Study of Cellular Uptake of Modified Oligonucleotides by Using Time-Resolved Microspectrofluorimetry and Florescence Imaging

P. Praus,¹ E. Kočiřová,¹ P. Mojzeř,¹ J. řtěpánek,¹ and F. Sureau²

¹*Faculty of Mathematics and Physics, Institute of Physics, Charles University in Prague, Ke Karlovu 5, 121 16, Prague 2, Czech Republic*

²*Laboratoire Jean Perrin, Case Courrier 138, Université P.&M. Curie, 4 Place Jussieu, 75231 Paris cedex 05, France*

Correspondence should be addressed to P. Praus, praus@karlov.mff.cuni.cz

Copyright © 2012 P. Praus et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract. Fluorescence microimaging and homodyne phase-resolved confocal microspectrofluorimetry were used to monitor the transport of antisense oligonucleotide into cancer MCF7 cells and the subsequent intracellular distribution. Phosphorothioate analog of 15-mer oligoadenylate (dA₁₅) labeled by ATTO 425 was complexed with 5,10,15,20-tetrakis (1-methyl-4-pyridyl) porphyrin (H₂TMPyP4) as an uptake-mediating agent. Fluorescence lifetime data within a broad spectral range have revealed properties of both components inside the cell. H₂TMPyP4 lifetime inside the cell is not influenced in this malignant cell line, while the lifetime of modified oligonucleotide was found to be slightly shortened.

Keywords: Porphyrin, fluorescence lifetime, time-resolved microspectrofluorimetry, fluorescence imaging

1. Introduction

Target delivery of drugs and their controlled release represent the most challenging problems of the current biomedical research. The most serious obstacle complicating the use of negatively charged synthetic oligonucleotides in antisense, antigene, and aptamer strategies [1, 2] consists in the necessity to transport them through the cell membrane against potential gradient. Various penetration enhancers have been thus suggested, realized, and studied as delivery agents, including water-soluble cationic porphyrins forming reversible ionic complexes with polyanionic oligonucleotides [3–5]. Due to effective charge compensation without covalent attachment to the transport agent, the complexed oligonucleotides can penetrate into the cells more easily, and they are not deprived to form stronger and more specific complexes with their natural targets inside the cells. To evaluate effectiveness of the delivery process and to track subsequent behavior of the complex and its constituents inside the living cells, microspectroscopic techniques sufficiently sensitive-to-low concentrations are needed. Using

the fluorescence-labeled oligonucleotides and innately fluorescent porphyrins as delivery agents, useful information can be acquired by a combination of two complementary microfluorescence techniques, the fluorescence microimaging and the frequency domain microspectrofluorimetry [6].

In the present study, the approach was tested using the ATTO 425-labeled phosphorothioate oligoadenylate as an oligonucleotide transported and the fluorescent H₂TMPyP4 porphyrin as a delivery agent. Lifetimes of both fluorophores inside the living cells were studied by a fluorescence microspectrometer with two-stage gain modulated image intensifier specially designed for such studies. Fluorescence micro-imaging was used to monitor transport of the complex through the cellular membrane and to visualize subsequent distribution of the respective components in the intracellular compartments.

2. Experimental

Detailed description of our experimental setup can be found in [6]. Here, we would like to summarize the main technical facts of the setup. An Optiphot-2 epifluorescence microscope equipped with a Nipkow wheel coaxial-confocal attachment (Technical Instruments, Germany) with TE-cooled CCD camera (MicroMAX, Princeton Instruments, USA) was used to obtain fluorescence micro-images. The microspectrofluorometer has been built on a phase modulation principle by using a homodyne method. The fluorescence lifetime can be determined for all emission wavelengths by acquiring several spectral images (regularly 6) at different excitation and detector gain modulation phase shifts. To resolve multiple components of the fluorescence lifetime, the spectra acquisition was performed for the frequency interval from 10 to 180 MHz. Laser diode module (Omicron LDM 442.50.A350) with 50 mW output (attenuated 1 to tens μ W at the sample level) was used for the excitation at 442 nm wavelength. This excitation wavelength matches ATTO 425 excitation maximum and the porphyrin Soret band very well.

