Towards Peptide and Protein Recognition by Antibody Mimicking Synthetic Polymers – Background, State of the Art, and Future Outlook*

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Antibody–peptide/protein interactions are instrumental for many processes in the pharmaceutical and biotechnology industries and as tools for biomedical and biochemical research. The recent development of molecularly imprinted polymer nanoparticles displaying antibody-like recognition of peptides and proteins offers the possibility for substituting antibodies with these robust materials for applications where the structural integrity and function of antibodies is compromised by temperature, pH, solvent, etc. The background to the development of this class of antibody-mimicking material and the state-of-the-art in their synthesis and application is presented in this review.

Introduction – Antibodies and Biomimetic Recognition Systems

The interaction of antibodies with peptidic antigens has provided the basis for medicines,[1] diagnostics,[2] separation techniques,[3] and tools for biomedical and biochemical research.[4] The importance of antibody–peptide/protein interactions in research and for the pharmaceutical and biotechnology industries has driven efforts to develop alternatives to antibodies in the form of materials with comparable, or enhanced, recognition properties. This search has been motivated by the relatively narrow window of operation of antibodies with respect to temperature, pH, organic solvents, and ionic strength along with their susceptibility to enzymic degradation and the cost of their production.

Over recent decades, several strategies have evolved with the aim of producing materials with recognition characteristics reminiscent of those displayed by antibodies. The development of phage display[5] and aptamer technologies, e.g. SELEX,[6] are examples of highly successful approaches that have contributed new functional materials for the types of applications where antibodies have traditionally been used. Nonetheless, as with antibodies, the general limitations inherent

After Ph.D. studies at the University of Melbourne (1989), Ian A. Nicholls pursued post-doctoral research at Cambridge and Lund universities and held a series of lecturing positions at Lund and Kalmar universities. In 2000, he was appointed to a chair in chemistry at the University of Kalmar (now Linnaeus University). His current research is focused on biomimetic recognition systems and their use for chemical catalysis, sensing, and therapeutic applications.

Jesper Wikander completed his M.Sc. in biomedical chemistry and Ph.D. in organic chemistry (2004) at the University of Kalmar (now Linnaeus University). His Ph.D. and subsequent research has been focused on establishing the mechanisms underlying molecularly imprinted polymer–ligand recognition behaviour and using molecularly imprinted materials in biosensing and biomaterials applications.

*This paper is dedicated to Professor Paul F. Alewood in recognition of his significant contributions to the field of peptide chemistry and his importance for so many in his roles as academic leader, collaborator, mentor, and friend.
Molecular imprinting has been defined as: 'Molecular Imprinting: an alternative to antibodies in a range of application areas. The construction of de novo design of discrete molecular structures capable of the selective recognition of single amino acids or small peptides is a daunting task, requiring significant computational and synthetic effort. While some success has been achieved using small targets, extrapolation to larger peptides or protein targets remains at this point a significant challenge. In parallel to these developments, polymer synthesis in the presence of molecular templates has been employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a polymerization or polycondensation process, with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by the templating species (Fig. 1).

Although examples of molecular imprinting in silica had been reported as early as the 1930s, it was not until 1972 that the first organic molecularly imprinted polymer was presented by Wulff.[10] Most of the subsequent work on the development of the molecular imprinting technique was performed using monosaccharide and, in particular, amino acid derivatives as templates.[11] However, it was the 1993 report[12] by the Mosbach group on MIPs selective for theophylline and diazepam and the similarity of their cross-reactivity profiles to those of their corresponding antibodies that awakened interest in using this technique to develop synthetic polymers with antibody-like behaviour. Importantly, these highly cross-linked polymers[13] were very robust offering excellent physico-chemical stability and accordingly long shelf-life without the need for refrigeration.[14] The first examples of the molecular imprinting of smaller (di- to penta-peptides) appeared in the mid 1990s,[11,15] however, attempts to work with larger peptides and proteins proved problematic.[16] The challenges posed by recognition site heterogeneity, incompatibility with larger targets due to physical entrapment, and difficulties in using the technique in aqueous media impeded progress.

