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Tools and methodologies capable of isolating and identifying a target molecule for a bioactive compound

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ABSTRACT

Elucidating the mechanism of action of bioactive compounds, such as commonly used pharmaceutical drugs and biologically active natural products, in the cells and the living body is important in drug discovery research. To this end, isolation and identification of target protein(s) for the bioactive compound are essential in understanding its function fully. And, development of reliable tools and methodologies capable of addressing efficiently identification and characterization of the target proteins based on the bioactive compounds accelerates drug discovery research. Affinity-based isolation and identification of target molecules for the bioactive compounds is a classic, but still powerful approach. This paper introduces recent progress on affinity chromatography system, focusing on development of practical affinity matrices and useful affinity-based methodologies on target identification. Beneficial affinity chromatography systems with using practical tools and useful methodologies facilitate chemical biology and drug discovery research.

1. Introduction

Historically, commonly used pharmaceutical drugs have been explored and discovered on the basis of their beneficial biological activities from the screening of a crude mixture involving natural products.[1,2] In addition, cell-based phenotypic screenings of natural products and synthetic compounds have also contributed to the discovery of promising drug candidates. An essential part of modern drug discovery research had been established at the beginning of the 19th century when it became possible to isolate pharmacologically active substances that are responsible for medication. Since then, drug discovery research has evolved dramatically with advances in research fields of synthetic organic chemistry, biochemistry, and molecular biology.

After the completion of the Human Genome Project drug discovery research has focused on an approach that includes identification and characterization of molecular and cellular functions of a wide variety of proteins encoded by eukaryotic and prokaryotic genomes.[3] Thus, bioactive compounds become more important not only as therapeutic agents to treat diseases and disorders but also as useful chemical tools to examine their complex biological processes in vitro and in vivo. Especially, among bioactive compounds, natural products isolated from plants, animals, and marine products show a large spectrum biological activity and many varieties of biologically active natural products have played important role in development of pharmaceutical drugs.[4,5] In fact, during past few decades, useful pharmaceutical drugs inspired from the biologically active natural products have been synthesized.[6] Following this trend, current research interests on natural products focuses on acquirement of knowledge on their molecular targets and mechanism of action, in order to understand how they function in vitro and in vivo, as well as their isolation, structural determination, and total syntheses. Many varieties of pharmaceutical drugs including fully synthetic products and natural-based products have been developed. On the basis of the concept that a bioactive compound interacts specifically with a certain protein to exert its effect, finding the certain protein related to a particular disease and disorder becomes important. Nevertheless there are still many bioactive compounds whose target molecules have yet to be revealed.[7] Therefore, a host of bioactive compounds have been utilized without a thorough total knowledge of their target proteins or their precise biological and pharmacological mechanism of action. Lack of understanding on pharmacological profiles of the pharmaceutical drugs often causes not only unwanted side effects and unexpected adverse reactions but also treatment failure, and that in turn results in limited efficacy for certain patients. Consequently, discovery of novel target molecules and reappraisal of known target molecules are essential for efficient modern drug discovery research. Moreover, precise identification and secure acquisition of new target molecules for bioactive compounds becomes more important in order to elucidate and understand their mechanism of action in the cells and the living body. To achieve this goal, development of practical tools and useful methodologies

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to identify target molecules for the bioactive compounds of interest has been desired.

Currently, identification of target molecules for bioactive compounds of interest is becoming popular in advancing biomedical research.[8–10] Chemical proteomics is an efficient and promising approach capable of directly and comprehensively analyzing certain proteins that bind specifically with bioactive compounds of interest by means of affinity chromatography combined with identification through mass spectrometry.[2,3,11] Significant progress in cutting-edge chemical proteomics has enabled to accelerate the clinical validation of pharmaceutical drug candidates and the discovery of the novel target molecules for bioactive compounds.[12] Affinity chromatography is a useful method capable of isolating and identifying target molecules for a specific ligand, utilizing affinity between biomolecules such as antigen–antibody reactions, DNA hybridization, and enzyme–substrate interactions.[13,14] Since the development of affinity chromatography in the early 1950s, various types of target proteins for bioactive compounds have been isolated and identified.[15] Especially, the discovery of FKBP12 (FK506 binding protein 12)[16] and HDAC (histone deacetylase)[17] as specific binding proteins of FK506 and trapoxin by taking advantage of affinity matrices demonstrated the effectiveness of affinity chromatography for isolation and identification of the target protein for the bioactive compounds of interest (Table 1). Since then, affinity chromatography has been gaining renewed attention as a widely applicable technique for discovering the target proteins for bioactive compounds. However, in spite of many successful examples of isolating and identifying the target proteins for the bioactive compounds of interest by taking advantage of affinity chromatography, several drawbacks in affinity chromatography system, such as non-specific binding of irrelevant proteins during affinity purification and chemical modification of bioactive compounds of interest used as ligands, have often limited its extensive application. Following this trend, recent studies on affinity chromatography mainly focus on development of systematic and useful methodologies. Since there are many comprehensive and excellent reviews[1–5,7–12,18,19] on isolation and identification of the target molecules for the bioactive compounds of interest taking advantage of a wide variety of affinity chromatography systems, in this review we would like to introduce recent affinity chromatography systems, focusing on development of practical and useful methodologies with and without affinity matrices.

2. Outline of affinity chromatography isolating and identifying the target molecule for the bioactive compound

Among the large number of target identification methods developed to date, affinity chromatography system is the most widely utilized method.[1–3,12,18,19] In fact, a wide variety of target proteins for the bioactive compounds including synthetic pharmaceutical drugs and biologically active natural products have been isolated and identified through various kinds of affinity chromatography systems.[1,15,19] In affinity chromatography, bioactive compounds of interest are used as ligands that play a role as functional probes to explore their target molecules. Current affinity chromatography system requires chemical modification for immobilization of ligands on affinity matrices or for labeling ligands with an affinity tag (mainly biotin), a fluorophore, a photoreactive group, or a radioisotope. Moreover, appropriate preparation of affinity matrices on which ligands are immobilized properly often determines efficient and successful isolation of target molecules. Therefore, once moieties essential for activity (pharmacological active site) of the bioactive compounds of interest are determined based on in-depth structure–activity relationship (SAR) studies, they are derivatized chemically without losing their activity into functionalized compounds as ligands, which are equipped with affinity tag such as biotin, fluorophore, radioactive elements, and/or reactive groups to be immobilized on affinity matrices. The compounds with reactive groups such as carboxylic acid and amine are widely utilized for the ligands to be immobilized through covalent bonding on affinity matrices. The cases of ligands with biotin utilize avidin-immobilized matrices by means of strong affinity between biotin and avidin.[20]

Typical outline of affinity chromatography system using mobile affinity matrices is shown in Figure 1.[2,3,12,19] Ligand-immobilized affinity matrices are incubated with protein libraries such as crude cell lysates, followed by extensive washing with buffer to remove non-specific bound proteins from the matrices. Later, specifically bound proteins are eluted with either excess amount of free ligand or high salt buffer, or protein denaturing agents. Eluted samples that include specifically bound proteins are analyzed by SDS–PAGE, followed by protein identification by means of mass spectrometry after treatment of digestive enzyme. Subsequently, binding activity of the identified proteins with the ligand is confirmed. Finally, the identified proteins are validated as actual target proteins by independent biochemical, molecular, and cellular assays confirming activity of the ligands.

