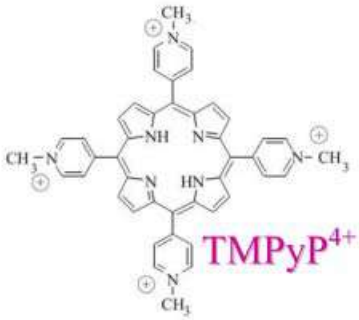
Olga Ryazanova

THE MOLECULAR SPECTROSCOPY AS A POWER TOOL FOR STUDYING THE PORPHYRIN–DNA INTERACTION

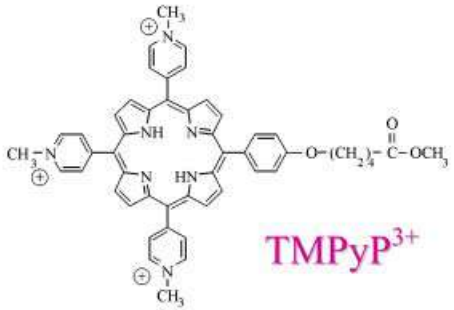
B. Verkin Institute for Low Temperature Physics and Engineering of NAS of Ukraine, 47 Nauky Ave., 61103, Kharkiv, Ukraine

NBP 2025, 6-9 October 2025, Kharkiv, Ukraine

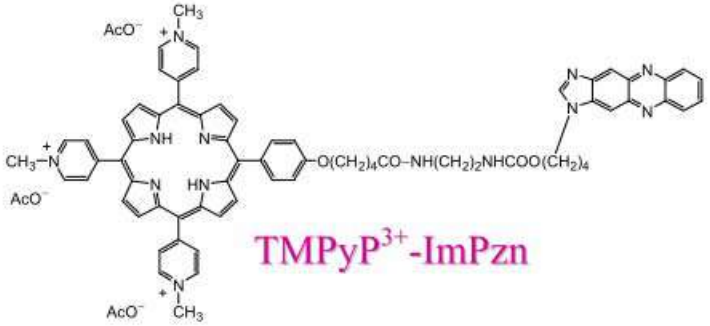
CATIONIC PORPHYRINS are macrocyclic compounds with extended planar chromophore and unique spectroscopic and photophysical properties. They selectively accumulate in the tumor cells and have a high extinction coefficient in the red region of the spectrum where the transparency of tissues to light increases considerably → **photosensitizer for PDT of cancer**.



$TMPyP^{4+}$



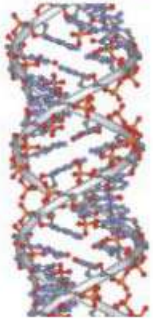
$TMPyP^{3+}$



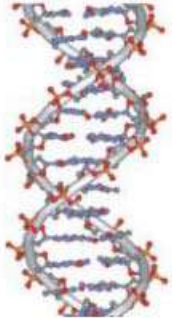
$TMPyP^{3+}$-ImPzn

Double-stranded polynucleotides

A-form (*ds*-RNA and DNA·RNA hybrids)



B-form (*ds*-DNA)



- quite water-soluble
- intensively fluorescent chromophores
- readily derivatized
- cationic group facilitate their binding to nucleic acids
- G-quadruplex binding ligands and stabilizers
- human telomerase inhibition IC_{50} = 6.5 μM
- side chain give the possibility to conjugate the dye with other one

G-quadruplexes

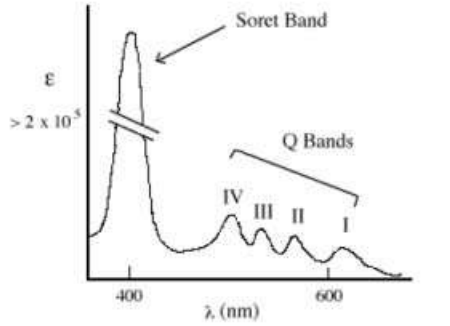
poly(G), 5'-d[AGGG(TTAGGG)$_3$]-3'

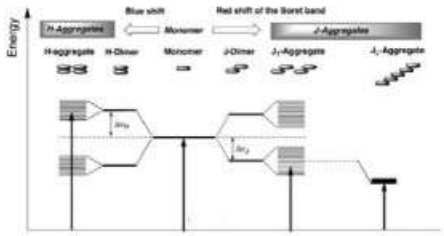
Cheong C., Moore P.B. *Biochemistry* **31** (36) (1992) 8406-8414.

MOLECULAR SPECTROSCOPY TECHNIQUES are very effective for studying porphyrin – DNA/RNA interaction.

ABSORPTION SPECTROSCOPY (AS)

UV-Vis absorption spectroscopy (spectrophotometry) is one of the main experimental methods to study the complex formation between porphyrins and nucleic acids, to determine its composition, apparent binding constant and thermodynamic characteristics (changes in enthalpy, entropy, and free Gibbs energy). The advantages: a possibility to identify complexes without their damage; simplicity; sensitivity; consistency of results and low-cost.





J. Phys. Org. Chem. 2004; **17:** 890–897

Bouguer–Lambert–Beer law: $D(\lambda) \equiv \lg\left(\frac{I_0}{I}\right) \equiv \lg\left(\frac{1}{T}\right) = C\varepsilon(\lambda)l$

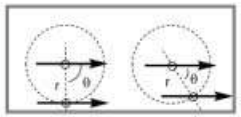
Registration of changes in shape, position and intensity of the porphyrin Soret absorption band (and other bands) caused by the nucleic acids addition gives the information about porphyrin-DNA binding modes. [Fiel R. *J Biomol Struct Dynam* 6 (1989)1259-1274. & Pasternack R. et al. *Biochemistry* 22(10) (1983) 2406–2414].

Intercalation of porphyrin between DNA base pairs (GC-regions, at high *P/D*): hypochromism of DNA UV–absorption band and the porphyrin Soret band (> 40%), red shift of the Soret band > 15 nm [1, 2].

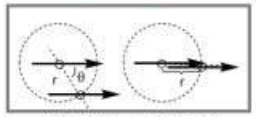
Monomeric groove binding of the porphyrin to DNA (AT-regions, at high *P/D*): no or minor changes of the porphyrin UV-VIS spectrum.

