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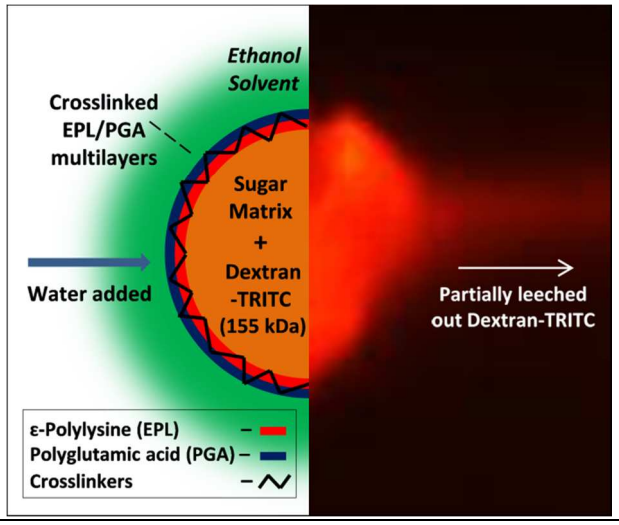
Using only “Generally Recognised as Safe” (GRAS) materials for assembly, edible polyelectrolyte multilayers (EPL/PGA) are deposited onto a sugar (maltotriose)-dextran matrix and crosslinked (left-half). By adding water to dissolve the sacrificial sugar template, an edible cargo-loaded hollow microcapsule is created (right half).



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Edible Polyelectrolyte Microcapsules with Water-Soluble Cargo Assembled in Organic Phase

Cite this: DOI: 10.1039/x0xx00000x

Houwen Matthew Pan,[a] Arjun Subramanian,[a] Christopher J. Ochs,[b] Jean-Yves Dewavrin,[c] Sebastian Beyer*[b] and Dieter W. Trau*[ad]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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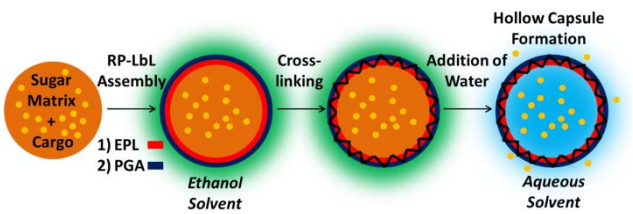
Edible polyelectrolyte microcapsules are constructed from food-grade materials classified as "Generally Recognised as Safe" (GRAS). ε-polylysine (EPL) and polyglutamic acid (PGA) are deposited onto water-soluble sugar-cargo mixtures by an organic-solvent based assembly technique and crosslinked. Cargo-loaded edible microcapsules are created by adding water to dissolve the sacrificial sugar template.

Polyelectrolyte multilayer (PEM) capsules are functional carriers created by the Layer-by-Layer (LbL) polyelectrolyte self-assembly technique.[1, 2] These capsules have become a widely studied subject with potential applications in delivery of therapeutics,[3, 4] biomimetics,[5] and bioreactors.[6, 7] Traditionally, PEM capsules are prepared from synthetic polyelectrolytes such as poly(allylamine hydrochloride), poly(styrene sulfonate), poly(acrylic acid) and poly(diallyldimethylammonium chloride).[8, 9] To shorten the transition from research to biomedical applications, many PEM capsules are now assembled from naturally-derived materials. Common examples include poly(lysine)/poly(glutamic acid) (PLL/PGA),[10] dextran sulfate/poly(arginine) (DS/pARG)[11] and poly(lysine)/hyaluronic acid (PLL/HA)[12] polyelectrolyte capsules. However, due to strict regulatory procedures, time consuming and rigorous testing is still required to prove their safety and effectiveness.[13] To overcome this barrier, materials designated "Generally Recognized as Safe" (GRAS) by the U.S. Food and Drug Administration (FDA) can be used. PEM capsules constructed from selected GRAS materials will be safe for consumption and biocompatible. This will greatly facilitate their integration into both food and biomedical industries. Up to date, chitosan/alginate polyelectrolytes are the only known GRAS substances used in LbL assembly of PEM capsules.[14] However, capsules assembled via aqueous phase LbL suffer severe loss of water-soluble cargo during encapsulation,[15] making them less attractive for industrial applications.

