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Intracellular localization and dynamics of Hypericin loaded PLLA nanocarriers by image correlation spectroscopy

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ABSTRACT

The study of cell-nanoparticle interactions is an important aspect for understanding drug delivery using nanocarriers. In this regard, advances in fluorescence based microscopy are useful for the investigation of temporal and spatial behavior of nanoparticles (NPs) within the intracellular environment. In this work, we focus on the delivery of the naturally-occurring hydrophobic photosensitizer Hypericin in human lung carcinoma A549 cells by using biodegradable poly L-lactic acid NPs. For the first time, Hypericin containing NPs are prepared by combining the miniemulsion technique with the solvent evaporation method. This approach yields an efficient loading of the NPs with Hypericin and allows for additional cargo molecules. To monitor the release of Hypercin from the NPs, an additional fluorescent lipophilic dye Coumarin-6 is incorporated in the NPs. Temporal and spatiotemporal image correlation spectroscopy is used to determine the fate of the NPs carrying the potential cargo. Both directed and non-directed motions are detected. By using image cross-correlation spectroscopy and specific fluorescent labeling of endosomes, lysosomes and mitochondria, the dynamics of the cargo loaded NPs in association with the organelles is studied.

1. Introduction

Engineered nanoparticles (NPs) create a coherent interest to develop nanomaterials for biomedical applications such as imaging, diagnostics and therapeutics [1,2]. Theranostic nanomedicine takes advantage of the high capacity of nanosystems to carry loads like imaging and therapeutic agents [3]. The theranostic nanocarriers capable of diagnosis and therapy have the potential to revolutionize the drug development process and change the landscape of the pharmaceutical industry [1–4]. They can travel through complex biological environments full of steric and adhesive obstacles and increase the selective drug delivery [4]. However, the therapeutic efficacy and intracellular behavior of NPs depend primarily on how they interface to biomolecules and their surroundings [5–7]. NPs interact not only passively with cells but also actively modulate the molecular processes that are essential for regulating cell functions [5,8]. These perturbed activities may or may not, late in time, reveal to be stressful for the cells. Understanding NP-biointeractions requires knowledge about the dynamic behavior of nanomaterials during their cellular uptake, intracellular traffic, mutual reactions with cell organelles, fate of nanomaterials and respective cellular end points [6–15]. The nanocarrier pharmocology at the microscale can shed light on the uptake mechanisms and intracellular trafficking of the nanocarriers [16]. As intracellular location and interactions of NPs correlate with their characteristics [17,18], every (new) nanomaterial has to be tested separately [6,8]. In this regard, fluorescence based techniques, proteomic mass spectrometry and pH-reporting (in the physiological range of 5.0–7.4) nanosensors provide insights on the NP uptake mechanisms and intracellular trafficking [19–27]. Recently, combination of complimentary techniques such as mass spectrometry and imaging analysis has been used to shed valuable insight on the intracellular trafficking of NPs [19,20].

Photodynamic therapy and diagnosis (PDT and PDD) are currently undergoing intensive clinical investigations as adjuvant treatment for oncological diseases [28–32]. PDT is based on the use of photochemical reactions mediated through an interaction between a photosensitizer, photoexcitation at a characteristic wavelength of light and production of reactive oxygen species (ROS) [28–30,33]. Studies have also shown that PDD enhances the visual contrast between benign and malignant tissue because of selective emission of fluorescence from the cancer cells [31,32]. Hypericin is a naturally-occurring, potent photosensitizer with several advantages over common photosensitizers, such as a broad excitation spectrum in the visible region, a high quantum yield of fluorescence and singlet oxygen generation upon photoexcitation, good photostability and a relatively low dark toxicity [28–30]. However,

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bioavailability is limited by its hydrophobic nature [28–30]. The efficacy of poly L-lactic acid NPs to carry Hypericin (henceforth PLLA-Hyp NPs) for the benefit of improving PDD and PDT of ovarian cancer has been reported [30]. PLLA has been approved by U.S. Food and Drug Administration (FDA) for clinical use [34] due to its unique properties such as established safety, and biocompatibility suitable for drug delivery applications [17,18]. PLLA being an ester can be degraded in an acidic environment as well as by enzymes such as esterases. The various cellular components maintain their own characteristic pH values ranging from 4.5 in the lysosome to about 8 in the mitochondria [8,21,22]. Therefore, the inherent pH conditions in addition to the presence of enzymes have influence on the degradation of the PLLA-Hyp NPs within the cells. Some studies have shown that PLLA-Hyp NPs exhibit cancer selective targeting, enhanced reduction of cancer cell viability together with no significant reduction in viability of normal cells [17,18,30,35,36].

Although PLLA NPs have been previously well investigated for the medical applications, more attention has to be devoted specifically to understand their intracellular dynamics [8,37,38]. The goal of the present study is to investigate the intracellular dynamics and localization of PLLA-Hyp NPs in living human lung carcinoma A549 cells. To the best of our knowledge, no other attempts to study this aspect have been reported and for the first time different variants of image correlation spectroscopy (ICS) [24,26,39] will be used for this NP platform.

