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Immobilization of Active Facilitated Glucose Transporters (GLUT-1) in Supported Biological Membranes

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ABSTRACT

Membrane fragments or membrane proteins within a lipid mixture were immobilized over metal electrodes. This procedure has been developed to study biological membranes without interferences from cell machinery. To obtain a smooth hydrophilic biomembrane support and a mode of binding of the membrane, either a crosslinked gel or an aromatic polyamine–polymer doped with avidin was deposited at the metal electrode by electropolymerization. This layer (less than 10 nm thick) also served as a submembrane compartment. The facilitated glucose transporter (GLUT-1) purified from human erythrocytes was integrated into a lipid membrane containing artificial biotinylated lipids and reacted with the activated surface of the glucose sensitive electrode. It was demonstrated that the lipid layer was attached to the polymer-containing avidin and could only be removed by detergent extraction. The presence of an active membrane transporter was demonstrated by electrochemical detection of glucose in the submembrane compartment, and by inhibition of glucose transport with the specific inhibitor Cytochalasin-B.

Index Entries: Biomembrane; facilitated glucose transporter (GLUT-1); avidin/biotin; polymer supported membrane.

INTRODUCTION

Biological membranes are highly selective, but rather unstable. Stabilization by immobilization can be of great help for membrane-based biosensors and for membrane research in general *(1–3)*. Like liposomes *(4)*, immobilized membranes should have a more or less enclosed submembrane compartment to study membrane transport processes. However, it is very difficult to obtain large membrane patches without any leakage *(5)*. To perform membrane transport experiments, at least analyte diffusion through pores and cracks should be smaller than transport effects to be studied. Unlike liposomes, which form spontaneously from biological membranes immersed in an aqueous environment, flat membrane patches have to be attached chemically to a solid support *(6)*. Alternatively, membrane patches can be composed of entirely artificial components, chosen to meet certain chemical requirements.

One way to test transmembrane flux is to construct a patch clamp-like design measuring membrane-penetrating ions caused by a change in transmembrane current or potential *(7,8)*. Another way to test membrane transport and to construct a sensor-like device is to immobilize an enzyme specific for the membrane-penetrating substance at an electrode, where an electrochemical response can be detected at a certain applied potential. Without the membrane barrier, a variety of biological substances yield a very high unspecific background of oxidation current. To meet these problems of interfering signals and electrode fouling (which arise when complex solutions have to be analyzed that contain physiological compounds), a variety of diffusion-limiting polymers had been tested, but none of them was selective for glucose only. For the electrochemical determination of glucose within the submembrane compartment, glucose oxidase (GlOx) was immobilized at the sensing electrode in a polymer matrix. This well-established technique was used to avoid interferences from the analyte detection system. The response in this type of electrochemical electrode is generated by the reduction of hydrogen peroxide, which is produced during the oxidation of glucose together with gluconic acid. To quantify the number of membrane defects, the unspecific flux of an ionic compound like ascorbate was monitored by electrochemical oxidation at the submembrane electrode.

The study done intended to find new methods useful for stabilizing membrane fragments on artificial supports, and as an alternative for studying membrane effects without interferences from cell metabolism or receptor effects. As a novel approach for studying membrane proteins in general, and for more selective glucose measurements in particular, we propose here to deliver the analyte to the immobilized enzyme via a transport protein for glucose (GLUT-1 from human erythrocytes), which is embedded in an artificial lipid membrane. We demonstrate the suppression of nonspecific signals,

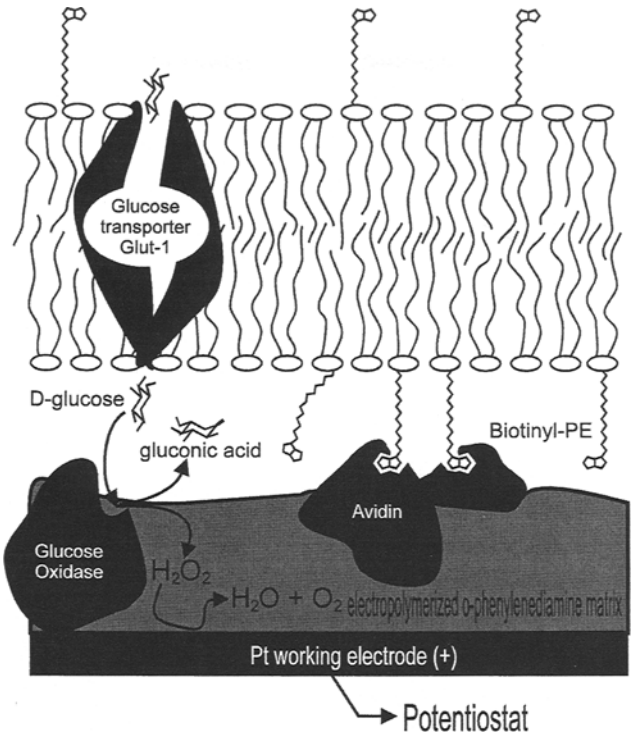

Fig. 1. Glucose transporter-enriched membrane patch attached to a *o*-phenylene diamine polymer on a electrochemical sensor chip.

which implies an increase of the glucose selectivity. The scheme of the sensor setup is shown in Fig. 1.

