Fluorescent Visualization of Nucleolar G-Quadruplex RNA and Dynamics of Cytoplasm and Intranuclear Viscosity

Le Yu¹², Peter Verwilst³, Inseob Shim², Yu-Qiang Zhao¹, Ying Zhou* & Jong Seung Kim*²

¹College of Chemical Science and Technology, Yunnan University, Kunming 650091, ²Department of Chemistry, Korea University, Seoul 02841, ³KU Leuven, Rega Institute for Medical Research, Medicinal Chemistry, B-3000 Leuven

*Corresponding authors: yingzhou@ynu.edu.cn; jongskim@korea.ac.kr; ¹L. Yu and P. Verwilst contributed equally to this work.

Cite this: CCS Chem. 2020, 2, 2725–2739

The nucleus, the locus of ribosome biogenesis, was found to be the predominant intracellular target of a new fluorescent probe, V-P1. In solution, the probe demonstrated both a selectivity to RNA G-quadruplexes and a sensitivity to the viscosity, while G-quadruplex binding did not disturb the viscosity sensing. In cells, confocal and fluorescence lifetime imaging, combined with digestion and competition experiments, lent support to the hypothesis of an RNA-based G-quadruplex as the intracellular target, postulated to be nucleolar ribosomal RNA (rRNA). The probe demonstrated a high sensitivity to viscosity in both the cytoplasm and the nuclear compartment and was used to precisely interrogate the viscosity changes resulting from diverse stimuli, such as temperature, monensin treatment, and etoposide-induced apoptosis. Owing to the putative rRNA G-quadruplex binding in vitro and in vivo, and further combined with a relatively low degree of toxicity, the dye enabled the interrogation of cytoplasm and intranuclear viscosity changes under diverse conditions and found applications in studying the influence and significance of cytoplasm and intranuclear viscosity as well as in gaining insight into the native secondary structure of rRNA in nucleoli.

Keywords: fluorescence imaging, G-quadruplex RNA, intranuclear viscosity, nucleoli

Introduction

Ribosome biogenesis in the nucleoli is controlled by RNA polymerase I, transcribing ribosomal RNA (rRNA) genes into pre-rRNAs, that are further processed into mature rRNA. As ribosomal availability is directly linked to a cell’s ability for de novo protein synthesis and thus regulates cell growth and proliferation,⁴ rRNA is a crucial component of the cellular machinery and an attractive, albeit underexplored, target for drug design.⁵

Despite the importance of this species, several key aspects of the regulation of rRNA biosynthesis and general structural properties of this polynucleotide are still being discovered. The GC-rich rDNA is known to have a
propensity toward the formation of DNA G-quadruplexes, a noncanonical planarly orientated sheet of four guanines bonded in a Hoogsteen hydrogen-bonding arrangement, stacked, and further stabilized by interspersed cations such as Na\(^+\) and K\(^+\). These G-quadruplexes have been demonstrated to play an important role in the regulation and epigenetics of rRNA transcription.\(^9\) Apart from the well-known G-quadruplex DNA structures, which have been studied extensively by Teulade-Fichou et al.\(^5\)-\(^9\), RNA has been shown to arrange in G-quadruplex structures as well, and may be even more prone to do so, as this single-stranded polynucleotide is not maintained in a (competitive) double-stranded architecture.\(^10\) While mature human rRNA contains several guanine rich regions, much less is known about its secondary structure and its potential function. Recent evidence regarding the presence of rRNA G-quadruplexes in vitro has come to light,\(^11\)-\(^14\) and some tentative evidence pointing toward RNA G-quadruplexes in the nucleoli in vivo has been recently reported as well.\(^15\)

