Detection of cardiac troponin I using nuclease resistant oligonucleotides

PhD thesis outline

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Budapest
2018

Introduction

Acute myocardial infarction is leading cause of death in the middle-aged and older population worldwide. Thus, prompt, on-site diagnostic is the prerequisite for timely triage of patients with acute chest pain. In modern laboratory diagnostics the kinetic measurement of creatine kinase, lactate dehydrogenase-1 and aspartate aminotransferase enzymes has completely replaced by sensitive, immunoassay-based detection of specific biomarkers. Presently, the most specific markers of myocardial injury are cardiac troponin proteins, which have been considered as 'gold standards'. The 80 kDa heterotrimer troponin complex is composed of I, T and C subunits with different properties and functions. The inhibitory subunit of the complex is troponin I, the C subunit has a Ca^{2+} binding domain, while TnT anchors the complex on tropomyosin. Each of these three subunits of the troponin complex has muscle type specific isoforms. However, the troponin C is present in only two isoforms in the three different striated muscle types; in adult myocardium and slow skeletal muscle the same TnC protein is expressed. In contrast, both TnI and TnT subunits are encoded by three genes i.e., slow skeletal, fast skeletal and cardiac muscle specific isotypes. Therefore, the appearance of cardiac TnI and TnT proteins in the systemic circulation is a highly selective indicator of cardiomyocyte necrosis.

In recent years, it has been demonstrated that short single-stranded oligonucleotides can selectively bind to their target molecules with high affinity thus they are promising new alternatives to antibodies. Nucleic acids are macromolecules composed of nucleotide units which, in addition to storing genetic information, play an indispensable role in protein synthesis, translation and transcription. It is known that nucleic acids, although only 4 nucleotides are involved in their construction, are able to form stable, but also dynamic structures and supramolecular complexes by self-organization, and both RNA and DNA can bind to their target molecules with high affinity. In the 1990s, two research groups independently recognized the possibility of producing selective single-stranded oligonucleotides by *in vitro* methods. Tuerk and Gold generated a pool of RNAs

randomised at eight specific positions and subjected to selection for binding to Gp43 T4 DNA polymerase. The screening technology that is employed to identify high-affinity RNA sequences has been described as Systematic Evolution of Ligands by EXponential Enrichment (SELEX) method. Working independently, Ellington and Szostak also successfully selected RNA molecules, which were capable for selective binding of small organic dyes. Ellington coined the term 'aptamer', derived from the combination of the Latin word aptus ('to fit') and the Greek word meros ('part') for short, single-stranded oligonucleotides of selective binding capacity. Aptamers have been developed against hundreds of target molecules, including inorganic and organic small molecules, peptides, proteins, carbohydrates, lipids and even such complex ligands as viruses, organelles, bacteria, and cells.

During the selection and synthesis of aptamers there is no need for immunization of animals and the use of living cells; thus, theoretically the oligonucleotides specific to targets with low immunogenicity or even toxic molecules can also be produced. In addition, the conditions of the selection can be freely chosen in accordance with the intended practical application of the aptamer, thereby ensuring the functionality and specific binding of the molecules to their ligands in prevailing conditions. *In vivo* application of aptamers does not generate immune response, and the therapeutic effect may be deactivated by the addition of the corresponding complementary oligonucleotide. Due to their chemical structure, nucleic acids are more stable than protein molecules; they are more resistant to physical-chemical effects and can be regenerated after denaturation and simply modified with different functional groups or labels.

One of the main limiting factors for therapeutic and diagnostic use of aptamers is that nucleic acids are inherently susceptible to exo- and endonuclease catalysed degradation. Because these nucleases are abundant in biological fluids, in recent years several modifications of nucleotides were introduced to increase the stability of aptamers. An effective approach for the generation of highly stable molecules is the use of mirror image aptamers, so-called Spiegelmers. These oligonucleotides are composed of L-nucleotides –instead of the natural D-nucleotides- thus are not recognized by nucleases and display exceptional serum

stability. Hitherto, Spiegelmers have been used for therapeutic purposes with promising results but their diagnostic exploitation has not been explored, yet.

