Affinity as a tool in life science

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The use of affinity-based tools has become invaluable as a platform for basic research and in the development of drugs and diagnostics. Applications include affinity chromatography and affinity tag fusions for efficient purification of proteins as well as methods to probe the protein network interactions on a whole-proteome level. A variety of selection systems has been described for in vitro evolution of affinity reagents using combinatorial libraries, which make it possible to create high-affinity reagents to virtually all biomolecules, as exemplified by generation of therapeutic antibodies and new protein scaffold binders. The strategies for high-throughput generation of affinity reagents have also opened up the possibility of generating specific protein probes on a whole-proteome level. Recently, such affinity proteomics have allowed the detailed analysis of human protein expression in a comprehensive manner both in normal and disease tissue using tissue microarrays and confocal microscopy.

INTRODUCTION

Life is based on specific interactions between biomolecules. The underlying affinities form the basis for molecular recognition events that make up the complex machinery of all living organisms, including man. In fact, the genome project (1,2) has taught us that the number of protein-coding genes is probably as few as between 21,000 (3) and 23,000 (4) and this reinforces the notion that cellular processes are built up by complex networks of specific interactions. The affinities between various molecules in biological systems range from low-affinity interactions to very high-affinity interactions in the picomolar range. The interactions can be transient, such as the molecules in signal pathways, or very stable, such as heterodimer-forming protein complexes or multicomponent organelles, such as ribosomes or proteosomes.

In life science, affinity has been used as a tool to study cellular processes in normal and disease tissues, but it has also been used to develop products for diagnostics and therapeutics. In fact, most pharmaceutical and diagnostic assays are based on affinity between a product and a biomolecular target. In this review, some of the development and use of affinity reagents during the last 25 years will be discussed, although it is important to note that the review is by no means meant to be comprehensive, but rather will show some examples of affinity applications in the field of "biotechniques."

Affinity Chromatography

The use of affinity for purification of proteins through chromatography was first described in the late 60s (5). The method relies on the use of an affinity ligand coupled to a matrix to allow specific capture of the product from a complex mixture. In this way, an essentially pure product can be obtained with a single operation. The most frequent use of affinity chromatography during the last decade has been the purification of antibodies using recombinant protein A (6) or protein G (7). Most monoclonal antibodies used for research and diagnostics and essentially all therapeutic antibodies used to treat patients have been purified using affinity chromatography (8). Recently, protein engineering and design have been used to create new affinity reagents more suitable for affinity chromatography, as exemplified by the protein A derivative engineered to be stable during industrial "cleaning-in-place" procedures involving 0.1 M NaOH (9).

Another application of affinity capture is the technique most commonly called immunoprecipitation (10), which is based on the use of a specific antibody coupled to a solid matrix to capture the protein targets, often in a complex with its interaction partners. This technique has become very popular, with applications ranging from molecular profiling of protein modifications to pathway mapping and network analysis (11). Affinity purification has also been used to facilitate analysis of plasma and serum samples based on affinity capture to remove the most abundant proteins in sera, such as albumin and IgG (12) or transferrin (13). This affinity procedure allows, for some cases, a more sensitive analysis in proteomics efforts aimed at discovering biomarkers useful for distinguishing patients with a particular disease.

Affinity Tags

With recombinant DNA methods it is possible to create fusion proteins consisting of a protein to be studied and various tags used for detection and purification (14). Such affinity tags have been used for the generation of purified fusion proteins in a multitude of applications, including structural genomics, antibody generation, and

