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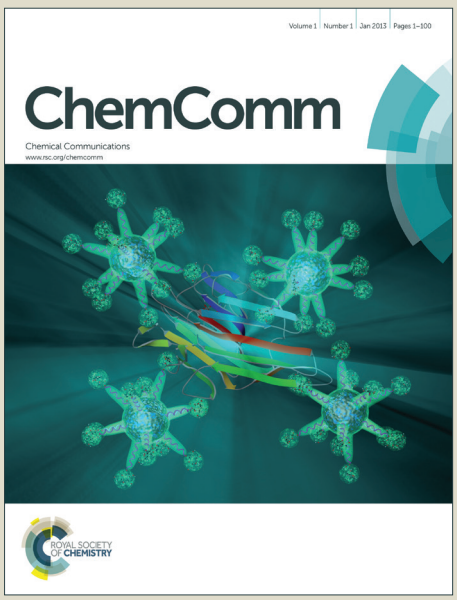

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Rational approach for generating cardiac troponin I selective Spiegelmers

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We report the first protein selective Spiegelmers of diagnostic relevance by rational identification of a target epitope and reverse screening of Spiegelmer candidates following the selection procedure Application of the presented approach resulted in isolation of cardiac troponin selective Spiegelmers with low nanomolar dissociation constant and functionality in serum.

Aptamers are oligonucleotide ligands that bind to their target with high selectivity and affinity, rivaling the characteristics of antibody-antigen interactions.[1] Furthermore, due to their *in vitro* selection procedure, entirely controlled chemical synthesis, and small size, aptamers are superior to antibodies in many terms thus have a great potential for therapeutic and diagnostic applications.[2, 3] Despite of the large number of innovative detection schemes, breakthrough in practical and commercial exploitation of aptamers still awaits for further developments. Hitherto, only mycotoxin and vascular endothelial growth factor selective aptamers have been marketed for food analysis and treatment of age-related macular degeneration, respectively.[4, 5] The development of the latter exposes one of the main limitations of their application as therapeutic agents and diagnostic receptors. Namely, oligonucleotides are susceptible to the ubiquitously present nucleases thus rapidly degrade in the body fluids.[6] Therefore, techniques emerged for generation of aptamers built from modified nucleotides with enhanced nuclease resistance. Although different unnatural nucleotides have been successfully applied to increase the half-life of aptamers, none of them seems to be entirely nuclease resistant.[7] To date, the only exceptions demonstrating seemingly complete resistance to enzymatic degradation are those composed of L-ribose or L-2'-deoxyribose units, i.e., Spiegelmers.[8] These oligonucleotides are enantiomers of natural RNA and DNA molecules, possess the chemical properties of aptamers, but unsusceptible to nuclease digestion. Generation of Spiegelmers is a more elaborate procedure than selection of aptamers and requires the equivalent mirror-image of protein targets. Due to the limitations of peptide synthesis, production of D-enantiomer of proteins is practically not feasible. Consequently, most of the published Spiegelmers have been selected for cytokines and peptide hormones.[9, 10] Notwithstanding, considering that aptamers and Spiegelmers do not recognize their full protein targets but contact them via definite amino acid motifs, protein selective Spiegelmers can be isolated without application of proteins. [11] This approach follows the rationality of antibody generation by using peptides of identified epitopes of protein molecules. Feasibility of this method has been demonstrated with isolation of Spiegelmers for a bacterial enterotoxin using 25-mer D-amino acid peptide.[12] We developed this method further to produce Spiegelmers for cardiac troponin I (cTnI), one of the gold standard biomarkers of acute coronary syndrome (Fig. 1).

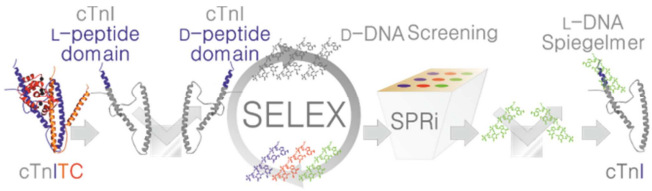


Figure 1. Schematics of cTnI selective Spiegelmer generating protocol.