Formation of the extended porphyrin aggregates (with/without self-stacking) externally bound to DNA backbone results in the hypochromism and blue shift of the Soret band in the case of *H*-aggregates and its red shift for *J*-aggregates.

***H*-aggregates** ***J*-aggregates**



$54.7° \le \theta < 90°$

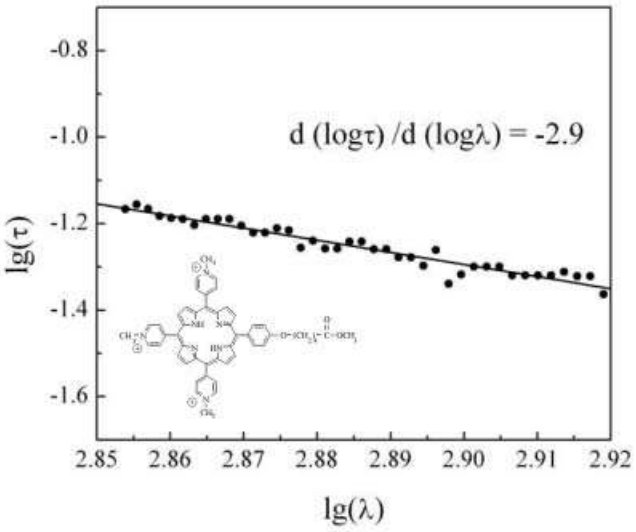


$0 \le \theta < 54.7°$

Stabilization of porphyrin aggregates:

electrostatic forces π-π stacking H-bonding
van der Waals forces hydrophobic interaction

Estimation of the size of the $TMPyP^{3+}$-poly(P) aggregates at *P/D* = 5 from the solution turbidity data in the range of λ = 710 - 830 nm using the method of Doty and Steiner [P. Doty, R.F. Steiner, *J. Chem. Phys.*18 (1950) 1211–1220].



The dependence of τ on λ was registered by measuring the absorbance in the range of λ = 710-830 nm, where the porphyrin does not absorb.

$$-\frac{d\log\tau}{d\log\lambda} = 4 - \frac{d\log Q}{d\log\lambda} = 4 - \beta \qquad \tau = 2.303A$$

$$\tau = \ln\frac{I_0}{I} \qquad A = \lg\frac{I_0}{I}$$

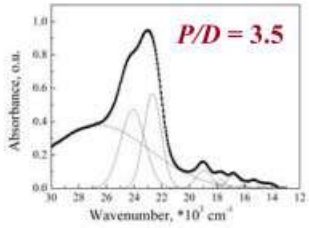
τ - solution turbidity
Q - particle dissipation factor
λ - wavelength of the light in vacuum

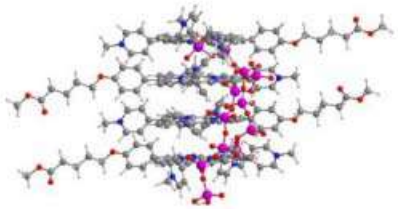
$$\frac{d\log\tau}{d\log\lambda} = -2.9 \qquad \beta = 1.1$$

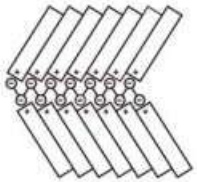
It is most probably that the aggregates adopt a conformation of the **polydisperse coils**

$D/\lambda' \approx 0.8$ D - coil diameter $\lambda' = 770$ nm - the mean value of light wavelength in water solution over the measured wavelength range

$D \approx 620$ nm







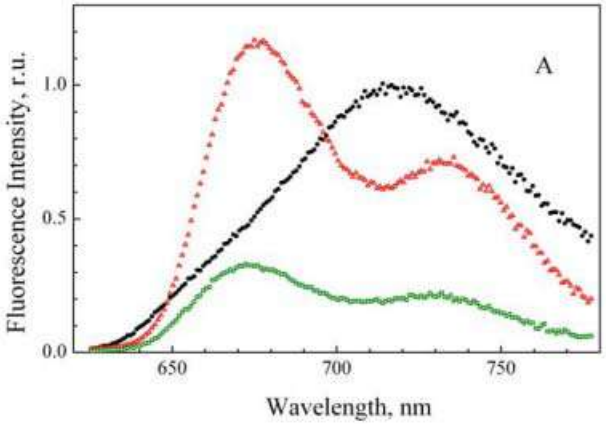
Possible arrangement of two continuous $TMPyP^{3+}$ stacks on the template of single poly(P) chain.

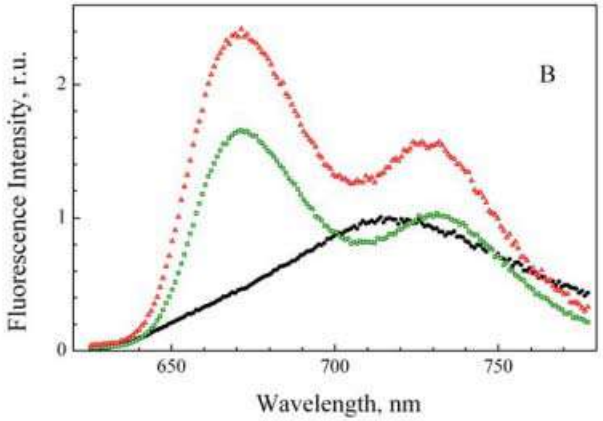
Zozulya V., Ryazanova O.A. et al. *Biophys. Chem.* 185(1) (2014) 39-46.

