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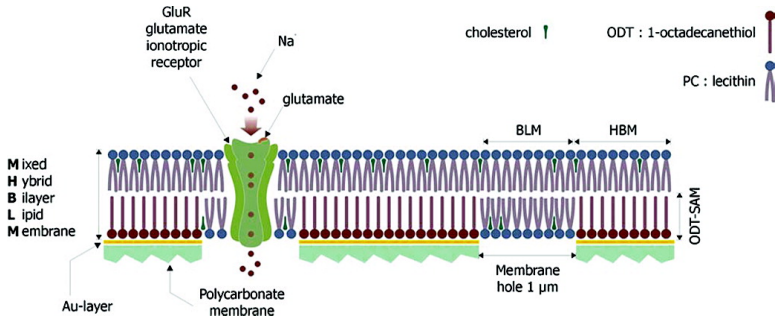

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Glutamate Receptor Incorporated in a Mixed Hybrid Bilayer Lipid Membrane Array, as a Sensing Element of a Biosensor Working under Flowing Conditions

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Abstract: The realization of a reliable receptor biosensor requires stable, long-lasting, reconstituted biomembranes able to supply a suitable biomimetic environment where the receptor can properly work after incorporation. To this end, we developed a new method for preparing stable biological membranes that couple the biomimetic properties of BLMs (bilayer lipid membranes) with the high stability of HBMs (hybrid bilayer membranes); this gives rise to an innovative assembly, named MHBLM (mixed hybrid bilayer lipid membrane). The present work deals with the characterization of biosensors achieved by embedding an ionotropic glutamate receptor (GluR) on MHBLM. Thanks to signal (transmembrane current) amplification, which is typical of natural receptors, the biosensor here produced detects glutamate at a level of nmol L^{-1}. The transmembrane current changes linearly vs glutamate up to 100 nmol L^{-1}, while the limit of detection is 1 nmol L^{-1}. In addition, the biosensor response can be modulated both by receptor agonists (glycine) and antagonists (Mg^{2+}) as well, and by exploiting the biosensor response, the distribution of different kinds of ionotropic GluR present in the purified sample, and embedded in MHBLM, was also evaluated. Finally, one of the most important aspects of this investigation is represented by the high stability of the biomimetic system, which allows the use of biosensor under flowing conditions, where the solutions flow on both biomembrane faces.

Introduction

In biological systems, the cellular membranes play a key role in signal trasduction: this attracted the increasing interest of researchers working on biosensors technology; clearly, the perspective of a successful utilization of cellular membranes and of proteins embedded therein as substitutes of traditional biological components used so far (enzymes, antibodies, whole cells, tissues, etc.) is highly attractive.[1−4]

Biological receptors are important because they act as (i) natural targets for toxins and (ii) mediators in physiological processes.[5] Therefore, they can be successfully employed for identifying and quantifying several substances of biological, clinical, and environmental interest and applied in the development of specific drugs as well. In addition, the biosensors based on embedded receptors may be utilized in the study of real time receptor−ligand interactions.[6]

To gain molecular recognition, receptors must be incorporated in a reconstituted biomembrane able to mimic the natural environment in which the selective interaction with substrate produces a membrane disruption that can be electrochemically trasducted,[1,2,7−12] as in the case of ion-channel-linked receptors, where the specific ligand−receptor interaction transiently opens or closes the ion gate, thus changing the membrane ion permeability.[13]

In the last years, various natural receptors, including glutamate ionotropic receptor,[14−19] nicotinic acetylcholine receptor ion-

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channel,[20,21] Na^+/D-glucose cotransporter,[22] lactose/H^+ cotransporter,[4,23] Na^+,K^+-ATPase,[24] and DNA,[25] have been tested as sensing elements. Semiartificial receptors were also obtained by modifying well-known peptides such as Val[26] and GramD[27] [widely used for testing reconstructed bilayer lipid membranes (BLMs)] achieving biosensors for detecting DNA[28] or antibodies.[13,29] Receptor-based biosensors were also obtained by embedding fully artificial receptors into BLMs.[30]

Studies on receptor-based biosensors are mainly focused on practical aspects, including analytical applications, or shed deeper light on yet partially unknown working mechanisms. One example is the application of the receptor for insulin,[31] and more complex systems are based on the nicotinic receptor for acetylcholine.[32,33] However, at present the glutamate receptor is surely one of the less known systems, even if highly intriguing, being very important in the neurobiology field.

Glutamate is the main excitatory neurotransmitter of the central nervous system of vertebrates,[34] being involved in fundamental mechanisms of learning, memory, formation, and plasticity of the synaptic endings and in the development of the central nervous system.[35] Glutamate and all its analogous species, such as kainic acid, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA), and *N*-methyl-D-aspartate (NMDA), are also powerful neurotoxins,[36,37] and the disfunction of glutamate receptors (classified in Figure 1) is probably implicated in serious neurological disorders, including epilepsy, ischemic stroke, Parkinson's and Alzheimer's diseases, and Huntington's chorea.[38−41]

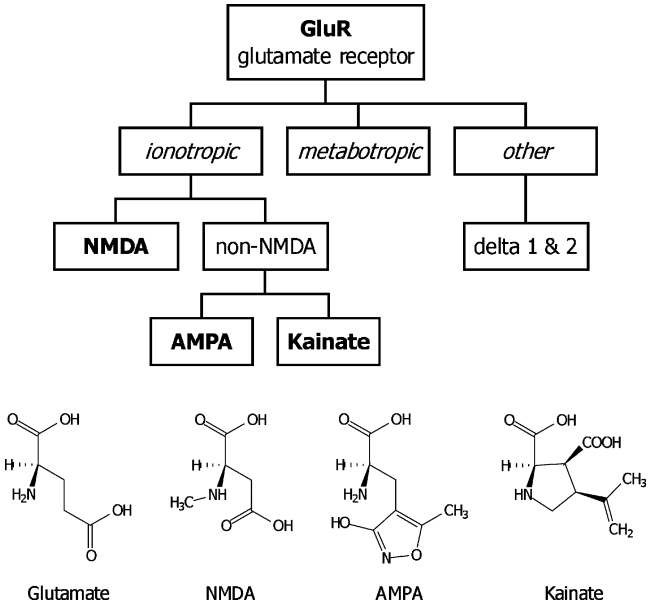

Figure 1. Classification of different types of glutamate receptors based on the most active ligand to which they are bound and the chemical structures of the ligands.

