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Longitudinal monitoring of skin accumulation of nanocarriers and biologicals with fiber optic near infrared fluorescence spectroscopy (FONIRS)

James I. Griffin [a], Michael J. Benchimol [b], Dmitri Simberg [a,*]

[a] The Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 East Montview Blvd., Aurora, CO 80045, USA
[b] Sonrgy Inc., 10655 Sorrento Valley Rd., San Diego, CA 92121, USA

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ABSTRACT

Systemically injected drug delivery systems distribute into various organs and tissues, including liver, spleen and kidneys. Recent reports pointed out a significant accumulation of systemically injected nanoparticles in the skin. Skin constitutes the largest organ in the body with important immune functions, and accumulation of drug delivery systems could have significant implications for skin toxicity in living subjects. Fiber optic-based near-infrared spectroscopy (FONIRS) setup was developed and tested for measuring of NIR (760 nm excitation) emission spectra in the skin. Ex vivo spectral measurements of NIR fluorescence through the skin showed linear response down to 34 femtomole of dye DiR. Following systemic injection of IRDye 800 labeled 500 kDa dextran, FONIRS detected an immediate and stable accumulation of fluorescence in the skin. Longitudinal monitoring of skin accumulation and elimination of IRDye 800-labeled therapeutic anti-epidermal growth factor antibody (cetuximab) showed significant signal in the skin after the antibody cleared from circulation. Comparison of skin accumulation of DiR labeled, long-circulating PEGylated liposomes with short-circulating non-PEGylated liposomes showed much higher accumulation of PEGylated liposomes that persisted several days after the liposomes cleared from blood. Measurements with FONIRS enabled to estimate skin concentration of liposomes (percent of injected dose per gram). This simple and practical approach can be used to monitor accumulation of drug delivery systems in preclinical and clinical studies.

1. Introduction

Modern pharmaceutical science has been witnessing an exciting renaissance in drug delivery technologies. In particular, engineered nanoparticles, polymers, and biologicals have a promise to greatly improve safety and efficacy of treatment of diseases. At the same time, these compounds have not demonstrated precise selectivity for diseased tissues alone; e.g., tumors, and often distribute throughout the body into healthy tissues and organs [1]. In addition to the main clearance organs (liver, spleen, and kidneys), deposition in other tissues could have a significant impact on safety and toxicity in human patients [2]. For instance, one of the most frequent and peculiar dose limiting toxicities of liposomal doxorubicin is skin toxicity, manifested by severe pain and ulceration in palms, feet, and in areas of pressure (palmar-plantar erythrodysesthesia, *aka* hands and feet syndrome) [3,4]. This phenomenon is likely caused by accumulation of long-circulating liposomes in the dermis and deposition of an anticancer drug [4]. Accumulation in the skin is not limited to liposomes, and recent evidence suggests that gold nanoparticles, silica and quantum dots distribute into the skin following systemic injection [5,6]. Biologicals are also known to accumulate in the skin as exemplified by severe dose-limiting skin toxicity of anti-EGFR antibody cetuximab (Erbitux™) used to treat lung and colorectal cancers [7].

In the light of the above evidence, there is an immense need for non-invasive tools to monitor deposition of xenobiotic drug delivery systems in humans. Of all organs that accumulate nanocarriers, skin is of high interest because it is the largest organ in the body (~16% body weight) and it performs multiple functions including immunity. Skin is easy to monitor and access, and skin color has been historically used to assess accumulation of drugs in the body, for example, blue color (chrysiasis) due to colloidal gold in arthritis, discoloration due to chloroquine, and skin pigmentation due to tetracycline antibiotics [8]. Albeit skin biopsies can be used to measure skin exposure, they are not convenient and cannot sample the entire body. Most nanoparticles except quantum dots and gold [9,10] produce very little if any intrinsic fluorescence or Raman spectra of sufficient intensity to enable measurements in the skin. However, drug delivery systems can be easily tagged with fluorescent or Raman tags [10]. Raman spectroscopy has certain limitations due to low sensitivity and usually only works well for photonic

* Corresponding author.
E-mail address: dmitri.simberg@ucdenver.edu (D. Simberg).

