Controlled enhancement of transmembrane enzyme activity in polymer cushioned supported bilayer membranes

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Reconstitution of transmembrane proteins into supported lipid bilayer (SLB) membranes has often been hampered by strong interactions of protein domains with the underlying solid support leading to loss of both activity and mobility within the plane of the lipid bilayer. Polymer cushioned SLBs can overcome this by avoiding direct contact with the support. To extend this approach we developed an anionic polymer cushion system that allows tunable lipid mobility as well as functional integration of the transmembrane protein β-amyloid precursor protein cleaving enzyme (BACE) into SLBs. Fluorescence recovery after photobleaching analysis revealed a homogeneous distribution and high lateral mobility of the reconstituted BACE in cushioned SLBs while an impaired mobility and inhomogeneous clustering of reconstituted BACE were found in SLBs on silicon oxide substrates. The cushioning of SLBs led to increased incorporation and enhanced enzymatic activity of the reconstituted BACE with a direct correlation between lipid mobility and BACE activity. The utilized polymer cushion system allows the successful reconstitution of transmembrane proteins within SLBs with tunable properties.

Introduction

There is a strong interest among biologists to test reconstituted proteins *in vitro* with the help of biophysical analytical techniques as they provide a high level of precise functional determination. One of those methods is the reconstitution of transmembrane proteins into polymer cushioned supported lipid bilayer (SLB) membranes. SLBs on polymer supports receive increasing attention as they maintain the biological activity of the incorporated protein species and prevent denaturation of transmembrane proteins.[1,2] SLBs have been reconstituted on 'hard' solid supports (silica and mica surfaces) by lipid vesicle deposition and Langmuir–Blodgett techniques[3,4] and were subject of intense research since the pioneering work of McConnell *et al.*[5,6] However, on 'hard' supports the integration is limited to peripherally associated membrane proteins or proteins with lipid anchors (*e.g.* peripherally integrated membrane associated proteins and glycosylphosphatidylinositol (GPI)-anchored proteins). These membrane proteins are diffusing within the outer membrane leaflet,[7,8] but diffusion within the inner membrane is obstructed by protein surface interactions. In contrast, the functional integration of transmembrane proteins with demanding large extracellular and/or intracellular domains (size > 2 nm) is not possible on hard supports. The extramembrane domains will certainly be impaired by strong intermolecular interactions with the 'hard wall' and might precipitate upon contact with the 'hard wall'. Therefore polymer cushions and tethers allow the successful integration of large transmembrane proteins[1,9,10] as the polymer provides better solvent accessibility and a more feasible space for the protein's motion. The challenge for successful SLB reconstitution on polymer supports is the need to meet the inevitable prerequisites, such as hydrophilicity, a balance between electrostatic repulsion and attraction, and a lubricating water layer between the substrate surface and the SLB. Several strategies have been developed to achieve this aim, for example, pH responsive poly[2-(dimethylamino)ethyl methacrylate-*block*-methyl methacrylate] (PDMMA) diblock copolymer cushions,[11] poly(ethylene imine) (PEI) cushions,[12,13] and functional tethered lipopolymers,[10,14] *e.g.* poly(ethylene glycol) tethered lipopolymers.[15,16] Those approaches on polymer cushions (PDMMA and PEI) were mostly limited to a thickness of up to ~10 nm, thus limiting the size for the functional integration of transmembrane proteins. Tethered bilayers are another interesting approach: but these bilayers have the disadvantage of decreasing the lateral mobility of both bilayer lipids and integrated transmembrane proteins due to the grafting of lipopolymer molecules to the substrate surface. Hence, many of those studies have been focused on the integration and the functionality of ion channels[17] where free protein mobility is not a concern.

We overcome these limitations by utilizing a new polymer cushion system which is based on maleic anhydride copolymer thin films (Fig. 1).[18] We recently showed that copolymer films with the comonomer units ethene, propene, and octadecene can be used to tune lipid mobility in SLBs in dependence on the hydrophilicity of the polymer cushion layer.[19] Both the degree of hydrophilicity and swelling of the anionic polymer cushions with film thicknesses of up to 60 nm were found to determine the

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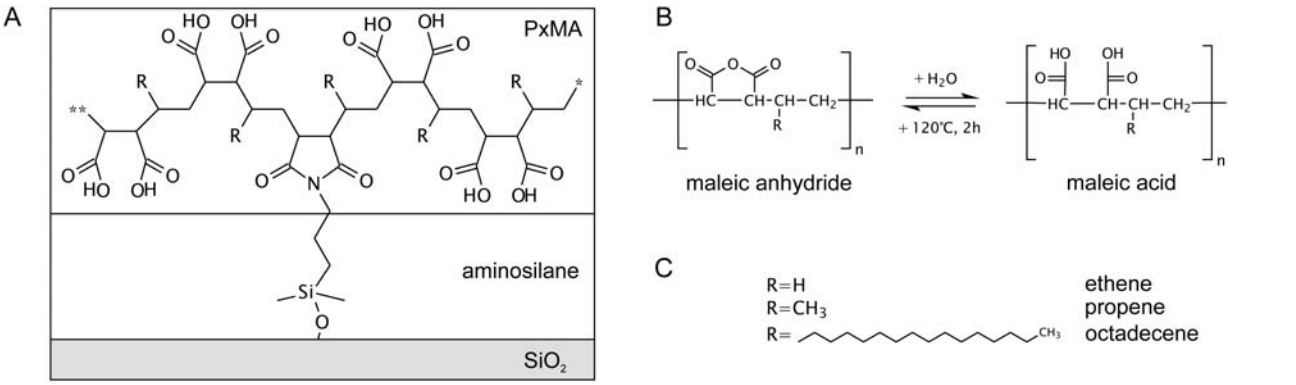

