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Review

Frontal affinity chromatography in characterizing immobilized receptors

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ABSTRACT

The state-of-the-art in frontal affinity chromatography (FAC) applied to receptor of pharmaceutical interest is here reported. This review will first discuss the principles of FAC for ligand characterization (K_d determination) and for screening studies, and will examine the different strategies that have been followed for the immobilization of a broad range of receptors (cytosolic and membrane receptors). Several reported applications will then be presented demonstrating that FAC is an interesting tool enabling convenient and efficient screening in the identification of new potential ligands. Moreover new applications of FAC including dual binding site assay, receptor subtype characterization, and multi-receptor binding experiments will be underlined.

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1. Introduction

In the last years the rapid scientific advances in human genomics and proteomics have brought to the identification of new therapeutic targets while the development of high-throughput synthesis techniques has generated hundreds or even thousands of new chemical entities with potential therapeutic activities. Although remarkable advances in generating complex libraries of molecules have been made, the analytical process of analyzing and screening the huge amount of generated compounds is still a challenge. To keep up with the synthetic process, analytical techniques should be able to screen a large number of compounds in a high-throughput manner with precision and accuracy. Thus, high-throughput screening (HTS) of a large number of molecules towards potential targets has now become an integral component of modern drug discovery and over the past decade demands on new analytical supports for drug discovery have intensified [1–5].

Typical methods used for HTS are often solution based. However, solution-based assays have limited versatility in terms of format, detection method, and extent of miniaturization. Solid phase assays are not only able to overcome these limitations, but also present additional advantages such as the potential to reuse proteins and

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to develop novel assay modes such as the methods based on affinity chromatography [6].

Affinity chromatography is defined as a liquid chromatographic technique that makes use of a "biological interaction" for the separation and analysis of specific analytes within a sample [7,8].

The retention of solutes in this method is based on the same type of specific, reversible interactions that are found in biological systems, such as the binding of a ligand to a receptor. "Bioaffinity chromatography" and "biointeraction chromatography" are the terms used to specify whether the affinity ligand is a biological molecule.

The type of support used for the immobilization of the biological species can be used to distinguish between one affinity method and another: in "low-performance affinity chromatography", the support usually is a large diameter, non rigid gel (i.e. agarose, dextran, or cellulose) while in "high-performance affinity chromatography" (HPAC), the support consists of small, rigid particles based on silica or synthetic polymers that can be used in HPLC systems [9,10].

Two general ways can be used in HPAC experiments: zonal elution and frontal analysis. In both of these formats, the protein of interest is used as the immobilized ligand and an injection (zonal) or application (frontal) of analyte is made onto the affinity column. By examining the elution time or volume of the analyte after it has passed through the column, it is possible to obtain information on the equilibrium constants that describe the binding of the analyte to the affinity ligand.

Frontal chromatography is a quantitative method developed in 1975 by Kasai and Ishii [11]. The above mentioned experimental approach, when applied on a bioaffinity column is termed frontal affinity chromatography (FAC). With this technique molecular interactions can be discovered and characterized as the stationary phase is constructed around one of the components of a binding event. As ligands flow through the column and bind with the target, individual ligands are retained in the column on the basis of their affinity for the target and detected as characteristic breakthrough curves. The binding is established in the chromatographic column and the mobile phase containing potential ligand(s) may be monitored using any detector appropriate for the compounds being tested for a given application: from low-throughput interaction characterization (i.e. K_d determination) to HTS (i.e. ligands ranking). When a single compound has to be assayed, then optical detection (UV–vis) may suffice. However, FAC does not separate compounds, so the detection of multiple binding events requires either the selective labeling of a marker ligand and the use of a suitable detector (radio-detector), or a detector capable of discriminating between coeluting ligands. In this context frontal affinity chromatography coupled to mass spectrometry (FAC–MS) can be considered the most flexible and generalized strategy for ligand binding studies [12].

From a discovery standpoint, aside from the utility of the frontal analysis method to support precise and accurate K_d measurements on single ligands, interfacing FAC to MS enables the screening of compound mixtures and provides the opportunity to rank-order binding strengths in a single experiment as each compound has a unique m/z value [13–16].

Bioaffinity chromatography has been successfully applied in many pharmacological studies and to a wide range of targets including receptors, transporters, plasma proteins and enzymes [7,17–19]. The present review is focused on receptor of pharmaceutical interest and will examine the principles, potentialities and recent advancements of frontal affinity chromatography as a platform technology for receptor–ligand interaction analysis in drug discovery. The next items considered will include a summary of the most significant and recent FAC systems based on receptor of pharmaceutical interest including trans-membrane ligand gated ion channel receptors, trans-membrane G-protein coupled receptors, soluble nuclear receptors, and kinase receptors. The applications of FAC systems will be described and the unique advantages of each system will be highlighted.

2. Approaches for studying drug–protein binding by HPAC

As described in the introduction, zonal elution and frontal analysis are the two principal experimental approaches used to characterize drug–protein binding in HPAC [7,20].

The zonal elution theory will not be treated in this context as out of the scope of this review while the potentialities of frontal affinity chromatography will be considered focusing the discussion on targets of pharmaceutical interest.

The theoretical and experimental approaches in FAC have been well described in many papers [13–16]. Briefly, the protein target is immobilized on a solid support that is packed in a chromatographic column. As the analyte under investigation flows through the column and binds with the target, it is retained in the column on the basis of its affinity for the target and detected as characteristic breakthrough curves. The saturation of the target by the analyte produces a vertical rise in the chromatographic trace, which ends, or plateaus, when the target is saturated. If fast association and dissociation kinetics are present in the system, then the mean positions of the breakthrough curves can be related to the concentration of applied solute, the amount of ligand in the column, and the association equilibrium constants for solute–ligand binding. The mean position (inflection point) of the breakthrough curves is the experimental parameter used for low-throughput characterization of a single analyte (K_d determination) and high-throughput screening of ligands mixtures (relative affinities determination).

2.1. Measurements of binding constants by frontal analysis

By FAC it is possible to accurately determine the affinity constant between an immobilized biomolecule and an analyte by continuously infusing analyte samples at different concentrations through the stationary phase and monitoring breakthrough times. The mean position of the breakthrough curves shifts to shorter breakthrough times as the analyte concentration increases. The simplest binding event involves the interaction of an analyte with a single class of binding sites. There may be multiple, equivalent sites in a given target molecule but the model assumes their independence. In the basic equation of FAC (Eq. (1)) two variables are present: $[A]_0$ (ligand infusion concentration) and $V - V_0$ (breakthrough volume V for the analyte corrected by the breakthrough volume of the analyte in the absence of the binding event V_0). True measurements of V_0 requires the ablation of the specific binding or the application of a saturating analyte concentration. By analyzing changes in $V - V_0$ vs. $[A]_0$ by means of Lineweaver–Burk type double reciprocal plot or standard nonlinear regression analysis, B_t (corresponding to the dynamic capacity of the affinity column for the ligand), and dissociation constant K_d (in M, corresponding to the dissociation constant for the interaction) are obtained.

$$(V - V_0) = B_t \times ([A]_0 + K_d)^{-1} \quad (1)$$

When competitive or non competitive mechanisms must be analyzed a key requirement is the existence of a compound which is known to bind to the target, the marker ligand [20].

Once it has been established that the immobilized target specifically binds the marker ligand, it is possible to calculate the affinity of the marker or of a competitive analyte (displacer) using frontal displacement chromatography. In the displacement studies, increasing concentrations of a detectable marker or of the competitive analyte, in the presence of constant concentrations of marker, are added to the mobile phase and the effects on the breakthrough

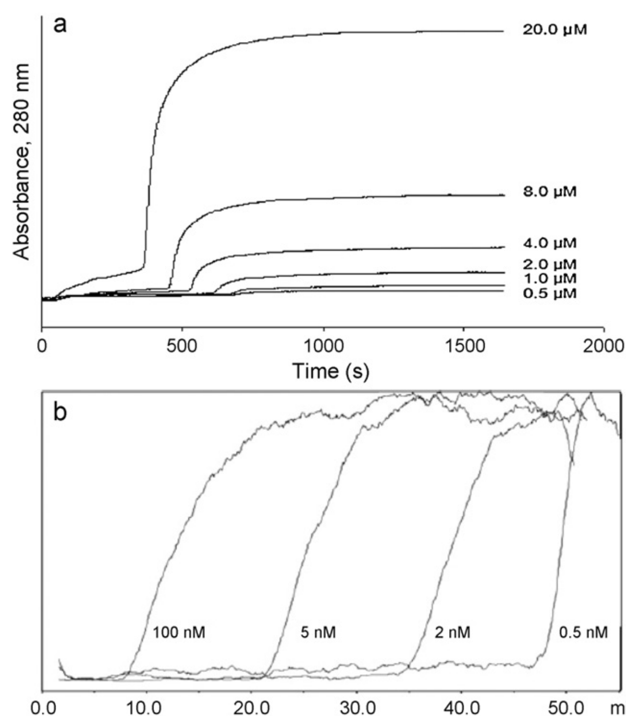

Fig. 1. Typical frontal affinity chromatography curves obtained frontal chromatography (a) and frontal displacement chromatography (b). (a) Typical frontal curves obtained on an HSA column during frontal analysis experiments with *S*-verapamil being applied as the analyte for K_d determination. The concentrations that are shown represent the concentration of *S*-verapamil that was applied. K_d value is derived using Eq. (1). Reprinted with permission from Ref. [9]. (b) Frontal displacement chromatography study for K_d determination of mepyramine. The chromatographic traces produced by [^{3}H]-mepyramine [50 pM] on the CMAC (1321N1P2Y1) column after the addition of mepyramine to the running buffer in 0.5 nM, 2 nM, 5 nM and 100 nM concentrations. K_d value is derived using Eq. (2). Reprinted with permission from Ref. [49].

volumes of the marker are determined. The relationship between displacer concentration [*D*] and marker retention volume can be established using Eq. (2) and can be used to determine the K_d value of the displacer as well as the number of active binding sites.

$$(V - V_0) = B_t \times (K_d + [D])^{-1} \quad (2)$$

where *V* is the retention volume of displacer ligand and V_0 is the retention volume of displacer ligand when the specific interaction is completely suppressed. From the plot of $[D] \times (V - V_0)$ versus [*D*], dissociation constant values for displacer analyte can be obtained.

As an example Fig. 1 reports typical frontal curves with *S*-verapamil being applied as the analyte to a HSA column for K_d determination and typical frontal displacement chromatography curves for mepyramine K_d determination on 1321N1P2Y1 column using [^{3}H]-mepyramine are marker ligand [9,49].

