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Article

Effects of Esterified Styrene−Maleic Acid Copolymer Degradation on Integral Membrane Protein Extraction

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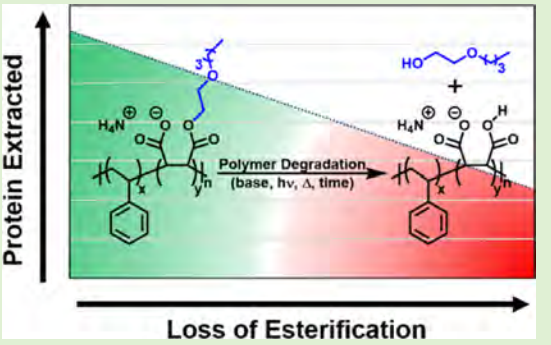
ABSTRACT: The detergent-free extraction of integral membrane proteins using styrene−maleic acid copolymers (SMAs) has shown promise as a potentially effective technique to isolate proteins in a more native-like conformation. As the field continues to develop, the protein selectivity and extraction efficiency of many analogues of traditional SMAs are being investigated. Recently, we discovered that the monoesterification of SMAs with alkoxy ethoxylate sidechains drastically affects the bioactivity of these copolymers in the extraction of photosystem I from the cyanobacterium *Thermosynechococcus elongatus*. However, subsequent investigations also revealed that the conditions under which these esterified SMA polymer analogues are prepared, purified, and stored can alter the structure of the alkoxy ethoxylate-functionalized SMA and perturb the protein extraction process. Herein, we demonstrate that the basic conditions required to solubilize SMA analogues may lead to deleterious saponification side reactions, cleaving the sidechains of an esterified SMA and dramatically decreasing its efficacy for protein extraction. We found that this process is highly dependent on temperature, with polymer samples being prepared and stored at lower temperatures exhibiting significantly fewer saponification side reactions. Furthermore, the effects of small-molecule impurities and exposure to light were also investigated, both of which are shown to have significant effects on the polymer structure and/or protein extraction process.



INTRODUCTION

The field of detergent-free, integral membrane protein isolation using styrene−maleic acid copolymers (SMAs) has grown rapidly over the last decade.[1] SMAs have enabled the isolation of membrane proteins and their surrounding lipids into discrete styrene−maleic acid lipid particles (SMALPs).[2] Compared to proteins isolated via detergent-facilitated extraction, integral membrane proteins incorporated into SMALPs have been proposed to retain a more native conformation and have enabled researchers to probe their structures using techniques like cryogenic electron microscopy (Cryo-EM).[3] As this field continues to advance, a better understanding of how structural alterations of the SMA copolymer scaffold impact the protein extraction process will be required to design next-generation SMA copolymers and derivatives thereof. This goal will be aided by in-depth SMA structure−property relationship studies and mechanistic investigations.

Prior structure−property relationship studies have shown that both the molecular weight[4] and styrene−maleic anhydride incorporation ratio[5] of an SMA can each affect the selectivity and the efficiency of the protein extraction process. It has also been shown that alternative monomer designs in SMA copolymer analogues may also impact the protein extraction process. For example, amphiphilic copolymers with hydrophobic moieties other than styrene, such as diisobutylene,[6] naphthalene,[7] and stilbene,[8] have been examined and shown to form lipid nanoparticles. Other studies have focused on altering the hydrophilic maleate groups, wherein SMA analogues have been synthesized via the esterification of the maleates with different chemical moieties, including ammonium functionalities and cyclohexyl groups.[9,10] These modifications have been shown to alter properties such as pH and divalent cation tolerance of the resulting polymers. We recently reported the synthesis of esterified SMA analogues, prepared via an esterification with alkoxy ethoxylate functionalities of various lengths, and highlighted how the inclusion of these sidechains can improve the extraction efficiency and selectivity for trimeric photosystem I (PSI) in the cyanobacteria, *Thermosynechococcus elongatus* (*Te*).[11]

During subsequent studies investigating the use of alkoxy ethoxylate-functionalized SMA analogues, we discovered variation in activity between multiple samples of these polymers. Although the polymers used were identical prior

