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Hydrophobic Functionalization of Polyacrylic Acid as a Versatile Platform for the Development of Polymer Lipid Nanodisks

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Polymer nanodisks have shown great potential as membrane mimetics that enable the study of functional membrane protein structural biology and also have a wider application in other fields such as drug delivery. To achieve these research goals, the ability to have a cheap, simple, fully customizable platform for future nanodisks technology applications is paramount. Here, a facile functionalization of polyacrylic acid (PAA) with varying hydrophobic groups that form nanodisks at different sizes is successfully demonstrated. The study shows that the choice of hydrophobic group can have a noticeable effect on the polymer solubilization properties and polymer-induced perturbation to the encased lipid bilayer. Due to this robust, tunable chemical synthesis method, PAA is an exciting platform for the future optimization of the hydrophobic, hydrophilic, or direct purposed functionalizations for polymer nanodisks.

Polymer nanodisks have emerged as a new native-like membrane mimetic in recent years and are routinely being used for the study of membrane proteins (MPs).[1–3] A major advantage of using polymers is that they can directly extract MPs from their native environment as shown by the styrene-maleic acid copolymers (SMA).[1,2,4–6] Despite the great potential, polymer nanodisks have suffered from some major drawbacks due to the intrinsic chemical properties of SMA inherent to its hydrophobic and hydrophilic units.[2,7] Several studies have already shown an enhancement of SMA's stability by the modification of hydrophilic functional units.[7–12] The most common hydrophobic group used in SMA polymer nanodisks is the styrene moiety. It is known that styrene has a strong absorption in the UV region that can interfere with various biophysical techniques, and can also have nonspecific interactions with other aromatic groups from the protein.[13,14] Recently, two styrene-free polymers (diisobutylene maleic acid co-polymer,[14] DIBMA, and polymethacrylate[13]) have been shown to form nanodisks; however there has been no systematic investigation comparing the effects of varying the hydrophobic functional groups used in the formation of nanodisks. In this study, we employed a simple and robust modification method to vary the hydrophobic groups on a commercially available low molecular weight polyacrylic acid (PAA) which allowed us to observe how alkyl-PAA affected the formation, stability, and other properties of nanodisks.

In the past hydrophobically modified PAAs were shown to act as amphipols which have the ability to form pores in a membrane and solubilize MPs.[15,16] We hypothesized that modifying a short chain PAA with relatively short alkyl groups (4–6 carbons), as compared to the long alkyl groups (>8 carbons) used in the formation of amphipols,[17] would produce an amphiphilic polymer with the ability to form nanodisks. To test this hypothesis, we used a low molecular weight (M_w = 1800 Da) PAA as the starting material. Different hydrophobic groups (butyl, pentyl, hexyl, and neopentyl) were chosen to functionalize PAA. To achieve this, we applied a simple condensation reaction scheme to PAA using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride as our coupling reagent and the appropriate alkyl amine as the reactant (**Figure 1**a; Supporting information). The resulting polymer was characterized using Fourier transform infrared spectroscopy (FT-IR), proton-nuclear magnetic resonance spectroscopy (^{1}H-NMR), and carbon-13-cross polarization magic angle spinning (^{13}C-CPMAS) solid-state NMR experiments. FT-IR spectra show amide stretching frequency (≈1640 cm^{-1}) for the products confirming the successful completion of the coupling reaction (Figure 1b). This was further confirmed by the carbonyl carbon resonance (≈185–180 ppm) and the appearance of new peaks in the aliphatic region (CH_2 ≈32 ppm, CH_3 ≈17 ppm, and quaternary C≈54 ppm) in ^{13}C-CPMAS NMR spectra of the synthesized polymers as compared to PAA starting material (Figure 1c). ^{1}H-NMR spectra were used to estimate the extent of functionalization by integration (Figure S1, Supporting Information) to be ≈40–50%, within the range of optimal hydrophobic to hydrophilic ratio as seen previously for nanodisks.[13]

