

# Encapsulation of Biomacromolecules within Polymersomes by Electroporation

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Polymersomes are formed by the self-assembly of amphiphilic block copolymers in aqueous solution.<sup>[1]</sup> Polymersomes, like other vesicular structures, combine the unique ability to encapsulate hydrophilic compounds within their lumen as well as hydrophobic and amphiphilic molecules within their membrane.<sup>[1]</sup> The macromolecular nature of the polymer-some building blocks leads to the formation of non-ergodic structures with no detectable exchange of individual copolymer chains with their environment.<sup>[2]</sup> This makes polymersomes much more stable than their low-molecular-weight surfactant liposome counterparts.<sup>[3]</sup> Given this important advantage and their wholly synthetic nature, polymersomes are potential candidates for many applications, ranging from drug delivery to nanoreactors.<sup>[1b]</sup> However, one of the limitations in developing commercial applications for polymersomes is their associated high stability and thus the relatively high energy penalty required for their formation.<sup>[4]</sup> This problem is typically overcome using various processing techniques, such as solvent exchange, electroformation, ultrasound, and extrusion under high shear.<sup>[4]</sup> However, these strategies are often not compatible with the encapsulation of sensitive biomolecules, especially if good size control is required.

In principle, this problem can be addressed by exploiting the supramolecular nature of polymersomes to temporarily destabilize their membrane so as to allow in situ loading of preformed polymersomes. Herein we explore this approach

using electroporation. This is a well-known process whereby cellular membrane can be permeabilized by applying an external electrical field.<sup>[5]</sup> When this external electric field exceeds a certain threshold, the phospholipids that comprise the cellular membrane rearrange to form a nanoscopic pore that allows the passage of ions, molecules, and even macromolecules.<sup>[5]</sup> Electroporation is widely used in molecular and cellular biology for introducing plasmid DNA (pDNA) (and other biomolecules) into live cells.<sup>[5,6]</sup> When an applied external field exceeds the capacity of the cell membrane, the temporary osmotic pressure forces the phospholipids to rearrange, stabilizing the formation of hydrophilic pores and the consequent diffusion of water and its solutes. The critical transmembrane potential at which poration occurs depends on cell membrane compositions as well as cell size, with large cells having much lower thresholds than small organelles.<sup>[5,6]</sup> The formation of these transient pores occurs within milliseconds, whereas resealing happens over time scales of minutes. The extension as well as the lifetime of these pores can be controlled using square-wave pulse generators, where pulse amplitude and pulse length can be modulated to optimize poration and diffusion of solute across the membrane.<sup>[5,6]</sup>

Herein we present electroporation as a new approach to encapsulate biological macromolecules into polymersomes by exploiting their supramolecular nature. Polymersome membranes have already been reported to undergo electroporation by Aranda-Espinoza et al.<sup>[7]</sup> The breakdown potential ( $V_c$ ) increased with the hydrophobic thickness  $d$  of the membrane. The  $V_c$  is 9 V for polymersomes with  $d = 15$  nm, compared to 1 V for liposomes with  $d = 3$  nm. In this present study, proteins (bovine serum albumin, BSA; myoglobin, Mb), antibodies (immunoglobulin G, IgG), nucleic acids (pDNA or small interfering RNA, siRNA), and enzymes (Lysozyme, Lz) are used as model biological macromolecules and loaded into poly(2-(methacryloyloxy)ethyl phosphorylcholine)-b-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA) polymersomes, which combine the highly biocompatible nature of the PMPC block with the pH-responsive nature of PDPA block.<sup>[8]</sup> We have reported using such PMPC-PDPA polymersomes for the delivery of DNA,<sup>[9]</sup> antibodies,<sup>[10]</sup> small molecules,<sup>[11]</sup> and nanoparticles<sup>[11]</sup> within live cells with no effect on their metabolic activity across several cell types.<sup>[8b,11,12]</sup> We have furthermore shown that cellular uptake can be effectively controlled by size, surface chemistry, and surface topology,<sup>[12,13]</sup> making this particular polymersome formulation very promising for several biomedical applications. More importantly, the pH-sensitive nature of PMPC-PDPA polymersomes allows encapsulation