2.2. Screening studies by FAC

Although FAC is often used to look at the binding of a single analyte at a time, this technique can be combined to mass detection for the simultaneous study of multiple solutes. This can be accomplished by using detection at selected *m*/*z* values to generate separate frontal analysis curves for each analyte in the mixture allowing compounds ranking based on their relative binding strength for the column. FAC–MS allows two screening possibilities, with and without an indicator. The concept of using an indicator has increased the capability of FAC–MS. An indicator is a compound detectable by MS that binds with a known affinity to a specific site on the immobilized protein target with a defined

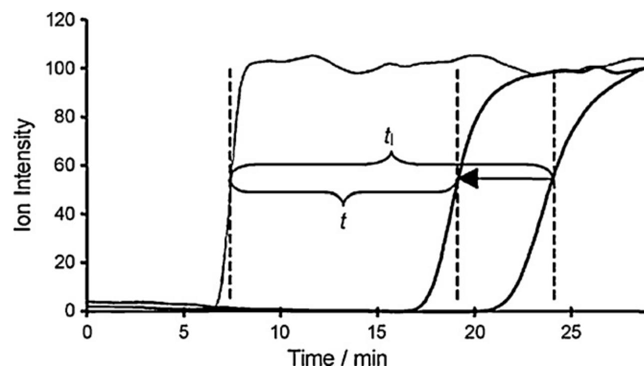

Fig. 2. Typical FAC curves obtained using the "indicator" screen method. Reprinted from Ref. [21].

breakthrough volume under constant chromatographic conditions. In screening studies using an indicator, a ligand or a mixture of ligands in the presence of the indicator and a void marker (a compound that has no affinity for the immobilized protein target and gives the same elution front whether the target protein is present in the column or not), is infused over the stationary phase and only the indicator and the void marker are monitored by MS.

The indicator method determines the extent (or percentage) to which a ligand(s) shifts an indicator for a particular target. With this method, the FAC–MS readout (percentage shift of the indicator) can be used to rank the binding of a series of ligand or ligand mixtures: the greater the percentage shifts, the greater the degree of competition for the indicator. With this technique it is also possible to rank mixtures and only those mixtures in which a significant displacement (or shift) of the indicator is observed merit further investigation and require deconvolution [21].

The % shift graphically shown in Fig. 2 is quantified from Eq. (3):

$$\%\,\text{Shift} = \frac{t_I - t}{t_I - t_{NSB}} \times 100\% \quad (3)$$

where *t* is the breakthrough time difference, measured at the inflection point, of the sigmoidal fronts between the indicator and a void marker in the presence of any competing ligand(s); t_{NSB} is the nonspecific binding breakthrough time difference in the absence of immobilized protein (and is a constant for the indicator used); and t_I is the breakthrough time difference in the absence of any competing ligands.

The use of a void marker and MS detection also allows ligands to be evaluated simultaneously in an equimolar mixture in a direct readout fashion (Q1 scan) and in a single experiment [22].

The Q1 scan FAC–MS screening method offers a very convenient way of measuring the relative binding strengths of ligands by monitoring their respective *m*/*z* values through extrapolation of individual breakthrough curves. In this approach, a sample consisting of an equimolar mixture of compounds is continuously infused through the column and the order of elution parallels the order of affinity, with the stronger ligand eluting last (Fig. 3). Mixtures arising from natural product extracts may also be screened by this technique [23,24].

3. Preparation of receptor-based stationary phases

The immobilization of the biological target is the first step for the development of an affinity chromatographic method. The primary concern in the preparation of a bioaffinity column is the choice of an immobilization procedure that can preserve receptor binding capacity. Indeed, the immobilized receptor should ideally mimic the interactions it has within its natural environment. Thus, it is important to select an immobilization method that will place the

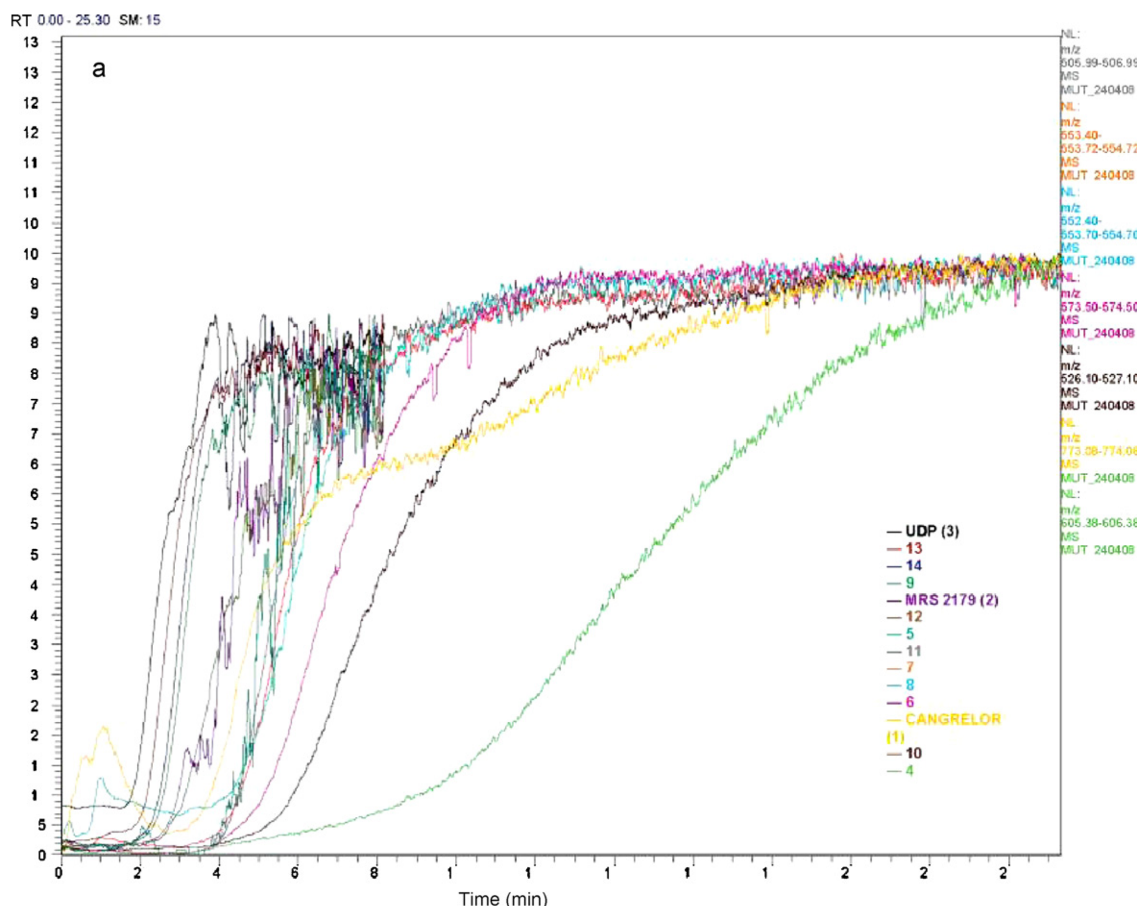

Fig. 3. Q1 scan FAC–MS screening method. Extracted breakthrough curves obtained by infusing a mixtures of potential ligands in the presence of three reference compounds each at 1 μM concentration through a GPR17 receptor stationary phase.
Reprinted from Ref. [51].

receptor on a support with the proper orientation and structure for binding to its ligands.

The immobilization of protein molecules has been widely studied and a variety of covalent and non-covalent immobilization techniques with different chromatographic supports have been developed [7,25]. The choice of the immobilization support (silica particles, controlled pore glass beads, sepharose, etc.) and immobilization method (covalent and non covalent) depends on the type and on the source of receptor under investigation (recombinant and purified receptors or membrane-bound receptors). Due to the mechanical properties of silica, silica particles have been selected as chromatographic support for affinity chromatography in many works. When this material is used the first step involves activation of the silica, followed by coupling of the protein to the activated support. Functional groups on proteins that can be used in such a process include amine, sulfhydryl, hydroxyl, or carbonyl groups. Of these, the use of amine groups is the most common and a complete summary of these methods has been given by Hage et al. [10,26].

The covalent methods are the most common approach for immobilizing cytosolic receptors to silica and different immobilization chemistries have been utilized. However, the covalent immobilization can result in several limitations, including loss of protein activity and unfavourable orientation of the receptor binding site. Moreover, the covalent immobilization is not generally applicable to membrane-associated receptors (ion-channel-linked receptors, G-protein-linked receptors and enzyme-linked receptors) and alternative entrapment technologies must be considered. In fact the structure of integral membrane proteins relies on hydrophobic interactions internal to the lipid bilayer, as well as hydrophilic interactions on either side of the lipid membrane. This requirement makes immobilization of membrane receptors difficult.

Since membrane receptors are the most popular targets for drug development, different immobilization strategies have been used to maintain membrane integrity and to preserve the receptor in its active form. We will report the most employed approaches for transmembrane receptors non covalent immobilization.

Cellular membrane chromatography (CMC) or cellular membrane affinity chromatography (CMAC) is a technique based on the immobilization of cell membranes containing the target transmembrane protein onto a stationary phase. The first cellular membrane column was described by Lundahl's group [27,28], after this work many applications of CMC have been described by Wainer group [20].

In these works, membranes obtained from cell lines that express a target membrane receptor (mainly G-protein coupled receptors and ion channels) have been mostly bound on immobilized artificial membrane (IAM) stationary phase. The IAM liquid chromatographic stationary phase was developed by Pidgeon et al. [29]. It is comprised of silica particles (12 μm i.d. with 300 Å pores) to which a monolayer of phospholipid analogues, with functional head groups, have been covalently coupled (Fig. 4). The specific feature of IAM is that this material is physically and chemically similar to cell membranes and, therefore, mimic fluid phospholipid bilayers. Thus, membrane proteins and receptors can be immobilized on a native hydrophobic environment.

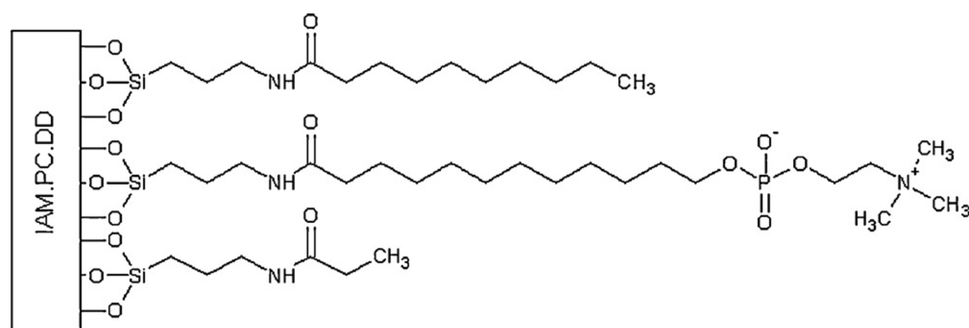

Fig. 4. The chemical structure of the monolayer that is covalently bound to the silica on the IAM-PC particles (12 μm, 300 Å pores).
Reprinted from Ref. [20].

The preferred source for cellular membranes containing the target transmembrane protein is a transfected cell line. The advantage of using an engineered cell line is that the non-transfected cell line can be used as a control to identify a specific interactions with all the components of the biological materials. For the preparation of the receptor-column the cells (10^6 to 10^7 cells) are homogenized and solubilized using a suitable detergent. After solubilization, the membrane preparation is mixed with the IAM stationary phase and dialysed. This results in the removal of detergent and in the immobilization of the lipid bilayers that contain the transmembrane protein onto the IAM stationary phase. More recently the same membrane preparation has been immobilized on the inner surface of silica capillaries to create open tubular chromatographic columns (OTC). This is the preferred approach when non-specific interactions with the IAM support are equivalent to or greater than the specific interactions between these ligands and the target protein. The immobilization procedure used for the preparation of a receptorial OTC is often based on non-covalent interaction of avidin with biotin.