However, isolation and identification of target proteins for bioactive compounds of interest by means of affinity chromatography is no easy task because the situation that some of non-specifically bound proteins cover specifically bound proteins with the ligands including expected target proteins is frequently encountered. Therefore, various techniques have been utilized in order to isolate and identify the target protein candidates more efficiently and surely.

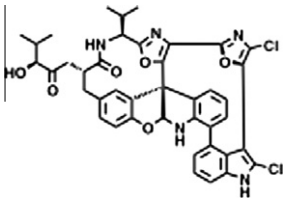
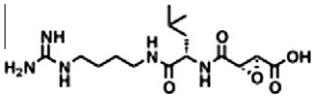
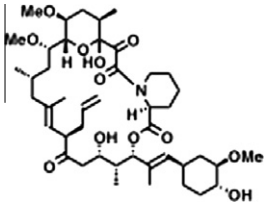
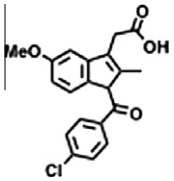
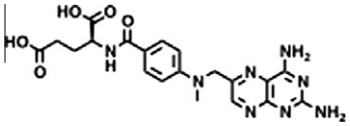
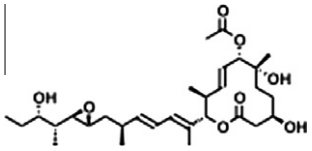
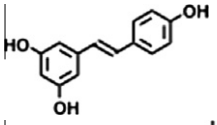
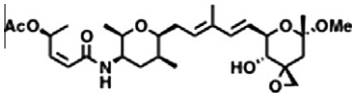
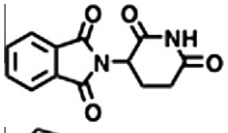
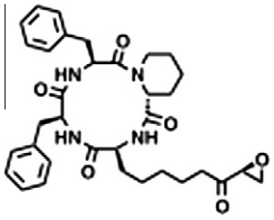
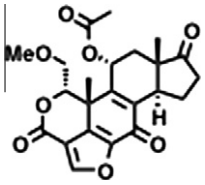
3. Tactics to discriminate the target proteins from non-specific binding of irrelevant proteins

Well-designed negative control experiments are important in distinguishing target proteins from non-specific or irrelevant binding protein. One tactics is use of an inactive analog of the bioactive compound (ligand). In this approach, design and selection of inactive molecules based on SAR studies of the ligand is key point. If an inactive molecule is available and/or can be immobilized on the affinity matrices, its use enables to discriminate anticipated target protein from non-specific binding proteins by comparison of results of affinity chromatography. The most appropriate negative control molecules would be pharmacologically inactive and chemically analogous molecules with physical properties similar to the ligand. In the case where biologically active natural products are used as the ligands, their enantiomers or diastereomers can often be used as inactive molecules, because many of them are chiral and the bioactivity of chiral molecules is dependent on the change of their chiral centers, namely their enantiomers or diastereomers sometimes lose the activity (Fig. 2).[19]

Since affinity chromatography systems make use of specific affinity between the ligands and certain proteins, the specific affinity sometimes change when buffer compositions in affinity chromatography change. Basically, target proteins for bioactive compounds of interest possess higher affinity than non-specific bound proteins. Therefore, comparison of affinity of some proteins for the ligands under different salt concentration will give information on specifically bound proteins with the ligands.

Elution of bound proteins with protein denaturing agents often gives complicated results due to occurrence of a large amount of non-specific binding proteins, and increase of non-specific binding protein becomes hard to find specifically bound proteins with the ligands including target protein candidates. Therefore, as an alternative tactics that dose not use protein denaturing agents, elution

Table 1
Selected examples of the target protein(s) isolated and identified through affinity chromatography. Contents are listed in alphabetical order of the bioactive compounds

Bioactive compound	Molecular structure	Target protein	Reference
Diazonamide A (anticancer drug)		Ornithine-δ-aminotransferase	73
E-64 (cystein protease inhibitor)		Cathepsin B, H, L	74
FK506 (immunosuppressive drug)		FKBP12 calcineurin	16
Indomethacin (antitumor drug)		Glyoxalase 1	61
Methotrexate (anticancer drug)		Dihydrofolate reductase Deoxycytidine kinase	22
Pladienolide B (antitumor drug)		Splicing factor 3b	75
Resvertatrol (health benefits including life span)		Tif1 (yeast elF4A)	68
Spliceostatin A (splicing inhibitor)		Splicing factor 3b	76
Thalidomide (sedative, anticancer drug)		Cereblon	54
Trapoxin B (antitumor drug)		HDAC (histone deacetylase)	17
Wortmannin (PI 3-kinase inhibitor)		Polo-like kinase 1 Polo-like kinase 3	77 78

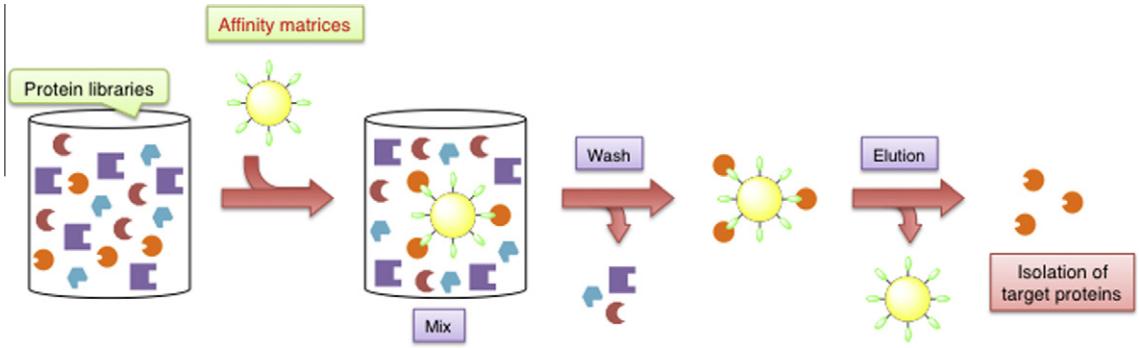


Figure 1. Outline of affinity chromatography using mobile affinity matrices.

of specifically bound proteins with a large amount of free ligands has been utilized. Viability of the selective elution often depends on solubility of the ligands and the use of ligand solution of as high concentration as possible is desired for successful selective elution. However, since most of commonly used pharmaceutical drugs utilized as the ligands in affinity chromatography are hydrophobic or liphophilic, low water solubility of the ligands is a hurdle for the selective elution. To resolve the difficulty that the ligands are less soluble in water, some hydrophilic organic solvents such as DMSO (dimethyl sulfoxide) have been used to assist improvement of water solubility of the ligands, on the assumption that the organic solvents does not affect the binding activity between the ligands and their target proteins (Fig. 2).

