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Two-photon fluorescence imaging of RNA in nucleoli and cytoplasm in living cells based on low molecular weight probes

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ABSTRACT

Two low molecular weight, indole-based, mono-cationic probes, were designed and synthesized. According to their spectral response to ribonucleic acid *in vitro* and direct fluorescence imaging in living cell lines, the two compounds were identified as ribonucleic acid-selective fluorescent turn-on probes with large two-photon excited fluorescence action absorption cross-sections when binding to ribonucleic acid. Moreover, both dyes with good membrane permeability have been successfully used to image ribonucleic acid in nucleoli and cytoplasm in living cells by confocal and two-photon fluorescence microscopy. Furthermore, the dyes possess good counterstain compatibility with Hoechst 33342, important deoxyribonucleic acid-staining dyes for ribonucleic acid–deoxyribonucleic acid colocalization.

1. Introduction

In-vivo, fluorescent imaging of ribonucleic acid (RNA) in nucleoli and cytoplasm is of great significance in biochemistry and biomedicine [1,2]. For example, Knowles et al., used nucleic acid (NA)stain SYTO 14 to visualize the translocation of endogenous RNA in living cells, and they found that labeled RNA was distributed nonrandomly as discrete granules in the neuronal processes [3]. Fluorescent imaging of RNA in parasites increases parasite detection, improves the spatial and temporal resolution of the parasite under drug treatments, and resolves the problems of morphological changing in an individual cell [4]. Previously, several classes of molecular probes have been developed for RNA detection in living cells, including (a) oligodeoxyribonucleotides (ODN) (Chart. S1) probes [5]; (b) linear fluorescence resonance energy transfer (FRET) probes [6]; (c) dual-labeled oligonucleotide hairpin probes (e.g., molecular beacons) [7]; (d) dual FRET molecular beacons [8]; (e) autoligation probes [9]; and (f) probes using fluorescent proteins as reporters [10,11]. However, because most of the RNA fluorescent probes have no membrane-permeability, they have to be injected into a target living cell in a typical *in-vivo* fluorescent imaging process [12–14]. Such a microinjection technology is destructive to living cells and may cause biological malfunctions [12,15], it is necessary to develop a low-molecular-weight fluorescent probe for imaging RNA in living cells. Even though there are many commercially available dyes with membrane-permeability for various organelles such as the nucleus [16] and mitochondria [17], preparation of RNA fluorescent probes with membrane permeability is still a challenge. Some of the facts that restricted its development include the diminished affinity to RNA than to double-stranded deoxyribonucleic acid (DNA) [18], the non-specificity in binding with proteins due to the hydrophobicity of probe molecules. Moreover, there is limited knowledge of the interaction mechanisms between RNA and fluorescent probes, including outside, groove and intercalative binding [19], compared with the wealth of papers on DNA biosensors. All increased the difficulties to develop an RNA biosensor, which also promotes the demand for a library of compounds. Li and coworkers screened a large combinatorial library of 1336 fluorescent styryl molecules but

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ultimately obtained only three RNA probes [20]. In another work just three dyes displaying high affinities to RNA were sifted from 125 fluorescent molecules [4]. Even though Molecular Probes Co. offers a commercially available RNA probe "SYTO RNA-Select" [21] for RNA imaging in living cells, its chemical structure has not been described.

Two-photon fluorescence microscopy (TPM) has advantages over traditional techniques such as lower photodamage, reduced photobleaching, higher detection sensitivity, as well as diminished image distortion [22–27]. TPM has an intrinsically high axial resolution without the need of a confocal pinhole in the detection path. Also the technique will reduce cell damage by using either longer wavelength excitation light, which avoids damaging ultraviolet (UV) [28], or blue excitation light. It also reduced out-of-focus irradiations [29,30]. Therefore, TPM can be used for repetitive imaging of living cells without severely damaging the cellular vitality. The fast development of two-photon excited fluorescence (TPEF) probes to different targets in living cells has attracted a lot of attention and many researchers [31–34], including us [35], have contributed to this area. Amongst all of these, the only one on live-cell RNA TPEF probes was given by Ohulchanskyy et al., who found that the conventional NA dye 4-((1-methylbenzothiazolyliliden-2) methyl)-1,2,6-trimethylpyridinium perchlorate (cyan 40) (Chart. S2) could be used to image RNA in nucleoli in TPM [36]. However, its two-photon excited fluorescence action absorption cross-sections ($\Phi \times \delta$), a crucial parameter for TPEF probes, was not given. Given the limited number of suitable dyes we were keen to explore new RNA-selective biosensors that can be used for both confocal microscopy and TPM in living cells.

