

***Searching for Molecular Solutions* – Additional Material**

CHAPTER 7

These Files contain additional material relevant to **Chapter 7** of *Searching for Molecular Solutions*. The page numbers of the book pertaining to each section are shown in the Table below, the corresponding page number for this file, and the title of each relevant section.

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Section A10: **Intrabodies**

This section provides more detail on intracellular antibodies; cited within p. 268 of *Searching for Molecular Solutions*.

Antibodies Inside

Harnessing the high specificity of antibodies in the intracellular environment has long been an aspiration of many researchers, as a tool for manipulating cellular pathways with a vast array of potential applications. This was spurred by early observations that pre-formed antibodies physically injected intracellularly could bind and inhibit targets of interest ¹. For full realization of antibodies as intracellular tools, though, *in situ* expression and function are required. It was noted in Chapters 6 and 7 of *Searching for Molecular Solutions* that there is much potential utility for functional RNA aptamers transcribed within living cells, or ‘intramers’. Antibodies expressed intracellularly have been termed ‘intrabodies’, a coinage which seems to have endured ♥.

When considering how to make intrabodies, it is necessary to note that the natural functions of immunoglobulins are readily definable as falling into two broad areas: as cell surface receptors and as extracellular circulating binders of non-self foreign antigens. Antibodies are not, therefore, naturally designed to function as binding proteins within the cytoplasm or nucleus of living cells.

♥This is consistent with the simple formula ‘X-bodies’ for new types of antibody creations, which is somewhat reminiscent of the origin of words like ‘cheeseburger’ (Forming a new word by splitting and recombining an earlier word (cheese + Ham/burger) even if technically inappropriate. But pedantry over word origins has never stopped anyone in the past, and never will). The only semantic problem likely to arise is when non-antibody frameworks are used as platforms for intracellular binding proteins. If these are included within the definition of ‘intrabodies’, the relationship of the word to ‘antibodies’ becomes rather indirect.

Indeed, there are *a priori* reasons to expect that antibodies should not function optimally (if at all) in the intracellular environment. Disulfide bonds stabilize the pairing of immunoglobulin heavy and light chains (Fig. 7.2 of *Searching for Molecular Solutions*), and conserved disulfides in the framework segments of variable regions assist in their stabilization as well. The cytoplasmic environment has reducing (anti-oxidizing) characteristics which disfavor disulfide bond formation and preservation, and additional problems of correct folding, aggregation and stability might be anticipated. Yet antibody designers have risen to these challenges and provided workable solutions, once again largely through diversification and selection from libraries.

A question at a fundamental level could ask, 'Is there an inherent requirement for disulfide bonds or other features of antibody natural design which might retard intracellular antibody applications, or are they dispensable?' In other words, even if a majority of antibody frameworks are incompatible with the intracellular environment, might not some rare variations on the immunoglobulin theme overcome such restrictions? Rational approaches can certainly be undertaken towards removing the need for variable region stabilization by (normally invariant) disulfides ². At the same time, optimization of antibody intracellular design can readily be approached by empirical directed evolutionary selection. It is possible to design powerful screening and selection processes for intracellular binding functions of antibodies. Consider an antibody whose specific binding is capable of re-activating a defective enzyme, which in turn allows rapid screening for new activity. Such a scenario exists in the case of certain defective mutants of β -galactosidase, and this effect was exploited for the identification of scFv fragments (Figs. 7.2, 7.3 of *Searching for Molecular Solutions*, and the same ftp site for color version of the latter) which could perform this function within *E. coli* cells ³. Antibody fragments passing this functional screen showed chemical reduction of cysteine residues, meaning that disulfide bond formation was not necessary for correct variable region folding of such binding molecules. The intracellular dispensability of disulfide bonds was confirmed in another antibody

system ⁴, and by directed evolution it was possible to isolate intracellular scFv fragments where all possibility of disulfide formation was removed by replacement of the participating cysteines with other residues ⁵.

In Chapter 7 of *Searching for Molecular Solutions* it was noted that antibody libraries based on single structural frameworks had sufficient binding diversity for most purposes ^{6,7}. This is particularly important for intrabodies, as it is largely the framework regions which confer the desired retention of binding under intracellular conditions. Specific framework formats have thus been evaluated and optimized for their intracellular robustness, for use in specialized library construction ⁸⁻¹⁰. Since the property of compatibility with intracellular expression is not directly linked with antigen-binding specificity, screening of antibody frameworks for their stabilities within cells need not include antigen recognition *per se*. Accordingly, an assay has been devised for intracellular stabilities of scFv fragments, based on their fusion with a selectable marker protein. Where enhanced stabilities of specific scFvs occurs, it also tends to promote stability of the linked fusion protein, allowing selection of candidate scFv molecules with the desired intracellular properties ¹¹. It has also been noted that fusion of scFv fragments with certain specific proteins in itself has stabilizing properties for the scFv in the intracellular environment [♥], but obligate reliance on this effect imposes a permanent requirement for such protein tags, and a potential limitation in some circumstances.

[♥]These are *E. coli* maltose binding protein ¹² and the Fc regions of immunoglobulins ¹³. Fusions of this type may enhance the intracellular folding of the attached scFv proteins by (directly or indirectly) having a chaperone effect ¹².