The present study describes the attachment of an erythrocyte-derived membrane containing the well-studied glucose transporter to an electrochemical glucose sensor and its use for a more selective detection of glucose. This technique is not limited to the sugar transport system, but may lead to biosensors designed to detect molecules that can interact with membrane receptors.

MATERIALS AND METHODS

Chemicals

GlOx (EC 1.1.3.4., *Aspergillus niger*) was purchased from Boehringer Mannheim (Mannheim, Germany), *o*-phenylenediamine (Fluka, Buchs, Switzerland) was recrystallized twice from dichloromethane. During the first dissolving step, activated charcoal was added to remove oxidation products. All other chemicals used were purchased from Fluka, Aldrich

(Vienna, Austria) Merck (Darmstadt, Germany), or Sigma (St. Louis, MO) in analytical grade.

Sensors were produced by lift-off photolithography evaporating platinum on polished Ti-activated Al_2O_3-wafers. The electrodes were conditioned by cyclovoltammetry in 0.5 *M* H_2SO_4 for more than 15 cycles, stored under water, and washed in HCl for 30 min just before use. The working electrodes were 1 × 1.8 mm in size.

Protein and Lipid Assay

Proteins within the lipid vesicles were quantified by a Lowry assay which had been modified for membrane proteins *(19)*. The amount of lipid in the vesicles was determined from dry wt.

Immobilization via Electropolymerization

For the immobilization of GlOx three different methods were tested:

1. Crosslinking of 10% w/v GlOx and 10% w/v avidin in a 3% w/v solution of glutaraldehyde (GA). Without applying an electrical potential, this solution was left on the electrodes for 15 min. Afterward, the crosslinking solution was rinsed off and electropolymerization was performed as described at the bottom of this page.
2. Adsorption from a solution containing 10% w/v GlOx, and 15% w/v avidin at the electrode for 30 min without *o*-phenylene diamine present. The immobilization mixture was then made 15 m*M* in *o*-phenylene diamine, and the potential for polymerization was applied.
3. Adsorption of the proteins in the polymerization mixture at the electrode for 30 min followed by electropolymerization of *o*-phenylene diamine.

All solutions were 100 m*M* in sodium phosphate and 10 m*M* in potassium chloride at pH 7.5.

With slight modifications, electropolymerization of *o*-phenylene diamine on the protein-covered platinum electrodes was performed as described in refs. *(9–12)*. In brief, the electrodes were covered with 1 mL of a solution that was 20 m*M* in *o*-phenylene diamine. The concentration of avidin or streptavidin in this solution ranged from 5 to 15% w/v avidin. In experiments with glutaraldehyde crosslinking, this buffer was made 1% w/v of GlOx. As soon as *o*-phenylene diamine was present, a N_2 atmosphere was maintained to avoid oxidation of the monomer.

Electropolymerization was performed by cyclovoltammetry at a potential of –100–850 mV at a scan speed of 19 mV/s. After 10 cycles, the difference of the current at 850 mV between two subsequent cycles was

lower than 0.2 mA and the maximum resistance increased from 34 kOhm in the first cyclovoltammogram (CV) to more than 90 kOhm in the last CV.

Lipid Preparations

Total lipid extract from human erythrocyte ghosts was added to 10 ml of suspended ghost membranes (4–6 mg/mL protein) 37.5 mL CH_3OH: $CHCl_3$ (2:1), and the mixture was shaken at 4°C for 1 h. Phase separation (into two clear phases and a turbid interphase) was achieved by centrifugation at 12,000*g* (Sorvall SS-34) for 20 min. The supernatant was then extracted again with 47.5 mL of CH_3OH:$CHCl_3$:H_2O (2:1:0.8), and the mixture was centrifuged. Twenty-five mL of $CHCl_3$ and 25 mL of H_2O were added to the combined extracts and the mixture was centrifuged. The lower phase was withdrawn into a sealable bottle and dried under N_2 at room temperature. The dried lipid extract was kept under N_2 at –20°C.

Membrane Biotinylation

For the introduction of biotin sites, we first used the biotinylation of transporter-containing vesicles. This was carried out using sulfo-*N*-hydroxy succinimide esters of *N*-biotinyl caproic acid *(13)*. However, the best results in binding the vesicles to the sensor were achieved when biotinylated phosphatidylethanolamine was mixed into the GLUT-1-containing vesicles by sonication and a subsequent freeze–thaw step.