The modulation of different substrates on the glutamate receptor (GluR) response is quite complex and not fully understood so far. For example, the NMDA receptor is a complex and intriguing macromolecule: opening of the intrinsic ion channel requires simultaneous binding of the coagonists glutamate and glycine to unique recognition sites.[34,42] The NMDA receptor ion-channel is permeable to cations, primarily to Na^+, K^+, and Ca^{2+}, but the ions influx is blocked by extracellular Mg^{2+}.[43] The use of ion-channel receptors (particularly GluR) as biological components of biosensors has been pioneered by Umezawa and co-workers 15 years ago.[15] In particular, they defined the first example of biosensor for glutamate based on the glutamate ionotropic receptor embedded in a reconstituted biomembrane;[14] this system, working in batch, displayed a remarkable amplification of the signal, allowing a limit of detection for glutamate as low as 3×10^{-8} mol L^{-1}. Despite the interest in developing such kinds of biosensors, only a few research groups have been engaged till now on this matter, and a lot of work still remains to be done, particularly on practical applications.

The need to have long-lasting and biomimetic reconstituted biological membranes, where receptors can be hosted in, is the main drawback preventing the development of such kind of biosensors. However, although several approaches[44] have been attempted in biomembrane reconstitution, they can be grouped essentially in two alternative approaches: unsupported BLMs (highly biomimetic but low stability) or supported hybrid bilayer membranes (HBMs) (highly stable but poorly biomimetic).[45]

Different attempts have been made in order to stabilize BLMs.[46−49] Worth of note are those ones based on microporous

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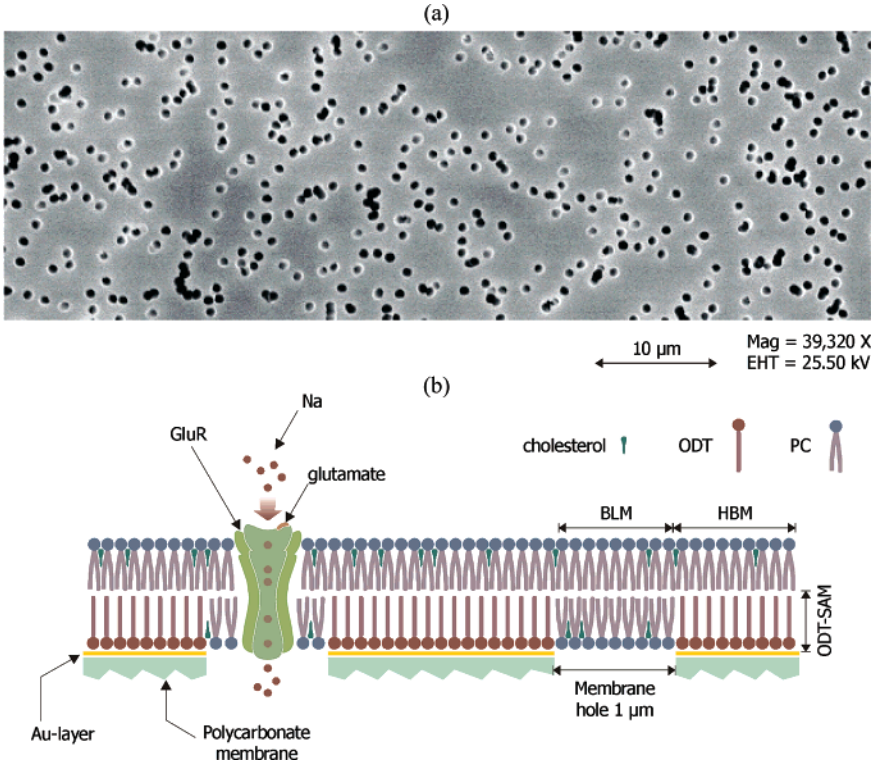

Figure 2. MHBLM array obtained by coupling a HBM structure to several BLMs working in parallel: (a) SEM image (obtained in secondary electron with an electron beam of 25.5 kV, magnification 39 000×) of ODT-SAM on Au-covered polycarbonate membrane showing regular holes of 1 μm diameter; (b) scheme of MHBLM incorporating GluR.

membranes,[50,51] like polycarbonate,[47,52] glass fiber,[46−48,52,53] or others[54−56] sometime following rather different approaches. Nikolelis et al.[46−48,52−56] also used such kinds of membranes with an approach making it possible to adopt flowing conditions for detection. In this approach, the authors essentially coupled microporous membranes to a classical BLM assembled on a SaranWrap film hole. The solution was flowed only on the microporous membrane side opposite to that one facing the BLM (motionlessly), so that the substrate reached the BLM by diffusing through the membrane pores.

At the beginning, we tried to prepare stable and reliable reconstituted BLMs through classical approaches, such as BLM, HBM, and salt-bridge HBM,[54,55] but no noteworthy result was achieved with respect to the state-of-art.

Hence, to couple the long-term stability of HBM with the biomimetic properties of BLM, we devised an innovative system based on the use a multiporous polycarbonate membrane (Figure 2a) suitably covered with an ODT-SAM (octadecanethiol self-assembled monolayer)-supporting Au layer. Such an assembly, named MHBLM and depicted in Figure 2b, consists of many (an array) BLMs (self-assembled in correspondence to membrane holes embedded in a HBM-type structure. Such a system has been developed[57] and improved[58] over the past few years by using oligopeptides (such as Val and GramD, widely described in the literature[27,59,60]) as testing probes.

In the present paper, we report the incorporation of a natural receptor in the MHBLM (Figure 2b), working as a glutamate receptor-based electrochemical biosensor.

The aim of the present work is (i) to obtain a GluR-based biosensor, (ii) to use it under flowing condition, (iii) to detect the receptor's agonist at very low concentration (by exploiting the typical signal amplification of natural receptors), and (iiii) to study the effect of receptor's coagonist and antagonist, in the perspective of future applications of the device.

Experimental Section

Materials. All reagents were of analytical grade unless differently indicated. Ethanol 95%; *n*-hexane; isobutyl alcohol; and lithium, ammonium, and sodium chloride were obtained from Carlo Erba (Milan, Italy). 1-Octadecanethiol (ODT, 95%) for GC and valinomycin were supplied by Fluka (Buchs, Switzerland). L-α-Phosphatidylcholine (PC, 99%) (also called lecithin) from fresh, frozen egg yolk, GramD, and tri-hydroxymethylaminomethane (Tris) were purchased from Sigma (St. Louis, MO). Polycarbonate membranes characterized by regular holes (diameter 1 μm, hole density 10^5−10^8 pores cm^{-2} according to pore size), were obtained from Whatman (Kent, England). Ultrapure deionized water from a Millipore Milli-Q system ($\rho \geq 18$ MΩ cm) was used throughout the experiments.

