Immunoassays: Biological Tools for High Throughput Screening and Characterisation of Combinatorial Libraries

M. Ângela Taipa*

IBB – Institute for Biotechnology and Bioengineering, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais 1049 – 001, Lisboa, Portugal

Abstract: In the demanding field of proteomics, there is an urgent need for affinity-catcher molecules to implement effective and high throughput methods for analysing the human proteome or parts of it. Antibodies have an essential role in this endeavour, and selection, isolation and characterisation of specific antibodies represent a key issue to meet success. Alternatively, it is expected that new, well-characterised affinity reagents generated in rapid and cost-effective manners will also be used to facilitate the deciphering of the function, location and interactions of the high number of encoded protein products. Combinatorial approaches combined with high throughput screening (HTS) technologies have become essential for the generation and identification of robust affinity reagents from biological combinatorial libraries and the lead discovery of active/mimic molecules in large chemical libraries. Phage and yeast display provide the means for engineering a multitude of antibody-like molecules against any desired antigen. The construction of peptide libraries is commonly used for the identification and characterisation of ligand-receptor specific interactions, and the search for novel ligands for protein purification. Further improvement of chemical and biological resistance of affinity ligands encouraged the "intelligent" design and synthesis of chemical libraries of low-molecular-weight bio-inspired mimic compounds. No matter what the ligand source, selection and characterisation of leads is a most relevant task. Immunological assays, in microtiter plates, biosensors or microarrays, are a biological tool of inestimable value for the iterative screening of combinatorial ligand libraries for tailored specificities, and improved affinities. Particularly, enzyme-linked immunosorbent assays are frequently the method of choice in a large number of screening strategies, for both biological and chemical libraries.

Keywords: Antibody engineering, combinatorial chemistry, biological libraries, chemical synthesis, high throughput screening, immunoassays, ELISA.

INTRODUCTION

The explosion in genome sequencing, and in subsequent DNA array experiments, has provided extensive information on gene sequencing, organization and expression. This has resulted in the need to perform similarly broad experiments on all the proteins encoded by a genome, namely the human genome [1]. Taking into account that the human genome contains ≈ 25000 genes, and that their products are found in different splice variants and produce proteins with post-translational modifications, it is expected that there are at least 100000 different protein products to be investigated [2]. The endeavour of this task requires wide panels of appropriate tools based on highly specific affinity ligands, such as antibodies and genetically engineered related molecules [3] that can provide high throughput, high sensitivity and resolution as well as the possibility of identifying post-translational modifications in proteins. Alternatively, it is also expected that bioengineered/synthetic affinity ligands for protein/antibody separation and selection will gain increasing importance in the proteome analysis as affinity reagents for the enrichment or depletion of proteins or groups of proteins by affinity chromatography [4].

*Address correspondence to this author at the IBB – Institute for Biotechnology and Bioengineering, Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais 1049 – 001, Lisboa, Portugal; Tel: +351.21 841 9065; Fax: +351.21 841 9062;
E-mail: angela.taipa@ist.utl.pt

Due to their remarkable properties regarding affinity and specificity of molecular recognition, antibodies have become invaluable tools in research, diagnostic and clinical applications. As such, this class of proteins is routinely used in biochemical and biological research as analytical reagents for qualitative or quantitative determination of molecules in a wide variety of assays [5]. Antibody-based (or immuno-) assays are the fastest growing technology for the analysis of biomolecules, and are now the most used and potent reagents available for facing the challenging task of analysing the human proteome [6].

The first milestone for the generalised use of immunoassays in biomedical research and clinical chemistry was the development of hybridoma technology, described in 1975 by Köhler and Milstein [7], which made possible an almost universal method to produce monoclonal antibodies against any desired antigen. Monoclonal antibodies are produced by fusion of spleen cells from an immunized animal with myeloma cells, to obtain hybridoma cells that are further selected and screened for the production of anti-target antigen antibodies. Hybridoma cells can be continuously grown, producing exactly the same antibody molecule for months or years. The mouse hybridoma technology has therefore paved the way for the exploitation of high affinity and specificity of antibody molecules in many different applications, and for the emergence of antibodies as effective therapeutics in inflammation, cancer, autoimmune, and infectious diseases [8-10].

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During the 1980's, the integration of hybridoma technology with recombinant DNA technology and the development of new and valuable screening tools such as phage display techniques enabled the design and construction of human-like antibodies with desirable affinity/specificity, and low human immunogenicity [11-14]. The immense possibilities offered by hybridoma technology combined with protein-engineering display techniques meant it was possible to treat antibodies more like a 'chemical' instead of a variable biological product [5]. This has granted antibodies a distinguished position as therapeutic agents and as the most used ligand in industrial downstream processing of therapeutic proteins. For such applications antibodies have to be produced and purified to reach highly demanding purity criteria [14, 15]. Affinity chromatography is traditionally the method of choice for the purification of antibodies. The need for antibodies with unique specificities and designed for single applications, has encouraged the search for novel purification methodologies and novel affinity ligands for antibody capture [16]. Further advances over the 1990's in molecular modelling *in silico*, genetic engineering and biological and chemical combinatorial methods, combined with high throughput screening (HTS) technologies provided important means for generating and selecting new biologically active molecules, tailored to specific biotechnological needs. These molecules often display enhanced robustness, resistance, stability and cost-efficient production as compared to their natural templates and include engineered antibody-like molecules such as affibodies [17, 18], novel antibody-mimic domains based on resistant protein scaffolds [19-21] small peptides [15, 22], and triazine-based synthetic affinity ligands that mimic the interaction of natural specific receptors with their complementary proteins [23]. All these molecules also have a broad spectrum of potential applications as antibody substituents in diagnostic [24], separomics/proteomics [4] and therapy [25-27].