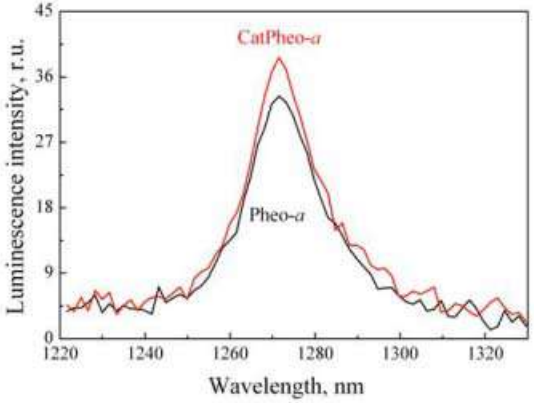
POLARIZED FLUORESCENCE SPECTROSCOPY (PFS)

have an exceptionally high sensitivity compared to absorption spectroscopy (10^{-6} - 10^{-7} M of fluorescent substance is sufficient for registration) and provide unique opportunities for studying the binding of porphyrins to nucleic acids, to obtain information on their excited states, photochemical reactions, the dynamics of fast molecular processes, the structure and properties of complexes formed, binding sites, interactions with the solvent, the degree of flexibility, intermolecular distances and rotational diffusion of macromolecules [Lakowicz JR (2006) Principles of Fluorescence Spectroscopy (3rd ed.), Springer, New York., Valeur, B. Molecular fluorescence: principles and applications / B. Valeur. -Wiley-VCH Verlag GmbH, 2001.- 399 p].

Registration of changes in the shape, position and intensity of the porphyrin emission band, as well as its fluorescence polarization degree caused by the nucleic acids addition was performed.







Fluorescence spectra of $TMPyP^{3+}$ in a free state (●) and (A) bound to poly(G)·poly(C) at $P/D = 3.1$ (■) and $P/D = 399$ (Δ) (B) to poly(A)·poly(U) at $P/D = 3.1$ (■) and $P/D = 265$ (Δ) in 5 mM PBS buffer, $C_{dye} = 10$ μM, $\lambda_{exc} = 500$ nm.

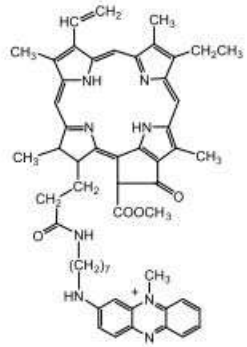
Luminescence spectra of singlet oxygen $^{-}$ generated by **the CatPheo-*a*** and **Pheo-*a*** in ethanol solution, λ_{exc}=665 nm.

O. Ryazanova et al., J. Fluoresc., **25(4),** 1013 (2015)
O. Ryazanova et al. , J. Fluoresc. (2024). doi: 10.1007/s10895-024-04000-4

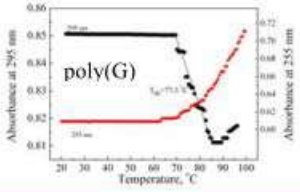
FLUORIMETRIC TITRATION (FT) is a powerful tool to study the porphyrin binding to nucleic acids.

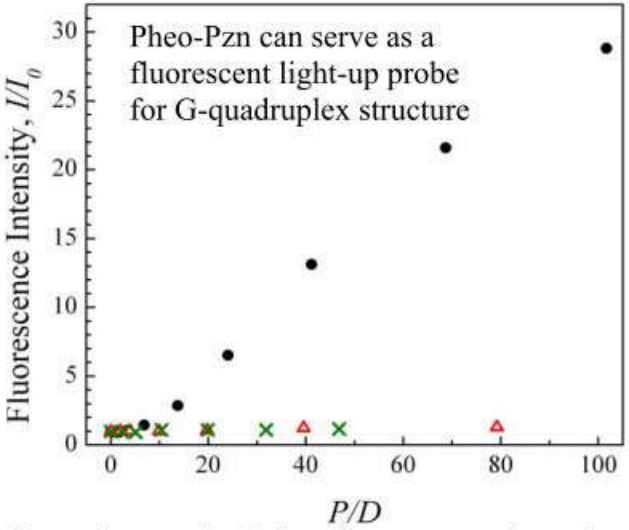
see Eftink M.R. Fluorescence methods for studying equilibrium macromolecule-ligand interactions. Method Enzymol 278 (1997) 221-257. doi: 10.1016/s0076-6879(97)78013-3.

The values of porphyrin fluorescence intensities and polarization degree at a fixed wavelength (usually, in the free porphyrin fluorescence band maximum) at different molar phosphate-to dye ratios (*P/D*) are compared. Also the dependence of integral intensity of the porphyrin emission band on *P/D* has to be studied. For this purpose the porphyrin sample is added with increasing amounts of the concentrated polymer stock solution containing the same dye content.

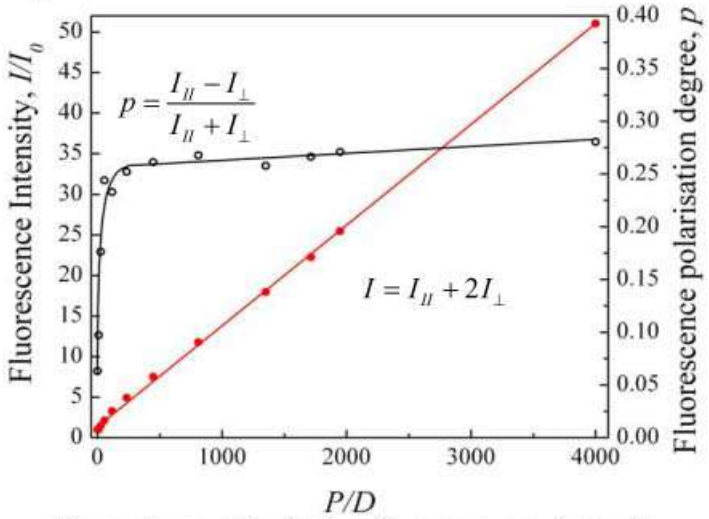


Molecular structure of Pheophorbide a – aminophenazinium conjugate (Pheo-Pzn).