Purification and Reconstitution of Purified Transporter into Lipid Vesicles

Purification of the glucose transporter was done using the protocol given by Baldwin et al. *(14)*. Outdated human-blood erythrocyte concentrate was obtained from the local blood bank. After purification of the transporter, reconstitution into vesicles was achieved by dialysis of a concentrated detergent solution. In order to introduce biotinylated lipids into these vesicles, a $CHCl_3$ solution of the desired lipid composition was dried to a homogenous film under N_2. Usually, a lipid extract from human erythrocytes with or without biotinylated dioleoyl-phophatidylethanolamine was used to prepare the sensor membranes. Vesicles were prepared by adding 0.1 *M* sodium phosphate buffer, pH 7.0 or a GLUT-1-containing vesicle suspension, to the dried film and suspending the vesicles by gentle agitation. In order to obtain vesicles of uniform size and composition, sonication was performed in a bath sonicator for 5 s, followed by a freeze–thaw step *(15–17)*. This suspension was applied as a droplet to the sensor surface. Usually, 20 min of incubation was enough to observe the effects caused by the coating of the sensor with a lipid membrane. Before measurements were carried out, the vesicle suspension was care-

fully removed, while paying attention to avoid turbulences at the wet electrode. According to atomic force micros-copy (AFM) studies, a few vesicles remain as intact spheres, but they are destroyed during the first scan.

The relative response to glucose depended on the quality of the transporter purification. Sensors produced from the same batch of vesicles did not differ by more than 10% in their glucose response.

Langmuir-Blodgett Technology

All experiments were performed on a Nimia trough (Teflon) *(18)*. The pressure was measured by a Wilhelmy balance (int. standard ISO 304 model 601S), using EtOH soaked and ultrapure H_2O washed filter paper plates provided by Nimia. The trough had a cover to minimize evaporation and was placed underneath a Plexiglas shielding to avoid dust and air circulation. The trough was cleaned with $CHCl_3$, EtOH, and ultrapure water. When proteins were used in an experiment, the trough was cleaned with 4 *M* urea, water (5–7 times), and by the usual solvent cleaning procedure. Solvents were of HPLC grade (Merck). The temperature was adjusted to 21°C by a cryostat 1 h before each measurement. After filling the trough with ultrapure water, a compression was performed to detect any impurities on the trough. If the pressure change was less then 0.2 mN/m, the experiment was performed without further cleaning. Lipids were dissolved in $CHCl_3$ and small droplets of the lipid solution were spread at the surface of the trough. After evaporation of the solvent (10 min), the film was compressed using a barrier speed up to 20 cm^2/min.

Electrochemical Assay Conditions

The glucose response was measured in 100 m*M* sodium phosphate buffer, pH 7.4 *(20)*. All solutions containing glucose, cytochalasin-B, or ascorbate were prepared in this buffer. Cyclovoltammetry was simultaneously performed on unmodified platinum and GlOx-containing electrodes between –200 and +200 mV in a 1 mL cell. Exchange of solutions was usually done by pipeting directly into the cell. During pipeting, a slight disturbance of the CV was observed, which is caused by the disturbance of the diffusion equilibrium. However, the shape of the CV was reestablished during the next two cycles. To monitor the effects of an attached membrane, the sensors were always characterized before and after the coating with lipids.

RESULTS AND DISCUSSION

Lipid Composition

In order to find a proper lipid composition for the vesicles to be used for coating, and to test the transporter activity upon integration into the

artificial membrane, isotherms for different lipid mixtures were measured on a Langmuir-Blodgett trough. The fusion of transporter-containing vesicles was performed subsequently. Lipid films consisting of dipalmitoyl phosphatidyl ethanolamine (DPPE), DPPC (DPP-choline), and biotinylated PE can be exposed to a maximum pressure of around 60 mN/m before they collapse. One can recognize a small step on the isotherms of DPPE/DPPC/B-DOPE films, when they are compressed through the phase transition.

According to the literature, this high pressure implies a good stability for these films on a planar surface. Since the transporter was reconstituted into vesicles, which have an optimal curvature for their lipid composition, we also studied the isotherms for a $CHCl_3$ lipid extract from erythrocyte ghost membranes.

Although the maximum pressure was about 20 mN/m lower than in films made from DPPC, DPPE, or B-DOPE (indicating that the film cannot occupy its physical state driven by thermodynamics on the planar surface of the LB trough), this extract from erythrocytes (EE) was suitable for the following reasons: First, because of the steep pressure increase for the extract (compared to the synthetic mixtures), we assume that the lipids are not uniformly distributed on the surface after their application to it, but rather form islands structures (*see* Fig. 3). This attribute can be an advantage when fusion of vesicles should occur. Previous studies showed that membranes composed of many different lipids also have a better flexibility, and therefore render a higher activity of proteins integrated into the membrane *(21–24)*. Second, we assume that a plasma membrane-like lipid mixture can more easily adopt to different curvatures on a surface like our sensor, which is not perfectly smooth *(25)*.

For a film consisting of erythrocyte extract and biotinylated DOPE (30 mol%), we observed a step at a phase-transition pressure typical for a pure B-DOPE film (at 46.9 mN/m). This indicated that a small area of B-DOPE monolayer was formed between the EE-islands (therefore, the slope of the pressure area isotherm is less steep, compared to the EE isotherm, Fig. 3). When compressing further, the pressure in the liquid increased. At about 52 mN/m, the film collapsed.

