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Review

Multiple protein stationary phases: A review☆

N.S. Singh[a], K-L. Habicht[b], K.S.S. Dossou[a], R. Shimmo[b], I.W. Wainer[a], R. Moaddel[a,∗]

[a] Biomedical Research Center, National Institute on Aging, National Institutes of Health, 251 Bayview Boulevard, Suite 100, Baltimore, MD 21224, USA
[b] Department of Natural Sciences, Institute of Mathematics and Natural Sciences, Tallinn University, Narva mnt. 29, 10120 Tallinn, Estonia

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Cellular membrane affinity chromatography stationary phases have been extensively used to characterize immobilized proteins and provide a direct measurement of multiple binding sites, including orthosteric and allosteric sites. This review will address the utilization of immobilized cellular and tissue fragments to characterize multiple transmembrane proteins co-immobilized onto a stationary phase. This approach will be illustrated by demonstrating that multiple transmembrane proteins were immobilized from cell lines and tissue fragments. In addition, the immobilization of individual compartments/organelles within a cell will be discussed and the changes in the proteins binding/kinetics based on their location.

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Contents

1. Introduction 64
2. Multiple ligand-gated ion channel columns 65
3. Multiple G-protein coupled receptor columns 66
4. Multiple ATP-binding cassette transporter columns 66
5. Membrane-enclosed organelle columns 66
6. Conclusions 67
Acknowledgements 67
References 68

1. Introduction

Cell surface transmembrane receptors and transporters are key therapeutic targets in drug discovery and development [1–3] and include large families of proteins such as G-protein coupled receptors (GPCRs), ligand-gated ion channels (LGICs) and the ATP-binding cassette superfamily (ABC transporters). GPCRs make up almost half of the current therapeutic target receptors and 36% of currently marketed drugs were developed as GPCR agonists or antagonists [4]. LGICs also represent a class of important therapeutic targets such as nicotinic acetylcholine receptors (nAChRs) and N-methyl-D-aspartate receptors (NMDARs) while the ABC transporters such as P-glycoprotein play essential roles in systemic and central bioavailability and are involved in an aspect of multiple drug resistance to therapeutic agents.

The broad range of cellular and pharmacological functions associated with these receptors and transporters has resulted in the development of a large number of in vitro assays for the high-throughput screening of small molecules against a specific receptor. The most commonly used methods for binding affinity determination include classic binding assays, functional assays, monolayer efflux assays and surface plasmon resonance (SPR) [5,6]. The classical binding assay approach is based on the concept of competitive interaction of a known analyte and a ligand for the same receptor binding site, while the functional assays allow the determination of a compound's effect on inhibition of transport, cell proliferation,

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∗ Corresponding author at: Bioanalytical and Drug Discovery Unit, Laboratory of Clinical Investigation, National Institute on Aging, National Institutes of Health, Suite 100, 8B131 Biomedical Research Center, 251 Bayview Boulevard, Baltimore, MD 21224-6825, USA. Tel.: +1 410 558 8294; fax: +1 410 558 8409.
E-mail address: moaddelru@grc.nia.nih.gov (R. Moaddel).

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mobilization of calcium, agonistic or antagonistic properties of the ligand, etc. [7,8]. These approaches allow quantitative determination of the binding affinity of a ligand for its receptor providing valuable information on the potency of the ligand, including effective concentration and selectivity for the targeted receptor.

However, the screening of chemical libraries as well as individual compounds against a single (orthosteric) binding site on the protein target does not always produce an adequate or comprehensive pharmacological profile [9]. For example, the majority of GPCRs possess allosteric binding sites and multiple conformations that can lead to increased or reduced activity or to distinctly different activities via alterations in intracellular signaling cascades. Differences can also arise from slight changes in amino acid residues that produce closely related proteins with widely ranging affinities and tissue expression. This is exemplified by the family of nicotinic acetylcholine receptors (nAChRs) which are composed of a combination of α and β subunits producing a family of structurally related LGICs with a broad range of affinities for the same agonists and antagonists. In addition, transmembrane receptors are expressed in a variety of membrane environments and the composition of the membrane can dramatically affect the function and selectivity of the target protein. This is illustrated by differences in the binding of ligands to the breast cancer resistance protein (BCRP), an ABC transporter, observed between cellular membrane-expressed BCRP and nuclear membrane-expressed BCRP [10].

One approach that provides for the direct measurement of multiple binding sites including orthosteric and allosteric sites, multiple binding configurations as well as subtype ligand interactions is bioaffinity chromatography, where the target biopolymer (protein) is immobilized onto silica based stationary phase. The use of this technology with isolated proteins and enzymes has been extensively reviewed [11–19]. In this review we address the utilization of an immobilized cellular and tissue fragments to characterize multiple proteins co-immobilized onto a stationary phase. The initial studies were carried out by Per Lundahl's group [20–22], where they immobilized the glucose transporter, GLUT1, through the incorporation of red blood cell membranes in proteo-liposomes [20]. This was quickly expanded by Wainer's group to include the immobilization of the transmembrane neuronal nicotinic receptor [23], onto the surface of the immobilized artificial membrane (IAM) stationary phase (12 μm, 300 Å pore) developed by Pidgeon and Venkataram [24]. The general experimental approach associated with this technology has been reviewed [25] and will not be discussed here, rather this review will concentrate on the study of the immobilization of different families of proteins and the co-immobilization of multiple receptors and differences produced by the cellular membrane environment.

