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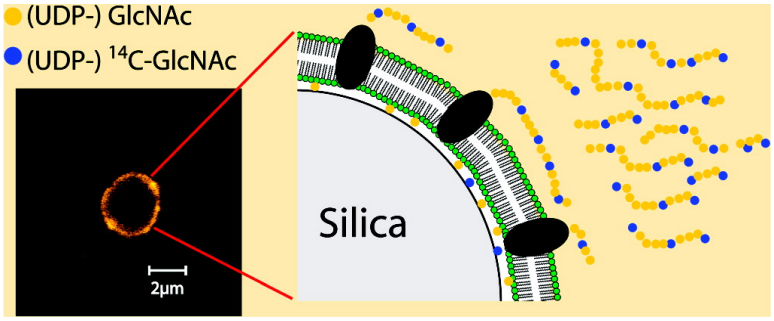

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Quantitative in Vitro Biopolymerization to Chitin in Native Chitosomal Membranes Supported by Silica Microparticles

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Abstract: To investigate the unknown physical mechanisms of chitin biosynthesis quantitatively, we designed a quantitative in vitro biopolymerization assay by deposition of native chitosomal membranes from *Saccharomyces cerevisiae* onto solid silica microparticles of a defined size ($\varnothing = 3\ \mu$m). The homogeneous coating of particle surfaces with native chitosomal membranes observed by confocal microscopy agrees well with the surface coverage calculated by the phosphate analysis. The amount of the synthesized chitin polymers is determined by radioactive assays, which demonstrate that chitin synthase in particle-supported membranes retains its specific enzymatic activity. In comparison to planar substrates, particle supports of defined size (and thus surface area) enable us to amplify the signals from immobilized proteins owing to the much larger surface area and to the capability of concentrating the sample to any given sample volume. Moreover, the large density of particle supports offers unique advantages over purified chitosomes in the quick separation of particle-supported membranes and materials in bulk within 1 min. This allows for the termination of the polymerization reaction without the disruption of the whole membranes, and thus the chitin polymers released in bulk can quantitatively be extracted. The obtained results demonstrate that the native biological membranes on particle supports can be utilized as a new in vitro biopolymerization assay to study the function of transmembrane enzyme complexes.

Introduction

Many fundamental biological processes are governed by integral or peripheral membrane proteins, such as the regulation of gene transcription by G-proteins.[1−3] One of the conventional strategies to model structures and functions of biological membranes utilizes phospholipid bilayers deposited onto solid substrates (so-called solid-supported membranes).[4,5] Supported membranes incorporating membrane proteins have provided insight into many biological processes, such as allogeneic stimulation of T cell lymphocytes[6] or formation of immunological synapse.[7] Moreover, the use of cells or vesicles in conjunction with supported membranes allows for the study of interplays of generic and specific forces in cell adhesion.[8] More recent studies demonstrated that the lipid membranes on polymer supports (polymer-supported membranes)[9,10] can offer unique advantages to avoid direct mechanical contacts between membrane proteins and the underlying substrates.[11,12]

Nature stringently controls the orientation and the population of transmembrane proteins, and it is practically difficult to replicate the complex molecular composites using artificial membranes. Thus, to mimic more closely the complexity of natural membranes, the deposition of native biomembranes provides a possibility. In our recent accounts, we demonstrated that the polymer supports actually play an active role to adjust the membrane−surface interaction, which enables the homogeneous spreading of natural membranes of human erythrocytes and sarcoplasmic reticulum without losing their asymmetric protein orientation.[13,14] Interestingly, although erythrocytes do not adhere or spread over bare glass slides, they readily coat the surface of bare silica microparticles with no polymer coating.[15] Actually, this finding can qualitatively be understood within a framework of wetting and adhesion. When cells are in contact with the highly curved surface, the local contact angle Θ between the cell and the particle is larger than that on planar substrates. This might result in a larger free energy of cell

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adhesion that can be represented as $W_{ad} = \gamma(1 - \cos \Theta)$, where γ is the membrane tension.

In fact, membranes supported on microparticles (particle-supported membranes) are advantageous over membranes on planar supports. The increase in surface area associated with placing membranes on microparticles will significantly increase both efficiency and speed to detect specific analytes interacting with membranes and membrane proteins.[16] Suspensions of membrane-coated microparticles can readily be concentrated in any given detection volume, which amplifies the spectroscopic signals.[17]

Transmembrane enzyme complexes, such as glycosyltransferases, produce various cell surface polymers, such as cellulose, hyaluronan, chitin, and pectin.[18−21] An increasing number of evidences point out that various parameters, such as the distinct chemistry of the monomers, postsynthetic modifications, and enzymatic kinetics, determine the structure and the mechanical properties of the resulting polymer fibers that finally regulate their biological functions.[22−29] Among these biopolymers, chitin is an extracellular component found in cell walls of fungi and invertebrates[30−32] and is also known to contribute to biomineralization processes.[33,34]