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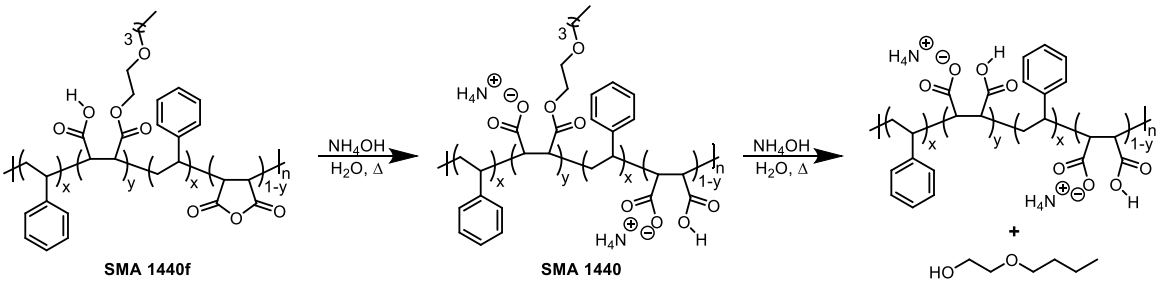
Scheme 1. Preparation of SMA 1440 via the Aqueous Solubilization of SMA 1440f and the Hypothesized Saponification of SMA 1440



to solubilization, ^{1}H NMR spectroscopy revealed a difference in the quantity of attached ester sidechains after polymer solubilization into aqueous solution at elevated temperatures (80 °C). We hypothesized that this difference in the esterification percentage resulted from undesirable saponification that occurs during solubilization (Scheme 1) and that the conditions in which the polymers are prepared and/or stored likely contribute to the overall extent of saponification. With the growing interest in esterified SMAs and other SMA analogues, it is essential to understand how copolymer synthesis, solubilization, and storage conditions impact the copolymer structure, and subsequently, the protein extraction process.

To probe the relationship between various reaction conditions and the degradation of esterified SMAs, we present a fundamental study designed to investigate how the exposure of SMA 1440 (an SMA partially esterified with 2-butoxyethanol) to temperatures approaching 80 °C and ambient light impact polymer purity, structure, and bioactivity. Furthermore, we will differentiate the effect of SMA 1440 losing esterification with the concomitant effect(s) that the released, free alcohols in solution may have on protein solubilization and/or functional properties such as PSI-chlorophyll retention. Together, this work suggests that polymer synthesis, solubilization, and storage are each critical factors dictating the reproducibility of SMA copolymer protein extraction activity and suggest that impurities in the polymer sample may have a deleterious effect on the protein extraction process and protein structure/function.

EXPERIMENTAL SECTION

General Materials and Methods. SMA 1440 flake (SMA 1440f) was obtained as a gift from Total Cray Valley (Exton, PA) and was purified via precipitation into MeOH (×3). NH_4OH was obtained from Fisher Scientific and used as received. Water was purified using a MilliQ Biocel reverse osmosis system with a resistance of >18 MΩ to mitigate the impact of trace ions. ^{1}H NMR spectroscopy was performed using a Varian 500 MHz NMR spectrometer, with 10 s between scans. Chemical shifts are reported with respect to residual solvent peaks.

SMA Solubilization and Quantification of Esterification. The solubilization of SMA 1440f was performed by combining the polymer sample (15 wt %), water (80 wt %), and a solution of 30% NH_4OH in water (5 wt %). This solution was then stirred at temperatures ranging from 40 to 80 °C for reaction times between 1 and 168 h while sealed in vials. The samples were then lyophilized to remove water, redissolved in tetrahydrofuran (THF), and purified via precipitation into hexanes. This precipitation process removes any cleaved alcohol sidechains from the polymer sample. The degree of esterification for each sample was quantified using ^{1}H NMR, as described in a previous report.[11]

Determination of Critical Aggregation Concentration. The critical aggregation concentrations for different polymer samples were measured by adopting a previously reported procedure.[12] Briefly, each polymer sample was diluted to 1.5 wt % using a standard Tris-Cl (pH = 9.5, at room temperature) buffer. These solutions were placed into a 96-well plate and diluted fivefold across each column of the plate (×12). Nile Red solution was added to each well at a final concentration of 1 μM. Then, each plate was excited at 550 nm, and the fluorescence emission was measured between 580 and 700 nm in 1 nm increments. The resultant emission spectra were fit to a Gaussian function using OriginPro 8.1, and the peak emission value from the Gaussian fit was plotted against polymer concentration for each specific sample. Each resulting data series was then fit using a standard sigmoidal line of best fit without locking any variables, also using OriginPro 8.1.