Fig. 1 (A) Schematic of covalently immobilized maleic acid copolymer layers. (B) The reversible hydrolysis between maleic acid and anhydride functionalities. (C) The side chains R vary with the type of comonomer unit (octadecene, propene or ethene).

mobility of the bilayer lipids with diffusion coefficients that range from 0.26 to 1.1 $\mu m^2 s^{-1}$.

We now show that this highly swollen anionic polymer cushion system can be advantageously applied to facilitate the functional integration of transmembrane proteins into SLBs. As a relevant example we use the β-amyloid cleaving enzyme (BACE), which plays an active role in Alzheimer's Disease (AD) as it is—together with γ-secretase—involved in the process of amyloid precursor protein (APP) degradation, which is thought to be one of the hallmarks of AD.[20–22] The cleavage of APP causes the formation of a β-amyloid peptide, which can further polymerize into oligomers and lead to plaque formation and deposition in between neuronal cells. Because of the central role of BACE in AD it is of medical interest to study its function in more detail using different *in vitro* techniques. Using fluorescence microscopy, fluorescence recovery after photobleaching (FRAP), and *in situ* activity assays we demonstrate a homogeneous integration, high mobility, and superior enzymatic activity of the incorporated BACE in the polymer cushioned SLBs when compared to BACE in silica SLBs.

Experimental

Liposome preparation and SLB formation

Two distinct lipid mixtures were used for SLB formation. Small unilamellar lipid vesicles (SUVs) were prepared by the common extrusion technique: egg phosphatidylcholine (PC), egg phosphatidylserine (PS), egg phosphatidylethanolamine (PE), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE, Avanti Polar Lipids, Alabama, USA), and sulforhodamine 1,2-dihexadecanoyl-glycero-3-phosphatidylethanolamine triethylammonium salt (Rho-PE) were dissolved in chloroform. The lipids PC : PE : PS were mixed in the molar ratio 6 : 2 : 2, respectively. The chloroform was removed under a stream of argon and the lipids were dried in vacuum overnight. The dried lipid cake was hydrated in pH 4 saline solution (140 mM NaCl with 1 mM $MgCl_2$ at pH 4) with a concentration of 4 mg mL^{-1} (overnight) and the mixtures were extruded (Mini Extruder, Avanti Polar Lipids, Alabama, USA) at least 31 times through 50 nm diameter pores of a polycarbonate membrane (Whatman Ltd., UK) following the procedure of Hope *et al.*[23] The average size of the used SUVs was determined to be in the range of 80–100 nm by dynamic light scattering. The stock solution was stored below 4 °C and consumed within two days. All lipids and chemicals—if not else stated—were purchased from Sigma-Aldrich and used without further purification. SLB formation was performed by direct suspension of a 0.2 mg mL^{-1} liposome solution on top of silicon oxide surfaces or the polymer supports.

Preparation of polymer supports

Thin films of poly(octadecene-*alt*-maleic anhydride) (POMA) (Polysciences Inc., Warrington, PA) (M_w 50 000), poly(propene-*alt*-maleic anhydride) (PPMA) (Leuna-Werke AG, Germany) (M_w 6000), and poly(ethene-*alt*-maleic anhydride) (PEMA) (Aldrich, Munich, Germany) (M_w 125 000) were produced by spin coating (RC5, Suess Microtec, Garching, Germany) 0.08%, 0.06%, and 0.03%, respectively, copolymer solutions in tetrahydrofuran (Fluka, Deisenhofen, Germany), methyl ethyl ketone (Fluka), and 1 : 1 THF : acetone (Acros Organics, Geel, Belgium), respectively, on top of square glass coverslips (Corning B.V. Life Sciences, Netherlands) (compare Fig. 1). The polymer films were spin-coated at 4000 rpm and an acceleration of 1500 rpm s^{-1} for 30 s. Before polymer coating the coverslips have been freshly oxidized in a mixture of aqueous solutions of ammonia (Acros Organics, Geel, Belgium) and hydrogen peroxide (Merck, Darmstadt, Germany) and were subsequently surface-modified with 3-aminopropyltriethoxysilane (ABCR, Karlsruhe, Germany) prior to spin-coating of the copolymer solutions to allow a covalent fixation of the thin copolymer films.[18] Stable covalent binding of the polymer films to the glass carriers was achieved by annealing at 120 °C for 2 h. The polymer films were thoroughly characterized with respect to water contact angle, film thickness, surface roughness, and chemical composition in dry and wet conditions, as recently published.[18,19] For comparative studies silica glass slides were cleaned as explained above. Prior to the direct application of lipid vesicle solution silica surfaces have been treated in an oxygen plasma chamber (Harrick Plasma, Ithaca, USA) for two minutes.