Objectives

The main objective of our work was to identify nuclease-resistant oligonucleotides, which selectively bind to the cardiac troponin I, a biomarker of the acute myocardial infarction. Furthermore, we aimed to confirm the applicability of the selected Spiegelmers in complex biological matrix. The specific goals of the PhD project were as follows:

1. Production of two human troponin I proteins (sTnI and cTnI) required for the characterisation of Spiegelmers by using wheat germ-based *in vitro* translation system
2. Determination of the D-peptide sequence to be used for selection
3. Selection of D-oligomers from random DNA oligonucleotide library
4. Development of a label-free method for the screening of sequences identified by the SELEX procedure
5. Development a highly sensitive, bead-based homogenous sandwich assay
6. Testing the applicability of selected Spiegelmer in human serum as an antibody replacing receptor

Methods

Production of proteins by *in vitro* translation

According to the published data, the recombinant full-length troponin I protein has very low expression level in bacteria therefore the target proteins were produced in wheat germ based *in vitro* translation system. Following the amplification of the troponin I genes, constructs suitable for the synthesis of GST-tagged proteins were produced by ligation independent cloning, and mRNA was

synthesized by *in vitro* transcription. The wheat germ extract was mixed with the mRNA transcript and layered below the translation feeding buffer to form a bilayer *in vitro* translation system. Following the incubation the efficiency of translation was checked by SDS-PAGE and Coomassie blue staining. The production of GST-tagged troponin I-proteins was also confirmed by immunoblot technique**,** whereby the synthetized proteins were blotted onto a PVDF membrane, and then the primary antibodies and HRP-conjugated secondary antibodies were incubated with the membrane and detected by chemiluminescence technique.

Selection of Spiegelmers

As a first step of our work, we designed a DNA library containing 40 nucleotides of randomized sections, from which we planned to select the aptamers specific for the D-peptide, representing the enantiomer pair of troponin I. Selection of Spiegelmers cannot be performed by adapting the conventional aptamer selection technique, because the polymerases used during the process are not capable of recognizing the mirror-image nucleotides. In addition, after the last selection step, sequencing the library requires cloning into bacteria and an additional amplification step is required. For all of these reasons, during the selection the mirror image pair of the target molecule was used.

In order to determine the sequence of the peptide to be used for the selection, a comparative analysis of the highly homologous heart muscle and slow skeletal troponin sequences was performed. Considering the constraints of reliable troponin detection, we chose the epitope corresponding to positions 28–36 of cTnI. The D-enantiomer peptide of the assigned amino acid sequence was commercially synthesized and covalently linked onto reactive bromoacetyl paramagnetic particles.

First, selection buffer containing the oligonucleotide library stock solution was incubated with cysteine coated, peptide-free beads to eliminate the oligonucleotides which could bind either to matrix of magnetic beads or to cysteine. Following the counter-selection, the supernatant was used for the first round of SELEX and the ssDNA pool was incubated with bead bound D-peptide.

The non-specifically bound oligonucleotides were discarded by washing the resins with PBS solution. The putative Spiegelmer enantiomers were amplified by PCR and the selected sense ssDNA strands were separated from the biotinylated antisense ssDNA by alkaline denaturation after affinity purification with streptavidin-coated paramagnetic beads. Selection cycle was repeated nine times with gradually decreasing peptide concentration, incubation time and more vigorous washing condition to increase the affinity of selected aptamers. Following the last selection step, the PCR products were inserted into a cloning vector, transfected into competent cells, and the sequence of 85 inserts was determined by Sanger sequencing.

