Twenty years since ‘antibody mimics’ by molecular imprinting were first proposed: A critical perspective

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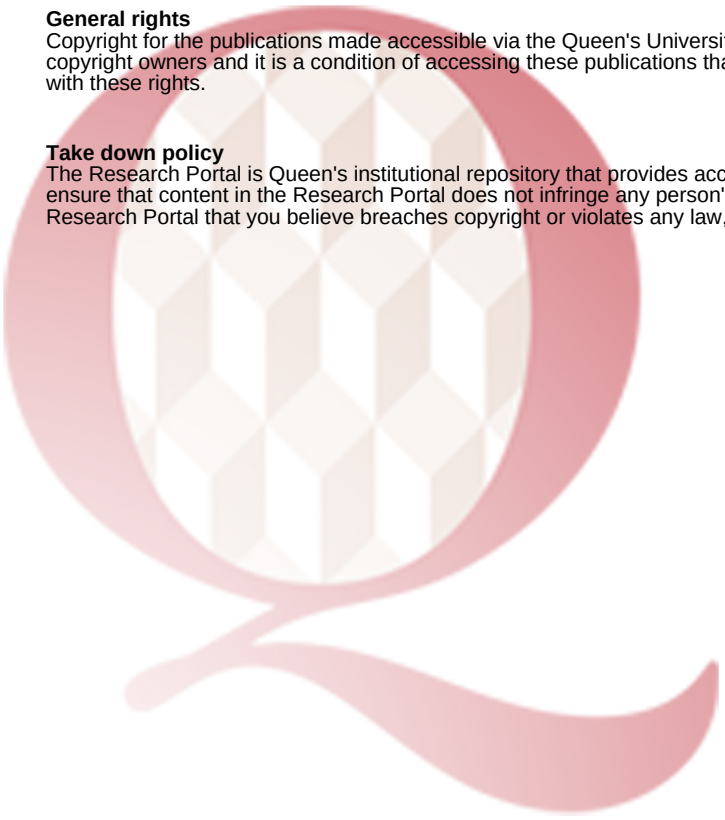

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Twenty years since *'antibody mimics'* by molecular imprinting were first proposed: A critical perspective

Abstract
In February 1993, the group of Klaus Mosbach published their milestone study in Nature where, for the first time, non-covalent molecular imprints were employed in a competitive binding assay. In this seminal piece of work, and also for the first time, they refer to molecularly imprinted polymers as being *'antibody mimics'* and hypothesised that these synthetic materials could one day provide *'a useful, general alternative to antibodies'*. This perspective article examines how far we have come in the 20 years since this publication in terms of realising this hypothesis and poses the question of whether we actually need molecularly imprinted polymers to be a general alternative to antibodies.

Keywords
Antibody mimic • Molecular imprinting • Plastic antibodies

Jenna L. Bowen[1], Panagiotis Manesiotis[2], Chris J. Allender[1]

[1]Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, King Edward VII Avenue, Cardiff, CF10 3NB, United Kingdom

[2]Department of Chemistry and Chemical Engineering, Queen's University Belfast, David Keir Building, Stranmillis Road, Belfast BT9 5AG, United Kingdom

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August 2012 marked the 40th anniversary of Günter Wulff's inaugural molecular imprinting paper that demonstrated imprinting within an organic polymer [1]. Some twenty years later, in February 1993, the group of Klaus Mosbach published their milestone study in Nature where, for the first time, non-covalent molecular imprints were employed in a competitive binding assay for the detection/quantitation of theophylline and diazepam in human serum [2]. Cited almost 1,200 times, this paper helped popularise the technique of imprinting as a means of generating synthetic recognition materials and, decades after the first report of molecular imprinting, spawned the phrase 'antibody mimics'; a term now synonymous with the technology. The authors hypothesised that the technology would, one day, provide *a 'useful, general alternative to antibodies'*.

