The Rational Development of Molecularly Imprinted Polymer-Based Sensors for Protein Detection

Michael J. Whitcombe,[a] Iva Chianella,[a] Lee Larcombe,[a] Sergey A. Piletsky,[a] James Noble,[b] Robert Porter,[b] Adrian Horgan,[b]

[a]Cranfield Health, Cranfield University, Cranfield, Bedfordshire, MK43 0AL. [b]National Physical Laboratory, Hampton Road, Teddington, TW11 0LW.

Abstract

The detection of specific proteins, as biomarkers of disease, health status, environmental monitoring, food quality, control of fermenters and civil defence purposes means that biosensors for these targets will become increasing more important. Among the technologies used for building specific recognition properties, molecularly imprinted polymers, molecularly imprinted polymers (MIPs) are attracting much attention. In this *critical review* we describe many of methods used for imprinting recognition for protein targets in polymers and their incorporation with a number of transducer platforms with the aim of identifying the most promising approaches for the preparation of MIP-based protein sensors.

1. Introduction

The selective detection and quantification of protein targets has wide applicability to a number of fields, including clinical diagnostics and therapeutic monitoring, control of bioreactors and the detection of organisms and toxins, including bio-terror agents. While laboratory-based methods such as HPLC-MS and immunoassay can provide precise measurements, they are generally too slow for situations when a rapid response or intervention is needed. Under these circumstances a biosensor can provide faster analysis with a direct read-out without the need to transport samples to a laboratory. Biosensors in which the recognition element is a molecularly imprinted receptor, rather that a biomolecule, offer a number of advantages; notably greater long-term storage stability, potential re-usability, resistance to microbial spoilage and custom synthesis of selective receptors without the need to inoculate laboratory animals. The literature on molecularly imprinted polymers (MIPs) for protein detection however contains a multitude of potential methods which is compounded by the numerous methods proposed for the transduction of the binding event to a useful signal. In this review we will present a critical assessment of the methods of protein imprinting and MIP-based biosensor development with the aim of identifying the most promising strategies for the creation of a sensitive and selective MIP-based biosensor for a protein target.

1.1 Key requirements of a biosensor for the detection of a specific protein

Soper *et al*. (2006) defined a biosensor as a:

"..bioanalytical device incorporating a biological material or a biomimic (e.g., tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.), intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric or magnetic. The usual aim of a biosensor is to produce either discrete or continuous digital electronic signals, which are proportional to a single analyte or a related group of analytes."[1]

The key requirements of a biosensor for detection of a protein are that it should be specific for its target and capable of measuring the analyte concentration over the required analytical concentration range. This requires that the recognition element (the "biological material or biomimic" in the above definition) is capable of selective binding of the target analyte with high affinity. Additionally there is an increasing need for biosensors to be resistant to a variety of chemical environments and tolerant to extremes of temperature and humidity and to resist bio-fouling. The vast majority of biosensors rely on biological reagents, including those of animal origin (enzymes and antibodies), to obtain the required specificity and selectivity for an assay. The selection of a suitable recognition element enables the assay to be both accurate and precise in its measurement. In nature the selectivity of these molecules has arisen through the evolutionary need to protect the organism or to regulate other processes to ensure survival. The latter role is filled by enzymes, which catalyse a huge range of metabolic and regulatory functions within the organism in a very specific and selective manner. These qualities have led to the utilisation of enzymes in devices such as the glucose biosensor.[2] Another important class of bio-molecule used in sensing are the antibodies. These are typified by immunoglobulin G (IgG), which is the familiar Y-shaped molecule which possesses two highly specific and selective binding domains at the end of the arms of the "Y". IgG was first employed as the recognition element by Yalow and Berson in a radio-immunoassays format,[3] and is now widely employed in a range of biosensor and bioassay configurations. Whilst these bio-molecules fulfil the requirements of specificity, selectivity and ease of use, such as the ability to absorb or chemically couple to solid surfaces with little denaturation,[4] they have limited stability to extremes of temperature (denaturing above ~40 ºC) and humidity as well susceptibility to damage by shear forces, such as those encountered in continuous monitoring situations. Recent efforts have included studies with antibodies and antibody fragments from camelids such as the llama[5] or from sharks[6] to overcome some of these issues. These antibodies differ from conventional IgG since they consist of only a single heavy chain which imparts greater temperature stability without sacrificing specificity or selectivity. The only drawback is that these proteins still have a tendency to unfold at higher temperature but re-fold to their original shape when the temperature is lowered, with the consequence that the reliability of assays utilising these molecules will diminish at operating temperatures above around 40 ºC. It is

for these reasons that biomimetic materials, such as molecularly imprinted polymers (MIPs) are of interest, since they hold the promise of selective recognition and robustness to a range of hash operating conditions not tolerated by their biological counterparts.

1.2 Molecularly imprinted polymers

As mentioned in the previous section, the construction of many current and future diagnostic devices relies almost exclusively on the use of sophisticated biological receptors, such as enzymes, antibodies and DNA, as the chemical or biochemical recognition element.[7-9] Due to their biological origins, these biomolecules may suffer from some inherent limitations when used in sensors and assays; for example, poor reproducibility, instability during the manufacturing process and problems associated with sterilisation. Synthetic receptors therefore may offer a promising alternative to antibodies and other biological receptors for use in biosensors.[10] The most generic and cost-effective technique for preparing synthetic receptors, which combines high affinity and specificity with robustness and low manufacturing costs is molecular imprinting.[11]

Molecular imprinting can be defined as the formation of specific recognition sites (with binding or catalytic properties) in a material through its interaction with a template, where the template directs the positioning and orientation of the material's structural components by a self-assembly mechanism (Figure 1). The material itself could be a linear sequence (in the most general sense, even DNA replication is a type of imprinting process), polymeric (organic MIPs and inorganic imprinted silica gels) or 2-dimensional surface assemblies (grafted monolayers). MIPs have a range of advantages when compared with natural biomolecules (Table 1). Imprinted polymers can in theory be prepared for any kind of substances. The best results however were obtained for molecules with molecular weights in the range of 200-1200 Da.

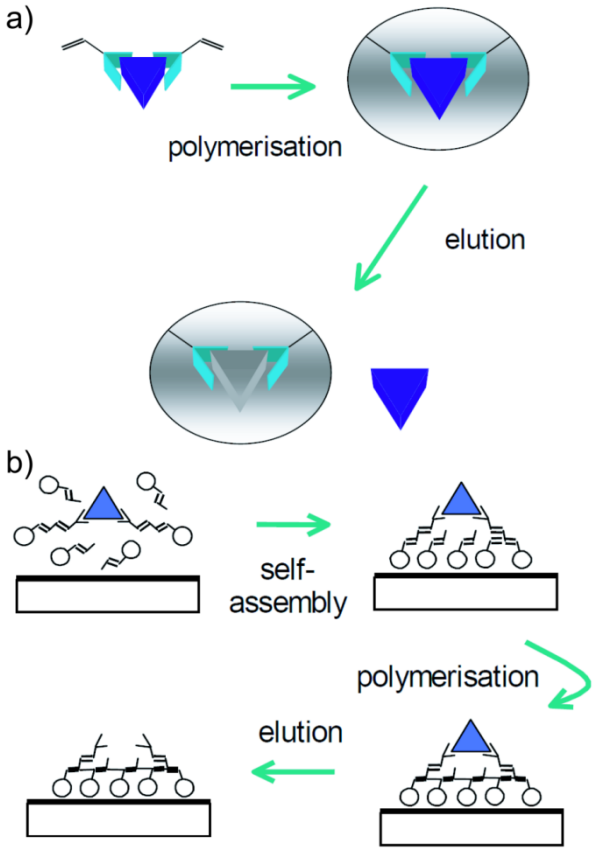

Figure 1: Scheme of (a) three-dimensional and (b) two-dimensional imprinting polymerisation (courtesy of VTT, Finland).

Table 1: Comparison of natural biomolecules used in sensors and MIPs.

Natural biomolecules	**MIPs**
Low stability	Stable to low/high pH, pressure and temperature (<180 ºC)
High price of enzymes and receptors	Inexpensive and easy to prepare
Generally poor performance in non-aqueous media	MIPs can work in organic solvents

Different natural biomolecules have their own operational requirements (pH, ionic strength, temperature, substrate etc.)	Due to their minimal operational requirements, the design of MIP-based multisensors is a relatively easy task
Natural receptors and enzymes exist for only a limited number of important analytes	In principle, MIPs can be prepared for practically any compound
Poor compatibility with micromachining technology and miniaturisation	Polymers are fully compatible with micromachining technology
Soluble	Most MIPs are insoluble co-polymers

Many hundreds of examples of successful imprinting have been demonstrated, including MIPs for inorganic ions, drugs, nucleic acids, proteins and even microbial cells.[12] The resulting polymers are robust, inexpensive and, in many cases, possess levels of affinity and specificity suitable for industrial applications. The quality of the synthesised materials depends to a large degree on the type of template. For example, it is relatively easy to imprint rigid and stable lipophillic drug molecules with several polar functional groups.[13] Other factors which affect the quality of MIPs include: monomer composition, solvents, polymerisation time, temperature, even the application of a magnetic field.[14] Imprinted polymers can be produced in bulk, in thin film format and as membranes[15] or as a suspension of spherical particles with well-defined size.[16] The high specificity and stability of MIPs renders them promising alternatives to enzymes, antibodies, and natural receptors for use in sensor technology.[17,18] The growing interest in MIPs can be illustrated by the fact that almost 10% of all papers published on the subject of "biosensor" nowadays are MIP-related.

The main driving forces for the substitution of antibodies with MIPs in sensors are the high stability of the polymers and their low price. Typically the lifetime of antibodies is restricted to a 6-12 months window. They may need to be stored in a refrigerated environment, and antibody-based sensors usually cannot be regenerated for more than about 10 cycles. In contrast, MIPs can be stored at ambient temperature for years without any noticeable loss in affinity. They can be autoclaved and regenerated many times using strongly acidic or basic wash steps or by washing with organic

solvents. All these features make them very attractive for use in robust sensors and assays. MIPs are also two orders of magnitude cheaper then antibodies. A range of MIPs for different targets are available commercially at a price varying from \$0.1-0.5 mg^{-1}. By comparison, the cost of antibodies varies depending on the target but typically lies in the range \$100-1000 mg^{-1}. Even relatively "cheap" immunoaffinity cartridges with a low density of immobilised antibodies still demand a relatively high price by comparison with MIPs, costing between \$10 to \$100 mg^{-1}.

There are however some perceived limitations associated with the development of MIP sensors: (i) absence of a general procedure for MIP preparation; (ii) difficulty in integrating them with a transducer; (iii) difficulty in transforming the binding event into an electric signal. Most of these problems however have been addressed in recent years.[19,20]

1.2.1 MIP design

One of the major problems in MIP design is the choice of an optimal polymerization protocol for the development of MIPs. This is mainly because of the need to select and optimise a multitude of variable parameters such as the types of monomer to be used in polymer synthesis (more than 4 thousands polymerisable compounds are commercially available), solvents, temperature, pressure etc.[21] The most advanced approaches available for the selection of appropriate functional monomers include combinatorial and computational methods.[22] In combinatorial approaches the best composition is selected on the basis of simultaneous synthesis and testing of tens to hundreds of imprinted polymers prepared on a small scale.[23-25] In the computational approach the monomer screening is performed virtually.[26,27] These two approaches permit the creation of MIPs with affinities and selectivities comparable to those of antibodies,[26] (Table 2).

In relation to the molecular imprinting of proteins and cells there is currently insufficient evidence from the literature to judge whether combinatorial or computational approaches might offer any substantial advantage. In contrast to the recognition of small molecules, which relies on strong electrostatic interactions, recognition of large biomolecules and cells is achieved through shape

complementarity combined with (weak) van der Vaals interactions and the formation of hydrogen bonds.[28] Thus, most often, imprinting of these species is successfully performed using weakly cross-linked single monomers such as acrylamide or aminophenyl boronic acid.[29,30]

Table 2. Affinity and sensitivity range of computationally designed molecularly imprinted polymer in comparison with antibodies raised against the same target.[26]

Receptor	**Kd, nM**	**Sensitivity range, μg L^{-1}**
Computational MIP	0.3 ± 0.08	0.1-100
Monoclonal antibody	0.03 ± 0.004	0.025-5
Polyclonal antibody	0.5 ± 0.07	0.05-10

The most significant benefits stemming from the use of combinatorial or computational approaches in MIP design are reductions in both time and cost involved in the development process. Depending on the number of tests required, the typical time needed to develop a novel MIP can be as short as 3-4 weeks.[31]

1.2.2 Integration with sensors

The integration of MIPs with sensors can be achieved by *in situ* polymerisation, using either photochemical or thermal initiation,[32] or by surface grafting with chemical or UV initiation.[33,34] The advantage of this latter approach lies in the possibility of controlled modification of inert electrode surfaces with thin films of specific polymers. Polymers can also be electropolymerised on the surface of physical transducers. For example, the electropolymerisation of phenylenediamine in the presence of glucose was successfully used in the construction of an imprinted sensor for the conductometric detection of this compound[35] (Figure 2).

Basically, polymers can either be produced separately, for later integration with existing (electrochemical, piezoelectric or optical) sensors, or be produced by *in situ* methods directly on the sensor surface. For ease of quality control it is better to produce polymers separately, in a dedicated laboratory in the form of membranes or powders, followed by extraction of the template and characterisation of the materials obtained. This will follow the modern trend in biosensors development whereby the suppliers of biological components are "separated" from the manufacturing of detectors and sensor assembly. This model would be difficult to apply for applications where MIPs need to be deposited as thin layers, of the order of nanometres, e.g. in evanescent wave sensors.[36] One possible compromise could be the development of methods for the production of MIP nanoparticles, "plastic antibodies", which can be handled by sensor manufacturers in much the same way as traditional (natural) antibodies or enzymes.[37,38] In order to prove the feasibility of this approach, there is a need for the development of generic and versatile procedures for the synthesis of MIP nanoparticles for various targets. One potential advance in this area could be the development of an automated "nanoreactor" for the reproducible synthesis of monodisperse MIP nanoparticles. A reactor similar to that proposed here has recently been produced for the preparation of MIP microparticles (Figure 3) and it may be possible to tune its characteristics for the production of nanoparticles.[39]

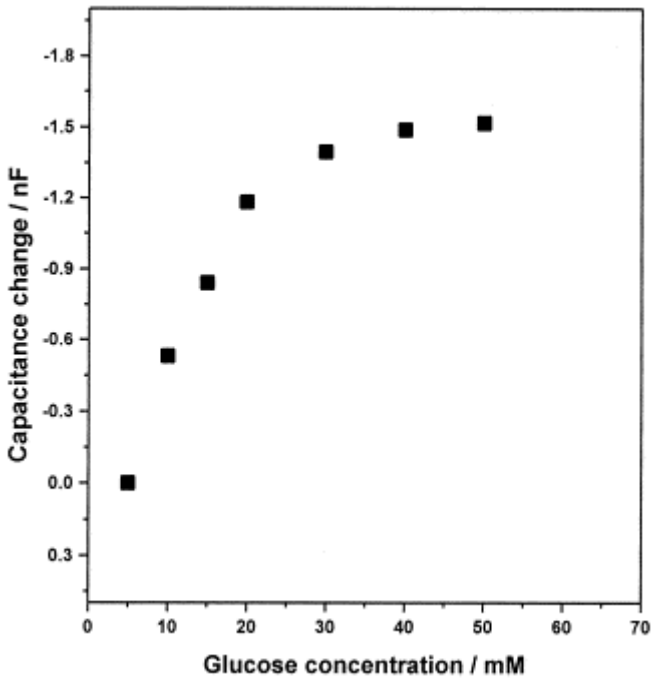

Figure 2. Impedance response of the phenylenediamine film imprinted with glucose.[35] Reprinted from *Biosensors and Bioelectronics*, Vol. 16, Issue 3, Z. L. Cheng, E. K. Wang and X.R. Yang, Capacitive detection of glucose using molecularly imprinted polymers, 179-185, Copyright (2001), with permission from Elsevier.

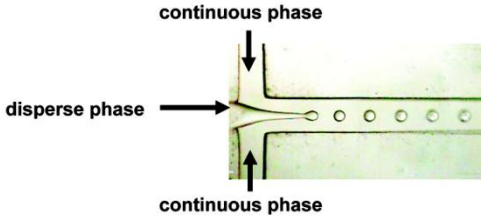

Figure 3. Flow-focussing microreactor for synthesis of MIP microparticles. Reprinted with permission from Ref. 39. Copyright 2009 American Chemical Society.

1.2.3 Detection of binding

Detection of binding is generally realised using electrochemical, piezoelectric and optical transducers. Immunosensor-type devices are the most common examples of MIP sensors.[40] Analyte detection in these devices is based on measurements of the template concentration adsorbed by a MIP, immobilised on the detector surface. Receptor sensors exploit the ability of a MIP to change its conformation upon binding with the template, leading to changes in a measurable property, such as conductivity, permeability or surface potential.[17,41] Alternatively, sensors can be designed that use the ability of a functional monomer to change its properties upon interaction with the template; in most cases, changes in fluorescence are employed.[42,43] In one example of this type of material, a fluorescent sensor for cAMP detection was constructed using the environmentally-sensitive dye, *trans*-4-[*p*-(*N*,*N*-dimethylamino)styryl]-*N*-vinylbenzylpyridinium chloride)[44] (Figure 4). The resulting polymer displayed both functions of template recognition and sensing.

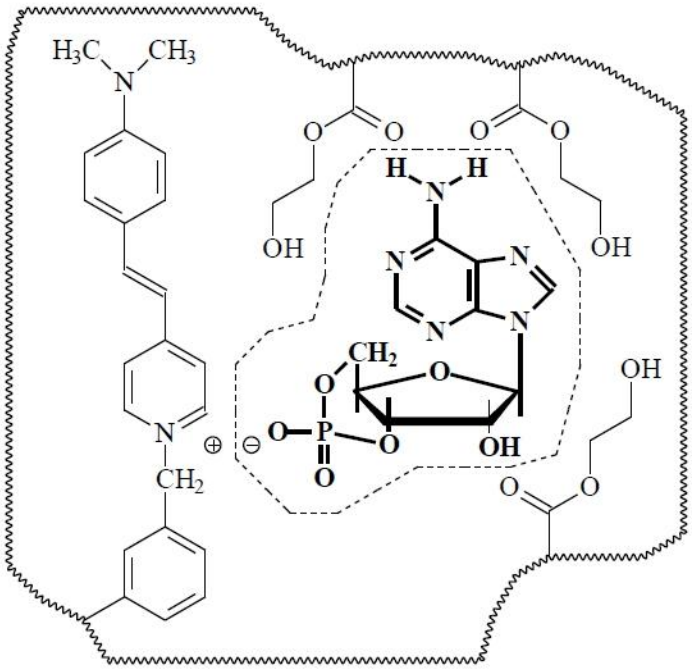

Figure 4. Schematic representation of polymer binding site for cAMP with signalling trans-4-[*p*-(*N*,*N*-dimethylamino)styryl]-*N*-vinylbenzylpyridinium monomer.[44] Reproduced with permission from: Piletsky S, Turner A. A New Generation of Chemical Sensors Based on MIPs. In: Piletsky S, Turner A, eds. Molecular Imprinting of Polymers. Austin: Landes Bioscience, 2006:64-79.

