Review

Immunochemical binding assays for detection and quantification of trace impurities in biotechnological production

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New, highly sensitive, biosensor concepts make it possible to assay biomacromolecules at concentrations that previously were far below the limit of detection. The previous generation of assays used in quality control situations during biotechnological production was designed primarily for monitoring target molecules, which typically appeared in high concentrations. Hence, novel analytical techniques with high sensitivity should become increasingly important in meeting the demands from regulatory agencies with regard to declaring levels of impurities in biopharmaceuticals. Such techniques also open up opportunities for a range of other challenging measurements, for example, in the area of biohazards. This review describes the development of immuno-based biosensors and exemplifies these by presenting analyses of common impurities in biopharmaceutical production.

Introduction

The development in protein purification has to a large extent been driven by the pharmaceutical industry. The need for high purity of the preparations that are to be injected into patients has been very high, and therefore, extensive resources have been spent on developing high-resolution techniques in the downstream area [1].

When reviewing the advances within the area of process analytical technology, it becomes clear that ground-breaking bioanalytical developments have been few in recent decades. Indeed, the use of immunoassays for process control was for a long time regarded as a futuristic dream, even though some early reports clearly indicated the possibilities [2–4]. However, the rising demands with regard to characterization of the low levels of impurities that remain in pharmaceutical preparations have again placed the focus on development of high-sensitivity analytical techniques that are relatively fast, convenient to use, and possible to integrate in the production process for at- or online measurements.

Sensitivity is needed

Impurities are typically present only in minute amounts, which makes it necessary for the analytical procedures to be adapted to the detection and quantification of extremely low concentrations of the target molecules (in this case the impurity). Conventional immunochemical binding assays often operate in the concentration range 10^{-9}–10^{-12} mol/l. However, such a detection limit would in many cases not be sufficient as even higher sensitivity (i.e. at sub-picomolar concentrations) might be required [5,6].

A draw-back with highly sensitive analytical procedures is that any non-specific responses, for example, non-selective binding to the recognition element, dramatically change the analytical outcome. One way to compensate for this is to employ biosensor surfaces that have been designed specifically for the purpose of reducing to an absolute minimum any non-specific binding [7–9]. Another approach is to develop assays that are so sensitive that it becomes possible to dilute the sample by several orders of magnitude, thereby significantly reducing the influence of non-specific binding [10].

Speed is desirable

The presence of certain impurities in a biotechnical production process might vary considerably over time. [11]. Also, the presence of proteases is often time dependent, and this can lead to the appearance of truncated proteins. Therefore, it is important to analyze samples as rapidly as possible and preferably in real time, so that it will be possible to change conditions quickly in order to react to observed increases in the concentration of key impurities by, for example, stopping cultivation and starting harvesting [12]. Likewise, it would certainly be highly desirable to be able to monitor downstream processes, not only broadly via UV absorbance at 280 nm or by conductivity of the eluting buffer, but also by more specific means [2,13–15]. The development towards online immunoassays for process control started some 25 years ago, but it was not regarded as necessary until the process analytical technology (PAT) initiative by the US FDA was presented in 2004 (Box 1) [16].

Convenience a demand

Immunoassays and other sensitive analytical methods must be convenient to use. The traditional quality analyses that involve SDS-PAGE for homogeneity analyses still fulfil a need, even if they are laborious and time consuming [17]. New developments within affinity separation combined with HPLC for the characterization of homogeneity

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Box 1. The PAT initiative

The PAT initiative is designed to support the use of more sophisticated pharmaceutical manufacturing techniques with an emphasis on advanced process monitoring and control [16] to:

- Encourage the early adoption of new technological advances by the pharmaceutical industry
- Facilitate industry application of modern quality management techniques, including implementation of quality systems approaches, to all aspects of pharmaceutical production and quality assurance
- Encourage implementation of risk-based approaches that focus both industry and Agency attention on critical areas
- Ensure that regulatory review and inspection policies are based on state-of-the-art pharmaceutical science
- Enhance the consistency and coordination of the FDA drug quality regulatory programmes, in part, by integrating enhanced quality systems approaches into the Agency business processes and regulatory policies concerning review and inspection activities (Figure I)

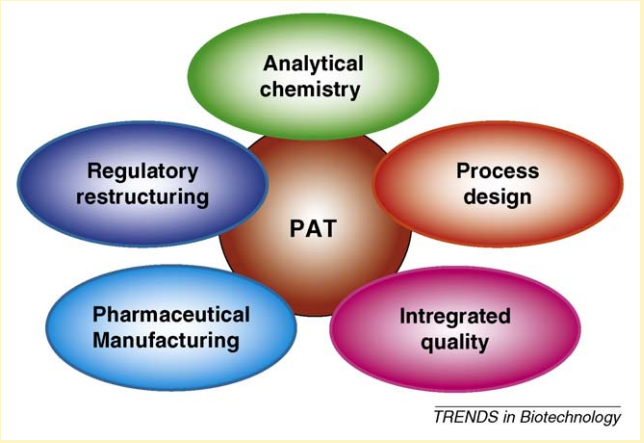

Figure I. Process analytical technology (PAT).

and quantification of target proteins hold great promise for the development of quick and medium-sensitivity analyses [18]. Furthermore, immunochemical binding assays increasingly have been streamlined over the years, but convenience and speed have been obtained at the expense of sensitivity, which however, is not fully compatible with the demands linked to the assaying of impurities in pharmaceutical preparations.