The reader can refer to an excellent review addressing the main issues in the development of cellular membrane affinity columns [30].

An alternative stationary phase for CMC prepared by immobilizing cell membranes containing special receptors on a silica carrier has proven to be an interesting method for studying drug–receptor interactions and screening active compounds [31–35].

In this method cells from tissues or cultured cells are dissociated by a hypoosmotic solution, centrifuged to remove nuclei and then centrifuged again to yield the cell membrane. Adsorption of the cell membrane on the activated silica is performed under vacuum and ultrasonication so that the cell membrane is distributed uniformly on the silica surface. The phospholipids of the living cell membrane are able to fuse spontaneously with each other (self-fusion) on the silica surface in the aqueous solution until a resealed cell membrane layer is obtained. The supernatant in the reaction mixture is then removed by centrifugation and the cellular membrane stationary phase is then washed with Tris–HCl buffer until no residual free cell membrane is detected on its surface. The purity of the cell membrane is verified using a scanning electron microscope. An ideal image and actual micrograph of the cellular membrane stationary phase are shown in Fig. 5.

In the last years the potentiality of monolithic supports has been shown and to date, several monoliths based on organic and inorganic polymers have been prepared. Thus, is not surprising that this material has been applied in affinity chromatography [26]. Bioimmobilization strategy for monolithic columns, that has proven useful in solid-phase screening technologies, is the entrapment of the receptor into an inorganic silicate matrix via the sol–gel process [36]. The sol–gel process is a low temperature inorganic polymerization reaction that occurs in aqueous solvents under mild pH conditions. Sol–gel entrapment methods basically involve forming a protein-doped sol and then infusing it into a capillary column prior to gelation to produce a monolithic bed that contains entrapped proteins.

A bioaffinity column can be prepared with different capacities and dimensions. Typical column dimensions are reported in Table 1. Minimizing protein and analyte consumption is an important issue since the amount of obtainable receptor can be very small and the availability of novel compounds limited, thus micro columns and open tubular capillary columns are more attractive [30]. Depending on column dimension or on the use of the open tubular approach, the ligand(s) infusion can be carried out using conventional HPLC

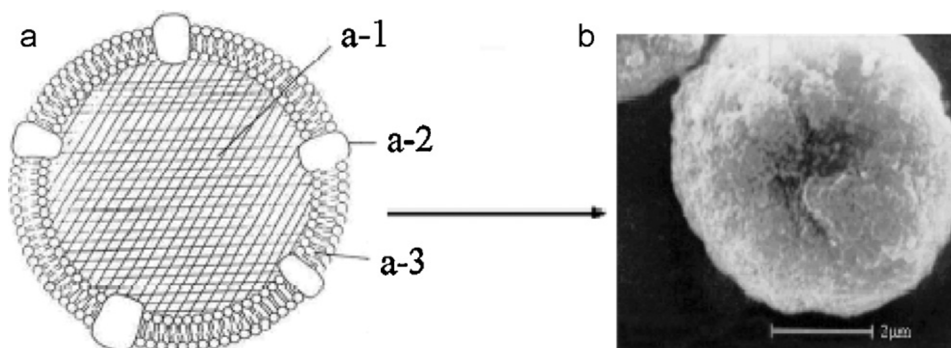

Fig. 5. Cell membrane chromatography. (a) Ideal image of the cellular membrane stationary phase. a-1, silica carrier; a-2, membrane receptor; a-3, phospholipid layer. (b) Actual micrograph of the cellular membrane stationary phase.
Reprinted with permission of Ref. [31].

Table 1
Application of FAC to receptors of pharmaceutical interest.

Ion channels				
Receptor type and receptor source	Support and column dimension	Type of study	Screening application	Ref.
Membrane receptors				
α3/β4 nAChR from KXα3β4R2 cell line	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.7 cm)	Column characterization: K_d determination of known ligands using frontal displacement chromatography with [^{3}H] epibatidine as marker ligand. Chromatographic system: HPLC–radio-detector	NO	[39]
α3-Subunits, β4-subunits and subunit combinations α3 + β4 from transfected human embryonic kidney cell lines and α4β4 nAChRs from rat brain membrane	IAM particles, 12 μm-300 Å, and liposome Superdex 200 gel beads	Column characterization: determination of [^{3}H] epibatidine and nicotine binding affinities by frontal displacement chromatography of individual subunits and combinations. Comparison of the two immobilization techniques. Chromatographic system: HPLC–radio-detector	NO	[40]
α3β2, α3β4, α4β2 and α4β4 nAChR	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.5 cm)	Columns characterization: K_d determination of known ligands using frontal displacement chromatography with [^{3}H] epibatidine as marker ligands. Chromatographic system: HPLC–radio-detector	NO	[38]
α3β4 and α4β2 nAChR	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.5 cm)	Single displacement chromatography studies to qualitatively rank compounds using [^{3}H] epibatidine as marker ligands. Chromatographic system: HPLC–radio-detector	Screening of 9 conformationally constrained nicotines and anabasines	[41]
α7 and α4β2 nAChRs from SH-EP1, SH-EP1-pCEP4-hα4β2 and SH-EP1-pCEP4-hα7 cell lines	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.8 cm)	Optimization of the immobilization protocol. Chromatographic system: HPLC–radio-detector	NO	[42]
nAChRs from *Torpedo californica*	Capillary-scale monolithic membrane receptor column prepared with the sol–gel method (250 μm i.d. × 100 cm fused-silica capillary)	Optimization of column preparation through multivariate factorial analysis. Column characterization: epibatidine affinity determination by frontal chromatography. Chromatographic system: FAC/MS/MS	NO	[36]
nAChRs, $GABA_A$, NMDA receptors from rat brain	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.8 cm)	Column characterization: K_d determination of known ligands for the three receptors. Chromatographic system: HPLC–radio-detector	NO	[43]
L-type calcium channel from rat vascular smooth muscle cell	Silica particles, 5 μm (0.2 i.d. × 1 cm)	Column characterization: K_d determination of known calcium antagonists. Chromatographic system: HPLC–DAD-detector	NO	[45]
G-protein-coupled receptors				
α_{1D}-Adrenergic receptor from rat aorta cell membrane	Silica particles, 7μm 100 Å (0.12 i.d. × 1 cm)	Column characterization: K_d determination of known ligands using frontal chromatography. Chromatographic system: HPLC–UV–vis	NO	[35]
β_2-Adrenergic receptor, HEK-293 cell line expressing the β_2-receptor	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.8 cm)	Column characterization: K_d determination of known radio-labeled ligands using frontal displacement chromatography. Chromatographic system: HPLC–radio-detector	NO	[47]
$P2Y_1$ receptor from 1321N1 cells stably transfected with $P2Y_1$ receptor	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.5 cm)	Column characterization: K_d determination of known ligands using frontal displacement chromatography. Chromatographic system: HPLC–radio-detector	NO	[48]
$P2Y_1$ and histamine subtype 1 receptors from 1321N1 cells stably transfected with $P2Y_1$	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.5 cm)	Column characterization: K_d determination of known ligands using frontal displacement chromatography. Chromatographic system: HPLC–radio-detector	NO	[49]

Table 1 (*Continued*)

Receptor type and receptor source	Support and column dimension	Type of study	Screening application	Ref.
Ion channels				
GPR17 receptor from 1321N1 cells transiently transfected with GPR17 receptor	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.5 cm)	Column characterization: K_d determination of known ligands using frontal chromatography and ranking affinity experiments using known ligands. Chromatographic system: HPLC–MS-MS	NO	[50]
GPR17 e mutated GPR17 receptors from 1321N1 cells transiently transfected with GPR17 receptor	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.5 cm)	Combination of a FAC–MS screening experiment with *in silico* studies to gain insights into the structure requirement of GPR17 ligands. FAC–MS ranking experiments and comparison with functional assay data. Chromatographic system: HPLC–MS-MS	Screening of new potential ligands (nucleotide) derivatives)	[51]
μ and κ opioid receptors from Chinese hamster ovary cells stably transfected with human μ and κ opioid receptors	IAM particles, 12 μm-300 Å (0.5 i.d. × 1.8 cm)	Column characterization: K_d determination of known ligands using frontal displacement chromatography. Chromatographic system: HPLC–radio-detector	NO	[52]
Enzyme-linked receptors				
Recombinant His-tagged EphB2 (kinase domain)	Controlled-pore glass. Immobilization with biotin-streptavidin system. Capillary column (250 μm i.d. × 2.5 cm)	FAC–MS experiments using the indicator screening method and comparison with ELISA data. Chromatographic system: HPLC–MS-MS	Screening of 10 known quitazoline kinase inhibitors	[21]
Recombinant His-tagged EphB2 (kinase domain)	Controlled-pore glass. Immobilization with biotin-streptavidin system. Capillary column (250 μm i.d. × 2.5 cm)	Combined application of high-throughput virtual screening and FAC–MS screening using the indicator method. Chromatographic system: HPLC–MS-MS	Screening of 468 virtually selected compounds.	[53]
Human recombinant PKCα and recombinant His-tagged EphB2	Controlled-pore glass. Immobilization with biotin-streptavidin system. Capillary column (250 μm i.d. × 2.5 cm)	Development of a FAC–MS method to simultaneously monitor binding at the ATP and substrate binding sites and measure ligand to both active and inactive kinases. Chromatographic system: HPLC–MS-MS	NO	[54]
	Immobilization	**Study**	**Screening application**	**Ref.**
Nuclear receptors				
Recombinant His-tagged human estrogen receptor ligand binding domain	Covalent immobilization on silica particles (0.4 i.d. × 2 cm)	Column characterization: K_d determination [^{3}H]-Estradiol using frontal displacement chromatography. Chromatographic system: HPLC–radio-detector and HPLC–MS	Screening of six known ligands by zonal elution chromatography and MS detection	[17]
Ligand binding domains of ERRα and ERRγ receptors	Covalent immobilization on aminopropyl silica (12 μm, 300 Å pores) and on the inner surface of silica capillaries. Column: 0.5 i.d. × 5 cm Capillaries: 100 μm i.d. × 25 cm	Columns and capillaries characterization: K_d determination of diethylstilbestrol using frontal chromatography. Chromatographic system: HPLC–MS	NO	[57]
Ligand binding domains of human ERRβ receptor	Covalent immobilization on controlled pore glass beads	Development of an automated ligand screening FAC–MS system with 2 columns. FAC–MS-MS	HTS of a 100 ligand mixture	[22]

systems for high flow rates or simple syringe pumps for low flow rates.

4. Applications

Membrane or transmembrane receptors play many important roles in cellular processes in both health and disease state and they represent the target for over 75% of pharmaceuticals in use today [37]. Cellular roles include communication between cells, communications between organelles and cytosol, and ion transport.

Membrane receptors are diverse due to their different structure and functions in cell and this serves as a basis for their classification. Three different classes can be categorized, which are ion channel receptors, G-protein-coupled receptors, and enzyme-linked receptors.

A summary of the most significant applications of frontal affinity chromatography based on immobilized membrane and cytosolic receptors is given in Table 1.

