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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 399–406
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Review

Conformational mobility of immobilized proteins

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Received 17 August 2006; accepted 20 August 2006
Available online 13 November 2006

Abstract

Cellular membrane fragments have been immobilized on the surface of a silica-based liquid chromatographic support and on the surface of glass capillaries to create immobilized receptor and drug transporter columns. These columns have included phases containing one subtype of the nicotinic receptor (α3β2, α3β4, α4β2, α4β4) and the P-glycoprotein transporter. A key question in the application of these columns to drug discovery and development is the ability of the immobilized receptor or transporter to undergo ligand and/or co-factor induced conformational changes. Using frontal affinity chromatographic techniques and non-linear chromatographic techniques it has been demonstrated that the immobilized nicotinic receptors undergo agonist-induced conformational shifts from the resting to desensitized states with corresponding changes in binding affinities and enantioselectivities. Ligand-induced allosteric interactions and ATP-driven conformational changes have also been demonstrated with the immobilized Pgp stationary phase. The results demonstrate that the immobilized proteins retained their ability to undergo conformational mobility and that this is an attractive alternative to allow for the full characterization of multiple protein conformations.
Published by Elsevier B.V.

Keywords: Nicotinic receptor; P-glycoprotein transporter; Conformational activity; Affinity chromatography; Displacement chromatography

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1. Introduction

In recent years, there has been increased interest in the conformational mobility of proteins as part of drug discovery programs. In the past, little importance was placed on this aspect of binding, but it is now known, that proteins can exist in multiple conformations. Two models dominate, the ligand-induced model and conformational selection model [1]. The ligand-induced model states that the ligand binds to the lowest energy conformation of the protein inducing a conformational change to accommodate the ligand. The second model, the conformational selection model, states that proteins exist in multiple conformations at all times. The ligand then selectively binds to one of the conformations increasing the population of that conformation over the others and subsequently eliciting its response. In any case it has been shown that upon binding of the ligand, the protein-ligand

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0731-7085/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jpba.2006.08.021

complex assumes a new conformation that has lower energy than the protein alone.

Conformational mobility appears to be an essential requirement for proper protein function. For example, the binding of a substrate to an enzyme produces conformational changes which lead to the transitional state, and subsequently formation and release of the product [1]. For transmembrane proteins, such as ligand gated ion channels, LGIC, agonist binding produces conformation changes that open an ion channel and facilitate the transfer of ions from one side of the cellular membrane to the other.

The structural requirements for the binding of agonists to target proteins, both on the molecular and protein levels have been extensively studied [1]. The structural requirements for competitive inhibitors, compounds which bind at the agonist binding site but do not activate the receptor, have also been extensively investigated. A third class of ligands, allosteric modifiers, contains compounds that bind at a site or sites on the receptor other than the active site [2,3]. The binding of these compounds produces conformational changes in the protein, which either increase (cooperative allosteric) or decrease (anti-cooperative allosteric) the activity of the agonist. Allosteric modifiers represent a new class of therapeutic agents which have not been extensively studied [4]. A key aspect to the successful exploitation of allosteric modifiers is the understanding and measurement of the conformational changes they produce in the target receptor.

Ligand-induced conformational changes in isolated proteins have been studied using multiple techniques, including the steady state and time-resolved fluorescence, far-UV circular dichroism and NMR relaxation studies [5,6]. Although these methods provide detailed information about the secondary structure and can provide very important functional data, they do not provide extensive binding data for a set of ligands to the different conformations. The spectroscopic techniques, in particular CD have been used to monitor the formation of the drug/protein complex, thus, providing direct data on the binding interaction between the protein and a drug [7]. In these cases, they are looking at gross changes in the molecule. However, if the binding site is small relative to the overall size of the protein, these techniques are unable to detect conformational changes associated with ligand binding, cf. [8].

Currently, quantitative analysis of the immobilized conformation can be carried out, but it often requires immunoprecipitation analysis, SDS-PAGE and Western blotting for conformational analysis, which is time consuming and laborious [9]. Another approach to the study of affect of protein conformation on ligand binding utilizes surface plasmon resonance (SPR). An example of this method is the characterization of the binding properties of the active conformation of the Bax protein, a protein involved in the mitochondrial pathway for apoptosis [9]. In this study, the Bax protein was immobilized using a monoclonal antibody that recognizes only its active conformation and then studied using SPR. However, only the active conformation could be studied in these experiments and the immobilized protein was not conformationally mobile.

An alternative technique for the measurement of ligand-induced conformational changes is bio-affinity chromatography, which utilizes liquid chromatographic stationary phases containing immobilized proteins in a flow system. Initial studies with immobilized human serum albumin (HSA) demonstrated that the chromatographic retention of a compound on the HSA stationary phase correlated with the compound's binding affinity to the immobilized protein [10]. It was also demonstrated that the HSA column could also be used to identify positive allosteric effects on binding affinity, such as the S-warfarin induced increase of the binding affinity of *S*-oxazepam hemmisuccinate [11] and the ibuprofen induced increase in the affinity of lorazepam as well as the negative allosteric effects of *S*-oxazepam on the binding of *R*-oxazepam [12].

