Synthesis and characterization of block copolymers of styrene-maleic acid with acrylamide and *N,N*-dimethylacrylamide

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Abstract

Styrene-maleic acid (SMA) block copolymers with either acrylamide (AM) or *N*,*N*-dimethylacrylamide (DMA) have been synthesized via a 3-step process comprising: (1) photopolymerization of styrene and maleic anhydride in solution to yield an alternating styrene maleic anhydride (SMAnh) copolymer, (2) copolymerization of SMAnh with either AM or DMA to yield SMAnh-*b*-AM and SMAnh-*b*-DMA block copolymers and (3) hydrolysis of the anhydride groups to yield water-soluble SMA-*b*-AM and SMA-*b*-DMA block copolymers as the final products. With a view to their intended application in membrane protein solubilization, molecular weights are controlled to below 10,000 by the synthesis conditions employed in step (1), including using carbon tetrabromide (CBr_4) as a chain transfer agent. The CBr_4 also plays an important role in step (2). By terminating the SMAnh chain radicals from step (1) with C-Br bonds that are photolytically active, SMAnh chain radicals can be regenerated to act as macroinitiators for the polymerization of AM or DMA in step (2). Finally, following step (3) and due to the pH-dependency of the SMA chain conformation in solution, a pH of 7-8 is found to be optimal for enabling the final products to be precipitated in a solid form that is completely soluble in water.

KEYWORDS

Styrene-maleic acid, styrene-maleic anhydride, acrylamide, *N*,*N*-dimethylacrylamide, block copolymers, membrane protein solubilization

1 INTRODUCTION

In recent years, there has been a growing interest in the use of water-soluble styrene-maleic acid (SMA) copolymers in membrane protein (MP) solubilization and stabilization.[1-6] Due to their amphipathic structure, SMAs can solubilize MPs directly from cells or from crude plant extracts. Their amphipathic properties are due to the combination of hydrophobic styrene units and hydrophilic carboxylic acid / carboxylate ($COOH/COO^-$) groups being joined together in the same chain. These properties also enable SMAs to exhibit hypercoiling behavior depending on the balance between the charge repulsion of the COO^- groups and the hydrophobic interactions of the styrene units. This hypercoiling, or hydrophobically associating, behavior has also led to potential applications of SMAs in targeted drug delivery and as lung surfactants in the treatment of respiratory distress syndrome in pre-term infants.[7,8]

Detergents (surfactants) have conventionally been used for MP solubilization leading to the formation of spherical micelles. However, the use of detergents has certain disadvantages which can lead to inactivation or aggregation of the protein. In contrast, SMAs act differently in that they interact with lipid membranes resulting in the formation of discoidal nanoparticles. These discoidal nanoparticles are often referred to in the literature as SMA-lipid particles (SMALPs).[9-12] In this behavior, they show a biomimetic resemblance to Surfactant Protein C (SPC), a pulmonary surfactant protein essential for lung function after birth.[7,8] An illustration of an SMA-lipid particle is shown in Figure 1, showing how the SMA wraps itself around a phospholipid bilayer containing the encapsulated membrane protein.

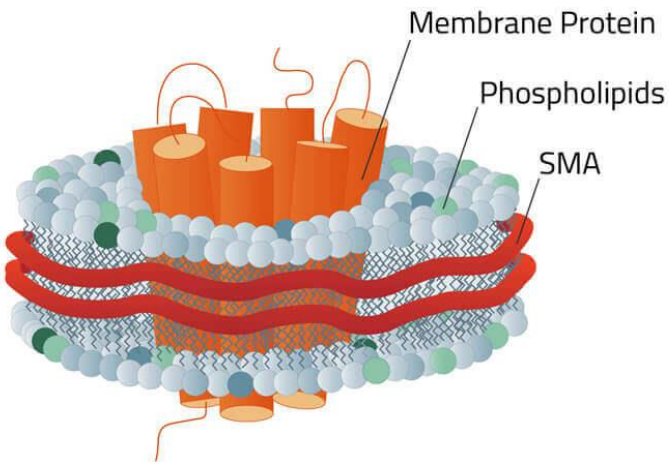
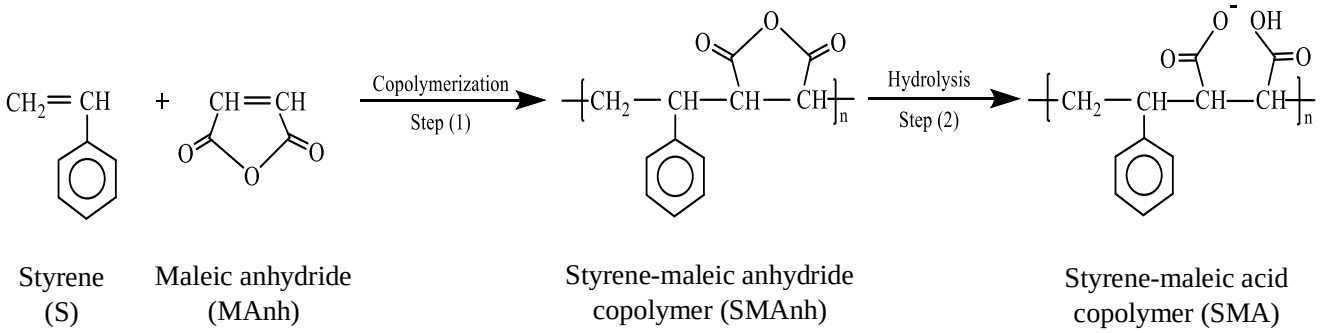


FIGURE 1 Illustration of a discoidal SMA-lipid particle (SMALP) showing the SMA arranged around a phospholipid bilayer encapsulating the membrane protein

SMA copolymers are synthesized via a two-step reaction: (1) radical copolymerization of styrene and maleic anhydride to yield a styrene-maleic anhydride (SMAnh) copolymer followed by (2) base-catalyzed hydrolysis of the anhydride groups to yield the SMA copolymer. This two-step reaction is shown in Scheme 1. When the styrene to maleic anhydride comonomer mole ratio in step (1) is 1:1, as is often the case, the SMAnh product obtained has a predominantly alternating monomer sequencing due to the extremely low monomer reactivity ratios.[13] However, when the styrene in step (1) is present in excess, the monomer sequencing becomes more complex. Thus, the synthesis conditions used in step (1) determine the copolymer composition, monomer sequencing and molecular weight. In the hydrolysis step (2), the degree of neutralization of the anhydride groups is an important factor which influences the chain conformation of the SMA in aqueous solution.[14] This will be discussed in more detail in section 2.5.



Step (1) : In solution (toluene or THF) using either thermal or photoinitiation
Step (2) : Base-catalyzed hydrolysis by refluxing in aqueous solution

SCHEME 1 Two-step synthesis of styrene-maleic acid (SMA) copolymers

However, there are still some problems associated with SMA copolymers in membrane protein extraction. For example, differences in the ease of protein solubilization, problems relating to binding to affinity purification resins, and sensitivity to low pH and divalent cations have all been reported.[2,15,16] Up until now, most of the structural variations in SMA synthesis have focused either on using alternatives to styrene[17], or more usually on varying the styrene to maleic acid mole ratio (1:1, 2:1, 3:1, etc.) and average molecular weight. In this present work, SMA synthesis is extended as the beginning of a research programme to more closely mimic the structure and behavior of Surface Protein C (SPC) by adding another monomer M at the end of step (1) to form a SMAnh-*b*-M block copolymer so that, after the hydrolysis step (2), the final product will be a SMA-*b*-M block copolymer.

In order to preserve the water solubility of the final block copolymer product, M needs to be a monomer that itself yields a water-soluble polymer. Hence, the monomers chosen for this study were acrylamide (AM) and *N*,*N*-dimethylacrylamide (DMA) with the aim of producing water-soluble SMA-*b*-AM and SMA-*b*-DMA block copolymers. This type of structural variation, which is the first step towards the generation of a closer structural analogue of SPC,[18] has not been previously reported. As far as we are aware, the closest structure that has been reported is an SMA graft copolymer with *N*-isopropylacrylamide (NIPAM).[19]

The motivation for this study is that it is anticipated that the addition of non-hypercoiling AM and DMA blocks to hypercoiling SMA chains may have some interesting effects on the SMA's membrane solubilization efficacy. For example, the effect that they have on the balance between the SMA's hydrophilic and hydrophobic interactions in aqueous solution, particularly with varying pH, is likely to be of particular importance. Although membrane solubilization is not a part of this present study, it is with this application in mind that these novel block copolymers are currently being investigated.

2 EXPERIMENTAL

2.1 Chemicals

Styrene (Merck, ≥ 99%) and *N*,*N*-dimethylacrylamide (Aldrich, 99%) were purified by vacuum distillation before use. Maleic anhydride (Merck, ≥ 99%) and acrylamide (Sigma, ≥ 99%) were used as supplied. 2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Aldrich, 98%) photoinitiator and carbon tetrabromide (Aldrich, 99%) chain transfer agent were also used as supplied, as were the tetrahydrofuran (RCI Labscan, 99.8%), methanol (RCI Labscan, 99.9%) and acetone (RCI Labscan, 99.5%) solvents.

2.2 Instrumentation

Structural characterization of the copolymer products was carried out using a Bruker Avance 500 MHz Nuclear Magnetic Resonance Spectrometer. Proton ^{1}H-NMR spectra were obtained using either d_6-DMSO or D_2O as the solvent. For thermal analysis, a Perkin-Elmer DSC7 Differential Scanning Calorimeter was used to determine glass transition temperatures (T_g), while a Perkin-Elmer TGA7 Thermogravimetric Analyzer was used for studying thermal stability. The DSC and TGA heating rates were 10°C/min and 20°C/min respectively, both

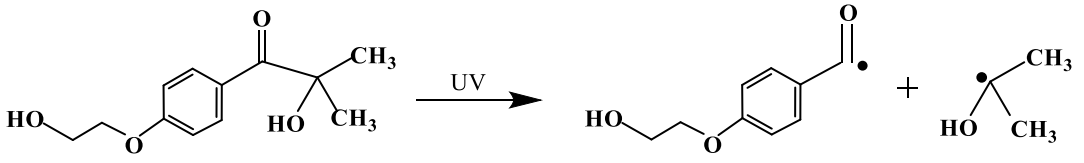
under an inert nitrogen atmosphere. Samples sizes were in the range of 3-5 mg for DSC and 7-10 mg for TGA.

Average molecular weights were determined by dilute-solution viscometry using calibrated Mark-Houwink equations reported in the literature.[14,20] These equations for SMAnh and SMA were derived via calibration of the dilute-solution viscometry data using absolute methods (e.g., light scattering) for the molecular weight determination of fractionated samples. For SMA, the equation was also specific for the degree of neutralization.[20] Consequently, dilute-solution viscometry was preferred to gel permeation chromatography (GPC) and was found to give more accurate results when used to test commercial SMAnh samples of accurately known molecular weight.

