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Perspective

Affinity maturation: highlights in the application of *in vitro* strategies for the directed evolution of antibodies

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Affinity maturation is a key technique in protein engineering which is used to improve affinity and binding interactions *in vitro*, a process often required to fulfil the therapeutic potential of antibodies. There are many available display technologies and maturation methods developed over the years, which have been instrumental in the production of therapeutic antibodies. However, due to the inherent limitations in display capacity of these technologies, accommodation of expansive and complex library builds is still a challenge. In this article, we discuss our recent efforts in the affinity maturation of a difficult antibody lineage using an unbiased approach, which sought to explore a larger sequence space through the application of DNA recombination and shuffling techniques across the entire antibody region and selections using ribosome display. We also highlight the key features of several display technologies and diversification methods, and discuss the strategies devised by different groups in response to different challenges. Particular attention is drawn to examples which are aimed at the expansion of sequence, structural or experimental diversity through different means and approaches. Here, we provide our perspectives on these methodologies and the considerations involved in the design of effective strategies for the directed evolution of antibodies.

Antibodies are an increasingly important class of therapeutic molecules. Over the past decades we have seen a tremendous increase in the number of antibody drugs that were approved for clinical use in almost all disease areas, as we learnt to harness their unique properties to address different therapeutic needs [1,2]. There are many ways to generate antibodies *in vivo* and *in vitro*, the most common being immunisation and phage display; but the antibodies isolated from these methods often require extra steps to improve their affinities and/or drug-like properties, to fulfil the potency required in a therapeutic setting. *In vitro* affinity maturation usually involves a diversification of the antibody base sequence, followed by stringent selections to isolate higher-affinity binders, a directed evolution process much like the somatic hypermutation that naturally occurs in mammalian B cells [3]. With a wide choice of available technologies, there is a myriad of possible paths when it comes to affinity maturation, but how does one decide which approach to take?

In a recent report, we described the use of an unbiased approach to affinity maturation for the optimisation of an inhibitory antibody specific to Arginase 2 (ARG2) [4]. The premise of the project was to generate a therapeutic which neutralises the extracellular ARG2 secreted by cancer cells, thought to mediate an immunosuppressive response through the depletion of arginine [5]. In this context, high-affinity binding and a potent inhibitory effect are essential properties for the therapeutic, which is ideal for an antibody approach. An antibody candidate was first isolated from AstraZeneca's naïve phage display libraries [6], which showed specific binding to human ARG2 and inhibitory activity in an enzymatic assay *in vitro* [7]. This antibody was then taken through a comprehensive affinity

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maturation process, in which each of the six complementarity-determining regions (CDRs) were targeted for diversification and taken through selections in parallel. Surprisingly, little improvement in antibody affinity or potency were obtained. Moreover, a random error-prone mutagenesis approach was also unsuccessful, suggesting that this was a difficult antibody to affinity mature.

Given their ineffectiveness, we thought that perhaps the approaches taken, which only focused on small regions of the antibody at a time, were insufficient to produce appreciable improvements to this antibody lineage. Taking inspiration from the somatic recombination process *in vivo*, in which modular segments of immunoglobulin domains are rearranged to generate a diverse repertoire, we used antibody chain shuffling [8–10] and a staggered-extension process (StEP) [11,12] to recombine mutations sampled from all six CDRs (Figure 1). This recombination created fresh combinatorial diversity and produced antibody variants with mutations that span the length of the antibody construct in an unbiased way. Ribosome display was used for the selection of these libraries because it is capable of displaying such diverse library builds. Candidates with the most improved characteristics were identified and subsequently fine-tuned via a pool maturation technique to obtain further affinity gains. This resulted in a panel of high affinities antibodies, with over 50-fold improvements in affinity and potency estimated in the final leads.