Several recent publications have described the development of catalytic sensors based on MIPs. In one such study we have used a transition state analogue approach to prepare a MIP with catalytic properties similar to the enzyme tyrosinase in the form of an electrochemical sensor for the measurement of catechol and dopamine concentration.[45] Further developments in catalytic receptors based on MIPs will depend largely on the successful preparation of MIP-based catalysts which can react with water-soluble and practically important analytes.[46,47]

In relation to protein detection, the integration of MIPs with acoustic, surface plasmon resonance or surface enhanced Raman sensors might seem to be the most promising platforms.[48,49] These sensors do not require the use of enzyme or fluorescent labels and could be used for the direct measurement of analyte concentrations. Alternatively, fluorescent, gold coated or magnetic MIP nanoparticles can be used in lateral flow sensor devices as a direct replacement for antibodies.[50-53]

2. Template selection

When considering proteins as targets for detection by MIP-based sensors, the question arises what is the best material to use as a template in order to obtain the required specificity in recognition of the target? Since some of these problems are common to decisions about how to raise effective antibodies to peptides and proteins, we will first consider in this section what tools are available to those working in biological aspects of molecular recognition, followed by a review of how the imprinting has been approached.

2.1 Protein structure, antigenicity and tools for structural analysis

The antigens bound by antibodies can be classified into groups of smaller, and individually non-immunogenic, molecules termed haptens, and larger molecules – often proteins. Key early work detailing the binding of antibodies and the complementarity determining region (CDR) began with the investigation of specificity by Lansteiner and van der Scheer,[54] the binding of small molecules within the hapten binding cleft by Amzel *et al*.[55] and the interaction with larger proteins by Amit *et al*.[56] Unlike larger protein molecules, haptens do not induce the formation of antibodies due to the inability of B-cells to process the small fragments for presentation to T-helper cells in order to trigger the production of various B-cell receptor ligands and cytokines necessary for B-cell clonal expansion, but can be used to do so when complexed with a larger molecule, such as a protein.

Molecular Imprinted Polymers (MIPs) do not rely on such complex biological signalling for their production, thereby providing opportunities to design receptors for a range of molecule sizes, including the opportunity to produce receptors to pharmacologically active, or otherwise toxic compounds. The aim, when designing synthetic receptors, is to mimic – or even improve upon – the specificity of natural antibodies. The binding of antigens to antibodies is well understood, and the nature of that interaction (including the important aspects we must consider when attempting to design such interactions) is as relevant to synthetic receptors, such as MIPs, as it is to the natural molecules.

Regions of a protein that can be classified as antigenic determinants within the structure of a globular protein have been called epitopes, a term coined by Niels Jerne, winner of the 1984 Nobel Prize for medicine (immunology). It is helpful to also use the term to describe the target region of a protein for synthetic receptors.[57] Early studies[58,59] showed that natural epitopes of proteins are often found at the surface, and this observation appears to hold. Whilst it is clear that only surface residues are available to be involved in epitopes, there are several structural arrangements by which this is possible.

The residues which comprise an epitope must be present in a conformation which will be accessible to the antigen-binding region (paratope) of an antibody, or to the binding site of a synthetic receptor. The residues in the epitope may achieve such a conformational arrangement through spatial proximity brought about by the tertiary structure of the globular protein; by their positions within a repeating motif of secondary structure such as an alpha helix or beta sheet; or simply by being adjacent residues in the primary sequence in a loop or coil region.

Obviously, these arrangements are often mutually exclusive when surface availability is considered. Residues which form an epitope should exhibit both spatial proximity within the confines of the

conformation of the receptor binding site, and surface availability for binding. It is likely therefore, that the epitope brought about by tertiary conformation will be the most common for natural antigenicity, given that it is defined by the folded and naturally occurring structure of the protein.

Epitopes which are determined simply through proximity of adjacent residues are, conceptually, perhaps the least likely to be observed in natural situations. The arrangement of residues must survive the formation of secondary structure motifs, and subsequent folding of the protein into tertiary structure. Therefore, for such an epitope to be present in a naturally folded protein, it is unlikely for the residues which comprise it to be particularly hydrophobic. A highly hydrophobic residue is more likely to be either internalised within the structure, or possibly situated within an alpha helix. The helical structure would be either internal, or through its structure have removed the linear availability through the helical turns.

Although, a beta sheet would be more likely to retain the local linear structure of such an epitope, the characteristics of the other residues within the sheet will influence its position within the tertiary structure. Whilst this does not necessarily mean that the availability of the linear epitope will be lost, it does mean that the characteristics of the residues which are adjacent to the epitope in the sequence will play a large role in determining its availability.

It has been suggested that segmental mobility, or flexibility, is linked to antigenicity,[60,61] which would indicate that the most suitable epitope sites would occur in loop regions of the protein structure. It has subsequently been hypothesised however, that any region of a protein that is accessible at the surface can potentially interact with an antibody.[62] More recent studies of antigen-antibody binding have suggested that flexibility of the CDR loop regions of the antibody is important for antigen binding.[63] This flexibility allows for an ‘induced fit’ mechanism for antigen binding, which could logically be

aided by flexibility of the antigen structure. Therefore, whilst any surface-available region of a protein may have the potential to interact with an antibody, perhaps regions of higher mobility or flexibility will afford better affinity with an antibody.

The flexibility that aids antibody-antigen binding is likely to be similarly beneficial to the interaction between protein targets and synthetic receptors. The dynamic nature of biomolecules means that some movement of the structure is likely either through variations in temperature, solution concentration or pressure. The same is, of course, true of the receptor, synthetic or otherwise. MIPs may be rigid, highly cross-linked structures or less cross-linked, flexible structures with variable solution content, tending towards hydrogels.[64,65] A flexible MIP may mimic the 'induced fit' model of the antibody CDR, although the nature of the polymer may negate some of the properties leading to robustness and longevity inherent in a rigid polymer. By utilising a highly flexible region for the target, some of the benefits of the molecular dynamics of the protein can still be exploited even in combination with a rigid synthetic MIP receptor.

Whole proteins can be used to raise antibodies or produce synthetic receptors but, whilst this method ensures that the receptor is capable of binding the natural form of the protein, there are some drawbacks. The main difficulty is in obtaining a purified sample of the protein target with which to prepare the receptor. Others may include the solubility of the highly hydrophobic transmembrane domains of membrane proteins, the size of the protein, and toxic or otherwise detrimental effects caused by the protein on the host animal when considering immunisation methods for producing antibodies.

Some of these drawbacks may be overcome by the use of recombinant proteins, but all can be avoided by cleavage or synthesis of a fragment of the target protein to create a new target substructure. A

receptor for this smaller fragment will still be capable of binding to the native protein, as long as it can be ensured that the fragment is a peptide that would ordinarily be available at the surface. For artificial receptors such as MIPs, the fragment can be used alone or conjugated to a larger entity, (such as a protein, which is necessary when producing antibodies, or, for example a micro- or nano-particle or planar surface in the case of MIPs) to influence orientation and cavity generation. When a carrier protein is used, a purification step would be required to ensure that affinity for the peptide can be selected for, rather than binding to the carrier.

The use of small peptides as 'synthetic antigens' is attributed to pioneering work by Michael Sela and Ruth Arnon during a career of joint publications spanning 40 years. Their use of synthetic antigens as a research tool led to better understanding of immunological processes, and specifically autoimmune disease, culminating in the development of Copaxone, a drug indicated for the treatment of relapsing-remitting multiple sclerosis.[66] Earlier work investigated the use of synthetic antigens comprised of poly-amino acids. One such experiment showed that a multi-chain copolymer of poly-DL-alanine attached to polypeptides containing L-tyrosine and L-glutamic acid, was strongly antigenic in rabbits.[67] Such a synthetic polypeptide antigen was subsequently used to demonstrate the use of Sephadex columns for the purification of antibodies – a technique which is now widely used.[68]

The activity of antibodies raised to a synthetic antigen was demonstrated for the first time in 1979 when a fragment of an MS-2 bacteriophage coat protein was used as a synthetic antigen to inoculate a rabbit. This approach was successful in raising antibodies reactive to the native protein.[69] The ability to produce protein-specific antibodies from genomic sequence-derived synthetic peptide antigens was further investigated by Lerner *et al*.[70] The major outer envelope protein of the hepatitis B virus was chosen, as the gene sequence was known and the virus was considered to be of interest. The (at the time unpublished) computational method of Kyte and Doolittle,[71] was used to investigate the hydrophobicity of the protein, and peptide sequences were chosen from the less hydrophobic regions.

Antibodies were produced which reacted to the native protein, the authors speculating that similar techniques could produce excellent vaccines.

This work subsequently prompted further investigation of the use of synthetic peptides to raise antibodies to important viral targets. Synthetic antigens were chosen from the translated amino acid sequence of foot and mouth disease virus after analysis using structure prediction algorithms that was cross-referenced with known information regarding serotypes and experimentally-derived natural epitopes. The resulting antibodies were shown to specifically bind and neutralise the virus.[72]

So, in order to raise antibodies or design synthetic receptors, a suitable target is required, and there is the choice of either using the whole protein, or a fragment of it as the antigen.

If only a fragment is to be used, then there arises the question of how to select the fragment. Natural epitopes can be elucidated through experimentation, synthesised and used. Alternatively, any peptide fragment may be used if it can be assured that it will be available for binding in the final structure. Additionally, the selection of a novel peptide provides the possibility of targeting regions of the protein which have not been demonstrated to be antigenic *in vivo*, but which may offer improved access or affinity for detection in sensing applications.

The simplest way to decide if a fragment will be solvent-accessible is to look at its position within the tertiary structure of the protein. This is the most suitable approach if X-ray or NMR structural data is available in the Protein Databank.[73] However, as there are currently only around 61,000 structures deposited in the database – including many duplications and encompassing many species – it is clear

that there is a significant possibility that a target that appears suitable from other experimental or "–omics" approaches may not have a solved structure (www.pdb.org).

When a protein has no experimentally determined structure, the best method for trying to obtain a model of the structure is to compare the sequence to that of a protein of known structure. This can be especially successful when the two proteins in question belong to the same family of functional proteins. The databases SCOP (Structural Classification of Proteins)[74] and CATH (Class, Architecture, Topology and Homology)[75] are both linked through the entries in the PDB, and provide a classification of protein structures into functional and structural subdomains. Such information can be useful when comparing an unknown sequence to partial homologous structures.

When attempting to model tertiary structure based on homology, there are several steps which should be undertaken. Initially, a sequence alignment against proteins of known structure should be performed, followed by matching the unknown sequence onto the structure of the homologue. Finally, optimisation of the structure, in particular modelling of sidechain torsions and interactions and energy minimisation of loops needs to be done to refine the model. In order to search for homology to known structures, the Fugue tool (tardis.nibio.go.jp/fugue/)[76] can be used to search the Homstrad database (tardis.nibio.go.jp/homstrad/)[77] of curated protein alignment structures, using other software used for the structure determination and optimisation. Alternatively the web-based Swiss-Model (swissmodel.expasy.org/)[78] can be used to align against Homstrad and determine a model in one step.

If the protein in question has no significant homology with another protein of known structure, the next logical step is to try and predict elements of the secondary structure from the primary amino acid sequence. The current practice of protein secondary structure prediction is dominated by the use of neural networks or complex statistical analysis for the determination of residue conformation states,

as well as older propensity-based statistical approaches which, whilst being somewhat inaccurate, have the benefit of ease of use and being well understood by their end users. Such methods have seen continued effort at improvement over the years, but it should generally still be considered a 'last option' approach to resort to predictions of structure from sequences in the absence of homology.

One notable methodology which has been adapted for use as part of an on-line tool (www.embl-heidelberg.de/predictprotein/predictprotein.html) is that of Rost.[79] Available as the PredictProtein server,[80] there are several methods available including; PHDsec for secondary structure; PHDacc for solvent accessibility; and PHDhtm for predicting transmembrane helix domains,[81] all of which use neural networks for analysis. The author reports accuracies of 72 percent for the PHDsec algorithm, and a significant 95 percent for the predictions of PHDhtm.

Despite the growing availability of on-line tools and the popularity of the more complex methods, there remains a niche application for some of the 'simpler' approaches, relying on statistical analysis of known structures. These form the basis of many other secondary structure prediction, and antigenic region prediction algorithms.

One such well-known algorithm based on statistical propensities is that of Garnier *et al*.[82] The GOR algorithm uses sets of experimentally-derived scales defining the propensities of amino acids to form helix, sheet, turn or coil conformations. The propensity for a given confirmation is compared for each amino acid in a sequence, which is then unambiguously assigned to a particular state based on the highest of the propensity values. In addition to the statistical propensity approach used by GOR, analysis of protein hydrophobicity has also been used to establish general structural features of proteins based on empirical evidence of hydrophobic/hydrophilic behaviour of residues. The original use of this approach was published by Hopp and Woods[83] who proposed the use of hydrophilicity as

an indication of potential antigenic domains due to their predicted solvent accessibility. The Hopp-Woods scale was applied using a program originally written in Fortran. The single scale of author-modified hydrophilicity scales, taken from Levitt,[84] was applied using a sliding window, equivalent to the suggested size of a hexapeptide antigenic structural motif.

A similar approach was detailed by Kyte and Doolittle,[71] where experimentally-determined values for residue hydrophobicity were used to identify internal or transmembrane regions of a protein structure. The Kyte-Doolittle scale was implemented using an original program written in C. A single scale of assigned hydrophilic/hydrophobic values derived by the authors from water/vapour, water/ethanol, and ethanol/vapour free energies for each amino acid, and applied using a sliding window of 7, 9, 11, or 13 residues, arbitrarily assigned depending on the size of the structural motif being predicted. The hydrophobicity/hydrophilicity analysis techniques are still used, but more often in combination with other structure prediction techniques based on propensities of residues to occur in particular structural elements.

A list of protein structure prediction algorithms (and other computational analysis tools) is maintained at the ExPASy (Expert Protein Analysis System) proteomics server from the Swiss Bioinformatics Institute (www.expasy.org/tools), serving as an excellent starting point for prediction of protein structural characteristics.

In addition to the many algorithms which have been written to predict structural conformations, there are others which attempt to predict antigenic regions and epitopes of proteins such as the Hopp-Woods analysis,[83] mentioned above. It is normal for structural prediction algorithms to be used to predict the structure only, and antigenic region prediction methods to be used to identify potential epitopes only. In the case where multiple algorithms have been used together, and structural and

antigenic prediction methods combined, the full structural analysis is used and added to the predictions of antigenicity.

One such method is that developed by Parker *et al*.,[85] derived from high-performance liquid chromatography (HPLC) of 20 model synthetic peptides, each containing a motif specific to each of the 20 naturally occurring amino acids found in proteins. The retention time of the peptide was measured and attributed as a measure of hydrophilicity to each amino acid. This scale of hydrophilicities was applied using a sliding window assignment modified from Hopp-Woods, in combination with a scale of accessibility taken from Janin *et al*.,[86] and accessibility values from Karplus and Schulz,[87] as a measure of potential antigenicity. Another example is that of Pellequer and Westhof,[88] which includes analyses from 22 different scales including hydrophilicity, accessibility, flexibility and secondary structure propensity.

Perhaps the most well known method which uses a combination of different algorithms is the Antigenic Index.[89] The original algorithm was written in the common, mathematically oriented Fortran for the VAX platform. The Antigenic index is calculated from numerical expressions of results from slightly author-modified versions of the Hopp-Woods, Janin *et al*, Karplus and Schultz, GOR, and Chou-Fasman[90] algorithms.

One notable single approach that has not been incorporated into multiple approaches is that of Kolaskar and Tongaonkar.[91] The method incorporates a scale of statistical antigenic propensities derived from an analysis of 34 different proteins, with experimentally determined epitope regions, applied using a sliding window of seven residues. It is of note primarily because it is so easily available to molecular biologists through its inclusion with the European Molecular Biology Open Software Suite (EMBOSS) as the programme ‘antigenic’ (www.emboss.sourceforge.net). The suite is

widely available and includes many tools for simple and routine bioinformatic analysis useful to biological researchers.[92] Despite being a simple approach, it is popular due to its accessibility, and the reported accuracy of 75 percent.

Authors report a wide range of accuracy values for these computational prediction methods, and often the performance is evaluated with widely different approaches. Simple methods tend to be fairly inaccurate, particularly when testing is re-visited with new evaluation techniques and datasets. Clearly, having experimentally determined structural data is the ideal, but where prediction methods must be used there is often a compromise to be made between speed and accuracy. It is important to consider the methods carefully and be aware of the limitations of the technique. Although perhaps obvious, one of the most important things to remember regarding any form of structure prediction is that an algorithm which is reported as having 70 percent accuracy is in effect 30 percent inaccurate. Of interest perhaps, is the commentary on pitfalls of protein sequence analysis by Rost and Valencia,[93] which discusses many issues surrounding the successful use and interpretation of structure predictions.

2.2 Methods of imprinting proteins

The earliest attempts at imprinting proteins used whole protein molecules as the template. These approaches were based on forming a few spatially-separated interactions with specific side chain residues on the surface of the protein; such as copper-mediated binding of histidine imidazole groups.[94] In this example a "surface-imprinting" approach was used to avoid encapsulating the template, which was dissolved in a DMF/water mixture. *N*-(4-vinyl)-benzyl iminodiacetic acid groups were employed as functional monomer units to chelate copper (II) ions. The chelate groups were captured at the surface of vinyl-functionalised silica particles. The imprinted material was shown to separate RNase A (template protein) from Lysozyme in an HPLC experiment. This approach was similar to that proposed earlier by Arnold and co-workers, who demonstrated the use of copper (II)

iminodiacetate groups to recognise a series of *bis*-imidazoles as protein surrogates.[95-100] There are a number of potential problems with this approach, the first being the use of aqueous DMF as the solvent for imprinting a protein. This is by no means a "kind" solvent mixture and many proteins would be denatured under these conditions. The use of a small number of strong interactions is also not ideal. While there appeared to be separation under dynamic (chromatographic) conditions, any protein with surface-exposed imidazole residues would be expected to bind reasonably tightly to copper sites on the silica surface. It is likely that non-specific binding would therefore be high for a large range of protein molecules. The lack of a three-dimensional element to the surface-bound "polymer" is also a limiting factor for good protein recognition (Figure 5).

