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Biocompatible photoresistant far-red emitting, fluorescent polymer probes, with near-infrared two-photon absorption, for living cell and zebrafish embryo imaging

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ABSTRACT

Exogenous probes with far-red or near-infrared (NIR) two-photon absorption and fluorescence emission are highly desirable for deep tissue imaging while limiting autofluorescence. However, molecular probes exhibiting such properties are often hydrophobic. As an attractive alternative, we synthesized water-soluble polymer probes carrying multiple far-red fluorophores and demonstrated here their potential for live cell and zebrafish embryo imaging. First, at concentrations up to 10 μM, these polymer probes were not cytotoxic. They could efficiently label *living* HeLa cells, T lymphocytes and neurons at an optimal concentration of 0.5 μM. Moreover, they exhibited a high resistance to photobleaching in usual microscopy conditions. In addition, these polymer probes could be successfully used for *in toto* labeling and *in vivo* two-photon microscopy imaging of developing zebrafish embryos, with remarkable properties in terms of biocompatibility, internalization, diffusion, stability and wavelength emission range. The near-infrared two-photon absorption peak at 910 nm is particularly interesting since it does not excite the zebrafish endogenous fluorescence and is likely to enable long-term time-lapse imaging with limited photodamage.

1. Introduction

In the last ten years, the number of commercially available exogenous probes dedicated to fluorescence bio-imaging has known an exponential increase. The most important criteria required by the biologist for the choice of a probe are the biocompatibility, the brightness and the photostability [1]. Other relevant characteristics for bio-imaging are the absorption and emission wavelengths. In fact, probes emitting in the far-red (650–750 nm) and near-infrared (750–950 nm) wavelength range are highly desirable for numerous reasons [2]. First, light absorption/scattering by cells and tissues is very low between 650 and 950 nm, that is called the biological optical window. Second, cell autofluorescence is mainly occurring in the UV and visible wavelength range, although for some cells like human osteoblasts

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and hepatocytes, plant cells or zebrafish embryonic cells, autofluorescence can be detected up to 700 nm, which complicates the optical observations [3]. If, in addition, the probe exhibits high two-photon absorption (TPA) efficiency in the near-infrared range, investigation of cells and cellular functions in deep tissue sections and organs can be achieved by two-photon microscopy (TPM). Moreover, TPM can be used for long-term live cell and deep tissue microscopy imaging with reduced phototoxicity and photobleaching since two-photon excitation is spatially localized [4].

One strategy to get exogenous probes with improved brightness and photostability, consists in using fluorescent *polymer* probes instead of *molecular* ones. Generally, this can be achieved by encapsulating numerous molecular dyes inside biocompatible polymer nanoparticles [5]. It can also be achieved by using intrinsically fluorescent π-conjugated polymers [6], encapsulated inside nanoparticles [7] or formulated as polymer dots (Pdots) which have a much smaller size (5–25 nm) than nanoparticles [8]. An alternative strategy to get fluorescent polymer probes of small size consists in covalently binding dyes along a biocompatible polymer chain [9,10]. However, whatever the chosen synthetic route, very few polymer probes emit in the far-red range.

Recently, some polymer dots have been synthesized from donor-acceptor polymer blends to reach visible absorption range and far-red emission and have been used for *in vivo* tumor imaging [11]. Using the dye-polymer binding strategy, Lucas et al. [12] prepared polylysine (PLL) or poly(dimethylaminoethyl methacrylate) (PDMAEMA) based polymers labeled with Cy5 dye ($\lambda_{max\ Emission} = 660$ nm). These fluorescent cationic polymers were used to complex oligonucleotides and to follow the intracellular dissociation of the polyplexes by confocal imaging. Saad et al. [13] carried out the binding of Cy5.5 dye ($\lambda_{max\ Emission} = 710$ nm) on the functional groups of a PAMAM dendrimer, further used as nanocarrier for *in vivo* drug delivery. However, in these examples, the polymer structure and/or the dye binding were not controlled and none of those polymer probes were used for two-photon imaging.

In a recent article [14], we reported the design and synthesis of well-defined dye-polymer conjugates, with control over both the polymer chain structure (controlled size, low polydispersity, controlled composition and microstructure) and the dye binding (controlled number of dyes per chain). The remarkable structural homogeneity of these polymer probes was expected to lead to very reproducible bio-labeling results. The chosen dye [15] was emitting in the far-red range, possessed a large Stokes shift and, in addition, was expected to be efficiently excited in the two-photon mode. However, as most of the probes that exhibit interesting TPA properties, it was a hydrophobic fluorophore. Our recent experience with hydrophobic TPA dyes (emitting in the visible range) demonstrated that it is indeed possible to get water-soluble and biocompatible dye-polymer conjugates provided that a biocompatible polymer backbone is used and that the number of dyes per chain is controlled [16]. By tuning the nature of the groups along the polymer chain and the number of far-red dyes per chain, we could synthesize fluorescent polymer probes that were water-soluble and exhibited a much higher brightness than the corresponding molecular dye (up to 10 fold).

