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COMMUNICATION

A cationic Zn^{II} porphyrazine induces a stable parallel G-quadruplex conformation in human telomeric DNA†

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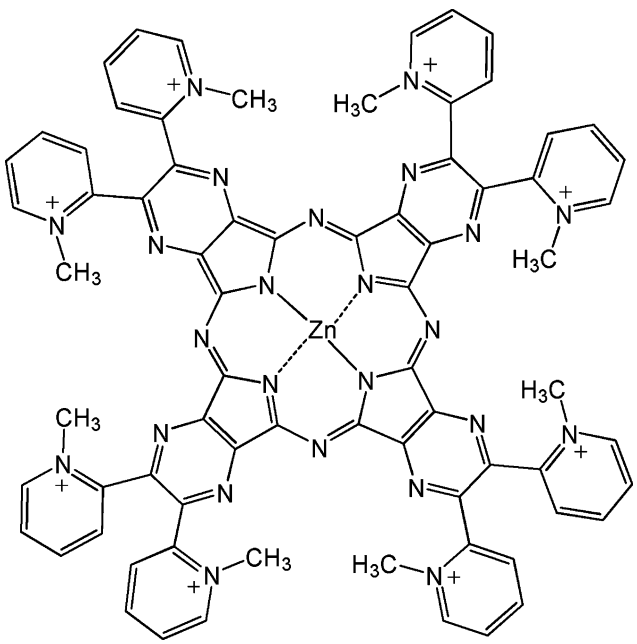
A *water soluble* Zn^{II} porphyrazine drives the conformational equilibrium of the G-quadruplex of a human telomeric sequence exclusively towards a parallel conformation upon complexation.

Guanine-rich DNA sequences can adopt G-quadruplex structures consisting of π–π stacked planes of four guanosines, cyclically bound to each other *via* eight hydrogen bonds according to the Hoogsteen motif. Formation of the G-quadruplex (G4) structure is favoured by the presence of monovalent cations like Na^+ and K^+.[1,2] Guanine-rich sequences are present in important regions of the human genome, like the telomeres at the end of the chromosomes and the promoter regions of several oncogenes. The human telomere possesses a single stranded overhang at the 3′end consisting prevalently of repeats of the GGGTTA sequence.[1–6] Cellular senescence is related to telomere shortening during cell replication and an enzymatic mechanism for telomere maintenance is mediated by a reverse transcriptase, called telomerase. Telomerase is overexpressed in about 85% of rapidly replicating tumour cells, thereby guaranteeing their "immortality".[7] It has been proven that the activity of this enzyme critically depends on the structural organization of these guanine-rich sequences.[7–11] In fact some ligands that bind to the G-quadruplex in solution stabilizing the structure, also interfere with the biological processes involving guanine-rich sequences.[12,13] This has been observed not only for the telomeric sequences but also for guanine-rich sequences present in the promoter regions of some oncogenes.[4,14] Consequently guanine-rich sequences have become a very promising target for the development of new antitumoral drugs and attracted a lot of research interest during the last decade.[3–7,12,13]

Some of us have investigated novel series of porphyrazine macrocycles, among them tetrakis-2,3-[5,6-di(2-pyridyl)pyrazino]porphyrazine and its $Mg^{II}(H_2O)$, Mn^{II}, Co^{II}, Cu^{II}, and Zn^{II} complexes.[15,16] By quaternization of the pyridine N atoms with methyl iodide both the free-base and the metal complexes generate the related *water soluble* octacation derivatives neutralized by I^- ions.[17,18] We focused on the octacationic Zn^{II} complex, called $[PzZn]^{8+}$ (see Scheme 1), potentially able to act as a bimodal drug. In fact, this complex may operate both as a photosensitizer producing singlet oxygen, $^1O_2$, of interest for photodynamic therapy, and as a G-quadruplex ligand, having an aromatic planar tetrapyrazinoporphyrazine core with dimensions similar to the G4 tetrad. Moreover the presence of the positive charges is expected to favour DNA binding due to the electrostatic interaction with the negatively charged DNA backbone.[19–21]



Scheme 1 Tetrakis-2,3-[5,6-di(2-(*N*-methyl)pyridiniumyl)pyrazino]porphyrazinato-Zn^{II}, $[PzZn]^{8+}$.

