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# Influence of hydrophobic groups attached to amphipathic polymers on the solubilization of membrane proteins along with their lipids.

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**KEYWORDS:** *amphipols, extraction, lipids, membrane proteins, SMA copolymers, surfactants.*

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**ABSTRACT:** One of the biggest challenges in membrane protein (MP) research is to secure physiologically relevant structural and functional information after extracting MPs from their native membrane. Amphipathic polymers represent attractive alternatives to detergents for stabilizing MPs in aqueous solutions. The predominant polymers used in MP biochemistry and biophysics are amphipols (APols), one class of which, styrene maleic-acid (SMA) copolymers and their derivatives, has proven particularly efficient at MP extraction. In order to examine the relationship between the chemical structure of the polymers and their ability to extract MPs, we have developed two novel classes of APols bearing either cycloalkane or aryl (aromatic) rings, named CyclAPols and ArylAPols, respectively. The effect on solubilization of such parameters as the density of hydrophobic groups, the number of carbon atoms and their arrangement in the hydrophobic moieties, as well as the charge density of the polymers was evaluated. The membrane-solubilizing efficiency of the SMAs, CyclAPols and ArylAPols was compared using as models *i*) two MPs, BmrA and a GFP-fused version of LacY, overexpressed in the inner membrane of *Escherichia coli*, and *ii*) bacteriorhodopsin, naturally expressed in the purple membrane of *Halobacterium salinarum*. This analysis shows that, as compared to SMAs, the novel APols feature an improved efficiency at extracting MPs while preserving native protein-lipid interactions.

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Integral membrane proteins (MPs) play an important role in cellular processes, and represent key targets for therapeutic drugs<sup>1</sup>. Naturally embedded in the lipid bilayer of biological membranes, MPs extensively interact with lipids, a synergetic relationship that stabilizes the protein and often modulates its function<sup>2</sup>. Many structural and functional investigations require extracting MPs from their native environment. This critical step is traditionally achieved using small molecular detergents above their critical micelle concentration. However, detergents fall short of mimicking the lipid bilayer in terms of molecular composition, lateral pressure and thickness<sup>3,4</sup>, leading to the design of mild detergents with improved properties<sup>5</sup>. A promising alternative to detergents is provided by macromolecular amphiphiles that can stabilize MP/lipid assemblies as water-soluble particles. The use of amphipathic polymers in MP biochemistry and biophysics has been introduced more than two decades ago with the concept of amphipols (APols)<sup>6–8</sup>. The impact of amphipathic polymers in structural biology is now significant thanks to the recent breakthrough of single-particle electron cryo-microscopy (cryo-EM)<sup>9</sup>.

Because the efficiency of APols at dispersing the membrane components was shown to be lower than that of detergents, APols were originally used to stabilize MPs after solubilizing and purifying them with conventional detergents<sup>8,10</sup>. Only recently have amphipathic polymers been used as a means of extracting MPs directly from membranes, an appli-

cation primarily initiated with the use of styrene maleic acid (SMA) copolymers<sup>11–14</sup>. SMAs of relatively small average molecular mass ( $\bar{M}_w \approx 7,500-9,500 \text{ g}\cdot\text{mol}^{-1}$ ;  $\bar{M}_n \approx 3,000-3,800 \text{ g}\cdot\text{mol}^{-1}$ ) and with a styrene to maleic acid ratio of either 2:1 or 3:1 are most frequently used to this end<sup>15,16</sup>. SMAs have been used for studying a wide range of MPs<sup>17,18</sup>. However, they present a set of limitations that has prompted the development of other macromolecular amphiphiles, including SMA derivatives<sup>19</sup>. Diisobutylene maleic acid (DIBMA) copolymers were shown to extract MPs<sup>20</sup>, but have been reported to provide lower protein yields of reduced purity as compared to SMAs<sup>21</sup>. Polymethacrylate copolymers<sup>22</sup> and poly(acrylic acid-co-styrene) (AASTY) copolymers<sup>23</sup> represent interesting alternatives, even if the applicability of the latter to cryo-EM is currently limited to low resolution data<sup>23</sup>. Poly(acrylic acid) (PAA) represents also an interesting polymer's backbone, which was modified with octyl and isopropyl side chains to design the most characterized APol to-date, named A8-35 (ref.<sup>24</sup>). The efficiency of A8-35 at extracting MPs varies, however, from one MP and from one membrane to another. Recently, we designed and tested PAA-based APols harboring cycloalkane side chains. The presence of cyclic side groups dramatically improved protein extraction efficiency from lipid membranes, while keeping the MPs in a native conformation<sup>25</sup>. As a result, cycle-containing APols may be interesting alternatives to SMAs.

Here, we report on membrane-solubilizing properties of a series of PAA polymers grafted with cycle-containing hydrophobic side groups. The chemical structures of the polymers were modulated by varying *i*) the nature of the cycles (saturated vs. aromatic), *ii*) the density of the hydrophobic groups grafted along the PAA's backbone, *iii*) the number of carbon atoms forming the hydrophobic moieties, *iv*) the molecular arrangement of carbon atoms within the hydrophobic groups (comparison of constitutional isomers), and finally *v*) the density of charges. The copolymers were grouped into two families according to the nature of the cycles: those with aromatic (aryl) rings (ArylAPols) and those with cycloalkane groups (CyclAPols).

The capacity of this library of PAA-based co-polymers to extract MPs directly from membranes was explored using two model membranes: the purple membrane of *Halobacterium salinarum*, known for its resistance to solubilization by polymers, and the membrane of *Escherichia coli*, which is one of the most widely used host cells for producing recombinant MPs<sup>26,27</sup>. The target MPs used to assess the efficiency of polymers at extracting MPs were bacteriorhodopsin (BR) in the case of the purple membrane, and, in the case of *E. coli* membrane, two overexpressed MPs, the multidrug ATP-binding cassette (ABC) transporter BmrA and a GFP-fused version of the lactose permease LacY (LacY-GFP). This subset of MPs, which differ in terms of size, number of transmembrane  $\alpha$ -helices, oligomeric state, and function, was used to explore the generality of the properties of the polymers tested in this study. The most promising polymers in terms of yield of extracted MPs were used to purify BmrA. The co-purified lipids were characterized by reversed-phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Native lipids were found to be extensively retained by the target MP as compared to extraction performed with detergent *n*-dodecyl  $\beta$ -D-maltoside (DDM).

## Experimental section

**Chemicals.** SMA (2:1) and SMA (3:1) were obtained from ProFoldin (SMA21S) and Polyscope (Xiran SL25010 P20), respectively. DIBMA, DDM, and octylthioglucoside (OTG) were from Anatrace. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was from Avanti Polar Lipids.

**Synthesis of polymers.** ArylAPols and CyclAPols were obtained after a single step of PAA modification with cyclic, hydrophobic groups as previously reported<sup>25</sup>. Briefly, the formation of an amide bond between the free amine group of aromatic or cycloalkane moieties with one of the carboxylate functions of the PAA ( $\bar{M}_w \approx 5.5$  kDa; Acros) was performed in *N*-methylpyrrolidone (NMP; SDS) in the presence of dicyclohexylcarbodiimide (DCC; Sigma). For ArylAPols and CyclAPols bearing also isopropylamine groups, a second step of PAA modification was performed in NMP in the presence of 1-hydroxybenzotriazole (HOBt)/DCC as reported for the standard chemical route used for the preparation of A8-35 (ref.<sup>24</sup>). In each case, the resulting polymer was purified by four cycles of precipitation/solubilization in water at acidic and basic pH, respectively. The final polymer solution was adjusted at pH 8-9, dialyzed against deionized water and freeze-dried. The degrees of grafting of side groups were determined by <sup>13</sup>C and <sup>1</sup>H NMR analysis of polymers, and by potentiometric titration of polymers. (For more details see SI).

## Solubilization of membranes upon addition of polymers.

The membrane suspensions used in this study were isolated from the archaeon *H. salinarum* and the bacterium *E. coli* (see SI). The solubilization of the *H. salinarum* purple membrane enriched with DMPC was performed at room temperature for 24h, and the solubilized BR was quantified by absorbance at 554 nm as previously described<sup>11,25</sup>. For the solubilization of *E. coli* membranes, the concentration of MPs in each membrane preparation was first adjusted to 2 g·L<sup>-1</sup> and the concentration of added polymers was varying from 0.1 to 2.0%, corresponding to a total MP/polymer (w/w) ratio of 1:0.5 to 1:10. After 2h incubation at 4°C, the samples were centrifugated (30 min. at 200,000 × g). The supernatants and pellets were loaded on 12%-acrylamide gels. After migration, the gels were either stained with Coomassie blue or visualized with a Typhoon TLA 9500 (GE Healthcare) to detect fluorescence of LacY-GFP within the gel. The fluorescence of the bands was quantified using Image J software. (For more details see SI).

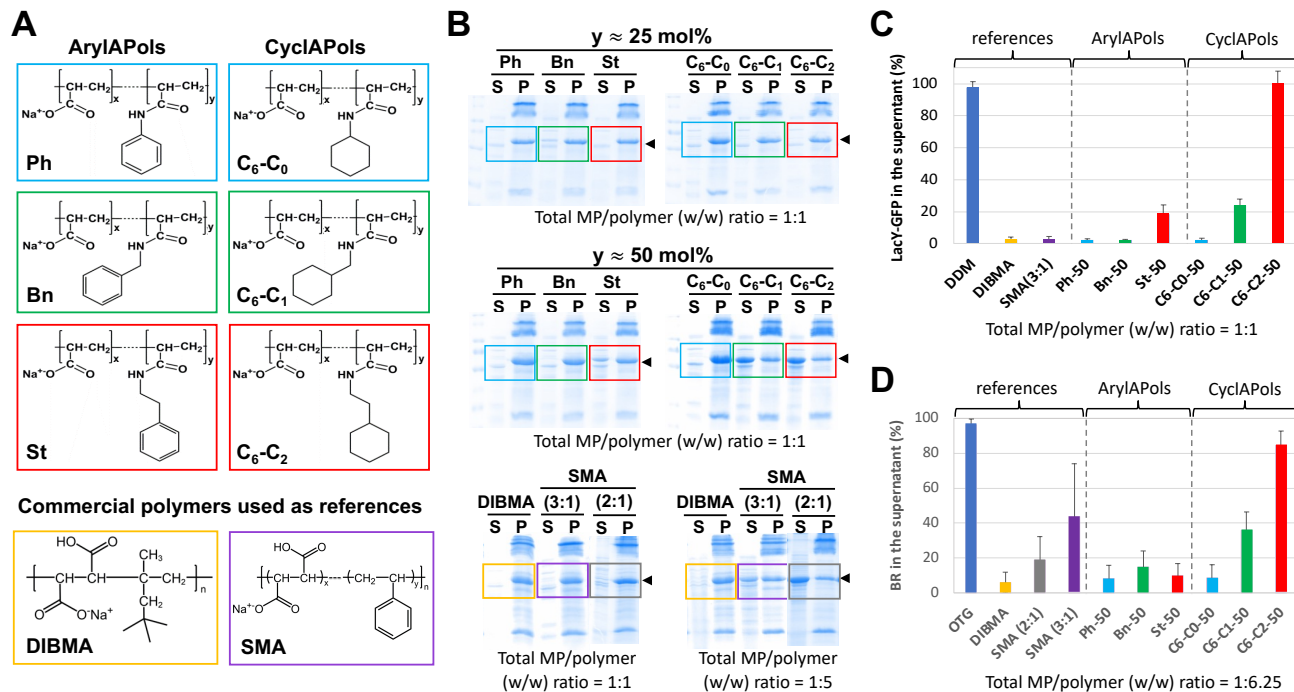
**BmrA purification and lipid analysis.** Polymer-solubilized BmrA was purified by affinity with an Ni<sup>2+</sup>-NTA resin, pre-equilibrated with buffer containing 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. Elution was performed with buffer supplemented with 300 mM imidazole. Fractions containing BmrA were dialyzed using 10,000 kDa cut-off dialysis cassette (Thermo Scientific). For DDM-solubilized BmrA, the same procedure was performed except that all the purification buffers were supplemented with 0.05% DDM.

Co-purified lipids (3 independent replicas) were extracted as reported in ref<sup>28</sup> and freeze-dried. Chloroform-resuspended phospholipids were injected in a mass spectrometer equipped with a dual detection system consisting of a Corona-CAD Ultra detector and an LTQ-Orbitrap Velos Pro MS detector (Thermo Scientific). Each lipid species, separated by their polar headgroup, was quantified with Corona-CAD detection based on a calibration curve of commercial phospholipids of known concentrations. Identification of phospholipids species was performed with high-resolution electrospray ionization (ESI) negative mode MS (full scan) and MS<sup>n</sup> fragmentations. Data analysis was performed using the Thermo Scientific XCalibur processing software.

## Results and Discussion

### Synthesis and chemical parameters of polymers into play.

Two families of copolymers resulting from the hydrophobic modification of a commercial PAA precursor were produced. The first family, named ArylAPol, gathers PAA modified with aromatic cycles, such as phenylamine (Ph), benzylamine (Bz) and styrenylamine (St) moieties. The second family, named CyclAPol, comprises PAA modified with cycloalkane moieties such as cyclohexylamine (C<sub>6</sub>-C<sub>0</sub>), cyclohexylmethylamine (C<sub>6</sub>-C<sub>1</sub>), and cyclohexylethylamine (C<sub>6</sub>-C<sub>2</sub>) (Fig. 1A). For both families of ArylAPols and CyclAPols, the number of carbon atoms in the side groups was either 6, 7 or 8, which enabled us to modulate the molecular volume and hydrophobicity of the grafted moieties. For ArylAPols, the aromatic ring was invariably formed by 6 carbon atoms, and a short (methyl or ethyl) substituent was used either as a spacer between the cycle and the polymer's backbone (Fig. 1A), or a *para* end group (Fig. 2A). In the case of CyclAPols, both short spacers and variation of the size of the cycle were used to produce a set of polymers



**Figure 1. Comparison of the efficiency of polymers to extract MPs directly from membranes according to the chemical structure of hydrophobic side groups and their density along the polymer's backbone.** (A) Chemical structures of ArylAPols (left) and CyclAPols (right). (B) Extraction of BmrA from *E. coli* membrane by polymers modified with either (*top*) 25 mol% or (*middle*) 50 mol% of hydrophobic groups. The total MP/polymer mass ratio used was 1:1, corresponding to a final concentration of polymer of 0.2%. (*Bottom*) Extraction of BmrA using DIBMA, SMA (2:1) and SMA (3:1) at 1:1 and 1:5 total MP/polymer mass ratios. (C) Quantification of LacY-GFP extracted from *E. coli* membrane and (D) BR extracted from the DMPC-fused purple membrane of *H. salinarum* by polymers modified with 50 mol% of side groups. A color code was used to compare more easily the efficiency of polymers at extracting MPs according to the number of carbon atoms present in the hydrophobic groups (blue, green, and red boxes stand for 6, 7 and 8 carbon atoms, respectively).

with a variety of structural features (Fig. 1A and 2A).

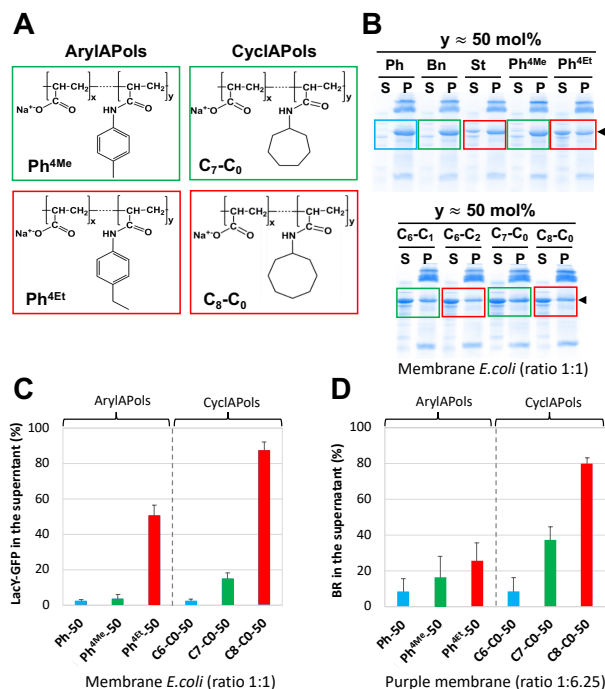
The degree of grafting of polymers was also considered using PAA modified with either 25 or 50 mol% of hydrophobic groups (within the experimental errors; see Table S1). As a result, the charge density of polymers was equal to either 75 or 50 mol%, respectively. Charge density can be modulated without change of the percentage of added cyclic groups *via* introduction of isopropyl grafts. To this end, isopropyl moieties were grafted along the polymer's backbone over a second step of PAA modification (Fig. 3A). For a few number of polymers, the charge density was accordingly lowered from 50 to 35 mol% of sodium carboxylates (*i.e.* to reach the same degree of free carboxylate groups as the standard APol A8-35). All the polymers were soluble in water at a final concentration of 10% (100 g·L<sup>-1</sup>).

**Solubilization by polymers of biological membranes.** The solubilization of two *E. coli* membrane preparations, containing either BmrA or LacY-GFP, was implemented under the same conditions to compare the relative efficiency of polymers, namely a membrane suspension diluted at a total MP concentration of 2 g·L<sup>-1</sup> supplemented with different concentrations of polymers, and incubation for 2 hours at 4°C. The total MP/polymer mass ratio of 1:1 was chosen to be discussed hereafter based on the following considerations: using higher concentrations of polymer may result in higher extraction/solubilization yields (see Fig. S1), but an excess of polymer would limit the selectivity of extraction (as with detergents) and make most of polymers comparable. As the main goal was to minimize the amount of polymer needed at

the extraction step, comments below will focus on extraction conditions for which not all polymers reach maximal extraction, best suited for comparison. To that regard, modifying polymer concentrations to have MP/polymer ratios ranging between 1:0.5 and 1:10 (see data in SI) did not significantly change the relative efficiencies of representative polymers. Data obtained with a MP/polymer mass ratio of 1:1 (corresponding to a final polymer concentration of 0.2%) are accordingly representative of the ranking of relative extraction capacities of polymers as a function of the diversity of structural parameters. Considering that hydrophobicity plays a predominant role, the size and hydrophobicity of side groups, as well as the degree of modifications of the macromolecules, will be the first parameters to be commented.

**1. Impact of the degree of grafting.** The solubilization of *E. coli* membrane containing BmrA was achieved in parallel with ArylAPols and CyclAPols modified with a density of cyclic, hydrophobic groups of either 25 or 50 mol%. Using 25mol%-modified ArylAPols, BmrA was essentially present in the pellets of all the samples (irrespective of the concentration of polymers used from 0.2 to 2.0%; Fig. 1B and S1A, *top*). In contrast, with a degree of modification of 50 mol%, ArylAPols (in particular the *St* one) were able to extract a significant fraction of BmrA at the highest concentration of 2.0% (Fig. S1A, *bottom right*). Their extraction efficiency was considerably lower when concentrations were 2- or 10-times lower (Fig. 1B and S1A, *bottom middle*).

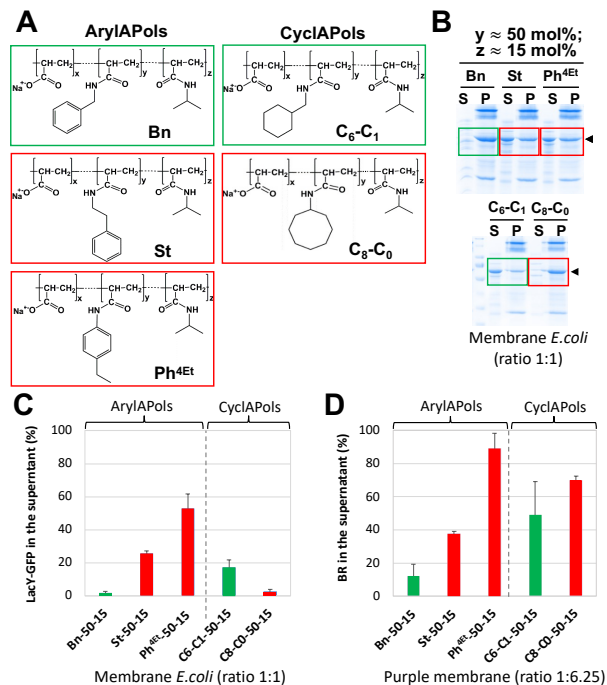
For CyclAPols modified with 25 mol% of hydrophobic groups, a small fraction of BmrA was present in the superna-



**Figure 2. Impact of the chemical structure of hydrophobic side groups grafted at 50 mol% along the polymer's backbone on the efficiency of polymers to extract MPs.** (A) Chemical structures of polymers. (B) Direct extraction of BmrA and (C) LacY-GFP from *E. coli* membrane upon addition of polymers. (D) Quantification of BR extracted from the DMPC-fused purple membrane of *H. salinarum* by polymers. (Blue, green, and red boxes stand for 6, 7 and 8 carbon atoms, respectively).

tant in only one condition: C<sub>6</sub>-C<sub>2</sub>-25 at 2.0% (Fig. S1B, *top right*). However, at lower concentration of C<sub>6</sub>-C<sub>2</sub>-25, BmrA was found in the pellet. In contrast, with 50 mol% of hydrophobic groups (specifically C<sub>6</sub>-C<sub>2</sub>-50 and C<sub>6</sub>-C<sub>1</sub>-50), a significant efficiency to extract BmrA was observed at much lower concentrations of polymers (Fig. 1B and S1B, *bottom*). This indicates that polymers with a higher hydrophobicity (50 vs. 25 mol%) solubilized the membranes better. Similarly, the results of extraction of BR from the DMPC-fused purple membrane obtained with SMA (2:1) and (3:1), which differ only in their density of styrene vs. maleic acid groups, indicated that a higher fraction of hydrophobic groups may enhance the ability of the polymers to extract MPs (Fig. 1D). This observation can be explained by a stronger interaction of copolymers with biological membranes when the density of hydrophobic side groups is high, as previously shown with liposomes<sup>29</sup>.

