Amphiphilic block copolymers significantly influence functions of bacteriorhodopsin in water†

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This paper investigates the effects of a macromolecular amphiphile poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) (PEO–PPO–PEO) on a photoresponsive membrane protein, bacteriorhodopsin (BR). After incubation of BR in EO23–PO65–EO23 (P123) solution, BR maintained its function as a light-driven proton pump; however, the rate of proton uptake and lifetime of the M intermediate in the photocycle of BR upon illumination were, under appropriate conditions, prolonged by about three orders of magnitude compared with that of native BR, even at neutral pH. Measurements using circular dichroism spectroscopy and dynamic light scattering indicated that BR molecules were still in a trimer state after treatment with the copolymers. This is quite different to BR, which showed a much slower photoresponse during drying. The BR–P123 assemblies did not exhibit significantly different photoresponsive behavior with changes to the water content, which implied that in the case of dried BR films or dried BR–polymer films, the elongation of the M decay may be caused not by lack of water molecules necessary for proton transfer, but by protein immobilization. Determination of the critical micelle concentration of P123 with and without BR revealed that this prolongation effect is closely related to the formation of micelles. The above phenomenon was also observed with 6 other Pluronic copolymers. In solutions of small molecular detergents, such as Triton X-100, the photoresponse of BR was prolonged as well; the extent of prolongation was, however, much less than in solutions of macromolecular amphiphiles. The formation of a local polymer coating due to self assembly of the copolymer and protein molecules might be responsible for this very significant prolongation effect, which is beneficial for the potential application of BR as an information material.

Introduction

Self assembly is one of the most exciting and fast developing fields in natural science.1–5 One such area in the study of self assembly is that of biomembranes containing membrane proteins. While self assemblies of various membrane proteins and lipids or small molecular detergents have been reported by many researchers,6–8 the effects of synthetic polymers on proteins, especially on protein functions, have been less widely investigated. This paper employs an amphiphilic block copolymer to treat a photoresponsive protein, bacteriorhodopsin (BR). Significant influence on the underlying protein function will be reported, which might be related to the self assembly between the copolymers and membrane proteins.

BR is the only protein present in the purple membrane (PM) isolated from Halobacterium salinarium.9 A BR molecule consists of 248 amino acids forming seven α-helices and a retinal chromophore bound to lysine-216 via a protonated Schiff-base linkage,10 as shown in Scheme 1 (a). Like some other retinal-containing membrane proteins,11–19 BR functions as a light-driven proton pump,20,21 and is an excellent model protein for photoresponsive supramolecular assemblies.22–26 The photoelectric response of BR is accompanied by a photochromic response. Upon absorption of light, the configuration of the retinal is changed from the all-trans to 13-cis, which triggers a remarkable series of intermediates and ultimately results in the transient deprotonation and reprotonation of the retinal Schiff

Scheme 1 Schematics of (a) a BR molecule surrounded by lipids in the PM, and (b) the chemical structure of the triblock copolymer PEO–PPO–PEO. BR is a transmembrane protein composed of seven α-helices and one retinal.
base and a net proton transport from the cytoplasmic to the extracellular side.\textsuperscript{10,27} Most of the steps in the photocycle are reversible.\textsuperscript{28} Those intermediates are called K, L, M, N and O. They differ by their lifetimes and wavelengths of maximum absorption.\textsuperscript{10,28} The M intermediate with an absorption peak at 412 nm is the only one in which the Schiff base is deprotonated. Upon deprotonation of the Schiff base of BR, the absorption peak significantly blue-shifted to 412 nm from the 570 nm in the initial ground state. This photochromic property provides a prototype for potential applications, such as spatial light modulation and real-time holographic image processing.\textsuperscript{29–32}

The lifetime of the M intermediate is several milliseconds in native PM suspensions.\textsuperscript{27} Prolongation of the M intermediate’s life time is desirable in many optical applications in order to enhance the diffraction efficiency etc.\textsuperscript{30,31} The lifetime of the M intermediate depends on the rate of reprotonation of the Schiff base from an internal donor and also on reprotonation of this donor upon proton uptake. The decay of M in water suspensions is typically biphasic.\textsuperscript{13,14} In native BR, proton transport from the internal proton donor, D96, to the Schiff base in about 3 ms, which makes the fast phase of M decay to the N intermediate in a reversible transition (M ⇌ N). The slow phase is connected to proton uptake from the bulk and subsequent reprotonation of D96 causing the decay of the N intermediate and part of M.\textsuperscript{37,14}