rRNA plays an important role in cellular proliferation and, together with G-quadruplex rDNA, G-quadruplex rRNA is likely intricately involved in regulatory cellular processes. Thus, the design of fluorescent probes specific to either of these two secondary structures is highly important and could readily find applications. Currently, only a handful of fluorescent probes with in vitro interactions with RNA G-quadruplexes have been reported. Pyridostatin (PDS) has been shown to interact with both DNA and RNA G-quadruplexes,\(^16\) while a carboxylated analogue does demonstrate a higher selectivity for RNA G-quadruplexes.\(^17\)-\(^19\) Furthermore, a decorated acridine, napthalene, and cyanine demonstrated the visualization of RNA G-quadruplex structures. However, all these studies were directed at human telomeric RNA structures or cytosolic targets.\(^20\)-\(^22\) Thiazole orange (TO), a well-known sensor for the detection of DNA, exhibits an obvious enhancement of fluorescence upon binding to DNA and shows a high affinity with G-quadruplex DNA compared with other forms of DNA.\(^9,23\) Thioflavin T, a prototypical viscosity and protein aggregation sensor, was demonstrated to localize to the nucleoli and may have G-quadruplexes as the intracellular target.\(^10,24\)-\(^26\) Sun et al.\(^27\) and Shivalingam et al.\(^28\) reported a G-quadruplex DNA sensor with a preferential localization to the nucleus in general and nucleoli, respectively.

To design probes that can dynamically interact with biological species without the need to wash before imaging, probes must be equipped with a means to prevent fluorescence in the absence of analyte binding. Among the different mechanisms, molecular rotation has been found to fulfill this function well,\(^29\) and in the context of polynucleotide sensing has also been proven to be highly effective, for example, in ethidium bromide and propidium iodide. Furthermore, the presence of a molecular rotor generally also allows for the determination of the viscosity in the immediate vicinity of the probe, as the ease of molecular rotation corresponds directly to the probes excited-state behavior.

In this work, we designed a small molecule probe for the elucidation of G-quadruplex RNA in living cells, based on several design aspects of polynucleotide targeting chemical probes. Our probe, V-P1, was equipped with a molecular rotor to enable bright fluorescence in the presence of the analyte, further allowing for the determination of dynamic changes is the viscosity of the cytosol, and to a lesser degree the nuclear compartment, by both fluorescence intensity and lifetime imaging (Scheme 1).

**Experimental Methods**

**Materials and instrumentation**

All reagents were purchased from Shanghai Titan Scientific Co., Ltd. (Shanghai, China) and were used without further purification. Monensin was purchased from Shanghai Titan Scientific Co., Ltd. (Shanghai, China). All different types of DNA, RNA, and DNA and RNA G-quadruplex structures were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Deionized water was used throughout all experiments. Flash chromatography was implemented on silica gel (200–300 mesh). \(^1\)H and \(^13\)C NMR spectra were recorded using a Bruker DRX 400 spectrometer (Bruker Corporation, Billerica, Massachusetts, USA); mass spectrometry was recorded with an Agilent 1100 LC-MSD TOF mass spectrometer (Agilent technologies inc., Santa Clara, California, USA). The fluorescence spectra were performed on a
F97XP FL spectrophotometer (Lengguang technologies inc., Shanghai, China) with the 1 cm standard quartz cell. Excitation and emission slit widths were 5 nm x 5 nm. The UV-vis spectra were obtained using a UV-240IPC spectrophotometer (Shimadzu Corporation, Kyoto, Kyoto, Japan).

Viscosity analysis
Viscous solutions were prepared by mixing methanol and glycerol in different volume proportions. The viscosity of each sample was measured with an NDJ-5S rotational viscosimeter. About 50 μL of a V-P1 stock solution (2.0 mM in MeOH) was added to the methanol-glycerol mixture (4.95 mL) to give a final concentration of 20 μM. The resulting solutions were shaken for 30 min and then kept still for 30 min at 25 °C, after which the fluorescence was recorded. The quantitative relationship between fluorescence intensity and the viscosity of the solution was described by Förster-Hoffmann eq1 as follows:

$$\log(\text{I}_s) = C + x \log \eta$$

where $\text{I}_s$ is the fluorescence intensity; $\eta$ stands for the viscosity of solution; $C$ is a concentration- and temperature-dependent constant; and $x$ is a sensor- and temperature-dependent constant.

Fluorescence quantum yields measurements
The relative fluorescence quantum yields were identified with Rhodamine B ($\Phi_r = 0.97$) in pure ethanol as a reference and calculated utilizing the following equation:

$$\Phi_x = \frac{\Phi_r(F_x/F_s)(A_x/A_s)(\lambda_{\text{exs}}/\lambda_{\text{ex}})(n_x/n_s)^2}{A_s}$$

where $\Phi$ represents quantum yield; $F$ is the integrated area under the corrected emission spectrum; $A_s$ stands for absorbance at the excitation wavelength; $n$ is the refractive index of the solvent [because of the low concentrations of the solution (10^{-6}–10^{-3} mol/L), the change of refraction coefficient in solution can be ignored]; $\lambda_{\text{exs}}$ is the excitation wavelength; and the subscripts x and s represent the unknown and the reference, respectively.