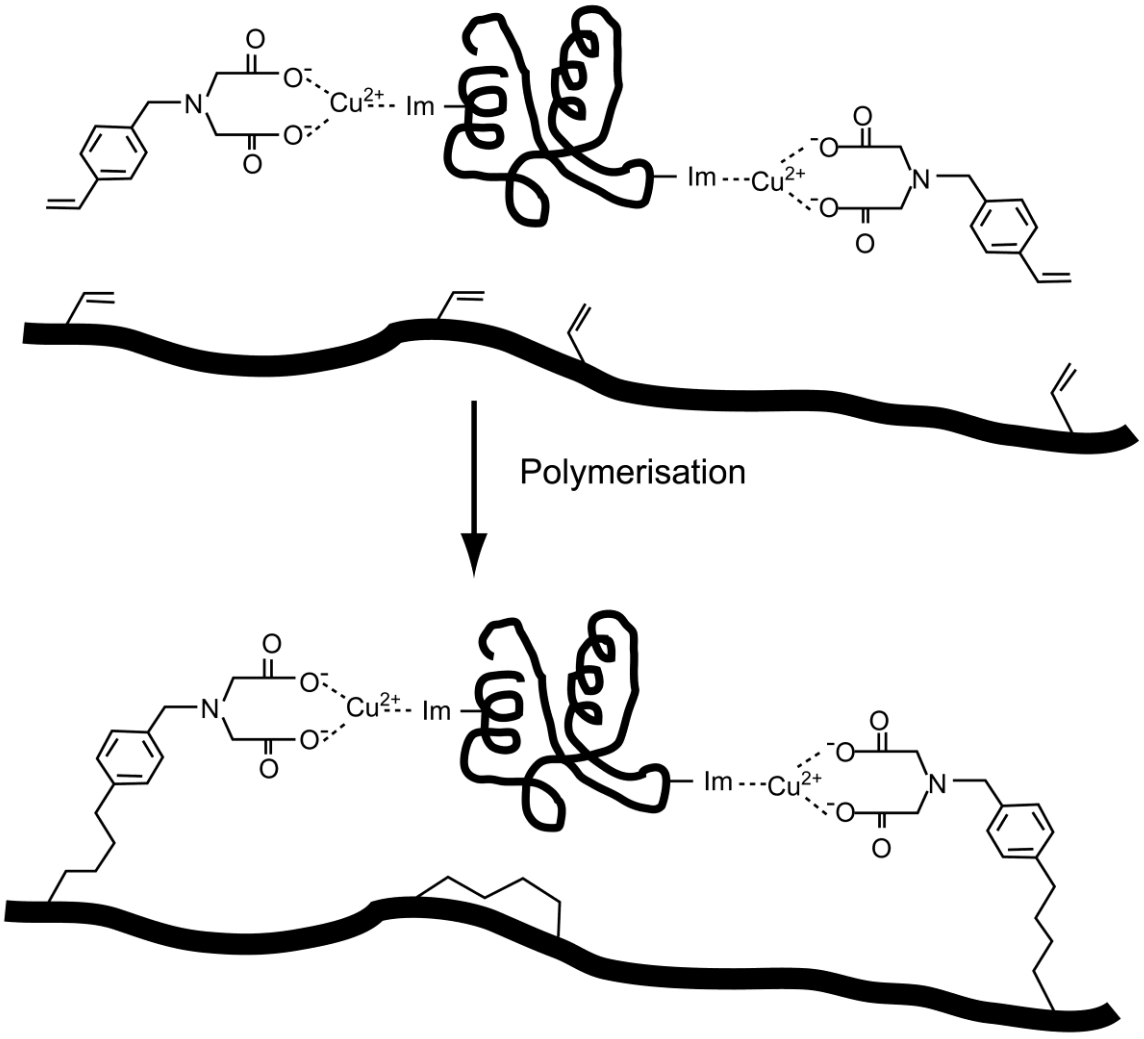

Figure 5. Proposed "surface imprinting" of RNase A *via* copper (II) *N*-(4-vinyl)-benzyl iminodiacetate groups at the surface of silica particles functionalised with double bonds. Adapted from Kempe *et al*.[94]

Polymerisation at the surface of silica particles was also employed by Burow and Minoura to prepare MIPs for glucose oxidase (GOD).[101] The aim was to build a thin layer of polymer from a mixture of water-soluble cross-linking monomers (*N*,*N*'-1,2-dihydroxyethylene-bis(acrylamide) and *N*,*N*'-(methylene)-bisacrylamide) in a phosphate-buffered solution of the enzyme template. This approach attempted to provide for 3-dimensional recognition and avoided the use of a few strong interactions for potentially many weaker ones and was reasonably successful in imparting specific binding to the MIP. Rebinding in batch mode showed selectivity for GOD with respect to glucose dehydrogenase (GHD) on the MIP and a BSA-imprinted polymer showed the same non-specific interactions with GHD.

The group of Hjertén[102] reported imprinting of whole protein in acrylamide gels prepared with much lower levels of cross-linking. These "artificial gel antibodies" as they were later called are relatively soft material possessing pores large enough for proteins to diffuse through the polymer, being similar in composition to polyacrylamide gels used for protein separation. Hemoglobin, cytochrome C and transferrin were used as templates in this initial study. The imprinted gels were granulated by passage through 100-mesh sieves and packed into columns in Pasteur pipettes. These small affinity columns showed selective binding of the template proteins from mixtures, with high retention of the template. High selectively was also demonstrated by the ability to adsorb myoglobin from horse but not whale myoglobin on the horse myoglobin-imprinted column, demonstrating selectivity for two proteins with similar sequences and 3D structures.[103,104] The same approach has also been used to prepare imprinted gel within the pores of particles made from a more rigid gel (agarose) in order to improve the flow rate in chromatography.[105] "Gel antibodies" prepared by the same approach have been employed in an

electrophoretic migration technique with bacteria[106] and viruses[107] employed as templates, as well as proteins.[108,109] The migration of the gel particles in a rotating tube under an electrophoretic field depends on their overall charge. Since the acrylamide particles are neutral they only become charged and can migrate in the electric field when they rebind their respective templates. The use of the materials in clinical diagnosis and the detection of protein biomarkers was recently demonstrated[110,111] and the approach was also shown to be applicable to the preparation of enzyme reactor beds.[112] The later paper also suggests that "renewable biosensors" for viruses, bacteria and spores could easily be created using the gel antibody approach.

A "whole" protein approach was also reported by Venton and Gudipati,[113] using a mixture of silane monomers, 3-aminopropyltriethoxysilane and tetraethylorthosilicate (1:3), to prepare a siloxane polymer in the presence of urease or bovine serum albumin (BSA). The polymers became entrapped in the polysiloxane matrix and were also probably covalently bound to the polymer through reactive side chain residues. Template species were removed by digestion with pronase and a moderate selectivity in rebinding was seen in binding experiments with the two proteins and their respective imprinted polymers. Attempts to reproduce this result with the protein couple haemoglobin and myoglobin did not show any selectivity however.

Compared with other methods, the use of whole protein in solution presents few advantages as a general approach to preparing MIPs for sensor applications. The main advantage is that the template structure will most accurately reflect that of the target (assuming the target and template are the same protein) however this is largely outweighed by a number of disadvantages namely: the template may easily become entrapped or covalently bound to the polymer, it may be difficult to maintain the native conformation of the protein throughout the polymerisation process and the large imprinted sites may be seen as general nanopores, able to bind a range of smaller polypeptides, resulting in reduced

selectivity. For these reasons, a number of other strategies, involving surrogate templates, have been employed.

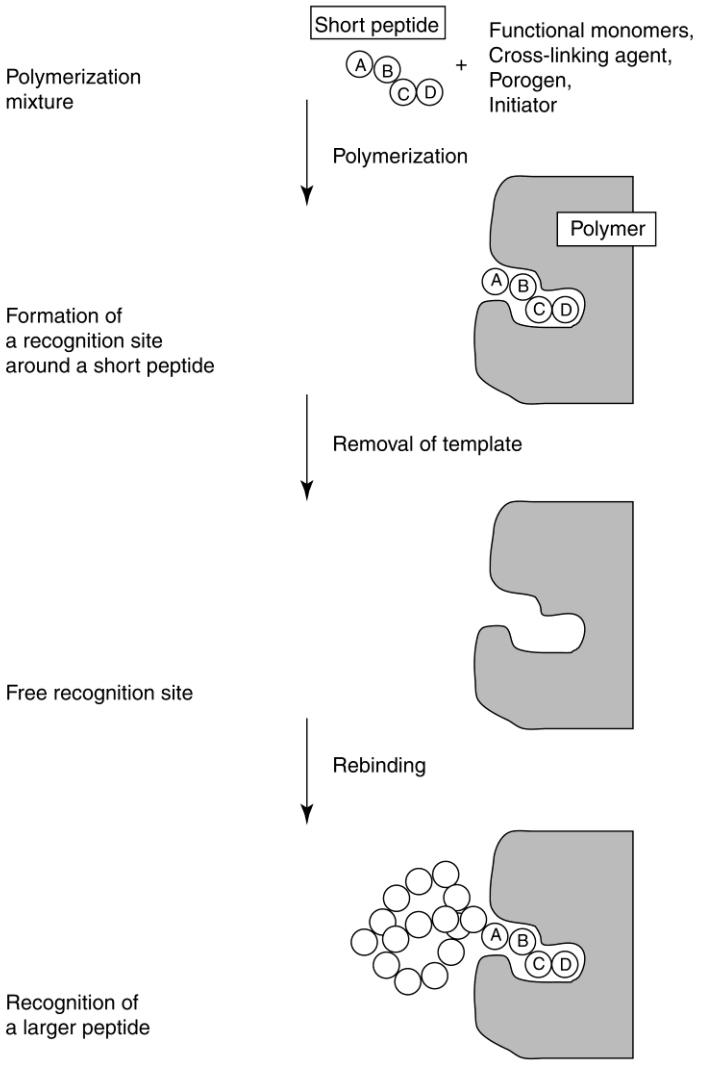

Figure 6. Schematic diagram of epitope imprinting: a short peptide sequence is used as the template to create selective binding sites for a larger peptide or protein. Adapted from Rachov and Minoura.[57]

An alternative to the use of whole protein as template is the "epitope approach", originally proposed by Rachov and Minoura.[57,114-116] In this method a peptide with the same sequence as one of the terminal chains of the target protein (the epitope) is used as a surrogate template for the whole protein

(Figure 6). This approach is analogous to protein recognition by antibodies, where an "epitope" of the immunogenic protein is the site of antibody binding, not the whole protein. In the imprinting context the template peptide generally represents one of the terminal sequences of the primary protein structure, although an antibody epitope could be any surface-accessible region of the target protein. Terminal peptides make better imprinting targets because their structure is unambiguously defined and relative to other regions of the target protein, they will have fewer interactions with the protein secondary structure, which may hinder or frustrate binding. The minimum length of peptide necessary to create "unique" recognition for the target protein has been estimated to be around 9 amino acids according to Nishino *et al*.[117] These authors also state that an exposed C-terminus is preferable, since this site is less prone to post-translational modifications.[118,119] While Nishino *et al*.[117] used nonapeptide epitopes as template, peptides as small as tri-[120] and tetra-peptides[114] and as large as 15-[121] or 16-[122] amino-acid units have been used to target other proteins.

The surface imprinting method applied by Nishino and co-workers[117] (Figure 7) was shown to be effective at selective extraction of the target protein from mixtures, such that Cytochrome c (Cyt c), alcohol dehydrogenase (ADH) and bovine serum albumin (BSA) were found to be selectively captured by polymers imprinted with their respective C-terminal peptide epitopes from mixtures containing five proteins. The templates were: AYLKKATNE (Cyt c from bovine-heart, amino acids 97–104), AYLKKATNE (ADH from yeast, amino acids 339–347) and VVSTQTALA (BSA amino acids 599–607). Each was immobilised by its N-terminus on a planar Si/SiO_2 surface through coupling to a carboxy-functionalised silane linker. Photopolymerisation of an acrylamide/ethylene-*bis*-acrylamide polymer thin film at the surface produced the imprinted layer which could be peeled-off the template assembly to reveal the binding surface. Rebinding was also shown to be sensitive to substitution of one amino acid residue and non-specific binding to the imprinted surface was low.

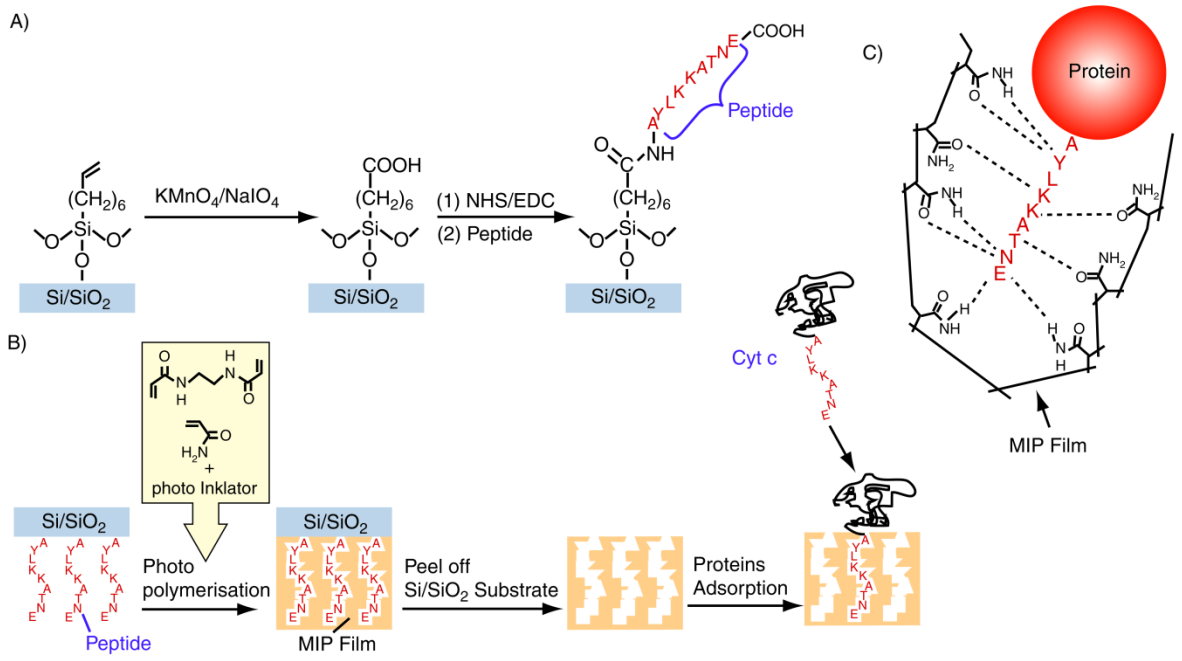

Figure 7. The surface-bound epitope approach employed by the group of Shea using C-terminal nonapeptides as templates for protein imprinting. Selective binding of the target proteins (Cyt c, ADH and BSA) was demonstrated from mixtures of Cyt c, ADH, BSA, carbonic anhydrase and trypsin inhibitor. Adapted from Nishino *et al.*[117]

A number of advantages therefore stem from using terminal peptide epitopes as the template, namely: the conformation of whole protein does not need to be retained, allowing harsher solvent and temperature conditions to be used; selectivity in protein recognition can be controlled by the choice of epitope and its length; template removal is far more easily achieved than with whole protein and highly selective imprints can be obtained. On the other hand, a good knowledge of the protein structure is necessary, but as we have seen from the earlier discussion, there are a number of computational and database-based methods available which can help. Another limitation is that custom synthesis may be required in order to prepare the templates which may be relatively costly and time-consuming.

2.3 Template presentation

For the imprinting of small molecules it is usually sufficient to dissolve the template, monomers and initiator in a porogenic solvent prior to the formation of MIPs, whether the resultant material is to be a monolith, bead, film or membrane. This approach can be followed when imprinting proteins or their epitopes, but the results are likely to be suboptimal due to a number of phenomena: protein entrapment, resulting in poor template removal and steric hindrance limiting rebinding of bulky protein molecules being just two. For these reasons it is preferable to form imprints for protein binding at surfaces or interfaces between phases to overcome these problems.

Imprinting methods at a planar surface or interface can be termed 2-dimensional approaches. These include the capture of protein complexes at existing surfaces, as in the example shown in Figure 5,[94] and the organisation of surfactant-like molecules in Langmuir monolayers.[123] While these 2-D imprints can show some selectivity, it is unlikely to ever be a suitable method for sensing applications, as non-specific binding is always likely to be high with such materials.

A degree of three-dimensional imprinting of whole protein can be achieved with "surface imprinting" methods. A good deal of ingenuity has been shown in the devising of methods for the partial embedding of protein templates in polymer without complete encapsulation. These methods include contact printing and interfacial methods. A number of these are summarised in the graphic below (Figure 8). These examples cover a wide range of materials in which the imprints are made: plasma-polymer,[124] metal,[125] polysilanes,[126] poly(acrylamide)[127] and poly(dopamine)[128] as well as a diverse set of techniques to prepare imprints on planar surfaces,[124,125,127] beads[126] and nanowires[128] with various degrees of complexity involved in their realisation. That these lengths are worth going to can be related to the likely cost of templates and their efficient use as well as the ready accessibility of the imprint sites. The same considerations equally apply to the imprinting of epitopes as to whole proteins, the templates may also be expensive and/or available in small amounts and the target is still

a bulky protein molecule (See Figure 7). An interesting strategy for the imprinting of peptides at surfaces was reported by Titirici *et al*.[129] using solid-phase synthesis on a porous silica bead to produce the template peptide *in situ*, before the imprinting polymerisation was carried out in the pores of the silica. Etching away the macro-template (silica bead) gave (negative) replicas of the pore structure of the template particle with surface accessible imprint sites. The imprints were shown to be capable of binding larger peptides with the same terminal sequence as the template peptide (epitope).[130] This method is a development of that shown for small molecules[131,132] and is also similar in concept to the nanowire method, shown in Figure 8.

When considering the pros and cons of bulk vs. surface imprinting methods, the following should be considered: Bulk imprinting is a fairly simple approach compared to surface-imprinting methods, which may require much more complex preparation procedures. Bulk methods are inefficient in their use of potentially expensive templates, whereas surface-confined templates may be used more efficiently and, although as yet largely unproven, could possibly enable template reuse though a number of imprinting cycles. Template removal should be much easier to achieve in surface-confined imprinting, providing good access to the imprinted sites, whereas MIPs prepared in bulk may suffer from poor template extraction, slow binding kinetics and template entrapment and/or bleeding. Surface methods may also offer lower non-specific binding but ultimately the number of binding sites created will be limited by the amount of surface available. Whether this would prove to be a limitation will depend on the ultimate application of the material and (for a sensor) the signal transduction method used.

