

PEGylation of Membrane Proteins Like Bacteriorhodopsin as a Tool to Increase Their Stability toward Ethanol

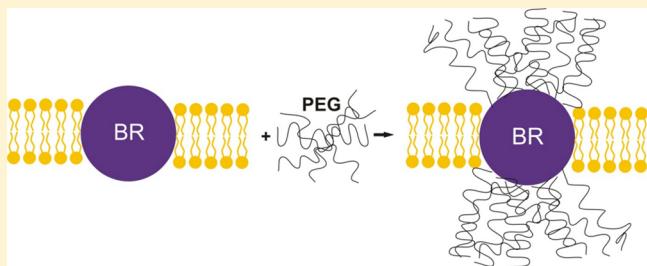
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Supporting Information

ABSTRACT: Protection of biological compounds, for example, enzymes, viruses, or even whole cells, against degradation is very important for many applications. Embedding of such compounds into polymer matrices is a straightforward common method. However, in biotechnology and medicine there is a great interest to prepare micro- and nanosized shells around the biocomponents in order to protect them and having only a minor increase in size. The PEGylation of biological macromolecules has gained attention because degradation by proteolytic enzymes is significantly retarded and, in turn, their bioavailability is enhanced. We found that PEGylation is also a powerful tool to protect biomaterials from degradation by small organic solvent molecules, in particular, ethanol. Methoxy-polyethylene glycol (MPEG) modified BR survives exposure to significant concentrations of ethanol, up to 30%, and preserves its photochromism, whereas unmodified PM is instantaneously denatured at such concentrations. This is useful for potential technical applications of BR but is of relevance for many other applications where biomaterials and, in particular, biomembranes may be exposed to solvents.



■ INTRODUCTION

PEGylation was introduced by Davies and Abuchowsky¹ in the 1970s and since then has developed into a state-of-the-art method to enhance the biological activity or availability of biological compounds by retarding their biodegradation.² Examples have been reported for enzymes, DNA, several drugs, and so on.^{3,4} PEGylation is an alternative technique to the embedding of biological compounds into, for example, silica sol-gel matrices,^{5–7} organoclays,⁸ and polymers.^{9,10} The coupling of PEG to a biomacromolecule such as polynucleotides or polypeptides¹¹ requires the activation of PEG by introducing a linker molecule that then allows functionalization of the biomaterial.

We now investigated whether PEGylation also serves as a diffusion barrier against low molecular weight solvent molecules. The purple membrane (PM) from *Halobacterium salinarum* is an excellent test object. PM comprises bacteriorhodopsin (BR) as the only protein and lipids in a stoichiometric ratio of 10 lipids per BR.¹² PM is a sensitive example because even minor amounts of ethanol destroy the membrane assembly and the BR is denatured, which is easy to recognize as it changes its intense purple color to pale yellow irreversibly. The functional intactness of BR is also easy to visualize using the photochromism of BR. Exposure to yellow light converts the purple to a yellow colored intermediate and flashing with blue light returns it back to purple.¹³

We found surprisingly that PEGylation of PM forms an effective diffusion barrier for low molecular organic molecules like ethanol. As a result, PEGylated PM becomes immune toward

short-term exposure to, for example, ethanol, which destroys the crystallinity of PM patches.¹⁴ Such short-term exposures may occur in technical applications of BR, for example, inkjet printing with bacteriorhodopsin.¹⁵

For this study we used PM containing the BR variant BR-D96N, which has an increased light sensitivity¹⁶ because of the substitution of Asp-96 with Asn.

■ METHODS AND MATERIALS

Chemicals. Chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Fisher Scientific (Leicestershire, United Kingdom), or Fluka (Buchs, Switzerland) and were used as received. Methoxy-polyethylene glycol activated with cyanuric chloride (MPEG) was obtained from Sigma-Aldrich (Taufkirchen, Germany).