Sequence and structural comparison of the parental and affinity-matured antibodies revealed that the antibody has gone through some very extensive changes during the affinity maturation process [4]. Substantial mutations across several regions of the antibody were translated into a large epitope shift that facilitated increases in the interface area and shape complementarity to the antigen, whilst preserving the key contacts of a hydrophobic cleft that is essential for its inhibitory mechanism [7]. Essentially, these changes have enabled the antibody to re-orient itself into a position allowing for superior binding without changing the observed mechanism of action, and in doing so seemed to have overcome some initial limitations of the parent. Indeed, there is evidence to suggest significant negative cooperativity in the binding mode of the parent antibody to trimeric ARG2, which was aptly resolved after affinity maturation. Such issues were unlikely to be overcome by small or focused changes, which might have been why the standard methods were ineffective. In contrast, the multiple changes applied simultaneously across the antibody provided sufficient scope to explore a larger region of combinatorial space, to escape the restrictions of the parental antibody and provide an improved binding solution.

It is extraordinary how such a solution would have presented itself through selections; nor could we have predicted or designed this. Based on the structural data, we might have attempted to improve the binding interactions at or around the key contacts of the hydrophobic cleft, which would have been unproductive or counterproductive. Indeed, our results suggested that mutations to CDRH3 of the antibody, which formed a large part of the hydrophobic cleft, were not tolerated and were rapidly eliminated during selections of the unbiased recombination libraries. There is existing evidence to suggest that feasible regions for affinity maturation are often not involved in key contacts, but lie in positions that provide indirect effects or establish fresh new interactions with its antigen [13–15]. Moreover, improved affinities can arise from multiple diverse mechanisms which are often unpredictable [15–17]. *In vitro* selections provide a way to probe the vast number of binding possibilities, with the potential to find the best available binding solution without requiring prior assumptions or dictation.

Given its utility, it is important that efforts are made to find the most suitable affinity maturation strategy for any given application. A diagram highlighting several features and considerations in the use of different technologies is shown in Figure 2. Generally, a key objective of affinity maturation is to maximise the sequence diversity of the initial library repertoire, as this would be translated to a higher structural diversity from which superior binders may be selected. However, we are often limited by the display capacity of the technology used. Methods with a cellular requirement, such as phage and yeast display, have a lower display size (typically 10^8–10^9) due to limitations in transformation efficiency [18–23]. It is not possible to sample every mutation at every position at the same time, which would require a theoretical capacity of ~10^{78} for ~60 positional variants across six CDRs. Besides the common compromise of limiting mutations to certain CDRs, there have been reports of alternative strategies to overcome this obstacle. Tiller *et. al.* recognised that combinations of mutations from different CDRs is important at an early stage [13]. To accommodate early re-combinatorial changes, they used alanine scanning to narrow down the permissive sites for mutagenesis, before generating libraries with restricted amino acid variants based on natural CDR diversity. Similarly, the 'look-through mutagenesis' method restricts the number of amino acid variants to nine representative residues based on side-chain chemistry, which allowed for the simultaneous mutation of positions in up to three CDRs [24]. Based on amino acid