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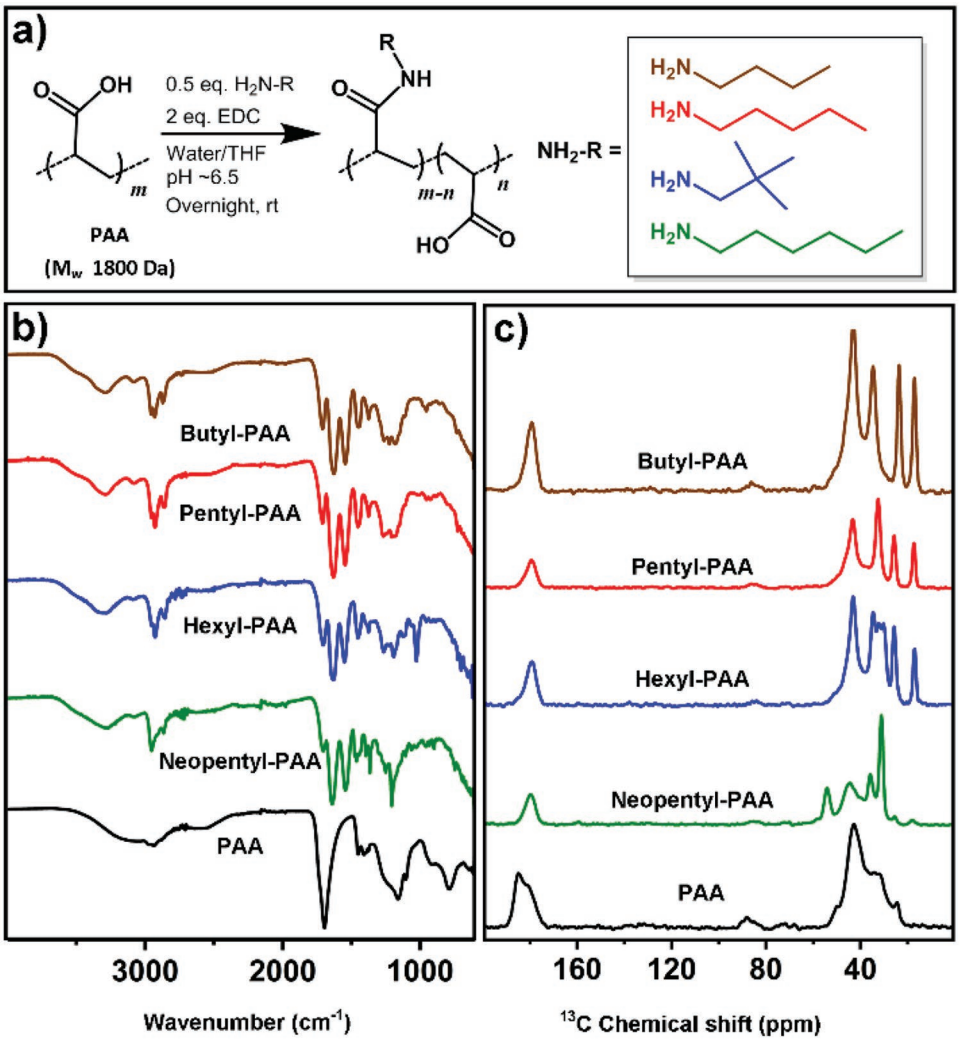

Figure 1. Synthesis and characterization of PAA polymers. a) General reaction schematic of PAA functionalization. b) FT-IR and c) ^{13}C-CPMAS solid-state NMR spectra of functionalized alkyl-PAA.

The resulting polymers were mixed with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) at different polymer:lipid weight ratios (0.25:1, 0.5:1, 1:1). Static light scattering (SLS) measurements were used to monitor the solubilization of DMPC multilamellar vesicles (MLVs). While the Pentyl-PAA, Neopentyl-PAA, and Hexyl-PAA were found to solubilize DMPC vesicles into small particles, the Butyl-PAA needed a much higher amount of polymer to achieve solubilization (>1:1) and therefore was not used in further studies (Figure S2, Supporting Information). In order to investigate the size distribution of the polymer nanodisks, we used size exclusion chromatography (SEC) and dynamic light scattering (DLS; **Figure 2**). Polymers were mixed with DMPC MLVs at appropriate weight ratios and then incubated overnight at 32 °C. SEC chromatograms showed the presence of two peaks: nanodisks eluted within the region of 9–12 mL whereas the free polymer eluted at 15–20 mL. All three polymers showed size tunability by varying the polymer:lipid ratio. We concluded this by the observation of a shift in the retention volume of the nanodisk's peak and no shift in the retention volume of the free polymer peak in the SEC profiles. Neopentyl-PAA and Pentyl-PAA nanodisks showed a major increase in the intensity of the polymer peak only at high polymer:lipid ratios. Hexyl-PAA showed the presence of new peaks (8.5 and 14.3 mL) and no shift in the nanodisk's peak at high polymer:lipid ratio, suggesting a saturation point in the polymer:lipid ratio needed to form nanodisks was reached (Figure 2a–c). The corresponding DLS profiles showed size variation as a function of polymer:lipid ratio (Figure 2d–f, **Table 1**) and were in good agreement with SEC observations similar to previous observations using SMA derivatives.[7,8]