A key advantage of bio-affinity chromatography is the ability to use this technique with both cytosolic and transmembrane proteins. Since transmembrane proteins constitute the largest family of therapeutic drug targets, this is a key advantage. In addition, it has been demonstrated that this approach can also be used to investigate induced conformational changes in the immobilized target. This aspect of bio-affinity chromatography will be explored in this review using data from the studies of the nicotinic acetylcholine receptor (a LGIC) and P-glycoprotein (a transmembrane drug transporter).

2. Methodologies

2.1. Immobilization of solubilized components of the cellular membranes on the IAM stationary phase

The amount of cellular membranes used to produce the membrane-column from transfected cells is dependent on the level of expression of the target protein and can range between 10^6 and 10^7 cells [13]. The necessary amount of cells is initially homogenized to lyse the cells. The solution is subsequently centrifuged and the pellet is solubilized overnight in the presence of protease inhibitors, peptidase inhibitors, salts and detergent and in some case in the presence of polyhydroxy compounds for protein stability. The resulting solution is centrifuged to remove insoluble proteins and the solubilized solution is then mixed with immobilized artificial membrane (IAM) particles, which contain phosphatidyl choline head groups. After mixing for 1 h, the suspended particles are dialyzed for a period of 1–3 days, depending on the protein stability in the environment. After dialysis, the particles are collected and washed by centrifugation. The protein-rich stationary phase is then packed into a HR 5/2 column (Amersham Pharmacia Biotech, Uppsala, Sweden) to yield a 150 mm × 5 mm (i.d.) chromatographic bed and washed for a period of at least 6 h before starting the frontal analysis experiments.

2.2. Immobilization of solubilized components of the cellular membranes on the surface of open tubular columns

Cellular membranes can also be directly immobilized on the surface of silica capillaries to create open tubular chromatography columns [14]. This approach utilizes a significantly smaller amount of cells, 10^4 to 10^5 cells, which are homogenized and solubilized in the same manner, with the exception that a much

smaller amount of solubilization buffer is used. The silica surface undergoes a series of chemical reaction to allow for the immobilization of avidin. A biotin molecule with a long lipophilic arm is then bound to the avidin, allowing its arm to insert into solubilized cellular membranes. These columns have shown to be stable for up to 4 weeks and are a great advantage over the IAM stationary phase, due to the dramatic decrease of potential non-specific binding sites.

2.3. Characterization of the immobilized protein stationary phases by frontal chromatography

The characterization of all the protein-stationary phases is carried out by frontal affinity chromatography. Frontal affinity chromatography is based upon the effect of escalating concentrations of a competitive binding ligand on the retention volume of a receptor-specific marker [15]. The data obtained from these experiments is similar to data produced by competitive binding studies and can be analyzed using methodology similar to Scatchard analyses to determine K_d and P values, where P is equivalent to B_{max}.

This technique is used to characterize the binding of small molecules to the immobilized membrane-bound target and to determine binding affinities (K_d) and the number of active binding sites on the column (B_{max}) [15]. A key requirement of this approach is the existence of a compound which is known to bind to the target, the marker ligand. In frontal affinity chromatography, the marker ligand is placed in the mobile phase and passed through the column. The frontal regions are composed of the relatively flat initial portion of the chromatographic traces, which represent the nonspecific and specific binding of the marker to the cellular membranes and the target. The saturation of the target by the marker produces a vertical rise in the chromatographic trace, which ends, or plateaus, when the target is saturated (Fig. 1) [16].

Once it has been established that the immobilized target specifically binds the marker ligand, it is possible to calculate K_d values of the marker or other ligands for the target receptor using displacement chromatography. In the displacement studies, increasing concentrations of the marker or other test ligands are added to the mobile phase and the effects on the retention volumes, measured at the midpoint of the breakthrough curves, are determined, cf. Fig. 1.The relationship between displacer concentration and retention volume can be established using Eq. (1) and can be used to determine the K_d value of the displacer and the number of active binding sites, B_{max} [15]:

$$[\text{Dis}](V - V_{min}) = B_{max}[\text{Dis}](K_{Dis} + [\text{Dis}])^{-1} \quad (1)$$

where V is retention volume of displacer, V_{min} the retention volume of displacer when the specific interaction is completely suppressed (this value can be determined by running the marker ligand with a high concentration of displacer). From the plot of [Dis] $(V - V_{min})$ *versus* [Dis], dissociation constant values, K_d, for the displacer can be obtained. The data is normally analyzed using non-linear regression employing a standard program such as Prism 4 software (Graph Pad Software, Inc., San Diego, CA, USA) running on a personal computer.

