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Review

Trends in therapeutic antibody affinity maturation: From *in-vitro* towards next-generation sequencing approaches

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ABSTRACT

Current advances in antibody engineering driving the strongest growth area in biotherapeutic agents development. Affinity improvement that is mainly important for biological activity and clinical efficacy of therapeutic antibodies, has still remained a challenging task. In the human body, during a course of immune response affinity maturation increase antibody activity by several rounds of somatic hypermutation and clonal selection in the germinal center. The final outputs are antibodies representing higher affinity and specificity against a particular antigen. In the realm of biotechnology, exploring of mutations which improve antibody affinity while preserving its specificity and stability is an extremely time-consuming and laborious process. Recent advances in computational algorithms and DNA sequencing technologies help researchers to redesign antibody structure to achieve desired properties such as improved binding affinity. In this review, we briefly described the principle of affinity maturation and different corresponding *in vitro* techniques. Also, we recapitulated the most recent advancements in the field of antibody affinity maturation including computational approaches and next-generation sequencing (NGS).

1. Introduction

During the last decades, monoclonal antibodies (mAbs) have been developed as key therapeutic agents for treating several diseases [1]. To date, more than 50 recombinant mAbs have been approved by the Food and Drug Administration (FDA). Moreover, there are over 570 therapeutic antibodies that are at the various phases of clinical validation [2]. The ability of antibodies to accurately recognize a specific part of a given antigen makes them ideal agents for therapeutic applications [3]. In general, therapeutic antibodies are comprehensively engineered to have optimized biological and physicochemical properties with regards to affinity, specificity, and stability [4]. The successful development of a therapeutic mAb mainly entails sequences humanization and affinity maturation [5]. The methods for antibody affinity maturation are extensively studied and still it is an active filed of research. This is probably due to the concept that enhancing the affinity of an antibody probably elicit higher biological activity which in turn would decrease dose of injections, limit related sides effects and finally reduce pharmaceutical costs [6].

In the human body, during a course of immune response affinity maturation increases antibody activity by several rounds of somatic hypermutation and clonal selection in the germinal center [7,8]. The final outputs are antibodies representing higher affinity and specificity against a particular antigen. In the pharmaceutical industry, *in vitro* mutagenesis is a widely used strategy for enhancing the affinity of therapeutic antibodies. Mainly, there are two approaches for *in vitro* mutagenesis; targeted and random mutagenesis. In targeted mutagenesis a set of selected residues within the complementarity determining region (CDR) loops of an antibody would be mutated. In random mutagenesis, sequences of variable fragment (Fv) would be mutated randomly [9]. A major shortcoming to these methods is limited size of variant library. Indeed, it is not feasible to produce all possible combinations of CDR mutant forms [10]. In addition, there is no guaranty to assure that replacing residues of the CDR will improve binding affinity. Therefore, it is difficult to identify which peripheral residues should be considered for mutagenesis [11].

Computational approaches have been widely accepted as useful tools for antibody engineering. These methods assist researchers to

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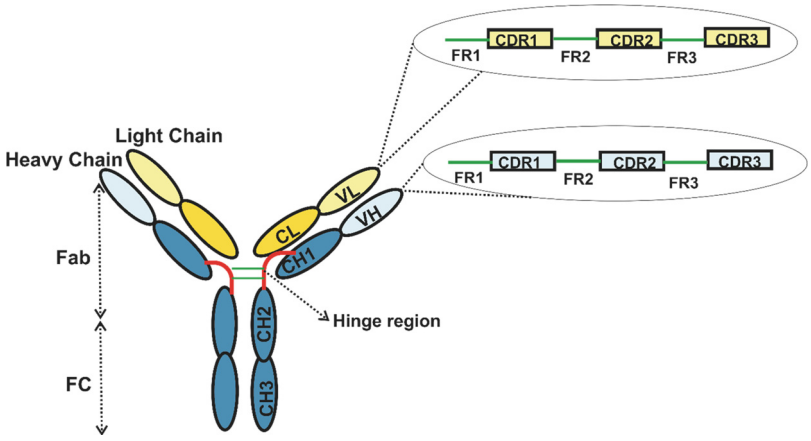