4.1. Frontal affinity chromatography systems based on membrane receptors

4.1.1. Ionic channels

4.1.1.1. Neuronal nicotinic acetylcholine receptors. Neuronal nicotinic acetylcholine receptors (nAChRs) are a family of ligand

Table 2
Comparison of K_d values obtained by frontal displacement chromatography and a conventional binding assay. Taken with permission from Ref. [39].

Ligand	K_d[a] (nM)	K_d[b] (nM)
(±)-Epibatidine	0.27 ± 0.05	0.38 ± 0.07
A85380	17.2 ± 0.5	73.6 ± 6.3
(−)-Nicotine	88 ± 33	475 ± 52
Carbachol	1280 ± 30	3839 ± 276
Atropine	14,570 ± 2600	

[a] Frontal chromatography with α3/β4-IAM stationary phase (0.5 × 1.3 cm).
[b] Binding assay using cell membrane homogenates.

gated ion channels found in different locations of the central and peripheral nervous systems. nAChRs play an important role in the regulation of synaptic activity, thus they are key targets in drug discovery for a number of diseases including Alzheimer's and Parkinson's diseases.

nAChRs consist of five trans-membrane subunits oriented around a central pore. At present, 12 different neuronal subunits have been identified, 9α subunits (α2–α10) and 3β subunits (β2–β4): these subunits form channels of a wide variety of homomeric and heteromeric nAChRs.

It has been reported that nAChRs contain multiple-binding domains which can accommodate different classes of endogenous and exogenous compounds.

A key goal in the development of therapeutic agents aimed at the nAChRs is to produce sub-type specific agonists in order to overcome the non-selective pharmacological profile of nicotine. The activity of new compounds acting as nAChRs agonist can be determined using binding and functional assays. However, a more elegant and simple approach, to the study of the binding of agonists and antagonists to nAChRs, is bioaffinity chromatography.

In particular for nAChRs, cellular membrane fragments obtained from cell lines expressing the target nAChR have been immobilized on the surface of IAM liquid chromatography stationary phase and the resulting nAChRs-IAM stationary phase used to create the desired column. Most of the pioneeristic work on nAChRs columns has been carried out by Wainer and coworkers. The columns were exploited in both zonal and frontal affinity mode or in non linear chromatographic studies to characterize ligand–nAChRs interactions. In this review, we will limit our discussion to frontal affinity chromatography experiments.

Frontal analysis has been used to study α3β2 [38], α3β4 [39], α4β2 [40,41], α4β4 [38] and α7 [42] nAChRs. In these studies, FAC has been used mainly to characterize the immobilized nAChRs in order to demonstrate that the immobilization procedure does not affect the conformational flexibility of the receptor before starting any on-line screening of pools of drug candidates. In particular, relative binding affinity (K_d determination) of four ligands (epibatidine EB, nicotine, carbachol and atropine) analyzed on α3 receptor column [39] where calculated by frontal displacement chromatography with radio-detection using radiolabeled EB as the marker ligand and it has been found that the rank order of the ligands was in agreement with the data obtained from traditional binding assays (Table 2). An example of frontal displacement chromatography is reported in Fig. 6. The retention volume of 60 pM [^{3}H]EB decreased from 23 to 18 ml when a 60 nM concentration of the nAChR–ligand (−) nicotine was added to the mobile phase (C) and fell to 0.9 ml when the (−) nicotine concentration was increased to 1000 nM (D) [39]. In this work, it also was studied the influence of the receptors coverage on the surface of the IAM stationary phase and it has been established that a lower concentration of nAChRs can lead to an alteration of the quantitative determination of K_d but the relative order of affinities is consistent for all the prepared columns.

In a subsequent paper [40], the same research group has immobilized recombinant nAChRs α3-subunits, β4-subunits and subunit

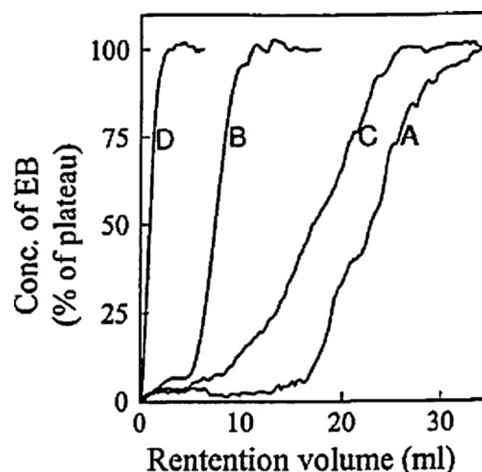

Fig. 6. Elution profiles of [^{3}H]epibatidine on a3/b4 nAChR-IAM stationary phase (0.5 cm × 1.7 cm). 60 pM [^{3}H]epibatidine (A), 450 pM [^{3}H]epibatidine (B), and 60 pM [^{3}H]epibatidine in the presence of 60 nM (−) nicotine (C) and 1000 nM (−) nicotine (D) were included, respectively, in eluent A: 50 mM Tris–HCl, pH 7.4. Flow rate, 0.4 ml/min.
Reprinted with permission of Ref. [39].

combinations (α3β4 and α4β2) for evaluating the drug binding affinities of individual subunits and their combinations. The results obtained clearly indicate that the independent α3 and β4-subunits do not present specific binding of EB while the intact subtype complexes bind the natural ligand. The effect of the mobile phase pH and ionic strength on the binding affinity of α3β4 IAM stationary phase was also investigated and the data obtained where used to derive information about the ligand–receptor interactions.

For α3β2, α3β4, α4β2 and α4β4 stationary phases, epibatidine, nicotine and cytosine were used to characterized the prepared columns [38]. The K_d values of the three test ligands were determined by frontal displacement affinity chromatography and compared with the K_d values reported in literature using filtration assay and the data produced by the chromatographic approach, also in study were similar. The authors underline that the K_d values for epibatine represent a qualitative estimation of the affinities. Indeed, quantitative measure of K_d can be obtained when the concentration of the marker ligand is significantly lower than the biding affinity of the marker; thus the ability of frontal affinity chromatography to determine K_d values is limited by the sensitivity of the detector.

In an other study [41] α3β4 and α4β2 nAChRs columns were used to quantitatively rank compounds according to their EC_{50} values. A small library of nine novel compounds was selected and each single compound was infused on the two columns using EB as a marker ligand. The results obtained demonstrated that affinity displacement chromatography based on immobilized nAChRs stationary phase can be used for on-line screening of compounds (Table 3).

An important concern in receptor-based column preparation is the influence of the source of cellular membrane on affinity data; in one of the latest work, Wainer end co-workers have recently studied the effect of cell type (SH-EP1-pCEP4-hα7 and α7 HEK-293) on the preparation of α7 nAChR column [42]. Moreover, a different receptor type (α4β2) was considered. It appeared that both cell type and receptor type affect the protocol required to produce stable columns and the membrane localization of the receptor and the expression system are important aspects to be considered during the creation of a receptor column. An important experimental aspect to consider is the quantity of immobilized functional receptor, which is determined by the level of expression of the target and the amount of the solubilized protein. In these works the amount of cells required to produce viable nAChR columns ranged from

Table 3
Comparison between the agonist activity of nicotine and anabasine derivatives, expressed as the EC50 value, determined using a cell line expressing the α3β4 neuronal nicotinic acetylcholine receptor (α3β4 nAChR) and their effect on the retention of epibatidine expressed as Δml, calculated as breakthrough volume of EB alone minus the breakthrough volume of EB after the addition of the test ligand, on the immobilized α3β4 nAChR and α4β2 nAChR liquid chromatography stationary phases. Reprinted with permission from Ref. [41].

Sample	EC_{50} (μM)	Δml α3β4	Δml α4β2
[^{3}H] EB	$(28.3 \pm 1.6) \times 10^{-3}$	–	–
TKS-9	26.4 ± 1.8	1.60	0.34
TKS-6	20.8 ± 2.9	0.25	0.28
TKS-8	>300	0.28	0.26
TKS-7	13.8 ± 3.0	0.24	0.20
Nicotine	19.8 ± 1.6	0.16	0.26
TKS-2	18.2 ± 3.4	0	−0.05
Carbachol	>1000	−0.04	−0.06
TKS-4	>1000	−0.04	−0.07
TKS-1	>1000	−0.04	0.17
TKS-3	>1000	−0.12	0.13
TKS-5	>1000	−0.32	0.18

1×10^6 (α3β4 subtype) to 30×10^6 (α7 subtype). The length of the columns ranged from 13 to 18 mm.

The extensive and very complete work on nAChRs illustrates that IAM is an ideal support for this type of receptors, indeed it has been demonstrated that chromatographic data obtained with nAChRs-IAM columns using displacement frontal affinity approach correlate well with the compound's binding affinity (K_d) obtained with a traditional assay. Furthermore, the FAC experiments carried out with nAChRs-IAM columns produced reproducible results and the columns were stable for at least 6 months.

However, some issues still exist such as low binding capacities and low resolution breakthrough profiles for such columns. The low binding capacities are innate to membrane receptors, because the receptors are expressed at low levels and can only be purified in small amounts. Low resolution breakthrough profiles are typically the result of structural inhomogeneity of the column, since multiple flow paths will inevitably result in broadening of the analyte fronts. Another limitation is related to the use of a radio-labeled marker ligand (i.e. [^{3}H-EB]) with on-line scintillation detection. In addition, this approach was only applied to single displacement experiments to qualitatively rank pool of compounds but no ranking experiments in mixture and in a single run were carried out.

Some of these limitations were overcome in recent works where new column materials (monolithic stationary phases) and advanced detection methods (MS) have been applied in FAC studies.

nAChR from *Torpedo californica* has been chosen as the model transmembrane receptor for the development of monolithic membrane-receptor columns. The sol–gel method has been used for receptor immobilization and optimized by multivariate factorial analysis for the formation of a capillary-scale nAChR monolithic silica columns [36]. Using FAC/MS/MS ligand–receptor interactions were investigated and it was determined that the immobilized receptor-doped columns retained 100% of the receptor in active form, and that the binding constant for the nanomolar agonist epibatidine was very close to that obtained from solution-based experiments. These results indicate that monolithic receptor columns, which represent a second generation of column material, in combination with FAC–MS methodology are useful for screening compound also in mixtures.

Multiple receptor stationary phase based on ligand-gated ion channels such as nicotinic, $GABA_A$ and NMDA receptors have been reported [43]. Solubilized rat forebrain tissue was immobilized on IAM stationary phase to prepare a column containing more than one functioning receptor. Binding affinities were obtained and the results of this study demonstrate that the co-immobilized receptors act independently using displacement chromatography.