2.4. Characterization of the immobilized protein stationary phases by non-linear chromatography

A second approach to the characterization of immobilized proteins is non-linear chromatography (NLC), which utilizes zonal chromatographic techniques. While the ideal chromatographic peak shape is Gaussian, in liquid chromatography the actual peak profiles are often asymmetrical. The observed asymmetry can arise from a variety of sources including extra column effects, heterogeneity of the stationary phase, heterogenous mass transfer or a non-linear isotherm [17]. While peak tailing is a problem in analytical separations, concentration-dependent asymmetry can be used with NLC techniques to characterize the separation processes occurring on the column. When immobilized proteins are involved in the chromatographic process, the observed dissymmetry can be used to calculate the binding kinetics and equilibrium constants of the protein–ligand binding process, cf. Fig. 2 [18].

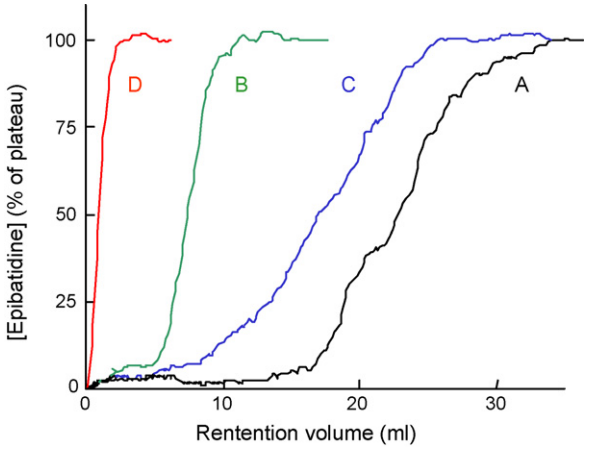

Fig. 1. The elution profiles of [^{3}H]-epibatidine on α3β4 NR-IAM stationary phase, where A = 60 pM, B = 450 pM, C = 60 pM [^{3}H]-epibatidine in the presence of 60 nM (−)-nicotine; D = 60 pM [^{3}H]-epibatidine in the presence of 1000 nM (−)-nicotine. Reprinted with permission from Zhang et al. [16].

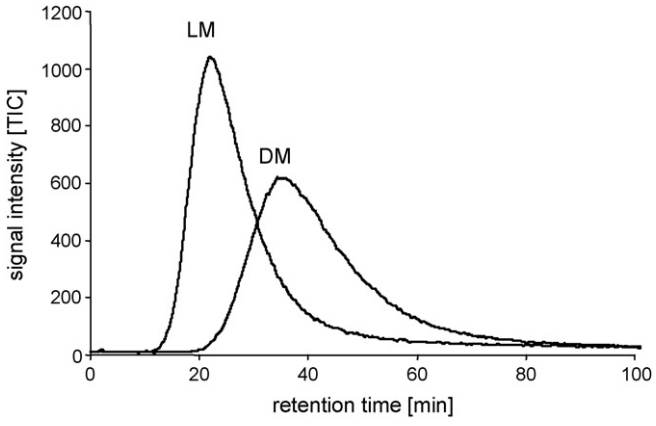

Fig. 2. The comparison of peak profiles of DM and LM obtained in independent experiments consequent injections. Reprinted with permission from Jozwiak et al. [18].

In the data analysis, the retention time t_R is calculated as the time to reach the apex of the peak and the chromatographic peak are then fitted using non-linear modeling employing PeakFit v4.11 for Windows Software (SPSS Inc., Chicago, IL). The mathematical approach for NLC modeling was derived from the Impulse Input Solution as described by Eq. (2) (PeakFit User's Manual, pp. 8–25):

$$y = \frac{a_0}{a_3}\left[1 - \exp\left(-\frac{a_3}{a_2}\right)\right] \times \left[\frac{\sqrt{a_1/x}I_1(2\sqrt{a_1 x}/a_2)\exp((-x-a_1)/a_2)}{1 - T(a_1/a_2, x/a_2)[1-\exp(-a_3/a_2)]}\right] \quad (2)$$

where y is the intensity of signal, x the reduced retention time:

$$T(u, v) = \exp(-v)\int_0^u \exp(-t)I_0(2\sqrt{vt})\,dt$$

The T function acts as a "switching" function, which produce the skew in the peak profile when the column is overloaded.

$I_0()$ and $I_1()$ are Modified Bessel functions, a_0 the area parameter, a_1 the center parameter, reveal to true thermodynamic capacity factor, a_2 the width parameter, a_3 is the distortion parameter.