2. Multiple ligand-gated ion channel columns

The immobilization of solubilized tissue onto the IAM stationary phase was initially demonstrated using solubilized rat brain tissue [11], where the rat brains were homogenized and solubilized and the resulting membrane fragments were immobilized onto an IAM stationary phase. It was clearly demonstrated that the immobilization of solubilized rat forebrain membrane fragments resulted in the co-immobilization of nicotinic receptors (nAChR), γ-amino-butyric acid receptors (GABA), and *N*-methyl D-aspartate (NMDA) receptors. Each receptor was characterized by frontal affinity chromatographic studies to determine the binding affinities. The resulting binding affinities correlated with those reported in literature [11]. As each receptor identified had a selective ligand, additional studies were carried out to determine whether the receptors were independent of each other. For example, the nAChR was characterized in the absence and in the presence of a saturating concentration of flunitrazepam, a marker used for the $GABA_A$ receptor. The study did not result in any changes in the elution volumes with or without the inhibitor for the other receptor, indicating that the marker ligands were specific for the immobilized receptors and that the immobilized receptors were independent of each other. Specifically, no decrease in the elution volume of [^{3}H]-epibatidine ([^{3}H]-EB) was observed when [^{3}H]-EB was injected on the column with or without saturating concentration of flunitrazepam in the mobile phase. In a subsequent study, human brain tissue was immobilized and the resulting column characterized. In this case, only the nicotinic receptor was probed and a biphasic curve was obtained indicating the presence of multiple sites for epibatidine. The frontal chromatographic studies demonstrated that at least two subtypes of the nicotinic receptor were immobilized, which were believed to be the homomeric α7 nAChRs and heteromeric $\alpha_x\beta_y$ nAChRs.

The presence of multiple receptors on the cellular membrane affinity chromatographic columns was not restricted to membrane fragments obtained from tissues. Cellular membranes from the endogenous cell lines, 1321N1 and A172 astrocytoma cell lines, were also immobilized onto the IAM stationary phase [12] and also resulted in a multiple receptor column. Specifically, the presence of multiple ligand-gated ion channels, including $GABA_A$ and NMDA receptors and multiple subtypes of the nAChR, was demonstrated and characterized by frontal affinity chromatography. Similar to what was observed in the human brain column, the frontal chromatogram of epibatidine resulted in a biphasic curve (Fig. 1).

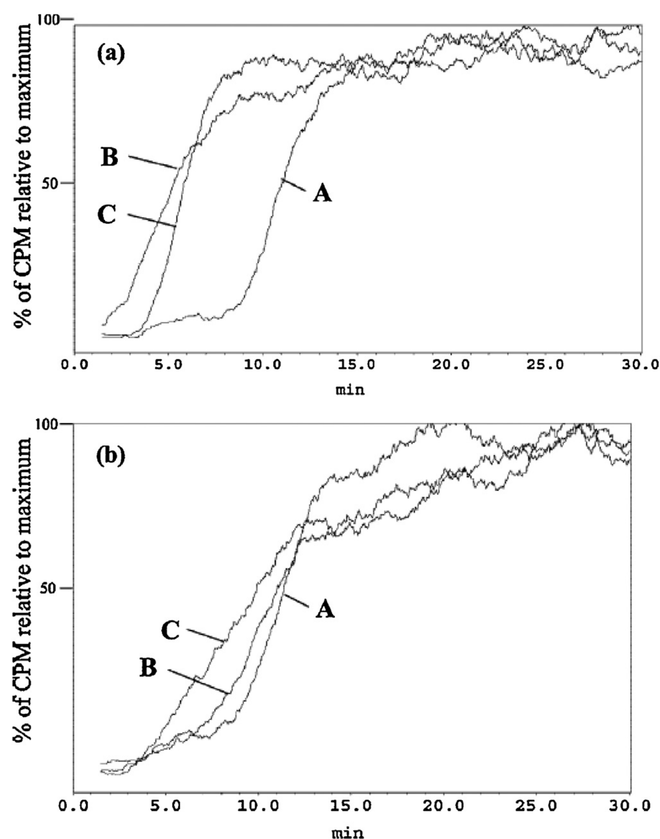

Fig. 1. The chromatographic traces obtained for 60 pM [^{3}H]-EB on the CMAC(1321N1) column (panel A) and CMAC(A172) column (panel B) where A is the trace obtained using a mobile phase composed of ammonium acetate [10 mM, pH 7.4]; B is the trace obtained after the addition of 5 nM α-BTx to the mobile phase; and C is the trace obtained after the addition of 1 nM κ-BTx to the mobile phase. Reprinted with permission from [12].

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