ChemComm

Self-Assembly/Disassembly of Giant Double-Hydrophilic Polymersomes at Biologically-Relevant pHs

Journal:	*ChemComm*
Manuscript ID	CC-COM-06-2018-005155.R1
Article Type:	Communication

Journal Name

COMMUNICATION

Self-Assembly/Disassembly of Giant Double-Hydrophilic Polymersomes at Biologically-Relevant pH

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

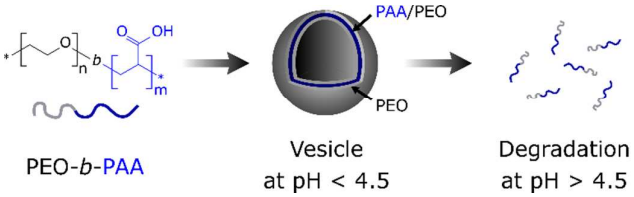
www.rsc.org/

Sun Hae Ra Shin,[a] Patrick T. McAninch,[a] Ian M. Henderson,[a,b] Andrew Gomez,[c] Adrienne C. Greene,[a] Eric C. Carnes [d] and Walter F. Paxton*[a]

Self-assembled giant polymer vesicles prepared from double-hydrophilic diblock copolymers, poly(ethylene oxide)-*b*-poly(acrylic acid) (PEO-PAA) show significant degradation in response to pH changes. Because of the switching behavior of the diblock copolymers at biologically-relevant pH environments (2 to 9), these polymer vesicles have potential biomedical applications as smart delivery vehicles.

Self-assembly of vesicles from amphiphilic block copolymers in polar media is typically driven by the phase separation of each block to form a hydrophobic membrane and hydrophilic corona that partitions internal components from external fluid.[1-2] Owing to the chemical versatility of polymeric building blocks, polymer vesicles have been explored for various applications in catalysis as nanoreactors[3-4] and in the biomedical field as diagnostic imaging probes, artificial organelles, and therapeutic delivery vehicles.[5] For effective delivery of therapeutics, it is highly desirable to design smart polymer vesicles that can release payloads in a controlled manner at target sites and under specific environmental conditions.[6-7]

Since polymer vesicles can load both hydrophilic cargos within the aqueous interior and hydrophobic cargos within the membrane, the integrity of the membrane plays a key role in the release of contents encapsulated in polymer vesicles. Consequently, one strategy to control the release of vesicle contents aims to disintegrate the membrane or to create pores in response to stimuli such as temperature, pH, redox, light or external fields.[8] Compared to other stimuli, pH-responsive systems have been studied extensively[9-11] because some of these systems can exploit biologically-relevant differences in pH. For example, the human gastrointestinal tract has different pH values in the stomach (pH = 1.0–2.5), small intestine (pH = 6.2–7.9), and colon (pH = 5.2–7.0).[12] Even within a cell, cytosol is nearly neutral (pH = 7.4) while the lysosome is acidic (pH = 4.5–5.0).[8] Therefore, biological changes in pH can be used to trigger the release of encapsulated contents to a certain tissue or cellular compartment.



Scheme 1 Formation of PEO-PAA polymer vesicles

[a] *Center for Integrated Nanotechnologies, Sandia National Laboratories, Albuquerque, NM 87185, USA. Email: wfpaxto@sandia.gov*
[b] *Omphalos Bioscience LLC, Albuquerque, NM 87110, USA.*
[c] *Nano and Micro Sensors, Sandia National Laboratories, Albuquerque, NM 87185, USA.*
[d] *Office of Research and Economic Development, University of Nebraska – Lincoln, Lincoln, NE 68588, USA.*

† Electronic Supplementary Information (ESI) available: Experimental details, z-stack of PEO-PAA vesicles, solubility of PEO-PAA at different pH, ζ potential distributions, cryoEM images, size distribution, and yields of PEO-PAA vesicles, ^{1}H NMR spectra of as-received PEO-PAA, swollen polymer droplets, and dye encapsulation efficiency and release from PEO-PAA vesicles (PDF). See DOI: 10.1039/x0xx00000x

