(54) Title: SUBSTRATE FOR A DEVICE FOR ELECTROPHYSIOLOGICAL MEASUREMENTS
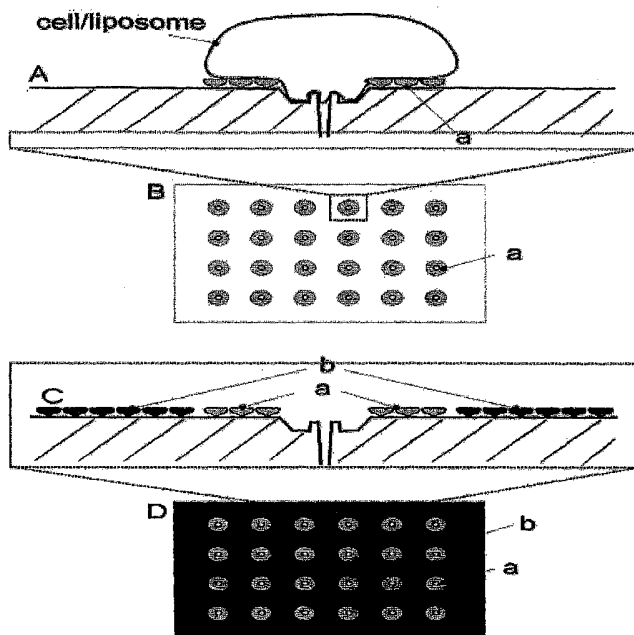
(57) Abstract: A substrate for a device for electrophysiological measurements comprising at least one perforation, wherein the substrate is covered at least in part with at least one protein and/or glycoprotein layer.

Substrate for a device for electrophysiological measurements

This invention relates to a substrate for a device for elec-
trophysiological measurements.

In recent years the patch-clamp technique became state of
the art for electrophysiological measurements of ion channels
and transporters located in cells, cell fragments or artificial
lipid membranes. The classic patch-clamp technique uses a glass
pipette, which is gently pressed against the lipid membrane. A
very small area is isolated, which is investigated (Sakmann &
Neher, 1995). The electric currents through reconstituted ion
channels and pores are recorded in a highly specific and sensit-
ive way. However, classic patch-clamping is time-consuming and
not convenient for practical devices and automation. Recently,
automated patch-clamp devices ("patch clamp on a chip" (Sigworth
& Klemic, 2002)) were introduced, using planar microstructured
chips made of PDMS (Klemic et al., 2002), silicon (Pantoja et
al., 2001; Schmidt et al., 2000) or glass (Fertig et al., 2002;
Brüggemann et al., 2003) for recording from whole cells (Fertig
et al., 2002; Klemic et al., 2002), liposomes (Schmidt et al.,
2000) and painted artificial membranes (Pantoja et al., 2001).

The planar chip replaces the pipette. Patch-clamp-on-a-chip
enables simultaneous patch-clamp recordings from many liposomes
or cells. The decisive factor in this system is the formation of
a high-resistance gigaohmic seal between the chip and the lipid
membrane. Lower resistances would lead to a low signal to noise
ratio. In all systems difficulties with seal formation were ob-
served, presumably because of non-sufficient contact area (nar-
row sidewalls, sharp corners of the etched aperture).

It is an object of the present invention to provide a sub-
strate for a device for electrophysiological measurements which
allows an accurate positioning of the membranous sample on the
substrate and supports the formation of a high-resistance
gigaohmic seal between a substrate and a membranous sample.

Therefore the present invention provides a substrate for a
device for electrophysiological measurements comprising at least
one perforation, wherein the substrate is covered at least in
part with at least one protein and/or glyoprotein layer.

According to the present invention "the at least one perfor-
ation" can exhibit different three dimensional shapes. For in-

stance the perforation has the form of a frustum of a cone or a
pyramid, of a cylinder , of a wedge, of a prism or of a cube.

According to the present invention "substrate" is defined as
a structural component of a device for electrophysiological
measurements whereon a membranous sample can adhere. Furthermore
the substrate may function additionally as a barrier between two
electrode compartments of a patch clamp device.

The "protein layer" according to this invention refers to
all kind of proteins capable to be immobilised on the surface of
a substrate to be used in apparatus for electrophysiological
measurements. Examples for suitable proteins are S-layer pro-
teins, antibodies, other proteins and glycoproteins and frag-
ments thereof. Of course also polypeptides and peptides may be
immobilised on the surface of a substrate.

The substrate according to the present invention may be used
in apparatus for electrophysiological measurements employing the
path clamp technique. Such apparatus are disclosed for example
in the WO 96/13721 A1, WO 99/66329 A1 and US 2002/0108869 A1.
The presence of more than one perforation enables the substrate
to be used also in high throughput screenings. Furthermore the
invention enables standardized and reproducible spatial binding
properties of membranous samples for electrophysiological meas-
urements.

The protein layer (glyosylated or non-glycosylated) can be
bound covalently or non-covalently, e.g. by hydrophobic or hy-
drophilic interactions, to the surface of the substrate. Fur-
thermore the substrate can be covered entirely or partially with
a protein layer, whereby the protein layer may comprise more
than one protein species.

The at least one protein layer provides anchoring/adhesion
sites for membranous samples, e.g. cells, cell fragments, lipo-
somes and other lipid membrane aggregates, on a substrate which
can be used in patch-clamp devices known in the state of the
art. Furthermore the protein layer plays an important role in
the high-resistance gigaohmic seal formation between the lipid
membrane and the chip surface. Moreover the proteins on the sur-
face of the substrate can be used as a pre-blocking inert coat-
ing, in order to prevent interfering effects and unspecific ad-
sorption of cells, liposomes and other lipid aggregates. The in-
vention enables a sensitive electrical acquisition of transmem-

brane proteins and is a further step toward high-throughput screening for drug discovery efforts.

According to a preferred embodiment the at least one protein layer is modified with at least one functionality. A modification of the protein layer with at least one functionality enables the substrate to interact with e.g. the membranous sample. The interaction of the at least one protein layer with the membranous sample is helpful in stabilizing the membrane on the substrate and furthermore to create a gigaohmic seal. The modification of the protein layer can occur chemically (by covalent or by non-covalent binding) or by genetic engineering (by creating e.g. fusion proteins).

The possibility to create a broad spectrum of recombinant proteins which can be employed in the formation of a protein layer (e.g. S-layer fusion proteins) enables specific interactions between the protein and specific groups (ligands, receptors) on the liposome, lipid bilayer or cell surface. Thus, a protein layer, in particular an S-layer, allows a tight and specific binding of unlimited types of liposomes, lipid bilayer aggregates, whole cells or cell fragments in an extremely gentle way. Thereby gigaohmic seal formation (with or without a gentle suction) is greatly facilitated.

The binding mechanisms and functionalities exhibited by the protein layer are the following (e.g. S-layer protein(s)):

*) Lattice of native or recombinant S-layer proteins, or a mixture of native and recombinant S-layer proteins (co-re-crystallization):
1) unmodified S-layer lattice; utilization of the intrinsic binding affinities (non-covalent interactions).
2) chemically modified S-layer lattice providing specific physico-chemical properties and binding affinities.
3) Attachment of cells/liposomes/lipid aggregates due to topographical features of the S-layer lattice (edge sharpness of zone/chip boundaries and geometry).

*) Reactive groups/receptors on the S-layer recognize ligands in the lipid membrane; reactive groups/receptors in the lipid membrane recognize an S-layer-bound ligand;
e.g. antigen-antibody, ligand-receptor, lectine-carbo-

- 4 -

hydrate-interactions, metal-affinity-tags (e.g.
histidine); S-layer/streptavidin fusion protein lattice -
specific binding via biotinylated molecules in cells/lipo-
somes/lipid aggregates.

According to another preferred embodiment the at least one
functionality is selected from the group consisting of proteins,
polypeptides, peptides, ligands, chemical groups and mixtures
thereof. The functional groups of the at least one functionality
may comprise all functional groups knwon in the state of the
art, which can be used for protein modifications. For instance
these groups comprise proteins (e.g. avidin, streptavidin, lect-
ine, antibodies and fragments thereof), ligands (e.g. carbo-
hydrates, biotin) and chemical groups (e.g. -OH, -COOH, -NH$_2$).
For further details see Wong (1991) and Schalkhammer (2002).

Preferably the at least one protein layer is composed of at
least one S-layer. The chemical and structural features of S-
layer proteins turned out to be advantageous for a substrate to
be used in patch clamp applications.

