Biosensors and Bioelectronics 25 (2010) 2302–2308

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A new assay design for clinical diagnostics based on alternative recognition elements

Christiane Albrecht[a,*], Peter Fechner[a], Dmytro Honcharenko[b], Lars Baltzer[b], Günter Gauglitz[a]

[a] Eberhard Karls University of Tuebingen, Institute of Physical and Theoretical Chemistry (IPTC), Auf der Morgenstelle 18, 72076 Tuebingen, Germany
[b] University of Uppsala, Department of Biochemistry and Organic Chemistry, Box 576, 75123 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 8 January 2010
Received in revised form 18 February 2010
Accepted 16 March 2010
Available online 24 March 2010

Keywords:
Immunoassays
(biological) Recognition elements
Kinetics
Clinical diagnostics

ABSTRACT

Herein, we present a new sandwich assay design containing a high affinity polypeptide scaffold as immobilized capture element and an antibody for detection.

These polypeptide scaffolds provide a good affinity towards one antigen and can be linked to biosensor surfaces without affecting their binding capabilities. Furthermore, the small peptides are very stable, which allows for regenerating the surface several hundreds of times and thus for reuse of the biosensor. Moreover, these receptors can be synthesized with different affinities towards one antigen, which has been proven by characterizing them using a label-free detection method RIfS (reflectometric interference spectroscopy) for collecting kinetic data. Polypeptide scaffolds with different affinities have been chosen and characterized.

Upon these results, sandwich-type assays have been set-up using a fluorescently labelled antibody as detection element. Thereby could be shown, that the working range of the assay can be shifted according to the affinity of the used capturing polypeptide scaffold. The scaffolds with a higher affinity towards the antigen can detect lower concentration, and in contrary, scaffolds with lower affinities can detect higher concentrations.

In consequence, using this new sandwich-type assay, we avoid the complex procedure to immobilize antibodies in correct orientation, but simultaneously keep this well-known recognition element in the assay for detection. Furthermore, in addition to all the acknowledged properties of immunoassays, we add the possibility of tuning the working range of assays in distinct manner according to request.

1. Introduction

During the last decades, immunoassays have become a more and more valuable tool in many areas of analytics (Borrebaeck, 2000). They take advantage of the positive abilities of antibodies, like their high affinities and their high selectivity for one antigen, but also suffer from their drawbacks e.g. the rather unstable structure. Antibodies can be made against many kinds of targets, against very small organic molecules as well as against huge proteins, which puts them up for a large area of applications e.g. water analytics and even more important clinical diagnostics (Ramirez et al., 2009).

For these immunoassay applications, the selection of the assay format has a significant impact on practical handling and the sensitivity of the testing results. Typically used assay formats are direct, competitive, displacement and sandwich-type formats (Sapsford et al., 2002). In clinical diagnostics, the sandwich-type assays are still the most commonly used ones due to lower limits of detection compared to other assay types, which are essential for a reliable analysis of many parameters (Jaras et al., 2007). The major drawback of this assay format is the necessity to immobilize the capture antibody on the surface. Antibodies are bio recognition elements, which have been evolving and have been specified over ages, whereby their tertiary structure is crucial for the detection of the antigen. Nevertheless, this highly specified tertiary structure of antibodies is rather susceptible and until now, many immobilization strategies have been developed (Barlen et al., 2009), but no ideal solution to immobilize antibodies without losing a big amount of the binding activity could be found yet. An additional difficulty is to guarantee the correct orientation of antibodies on the surface with the binding sites exposed to the antigen in solution. The most common immobilization procedures such as bonding via amino or carboxyl functions are not site-directed but result in statistically orientated immobilization. This leads to a loss in function and sensitivity as well as a loss in stability of the sensor surface. This reveals another difficulty in handling antibody surfaces. Due

* Corresponding author. Tel.: +49 0 7071 29 74668; fax: +49 0 7071 29 5772.
E-mail address: christiane.albrecht@uni-tuebingen.de (C. Albrecht).

0956-5663/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2010.03.022

to the delicate tertiary structure, antibodies have to be stabilized when spotted during the immobilization procedure as well when stored for longer time periods. There have been several attempts to overcome these problems, such as reducing antibodies down to their Fab fragments (Oshannessy and Hoffman, 1987) or building up artificial constructions like molecular imprinted polymers (MIPs) or plastic antibodies (Haupt and Mosbach, 1998). However, none of these attempts led to satisfactory results in sensitivity and stability.

To phrase the requirements of an ideal recognition element for immobilization, it needs a high affinity and specificity towards the antigen, which is not affected or reduced by being immobilized. Furthermore, the recognition element on the surface should be stable enough to be stored and not losing functionality within time. There are small-structured recognition molecules such as DNA which provide a high stability and represent a guide for new, rationally designed recognition elements. Here we present an immunoassay set-up using a small and stable peptide sequence as immobilized capture element (Baltzer, 2007). These are small helix-loop-helix motifs, which contain natural binders of the target analyte. These are easily accessible while concerted modifications made for immobilization at the artificial helices do not affect binding properties. Due to their small size, the peptides are very stable and can be stored without any further treatment.

