Theses of Ph.D. dissertation

ARTIFICIAL METALLONUCLEASES - MOLECULAR TOOLS FOR GENE THERAPY OF CANCER

HEBA ALAA ELDEEN HOSINY ABD ELHAMEED

Supervisor:
DR. BÉLA GYURCSIK
Associate professor

Doctoral School of Chemistry

Department of Inorganic and

Analytical Chemistry

Faculty of Science and Informatics

University of Szeged, Hungary

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1. INTRODUCTION

All cancer cells contain multiple genetic mutations that allow them to grow progressively and exhibit the characteristics of malignancy. Therefore, targeting the cancer cell genome is an attractive approach of cancer therapy. The ability to redeem such cancer-associated mutations requires a reagent which should induce correction of genetic changes in a highly specific manner without off-target effects. The reagent would also require efficient delivery into all or nearly all cells of a tumor. In the last decades, novel genetic-editing technologies have been developed, based on artificial nucleases (ANs). These ANs have important potential clinical applications including the treatment of genetic diseases, viral infections, and cancer. These new classes of reagents, which can specifically target nucleotide sequences within cellular genomes, are of four major types: meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regulatory interspaced short palindromic repeat (CRISPR) and its associated nuclease 9 (Cas9) system. However, all of the above artificial nucleases have various levels of cytotoxicity.

Therefore, the goal of our research group is to develop a regulated zinc finger nuclease, which is based on Colicin E7 metallonuclease domain (NColE7) instead of the widely used FokI nuclease domain in the ZFNs and TALENs. Accordingly, new ANs should be developed, in which the various parts of the enzyme regulate the catalytic activity so that the DNA hydrolysis only occurs when the enzyme binds to its specific target site. In addition, any damage of the enzyme should cancel its function, rather than lead to non-specific cleavages.

On this path, the development of such an AN includes various approaches presented in this thesis. (i) The optimization of NColE7 based AN depends largely on understanding of the intramolecular

regulation of the NColE7 nuclease within the native and modified variants, including the zinc finger – NColE7 ANs. (ii) The design of CRISPR/Cas9 ANs to target and knockout selected oncogenes promotes the selection of the most efficient DNA recognition sites for the new types of ANs. It also allows for comparison of different types of ANs. (iii) The optimization of gene delivery systems using high molecular weights poly(ethylenimine) (PEI) modified into a water-soluble lipopolymer (WSLP) increases the bioavailability and efficiency of the ANs.

The results of my PhD work are intended to contribute to the development of an artificial metallonulease with improved specificity and effectivity for potential gene therapy.

2. AIMS AND OBJECTIVES

The aim of the research conducted in the Bioinorganic Chemistry Research Group of the Chemistry Institute at University of Szeged is to develop an effective artificial nuclease as a promising molecular tool for gene therapy. I have joined this research team in 2016. At that time the research has focused on the design of a novel zinc finger artificial nuclease based on the NColE7 nuclease domain instead of the NFokI nuclease domain. Therefore, my work is aimed at optimization of the newly designed ZFNs for targeting oncogenes in cancer cells, as well as at comparison of these artificial nucleases with the currently widely used CRISPR/Cas9 system. Furthermore, we decided to develop a novel drug delivery system to increase the efficiency of the designed artificial nucleases by their delivery into the target cells.

The summary of my aims is shown in **Figure 1,** which explains the interdisciplinary nature of the research including chemistry and biology. According to this scheme, my research consists of three main steps: (i) optimizing of a promising artificial nuclease, (ii) development

of an effective drug delivery system and (iii) studying of the behavior of these systems in cancer cells.

