

Myelin basic protein binding is modulated by leaflet asymmetry and lipid composition

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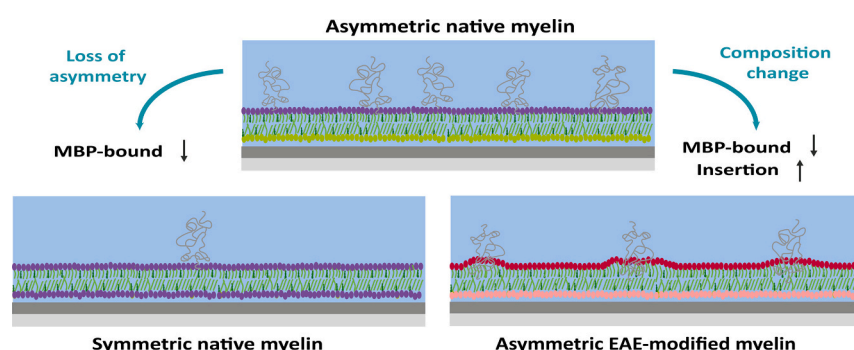
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HIGHLIGHTS

- Silicon-supported asymmetric myelin bilayers capture realistic lipid complexity.
- Neutron reflectometry with deuterated lipid reporters resolves leaflet asymmetry.
- Native-like asymmetric myelin membranes show higher Myelin Basic Protein coverage.
- Thermal lipid redistribution or symmetric bilayers sharply reduce adsorbed protein.
- Bilayer contact compacts protein; disease-mimetic lipids widen membrane spacing.

GRAPHICAL ABSTRACT



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ABSTRACT

Compositional asymmetry between lipid bilayer leaflets is a defining feature of biological membranes and can modulate protein binding. Here, we investigate how leaflet-specific composition affects the interaction between Myelin Basic Protein and biomimetic myelin membranes using neutron reflectometry. Asymmetric supported myelin bilayers containing deuterated cholesterol in the cytoplasmic leaflet enabled resolution of structural asymmetry. Neutron reflectometry measurements show that Myelin Basic Protein binds preferentially to asymmetric supported bilayers mimicking native myelin, whereas *Experimental Autoimmune Encephalomyelitis*-modified compositions exhibit weaker binding and more pronounced protein insertion. Disruption of

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asymmetry—either by thermally induced lipid redistribution or by using symmetric cytoplasmic myelin—leads to a marked reduction in Myelin Basic Protein binding, despite increased membrane charge. Following vesicle adsorption and formation of a second bilayer, the protein layer narrows to near *in vivo* thickness in both systems, with the diseased condition exhibiting a wider inter-bilayer spacing. These results underscore the relevance of lipid asymmetry and composition in governing protein–membrane interactions, with implications for the molecular basis of myelin stability and demyelination.

1. Introduction

Biological membranes play a central role in maintaining cellular integrity, enabling signal transduction, and regulating metabolic processes. A thorough understanding of the physicochemical properties of membrane constituents—and their interactions with both peripheral and integral membrane proteins—is essential to elucidate the molecular mechanisms underlying physiological function and disease. Among the various pathologies linked to altered membrane architecture, neurodegenerative disorders involving myelin sheath degradation stand out for their complexity and their significant impact on human health and disease [1].

Myelin is a multilamellar lipid membrane that surrounds neuronal axons in the central and peripheral nervous systems, providing electrical insulation and allowing the rapid saltatory propagation of action potentials [2]. It contains a diverse mixture of lipids, differing in head-group chemistry, acyl chain length, and saturation. Cholesterol is also abundant, accounting for approximately 35 mol% of the membrane [3]. The cytoplasmic leaflet faces the axoplasm, whereas the periplasmic leaflet is oriented toward the extracellular apposition; these two leaflets differ in lipid composition, giving rise to a pronounced bilayer asymmetry [4].

The structure of myelin *in vivo* has long been the subject of extensive investigation, particularly through the use of X-ray techniques [5,6]. These studies have demonstrated that, under proper cryoprotective conditions, the ultrastructure of myelin can be preserved with minimal alteration, allowing compacted and native-period membrane phases to coexist within the sheath. Purified myelin membranes from living organisms have also been widely studied using various model membrane approaches [7–9].

In recent years, research has moved toward biomimetic systems that more accurately represent the lipid complexity and leaflet asymmetry of native myelin [10,11]. These models have been employed to simulate both healthy and pathological states, for example by using lipid compositions derived from Experimental Autoimmune Encephalomyelitis (EAE), the standard animal model for Multiple Sclerosis (MS).

Myelin compaction depends on Myelin Basic Protein (MBP), which anchors apposed cytoplasmic leaflets of oligodendrocyte membranes and helps maintain the close packing of compact myelin [12]. MBP–membrane interactions have therefore been examined in liposomes and supported bilayers that reproduce key features of the cytoplasmic myelin interface [13–15]. These studies show that lipid composition influences how the membrane responds to the protein. Membranes with disease-related lipid profiles are more prone to MBP-induced instabilities, including loss of compact stacking, with features reminiscent of demyelination [16,17]. In SAXS and SANS experiments from our group, MBP induced aggregation of both native cytoplasmic and EAE-modified myelin vesicles, but only vesicles with the native composition developed multilamellar stacks over time [18]. Native cytoplasmic myelin membranes also bind MBP more strongly than EAE-modified membranes, which have lower bending stiffness [18–22]. MBP binding, then, cannot be reduced to surface charge. In myelin-like systems, the interaction also depends on sphingomyelin/cholesterol-dependent packing and membrane mechanics [23,24].

Most of this work, however, has focused on symmetric cytoplasmic models, leaving the specific contribution of transbilayer lipid asymmetry to MBP recruitment much less clear. The architecture of myelin

makes it difficult to disentangle the contributions of each leaflet and their coupling to MBP. Leaflet-asymmetric model membranes—ranging from asymmetric liposomes and giant unilamellar vesicles (GUVs) to supported bilayers—provide a controlled route to address this problem by tuning the composition of the two monolayers independently [25–31]. Work in this area is also beginning to clarify how transbilayer asymmetry reshapes bilayer organization and modulates membrane–protein interactions [32,33]. Recent studies further suggest that leaflet asymmetry can influence protein insertion [34], peptide activity [35], and membrane protein folding [36], indicating that the transbilayer distribution of lipids can be functionally relevant even when the overall bilayer composition remains unchanged. A quantitative mechanistic understanding of these effects, however, remains limited for multicomponent mixtures.

In the present work, a combination of Langmuir–Blodgett (LB) and Langmuir–Schaefer (LS) techniques was employed to fabricate planar asymmetric myelin membranes on silicon substrates. The incorporation of a deuterated lipid such as d_{45} -cholesterol in the cytoplasmic leaflet enabled contrast variation in neutron reflectometry (NR) experiments for resolving subtle differences in the bilayer composition. A similar approach was used in Clifton et al. [37] for studying the effect of asymmetry in a Gram-negative bacterial outer membrane biomimetic model. NR was used to examine how the structure normal to the interface is modified when native and EAE-modified asymmetric myelin interact with MBP. The stability of the asymmetry was tested at different temperatures and compared with symmetric cytoplasmic myelin membranes in terms of the protein binding affinity. The out-of-plane structure was finally characterized when a second bilayer was deposited on the asymmetric SMB-MBP system.

These results provide new insights into how lipid complexity and membrane asymmetry modulate MBP–myelin interactions, offering a molecular perspective that is crucial to demyelination, human health and disease.

2. Materials and methods

2.1. Materials

The biomimetic myelin membranes employed in this study were prepared from natural porcine brain lipid extracts obtained from Avanti Polar Lipids (Alabaster, AL, USA): Brain PC (840053), Brain PE (840022), Brain PS (840032), Brain SM (860062), Cerebrosides (Brain) (131303), and Sulfatides (Brain) (131305). Cholesterol was purchased separately as Sigma-grade cholesterol (Sigma-Aldrich, C8667, $\geq 99\%$). Because the brain-derived lipids are natural extracts, each lipid class is itself a mixture of molecular species rather than a single defined compound. The manufacturer-reported average fatty-acid distributions of the extracts used here are summarised in Table S1. In those distributions, the category “Unknown” denotes unresolved minor acyl-chain species within the corresponding natural extract. For the main asymmetry-resolved datasets and all subsequent temperature- and protein-binding experiments, the cytoplasmic leaflet contained either protiated cholesterol or its deuterated analogue, d_{45} -cholesterol ($79 \pm 2\%$ D enrichment), sourced from the National Deuteration Facility (NDF, ANSTO, Lucas Heights, NSW, Australia). In the two additional native control bilayers used to validate leaflet asymmetry at 15°C , the deuterated cytoplasmic reporter lipid was either 18:0 PE- d_{70} [1,2-

distearyl-d70-sn-glycero-3-phosphoethanolamine] or 18:0 PC-d83 [1,2-distearyl-d70-sn-glycero-3-phosphocholine-1,1,2,2-d4-N,N,N-trimethyl-d9], also obtained from Avanti Polar Lipids (Alabaster, AL, USA).

3-(N-morpholino)propanesulfonic acid (MOPS; 99%, Alfa Aesar, CAS 1132-61-2; cat. no. A12914) and sodium chloride (NaCl; ACS reagent, $\geq 99.0\%$, powder, Sigma-Aldrich, CAS 7647-14-5) were used to prepare MOPS/NaCl buffer composed of 10 mM MOPS and 150 mM NaCl at pH/pD 7.4 in the required H₂O/D₂O solvent contrast. Unless otherwise stated, all MOPS-based buffers used for NR, MBP-binding, liposome, DBL and DSC experiments contained 150 mM NaCl. Myelin basic protein (MBP; bovine, purified; Sigma-Aldrich, cat. 13-104) was used as supplied, stored at -20°C , and diluted in the corresponding MOPS/NaCl buffer immediately before experiments to a final concentration of $c_{\text{MBP}} = 0.10\text{ mg/mL}$.

For fluorescence microscopy, 18:0 Cyanine 5 PE (Avanti 810345C) and 18:1 PE-TopFluor AF594 (Avanti 810387C) were used as fluorescent reporter lipids for the periplasmic and cytoplasmic monolayers, respectively. MBP was labelled with an Atto 488 Protein Labelling Kit (Sigma-Aldrich, 38371).

2.2. Lipid mixture preparation

Individual lipids were pre-dissolved in high-purity solvents (chloroform or chloroform:methanol 2:1 v/v) purchased from Sigma-Aldrich ($\geq 99.5\%$, Sigma-Aldrich, St. Louis, MO, USA) and mixed at appropriate molar ratios to yield final concentrations of 1 mg/mL for Langmuir monolayer deposition or up to 10 mg/mL for liposome preparation. Native and EAE-modified biomimetic myelin compositions were prepared according to previously reported cytoplasmic and periplasmic leaflet molar ratios (Table 1) [22].