Instruments. Reconstruction of MHBLM and experimental measurements were performed by a custom-made apparatus called a

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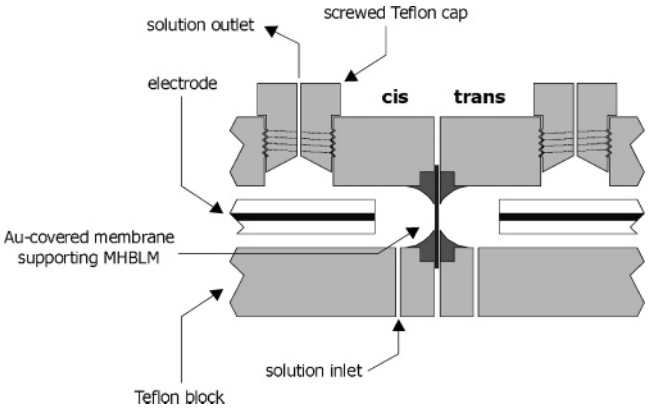

Figure 3. Assembly of measurement apparatus (bicell). Different electrodes were employed for measurements: Au electrodes were used for continuous conductance measurements, while Ag,AgCl/Cl^- electrodes were used for step-by-step voltammetric measurements.

"bicell", whose assembly was described in previous papers.[57,58] In this research, the bicell was further improved (see Figure 3 for details) in order to carry out both MHBLM reconstruction and experimental measures, under flowing conditions.

Deposition of a gold layer onto one side of the polycarbonate membranes was performed by a PS3 coating unit (Agar Aids).

For conductance measurement, two Au electrodes (2 mm diameter), cleaned as previously described,[61] were plugged in the bicell and connected to an Amel model 160 conductivity meter (Amel Instruments, Milan, Italy). Data were continuously recorded on a personal computer by custom-made connecting cable and software. Conversely, for voltammetric measurements, the electrodes were replaced by two custom-made Ag,AgCl/3 mol L^{-1} Cl^- electrodes in contact with cis and trans solution through 3 mol L^{-1} NaCl salt bridges, and data were recorded with a Princeton Applied Research potentiostat/galvanostat model 273 (EG&G, Princeton NJ), driven by a PAR 270 electrochemical software.

All experiments were carried out inside a well-grounded Faraday cage (40 × 40 × 40 cm) to avoid electrical interference with the bicell placed on an antivibrating support to avoid mechanical shocks. A Crison pH-meter, model 2002 (Crison, Allela, Spain), was used for pH measurements.

Procedures. Extraction and Purification of GluR. GluR was extracted and purified from a natural source, according to a modified procedure reported in the literature,[62−64] and it entailed the following steps: homogenization of rat brains, ultracentrifugation, affinity chromatography, dialysis, and concentration. The GluR so obtained, was finally identified by SDS-PAGE (sodium dodecyl sulfate polyacrilamide gel electrophoresis) and quantified by Bradford assay. Details of different steps are here described.

Preparation of Homogenate. Eight brains from 30−45-day-old male rats (Sprague−Dawley) were taken and homogenized (tissue homogenizer GHL-OMNI-international) in sucrose (0.32 mol L^{-1}), $CaCl_2$ (0.05 mmol L^{-1}), and HEPES (0.2 mmol L^{-1}) in the presence of proteases inhibitors (Roche cat. #1697498). The homogenate was centrifuged for 5 min at 1000*g* and the supernatant was collected. The pellet was washed once more in the same buffer to improve the recovery, centrifuged as above, and finally discarded. The resulting supernatants were combined and centrifuged at 38 900*g* for 15 min, and the pellet was lysed by osmosis in 10 mL of Tris/acetate (1 mmol L^{-1}, pH 8).

The suspension was stratified on 10 mL of 1.2 mol L^{-1} sucrose in 1 mmol L^{-1} HEPES (pH 7,4) and centrifuged at 302 000*g* for 30 min. The gradient interface, containing synaptic plasma membranes (SPMs) and myelin, was taken, diluted to 10 mL, and layered on 0.9 mol L^{-1} sucrose in 1 mmol L^{-1} HEPES (pH 7,4). A further centrifugation at 302 000*g* for 30 min allowed myelin to be discarded at the interface while the pellet (SPMs) was dissolved in 30 mL of 10 mmol L^{-1} (pH 7) phosphate buffer (PB) + sodium cholate 2% w/w in the presence of proteases inhibitors.

Affinity Batch Chromatography. About 30 g of glass fibers was ground, washed abundantly with hot concentrated HNO_3, and rinsed first with water and then with acetone and finally once again with water. Glass fibers were mixed with 25 mL of 22% bovine albumin, 3.5 mL of 25% glutaraldehyde solution, and 84 mL of 10 mmol L^{-1} (pH 7) PB and incubated at −30 °C overnight.[65] The frozen cake was defrosted, washed with PB, and incubated again with 150 mL of 0.05 mol L^{-1} sodium glutamate + 3% glutaraldehyde at 0 °C for 24 h. The obtained stationary phase was then washed with 100 mL of PB + 2% cholate and stored at 0 °C until use.

The pellets previously obtained were resuspended in 30 mL of PB + 2% w/w sodium cholate solution, added to the affinity chromatographic support, and incubated at 0 °C for 24 h. The stationary phase was then washed with 50 mL of PB + sodium cholate 2% in the presence of proteases inhibitors and dried by filtration (at 4 °C in a thermostated room). GluR was recovered by treating the stationary phase with 100 mL of PB + 2% sodium cholate + 1 mol L^{-1} KCl (always in the presence of proteases inhibitors) at 0 °C overnight.

The eluted solution was dialyzed at 0 °C until the absence of chlorides was detected and then concentrated to a volume of 3 mL by ultrafiltration at 0 °C (molecular weight cutoff = 20 kDa). The GluR solution was divided into 150 μL (2.1 μg μL^{-1}) aliquots and store at −20 °C.