2.3 Photopolymerization apparatus and conditions

Copolymerizations were carried out in solution in tetrahydrofuran (THF) under nitrogen in a closed system with external UV light irradiation from a Philips Solarium Model MD 1-15 UV lamp. The lamp comprised four parallel Cleo 15 W fluorescent tubes that emitted UV light in the 300-415 nm wavelength range.

The photoinitiator used was 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (HHEMP) which is more commonly referred to in the literature by its trade name of Irgacure® 2959. It tends to be favoured for use in biomedical applications since it has been shown to be biocompatible for cell encapsulation.[21] The mode of photodissociation of Irgacure 2959 to give free radicals is shown in Scheme 2. The benzoyl radical is the more reactive of the two radicals formed and is considered to be the initiating radical in polymerization.



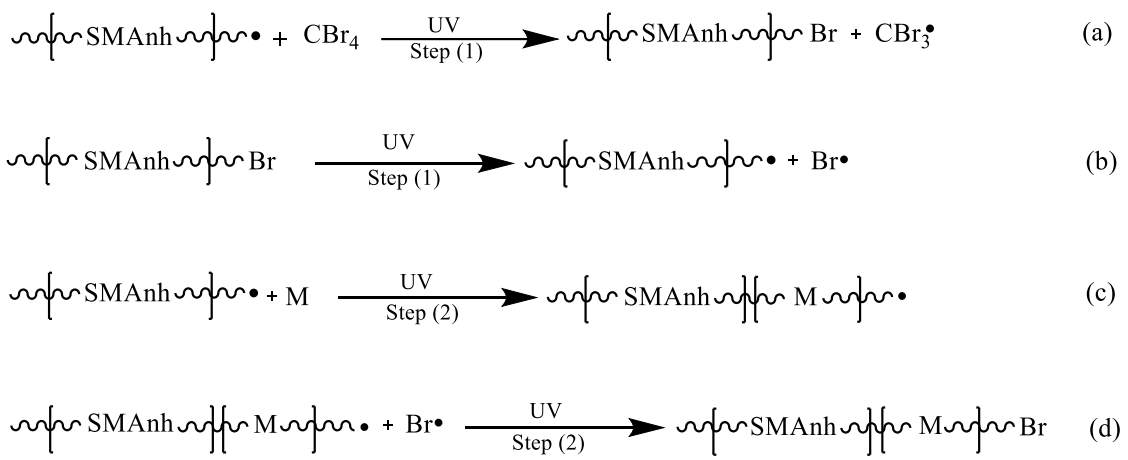
Irgacure 2959 benzoyl radical ketyl radical

SCHEME 2 Photodissociation of Irgacure 2959

Tetrabromomethane (CBr_4) was added as a chain transfer agent. Also known as carbon tetrabromide, CBr_4 is well-known as having an especially high chain transfer constant in radical polymerization due to its weak C-Br bonds. In this work, the CBr_4 served two distinct purposes.

The first purpose was to control the molecular weight of the SMAnh copolymer from Step (1) to below 10,000 g mol^{-1} since it has been reported that SMAs are most efficient in membrane insertion and solubilization below this limit.[22-24] The reason for this is that it is sterically more difficult for higher molecular weight and hence longer chain SMAs to cover a large membrane area while at the same time achieving insertion of all of the styrene units' hydrophobic phenyl groups. However, more in-depth studies of the effect of SMA molecular weight have indicated that the optimum molecular weight range may be as low as 2,000-6,000 g mol^{-1}.[22]

The second purpose of the CBr_4 was to functionalize the SMAnh chains with terminal C-Br bonds which, being relatively weak bonds, could undergo further photolysis, thereby regenerating SMAnh chain radicals to act as macroinitiators for the addition of another monomer M. This sequence of reactions is shown in Scheme 3 where M in this work was either acrylamide (AM) or *N*,*N*-dimethylacrylamide (DMA). This was the methodology used for the synthesis of the SMAnh-*b*-AM and SMAnh-*b*-DMA block copolymers described in this paper. It is based on the assumption that the only way that monomer M can polymerize in step (c) is via initiation by the SMAnh chain radicals regenerated via step (b). The bromine radical Br• is not effective as an initiator. Interestingly, a similar methodology to this but using bromoform ($CHBr_3$) instead of CBr_4 to prepare block copolymers of DMA and *N*-isopropylacrylamide (NIPAM) in an aqueous system has recently been reported.[25]



SCHEME 3 (a) SMAnh chain transfer to CBr_4, (b) regeneration of SMAnh chain radical, (c) block copolymerization of momoner M, (d) final block copolymer

2.4 Synthesis of styrene-maleic anhydride (SMAnh) copolymers

In a typical synthesis, weights as close as possible to 7.811 g (0.075 mol) styrene (S), 7.354 (0.075 mol) maleic anhydride (MAnh), 0.336 g (0.0015 mol) Irgacure 2959 and 0.995 g (0.0030 mol) CBr_4 were dissolved in 150 ml THF as solvent. Photopolymerization was carried out by means of UV irradiation of the solution in a closed conical flask under a nitrogen atmosphere with magnetic stirring at room temperature for 6 hr. Thus, the polymerization conditions were: [S]:[MAnh] = 1:1 (mol ratio), [S+MAnh] = 1 M, [Irgacure] = 1 mol %, and [CBr_4] = 2 mol %. These conditions were predetermined in a series of experiments as being suitable for obtaining the SMAnh copolymer in high yield (~ 90%) and with an average molecular weight in the required range (< 10,000).

Since the SMAnh copolymer was soluble in THF, it remained in solution throughout the synthesis. It was isolated by precipitation from solution into a 5-fold excess of chilled methanol before being filtered off, washed with more methanol, and dried to constant weight in a vacuum oven at 60°C. The final product was obtained as a finely divided white powder in over 90% yield (Table 1). The copolymer's solubility in THF was essential from the point of view of allowing another monomer to be added into the solution for block copolymerization, as will be described in section 2.6. The % yield was calculated from the equation below:

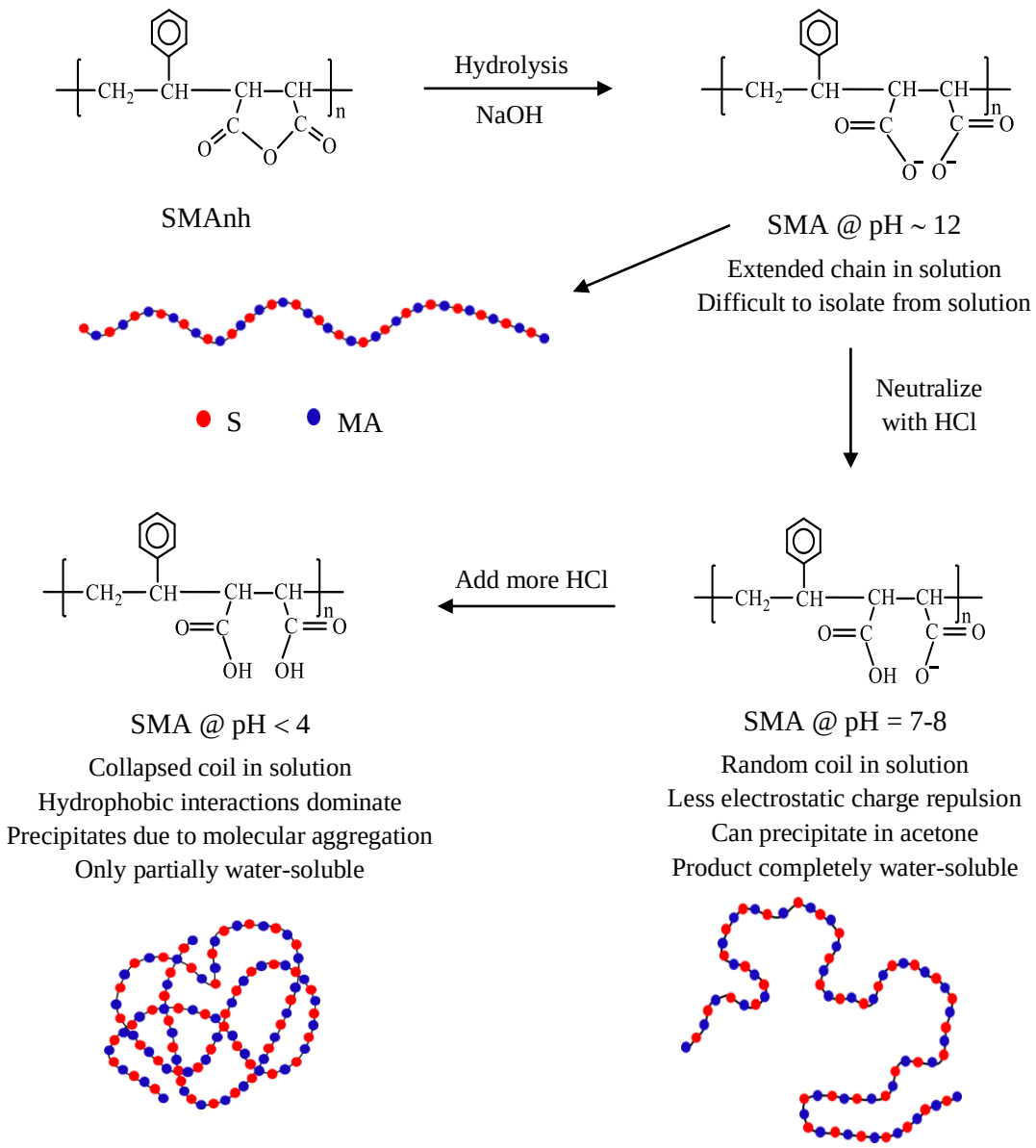
$$\%\, Yield \;=\; \frac{Final\ wt.\ of\ dry\ SMAnh\ product}{Initial\ wt.\ of\ S + MAnh\ monomers} \times 100\ \%$$

2.5 Hydrolysis of SMAnh to styrene maleic acid (SMA)

Various experimental procedures have been reported in the literature for the hydrolysis of SMAnh to SMA.[17,26-28] In this present work, approximately 4 g SMAnh were weighed out accurately and refluxed gently in 80 ml 0.5 M aqueous NaOH solution with magnetic stirring for 3 hr. The initially insoluble SMAnh gradually dissolved over a period of approximately 20 min to give a pale yellow solution as it hydrolyzed to SMA. After leaving the solution (pH~ 12) to cool to room temperature, it was then titrated with 0.5 M HCl to pH 7-8. Finally, the SMA product was isolated by precipitation into a 5-fold excess of chilled acetone after which it was filtered off, washed with a minimum amount of cold water, and dried to constant weight in a vacuum oven at 60°C. The final product was obtained as a finely divided off-white powder, also in high yield (Table 1) although slightly less than the yield of SMAnh.