ICS is a fluorescence based microscopic technique most appropriate for the study of biomolecular interactions and the measurement of molecular diffusion and directed transport (flow) on time scales ranging from microseconds to milliseconds [23,24,26,40]. Whereas fluorescence correlation spectroscopy (FCS) is based on the temporal fluctuations at a single spot, ICS also utilizes the spatial information within a significant, homogeneous area of the specimen [24,25,39,41,42]. ICS allows for relatively slow intracellular dynamics as compared to FCS [24,43], and to determine the flow and diffusion of small particles at a higher density as compared to single particle tracking (SPT) [19]. ICS implementations are subdivided according to whether fluorescence fluctuation information in space and/or time is analyzed within the image series. Temporal ICS (TICS) involves the correlation analysis of the fluorescence fluctuations in time recorded in the pixels of an image time series allowing for the determination of diffusion related processes [10,12,39,41]. ICS extended for spatiotemporal image correlation spectroscopy (STICS) correlates fluorescence fluctuations in space and time. STICS depends on both diffusion and flow directions of fluorescently tagged macromolecules or particles enabling the mapping of the velocity vectors [25,44,45].

The intracellular trafficking of NPs and their association with cell organelles is essential for understanding the dynamics of the intracellular drug delivery process. Therefore, the intracellular motions of PLLA-Hyp NPs and released Hypericin in association with individual early endosomes, late endosomes, lysosomes, mitochondria and nucleus contents are explored. To assess the dynamics of the associated motions of the loaded NPs and organelles, STICS is used via cross-correlation analyses (STICCS) of two fluorescence detection channels [25,27].

The fluorescence lifetime is highly dependent on the surrounding microenvironment and can therefore report on local interactions of the fluorophores [46]. The fluorescence lifetime is independent of concentration and artifacts such as photobleaching. In fluorescence lifetime imaging microscopy (FLIM) a map of the fluorescence lifetime over space is obtained [47]. FLIM experiments have been reported for Hypericin in pharmaceutical preparations [46]. Although the aim of the present study is not to elaborate on the phototoxicity of the Hypericin itself but to study the dynamics of the NPs carrying the Hypericin, we use FLIM in this work as a tool to monitor Hypericin release from the NPs.

To monitor the degradation of the PLLA-Hyp NPs and subsequent release of Hypericin, a suitable control dye that can be co-localized at the beginning of the observations is required. To achieve the aforementioned goal, a careful design of nanocarrier with mono- and dual-labeling is crucial; here dual-labeled particles allow for the co-localization of Hypericin and the control dye. Previously, PLLA-Hyp particles were prepared by nanoprecipitation method, where, despite small Hypericin loading, low entrapment efficiency was reported [30]. For the present study, sufficiently high loading of dyes with decent encapsulation efficiency and homogeneous distribution of dye in the particle dispersion (especially considering dual-labeling options) are required. Therefore, to overcome the aforementioned challenges, in this work labeled polymer particles are prepared by combining the versatile miniemulsion technique with the emulsion/solvent evaporation method [48–50]. This procedure allows for reliable entrapment of the different hydrophobic dyes in case of both mono- and dual-labeled particles. It is worth to note that such a dual-labeled NP design containing therapeutic Hypericin has not been envisaged before. In this work, the green-fluorescent lipophilic dye Coumarin-6 was chosen as a control. Although, this dye has been used as a drug model before [48–50], in here, the inclusion of the dye is only to mark the onset of the Hypericin release as it can be discriminated spectrally from Hypericin.

As lung cancer is the leading cause of cancer-related mortality worldwide and patients with this cancer have a poor five-year survival rate less than 20% [51], the human lung cancer carcinoma A549 cell line was employed here. The latter serves as a good model cell line, as translational research can be relied upon for the diagnostic and therapy of lung cancer [51,52]. Moreover, as A549 cells are adherent and flat, they are suitable cells for imaging [52].

2. Materials and methods

2.1. Materials

The anionic surfactant sodium dodecyl sulfate (SDS) was purchased from Merck. PLLA (Mw = 101,700 g/mol, inherent viscosity: 1.0 dl/g) was delivered by Sigma-Aldrich. Chloroform ($CHCl_3$) was bought from AnalaR NORMAPUR. Hypericin was obtained from HWI analytic GMBH pharma solutions and Coumarin-6 from Sigma-Aldrich. Polyvinylpyrrolidone (PVP, Mw = 40,000 g/mol) was delivered by Sigma-Aldrich Corporation. The Amicon ultra filter membrane tubes, which are used for washing the NPs, were acquired from Millipore. Milli-Q water was used for the sample preparation. The transmission electron microscopy (TEM) grids were bought from Electron Microscopy Science.

For the cell preparation, eagle's medium with GlutaMAX and penicillin/streptomycin were purchased from Gibco, Paisley, UK. Fetal bovine serum (FBS) was obtained from Biochrom AG, Germany. μ-Slide 8 well petridishes were bought from Ibidi GmbH, Martinsried, Germany. Organelle specific dyes, early endosomes (Rab5a-GFP) and late endosomes (Rab7a-GFP) (CellLight® Reagents, BacMam 2.0), LysoTracker® Green DND-22 and MitoTracker® Green FM were purchased from molecular probes. Hoechst 33442 and nocodazole were delivered by Sigma-Aldrich Corporation.

2.2. NPs characterization methods

The solid content of the samples was determined thermogravimetrically. The size and size distribution of all NPs were characterized by dynamic light scattering (DLS) using a Brookhaven Instruments ZetaPALS. The morphology of the NPs was imaged using a TECNAI spirit TEM of FEI operating at an accelerating voltage of 120 kV. The sample was prepared by air drying the diluted sample in water on a carbon-coated copper grid. The absorption spectra of the NPs were measured using the Agilent Cary500 Scan UV–Vis-NIR spectrophotometer. The emission spectrum of the NPs was obtained using the Horiba-JobinYvon FluoroLog-3 spectrofluorometer. The optical spectra were measured from the NP dispersions of known solid content in order to determine the amount of encapsulated dye (see Figs. S1 and S2, Tables S1 and S2 in Supplementary Material).