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SAXS structure studies of the aptamer to the Oncolytic Virus VV-GMCSF-Lact

Roman V. Moryachkov*
Federal Research Center KSC SB RAS
Krasnoyarsk, Russian Federation
Daria O. Malysheva†
Maya A. Dymova‡
Vladimir A. Richter§
Institute of Chemical Biology and Fundamental Medicine SB RAS
Novosibirsk, Russian Federation
Irina A. Shchugoreva¶
Anna S. Kichkailo‖
Federal Research Center KSC SB RAS
Krasnoyarsk, Russian Federation
Prof. V. F. Voino-Yasenetsky Krasnoyarsk State Medical University
Krasnoyarsk, Russian Federation

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Abstract. Oncolytic viral therapy is a relatively young but rapidly developing area in cancer therapy. Some viruses can be used to combat cancer cells due to their cytotoxic action, but it is desirable to inhibit the influence of the human immune system on them. Aptamers, short oligonucleotides capable of specifically binding to their molecular targets, can perform a dual role: binding to oncoviruses and blocking their receptors through which the immune system attacks them, and also delivering them to another target — cancer cells, to increase the effectiveness of the virus. For such a complex task, it is critically important to know the three-dimensional structure of such bifunctional molecules as aptamers. This work presents a study of the NV14t_56 aptamer to the oncolytic virus VV-GMCSF-Lact using the small-angle X-ray scattering method in solution, determination of the structural characteristics extracted using this method, and validation of the molecular model constructed on the basis of the predicted secondary structure.

Keywords: aptamer, small-angle X-ray scattering, SAXS, tertiary structure, virus, VV-GMCSF-Lact, molecular dynamics, molecular simulations

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Introduction

Oncolytic virotherapy is a promising approach for the treatment of oncological diseases due to the selectivity of tumor cell damage, the possibility of immunomodulation of the patient's body

*mrv@iph.krasn.ru https://orcid.org/0000-0002-0409-779X
†d.malysheva@g.nsu.ru https://orcid.org/0009-0005-1210-1815
‡maya.a.rot@gmail.com https://orcid.org/0000-0002-7281-9096
§richter@niboch.nsc.ru https://orcid.org/0000-0001-5849-5892
¶Shchugorevai@mail.ru https://orcid.org/0000-0003-4207-1627
‖annazamay@yandex.ru https://orcid.org/0000-0003-0690-7837

and use in combination with chemo- and radiation therapy. The recombinant virus VV-GMCSF-Lact is a genetically modified vaccinia virus developed by the ICBFM SB RAS jointly with the SSC Vector Rospotrebnadzor, where clinical trials are being conducted for oncolytic virotherapy of breast cancer (ClinicalTrials.gov identifier: NCT05376527). Currently VV-GMCSF-Lact's antitumor efficacy against glioblastoma cell-line-derived and patient-derived xenografts has been shown [1]. To study the antitumor efficacy of the virus against brain tumors, it is advisable to study not only the possibility of intracranial, but also intravenous administration of the drug as it is less invasive. The success of virotherapy depends on the virus gaining access to tumor cells and circulating as long as possible, avoiding the action of the immune system [2]. However, when the virus is introduced into the body, it provokes the production of virus-neutralizing antibodies, which can reduce its antitumor effect. To protect the virus from the immune system, it is necessary to shield it. Previous studies have shown that aptamers can shield the vesicular stomatitis virus from neutralizing antibodies by covering its membrane [3]. The spatial structure of several DNA aptamers, potential shielding agents for the recombinant virus VV-GMCSF-Lact, was obtained using molecular modeling, with further experimental verification of their affinity [4]. The aptamer NV14t_56 showed the highest binding properties to the virus, and hence was the one to undergo further characterisation of its interactions with VV-GMCSF-Lact [5]. Modeling of the secondary structures of this aptamer was performed using the mFold program for folding nucleic acids, and 3D model calculations were conducted using molecular dynamics simulation methods [4, 5]. To elucidate the mechanism of these interactions, the three-dimensional structure of the aptamer in the apo state must be determined. Common methods used in structural biology, such as X-ray crystallography, cryo-electron microscopy (Cryo-EM) and nuclear magnetic resonance (NMR) spectroscopy, each have several limitations when it comes to studying aptamers. X-ray crystallography, for instance, is limited by the difficulty of crystallizing the nucleic acids. Although co-crystallization with a binding partner is sometimes possible [6, 7], crystallization of the aptamer on its own is often hindered by the presence of phosphate groups. Moreover, crystallization can lead to conformational changes of the molecule. A key limitation of cryo-EM is molecular mass: the minimum required mass is about 100 kDa, which is 5 or more times larger than the average size of aptamers (3–20 kDa). But this obstacle can be overcome by placing the aptamer on a larger RNA scaffold, which increases the contrast for cryo-EM to determine the tertiary structure of the aptamer [8]. NMR-spectroscopy has been used in aptamer research [9, 10] as this method allows to study the molecule in native conditions and both determine the structure of the molecule at atomic resolution and observe dynamic conformational changes. However it is a method that requires large sample amounts (5–25 mg), which isn't always feasible for candidate-molecules at early stages of research. An alternative approach is small-angle X-ray scattering (SAXS) — a powerful technique that uses the scattering of a beam of X-rays to probe the structure, morphology, and arrangement of submicron-sized particles under native conditions and is particularly useful for studying systems at the nanometer scale (10–20 Å) [11]. The essence of the method is to record the elastic scattering of X-rays by a sample at very small angles (usually 0.1–10°, measured from the beam axis). The method is characterized by low resolution [12], however, this resolution is sufficient for comparison with and verification of in silico models, and requires small sample amounts. *Ab initio* bead modeling is performed in programs such as DAMMIN, part of the ATSAS package [13], in which different bead models are iteratively generated until their SAXS plot is no longer statistically significantly different from the experimental SAXS plot. This method can be used in DNA/RNA structural biology to determine the geometric parameters of nucleic acids (Rg, Dmax, Porod volume), shape (low resolution), molecular weight, oligomeric state [14].

