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Research paper

Two-photon fluorescence correlation spectroscopy as a tool for measuring molecular diffusion within human skin

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ABSTRACT

There is a need for tools enabling quantitative imaging of biological tissue for pharmaceutical applications. In this study, two-photon fluorescence microscopy (TPM) has been combined with fluorescence correlation spectroscopy (FCS), demonstrating proof-of-principle providing quantitative data of fluorophore concentration and diffusion in human skin. Measurements were performed on excised skin exposed to either rhodamine B (RB) or rhodamine B isothiocyanate (RBITC), chosen based on their similarity in fluorescence yield and molecular weight, but difference in chemical reactivity. The measurements were performed at tissue depths in the range 0 and 20 μm, and the diffusion coefficients at skin depths 5 and 10 μm were found to be significantly different ($P < 0.05$). Overall median values for the diffusion coefficients were found to be 4.0×10^{-13} m^2/s and 2.0×10^{-13} m^2/s for RB and RBITC, respectively. These values correspond to the diffusion of a hard sphere with a volume eight times larger for RBITC compared to RB. This indicates that the RBITC have bound to biomolecules in the skin, and the measured signal is obtained from the RBITC-biomolecule complexes, demonstrating the potential of the TPM–FCS method to track molecular interactions in an intricate biological matrix such as human skin.

1. Introduction

The focus of this paper is to investigate the interactions between complex biological tissue and active compounds in a non-invasive manner. Two-photon fluorescence microscopy (TPM) has proven to be an important pharmaceutical tool for visualising the distribution and penetration of various fluorescent compounds into highly light scattering biological matter, for example, human skin [1–7]. The major limitation, however, is the fact that TPM mainly shows the distribution of fluorophores, and the possibility of obtaining quantitative data is poor. In a previous study, we demonstrated that TPM can be combined with fluorescence correlation spectroscopy (FCS) to non-invasively track the diffusion of sulphorhodamine B (SRB) in human skin and measure the fluorophore concentration with sub-cellar resolution [8]. In the present paper, we apply the same technique to quantitatively track the diffusion of two fluorescent analogues with different chemical reactivity and to demonstrate proof-of-principle of the method for, for example, pharmaceutical applications.

FCS is based on measuring the fluorescence fluctuations occurring from fluorophores diffusing in and out of the focal volume in laser scanning microscopy [9]. Generally, the method is based on one-photon excitation, but the combination with two-photon excitation gives a powerful method for measuring diffusion of fluorophores in biological samples, for example, cells and tissues [10–13]. FCS has demonstrated to be a very sensitive method that works best at low concentrations between sub-nanomolar and micromolar. The high sensitivity at low concentrations also makes the measurements challenging when performed in biological tissue. Thus, only a limited number of studies applying FCS on tissue have been published [8,12].

In the development of topical drug delivery systems, analysis of the cutaneous absorption and distribution of topically applied formulations is fundamental. Mechanistic studies are also critical in order to improve the understanding of the underlying processes governing cutaneous absorption. This is equally important in toxicological studies related to skin exposure of hazardous compounds, for example, contact allergy. Contact allergy is caused by skin exposure to small reactive molecules called haptens, which reacts with skin macromolecules, forming immunogenic complexes triggering the allergic reaction [14]; however, it is not clear, where in the skin, these complexes are formed. Thus, one of the key questions when it comes to pharmacological studies of the human

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skin is to answer how reactive compounds enter the skin barrier and to determine where they react with endogenous biomolecules. Earlier studies using TPM have demonstrated specific localisations of fluorescent haptens in the epidermis. [2,15]. However, these studies were only based on visualising the epidermal distribution of the compounds after topical drug delivery using TPM; thus, it could not be discerned if the formed hapten-protein complexes could diffuse further or if it is only in its unreacted form the diffusion takes place.

The aim of the present study was to demonstrate proof-of-principle applying FCS in combination with TPM for tracing reactive compounds when applied to human skin. Two structural analogues, that is, rhodamine isothiocyanate (RBITC) and rhodamine B (RB), were chosen as fluorescent model compounds, because of their difference in reactivity [16]. It is known that isothiocyanates react with aminoacids and act as skin sensitisers [16,17]. RBITC has earlier been found to be allergenic in experimental studies [18], while RB is unreactive and non-sensitising [19]. The results show that the diffusion of RBITC is substantially slower than for RB in skin, implying that the measured signal is obtained from the RBITC-biomolecule complexes. Thus, the study demonstrates the potential of the TPM–FCS method to non-invasively track molecular interactions in tissue.

2. Materials and methods

2.1. Chemicals

RB (MW: 479 Da) and RBITC (MW: 536 Da) were both purchased from Sigma–Aldrich, Steinheim, Germany. The compounds were dissolved in PBS to a concentration of 100 nM for the skin experiments and 200 nM for the measurements in solution. The choice of concentrations for the skin measurements was found to be crucial in order not to saturate the fluorescence signal.