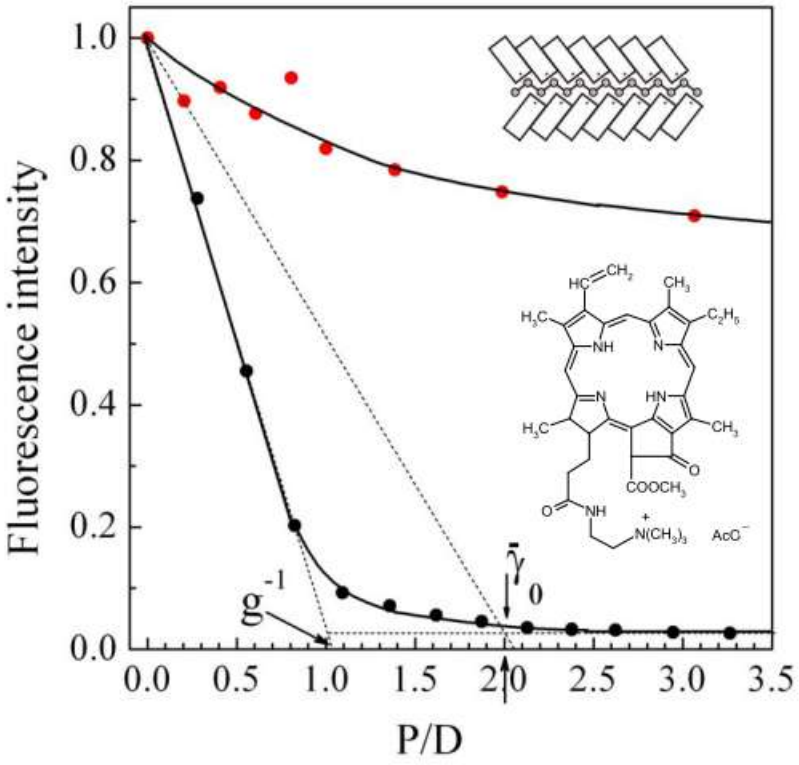
Dependence of relative fluorescence intensity, I/I_0 of **Pheo-Pzn** on *P/D* upon titration with poly(A)·poly(U) (Δ), poly(G)·poly(C) (×) and poly(G) (●) in 2 mM cacodylate buffer with 6.6% of ethanol, C_{dye}=10 μM, λ_{exc} = 633 nm, λ_{obs} = 670 nm



Dependence of relative fluorescence intensity (●), I/I_0, and fluorescence polarization degree, p, of **Pheo-Pzn** on *P/D* upon titration by quadruplex poly(G) in 2 mM cacodylate buffer with 2% of ethanol, C_{dye}=10 μM, λ_{exc} = 633 nm, λ_{obs} = 670 nm

Ryazanova O.A. et al. *J. Mol. Struct.* 1214 (2020), id.128218.

Parameters of cooperative binding of cationic Pheoporbide *a* to inorganic polyphosphate estimated by Schwarz's method from fluorescence titration curve according [*Schwarz G. Eur. J. Biochem. (1970), vol.12, p. 442-453*].



Initial part of fluorescence titration curves of CatPheo-*a* with poly(P) measured in aqueous buffered solution containing 2 mM Na^+ (●) and 0.15 M Na^+ (●), $C_{dye} = 1.3 \cdot 10^{-5}$ M. Excitation wavelength is 633 nm.

A number of binding sites per monomer unit of PPS:

g = 1.00

The cooperative binding constants are:

$$K = \frac{1}{\overline{\gamma}_0 \cdot C_T} \qquad K = qK^+$$

$K \approx 2 \cdot 10^6$ M^{-1} (in solution with 2 mM Na^+)

$K^+ = 1800$ M^{-1}

for binding of monocationic dyes to polyanionic lattice

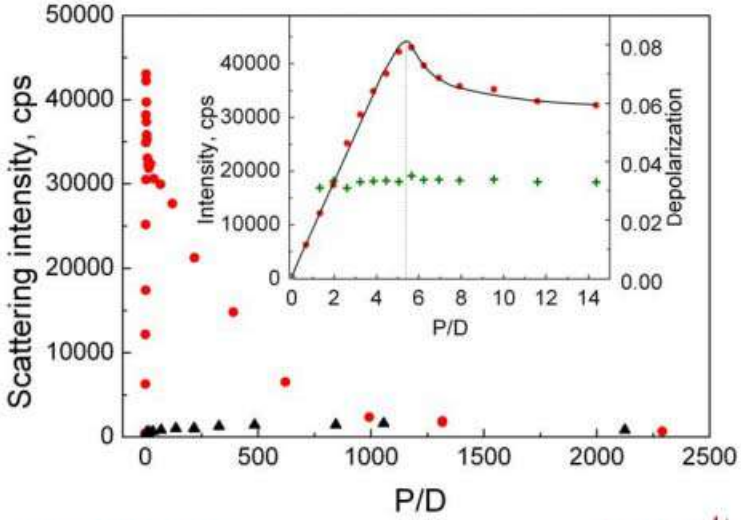
The cooperativity parameter:

$q \approx 1000$

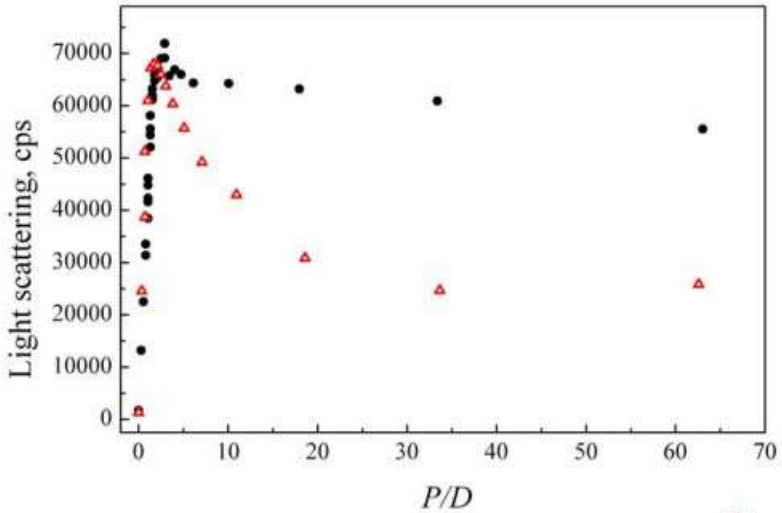
Ryazanova O. et al. Annals of the New York Academy of Sciences 1130 (2008) 293-299.