Experiments taking advantage of competitive inhibition of binding by ligands have also been used as an alternative tactics selecting target proteins. Ligand-immobilized affinity matrices are incubated with protein libraries with which excess amounts of free ligands are premixed, then specific proteins with lower recovery rate in this experiment are more likely to become target protein candidates to be validated when binding of free ligands with target proteins in the protein libraries competes with that of the ligands on the matrices with the target proteins. This experiment using excess amounts of free ligands also has the same problem as in the case of selective elution with the ligands. If the ligands show low water solubility, it also becomes difficult to conduct the competition experiment (Fig. 2).

Recently, Tanaka and coworkers have reported an alternate serial affinity chromatography strategy for distinguishing specific binding proteins from non-specific ones.[21] The cell lysates are mixed with the ligand-immobilized affinity matrices, which are subsequently removed, and then fresh ligand-immobilized affinity matrices are incubated with the same lysates. While both affinity matrices capture the same amount of non-specific background binding proteins, the first affinity matrices should be preferentially enriched for specific target proteins.

4. Immobilization of the ligands on affinity matrices

In performing efficient affinity chromatography, use of proper molecule is important for not only matrix-based one but also non-matrix-based one. Deep understanding on molecular structure of bioactive compounds of interest (ligands) is helpful for designing suitable ligand. As described above, usually the ligand is immobilized on affinity matrices without losing its activity based on identification of its biologically active site through SAR studies. If the ligand is immobilized on the matrices at inappropriate position, it is possible that anticipated target proteins might not be obtained.[19] Therefore, success and failure of affinity chromatography with the matrices often depends on proper immobilization of the ligand on the matrices. In contrast, there is the case that slight difference in immobilization position of the ligands on affinity matrices provides successful isolation of completely different target proteins for the ligand.[22] In elucidating the mechanism of action of anticancer agent MTX (methotrexate),[23,24] we successfully isolated completely different two target proteins for MTX using affinity magnetic beads, FG beads (described later). Using different immobilization position in MTX, we prepared two types of beads: (1) immobilization using side chain of glutamic acid moiety in MTX and (2) immobilization using α-carboxylic acid of glutamic acid moiety in MTX. Affinity purification using FG beads on which MTX is immobilized at side chain of glutamic acid provided DHFR (dehydrofolate reductase), as a known target protein for MTX. On the contrary, affinity purification using FG beads on which MTX is immobilized at α-carboxylic acid of glutamic acid provided novel target protein dCK (deoxycytidine kinase) for MTX. These results indicate that immobilization position of ligand on the affinity matrices determines isolation of target proteins in affinity chromatography.

Recently, Osada and coworkers have developed affinity chromatography system involving immobilization of ligands on affinity matrices taking advantage of a photoaffinity reaction.[25] In this affinity chromatography system, they used agarose beads in which immobilization position of the ligands is not specific. Even though the ligands are immobilized on agarose beads independently of their biologically active site, they demonstrated that the agarose beads had enough ability to carry out affinity chromatography to isolate and identify the target proteins for the ligands. They isolated and identified the target proteins of biologically active natural products using this affinity chromatography system.[26]

5. Practical affinity matrices to capture the target protein efficiently and selectively

Emergence of non-specific binding of irrelevant proteins is a major issue to be solved in affinity chromatography. Hence, successful isolation and identification of target proteins for bioactive compounds of interest through affinity chromatography often depends on appropriate choice of affinity matrices (Table 2). Properties required for practical matrices in affinity chromatography include physical/chemical stability, proper functional groups for ligand immobilization, and low non-specific binding proteins. Furthermore, considerable efforts have been made for reduction of non-specific binding protein.[27] Extensive washing conditions are often required in order to surely remove non-specific binding proteins from affinity matrices. However, it might be possible to lose expected target proteins with low affinity during the extensive washing process.[3,19] Therefore, development of practical affinity

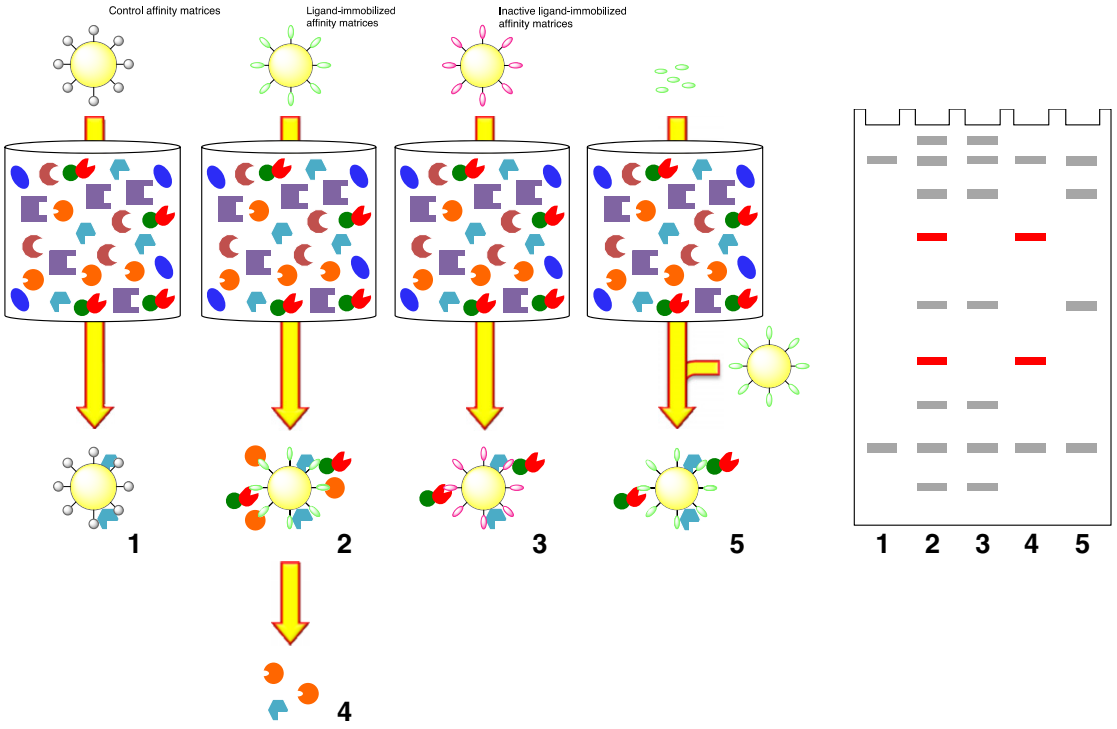


Figure 2. Typical band patterns of samples obtained through affinity chromatography using ligand-immobilized affinity matrices. Red bands are specific binding proteins and gray bands are non-specific and irrelevant proteins. Lane 1: Samples obtained by use of control affinity matrices on which the ligand is not immobilized. Ideally, no band is observed. However, several bands often appears as non-specific binding proteins to the matrices. Lane 2: Samples obtained by elution with protein denaturing agents such as SDS with use of ligand-immobilized affinity matrices. Often, target proteins as well as non-specific and irrelevant proteins are eluted. Lane 3: Samples obtained by use of affinity matrices on which inactive analog is immobilized. By comparison with the results of affinity purification with bioactive compound-immobilized matrices, target proteins is deduced. Lane 4: Samples obtained by elution with free bioactive compounds with use of ligand-immobilized affinity matrices. The elution experiment with excess amounts of the bioactive compounds sometimes provides valuable knowledge on the target proteins. Lane 5: Samples obtained by competition experiments. By combination with the results of elution experiments with excess free bioactive compounds and other experiments on affinity chromatography, more secure information on target proteins is obtained.

matrices that reduce non-specific bound proteins at a maximum has been desired.