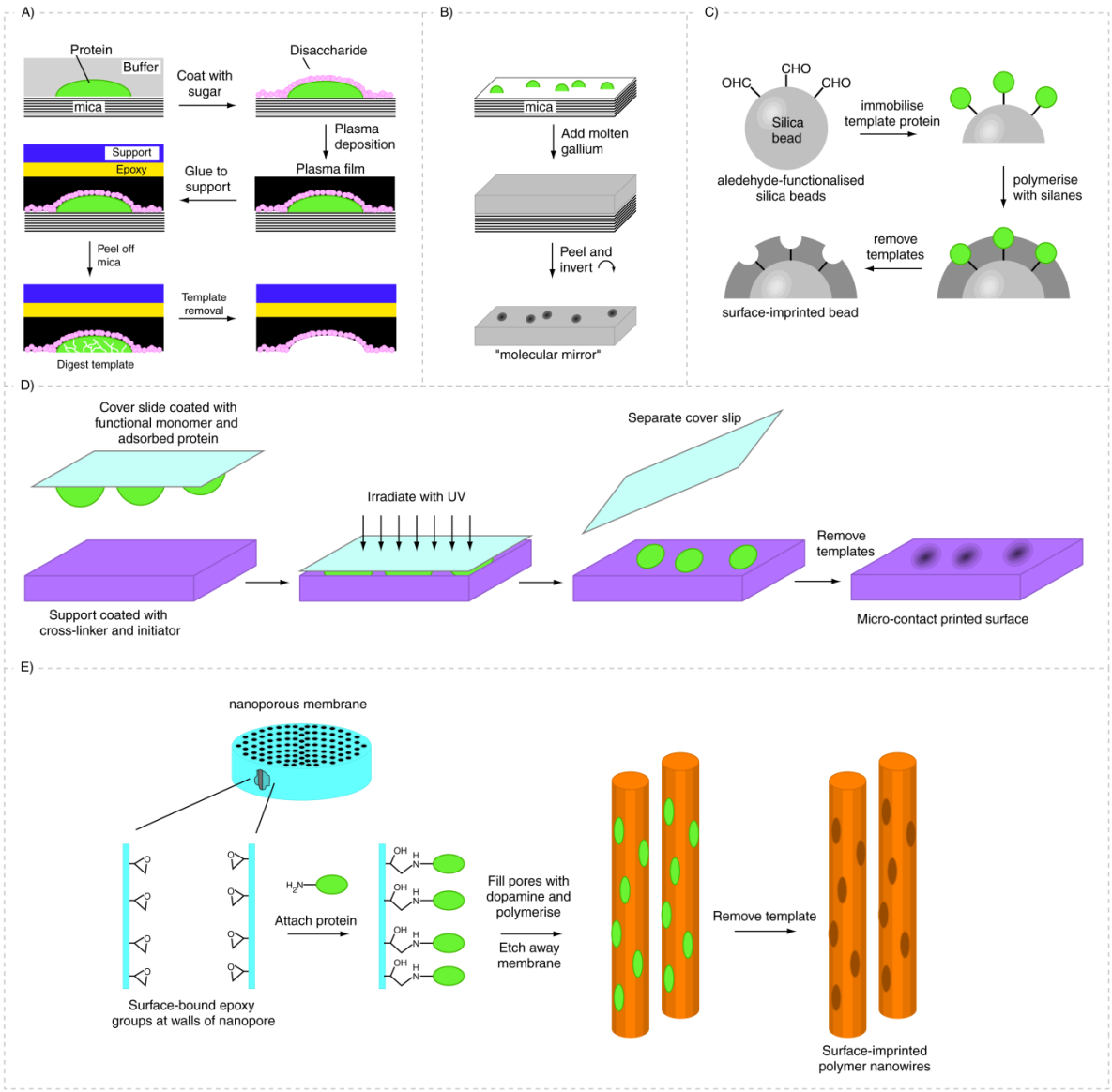

Figure 8. Various protein surface-imprinting methods: A) Method of the group of Ratner[124,133] involving imprinting in a plasma-polymerised film using disaccharide units as the species providing functional group interactions with the protein surface. Adapted from Shi *et al.*[124] B) The formation of "molecular mirrors" by casting replicas of protein molecules adsorbed on mica surfaces using molten gallium.[125] C) Surface grafting of polymer around template molecules immobilised on silica beads, according to the method of Shiomi *et al.*[126] A similar strategy was employed by Bonini *et al.* in the surface imprinting of human serum albumin (HSA) using poly(3-aminophenylboronic acid) as the recognition material.[134] D) Surface micro-contact imprinting method developed by the group of Chou.[127] E) The formation of surface protein-imprinted polymer nanowires by hierarchical templating

using proteins anchored to the inner walls of membrane nanopores as molecular templates. Adapted from Ouyang *et al.*[128]

2.4 Environmental factors

For the imprinting of whole protein, careful control over pH and salt concentration may be required to preserve the native conformation of the template protein and control the charge state of the protein. Such precise control may not be necessary for epitope imprinting however, depending on the functionality of the constituent amino acids and indeed whether aqueous or non-aqueous polymerisation conditions are utilised.

Uysal and co-workers[135] prepared haemoglobin-imprinted polymers at different pH (4.0, 6.8 and 8.0) to study the effect of pH on the imprinting process. They used *t*-butylacrylamide, acrylamide and itaconic acid (ITA) as functional monomer. It was found that the greatest binding for the template protein was shown by the polymer imprinted at pH 4.0, below the isoelectric point of the template, (pI = 6.8) where the protein carried a net positive charge, enabling its interaction with the negatively charged ITA residues.

3 Polymer selection

The recognition properties of MIPs are highly dependent on the quality of the binding sites contained in the matrix and this applies equally to MIPs imprinted with proteins. Key factors governing the quality of the imprinted cavities are the choice of monomers and cross-linkers as well as their amounts. The following sections describe strategies for monomer selection and survey of a number of

recent papers showing the diversity of compositions used to imprint proteins and other biological targets. This is followed by a consideration of the cross-linkers used in the imprinting of proteins.

3.1 Choice of functional monomer

There are many monomers available for interaction with template functionality and in general their selection can be made by selection, based on chemical intuition according to the structure of the template, or on the basis of molecular motifs for which designed small molecule receptors have been published.[136-138] Alternatively a rational selection can be made, either using a combinatorial (experimental) approach,[23,24] a chemometric experimental design[24] or by computational methods.[26,139-142] In the combinatorial and chemometric approaches libraries of MIPs with different compositions are prepared, either as a comprehensive (combinatorial) or representative (chemometric) set, varying parameters such as functional monomer, monomer-template ratio, solvent, cross-linker etc. Evaluation of the polymer properties, such as extent of binding of the template, can be made using semi-automated approaches. Computational methods however use computer modelling in an attempt to predict the locations and strength of the interactions of monomer with template. Selection of which monomer to use can then be based on criteria such as binding energies and number of points of interaction. Selection of monomers for protein imprinting, however, is complicated by factors which do not apply when small molecules are to be imprinted. Firstly, as most proteins are not soluble in non-polar or medium polarity solvents, only monomers which are soluble in polar solvents (ideally water) can be selected. Even when the protein target can be dissolved in an organic solvent, it would be unwise to use it the production of MIPs, since biomolecules are highly likely to undergo conformational changes[143] such that the resulting imprint will not be representative of the native protein target in aqueous solution, where the sensor is most likely to be required to perform. Disregarding monomers soluble in apolar organic solvents, many of the remaining monomers of the functional monomer "pool" contain ionisable groups (strong acids and bases). Whether polymers containing these highly charged monomers are helpful in the imprinting of proteins has been of some

debate. In fact whereas, relatively strong non-covalent interactions can arise from electrostatic interaction of opposing charges on polymer and template, a net positive or negative charge on the polymer can also lead to high levels of non-specific interaction for all species carrying the opposite charge, since many of the charged monomers will be randomly distributed over the polymer surface, as well as being specifically located in the imprinted sites. This situation is generally incompatible with cases where the target protein must be detected from a mixture of similar molecules, as is usually the case for biomedical use. In fact smaller charged species, such as physiological salts and buffers could interfere with recognition based largely on charge. For these reasons the majority of successful protein imprinting experiments have used neutral, water soluble monomers such as acrylamide and its derivatives or other neutral monomeric species (see Table 3).

Table 3. Examples of protein imprinting taken from the literature and the functional monomer composition used in the imprinting step.

Monomers	**Protein/Template**
2-methacryloyloxyethyl phosphorylcholine and 2-methacryloyloxyethyloxy carbonyl 4-phenylazide	Fibronectin[144]
chitosan and acrylamide	haemoglobin and bovine serum albumin (BSA)[145-148](see however Ref. 149)
Acrylamide	bovine haemoglobin,[150-152] *Staphylococcus aureus* protein A,[153] cytochrome C,[154] lysozyme,[155,156] human serum albumin,[157] BSA[156]
acrylamide (hydrogels)	cytochrome C, transferrin and haemoglobin[102]
acrylamide, methacrylic acid and 2-dimethylamino ethyl methacrylate	Lysozyme[158]
acrylamide and 4-vinylpyridine	BSA[159]

acrylic acid* and acrylamide	Anthrax protective antigen[160]
acrylic acid,* acrylamide and *N*-benzylacrylamide	Protein 1 of flavivirus (Dengue Virus)[121,161]
acrylamide and epichlorhydrin cross-linked chitosan	albumin[162]
N-isopropylacrylamide, acrylamide and methacrylic acid *	lysozyme and cytochrome c[163]
3-aminopropyltrimethoxysilane and trimethoxypropylsilane	Haemoglobin[126],
11-mercapto-1-undecanol	myoglobin and haemoglobin,[164] carcinoembryonic antigen[165]
3-aminophenylboronic acid	bovine haemoglobin and BSA,[159,166-169] papain and trypsin,[170] microperoxidase, horseradish peroxidise, lactoperoxidase and haemoglobin,[171] β-lactoglobulin,[172] lysozyme and haemoglobin,[173] lysozyme or cytochrome C[174,175]
Poly(ethylene-co-ethylene alcohol)	albumin and lysozyme[176]
Poly(ethylene-co-vinyl alcohol)	α-amylase[177,178]
N-[3-(dimethylamino) propyl] methacrylamide and *N*-isopropylacrylamide	BSA[179,180]
3- aminopropyltriethoxysilane	human serum albumin[134]
3-aminopropyltriethoxysilane and tetraethoxysilane	Ricin[181],
3-aminopropyltrimethoxy siloxane and tetraethoxysiloxane	BSA[182]

aminopropyltrimethoxysilane and tetraethyl orthosilicate	bovine haemoglobin[53]
3-aminosilane and tetraethosysilane and octyltrimethoxysilane	BSA[183]
3-aminosilane and tetraethosysilane and octyltrimethoxysilane	*Staphylococcus* enterotoxin B[184]
phenyltrimethoxysilane and methyltrimethoxysilane	human serum albumin[185]
acrylamide , 3- aminopropyltriethoxysilane	albumin, hemoglobin and cytochrome C[186]
acrylamide, methacrylic acid* and 2-(dimethylamino)ethyl methacrylate	lysozyme[187]
Poly(ethyleneglycol dimethacrylate) and methacrylic acid*	Ovalbumin[188]
methacrylic acid,* Poly(ethyleneglycol dimethacrylate)	creatine kinase[189]
acryloyl-β-cyclodextrin, acrylamide	lysozyme, haemoglobin[190,191]
methylmethacrylate and tetraethylene glycol dimethacrylate	myoglobin[192]
N-isopropylacrylamide, acrylamide and 4-vinylpyridine	BSA[193]
N-methacryloyl-L-tyrosine methyl ester and 2-hydroxyethyl methacrylate	Hepatitis B surface antibody[194]
N-isopropylacrylamide and 2-acrylamido-2-methyl propane sulfonic acid*	myoglobin[195]
3-methacrylamido propyltrimethyl ammonium or 2-acrylamido-2-methyl propane sulfonic	bovine hemoglobin[196]

acid*	
N-methacryloyl-L-histidine methylester	lysozyme[197]
methacrylic acid* and ethylene glycol dimethylacrylate	BSA[198]
methacrylic acid* and trimethylolpropane	cell adhesion proteins[120]
methyl methacrylate, methacrylic acid* styrene, tetraethylene glycol dimethacrylate and ethyleneglycol dimethacrylate as cross linkers	myoglobin, lysozyme and ribonuclease A[199,200]
N-[3-(dimethylamino) propyl]methacrylamide and methacrylic acid*	BSA and lysozyme[201]
Agarose	BSA[202]
acrylic acid*	lysozyme[203]
methyl methacrylate	ribonuclease A, BSA, lysozyme[204]
methyl methacrylate and ethylene glycol dimethacrylate	ribonuclease A[205]
Styrene, divinylbenzene and methacrylic acid*	lysozyme and Tobacco Mosaic Virus[28]
styrene	ribonuclease A[206]
2-acrylamido benzamide	CYP2D6 isomer of Cytochrome P450[207]
6-(*N*-acrylamido)-6-deoxy-β-cyclodextrin	angiotensin II[208]
N-acryloyl *p*-aminobenzamidine and acrylamide	trypsin[209]
O-(4-nitrophenylphosphoryl) choline	C-reactive protein[127]
Poly(vinyl alcohol)-based assistant recognition polymer chains (ARPCs),	BSA[210,211]

*Charged monomer

Table 3, above lists the functional monomers used in the molecular imprinting of proteins. These can be roughly divided into: acrylic monomers, such as acrylamide, used to produce polymers, either in water (e.g. hydrogels) or in organic solvents; silane-based monomers, to create sol-gels, organically-

modified silicas (ORMOSILs) and related materials; monomers such as 3-aminophenyl boronic acid, used to make imprinted polyaniline; and thiol derivatives and surfactants used in the formation of "2-D" imprints at interfaces or on gold surfaces, the latter mainly for sensor applications. The majority of the monomers used are either neutral compounds or weak acids or bases, which will be uncharged over a relatively wide pH range. There are relatively few examples (marked with a star in the table) where monomers that are likely to be significantly charged at neutral pH, were used for imprinting.

Janiak *et al.*,[196] recently looked at the effect of charge density on the recognition properties of molecularly imprinted hydrogels, prepared using either positively or negatively charged monomers (3-methacrylamido propyltrimethyl ammonium or 2-acrylamido-2-methyl propane sulfonic acid) respectively. The gels showed a decrease in recognition ability with increasing charge density, which also affected the swelling behaviour of the gels. The authors also showed that surfactants, generally employed in the template removal process, were responsible for some of the binding interactions seen.

Ou *et al.*[187] have shown that when hydrogels (polyacrylamide materials produced in aqueous environments) imprinted with lysozyme were prepared using acrylamide as the only monomer, the protein molecules were partially incorporated through covalent bonding to the polymer matrix, occurring during the polymerisation, and could not be removed. This limited the number of recognition sites available for specifically rebinding the template. This phenomenon could be reduced, however, by the inclusion of methacrylic acid into the hydrogel compositions. This was attributed to the acidic monomer interacting through electrostatic forces with groups on the protein responsible for formation of covalent bonds, making them unavailable to attack by radicals during polymerisation. Chen *et al.*[163] also used a small amount of methacrylic acid as copolymer, alongside *N*-isopropylacrylamide as the major functional monomer, in order to synthesise hydrogels specific for lysozyme and cytochrome C. Matsunaga *et al.*[203] similarly produced lysozyme-imprinted hydrogels films on a sensor surface using an acidic monomer, acrylic acid. In addition to the acidic monomer,

the zwitterionic monomer, 2-methacryloylethyl phosphorylcholine, was included in their polymer composition in order to reduce non-specific binding to the hydrogels. The same group[212] investigated the role of acrylic acid in the imprinting of lysozyme on the surface of silica beads using acrylamide and *N*,*N*-methylene-*bis*-acrylamide based polymers. They concluded that a ratio of 5:1, acrylamide:lysozyme was optimal and that in the absence of acrylic acid there was no binding specificity in the polymers, whereas too much of the monomer gave rise to high non-specific binding. On the other hand Zhang *et al*.[190] were able to produce MIPs for lysozyme using only a mixture of acrylamide and acryloyl-β-cyclodextrin, another neutral monomer. The authors managed to avoid the problem of lysozyme incorporation into the matrix by covalently immobilising the template onto silica beads, making it less available to radical attack. The material synthesised by Zhang and colleagues showed high specificity and excellent selectivity for rebinding of the template with respect to other proteins (cytochrome C, BSA, avidin and methylated bovine serum albumin).

Another study that lends weight to the proposition that acrylamide (usually with methylene-*bis*-acrylamide as cross-linker) is the monomer of choice for protein imprinting, is that reported by the group of Ulbricht.[213] They showed that highly cross-linked layers of polyacrylamide grafted on sensors, strongly reduces the adsorption of protein (BSA) compared to bare sensor surfaces, making this material very promising for the development of sensors for protein detection.

Turan *et al*.[195] produced hydrogels with specificity for myoglobin, prepared using *N*-isopropylacrylamide along with the strongly acidic monomer, 2-acrylamido-2-methyl-propanesulfonic acid (AMPSA) as the functional co-monomer. Although the authors showed that this composition produces selective hydrogels in model solutions, it would been interesting to see the behaviour of their material in a complex biological fluid. This would make it possible to assess whether the presence of negative charges, due to the presence of AMPSA, would lead to high levels of non-specific binding.

Other examples, cited in Table 3, involving the use of charged monomers, are those in which surface imprinted layers, thin films or self-assembled monolayers (SAMS) have been prepared. These can

generally be classed as 2-dimensional or 2-D MIPs. In these 2-D MIPs, non specific binding, which can result from the presence of charges generally distributed over the surface of porous polymer particles, is minimised by limiting the amount of available surface. Molecularly imprinted films specific for creatine kinase and ovalbumin were made by 'micro-contact imprinting' by the group of Chou[188,189] using methacrylic acid as monomer. These molecularly imprinted films were able to maintain their recognition capabilities even when tested in undiluted serum. Tai *et al*.[121,160,161] produced MIP films based on a mixture of acrylamide and acrylic acid for a variety of targets onto quartz crystal microbalance sensors. These examples have therefore shown that for sensor applications, where 2-D MIPs films are used for the recognition of target analytes, using acrylamide as the principal functional monomer, accompanied by a small amount of a strongly basic or acidic co-monomer, can produce better sensing materials. The implication is that combinatorial approaches and computational modelling can also be used in the design of polymer compositions for protein imprinting, as well as for small molecule targets, to help in the selection of the "best co-monomer" for a specific target protein (or peptide).

For completeness some of the more unusual systems reported for protein imprinted should be mentioned here. The first is the use of so-called "assistant recognition polymer chains" (ARPCs), used in the imprinting of BSA[210,211] and cloned pig cyclophilin 18.[214] These are poly(vinyl alcohol) chains modified, with polar functional groups and double bonds, which are allowed to interact with the target protein before being immobilised onto the surface of a macroporous adsorbent sphere through a polymerisation process. The use of a range of ARPC structures allows a selection of the best binding chains on the basis of competition before polymerisation. Another unusual approach is the photochemically-induced templating of proteins into a polymer bearing *cis-trans* photoisomerisable azobenzene side-chains.[215] Irradiation of the polymer in the presence of proteins,[215-217] viruses[218] or polymer beads[215] causes the polymer to deform as it undergoes repeated photoactivated conformational changes, resulting in immobilisation of proteins and/or the formation of photo-imprints. While scientifically interesting, the system is unlikely to be relevant to sensor construction as the polymers remain photo-active after template removal and would therefore be prone to "erasure"

of the imprints through stray light activation. The final system worth mentioning is the "antibody replicas" prepared by the group of Dickert.[219,220] In this case imprints of antibodies for a specific target were prepared in particles of poly(vinylpyrrolidone-*co*-methacrylic acid) crosslinked with *N*,*N*'-(1,2-dihydroxyethylene)-bisacrylamide. The antibody-imprinted particles were used as a stamp in the formation of a secondary imprint as a polymer layer on the surface of a sensor chip. Remarkably the plastic replica antibodies were claimed to show improved selectivity and sensitivity on quartz crystal microbalance sensors when compared to their natural counterparts (the original templates).[219] This approach has the advantage that potentially many copies of the antibody replicas can be prepared, however it suffers from the disadvantage that antibodies must first be prepared as the template species, which tends to negate many of the attractions of using imprinting in the first place, in particular dispensing with the need to use laboratory animals and/or expensive and time-consuming biological processing. Clearly this approach is relevant to sensor preparation, but at the moment is a curiosity.