Obtained chromatograms are extracted to an Excel worksheet as a set of two columns: retention time and signal intensity. Such chromatogram profiles are further smoothed and in next step fitted to the NLC function by adjusting the a_x parameters of Eq. (2). Minimization procedure was repeated at least three times in order to find the global minimum. The set of NLC parameters (a_0, a_1, a_2 and a_3) was collected for each profile.

The NLC parameters were further processed for the calculation of the k', K_a, k_a and k_d values. These parameters were calculated using the following relationships (PeakFit User's Manual, pp. 8–26):

- $k' = a_1$ is the real thermodynamic capacity factor;
- $k_d = 1/a_2 t_0$ is the solute desorption constant rate (t_0 is the dead time of a column);
- $K_a = a_3/C_0$ is the equilibrium constant for adsorption (C_0 is a concentration of solute injected multiplied by a width of the injection pulse (as a fraction of column dead volume));
- $k_a = k_d K_a$ is the solute adsorption constant rate.

3. Nicotinic receptor

The nicotinic receptor is the largest member and the most well characterized member of the LGIC superfamily. It is subdivided into the muscular and neuronal nicotinic receptor family, where the neuronal nicotinic receptors are being targeted for possible treatments for Alzheimer's Disease, Parkinson's disease, nicotine addiction, short term memory, ulcerative colitis, pain and gastrointestinal tract motility [19].

The primary binding sites for agonists and competitive antagonists are located on the extracellular portion of the receptor in a pocket located at the interface between the N-terminals of the α and β subunits [20]. Binding of the agonists into these domains induces the cascade of conformational changes of the receptor, which leads to the opening of the channel and ion flux. There is also an additional binding site of increasing importance, namely the non-competitive inhibitor (NCI) binding site. NCIs are targeted in drug discovery and development for their potential treatment of disease and for the prevention of adverse effects of certain drugs. The high affinity site of NCIs is located in the channel lumen within the pore. At this site, NCIs inhibit nAChR function by a reversible channel blockade or shorten channel opening time in a voltage-sensitive manner [21].

The nicotinic receptor exists in four inter-convertible states including a low affinity resting state, an intermediate state and a high affinity desensitized state [22]. The desensitized state is a result of agonist-induced conformational changes. A key issue in the utilization of immobilized nAChR columns in the study of agonist-induced conformational changes is whether or not the immobilized receptor retains its conformational flexibility. This question has been recently investigated by our laboratory [20].

The standard frontal affinity chromatographic techniques used to characterize the immobilized nAChR employ the specific nAChR agonist epibatidine, EB, as the marker ligand. The data obtained by these studies suggested that the immobilized nAChR was in the desensitized state, which was confirmed by comparing the relative magnitude of the K_d values obtained by chromatographic and membrane filtration assays, Table 1 [20]. The question was whether the nAChR was immobilized in the desensitized state or if the exposure to EB converted the receptor into this state.

The experimental format of the frontal affinity chromatography studies made it difficult to answer this question. The approach used to investigate agonist-induced conformational changes in the nAChR was NLC and the test ligands were NCIs [20]. NCIs were chosen as they bind to specific sites on the nAChR, but the binding does not induce conformational changes in the nAChR, rather inhibits them. The objective was to determine agonist-induced changes in the conformation of the nAChR by determining the corresponding changes in the affinities of the NCI probes. The columns used in this studied contained either the α3β2, α3β4, α4β2 or α3β4 nAChR [20]. The NCIs chosen for this study were (*R*)- and (*S*)-mecamylamine (MCM), dextro- and levomethorphan (DM, LM) and pencyclidine (PCP). A similar mechanism of action has been proposed for all of these compounds wherein they act in the internal lumen of the receptor [23].

Each of the respective columns was produced and the retention times of the NCIs obtained using NLC techniques, pre-EB results. The columns were then exposed to EB and the test ligands were chromatographed using the same mobile phase

Table 1
Binding affinities (nM) calculated by frontal affinity chromatography using the immobilized nicotinic receptor columns, compared to binding affinities calculated by filtration assays [20]

	α3β2	α3β4	α4β2	α4β4
Epibatidine	0.001 (0.035)	0.086 (0.57)	0.005 (0.061)	0.042 (0.16)
Nicotine	2.18 (47)	80 (440)	16.4 (10)	0.387 (40)
Cytisine	2.43 (37)	7.6 (220)	6.09 (1.5)	0.237 (2.1)

Table 2
Test of the retention of non-competitive inhibitors on four different columns before and after exposure to epibatidine