RESONANCE LIGHT SCATTERING (RLS)

RLS is an effective, highly sensitive and selective method for studying chromophore arrays with strong electronic coupling between chromophores. It was proposed in 1993 by R.F. Pasternak [Pasternack et al., J. Am. Chem. Soc. 1993 Vol. 115, P. 5393-5399. // R.F. Pasternack et al., Science 1995 Vol. 269 P. 935-939] **as an tool for studying the aggregation of porphyrin dyes giving information on the size, shape, and aggregation number of supramolecular aggregates of organic dyes (including heteroaggregates). RLS experiments are usually performed at wavelengths away from absorption bands, but for species that aggregate, enhancements in light scattering of several orders of magnitude can be observed at wavelengths characteristic of these species. For example,** at λ_{exc}= λ_{obs}= **500 nm.**



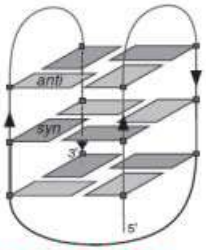
RLS intensity at λ=500 nm *vs P/D* for $TMPyP^{4+}$-poly(P) in deionized water (●) and in water with 0.14 M NaCl (▲) [Zozulya V., Ryazanova O. et al. *J. Fluoresc.* **20(3)** (2010), 695-702].



RLS intensity at λ=500 nm *vs P/D* for $TMPyP^{3+}$ with poly(A)·poly(U) in 5 mM phosphate buffer without (●) and with 0.14 M NaCl (▲) [Ryazanova O. et al. *J. Fluoresc.* (2024), doi: 10.1007/s10895-024-04000-4].

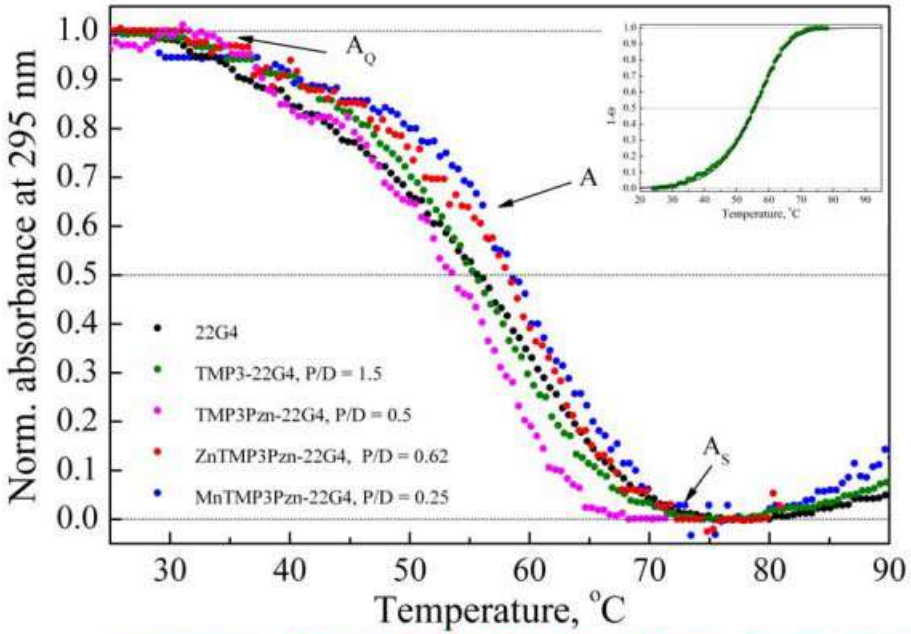
MELTING WITH ABSORPTION AND FLUORESCENCE REGISTRATION

The porphyrin intercalated into DNA stabilizes its double helix against thermal denaturation. It the case of G-quadruplexes the situation is ambiguou



22G4 (Tel22)

repeated non-coding DNA sequence **5'-d[AGGG(TTAGGG)$_3$]-3'** which is presented in the telomeric ends of human chromosomes.



Normalized absorption melting curves for **22G4**, **TMPyP^{3+}+22G4**, **TMPyP^{3+}-Pzn+22G4**, **ZnTMPyP^{3+}-Pzn** and **MnTMPyP^{3+}-Pzn** measured at 295 nm in 2 mM phosphate buffer with 0.1 M NaCl, 0.5 mM EDTA. Oligomer concentration was 10-20 μM in strands. [Zozulya V., Ryazanova O. et al. *Int. Rev. Biophys. Chem.***2(4)** (2011) 112-119.

To evaluate the effect of the porphyrin and porphyrin-phenazine conjugate on thermodynamic parameters of G-quadruplex formation by Tel22 the experimental transition curves were fitted to the equation based on **all-or-none binding model** [L.A. Marky, K.J. Breslauer, Biopolymers (1987) v. 26, pp. 1601–1620]:

$$\Theta(t) = \frac{A_S - A}{A_S - A_Q}$$

Θ - fraction of the oligomer strands forming G4

$$\ln \frac{\Theta}{(1-\Theta)^2 C} = \frac{\Delta S}{R} - \frac{\Delta H}{RT}$$

$$\Delta G = \Delta H - T\Delta S \qquad K_{eq} = e^{-\Delta G / RT}$$

Thermodynamic Parameters of the G-Quadruplex Formation by Tel22 with/without CatPheo-*a* calculated from absorption melting curves

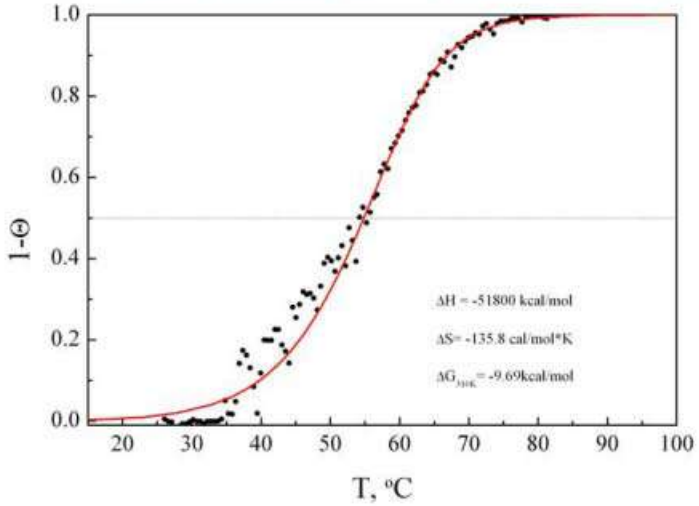


Fig. 7 Transition **4→1** for **CatPheo-*a* + Tel22** complex. Solid lines represent the best fits to the experimental data obtained using two-state model.