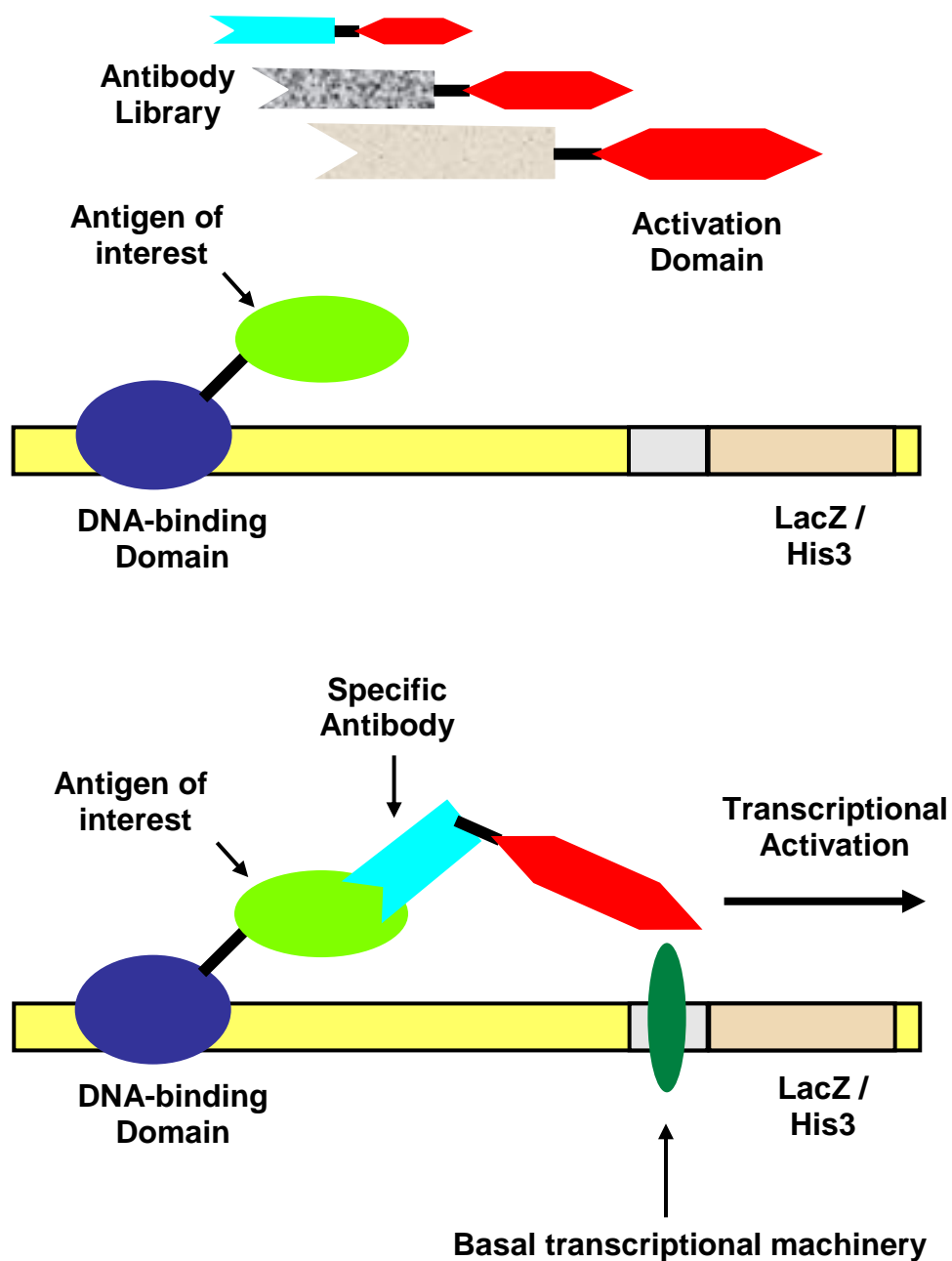


Fig. 7A10.1

Depiction of screening *in vivo* antibody libraries by yeast two-hybrid system, based on the modularity of protein domain function. A fusion of a polypeptide antigen of interest is made with a DNA-binding domain whose target sequence is upstream of a marker (*his3*)

which allows yeast growth on a selective medium, or a marker (*lacZ*) which allows chromogenic screening based on enzyme activity. When an antibody library fused to a powerful transcriptional activation domain is co-expressed in the cell, only antibodies with the desired specificity and intracellular activity will allow the activation domain to interact with the basal transcriptional machinery (striped oval) and activate the selectable / screenable markers.

A common strategy employed in the above β -galactosidase activation study ³ has been to initially identify antibody fragments capable of satisfying the desired binding function *in vitro*, as a starting-point for finding corresponding antibodies whose function is retained in an intracellular setting. Within this process, in principle one can take a single representative which has *in vitro* functionality and use this for subsequent mutation and selection towards intracellular activity. Alternatively, it is possible to screen a sublibrary of *in vitro* binders for those which retain capability as intrabodies. The latter two-phase ‘capture’ approach has commonly used a variation on the yeast two-hybrid system [▼] for *in vivo* sublibrary evaluation, and its essential features as relevant to intracellular selection and screening of intrabodies ¹⁴ are schematically depicted in Fig. 7A10.1 above.

The size of intrabodies is also relevant to their intracellular roles and transport, and with this in mind we might hark back to the camelid VHH and shark IgNAR single domain antibodies considered within Chapter 7 of *Searching for Molecular Solutions*. VHH domains have indeed been exploited as intrabodies ¹⁵, and IgNARs proposed for analogous roles ¹⁶. Yet somewhat ironically, despite their great utility in many conventional roles, these kinds of recognition molecules may not be necessary for the straightforward goal of obtaining workable single domain intrabodies. It has been found possible to use single domains (V_H or V_L) from

▼ This important *in vivo* technique for finding participants in molecular binding interactions is described in more detail in the file SMS–CitedNotes–Ch4/Section9; from the same ftp site).

scFv proteins previously optimized as intrabodies, and successfully apply them for corresponding intracellular use against the same antigens ¹⁷. This suggested that optimization for intracellular folding and function was the most important factor in single antibody domain activities under these conditions. Moreover, intradomain disulfide bonds were dispensable for such single domain intrabodies, as for their scFv equivalents ¹⁸. Although isolated V_H and V_L domains might fail to attain the specificity and affinity of their original V_H: V_L dimers, direct *in vivo* selection ♥ of specific binding could be achieved through diversified single domain libraries in yeast ¹⁷.

There are certain other requirements which need to be satisfied if intrabodies are to realize their promise for most applications. An important issue to pay heed to is the fact that a eukaryotic cell is anything but a simple bag of 'protoplasm', but is divided into many well-demarcated compartments. The nuclear/cytoplasmic divide is the most familiar, but we must also recall fundamentally important organelles such as mitochondria, chloroplasts, and the endoplasmic reticulum (the latter is important for functions including transmembrane protein biosynthesis and protein secretion) ²⁰. The cellular location of an intrabody target will determine whether intrabody transport into one of these compartments is warranted (with or without retention, in the case of the endoplasmic reticulum). Clearly, if a target protein for an intrabody is channeled into a cellular compartment from which the intrabody is excluded, biological effects of the intrabody expression will be minimal if present at all. Distinct peptide signals are known in each case which direct a polypeptide into the nucleus or a specific organelle, and these peptide tags can be appended to any intracellularly-expressed protein as desired, including intrabodies ²¹.

♥ Direct *in vivo* selection stands in contrast to the 'capture' method referred to above, where initial selection for an antigen-binding sublibrary is performed *in vitro*. Direct selection *in vivo* has nonetheless previously been used with scFv fragments in a two-hybrid assay format ¹⁹.

