Characterization of a tethering system for biosensor applications

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ABSTRACT

The immobilization of brain dopamine receptors (primary targets in the treatment of schizophrenia, Parkinson's disease, and Huntington's chorea) on a solid support can represent an appealing strategy for the realization of biosensors for dopamine and dopaminergic drugs. Our final goal is the realization of an optical biosensor that allows parallel quantitative analysis, with different receptors, of neurotransmitters and related drugs. In our scheme, the active layer is constituted by dopaminergic receptors immobilized in biomimetic membranes tethered to a sensor surface. In order to preserve the biological activity of the neuroreceptors, the fabrication is carried out by soft lithography methods.
In this report, we have characterised the active layer by scanning probe techniques, in order to test its quality and integrity, and by optical experiments involving the signal emitted by specific fluorescent ligands

Keywords: dopaminergic receptors, tethered bilayer, scanning probes microscopy, fluorescence spectroscopy

1 INTRODUCTION

Dopamine receptors belong to a large superfamily of neurotransmitters that are coupled to their specific effector functions via guanine nucleotide regulatory (G) proteins. Until recently, the dopaminergic system was thought to consist of two receptors, D1 and D2. In the last few years, the application of molecular biology techniques has led to the identification of three new dopamine receptors [1] However, the original classification of two main groups of dopamine receptors, namely D1-like and D2-like dopamine receptors, still stands. All five known and cloned dopamine receptors fall into these two classes [2].

Dopamine receptors contain seven transmembrane domains that form a narrow hydrophobic cleft surrounded by three extracellular and three intracellular loops. The N-terminal domain is extracellular while the C-terminus projects into the cytosol [3]. Dopamine receptor antagonists are used as neuroleptics in the treatment of psychotic syndromes, and agonists for Parkinson's disease [4]. A current hope is that the therapy of these diseases is improved by selectively targeting a particular type of dopamine receptor [5]. The development of novel platforms for high throughput screening, coupled to the availability of cloned receptors, would greatly facilitate the search for new drugs. In order to achieve this, we have designed a novel type of biosensor for the analysis of the interactions between dopamine receptors and their ligands.

2 EXPERIMENTAL

2.1 Materials

Membrane preparations containing D1 and D2 dopamine receptors, sodium cholate and phospholipids were purchased from SIGMA. BODIPY FL SCH 23390 and PPHT (respectively a D1- and a D2-antagonist) were purchased from Molecular Probes.
The preparation of the cholesteryl polysulfides derivatives, is detailed in ref 6.

2.2 Solubilization and reconstitution procedure

Membranes (0.5 mg of proteins) were dialyzed against 50 mM Tris HCl pH 7.4 containing 1 mM PMSF and diluted 1:2 with Buffer A (50 mM Tris HCl pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 1 mM PMSF). In order to obtain a high yield of receptor recovery [7], the antagonists BODIPY FL SCH 23390 and BODIPY FL PPHT were added to 1 µM and, after an incubation period of 20 min at 37°C, the membranes were centrifuged at 18,000 g for 15 min. The ligand-occupied membranes isolated were resuspended in buffer S (50 mM Tris HCl pH 7.4, 5 mM KCl, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 1 M NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 1 mM PMSF).
Phosphatidylethanolamine and phosphatidylcholine in a ratio of 1:2 (already sonicated in 10 mM Tris HCl pH 7.4 containing 10 µg of 3,5-di-t-butyl-4-hydroxytoluene (BHT) per mg of phospholipids and solubilized with 1% sodium cholate) were added to a final concentration of 1.2 mg/ml. This phospholipid

ratio should be better able to promote receptor recovery during solubilization and reconstitution [8]. After 10 min incubation on ice, sodium cholate was added to a final concentration of 1%. The suspension was maintained on ice for an additional 15 min and then centrifuged at 31300 g for 40 min. 70-80% of total membrane proteins were extracted in the supernatant, after this cholate-solubilization. The supernatant was dialyzed overnight against buffer S in order to remove the detergent and to reconstitute the cholate-solubilized dopamine receptors into proteoliposomes.