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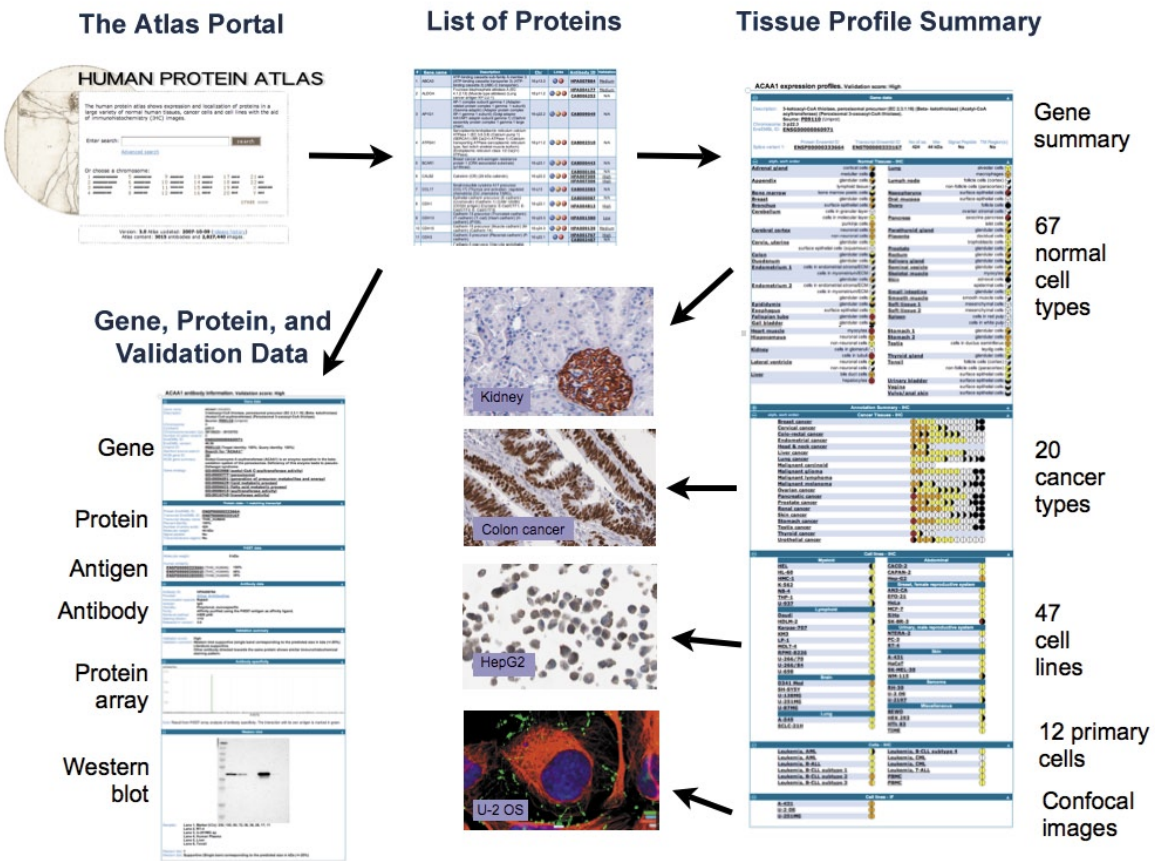

Figure 1. The Human Protein Atlas. This portal (www.proteinatlas.org) contains data demonstrating the expression and localization of proteins in a large variety of normal human tissues, cancer cells, and cell lines with the aid of immunohistochemistry (IHC) images generated with specific affinity reagents (antibodies). The database contains validation data (protein arrays and Western blots) for all the "in-house" generated antibodies and confocal images in three cell lines for some antibodies. Each of the original 2.8 million images can be accessed through the tissue profile summary page, while the gene, protein, and validation data can be accessed through the antibody info page.

interaction analysis. The first affinity tag was described in 1983 (15) and during the last 25 years many alternative systems have been described (Table 1), all having advantages and disadvantages depending on the application and the requirement for specificity, solubility, and the binding and elution conditions. The most often used affinity tag today is probably the His-tag, consisting of a short peptide of histidine residues, which allows a convenient affinity chromatography step using metal-chelating chromatography (16).

An important application of affinity tags is the study of protein networks using tandem affinity protein (TAP) fusions (17). With the aid of two affinity tags, it is possible to capture proteins interacting with the target molecule and by careful elution yield a mixture of proteins corresponding to interactive partners that can subsequently be identified with mass spectrometry. Recently, this procedure has been used to create a proteome-wide network map of yeast (18) and it can be envisioned that the procedure could be used to study in a proteome-wide manner the interactions in humans and model organisms, such as in rodents. In this context, a complementary affinity-based technology for protein interaction analysis is the two-hybrid system (19) based on a selection system that senses the interaction between two affinity partners, one of which is fused to a DNA-binding domain ("bait") and the other to a transcriptional activation domain ("prey"). In such a way, whole proteomes can be probed for interactions to a particular protein and this can be extended to create proteome-wide network maps (19).