Solubilization and Isolation of Photosystem I from Thylakoid Membranes. *Te* membranes were prepared as 1 mg/mL chlorophyll solutions using established protocols.[13] The prepared membrane suspension was separated into aliquots (500 μL) and incubated with a solubilized SMA 1440 sample (1.5 wt %) for 3 h at 40 °C, while shaking (250 rpm, orbital shaker) in the dark. The resulting samples were centrifuged (190,000×*g*) to pellet the remaining insoluble thylakoid membrane fragments. After 15 min, the supernatant was carefully removed with a flame-drawn Pasteur pipette for analysis.

The solubilization efficiency (SE) of this protein extraction was determined as previously reported.[11] Isolated supernatants were diluted (×100) and chlorophyll was extracted with 90% methanol at 65 °C for 2 min. The absorbance of the extracted chlorophyll was measured at 665 nm, using a dual-beam benchtop spectrophotometer (Evolution 300, Thermo Scientific), to determine the chlorophyll concentration. The SE was calculated based on the recovered chlorophyll concentration as compared to the starting thylakoid chlorophyll concentration (1 mg/mL).

RESULTS AND DISCUSSION

Esterification Loss as a Function of Solubilization Time and Temperature. In these studies, SMA 1440f was subjected to hydrolysis at various temperatures for increasing reaction times to investigate possible degradation (Scheme 1). This polymer has a molecular weight (M_n) of 6.3 kg/mol and dispersity ($Đ$) of 1.8 (Figure S1). Furthermore, it has a 1.5:1 ratio of styrene−maleic anhydride and is partially esterified with 2-butoxyethanol, according to the manufacturer. It was experimentally determined that the specific batch of SMA 1440f used in this study has ∼48% of the maleate groups monoesterified with 2-butoxyethanol, as shown in Figure S2. The solubilization of SMA and related analogues in aqueous base (NH_4OH) is commonly performed at temperatures as high as 100 °C;[14] however, we have routinely performed this task at 80 °C in previous studies.[11] We hypothesized that the combination of basic conditions and high temperatures used to solubilize the polymer may lead to the deleterious

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saponification of the esterified maleates. To test this hypothesis, the loss of esterification as a function of solubilization time was investigated (Figure 1). Therein,

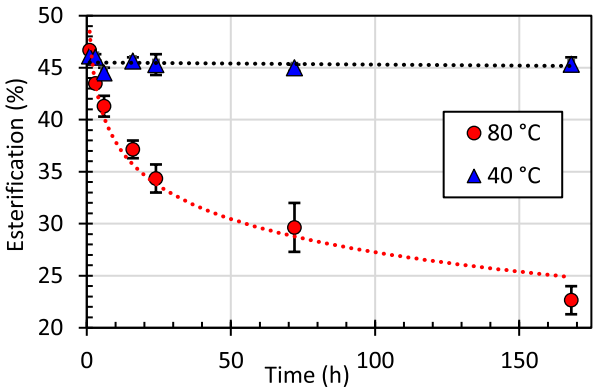

Figure 1. Monitoring SMA 1440 esterification percentage as a function of solubilization time at 80 °C (red circles) and 40 °C (blue triangles), in the presence of aqueous NH_4OH.

SMA 1440f samples were reacted with NH_4OH at either 40 or 80 °C for times ranging from 1 to 168 h, before being purified and characterized via ^1H NMR spectroscopy (Figures S3 and S4).

Figure 1 shows that solubilization of SMA 1440f at 80 °C results in a loss of 2.7, 12, and 60% of its esterified maleate groups over a solubilization times of 1, 6, and 168 h, respectively. This result demonstrates that esterification loss at 80 °C may be significant and that reaction times at this temperature should be minimized to prevent esterified polymer degradation.

In contrast, we have routinely found that many SMA derivatives may be readily solubilized at lower temperatures, such as 40 °C, yet will require slightly longer solubilization times. To probe how lower temperatures effect esterification loss, a sample of SMA 1440f was solubilized at 40 °C in aqueous NH_4OH solution, and the loss of esterification was quantified via ^1H NMR spectroscopy (Figure 1). As seen in Figure 1, only ~5% of the sample's total esterified sidechains are lost after solubilization times of 168 h at 40 °C, an order of magnitude lower than the 60% loss observed when solubilization is carried out at 80 °C.