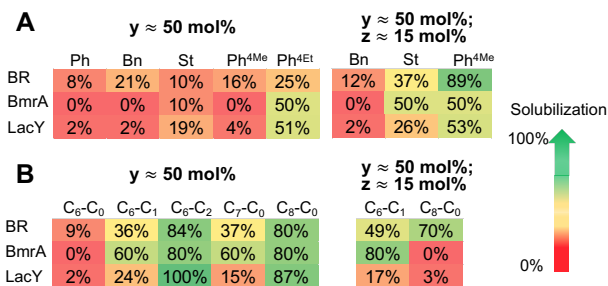
**2. Impact of the (total) number of carbon atoms in side groups.** Next, we compared the efficiencies of polymers having the same degree of modification of 50 mol% and used at the same concentration. The hydrophilic-lipophilic balance of a molecule primarily increases with the number of aliphatic and/or aromatic carbon. In this line, increasing the total number of carbon atoms per side group was observed to enhance significantly the yield of MP solubilization. Namely for ArylAPols, BmrA was collected in the supernatant in the presence of St-50 at either 0.2% or 1% (side groups of 8 carbon atoms), whereas it was essentially in the pellet with ArylAPols containing side groups of 7 or 6 carbon atoms



**Figure 3. Impact of the charge density along the polymer's backbone on the efficiency of polymers to extract MPs.** (A) Chemical structures of polymers. (B) Direct extraction of BmrA and (C) LacY-GFP from *E. coli* membrane by polymers. (D) Quantification of polymer-extracted BR from the DMPC-fused purple membrane of *H. salinarum*.

(Bn-50 and Ph-50; Fig. 1B and S1A, *bottom middle*). The better efficiency of St-50 was confirmed with LacY-GFP (Fig. 1C). The difference was less pronounced on the purple membrane, but in this case error bars were comparable to the average % MP extracted, suggesting a poor efficiency with all polymers (Fig. 1D). Accordingly, polymers could be ranked as Ph-50 < Bn-50 < St-50 based on global efficiency. Similarly, an increase of the number of carbon atoms in the *para* position resulted in higher extraction of MPs and the global ranking was Ph-50 < Ph<sup>4Me</sup>-50 < Ph<sup>4Et</sup>-50 (Fig. 2). With CyclAPols, extraction of BmrA was observed with C<sub>6</sub>-C<sub>1</sub>-50 and C<sub>6</sub>-C<sub>2</sub>-50 but not upon addition of C<sub>6</sub>-C<sub>0</sub>-50 (Fig. 1B). Based on the quantity of BmrA left in the pellets, the capacity of C<sub>6</sub>-C<sub>2</sub>-50 to extract BmrA appears higher than that of C<sub>6</sub>-C<sub>1</sub>-50. The same result was obtained with both LacY-GFP and BR, showing a linear enhancement of the efficiency of CyclAPols to extract MPs (C<sub>6</sub>-C<sub>0</sub>-50 < C<sub>6</sub>-C<sub>1</sub>-50 < C<sub>6</sub>-C<sub>2</sub>-50, Fig. 1C and 1D). Similarly, an increase of the size of the aliphatic ring provided a similar enhancement of extraction with the ranking of C<sub>6</sub>-C<sub>0</sub>-50 < C<sub>7</sub>-C<sub>0</sub>-50 < C<sub>8</sub>-C<sub>0</sub>-50 (Fig. 2).

Altogether, these results suggest that side groups with typically 7 or 8-carbon atoms are required to achieve a significant extraction yield. The aromatic or aliphatic nature and constitutional isomers certainly affect the yield of solubilization, although to a lesser extent than the total number of atoms (*i.e.* the global size of hydrophobic side groups). Note that CyclAPols modified with cyclic, hydrophobic groups comprising more than 8 carbon atoms were capable to extract MPs but without showing a significant improvement as compared to C<sub>6</sub>-C<sub>2</sub>-50 and C<sub>8</sub>-C<sub>0</sub>-50 (not shown). This apparent threshold in the extraction efficiency of polymers may



**Figure 4. Overview of the efficiency of ArylAols (A) and CyclAPols (B) to extract MPs directly from the membrane.** All the data presented in Figures 1, 2 and 3 were combined with a color code that goes from red (no extraction) to green (complete extraction).

be due to the lower solubility (as observed in EtOH/water) and/or higher propensity of self-aggregation of polymers carrying too many hydrophobic groups.

**3. Impact of the aromatic nature of the cycle.** To evaluate the impact of the nature of the cycle (saturated vs. unsaturated), ArylAPols and CyclAPols with the same degree of hydrophobic modifications (either 25 or 50 mol%) and with hydrophobic moieties comprising 6-carbon cycles and the same total number of carbon atoms (either 6, 7 or 8 atoms) were compared. Under the same experimental conditions (here with 2.0% polymer), St-25 appeared markedly less effective compared to C<sub>6</sub>-C<sub>2</sub>-25 (see, for instance, the presence or not of BmrA in the supernatants in Fig. S1A and S1B). The comparison of other ArylAPols and CyclAPols having the same spacer between the cycle and the polymer backbone (and modified at 50 mol%) showed also a higher extraction with aliphatic polymers compared to aromatic ones (here compare Bn-50 vs. C<sub>6</sub>-C<sub>1</sub>-50, and St-50 vs. C<sub>6</sub>-C<sub>2</sub>-50; Fig. 1B). The same result was obtained with the two other MPs (Fig. 1C and 1D). Therefore, copolymers modified with saturated cycles showed a markedly higher solubilization efficiency as compared to similar polymers modified with aromatic cycles. The flexibility of saturated cycles may play a role in perturbing the organization of lipids after insertion within the membrane, as opposed to flat aromatic rings that can form dense assemblies without perturbing lipid orientation<sup>30</sup>. Also, saturated compounds are more hydrophobic and can insert deeper into the membrane than unsaturated cycles, which present a  $\pi$ -electronic structure associated with quadrupolar moments<sup>31</sup>.

**4. Impact of constitutional isomers.** As steric hindrance with the polymer chain and flexibility of spacers between the cycles and the polymer backbone may affect interactions with MPs and lipids, we compare here side groups that differ only in the position of carbon atoms. For ArylAPols, this effect is expected to depend on the position of methyl or ethyl substituents. The amount of BmrA and LacY-GFP extracted by addition of Ph<sup>4Me</sup>-50 compared to Bn-50 was unchanged, and remained essentially very low (< 15-20%) in both cases (Fig. 2B and 2C). In contrast, the percentage of extracted MPs was significantly higher with Ph<sup>4Et</sup>-50 compared to St-50 (Fig. 2B and 2C). Using BR as target MP, the improved ability of Ph<sup>4Et</sup>-50 to extract MPs was confirmed (Fig. 2D), suggesting that ethyl positioned as a dangling end

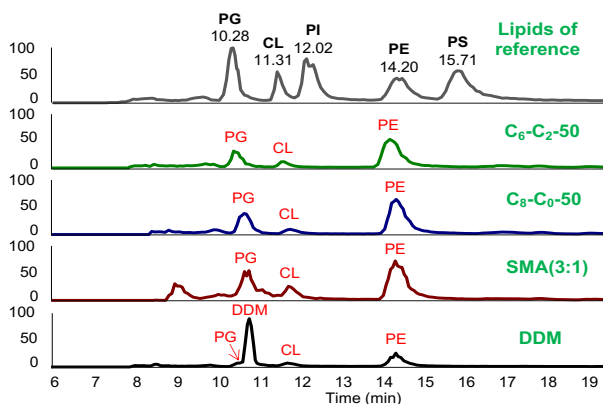
enhanced extraction compared to an ethyl spacer. One can speculate that the flexibility of the ethyl end group likely contributes to perturbing the lipids.

In the case of CyclAPols, we compared side groups having a short spacer with fully cyclic ones having the same total number of carbon atoms. The amounts of extracted BmrA by solubilization with C<sub>6</sub>-C<sub>1</sub>-50 and C<sub>7</sub>-C<sub>0</sub>-50 in one hand, and by C<sub>6</sub>-C<sub>2</sub>-50 and C<sub>8</sub>-C<sub>0</sub>-50 in the other hand, revealed no significant differences (Fig. 2B). The same conclusion was drawn using LacY-GFP (Fig. 2C) or BR (Fig. 2D). Therefore, CyclAPols whose side groups differed only by the arrangement of one carbon atom in different isomers extracted MPs to comparable extents. As with the aromatic groups (Ph<sup>4Me</sup>-50 compared to Bn-50), this suggests that a short (specifically one-carbon atom) spacer does not contribute significantly to steric or flexibility effects. For an identical number of carbon atoms, a flexible ethyl end was shown to increase the efficiency of aromatic grafts better than ethyl spacers, whereas the addition of a methyl group in various positions (as a spacer or a *para* end group) did not significantly affect extraction yields.

**5. Impact of the charge density.** Knowing that the ionization state of polymers affects their hydrophobic/hydrophilic balance, a few number of ArylAPols and CyclAPols with a lower charge density have been tested for their capacity to extract MPs. To reach the same degree of free carboxylate groups than that of A8-35, that is 35 mol%, isopropyl moieties were grafted at 15 mol% over a second step of PAA modification (Fig. 3A). For the resulting ArylAPols and CyclAPols, no real improvement was observed as regards to their capacity to extract BmrA (Fig. 3B), nor LacY-GFP (Fig. 3C). In the case of C<sub>8</sub>-C<sub>0</sub>-50-15, lowering the charge density from 50 to 35 mol% was detrimental as BmrA was not present in the supernatant anymore. However, for ArylAPol Ph<sup>4Et</sup>-50, reducing the charge density significantly improved MP extraction (cf. Ph<sup>4Et</sup>-50 vs. Ph<sup>4Et</sup>-50-15; Fig. 3D).

As an outcome of this study, several ArylAPols and CyclAPols have emerged as providing an improved extraction of MPs at low concentrations of polymer (that is 0.2%) as compared to SMA (2:1), SMA (3:1), and DIBMA copolymers. Of all the copolymers tested, CyclAPols C<sub>6</sub>-C<sub>2</sub>-50 and C<sub>8</sub>-C<sub>0</sub>-50 are the polymers that showed the greatest ability to extract MPs, regardless of the nature of the target MP (Fig. 4). Like SMA copolymers, ArylAPols absorb in the UV range due to the presence of unsaturated cycles. CyclAPols are not only more efficient extractants, but also UV-transparent at the wavelengths of protein absorption. This property makes CyclAPols more convenient for MP biochemistry.

**Identification and quantification of co-purified lipids in CyclAPol-extracted BmrA samples.** A recent study has reported that phospholipids were present in samples of Acrb solubilized and purified in CyclAPols<sup>32</sup>. However, the lipid analysis, performed by TLC, did not permit to accurately identify and quantify lipid species. Here, after solubilization of *E. coli* membrane with CyclAPols C<sub>6</sub>-C<sub>2</sub>-50 and C<sub>8</sub>-C<sub>0</sub>-50, BmrA was purified upon a single step of affinity chromatography. The purity of BmrA in the elution fractions was checked by SDS-PAGE (Fig. S2). The yield of purified



**Figure 5. Mass spectrometry analysis of phospholipids co-purified with BmrA in different surfactants.** Chromatographic profiles of phospholipids obtained with an ESI-MS detector. Lipids of reference is a mix of phosphatidyl glycerol (PG), cardiolipin (CL), phosphatidyl inositol (PI), phosphatidyl ethanolamine (PE), and phosphatidyl serine (PS). DDM was assigned based on its  $m/z$  value. For experimental details see SI.

BmrA with CyclAPols was slightly lower but comparable to that obtained with DDM, whereas it was much better than that obtained with SMA (3:1). Next, from the elution fractions containing BmrA, the co-purified phospholipids were extracted and characterized by liquid chromatography-mass spectrometry (LC-MS/MS). Lipid assignment was performed on retention time compared to lipids of reference (Fig. 5),  $m/z$  value (Fig. S3 and S4), and lipid fragmentation.

The total lipid extract of our *E. coli* membrane preparation contains the three major phospholipid classes, namely phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG) and cardiolipin (CL), in the following (w/w) proportions: 68.7%, 19.3% and 12.0%, respectively, which is in agreement with published data<sup>33</sup>. In each CyclAPol-purified BmrA sample, these three lipid species were found (Fig. 5). Under the same experimental conditions, these lipid species also co-purified with BmrA upon solubilization with SMA (3:1) or with DDM. Lipid fragmentations showed a majority of PE, PG and CL with acyl chain lengths of 12 to 19 carbon atoms and 0 to 1 unsaturated bound. This analysis reveals a similar lipid distribution for all samples, indicating no specific selection of lipids with one amphipathic environment.

Quantification of phospholipids in each sample revealed, however, differences between surfactants in terms of the total number of co-extracted lipids (Table S2). Unsurprisingly, the lowest number of phospholipids was co-extracted with DDM-solubilized BmrA. Considering the error bars, the number of co-extracted lipids obtained under this condition is consistent with that reported in a previous study ( $\sim 12$  lipids per dimer of BmrA according to ref.<sup>34</sup> vs.  $\sim 7 \pm 5$  lipids in the present study). The total number of lipids extracted along BmrA with SMA (3:1) corresponds to  $\sim 11$  lipid molecules, whereas with CyclAPols this number was  $\sim 14$  lipids (Table S2). Altogether, these results indicate that CyclAPols are mild surfactants that can preserve protein/lipid interactions better than DDM and to the same extent as SMA, if not better.

From the literature, previous studies have reported an estimation of the number of lipids present in SMALPs large enough to cover the transmembrane domain of MPs. For

instance, analysis of purified SMALPs of the bacterial reaction center or aquaporin Z revealed a content of around 150 and 140 lipids, respectively<sup>35,36</sup>. In our study, the number of lipids co-purified with BmrA, whether in SMA or in CyclAPol, is too small to form a complete layer of lipids covering the transmembrane surface of the protein. This variability in the protein to lipid ratios of SMALPs may arise from technical differences in sample preparation and/or analysis, emphasizing that more data are needed. In our experiments, we cannot completely exclude that a fraction of BmrA-bound lipid molecules be lost upon lipid extraction and their actual number be underestimated. Given that APols are compatible with native MS<sup>37,38</sup>, this approach would deserve to be tested to access to the number of MP-bound lipids without resorting to lipid extraction.

To sum up, the development of mild surfactants able to efficiently extract MPs from their native membrane environment while retaining MP-bound lipids is of paramount importance. Yet, the combination of these two properties is somewhat antinomic, because the surfactant must be able, on the one hand, to disperse membrane components and, on the other hand, to preserve as many protein-lipid interactions as possible. SMA co-polymers are much less efficient than detergents at solubilizing MPs, but better at retaining native lipids<sup>36,39,40</sup>, which makes them very interesting mild surfactants. Efforts nevertheless are still required to improve extraction yields, which is of importance when levels of MP expression are low. The goal of the present study was to develop a novel library of solubilizing polymers and use it to explore the relationship between their chemical structure and their ability to extract MPs without delipidating them.

A systematic and quantitative analysis of MP extraction was recently undertaken with SMA derivatives<sup>41</sup>, but was limited to polymers carrying aromatic cycles. In our case, two sets of polymers bearing either aromatic (ArylAPols) or cycloalkane groups (CyclAPols) were screened for their ability to extract MPs. Whenever a significant fraction of MPs could be solubilized, the three MPs tested, which feature different sizes, oligomeric states and functions, reported similar effects of variations of the chemical structure of the polymers. The major contribution that improved efficiency was the global hydrophobicity of the polymers, which was adjusted by playing on both the degree of modification and the number of carbon atoms in the side groups. Replacing unsaturated with saturated cycles is also a parameter that modulates the capacity of the polymers to efficiently extract MPs from membranes. Therefore, the hydrophilic-lipophilic balance appears to be important, as it has been shown in the design of new detergents for MP studies<sup>42</sup>.

**Conclusion.** Short, flexible amphipathic polymers have emerged as innovative tools for MP extraction and study. However, copolymers tend to be much less efficient at extracting agents than detergents. Developing polymers with improved ability at solubilizing MPs while preserving protein-lipid interactions would be extremely beneficial.

In this study, we showed that modulating the density of cyclic, hydrophobic groups along the PAA's backbone, varying the number of carbon atoms in the hydrophobic groups and, finally, replacing unsaturated with saturated

cycles are all parameters that modulate the capacity of the polymers to efficiently extract MPs from membranes. Hydrophobicity can be further fine-tuned via small structural adjustments. Among the copolymers tested, two CyclAPols C<sub>6</sub>-C<sub>2</sub>-50 and C<sub>8</sub>-C<sub>0</sub>-50 have shown to be better than SMAs at extracting prokaryotic MPs, while retaining more lipids. Knowing that extraction efficiencies can vary between prokaryotic and eukaryotic MPs, additional work would be needed to confirm this property. The CyclAPols (now supplied by Cube Biotech) have recently been validated as appropriate amphipathic environments for determining high-resolution structures of MPs by cryo-EM<sup>32</sup>, which bodes well for their usefulness. One may add that APol A8-35 can be chemically modified with different types of functionalities, such as affinity tags<sup>43–45</sup> or fluorophores<sup>46</sup>, without affecting its MP-stabilizing properties. Functionalizing CyclAPols would similarly widen the scope of their applications. These polymers therefore have the prospect of providing biochemists and structural biologists with versatile tools for extraction, handling and studying MPs.

## ASSOCIATED CONTENT

### Supporting Information

<sup>1</sup>H and <sup>13</sup>C NMR spectra of ArylAPols and CyclAPols, and chemical composition. SDS-PAGE analysis of protein contents in the supernatants and pellets after *E. coli* membrane solubilization by polymers. SDS-PAGE analysis of BmrA samples after affinity purification. LC-MS/MS of lipid extracts and lipid quantification. The Supporting Information is available free of charge on the ACS Publications website.

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### Author Contributions

FG, AM, and MZ designed CyclAPols and ArylAPols. AM synthesized CyclAPols and ArylAPols with the help of FG. AM performed the purple membrane solubilization with the help of CLB. BM and MZ performed the solubilization of *E. coli* membranes. BM performed the purification of BmrA and the lipid extraction for quantification by LC-MS/MS with the help of BP and AS. All authors wrote the manuscript and have given approval to the final version. ‡A.M and B.M. contributed equally to this work.

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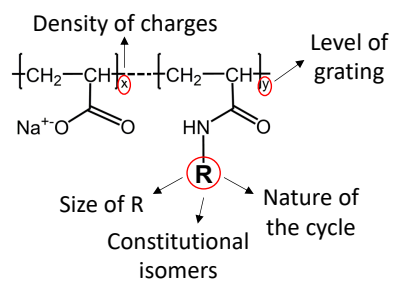


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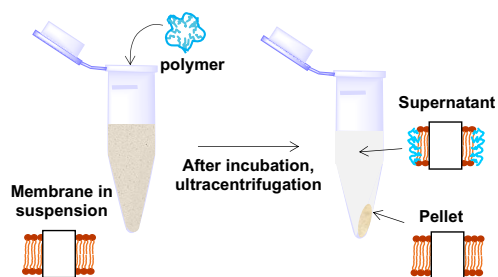
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## 1. Library of poly(acrylic acid) polymers bearing cycle-containing hydrophobic side groups

Modulated parameters:



## 2. Extraction of membrane protein-lipid complexes



## Supporting Information

# Influence of Hydrophobic Groups Attached to Amphipathic Polymers on the Solubilization of Membrane Proteins along with Their Lipids

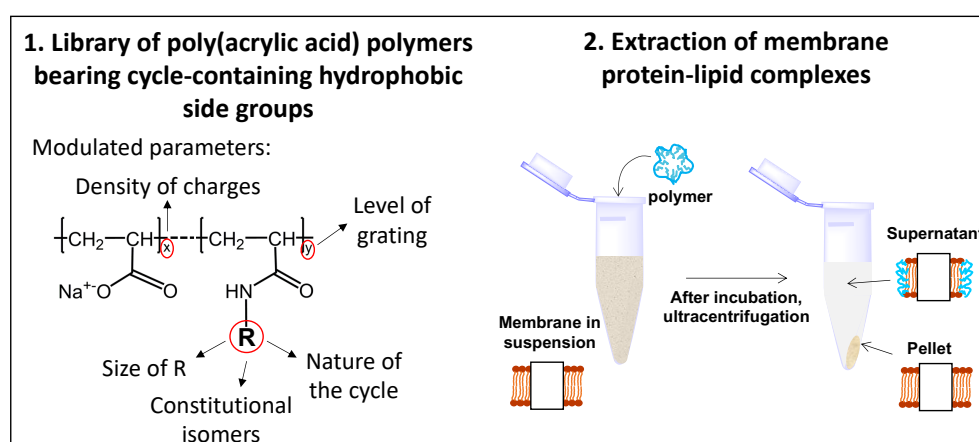
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## Materials and Methods:

### 1. Nomenclature of CyclAPols and ArylAPols

The nomenclature used for CyclAPols is as follows:  $C_nC_m-y-z$  with  $n$  the number of carbon atoms forming the cycle ( $n$  could be 6, 7 or 8),  $m$  the number of carbon atoms between the amide bond and the cycle ( $m$  could be 0, 1 or 2),  $y$  the grafting percentage of hydrophobic moieties ( $y$  could be 25% or 50%), and  $z$  the grafting percentage of isopropyl moieties ( $z$  could be 0% or 15%). For ArylAPols, the unsaturated cycle (with invariably 6 carbon atoms) and the number of carbon atoms between the amide bond and the ring are noted as follows: Ph- $y-z$  (Ph for phenyl), Bn- $y-z$  (Bn for benzyl), and St- $y-z$  (St for styrenyl),  $y$  remains the grafting percentage of hydrophobic moieties ( $y$  could be 25% or 50%), and  $z$  the grafting percentage of isopropyl groups ( $z$  could be 0% or 15%). With a methyl or ethyl group in the *para* position of the phenyl ring, the nomenclature becomes Ph<sup>4Met</sup>- $y$  and Ph<sup>4Et</sup>- $y$ , respectively.

### 2. Characterization of the chemical composition of CyclAPols and ArylAPols

The degree of grafting of side groups were determined by <sup>13</sup>C and <sup>1</sup>H NMR analysis of polymers dissolved at 100 mg·mL<sup>-1</sup> in deuterated methanol, and by potentiometric titration of polymers in a water/ethanol mixture (20:80, v/v). The percentages of unmodified carboxylic groups ( $x$ ), cyclic hydrophobic groups ( $y$ ) and isopropyl groups ( $z$ ), deduced from both <sup>13</sup>C and <sup>1</sup>H NMR analysis and pH-titration, were averaged. The number-average molar masses ( $\bar{M}_n$ ) of sodium salts of polymers were calculated with the average degree of polymerization ( $\bar{X}_n$ ) of the commercial PAA, which is close to ~35 as previously determined by size exclusion chromatography (SEC) analysis in organic solvent<sup>1</sup>, and the averaged percentage of each unit ( $x$ ,  $y$  and  $z$ ; Table S1).