Various strategies have been explored to help overcome these issues and to gain insight into the mechanisms underlying the molecular imprinting process, including: the use of
thermodynamic treatments,[17] spectroscopic studies,[18] combinatorial polymer library synthesis,[19] and molecular modelling,[20] especially full system molecular dynamics studies.[21] New polymerization formats were also investigated in attempts to circumvent the difficulties associated with heterogeneity, entrapment, and incompatibility with polymerization solvents. These approaches included the imprinting of surfaces were the surface is decorated with immobilized template,[22] the use of liquid–liquid interfaces as explored through emulsion polymerization,[23] precipitation polymerization,[24] imprinting in hexagonal phases and micelles,[25] and through the immobilization of a template on sacrificial solids.[26] The use of an epitope from the target protein has been successfully used to limit the impact of template entrapment, possible influence on protein three-dimensional structure, and even the cost associated with using some proteins.[27]

The past decade has witnessed the development of MIP nanoparticles (nanoMIPs) with antibody-like recognition behaviour, and in the literature these are sometimes referred to as molecularly imprinted nanogels. The establishment of this new class of MIPs, a significant development in the field, was in part informed through the types of studies described above, although in particular through step changes in polymer design and synthesis methodologies. In this review we present the development and state-of-the-art of oligopeptide targeted molecularly imprinted nanoparticle synthesis.

### From the Molecular Imprinting of Small Molecules to that of Larger Peptides and Proteins

The success obtained in the use of highly crosslinked, most often acrylate-based, polymers for the imprinting of small organic structures was not readily replicated when using water soluble biomacromolecular structures, in particular larger peptides and proteins. Despite evidence of recognition and selectivity in some cases (Table 1), these systems were plagued by template entrapment, low yields, and reproducibility issues.[28] While the imprinting of surfaces helped avoid the issue of entrapment and even provided the potential for control of the orientation of template presentation to the polymerization mixture, the low yields of selective binding sites and the general incompatibility of these templates with the polymers traditionally deployed in molecular imprinting studies remained problematic.

Shea and colleagues provided a step change in the molecular imprinting of biomacromolecular structures through the use of combinations of predominantly acrylamide-based monomers containing functionalities reminiscent of those found in proteins (hydrophobic, polar-neutral, polar-ionizable): \( N\)-isopropylacrylamide (NIPAM), acrylamide, acrylic acid, \( N\)-\( t\)-butylacrylamide, and \( N,N'\)-methylenbisacrylamide as cross-linking monomer.[33] A notable feature of these polymers was the significantly lower degrees of crosslinking used relative to traditional MIP synthesis protocols, which facilitates the separation of template from the polymer nano-gel particles as can the thermoresponsive \( N\)-isopropylacrylamide, the dominant monomer, using temperature variation. This polymerization system was successfully used for the precipitation polymerization of nanoparticles imprinted with melittin, the 26-amino acid bee venom peptide, which were in later studies demonstrated to inhibit the inflammatory response of this toxin in a mouse model.[34]

Others have subsequently used the ‘Shea-protocol’ and adaptations thereof for the imprinting of several smaller structures.[35] Nonetheless, despite the impressive results obtained using this polymer system, imprinted nanoparticle preparation in this manner necessitates separation of unreacted monomers and laborious affinity purification and dialysis steps. This, together with relatively low yields of high affinity nanoparticles, limits the large-scale use of these materials.[28]

### High Throughput Nano-MIP Synthesis

The Piletsky group’s development of a solid-phase approach for the synthesis of molecularly imprinted nanoparticles constituted a paradigm shift for the sector.[36] In their seminal paper Canforotta et al.[36] presented the method as being comprised of three stages: 1) the activation of glass beads and immobilization of the template, 2) polymerization, and 3) affinity purification (Fig. 2).

The distinct advantages of the solid-phase molecular imprinting strategy over that of solution phase polymerization include: 1) the possibility of controlling template orientation, which can provide more homogenous recognition site populations, 2) the possibility of recovering and re-using the immobilized template in subsequent syntheses, 3) the combination of an affinity (immobilized template) stationary phase and flow system in conjunction with affinity purification, which in concert with the use of the thermoresponsive NIPAM polymers provide high affinity nano-MIPs with improved yields and free from template, which can eliminate the issue of template bleeding.[37]

This encouraging development saw this approach, and adaptations thereof, applied to the preparation of a range of small molecule targeted nano-MIPs by the Piletsky group and others e.g. cocaine,[38] biotin,[39] methyl parathion,[40] gentamicin,[41] and vancomycin.[42] The high affinities (pM to nM) of the nano-MIPs for their templates lead to the exploration of their use in a range of applications, e.g. in sensing,[43] and as vehicles for drug delivery of e.g. carbazole derivatives,[44] paclitaxel anti-cancer drugs,[45] quercetin,[46] and \((R)\)-thalidomide.[47] Effort has recently been directed to developing nanoparticles selective for peptides and proteins, e.g. insulin,[48] haemoglobin,[49] cadherin,[50] and immunoglobulin G.[51] It is important to note that the automation of this synthesis strategy has enhanced the potential for high volume production of nanoMIPs, although yields obtained using glass beads as supports are low, driving the search for higher yielding alternatives.