If the content of biotinylated lipids was lower than 20%, a step at the transition point was no longer observed in the isotherm. To introduce the GLUT-1 into the supported membrane, we investigated the fusion of transporter-enriched vesicles into the layer. We performed these experiments at a pressure around 20 mN/m. Within this pressure range of the isotherm, any addition of material caused a significant pressure change (the slope was already very steep). Although there was a low surface pressure, vesicles (internal surface pressure of vesicles depends on lipid composition and curvature) are able to fuse into the lipid membrane (vesicles have to fuse

against the surface pressure). The amount of fusion depended on the batch of blood used to purify the transporter. The formation of the lipid layer was investigated by transmission electron microscopy (TEM). Upon application of vesicles, a pressure increase was measured that was proportional to the amount of vesicles injected. Vesicles that simply adsorbed to the planar layer (causing a pressure increase) were not detected in TEM, and therefore supported the argument that fusion had occurred (integrated GLUT-1 was used as marker protein; data not shown). After the fusion experiment, when the film was stable again, glucose was injected into the subphase to a final concentration of 1 m*M*. A further pressure increase was measurable. Since this effect can only be observed when the transporter is present, we concluded that we actually monitored the conformational change of the transporter caused by the binding of glucose (Fig. 2). Since we calculated a transporter:glucose ratio of more than 2, this effect might also be enhanced because of a physical phase change in the lipid film induced by the conformational change of the transporter, or by a selective interaction of glucose with the transporter. According to these data, we were able to show that the transporter is active in a membrane of this composition.

Supported Lipid Membranes

To obtain a stable membrane, the first and basic development was to compare several methods to achieve a stable linkage between the lipid membrane and the solid sensor support. In general, these experiments aimed to covalently bind lipid-like long C-chains to the surface. Since the GlOx has to be placed underneath the lipid membrane, we first attempted to modify the surface of the polymer where GIOx was immobilized. Two strategies to form the polymer support proved to be most useful: polyaddition and electropolymerization.

Supported Lipid Membranes Prepared by Polyimine Polyaddition

A variety of polymers had been used to fill casted microwells on thin-film sensors. Because of easy and quick procedures, gels were prepared by polyaddition, which is not blocked by free oxygen as most polymerizations of, e.g., acrylic compounds are blocked.

Nevertheless, crosslinking with glutaraldehyde is a well-established method for immobilization of proteins and various ligands; the chemical reactions are not well understood and mainly simplified as linear imine bonds or polymers. Coupling of amines with glutaraldehyde starts at a above pH 5.0 and is optimal at pH 7.0–8.0. A further increase in pH does not only increase imine bond formation, but significantly promotes autopolymerization of glutaraldehyde by aldol formation, introducing very unstable aldol linkages. Incubation of monoamines with glutaraldehyde at a pH around