Detection and Quantification of GluR. GluR was detected by gel electrophoresis (SDS-PAGE, Laemmli method[66]), comparing the R_f for the obtained protein (70−80 kDa) to that one of some selected standard in the range 30−120 kDa [phosphorylase B (112 kDa), BSA (84 kDa), ovoalbumine (53.2 kDa), carbonic anhydrase (34.9 kDa), tripsine inhibitors (28.7 kDa), lisozime (20.5 kDa)]. Acrylamide concentration was 2.5% in the gel (or in the stacking gel) and 12% in the separation gel, and the electrophoretic run was performed by a Mini-protean II, starting with 100 V. The applied potential was then raised to 180 V, when the proteins entered the separation gel. After the proteins separation, the gel was treated with 0.1% Coomassie Brilliant Blue R (water:methanol:acetic acid = 5:5:2) for 30 min and then it was destained by immersion in water:methanol:acetic acid = 8:1:1. In Figure 4 an example of the electrophoretic detection of isolated GluRs is reported.

Quantification of GluR was performed by the Bio-Rad Protein Assay Kit (based on the Bradford assay[67]). Protein content in the examined solution was calculated using a calibration curve obtained by plotting the absorbance values (at λ = 595 nm) of differently concentrated protein standard (bovine serum albumin) solutions.

MHBLM Formation. MHBLM spontaneously self-assembles over a ODT-SAM according to the "dilution procedure" described in detail in previous papers.[57,58] In the present work, cholesterol was introduced as a component of the membrane to be reconstituted; in particular, the solution used to form MHBLM was 25% w/w in cholesterol and 75% w/w in phosphatidyl choline (in *n*-hexane/isobutyl alcohol 10:1). Thus, after having spread 100 μL of MHBLM-forming solution onto ODT-SAM, the bicell was closed and rapidly turned to the horizontal position. Immediately, both compartments of the bicell were simultaneously filled (the volume of each compartment is approximately 2 mL) with Tris

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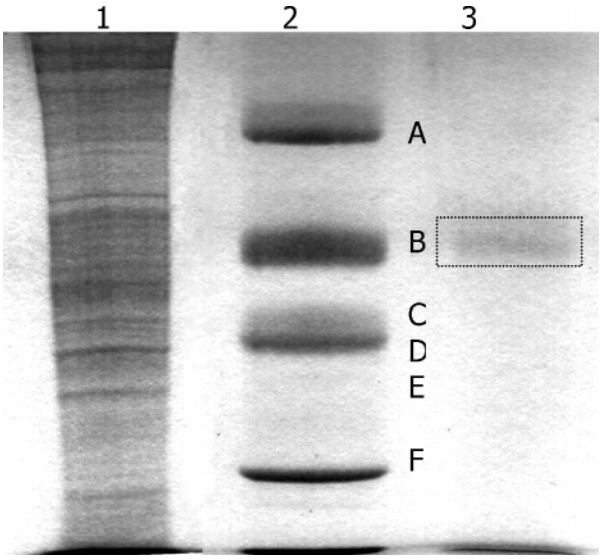

Figure 4. SDS-PAGE electropherograms of (1) brain extract before affinity chromatography, (2) calibration standards, (3) GluR after purification procedure. Standards used: A, phosphorylase B (112 kDa); B, BSA (84 kDa); C, ovoalbumine (53.2 kDa); D, carbonic anhydrase (34.9 kDa); E, tripsine inhibitors (28.7 kDa); F, lisozime (20.5 kDa).

buffer by means of a peristaltic pump operating at a flow rate = 0.5 mL min^{-1}. After formation, the MHBLM was allowed to stabilize for at least 30 min and then 50 mL of Tris was pumped (flow rate = 0.5 mL min^{-1}) in the bicell.

Incorporation of GramD and Val. GramD was incorporated simply by diffusion in previously formed MHBLMs. GramD (25 μL of 2 mmol L^{-1} ethanolic solution) was added to Tris buffer solution (≈2 mL) used for MHBLM. In particular both cis and trans bicell compartments were filled with 25 μmol L^{-1} GramD in Tris buffer and left to stand for 30 min. This time is sufficient to get GramD incorporation into biomimetic structures such as MHBLM.[8,27,68] Conversely, incorporation of Val into MHBLM was obtained simultaneously to the MHBLM self-assembling. The phospholipid−cholesterol solution made 1 mg mL^{-1} in valinomycin was spread on the SAM, and after that, the dilution step was carried out.

Incorporation of GluR. GluR is incorporated in the dilution step during MHBLM self-assembling. In particular, an aliquot of GluR was added to 3 mL of aqueous Tris buffer (final concentration of the purified GluR ≈ 100 μg mL^{-1}) that was flowed into the cis compartment; in the meantime, into the trans compartment was flowed the same volume of Tris buffer. Afterward the flow was stopped for approximately 0.5 h to let self-assembling of a stable MHBLM embedding GluR occur.

Measurements. All conductance measurements (by conductivimeter) were carried out under flowing conditions: solutions of different concentrations of the same components were flowed in both compartments at the same flow rate (0.5 mL min^{-1}) in such a way as to have rigorously the same hydrostatic pressure in both compartments to avoid membrane breaking. Although this approach could be less accurate due to the presence of undesired contributions, we previously confirmed its reliability by voltammetry.[57]

Measurements on the GluR−MHBLM system were carried out step-by-step by cyclic voltammetry (CV), scanning the potential at 2 mV s^{-1} in the range ±50 mV, with respect to the equilibrium value. The solutions flowing in the cis compartment differed by those flowing in the trans compartment by the presence of the analyte/s to be studied at suitable concentration/s. The flow was stopped and the CV was recorded.

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Table 1. Conductance Values, Measured in Tris Buffer, after MHBLM Formation and after Addition of Na^+ (Final Concentration 10 mmol L^{-1}) and Average Lifetime for MHBLMs Containing Different Percentages of Cholesterol

PC/cholesterol ratio	conductance value (μS cm^{-2})		average lifetime (min)
	initial	after Na^+ addition	
PC only	17−25	25−50	30−40
6:1	17−25	25−50	30−40
5:1	13−17	25−35	75−85
4:1	7−13	17−21	200−220
3:1	1−6	4−8	>600
2:1	13−17	21−30	60−70

Results and Discussion

To be successful, our investigation requires reconstituted biomembrane that must be characterized by (i) good biomimetic properties, which provide a suitable environment for a correct behavior of the embedded GluR, and (ii) enhanced stability, which enables the system to be used under flowing conditions.