With the availability of this stable capture element for immobilization, a new design for sandwich-type assays can be set up. In this paper, we present a completely new sandwich-type assay design for detection of C-reactive protein (CRP), using a tailored binder as capture element on the surface and an antibody as detection element. By this, disadvantages of immobilized antibodies are avoided by replacing them, but their long evolved abilities are still kept in the assay by keeping them as secondary detection element. This heterogeneous assembly in recognition elements is a new approach in assay development. Therefore, the new capture elements have been first characterized by determining their affinity constants towards CRP using reflectometric interference spectroscopy (RIfS), a label-free detection method.

Applications in clinical diagnostics are often using fluorescence based detection methods due to higher sensitivity and better elimination of background noise which is inevitable in complex matrices like human plasma or serum. Therefore, the heterogeneous sandwich assay was set upon a TIRF (total internal reflection fluorescence) based platform. Complete calibration curves were carried out for two different scaffold binders and it could be shown that the different affinities of the binders influence the working range of the assay.

2. Material and methods

2.1. Materials

Common chemicals of analytical grade were purchased from Sigma–Aldrich, Deisenhof, Germany, or Merck KGaA, Darmstadt, Germany. Aminodextran with 100 KDa molecular weight was purchased from Innovent e.V., Jena, Germany. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was used as a buffer solution (10 mM) containing 5 mM $CaCl_2$.For regeneration of the biosensor surface, a 10 mM glycine solution at a pH of 2.5 was used.

The C-reactive protein (CRP) and the two monoclonal antibodies from mouse (C5-clone M86005M) were purchased from Exbio, Prague, Czech Republic. The conjugation service of the detection antibody with DY647 was also kindly provided by Exbio, Prague, Czech Republic.

2.2. Conjugate binders

The design principles have been described in detail elsewhere (Baltzer, 2007). Briefly, all proteins which are detected in clinical diagnostics have a natural binder. In our case, it is a small organic molecule which binds to its target protein with high specificity. The conjugate binders consist of a stable polypeptide scaffold with 42 amino acid residues which have no affinity for the target protein. They were designed to form helix-loop-helix motifs based on amphiphilic helices because of their ability to present well-defined interaction surfaces in the folded state. The actual binder, the small organic molecule, can be attached to one of the amino acid residues. Furthermore, other linkers for labeling or immobilization can be attached at other positions of the protein scaffold without affecting the binding capability and affinity of the organic molecule. The mechanism of binding can be described as an adapted fit where the small molecule binds to its specific binding site and the polypeptide seeks the interaction of the lowest free energy that is compatible with small molecule complexation to its binding site and the spacer length. The binding energy comes most likely from hydrophobic interactions between polypeptide and protein and selectivity is provided by charge–charge interactions.

The obtained binders vary with regard to affinities due to the variable charge complementarity between target protein and polypeptide and with regard to the position to which the organic molecule is attached to.

The resulting binder molecules are robust and small with molecular weights of around 5 kDa while being capable of high affinities and specificities. Their chemical origin ensures that there is no prior relationship to any biomolecule and therefore, the probability of non-specific binding is low. They are prepared by chemical synthesis and modifications of amino acids are introduced specifically under full synthetic control. The batch-to-batch variation is negligible as they are prepared by chemical synthesis and found to be chromatographically pure.

The polypeptides were prepared by solid phase peptide synthesis using standard Fmoc protocols and PAL-PS as well as PEG-PS resins. They were purified by reversed phase HPLC and identified by MALDI-TOF MS. The small molecule warhead was prepared for conjugation by attaching an aminohexanoic residue and converting the carboxcylic acid to a p-nitrophenyl ester. The active ester was reacted with each polypeptide in pure DMSO solution to form an amide at the side chain of a lysine residue.

The binder molecules for CRP have been described in detail elsewhere (Tegler et al., in preparation and see supplementary material). In short, they were prepared based on the fact that phosphocholine binds CRP with 5 μM affinity. The phosphocholine group was linked by an aminohexanoic acid spacer to the side chain of a lysine residue. Here, one of the CRP binder molecules has been used in combinations with an antibody to form an assay for the identification and quantification of CRP (Fig. 1).

2.3. Set-ups

2.3.1. Reflectometric interference spectroscopy (RIfS)

For kinetic evaluation of the conjugate binders, a biosensor based on reflectometric interference spectroscopy (RIfS) was used. A detailed description of this technique can be found in Schmitt et al. (1997). In short, white light is guided to the transduction layer via an optical fiber and is partially reflected at each interface. The beams superimpose and build a characteristic interference spectrum which is detected using a diode-array spectrometer. Molecules bound to the surface change the physical thickness as well as the refractive index of the toggling layer, causing a shift in the reflectance spectrum. Thus, interaction between the antibody