In this communication we report on the ability of $[PzZn]^{8+}$ to form stable complexes with telomeric DNA in the G-quadruplex form and on the unique properties of these complexes. In particular we investigated the interaction of $[PzZn]^{8+}$ with the telomeric 22-mer sequence 5′-d[AGGG(TTAGGG)$_3$]-3′. This sequence is known to adopt a "basket" G4 structure in Na^+ rich solutions, with pairs of parallel and antiparallel GGG tracts linked by one

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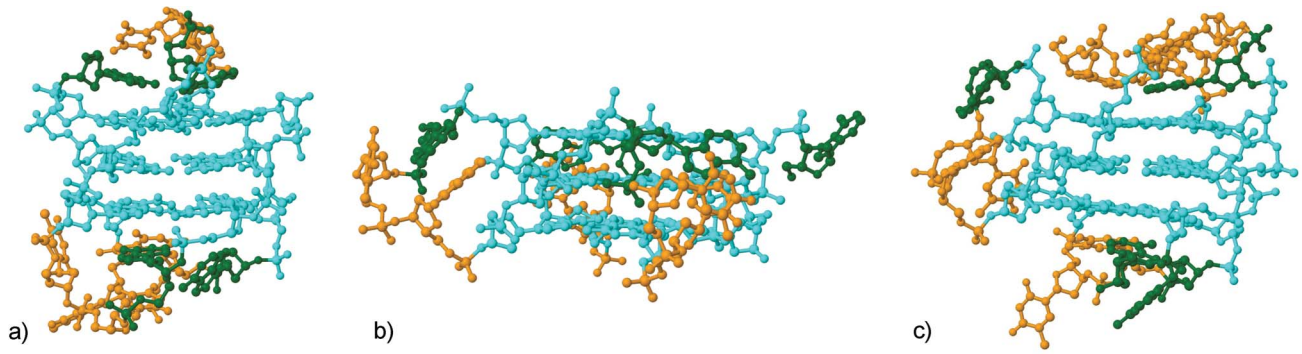

Fig. 1 Cartoon representation of G4 conformations of (a) the 22-mer in Na^+ rich solutions (basket conformer, NDB 143D), (b) the 22-mer in the crystal structure in the presence of K^+ (parallel conformer, NDB 1KF1), and (c) a 24-mer in K^+ rich solutions (3+1 hybrid conformer, NDB 2GKU). G, cyan, T, orange-brown, A, green.