To evaluate the effect of CatPheo-a on thermodynamic parameters of Tel22 folding the experimental transition curves were fitted with equation based on all-or-none binding model [*L.A. Marky, K.J. Breslauer, Biopolymers 26 (1987) 1601–1620*]

$$\Theta(t) = \frac{A_{SS} - A}{A_{SS} - A_{G4}} \qquad \ln \frac{\Theta}{(1-\Theta)^2 C} = \frac{\Delta S}{R} - \frac{\Delta H}{RT}$$

Θ – fraction of the oligomer strands forming G4

ΔH – enthalpy change ΔS – entropy change

C – total molar concentration of Tel22 strands

ΔG – Gibbs standard free energy change

Compound	T_m, °C	C, μM (in strands)	*P/D*	-ΔH, kcal/mol	-ΔS, cal/mol·K	-ΔG [a], kcal/mol	K_{eq} [a], M^{-1}
Tel22	55.6	17.5	0.0	52	134.9	10.15	$1.4 \cdot 10^7$
CatPheo-*a* + Tel22	54.7	30.0	0.7	51.8	135.8	9.69	$6.7 \cdot 10^6$

[a] *Data were calculated at t = 37 °C using equations* $K_{eq} = e^{-\Delta G/RT}$ $\Delta G = \Delta H - T\Delta S$

Resonance Raman Spectroscopy (RRS)

RRS is a powerful and versatile high-resolution technique for the study of vibrational and electronic structures of chromophoric molecular systems, interactions of drugs (porphyrins) with biological macromolecules (nucleic acids) under various conditions (changes in pH, ionic strength, or the presence of competing ligands) etc. RR spectra are obtained by irradiation of the sample with a monochromatic light source whose energy is close to that of an electric-dipole-allowed electronic absorption band. Most of the Raman bands are attenuated by the absorption, but some bands may be greatly enhanced. This effect arises from a coupling of the electronic and vibrational transitions, and the vibrational modes that do show enhancement are localized on the chromophore, that is, on the group of atoms that give rise to the electronic transition.

The technique allows researchers to observe shifts in vibrational modes associated with the porphyrin ring and the nucleobases, which can indicate changes in electronic environments and molecular conformations upon binding. For instance, changes in the intensity and position of specific peaks can reveal information about the stacking interactions or intercalative binding.

ADVANTAGES: • RRS is applicable to aqueous solutions under biological conditions;

• it is possible to resonance-enhance chromophores (drug and nucleic acid) vibrations separately by tuning the laser wavelength to that of the electronic transition of each chromophores;

• it requires only a very small volume (~|μL) of a dilute solution (10^{-3} – 10^{-5} M/L) since chromophore vibrations are enhanced by a factor of 10^4 – 10^5 under resonance conditions.

Blom N., Odo J., Nakamoto K., Strommen D.P. Resonance Raman studies of metal tetrakis(4-N-methylpyridyl)porphine: band assignments, structure-sensitive bands, and species equilibria, J. *Phys. Chem*., 1986, V. 90, P. 2847–2852.

Strommen D.P., Nakamoto K. Resonance Raman spectroscopy. J. Chem. Educ. 54(8) (1977) 474-478. doi: 10.1021/ed054p474

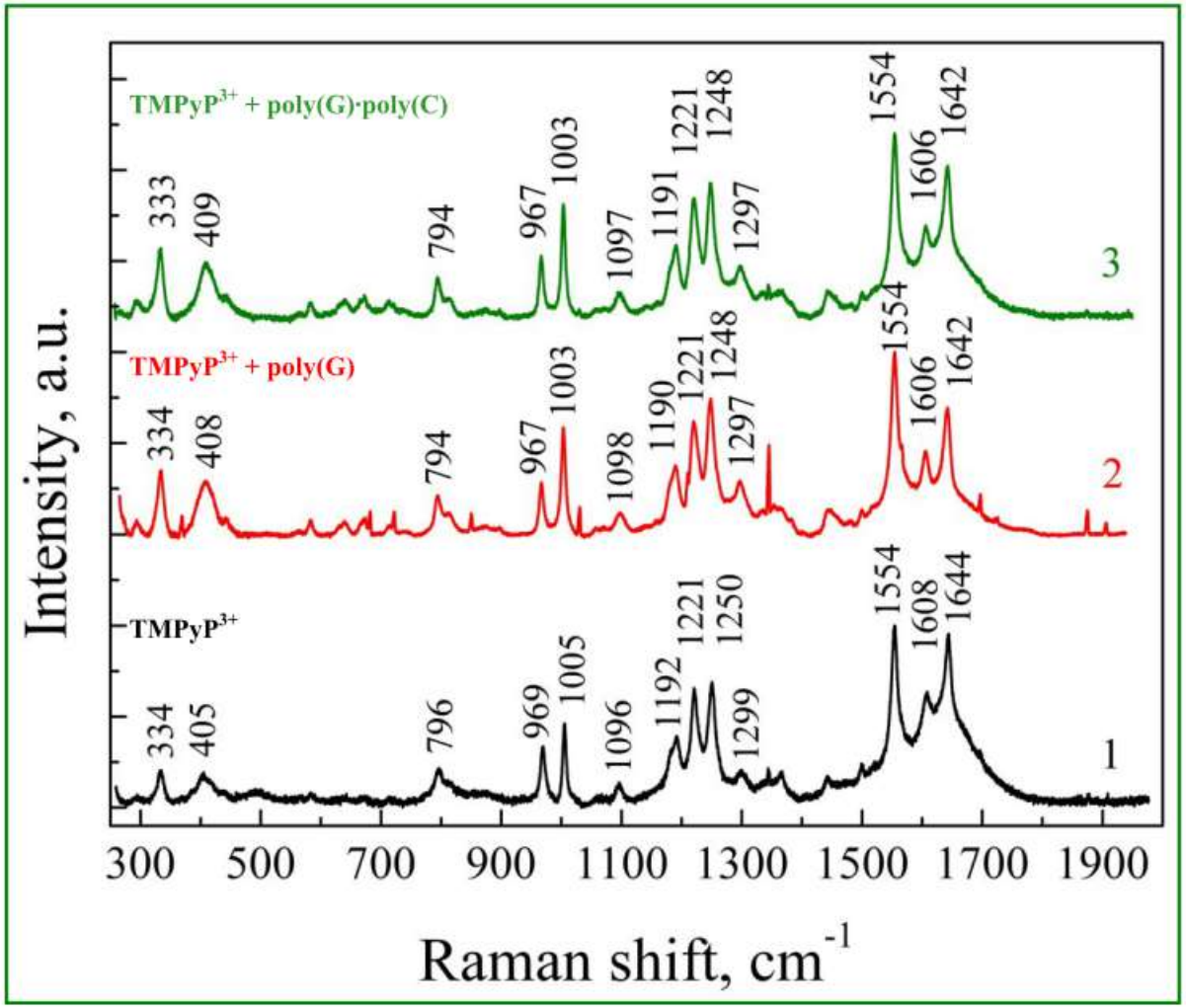
Morris M. D., Wallan D.J. Resonance Raman spectroscopy. Current applications and prospects. *Analytical Chemistry*, 51(2) (1979) 182A–192A.