Although cTnI is released into the circulation upon myocardial injury, its detection is hampered by multiple factors among which are proteolytic degradation, phosphorylation, complexing with other molecules and cTnI-specific autoantibodies circulating in patients' blood,[13] as well as cross-reactivity of c-TnI receptors with skeletal muscle specific troponins (sTnIs).[14] To alleviate these difficulties of troponin measurement, we carefully determined a 9-mer peptide hallmark sequence of cTnI. In comparison to sTnIs, cTnI possesses an N-terminal extension thus assigning an amino acid sequence from this region is expected to ensure the troponin I discriminating capacity of selected Spiegelmers.[15] Although the N-terminal domain is an optimal choice in terms of selectivity of troponin I isoforms, both terminals of cTnI are extremely susceptible to proteolytic degradation; therefore, determination of cTnI concentration using receptors that bind to the N-terminal of the protein could result in misleading data.[16] Further

important factors influencing accurate detection of cTnI are its interaction with troponin C and phosphorylation by Protein Kinase A.[17] Considering these constraints of reliable troponin detection, we choose the epitope corresponding to positions 28-36 of cTnI. The D enantiomer peptide of assigned amino acid sequence was synthesized (D-cTnI peptide) and N-terminally extended with a cysteine to covalently link onto reactive bromoacetyl paramagnetic particles. Prior to selection of specific aptamers, the single-stranded DNA library of 10^{14} variants was challenged with unmodified paramagnetic beads to remove the nonspecifically interacting ssDNAs and obtain the initial selection library. The D-cTnI peptide specific oligonucleotides were isolated basically according to previously described SELEX procedure by using the peptide-coated paramagnetic beads. [18] The selection cycle was repeated nine times with gradually decreasing peptide-coated particle concentration and incubation time combined with increased stringency washing to enhance the affinity of selected ssDNAs (see SI). 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. Analysis of sequencing results indicated a successful selection by enrichment of oligonucleotide library. Out of cloned inserts, 32 had the same sequence, 6 appeared twice, and the rest represented orphan sequences (Table S1.).

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 been also 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.[19] Consequently, in order to designate the most auspicious 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 introduced a prescreening step with a reverse approach to identify the most promising Spiegelmers, i.e., to characterize the interaction of isolated D-oligonucleotides and D-cTnI peptide ligand by using surface plasmon resonance imaging (SPRi). Moreover, to evade the time consuming and costly chemical oligonucleotides synthesis, the evaluated D-oligonucleotides were produced by PCR using biotinylated forward and non-modified reverse primer of selection library. The obtained fragments of the representative amplification reactions were microspotted with solid contact pin onto ExtrAvidin coated SPR chips and directly converted into ssDNA by alkali denaturation for interaction analysis. A different concentration of D-cTnI peptide in selection buffer was flown over the sensor slides to estimate the affinity between selected oligonucleotides and D-cTnI peptide (Fig. 2).

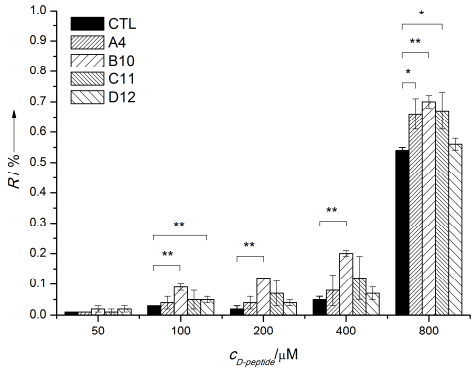
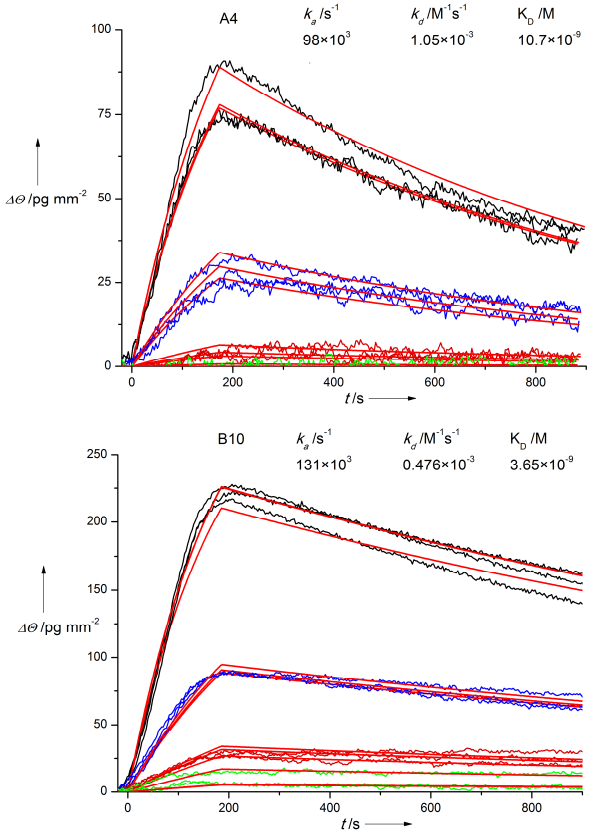


