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

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Polymer nanodiscs 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 nanodiscs technology applications is paramount. Here, we successfully demonstrated a facile functionalization of polyacrylic acid (PAA) with varying hydrophobic groups that form nanodiscs at different sizes. Our 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 future optimization of the hydrophobic, hydrophilic, or direct purposed functionalization's for polymer nanodiscs.

Polymer nanodiscs 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 membrane proteins from their native environment as shown by the styrene-maleic acid copolymers (SMA).[1, 2, 4-6] Despite the great potential, polymer nanodiscs 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 nanodiscs 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 nanodiscs; however there has been no systematic investigation comparing the effects of varying the hydrophobic functional groups used in the formation of nanodiscs. In this study, we employed a simple and robust modification method to vary the hydrophobic groups on a commercially available low molecular weight PAA which allowed us to observe how alkyl-PPA affected the formation, stability, and other properties of nanodiscs.

In the past hydrophobically modified PAAs were shown to act as amphipols which have the ability to form pores in a membrane and solubilize membrane proteins.[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 nanodiscs. 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 (EDC) as our coupling reagent and the appropriate alkyl amine as the reactant (**Figure 1a**, **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)** to be ~40-50%, within the range of optimal hydrophobic to hydrophilic ratio as seen previously for nanodiscs.[13]

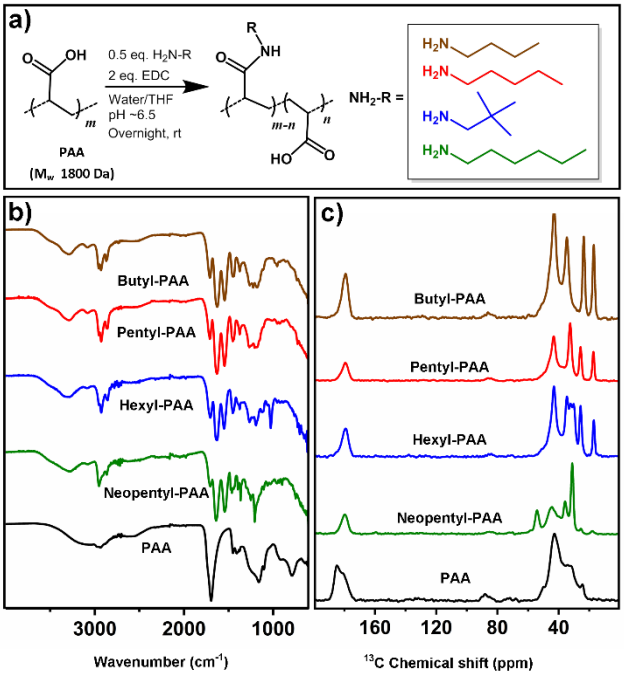

Figure 1. Synthesis and characterization of polyacrylic acid polymers. a) General reaction schematic of PAA functionalization. b) FT-IR and c) ^{13}C-CPMAS solid-state NMR spectra of functionalized alkyl-PAA.

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**). In order to investigate the size distribution of the polymer nanodiscs, 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: nanodiscs

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 nanodisc's peak and no shift in the retention volume of the free polymer peak in the SEC profiles. Neopentyl-PAA and Pentyl-PAA nanodiscs 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 mL and 14.3 mL) and no shift in the nanodisc's peak at high polymer:lipid ratio, suggesting a saturation point in the polymer:lipid ratio needed to form nanodiscs was reached (**Figure 2 a-c**). The corresponding DLS profiles showed size variation as a function of polymer:lipid ratio (**Figure 2(d-f), Table 1**) and were in good agreement with SEC observations similar to previous observations using SMA derivatives.[7, 8]