Since the aim of this work was to obtain a completely water-soluble SMA, particular attention was focused on the degree of neutralization of the hydrolyzed SMA during titration

with HCl acid. In order to (a) keep the SMA in solution, (b) enable the SMA to be precipitated in acetone and (c) obtain a completely water-soluble product, it was found that a pH of 7-8 in which the SMA adopts a random coil conformation in solution gave the best results. The pH-dependency of SMA's chain conformation in aqueous solution is visualized in Scheme 4 based on literature reports.[1,14] It has also been reported that, for membrane solubilization, SMAs are commonly used at a pH of 7-8.[1] This is because a random coil conformation facilitates the required balance between the electrostatic and hydrophobic forces necessary for effective membrane insertion. In addition, pH neutralization also protects the extracted proteins from precipitation or denaturation.

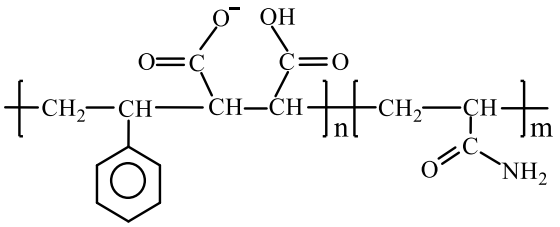


SCHEME 4 pH-dependency of SMA's chain conformation in aqueous solution

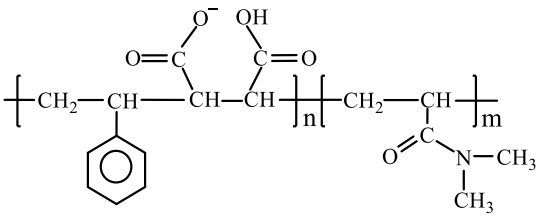
2.6 Block copolymerization with acrylamide or *N,N*-dimethylacrylamide

For block copolymerization, the SMAnh copolymer was first synthesized according to the procedure described previously in section 2.4 (Step 1). Then, with the SMAnh copolymer still in solution, an equimolar amount of either acrylamide (AM) (10.662 g = 0.150 mol) or *N,N*-dimethylacrylamide (DMA) (14.869 g = 0.150 mol) was added to the solution and UV irradiation continued for a further 6 hr (Step 2). In the case of AM, a white precipitate soon formed during irradiation which, at the end of the 6 hr period, was filtered off, washed with methanol and vacuum dried to constant weight. The filtrate was also found to contain a soluble product which could be precipitated in methanol. In the case of DMA, no precipitation occurred during irradiation and so the soluble product as a whole was precipitated in methanol.

Hydrolysis of the SMAnh-*b*-AM and SMAnh-*b*-DMA block copolymers to SMA-*b*-AM and SMA-*b*-DMA was carried out via the procedure described previously in section 2.5. As before, neutralization of the hydrolysis solution was carried out to pH 7-8. The products obtained were completely water-soluble, finely divided white powders.



SMA-*b*-AM



SMA-*b*-DMA

3 RESULTS AND DISCUSSION

3.1 Characterization of the SMAnh and SMA copolymers

Since the initial S:MAnh comonomer mole ratio used in this work was 1:1 and with monomer reactivity ratios of 0.02 for styrene (S) and 0.01 for maleic anhydride (MAnh),[29] it is now generally accepted that, under these conditions, the SMAnh copolymer formed is an almost exclusively alternating copolymer with the same 50:50 mol % composition as the comonomer feed.[13,30] This can be confirmed by ratioing the peak areas of the aromatic and aliphatic protons in the ^{1}H-NMR spectrum.[31,32] Since hydrolysis of SMAnh to SMA does not affect the

copolymer composition, it can be assumed that the SMA has the same 50:50 S:MA mol % ratio as the S:MAnh ratio in SMAnh.

The ^{1}H-NMR spectra of the SMAnh and SMA copolymers are compared in Figure 2 showing the various proton assignments. As expected, the spectra are quite similar in appearance and conform to their respective structures. The most obvious difference is that the MA proton peak (d,e) at δ3.0-3.8 is much clearer in the SMA spectrum since it is well separated from the D_2O solvent peaks, unlike the MA peak in the SMAnh spectrum which is masked by the HOD peak in d_6-DMSO. Regarding composition, the ratio of the peak area integrations I_c and I_{de} in Figure 2(b) of I_c : I_{de} = 1.00 : 0.38 corresponds closely to the 5 : 2 ratio of the phenyl protons (c) in styrene and the methine (d,e) protons in maleic acid.

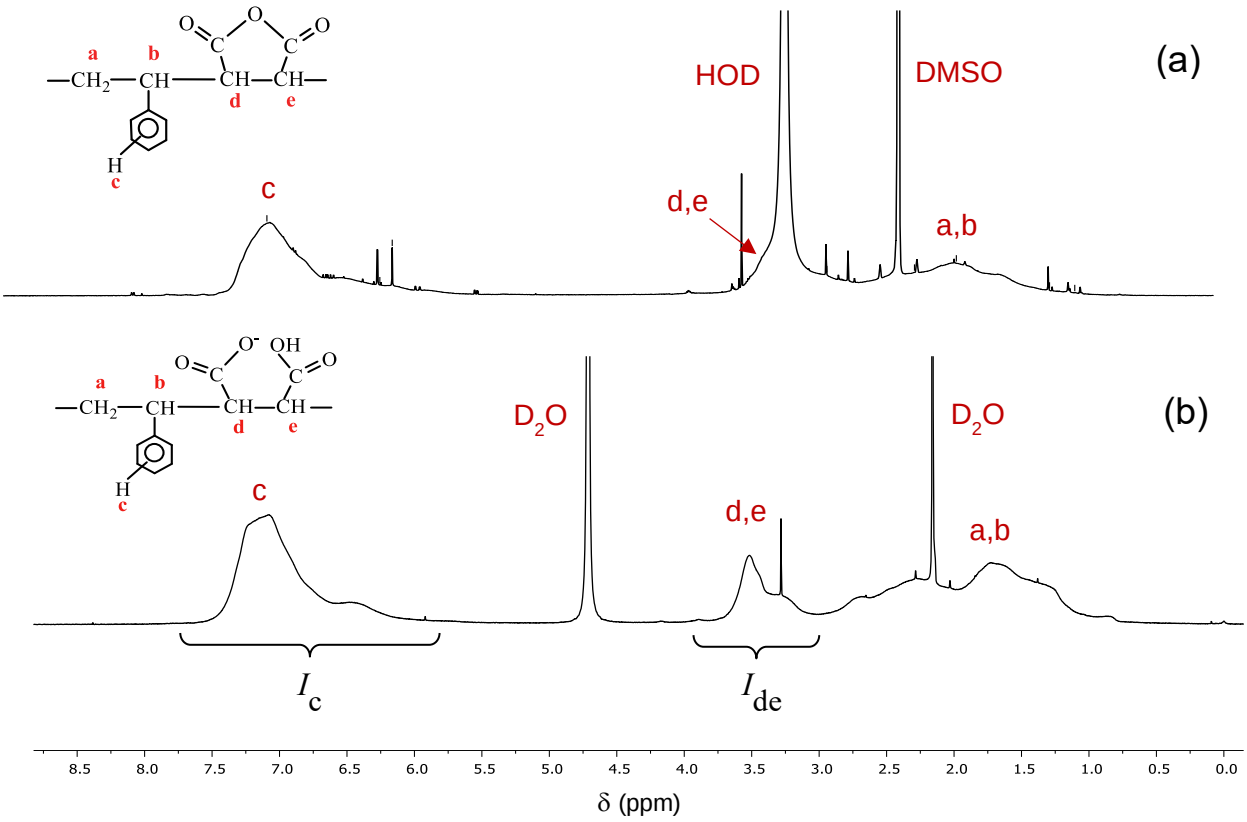


FIGURE 2 ^{1}H-NMR spectra of (a) SMAnh recorded in d_6-DMSO as solvent and (b) SMA recorded in D_2O as solvent

For molecular weight determination, dilute-solution viscometry was the method used. For both SMAnh and SMA, well-calibrated Mark-Houwink equations are available in the literature which yield either the weight-average molecular weight, M_w, or the viscosity-average

molecular weight, M_v. According to Chow,[20] the equation for SMAnh in tetrahydrofuran (THF) as solvent at 25ºC yields M_w, as given by Eq. (1).

$$[\eta] = 3.98 \times 10^{-4} M_w^{0.596} \text{ dl/g} \quad (1)$$

The intrinsic viscosity, [η], and M_w values for the SMAnh sample described in section 2.4 are listed in Table 1. In order to confirm the accuracy of this viscometric method and Eq. (1), the M_w value of a commercial SMAnh sample (SMA® 1000P; Total Cray Valley, USA) of known M_w (5,500) was also determined in the same way and was found to be in close agreement (5,900) with the manufacturer's stated value. In comparison, gel permeation chromatography (GPC), which was also used as an alternative method, gave a much lower M_w value (2,100). Hence, dilute-solution viscometry was the preferred method.

In contrast, the Mark-Houwink equation for SMA in 0.02 M aqueous NaCl solution at 25°C yields M_v rather than M_w and was derived from the conformational studies of Ohno and co-workers,[14] as shown in Eq. (2).

$$[\eta] = 3.73 \times 10^{-4} M_v^{0.67} \text{ dl/g} \quad (2)$$

The [η] and M_v values for the SMA sample described in section 2.5 are also listed in Table 1. The M_w and M_v values for SMAnh and SMA are seen to be well below the previously mentioned upper limit of 10,000 recommended for membrane solubilization.

For thermal analysis, the DSC thermograms of SMAnh and SMA are compared in Figure 3. Both SMAnh and SMA exhibit broad glass transitions (T_g) with mid-points of around 155°C and 165°C respectively (Table 1). The T_g of SMAnh of 155°C is consistent with values reported in the literature of around 130-160°C.[33] The T_g of SMA is less well documented due to its variation with the degree of neutralization of the acid groups. However, whatever the degree of neutralization, it is generally assumed that the slightly higher T_g of SMA compared to SMAnh is due to the intermolecular polar interactions of the $COOH/COO^-$ groups in SMA.