A family of integral membrane proteins, chitin synthase, catalyzes the ATP-enhanced polymerization of UDP−*N*-acetylglucosamine (UDP−GlcNAc) monomers and translocates the β(1−4)-linked polymer chain into the extracellular space.[35−37] Chitin synthase can also be found in intracellular precursor vesicles, so-called chitosomes.[3,38,39] Chitin oligomers of more than 10 monomers prepared according to standard methods[40] are hardly soluble in water and spontaneously assemble into fiber crystals (Weiss et al., unpublished results). The crystal morphology of biogenic chitins (α-, β-, γ-chitin, determined by fiber diffraction studies) is under distinct control of the organisms.[41−44] However, the exact physical mechanism of chitin biosynthesis is still poorly understood.[45]

As demonstrated by Kobayashi et al., enzymatic polymerization has been defined as chemical polymer synthesis in vitro (in test tubes) via nonbiosynthetic (nonmetabolic) pathways catalyzed by an isolated enzyme.[46] In this study, instead of widely used large-scale biotechnological fermentation or covalent linkage of soluble enzymes,[47] we apply the concept of supported membranes to immobilize the transmembrane chitin synthase complex on solid supports. Here, to quantify the small amount of chitin synthesized by chitin synthase, we choose particle-supported membranes as the model system. Chitosomal membranes isolated from the yeast *Saccharomyces cerevisiae* are deposited on silica microparticles, and the composition of the immobilized proteins and their functions are fully characterized. With the use of highly sensitive radiochemical assays, the quantity of synthesized chitin polymers can be calculated. This is the first report that the function of chitin synthase can be maintained by deposition of natural chitosomal membranes on particle supports, which can be used as a new biophysical tool to study biopolymer synthesis in a quantitative manner.

Experimental Section

All chemicals used were of p.A. quality, if not otherwise specified. Deionized ultrapure water, termed Milli-Q (Millipore, Eschborn, Germany), was used for preparation of all buffer solutions.

Isolation of Chitosomal Membranes. Chitosomal membranes were isolated as previously described[38,48] with minor modifications. In brief, yeast cells (*S. cerevisiae* wild type, strain 34/70) were grown in a 2 L scale from 4 × 4 mL precultures (20 h) in YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) at 30 °C for 20 h with shaking at 165 rpm from a cell density of 5×10^5 to 3×10^7 cells/mL. Cells (late log phase) were harvested by 10 min centrifugation at 500×*g*, 4 °C, and washed twice with 150 mL of TM buffer (100 mM Tris/HCl, pH 7.5, 40 mM $(CH_3CO_2)_2Mg$). All subsequent manipulations were performed at 4 °C using TM buffer. An amount of 12 g of cells was resuspended in 14.5 mL of TM buffer and vigorously vortexed in the presence of 27.4 g of glass beads (0.25−0.5 mm, Carl-Roth GmbH, Karlsruhe, Germany) for at least 8 × 2 min with brief interruptions for cooling. Glass beads were removed by slow sedimentation, and cell walls and unruptured cells were removed from the crude extract by 10 min of centrifugation at 1000×*g*. The cell-free supernatant (18 mL) was centrifuged in a Beckman 75Ti rotor for 45 min at 54 000×*g*. The obtained membrane pellet was used as a positive control for the chitin synthase activity assays. The vesicular supernatant, which still contained ribosomal contaminations, was treated with 80 μg/mL ribonuclease (Carl-Roth GmbH, Karlsruhe, Germany) for 30 min at 30 °C. The ribosomal protein precipitate was removed by 20 min of centrifugation at 10 000×*g*. A volume of 10 mL of supernatant was filtered through a membrane (Millex HV45, Millipore, Eschborn, Germany) and concentrated for 10 h under 1.5 bar of N_2 to a final volume of 1.2 mL with an Ultracel Amicon YM100 ultrafiltration disc (Millipore, Eschborn, Germany: molecular weight cutoff, 100 kDa). The concentrated vesicle suspension was centrifuged at 13 200×*g*, and a 1 mL portion of supernatant was filtrated through a Millex HV45 filter and subjected

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to FPLC, equipped with a HiPrep 26/60 Sephacryl S-300/HR size exclusion column (Pharmacia, Freiburg, Germany) equilibrated in TM buffer, supplemented with 150 mM NaCl. After a 50 min run time, the UV_{260} signal arose, and 6.5 mL fractions were collected at a flow rate of 1.3 mL/min. Fractions with the highest chitin synthetase activity (~5 nmol/min per fraction) were stored in 100 μL aliquots shock frozen in liquid nitrogen at −80 °C. Only fresh, never refrozen aliquots were thawed gently on ice for immediate use.

Prior to the deposition of chitosomal membranes on particles, the size distribution of freshly thawed chitosome samples was first characterized by dynamic light scattering (HPPS5001, Malvern Instruments GmbH, Herrenberg, Germany) in various dilution series. The turbid chitosome samples with a polydispersive size distribution were separated by a mild centrifugation (1000×*g*) for 1 min into sedimented pellets and supernatants, and the latter fraction (supernatant) was used to coat the particle surfaces.