Fig. 1. Schematic structure of a whole antibody. An antibody molecule is composed of two light chain and heavy chain that form "Y" shape. Each chain contains a variable domain and a constant domain. The variable parts of antibodies are responsible to recognize antigens.

screen libraries and also to optimize antibody pharmacokinetic properties such affinity, specificity as well as stability [12]. Although *in silico* methods are generally not regarded as an alternative for "wet lab" experiments, but they can help to produce testable assumptions. In the context of antibody affinity maturation, the rational methods facilitate targeting specific epitopes of interest and evaluating multiple biophysical properties through screening of libraries.

In this review, we briefly described the principle of antibody affinity maturation and different corresponding *in vitro* techniques. Also, we recapitulated the most recent advancements in the field of antibody affinity maturation including computational approaches and NGS.

2. Structure of antibody

An antibody is composed of four polypeptide chains: two homologous heavy (H) and two uniform light (L) chains [13]. The heavy chain consists of a variable domain (VH) and three constant domains (CH1, CH2, and CH3) [14]. The light chain is composed of a variable domain (VL) and only one constant domain (CL) (Fig. 1) [14]. Heavy and light chains join together by several disulfide bonds (Fig. 1) and form a basic Y-shaped structure. The two 'arms' of the Y-shaped antibody molecule are known as fragment antigen binding (Fab) region [15]. Each Fab consists of two variable domains (VH and VL) at the N-terminus and two constant domains (CH1 and CL) at the C-terminal region. The two VH and VL domains form the FV fragment which is responsible for antigen binding interactions [16]. The FV fragment contains six CDR loops, three in the light chain (CDR-L1, CDR-L2, CDR-L3) and three in the heavy chain (CDR-H1, CDR-H2, CDR–H3) which are supported by highly conserved β-sheet frameworks [17]. Although the variable structures are extremely conserved, the CDR loops, particularly CDR-H3, differ extensively both in terms of sequence and structure [18]. All CDRs are being folded in order to make "paratopes", the antigen binding sites [19,20]. Due to its structural diversity, the CDR-H3 loop of antibodies plays a pivotal role in antigen recognition and is a main contributor to binding strength [21].

The heavy chain CH2 and CH3 domains constitute the Fc region. This region is responsible for mediating antibody-dependent cytotoxicity effects including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC). In addition, the Fc region is critical for increasing antibody stability and contributes to the considerable long half-life of antibodies [22,23].

3. Naïve antibody affinity maturation

Antibody function depends on their binding characteristics. Affinity and avidity are the two main determinants of antibody-antigen binding. Affinity refers to specific binding strength between antibody paratopes and corresponding epitopes within an antigen [24]. Avidity is defined as the overall number of antibody binding sites as well as the number of epitopes in a single antigen [25].

The capability of the humoral immune system to produce high-affinity antibodies is based on its ability to generate a large repertoires of B-cells. Such repertoires include extensive collection of antibodies with different binding affinity and specificity [26]. There are two main mechanisms for affinity maturation of naïve (germline, unmutated) antibody; somatic hypermutation (SHM) and clonal selection. With second exposures to the same antigen, a naive B cell would enter to germinal center and undergoes SHM in the CDR of the immunoglobulin genes. Consequently, the mutations modify binding specificity and affinity of antibodies [27]. Later in clonal selection process (Fig. 2), follicular dendritic cells present the antigen to the B cells that have undergone SHM. Through the positive selection process, only B cell progeny producing antibody with highest affinity for the antigen are preferred for selection and then survived. The B cell progeny undergone SHM but have not optimal affinity to the antigen will be out-competed and then discarded [26,27].