Czernuszewicz R.S., Zaczek M.B. Resonance Raman Spectroscopy. (2011) In: Encyclopedia of Inorganic and Bioinorganic Chemistry. USA: John Wiley & Sons Ltd.; 2011. doi:10.1002/9781119951438.eibc0303

Stretching vibrations of porphyrin macrocycle: 970, 1005 cm^{-1} 1-2 cm^{-1} downshift

Bending vibration of porphyrin macrocycle: 334 cm^{-1} 1 cm^{-1} downshift and 2-fold intensity increase

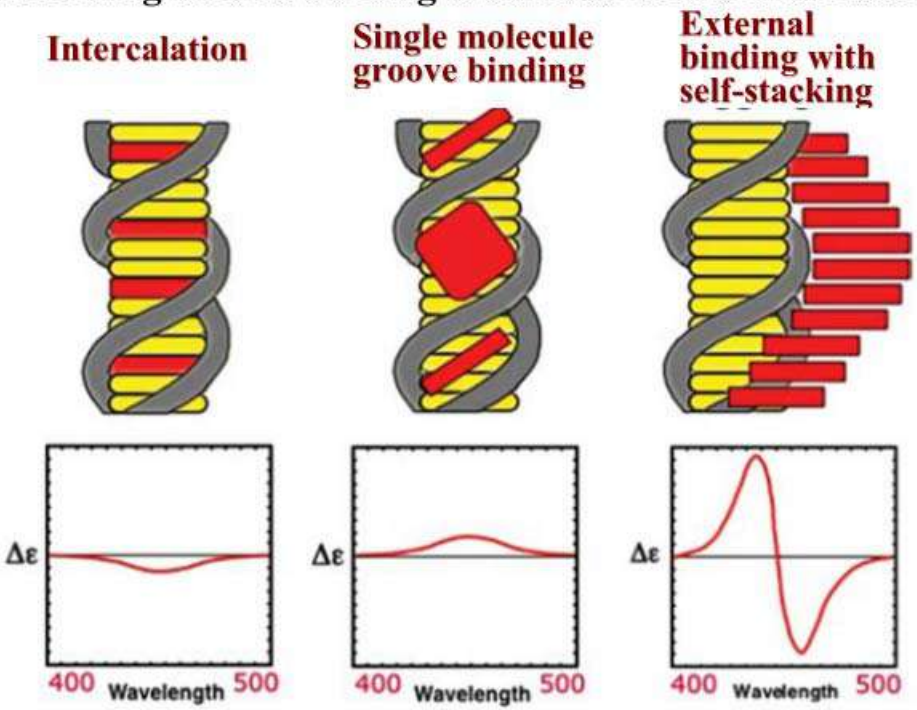
N-methylpyridyl group vibrations: 796, 1250, 1644 cm^{-1} 1-2 cm^{-1} downshift



Fragments of resonance Raman spectra of **$TMPyP^{3+}$** in a **free state** (trace 1) and bound to **poly(G)** (trace 2), **poly(G)·poly(C)** (trace 3) at *P/D* = 3.1, C_{dye} = 50 μM, λ_{exc} = 457.9 nm. [Ryazanova O., Zozulya V. et al. (2016) *Methods Appl. Fluoresc.*, **4(3)** 034005] – **formation of the porphyrin aggregates externally bound to NA backbone with self-stacking at low *P/D* ratios**.

INDUCED CIRCULAR DICHROISM (ICD)

ICD is a powerful tool to detemine the porphyrin-DNA(RNA) binding type. The porphyrins represent chiroptical conformational probes. The type of ICD signals in the Soret region provides precise diagnostic insights into binding mechanisms and molecular interactions.



$TMPyP^{4+}$ porphyrin is not optically active, but a CD spectrum is induced when the porphyrin binds to a chiral polymer like a DNA or RNA. The sign of the induced signal (for monodispersed porphyrins) is a reliable indicator of the binding mode. [R.F. Pasternack, Circular Dichroism and the Interactions of Water Soluble Porphyrins with DNA—A Minireview. Chirality 15 (2003) 329–332].

POPRHYRIN - DNA(RNA) BINDING MODES AND THEIR FINGERPRINTS

Low *P/D* ratios

EXTERNAL BINDING OF PORPHYRIN TO DNA/RNA PHOSPHATE BACKBONE

It depends strongly on the Na^+ content in the solution

WITH SELF-STACKING (highly cooperative process)

WITHOUT SELF-STACKING

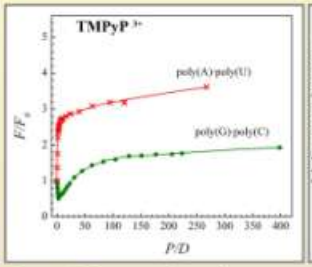
Examples:

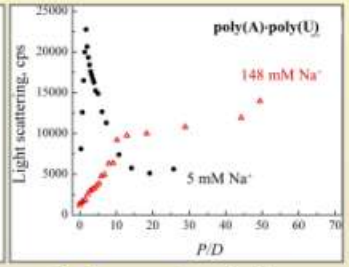
$TMPyP^{3+}$ + poly(G)·poly(C) [2]