Sugar-based affinity matrices, such as agarose[28] or sepharose,[29] have been frequently used to isolate the target molecules in the research fields of biochemistry and molecular biology. AffiGel®,[28] an agarose derivative, is one of the most common affinity matrices. Their hydrophilic character assists reduction of the non-specific binding protein. So far, target proteins for various bioactive compounds have been isolated and identified through affinity chromatography using AffiGel®. However, highly hydrophilic and chemically fragile properties of sugar-based affinity matrices often restrict preparation of ligand-immobilized matrices, since many of commonly used pharmaceutical drugs are either hydrophobic or at least less hydrophilic, their efficient immobilization on the matrices requires conditions using various organic solvents. In contrast, functional organic polymers are basically stable to various organic solvents. Therefore, use of the functional organic polymers as main components of affinity matrices is advantageous to preparation of affinity matrices bearing hydrophobic ligands such as commonly used pharmaceutical drugs. Toyopearl®,[30] a polymethacrylate derivative, has been utilized as alternative matrices to AffiGel® in affinity chromatography, which is stable to organic solvents.[31] However, affinity chromatography using methacrylate-based matrices often show high level of non-specific binding protein in comparison with cases of AffiGel®.[27] Therefore, development of hydrophilic methacrylate-based affinity matrices with low non-specific binding protein has been desired. Tanaka, Hosoya, and coworkers developed novel polymethacrylate-based monolithic affinity matrices based on their earlier studies on the reduction of non-specific binding protein. The monolithic affinity matrices possess enough hydrophilicity and show low non-specific binding protein (Fig. 3).[32–35] The matrices are composed of polymethacrylates made from three kinds of methacrylates with ethylene glycol units. They examined performance of the monolithic affinity matrices through comparison with conventional methacrylate-based matrices (Toyopearl®) using specific carbonic anhydrase II (CA II) inhibitor, benzenesulfonamide, as a model ligand. As a result, they observed that benzenesulfonamide-immobilized monolithic affinity matrices captured CA II and non-specific binding of irrelevant proteins reduced considerably.[34]

We have examined the development of practical matrices in affinity chromatography over long periods.[36] In order to resolve issues that sugar-based or methacrylate-based affinity matrices possess inherently, organic polymer-based and non-porous bead with submicron size was adopted because it offers a large surface area for binding to proteins, is highly mobile, and can be easily resuspended in buffer.[37] In addition, functionalized organic polymer bead that allows immobilization of hydrophobic ligands of interest including commonly used pharmaceutical drugs would contribute to efficient isolation of target proteins. Given these reasons, we examined development of practical affinity matrices based on functional organic polymer. At the time when the development of original affinity beads was launched, affinity chromatography to purify specific transcription factors using DNA-immobilized agarose gel had several issues such as non-specific binding protein to the gel and low efficiency of affinity purification. After careful consideration and numerous attempts to resolve these issues, we successfully developed novel latex affinity beads with 200 nm in

Table 2
List of selected affinity matrices utilized for affinity chromatography (including commercially available ones)

Name	Components	Availability
AffiGel®	Agarose (carbohydrate)	Bio-Rad Laboratories
Toyopearl®	Polymethacrylate	Tohso Bioscience GmbH
Sepharose®	Agarose	GE Healthcare
TentaGel®	Polystyrene	Sigma–Aldrich (Rapp Polymere)
AQUAFIRMUS®	Polymethacrylates with ethylene glycol unit	IEDA CHEMICALS Co., Ltd
SG beads	Polystyrene and polyGMA	—
FG beads	Polystyrene, polyGMA, and fertile	Tamagawa Seiki Co., Ltd
DynaBeads®	Hydrophilic polymer-coated magnetic iron oxide	Invitrogen

diameter, which were composed of a copolymer of styrene and glycidyl methacrylate (GMA) as the core and polyGMA as the shell (Fig. 4a and b).[36,38,39] The beads are called 'SG beads', which are named after *styrene* and *GMA*. Compared to conventionally used agarose-based affinity matrices, SG beads possess several advantages such as high dispersibility in water, physical/chemical stability, moderate hydrophilicity, and homogenous immobilization of the ligand on the beads' surface. Among these advantages, extremely low non-specific binding proteins are the most notable feature. The performance of SG beads as affinity matrices was demonstrated by successful single step purification of specific transcription factors using DNA-immobilized SG beads from crude nuclear extracts of HeLa cells.[40–44] In contrast to affinity chromatography with column or conventional matrices, affinity chromatography system with SG beads can be performed more easily within several hours. Next, isolation and identification of target proteins for the bioactive compounds of interest through affinity chromatography using SG beads was examined. However, initial attempts using ligand-immobilized SG beads were unsuccessful. We reasoned that direct immobilization of ligands on the surface of the beads considerably reduced the efficiency of affinity purification due to steric repulsion between anticipated target proteins and matrices bearing the ligand. This issue was resolved by the introduction of a proper hydrophilic linker on the beads, and we found that ethylene glycol diglycidyl ether (EGDE) was the best linker for efficient isolation of the target proteins.[38,39] Then, we evaluated performance of SG beads as affinity matrices through affinity purification of well-studied FKBP12, which is the target protein for the immunosuppressive drug FK506.[16,45,46] Results of affinity purification of FKBP12 with SG beads from crude cell lysates provided very clean single band on SDS–PAGE gel, while the case using conventional agarose beads showed significantly higher background including FKBP12. These results indicate that SG beads have enough binding efficiency of a target protein and show the property of low non-specific binding proteins (Fig. 4c).

Following the success of development of SG beads, we examined original affinity magnetic beads, which take over all advantage from SG beads. Although SG beads were useful affinity matrices compared to conventional ones, there are a few drawbacks in affinity chromatography using SG beads such as requirement of centrifugal separation process. The process of centrifugal separation followed by resuspension of SG beads sometimes takes a long time and might lose the activity of the target protein. Therefore, we turned our attention to magnetic force and expected that magnetic manipulation of the beads like SG beads by an external magnetic field improve the efficiency in affinity chromatography. Then, affinity polymer-coated magnetic bead which possesses SG beads' advantages was designed. After careful examination of conditions, we created novel polymer-coated magnetic beads through encapsulation of moderately dispersed ferrite (magnetic iron oxide) nanoparticles into organic polymer.[36,47] Compared to conventional magnetic beads, prepared magnetic affinity beads are structurally unique in terms of containing large size ferrite nanoparticles. The beads are called 'FG beads', which are named after *ferrite* and *GMA*. The size of FG beads was approximately 200 nm in diameter, nearly the same size as SG beads (Fig. 5a and b). Very recently, more magnetically responsive FG beads with 140 nm in diameter were also developed. FG beads not only enable easy collection by magnet but also show the beneficial features including high dispersibility in water and highly low non-specific binding protein.[36,47] The performance of FG beads as practical affinity matrices was confirmed through affinity purification of the target proteins for anticancer agent MTX, DHFR.[23,24] Hence, we concluded that FG beads are high performance matrices in affinity chromatography with respect to purity of the target proteins for the bioactive compounds and extremely low non-specific binding of irrelevant proteins (Fig. 5c).[36,47,48] In addition, comparison of FG beads with commercially available magnetic affinity matrices including Dynalbeads®, micrometer-sized polymer-coated magnetic beads,[49] revealed superiority of FG beads in affinity chromatography. To date, we have isolated and identified new target proteins for various bioactive compounds of interest using SG beads and FG beads, and elucidated their mechanism of action.[22,36,50–56] One of notable achievements on isolation and identification of the target proteins for the bioactive compounds of interest through affinity chromatography with FG beads is elucidation of the mechanism of action of thalidomide.[54] Although thalidomide had been known as typical drugs that causes serious adverse effects, recently it has been revised as a therapeutic agent for a certain leprosy and multiple myeloma[57,58] and has been prescribed