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materials [11]. On the other hand, fluorescence is arguably one of the cheapest and attractive imaging modalities that allows penetrating several millimeters of tissue in the near-infrared (NIR) window [12]. Several commercially-available NIR fluorophores with high quantum yield have been clinically approved or are at different stages of clinical testing (i.e., IRDye 700 conjugated anti-EGFR antibody (clinical trial identifier NCT02422979) and indocyanine for fluorescence guided surgery) [13,14], making fluorescent tagging of drug delivery systems an attractive, clinically viable option.

Several in vivo fluorophore detection systems based on NIR fluorescence spectroscopy, digital imaging, and luminescence have been described [15]. Compared to imaging, spectroscopy is a very attractive modality because of its speed, low cost, and sensitivity [15,16], but its utility for skin monitoring has not been investigated before. Here, we designed a portable fiber optic-based NIR spectrometry setup (FONIRS) and demonstrated feasibility of longitudinal monitoring of skin accumulation of fluorescently tagged drug delivery systems.

2. Materials and methods

2.1. Materials

IRDye 680RD NHS Ester (IRDye 680) and IRDye 800CW NHS Ester (IRDye 800) were ordered from Li-COR (Lincoln, NE, USA). Amino-dextrans 500 kDa and 3 kDa were from Thermo Fisher. Egg phosphatidylcholine and DSPE-PEG 2000 were from Avanti Polar Lipids (Alabaster, AL, USA), DiR (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindotricarbocyanine Iodide) was from Biotium (Hayward, CA, USA). Whatman Nucleopore Track-Etch Membranes (0.2 μm pore size) were from Sigma-Aldrich (St. Louis, MO, USA). Cetuximab (ERBITUX™) was obtained from the University of Colorado Cancer Center pharmacy. Nitrocellulose membrane was from Bio-Rad. Collagenase type IV was from Sigma.

2.2. FONIRS

The system consisted of a Maya 2000 Pro spectrometer with OceanView 1.5.0 software (both Ocean Optics) connected to a custom-built 600 μm-excitation/emission probe via fiber-optic bundle (all from Ocean Optics, San Jose, CA). The system can perform extremely fast measurements of entire spectra with a handheld probe and has the ability to analyze and integrate spectra over a desired range for comparative spectroscopy. The FONIRS system used LED with a peak emission wavelength of 760 nm as an excitation light source (Prizmatix Ltd., Givat-Shmuel, Israel). The in-line BrightLine® filters (Semrock Inc., Rochester NY) were 785 nm short-pass (excitation) and 785 nm long-pass (emission) for 760 nm LED. The emission was collected over a range of 800–840 nm. The reported integrated fluorescence (IF) values are an integration of the spectral intensity over the appropriate range.

2.3. In vitro and ex vivo sensitivity with FONIRS and Li-COR

DiR dye was dissolved in DMSO, and the concentration was quantified with a Thermo Scientific BioMate 3S Spectrophotometer. The dye was then serially diluted in deionized water in a 96-well plate; each well contained 100 μL of the diluted dye solution. Alternative, the dye dilutions were applied as 5 mm spots on a nitrocellulose membrane, and integrated fluorescence emission over the specified wavelength range was measured using FONIRS. In addition, the membranes were scanned with Li-COR Odyssey, and the integrated density of signal in the 800 nm channel, 16-bit, was measured using ImageJ software.

