Pulsed EPR Spectroscopy in Cells

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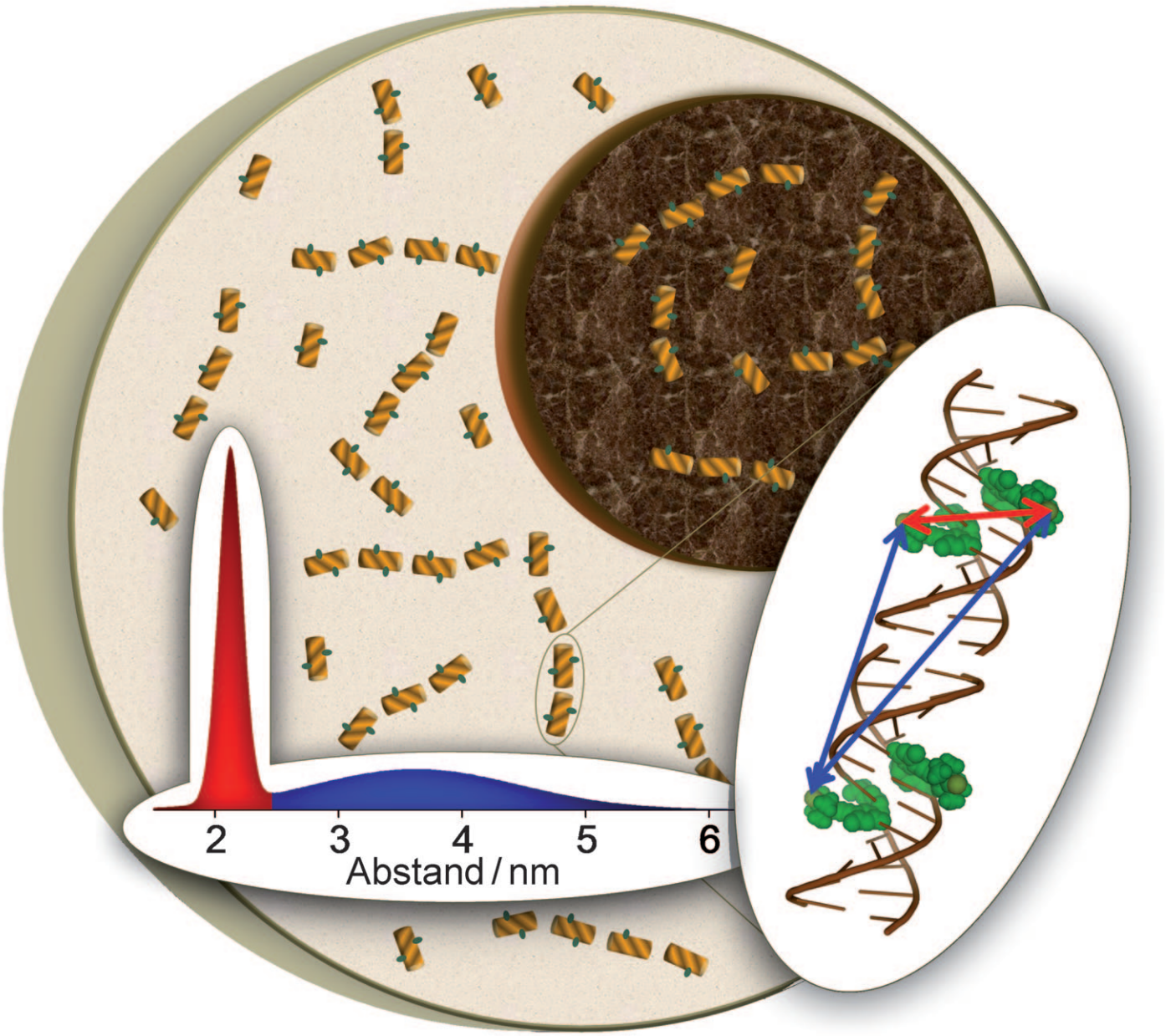
Long-Range Distance Measurements on Nucleic Acids in Cells by Pulsed EPR Spectroscopy**

*Ivan Krstić, Robert Hänsel, Olga Romainczyk, Joachim W. Engels, Volker Dötsch, and Thomas F. Prisner**



The tertiary structure and conformational dynamics of ribonucleic acids (RNAs) are essential for their function as biological catalysts, regulators, and structural scaffolds.[1] X-ray crystallography, NMR, continuous-wave (cw) EPR, and fluorescence spectroscopies are well-established methods to investigate their structural and dynamic properties.[2] Additionally, over the past few years pulsed electron–electron double-resonance (PELDOR)[3] spectroscopy has been used to determine distances in nucleic acid (NA) molecules in the range of 1.5–7 nm. The method measures the magnetic dipole–dipole interaction between two nitroxide spin labels covalently attached to the oligonucleotide molecule.[4] PELDOR spectroscopy has proven to provide very accurate distances and distance distribution functions for double-labeled oligonucleotides.[5] Thus, we can obtain important information on the tertiary structure as well as on the conformational flexibility of the molecule.[6] The structure and dynamics of NAs depend on environmental factors, such as the concentration of ions, small molecules, molecular crowding, viscosity, and interactions with proteins.[7] Therefore, it is important to investigate whether the in vitro determined NA structure reflects the intracellular (in vivo) conformation.

