# A simple and convenient method for the hydrolysis of styrene-maleic anhydride copolymers to styrene-maleic acid copolymers

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ABSTRACT

Styrene-maleic acid (SMA) copolymers are increasingly gaining attention in the membrane protein field due to their ability to solubilize lipid membranes into discoidal nanoparticles. The copolymers are synthesized as styrene-maleic anhydride (SMAnh), and need to be converted to the free acid form (SMA) before they are capable of solubilizing membranes. This hydrolysis reaction is traditionally performed under rather cumbersome reflux conditions. Here we report an alternative method for the hydrolysis reaction using simple and readily available equipment found in virtually all biochemical laboratories, namely an autoclave. Based on the results we propose an optimum set of standard conditions for the hydrolysis reaction, that should make the method easily accessible to a wide scope of researchers.

## 1. Introduction

The use of styrene-maleic acid (SMA) copolymer as an alternative to detergents for the solubilization of membrane proteins is increasing rapidly. This is due to the ability of the polymer to solubilize membrane proteins into discoidal nanoparticles, referred to as native nanodiscs or SMA lipid particles (SMALPs), while retaining part of the native membrane environment (see e.g. (Knowles et al., 2009)(Esmaili and Overduin, 2018)(Stroud et al., 2018)(Dörr et al., 2016)). SMA is derived from styrene-maleic anhydride (SMAnh) copolymers, which are synthesized from styrene and maleic-anhydride comonomers in a free-radical polymerization reaction (Barron et al., 1984)(Hall et al., 2018). To be able to solubilize membranes, the hydrophobic SMAnh then needs to be converted to the water-soluble amphiphilic membrane-active acid derivative (SMA) in a hydrolysis reaction (see Fig. 1).

Traditionally, this hydrolysis reaction is performed under refluxing conditions (Lee et al., 2016). There are several factors that influence the rate of the hydrolysis of the anhydride to the acid, including particle size (i.e., the reaction is faster for the copolymer in the powder than in the granulate form), temperature, and the use of base (either NaOH or KOH), see e.g. (Dörr et al., 2016).

Only recently some SMA preparations have become commercially available in the hydrolyzed form as solutions (i.e., Xiran® SL). However, not all of the different SMA variants are available in the acid form. Furthermore, one may wish to acquire the copolymers in the anhydride form, e.g. for characterization, fractionation, purification or modification, and then perform hydrolysis in a later stage. Unfortunately, the traditional hydrolysis requires the use of equipment and glassware that is not found in all laboratories, and that is overall a rather cumbersome procedure. Here we explore the use of a piece of equipment that is ubiquitous in all biochemistry laboratories: the autoclave (pressure cooker) as an alternative SMAnh hydrolysis system. While autoclaves are generally used for sterilization purposes, they can also be used to perform chemical reactions, such as SMAnh hydrolysis (Dominguez Pardo et al., 2018) at elevated temperatures and pressures. As the pressure inside of the autoclave is higher than atmospheric pressure it is possible to heat the solvent, in this case water, above its normal boiling point of 100 °C, thereby also accelerating the reaction time.

Here we use different conditions to investigate details of the hydrolysis reaction of SMAnh using an autoclave. The SMAnh copolymer used in this study has a styrene-to-maleic acid ratio of 2:1 (Xiran SP 30010), as previous research has found this to be the most favorable for lipid membrane solubilization as well as for the stability of the resulting SMALPs (Grethen et al., 2017)(Dominguez Pardo et al., 2017)

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**Fig. 1.** Reaction scheme demonstrating the hydrolysis reaction whereby SMAnh is converted into SMA, illustrated here for a SMA 1:1 (styrene-*alt*-maleic acid) copolymer. The hydrolysis is performed under alkaline aqueous conditions.

(Scheidelaar et al., 2016)(Morrison et al., 2016). Based on the results we will propose an optimal set of conditions for the hydrolysis reaction, including time, temperature, and amount and nature of the base used. This will make the method more easily accessible to the growing number of researchers exploring the use of SMA for solubilization and characterization of membrane proteins.

## 2. Materials and methods

SMAnh copolymer (Xiran SP 30010, $M_w = 10$ kDa) was a kind gift from Polyscope Polymers (NL). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (di-14:0 PC, DMPC) was purchased from Avanti Polar Lipids (USA). All other chemicals were purchased from Sigma-Aldrich (DE). MilliQ water is simply referred to as water, or $H_2O$.

### 2.1. SMAnh hydrolysis reactions

#### 2.1.1. Reflux
As a control experiment SMAnh copolymer was hydrolyzed under reflux conditions as described in (Lee et al., 2016). Briefly, the starting material (SMAnh, 1 g) was suspended at 10% (w/v) in $H_2O$ containing 1 mol equivalent of NaOH (NaOH-to-carboxylic acid groups). The round-bottomed flask was connected to the reflux condenser and the reaction mixture heated to reflux (100 °C) with constant stirring using a Teflon coated magnetic stir bar. The mixture was refluxed for 3 h, during which time the solid material completely dissolved and the solution took on a slight yellow color. For work-up, the reaction mixture was allowed to cool down to room temperature and the SMA product was precipitated by the addition of HCl (4 mL, 1 M). The precipitated polymer was centrifuged and the resulting pellet was washed twice with dilute HCl (~ 25 mL, 10 mM), then once with $H_2O$ (~ 25 mL). After the final centrifugation step, the supernatant was removed and the pellet, consisting of styrene-maleic acid copolymer, was dried under a constant flow of $N_2$ with heating (~ 40 °C). Finally, the SMA product was dried further in a vacuum desiccator.