Fluorescence lifetime detection
Solvents of variable viscosity were prepared as mentioned above. A fluorescence lifetime measuring apparatus (Shimadzu) was used to acquire the fluorescence lifetimes of V-P1, with the excitation wavelength at 580 nm and emission at 650 nm. An excellent straight fitting was obtained, and the quantitative relationship between the fluorescence lifetime of V-P1 and the viscosity of the solution is described by Förster–Hoffmann eq2 as follows:

$$\log \tau = C + x \log \eta$$

where $\tau$ is the fluorescence lifetime; $\eta$ stands for the viscosity of solution; $C$ is a concentration- and temperature-dependent constant; and $x$ is a sensor- and temperature-dependent constant.

The investigation of selectivity
Considering the complexity of the intracellular environment, potential interference by various ions and bio-analytes toward V-P1 was also monitored, including anions (ONOO-, NO2-, and ClO-), cations (Co^{2+}, Fe^{2+}, Hg^{2+}, Ag^+, and Ni^{2+}), reactive oxygen species (H2O2 and TBHP), and thiols (GSH, Hcy, and Cys). The stock solutions of these biologically relevant analytes (1.0 mM each) were prepared in trice distilled water. Stock solutions of V-P1 (2.0 mM) were prepared in methanol. For the measurements of fluorescence spectra, the excitation wavelength was set at 590 nm with excitation and emission slit widths being 5 nm x 5 nm. The experiments were performed using 20 μM of V-P1 in phosphate-buffered saline (PBS) solutions (pH = 7.4, 0.01 M, 1% methanol, 25 °C) with 50 μM of each analyte.

The ds-DNA and ss-RNA were formulated into a 0.4 mg/mL stock solution in 10 mM Tris–HCl (pH 7.4) buffer solution. ss-DNA and all types of DNA and RNA G-quadruplexes were formulated into a 400 μM stock solution in 10 mM Tris–HCl (pH 7.4) buffer solution. About 40 μL of a V-P1 stock solution (1.0 mM in MeOH) was added to the Tris–HCl (100 mM KCl, pH 7.4) solvent (4 mL) to give a final concentration of 10 μM. Then the fluorescence and absorbance titration spectra of these DNA, RNA, and G-quadruplexes were measured.

Competition experiment between viscosity and G-quadruplex RNA
Viscous solutions were prepared by mixing Tris–HCl buffer (100 mM KCl, pH 7.4) and glycerol in different volume proportions. The viscosity of each sample was measured with an NDJ-5S rotational viscosimeter. About 30 μL of a V-P1 stock solution (1.0 mM in MeOH) was treated with 60 μL R-570NT (1.0 mM in Tris–HCl buffer, 100 mM KCl, pH 7.4) first, then was added to the Tris–HCl/glycerol mixture (3 mL) to give a final concentration of 10 μM. The resulting solutions were shaken for 30 min and then kept still for 30 min at 25 °C, after which the fluorescence and fluorescent lifetime were recorded.

Circular dichroism spectra determination
Circular dichroism (CD) was utilized to explore the structural deformations occurring in V-P1 in binary solvent mixtures with different viscosity and with G-quadruplexes. To binary solvent mixtures with different
v viscous and healthy. The culture medium was amended with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO2 and 95% air at 37 °C. The cells were seeded onto 24-well flat-bottomed plates (1.0 × 10^4 each well). When the density of the cell reached 70–80% of confluence, the subculturing was conducted. The culture medium was replaced every 2–3 days after PBS washing. All the details about the experiments of cell imaging are presented in the Supporting Information.

Results and Discussion

Design and synthesis of V-P1

Our strategy to develop the multifunctional probe V-P1 was to combine a fluorescent cationic chromenylum ring that targeted the polynucleotides, with a N,N-dimethylaniline ring through a C–C bond to form a rotor that could produce fluorescence quenching in low-viscosity environments and demonstrate a fluorescence enhancement in highly viscous media. V-P1 was synthesized by a condensation reaction between 4-(diethylamino)salicylic aldehyde and 1-[4-(dimethylamino)phenyl]ethanone and was isolated in a moderate yield of 62% (Schemes 1a and 1b and Supporting Information Figures S1–S3).