Screening and characterization of Spiegelmers

In order to designate the most promising aptamers, the isolated oligonucleotides have to be evaluated individually in terms of their target-binding properties. To avoid the synthesis of each Spiegelmer candidates, we have developed a novel method to identify the most promising Spiegelmers in collaboration with the Department of Inorganic and Analytical Chemistry at Budapest University of Technology and Economics. The developed approach provides a method for cost-effective pre-screening of sequences without the synthesis of Spiegelmer candidates. To pre-screen the selected oligonucleotides, the intensity of the interaction between the isolated D-oligonucleotide and the D-cTnI peptide was assessed by surface plasmon resonance imaging (SPRi). In order to simplify the procedure, the candidate oligonucleotides were amplified by PCR and the double-stranded, biotinylated DNA products were denatured by alkali treatment after their immobilization on streptavidin-coated SPR chip. Following the pre-screening, the most promising sequences were converted into Spiegelmers by synthesizing them form L-nucleotides. The thiol labelled Spiegelmers were linked to the gold surface of the sensor chip and kinetics of Spiegelmer-cTnI protein interactions were examined. (Figure 1)

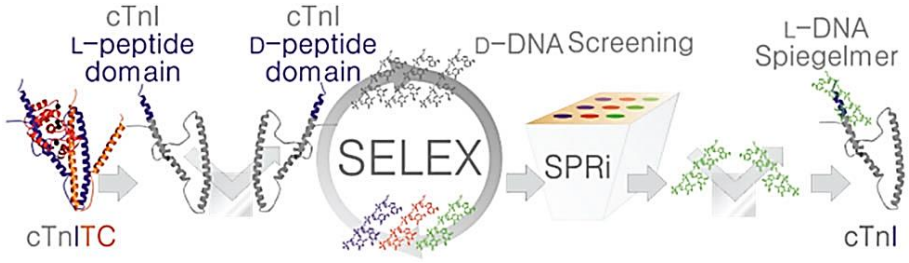

Figure 1: Schematic illustration of Spiegelmer selection

ALPHA

In the present study, we studied the analytical application of our Spiegelmer to test whether it can substitute antibodies of sandwich type assays for quantitative assessment of cTnI in human serum. To implement these proof-of-principle studies, we used a highly sensitive, bead-based homogenous proximity assay, the so-called ALPHA (Amplified Luminescent Proximity Homogeneous Assay). This method relies on selective antibody coated donor and acceptor beads that are brought into proximity through binding to the pertinent analyte. Upon light exposure at 680 nm, the donor bead released singlet oxygen reaches the acceptor beads in vicinity and reacts with the 1,4-oxathine derivative in the acceptor. The cascade of chemical reactions leads to chemiluminescence, which further activates the anthracene derivative in the same bead. As a result, final fluorophores emit light shorter than wavelengths of excitation, ranging from 520 to 620 nm. For measuring cTnI we selected streptavidin- and Protein A-covered donor and acceptor beads, respectively. Accordingly, Spiegelmer was synthesized with the biotin modification at the 5 'end, while the second receptor molecule was a monoclonal antibody recognizing the cTnI protein. Since our previously generated Spiegelmer was directed against an N terminal peptide sequence of cTnI, we chose a commercial antibody that was raised against a C terminal epitope of our protein of interest. Prior to measurements in human serum, the antibody was first

incubated with the acceptor bead and then crosslinked between the Fc region of the antibody and Protein A molecules with dimethyl pimelimidate (Figure 2).

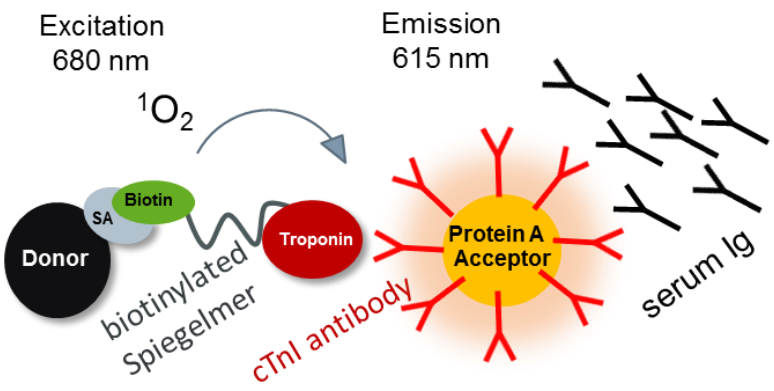

Figure 2: Study of the functionality of Spiegelmers in complex medium by ALPHA method.