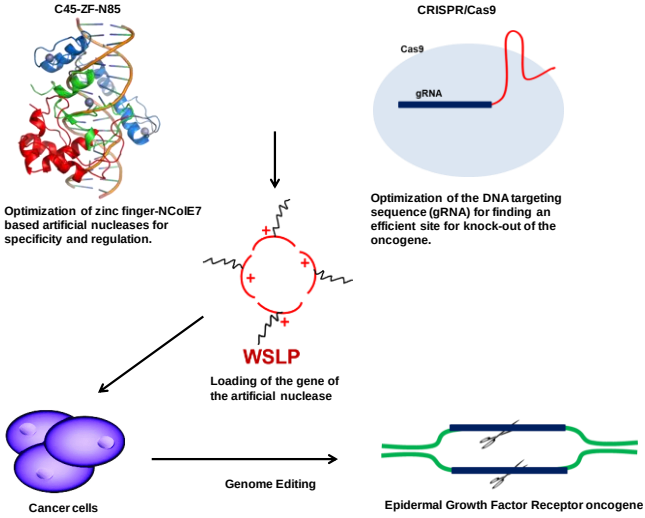

Figure 1. The schematic demonstration of the aims of my PhD research project.

During my PhD studies I focused on the following objectives:

1- Construction of a new protein expression system for effective purification of artificial nucleases

The production of proteins in high yield and purity is usually difficult and requires multistep chromatographic methods often leading to a significant loss of target proteins. In our laboratory, the synthesis of proteins with precisely determined sequences is very important because the computer design of artificial nucleases requires the reproduction of the protein sequence. Therefore, a strategy was elaborated by means of which the designed artificial nucleases can expressed and purified by immobilized metal ion affinity

chromatography, but without any additional remaining amino acids at the termini of the protein after removal of the hexahistidine affinity tag.

2- Improving of the newly designed NColE7-based zinc finger nuclease

The metal ion binding hexahistidine affinity tag may influence and thus, modulate the catalytic activity of the NColE7 metalloenzyme. Therefore, we decided to explore this effect for development of a novel regulation mechanism. The regulation may also be achieved in the novel zinc finger nucleases by inactivating the nuclease domain itself in the absence of a specific DNA target sequence. To better understand the possible regulation mechanisms, the expression and purification of the promising AN variant (C45-ZF-N85), its mutants, as well as 6×His tagged NColE7 mutants was foreseen followed by the study of their solution structure by circular dichroism spectroscopy and their specificity and/or regulation by *in vitro* catalytic experiments.

3- Design of CRISPR/Cas9 artificial nucleases for oncogene targeting

I planned to apply the CRISPR/Cas9 system to target epidermal growth factor receptor (EGFR) oncogene. Designing various recognition sequences within the gene would allow for detection of the most efficient artificial nuclease target sites, which can also be targeted by the new NColE7-based ANs upon redesign of the ZF domain.

4- Development of a novel drug delivery system for artificial nucleases

The success of the selected genome editing technique is influenced by the effectivity of the delivery system used to carry the nuclease to the target cells. Chemical delivery methods are being considered to be the most promising. For this purpose, we decided to

synthesize a water-soluble lipopolymer from a high molecular weight poly(ethylenimine) by cholesterylation, to characterize its DNA loading properties and study its gene delivery i.e., transfection efficiency in cancer cells.

3. PRECEDING RESEARCH

Recently, a novel zinc finger AN was designed in our laboratory, based on the NColE7 nuclease domain and various models were designed providing the basis for a new approach towards integrated artificial nucleases. The resulting construct was expected to act as a regulated molecule however the optimization of such ANs can lead to safe gene editing enzymes. These results inspired further studies and improvement of our newly AN construct to be more specific and regulated. No preceding research was carried out in our laboratory concerning the CRISPR/Cas9 AN and drug delivery systems.

4. METHODS

Various methods of bioinorganic chemistry and molecular biology were used in this work. Recombinant DNA technology was applied to produce functional DNA molecules carrying the genes of the designed proteins. The specific mutations in these genes and in the plasmids (e.g. the modification of the pET-21a plasmid for protein expression with SXH cleavage motif) were introduced by Polymerase Chain Reaction (PCR) technique. The proteins were expressed in *E.coli* bacteria and then purified from the bacterial culture by cation exchange or immobilized metal ion affinity chromatography.

