

Semi-synthetic antibody molecules having enhanced affinity and selectivity

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1] Field of the Invention, and a note on this paper.

This paper is brief, eliminating or abbreviating much material which would be worthy of discussion in a professional scientific publication. It is intended for a non-specialist reader, though biochemically aware. Some potentially controversial statements are made without explanation or argument to facilitate an initial grasp of the whole picture. Reference is made to higher-level material.

The invention provides for hybrid 'antibody' molecules or conjugates, assembled in such a way that the two or more binding units of the conjugate can be brought to bear simultaneously upon two distinctly different epitopes on the same target, whether that target is a molecule, a cell or another supramolecular entity. This requires greater flexibility than is found between the Fab units of natural antibodies. Binding units of the conjugate are typically but not necessarily different, having distinct intrinsic specificities, and there is no limitation to antibodies as the source material.

It is expected that conjugates made according to the methods of the invention will be prepared extemporaneously, for immediate use, to attack cells in the body such as cancer or other disease-causing cells, and tailored to the exact requirements for an attack on such cells in a particular individual patient, having regard to the epitopes present on those particular clones of undesirable cells which may differ critically from similar cells in another patient. They will also be useful in making chemical reagents of enhanced power to discriminate between similar molecules, for laboratory or industrial use.

2] Background.

a] General Immunochemistry.

Though antibodies are commonly described in elementary texts as being marvellously specific and strong-binding, they are actually quite modest in these properties, most Fab units having affinity constants beginning at about 10^6 and ranging up to 10^9 l/mol. Much experimental effort has been devoted to producing antibody-like molecules with greater affinity, for example phage display may achieve 10^{11} l/mol in favourable cases; still modest compared with biotin-avidin binding. Such constants, involving the reaction of one target molecule with one antibody binding site, are often incorrectly equated with 'intrinsic' affinity constants.

b] Affinity and polyvalency.

The prototypical antibody is bivalent and in some classes of antibody there are two or up to five such bivalent structures in a single antibody molecule. Discussion here concentrates on the simple bivalent kind of molecule but can be readily extended. There must be evolutionary advantage in polyvalency and it is presumably connected

with the structure of many natural targets, yielding enhanced selectivity for a homopolyvalent target, but any proposition of that kind is inherently difficult to prove. The binding sites of a natural antibody are identical and a bivalent molecule is represented here as XX, YY, etc. Corresponding bivalent targets are here represented as xx, yy, etc.

Affinity can only be described simply when it relates to a reaction between two molecules to yield a single product and all remain in solution. Using examples relevant to this present discussion, XX may react with molecules x or xy to yield products XX.x and XX.xy. In each case the reaction is monovalent; one binding site reacts with one epitope. Possibly, too, a monovalent reaction might occur with the bivalent target xx because the second part of the reaction is impossible for steric reasons. That product we write XX.xx. In each case an equation may be written defining an affinity constant in terms of the concentrations [strictly, chemical potentials] of antibody, target and product. If a product can form such that both binding sites of an antibody are able to react simultaneously with both epitopes of a bivalent target, the product must be written differently, to emphasise the pattern of bonding, say, (XX):(xx). We may reasonably expect the affinity constant for the reaction yielding (XX):(xx) to be greater than that which yields XX.xx, and it is still a true affinity constant since the reaction involves two initially independent molecules.

This predicted increase in affinity has been shown experimentally though there is controversy and ample room for confusion. In the immunology literature, commonly, the word 'affinity' is accorded unique status in relation to the monovalent reaction, which leads to the following defects in one publication or another,

- i] describing avidity as if it arose only from polyvalent binding,
- ii] devaluing or failing to grasp the significance of polyvalency per se,
- iii] assuming that binding is independent of other parts of the molecule.

References on the topic may be found at <http://www.trcboyde.net/publications-co-selection-co-bodies-co-affinity.html>. Roughly speaking, increased affinity is indeed found, according to naïve expectation, provided that either the antibody or the target are sufficiently flexible to allow multi-site binding to occur. Binding energies of individual sites are not simply additive.

c] Avidity.

