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COMMUNICATION

A 2,7-carbazole-based dicationic salt for fluorescence detection of nucleic acids and two-photon fluorescence imaging of RNA in nucleoli and cytoplasm†

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A new carbazole-derived dicationic compound, namely 2,7-bis(1-hydroxyethyl-4-vinylpyridinium iodine)-*N*-ethyl-carbazole (2,7-9*E*-BHVC), with a large two-photon action absorption cross section in nucleic acids has been obtained. Moreover, it possesses the potential of imaging RNA in nucleoli and cytoplasm in two-photon fluorescence microscopy and exhibits good counterstain compatibility with the commercial fluorescent nucleic dye DAPI.

Introduction

Both RNA and DNA are important biomacromolecules.[1,2] However, compared to the many fluorescent probes imaging DNA,[3–7] RNA probes for cell imaging are rarely reported. Molecular Probes Co., which sells various DNA probes such as DAPI, series of Hoechsts, and propidium iodide, only offered a classical commercial RNA probe "SYTO RNA-Select" for imaging RNA in living cells, but its chemical structure has not been described.[8] Li and coworkers screened a large combinatorial library of 1336 fluorescent styryl molecules and only ultimately obtained three RNA probes.[9] Yu *et al.* reported a europium complex for nucleoli imaging.[10] At the same time, Turro's team also constructed two RNA probes by covalently linking ruthenium(II)[11] or fluorescein[12] to phenanthridine derivatives, which are the oldest and most often used fluorophores intercalating with DNA or RNA. The possible reasons why RNA probes are rare could be at least three-fold: first, small nucleic-acid binding molecules generally have better affinity for double-stranded DNA than for RNA; second, hydrophobic biosensors including nucleic acid (NA) probes may also non-specifically bind with proteins and membranes in cells; third, knowledge of the interaction mechanism between RNA and fluorescent probes is still not enough compared with the wealth of deeper understanding of DNA biosensors, including outside, groove and intercalative binding.[13–15] Thus it is necessary to find new RNA fluorescent probes.

Recently, two-photon fluorescence microscopy (TPM) has become an indispensable tool in many biological laboratories due to its unique advantages in bioimaging[16–19] and various two-photon fluorescent probes have been investigated intensively and extensively.[20–24] To our knowledge, only one two-photon RNA probe has been reported: Ohulchanskyy *et al.* found that conventional NA dye cyan 40 can be used to image RNA in nucleoli by TPM.[25] However, its two-photon action absorption cross section ($\Phi\times\delta$), a crucial parameter for two-photon fluorescent probes, is not reported. Recently, we have reported a series of two-photon DNA and mitochondria probes from carbazole-containing cationic compounds, especially 3,6-carbazole-dicationic salts, they have been successfully used to image nuclei in plant cells and tissues and mitochondria in living cells by TPM.[26,27] The works of Chang and ourselves both demonstrated that structural changes in carbazole cationic compounds can practically influence their cellular responses and become different fluorescent probes.[26–28] According to the idea mentioned above, an isomer of 3,6-carbazole dicationic salt, 2,7-carbazole dicationic salt (2,7-9*E*-BHVC), was synthesized (Chart 1), and our experimental results have shown that 2,7-9*E*-BHVC is a two-photon turn-on fluorescent probe for NA, and its $\Phi\times\delta$ are 1.67 GM and 30 GM in the absence and presence of NA, respectively. Moreover, the staining results from both wide-field fluorescence microscopy and TPM have shown that 2,7-9*E*-BHVC is able to image the RNA in nucleio and cytoplasm.

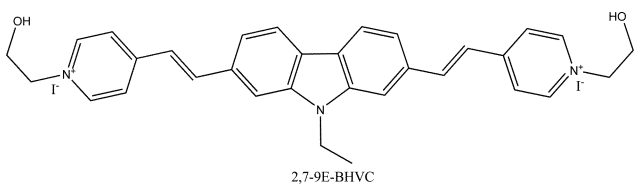

Chart 1 Chemical structure of 2,7-9*E*-BHVC.