S-layer proteins are routinely utilised in several technical
applications. For instance the EP 0 306 473 B1 discloses the use
of S-layer proteins as a carrier for haptens, immunogenic or im-
munostimulant substances. The immobilisation of molecules, par-
ticularly of proteins, on S-layer proteins is described in the
EP 0 362 339 B1. The WO 01/81425 A1 discloses the use of a
structure comprising carbohydrates (secondary cell wall polymer)
as an anchoring means for S-layer proteins in order to bind them
to a solid support. In the WO 02/097118 A1 the production of a
layer of functional molecules on a substrate using S-layer pro-
teins is disclosed. Therein the protein layer on the surface of
a carrier is formed by creating an electrochemical potential
difference between the solution and the surface. The
EP 0 189019 B1 discloses the making and the use of an ultra-
filtration membrane employing S-layer proteins.

S-layer protein species from different organisms can be
used. A protein species is used as a native, a recombinant, or a
recombinant protein optionally with distinct functional domain
(s), e.g. streptavidin, ligand, affinity tag. The S-layer is
composed of identical S-layer proteins, a mixture of at least
two species, or different protein species with each comprising

one or more specific functionalities. The ratio of the distinct
proteins can be varied. The S-layer can be native or chemically
modified. Furthermore S-layers can be recrystallized as mono-,
bi- or multilayers.

Dependent on the S-layer protein species employed, distinct
surface properties are obtained after recrystallization:

*) intrinsic functionalities of native isolated S-layer pro-
   teins and recombinant S-layer proteins with no fused
   functional domain: e.g. charge density and distribution.
   In many cases the recombinant S-layer proteins that are
   predominantly expressed in E. coli show a different
   charge distribution than the native protein isolated from
   the bacterial cell wall.
   With regard to their orientation in vivo, a common fea-
   ture of S-layers is their smoother charge-neutral outer
   surface and a more corrugated, net-negatively charged in-
   ner surface (Pum et al., 2000). Hence, the orientation of
   the S-layer proteins on the substrate defines the
   physico-chemical properties.
*) functionalities after chemical surface modification of
   native S-layers; e.g. change in charge and charge dens-
   ity, introduction of chemically reactive groups.
*) artificially inserted functional domains : genetically en-
   gineered S-layer fusion proteins, chemically fused func-
   tional domains.
*) inert surface character: neutrally charged surfaces. These
   types of S-layers show no binding affinity or unspecific
   binding.

Two-dimensional crystalline bacterial surface layers (S-lay-
ers) can be recrystallized in vitro into coherent lattices on a
great variety of surfaces and interfaces, including silicon or
glass (Sleytr et al., 2002, 2003; Pum et al., 2000). Lab-on-a-
chip-technologies were facilitated with the patterning of S-lay-
ers on solid supports by microlithography (Neubauer et al.,
1997, 1998; US 6296700 B1) or micromoulding in capillaries
(Györvary et al., 2003a). Moreover, functional groups of the at
least one functionality on S-layers interact with the lipid head
group region of phospholipids, leading to a stabilization of

- 6 -

lipid membranes. Stabilization was demonstrated on lipid membranes (Schuster et al., 1999) (Wetzer et al., 1997, 1998; Gufler et al., 2004) and on liposomes (Küpcü et al., 1995; Mader et al., 1999). S-layers do not penetrate the hydrophobic region of mono- (e.g. tetraetherlipid) or bilayer membranes; therefore they have no impact on the integrity of the lipid aggregates, cells or cell membranes. S-layers provide a natural environment for lipid membranes and biological materials: e.g. in many archaeal organisms the S-layer is the only component external to the cytoplasmic membrane (Baumeister & Lembcke, 1992). A broad range of S-layer proteins with different physico-chemical surface properties and functionalities are available. The implementation of recombinant S-layer- (US 2002/0168728 A1) and S-layer-fusion proteins exhibiting functional domains, enables the application of specific binding mechanisms (Moll et al., 2002). Due to the high regularity of the crystal lattice, functionalities repetitively arranged in well defined positions and orientations can be obtained. Furthermore a significant reduction in surface roughness of the substrate is observable after recrystallization (Gufler et al., 2004).

According to a preferred embodiment of the present invention the substrate comprises an electrically insulating material. It is a basic requirement of devices for electrophysiological measurements, e.g. patch clamp devices, that the current can only pass the susbstrate through the pores, transporters and channels part of a membranous structure present in the vicinity of a perforation of the substrate. Therefore substrates used for patch clamp experiments may comprise electrically insulating material. The surface of the substrate or the entire substrate may comprise an electrically insulating material.

Preferably the electrically insulating material material is selected from the group consisting of silicon, glass, solid state polymers and mixtures thereof. Microfabricated chips made of silicon, glass, solid state polymers or other materials are preferably used. Transparent chips, made e.g. of glass or synthetic polymers, can be used for simultaneous electrophysiological and optical or spectroscopical measurements (e.g. fluorescence measurements, microscopical methods). According to another embodiment part of the substrate may also comprise an electrode. In this case part of the substrate is electroconductive.

The perforation is preferably 100 nm to 10 µm, preferably 500 nm to 9 µm, more preferably 1 µm to 8 µm, particularly 2 µm to 7 µm wide. The perforations of the chips are fabricated e.g. by nano- and micro-lithographic methods, laser or micromoulding techniques. The dimension of the perforation is adjusted according to the size of the membranous sample, whereby different perforations of the substrate can possess differing dimensions. This demonstrates once more the flexible use of the substrate according to this invention.

"Wide" according to this invention refers to the physical dimensions of the perforations. For instance the term "wide" for a circular perforation refers to its diameter and "wide" for a quadratic perforation is referred to the distance between the nooks of such a perforation.

According to a preferred embodiment the substrate is substantially planar. The geometry of a substrate can be manifold. For instance the substrate can be planar like chips or exhibit a spherical form.

According to another preferred embodiment the substrate is chemically or physically modified. Not only the protein layer on the surface of the substrate can be modified, but also the surface of the substrate itself. Such modifications allow creating zones in which e.g. no protein layer can adhere and other zones where an interaction between surface of the substrate and the membranous sample is prevented. Other modifications may prevent an unspecific binding of the membranous sample on the surface of the substrate, where no protein layer is present.

Such a modification is preferably selected from the group consisting of ionizing radiation, atomic radicals, corona treatment, silane groups, chemical groups and mixtures thereof. Chemical groups may comprise carbohydrates (e.g. secondary cell wall polymer), biotin and reactive groups. Other chemical groups and modification methods are disclosed in several publications and textbooks (Schalkhammer, 2002; Wong, 1991).

According to another aspect of the present invention the substrate can be used for electrophysiological measurements, preferably for patch clamp measurements. Especially apparatus for patch clamp experiments can employ a substrate as disclosed herein.

Preferably the substrate according to the present invention

can be used for a device for simultaneous electrophysiological
measurements, preferably for patch clamp measurements, and op-
tical or spectroscopical measurements, if the substrate com-
prises a transparent electrically insulating material.

Another aspect of the present invention relates to a method
for measuring an electrical property of a membranous sample us-
ing a substrate as disclosed.

According to a preferred embodiment the membranous sample is
selected from the group consisting of cells, cell fragments,
cell membranes, liposomes, artificial lipid membranes, lipid
layers and mixtures thereof. The membranous sample comprises
next to lipids and other cell membrane components particularly
transporters and ion channels.

The substrate of the present invention may be preferably
used in a method for the simultaneous electrophysiological and
optical or spectroscopical measurements of a membranous sample.

Another aspect of the present invention relates to kit for
the electrophysiological measurement of a membranous sample com-
prising a substrate according to the present invention and a
device for electrophysiological measurements, preferably a patch
clamp device.

The present invention is further illustrated by the follow-
ing example and figures, yet without being restricted thereto.

Fig.1 shows two examples of substrate - and perforation (a)-
structures (sections: 1A and 1B; top view: 1C). The substrate is
provided with a plurality of perforations.

Fig.2 shows S-layer coatings. The S-layer adhesion site (a)
for the positioning of cells/liposomes/artificial membranes is
recrystallized around the aperture (section: 2A, top view: 2B).
An inert coating (b) of the residual chip surface can be per-
formed by using another S-layer protein species (section: 2C,
top view: 2D). The S-layer adhesion sites (Fig. 2, marked with
a) can be structured or non-structured. The S-layer patterns can
be performed simple, economical, reproducible and versatile.
Structuring can be performed by (i) nano- and micromoulding
techniques (Györvary et al., 2003a), (ii) microcontactprinting
or (iii) local depletion of previously generated coherent S-lay-
er lattices (microlithography, e.g. ion beam, deep UV) (U.S.
Patent: US 6296700 B1). The mould is placed onto the chip sur-
face. The mould can either be prefilled with the distinct S-lay-

er solution(s) or the protein solution(s) is (are) injected af-
terwards into the cavity(ies).