2.3. Deposition of asymmetric supported myelin bilayers (SMB) for NR

Solid supports were single crystals of silicon ($8 \times 5 \times 1\text{ cm}^3$) polished on one large face. They were cleaned before use with chloroform, acetone, ethanol and pure water in that sequence. They were finally treated by UV-ozone (Jelight-type) for 15 min immediately prior to use.

The inner monolayer was transferred via LB method and the outer monolayer via the LS technique, as described in Rondelli et al. [38]. SMB were prepared using Langmuir troughs (KSV NIMA, Biolin Scientific, Espoo, Finland) at the Partnership for Soft Condensed Matter (PSCM, Grenoble, France) and at the ISIS Neutron and Muon Source (Rutherford Appleton Laboratory, Harwell Campus, Didcot, UK). For the LB deposition, a lipid monolayer corresponding to the periplasmic lipidic composition—comprising native myelin lipids and protiated ovine cholesterol—was spread from the organic lipid solution onto the air–water interface using ultrapure water as the subphase. The monolayer was then compressed to a final surface pressure of 35 mN/m, which was selected as it approaches to lateral stress conditions similar to those in in-vivo membranes [39]. A silicon wafer initially immersed (before the lipid spreading) into the subphase was vertically lifted through the

monolayer at 4 mm/min under constant surface pressure. After trough cleaning and subphase renewal, the cytoplasmic leaflet (containing either protiated or deuterated cholesterol) was deposited at the air–water interface and compressed under identical conditions. Prior to LS transfer, a custom-built levelling system was used to align the substrate parallel to the air–water interface. LS transfer was performed by lowering the LB-coated crystal onto the new monolayer at 3 mm/min and finally closing the solid-liquid flow cell used for NR measurements submerged at the base of the trough.

All depositions were carried out at 15°C , and samples were stored under the same conditions until NR measurements, which were performed at $15\text{--}37^\circ\text{C}$.

2.4. Liposome preparation

Lipid mixtures dissolved in organic solvent were evaporated under an argon stream, followed by vacuum annealing overnight at 50°C . The dried lipids were rehydrated in MOPS/NaCl buffer prepared in D₂O (10 mM MOPS, 150 mM NaCl, pD 7.4) and shaken until fully detached from the glass surface. Residual material was removed by pipetting if necessary. The mixture was bath-sonicated for 30 min at 40°C and subjected to five freeze–thaw cycles. To remove large aggregates, it was centrifuged through a $0.45\text{ }\mu\text{m}$ filter at $10,000 \times g$ for 10 min. The final solution was extruded twenty times through a 100 nm membrane at 50°C .

2.5. Differential scanning calorimetry (DSC)

Thermotropic transitions of the four leaflet-mimicking lipid mixtures (native cytoplasmic, native periplasmic, EAE cytoplasmic, and EAE periplasmic; Table 1) were characterized by differential scanning calorimetry using a multicell DSC instrument (MC-DSC, TA Instruments) at the PSCM (ILL, Grenoble). Liposome suspensions were prepared as described above and measured at a lipid concentration of 3 mg/mL in MOPS/NaCl buffer (10 mM MOPS, 150 mM NaCl, pH 7.4). Prior to loading, samples and reference buffer were degassed for 10 min. Thermograms were acquired between 10 and 50°C at a scan rate of 1°C min^{-1} , using three heating/cooling cycles. Thermotropic parameters were extracted from the second heating scan to minimize thermal-history effects. Raw heat-flow curves were corrected by subtracting the corresponding buffer reference scan and applying a baseline correction defined from pre- and post-transition regions.

2.6. NR measurements

NR measurements were conducted at two complementary time-of-flight instruments: INTER [40] at the ISIS Neutron and Muon Source (Rutherford Appleton Laboratory, Didcot, UK), and FIGARO [41] at the Institut Laue-Langevin (ILL, Grenoble, France). On INTER, the accessible wavelength range spanned from 1.5 to $17\text{ }\text{\AA}$ with a momentum transfer (Q) resolution ($\Delta Q/Q$) of 3.5%, using incident angles of 0.7° and 2.3° . On FIGARO, measurements were performed using a wavelength band of 2 to $20\text{ }\text{\AA}$ and a resolution of 7%, with incident angles of 0.7° and 3.0° . In both setups, beam footprints were adapted to the silicon substrate dimensions and defined by slit geometry and beam divergence, yielding illuminated areas of approximately 60 mm in length and 35 mm (INTER) or 25 mm (FIGARO) in width. All measurements were carried out in time-of-flight mode, with beam alignment and data acquisition optimized to ensure consistent Q_z coverage across both instruments.

Both symmetric bicytoplasmic and asymmetric SMB were prepared via sequential LB and LS depositions as described above and then sealed within the sample cell prior to beamline installation. Once in place, the cell was connected to the automated solvent exchange system. Solvent contrast variation was performed using an automated HPLC pump at a flow rate of 1 mL/min for a total exchange volume of 20 mL. Initially, bare silicon substrates were characterized in four isotopic contrasts using the same MOPS/NaCl buffer composition (10 mM MOPS, 150 mM

Table 1

Native and EAE-modified cytoplasmic/periplasmic molar lipid fractions (%). Values are target compositions used to formulate monolayers/bilayers.

Lipid type	$\phi_{\text{nat, cyt}}$ (%)	$\phi_{\text{nat, per}}$ (%)	$\phi_{\text{EAE, cyt}}$ (%)	$\phi_{\text{EAE, per}}$ (%)
Phosphatidylcholine (PC)	25.9	18.2	20.1	13.1
Phosphatidylethanolamine (PE)	29.0	9.0	32.9	9.6
Phosphatidylserine (PS)	7.0	1.1	7.4	6.9
Sphingomyelin (SM)	6.2	4.2	2.2	1.3
Cholesterol	31.9	33.5	37.4	38.3
Cerebrosides	–	24.4	–	25.2
Sulfatides	–	9.6	–	5.6

NaCl, pD/pH 7.4) prepared in different H₂O/D₂O mixtures having specific neutron scattering length densities (SLD): 100% D₂O (SLD: $6.35 \times 10^{-6} \text{ \AA}^{-2}$), '4-matched water' 66% D₂O/ 34% H₂O (4 MW; SLD: $4.00 \times 10^{-6} \text{ \AA}^{-2}$), 'silicon-matched water' 38% D₂O/ 62% H₂O (SMW; SLD: $2.07 \times 10^{-6} \text{ \AA}^{-2}$), and 100% H₂O (SLD: $-0.56 \times 10^{-6} \text{ \AA}^{-2}$). The SMB were then characterized under the same four contrasts. MBP was incubated at $c_{MBP} = 0.10 \text{ mg/mL}$ for 30 min before flushing 20 mL of buffer to remove unbound protein following the protocol described in Krugmann et al. [18]. The resulting bilayer-protein complexes were measured in the D₂O-based MOPS/NaCl contrast. Finally, liposomes were injected into the cell containing the SMB/MBP coated wafers to form double bilayers (DBL) which were also measured in D₂O.

2.7. NR data analysis

Reflectivity profiles were collected from a series of myelin bilayer samples in which isotopic contrast was introduced through a selectively labelled component of the cytoplasmic leaflet. Specifically, for the main native and EAE datasets, the non-labelled cytoplasmic leaflet contained protiated cholesterol and the labelled cytoplasmic leaflet contained its deuterated analogue d₄₅-cholesterol, while the periplasmic leaflet composition remained unchanged in both cases. In two additional native control bilayers used only to validate leaflet asymmetry at 15 °C, the selectively deuterated cytoplasmic reporter lipid was instead 18:0 PE-d70 or 18:0 PC-d83; these control datasets were measured only in D₂O and H₂O.

The base SMB was modelled as six layers (Fig. 1): native silicon oxide, interfacial water, periplasmic heads and tails (p_h and p_t), cytoplasmic tails and heads (c_t and c_h). Upon MBP addition, a seventh layer was introduced to represent the adsorbed protein, and in the final configuration with vesicle adsorption, an eighth layer accounting for the second bilayer was incorporated.

Data from FIGARO were processed using COSMOS [42] within the LAMP suite (ILL), while data from INTER were reduced using the Mantid framework (ISIS). Data fitting was performed using the Anaklasis package [43], which allows co-refinement of multiple contrasts using a stratified layer model. A global scaling factor was introduced to account for minor attenuation effects and potential diffuse scattering, particularly in late-stage samples containing vesicles.

For each dataset, layer thicknesses (t) and hydration fractions (defined as solvent volume fraction, ϕ_w) were fitted within physically

reasonable ranges. To resolve leaflet asymmetry in selectively deuterated samples, the acyl chain SLD were fitted independently for each leaflet. Headgroup SLD were kept at their theoretical values, while head and tail thicknesses were allowed to vary independently for inner and outer leaflets (heads: 7–12 Å; tails: 12–17 Å). Hydration was leaflet-specific (heads: 0–0.7; tails: 0–0.5). A single lipid-lipid roughness of $\sigma = 3.0 \pm 0.5 \text{ \AA}$ was applied; Si/SiO₂ and MBP roughnesses were fitted separately and kept within $\sigma = 0\text{--}4 \text{ \AA}$ and $0\text{--}15 \text{ \AA}$, respectively. The SiO₂ thickness and the interfacial-water gap were refined per dataset with bounds $t = 9\text{--}15$ and $2\text{--}10 \text{ \AA}$, respectively. For the d₄₅-cholesterol-labelled samples, the outer-tail SLD was allowed to vary between 1.6 and $3.0 \times 10^{-6} \text{ \AA}^{-2}$, and the inner-tail SLD between -0.5 and $0.5 \times 10^{-6} \text{ \AA}^{-2}$ (broadened to $-0.5\text{--}1.8 \times 10^{-6} \text{ \AA}^{-2}$ for the 37 °C series). The additional native control bilayers containing 18:0 PE-d70 or 18:0 PC-d83 in the cytoplasmic leaflet were fitted analogously to the d₄₅-cholesterol datasets, with independent refinement of inner and outer headgroup and tail thicknesses and independent fitting of the acyl-chain SLDs in both leaflets; in these two controls, the outer-tail SLD was allowed between 3.2 and $3.6 \times 10^{-6} \text{ \AA}^{-2}$ and the inner-tail SLD between -0.5 and $0.5 \times 10^{-6} \text{ \AA}^{-2}$, while headgroup SLDs were fixed to the corresponding theoretical values listed in Table 2.

For datasets recorded after MBP addition, the structural parameters of the underlying SMB were initialised from the protein-free best fits (Table 3) and then allowed to relax to accommodate potential protein insertion. Unless noted otherwise, thickness, hydration and SLD for each leaflet were permitted to vary by up to $\pm 20\%$ relative to their protein-free values. MBP SLD was computed from sequence using Biomolecular SLD calculator [44] assuming 90% exchange of labile hydrogens, and MBP thickness was fitted within $t = 60\text{--}100 \text{ \AA}$.