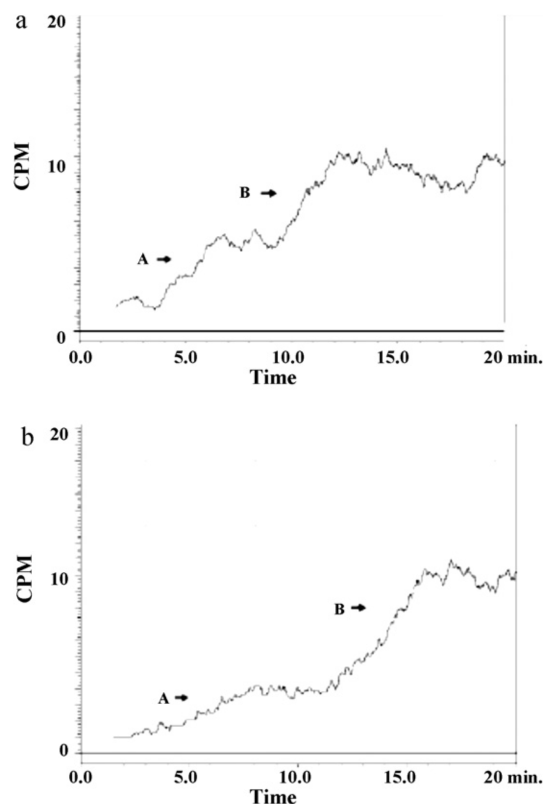

Fig. 7. Biphasic frontal curves obtained for 7.5 pM [^{3}H]-EB on the CMAC(1321N1) column (panel a) and CMAC(A172) column (panel b) where A represents the binding to the homomeric nAChR receptor and B represents the binding to the heteromeric nAChR.
Reprinted from Ref. [44].

Cellular membranes obtained from native cell lines (1321N1 and A172 astrocytoma), instead of stably transfected cell lines were used to create a cellular membrane affinity column based on IAM supports [44]. The columns were characterized using frontal affinity chromatography with [^{3}H]-epibatidine as the marker ligand and epibatidine, nicotine, and methyllycaconitine as the displacers. The biphasic chromatographic traces obtained using [^{3}H]-epibatidine on the two columns (Fig. 7) suggest the presence of two distinct binding interactions due to the presence on the columns of homomeric alpha7 nicotinic acetylcholine receptors (alpha7 nAChR) and heteromeric nicotinic acetylcholine receptors (alpha(x)beta(y) nAChRs). This result was confirmed by the addition of subtype-specific inhibitors, alpha-bungarotoxin (alpha7 nAChR) and kappa-bungarotoxin (alpha(x)beta(y) nAChR) to the mobile phase. The presence of two additional ligand-gated ion channels (LGICs), gamma-aminobutyric acid (GABA(A)) and N-methyl-D-aspartic acid (NMDA), was established using frontal affinity chromatography with flunitrazepam and diazepam (GABA(A) receptor) and MK-801 and NMDA (NMDA receptor). The presence of the four LGICs was confirmed using confocal microscopy and flow cytometry. The results indicate that the CMAC(1321N1) and CMAC(A172) columns contain four independently functioning LGICs, that the columns can be used to characterize binding affinities of small molecules to each of the receptors, and that the CMAC approach can be used to probe the expression of endogenous membrane receptors.

The extensive work carried out on nicotinic receptors demonstrates that the resulting stationary phases can be successfully used in different applications. The K_d-values determined chromatographically were comparable to those found using standard

membrane affinity binding techniques. All these studies also indicate that the nAChR columns can be used to identify both competitive and non-competitive ligands, to detect differences in the binding affinity of a compound between the receptor subtypes and for the on-line screening of ligands to the nAChRs.

4.1.1.2. L*-calcium channel.* Recently a stationary phase based on L-type calcium channels (L-CCs) has been described and used in frontal chromatographic experiments [45]. L-CCs are widely expressed in cardiovascular tissues and represent the critical drug-target for the treatment of several cardiovascular diseases. In this work, vascular smooth muscle cells, rich in L-CCs, have been used to prepare a column based on activated silica (CMC approach) that has been employed in frontal affinity studies for the determination of K_d of different calcium-antagonist (verapamil and some dihydropyridines, namely amlodipine, nitrendipine, nimodipine) known to bind and block L-CCs. The affinity rank order of four ligands determined with CMC column was consistent with the K_d values reported in literature. In addition displacement experiments were performed in order to validate the ability of the system to discriminate between the different binding sites.

4.1.2. G-protein-coupled receptors (GPCRs)

G-protein-coupled receptors (GPCRs) convey the majority (80%) of signal transduction across cell membranes. They are activated by diverse ligands. All GPCRs are located within the plasma membrane and have a common architecture consisting of seven-transmembrane (TM) domains, connected by extracellular and intracellular loops. One of the very special features of GPCRs is that they are highly druggable, to the extent that more than one third of all current therapeutics are directed at them [46].

4.1.2.1. Adrenergic receptors stationary phases. The receptors for endogenous catecholamine, designed as adrenoreceptors (AR), are classified in three major types (α_1, α_2 and β) each of which is further divided into at least three subtypes α_1(α_{1A}, α_{1B}, α_{1D}) α_2 (α_{2A}, α_{2B}, α_{2C}), β (β_1, β_2, β_3). These receptors are of particular therapeutic interest because they mediate a variety of physiological effects in many tissues, thus is not surprising that a certain number of works have been published on the development of bioaffinity methods for the rapid investigation of the interactions between ligands and these receptors.

Yu et al. have firstly reported the preparation of cell membrane stationary phase consisting of silica particle coated with cell membranes obtained from transfected cell lines (HEK293) overexpressing subtype receptors α_{1A} and α_{1B}. In a first paper the use of cell lines stably overexpressing α_{1A} and α_{1B}-adrenoreceptors subtypes (human embryonic kidney 293 cell lines or HEK293) for the preparation of a cell membrane stationary phase using the CMC technique was described and the retention times of nine α_1 adrenoreceptor ligands were determined. The rank order of capacity factors was consistent with the affinity rank order obtained from radioligand binding assays [33]. In a second work the same authors described the preparation of cell-membranes stationary phases prepared from homogenized tissue, from cells in primary culture, and from cell lines stably overexpressing subtype receptors [34]. Also in this study the retention times of the nine α_1 adrenoreceptor ligands were determined and compared on the three columns. In general, all nine test ligands had greater affinity towards the HEK293 α_{1A} stationary phase.

The utility of these columns to determine the relative affinity of known ligands was only demonstrated by zonal elution experiments [33,34]. Instead, in a more recent paper, frontal affinity chromatography has been used to study quantitatively ligand–receptor interactions in the determination of K_d values of six test ligands of α_{1D} receptor [35]. The stationary phase was prepared by immobilizing rat aorta cell membranes on silica and the columns, with different inner diameters, were tested. The frontal chromatographic curves obtained for these columns showed that the FAC analysis time was shortened by reducing the inner diameter, and the small-inner-diameter column can increase sensitivity. The K_d values determined by the CMC system were not consistent with the binding affinity constants reported previously, especially the high binding affinity ligands (i.e. Prazosin). However, the affinity rank order of the six ligands was consistent with that reported previously, and the calculated K_d values were positively correlated with the binding affinity constants reported in the literature.

β_2-ARs obtained from HEK293 cells have been immobilized on IAM by Wainer research group to prepare columns to be used for the on-line determination of ligand–receptor binding interactions [47]. The procedures for membrane preparation and synthesis of a stable stationary phase were established, then the characterization of the stationary phase was carried out using the dissociation constant and the number of active binding sites. This was accomplished using frontal chromatography with [^{3}H]-CGP and (*R,S*)-[^{3}H]-propranolol as the marker ligands. As observed in other works, the chromatographic approach provides at least a qualitative assessment of ligand–β_2-AR binding interactions and it is an alternative method to the investigation of ligand–β_2-AR interactions.

4.1.2.2. Purinergic receptors stationary phases. A P2Y1-immobilized artificial membrane (P2Y1-IAM) liquid chromatography stationary phase prepared from 1321N1 cell lines was developed and applied to the determination of the affinity constants of some known ligands [48].

The chromatographic experiments were carried out using a chromatographic system that includes a radioflow detector and frontal displacement affinity chromatography technique.

In this study, the characterized P2Y1 agonist ^{3}H-labeled 2-MeSADP was used as the marker ligand. The results demonstrate that the 1321N1 cell membrane fragments were successfully immobilized on the IAM stationary phase and that the immobilized P2Y1 receptor maintained its ability to bind 2-MeSADP. The K_d values of MRS-2179, a competitive inhibitor of P2Y1, and ATP, a P2Y1 agonist, was also determined with a good correspondence with the same values reported in literature. Applications of the stationary phase in screening studies have not been reported so far.

The 1321N1 cell line used for the preparation of P2Y1 column contains functional histamine 1 receptors (H1Rs). Based on this evidence the same column was used to investigate whether the histamine subtype 1 receptor was also present in the P2Y1 column, if the same column could be used to identify H1R ligands and if the expression on the P2Y1 receptor in the 1321N1 cell line altered the characteristics of the H1R [49]. The presence of functional H1Rs on the column was established by displacement frontal chromatography using the H1R antagonist [^{3}H]-mepyramine. Moreover the affinity constants of histamine were calculated with a good agreement with the same data reported in literature. To determine if the observed binding interactions with P2Y1 and H1R represent independent functioning of the two GPCRs, the ability of the P2Y1 marker 2-MeSADP to displace the H1R marker mepyramine and the ability of mepyramine to displace 2-MeSADP was investigated. The results from this study demonstrate for the first time that a multiple-GPCR column was created and that the two GPCRs, P2Y1 and H1 receptors maintain their ability to independently bind receptor–selective ligands.

The preparation of a stationary phase based on GPR17 receptor, belonging to the purinergic cluster, was also described [50]. This receptor is dually activated by both uracil nucleotides and cysteinyl-leukotrienes. GPR17 was found to be highly expressed in organs typically undergoing ischemic damage (i.e. brain, heart

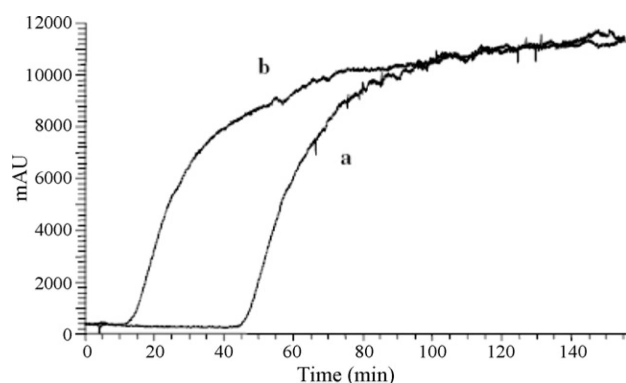

Fig. 8. Frontal chromatographic profiles of cangrelor (1 μM) under optimized conditions on GPR17(+)-IAM column (a) and GPR17(−)-IAM column (b).
Reprinted with permission from Ref. [50].

and kidney) thus it can represent an important target for new therapeutic approaches in acute and chronic neurodegenerative diseases. GPR17 was entrapped on the surface of an immobilized artificial membrane. An optimization study was carried out increasing the membrane/IAM ratio in order to increase receptor density and reduce the unwanted non-specific binding. Fig. 8 reports the frontal chromatographic profiles of the ligand cangrelor in the optimized conditions on the GPR17(+)-IAM column (chromatogram a) and the GPR17(−)-IAM column (chromatogram b). The stationary phase was validated in the determination of the affinity constants of three known selected ligands and for its ability to rank the same ligands in mixture in a single experiment by FAC–MS. A second paper by the same author reports an application of the GPR17 stationary phase a drug discovery screening study [51]. In particular the application of FAC–MS approach, along with a molecular modelling study, to the screening of potential drug candidates towards GPR17 was described [51]. The stationary phase was used to screen a library of nucleotide derivatives by FAC–MS in order to select high affinity GPR17 ligands. The chromatographic results obtained have been confirmed with a reference functional assay ([^{35}S]GTPγS binding assay) (Table 4). The simplicity of the method allowed to explore the binding mode of the purinergic component of the receptor by extending this approach to a column where an artificial GPR17 receptor bearing a mutation in a key aminoacid for ligand recognition has been immobilized. The comparison of the breakthrough times determined on wild-type and mutated receptors led to the conclusion that the mutation is not silent. Table 4 reports the % variation of the breakthrough time determined on mutated GPR17-IAM with respect to the wild-type GPR17-IAM column. The ligands can be divided into two groups depending on the sign of the breakthrough time variation. It is speculated that analytes with a positive variation most likely interact with the receptor binding pocket through hydrophobic interactions, while for compounds with a negative variation electrostatic interactions are predominant. Comparison of the different chromatographic behaviour of the nucleotide derivatives in the library together with the *in silico* studies has been used to gain insight into the structure requirement of GPR17 ligands. A high affinity ligand was selected with this technique.