In this article, we demonstrate the bio-imaging potential of these far-red emitting fluorescent polymer probes first *in cellulo* and then *in toto* using the zebrafish (*Danio rerio*). The latter has been largely validated as a valuable vertebrate model well suited for live imaging. Mainly, the zebrafish embryo exhibits overall tissue transparency and fertilization is external, which enables live imaging at all early embryonic stages. In a first step, we performed qualitative and quantitative cytotoxicity studies of the polymer probes in living cells. Then, we investigated their internalization pathway and extended the study to various human cell lines. We also evaluated the resistance of the probes to photobleaching and showed that they can be advantageously used under two-photon excitation in the near-infrared for live cell imaging. Then, we demonstrated that such polymer probes were non-toxic to zebrafish embryos, efficiently diffused in the embryo depth and labeled sub-cellular structures, thus providing an efficient counterstain of tissues for live two-photon imaging.

2. Materials and methods

2.1. Synthesis of the polymer probes

The detailed synthesis of the fluorescent polymer probes has been described in a previous article [14].

2.2. UV–Visible absorption and fluorescence spectroscopy

UV–Visible spectra were recorded on a Jasco V-670 spectrophotometer at ambient temperature using 1 cm quartz cells. The fluorescence emission spectra were recorded using a Horiba–Jobin Yvon Fluorolog-3® spectrofluorimeter at 298 K, using a 1 cm quartz cells. The steady-state luminescence was excited by unpolarized light from a 450 W xenon CW lamp and detected at right angle (90°) for diluted solutions (DO < 0.1) by a red-sensitive Hamamatsu R928 photomultiplier tube.

2.3. Cell culture

Human HeLa cells used in this study were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin/streptomycin) [17]. Jurkat T cells (Human T cell leukemia cell line) were grown in RPMI-1640 medium (GIBCO) supplemented with glutamax, 10% FCS and antibiotics. The neuroblastoma cell line SH-SY5Y (ATCC # CRL-2266) was grown in a 1:1 mixture of Eagle Minimum essential medium (PAA E15-832) and F12 medium (PAA E15-890) supplemented with glutamax, 10% FCS and antibiotics. For differentiation studies, cells were treated for 4 days with retinoic acid (RA) at 10 μM in regular culture dishes, then transferred to chambered coverslips (μ-Slide 8 well, ibiTreat – 12,500 cells/well) and finally further differentiated with brain derived neurotrophic factor (BDNF) at 0.05 μg/ml for 2–3 days. Prior live cell imaging, the medium was replaced with phenol red free medium to reduce autofluorescence.

In order to label specific cellular compartments, HeLa cells were transfected with plasmids expressing endosomal markers described elsewhere [17,18] 24 h prior to probe incubation.

2.4. Cytotoxicity assays

To evaluate polymer probe cytotoxicity, 0.5×10^5 Jurkat cells were plated on a 24 well plate (Corning 3524 Costar 24 Well Clear TC-Treated Microplates) and incubated at 37 °C with different concentrations of various polymer probes (solubilized in the cell culture medium MEM-alpha, PAA) during 7 h or 32 h (no polymer probe for the control experiment).

A qualitative evaluation was carried out using an inverted microscope Leica DM IRBE with a EB-CCD camera (C4880 Hamamatsu, Ichinocho, Japan) controlled by the HIPIC Software (Hamamatsu). The cell observations were performed using a 40x oil immersion objective lens and the images processed with ImageJ freeware.

A quantitative evaluation was performed using a BD FACS Canto II flow cytometer equipped with the BD FACS Diva Software (10,000 cells per condition). Cell viability was determined using the granulometry mode of the FACS system that distinguishes viable cells from granular non-viable cells.

2.5. Internalization assays

Cells were plated on chambered coverslips (μ-Slide 8 well, ibiTreat – 12,500 cells/well) and allowed to adhere overnight. In the case of the Jurkat T cells, cells were allowed to adhere on polylysinated coverslips 30 min prior to the addition of the polymer probe. Cells were then incubated for 1h30 to 2 h with different concentrations of polymer probes solubilized in the cell culture medium (MEM-alpha or RPMI). Cells were washed twice with PBS 1X to remove the residual (non internalized) polymer probe and visualized using inverted microscope LEICA DMI4000 with an incubation chamber (CO_2 and temperature control), consisting of a confocal "spinning disk" head (Yokogawa CSU22), an EMCCD camera (Photometrics Quantem 512). This system is composed of 4 laser lights (405, 491, 561 and 635 nm) and controlled by the Metamorph Software (Molecular Devices). The cell observations were performed using the 63× water immersion objective lens (NA = 1.2). Generally, the exposition time was 800 ms. In the particular case of the Z-stacks, the exposition time was 1300 ms. Images were processed with ImageJ freeware.

2.6. Resistance to photobleaching assays

Cells were plated on chambered coverslips (μ-Slide 8 well, ibiTreat – 12,500 cells/well) and allowed to adhere overnight. They were then incubated for 3 h with the polymer probe (P3, 1 μM) and 1 h with the commercial probe LysoTracker® Red DND-99 (75 nM) solubilized in the cell culture medium (MEM-alpha, PAA). Cells