For DBL fits, a second bilayer was added above the first, separated by an interstitial solvent layer. This solvent layer was modelled to allow for membrane fluctuations and is expected to have an SLD close to the buffer (D₂O). SMB parameters obtained at the MBP/SMB stage were kept fixed and the MBP volume fraction was allowed to vary by $\pm 20\%$ around the pre-adsorbed value. The second bilayer was modelled as a single homogeneous slab with adjustable SLD between 0.3 and $0.9 \times 10^{-6} \text{ \AA}^{-2}$, a thickness t between 45 and $55 \text{ \AA}$, and a high roughness ($\sigma = 0\text{--}7 \text{ \AA}$), to minimize the number of free parameters.

Unless noted otherwise, reported uncertainties are 1σ (68%) from the covariance matrix of the Levenberg-Marquardt minimizer in Anaklasis, with fits weighted by pointwise experimental errors and after convolution with the instrumental resolution appropriate to each dataset. For derived quantities (e.g., total bilayer and acyl region thicknesses and their temperature differences), we propagated the full joint covariance (i.e., including parameter correlations).

Table 2

Theoretical neutron SLD ($\times 10^{-6} \text{ \AA}^{-2}$) for buffers, substrate and membrane-associated layers used in the slab model at pH/pD 7.4. Values are fixed during fitting unless stated otherwise.

Lipid/solvent	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)
D ₂ O MOPS/NaCl buffer (pD 7.4)	6.35
4 MW MOPS/NaCl buffer (pH 7.4)	4.00
SMW MOPS/NaCl buffer (pH 7.4)	2.07
H ₂ O MOPS/NaCl buffer (pH 7.4)	-0.56
Silicon	2.07
Silicon oxide	3.41
Native protiated c_h	1.79
Native protiated c_t	-0.09
Native protiated p_t	-0.21
Native protiated p_h	1.92
Native deuterated c_t	2.55
EAE protiated c_h	1.76
EAE protiated c_t	-0.16
EAE protiated p_t	-0.04
EAE protiated p_h	1.99
EAE deuterated c_t	2.42
18:0 PC-d83 c_h	2.90

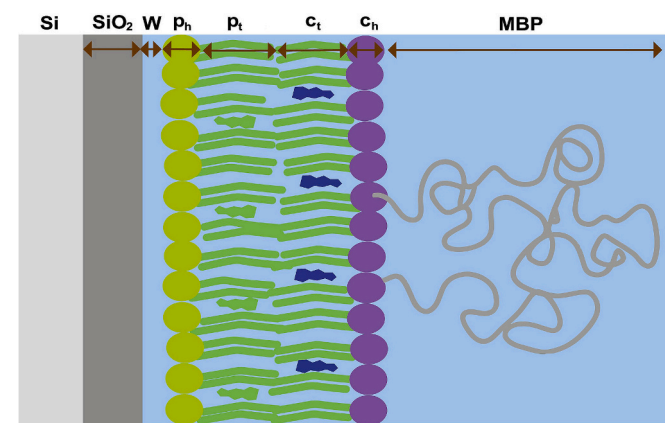


Fig. 1. Schematic of the asymmetric SMB used in NR. From the substrate to the bulk: silicon (Si), silicon oxide (SiO₂), an interfacial water layer (W), inner periplasmic heads (p_h) and tails (p_t), and outer cytoplasmic tails (c_t) and heads (c_h). Where indicated in the manuscript, the cytoplasmic tail contains d₄₅-cholesterol (blue) to provide isotopic contrast. "Inner/outer" designate periplasmic/cytoplasmic sides relative to the substrate. Drawings are not to scale; colours denote model layers only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3

Fitted structural parameters for asymmetric SMB (native and EAE-modified) at 15 °C: layer thickness (t), surface coverage ($\eta = 1 - \phi_w$) and SLD ($\times 10^{-6} \text{ \AA}^{-2}$). SMB denotes the total bilayer thickness ($pH + p_t + c_t + c_h$). Uncertainties are 1σ from the fit covariance; for the totals, errors were obtained by covariance-aware propagation.

Layer	Native protiated			Native deuterated		
	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)
SiO ₂	13.1 ± 1.9			13.4 ± 2.5		
Water	4.7 ± 1.4			4.6 ± 1.1		
p_h	9.8 ± 1.6	0.93 ± 0.05		10.5 ± 2.1	0.92 ± 0.02	
p_t	16.6 ± 1.9	0.93 ± 0.03	−0.08 ± 0.51	16.2 ± 1.1	0.91 ± 0.02	−0.06 ± 0.58
c_t	15.3 ± 1.3	0.84 ± 0.04	−0.18 ± 0.71	16.3 ± 1.4	0.82 ± 0.04	2.41 ± 0.62
c_h	11.1 ± 1.1	0.77 ± 0.03		10.1 ± 1.9	0.79 ± 0.03	
SMB	52.8 ± 5.9			53.1 ± 6.5		
Layer	EAE protiated			EAE deuterated		
	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)
SiO ₂	12.9 ± 1.8			13.6 ± 2.1		
Water	4.5 ± 0.5			4.4 ± 0.8		
p_h	9.8 ± 1.1	0.88 ± 0.08		10.5 ± 1.1	0.91 ± 0.03	
p_t	14.9 ± 1.8	0.90 ± 0.03	−0.09 ± 0.38	14.1 ± 1.7	0.93 ± 0.05	0.05 ± 0.45
c_t	14.6 ± 1.0	0.86 ± 0.05	−0.15 ± 0.44	13.8 ± 1.3	0.83 ± 0.02	2.35 ± 0.42
c_h	9.6 ± 1.9	0.71 ± 0.06		9.8 ± 1.6	0.77 ± 0.01	
SMB	48.9 ± 5.8			48.2 ± 5.7		

2.8. Fluorescence microscopy of SMB

Equivalent SMB for fluorescence microscopy were prepared on glass substrates by sequential LB/LS deposition at 20 °C using a Langmuir trough (Nima Technology Ltd., Coventry, UK) at a target surface pressure of 35 mN m^{−1}. Substrates were sonicated in isopropanol for 10 min, rinsed three times with ultrapure water, and plasma-cleaned for 2 min immediately before deposition. 18:0 Cyanine 5 PE and 18:1 PE-TopFluor AF594 were added at 0.1 mol% to the periplasmic and cytoplasmic mixtures, respectively.

For protein-binding experiments, MBP labelled with Atto 488 was added under the same conditions as those employed in the NR measurements. Samples were imaged on a widefield microscope (AxioObserver; Carl Zeiss) equipped with a Colibri 7 light source, an Axiocam 712 mono camera, and a Plan-Apochromat objective (20×/0.8 NA; Carl Zeiss), using the appropriate filter sets. Sequential acquisition was used for the Atto 488, Cyanine 5 and AF594 channels. Z-stacks were acquired (35 planes, 0.29 μm step), and representative focal planes are shown. Two independently prepared samples were examined for each condition. Unless otherwise stated, only linear brightness and contrast adjustments were applied equally within each channel.

3. Results and discussion

To elucidate the interaction between MBP and SMB with high structural precision, we employed NR. This technique offers exceptional sensitivity to variations in the atomic composition normal to the membrane plane, enabling the reconstruction of depth-resolved SLD profiles

with sub-nanometre resolution. Under specular reflection conditions—where the angle of incidence equals the angle of reflection—the measured intensity provides direct insight into the stratification and composition of interfacial layers. This makes NR particularly well suited for probing the vertical architecture of model membrane systems, such as supported bilayers, and for assessing subtle structural rearrangements induced by protein binding.

3.1. Validation of membrane asymmetry in SMB

To emulate CNS myelin, our model membranes comprise the major glycerophospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine), sphingolipids (sphingomyelin, cerebroside, and sulfatides), and cholesterol, arranged asymmetrically between cytoplasmic and periplasmic leaflets as reported in Min et al. [22] (full molar profiles in Table 1).

Asymmetric SMB with the native myelin lipid composition were initially assembled at 15 °C using a combination of the LB and LS techniques (see Materials and Methods for details).

The periplasmic leaflet was first deposited via the LB method to form the initial Langmuir monolayer, after which the bilayer was completed by transferring the cytoplasmic leaflet using the LS technique. In the adopted geometry, the periplasmic leaflet is oriented toward the silicon substrate, while the cytoplasmic leaflet interfaces with the bulk aqueous phase (see Fig. 1). NR measurements were conducted on two bilayer systems: one comprising a fully protiated cytoplasmic leaflet, and the other in which ovine cholesterol was selectively replaced with its deuterated analogue, d₄₅-cholesterol. The d₄₅-cholesterol was selected as the deuterated component because it was available with a well-defined deuteration level ($\sim 79 \pm 2\%$), allowing for accurate calculations of the SLD. Moreover, unlike phospholipids (used here as natural mixtures), cholesterol is a single, well-defined molecule, making selective deuteration straightforward. A summary of the SLD values for all lipid and solvent components used in this study is provided in Table 2.

Each bilayer was characterized under four isotopic solvent contrasts—D₂O-buffer, four-matched water (4 MW, 66% D₂O/34% H₂O), silicon-matched water (SMW, 38% D₂O/62% H₂O) and H₂O-buffer—yielding a total of eight distinct reflectivity profiles. Fig. 2 presents the resulting data, the corresponding model fits, and the derived SLD profiles for both the unlabelled and d₄₅-cholesterol-labelled bilayers.

The primary aim of these experiments was to assess whether membrane asymmetry is retained upon assembly at 15 °C. As summarised in Table 2, the SLD contrast between the acyl chain regions of the periplasmic and cytoplasmic (c_t and p_t) leaflets is negligible when both are protiated. In contrast, the introduction of deuterated cholesterol in the cytoplasmic leaflet produces a marked divergence in both the reflectivity curves and the derived SLD profiles as shown in Fig. 2, thereby providing evidence that asymmetry is maintained in native myelin membranes under these conditions. As expected from contrast variation, the non-deuterated bilayer (●) shows larger Kiessig fringes in D₂O and 4 MW, whereas the deuterated one (□) dominates in H₂O, reflecting the reversal of ΔSLD with the solvent.

Using the six-layer model described in the Methods section, we fitted the eight contrasts per membrane. For the fitting procedure, all SLD values were held constant except for the SLD of the acyl chain regions, which was refined within a narrow range. From the hydration fraction ϕ_w we calculated a surface coverage fraction η defined as $\eta = 1 - \phi_w$. The adjusted structural parameters, including η , thickness (t) and SLD of the acyl chains, are summarised in Table 3.