Following degradation at 80 °C, each polymer sample discussed above was lyophilized and purified via precipitation to remove any sidechains cleaved via saponification. These samples were solubilized into aqueous solution at room temperature to prevent further degradation and used to isolate PSI from *Te* (Figure 2) to highlight how this loss of esterification can affect the protein extraction process. These results suggest that the solubilization of esterified SMA analogues at high temperature, and subsequent loss of sidechains from the sample, significantly impacts the extraction process, lowering the yield to 50% of the chlorophyll extracted compared to a polymer sample with no degradation.

Additional Impact of Light Exposure and Elevated Temperature during Solubilization. In addition to the loss of esterification discussed above, we also noticed gelation and a yellowing of our samples that increased as the SMA 1440f samples were solubilized at elevated temperatures for increasing lengths of time. Prior literature suggests that SMA copolymers can undergo photodegradation and subsequent

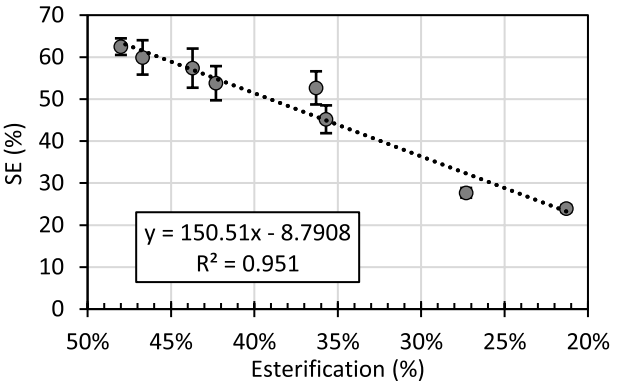

Figure 2. Percent solubilization efficiency of SMA 1440 polymers with varying degrees of esterification, resulting from increased reaction times.

cross-linking,[15] which we hypothesized could explain our observations regarding yellowing and vitrification. To probe this observation, four SMA 1440f samples were solubilized for 168 h each, two at 80 °C and two at 40 °C. Additionally, one sample at each temperature was shielded from light in an attempt to decouple the role of heat and light in this degradative process. These four samples, post-heating, can be seen in Figure 3.

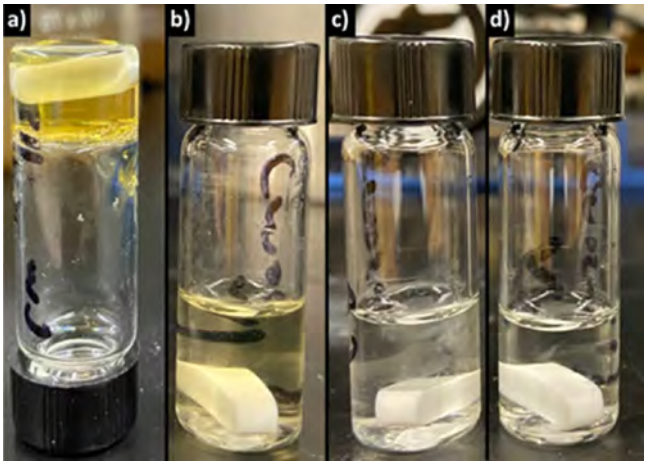

Figure 3. SMA 1440 samples heated for 168 h at (a) 80 °C (shown inverted), (b) 80 °C while shielded from light, (c) 40 °C, and (d) 40 °C while shielded from light.

Qualitatively, the sample of SMA 1440f heated for 168 h at 80 °C while uncovered experienced the greatest yellowing and fully vitrified, despite starting as a clear solution with low viscosity (Figure 3a). When heated at 80 °C for 168 h and shielded from light, the solution still turned yellow in color, but did not vitrify or exhibit a notable increase in viscosity (Figure 3b). The observed yellowing of these samples was also examined by UV−visible spectroscopy, wherein an increase in absorbance was observed between 225 and 245 nm (Figure S7). This observation is consistent with the photodegradation of similar polymers reported in previous literature studies.[15,16]

In contrast to the samples solubilized at 80 °C, both samples heated for 168 h at 40 °C did not appear to visibly change in color or experience any vitrification, irrespective of light exposure (Figure 3c, d). While these results do not provide direct evidence of SMA 1440 degradation and cross-linking at

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elevated temperatures (i.e., 80 °C), they do indicate that both temperature and light exposure may play a critical role in the SMA degradation process. Furthermore, these results strongly suggest that SMA copolymers and their analogues, regardless of their identity, should be prepared, solubilized, and stored in a way that limits exposure to light and elevated temperatures.