3.2 Cross-linkers and cross-linking

There are two principal approaches to protein imprinting, either the use of a relatively high degree of cross-linking to make rigid or semi-rigid polymeric materials or to use lower amounts of cross-linking agent to prepare soft hydrogel materials that need to remain hydrated to retain memory for the template. A compromise situation can arise when grafting low cross-linked polymer to a solid support, such as a porous membrane, since this adds a local degree of order at the interface, maintaining the structure more efficiently than an unsupported soft gel.

For sensing purposes, soft hydrogels are unlikely to be practicable, since they require constant hydration and are readily damaged. Byrne and Salian recently reviewed the area of protein imprinting in hydrogel polymers.[221] The authors predict a rapid rise in the number of publication in imprinted

hydrogels, based on current trends, although there are clearly some problems associated with working with soft gel materials.

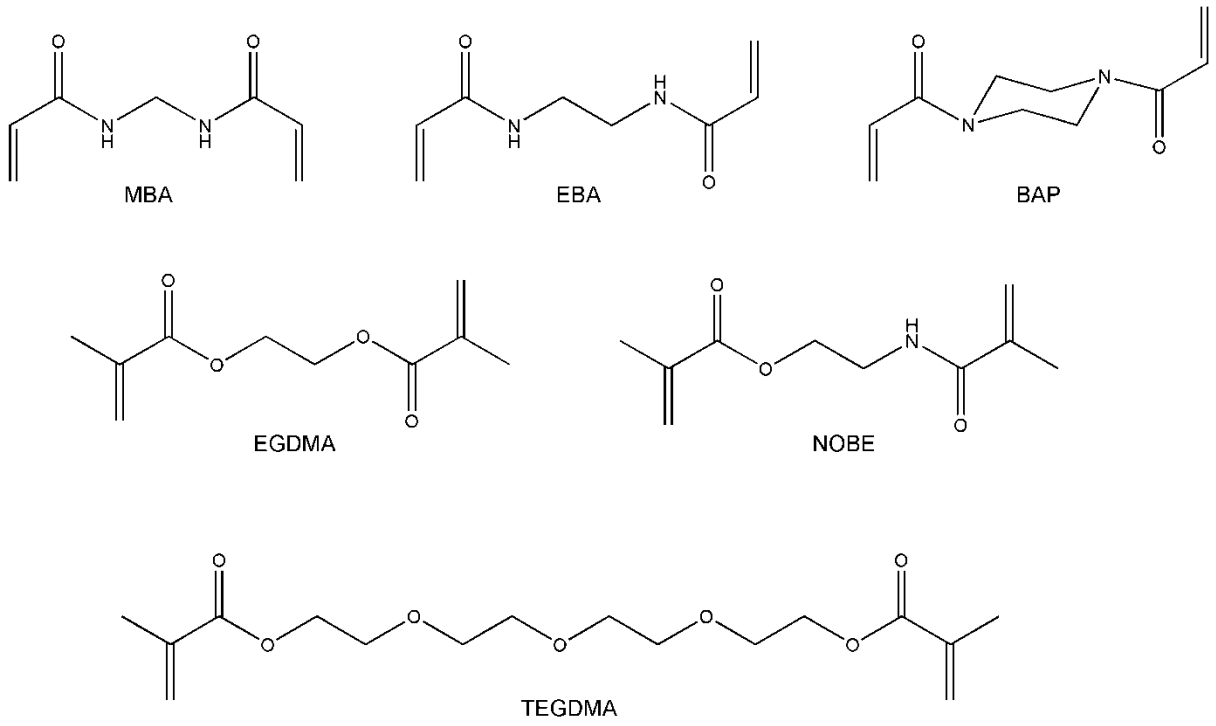

Figure 9. Structures of some of the cross-linkers mentioned in this section: MBA: methylene-*bis*-acrylamide; EBA: ethylene-*bis*-acrylamide; BAP: *bis*-acryloylpiperazine; EGDMA: ethyleneglycol dimethacrylate; NOBE: *N*,*O*,-*bis*-methacryloyl ethanolamine; TEGDMA: tetraethylene glycol dimethacrylate.

For aqueous-based polymerisation there are relatively few compounds available for use as cross-linker, the principal candidates are: methylene-*bis*-acrylamide (MBA), ethylene-*bis*-acrylamide (EBA) and *N*,*N*'-*bis*-acryloylpiperazine (BAP).

MBA is commonly used in the cross-linking of polyacrylamide gels for electrophoresis applications and has been used, for example in the "gel antibodies" of Hjertén.[103] The monomer performs well as an aqueous-compatible cross-linker but suffers from relatively poor solubility, preventing one from achieving relatively high levels of cross-linking when used alone. EBA is more soluble in water and BAP has an even higher solubility.

Burow and Minoura[101] used MBA in combination with *N*,*N*'-1,2-dihydroxyethylene-*bis*(acrylamide) in the imprinting of glucose oxidase on the surface of silica beads. EBA was used as the cross-linker in the work of Nishino[117] on epitope imprinting at a surface layer. The cross-linker was used at the level of 10%, and polyethylene glycol 200-diacrylate was also added at the level of 0.1 mol%. El Kirat and co-workers[154] compared the binding capacity and specificity of a number of cytochrome c imprinted polymers, prepared using a range of cross-linkers, including MBA, EBA and BAP. The optimum varied for each cross-linker studied, but was in the range of 3.3 to 6.6% in each case. The highest binding was seen in the case of 6.6% EBA, which also showed reasonably low non-specific binding.

Lin *et al*.[199] based the selection of cross-linker in their work on the microcontact imprinting of myoglobin by finding the cross-linker with the least affinity for their template. On this basis tetraethylene glycol dimethacrylate (TEGDMA) was chosen as cross-linker. Methyl methacrylate was chosen as functional monomer and the MIP showed excellent specificity and good selectivity for the template.

In organic solvents, the cross-linker of choice is usually ethyleneglycol dimethacrylate (EGDMA) although the use of hybrid cross-linkers[222] is an interesting option. In particular *N*,*O*-*bis*-methacryloyl ethanolamine (NOBE)[223] has proved useful in the preparation of OMNiMIPs (one monomer

molecularly imprinted polymers),[224] which have shown good performance in the enantioselective imprinting of amino acid derivatives and other chiral templates.[225] More recently this approach was shown to give specific recognition materials for peptides, indicating that NOBE may be useful for applications in epitope imprinting for binding larger protein targets.[226]

4 Polymer preparation

The choice of thermal or photochemical polymerisation conditions may be dictated by the polymer format. For example surface grafting, which is preferable for sensor applications, is often conveniently carried out by photochemically-activated graft polymerisation, using surface-bound initiators. While high temperatures can be reached inside bulk polymerisations,[227] which would be damaging to protein templates, this should not be the case with thin polymer films where the heat can be more efficiently dissipated. The polymerisation time and initiator concentration can be optimised by experiment, although low initiator concentrations and longer polymerisation times have been shown to be beneficial in the imprinting of low molar mass compounds.[228]

Once polymerisation is complete the template molecule (the protein or peptide) must be extracted with a suitable solvent in order to allow the specific recognition sites created by the imprinting process to be available for binding. The washing conditions used for template removal need to be carefully selected in order to preserve the integrity of the imprint sites, while removing as much template as possible. Mildly acidic and/or basic solutions, as well as various surfactants and enzymatic digestion methods have been employed for the removal of protein templates. Mildly basic conditions (a solution of NaOH/NaClO (0.5/1.0%) for 0.5-2 h) were used by Shi *et al*.[124] to dissolve and extract the protein templates from a disaccharide-based MIP layer. Successful rebinding of the template proteins suggested that indeed the mild washing solution succeeded in freeing specific binding sites without any damage. Janiak *et al*.[196] pointed out that surfactants, also widely used for template removal, in addition to washing out the template molecule, can be responsible for some of the binding properties attributed to imprinting. This also highlights the need for careful use of controls when interpreting binding to specific interactions with MIPs. Protein digestion approaches, using

enzymes such as pronase[113] or trypsin,[150] run the risk of leaving template fragments attached to the polymer. The use of enzymes, even those that are relatively small proteins, also means that some template will be inaccessible to the removal agent, especially if MIPs are in a "bulk" or 3-dimensional format. This should be less of an issue for very thin (2-D) MIP layers. Surface imprinting approaches, such as the microcontact printing method, can simplify template removal, since most is peeled away with the substrate used to present the templates to the polymerisation mixture.

Regeneration of the imprint sites after a binding event involves very similar issues to those concerned with template removal. If the MIPs are to be used in reusable sensors, an efficient regeneration step will be required. In many practical applications of sensors (e.g. for medical use) a disposable (one-shot) system would be preferable, both for operational and safety reasons. In this case how to regenerate the MIP would no longer be an issue.

A fundamental feature of a successful sensor is its selectivity: insufficient selectivity can lead to false negatives. In the case of MIP-based sensors the imprinting approach must be selected so as to maximise selectivity. In protein recognition, selectivity impinges on the ability of the MIP sensor to recognise only the target protein or a protein family. In practical terms, this means that the affinity of the polymer for the target must be considerably greater than for any non-target protein. Despite the numerous examples reported in the literature, in our opinion, it will be highly unlikely that sufficient selectivity would be achieved using a whole protein approach. Probably the best way to ensure a high degree of selectivity is to prepare MIPs for use in biosensors using the epitope approach,[57,114-116] as described above. In this case maximum selectivity in binding can be achieved through selection of a peptide epitope of sufficient length such that it is as close as possible to being a unique identifier of the target protein as possible, within the bounds of probability; but not so long that binding will be sterically hindered or require considerable reorganisation of the protein structure.

Whereas high selectivity avoids false negatives, a high specificity (or low non-specific binding to the non-imprinted regions of the transducer coating) will avoid false positives. The specificity will therefore depend on the exposed surface area of the polymer, its functionality and surface charge,

among other factors. As it has been already explained above, it is suggested that the avoidance of charged monomers (and initiator) might help in reducing non-specific interactions. This also applies to the use of charged surfactants for the removal of template. A dense array of epitope-imprinted sites, as described by Nishino *et al*.[117] is also to be recommended.

4.1. Polymer format for sensing applications

The format of the polymer used in protein imprinting is usually dictated by the method of polymerisation and how the template is presented (surface or interfacial imprinting approaches) or the application (surface-grafted films on transducer surfaces or beads for chromatographic separations).

Several examples of surface-grafted protein imprinting films are reported in the literature, where either the protein or the peptide, representing part of the protein (epitope approach), are mixed with monomers and in some cases also with a cross-linker (e.g. *N*,*N*-methylene-*bis*-acrylamide) and polymerised *in situ* to form grafted films on transducer surfaces or on supporting particles. Different type of monomers have been used for this type of surface-grafted sensor and among the most common are: thiol derivatives;[164,165] silane derivatives;[53,126,182,184,185] boronic acid;[157,166,167,170] and acrylics such as acrylamide or *N*-isopropylacrylamide.[121,154-156,160,162,180,203]

Surface-constrained templates, as required by contact imprinting[127,189,199] and surface confined epitope approaches[117] are probably the most readily adapted to incorporation with transducer surfaces. In these cases a planar thin film results from a casting process, polymer-forming components being confined as a thin film between surfaces, one treated to bind to the polymer (corresponding to the transducer surface) and the other, bearing the template structure, allowing easy release.

Bulk materials are the least suitable for sensor development, since ground and irregular particles are difficult to immobilise on transducers, resulting in an uneven distribution of imprinted sites over the transducer surface and poor connectivity between polymer binding and the transduction mechanism. A compromise between “bulk imprinting” and “surface imprinting” can come from the immobilisation of spherical particles, which can be surface-imprinted, onto the transducer surface. In particular, nanoparticles would allow for more efficient receptor immobilisation and they can be

processed as if they were the plastic equivalent of antibodies. The analogy with antibodies can even extend to the use of affinity-based methods for the selection of fractions with the highest binding affinity and gel permeation chromatography to select particle fractions with a selected size distribution.[37] There are also methods for the preparation of surface-confined whole protein binding sites on microparticle beads, used as supports.[126,134] An alternative approach is to imprint the protein at the interface in mini-emulsion polymerisation.[204,205]

Other particle-based approaches include the deposition of thin shell layers over core particles, which ensure that the imprint sites (which resemble bulk imprints) remain within close proximity to the surface.[155,166] Particle-based approaches to protein imprinting have been reviewed by Tan and Tong.[229] The incorporation of particles of larger sizes with transducer surfaces will still involve some of the difficulties inherent with bulk polymers, nanoparticles, particularly soluble nanogels, however, could be treated as if they were biological molecules to some extent, for which many methods of transducer integration are known.

5 Signal transduction

When a protein template binds to its imprinted polymer the resulting physical and chemical changes can be exploited to measure the event, i.e. signal transduction. The transducer can either be an external component added to the system to monitor binding, for example an electrochemical tag, or can be integrated as part of the polymer matrix. Integrated transducer systems allow reagentless sensing. Reagentless sensing is a current driver in sensing technology due to the decreased time to result, reduced assay complexity and fewer steps associated with generating a signal. Here we describe sensor techniques using either polymers prepared on planar surfaces, as shells over nanoparticle cores, or synthesised as nanoparticles. Such preparations are favourable for use in sensing as the binding element can be efficiently coupled to the transducing element, and there is an

adequate number of binding sites exposed on the polymer surface, resulting in improved sensitivity and response time.

A significant advantage of MIPs over biologically-derived affinity agents are their long-term stability, relative insensitivity to harsh reagents and the ability to measure multiple "repeated" binding reactions. The continuous detection of analytes over prolonged periods and in a reagentless system is an obvious application for MIPs and has been exploited to detect the pesticide carbaryl in water (≥ 200 binding cycles with good assay stability for at least 4 months) using the native fluorescence of the analyte.[230] We will focus this discussion on signal transduction mechanisms that can be applied to reagentless sensing, however where sensitivity is a major issue an external transducer, or competition assay (e.g. for ribonuclease A[231]), can be used to amplify the signal, improving the limit of detection.

Various reviews of MIP signal transduction mechanisms have been published,[232] however many of these methods are for small molecules and depend on specific chemical functionalities for signal transduction that are not applicable to general protein analysis. These transduction techniques include; electrochemical, fluorescent quenching, IR and Raman spectroscopy. Unless the protein template contains an electro-active moiety, metal complex, or is intrinsically fluorescent (aromatic fluorescence is not suitable due to strong background and low quantum efficiency), reagentless protein detection transduction systems would appear to be limited. These include optical, or acoustic measurement of changes in the polymer thickness, refractive index, bulk properties, or swelling upon binding; or electrical changes at, or within the polymer upon binding, that can result in: shielding, or re-organisation of the polymers within the MIP layer, a change in the dielectric constant, or in charge distribution at the electrode.

5.1 Signal transduction using polymers

Polymers can be either selected, or designed to incorporate the transducing element of the sensor, thereby limiting the need for additional sensor components. For many of the electrical and electrochemical techniques described, the use of conducting polymers[233] within the MIP polymerization reaction is required. The intrinsic conduction of a polymer is apparent upon oxidation,

or reduction ‘doping’ of the conjugated backbone. Doping can also change the optical properties of the conducting polymer in the UV-vis and NIR regions leading to new applications for optical sensing. Such conducting polymers are also influenced by protonation/deprotonation and conformational changes in the receptor/sensor layer that alter ion diffusion and therefore conducting properties.

Libraries of polymers used for selection and modelling will therefore be biased to include a proportion of conducting polymers for electrical/electrochemical sensors. Conducting polymers that have previously been used in the development of imprinted receptors include: polypyrrole, polyaniline and benzophenone. The application, use and incorporation of conducting polymers in electrochemical sensors has been reviewed.[234] Energy transfer associated with conducting polymers can also be used as the basis for fluorescence-based sensors. Fluorescent mechanisms that have been used in MIP-based sensors include; photo-induced electronic transfer (PET), replacement of coordinating ligands, changes in rigidity, complexation and alteration of quenching efficiency. Unless significant changes in fluorescent quenching are observed, the background signal from unbound conducting polymers will reduce the sensitivity of the assay, potentially limiting its use for protein analysis.

5.2 Electrical and electrochemical sensors

Electrochemical techniques relevant to sensing include; voltammetry and amperometry, potentiometry and impedometry transduction methods. These techniques have been used in the development of MIP-based sensors, with the majority of examples involving the detection of small molecule electro-active analytes,[235,236] as opposed to proteins. However notable examples include: an immunosensor-based detection of prostate-specific antigen (PSA) through oxidation of Trp and Tyr residues;[237] a MIP sensor for chiral amino acid recognition that could be adapted for proteins[201] and a potentiometric sensor for selective detection of either myoglobin or haemoglobin in complex protein mixtures.[164] The potentiometric sensor displayed protein detection in the low μg/ml range and specificity when

compared to proteins of similar size and charge. Binding of charged protein to an insulating layer of MIP over an electrode alters the surface potential, allowing binding to be measured potentiometrically.[164] The sensor response depends on the isoelectric point of the protein and the pH of the sample matrix, however this dependency may be exploited to enhance specificity.

Capacitance/Impedance sensors can detect analyte binding to imprinted polymers through changes in the thickness, and/or dielectric constant of the insulating layer.[238,239] Polymers used for imprinting in capacitive sensors include those prepared from; phenol, MBA, AMPSA and *o*-phenylenediamine). There are limited examples of MIP-based capacitance sensors for protein analytes, however an immunosensor for detection of cancer antigen 125 was shown to display good sensitivity, dynamic range and the ability to perform repeated measurement using diluted serum samples, when compared with SPR and conventional ELISA.[240] Additional small molecule sensor data and macromolecule studies suggest capacitance can be applied for sensitive protein detection.

Impedance-based spectroscopy has been used to detect a virus binding to MIPs,[241] and therefore could be employed to detect specific proteins associated with organisms, or larger complexes. An interdigitated electrode-based capacitor was used to monitor changes in the dielectric properties of the MIPs (co-polymer of methacrylic acid and *N*-vinyl pyrrolidone) occurring upon binding the virus. The resulting reusable sensor facilitates continuous, rapid monitoring of complex biological samples, albeit with lower detection limits when compared with ELISA.

Capacitance-based sensors can be prepared inexpensively in bulk using, for example, field-effect transistor (FET) technology. Multi-analyte detection can be facilitated by the use of interdigitated electrode-based capacitors, making this a flexible technique for protein detection. However other techniques, notably optical and electrochemical sensing, offer better sensitivity and specificity as various analytes can interfere with the polymer layer.