4.1.2.3. Opioid receptors. Opioid receptors are members of the superfamily of G-protein-coupled receptors and exist as the μ, δ and κ subtypes. Liquid chromatographic stationary phases containing either the human μ and κ opioid receptor subtypes have been developed. This work must be considered the first example of the use of immobilized GPCRs in a chromatographic system [52]. The receptors were obtained from Chinese hamster ovary cells stably transfected with human mu or kappa receptor subtypes. The receptors were isolated through partial solubilization with sodium cholate detergent, and the partially purified receptor complex was immobilized in the phospholipid analogue monolayer of an immobilized artificial membrane liquid chromatographic stationary phase. The resulting phase was packed into a glass column and used in the on-line chromatographic determination of drug/ligand binding affinities to the immobilized opioid receptors using the known mu antagonist naloxone and the kappa agonist U69593. The results indicate that the immobilized receptors retained their ability to specifically bind ligands.

4.1.3. Enzyme-linked receptors

4.1.3.1. Receptor protein kinases stationary phases. Receptor tyrosine kinases (RTK) are targets for treatment of a number of diseases and disorders. Overexpression and/or overactivation of RTKs has been implicated in a number of cancers and the identification of small molecule inhibitors for RTKs is currently a viable strategy for therapeutic intervention. The research group from Protana Inc. has proposed the use of FAC–MS to identify new inhibitors for erythropoietin-producing hepatocellular (Eph) RTK family member, EphB2 that is overexpressed in a number of cancers such as colorectal cancer and has been proposed as important in angiogenesis [21,53,54]. In these papers, the streptavidin/biotin complex was used for the immobilization of recombinant His-tagged EphB2 (kinase domain) [21,53,54]. Biotinylated anti-His tag monoclonal antibodies was incubated with streptavidin-coated controlled-pore glass (CPG) beads. Afterward EphB2 was added and the receptor-CPG beads were packed into FAC–MS capillary columns. The EphB2-column was tested towards 10 known quinazoline kinase inhibitors expected to bind in the ATP binding-site using

Table 4
FAC–MS data obtained on GPR17-IAM and mutated GPR17-IAM columns in comparison with [^{35}S]GTPγS binding results. Data taken from Ref. [51].

	GPR17-IAM[a]			Mutated-GPR17-IAM[a]			
Cpd	t^b (min)	EC_{50}	IC_{50}[c]	t^b (min)	EC_{50}	IC_{50}[c]	$(t_m/t_w \times 100) - 100^d$ (%)
UDP	2.69	1.14 ± 0.2 μM		2.15	3.0 ± 0.3 μM		−20.1
13	2.79	945 ± 48 nM		2.51	1.87 ± 0.20 μM		−10.0
12	3.55		582 ± 57 nM	3.54			−0.3
MRS 2179	3.62		508 ± 29 nM	3.47		227 ± 22 nM	−4.1
5	3.69		112 ± 7 nM	4.08			+10.6
7	4.5	11 ± 1 nM		5.27			+17.1
8	4.77	1.7 ± 0.1 nM		5.79			+21.4
6	5.67	1.4 ± 0.1 nM		7.08			+24.9
Cangrelor	10.6		0.7 ± 0.02 nM	13.16		0.15 ± 0.01 nM	+24.1
4	13.07	36 ± 3 pM		16.07	25.4 ± 1.0 pM		+23.0

[a] Columns: GPR17-IAM (19.5 million cells), mutated GPR17-IAM (19.5 million cells).
[b] t is the breakthrough time of the ligand with the immobilized GPR17.
[c] *versus* UDP-glucose 10 μM.
[d] The breakthrough time variation % was calculated on mutated GPR17-IAM (t_m) with respect to GPR17-IAM column (t_w).

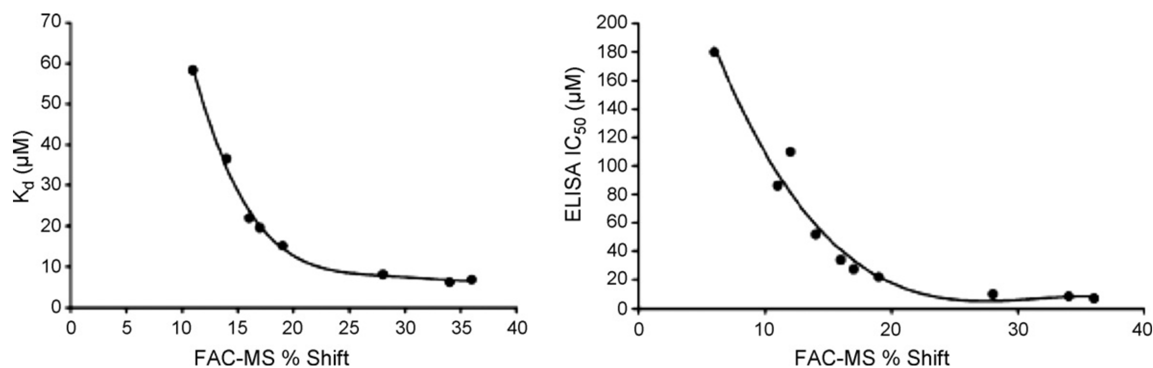

Fig. 9. Correlation of the kinase inhibitors FAC–MS % shift with K_d (left panel) and ELISA IC50 (right panel).
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the indicator screening method (Eq. (3)) monitoring the breakthrough time for a selected indicator (WHI-P180). Each inhibitor was also analyzed for K_d determination. The results were compared to functional IC_{50} activity values and a good correlation was obtained between FAC–MS indicator % shift and both IC_{50} value and K_d constant allowing the validation of the FAC–MS system for further screening studies (Fig. 9) [21].

An extension of this work is the combined application of the high-throughput virtual screening and FAC–MS screening approaches in the discovery of hits against EphB2 [53]. The high-throughput virtual screening based on docking of a diverse drug-like compound library against the crystal structure of the EphB2 kinase domain was followed by physically ranking of virtually identified hits by FAC–MS using the previously validated system [21]. Of the 500 virtually selected compounds only 468 were available for purchase and were randomly combined into 52 mixtures of nine compounds. To profile individual mixtures the indicator method was used. Mixtures that generated the largest % shift were selected for deconvolution. The compounds in these four mixtures were run individually in the presence of the indicator. 12 compounds were selected, and IC_{50} values for these compounds were also determined using an ELISA assay. Once again a good correlation between FAC–MS % shift and IC_{50} values was obtained indicating that % shifts are a good surrogate for IC_{50} values.

A simultaneous ATP and substrate site FAC–MS assay kinase assay (dual binding site assay) was developed by the same authors [54].

Human recombinant protein kinase Cα (PKCα) was selected as the target protein for the commercial availability of the substrate site ligands that can act as indicators. The PKC inhibitor peptide 19–36 and chelerythrine chloride were selected for the PKCα kinase substrate site. The ligands for the ATP binding site were identified to be PD153035 and WHI-P180. Chelerythrine chloride and PD153035 were established as dual indicators for their two respective binding sites. When WHI-P180 was added to the infuse containing the void marker and the two indicators, only the ATP binding site indicator PD153035 shifted to the left indicating that WHI-180 is an ATP-competitive ligand for PKCα. WHI-180 had no effect at the substrate binding site of PKCα since the breakthrough time of chelerythrine chloride remained unchanged. An analogous situation occurred for the substrates binding site when PKC inhibitor peptide 19–36 was added and only chelerythrine chloride shifted to the left. The same results were further confirmed mixing all ligands together.

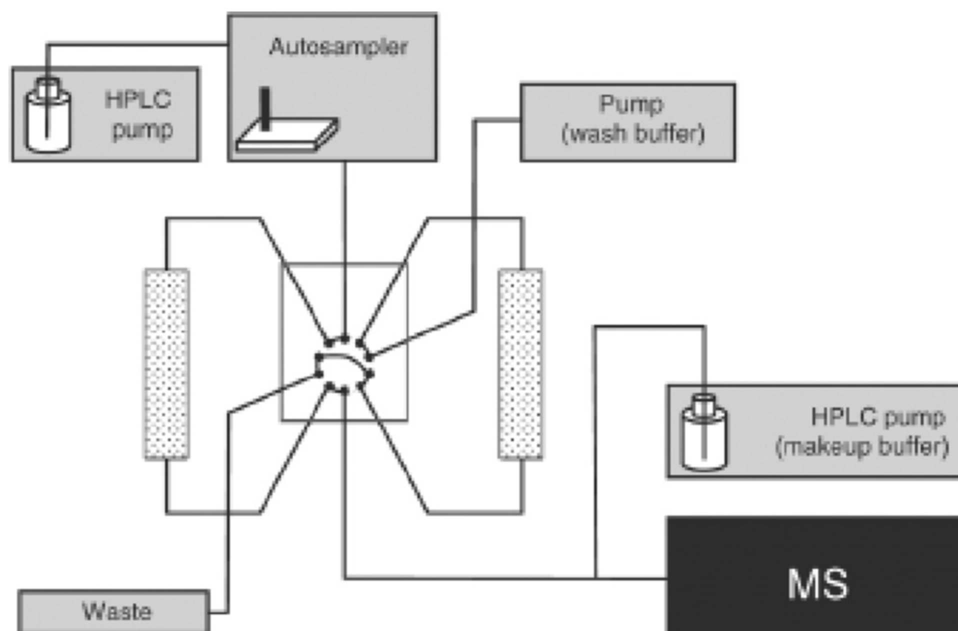

Fig. 10. Example of automated frontal affinity chromatography–mass spectrometry system. A high-performance liquid chromatography micropump containing the running buffer is connected in series to the autosampler and then to the column switcher equipped with 1 or 2 columns and then connected to the mass spectrometer.
Reprinted with permission from Ref. [22].