Isolation of Purple Membrane. Purple membrane (PM) was purified according to standard procedures,¹⁷ lyophilized, and stored at room temperature until use. PM comprising the BR variant BR-D96N, where Asp-96 (D) is replaced by Asn (N), was used throughout all the experiments due to its enhanced photochromic properties,¹⁶ which are very useful for the photochromism tests.

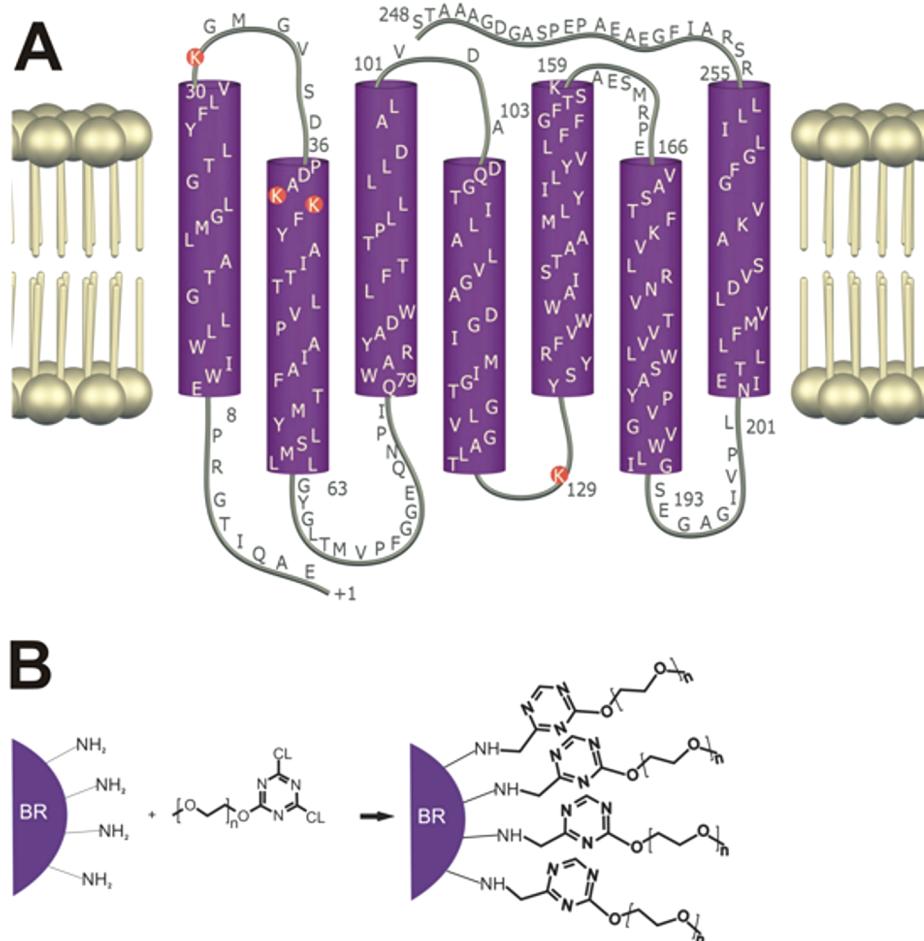
General Procedure for PEGylation. Sodium carbonate buffer (0.2 M) of pH 8.3 was obtained from mixing 0.2 M solutions of sodium bicarbonate and sodium carbonate. In 5 mL

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Scheme 1. (A) Peptide Sequence of Bacteriorhodopsin (BR) Comprises Four Lysines (Marked in Red) That Are Primarily Accessible for Chemical Modification; (B) Schematic Illustration of the PEGylation of BR Lysines with Cyanuric Chloride Activated PEG ($n \approx 110$)



of this buffer, 200 mg PM ($7.41 \mu\text{M}$) was suspended and 705.5 mg MPEG (0.14 mM, $M_w \approx 5000 \text{ g mol}^{-1}$) and slowly added. After 24 h of incubation at 50°C , the PM was removed from the PEGylation solution by pelleting the PM (Sorvall Super T 21, SL-505 bucket rotor, 45 min, 18500 rpm, 24°C) and resuspending in 10 mL of distilled water. The PEGylated material was stored at -20°C until further use.