4. Affinity maturation of therapeutic antibodies

Engineering of therapeutic antibodies to optimize their binding affinity can decrease injection dose, limit adverse effects, and significantly reduce cost of therapy [28–30]. In general, therapeutic antibodies are expected to have strong affinity in the range of 1 nM or less for binding to their specific epitopes. Therefore, it is common that they undergo one or numerous cycles of affinity maturation to achieve desired affinity [31].

The main sources of the industrial therapeutic antibodies are transgenic mice, humanized antibodies and recombinant antibodies originated from phage display [32]. The benefit of transgenic antibodies over those that obtained from phage display libraries is that they had been undergone *in vivo* affinity maturation through multiple rounds of somatic SHM [33]. This process generally obviates the requirement for further *in vitro* affinity maturation and thereby it shortens antibody development timeline in the laboratory. Sometimes, an *in vivo*-derived antibody may has no optimal affinity to the relevant antigen. So, it should be engineered by *in vitro* methods to obtain desired affinity [34]. Moreover, it is acknowledged that humanization of non-human antibodies could lead to a decrease in their binding affinity [35]. Therefore, humanized antibodies may require further *in vitro* process in order to enhance their affinity.

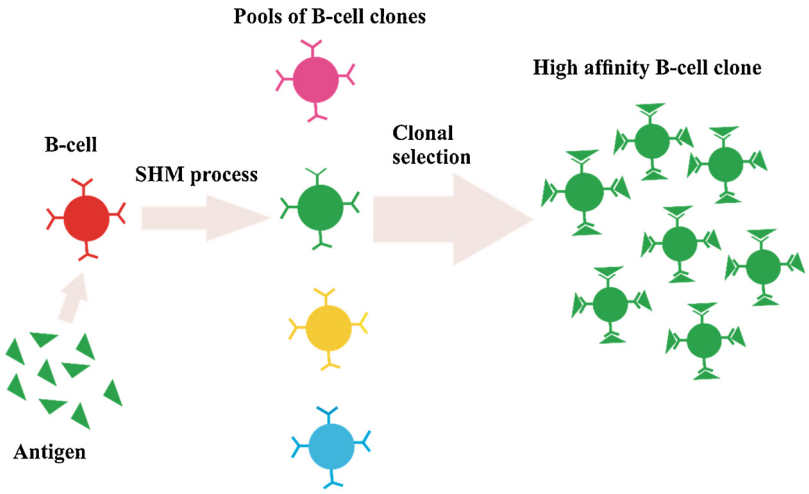

Fig. 2. Clonal Selection. In the process, an antigen will be present to the B cells that had undergone SHM. By positive selection process, B cell progeny producing antibody with highest affinity for the antigen are preferred for selection and then survived. The B cell progeny that had undergone SHM but have low affinity to the antigen will be out-competed and would be discarded.

4.1. Approaches of in vitro antibody affinity maturation

To date, two methods have been developed for *in vitro* affinity maturation of antibodies including random and targeted mutagenesis. Random mutagenesis is incredibly a powerful tool to produce a wide range of mutant from native antibodies. In this method, saturation mutagenesis or error-prone PCR are used to introduce mutations into CDR sequence [36,37]. Targeted mutagenesis method applies alanine-scanning or site-directed mutagenesis to make a small size library of the specific variants [37]. After mutagenesis, an affinity screening procedure will be done through a display panning technology to select the best matured antibody. The most common display technology is phage display, however, a variety of other display systems have been developed such as yeast surface display, ribosome display, and *E. coli* surface display [38]. The combinatorial strategies in antibody affinity maturation require a relatively large size library. However, the size of library is almost limited to a small number of successful cellular transformations [39]. Cell-free ribosome display systems can display larger libraries up to 10^4 members and identify antibodies with picomolar affinity [40].