In native PM, BR molecules are organized into a two-dimensional hexagonal lattice of trimers that are surrounded by lipids. Once PM was partially delipidated or the trimer structure was eliminated by detergents,\textsuperscript{35–41} BR exhibited spectral properties and other features different from that of its native state. For example, the longer intermediate lifetimes and slower proton pumping rate of BR monomers (here, the term “monomer” means a single protein in contrast to BR trimer,\textsuperscript{35} different from the meaning in polymer chemistry\textsuperscript{42}) the detergents used for solubilization or delipidation of a membrane in biochemistry are usually small or moderate molecular weight amphiphiles, such as Triton X-100\textsuperscript{43} and several others.\textsuperscript{36–44} Some synthetic macromolecules are also amphiphilic. It is interesting to know what will happen if BR is treated by a macromolecular amphiphile.

Triblock copolymers of PEO–PPO–PEO, are water soluble nonionic macromolecular surfactants known as Pluronics or Poloxamers.\textsuperscript{44,45} The copolymer is sometimes abbreviated as EOn–POm–EOn, as shown in Scheme 1 (b), where n and m represent the number of repeat units in the copolymer blocks. The amphiphilic nature of this type of copolymer makes them very useful for stabilization and emulsification, pharmaceutical, and separation applications.\textsuperscript{46} In our previous communication,\textsuperscript{46} a diacylated Pluronic copolymer was utilised as a macromonomer to prepare a chemically-crosslinked hydrogel encapsulating BR, and we surprisingly found that the photochromic response of BR was prolonged by over 30 min, even under high water content conditions by a combination of gene mutation and copolymer modification. Therefore, replacing a detergent by a macromolecular amphiphile had a significant effect on the lifetime of the M intermediate of BR.

While that communication reports a novel phenomenon, this full paper investigates the origin of this phenomenon. Here, we expand the study of the effect of copolymers on protein functions in four ways: (1) we will use native BR instead of a gene-engineered D96N mutant; (2) effects on both photoelectric and photochromic responses of BR are investigated instead of merely the photochromic response; (3) EOn–POm–EOn (P123) and another 6 Pluronic copolymers are investigated as model polymers instead of just diacylated F127; (4) the M decay behaviors of BR–P123 assemblies in solution and as a dried PVA film are compared. The effects of synthetic polymers on the function of BR described in the present paper would thus show several universal features, and the underlying reasons will be partially discussed.

### Materials and methods

The PM was isolated from strain R1M1, and bacterium culture and membrane isolation procedures were performed as in Ref. 47. The pluronic copolymers listed in Table 1 were donated by BASF Corporation. Triton X-100, PEO-5K and poly(vinyl alcohol) (PVA) were purchased from Sigma-Aldrich. The molecular weight of PVA is 31 000–50 000 with 87–89% of the side groups hydrolyzed. These chemicals were used without further purification.

### Table 1 The Pluronics examined, rate constants and time constants of the M decay of BR in different solutions

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>meta</th>
<th>para</th>
<th>HLB$^a$</th>
<th>CMC</th>
<th>$k_{fast}$/s$^{-1}$</th>
<th>$k_{slow}$/s$^{-1}$</th>
<th>$\tau_{fast}$/s</th>
<th>$\tau_{slow}$/s</th>
<th>$A_{slow}$</th>
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<tr>
<td>F127</td>
<td>12600</td>
<td>65</td>
<td>99</td>
<td>70</td>
<td>18–23$^{a}$</td>
<td>0.7$^e$</td>
<td>13.2</td>
<td>3.33</td>
<td>0.49</td>
</tr>
<tr>
<td>P123</td>
<td>5750</td>
<td>65</td>
<td>23</td>
<td>30</td>
<td>7–12$^{a}$</td>
<td>0.03$^e$</td>
<td>6.0</td>
<td>1.15</td>
<td>0.14</td>
</tr>
<tr>
<td>F108</td>
<td>14600</td>
<td>56</td>
<td>129</td>
<td>80</td>
<td>&gt;24$^{a}$</td>
<td>4.5$^e$</td>
<td>15.3</td>
<td>2.63</td>
<td>0.41</td>
</tr>
<tr>
<td>F88</td>
<td>11400</td>
<td>39</td>
<td>104</td>
<td>80</td>
<td>&gt;24$^{a}$</td>
<td>10–15$^{d}$</td>
<td>20.0</td>
<td>1.28</td>
<td>0.31</td>
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<tr>
<td>P85</td>
<td>4600</td>
<td>39</td>
<td>26</td>
<td>50</td>
<td>12–18$^{a}$</td>
<td>4$^e$</td>
<td>7.7</td>
<td>1.69</td>
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<tr>
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<td>39</td>
<td>22</td>
<td>40</td>
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<td>2.6$^e$</td>
<td>7.1</td>
<td>1.08</td>
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<tr>
<td>F68</td>
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<td>76</td>
<td>80</td>
<td>&gt;24$^{a}$</td>
<td>&gt;15$^{e}$</td>
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<tr>
<td>PEO-5K</td>
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<td>57</td>
<td>100</td>
<td>——</td>
<td>——</td>
<td>5.25</td>
<td>313</td>
<td>100</td>
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<tr>
<td>Triton X-100</td>
<td>647</td>
<td>0</td>
<td>57</td>
<td>100</td>
<td>——</td>
<td>13.5$^b$</td>
<td>0.013</td>
<td>1.02</td>
<td>159</td>
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</table>