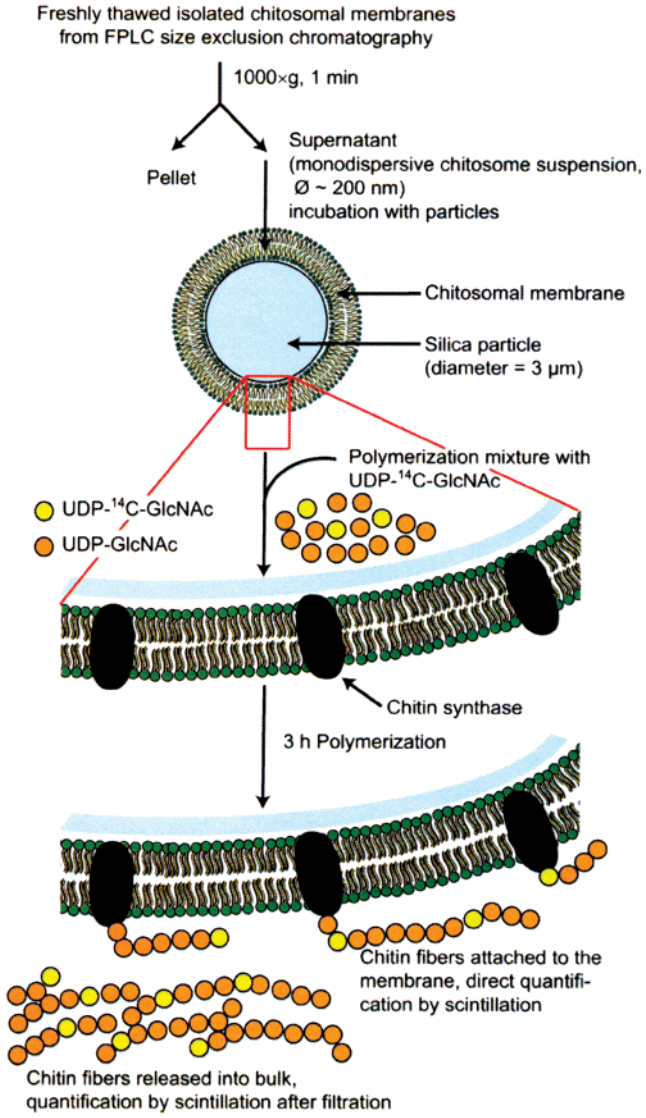
Analysis of Membrane Protein, Enzyme Activity Assays. Protein composition in chitosomal membranes was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS−PAGE) according to the procedure of Laemmli.[49] Protein bands were detected by coomassie brilliant blue staining, with subsequent destaining in 25 vol % methanol and 10 vol % acetic acid. Protein reference samples and chitosome suspensions were mixed with Laemmli buffer (65 mM Tris/HCl, pH 6.8, 3.3 wt % SDS, 5 vol % mercaptoethanol, and 10 vol % glycerol) in a 1:1 volume ratio and boiled for 5 min. After cooling, about 20 μL of the sample was applied on each gel lane. The membranes were detached from silica particles by boiling the sample in Laemmli buffer for 5 min. After the sedimented particles were removed, the solubilized proteins were subjected to SDS−PAGE. The protein concentration of each fraction was determined according to the method reported by Bradford[50] and Lowry et al.[51]

Selected protein components of the chitosomal membrane fractions were identified by a MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA).[52] The protein bands of interest in the SDS gel were cut off and subjected to in-gel tryptic digestion. Then, the sample was processed by cyanohydroxycinnaminic acid (CHCA) for mass spectrometry experiments.

The chitin synthetase activity in the isolated chitosomal membranes was studied according to the established protocol[35,48,53] with some minor modifications. In brief, chitosomal chitin synthase in its zymogenic precursor form[48] in 100 μL of chitosome suspension was shortly activated by incubating with 10 μg of trypsin for 15 min at 30 °C, and trypsin inhibitor (15 μg) was added prior to the polymerization. A standard polymerization assay was performed in a final volume of 150 μL containing 50 mM KH_2PO_4/NaOH, (pH 6.5), 0.17 mM ATP/$MgCl_2$, 17 mM GlcNAc, 1.7 mM UDP−GlcNAc, and 0.3 μM (12.5 nCi) UDP−^{14}C-GlcNAc, which is referred to as a "polymerization mixture" in the following. After 2 h of incubation at 30 °C, the reaction was stopped by the addition of 150 μL of 0.2 wt % SDS, which results in the disruption of the membranes. The synthesized chitin polymers, which include polymers attached to the membranes and those released in bulk, were collected by filtration of the whole reaction mixture through a Whatman GF/C glass fiber filter (Whatman, Dassel, Germany) preimpregnated with 1 wt % GlcNAc and washed three times with a 10 mL portion of 70 vol % ethanol to remove traces of ^{14}C monomers. The filter was dried for 15 min at 70 °C, and the radioactivity (and thus the quantity) of chitin polymers on the filter was determined by standard liquid scintillation counting.

Fabrication and Characterization of Chitosomal Membranes on Particle Supports. As depicted in Scheme 1, a 600 μL portion of the supernatant (total lipid concentration: 50 μg/mL) suspended in TM buffer with 150 mM NaCl was incubated with 10 mg of silica microparticles (∅ = 3 μm) for 60 min at 20 °C under a slow overhead rotation. Unbound chitosomal membrane fractions were removed by extensive washing with TM buffer by low gear forces in a benchtop centrifuge for 5 s.