Analytical Density Gradient Centrifugation. Final purification of PEGylated PM was carried out via a five phase sucrose density gradient with 80 mL of (D+)-sucrose solutions with concentrations of 34, 38, 39, 41, and 50% were prepared and checked for their correct refractive index. From highest to lowest density, 2.2 mL of each solution were funneled into an ultra centrifugation tube (12.5 mL; Kontron 9091–90200) via a peristaltic pump to form separate layers. A 100 μL aliquot of PM material (unmodified PM: OD 21, PEGylated PM: OD 18) was placed on sucrose density gradients and tared with distilled water. The buckets (Sorvall Instruments) were centrifuged in a Sorvall WX ultra 80 centrifuge in a Sorvall Surespin 630/36 rotor at 4°C and 25000 rpm for 19 h.

Mass Spectroscopy. Samples were prepared according to Hufnagel et al.¹⁸ First, 10 nmol PM is denatureated by the addition of 1 mL of ethanol in an Eppendorf cup, vortexed for 2 min, and sonicated (Ultrasound disintegrator Sonifier II, Branson, UK; microtip, 1 s pulses with an 25% amplitude, 5 s pulse off, 5-times repeated). Then, 500 μL of hexane was added

and the sample was centrifuged for 3 min at 5000 rpm at room temperature (Biofuge 13, Heraeus Instruments). The supernatant was discarded, 1 mL of hexane was added, vortexed, and sonicated again, as described above. After pelleting in the centrifuge, the supernatant was carefully discarded and the remaining white-colored pellet was dried in an argon flow for 15 min. Finally, the pellet was dissolved in a mixture comprising 200 μL of chloroform, 200 μL of methanol, 175 μL of distilled water, and 10 μL of methanoic acid and used for analysis.

Mass spectrometry (MS) was performed on a Qstar Pulsar I mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with an electrospray ionization source (ESI). Samples were continuously injected from a Hamilton syringe with a flow rate of 25 $\mu\text{L}/\text{min}$.

The following ESI parameters were used: ion spray voltage 5 kV, declustering potential (DP1) 80 V, focusing potential (FP) 220 V, declustering potential 2 (DP2) 15 V. The parameters for ionization (N_2) and protective gas (N_2) were 25 (arbitrary units). For deconvolution purposes, the “Bayesian Protein Reconstruct tool” as part of the BioAnalyst 1.1.5 software package was used. Measurements were performed in positive mode.

Ethanol Stability and Photochromism Testing. The main adsorption peak at 570 nm refers to the B state of BR indicating the structural intactness of the material. After the addition of an ethanol–water mixture to the dispersion of

PEGylated PM UV-vis spectra (Lambda 35, PerkinElmer Instruments, U.S.A.) were measured.

To detect whether the PEGylated PM retained its photochromic activity, we measured modified PM dispersed in 0.1 M borate buffer (pH 8.5) after light irradiation with blue light to initiate the B state and yellow light to achieve the M^{II} state by holding into the beam of lamp system consisting of a blue light ($\lambda = 565$ nm) and yellow light ($\lambda = 400$ nm) for 1 min.