Confocal epifluorescence upright microscope (Zeiss UMSP-80) is used with a high numerical aperture water-immersion 63x objective (Zeiss Neofluar, NA 1.2). Fluorescence signal collected from the pinhole is focused on the entrance slit of the Jobin-Yvon HR640 spectrograph equipped with a 100 line/mm grating. Spectral detection window (375 nm wide) is covering both the excitation wavelength (reference) and the fluorescence emission. The detection part consists of a gain modulated image intensifier which is optically coupled with the CCD detector (1024 $\times$ 1024 pixels). USB communication interface is used to connect the CCD controller to the control computer, which also drives directly both high-frequency digital synthesizers (modulation frequency, the phase, and the amplitude of the output signal) *via* serial interface.

The LIFLIM software (Lambert Instruments) is used for the setup control and data acquisition. In-house software (PHR) has been written to calculate the phase shift and the demodulation spectral dependence of the fluorescence signal from the acquired data correctly. It also transforms the data into a global fitting procedure, where subsequent lifetime calculation and spectral decomposition are performed.

3. Sample Preparation

Synthetic phosphorothioate oligonucleotide (15-mers adenylate) labeled by ATTO 425 at the 3'-end (ATTO-dA₁₅) was synthesized by Biomers, Germany. The 5,10,15,20-tetrakis (1-methyl-4-pyridyl) porphyrin (H₂TMPyP4) was purchased from Sigma-Aldrich, Co.

Figure 1: Fluorescence microimages of MCF-7 cells incubated overnight with ATTO-dA₁₅/H₂TMPPyP4 complex obtained with different emission filters. (a): H₂TMPPyP4 emission (610–680 nm); (b): details of the porphyrin and ATTO 425 emission from the cell nucleus and nucleoli; (c): ATTO-dA₁₅ emission (430–500 nm).

MCF-7 cancer cells (human breast adenocarcinoma cell line) used in this study were treated as usual; cultured in 25 cm² flasks at 37°C in a humidified 5% CO₂ atmosphere. Growth medium with Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% calf fetal serum, 2 mM L-glutamine, streptomycin (0.1 mg/mL), penicillin (100 U/mL), and sodium pyruvate was used. Cells were subcultured in Petri dishes 48 hours before experiment and incubated overnight with solution of oligonucleotide/porphyrin complex (mixture of stock solutions of H₂TMPPyP4 and modified oligonucleotide ATTO-dA₁₅ in final concentration 3.5×10^{-5} M and 6×10^{-6} M, resp.).

4. Experimental Results

In this study, we have focused on internalization of modified oligonucleotide into the cancerous cell line MCF-7 by means of cationic H₂TMPPyP4. The cellular uptake of labeled phosphorothioate ATTO-dA₁₅ was mediated by H₂TMPPyP4, and it has been already fruitfully used in our previous studies [7, 8]. The fluorescence micro-imaging of the typical distribution of both fluorophores inside the MCF-7 cells is seen in Figure 1. Similarly to the cellular uptake to 3T3 cell line [7], the H₂TMPPyP4/ATTO-dA₁₅ complex is accumulated in the nuclei of MCF-7 cells, the oligonucleotide being more preferentially concentrated into the nucleoli. Only a small fraction of the oligonucleotide and porphyrin remains in the cytoplasm, as evident from combined picture showing in detail porphyrin and oligonucleotide fluorescence emission of a single cell (Figure 2, middle).

Time-resolved microfluorescence spectra were collected separately from the cellular nucleus and from the cytoplasm. Both fluorophores were excited by 442 nm laser line that is close to their absorption maxima. Resulting two-component emission experimental spectrum coming from the cellular nucleus is shown in Figure 2. Peak of elastic scattering serves as a zero lifetime reference. Important advantage for further spectrum analysis is the fact, that both ATTO-dA₁₅ and H₂TMPPyP4 spectral contributions are clearly separated. Global fitting procedure has provided only two lifetimes, one for each fluorophore. The ATTO-dA₁₅ fluorescence in the nucleus can be characterized by a single lifetime of 3.5 ns, whereas the lifetime in the solution was 4 ns. Consequently, the ATTO lifetime seems to be slightly shortened in the MCF-7 cellular environment. It should be emphasized that the same ATTO lifetime was obtained both for the oligonucleotide in the nucleus and in the cytoplasm. The fluorescence of H₂TMPPyP4