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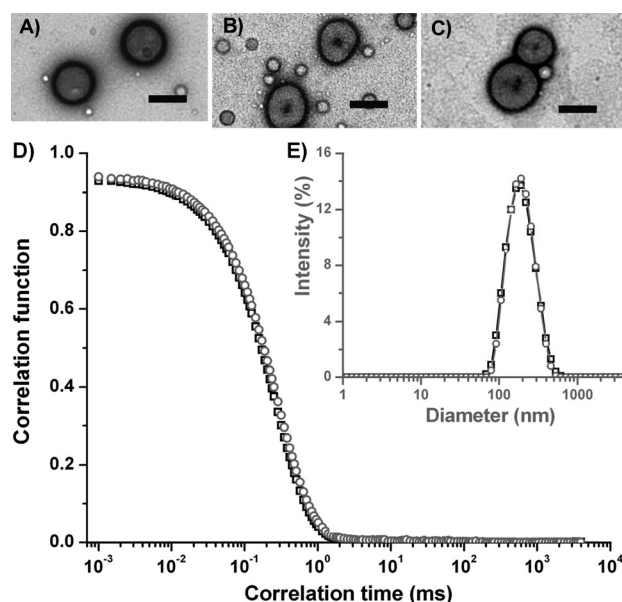


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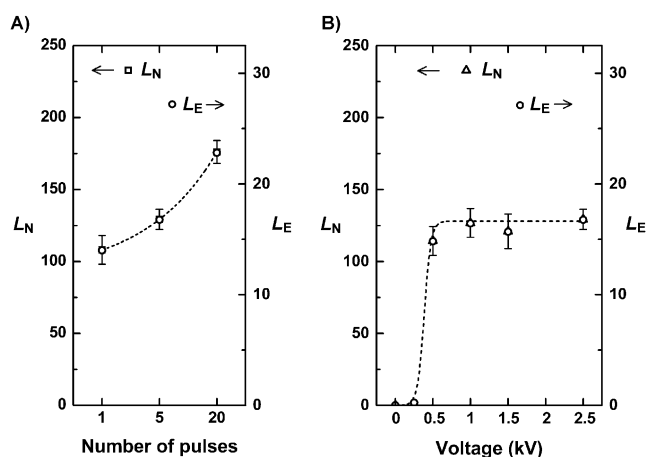
by using a pH switch to control copolymer self-assembly,<sup>[8b,9]</sup> which makes it an appropriate model system to compare with other encapsulation strategies (for details of the encapsulation of biomacromolecules within polymersomes using the traditional pH-switch method, see the Supporting Information, Figure S1).

Electroporation depends on several parameters, such as the applied voltage ( $V_a$ ), the number of pulses, duration, frequency, and also the properties of the solution.<sup>[6,14]</sup> Membrane permeabilization only occurs at a characteristic critical field strength. Above this threshold value, its extent is controlled by the number (and duration) of the electrical pulses.<sup>[15]</sup> It has been observed that the critical threshold also depends on the cell size, with submicrometer vesicles requiring much higher voltages.<sup>[16]</sup> Accordingly,<sup>[7]</sup> we applied the maximum voltage ( $V_a = 2500$  V) achievable for our electroporator to induce polymersome membrane rupture and examined whether electroporation affected the original polymersome morphology and size distribution. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) studies confirmed that the morphology and size distribution of the PMPC–PDPA polymersomes before (Figure 1A) and after (Figure 1B,C) electroporation remains essentially unchanged. These data indicate that electroporation only temporarily disrupts the membrane, which self-heals without inducing inter-polymersome interactions that might otherwise cause precipitation, fusion, or other unwanted changes in polymersome morphology.

As shown in Figure 2A, we examined the feasibility of encapsulating a model protein, BSA, within polymersomes and explored the effect of varying the applied voltage and the number of pulses on the encapsulation efficiency (further



**Figure 1.** TEM images obtained for PMPC–PDPA polymersomes a) before and B, C) after electroporation at 2500 V to encapsulate either BSA (B) or siRNA (C). Scale bars = 100 nm. D) Correlation function  $C(\tau)$  for PMPC–PDPA polymersomes before ( $\square$ ) and after ( $\circ$ ) electroporation as measured by DLS. E) The corresponding size distribution.

