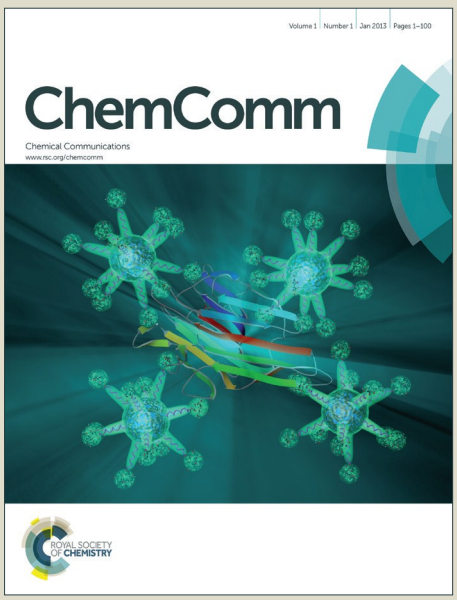
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

Rational design of degradable polyanion for layer-by-layer assembly for encapsulation and release of cationic functional biomolecules

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

DOI: 10.1039/x0xx00000x

www.rsc.org/

Ryuichi Hiraoka,[a] Yuichi Funasaki,[a] Jun Ishii,[b] Tatsuo Maruyama*[a]

A novel degradable polyanion, poly(phthalic ethylene glycol ester), was synthesized in one pot in a single step. The degradable polyanion assembles with various polycations to form layer-by-layer films that can encapsulate physiologically active biomolecules. Polyanion degradation can induce film disassembly and release of the encapsulated functional protein.

There is a high demand in the biomedical and bioengineering fields for the development of new materials and methods that control drug release. Controlled release of drugs and biological agents has been explored using various approaches involving synthetic and natural polymers.[1-6] Layer-by-layer (LbL) assembly of oppositely charged polymers is a powerful method for readily imparting various functional properties to the surfaces of solid and soft materials.[7-13] This enables chemical functionalization of the surfaces of implantable devices and scaffolds for tissue engineering.[14,15] For example, LbL enables the encapsulation of a broad range of small and large functional molecules in polyelectrolyte multilayered films.[23-28] The encapsulated substances can be released from the films depending on the polyelectrolyte molecule and the conditions.[16-22] Lynn et al. developed a degradable polycation, poly(β-amino ester) (PBAE), and achieved controlled release under physiological conditions of encapsulated DNA (polyanion) from a multilayered film of the PBAE. [29-31] They also reported localized delivery of plasmid DNA and its transfection of cells using LbL assembly. PBAEs are designed for assembly with polyanions, but various bioactive polycations (e.g., polycationic proteins and peptides) can not be encapsulated in the LbL assembly of PEAEs. Hammond et al. used a naturally derived degradable polyanion, poly(β-L-malic acid), which was extracted from eukaryotic microbes and purified, to encapsulate polycationic proteins in LbL assembly,

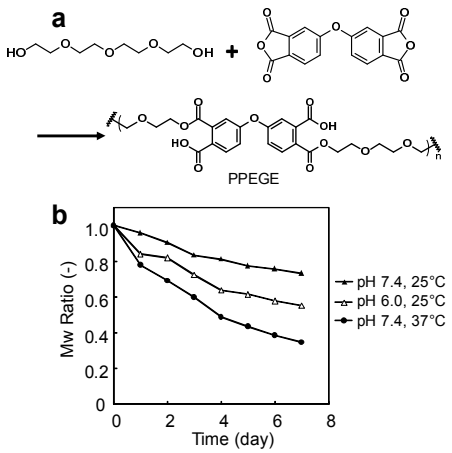


Fig. 1. a) One-step synthesis of degradable polyanion, poly(phthalic ethylene glycol ester) (abbreviated PPEGE) and b) degradation of PPEGE in aqueous solution (phosphate buffer). Degradation degree is expressed as relative M_w over time.

and achieved controlled release of proteins based on hydrolytic degradation of poly(β-L-malic acid).[32,33] However, to our knowledge, there is no report of degradable synthetic polyanion that can be available for LbL assembly.