The requirements for analyses to be rapid and highly sensitive can be difficult to fulfil. Reuse of antibodies is a prerequisite for the immuno-based biosensors used for process control. This however requires the regeneration of the immobilised antibody by stripping off the bound antigen, which is often a rate-limiting step. Thus, it has been argued that weak-affinity antibodies are better suited for such applications. In trials, it has become clear that only weakly binding antibodies are easier to deal with when it comes to dissociation. However, these weaker antibodies, generally speaking, result in less sensitive analyses. Thus, what can be gained in speed is lost in sensitivity [19].

Another issue associated with process monitoring is the question of whether discrete assays or continuous monitoring should be used. With discrete assays, high analysis frequency, or in other words, short analytical cycles are needed. Continuous monitoring on the other hand either involves operations with a large excess of binding sites, or exploitation of affinity interactions with fast binding or fast dissociation [20–22]. Furthermore, continuous assays are based on label-free assays, which restrict considerably the choice of assay methods that can be applied in continuous monitoring.

Table 1 contains a list of the most common analytes that can appear as contaminants and their typical concentrations.

Assay methods

When setting up an analytical assay with the objective of it being fast and convenient, the possibility to monitor directly the underlying binding reaction is highly advantageous. The early generations of immuno-based assays required labelled reagents. However, because of the complexities involved, it is certainly preferable to avoid their use and instead monitor the binding reaction directly.

Described below are recently developed methods that have shown great potential with regard to their use in process monitoring. We will discuss three different sensor platforms that are based on the use of electrodes that have been modified using monomolecular layers of alkylthiols, that is, capacitive biosensors, surface plasmon resonance (SPR) sensors, and quartz crystal microbalance (QCM) (Figure 1). Despite it being based on labelled reagents, flow-ELISA is also included here because it is the most used technique to date. Typical sensitivities and dynamic ranges obtained for the different methods discussed are presented in Table 2.

Capacitive biosensors

The assaying principle of capacitive biosensors is simple and does not require the use of labelled compounds. A metal electrode, often made from compact gold of high purity, is covered by a monomolecular layer of alkylthiols in a so-called self-assembled monolayer (SAM) [23,24]. Among the alkylthiols, some molecules are introduced with

Table 1. Typical contaminants in bioproduction

Impurity	Concentration allowed	Analysis method used today	Biosensor option	References
Host cell protein	ng/mg	SDS-PAGE, Western blot	NR	[39,42]
DNA	pg/mg	Q-PCR	Capacitive	[10,44–46]
Endotoxin	Dependent on dose, duration and route of administration	LAL assay	Capacitive	[6,50–52]
Leaking affinity ligand	pg/ml	ELISA	Calorimetric immunoassay	[53,54]
Protein variants	mg/ml	SDS-PAGE, LC-MS	NR	[55–57]

NR: not reported.