The polymer nanodisks were further characterized using transmission electron microscopy (TEM). The TEM images of the polymer nanodisks with differing weight ratios clearly showed the presence of disk shaped particles of varying sizes complementary to SEC and DLS results (Figure 2g–o). These results confirmed that these polymers can form nanodisks and the size of nanodisks can be controlled by varying the polymer:lipid ratio.

The stability of the nanodisks to the presence of divalent metal ions and pH were tested using SLS. Akyl-PAA polymers

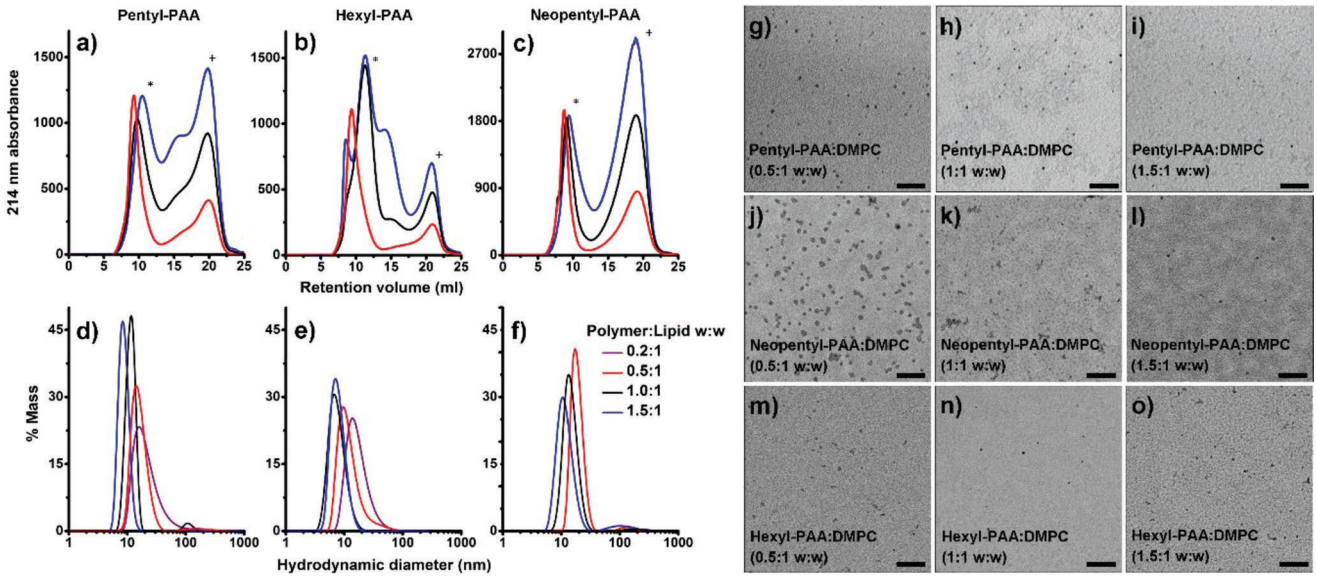

Figure 2. Characterization of PAA polymer nanodisks. a–c) SEC and d–f) DLS profiles of nanodisks prepared at the indicated polymer:lipid ratios. (*) denotes nanodisks fractions collected and (+) denotes free polymer fraction. TEM images g–o) of samples prepared at the specified polymer:lipid ratio; scale bar represents 200 nm.