5.3 Optical and Acoustic Sensors

Optical and acoustic sensors that essentially measure changes in the mass of the sensing layer, or changes in the refractive index at a surface are both suited to protein analysis. The majority of published articles involving the detection of protein- or cell-based analytes use the quartz crystal microbalance (QCM), (acoustic) or surface plasmon resonance (SPR), (optical) sensor platforms, due to the sensitivity achievable with these techniques compared to other methods. An additional bonus arising from using these planar transducers is the relative ease of preparing MIPs on their surfaces. Quartz crystal microbalances use a thin disc of quartz, cut at a specific crystal plane, sandwiched between a pair of (usually gold) electrodes as the transducer. The piezoelectric crystal oscillates at MHz frequencies when an electric field is applied to the crystal. Materials that are acoustically coupled to the sensor surface, i.e. due to binding rather than proximity, are detected through changes in the oscillation frequency. This offers an advantage over other sensors that measure bulk changes in refractive index. In QCM the change in resonance frequency is proportional to the mass of molecules bound to the surface. Ligand binding events that alter the conformation or the rigidity of the imprinted polymer layer can significantly enhance the resulting signal and can be analysed independently as shear modulus, viscosity or density changes. The use of piezoelectric sensors to detect low-molecular weight analytes, employing MIPs immobilised to the gold electrodes, has been reviewed.[242] A MIP-based QCM sensor has been developed for the detection of anthrax protective antigen.[160] The sensor displayed excellent sensitivity, in the picomolar range, however limited data about specificity and the influence of potential interferences was provided, therefore the long term applications of this sensor format cannot be assessed. Other examples of protein analytes detected using QCM include native trypsin (reported detection limit 100 ng/ml),[243] micro albumin in clinical samples[244] and β-lactoglobulin.[172] QCM provides a flexible technique to measure protein analytes through changes to the mass and physicochemical properties of the polymer-sensing surface. The need for acoustic coupling to the sensor surface and the relative mass measurement makes this technique less prone to interferences. QCM instruments can be adapted for continuous sensing, for prolonged periods as highlighted by the on-line detection of pesticides.[245]

The SPR phenomenon requires that the recognition element be coupled to a metallic surface (typically silver or gold), or to nanoparticle "island" substrates. The use of SPR in biosensing has been reviewed.[246] In brief, SPR is sensitive to changes in the refractive index of the sensor layer (to a distance of around 1000 nm), as a result of analyte binding gives rise to distinct detection modes; including changes in intensity and a shift in the SPR angle. Examples of protein analytes detected using SPR sensors include lysozyme[203] and Hepatitis B surface antibody (HBsAb).[194] HbsAb is a biomarker for asymptomatic HBV infection. It was shown that HbsAb can be detected using a 2-hydroxyethyl methacrylate, *N*-methacryloyl-L-tyrosine methyl ester-based MIP in diluted serum from patients. A significant issue associated with long-term measurements with MIPs in biological matrices, is fouling by non-specific protein adsorption, however the authors report that the SPR sensor has a significant resistance to this biofouling. Albeit for a limited number of samples, a good relationship was observed between the MIP-based SPR assay and a commercially available immunoassay.[194] SPR technology has previously required expensive optical equipment not suited for sensing purposes. However recent advances in the availability of low-cost sensing devices have made SPR more portable and economically viable for general sensoring requirements.

Changes in the flexibility and swelling of polymers associated with ligand binding can be exploited to measure proteins, albeit with low sensitivity. An example is the detection of BSA, at the level of 1 mg/ml, through changes in the Bragg shift of imprinted polymer-based photonic crystals.[198,247,248] Various optical formats exist to measure ligand-induced changes in swollen polymers and hydrogels, however these methods are very sensitive to changes in buffer composition, e.g. pH and ionic strength, limiting the potential use of such technology in protein sensing.

5.4 Nanotechnology

The use of nanoparticles, either prepared on a substrate or in solution, can be applied to MIP-based biosensors through adaptation of the transduction routes described above, or through novel mechanisms. The techniques and examples given are mainly based on the detection of small

molecules, or immunosensor technology, both of which could be applied to the sensing of proteins or peptides using MIP technology. Nanoparticles offer many advantages in MIP-based sensing including: improved signal sensitivity, increased sensor surface area (making removal of the template easier) and novel signal transduction mechanisms.[249,250] Gold nanoparticle aggregation is routinely used in lateral flow devices, which highlights that such technology can be readily adapted to inexpensive and disposable platforms for diagnostic applications.

Compared to planar surfaces, nanostructures can be engineered with high surface to volume ratios, increasing the number and ratio of imprinted sites that are accessible for binding, and therefore binding capacity of the MIP. The grafting of MIPs to the surface of carbon nanotubes (CNTs),[251,252] to enhance the surface area of electrochemical[253,254] or electrical transducers has been described. An example is the CNT nanoarray, coated with a caffeine-imprinted polypyrrole layer, for which a 15-fold increase in sensitivity was reported, compared to similarly imprinted planar surfaces. [251] An electrochemical sensor, using TNT-imprinted Au nanoparticles has been used for the sensitive detection of the template, with a reported limit of detection of 46 ppt, (~200 pM).[255] Silver nanoparticles have been used to enhance electron transfer in a MIP-based amperometric sensor for the insecticide, dimethoate.[256] Improvements in the effective receptor surface area could be considered more significant for the sensing of large molecules, such as proteins, where mass transfer to the surface and limited diffusion into deep polymer layer play an important role in binding kinetics.

Nanoparticles can be used to increase the effective surface area of a sensor surface. Examples include the use of gold colloids, directly immobilised to a polymer surface, in an immuno-capacitance sensor[257] and an electric tunnelling assay,[258] both for the detection of cholera toxin. The excellent sensitivity obtained in these assays and the ability to measure in diluted samples suggests these sensors could be adapted for MIP-based sensing.

MIP nanoparticles can either be prepared directly, for example by microemulsion polymerisation or by depositing a shell of polymer over a pre-formed nanoparticle, such as for example quantum dots (QDs), or gold colloids. Many of the advantages of using either core-shell nanoparticles, or

nanostructured surfaces with MIP's will only be apparent if the polymer layer can be prepared sufficiently thin and with a regular thickness. A QCM sensor for the detection of the binding of imprinted polymer nanoparticles to the peptide melittin, immobilised on the QCM chip has been reported.[38] In this format the MIP-nanoparticle behaved similarly to antibodies using in immunoassays, showing the validity of this approach for sensing applications.

Nanoparticles bearing surface-confined peptide imprints prepared by inverse microemulsion polymerisation were similarly shown to bind to peptide targets immobilised on QCM chips.[259] The nanoparticles were prepared with a hydrophobic "tail" in order to confine them at the water-in-oil interface and engineer the imprint sites to accept the target peptide in only one orientation. The ability to selectively bind from only one terminus of the peptide sequence facilitates protein detection by providing more uniform imprints which can target a specific protein chain terminus. The resulting MIP nanoparticles bound the target peptide, melittin, with dissociation constants in the range 90-900 nM. The nanoparticles were prepared from acrylamide and ethylene-*bis*-acrylamide as functional monomer and cross-linker respectively. It would be possible to hydrophobically modify either end of a peptide in order to select binding of either the exposed N- or C-terminus of the target protein. It would be interesting to see whether peptides, hydrophobically modified at both ends, imprinted in the same manner, would allow intra-protein sequences to be targeted, for example surface exposed loops. Other methods for the preparation of imprinted nanoparticles for sensor applications have been described: polymer[260] and superparamagnetic particles,[261,262] Quantum dots[43,176,263] and nanowires.[186,264,265] The MIP nanoparticles described could be used in various assay formats and devices, for example as detection (fluorescent, electrochemical), or capture/separation agents (magnetic) in a sandwich complex, or in combination with other binding agents.

Localized surface plasmon resonance (LSPR) can be obtained from metals, alloys or semiconductors, however most examples reported have involved either gold or silver. LSPR nanoparticles display scattering spectra whose peak wavelength depends on the particle composition, size, shape, orientation and local dielectric environment.[266] The latter property can be exploited for biosensing applications. This has been demonstrated for the binding of conconavalin A to mannose-

functionalized nanoparticles.[267] LSPR has been demonstrated for TNT imprinted gold nanoparticles, cross-linked and electropolymerized on a gold surface.[255] A detection limit of 10 fM was achieved, associated with changes in the dielectric properties of the *bis*-aniline polymer upon the formation of a π-donor-acceptor complex with the analyte. Nanoparticle islands of gold on a glass substrate were employed for enhanced SPR, facilitated by plasmon coupling to gold nanoparticles embedded in a cholesterol-imprinted polymer film.[268] Re-binding of cholesterol led to a shift in the SPR angle. Interestingly the binding of structurally similar compounds led to smaller shifts, indicating an enhanced discrimination mode. A similar polymer swelling concept, using surface-enhanced Raman scattering (SERS) has been reported for adrenaline.[269] Assays of this type can be limited by slow protein diffusion and interferences that alter polymer flexibility/swelling. SERS from Au nanoparticles is a sensitive and specific sensor technique for the determination of compounds with unique Raman spectra, allowing for their detection within complex mixtures.[270,271] However the enhancement of protein, peptide, or specific amino acids is not as efficient and therefore has not been significantly exploited in sensor development.

QDs embedded into polymer films have been reported to detect small molecules such as uracil, caffeine[272,273] and guanosine.[274] Spectrally distinct QDs with a polymer shell imprinted with creatine, lysozyme and albumin were used for sensing in urine.[176] The QDs were mixed with the template and poly(ethylene-co-vinyl alcohol) at different ratios to prepare the mixed QD/MIP nanoparticles. Specific binding of the template resulted in quenching of phosphorescence. The unique spectrum of each QD allowed for multiplexed analysis. However significant cross-reactivity for creatine with the lysozyme and albumin sites was observed. The authors presented data on rebinding, long-term stability and a limited comparison with a commercial clinical analyzer. The reported limits of detection for albumin and lysozyme, ~ 900 and 200 ng/ml respectively, compares favourably with other techniques, however the long-term stability of QDs and the limited fluorescent dynamic range of the assay could limit the use of such sensors.

A MIP-based sensor has been reported that detected dipicolinic acid (DPA), a marker for bacterial endospores, through analyte-induced fluorescent quenching of gold-silver nanoclusters.[275] This sensor should be more photostable than conventional fluorescent approaches, however the quenching mechanism may be specific to DPA, or other small acidic compounds and therefore not applicable to protein-based sensing.

Dynamic light scattering[276] and electrophoresis[107-109] can be used to detect changes in the physical properties of nanoparticles (net charge and size) upon protein binding to an adsorbed receptor. Polyacrylamide microparticles imprinted with human haemoglobin were used to specifically detect the template using free-zone electrophoresis in a revolving capillary.[109]

Optical and acoustic techniques still dominate the literature for protein detection using MIPs, based on the flexible nature of the techniques. However as advances in MIP development in relation to specificity, re-binding and fouling of the polymer surface improve for protein analytes, it is expected that more robust/inexpensive techniques, for example conductance, will be required for remote/continuous sensing, thereby realising the stability advantages that MIPs confer. We predict that initially nanoparticles will have most impact in improving the sensitivity of current transduction techniques through enhancements in effective surface area and electron transfer efficiency.

6 Conclusions and recommendations

The methods and techniques used in molecular imprinting cover a very broad canvas. This is especially noticeable when considering methods of imprinting proteins, largely due to the additional constraints involved in working with these challenging templates. In our opinion, the most successful strategy for imprinting protein targets involves the use of small to medium peptides as the template, representing an epitope of the target protein. So far the epitopes chosen have been linear peptides, identical in sequence to one of the terminal peptide chains of the target protein; this however is a fairly narrow definition of epitope in terms of the regions of proteins recognised by antibodies. In this

case more diverse regions of the protein can be the target for antibody binding, whether strategies for designing epitope templates, that can be used to imprint recognition properties for surface regions or loops of the target, can ever be successfully devised remains to be seen. The problems of entrapment and covalent immobilisation of whole proteins suggests that only strategies aimed at surface imprinting, at interfaces or by stamping/printing approaches, stand any chance of competing with those based on epitope imprinting. As far as polymer systems are involved, soft hydrogels are probably not recommended over more rigid systems for use in sensor applications. Nano-structured materials and thin films are important formats for sensing applications, in fact MIP nanoparticles can be considered as direct replacements for antibodies, and this is expected to be a growing trend since they have been demonstrated to act as such *in vivo*, neutralising the toxic effect of a component of bee venom in mice.[277]

Selection of monomer is important, and computational and combinatorial methods can help. Clearly there are cases where a small amount of ionisable or ionised monomer aids in selectivity and specificity of recognition, however too much has a detrimental effect. The majority of functional monomer should however be neutral, with acrylamide being the clear favourite, imparting recognition properties for a wide range of peptide and protein templates. For the sensing platform, a reagentless system would be preferable, although a sensing system for proteins will, by necessity, involve some form of liquid handling. In transduction, SPR and QCM-based sensors and their allied techniques are attractive, as a number of instruments are readily available for use with easily modified sensor chips. For electroactive proteins, electrochemical detection would be possible. Such sensors can be sensitive and compact, however very few proteins are likely to be redox active in the manner required for detection. Other electrical properties, e.g. capacitance or impedance, can be used and may provide the required sensitivity.

Acknowledgements

We wish to thank the Home Office, OSCT (UK) for financial support and guidance during the preparation of this manuscript.

References

1. S. A. Soper, K. Brown, A. Ellington, B. Frazier, G. Garcia-Manero, V. Gau, S. I. Gutman, D. F. Hayes, B. Korte, J. L. Landers, D. Larson, F. Ligler, A. Majumdar, M. Mascini, D. Nolte, Z. Rosenzweig, J. Wang, and D. Wilson, *Biosens. Bioelectron.*, 2006, **21**, 1932.
2. A. E. G. Cass, G. Davis, G. D. Francis, H. A. O. Hill, W. J. Aston, I. J. Higgins, E. V. Plotkin, L. D. L. Scott, and A. P. F. Turner, *Anal. Chem.*, 1984, **56**, 667.
3. R. S. Yalow and S. A. Berson, *Nature*, 1959, **184**, 1648.
4. S. Thobhani, S. Attree, R. Boyd, N. Kumarswami, J. Noble, M. Szymanski, and R. A. Porter, *J. Immunol. Methods*, 2010, **356**, 60.
5. D. Kastelic, S. Frkovic-Grazio, D. Baty, G. Truan, R. Komel, and D. Pompon, *J. Immunol. Methods*, 2009, **350**, 54.
6. J. L. Liu, G. P. Anderson, J. B. Delehanty, R. Baumann, A. Hayhurst, and E. R. Goldman, *Mol. Immunol.*, 2007, **44**, 1775.
7. S. Subrahmanyam, S. A. Piletsky, and A. P. F. Turner, *Anal. Chem.*, 2002, **74**, 3942.
8. C. A. K. Borrebaeck and C. Wingren, *J. Protem.*, 2009, **72**, 928.
9. I. E. Tothill, *Semin. Cell Dev. Biol.*, 2009, **20**, 55.
10. A. P. F. Turner, B. N. Chen, and S. A. Piletsky, *Clin. Chem.*, 1999, **45**, 1596.
11. A. G. Mayes and M. J. Whitcombe, *Adv. Drug Deliv. Rev.*, 2005, **57**, 1742.
12. C. Alexander, H. S. Andersson, L. I. Andersson, R. J. Ansell, N. Kirsch, I. A. Nicholls, J. O'Mahony, and M. J. Whitcombe, *J. Mol. Recogn.*, 2006, **19**, 106.
13. I. A. Nicholls and H. S. Andersson, in *Molecularly Imprinted Polymers: Man-Made Mimics of Antibodies and their Applications in Analytical Chemistry*, ed. B. Sellergren, Elsevier, Amsterdam, 1st edn., 2001, vol. 23, ch. 3, pp. 60-70.
14. E. V. Piletska, A. R. Guerreiro, M. J. Whitcombe, and S. A. Piletsky, *Macromolecules*, 2009, **42**, 4921.
15. T. A. Sergeyeva, L. A. Gorbach, O. A. Slinchenko, L. A. Goncharova, O. V. Piletska, O. O. Brovko, L. M. Sergeeva, and G. V. Elska, *Mater. Sci. Eng. :C*, 2010, **30**, 431.
16. J. Haginaka, *J. Chromatogr B*, 2008, **866**, 3.

17. S. A. Piletsky, E. V. Piletskaya, T. L. Panasyuk, A. V. Elskaya, R. Levi, I. Karube, and G. Wulff, *Macromolecules*, 1998, **31**, 2137.
18. A. L. Jenkins, O. M. Uy, and G. M. Murray, *Anal. Chem.*, 1999, **71**, 373.
19. F. G. Tamayo, E. Turiel, and A. Martín-Esteban, *J. Chromatogr. A*, 2007, **1152**, 32.
20. S. A. Piletsky and A. P. F. Turner, *Electroanalysis*, 2002, **14**, 317.
21. A. R. Koohpaei, S. J. Shahtaheri, M. R. Ganjali, A. R. Forushani, and F. Golbabaei, *Talanta*, 2008, **75**, 978.
22. I. A. Nicholls, H. S. Andersson, C. Charlton, H. Henschel, B. C. G. Karlsson, J. G. Karlsson, J. O'Mahony, A. M. Rosengren, K. J. Rosengren, and S. Wikman, *Biosens. Bioelectron.*, 2009, **25**, 543.
23. F. Lanza and B. Sellergren, *Anal. Chem.*, 1999, **71**, 2092.
24. T. Takeuchi, D. Fukuma, and J. Matsui, *Anal. Chem.*, 1999, **71**, 285.
25. M. Valtchev, B. S. Palm, M. Schiller, and U. Steinfeld, *J. Haz. Mater.*, 2009, **170**, 722.
26. I. Chianella, M. Lotierzo, S. A. Piletsky, I. E. Tothill, B. N. Chen, K. Karim, and A. P. F. Turner, *Anal. Chem.*, 2002, **74**, 1288.
27. C. Herdes and L. Sarkisov, *Langmuir*, 2009, **25**, 5352.
28. O. Hayden, R. Bindeus, C. Haderspöck, K. J. Mann, B. Wirl, and F. L. Dickert, *Sens. Actuators B*, 2003, **91**, 316.
29. A. Bossi, F. Bonini, A. P. F. Turner, and S. A. Piletsky, *Biosens. Bioelectron.*, 2007, **22**, 1131.
30. N. M. Bergmann and N. A. Peppas, *Ind. Eng. Chem. Res.*, 2008, **47**, 9099.
31. S. Piletsky, E. Piletska, K. Karim, G. Foster, C. Legge, and A. Turner, *Anal. Chim. Acta*, 2004, **504**, 123.
32. O. Y. F. Henry, S. A. Piletsky, and D. C. Cullen, *Biosens. Bioelectron.*, 2008, **23**, 1769.
33. S. A. Piletsky, E. V. Piletska, B. N. Chen, K. Karim, D. Weston, G. Barrett, P. Lowe, and A. P. F. Turner, *Anal. Chem.*, 2000, **72**, 4381.
34. M. M. Titirici and B. Sellergren, *Chem. Mater.*, 2006, **18**, 1773.
35. Z. L. Cheng, E. K. Wang, and X. R. Yang, *Biosens. Bioelectron.*, 2001, **16**, 179.
36. V. Chegel, M. J. Whitcombe, N. W. Turner, and S. A. Piletsky, *Biosens. Bioelectron.*, 2009, **24**, 1270.
37. A. R. Guerreiro, I. Chianella, E. Piletska, M. J. Whitcombe, and S. A. Piletsky, *Biosens. Bioelectron.*, 2009, **24**, 2740.