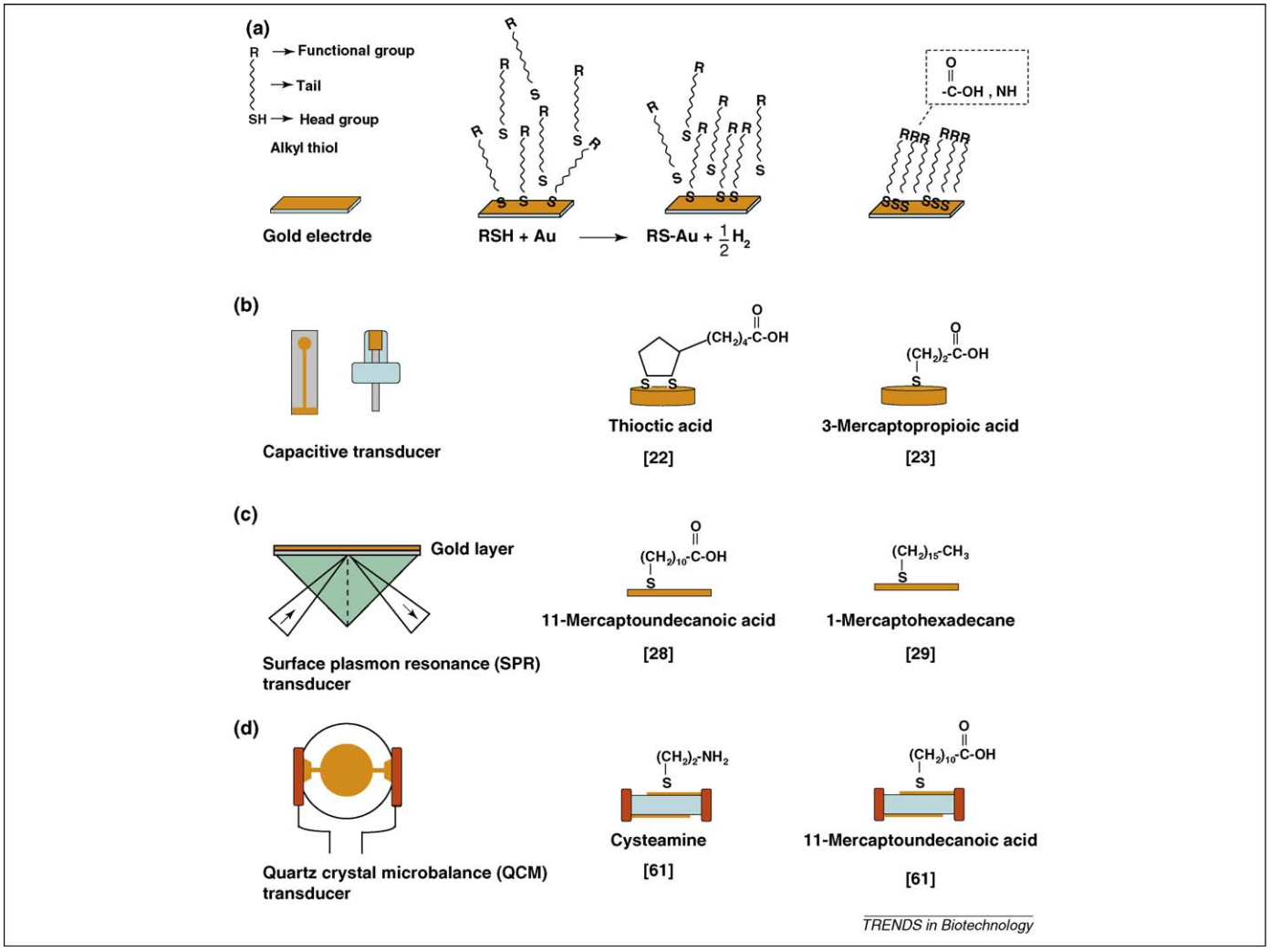

Figure 1. Biosensors based on SAMs. **(a)** General schematics of the self-assembly of a functionalized (-R) alkylthiol monolayer. Examples of active –R groups are shown in the figure. The high affinity between sulphur and gold induce spontaneous formation of the SAM, which is utilised extensively within bioanalytical chemistry. Three different analytical concepts are illustrated here: **(b)** capacitive biosensors [22,23]; **(c)** SPR analysis [28,29]; and **(d)** QCM technology [61]. For these techniques, gold transducer (shown in orange) is of central importance. The thiol-based molecules arrange themselves as monolayers onto the gold surface, which results in a highly insulating layer that protects against, for example, electron transfer, while at the same time offering multiple anchor points for ligand binding.

terminal amino- or carboxyl groups, or alkylthiols to which functional groups have already been attached, as shown in Figure 1 [25,26]. Receptor molecules are bound covalently to these groups and the capacitance is measured. When a target molecule binds to the receptor, displacement of the counter ions (i.e. the diffuse layer) around the capacitive electrode results in a change of the sensor capacitance (Figure 2). The more target molecules bound to the affinity layer, the greater is the achieved displacement and the decrease in the registered capacitance signal [27]. The assay requires that signals from three or four electrodes need to be measured simultaneously. In addition to the gold electrode that contains the immobilised antibodies, one or two platinum electrodes are required as references, together with an Ag/AgCl-electrode to control the baseline drift. In the preferred sensor configuration, the electrode assembly is placed as tightly as possible and the entire unit is then connected to a continuous flow system. The data presented in this review are obtained using such a configuration.

SPR

In SPR, the registered signals are based on optical measurements [28,29]. A light beam is sent through a glass prism at a certain angle relative to the surface of the prism, which has been modified with a SAM. This allows for different chemistries to be used to immobilise the biochemical receptor molecules at the sensor surface.

Table 2. Analytical methods used for monitoring impurities in protein pharmaceuticals

Analytical techniques	Analytical performance		References
	Linear dynamic range (ng/ml)	Limit of detection (ng/ml)	
Capacitive biosensor	0.001–1	0.001 (fg/ml)	[6]
SPR	100–10 000	20	[58]
QCM	50–1000	16	[59]
Flow-injection ELISA	0–300 000	NR	[60]

NR: not reported.

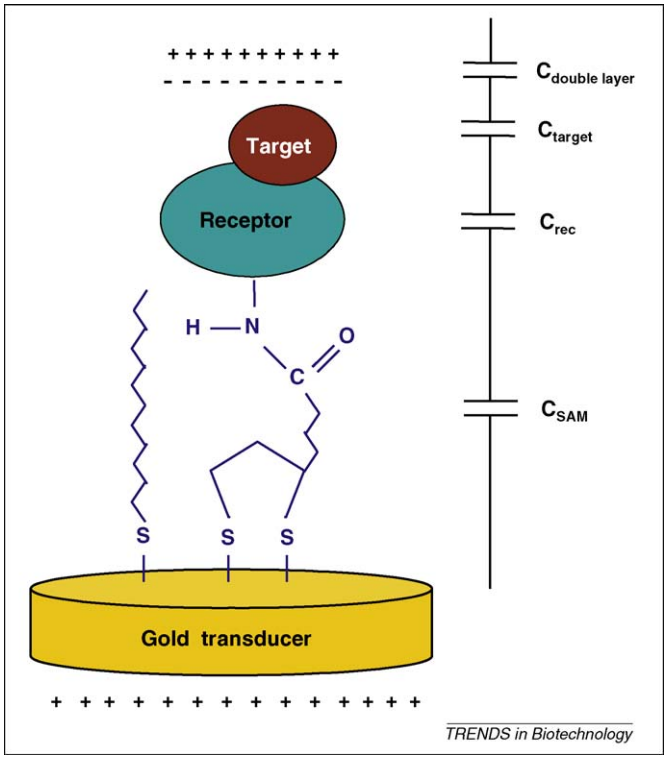

Figure 2. Principle of a capacitative transducer. Immobilisation of the receptor molecule on the transducer surface is achieved via a SAM of functionalised alkylthiols. Intermittent potential pulses (e.g. +50 mV) applied to the gold transducer results in the formation of an electrical double layer in the bulk electrolyte, where charge separation strictly depends on the insulation capacity of the SAM. Binding interactions between the target molecule and the receptor force the double layer of counter ions further out from the gold transducer, which results in a capacitance change proportional to the target molecule concentration.