Fig.3 shows possible architectures of S-layer adhesion
sites. Areas of tight contact and local specific interactions (=
adhesion sites) between the cells/liposomes/artificial membranes
may be designed next to each other (e.g. bands, rings, patches
etc., hereafter referred to as "zones"). Some examples of pos-
sible patterns of S-layers around the chip aperture are illus-
trated in Fig. 3. The rings, squares/rectangles and bands can be
thin (a,f) or broad (b,g). The apertures can be surrounded by a
single S-layer zone (a,b,f,g) or by multiple zones (c,d e, h ,
i ,j). The distance between two zones (d,i) as well as the
thickness of the different zones (e, j) can be varied. The
rings, rectangles or bands can be continuous (a-e, f-j) or non-
continuous (k-n). Different zones may be covered with different
types of S-layers.

A further structuring embodiment is obtained by time-depend-
ent control of the recrystallization process. Starting from a
few randomly distributed nucleation sites, 2-dimensional growth
of S-layer domains takes place in all directions, until neigh-
bouring areas meet (Györvary et al., 2003a). Thus, the dimension
and the number of crystallites generated per unit area (and
hence, the area devoid of S-lattice) can be controlled by the
time of incubation (= recrystallization time): Fig. 3; (o) short
recrystallization time, (p) longer recrystallization time. When
different types (mixtures) of S-layer proteins are used for
coating (recrystallization), individual crystallites will as-
semble next to each other (Sleytr, 1975).

Fig.4 shows the composition of the S-layer lattice (protein
type, lattice symmetry, functionality) within the adhesion site
(A, B, C, D) and within one zone (E, F, G). Variable architec-
tures (as shown in Fig.3) exhibiting zones of S-layer lattices
composed of proteins from either the same (Fig. 4A) or different
(Fig. 4B) organisms (native or recombiant; single or variable
functionalities) can be used. Different zones of an S-layer ad-
hesion site can be composed of one functionality (Fig. 4C) or
variable functionalities (Fig. 4D). One zone can be composed of
an S-layer lattice with one single functionality (Fig. 4E) or a
mixture of at least two functionalities (same S-layer proteins,
but different fused functionalities) (Fig. 4F). One zone can be

composed of S-layer proteins from different organisms exhibiting
varying lattice symmetry (pA, pB, pC) and/or functionalities
(Fig. 4G).

Fig.5 shows the fabrication of an S-layer adhesion site by
using the micromoulding technique (e.g. PDMS mould).

Fig.6 shows additional modification of the substrate surface
devoid of S-layer lattice within the adhesion site.

Fig.7 shows S-layer proteins as means for smoothing the
sharp corners of the chip perforations.

Fig.8 shows the closing- and opening-behaviour of single
gramicidin pores reconstituted in anchored and clamped giant
liposomes.

EXAMPLE: **The application of 2-dimensional protein crystals
for gramicidin ion channel recordings from giant liposomes
placed on a planar chip-device**

Two-dimensional bacterial surface layer (S-layer) proteins
were applied for coating of planar silicon chips and subsequent
binding of giant unilamellar vesicles (GUVs) that were function-
alized with gramicidin. The GUVs contained a low amount of biot-
inylated lipids. A genetically engineered S-layer protein that
exhibited a fused heterotetrameric streptavidin domain was re-
crystallized around the aperture of the microstructured silicon
chip forming an S-layer adhesion site. Successful binding via
biotin-streptavidin interactions between GUV and the S-layer-
streptavidin fusion protein layer (one GUV per aperture) was ob-
served. Gigaseal formation was monitored by resistance measure-
ments. The closing- and opening-behaviour of single gramicidin
pores reconstituted in the anchored and clamped giant liposomes
was observed. The additional coating of the residual chip sur-
face (around the S-layer streptavidin fusion protein adhesion
site) by an inert coating of wildtype SbpA, significantly dimin-
ished unspecific adsorption and/or fusion processes of GUVs.

1. MATERIAL AND METHODS

1.1 Chemicals

The phospholipids EggPC and Biotin-cap-DPPE (Avanti Polar
Lipids, Alabaster, AL) were dissolved and stored at -20°C in
chloroform (Merck, Darmstadt, Germany) at a concentration of
0.1M. As a standard buffer an aqueous solution of 1M KCl was
used. The buffer was prepared in MilliQ-water (Millipore, minim-
um resistance >18MOhms cm) and thoroughly degassed and filtered

through a 0.2-μm filter (Sartorius AG, Göttingen, Germany) be-
fore use. All chemicals were purchased from Sigma Aldrich (Vi-
enna, Austria).

1.2 Chip (substrate) preparation and treatment

A silicon wafer (NMRC, Cork, Ireland, 1x1 cm², contact angle
64°) was structured by standard lithographic techniques and an-
isotropic ion etching, resulting in an aperture with a diameter
of ~ 1-7 μm. The aperture was placed in the centre of the wafer
(one pore/chip). The chips were cleaned by ultrasonic treatment
twice for 20 min in 2% Hellmanex (Hellma, Müllheim, Germany) and
twice for 20 min in absolute ethanol. After each sonication
step, the slides were rinsed thoroughly in MilliQ-water and fi-
nally dried in a stream of dry nitrogen. Cleaned chips were
stored in clean air until usage.

1.3 Preparation of S-layer protein solutions

Growth, cell wall preparations, and extractions of the S-
layer protein SbpA from the gram-positive strain B. sphaericus
CCM 2177 (Czech Collection of Microorganisms) were performed as
described elsewhere (Sleytr et al., 1986). The wildtype SbpA
stock solution was diluted with to a final concentration of 0.1
mg/ml. Molecular cloning, gene expression, isolation, refolding
and purification procedures of S-layer streptavidin fusion pro-
teins (rSbpA/strp) were described elsewhere (Moll et al., 2002)
(Huber et al., submitted). rSbpA/strp proteins recrystallized on
hydrophobic silicon as a monolayer with the streptavidin domain
exposed to the aqueous solution. An appropriate amount of
rSbpA/strp was desintegrated in 4M GHCl for 5 min. The protein
was dialysed against MilliQ-water for 2 h. The protein solution
was adjusted to a concentration of 1mg/ml with MilliQ-water,
centrifuged in an Eppendorf Centrifuge and stored at 4°. For re-
crystallization rSbpA/strp was diluted with recrystallization
buffer (0.5 mM Tris (pH = 9.0) containing 10mM CaCl$_2$) to a final
concentration of 0.1 mg/ml. Recrystallization was performed from
2 to 4h.

*1.4 Preparation of giant unilamellar liposomes (GUVs) func-
tionalized with gramicidin*

Preparation of giant unilamellar functionalized liposomes
was performed by a slightly modified dehydration-rehydration
procedure according to Riquelme et al. (1990). In a first step
unilamellar proteoliposomes were prepared. Chloroformic solu-

- 12 -

tions of EggPC and Biotin-cap-DPPE (molar ratio 98:2) were added
into a glass flask. An ethanolic solution of Gramicidin D, which
predominantly (80%) consists of gramicidin A (Sigma, Deisen-
hofen, Germany) was added (molar lipid/protein-ratio = 200:1).
The organic solvent was removed in a rotary evaporator for at
least 4h. The dried film was hydrated to a final lipid concen-
tration of 1% (w/v) by adding a standard buffer containing 2%
CHAPS (Sigma, Vienna, Austria). The detergent was either removed
by dialysis or by addition of Bio Beads SM-2 (Sigma, Vienna,
Austria). Glass microscopic slides (ELKA, Germany) were cleaned
in Hellmanex and absolute ethanol (ultrasonic treatment), thor-
oughly rinsed with MilliQ-water and dried in a stream of nitro-
gen. Small drops (~ 20 µl) or wide stripes (for larger amounts)
of the vesicle suspension were deposited onto the glass slides.
The suspension was partially dehydrated for 3 h/4°C in a desic-
cator. Giant liposomes exhibiting a size distribution from 20 µm
to 80 µm were obtained by rehydration: an appropriate amount of
standard buffer solution was deposited on the top of the dehyd-
rated drop. Size and size distribution of GUVs were examined by
transmission electron microscopy (Philips, Eindhoven, The Neth-
erlands) of negatively stained samples. Formvar/carbon coated EM
copper grids (Athene old/300 mesh) were deposited on a drop of
the sample (5min). Afterwards the sample was fixed with 2.5%
glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0 and
stained in 2% uranyl acetate.