$^a$ HLB data of Pluronics from Ref. 45. $^b$ HLB data of Triton X-100 from Ref. 56. $^c$ CMC data from Ref. 49. $^d$ CMC of F88 at 25 °C is between 10–15 wt% . $^e$ CMT of 15 wt% F68 is 27 °C, so micelles were formed for F68 with a concentration of 20 wt% at 35 °C. $^f$ Molar ratio: Pluronic/BR = PEO-5K/BR = Triton X-100/BR = 750.

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A PM suspension with a concentration of 20 mg mL\(^{-1}\) as a stock solution was sonicated 10 times in an ice bath with a sonication period of 5 s and an interval of 30 s. Then 25 µL aliquots of a stock PM suspension were mixed with Pluronics or other chemicals to achieve the required concentrations. Incubation of the PM in Pluronic solutions was carried out by stirring the mixtures for about 5 days, while incubation of the PM in Triton X-100 was carried out in the dark for 2 days, without stirring. The incubation experiments were done at room temperature (about 25 °C), unless otherwise stated. Centrifugation of the samples at 20 000 rpm (~50 000 g) for 45 min was performed at 4 °C on a Shanghai Zhizheng GL21R high-speed centrifuge. PM and Pluronics usually did not dissolve in the water but remain in suspension. Nevertheless, we do not strictly distinguish between the terms “solution” and “suspension” in the present paper.

The flash-induced absorption changes were measured on a home-made kinetic spectrophotometer at 25 °C, which was built following the model described by Govindjee et al.\(^{39}\) The decay kinetics of the M intermediate was measured by the absorption changes at 412 nm. The flash-induced proton release and uptake was detected by the absorption change of a pH-sensitive dye, 8-hydroxy, 3,6-pyrenetrisulfonate (pyranine) at 456 nm.

Absorption spectra in the near UV-vis range were measured on a spectrophotometer from Beijing Puxi Corporation at 25 °C. For dark adaptation (thermal equilibration of all-trans and 13-cis-15-anti configuration of the chromophore), samples were placed in the dark for 24 h before examination; light adaptation was achieved by illuminating samples for 10 min with a tungsten-halogen lamp (about 30 mW cm\(^{-2}\)) for 5 min. Circular dichroism (CD) spectra were measured on a JASCO J-715 spectropolarimeter at 25 °C.

The critical micelle concentration (CMC) of P123 was determined by the dye solubilization method.\(^{49–52}\) A solution of 1,6-diphenyl-1,3,5-hexatriene (DPH) in methanol (10 µL at 0.4 mM) was injected into the P123 solutions (2.0 mL) with various concentrations and the solutions were equilibrated for 12 hours before measurements were taken. The absorption spectra of these samples were recorded from 300–450 nm at 25 °C. The CMC value was estimated as the crossing point when the extrapolation of the absorbance at 377 nm with respect to that of 400 nm was plotted against polymer concentration. The absorption difference was used in order to compensate the scattering effect.

Dynamic light scattering (DLS) measurements were performed on a light scattering spectrophotometer (Autosizer 4700, Malvern) with a vertically polarized incident beam at 532 nm supplied by an argon-ion laser at 25 °C with a scattering angle of 90°. Before the measurements, all the samples were filtered through a 1 µm filter (Millipore). The Stokes–Einstein equation was employed to calculate the hydrodynamic radius of the micelles. The intensity–intensity time correlation function was analyzed with the CONTIN algorithm.

Observations with transmission electron microscopy (TEM) were performed in a JEM-2011 electronic microscope with an accelerating voltage of 200 kV. Before sample preparation, a mixed solution was filtered by passing them through a 1 µm filter. 5 µL of the solution was dropped on copper grids coated with a thin carbon film, and then allowed to dry under infrared light.