In the present study, we synthesized a novel degradable polyanion that can assemble with polycations (especially polycationic enzymes) to form LbL films, and showed that the LbL film can encapsulate and release a polycationic antibacterial enzyme. Synthetic polymers are preferred in therapeutic applications because they minimize the risk of contamination by pathogenic agents (viruses, pathogenic proteins, etc.).[34] A degradable synthetic polyanion for LbL assembly must meet the following design criteria: (i) it can be synthesized via a simple procedure in considerable yields, (ii) it has high solubility in water and forms a polyelectrolyte complex with polycations, (iii) the degradation rate is suitable for LbL assembly in aqueous solution (minimizing degradation), and (iv) degradation in aqueous solution induces disassembly of the LbL film. To satisfy these criteria, we designed a degradable polyanion, poly(phthalic ethylene glycol ester)

[a] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan. E-mail: tmarutcm@crystal.kobe-u.ac.jp
[b] Organization of Advanced Science and Technology, Kobe University.
† Electronic supplementary information (ESI) available: The materials, experimental procedures, and additional results. See DOI: 10.1039/x0xx00000x

(PPEGE), which has ester bonds as the degradable part and carboxy groups as the anionic part (**Fig. 1a**). PPEGE was synthesized via ring-opening polymerization between 4,4-oxydiphthalic anhydride (ODPA) and poly(ethylene glycol) 200 (PEG 200). This ring-opening polymerization enables simultaneous introduction of ester bonds and carboxy groups into the polymer backbone. This is a one-pot, single-step reaction, without any protection (or deprotection) of monomer functional groups.

We investigated the synthesis of PPEGE under various conditions (**Table S2**). First, the effect of the PEG 200 concentration was examined. Although the reaction theoretically progresses via ring-opening polymerization with an ODPA/PEG 200 molar ratio of 1:1, a molar ratio of 0.50:0.48 gave the largest weight-average molecular weight (M_w = 1.56 × 10^4), with a yield of 57%. This could be related to the ODPA purity. Carboxylic acid anhydrides are sensitive to small amounts of water; therefore a small portion of ODPA could have been hydrolyzed prior to the reaction, although special care was taken to avoid water contamination of the experimental setup. The reaction time also affected the PPEGE M_w; 3 h gave the largest M_w under the present conditions. A reaction time longer than 3 h resulted in a low M_w, possibly because of hydrolysis of the produced PPEGE.

ODPA was not soluble in *N*,*N*-dimethylformamide (DMF) at the target concentration (0.48 M) in the absence of *N*,*N*-diisopropylethylamine (DIEA); therefore DIEA was added to the ODPA/DMF solution. The addition of DIEA increased the M_w, and a DIEA concentration of 0.9 M produced PPEGE with the largest M_w (2.01 × 10^4), in a yield of 53%. There are two isomeric forms of the ester produced from ODPA and PEG 200 (**Fig. S1**). ^{1}H NMR spectroscopy indicated that the ratio of form 1 to form 2 was 3:1. PPEGE with the largest M_w (i.e., 2.01 × 10^4) was used in the following experiments.

The solubility of PPEGE in phosphate buffer (0.1 M, pH 6.0) was more than 10 g L^{-1} at 25 °C. We observed that a polyelectrolyte complex was formed when equivalent volumes of 1 wt% PPEGE and 1 wt% chitosan solutions were mixed (**Fig. S2**).

The degradation of PPEGE in aqueous solution was investigated (**Fig. 1b**). At 37 °C and pH 7.4 (physiological conditions), the M_w of PPEGE decreased to 34% relative to the initial M_w after 7 d. At a lower temperature (25 °C), the M_w of PPEGE after 7 d was 73% relative to that of the initial PPEGE. Matrix-assisted laser desorption ionization time-of-flight mass spectrometric (MALDI-TOF/MS) analysis of the degraded PPEGE showed the presence of low-molecular weight PPEGE (875–1650), indicating hydrolysis of ester bonds in PPEGE (**Fig. S3**). The m/z values of the degradation products indicate the presence of the short oligomers comprising ODPA and PEG 200. Under the conditions for LbL assembly (25 °C and pH 6.0, see later), the degradation rate was slower than that under physiological conditions. These results show that PPEGE degradation is based on ester hydrolysis in the polymer backbone. It should be noted that degradation of dry PPEGE can be prevented at −20 °C for more than 6 months.