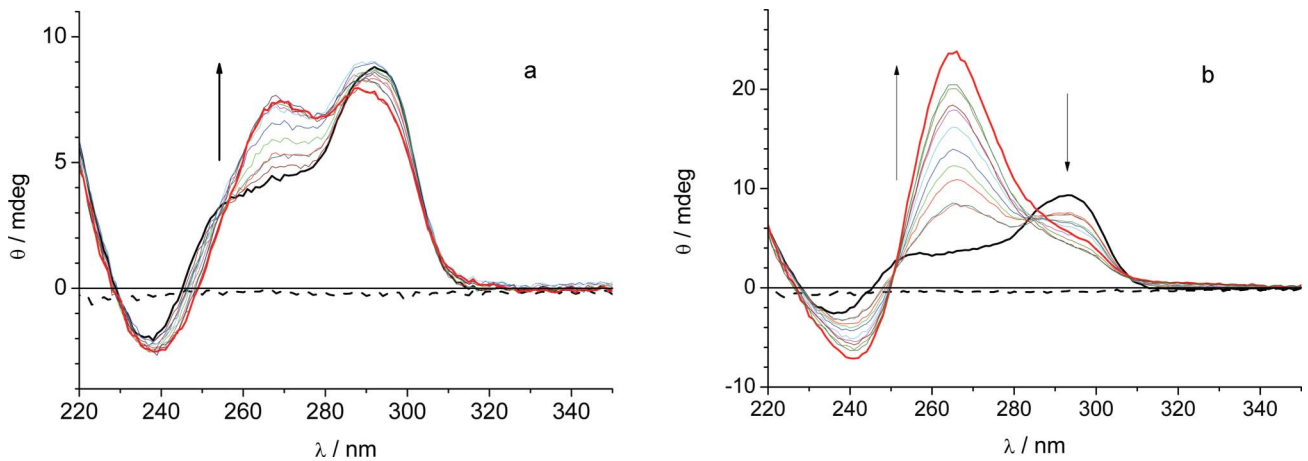

Fig. 2 Ellipticity (θ) of 3×10^{-6} M 22-mer solutions containing increasing concentrations of $[PzZn]^{8+}$ in TRIS/KCl buffer, pH 7.4, d = 1.0 cm, 295 K; $[[PzZn]^{8+}] = (0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8) \times 10^{-6}$ M; (a) the mixtures shortly after preparation; (b) the same mixtures kept in the dark for three days and then heated at 85 °C and cooled down. Note: ellipticity of $[PzZn]^{8+}$ solution: dashed line.

diagonal and two lateral loops (Fig. 1a).[22] The same sequence assumes a propeller-type G4 folding in a K^+ containing crystal, with four parallel GGG tracts and three TTA loops of the double-chain-reversal type (Fig. 1b).[23] NMR studies in K^+ rich solutions showed the same 22-mer adopts a "(3+1) hybrid" structure, as major conformer, featuring an arrangement of three parallel and one antiparallel GGG tract, joined by one double-chain-reversal loop and two lateral loops (see Fig. 1c for example of hybrid conformation).[24,25]

Binding of $[PzZn]^{8+}$ to the 22-mer was monitored with several spectroscopic techniques. The changes in the absorption, fluorescence and circular dichroism (CD) clearly showed that association of $[PzZn]^{8+}$ to the 22-mer occurs. CD allowed us to directly evidence the effect of the $[PzZn]^{8+}$ binding on the G4 conformation.

A 3×10^{-6} M solution of $[PzZn]^{8+}$ in 0.01 M TRIS buffer of pH 7.4 with 0.1 M KCl at 295 K exhibits a weak fluorescence band with maximum at 662 nm and a shoulder at 726 nm. This emission is completely quenched upon addition of 22-mer. An electron transfer process from the guanosine residues to the porphyrazine may be the reason for fluorescence quenching as documented already in previous literature (see SI, paragraph S3).[26]

We titrated the 22-mer with $[PzZn]^{8+}$ monitoring CD at 295 K in the 220–350 nm range, dominated by the G4 signal.[27] Fig. 2a and 2b show the ellipticity changes observed shortly after preparation of the mixtures, and after three days the solutions were stored in the dark at room temperature and subsequently heated. The spectrum of the 22-mer (black) exhibits the typical features of a mixture of 3 + 1 hybrid and basket conformers as main and minor species, respectively.[24,25,28] Addition of $[PzZn]^{8+}$ leads to an intensity increase of both the positive shoulder at 265 nm and the negative signal at 238 nm, the latter slightly shifting to the red, with a concomitant decrease of the signal at 292 nm. These changes become much more evident after storage of the sample in the dark and after heating (see also Fig. S2 in SI). Noticeably, the CD spectrum of the sample with the highest $[PzZn]^{8+}$ concentration, after heating at 85 °C, exhibits almost exclusively the spectral features of the parallel G-quadruplex conformation, characterized by the intense positive signal around 265 nm and the negative signal at 245 nm.[4,29]

In order to gain further insights into the effect of the $[PzZn]^{8+}$ binding on the G4 conformational equilibrium we performed melting experiments monitoring the ellipticity of a mixture at 290 nm and 268 nm and of the free 22-mer at the same wavelengths. The results are shown in Fig. 3a.