BR–P123 assemblies were collected after centrifugation and then re-suspended. PVA was added into the re-suspended solution. The final concentrations of PVA and BR were 80 mg mL\(^{-1}\) and about 5 mg mL\(^{-1}\) respectively. In a control experiment, PVA was dispersed in a native PM solution. Then 0.5 mL of the mixtures were dropped onto a clean glass slide and dried in a sealed container with a saturated NaCl solution at 4 °C until dry films formed.

**Results**

**Both photoelectric and photochromic responses of BR remaining in a P123 solution**

As a light-driven proton pump, the basic biological activity is its photoelectric response and photochromic response. The photochromic property of BR could be characterized by detection of the absorption changes at 412 nm after a flash illumination, which indicates the formation and thermal decay of the M intermediate. The proton-pumping behavior of PM in a suspension could be indirectly detected from the change in the transient bulk pH triggered by a flash, with addition of a pH-sensitive dye, pyranine, before the measurement. The absorption peak of pyranine is around 456 nm, in which BR itself has no significant absorption. The transient pH change was generated because BR releases a proton to medium first (upon formation of the M intermediate) and takes up another proton from the bulk later in the photocycle. Flash-induced kinetic traces of the M intermediate and proton-pumping behavior of BR in 5 wt% P123 are shown in Fig. 1. Our measurements demonstrate that BR keeps its biological activity after treatment with macromolecular amphiphiles.

However, the kinetics of M decay and proton uptake are severely slowed. The M decay of BR in the 5 wt% P123 solution was prolonged by about 10 s at neutral pH, which was nearly 3 orders of magnitude longer when compared to that of BR in the

![Fig. 1 Flash-induced kinetic traces of the formation and decay of the M intermediate followed by absorption changes at 412 nm and proton-pump activity indicated by absorption changes of pyranine at 456 nm. The concentrations of PM, P123 and pyranine were 0.5 mg mL\(^{-1}\), 5 wt% and 0.1 mM, respectively. The aqueous medium contained 50 mM NaCl and 10 mM KCl at pH 7.05. The samples were incubated for 5 days before the measurement were taken.](image-url)
constants of decay ($k$) intermediate ($s$)

Here, $t$ is the incubation time.

The decay in Fig. 2 (a). With increasing incubation time, the M decay of BR in a 5 wt% P123 solution after various incubation times are shown in Fig. 2 (a). Flash-induced kinetic traces of the M intermediate of BR in a P123 solution with varying incubation time. (b) $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ versus incubation time $t$ in double-logarithm coordinates. Concentrations of the PM and P123 were 0.5 mg mL$^{-1}$ and 5 wt% (the molar ratio of P123–BR was 625). The samples were in 10 mM phosphate buffered saline (PBS) solutions at pH 7.0. The solid lines in (b) are just guides for the eye.

native state (about 10 ms). This is beneficial for some potential applications of BR. The proton uptake of BR in the P123 solution, as detected by the pH-sensitive dye, pyranine, was also slowed in accordance with the prolonged decay of the M intermediate, compared with a few milliseconds of BR in the native state and at 546 nm in the dark-adapted state, and is 6 nm blue-shifted with a molar ratio of P123–BR of 2300 in our experiment.

Change of thermal decay of the M intermediate with incubation time

Flash-induced kinetic traces of the M intermediate of BR in a 5 wt% P123 solution after various incubation times are shown in Fig. 2 (a). With increasing incubation time, the M decay of BR in the P123 solution was gradually slowed down. The decay component could be fitted with a bi-exponential function as follows:

$$Y(t)/Y_{\text{max}} = A_{\text{fast}}\exp(-k_{\text{fast}}t) + A_{\text{slow}}\exp(-k_{\text{slow}}t) = A_{\text{fast}}\exp(-t/\tau_{\text{fast}}) + A_{\text{slow}}\exp(-t/\tau_{\text{slow}})$$

(1)

Here, $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ are the time constants of the fast-decay and slow-decay components. $A_{\text{fast}}$ and $A_{\text{slow}}$ represent the corresponding fractions ($A_{\text{fast}} + A_{\text{slow}} = 1$). The lifetime of the M intermediate ($\tau$) is simply the reciprocal of the corresponding rate constants of decay ($k$). The time constants shown in Fig. 2 (b) exhibit an abrupt increase after 10 h of incubation. No significant increase of the M lifetime (both of $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$) was observed after 2 days. All of the following data were collected after reaching an equilibrium state (usually with incubation times of 5 or 6 days).

pH dependence of the M lifetime of BR in P123 solutions

In native PM suspensions, the lifetime of the M intermediate of BR is sensitive to pH, although $\tau_{\text{fast}}$ was not significantly influenced, the change of $\tau_{\text{slow}}$ was over 2 orders of magnitude when the pH reached 11, compared to that at pH $\approx 7$. While the M decay of native BR is, as revealed by $\tau_{\text{slow}}$, quickest at neutral pH, the decay time in the presence of 5 wt% P123 monotonically increases with pH (open triangles in Fig. 3). The ratio of the M decay time with and without P123 is thus most striking at neutral pH.