#### 2.1.2. Autoclave
SMAnh was hydrolyzed in an autoclave similarly as described in (Dominguez Pardo et al., 2018). SMAnh copolymer (1 g) was placed in a Schott bottle and suspended at 10% (w/v) in $H_2O$ containing 1 mol equivalent of NaOH. For titration experiments samples of SMAnh (0.5 g) were suspended at 10% (w/v) with varying amounts of base (NaOH or KOH). The reaction mixtures were subjected to' standard' autoclave sterilization cycles, each consisting of 15 min at 125 °C, in a CertoClav (Traun, Austria) table top autoclave, for a total of 3 cycles. After each cycle the mixtures were allowed to cool down to room temperature and the pH determined. After the final heating cycle the crude reaction mixtures were worked-up as described above (2.1.

Reflux).

### 2.2. Stock solutions of hydrolyzed copolymers

The hydrolyzed SMA copolymers were dissolved in an alkaline aqueous solution (0.6 M NaOH) to a concentration of 5% (w/v). This stock solution was used directly for lipid solubilization studies, or diluted further with $H_2O$ for ultraviolet (UV) spectroscopy experiments.

### 2.3. Ultraviolet spectroscopy

UV spectra were obtained for diluted SMA solutions (final concentration 0.025% (w/v)) in a total volume of 1 mL of $H_2O$. Measurements were performed using quartz cuvettes, with 10 mm path length, that were equilibrated at 21 °C. Scans were recorded in the wavelength range of 230–290 nm, at a speed of 120 nm/min, with data points measured every 0.25 nm. Measurements were performed using a Lambda 18 Spectrophotometer (PerkinElmer, USA).

### 2.4. Infrared spectroscopy

Attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR) was performed on solid samples of the SMAnh starting material, as well as the hydrolyzed SMA products. Spectra were recorded in the range of 500-4000 $cm^{-1}$ and taken as an average of 4 scans, with a resolution of 1 $cm^{-1}$. Data was obtained using a PerkinElmer Spectrum Two FT-IR spectrometer with an UATR accessory, ATR correction was not performed. Relative maleic anhydride/acid ratios were obtained from the ratio $A_{1775cm-1}/A_{1705cm-1}$, corresponding to the vibration modes of C=O anhydride stretching at 1775 $cm^{-1}$ (Ravula et al., 2017b)(Al-sabagh et al., 2009), and the C=O acid stretching at 1705 $cm^{-1}$ (Jamshad et al., 2015b)(Ravi et al., 2013).

### 2.5. Preparation of multilamellar vesicles

Phospholipid (DMPC) stock solution was prepared in chloroform at a concentration of 20 mM. The solvent was removed with mild heating (~ 35 °C) under a stream of $N_2$. The resulting lipid film was dried further in a vacuum desiccator for at least 1 h. Next, the lipid film was hydrated with buffer (Tris-HCl 50 mM, NaCl 150 mM, pH 8) to a concentration of 20 mM and incubated for 1 h at 37 °C (T > $T_m$), well above the $T_m$ of DMPC (24 °C) (Lewis et al., 1987). The sample was then subjected to 10 freeze-thaw cycles, using a cold bath of dry ice in ethanol and thawing in a water bath at 37 °C (T > $T_m$).
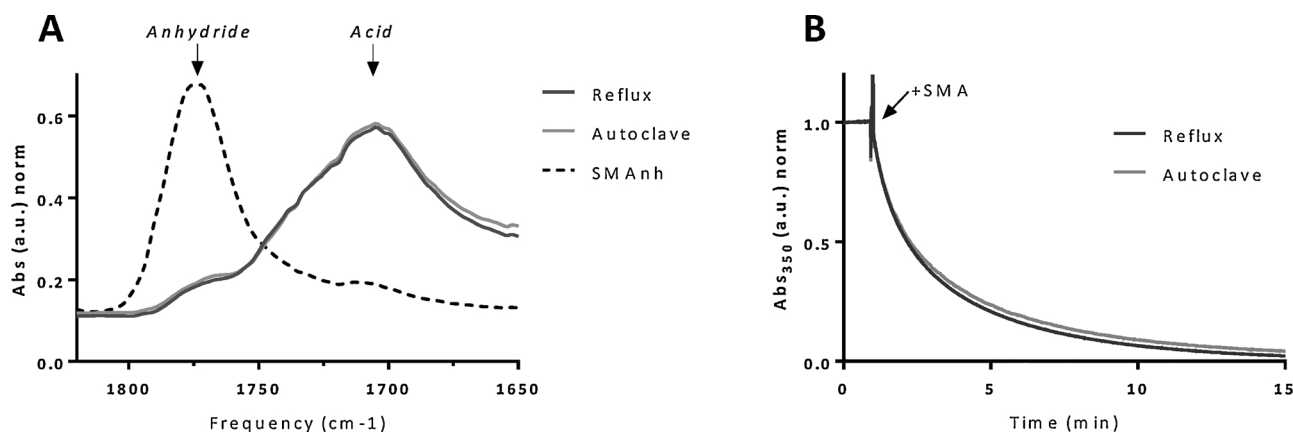
### 2.6. Kinetics of solubilization of phosphatidylcholine vesicles