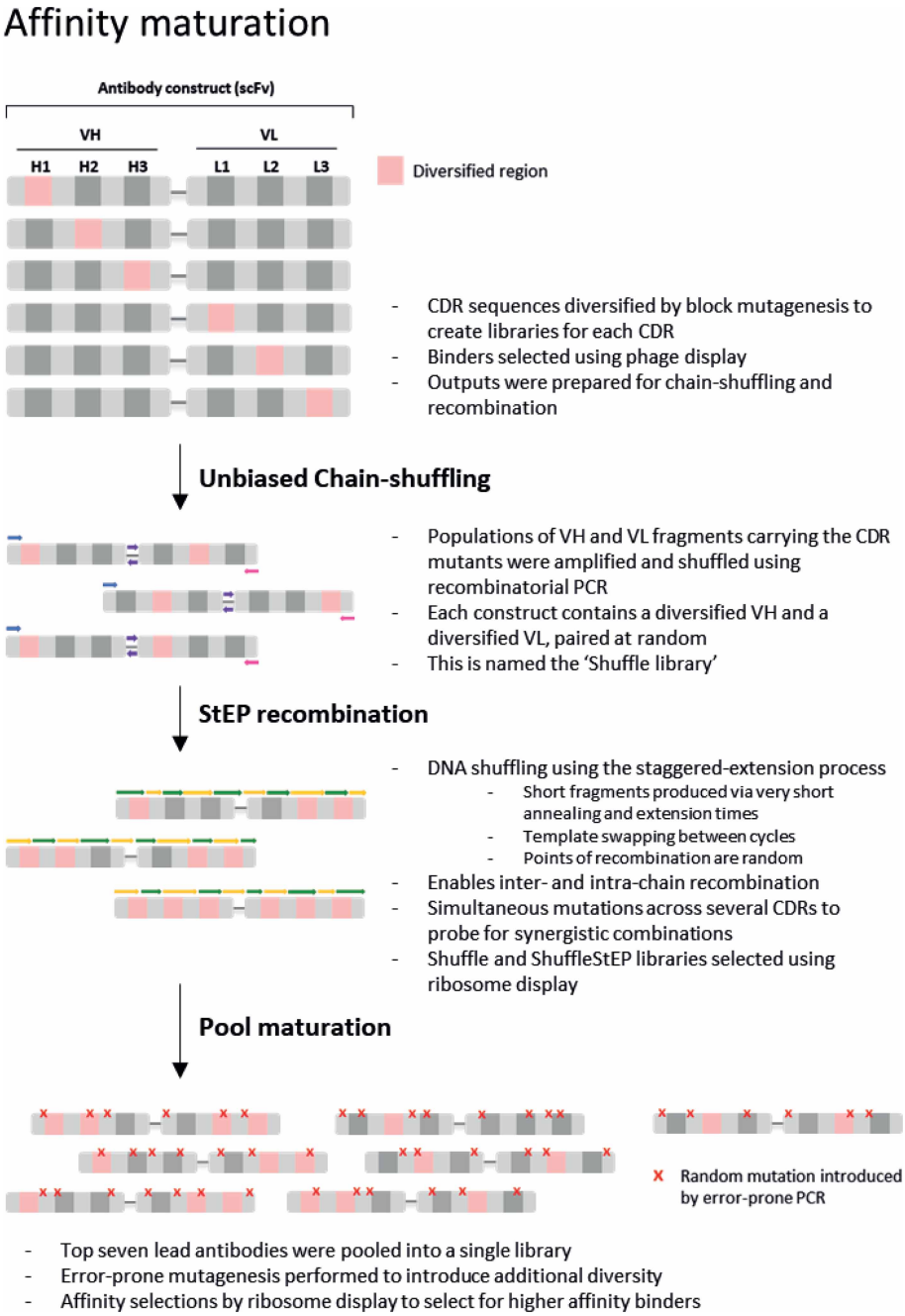

Figure 1. Overview of the affinity maturation process in the optimisation of an ARG2-inhibitory antibody.
The antibody construct, in the single-chain variable fragment (scFv) format, was diversified and recombined in a comprehensive affinity maturation process. Mutations sampled from all six CDRs were recombined and shuffled in an unbiased way via chain-shuffling and StEP recombination, and selected using ribosome display. The most improved leads were then pooled and diversified by error-prone PCR, which introduced random mutations through the length of the construct. The resulting antibodies from these selections have a high number of mutations, which are scattered throughout the length of the constructs and across different CDRs.

usage analyses, Gonzalez-Munoz et al. [25] selected a subset of seven amino acid variants that were typically over-presented from large datasets of affinity-matured antibodies. This tailored diversification approach allowed them to cover more positional ground with fewer library builds, with comparable effectiveness to full amino acid randomisation.

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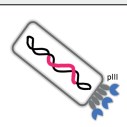
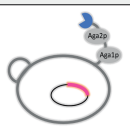
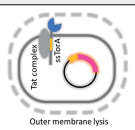
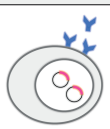
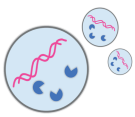
A