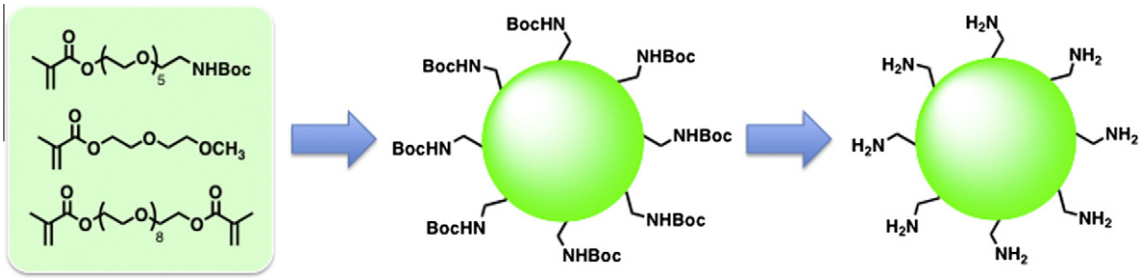


Figure 3. Preparation scheme of monolithic affinity matrices.[34]

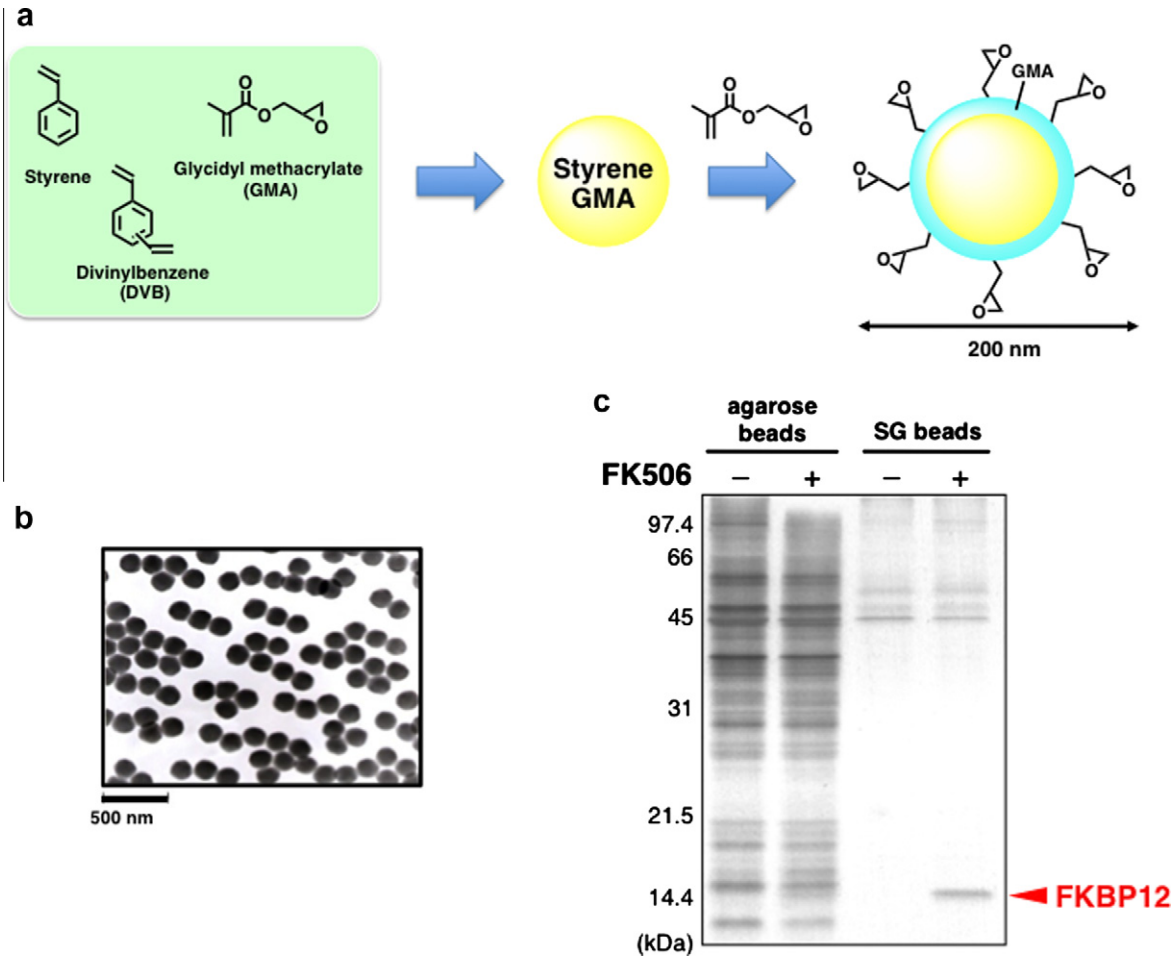


Figure 4. Latex affinity matrices SG beads: (a) Preparation scheme of SG beads; (b) Transmission electron microscopy image of SG beads; (c) The result of affinity purification of FKBP12 using FK506-immobilized agarose beads and SG beads from crude cell lysates. Bound proteins in eluted samples were subjected to SDS–PADE and visualized by silver staining.

under strict control in order to avoid its serious side effects. Nevertheless, the exact mechanism of action of thalidomide including its target protein has been unclear. In order to clarify the mechanism of action of thalidomide, we first examined affinity purification of specifically bound proteins with thalidomide using thalidomide-immobilized FG beads. As a result, damage specific DNA binding protein 1 (DDB1) and celebron (CRBN) were isolated as specifically bound proteins with thalidomide-immobilized FG beads (Fig. 6). Then, in vitro binding assay with recombinant proteins revealed that CRBN interacted with thalidomide directly. Through in-depth biochemical analyses, we found out mutant $CRBN^{YW/AA}$ that kept biochemical functions of wild-type CRBN, but showed extremely low thalidomide-binding activity. Finally, we demonstrated that CRBN is a *bona fide* target protein for thalidomide through animal experiments using zebrafish and chick/chicken with mutant $CRBN^{YW/AA}$.[54]

6. Linker to improve efficiency of affinity purification to isolate the target proteins

In performing affinity chromatography with affinity matrices, use of proper linker affects the success of obtaining the target proteins for the bioactive compounds of interest. Moreover, appropriate choice of linker is considerably important for diminishing non-specific binding protein. So far, a variety of linkers with polymethylene chain and polyethylene glycol (PEG) chain have been utilized. However, since increase of hydrophobicity leads to enhancement of non-specific binding protein, use of hydrophilic linkers with PEG unit is preferable because of desirable physical properties of PEG unit.[27,59] Linker length is also an important factor that determines the success of affinity chromatography. We have used EGDE as a workable linker in affinity chromatography using SG beads and FG beads, as described above.