The resulting fits reveal high surface coverage for both bilayers. The periplasmic leaflet exhibits consistent coverage of 91–93%, regardless of cytoplasmic composition and the cytoplasmic leaflet shows slightly lower values, close to 80%. These values indicate a high, but not complete, bilayer coverage; residual defects or uncovered regions therefore cannot be excluded, particularly in the outer leaflet. The t values obtained are in good agreement with expectations, resulting in a total

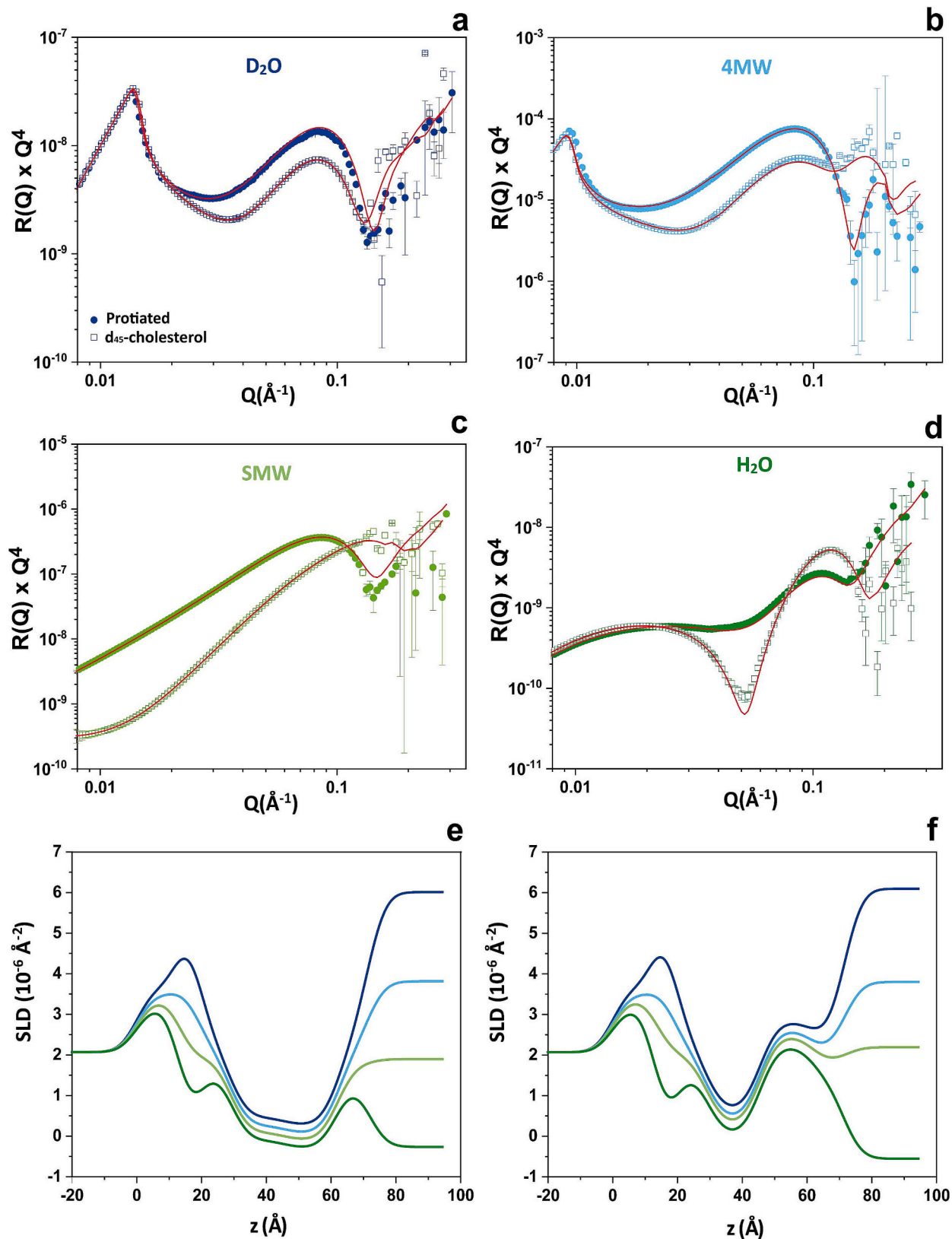


Fig. 2. Neutron reflectivity (NR) and global co-refinement fits for a native asymmetric SMB with either a protiated or a d₄₅-cholesterol-labelled cytoplasmic leaflet under four solvent contrasts: (a) D₂O, (b) 4 MW, (c) SMW, and (d) H₂O. Symbols denote experimental data—filled circles: all-protiated, open squares: d₄₅-cholesterol-labelled—and colours encode the solvent contrast (D₂O: dark blue; 4 MW: light blue; SMW: olive; H₂O: green). Solid lines are the best co-refinement fits. Error bars represent 1 σ . (e,f) Reconstructed SLD profiles (same colour coding). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bilayer thickness of around $t = 53 \text{ \AA}$, comparable to those reported for myelin lipids using SAXS and Brewster Angle Microscopy (BAM) [45]. Additionally, the thickness of the interfacial water layer between the silicon oxide and the bilayer ranged from $t \sim 4.5\text{--}5 \text{ \AA}$.

To test whether the asymmetric assembly introduced any interleaflet exchange during LS deposition, we relaxed the usual constraint on the acyl chain SLD and fitted them with bounds around their theoretical values in the d-labelled bilayer. For the d-labelled bilayer the cytoplasmic-tail SLD was $2.41 \pm 0.62 \times 10^{-6} \text{ \AA}^{-2}$ versus the theoretical $2.55 \times 10^{-6} \text{ \AA}^{-2}$ ($\approx 5\%$ lower), while the periplasmic-tail SLD increased from -0.21 to $-0.06 \pm 0.58 \times 10^{-6} \text{ \AA}^{-2}$; both shifts lie within uncertainty. Such minor offsets likely reflect natural variability in fatty-acyl composition and differences between assumed and effective molecular volumes, and/or slight mixing during LS transfer, rather than genuine interleaflet exchange at 15°C . No systematic drift in either leaflet's SLD was detected over $\sim 9 \text{ h}$ at 15°C , arguing against cholesterol flip-flop under these conditions.

It was then examined whether the retention of leaflet asymmetry at 15°C in native SMB could also be verified with some phospholipid reporters. Therefore, two additional native asymmetric SMB in which the deuterated cytoplasmic reporter lipid was either 18:0 PE-d70 or 18:0 PC-d83 were prepared. PE and PC were selected because they are the two major glycerophospholipid classes in the cytoplasmic leaflet and, unlike PS, their substitution does not directly alter the anionic character of the membrane surface relevant to MBP binding. In D_2O and H_2O , both reporter bilayers show clear contrast-dependent deviations from the protiated reference and distinct reconstructed SLD profiles when analysed with the same six-layer model (Fig. 3), demonstrating that the retention of leaflet asymmetry at 15°C in native SMB is not unique to cholesterol but is also evident with these representative phospholipid reporters. The fitted structural parameters are summarised in Table S2. These controls serve as an orthogonal validation of low-temperature asymmetry, rather than as molecularly exact substitutes for the natural brain PE and PC extracts, because 18:0 PE-d70 and 18:0 PC-d83 are defined synthetic lipids with saturated chains. The subsequent temperature-dependent and MBP-binding experiments were accordingly continued with d_{45} -cholesterol as the primary reporter lipid, since it provides strong contrast while introducing the smallest conceptual perturbation to the heterogeneous phospholipid composition of the native and EAE myelin mixtures.

The same d_{45} -cholesterol-based procedure was then applied to the asymmetric EAE-modified myelin membrane. Reflectivity profiles were successfully collected for the four previously described contrasts (Fig. S1), leading to good fits for both the all-protiated and d_{45} -

cholesterol-labelled bilayers in each contrast. As with the native SMB, the structural values obtained are virtually identical between the deuterated and protiated versions, reinforcing the notion that d_{45} -cholesterol does not substantially alter the membrane's structural properties. Compared with native SMB ($\sim 53 \pm 6 \text{ \AA}$), the EAE system is slightly thinner ($\sim 48 \pm 6 \text{ \AA}$) (Fig. 4; Table 3).

3.2. Thermal destabilisation of membrane asymmetry in SMB

Fig. 5a shows the reflectivity curves for a native asymmetric SMB featuring a d_{45} -cholesterol-labelled outer leaflet, recorded at four temperatures between 15 and 37°C , approaching physiological conditions. After each target temperature was reached, the sample was equilibrated for 15 min prior to measurement. Data were collected under two solvent contrasts (D_2O and H_2O -buffer), and comparative reflectivity profiles in D_2O are plotted. Beyond serving as a physicochemical benchmark, this series tests whether cholesterol asymmetry persists at 37°C .

Using the same six-layer interfacial model described earlier, the thickness and SLD of the acyl chain regions in both leaflets were refined to generate structural profiles of the bilayers at each temperature (Fig. 5b). To minimize the number of fitting parameters and based on the similarity in SLD values for the polar headgroup regions across the bilayer, both the SLD and t of these polar layers were fixed to the values reported in Tables 2 and 3, respectively. Between 15 and 20°C , structural changes are within error. With increasing temperature, the acyl chain region thins modestly (about $4 \text{ \AA}$ from 15 to 37°C ; Table S3), while the leaflet SLD contrast ($\Delta\text{SLD} = \text{SLD}_{\text{Ct}} - \text{SLD}_{\text{Pt}}$) is strongly reduced from 15 to 37°C for both compositions, indicating cholesterol redistribution (Fig. 5c; Table 4). The EAE-modified SMB appears to show an earlier loss of contrast, with ΔSLD already near zero at 30°C , whereas the native system decays more gradually (Fig. 5c; Table 4). Changes at intermediate temperatures are small and, within the propagated uncertainties, do not resolve composition-specific differences; we therefore focus on the robust $15 \rightarrow 37^\circ\text{C}$ decrease. EAE-modified counterparts of panels (a) and (b) are provided in the Supporting Information (Fig. S2).