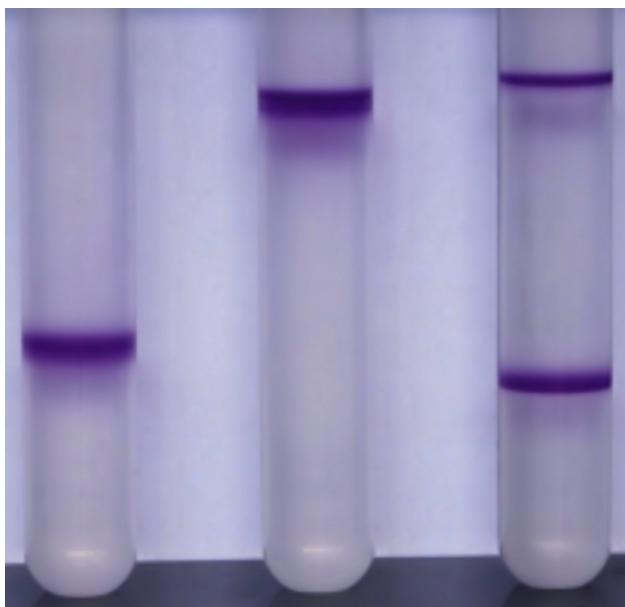


Figure 1. Density gradient centrifugation of unmodified and PEGylated PM. Sucrose gradients loaded with unmodified BR-D96N (left), PEGylated BR-D96N (middle), and a mixture of unmodified and PEGylated BR-D96N (right) after density gradient ultracentrifugation. Both materials show sharp bands, but with significantly different buoyant densities, 1.18 g mL^{-1} for unmodified PM (left) and 1.16 g mL^{-1} for PEGylated PM (middle).

Determination of Particle Size and Zeta Potential.

Particle size and the zeta potential were measured using a Delsa NanoC Particle Analyzer (Beckman Coulter). For these measurements we used solutions of unmodified PM (OD = 10) and PEGylated PM (OD = 10) in distilled water.

RESULTS AND DISCUSSION

PEGylation of BR is easily accomplished by using methoxy-polyethylene glycol activated with cyanuric chloride (Scheme 1), which primarily reacts with lysine and N-terminal amino groups.¹³ For BR, four lysines are known to be accessible for easy modification, these are lysine 30, 40, 41, and 129.^{19–23} Lysine 129 is the only one on the extracellular side of the membrane, whereas lysines 30, 40, and 41 are located on the cytoplasmic side of the membrane (see Scheme 1). All other lysines in BR are located within the membrane bilayer and are not accessible to aqueous reagents. Careful adjustment of the pH during the reaction time is essential to buffer the generated hydrochloric acid. A pH value of pH = 8.3 revealed to be optimal for the reaction. In a typical procedure, $7.41 \mu\text{M}$ PM (200 mg) and 0.14 mM (705.5 mg) activated PEG were dissolved in 5 mL 0.2 M sodium carbonate buffer pH = 8.3, heated to 50 °C, and stirred for 24 h. The reaction was stopped by pelleting the resulting product by centrifugation. The pellet was dissolved in distilled water and finally purified by preparative density gradient ultracentrifugation.

Analytical sucrose density gradients of unmodified and PEGylated PM are shown in Figure 1. It reveals that a significant decrease in buoyant density ($\rho = 1.16 \text{ g mL}^{-1}$) compared to unmodified PM ($\rho = 1.18 \text{ g mL}^{-1}$)²⁴ accompanies the PEGylation process.

The mass spectra (Figure 2) indicate that up to four PEG molecules were coupled to BR (see also Supporting Information).

PM containing the BR variant BR-D96N shows a prolonged lifetime of the M^{II} intermediate resulting in an enhanced photochromic effect.¹⁶ In Figure 3A the absorption spectra of the unmodified and the PEG-modified PM after light exposure to