1.5 Recrystallization of S-layer proteins on the chip

An S-layer adhesion site composed of rSbpA/strp was recrys-
tallized around the micromachined aperture on the chip by a
soft-lithographic technique, micromoulding in capillaries, as
previously described (Györvary et al., 2003a). For mold forma-
tion, mesastructures mold masters in photoresist (AZ 9260;
Clariant) on 4-in. silicon wafers were fabricated by photolitho-
graphic methods. A master pattern of two horseshoe-like struc-
tures was chosen. The horseshoe structures were arranged to each
other such that an almost continuous ring with two inlets was
formed. Poly(dimethylsiloxane), PDMS (Sylgard 184; DOW corning),
is used to fabricate the molds from these masters using the
standard procedure (Kumar et al., 1994) (Kim et al., 1995). The
mold was carefully placed onto the manufactured silicon chip
(microscopical control) such that the aperture was circularily

surrounded by the two horseshoe-like capillaries. Starting from inlets the microchannels (20 µm channel width) were filled with the rSbpA/strp recrystallization solution. After self-assembly and recrystallization of rSbpA/strp proteins the mold was removed under MilliQ-water, leaving the patterned rSbpA/strp arrays behind. Additionally, in some experiments the residual chip surface was coated with wildtype SbpA, which exhibited low unspecific binding surface properties. SbpA formed a monolayer on hydrophobic silicon. The recrystallization quality and integrity of the S-layer was controlled by Atomic Force Microscopy (AFM) analysis. AFM images were recorded in contact mode in liquid with a Nanoscope III AFM (Nanoscope III, Digital Instruments, Santa Barbara, CA). Oxide-shaped silicon nitride tips (Nano-Probes, Digital Instruments) with a nominal spring constant of 0.06 N/m were used. A 100 mM NaCl solution was used for imaging.

1.6 Experimental set-up

The voltage-clamp setup was mounted on a steel core breadboard (CS-11-4, Newport, Darmstadt, Germany). The headstage of the patch clamp amplifier (EPC 9, HEKA Electronic, Lambrecht/Pfalz, Germany) was fixed on a translation stage (M-426, Newport, Darmstadt, Germany) which could be moved vertically (Motorizer 860 A, Newport, Darmstadt, Germany; regulated DC power supply: Kenwood PR 18-1.2, Tokyo, Japan). The headstage of the amplifier, together with measurement chamber were placed within a grounded Faraday cage. The S-layer covered chip was placed horizontally into a home-made teflon-chamber. The chip was sealed into the chamber by a silicone paste (Bayer AG, Leverkusen, Germany) und thus divided the chamber into two compartments. Ag/AgCl redox electrodes were used for voltage-clamp recordings. The reference electrode was placed in the lower, the working electrode in the upper compartment. The lower compartment comprised a vacuum device in order to apply a suction to the clamped liposome. The S-layer covered surface was facing the upper compartment. During this procedure, desiccation of the S-layer was carefully prevented. After placing the chip, the chamber was filled with the standard buffer solution. A small amount of the freshly prepared biotinylated gramicidin-GUV suspension was pipetted onto the S-layer. A single liposome was bound to the rSbpA/strp adhesion zone. After application of a gentle suction, seal formation was monitored by resistance measurements.

1.7 Signal recording and data acquisition

The current response of a given voltage function was measured at 25°C to calculate the electrophysiological parameter of the clamped liposome patch. Conductance was measured via rectangular voltage pulses of up to 200mV across the membrane. All the data handling (voltage stimulation, data acquisition, data storage, data analysis) was performed on a Power Macintosh 7600/120 computer (Apple Computer GmbH, Ismaning, Germany). The setting of the two built-in Bessel filters of the EPC 9 amplifier for the current-monitor signal was 10 and 1.5 kHz, respectively. The data analysis was performed by the Pulse + PulseFit software 7.89/Quadra (HEKA Elektronik, Lambrecht/Pfalz, Germany). Correlations of conductance vs. time and statistical analysis (ANOVA) were performed by using Jandel SIGMA PLOT program for Windows.

2. RESULTS AND DISCUSSION

A prototype of a planar S-layer-based patch-clamp-chip was implemented. GUVs functionalized with biotinylated lipids and gramicidin were chosen as an appropriate system for single channel recordings. The size of GUVs varied from ~20-80 μm, the size distribution was fairly balanced. In some cases, multilamellar vesicles were obtained, which were not used for the experiments. The GUVs were used immediately after preparation or for later experiments devided into aliquots and stored at -80°C until usage.

The recrystallization of rSbpA/strp and wildtype SbpA was monitored by Atomic Force Microscopy. All types of S-layers used in this example recrystallized on silicon chip as monolayered S-layers with the functional (for rSbpA/strp) or the inert surface (for wildtype SbpA) exposed to the aqueous solution. Recrystallization of S-layers on silicon as well as the microstructuring of S-layers by using the micromoulding technique (PDMS moulds) are well-established (Györvary et al., 2003a, 2003b). Diameters of microstructured S-layer adhesion sites ranging from 60-100 μm were obtained. Optionally, the residual chip surface around the rSbpA/strp adhesion site was coated with wildtype SbpA. The outer surface of SbpA is charge-neutral. On chips without a wildtype SbpA layer, adhesion and/or fusion of GUVs to larger lipid aggregates or bilayered structures were observed. A significant decrease in unspecific adsorption and/or fusion processes of GUVs was achieved. Since liposomes only adsorbed slightly and at

a very low amount, they could be washed away easily by gentle
rinsing of the upper compartment of the measurement cell. In the
majority of experiments a single GUV was bound per S-layer adhe-
sion site. In some experiments either the GUV was not centered
and thus no gigaseal was obtained or more smaller GUVs per adhe-
sion site were bound.

Functional studies of single membrane-active peptides and
membrane proteins like e.g. ion channels require the reduction
of electrical background noise and consequently a high electric-
al insulation of the surrounding membrane patch, the so-called
"giga-seal" ($R>10^9$ Ω). The arrangement of the giant liposomes on
the perforation is achieved by the linkage of biotinylated lip-
ids within the giant liposomes with the patterned S-layer-strep-
tavidin fusion protein. The tight seal, however, is obtained
manually by suction. Stable membranes on the chip with seal res-
istances of $R>10^9$ Ω were obtained in 17 out of 19 experiments.

The challenge of preparing lipid membranes on silicon chips
is the recording of single ion carriers and channels. The gener-
al functionality of this concept was demonstrated with gramicid-
in, a peptide known to self-integrate into one leaflet of a lip-
id bilayer and to form single channels if dimerization occurs.
The closing- and opening-behaviour of single gramicidin pores
reconstituted in the anchored and clamped giant liposomes was
observed (Fig. 8). As a driving force for ion flux, a voltage
typically around 200 mV was applied across the bilayer. Gram-
icidin is selective for monovalent cations and as KCl solution
was used, K+ currents were recorded. There were clearly five
different levels (on horizontal) visible, the lowest represent-
ing all channels closed, and each additional level the opening
of a channel unit (corresponding to a peptide dimer). Also most
channels had the same amplitude of about 8.4 pA, some channels
were smaller, i.e., carried less current or transports fewer
ions per unit time. This may be due to impurities in Gramicidin
D preparation as this preparation is known to contain also a few
proportions of other peptides.

The present example shows that stable gigaohm seals over mi-
crometer-sized holes can be obtained. The self-positioning of
biotinylated giant liposomes can easily be achieved by the high
binding affinity of biotin to the streptavidin hetrotetramers
which are presented in a well-defined and oriented way by the S-

layer lattice. Thus, no suction is necessary for the positioning
of the target molecules, which is always problem as this leads
often to clogging of the aperture. If the target molecule is on
the right position, suction guarantees the giga-seal formation
between the liposome and the aperture. The application potential
of this new technique (positioning by the S-layer fusion - lipid
anchor molecule interaction and subsequent seal formation by
suction of giant liposomes) was clearly demonstrated by measur-
ing the transmembrane current through single ion channels, or in
other words by tracing single molecules.

References:

Baumeister,W. and G.Lembcke. 1992. Journal of Bioenergetics and Biomembranes 24:567-575.

Brüggemann,A., George,M., Klau,M., Beckler,M., Steindl,J., Behrends,J.C., Fertig,N. 2003. Assay and Drug Development Technologies 1(5):665-673.

Fertig,N., M.Klau, M.George, R.H.Blick, and J.C.Behrends. 2002. Applied Physics Letters 81:4865-4867.

Gufler,P., D.Pum, U.B.Sleytr, and B.Schuster. 2004. Biochimica et Biophysica Acta 1661:154-165.

Györvary,E.S., A.O'Riordan, A.J.Quinn, G.Redmond, D.Pum, and U.B.Sleytr. 2003a. Nano Letters 3:315-319.