Spectral properties

The absorbance and emission maxima of V-P1 in methanol were 603 and 648 nm, respectively (Supporting Information Figure S4), indicating a Stokes shift that was sufficiently large to enable imaging with a high degree of signal to noise. The fluorescent response of V-P1 as a function of the solvent viscosity was studied in solutions of methanol with different proportions of glycerol. The fluorescence intensity of V-P1 clearly increased with increasing proportions of glycerol (Figure 1a). The viscosity of the samples tested increased from 1.3 cP (pure methanol) to 433.0 cP (90% glycerol). (The viscosities of the mixtures used are listed in Supporting information Table S1.) V-P1 exhibited a weak emission when it was excited at 590 nm in a low-viscosity solution (100% methanol, 1.3 cP), with a low quantum yield of 0.09 and a high molar extinction coefficient (ε = 0.7 × 10^4 L mol^-1 cm^-1). In 90% glycerol, a high quantum yield of 0.88 and molar extinction coefficient of 0.85 × 10^4 L mol^-1 cm^-1 were observed, while the absorbance of these V-P1 solutions remained largely unchanged (Supporting Information Figure S5). Thus, a 10-fold fluorescence intensity increase was achieved for V-P1 in this viscosity range (Figure 1a).

As shown in the inset, a good linear proportionality between the fluorescence intensity I_{E550} (log I_{E550}) and viscosity (log η), with a correlation coefficient of 0.9903 was
observed, demonstrating V-P1 could potentially be applied for quantitative viscosity measurements.

As the viscosity is inversely related to the temperature, we tested the influence of the temperature toward the fluorescence emission of V-P1 (Figure 1b). In the 45 to −5 °C temperature range, the emission intensity at 650 nm increased about fourfold. Importantly, an excellent linear relationship existed between I_{650} and the temperature (R² = 0.9985).

Meanwhile, when the viscosity (η) in the mixed solvent increased from pure methanol (1.3 cP) to 90% glycerol (433 cP), the fluorescence lifetime (τ) gradually extended from 371 to 2200 ps with a good linear relationship between log τ and log η (R² = 0.9919) and a steep slope (0.429), indicating that sensor V-P1 can also quantitatively detect the viscosity by measuring the fluorescence lifetime changes (Figure 1c).

As viscosity sensors can also be sensitive to the solvent polarity, fluctuations in the polarity can affect the accuracy of microenvironment viscosity determinations. Thus, the influence of solvent polarity on the fluorescence of V-P1 was determined using virtually isoviscous 1,4-dioxane/water mixtures with large polarity differences. Upon polarity changes, V-P1 demonstrated only slight fluorescence fluctuations, meaning the polarity influence was negligible in the test system (Supporting Information Figure S6 and Table S2). Thus V-P1 is endowed with the critical characteristic of detecting microenvironmental viscosity without disturbance from the polarity. Furthermore, only relatively small fluorescence changes could be observed in different organic solvents (Supporting Information Figure S7), and the fluorescence intensity remained stable in the pH 3–8.5 range (Supporting Information Figure S8), demonstrating the absence of pH effects under physiological pH conditions.

To evaluate the possibility of V-P1 microviscosity sensing in complex cellular microenvironments, several potential interferents were tested, such as metal ions, anions, thiols, reactive oxygen species, and bioactive small molecules. The selectivity of V-P1 is exhibited in

Figure 1 | (a) The fluorescence spectra of V-P1 (20 μM) in mixed solvents with different proportions of methanol–glycerol at 25 °C. Inset: Linear relationship of log I_{650} and log η (R² = 0.9903). (b) The fluorescence spectra of V-P1 (20 μM) at different temperatures in ethanol–glycerol (30/70, v/v), excited at 590 nm. Inset: Linear relationship of I_{650} and temperature (R² = 0.9985). (c) Fluorescence lifetime of V-P1 (20 μM) in mixed solvents with different proportions of methanol–glycerol (λ_em = 650 nm). Inset: Linear relationship of log τ and log η (R² = 0.9919). (d) Fluorescence intensity (λ_em = 650 nm) of V-P1 (20 μM) in the presence of various analytes (50 μM n: MeOH/glycerol mixture with viscosity of 433 cP). Bars denote the average of n = 3 independent measurements, while error bars denote the standard deviation.
Figure 1d. All potential interference factors that were tested produced a negligible fluorescent effect, compared with the large fluorescent enhancement of the sensor at a viscosity of 433 cP.