Aliquots (700 μL) of 0.5 mM dispersions of DMPC multilamellar vesicles (MLVs) in buffer (Tris-HCl 50 mM, NaCl 150 mM, pH 8) were left to equilibrate for at least 1 min, at 15 °C (T < $T_m$). The temperature was controlled using a Peltier cuvette holder and the suspensions were constantly stirred with a Teflon coated magnetic stir bar. 15 μL of SMA 5% (w/v) was added to yield a final SMA-to-lipid mass ratio of ~ 3. Solubilization kinetics was followed at a fixed wavelength of 350 nm by monitoring the decrease of the apparent absorbance (Scheidelaar et al., 2015). Absorbance values were recorded every 0.4 s for a total of 15 min using a Lambda 18 spectrophotometer (PerkinElmer, USA).

## 3. Results and discussion

### 3.1. Extent of polymer hydrolysis and functional activity of hydrolyzed polymer are independent of the method used to perform the hydrolysis reaction

Two different methods were used for the hydrolysis of SMAnh to SMA. The reaction conditions were similar, i.e., one equivalent of

**Fig. 2.** (**A**) IR analysis of hydrolyzed SMA polymers obtained using different hydrolysis methods (reflux vs autoclave), compared to the anhydride (SMAnh) starting material (dashed line). Indicated are the positions of the peak corresponding to the *anhydride* (C=O stretching 1775 cm$^{-1}$) versus the peak of the *acid* (C=O stretching 1705 cm$^{-1}$). (**B**) Phospholipid membrane solubilization as tested on multilamellar vesicles (MLVs) composed of DMPC. Hydrolyzed SMA from either the reflux setup, or autoclave was used to solubilize MLVs at 15 °C (gel phase) over a period of 15 min, polymers were added at the 1 min mark. The process was followed at a wavelength of 350 nm and all measurements were normalized to the absorbance of the MLVs at the start of the experiment. The maximum error found from three experiments was in the range of ~ 6%.

sodium hydroxide to give an alkaline aqueous environment with a polymer concentration of 10% w/v. What differed between the techniques was the apparatus used to heat the reaction mixture. The first method involved the use of a reflux setup (heated to reflux, 100 °C). This setup required several hours of reacting to completely dissolve the polymer despite the vigorous stirring. The second method was the use of an autoclave (heating to 125 °C). Although the mixture in the autoclave had no stirring, the polymer was dissolved within two standard cycles (total of 30 min at 125 °C). Since hydrolysis implies that the polymer becomes water soluble, these results suggest that the temperature of the reaction is more important for efficient hydrolysis than stirring of the reaction mixture. Indeed, when the reaction was performed in an oven at 80 °C with constant mixing, the polymer required several days to dissolve.

The polymers were next analyzed by IR spectroscopy (Fig. 2A). Specifically, by comparing the intensities of the peaks at 1775 cm$^{-1}$ and 1705 cm$^{-1}$ one can obtain information on the extent of hydrolysis. These peaks correspond to the stretching of the carbonyl (C=O) in the anhydride and in the acid form, respectively. The SMAnh starting material has a major peak at 1775 cm$^{-1}$, while the polymers after refluxing or autoclave treatment showed a high intensity at 1705 cm$^{-1}$, indicating conversion to the hydrolyzed form. Importantly, the hydrolyzed products have identical spectra regardless of which method was used to perform the reaction, demonstrating that both of the methods are equally suitable to perform the hydrolysis.

Two other features are notable from the spectra in Fig. 2A. First, the hydrolyzed products have a shoulder at the position corresponding to the anhydride. This may be due to weak aromatic signals from C=C in mono-substituted rings such as observed for polystyrene (Olmos and Gonza, 2014), which is obscured by the C=O stretch vibration in the anhydride form. In any case it is unlikely to correspond to unhydrolyzed product as this spectrum seems to correspond to that of the end product after hydrolysis under many different conditions (see e.g. forthcoming spectra). Second, the anhydride form has a small shoulder at 1705 cm$^{-1}$, indicating that it already has some of the anhydride rings hydrolyzed. This is important to consider when functionalizing the polymer by amide/ester connections, as only the anhydride will be readily reactive to nucleophiles (R-NH$_2$ or R-OH); thus if one desires to have 100% conversion then all of the material should be converted to the anhydride form first. To achieve this the acid needs to be activated using reagents such as acetic anhydride or acetyl chloride (Manoni et al., 2012)(Afri et al., 2014). Similarly, when the polymer has been modified with primary amines to form an amide linkage, the ring can be
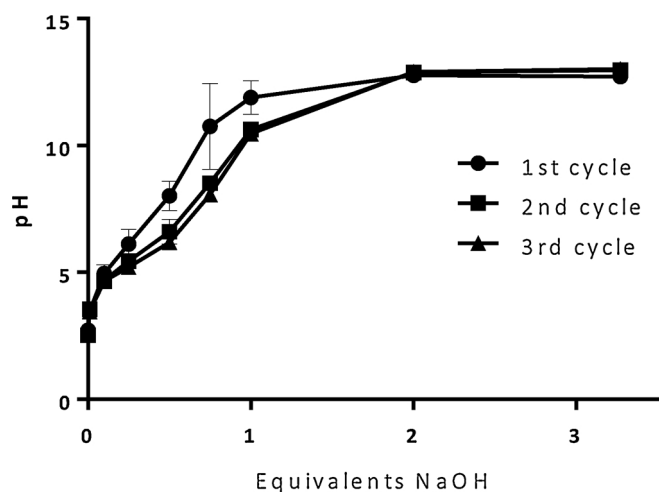
closed again under the same conditions to form the maleimide moiety (Ravula et al., 2017a)(Ravula et al., 2018).