There are several studies used mutagenesis approaches to improve binding affinity of therapeutic mAbs. Larsen et al. [41] applied *in vitro* mutagenesis methods with surface plasmon resonance screening techniques for affinity maturation of belatacept. This is an anti-CTLA4 (CD158) antibody for treatment of rheumatoid arthritis disease. They showed that substitutions of the two amino acids at CDRs of the antibody, significantly improved its affinity for binding to both CD80 and CD86. Also, these substitutions increased antibody related T cell inhibition over ten-fold.

Wu et al. [42] experimentally changed 13 amino acid residues in palivizumab sequence to increase the affinity. The affinity matured antibody was called Motavizumab. It is a monoclonal antibody for prophylaxis against respiratory syncytial virus (RSV). Although their findings showed a remarkably improved affinity of the antibody, its potency against RSV reduced as 26% in phase III clinical trials [42,43]. Therefore, one should keep in mind that an improved affinity would not always lead to higher clinical efficacy. A list of studies [44–49] used *in vitro* approaches for affinity maturation is presented in the Table 1.

4.2. Challenges in experimental antibody affinity maturation

It is noteworthy to mention outstanding challenges related to *in vitro* affinity maturation of antibodies. However, it is possible to apply saturation mutagenesis to investigate all possible single mutation in antibody CDRs, single mutations do not lead to significant gains in affinity. Thus, it is needed to make sub-libraries in order to have combinations of single mutations that cooperatively result in a substantial increase in binding affinity. Making such libraries is very time consuming and hard labor process [50].

With the site-directed mutagenesis approach, researchers try to find and mutate residues possibly involve in antibody-antigen interaction. Indeed, the actual binding site generally includes multiple CDRs and precise mapping of the paratropes is not a trivial task. Nevertheless, there is no assurance if the substitution of interacting residues result in enhanced binding affinity or not [11].

The serious challenge of *in vitro* approaches is identifying of a highly optimized target from a large size library. For instance, a library size of greater than 10^{39} would be needed to encompass all possible combinations of single and multiple mutations at all CDRs residues of a usual variable domains. Therefore, it is unfeasible to assess all possible models. Hence, only an extremely small subsection of the possible single and multiple mutations could be evaluated by display approaches [39,51,52].

The last but not the least challenge during *in vitro* affinity maturation process is the reduction of specificity and stability of the antibodies. During affinity maturation process, the number of highly interactive residues such as aromatic amino acids would increase at the CDR regions. These kind of amino acids could enhance the risk of non-specific binding [53–55].

Table 1
Affinity matured therapeutic antibodies by different *in vitro* approaches.

Antibody	Mutagenesis approach	Screening method	Fold of affinity improvement (KD)	Year	Ref.
Anti-CD22	Randomizing hotspots mutagenesis	–	7 fold	2004	[44]
Anti-TNFα(tumor necrosis factorα)	Saturation mutagenesis	ELISA	500 fold	2012	[45]
Anti-TfR (transferrin receptor)	Error Prone PCR	Yeast Display system	3–7 fold	2012	[46]
Anti-Fas single chain variable fragment (scFv)	Random mutagenesis	Yeast Display system	22 fold	2008	[47]
Anti-ErbB2 (receptor tyrosine kinase 2)	Saturation mutagenesis	phage display	158 fold	2015	[48]
Antibody fab fragments	Error Prone PCR	Yeast display system	10 fold	2003	[49]

5. *In silico* antibody affinity maturation

One of the most important advantages of computer-aided methods is their ability to search all designed variants in a virtual library (~10^{40} members) in a short time and a cost-effective manner. This is while, in experiment screening processes of a library containing 10^{10} sequences will last for several weeks [56,57]. Another benefit of *in silico* analysis methods is a better understanding of antibody-antigen interactions and structural analysis through different algorithms [12,57]. Whenever a three-dimensional crystallographic structure of antigen-antibody is available, it is possible to identify residues involved in intermolecular interaction. These data will help the researchers to select candidate residues suitable for mutagenesis in order to improve affinity [11,58]. On the other hand, a considerable number of modeling and simulation software are available to predict the 3D structure of antibody- antigen structure if the crystal structure of the molecules have not been determined [12].