When the incident light hits the surface from a specific angle, it is reflected. However, the angle of reflection is influenced by the thin layer directly above the SAM surface, and this effect can be correlated to the refractive index of this closely associated layer. Biochemical receptor molecules that are immobilised within this layer result in a given refractive index. Upon their binding to the target molecules, the refractive index of this thin layer changes and subsequently also the angle by which the incoming light is reflected. This is the basis for measurements using SPR.

SPR-based sensors frequently have been employed to provide information on molecular interactions between target proteins and their ligands [30]. The use of SPR for continuous monitoring of affinity interaction using weak-affinity antibodies has been demonstrated [31]. SPR is an elegant method, but at present it may not be sufficiently sensitive to address the impurities present in very low concentrations.

QCM

The third analytical method that is based on SAMs on gold surfaces is the so-called QCM. A piezoelectric quartz crystal that is vibrating with a certain frequency when placed in an electrical field can be modified with a surface-bound SAM. With biochemical receptors immobilised onto the SAM, the gravimetric characteristics of this layer change, thus causing viscoelastic effects that influence the vibrating frequency according to the Sauerbrey relation [32]. The observed shift in frequency can be correlated to the amount of material being deposited on the crystal.

Similar to SPR, analytical systems based on QCM provide means for quantitative determination of the analyte [33] and the possibility to obtain information about kinetic parameters from macromolecular interactions [34]. In Table 2, a comparison of the three methods is given. The sensitivity of the capacitive sensor is better for monitoring low concentrations, however, at present, the availability of good instrumentation is in favour of SPR and to some extent QCM.

Flow-injection ELISA

ELISA is a well-established analytical method that exploits binding between antigens and antibodies and requires the use of labelled reagents. For a review of different variants of ELISA, please see Ref. [35]. In a competitive assay format, the native antigen present in a sample is mixed with a fixed amount of labelled antigen, and the quantity of labelled antigen bound to the surface is analyzed after competitive binding has taken place. Higher amounts of bound labelled antigen correlate with a lower concentration of native antigen in the sample to be analyzed. In a sandwich concept of the ELISA format, a primary antibody is used to bind the antigen to which a labelled secondary antibody is added in excess, which will only bind to the already trapped antigen. Furthermore, when analyzing for a specific target antigen, it is also possible to utilise a standard immobilised antigen in a competitive approach.

These methods originally were very labour intensive because of the need for many different pipetting steps. This caused large variations in analysis results. Nowadays, these variations can be overcome with the use of pipetting robots. Moreover, when ELISA was first evaluated with regard to its use in process monitoring, it was important to obtain rapid analysis results. These were achieved by using only very short but accurate exposure times between antigen and antibody with the use of flow injection to warrant reproducibility. In this flow-injection ELISA, the immobilised antibody is placed into a small column that is connected to a continuous, constant flow of buffer. The sample and other reagents are introduced as short defined pulses into the system, thereby minimising the variation in experimental conditions. A schematic presentation of an assay cycle of flow-ELISA is shown in Figure 3.

Automated analysis using the flow-ELISA methodology has been reported for the monitoring of cell culture processes [36], fermentation [37], and downstream processing [38].

Examples for detection and quantification of trace amounts of impurities

Host cell protein

Most pharmaceutical proteins/peptides produced today are the result of genetic engineering, in which the respective genes have been cloned and expressed in a host organism. Thus, when target molecules are isolated from the cultivation broth, certain traces of the host cell proteome always prevail [39,40]. Related to present regulations [41], the