Subtype column	Compound	Pre-EPI	Post-EPI	Difference
	(*R*)-MCM	5.54(±0.04)	6.16(±0.03)	0.62[a]
	(*S*)-MCM	5.60(±0.02)	6.19(±0.01)	0.60[a]
α3β2	PCP	15.77(±0.09)	16.52(±0.05)	0.76[a]
	DM	42.79(±0.86)	44.35(±0.56)	1.56
	LM	42.46(±0.23)	45.42(±0.39)	2.96[a]
	(*R*)-MCM	6.07(±0.06)	6.46(±0.05)	0.39[a]
	(*S*)-MCM	6.13(±0.02)	6.56(±0.003)	0.44[a]
α4β2	PCP	16.79(±0.24)	17.46(±0.07)	0.67[a]
	DM	45.62(±1.22)	46.65(±0.24)	1.02
	LM	44.94(±0.21)	46.52(±0.75)	1.58[a]
	(*R*)-MCM	7.32(±0.33)	8.05(±0.12)	0.72[a]
	(*S*)-MCM	7.48(±0.13)	8.38(±0.06)	0.91[a]
α3β4	PCP	22.65(±0.19)	27.61(±0.04)	4.96[a]
	DM	84.09(±0.79)	96.60(±0.08)	12.51[a]
	LM	79.31(2.26)	87.57(±0.72)	11.26[a]
	(*R*)-MCM	5.53(±0.01)	6.49(±0.06)	0.96[a]
	(*S*)-MCM	5.59(±0.02)	6.60(±0.05)	1.01[a]
α4β4	PCP	15.11(±0.15)	17.49(±0.19)	2.38[a]
	DM	41.86(±0.36)	44.08(±0.22)	2.22[a]
	LM	41.84(±0.40)	44.3(±0.34)	2.46[a]

Comparison of the retention times (min) in zonal chromatography obtained before (pre-EPI) and after (post-EPI) experiments of five compounds: (*R*)-mecamylamine [(*R*)-MCM], (*S*)-mecamylamine [(*S*)-MCM], phencyclidine (PCP), dextromethorphan (DM) and levomethorphan (LM) [20].
[a] Represents a significant difference.

conditions used before exposure to EB, post-EB results [20]. The post-EB retention times of the NCIs were increased relative to the pre-EB retention times on each column with the exception of DM on the α3β2 nAChR and α4β2 nAChR columns where the changes did not reach significance, Table 2.

The data was consistent with the existence of two conformationally different states and suggest that the NCIs bound to two forms of the nAChR, the resting (pre-EB) and desensitized (post-EB) states. This is consistent with the previous observations that retention times on the immobilized nAChR column reflect the affinity of the solute for the nAChR [20] and that the desensitized state is a high affinity state relative to the resting state. A change in the affinity of the nAChR for the test NCIs was also demonstrated by the NLC analysis of the data from the pre-EB and post-EB forms of the α3β4 nAChR column. The results indicated that the post-EB dissociation rate constants, k_d, were significantly decreased relative to the pre-EB data: from $6.95(\pm0.01)\,s^{-1}$ to $5.68(\pm0.01)\,s^{-1}$ (PCP), from $1.92(\pm0.01)\,s^{-1}$ to $1.69(\pm0.01)\,s^{-1}$ (LM), from $1.84(\pm0.01)\,s^{-1}$ to $1.67(\pm0.01)\,s^{-1}$ (DM). Previous thermodynamic studies on the α3β4 nAChR column have demonstrated that differences in k_d values are a reflection of differences in the enthalpies (ΔH) of the NCI-α3β4 nAChR complexes [23]. Thus, these results reflect an increased stability of the NCI-nAChR complexes on the post-EB column.

The enantioselectivities of the pre-EB and post-EB columns were also examined using (*R*)- and (*S*)-MCM and DM and LM as the test ligands [20]. An enantioselective separation of MCM was obtained only on the post-EB α4β2 nAChR and α3β4 nAChR columns, and the enantioselective separation of DM and LM increased after exposure of the α3β4 nAChR column to EB. The observed enantioselectivities were consistent with the reported enantioselective interactions of (*R*)-MCM and (*S*)-MCM [24] and DM and LM [25] with the nAChR.

Since enantiomers have the same physicochemical properties [26], a chromatographic chiral separation in a system containing an immobilized chiral selector is the result of specific interactions with the selector [27]. In these studies, the only difference in the pre-EB and post-EB experimental conditions was the exposure of the respective columns to EB. Thus, the changes in the observed enantioselectivities must reflect an EB-induced change in the chiral selectors, the nAChRs.

The existence of multiple conformations were directly observed using a α3β4 nAChR column prepared using 50×10^6 cells, a 50-fold increase [20]. When DM was chromatographed on the column prior to exposure to EB, a bimodal peak profile was observed, with a dominant fronting peak, Fig. 3a [20]. The same profile was observed with LM. The column was then exposed to EB and DM and LM were chromatographed again on the column. In both cases, the bimodal peak collapsed into a single, asymmetric peak, Fig. 3b. Thus, the initial chromatograms suggest that when the nAChRs were immobilized they were predominantly in the resting state, but that some were present in the desensitized state. Exposure to EB altered this distribution as the majority of the nAChRs were conformationally changed to the desensitized state.