Because of the high sensitivity of EPR spectroscopy, it can also be applied to in vivo systems. In the past, nitroxide labels have been used for in vivo EPR applications to determine the oxygen concentration, pH value, redox state, molecular mobility, and polarity of the local environment, and for spatial mapping of the free-radical metabolism.[8] Furthermore spin traps have been used for detection of nitric oxide, superoxide, and other reactive oxygen species.[8] Recently, first distance measurements on a double-spin-labeled protein (ubiquitin) in cells were reported.[9] Here, we applied for the first time the PELDOR method to determine structural aspects of RNA and DNA molecules inside living *Xenopus laevis* oocytes.

[*] I. Krstić,[+] Dr. T. F. Prisner
Institute of Physical and Theoretical Chemistry
and Center for Biomolecular Magnetic Resonance (BMRZ)
Goethe University Frankfurt
Max-von-Laue-Str. 7, 60438 Frankfurt am Main (Germany)
Fax: (+49) 69-798-29-404
E-mail: prisner@chemie.uni-frankfurt.de
Homepage: http://www.prisner.de
R. Hänsel,[+] Dr. V. Dötsch
Institute of Biophysical Chemistry
and Center for Biomolecular Magnetic Resonance (BMRZ)
Goethe University Frankfurt
Max-von-Laue-Str. 9, 60438 Frankfurt am Main (Germany)
Dr. O. Romainczyk, Dr. J. W. Engels
Institute of Organic Chemistry and Chemical Biology
Goethe University Frankfurt
Max-von-Laue-Str. 7, 60438 Frankfurt am Main (Germany)
[+] These authors contributed equally to this work.

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Mature *Xenopus laevis* oocytes (stage VI) arrested at the G2M transition of the cell cycle are routinely used for studying cellular and developmental biology as well as for in-cell NMR experiments.[10] We recently demonstrated that DNA and RNA hairpins and DNA quadruplexes can be observed in *Xenopus laevis* oocytes by in-cell NMR spectroscopy.[11] In these experiments we showed that the conformation of a telomeric G-quadruplex sequence in vitro is different from that in a cellular environment. Here, we have used PELDOR spectroscopy on a double-labeled 12 base pair (bp) DNA duplex,[5b] a 14-mer cUUCGg tetraloop hairpin RNA,[12] and the 27-mer neomycin-sensing riboswitch[13] to obtain long-range distance constraints on such systems in *Xenopus laevis* oocytes and to compare them with in vitro measurements.

The reduced lifetime of nitroxide radicals under in vivo conditions is a major obstacle for such measurements. Although it may not be the primary source of metabolism in vivo, reduction by ascorbic acid can serve as a screening test for the nitroxide reactivity. The reduction kinetics in ascorbic acid solution depend on the ring structure of the nitroxide: in general, the reduction of five-membered pyrrolidine and pyrroline derivatives is significantly slower than the reduction of a six-membered piperidine derivative.[14] Because the five-membered-ring nitroxide spin label 2,2,5,5-tetramethyl-pyrrolin-1-oxyl-3-acetylene (TPA), which we used as a spin label for the NA molecules, is poorly water soluble, we used its precursor 2,2,5,5-tetramethyl-pyrroline-1-oxyl-3-carboxylic acid amide (TPOA) for determining its in-cell reduction kinetics at room temperature relative to the in-cell reduction kinetics of the six-membered-ring representative 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL). The chemical structures of the nitroxide radicals used in our study are depicted in Figure S1 in the Supporting Information.

Nitroxide reduction in *Xenopus laevis* oocytes follows the general trend described above (Figure S2 in the Supporting Information). Accordingly, the six-membered piperidine-type nitroxide TEMPOL was metabolized much faster in the oocyte extract (lifetime 3.8 ± 0.1 min) than the five-membered pyrroline nitroxide radical TPOA (lifetime 46.0 ± 0.1 min), leading to the corresponding diamagnetic hydroxylamine moiety. The persistence of the spin label attached to different NAs against reduction in cells was also studied by cw EPR spectroscopy. Figure 1 shows that the in-cell reduction kinetics for the TPA spin label attached to NAs is much slower than the in-cell reduction kinetics for free TPOA. This effect can presumably be ascribed to steric protection of the N–O part of the radical by the NA structure or to electrostatic repulsion of the negatively charged reducing agent by the NA molecule. Steric shielding was also reported as the protective effect of replacing methyl with ethyl groups in neighboring positions to the NO moiety of the imidazoline, imidazolidine, and piperidine nitroxide radicals in aqueous solution of both ascorbic acid and rat blood samples.[15] The in-cell lifetimes of the tetraethyl-substituted pyrrolidine- and pyrroline-based nitroxide spin labels attached to NA molecules are expected to be even larger.