38. Y. Hoshino, T. Kodama, Y. Okahata, and K. J. Shea, *J. Am. Chem. Soc.*, 2008, **130**, 15242.
39. D. Roeseling, T. Tuercke, H. Krause, and S. Loebbecke, *Org. Process Res. Dev.*, 2009, **13**, 1007.
40. M. Ávila, M. Zougagh, A. Ríos, and A. Escarpa, *TrAC, Trends Anal. Chem.*, 2008, **27**, 54.
41. A. Aghaei, M. R. Milani Hosseini, and M. Najafi, *Electrochim. Acta*, 2010, **55**, 1503.
42. D. L. Rathbone, D. Q. Su, Y. F. Wang, and D. C. Billington, *Tet. Lett.*, 2000, **41**, 123.
43. H. F. Wang, Y. He, T. R. Ji, and X. P. Yan, *Anal. Chem.*, 2009, **81**, 1615.
44. P. Turkewitsch, B. Wandelt, G. D. Darling, and W. S. Powell, *Anal. Chem.*, 1998, **70**, 2025.
45. D. Lakshmi, A. Bossi, M. J. Whitcombe, I. Chianella, S. A. Fowler, S. Subrahmanyam, E. V. Piletska, and S. A. Piletsky, *Anal. Chem.*, 2009, **81**, 3576.
46. J. Q. Liu and G. Wulff, *J. Am. Chem. Soc.*, 2008, **130**, 8044.
47. N. Kirsch, J. Hedin-Dahlström, H. Henschel, M. J. Whitcombe, S. Wikman, and I. A. Nicholls, *J. Mol. Catal. B*, 2009, **58**, 110.
48. A. Pietrzyk, S. Suriyanarayanan, W. Kutner, R. Chitta, and F. D'Souza, *Anal. Chem.*, 2009, **81**, 2633.
49. M. Bompart, L. A. Gheber, Y. De Wilde, and K. Haupt, *Biosens. Bioelectron.*, 2009, **25**, 568.
50. J. L. Urraca, M. C. Moreno-Bondi, G. Orellana, B. Sellergren, and A. J. Hall, *Anal. Chem.*, 2007, **79**, 4915.
51. A. L. Medina-Castillo, G. Mistlberger, J. F. Fernandez-Sanchez, A. Segura-Carretero, I. Klimant, and A. Fernandez-Gutierrez, *Macromolecules*, 2010, **43**, 55.
52. M. Frasconi, R. Tel-Vered, M. Riskin, and I. Willner, *Anal. Chem.*, 2010, **82**, 2512.
53. X. W. Kan, Q. Zhao, D. L. Shao, Z. R. Geng, Z. L. Wang, and J. J. Zhu, *J. Phys. Chem. B*, 2010, **114**, 3999.
54. K. Landsteiner and J. van der Scheer, *J. Exp. Med.*, 1936, **63**, 325.
55. L. M. Amzel, R. J. Poljak, F. Saul, J. M. Varga, and F. F. Richards, *Proc. Natl. Acad. Sci. USA*, 1974, **71**, 1427.
56. A. G. Amit, R. A. Mariuzza, S. E. V. Phillips, and R. J. Poljak, *Science*, 1986, **233**, 747.
57. A. Rachkov and N. Minoura, *Biochim. Biophys. Acta*, 2001, **1544**, 255.
58. M. Reichlin, *J. Mol. Biol.*, 1972, **64**, 485.

59. E. M. Prager and A. C. Wilson, *J. Biol. Chem.*, 1971, **246**, 5978.
60. E. Westhof, D. Altschuh, D. Moras, A. C. Bloomer, A. Mondragon, A. Klug, and M. H. V. Vanregenmortel, *Nature*, 1984, **311**, 123.
61. J. A. Tainer, E. D. Getzoff, H. Alexander, R. A. Houghten, A. J. Olson, R. A. Lerner, and W. A. Hendrickson, *Nature*, 1984, **312**, 127.
62. D. C. Benjamin, J. A. Berzofsky, I. J. East, F. R. N. Gurd, C. Hannum, S. J. Leach, E. Margoliash, J. G. Michael, A. Miller, E. M. Prager, M. Reichlin, E. E. Sercarz, S. J. Smithgill, P. E. Todd, and A. C. Wilson, *Ann. Rev. Immunol.*, 1984, **2**, 67.
63. J. M. Rini, U. Schulze-Gahmen, and I. A. Wilson, *Science*, 1992, **255**, 959.
64. M. E. Byrne, K. Park, and N. A. Peppas, *Adv. Drug Deliv. Rev.*, 2002, **54**, 149.
65. N. M. Bergmann and N. A. Peppas, *Prog. Polym. Sci.*, 2008, **33**, 271.
66. Teva Pharmaceuticals Ltd. Copaxone: Summary of Product Characteristics. 2004; Last updated April 2009. Available from the electronic Medicines Compendium, http://www.medicines.org.uk, last accessed 23/6/2010
67. M. Sela and R. Arnon, *Biochim. Biophys. Acta*, 1960, **40**, 382.
68. D. Givol, S. Fuchs, and M. Sela, *Biochim. Biophys. Acta*, 1962, **63**, 222.
69. H. Langbeheim, R. Arnon, and M. Sela, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 4636.
70. R. A. Lerner, N. Green, H. Alexander, F. T. Liu, J. G. Sutcliffe, and T. M. Shinnick, *Proc. Natl. Acad. Sci. USA-Biol. Sci.*, 1981, **78**, 3403.
71. J. Kyte and R. F. Doolittle, *J. Mol. Biol.*, 1982, **157**, 105.
72. E. Pfaff, M. Mussgay, H. O. Bohm, G. E. Schulz, and H. Schaller, *EMBO J.*, 1982, **1**, 869.
73. H. M. Berman, P. E. Bourne, and J. Westbrook, *Curr. Proteomics*, 2004, **1**, 49.
74. A. G. Murzin, S. E. Brenner, T. Hubbard, and C. Chothia, *J. Mol. Biol.*, 1995, **247**, 536.
75. C. A. Orengo, A. D. Michie, S. Jones, D. T. Jones, M. B. Swindells, and J. M. Thornton, *Structure*, 1997, **5**, 1093.
76. J. Y. Shi, T. L. Blundell, and K. Mizuguchi, *J. Mol. Biol.*, 2001, **310**, 243.
77. K. Mizuguchi, C. M. Deane, T. L. Blundell, and J. P. Overington, *Protein Sci.*, 1998, **7**, 2469.
78. K. Arnold, L. Bordoli, J. Kopp, and T. Schwede, *Bioinformatics*, 2006, **22**, 195.
79. B. Rost, *Methods Enzymol.*, 1996, **266**, 525.
80. B. Rost and J. F. Liu, *Nucleic Acids Res.*, 2003, **31**, 3300.
81. B. Rost, P. Fariselli, and R. Casadio, *Protein Sci.*, 1996, **5**, 1704.

82. J. Garnier, D. J. Osguthorpe, and B. Robson, *J. Mol. Biol.*, 1978, **120**, 97.
83. T. P. Hopp and K. R. Woods, *Proc. Natl. Acad. Sci. USA-Biol. Sci.*, 1981, **78**, 3824.
84. M. Levitt, *J. Mol. Biol.*, 1976, **104**, 59.
85. J. M. R. Parker, D. Guo, and R. S. Hodges, *Biochemistry*, 1986, **25**, 5425.
86. J. Janin, S. Wodak, M. Levitt, and B. Maigret, *J. Mol. Biol.*, 1978, **125**, 357.
87. P. A. Karplus and G. E. Schulz, *Naturwissenschaften*, 1985, **72**, 212.
88. J. L. Pellequer and E. Westhof, *J. Mol. Graphics*, 1993, **11**, 204.
89. B. A. Jameson and H. Wolf, *Comput. Appl. Biosci.*, 1988, **4**, 181.
90. P. Y. Chou and G. D. Fasman, *Adv. Enzymol. Relat Areas Mol. Biol*, 1978, **47**, 45.
91. A. S. Kolaskar and P. C. Tongaonkar, *FEBS Lett.*, 1990, **276**, 172.
92. P. Rice, I. Longden, and A. Bleasby, *Trends Genet.*, 2000, **16**, 276.
93. B. Rost and A. Valencia, *Curr. Opin. Biotechnol.*, 1996, **7**, 457.
94. M. Kempe, M. Glad, and K. Mosbach, *J. Mol. Recogn.*, 1995, **8**, 35.
95. P. K. Dhal and F. H. Arnold, *J. Am. Chem. Soc.*, 1991, **113**, 7417.
96. P. K. Dhal and F. H. Arnold, *Macromolecules*, 1992, **25**, 7051.
97. S. Mallik, R. D. Johnson, and F. H. Arnold, *J. Am. Chem. Soc.*, 1993, **115**, 2518.
98. S. Mallik, S. D. Plunkett, P. K. Dhal, R. D. Johnson, D. Pack, D. Shnek, and F. H. Arnold, *New J. Chem.*, 1994, **18**, 299.
99. S. Mallik, R. D. Johnson, and F. H. Arnold, *J. Am. Chem. Soc.*, 1994, **116**, 8902.
100. S. Vidyasankar, P. K. Dhal, S. D. Plunkett, and F. H. Arnold, *Biotechnol. Bioeng.*, 1995, **48**, 431.
101. M. Burow and N. Minoura, *Biochem. Biophys. Res. Commun.*, 1996, **227**, 419.
102. J. L. Liao, Y. Wang, and S. Hjertén, *Chromatographia*, 1996, **42**, 259.
103. S. Hjertén, J. L. Liao, K. Nakazato, Y. Wang, G. Zamaratskaia, and H. X. Zhang, *Chromatographia*, 1997, **44**, 227.
104. M. Rezeli, F. Kilar, and S. Hjertén, *J. Chromatogr. A*, 2006, **1109**, 100.
105. D. Tong, C. Hetenyi, Z. Bikadi, J. P. Gao, and S. Hjertén, *Chromatographia*, 2001, **54**, 7.
106. I. Bacskay, A. Takátsy, A. Végvári, E. Elfwing, A. Ballagi-Pordány, F. Kilár, and S. Hjertén, *Electrophoresis*, 2006, **27**, 4682.
107. A. Takátsy, J. Sedzik, F. Kilár, and S. Hjertén, *J. Sep. Sci.*, 2006, **29**, 2810.
108. A. Takátsy, A. Kilár, F. Kilár, and S. Hjertén, *J. Sep. Sci.*, 2006, **29**, 2802.
109. A. Takátsy, Á. Végvári, S. Hjertén, and F. Kilár, *Electrophoresis*, 2007, **28**, 2345.
110. N. Ghasemzadeh, F. Nyberg, and S. Hjertén, *J. Sep. Sci.*, 2008, **31**, 3945.

111. N. Ghasemzadeh, F. Nyberg, and S. Hjertén, *J. Sep. Sci.*, 2008, **31**, 3954.
112. M. Hjertén, M. Rezeli, F. Kilár, and S. Hjertén, *J. Biochem. Biophys. Meth.*, 2008, **70**, 1188.
113. D. L. Venton and E. Gudipati, *Biochim. Biophys. Acta*, 1995, **1250**, 126.
114. A. Rachkov and N. Minoura, *J. Chromatogr. A*, 2000, **889**, 111.
115. A. Rachkov, N. Minoura, and T. Shimizu, *Anal. Sci.*, 2001, **17**, i609.
116. N. Minoura, A. Rachkov, M. Higuchi, and T. Shimizu, *Bioseparation*, 2002, **10**, 399.
117. H. Nishino, C. S. Huang, and K. J. Shea, *Angew. Chem. Intl. Ed.*, 2006, **45**, 2392.
118. B. Polevoda and F. Sherman, *J. Biol. Chem.*, 2000, **275**, 36479.
119. B. Polevoda and F. Sherman, *J. Biol. Chem.*, 2001, **276**, 20154.
120. A. Rechichi, C. Cristallini, U. Vitale, G. Ciardelli, N. Barbani, G. Vozzi, and P. Giusti, *J. Cell. Mol. Med.*, 2007, **11**, 1367.
121. D. F. Tai, C. Y. Lin, T. Z. Wu, J. H. Huang, and P. Y. Shu, *Clin. Chem.*, 2006, **52**, 1486.
122. M. E. Brown and D. A. Puleo, *Chem. Eng. J.*, 2008, **137**, 97.
123. N. W. Turner, B. E. Wright, V. Hlady, and D. W. Britt, *J. Colloid Interface Sci.*, 2007, **308**, 71.
124. H. Q. Shi, W. B. Tsai, M. D. Garrison, S. Ferrari, and B. D. Ratner, *Nature*, 1999, **398**, 593.
125. A. Bossi, C. Rivetti, L. Mangiarotti, M. J. Whitcombe, A. P. F. Turner, and S. A. Piletsky, *Biosens. Bioelectron.*, 2007, **23**, 290.
126. T. Shiomi, M. Matsui, F. Mizukami, and K. Sakaguchi, *Biomaterials*, 2005, **26**, 5564.
127. P. C. Chou, J. Rick, and T. C. Chou, *Anal. Chim. Acta*, 2005, **542**, 20.
128. R. Z. Ouyang, J. P. Lei, and H. X. Ju, *Chem. Commun.*, 20085761.
129. M. M. Titirici, A. J. Hall, and B. Sellergren, *Chem. Mater.*, 2003, **15**, 822.
130. M. M. Titirici and B. Sellergren, *Anal. Bioanal. Chem.*, 2004, **378**, 1913.
131. E. Yilmaz, K. Haupt, and K. Mosbach, *Angew. Chem. Intl. Ed. Engl.*, 2000, **39**, 2115.
132. M. M. Titirici, A. J. Hall, and B. Sellergren, *Chem. Mater.*, 2002, **14**, 21.
133. H. Q. Shi and B. D. Ratner, *J. Biomed. Mater. Res.*, 2000, **49**, 1.
134. F. Bonini, S. Piletsky, A. P. F. Turner, A. Speghini, and A. Bossi, *Biosens. Bioelectron.*, 2007, **22**, 2322.
135. A. Uysal, G. Demirel, E. Turan, and T. Çaykara, *Anal. Chim. Acta*, 2008, **625**, 110.
136. C. Lübke, M. Lübke, M. J. Whitcombe, and E. N. Vulfson, *Macromolecules*, 2000, **33**, 5098.

137. K. Tanabe, T. Takeuchi, J. Matsui, K. Ikebukuro, K. Yano, and I. Karube, *J. Chem. Soc. Chem. Commun.*, 19952303.
138. A. J. Hall, P. Manesiotis, M. Emgenbroich, M. Quaglia, E. De Lorenzi, and B. Sellergren, *J. Org. Chem.*, 2005, **70**, 1732.
139. K. Karim, F. Breton, R. Rouillon, E. V. Piletska, A. Guerreiro, I. Chianella, and S. A. Piletsky, *Adv. Drug Deliv. Rev.*, 2005, **57**, 1795.
140. S. Monti, C. Cappelli, S. Bronco, P. Giusti, and G. Ciardelli, *Biosens. Bioelectron.*, 2006, **22**, 153.
141. L. Q. Wu and Y. Z. Li, *J. Mol. Recogn.*, 2004, **17**, 567.
142. B. C. G. Karlsson, J. O'Mahony, J. G. Karlsson, H. Bengtsson, L. A. Eriksson, and I. A. Nicholls, *J. Am. Chem. Soc.*, 2009, **131**, 13297.
143. S. K. Chowdhury, V. Katta, and B. T. Chait, *J. Am. Chem. Soc.*, 1990, **112**, 9012.
144. K. Fukazawa and K. Ishihara, *Biosens. Bioelectron.*, 2009, **25**, 609.
145. T. Y. Guo, Y. Q. Xia, G. J. Hao, M. D. Song, and B. H. Zhang, *Biomaterials,* 2004, **25**, 5905.
146. T. Y. Guo, Y. Q. Xia, J. Wang, M. D. Song, and B. H. Zhang, *Biomaterials*, 2005, **26**, 5737.
147. Y. Q. Xia, T. Y. Guo, M. D. Song, B. H. Zhang, and B. L. Zhang, *Reac. Func. Polym.*, 2008, **68**, 63.
148. Y. Q. Xia, T. Y. Guo, H. L. Zhao, M. D. Song, B. H. Zhang, and B. L. Zhang, *J. Biomed. Mater. Res. Part A*, 2009, **90A**, 326.
149. G. Q. Fu, H. Yu, and J. Zhu, *Biomaterials*, 2008, **29**, 2138.
150. D. M. Hawkins, D. Stevenson, and S. M. Reddy, *Anal. Chim. Acta*, 2005, **542**, 61.
151. D. M. Hawkins, A. Trache, E. A. Ellis, D. Stevenson, A. Holzenburg, G. A. Meininger, and S. M. Reddy, *Biomacromolecules*, 2006, **7**, 2560.
152. D. M. Hawkins, E. A. Ellis, D. Stevenson, A. Holzenburg, and S. M. Reddy, *J. Mater. Sci.*, 2007, **42**, 9465.
153. J. Pan, X. H. Xue, J. H. Wang, H. M. Xie, and Z. Y. Wu, *Polymer*, 2009, **50**, 2365.
154. K. El Kirat, M. Bartkowski, and K. Haupt, *Biosens. Bioelectron.*, 2009, **24**, 2618.
155. L. Qin, X. W. He, W. Zhang, W. Y. Li, and Y. K. Zhang, *J. Chromatogr. A*, 2009, **1216**, 807.
156. S. L. Lu, G. X. Cheng, and X. S. Pang, *J. Appl. Polym. Sci.*, 2006, **100**, 684.
157. A. Bossi, M. Andreoli, F. Bonini, and S. Piletsky, *Anal. Bioanal. Chem.*, 2007, **389**, 447.