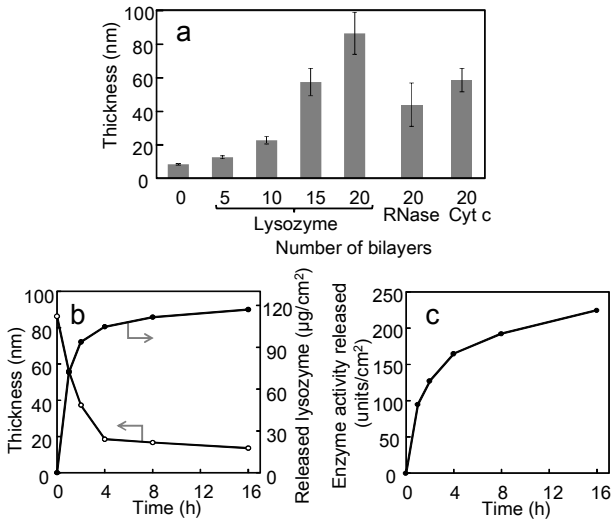


Fig. 2. a) Ellipsometric thickness of dry PPEGE/lysozyme film as function of number of bilayers. RNase and Cyt c represent ribonuclease A (from bovine pancreas) and cytochrome c (from horse heart). b) Release of lysozyme (black circles) on PPEGE/lysozyme film degradation (open circles) in pH 7.4 PBS at 37 °C. c) Enzymatic activity released from PPEGE/lysozyme film. Enzymatic activity (unit) is defined as follows. One unit produces a decrease in $Abs_{450\,nm}$ of 0.001 per min at pH 7.4 (1.0 mL PBS) and 25 °C, using *Micrococcus lysodeikticus* (0.25 mg mL^{-1}) as a substrate.

The use of PPEGE as a polyanion in an LbL film was shown by deposition of PPEGE/polycationic protein on a planar silicon substrate. We used lysozyme (pI of 11[35]), which is an antibacterial enzyme, as a model polycationic protein. Prior to preparation of LbL films, lysozyme was conjugated with fluorescein isothiocyanate.[36,37] Initially, 10 bilayers of poly(sodium 4-styrenesulfonate)/polyethyleneimine $[(PSS/PEI)_{10}]$ were deposited on surface-aminated silicon substrates, using a dipping method, to prepare a charged base surface suitable for PPEGE adsorption.[29] In **Fig. 2a**, 0 bilayer (ca. 8 nm) indicates the $(PSS/PEI)_{10}$ film in dry form; this layer helped deposition of PPEGE/lysozyme films on the silicon substrate.

The LbL assembly of PPEGE/lysozyme on the $(PSS/PEI)_{10}$ bilayers was performed at pH 6.0 and 25 °C. Under these conditions, the PPEGE degradation rate was slow enough to allow assembly (half-life $t_{1/2}$ > 8 d). The thickness of the $(PPEGE/lysozyme)_n$ film increased with increasing numbers of bilayers; the $(PPEGE/lysozyme)_{20}$ film had a thickness of about 86 nm (**Fig. 2a**). Film growth was exponential with the number of bilayers at the beginning of the film preparation; this has also been observed for LbL films composed of other weakly charged polyelectrolytes.[38-40] The sequential growth of the film thickness suggests successful preparation of LbL films composed of PPEGE and lysozyme. The surface morphology of the dried $(PPEGE/lysozyme)_{20}$ film was observed using an atomic force microscope (AFM). The AFM observation reveals the relatively smooth surface of the film (**Fig. S4**). Particulates observed on the surface would be crystalline salt.

We used two other polycationic proteins (ribonuclease A and cytochrome c) to explore the versatility of $(PPEGE/polycationic\ protein)_n$ films. The pI values of ribonuclease A (from bovine pancreas) and cytochrome c (from horse heart) are 9.6[41] and 10.2–10.4,[42] respectively. The thicknesses of the

