Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Journal of Materials Chemistry B

COMMUNICATION

Fabrication of stimulus-responsive diatom biosilica microcapsules for antibiotic drug delivery

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

DOI: 10.1039/x0xx00000x

www.rsc.org/MaterialsB

R. B. Vasani,[a] D. Losic,[b] A. Cavallaro[a] and N. H. Voelcker[a]

In this report, we employed surface-initiated activators regenerated by electron transfer based atom transfer radical polymerisation to graft thermo-responsive copolymers of oligo(ethylene glycol) methacrylates from the surface of diatom biosilica microcapsules. We demonstrate the application of the resulting composites for thermo-responsive drug delivery.

Diatomaceous earth (DE) are the fossilised remains of single cell photosynthetic algae that are found abundantly in marine and fresh-water systems. Diatoms produce complex three-dimensional (3-D) silica-based porous shells called frustules with ordered pore structures, in species-specific patterns.[1, 2] Diatoms have been studied extensively for applications in nanofabrication, optics, water purification, solar cells, biosensing and drug delivery owing to their uniqe porous structure, good mechanical properties, biocompatibility, tailor-able surface chemistry and low cost.[3-7] The concept of drug delivery from diatoms has recently been demonstrated, where the hollow structure of the biosilica frustules acted as microcapsules for loading therapeutics.[5, 8] These studies showed that drug release from the frustules was dependent on the pore size and architecture of the biosilica structure and can to some extent be controlled by altering their surface chemistry.[5] More recently, diatom frustules broken into nanoparticles were shown to be capable of transport through cellular membranes,[9] and were used as non-toxic carriers of siRNA into cancer cells.[10] A study by Kumeria et al. described a system based on a combination of diatom frustules with graphene oxide layers to achieve pH-dependent drug release.[7] However, even though certain progress has been made in this area, the problem of achieving controllable drug release from diatom biosilica microcapsules still remains to be addressed.

A strategy for controlled release *via* the utilisation of stimulus-responsive polymers that act as actuators capable of blocking or opening the pore apertures in response to environmental changes is well-established for nanoporous drug carriers including porous silicon, synthetic mesoporous silica and polymeric membranes.[11-19] However, to the best of our knowledge this concept has not yet been applied to diatom biosilica materials. An advantage of the DE microcapsules over other synthetic porous silica/silicon based materials is that DE is an inexpensive material, abundantly available in nature and can be easily processed with low energy cost and without excessive waste or use of toxic materials, such as in the case for synthetic silica materials.