	Cellular Requirement				*DNA / RNA template*		
	Phage Display	**Yeast Display**	**Bacterial (*E. coli*) Display**	**Mammalian Cell Display**	***In vitro* compartmentalisation**	**Ribosome display**	**mRNA Display**
Genotype-phenotype Linkage							
	Genetic fusion to Protein III, expressed on the surface of M13 phage	Display on yeast cell surface via fusion with cell-surface anchor proteins (e.g. agglutinin protein Aga2p)	Protein anchored on inner periplasmic membrane via the Tat transport system & signal peptide ssTorA. Outer membrane is lysed	Episomal vectors transfected into mammalian cells (e.g. HEKs) expressing IgGs on cell surface	Physical encapsulation of DNA/RNA template with protein expressed in artificial cells (e.g. emulsion droplets)	mRNA and translated protein linked via a ribosome stalled in translation	Covalent attachment of nascent peptide chain to mRNA via a 3' puromycin
Typical Display Size	~10^8 - 10^{10}	~10^7 - 10^9	~10^9	~10^7	~10^8	~10^{12} - 10^{13}	~10^{12} - 10^{13}
Features and Considerations	Robust and well-established technology; phage stable and tolerant to selections under harsh conditions	Selection of binders via fluorescence activated sorting (FACS) allows for multi-parameter screening (e.g. binding & expression)	Displayed proteins are generally well-folded due to quality control mechanism of Tat pathway. Other formats (e.g. with chaperones) available	Facile Induction of somatic hypermutation via AID; simultaneous display and secretion of IgGs, no reformatting requirements	Multiple formats available; e.g. microbead, DNA, SNAP display etc. *In vitro* transcription-translation; no selection bias	Large display capacity to accommodate complex library builds, cell-independent and no selection bias, directed evolution	Stable covalent linkage enabling selections under wide range of conditions, large library size and no selection bias
	Low display capacity. Selection bias favoring selection of high-expressing clones (as opposed to strong binders)	Low transformation efficiency leading to limited library size	Low transformation efficiency. Lysis requirement lowers *E. coli* stability which can restrict selection conditions and efficiency	Practical limitations in cell handling and transfection densities leading to small display size	Library size must be kept low to ensure spatial segregation of individual clones	Possible affinity ceiling; low-affinity variants may not be selected	Potential difficulties in displaying large proteins and membrane proteins
Ref	18, 19	20, 21	23	44-47	22	26, 27	28, 29

B

	Error-prone mutagenesis	**Saturation / Block Mutagenesis**	**CDR recombination**	**DNA Shuffling / StEP**	**Computational Approaches**
Description	PCR under conditions which lowers fidelity of *Taq* polymerase, resulting in point mutations scattered across length of construct	Mutagenic oligonucleotides with degenerate codons used for sequence randomisation, usually in blocks of 6-10 residues at a time	Recombination of mutations from two or more selected CDRs, using standard molecular biology methods	Random shuffling of DNA segments between populations of mutational variants, producing fresh combinations	Design of mutational changes to optimise binding interactions based on protein modelling and predictions
Approach	Random, unbiased	Targeted, focused	Combinatorial	Random, unbiased	Rational design, focused
Features and considerations	Adjustable mutation rates. Usually used as a gentle method of mutagenesis	Deep scanning of amino acid repertoire, but typically focused on small regions of the antibody at a time	Explores across a larger sequence space. However mutational effects not always synergistic or additive	Reshuffling of diversity gives chance of producing unexpected synergy. Large display capacity required	Structural information and advanced design methodologies required

Figure 2. Comparison of (A) different display technologies and (B) *in vitro* diversification and maturation methods.
A variety of technologies and methods with different strengths and limitations; features which may guide the design of an effective affinity maturation strategy.

Cell-free systems such as ribosome and mRNA display have a much higher display capacity in the range of 10^{12}–10^{13} [26–29], and are favoured methods for the exploration of larger sequence space. Direct comparisons of phage and ribosome display methods have suggested higher diversity and affinity gains in the outputs of the latter [30,31], which lends itself to a broader range of applications. A recent study described the use of insertion and deletion (InDel) mutagenesis to create large diversified libraries in the affinity maturation of an anti-IL-13 antibody [32]. Random in-frame InDels were introduced using a transposon-based system and selected using ribosome display, which uncovered positions of tolerance and allowed for the exploration of loop length

variation on maturation outcomes. This is a particularly interesting application as increasing evidence have shown the importance of InDels in antibody maturation, both *in vivo* and *in vitro* [33–35]. Lengths of CDRs, particularly that of CDR3, vary considerably in nature [36–39], yet much of our focus during antibody engineering have tended to remain on point substitutions. There is evidence that unconventional loop lengths may confer advantages for antibodies against challenging antigens, such as G-protein-coupled receptors (GPCRs) [40,41] and rapidly evolving pathogens (influenza and HIV-1) [35,42]. The likely expansion of structural or conformational diversity that may be attained through length diversification makes it an attractive strategy for affinity maturation.