In this study, we report on the organic phase assembly and characterization of polyelectrolyte microcapsules made entirely from GRAS-approved materials. PEMs were assembled onto highly water-soluble templates by a Reverse-Phase LbL (RP-LbL) encapsulation process.[16, 17] Unlike aqueous LbL assemblies, RP-LbL uses organic solvents instead of water to dissolve the polymer to be coated. This solved the problem of template dissolution and payload leakage during encapsulation.[18, 19] Assembled in an organic phase and consisting only of GRAS substances, the edible microcapsules presented herein have great potential as enzyme or drug delivery vehicles.



Scheme 1 Reverse-Phase Layer-by-Layer (RP-LbL) assembly of ε-polylysine (EPL) and polyglutamic acid (PGA) on sugar-cargo particles, followed by crosslinking and template dissolution to form cargo-loaded hollow capsules.

ε-polylysine (EPL) and polyglutamic acid (PGA) were chosen as polyelectrolyte pair for PEM assembly as they are both GRAS substances,[20, 21] soluble in ethanol and have excellent biocompatibility.[10] Low molecular weight sugar particles are ideal templates for edible microcapsules as they

are known to protect proteins against denaturation in aqueous solutions.[22] Furthermore, the sugar-protein mixture can be spray-dried into a homogeneous template,[23] enabling automated and high throughput fabrication of cargo-loaded templates. EPL/PGA multilayers were assembled onto sugar templates by stepwise deposition of EPL (odd layers) and PGA (even layers) in ethanol containing 5 mM HCl (Scheme 1). The assembled layers were further crosslinked to strengthen the polymer capsule and control capsule degradation. As a final step, hollow polyelectrolyte microcapsules were formed by dissolving the sugar templates with water.

For characterization, silica or polystyrene particles were used as templates for PEM assembly. Fluorescence intensity, zeta-potential and atomic force microscopy (AFM) measurements were used to confirm multilayer buildup (Fig. 1). Fluorescence intensity increased with each deposition of TRITC-conjugated EPL (odd layers) and decreased for non-fluorescent PGA (even layers) (Fig. 1A). Similarly, the general trend of zeta-potentials displayed an alternating increase and decrease in value every deposition cycle. These results indicate the assembly of EPL and PGA for each deposition. AFM measurements of EPL/PGA film on planar silicon substrates also indicate an increase in film thickness and surface roughness (root mean square, RMS) with each coating (Fig. 1B).

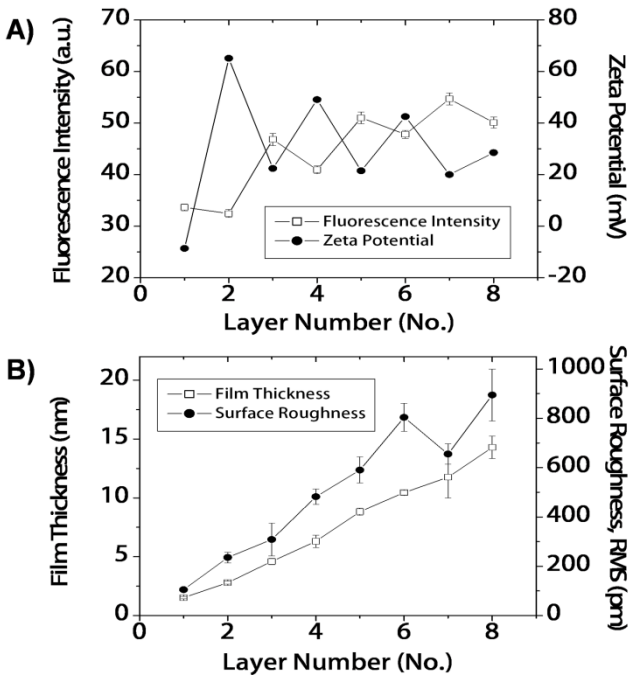


Fig. 1 RP-LbL assembly of polyelectrolytes as monitored by (A) fluorescence intensity and zeta-potential measurements of coated particles, and (B) AFM measurements of LbL film thickness and surface roughness on planar substrates.