Esterification Loss during SMA Storage in Basic Aqueous Media. Not only are SMA polymers solubilized under basic conditions, but they are also often stored as basic aqueous solutions. To investigate the potential of further esterification loss during storage, a sample of SMA 1440f was solubilized (15 wt %) in water (80 wt %) with an aqueous solution of 30% NH_4OH in water (5 wt %) at 80 °C for 1 h. This solution was then stored at room temperature and shielded from light. Once a month, an aliquot was taken from the solution, lyophilized, purified via precipitation into hexanes, and characterized via ^1H NMR spectroscopy to determine its extent of esterification and hence quantify any loss in the esterification percentage (Figure S5). Interestingly, after 10 months of storage at ambient conditions, no noticeable loss of overall esterification was observed. This result confirms that while esterified SMA analogues are susceptible to esterification loss during solubilization at elevated temperatures, they appear to be stable in basic aqueous solution at room temperature for extended periods of time.

Effect of Free Alcohols on Protein Solubilization. The data described in this report, and in the existing literature,[11] have shown that the degree of esterification is crucial to the overall efficiency of the protein extraction process. Deleterious saponification reactions can cleave esterified sidechains during polymer solubilization, not only lowering the overall ester content of the SMA copolymer but also resulting in the release of free 2-butoxyethanol in the polymer solution. To determine if these released small-molecule alcohols impact the protein extraction process, a systematic series of SMA 1440 solutions were spiked with varying amounts of 2-butoxyethanol prior to protein extraction. Each of the samples in this experiment were solubilized at room temperature to prevent any unwanted saponification side reactions that might occur at elevated temperatures. Each SMA 1440 solution (15 wt %) was prepared using an aqueous 30% NH_4OH solution (5 wt %), 2-butoxyethanol (x wt %), and H_2O ((80−x) wt %). These polymer solutions were then diluted tenfold using a standard Tris-Cl (pH = 9.5, at room temperature) buffer to a final concentration of 1.5 wt % SMA and used to extract PSI from *Te* thylakoids. As a note, these extractions were performed using a separate batch of *Te* membranes than that were used for the study outlined in Figure 2, which accounts for the slight SE discrepancy observed between these two experimental data sets.

The impact of added 2-butoxyethanol, which mimics free small-molecule alcohols released into the polymer solution during solubilization at elevated temperatures, is shown in Figure 4. These results suggest that while SE percentages are relatively consistent (50−60% SE) for samples spiked with ≤4 wt % 2-butoxyethanol, large amounts of free 2-butoxyethanol in solution (≥5 wt %) significantly impact SE. For example, only ∼10% SE was observed for extractions containing 6 wt % 2-butoxyethanol, becoming largely ineffective at isolating PSI from *Te* (Figure 4).

Previous literature has suggested that the first step of SMA-based protein solubilization is that the SMA copolymer chains

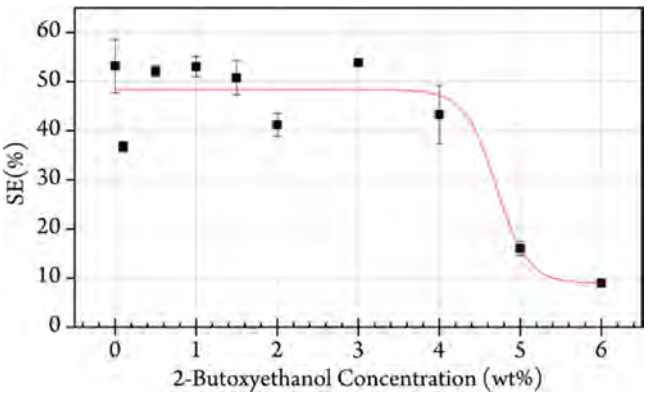

Figure 4. Effect of free 2-butoxyethanol on the SE of chlorophyll-containing proteins from thylakoids. Polymer solutions prepared in the following ratios (in wt %): 15% SMA 1440, 5% NH_4OH solution (30% in H_2O), x% 2-butoxyethanol, and (80−x)% H_2O. After preparation, each solution was diluted tenfold to arrive at the final 2-butoxyethanol concentrations plotted on the x-axis.