Electrophoretic metods were applied to separate and detect biological macromolecules. The proteins were investigated by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis), while agarose gel electrophoresis was used in experiments with DNA,

including the monitoring of the DNA cleavage in nuclease activity assays of KGNK-6×His and the new ZFNs. The plasmid DNA (pDNA) loading of the new water-soluble lipopolymer (WSLP) was also verified by agarose gel electrophoresis.

The concentrations of nucleic acids and proteins were determined by UV absorption spectroscopy.

Electrospray Ionization Mass Spectrometry (ESI-MS) was a tool to validate the expressed proteins. It has been applied to determine the molecular weight of the purified proteins, as well as for monitoring their metal ion binding ability.

Circular dichroism spectroscopy was applied to investigate the secondary structure of the purified proteins in solution.

I applied various purification kits to obtain and purify plasmid or genomic DNA and RNA.

Several bioinformatics on-line tool helped my research. The new gRNAs were designed by the Guide Design Resources (http://crispr.mit.edu/); ExPaSy (https://www.expasy.org/resources) was used to estimate the protein parameters, translate DNA sequences to protein sequence; CodonCode Aligner software (https://www.codoncode.com/aligner/) was applied for the analysis of the sequence data and Tracking of Indels by Decomposition tool (https://tide.nki.nl/) was applied for the analysis of CRISPR/Cas9 activity; ZF redesign was carried out using Zinc Finger Tools (https://www.scripps.edu/barbas/zfdesign/zfdesignhome.php).

WSLP was prepared from high molecular weight poly(ethylenimine) and cholesteryle chloroformate. WSLP and WSLP/pDNA complexes were characterized by dynamic and static light scattering, scanning electron microscopy, and transmission electron microscopy.

The specific charge of PEI and WSLP determined by the particle charge detector (PCD) formed the basis of the molecular weight, i.e. cholesterylation efficiency estimation for WSLP.

The ability of mitochondrial dehydrogenase enzymes in viable cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into purple formazan crystals (MTT assay) revealed the cytotoxicity of WSLP and of WSLP/pDNA on A549 human lung cancer cells and HeLa cervical adenocarcinoma cells.

Flow cytometry and fluorescence microscopy were applied for monitoring of the success of the plasmid delivery into the mammalian cancer cells (transfection).

Genomic DNA sequencing and quantitative real-time PCR were used to check the EGFR knockout efficiency of CRISPR/Cas9 in A549 cell lines.

5. NEW SCIENTIFIC RESULTS

1.Design of a new DNA construct for affinity based purification of ΔN4-NColE7

A new DNA construct (pET-21a*-SRHS) was established for affinity purification of proteins with native or precisely designed sequence. This new purification method avoids any remnant amino acid residues after the removal of the affinity tag, while overcoming several disadvantages of the proteases (sensitivity, high price). This new DNA construct can be adopted to work with various C-terminal affinity tags. [1]

2.Modulation of the catalytic activity of NColE7 by the 6×His tag

2.1. It was found that the pET-21a*-SRHS vector could also carry the gene of the toxic NColE7 and R447G-NColE7 (KGNK) mutant in

DH5α bacterial cells. Due to the inhibitory effect of the C-terminal 6×His tag on the catalytic activity, I could express a new KGNK-6×His protein directly, and then purify it by using metal ion affinity chromatography. Nevertheless, the 6×His attachment couldn't completely inhibit the nuclease activity of the KGNK protein, which is also reflected in the relatively low yield of the expressed protein. The secondary structure analysis of this new protein by CD spectroscopy revealed that its α-helical content corresponds to that expected for a functionally folded form. [2]

2.2. Metal ion binding ability of the KGNK-6×His protein assessed using ICP-MS measurement revealed that the new protein was obtained in its Zn^{2+}-loaded form containing an equivalent amount of Zn^{2+} ions. Thus, the metal ion binding in the active site is characterized by similar high affinity as the NColE7 itself. I showed that the KGNK-6×His protein was able to bind more Zn^{2+} ions in the presence of 4× Zn^{2+}-escess. Even the Zn_6P metal-protein comnplex was deetcted by mass spectrometry. Based on these measurements the relative amounts of the complexes containing various number of metal ions were estimated versus the added Zn^{2+} equivalents. [2]