Molar ratio (P123–BR) dependence of the absorption of BR and lifetime of the M intermediate

The absorption spectra of the light-adapted BR in P123 solutions at neutral pH are shown in Fig. 4 (a). With increasing copolymer concentration, the wavelength of the absorption maximum was gradually blue-shifted along with the decrease of the absorption peak. This blue shift was observed upon treatment of PM with small molecular detergents, such as Triton X-100. With increasing molar ratio of P123–BR over the range 1–313, the absorption maximum of light-adapted BR gradually shifted from 567 to 547 nm, while no blue-shift was observed with further addition of P123 up to a concentration of 18.5 wt%, corresponding to a molar ratio of P123–BR of 2300 in our experiment.

Fig. 4 (b) shows the dependence of the wavelengths of the absorption maximum of light-adapted and dark-adapted BR upon the molar ratio of P123–BR. The absorption maximum of light-adapted and dark-adapted BR in the native state are at 567 nm and 555 nm, respectively. For BR solubilized by Triton X-100, the absorption maximum is at 552 nm in the light-adapted state and at 546 nm in the dark-adapted state, and is 6 nm blue-shifted. In the case of P123, the absorption maximum was shifted to 547 nm in the light-adapted state and 543 nm in the dark-adapted state, and is 4 nm blue-shifted with a molar ratio of
P123–BR over 313. The difference between the maximum absorption wavelengths between light-adapted and dark-adapted states gradually decreased with an increase of the molar ratio of P123–BR up to 313, and no decrease was observed with further addition of P123.

We incubated the PM in P123 solutions of different concentrations, and then recorded the corresponding flash-induced kinetic traces of the M intermediate. The decay profiles of the M intermediate were fitted by Eqn (1), and the resulting time constants were plotted against the molar ratio of P123–BR in double-logarithm coordinates, as shown in Fig. 5. The figures exhibit an abrupt increase in the time constants when the molar ratio of P123–BR was in the range of 30–300, in which the fast decrease of the absorption maxima of light-adapted and dark-adapted BR took place, and a plateau of τ was seen when the molar ratio was over about 300.

Fig. 4 (a) Absorption spectra of BR in P123 solutions at indicated molar ratios of P123–BR, and (b) the dependence of the wavelengths of the absorption maxima of light-adapted and dark-adapted BR upon molar ratio of P123–BR. The arrows in (b) show the amplitude of the light–dark adaptation changes at corresponding molar ratios of P123–BR. A blank absorption of a P123 solution is also shown by the dashed line in (a). All samples were in 10 mM PBS at pH 7.0. The concentration of PM was kept at 0.5 mg mL⁻¹, and thus a molar ratio of P123–BR of 1250 refers to a P123 concentration 10 wt%. The solid lines in (b) are just guides for the eye.

P123–BR over 313. The difference between the maximum absorption wavelengths between light-adapted and dark-adapted states gradually decreased with an increase of the molar ratio of P123–BR up to 313, and no decrease was observed with further addition of P123.

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Fig. 5 Molar-ratio dependence of time constants (τ_fast and τ_slow) of the fast and slow component of the M decay of BR. All samples were in 50 mM NaCl and 10 mM KCl at pH 7.0 ± 0.1 (adjusted by 0.1 M NaOH and 0.1 M HCl), and the concentration of PM in all of the samples was 0.75 mg ml⁻¹. These measurements were taken at 25 °C. The solid lines in the figures are just guides for the eye.

Fig. 6 (a) Absorption difference Δ(OD₃₇₇ – OD₄₀₀) of the hydrophobic dye DPH versus concentration of P123 in water with or without BR. The CMC of P123 is determined at 0.046 wt%. The CMC values became 0.23 and 0.37 wt% after the addition of 0.5 and 1.0 mg mL⁻¹ of BR. In obtaining Δ(OD₃₇₇ – OD₄₀₀), the value of (OD₃₇₇ – OD₄₀₀) of the dye aqueous solution was used as subtrahend for that of the P123 solutions with dye in the case of no BR; in the presence of BR, (OD₃₇₇ – OD₄₀₀) of P123 solutions with BR but without dye was used as subtrahend for that of the P123 solutions with both BR and dye. (b) The absorption difference of DPH in solution, time constants of the M decay and proton uptake as a function of the molar ratio P123–BR. τₘ and τ_proton are time constants of the M decay and proton uptake was fitted by a single exponential equation. The concentrations of PM in measurements of dye absorption were 0.5 and 1.0 mg mL⁻¹, and in measurements of Ω lifetime and proton pump, 0.75 mg mL⁻¹. 4 μM DPH was used as a spectroscopic probe and measurements were carried out at 25 °C. All samples were in 10 mM PBS at pH 7.0. The solid lines in (b) are just guides for the eye.
The abrupt increase of the M lifetime and proton-uptake rate with P123 concentration is related to formation of macromolecular micelles