IV. Results

Selection of cTnI-specific Spiegelmers

After obtaining the sequences of isolated oligonucleotides, we made multiple sequence alignment to find the identical sequences and similar patterns. The base sequences of the variable regions of the oligonucleotides were subjected to comparative analysis with the software ClustalX2, and the results confirmed the enrichment of certain sequences. Out of cloned 96 inserts, 32 had the same sequence, 6 appeared twice, and the rest represented orphan sequences. According to the theory of SELEX, the selection procedure results in enrichment of those aptamers that bind their target molecules with the highest affinity. However, it has also been reported that the selection could be distorted by intrinsic differences in the amplification efficiency of nucleic acid templates. Therefore, the most

abundant oligonucleotides of SELEX do not necessarily represent the highest affinity aptamers. Consequently, in order to designate the most promising aptamers, the isolated oligonucleotides have to be evaluated individually in terms of their target-binding properties.

Screening and characterisation of Spiegelmers

Using our novel approach, the interaction of D-peptide ligand and D oligonucleotides was investigated. According to these results, all studied oligonucleotides can bind the selected peptide ligand. Of note, the most abundant oligonucleotide demonstrated the second smallest affinity for the D-cTnI peptide affirming the previous finding that the most frequently represented sequence is not necessarily the best aptamer candidate. Following the pre-screening of Spiegelmer candidates, the affinities between two L-oligonucleotides and the purified target proteins were measured. The equilibrium dissociation constants of both Spiegelmers and the troponin protein were in the low nanomolar range and in agreement with the results of the D-DNA strand pre-screening, the Spiegelmer equivalent of the most abundant oligonucleotide possessed a lower affinity towards the cTnI protein. Thus, the obtained data confirmed the results of oligonucleotide screening experiment implying pertinence of the proposed approach for identification of the most promising Spiegelmer candidates.

Having demonstrated the high cTnI affinity of selected Spiegelmers, we embarked on evaluating their selectivity. cTnI is a positively charged protein under physiological conditions (pI 10.31); thus, it could interact with the inherently negatively charged Spiegelmers by formation of non-selective, electrostatic interactions. To investigate this possibility, we measured the interaction of Spiegelmers with lysozyme (pI 11.35) using the Spiegelmer spotted sensor slide. No increase in the SPR signal was detected indicating the specificity of Spiegelmer-cTnI interaction. Although our target peptide of the selection

procedure is specific for cTnI, we scrutinized the troponin I selectivity of Spiegelmers. To this end, the modified SPR sensor chips were challenged with purified sTnI. The measurements again revealed lack of interaction indicating that the analysed Spiegelmers can discriminate the troponin I isoforms.

Following cardiac cell death, the majority of cTnI is released into the circulation as a part of the ternary complex (cTnI–cTnT–TnC); therefore, the monomer and also the complex forming cTnI have to be detected to obtain diagnostically valuable data. To evaluate the cTnI complex perceiving capability of Spiegelmers, SPRi interaction analysis of Spiegelmer modified sensor chips with the cTnI–cTnT–TnC protein complex, the generally accepted reference material of cTnI measurment by the AACC (American Association for Clinical Chemistry), was performed. The measurements showed obvious interactions with both Spiegelmers. Finally, to assess if the selected Spiegelmers meet the stability requirement of an ideal diagnostic receptor, the Spiegelmer spotted chip was desiccated and stored at 4 °C for six months, then challenged with cTnI. The SPRi measurements revealed no loss of sensitivity of the Spiegelmer modified sensor slides. Furthermore, the chips withstood multiple harsh regeneration steps using 20 mM NaOH, as shown by the unchanged SPR responses of subsequent cTnI measurements.