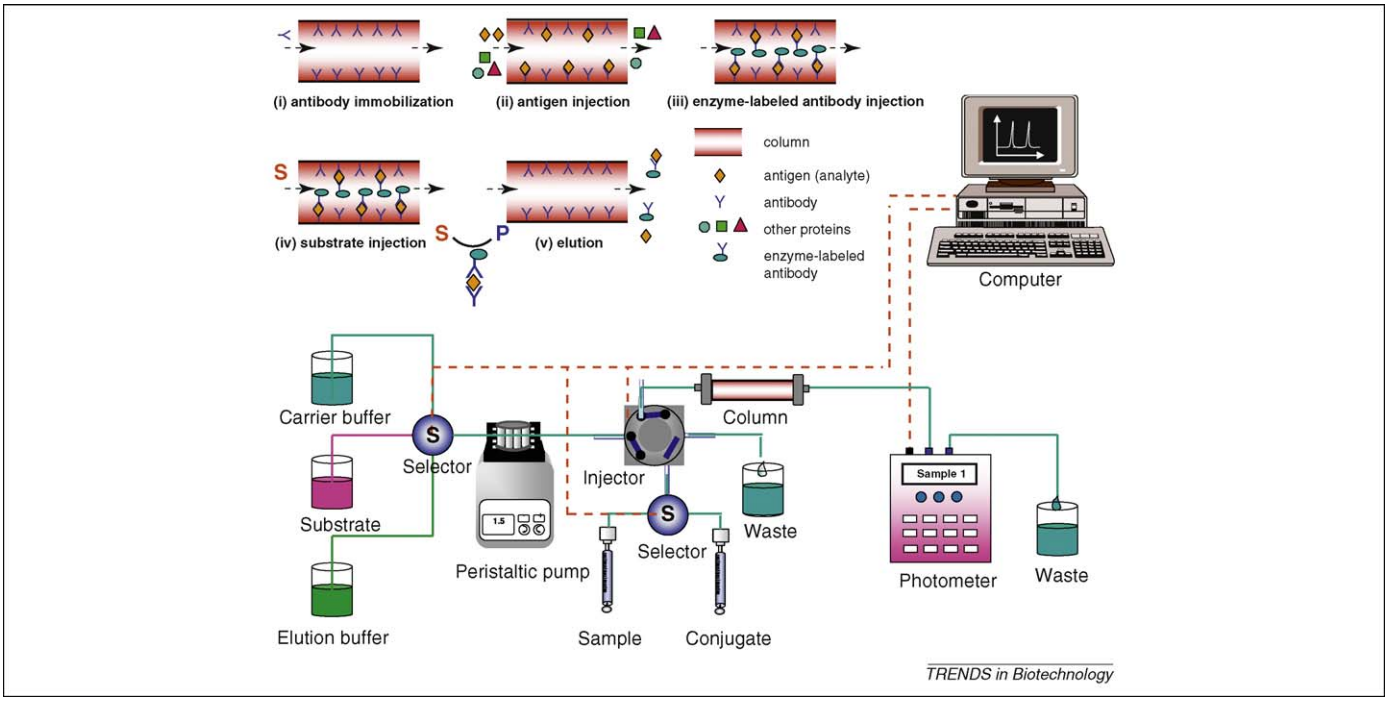

Figure 3. Schematic diagram of the set up of the flow-injection ELISA system. Two selection valves are employed. One is used to switch substrate and buffer flows, the other to select sample and conjugate. A column is placed after the injection valve that contains immobilised primary antibody (i). Then the antigen (analyte) is injected and bound to the immobilised primary antibody (ii). After that, enzyme-labelled antibody is injected as a conjugate, which binds to the antigen in a sandwich formation (iii). Thereafter, the substrate is injected (iv) and the product from the enzymatic reaction is detected by a photometer with an integrated quartz flow-cell. After regenerating the binding element (e.g. at low pH), the column, after reconditioning, is ready for a new analytical cycle (v).

analytical challenge of the process therefore constitutes a number of different factors: (i) impurities are, at least towards the end of the purification protocol, present in only very low concentrations; (ii) an impurity is often not a well-defined molecule, but rather a set of molecules that originates from the host cell proteome; and (iii) the ratio between different components comprising the impurities might vary over time because the different isolation and purification steps might remove some of the impurities more efficiently than others. This also implies that an impurity that was initially present only in a very low concentration could become a major contaminant later on in the downstream process [42]. These analytical challenges could be met by using polyclonal antibodies against the entire proteome of the host cell. However, such an approach is also associated with some uncertainty as it is difficult, if not impossible, to make sure that a complementary set of antibodies to the impurities is available. Since there are many potential impurities, it is advantageous to base an ideal assay on direct recognition between polyclonal antibodies and the impurities. However, it could be argued that a cocktail of monoclonal antibodies is equally good in this respect. This might be true if the number of monoclonal antibodies is sufficiently large to recognise the major part of all impurities.

Such an assay was set up using *Escherichia coli* as a model organism. The production of a recombinantly expressed enzyme was followed in parallel to measure the levels of all host cell proteins. The assay was highly sensitive with a detection limit of 8×10^{-18} mol/l. The levels of host cell proteins were initially quite high, as expected, but as the downstream processing continued, fewer host cell proteins were detectable in the product. The entire assay took only approximately 25 min, which makes it feasible for monitoring events during downstream processing (unpublished observations). This analysis time could however be further shortened, and it might also become possible to use capacitive biosensors for continuous monitoring [22].

The golden standard for determining impurities of host cell proteins remains ELISA, but it will remain far behind with regard to sensitivity. Nevertheless, it should be stressed that the use of continuous flow-injection systems offers promising possibilities for online sample handling, which are relevant for a number of steps in a process such as cell lysis, addition of reagents, and dialysis [43,44].

As there are only a few established host organisms for the production of recombinant proteins/peptides, it would be relatively straight forward to develop assays for the analysis of the proteomes of these few host cells within a product stream, similar to that presented above for *E. coli*.

DNA

It is a complicated and challenging task to remove DNA completely when purifying a biomolecule, and together with the need to trace any genetic material remaining from the host, requires the quantification of DNA within a process.

The analysis of DNA *per se* is an interesting challenge regarding the choice of an affinity ligand to trap DNA on a sensor surface. Several options are at hand: (i) oligonucleotides that will form base pairs with the DNA present [34]; (ii) histones that interact with larger pieces of DNA [10]; (iii) repressor proteins that recognise certain sequences

[45]; and (iv) anion exchangers that trap all nucleic acids, but perhaps also other molecules. In the latter case, efficient washing procedures are of utmost importance.

At present, there is no clear-cut recipe for a generally applicable DNA assay and the most suitable assay needs to be evaluated on the basis of each individual case. However, among the different possibilities mentioned above, oligonucleotides [34], histones [10] and repressor proteins [45] have been used successfully as ligands on the capacitive biosensor transducer, and it is suggested that these sensors types could be used with exceedingly high sensitivity (limit of detection down to 10^{-20} mol/l).

It can be expected that suitable DNA-based assays are based on general recognition elements, such as containing cocktails of oligonucleotides for forming base pairs with numerous DNA fragments, or perhaps including ion exchangers in combination with a strict washing regime as mentioned above. Such an approach was used successfully for the quantification of plasmid DNA in a flow-injection binding assay [39].

An alternative technology for the quantification of DNA is the various available formats of PCRs. PCR allows amplification of the different DNA present as impurities, and their quantification and/or identification [46].