5.1. Principles of in silico antibody affinity maturation

Recent advances in computational prediction of antibody 3D structure has led to developing several useful methods for redesigning of antibodies with improved biophysical properties [59–61]. These approaches are based on screening a large number of models and selecting the most optimized ones. Principle of the behind statistical models is standing on different exhaustive algorithms including Monte Carlo and Dead-End Elimination (DEE). Monte Carlo-based algorithm has been widely used to screen libraries containing a large number of different residues with several conformations. Taking advantages of the algorithm, mutants could be rapidly screened based on energy estimation and biophysical properties [62]. DEE is also a great algorithm applied for efficient sampling of amino acids and side chain rotamers to decrease the search space of structure-based protein design [63,64]. The next critical step is quality filtration based on energy assessments such as solvent treatment and electrostatic interactions. The Poisson-Boltzmann equation is commonly used algorithm for energy evaluation in the search algorithms [65].

5.2. General approaches in computational antibody affinity maturation

The general strategy for rational design is to generate considerable subsets of antibodies and then using computational search algorithms to select a small number of optimized models for further assessment.

The essential criteria that would be considered in antibody engineering are physicochemical properties and stabilizing energies of each residues [66]. Having a comprehensive understanding of the CDR residues roles is fundamental in antibody rational engineering [67]. Commonly, information about the CDR residues is obtained by analyzing 3D structure of an antibody in complex with antigen [68]. In fact, accurate computational modeling of antibody loop regions is quite challenging and difficult. Five of six CDR loops (CDR-L1, CDR-L2, CDR-L3, CDR-H1 and CDR-H2) generally assume to have well-characterized canonical conformations. Therefore, it is possible to get reliable sequence-based structure predictions for these CDR loops [69]. In contrast to these canonical loops, CDR-H3 has non-canonical conformation and comprises more diverse structures [70]. Thus, computational modeling of the non-canonical loop is problematic which requires more optimization strategies [71].

After modeling of antibody and antigen, it needs to specify residues that have role in the binding interactions between antibody and antigens. Whenever the 3D structure of an antibody-antigen complex is not determined, docking methods can be applied to predict the binding interactions [72]. Now days, computational docking analysis become routine step in antibody-antigen interaction analysis. Docking analysis measures the free energy difference between the native and bound state of antibody and antigen [73].

Molecular dynamics (MD) simulation is a computational tool which simulates the dynamic behavior of molecular systems as a function of time, considering all the components in the simulation box as flexible [74]. By using different force fields, MD can simulate time-dependent trajectories of antibody structures and provide alternative candidates that can be examined through further experimental assessments [75].

The following are examples of studies used MD simulations in their experiments to engineer the affinity of antibodies. Clark et al. [76] applied an *in silico* affinity maturation approach to engineer a therapeutic antibody targeting integrin-1 (VLA1). In the experiment, they found an improved kinetic affinity (KD) of the antibody by 10-fold using CHARMM force field during 10 ps (ps). Barderas et al. [11] reported 454-fold improvement in affinity of a mutant antibody over the parental molecule that achieved with a structure-based computational method. Ahmed et al. [77] enhanced affinity of anti- Ganglioside GD2 murine MoAb 3F8 antibody based on *in silico* scanning mutagenesis using CHARMM force field methods. The affinity improvement led to 9-fold increased antibody-dependent cell-mediated cytotoxicity on GD2-positive tumor cell lines.