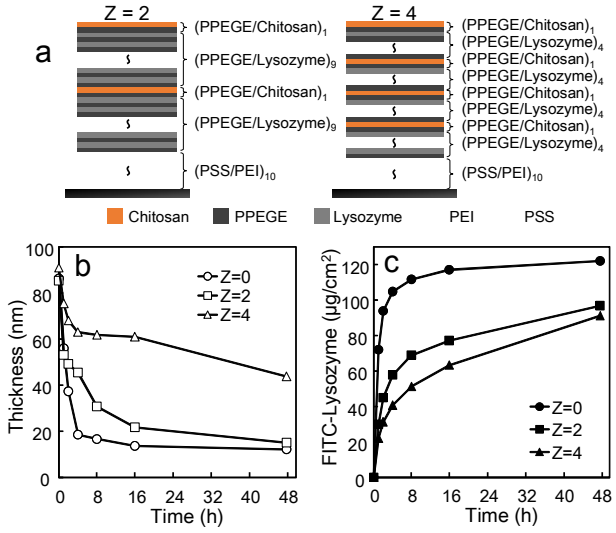


Fig. 3. a) Schematic diagram of introduction of PPEGE/chitosan bilayers into PPEGE/lysozyme LbL films. b) Effect of PPEGE/chitosan bilayers on film degradation over time. c) Effect of PPEGE/chitosan bilayers on lysozyme release. *Z* indicates number of PPEGE/chitosan layers.

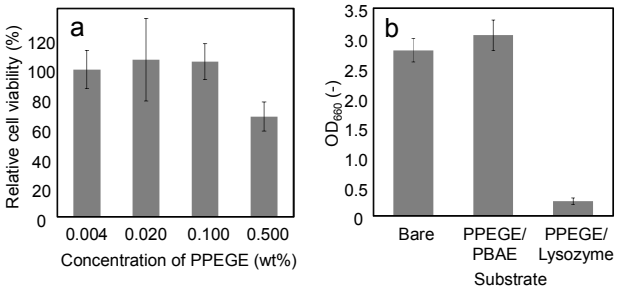


Fig. 4. a) Cytotoxicity of PPEGE to HeLa cells. Viability of HeLa cells, relative to that without PPEGE, is expressed as a function of PPEGE concentration. Cytotoxicity was evaluated using Cell Counting Kit-8 (see **ESI**). b) Growth of *B. subtilis* on bare silicon substrates (control), PPEGE/PBAE films, and PPEGE/lysozyme LbL films. Culture medium (0.5 mL) containing *B. subtilis* (OD_{660} = 0.08) was dripped on substrates (15 × 30 mm). After 5 h incubation at 37 °C, the turbidity of the culture medium was measured at 660 nm.

(PPEGE/ribonuclease A)$_{20}$ and (PPEGE/cytochrome c)$_{20}$ films were 44 and 58 nm, respectively (**Fig. 2a**), indicating successful preparation of LbL films using PPEGE and these polycationic proteins.

We then investigated erosion of the LbL film and the release of encapsulated lysozyme from the film. The (PPEGE/lysozyme)$_{20}$ film was incubated in pH 7.4 phosphate-buffered saline (PBS) at 37 °C. The film thickness gradually decreased to 14 nm in 16 h (**Fig. 2b**), indicating that PPEGE degradation induced disassembly of the LbL film and that non-degradable bilayers, (PSS/PEI)$_{10}$, remained on the silicon substrate. Lysozyme was simultaneously released into the solution (**Fig. 2b** and **c**). The total amount of lysozyme released at 16 h was 118 μg cm^{-2}, and it had an enzymatic activity of 220 units cm^{-2}, which was 75% of the intrinsic activity. This indicates that lysozyme was encapsulated in the LbL film without significant loss of enzymatic activity. Erosion of the film and lysozyme release were faster than PPEGE degradation (**Fig. 1b**), implying that partial degradation of PPEGE accelerated disassembly of the LbL film.

In the investigations, the release of lysozyme from the film was almost complete within 4 h. Hammond et al. described the burst release of encapsulated substrates from LbL films and reported the significance for controlled release.[32, 33] We introduced several PPEGE/chitosan bilayers into the PPEGE/lysozyme LbL films to delay film erosion and lysozyme release (**Fig. 3a**). **Fig. 3b** shows that film erosion was changed by the introduction of PPEGE/chitosan bilayers, although the initial film thicknesses were almost the same. Lysozyme release was also delayed by increasing the number of PPEGE/chitosan bilayers (**Fig. 3c**). These results indicate that the introduction of relatively stable bilayers into the films can control lysozyme release from degradable LbL films.

The biocompatibility of PPEGE is important in biomedical applications of PPEGE/polycation LbL films. We evaluated the cytotoxicity of PPEGE to HeLa cells. HeLa cells were cultured for 16 h in Dulbecco's modified Eagle's medium containing PPEGE at various concentrations. The PPEGE toxicity was negligible at PPEGE concentrations less than 0.1 wt% (**Fig. 4a**).