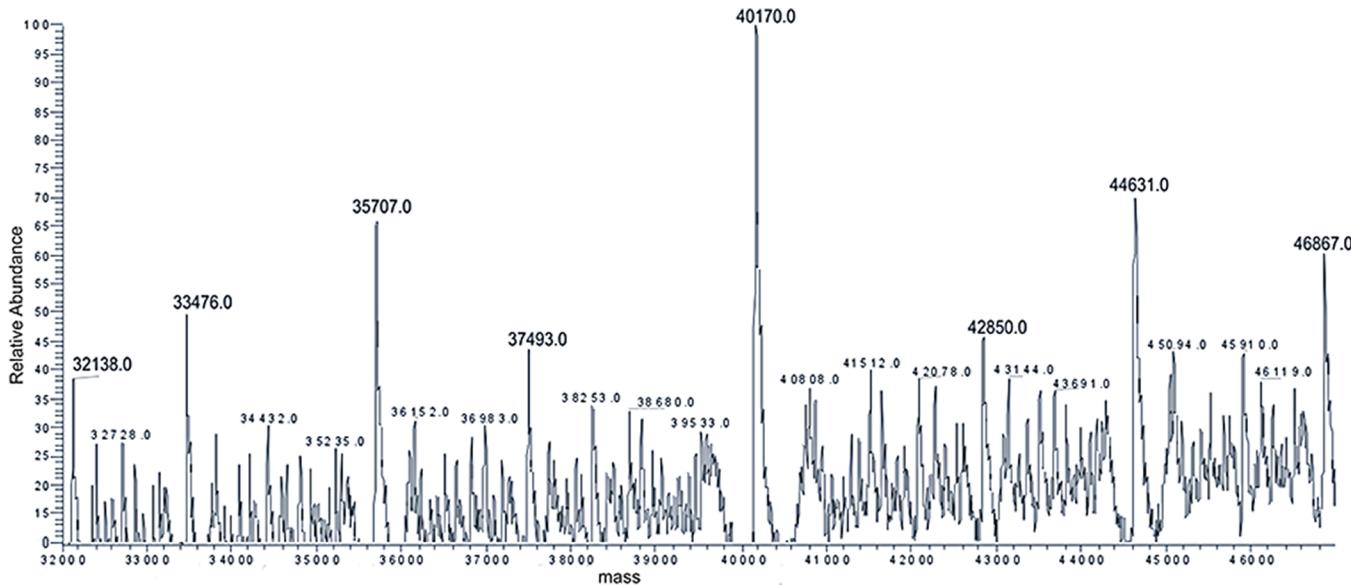


Figure 2. Deconvoluted ESI mass spectrum of PEGylated BR. The molecular weight of unmodified BR is 27684 Da. PEG in average has a molecular weight of 5000 Da. Numerous coupling peaks are observed, but some of them are dominant. Up to four times coupling of PEG to PM-embedded BR is observed.