Selection Systems for Affinity

In the early 90s, Winter and colleagues described the use of new technology for linking genotype and phenotype to select for new affinity molecules in vast libraries using in vitro selection principles (20,21). The strategy was based on phage display, an elegant technique for creating surface-displayed libraries of peptides or proteins (22). During the last 15 years, many libraries based on these principles have been described, often based on antibody fragments, such as Fabs or single-chain Fv-fragments

(23). Furthermore, a large number of alternative ways to generate and handle complex combinatorial libraries have been developed (Table 2), including ribosomal display (24), yeast display (25), bacterial display (26), microbead display (27), protein complementation assays (28), and nucleic acid–based assays (29). These methods are all characterized by differences with respect to, among others, intracellular or extracellular expression, library size limitations, possibilities to use fluorescent-activated cell sorters

Table 1. Examples of Affinity Tags

Affinity Tag	Ligand	Tag Size (kDa)
Protein A domain	IgG	7
Beta-galactosidase	TPEG	110
Arginine residues	Ion-exchange	1–2
Histidine residues	Metal ions	1–2
MBP	Maltose	40
GST	Glutathione	26
Protein G	Albumin	6
Flag peptide	Antibody	1
Strep tag	Streptavidin	1
Zbasic	Ion-exchange	7

Some examples of affinity tags used for purification of fusion proteins ordered by the year of first publication. The size refers to the minimal size, although the tag is in some cases larger. For details and references, see References 14 and 56.

Table 2. Selection Systems for Affinity Reagents

Selection System	Comment	Selection Principle
Phage display	Surface of *E.coli* phage	Affinity capture
Bacterial display (Gram-)	Surface of *E.coli*	Affinity capture and flow sorter
Bacterial display (Gram+)	Surface of *staphylococci*	Flow sorter
Yeast display	Surface of yeast	Flow sorter
Ribosomal display	In vitro translation	Affinity capture
Protein complementation assay (PCA)	In vivo using *E.coli*	Growth on selective media
Bead display	In vitro translation	Flow sorter
mRNA peptide fusion	In vitro translation	Affinity capture
Plasmid display	In vivo using *E.coli*	Affinity capture
Nucleic acid–based	PCR-based	Affinity capture

For details and references, see References 57 and 33.

Table 3. Examples of Different Types of Affinity Reagents

Type of Affinity Reagent	Origin (Typical)	Size (kDa)
Polyclonal antibody	Rabbit serum	150
Monospecific antibody	Antigen-based affinity purified serum	150
Monoclonal antibody	Hybridoma cells	150
Recombinant antibody fragments	Combinatorial libraries	25–50
Scaffold binder	Combinatorial libraries	5–20
Nucleic-acid scaffold (aptamers)	Combinatorial libraries	8
Peptides	Combinatorial chemical synthesis	1–2
Small molecules	Chemical synthesis	<1

(FACS), and the option for either in vitro or in vivo expression. Since the size of the library is directly linked to the likelihood to select high-affinity binders, many examples of libraries with more than 10^9 members have been described (23). However, to obtain very high affinities in the subnanomolar range, it is usually necessary, even for large libraries, to follow up the initial selection with an affinity maturation step involving careful protein design and selection using a purpose-built secondary library (30). These selection methods have created many affinity reagents used to probe biological questions in various fields of bioscience and it is most likely possible to generate affinity reagents with high affinity to virtually all biomolecules.

Protein Scaffolds for Affinity

During the last decade, new alternative protein scaffolds have been developed (30), often smaller in size as compared with the antibody fragments and lacking cysteines that can cause problems in folding. As an example, the affibody class of binders is based on the 58 amino acid protein A domain (32), and a large number of binders to a wide variety of protein targets have been selected using phage display (33). The small size of the affibody scaffold also allows chemical synthesis of the selected binder and such a binder has been used for tumor targeting in molecular image analysis using PET and SPECT cameras of human breast cancer patients (34). Other protein scaffolds include ankyrins, anticalins, Zn-fingers, and fibronectins (35).

Therapeutic Affinity Reagents

In the field of biopharmaceuticals, the last decade has seen the advent of a steady stream of therapeutic antibodies for various disorders (8). Originally, mouse monoclonals generated by classical hybridoma technology (36) were used, but it subsequently was shown that these affinity reagents of animal origin could be "humanized" using protein engineering (37). The development of in vitro selection systems made it possible to generate human antibodies without any mouse-derived regions, although the use of antibody fragments in these settings made it necessary to "graft" the fragment onto the framework of the complete IgG chains to generate a recombinant human monoclonal. The affinity reagents based on these combinatorial methods have led to a multibillion dollar industry within the field of cancer, autoimmune diseases, and cardiovascular disorders (8).