As for conventional antibodies, all applications of intrabodies must necessarily involve a molecular recognition and binding event. Most of time, the aim of such an interaction is the blocking of some undesirable cellular activity. If that indeed is the sum total of the proposed function for the intrabody, a modern molecular biologist would immediately respond that alternative technologies are available for achieving such ends. By this I am not referring to the intramer / intrabody choice (referred to in Chapter 6 and 7 of *Searching for Molecular Solutions*) but the knock-down of protein expression through the targeting of corresponding mRNAs by RNA inhibition (RNAi ♥) or other ways of down-regulating gene expression at the RNA or transcriptional level. To most workers at the present time, RNAi-based approaches would appear greatly superior in terms of simplicity of design and perhaps performance as well, although in the latter regard more information about intrabody efficiency is required. Yet there is more to intrabody 'protein-i' (as opposed to RNAi ²²) than first meets the eye. It is possible that intrabodies will offer advantages for the inhibition of specific isoforms of closely-related proteins, or where protein targets have long half-lives ²³. In particular, though, intrabody inhibition has potential to show great finesse in protein functional control *, as opposed to the relative battering-ram approach of total expression ablation at the RNA level.

There are numerous precedents where it is not desirable to prevent expression of a specific protein entirely, but rather modify its properties or functions in specific ways. Good examples in this regard are protein misfolding or aggregation syndromes, such as prion and amyloid-forming neurological diseases. Prevention of a misfolding cascade by intracellular binding specifically to a pathological alternative protein conformation would offer a potential therapy for these serious clinical conditions ²⁴, and scFv antibodies with specific high-affinity towards misfolded prion proteins have been obtained through *in vitro*

♥ RNAi is briefly considered in Chapter 9 of *Searching for Molecular Solutions*, and (in the context of global screening), also in the file SMS–CitedNotes-Ch9/Section 29; from the same ftp site.

*In this respect, intramers could offer equivalent performance as for intrabodies.

directed evolution ²⁵. Intrabody-based strategies for other neurological disorders have been devised where protein aggregation is a prominent feature ^{26,27}. By means of judicious tagging with one of the above-mentioned subcellular localization peptides, an intrabody could also in principle re-direct the entry of a protein from one cellular compartment to another. Where proteins possess more than one functional domain (as is frequently the case), intrabodies have the potential to selectively modify protein behavior by specifically binding only to relevant site(s) on the protein molecule ²³, in manner impossible for RNAi approaches.

Even given the existence of alternative potential therapies, intrabodies have been investigated as anti-cancer agents ^{28,29}. Intrabody-mediated inhibition of the Ras oncogene has shown reversal of tumorigenic phenotypes in experimental models ^{30,31}. Applications for intrabodies in the growing field of gene therapy have also been envisaged ^{32,33}. Perhaps the most significant feature of intrabodies (and by inference, intramers as well) is their potential for targeting biomolecules which were hitherto regarded as 'undruggable' by classic low-molecular weight compounds ³⁴.

Section A11: ***Natural catalytic Antibodies***

Relevant to the section on catalytic antibodies ('Antibodies as Catalysts') beginning on p. 246 of *Searching for Molecular Solutions*.

There is a flip side to the increasing levels of artificial tinkering with catalytic antibodies (rational or otherwise), and that is to wonder whether there are natural precedents to antibody-mediated catalysis. From what we have seen already, at first this might seem an unlikely prospect. To overcome activation energy barriers, an enzyme must bind a chemical transition state for a reaction with higher affinity than the corresponding substrate 'ground state'. Antibodies selected and evolved *in vivo* against normal antigens in their energetic ground states would thus not be expected to exhibit catalysis, and indeed might inhibit rather than promote any such reactivity. This is consistent with the theory of generation of artificial catalytic antibodies with transition state analog haptens, as successfully applied^{35,36}. Yet a body of evidence exists documenting the existence of naturally-formed antibodies with protease³⁷⁻³⁹, kinase⁴⁰, and nuclease activities⁴¹. In the case of antibody-mediated proteolysis, it has been proposed that variable region high-affinity recognition of a peptide in the ground state does not preclude the possibility of corresponding transition-state stabilization by the same immunoglobulin combining site, through different interactions⁴². In this view, interactions mediating ground state binding are not necessarily lost during formation and stabilization of the transition state⁴². Indeed, immunization with a chemically-normal biological peptide (vasoactive intestinal peptide; VIP) has been found to elicit antibody light chains with anti-VIP proteolytic activity^{38,39}. Residues likely to participate in this catalysis were identified (serine, histidine and aspartic acid), and found to be present in the

germline gene from which the active light chain was derived ^{♥ 39}. Serine-histidine 'dyads' are well-characterized as catalytic effectors in the serine protease family, and shown (by structural studies) to be located in the binding site of an artificially-generated esterase antibody ⁴³. These observations underscore the viewpoint that artificial catalytic antibodies may benefit from underlying germline residue configurations which are inherently conducive to catalysis ^{* 45}.

Additional impetus towards the study of naturally-formed catalytic antibodies has come from observations that they are elevated in certain pathological states, especially autoimmune diseases ^{45,46}. An autoantibody which combines recognition of its target with cleavage, and shows significant enzymatic turnover, could prove more of a pathological burden than antibody exhibiting binding alone. The reason for the association of catalytic antibodies with autoimmune disease is uncertain, but some interesting possibilities have been raised. It was noted in *Searching for Molecular Solutions* (Chapter 7) that specific anti-idiotypic antibodies can recapitulate enzyme active sites (Fig. 7.7), and an autoantibody against a self-enzyme could thus provoke a secondary anti-idiotypic response with a 'mimic' catalytic activity of the original enzyme ⁴⁷. From a theoretical point of view, binding of target ligand by highly active catalytic B cell immunoglobulin receptors might result in product formation and release before receptor-mediated signaling could occur (and in turn before B cell proliferation and clonal selection could take place). If so, this would normally serve to mitigate the formation of deleterious highly reactive catalytic antibodies *in vivo*. It has been postulated that certain phenotypes conducive to autoimmune states might favor retention of signaling by highly catalytically active B cell surface immunoglobulins, leading to

[♥]This indicates that the putative catalytic residues were not introduced through a somatic mutational event.