Immense progress in HTS strategies, which possess the "power of numbers" for the resolution, characterisation, and purification of target active molecules, have encompassed the advances in genetic engineering to allow the screening of large combinatorial libraries of either biological or chemical origin. The screening strategies are often based in the immunoafinity concept, relying on the specific, reversible and non-covalent interaction between an antibody and a biological target, or its analogue. ELISA (enzyme linked immunosorbent assays) assays in 96w microtitre plates have been so far the most used HTS methodology for the screening and characterisation of combinatorial libraries. In the last decades, improvements in automation devices have resulted in reduced assays time without compromising specificity and sensitivity [2, 28]. Furthermore, antibody engineering and phage-display antibodies led to greater assay flexibility, allowing the design of antibody-like molecules with improved affinity and fine specificity, fused to useful molecular markers, which today also hold great promise within the area of microarray technology [5, 6, 29].

THE ANTIBODY MOLECULE

Antibodies or immunoglobulins are a group of bifunctional glycoproteins with unique structural features that play a central role in the functioning and regulation of the immune system in mammals. Structurally, immunoglobulins are glycoproteins with a common Y-shaped building block comprised of two identical light chains (L) (~25 kDa) and two identical heavy chains (H) (~50 kDa), associated by disulphide covalent forces and by non-covalent interactions. Each chain is composed of constant (C_L and C_H) and variable domains (V_L and V_H). These form two antigen-binding fragments (Fab) and a constant region involved in the effector function and biodistribution of the antibody (Fc), linked *via* the flexible hinge region (Fig. **1**).

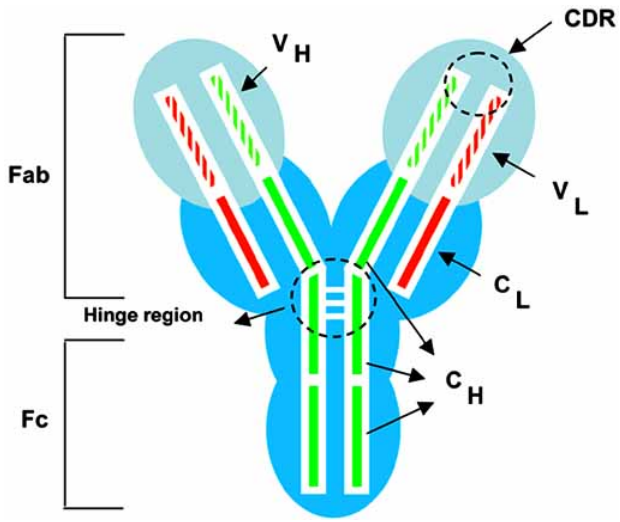

Fig. (1). Simplified schematic representation of the several domains of the Y-shaped building block of immunoglobulins, comprising two identical heavy chains (H) and two identical light chains (L). CDR represents the complementary determining region, a region of amino acid hypervariability within the variable domains of both light and heavy chains that is responsible for the specific molecular recognition of the antigen.

All the antibody or immunoglobulin domains have the same conformation, consisting of a "sandwich" of two β pleated sheets, each containing anti-parallel β-strands of amino acids. The chains fold into a tertiary structure of compact globular domains (40x25x25 Å) that interact to form the quaternary structure, and thus create the biological activity of the molecule. Five distinct classes of immunoglobulin molecules (corresponding to heavy chain isotypes: α (IgA), δ (IgD), ε (IgE), μ (IgM) and γ (IgG)) are recognised in higher mammals and differ in size, charge, biological properties, amino acid composition and carbohydrate content [30]. Immunoglobulin oligosaccharide chains are extremely heterogeneous and differ in both composition and structure. Carbohydrate components can facilitate the maintenance of the structure of the C_H2 domains and are also crucial for the bioactivity of the molecule as they are involved in IgG interactions with ligands and receptors [31].

ANTIBODY ENGINEERING: GENETIC AND COMBINATORIAL TOOLS

The increasing knowledge of antibody gene structure, *in vivo* expression, and regulation has made possible the generation of antibody-derived structures, which were denominated as "man-made" antibodies [32]. Genetic engineering of the basic immunoglobulin structure allowed the combination of target regions of antibodies into small functional monomeric or multimeric proteins such as Fab and $F(ab')_2$ frag-

ments, single chain variable fragments (ScFv) which are heterodimers of V_H and V_L stabilised by an hydrophilic linker - and combination of these as diabodies and triabodies [reviewed in 14, 18].

The notion that immunoglobulins owe their function to the hypervariable composition of a conserved framework region has also attracted considerable attention in the context of generating novel types of ligand receptors mimicking antibody specificity and affinity [19, 20, 33, 34]. Using combinatorial chemistry to create novel binding molecules based on protein frameworks or 'scaffolds' is a concept that has been strongly promoted during the past years in both academia and industry (recently reviewed in [35]). Non-antibody recognition proteins derive from different structural families and mimic the binding principle of immunoglobulins to varying degrees. For example, peptides with known affinity towards a certain target can be fused to, or inserted to, a carrier protein to combine its binding properties with desired favourable characteristics (e.g. effective tissue penetration for the treatment of solid tumours, or intracellular activity for targeting cellular signal pathways) of the scaffold [35]. Combinatorial engineering of immunoglobulin-binding domains such as the α helical bacterial receptor derived from staphylococcal Protein A, allowed the generation of novel binding proteins called affibodies, which present a secondary structure similar to the scaffold domain and specific affinity for a wide range of targets [17, 18]. The 10^{th} human fibronectin type III (^{10}Fn3) domain is one of the protein scaffolds used in recent years to design, and select, *in vitro*, a wide range of proteins that bind with high affinity and specificity to a variety of macromolecular targets [36, 37]. The fibronectin III domain constitutes a small (94 amino acids), monomeric natural β-sandwich protein that is made up of seven strands with three connecting loops at one end of the β-sheet. Randomisation of three N terminal loops in the tenth of the 15 repeating units in human fibronectin showed that this domain can be used as a scaffold to obtain different protein binding variants with detectable biological activity [35]. Recently, high-affinity antibody mimics (Kd ≤ 350 pM) based on the ^{10}Fn3 scaffold have been generated and selected by evolution of an interloop disulphide bond, using a yeast surface display combinatorial approach [21].