Double-hydrophilic copolymers containing different hydrophilic blocks can self-assemble to form stable vesicles in aqueous solution despite having no apparent hydrophobic block.[13-15] In response to changes in solution pH or temperature, one of the blocks becomes less hydrophilic rendering the copolymer able to assemble thermodynamically favoured structures like those formed by conventional amphiphiles. Self-assembly of double-hydrophilic copolymers consisting of poly(ethylene oxide) (PEO) and poly(acrylic acid) (PAA) is intriguing because not only is PAA responsive to changes in pH, but also PAA can participate in hydrogen bonding interactions with itself or PEO under appropriate pH conditions.[16-17] The hydrogen bonding between PEO and PAA blocks produces pH-sensitive nanoaggregates,[18-19] and previous reports have shown that phase separation of PEO-PAA copolymers, with appropriate additives, can also be induced to form micelles (with $CaCl_2$[20] or oligochitosan[21]) and vesicles (with α-cyclodextrin[22]). Surprisingly, despite the ability of PEO-PAA copolymers to form nanoaggregates that appear as hollow capsules,[19] the formation of pH-switchable vesicular structures that are amenable to visualization of membrane dynamics via optical microscopy has not been reported.

Here, we present a class of pH-sensitive polymer vesicles prepared by the self-assembly of double-hydrophilic PEO-PAA diblock copolymers (Scheme 1). To the best of our knowledge, this is the first work that shows the formation of micron-sized vesicles of PEO-PAA copolymers. We survey the self-assembled morphologies of PEO-PAA copolymers with different PEO fractions and demonstrate substantial degradation of giant polymer vesicles by adjusting solution pH. Furthermore, these vesicles are capable of encapsulating and releasing small molecule cargo, suggesting that PEO-PAA vesicles have great potential as carriers for smart drug delivery applications.

Table 1 Details of double hydrophilic diblock copolymers

Designated name	Formula[a]	f_{EO}[b]
EA1	EO_{45}-AA_{56}	0.33
EA2	EO_{45}-AA_{21}	0.57
EA3	EO_{68}-AA_{18}	0.70

[a]Determined based on peak integration of AA block with respect to EO block (ESI†). [b]Mass fraction of EO block.

There are many strategies for preparing giant polymer vesicle assemblies.[23-25] The polymer vesicles described here were prepared by the gel-assisted rehydration method (ESI†).[26] Giant PEO-PAA (**EA1**) vesicles were observed by fluorescence microscopy when the polymer film was rehydrated with pH 2.3 solution (Fig. 1a,b). Confocal microscopy of one representative vesicle revealed a thin membrane surrounding a dark center. Intensity profile across the object showed pronounced intensities near the edges. A series of images that were taken at different focal planes (z-stack) showed a membrane surrounding a dark center throughout the focal planes (Fig. S1). The results suggest that

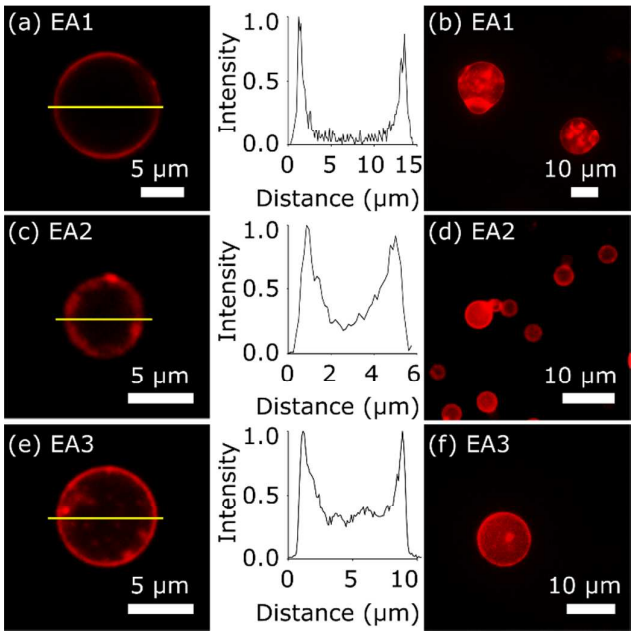


Fig. 1 Formation of PEO-PAA vesicles. (a,c,e) Confocal micrographs, normalized intensity profiles, and (b,d,f) epifluorescence micrographs of **EA1**, **EA2**, **EA3** vesicles at pH 2.3