The results suggest that membranes obtained from each of the expressed cell lines contained nAChRs that were predominately in the resting state. The immobilization process did not significantly alter this population, nor did it affect the sensitivity of the immobilized nAChRs to agonist-induced desensitization. Data from a number of other studies have also demonstrated that the transition from the desensitized state to the resting state also occurs. This transformation is a time-dependent process that took 4 weeks for the immobilized α3β4 nAChR. Thus, the immobilized nAChR columns can be used to study different conformational states of the receptor and the consequences of agonist-induced desensitization.

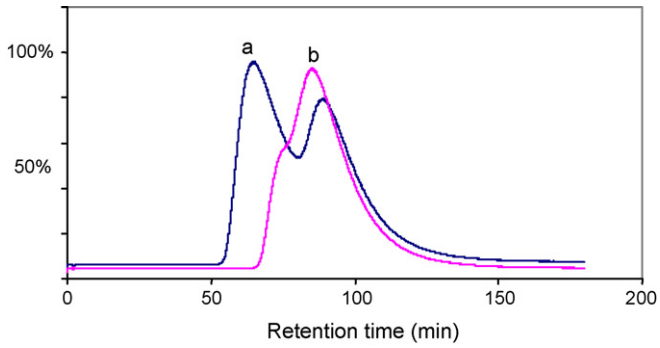

Fig. 3. Peak profile of dextromethorphan on the α3β4 nAChR column containing a larger amount of protein (a); and the peak profile of dextromethorphan after re-exposure to epibatidine (b). It was chromatographed on the column prior to exposure to EB, a bimodal peak profile was observed, with a dominant fronting peak, Fig. 3a. Reprinted with permission from Moaddel et al. [20].

4. P-glycoprotein transporter (Pgp)

The P-glycoprotein transporter (Pgp) is an efflux drug transporter that is expressed in the gastrointestinal epithelium, the kidney, capillary endothelial cells, resistant tumor cells and is a component of the blood brain barrier [14]. Transport by Pgp has been associated with reduced systemic bioavailability of orally administered drugs and the overexpression of Pgp in tumor cells has been linked to multiple-drug resistance in cancer chemotherapy [28]. The transporter is involved in the efflux of a number of substrates including agents used in the treatment of cancer, inflammation, immunosuppression, HIV/AIDS and hypertension. Thus, the determination of interactions between lead drug candidates and Pgp is a key aspect of the drug development process and in the development of new anti-cancer therapies.

One approach to the identification of Pgp substrates and inhibitors is online screening using immobilized Pgp columns in both the IAM liquid chromatographic and open tubular column formats [29,14]. This approach utilizes cellular membranes that do not contain Pgp obtained from the MDA435/LCC6 cell line and cellular membranes that contain Pgp obtained from the MDA435/LCC6MDR1 cell line [29]. Frontal displacement chromatography experiments have demonstrated that K_d values obtained on the IAM-Pgp columns correlate with those obtained using standard membrane binding techniques, Table 3 [29]. In addition, it has been demonstrated that there is no significant difference in the ability of the Pgp open tubular column to identify Pgp substrates as compared to Caco-2 permeability studies, the industry standard [30].

Pgp is thought to cycle through 4 ATP-catalyzed phases, Fig. 4 [29]. This hypothesis has been supported by infrared attenuated total reflection spectroscopy studies, which demonstrated that the addition of 3 mM ATP induced a conformational change in the tertiary structure of Pgp [31]. In Phase 1, when ATP is not bound to the transporter, Pgp binds substrates with a high affinity, and after binding ATP, the protein shifts to a low substrate affinity-state (Phase 3), in which it binds noncompetitive inhibitors with a high affinity. In addition to ATP-catalyzed conformational changes, there is experimental evidence for the existence of multiple binding sites on the Pgp molecule and for allosteric interactions between these sites [32].

Table 3
The K_d values calculated using fronal affinity chromatography on the immobilized Pgp-IAM stationary phase, reprinted from ref [29].

	K_d (experimental)	K_d (literature)
Vinblastin	23.5 ± 7.8 nM	36.0 ± 5.0 nM
Doxorubicin	15.0 ± 3.2 μM	31.0 ± 7.3 μM
Verapamil	54.2 ± 4.6 μM	0.45 ± 0.05 μM
Cyclosporin A	62.5 ± 5.6 nM, 97.9 ± 19.4 nM	18.0 ± 3.6 nM

Because of the differences in binding selectivity and affinity between the two ATP-induced states and the possibility for cooperative and anti-cooperative allosteric interactions, the online screening approach must be able to identify these interactions. Therefore, it is necessary that the immobilized Pgp retain its ATP- and ligand-induced conformational mobility. This property has been investigated using the Pgp-IAM column [29,33].