Tanaka and coworkers have studied systematically various kinds of hydrophilic linkers to reduce non-specific protein binding in affinity chromatography using some commercially available matrices.[27,60] By use of hydrophilic linker with proper length, the methacrylate-based affinity matrices achieved the same ability to capture the target proteins and the same levels of non-specific binding protein as AffiGel®.[27] They also investigated several hydrophilic materials with PEG units, glucose derivatives, or tartaric acid derivatives as linkers to reduce non-specific binding protein using methacrylated-based affinity matrices Toyopearl®,[60] then they found that introduction of the hydrophilic spacer derived from tartaric acid effectively reduced non-specific binding proteins. Furuya and coworkers investigated effect of PEG-based hydrophilic linkers with different length on Toyopearl®, and found that linker with more than 10 ethylene glycol units considerably diminish non-specific binding proteins without loss of capture ability of the target proteins.[59] Uesugi and coworkers examined the effects of PEG-based linker on recovery rates of target proteins from affinity matrices and found that linker with more than 6 ethylene glycol

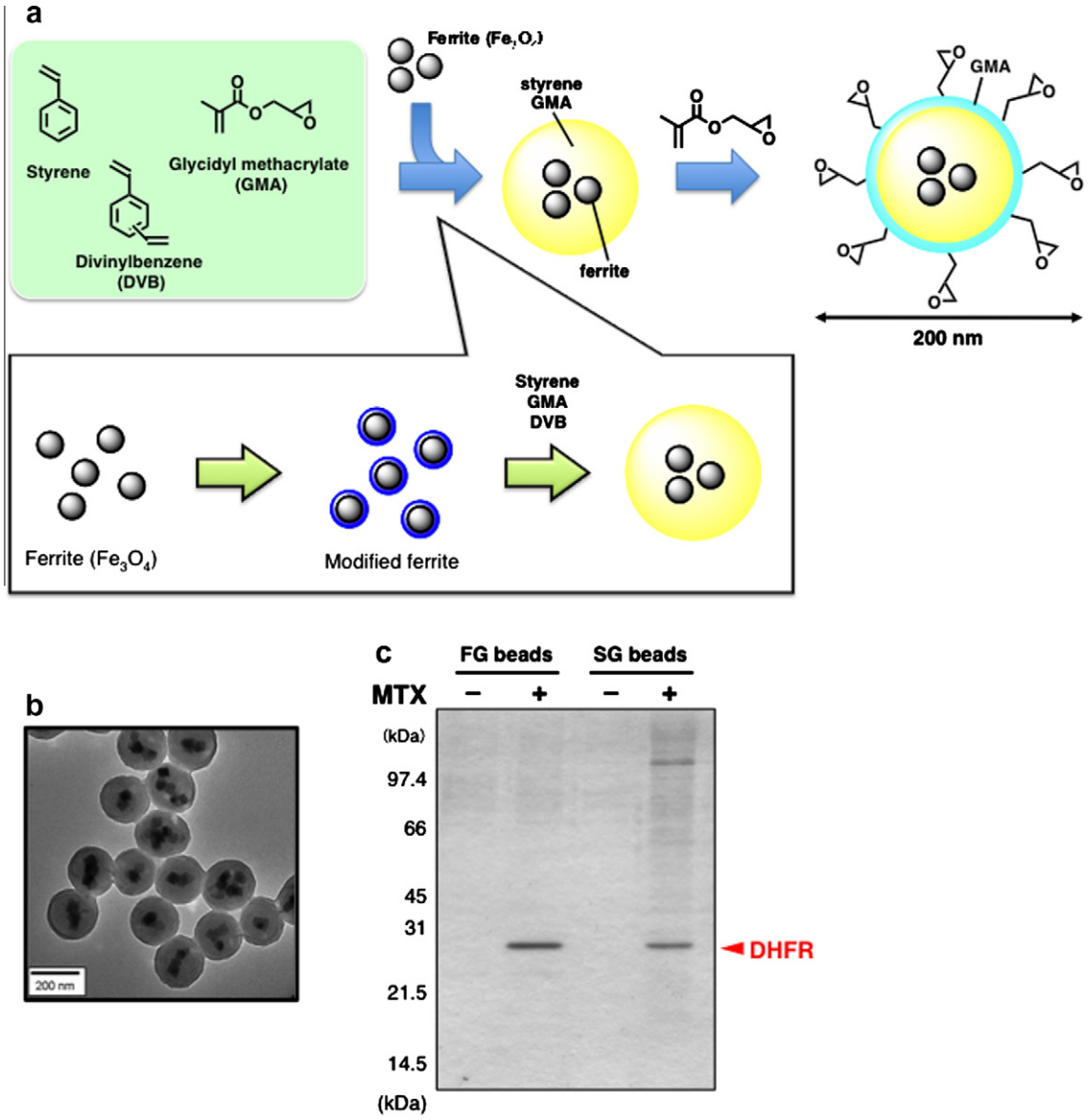


Figure 5. Magnetic affinity matrices FG beads: (a) Preparation scheme of FG beads; (b) Transmission electron microscopy image of FG beads; (c) The result of affinity purification of DHFR using MTX-immobilized FG beads and SG beads from crude cell lysates. Bound proteins in eluted samples were subjected to SDS–PADE and visualized by silver staining.

units showed higher recovery of the target proteins from cell lysates.[19,61] Furthermore, they found that elongation of the linker by insertion of a rigid polyproline helix improved the capacity of affinity purification.

As other linker type in affinity chromatography, chemically cleavable linkers have been introduced recently. In isolation and identification of specifically bound proteins to the ligands, contamination of samples for mass spectrometry has frequently occurred due to use of harsh conditions in obtaining the samples. To avoid this issue, various kinds of chemically cleavable linkers have been developed.[62]

7. Activity-based protein profiling (ABPP)

A chemical proteomic approach that accesses target proteins taking advantage of reactive functional groups of molecules of interest also constitutes a powerful means for identifying unknown target molecules. Activity-based protein profiling (ABPP) is an alternative chemical proteomics approach for selective identification of enzyme activity[9,63–65] and utilizes small molecule (functionalized probe) containing reactive functional groups that can covalently attach to catalytic site in an enzyme active site. Covalent linkage between the functionalized probe based on the small molecule of interest and its target protein is important factor for achieving ABPP.[66]

The key to ABPP is molecular design of tag functionalized probe. The functionalized probe is basically composed of three elements (Fig. 7a).[63] The first element of the probe is a reactive functional group (mainly electrophiles) that has a high affinity for active sites of the target enzyme. The second one is a spacer, which is usually a short hydrophilic PEG chain that separates the reactive group from a tag. The third one is the tag, which serves for the enrichment, identification, and visualization of the target enzymes. Based on molecular architecture of the bioactive compound of interest,