Those who work every day with natural antibodies or antisera are justifiably scornful of those who wish to deal only in terms of affinity. Instead, they use 'avidity', which is a fully valid concept scientifically though highly dependent on operational conditions. Some of the phenomena which add to or distort binding behaviour away from predictions based on monovalent affinity alone are:- i] polyvalency as discussed above; ii] formation of multimolecular complexes in addition to the bimolecular products discussed above; iii] presence in the reagents of several types of antibody of differing intrinsic affinity and other properties; iv] crosslinking reactions of many kinds; v] precipitation of insoluble products; vi] consequential biological reactions.

Affinity-based theory is appropriate for a description of the invention which is the subject matter of this paper. An alternative theory of enhanced selectivity, in avidity terms, will be developed when time permits.

d] Hybrid Antibody Conjugates with two different binding sites.

The first successful attempts to make such things were over forty years ago. All early work was intended to produce hybrid antibodies, eg XY, binding to distinct epitopes, e.g. x and y, on separate targets (bringing them together in a triple complex), so that the product may be written x.XY.y. Many different chemical and biological methods have been used to produce such hybrids. Only in very recent years has the idea been discussed that a flexible conjugate antibody molecule might have certain advantages in reacting with two distinct epitopes on a single target to yield (XY):(xy).

e] The definition of selectivity.

In common use, the word specificity is ambiguous, meaning qualitatively the identity of the target that an antibody reacts with (e.g. X is 'specific for x') or quantitatively that an antibody reacts better than another with one particular target (e.g. X is 'more specific' than Z for target x). For clarity it is best to use different words for these two senses, and it will be appreciated that an element essential for precise expression is a standard for comparison, which in this case is the species named y. Accordingly, a definition of selectivity or 'discriminatory power' is proposed as the ratio of the affinity constants of an antibody or other ligand molecule for two named targets, e.g. x and y. Then the relative selectivities of two different antibodies may be expressed in a precise fashion provided that measurements are made with the same two targets.

f] Selectivity, specificity, and valency of reaction.

It will be obvious that a reaction to produce (XY):(xy), such as in 2[d], introduces an entirely new specificity, namely that for the complete target xy rather than only for either of its component epitopes. When the quantitative measure 'selectivity' is brought into play, we can see that XY should have greater selectivity for the target xy than either XX or YY. It is capable of discriminating for xy in a solution containing free x and/or y.

Important considerations arise about specificity and selectivity of homopolyvalent antibodies. This is explored at <http://www.trcboyde.net/publications-co-selection-co-bodies-co-affinity.html>.

3] Essential content of the present invention.

The first step is to make 'adducts' in which oligonucleotides are attached covalently to antibody fragments or other binding proteins which have been prepared so as to contain only one epitope binding site. We may represent such adducts as X $\div\div\div\div$, Y $\div\div\div\div$, etc.

The nucleotide sequences are so chosen that when two suitable adducts are mixed, hybridization occurs along part only of the oligonucleotide strands, the remaining parts remaining single-stranded and therefore flexible in the reaction product, which is in fact a conjugate, or hybrid antibody, having two distinct intrinsic specificities. Such a conjugate might be written X $\div\div\div\div$ ~~~~~ $\div\div\div\div$ Y, where the duplex is shown as ~~~~~ and the single-stranded segments as $\div\div\div\div$, irrespective of base

sequence. The whole nucleic acid structure is known as a linker. A simple example showing actual base sequences is Xaaaaagggggg.ccccccaaaaY. (The ggg... overlaps and hybridizes with ...ccc.)

Because of the presence of aaaa sequences, which cannot hybridise with anything else, the conjugate is flexible enough to allow a heterobivalent reaction, to produce (XY):(xy), even if the target is completely rigid.

4] Advantages of the invention.