1. Materials and methods

1.1. Oligonucleotides sequence

The DNA aptamer (NV14t_56) used in this work was synthesized at Lumiprobe RUS Ltd. (Moscow, Russia) and had the primary structure:
5'-GTAACCACGCCATCACCCTATTATCTCATTATCTCGTTTTCCCTATGCGGCATAGG-3'.
The aptamer consists of 56 nucleotides, molecular weight estimated by the nucleotide sequence is 17 kDa. Aptamers were pre-renatured before use. To achieve this, fluorescently labeled DNA aptamers were heated to 95 °C for 5 min, ice-cooled for 2 min, and incubated at 37 °C for 15 min. Aptamer solution samples were taken for SAXS measurements at three concentrations: 2.5, 5.0 and 10 mg/ml. 1 mM Tris-HCl, pH 8.5, was used as a buffer solvent.

1.2. Small-Angle X-ray Scattering (SAXS)

SAXS experimental data were obtained using the HECUS S3-MICRO instrument (Hecus X-ray Systems, Graz, Austria) at the NRC «Kurchatov Institute» according to the proposal №2597. HECUS S3-MICRO is a lab-based small instrument for combined SAXS and WAXS (wide-angle X-ray scattering) measurements for solids, gels, polymers, thin films, mesoporous materials, nanoparticles, macromolecular solutions. It has the X-ray 50 Watt microsource based on a Cu K-edge anode with the momentum transfer range: $0.003 < s < 0.6$ Å^{-1}. It can simultaneously measure at small angle (0.2°–8°) and wide angle (18°–28°) with the X-ray Dectris Pilatus 100K detector. It provides a beam size on the sample of about 50 x 200 μm^2 and a photon flux up to 4 x 10^8 photons/s/mm^2.

Compared to other synchrotron-based sources, the HECUS S3-MICRO provides lower but more stable and constant X-ray intensity and spatial beam localization, in contrast to changing X-ray beam center at the synchrotron KISI-Kurchatov due to the reduction of the electron current in the user mode. SAXS measurements were performed for all three concentrations of the aptamer solution (2.5, 5.0 and 10 mg/ml). For each concentration 24 shots were recorded with the exposure time 3000 s with the whole resulting exposition of 20 hours for each sample. The same number of SAXS records were measured for the buffer solution. SAXS data curves showed stability of the samples during whole measurement time without any change of the form and slope of the scattering signals from the first to last record within the signal-to-noise range.

SAXS experimental data were processed and analyzed by standard pipelines described in the publications [15–17].