2.3. The inhibitory effect of the 6 × His fusion tag on the nuclease activity of KGNK-6×His protein proved to be a complex process via both coordinative and non-specific steric interactions. The modulatory effect of Zn^{2+} ion was observed in the *in vitro* catalytic activity experiments. The DNA cleavage ability of the 6×His tagged enzyme was first enhanced by an increase of metal ion concentration, while high excess of Zn^{2+} ions caused a lower rate of the DNA cleavage. The increase of the DNA cleavage ability of the enzyme upon increasing metal ion-to-protein ratio suggested that the added metal ion can compete for the 6×His sequence, which originally may bind to the free

site of the catalytic Zn^{2+} ion accounting for the coordinative inhibition. Thus, by binding to the 6×His sequence, the added metal ion enhances the catalytic activity. Upon saturating the histidines outside the catalytic center with one or two Zn^{2+} ions, H545 may also be metallated on further addition of metal ion, preventing the generation of the OH^- nucleophile by the side-chain of this histidine residue. The higher is the metal ion excess the higher is the probability of this type of coordination. [2] Using these results other similar metalloenzymes can be modulated without changing the core enzyme sequence.

3. Construction and optimization of CRISPR/Cas9 system for targeting the epidermal growth factor receptor (EGFR, ErbB1) oncogene

3.1. CRISPR/Cas9 system was used in my PhD research work to detect which target will be suitable for the practical application of the new zinc finger nuclease. For this, new guide RNA sequences were designed and inserted into pX458 plasmid to target lung cancer through the EGFR oncogene.

3.2. EGFR knock-out efficiency was determined by genomic DNA sequencing of the DNA extracted from A549 cells. CodonCode Aligner and TIDE analysis of CRISPR/Cas9 activity detected the difference between the control and treated DNA by determining the possibility of insertions or deletions leading to frameshift mutations. The mutation efficiency proved to be 7.5% after seven days incubation period so further improvements are needed to enhance the gene therapy efficiency by ANs.

4.Delivery of the artificial nucleases into the mammalian cells

4.1. A water-soluble cationic lipopolymer (WSLP) was synthesized as a novel drug delivery system to improve the efficiency of the artificial nuclease action in cells. WSLP was prepared by the modification of high molecular weight branched poly(ethylenimine) (PEI) with lipophilic cholesteryl chloroformate. The cholesterylation was efficient, in average ~ 10% of the PEI nitrogens were reacted. This kind of modification of a cytotoxic high molecular weight PEI retains the positive charges for strong interaction with DNA, while decreasing the toxicity of the preparation. [3]

4.2. The interaction between the lipopolymer and DNA, monitored by agarose gel electrophoresis, clearly showed the lack of the free DNA at 5/1 and higher N/P ratios of the WSLP and DNA corresponding to the overall positive charge of the particles at this N/P ratio. Electron microscopy also proved that the DNA is condensed by the cationic WSLP, being important for the protection of the carried DNA against DNA degrading and damaging agents. [3]

4.3. The specific charge of the polymers was determined by the titration of sodium dodecyl sulfate (SDS) by PEI and WSLP in particle charge detector cell. The specific charges of PEI and WSLP were calculated, to be q(PEI) = 15.38 mmol g^{-1} and q(WSLP) = 6.76 mmol g^{-1}, respectively. The theoretically maximal specific charge of the applied PEI polymer is 23.22 mmol g^{-1}, suggesting that ~ 66% of the nitrogens were protonated in PEI under the measurement conditions. From the specific charge data the M_w of the WSLP could be estimated to be between 269.7 and 316.5 kDa. [3]

4.4. WSLP and the WSLP/DNA adducts did not show significant toxicity at concentrations of ~ 150 ng per well. However, the toxicity was dose and cell-line dependent. [3]

4.5. A549 and HeLa cells were more efficiently transfected using the new WSLP than with Lipofectamine 3000 standard transfection reagent under the applied conditions. [3]

5.Development of specific and regulated NColE7-based zinc finger artificial nucleases

5.1. New C45-ZF-N85, C45-ZF, as well as W/A and R/G mutants of C45-ZF-N85 proteins were successfully expressed and purified by cation exchange chromatography. The analysis of the CD spectrum revealed the expected secondary structure of C45-ZF-N85, while ESI-MS approved the M_w to be 26057.0 Da.