Figure 2. Binding of D-cTnI peptide at different concentrations to selected D-oligonucleotides as determined by SPRi. The selected, biotinylated oligonucleotides (A4, B10, C11 and D12) were immobilized by microspotting their 10 μM solution onto an ExtrAvidin modified SPRi sensor chip. * (P<0.05) and ** (P<0.01) denotes significant difference in reflectivity change between tested oligonucleotides and control. Error bars show the standard error, n=3.

According to these results, all studied oligonucleotides can bind the peptide ligand of selection. Of note, the most abundant oligonucleotide (A4) 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.

To investigate whether the binding properties observed during prescreening of selected D-oligonucleotides with the mirrored cTnI epitope peptide are indicative of the Spiegelmer-protein binding, both the most auspicious (B10) and the most abundant (A4) sequences were synthesized from L-nucleotides with 5' terminal thiol labelling. Next, the thiolated Spiegelmers were spotted onto bare gold sensor slides to characterize their cTnI protein binding kinetics by SPRi. The Spiegelmer spotted chips were blocked with (11-mercaptoundecyl)tetra(ethylene glycol) (HS-TEG),[20] then incubated with various concentration of cTnI protein in selection buffer (Fig. 3).

Figure 3. SPR binding curves of cTnI protein at various



concentrations to surface immobilized A4 (top) and B10 (bottom) Spiegelmers. cTnI was injected in selection buffer at 125, 250, 500 and 1000 ng mL-1 concentrations (bottom-up).

The equilibrium dissociation constant of both Spiegelmers and troponin protein were in the low nanomolar range (K_D = 3.5 nM for B10 and K_D = 10.7 nM for A4) and in agreement with the results

of the D-DNA strand prescreening, the Spiegelmer equivalent of the most abundant oligonucleotide (A4) possessed the lower affinity to cTnI protein. Thus, the obtained data confirmed the result of oligonucleotide screening experiment implying pertinence of the proposed reverse 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) -a protein notorious for non-specific binding-[21] using the Spiegelmer spotted sensor slides (Fig. 4). No SPR signal increase was detected indicating that the electrostatic intermolecular forces between the Spiegelmer and cTnI are disrupted at the physiological ionic strength. The raised cTnI level of blood is a very specific indicator of cardiac muscle damage however false positive values can be measured if the applied diagnostic receptor cross-reacts with the closely homologues skeletal muscle troponin I (sTnI) isoforms. 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 analyzed Spiegelmers can discriminate the troponin I isoforms (Fig. 4).

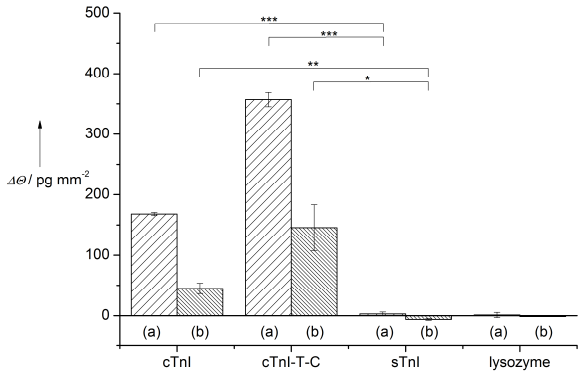


Figure 4. Selectivity of B10 (a) and A4 (b) Spiegelmers expressed through the amount of protein bound to the Spiegelmer modified surfaces upon contacting them with 1 µg mL^{-1} solutions of cTnI, troponin complex (cTnI-cTnT-TnC), sTnI and lysozyme was determined by SPRi. Error bars show the standard error, n=3. * (P<0.05), ** (P<0.01) and *** (P<0.0005)