were found to have very similar stability properties toward pH and metal ions as compared to SMALP due to the presence of carboxylic groups as the hydrophilic functional group (Table 1, Figures S3–S5, Supporting Information). Due to the absence of aromatic moieties, the akyl-PAA polymers showed no absorbance at 254 nm (Figure S6, Supporting Information). The effect of polymer hydrophobic group on lipid bilayer properties was studied using differential scanning calorimetry (DSC) experiments. The DSC profiles of most polymer-DMPC-nanodisks showed a typical gel to liquid crystalline phase transition temperature (T_m) in the range of ≈25–27 °C, which is very close to pure DMPC's T_m = 24 ± 1 °C, suggesting the preservation of lipid dynamics upon the formation of nanodisks (Table 1, Figure S7, Supporting Information). Pentyl-PAA showed only a 1 °C change in the phase transition temperature between the low (0.2:1 w:w) and high (1:1 w:w) polymer:lipid ratios, signifying only a minor perturbation of the lipid bilayer. Neopentyl-PAA nanodisks were seen to have both a similar major transition temperature (≈26 °C) and a minor lower transition temperature at ≈19 °C. This observation may be interpreted as Neopentyl-PAA increasing the disorder of those lipids located close to the polymer-belt of the nanodisks. Hexyl-PAA at a low polymer:lipid ratio exhibited a similar behavior to that observed for Pentyl-PAA, whereas at a higher polymer:lipid ratio the transition temperature is shifted from ≈26 to ≈22 °C and is significantly broadened. This change is likely due to a strong perturbation of the lipid bilayer by the longer alkyl chain.

Since the size of PAA polymer based nanodisks were easily tunable, we prepared macro-nanodisks and examined their ability to spontaneously align in the presence of a magnetic field. All three types of polymer macro–nanodisks were tested with the lowest polymer:lipid ratio possible (Table 1) using ^{31}P and ^{14}N static solid-state NMR experiments. The ^{31}P-NMR spectra were recorded at different temperatures ranging from 280 to 320 K. ^{31}P-NMR showed the appearance and disappearance of two main peaks at ≈–1.5 ppm and in the ≈–12 to ≈–14 ppm region as a function of temperature. The peak at ≈–1.5 ppm is due to the fast tumbling of isotropic nanodisks, whereas the peak at ≈–12 to ≈–14 ppm is indicative of macro–nanodisks with the lipid phosphate head groups aligned perpendicular to the magnetic field axis. Pentyl-PAA macro-nanodisks showed an isotropic peak (≈–1.5 ppm) at 280 K. Partial alignment of the nanodisks was seen (≈–12 ppm) at 285–290 K, and complete alignment above 295 K (**Figure 3**a). Hexyl-PAA macro–nanodisks showed alignment similar to Pentyl-PAA macro–nanodisks, however, they required a higher temperature (300 K) to fully align, and at 320 K a small isotropic peak was observed. Neopentyl-PAA macro–nanodisks had similar characteristics of Pentyl-PAA macro–nanodisks at lower temperatures (<310 K) above which a large isotropic signal was observed

Table 1. Properties of alkyl-PAA-DMPC-nanodisks.

Hydrophobic group	Polymer/lipid (w/w)	Size[a)] [nm]	PDI	Elution volume [mL]	pH stability[b)]	Mg^{2+}, Ca^{2+} stability[c)] [$\times 10^{-3}$ M]	DSC transition temperature [°C]
Pentyl	0.2, 0.5, 1, 1.5	16, 14, 12, 8	1.7, 0.25, 0.11, 0.14	nd, 9.3, 9.7, 10.5	>6	5.5, 3.5	26, nd, 25, nd
Hexyl	0.2, 0.5, 1, 1.5	14, 10, 7, 7	1.0, 0.63, 0.31, 0.27	nd, 9.3, 11.3, 11.3	>6	2, 2	26, nd, 22, nd
Neopentyl	0.3, 0.5, 1, 1.5	nd, 17, 13, 10	nd, 0.39, 0.23, 0.27	nd, 8.7, 9.1, 9.5	>6.5	5.5, 2	28, nd, 26, nd

[a)]hydrodynamic diameter measured from DLS; [b,c)]Measured from SLS. nd denotes not determined. polydispersity index (PDI) from DLS.

