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# Aptamer–antibody on-chip sandwich immunoassay for detection of CRP in spiked serum

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ABSTRACT

This study describes a RNA aptamer-based biochip with high affinity and specificity for C-reactive protein (CRP). CRP, which exists in concentrations of 1–3 mg/l in the serum of healthy patients, has been identified as a reliable biomarker for inflammation and as a potential marker for sepsis and tissue necrosis. The CRP-specific aptamer was covalently immobilized with its 5′-end on ARChip Epoxy. The detection of bound CRP was carried out optically using labelled secondary antibody in a sandwich format. Assay conditions were optimized with respect to the CRP binding buffer (buffer system, pH and additives) and $Ca^{2+}$ concentration (10 mM). Moreover, two sandwich immunoassay formats were tested, the one using dye-labelled antibodies and the other with biotin-modified antibodies/Dy647-labelled streptavidin. In comparison with an antibody-based chip assay, the aptamer chip is superior in terms of CRP measuring range (10 μg/l to 100 mg/l) in human serum whereas antibody-based chips result in superior data reproducibility (CV of 8–15%). In contrast to antibody chips, aptamer microarrays provide the unique potential of detecting CRP in serum samples of low risk patients (1–3 mg/l) as well as high risk patients (>500 mg/l), furthermore elevated CRP levels (20–350 mg/l) with acceptable recovery (70–130%) by including only one serum sample dilution step (1:100) for the complete measuring range.



## 1. Introduction

The fast and reliable detection and profiling of multiple protein biomarkers in biological samples such as blood and serum is an essential and powerful technique in the diagnosis of diseases and in the monitoring of consecutive therapeutic treatments.

C-reactive protein (CRP), a widely accepted biomarker for inflammation, is an acute-phase protein which increases by a factor of 1000 in response to inflammatory stimulus. In serum as well as in purified state, CRP has a variable molecular weight ranging from 110 to 144 kDa (Oliveira et al., 1977). A CRP level of up to 3 mg/l is considered normal, whereas a higher level is considered abnormally elevated (16 mg/l: Gul et al., 2007; 5 mg/l: Meyer et al., 2006). Due to the risk of severe consequences for patients with high CRP concentrations, bioanalytical devices of high sensitivity are required that allow a fast and precise diagnosis of disease at an early stage.

One well-established and effective method for detecting immunoproteins, such as CRP, is based on the colloidal agglutination of antibody-coated particles upon addition of the antigen (Watkin et al., 2007; Deegan et al., 2003; Ortega-Vinuesa et al., 1997; Rogowski et al., 2005). In fact, some commercial assays (Bayer, Dade Behring, Roche Diagnostics) are based on either immunoturbidimetric (Watkin et al., 2007; Rogowski et al., 2005) or nephelometric detection (Rogowski et al., 2005) using polystyrene latex particles for antibody immobilization.

Detection of CRP on planar surfaces may be performed by classic ELISA (Wu et al., 2002) or using biochips, including both those which function under "microfluidic" conditions (kinetic: Shin et al., 2007; Wolf et al., 2004; Hu et al., 2006; Meyer et al., 2006; Christodoulides et al., 2002; Roper et al., 2006) and others which do not (static measurement: Gul et al., 2007; Abdo et al., 2005). The latter offer the unique advantage of highly parallel assay format and high throughput capacity. Most of the on-chip immunoassays reported in the literature use the sandwich format and perform detection either by absorbance (Christodoulides et al., 2002), fluorescence (Shin et al., 2007; Wolf et al., 2004; Abdo et al., 2005) or SPR (Hu et al., 2006; Meyer et al., 2006; Vikholm-Lundin and Albers, 2006; Bini et al., 2008). The assay principle is usually based on the formation of an antibody–antigen complex. Moreover, alternative approaches have been recently reported, such as those relying on the interaction between phosphocholine and CRP (Deegan et al., 2003; Roper et al., 2006), on the use of a chemically modified thermoresponsive copolymer (Raj et al., 2007) or on a CRP-reactive aptamer (Bini et al., 2008; Kim et al., 2004).

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**Table 1a**
Tested aptamer (1–10) and antibody print buffers (7 and 10), %CV and fluorescence signals (a.u.) at 1 mg/l CRP using the aptamer

| Print buffer | Print buffer composition | Fluorescence signals (a.u.) | %CV |
|---|---|---|---|
| **1** | **10 mM phosphate (pH 8.4)/150 mM NaCl/0.2 mM EDTA** | **9403** | **15** |
| **2** | **Nexterion Spot: 1× PBS (pH 8)/0.005% sarcosyl/0.1% Tween-20** | **8416** | **13** |
| 3 | 0.1 M boric acid (pH 9)/150 mM NaCl/10 mM EDTA | 1524 | 25 |
| 4 | 1× PBS (pH 8)/1% glycerol (v/v) | 926 | 24 |
| 5 | 0.1 M phosphate (pH 8)/10% glycerol (v/v) | 4156 | 22 |
| 6 | 20 mM Hepes (pH 7.4)/150 mM NaCl/2 mM $CaCl_2$/0.005% Tween-20 | 1006 | 31 |
| 7 | 1× PBS (pH 7.2)/0.01% sodium-deoxycholate | 1712 | 30 |
| 8 | 1× DPBS (pH 7.2)/0.9 mM $CaCl_2$/0.5 mM $MgCl_2$/0.1% Tween-20/1% glycerol (v/v) | 865 | 35 |
| 9 | 20 mM Tris (pH 7.4)/150 mM NaCl/5 mM $CaCl_2$ | 986 | 30 |
| 10 | 1× PBS (pH 7.2)/0.005% CHAPS/0.01% BSA | 1865 | 28 |