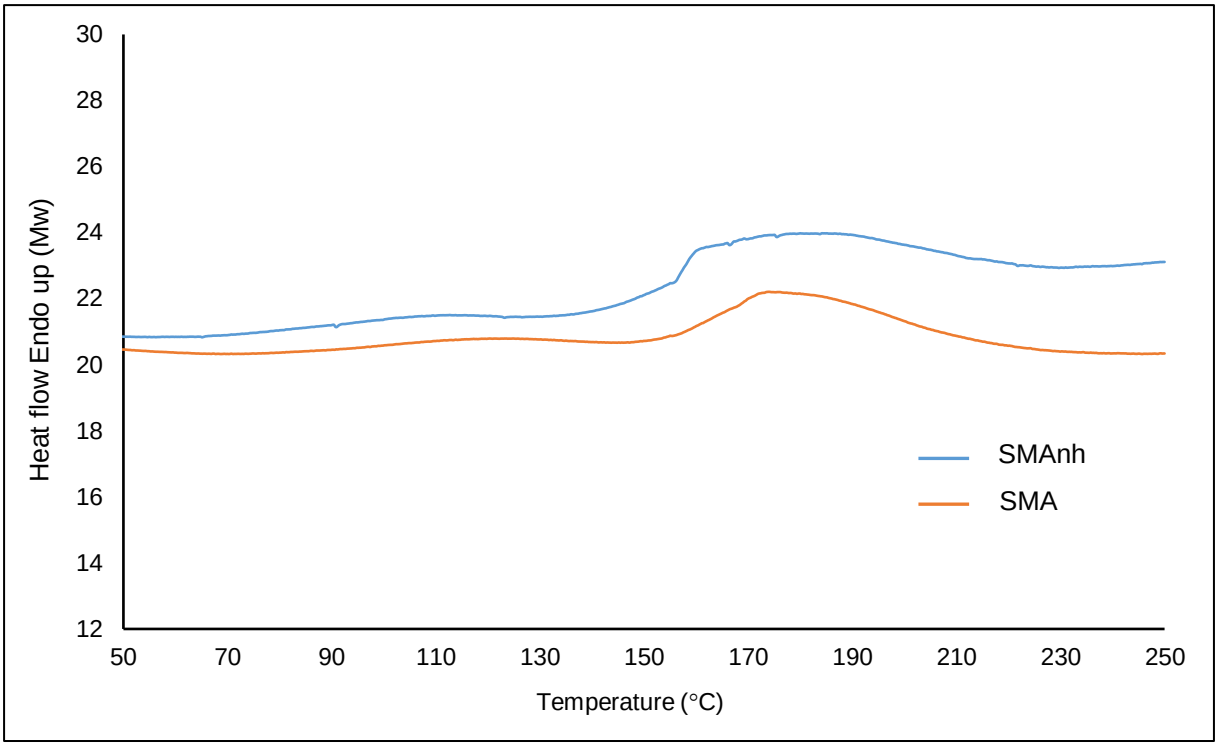


FIGURE 3 DSC thermograms of the SMAnh and SMA copolymers (Heating rate = 10°C/min)

TABLE 1 Comparison of the properties of the synthesized SMAnh and its subsequent SMA hydrolysis product

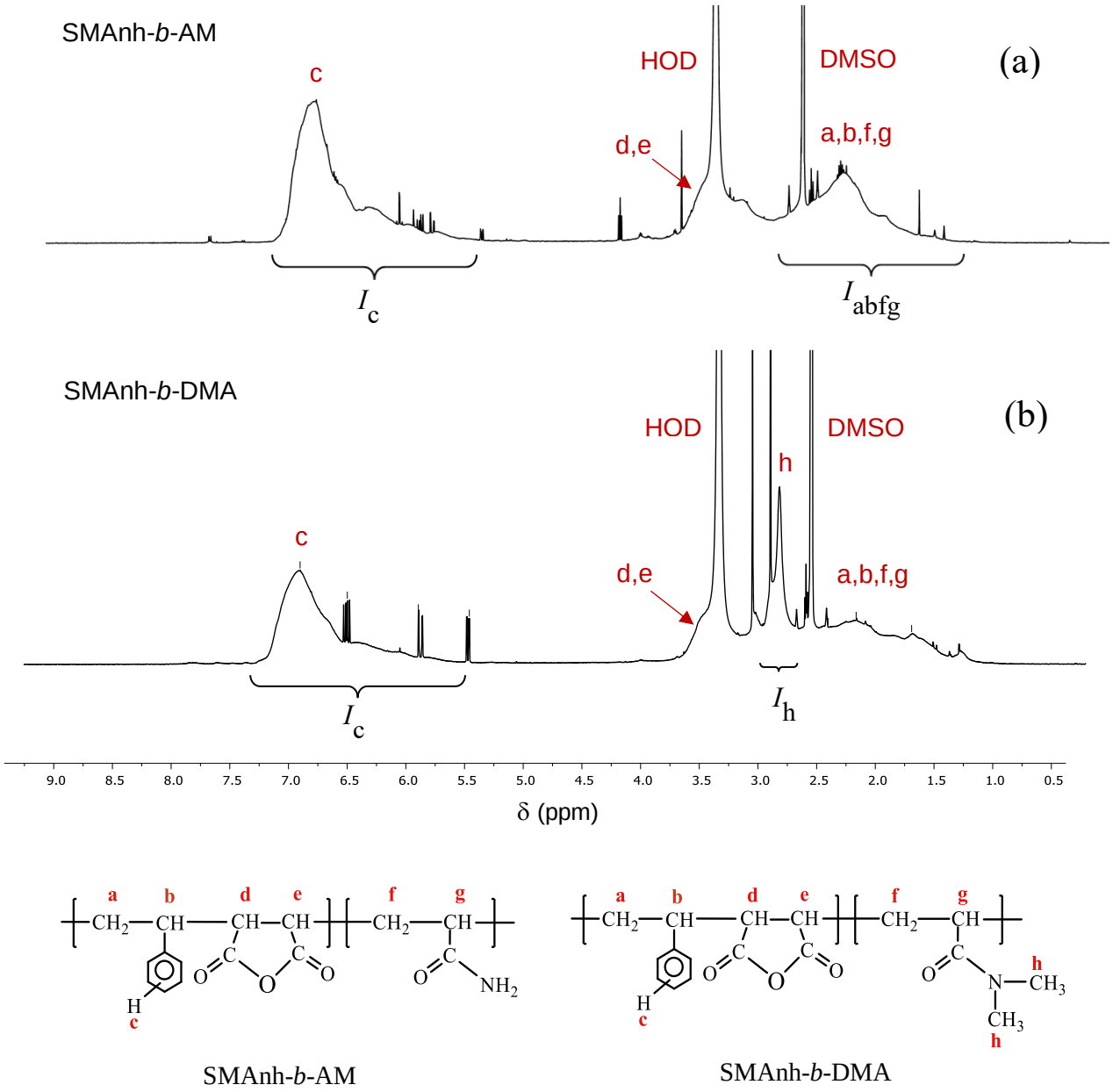
PROPERTIES	SMAnh	SMA
Yield, %	95 [a)]	88 [a)]
Copolymer composition, mol %	50 : 50	50 : 50
Intrinsic viscosity, [η] dl/g	0.081 [b)]	0.122 [c)]
Weight-average mol. wt., M_w g/mol	7.47×10^3 [d)]	-
Viscosity-average mol. wt., M_v g/mol	-	5.67×10^3 [e)]
Glass transition temperature (mid-point), T_g °C	155	165
Soluble in water	No	Yes

a) Relative to the initial combined weight of S + MAnh
b) Determined in THF as solvent at 25°C
c) Determined in 0.02 M aqueous NaCl as solvent at 25°C
d) Calculated from $[\eta] = 3.98 \times 10^{-4} M_w^{0.596}$ dl/g
e) Calculated from $[\eta] = 3.73 \times 10^{-4} M_v^{0.67}$ dl/g

3.2 Characterization of the SMAnh-*b*-AM block copolymer

As described in section 2.6, addition of acrylamide (AM) (in Step 2) to the still reactive SMAnh copolymer in solution (from Step 1) followed by continued UV irradiation produced two products, the first of which precipitated out from solution as it was formed while the second remained in solution and was later precipitated in methanol. Subsequent ^{1}H-NMR analysis of the soluble product showed that its spectrum was identical to that of SMAnh in Figure 2(a) with no evidence of AM addition. Hence, it could be concluded that this soluble product was simply unreacted SMAnh from Step 1.

In contrast, the spectrum of the product which precipitated out from solution as it was formed, as shown in Figure 4(a), exhibited enhanced peaks in the aliphatic proton region from δ1.5-3.0 when compared with the SMAnh spectrum. This was due to the addition of AM blocks to the SMAnh chains in forming an SMAnh-*b*-AM block copolymer. The AM blocks were also responsible for the copolymer precipitating out of solution since polyacrylamide (PAM) is known to be insoluble in THF.



SMAnh-*b*-AM

SMAnh-*b*-DMA

FIGURE 4 ^{1}H-NMR spectra of (a) SMAnh-*b*-AM and (b) SMAnh-*b*-DMA recorded in d_6-DMSO as solvent

From the ^{1}H-NMR spectrum in Figure 4(a), the SMAnh-*b*-AM copolymer composition (SMAnh : AM) (mol %) in Table 2 can be estimated from the ratio of the peak area integrations of the aromatic (I_c) and aliphatic (I_{abfg}) protons. If it is assumed that the S : MAnh ratio in the SMAnh blocks is 1:1, the number of S units will be equal to the number of SMAnh units. Therefore, the intensity of the aromatic proton peak (I_c) alone is sufficient to be able to estimate the amount of SMAnh relative to the amount of AM via the following calculation:

$$\text{SMAnh : AM} = I_c/5 : [I_{abfg} - 3/5(I_c)]/3 = 0.200 : 0.240$$

$$\text{SMAnh : AM} = 46 : 54$$

Note: The *I* peak area integrations in Figure 4 exclude any overlapping DMSO solvent peaks.

As also shown in Table 2, the SMAnh-*b*-AM block copolymer was obtained in only 50% yield. The remaining 50% yield was lost mainly as a result of the block copolymer precipitating out of solution and taking with it the propagating chain ends, thereby leaving a surplus of unreacted AM monomer in solution. It is also possible that there could have been a fraction of the SMAnh chains which were not Br-terminated from Step 1 and which were therefore unreactive in Step 2.

Since the SMAnh-*b*-AM block copolymer was insoluble in THF, viscometry was performed in dimethyl sulfoxide (DMSO) as solvent at 25°C. In the absence of a Mark-Houwink equation, an actual molecular weight value could not be calculated. However, the fact that the block copolymer's intrinsic viscosity [η] of 0.033 dl/g (Table 2) was significantly higher than that of its SMAnh precursor ([η] = 0.020 dl/g), also measured in DMSO, indicated that its molecular weight was also higher due to the AM block addition.

One of the clearest indications of block formation in a copolymer is the presence of two T_gs, one for each block. However, as the DSC thermogram of SMAnh-*b*-AM in Figure 5 shows, only the T_g of the SMAnh block is observed with a mid-point of around 155°C, similar to that of SMAnh alone (Table 2). The apparent absence of a T_g for the AM block is probably due to either (a) the AM blocks being too short and/or (b) overlap with the SMAnh block transition (PAM has a reported T_g of 160-170°C depending on the reference source). The broad peak between 70-100ºC is probably due to the loss of moisture from the sample.