Scheme 1. Deposition of Isolated Chitosomal Membranes on Silica Microparticles and the Following Chitin Synthase Activity Assays



The concentrations of phospholipids in (a) original supernatants (i.e., the sample before deposition), (b) supported membranes, and (c) unbound membranes in bulk were measured by the phosphate assay of Bartlett.[54] An amount of 1 mg of solid beads was incubated with excess amount of membranes to achieve a high surface coverage. A 0.3 mL portion of each sample was placed in a test tube, and water was evaporated by heating the sample for 5 min at 180 °C. After addition of 0.3 mL of concentrated perchloric acid (70 vol %), the sample was heated at 180 °C for another 30 min in order to mineralize any phosphate residue. The sample was resuspended with 1.4 mL of H_2O and 0.2 mL of ammonium molybdate (2.5 wt %) and vigorously vortexed. After addition of 0.2 mL of ascorbic acid, the sample was heated at 100 °C for 5 min. The phosphate concentration was deduced from the optical absorption recorded at 797 nm using a UV−vis spectrophotometer (Perkin-Elmer, Rodgau, Germany). The range of

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standard calibration was 0−100 nmol. Three independent measurements were performed.

For microscopic observation of chitosomal membranes on silica microparticles, TRITC−DHPE (*N*-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, Molecular Probes, Paisley, U.K.) was doped into chitosomal membrane extracts. The ethanol solution of TRITC−DHPE (0.1 mM) was added to the chitosomal membrane suspensions at a molar ratio of [dye]/[lipid] = 1:500 and incubated for 2 h at room temperature.[55] The fluorescently labeled membranes were used for the deposition without any further purification. The sample was placed on a glass coverslip, coated with a thin agarose film to avoid the evaporation of water. Differential interference contrast (DIC) and fluorescence images are collected with a laser scanning confocal microscope (LSM510-Meta, Carl Zeiss, Jena, Germany) equipped with a Plan-Apochromat 100× oil immersion objective (N.A. = 1.4).

The enzymatic activity of chitin synthase in particle-supported membranes was evaluated almost in the same manner as that for chitosome membrane extracts. Prior to the membrane deposition, naked silica particles (10 mg) were treated with the polymerization mixture (600 μL, 125 nCi). The particles were separated from the bulk polymerization mixture by a brief centrifugation (1 min, 1000×*g*) and incubated with a 600 μL portion of monodispersive chitosomal vesicle suspensions (supernatants) for 2 h at 20 °C (Scheme 1). Owing to the large density of silica particles ($\rho_{silica} \sim 2.6$ g/cm^3), the unbound membranes bulk can easily be separated by a gentle sedimentation (1×*g*, 15 min). After washing the particle-supported membranes twice with TM buffer, the sample was resuspended in the polymerization mixture (600 μL) with 6 μg of trypsin. After 15 min, 9 μg of trypsin inhibitor was added, and the polymerization was run for 3 h. The particle-supported membranes were readily separated by a brief centrifugation (100×*g*, 1 min), resuspended in TM buffer (150 μL), and subjected directly to the liquid scintillation counting to quantify the radioactivity of chitin polymers attached to the membranes. The chitin polymers released into bulk were collected by filtering the 100×*g* supernatant through a GF/C glass fiber filter, and the sample was washed, dried, and subjected to scintillation counting. It should be noted that the quick and easy (100×*g*, 1 min) separation of the membrane-bound polymers and polymers released in bulk is a unique advantage of the supported membrane assay over the assay using purified chitosome membrane extracts in suspension, which requires the disruption of the membrane with detergents to terminate the polymerization reaction.

The background radioactivity from nonspecific adsorption of UDP−^{14}C-GlcNAc monomers onto the particles was determined by treating naked silica particles in the identical manner.[56] As a negative control, another native membrane that has no chitin synthase or specific interaction with GlcNAc was deposited on silica particles. Based on our previous works,[15] we chose particle-supported human erythrocyte ghost membranes and carried out exactly the same enzymatic activity assays.

Results and Discussion

Characterization of Isolated Chitosomal Membrane Extracts. When the shock-frozen chitosome samples are freshly thawed, the suspension often seems turbid, suggesting the aggregation or fusion of chitosome vesicles caused by freeze-thawing. This trend becomes more prominent when several freeze-thawing cycles are repeated. Here, the size distribution of the complete fraction of the freshly thawed chitosome vesicles was calculated from the autocorrelation function measured by dynamic light scattering (Figure 1A). The obtained size histogram clearly indicates that there are two major populations in the size distribution; one is about 0.2−1 μm, and the other is 3−7 μm. The optical microscopy image indicates that the latter coincides with clotted membrane aggregates. On the other hand, the site of the former fraction seems reasonable from the size of other chitosomes.[38] Since such membrane aggregates often nonspecifically interconnect particles and disturb the fabrication of homogeneous supported membranes, it is desired to separate these two fractions. After careful optimization of the force and time for the centrifugation, we found that a gentle centrifugation of the complete fraction (at 1000×*g* for 1 min) results in a white pellet and an supernatant suspension (Scheme 1). As presented in Figure 1B, the supernatant obtained by a gentle centrifugation exhibits a monodispersive size distribution that has the peak at 243 nm and the full width at a half-maximum (fwhm) of 42.6 nm. In contrast, the resuspended pellet fraction remains turbid, which matches well with the randomly clotted membrane aggregates found by optical microscopy.