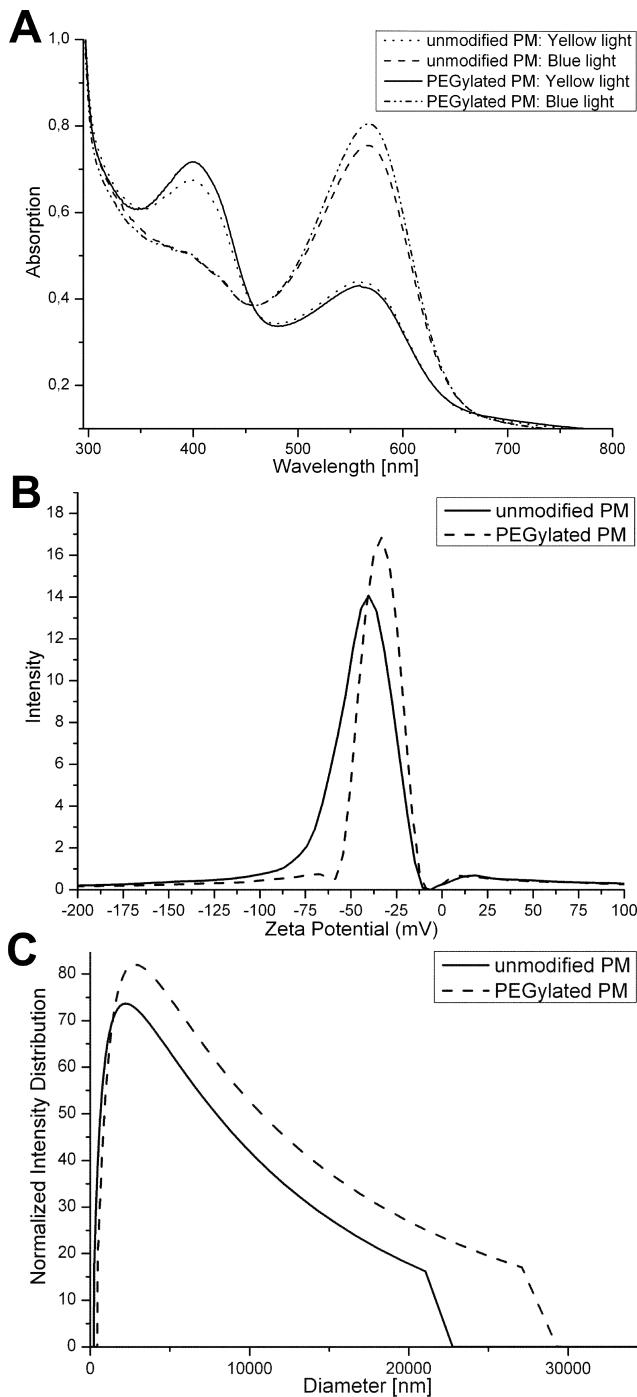


Figure 3. (A) Absorption spectra of unmodified and PEGylated BR-D96N after flashing with blue and yellow light indicating the preservation of its photochromic properties. (B) Zeta potential of PEGylated material in comparison to unmodified PM shows a significant decrease of about 25% from -41.73 to -31.27 mV for PEGylated BR. (C) Dynamic light scattering experiments show that PEGylation of the PM material causes an increase of about 50% in hydrodynamic radius.

yellow and blue light are shown. The observed spectral changes indicate that the PEGylated material is fully functional and behaves identical to the unmodified PM.

The zeta potential of the PEGylated PM is about 0.8 mV shifted toward neutral as PEG and the bound water molecules shield the negative surface charges. We found for unmodified PM

a zeta potential²⁵ of -41.73 mV and for PEGylated BR a zeta potential of -31.27 mV.

Size measurements of both PM types by dynamic light scattering show that the hydrodynamic radius increases significantly by about 50% during PEGylation. Due to the shape of PM, a thin flexible membrane, the absolute values are not rateable, only the relative changes are relevant.

Finally we tested the tolerance toward various concentrations of ethanol, methanol, propanol, 2-propanol, and acetone. Only for methanol and ethanol a significant protecting effect was observed. The spectral changes observed for different ethanol and methanol concentrations may be found Supporting Information. The absorption band at 570 nm of unmodified PM decreases fast even in 30% ethanol (Figure 4). The