To place this temperature-dependent loss of contrast in a thermotropic framework, DSC thermograms were acquired for symmetric liposomes prepared from the four leaflet-mimicking compositions. The cytoplasmic mixtures exhibit broad endothermic maxima centred near 30°C , whereas the periplasmic mixtures show broader and less well-defined endotherms extending over a wider temperature interval and shifted to higher temperature (Fig. S3). Such broad profiles are expected for cholesterol-rich, multicomponent myelin extracts [8]. These data situate 15°C below the main transition interval of the mixtures, whereas

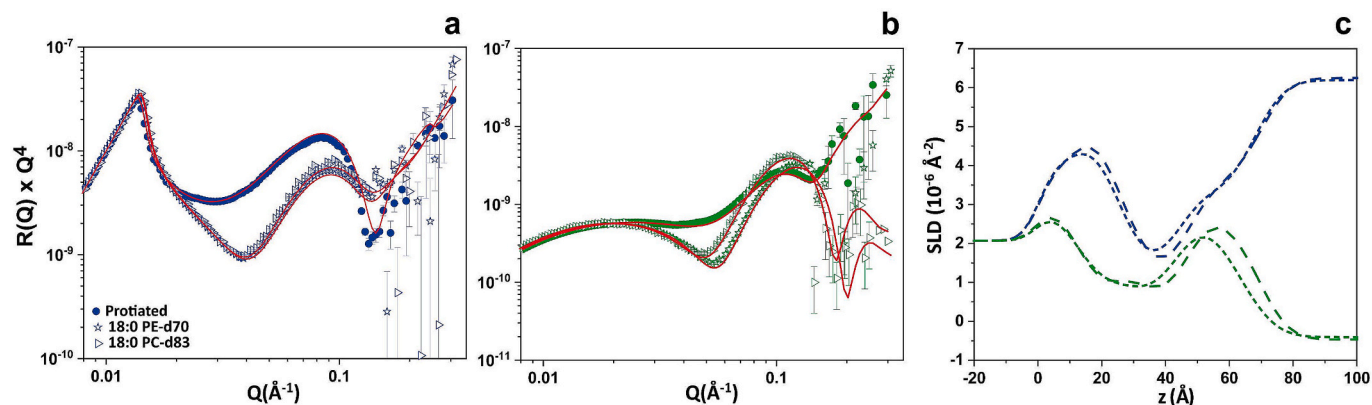


Fig. 3. Independent NR validation of 15°C leaflet asymmetry in native asymmetric SMB using phospholipid reporter lipids. Reflectivity curves in (a) D_2O and (b) H_2O are shown for a native asymmetric bilayer with a protiated cytoplasmic leaflet (filled circles), or with the deuterated cytoplasmic reporter lipid 18:0 PE-d70 (stars) or 18:0 PC-d83 (open triangles). Red lines are the best fits. (c) Reconstructed SLD profiles for the two phospholipid-reporter bilayers; blue profiles correspond to D_2O and green profiles to H_2O , with short-dashed lines for 18:0 PE-d70 and long-dashed lines for 18:0 PC-d83. The fitted structural parameters are listed in Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

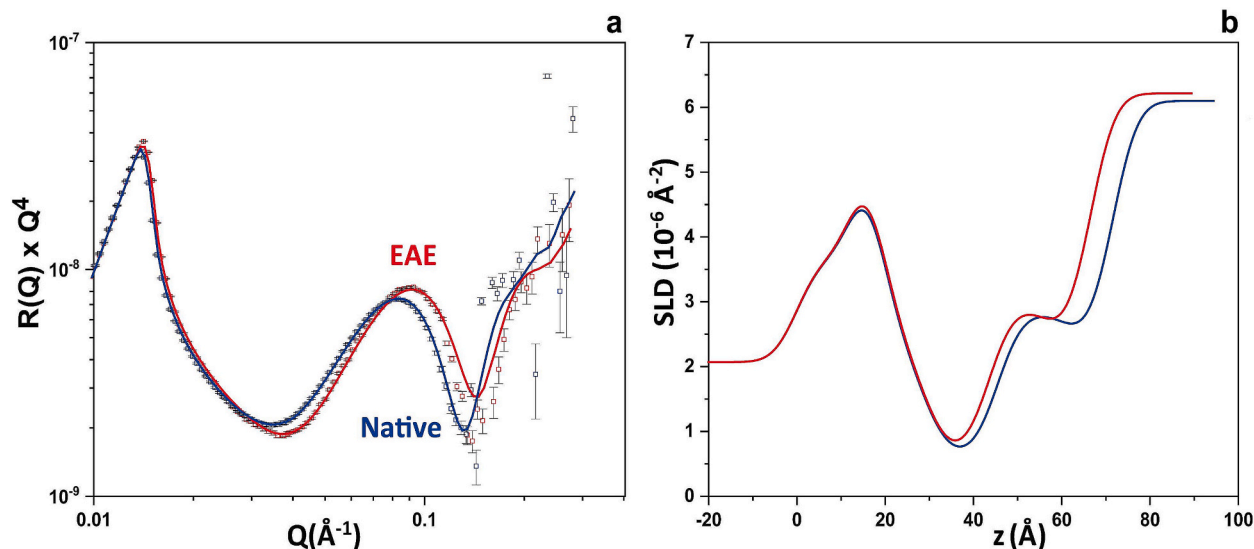


Fig. 4. Comparison of NR (a) and corresponding SLD profiles (b) for native (blue) and EAE-modified (red) asymmetric SMB with a cytoplasmic leaflet containing d_{45} -cholesterol in D_2O at 15°C . Solid lines are co-refinement fits. Fitted parameters with 1σ uncertainties are reported in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

37°C lies within or slightly above it, indicating a more ordered membrane state at low temperature and a more fluid one closer to physiological temperature.

The progressive decrease in acyl-chain SLD contrast between leaflets upon heating is interpreted specifically as redistribution of the deuterated cholesterol reporter within the supported bilayer, rather than as a direct measure of transbilayer exchange for all lipid classes. The NR readout thus indicates that the leaflet-resolved distribution of the cholesterol analogue remains effectively “frozen” on the experimental timescale at low temperature, but relaxes toward symmetry as the membrane becomes more dynamically permissive at elevated temperature. The temperature series was not acquired in a time-resolved manner, so no flip-flop rate constants are extracted here; instead, NR provides a sensitive measure of whether cholesterol redistribution is negligible or appreciable on the experimental timescale. This interpretation is consistent with earlier NR work on asymmetrically deposited phospholipid bilayers, where substantial interleaflet exchange becomes prominent once the membrane enters the fluid regime [46]. Surface-sensitive vibrational spectroscopy and molecular simulations likewise show that transbilayer migration accelerates in more fluid membranes [47,48], with cholesterol flip-flop predicted to become rapid near physiological temperature [47].

It should also be noted that persistence of compositional asymmetry in SMB is not a generic property of asymmetric membranes, but depends strongly on the deposition route, support interactions, and bilayer integrity. Using fluorescence interference contrast microscopy, Crane et al. [49] showed that LB/LS-prepared bilayers can exhibit substantial interleaflet mixing, with cholesterol promoting leaflet coupling, whereas LB/vesicle-fusion routes may retain asymmetry more effectively over comparable time windows. Support-lipid interactions can further modify the proximal leaflet composition and may either accelerate asymmetry loss or favour enrichment of particular lipid species near the substrate [50,51].

The present observations should therefore be read as a quantitative statement about cholesterol redistribution in this specific LB/LS-prepared, silicon-supported myelin mimic: leaflet contrast is stable at 15 – 20°C over hours, yet relaxes markedly on heating toward 37°C . Biological membranes, by contrast, maintain lipid asymmetry through active transport and structural constraints; the loss of asymmetry in a protein-free supported bilayer at physiological temperature accordingly underscores the need for such maintenance mechanisms in vivo [52,53].

3.3. Differential MBP binding to native and EAE-modified asymmetric SMB

As a next step, MBP was added to the liquid cell at a concentration of $c_{MBP} = 0.1 \text{ mg/mL}$ for both the native and EAE-modified asymmetric SMB. The experiments were conducted at 15°C . MBP interacts with the cytoplasmic surface forming a protein-rich layer atop the bilayer. Reflectivity data in D_2O for both systems in the presence of MBP, along with their corresponding SLD profiles and schematic illustrations of the membrane architecture, are shown in Fig. 6.

These datasets were modelled using a sequential slab structure comprising water – $p_h - p_t - c_t - c_h$, with an additional MBP layer positioned on the solvent-facing side. The MBP layer was fitted with two free parameters: its thickness t_{MBP} and its coverage η_{MBP} . The underlying SMB structural parameters were initialised from the protein-free fits (Table 3) and relaxed according to the constraints defined in NR data analysis (Materials and Methods); results are summarised in Table 5. As usual for specular NR, these MBP parameters are laterally averaged effective slab quantities over the illuminated footprint and therefore describe the average adsorbed layer rather than the local lateral distribution of MBP.

The $R(Q) \times Q^4$ plots (Fig. 6a,b) show composition-dependent responses to MBP: in native SMB the minima remain essentially fixed in Q , whereas in EAE they shift to lower Q and SLD fits indicate bilayer thickening; accordingly, we analyze the two datasets separately.

For native myelin, the model yields an effective MBP layer with $t_{MBP} \approx 80 \text{ \AA}$ and a surface concentration (defined as $c_{MBP/SMB} (\%) = \eta_{MBP} \times 100$) of $c_{MBP/SMB} = 25 \pm 4\%$ vol. The bilayer structural parameters remain almost unchanged compared to the protein-free system ($t = 53.1 \pm 6.5 \text{ \AA} \rightarrow 53.9 \pm 5.7 \text{ \AA}$ and $SLD_c = 2.41 \pm 0.62 \rightarrow 2.47 \pm 0.44 \times 10^{-6} \text{ \AA}^{-2}$, respectively), suggesting minimal insertion of MBP into the bilayer (Fig. 6c). This result aligns with previous NR data on recombinant MBP, which showed a similarly thick protein layer with $\sim 25\%$ volume occupancy atop model bilayers, supporting a conserved surface binding mode under native-like conditions [20].

In contrast, some differences are observed for the EAE-modified asymmetric SMB. The protein layer is thinner by approximately $10 \text{ \AA}$, and the surface enrichment is markedly lower, with $c_{MBP/SMB} = 10 \pm 4\%$ vol (Fig. 7a). Upon MBP binding, the total thickness and SLD of the outer leaflet increase relative to the bare SMB ($t = 48.2 \pm 5.7 \text{ \AA} \rightarrow 53.5 \pm 6.0 \text{ \AA}$ and $SLD_c = 2.35 \pm 0.42 \rightarrow 3.01 \pm 0.88 \times 10^{-6} \text{ \AA}^{-2}$, respectively), which indicates a more pronounced protein penetration into this leaflet