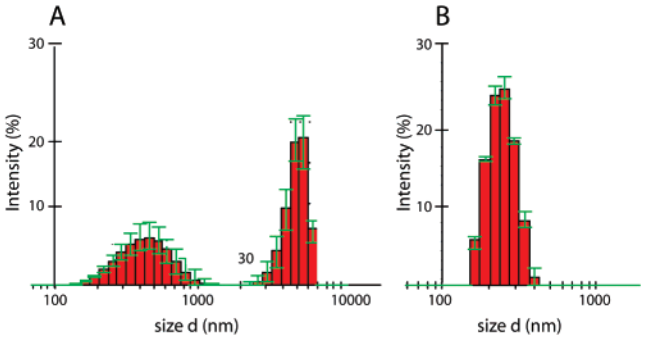

Figure 1. Size distributions of isolated chitosomal membrane extracts measured by dynamic light scattering: (A) the histogram of freshly thawed membranes (i.e., the complete fraction) and (B) the supernatant after the removal of membrane aggregates (pellet) by mild centrifugation. The centrifugation results in a monodispersive and suspension of chitosomes (supernatant) with an average diameter of 243 nm. In the following experiments, the latter fraction was used for coating silica particles.

In the next step, the compositions of proteins in the chitosome samples are analyzed by SDS−PAGE. Figure 2A represents the protein patterns after electrophoresis of different chitosome fractions in 7 wt % polyacrylamide gel stained with coomassie blue. First, it should be noted that the band corresponding to the molecular weight of 116 kDa, identified as chitin synthase (*S. cerevisiae* Chs3) by MALDI-TOF/TOF, can be found in all three fractions. On the other hand, the major band close to the 200 kDa molecular weight standard is predominantly present in the complete fraction and the supernatant but not in the pellet. This band can be identified as fatty acid synthase (FAS), which is a soluble multienzyme complex. The other bands can be identified as membrane proteins, such as vesicle coat protein at a molecular weight of 187 kDa and vesicle fusion protein at 131 kDa. As seen in Figure 2A, the protein compositions of the supernatant and pellet seem almost identical to that of the complete chitosome fraction, except for FAS impurities. The presence of FAS impurity can be attributed to the difficulty in separating FAS (diameter of the whole complex is ∼25 nm) from monodispersive chitosomal membrane extracts (diameter of about 240 nm) by size exclusion chromatography. Thus,

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(56) Naked silica particles were incubated with bovine serum albumin (BSA) solution (5 mg/mL in TM buffer) for 60 min and treated identically to the particle-supported membrane samples. Throughout this series of experiments, the background signal always remains below 50 cpm. Here, we use solid (and thus nonporous) silica particles, whose surface area is comparable to that calculated from the radius. The surface topography of the same particle (measured by scanning electron microscopy) is presented in ref 15.

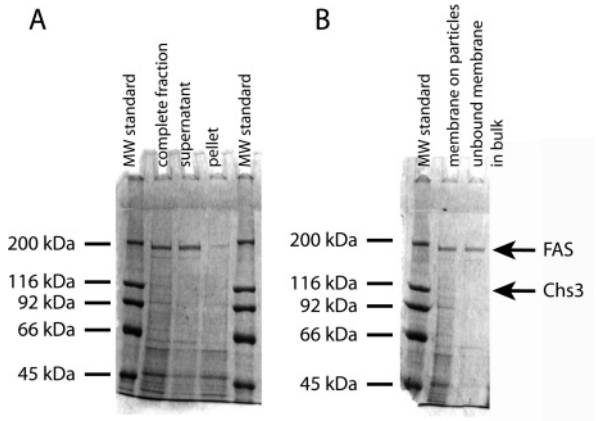

Figure 2. Protein distributions after electrophoresis of different chitosome fractions on 7 wt % polyacrylamide gel, stained with coomassie blue. Panel A represents the protein patterns for the complete fraction (prior to 1000×*g* centrifugation, see Scheme 1), the supernatant, and the pellet. Note that the band of chitin synthase (Chs3) at 116 kDa can be found in all the fractions. Panel B shows the protein patterns in the particle-supported membranes and the unbound membranes in bulk. It should be noted that almost all membrane proteins appear in particle-supported membranes but not in unbound membranes.

despite the presence of FAS impurity, the monodispersive supernatant fraction retains chitin synthase that is necessary for the chitin synthesis.

Characterization of Chitosome Membranes on Particle Supports. The successful deposition of chitosomal membranes on silica microparticles can be checked by the analysis of protein compositions in the particle-supported membranes. After incubating the particles with membrane extracts for 2 h, the bead suspension (0.3 mL, containing 4 mg of silica particle) was washed with TM buffer and subjected to SDS−PAGE (Figure 2B). For comparison, the protein composition in the unbound membranes remaining in bulk is also presented in the next lane. It is notable that all the membrane proteins, i.e., except for FAS, were predominantly present in the lane of the particle-supported membranes. In contrast, the lane corresponding to the unbound membranes merely displays FAS and one more soluble protein of a smaller molecular weight (∼60 kDa). This implies that a large amount of chitosome membranes preferably adsorbs on silica microparticles. The presence of the chitin synthase in particle-supported membranes is also proven by the subsequent MALDI-TOF/TOF analysis, which corresponds well with the sample composition as previously characterized by electron microscopy.[38]

Table 1 summarizes the identification of chitin synthase in chitosomal membranes supported on silica particles by MALDI-TOF/TOF. No other glycosyltransferases were found in the list of the 15 most likely protein candidates as deduced from yeast genome database comparisons.