2.3 Deposition procedure

Au(111)-on-mica substrates (Molecular Imaging, AZ) were annealed by ethanol flame and cleaned by O_2 plasma treatment, in order to remove organic residues on the surfaces. They were then immersed into a freshly prepared solution (0.5mg/mL) of cholesteryl derivatives in acetone for 24 h, rinsed abundantly with the same solvent and dried in an N_2 stream.

2.4 Imprinting procedure

A few drops of a freshly prepared solution of cholesteryl derivatives in acetone (0.5mg/ml) were applied to the surface of the PDMS stamps, which were then dried under a nitrogen stream until the solvent had completely evaporated. The polysulfide-coated elastomeric elements were then placed under their own weight onto gold substrates. After peeling-off the replicas, the patterned surfaces were vigorously rinsed several times with acetone in order to remove unattached molecules.

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2.5 Preparation of the biomimetic membrane

Cholesteryl-derivatized substrates were incubated in a freshly prepared solution of proteoliposomes in buffer S for 30' at room temperature and then rinsed several times with the same buffer.

3 RESULTS AND DISCUSSION

The fabrication of an artificial membrane anchored to a substrate and designed for incorporation of biomolecules requires a stable tether that guarantees a mechanically and chemically robust attachment of the lipid film to the support and also acts as a spacer, determining a spatial and functional decoupling between the membrane and the solid substrate [9]. In fact, the lipid–substrate interactions and their specific dependence on the separation distance may directly affect the folding and functionality of some proteins that are suitable for sensor applications. In particular, cholesteryl derivatives, composed by two cholesterol units attached to a polysulfide through a diethylene glycol chain, exhibit suitable terminations for anchoring phospholipids on a gold substrate, thus allowing the formation of biomimetic immobilized bilayers and retaining a hydrophilic environment on the solid support (Fig. 2) [10,11]. A typical fluid tapping-mode AFM (TM-AFM) image of layers of the cholesteryl derivative obtained by deposition onto gold surfaces from solution is shown in Fig. 2. To further favor the correct insertion and folding of the transmembrane receptor, a patterning of the cholesteryl layer could be required and microcontact printing (μCP) was used for this purpose (Fig. 1).

Resolution, contrast and possible surface diffusion of these patterns on the biosensor surface were analysed by Electric Force Microscopy (EFM), which monitors the surface chemical composition [12]. Fig. 3 shows an EFM image of the patterned thiolipid onto the gold surface. Periodic stripes of thiolipids with a width of 2 μm are visible that faithfully reproduce the master features.

Biomimetic membranes were obtained by fusing proteolipomes containing D1/D2 receptors with cholesteryl-derivatised gold substrates and imaged by fluid TM-AFM in bidistilled water. The uniformity of these membranes was high (Fig. 4, region "A"), while the cross-sectional profile reveals a difference in height between the membrane and the cholesteryl layer of about 5 nm (Fig. 5). This agrees well with the thickness of a phospholipid bilayer [13]. In this case, the AFM morphological analysis was not sufficient to visualize directly the receptor inserted in the membrane, as its expected diameter is ~ 4 nm.

To confirm the presence of neuroreceptors in the biomimetic membrane, binding of fluorescent ligands was evaluated. Fig.6 and Fig 7 show the photoluminescence (PL) spectra of biomimetic membranes prepared with liposomes only (control) or with liposomes containing dopamine receptors, after incubation respectively with the D1-antagonist Bodipy SCH-23390 and the D2 antagonist Bodipy FL PPHT (the emission peak of the fluorophore is ~ 510 nm). In both cases, the signal obtained with membranes with receptors was at least 5 times higher than that with membranes without receptors. This preliminary data clearly demonstrate the retention of the ligand binding capability by the immobilized membrane receptors.

4 CONCLUSIONS

Lipid bilayers are very important, since they are the natural environment of membrane proteins. In this work, we investigated deposited and patterned cholesteryl derivatives and biomimetic membranes by scanning probe microscopy techniques, showing high-quality bioactive layers. Based on fluorescence binding assays, the immobilized receptors are functional.