BIOLOGICAL COMBINATORIAL LIBRARIES

Biological combinatorial libraries arose with the dawn of display techniques as a platform for the presentation of several biomolecules. Phage display has been applied in many fields of biological and medical sciences, and used to generate biological combinatorial libraries of different sizes. The use of filamentous phages that infect *Escherichia coli* has proven to be a very powerful technique to display millions or even billions of different peptides or proteins as coat-protein fusions. These include the expression and selection of peptides binding to antibody epitopes [38-40], the selection of peptides for protein purification [41, 42], the expression and affinity maturation of proteins, as, for example, multivalent avimer proteins, a class of binding proteins evolved from a large family of human extracellular receptor domains (human A-domains) with high therapeutic potential [34], and the selection of specific antibodies from large naïve, immune libraries or synthetic libraries [43-45].

Numerous display methods have been developed to obtain recombinant antibodies, as recently reviewed in [3]. Antigen-specific antibodies can be directly selected either from antibody gene repertoires expressed *in vivo* on the surface of filamentous bacteriophages [43], bacterial [46-48], and yeast cells [48-52] or *in vitro* by ribosome display [53-55] or puromycin display [56]. However, phage display is undoubtedly the most successful and commonly used method for combinatorial antibody display and screening [2, 3, 43, 57].

Phage Display

Phage display can mimic the strategy used by the humoral immune system to produce fully human antibodies or antibody fragments *in vivo* and can exempt immunisation and hybridoma construction. A prerequisite for such an approach is the ability to isolate and clone the variable (V) region genes from the immunoglobulin heavy and light chains to display them functionally on a phage. According to the source of the immunoglobulin genes, antibody phage libraries can be classified as immune (where the variable (V) genes are isolated from IgG-secreting plasma cells from immunised donors) or "single pot", that comprise non-immune naïve, semi-synthetic and fully synthetic libraries [6, 58]. In the case of naïve libraries, the size of the library influences directly the affinity and specificity of the isolated antibodies, with Kd values ranging from 10^{-6} to 10^{-7} for smaller libraries to 10^{-9} for larger ones [59]. However, when selected antibodies are used as the basis for subsequent libraries and selection, affinities can be further increased to levels unobtainable in natural immune systems. Affinity and specificity can be improved by *in vitro* affinity maturation [57, 60] or the use of semi-synthetic strategies by selecting one or more antibody frameworks as a scaffold and randomising sequences within the CDR loops [11]. To date, "single pot" antibody libraries with a theoretical diversity of 10^{11} have been generated, with affinities ranging from 10^{-6} to 10^{-11}, being useful sources for selecting antibodies for proteome research [6].

Recombinant antibodies are generally displayed in non-lytic filamentous phages, fd or M13, that infect strains of *Escherichia coli* containing the F conjugative plasmid. Phage particles attach to the tip of F pillus that is encoded by genes on this plasmid, and the phage genome, a circular stranded DNA molecule, is translocated into the cytoplasm. The genome is replicated involving both phage and host derived proteins, and packaged by the infected cell into a rod-shaped particle which is released into the medium. Though fusion to different phage coat proteins can be used (pIII, PVI and PVIII) [43] most successful phage antibody libraries, against a wide variety of antigens, have been created by cloning antibody variable genes upstream of the gene of the pIII coat protein gene and using phage or phagemid as the display vehicles [3, 58, 61]. Phagemids are small plasmid vectors that carry gene III with appropriate cloning sites and a packaging signal, providing high transformation efficiencies and, therefore, are ideally suited for generating very large combinatorial repertoires [43, 58]. Strategies as the addition of helper phage systems [62] and, more recently, engineering *E. coli* cell lines [63] have been developed to improve the enrichment of specific antibodies in phaghemid-based combinatorial libraries. A general scheme for the lead isolation of