Györvary,E.S., O.Stein, D.Pum, and U.B.Sleytr. 2003b. Journal of Microscopy 212:300-306.

Kim, E; Xia, Y. and Whitesides, G. M. 1995. Nature 376:581-584.

Klemic,K.G., J.F.Klemic, M.A.Reed, and F.J.Sigworth. 2002. Biosensors and Bioelectronics 17:597-604.

Kumar,A., H.A.Biebuyck, and G.M.Whitesides. 1994. Langmuir 10:1498-1511.

Küpcü,S., M.Sára, and U.B.Sleytr. 1995. Biochimica et Biophysica Acta 1235:263-269.

Mader,C., S.Küpcü, M.Sára, and U.B.Sleytr. 1999. Biochimica et Biophysica Acta 1418:106-116.

Moll,D., C.Huber, B.Schlegel, D.Pum, U.B.Sleytr, and M.Sára. 2002. Proceedings of the National Academy of Sciences of the United States of America 99:14646-14651.

Neubauer,A., S.Pentzien, S.Reetz, W.Kautek, D.Pum, and U.B.Sleytr. 1997. Sensors & Actuators B 40:231-236.

Neubauer,A., W.Kautek, S.Pentzien, S.Reetz, M.Sahre, T.Solomun, R.Korntner, D.Pum, and U.B.Sleytr. 1998. PTB-Berichte F-34:75-81.

Pantoja,R., D.Sigg, R.Blunck, F.Bezanilla, and J.R.Heath. 2001. Biophysical Journal 81:2389-2394.

Pum,D., A.Neubauer, E.Gyoervary, U.B.Sleytr. 2000. Nanotechnology 11:100-107.

Riquelme,G., E.Lopez, L.M. Garcia-Segura, J.A.Ferragut, and J.M.Gonzalez-Ros. 1990. Biochemistry 29:11215-11222.

Sakmann, B., and E.Neher. 1995. Single channel recording (2nd edition). New York, London:Plenum Press.

Schalkhammer, T.G.M.(ed.) 2002. Analytical Biotechnology. Basel, Boston, Berlin:Birkhäuser Verlag.

Schmidt,C., M.Mayer, and H.Vogel. 2000. Angewandte Chemie Int. Ed. Engl. 39:3137-3140.

Schuster,B., U.B.Sleytr, A.Diederich, G.Bähr, and M.Winterhalter. 1999. European Biophysics Journal 28:583-590.

Sigworth,F.J. and K.G.Klemic. 2002. Biophysical Journal 82:2831-2832.

Sleytr,U.B. 1975. Nature 257:400-402.

Sleytr,U.B., M.Sára, Z.Küpcü, and P.Messner. 1986. Archives of Microbiology 146:19-24.

Sleytr,U.B., D.Pum, E.Györvary, B.Schuster, S.Küpcü, and M.Sára. 2002. Progress in Colloid and Polymer Science 121:57-61.

Sleytr,U.B., B.Schuster, and D.Pum. 2003. IEEE Engineering in Medicine and Biology Magazine 22:140-150.

Wetzer,B., D.Pum, and U.B.Sleytr. 1997. Journal of Structural Biology 119:123-128.

Wetzer,B., A.Pfandler, E.Györvary, D.Pum, M.Lösche, and U.B.S-leytr. 1998. Langmuir 14:6899-6906.

Wong, S.S. 1991. Chemistry of protein conjugation and cross-linking. Boca Raton, Ann Arbor, Boston, London:CRC Press,Inc.

Claims:

1. A substrate for a device for electrophysiological measurements comprising at least one perforation, characterised in that the substrate is covered at least in part with at least one protein and/or glycoprotein layer.

2. A substrate according to claim 1, characterised in that the at least one protein layer is modified with at least one functionality.

3. A substrate according to claim 2, characterised in that the at least one functionality is selected from the group consisting of proteins, polypeptides, peptides, ligands, chemical groups and mixtures thereof.

4. A substrate according to any one of claims 1 to 3, characterised in that the at least one protein layer is composed of at least one S-layer.

5. A substrate according to any one of claims 1 to 4, characterised in that the substrate comprises an electrically insulating material.

6. A substrate according to claim 5, characterised in that the electrically insulating material is selected from the group consisting of silicon, glass, solid state polymers and mixtures thereof.

7. A substrate according to any one of claims 1 to 6, characterised in that the perforation is 100 nm to 10 μm, preferably 500 nm to 9 μm, more preferably 1 μm to 8μm, particularly 2 μm to 7 μm wide.

8. A substrate according to any one of claims 1 to 7, characterised in that the substrate is substantially planar.

9. A substrate according to any one of claims 1 to 8, characterised in that the substrate is chemically or physically modified.

10. A substrate according to claim 9, characterised in that the modification is selected from the group consisting of ionizing radiation, atomic radicals, corona treatment, silane groups, chemical groups and mixtures thereof.

11. Use of a substrate according to any one of claims 1 to 10 for a device for electrophysiological measurements, preferably for patch clamp measurements.

12. Use of a substrate according to any one of claims 1 to 10 comprising a transparent an electrically insulating material for a device for simultaneous electrophysiological measurements, preferably for patch clamp measurements, and optical or spectro-scopical measurements.

13. A method for the electrophysiological measurement of a membranous sample using a substrate according to any one of claims 1 to 10.

14. A method according to claim 13, characterised in that the membranous sample is selected from the group consisting of cells, cell fragments, cell membranes, liposomes, artificial lipid membranes, lipid layers and mixtures thereof.

15. A method for the simultaneous electrophysiological and optical or spectroscopical measurements of a membranous sample using a substrate according to any one of claims 1 to 10.