We previously demonstrated that the MHBLM approach[57,58] confers interesting features to biomembranes; nevertheless, further improvement is possible if some experimental parameters are optimized; we cite here (i) the biomembrane composition, which can be changed by varying the components used for membrane assembly, (ii) the apparatus geometry, and (iii) the experimental procedure followed for MHBLM assembling.

It is known that cholesterol is an important constituent of natural biological membranes.[8,69] Its addition to the phospholipid solution used for biomembrane reconstitution, it is expected to improve the membrane stability. To optimize the PC/cholesterol ratio, solutions with different PC/cholesterol ratios were used for MHBLMs assembling, and the electrical insulating properties of the membranes were tested as evaluating criteria. To this issue, the conductance was first measured in Tris buffer (just after MHBLM formation; initial conductance) and then after Na^+ (up to 10 mmol L^{-1} as final concentration) was added to the solution. The lower the transmembrane conductance, the better the MHBLM properties (i.e., higher blocking feature and longer lifetime).[57,58]

The results obtained at different PC/cholesterol ratios are reported in Table 1: they show that the best results are achieved at 3:1 PC/cholesterol ratio. It is noteworthy that, in this case, the initial conductance value, measured just after MHBLM formation, is the lowest, so it remains even after Na^+ addition, as expected for an efficient (highly blocking) membrane. The lifetime of different MHBLM was also simultaneously evaluated; it appears evident that the use of a solution with 3:1 PC/cholesterol guarantees a good stability of the MHBLMs for at least 600 min (as said, stability is a primary requirement for reliable applications). The membrane stability was tested within 1 day, so longer lifetimes could not be excluded a priori. Since longer lifetime was observed in experiments carried out on GluR-MHBLMs (see below), the 3:1 (PC/cholesterol ratio) was adopted in our experiments.

Besides the biomembrane composition, the experimental procedure used for its assembly was determined. In previous works (mainly operating in static solution), the membrane reconstruction[57,58] was carried out by manual addition of known volumes of Tris buffer solution into both bicell compartments,

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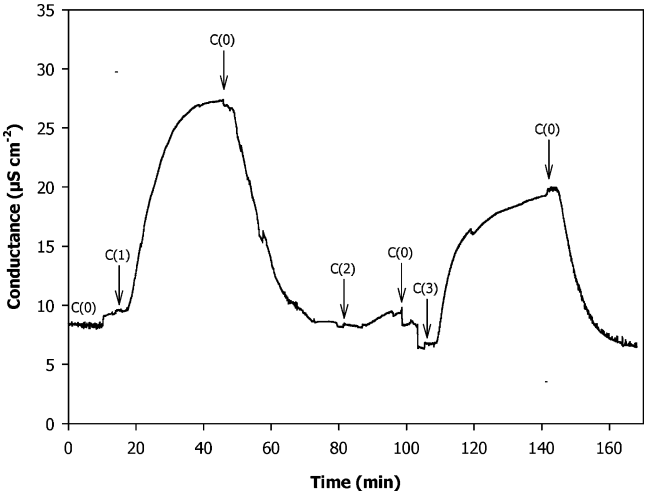

Figure 5. Conductance (measured by conductivimeter) of GramD-MHBLM as a function of different 4 mmol L^{-1} ions present in Tris buffer flowing into cis and trans bicell compartments. Experiment starts by flowing into bicell compartments only Tris buffer [C(0)], and then flowing solution composition was changed by adding different ions [C(1) = NH_4^+, C(2) = Li^+, C(3) = K^+] to Tris buffer; between one ion and the next, the bicell was washed by flowing Tris buffer alone.

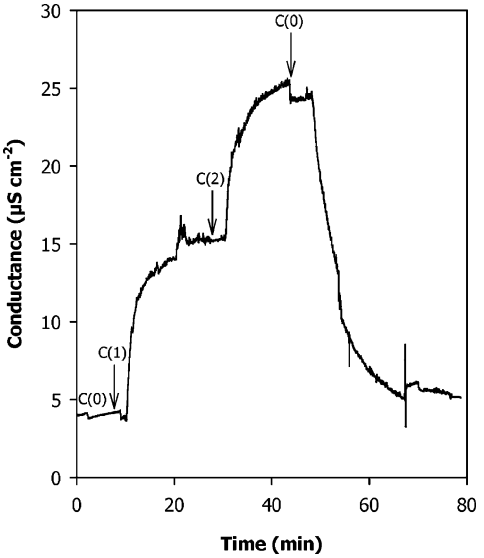

Figure 6. Conductance (measured by conductivimeter) of Val−MHBLM as a function of the K^+ concentration present in Tris buffer flowing into cis and trans compartments. The experiment starts by flowing into bicell compartments only Tris buffer [C(0)], and then Tris buffer flowing in two compartments was made first 20 mmol L^{-1}[C(1)] then 40 mmol L^{-1} [C(2)] in K^+. At the end, Tris buffer alone was once again flowed.

paying attention to maintain the same hydrostatic pressure. In this work, the bicell was modified to make possible adopting flowing conditions (see Figure 3); here, during membrane reconstruction buffer solution was added by controlling the flow inside the bicell (as described in detail in the Experimental Section). With respect to previous work, this approach increases the percentage of success in MHBLMs reconstitution. It must be considered that addition of aqueous buffer under controlled flowing conditions is much more reliable and reproducible than the manual method adopted earlier. In particular, during MHBLM formation the level of solution inside cis and trans bicell compartments is always the same, as well as the hydrostatic pressure on both membrane faces; this favors membrane self-assembling and avoids membrane breaking. This accounts for a successful MHBLM reconstruction percentage close to 100% and appears of great importance, particularly when expensive and/or hard to extract receptors are to be incorporated. Contrary to previous reports, in the present approach, the flowing solutions are in direct contact with both sides of the biomimetic membrane.

The behavior of reconstructed biomembranes under flowing conditions was investigated by measuring the conductance of the solutions flowing in cis and trans bicell compartments, properly separated by MHBLM incorporating GramD or Val (two probes largely utilized to check reliability reconstructed biomembranes).[27,59,60]

The results obtained for GramD-MHBLM and Val-MHBLM under flowing conditions are shown in Figures 5 and 6. Conductance measurements were carried out in a continuous direct mode as described in the Experimental Section.