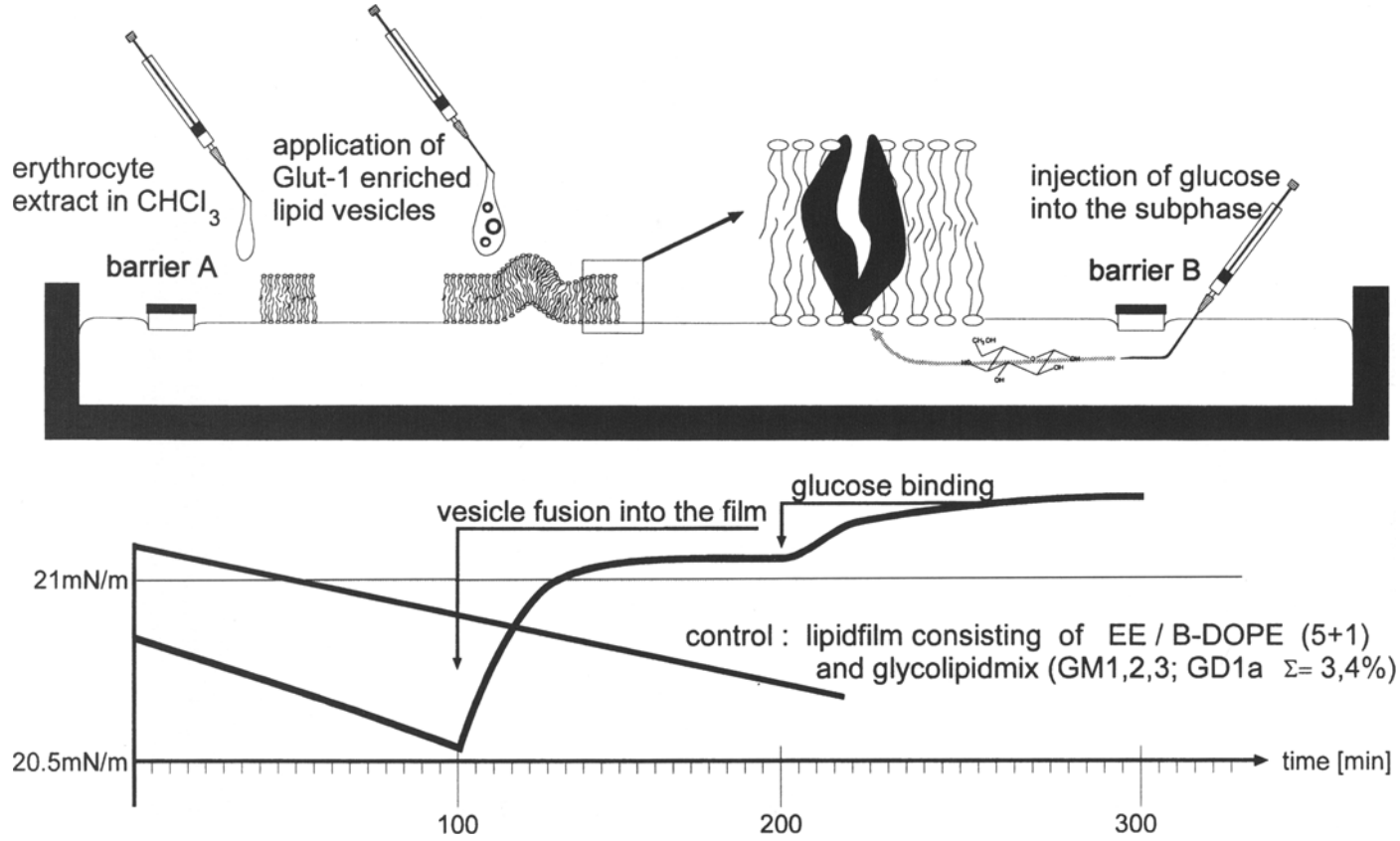

Fig. 2. Monitoring a conformation change in a facilitated glucose transporter-containing membrane caused by the binding of glucose to specificity sites (erythrocyte lipid film on an LB-trough).

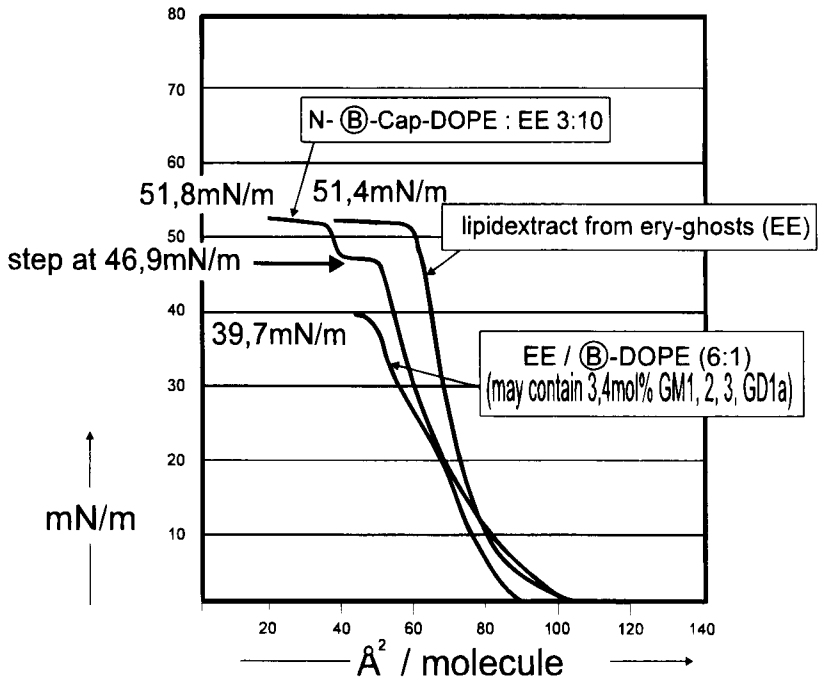

Fig. 3. Pressure/area isotherms on water at 21°C of a lipid extract from erythrocyte-ghosts, 40 µL (4,986 10^{-8} mol, average MW: 596,81) B-Cap-DOPE + ery-extract, 30% + 70% (2,672 10^{-8} mol + 6,233 10^{-8} mol).

7.0–9.0 did not result in a small molecule (created by coupling of two amines to glutaraldehyde via imine bonds), but resulted in high mol wt polymers. Size distribution of polyimines was determined by HPLC size-exclusion chromatography on TSK-GSWP 2000 or 3000. Using a spectrophotometric assay, the ratio of aldehyde to amine was found to be 1:1, instead of 2:1. This, and the spectrum (and color) of the polymer, clearly indicated the formation of five- or six-membered rings. IR-spectroscopy indicated OH at 3400 cm^{-1}, HC = C at 3050 cm^{-1}, CH_2 at 2920 cm^{-1}, CH_2-N at 2850 cm^{-1}, COH at 1710 cm^{-1}, C = C-C-N at 1650 cm^{-1}, C = C and NH at 1590 cm^{-1}, CH_2 at 1450 cm^{-1}, and OH at 1390 cm^{-1}. Free aldehyde, having strong absorption bands around 1750 cm^{-1}, was nearly invisible in high mol-wt products.