In contrast to its DSC curve, the TGA curve of the SMAnh-*b*-AM in Figure 6 shows a quite different profile compared to that of its SMAnh precursor. The gradual weight loss below 300°C is indicative of the presence of the thermally less stable AM blocks. PAM shows a similar weight loss profile. This characteristic weight loss in PAM is reported to be due mainly to the elimination of ammonia between adjacent amide groups to form imide groups.[34]

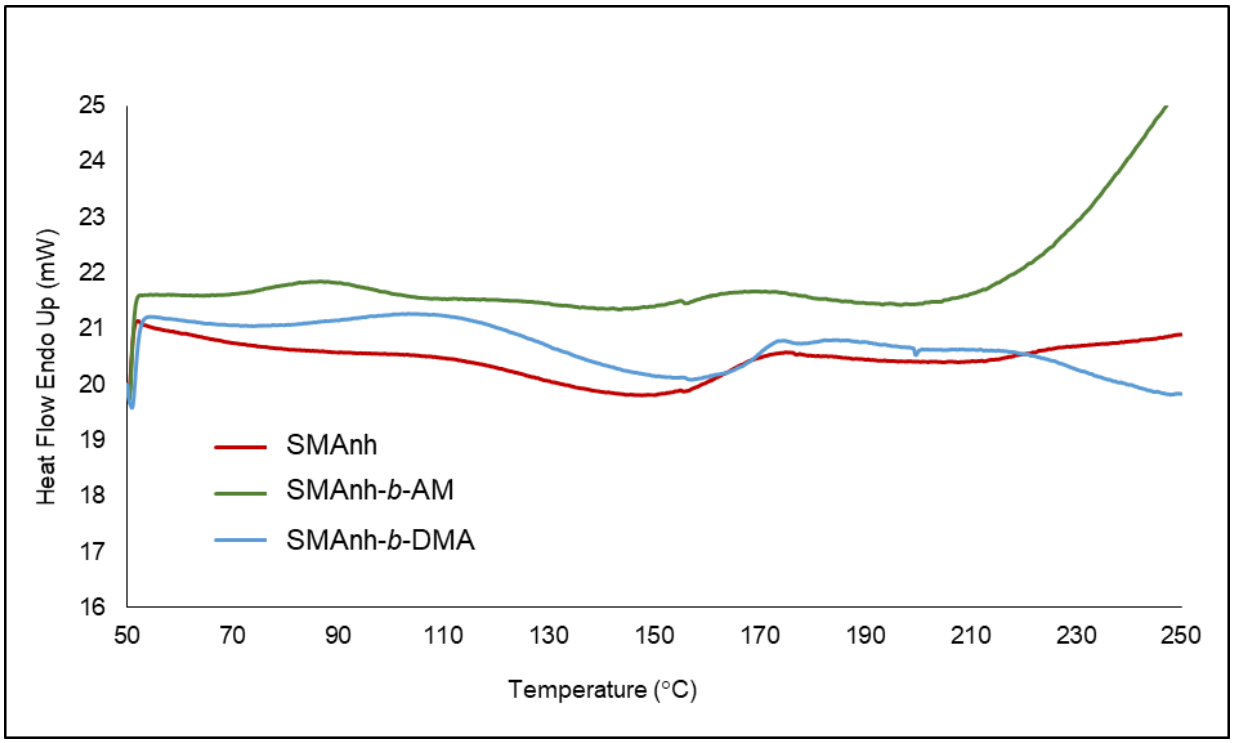


FIGURE 5 DSC thermograms of SMAnh, SMAnh-*b*-AM and SMAnh-*b*-DMA (Heating rate = 10°C/min)

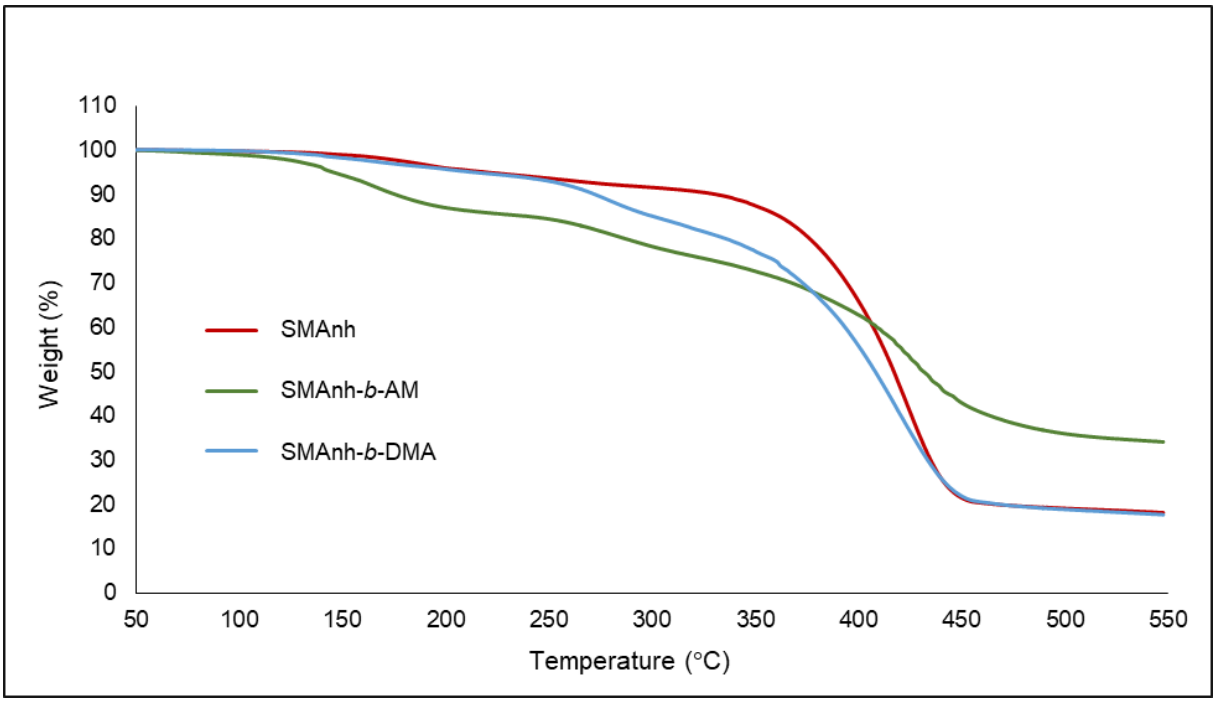


FIGURE 6 TGA curves of SMAnh, SMAnh-*b*-AM and SMAnh-*b*-DMA (Heating rate = 20°C/min)

TABLE 2 Comparison of the properties of the SMAnh (Step 1) and SMAnh-*b*-AM (Step 2) block copolymer

PROPERTIES	SMAnh	SMAnh-*b*-AM
Yield, %	95 [a)]	50 [a)]
Copolymer composition, S : MAnh (mol %)	50 : 50	-
Copolymer composition, SMAnh : AM (mol %)	-	46 : 54
Intrinsic viscosity, [η] dl/g	0.020 [b)]	0.033 [b)]
Weight-average mol. wt., M_w g/mol	5.58×10^3 [c)]	-
Glass transition temperature (mid-point), T_g °C	160	155
Thermal degradation weight loss up to 300°C, %	< 10	20
Soluble in THF	Yes	No

a) Relative to the initial combined monomer weights

b) Determined in DMSO as solvent at 25°C

c) Determined in THF as solvent at 25°C and calculated from Eq. (1)

3.3 Characterization of the SMAnh-*b*-DMA block copolymer

As described previously in section 2.6 and in contrast to acrylamide (AM), addition of *N*,*N*-dimethylacrylamide (DMA) (in Step 2) to the SMAnh copolymer in solution in THF (from Step 1) followed by continued UV irradiation produced no precipitation. This was to be expected since poly(*N*,*N*-dimethylacrylamide) (PDMA), like SMAnh, is soluble in THF. Consequently, the final product had to be recovered by precipitation in chilled methanol.

The ^{1}H-NMR spectrum of the final product is shown in Figure 4(b). The presence of the dimethyl protons in DMA gives rise to a well-separated and clearly defined peak (h) at δ2.7-3.0 which effectively confirms of the addition of the DMA blocks. The copolymer composition (SMAnh : DMA) (mol %) in Table 3 can be estimated from the ratio of the peak area integrations of the phenyl (I_c) and dimethyl (I_h) protons via the following equation:

$$\text{SMAnh : DMA} = I_c/5 : I_h/6 = 0.240 : 0.102$$

$$\text{SMAnh : DMA} = 70 : 30$$

which indicates a DMA content which is much less than in the comonomer feed ratio (mol %) of S + MAnh (Step 1) : DMA (Step 2) = 50 : 50.

It is also significant to note that, despite the final SMAnh-*b*-DMA product remaining in solution, the % yield decreased to 44% (Table 3). Again, there could have been a fraction of SMAnh chains which were not Br-terminated from Step 1 but it is thought that the main reason for this reduced yield is the lower monomer reactivity of DMA (compared to AM) towards the SMAnh chain radicals. The lower DMA content (30 mol %) in the copolymer supports this view. The lower monomer reactivity of DMA is a result of the steric hindrance effect of the *N*,*N*-dimethyl groups. This is reflected in the respective reactivity ratios (r) of the styrene-acrylamide and styrene-dimethylacrylamide comonomer combinations.[35]

Styrene (S) - Acrylamide (AM)	$r_S = 1.17$	$r_{AM} = 0.58$
Styrene (S) - Dimethylacrylamide (DMA)	$r_S = 1.15$	$r_{DMA} = 0.12$

For the SMAnh-*b*-DMA copolymer's molecular weight, although an absolute value could not be determined, its higher intrinsic viscosity [η] of 0.067 dl/g compared to 0.040 dl/g for its SMAnh precursor (Table 3) was evidence of chain extension. Dioxane was found to be an effective common solvent for both SMAnh-*b*-DMA and SMAnh.

The DSC thermogram of SMAnh-*b*-DMA is shown in Figure 5 and exhibits a T_g centered at around 170°C due to the SMAnh blocks. A separate T_g due to the DMA blocks would appear at a much lower temperature (the T_g of PDMA is typically quoted as 89°C) but, as in the SMAnh-*b*-AM curve, the change in baseline at around 80°C is more likely to be loss of moisture. Considering the low DMA content (30%) in the SMAnh-*b*-DMA and the likelihood that the DMA blocks will be short in length, the absence of a separate T_g is not surprising.

In contrast to the weight loss profile of SMAnh-*b*-AM, the TGA curve of SMAnh-*b*-DMA in Figure 6 shows a similar profile to that of SMAnh up to 250°C. The afore-mentioned ammonia elimination from the AM blocks in SMAnh-*b*-AM leading to imidization is sterically hindered by the *N*,*N*-dimethyl groups in DMA. Hence, the SMAnh-*b*-DMA weight loss profile resembles that of SMAnh until the DMA component starts to degrade at above 250°C.