To demonstrate the capability of FAC–MS to identify binders of an active kinase, both the active and inactive forms of EphB2 were evaluated under similar conditions. To validate the ability to screen the inactive EphB2 mutant kinase, it was demonstrated that the ATP binding site was similar in both proteins using a series of four known commercially available kinase inhibitors run in mixture on the two columns. By analyzing both active and inactive forms, the FAC–MS results for the active protein act as a reference and provide confidence that any observed binding for the inactive is real. Although the structure of this inactive EphB2 has been solved, until now it would have been impossible to use this variant of EphB2 directly in a traditional functional assay.

This study represents a novel application of FAC–MS that can be applied in areas where traditional screening assays are not applicable such as determining binding site information and screening with inactive conformations of proteins.

4.2. Frontal affinity chromatography systems based on nuclear receptors

The nuclear receptors (NRs) are a family of transcription factors that bind and respond to certain steroids and other signaling molecules. For the purposes of drug discovery, NRs have favourable attributes, with most containing protected internal cavities predisposed for occupation with hydrophobic molecules of volumes typical of drugs. Moreover, these transcription factors control central pathways impacting a wide range of pathophysiologies ranging from cancers to metabolic diseases [55,56].

4.2.1. Human estrogen receptor (hER)

Estrogen receptors are promising targets for therapeutic purposes such as treating breast cancer, osteoporosis, cardiovascular disease, obesity, as well as disorders of the central nervous system. For the purpose of drug discovery, frontal and zonal affinity chromatography can be used as HTS approach for the evaluation of new natural and synthetic ER ligands. A first study reported by Wainer and co-workers describes the immobilization of hER ligand binding domain onto a column via coordinating complex covalently linked to a silica backbone with a hydrophilic spacer [17]. The stationary phase was connected to an on-line scintillation detector and frontal chromatographic experiments were conducted using [^{3}H]-estradiol and the obtained K_d value was in agreement with previously reported data. The same column was also connected to a mass spectrometer and six known estrogen receptor ligands where analyzed by zonal elution approach. The same research group has recently published a paper on the characterization of chromatographic columns containing the ligand binding domain of estrogen related receptor α and estrogen related receptor [58]. In this paper, the ligand binding domains of the ERRα and ERRγ were covalently immobilized onto the surface of an aminopropyl silica liquid chromatography stationary phase to create the ERRα-silica and ERRγ-silica columns and onto the surface of open tubular (OT) capillaries activated with aminopropyltrimethoxysilane to create the ERRα-OT and ERRγ-OT columns. The receptors were successfully immobilized as demonstrated by the consistency of the chromatographically determined K_d values for diethylstilbestrol, obtained by frontal displacement chromatography, with the previously reported IC_{50}.

FAC–MS experiments were also carried out using microaffinity columns based on the ligand binding domain of hERβ [58]. hERβ was first biotinylated and then immobilized to streptavidin-coated controlled pore glass beads packed in a capillary column. Dissociation constants of low nanomolar range (i.e. β-estradiol) were reliably determined using the staircase procedure.

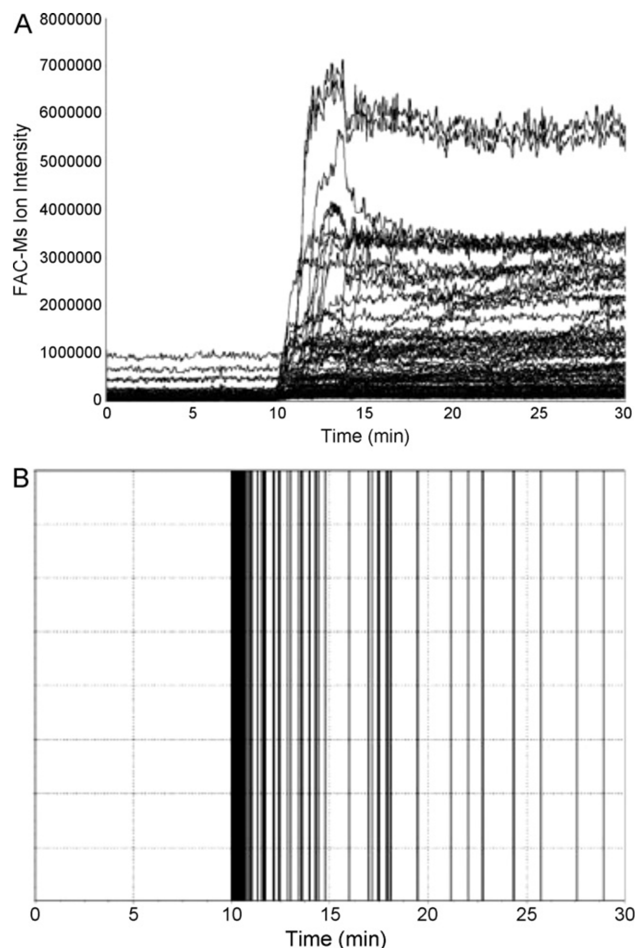

Fig. 11. (A) Frontal affinity chromatography–mass spectrometry (FAC–MS) Q1 scan of a 100-ligand mixture of ligands each at a concentration of 1 μM and infused through the hERβCBX column using the mass spectrometer (MS) in positive mode with the *m*/*z* range from 100 to 1200 scanned. (B) Bar diagram showing the inflection point of the breakthrough front curves for the FAC–MS chromatogram of (A). Reprinted with permission from Ref. [22].

To push FAC–MS more to the forefront as a moderate primary HTS system, automation has been addressed by using hERβ affinity columns prepared by covalent attachment of the ligand–receptor domain to the free carboxyl groups of controlled pore glass beads using N-ethyl-N′-[3-dimethylaminopropyl] carbodiimide and N-hydroxy succinimide [22]. The stationary phase was packed in capillary columns. An automated multiple ligand screening FAC–MS system using two columns was developed and the Q1 scan FAC–MS screening method, in which multiple ligands are analyzed simultaneously, was followed (Fig. 10).

To detail FAC–MS automation as well as the value of the Q1 scan method, 100 compounds were mixed together and the mixture was infused through the hERβ column. Because the molecular weights for each of the 100 ligands were known, using proprietary software for data management, the individual breakthrough fronts were identified and a bar diagram was created to better visualize the breakthrough times (Fig. 11). The total runtime was 30 min to screen 100 compounds, and because two columns were used the authors extrapolated that in a continuous 24 h operation with 200 ligands/mixture, the number of ligands that can be screened could approach 10,000 suggesting that FAC–MS should be considered as a moderate-throughput binding assay offering the real possibility to generate preliminary binding SAR information from diverse or closely related libraries.

5. Concluding remarks

The contribution of frontal affinity chromatography, based on immobilized receptors, for the screening of compounds alone or in mixture has been reported.

The survey indicates that different immobilization protocols and on different supports can be selected for cytosolic and membrane receptors and alternative entrapment technologies have been described for membrane-associated receptors.

FAC assay offers not only high precision and accuracy in the measurement of binding constants but also high-throughput screening using a multidimensional detector such as a mass spectrometer.

The reported applications demonstrate that FAC offers new entry points including dual binding site assay, receptor subtype characterization and multi-receptor binding experiments. Moreover, the FAC method can be easily extended to receptors bearing mutations in key amino acids for ligand recognition. The comparison of the breakthrough times determined on wild-type and mutated receptors can help for a better characterization of the ligand binding site.

Since today, frontal affinity chromatography remains of academic interest and the published studies are mainly focused on proof of concept. Receptor columns for FAC studies are still not fully characterized in terms of stability and robustness and are not commercially available. However, in our opinion, this approach can re-shape the ways that drug–protein interactions studies will be performed in drug discovery initiatives as it has been demonstrated that FAC fits for the purpose of the screening of large libraries. The automation and miniaturization of existing prototype systems will be the future trends.