shift from a globular, random-coil structure to an extended chain conformation.[17] We hypothesized that small molecules, such as 2-butoxyethanol, may interact with SMA 1440 molecules or aggregates thereof in solution, potentially disrupting this conformational change and resulting in a lower SE, as we observed at higher 2-butoxyethanol concentrations. While previous studies suggest that the critical aggregation concentration (CAC) of SMA 1440 should be orders of magnitude lower than the concentrations used during the protein extraction studies described herein,[11] any observed changes in SMA 1440 CAC may provide some indication of interaction(s) between free 2-butoxyethanol and the polymer.

Indeed, our studies revealed that the addition of free 2-butoxyethanol to a solution of SMA 1440 generally increases the CAC of this polymer. Therein, we found that in all SMA 1440 solutions with any amount of free 2-butoxyethanol present, Nile Red does not fully blueshift until a polymer concentration of 0.06 wt % (Figure 5). For comparison, Nile Red exhibits a full blueshift in pure SMA 1440 solution at a

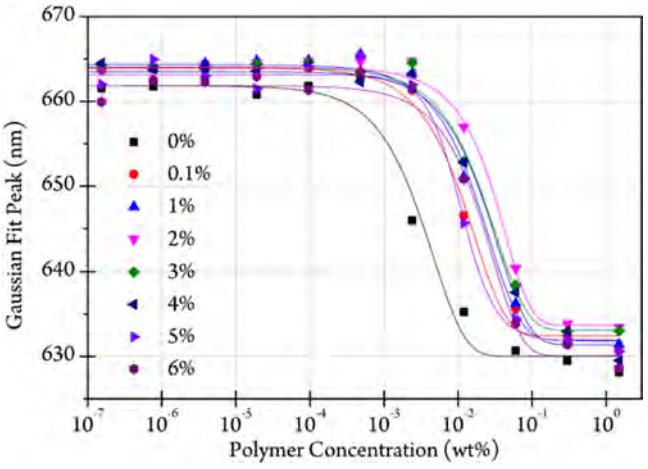

Figure 5. Monitoring the blueshift of Nile Red emission as a function of increasing polymer concentration as an indicator of each sample's CAC. Plot of the peaks of Gaussian curves fit to the fluorescence emission maxima of Nile Red in solutions of SMA 1440 and 2-butoxyethanol. Data labels in the legend refer to the concentration of 2-butoxyethanol (wt %) in the original polymer solution.

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concentration of 0.012 wt %. While the protein extraction studies are performed at polymer concentrations (1.5 wt %) well above the increased CAC, these findings suggest that free 2-butoxyethanol may interact with SMA 1440 in aqueous solution. Additionally, control experiments showed that 2-butoxyethanol alone did not provide a sufficiently hydrophobic environment for the blue shift of Nile Red's fluorescence (Figure S9). Based on these experimental results, we hypothesize that interactions between free 2-butoxyethanol and SMA 1440 may potentially be linked to the reduction in SE observed in Figure 4.

To further investigate the effects that undesired saponification has on the CAC, all SMA 1440 polymer samples that were solubilized at 80 °C, which underwent varying degrees of saponification, were purified to remove small-molecule byproducts. These purified polymers were then subjected to similar CAC studies, but with no free small-molecule 2-butoxyethanol added (Figure S10). The resulting data show that in the absence of 2-butoxyethanol, sidechain density has a minimal impact on the CAC of this polymer. This observation further supports the potential that free 2-butoxyethanol may interact with solubilized SMA 1440.

Effect of Free Alcohols on the Protein−Chlorophyll Content. Finally, we also hypothesized that free 2-butoxyethanol may interact with proteins within the *Te* membrane, potentially releasing free chlorophyll into solution, rather than remaining coordinated within PSI. To investigate this relationship, previously isolated PSI-DDM (Figure 6) and PSI-SMA (Figure 7) samples were added to buffer solutions and spiked with varying amounts of 2-butoxyethanol. These solutions were then incubated for 3 h at 40 °C and subjected to centrifugation (190,000×*g*). At this point, some materials had formed a green pellet, resulting in an overall loss of chlorophyll as seen for some samples in Figures 6b and 7b. This effect is especially pronounced in PSI-DDM solutions exceeding 6 wt % added 2-butoxyethanol. We hypothesize that the addition of 2-butoxyethanol could disrupt the DDM micelle, causing the trimeric PSI complexes to aggregate and crash out of solution. Furthermore, the preexisting SMA polymer belt surrounding PSI[2] may protect the complex by either preserving the protein complexes in the more native environment or preventing self-association due to the charged maleate groups on the polymer chain.