PLL/PGA polyelectrolyte multilayers assembled from aqueous LbL under acidic conditions have been reported to exhibit exponential film growth even at lower layer numbers.[24, 25] This phenomenon is attributed to freely diffusing polyelectrolytes within the film that are able to form complexes at the interface every assembly cycle. In contrast to these reports, AFM measurements of EPL/PGA film assembled in ethanol by RP-LbL followed a linear growth profile of about 2nm per layer (Fig. 1B). Furthermore, film surface roughness was more than ten-fold lower than exponential growth systems.[26] We attributed this to the lack of "free" ionic PGA chains required for exponential growth. The high acidity significantly suppressed the deprotonation of PGA and the partial solubility of PGA in ethanol further reduced the pool of excess ionic PGA chains. The low ionization of PGA on the template surface was confirmed by the absence of a negative charge reversal (Fig. 1A). The suppressed auto-dissociation of PGA is known to enhance hydrogen bonding and hydrophobic interactions[24] during assembly. The resulting multilayer assembly is thus likely driven by a combination of electrostatic, hydrogen bonding and hydrophobic interactions.

Table 1 Degree of ionization of polyelectrolytes/complexes assembled in water/ethanol (estimated from IR peak area).

Polymer (Dried from Solvent)[a]	***Solvent***	***Functional Group / IR Peak (cm^{-1})***	***Degree of Ionization***
EPL-HCl	Acidified[b] water, (~pH 2)	NH_3^+ / 3254	100%
PGA-Na	Neutral water, (~pH 7)	CO_2^- / 1405	100%
EPL/PGA (RP-LbL complex)	Acidified[b] ethanol	NH_3^+ / 3254	61%
		CO_2^- / 1405	27%
EPL-HCl/ PGA-Na (LbL complex)	Acidified[b] water	NH_3^+ / 3254	75%
		CO_2^- / 1405	28%

[a] Samples were prepared in the specified solvent and dried for FTIR analysis.
[b] Solvents acidified with 5 mM HCl.

FTIR spectroscopic studies were carried out to further investigate the nature of interactions within EPL/PGA complexes assembled in ethanol. The degree of ionization for EPL/PGA and EPL-HCl/PGA-Na were determined by referencing EPL-HCl and PGA-Na as completely dissociated compounds (Table 1). For detailed sample preparation and calculations refer to section 1.8 in ESI†. N-H stretching vibrations were observed at 3332 cm^{-1}, for pure EPL dried from ethanol, which shifted to 3254 cm^{-1} for $N\text{-}H^+$ stretching vibrations (ESI, Fig. S1B† and Table S1†). Referencing EPL-HCl (apparent pK_a=9) as 100% ionization at ~pH 2,[27, 28] the degrees of ionization for EPL/PGA and EPL-HCl/PGA-Na were calculated to be 61% and 75%, respectively. The degree of ionization for RP-LbL complexes was only slightly lower than that for LbL complexes. This finding partially supported assumptions that electrostatic interactions were also involved in RP-LbL assembly. The peak at 1405 cm^{-1} was attributed to $-CO_2^-$ symmetric stretching vibrations (ESI, Fig. S1B† and Table S1†). Similarly, PGA-Na (apparent pK_a=5) was

referenced as 100% ionization at ~pH 7. The degrees of ionization of 27% and 28% were obtained for EPL/PGA and EPL-HCl/PGA-Na, respectively. Despite the suppressed deprotonation of PGA in ethanol, a similar degree of ionization was obtained for RP-LbL and LbL complexes. This was attributed to the ability of EPL and PGA groups to form ionic complexes in ethanol via acid-base reactions. In summary, results from FTIR spectroscopy confirmed that besides secondary interactions, electrostatic forces also played a role in stabilizing RP-LbL assembled complexes.