For BACE-activity measurements polystyrene 96-well plates (Greiner Bio-One, Germany) were modified to prepare polymer functionalized substrate surfaces. For this purpose 96-well plates were dried overnight in a vacuum oven and modified by

ammonium–plasma for 300 s at 400 W, a pulse frequency of 1000 Hz, a duty cycle of 5%, an ammonia gas flow of 15 standard cubic centimeters per minute, and a pressure of 7×10^{-3} mbar. Immediately after plasma modification the surfaces were immersed in solutions of hydrolyzed 0.1% PEMA (in water), 0.1% PPMA (in water), or 0.1% POMA (in isopropanol). The surfaces were dried at RT, followed by annealing at 90 °C for at least 48 h to achieve covalent attachment of the copolymers to the amine groups of the plasma modified polystyrene plates. Subsequently the surfaces were rinsed extensively with water and placed in water for 24 h to remove unbound polymer and gently dried under a stream of nitrogen. Prior to the sample application the surfaces were hydrolyzed in water for 24 h to convert maleic anhydride bonds into maleic acid groups.

BACE production and purification

The protocol follows in general the purification of BACE by Kalvodova *et al.*[24] with minor changes. The preparation was kept on ice at 4 °C. Briefly, 1 litre SF+ cells were harvested 64 h after infection with BACE baculovirus. The cells were resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 250 mM sucrose, protease inhibitors with E64, pH 7.25). The cells were lysed with a dounce and the extract was centrifuged for 45 min at 140 000*g*. The pellet was resuspended in 50 mM HEPES, 150 mM NaCl, 1% Triton X-100 (TX-100), 5% Glycerol, protease inhibitors with E64, pH 7.25 and incubated for 30 min at 4 °C. The pellet was spun down for 45 min at 180 000 × *g* and the supernatant was loaded on a 2 mL IgG column. After 15 min of incubation, the column was extensively washed with running buffer (50 mM HEPES, 150 mM NaCl, 0.5% Triton, 5% Glycerol, 0.2 mM EDTA, pH 7.25), whereupon the column volume was replaced at least 10 times. After washing, the column was incubated with 50 μL of PreScission protease (1 mg mL^{-1}) for 1.5 h at 4 °C. BACE was collected by eluting with running buffer. For fluorescence experiments BACE was either labeled with Alexa488 or Alexa647 (Molecular Probes, Oregon, USA), without loss of enzymatic activity. BACE was stabilized in solution with 0.05% TX-100.

BACE integration

BACE in different solution concentrations (6, 12, and 22.7 nM) was directly added onto previously formed SLBs by applying octylglycoside (OGL) (0.5 mM) well below the critical micelle concentration (CMC) (25 mM) to soften the SLB as described below, followed by extensive rinsing to remove unbound detergent (10 cycles). The detergent concentration of TX-100 was kept constant at 0.0015% for all solutions. SLBs have been incubated with the protein for 10 min. After incubation the SLBs were extensively rinsed with HEPES buffer (pH 7.2) (at least 10 cycles) to remove non-incorporated BACE.

A complementary approach of confocal laser scanning microscopy (cLSM) and ^{125}I-labeled BACE adsorption analysis was directed to quantify the integrated amount of BACE in SLBs. To correlate the fluorescence intensities BACE was adsorbed on POMA surfaces with fluorescently labeled BACE (cLSM) and ^{125}I-labeled BACE (radio labeling). BACE was ^{125}I-labeled following an established protocol (Pierce, Rockford, IL). Briefly, 1 mCi $Na^{125}I$ was dissolved in 100 μL PBS and 1 iodobead was added into the solution. After incubating the iodobead in the $Na^{125}I$ solution for 5 min 100 μL BACE solution was added (conc. approx. 1 mg mL^{-1}). The protein solution was incubated for 20 min, and the reaction was stopped by removing the iodobead from the solution. Unreacted $Na^{125}I$ was removed on a NAP-5 column (GE Healthcare, Freiburg, Germany). The end product ^{125}I-labeled BACE at concentrations between 6 and 22.7 nM was incubated on POMA substrates for 10 min and the adsorbed amount was determined after several rinses in PBS by a gamma-counter (UMo LB 123, Berthold Technologies).

Confocal laser scanning microscopy

SLBs have been analyzed with 0.1 mol% fluorescently labeled NBD-PE or Rho-PE. FRAP experiments and distribution analysis of the SLB with and without incorporated BACE have been undertaken on a Leica confocal microscopy setup (Leica, Heidelberg, Germany) with Ar and HeNe excitation laser lines (488 nm, 543 nm, 633 nm) for the excitation of NBD-PE, BACE-Alexa488, Rho-PE, and BACE-Alexa647, respectively.

The diffusion coefficients of lipid and BACE mobility were estimated from FRAP measurements using the approach of Soumpasis.[25] The bleaching area and the diffusion coefficient were subjected to corrections using the approach suggested by Weiss and Nilsson.[26,27]

BACE activity assay

For BACE activity measurements polystyrene 96-well plate was used consisting of silica and polymer modified coated surfaces (see polymer preparation). SLBs (mixtures PC, PE and PS) were formed as previously described and BACE was integrated from solution. The amyloid precursor substrate analogue FS-1 from BACHEM (Torrance, CA, USA) was cleaved by BACE in a FRET-based activity assay. The solution was replaced by sodium acetate buffer (pH 5.4). The measurements were conducted on a Fluoroskan Ascent CF plate reader (Thermo Fisher Scientific, USA) at 37 °C. Upon cleavage the substrate emits a fluorescence signal at 485 nm while excited at 355 nm.[28] The FS-1 substrate was dissolved in DMSO and no more than 1 μL of substrate was added on top of the SLB. Activity measurements were repeated at least 3 times.