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† Electronic supplementary information (ESI) available: Experimental details and characterization of 2,7-9*E*-BHVC, measurement equipment and materials, measurement of two-photon absorption cross section, cell culture and staining, wide-field fluorescence microscopy imaging and two-photon fluorescence microscopy imaging experiment, UV–vis absorption and fluorescence spectra of 2,7-9*E*-BHVC in various solvents, photophysical properties of 2,7-9*E*-BHVC. See DOI: 10.1039/c1ob05123g

Results and discussion

The synthesis (Scheme S1†) and characterization of 2,7-9*E*-BHVC are shown in the ESI.† Fig. S1† shows the solvatochromic studies of 2,7-9*E*-BHVC. Its one-photon and two-photon fluorescence intensities are high in organic solvents and very low in aqueous solution. The appreciable solvent effect implies that the intramolecular charge transfer state is predominant in the photophysical behavior of 2,7-9*E*-BHVC, which is similar to our other carbazole derivatives.[26,27] Accordingly, the weak emission of 2,7-9*E*-BHVC in water can be attributed to the strong interaction of the molecules at excited state with the water environment, which induces the formation of twisting intramolecular charge transfer state, resulting in the increase of nonradiative decay.[26,27,29] On the other hand, the self-aggregation of 2,7-9*E*-BHVC with low solubility in water could also result in self-quenching. It is well known that the concentration-quenching effect is common for many aromatic compounds due to the formation of sandwich-shaped excimers and exciplexes aided by the collisional interactions between the aromatic molecules in the excited and ground states.[30,31]

Subsequently, the interaction of 2,7-9*E*-BHVC with NA was carefully studied (Fig. 1a). When the compound was mixed with NA in a 100 : 1 [NA]/[dye] ratio, both DNA and RNA induced marked hypochromism of the absorbance (*ca.* 18%) and red shift (*ca.* 18 nm) of the absorption maximum, which suggests that the dye interacts apparently with NA.[13,14] Moreover, 2,7-9*E*-BHVC is only weakly fluorescent in tris-HCl buffer, whereas its fluorescence intensity shows an approximately 25 fold enhancement in NA. The mechanism of the light-switch effect of the dyes binding to NA could be explained by two factors: firstly, the restricted action on the torsional motion of vinyl groups that is derived from the interactions between the cationic N-pyridinium unit and the anionic phosphate of NA; secondly, the dye is protected from water in the hydrophobic environment offered by the higher-order structure of NA. In addition, the gradual change of fluorescence with addition of NA is shown in Fig. 1b, in which the fluorescence of 2,7-9*E*-BHVC enhances sharply at the low ratio (<40 : 1 [NA]/[dye] ratio) and then tends to mildly increase until saturation point is reached (from 60 : 1 to 180 : 1 [NA]/[dye] ratio). The whole process of fluorescence enhancement with the addition of RNA is identical to that of DNA. Furthermore, the two-photon fluorescence spectra of 2,7-9*E*-BHVC in tris-HCl buffer, DNA and RNA solutions were examined at 800 nm which is an optimal excitation wavelength provided by a commercial mode locked Ti:sapphire laser source (Fig. 2a). 2,7-9*E*-BHVC exhibits bright two-photon induced fluorescence both in DNA and RNA and the

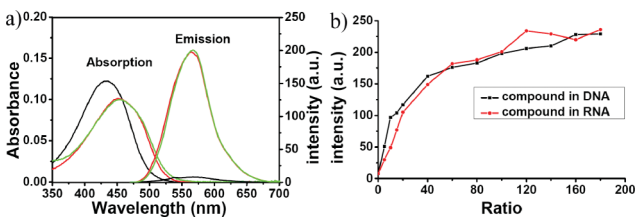

Fig. 1 a: The UV–vis absorption and single-photon excited fluorescence spectra of 2,7-9*E*-BHVC in buffer (black), DNA (red) and RNA (green) at 100 : 1 [NA]/[dyes] ratio. b: Fluorometric titration curve of 2,7-9*E*-BHVC, fluorescence intensity *versus* the [NA]/[dyes] ratio from 0 to 180 : 1. Compound concentration: 2.0×10^{-6} M.

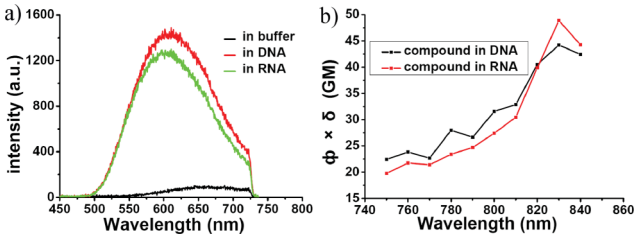

Fig. 2 a: Two-photon excited fluorescence spectra of 2,7-9*E*-BHVC in the absence and in the presence of DNA or RNA with 100 : 1 [NA]/[dyes] ratio. b: Two-photon action absorption spectra of 2,7-9*E*-BHVC in DNA and RNA with 100 : 1 [NA]/[dyes] ratio. Compound concentration: 1.0×10^{-5} M.