High mol-wt linear polymers were synthesized from ethanol amine, amino butyric acid, amino capronic acid, aniline, and all amino acids. Using amino acid precursors, pseudo-protein polymers were synthesized. Polymerization of amino acids with glutaraldehyde was achieved with (in order of decreasing polymerization speed) Ser, Cys > Gly, Lys, Arg > Phe, Thr, Met > Val, Leu, Ile, Trp, His, Asp, Glu, and Gln.

Based on these results, the use of diamines should produce crosslinked gels instead of linear polymers. This hypothesis was verified using

diamines of butane and hexane. Crosslinked, brown hydrogels were obtained between pH 4.0 and 10.0. After a primary crosslinking step, the gels absorb oxygen from air, changing their color and absorption around 620 nm. The oxidized polymer exhibited increased stability with respect to hydrolytic cleavage. The polymer matrix is rather stable in aqueous buffers, diluted acids, alkaline solutions, and organic solvents. At a very high pH, the gel collapses by further crosslinking. Prolonged exposure to high concentrations of hydrogen peroxide attacked the polymer backbone, which was monitored by bleaching of the brown polymer color. Reduction of imine bonds to amine bonds by sodium borohydride was only achieved, with soluble polymer precursor bleaching the dark brown color of the solution. Incubation of the crosslinked polymer gels with $NaBH_4$ did not change the dark brown color nor the chemical properties of the gel matrix.

Glutaraldehyde/diamine gels were cocrosslinked with various enzymes and proteins (GlOx, glutamate oxidase, glutaminase, papain, lipase from Candida rugosa, avidin, and so on) and proved to be a highly stable support for protein immobilization and stabilization.

Two matrices were chosen for most of the experiments: diamino hexane/glutaraldehyde and a lysine/polylysine/glutaraldehyde copolymers. Thus, because of the use of diamines, about 10 min after mixing, a highly crosslinked hydrogel was formed. After limited hydrolysis, free amino groups were used for the modification of lipid binding. Reactions with palmitoyl chloride and an activation via *N*-hydroxy succinimide proved to be useful. Gels were either applied with a micropipet by spin coating or using a well structure produced by photolithography of a polyvinyl-pyrrolidone/bisazide or a photoresist film.

GlOx was polymerized, in a crosslinked gel adding a 0.1% solution to the polymerization cocktail. The enzyme was active within the polymer matrix for more than 3 mo of continuous measurement.

To obtain a multipoint attachment of the lipid membrane to the gel support, well-defined anchor points were introduced by avidin/biotin technology, and proved to be advantageous. Incorporation of avidin was achieved in a way similar to GlOx and tested by the interaction of avidin with biotin-labeled peroxidase.

Vesicles used for coating the sensor contained biotinylated phosphatidyl ethanolamine lipids. Avidin/biotin provided a ligand-binding strength that was almost covalent-like. Furthermore, a 8-C spacer between the biotin and the phosphatidyl ethanolamine (PE) headgroup facilitated the adaptation of the lipid anchor to the laterally diffusing lipids in the membrane. The scheme of the sensor's architecture used was similar to the one shown in Fig. 1.

Using chemical crosslinking with amino groups of the polymer matrix, we observed attachment of lipid membranes to the gel surface.

Nevertheless, the sensor characteristic was not satisfactory because of a large number of cracks and pores. Data gained by incorporation of avidin within the polymerization mix of glutaraldehyde and diaminohexane proved that the stability of the attached lipid film significantly increased, compared to the chemical coupling approach. Also, these gels exhibited extraordinary properties for immobilization of enzymes in various biosensors; the use of ultrathin nonswelling layers would increase reproducibility and stability of the lipid membrane layer.

Supported Lipid Membranes Prepared by o-Phenylene Diamine Electropolymerization

To produce ultrathin stable layers with thickness in the nm-range, we tested electropolymerization of different compounds. Because of the fact that polypyrrole, polythiophene, and polyaniline gave very rough structures, we switched to nonconducting polyamines and phenols. The electropolymerization of *o*-phenylene diamine resulted in a very thin and homogenous film. Combining this electropolymer with avidin resulted in a new support for the attachment of biomembranes doped with biotinylated lipids. Membranes were prepared as described in Materials and Methods.

To quantify the leakage of the supported lipid membranes, we measured different concentrations of ascorbate, which penetrates very slowly through intact lipid membranes. We chose ascorbate instead of, e.g., uric acid or cysteine, because it is the compound that is responsible for most of the glucose sensor background in biological fluids. As seen in Table 1, this signal can be significantly reduced regardless of the presence of GLUT-1 in the membrane.