Results and discussion

Bilayer formation

We have shown that SLBs of a mixture of naturally occurring lipids can be formed on maleic acid copolymer supports with a cushion thickness of up to 60 nm.[19] Moreover, we found that the diffusion behavior of lipids strongly depended on the physicochemical properties of the underlying substrate (see Table 1) with increasing surface hydrophilicity and charge enhancing lipid mobility. Thereby the lipid mobility could be adjusted from 0.26 to 1.1 $\mu m^2\ s^{-1}$ proposing a tool for modulating the function of SLBs containing reconstituted transmembrane proteins for specific applications.

Table 1 Summary of polymer substrate properties

	POMA	PPMA	PEMA
Thickness[a] pH 7.4 (±0.5 nm) (hydrolyzed)	4 nm	25 nm	60 nm
Thickness[a] pH 3 (±0.5 nm) (hydrolyzed)	4 nm	20 nm	45 nm
COOH group surface density[b]	6×10^{13} cm^{-2}	7×10^{14} cm^{-2}	1.2×10^{15} cm^{-2}
Water contact angle[c] (±3 deg)	100°	38°	26°
Lipid diffusion coefficient[d]	0.26 ± 0.1 μm^2 s^{-1}	0.6 ± 0.12 μm^2 s^{-1}	1.1 ± 0.2 μm^2 s^{-1}

[a] Determined by QCM-D. [b] Determined by XPS after methionine amide conversion. [c] Measurement of hydrolyzed copolymer surfaces. [d] Determined by FRAP measurements, data taken from ref. 18 and 19.

BACE integration

In order to investigate how membrane protein function was influenced by the nature of the substratum, we introduced the integral membrane protease BACE into SLBs. There are several strategies for integrating proteins into SLBs including (i) reconstitution of proteins into SUV (proteoliposomes) followed by their deposition and fusion onto the supporting surface,[29] (ii) fusing proteoliposomes to the outer leaflet of a Langmuir–Blodgett deposited lipid monolayer,[30] or finally, (iii) direct incorporation of detergent solubilized proteins into a pre-formed bilayer (termed the detergent removal technique).[31] The latter method proved best for BACE incorporation. First, the bilayer was softened by incubation for 5 min with a low concentration (0.5 mM) of the detergent OGL, which was proved in earlier experiments to leave the SLB integrity mainly unaffected. Then, after rinsing with buffer, solutions containing various concentrations of BACE were added and incorporation was monitored by fluorescence microscopy over 10 min. BACE started to spontaneously integrate into SLBs supported by silica surfaces and increasing levels were detected over time (Fig. 2A–C). Accordingly, a monotonic increase in BACE fluorescence and a slight decrease in lipid fluorescence were observed (Fig. 2D and E). The integration of BACE into silica supported SLBs appeared to occur in three stages: (i) adsorption of BACE from the bulk solution onto the distal lipid leaflet (t < 1 min), (ii) insertion of single BACE molecules with (iii) the possible subsequent adsorption and formation of BACE clusters (t > 2 min). It was difficult to experimentally distinguish between adsorption and insertion stages. Therefore, the formation of BACE clusters might have already occurred during the process of insertion.

As the clusters observed on silica supports likely represented inactive or less active aggregates of BACE, the same experiment was performed by BACE insertion into SLBs pre-formed on polymer supports (Fig. 3). Over time, the integration of BACE (at 6 nM) on cushioned SLBs caused a monotonic decrease in lipid fluorescence and a monotonic increase of BACE fluorescence until saturation was reached between 4 and 5 min after addition (Fig. 2D and E). No further uptake was observed between 5 and 10 min. Hence, this observation was in accordance with the results for the integration of BACE on silica substrates. In contrast to silica SLBs, polymer cushioned SLBs showed little if any clustering of BACE molecules upon insertion (compare Fig. 3C). BACE was found to be homogeneously distributed within polymer cushioned SLBs.

The timelapse studies in Fig. 2 and 3 indicated the effective integration of BACE into the SLB. The simultaneous increase in the fluorescence signal of BACE and decrease of the fluorescence signal of the lipids suggested for the insertion of BACE. As shown later, differences in the mobility of integrated BACE confirmed the incorporation of the protein in SLBs as the