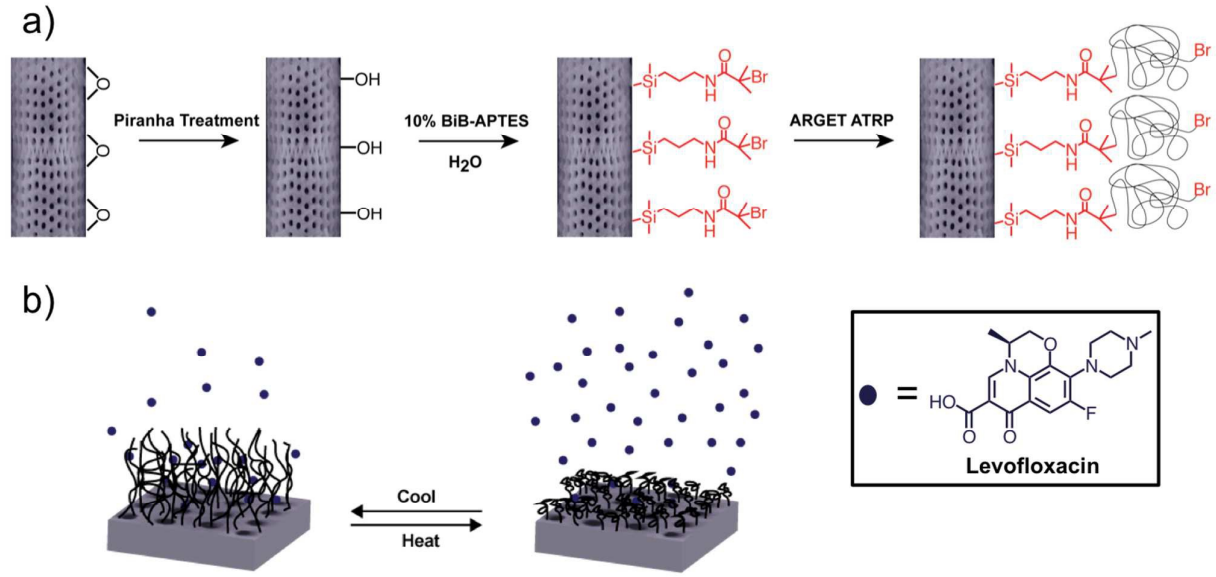
Previous studies have demonstrated that oligo(ethylene glycol) methacrylates (O(EG)MA) can be polymerised to attain stimulus - responsive polymers that react to changes in the temperature of the environment, i.e. they are thermo-responsive.[20] A unique property of these polymers is that the temperature of transition, known as the lower critical solution temperature (LCST), is dependent on the number of ethylene glycol units present in the monomer unit.[21, 22] For example, a homopolymer produced from the monomer with two units ($O(EG)_2MA$) is reported to have a LCST of 26 °C while a homopolymer produced from the monomer of molecular weight of 300 (4 - 5 units) ($O(EG)_{4\text{-}5}MA$) responds at a transition temperature of around 65 °C.[23] On heating above the LCST, the polymers in solution collapse into globules. Random copolymers produced using two distinct oligo(ethylene glycol) methacrylate monomers of different molecular weights have been shown to possess LCST transitions dependent on the ratio of the two monomers present in the chain.[20] This property offers a rather unique opportunity as it allows to 'dial in' the LCST of the copolymer according to the requirements of the application. However, to obtain narrow LCST ranges, it is imperative that the ratio of the two monomers used be constant.[24] This is best achieved using controlled radical polymerisation techniques such as activators regenerated by electron transfer based atom transfer radical polymerisation (ARGET-ATRP).

In this communication, we describe a method of grafting

[a] *ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Mawson Institute, University of South Australia, SA 5095, Australia*
[b] *School of Chemical Engineering, The University of Adelaide, SA 5000, Australia*
Electronic Supplementary Information (ESI) available: SEM and AFM images of diatoms and detailed experimental procedures. See DOI: 10.1039/x0xx00000x

Journal of Materials Chemistry B

COMMUNICATION



Scheme 1 a) Process of diatom biosilica microcapsule functionalisation and b) drug release from thermo-responsive polymer-grafted biosilica frustule.

stimulus-responsive O(EG)MA co-polymers from the surface of diatom microcapsules using aqueous ARGET-ATRP. We demonstrate the application of these modified diatoms for controlled release of levofloxacin (LVX), a broad-spectrum antibiotic that is used to treat bacterial infections including those in chronic wounds.[25, 26] We also examine the thermo-responsive antibacterial activity of the system against two common wound pathogens, namely, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using a zone of inhibition assay.

DE material composed of fresh water *Aulacoseria sp* diatoms was obtained from Mount Sylvia Pty. Ltd. This species of diatoms typically produces cylindrical frustules, approximately 10 – 20 μm in length and 4 – 5 μm in diameter.[8] Fig. S1 shows an SEM image of a typical *Aulacoseria sp.* fusttule. The samples were purified to obtain whole un-fractured diatoms structures in the form of hollow microcapsules with either one or both ends open, using a previously described method.[27] The purified diatom microcapsules were then functionalised using a 3-step process schematically illustrated in Scheme 1a. First, the diatoms were treated with Piranha solution for 1 hour in order to generate hydroxyl groups on the surface. The diatom microcapsules were then reacted with a silane-based ATRP initiator 3-(2-bromoisobutyramido)propyl(triethoxy)silane (BiB-APTES), synthesised in-house,[11] using a previously reported aqueous silanisation method for 48 hours.[28] Oligo(ethylene glycol) methacrylate copolymers were first synthesized in solution by means of aqueous ARGET-ATRP using a 25% methanol-water mixture as the solvent. The other components of the reaction were: a $CuBr_2$/bipyridyl system as catalyst, ascorbic acid as reducing agent and ethyl 2-bromoisobutyrate (EBiB) as initiator.