Figure 4 shows the conductance of GramD-MHBLM as a function of time when different solutions flow in the bicell compartments. Once GramD is incorporated, the signal is stabilized at low conductance values due to the base electrolyte (Tris buffer) flowing through the cell compartments. When the flowing solution contains also NH_4^+ (NH_4Cl), the conductance value raises as GramD ion-channels in the MHBLM can be permeated by NH_4^+. Conversely, no significant conductance change is detected when NH_4^+ is added in the absence of GramD, which indicates that the MHBLM preserves its blocking feature. When the Tris buffer (without NH_4^+) is once again flowed, the conductance comes back to the initial value, indicating that MHBLM is not damaged by the flowing solution. A similar behavior is observed when either Li^+ or K^+ (at the same concentration) is flowed; however, the conductance attains a different value according to the selectivity coefficient of GramD toward these ions.[70]

The behavior of GramD−MHBLM provides evidence that, in correspondence of polycarbonate membrane holes, true BLMs (not extended or multilayer ones[50]) are formed. The length of GramD molecule is the same as that of phosphatidilcholine molecules forming the biomembrane layers; thus, an ion channel crossing the bilayer, through which NH_4^+ ions (or other permeating ions) can go through (transmembrane current), can be formed only when two GramD molecules (present in two layers and able to move laterally in it) are aligned.[27] If double bilayers or multibilayers would be formed, even assuming that GramD is present in every layer, the probability that $2n$ (with $n \geq 2$) GramD molecules in $2n$ layers would align to form an ion channel would be extremely low, just like the transmembrane current.

Figure 6 shows the conductance of a Val−MHBLM vs the concentrations of K^+. It can be observed that in the flowing solution the conductance changes proportionally to the K^+ concentration; moreover, the signal rapidly raises when the potassium ion is introduced in the bicell, until the conductance reaches a stationary state. When the solution composition is reverted to Tris buffer, the system shows itself as reversible and the conductance value goes back to its initial value, showing again that the MHBLM structure is conserved.

Results shown in Figures 5 and 6 are just given to show that, thanks to its very high stability, MHBLM preserves selectivity of GramD and Val incorporated under flowing conditions. In

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addition, as far as the selectivity is concerned, our findings are practically identical to those obtained for the same peptides under static conditions.[57,58] The figures also show that some time is needed to detect a change of the signal after the solution is changed; this is the time needed for solution exchanging in both tubing and in the cell compartments (approximately 2 mL volume).

Flowing conditions give some advantages versus static (batch) conditions. In fact, in the case of a biomembrane unable to operate under flowing conditions, the reversibility of the signal is usually checked[27] upon dilution of the analyte in the cell. This is mandatory, since it is hard (when operating in batch conditions) to achieve in the cell a complete change of the solution without destroying the reconstructed biomembrane. Conversely, in our measurements the ion concentration can be changed (and it is possible to revert to the initial conditions in the presence of the supporting electrolyte alone), preserving the biomembrane functionality. In addition, satisfactory results are obtained when the same biomembrane is used to check different analytes, the only limitation being given by the membrane stability.

The results above-reported demonstrate that MHBLMs (and modified MHBLMs reconstructed as described in this research) are highly stable and reliable. Moreover, further experiments carried out by leaving the cell under flow during the time it is working and stopping the flow overnight have shown that MHBLM preserves its blocking features up to 6 days later. These features are very encouraging in view of further practical applications based on embedded receptors.

Incorporation of peptides/receptors inside biomembranes may be achieved by different procedures. Concerning the insertion into MHBLM of GramD and Val (see also Experimental Section), the former is easily incorporated in assembled biomembranes by diffusion, while the latter is added at the beginning of the MHBLM assembling procedure. This means that Val has to be present in the phospholipid + cholesterol solution spread onto the ODT-SAM. Another possibility is the fusion between biomembranes and liposomes,[8] even if in some cases the process could be unwieldy.[71,72] In our research, purified GluR, available as frozen aqueous solution aliquots (at the concentration of 2.1 μg μL^{-1}), was incorporated during the dilution step. In particular, one aliquot (150 μL) was added to the Tris buffer (volume 3 mL) and then flowed into the cis compartment during the membrane self-assembling procedure; simultaneously the same volume of Tris buffer was flowed in the trans compartment. As the bicell compartments becomes full, the peristaltic pump was stopped and the system stabilized for 1 h. During this period, the biomembrane self-assembles, squeezing away organic solvent residues,[73] and GluR incorporation occurs. After that, stabilization was achieved, the flow was restarted, and the Tris buffer flowed in bicell compartments; after 20 min, GluR−MHBLM was ready to be used. The GluR−MHBLM reconstruction is a highly successful process (>80%).

The GluR insertion in MHBLM (see Experimental Section) was investigated by then use of a conductivimeter: this is a not accurate approach (due to the presence of undesired contributions) but was used as an up-front method to check membrane stability and GluR incorporation, under a variety of experimental conditions. Hence, the GluR−MHBLM system was investigated by CV (as described in Experimental Section).[74] Figure 7 shows the signal recorded in the absence (broken line) or in the presence of 50 nmol L^{-1} glutamate (solid line), and the slope of the curves gives the transmembrane conductance. Reported in the figure are the regression curves fitting three CV runs, which were recorded according to a proper setup (described in the Experimental Section) from which conductance values were evaluated. It appears evident that in the absence of glutamate, the system is blocked, despite the presence, in both cell compartments, of 50 mmol L^{-1} Na^+ (able to permeate the ion-channel formed by "open" glutamate ionotropic receptor). This demonstrates that the MHBLM is correctly formed, and glutamate receptors are "closely" positioned.

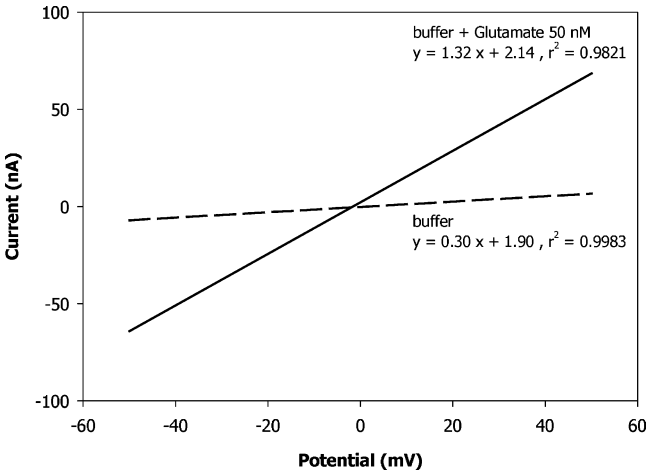

Figure 7. Regression curves fitting CVs recorded on GluR-modified MHBLM, without (dotted line) and in the presence (solid line) of 50 nmol L^{-1} glutamate into the solution flowing in the cis bicell compartment; Tris buffer + 50 mmol L^{-1} Na^+ (NaCl) was always present in solution flowing in cis and trans compartments.