16. A kit for the electrophysiological measurement of a membranous sample comprising a substrate according to any one of claims 1 to 10 and a device for electrophysiological measurements, preferably a patch clamp device.
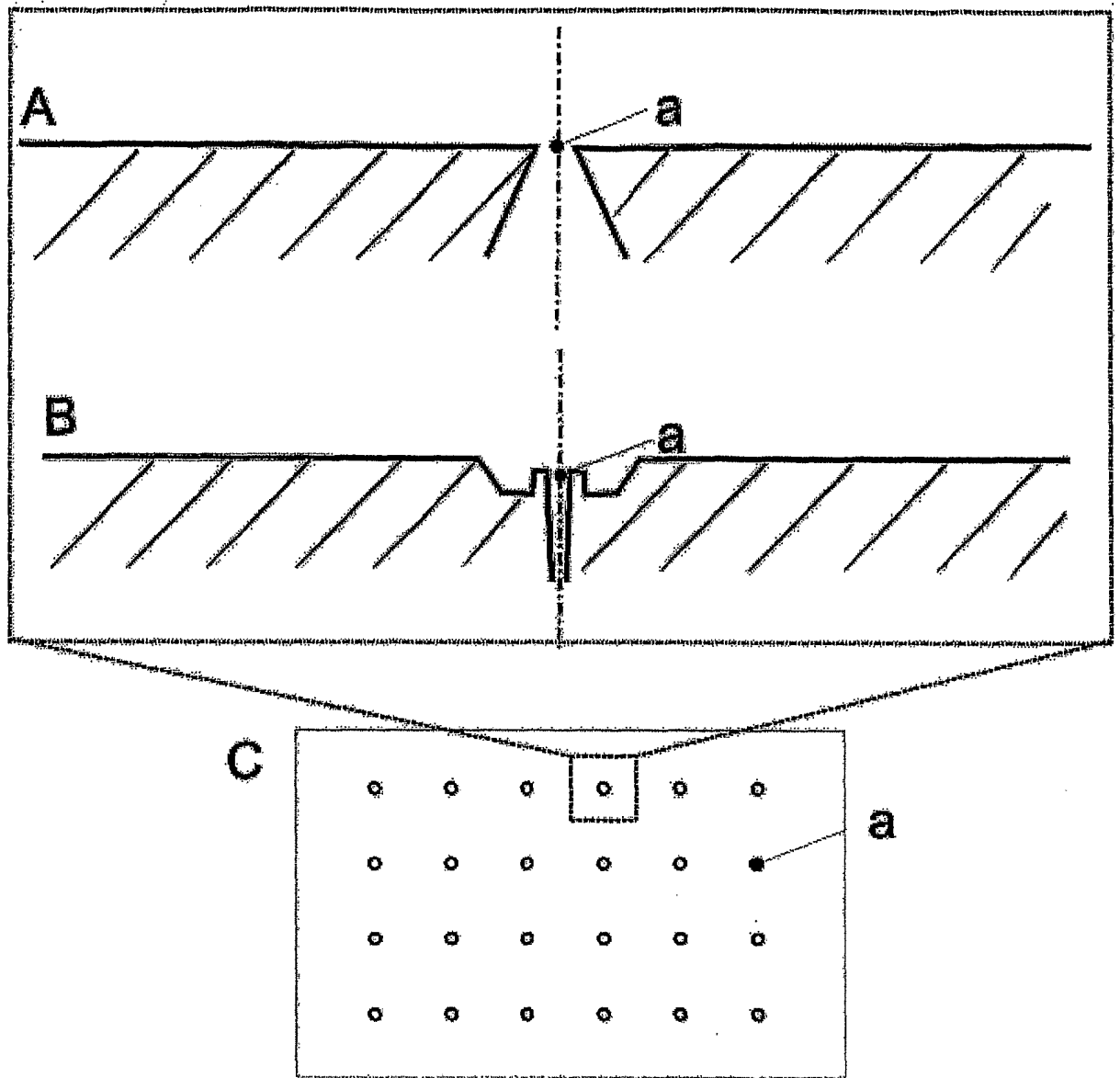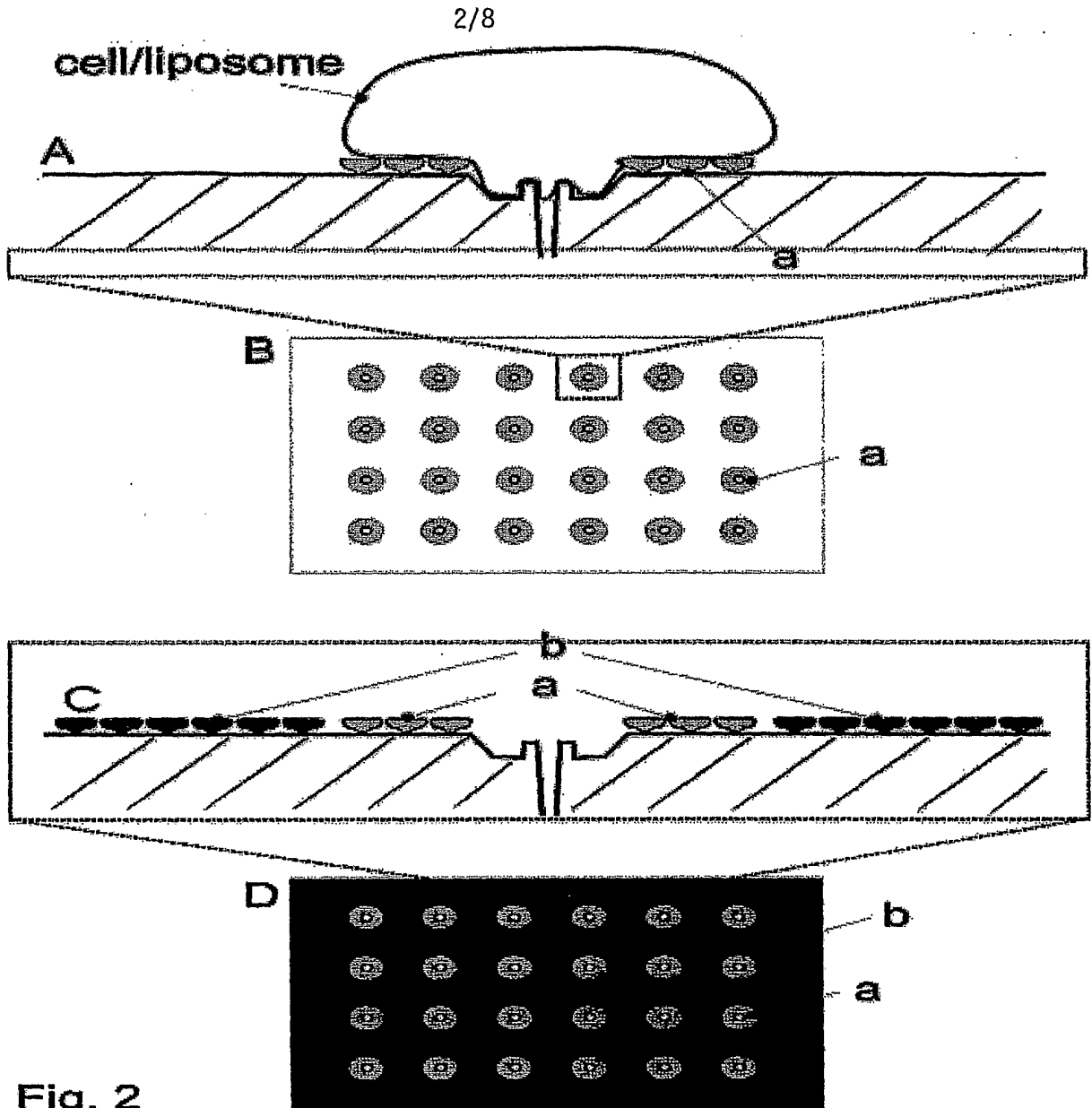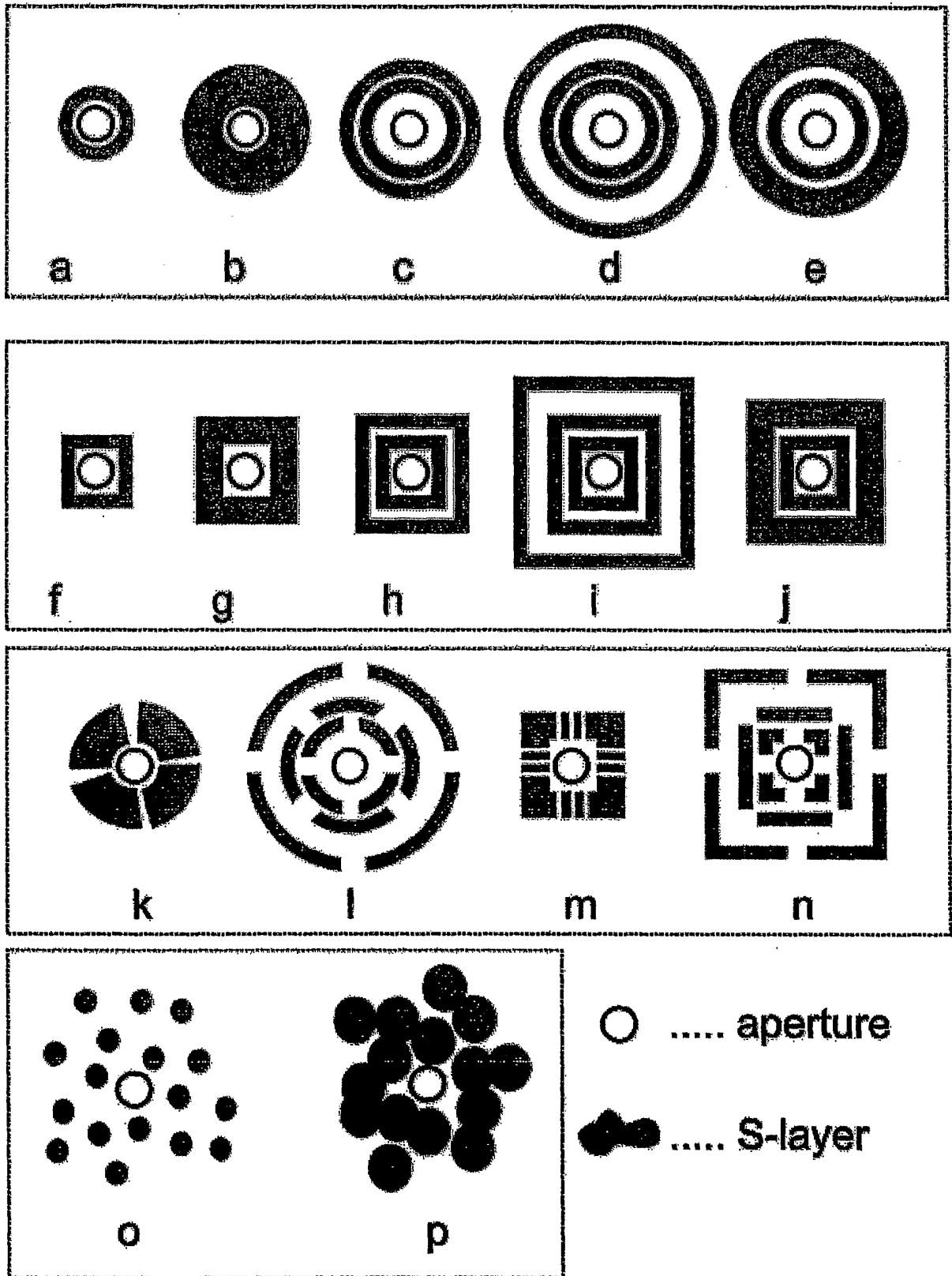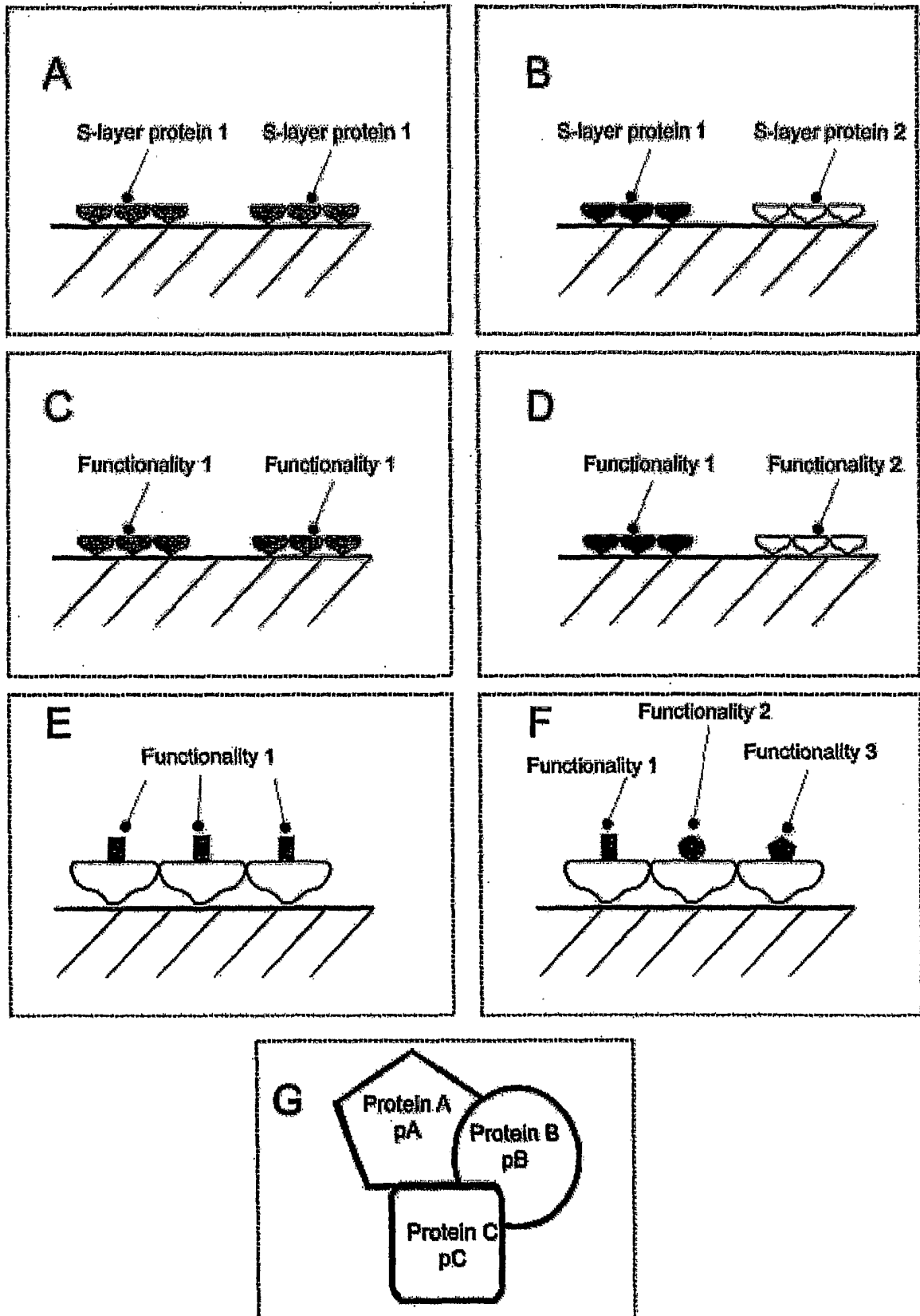
Fig. 1