### 3. Preparation of membrane suspensions

The purple membrane was purified from *H. salinarum* as previously reported<sup>2</sup>. The purple membrane was diluted at a concentration of BR of 0.5 g·L<sup>-1</sup> with buffer containing 20 mM sodium phosphate, 100 mM NaCl, pH 7.0. Large unilamellar vesicles of DMPC were mixed to the purple membrane at a BR:DMPC mass ratio of 1:5, as indicated in ref.<sup>3,4</sup>. The sample was sonicated in the dark for 30 min with a bath sonicator (VWR), and then stored at -80°C until usage.

The expression vectors encoding for BmrA and LacY-GFP (kindly provided from Jean-Michel Betton, Institut Pasteur, Paris) were used to transform *E. coli* C43 (DE3) strain and C41 (DE3) strain, respectively. Bacterial cells were grown at 37°C in Luria-Bertani broth supplemented with either ampicillin or kanamycin, depending on the plasmid. The protein expression was induced with 0.5 mM isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) for 4h before harvesting the cells by centrifugation (20 min at 3,500  $\times g$ ). The cells were broken with a Cell Disruptor (Constant Systems). Unbroken cells and cellular debris were removed by centrifugation (20 min at 10,000  $\times g$ ). The membranes were harvested from the supernatant by a high-speed centrifugation (1h at 100,000  $\times g$ ). The pellet containing the membranes was resuspended in Tris/NaCl buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). The concentration of total MPs in the membrane preparations was determined with a colorimetric assay (Pierce) before storage at -20°C.

#### 4. Solubilization of membranes upon addition of polymers

The solubilization of the DMPC-fused purple membrane was performed at a fixed BR/polymer mass ratio of 1:6.25. A control condition was performed by solubilizing the DMPC-fused purple membrane with 100 mM octylthioglucoside (OTG) detergent. The samples were incubated overnight at room temperature in the dark under shaking before centrifugation (20 min. at  $200,000 \times g$ ). The quantification of native BR in the supernatants was evaluated with the ratios of absorbance values at 554 nm measured before and after centrifugation.

The solubilization of *E. coli* membranes was performed in the presence of varying concentrations of polymers, pre-solubilized at  $100 \text{ g}\cdot\text{L}^{-1}$  (w/w) in deionized water, and with 1% DDM detergent as a control condition. After 2h incubation at  $4^\circ\text{C}$  of the *E. coli* membranes with polymers, the samples were centrifugated (30 min. at  $200,000 \times g$ ). The supernatants containing solubilized MPs were collected and the pellets containing insoluble material were resuspended in the same volume with the same Tris/NaCl buffer containing 5% (w/v) SDS. The supernatants and pellets were loaded on 12%-acrylamide gels. In the case of BmrA samples, the gels were stained with Quick Coomassie blue (NeoBiotech), whereas in the case of LacY-GFP samples, the gels were visualized with a Typhoon TLA 9500 (GE Healthcare) under the excitation and emission wavelengths of Alexa Fluor 488 (corresponding to 495 nm and 519 nm, respectively) to detect fluorescence of LacY-GFP within the gel. The fluorescence of the bands was quantified using Image J software. The fluorescence measured in each supernatant was normalized with the fluorescence quantified for the 1%-DDM supernatant, which was defined as the maximum of the solubilization.

#### 5. Extraction co-purified lipids for LC-MS/MS analysis

Co-purified lipids were extracted using a procedure adapted from Bligh and Dyer<sup>5</sup>. Briefly, chloroform and methanol were added to each BmrA sample purified in Tris/NaCl buffer to reach a final chloroform/methanol/water volume ratio of 1:2:0.8. Samples were stirred for 2 h at room temperature. Non-soluble material was sedimented by short centrifugation (5 min. at 14,000 rpm), after what the supernatant was collected and mixed volume to volume with chloroform/methanol 1:1 (v/v). After centrifugation (5 min. at 14,000 rpm), the lipid-containing organic phase was collected, dried under an argon stream and stored at  $-20^\circ\text{C}$  under inert atmosphere.

For LC-MS/MS analysis, extracted phospholipids were resuspended in chloroform containing an internal standard (absent in *E. coli* membrane that is phosphatidylcholine) at a known concentration to normalize the chromatograms. Retention time of lipids was calibrated using commercial PE, PG and CL standards. The method of lipid analysis was performed as reported in ref.<sup>6</sup>.

## References

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- (2) Oesterhelt, D.; Stoekenius, W. Rhodopsin-like Protein from the Purple Membrane of Halobacterium Halobium. *Nat New Biol* **1971**, *233* (39), 149–152.
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- (5) Bligh, E. G.; Dyer, W. J. A Rapid Method of Total Lipid Extraction and Purification. *Can J Biochem Physiol* **1959**, *37* (8), 911–917.
- (6) Moulin, M.; Solgadi, A.; Veksler, V.; Garnier, A.; Ventura-Clapier, R.; Chaminade, P. Sex-Specific Cardiac Cardiolipin Remodelling after Doxorubicin Treatment. *Biol Sex Differ* **2015**, *6*, 20.

**Additional tables:**

<b>ArylAPols</b>	CO <sub>2</sub> <sup>-</sup> (x <sub>1</sub> / x <sub>2</sub> , %)	Hydrophobic groups (y <sub>1</sub> / y <sub>2</sub> , %)	Isopropyl groups (z <sub>1</sub> / z <sub>2</sub> , %)	x (%)	y (%)	z (%)	$\bar{M}_n$ (kg.mol <sup>-1</sup> )	Yield (%)
Ph-25	78 / 75	22 / 25	0 / 0	76.5	23.5	0	3.73	42
Ph-50	56 / 49	44 / 51	0 / 0	52.5	47.5	0	4.17	76
Ph <sup>4Me</sup> -50	52 / 55	48 / 45	0 / 0	53.5	46.5	0	4.38	58
Ph <sup>4Et</sup> 50	51 / 55	49 / 45	0 / 0	53	47	0	4.62	42
Ph <sup>4Et</sup> -50-15	37 / 42	48 / 43	15 / 15	39.5	45.5	15	4.68	69
Bn-25	76 / 77	24 / 23	0 / 0	76.5	23.5	0	3.84	73
Bn-50	55 / 46	45 / 54	0 / 0	50.5	49.5	0	4.45	93
Bn-50-15	40 / 39	47 / 48	13 / 13	39.5	47.5	13	4.49	83
St-25	73 / 70	27 / 30	0 / 0	71.5	28.5	0	4.10	78
St-50	50 / 49	50 / 51	0 / 0	49.5	50.5	0	4.72	88
St-50-15	46 / 37	44 / 53	10 / 10	41.5	48.5	10	4.49	83

<b>CyclAPols</b>	CO <sub>2</sub> <sup>-</sup> (x <sub>1</sub> / x <sub>2</sub> , %)	Hydrophobic groups (y <sub>1</sub> / y <sub>2</sub> , %)	Isopropyl groups (z <sub>1</sub> / z <sub>2</sub> , %)	x (%)	y (%)	z (%)	$\bar{M}_n$ (kg.mol <sup>-1</sup> )	Yield (%)
C <sub>6</sub> -C <sub>0</sub> -25	72 / 67	28 / 33	0 / 0	69.5	30.5	0	3.92	44
C <sub>6</sub> -C <sub>0</sub> -50	48 / 44	52 / 56	0 / 0	46	54	0	4.41	66
C <sub>6</sub> -C <sub>1</sub> -25	74 / 67	26 / 33	0 / 0	70.5	29.5	0	3.04	82
C <sub>6</sub> -C <sub>1</sub> -50	52 / 47	48 / 53	0 / 0	49.5	50.9	0	4.58	76
C <sub>6</sub> -C <sub>1</sub> -50-15	37 / 37	50 / 50	13 / 13	37	50	13	4.65	80
C <sub>6</sub> -C <sub>2</sub> -25	78 / 73	22 / 27	0 / 0	75.5	24.5	0	4.04	74
C <sub>6</sub> -C <sub>2</sub> -50	47 / 46	53 / 54	0 / 0	46.5	53.5	0	4.92	98
C <sub>7</sub> -C <sub>0</sub> -50	53 / 46	47 / 54	0 / 0	49.5	50.5	0	4.58	74
C <sub>8</sub> -C <sub>0</sub> -50	52 / 46	48 / 54	0 / 0	49	51	0	4.84	96
C <sub>8</sub> -C <sub>0</sub> -50-15	44 / 40	46 / 50	10 / 10	42	48	10	4.77	40

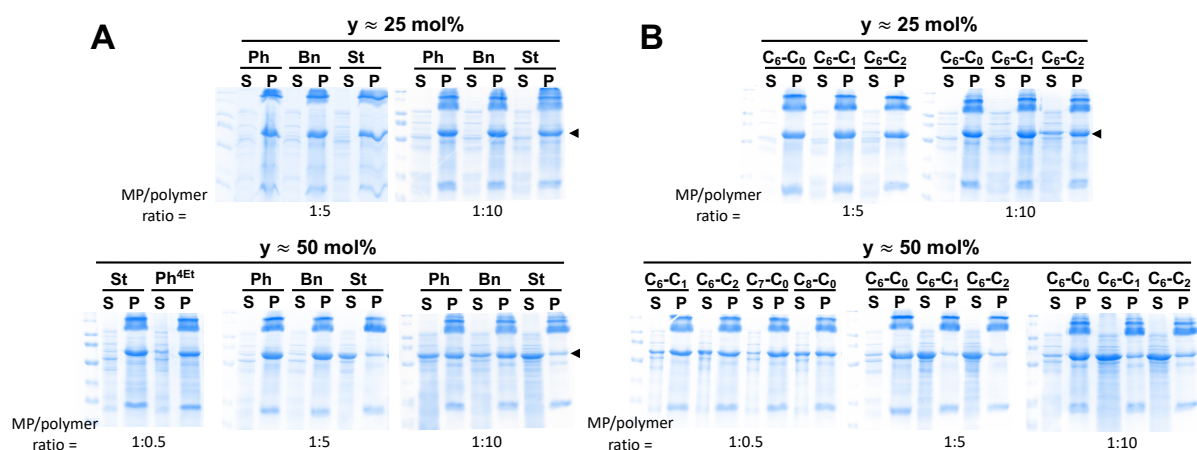
**Table S1. Chemical compositions and number-average molecular masses ( $\bar{M}_n$ ) of ArylAPols and CyclAPols synthesized in this study.** The percentages of grafting and free carboxylates of each polymer were deduced from an average of <sup>1</sup>H and <sup>13</sup>C NMR analysis (yielding x<sub>1</sub>, y<sub>1</sub> and z<sub>1</sub>) and from acid-base titrations (yielding x<sub>2</sub>, y<sub>2</sub> and z<sub>2</sub>). The values were averaged, giving the final x, y and z values. Since these values are close to the expected grafting levels, the theoretical grafting percentages have been carried over into the writing of the names of ArylAPols and CyclAPols for simplification.

	PG	CL	PE	Total lipids	lipid-to-BmrA ratio (w/w)
<b>C<sub>6</sub>-C<sub>2</sub>-50</b>	1.9±0.6	0.6±0.0	11.2±2.0	13.9±2.6	0.076±0.014
<b>C<sub>8</sub>-C<sub>0</sub>-50</b>	1.5±0.3	0.6±0.2	11.6±0.4	13.8±0.6	0.075±0.006
<b>SMA (3:1)</b>	1.6±0.4	0.3±0.1	7.4±3.0	11.4±0.3	0.052±0.019
<b>DDM</b>	1.0±1.1	0.6±0.2	4.8±4.0	7.3±5.1	0.038±0.030

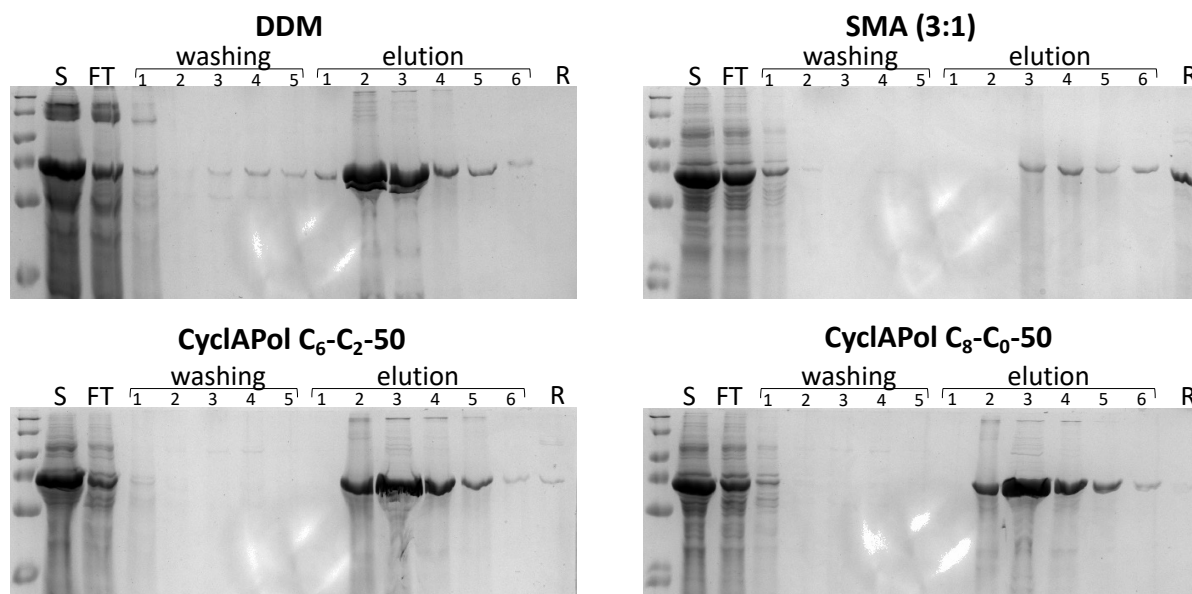
**Table S2. Quantification of phospholipids co-purified with BmrA.** After solubilization of the *E. coli* membrane with different surfactants, BmrA was purified by affinity chromatography. The elution fractions containing BmrA were treated following a procedure adapted from Bligh and Dyer<sup>5</sup>. Then, the lipid extracts were analyzed by LC-MS/MS equipped with a Corona-CAD and an LTQ-Orbitrap detector. For each lipid species, the number of phospholipid molecules were calculated per dimer of BmrA (3 independent replicas of each sample).



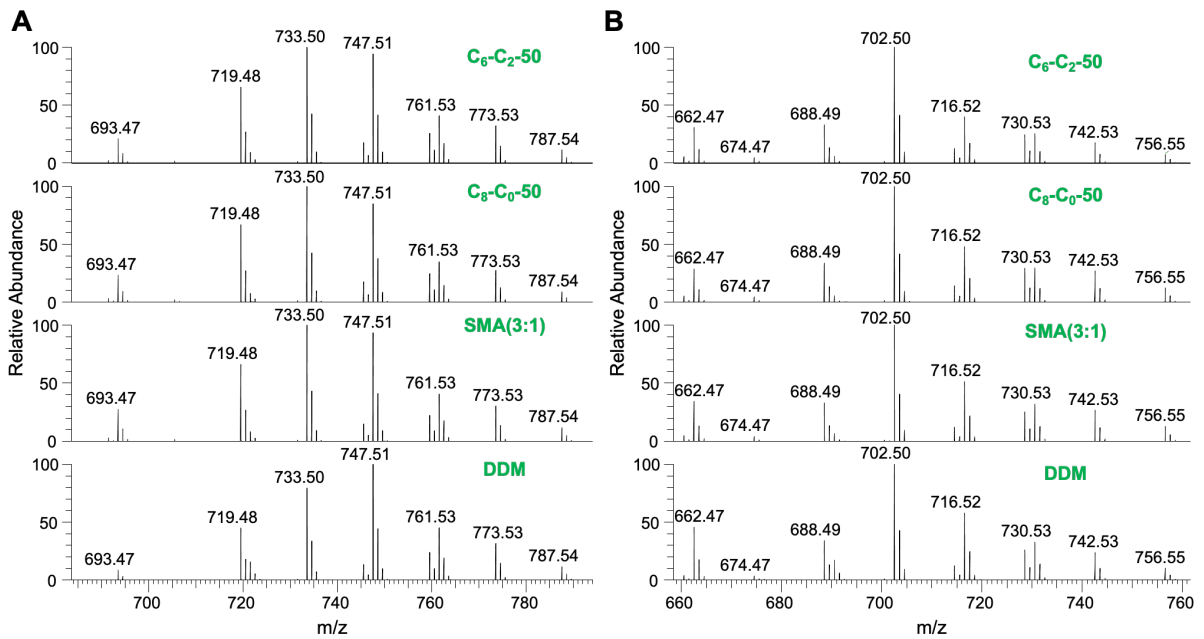
## Additional figures:



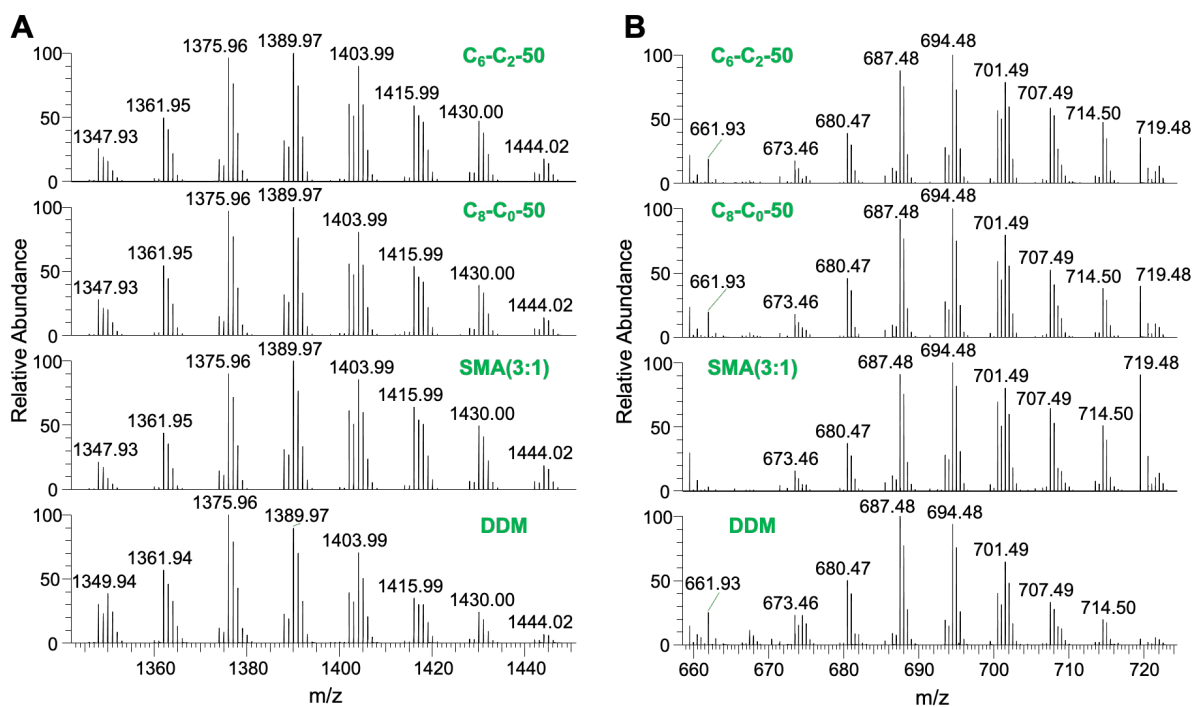
**Figure S1.** Comparison of the efficiency of polymers to extract BmrA from the *E. coli* membrane according to the chemical structure of hydrophobic groups and their density along the polymer's backbone. (A) Direct extraction of BmrA from the *E. coli* membrane by ArylAPols modified with either 25 mol% (*top*) or 50 mol% (*bottom*) of hydrophobic side groups. The total MP/polymer mass ratios used were 1:0.5 (*left*), 1:5 (*middle*), and 1:10 (*right*), corresponding to a final concentration of polymer of 0.5, 1.0 and 2.0%, respectively. After 2 h of incubation at 4°C, the samples were centrifuged (30 min at 200,000  $\times g$ ). The protein content in the supernatants (S) and pellets (P) was analyzed by SDS-PAGE. (B) Direct extraction of BmrA from the *E. coli* membrane upon addition of CyclAPols modified with either 25 mol% (*top*) or 50 mol% (*bottom*) of hydrophobic side groups. The experimental conditions used (final concentrations of polymers, incubation time, etc.) were identical to those used for the ArylAPols.



**Figure S2.** Purification of BmrA in different surfactants. The *E. coli* membrane suspension was incubated with either 1% DDM, 1% SMA (3:1) and 0.2% CyclAPols C<sub>6</sub>-C<sub>2</sub>-50 or C<sub>8</sub>-C<sub>0</sub>-50. After 2 h of incubation at 4°C, the samples were centrifuged (30 min at 200,000 ×g). The protein in the supernatant (S) was loaded on an Ni<sup>2+</sup>-NTA column pre-equilibrated with Tris/NaCl buffer and the flow-through (FT) recovered. After a washing step with Tris/NaCl buffer, the protein was eluted with Tris/NaCl buffer containing 300 mM imidazole. An aliquot of the Ni<sup>2+</sup>-NTA resin (R) was also analyzed by SDS-PAGE in order to check the presence or absence of aggregated protein in the resin. For the purification with DDM, all the buffers contained 0.34 mM DDM. All the gels were Coomassie blue stained.



