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Epitope Determination of DNA and RNA Aptamers as Antibody Alternatives by Affinity-Mass Spectrometry Open New Perspectives for Peptide Biomarkers and Molecular Diagnostics

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Introduction

Aptamers are short single stranded DNA or RNA oligonucleotides that have recently gained attention as new therapeutic structures vis-à-vis classical IG-type antibodies. In contrast to antibodies, aptamers are chemically synthesized and show a number of unique features, e.g. in bioassays and drug development. As "chemical antibodies", aptamers are less immunogenic and do not interfere with cell viabilities, since they specifically bind and release cells, suggesting their potential for the evaluation of biomarkers. Aptamers are typically produced using the SELEX (Systematic Evolution of Ligands by EXponential Enrichment) procedure [1] with high affinities towards a biomolecular target. The production of aptamers solely in vitro, their chemical synthesis, and high affinity to specific targets have created high interest for development of alternatives to antibodies. However, determinations of aptamer epitopes of protein antigens, as key criteria for their functional specificity have rarely been achieved.

We report here the first epitope and affinity determinations illustrated for DNA protein aptamers in comparison to monoclonal and polyclonal antibodies. Epitopes were identified for two DNA aptamers (60 and 64 bases) against C-MET, a cancer diagnostic protein, obtained by SELEX from a random DNA library [2]. Proteolytic affinity-mass spectrometry in combination with SPR biosensor analysis (PROTEX-SPR-MS) was used as the principal tool for epitope determination [3]. Moreover, a molecular comparative study of a DNA aptamer and a monoclonal antibody against tumor necrosis factor-alpha protein (TNFα) revealed identical epitope peptides, associated with high affinities (K_D, 7 and 13 nM). The molecular identification of DNA aptamer epitopes from specific protein targets, reported here for the first time, indicate that they have comparable recognition properties to the structure-based recognition of variable antibody sequences.

Materials and Methods

Proteins and selection of aptamers
Synthetic DNA aptamers of C-Met (CNL0003 and CNL0004) consisting of 60 and 64 bases [4] were obtained by SELEX from a large DNA library. A DNA aptamer of TNFα (VR11) [5] composed of 26 bases was produced using the filter SELEX procedure with recombinant TNFα. All aptamers were prepared with 5'-amino groups (5AmMC12).

Proteolytic digestion
Tryptic digestion was performed with an enzyme-substrate ratio of 1:100. High pressure digestion was performed with a Barocycler 2320EXT instrument (Pressure Biosciences, Boston/USA) [2]. Affinity microcolumns were prepared with NHS/EDC-activated Sepharose microbeads, using 40 µg aptamer.

Epitope identification and affinity determinations
Epitope analyses were carried out by epitope-extraction- MS, using 10 µg of tryptic digestion mixture incubated with the aptamer or antibody immobilized on Sepharose resin. After incubation for 2 hrs, the affinity column was washed with ammonium bicarbonate until no background signal was observed

by MALDI-MS. Epitope fractions were eluted with 100 µl 0.1% TFA. MALDI-MS was performed with a Bruker Autoflex III Smartbeam-MS (Bruker Daltonics, Bremen, Germany). SPR Analysis was performed with an Ametek 2CH7500 instrument using 1 x 1 cm gold chips with self-prepared 40 nm gold layer. Affinity determinations and K_D values were obtained with the TraceDrawer 1.7.1 software. Epitope peptides were synthesized by Fmoc-SPPS (Applied Biosystems ABI-433A synthesizer).

Epitope identification and affinity determination using PROTEX-SPR-MS

Figure 1 shows the analytical platform of the SPR-MALDI-MS combination, comprising an autosampler connected to an SPR chip, connection of autosampler and SPR, and a transfer line from SPR to MALDI-MS. The SPR-MS combination can utilize all major biosensor types and microfluidic systems such as prism-based SPR and SAW-biosensors. An SPR fluidic module compatible with both ESI-MS and MALDI-MS has been optimized for protein digestion mixtures containing the immobilized antibody or aptamer. The SPR sensorgram is determined in a first step, and K_D determinations of the chip- immobilized antibody or aptamer interacting with the epitope peptide(s) is performed at different concentrations over the SPR chip. Following removal of unbound components by washing, the epitope(s) are eluted at slightly acidic aqueous conditions and spotted on the MALDI target plate (Figure 2).