5 FIGURES

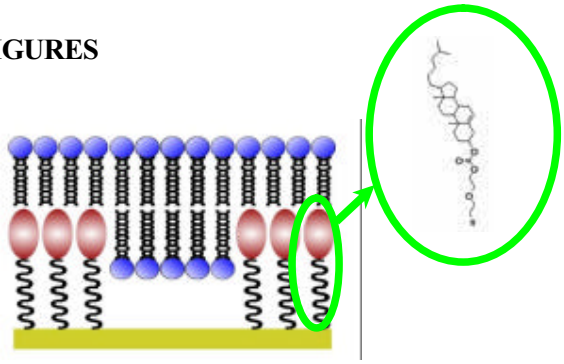

Fig. 1. Schematic model of lipid bilayer on μ-patterned tethering molecules (features not in scale).

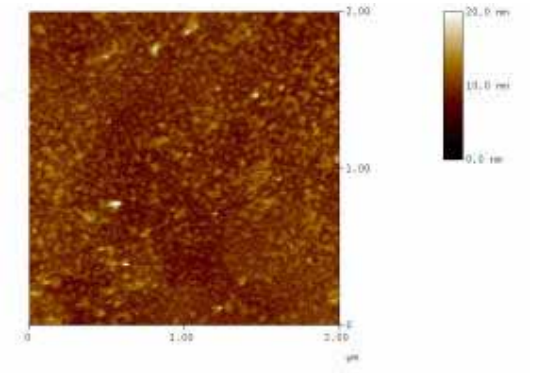

Fig. 2. Fluid TM-AFM image of an Au (111) surface with cholesteryl derivatives (in water). Scan size=2×2 μm^2.

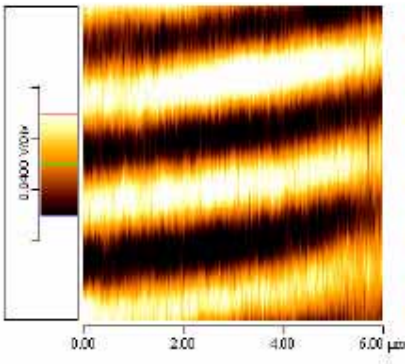

Fig. 3. EFM image of Au (111) surface with patterned cholesteryl derivatives. Scan size=6×6μm^2.

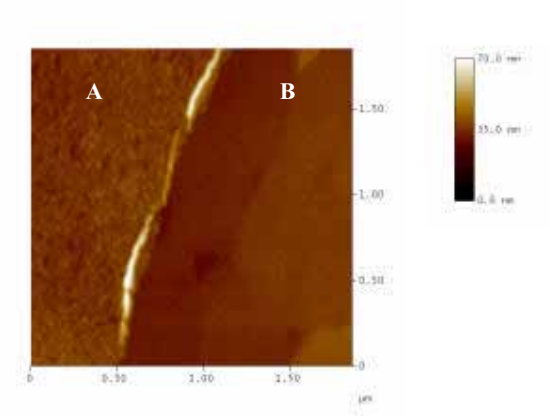

Fig. 4. Fluid TM-AFM image of an Au (111) surface with the tethered membrane (in water). Region "A" corresponds to the lipid bilayer while region "B" to the tethering layer of cholesteryl derivatives.

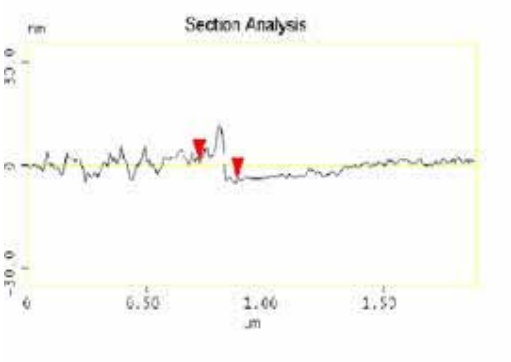

Fig. 5 The cross-sectional profile of the sample imaged in Fig. 4 reveals a difference in height of ~50 nm consistent with a phospholipid bilayer . Scan size=1.75×1.75 μm^2.

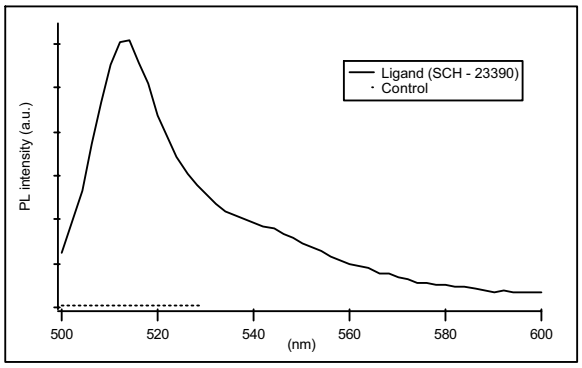

Fig. 6 Photoluminescence (PL) spectrum of the biomimetic membrane prepared with liposomes only (control) or with D1 Receptor containing liposomes, after incubation with the antagonist Bodipy SCH-23390.