The homogeneity of the chitosomal membranes on particle supports can be monitored by confocal microscopy. Here, the chitosome membrane extracts are labeled with TRITC−DHPE. Figure 3 represents the confocal fluorescence image (panel A) and the corresponding DIC image (panel B) of the fluorescently labeled chitosomal membranes on silica microparticles. Despite the presence of some fluorescent "spots" corresponding to adhered membrane aggregates on the surface (indicated by white arrows), the continuous fluorescence contour at the particle rim implies that the particle surfaces are continuously coated with

Table 1. Identification of Chitin Synthase in Chitosomal Membranes Supported on Silica Particles by MALDI-TOF/TOF[a]

CM	OM	Sequence	M
992.473	992.509	ERQMAWR	O
1004.502	1004.518	HSVGSGAPHR	
1022.588	1022.513	ASTFDLLKK	
1439.745	1439.834	DSQIILMSFLEK	O
1687.855	1687.867	VFPDALTHMVAEMVK	
1716.904	1716.879	FIIACYFRWTVAR	P
1773.924	1773.938	DPLIMGLCGETKIANK	P
1886.867	1886.912	FDDFSWGDTRTIAGGNK	
1894.830	1894.968	DGDVDNFEESSTQPINK	
2185.121	2185.073	DSQIILMSFLEKITFDER	
2200.986	2201.044	AFESVFGSVTCLPGCFS**MYR**	
2273.150	2273.186	AVHSRNPSTLLPTSSMFWNK	
2289.145	2289.168	AVHSRNPSTLLPTSSMF**WNK**	O

FASTA sequence of ChS3:

>NP_009579
MTGLNGDDPDDYYLNLNQDEESLLRSRHSVGSGAPHRQGSLVRPERSRLN
NPDNPHFYYAQKTQEQMNHLDVLPSSTGVNPNATRRSGSLRSKGSVRSKF
SGRETDSYLLQDMNTTDKKASVKISDEGVAEDEFDKDGDVDNFEESSTQP
INKSIKPLRKETNDTLSFWQMYCYFITFWAPAPILAFCGMPKKERQMAWR
EKVALISVILYIGAIVAFLTFGFTKTVCSSSKLRLKNNEVSTEFVVINGK
AYELDTSSRSGIQDVEVDSDTLYGPWSDAGKDASFLFQNVNGNCHNLITP
KSNSSIPHDDDNNLAWYFPCKLKNQDGSSKPNFTVENYAGWNCHTSKEDR
DAFYGLKSKADVYFTWDGIKNSSRNLIVYNGDVLDLDLLDWLEKDDVDYP
VVFDDLKTSNLQGYDLSLVLSNGHERKIARCLSEIIKVGEVDSKTVGCIA
SDVVLYVSLVFILSVVIIKFIIACYFRWTVARKQGAYIVDNKTMDKHTND
IEDWSNNIQTKAPLKEVDPHLRPKKYSKKSLGHKRASTFDLLKKHSSKMF
QFNESVIDLDTSMSSSLQSSGSYRGMTTMTTQNAWKLSNENKAVHSRNPS
TLLPTSSMFWNKATSSPVPGSSLIQSLDSTIIHPDIVQQPPLDFMPYGFP
LIHTICFVTCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGN
DKTTPEIALGMMDDFVTPPDEVKPYSYVAVASGSKRHNMAKIYAGFYKYD
DSTIPPENQQRVPIITIVKCGTPAEQGAAKPGNRGKRDSQIILMSFLEKI
TFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADTKVFPDALTHMVA
EMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFGSV
TCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDNVTNTLHKKNLLL
LGEDRFLSSLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINST
VHNLFELVLIRDLCGTFCFSMQFVIGIELIGTMVLPLAICFTIYVIIFAI
VSKPTPVITLVLLAIILGLPGLIVVITATRWSYLWWMCVYICALPIWNFV
LPSYAYWKFDDFSWGDTRTIAGGNKKAQDENEGEFDHSKIKMRTWREFER
EDILNRKEESDSFVA*

[a] A combination of 12 different peptides obtained after in-gel tryptic digestion from an isolated ∼116 kDa protein band were identified and correlated with the sequence of Chs3 (S. cerevisiae) [Acc. No. NP_009579 gi: 6319497], theoretical molecular weight ∼132 kDa. CM = calculated peptide mass; OM = observed peptide mass; M = peptide modification, either oxidation of methionine residues (O) or propionamide modification of cysteine residues (P). The different peptides are highlighted by identical colors in the table and in the FASTA sequence of Chs3.