In order to impart additional levels of control over capsule degradation, various crosslinkers were tested on the capsules. We compared the crosslinking efficiency of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), transglutaminase (TG) and Genipin. Genipin and TG were selected due to their GRAS classification[29] and wide use in the food industry as blue food colorant[30] (Genipin) and as "meat glue" for binding proteins[31] (TG). TG and EDC, through different reaction mechanisms, achieve crosslinking by forming covalent bonds between acid and amine groups. In contrast, Genipin forms crosslinks only between amine groups.[32]

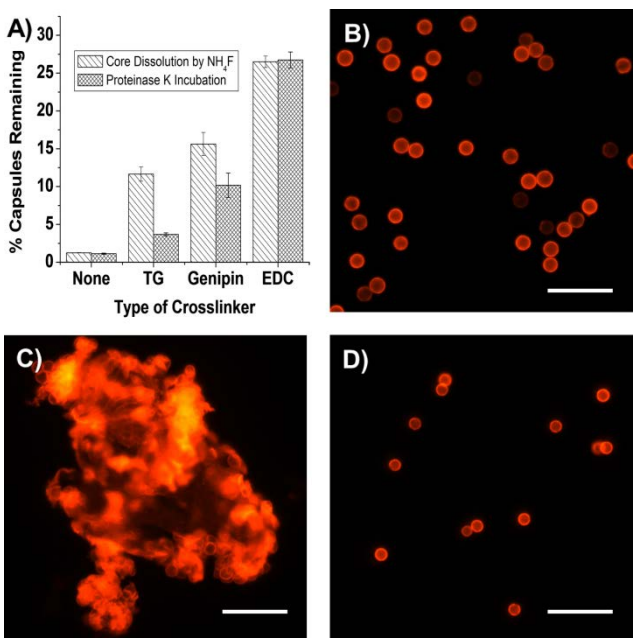


Fig. 2 (A) Percentage of capsules remaining after NH_4F treatment, followed by proteinase K degradation for EPL/PGA microcapsules crosslinked with different crosslinkers. Fluorescent images of EPL-TRITC/PGA capsules (B) before and (C and D) after NH_4F and proteinase K treatment for (C) non-crosslinked and (D) EDC-crosslinked capsules. All scale bars represent 25 µm.

Bilayer-coated silica particles were crosslinked with an excess of TG, Genipin or EDC at 37 °C for 1.5 hours at pH 7.4. Non-crosslinked particles were used as negative control. The coated particles were then treated with NH_4F to dissolve the silica core, followed by two hour incubation with proteinase K at 37 °C. EDC-crosslinked capsules exhibited the best colloidal stability after capsule formation and also the strongest resistance to proteolytic degradation. Weaker crosslinking was observed for Genipin-crosslinked and TG-crosslinked capsules (Fig. 2A). Fluorescence imaging of capsules revealed that non-crosslinked capsules became severely aggregated after degradation (Fig. 2C). We attributed this to the formation of defects in the PEM membrane, leading to the blending of polymers between capsules. A similar phenomenon had been reported for salt-induced defects in PEM capsules.[33] After crosslinking, capsules remained intact and improved in colloidal stability, accompanied by a decrease in diameter from ~5 µm to ~3 µm (Fig. 2D). The survival rate of crosslinked capsules increased by more than two-fold for higher number of EPL/PGA depositions (ESI, Fig. S2†). The use of different crosslinkers and number of depositions presents a facile strategy for tailoring capsules to be either biodegradable or biostable delivery vehicles.