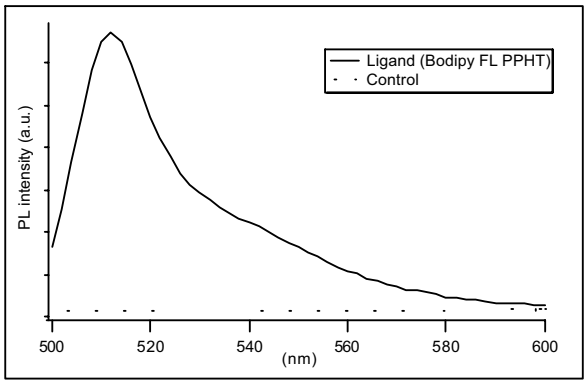

Fig. 7 Photoluminescence (PL) spectrum of the biomimetic membrane prepared with liposomes only (control) or with D2 Receptor containing liposomes, after incubation with the antagonist Bodipy FL PPHT.

REFERENCES

[1] H.S. Bracha, D.J. Seitz, J. Otemaa and S.D. Glick, Rotational movement (circling) in normal humans: Sex difference and relationship to hand, foot and eye preference. Brain Res., 411, 231, 1987.

[2] P.F. Spano, S. Govoni and M. Trabucchi, Studies on the pharmacological properties of dopamine receptors in various areas of the central nervous system, Adv. Biochem. Psychopharmacol., 19, 155, 1978.

[3] D.R. Sibley and F.J. Monsma, Molecular biology of dopamine receptors, Trends Pharmacol. Sci., 13, 61, 1992.

[4] A. Meco, A. Alessandri, N. Locuratolo, A. Rum and F. Quaranta, Nuovi neurolettici "atipici": interazioni con il sistema extrapiramidale ed effetti collaterali. Giornale di Neuropsicofarmacologia, Anno XXII, n.1/2000

[5] P. Seeman and H.H.M. Van Tol, Dopamine receptor pharmacology. Curr. Opin. Neurol. Neurosurg., 6, 602, 1993.

[6] G. Ciccarella, A. Maffei, G. Vasapollo, L. Blasi, D. Pisignano, R. Rinaldi, Use of cholesteryl polysulfides in self-assembly and soft lithoghaphy on Au (111) and ITO, Appl. Surf. Sci. 2005 (In press).

[7] A. Sidhu, Solubilization and reconstitution of the D1 dopamine receptor: potentiation of the agonist high-affinity state of the receptor, Biochem., 27, 8768,1988.

[8] P. Balen, K. Kimura and A. Sidhu, Specific Phospholipid Requirements for the Solubilization and Reconstitution of D1 Dopamine Receptors from Striatal Membranes, Biochem. 33, 1539, 1994

[9] E. Sinner and W. Knoll, Functional tethered membranes, Curr. Opin. Chem. Biol., 5, 705, 2001.

[10] A.T.A. Jenkins, R.J. Bushby, N. Boden, S.D. Evans, P.F. Knowles, R.E. Miles, Q. Liu and S.D. Ogier, Ion-selective lipid bilayers tethered to microcontact printed self-assembled monolayers containing cholesterol derivatives, Langmuir, 14, 4675, 1998.

[11] N. Boden, R.J. Bushby, S. Clarkson, S.D Evans, P.F. Knowles and A. Marsh, The design and synthesis of simple molecular tethers for binding biomembranes to a gold surface, Tetrahedron, 53, 10939, 1997.

[12] H. Takano, S. Wong, J.A. Harnish and M.D. Porter, Mapping the subsurface composition of organic films by electric force microscopy, Langmuir 16, 5231, 2000.

[13] L.A. Kung, J.T. Groves, N. Ulman and S.G. Boxer, Printing via Photolithography on Micropartitioned Fluid Lipid Membranes, Adv. Mater., 12, 731, 2000.