References

[1] L.M. Mayr, D. Bojanic, Novel trends in high-throughput screening, Curr. Opin. Pharmacol. 9 (2009) 580–588.
[2] P. Gribbon, A. Sewing, High-throughput drug discovery: what can we expect from HTS? Drug Discov. Today 10 (2005) 17–22.
[3] J.-P. Renaud, M.-A. Delsuc, Biophysical techniques for ligand screening and drug design, Curr. Opin. Pharmacol. 9 (2009) 622–628.
[4] G.M. Keserü, G.M. Makara, Hit discovery and hit-to-lead approaches, Drug Discov. Today 11 (2006) 741–748.
[5] C.E. Whitehurst, D.A. Annis, Affinity selection-mass spectrometry and its emerging application to the high throughput screening of G protein-coupled receptors, Comb. Chem. High Throughput Screen. 11 (2008) 427–438.
[6] K.F. Geoghegan, M.A. Kelly, Biochemical applications of mass spectrometry in pharmaceutical drug discovery, Mass Spectrom. Rev. 24 (2005) 347–366.
[7] J.E. Schiel, K.S. Joseph, D.S. Hage, Biointeraction affinity chromatography: general principles and recent developments, Adv. Chromatogr. 48 (2010) 145–193.
[8] D.S. Hage, Affinity chromatography: a review of clinical applications, Clin. Chem. 45 (1999) 593–615.
[9] D.S. Hage, A. Jackson, M.R. Sobansky, J.E. Schiel, M.J. Yoo, K.S. Joseph, Characterization of drug–protein interactions in blood using high-performance affinity chromatography, J. Sep. Sci. 32 (2009) 835–853.
[10] J.E. Schiel, R. Mallik, S. Soman, K.S. Joseph, D.S. Hage, Applications of silica supports in affinity chromatography, J. Sep. Sci. 29 (2006) 719–737.
[11] K. Kasai, S. Ishii, Quantitative analysis of affinity chromatography of trypsin. A new technique for investigation of protein–ligand interaction, J. Biochem. 77 (1975) 261–264.
[12] D.C. Schriemer, D.R. Bundle, L. Li, O. Hindsgaul, Micro-scale frontal affinity chromatography with mass spectrometry detection: a new method for the screening of compound libraries, Angew. Chem. Int. Ed. 37 (1998) 3383–3387.
[13] E.S. Ng, D.C. Schriemer, Emerging challenges in ligand discovery: new opportunities for chromatographic assay, Expert Rev. Proteomics 2 (2005) 891–900.
[14] E.S. Ng, N.W. Chan, D.F. Lewis, O. Hindsgaul, D.C. Schriemer, Frontal affinity chromatography–mass spectrometry, Nat. Protoc. 2 (2007) 1907–1917.
[15] E. Calleri, C. Temporini, G. Caccialanza, G. Massolini, Target-based drug discovery: the emerging success of frontal affinity chromatography coupled to mass spectrometry, ChemMedChem 4 (2009) 905–916.
[16] N. Chan, D. Lewis, M. Kelly, E.S.M. Ng, D.C. Schriemer, in: K.T. Wanner, G. Höfner (Eds.), Mass Spectrometry in Medicinal Chemistry, 2007, pp. 217–246 (Chapter 6).
[17] R. Moaddel, L. Lu, M. Baynham, I.W. Wainer, Immobilized receptor- and transporter-based liquid chromatographic phases for on-line pharmacological and biochemical studies: a mini-review, J. Chromatogr. B 768 (2002) 41–53.
[18] D.J. Winzor, Determination of binding constants by affinity chromatography and enzymes, J. Chromatogr. A 1037 (2004) 351–367.
[19] C. Bertucci, M. Bartolini, R. Gotti, V. Andrisano, Drug affinity to immobilized target bio-polymers by high-performance liquid chromatography and capillary electrophoresis, J. Chromatogr. B 797 (2003) 111–129.
[20] R. Moaddel, I.W. Wainer, Development of immobilized membrane-based affinity columns for use in the online characterization of membrane bound proteins and for targeted affinity isolations, Anal. Chim. Acta 564 (2006) 97–105.
[21] J.J. Slon-Usakiewicz, W. Ng, J.E. Foster, J.-R. Dai, E. Deretey, L. Toledo-Sherman, P.R. Redden, A. Pasternak, N. Reid, Frontal affinity chromatography with MS detection of EphB2 tyrosine kinase receptor. 1. Comparison with conventional ELISA, J. Med. Chem. 47 (2004) 5094–5100.
[22] W. Ng, J.-R. Dai, J.J. Slon-Usakiewicz, P.R. Redden, A. Pasternak, N. Reid, Automated multiple ligand screening by frontal affinity chromatography-mass spectrometry (FAC–MS), J. Biomol. Screen. 12 (2007) 167–174.
[23] L. Zhu, L. Chen, H. Luo, X. Xu, Frontal affinity chromatography combined online with mass spectrometry: a tool for the binding study of different epidermal growth factor receptor inhibitors, Anal. Chem. 75 (2003) 6388–6393.
[24] H. Luo, L. Chen, Z. Li, Z. Ding, X. Xu, Frontal immunoaffinity chromatography with mass spectrometric detection: a method for finding active compounds from traditional Chinese herbs, Anal. Chem. 75 (2003) 3994–3998.
[25] H.S. Kim, D.S. Hage, Immobilization methods for affinity chromatography, in: D.S. Hage (Ed.), Handbook of Affinity Chromatography, CRC Press/Taylor & Francis, 2005 (chapter 3).
[26] R. Mallik, D.S. Hage, Affinity monolith chromatography, J. Sep. Sci. 29 (2006) 1686–1704.
[27] E. Brekkan, A. Lundqvist, P. Lundahl, Immobilized membrane vesicle or proteoliposome affinity chromatography. Frontal analysis of interactions of cytochalasin B and D-glucose with the human red cell glucose transporter, Biochemistry 35 (1996) 12141–12145.
[28] I. Gottschalk, C. Lagerquist, S.-S. Zuo, A. Lundqvist, P. Lundahl, Immobilized-biomembrane affinity chromatography for binding studies of membrane proteins, J. Chromatogr. B 768 (2002) 31–40.
[29] C. Pidgeon, C. Marcus, F. Alvarez, in: T.O. Baldwin, J.W. Kelly (Eds.), Applications of Enzyme Biotechnology, Plenum Press, New York, 1992.
[30] R. Moaddel, I.W. Wainer, The preparation and development of cellular membrane affinity chromatography columns, Nat. Protoc. 4 (2009) 197–205.
[31] L. He, S. Wang, G. Yang, Y. Zhang, C. Wang, B. Yuan, X. Hou, Progress in cell membrane chromatography, Drug Discov. Ther. 1 (2007) 104–107.
[32] B.X. Yuan, J. Hou, G.D. Yang, L.M. Zhao, L. He, Comparison of determination of drug-muscarinic receptor affinity by cell membrane chromatography and by radioligand binding assay with the cerebrum membrane of the rat, Chromatographia 619 (2005) 381–384.
[33] W. Yu, B. Yuan, X. Deng, L. He, Z. Youyi, H. Qide, The preparation of HEK293 α_{1A} or HEK293 α_{1B} cell membrane stationary phase and the chromatographic affinity study of ligands of α_1 adrenoreceptor, Anal. Biochem. 339 (2005) 198–205.
[34] Y. Wang, B. Yuan, X. Deng, L. He, S. Wang, Y. Zhang, Q. Han, Comparison of alpha1-adrenergic receptor cell-membrane stationary phases prepared from expressed cell line and from rabbit hepatocytes, Anal. Bioanal. Chem. 386 (2006) 2003–2011.
[35] A. Zeng, B. Yuan, C. Wang, G. Yang, L. He, Frontal analysis of cell-membrane chromatography for the determination of drug-α_{1D} adrenergic receptor affinity, J. Chromatogr. B 877 (2009) 1833–1837.
[36] J.M. Lebert, E.M. Forsberg, J.D. Brennan, Solid-phase assays for small molecule screening using sol–gel entrapped proteins, Biochem. Cell Biol. 86 (2008) 100–110.
[37] Y. Landry, J.-P. Gies, Drugs and their molecular targets: an updated overview, Fundam. Clin. Pharmacol. 22 (2008) F1–F18.
[38] R. Moaddel, K. Jozwiak, K. Whittington, I.W. Wainer, Conformational mobility of immobilized alpha3beta2, alpha3beta4, alpha4beta2, and alpha4beta4 nicotinic acetylcholine receptors, Anal. Chem. 77 (2005) 895–901.
[39] Y. Zhang, Y. Xiao, K.J. Kellar, I.W. Wainer, Immobilized nicotinic receptor stationary phase for on-line liquid chromatographic determination of drug–receptor affinitie, Anal. Biochem. 264 (1998) 22–25.
[40] WainerF I.W., Y. Zhang, Y. Xiao, K.J. Kellar, Liquid chromatographic studies with immobilized neuronal nicotinic acetylcholine receptor stationary phases: effects of receptor subtypes, pH and ionic strength on drug–receptor interactions, J. Chromatogr. B 724 (1999) 65–72.
[41] R. Moaddel, K. Jozwiak, R. Yamaguchi, C. Cobello, K. Whittington, T.K. Sarkar, S. Basak, I.W. Wainer, On-line screening of conformationally constrained nicotines and anabasines for agonist activity at the alpha3beta4- and alpha4beta2-nicotinic acetylcholine receptors using immobilized receptor-based liquid chromatographic stationary phases, J. Chromatogr. B 813 (2004) 235–240.
[42] R. Moaddel, R.V. Oliveira, T. Kimura, P. Hyppolite, M. Juhaszova, Y. Xiao, K.J. Kellar, M. Bernier, I.W. Wainer, Initial synthesis and characterization of an alpha7 nicotinic receptor cellular membrane affinity chromatography column: effect of receptor subtype and cell type, Anal. Chem. 80 (2008) 48–54.
[43] R. Moaddel, J.-F. Cloix, G. Ertem, I.W. Wainer, Multiple receptor liquid chromatographic stationary phases: the co-immobilization of nicotinic receptors, the g-amino-butyric acid receptors, the N-methyl D-aspartate receptors, Pharm. Res. 19 (2002) 104–107.
[44] T. Kitabatake, R. Moaddel, R. Cole, M. Gandhari, C. Frazier, J. Hartenstein, A. Rosenberg, M. Bernier, I.W. Wainer, Characterization of a multiple ligand-gated ion channel cellular membrane affinity chromatography column and identification of endogenously expressed receptors in astrocytoma cell lines, Anal. Chem. 80 (2008) 8673–8680.

[45] H. Du, J. He, S. Wang, L. He, Investigation of calcium antagonist-L-type calcium channel interactions by a vascular smooth muscle cell membrane chromatography method, Anal. Bioanal. Chem. 397 (2010) 1947–1953.
[46] R. Heilker, M. Wolff, C.S. Tautermann, M. Bieler, G-protein-coupled receptor-focused drug discovery using a target class platform approach, Drug Discov. Today 14 (2009) 231–240.
[47] F. Beigi, K. Chakir, R.-P. Xiao, I.W. Wainer, G-protein-coupled receptor chromatographic stationary phases. 2. Ligand-induced conformational mobility in an immobilized β_2-adrenergic receptor, Anal. Chem. 76 (2004) 7187–7193.
[48] R. Moaddel, E. Calleri, G. Massolini, C.R. Frazier, I.W. Wainer, The synthesis and initial characterization of an immobilized purinergic receptor (P2Y1) liquid chromatography stationary phase for online screening, Anal. Biochem. 364 (2007) 216–218.
[49] R. Moaddel, H.K. Musyimi, M. Sanghvi, C. Bashore, C.R. Frazier, M. Khadeer, P. Bhatia, I.W. Wainer, Synthesis and characterization of a cellular membrane affinity chromatography column containing histamine 1 and P2Y1 receptors: a multiple G-protein coupled receptor column, J. Pharm. Biomed. Anal. 52 (2010) 416–419.
[50] C. Temporini, S. Ceruti, E. Calleri, S. Ferrario, R. Moaddel, M.P. Abbracchio, G. Massolini, Development of an immobilized GPR17 receptor stationary phase for binding determination using frontal affinity chromatography coupled to mass spectrometry, Anal. Biochem. 384 (2009) 123–129.
[51] E. Calleri, S. Ceruti, G. Cristalli, C. Martini, C. Temporini, C. Parravicini, R. Volpini, S. Daniele, G. Caccialanza, D. Lecca, C. Lambertucci, M.L. Trincavelli, G. Marucci, I.W. Wainer, G. Ranghino, P. Fantucci, M.P. Abbracchio, G. Massolini, Frontal affinity chromatography-mass spectrometry useful for characterization of new ligands for GPR17 receptor, J. Med. Chem. 53 (2010) 3489–3501.
[52] F. Beigi, I.W. Wainer, Syntheses of immobilized G protein-coupled receptor chromatographic stationary phases: characterization of immobilized mu and kappa opioid receptors, Anal. Chem. 75 (2003) 4480–4485.
[53] L. Toledo-Sherman, E. Deretey, J.J. Slon-Usakiewicz, W. Ng, J.R. Dai, J.E. Foster, P.R. Redden, M.D. Uger, L.C. Liao, A. Pasternak, N. Reid, Frontal affinity chromatography with MS detection of EphB2 tyrosine kinase receptor. 2. Identification of small-molecule inhibitors via coupling with virtual screening, J. Med. Chem. 48 (2005) 3221–3230.
[54] J.J. Slon-Usakiewicz, J.R. Dai, W. Ng, J.E. Foster, E. Deretey, L. Toledo-Sherman, P.R. Redden, A. Pasternak, N. Reid, Global kinase screening. Applications of frontal affinity chromatography coupled to mass spectrometry in drug discovery, Anal. Chem. 77 (2005) 1268–1274.
[55] J.T. Moore, J.L. Collins, K.H. Pearce, The nuclear receptor superfamily and drug discovery, ChemMedChem 1 (2006) 504–523.
[56] P. Fechner, G. Gauglitz, J.-A. Gustafsson, Neclear receptors in analytics—a fruitful joint venture or wasteful futility? Trends Anal. Chem. 4 (2010) 297–305.
[57] M. Sanghvi, R. Moaddel, C. Frazier, I.W. Wainer, Synthesis and characterization of liquid chromatographic columns containing the immobilized ligand binding domain of the estrogen related receptor alpha and estrogen related receptor gamma, J. Pharm. Biomed. Anal. 53 (2010) 777–780.
[58] N.W. Chan, D.F. Lewis, P.J. Rosner, M.A. Kelly, D.C. Schriemer, Frontal affinity chromatography–mass spectrometry assay technology for multiple stages of drug discovery: applications of a chromatographic biosensor, Anal. Biochem. 319 (2003) 1–12.