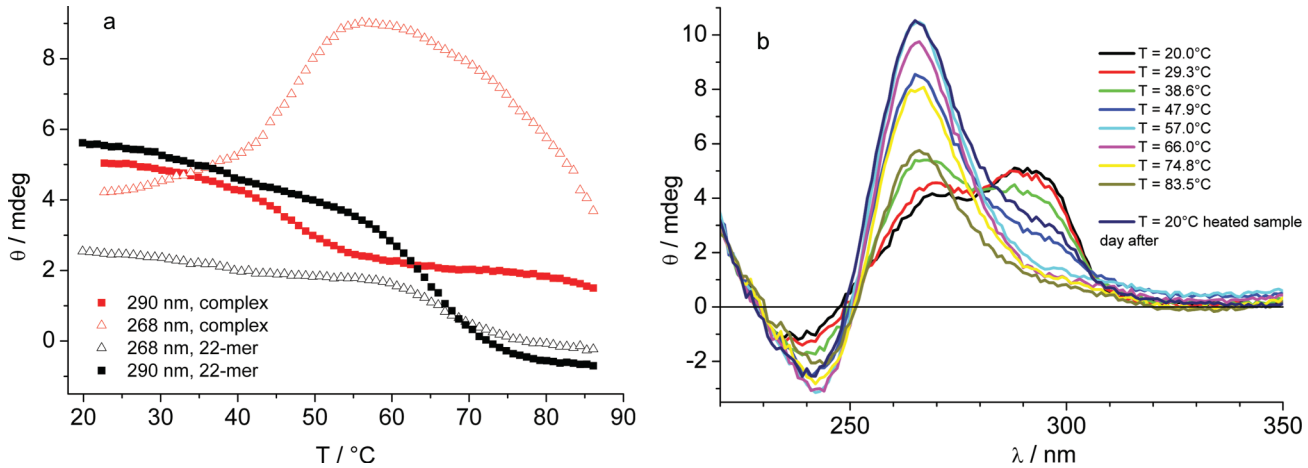

Fig. 3 (a) Temperature dependence of ellipticity $\theta(T)$ at 268 nm (Δ, △) and 290 nm (■, ■) of 22-mer solution (3×10^{-6} M, black) and of a mixture (red) of 22-mer (2.0×10^{-6} M) and $[PzZn]^{8+}$ (6.0×10^{-6} M) in 10 mM Na cacodylate buffer of pH 7.4 with 100 mM KCl. Note: temperature change of 1 °C every 5 min; error on the readings is ±0.05 mdeg. (b) CD spectra of the mixture at different temperatures.

We calculated $d\theta/dT$ from the data in Fig. 3a to evidence the melting temperatures. The free 22-mer melts at *ca.* 65 ± 2 °C, in agreement with data reported in literature.[30] The mixture $[PzZn]^{8+}$/22-mer presents instead two transition points. The first one at *ca.* 48 ± 2 °C, evidenced both at 268 and 290 nm, and a second one above 85 °C. We also registered the CD spectra of the mixture in the UV region at various temperatures (see Fig. 3b). It is evident from the spectral evolution that a complex with a less stable conformation, most likely the 3+1 hybrid one, undergoes a transition at *ca.* 48 °C, assuming the parallel G-quadruplex structure characterized by the positive and negative peaks at 265 nm and 245 nm, respectively, prominent in the spectrum at 66 °C. Only for temperatures higher than 65 °C the parallel complex starts to disappear, but it is still surviving in part at $T >$ 85 °C. After cooling the complex maintains the spectral features of the parallel conformer indicating that this species, once formed, is extraordinarily stable. This behaviour is not exhibited by the 22-mer alone, which only shows decrease of the CD signal with increasing temperature (see SI, Fig. S3).

The results obtained for $[PzZn]^{8+}$ are similar to those reported for a positively charged phthalocyanine derivative[31] and very different from those reported for binding of a tetramethylpyridiniumporphyrazine (both as free base and Zn^{II} complex), which in the presence of K^+ preferentially induces formation of the antiparallel basket conformation in the complex.[20] All these compounds have a planar aromatic core of similar size. Nevertheless they differ for the position of the positive charges, residing on the core in the case of the tetramethylpyridiniumporphyrazine and on the peripheral substituents in the other cases. Most likely in the case of $[PzZn]^{8+}$ π–π stacking of the planar aromatic nucleus with the G-tetrad is optimized in the parallel conformation. Actually in this geometry the three propeller loops do not confer substantial steric hindrance to ligand stacking. Moreover the presence of the positive charges in the peripheral substituents allows optimization of the electrostatic interactions with the G4 phosphate groups present at the periphery of the top and bottom tetrad.[23] This represents an extra driving force to form the parallel G4 complex compared to the tetramethylpiridiniumporphyrazine. At room temperature the equilibrium evolves only slowly in favour of the parallel G-quadruplex complex. Upon warming the solution the equilibration process accelerates leading eventually to almost exclusively parallel conformers, characterized by a high stability as testified by a melting temperature above 85 °C and persistence after cooling.