These components were added to 5 mL of the solvent in the molar ratio of 100/0.01/0.20/2.72 (monomer/$CuBr_2$/ascorbic acid/EBiB). Following this, the monomers $O(EG)_2MA$ and $O(EG)_{4\text{-}5}MA$ were either added to the solution on their own or at a molar ratio of 1/0.16 $O(EG)_2MA/O(EG)_{4\text{-}5}MA$ (P1). Polymerisation reactions were carried out for 1 hour at 25 °C under constant bubbling of argon gas. Once the reaction was completed, the polymerisation mixture was diluted with tetrahydrofuran and passed through a short column of neutral aluminium oxide to remove the catalyst. The polymer chains were then precipitated out in excess of diethyl ether. Cloud point (CP) measurements (Fig. 1a) were performed in Milli-Q water (18 MΩ.cm) using a Hewlett-Packard 8452 diode array UV-Vis spectrophotometer. The CP of P1 was found to be around 36 °C with a sharp transition (ΔT = 4.2 °C) and little hysteresis between the heating and cooling cycles. For the homopolymers $O(EG)_2MA$ and $O(EG)_{4\text{-}5}MA$, CP values were determined to be 27 ˚C and 67 ˚C, respectively

A grafting-from approach namely, surface initiated – ATRP (SI-ATRP) was employed to graft P1 from the diatom surface. This technique was used in order to generate high-density polymer layers on the surfaces.[29] The grafting of P1 from the surface of the diatom frustules was performed by employing a similar protocol to the one described above with the exception that 20 mg of BiB-APTES-functionalised diatoms were used as

the initiator instead of EBiB. Following the reaction, the polymer-modified diatom structures were separated from the reaction mixture using centrifugation, washed three times with ethanol, collected by centrifugation and dried overnight at room temperature.

Fourier-transformed infrared (FTIR) spectra of the surface were acquired in reflectance mode on a Nicolet iN10 microscope (Thermo scientific) after each modification step to study the changes in the surface chemistry. The bare frustule surface (Fig. 1b (i)) shows one large peak at around 1100 cm^{-1} (1) that corresponds to Si-O vibrations of silica,[11] and also a peak at 1635 cm^{-1} (2) that can be attributed to the stretching vibration of zeolitic water.[30] The broad peak around 3400 cm^{-1} (7) corresponds to free –OH groups from adsorbed water. Following the aqueous silanisation reaction (Fig. 1b (ii)), a small peak appears at 1565 cm^{-1} (3) along with a larger peak at 1654 cm^{-1} (4) that can be attributed to the N-H and C-O vibrations of the amide present in the BiB-APTES.[11] The spectrum of the polymer-modified frustule surface (Fig. 1b (iii)) shows the peak at 1100 cm^{-1} of the silica surface as well as the amide peaks from the BiB-APTES and additionally also shows the appearance of a new peak 1730 cm^{-1} (5) which can be assigned to the stretching vibration of the carbonyl present in the polymethacrylate backbone of the polymer.[31] The three peaks present between 2800 – 3000 cm^{-1} (6) were attributed to the C-H stretching vibrations from the polymer chains. SEM characterisation provided corroborating evidence for the presence of the polymer film on the surface of the diatoms. Fig. 1c-e shows SEM images of the diatom microcapsule surface before modification, after BiB-APTES modification and after grafting of P1 from the surface. The frustules of *Aulacoseria sp.* are typically cylindrical in shape and have pore openings in the order of 200 – 300 nm. In Fig. 1e, the coating of polymer is clearly observed, blocking the pores of the P1-modified diatoms. AFM measurements (Fig. S2a and b) also confirmed the presence of the polymer layer on the diatoms.