^{*}In other words, at least some antibodies selected for recognition of a hapten transition state analog may have a germline bias towards bearing catalytically useful residues. Certainly, cases have been reported of antibodies with better catalytic performance than expected from the immunization hapten alone ⁴⁴.

production of antibodies with the same damaging self-reactivities ⁴². Irrespective of the pathways leading to autoimmunity, the other side of the coin is the possibility that naturally-acquired abzymes might (at least in some circumstances) also have a beneficial role as part of the adaptive immune system ⁴⁸.

The above instances of natural antibody catalysis share the usual feature of specific antigen recognition as an intrinsic feature of the catalytic process. But a very remarkable finding in relatively recent times has thrown a quite different quality over the whole field. Evidence has accrued to suggest that all antibodies possess an inherent catalytic capacity which is quite distinct from their talents for antigen recognition. This catalysis uses singlet oxygen [▼] to produce the strongly oxidizing molecules hydrogen peroxide ⁵⁰ and even triatomic oxygen, or ozone ⁵¹, using the oxidation of water itself as the electron source for the process ⁵². Other non-immunoglobulin proteins show evidence for production of hydrogen peroxide from singlet oxygen ⁵⁰, but only immunoglobulins and α / β T cell receptors were found to exhibit true catalytic turnover in this regard ⁵². (Yet not all members of the immunoglobulin superfamily were correspondingly active). Any property common to all antibodies immediately informs us that it cannot by definition be restricted to hypervariable CDRs in the same manner as for antigen recognition. Structure –function studies have pointed to conserved residues at the interfaces of the immunoglobulin variable and constant regions as being involved in the oxidative catalytic mechanism ^{53,54}. Integrating these findings into the broad field of antibody catalysis as a whole, an artificially-derived abzyme would be

[▼] Note that the 'singlet' state refers to a specific electronic configuration of oxygen as a diatomic molecule. As a consequence of its electronic structure, diatomic oxygen can exist in a stable ground 'triplet' state, where electrons in a molecular orbital have parallel spins (denoted as $^3\text{O}_2$) and an excited 'singlet' state ($^1\text{O}_2^*$) characterized by molecular orbital electrons with antiparallel spin. The latter $^1\text{O}_2^*$ species is short-lived (several microseconds in aqueous solvent) and much more reactive than triplet oxygen ⁴⁹?. The 'triplet' and 'singlet' terms refer to the numbers of electronic spin alignments possible.

expected to exhibit *two* enzymatic capabilities sited in separate regions of the molecule: the selected catalysis itself, associated with the binding site in the variable regions, and the innate oxidative catalysis at variable and constant region interfaces.

This quite surprising generalized catalytic feature of antibodies raises many interesting questions. Biological sources for the required singlet oxygen are known from the 'respiratory burst' of neutrophils and macrophages⁵⁵⁻⁵⁷, this is a productive area for further study⁵⁸. But at a more fundamental level, it has been speculated that intrinsic oxidative antibody catalysis may be yet another (and ancient) facet of the innate immune system, upon which the adaptive immune system antibody arm has been superimposed^{52,57}. It is even conceivable that the facility for intrinsic catalysis has played an evolutionary hand in the shaping of the immunoglobulin fold⁵². While the effective role of antibody intrinsic catalysis in present-day immune functioning has been questioned⁵⁹, it is reasonable to suppose that an antibody which can combine recognition with destruction of its target would have increased physiological significance. In this context it is notable that antibodies themselves appear to be inherently resistant to the reactive-oxygen products of their own intrinsic oxidative catalysis⁵².

Section A12: ***Molecular Imprinting Notes***

Relevant to the section dealing with molecular imprinting; commencing on p. 258 of *Searching for Molecular Solutions*.

This section provides some additional detail on certain specific aspects of molecular imprinting.

A Case of Illusory Imprinting

Not all views of imprinting have stood the test of time. Consider the following case history: In 1988, a report in the high-profile journal *Nature*⁶⁰ suggested another and very peculiar type of imprinting, soon checked and quite discredited. We can spend a minute looking at these proceedings, because it serves to reinforce some general points that are relevant to real molecular imprinting. In this saga, the authors of the paper in question used a system where antibodies directed against another immunoglobulin (IgE) could trigger 'degranulation' and release of histamine from basophils, via surface IgE on these cells which becomes cross-linked by bound anti-immunoglobulin. This in itself is not at all controversial, but it was reported that the responsible anti-IgE could be diluted by astronomical factors (up to 10^{120}) without losing activity, such that there could not possibly be any of the original immunoglobulin molecules remaining in the water-based solvent used for the (supposedly successful) assays. It was proposed that the immunoglobulin somehow left an 'imprint' in solvent water molecules, which was retained as a 'memory' of the original molecule, and which could still perform the original (assayable) function. This was independently investigated, and it was understandably found that the authors' conclusions did not hold water, so to speak⁶¹. Yet from a purely imprinting point of view there is an additional problem with the original report, from a different logical stance to the obvious dilution

conundrum. As a thought experiment, we can momentarily suspend our very justifiable disbelief and imagine that around a macromolecular solute (such as anti-IgE immunoglobulin) a shell of water molecules is formed which is maintained in a stable configuration even in the absence of the solute. But it has been pointed out ⁶² that even if this ‘imprint’ was possible, it would not reproduce the shape (or function) of the anti-IgE, but rather its original target, IgE itself (Therefore the ‘water imprint’ should not trigger basophils in the assay). By the same impeccable logic, a real molecular imprint (as in *Searching for Molecular Solutions* Figs. 7.10; 7.11;) can never hope to reproduce the function of the template target, although it might indeed mimic a natural receptor for the same target molecule, if it exists. In turn, these kinds of thoughts are reminiscent of immunoglobulin anti-idiotypic networks considered in Chapter 3, and ‘internal images’ of molecular shapes recognized by primary antibodies. In a real idiotypic network, an antibody against an internal image of an epitope associated with the original IgE might indeed trigger basophils [▼]. (Although of course, as noted above, an antibody network is not generated by ‘instructive’ mechanisms as for true molecular imprints).

Non-covalent Imprinting

This refers to p. 261 of *Searching for Molecular Solutions* where covalent / non-covalent imprinting methods were noted. A figure (Fig. 7A12.1) providing more detail of the non-covalent process is provided below:

[▼]Apart from the inherent challenge to established scientific principles, another reason for the controversy over the original ‘water memory’ paper ⁶⁰ was that it appeared to give succor to homeopathy, which subscribes to the ultra-dilution principle for medicines. If a ‘water imprint’ itself could imprint water, then the ‘anti-imprint’ itself could generate another imprint, *ad infinitum*.....a sort of homeopathic ‘anti-idiotypic network’. While this of course is nonsensical, an ‘anti-idiotypic’ approach has indeed been used in real molecular imprinting, where the initial imprinted binding cavity is used to specify synthesis of a molecular mimic of the original template ^{63,64} (as noted further below).