One possible application of PPEGE/lysozyme LbL films is as implantable biomaterials that release antibacterial activity. The antibacterial activity of the PPEGE/lysozyme LbL film was evaluated by placing a *Bacillus subtilis* suspension on the film and incubating for 5 h. PBAE,[43, 44] which is a degradable and non-toxic polycationic material, was used to prepare a PPEGE/polycation LbL film without an antibacterial agent, for use as a positive control. Formation of the PPEGE/PBAE LbL film was confirmed using ellipsometry (**Fig. S5**). *B. subtilis* grew on a bare silicon substrate and on the PPEGE/PBAE film [the initial optical density at 660 nm (OD_{660}) of the *B. subtilis* suspension was 0.08]. In contrast, *B. subtilis* growth on the PPEGE/lysozyme film was significantly inhibited (**Fig. 4b**). These results show that the PPEGE/lysozyme LbL film had antibacterial activity derived from the released lysozyme.

In conclusion, we designed and synthesized a novel hydrolytically degradable polyanion, PPEGE. PPEGE was easily synthesized in a single step via ring-opening polymerization between ODPA and PEG 200. PPEGE was degraded under physiological conditions, and had low cytotoxicity to mammalian cells. PPEGE can assemble with polycationic proteins, and with chitosan and PBAE, to form thin LbL films. The LbL film was eroded in aqueous solution and released the encapsulated protein, with maintenance of the enzymatic activity. LbL assembly is based on positively and negatively charged polyelectrolytes. A degradable synthetic polycationic material, PBAE, has already been reported to be useful in LbL films that can encapsulate and release polyanionic substances, and the development of a degradable, low-toxicity, synthetic polyanion will increase potential applications of LbL assembly with degradable synthetic polycationic materials.

We thank Prof. T. Nishino and Prof. M. Mizuhata for their technical help. This work was supported by Special Coordination Funds for Promoting Science and Technology,

Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan.