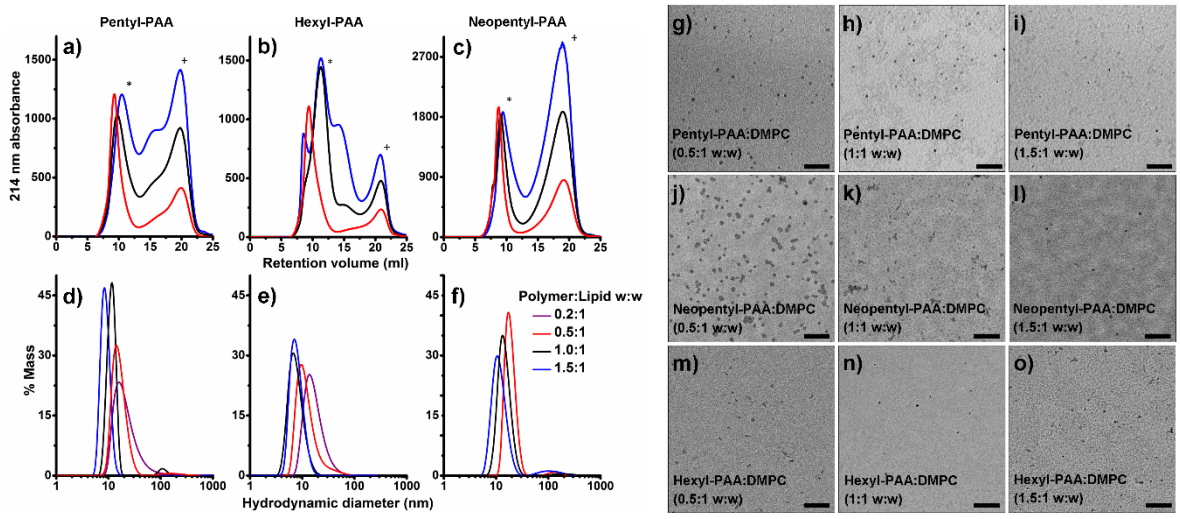

Figure 2. Characterization of PAA polymer nanodiscs. SEC (a-c) and DLS (d-f) profiles of nanodiscs prepared at the indicated polymer:lipid ratios. (*) denotes nanodiscs fractions collected and (+) denotes free polymer fraction. TEM images (g-o) of samples prepared at the specified polymer:lipid ratio;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

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

The stability of the nanodiscs to the presence of divalent metal ions and pH were tested using SLS. Akyl-PAA polymers were found to have very similar stability properties towards pH and metal ions as compared to SMALP due to the presence of carboxylic groups as the hydrophilic functional group (**Table 1, Figure S3-S5**). Due to the absence of aromatic moieties, the akyl-PAA polymers showed no absorbance at 254 nm (**Figure S6**). 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-nanodiscs 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 nanodiscs (**Table 1, Figure S7**). 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 nanodiscs 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 nanodiscs. 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.

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

Hydrophobic group	Polymer/Lipid (w/w)	Size[a] (nm)	PDI	Elution volume (mL)	pH stability[b]	Mg^{2+}, Ca^{2+} stability[c] (mM)	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

ahydrodynamic diameter measured from DLS. b, c measured from SLS. nd denotes not determined. polydispersity index (PDI) from DLS.

Since the size of PAA polymer based nanodiscs were easily tunable, we prepared macro-nanodiscs and examined their ability to spontaneously align in the presence of a magnetic field. All three types of polymer macro-nanodiscs 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 nanodiscs, whereas the peak at ~-12 to ~-14 ppm is indicative of macro-nanodiscs with the lipid phosphate head groups aligned perpendicular to the magnetic field axis. Pentyl-PAA macro-nanodiscs showed an isotropic peak (~-1.5 ppm) at 280 K. Partial alignment of the nanodiscs was seen (~-12 ppm) at 285-290 K, and complete alignment above 295 K (**Figure 3a)**. Hexyl-PAA macro-nanodiscs showed alignment similar to Pentyl-PAA macro-nanodiscs, however, they required a higher temperature (300 K) to fully align, and at 320 K a small isotropic peak was observed. Neopentyl-PAA macro-nanodiscs had similar characteristics of Pentyl-PAA macro-nanodiscs at lower temperatures (<310 K) above which a large isotropic signal was observed 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-nanodiscs with lipid bilayer normal oriented perpendicular to the magnetic field. (**Figure 3b**).

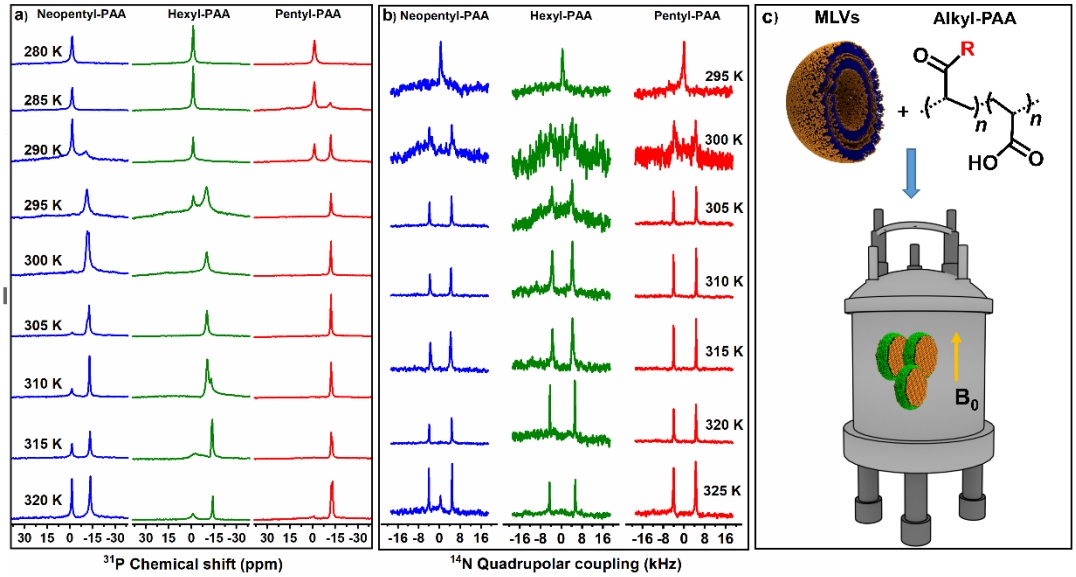

Figure 3. Magnetic-alignment of PAA based macro-nanodiscs. ^{31}P (a) and ^{14}N (b) NMR spectra of macro-nanodiscs prepared from Neopentyl-PAA:DMPC (0.3:1 w/w), Hexyl-PAA:DMPC (0.2:1 w/w), and Pentyl-PAA:DMPC (0.2:1 w/w) at the indicated temperatures. (c) Schematic representation of magnetic-alignment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

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

In conclusion, we have successfully demonstrated the facile functionalization of PAA with differing hydrophobic groups that form nanodiscs 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 nanodiscs. This allowed us to systematically probe the important effects of differing hydrophobic functionalization on the intrinsic properties polymer nanodiscs, 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 nanodiscs.