The Vlatakis *et al.* paper was the first demonstration of MIPs being successfully used as alternatives for antibodies in competitive binding assays. The results reported were, and still are, impressive with molecular imprinted polymer (MIP) dissociation constants (K_d) in the nanomolar range and when challenged with a range of structurally related competitive ligands, cross-reactivity profiles correlating with those observed with antibodies (Table 1). However, the key difference between the MIP assay and a typical immunoassay was the environment in which the assays were performed. Antibodies have the ability to efficiently bind their antigen with high affinity directly from complex biological milieu, whereas the MIP system required extraction of drug into organic solvents, namely acetonitrile/acetic acid and toluene/heptane mixtures, in order to optimise performance. As a consequence, the MIP assay took longer and was more resource intensive than conventional ELISA – type approaches. Nonetheless, should the authors have sought to apply their antibody assay system in the organic phase, under the same experimental conditions in which the MIP assay was shown to be so effective, they would have been met with failure; a point often under-stated when MIP researchers are striving to mimic biological assays. The polymers described in the paper

Table 1. Cross reactivity data comparing the performance of the MIP employed in the competitive binding studies published by Vlatakis et al. to that of an antibody [2].

	Cross-reactivity (%)	
	MIP	Antibody
Theophylline and related substances		
theophylline	100	100
3-methylxanthine	7	2
caffeine	< 1	< 1
theobromine	< 1	< 1
uric acid	< 1	< 1
Diazepam and related substances		
diazepam	100	100
alprazolam	40	44
desmethyldiazepam	27	32
clonazepam	9	5
lorazepam	4	1

* *E-mail: allendercj@cf.ac.uk*

possessed a range of binding sites of varying affinities, making them polyclonal as opposed to monoclonal mimics but of course, being simple synthetic polymers, they would have been considerably more stable and more readily prepared than their biological counterparts.

In the 20 years following this milestone publication we have seen a huge increase in the number of researchers involved in the field of molecular imprinting with an associated dramatic increase in the number of publications (Figure 1) [3]. However, in spite of the significant interest the technology has attracted, have we come any closer to Vlatakis *et al.*'s hypothesis that one day MIPs would become *'useful, general alternative to antibodies'*?

Table 2 summarises some of the qualitative and quantitative features of antibodies and MIPs, upon which comparisons between the two receptor types are usually based. Apart from their well-documented high selectivity, antibodies also have the edge in terms of affinity, with K_d values often in the picomolar range as compared to low micro - nanomolar for MIPs. This is a crucial point in that assay sensitivity is directly linked to the affinity of the receptor and therefore detection limits for a MIP assay will, at best, be nanomolar but more commonly micro- or even millimolar. However, in all other aspects of this comparison MIPs seem to have a clear advantage: their preparation is much simpler, quicker and cheaper compared to their natural counterparts, their application range is much wider in terms of solvent, pH and temperature conditions, while their stability over time and reusability are exceptional compared to any other affinity sorbent.

Researchers aiming to capitalise on the benefits listed in Table 2 have adapted and employed molecular imprints to a variety of applications spanning most, if not all, *ex vivo* processes where selective recognition/binding is required and where an antibody would be typically used. Thus, at the time of writing, the database of imprinting literature reports more than 8,400 publications [4] associated with a highly diverse range of 'antigens' or templates as they are commonly termed in molecular imprinting. This large library of molecules can be broadly divided in two categories based on molecular size. There are the small molecules with molecular weights (M.W.) up to 1,000 Da, which among others include environmental pollutants [5], food additives or contaminants [6], pharmaceuticals [7] and drugs of abuse [8,9], and the larger molecules with M.W. up to or exceeding 100,000 Da, typically small to medium size peptides and proteins [10].