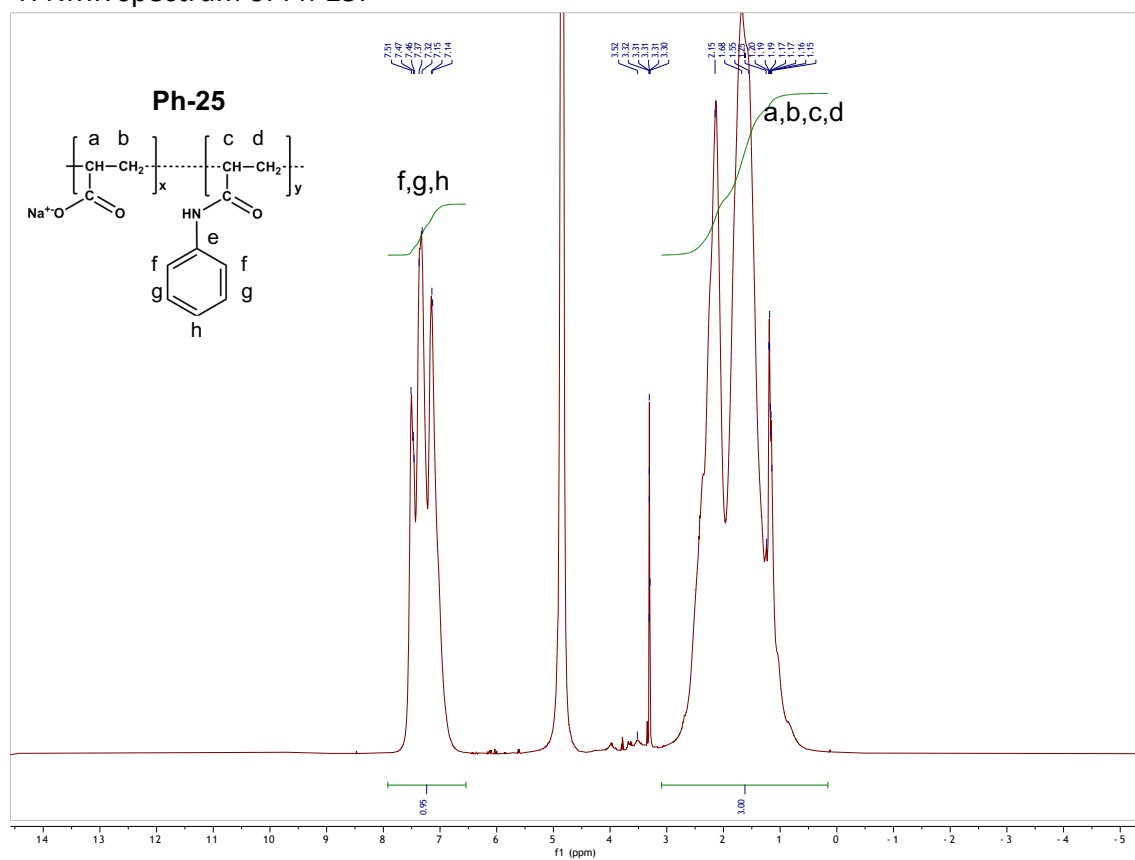
**Figure S3.** ESI-MS/MS analysis of the phospholipid content in BmrA samples. (A) Full scans of phospholipids eluting at position 10.3-10.8 min in chromatograms of Fig. 5 and identified as PG. (B) Full scans of phospholipids eluting at position 14.1-14.5 min in chromatograms of Fig. 5 and identified as PE.



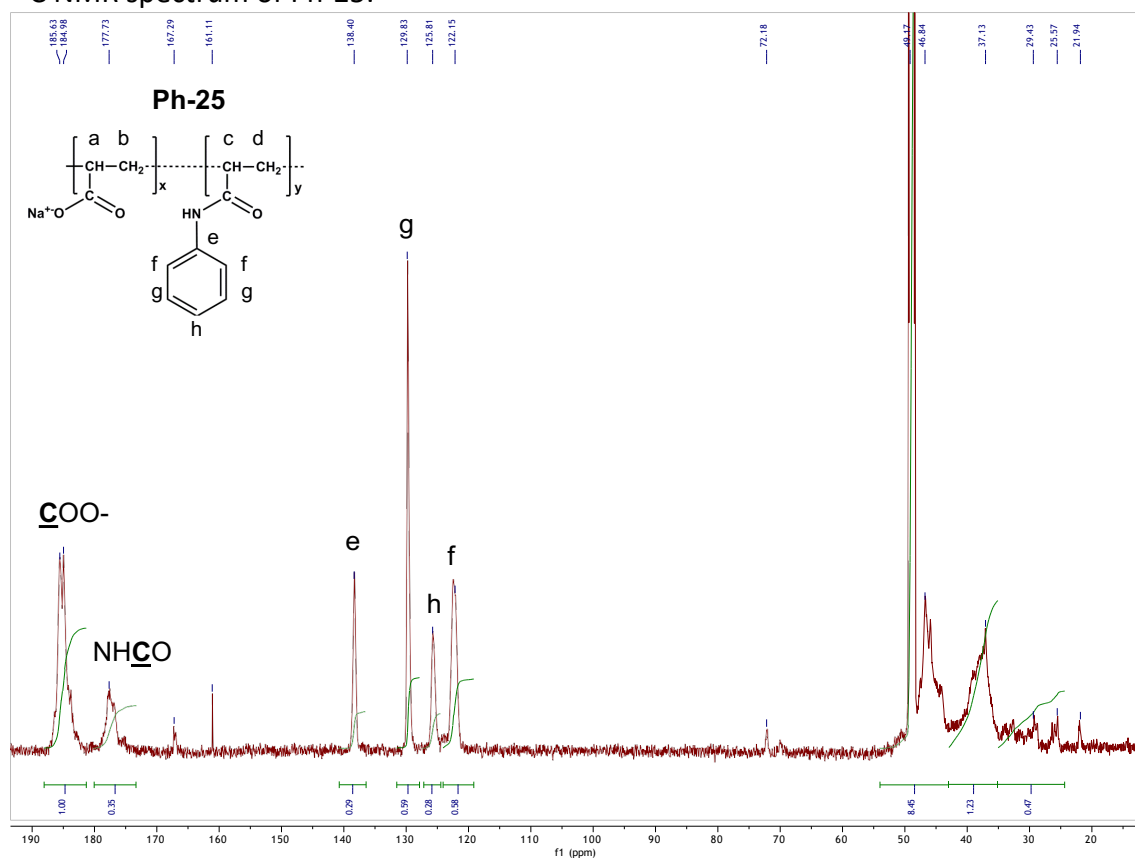
**Figure S4.** ESI-MS/MS analysis of the cardiolipin content in purified BmrA samples. Full scans of monocharged cardiolipins eluting at position 11.4-11.8 min in chromatograms of Fig. 5 (A) and double-charged cardiolipins (B).

# <sup>1</sup>H and <sup>13</sup>C NMR analysis of ArylAPols:

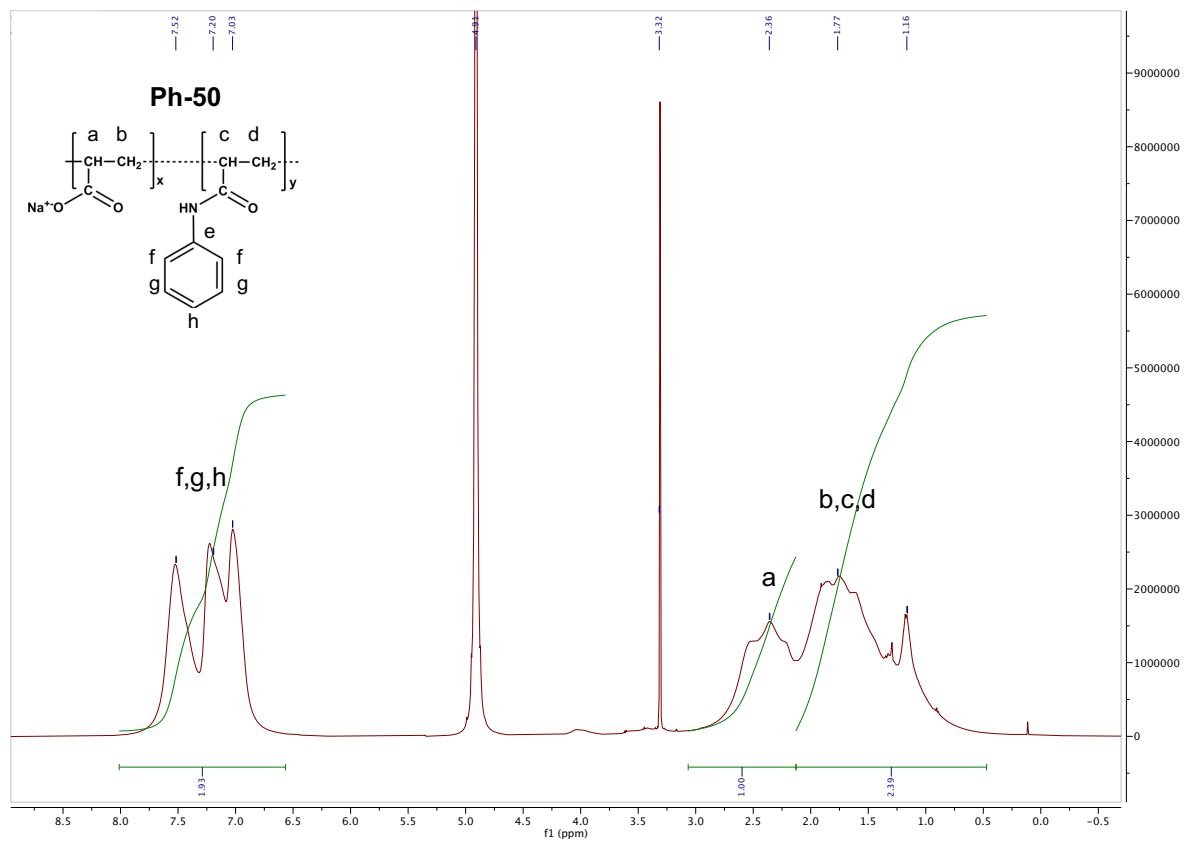
<sup>1</sup>H NMR spectrum of Ph-25:



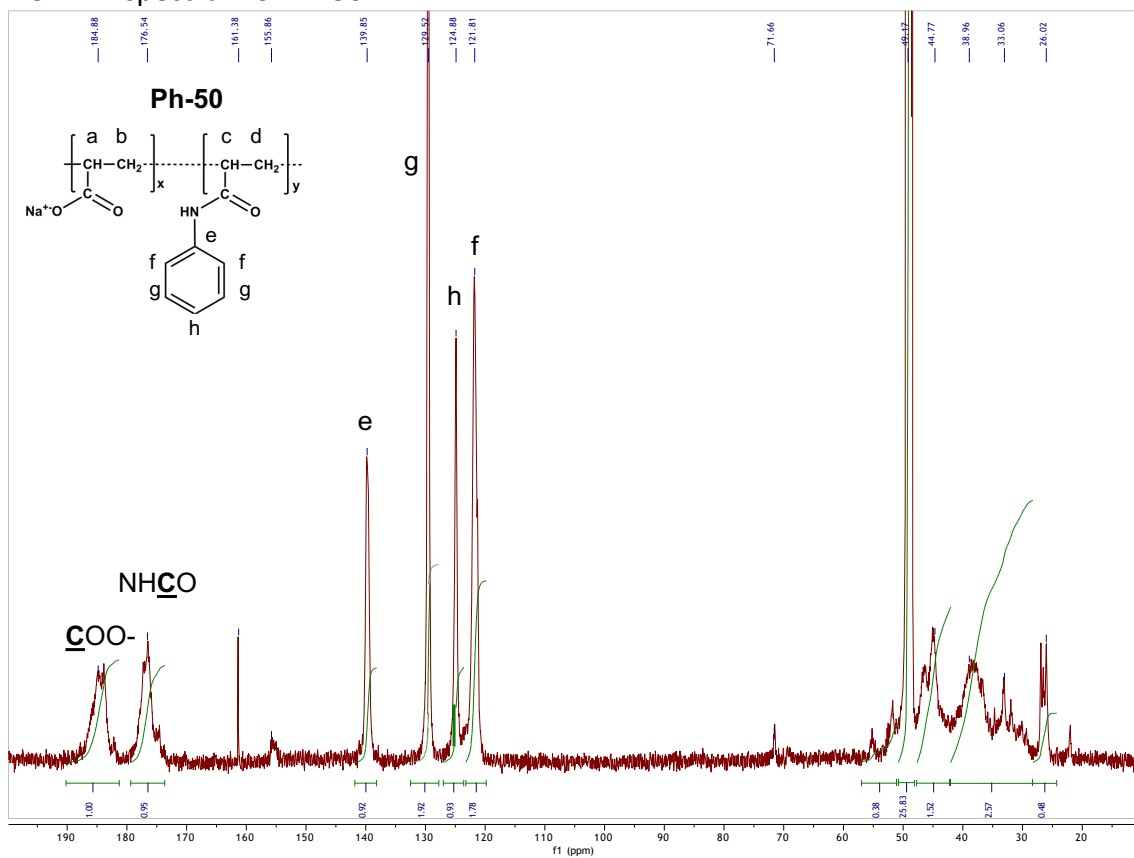
<sup>13</sup>C NMR spectrum of Ph-25:



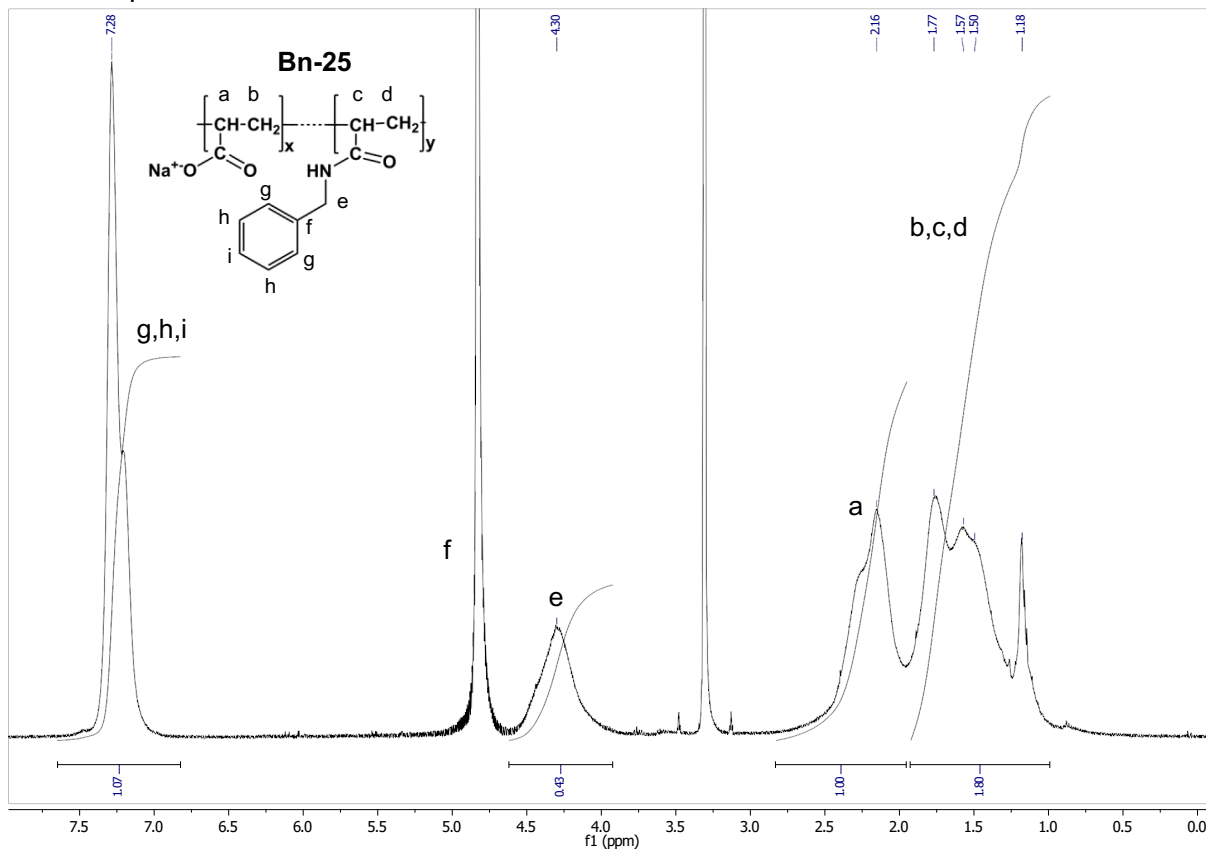
<sup>1</sup>H NMR spectrum of Ph-50:



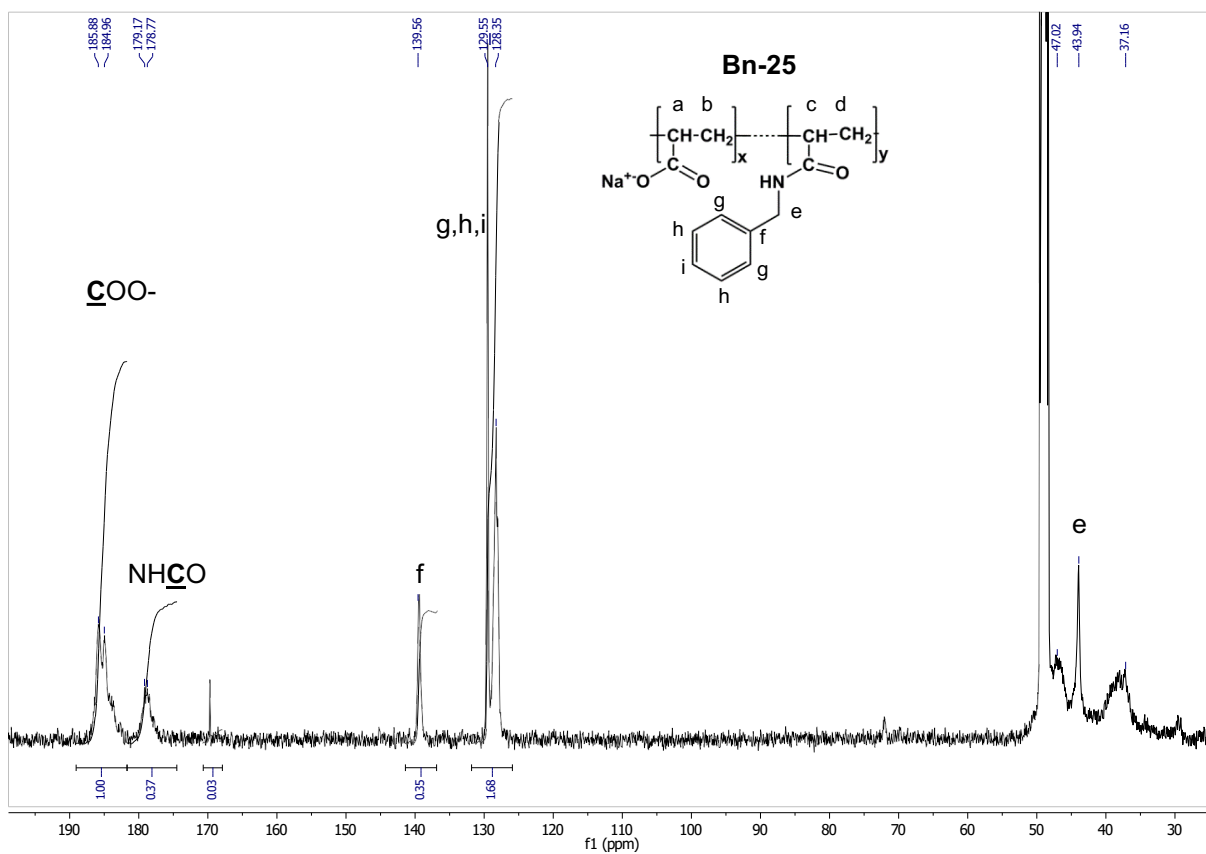
<sup>13</sup>C NMR spectrum of Ph-50:



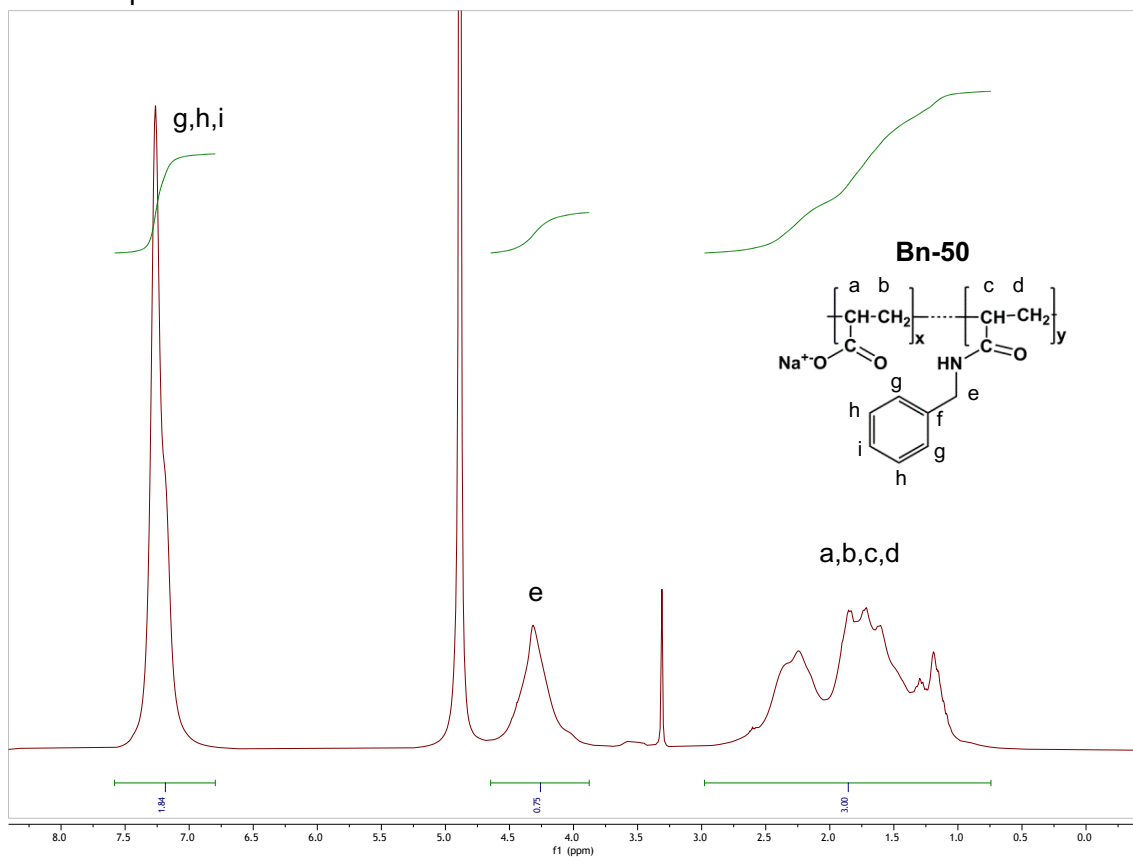
$^1\text{H}$  NMR spectrum of Bn-25:



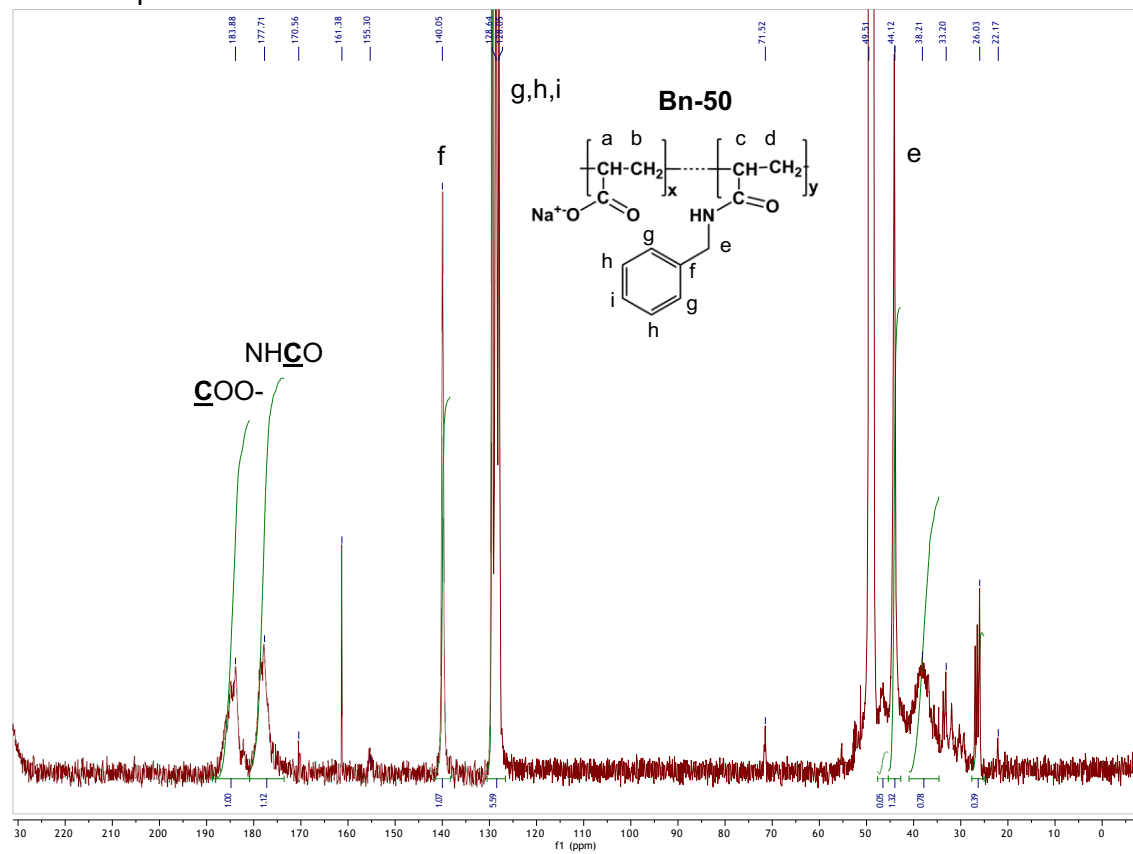
$^{13}\text{C}$  NMR spectrum of Bn-25:



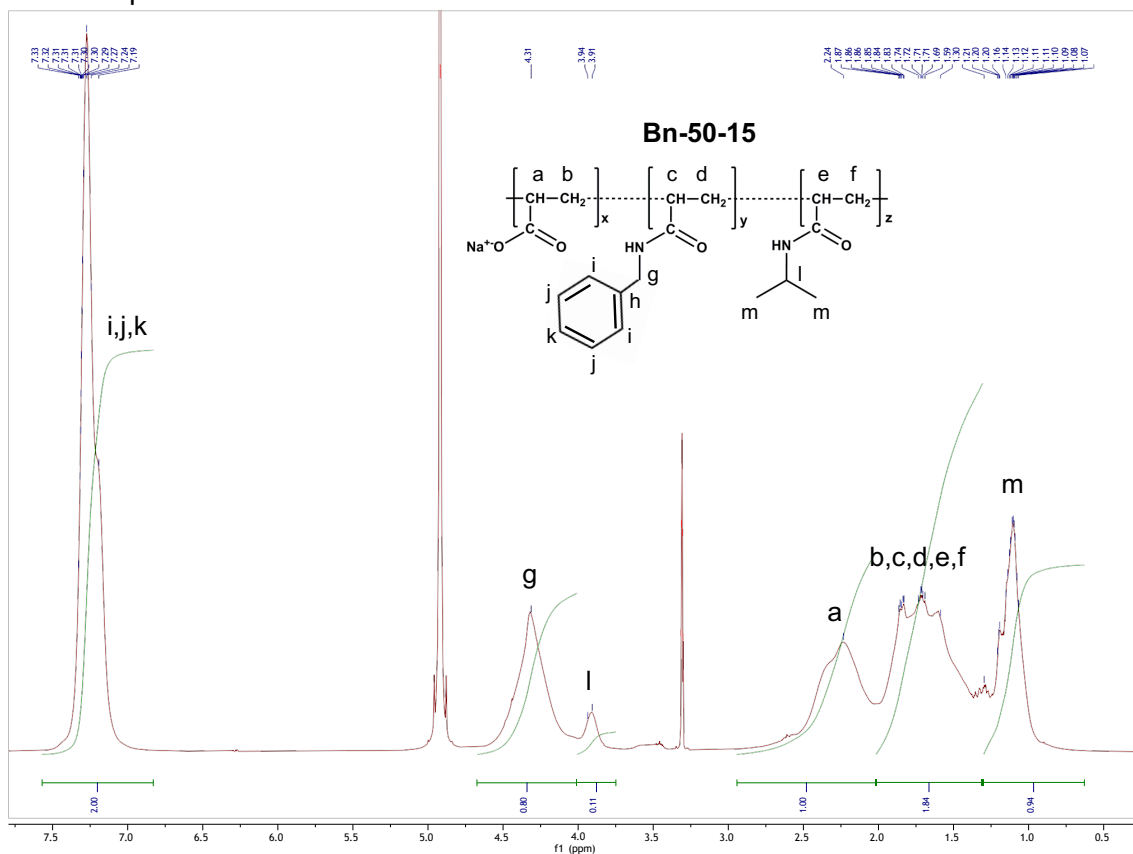
$^1\text{H}$  NMR spectrum of Bn-50:



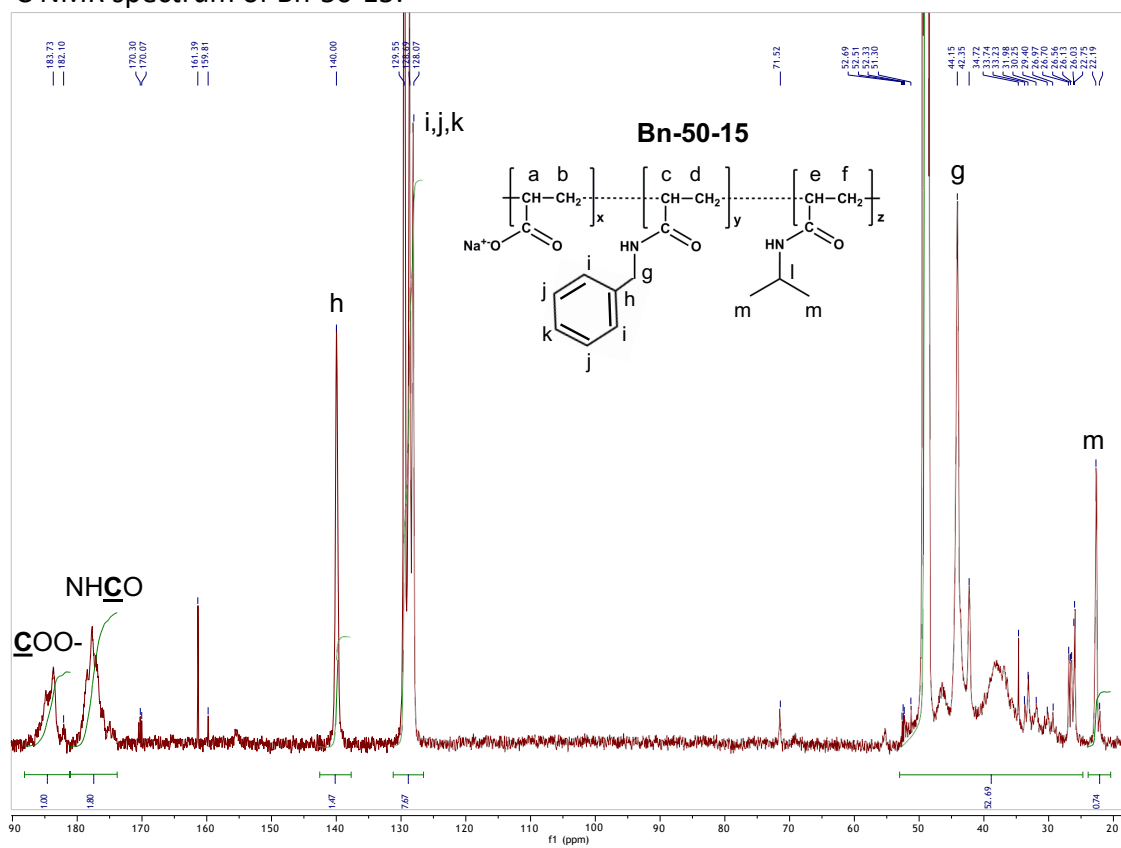
$^{13}\text{C}$  NMR spectrum of Bn-50:



<sup>1</sup>H NMR spectrum of Bn-50-15:

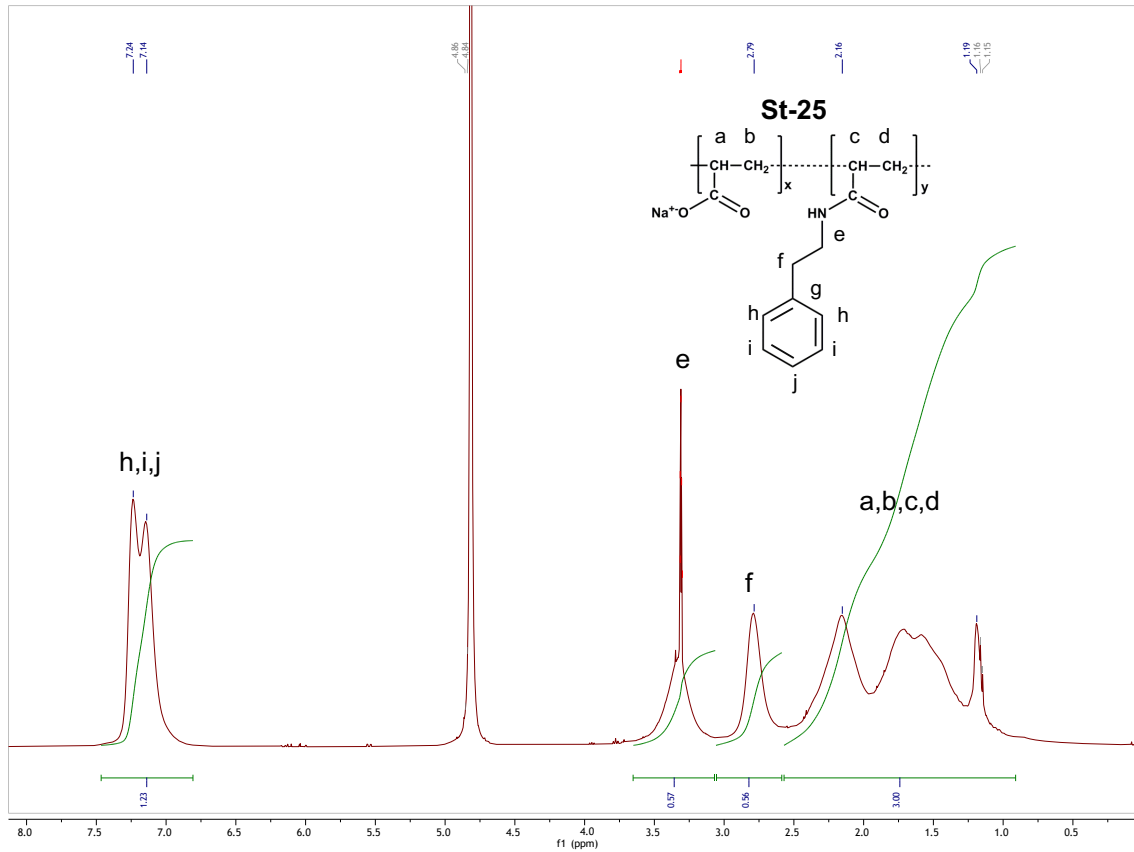


<sup>13</sup>C NMR spectrum of Bn-50-15:

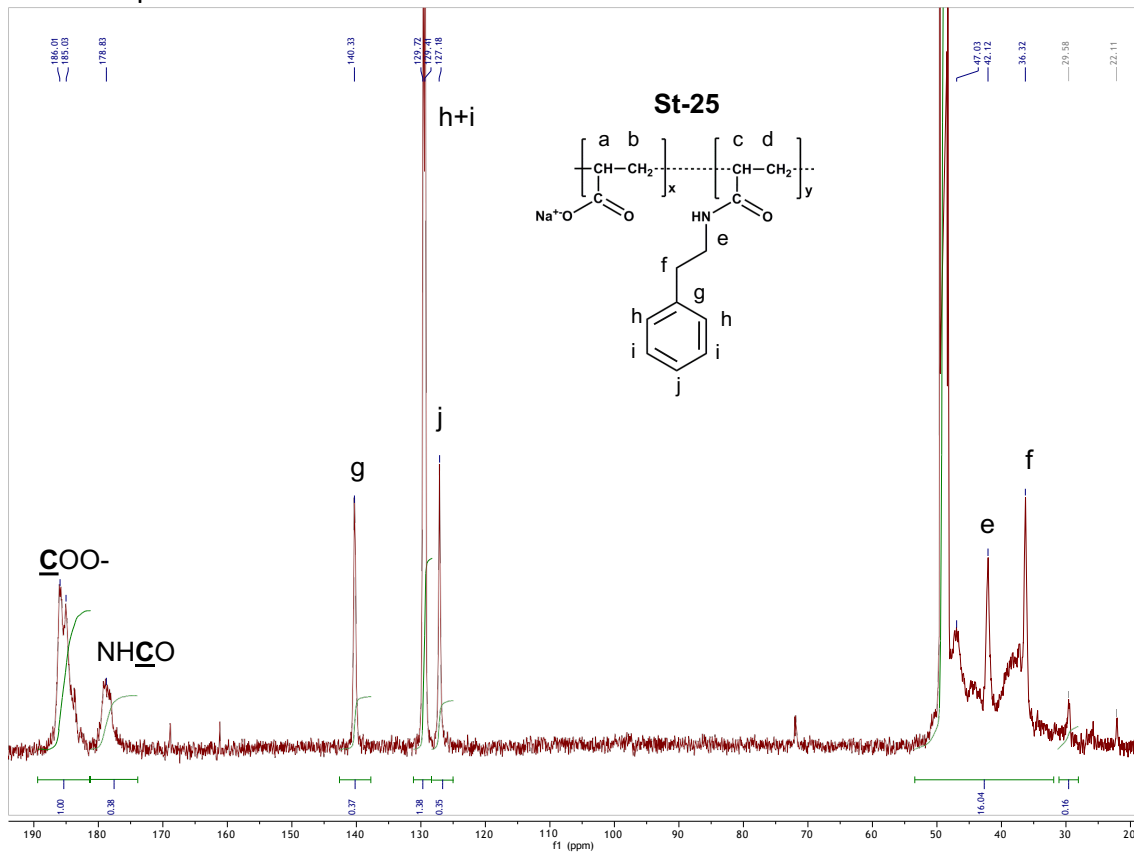




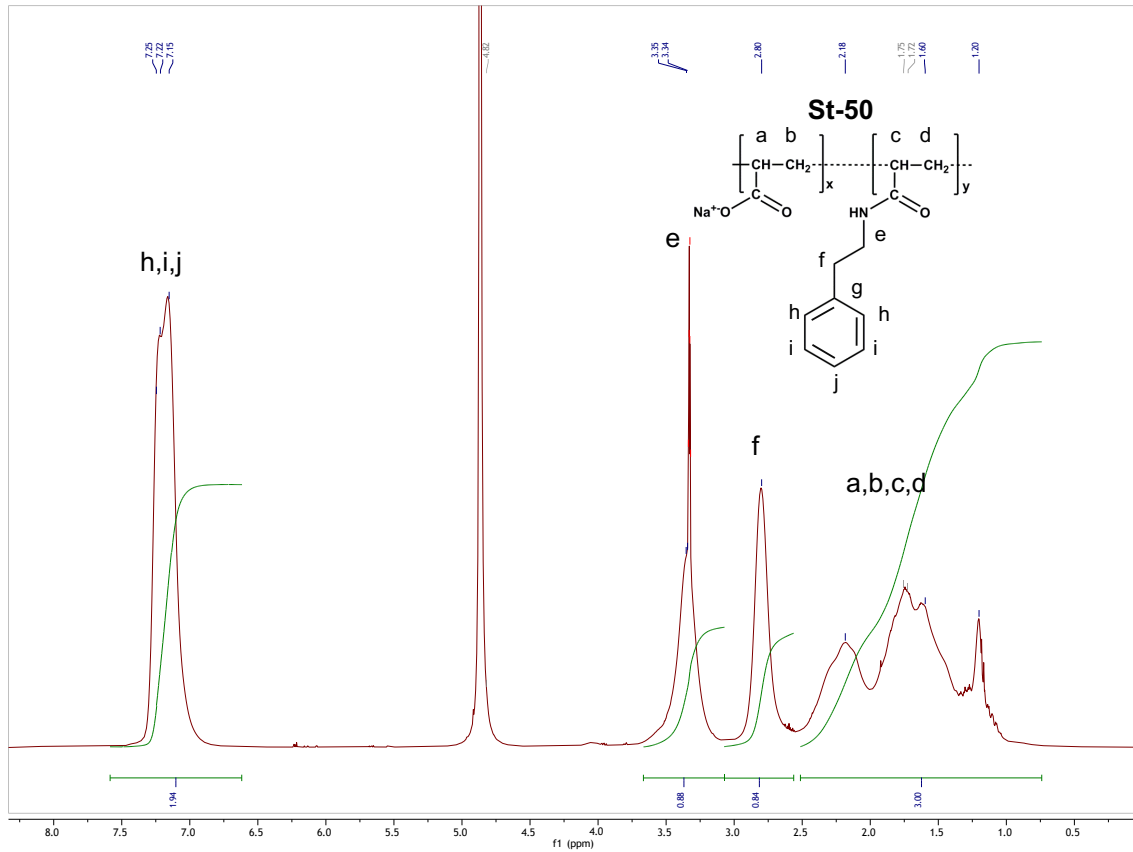
<sup>1</sup>H NMR spectrum of St-25:



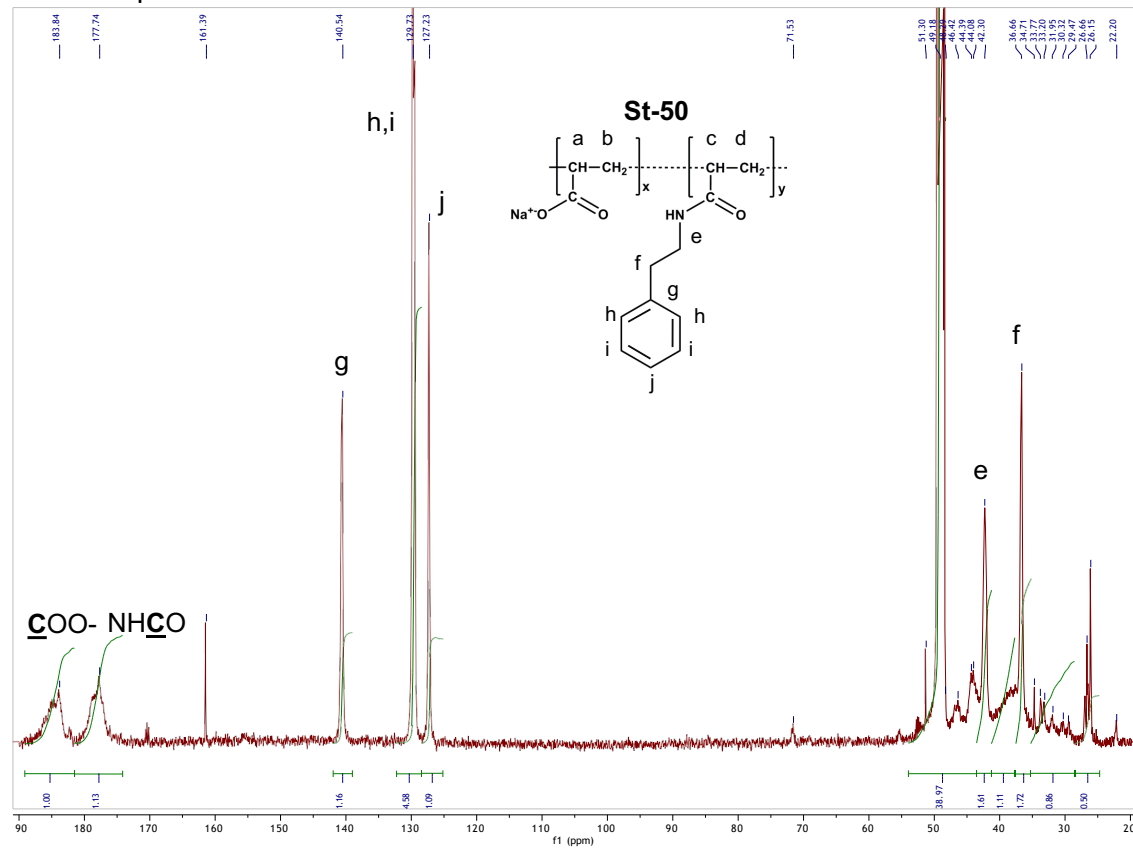
<sup>13</sup>C NMR spectrum of St-25:



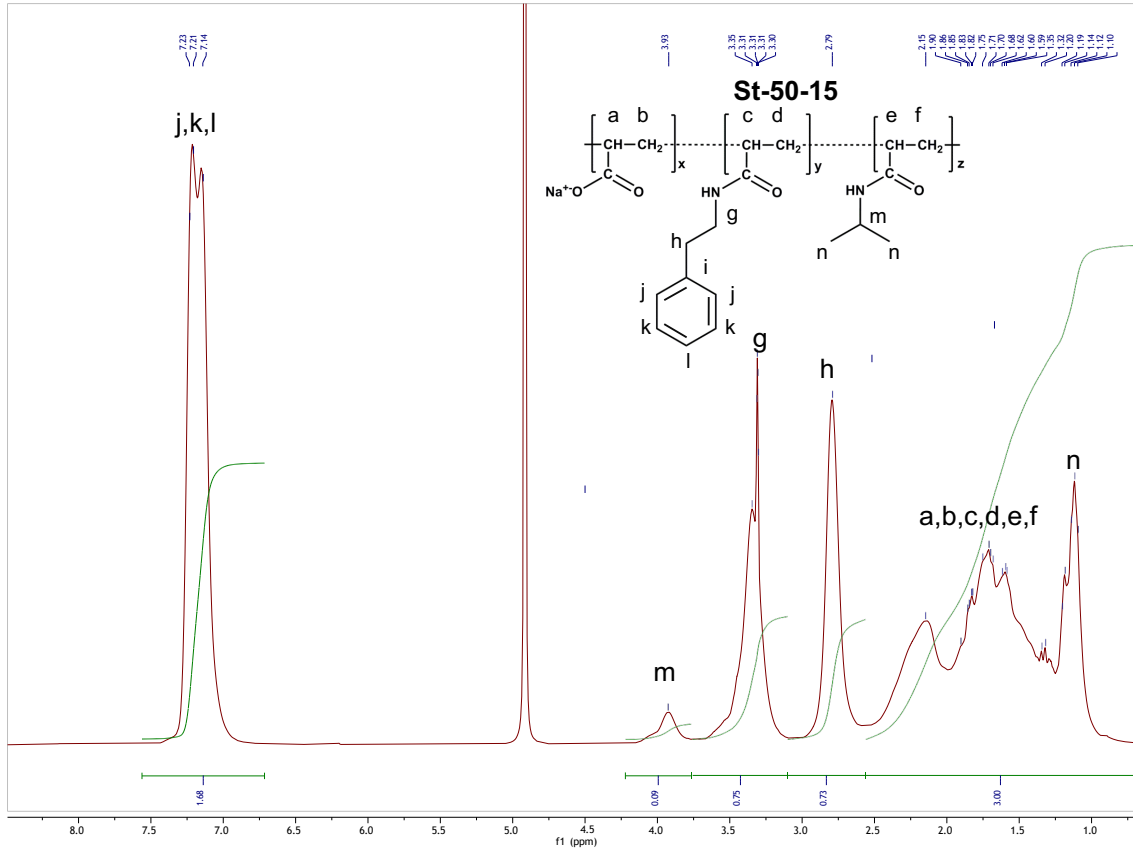
<sup>1</sup>H NMR spectrum of St-50:



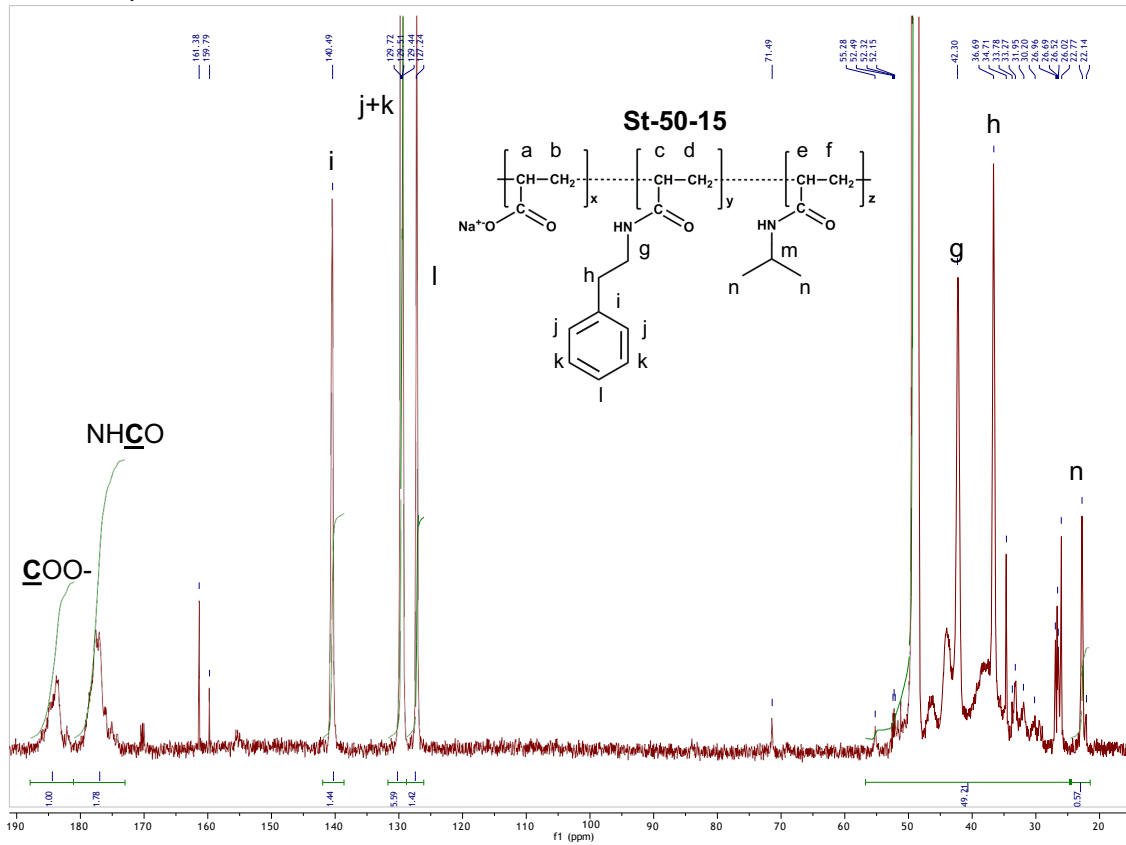
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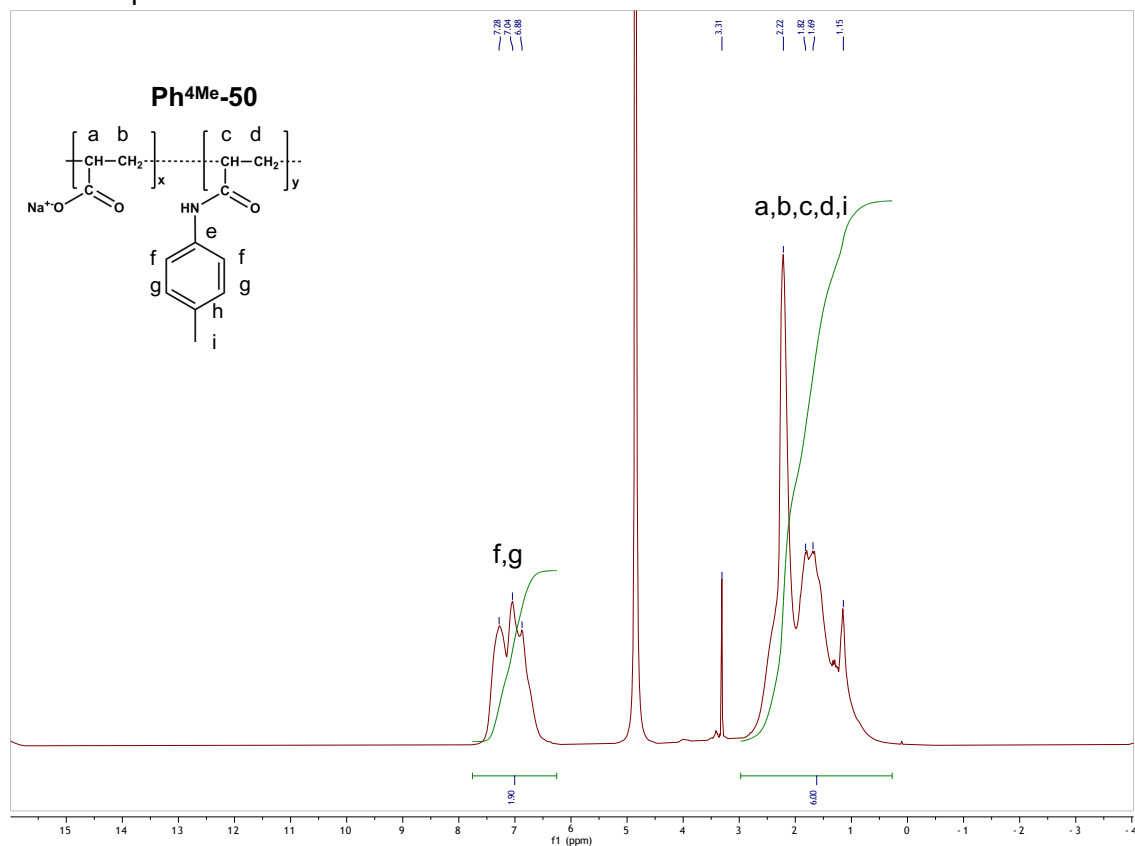
<sup>1</sup>H NMR spectrum of St-50-15:



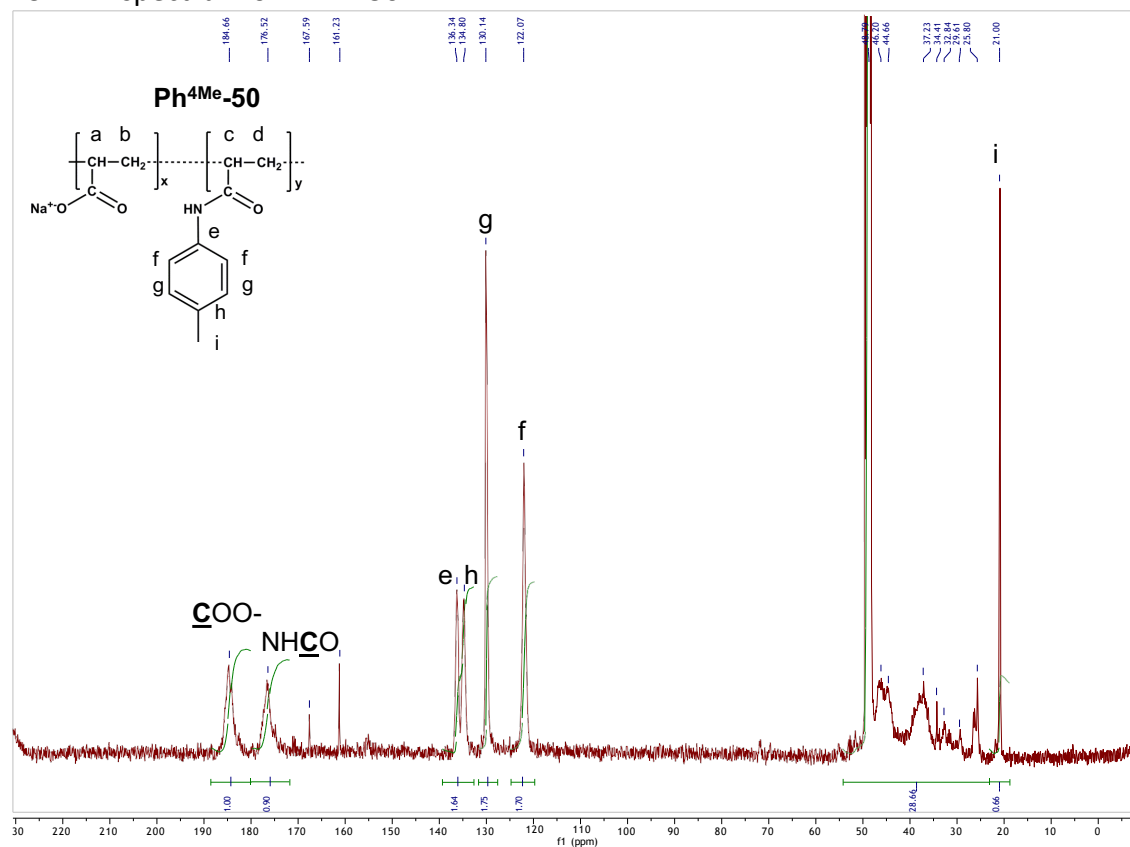
<sup>13</sup>C NMR spectrum of St-50-15:



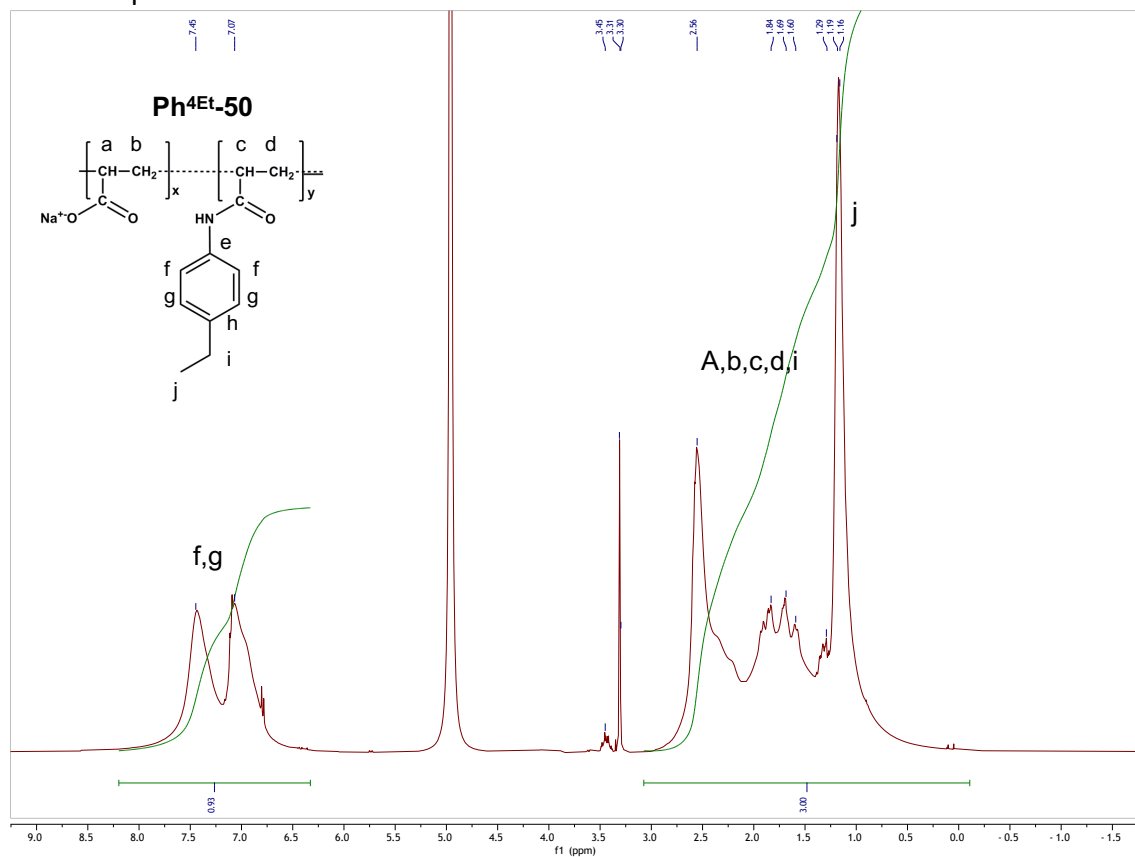
$^1\text{H}$  NMR spectrum of Ph<sup>4</sup>Me-50:



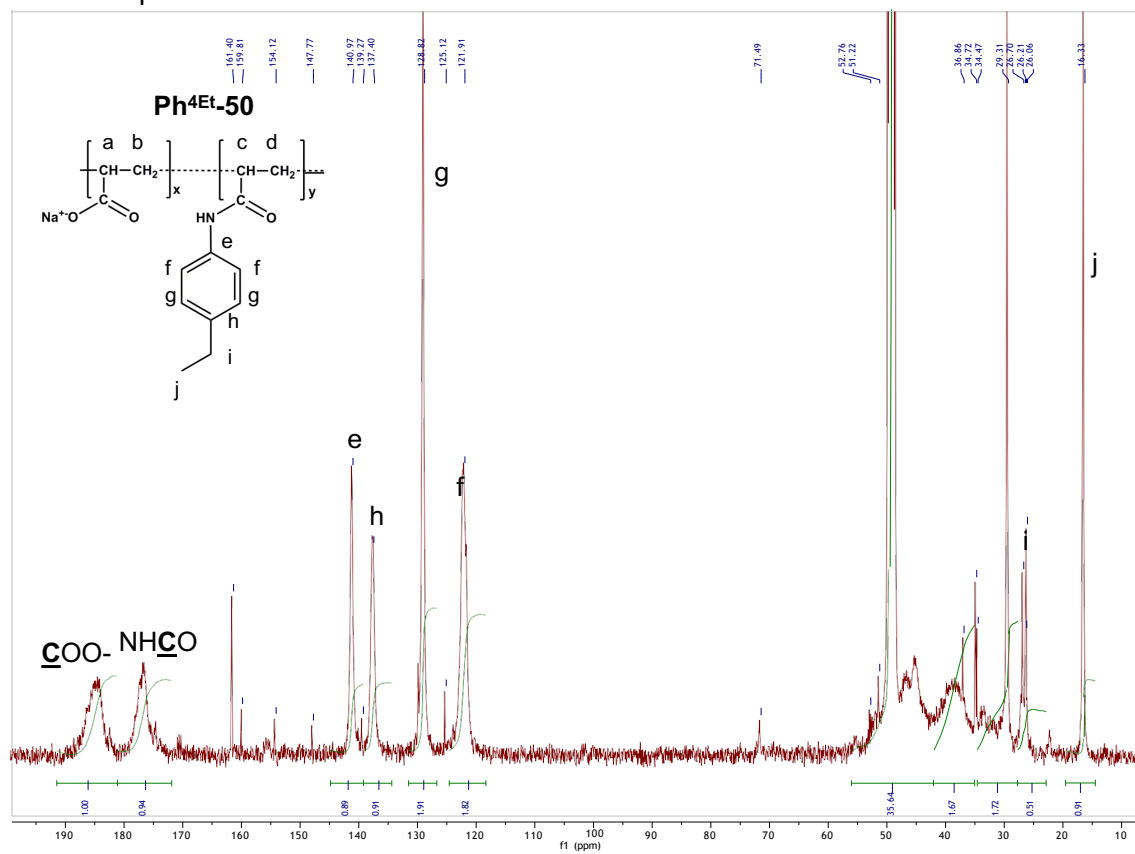
$^{13}\text{C}$  NMR spectrum of Ph<sup>4</sup>Me-50:



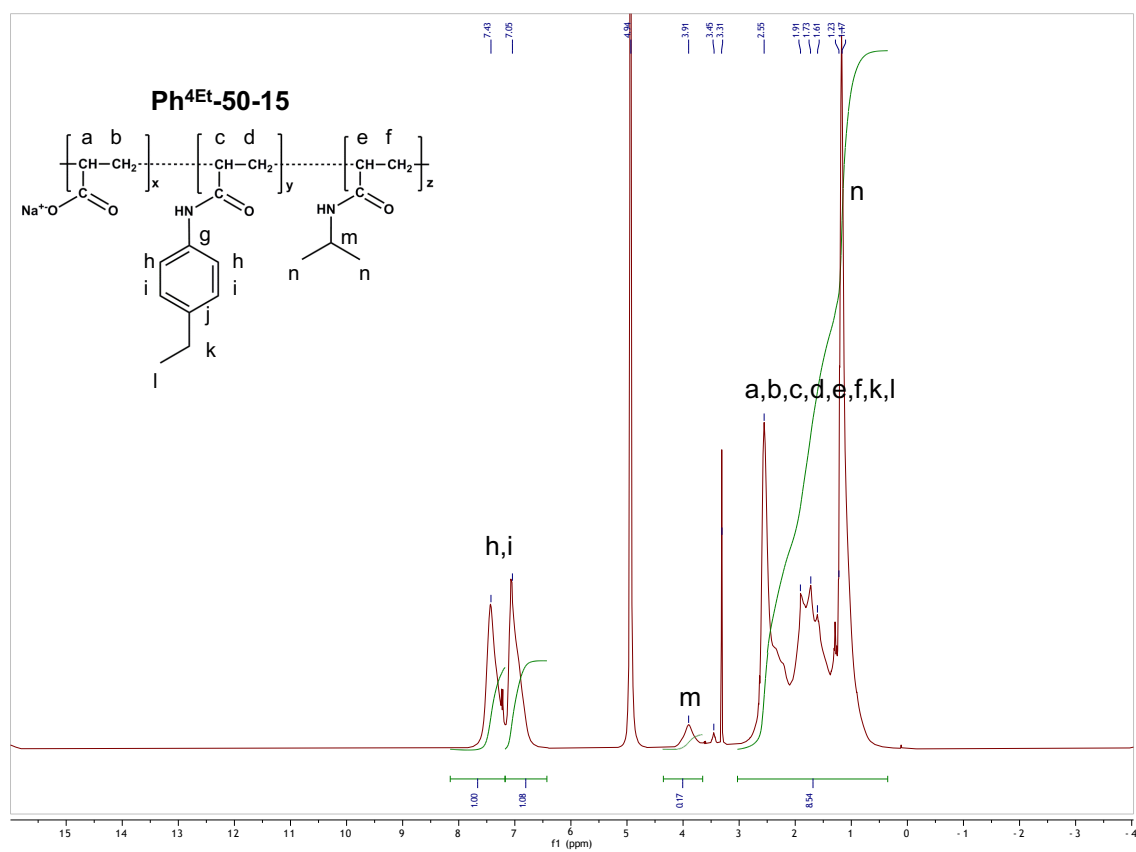
$^1\text{H}$  NMR spectrum of  $\text{Ph}^{4\text{Et}}\text{-50}$ :



$^{13}\text{C}$  NMR spectrum of  $\text{Ph}^{4\text{Et}}\text{-50}$ :

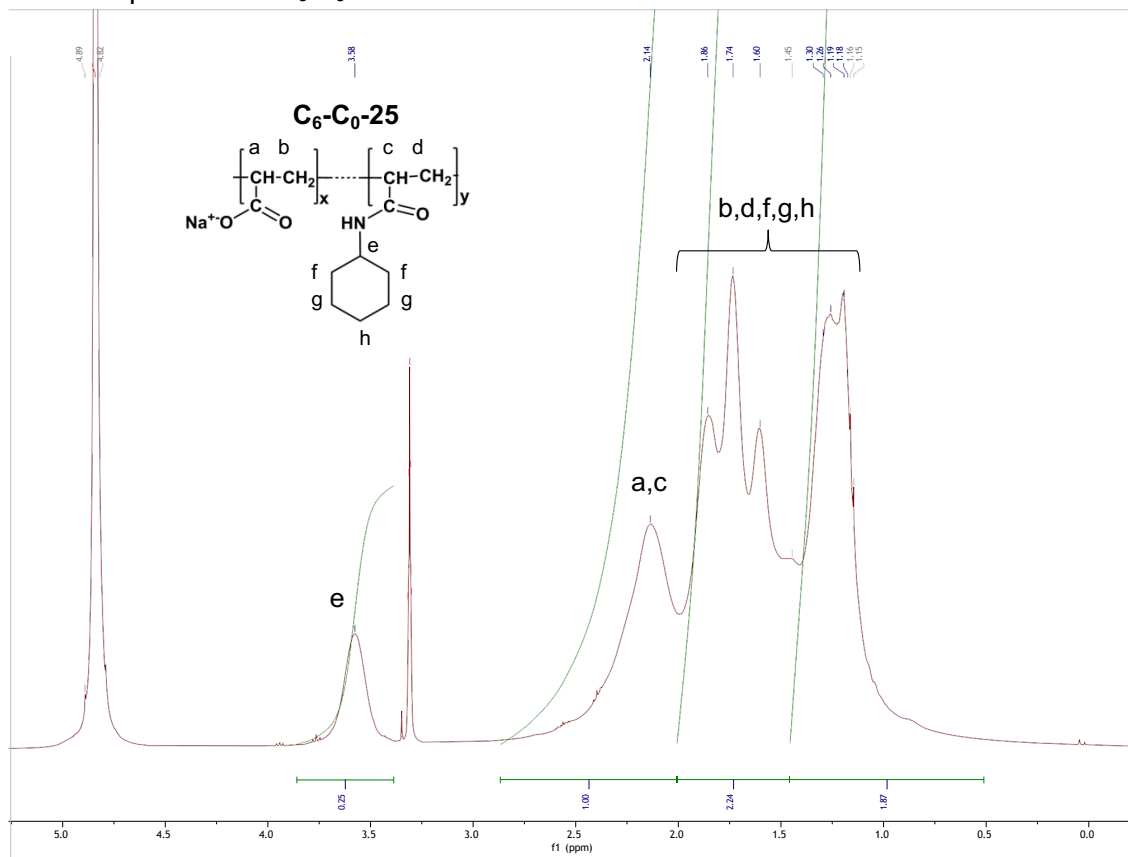


$^1\text{H}$  NMR spectrum of Ph<sup>4Et</sup>-50-15:

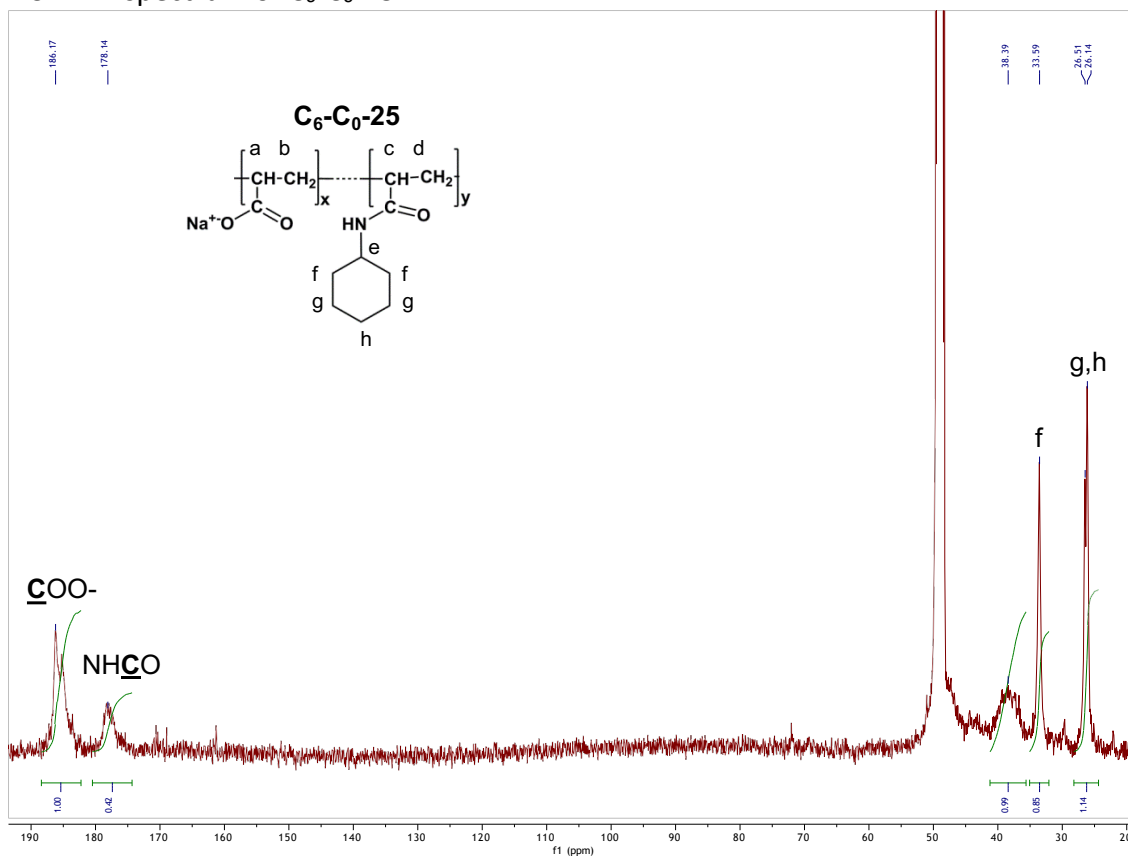


# $^1\text{H}$ and $^{13}\text{C}$ NMR analysis of CyclAPols:

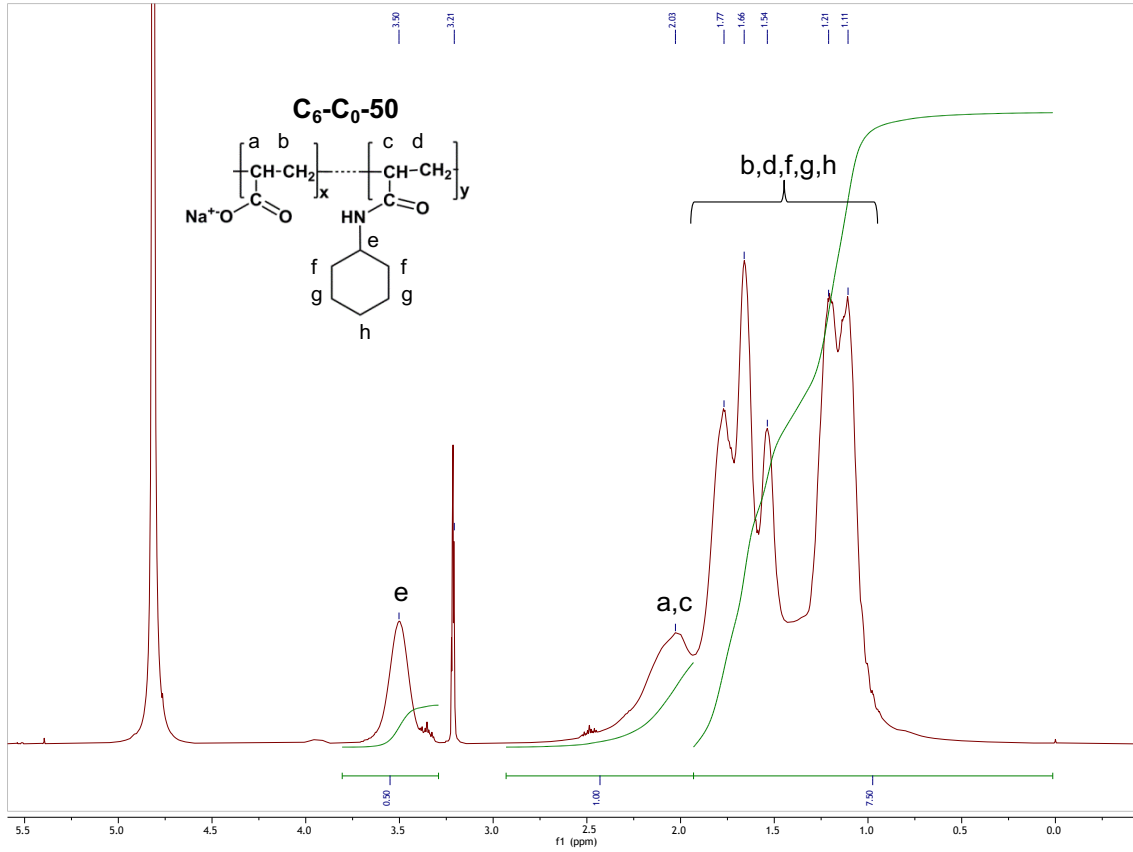
$^1\text{H}$  NMR spectrum of  $\text{C}_6\text{-C}_0\text{-25}$ :



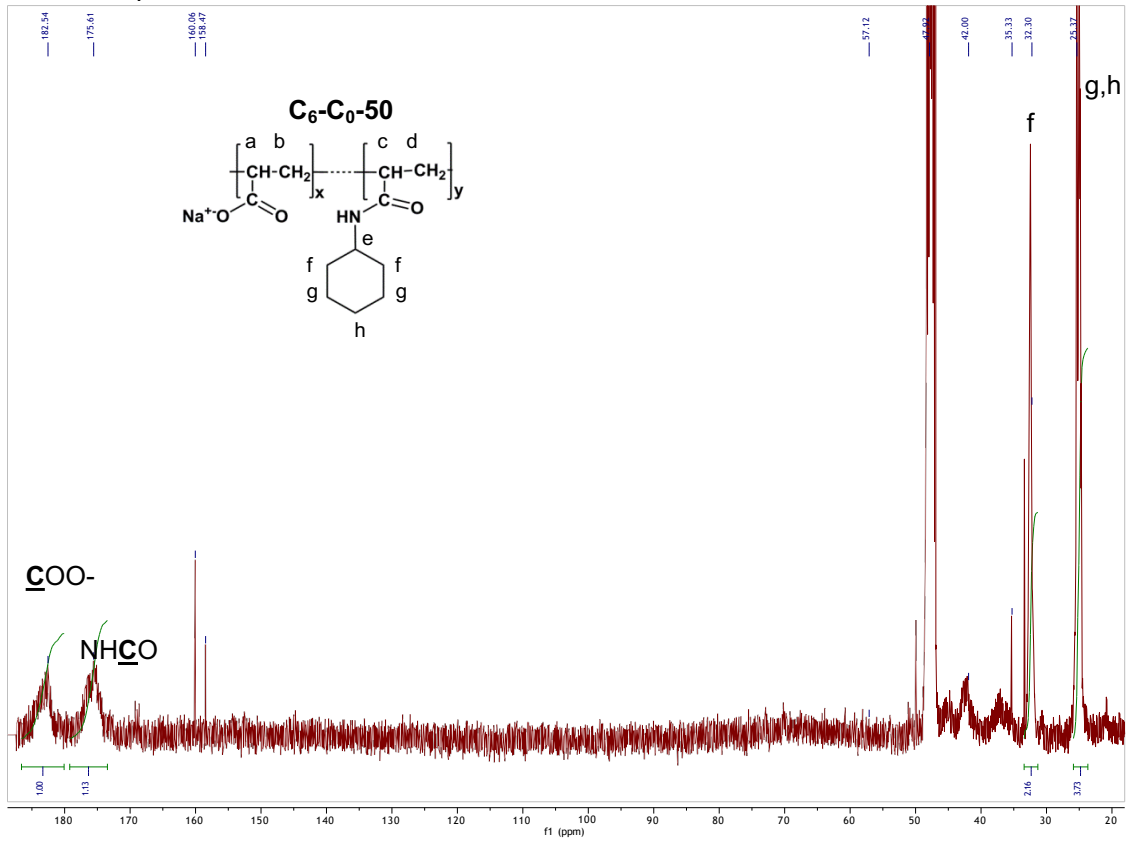
$^{13}\text{C}$  NMR spectrum of  $\text{C}_6\text{-C}_0\text{-25}$ :



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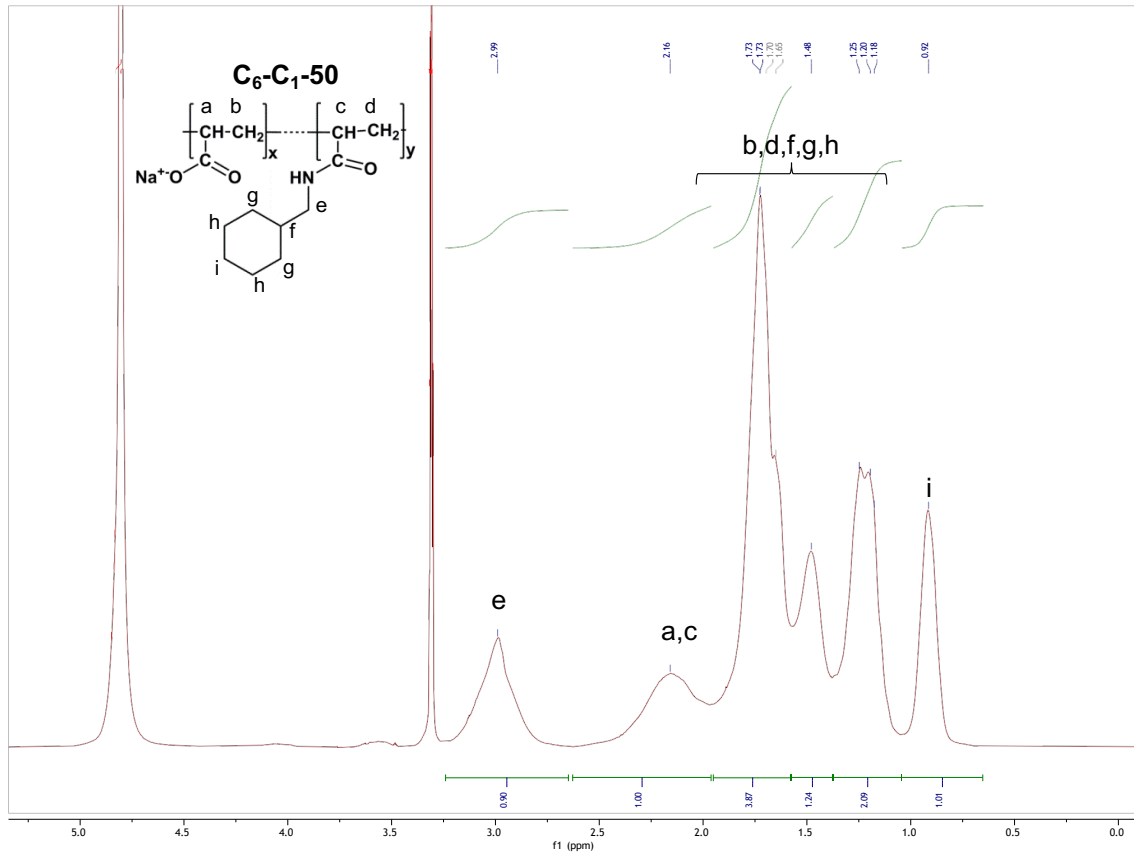
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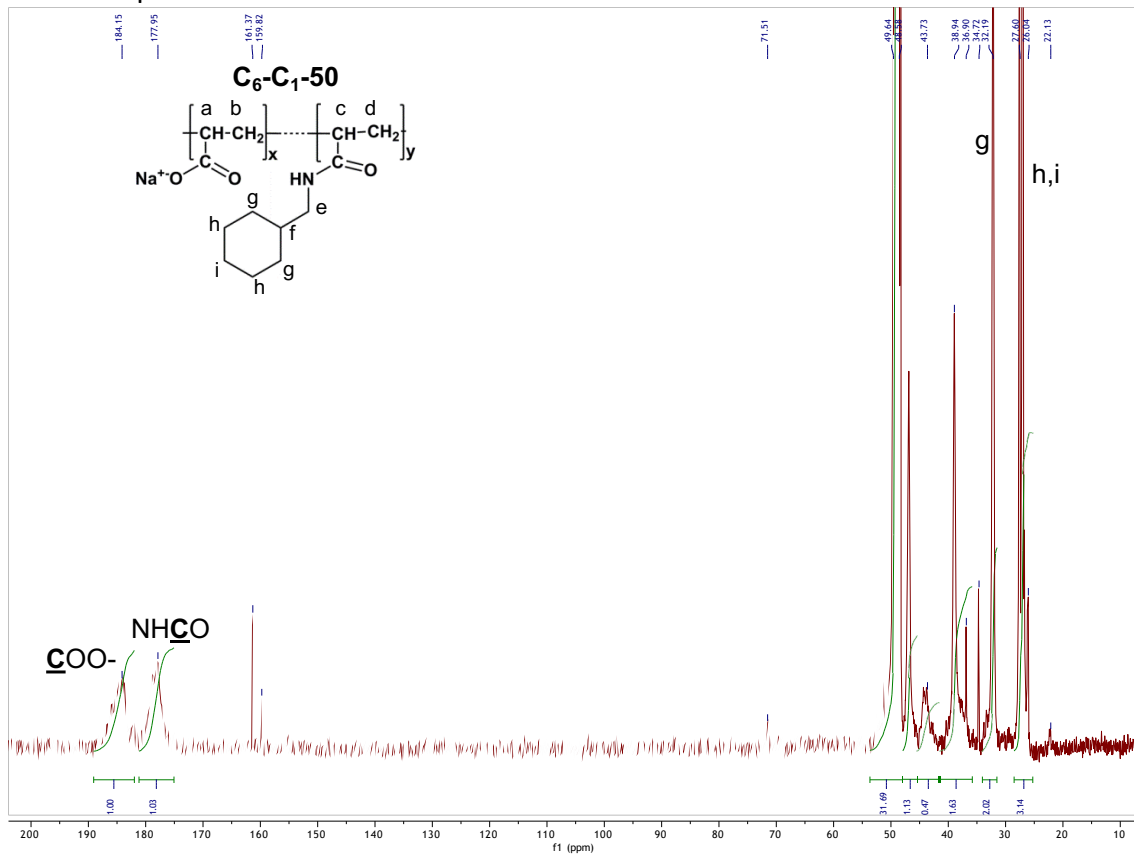




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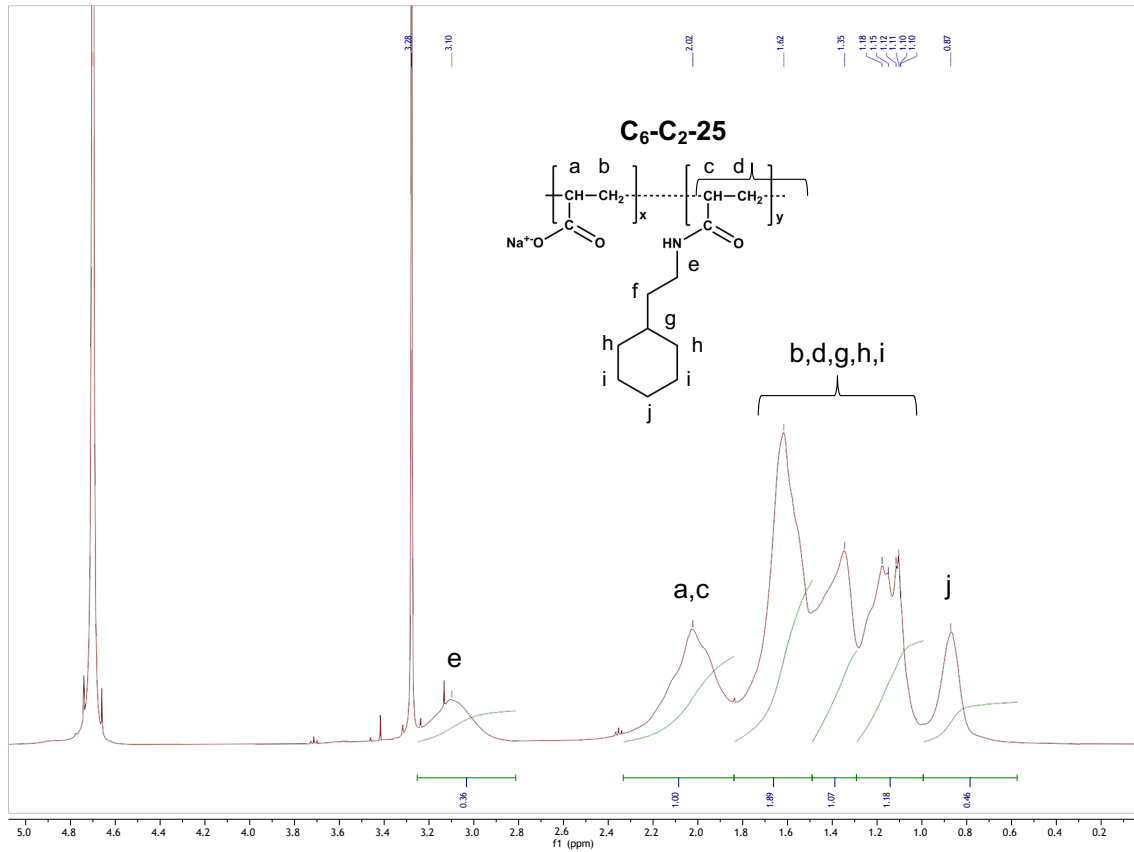


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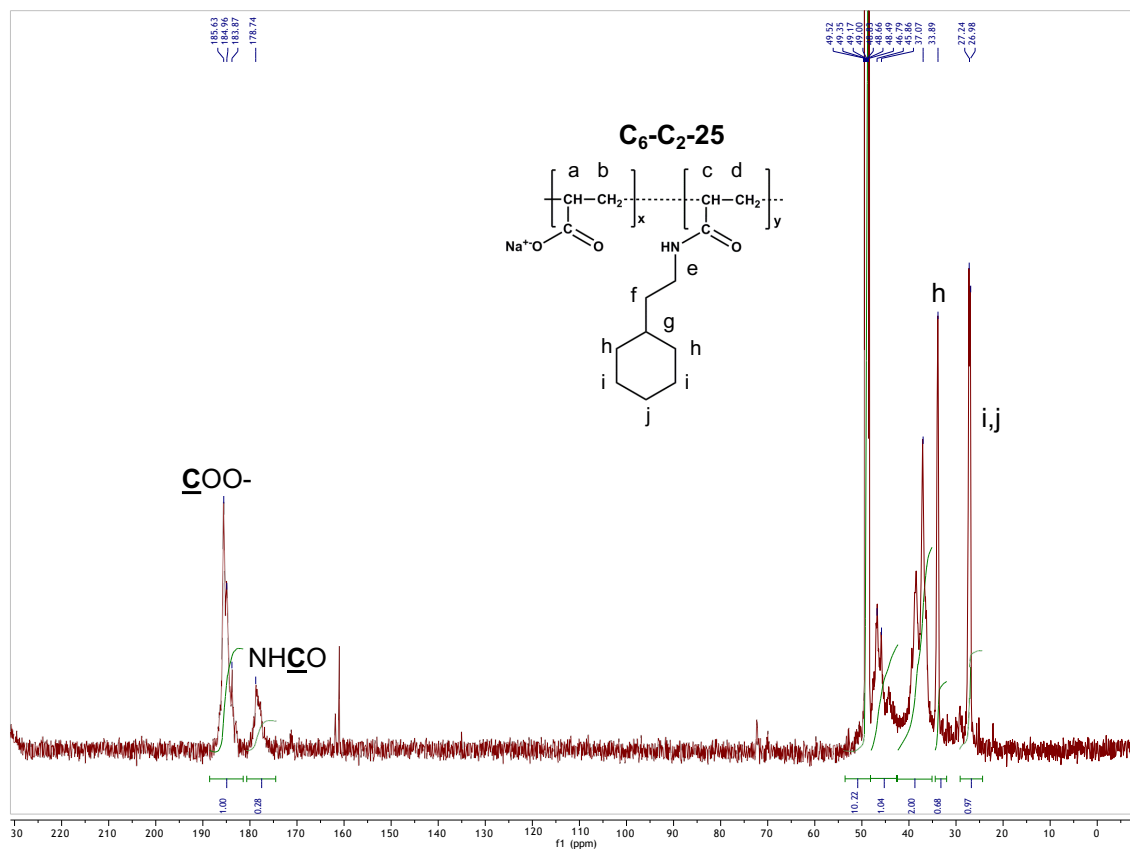