maximum emission appears at about 600 nm. According to the measurement data, in tris-HCl buffer, its $\Phi \times \delta$ is 1.67 GM, but the values become 31.56 GM and 27.42 GM in DNA and RNA, respectively (Table S1†), which show *ca.* 18-fold enhancement. This demonstrated that 2,7-9*E*-BHVC can be used as a two-photon fluorescence "light-switch" for NA. Moreover, taking into account that the 2,7-9*E*-BHVC in NA solution has no linear absorption in the spectral range of 700–900 nm and the maximum absorption wavelength is at 450 nm, the two-photon action spectra of 2,7-9*E*-BHVC in NA solution were determined by Ti:sapphire laser sources tuneable at 750–840 nm (Fig. 2b). The maximum $\Phi \times \delta$ of the compound in DNA and RNA solutions are about 44.22 GM and 48.89 GM at 830 nm. It is worth understanding that 2,7-9*E*-BHVC possesses a larger $\Phi \times \delta$ in comparison with commercial NA dyes, such as DAPI-DNA (2.18 GM)[26] and EB-DNA (0.91 GM).[32] Therefore, one can reasonably expect that, once the compound binds to NA particularly in an intracellular environment, it will be a good two-photon fluorescent NA probe.

Fig. 3 shows the wide-field fluorescence microscopy image of pretreated HeLa, SiHa and MS1 cells stained with 2,7-9*E*-BHVC. The green fluorescence of 2,7-9*E*-BHVC mainly localizes at the cytoplasm and nucleoli accompanied with faint nuclei distribution in all three kinds of cells. Additionally, as shown in Fig. 4, after treatment with ribonuclease A (RNase) which only hydrolyzes the RNA and does not influence the DNA, the fluorescence of 2,7-9*E*-BHVC in cytoplasm and nucleoli dramatically diminished and tended to redistribute to the nucleoli (Fig. 4c) in contrast to the untreated sample (Fig. 4a). However, there was no apparent effect on the staining result of DAPI when digested with RNase, because the DAPI tends to stain double-stranded DNA rather than RNA. The digested results reconfirm that 2,7-9*E*-BHVC prefers RNA to DNA in the complex internal environment of cells, although there was no apparent disparity in solution fluorescence measurements. A similar phenomenon was also reported by Chang[9] and Ohulchanskyy,[25] but the mechanism

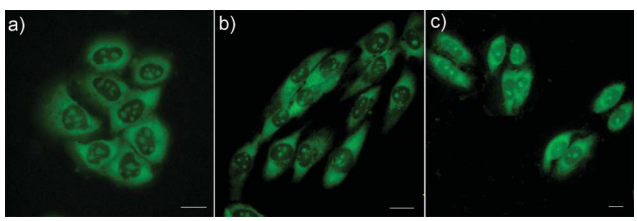

Fig. 3 Wide-field fluorescence images of (a) HeLa, (b) SiHa and (c) MS1cells stained with 5 μM 2,7-9*E*-BHVC for 30 min. Bar = 20 μm.

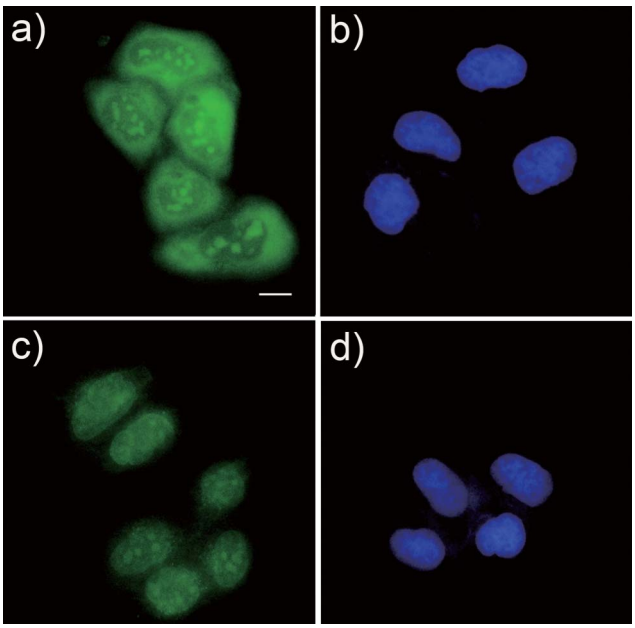

Fig. 4 The RNase digest experiment. Wide-field fluorescence imaging of cells stained with (a) 2,7-9*E*-BHVC and (b) DAPI. Cells stained with (c) 2,7-9*E*-BHVC and (d) DAPI followed by treatment with RNase (30 μg ml^{-1}) for 2 h at 37 °C. Bar = 20 μm. Conditions: 5 μM, 2,7-9*E*-BHVC; 1 μM, DAPI; incubation time: 30 min.