Fab antibody fragments or other binding proteins of low intrinsic affinity [and therefore easily obtainable] may be combined in a conjugate to yield a hybrid of very high affinity indeed, having a novel specificity and greatly enhanced selectivity for the chosen target. Moreover, it turns out that nucleic acid linkers are neat and advantageous in that,

- 1] adducts are rather easily made in high yield, and are easily purified,
- 2] may be of fairly low molecular weight, e.g. about 20kDa,
- 3] readily self-assemble forming a link that is as stable as necessary,
- 4] having also a flexible segment that is as long as necessary.
- 5] Moreover, hybrids can be made with more than 2 Fab's,
- 6] plus additional components including cellular toxins or labels,
- 7] all in a few moments,
- 8] tailored to suit the particular case,
- 9] even within the body, on site, in vivo,
- 10] thus and otherwise avoiding problems of tissue and cellular penetration

The reaction to form a given hybrid from pre-formed adducts is very easy and quick. As indicated, the concept is readily extendable to hybrids made up of more than two components and if a library of adducts is maintained, directed against an appropriate range of target epitopes, then these adducts can be combined (merely by mixing the solutions) to yield a far greater number of hybrids each having its own novel (combined) specificity and very high selectivity for the chosen constellation of epitopes on its heteropolyvalent target. This would permit the instant preparation of a hybrid directed against a peculiar and unusual combination of target epitopes, as might be, for example, borne on the cell surfaces of an individual patient's own cancer; a combination peculiar to himself and for which a pre-formed antibody combination might not be suitable.

Notwithstanding the very high affinities of the hybrids, they may be dismantled merely by a change of ionic concentrations in the solution. And this process itself is reversible: new combinations of adducts may be formed from the original adducts as readily as at first. This is applicable in the laboratory, not within a patient's body where the ionic concentrations are those needed for life.

Another means of dismantling the hybrids is available by the use of restriction enzymes which will attack particular nucleotide sequences, either in the double- or the single-stranded segments, according to the enzyme used. This mode is not reversible.

5] Uses of the invention.

In the development of antibody-based drugs for killing cancer cells, a profound limitation has been the low affinity and selectivity of the antibodies available. In addition to the possibility of unique and novel epitopes, a cancer cell may have a combination of epitopes on its surface which is thought to characterize

that cancer, and yet they are present also at lower density on the surface of normal cells. An antibody against one such epitope is not sufficiently specific as an agent against the cancer although an antibody against a combination of epitopes may well be. Also, molecules bearing candidate epitopes may be present in solution in the body fluids, having been released from cell surfaces; consequently a monospecific antibody administered to the patient will be taken up with reactions against this abundant and readily accessible form of epitope. In each case, the use of the hybrids of the invention will overcome the difficulty because of the presence of several intrinsic specificities and it is preferable that each should be of low intrinsic affinity so as to allow for ready re-association of binding sites that happen to be at first disadvantageously bound. The overall affinity and selectivity can be ramped up without apparent limit.

A significant feature of cancer, adding to the potential usefulness of hybrid antibodies in treatment, is the occurrence of numerous mutations which may have nothing to do with tumour function, but may offer targets for antibody binding. See <http://www.trcboyde.net/nature-of-cancer.html>, which discusses recent references.

Similar considerations apply to non-cancer therapeutic applications and to non-therapeutic applications such as rapid diagnosis of microbial infections.

Immunoassay.

It is now understood that for highly specific assays, two distinct antibodies should be used, one for capture of the target molecule and the other for giving rise to the signal after having bound to the captured target at a different epitope site - 'sandwich' assays. This is an earlier application of the principle that specificity, or as we prefer to say, selectivity, may be improved by the combination of two intrinsic specificities and it is perhaps surprising that it did not lead at once to recognition of selectivity as a concept. Hybrids of this invention may be used in place of other antibodies as one or both slices of bread in the sandwich. Immunoassay is a multi-billion dollar business.

Histopathology.

An antibody construct of enhanced selectivity for a combination of epitopes must eventually overtake the kinds of reagent currently in use. This would include for the recognition of cancers appropriate to be treated with the hybrids of this invention even if for no other applications.

Industry.

Purification of biological molecules is now a multi-billion dollar business in support of pharmaceutical and laboratory applications - perhaps soon more widely. Hybrids of this invention lend themselves to use in this work because they are readily purified before use, highly selective, readily immobilized e.g. on a chromatography column, readily separated from the product or liberated from the column, and readily purified for re-use.

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