried out according to protocols described in previous publications (Desai et al., 2016; Nakamura et al., 2007; Rosenholm et al., 2009a). In a typical synthesis, co-condensation of 3-aminopropyltrimethtoxysilane (APTMS; Sigma-Aldrich) (10 mol%), together with tetramethoxy orthosilicate (TMOS; ≥98%, Fluka), was performed by first mixing the silica-sources and then adding them to an alkaline (sodium hydroxide solution; ≥98%, Sigma-Aldrich) water/methanol solution, containing hexadecyltrimethylammonium chloride (CTACl; 25 wt.% in water; Sigma-Aldrich) as a structure-directing agent. The sol was stirred over night at room temperature (RT), after which the particles were separated by centrifugation and extracted three times with acidic (hydrochloric acid, HCl; 37–38%, Bruker) ethanol and dried under vacuum at RT. The particle surface was further conjugated with polyethyleneimine, PEI, which was grown onto the silica surface by hyperbranching surface polymerization of aziridine (98%, Menadiona, Spain) (Desai et al., 2016). In a typical conjugation procedure 100 mg particles were suspended in anhydrous toluene (purity 99.8%, Sigma-Aldrich) and kept under inert atmosphere. A catalytic amount of concentrated acetic acid (5.5 μl), along with 55 μl aziridine was added while stirring vigorously. The suspension was refluxed over night at 348 K. The particles were then separated by filtration, washed with toluene and vacuum dried at 75 °C. 5 wt.% of the hydrophobic dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI or $DiIC_{18}$(3) (Molecular Probes®, Invitrogen)) or the gamma(γ)-secretase inhibitor (GSI) drug *N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT) (≥98.5%, Sigma-Aldrich) was physicochemically adsorbed into the particles' pores for the purpose of mimicking (DiI) or serving as (DAPT) a hydrophobic drug, respectively.

2.2. Characterization methods

Dynamic light-scattering (DLS) and zeta potential (ZP) measurements were performed using a ZetaSizer NanoZS® (Malvern, Worcestershire, UK) setup in 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer at pH 7.2. Measurements were performed at 25 °C, using a monochromatic laser with a working wavelength of 632.8 nm, using non-invasive back-scatter (NIBS) with the detector positioned at 173° relative to the laser beam. Scanning electron microscopy (SEM) was performed with a LEO Gemini 1530® (Zeiss, Oberkochen, Germany) with a Thermo Scientific UltraDry Silicon Drift Detector (SDD) to verify the particle size, size distribution and morphology of the particles. The instrument was equipped with a SE (secondary electron), BSE (backscattered electron) and in-lens detector. The aperture was kept at vacuum during analysis. The samples were dropped as acetone suspensions onto double sided copper tape, allowed to dry, and subsequently sputtered with carbon and mounted onto a sample holder. Transmission electron microscopy using the bright-field imaging mode (TECNAI-20® operating at 200 kV, Philips, Eindhoven, The Netherlands and JEM-1400Plus operating at 120 kV, JEOL Ltd., Tokyo, Japan) was used to confirm a mesoporous structure. The TEM samples were prepared by casting a drop of the nanoparticle suspension (acetone) onto copper grids covered with carbon films and letting them dry. The mesoscopic ordering of the pores was determined by X-ray powder diffraction using a Kratky compact small-angle system (Hecus Braun Graz, Graz, Austria). The system was equipped with a position-sensitive detector consisting of 1024 channels with a width of 55.5 mm. A Seifert ID-300 X-ray generator, with the maximum operating intensity of 50 kV and 40 mA provided the Cu K_α radiation at the wavelength, $\lambda = 1.542$ Å. The sample-to-detector distance was 277 mm. The sample holder was kept under vacuum during the