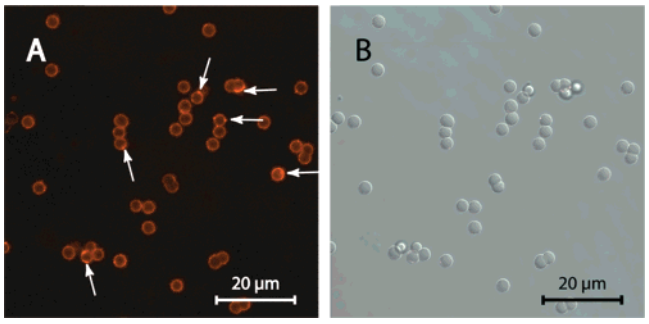

Figure 3. Confocal fluorescence (A) and the corresponding DIC (B) images of silica particles (Ø = 3 μm) after incubation with isolated chitosomal membrane extracts (Ø = 240 nm). Monodispersive membranes (the supernatants) are labeled with TRITC−DHPE lipids. A continuous fluorescence contour at the rim found for all the particles indicates a homogeneous coating with natural chitosomal membranes.

fluorescently labeled native chitosomal membranes. The comparison of the fluorescence and DIC images at several different

locations further confirms that all the beads captured by the microscopy are coated with chitosomal membranes.

To roughly estimate the particle surface coverage with the chitosomal membranes, the amount of lipid molecules on the particles are determined by phosphate analysis. After subtraction of the intrinsic phosphate contamination from naked silica particles, the amount of phosphate residues in the chitosomal membranes supported by 1 mg of silica particles is calculated out of three independent measurements to be 5.0 ± 1.2 nmol. As every phospholipid molecule has one phosphate headgroup, this value is taken as the amount of phospholipids on particles. From the bead radius ($r = 1.5$ μm) and the density of silica ($\rho_{silica} = 2.6$ g/mL), the accessible surface area of the certain weight of silica particles can be calculated. If one takes the mean area per one lipid molecule in a fluid phase (60 Å^2),[57] the calculated area of a lipid bilayer agrees reasonably well with the total surface area of silica particles within experimental errors (±20%). Thus, both optical microscopy and phosphate analysis consistently demonstrate the formation of continuous chitosomal membranes on particle supports.

Quantitative Evaluation of in Vitro Chitin Synthesis. Instead of commonly used enzyme activity assays in bulk solution, we use a highly sensitive radiochemical assay to determine the amount of chitin polymers synthesized in particle-supported chitosomal membranes. As mentioned in the previous section, the polymerization mixture with UDP−^{14}C-GlcNAc is added after a short-term of proteolytic trypsin activation of zymogenic chitin synthase. After the polymerization reaction is terminated, the particle-supported membranes can readily be separated from the bulk by a gentle sedimentation (100×g, 1 min) owing to the large density of silica particles. As schematically illustrated in Scheme 1, the presence of two fractions of chitin polymers can be expected: chitin polymers attached to the membranes, and chitin polymer released into bulk. The sum of these two fractions coincides with the total amount of the synthesized chitin polymers.

Figure 4, parts A and B, represents the UDP−^{14}C-GlcNAc radioactivity values obtained from (Figure 4A) the chitin polymer fraction attached to the particle-supported membranes and (Figure 4B) the chitin polymer fraction released into bulk after 3 h of polymerization, respectively. As the radioactive product is selected according to size (filter pore size >1.2 μm), the most promising candidate among the possible products is chitin polymers. All the presented results have already been corrected by subtraction of the radioactivity from the naked silica particles, and the mean values of three independent experiments are presented for each data set. The obtained results demonstrate that not all of the synthesized chitin polymers are released into bulk, but some of them are still attached to the membrane.

To ensure that the observed radioactivity signals are from the chitin polymers specifically synthesized by chitin synthase, we carry out the same series of radioactivity assays on the same particles coated with the human erythrocyte ghost membranes, which have no chitin synthase or specific interaction with chitin (Figure 4, parts C and D).

If one compares the radioactivity values from the particle-supported membranes, the signal from erythrocyte membranes (22 ± 9 cpm, Figure 4C) is much smaller than that from chitosomal membranes (183 ± 35 cpm, Figure 4A). The clear difference confirms that the nonspecific adsorption of UDP−^{14}C-GlcNAc monomers to the both types of supported membranes is negligibly small. The larger amount of chitin fibers produced by immobilized chitin synthase is released into bulk (328 ± 56 cpm, Figure 4B). On the other hand, a relatively high background radioactivity can be detected from the bulk solution of the control sample (83 ± 15 cpm, Figure 4D). As erythrocyte membranes have no chitin synthase, it is reasonable to assign this signal as the nonspecific adsorption of radioactive monomers (UDP−^{14}C-GlcNAc) to the reaction vial under the chosen assay conditions. Therefore, for this series of experiments, the thresholds of specificity for chitins attached to particle-supported membranes and chitins released in bulk are set to be 20 and 100 cpm, respectively. The high control value for radioactivity in the bulk in comparison to the particle-supported membranes could be explained by taking into account that 10× more UDP−^{14}C-GlcNAc was used in this assay due to several orders of magnitude less chitin synthase complexes than in the regular bulk assays.