Supporting Information

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

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References

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

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

[3] J. M. Dörr, M. C. Koorengevel, M. Schäfer, A. V. Prokofyev, S. Scheidelaar, E. A. W. van der Cruijsen, T. R. Dafforn, M. Baldus, J. A. Killian, Proc. Natl. Acad. Sci. U.S.A. 2014, 111, 18607.

[4] 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.-H. Javed, N. P. Chmel, T. R. Dafforn, A. J. Rothnie, Biochem. J. 2016, 473, 4349.

[5] Z. Hu, J. C. S. Ho, M. Nallani, Curr. Opin. Biotechnol. 2017, 46, 51.

[6] 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, 3.

[7] T. Ravula, N. Z. Hardin, S. K. Ramadugu, S. J. Cox, A. Ramamoorthy, Angew Chem Int Ed Engl 2018, 57, 1342.

[8] T. Ravula, S. K. Ramadugu, G. Di Mauro, A. Ramamoorthy, Angew Chem Int Ed Engl 2017, 56, 11466.

[9] T. Ravula, N. Z. Hardin, S. K. Ramadugu, A. Ramamoorthy, Langmuir 2017, 33, 10655.

[10] S. Lindhoud, V. Carvalho, J. W. Pronk, M. E. Aubin-Tam, Biomacromolecules 2016, 17, 1516.

[11] M. C. Fiori, Y. Jiang, G. A. Altenberg, H. Liang, Sci Rep 2017, 7, 7432.

[12] S. C. L. Hall, C. Tognoloni, J. Charlton, E. C. Bragginton, A. J. Rothnie, P. Sridhar, M. Wheatley, T. J. Knowles, T. Arnold, K. J. Edler, T. R. Dafforn, Nanoscale 2018, 10, 10609.

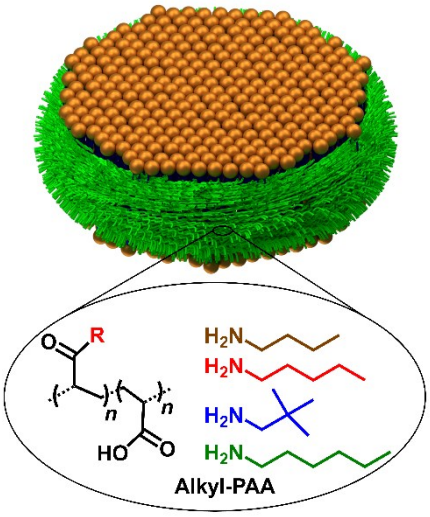
[13] K. Yasuhara, J. Arakida, T. Ravula, S. K. Ramadugu, B. Sahoo, J. I. Kikuchi, A. Ramamoorthy, J Am Chem Soc 2017, 139, 18657.

[14] A. O. Oluwole, B. Danielczak, A. Meister, J. O. Babalola, C. Vargas, S. Keller, Angew Chem Int Ed Engl 2017, 56, 1919.

[15] F. Vial, A. G. Oukhaled, L. Auvray, C. Tribet, Soft Matter 2007, 3, 75.

[16] Y. Gohon, F. Giusti, C. Prata, D. Charvolin, P. Timmins, C. Ebel, C. Tribet, J. L. Popot, Langmuir 2006, 22, 1281.

[17] J. L. Popot, T. Althoff, D. Bagnard, J. L. Baneres, P. Bazzacco, E. Billon-Denis, L. J. Catoire, P. Champeil, D. Charvolin, M. J. Cocco, G. Cremel, T. Dahmane, L. M. de la Maza, C. Ebel, F. Gabel, F. Giusti, Y. Gohon, E. Goormaghtigh, E. Guittet, J. H. Kleinschmidt, W. Kuhlbrandt, C. Le Bon, K. L. Martinez, M. Picard, B. Pucci, J. N. Sachs, C. Tribet, C. van Heijenoort, F. Wien, F. Zito, M. Zoonens, Annu Rev Biophys 2011, 40, 379.



Functionalized polyacrylic acid polymers with varying hydrophobic groups form lipid-nanodiscs. PAA polymer nanodiscs are size tunable by simply varying the lipid:polymer ratio and macro-nanodiscs exhibit magnetic alignment. The facile functionalization enabled the investigation of the effect of hydrophobic group of PAA on the nanodisc formation and lipid bilayer properties.