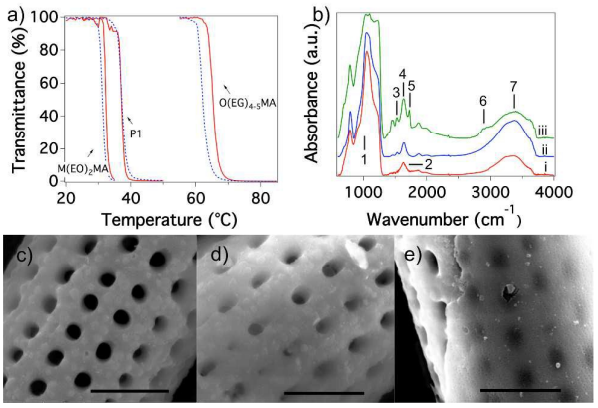


Fig. 1 a) Cloud point (CP) measurements on heating and cooling of an $O(EG)_2MA$ homopolymer (Mn – 157,632 Da), P1 (Mn – 124,769 Da) and $O(EG)_{4-5}MA$ homopolymer (Mn – 173,425 Da) showing CPs of 27 °C, 36 °C and 67 °C, respectively, b) FTIR spectra of the samples after (i) no modification, (ii) BiB-APTES functionalisation and (iii) modification with P1; SEM images (scale bar = 2 μm) of c) an unmodified frustule, d) a BiB-APTES functionalised frustule and e) a P1-modified frustule structure

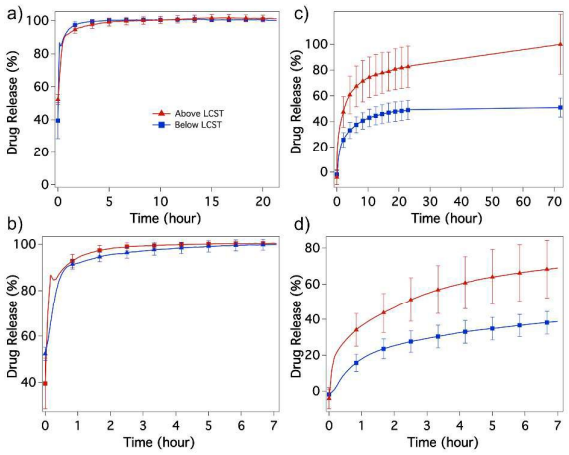


Fig. 2 Release curves of LVX from the diatom microcapsules below and above the LCST of the P1 copolymer. a) Total Release and b) first 7 hours of release from unmodified microcapsules, c) total release and d) first 7 hours of release from P1-modified microcapsules.

Following characterisation, an application of the modified diatom microcapsules for controlled drug delivery was evaluated using LVX as an antibiotic drug. This drug is commonly used as a first-line antibiotic for the initial treatment of infected wounds.[25] LVX is a fluoroquinone molecule that emits fluorescence at 454 nm when excited at a wavelength of 292 nm.

Drug loading into the diatom microcapsules was performed using the vacuum infiltration method.[32] For this method, a known weight of diatom microcapsules was immersed in a 100 mg/mL solution of LVX in dimethylformamide (DMF) and the solution was then frozen in liquid nitrogen and pumped under vacuum to degas it. Following this, the solution was allowed to warm back up to room temperature. The vial was then quickly brought back to atmospheric pressure, facilitating the infiltration of the drug solution into the porous diatoms. The frustules were collected by centrifugation, washed with water and acetone, and dried in an oven at 70 °C.

The kinetics of LVX release from the diatoms were investigated using a fluorescence spectrophotometer equipped with a Peltier system for temperature modulation. The release experiments were performed in PBS (pH 7.4) at temperatures both above and below the LCST of P1. Approximately 1 mg of diatom microcapsules was used for these studies. The results are graphically illustrated in Fig. 2. As is evident from the graph, the control samples (Fig. 2a), corresponding to LVX-loaded frustules with no polymer coating, released over 95% of their total payload of LVX within 2 hours. This is expected as the pores of the frustule are in the order of around 200 – 300 nm and therefore cannot form a good barrier to the release of a small molecular drug such as LVX. Fig. 2b shows the release profile of the control samples in the first 7 hours of release. A slight increase in the burst release was observed on heating the system to 45 °C that can be attributed to the higher diffusion kinetics at elevated temperatures. In contrast to this, the P1-modified diatoms

showed sustained release profiles with a very conspicuous difference in the release kinetics below and above the LCST of P1 (Fig. 2c). . Below the LCST, the expanded polymer blocked the pore openings and slowed the diffusion of the drug molecules. On the other hand, above the LCST the polymer layer had collapsed and opened the pores, allowing for quicker release of the entrapped molecules. The process is schematically represented in Scheme 1b. Fig. 2d depicts the first 7 hours of release from the P1-modified diatoms and also shows a clear difference in the amount of burst release observed at the two temperatures. This type of release profile is consistent with previously reported results for thermo-responsive polymer-coated porous systems.[11]