Next, it was investigated whether the hydrolyzed polymers obtained from the different methods for the hydrolysis are equally suited to solubilize lipid bilayers. To test this, synthetic model membranes consisting of DMPC (di-14:0 PC) MLVs were used at 15 °C, where the lipids are in the gel phase (Lewis et al., 1987) and solubilization is slowed down to provide a convenient time window (Scheidelaar et al., 2015). The solubilization kinetics were followed with a turbidimetry assay, which monitors the decrease in apparent absorbance of UV light due to reduced scattering as the particles become smaller (Fig. 2B). It can be seen that the polymers behave comparably, with no significant differences in the time traces. An average error of three experiments was observed of less than ~ 6%, which is mainly attributed to small fluctuations between experiments (e.g., exact moment of injection or efficiency of mixing). Thus, the polymers are equally capable of solubilizing lipids, with complete solubilization of DMPC vesicles after 15 min.

We would like to emphasize here, that the method of hydrolysis by itself indeed should not affect the properties of the hydrolyzed product. Therefore the lipid solubilizing capability of these polymers should be similar as for the SMA polymers used in previous studies on membrane solubilization, i.e. they should be able to solubilize bilayers of lipids with different head groups, acyl chain lengths, and degrees of unsaturation (Scheidelaar et al., 2015)(Dominguez et al., 2017)(Stroud et al., 2018)(Arenas et al., 2016), as well as native biological membranes from various sources, including bacteria (Sun et al., 2018)(Swainsbury et al., 2017), yeast (Jamshad et al., 2015a), and mammalian cells (Dörr et al., 2017).

### 3.2. Aqueous hydrolysis is improved by hydroxide ions, with an optimum at sub-stoichiometric concentrations

To investigate the role that hydroxide ions play in the hydrolysis reaction of SMAnh to SMA, titration experiments were performed. SMAnh was subjected to varying amounts of hydroxide (0–3.3 mol equivalents NaOH-to-carboxylic acid groups). The reactions were performed in the autoclave using a total of three standard cycles, where each cycle consists of heating at 125 °C for 15 min. After each cycle the pH was measured (Fig. 3). Two important conclusions can be drawn from the results. First, the progress of the reaction at a specific base concentration can be followed by monitoring the decrease in pH. This is because hydroxide ions are consumed by either reacting with the

**Fig. 3.** Residual pH in the solution upon titration of polymer solutions with varying amounts of base (NaOH) after an increasing number of cycles in the autoclave (circles = 1, squares = 2, and triangles = 3). Error bars represent the standard deviation from three independent experiments, trendlines are added between the data points to help guide the eye.

anhydride ring (i.e., as nucleophiles), and/or deprotonating the resulting carboxylic acids that are formed when the anhydride is ring is opened (i.e., as base). This loss of hydroxide ions results in a decrease of pH. As there is an initial drop in pH between the first and second cycle, but no difference in pH between the second and third cycle, this means that the hydrolysis reaction is complete after the second autoclave cycle. Second, the final pH of the solution can be conveniently controlled by adding a specific amount of base. For example, if one wishes to achieve a final pH of 8 at the completion of hydrolysis, then approximately 0.6 equivalents of hydroxide should be used. This results in the polymer being exposed to less harsh conditions, as well as having a much higher atom efficiency (i.e., less waste of material, since less base is required for performing the reaction and less acid is required to acidify for work-up). Furthermore, it is then possible to directly use the hydrolyzed copolymer without performing a work-up (precipitation). However, one should then keep in mind that all impurities such as unpolymerized comonomers, initiator, etc. will remain in the mixture. If the mixture is slightly turbid it may be filtered through a 0.45 μm filter to remove any undissolved impurities. Another alternative, although time consuming, would be to perform dialysis to remove any low molecular weight impurities (Arenas et al., 2016).