Pluronic molecules tend to form micelles with increasing concentration or upon elevating temperature. It is interesting to establish whether there is a relationship between the slowing of the M decay and the proton uptake of BR with the micelle formation of P123. We first determined the CMC of P123 solutions in the presence of PM using DPH as a hydrophobic probe. The apparent CMC values for the P123 in solutions with PM were larger than that without PM, as shown in Fig. 6 (a); and the CMC determination curves of P123 at two different concentrations of PM basically overlapped with each other when they were plotted as the molar ratio of P123–BR, as shown in Fig. 6 (b). It implies that the block copolymer and PM might be co-assembled.

The curve of CMC determination and the time constants of the M intermediate and proton uptake are also plotted together as a function of the molar ratio of P123–BR in Fig. 6 (b). The point corresponding to the abrupt increase of the time constant of M lifetime and proton uptake is around the CMC of the Pluronics in the presence of PM, which indicates that the prolongation effect might be closely related to the micelle formation of Pluronics.

Due to the thermosensitivity of Pluronics, their micelle formation depends not only on the concentration, but also on the temperature. For example, for a 5 wt% P123 solution and a 15 wt% F68 solution, the critical micelle temperature (CMT) was 12.5 °C and 27 °C, respectively.49 We examined the M kinetics of BR in a 5 wt% P123 solution and a 15 wt% F68 solution at stirring temperatures below and above their corresponding CMT. As shown in Fig. 7, the M decay of BR is prolonged at temperatures above the CMT. BR itself exhibits a faster M decay with rising temperature in the absence of Pluronics.54 So, the significant prolongation of the M decay at temperatures above CMT might be attributed to micelle formation.

Micelle observations via dynamic light scattering (DLS) and transmission electron microscopy (TEM)

We measured the sizes of the PM in different concentrations of P123 solutions by DLS. Fig. 8 (a) shows the distribution of the apparent hydrodynamic radius, if membrane patches or micelles were regarded as a Stokes sphere in water. With the addition of the P123, a single peak was present at first, which corresponds to the PM. Another peak could be seen, when P123–BR reached 31, which is close to the CMC in Fig. 6. The concentration of Triton used in (b) was 1.0 wt% (molar ratio of Triton X-100/BR = 750). The concentration of PM in the P123 solutions was 0.5 mg mL⁻¹, and that of PM in Triton X-100 solution was 0.75 mg mL⁻¹. All samples were in 10 mM PBS at pH 7.0. Measurements were taken at 25 °C.
PM patches in a 5 wt% P123 solution were visualized by TEM, as shown in Fig. 9 (a). The nanoparticles on the PM patches might be P123 micelle aggregates. Those particles shown in Fig. 9 (b) are similar to P123 micelles, as shown in (c). The PM is not completely covered by micelles of P123 in (a), which may be caused by shrinking when the samples were dried under IR light during preparation of the sample for TEM observations.

**M decay of BR–P123 assemblies in dried films**

Usually, a long lifetime of the M intermediate can be achieved in dehydrated BR and dried BR–polymer composite films. In the present study, BR–P123 assemblies collected by centrifugation of the PM treated in 5 wt% P123 were embedded into the PVA films. The kinetic traces of the flash-induced M intermediate of BR–P123 assemblies in solution and in a dried PVA film are shown in Fig. 10. The decay of the M intermediate of BR in P123 solutions is similar to that of the dry BR–PVA film and also to that of the BR–P123 assemblies embedded into a PVA film. It is surprising that dehydration has little influence on the M decay of the BR–P123 assemblies.

**M lifetime of BR in solutions of other chemicals**

We also incubated PM in several other Pluronic molecules with different degrees of polymerization of EO and PO, as listed in Table 1. The M decays of BR treated by those Pluronic molecules were slowed almost to the same extent, as shown in Fig. 11. The molar ratio of Pluronic to BR was 750. The concentrations of Pluronics, with the exception of F68, were above their corresponding CMC values at 25 °C, as shown in Table 1. Incubation of PM in F68 solution was carried out at 35 °C in order to facilitate the formation of micelles.