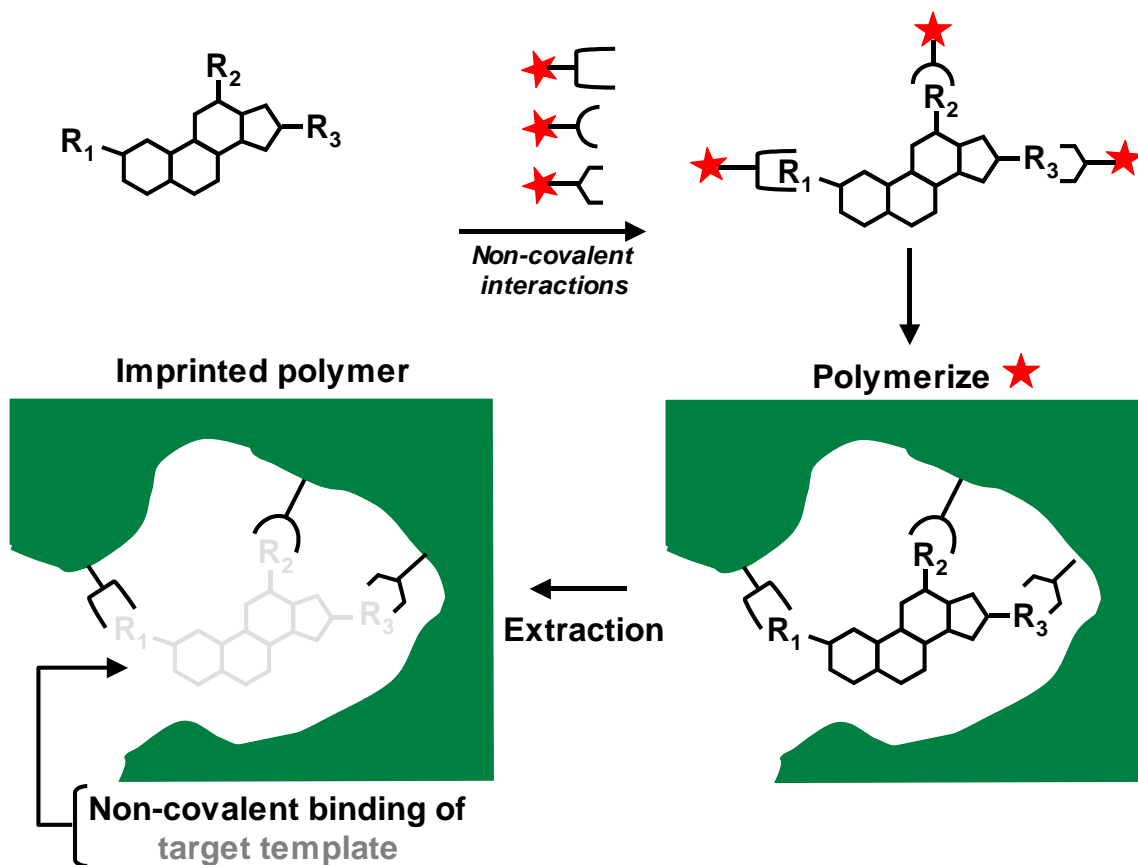


Fig. 7A12.1

Molecular imprinting using non-covalent interactions with the target. Red stars denote chemical groups (containing a C=C double bond) suitable for co-polymerization with monomer and cross-linker used to obtain the polymerized matrix. Non-covalent process uses polymerizable groups which self-assemble into the target template via non-covalent interactions as indicated.

'Anti-idiotypic' Imprinting

This refers to pp. 263-264 of *Searching for Molecular Solutions* where the notion of 'anti-idiotypic' imprinting was described. A figure (Fig. 7A12.2) providing more detail of this process is provided below:

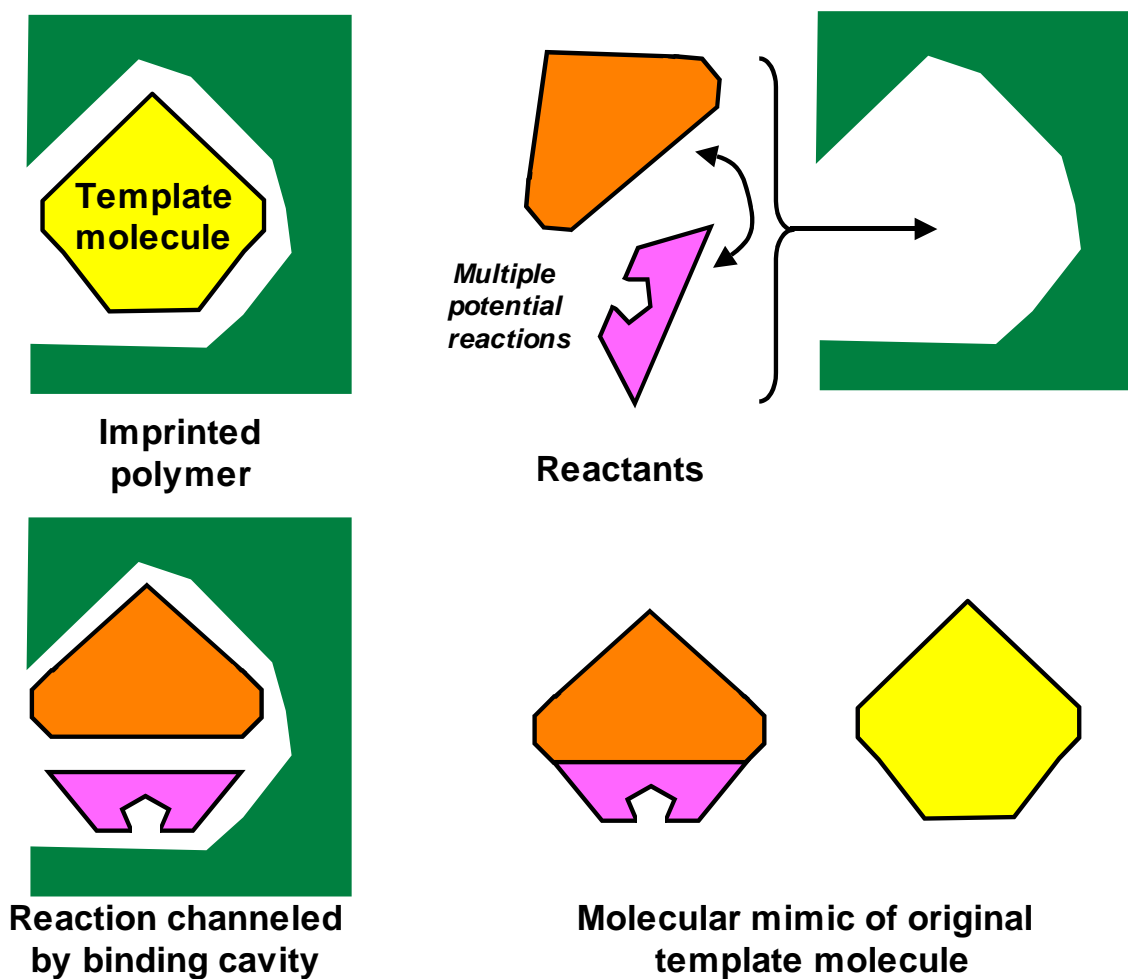


Fig. 7A12.2

Fig. 7A12.2. Synthesis directed by an imprinted polymer binding cavity, or ‘anti-idiotypic’ imprinting. Potential reaction pathways of reactants are channeled in the desired direction through the shape of the imprinted cavity.

‘Induced-Fit’ Binding with Linear Polymers

Let’s briefly consider another interesting avenue towards recognition of proteins by artificial polymers which is allied to this field but technically not an imprinting process as such. If a linear (non-cross-linked) polymer is equipped with side-chain functional groups which can recognize amino acid residues on a protein surface, then it might be expected that a polymer with just the right configuration of functional groups might bind proteins in a specific manner (Fig. 7A12.3). Such polymers might have little or no pre-existing conformation of a defined nature, but assume a specific structure upon wrapping around a protein surface, a form of the ‘induced fit’ concept which we have visited previously in the context of antibodies and aptamers (Chapters 3 and 6). This has been investigated using a limited set of copolymers each composed of three different monomers, showing that certain copolymers of specific compositions exhibit strong differential protein binding ⁶⁵. (In other words, different combinations of polymerized monomers favor some proteins over others by the nature of each protein’s specific surface structural features). Screening of more diverse copolymer libraries may allow definition of ‘induced fit’ binders with desirable specificities ⁶⁵, and rational design of copolymers based on known protein 3-D structures may attain the same end.

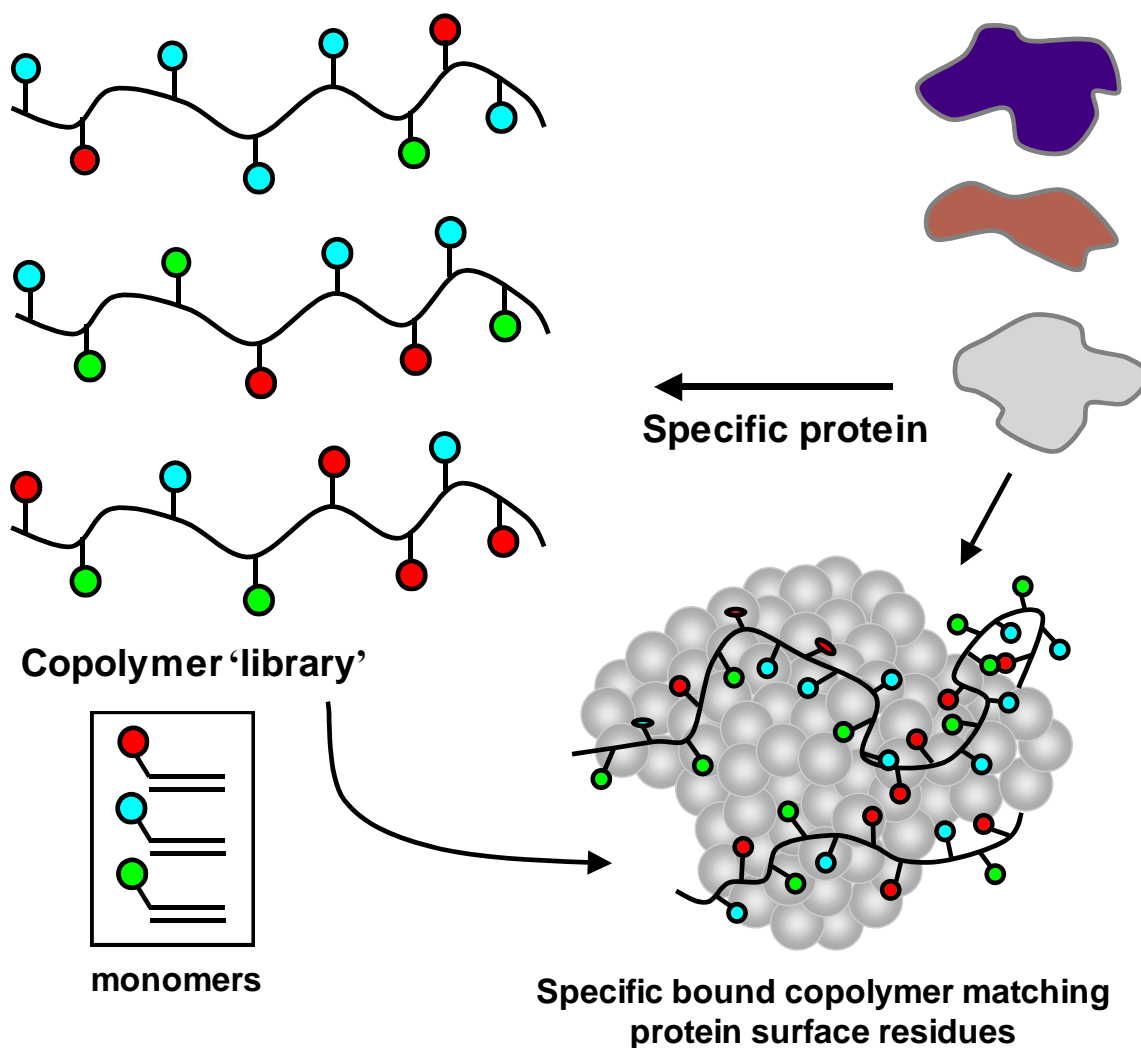


Fig. 7A12.3

Protein binding by linear polymers with different functional groups tailored for interaction with protein surface residues. In this schematic a copolymer of three monomers with different functional group substitutions (tailored for interaction with certain amino acid residues) are copolymerized, resulting in a library of polymers with random monomer sequences. The chemical nature of the functional groups and their relative ratios will favor certain proteins over others, and of these some may interact with specific polymers within the total polymer population.