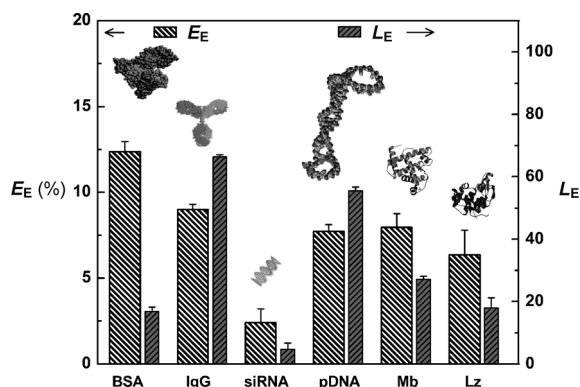


**Figure 2.** Effect of varying A) the number of pulses ( $V_a = 2500$  V) and B) the applied voltage (5 pulses) on BSA encapsulation efficiency by electroporation  $L_N$  = loading number,  $L_E$  = loading efficiency. Initial loading concentration of BSA:  $2.5 \text{ mg mL}^{-1}$ .

results are also summarized in the Supporting Information, Table S1). Each experiment was performed at BSA and polymersome concentrations of  $2.5 \text{ mg mL}^{-1}$  and  $10 \text{ mg mL}^{-1}$ , respectively, in 0.1 M phosphate buffer solution (PBS). After electroporation, the BSA-loaded polymersomes were purified by size-exclusion chromatography (SEC) according to a previously published procedure.<sup>[9a]</sup> The concentration of the encapsulated BSA was determined by UV/Vis spectroscopy (Supporting Information, Figures S2 and S3). Electroporation allows effective encapsulation of BSA within PMPC–PDPA polymersomes. The number of BSA molecules per polymersome ( $L_N$ ) and the loading efficiency ( $L_E$ , expressed as the ratio between the encapsulated BSA number per polymersome and the theoretical encapsulated BSA number per polymersome) are both given in Figure 2A. The latter parameter, also known as statistical encapsulation, is calculated as the number of proteins encapsulated when the BSA concentration within the polymersomes is equal to the bulk BSA concentration (for this calculation, see the Supporting Information). This is a theoretical upper limit as it corresponds to a BSA concentration gradient of zero across the polymersome membrane. If this loading efficiency ( $L_E$ ) is higher than unity (the loaded BSA number is higher than the maximum value by diffusion), it would suggest a positive interaction between the polymersomes and the encapsulated molecules, as previously reported for DNA encapsulation using the pH switch method.<sup>[8b,9]</sup> The data shown in Figure 2A indicate that the loading efficiency varies from 14 % to 23 % depending on the number of pulses, suggesting that electroporation allows loading efficiencies much higher than the theoretical limit. As shown in the Supporting Information, Figure S9, BSA does not undergo denaturation after 20 pulses. More importantly, the maximum encapsulation efficiency ( $E_E$ ) is 17.1 %. This value achieved with 20 pulses (Supporting Information, Table S1) is very close to that obtained using the pH switch method. In a comparative study, BSA ( $2.5 \text{ mg mL}^{-1}$ ) was loaded into the PMPC–PDPA polymersomes ( $10 \text{ mg mL}^{-1}$ ) using a pH switch, and the  $E_E$  of BSA was found to be 23.9 %.

In particular, increasing the number of pulses considerably increases the number of BSA molecules encapsulated within a single polymersome, while the applied voltage has almost no effect on the encapsulation efficiency. Moreover, the data suggest that polymersome membrane poration occurs at voltages as low as 500 V. Electroporation occurs when an applied external field exceeds the capacity of the cell membrane. Transient hydrophilic pores are formed and the external material enters the cell membrane owing to the osmotic pressure.<sup>[17]</sup> For phospholipid membranes, the pore lifetime has been estimated to be less than one millisecond, whereas pore resealing occurs over a time scale of seconds.<sup>[18]</sup> A higher applied voltage leads to an increase in the transient membrane area where permeabilization occurs. In such areas, the extent of permeabilization is controlled by the number of pulses.

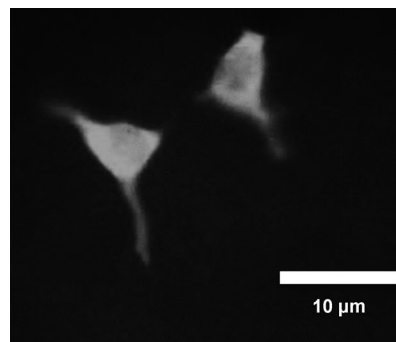
In addition to BSA, we demonstrate that electroporation is also applicable to other biomolecules, such as Mb, Lz, IgG, siRNA, and pDNA. The biomacromolecule concentration and its loading efficiency are characterized by UV/Vis spectroscopy, high-performance liquid chromatography (HPLC), and fluorescence spectrophotometry (Supporting Information, Figures S4–S8). As shown in Figure 3, each