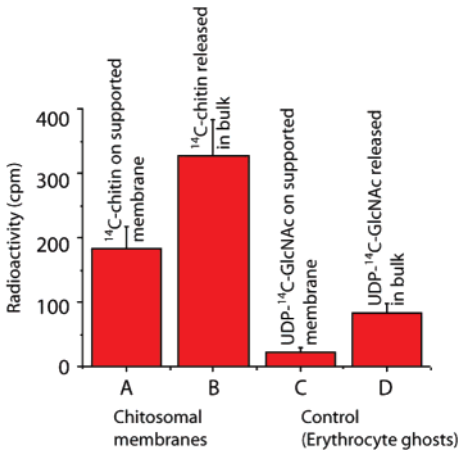

Figure 4. Quantitative measurement of the radioactivity from the ^{14}C-chitin polymers (A) attached to the particle-supported chitosomal membranes and (B) released into bulk. All the presented values are the mean values of three independent measurements after subtraction of the background signals. As the negative (and thus nonspecific) control system, the same assay with equal amount of UDP−^{14}C-GlcNAc was tested by deposition of erythrocyte ghost membranes (with no chitin synthase or specific interaction with chitin) on the same particles (C and D). The clear differences found between the specific (A and B) and nonspecific (C and D) systems confirm the specificity of biopolymerization in particle-supported chitosomal membranes.

For both chitin fractions, the clear differences between specific (chitosomal membranes) and nonspecific (erythrocyte membranes) systems verify the specificity of the assays used in this study. Taking these specificity thresholds into account, the obtained results suggest that about more than a half of the synthesized chitin polymers are released into bulk. In fact, the easy separation of the polymers on membranes from the bulk is one of the advantages of the particle-supported membranes for the quantitative study of biopolymerization in in vitro systems.

Since chitosomal membranes are deposited on particles of defined size and weight concentration, it is straightforward to evaluate the enzymatic function of chitin synthase in particle-supported membranes. As presented above, the confocal fluorescence image indicates that almost all the particles are continuously coated with chitosomal membranes, and the surface coverage calculated from the phosphate analysis suggests almost

(57) Chapman, D., Ed. *Biological Membranes*; Academic Press: New York, 1968.

a complete (120%) coverage of the bead surfaces. By assuming the weight balance between phospholipids and proteins to be 1:1,[58] the amount of protein on 1 mg of the particle supports can be calculated to be 3.5 μg. In fact, this value corresponds well with the protein amount on solid particles determined by the Bradford method (6.7 μg).

After the correction of the measured radioactivity by the background and the threshold level, the calculated amount of GlcNAc monomers polymerized into chitin polymers by 1 μg of immobilized protein is 5 pmol/h. By assuming that the fraction of chitin synthase (MW 132 000 g/mol) in the immobilized protein is 0.1−1%, the turnover ratio can roughly be estimated to be in the range of 1−11 min^{-1}. In comparison with the enzyme activity assays using chitosomes in suspension, the calculated enzymatic activity on particle supports is found to be approximately 1 order of magnitude smaller than that in suspension, which is 50 pmol/h. The lower enzymatic activity found on particle supports can be attributed to several possible scenarios, including (i) the diffusion-limited feed of activated UDP−^{14}C-GlcNAc monomers to the particle-supported membranes, (ii) the loss of short chitin oligomers through the filters, (iii) presence of multilayer stacks or local defects, or (iv) the decrease in the enzymatic activity on particle supports. Most of these problems can potentially be overcome by the use of finer filter pores or by providing a larger water reservoir under the membranes using polymer supports[10] or silica particles with porous surfaces.[15] Recently, we preliminarily succeeded in the deposition of chitosomal membranes on porous silica particles (data not shown) despite difficulties in determination of the accessible surface area (and hence the surface coverage). The density of porous particles (ρ_{porous} = 2.25 g/mL) is slightly smaller than that of solid particles (ρ_{silica} = 2.6 g/mL) so that one can use larger particles without undesired precipitation of the particles.[59] In fact, this would also offer further flexibilities to study the influence of the geometry and topography of the surface (e.g., particle size, pore size, and porosity) on the synthesis and self-assembly of chitin polymers in vitro.

(58) Guidotti, G. *Annu. Rev. Biochem.* **1972**, *41*, 731−752.
(59) To our knowledge, the largest size of monodispersive, smooth silica particle is ∅ = 3 μm. We have tried polydispersive glass beads (particle diameter ranges from 0.1 to 100 μm) but found that sedimentation of large particles often disturbs the homogeneous surface coating.

Conclusions

This study demonstrates that monodispersive, solid silica particles can be utilized as solid supports of a defined surface area for the deposition of native chitosomal membrane extracts. Particle-supported membranes can be used for quantitative investigation of in vitro biopolymerization by transmembrane chitin synthase complex, which can be considered as an alternative biophysical approach to large-scale fermentation or covalent linkage of soluble enzymes. Both confocal fluorescence microscopy and the phosphate analysis suggest that particle surfaces are almost fully covered with native chitosomal membranes. In contrast to planar solid supports, particle supports possess much larger surface area. Moreover, the large density of particle supports with respect to the membranes allows for quick separation of supported membranes from materials in bulk (within 1 min), which offers several unique advantages over chitosomal membrane extracts; for instance, the particle-supported membranes can readily be concentrated so to amplify the radioactive (or spectroscopic) signals from the membrane. Moreover, in contrast to the membrane-based assays, the polymerization reaction in particle-supported membranes can be terminated without the disruption of the whole membranes. Highly sensitive radioactive assays of concentrated samples enable one to identify small amounts of chitin polymers attached to the supported membranes as well as those in bulk in a quantitative manner. Thus, the well-defined, in vitro model system established in this study can potentially be used as a new biophysical tool for quantitative evaluation of the function of transmembrane enzyme complexes.

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