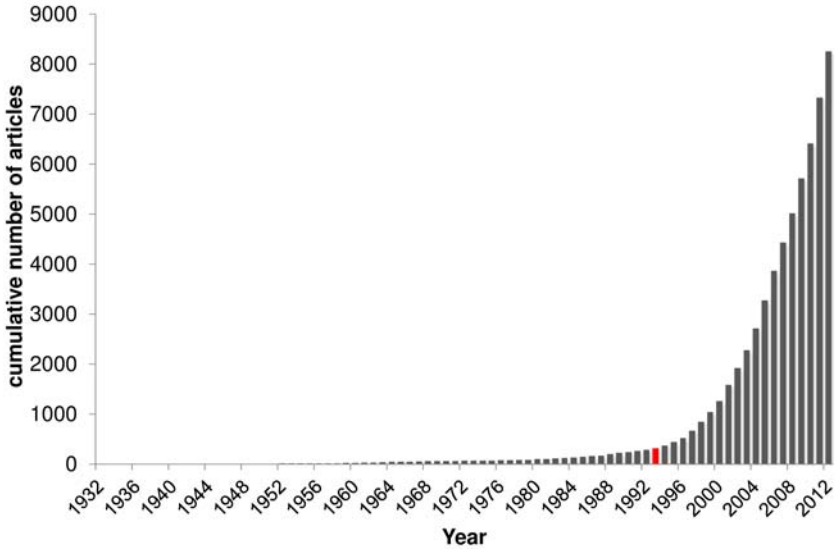

Figure 1. Cumulative number of MIP articles published since 1932. Data generated from www.mipdatabase.com (accessed on 05/02/2013) [4]

Table 2. Typical characteristics of antibodies and MIPs.

	Antibodies	MIPs
Affinity	10^{-7} – 10^{-11} M	10^{-3} – 10^{-10} M
Application	Physiological conditions	Organic or aqueous media
Capacity	~ 6 µmol.g^{-1}	~ 0.1 – 10 µmol.g^{-1}
Cost	£100's for µg quantities	£10's for g quantities
Production	Animal host, months	2 – 3 days
Reusability	Not usually	100's of times
Stability	Narrow temperature and pH range	Wide temperature and pH range
Storage time	Limited	Stable over period of years

Akin to the publication by Vlatakis *et al.*, the bulk of MIP literature refers to polymers molecularly imprinted with substances that fall into the first category of small molecules. These are typically soluble in aprotic, low-polarity organic solvents (such as toluene, chloroform or acetonitrile), have a definite, often conformationally restricted, shape and confined functionality that can be matched to one or more commercially available or custom made functional monomers and in most cases are light and/or heat stable. These properties make them ideal candidates for a textbook imprinting protocol whereby functional monomer(s), cross-linker(s) and initiator are mixed with the template in the solvent of choice and the resulting homogenous solution is thermally or photo-chemically polymerised to form a monolithic imprinted polymer [11]. Subsequent grinding, sieving and removal of the template, by solvent extraction, produces a 'plastic antibody' [12] in relatively good yields, short preparation time and at low cost. Conversely, antibody production is time and resource intensive, giving rise to an expensive product where reproducibility and consistency are increasingly of concern. Despite an ever growing demand, serviced by abundant commercial suppliers, significant quality issues concerning both antigen origin and antibody characterisation, have as yet to be addressed [13,14]. An important point to consider when discussing antibody production and supply is immunogenicity. This is a propensity for an antigen to stimulate an immune response in a host animal that is in general a function of molecular weight. Therefore, whereas conventional molecular imprinting favours antigens (templates) with M.W. < 1000, antibody production generally requires antigens with molecular weights exceeding 6,000 Da [15].

With the aim of producing more 'antibody-like' materials, researchers have invested considerable effort in developing water-compatible MIPs to facilitate their direct application in the analysis of aqueous samples, including samples of biological origin, thus minimising sample pre-treatment and doing away with non-polar organic solvent extraction steps from assay protocols. This has been achieved by the use of hydrophilic building blocks, post-modification of the materials by grafting of hydrophilic layers or chemical passivation [16] or, in more ambitious cases, by imprinting directly in water using water-soluble monomers and cross-linkers [17,18]. Such materials have been shown to outperform their biological counterparts in real sample applications, as they are capable of retaining their function in environments that fall outside of normal physiological conditions e.g. extremes temperatures and pH values [19].

To date, the imprinting of larger molecules, biological macromolecules being the most important member in this category, has been hindered by a number of complicating factors. The size, complexity, conformational flexibility and environmental sensitivity of such molecules, coupled with poor target specificity and the lack of recognition by conventional imprinted polymers outside organic media, has made this area of molecular imprinting particularly challenging and is one where true antibody mimicry is quite some way off. However, some success has been achieved in the field through careful design and optimisation of the imprinted system [18,20,21]. Conventional bulk imprinting approaches and the synthesis of imprinted soft gels both suffer from inefficient removal of the template and poor mass transfer upon re-incubation with their target species [22]. As a result, efforts have primarily focused on the use of surface imprinting approaches to allow for unhindered access to recognition sites. Early studies employed metal ion co-ordinated imprinting as an approach for the recognition of proteins [23-25] and although the systems were efficient at rebinding their templates, the application of this imprinting technique is limited to proteins that express histidine residues on their surface. Hierarchical imprinting has also been used to generate recognition elements for peptides. The use of a sacrificial solid support such as silica, allows for the generation of surface confined binding sites that are more homogenous than those in conventional imprinted polymer systems [26,27]. This homogeneity arises from the fact that the template is immobilised during the imprinting process, eliminating the probability for random incorporation in the polymer matrix and limiting the number of different possible complexes, however the main drawback of such an approach is the necessity for harsh conditions to bring about removal of the solid support.