TABLE 3 Comparison of the properties of the SMAnh (Step 1) and SMAnh-*b*-DMA (Step 2) block copolymer

PROPERTIES	SMAnh	SMAnh-*b*-DMA
Yield, %	95 [a)]	44 [a)]
Copolymer composition, S : MAnh (mol %)	50 : 50	-
Copolymer composition, SMAnh : DMA (mol %)	-	70 : 30
Intrinsic viscosity, [η] dl/g	0.040 [b)]	0.067 [b)]
Weight-average mol. wt., M_w g/mol	4.90 x 10^3 [c)]	-
Glass transition temperature (mid-point), T_g °C	160	165
Thermal degradation weight loss up to 300°C, %	< 10	~10

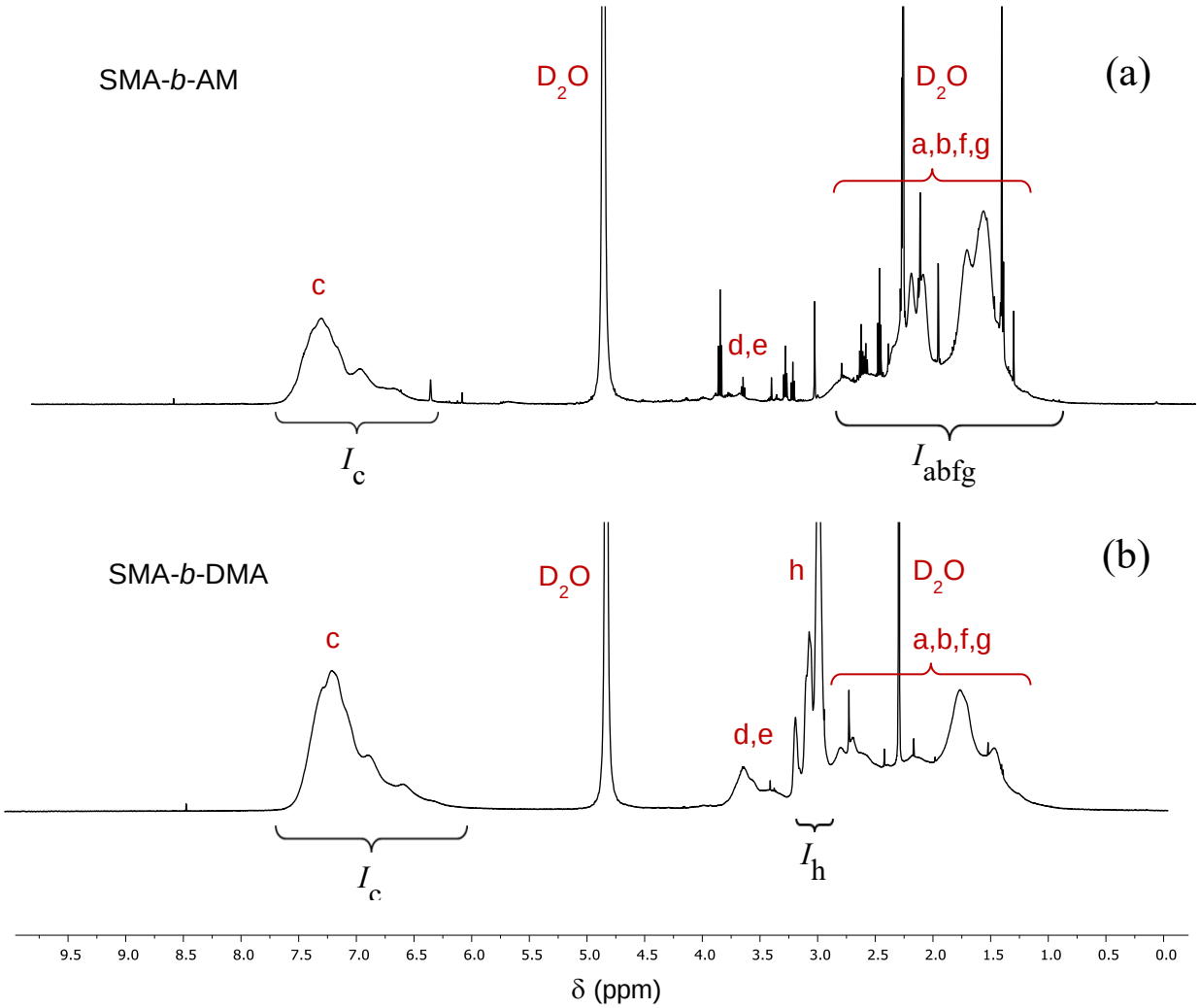
a) Relative to the initial combined monomer weights

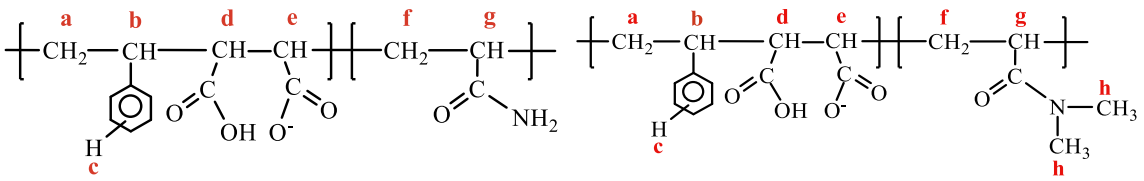
b) Determined in dioxane as solvent at 25°C

c) Determined in THF as solvent at 25°C and calculated from Eq. (1)

3.4 Characterization of the hydrolyzed SMA-*b*-AM and SMA-*b*-DMA block copolymers

The anhydride groups of the SMAnh-*b*-AM and SMAnh-*b*-DMA block copolymers were hydrolyzed according to the procedure described previously for SMAnh in section 2.5. Following their precipitation, filtration and drying, the SMA-*b*-AM and SMA-*b*-DMA products were obtained in 90% and 88% yield respectively and were completely water-soluble (Table 4). Their ^{1}H-NMR spectra, recorded in D_2O as solvent, are compared in Figure 7 from which their copolymer compositions (mol %) can be estimated from the relevant peak area integrations.





SMA-*b*-AM SMA-*b*-DMA

FIGURE 7 ^{1}H-NMR spectra of (a) SMA-*b*-AM and (b) SMA-*b*-DMA recorded in D_2O as solvent

For SMA-*b*-AM in Figure 7(a)

$$\text{SMA : AM} = I_c/5 : [I_{abfg} - 3/5(I_c)]/3 = 0.200 : 0.415$$

$$\text{SMA : AM} = 33 : 67$$

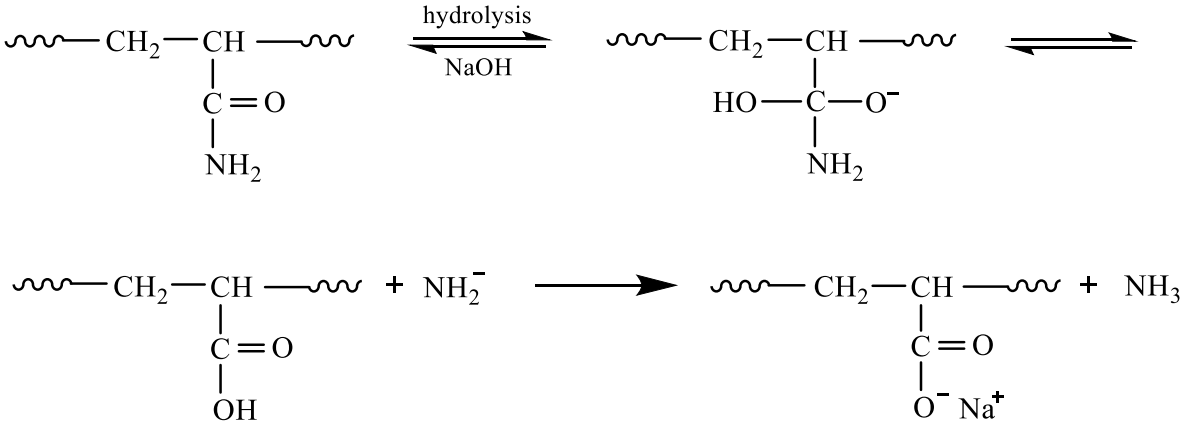
For SMA-*b*-DMA in Figure 7(b)

$$\text{SMA : DMA} = I_c/5 : I_h/6 = 0.200 : 0.092$$

$$\text{SMA : DMA} = 68 : 32$$

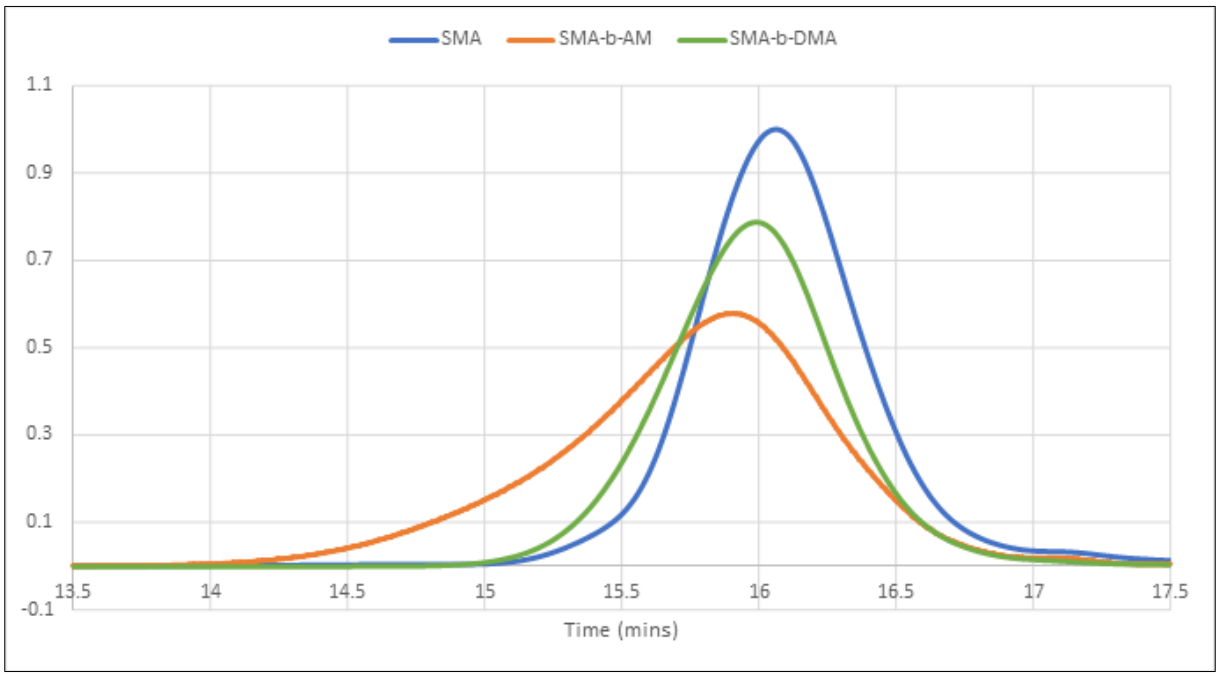
Note: The *I* peak area integrations in Figure 7 exclude any overlapping D_2O solvent peaks.