Lastly, we demonstrated the formation of hollow microcapsules from edible templates. Maltotriose (3-glucose units, 504 Da) was mixed with 1 wt% dextran-TRITC (155 kDa), freeze-dried and used as template material. The maltotriose-dextran particles were coated with 8 layers of EPL/PGA via RP-LbL and crosslinked with EDC or Genipin. Next, water was gradually added to dissolve the sugar template to form hollow microcapsules. A fraction of the encapsulated dextran rapidly leeched out together with the maltotriose template. The hollow capsules appear fluorescent due to adsorption of dextran-TRITC onto the remaining capsule material (ESI, Fig. S3†). However, the stability of EDC-crosslinked capsules is much higher than Genipin-crosslinked capsules as they remained intact even after complete re-dispersion in water (ESI, Fig. S4†). Future studies aim to utilize spray-drying techniques to produce spherical and monodisperse sugar templates. This would also improve the overall stability of capsules formed.

In summary, we show for the first time that PEM capsules can be assembled exclusively from GRAS-approved materials using RP-LbL. We found that a combination of electrostatic, hydrogen bonding and hydrophobic interactions is stabilizing the assembly of EPL/PGA in acidified ethanol. Most importantly, we established that layer number and the use of different crosslinkers can be used to tailor capsule degradation. We anticipate that our work on GRAS-microcapsules will open up new avenues for the use of polyelectrolyte multilayer capsules and accelerate the transition from research to commercial application.

Acknowledgements

This work was supported by research grants R-397-000-154-112 and R182-000-200-112 from National University of Singapore and by the National Research Foundation Singapore through the Singapore MIT Alliance for Research and Technology's SMART Scholars fund S-900110. The authors also thank research interns Pally Chan and Joelyn Liew for their valuable assistance and AJINOMOTO for providing food-grade transglutaminase.

Notes and references

[a] Department of Biomedical Engineering, National University of Singapore, Singapore 117574, Singapore.
E-mail: bietrau@nus.edu.sg; Website: www.biosingapore.com
[b] Singapore-MIT Alliance for Research and Technology (SMART) Center, BioSystems and Micromechanics (BioSym) Interdisciplinary Research Group, Singapore 138602, Singapore.
Email: sebastian@smart.mit.edu
[c] NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore.
[d] Department of Chemical & Biomolecular Engineering, National University of Singapore, Singapore 117576, Singapore.

† Electronic Supplementary Information (ESI) available: Experimental methods; FTIR spectroscopy data; fluorescent images of EDC crosslinked sugar templates; crosslinking efficiency data for different layer numbers. See DOI: 10.1039/c000000x/