158. M. Haruki, Y. Konnai, A. Shimada, and H. Takeuchi, *Biotechnol. Prog.*, 2007, **23**, 1254.
159. H. F. Wang, Y. H. He, X. W. He, W. Y. Li, L. X. Chen, and Y. K. Zhang, *J. Sep. Sci.*, 2009, **32**, 1981.
160. D. F. Tai, M. H. Jhang, G. Y. Chen, S. C. Wang, K. H. Lu, Y. D. Lee, and H. T. Liu, *Anal. Chem.*, 2010, **82**, 2290.
161. D. F. Tai, C. Y. Lin, T. Z. Wu, and L. K. Chen, *Anal. Chem.*, 2005, **77**, 5140.
162. B. Okutucu, F. Zihnioglu, and A. Telefoncu, *J. Biomed. Mater. Res. Part A*, 2008, **84A**, 842.
163. Z. Y. Chen, Z. D. Hua, L. Xu, Y. Huang, M. P. Zhao, and Y. Z. Li, *J. Mol. Recogn.*, 2008, **21**, 71.
164. Y. T. Wang, Y. X. Zhou, J. Sokolov, B. Rigas, K. Levon, and M. Rafailovich, *Biosens. Bioelectron.*, 2008, **24**, 162.
165. Y. T. Wang, Z. Q. Zhang, V. J. Jain, J. J. Yi, S. Mueller, J. Sokolov, Z. X. Liu, K. Levon, B. Rigas, and M. H. Rafailovich, *Sens. Actuators B*, 2010, **146**, 381.
166. Y. Lu, C. L. Yan, and S. Y. Gao, *Appl. Surf. Sci.*, 2009, **255**, 6061.
167. L. Li, X. W. He, L. X. Chen, and Y. K. Zhang, *Sci. China Ser. B Chem.*, 2009, **52**, 1402.
168. H. F. Wang, W. Y. Li, X. W. He, L. X. Chen, and Y. K. Zhang, *Acta Chim. Sin.*, 2007, **65**, 43.
169. H. F. Wang, W. Y. Li, X. W. He, L. X. Chen, and Y. K. Zhang, *Reac. Func. Polym.*, 2008, **68**, 1291.
170. Y. Lu, C. L. Yan, X. J. Wang, and G. K. Wang, *Appl. Surf. Sci.*, 2009, **256**, 1341.
171. A. Bossi, S. A. Piletsky, E. V. Piletska, P. G. Righetti, and A. P. F. Turner, *Anal. Chem.*, 2001, **73**, 5281.
172. N. W. Turner, X. Liu, S. A. Piletsky, V. Hlady, and D. W. Britt, *Biomacromolecules*, 2007, **8**, 2781.
173. C. L. Yan, Y. Lu, and S. Y. Gao, *J. Polym. Sci. A,Polym. Chem.*, 2007, **45**, 1911.
174. J. Rick and T. C. Chou, *Biosens. Bioelectron.*, 2005, **20**, 1878.
175. J. Rick and T. C. Chou, *Biosens. Bioelectron.*, 2006, **22**, 544.
176. H. Y. Lin, M. S. Ho, and M. H. Lee, *Biosens. Bioelectron.*, 2009, **25**, 579.
177. D. Silvestri, C. Cristallini, G. Ciardelli, P. Giusti, and N. Barbani, *J. Biomater. Sci. Polym. Ed.*, 2005, **16**, 397.

178. D. Silvestri, N. Barbani, M. L. Coluccio, C. Pegoraro, P. Giusti, C. Cristallini, and G. Ciardelli, *Sep. Sci. Technol.*, 2007, **42**, 2829.
179. Z. D. Hua, Z. Y. Chen, Y. Z. Li, and M. P. Zhao, *Langmuir*, 2008, **24**, 5773.
180. Z. D. Hua, S. Zhou, and M. P. Zhao, *Biosens. Bioelectron.*, 2009, **25**, 615.
181. S. Pradhan, M. Boopathi, O. Kumar, A. Baghel, P. Pandey, T. H. Mahato, B. Singh, and R. Vijayaraghavan, *Biosens. Bioelectron.*, 2009, **25**, 592.
182. F. Li, J. Li, and S. S. Zhang, *Talanta*, 2008, **74**, 1247.
183. X. Zhou, X. W. He, L. X. Chen, W. Y. Li, and Y. K. Zhang, *Chin. J. Anal. Chem.*, 2009, **37**, 174.
184. W. Yao, B. Ning, H. Y. Zhou, Y. J. Fang, and Z. X. Gao, *J. Sep. Sci.*, 2008, **31**, 413.
185. Z. H. Zhang, Y. M. Long, L. H. Nie, and S. Z. Yao, *Biosens. Bioelectron.*, 2006, **21**, 1244.
186. Y. Li, H. H. Yang, Q. H. You, Z. X. Zhuang, and X. R. Wang, *Anal. Chem.*, 2006, **78**, 317.
187. S. H. Ou, M. C. Wu, T. C. Chou, and C. C. Liu, *Anal. Chim. Acta*, 2004, **504**, 163.
188. W. X. Su, J. Rick, and T. C. Chou, *Microchem. J.*, 2009, **92**, 123.
189. Y. W. Chen, J. Rick, and T. C. Chou, *Org. Biomol. Chem.*, 2009, **7**, 488.
190. W. Zhang, L. Qin, X. W. He, W. Y. Li, and Y. K. Zhang, *J. Chromatogr. A*, 2009, **1216**, 4560.
191. W. Zhang, L. Qin, R. R. Chen, X. W. He, W. Y. Li, and Y. K. Zhang, *Appl. Surf. Sci.*, 2010, **256**, 3000.
192. P. C. Liao, Y. C. Tyan, C. Y. Wang, J. F. Hsu, T. C. Chou, and H. Y. Lin, *J. Biomed. Mater. Res. Part A*, 2009, **91A**, 597.
193. S. H. Li, J. Wang, and M. P. Zhao, *J. Sep. Sci.*, 2009, **32**, 3359.
194. L. Uzun, R. I. Say, S. Ünal, and A. Denizli, *Biosens. Bioelectron.*, 2009, **24**, 2878.
195. E. Turan, G. Özçetin, and T. Caykara, *Macromol. Biosci.*, 2009, **9**, 421.
196. D. S. Janiak, O. B. Ayyub, and P. Kofinas, *Macromolecules*, 2009, **42**, 1703.
197. N. Bereli, M. Andaç, G. Baydemir, R. Say, I. Y. Galaev, and A. Denizli, *J. Chromatogr. A*, 2008, **1190**, 18.
198. X. B. Hu, G. T. Li, J. Huang, D. Zhang, and Y. Qiu, *Adv. Mater.*, 2007, **19**, 4327.
199. H. Y. Lin, J. Rick, and T. C. Chou, *Biosens. Bioelectron.*, 2007, **22**, 3293.
200. H. Y. Lin, C. Y. Hsu, J. L. Thomas, S. E. Wang, H. C. Chen, and T. C. Chou, *Biosens. Bioelectron.*, 2006, **22**, 534.
201. J. Y. Huang, Z. X. Wei, and J. C. Chen, *Sens. Actuators B*, 2008, **134**, 573.

202. Y. Lin, S. Q. Tang, X. Mao, and L. Bao, *J. Biomed. Mater. Res. Part A*, 2008, **85A**, 573.
203. T. Matsunaga, T. Hishiya, and T. Takeuchi, *Anal. Chim. Acta*, 2007, **591**, 63.
204. C. J. Tan, S. Wangrangsimakul, R. Bai, and Y. W. Tong, *Chem. Mater.*, 2008, **20**, 118.
205. C. J. Tan and Y. W. Tong, *Langmuir*, 2007, **23**, 2722.
206. C. Y. Hsu, H. Y. Lin, J. L. Thomas, B. T. Wu, and T. C. Chou, *Biosens. Bioelectron.*, 2006, **22**, 355.
207. D. L. Rathbone, A. Ali, P. Antonaki, and S. Cheek, *Biosens. Bioelectron.*, 2005, **20**, 2353.
208. S. H. Song, K. Shirasaka, M. Katayama, S. Nagaoka, S. Yoshihara, T. Osawa, J. Sumaoka, H. Asanuma, and M. Komiyama, *Macromolecules*, 2007, **40**, 3530.
209. A. A. Vaidya, B. S. Lele, M. G. Kulkarni, and R. A. Mashelkar, *J. Appl. Polym. Sci.*, 2001, **81**, 1075.
210. M. J. Guo, Z. Zhao, Y. G. Fan, C. H. Wang, L. Q. Shi, J. J. Xia, Y. Long, and H. F. Mi, *Biomaterials*, 2006, **27**, 4381.
211. M. J. Guo, T. Gao, Z. Fan, J. X. Yao, J. J. Xia, and H. F. Mi, *Sci. China Chem.*, 2010, **53**, 905.
212. T. Matsunaga, T. Hishiya, and T. Takeuchi, *Anal. Lett.*, 2007, **40**, 2633.
213. H. Yang, D. Lazos, and M. Ulbricht, *J. Appl. Polym. Sci.*, 2005, **97**, 158.
214. Y. Long, Y. Sun, Y. Wang, X. C. Xing, Z. Zhao, C. H. Wang, Y. G. Fan, and H. F. Mi, *Chin. Sci. Bull.*, 2008, **53**, 2617.
215. T. Ikawa, F. Hoshino, T. Matsuyama, H. Takahashi, and O. Watanabe, *Langmuir*, 2006, **22**, 2747.
216. M. Narita, F. Hoshino, M. Mouri, M. Tsuchimori, T. Ikawa, and O. Watanabe, *Macromolecules*, 2007, **40**, 623.
217. M. Narita, T. Ikawa, M. Mouri, M. Tsuchimori, F. Hoshino, and O. Watanabe, *Jpn. J. Appl. Phys.*, 2008, **47**, 1329.
218. T. Ikawa, Y. Kato, T. Yamada, M. Shiozawa, M. Narita, M. Mouri, F. Hoshino, O. Watanabe, M. Tawata, and H. Shimoyama, *Langmuir*, 2010, **26**, 12673.
219. R. Schirhagl, P. A. Lieberzeit, D. Blaas, and F. L. Dickert, *Adv. Mater.*, 2010, **22**, 2078.
220. R. Schirhagl, D. Podlipna, P. A. Lieberzeit, and F. L. Dickert, *Chem. Commun.*, 20103128.

221. M. E. Byrne and V. Salian, *Int. J. Pharm.*, 2008, **364**, 188.
222. M. Sibrian-Vazquez and D. A. Spivak, *J. Polym. Sci. A,Polym. Chem.*, 2004, **42**, 3668.
223. M. Sibrian-Vazquez and D. A. Spivak, *J. Am. Chem. Soc.*, 2004, **126**, 7827.
224. R. Simon, S. Houck, and D. A. Spivak, *Anal. Chim. Acta*, 2005, **542**, 104.
225. A. C. Meng, J. LeJeune, and D. A. Spivak, *J. Mol. Recogn.*, 2009, **22**, 121.
226. K. Yoshimatsu, K. LeJeune, D. A. Spivak, and L. Ye, *Analyst*, 2009, **134**, 719.
227. S. A. Piletsky, I. Mijangos, A. Guerreiro, E. V. Piletska, I. Chianella, K. Karim, and A. P. F. Turner, *Macromolecules*, 2005, **38**, 1410.
228. I. Mijangos, F. Navarro-Villoslada, A. Guerreiro, E. Piletska, I. Chianella, K. Karim, A. Turner, and S. Piletsky, *Biosens. Bioelectron.*, 2006, **22**, 381.
229. C. J. Tan and Y. W. Tong, *Anal. Bioanal. Chem.*, 2007, **389**, 369.
230. I. Sánchez-Barragán, K. Karim, J. M. Costa-Fernández, S. A. Piletsky, and A. Sanz-Medel, *Sens. Actuators B*, 2007, **123**, 798.
231. A. Menaker, V. Syritski, J. Reut, A. Öpik, V. Horváth, and R. E. Gyurcsányi, *Adv. Mater.*, 2009, **21**, 2271.
232. L. Basabe-Desmonts, D. N. Reinhoudt, and M. Crego-Calama, *Chem. Soc. Rev.*, 2007, **36**, 993.
233. M. Gerard, A. Chaubey, and B. D. Malhotra, *Biosens. Bioelectron.*, 2002, **17**, 345.
234. U. Lange, N. V. Roznyatovskaya, and V. M. Mirsky, *Anal. Chim. Acta*, 2008, **614**, 1.
235. A. Merkoçi and S. Alegret, *TrAC, Trends Anal. Chem.*, 2002, **21**, 717.
236. M. C. Blanco-López, M. J. Lobo-Castañón, A. J. Miranda-Ordieres, and P. Tuñón-Blanco, *TrAC, Trends Anal. Chem.*, 2004, **23**, 36.
237. J. Okuno, K. Maehashi, K. Kerman, Y. Takamura, K. Matsumoto, and E. Tamiya, *Biosens. Bioelectron.*, 2007, **22**, 2377.
238. T. L. Panasyuk, V. M. Mirsky, S. A. Piletsky, and O. S. Wolfbeis, *Anal. Chem.*, 1999, **71**, 4609.
239. T. L. Delaney, D. Zimin, M. Rahm, D. Weiss, O. S. Wolfbeis, and V. M. Mirsky, *Anal. Chem.*, 2007, **79**, 3220.
240. S. Suwansa-ard, P. Kanatharana, P. Asawatreratanakul, B. Wongkittisuksa, C. Limsakul, and P. Thavarungkul, *Biosens. Bioelectron.*, 2009, **24**, 3436.
241. G. M. Birnbaumer, P. A. Lieberzeit, L. Richter, R. Schirhagl, M. Milnera, F. L. Dickert, A. Bailey, and P. Ertl, *Lab Chip*, 2009, **9**, 3549.

242. Y. Uludag, S. A. Piletsky, A. P. F. Turner, and M. A. Cooper, *FEBS J.*, 2007, **274**, 5471.
243. O. Hayden, C. Haderspöck, S. Krassnig, X. H. Chen, and F. L. Dickert, *Analyst*, 2006, **131**, 1044.
244. T. Y. Lin, C. H. Hu, and T. C. Chou, *Biosens. Bioelectron.*, 2004, **20**, 75.
245. X. Y. Bi and K. L. Yang, *Anal. Chem.*, 2009, **81**, 527.
246. M. Piliarik, H. Vaisocherová, and J. Homola, *Meth. Mol. Biol.*, 2009, **503**, 65.
247. X. B. Hu, Q. An, G. T. Li, S. Y. Tao, and J. Liu, *Angew. Chem. Intl. Ed.*, 2006, **45**, 8145.
248. X. B. Hu, G. T. Li, M. H. Li, J. Huang, Y. Li, Y. B. Gao, and Y. H. Zhang, *Adv. Funct. Mater.*, 2008, **18**, 575.
249. G. J. Guan, B. H. Liu, Z. Y. Wang, and Z. P. Zhang, *Sensors*, 2008, **8**, 8291.
250. S. Tokonami, H. Shiigi, and T. Nagaoka, *Anal. Chim. Acta*, 2009, **641**, 7.
251. C. L. Choong, J. S. Bendall, and W. I. Milne, *Biosens. Bioelectron.*, 2009, **25**, 652.
252. H. Y. Lee and S. Kim, *Biosens. Bioelectron.*, 2009, **25**, 587.
253. P. Y. Chen, P. C. Nien, C. W. Hu, and K. C. Ho, *Sens. Actuators B*, 2010, **146**, 466.
254. Z. H. Zhang, Y. F. Hu, H. B. Zhang, L. J. Luo, and S. Z. Yao, *J. Electroanal. Chem.*, 2010, **644**, 7.
255. M. Riskin, R. Tel-Vered, O. Lioubashevski, and I. Willner, *J. Am. Chem. Soc.*, 2009, **131**, 7368.
256. D. Du, S. Z. Chen, J. Cai, Y. Tao, H. Y. Tu, and A. D. Zhang, *Electrochim. Acta*, 2008, **53**, 6589.
257. S. Loyprasert, M. Hedstrom, P. Thavarungkul, P. Kanatharana, and B. Mattiasson, *Biosens. Bioelectron.*, 2010, **25**, 1977.
258. J. M. Slocik, S. N. Kim, T. Auvil, E. R. Goldman, J. Liu, and R. R. Naik, *Biosens Bioelectron*, 2010, **25**, 1908.
259. Z. Y. Zeng, Y. Hoshino, A. Rodriguez, H. S. Yoo, and K. J. Shea, *ACS Nano*, 2009, **4**, 199.
260. K. G. Yang, M. M. Berg, C. S. Zhao, and L. Ye, *Macromolecules*, 2009, **42**, 8739.
261. M. H. Lee, J. L. Thomas, M. H. Ho, C. Yuan, and H. Y. Lin, *ACS Appl. Mater. Interf.*, 2010, **2**, 1729.
262. C. H. Lu, Y. Wang, Y. Li, H. H. Yang, X. Chen, and X. R. Wang, *J. Mater. Chem.*, 2009, **19**, 1077.

263. M. H. Lee, Y. C. Chen, M. H. Ho, and H. Y. Lin, *Anal. Bioanal. Chem.*, 2010, **397**, 1457.
264. H. H. Yang, S. Q. Zhang, F. Tan, Z. X. Zhuang, and X. R. Wang, *J. Am. Chem. Soc.*, 2005, **127**, 1378.
265. A. V. Linares, F. Vandevelde, J. Pantigny, A. Falcimaigne-Cordin, and K. Haupt, *Adv. Funct. Mater.*, 2009, **19**, 1299.
266. J. N. Anker, W. P. Hall, O. Lyandres, N. C. Shah, J. Zhao, and R. P. Van Duyne, *Nat. Mater.*, 2008, **7**, 442.
267. C. R. Yonzon, E. Jeoung, S. Zou, G. C. Schatz, M. Mrksich, and R. P. Van Duyne, *J. Am. Chem. Soc.*, 2004, **126**, 12669.
268. I. Tokareva, I. Tokarev, S. Minko, E. Hutter, and J. H. Fendler, *Chem. Commun.*, 20063343.
269. J. Matsui, K. Akamatsu, S. Nishiguchi, D. Miyoshi, H. Nawafune, K. Tamaki, and N. Sugimoto, *Anal. Chem.*, 2004, **76**, 1310.
270. J. F. Li, Y. F. Huang, Y. Ding, Z. L. Yang, S. B. Li, X. S. Zhou, F. R. Fan, W. Zhang, Z. Y. Zhou, D. Y. Wu, B. Ren, Z. L. Wang, and Z. Q. Tian, *Nature*, 2010, **464**, 392.
271. K. Kantarovich, I. Tsarfati, L. A. Gheber, K. Haupt, and I. Bar, *Anal. Chem.*, 2009, **81**, 5686.
272. C. I. Lin, A. K. Joseph, C. K. Chang, and Y. D. Lee, *J. Chromatogr. A*, 2004, **1027**, 259.
273. C. I. Lin, A. K. Joseph, C. K. Chang, and Y. D. Lee, *Biosens. Bioelectron.*, 2004, **20**, 127.
274. S. E. Diltemiz, R. I. Say, S. Büyüktiryaki, D. Hür, A. Denizli, and A. Ersöz, *Talanta*, 2008, **75**, 890.
275. A. Gültekin, S. E. Diltemiz, A. Ersöz, N. Y. Sariözlü, A. Denizli, and R. I. Say, *Talanta*, 2009, **78**, 1332.
276. H. Jans, X. Liu, L. Austin, G. Maes, and Q. Huo, *Anal. Chem.*, 2009, **81**, 9425.
277. Y. Hoshino, H. Koide, T. Urakami, H. Kanazawa, T. Kodama, N. Oku, and K. J. Shea, *J. Am. Chem. Soc.*, 2010, **132**, 6644.