Notes and references

1 T. P. Richardson, M. C. Peters, A. B. Ennett and D. J. Mooney, *Nat Biotechnol*, 2001, **19**, 1029-1034.
2 A. C. Richards Grayson, I. S. Choi, B. M. Tyler, P. P. Wang, H. Brem, M. J. Cima and R. Langer, *Nat. Mater.*, 2003, **2**, 767-772.
3 J. M. Saul, M. P. Linnes, B. D. Ratner, C. M. Giachelli and S. H. Pun, *Biomaterials*, 2007, **28**, 4705-4716.
4 L. D. Shea, E. Smiley, J. Bonadio and D. J. Mooney, *Nat Biotechnol*, 1999, **17**, 551-554.
5 J. T. Santini, Jr., M. J. Cima and R. Langer, *Nature*, 1999, **397**, 335-338.
6 J. M. Lu, X. W. Wang, C. Marin-Muller, H. Wang, P. H. Lin, Q. Z. Yao and C. Y. Chen, *Expert. Rev. Mol. Diagn.*, 2009, **9**, 325-341.
7 J. J. Richardson, M. Bjoernmalm and F. Caruso, *Science*, 2015, **348**, 411-+.
8 E. M. Shchukina and D. G. Shchukin, *Curr. Opin. Colloid In.*, 2012, **17**, 281-289.
9 Y. Xiang, S. Lu and S. P. Jiang, *Chem. Soc. Rev.*, 2012, **41**, 7291-7321.
10 O. Azzaroni and K. H. A. Lau, *Soft Matter*, 2011, **7**, 8709-8724.
11 S. Srivastava and N. A. Kotov, *Acc. Chem. Res.*, 2008, **41**, 1831-1841.
12 K. Ariga, Y. Yamauchi, G. Rydzek, Q. M. Ji, Y. Yonamine, K. C. W. Wu and J. P. Hill, *Chem. Lett.*, 2014, **43**, 36-68.
13 R. M. Iost and F. N. Crespilho, *Biosens. Bioelectron.*, 2012, **31**, 1-10.
14 G. Decher, J. D. Hong and J. Schmitt, *Thin Solid Films*, 1992, **210**, 831-835.
15 G. Decher, *Science*, 1997, **277**, 1232-1237.
16 K. Ariga, Y. Lvov and T. Kunitake, *J. Am. Chem. Soc.*, 1997, **119**, 2224-2231.
17 C. Vodouhe, M. Schmittbuhl, F. Boulmedais, D. Bagnard, D. Vautier, P. Schaaf, C. Egles, J. C. Voegel and J. Ogier, *Biomaterials*, 2005, **26**, 545-554.
18 Z. W. Mao, L. Ma, J. Zhou, C. Y. Gao and J. C. Shen, *Bioconjug. Chem.*, 2005, **16**, 1316-1322.
19 A. Dierich, E. Le Guen, N. Messaddeq, J. F. Stoltz, P. Netter, P. Schaaf, J. C. Voegel and N. Benkirane-Jessel, *Adv. Mater.*, 2007, **19**, 693-+.
20 D. M. Lynn, *Adv. Mater.*, 2007, **19**, 4118-4130.
21 R. C. Smith, M. Riollano, A. Leung and P. T. Hammond, *Angew. Chem. Int. Ed.*, 2009, **48**, 8974-8977.
22 O. S. Sakr and G. Borchard, *Biomacromolecules*, 2013, **14**, 2117-2135.
23 S. A. Sukhishvili and S. Granick, *J. Am. Chem. Soc.*, 2000, **122**, 9550-9551.
24 C. Schuler and F. Caruso, *Biomacromolecules*, 2001, **2**, 921-926.
25 X. P. Qiu, S. Leporatti, E. Donath and H. Mohwald, *Langmuir*, 2001, **17**, 5375-5380.
26 X. Y. Shi and F. Caruso, *Langmuir*, 2001, **17**, 2036-2042.
27 S. A. Sukhishvili and S. Granick, *Macromolecules*, 2002, **35**, 301-310.
28 M. Macdonald, N. M. Rodriguez, R. Smith and P. T. Hammond, *J. Control. Release*, 2008, **131**, 228-234.
29 E. Vazquez, D. M. Dewitt, P. T. Hammond and D. M. Lynn, *J. Am. Chem. Soc.*, 2002, **124**, 13992-13993.
30 J. T. Zhang, L. S. Chua and D. M. Lynn, *Langmuir*, 2004, **20**, 8015-8021.
31 C. M. Jewell, J. T. Zhang, N. J. Fredin and D. M. Lynn, *J. Control. Release*, 2005, **106**, 214-223.
32 B. B. Hsu, K. S. Jamieson, S. R. Hagerman, E. Holler, J. Y. Ljubimova and P. T. Hammond, *Angew. Chem. Int. Ed.*, 2014, **53**, 8093-8098.
33 B. B. Hsu, S. R. Hagerman, K. Jamieson, J. Veselinovic, N. O'Neill, E. Holler, J. Y. Ljubimova and P. T. Hammond, *Biomacromolecules*, 2014, **15**, 2049-2057.
34 M. R. Jackson, *Am. J. Surg.*, 2001, **182**, 1S-7S.
35 V. Z. Spassov, A. D. Karshikov and B. P. Atanasov, *Biochim. Biophys. Acta*, 1989, **999**, 1-6.
36 M. Takano, Y. Koyama, H. Nishikawa, T. Murakami and R. Yumoto, *Eur. J. Pharmacol.*, 2004, **502**, 149-155.
37 Y. Hao, C. Liang, A. Moriya, H. Matsuyama and T. Maruyama, *Ind. Eng. Chem. Res.*, 2012, **51**, 14850-14858.
38 C. Picart, P. Lavalle, P. Hubert, F. J. G. Cuisinier, G. Decher, P. Schaaf and J. C. Voegel, *Langmuir*, 2001, **17**, 7414-7424.
39 P. Lavalle, C. Gergely, F. J. G. Cuisinier, G. Decher, P. Schaaf, J. C. Voegel and C. Picart, *Macromolecules*, 2002, **35**, 4458-4465.
40 C. Picart, J. Mutterer, L. Richert, Y. Luo, G. D. Prestwich, P. Schaaf, J. C. Voegel and P. Lavalle, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 12531-12535.
41 C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.*, 1956, **78**, 5287-5291.
42 M. A. Marini, G. E. Marti, R. L. Berger and C. J. Martin, *Biopolymers*, 1980, **19**, 885-898.
43 D. M. Lynn and R. Langer, *J. Am. Chem. Soc.*, 2000, **122**, 10761-10768.
44 Y. Funasaki, E. Tsuchiya and T. Maruyama, *Colloid Polym. Sci.*, 2014, **292**, 3049-3053.