To gain further insights into the complexation process we titrated $[PzZn]^{8+}$ with the 22-mer following the CD signal in the 215–350 nm range (Fig. 4a).[32] Global analysis of the multi-wavelength data set corresponding to the spectra of the different mixtures in Fig. 4a with the commercially available program SPECFIT/32 (see SI, paragraph S6) allowed us to determine the best complexation model (stoichiometry and binding constants of the most stable complexes) as well as the individual CD spectra of the associated species. We included in the analysis the $[PzZn]^{8+}$ dimerization equilibrium with $\log(K_d/M^{-1}) = 6.6 \pm 0.5$ (for more information see SI, paragraph S7). When using a model with two complexes of 1 : 1 and 2 : 1 ($[PzZn]^{8+}$:22-mer) stoichiometry in equilibrium with the free components in solution convergence was reached with a satisfactory quality of the best fit to the experimental data (Durbin Watson factor of 1.9). The minimization procedure did not attain convergence with other binding models. The optimized binding constants are $\log(K_{11}/M^{-1}) = 6.4 \pm 0.3$ and $\log(K_{21}/M^{-1}) = 12.4 \pm 0.4$. The calculated CD of the individual complexes is shown in Fig. 4b. The spectral profiles for both the 1 : 1 and the 2 : 1 stoichiometry possess qualitative features characteristic of the parallel G4 conformation, in agreement with the results in Fig. 3. The CD spectrum for the 2 : 1 stoichiometry evidences the exclusive presence of the parallel conformation and the $\Delta\varepsilon$ values agree well with values reported in literature.[29] The spectrum for the species with 1 : 1 stoichiometry shows a shoulder at 290 nm that may indicate the presence of some other conformation along with the parallel one.

The CD signal was also registered in the visible region for the same mixtures (Fig. 4c). $[PzZn]^{8+}$ is not chiral, thus the signal observed in this range is induced by the complexation to 22-mer. Analysis of the CD titration data in the visible region including the