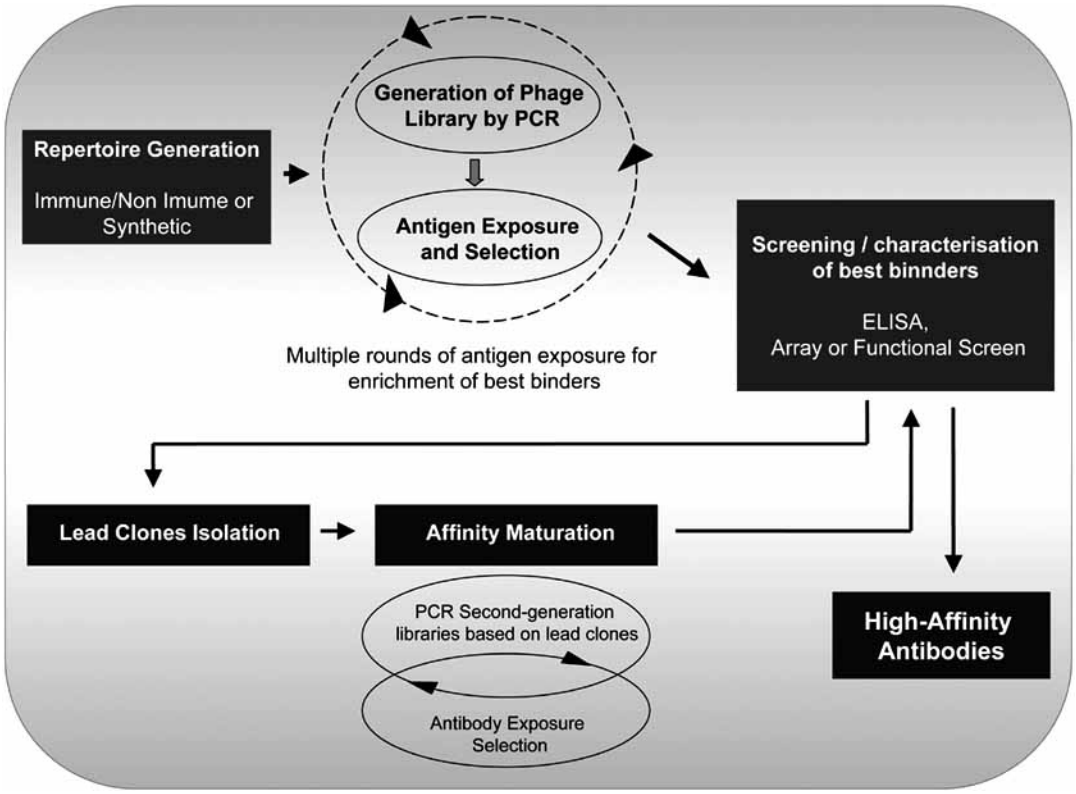

Fig. (2). Isolation of lead antibody fragments from a phage-display library involves several steps, which include the generation of the library from a repertoire of antibody variable genes, the production of antibody-displaying phages, and the selection of high affinity binders by exposure to antigen. Lead antibodies can be identified by ELISA assays, microarrays or biological-activity screening in cell-based or receptor assays. Although screening thousands of clones can yield a greater diversity of "binders" after a single round, generally several (two-to-three) rounds of enrichment of the preliminary library and *in vitro* affinity maturation of the gene repertoire of lead clones are required to isolate antibodies that bind with high affinity and specificity to the target antigen (adapted from [20]).

antibody fragments by phage display is shown in Fig. **2**. Due to limitations of the *E. coli* folding machinery, complete IgG molecules can hardly be expressed in *E. coli* and displayed on the surface of phages. Therefore, smaller antibody fragments such as Fab, ScFv fragment or diabody fragments are mainly used for antibody phage display. However, once a Fab or scFv fragment with high affinity and specificity for a target antigen has been obtained, it is possible to genetically reconstruct these fragments into an intact fully human antibody for therapeutic application [64].

Yeast Surface Display

Among the display techniques, yeast surface display has emerged as a powerful platform for the combinatorial engineering of a variety of proteins, including antibody fragments, for improved affinity, specificity, expression and stability (recently reviewed in [52]). The fundamentally different folding capacities of prokaryotic and eukaryotic cells restrict the presentation of some correctly folded proteins on bacteria, in particular if they are large and of mammalian origin. A typical example is antibody fragments consisting of at least two domains, and sometimes two independent polypeptide chains (Fabs), requiring disulphide bond formation [48]. Yeast display system offers an alternative to phage display. It is based on a carboxyterminal fusion to the Aga 2p subunit of *Saccaromyces cerevisiae a*-agglutinin receptor. Through two disulphide bridges, the Aga 2p fusion protein is bound to the Aga 1p subunit of the same receptor that anchors the assembly to the yeast cell wall [49]. This display system has been used for the successful display and engineering (by directed evolution) of several proteins, including the single-chain T cell receptor (scTCR) [65], the epidermal growth factor receptor (EGFR) [52], and fibronectin-based high-affinity antibody mimics [21].

One of the most successful applications of yeast surface display has been the selection and affinity maturation of antibody fragments, both scFv fragments [49-51, 66] and Fab [67, 68]. Recently, yeast display has also been used for clinical applications such as the assessment of tumour-specific antibody responses in cancer patients and the screening of novel tumour antigens not previously detected from prokaryote-displayed libraries. In addition, several groups have isolated novel lead antibodies binding to a variety of targets from immune and non-immune human scFv libraries [52]. A recent study compared yeast surface display and phage display using identical immune ScFv antibody libraries and target antigens [69]. It was found that yeast display is able to sample a considerable more fully immune ScFv library repertoire, while also being less labour intensive. A main advantage of combinatorial yeast display over phage display arises from the employment of a eukaryotic host system possessing the secretory biosynthetic machinery for efficient protein folding and N-linked glycosylation, and the ability to characterise isolated antibody clones directly in display format by flow-cytometry analysis [52, 66].

CHEMICAL COMBINATORIAL LIBRARIES

The field of combinatorial library chemistry was pioneered by synthesis of the first limited peptide library in 1984 [70], and has since become a powerful tool that not only facilitates new drug discovery but also provides important information for the fundamental understanding of molecular recognition. Nowadays, combinatorial chemistry enables the synthesis of a myriad of small molecules with biological and therapeutic applications, in a time and resource effective manner (recently reviewed in [71]). Several formats can be used to undertake combinatorial synthesis, namely liquid-phase (in solution or using soluble polymers as supports), solid-phase or surfaces. Solid-phase chemistry, utilising "mix-and-split" strategies, is a particular powerful method for generating large libraries of compounds [72], and continues to hold a dominant position in combinatorial chemistry. Generally, compounds being synthesised are attached, through a linker group, to insoluble, functionalised polymeric materials or beads, facilitating their separation from excess reagents, soluble reaction by-products or solvents [73]. A sample of beads is divided among several reaction flasks and a different reaction is performed in each flask. The beads from all flasks are then pooled and redivided into a second set of flasks containing equal amounts of beads carrying the products of the first substitution. When a second reaction is performed, each of the products of the first reaction is a substrate and all possible products are formed simultaneously. Each bead carries a single product, and libraries from 10^3 to 10^{10} compounds can easily be generated by this methodology [72, 73]. The ability to drive reactions to completion using large excesses of removable soluble reagents is one of the reasons behind the extensive use of solid-phase chemistry. However, in drug discovery the understanding of advantages and disadvantages of both solid and solution combinatorial chemistry, and the ability to apply either when most appropriate to a particular target gives the greatest potential to drive lead discovery and optimisation forward [74, 75].

Combinatorial methods, based either on solid or solution chemistry, have been utlised for several applications as in the discovery of better catalysts [76] or active small molecules of agrochemical relevance [77]. A major use of chemical combinatorial approaches has been, however, in the area of medicinal chemistry [78], in the discovery and screening of novel active chemical compounds for binding specific cell receptors [79, 80], the design of specific and potent bacterial endotoxin sequestrants [81] or the design, synthesis and evaluation of novel anticancer agents based on natural products-templated ligand libraries with cytotoxic activity [82]. Also, small-molecule microarrays are emerging as a tool for identifying proteins and/or drugs with pharmacological interest [83].