has not been elucidated clearly. The possible reasons for this puzzling state are two-fold: 1) some specificity of intracellular distribution and organization of RNA and DNA molecules can cause a difference in the affinity of 2,7-9*E*-BHVC to DNA and RNA inside cells[25] and 2) the structure of NA in solution may not exactly reflect their real state in cells. Further investigations are being executed to clarify this point.

The TPM imaging data of HeLa and SiHa cells stained with 2,7-9*E*-BHVC are shown in Fig. 5, and the position, shape and amounts of the nucleoli labeled by 2,7-9*E*-BHVC are the same with the most dense, dark-phase region of the nucleus in DIC images which definitely represents nucleoli. From Fig. 5, one can also see that HeLa cells have larger amounts of nucleoli than SiHa and the distribution of the nucleoli in the two kinds of cells are significantly

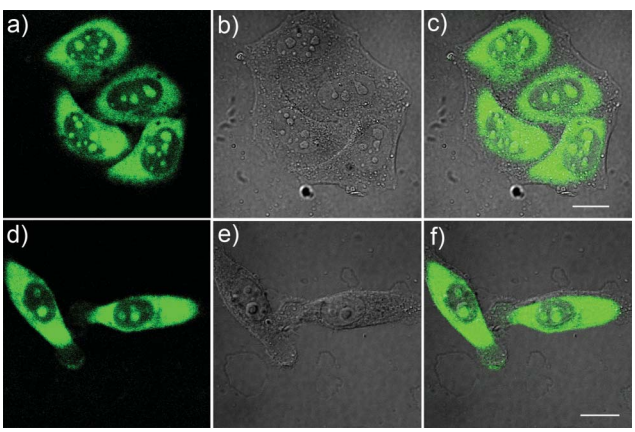

Fig. 5 Imaging of HeLa (a, b and c) and SiHa (d, e and f) cells stained with 5 μM 2,7-9*E*-BHVC for 30 min. a and d: TPM fluorescence imaging. b and e: DIC imaging. c and f: merged picture. Bar = 20 μm.

different which may reflect the distinguishment of transcriptional activity in different cell lines.

Fig. 6 shows the counterstain result of 2,7-9*E*-BHVC and DAPI. The green fluorescence from nucleoli and cytoplasm is easily discriminated from the nuclear zone with blue fluorescence, and it means that 2,7-9*E*-BHVC has very good counterstain compatibility with DAPI. The characteristics of 2,7-9*E*-BHVC could help to image RNA distribution in relation to DNA in cells and probably to reveal different patterns of RNA-DNA colocalization which are cell type dependent.

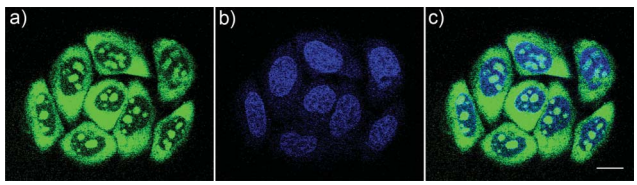

Fig. 6 TPM fluorescence imaging of HeLa cells stained with 5 μM 2,7-9*E*-BHVC for 30 min followed by staining with 1 μM DAPI for 30 min. a: fluorescence of 2,7-9*E*-BHVC. b: fluorescence of DAPI. c: merged picture of a and b. Bar = 20 μm.

Conclusions

In conclusion, we have developed a carbazole-centered dicationic compound 2,7-9*E*-BHVC based on an A–π–D–π–A structure. Very importantly, when binding to NA, 2,7-9*E*-BHVC possesses bigger $\Phi \times \delta$ and notable two-photon fluorescence enhancement in comparison with itself. At the same time, the experimental results from wide-field fluorescence microscopy and TPM showed it preferred RNA to DNA in cells. Moreover, it has good counterstain compatibility with DAPI and is suitable for DNA-RNA colocalization experiments.

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