Processing of the SAXS data carried out in the ATSAS program suite (BioSAXS Svergun group, EMBL, Hamburg, Germany) [18]. Initial visual analysis and evaluation of the SAXS curves, scaling, averaging, subtraction of the buffer signal, visualization of the Guinier and Kratky plot were done using the program PRIMUS [13] from the ATSAS package. Pair distance distribution function (PDDF), p(r), was calculated and constructed in the GNOM program [19] implemented into the PRIMUS. Bead models based on the p(r) were constructed using the programs DAMMIN [20] and DAMMIF [21], fit of the SAXS data and theoretical SAXS curve calculated from the molecular model was done in the program CRYSOL [22].

2. Results and discussion

When calculating such complex structures as biological macromolecules, it is necessary to take into account the conformational features that are dictated by various chemical bonds: covalent, hydrogen, Van der Waals and others. As a result, we come from a schematic secondary structure to a more complex tertiary, spatial structure, which can be characterized by physical parameters, as a nanoparticle: determine its dispersed state, particles form a monodisperse or polydisperse

colloidal solution, that is, they are in a free state or form oligomers, aggregates; the size and mass of the particle, the volume it occupies in space and the distribution of the particle material in space, which is described, for example, by the radius of gyration.

In work [5] we comprehensively characterized the aptamers to the oncolytic virus, the secondary and spatial structure has been predicted. In the current work we validated the atomic structure of NV14t_56 DNA aptamer in solution using the SAXS method and obtained the structural parameters of this molecule. From three aptamer concentrations measured we chose the intermediate 5 mg/ml, since the lower concentration 2.5 mg/ml showed high noisy SAXS signal even on the middle angles. The higher concentration 10 mg/ml showed the interparticle interaction represented in increasing the scattering intensity on the low angles, that would aberrate the calculations and interpretation of the SAXS data. The middle concentration appeared to be a better set of data. The useful scattering signal was recorded in the momentum transfer range $0.3 < s < 3$ nm^{-1}.

Linearity of the Guinier region ($sRg < 1.3$) confirmed the monodisperse composition of the aptamer solution, radius of gyration of the aptamer molecule calculated from the angle of the Guinier plot (in the coordinates ln I vs. s^2) is 2.66 ± 0.12 nm (Fig. 1a). The Indirect Fourier transform of the SAXS curve resulting in the PDDF p(r) is shown in Fig. 1b, that describes a distribution of the intraparticle distances between scattering centers. The maximum value r on the plot denotes the size of the molecule Dmax = 10.5 nm. The inserted plot in Fig. 1b is the Kratky plot in coordinates I^2s vs. s shows the bell-shaped form of the graph that demonstrates the compact conformation of the molecule. According to the length of the oligonucleotide sequence, Dmax and Rg values we already may suppose that the molecule of the aptamer is folded in half of its whole chain. The function p(r) shows the most represented distance about 2 nm, which corresponds to the width of double-stranded DNA.