1 B. V. Parakhonskiy, A. M. Yashchenok, M. Konrad and A. G. Skirtach, *Adv. Colloid Interface Sci.*, 2014, **207**, 253-264.
2 T. A. Kolesnikova, A. G. Skirtach and H. Möhwald, *Expert Opin. Drug Deliv.*, 2013, **10**, 47-58.
3 C. J. Ochs, G. K. Such, B. Städler and F. Caruso, *Biomacromolecules*, 2008, **9**, 3389-3396.
4 M. B. Thomas, M. Vaidyanathan, K. Radhakrishnan and A. M. Raichur, *J. Food Eng.*, 2014, **136**, 1-8.
5 T. Boudou, T. Crouzier, K. Ren, G. Blin and C. Picart, *Adv. Mater.*, 2010, **22**, 441-467.
6 W. C. Mak, K. Y. Cheung and D. Trau, *Adv. Funct. Mater.*, 2008, **18**, 2930-2937.
7 W. C. Mak, K. Y. Cheung and D. Trau, *Chem. Mater.*, 2008, **20**, 5475-5484.
8 A. G. Skirtach, A. M. Yashchenok and H. Mohwald, *Chem. Commun.*, 2011, **47**, 12736-12746.
9 W. Tong, X. Song and C. Gao, *Chem. Soc. Rev.*, 2012, **41**, 6103-6124.
10 A. Yu, I. R. Gentle and G. Q. Lu, *J. Colloid Interface Sci.*, 2009, **333**, 341-345.
11 S. De Koker, B. G. De Geest, S. K. Singh, R. De Rycke, T. Naessens, Y. Van Kooyk, J. Demeester, S. C. De Smedt and J. Grooten, *Angew. Chem., Int. Ed. Engl.*, 2009, **121**, 8637-8641.
12 A. Szarpak, D. Cui, F. d. r. Dubreuil, B. G. De Geest, L. J. De Cock, C. Picart and R. Auzély-Velty, *Biomacromolecules*, 2010, **11**, 713-720.
13 S. De Koker, L. J. De Cock, P. Rivera-Gil, W. J. Parak, R. Auzély Velty, C. Vervaet, J. P. Remon, J. Grooten and B. G. De Geest, *Adv. Drug Deliv. Rev.*, 2011, **63**, 748-761.
14 C. Peng, Q. Zhao and C. Gao, *Colloids Surf., A*, 2010, **353**, 132-139.
15 M. L. De Temmerman, J. Demeester, F. De Vos and S. C. De Smedt, *Biomacromolecules*, 2011, **12**, 1283-1289.
16 H. M. Pan, S. Beyer, Q. Zhu and D. Trau, *Adv. Funct. Mater.*, 2013, **23**, 5108-5115.
17 S. Beyer, W. C. Mak and D. Trau, *Langmuir*, 2007, **23**, 8827-8832.
18 S. Beyer, J. Bai, A. M. Blocki, C. Kantak, Q. Xue, M. Raghunath and D. Trau, *Soft Matter*, 2012, **8**, 2760-2768.
19 W. C. Mak, J. Bai, X. Y. Chang and D. Trau, *Langmuir*, 2008, **25**, 769-775.
20 *GRAS Notices Inventory*; GRN No. 339, U.S. Food and Drug Administration, 2010.
21 *GRAS Notices Inventory*; GRN No. 336, U.S. Food and Drug Administration, 2011.
22 J. C. Lee and S. N. Timasheff, *J. Biol. Chem.*, 1981, **256**, 7193-7201.
23 J. Elversson and A. Millqvist-Fureby, *J. Pharm. Sci.*, 2005, **94**, 2049-2060.
24 J. Zhou, B. Wang, W. Tong, E. Maltseva, G. Zhang, R. Krastev, C. Gao, H. Möhwald and J. Shen, *Colloids Surf., B*, 2008, **62**, 250-257.
25 L. Richert, Y. Arntz, P. Schaaf, J.-C. Voegel and C. Picart, *Surf. Sci.*, 2004, **570**, 13-29.
26 P. Lavalle, C. Gergely, F. J. G. Cuisinier, G. Decher, P. Schaaf, J. C. Voegel and C. Picart, *Macromolecules*, 2002, **35**, 4458-4465.
27 F. Bordi, C. Cametti and G. Paradossi, *Biopolymers*, 2000, **53**, 129-134.
28 M. Rozenberg and G. Shoham, *Biophys. Chem.*, 2007, **125**, 166-171.
29 *GRAS Notices Inventory*; GRN No. 95, U.S. Food and Drug Administration, 2002.
30 J.-E. Park, J.-Y. Lee, H.-G. Kim, T.-R. Hahn and Y.-S. Paik, *J. Agric. Food Chem.*, 2002, **50**, 6511-6514.
31 M. Kieliszek and A. Misiewicz, *Folia Microbiol.*, 2014, **59**, 241-250.
32 L.-q. Yang, Y.-q. Lan, H. Guo, L.-z. Cheng, J.-z. Fan, X. Cai, L.-m. Zhang, R.-f. Chen and H.-s. Zhou, *Acta Pharmacol. Sin.*, 2010, **31**, 1625-1634.
33 R. Zhang, K. Kohler, O. Kreft, A. Skirtach, H. Mohwald and G. Sukhorukov, *Soft Matter*, 2010, **6**, 4742-4747.