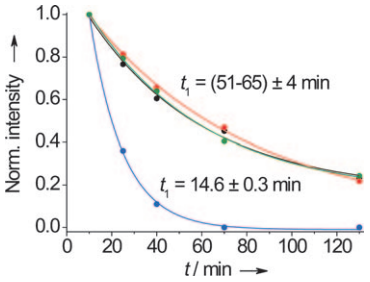

Figure 1. Time versus intensity curves displaying the in-cell reduction kinetics for the spin label TPA attached to a 27-mer neomycin-sensing riboswitch (black), a 14-mer RNA hairpin (red), a 12 bp duplex DNA (green), and for the free spin label TPOA (blue).

In vitro PELDOR experiments were carried out in intra-oocyte buffer[11] with a final concentration of 150 μM double-labeled oligonucleotide. For the in-cell experiments, injection of 30–50 nL of 2.5–5 mM double-labeled NAs in roughly 50 oocytes takes up to 10 minutes (about 10 s for each oocyte, respectively). Subsequent washing of the oocytes with a buffer, transfer into an EPR tube, and settling by gravity take another 5 min. Samples were then either directly frozen in liquid nitrogen or first incubated at room temperature for additional 30 and 60 min. The shortest possible incubation time was thus on average about 10 minutes, whereas because of the spin-label reduction, the longest possible incubation time was in total 70 minutes. Samples prepared with the shortest incubation time showed a faster transverse relaxation than samples prepared with longer incubation times (Figure S4 in the Supporting Information), limiting our observation time window in PELDOR experiments to a maximum of 1.5 μs. Nevertheless, this time is sufficiently long to observe dipolar oscillation for all measured NA molecules which makes reliable interpretation of the data possible (Figures 2 and 3). PELDOR time traces for all three NAs show clearly visible oscillations, indicating well-structured molecules. The faster transverse relaxation of the shortest incubated in-cell samples is caused by the high local concentration of spins[16]

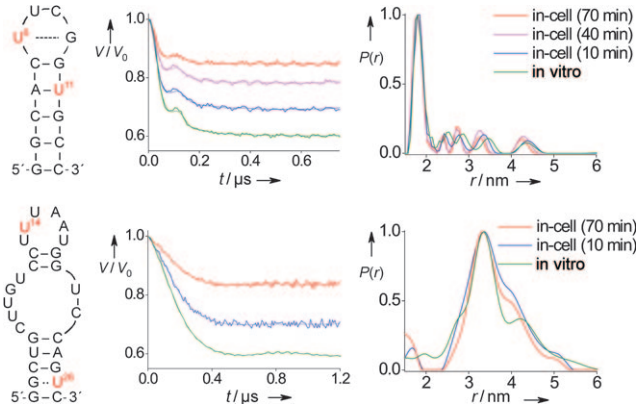

Figure 2. Secondary structures (with spin-labeled nucleotides in red), baseline-corrected PELDOR time traces, and distance distribution functions for the double-labeled 14-mer cUUCGg tetraloop hairpin RNA (upper panel) and the 27-mer neomycin-sensing riboswitch (lower panel). The in-cell data after different incubation times are compared with in vitro data.

immediately after injection of the concentrated stock solution of the labeled NAs into oocytes. Increasing the incubation time allows for translational diffusion of the spin-labeled oligonucleotides and in parallel for spin-label reduction in cells, decreasing the local spin concentration. This leads to a longer transverse relaxation time, comparable to the transverse relaxation time of the in vitro samples.

The PELDOR time domain signal is the product of two contributions: intermolecular and intramolecular dipolar interactions. The former encodes information about the local spin concentration and the local dimensionality of the spatial distribution of spin labels and is described by a monotonically decaying function. The latter contribution yields information about intramolecular spin–spin distances, distance distributions, the labeling efficiency, and oligomeric state.[17] The PELDOR time traces after removal of the intermolecular background function as well as the distance distribution functions obtained from Tikhonov regularization[18] for double-labeled RNAs are shown in Figure 2. The modulation depth of the in vitro measurements of about 40 % (defined as the decay of the normalized PELDOR time traces at the maximum evolution time) indicates a labeling efficiency of around 90 %. Distances of 1.8 nm for the cUUCGg tetraloop RNA hairpin and 3.4 nm for the neomycin riboswitch are obtained from the in vitro time traces. The widths of the distance distributions are 0.27 nm for the hairpin and 0.7 nm for the riboswitch. This indicates a rather rigid structure of the RNA hairpin, where U6 is *trans*-wobble base-paired with G9, and U11 is located in the rigid canonical closing stem.