To produce a smooth surface at the sensor, we electropolymerized *o*-phenylene diamine while adsorption of the proteins to the electrode took place. The electropolymerization of *o*-phenylene diamine, resulted in a very thin and homogenous film of 3–10 nm thickness (according to atomic force microscopy [AFM] studies). This is an important advantage of this polymer, because avidin (or streptavidin) are not covered under such a thin film, and the binding sites for biotin are therefore free and accessible.

Immobilization of GlOx and avidin was done using the three different methods described in Materials and Methods. On sensors with the glutaraldehyde-crosslinked GlOx, we observed a higher sensitivity to glucose, compared to the sensors in which adsorption was used to immobilize the enzyme. This was due to a higher amount of enzyme which was entrapped. The characteristics after the attachment of lipid vesicles without GLUT-1 were similar for both methods. When GLUT-1-enriched vesicles were used for coating, we measured a higher response on sensors

Table 1
Electrochemical Oxidation of Ascorbate on Polymer-Coated Platinum Eletrodes

Sensor chip	Electrochemical background	Electrochemical response to ascorbate	Change in capacitance in %
Without lipid coating	25	52	
After lipid coating (with or without GLUT-1 transporter)	12	22	133%

Lipid coating was performed using a lipidextract from erythrocyte and B-DOPE (70:30 mol%); vesicle suspension in 0.1*M* Na-phosphate buffer, pH 7.4; GLUT-1 vesicles contained ~115 μg of protein and ~700 μg of total lipid (85:15 mol%).

bearing the glutaraldehyde-crosslinked enzyme than before the coating. AFM analysis showed that the crosslinking step with glutaraldehyde resulted in a very rough surface, where large aggregates were observed. Results indicated that the surface structure was not suitable for the attachment of a lipid membrane.

Consequently, GlOx and avidin were immobilized by adsorption and subsequent electropolymerization. The surface after the electropolymerization was rather smooth and a good support for AFM studies at higher resolution. No matter if GlOx was present at higher or lower concentrations, sufficient attachment of the membrane occurred only if a solution with more than 10% of avidin (w/v) was used. For method 3, 10% avidin was present in the polymerization mixture. A higher sensitivity was obtained when the proteins were directly adsorbed from the *o*-phenylene diamine solution.

After characterizing the response to glucose of the noncoated sensors, we applied the biotinylated vesicles to build up the selectivity barrier containing GLUT-1. The facilitated glucose transporter is a membrane-spanning protein located in the erythrocyte plasma membrane, which enhances the diffusion of D-glucose into the erythrocyte *(26–31)*. After solubilization, it can be reconstituted into vesicles containing the erythrocyte lipids or into artificial liposomes of different lipid composition *(32–34)*. After purification and reconstitution, the K_m for the equilibrium exchange is 35 m*M*, with a V_{max} of 50 mmol/mg of protein/min *(35)*. Cytochalasin-B and *o*-ethyliden glucose are known to be selective and strong inhibitors binding directly to the glucose transporter protein.

After every set of measurements, the cell was washed, and, to detect changes during the assay, a zero response cyclovoltammogram was taken. The zero response showed a slight increase in the resistance when the same sensor was measured for longer than 15 min (from 1.66 MΩ to 1.74 MΩ).

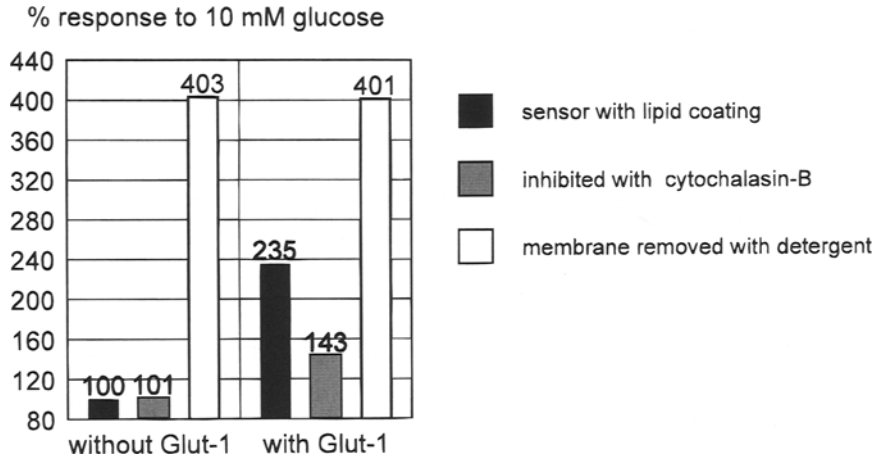

Fig. 4. Effects of GLUT-1 integration into electropolymer-supported lipid membranes (data are averaged from different measurements, sensor deviations were in the range of 20%). To avoid membrane stress induced by a flowthrough system, measurements were carried out in an open micro-chamber by pipeting the desired buffer solution onto the chip. Signals increased with increasing glucose concentrations, and were reversible and stable immediately after pipeting.