**Fluorescence enhancements in the presence of oligonucleotides**

As shown in Figures 2a and 2b, in Tris–HCl buffer (20 mM, pH 7.4, containing 100 mM K⁺) the probe (10 μM) exhibited a specific absorption peak with a maximum at 570 nm and a relatively weak emission peak located at 637 nm. Upon addition of G4-RNA (R-6960NT, up to 10 μM) to the buffered V-P1 solution, the absorption peak gradually shifted to 605 nm with an obvious isosbestic point at 580 nm (Figure 1b), suggesting a strong interaction between V-P1 and R-6960NT. In accordance with the change in the absorption spectra, the fluorescence of V-P1 also displayed a remarkable change. The emission peak gradually shifted to 655 nm with an obvious enhancement of the intensity (Figure 1a). In addition, the reverse titration between V-P1 and R-6960NT also produced similar results, which further indicated an interaction of V-P1 with R-6960NT (Figure 2c).

As a potential fluorescent probe for oligonucleotides, the fluorescent responses of V-P1 toward different types of DNA and RNA structures were tested. As shown in Figure 2d, the fluorescence intensity of V-P1 at 650 nm in the presence of R-6960NT was approximately six times higher than other oligonucleotides, such as ds-DNA, ss-RNA, ss-DNA (mpu22), and G4-DNA (c-kit1). Clearly, these potential competing species did not interfere with the measured fluorescence sensitivity of V-P1 toward R-6960NT. These results indicate that V-P1 has the potential to bind with specific G4-RNA even in a complex biological environment.

To further explore the selectivity of V-P1, different sequences of DNA, RNA, DNA and RNA G-quadruplex
were used for the next experiments (Figure 3). As shown in Figures 3c and 3d, while the fluorescence enhancement in the presence of ss-DNA or ds-DNA and ss-RNA was negligible, obvious increases in fluorescence intensities were observed for G-quadruplex structures, especially for RNA G-quadruplexes (Figures 3b and 3d). Representative examples of binding isotherms to DNA G-quadruplex (c-kit2), RNA G-quadruplex (R-570NT), and ss-DNA (mpu22) are found in Figures 3a–3c, while the binding isotherms for all tested sequences listed in Figure 3d are found in Supporting Information Figures S9–S22, and the sequence identity of the tested oligonucleotides is listed in Supporting Information Table S3.

The obvious differences in these responses most likely arose from architectural differences in the quadruplex structures. A stronger fluorescence enhancement with RNA G-quadruplex may result from the more base-constrained RNA G-quadruplex as compared with DNA G-quadruplex structures. Fluorescence titrations of V-P1 with five RNA G-quadruplexes and seven DNA

![Figure 3](image_url)

**Figure 3** | (a–c) Fluorescence spectra of V-P1 (10 μM) upon the addition of increasing concentrations of c-kit2, R-570NT, and mpu22 in Tris–HCl (20 mM, pH 7.4) buffer solutions containing K+ (100 mM). Inset: the Benesie-Hildebrand fitting of the titration curves and the fluorescence fitting curve of V-P1 with the oligonucleotides (for panels a and b) and fluorescence intensity at 650 nm for panel c. Bars denote the average of n = 3 independent measurements, while error bars denote the standard deviation. (d) Fluorescent response of V-P1 (10 μM) at the respective fluorescent maxima in the presence of various types of oligonucleotides (10 μM) in Tris–HCl (20 mM, pH 7.4) buffer solution with K+ (100 mM). Bars denote the average of n = 5 independent measurements, while error bars denote the standard deviation. (e) The extrapolated K_d values of V-P1 with various DNA/RNA G-quadruplexes.
G-quadruplexes were tested, and the calculated binding constants are summarized in Figure 3e, with $K_d$ values ranging from 1.82 to 7.66 $\mu$M and from 1.15 to 47.65 $\mu$M for DNA G-quadruplexes and RNA G-quadruplexes, respectively.