5.2. The nuclease activity experiments demonstrated the specific DNA recognition and cleavage under the applied experimental conditions. The C45-ZF-N85 AN could cleave the Z0 plasmid containing the recognition site of the zinc finger domain, but could not cleave the pGEX-6P-1 plasmid that does not contain the specific recognition site.

5.3. The truncated version C45-ZF, did not cleave the plasmid DNA, independently of the DNA sequence. By this, we could prove that the catalytic domain does not function in the absence of the activator domain, thus the C45-ZF-N85 AN has a positive allosteric regulation.

5.4. The redesign of the zinc-finger part of the new ANs to target the ErbB1 oncogene has been initiated through several steps of single finger mutations. Comparison of the DNA cleavage properties of

several successfully modified full length and truncated AN versions within BL21(DE3) bacterial cells proved that the AN design is robust, so that the DNA recognition domain is exchangeable.

6. PUBLICATION LIST

Identification number in the Hungarian Collection of Scientific Publications (MTMT): 10069615

Publications related to the dissertation

[1] **H.A.H. Abd Elhameed**, B. Hajdu, R.K. Balogh, E. Hermann, E. Hunyadi-Gulyás, B. Gyurcsik: Purification of proteins with native terminal sequences using a Ni(II)- cleavable C-terminal hexahistidine affinity tag.
Protein Expr. Purif., 159, 53–59 (2019)
IF= 1.291

[2] **H.A.H. Abd Elhameed**, B. Hajdu, A. Jancsó, A. Kéri, G. Galbács, E. Hunyadi-Gulyás, B. Gyurcsik: Modulation of the catalytic activity of a metallonuclease by tagging with oligohistidine.
J. Inorg. Biochem., 206, 111013 (2020)
IF= 3.2

[3] **H.A.H. Abd Elhameed**, D. Ungor, N. Igaz, M.K. Gopisetty, M. Kiricsi, E.Csapó, B. Gyurcsik: High molecular weight PEI-based water-soluble lipopolymer for transfection of cancer cells.
Macromolecular Bioscience, 2000040 (2020)
IF= 2.895

ΣIF = 7.386

Presentations at international conferences

1. **H.A.H. Abd Elhameed**, B. Hajdu, Z. Fabian, E. Hermann, W. Bal, B. Gyurcsik: Affinity protein purification resulting in protein sequence without remaining amino acid residues. *Talking molecules: the network that shape the living world. Plenary meeting of the Association of Resources for Biophysical Research in Europe-Molecular BioPhysics in Europe (ARBRE-MOBIEU) COST Action CA 15126*, Warsaw, Poland, 19-21 March, (2018)-poster

2. **H.A.H. Abd Elhameed**, B. Hajdu, Z. Fabian, E. Hermann, W. Bal, B. Gyurcsik: Affinity protein purification resulting in protein sequence without remaining amino acid residues. *Biotechnology and Research conference*, Rome, Italy, 25-27 April, (2018)-poster

3. **H.A.H. Abd Elhameed**, Bálint Hajdu, Enikő Hermann, Mohana Krishna Goppisetty, Mónika Kiricsi, Ditta Ungor, Edit Csapó, Wojciech Bal, Béla Gyurcsik: Metal ions as regulatory elements of artificial nucleases. *ISMEC2019, International Symposium on Metal Complexes*, Hajdszoboszló, Hungary, 11-14 June, (2019)-lecture

4. **H.A.H. Abd Elhameed**, Béla Gyurcsik, Mohana Krishna Goppisetty, Mónika Kiricsi, Ditta Ungor, Edit Csapó: Artificial Nucleases-Molecular Tools for Gene Therapy. *The 2nd International Conference "Plant Genome Editing & Genome Engineering*, Vienna, Austria, 5-6 July, (2019)-lecture