Application of Spiegelmers in sandwich type ALPHA assay

To test applicability of Spiegelmer-antibody sandwich assay, translational mixtures containing the GST tagged cardiac or skeletal troponin I was added to the ALPHA mixture in various dilutions. A significantly higher signal intensity measured with cardiac troponin I suggests that Spiegelmer is capable of selective detecting its target molecule even in a complex matrix. Our results suggest that the high ALPHA signal detected in case of translated cTnI was not the consequence of unspecific binding of the Spiegelmer. As a proof of their potential diagnostic

receptor application, the Spiegelmers were tested for the selective recognition of cTnI in human serum. However, due to the enhanced matrix effect in homogeneous systems, we needed to further optimize the measuring process and immobilize the antibodies onto ALPHA acceptor beads. The obtained data demonstrated low standard deviation of the parallel samples and the plotted luminescence signal intensity showed good linearity in the ng/ml range of troponin. To answer whether the Spiegelmer in the optimized system can distinguish the troponin I skeletal muscle and myocardial isoforms, a human sTnI protein was added to the troponin-free serum at a final concentration of 50 ng/ml. The detected fluorescence signal corresponded to the background, which also demonstrated a high selectivity of our method.

Conclusions

In the course of our work, we aimed at producing human cTnI protein selective Spiegelmers for diagnostic purposes. According to our results we can conclude:

1. Protein selective Spiegelmers can be effectively produced by a rational identification of relevant protein epitopes. The success of this procedure is demonstrated by Spiegelmers that could recognize both troponin I monomer and heterotrimer I-T-C complex. Based on the SPRi analysis, the values of the dissociation constants are within the nanomolar range thus their affinity is similar to those of antibodies currently employed in diagnostics.

2. Using our novel approach the pre-screening of the candidate sequences can be accomplished without costly synthesis of the Spiegelmers. Our results indicate that measuring the interaction of the D-peptide and the D-oligomers enables the selection of the most promising Spiegelmer candidates.

3. Based on our results we can conclude that most abundant oligomers do not necessarily have the greatest affinity in the selected library.

4. The superiority of the cTnI detecting capabilities of the Spiegelmers is highlighted by the fact that selective determination was achieved. The synthetic receptors can distinguish between nearby homologues of the troponin I proteins and are not bound to protein with high pI, like lysozyme. As a preferred feature of the Spiegelmer, it can be emphasized that it will retain its selectivity in complex medium like wheat germ extract containing translational mixture and human serum.

5. The developed ALPHALisa protocol signifies the applicability of Spiegelmer receptors as antibody replacing molecules in sandwich assays. Thus, our proof-of-principle studies could pave the way for the development of completely oligonucleotide-based, biomarker detecting devices.

Publications

Publications related to doctoral thesis:

1. **Z. Szeitner**, G. Lautner, S.K. Nagy, R.E. Gyurcsányi, T. Mészáros. (2014) A rational approach for generating cardiac troponin I selective Spiegelmers. Chem Commun (Camb), 50: 6801–4. IF: 6,834
2. **Z. Szeitner**, A. Doleschall, M. Varga, K. Keltai, K. Révész, R.E. Gyurcsányi, T. Mészáros. (2017) Spiegelmers as potential receptors for cTnI diagnostics. Anal Methods, 9: 5091–5093. IF:1,9
3. **Z. Szeitner**, J. András, R.E. Gyurcsányi, T. Mészáros. (2014) Is less more? Lessons from aptamer selection strategies. J Pharm Biomed Anal, 101: 58–65. (review) IF:2,979

Publications not related to doctoral thesis:

1. K. Percze, Z. Szakács, É. Scholz, J. András, **Z. Szeitner**, C.H. van den Kieboom, G. Ferwerda, M.I. de Jonge, R.E. Gyurcsányi, T. Mészáros. (2017) Aptamers for respiratory syncytial virus detection. Sci Rep, 7: 42794 IF:4,259