Recently emerging software such as GROMACS provides more accurate and longtime evaluation of antibody-antigen complexes. This software assists researchers to gain further details about binding interactions, stability and make calculation of non-covalent energies (hydrophobic, electrostatic, non-polar and binding energy) easier [78]. Corrada et al. [79] examined internal energetics and dynamics of mutated anti-VEGF antibodies with MD simulation by GROMACS. They found modulation of internal stabilizing energy and variation in the interaction energy are correlated with experimentally determined affinities. Accordingly, the group introduced a method to investigate the effects of mutations on stability, conformational properties and affinity towards the antigen. Mahajan et al. [57] enhanced KD affinity by 202-fold of an antibody against α-synuclein antigen. They applied electrostatic optimization methods to identify potential affinity-enhancing mutations with a particular focus on alleviating electrostatically suboptimal contacts at the binding interface. Ebrahimi et al. [80] used a computational method for affinity enhancement of a nanobody against placental growth factor (PlGF). They monitored all interactions between the 3D structure of anti-PlGF nanobody and the target. To determine the most important CDR residues in the binding, they considered stabilizing energies, inter and intra molecules distance, bonds formation or breakage and stability of the complex. The Table 2 provides a list of studies [11,57,76,77,79–83] used computational approach to increase binding affinity.

5.3. Challenges in computational antibody affinity maturation

It is conceivable that a number of limitations may have influenced the result obtained from computational analysis. As proposed by a recent study, CDR-H3 loop structures are not stable in solution and it would cause a remarkable fluctuation in energy levels. Accordingly, the major problem in antibody–antigen docking and MD simulation is the less favorable free energies comparing with other protein-protein complexes [84]. Moreover, concerns about docking approaches (rigid-body and flexible docking) worth to be mentioned here. While most of docking servers are using rigid-body docking, theoretically flexible docking provides more accurate results. Flexible docking algorithm considers all possible structural adaptations, but this is extremely time-consuming and computation demanding in research [85]. Currently, majority of docking server developers focus has been directed to providing flexibility or softness in the rigid-body docking algorithms. Moreover, they are developing a scoring function to efficiently distinguish correct docking structures from lots of false positives [86]. In the case of MD simulation, there are a number of inevitable limitations, especially with respect to the force field accuracy [75]. In contrast to new advances in algorithms, MD predictions still have evident deviation from the experimental data.

Table 2
Affinity matured therapeutic antibodies by different *in silico*-based approaches.

Antibody	*In silico* method	Simulation software/force field/ time	Type of energy prediction	Fold of affinity improvement	Year	Ref.
Anti-I-domain of the integrin VLA1	Targeted CDRs engineering with Structural-based on simulation	ICE/CHARMM/10 ps	Binding Energy	10 fold (KD)	2004	[76]
Aanti-gastrin TA4 scFv	Targeted CDR3 engineering MD-based docking	Manual software/20 ps	–	454 fold (KD)	2008	[11]
Anti-GD2 murine MoAb 3F8	Targeted CDRs engineering by molecular docking simulations	CDOCKER/CHARMm	electrostatic,van der Waals, entropic, free energy of binding	9 fold (IC50)	2013	[77]
Anti-CD4 (Ibalizumab)	Targeted CDR engineering by molecular docking	ZDOCK	Binding energy	Energy enhancement	2019	[81]
Anti-MCP-1 (monocyte chemo-attractant protein-1	All CDRs residues substitution by other 19 amino acids	MOE/AMBER99	Electrostatic energy	4.6 fold (KD)	2014	[82]
Anti-VHNAC1 (nonamyloid component of human α-synuclein)	Targeted CDR engineering by MD simulation	GROMACS/CHARMM27/20 ns	Electrostatic free energy of binding	202 fold (KD)	2018	[57]
Anti- VEGF (Vascular endothelial growth factor)	Targeted CDR engineering by MD simulation	GROMACS /GROMOS96 43A1/ 50 ns	Electrostatic free energy of binding	38 (KD)	2013	[79]
Anti-PIGF (placental growth factor)	Targeted CDR engineering by MD simulation	GROMACS /GROMOS96 43A1/ 50 ns	Binding free energy	Energy enhancement	2018	[80]
Anti-CD20	Targeted CDR engineering by MD simulation	GROMACS/GROMOS96 43A1/20 ns	Electrostatic free energy of binding	Energy enhancement	2018	[83]