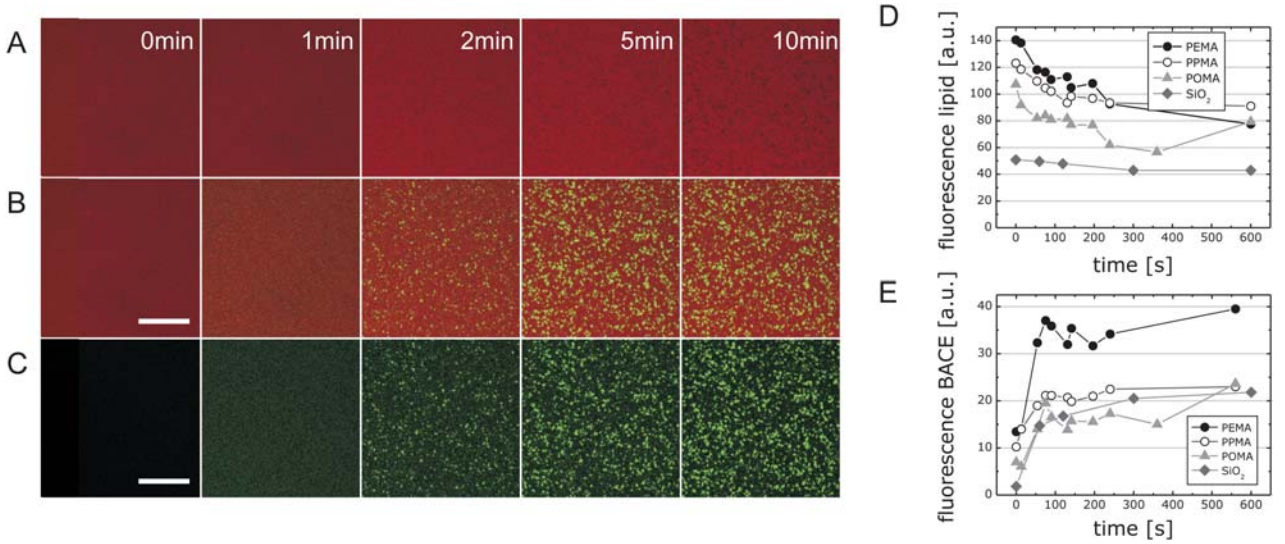

Fig. 2 Timelapse of BACE integration into a SLB on a silica surface: images acquired after the indicated times of incubation of the SLB with 6 nM BACE: (A) lipid (red), (B) merge, and (C) BACE in green (bar size 10 μm). Fluorescence intensity *vs.* time of (D) lipid and (E) BACE for the integration of BACE into SLBs at a BACE concentration of 6 nM in solution.

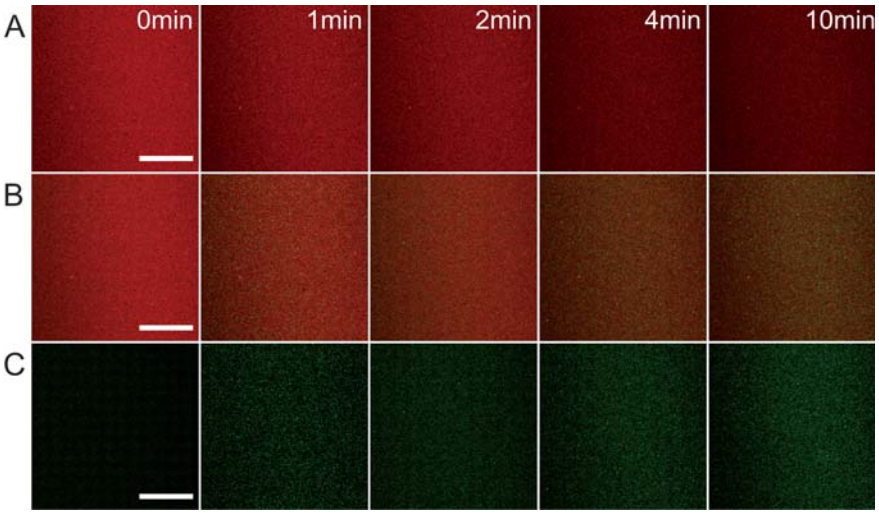

Fig. 3 Timelapse of BACE integration into a SLB on a polymer cushion substrate (PEMA): images acquired after the indicated times of incubation of the SLB with 6 nM BACE: (A) lipid (red), (B) merge, and (C) BACE in green (bar size 10 μm).

observed effects can only be explained by protein interactions with the support. Hence, these data clearly show that BACE readily integrated into SLBs by the detergent removal technique. Moreover, polymer cushioned SLBs allowed for a more homogeneous distribution of BACE molecules.

We intended to determine the relative levels of BACE incorporated in SLBs supported by different surfaces to evaluate if the charge and hydrophilicity of the cushion affected the recruitment. We quantified the amount of integrated BACE over a range of concentrations from 6 to 22.7 nM. A strong linear dependence of the amount of integrated BACE on BACE solution concentration was only detected for PEMA surfaces (Fig. 4A). Only minor increases in BACE incorporation from solutions of increasing concentration were observed on the other surfaces. On silica, incorporation was nearly independent of the solution concentration. Moreover, in the order SiO_2 < POMA < PPMA < PEMA, an increase in the absolute BACE amount was observed (Fig. 4A). Strikingly, the order of this series was identical to the mobility of the lipids on the different substrates except for the mobility on the hard silica support (Table 2). (We measured the lipid diffusion coefficients (Table 2) for each SLB after the integration of BACE and the observed values were comparable to the previously determined lipid mobilities (Table 1, ref. 19). This fact supported the above finding that the application of detergent did not significantly alter the integrity and properties of the SLBs.) The polymer cushions apparently provide a better environment for the integration of BACE at all concentrations. The best integration was found on PEMA substrates which might correlate to the higher lipid mobility of SLBs on PEMA. However, lipid mobility alone is not a sufficient determinant for an efficient BACE incorporation as BACE did not homogeneously integrate into SLBs on silica. Furthermore, one can even correlate the efficacy of BACE integration with the thickness of polymer cushion (Table 1). A higher thickness of the