The tertiary structure of the riboswitch shows a somewhat larger flexibility, in agreement with reported NMR[13] and EPR[6d] data. All in-cell time traces show progressively smaller modulation depths for longer incubation times. This originates from the partial loss of dipolar-coupled spin pairs as a result of in-cell nitroxide reduction. Nevertheless, the signal-to-noise ratio is sufficient for a quantitative interpretation of the PELDOR data. The observed oscillations in the time trace for the hairpin RNA sample result again in one prominent peak in the distance distribution function, with no alteration in the mean distance and peak width. This suggests that the overall structure of the 14-mer cUUCGg tetraloop hairpin RNA remains rigid and unaltered in the cellular environment even after one hour of incubation. Similar conclusions can be drawn for the double-labeled neomycin-riboswitch. Despite slight broadening in the distance distribution for the in-cell measurements, the mean intramolecular distance persists. This indicates that the global architecture of the 27-mer neomycin-sensing riboswitch is preserved inside *Xenopus laevis* oocytes.

Figure 3 compares the in vitro and in-cell PELDOR results of the spin-labeled DNA duplex. In contrast to the RNA samples, the in-cell time traces show a change in the oscillation pattern relative to the time traces of the in vitro experiments. The in vitro time trace can be fitted by a single Gaussian distance distribution function with a mean value of 2.1 nm and a width of 0.14 nm, whereas the in-cell time traces can be fitted only by a superposition of two Gaussian distance distribution functions. The short distance is the same as in the

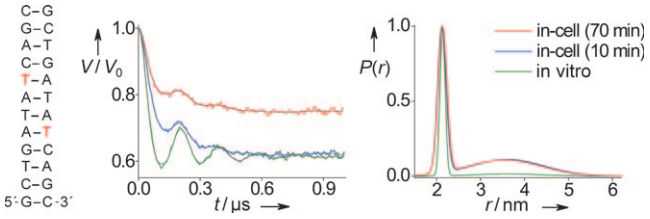

Figure 3. The secondary structure (with spin-labeled nucleotides in red), baseline-corrected PELDOR time traces, and distance distribution functions for 12 bp double-labeled DNA in vitro and in cells (incubation times shown in the legend). The data were fitted with two Gaussian functions.

in vitro measurement, indicating that the duplex structure is undisturbed in cells. The longer distance (3.7 nm) with a very broad distribution (2 nm) may arise from the stacking of DNA molecules in cells. End-to-end stacking of 6–20 bp DNA duplexes and ordering into semirigid rod-shaped structures is a well-known phenomenon.[19] In-cell reduction of nitroxide labels attached to DNA occurs with the same rate as for RNAs, but the modulation depth in PELDOR experiments is deeper for the DNA sample. This increase in modulation depth can be ascribed to dipolar interaction of more than two coupled spins,[17] which would be in accordance with our assumption of DNA assembling. An additional argument for this assumption is that the best fit for the intermolecular background function of the in-cell DNA PELDOR time traces is a one-dimensional spin distribution, which is in agreement with a linear stacking of DNA.

To the best of our knowledge, this is the first in-cell application of PELDOR spectroscopy to NA molecules reported to date. We determined in-cell intramolecular distances in NA molecules with high precision by PELDOR. The fact that the distances measured in the in vitro and in-cell experiments were the same implies the existence of stable global structures of the 14-mer cUUCGg tetraloop hairpin RNA and the 27-mer neomycin-sensing riboswitch. In contrast, the double-stranded short DNA molecule showed some variation in the PELDOR time traces relative to the PELDOR time traces recorded by in vitro measurements, which were interpreted as the stacking of DNA duplexes. Because of the prolonged lifetime of the TPA nitroxide labels covalently attached to NA molecules PELDOR signals could be measured with good signal-to-noise ratios at incubation times of up to 70 minutes. The partial loss of coupled spin labels because of nitroxide reduction only led to a decrease in the modulation depth upon increased incubation time. The application of tetraethyl-substituted nitroxide radicals resistant to bioreduction will make possible an extension of the incubation time to follow biological processes in cells, such as diffusion, interaction with proteins, and other factors or chemical reactions. These results lay a foundation for the application of PELDOR spectroscopy for probing structural aspects of nucleic acids through the cell-cycle progression.

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