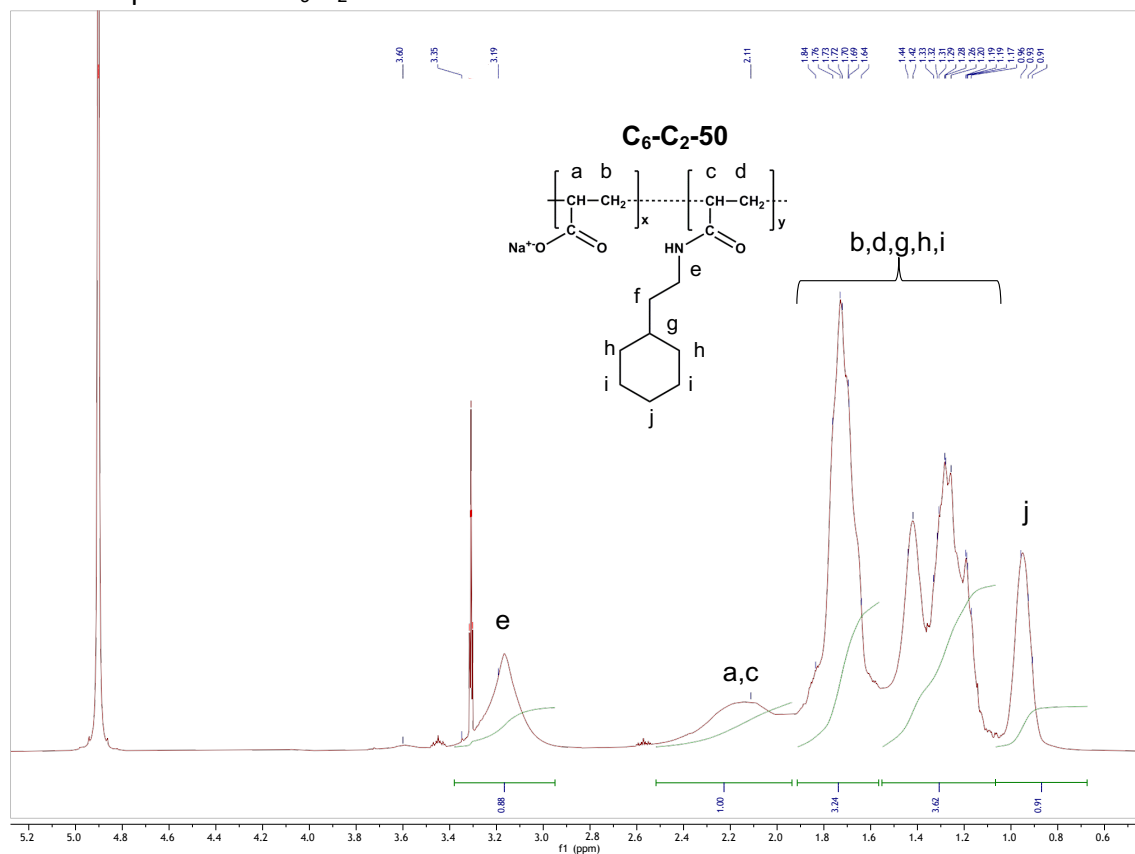
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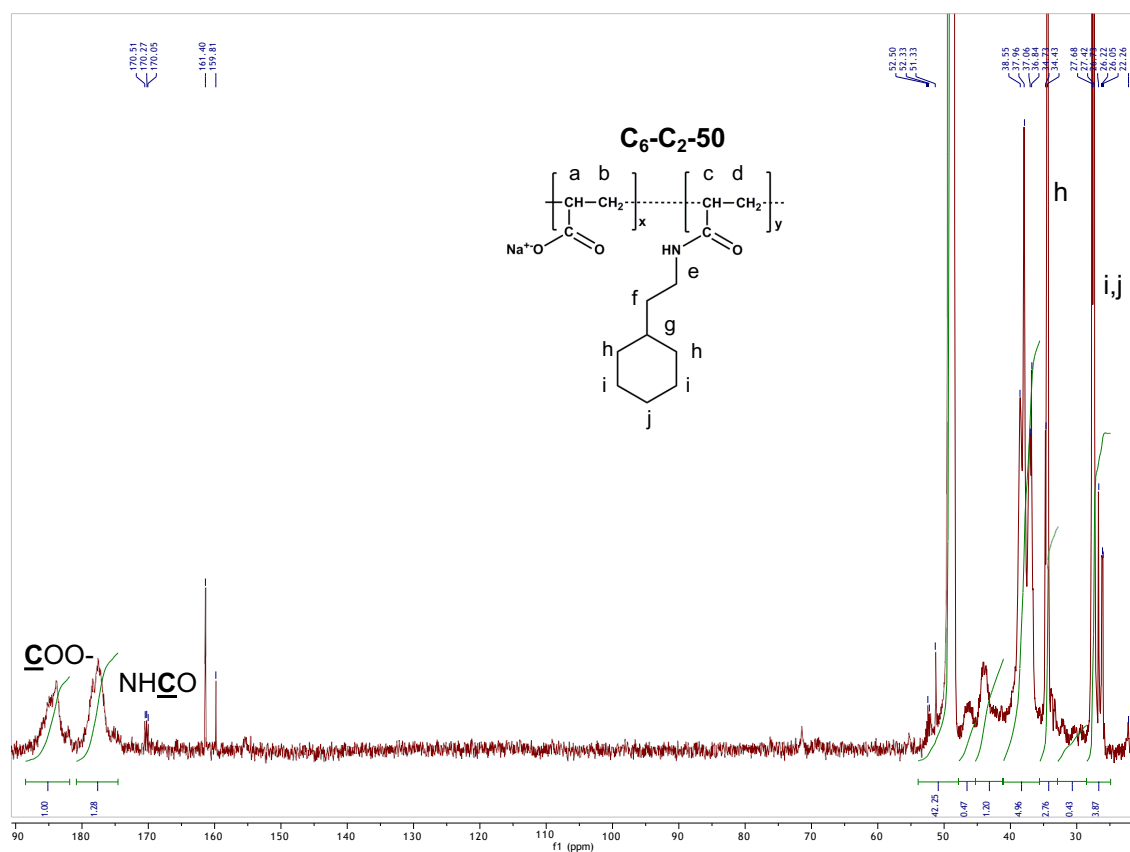
$^{13}\text{C}$  NMR spectrum of  $\text{C}_6\text{-C}_2\text{-25}$ :



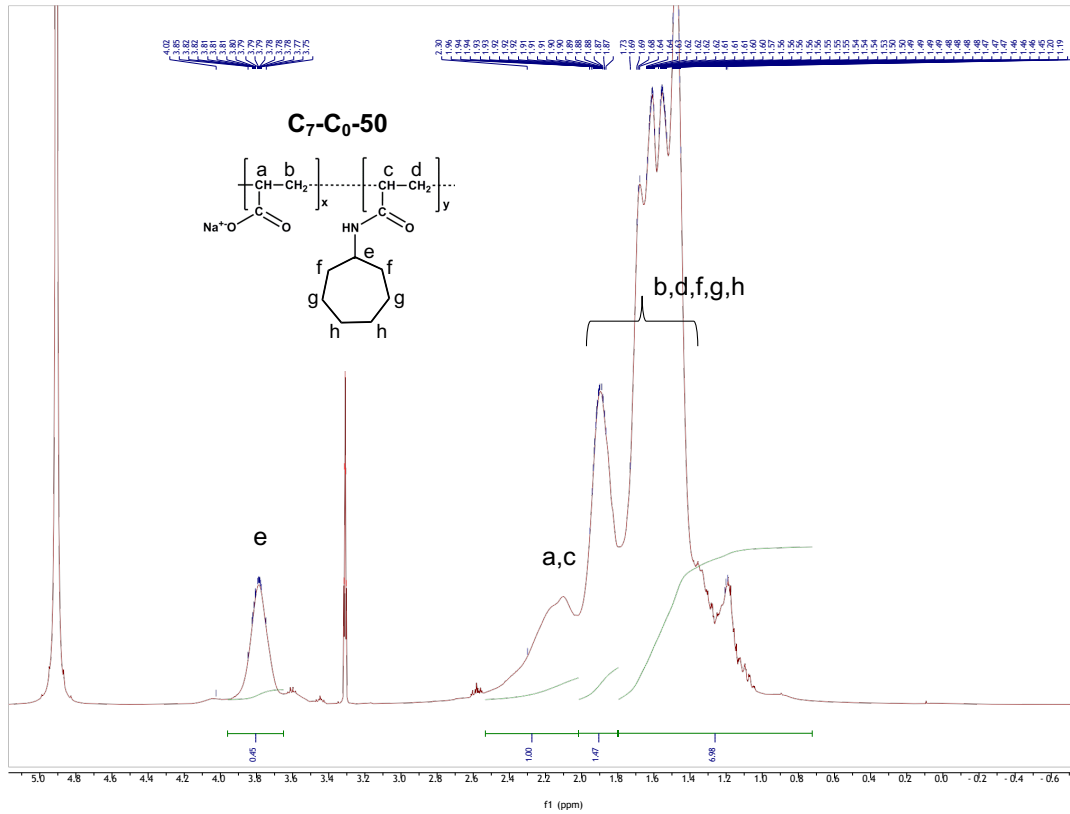
$^1\text{H}$  NMR spectrum of  $\text{C}_6\text{-C}_2\text{-50}$ :



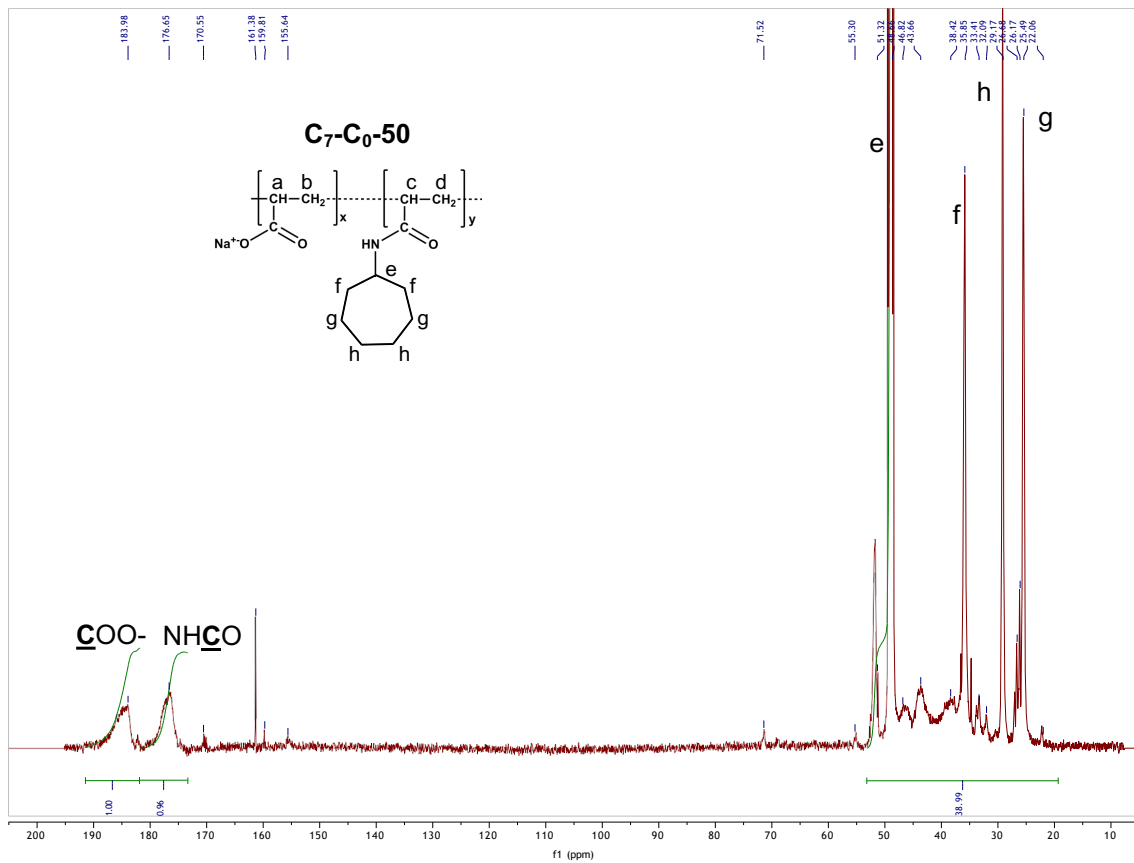
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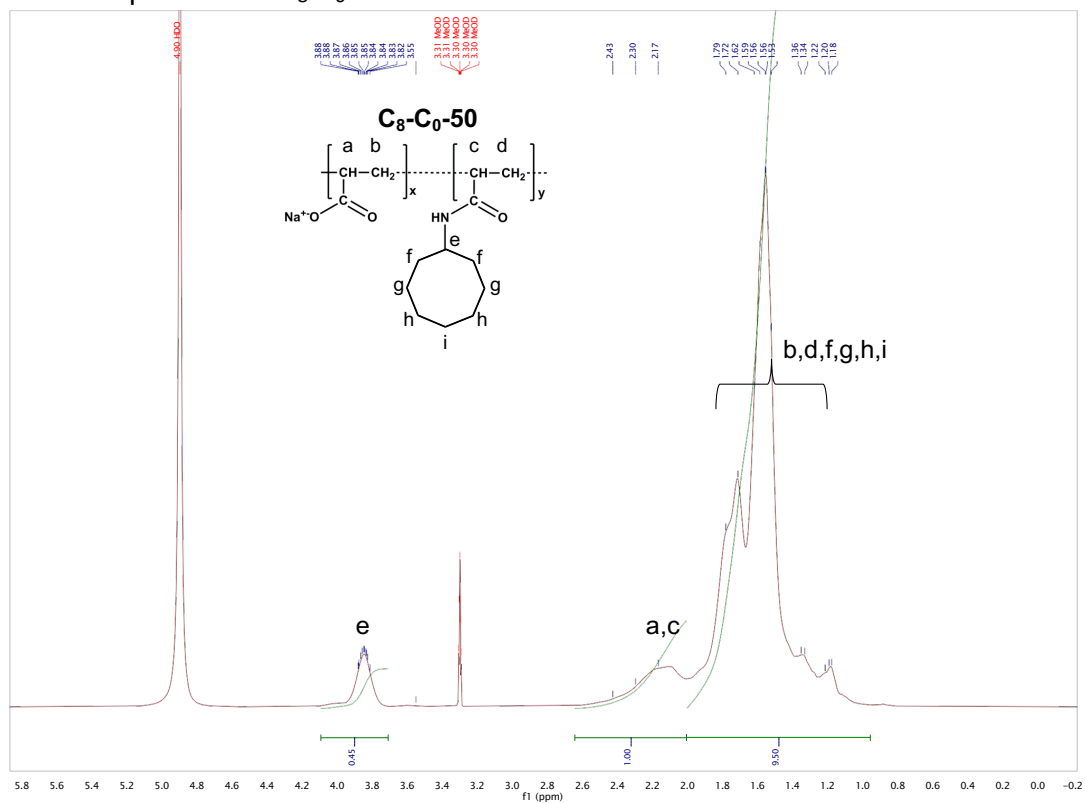
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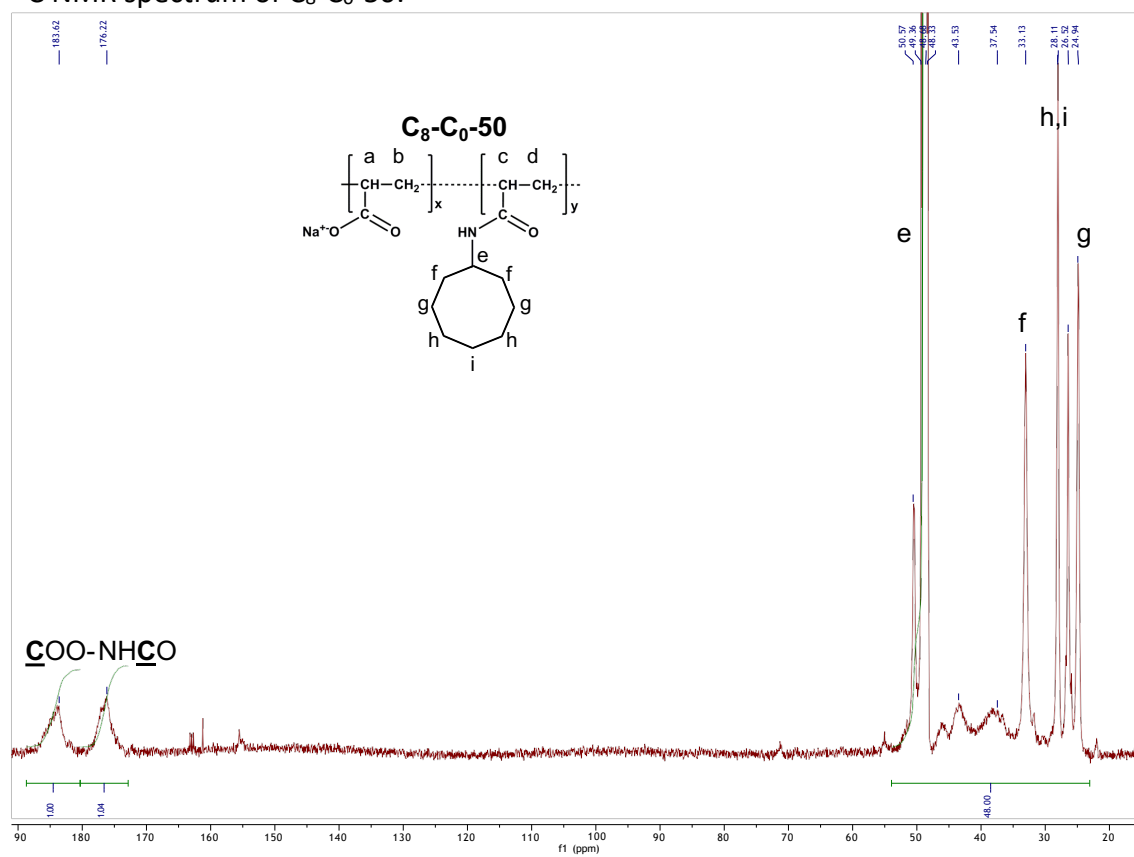
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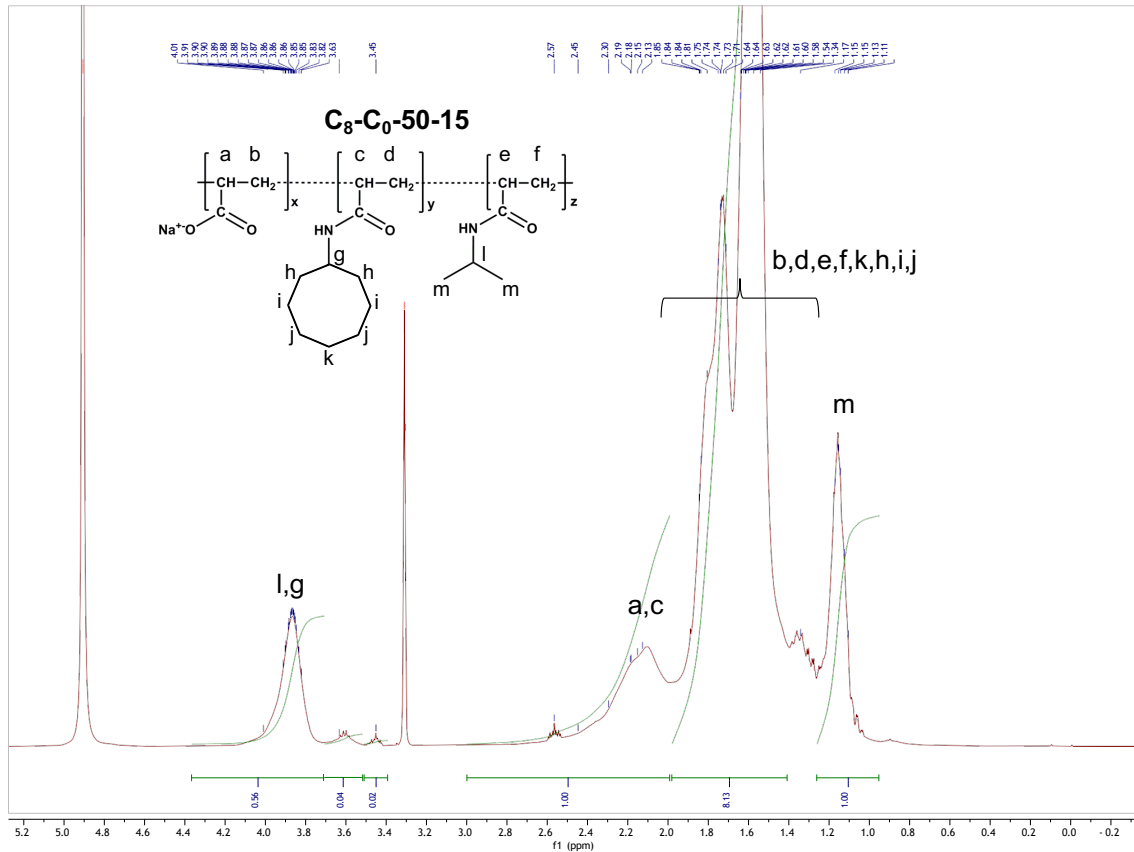
<sup>1</sup>H NMR spectrum of C<sub>8</sub>-C<sub>0</sub>-50:



<sup>13</sup>C NMR spectrum of C<sub>8</sub>-C<sub>0</sub>-50:



$^1\text{H}$  NMR spectrum of  $\text{C}_8\text{-C}_0\text{-50-15}$ :



$^{13}\text{C}$  NMR spectrum of  $\text{C}_8\text{-C}_0\text{-50-15}$ :