Affinity determinations of the aptamer complexes with the C-Met protein were carried out by SPR analysis on self-assembled monolayer (SAM) gold chips, by immobilization of either the aptamers or the protein on the chip surface using standard SAM technology [2]. Binding constants were determined with dilution series of protein and aptamers, respectively. The K_D determinations of the CLN00003 and CLN00004 aptamers revealed 223 nM and 535 nM, respectively. Epitope extraction by PROTEX-SPR-MS was confirmed by replications with an affinity column.

Epitope extraction of aptamer CLN0004 showed a single linear epitope. In contrast, the aptamer CLN0003 revealed an assembled epitope comprised of two peptides within adjacent sequence domains, C-Met [524-543] and [557-568]. The high affinities of epitope peptides were established by synthesis and SPR determinations (K_Ds, <1 µM). Structure modelling of the aptamers was consistent with the identified epitopes.

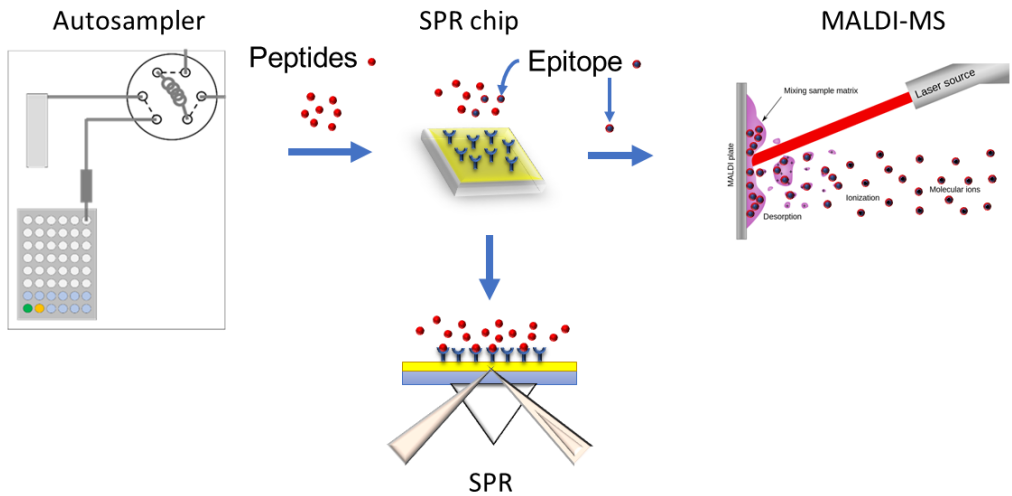

Fig. 1. Analytical platform for the Chip-SPR–MALDI-MS epitope analyzer. An autosampler/valve system connects the biosensor chip with the MALDI-target plate (multi-well plate) for the MALDI-MS analysis. Sample injection is performed on the SPR chip containing the immobilized antibody or aptamer. Peptide mixtures injected over the antibody channel provide the SPR sensorgram of binding epitope(s), and eluted epitope peptide(s) are spotted on the MALDI target plate. K_D determination of the antibody-bound protein and peptide fragments is performed by injecting a dilution series over the SPR chip.

Antibody- and DNA-Aptamer- complexes of Tumor-necrosis-factor-alpha reveal identical molecular epitopes

A comparative study of a DNA aptamer and a monoclonal antibody against tumor necrosis factor-alpha protein (TNFα) was performed by PROTEX-SPR-MS and provided the identification of identical epitope peptides.