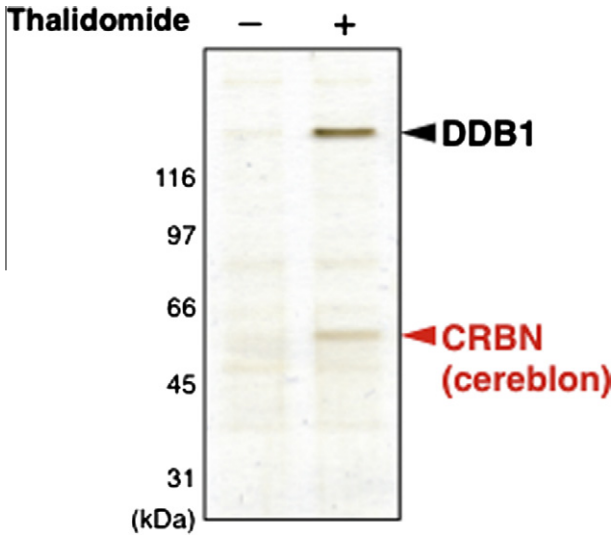


Figure 6. Isolation and identification of the target protein for thalidomide through affinity chromatography using FG beads. CRBN and DDB1 were purified from crude cell extracts using thalidomide-immobilized FG beads. Bound proteins in eluted samples were subjected to SDS–PADE and visualized by silver staining.

functionalized probe equipped with both reactive functional group and tag including fluorophore and chromophore is designed and prepared. The functionalized probe binds in the free active site of its target enzyme and provides the visualization and identification by fluorescence detection and SDS–PAGE (Fig. 7b). In addition, introduction of bioorthogonal reactions such as the Huisgen cycloaddition ('click' chemistry) or the Staudinger reaction into ABPP system expands its application range. Such bioorthogonal reactions show low reactivity towards DNA and proteins, and can be easily achieved in buffer (Fig. 7c).[63,67]

Biologically active natural products have served as lead structures of pharmaceutical drugs because of specific pharmacological activity derived from its unique molecular architecture, and they have been the basis for numerous approved pharmaceutical drugs. Moreover, as described before, current research interest on natural products moved to elucidation of their mechanism of action based on their molecular targets. Interestingly, there are many examples of the application of ABPP to biologically active natural products because they already show specific affinity with certain proteins, especially enzymes, and possess unique and characteristic molecular structures. Therefore, the biologically active natural products can be utilized as efficient scaffolds for isolating and identifying their target proteins and can be transformed chemically into functionalized probe with specifically reactive groups such as azido group and alkyne group, which is applicable to click chemistry.[63]

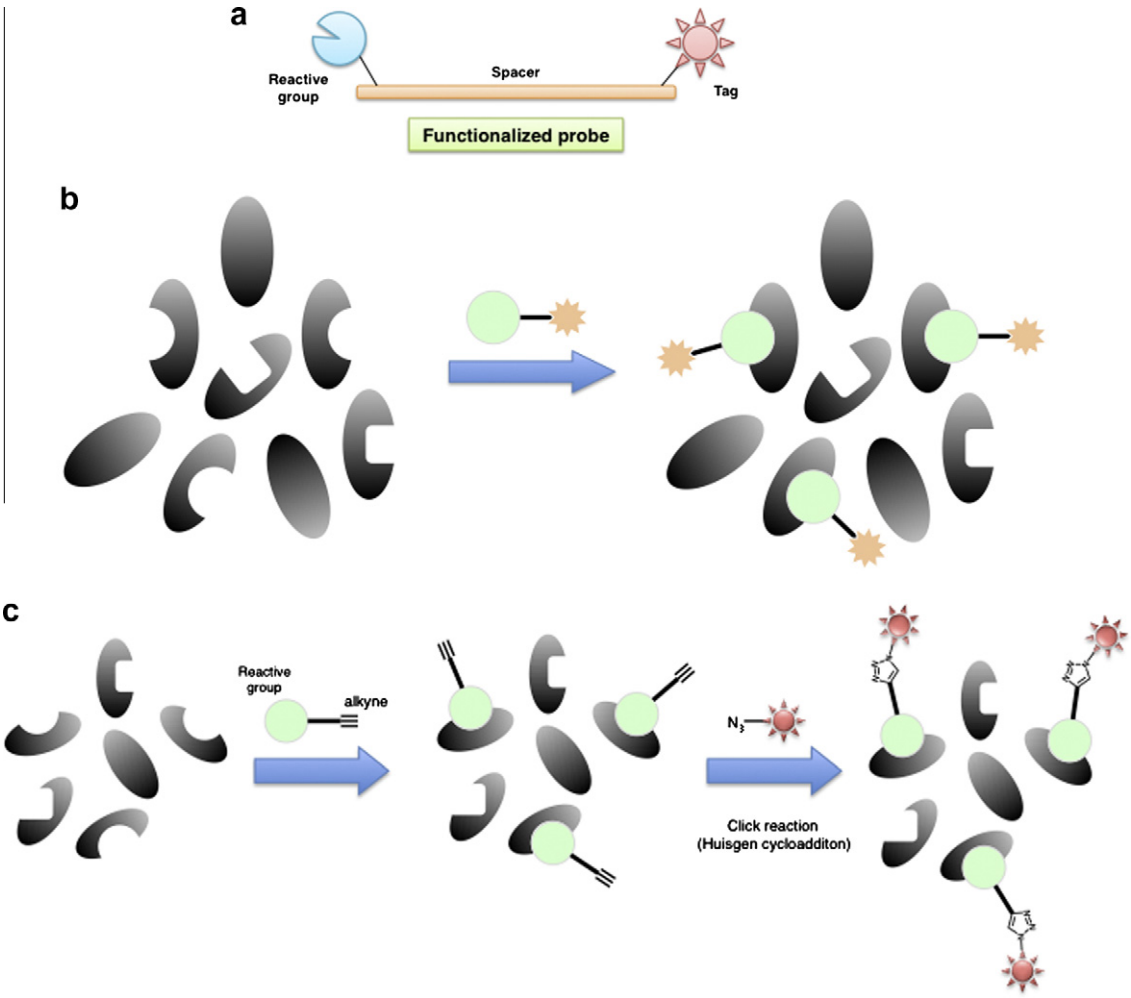


Figure 7. Activity-based protein profiling (ABPP). (a) Functionalized probe in ABPP. The probe contains a reactive group to capture target protein for the bioactive compound of interest, a tag to visualize, enrich, and identify the target protein, and a spacer with proper length to link the reactive group and the tag. (b) Scheme of ABPP. The functionalized probes are incubated with protein libraries. After linkage of the functionalized probes with specific proteins (target proteins), the proteins are detected taking advantage of property of the tag. (c) ABPP making use of 'click chemistry'.