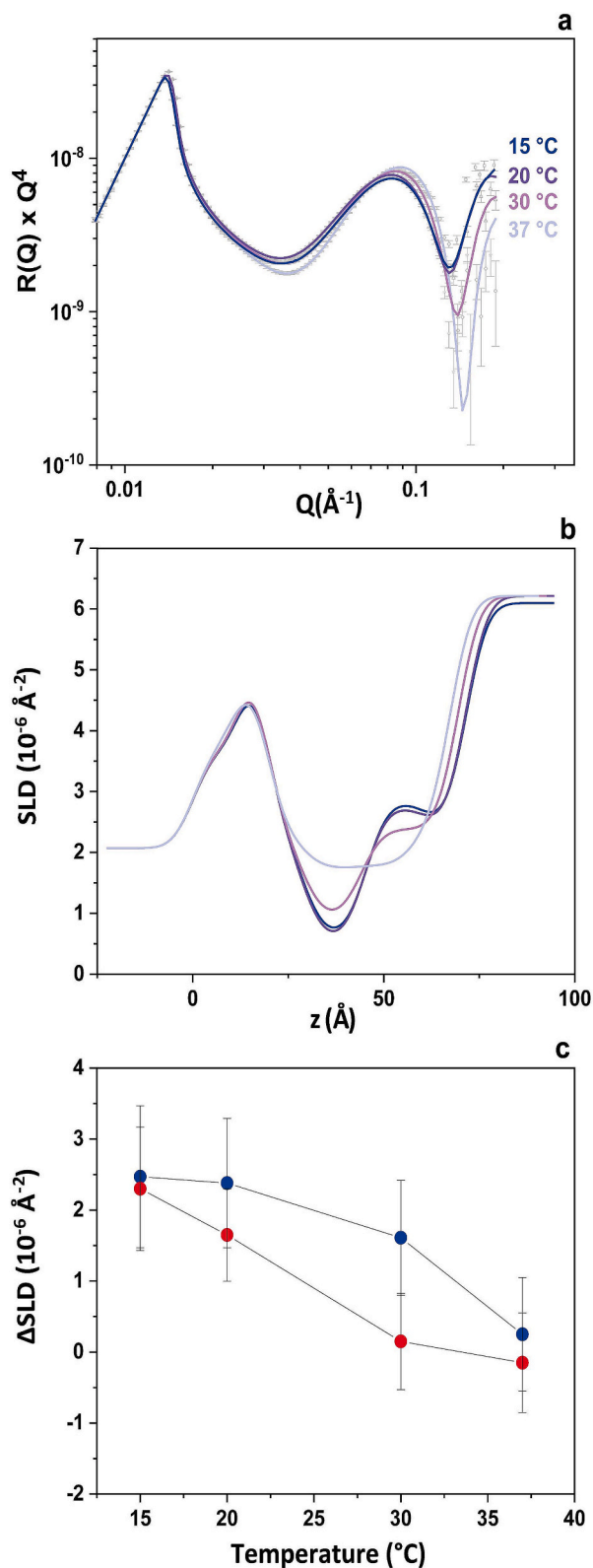


Fig. 5. Temperature-dependent evolution of an asymmetric native SMB with a cytoplasmic leaflet containing d_{45} -cholesterol (a) reflectivity in D_2O at 15, 20, 30 and 37 °C; (b) SLD profiles; (c) acyl-chain SLD contrast between leaflets ($\Delta\text{SLD} = \text{SLD}_t - \text{SLD}_p$) versus temperature for native (blue) and EAE-modified (red) SMB. Error bars in (c) are 1σ from the fit covariance with standard propagation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4

Temperature series of leaflet SLD and contrast ($\Delta\text{SLD} = \text{SLD}_t - \text{SLD}_p$). Leaflet acyl-chain SLD for the periplasmic (p_t) and cytoplasmic (c_t) leaflets and their contrast ΔSLD at 15, 20, 30 and 37 °C for native and EAE-modified asymmetric SMB. Uncertainties are 1σ from the fit covariance; Δ -type quantities use full covariance propagation.

T (°C)	SLD ($\times 10^{-6} \text{\AA}^{-2}$)					
	Native			EAE-modified		
	p_t	c_t	$c_t - p_t$	p_t	c_t	$c_t - p_t$
15 °C	-0.06 ± 0.38	2.41 ± 0.62	2.47 ± 1.00	0.05 ± 0.45	2.35 ± 0.42	2.30 ± 0.87
20 °C	0.01 ± 0.47	2.39 ± 0.44	2.38 ± 0.91	0.40 ± 0.36	2.05 ± 0.29	1.65 ± 0.65
30 °C	0.41 ± 0.39	2.02 ± 0.42	1.61 ± 0.81	0.90 ± 0.26	1.05 ± 0.42	0.15 ± 0.68
37 °C	1.11 ± 0.43	1.36 ± 0.37	0.25 ± 0.80	1.10 ± 0.39	0.95 ± 0.31	-0.15 ± 0.70

for the modified system (Fig. 6d).

3.4. Reduced MBP binding upon loss of membrane asymmetry

The interaction between MBP and SMB was further assessed under two additional conditions. First, an SMB was assembled by depositing two native cytoplasmic leaflets containing d_{45} -cholesterol at 15 °C. In the absence of MBP, both the bicytoplasmic and the asymmetric SMB matched in overall thickness (Table S4); nevertheless, the $c_{\text{MBP/SMB}}$ differed substantially. Although the protein-facing leaflet is identical in both systems, MBP binding is clearly enhanced in the presence of transbilayer asymmetry, suggesting that coupling plays a fundamental role in the protein recruitment. The reduced $c_{\text{MBP/SMB}}$ value (~ 3 vol%; Fig. 7) is consistent with the one reported by Krugmann et al. [19] for cytoplasmic myelin bilayers formed via vesicle fusion and is clearly lower than the 25% vol measured for the asymmetric native SMB (vide supra). A similar trend is observed for the EAE-modified composition, where $c_{\text{MBP/SMB}}$ decreases from 10% to about 5% vol between the asymmetric and the bicytoplasmic SMB. Reflectivity and SLD profiles remain mostly unchanged upon MBP addition (Fig. S4 and Table S4), consistent with negligible bilayer perturbation.

An MBP-bound asymmetric SMB prepared at 15 °C was then heated to 37 °C, a temperature at which cholesterol redistribution across the bilayer was shown to occur. Under these conditions, $c_{\text{MBP/SMB}}$ drops from 25% vol to $\sim 2\%$ vol for the native myelin and from 10% vol to $\sim 6\%$ vol for the EAE-modified one (Fig. 7a), reaching values comparable to those of the symmetric bicytoplasmic membranes. Addition of MBP caused only minor variations in the reflectivity and SLD profiles at 37 °C (Fig. S4 and Table S4). Both results thus point in the same direction: MBP binding is drastically reduced under both conditions—cholesterol redistribution at 37 °C and symmetric bicytoplasmic bilayers—compared to the former asymmetric SMB at 15 °C.

A thinning of the MBP layer (Fig. 7b) is observed in the EAE-modified asymmetric SMB relative to the native one. By contrast, t_{MBP} remains essentially unchanged for both the bicytoplasmic bilayer and the SMB at 37 °C, irrespective of lipid composition.

To test whether optical-scale lipid organization might play a role in protein binding, equivalent SMB were prepared on glass by sequential LB/LS deposition at low temperature ($T = 20$ °C) and a surface pressure of 35 mN m^{-1} , with Cyanine 5 PE (Cy5-PE) included in the first (periplasmic) transfer step and AF594-labelled PE in the second (cytoplasmic) step; protein binding was imaged with Atto488-labelled MBP at the same bulk MBP concentration used in NR (Fig. 8). In representative focal planes from the widefield z-stacks, the AF594 reporter extended across the field of view in all three SMB samples, with weak mottled intensity variations and occasional small dark spots (Fig. 8a,c,e; cropped enlargements in Fig. S5). The mottled contrast may reflect local reporter enrichment or small-scale compositional heterogeneity, whereas the

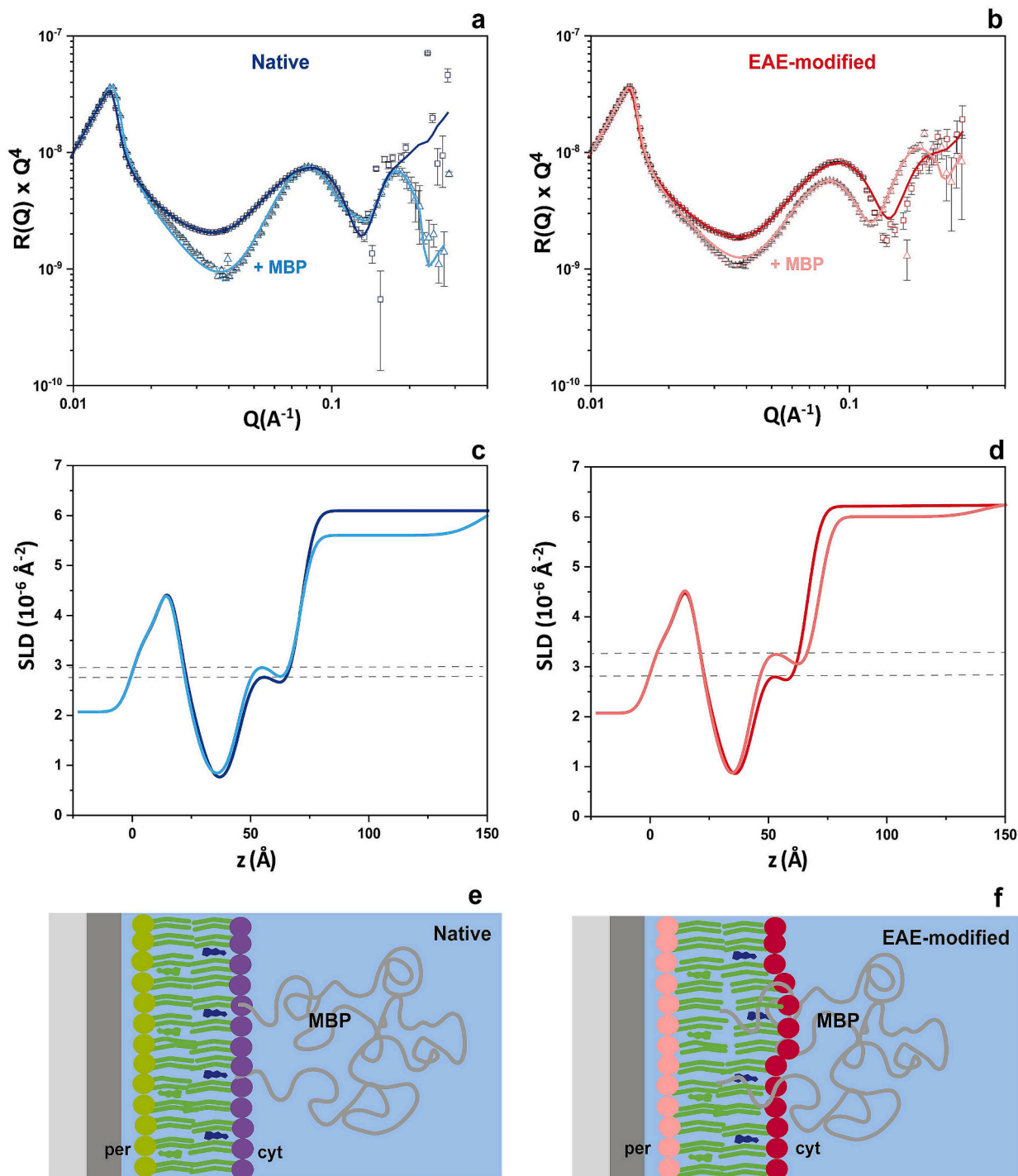


Fig. 6. NR data (a,b), SLD profiles (c,d), and schematics (e,f) for native (blue) and EAE-modified (red) asymmetric SMB before and after MBP addition at 15 °C (D_2O). MBP thickness t_{MBP} and coverage η_{MBP} are summarised in Table 5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

darker spots are compatible with local transfer/coverage defects. Neither type of contrast delineated sharp boundaries or percolating domains characteristic of well-defined optical-scale phase separation at 35 mN m⁻¹, consistent with previous results on model cytoplasmic myelin monolayers, where optically resolved Lo/Ld contrast is lost by ~20–30 mN m⁻¹, below the deposition pressure used here [21]. In the asymmetric membranes (Fig. 8a,c), the Cy5 reporter confirmed incorporation of lipids from the first transfer step; the corresponding full-field images are shown in Fig. S6. Occasional Cy5 puncta are also compatible with local probe aggregation. As the z-stacks were sampled every 0.29

μm, whereas the total bilayer thickness is ~5 nm, the two leaflets cannot be optically resolved separately.