<table>
<thead>
<tr>
<th>Target peptide/protein</th>
<th>Template</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Hemoglobin, cytochrome C, transferrin</td>
<td>Hemoglobin, cytochrome C, transferrin</td>
<td>[29]</td>
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<tr>
<td>Protein surface histidines</td>
<td>Bis-imidazole</td>
<td>[30]</td>
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<tr>
<td>RNase A</td>
<td>RNase A</td>
<td>[31]</td>
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<tr>
<td>Oxytocin</td>
<td>Z-oxytocin</td>
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<tr>
<td>Phage displayed hexapeptides</td>
<td>Yohimbine</td>
<td>[33]</td>
</tr>
<tr>
<td>( \beta )-Lactoglobulin</td>
<td>( \beta )-Lactoglobulin</td>
<td>[34]</td>
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Table 1. Examples of early studies of oligopeptide-MIP recognition systems
Glass beads with immobilised template + Polymerisation mixture

Low T: elution of unreacted monomers and low affinity NPs 60°C: elution of high-affinity MIP NPs

**Fig. 2.** A schematic illustration of the solid phase synthesis of nanoMIPs as developed by Piletsky et al. Reproduced from ref. [28] with permission.

**Fig. 3.** (a) Fluorescence intensity of displaced trypsin (T) or pepsin (P) imprinted nanogels (NG) immobilized on the corresponding magnetic nanoparticles (magNPs) after addition of incremental amounts of different proteins and incubation for 2 h. (b) Dynamic light scattering size distribution (left) and transmission electron microscopy image (right) of P-NG.
Further Developments

Recently, the use of much smaller magnetic nanoparticles (magNPs) instead of glass beads was perceived as a way to dramatically increase the surface area available for template immobilization, and at the same time provide a mechanism for separating the particles from the polymerization reaction mixture. In a proof-of-concept study,[2] the enzymes pepsin and trypsin were immobilized on functionalized magNPs and a polymer system similar to that developed by Shea[3] and including a low (~1 %) concentration of a fluorescent monomer (N-fluorescein acrylamide) was used to produce nanoMIPs targeting these proteins in improved yields relative to the use of glass beads. The protein-conjugated magNP templates were then used together with the nanoMIPs in a magnetic competitive fluorescence assay in a 96-well format. Both the pepsin and trypsin nanoMIPs demonstrated $K_D < 10 \, \text{pM}$ for their respective target protein and low cross-reactivity (Fig. 3b). The improved nanoMIP yields and possibility of their use in an integrated fluorescence assay together highlight the attractiveness of this technique for the rapid and efficient production of synthetic polymer nanoparticles with antibody-like recognition characteristics.

One of the most exciting areas for nanoMIP use is in vivo applications.[3] Drug delivery (see above) and imaging are areas of particular interest. With respect to the latter, nanoMIPs can be easily functionalized using fluorescent dyes or even quantum dots.[34,35] A good example of this was presented by the Haupt group[54] who combined sol–gel and boronate chemistries to produce HER2 nanomaterials. When used in in vivo studies, an 50 % suppression of HER2+ breast cancer tumour growth was observed relative to control groups.

Conclusion and Future Outlook

The development of synthetic polymers with antibody-like recognition behaviour constitutes a significant step toward synthetic alternatives to antibodies. This, in combination with the establishment of protocols for solid phase nanoMIP synthesis,[36] opens the use of these materials in several applications, where in vivo (therapeutic, diagnostic) and sensing appear to be those closest to commercial outcomes. The further development of this novel class of molecularly imprinted materials shall require the application of rational design strategies in combination with a better understanding of the broader interaction of the nanoMIPs in biological contexts, in particular with respect to distribution, toxicities, and bioavailability.

Our group’s general interest in peptide chemistry and molecular recognition phenomena was inspired though PhD studies (I.A.N.) under the guidance of Professor Paul Alewood working with peptide and peptide-mimetic design and development[56] and through Paul’s coordination of a decade-long student exchange program between the University of Queensland and University of Kalmar (since 2010 Linnaeus University) that engaged over 20 MSc students. Several of these students went on to successful academic careers at the University of Queensland, and many at other academic or industrial organizations around the globe. J.G.W. was one of the first students who participated in this program. Paul Alewood was awarded an honorary doctoral degree by our university in recognition of his significant scientific achievements and his contribution to the development of our university’s international profile.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

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