This change in the zero response did not significantly affect the response when glucose was measured at different times under identical conditions. Changes were in range of the system's noise (±7 nA). Typical data extracted from various CV measurements are shown in Table 1.

After incubation with sonicated vesicles, which contained between 15 and 30% (mol%) of biotinyl-cap-DOPE (di-oleoyl phosphatidyl ethanolamine) in an erythrocyte lipid extract, the zero response typically dropped to 40 μA. The response to glucose decreased to <7% of the value before the coating.

Using transporter-enriched vesicles, a similar decrease of the zero response was measured. The response to glucose was 235% of the value obtained for uncoated electrodes. All measurements were performed with sensor pairs that had the same characteristics before the coating took place. The amount of biotinylated lipids within the vesicles was 20 mol% or higher, which was enough to maintain a lipid coverage of the sensor during the measurements, including several steps of buffer change.

To prove transmembrane flux through facilitated glucose transports, the channels were blocked by binding of cytochalasin-B. Sensors without GLUT-1 did not exhibit any inhibition of glucose current upon cytochalasin-B addition. The use of transporter-saturating amounts of cytochalasin-B was preferred because of a high standard deviation of sensor signals with lower concentrations of the blocking agent.

When exposed to the detergent that was used in the purification of the

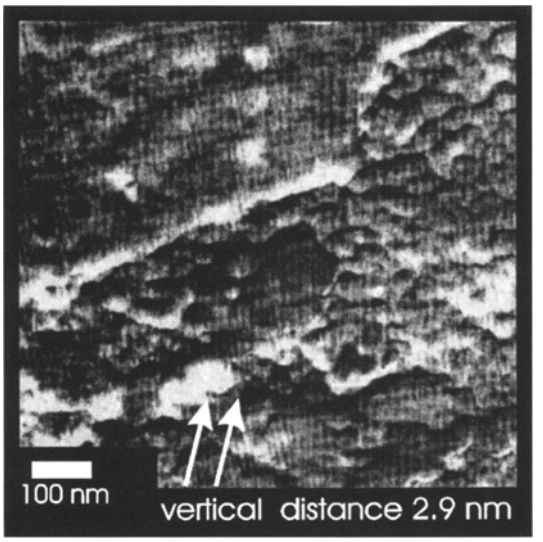

Fig. 5. AFM-scan of a facilitated glucose transporter-enriched membrane patch attached to an *o*-phenylene diamine polymer on a platinum sensor chip.

transporter (*n*-octyl-glycoside), the membrane was partly solubilized and detached from the polymer surface. The removal of the lipid membrane increased the glucose response, regardless if GLUT-1, containing vesicles or erythrocyte-extract vesicles were used for the coating. A summary of these characteristics is shown in Fig. 4.

Our experiments demonstrated that avidin/biotin coupling is not only a suitable method to attach a lipid membrane, but a functional lipid/protein assembly of biological origin to a solid sensing support. The coating of the sensor electrode with active membrane transporters was transduced to an electrochemical signal via enzymes included in the submembrane compartment.

Comparisons of the glucose response before the attachment of the lipid membrane and afterward not only indicated the lower conductivity of the coated sensor, but also showed that the glucose response depends on the presence of transporter in the vesicles used for coating. At the same time, interfering signals caused by ascorbate were significantly reduced. An inhibition of the glucose transport dependence of the glucose response on the presence of transporter were observed when a lipid coverage was obtained.

Although we did not achieve an electrical isolation of the electrode, as would be expected for a tight membrane, we could prove that the observed effects are caused by to filtering attributes (Fig. 4). Because of the fact that our electrode had an area of 1.8 mm^2, it was difficult to achieve a stable and faultless coverage, which was only demonstrated for significantly smaller black lipid membranes.

To elucidate factors that may be responsible for membrane defects, AFM studies of the supported membranes were done. As seen by the AFM (Fig. 5), the surface structure of the electrodes is very rough, so that the membrane has to cover areas where adaptation to different bending forces is required.

In order to obtain stable but fluid membranes attached to a solid or gel support, two fundamental problems had to be solved: First, a stable coupling of the lipid layer to the surface had to be achieved; second, it was necessary to prevent the second lipid layer of the membrane from floating on top of the first one. To overcome these limitations, we redesigned lipids to mimic the structure and properties of the cyclic tetraether-lipids from archaebacteria. Synthesis and characterization were published in Smetazko et al. (*36*, in press).

CONCLUSIONS

Summing up the results of the experiments, it could be proven that:

1. It was possible to incorporate artificial biotinylated lipids into a erythrocyte membrane.
2. This membrane could be deposited on, and attached to, smooth permeable supports by biotin–avidin bonds.
3. An electropolymerized *o*-phenylene diamine layer is a useful ion conducting support for lipid membranes.
4. Transporters incorporated in the membrane were functional on the artificial sensor chip.
5. The membrane assembly was mechanically stable and could be handled without additional care.

Based on these encouraging data, the construction of supported membrane sensors based on glucose and ligand-controlled ion channels is in progress.

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