Final report OTKA K-124697

One of the main goals of the project was to measure the phase diagram of the G-quadruplexes. This allows determination of several thermodynamical parameters, including volumetric ones.

We determined the phase diagram for several DNA and RNA oligos, which are able to form G-quadruplex (GQ) structure in vitro (Task.1.). [1-3]. The phase transition line in the pressure-temperature phase diagram was found to be linear in case of all the studied GQs (TBA, Htel, c-myc, KIT, VEGF, HepB1-3). This means the transition temperature was a linear function of the applied pressure (Fig. 1.). It was known that the proteins show elliptic like phase diagram, while lipid systems have simple linear phase transition lines. Our results show, that in case of nucleic acids no sign of the curved transition line can be observed. If the phase diagram is elliptic, the ellipse must be very large, and we see only small part of it. From thermodynamic point of view this means, that the second derivatives of the thermodynamic functions are small. Applying the Hawley-theory (which is used to describe the elliptic phase diagram of the proteins) , we can state, that the difference in the compressibility and heat capacity between the folded and unfolded states of GQ forming aptamers is very small.
Volumetric parameters are the ones which can be determined specifically from the pressure studies. We determined the unfolding volumes of several GQs: (Htel, c-MYC, KIT, VEGF, HepB1-3) [1-3].
For these measurements we used fluorescence (Förster Resonance Energy Transfer, FRET) and infrared spectroscopic experiments to investigate the conformational changes of GQs under variable pressure and temperature. The oligos were labeled with a FRET pair (FAM and TAMRA). These chromophores show energy transfer in the folded state, which is considerably reduced in case of random chain, i.e. when the quadruplex unfolds. We also constructed an optical setup, where we could investigate the infrared and fluorescence spectra of the GQs simultaneously, using the exact same sample. This way we could correlate the spectral changes in the infrared spectrum with the presence of the GQ structure proven by the FRET experiments. This was essential, because the literature of GQs from the point of view of IR spectroscopy is very weak and inconsistent. Using this method we identified conformation-sensitive vibrational bands of the infrared spectrum to use them for the detection of the folding-unfolding transition of GQs [1].
We also performed kinetic experiments to determine the activation parameters of the GQ folding. Unfortunately the folding of the oligos was too fast to obtain reliable kinetic parameters.
According to the research plan, we also investigated the complementary sequence of the GQ, that is known as i-motif, due to its intercalated nature (Task 5) [4]. It was found to be pressure sensitive. Although pH sensitivity of the i-motif complicated the evaluation of the data, we determined a volume change of the folding, which was negative and slightly smaller than the volume of one water molecule. Comparison of the infrared and fluorescence (FRET) results allowed to reveal the multistep nature of the unfolding transition. [4].
The research was extended from human GQ sequences, which were hypothesized in the sequences of viruses. Three oligos of the hepatitis B virus (HepB1-3) were investigated and they were found to be able to form quadruplexes. They were also fully characterized: their phase diagram, folding volume was determined and their stabilization by specific ligands was also quantitatively characterized [3, 5]. We also performed experiments on the RNA oligos of the SARS-CoV-2 virus, publication of these preliminary results is planned in next months [6].

Another important direction was to investigate the effect of the crowded environment on the stability of the GQ structures (Task 3). It is well known, that the macromolecular concentration in the cell is around 30-40%. This is known as macromolecular crowding. Since spectroscopic experiments (especially fluorescence measurements) are often performed in solutions of micromolar concentration, they might not be representative for the behavior of the molecules in the interior of the cell. In order to mimic the cellular environment, high concentration solutions have to be studied. One solution is adding so called crowding agents.
The infrared spectroscopic experiments allowed us to study the concentration dependence of the phase diagram of Htel. Increasing concentration of the investigated molecule can be considered as a kind of self-crowding effect. [1]

Role of the ions in the stabilization of the quadruplex structures.

Although our original idea to use lanthanides for stabilizing GQs and to use their fluorescence as marker of the folded GQ structure – despite of a series of experimental approaches and attempts - turned out to be impossible, we systematically studied the effect of monovalent ions in case of the newly discovered viral GQs. There is a common belief in the literature, that potassium ion stabilizes more than sodium, and lithium is unable to stabilize. This is well proven in case of TBA and Htel, where we found similar results too. Our experiments however on the hepatitis B oligos (HepB1-3) questioned this dogma, since in case of these oligos not only Na+ and K+, but Li+ and Rb+ ions are also able to stabilize these structures. In some cases Li+ was surprisingly the most potent stabilizer. [3, 5]

Effect of stabilizing ligands on the stability of GQ structure

There are a series of ligands, which were developed in order to stabilize those human GQs, which were believed to be potential targets in cancer therapy. Since their interactions with human GQs have been already characterized, we investigate the question, whether they are also potent stabilizer for the viral GQs? (see Task 6) We used TMPyP4, BRACO19, PhenDC3 as ligands.
Our measurements revealed that the above ligands all bind the three studied GQ forming oligos of the hepatitis B virus. (oligos HepB1-3). The stabilizing effect of the ligands was pronounced, but different. TMPyP4 and PhenDC3 were the most potent stabilizers, while BRACO19 stabilized the GQ structure only slightly [5]. Similar stabilization profile was found in case of the GQs of SARS-CoV-2.
Pressure studies on GQ-bound ligand systems showed stabilization of the folded GQ structure against pressure induced unfolding upon binding of TMPyP4. This indicates the reduction of the volume of the system, i.e. negative binding volume.

The results were published at several scientific conferences (12 conferences, 6 oral, 12 poster presentations), although in the last years of the project participation the conferences was limited due to the pandemic.

The above outlined results were published in several conferences and peer reviewed papers [1-5, 7].

1. Somkuti, J., M. Adanyi, and L. Smeller, *Self-crowding influences the temperature - pressure stability of the human telomere G-quadruplex.* Biophysical Chemistry, 2019. **254**: p. 106248.
2. Molnar, O.R., J. Somkuti, and L. Smeller, *Negative volume changes of human G-quadruplexes at unfolding.* Heliyon, 2020. **6**(12).
3. Somkuti, J., et al., *Pressure Perturbation Studies of Noncanonical Viral Nucleic Acid Structures.* Biology (Basel), 2021. **10**(11).
4. Somkuti, J., O.R. Molnar, and L. Smeller, *Revealing unfolding steps and volume changes of human telomeric i-motif DNA.* Physical Chemistry Chemical Physics, 2020. **22**(41): p. 23816-23823.
5. Molnar, O.R., et al., *Characterization of a G-quadruplex from hepatitis B virus and its stabilization by binding TMPyP4, BRACO19 and PhenDC3.* Sci Rep, 2021. **11**(1): p. 23243.
6. Somkuti, J., Grád, A., Molnár R.O., Cervenak, M., Smeller, L., *Manuscript under preparation.* 2022.
7. Smeller, L., *Biomolecules under Pressure: Phase Diagrams, Volume Changes, and High Pressure Spectroscopic Techniques.* International Journal of Molecular Sciences, 2022. **23**(10).