In one study, the chromatographic retentions of cyclosporin A (CysA), vinblastine (Vb) and verapamil (Ver) were determined alone, in conjunction with one of the other compounds and with and without ATP in the mobile phase [29]. When CysA was run alone, the observed retention volume was 7.8 ml, which reflected a low affinity for the immobilized Pgp transporter, Table 4. When 50 nM Vb was added to the mobile phase, the retention volume of CysA increased to 15.7 ml while the addition of 100 nM Vb resulted in an additional increase in the retention volume to 18.8 ml, Table 4. The results indicate that CysA and Vb bind at different sites on the Pgp molecule and that the binding of Vb to Pgp results in a conformational change in the Pgp molecule which results in an increased affinity for CysA, a positive allosteric effect. The data also demonstrates that this is a saturable, and therefore specific phenomenon, and that 50 nM Vb saturated the majority of the available binding sites.

The specificity of the CysA–Vb interaction was confirmed by the ability of Vb to displace itself and the lack of effect of Vb on the retention of Ver, Table 4 [29]. The first interaction demonstrates that Vb binds at a specific saturable site, while the

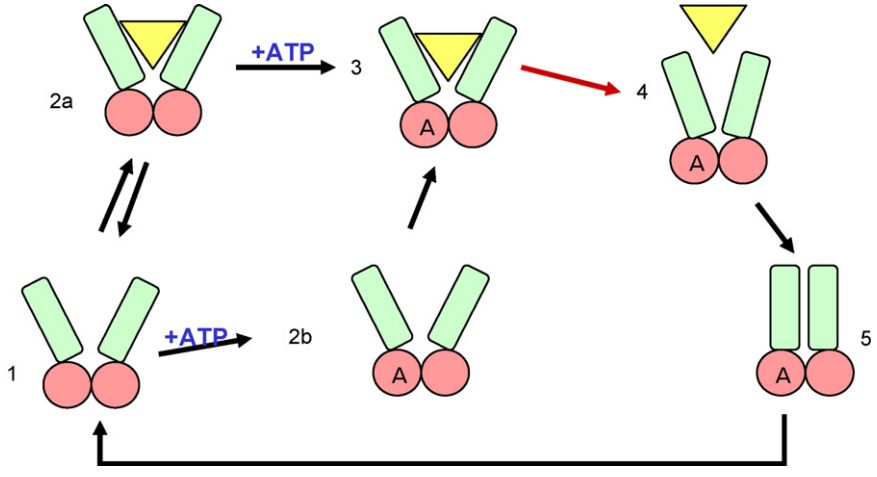

Fig. 4. The drug transporter Pgp is thought to cycle through 4 ATP catalyzed phases.

Table 4
Retention volumes (ml) of [^{3}H]vinblastine, [^{3}H]-verapamil and [^{3}H]cyclosporin A were obtained when (1) no ATP was present in the running buffer, (2) 3 mM ATP was added in the running buffer, (3) 50 nM cold vinblastine was supplemented in the sample (no ATP in the buffer), and (4) 100 nM cold vinblastin was in the sample (no ATP in the buffer) [29]

	No ATP	3 mM ATP	50 nM vinblastin (no ATP)	100 nM vinblastin (no ATP)
[^{3}H]-vinblastin	32.1	8.4	11.0	9.5
[^{3}H]-verapamil	34.2	5.9	34.1	34.0
[^{3}H]-cyclosporin A	7.8	17.5	15.7	18.8

fact that Vb does not displace Ver indicates that these compounds bind at separate sites on the Pgp molecule. However, unlike the Cys site, the Ver binding site is not affected by Vb-induced conformational changes. Thus, the immobilized Pgp column can be used to discern complex binding interactions.

The ability of the immobilized Pgp to undergo ATP-induced conformational changes was demonstrated by the affect on the retention of Vb, Ver and CysA by the addition of ATP to the mobile phase [29]. When 3 mM ATP was added to the running buffer, the retention volumes of Vb and Ver were significantly reduced, Table 3, Fig. 5. Vb and Ver are Pgp substrates and these results indicate that the immobilized Pgp molecule underwent an ATP-induced transition from Phase I, high substrate affinity, to Phase II, low substrate affinity. CysA is a non-competitive inhibitor of Pgp and the data demonstrate that this compound only binds to the Pgp conformation corresponding to Phase II of the transporter cycle. When the ATP was removed from the mobile phase, the effect was reversed indicating that the immobilized Pgp was able to complete the conformational cycle.

This ability of the immobilized Pgp to undergo ligand- and ATP-induced conformational changes was confirmed by the results of a study which examined the effect of mefloquine enantiomers, (+)-MQ and (−)-MQ, on the chromatographic retention of Vb and CysA [33]. When a 200 nM concentration of either (+)-MQ or (−)-MQ was added to the mobile phase, the specific retention of Vb was significantly decreased and no further reductions in retention were observed when the (+)-MQ and (−)-MQ concentrations were sequentially increased up to 1.0 μM. The addition of 3 mM ATP to the mobile phase containing 200 nM (+)-MQ or (−)-MQ also did not produce any further reduction in the retention volume of Vb. The results indicate that the affect of (+)-MQ and (−)-MQ on the binding of Vb was a specific saturable anti-cooperative interaction.