Endotoxins

A common problem with the use of *E. coli* as a production organism is the release of endotoxins into the medium upon processing, which, as glycolipids, are difficult to remove during the subsequent purification steps. Adsorption of endotoxins to different positively charged adsorbent media, including arginine–Sepharose, immobilised polymyxin or poly(ethyleneimine), has been utilised successfully [47–49]. The ligands present in these adsorbents could in principle also be used to develop an endotoxin-specific sensor. However, they are not particularly specific, which might lead to the binding of several other negatively charged molecules (e.g. nucleic acids). Alternatively, other ligands, such as *Limulus* lectin (from *Limulus* spp.), or *Limulus* amebocyte lysate (LAL), can be used in specific and sensitive endotoxin assays. LAL is an aqueous extract of blood cells (amebocytes) from the horseshoe crab *Limulus polyphemus* and is known to interact with bacterial endotoxins. This reaction forms the basis of the LAL assay, the underlying principle of which has a clot-forming cascade of serine proteases. This test was first developed in 1977 by the FDA and subsequently further developed into turbidimetric LAL and chromogenic LAL methods [50–52]. Recently, an ultra-sensitive capacitive biosensor assay was developed for the direct detection and quantitation of endotoxins present in fermentation fluids [6].

Leaking affinity ligands

For the purification of proteins, affinity-mediated separation, for example, chromatography, is often an attractive method of choice because of its high resolution and the possibility to remove a large proportion of the impurities in one single step. Affinity separation is often used towards the end of a purification scheme so that impurities, which have a great similarity to the target protein, can be removed. However, it could be even more advantageous to introduce affinity-mediated separation at an early stage in the downstream process because it could allow reduction of sample volumes, thereby facilitating subsequent steps and resulting in significant cost reductions.

Affinity chromatography is usually carried out using an affinity ligand that is covalently coupled to a porous matrix. This chemical bond is not perfectly stable, and the same might also be true for the matrix. Consequently, leakage of affinity ligands during affinity separation can occur [53]. It has been shown that small so-called 'polishing columns' might be able capture any released ligand material when operations are on a small scale. The economically most important affinity step used industrially today is the isolation of monoclonal antibodies using immobilised protein A. When passing the culture broth over the column, leakage takes place and the product stream is likely to be contaminated with protein A. To be able to quantify such a molecule that is present at only very low concentrations, a pre-enrichment step, for example, another affinity step aimed at capturing the leaking ligand, might be required [54]. To that end, an affinity sensor using immobilised IgG is readily able to detect protein A. An assay for the quantitative determination of leaking protein A molecules from affinity columns used for the isolation of monoclonal antibodies, for example, might nevertheless remain complicated, even if it were possible to detect protein A in very low concentrations. This is because if leakage occurs when IgG is processed, the probability for complex formation in free solution is high, and then binding of protein A to immobilised IgG might be hampered.

Proteins variants

Besides impurities caused by the presence of foreign molecules, variants (e.g. truncated molecules) or aggregates of the target protein might also cause problems [55–57]. This makes the separation challenge even more difficult as the molecules to be separated are mostly identical to the desired products. In these cases, it is often not beneficial to use affinity-mediated separation, but to prefer other chromatography steps such as ion exchange chromatography. This is because small changes in protein structure might induce minor changes, for example, exposure of charged groups on the surface of the protein molecule, that could affect their behaviour on the resins used in chromatography.

One strategy has been to trap initially all target proteins (including the variants) in an affinity capture step and subsequently to release and sort these by additional steps such as ion exchange interactions using HPLC, capillary electrophoresis, or LC-MS-MS. If impurities are related to truncated protein, an MS-based method is particularly valuable. For protein aggregates, size exclusion chromatography is the preferred method to separate the different molecular sizes before quantification.

Conclusion and outlook

The recent advances in bioanalytical methods are exciting and we are now approaching detection limits of concentrations, in which impurities in most cases will be completely irrelevant until it is proven to the contrary.

Further developments in the area might address convenience of use, speed of analysis, and the ability to analyse several target molecules simultaneously. Shrinking of the sensor units is certainly also anticipated, particularly if multi-analytical devices are to be developed. A limiting factor so far has been the lack of means to modulate the properties of the biological recognition elements, such as their affinity and stability. For example, biomolecules with high affinity require harsh conditions to release bound material, which in turn might induce denaturation. The use of weak binders might solve this problem, but at the expense of sensitivity of the assay. There have been reports of so called 'switch-molecules' that can change their affinity dramatically upon minor changes in the environment. However, it remains to be seen whether such molecules can be utilised in biosensors.

When suitable analytical methods have become available, it might be possible to monitor accurately cultivation processes and downstream operations. This will lead to better understanding of the process conditions that cause the release or production of impurities, and which might also be exploited to implement measures to reduce such events. This in turn might lead to unit operations that are carried out in a sequence that is determined ideal for efficient removal of trace impurities. This could improve dramatically current downstream processing that is governed mainly by the concentration of the target molecule and much less by the presence of impurities.