the objects are vesicles with hollow interiors. Though the centers of the resulting objects observed using epifluorescence microscopy looked less dark than analysis by confocal microscopy, these images still supported vesicle structures (Fig. 1b). The resulting vesicles were polydisperse with 8.0 ± 3.3 μm diameter.

At pH = 2.3, the carboxylic groups (–COOH) on the PAA blocks are protonated (pK_a of COOH ~ 4.5)[27] increasing the hydrogen bonding interactions with itself and the PEO block resulting in aggregation of the polymer chains, and providing a mechanism for phase segregation of the complex of otherwise soluble polymers (Fig. S2). By contrast, no vesicle formation was observed when the polymer film was rehydrated with pH 9.0 solution (Fig. S3). At this pH, the PAA block is fully deprotonated, substantially reducing its ability to donate hydrogen bonds in the complex, resulting in complete dissolution of both polymer blocks in basic aqueous solution.

We hypothesize that the protonated PAA block favourably forms a membrane to be concealed from its polar surroundings while the PEO block forms the corona under acidic conditions. To test this hypothesis, we investigated the outside surface property of the PEO-PAA and poly(ethylene oxide)-*b*-polybutadiene (PEO-PBD) vesicles by the ζ potential measurement (Fig. S4). The ζ potential of the PEO-PAA vesicles was measured to be -11.3 ± 0.6 mV at pH 2.4. In comparison, the ζ potential of PEO-PBD vesicles which the outside corona consists of only PEO blocks was measured to be -8.9 ± 0.7 mV at pH 2.4. The similar ζ potential values of PEO-PAA and PEO-PBD vesicles suggest that PEO block forms the corona in the PEO-PAA vesicles. By contrast, the ζ potential of poly(acrylic acid)-*b*-polystyrene (PAA-PS) vesicles in which the outside corona consists of only PAA blocks is reported to be ~ -6 mV

under the similar condition at pH 3.[28] Due to the neutral, protonated form of the PAA blocks at pH < 4.5, the ζ potential of PAA-PS vesicles is expected to be less negative than PEO-PBD vesicles at pH 2-3. Thus, the observed ζ potential for our vesicles was more consistent with a PEO, rather than a PAA, corona. We note that some degree of inter/intra molecular hydrogen bonding between the protonated PAA and the PEO is likely associated with self-assembly of the polymer to form these vesicle membranes,[29-30] and our understanding of the exact nature of the vesicle membranes is still incomplete.