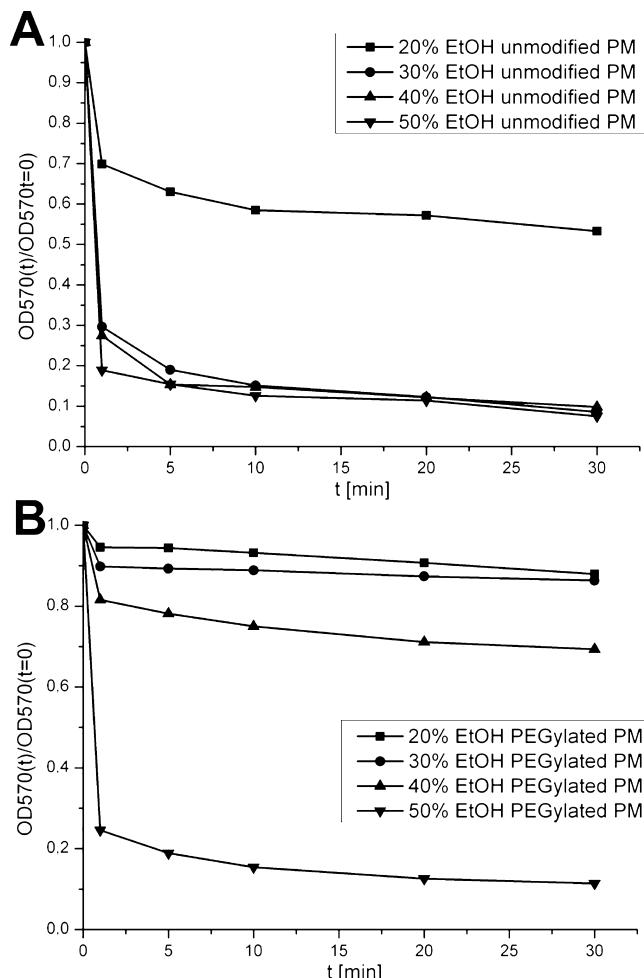


Figure 4. Time-dependent absorption changes of unmodified and PEGylated PM upon exposure to various ethanol concentrations. (A) Unmodified PM instantaneously denatures upon exposure to ethanol–water mixtures of 30% and beyond. (B) PEGylated PM is virtually unaffected by 20 and 30% ethanol and only slightly by 40% ethanol over 30 min.

irreversible loss of purple color is a clear indication for the denaturation of BR. However, PEGylated PM shows no major denaturation for ethanol concentrations up to 40%. From 50% onward it shows a clear decrease of the optical density which means denaturation. The initial drop in the curves is due to a small amount of nonprotected material in the samples. This may be omitted by further optimization of the PEGylation procedure.

Pelleting of PMs from ethanol–water test solutions and addition of fresh ethanol–water test solution did not show any further loss of absorption, that is, stability.

The color of BR and its photochromic properties depend on the integrity of its biological structure and the intactness of the membrane assembly. In Figure 5 unmodified and PEGylated PM

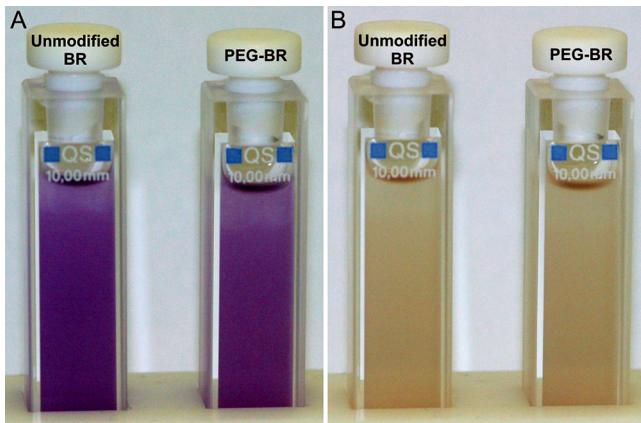


Figure 5. Photochromism of BR-D96N in borate buffer pH = 8.5. Unmodified and PEGylated purple membrane in its purple state after flashing with blue light (left) and after flash exposure to yellow light (right). No visible differences in the reversible bleaching characteristics of both materials is observed.

samples in alkaline buffer of pH 8.5 are shown before light exposure (left) and after light exposure (right). Upon light exposure, the PM reversibly changes its color to yellow. Due to the long lifetime of the M-state of the BR-D96N variant the lifetime of the yellow state is several tens of seconds and can be easily photographed. No differences in the color are observed between unmodified and PEGylated PM.

CONCLUSIONS

The easy procedure to prepare PEGylated purple membrane and the unexpected good protection of PM against ethanol concentrations of up to 40% make this method interesting for stabilizing PM against occasional short-term exposure to ethanol. Ethanol is the most prominent representative of low molecular weight water miscible solvents. Similar results were also obtained in a screening with methanol, but only little stabilization is obtained for propanol, 2-propanol, and acetone. The PEGylation yields nanoscaled protected PMs without enhancing the scattering as only minor size changes occur and the refractive index of the PEG–water hull is very close to the value of unmodified PM. This new alternative encapsulation of PM widens the possible applications of BR, such as a photochromic pigment in security inks.^{15,26} Further this result may be useful for membrane stabilization in general.