By contrast, at very low glutamate concentration (data of Figure 7 refer to 50 nmol L^{-1} glutamate), the receptors turn to the "open" condition, and Na^+ is able to pass through the membrane; an increased current is therefore detected. According to ionotropic receptors properties, even a very low agonist concentration (glutamate in this case) it is able to open the receptor ion channel and let a large number of ions to pass through. This entails a signal amplification, of interest in view of possible analytical applications.

The reversible behavior toward glutamate of the MHBLM-embedded GluR further confirms that true bilayers are really formed in correspondence of membrane holes, as already evidenced by experiments with GramD. The transmembrane current (proportional to the glutamate concentration) that is detected when the glutamate is present in the cis compartment indicates that Na^+ can cross the bilayer through the ion channel of GluR activated by the interaction with glutamate. No current would be detected in the presence of double or multibilayers, since both the size and structure of GluR would not allow it to make contact in two compartments.

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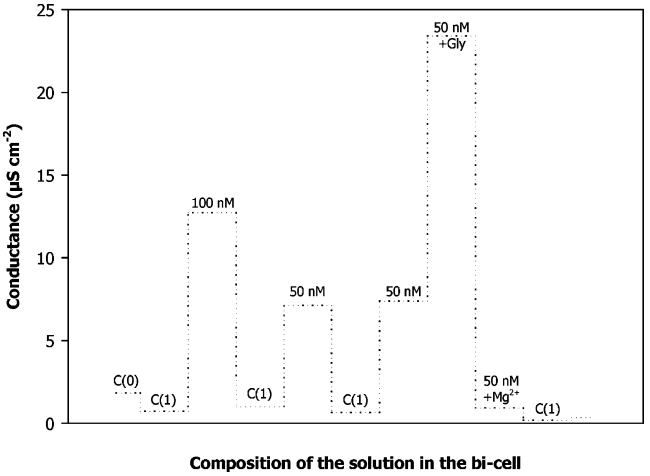

Figure 8. Successive conductance values, measured by CV, for GluR-modified MHBLM on differently concentrated glutamate solutions flowing into the cis bicell compartment in the presence and absence of glycine (50 nmol L^{-1}) or Mg^{2+} (50 nmol L^{-1}). Either Tris buffer [C(0)] or Tris buffer plus Na^+ [C(1)] was flowed in cis and trans compartments. C(0) = Tris buffer only, C(1) = Tris buffer + 50 mmol L^{-1} Na^+. Agonists (50 or 100 nmol L^{-1} glutamate and 50 nmol L^{-1} glycine) and antagonist (50 nmol L^{-1} Mg^{2+}) flowed in cis compartment were always dissolved in C(1) solution.

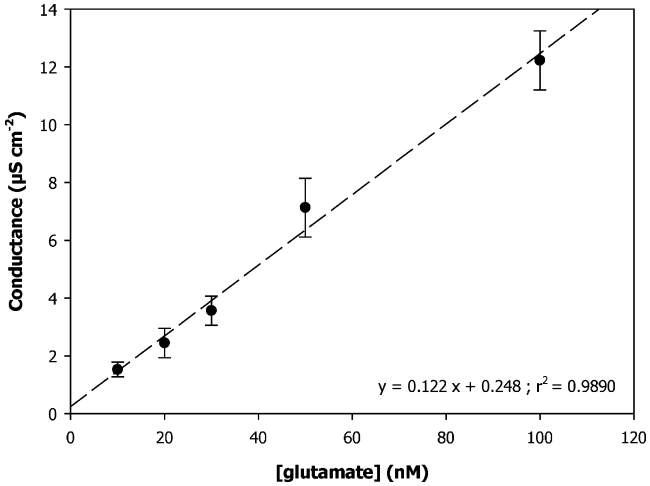

Figure 9. Glutamate calibration graph recorded under flowing conditions by CV of GluR-modified MHBLM. Other experimental conditions are the same as reported in Figure 7.

Figure 8 shows the conductance values at varying composition of the flowing solution into the bicell. As mentioned above, at the beginning of the experiment [C(0)] the membrane conductance is very low, even in the presence of 50 mmol L^{-1} Na^+ [composition C(1)], as expected for a blocking system. Then, when the ionotropic channels get "open" due to the addition of 100 nmol L^{-1} glutamate, and an increased conductance value is detected. Moreover, if the buffer solution [composition C(1)] missing the glutamate is again pumped into the bicell, the system displays a reversible behavior and the conductance comes back to the initial value, suggesting that the channels turn to the "closed" position. Upon changing the flowing solution with another containing 50 nmol L^{-1} glutamate, the signal gets stronger again. Withdrawal of glutamate reverts the system to initial conditions.

These results obtained indicate that (i) GluR−MHBLM shows a reversible behavior (the agonist−receptor reversible interaction is driven by mass action), (ii) the system is suitable to detect glutamate at a nmol L^{-1} concentration level (thanks to the amplifying capability of natural receptors), and (iii) response is proportional to the analyte concentration, as shown by the calibration curve (see Figure 9, where each point is the average value of at least three replicates and reported with its standard deviation). These features are of relevance for reliable receptor-based biosensors.

The linearity range extends from 10 to 100 nmol L^{-1} (which represents the highest value checked), and the limit of detection (calculated as three times the noise) is 1 nmol L^{-1}, 1 order of magnitude lower than that reported by Umezawa et al.,[14,15] who utilized GluR embedded in classical BLM and worked in batch.

Despite the exciting result of determining concentrations as low as nmol L^{-1} glutamate using a simple electrochemical system, the main interest in such kind of biosensor is the study of those substrates modulating the receptorial activation (response) as toxins or drugs. To this end, the response of GluR−MHBLM toward Mg^{2+} (antagonist) and glycine (coagonist) was taken into consideration.

As shown in Figure 8, addition of 50 nmol L^{-1} glycine to glutamate (50 nmol L^{-1}) enhances the signal (more than twice), in agreement with the coagonist role played by glycine for NMDA-type GluR.[34,43,75,76] The graph also shows that system reversibility is preserved against coagonist action. As above-mentioned, such a behavior is interpreted through the different action of glutamate and glycine toward the each kind of MHBLM-embedded GluR.

