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Research Paper

Multimodal non-linear optical imaging for the investigation of drug nano-/microcrystal–cell interactions

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ABSTRACT

Drug nano-/microcrystals are being used for sustained parenteral drug release, but safety and efficacy concerns persist as the knowledge of the *in vivo* fate of long-living particulates is limited. There is a need for techniques enabling the visualization of drug nano-/microcrystals in biological matrices. The aim of this work was to explore the potential of coherent anti-Stokes Raman scattering (CARS) microscopy, supported by other non-linear optical methods, as an emerging tool for the investigation of cellular and tissue interactions of unlabeled and non-fluorescent nano-/microcrystals. Raman and CARS spectra of the prodrug paliperidone palmitate (PP), paliperidone (PAL) and several suspension stabilizers were recorded. PP nano-/microcrystals were incubated with RAW 264.7 macrophages *in vitro* and their cellular disposition was investigated using a fully-integrated multimodal non-linear optical imaging platform. Suitable anti-Stokes shifts (CH stretching) were identified for selective CARS imaging. CARS microscopy was successfully applied for the selective three-dimensional, non-perturbative and real-time imaging of unlabeled PP nano-/microcrystals having dimensions larger than the optical lateral resolution of approximately 400 nm, in relation to the cellular framework in cell cultures and *ex vivo* in histological sections. In conclusion, CARS microscopy enables the non-invasive and label-free imaging of (sub) micron-sized (pro-)drug crystals in complex biological matrices and could provide vital information on poorly understood nano-/microcrystal–cell interactions in future.

1. Introduction

Intense nanotechnology-based drug delivery research over the last two decades has resulted in a variety of vectors available for the systemic or targeted release of therapeutic molecules [1,2]. In this context, drug nanocrystals consist of pure crystalline drug particles, typically having sizes between 100 and 1000 nm, onto which small amounts of biocompatible stabilizers (hydrophilic polymers

Abbreviations: λ_p, wavelength of the pump/probe laser beam; λ_s, wavelength of the Stokes laser beam; ω_p, vibrational frequency of the pump/probe beam; ω_s, vibrational frequency of the Stokes beam; BF, bright field; CARS, coherent anti-Stokes Raman scattering; DMEM, Dulbecco's modified Eagle medium; E-, epi-directed, backward; EDTA, ethylenediaminetetraacetic acid disodium salt dehydrate; F-, forward-directed; FWHM, full width at half maximum lateral resolution; H&E, hematoxylin and eosin; HBSS, Hank's balanced salt solution; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HIFBS, heat-inactivated fetal bovine serum; HPMC, hydroxypropyl methylcellulose; i.m., intramuscular(ly); IR, infrared; I.U., international unit; LAI, long-acting injectable; L-Glu, L-glutamine; N.A., numerical aperture; NEAA, non-essential amino acids; NIR, near infrared; OPO, optical parametric oscillator; ORO, Oil Red O; P338, poloxamer 338, Kolliphor® P 338; PAL, paliperidone; PBS, phosphate buffered saline; PEG, polyethylene glycol; PEST, penicillin G and streptomycin; PFA, paraformaldehyde; PLM, polarized light microscopy; PMT, photomultiplier tube; PP, paliperidone palmitate; PP-LAI, paliperidone palmitate long-acting injectable suspension, Xeplion®; PP-NC, paliperidone palmitate nano-/microcrystals; RAW 264.7, Abelson murine leukemia virus transformed monocytes/macrophages; ROI, region of interest; SHG, second harmonic generation; THP-1, human-derived acute monocytic leukemia monocytes/macrophages; TPEF, two-photon excited fluorescence; TPGS, D-α-tocopheryl polyethylene glycol 1000 succinate; TW20, polysorbate 20, Tween® 20.

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and/or surfactants) are adsorbed [3]. Their high surface area-to-mass ratio and high curvature increase the dissolution rate, thereby driving the drug release [4]. As a result, drug nanocrystals have been successfully implemented in the pharmaceutical arena and have evolved into an established formulation strategy for the oral bioavailability enhancement and systemic delivery of poorly water-soluble drugs [4]. The ability to tailor the drug dissolution rate also recently led to the development of nanosuspensions for sustained (*i.e.* several weeks or months) parenteral drug delivery [5–11]. By balancing the drug particle size distribution on one hand and the compound solubility and partition coefficient on the other hand, the latter of which can be modified by salt formation or prodrug derivatization, it is possible to achieve controlled dissolution rates that enable prolonged therapeutic plasma concentrations [12–14]. Despite the successful development of nanosuspensions, safety and efficacy concerns persist as the knowledge of the *in vivo* fate of nano- and microparticles remains limited [15,16].