The composition of the SMA-*b*-DMA (68:32) agrees closely with that of the SMAnh-*b*-DMA (70:30) from which it was made, which is as would be expected since hydrolysis does not change the block ratio. However, the same cannot be said of the composition of the SMA-*b*-AM copolymer (33:67) which shows a higher AM content than its SMAnh-*b*-AM precursor (46:54). The reason for this is unclear but is obviously related to an effect of the AM blocks which is not present in the DMA blocks. Apart from the AM blocks being longer and more polar, another difference is that, similar to the Anh groups, the amide groups in the AM blocks are also susceptible to alkaline hydrolysis, as shown in Scheme 5.[36] With enhanced polyelectrolyte character, this would certainly have a significant effect on the chain conformation of the SMA-*b*-AM in solution in water and similarly in D_2O. However, how this would lead to the SMA-*b*-AM NMR spectrum giving such a different composition to that of SMAnh-*b*-AM remains a subject for further investigation. In contrast to the AM blocks, the amide groups in the DMA blocks are less prone to hydrolysis. This is due to the effect of the *N,N*-dimethyl groups in deactivating the C=O group towards nucleophilic attack by the OH^- anion.



SCHEME 5 Mechanism of alkaline hydrolysis of the amide group in polyacrylamide

With regard to the molecular weights of the SMA-*b*-AM and SMA-*b*-DMA, although actual values of M_v could not be calculated, their intrinsic viscosities of 0.395 and 0.169 dl/g respectively (Table 4) are both significantly greater than the 0.122 dl/g of the SMA precursor (Table 1). This indicates molecular weight increases as a result of adding on the AM and DMA blocks, although more so in the case of AM. The aqueous GPC data in Figure 8 provides supporting evidence for this with the curves shifting to shorter elution times (higher mol. wts.) for the SMA-*b*-AM and SMA-*b*-DMA block copolymers compared to the SMA precursor.



SMA	M_n = 4010	M_w = 4571	PD = 1.14
SMA-*b*-AM	M_n = 5400	M_w = 7452	PD = 1.38
SMA-*b*-DMA	M_n = 4520	M_w = 5153	PD = 1.14

FIGURE 8 GPC curves and the corresponding M_n, M_w and polydispersity PD (= M_w/M_n) values for the SMA, SMA-*b*-AM and SMA-*b*-DMA block copolymers

Despite their polyelectrolyte character, their dilute-solution viscometry plots in Figure 9 show good linearity due to the charge shielding effect of the NaCl in solution. The negative slopes of the SMA-*b*-AM plots in Figure 9 reflect the polyelectrolyte behavior of the partially dissociated SMA which is further enhanced by the AM blocks due to the hydrogen bonding capability of the $-NH_2$ groups. This is less apparent in the SMA-*b*-DMA plots since hydrogen bonding in the DMA blocks is precluded by the dimethyl substitution in the $-N(CH_3)_2$ groups.

The DSC thermograms of the SMA-*b*-AM and SMA-*b*-DMA are compared in Figure 10 and show similarly broad transitions with mid-points at around 110°C which can be assigned to the SMA blocks. This T_g is considerably lower than the 160 and 165°C for the same samples before hydrolysis (Table 3). The reason for this is uncertain, although it could be due to the effects of the AM and DMA blocks interacting with the COOH/COO^- groups in the SMA blocks to increase chain separation, create more free volume, and thereby decrease the energy barrier to chain rotation. As was the case before hydrolysis, separate T_gs for the AM and DMA blocks were again not observed.

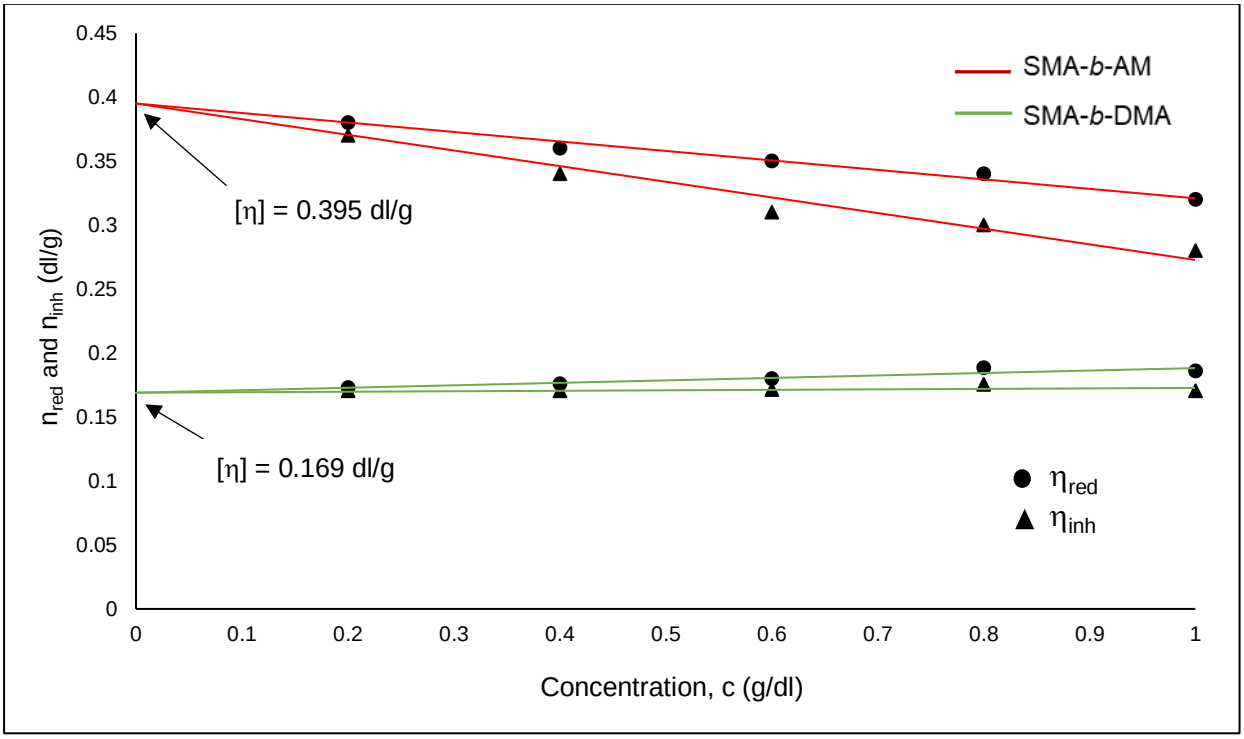


FIGURE 9 Double extrapolation plots of reduced viscosity η_{red} and inherent viscosity η_{inh} against concentration c for the SMA-*b*-AM and SMA-*b*-DMA block copolymers in 0.02 M aqueous NaCl at 25°C

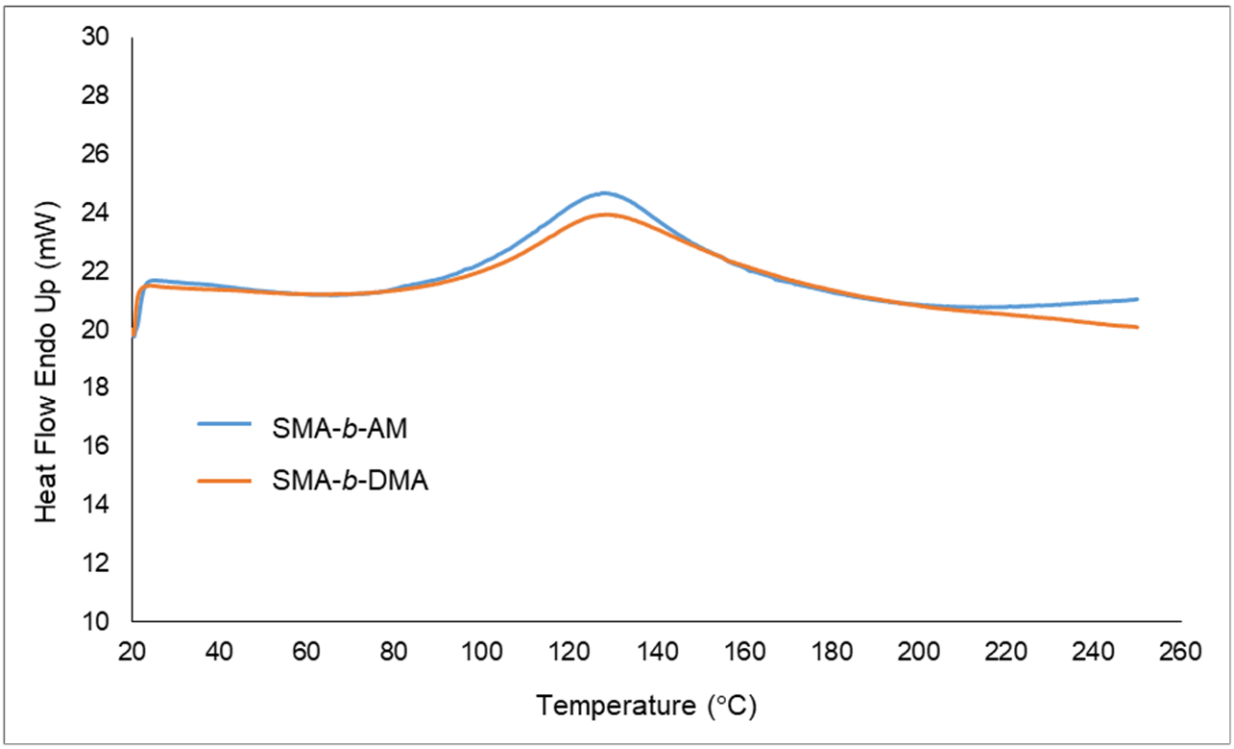


FIGURE 10 DSC thermograms of SMA-*b*-AM and SMA-*b*-DMA (Heating rate = 10°C/min)

TABLE 4 Comparison of the properties of the SMA-*b*-AM and SMA-*b*-DMA block copolymers after hydrolysis

PROPERTIES	SMA-*b*-AM	SMA-*b*-DMA
Yield, %	90 [a)]	88 [a)]
Copolymer composition, SMA : AM (mol %)	33 : 67	-
Copolymer composition, SMA : DMA (mol %)	-	68 : 32
Intrinsic viscosity, [η] dl/g	0.395 [b)]	0.169 [b)]
Glass transition temperature (mid-point), T_g °C	110	110
Soluble in water	Yes	Yes
pH of 1% w/v aqueous solution	8.55	8.81

[a)] Relative to an initial weight of 4 g of SMAnh-*b*-AM or SMAnh-*b*-DMA

[b)] Determined in 0.02 M aqueous NaCl as solvent at 25°C (Figure 9)

4 CONCLUSIONS

The synthesis of styrene maleic anhydride (SMAnh) copolymers is a straightforward process which is well documented in the literature. Due to their very low monomer reactivity ratios, styrene (S) and maleic anhydride (MAnh) combine to produce a 50:50 alternating copolymer starting from an equimolar S:MAnh comonomer ratio. With the monomer sequencing and final copolymer composition effectively predetermined, interest in this work focused on molecular weight control to M_w < 10,000 g.mol^{-1}. As described previously in section 2.4, this has been achieved through establishing the appropriate synthesis conditions including the use of carbon tetrabromide (CBr_4) as a chain transfer agent.