It is well-known that some DNA/RNA binding ligands are optically inactive and achiral, and while interacting with DNA/RNA, an enhanced CD signal can be observed. Therefore, CD was utilized to explore the structural deformations occurring in V-P1 with G-quadruplexes. On the one hand, as expected, utilizing binary solvent mixtures with different viscosity did not lead to any appearance of CD signals (Supporting information Figure S23a). This is because upon excitation of V-P1 in solution with a torsion angle of $\phi = 0^\circ$, the formation of a twisted shape can occur in both the left- and right-handed direction, as the 4-dimethylamino-phenyl group is fully symmetrical. However, by binding the G4 RNA, a degree of unidirectional molecular motion and axial chirality/atropisomerism in V-P1 was gained, which is also reflected in the CD spectrum (Supporting information Figure S23b). From the results above, it was clear that there was a net CD absorbance effect upon the addition of G4 RNA, consistent with an expected groove binding mode between V-P1 and G4 RNA (see Docking studies with G-quadruplex RNA).

As V-P1 exhibited fluorescence enhancements both in the presence of G-quadruplex oligonucleotides and increased viscosity, we next tested the potential influence of viscosity in the presence of oligonucleotides. In the presence of 10 $\mu$M R-570NT, a concentration in which fluorescence saturation for this oligonucleotide was reached, both the fluorescence intensity and lifetime were nonetheless clearly increased when the viscosity of the medium was increased (Figures 4a and 4b). When the viscosity was 433 cP, the lifetime reached 3.2 ns while that one in control was only 2.2 ns. Thus, clearly the binding with G4 RNA did not quench the response of V-P1 toward viscosity.

### Theoretical analysis of V-P1

A potential energy surface (PES) in function of the twisting angle of the pendent dimethylaniline was calculated for the ground state and first and second excited states of V-P1 in methanol. As shown in Supporting Information Figure S24, the ground-state geometry reached a minimum at a twisting angle of $\phi = 0^\circ$, whereas the excited states’ PES demonstrated a local minimum at $\phi = 0^\circ$ and a global minimum at $\phi = 90^\circ$. As such it was apparent that in low-viscosity solvents the freely rotating dimethylaniline will adopt a perpendicular orientation in the excited state, whereas in more viscous solvents the reduced mobility will result in slower rotation. The oscillator strength of the transition between highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) dramatically decreased with a larger twisting angle (Supporting information Figure S24). In analogy with previous reports, this typical twisted intramolecular charge-transfer (TICT) behavior arose from a decrease in orbital overlap between the ground state and first excited state with increased twisting angles. As shown in Supporting Information Figure S25, at a twisting angle of $\phi = 90^\circ$, the HOMO and LUMO orbitals were fully orthogonal with near-zero orbital overlap. Thus, in high viscosity samples or when conformationally restrained as a result of target binding, bright fluorescence can be observed. On the other hand, free rotation results in a dramatically quenched fluorescence.

### Docking studies with G-quadruplex RNA

Docking studies were performed to gain additional information on the potential binding mode of V-P1 to...
quadruplex oligonucleotides. As shown in Figures 5a–5f, the highest binding affinity of V-P1 to RNA quadruplex structures (PDB: 3IBK and 4XK0) was found to be in the groove between two parallel RNA strands, with a similar interaction in both model systems. As can be seen the three major cationic centers of the dye (the two nitrogen and the oxygen atoms) align with the anionic phosphate groups. Twisting angles in the structures are $\phi = 35^\circ$ and $37^\circ$ for 3IBK and 4XK0, respectively, within the highly emissive regime of the dye (Supporting Information Figure S24). An alternative binding through $\pi$–$\pi$ stacking with a telomeric G-quartet structure, analogous to a previously reported acridine ligand complex (PDB: 3MIJ, Supporting Information Figure S26) did not show any high-affinity interactions, and thus G-quartet stacking was not likely to be a dominant binding mode. As a result of the binding in the groove, V-P1 was maintained in a relatively flat conformation, and molecular rotation was impeded, resulting in a fluorescent molecule.

The lack of clear agreement between the fluorescence enhancement and binding affinity (Figures 3d and 3e) may be explained by the degree of molecular confinement, with a potentially higher mobility or a binding mode confining V-P1 in a twisted conformation in those complexes exhibiting a high binding affinity but a relatively low fluorescence enhancement.