8. Drug affinity responsive target stability (DARTS)

Most of currently used affinity-based target identification techniques including ABPP system require chemical modification of bioactive compounds of interest based on SAR studies. However, chemical modification of the bioactive compounds could limit effectiveness of affinity chromatography because their chemical modification is often time-consuming challenge. Then, to overcome this drawback in current affinity chromatography system, Huang and coworkers developed a simple target identification approach, drug affinity responsive target stability (DARTS), which takes advantage of susceptibility of the target protein upon ligand binding to protease.[15,68] This proteomic approach is based on the concept that a protein complex formed by specific binding of a bioactive compound (a ligand) with its target protein becomes stable to a certain protease. Stabilization of the protein complex induced by ligands has been exploited by numerous techniques in order to detect and analyze specific interactions between the ligands and their target proteins. Therefore, although this idea of ligand-induced resistance to proteolysis has been known, they demonstrat the potential of application of DARTS for target identification of a bioactive compound of interest without its chemical modification.

Whereas current affinity chromatography systems utilize positive enrichment by selectively accumulating the target proteins for tag bioactive compounds of interest along with precluding non-specific binding proteins, DARTS makes use of negative enrichment by digesting away non-target proteins while leaving behind the target proteins (Fig. 8). The procedure of DARTS involves separation of ligand-treated and control protein samples digested with varying amounts of protease by SDS–PAGE and analysis of each lane of the gel for bands that are more intense than the others. They demonstrate feasibility of DARTS taking advantage of specific interactions between FK506 and its target protein FKBP12 and reveal molecular mechanism of the biologically active natural product based on its target protein taking advantage of DARTS.[15,68]

The key advantage of DARTS is no requirement of chemical modification of bioactive compounds of interest and use of 'native' ones for binding with the protein libraries. In addition, DARTS does not require extensive washing conditions and has a potential to be applicable to analyze lower affinity interactions between the bioactive compounds of interest and their target proteins. Same as conventional affinity chromatography system, affinity of the bioactive compound to its target protein would be a limiting factor. Additionally, protein's susceptibility to proteolysis might be problematic point in DARTS. On this point, they investigated the protease for which is applicable to DARTS and found versatile proteases applicable to DARTS.

9. Outlook

Isolating and identifying target proteins for bioactive compounds of interest such as commonly used pharmaceutical drugs and biologically active natural products through affinity chromatography gives valuable information on biological reactions in the living body and also provides new insights for developing therapeutic agents. Eventually, the importance of affinity chromatography is increasing in phenotype-based drug discovery research. Basically, this review focuses on the affinity chromatography system with mobile affinity matrices and recent progress on affinity chromatography system. Apart from methodologies described here, other many practical tools and useful technologies on affinity chromatography[69–72] that have both merits and demerits. Thus, selection and

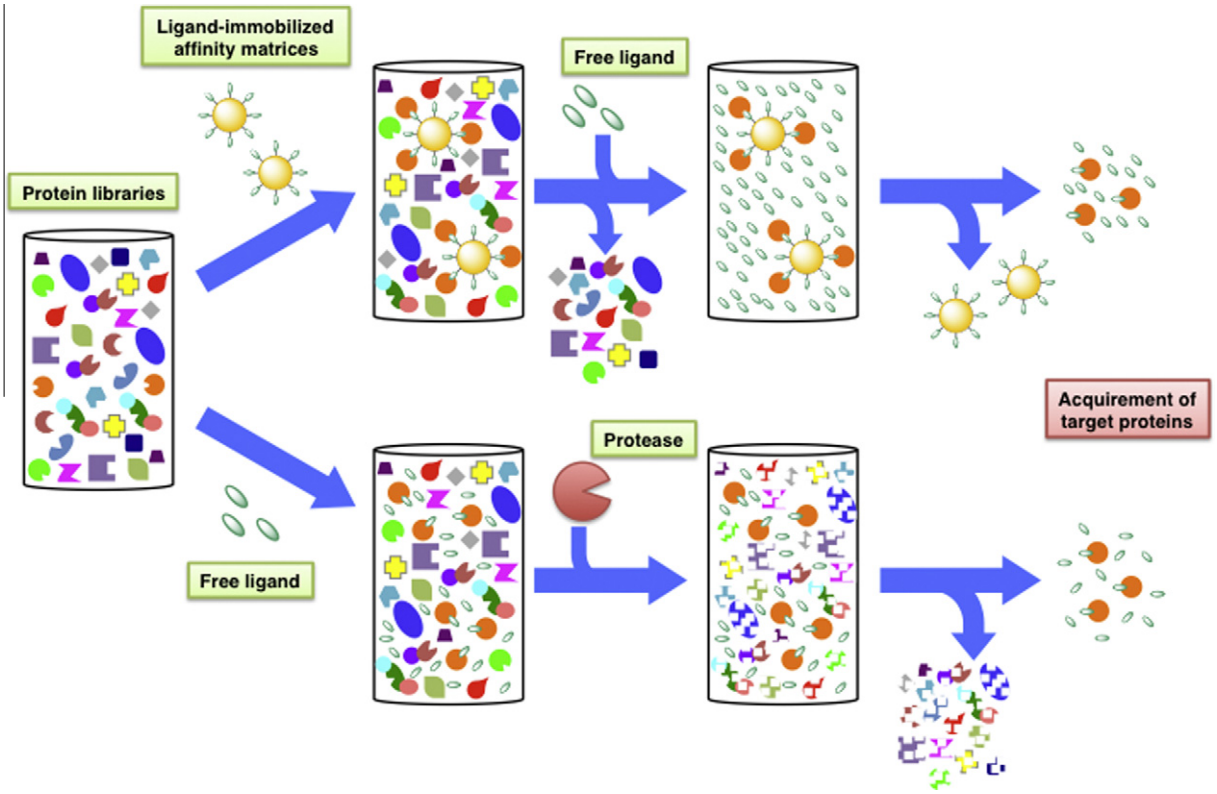


Figure 8. Comparison of affinity chromatography using mobile matrices with drug affinity responsive target stability (DARTS). Whereas affinity chromatography with mobile matrices utilizes positive enrichment of the target proteins for bioactive compounds with excluding non-specific bound proteins, DARTS utilizes negative enrichment of the target proteins using 'native' bioactive compounds and protease.

usage of suitable tools and methodologies according to property of the bioactive compounds of interest will be best in acquiring their target molecules through affinity chromatography. Recent progress on affinity chromatography system enables to understand more rapidly and more deeply functions of the bioactive compounds of interest, and thereby strongly drive drug discovery research. By using these thoughtful affinity chromatography systems, basic and applied research around chemical biology and drug discovery research will be promoted actively. Recent advances in various analytical technologies including highly sensitive mass spectrometry have enabled us to improve biochemical isolation and validation of the target proteins.[12]

Isolation and identification of target proteins for bioactive compounds of interest through affinity chromatography is just starting point of drug discovery research and chemical biology. Starting from the target proteins, much of knowledge on the bioactive compounds obtained from various experimental results including biochemical and non-biochemical analyses and animal experiments must be valuable for understanding their mechanism of action and the network of related biological reactions. Thus, affinity chromatography system using practical tools and/or useful methodologies contributes to the advancement of a wide range of research fields such as drug discovery research, proteome analysis, and chemical biology, and thereby target molecules isolated and identified through affinity chromatography would offer a new avenue for life science and life innovation.

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