Fig. 2

○ ..... aperture

⬤ ..... S-layer

Fig. 3

Fig. 4
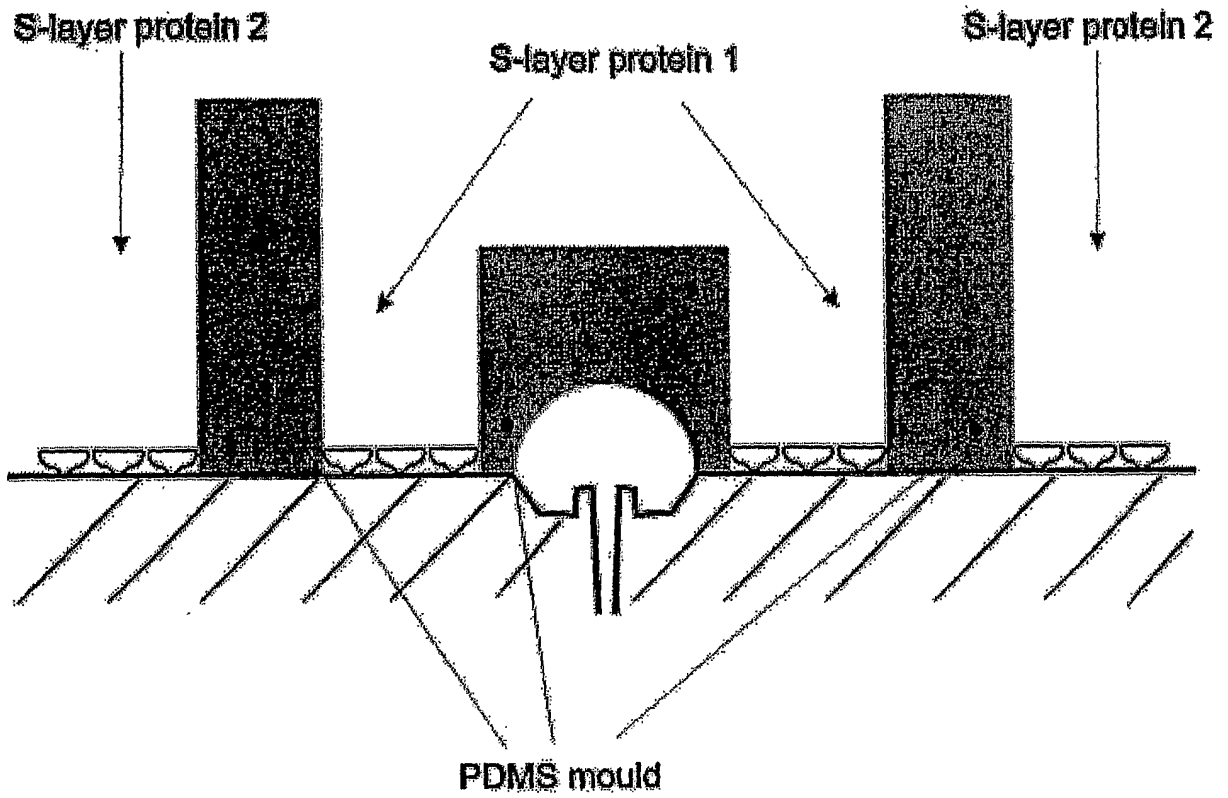
Fig. 5
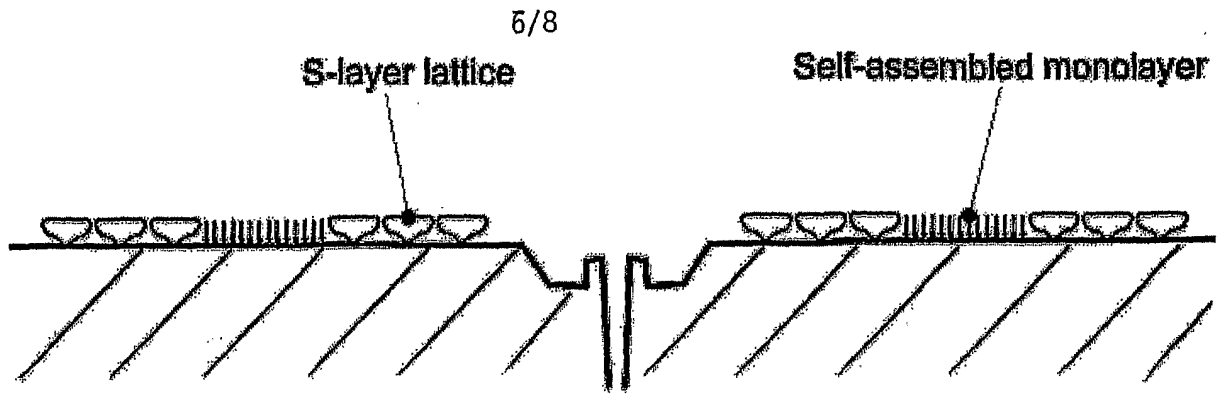
Fig. 6
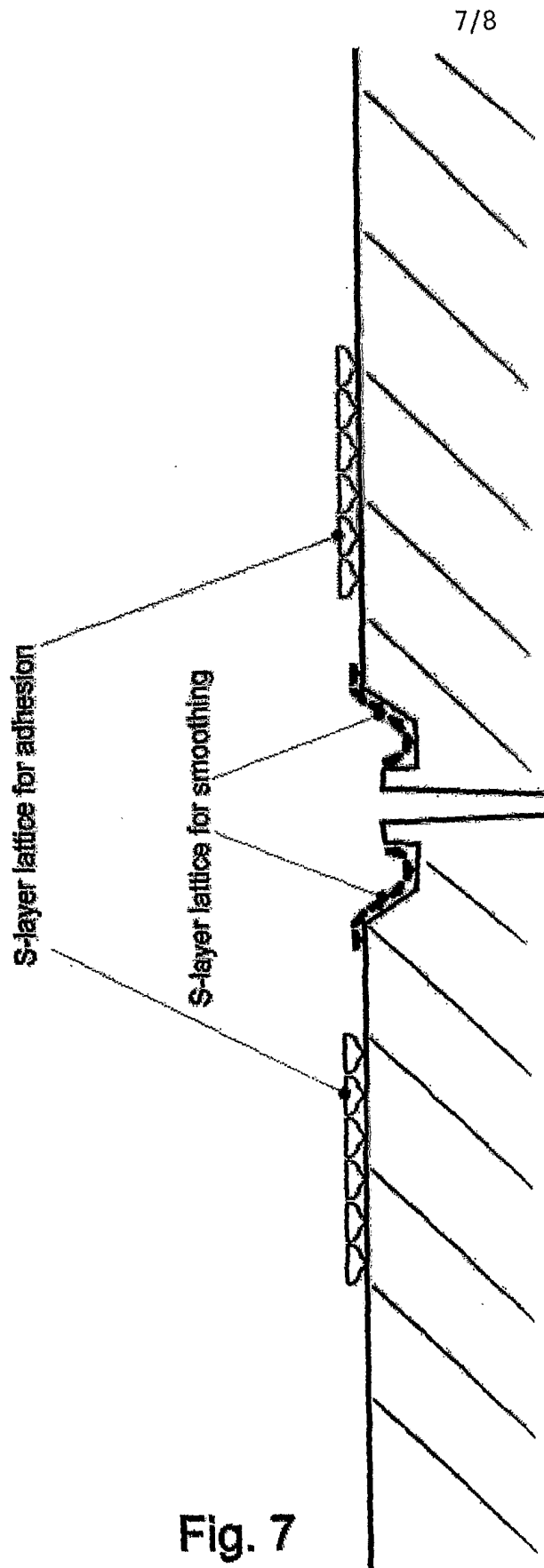
Fig. 7
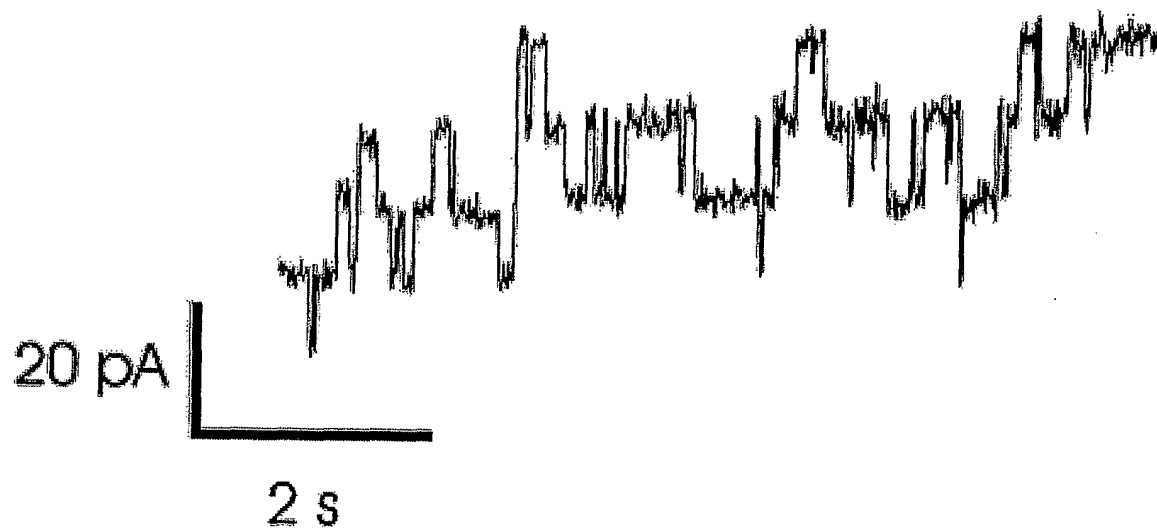
Fig. 8

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   G01N33/487   G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS, INSPEC, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 315 940 B1 (NISCH WILFRIED ET AL) 13 November 2001 (2001-11-13) abstract figures 1,3 column 5, line 40 - line 45 column 9, line 12 - line 18	1,5-7, 11-16
X	WO 02/04943 A (BRISTOL-MYERS SQUIBB COMPANY) 17 January 2002 (2002-01-17) the whole document in particular page 7, line 14 - page 8, line 11; page 12, lines 4-24; page 13, lines 12-28; Figures 1-3	1-3,5-16
Y		4

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 February 2005	18/02/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Thumb, W

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2003/146091 A1 (VOGEL HORST ET AL) 7 August 2003 (2003-08-07) the whole document in particular paragraphs 42, 50, 57, 60-67, 82-86	1-3,5-16
Y		4
X	US 2002/182627 A1 (WANG XIAOBO ET AL) 5 December 2002 (2002-12-05) the whole document in particular paragraphs 26, 47, 193, 337.	1-3,5-16
Y		4
Y	US 2004/033576 A1 (SLEYTR UWE B ET AL) 19 February 2004 (2004-02-19) the whole document	4
Y	PUM D ET AL: "The application of bacterial S-layers in molecular nanotechnology" TRENDS IN BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 17, no. 1, January 1999 (1999-01), pages 8-12, XP004155527 ISSN: 0167-7799 the whole document in particular page 10, col. 1, last paragraph - col. 2, paragraph 2.	4

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 6315940	B1	13-11-2001	DE	19712309 A1	20-05-1998
			WO	9822819 A1	28-05-1998
			EP	0938674 A1	01-09-1999
			US	2002074227 A1	20-06-2002
WO 0204943	A	17-01-2002	AU	7189801 A	21-01-2002
			CA	2413663 A1	17-01-2002
			EP	1311848 A2	21-05-2003
			JP	2004514417 T	20-05-2004
			MX	PA03000160 A	22-09-2003
			WO	0204943 A2	17-01-2002
			US	2002053915 A1	09-05-2002
US 2003146091	A1	07-08-2003	US	2003098248 A1	29-05-2003
			US	2002104757 A1	08-08-2002
			US	2002144905 A1	10-10-2002
			US	2003052002 A1	20-03-2003
			AT	205300 T	15-09-2001
			AU	746580 B2	02-05-2002
			AU	8237998 A	05-07-1999
			CA	2316966 A1	24-06-1999
			DE	59801410 D1	11-10-2001
			DK	1040349 T3	28-01-2002
			EP	1040349 A1	04-10-2000
			ES	2163284 T3	16-01-2002
			HK	1028450 A1	15-03-2002
			WO	9931503 A1	24-06-1999
			JP	3486171 B2	13-01-2004
			JP	2002508516 T	19-03-2002
			US	6758961 B1	06-07-2004
			AU	1047401 A	06-06-2001
			WO	0134764 A2	17-05-2001
			AU	8747201 A	02-04-2002
			WO	0224862 A2	28-03-2002
US 2002182627	A1	05-12-2002	CA	2441366 A1	03-10-2002
			EP	1372828 A2	02-01-2004
			WO	02077259 A2	03-10-2002
US 2004033576	A1	19-02-2004	AT	409423 B	26-08-2002
			WO	0181425 A1	01-11-2001
			AT	7322000 A	15-12-2001
			AT	275584 T	15-09-2004
			AU	5201001 A	07-11-2001
			CA	2407639 A1	01-11-2001
			DE	50103547 D1	14-10-2004
			EP	1285003 A1	26-02-2003
			JP	2003531390 T	21-10-2003