The hydrolyzed products from the titration experiments were further characterized and investigated by spectroscopic analyses (Fig. 4). IR spectroscopy (Fig. 4A) revealed that even in the complete absence of base there is still significant hydrolysis as shown by the strong reduction of the anhydride peak at $1775\,cm^{-1}$ and the high intensity of the acid peak at $1705\,cm^{-1}$. This finding is quite remarkable given that the polymer is not completely dissolved under these conditions. This demonstrates the high reactivity of the anhydride, that can be hydrolyzed by water alone. By the addition of catalytic amounts of hydroxide (0.01 – 0.25 eq.) the extent of hydrolysis is improved, with maximal hydrolysis achieved at ~ 0.5 equivalents and higher, where the peaks at $1775\,cm^{-1}$ and $1705\,cm^{-1}$ do not further change in intensity (Fig. 4A). It is important to note that even when the copolymers are completely hydrolyzed, there may be a clear plastic solid present on the bottom of the flasks. This is likely due to the absence of mixing during the reactions, and the solid material may be dissolved by incubating in a 100 °C water bath while swirling vigorously.

The lipid membrane solubilization efficacy of the polymers (Fig. 4B) appears to be rather similar after treatment with different amounts of base, especially if one takes into account the error of the experiments (~ 6%). Nonetheless, the polymers hydrolyzed with larger amounts of
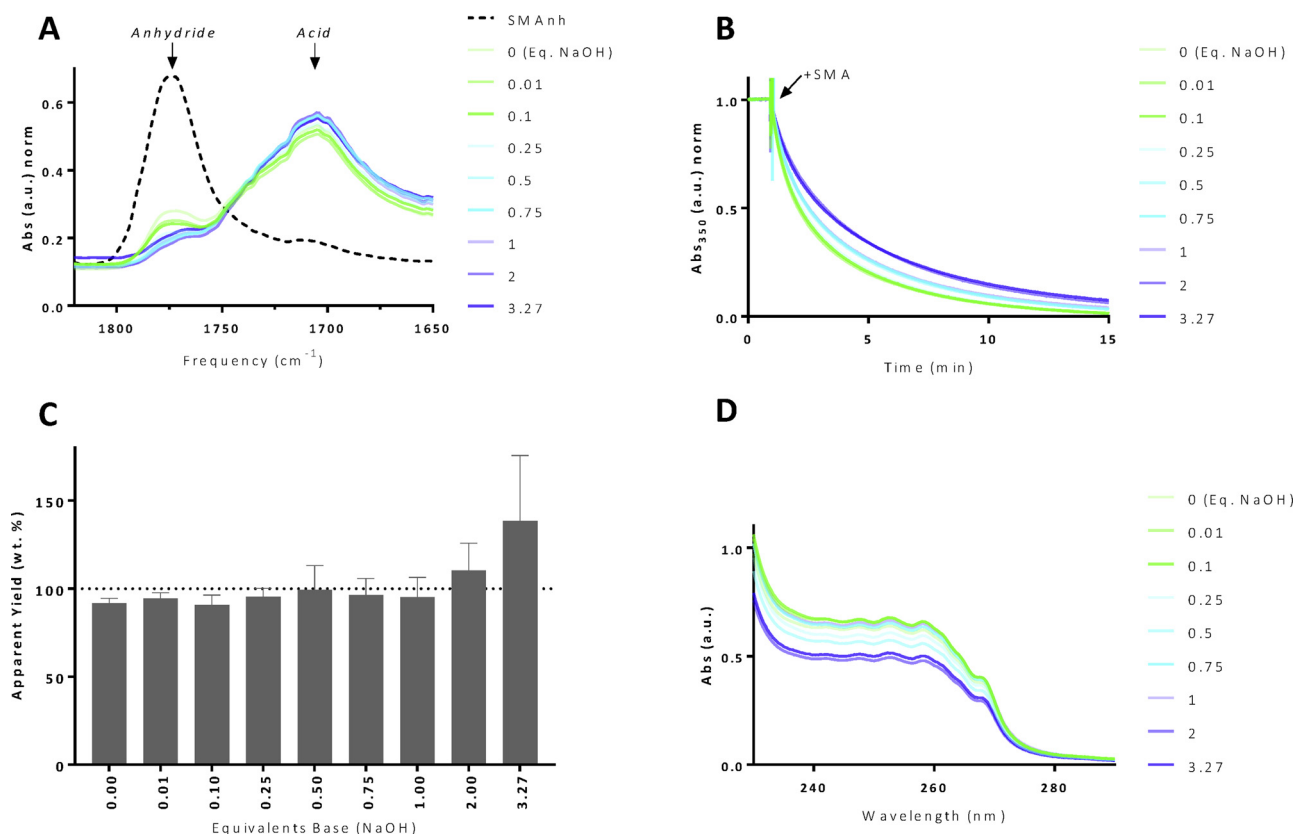
hydroxide (particularly the polymers hydrolyzed using an excess of base (≥ 2 eq.)), appear to be somewhat less functionally active. This phenomenon is probably due to an overestimation of the amount of polymer present in these samples, despite the fact that the polymer stocks were all dissolved at 5% w/v and used at a final concentration of ~ 0.1% w/v. An indication for this comes from the yields of the hydrolyzed polymers (Fig. 4C). Although most of the reactions gave near quantitative yields, when using excess base the apparent yields exceeds the theoretical maximum (100%). The reason for this may be that the excess base (NaOH) present will react with the hydrochloric acid used in the work-up to form sodium chloride, and despite several washing steps, the additional salt is probably trapped within the polymer precipitate. This hypothesis is validated by the UV spectra (Fig. 4D), which show that despite being at the same concentration (~ 0.025% w/v), the polymers hydrolyzed with larger amounts of base have lower levels of absorbance, indicating that less polymer is present. Indeed, when the spectra are normalized at $A_{259nm}$ they all show identical spectra (Figure S1). These findings demonstrate not only that an amount of hydroxide of around 0.6 equivalents is sufficient for efficient hydrolysis, but they also warn of the potential pitfall of using an excess of base such as is standard practice, as this may result in a product that consists not solely of polymer but also contains a significant fraction of salt.