After MBP addition, the native asymmetric SMB showed extended MBP-rich regions, whereas the EAE-modified asymmetric and bicytoplasmic SMB displayed weaker and more spatially restricted MBP fluorescence (Fig. 8b,d,f), consistent with the average MBP recruitment determined by NR. Because specular NR averages over the illuminated sample area, η_{MBP} and t_{MBP} should be interpreted as effective descriptors of the adsorbed layer rather than local properties of individual MBP-rich regions. The native/EAE difference may therefore reflect combined

Table 5

Fitted parameters for native and EAE-modified asymmetric SMB in the presence of MBP at 15 °C: layer thicknesses (t), surface coverage (η), and SLD ($\times 10^{-6} \text{ \AA}^{-2}$) for the SMB, and t_{MBP} with η_{MBP} for the protein. Values are best-fit $\pm 1\sigma$.

Layer	Native			EAE-modified		
	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)
p_h	10.1 $\pm$ 1.5	0.92 $\pm$ 0.02	1.96 $\pm$ 0.61	10.0 $\pm$ 1.1	0.89 $\pm$ 0.03	1.95 $\pm$ 0.48
	16.2 $\pm$ 1.3	0.91 $\pm$ 0.02	-0.08 $\pm$ 0.51	14.5 $\pm$ 2.1	0.90 $\pm$ 0.06	0.19 $\pm$ 0.71
c_t	17.3 $\pm$ 1.4	0.79 $\pm$ 0.04	2.47 $\pm$ 0.44	17.5 $\pm$ 1.6	0.77 $\pm$ 0.04	3.01 $\pm$ 0.88
	10.3 $\pm$ 1.5	0.75 $\pm$ 0.03	1.71 $\pm$ 0.52	11.5 $\pm$ 1.2	0.75 $\pm$ 0.05	2.01 $\pm$ 0.73
SMB	53.9 $\pm$ 5.7			53.5 $\pm$ 6.0		
MBP	78.9 $\pm$ 8.9	0.25 $\pm$ 0.04		68.6 $\pm$ 9.1	0.10 $\pm$ 0.04	

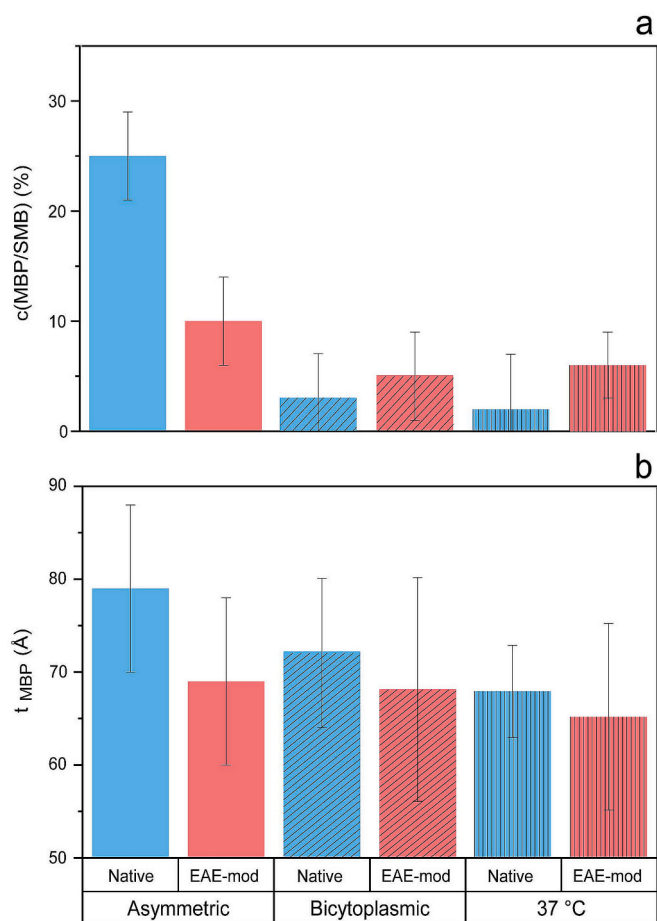


Fig. 7. MBP binding across membrane states—symmetric (15 °C), asymmetric (15 °C), and asymmetric heated (37 °C). (a) Surface concentration $c_{\text{MBP/SMB}}$ (%) (b) Protein-layer thickness t_{MBP} . Blue bars: native; red bars: EAE-modified. Bars show best-fit values; error bars are 1σ . Exact values in Tables 5 and S4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contributions from the lateral extent of MBP-rich regions, local protein density and vertical organization.

Overall, the reduced MBP recruitment upon loss of asymmetry is more consistently linked to leaflet arrangement and local packing/

hydration than to optically resolved lipid phase separation. Weak small-scale or sub-diffraction heterogeneity cannot be excluded, but these fluorescence images do not resolve it as a distinct phase-separated pattern.

3.5. Vesicle adsorption forming a double bilayer (DBL)

To test whether MBP-mediated adhesion persists upon bringing a second membrane into contact, we deposited cytoplasmic myelin vesicles onto pre-formed MBP/SMB of both lipidic compositions and promoted rupture/coverage by an osmotic D_2O shock. This system can be considered as a simple biomimetic model of an oligodendrocyte wrapping around an axon, with the cytoplasmic membrane leaflets held together by MBP [20].

After ~ 1 h, the reflectivity stabilised (Fig. 9a). In the raw $R(Q)$ curves, vesicle deposition shifts the Kiessig minima to lower Q and reduces their spacing, consistent with the addition of a ~ 50 Å slab and an increase in interfacial roughness; this effect is captured by the reconstructed SLD profiles (Fig. 9b).

Datasets were analysed with the DBL model defined in the Methods section: the underlying SMB (from the MBP/SMB stage) was kept fixed, the MBP volume fraction was allowed to vary within $\pm 20\%$ from its pre-adsorbed value, and the upper bilayer was represented by a single, highly rough slab with adjustable SLD and thickness. Under these constraints, the MBP layer compacts to $t_{\text{MBP/DBL}} = 38.1 \pm 6.7$ Å (native) and 48.6 ± 8.8 Å (EAE), while its coverage decreases modestly relative to the MBP/SMB state, from $\eta = 0.25 \pm 0.04$ to 0.20 ± 0.05 in native, and from $\eta = 0.10 \pm 0.04$ to 0.08 ± 0.03 in EAE-modified myelin (Tables 5 and 6). This behaviour indicates a rearrangement into a more compact adhesive layer as MBP bridges the two cytoplasmic leaflets.

The upper bilayer is incomplete and laterally disordered: the single-slab model returns an apparent coverage η_{DBL} of 0.68 ± 0.19 (native) and 0.72 ± 0.22 (EAE), and SLD of 0.66 ± 0.26 and $0.94 \pm 0.31 \times 10^{-6} \text{ \AA}^{-2}$, respectively (Table 6). The higher SLD in the EAE case is compatible with deeper MBP penetration or enhanced D_2O uptake at the protein-membrane interface; given the single-slab representation, these effects cannot be disentangled unambiguously.

The inter-bilayer spacing is on average ~ 10 Å larger in the EAE-modified myelin compared to the native one, which reinforces the idea that lipid composition tunes not only the protein recruitment but also the thickness of the periodic stack (Fig. 9c,d; Table 6).

4. Conclusions

To our knowledge, this is the first study focused on the design of silicon-supported asymmetric membranes with realistic myelin-like lipid complexity, providing a new platform for dissecting leaflet-specific contributions to membrane-protein interactions. Our experiments demonstrate that membrane asymmetry is not a neutral feature in the organization of myelin bilayers, but rather a determining factor that modulates interactions between myelin basic protein and the membrane.

Using neutron reflectometry, we showed that myelin basic protein binds preferentially to asymmetric supported myelin bilayers that mimic the native composition of myelin, whereas bicytoplasmic bilayers, despite bearing a higher net negative charge, exhibit significantly reduced protein binding. The reduced protein recruitment supports a mechanism in which leaflet asymmetry and interleaflet coupling tune the local packing and hydration landscape sensed by the protein. Fluorescence microscopy showed modest optical-scale heterogeneity in the lipid-reporter channel, but not sharply bounded or percolating domains, arguing against optically resolved lipid phase separation as the dominant origin of the binding differences. At the protein level, the laterally non-uniform MBP fluorescence indicates that the NR-derived values should be read as average descriptors of the adsorbed layer. We hypothesize that asymmetry pre-organizes an anionic, weakly hydrated