2.2. Sample preparation for diffusion experiment

Human skin was obtained as leftovers from breast reduction surgery at the Sahlgrenska University Hospital, University of Gothenburg, Gothenburg, Sweden. The skin tissues were collected after surgery, thereafter cut into 1 × 1 cm pieces and stored in −70 °C and used within 2–10 months. Before use, the skin was thawed in room temperature. For each experiment, two skin samples from the same donor were prepared and exposed to either a solution with RBITC or RB, to measure the compounds under similar conditions. A major part of subcutis was removed by a scalpel. The full thickness skin samples were mounted in flow-through diffusion chambers [20]. The acceptor compartments were filled with PBS and the donor compartments filled with either RB or RBITC test solution (100 nM). The diffusion chambers were covered with parafilm and aluminium foil. The acceptor compartments were kept at a constant temperature of 30 °C under constant stirring for 20 h. Then, the samples were thoroughly washed with PBS, dried with Kleenex® and mounted in imaging chambers. The imaging chamber consisted of a cover slip (No. 1.5 from Menzel–Gläser), with a measured thickness of 0.18 mm, and double-coated adhesive tape allowing sufficient space for the sample. Skin from three different donors was used in the experiments.

2.3. Cryosectioning

For one of the donors, the samples were dismounted from the imaging chamber and subject to cryosectioning after the FCS measurements. The samples were cryofixed in Tissue-Tech O.C.T (Sakura Finetek, Zoeterwoude, NL) and placed in −80 °C for no longer than 10 days. The sectioning was performed using a CM1950 cryostate, (Leica, Wetzlar, Germany). The tissue sections, 15 μm thick, were collected and mounted on microscope slides (Menzel–Gläser, Menzel GmbH, Braunschweig, Germany) using cover slips (No. 1.5 from Menzel–Gläser) and Gel Mount aqueous mounting medium (Sigma–Aldrich, Steinheim, Germany). The edges were sealed with clear nail polish.

2.4. Two-photon microscopy

The set up for TPM consisted of a modified inverted Zeiss Axiovert 135 TV microscope, a Bio-Rad MRC600 scan box and external GaAsP photomultiplier tubes (Hamamatsu, Japan), equipped with an emission filter (580/150) matching the 590 nm emission peaks of RBITC and RB. A 63× water immersion objective lens (Zeiss 'C-Apochromat' 63×/1.2 W Corr) with a working distance of 0.28 mm was used. For two-photon excitation, a tunable mode-locked femtosecond pulsed Ti:Sapphire laser (Tsunami, Spectra-Physics, California, USA) with 83 MHz repetition rate was employed. The laser was set to operate at 840 nm. The laser power was controlled by a Pockels cell (Conoptics, Connecticut, USA). Measurements were performed using laser powers in the rage 0.3–2 mW, measured through the objective lens. Generally, the power was increased when measuring deeper into the tissue.

The point spread function, corresponding to the focal volume, was measured by recording the intensity profile from orange fluorescent beads (PS-Speck Microscope Point Source Kit, Molecular Probes) with a diameter of 0.175 ± 0.005 μm and excitation/emission maxima at 540/560 nm. The resolution was determined as the full width at half maximum from the fluorescence signal from the beads and was found to be 0.42 μm in the lateral direction and 2.8 μm in the axial direction, similar to our earlier experiments [8].

The prepared skin samples were mounted in imaging chambers and placed on the microscope. The zero level of the skin was found by imaging the red fluorescence, and the *z*-level with the highest intensity was set as the surface. The single-plane images were acquired using a zoom factor of 1 (*xy*-pixel size 0.3 μm), a scan speed of 4 s per frame and a Kalman filter average of four frames. The focus was changed by using a step-motor sequentially with a step size of 0.5 μm. A *z*-stack could then be processed by the images from different depths.

2.5. FCS data collection

The FCS data was collected using the TPM described above using a ALV-5000 acquisition card (ALV-Laser Vertriebsgesellschaft m.b.H, Germany) for recording and autocorrelating the signal. Measurements were performed at the *z*-levels 0, 5, 10 and 20 μm by parking the laser at a point of interest, either in areas identified as intracellular or extracellular judging from the TPM image at each plane. The fluorescence fluctuations were recorded for 20 × 8 s at each measurement site, and 10 measurements were performed at each *z*-level. During the measurements, care was taken to keep all settings constant. As a control, FCS measurements were also performed in solution.

2.6. Confocal laser scanning microscopy

A confocal laser scanning microscope (Zeiss LSM 510 Meta) was used for imaging of the cryosectioned samples. Excitation was performed using a HeNe-laser (543 nm). A Plan-Neofluar 40×/1.3 Oil DIC objective lens was used. The size of the pinhole was set to correspond to an optical slice of 1.8 μm in the axial direction. The same setup was also employed to perform additional control FCS measurements in solution, but the objective lens was switched to a C-Apochromat water immersion lens (40×/1.2 W corr).