Table 2 Summary of diffusion coefficients (for BACE and lipid) and mobile fractions as determined by FRAP for BACE in SLBs on silica and polymer substrates

Substrate	Diffusion BACE coeff./μm^2 s^{-1}	Mobile fraction (%)	Diffusion lipid coeff./ μm^2 s^{-1}
Glass	0	0	2.55 ± 0.17
PEMA	0.11 ± 0.024	79 ± 19	1.24 ± 0.14
PPMA	0.04 ± 0.026	60 ± 12	0.62 ± 0.06
POMA	0.055 ± 0.039	70 ± 13	0.25 ± 0.08

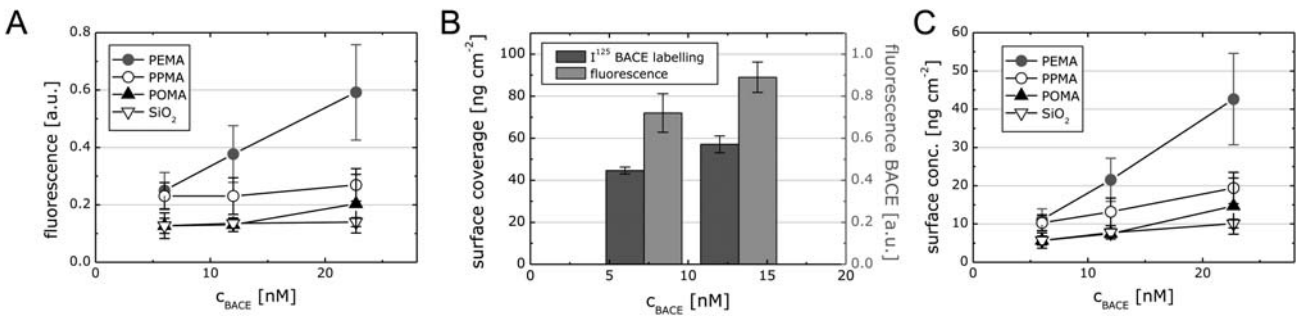

Fig. 4 Amount of BACE integrated from solutions at varying concentrations into SLBs on different supports. (A) Integration quantified by absolute fluorescence. (B) Comparison of fluorescence intensities of fluorescently labeled BACE with levels determined by ^{125}I-labeled BACE after adsorption of POMA surfaces in order to calibrate BACE concentrations in SLB experiments. (C) Calibrated surface concentration of BACE in SLBs.

swollen polymer cushion led to a better integration of BACE, which was possibly triggered by a decrease of protein–support interactions due to higher cushion swelling. This observation emphasizes the importance of polymer cushions for transmembrane protein integration in SLBs by decreasing or interrupting the direct protein interactions with the solid support.

To convert BACE fluorescence intensity measurements (Fig. 4A) into surface concentrations, ^{125}I-labeled BACE was adsorbed onto control POMA surfaces *i.e.* without overlaying SLBs. Based on the radio-labeling analysis, we were able to extract ratios for the BACE concentrations between the ^{125}I-labeled and the fluorescently labeled BACE protein adsorbed onto control POMA surfaces shown in Fig. 4B. These ratios were used to calculate the surface concentration of reconstituted BACE in the various polymer SLBs (Fig. 4C).

BACE mobility

One of the rationales for developing this platform for studying the function of transmembrane proteins integrated into SLBs was the premise that thick, hydrophilic cushions might prevent strong interactions or even irreversible adsorption to the substrate underlying the lipid bilayer. Hence, this characteristic should enhance the mobility and functionality of reconstituted transmembrane proteins. After the integration of BACE into SLBs, we used the FRAP method to examine the diffusion behavior of the lipids and the transmembrane protein in the different SLBs. When fluorescently labeled BACE was locally bleached by intense laser illumination, no recovery of fluorescence was observed for BACE incorporated into lipid bilayers supported by silica surfaces without polymer cushions. In contrast, rapid diffusion of BACE was detected in polymer cushioned SLBs although significantly lower than the lipid mobility (Table 2). We observed significant differences in BACE mobility between PEMA and PPMA/POMA cushions with a two-fold increase in PEMA SLBs. In addition, the mobile fractions were similar (from 60–80%) on all polymer cushioned SLBs. This implies that 20–40% of the protein species were immobile either due to strong interactions with the substrate support or due to interactions between large protein aggregates with low mobility. It is not clear if the existence of two populations of BACE—one with high mobility and the other stationary—reflects heterogeneities of the polymer cushion or clustering of BACE protein prior to integration in SLBs. The strong scatter in the data might suggest that a heterogeneous polymer cushion layer leads to partly mobile or otherwise strongly coupled BACE molecules. The other possible explanation is the clustering of BACE molecules in solution prior to the integration in SLBs causing decreased mobility and stronger support interactions due to the larger size of the aggregates.