References:

1. Stocks, M. Intrabodies as drug discovery tools and therapeutics. *Curr Opin Chem Biol* **9**, 359-65 (2005).
2. Uson, I. et al. X-ray crystallography reveals stringent conservation of protein fold after removal of the only disulfide bridge from a stabilized immunoglobulin variable domain. *Fold Des* **2**, 357-61 (1997).
3. Martineau, P., Jones, P. & Winter, G. Expression of an antibody fragment at high levels in the bacterial cytoplasm. *J Mol Biol* **280**, 117-27 (1998).
4. Tavladoraki, P. et al. A single-chain antibody fragment is functionally expressed in the cytoplasm of both *Escherichia coli* and transgenic plants. *Eur J Biochem* **262**, 617-24 (1999).
5. Proba, K., Worn, A., Honegger, A. & Pluckthun, A. Antibody scFv fragments without disulfide bonds made by molecular evolution. *J Mol Biol* **275**, 245-53 (1998).
6. Desiderio, A. et al. A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. *J Mol Biol* **310**, 603-15 (2001).
7. Lee, C. V. et al. High-affinity human antibodies from phage-displayed synthetic Fab libraries with a single framework scaffold. *J Mol Biol* **340**, 1073-93 (2004).
8. Donini, M. et al. Engineering stable cytoplasmic intrabodies with designed specificity. *J Mol Biol* **330**, 323-32 (2003).
9. Ewert, S., Honegger, A. & Pluckthun, A. Stability improvement of antibodies for extracellular and intracellular applications: CDR grafting to stable frameworks and structure-based framework engineering. *Methods* **34**, 184-99 (2004).
10. Philibert, P. et al. A focused antibody library for selecting scFvs expressed at high levels in the cytoplasm. *BMC Biotechnol* **7**, 81 (2007).
11. Auf der Maur, A., Tissot, K. & Barberis, A. Antigen-independent selection of intracellular stable antibody frameworks. *Methods* **34**, 215-24 (2004).
12. Shaki-Loewenstein, S., Zfania, R., Hyland, S., Wels, W. S. & Benhar, I. A universal strategy for stable intracellular antibodies. *J Immunol Methods* **303**, 19-39 (2005).
13. Strube, R. W. & Chen, S. Y. Enhanced intracellular stability of sFv-Fc fusion intrabodies. *Methods* **34**, 179-83 (2004).
14. Visintin, M., Tse, E., Axelson, H., Rabbitts, T. H. & Cattaneo, A. Selection of antibodies for intracellular function using a two-hybrid in vivo system. *Proc Natl Acad Sci U S A* **96**, 11723-8 (1999).

15. Verheesen, P. et al. Prevention of oculopharyngeal muscular dystrophy-associated aggregation of nuclear polyA-binding protein with a single-domain intracellular antibody. *Hum Mol Genet* **15**, 105-11 (2006).
16. Nuttall, S. D. et al. Isolation and characterization of an IgNAR variable domain specific for the human mitochondrial translocase receptor Tom70. *Eur J Biochem* **270**, 3543-54 (2003).
17. Tanaka, T., Lobato, M. N. & Rabbitts, T. H. Single domain intracellular antibodies: a minimal fragment for direct in vivo selection of antigen-specific intrabodies. *J Mol Biol* **331**, 1109-20 (2003).
18. Tanaka, T. & Rabbitts, T. H. Functional intracellular antibody fragments do not require invariant intra-domain disulfide bonds. *J Mol Biol* **376**, 749-57 (2008).
19. Auf Der Maur, A. et al. Direct in vivo screening of intrabody libraries constructed on a highly stable single-chain framework. *J Biol Chem* **277**, 45075-85 (2002).
20. Alberts, B. et al. *Molecular Biology of the Cell. 4th Edition*. (Garland Science, NY, 2004).
21. Cardinale, A., Filesi, I., Mattei, S. & Biocca, S. Intracellular targeting and functional analysis of single-chain Fv fragments in mammalian cells. *Methods* **34**, 171-8 (2004).
22. Manikandan, J., Pushparaj, P. N. & Melendez, A. J. Protein i: interference at protein level by intrabodies. *Front Biosci* **12**, 1344-52 (2007).
23. Lobato, M. N. & Rabbitts, T. H. Intracellular antibodies and challenges facing their use as therapeutic agents. *Trends Mol Med* **9**, 390-6 (2003).
24. Cardinale, A. & Biocca, S. Combating protein misfolding and aggregation by intracellular antibodies. *Curr Mol Med* **8**, 2-11 (2008).
25. Luginbuhl, B. et al. Directed evolution of an anti-prion protein scFv fragment to an affinity of 1 pM and its structural interpretation. *J Mol Biol* **363**, 75-97 (2006).
26. Lecerf, J. M. et al. Human single-chain Fv intrabodies counteract in situ huntingtin aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A* **98**, 4764-9 (2001).
27. Messer, A. & McLearn, J. The therapeutic potential of intrabodies in neurologic disorders: focus on Huntington and Parkinson diseases. *BioDrugs* **20**, 327-33 (2006).
28. Tellez, C., Jean, D. & Bar-Eli, M. Construction and expression of intracellular anti-ATF-1 single chain Fv fragment: a modality to inhibit melanoma tumor growth and metastasis. *Methods* **34**, 233-9 (2004).
29. Williams, B. R. & Zhu, Z. Intrabody-based approaches to cancer therapy: status and prospects. *Curr Med Chem* **13**, 1473-80 (2006).
30. Tanaka, T. & Rabbitts, T. H. Intrabodies based on intracellular capture frameworks that bind the RAS protein with high affinity and impair oncogenic transformation. *Embo J* **22**, 1025-35 (2003).