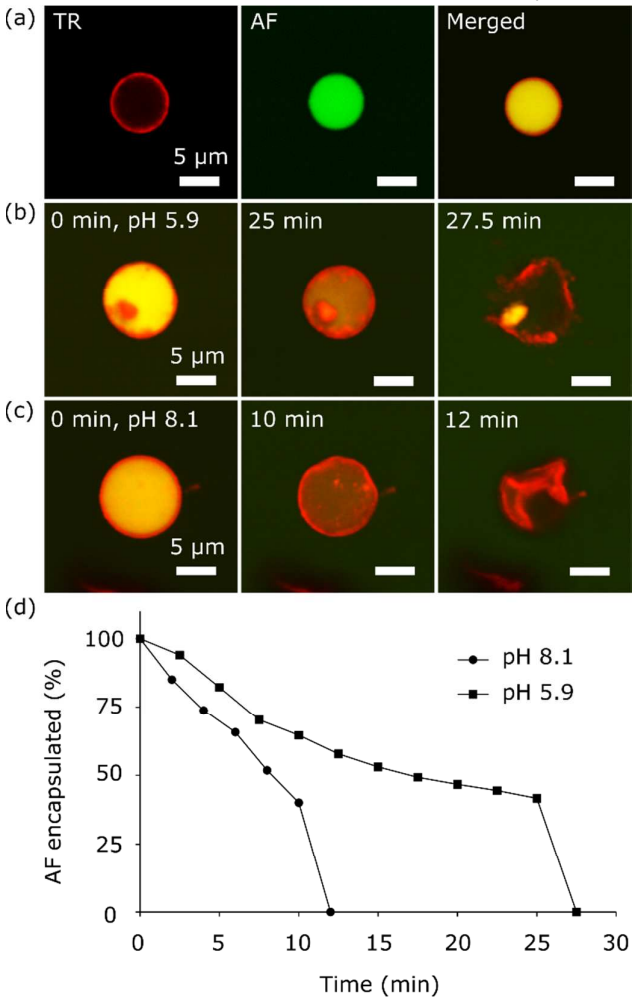


Fig. 2 Encapsulation and release of Alexa Fluor 488 (AF) dye from PEO-PAA vesicles labeled with Texas Red DHPE (TR). (a) Confocal micrographs of **EA3** vesicle with TR filter, AF filter, and merged channel show encapsulation of hydrophilic AF dye (green) within a giant polymer vesicle (red). (b,c) Confocal micrographs of a **EA3** vesicle with merged channel (b) at pH 5.9 and (c) at pH 8.1 show degradation of the vesicle and complete release of AF dye. (d) AF dye release profile as a function of time after addition of NaOH solution.

The morphology of aggregates of amphiphiles driven by the phase separation of two immiscible blocks, such as PEO-PBD, is generally predicted by the relative size of each block.[1, 31] Molecules with a hydrophilic mass fraction (f) > 0.5 are likely to form micelles whereas molecules with 0.25 < f < 0.45 are expected to form vesicles, but it has been noted that this empirical rule can depend strongly on copolymer composition and the conditions applied.[32] A series of PEO-PAA copolymers with different PEO block fractions (f_{EO}) was used to investigate their self-assembled morphologies. The number of monomer units in each block was determined by ^{1}H NMR (Fig. S5) and the result was summarized in Table 1. Based on the f_{EO}, **EA2** and **EA3** are expected to form micelles while **EA1** is expected to form vesicles. Surprisingly, fluorescence microscopy studies revealed giant vesicles for all PEO-PAA copolymers ($0.33 \leq f_{EO} \leq 0.70$) at pH 2.3 (Fig. 1c-f and Fig. S1). The objects from **EA2** (5.3 ± 1.1 μm diameter) and **EA3** (7.3 ± 1.2 μm diameter) each had a thin membrane surrounding a dark center suggesting vesicle structures. The membrane thickness was measured to be 23.6 ± 2.9 nm for **EA2** and 18.5 ± 2.8 nm for **EA3** from the cryo-EM images (Fig. S6). The suspensions also have some smaller aggregates (< 1μm) as quantified by DLS, which were not visible via optical microscopy (Fig. S7). These results show that these PEO-PAA diblock copolymers fall outside the empirical rule of predictive structures based on the hydrophilic block fraction. Previous reports have shown that hydrogen bonding interaction between PEO and PAA can provide a local environment that is substantially less polar than that of the aqueous medium.[29-30] Thus, we suspect that the hydrogen bonding facilitates the vesicle formation of PEO-PAA polymers with higher f_{EO}.

Several features of this system are worth pointing out. We note that the yield of vesicle formation depends on the preparation method that is employed. While gel rehydration produced vesicles, solvent exchange of a PEO-PAA solution from THF to pH = 2.3 water resulted in swollen polymer droplets (Fig. S8). In addition, the number of vesicles formed via rehydration on agarose layer was much less than PEO-PBD due to coexistence of the polymer droplets and hemispheres of vesicles (Fig. S9). The hemispheres were not fully detached from the agarose layer and the number of attached hemispheres was 4 times higher than the number of fully detached vesicles. Finally, the resulting vesicles were responsive to changes in pH, which we observed as a rapid degradation of giant vesicles when the pH of solution was increased to pH 10.2 (Fig. S10). We attribute the loss of vesicle integrity to deprotonation of the PAA block and dissolution of PEO-PAA polymer in basic aqueous solution. This response suggests the possibility of a controlled payload release.