As shown above [29], the addition of 3 mM ATP to the running buffer results in the specific retention of CysA. Under these conditions, the addition of increasing concentrations of (+)-MQ to the running buffer produced corresponding reductions in the retention of CysA [33]. This indicates that (+)-MQ and CysA competitively compete for binding at the same site on the Pgp molecule. However, the addition of (−)-MQ did not result in a reduction of the retention of CysA, which indicates that there is no competitive binding interaction between the two compounds. The data demonstrates that there is an enantioselective difference in the binding of (+)-MQ and (−)-MQ at the site that CysA binds.

It has been previously shown that Pgp can be chemically locked into the Phase II conformation using sodium vanadate. The applicability of this approach to studies on the IAM immobilized Pgp was investigated by initially exposing a Pgp-IAM column to ATP followed by the addition of sodium vanadate [13]. Under these conditions, the retention time of Vb fell from 40 min in the absence of sodium vanadate to 8 min in the presence of sodium vanadate, Fig. 6 [13]. These results support the supposition that sodium vanadate locked the immobilized Pgp

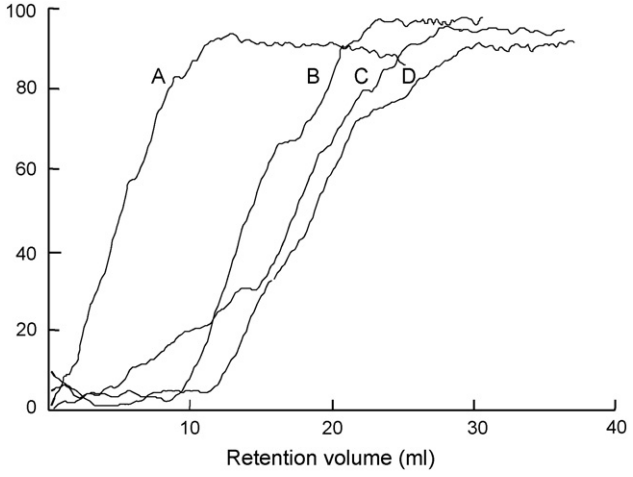

Fig. 5. Frontal affinity analysis of 1.0 nM [^{3}H]cyclosporin A. (A) [^{3}H]cyclosporine A was in the sample alone; (B) 50 nM cold vinblastine was supplemented in the sample; (C) 3 mM ATP was in the sample and running buffer; (D) 100 nM cold vinblastine was added in the sample. The running buffer was 50 mM Tris–HCl, pH 7.4. Reprinted with permission from Lu et al. [29].

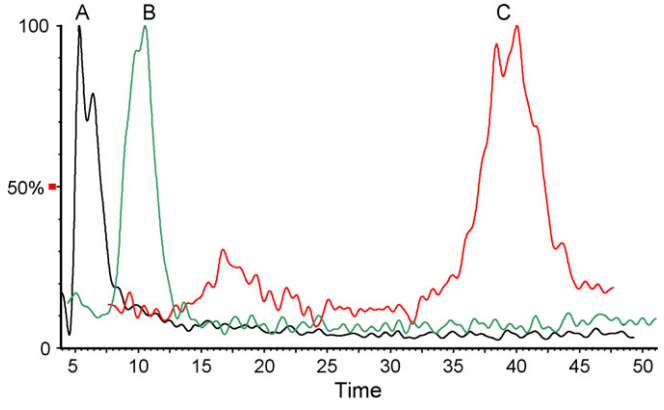

Fig. 6. PGP-SP (IAM) was packed into peek tubing (762 μm i.d. × 40 cm), with a flow rate at 50 μl/min. Zonal injections of 1 nM vinblastin were carried out in the presence of 1 μM vinblastin (A), on the column trapped in the first ATP cycle via the presence of vanadate in the mobile phase and alone (C). Reprinted with permission from Moaddel et al. [13].

transporter in Phase II. The conformational change was reversed by washing the sodium vandate and ATP from the column.

5. Conclusion

The data from the studies on the immobilized membrane liquid chromatography columns, namely the nicotinic receptors and the Pgp transporter, have demonstrated that the immobilized proteins retain their conformational mobility. This approach has an advantage over existing methods in that it can be used to study both cytosolic and transmembrane proteins. It can also be used to fully characterize a conformational state, as it is capable of locking a protein in a particular conformation. This approach can also be extended to be used in an open tubular column format with a variety of chromatographic techniques including frontal, zonal and non-linear chromatography.

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