We remind the reader that only GluRs able to interact with glutamate by affinity chromatography are isolated. Thus, although all MHBLM-embedded GluR can interact with glutamate, ion channel activation occurs (or not) according to the GluR properties.

In particular, the interaction with glutamate is sufficient to open the ion channel of non-NMDA GluR type; by contrast, the NMDA receptor requires also a simultaneous interaction with glycine.[34,43,75,76] Really, this last issue is much more complex: several additional processes may be involved, including desensitizing phenomena, due to glutamate and glycine.[77] In particular, in the continuous presence of glutamate, the NMDA receptors response is diminished in a time-dependent fashion that reflects negative allosteric coupling between the glutamate and glycine binding sites. At high glutamate concentration, desensitization is manifest as a decrease in glycine affinity. Alternatively, at high glycine concentration (in dialyzed cells or excised membrane patches), the NMDA receptor response is desensitized approximately 50−80%.[78]

Since we operated at relatively low glutamate and glycine concentration, it is our opinion that the above-mentioned model should fit the behavior of our GluR−MHBLM system, in consideration of its extreme simplicity in comparison with cellular membranes (where additional processes may take place).

Our data allow us to evaluate the abundance of different kinds [NMDA (N) and non-NMDA (nN)] of MHBLM-embedded

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GluR. Indicating as "R_a" the GluR−MHBLM response when only glutamate is present in the solution of the cis compartment and as "R_b" the conductance when glycine is also present at the same glutamate concentration, we should be able to evaluate the contribution of different kinds of receptors to the response. In truth, R_a should be ascribed to the nN only, while R_b should be attributed to all GluRs; therefore, the $R_b - R_a$ difference should represent the current change due to N receptor activation. By assuming the same output response factor for both receptors, the following relationship can be written:

$$\frac{R_a}{R_b - R_a} = \frac{[\mathrm{nN}]}{[\mathrm{N}]}$$

This relationship, applied to the data of Figure 8, allows one to gain the distribution of NMDA and non-NMDA MHBLM-embedded receptors: in particular, from the ratio determined (0.43), we obtain ≅30% for non-NMDA vs ≅70% for the NMDA type.

Finally, the inhibitory effect of Mg^{2+} (antagonist for GluR) was investigated. As said above, Mg^{2+} can penetrate the open NMDA channel but when inside it blocks the ion channel itself. As shown in Figure 8, by flowing a 50 nmol L^{-1} Mg^{2+} solution in cis compartment when all receptor channels are open, the conductance falls down to an initial value, thus showing that channels are blocked. If Mg^{2+} is removed, the conductance change remains negligible. This last observation indicates that Mg^{2+} acts as an inhibitor for GluR, but it cannot be rapidly removed from the channel by simple mass action.[76] At present, Mg^{2+} is considered a channel blocker only for the NMDA (but not for non-NMDA) receptor; thus, the addition of Mg^{2+} to a fully activated NMDA and non-NMDA mixture (as assumed for our system) should lower the conductance to that value reached in the presence of the activated non-NMDA-type only (being NMDA-blocked). Our findings are in disagreement with this view, since we observe a complete blockage of the system. At present, we cannot explain such behavior. Anyway, we are aware that GluR behavior is not fully understood due to the extreme complexity of this matter. On the other hand, our data may be of help to better elucidate these very complex biochemical processes.

Conclusions

The work presented here is an end point of our previous investigation on biomembrane reconstruction; at the same time, it represents a starting point for future research both theoretical studies and practical applications.

In particular, the following relevant results have been achieved:

(i) The described experimental procedure allows assembling of stable, reliable, and biomimetic reconstituted biological membrane practically without failures. The system is long lasting (up to 6 days) and stable enough to make possible its use even under flowing conditions (both aspects are very important in view of practical applications).

(ii) Conversely to previous approaches reported in the literature, where the solution at the best flowed on one side of the system, for the first time, here the solutions flow on both biomembrane faces.

(iii) MHBLM allows profitable incorporation of natural proteins, despite their size ("large" natural proteins require much space on both sides of the membrane), preserving their natural functionality, in contrast to other stable systems (as HBM or tethered BLM), which provide space only at one side, the other acting as an interface to the supporting system.

(iv) GluR, extracted from a natural source and properly purified, was incorporated in MHBLM and constitutes a biosensor exploiting the features of a natural receptor (such as high signal amplification, able to detect nmol L^{-1} glutamate concentration levels) and showing high reversibility (mass-action driven); to our knowledge this is the first example of a biosensor, working under flowing conditions, based on a natural receptor embedded in a reconstituted biomembrane.

(v) This biosensor, being based on an array of micro-BLMs embedding GluRs, allows the use of a quite common potentiostat/galvanostat for measurements in place of much more specialized instrumentation (e.g., patch-clamp amplifiers).

(vi) Analysis of the biosensor response vs agonist (glutamate), coagonist (glycine), and blocker (Mg^{2+}) demonstrates that embedded GluR preserves the features typical of a natural receptor.

(vii) The simplified system here defined appears suitable for evaluating the distribution of different kinds of GluRs present in the purified sample. Our GluR sample seems to be constituted mainly by NMDA type (about 70% of total); this is of interest in view of a possible biochemical application of the system (clearly, additional experiments with other substrates such as NDMA, AMPA, or kainic acid should be carried out for further confirmation).

These results show as the system is very promising for applications in analytical chemistry, neurobiology, health, and medicine. In particular, the flowing approach should allow continuous measurements of important physiological parameters and flow injection analysis as well, even though further improvements (reducing, for example, the volume of cell compartments) will be necessary. In addition, thanks to the possibility to flow solutions on both membrane sides, this system makes it possible to mimic what occurs in cellular membranes embedding integral proteins. This is very important for basic biochemical studies, as for instance for clarification of working mechanisms of membrane proteins.

Acknowledgment. The authors are greatly indebted both to Prof. Ingeborg Grgurina (Dipartimento di Scienze Biochimiche "A. Rossi Fanelli", Università di Roma "La Sapienza") and co-workers for their collaboration in proteins' detection and quantification and to Prof. Roberto Santucci (Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università di Roma "Tor Vergata") for critical reading and revision of the manuscript. This work was carried out thanks to financial support of MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca) of Italy.

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