Such techniques show great promise inasmuch as the receptors can be engineered in order to bind a wide range of non-nucleotide target molecules such as proteins, drugs, whole cells and small molecules. For example, aptamers which are single-stranded DNA or RNA oligonucleotides have been semi-synthetically produced using the SELEX technique (Tuerk and Gold, 1990; Ellington and Szostak, 1990) against a variety of diagnostically relevant marker molecules, among these cancer-associated proteins (McCauley et al., 2003), thrombin (Baldrich et al., 2004) and HIV-1 Tat protein (Minunni et al., 2004). Aptamers have displayed several advantages over antibodies in previous studies: they are highly chemically stable, show high detection sensitivity and selectivity and can be selected in vitro for nearly any given target (Song et al., 2008). In addition, while incorporating a site-specific modification into an antibody can prove difficult, this is easy with aptamers. Modifying them at their 3′- or 5′-end after synthesis is an established technique for improving immobilization as well as target capturing and has been demonstrated for a simple biotin group (Cho et al., 2006), a label molecule (McCauley et al., 2003) and a spacer (Bini et al., 2008). The fabrication and optimization of aptamer arrays for detection of lysozyme, ricin and thrombin was described previously (Cho et al., 2006). A single buffer was found for chip processing (PBS/5 mM $CaCl_2$/0.1% Tween-20), whereas for biotinylated aptamer immobilization on NeutrAvidin/streptavidin-coated slides, 10 mM phosphate (pH 7.4)/137 mM NaCl/3 mM KCl/5% glycerol worked best. A comprehensive study on the fabrication of aptamer arrays including probe preparation and modification, arraying techniques and optimization of assay procedures was reported for lysozyme detection in cell lysates using a direct assay format (Collett et al., 2005).

In contrast to those aptamer array developments our study was intentionally directed at single-stranded RNA aptamers to serve as alternative receptors for CRP detection in a combined aptamer–antibody chip. The study presents a chip-based sandwich immunoassay for CRP in diluted serum using an immobilized aptamer specific to CRP and a secondary fluorophor-labelled antibody for detection. The combined aptamer–antibody chip was compared with the respective antibody–antibody chip with regard to assay performance in serum (% recovery), sensitivity (LOD, LOQ) and data reproducibility (%CV).

## 2. Experimental

### 2.1. Materials and reagents

ARChips Epoxy are proprietary slides developed at ARC. Phosphate buffered saline (PBS) was purchased from Gibco. Tris methylaminomethane (Tris) was from Invitrogen, Hepes, boric acid and the buffer additives polyoxyethylene-sorbitan monolaurate (Tween-20), glycerol and the RNase inhibitor "RNase Zap" were purchased from Sigma; Nexterion Spot buffer (10×) from Peqlab and phosphate buffer (Sorensen's) was from Electron Microscopy Sciences. The print buffers were prepared in diethylpyrocarbonat (DEPC, from Sigma)-treated water and filtered through 0.20 μm pore size filters (VWR). DEPC water was prepared by adding 1 ml of DEPC to 1 l of MilliQ water, stirred overnight on a shaker at 37 °C and autoclaved prior to use. EDTA and $CaCl_2$ were obtained from Merck.

The single-stranded RNA aptamer specific for CRP was purchased from IBA Nucleic Acid Synthesis (Göttingen) and consists of 44 bases with the following sequence modified with an aminohexaethylene glycol group (HEGL) (18 atom spacer) at the 5′-end: 5′-GCC UGU AAG GUG GUC GGU GUG GCG AGU GUG UUA GGA GAG AUU GC-3′. The studied RNA aptamer shows a binding constant of 125 nM (Bini et al., 2008). Purified human CRP, monoclonal anti-CRP (C5), biotinylated- and Dy647-labelled anti-CRP (C7) were obtained from Exbio (Praha). CRP-free serum was purchased from HyTest (Finland). Dy647-Streptavidin was from Dyomics.

### 2.2. Chip fabrication and design

250 nM, 500 nM, 1 μM, 25 μM, 50 μM and 100 μM aptamer and 0.25 and 0.5 mg/ml monoclonal anti-CRP in print buffers as described in Table 1a were arrayed on ARChip Epoxy at a relative humidity of 70% using the contact spotter OmniGrid from GeneMachines (4 pins; pin SMP3, spotting volume: 0.6 nl/spot). Bench and spotter were carefully cleaned with an RNase inhibitor.

To thermally equilibrate single-stranded aptamer conformers, the RNA molecule was denatured at 70 °C for 4 min and immediately placed on ice to avoid refolding of the RNA strand. This was done just prior to spotting. The spot to spot distance was 400 μm and the array to array distance was 8950 μm. All probes were immobilized in triplicate and 12 identical arrays were spotted per slide. After spotting, the slides were kept at 4 °C for a minimum of four days to ensure complete receptor immobilization.

### 2.3. Chip processing

Surface blocking was performed in 1× PBS (pH 7.2)/0.1% Tween-20 for 30 min to remove any non-bound receptors and deactivate reactive surface groups. Afterwards the slides were washed twice in 1× PBS (pH 7.2) and dry-centrifuged for 4 min (900 rpm). After blocking, the slides were mounted into the Fast Frame (Whatman), composed of silicone chambers which create 16 separate reaction wells on each slide. On that way a high throughput processing of the microarray was enabled by pipetting and withdrawing the target and washing solutions with a multi-pipette.

Bound aptamers and monoclonal capture antibodies were incubated with human CRP at room temperature (RT) for 1 h. After CRP incubation, the slides were washed three times by pipetting 1×