Following cardiac cell death, majority of cTnI is released into the circulation as a part of ternary complex (cTnI-cTnT-TnC); therefore, the monomer and also the complex forming cTnI have to be detected to obtain diagnostically valuable data.[16] To evaluate the cTnI complex perceiving capability of Spiegelmers, SPRi interaction analysis of Spiegelmer modified sensor chips with commercial cTnI-cTnT-TnC protein complex was performed. The measurements showed obvious interactions with both Spiegelmers, and the SPR signals were higher than measured with monomer cTnI that is in accordance with the larger molecular weight of troponin complex (Fig. 3). These results suggest that the Spiegelmer probes detect both free and complex cTnI forms.

As a proof for their potential diagnostic receptor application, the Spiegelmers were tested for the selective recognition of cTnI in human blood serum. To this end, ten times diluted, troponin-free human serum was spiked with various amount of cTnI to measure the cardiac marker protein concentration dependent alterations of SPR signal. The results of analysis showed a cTnI concentration dependent signal increase for both Spiegelmers, and in concert with the previous experiments, the most auspicious Spiegelmer (B10) demonstrated higher sensitivity of cardiac troponin I detection (Fig. 5).

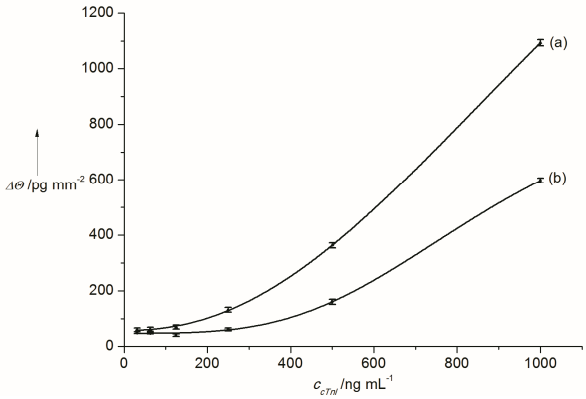


Figure 5. Calibration curve of B10 (a) and A4 (b) Spiegelmers measured in 10-fold diluted serum. Curves are referenced with HS-TEG modified surface and corrected with the blank (10-fold diluted cTnI free serum). Data points are fitted with Hill-equation for 1:1 interaction. Error bars show the standard error, n=3.

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 with 20 mM NaOH as shown by the unchanged SPR responses of subsequent cTnI measurements (Fig. S1).

The in serum stability and analytical performance of the selected Spiegelmers implies that they have the potential to be used as cTnI specific receptors in cardiovascular diagnostic assays. Label-free SPR measurements are appropriate for kinetic characterizations of affinity interactions but they are not sensitive enough for analytical cTnI assays in the diagnostically relevant range (0.04-100 ng/ml). However, what is important from the receptor development point of wiew is the K_D of the Spiegelmer-cTnI complex as the smaller this value the lower the expected LOD is. A thorough analysis of National Institute of Standards and Technology has revealed that the monoclonal antibodies of high sensitivity cTnI diagnostic kits possess at least an order higher K_D then our Spiegelmers. [22] Therefore, it is expected that implementing a label-based amplification or a more sensitive detection technique, that is currently in development in our laboratories, the relevant diagnostic range of cTnI will be achievable. The superiority of the cTnI sensing capabilities of the Spiegelmer-modified chips is highlighted also by the fact that selective determination was achieved at low serum dilutions while conventional ELISA methods may need higher dilutions and a set of monoclonal antibodies to avoid matrix effects and infer selectivity.[23]

In summary, here we showed an approach indicating that protein selective Spiegelmers can be effectively produced by a rational identification of relevant protein epitopes combined with SPRi screening of isolated Spiegelmer candidates. The selection

protocol resulted in isolation of a cardiovascular marker protein discriminatory Spiegelmers, which could be potentially applied in diagnostic assay development as demonstrated by blood serum measurements and lack of cross-reactivity to sTnI.

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† Electronic Supplementary Information (ESI) available: Experimental details of the Spiegelmer selection and screening, SPRi measurements, and stability of the Spiegelmers. See DOI: 10.1039/c000000x/

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