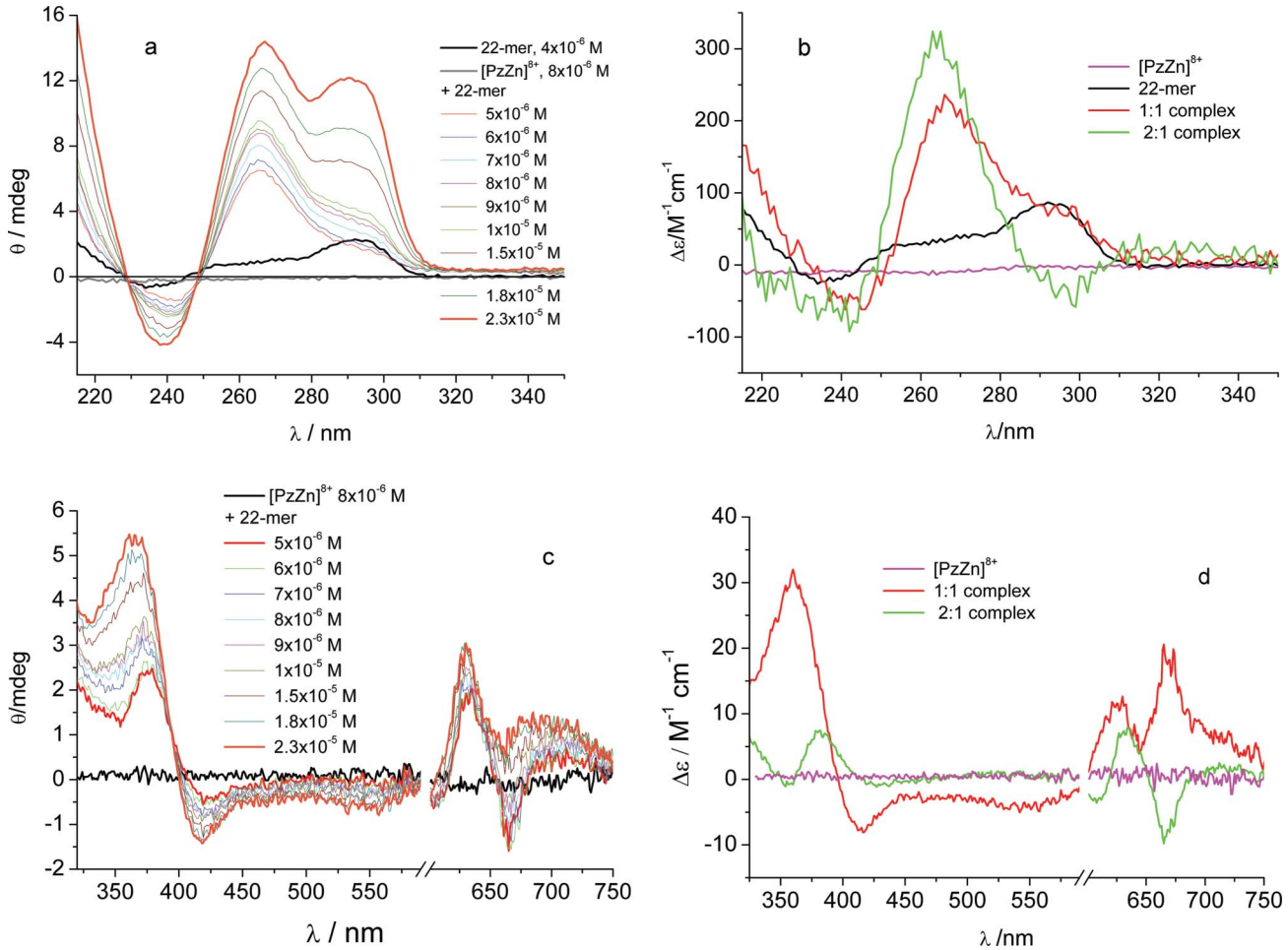

Fig. 4 (a) Ellipticity (θ) of 8×10^{-6} M $[PzZn]^{8+}$ solutions with increasing 22-mer concentration (0.5–2.3)$\times 10^{-5}$ M in TRIS/KCl buffer of pH 7.4 at 295 K, (a) $d = 0.2$ cm and (c) $d = 2$ cm, 320–600 nm and 600–750 nm range with 3 and 6 accumulations, respectively. (b) calculated CD spectra ($\Delta\varepsilon$) of the free compounds and complexes, $\log(K_{11}/M^{-1}) = 6.4 \pm 0.3$ and $\log(K_{21}/M^{-2}) = 12.4 \pm 0.4$. (d) calculated CD spectrum ($\Delta\varepsilon$) of the complexes, $\log(K_{11}/M^{-1}) = 6.2 \pm 0.2$ and $\log(K_{21}/M^{-2}) = 13.1 \pm 0.4$ Note: the solutions have been warmed up at 60 °C for 10 min and cooled before measurement.

dimerization equilibrium yielded results similar to those of the UV region (Durbin-Watson value of 1.5). The presence of complexes with 1 : 1 and 2 : 1 ($[PzZn]^{8+}$: 22-mer) stoichiometry was confirmed and the optimized binding constants, $\log(K_{11}/M^{-1}) = 6.2 \pm 0.2$ and $\log(K_{21}/M^{-2}) = 13.1 \pm 0.4$, were in good agreement within the error with those obtained in the UV region. The weakness of the CD of the 2 : 1 complex (Fig. 4d) compared to that of the 1 : 1 complex may be due to a cancellation effect. Actually, in a configuration where two porphyrazines stack as monomers on different diastereotopic faces of the top and bottom tetrads, they reasonably have CD of opposite sign.[33,34]