Solid-phase DNA Sequencing

Affinity techniques have also been shown to be very useful in the field of molecular biology and genomics. In the late 80s it was shown (38) that biotinylated double-stranded DNA could be immobilized to matrices containing streptavidin and that the nonbound DNA strand could be eluted efficiently with high concentrations of alkali without disturbing the biotin/streptavidin interaction. In this manner, single-stranded DNA could be generated conveniently from PCR-derived material in both soluble form (eluted strand) or immobilized to a solid phase (biotinylated strand). Recently it was shown that the strong affinity between biotin and streptavidin can be reversibly broken using aqueous solutions (39), providing a convenient approach for binding and quantitative elution of single-stranded DNA. The method of generating single-stranded DNA by affinity capture has found numerous applications in molecular biology, including mutational analysis, genotyping, and other DNA assays often using magnetic separation (40). The method to generate single-stranded templates was later used to develop solid-phase DNA sequencing based on the release of pyrophosphate as a consequence of the incorporation of a base using a polymerase and conversion of the released pyrophosphate to visible light using an enzyme cascade (41). This sequencing by synthesis method was subsequently named pyrosequencing (42) and it was further developed into a massive parallel DNA sequencing method called 454 sequencing (43). These new developments and similar massive sequence methods involving incorporation of fluorescent bases or sequencing by ligation (44) have led to a new era of genomics with significant improvements in throughput and cost for DNA sequencing, opening up new genome applications both for population-based analysis and metagenomics (45).

Affinity Proteomics

One of the great challenges in bioscience today is the need for well-validated affinity reagents to explore the human proteome. The generation of antibodies on a whole-proteome scale has been called affinity proteomics (46) and requires the selection and development of unit operations suitable for high-throughput performance. It has been suggested that a vision for an international effort (47,48) could be to generate paired antibodies (affinity reagents) to a representative protein from every human gene (49). The paired antibodies should preferably be produced against two separate and nonoverlapping epitopes of the same protein target to facilitate quality assessment of target specificity.

A list of different affinity reagents used in bioscience is shown in Table 3. The choice of affinity reagent depends on factors such as throughput, yield, and specificity requirements. For affinity proteomics, monospecific antibodies, in which the polyclonal antibody mixture is affinity-purified using synthetic peptides (50) or recombinant protein fragments (51), has proven useful (52). However, the rapid development of new selection methods (53) might make it possible in the future to create an almost endless variety of renewable affinity reagents in a high-throughput manner using in vitro selection principles. This emphasizes the need for high-quality antigens in milligram amounts, enough for both selection and subsequent screening and validation. So far, the most common antigens used for antibody generation are synthetic peptides and recombinant protein fragments, the latter exemplified by the Human Protein Atlas effort (52). Although conceptually attractive, the alternatives to generate full-length proteins or to isolate native proteins with

correct folding have been hampered by poor yields and the need for relatively cumbersome production procedures.

A Human Protein Atlas Based on Affinity Reagents

Based on affinity proteomics using both monospecific and monoclonal antibodies, a publicly available human protein atlas portal (www.proteinatlas.org) has been created for protein profiles in normal and cancer tissues (Figure 1). The present version of the atlas is based on the analysis of 3014 antibodies to 2618 human proteins and contains more than 2.8 million high-resolution immunohistochemistry images generated by tissue microarrays and manually annotated by certified pathologists (52). The atlas also contains confocal microscopy images of three human cell lines using four fluorescent probes giving more detailed data on subcellular localization (54). An advanced search function has recently been added to enable complex queries about proteins expressed in normal and/or cancer cells (55). The vision is to extend this atlas with up to 3000 new validated antibodies per year to generate a draft version of the complete human proteome by 2015 (49).

CONCLUSION

Affinity techniques, methodologies, and concepts have become invaluable tools in bioscience with applications spanning from protein purification, protein interaction mapping, development of biopharmaceuticals and diagnostics, as well as new developments in genomics and proteomics. These tools have been adopted, often as a consequence of creative use of molecular biology and protein engineering, and have led to many thousands of publications in the field of bioscience. It is likely that affinity-based principles will continue to be applied in the near future based not only on mimicking interactions found in nature but also on de novo molecular interactions generated by in vitro evolution or protein design. This reinforces the importance of affinity in the pursuit of biological knowledge as well as the goal of creating therapeutically useful products to benefit mankind.

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COMPETING INTERESTS STATEMENT

The author declares no competing interests.

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