$TMPyP^{3+}$ + poly(A)·poly(U) [1]

$TMPyP^{4+}$+poly(dG)·poly(dC) [7]

$TMPyP^{4+}$+poly(dA)·poly(dT) [7]





Quenching of the porphyrin fluorescence

Enhancement of the porphyrin emission

- substantial hypochromism (H) and red shift of Soret absorption band
- splitting of the emission band
- increase of the fluorescence polarization degree
- strong resonance light scattering due to formation of the dye aggregates
- bisignate ICD band in the Soret region (near 440 nm)
- increase in the fluorescence polarisation degree

Fluorescence enhancement upon binding at low *P/D*	Fluorescence quenching upon binding at *low P/D*
$TMPyP^{3+}$ + poly(A)·poly(U) [1]	$TMPyP^{3+}$ + poly(G)·poly(C) [2]
	$TMPyP^{3+}$ + poly(G) [2]
$TMPyP^{4+}$ + poly(A) [9]	$TMPyP^{3+}$ + poly(P) [10]
	$TMPyP^{4+}$ + poly(P) [11]
$TMPyP^{4+}$ + poly(A)·poly(U) [5]	$Zn^{2+}TMPyP^{4+}$ + poly(A)·poly(U) [12]
$TMPyP^{4+}$ + poly(dA)·poly(dT) [7]	$TMPyP^{4+}$ + poly(dG)·poly(dC) [7]

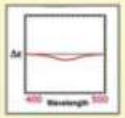
High *P/D* ratios

INTERCALATION OF PORPHYRIN BETWEEN NUCLEIC BASE PAIRS

was detected for GC-containing deoxypolynucleotides

For example, $TMPyP^{4+}$ +poly[(dG-dC)]$_2$ [3]

$TMPyP^{4+}$ + poly(G)·poly(C)



For DNA duplexes (B-form)

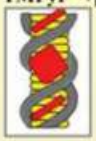
- Great changes in Soret absorption band maximum: H > 40%, red shift Δλ > 15 nm
- Fluorescence quenching
- Strong increase in the fluorescence polarization degree
- Negative ICD band in the Soret region (at ≈ 440 nm)
- Lengthening of DNA (RNA)
- Rise of the solution viscosity
- Energy transfer between DNA and porphyrin → 2-4-fold rise of fluorescence quantum yield upon excitation at 260 nm

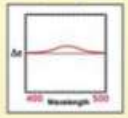
PORPHYRIN MONOMERIC INCORPORATION IN DOUBLE HELIX GROOVE

was detected for AT-containing deoxypolynucleotides

For example: $TMPyP^{4+}$ +poly[(dA-dT)]$_2$ [3]

$TMPyP^{4+}$+poly(dA)·(dT)





For DNA duplexes (B-form)

- No or minor changes in the porphyrin absorption spectra
- No or minor changes in the porphyrin fluorescence spectrum
- No energy transfer between porphyrin and DNA
- No lengthening of DNA
- Moderate increase in the fluorescence polarization degree, *p*.
- Positive ICD band in the Soret region (at ≈ 440 nm)
- Relatively low value of binding constant

EMBEDDING OF PORPHYRIN DIMERS IN THE DOUBLE HELIX GROOVE

was detected for RNA duplexes

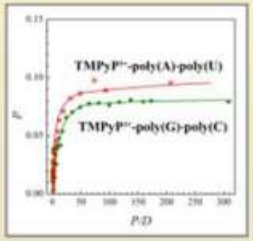
For example:

$TMPyP^{3+}$ + poly(G)·poly(C) [2]

$TMPyP^{3+}$ + poly(A)·poly(U) [1]

For RNA duplexes (A-form)

- Substantial changes in Soret absorption band maximum: H > 22 % red shift Δλ > 15 nm
- Moderate increase in the fluorescence polarization degree, *p*.
- Exciton coupled bisignate ICD band in the Soret region (at ≈ 440 nm)



Porphyrin + Polynucleotide	Reference
$TMPyP^{3+}$ + poly(A)·poly(U)	[1] Ryazanova O. et al. *J. Fluoresc.* doi:10.1007/s10895-024-04000-4
$TMPyP^{3+}$ + poly(G)·poly(C)	[2] Ryazanova O. et al. *Methods Appl Fluoresc.* 4(3) (2016) 034005.
$TMPyP^{3+}$ + [poly(dA-dT)]$_2$	[3] Andrews K. et al. *Biochemistry* 47(4) (2008) 1117–1125.
$TMPyP^{3+}$ + [poly(dG-dC)]$_2$	
$TMPyP^{4+}$ + poly(A)·poly(U)	[4] Uno T. et al. *Inorg. Chem.* 36(8) (1997) 1676–1683.
$TMPyP^{4+}$ + poly(G)·poly(C)	
$TMPyP^{4+}$ + poly(rA)·poly(dT)	
$TMPyP^{4+}$ + poly(rG)·poly(dC)	
$TMPyP^{4+}$ + poly(A)·poly(U)	[5] Tolstykh G. et al. *J. Mol. Str.* 1098 (2015) 342-350.

Porphyrin + Polynucleotide	Reference
$TMPyP^{4+}$ + poly(dA)·poly(dT)	[6] Pasternack R. et al. *Biochemistry* 22(10) (1983) 2406-2414.
$TMPyP^{4+}$ + poly(dG)·poly(dC)	
$TMPyP^{4+}$ + poly(dA)·poly(dT)	[7]. Kelly J.M. et al. *Nucleic Acids Res.* 13(1) (1985) 167–184.
$TMPyP^{4+}$ + poly(dG)·poly(dC)	
$TMPyP^{4+}$ + [poly(dA-dT)]$_2$	[3] Andrews K. et al. *Biochemistry* 47(4) (2008) 1117–1125.
$TMPyP^{4+}$ + [poly(dG-dC)]$_2$	
$TMPyP^{4+}$ +DNA (B-form)	[8] Oh Y.S. et al. *ACS Omega* 3 (1) (2018) 1315-1321.
$TMPyP^{4+}$ + DNA (A-form)	