Hydrolysis of SMAnh to SMA in aqueous NaOH solution is also straightforward but care needs to be taken if the SMA is to be physically isolated as a solid which is completely water-soluble. Precipitation from aqueous alkaline solution depends on the chain conformation of the SMA which in turn depends on the degree of neutralization with HCl acid. It is well known that SMA chain conformation in aqueous solution is determined by the balance between the hydrophilic interactions of the MA units and the hydrophobic interactions of the S units. When the former predominate, the chain adopts an extended chain conformation whereas when the latter predominate the chain collapses into a globular coil (as shown in Scheme 4). This hydrophilic-hydrophobic balance varies with pH and it was found in this work that an intermediate random coil conformation at a pH of 7-8 was best for enabling the SMA to be precipitated quantitatively in acetone. More importantly, the SMA precipitated in a form which could be completely re-dissolved in water. At a lower pH (< 4), it was found that the SMA started to precipitate out of solution due to the chains collapsing into globular coils leading to molecular aggregation and a final product that was only partially water-soluble.

Having synthesized both SMAnh and SMA to within the required molecular weight limit, the main objective of this work was then to synthesize block copolymers with acrylamide (AM) and *N,N*-dimethylacrylamide (DMA). In order to achieve this, CBr_4 was chosen as the chain transfer agent in Step 1 (section 2.4) so that the Br-terminated SMAnh chains would still be photolytically active in the presence of an added monomer (Scheme 3). As the results in Tables 2 and 3 have shown, SMAnh-*b*-AM and SMAnh-*b*-DMA were successfully obtained using this methodology. As expected, AM was a more reactive monomer than DMA towards the SMAnh chain radicals, resulting in SMAnh-*b*-AM being obtained in a higher yield and with a more even composition than SMAnh-*b*-DMA. Their combined NMR, viscometry, thermal analysis and solubility data has provided solid evidence of their block copolymer structures.

The different solubilities of the SMAnh and AM/DMA blocks has proved to be a particularly useful indicator of block formation.

Finally, as with SMAnh alone, hydrolysis of the anhydride groups to SMA-*b*-AM and SMA-*b*-DMA followed by neutralization to pH 7-8 and precipitation in acetone produced completely water-soluble final products. Water solubility is an essential requirement for the copolymers' intended use in aqueous-based membrane protein extraction. With this in mind, an intriguing aspect of the SMA-*b*-AM and SMA-*b*-DMA copolymers is that they comprise a hypercoiling SMA block attached to a non-hypercoiling AM or DMA block, the balance of which can be pH-controlled. What effect this combination will have on their efficiency in membrane protein extraction is of interest in the wider context of medicinal drug discovery. This is the subject of ongoing work which will be described in a future paper.

As mentioned in the 'Introduction', SMA block copolymers of this kind have not been previously reported in the literature. They are, as far as we are aware, novel materials. Whether or not their properties show any advantages over SMA alone in membrane protein extraction or any other pharmaceutical application remains to be seen but, from a purely structural point of view, they are materials of considerable interest.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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REFERENCES

[1] J. M. Dörr, S. Scheidelaar, M. C. Koorengevel, J. J. Dominguez, M. Schäfer, C. A. van Walree, J. A. Killian, *Eur. Biophys. J.* **2016**, *45(1)*, 3.

[2] D. J. K. Swainsbury, S. Scheidelaar, N. Foster, R. van Grondelle, J. A. Killian, M. R. Jones, *Biochim. Biophys. Acta Biomembr*. **2017**, *1859(10)*, 2133.

[3] A. J. Smith, K. E. Wright, S. P. Muench, S. Schumann, A. Whitehouse, K. E. Porter, J. Colyer, *Sci. Rep.* **2019**, *9*, 16408.

[4] P. Angelisová, O. Ballek, J. Sýkora, O. Benada, T. Čajka, J. Pokorná, D. Pinkas, V. Hořejší, *Biochim. Biophys. Acta Biomembr*. **2019**, *1861(1)*, 130.

[5] P. S. Orekhov, M. E. Bozdaganyan, N. Voskoboynikova, A. Y. Mulkidjanian, H-J. Steinhoff, K. V. Shaitan, *Langmuir* **2019**, *35(10)*, 3748.

[6] R. C. Arenas, J. Klingler, C. Vargasa, S. Keller, *Nanoscale* **2016**, *8*, 15016.

[7] S. R. Tonge, B. J. Tighe, *Adv. Drug Deliv. Rev.* **2001**, *53(1)*, 109.

[8] S. R. Tonge, B.J. Tighe (Aston University), U.S. 6,436,905 B1, **2002**.

[9] T. J. Knowles, R. Finka, C. Smith, Y-P. Lin, T. Dafforn, M. Overduin, *J. Am. Chem. Soc.* **2009**, *131*, 7484.

[10] S. J. Hesketh, D. P. Klebl, A. J. Higgins, M. Thomsen, I. B. Pickles, F. Sobott, A. Sivaprasadarao, V. L. G. Postis, S. P. Muench, *Biochim. Biophys. Acta Biomembr*. **2020**, *1862(5)*, 183192.

[11] B. D. Harding, G. Dixit, K. M. Burridge, I. D. Sahu, C. Dabney-Smith, R. E. Edelmann, D. Konkolewicz, G. A. Lorigan, *Chem. Phys. Lipids* **2019**, *218*, 65.

[12] Z. Stroud, S. C. L. Hall, T.R. Dafforn, *Methods* **2018**, *147*, 106.

[13] B. Klumperman, *Polym. Chem.* **2010**, *1*, 558.

[14] N. Ohno, K. Nitta, S. Makino, S. Sugai, *J. Polym. Sci.: Polym. Phys. Edn.* **1973**, *11(3)*, 413.

[15] K. A. Morrison, A. Akram, A. Mathews, Z. A. Khan, J. H. Patel, C. Zhou, D. J. Hardy, C. Moore-Kelly, R. Patel, V. Odiba, T .J. Knowles, M. U. Javed, N. P. Chmel, T. R. Dafforn, A. J. Rothnie, *Biochem. J.* **2016**, *473*, 4349.

[16] N. L. Pollock, S. C. Lee, J. H. Patel, A. A. Gulamhussein, A. J. Rothnie, *Biochim. Biophys. Acta Biomembr*. **2018**, *1860*, 809.

[17] A. A. Gulamhussein, R. Uddin, B. J. Tighe, D. R. Poyner, A. J. Rothnie, *Biochim. Biophys. Acta Biomembr*. **2020**, *1862(7)*, 183281.

[18] M. L. Kelley, R. MacCallum, M. Sternberg, *J. Mol. Biol.* **2000**, *299(2)*, 499.

[19] C. Yi, N. Liu, J. Zheng, J. Jiang, X. Liu, *J. Colloid Interface Sci.* **2012**, *380(1)*, 90.

[20] C. D. Chow, *J. Appl. Polym. Sci.* **1976**, *20*, 1619.

[21] J. W. Nichol, S. T. Koshy, H. Bae, C. M. Hwang, S. Yamanlar, A. Khademhosseini, *Biomaterials* **2010**, *31(21)*, 5536.

[22] J. J. Domínguez Pardo, M. C. Koorengevel, N. Uwugiaren, J. Weijers, A. H. Kopf, H. Jahn, C. A. van Walree, M. J. van Steenbergen,, J. A. Killian, *Biophys. J.* **2018**, *115*, 129.

[23] M. C. Fiori, W. Zheng, E. Kamilar, G. Simiyu, G. A. Altenberg, H. Liang, *Sci. Rep.* **2020**, *10*, 9940.

[24] S. Scheidelaar, M. C. Koorengevel, C. A. van Walree, J. J. Dominguez, J. M. Dörr, J. A. Killian, *Biophs. J.* **2016**, *111*, 1974.

[25] H. J. Hutchins-Crawford, P. Ninjiaranai, M. J. Derry, R. Molloy, B. J. Tighe, P. D. Topham, *Polym. Chem.* **2021**, *12*, 4317.

[26] A. H. Kopf, M. C. Koorengevel, C. A. van Walree, T. R. Dafforn, J. A. Killian, *Chem. Phys. Lipids* **2019**, *218*, 85.

[27] S. C. Lee, T. J. Knowles, V. L. G. Postis, M. Jamshad, R. A. Parslow, Y. Lin, A. Goldman, P. Sridhar, M. Overduin, S. P. Muench, T. R. Dafforn, *Nat. Protoc.* **2016**, *11*, 1149.

[28] D. Braun, H. Cherdron, H. Ritter, *Polymer Synthesis: Theory and Practice*, Ch. 5, Springer, Berlin, **2001**.

[29] J. Brandrup, E. H. Immergut, E. A. Grulke (Eds.), *Polymer Handbook*, 4th Edn., II/257, Wiley-Interscience, New York, **1999**.

[30] A. Rudin, P. Choi, *The Elements of Polymer Science and Engineering*, 3rd Edn., Academic Press, San Diego, **2013**.

[31] L. S. Bark, N. S. Allen (Eds.), *Analysis of Polymer Systems*, Applied Science, London, **1982**.

[32] M. Wang, X. Zhu, S. Wang, L. Zhang, *Polymer* **1999**, *40*, 7387.

[33] G. Schoukens, P. Samyn, *Thermochim. Acta* **2014**, *580*, 28.

[34] M. Elisa, S.R.e Silva, E. R. Dutra, V. Mano, J. C. Machado, *Polym. Degrad. Stab.* **2000**, *67(3)*, 491.

[35] J. Brandrup, E. H. Immergut, E. A. Grulke (Eds.), *Polymer Handbook*, 4th Edn., II/249, Wiley-Interscience, New York, **1999**.

[36] Q. Zhao, J. Sun, Y. Lin, Q. Zhou, *React. Funct. Polym.* **2010**, *70(9)*, 602.