Drug nano-/microcrystals have been shown to accumulate *in vivo* in cells of the mononuclear phagocyte system [17,18]. Unfortunately, *in vitro* data on nano-/microcrystal uptake and tolerability in cell cultures remain elusive, which is in contrast to the extensive knowledge on polymeric nanoparticles (<100 nm) [2,19]. Particle–cell interactions are not only paramount in relation to safety, but they are also capable of drastically affecting the drug release or the (pro)drug stability. The nano-/microcrystal–macrophage interactions and intracellular sequestration are especially relevant in case of injectable extended-release drug nano-/microcrystals, where prolonged interactions with the biological matrix reacting to the insertion of foreign particulate material are occurring. Recently, a rigorous histopathological evaluation of the administration sites and pharmacokinetic analysis following the intramuscular (i.m.) administration of extended-release paliperidone palmitate (PP) nano-/microcrystals in rats revealed a subclinical foreign body granulomatous reaction with extensive PP nano-/microcrystal accumulation within macrophages [18]. Similar observations in terms of inflammatory response were made after i.m. administration of two other long-acting injectable suspensions [20]. In the case of PP nano-/microcrystals, the complex *in vivo* disposition resulted in multiphasic systemic absorption of the active moiety paliperidone (PAL) [18]. Atypical pharmacokinetics of PAL have also been observed in humans; however, mechanistic explanations are still missing [7]. Modified drug release kinetics has also been attributed to the entrapment in liver Kupffer cells after intravenous administration of itraconazole nanocrystals in rats [17]. These findings highlight the interplay between formulation parameters and dynamic physiological variables. As the factors influencing particle uptake are multiple, it is imperative to systematically investigate the fate of these nanocrystal-based formulations, either *in vitro* using suitable cell models, or *in situ* through the examination of histological sections. This can only be achieved provided that suitable analytical tools are available.

Among conventional optical imaging modalities, confocal laser scanning fluorescence microscopy is one of the most widely implemented imaging methods in cellular biology. In spite of its versatility and high sensitivity, fluorescence microscopy suffers from a few major limitations, which hamper its routine application for the study of drug nano-/microcrystal disposition. Firstly, many pharmaceutical compounds, including the antipsychotic prodrug PP used in the present study, are non-fluorescent, precluding fluorescence-based detection without exogenous labeling. Such fluorescent markers can modify the properties of the molecular species under investigation and potentially perturb the cell function [21]. Furthermore, undesired matrix autofluorescence or non-specific dye adsorption might result in a strong background signal, rendering the visualization of nanoparticles in complex biological matrices challenging. Finally, photobleaching of the fluorophores makes quantitative data interpretation difficult, while the photolytic high laser energies and intrinsic toxicity of many dyes limit live cell imaging applications. Hence, alternative imaging techniques are required in order to visualize non-fluorescent drug nano-/microcrystals in a chemically-resolved manner and in relation to the cellular framework.

Vibrational spectroscopic imaging techniques such as infrared (IR) absorption and spontaneous Raman scattering microscopy offer intrinsic chemical selectivity without artificial labeling. However, IR microscopy has a comparatively poor spatial resolution (2–10 μm) and in biological samples, the interference from the strong water signal limits its applicability to [22]. Spontaneous Raman microscopy, on the other hand, is suitable for imaging in aqueous samples and is highly chemically specific. However, the intrinsically weak Raman scattering signal, can translate into extremely long acquisition times, restricting its use for real-time imaging of cellular systems [23]. In addition, the axial resolution, in particular, is insufficient for visualizing nanoparticles.

Coherent anti-Stokes Raman scattering (CARS) microscopy is a non-linear variant of Raman microscopy that is suitable for rapid, label-free and compound-specific imaging through probing of specific molecular vibrations. It involves at least two laser wavelengths, with the frequency difference between the two (denoted pump beam (ω_s) and Stokes beam (ω_p)) selected to create a beating frequency that matches a specific Raman-active vibrational mode of the target molecule. The excitation fields coherently amplify the specific vibrational mode, hence generating a strong and specific anti-Stokes signal upon illumination with a probe beam (ω_p). For more background and detailed information on the optical principles behind CARS microscopy, the reader is referred to some excellent reviews [21,22,24]. The non-linear nature of the CARS process comes with a range of advantages over conventional vibrational microscopy, thus fulfilling the stringent requirements for studying nano-/microcrystal–cell interactions. CARS microscopy is non-invasive and offers label-free chemical selectivity, video-rate acquisition, high spatial resolution and an intrinsic axial sectioning capability within a single imaging platform [25,26]. The submicron lateral resolution of CARS microscopy, which is limited in part by the wavelength of the near-infrared pump and Stokes beams, constitutes a clear advantage over IR microscopy. Theoretical aspects and experimental characterization of the spatial resolution of CARS microscopy have been reported by Cheng and co-workers previously [24]. In their work, the authors have shown that adjacent 200 nm polystyrene beads separated by 0.5 μm could be clearly resolved, indicating a lateral resolution of better than 500 nm.

CARS microscopy is nowadays relatively well established for imaging applications in the biological and (bio)medical fields, and in particular for the investigation of the lipid content or metabolism in living cells and tissues [22,27]. In contrast, CARS imaging has only recently gained interest in the pharmaceutical research and development setting and has resulted in several new applications in this field [28]. In the area of lipid-based formulations for example, multiplex CARS microscopy has been used for the imaging of the lipid digestion process and local phase behavior in lipid emulsion droplets containing lipophilic drugs [29]. CARS microscopy has also been successfully applied in the field of solid dosage forms, for example to visualize the three-dimensional drug distribution within and release from various polymer matrices [30–32]. The effect of processing conditions on the theophylline distribution within solid lipid matrices and the subsequent drug solid-state transitions occurring during dissolution have been investigated using CARS microscopy [33,34]. CARS imaging has also been used for the real-time monitoring of intrinsic dissolution experiments, allowing the correlation of the drug dissolution rate

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