In vitro fluorescence studies in HeLa cells

First, the cytotoxicity of V-P1 was determined in cells, with IC$_{50}$ values ranging from 9.30 to 23.46 $\mu$M in cancerous cells and $>80$ $\mu$M in BEAS-2B (Supporting Information Figure S27). This differential toxicity clearly hints at an intracellular target important to cellular proliferation. In the subsequent cell studies, the concentrations of V-P1 never exceeded 10 $\mu$M to avoid significant toxicity induction. To identify the subcellular location of V-P1, HeLa cells were costained with the commercial nuclear marker DAPI, MitoTracker Green (MTG) dye, and V-P1 (Supporting Information Figure S28). The fluorescent intensity of DAPI (4′,6-diamidino-2-phenylindole) from the blue channel and MTG in the green channel stayed almost the same over the course of the 25-min incubation time, while the red emission intensity of V-P1 increased. V-P1 is mostly localized in the nuclear compartment, while a small cytosolic fraction was observable as well. This fraction showed overlap with MTG, suggesting that this organelle might be targeted as well, which is in agreement with the previously reported high viscosity in mitochondria. As the highest fluorescence intensity originated from the nuclear compartment, we focused further studies on this crucial organelle.

To confirm the specific target of V-P1 in HeLa cells, deoxyribonuclease (DNase I), ribonuclease (RNase T1), and protease digestion tests were performed in fixed
HeLa cells. As shown in Figure 6a, DNase digestion did not decrease the fluorescence of V-P1, ruling out DNA as a major contributor to the nucleus-localized fluorescence of V-P1. Note the absence of DAPI fluorescence, confirming the digestion of DNA. Protease digestion was found to not lead to any significant reductions in fluorescence in V-P1, suggesting the target of this fluorophore is not a protein. RNase treatment however resulted in a dramatic reduction in the fluorescence of V-P1 (Figure 6b), demonstrating RNase as the main target in the nuclear compartment of HeLa cells. The remaining fluorescence was presumably a result of the viscosity sensing by V-P1.

In addition, a competition experiment with PDS, a generic G-quadruplex sensor binding parallel to G4 quartets (with significant π-π stacking),17–19 induced a significant reduction of V-P1 fluorescence in a solution containing R-S70NT, while virtually completely excluding V-P1 from nuclear localization (Supporting Information Figure S29). These results clearly suggest a G-quadruplex target in solution and in vitro. While docking experiments suggest V-P1 and PDS would have a different binding mode to G-quadruplex structures, the large conformational changes encountered in an RNA G-quadruplex with an acridine ligand versus the native structure (Supporting Information Figure S26) could readily explain the competitive behavior, despite different putative binding modes.

**In vitro viscosity sensing**

As demonstrated in solution studies (Figures 4a and 4b), the binding to RNA quadruplexes did not obstruct viscosity sensing of the probe, and to test whether this holds true in HeLa cells as well, HeLa cells incubated with V-P1 were subjected to viscosity-altering perturbances.

First, fixed HeLa cells were preincubated with V-P1 at 37 °C for 15 min and washed, to ensure equal V-P1 uptake. Then the cells were incubated at 5, 25, and 37 °C for 1 h, and DAPI was added. The fluorescence of these cells was recorded, demonstrating a clear reduction in intracellular fluorescence with increasing temperatures, while DAPI fluorescence remained constant. These results clearly suggest that V-P1, bound to RNA in the nucleus, remains sensitive to the viscosity (Figures 7a and 7b).

Previous studies have reported dramatic changes in the microenvironment of the cytoplasm and mitochondria of cells in the presence of apoptosis-inducing chemicals.49–51 Yet to the best of our knowledge, fluorescent tools to study the viscosity changes in the nuclear compartment under apoptosis are lacking. Thus, taking advantage of the nuclear localization of V-P1, we explored the fluorescent changes of the dye upon supplementation of 1.0 μM etoposide. As can clearly be observed, upon induction of apoptosis, the red emission in cells was strongly elevated over the course of 1 h (Figure 8a). Interestingly, by selecting an region of interest (ROI) in the cytoplasm and the nuclear compartment, an increase in fluorescence was observed in both cases. Still, the absolute change in fluorescence was considerably higher in the cytoplasm as compared with the nucleus (Figure 8a and 8b), suggesting the nucleus is less affected by a viscosity change than the cytoplasm under these conditions. In addition, we did not observe any of these phenomena in the cells of the control group (Supporting Information Figure S30). The induction of apoptosis under these conditions was confirmed using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Supporting Information Figure S31).