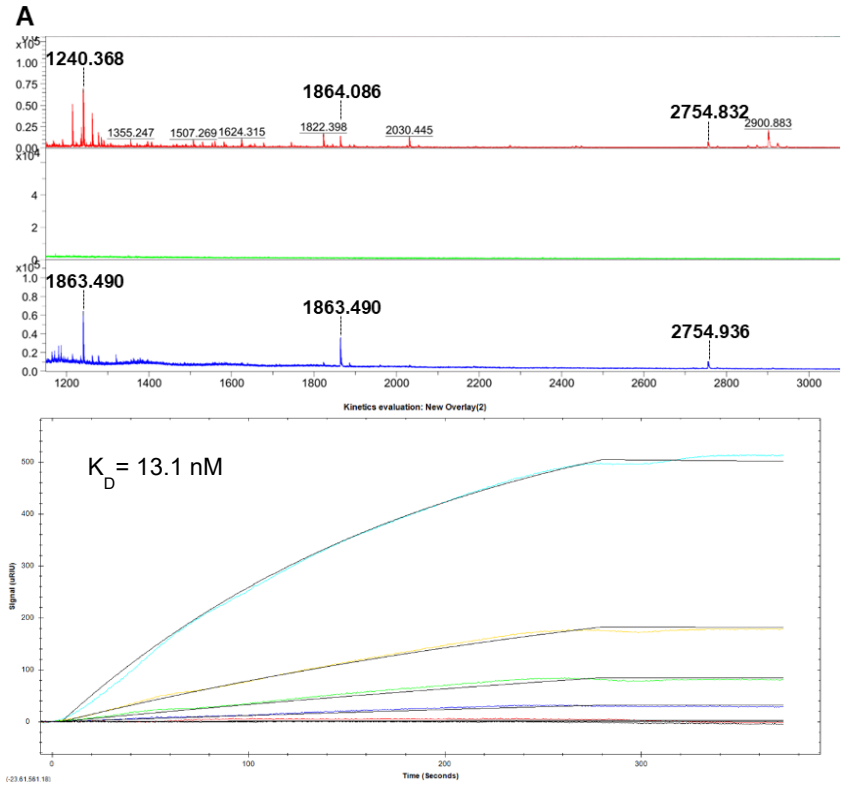

Fig. 2. (A) Epitope identification of TNFα- aptamer by proteolytic epitope extraction and sequential MALDI-MS analysis of supernatant, last wash and epitope elution fractions. B. Anti-TNF alpha antibody interacting with TNF alpha protein- Dilution series (28-950 nM) of intact TNF alpha protein. Identical molecular epitopes (Figure 2A), both associated with high affinities (K_D, 7 and 13 nM) for aptamer and antibody, respectively. (TNFα- [7-31] TPSDKPVAHVVANPQAEGQLQWLNR at $[M+H]^+$ = 1864.1 m/z and [66-82] GQGCPSTHVLLTHTISR by $[M+H]^+$ = 2755.6 m/z).

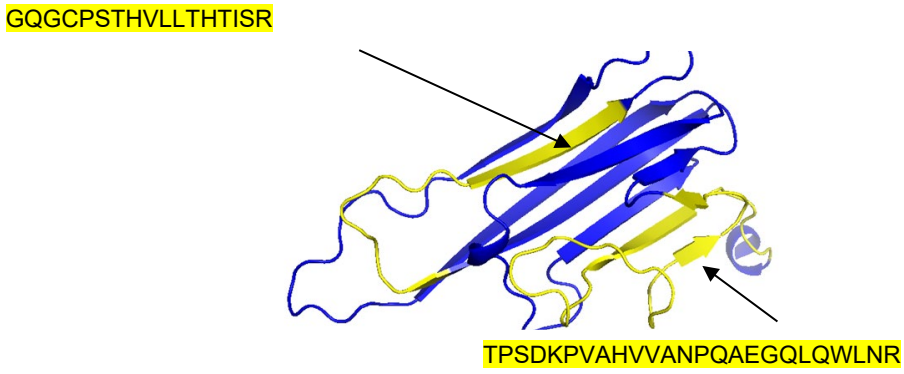

Fig. 3. Ribbon representation of TNFα protein with associated epitope identified (in yellow) from both aptamer and antibody. Epitopes are visualized on the TNFα monomer.

Summary and Conclusions

The molecular comparison of epitopes of TNFα -antibodies and aptamers identified here for the first time opens new perspectives for specific biomarker elucidation and clinical diagnostics. In contrast to antibodies showing both high immunogenicity and considerable stability problems, DNA aptamers exert high stability, specificities, and affinities. Moreover, aptamers generally have low immunogenicity.

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