31. Tanaka, T., Williams, R. L. & Rabbitts, T. H. Tumour prevention by a single antibody domain targeting the interaction of signal transduction proteins with RAS. *Embo J* **26**, 3250-9 (2007).
32. Chen, S. Y., Bagley, J. & Marasco, W. A. Intracellular antibodies as a new class of therapeutic molecules for gene therapy. *Hum Gene Ther* **5**, 595-601 (1994).
33. Mhashilkar, A. M. et al. Intrabody-mediated phenotypic knockout of major histocompatibility complex class I expression in human and monkey cell lines and in primary human keratinocytes. *Gene Ther* **9**, 307-19 (2002).
34. Visintin, M., Melchionna, T., Cannistraci, I. & Cattaneo, A. In vivo selection of intrabodies specifically targeting protein-protein interactions: A general platform for an "undruggable" class of disease targets. *J Biotechnol* **135**, 1-15 (2008).
35. Schultz, P. G. & Lerner, R. A. From molecular diversity to catalysis: lessons from the immune system. *Science* **269**, 1835-42 (1995).
36. Schultz, P. G., Yin, J. & Lerner, R. A. The chemistry of the antibody molecule. *Angew Chem Int Ed Engl* **41**, 4427-37 (2002).
37. Paul, S. et al. Natural catalytic antibodies: peptide-hydrolyzing activities of Bence Jones proteins and VL fragment. *J Biol Chem* **270**, 15257-61 (1995).
38. Sun, M., Gao, Q. S., Kirnarskiy, L., Rees, A. & Paul, S. Cleavage specificity of a proteolytic antibody light chain and effects of the heavy chain variable domain. *J Mol Biol* **271**, 374-85 (1997).
39. Gololobov, G., Sun, M. & Paul, S. Innate antibody catalysis. *Mol Immunol* **36**, 1215-22 (1999).
40. Karataeva, N. A., Buneva, V. N. & Nevinsky, G. A. Polysaccharide kinase activity of human milk IgG antibodies. *Biochemistry (Mosc)* **71**, 1207-21 (2006).
41. Shuster, A. M. et al. DNA hydrolyzing autoantibodies. *Science* **256**, 665-7 (1992).
42. Paul, S., Nishiyama, Y., Planque, S. & Taguchi, H. Theory of proteolytic antibody occurrence. *Immunol Lett* **103**, 8-16 (2006).
43. Zhou, G. W., Guo, J., Huang, W., Fletterick, R. J. & Scanlan, T. S. Crystal structure of a catalytic antibody with a serine protease active site. *Science* **265**, 1059-64 (1994).
44. Wirsching, P., Ashley, J. A., Benkovic, S. J., Janda, K. D. & Lerner, R. A. An unexpectedly efficient catalytic antibody operating by ping-pong and induced fit mechanisms. *Science* **252**, 680-5 (1991).
45. Hanson, C. V., Nishiyama, Y. & Paul, S. Catalytic antibodies and their applications. *Curr Opin Biotechnol* **16**, 631-6 (2005).
46. Gabibov, A. G., Ponomarenko, N. A., Tretyak, E. B., Paltsev, M. A. & Suchkov, S. V. Catalytic autoantibodies in clinical autoimmunity and modern medicine. *Autoimmun Rev* **5**, 324-30 (2006).

47. Bronshtein, I. B. et al. DNA-specific antiidiotypic antibodies in the sera of patients with autoimmune diseases. *FEBS Lett* **314**, 259-63 (1992).
48. Paul, S. et al. Antibodies as defensive enzymes. *Springer Semin Immunopathol* **26**, 485-503 (2005).
49. Cotton, F. A. & Wilkinson, G. *Advanced Inorganic Chemistry. 3rd Edition.* (Interscience / John Wiley & Sons, 1972).
50. Wentworth, A. D., Jones, L. H., Wentworth, P., Jr., Janda, K. D. & Lerner, R. A. Antibodies have the intrinsic capacity to destroy antigens. *Proc Natl Acad Sci U S A* **97**, 10930-5 (2000).
51. Wentworth, P., Jr. et al. Evidence for antibody-catalyzed ozone formation in bacterial killing and inflammation. *Science* **298**, 2195-9 (2002).
52. Wentworth, P., Jr. et al. Antibody catalysis of the oxidation of water. *Science* **293**, 1806-11 (2001).
53. Datta, D., Vaidehi, N., Xu, X. & Goddard, W. A., 3rd. Mechanism for antibody catalysis of the oxidation of water by singlet dioxygen. *Proc Natl Acad Sci U S A* **99**, 2636-41 (2002).
54. Zhu, X. et al. Probing the antibody-catalyzed water-oxidation pathway at atomic resolution. *Proc Natl Acad Sci U S A* **101**, 2247-52 (2004).
55. Steinbeck, M. J., Khan, A. U. & Karnovsky, M. J. Intracellular singlet oxygen generation by phagocytosing neutrophils in response to particles coated with a chemical trap. *J Biol Chem* **267**, 13425-33 (1992).
56. Steinbeck, M. J., Khan, A. U. & Karnovsky, M. J. Extracellular production of singlet oxygen by stimulated macrophages quantified using 9,10-diphenylanthracene and perylene in a polystyrene film. *J Biol Chem* **268**, 15649-54 (1993).
57. Nieva, J. & Wentworth, P., Jr. The antibody-catalyzed water oxidation pathway--a new chemical arm to immune defense? *Trends Biochem Sci* **29**, 274-8 (2004).
58. Babior, B. M., Takeuchi, C., Ruedi, J., Gutierrez, A. & Wentworth, P., Jr. Investigating antibody-catalyzed ozone generation by human neutrophils. *Proc Natl Acad Sci U S A* **100**, 3031-4 (2003).
59. Parren, P. W., Leusen, J. H. & van de Winkel, J. G. Antibody-catalyzed water oxidation: state-of-the-art immunity or ancient history? *Trends Immunol* **24**, 467-9 (2003).
60. Davenas, E. et al. Human basophil degranulation triggered by very dilute antiserum against IgE. *Nature* **333**, 816-8 (1988).
61. Maddox, J., Randi, J. & Stewart, W. W. "High-dilution" experiments a delusion. *Nature* **334**, 287-91 (1988).
62. Ball, P. *H₂O. A biography of water.* (Weidenfeld & Nicolson., 1999).

63. Mosbach, K., Yu, Y., Andersch, J. & Ye, L. Generation of new enzyme inhibitors using imprinted binding sites: the anti-idiotypic approach, a step toward the next generation of molecular imprinting. *J Am Chem Soc* **123**, 12420-1 (2001).
64. Zhang, H., Ye, L. & Mosbach, K. Non-covalent molecular imprinting with emphasis on its application in separation and drug development. *J Mol Recognit* **19**, 248-59 (2006).
65. Koch, S. J., Renner, C., Xie, X. & Schrader, T. Tuning linear copolymers into protein-specific hosts. *Angew Chem Int Ed Engl* **45**, 6352-5 (2006).