6. Antibody affinity maturation by NGS

Recent advances in next-generation DNA sequencing are opening a new avenue for the study of immune repertoires diversity. Moreover, the genome data is helpful to guide selection toward high affinity clones in recombinant antibody repertoires [87]. NGS allows researchers to have a closer look into sequences of the paired heavy and light chains from isolated naïve, antigen-specific B-cells, and antibody display repertoires [88]. The technology has been combined with protein display methods (phage or ribosome systems) to analyze huge libraries produced by mutagenesis techniques. Moreover, it has been applied for evaluating of sequence-function relationships to understand the concept of antibody binding to a specific ligand [89–91].Conventional Sanger sequencing technology is suitable to screen 10^2–10^3 clones to find high affinity antibodies originated from a display library [92]. By this method only a narrow snapshot of actual diversity will be provided [93]. Surprisingly, NGS approaches provide much deeper insights into the diversity of a library provided by over 10^7 sequences [93]. Recent advancements in antibody modeling in combination with NGS will enable us to understand more details about the immune system and engineering of antibody structures.

The Table 3 summarized examples of antibody affinity maturation studies [48,94–96] by NGS technology. Forsyth et al. [96] applied deep mutational scanning evaluation through NGS technology to analyze binding data of 1060 point variants in a single experiment. They produced independent VH and VL libraries containing over 1000 single amino acid substitutions at 59 different positions (32 in VH and 27 in VL domains). The variants were selected by mammalian cell display systems and analyzed by NGS. Finally, about 67 substitutions were found to be correlated with enhanced affinity and KD of the best mutant model increased over five-fold. In the another study, Hu et al. [48] improved affinity of an scFv fragment against ErbB2 by NGS technology. The CDR regions of the anti-ErbB2 antibody were subjected to saturation mutagenesis. Then, the phage display libraries were analyzed by NGS before and after panning. Data revealed a 158-fold enhancement of affinity over native type. Fujino et al. [95] had identified beneficial single amino acid substitutions and applied targeted mutagenesis to produce high quality combinatorial libraries. Using NGS analysis they identified a mutant with seven amino acids substitutions that showed 2110-fold increase in binding affinity as compared to the wild type antibody.

7. Conclusions

However, there are studies used traditional *in vitro* strategies to mature affinity of antibodies, it is often difficult to target specific epitopes on antigens by using display technologies alone. To obtain high epitope-specific antibodies, it is suggested to accompany display technologies with computational structural methods. Moreover, conventional Sanger sequencing technology has limited power to exploring large number of clones in order to identify high affinity ones. Recent advances in NGS technology have revolutionized the analysis of antibody repertoires as it is able to screen ˜10^7 clone. NGS is now promising enough for the development of therapeutic antibodies by allowing unprecedented insights into the library diversity and clonal enrichment. Besides, rational design strategies allow targeting specific epitopes of antigen to produce high affinity antibodies, decrease the time duration and costs of antibody engineering. More importantly, the approaches provide opportunity to consider different biophysical features of antibodies at the same time. Computational approaches in combination with *in vitro* display strategies and taking advantage of NGS platforms would permit biopharmacists to develop more effective mAbs.

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Table 3
Affinity matured therapeutic antibodies by various NGS-based approaches.

Antibody	Mutagenesis method	Screening method	Library size	NGS platform for deep sequencing	Fold of Affinity improvement (KD)	year	Ref.
Anti-VEGF	Saturation mutagenesis	Phage display	10^{10}	Illumina	25	2015	[94]
Anti-TNFα receptor (TNFaR)	Targeted	Ribosome display	10^{6}	Roche GS-FLX 454	2110	2012	[95]
Anti-ErbB2	saturation mutagenesis	Phage display	10^{8}	Illumina	158	2015	[48]
Anti-EGFR (Epidermal growth factor receptor)	Targeted	mammalian cell surface display	10^{8}	Roche/454 GS FLX	5	2013	[96]

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