Acknowledgements

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References

1 Anicetti, V. and Hancock, W.S. (1994) Analytical considerations in the development of protein purification processes. *Bioprocess. Technol.* 18, 11–36
2 Mattiasson, B. (1984) lmmunochemical assays for process control: potentials and limitations. *Trends. Anal. Chem.* 3, 245–250
3 Nilsson, M. *et al.* (1992) Process monitoring by flow-injection immunoassay – evaluation of a sequential competitive binding assay. *J. Chrom.* 597, 383–389
4 Degelau, A. *et al.* (1992) Immuno- and flow cytometric analytical methods for biotechnological research and process monitoring. *J. Biotechnol.* 25, 115–144
5 Labib, M. *et al.* (2008) A capacitive biosensor for detection of staphylococcal entrotoxin B. *Anal. Bioanal. Chem.* 393, 1539–1544
6 Limbut, W. *et al.* (2007) Capacitive biosensor for detection of endotoxin. *Anal. Bioanal. Chem* 389, 517–525
7 Zhou, Y. *et al.* (2004) Structural characterization of microcontact printed arrays of hexa(ethylene glycol)-terminated alkanethiols on gold. *Langmuir* 20, 6206–6215
8 Galaev, I.Y. *et al.* (1994) Interaction of Cibracron-blue with polymers – implications for polymer shielded dye-affinity chromatography of phospho-fructokinase from Baker's yeast. *J. Chromatogr.* 684, 45–54
9 Li, Y.C. *et al.* (2001) Separation of mistletoe lectins based on the degree of glycosylation using affinity chromatography. *J. Chromatogr.* 925, 115–121
10 Numnuam, A. *et al.* (2009) Capacitive biosensor for quantification of trace amounts of DNA. *Biosens. Bioelectron.* 24, 2559–2565
11 Svensson, M. *et al.* (2005) Control of endotoxin release in *Escherichia coli* fed-batch cultures. *Bioprocess. Biosyst. Eng.* 27, 91–97
12 Lausch, R. *et al.* (1993) Rapid capillary gel electrophoresis of proteins. *J. Chromatogr. A* 654, 190–195
13 Mattiasson, B. and Håkanson, H. (1991) Measurement and control in down-stream processing. In *Measurement and Control in Bioprocessing* (Carr-Brion, K.G., ed.), pp. 221–250, Elsevier Applied Science
14 Fenge, C. *et al.* (1991) On-line monitoring of monoclonal antibody formation in high density perfusion culture using FIA. *Cytotechnol.* 6, 55–63
15 Labib, M. *et al.* (2009a) A comparative study between three different analytical techniques for assay of cholera toxin. *Anal. Chim. Acta.* 634, 255–261
16 U.S. Food and Drug Administration (2004) Guidance for industry, PAT – A framework for innovative pharmaceutical manufacturing and quality assurance (http://www.fda.gov/cder/OPS/PAT.htm Washington, D.C. U.S.A.)
17 Oh-Ishi, M. and Maeda, T. (2002) Separation techniques for high-molecular-mass proteins. *J. Chromatogr. B* 771, 49–66
18 Jai, T. *et al.* (2008) Recent development of multi-dimensional chromatography strategies in proteome research. *J. Chromatogr. B* 866, 123–132
19 Mattiasson, B. *et al.* (1978) Non-equilibrium enzyme immunoassay of gentamycin. *Clin. Chem.* 24, 1770–1773
20 Ohlson, S. *et al.* (2000) Continuous weak-affinity immunosensing. *Trends Biotechnol.* 18, 49–52
21 Miyabayashi, A. and Mattiasson, B. (1990) A dual streaming potential device used as an affinity sensor for monitoring hybridoma cell cultivations. *Anal. Biochem.* 184, 165–171
22 Hedström, M. *et al.* (2005) Continuous measurements of a binding reaction using a capacitive biosensor. *Biosens. Bioelectron.* 21, 41–48
23 Limbut, W. *et al.* (2006) A comparative study of capacitive immunosensors based on self-assembled monolayers formed from thiourea, thiotic acid and 3-mercaptopropionic acid. *Biosens. Bioelectron.* 22, 233–240
24 Berggren, C. and Johansson, G. (1997) Capacitive measurements of antibody-antigen interaction in a flow system. *Anal. Chem.* 69, 3651–3657
25 Li, J.S. *et al.* (2005) A reusable capacitive immunosensor with a novel immobilization procedure based on 1,6-hexanedithiol and nano-Au self-assembled layers. *Sens. Actuators, B* 110, 327–334
26 Valiokas, R. *et al.* (2008) Self-assembled monolayers containing terminal mono-, bis-, and tris-nitrilotriacetic acid groups: Characterization and application. *Langmuir.* 24, 4959–4967
27 Berggren, C. *et al.* (1998) An immunological interleukin-6 capacitive biosensor using pertubation with a potentiostatic step. *Biosens. Bioelectron.* 13, 1061–1068
28 Suwansa-ard, S. *et al.* (2009) Comparison of surface plasmon resonance and capacitive immunosensors for cancer antigen 125 detection in human samples. *Biosens. Bioelectron.* doi:10.1016/j.bios.2009.04.008
29 Reno, F. *et al.* (1996) Self-assemble of short and long-chain alkyl thiols onto gold surfaces: A real-time study using surface plasmon resonance technique. *Can. J. Chem.* 74, 677–688
30 Suzuki, M. *et al.* (2002) Miniature surface-plasmon resonance immunosensors – rapid and repetitive procedure. *Anal. Bioanal. Chem.* 372, 301–304
31 Mandenius, C.F. *et al.* (2008) Monitoring of influenza virus hemagglutinin in process samples using weak affinity ligands and surface plasmon resonance. *Anal. Chim. Act.* 623, 66–75
32 Sauerbrey, G. (1959) The use of quartz oscillators for weighing thin layers and for microweighing. *Z. Phys.* 155, 206–222
33 Tang, D. *et al.* (2007) Quartz crystal microbalance immunoassay for carcinoma antigen 125 based on gold nanowire-functionalized biomimetic interface. *Analyst* 133, 933–938
34 Okahata, Y. *et al.* (2000) A highly sensitive 27 MHz quartz-crystal microbalance as a device for kinetic measurements of molecular recognition on DNA strands. *Anal. Sci.* 16, 1113–1119
35 Taylor, L. *et al.* (2009) Allergen immunoassays-considerations for use of naturally incurred standards. *Anal. Bioanal. Chem.* 395, 83–92
36 Rhee, J. *et al.* (1999) Flow-injection sandwich ELISA for bioprocess monitoring. *Autom. Methods Manage. Chem.* 21, 121–125
37 Nandakumar, R. *et al.* (2000) Quantification of nisin in flow-injection immunoassay systems. *Biosens. Bioelectron.* 15, 241–247
38 Nilsson, M. *et al.* (1991) Flow-injection ELISA for process monitoring and control. *Anal. Chim. Acta.* 249, 163–168
39 Eaton, L.C. (1995) Host cell contaminant protein assay development for recombinant biopharmaceuticals. *J. Chromatogr. A* 705, 105–114
40 Rathore, A.S. *et al.* (2003) Analysis of residual host cell proteins and DNA in process streams of a recombinant protein product expressed in *Escherichia coli* cells. *J. Pharm. Biomed. Anal.* 32, 1199–1211