In 2000, Rachkov and Minoura demonstrated what is termed 'the epitope approach' to molecular imprinting for the first time [28,29]. They imprinted a tetra-peptide sequence from oxytocin, a natural peptidic hormone, in acetonitrile containing 3% water. Importantly, it was found that recognition of the full peptide was possible using the epitopic tetra-peptide MIP, thus demonstrating the feasibility of using epitope sequences as templates when targeting recognition of macromolecules. However, despite delivering good chromatographic performance with high-acetonitrile mobile phases, when the water content was increased a significant reduction in retention time was observed due to the loss of hydrogen bonding interactions between the template and the polymer. Ken Shea's group further progressed this technique, achieving recognition of larger protein structures (cytochrome C, bovine serum albumin and alcohol dehydrogenase) through the imprinting of a nonapeptide sequence isolated from the C-terminus of the proteins [30]. Recently, the same group demonstrated the use of molecularly imprinted polymers in an *in vivo* system for the first time [31]. The polymers, imprinted with the 26 amino acid peptide mellitin (from bee venom), demonstrated affinities in the picomolar range and comparable to those achieved with antibodies [32,33]. To achieve such affinities for a biological macromolecule through polymerisation in wholly aqueous conditions is undisputedly a major advancement for the field of molecular imprinting.

Such examples offer clear evidence that molecular imprinting has evolved significantly over the past two decades; a result of the continuous and dedicated efforts of the researchers working in the field. So, are we any closer to preparing molecularly imprinted 'synthetic antibodies'? From a literal perspective this is a difficult question to answer. While we can safely say that we have witnessed the evolution of a new class of materials with unique properties that in specific cases match or exceed those of antibodies, a direct and general comparison between

the two classes of receptors is not appropriate or helpful. This is due to the fact that MIPs are typically highly cross-linked organic polymers, insoluble in any solvent, especially water, while antibodies are water-soluble biological macromolecules. Whilst both of these systems address the same problem, that is the need to bind a particular target molecule with good affinity and selectivity, they are fundamentally dissimilar in both their composition and mode of action. Hence, MIPs can achieve affinity and selectivity for their substrate similar to those of an antibody but typically in highly organic media where antibodies cannot function. Conversely, in physiological conditions where antibodies exhibit their full capabilities, most MIPs fail due to extreme hydrophobic interactions hindering any specific binding event. In the very few recent examples of soluble imprints [31,34] the yield of useful polymer is extremely low, typically a few milligrams - almost in antibody production quantities, and materials are laborious to prepare, thus eliminating the most important advantages of the technology: cost-effectiveness, simplicity and scalability. Furthermore, however 'antibody-like' soluble imprints are, they are still far from being a 'general alternative' as favourably oriented substrate functionality is required for the imprints to be successful.

Today, the perception of molecular imprinting by the wider research community remains mixed whilst its commercial impact is modest. It is perhaps worthy to reflect on where we might be should researchers have sought to target applications for which MIPs were naturally suited rather than for those where antibodies are better placed to deliver a working solution. The strength of molecular imprinting lies in its ability to deliver synthetic receptors capable of targeting low molecular weight molecules in non-polar environments. What they are not good at is being solubilised, labelled or linked to enzyme reporter systems. We should not forget that chemical and pharmaceutical industry rely strongly on the use of organic solvents in the majority of their processes, many of which take place at elevated temperatures and pH values outside the physiological window. An alien environment for any antibody, but a place where most MIPs operate best and could provide benefits beyond conventional analytical techniques. Lab-scale processes, be it a synthetic procedure that requires chiral separation or an analytical protocol that requires selective isolation of particular compounds, could benefit from the flexibility, adaptability and low-cost of MIPs: given a template, robust and selective receptors are accessible in 48-72 hours at only a fraction of the cost of an antibody.