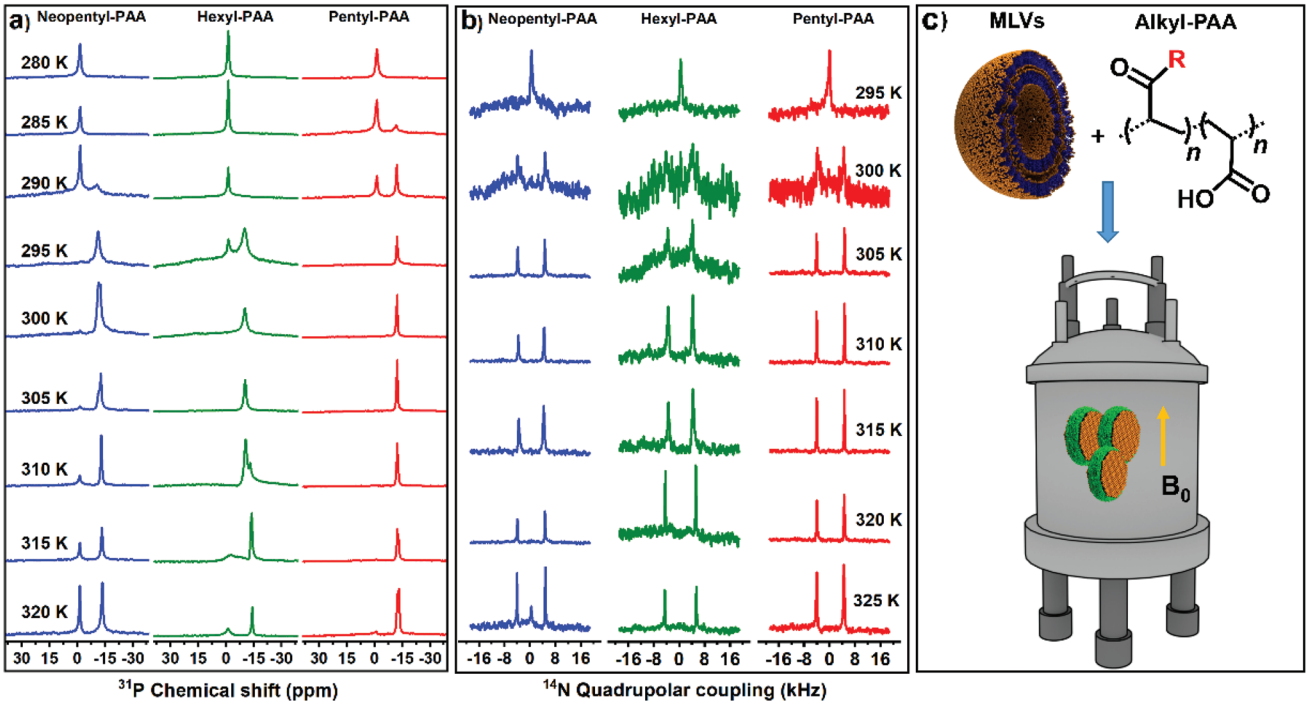

Figure 3. Magnetic alignment of PAA based macro-nanodisks. a) ^{31}P and b) ^{14}N NMR spectra of macro–nanodisks prepared from Neopentyl-PAA:DMPC (0.3:1 w/w), Hexyl-PAA:DMPC (0.2:1 w/w), and Pentyl-PAA:DMPC (0.2:1 w/w) at the indicated temperatures. c) Schematic representation of magnetic-alignment of macro–nanodisks.

suggesting less stability at higher temperature as compared to Pentyl-PAA. A similar trend was observed using ^{14}N-NMR. The quadrupolar coupling of ^{14}N nuclei is a direct measurement of the orientation of choline group (C—N bond vector) relative to the magnetic field direction. While at low temperature ^{14}N peaks were isotropic, at higher temperatures a quadrupolar coupling of 7–8 kHz was observed further confirming the magnetic alignment of macro–nanodisks with lipid bilayer normal oriented perpendicular to the magnetic field (Figure 3b).

To demonstrate that these new polymers can be used for direct MP extraction from cellular membranes, we incubated the polymer with cell lysate following a published protocol[2] (Supporting information). All three alkyl-PAA polymers showed similar efficacy as compared to SMALP as evident from the SDS-PAGE gel (Figure S8, Supporting information). These observations suggest that the hydrophobic modifications of PAA have the ability to extract MPs from their native environment.

In conclusion, we have successfully demonstrated the facile functionalization of PAA with differing hydrophobic groups that form nanodisks at varying sizes. By using a variety of techniques (SLS, DLS, SEC, TEM, DSC, and NMR) we have shown that the method of functionalization proved robust with multiple different sidechains that form nanodisks. This allowed us to systematically probe the important effects of differing hydrophobic functionalization on the intrinsic properties polymer nanodisks, which have yet to be seen. We show that the choice of hydrophobic group can have a noticeable effect on the polymer solubilization properties. Using these polymers we can control the extent of lipid bilayer perturbation which is vital for ensuring a more native like membrane environment. Due to this robust, tunable chemical synthesis method, PAA is an exciting platform for future optimization of the hydrophobic, hydrophilic, or direct purposed functionalization's for polymer nanodisks.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

functionalization, magnetic alignment, membrane mimetic, nanomaterials, polymer nanodisks

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