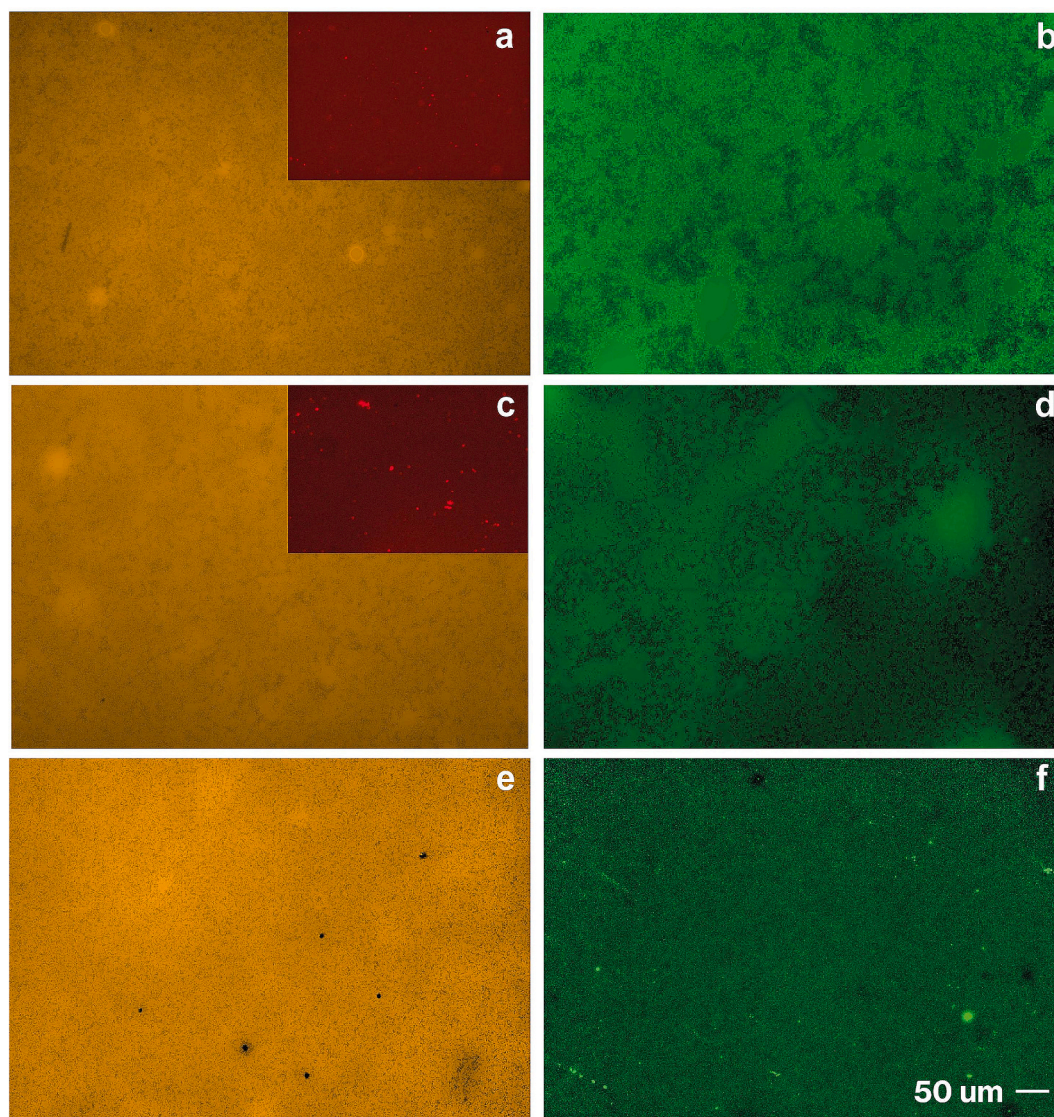


Fig. 8. Fluorescence microscopy of equivalent supported myelin bilayers prepared on glass by sequential LB/LS deposition, before and after MBP addition. (a,b) Native asymmetric SMB; (c,d) EAE-modified asymmetric SMB; (e,f) symmetric bicytoplasmic SMB. Panels (a,c,e) show AF594-labelled PE from the cytoplasmic deposition mixture in yellow; cropped enlargements of representative regions are provided in Fig. S5. In the asymmetric bilayers, the periplasmic reporter 18:0 Cyanine 5 PE is shown as red insets in (a,c); the corresponding full-field Cy5 images are provided in Fig. S6. Panels (b,d,f) show Atto488-labelled MBP after incubation at the same bulk concentration used in NR and removal of excess protein. Scale bar: 50 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cytoplasmic environment that enhances protein binding. Previous studies using both simple and more complex membrane systems have already shown that changes in compositional asymmetry can influence the mechanical properties of membranes [54,55] and their interactions with proteins [33,56].

Heating the supported asymmetric bilayers toward physiological temperature leads to loss of the leaflet-resolved contrast produced by d_{45} -cholesterol and is accompanied by a marked decrease in protein surface coverage. Asymmetric lipid distribution is known to be actively sustained in vivo by the coordinated action of flippases, floppases, and cytoskeletal scaffolds, often at considerable energetic cost [53]. When the mechanisms that sustain lipid asymmetry are compromised, lipid scrambling provides a plausible route to rapid compositional remodelling across the bilayer [57]. In that scenario, the reduced MBP recruitment observed upon loss of asymmetry may represent an early molecular consequence of such remodelling, with potential relevance under neuroinflammatory conditions. Given the protein's capacity to bridge cytoplasmic leaflets and condense anionic lipids, it is possible

that protein binding itself helps stabilize leaflet asymmetry in vivo by hindering interleaflet lipid exchange, a possibility which merits further investigation.

This interpretation must be framed within the constraints of the silicon-supported LB/LS model: the system provides controlled leaflet deposition and strong neutron contrast, but it lacks active lipid transport, membrane curvature, cytoskeletal scaffolding, and the full multi-lamellar topology of myelin; moreover, residual defects, support interactions, and high but incomplete leaflet coverage may facilitate passive redistribution [49–51].

In line with the results of Raasakka et al. [20] and Krugmann et al. [18,19], we found that the initial protein layer thickness reaches ~ 80 Å, more than twice the typical spacing of the intraperiod dense line reported for native myelin. This could reflect the extension of a single disordered protein molecule or the formation of an amorphous protein meshwork with lateral self-association [20,58,59], possibly involving dimers. We also observed that, while the protein inserts more deeply into the modified membranes, it does not promote the same level of

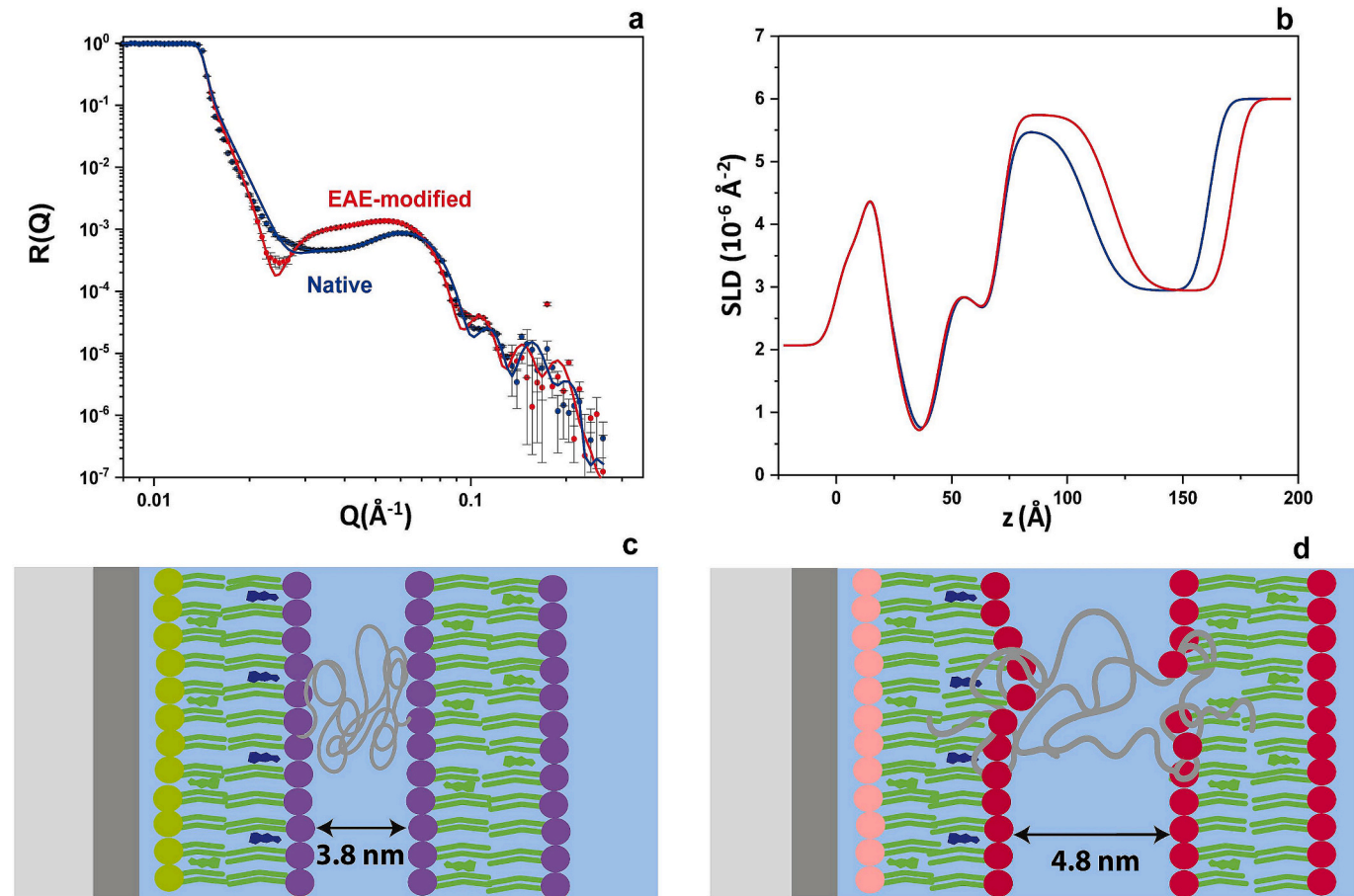


Fig. 9. Double-bilayer (DBL) assembly on pre-adsorbed MBP/SMB: (a) NR data and fits; (b) SLD profiles; (c,d) schematics for native (blue) and EAE-modified (red) compositions. The DBL is modelled as a single, rough slab; MBP compacts upon bridging. Fitted MBP and DBL parameters are listed in Table 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6

Fitted parameters for the MBP layer and the upper bilayer in the DBL configuration: MBP thickness ($t_{MBP/DBL}$) and coverage ($\eta_{MBP/DBL}$), and DBL apparent thickness (t_{DBL}), coverage (η_{DBL}) and SLD ($\times 10^{-6} \text{ \AA}^{-2}$). Values are best-fit $\pm 1\sigma$.

Layer	Native			EAE-modified		
	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)	t (Å)	η	SLD ($\times 10^{-6} \text{ \AA}^{-2}$)
MBP	38.1 ± 6.7	0.20 ± 0.05		48.6 ± 8.8	0.08 ± 0.03	
DBL	52.4 ± 8.9	0.68 ± 0.19	0.66 ± 0.26	54.6 ± 10.3	0.72 ± 0.22	0.94 ± 0.31

protein enrichment as in the native system. This points to an uncoupling between membrane insertion and adhesive function. Complementary double bilayer experiments provide a mechanical and biological perspective: after deposition of the second bilayer, the pre-adsorbed protein layer becomes reproducibly thinner, indicating a rearrangement into a more compact and adhesive conformation as the protein bridges both leaflets. This transition appears less effective in the Experimental Autoimmune Encephalomyelitis model, where the apparent intermembrane spacing is larger than in the native case. Altogether, these results support the idea that protein recruitment and structural adaptation are strongly influenced by the lipid environment, as previously proposed using similar biomimetic systems [17,18].

In summary, these findings support the view that myelin is not a passive lipid sheath, but a dynamic structure whose stability relies on a

tightly regulated interplay between protein conformation and lipid organization [12,60]. By providing nanoscale insight into myelin basic protein–membrane interactions under asymmetric and pathological conditions, our study offers mechanistic understanding of how membrane architecture governs protein function and, ultimately, myelin stability.

CRediT authorship contribution statement

Julio M. Pusterla: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alexandros Koutsoubas:** Writing – review & editing, Validation, Investigation, Formal analysis. **Nicolò Paracini:** Writing – review & editing, Resources, Investigation, Data curation. **Philipp Gutfreund:** Writing – review & editing, Resources, Investigation, Data curation. **Maximilian W.A. Skoda:** Writing – review & editing, Resources, Investigation, Data curation. **Stephen Hall:** Writing – review & editing, Resources, Investigation, Data curation. **Igor Graf von Westarp:** Writing – review & editing, Investigation. **Irina Apanasenko:** Writing – review & editing, Investigation. **Sabine Dieluweit:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Guillermo Beltramo:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Marina Cagnes:** Resources. **Stephan Förster:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Andreas M. Stadler:** Writing – review & editing, Supervision, Project administration, Investigation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcis.2026.141116>.

Data availability

Raw neutron reflectometry data are available at the ISIS and ILL portals under the DOIs listed in the Data availability section. Additional processed data are available from the authors on request.

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