ASSOCIATED CONTENT

Supporting Information

ESI-mass spectrum of PEGylated BR, UV-vis spectra of ethanol, and methanol stability tests are presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Abuchowski, A.; Van Es, T.; Palzuk, N. C.; Davis, F. F. *J. Biol. Chem.* **1977**, *252*, 3578–3581.
- (2) Diwan, M.; Park, T. G. *J. Controlled Release* **2001**, *73*, 233–244.
- (3) Roberts, M. J.; Bentley, M. D.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002**, *54*, 459–476.
- (4) Veronese, F. M.; Pasut, G. *Drug Discovery Today* **2005**, *10*, 1451–1458.
- (5) Gill, I.; Ballesteros, A. *J. Am. Chem. Soc.* **1998**, *120*, 8587–8598.
- (6) Frenkel-Mullerad, H.; Avnir, D. *J. Am. Chem. Soc.* **2005**, *127*, 8077–8081.
- (7) Gill, I.; Ballesteros, A. *Trends Biotechnol.* **2000**, *18*, 469–479.
- (8) Patil, A. J.; Muthusamy, E.; Mann, S. *Angew. Chem., Int. Ed.* **2004**, *43*, 4928–4933.
- (9) Ion, D. D.; Gonzalez, M.; Slaez, V.; Ramlon, J.; Rieumont, J. *IEE Proc. Nanobiotechnol.* **2005**, *152*, 165–168.
- (10) Yang, Z.; Mesiano, A. J.; Venkatasubramanian, S.; Gross, S. H.; Harris, J. M.; Russell, A. J. *J. Am. Chem. Soc.* **1995**, *117*, 4843–4850.
- (11) Kozlowski, A.; Harris, J. M. *J. Controlled Release* **2001**, *72*, 217–224.
- (12) Sasaki, T.; Demuru, M.; Kato, N.; Mukai, Y. *Biochemistry* **2011**, *50*, 2283–2290.
- (13) Konishi, T.; Berkeley, L. *Biochem. Biophys. Res. Commun.* **1976**, *72*, 1437–1442.
- (14) Mitaku, S.; Ikuta, K.; Itoh, H.; Kataoka, R.; Naka, M.; Yamada, M.; Suwa, M. *Biophys. Chem.* **1988**, *30*, 69–79.
- (15) Imhof, M.; Pudewills, J.; Rhinow, D.; Chizik, I.; Hampp, N. *J. Phys. Chem. B* **2012**, *116*, 9727–9731.
- (16) Tittor, J.; Wahl, M.; Schweiger, U.; Oesterhelt, D. *Biochim. Biophys. Acta* **1994**, *1187*, 191–197.
- (17) Oesterhelt, D.; Stoeckenius, W. *Meth. Enzymol.* **1974**, *31*, 667–687.
- (18) Hufnagel, P.; Schweiger, U.; Eckerskorn, C.; Oesterhelt, D. *Anal. Biochem.* **1996**, *243*, 46–54.
- (19) Sigrist, N.; Allergini, P.; Stauffer, K.; Schaller, J.; Abdulaev, N.; Rickli, E.; Zahler, P. *J. Mol. Biol.* **1984**, *173*, 93–108.
- (20) Singh, A. K.; Sonar, S. M. *Biochim. Biophys. Acta* **1988**, *955*, 261–268.
- (21) Wolber, P. K.; Stoeckenius, W. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2303–2307.
- (22) Seiff, F.; Wallat, I.; Westerhausen, J.; Heyn, M. P. *Biophys. J.* **1986**, *50*, 629–635.
- (23) Abercrombie, D. M.; Khorana, H. G. *J. Biol. Chem.* **1986**, *261*, 4875–4880.
- (24) Stoeckenius, W.; Kunau, W. H. *J. Cell Biol.* **1968**, *38*, 337–357.
- (25) Schibata, A.; Sakata, A.; Ueno, S.; Hori, T.; Minami, K.; Baba, Y.; Kamo, N. *Biochim. Biophys. Acta* **2005**, *1669*, 17–25.
- (26) Hampp, N.; Fischer, T.; Neebe, M. *Proc. SPIE* **2002**, *4677*, 121–128.