Incubation of PM in solutions of PEO-5K and Triton X-100 were carried out, and kinetic curves of the M intermediate in different chemical solutions are shown in Fig. 12. The lifetime of the M intermediate was only slightly prolonged in a PEO-5K solution. Although M decay was slowed down in the solution of small molecular detergent Triton X-100, the prolongation effect was much less significant than that in the P123 solution.
in solutions of small molecular detergents, such as Triton X-100, reported changes to the photochemical properties of BR, e.g. blue-shift of maximum absorption, prolongation of the M lifetime,35,37,39,40 which was explained by changes of the native lipid environment surrounding the BR molecules in PM. In the present paper, such phenomena were also observed for the PM treated by Pluronics (Figs 4 and 5). The results indicate that macromolecular amphiphiles cause similar changes and most likely could alter the native lipid environment of the BR to a large extent, in the process of co-assembly of a synthetic polymer and PM.

Unlike the PM treated by Triton X-100, no monomers of BR were formed when the PM was treated with P123 at the same molar ratio of detergent–BR (750), as indicated by both DLS and CD measurements (Figs 8 and 13). The prolongation effect of the Pluronic copolymers on the M decay and proton uptake was much more pronounced than that produced by Triton X-100 (Fig. 12). This cannot be interpreted from the state of BR (trimer versus monomer), but might be related to the self assembly of the PM and Pluronic copolymers.

As reported in other studies, self assembly of amphiphilic block copolymers could form a lipid bilayer-like structure in water.61,62 Pluronic molecules could even span across the lipid membrane with PPO blocks integrated into the hydrophobic region of the membrane and with their hydrophilic PEO blocks sticking out on two sides of the membrane; or the PPO blocks could be inserted into the membrane with the PEO blocks residing on the same side of the membrane.63–69 We anticipate that Pluronic molecules may also have their hydrophobic PPO block penetrated into the PM with the PEO block dangling at the membrane surfaces, after stirring the mixture of the PM and the copolymers over a long period of time (about 5 days) in these experiments.

It is worth mentioning that the assembly of the copolymers and the PM was rather stable, since the prolongation effect of the M lifetime still existed even after removing the excess Pluronic copolymers from the solution by centrifugation, as shown in Fig. S1 (ESI†). In contrast, the protein denaturation occurred after several days storage in the dark at room temperature after the PM was solubilized by small or moderate molecular detergents, such as Triton X-100, reported changes to the photochemical properties of BR, e.g. blue-shift of maximum absorption, prolongation of the M lifetime,35,37,39,40 which was explained by changes of the native lipid environment surrounding the BR molecules in PM. In the present paper, such phenomena were also observed for the PM treated by Pluronics (Figs 4 and 5). The results indicate that macromolecular amphiphiles cause similar changes and most likely could alter the native lipid environment of the BR to a large extent, in the process of co-assembly of a synthetic polymer and PM.

**Discussion**

**Assembly of Pluronics and PM**

The previous studies on the solubilization or delipidation of PM by small or moderate molecular detergents, such as Triton X-100, reported changes to the photochemical properties of BR, e.g. blue-shift of maximum absorption, prolongation of the M lifetime,35,37,39,40 which was explained by changes of the native lipid environment surrounding the BR molecules in PM. In the present paper, such phenomena were also observed for the PM treated by Pluronics (Figs 4 and 5). The results indicate that macromolecular amphiphiles cause similar changes and most likely could alter the native lipid environment of the BR to a large extent, in the process of co-assembly of a synthetic polymer and PM.

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**Prolonged decay of the M intermediate and proton uptake of BR treated by different amphiphiles**

Lipids in native PMs play an important role allowing the BR to function correctly and may bind water molecules ensuring a sufficient structural flexibility of the PM.71,72 Treatments of the PM with dilute detergents illustrate that even a minor delipidation of the membrane without disrupting the trimer structure of the BR could cause disruptions of the BR photocycle,73 a blue
shift of its absorption spectra and conformational changes of the BR proteins. A significant prolongation of the M decay and proton uptake was observed when the BR was solubilized into monomers or in heavily delipidated PMs by lipid-like detergents.