Although the anhydride ring is reactive enough to be spontaneously hydrolyzed by water, it is a lengthy process and the reaction is greatly accelerated by the use of hydroxide ions (Mishchenko et al., 1984). It is important to realize that the hydroxide can not only act as a nucleophile (taking part in the reaction) but also as a base, deprotonating the resulting carboxylic acids ($COOH \rightarrow COO^-$). This deprotonation of the acidic groups is important for dissolving the copolymer, particularly for the larger and/or more hydrophobic variants of the copolymer (Scheidelaar et al., 2016). The source of the hydroxide ion can be either potassium hydroxide or sodium hydroxide, and indeed both have been used in the past (Dörr et al., 2014)(Jamshad et al., 2015b). Therefore, we compared the effect of these two bases. To this end the same titration experiments were performed using potassium hydroxide instead of sodium hydroxide. Very similar results were obtained in terms of efficiency and kinetics of hydrolysis as well as functional activity as monitored by lipid solubilization (Figure S2). Nevertheless, as it appears that some of the salt will be trapped with the polymer, we recommend the use of sodium hydroxide as base instead of potassium hydroxide. The reasons are simply that NaOH is cheaper, that it has a smaller mass, thereby giving a smaller error in apparent polymer mass, and probably most importantly, that having a slight increase of sodium ions is generally less intrusive for experiments under physiological conditions.

## 4. Conclusions

The hydrolysis of SMAnh into the membrane active SMA form can easily be achieved through the use of an autoclave. Compared to refluxing, the method presented here uses more widely available equipment, is faster, and also has the advantage of being applicable for 'high-throughput'. The method would also be suitable for other amphipathic copolymers containing the maleic anhydride moiety (e.g. DIBMAnh). The use of hydroxide helps the hydrolysis reaction, although an excess of base is discouraged as it may lead to overestimation of the apparent yield and amount of polymer present. As a base, either NaOH or KOH can be used. We however recommend the use of NaOH, and specifically at sub-stoichiometric amounts of ~ 0.6 equivalents base to carboxylic acid groups. As an example: for a 2:1 (S:MA) copolymer, such as Xiran 30010, the polymer can be suspended at a concentration of 10% w/v in an aqueous solution of sodium hydroxide with a molar concentration of ~ 0.4 (based on a molecular weight of ~100 g/mol per maleic-anhydride monomer and a yield of two COOH groups upon hydrolysis). The suspension will have a high pH initially (pH ~13), but after hydrolysis is complete the final pH will be only moderately alkaline (pH ~8).

**Fig. 4.** (**A**) IR analyses of hydrolyzed SMA using various amounts of base, compared to the SMAnh starting material (dashed line). Indicated are the positions of the peak corresponding to the *anhydride* (C=O stretching 1775 cm$^{-1}$) versus the peak of the *acid* (C=O stretching 1705 cm$^{-1}$). (**B**) Phospholipid membrane solubilization as tested on MLVs composed of DMPC. Hydrolyzed SMA from titration experiments were used to solubilize MLVs at 15 °C (gel phase) over a period of 15 min, polymers were added at the 1 min mark. The process was followed at a wavelength of 350 nm and all measurements were normalized to the absorbance of the MLVs at the start of the experiment. The maximum error found from three experiments was in the range of ∼ 6%. (**C**) The apparent yield (weight %) for the polymers obtained from the titration experiments. Error bars represent the standard deviation from three independent experiments. (**D**) UV scans in the range of 230 to 290 nm of the hydrolyzed polymers. All spectra were measured at a final concentration of 0.025% (w/v) SMA.

Finally we wish to emphasize that the lipid solubilizing capability of the hydrolyzed polymers was tested here on DMPC vesicles as proof of 'bioequivalence'. Nonetheless, they will have the same ability to solubilize other types of membranes as SMA copolymers used in previous studies, as the method of hydrolyzation will not affect the chemical composition or properties of the hydrolyzed polymers.

## Conflicts of interest

The authors have no conflicts of interest to declare.

## Acknowledgements

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2018.11.011.

## References

Afri, M., Alexenberg, C., Bodner, E., Frimer, A.A., Jacob, A.I., Naqqash, M.E., 2014. NMR-based molecular ruler for determining the depth of intercalants within the lipid bilayer. Part V: a comparison of liposomes, bioliposomes and erythrocyte ghosts. Chem. Phys. Lipids 184, 52–60. https://doi.org/10.1016/j.chemphyslip.2014.10.002.