Titrating $[PzZn]^{8+}$ with the 22-mer in buffered solution we also followed the absorption changes in the 320–880 nm interval, where we observed exclusively the $[PzZn]^{8+}$ signal (Fig. 5). The Q-band peaking at 625 nm with a shoulder centered at 650 nm is attributed to the presence of the $[PzZn]^{8+}$ dimer, according to observations for its $Mg^{II}(H_2O)$ and Cu^{II} analogs in pure water.[17] The analysis of absorption titration data did not converge either for the complete data set in Fig. 5 or for a reduced data set with elimination of the spectra for the most diluted DNA solutions. When there is a porhyrazine excess of more than two we observe strong hypochromicity and distortion in the absorption spectra, consistent with scattering due to higher order aggregation of G4 complexes. On our opinion some binding of porphyrazines to grooves due to electrostatic interactions with the negatively charged phosphate groups might promote such phenomena with the porphyrazines acting as bridging ligands between parallel G4 units.[35] This type of interaction has also been evidenced in the crystal structure of TMPyP4 with parallel G4, where one molecule stacks with the tetrad and the second interacts with the groove.[36] The absorption changes observed along with the titration with DNA confirm that complexation occurs. In fact, the Soret peak undergoes a red shift of 10 nm as well as a strong hypochromic effect. The peak at 625 nm decreases strongly in intensity. Concomitantly the Q band shifts to the red showing maxima at 635 and 672 nm. The final spectrum (red) corresponds to a solution where more than 95% of $[PzZn]^{8+}$ is bound ($[1 : 1] = 5.6 \times 10^{-6}$ M and $[2 : 1] = 1.0 \times 10^{-6}$ M calculated with the UV CD binding constants, see Fig. S6 in SI). It is deduced that the complexed species in both stoichiometries are endowed with a Q band peaking at 635 and 672 nm. This is also confirmed by the visible CD spectra of the complexes, in particular of the 1 : 1

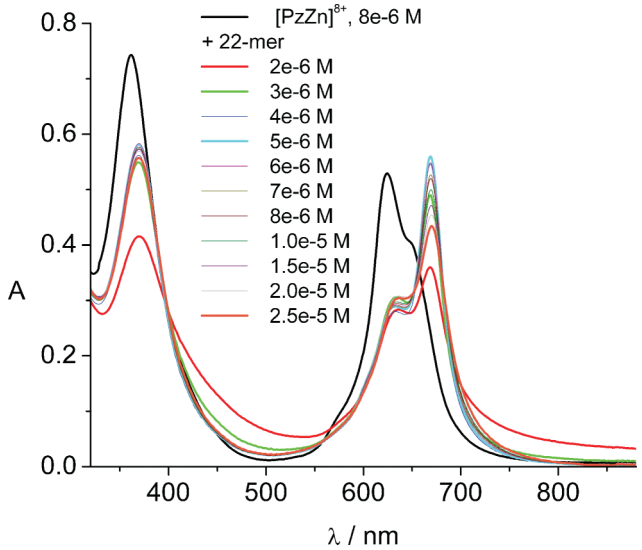

Fig. 5 Absorption spectra of a 8.0×10^{-6} M $[PzZn]^{8+}$ solutions with increasing 22-mer concentration (range 0.2–2.5)$\times 10^{-5}$ M in TRIS/KCl buffer, pH 7.4, d = 1.0 cm. Note: absorption spectrum of 8.0×10^{-6} M $[PzZn]^{8+}$ solution is black.

complex exhibiting a structured Q band with two maxima close to those of the absorption (see Fig. 4d).

In conclusion, our approach allowed us to obtain a clear picture of the binding of the porphyrazine $[PzZn]^{8+}$ to an unmodified human telomeric sequence in K^+ rich solution. In fact, accurate analysis of the circular dichroism data in the UV as well as in the visible regions yielded information on the complex stoichiometry and binding constants, taking into account also the dimerization equilibrium of the Zn^{II} species. The CD spectra of the complexes in 1 : 1 and 2 : 1 stoichiometry gave us important structural information on the G4 conformation in the complex. To the best of our knowledge ligand induced exclusive formation of the parallel G-quadruplex of a telomeric sequence in K^+ rich solution has only been observed for some phthalocyanine derivatives.[31,37] $[PzZn]^{8+}$ has good affinity for the parallel G-quadruplex as illustrated by the binding constants and the thermal stability of the complexes is remarkable. So $[PzZn]^{8+}$ has promising features as a G4 binding molecule. In the frame of the bimodal potential of this molecule as drug, mentioned in the introduction, we are currently investigating its photochemical behaviour in the presence of biomolecules and its biological activity *in vitro*.

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