Notably, in the last decade, the use of combinatorial chemistry, integrated with molecular modelling computational techniques, has allowed the generation of novel synthetic affinity ligands for protein and antibody purification [15, 16, 23]. These bio-inspired mimic compounds display enhanced robustness combined with high selectivity, stability and low-cost of production, and tend to replace naturally occurring receptor-binding proteins in biotechnological, clinical or diagnostic applications.

Peptides

Peptide libraries have assumed an important role in the identification and characterisation of ligand-receptor interactions. Large synthetic combinatorial libraries (SCL) of peptides may be chemically synthesised by different techniques ('split synthesis', 'peptides on beads', 'synthetic peptide combinatorial libraries' and photolithography), which can be used separately or combined [60, 84, 85]. Application of SCL of peptides is a relevant approach in both proteome and cancer research context [86]. The identification of synthetic mimics that are recognized by an antibody, regardless of whether the mimic retains apparent resemblance to immunogen, is of major interest in the development of more effective immunoassays and synthetic vaccine candidates [87]. Peptide SCLs have been extensively used for epitope and mimotope mapping of monoclonal antibodies against clinical relevant proteins and cell receptors [88-90]. Combinatorial chemical libraries of peptides are also are useful to screen for diagnostic agents that bind to disease-specific antibodies [24, 91] and as affinity ligands for protein purification [92]. An important application in this area has been the synthesis of mimic peptides for antibody purification [16, 93]. Difficulties in mimicking discontinuous epitopes with linear peptides has led to specific approaches (e.g. cyclization) for generating peptides with better mimic properties, increased resistance to enzymatic degradation and constrained flexibility [60]. A recent example reports the selection of a disulfide-bridged cyclic peptide with the general formula (NH_2-Cys_1-Phe-His-His-)$_2$-Lys-Gly-OH (named Fc-receptor mimetic – (FcRM) as an efficient ligand for the removal of antibodies from biological fluids to purities up to 90% [26]. Another relevant example has also been the search for staphyloccocal IgG-binding Protein A mimetic by the design of a tetrameric peptide library, where four identical peptide chains were assembled starting from a tetradentate lysine core [94]. A multimeric library has been produced by solid-phase peptide synthesis. After three screening cycles, the multimer (Arg-Thr-Tyr)$_4$-K_2-K-G, denoted PAM (protein A mimetic) or TG19318, was proven to be an efficient ligand for one-step purification of antibodies directly from crude sera up to 95% purity.

Biomimetic Ligands

In recent years, knowledge of the mechanisms involved in molecular recognition has encouraged the design of new and powerful bio-inspired ligands, for protein and antibody purification. Bio-inspired or 'biomimetic' ligands are synthetic compounds of full chemical nature, with similar molecular recognition properties of their biological model (template). The general concept behind this class of compounds - often referred as 'artificial proteins'- relies on the combination of molecular modelling, through the evaluation and study of available X-ray crystallographic structures of the target proteins or complexes of proteins, solid-phase combinatorial synthesis and high throughput screening techniques. A most effective way for obtaining these biomimetic ligands was pioneered by Lowe and co-workers [95], by integrating rational design with combinatorial synthesis and screening on the same support. Such integration avoids numerous indefinite factors that can be introduced upon immobilisation of solution-phase synthesised ligands on a matrix. Apart from the ligand properties and its ability to bind the target

protein, the nature of the support and the coupling chemistry can affect the overall binding process. Therefore, a procedure based on solid-phase combinatorial chemistry has been developed to construct libraries of triazine-based near-neighbour ligands interacting in a complementary affinity-like mode with protein surface-exposed key residues (Fig. **3**). The solid support utilised, agarose, has proven to satisfy both the exigencies for solid-phase synthesis and the properties required for ligand screening and application in affinity chromatography [23]. The triazine scaffold serves as the spatial framework for the display of attached functional groups and has shown to deliver effective protein binding ligands.

Using this strategy, a series of triazine-scaffolded bio-inspired molecules presenting affinity to different proteins or families of proteins have been designed, synthesised and fully characterised [96-101]. From a proteomics point of view, these synthetic ligands can represent a good alternative to biological ligands for depletion of complex biological mixtures to be further analysed by specific techniques. For example, affinity chromatography has been recently reported as an efficient methodology for the elimination of immunoglobulin content in crude samples to be further submitted to two-dimensional electrophoresis or mass spectrometry analysis [102]. Good candidates for such application include synthetic ligands mimicking the interaction of immunoglobulins with bacterial IgG-binding receptors, which can surmount problems associated with their biological templates while preserving affinity and specificity to target antibodies [4]. Ligand 22/8 (4-[4-Chloro-6-(3-hydroxy-phenylamino)-[1,3,5]triazin-2-ylamino]-naphthalen-1-ol), - named as 'artificial Protein A' – was designed to mimic the natural *Staphylococcus aureus* Protein A receptor in binding IgG from various sources, being able to separate IgG from human plasma to purities of 98-99% [97,98]. Ligand 8/7 (or 'artificial Protein L') has shown to mimic Protein L in terms of antibody separation performance, while binding to IgG1 with κ and λ isotypes [101]. Protein L is a bacterial receptor from Peptostreptococcus magnus strains, which binds to the Fab portion of immunoglobulins. The 'artificial Protein L' (4-[4-(4-carbamoyl-phenylamino)-6-chloro-[1,3,5]triazin-2-ylamino]-butyric acid) compared well with the natural receptor in the isolation of native immunoglobulins from different classes (human IgG, IgA and IgM), species (rabbit, goat), and sources, including a recombinant scFv antibody from crude extracts [103, 104].