Therefore the answer to the commonly asked question "why has molecular imprinting had so little commercial impact?" is that it has been 'marketed' as a solution to a problem that did not exist. Clearly, the competitor of the technology is not the antibody, but conventional separation science. The commercial opportunity perhaps lies in the development of MIP solutions to the problems of rapid and cost effective analysis of small molecules in non-polar environments. Of course existing technologies such as HPLC-MS are well entrenched, but opportunities for significant improvement in speed, selectivity and/or cost can be envisaged; these could be in the form of MIP sensors or highly-automated non-aqueous binding assays.

So maybe we need to rephrase the question and ask whether we actually need a 'general alternative to antibodies'. It would appear that perhaps we do not and a closer look at Figure 2 reveals that the research community has already made this decision. Although a steady increase in the number of publications comparing MIPs with antibodies has been observed since the original paper in 1993, the percentage of these publications to the total number of MIP articles has

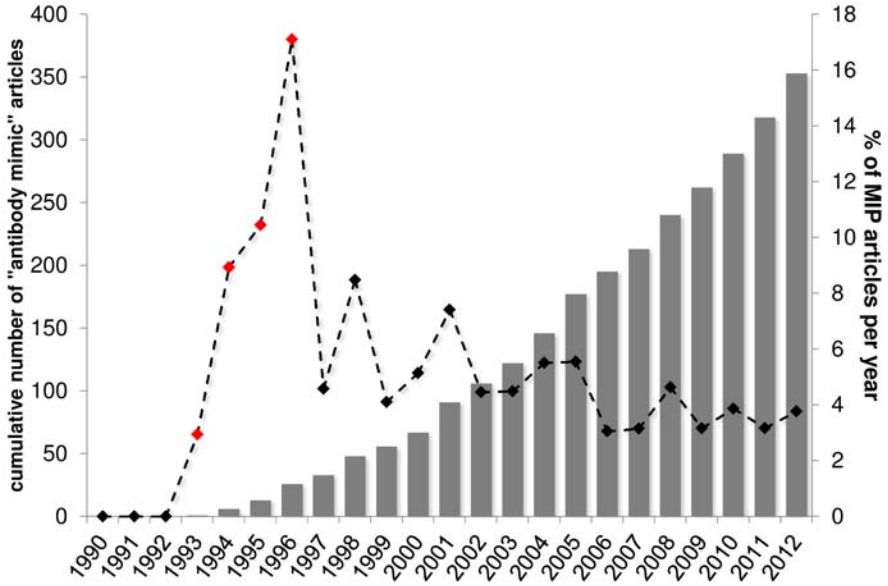

Figure 2. Cumulative number of articles comparing MIPs to antibodies since the use of the term "antibody mimics" in Vlatakis' paper (bars) and percentage of "antibody mimic" articles to the number of MIP articles published per year (dashed line). Data generated from www.mipdatabase.com (accessed on 05/02/2013) [4]

stabilised to around 4% over the past decade following a 3 year burst peaking at an impressive 17% (13 out of 76) in 1996.

In conclusion, MIPs and antibodies have co-existed for the past decades as two discrete types of receptors, each with its own advantages and disadvantages. For the time being it appears that they cannot, and perhaps should not, converge but simply continue to complement each other. Conceivably, in the not too distant future, a researcher will be able to choose their receptor of choice, synthetic or natural, depending on their desired application. After all, both MIPs and antibodies have been applied in affinity-based extractions, separations and sensing with varying degrees of success so that one should not have to completely replace one with the other, just pick whichever best solves the problem at hand.

By no means should this be considered as a failure for the field of molecular imprinting. Nature has perfected a remarkably rapid, highly adaptable and specific antibody production mechanism over millions of years of evolution, using combinations of around 20 building blocks. Yet, after just a few decades of research, man-made receptors, typically built using not more than two functional monomers, are now readily available that in many cases are capable of outperforming their natural counterparts. Given that the synthetic chemist's palette includes many more options coupled with the ingenuity and imagination exhibited by the multidisciplinary imprinting community over the past 20 years, we have every reason to believe that the full potential of molecular imprinting is yet to be revealed and look forward to the exciting future of this technology.

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