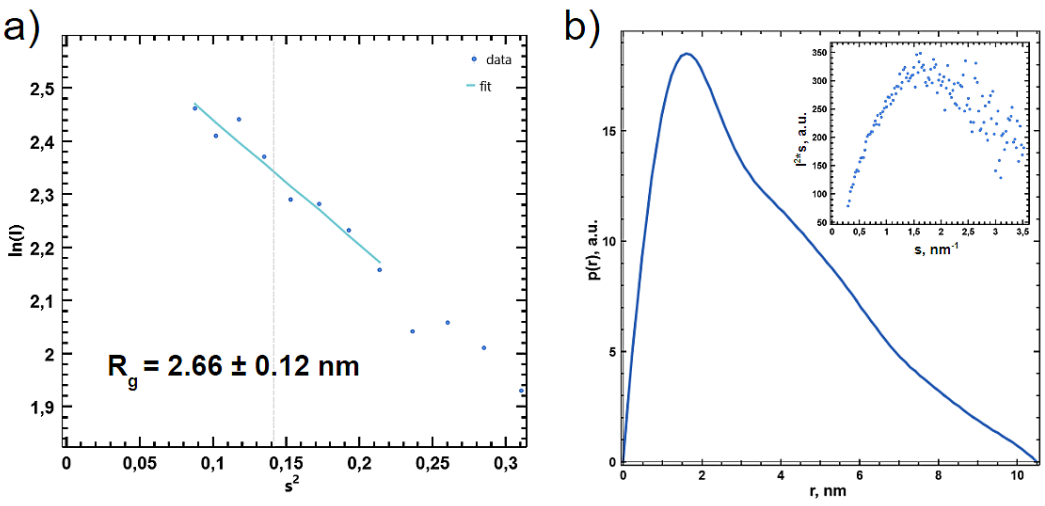

Fig. 1. SAXS data manipulations. a) Guinier plot of the initial part of the SAXS curve ("Guinier region"); b) PDDF function p(r) calculated from the SAXS data for the aptamer NV14t_56, Kratky plot in the coordinates I^2s vs. s is inserted

The mass of the molecule is estimated at 16.4 kDa with a Porod volume of about 18.3 nm^3, which is most likely due to the fact that the molecule has loop sections in the structure, into which water molecules could enter and increase the observed "looseness" of the structure.

It confirms that the molecule is not compactly folded, unlike oligonucleotides with large sections of complementary nucleotide pairs, but has breaks in the structure, which most likely have a great significance in the specific binding of the aptamer to the virus.

Using the p(r) function the 3-dimensional bead model of the overall electron density volume of the molecule was reconstructed in the programs DAMMIN and DAMMIF. The averaged from 20 constructed DAMMIF bead models final spatial structure is shown in Fig. 2b by green balls. This structure is aligned with the full-atomic model obtained using the molecular dynamics simulation.

Also the quantitative analysis was performed by the comparison of the SAXS experimental curve and theoretical SAXS curve calculated from the molecular dynamics. On Fig. 2a one can see the blue dots of the SAXS data, green line of the bead model corresponding to the calculated p(r) function, and red line of the theoretical SAXS curve of the molecular model.

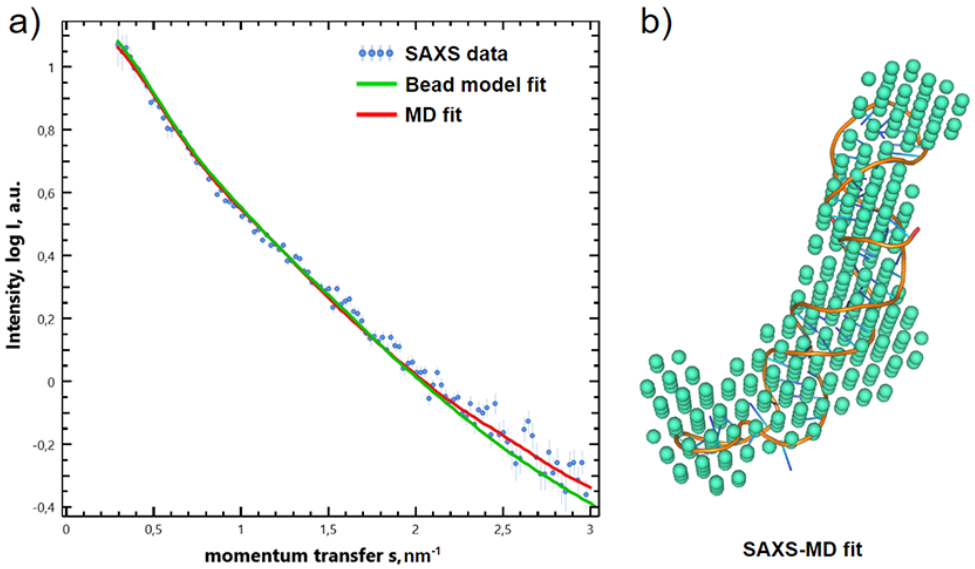

Fig. 2. Validation of the MD-result by the SAXS experimental data. a) Fit of the SAXS data (blue dots), SAXS curve corresponding to the bead model (green line) and theoretical curve calculated from the PDB-structure resulted from molecular dynamics simulation (red line). b) Matching the spatial molecular model of aptamer with the bead model constructed on the SAXS experimental data basis

A good coincidence of the graphs shows the agreement between theory and experiment, and confirms the validity of the suggested molecular model for further procedures, such as molecular docking and also structure analysis in order to search for active sites responsible for specific binding the aptamer with the target. Discrepancy between the experimental data and theoretical curve expressed in the χ^2 value is quite high, due to a small amount of experimental points and high level of signal deviations relative to average, while the theoretical curve is imaged in a more smooth manner. Such deviations are the result of averaging the noisy SAXS data sets.