IMMUNOLOGICAL METHODS: HTS TOOLS FOR THE SELECTION OF LEAD LIGANDS

Within the proteomic context, the development of HTS technologies is essential for the selection and screening of robust affinity reagents, either from biological or chemical combinatorial libraries. Immunological methods have proven to be a biological tool of inestimable value to reach this goal. Antibody-based assays are known for their versatility, sensitivity, specificity, high throughput and ease of automation. The interaction between of an antibody with its target (whether this a cell, tissue, a peptide or synthetic mimic ligand) is easily and rapidly monitored by classical assays involving the detection and measure of a complex formation by using a fluorescent-, radio- or enzyme- labelled antibody. Other biophysical techniques, such as NMR (nuclear magnetic resonance) and optical spectroscopy (fluorescence,

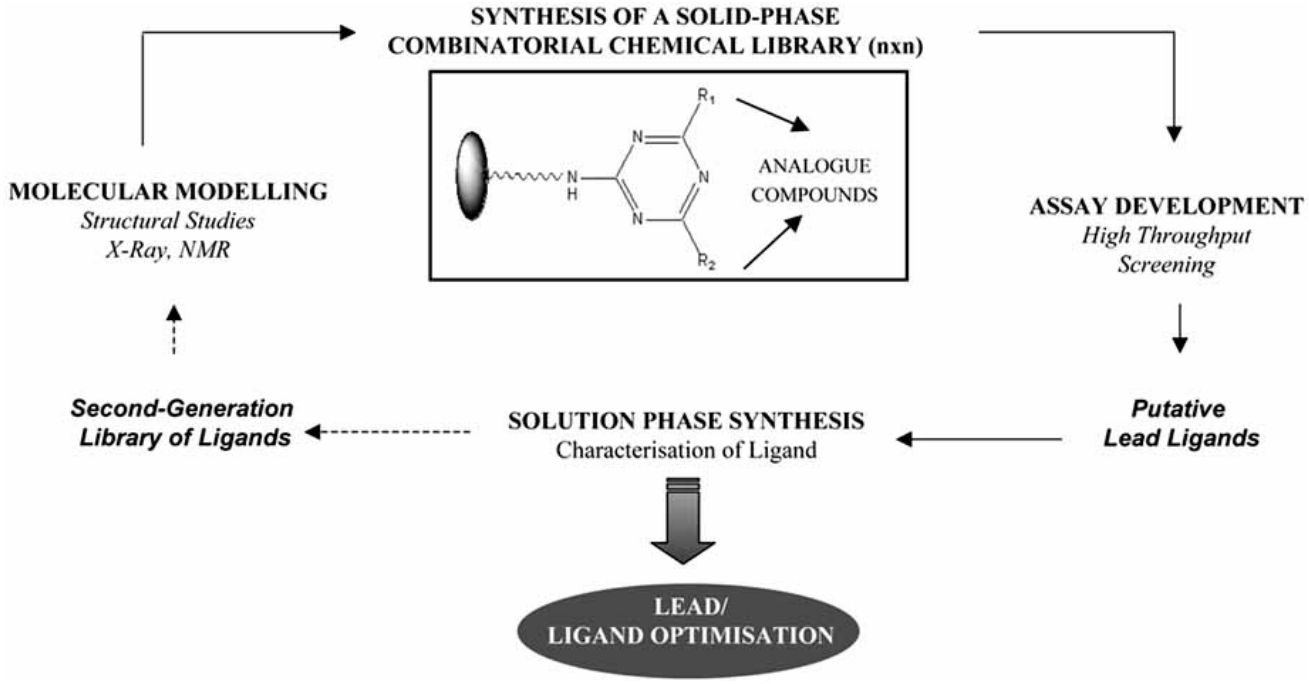

Fig. (3). A general research strategy for the generation of effective *de novo* designed triazine-based synthetic mimic ligands is based on i) molecular modelling based on structural information available on the interaction of target biomolecules and *in silico* design of *n* chemical analogues that mimic the key residues involved in the specific molecular recognition; ii) synthesis of a *nxn* member solid-phase combinatorial library in agarose; iii) development of high throughput assays for ligand screening; iv) solution-phase synthesis and further characterisation/optimisation of lead ligands. In some cases a second-generation library may be designed to improve the affinity/selectivity of selected putative lead ligands, allowing the synthesis of better mimics.

circular dichroism) combined with computer-assisted molecular modelling (CAAM) are also available to assist investigators to gain a better understanding of how antibodies can be used as tools for ligand library screening, and help to improve prediction, modelling and design of novel antigen-binding sites [22, 105] and the construction of antibody microarrays.

Despite the efforts and progress in developing efficient microarrays [5] and adapting selection methods to robotics [6] screening and characterisation of lead ligands of combinatorial libraries from both biological and chemical sources are frequently based on ELISA assays, in a classical or automated mode. Given the ubiquity of the microtiter plate and its derivatives, it is probable that this format will continue as the standard in HTS of antibody and synthetic libraries [3]. An automated, cost-effective, high throughput method based on a 96-well plate filtration assay for the screening of affinity ligands was recently reported [106]. This methodology has shown to be effective for producing lead compounds, and may have utility as a generic tool for the functional characterisation of novel proteins emerging from proteomics work.

Several methods have been reported and can be applied, alone or combined, for antibody phage display screening including biopanning, ELISA, antigen-coated magnetic particles, BIAcore sensor chips and selection by sorting procedures such as FACS [2, 3, 59, 61, 107]. However, most commonly, 96 phage antibodies (i.e., a single microtitre plate) are screened by ELISA using relevant and non-relevant antigens [3, 43]. If the antigen is not available, alternative strategies may involve the use of natural antigen sources (e.g. tissue sections, cell or tissue extracts) or small peptides (epitopes) [107]. Lou and co-workers have developed a successful and high throughput method for antibody-phage libraries screening based on the use of multipins in 96-well microtiter plates [61]. The method enables single aliquots of the library to be incubated with 96 different antigens simultaneously, using minimum volumes, with the advantage that fully library diversity can be assessed for each antigen. After several rounds of selection, a polyclonal ELISA is performed for each pool of amplified phage in order to identify positives that need to be further analysed at a monoclonal level [107].

Regarding peptide mimetics or synyhetic mimic triazine-based affinity ligands, the iterative, high throughput screening of large solid-phase combinatorial libraries is commonly performed by combination of several methods as "on-bead" fluorescence microscopy binding assessment, affinity chromatography, and quantitative/competitive ELISA assays for ligand selection and characterisation [26, 97, 98, 101].