Our BACE mobility data can be partially compared to a recent study of Purrucker *et al.*[32] who examined the lateral mobility of integrin cell membrane receptors in polymer tethered SLBs and found similar diffusion coefficients for the integrins. Nonetheless, they only observed mobile fractions of about 20% consistent with comparable work on cellulose cushioned SLBs.[33] The obtained results clearly support the beneficial behavior of unrestricted lipid and protein motion in polymer cushioned SLBs for our highly swollen polymer cushion system. The polymer supports employed in our study permit greater membrane lipid and protein mobility compared to previously described systems.

The correlation between lipid and BACE mobility leads us to two conclusions: (i) the cushioning of SLBs by swollen hydrophilic polymer layers is a prerequisite for the mobility of transmembrane proteins with demanding extramembrane domains (*i.e.* BACE, as it prevents the strong interaction and immobilization of the transmembrane protein on the hard support); and (ii) the mobility of transmembrane proteins decoupled from any strong interaction with a solid support is correlated to the mobility of surrounding lipids in the SLBs.

BACE activity

In addition to an enhanced lateral mobility, polymer cushioned SLBs should enhance the enzymatic activity of the incorporated transmembrane protein when compared to SLBs on solid surfaces. The activity of BACE at various concentrations was examined after integration into SLBs using a fluorescent peptide substrate mimicking the cleavage site of APP (FS-1, see Material and methods), a natural target of BACE proteolysis found in membranes.[20,21] The rates of substrate cleavage (fluorescence intensity) were found to be higher with increasing BACE concentrations (Fig. 5A). The general outcome of the measurements suggested that the higher the concentration of integrated BACE the higher the substrate conversion, compare Fig. 4C and 5A. PEMA SLBs always showed the highest substrate

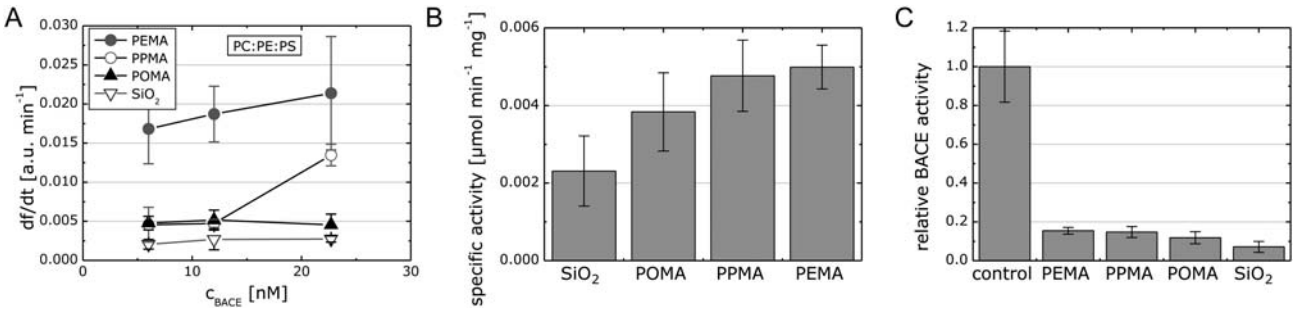

Fig. 5 (A) Activity measurements of integrated BACE in SLBs on the respective supports SiO_2, POMA, PPMA, and PEMA. BACE concentrations were 6, 12, and 22.7 nM. Comparison of absolute BACE activity (fluorescence per time step df/dt) in SLBs. (B) Mean specific activity calculated from the cleavage of the substrate FS-1 per BACE molecule in respect to the integrated BACE amount as estimated from ^{125}I-labeling data (see Fig. 4). (C) Comparison of relative BACE activity in solution (control) and of BACE incorporated into SLBs on different substrate surfaces.

conversion followed by PPMA SLBs which had comparable conversion rates to POMA SLBs at low BACE : lipid ratios. A minor increase in substrate cleavage was detected for silica SLBs with increasing BACE concentrations. We hypothesize that the higher substrate conversion rates on the polymer supports can be correlated to the functional effect of the polymer cushioned SLBs taking up higher amounts of BACE, preventing clustering, and maintaining a higher degree of lateral mobility of the integrated BACE species.

A conversion from the totally observed substrate into single enzyme activity enables the direct comparison of BACE activity between the different SLBs. The specific activity of BACE enzymes was calculated according to the specific conversion rate and the total mass of integrated BACE molecules (as determined from calibrated data in Fig. 4C) in dependence of the substrate, as presented in Fig. 5B. The results proved an enhanced BACE activity on the polymer cushions given the fact that one compares the activity per integrated molecule (Fig. 5B). BACE integrated in SLBs on a polymer cushion converted 1.5–2.5× more FS-1 substrate per minute than BACE in silica SLBs (Fig. 5B). Hence, polymer cushioned SLBs not only provided a higher mobility and homogeneous distribution of integrated BACE molecules but further preserved a higher enzymatic activity of BACE.