According to the previous literature, a long lifetime of the M intermediate of BR can be obtained by appropriate mutation, addition of some chemical agents like lanthanum ions and guanidine hydrochloride and in dried BR or in dry BR–polymer films. In this paper, we have, by addition of amphiphilic polymers, achieved a significant prolongation of the M decay of BR in solution, of the same order of that in a dry BR–polymer film, which is significantly longer when compared with that of BR in other detergent solutions. That prolongation may originate from a much more severe restriction of conformational changes of BR imposed by the copolymers. This argument was also supported by the similarity of the kinetics of the M intermediate between BR treated by P123 in solutions and BR–P123 assemblies in dehydrated PVA films (Fig. 10). We think that a local polymeric coating was formed on the surface of the PM in the P123 solution, and thus further embedding into a PVA film did not lead to a significant effect. This also implies that in dried BR or dried BR–polymer films, the elongation of the M decay may be caused not by a lack of necessary water molecules taking part in proton transfer, but by protein immobilization.

The photocyte of the BR accompanies a series of conformational changes of the retinal chromophore and the backbone of the protein. It has been reported that reprotonation of the Schiff base, proton uptake and M decay are coupled to the movements of helices in BR, especially helix F. In the present paper, penetrated copolymer molecules, which are much larger than native lipid, might lead to a less-mobile state of proteins in the PM, and the conformational change of BR during the photochemical reaction might be restricted, especially when the local polymer coating layer is formed on the surface of the PM. As is known, the decay of the M intermediate (formation of the N intermediate) in the photocyte corresponds to the proton transfer from the inner proton donor Asp96 to the Schiff base, which is followed by reprotonation of Asp96 (decay of N intermediate) by accepting a proton from the media and accompanying backbone conformational change. So, this conformational change may be heavily restricted by penetration of the copolymers into the membranes.

Another factor might come from some specific amphiphile–protein interactions. The methyl groups on the PPO chain may interact with some residues of this seven-helix membrane protein. Baldwin and Hubbell showed that the transition of meta I to meta II of bovine rhodopsin was inhibited when the protein was embedded in bilayers of lipids with short acyl chains or with n-alkyl chains; and the phenomenon was ascribed to unfavorable lipid–protein interactions, which exerted an effective pressure on the protein.

The hypothetic physical picture of the effects of the macromolecular amphiphiles on the PMs is presented in Fig. 14. BR molecules treated by macromolecular amphiphiles still maintain the trimeric structure, as in the PM, instead of separated monomers, as seen with Triton X-100. Part of the copolymer molecules may have its hydrophobic PPO block penetrate the PM with the PEO block dangling at membrane surfaces. Upon a sufficiently high concentration of amphiphiles, a local polymer coating is formed around two surfaces of the PM, and the photoresponse of BR is significantly slowed down due to the restriction of the copolymers to the conformational change of BR.

The self assembly of the macromolecular amphiphiles and the PM results in significant prolongation of the M decay and proton uptake of BR in high water content conditions. Under these conditions, it is very easy to modify conditions in the samples, such as the addition of other chemicals and to adjust the pH; in contrast, these operations are difficult to perform for a PM in a solid polymeric matrix. Further investigation into the detailed assembly structure between these macromolecular amphiphiles and PM is called for. We would like to highlight a series of multiplexing fluorescent sensors introduced by de Silva’s group, which could simultaneously monitor local proton concentration and polarity by emission intensity and wavelength. This technique might provide a useful tool for further exploring the interactions between amphiphile micelles and membrane proteins.

Conclusions

This article examines the photoelectric and photochromic responses of BR in the presence of a series of Pluronics representing amphiphilic block copolymers. While BR kept its biological activity, the flash-induced proton uptake and the M decay of BR treated by Pluronics were slowed down by about three orders of magnitude compared with that of native BR in solution at neutral pH. Different from treatment by non-macromolecular detergents, such as Triton X-100, BR molecules still exist in the form of trimers in the PM, instead of as separated monomers. The system of BR in Pluronic aqueous solutions is very stable compared to BR in small detergent aqueous solutions.

Self assembly of the copolymer molecules and the native membrane might be responsible for the significant prolongation effect. It is likely that the assembly of amphiphilic copolymers and the PM results in a local polymer coating on the surfaces of PMs, which restricts the conformational changes of BR in the photocyte. That is why the dehydration has little effect on BR–P123 assemblies and no further prolongation of the M lifetime was observed after the BR–P123 mixtures were imbedded into dried PVA films. The similarity of the M decay of BR–P123 assemblies in a solution and a dried PVA film implies that the slowing of the M decay of BR in a dried state might be caused by
the immobilization of protein movements instead of by a lack of water molecules necessary for proton transfer. This stable system of retinal proteins of significantly prolonged M decay under high water content is helpful for fundamental research of BR and potential applications of PM as information materials. Further investigation of the interactions between various functional membrane proteins and macromolecular amphiphiles remains an open and interesting topic.

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