ELISA-Based Methods

ELISA or enzyme linked immunosorbent assays involve the adsorption of an antigen, (protein, peptide or analogue) or an antibody on the bottom of a 96 well microtiter plate. ELISA tests can be developed in different formats, based either on competitive or non-competitive principles, depending on whether or not the antigen/analogue to be identified/quantified competes with a different labelled antigen for antibody binding sites [108]. Four common ELISA formats are represented in Fig. **4**.

Non-competitive assays are simple and based on the direct or indirect detection of a target, using primary or secondary antibody reagents labelled with an enzyme (ELISA) - or alternatively, in a more late sense, with a fluorescent or a radioactive compound. Competitive assays are frequently

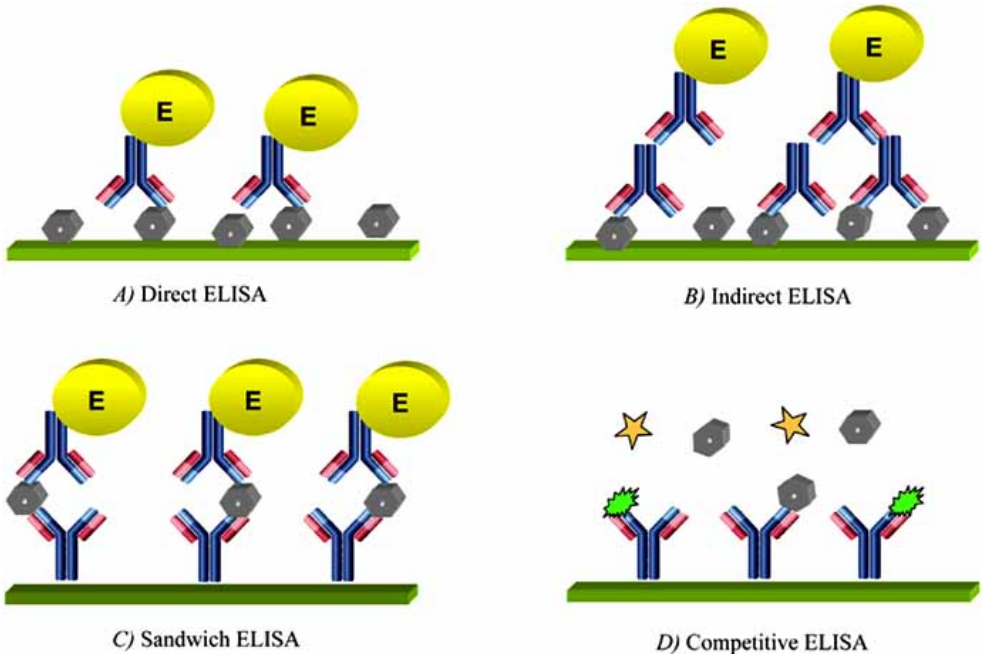

Fig. (4). Common formats for ELISA assays. (**A**) A direct assay is possible if the antigen is pure and a primary labelled antibody is available. (**B**) Indirect assays are performed when not a primary but a secondary enzyme-labelled anti-antibody is available for detection. (**C**) If the antigen is impure, a sandwich format is the best option. This assay requires two specific monoclonal antibodies that bind the antigen through two different epitopes, one that ensures antigen capture, and a second that acts as a detector. (**D**) Competition ELISA assays allow the monitoring of the strength of binding of antibodies to different antigens or to their synthetic mimic analogues.

used to characterise the interaction of synthetic/analogue affinity reagents with target molecules or the competition of lead peptides and natural antigens in epitope mapping [25]. Competitive ELISA (C-ELISA) assays allow the relative comparison of the strength of binding to target proteins, and give evidence on epitope recognition by different antibody molecules. In the case of synthetic/antibody mimics, C-ELISA assays can monitor if bioengineered or synthetic molecules compete with their natural biological template in binding to the same *locus* on the target protein surface [97, 98, 103]. Enzymes commonly used for antibody labelling are horseradish peroxidase (HRP) and alkaline phosphatase (AP), and a panoply of HRP- or AP- labelled primary and secondary antibodies and compatible substrate kits for ELISA assay development are commercially available. Table **1** summarizes examples of different types of combinatorial libraries where ELISA-based assays were used for lead-ligands selection and characterisation.

In a recent and interesting work, Vicennati and co-workers demonstrated that, aside from spectroscopic and chemosensor-based methods, techniques that exploit the specific binding of antibodies might be highly valuable for the high throughput screening of combinatorial libraries of enantioselective catalysts. As so, a general-analytical tool based on a sandwich immunoassay was developed and successfully used for the screening of catalysts for cross-coupling reactions [76]. Another example of a screening strategy based on a high throughput ELISA assay for the lead discovery of a bio-inspired synthetic ligand is illustrated in Fig. **5**.

The development of an artificial Protein L binding specifically to the Fab domain of human IgG involved the iterative screening of a 169-membered solid-phase combinatorial library of triazine-scaffolded ligands [101]. All the synthesised ligands were firstly assessed for binding to human IgG by a new "on-bead" FITC-based method [109]. "Scale-down" and high throughput analysis for selectivity against human IgG fragments was driven by their high cost and limited availability. Positive ligands for human IgG were assessed for binding to human Fab by a quantitative ELISA assay, combined with a micro-scale affinity chromatography. Putative leads binding to Fab were screened against human Fc using a similar methodology [101]. Ligand 8/7 which emerged as the lead from this screening strategy has proved to be highly selective to the Fab moiety of human IgG [103] and represents a synthetic mimic of Protein L with a universal affinity and specificity for small antibody fragments (scFv, Fab or $F(ab')_2$).