41 Wenzel, F. and Sonnega, C. (2005) The regulatory environment for biopharmaceuticals in the EU. *Mod. Biopharm.* 4, 1669–1708
42 Shukla, A. *et al.* (2008) Demonstration of robust host cell protein clearance in bio pharmaceutical downstream processes. *Biotechnol. Progr.* 24, 615–622
43 Canarelli, S. *et al.* (2002) On-line microdialysis of proteins with high-salt buffers for direct coupling of electrospray ionization mass spectrometry and liquid chromatography *J. Chrom. A.* 948, 139–149
44 Nandakumar, M. *et al.* (2001) Variations in plasmid content during *Escherichia coli* cultivations detected by on-line flow injection processing. *Biotechnol. Lett.* 23, 1135–1140
45 Bontidean, I. *et al.* (2001) Highly sensitive novel biosensor based on an immobilized lac repressor. *Angew. Chem. Int. Ed.* 40, 2676–2678
46 Aleksandrov *et al.* (2005) A PCR-based semiquantitative assay of DNA impurities in recombinant protein preparations. *Russ. J. Bioorg. Chem.* 31, 66–69
47 Hanora, A. *et al.* (2005) Capture of bacterial endotoxins using a super-macroporous monolithic matrix with immobilized polyethyleneimine, lysozyme or polymyxin B. *J. Biotech.* 118, 421–433
48 Shouwan, T. *et al.* (2006) Adsorption of endotoxin on polymyxin B immobilized affinity matrices. *Chin. J. Anal. Chem.* 34, 455–458
49 Teramoto, K. *et al.* (2002) Removal of endotoxin in blood by polymyxin B immobilized polystyrene-derivative fiber. *Ther. Apher. Dial.* 6, 103–108
50 U.S. Food and Drug Administration (1978) Guideline on the validation of *Limulus* amebocyte lysate test as end-product endotoxin test for human and animal parental drugs biological products, and medical device
51 Iwanaga, S. *et al.* (1992) Molecular mechanism of hemolymph clotting system in *Limulus*. *Thromb. Res.* 68, 1–32
52 Morita, T. (1981) A new (1,3)-β-D-glucan mediated coagulation pathway found in *Limulus* amebocytes. *FEBS Lett.* 129, 318–321
53 Wilchek, M. (1975) Structure of a soluble super-active insulin is revealed by nature of complex between cyanogen-bromide-activated Sepharose and amines. *Proc. Natl. Acad. Sci. U. S. A.* 72, 1055–1058
54 Stewart, D.J. *et al.* (1992) Development of an enzyme-linked immunosorbent-assay for C1 reactive blue-2 and its application to a comparison of the stability and performance of a perfluorocarbon support with other immobilized C1 reactive blue-2 affinity adsorbent. *J. Chromatogr.* 623, 1–14
55 Hellebust, H. *et al.* (1988a) Proteolyic degradation of fused protein A-beta-galactosidase in *Escherichia coli*. *J. Biotechnol.* 7, 185–198
56 Hellebust, H. *et al*. (1988b) Role of proteases in fermentation of the fused protein staphylococcal protein A human insulin-like growth factor-1 in *Staphylococcus aureus*. *Appl. Microbiol. Biotechnol.* 28, 258–262
57 Labib, M. *et al.* (2009b) A multipurpose capacitive biosensor for assay and quality control of human immunoglobulin G. *Biotechnol. Bioeng.* DOI:10.1002/bit.22395
58 Nakamoto, K. *et al.* (2008) Simultaneous on-chip surface plasmon resonance measurement of disease marker protein and small metabolite combined with immuno-and enzymatic reactions. *Chem. Lett.* 37, 698–699
59 Tsai, C.W. and Hsieh, K. (2007) QCM-based immunosensor for the detection of ochratoxin A. *Anal. Lett.* 40, 1979–1991
60 Wan, M. *et al.* (2002) An enzyme-linked immunosorbent assay for host cell protein contaminants in recombinant PEGylated staphylokinase mutant SY161. *J. Pharm. Biomed. Anal.* 255, 953–963
61 Liu, Y.C. *et al.* (2001) Comparison of difference protein immobilization methods on quartz crystal microbalance surface in flow injection immunosensor system. *Anal. Biochem.* 299, 130–135