To demonstrate that giant polymer vesicles enable delivery of cargos, we loaded a small molecule cargo within the polymer vesicles by simply rehydrating polymer film with a solution of hydrophilic fluorophore, Alexa Fluor 488 (AF). Successful encapsulation of the dye within the aqueous interior of the PEO-PAA (**EA3**) vesicle was confirmed by confocal microscopy with two filter sets (Fig. 2a): imaging the Texas Red (TR, red channel) revealed spherical structures with pronounced intensities near the edges, indicating vesicles, while the AF (green channel) revealed spherical structures with highest intensity near the middle of the object consistent with dye uniformly distributed throughout the interior of the vesicles. The AF encapsulation efficiency was 73 ± 11 % determined from the initial dye concentration and

concentration inside vesicles after rinsing (Fig. S11). To release the encapsulated AF, the pH of the vesicle suspension was increased to pH > 4.5 by addition of iso-osmolar NaOH solution. At pH 5.9 and pH 8.1, **EA3** vesicles were degraded and released AF dye demonstrating a pH responsive release (Fig. 2b,c and Fig. S12). To characterize the release efficiency, intensity of AF dye was monitored over time by confocal microscopy and normalized to the intensity at t = 0 (Fig. 2d). The encapsulated AF dye was rapidly and completely released within 30 min at biologically-relevant pH ranges.

Our experiments demonstrate that giant polymer vesicles can be successfully formed via self-assembly of double-hydrophilic PEO-PAA diblock copolymers. Owing to a pH-sensitive PAA block, the polymers self-assemble during gel-assisted rehydration to form vesicles at low pH < 4.5 and disassociate at higher pH > 4.5. In contrast with amphiphilic block copolymer based vesicles formed in methanol/water which are converted into micelles at high pH[11], double hydrophilic PEO-PAA copolymer based vesicles formed in aqueous acidic solutions are dissolved completely in basic environment. The ability of these polymers to form vesicles is much less sensitive to changes in relative length of the polymer blocks, compared to amphiphilic polymers. Taken together, the ability to encapsulate small molecule cargo and the pH-driven vesicle degradation makes these polymer vesicles very attractive as smart drug vehicle applications suitable for oral delivery.

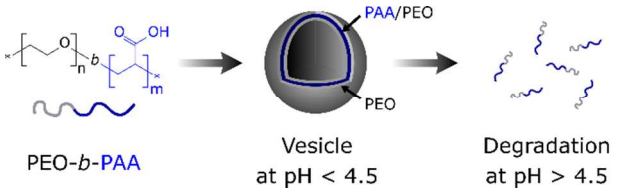
This work was performed in part, at the Center for Integrated Nanotechnologies, an Office of Science User Facility operated for the U.S. Department of Energy (DOE) Office of Science (project number 2017BC0053). Research was supported by the Laboratory Directed Research and Development program at Sandia National Laboratories, a multi-mission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International, Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA-0003525.