CONCLUSIONS

Combinatorial molecular technology and display systems combined with high throughput screening methodologies provide the means to generate variability in a diverse set of molecules such as engineered antibody-like molecules, novel antibody-mimic domains based on resistant protein scaffolds, small peptides, and synthetic affinity ligands that mimic the interaction of natural specific receptors with their complementary proteins. Antibody-based proteomics is nowadays the most powerful approach for the study of the human proteome. However, it is both desirable and to be expected that novel robust protein-specific affinity reagents, generated by rapid and cost-effective manners, will gain

Table 1. Examples of Application of ELISA-Based Assays for the Screening and Characterisation of Diverse Biological and Chemical Combinatorial Ligand Libraries

Target	Application	ELISA Selection Methods	Ref.
Phage-displayed peptides	Identification of peptide affinity ligand for Anti-tenascin –C Ab	Competitive ELISA	[40]
Phage-displayed multivalent avimer proteins	Antibody mimics with potential therapeutic application	Indirect ELISA (for specificity analysis)	[34]
ScFv antibody phage-library based on chicken V genes	Selection of ScFv recognising haptens, proteins and virus	Indirect, sandwich and competitive ELISAs	[45]
Phage-displayed Fab fragments	Selection of high-affinity Abs against human kallikrei tissue 1	Fab-automated indirect ELISA assay (primary screening)	[110]
Synthetic peptides	Epitope mapping: Peptides recognised by an Anti-carbohydrate Ab	Competitive ELISA	[25]
Synthetic peptides (mimotopes)	Binding human IgM with an unusual combining site	Indirect ELISA	[111]
Palladium-based catalysts for Sonogashira reaction	Selection of enantioselective catalysts for cross-coupled reactions	Sandwich ELISA	[76]
PAM (Protein A mimetic peptide)	Purification of Abs by targeting Fc region	Competitive ELISA	[94]
FcRM mimetic peptide	Purification of Abs	Indirect and Competitive ELISAs	[26]
Artificial protein A	Fc receptor synthetic mimic for Ab purification	Competitive ELISA	[97, 98]
Artificial protein L	Synthetic mimic for purification of Ab fragments (Fab, ScFv $(Fab)_2$)	Quantitative direct ELISA Competitive ELISA	[101, 103]

Abs = Antibodies.

increasing importance in proteome analysis. Discovery of most potent and selective binders from combinatorial libraries depends on the development and use of suitable and efficient screening strategies. Immunological methods have proven to be a biological tool of inestimable value to reach this goal. Antibody-based assays are known for their versatility, sensitivity, specificity, high throughput and ease of automation. Particularly, ELISA assays have been extensively used as HTS methodology for the screening and characterisation of lead ligands from both biological and chemical combinatorial libraries. Given the ubiquity of the 96 well microtiter plate and its derivatives, it is anticipated that this format will continue as a standard in high throughput screening and selection strategies.

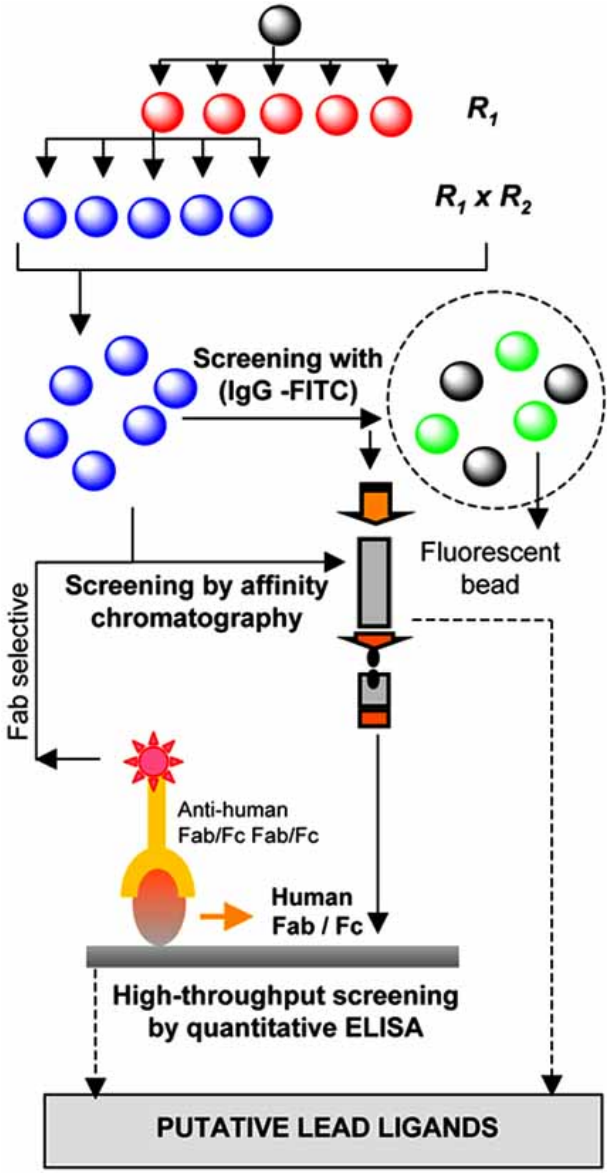

Fig. (5). Screening strategy for the lead discovery of a synthetic affinity ligand (artificial Protein L) mimicking the interaction between Protein L and the Fab portion of human IgG.

ABBREVIATIONS

Ab = Antibodies

AP = Alkaline phosphatase

CAAM = Computer-assisted molecular modeling

CDR = The complementary determining region

C_H = Heavy chain constant domain

C_L = Light chain constant domain

ELISA = Enzyme-linked immunosorbent assay

Fab = Antigen-binding fragment

Fc = Crystallizable fragment

FcRM = Fc-receptor mimetic

FITC = Fluorescein isothiocyanate

HRP = Horseradish peroxidase

HTS = High throughput screening

IgA = Immunoglobulin of class A

IgD = Immunoglobulin of class D

IgE = Immunoglobulin of class E

IgG = Immunoglobulin of class G

IgM = Immunoglobulin of class M

PAM = Protein A mimetic

ScFv = Single chain variable fragments

SCL = Synthetic combinatorial libraries

V_H = Heavy chain variable domain

V_L = Light chain variable domain

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