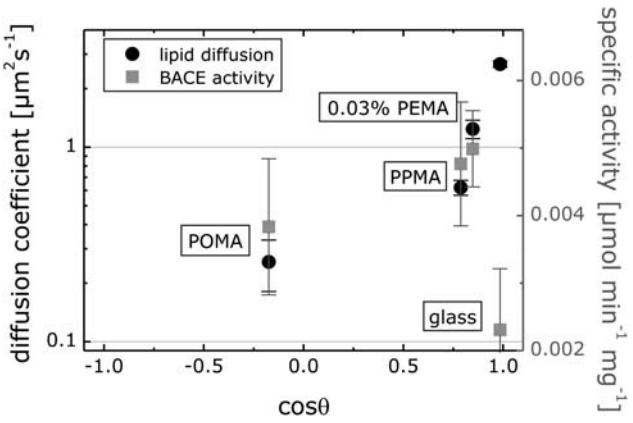

Fig. 6 Comparison between lipid diffusion and specific BACE enzymatic activity data in dependence of substrates interfacial energy expressed by cos θ as integrative measure of interfacial forces.

We also compared the BACE activity in SLBs on the different substrates with the solution enzymatic activity (Fig. 5C). The activity of the incorporated BACE protein ranges from approx. 16% (PEMA) to 8% (SiO_2) of the total solution activity. The activity of the incorporated BACE was significantly lower compared to the free enzyme. This difference can be attributed first of all to the type of activity assay, which favors the free access and high diffusivity of BACE in solution (when compared to the 2D presentation of BACE incorporated in SLBs). Additionally, differences may arise from the immobile fractions of incorporated BACE in the SLBs (see above) and errors in the estimation of incorporated BACE in the bilayer.

By plotting the specific BACE activity in SLBs and the diffusion coefficient data of lipids taken from Table 2 *versus* cos θ (θ—water contact angle of respective surfaces, see ref. 19) we can show the dependence of BACE activity and lipid mobility on the substrate properties (Fig. 6). cos θ is regarded as an approximated integrative measure of interfacial forces important in SLB formation like electrostatic interaction, hydration force, van der Waals interaction, and steric forces[34] as already discussed in ref. 19, which is used herein only to roughly characterize the different substrates. It can be clearly shown that the specific BACE activities unambiguously follow the trend of the lipid diffusion data—except for the activity data of BACE in silica SLBs. This supports our hypothesis that substrate controlled lipid mobility determines protein mobility and activity, as long as the extramembrane protein domains are efficiently shielded from the contact with the solid support by polymer cushions. The comparison shows that the careful adjustment/tuning of the polymer support can be used to obtain SLBs with enhanced transmembrane protein activity and lateral mobility.

Conclusions

In the present study, we compared the integration, mobility, and activity of the transmembrane protein BACE in SLBs on silica surfaces and polymer cushions with varying physico-chemical characteristics. The integration of BACE from solution by the detergent-removal technique resulted in an enhanced BACE uptake in SLBs on the more hydrophilic polymer cushions. Activity studies on the SLBs were consistent with the amount of

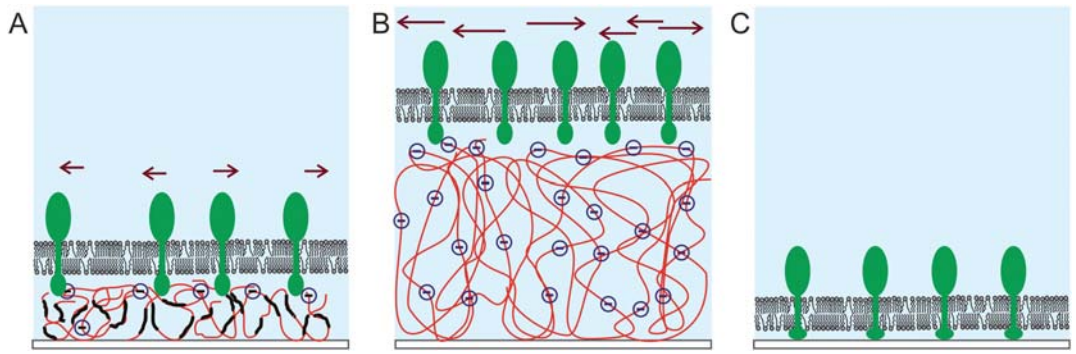

Fig. 7 Comparison between BACE mobility in polymer cushioned and silica SLBs, demonstrating the effects of a polymer cushion to enhance BACE mobility (arrows indicate the magnitude of diffusion coefficient and mobile fraction of BACE). The schematic drawings depict BACE integrated in a (A) more hydrophobic cushioned, (B) hydrophilic cushioned, and (C) silica/solid SLB.

integrated BACE. Normalization of the enzymatic activity data to the total integrated protein mass confirmed that the BACE activity is effectively enhanced on polymer cushioned SLBs in comparison to silica supports additionally correlating with BACE mobility in polymer cushioned SLBs (Fig. 6). These results corresponded to the dependence of BACE mobility on lipid mobility for polymer cushioned SLBs. We tried to summarize our observations schematically in Fig. 7: it could be convincingly shown for the integration of BACE in polymer cushioned SLBs that cushioning leads to a decoupling of the transmembrane protein from the solid support in the first place. In this case the protein's mobility and activity were directly correlated to the viscosity of the SLBs. On the contrary, the mobility and activity of BACE were strongly inhibited by the integration into SLBs on solid substrate surfaces.

Altogether, SLBs on maleic acid copolymer supports had significant beneficial effects with respect to the functional integration of the transmembrane protein BACE. The introduced polymer cushion system is therefore considered a valuable extension and will be further utilized for the *in vitro* reconstitution of functional proteins in model lipid membranes.

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