In our previous work, we have focused on small-molecule probes, carbazole derivatives, for selective imaging NA, cysteine and lysosome in live-cells and deep tissues [35,37,38]. These dyes possesses large $\Phi \times \delta$, thus they can luminescence more intensely under the some incident power. Despite the numerous desirable properties of small-molecule NA probes have been designed by us [37], many of them work well with DNA in plant tissue but are not suitable for RNA TP imaging in living human cells. Among them, possessing V-shaped conjugated structures inspired us to survey RNA markers, especially TPEF live-cell RNA sensors among various cationic salts with the particular chemical structure. The results indicate that this idea should be rational. In this ongoing study we synthesized and examined V-shape structures, namely 3,5-bis((*E*)-2-(pyridin-4-yl)vinyl)-1*H*-indole monoiodides (**INR1**/**INR2**) (Scheme 1).

2. Material and methods

2.1. Material

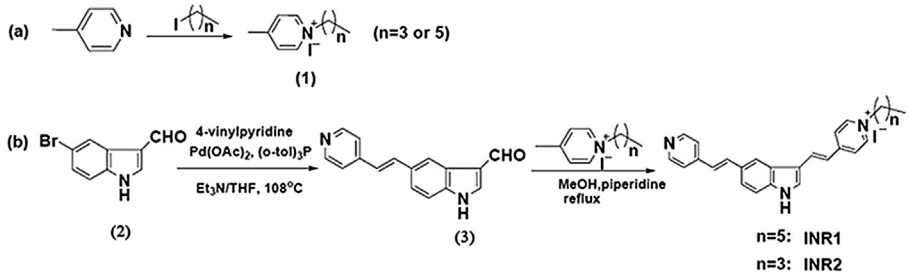
All chemicals used are of analytical grade, and 4-picoline, 1-iodobutane, 1-iodohexane and 5-Bromo-1*H*-indole-3-carbal dehyde were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Palladium (II) acetate and tri-*o*-tolylphosphine were purchased from **J&K Chemical** (Beijing, China). Tris and PBS were purchased from Seikagaku Corporation (Japan). The solvents used in the spectral measurement are of chromatographic grade. The double-stranded DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI)/Hoechst 33342 and SYTO RNA-Select were purchased from Molecular Probes. Calf thymus DNA and torula yeast RNA, which were used as the model of DNA and RNA, and ribonuclease A (RNase) were obtained from Sigma. Spectroscopic measurements of were performed in acetonitrile/Tris–HCl buffer solution (tris: 10 mM, KCl: 100 mM) with pH 7.2. TLC analyses were performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from Qingdao Ocean Chemicals.

2.2. Measurements

Nuclear magnetic resonance spectra (^{1}H and ^{13}C) were obtained on a Bruker Avanace 300/400 spectrometer. The HRMS spectra were recorded on Agilent Technologies 6510 Q-TOF LC/MS or ThermoFisher LCQ FLEET. The elemental analyses were performed on a Vario EI III instrument. The UV–visible–near-IR absorption spectra of dilute solutions were recorded on a Cary 50 spectrophotometer using a quartz cuvette having 1 cm path length. One-photon fluorescence spectra were obtained on a HITACHI F-2700 spectrofluorimeter equipped with a 450-W Xe lamp. Two-photon fluorescence spectra were recorded on a SpectroPro300i and the pump laser beam comes from a mode-locked Ti: sapphire laser system at the pulse duration of 200 fs, with a repetition rate of 76 MHz (Coherent Mira900-D). Confocal microscopic photos of photostability were obtained with Carl Zeiss Microscopy LSM780, The confocal microscopic image and differential interference contrast (DIC) image were taken with a 488 nm laser, CD Spectrometer Jasco J-810.

Wide-field fluorescence microscopy images were acquired with an Olympus IX71 inverted microscope coupling with a CCD and display controller software. The fluorescence of four dyes and DAPI/Hoechst 33342 were excited and collected through U-MNIBA3 and U-MWU2, respectively. The confocal microscopic image and differential interference contrast (DIC) image were taken with a 488 nm Arion laser. Fluorescence of **INR1**/**INR2** were collected with a beam splitter DM570 and BA510-540 nm bandpass emission filter combination. All of TPM microscopic photos were obtained with Olympus FV 300 Laser Confocal System with a 60× water objective (N.A. = 1.25) and photomultiplier tubes and Ti: sapphire laser (Coherent) was used to excite the specimen at 800 nm. The total power provided by laser source can be maintained stable and the incident power was examined with Power Monitor (Coherent) directly. A multiphoton emission filter (FF01-750; Semrock) was used to block the IR laser.

TPA cross-sections have been measured using the two-photon induced fluorescence method [40]. Fluorescein (pH = 13, cyan



Scheme 1. The synthetic routes to **INR1**/**INR2**.