Interestingly, the conformation of the aptamer in the molecular model fits quite well into the shape of the bead model. This suggests that the modeling takes into account both the hydrogen bonds between complementary nucleotides that hold the aptamer together in its structure, and the mobility of the free ends and loops, which is determined by their physical size and the presence of nearby chemical groups that also participate in the formation of the observed conformation

of the molecule. The more mobile part of the molecule chain from 41 to 56 nucleotide, due to the absence of the complementary connection in the secondary structure of the 3'-end of the aptamer with its 5'-end, is placed in the same domain of the molecule, that it's observed in the bead model.

Since the SAXS method gives only a low-resolution model, one cannot directly determine all the peculiarities in the aptamer structure. In this case we combine the experimental method SAXS, which is aimed to describe overall form of the molecule and confirm the conformation of the aptamer in solution, with the theoretical method of molecular modeling, that place definite restrictions on the folding and mobility of the molecular domains in the structure and can be validated by SAXS.

Conclusion

Small-angle X-ray scattering has recently been used for coarse-grained modeling of aptamer structures, as it requires small sample volumes and allows studying the structure under native conditions. The use of SAXS in combination with instrumental data can contribute to understanding the structure and dynamics of ligand-bound and apo states. The conformation of the aptamer molecule obtained in this work reveals the main sites that can bind to the target molecule, the viral surface protein.

In this work we investigated using the SAXS technique the spatial structure characteristics of the DNA aptamer NV14t_56 to the oncolytic virus VV-GMCSF-Lact, such as radius of gyration, maximum size, volume and estimated molecular weight of the aptamer molecule, and also performed the validation of the molecular model obtained using the molecular dynamics simulation of the known sequence and predicted secondary structure of the molecule. Comparison of three-dimensional models, as well as comparison of SAXS graphs, showed good agreement between the molecular model and the SAXS experiment.

Analysis of the overall structure of the aptamer and the nucleotides whose nucleobases are turned outward will provide information on which region or regions play a key role in recognizing and binding specific regions of the virus. Moreover, additional analysis of the virus-aptamer complex, which we hope will be performed in the near future, will allow us to more accurately determine the active sites of both structures under study. This will enable us to get a clearer picture of the resulting complex and use the data obtained to modify the aptamer, if necessary, to adjust or improve its properties.

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Структурные исследования методом МУРР аптамера к онколитическому вирусу VV-GMCSF-Lact

Роман В. Морячков
Федеральный исследовательский центр КНЦ СО РАН
Красноярск, Российская Федерация
Дарья О. Малышева
Майя А. Дымова
Владимир А. Рихтер
Институт химической биологии и фундаментальной медицины СО РАН
Новосибирск, Российская Федерация
Ирина А. Щугорева
Анна С. Кичкайло
Федеральный исследовательский центр КНЦ СО РАН
Красноярск, Российская Федерация
Красноярский государственный медицинский университет им. проф. В. Ф. Войно-Ясенецкого
Красноярск, Российская Федерация

Аннотация. Онколитическая вирусная терапия — сравнительно молодое, но быстро развивающееся направление в терапии рака. Некоторые вирусы могут использоваться для борьбы с раковыми клетками за счет своего цитотоксического действия, но желательно ингибировать влияние на них иммунной системы человека. Аптамеры — короткие олигонуклеотиды, способные специфически связываться со своими молекулярными мишенями, могут выполнять двойную роль: связываться с онковирусами и блокировать их рецепторы, через которые их атакует иммунная система, а также доставлять их к другой мишени — раковым клеткам, для повышения эффективности действия вируса. Для столь сложной задачи критически важно знать трехмерную структуру таких бифункциональных молекул, как аптамеры. В данной работе представлено исследование аптамера NV14t_56 к онколитическому вирусу VV-GMCSF-Lact методом малоуглового рентгеновского рассеяния в растворе, определение структурных характеристик, извлеченных с помощью этого метода, и валидация молекулярной модели, построенной на основе предсказанной вторичной структуры.

Ключевые слова: аптамер, малоугловое рентгеновское рассеяние, МУРР, третичная структура, вирус, VV-GMCSF-Lact, молекулярная динамика, молекулярное моделирование.