Figure 2: Lifetime-resolved fluorescence spectrum collected from the cell nucleus of the MCF-7 cell incubated with ATTO-dA₁₅/H₂TMPyP4 complex.

possesses only a single lifetime component nearby 6 ns. The single lifetime of the same value was found for the porphyrin in the nucleus, cytoplasm, and solution. Although the H₂TMPyP4 lifetime surprisingly exhibits no change with its intracellular localization, H₂TMPyP4 undoubtedly forms a complex with the modified oligonucleotide, facilitates transport into the cell, and probably changes its binding status in the nucleus and nucleoli.

5. Conclusion

Combination of two fluorescence techniques represents effective tool for reliable monitoring of the penetration and distribution of labeled oligonucleotides inside the living cells. Fluorescence micro-imaging can promptly confirm successful penetration and watch the chromophore's distribution in the cellular environment. The buildup homodyne time-resolved microspectrofluorometer has broad spectral window, and it is profitable for monitoring of multifluorophore molecular systems. H₂TMPyP4/modified oligonucleotide ATTO-dA₁₅ complex was found to penetrate successfully into MCF7 human epithelial breast adenocarcinoma with preferential accumulation in the cellular nucleus. H₂TMPyP4 emission lifetime is not influenced in this malignant cell line, while the oligonucleotide emission lifetime was found to be slightly shortened.

Acknowledgment

Financial support from The Grant Agency of the Czech Republic (Project no. P208/10/0941) is gratefully acknowledged.

References

- [1] E. Uhlmann and A. Peyman, "Antisense oligonucleotides: a new therapeutic principle," *Chemical Reviews*, vol. 90, no. 4, pp. 543–584, 1990.

- [2] J. Goodchild, "Oligonucleotide therapeutics: 25 years agrowing," *Current Opinion in Molecular Therapeutics*, vol. 6, no. 2, pp. 120–128, 2004.
- [3] L. Benimetskaya, G. B. Takle, M. Vilenchik, I. Lebedeva, P. Miller, and C. A. Stein, "Cationic porphyrins: novel delivery vehicles for antisense oligodeoxynucleotides," *Nucleic Acids Research*, vol. 26, no. 23, pp. 5310–5317, 1998.
- [4] J. Králová, M. Dvořák, and V. Král, "Novel cationic transport agents for oligonucleotide delivery into primary leukemic cells," *Journal of Medicinal Chemistry*, vol. 46, no. 11, pp. 2049–2056, 2003.
- [5] P. Praus, E. Kočišová, O. Seksek, F. Sureau, J. Štěpánek, and P. Y. Turpin, "Advanced microfluorescence methods in monitoring intracellular uptake of "antisense" oligonucleotides," *Current Organic Chemistry*, vol. 11, no. 6, pp. 515–527, 2007.
- [6] P. Praus, E. Kočišová, P. Mojzeš, J. Štěpánek, F. Sureau, and P. Y. Turpin, "Frequency domain fluorescence microspectrometry: application to cellular uptake and drug distribution," *Spectroscopy*, vol. 24, no. 3-4, pp. 303–307, 2010.
- [7] P. Praus, E. Kočišová, P. Mojzeš, J. Štěpánek, P. Y. Turpin, and F. Sureau, "Cellular uptake of modified oligonucleotides enhanced by porphyrins studied by time-resolved microspectrofluorimetry and fluorescence imaging techniques," *Journal of Molecular Structure*, vol. 993, no. 1–3, pp. 316–318, 2011.
- [8] P. Praus, E. Kočišová, P. Mojzeš et al., "Time-resolved microspectrofluorimetry and fluorescence imaging techniques: study of porphyrin-mediated cellular uptake of oligonucleotides," *Annals of the New York Academy of Sciences*, vol. 1130, pp. 117–121, 2008.