The resulting mixture was separated via sucrose density gradient to qualitatively investigate how trimeric PSI and free chlorophyll concentrations change when free 2-butoxyethanol is present in solution. Both Figures 6a and 7a show a qualitative decrease in PSI trimer concentration and an increase in free chlorophyll. This observation suggests that high 2-butoxyethanol concentrations may cause the noncovalently bound chlorophyll to be released from isolated PSI trimeric complexes.

CONCLUSIONS

As functionalized SMA analogues gain popularity as materials for protein extraction,[3] it is increasingly important to establish and understand proper protocols to prepare and store these materials. Improper handling procedures may lead to deleterious polymer degradation, potentially affecting the protein extraction process. In this study, we showed how two common environmental conditions, temperature and light, could each lead to the deleterious degradation of an ester-functionalized SMA derivative.

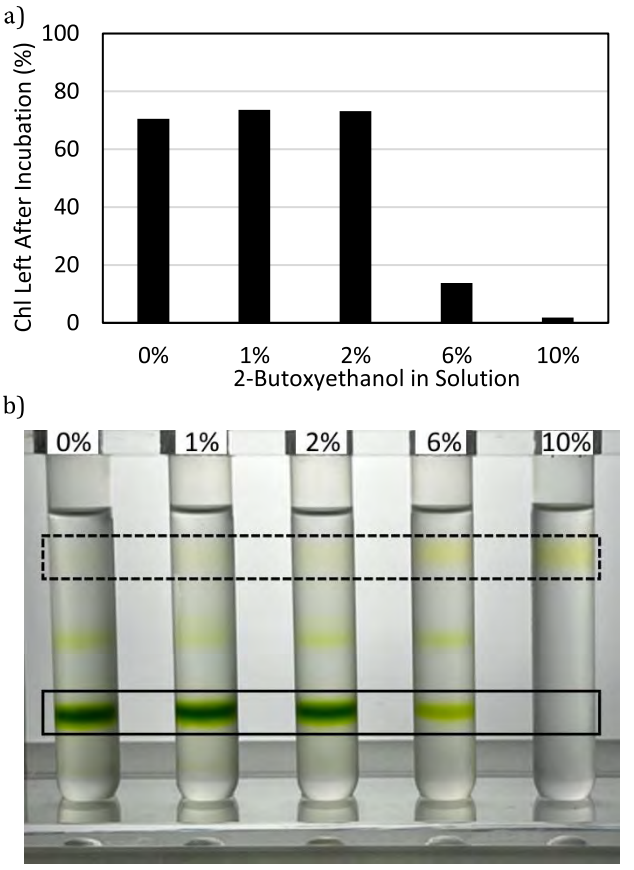

Figure 6. (a) Amount of chlorophyll remaining after incubation with 2-butoxyethanol, as compared to previously isolated PSI-DDM. (b) Sucrose density gradients of PSI-DDM that had been previously isolated using DDM-facilitated protein extraction and purification via sucrose destiny gradient, followed by incubation with 2-butoxyethanol. The labels at the top of the gradients refer to the total percent of the final solution that was 2-butoxyethanol. Bands marks with the dashed box are free chlorophyll and the bands marked with the solid box are trimeric PSI-DDM.

First, we observed that esterified SMA analogues are susceptible to base-catalyzed saponification side reactions at high temperatures, with a sample of SMA 1440f losing nearly 60% of its overall sidechains when solubilized at 80 °C for 168 h. In contrast, we found that solubilizing the same esterified polymer at lower temperature (40 °C, also for 168 h) decreases the number of sidechains lost (only ∼5%). While SMA 1440 is only one example of a functionalized SMA that is commercially available, a growing number of manuscripts have appeared in the recent literature in which additional functionalized SMA copolymers, and analogues thereof, are used for protein extraction. We anticipate that this study will highlight the need for precise protocols for the preparation, solubilization, and storage of similar polymers containing other hydrolysis-susceptible moieties so as to more accurately measure each polymer's protein extraction efficacy.