**Figure 3.** Encapsulation efficiencies  $E_E$  loading efficiencies  $L_E$  for BSA, IgG, siRNA, pDNA, Mb, and Lz within PMPC–PDPA polymersomes by electroporation at pH 7.3 (except Lz, loaded at pH 11). The applied voltage was 2500 V for BSA, IgG, pDNA, and Mb, and 1800 V for siRNA; 5 pulses (except siRNA: one pulse). Initial loading concentrations for BSA, IgG, siRNA, DNA, Mb, and Lz into the polymersomes solution were 2.5 mg mL<sup>-1</sup>, 5  $\mu$ g mL<sup>-1</sup>, 66.5  $\mu$ g mL<sup>-1</sup>, 180  $\mu$ g mL<sup>-1</sup>, 2.5 mg mL<sup>-1</sup>, and 2.5 mg mL<sup>-1</sup>, respectively.

biomacromolecule is successfully encapsulated within the PMPC–PDPA polymersomes. The  $E_E$  value of IgG also increases with the number of pulses, with values similar to BSA being obtained (Supporting Information, Figure S9). We tested the effect of electroporation on the IgG functionality using an ELISA assay and observed no negative effects (Supporting Information, Figure S11). For nucleic acid encapsulation, we initially examined the effect of electroporation on naked pDNA and siRNA, assessing their structural integrity by gel electrophoresis (Supporting Information, Figure S12 and S13). pDNA can withstand the electroporation conditions with no apparent degradation; only a conformational change was induced by the electroporation

process. Moreover, pDNA-loaded PMPC–PDPA polymersomes formed by electroporation can effectively transfect cells. HEK293T cells were incubated for 48 h with polymersomes encapsulating E2-Crimson, and good-quality far-red fluorescence images were obtained (Figure 4; Supporting Information, Figure S14). In contrast, control cells incubated



**Figure 4.** E2-Crimson expression in HEK293T cells 48 h after incubation with PMPC–PDPA polymersomes loaded with E2-Crimson plasmid DNA by electroporation.

with empty polymersomes (without any loading pDNA) did not display any fluorescence, as expected. This confirms that the plasmid is not degraded by electroporation and is able to produce functional protein after its polymersome-mediated delivery to the cell nucleus. Compared to pDNA, siRNA is much more sensitive and a lower voltage is required to ensure the integrity of the latter. Thus electroporation was performed using just a single pulse at a  $V_a$  of 1800 V to encapsulate siRNA. Despite these milder conditions compared to the BSA experiments, loading efficiencies ( $L_E$ ) calculated for IgG, pDNA, and siRNA macromolecules always significantly exceeded unity. Given that each of these species is negatively charged at pH 7.3 (see zeta potential data in the Supporting Information, Table S2), we postulate that electrophoretic motility drives the diffusion of these biomacromolecules within the polymersome lumen. The same anionic character of these biomacromolecules is critical to facilitate their encapsulation into PMPC–PDPA polymersomes by electrostatic interaction with the cationic PDPA chains at acidic pH.<sup>[8b,9]</sup>

A more challenging biomacromolecule is myoglobin. This protein has an isoelectric point at pH 6.8 and hence is positively charged at acidic pH (zeta potential of 6.5 mV at pH 6) and slightly negatively at neutral pH (zeta potential of –5.9 mV at pH 7.3). This protein is therefore very difficult to encapsulate using the pH switch with  $E_E$  of only 0.50 % owing to the repulsion interaction between the positive protein and PDPA chains at acidic pH. On the contrary, electroporation enable Mb encapsulation with  $E_E$  equal to 7.97 %. We explore the effect of the biomacromolecule charge using lysozyme (Lz) as model. This is positively charged at acidic and neutral pH (zeta potentials are 4.3 mV at pH 6.5 and 3.2 at pH 7.3, respectively). Using the pH switch method, Lz was encapsulated with a  $E_E$  value of 2.60 %. Using electroporation at pH 7.3 we achieved an encapsulation efficiency of 0.83 %.

However, when the pH of the solution was increased to 11, the zeta potential of Lz becomes negative ( $-7.8$  mV), and the  $E_E$  increased to 6.36 %, confirming that surface charge of the biomacromolecules is one of the dominant factors dictating encapsulation efficiency. Previous studies have shown that during electroporation, diffusion alone is the cause of transmembrane uptake for small molecules only. With increasing molecule size, uptake is affected by a combination of diffusion and electrophoretic forces.<sup>[17]</sup>

In summary, six biomacromolecules were successfully encapsulated within PMPC-PDPA polymersomes by electroporation. The morphology and the size of the polymersomes remain essentially unchanged by electroporation, which indicates efficient self-healing of the defects generated within the polymersome membranes after the applied high voltage pulses. The surface charge of the biomacromolecules plays a key role for during electroporation, since negatively charged molecules can be loaded much more than positively charged molecules. The encapsulation efficiency, loading number, and loading efficiency of the biomacromolecules increases dramatically as the number of pulses is increased, but is barely affected by the applied voltage. Electroporation provides a new and highly convenient approach for the efficient encapsulation of a range of delicate anionic biomacromolecules within polymersomes for drug delivery and gene therapy applications.