Ribosome display is also ideal for the selection of libraries diversified through DNA shuffling. In one example, this was achieved through the random digestion by DNAse I followed by enzymatic ligation to recombine point mutations accumulated from error-prone selections [43], with interesting parallels to our approach. Such recombination and shuffling methods have the potential to eliminate deleterious mutations, as a result of backcrossing with original template DNA segments in the sequence pool. Moreover, spontaneous mutations which occur through the numerous amplification steps through the selection and recovery cycles further adds to the diversity, promoting the simultaneous evolution of non-targeted regions, with favourable implications for directed evolution.

Progressive or continuous diversification at the same time as selections is a clear advantage for *in vitro* maturation, as it allows for the gradual emergence of epistatic and synergistic mutations, lessening the demand on the diversity of the initial library repertoire. This is exemplified in the case of mammalian cell display, which, despite having a small display size, can be induced to diversify *in vitro* through the addition of activation-induced cytidine deaminase (AID) [44–47]. Full-length IgG or Fabs are typically expressed on the mammalian cell surface, and between rounds of selections and sorting, AID can be introduced to induce somatic hypermutation *in situ*, without further requirements for reformatting or library builds. This can be viewed as an evolving library; with diversification concurrent with selections. Advancements in the use of gene editing techniques have also allowed for a more directed approach. A recent report describes the use of TALE nucleases and CRISPR-Cas9 to promote site-specific integration of antibody gene populations; allowing for the creation of large diversified libraries which were successfully used to affinity mature a PD1-blocking antibody using mammalian cell display [48].

The increased capacity for diversification is particularly advantageous for optimisation strategies seeking to cover a broader ground. While it is widely established that CDRs are major determinants of antigen recognition, there is evidence to suggest that framework (FR) regions may also play an important role. In particular, a loop identified in the FR-3 region has been known to exhibit CDR-like characteristics, in terms of sequence and structural diversity as well as antigen binding, and is sometimes referred to as the 'CDR4' [49–52]. Other FR regions in the VH and VL domains may also contribute to antigen binding, through direct antigen contact or distal effects [53–55]. Pairings of different VH and VL frameworks has been shown to affect antigen and Fc receptor (FcR) engagement [56], and conversely isotype selection for the constant region can also influence antigen binding [57–59]. Such observations remind us to think of an antibody as a whole protein during engineering, with interconnected domains that can exert influence on each other [60].

The numerous display and maturation strategies, each with their own unique features, provide us with an extensive toolkit to fulfil our antibody engineering needs. Exploration of a larger experimental and sequence space has the clear ability to provide a wider range of conceivable binding solutions, with the potential to deliver greater improvements. On the other hand it can also lead to increased functional divergence or thermostability trade-offs, which may require additional screening steps or compensatory mechanisms [61]. As exemplified by the many different approaches devised in response to different challenges, it is about choosing the right strategy for the antibody lineage under examination. Assessment of antigenic properties, choice of an optimal diversification strategy paired with appropriate display and screening methods, adaptation of selection strategy as the protein evolves; these are all important considerations which are key to the success of affinity maturation.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

Both authors contributed to the design, writing and revision of the manuscript.

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Abbreviations

AID, activation-induced cytidine deaminase; ARG2, Arginase 2; CDRs, complementarity-determining regions; Fab, antigen-binding fragment; FcR, Fc receptor; FR, Framework; GPCR, G-protein-coupled receptors; IgG, immunoglobulin; InDels, insertions and deletions; scFv, single-chain variable fragment; StEP, staggered-extension process.

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