Next, we discovered that exposure of SMA polymer solutions to light can also result in the degradation of SMA copolymers, an effect that is amplified at higher temperatures. For example, SMA 1440f samples that were heated at elevated temperatures turned yellow and began to gel because of the formation of strongly absorbing side products (in the visible spectrum) and potential cross-linking, respectively, each of

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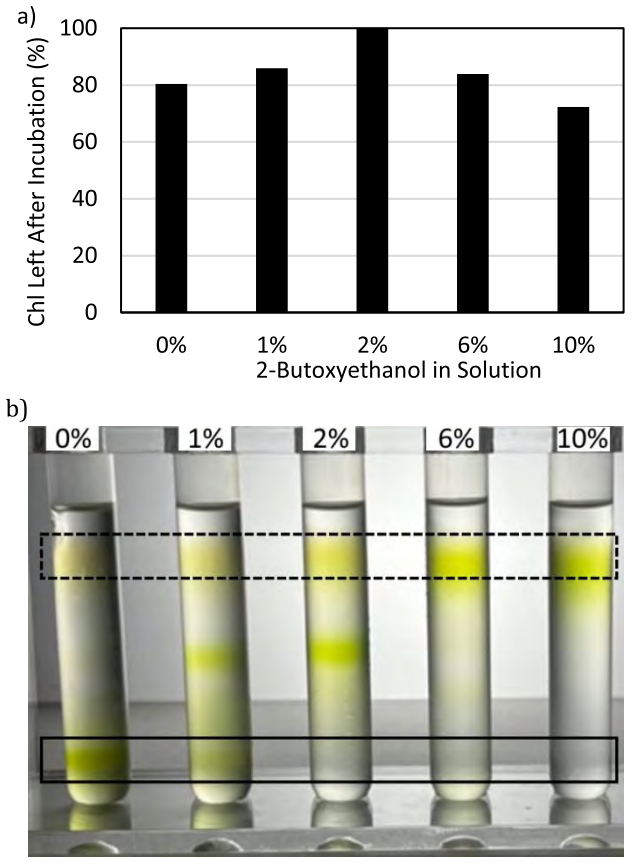

Figure 7. (a) Amount of chlorophyll remaining after incubation with 2-butoxyethanol, as compared to previously isolated PSI-SMA. (b) Sucrose density gradients of PSI-SMA that had been previously isolated using SMA-facilitated protein extraction and purification via sucrose destiny gradient, followed by incubation with 2-butoxyethanol. The labels at the top of the gradients refer to the total percent of the final solution that was 2-butoxyethanol. Bands marks with the dashed box are free chlorophyll and the bands marked with the solid box are trimeric PSI-SMA.

which are suggestive of SMA oxidation.[15] We found that the formation of discolored and cross-linked polymer solutions can be reduced by shielding the samples from light and solubilizing at lower temperatures (i.e., 40 °C). These findings further support that all SMA samples, regardless of identity, should be solubilized into aqueous solution while being protected from light.

Finally, because saponification of esterified SMA 1440 units results in the release of free 2-butoxyethanol molecules into solution, we investigated the effect that added 2-butoxyethanol has on the protein extraction process. Our data indicate that free 2-butoxyethanol in solution decreases the protein solubilization efficiency of SMA 1440, especially in solutions containing >4 wt % 2-butoxyethanol. Investigations of SMA 1440 CAC suggest that the decreased solubilization efficiency of partially saponified polymer samples may potentially be linked to interactions between free 2-butoxyethanol and the polymer chains, although further studies will be required to fully elucidate the mechanistic rationale for this behavior. Lastly, we showed that increasing concentrations of free 2-butoxyethanol lead to the release of noncovalently bound chlorophyll from PSI-SMALPS, further convoluting the quantification of protein extraction process. While it is currently unknown how specific this process may be to specific small molecules present in solution (e.g., 2-butoxyethanol), these findings highlight the importance of using high-purity, functionalized SMA materials to ensure consistent, reliable, and broadly comparable protein extraction data.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.2c00928.

NMR characterization of SMA 1440; GPC characterization of SMA 1440; and absorbance spectra of SMA 1440 (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SMA, styrene−maleic acid copolymer; SMALP, styrene−maleic acid lipid particle; Te, thermosynechococcus elonga-

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tus; SE, solubilization efficiency; PSI, photosystem I; wt %, weight percent

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