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- [1] a) D. E. Discher, A. Eisenberg, *Science* **2002**, 297, 967–973; b) C. LoPresti, H. Lomas, M. Massignani, T. Smart, G. Battaglia, *J. Mater. Chem.* **2009**, 19, 3576–3590.
- [2] a) S. Jain, F. S. Bates, *Science* **2003**, 300, 460–464; b) S. Jain, F. S. Bates, *Macromolecules* **2004**, 37, 1511–1523.
- [3] G. Battaglia, A. J. Ryan, *J. Am. Chem. Soc.* **2005**, 127, 8757–8764.
- [4] G. Battaglia, A. J. Ryan, *J. Phys. Chem. B* **2006**, 110, 10272–10279.
- [5] E. Neumann, M. Schaeffer, Y. Wang, P. H. Hofschneider, *EMBO J.* **1982**, 1, 841–845.
- [6] S. Y. Ho, G. S. Mittal, *Crit. Rev. Biotechnol.* **1996**, 16, 349–362.
- [7] H. Aranda-Espinoza, H. Bermudez, F. S. Bates, D. E. Discher, *Phys. Rev. Lett.* **2001**, 87, 208301.
- [8] a) J. Z. Du, Y. P. Tang, A. L. Lewis, S. P. Armes, *J. Am. Chem. Soc.* **2005**, 127, 17982–17983; b) H. Lomas, M. Massignani, K. A. Abdullah, I. Canton, C. Lo Presti, S. MacNeil, J. Z. Du, A. Blanazs, J. Madsen, S. P. Armes, A. L. Lewis, G. Battaglia, *Faraday Discuss.* **2008**, 139, 143–159.
- [9] a) H. Lomas, I. Canton, S. MacNeil, J. Du, S. P. Armes, A. J. Ryan, A. L. Lewis, G. Battaglia, *Adv. Mater.* **2007**, 19, 4238–4243; b) H. Lomas, J. Z. Du, I. Canton, J. Madsen, N. Warren, S. P. Armes, A. L. Lewis, G. Battaglia, *Macromol. Biosci.* **2010**, 10, 513–530.
- [10] a) M. Massignani, I. Canton, N. Patikarnmonthon, N. J. Warren, S. P. Armes, A. L. Lewis, G. Battaglia, *Nature Precedings* **2010**; b) G. Battaglia, M. Massignani, A. L. Lewis, Intracellular Antibody Delivery, WO2009138473, 2009.
- [11] M. Massignani, I. Canton, T. Sun, V. Hearnden, S. MacNeil, A. Blanazs, S. P. Armes, A. Lewis, G. Battaglia, *PLoS One* **2010**, 5, e10459.
- [12] M. Massignani, C. LoPresti, A. Blanazs, J. Madsen, S. P. Armes, A. L. Lewis, G. Battaglia, *Small* **2009**, 5, 2424–2432.
- [13] C. LoPresti, M. Massignani, C. Fernyhough, A. Blanazs, A. J. Ryan, J. Madsen, N. J. Warren, S. P. Armes, A. L. Lewis, S. Chirasatitsin, A. J. Engler, G. Battaglia, *ACS Nano* **2011**, 5, 1775–1784.
- [14] a) J. C. Knutson, D. Yee, *Anal. Biochem.* **1987**, 164, 44–52; b) H. Wolf, M. P. Rols, E. Boldt, E. Neumann, J. Teissie, *Biophys. J.* **1994**, 66, 524–531.
- [15] J. M. Escoffre, T. Portet, L. Wasungu, J. Teissie, D. Dean, M. P. Rols, *Mol. Biotechnol.* **2009**, 41, 286–295.
- [16] K. H. Schoenbach, S. J. Beebe, E. S. Buescher, *Bioelectromagnetics* **2001**, 22, 440–448.
- [17] E. Neumann, S. Kakorin, K. Toensing, *Bioelectrochem. Bioenerg.* **1999**, 48, 3–16.
- [18] T. Y. Tsong, *Biophys. J.* **1991**, 60, 297–306.