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 D. E. Discher and A. Eisenberg, *Science* **2002,** *297,* 967-973.
2 C. LoPresti, H. Lomas, M. Massignani, T. Smart and G. Battaglia, *J. of Mater. Chem.* **2009,** *19,* 3576-3590.
3 K. T. Kim, J. J. L. M. Cornelissen, R. J. M. Nolte and J. C. M. van Hest, *Adv. Mater.* **2009,** *21,* 2787-2791.
4 W. F. Paxton, D. Price and N. J. Richardson, *Soft Matter* **2013,** *9,* 11295-11302.
5 C. G. Palivan, R. Goers, A. Najer, X. Zhang, A. Car and W. Meier, *Chem. Soc. Rev.* **2016,** *45,* 377-411.
6 T. Anajafi and S. Mallik, *Therapeutic Delivery* **2015,** *6,* 521-534.
7 H. De Oliveira, J. Thevenot and S. Lecommandoux, *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2012,** *4,* 525-546.
8 X. Hu, Y. Zhang, Z. Xie, X. Jing, A. Bellotti and Z. Gu, *Biomacromolecules* **2017,** *18,* 649-673.
9 A. E. Felber, M. H. Dufresne and J. C. Leroux, *Adv. Drug Delivery Rev.* **2012,** *64,* 979-992.
10 G. Kocak, C. Tuncer and V. Butun, *Polym. Chem.* **2017,** *8,* 144-176.
11 E. Yoshida, *Colloid Polym. Sci.* **2015,** *293,* 649-653.
12 M. T. Cook, G. Tzortzis, D. Charalampopoulos and V. V. Khutoryanskiy, *J. Controlled Release* **2012,** *162,* 56-67.
13 J. Rodríguez-Hernández and S. Lecommandoux, *J. Am. Chem. Soc.* **2005,** *127,* 2026-2027.
14 J. Du, Y. Tang, A. L. Lewis and S. P. Armes, *J. Am. Chem. Soc.* **2005,** *127,* 17982-17983.
15 C. Feng, Z. Shen, L. Gu, S. Zhang, L. Li, G. Lu and X. Huang, *J. Polym. Sci., Part A: Polym. Chem.* **2008,** *46,* 5638-5651.
16 F. E. Bailey, R. D. Lundberg and R. W. Callard, *J. Polym. Sci., Part A: General Papers* **1964,** *2,* 845-851.
17 K. L. Smith, A. E. Winslow and D. E. Petersen, *Ind. Eng. Chem.* **1959,** *51,* 1361-1364.
18 J. Hao, G. Yuan, W. He, H. Cheng, C. C. Han and C. Wu, *Macromolecules* **2010,** *43,* 2002-2008.
19 E. Khousakoun, J.-F. Gohy and R. Jérôme, *Polymer* **2004,** *45,* 8303-8310.
20 H. R. Sondjaja, T. A. Hatton and K. C. Tam, *Langmuir* **2008,** *24,* 8501-8506.
21 J. Reboul, T. Nugay, N. Anik, H. Cottet, V. Ponsinet, M. In, P. Lacroix-Desmazes and C. Gerardin, *Soft Matter* **2011,** *7,* 5836-5846.
22 J. Liu, H. R. Sondjaja and K. C. Tam, *Langmuir* **2007,** *23,* 5106-5109.
23 B. M. Discher, Y. Y. Won, D. S. Ege, J. C. M. Lee, F. S. Bates, D. E. Discher and D. A. Hammer, *Science* **1999,** *284,* 1143-1146.
24 J. R. Howse, R. A. L. Jones, G. Battaglia, R. E. Ducker, G. J. Leggett and A. J. Ryan, *Nat. Mater.* **2009,** *8,* 507-511.
25 A. Li, J. Pazzi, M. Xu and A. B. Subramaniam, *Biomacromolecules* **2018,** *19,* 849-859.
26 A. C. Greene, I. M. Henderson, A. Gomez, W. F. Paxton, V. VanDelinder and G. D. Bachand, *PLOS ONE* **2016,** *11,* e0158729.
27 Pradip, C. Maltesh, P. Somasundaran, R. A. Kulkarni and S. Gundiah, *Langmuir* **1991,** *7,* 2108-2111.
28 L. Luo and A. Eisenberg, *Angew. Chem. Int. Ed.* **2002,** *41,* 1001-1004.
29 H. L. Chen and H. Morawetz, *Eur. Polym. J.* **1983,** *19,* 923-928.
30 V. V. Khutoryanskiy, A. V. Dubolazov, Z. S. Nurkeeva and G. A. Mun, *Langmuir* **2004,** *20,* 3785-3790.
31 Y. Mai and A. Eisenberg, *Chem. Soc. Rev.* **2012,** *41,* 5969-5985.
32 R. P. Brinkhuis, F. P. J. T. Rutjes and J. C. M. van Hest, *Polym. Chem.* **2011,** *2,* 1449-1462.



Double-hydrophilic diblock copolymers can assemble into pH-switchable containers for the encapsulation and release of cargo across biologically-relevant pH ranges.