Al-sabagh, A.M., El-din, M.R.N., Morsi, R.E., Elsabee, M.Z., 2009. Styrene-maleic anhydride copolymer esters as flow improvers of waxy crude oil. J. Pet. Sci. Eng. 65, 139–146. https://doi.org/10.1016/j.petrol.2008.12.022.

Arenas, R.C., Klingler, J., Vargas, C., Keller, S., 2016. Influence of lipid bilayer properties on nanodisc formation mediated by styrene/maleic acid. Nanoscale 8, 15016–15026. https://doi.org/10.1039/c6nr02089e.

Barron, P.F., Hil, D.J.T., Donnell, J.H.O., Sullivan, P.W.O., 1984. Applications of DEPT experiments to the 13C NMR of copolymers: poly(styrene-co-maleic anhydride) and poly (styrene-co-acrylonitrile). Macromolecules 17, 1967–1972. https://doi.org/10.1021/ma00140a016.

Dominguez, J.J., Jonas, P., Aditya, M.D., Ruud, I., Scheidelaar, S., Koorengevel, M.C., Subramaniam, V., Killian, J.A., 2017. Solubilization of lipids and lipid phases by the styrene–maleic acid copolymer. Eur. Biophys. J. 91–101. https://doi.org/10.1007/s00249-016-1181-7.

Dominguez Pardo, J.J., Dörr, J.M., Renne, M.F., Ould-Braham, T., Koorengevel, M.C., van Steenbergen, M.J., Killian, J.A., 2017. Thermotropic properties of phosphatidylcholine nanodiscs bounded by styrene-maleic acid copolymers. Chem. Phys. Lipids 208, 58–64. https://doi.org/10.1016/j.chemphyslip.2017.08.010.

Dominguez Pardo, J.J., Koorengevel, M.C., Uwugiaren, N., Weijers, J., Kopf, A.H., Jahn, H., Walree, C.A., Van, Steenbergen, M.J., Van, Killian, J.A., 2018. Membrane solubilization by styrene-maleic acid copolymers: delineating the role of polymer length. Biophys. J. 115, 129–138. https://doi.org/10.1016/j.bpj.2018.05.032.

Dörr, J.M., Coevorden-hameete, M.H., Van, Hoogenraad, C.C., Killian, J.A., 2017. Solubilization of human cells by the styrene–maleic acid copolymer: insights from fluorescence microscopy. BBA Biomembr. 1859, 2155–2160. https://doi.org/10.1016/j.bbamem.2017.08.010.

Dörr, J.M., Koorengevel, M.C., Schäfer, M., Prokofyev, A.V., Scheidelaar, S., 2014. Functional reconstitution of a tetrameric K + channel: the power of native nanodiscs. PNAS 111, 18607–18612. https://doi.org/10.1073/pnas.1416205112.

Dörr, J.M., Scheidelaar, S., Koorengevel, M.C., Dominguez, J.J., Schäfer, M., van Walree, C.A., Killian, J.A., 2016. The styrene–maleic acid copolymer: a versatile tool in membrane research. Eur. Biophys. J. 45, 3–21. https://doi.org/10.1007/s00249-015-1093-y.

Esmaili, M., Overduin, M., 2018. Membrane biology visualized in nanometer-sized discs formed by styrene maleic acid polymers. Biochim. Biophys. Acta Biomembr. 1860, 257–263. https://doi.org/10.1016/j.bbamem.2017.10.019.

Grethen, A., Oluwole, A.O., Danielczak, B., Vargas, C., Keller, S., 2017. Thermodynamics of nanodisc formation mediated by styrene/maleic acid (2:1) copolymer. Sci. Rep. 7, 1–14. https://doi.org/10.1038/s41598-017-11616-z.

Hall, S.C.L., Tognoloni, C., Price, G.J., Edler, K.J., Dafforn, T.R., Arnold, T., 2018. The influence of poly(styrene-co-maleic acid) copolymer structure on the properties and self- assembly of SMALP nanodiscs. Biomacromolecules 19, 761–772. https://doi.org/10.1021/acs.biomac.7b01539.

Jamshad, M., Charlton, J., Lin, Y., Routledge, S.J., Bawa, Z., Knowles, T.J., Overduin, M., Dekker, N., Dafforn, T.R., Bill, R.M., Poyner, D.R., Wheatley, M., 2015a. G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent. Biosci. Rep. 1–10. https://doi.org/10.1042/BSR20140171.

Jamshad, M., Grimard, V., Idini, I., Knowles, T.J., Dowle, M.R., Schofield, N., Sridhar, P., Lin, Y., Finka, R., Wheatley, M., Thomas, O.R.T., Palmer, R.E., Overduin, M., Govaerts, C., Ruysschaert, J.M., Edler, K.J., Dafforn, T.R., 2015b. Structural analysis of a nanoparticle containing a lipid bilayer used for detergent-free extraction of membrane proteins. Nano Res. 8, 774–789. https://doi.org/10.1007/s12274-014-0560-6.