**High-resolution confocal and fluorescence lifetime imaging in HeLa cells**

To further validate the sensing ability of the intranuclear viscosity, as well as to gain further information on the
exact target of V-P1 in the nucleus, additional high-resolution imaging was performed. As was immediately apparent from both the confocal and fluorescence lifetime imaging (FLIM) (Figure 9a), V-P1 predominantly localized to a distinct subdomain of the nucleus, identified as the nucleolus. In view of the preference for RNA G-quadruplexes in solution, the nucleolar localization and the dramatic reduction of fluorescence upon RNA digestion, as well as competition with PDS, combined with the in vitro formation of G-quadruplexes in

![Figure 7](image-url)

**Figure 7** | (a) Confocal images of fixed HeLa cells (4% FPA) incubated with V-P1 (1 μM) and DAPI (10 μM) for 15 min at 5, 25, and 37 °C, respectively. (b) Average fluorescence intensity of each group from panel (a). Bars denote the average of n = 3 measurements of random cells, while error bars denote the standard deviation. **p < 0.01.

![Figure 8](image-url)

**Figure 8** | (a) Confocal images of HeLa cells at different time points after the addition of 1.0 μM etoposide and V-P1 (1 μM) at 25 °C. (b and c) Average fluorescence intensity of cytoplasm and nuclear of the experimental group from the panel (a). Bars denote the average of n = 5 measurements of random cells, while error bars denote the standard deviation. ***p < 0.001.
we postulate that the molecular target of V-P1 is a G-quadruplex architecture in rRNA.

HeLa cells were subjected to increasing concentrations of monensin, a known cellular viscosity-inducing agent, and the changes in the viscosity of the cytosol, nucleoli, and nucleoplasm, as well as the whole cell average, were monitored using FLIM. As shown in the Figure 9b with the increase of monensin concentrations, the lifetime of the cytoplasm extended from 2.03 ns, to 2.35, 2.64, and 2.73 ns, which corresponded well with the previously reported viscosity change in the cytoplasm.54 Furthermore, in the presence of monensin, the nucleolus maintained a relatively stable lifetime. Importantly, in all groups, the nucleus showed a much longer lifetime (>4.10 ns) as compared with the nucleolus (<3.6 ns) and the cytoplasm (<3 ns), which can be rationalized by V-P1’s interaction with G4-RNA in the nucleus. All these results were consistent with the results in solution (Figures 1–3) and viscosity-dependent fluorescence (Figures 7 and 8) and further indicate that V-P1 can not only accurately detect the viscosity of the cytoplasm, but also make its way into the nuclear compartment and sense the intranuclear viscosity changes from the fluorescence lifetime variations.

Conclusion

We presented a viscosity sensitive sensor V-P1 and studied the photophysical properties of the dyes in solution, further supported by in silico experiments. The sensor was designed with selectivity for polynucleotides in mind, and we demonstrated that the probe exhibited a high fluorescence enhancement for RNA G-quadruplexes in solution, which could be rationalized by docking experiments. In HeLa cells, the probe demonstrated a predominant localization to the nucleoli, while viscosity changes in the cytoplasm and nucleoplasm could be detected using both changes in incubation temperature, apoptosis induction, and monensin treatment. Thus, owing to the putative rRNA G-quadruplex binding in vitro and in vivo, and further combined with a low degree of toxicity, the dye enabled the interrogation of cytoplasm and nucleoplasm viscosity changes under diverse conditions and could find applications in studying the influence and significance of cytoplasm and nucleoplasm viscosity as well as in gaining insight into the native secondary structure of rRNA.

Supporting Information

Supporting Information is available.

Conflict of Interest

There is no conflict of interest to report.

Funding Information

This work was supported financially by the National Natural Science Foundation of China (nos. 21672185, 22067019; Y.Z.), the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (CRI project no. 2018R1A2B1052702; J.S.K.), the Basic Science Research Program funded by the Ministry of Education (2017R1D1A1B03032561; P.V.), and the Korea Research Fellowship Program funded by the Ministry of Science and ICT through the National Research Foundation of Korea (no. 2016H1D3A1938052; P.V.).
Acknowledgments

This work was supported by CRI project (no. 2018R1A3B1052702; J.S.K) from the National Research Foundation of Korea (NRF, by the China Scholarship Fund (CSC no. 201907030009; L.Y), and by the Interne Fonds KU Leuven/Internal Funds KU Leuven (STG/19/029; P.V.).

References