5. **H.A.H. Abd Elhameed**, Bálint Hajdu, Mohana Krishna Goppisetty, Mónika Kiricsi, Ditta Ungor, Edit Csapó, Béla Gyurcsik: 6×HIS tag modulates the catalytic activity of NColE7 nuclease. *Living Molecules: towards Integrative Biophysics of the cell. Plenary meeting of the Association of Resources for Biophysical Research in Europe-Molecular BioPhysics in Europe (ARBRE-MOBIEU)*, Prague, Czech Republic, 24-26 February, (2020)-poster

Coauthor at international conferences

1. Z. Fábián, B. Hajdu, E. Hermann, **H.A.H. Abd Elhameed**, W. Bal, B. Gyurcsik: Affinity protein purification resulting in protein sequence without remaining amino acid residues. *ISMEC2018, International Symposium on Metal Complexes,* Florence, Italy 3-7 June, (2018)-poster

2. B. Gyurcsik, Z. Fábián, E. Hermann, E. Németh, B. Hajdu, R.K. Balogh, **H.A.H. Abd Elhameed**, C. Oostenbrink, K. Nagata: Development of novel zinc finger-based artificial nucleases. *43rd International Conference on Coordination Chemistry (ICCC2018),* Sendai, Japan 30 July - 4 August, (2018)

3. B. Gyurcsik, B. Hajdu, Z. Fábián, E. Hermann, E. Németh, R.K. Balogh, **H. A.H. Abd Elhameed**, C. Oostenbrink, K. Nagata: Multiple allosteric control in novel zinc finger-based artificial nucleases. *14th European Biological Inorganic Chemistry Conference (EuroBIC 14),* Birmingham, UK, 26-30 August, (2018)

4. B. Gyurcsik, B. Hajdu, E. Hermann, R.K. Balogh, H.A.H. Abd Elhameed: Intramolecular allosteric control of NColE7 metallonuclease based on the specificprotease action of nickel(II) ions. *Molecular Biophysics: ABC of the puzzle of Life, ARBRE-MOBIEU Plenary Meeting.* March 18-20, 2019, Zagreb, Croatia.

5. **H.A.H. Abd Elhameed**, B. Hajdu, B. Gyurcsik: Modulation of catalytic activity of the NColE7 metallonuclease. *ISMEC2019, International Symposium on Metal Complexes,* 11-14 June 2019, Debrecen, Hungary.

6. B. Gyurcsik, B. Hajdu, **H.A.H. Abd Elhameed**, W. Bal, K. Nagata: Metal ions as regulatory elements of artificial DNA cleaving enzymes. *15th International Symposium on Applied Bioinorganic Chemistry (ISABC15),* June 2-5, 2019, Nara, Japan.

7. E. Hermann, **H.A.H. Abd Elhameed**, E. Németh, R. Csáki, B. Hajdu, B. Gyurcsik: Purification and characterization of the C45-ZF-N85 artificial zinc-finger nuclease and its mutants. *Progressive Trends In Coordination, Bioinorganic and Applied Inorganic Chemistry, XXVII. International Conference on Coordination and Bioinorganic Chemistry (XXVII. ICCBIC)*, June 2-7, 2019, Smolenice, Slovakia.

8. B. Hajdu, **H.A.H. Abd Elhameed**, E. Hermann, R.K. Balogh, É. Hunyadi-Gulyás, K. Kato, K. Nagata, W. Bal, B. Gyurcsik: Applications of Ni(II)-induced peptide bond cleavage. *19th International Conference on Biological Inorganic Chemistry (ICBIC-19)*, August 11-16 2019, Interlaken, Switzerland.

9. B. Gyurcsik, B. Hajdu, **H.A.H Abd Elhameed**, W. Bal, K. Nagata: Metal ions as regulatory elements of artificial DNA cleaving enzymes. *Serbian Biochemical Society, Ninth Conference with international participation,* University of Belgrade – Kolarac Endowment 14-16.11.2019. Belgrade, Serbia.