Knowles, T.J., Finka, R., Smith, C., Lin, Y.-P., Dafforn, T., Overduin, M., 2009. Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. J. Am. Chem. Soc. 7, 7484–7485. https://doi.org/10.1021/ja810046q.

Lee, S.C., Knowles, T.J., Postis, V.L.G., Jamshad, M., Parslow, R.A., Lin, Y.P., Goldman, A., Sridhar, P., Overduin, M., Muench, S.P., Dafforn, T.R., 2016. A method for detergent-free isolation of membrane proteins in their local lipid environment. Nat. Protoc. 11, 1149–1162. https://doi.org/10.1038/nprot.2016.070.

Lewis, R.N.A.H., Mak, N., Mcelhaney, R.N., 1987. A differential scanning calorimetric study of the thermotropic phase behavior of model membranes composed of phosphatidylcholines containing linear saturated ijipid. Biochemistry 6118–6126. https://doi.org/10.1021/bi00393a026.

Manoni, F., Cornaggia, C., Murray, J., Tallon, S., Connon, S.J., 2012. Catalytic, enantio- and diastereoselective synthesis of butyrolactones incorporating quaternary stereocentres. Chem. Commun. 48, 6502–6504. https://doi.org/10.1039/c2cc32147e.

Mishchenko, V.F., Zubov, V.A., Yevdokimov, V.A., 1984. Synthesis and pproperties of hydrolyzed polymers of maleic anhydride. Polym. Sci. U.S.S.R. 25, 2254–2258.

Morrison, K.A., Akram, A., Mathews, A., Khan, Z.A., Patel, J.H., Zhou, C., Hardy, D.J., Moore-Kelly, C., Patel, R., Odiba, V., Knowles, T.J., Javed, M.-u.-H., Chmel, N.P., Dafforn, T.R., Rothnie, A.J., 2016. Membrane protein extraction and purification using styrene-maleic acid (SMA) copolymer: effect of variations in polymer structure. Biochem. J. 473, 4349–4360. https://doi.org/10.1042/BCJ20160723.

Olmos, D., Gonza, J., 2014. New molecular-scale information on polystyrene dynamics in PS and PS – BaTiO 3 composites from FTIR spectroscopy. PCCP 16, 24339–24349. https://doi.org/10.1039/c4cp03516j.

Ravi, B., Jegathesan, A., Prasad, B.N., Sadeshkumar, C., 2013. Spectral diffraction and optical studies of succinic-acetic acid single crystals. Rasayan J. Chem. 6, 334–341.

Ravula, T., Hardin, N.Z., Ramadugu, S.K., Cox, S.J., Ramamoorthy, A., 2018. Lipid bilayers formation of pH-Resistant monodispersed polymer – lipid nanodiscs. Angew. Chemie Int. Ed. 57, 1342–1345. https://doi.org/10.1002/anie.201712017.

Ravula, T., Hardin, N.Z., Ramadugu, S.K., Ramamoorthy, A., 2017a. pH tunable and divalent metal ion tolerant polymer lipid nanodiscs. Langmuir 33, 10655–10662. https://doi.org/10.1021/acs.langmuir.7b02887.

Ravula, T., Ramadugu, S.K., Di Mauro, G., Ramamoorthy, A., 2017b. Bioinspired, size-tunable self-assembly of polymer – lipid bilayer nanodiscs. Angew. Chemie Int. Ed. 1055, 11466–11470. https://doi.org/10.1002/anie.201705569.

Scheidelaar, S., Koorengevel, M.C., Pardo, J.D., Meeldijk, J.D., Breukink, E., Killian, J.A., 2015. Molecular model for the solubilization of membranes into nanodisks by styrene maleic acid copolymers. Biophys. J. 108, 279–290. https://doi.org/10.1016/j.bpj.2014.11.3464.

Scheidelaar, S., Koorengevel, M.C., Walree, C.A., Van, Dominguez, J.J., Do, J.M., Killian, J.A., 2016. Effect of polymer composition and pH on membrane solubilization by styrene-maleic acid copolymers. Biophys. J. 1974–1986.

Stroud, Z., Hall, S.C.L., Dafforn, T.R., 2018. Purification of membrane proteins free from conventional detergents : SMA, new polymers, new opportunities and new insights. Methods 147, 106–117. https://doi.org/10.1016/j.ymeth.2018.03.011.

Sun, C., Benlekbir, S., Venkatakrishnan, P., Wang, Y., Hong, S., Hosler, J., Tajkhorshid, E., Rubinstein, J.L., Gennis, R.B., 2018. Structure of the alternative complex III in a supercomplex with cytochrome oxidase. Nat. Lett. https://doi.org/10.1038/s41586-018-0061-y.

Swainsbury, D.J.K., Scheidelaar, S., Foster, N., van Grondelle, R., Killian, J.A., Jones, M.R., 2017. The effectiveness of styrene-maleic acid (SMA) copolymers for solubilisation of integral membrane proteins from SMA-accessible and SMA-resistant membranes. Biochim. Biophys. Acta Biomembr. 1859, 2133–2143. https://doi.org/10.1016/j.bbamem.2017.07.011.