2.4. Preparation of fluorescent dextran, liposomes, and antibody

Dextran was labeled with IRDye 800 as follows. PBS solution of 500 kDa amino dextran (10 mg/mL) was mixed with excess of IRDye800 for 1 h at room temperature, followed by 24 h of mixing at 4 °C. *t*-BuOH was used to precipitate the labeled dextran and to remove the unreacted dye. This precipitation was repeated several times to eliminate the residual non-bound dye. The labeled dextran was resuspended in PBS at 10 mg/mL. PEGylated and non-PEGylated liposomes were prepared with 0.154 mL of 25 mg/mL Egg PC, 0.065 mL of 4 mM DSPE-PEG 2000 (PEG liposomes only), and 0.002 mL of 12.3 mM DiR solutions in chloroform by evaporation-rehydration-vortexing method. The dry lipid cake was resuspended in 50 μL PBS and subjected to three "freeze-thaw" cycles. The solution was brought to 1 mL by adding PBS, and was extruded using an Avastin manual extruder through 200 nm-Whatman Nucleopore Track-Etch Membranes, through at least 15 extrusion cycles. Dialysis was performed with a 1000 kDa Float-A-Lyzer Dialysis Device (Thermo Fisher) into 1 L of PBS 3 times. A Zetasizer Nano (Malvern, UK) was used to confirm the size of the liposomes. To prepare liposomes loaded with dextran IRDye 680, dextran 3 kDa was labeled with the dye and purified as described above, and the liposomes were reconstituted with 10 mg/mL solution of dextran in PBS instead of PBS. The subsequent steps for liposome preparation were the same as above.

Cetuximab was labeled with IRDye800 as follows. One hundred microgram of cetuximab in 50 μL PBS was mixed with 2.2 μL of 3.02 mM IRDye800-NHS in DMSO and the reaction was maintained at 4 °C for 24 h. 100 kDa Amicon Ultra Centrifugal Filters were used to wash the antibody and remove unreacted dye. The labeling density was determined with Nanodrop 2000C (Thermo Scientific) by measuring absorbance at 280 nm (IgG) and 778 nm (IRDye800). The absorbance of the dye at 280 nm was experimentally determined as $0.0265 \times A_{778nm}$ and subtracted from the total absorption A280. The labeling density was found to be ~1 dye per antibody.

2.5. In vivo monitoring of skin fluorescence

The University of Colorado IACUC approved all animal experiments (protocol 103913(11)1D). BALB/c mice (6–8 weeks of age) were anesthetized with ketamine/xylazine (1 mg/0.16 mg per mouse) and the right flank was depilated using Nair Hair Removal Cream. Skin was washed with warm water to prevent irritation from leftover cream. Mice were injected 24 h later through the tail vein with labeled dextran, liposomes, or antibody. Blood was collected via the retro-orbital sinus from each mouse prior to injection and at different times post-injection. Two microliter spots were applied in duplicate on a 0.22 μm nitrocellulose membrane and scanned at 800 nm with Li-COR Odyssey. The spot integrated density of a 16-bit image was measured with ImageJ and plotted as a function of time with Prism (GraphPad, San Diego CA). A non-linear regression curve-fitting algorithm was used to fit the elimination profile into a mono- or bi-exponential decay to determine half-life. Skin fluorescence (integrated fluorescence) was measured with FONIRS as described in 3A in the depilated flank of each injected and non-injected mouse at the same time points as the blood drawing. Three skin scans per time point were taken, and each scan was the average of 2 scans with 800 ms integration time. The signal values at 1 min post-injection were set as 100% of injected fluorescence, and the data were normalized to this value. At different times post-injection, injected and non-injected (control) mice were sacrificed and the organs were isolated into a 12-well plate. Skin around the torso of each mouse was also collected after the final time point. Organs were scanned with Li-COR Odyssey (intensity of 1.0 at both wavelengths (700 nm and 800 nm)). Mean fluorescence of the organs was determined from 16-bit images using ImageJ software by subtracting the background, drawing a ROI around the organs, and using a measure function to determine mean gray value intensity per organ.

2.6. Skin digestion and quantification of fluorescence

To digest the skin, collagenase IV was prepared at 5 mg/mL in 0.1% Tween-20 solution in PBS. Pieces of skin (4 per mouse) were weighed, minced, and placed into Eppendorf tubes. Tissue was mixed with 1:5