An initial burst release of about 30% to 60% of the drug was observed below and above the LCST, respectively, followed by a more sustained release profile. This burst release could be due to the sudden expulsion of drug molecules adsorbed on the outer surface of the microcapsule or trapped in the polymer layer rather than inside the microcapsule. It was found that approximately 100% release of the drug occurred after 3 days of incubation above the LCST. In contrast, only approximately 50% release was observed for samples incubated below the LCST over that time frame.

When comparing the drug release results outlined here with previous work using diatom microcapsules, Sinn Aw *et al*. and Bariana *et al.*[5, 8] modified the surface chemistry of the microcapsules in an attempt to control the release of loaded therapeutics. However, while their approach was reasonably successful in altering the release kinetics, a large burst release was observed with approximately 40 – 90% of the loaded therapeutics released within a few hours, depending on the surface chemistry of the diatoms and the type of drugs used. In comparison, the microcapsules described here exhibit variable bursts of 30 – 60% of the payloads, depending on the temperature of the environment. Kumeria *et al.*[7] described a pH-responsive drug delivery system using diatom microcapsules coated with graphene oxide to control the release of indomethacin in different pH environments. The release behaviour in this case was governed by changes in the chemical interaction of the loaded drug with the graphene oxide in different pH buffers. In contrast, the delivery system described in this report relies on the polymer acting as a physical actuator to control the release of drugs from the microcapsules rather than chemical interactions. Whilst our study exemplifies the potential of this technology by demonstrating antibiotic release, it is likely that this controlled delivery approach is compatible with a range of therapeutics at a temperature defined by the oligo(ethylene glycol) methacrylate copolymer composition. Furthermore, while a temperature-responsive polymer was used here, it is also possible to use the same functionalisation strategy to graft other types of responsive polymers, including those that are triggered by pH changes or by certain wavelenghts of light.

An important factor for consideration when fabricating a drug delivery system is ensuring that the activity of the loaded therapeutic is still retained. We therefore performed zone of inhibition (ZOI) studies with LVX samples collected at different points during the release experiment, using two common wound pathogens, namely, *S. aureus* (gram positive) and *P. aeruginosa* (gram negative). Samples of the release solution (above and below the LCST) were collected at 0, 1 and 20 hours of release and 50 μL aliquots were pipetted onto filter discs. The results are depicted in Fig. 3a. Both types of bacteria were found to be susceptible to the action of the released LVX, thereby proving the activity of the loaded therapeutic. It should also be noted that *S. aureus* showed higher susceptibility to LVX than *P. aeruginosa*. This is an expected trend as *S. aureus* has a lower minimum inhibitory concentration (MIC) (0.25 μg/mL) as compared to P. aeruginosa (0.50 μg/mL).[33, 34] A clear increase in the ZOI with release time was observed for both species of bacteria. In all cases, but particularly after 1 hour, the ZOI values were higher for LVX released from P1-modified diatoms at 45 ˚C in comparison to release at 25 ˚C (Fig. 3 b-e). After 1 hour of LVX release, the zone diameter at 45 ˚C plateaus at around 22 mm due to the diffusion limit of LVX through the agar, leading to a reduction in the difference between the ZOI diameters between the 25 ˚C and 45 ˚C release conditions. It should also be noted that solutions (50 μL) containing the respective MIC concentrations for the two species of bacteria did not result in measurable ZOI when 50 μL aliquots were applied to the ZOI discs, indicating that the LVX release from the diatoms was at concentrations higher than the MICs. ZOI tests performed using buffer incubated with diatoms not loaded with LVX did not show inhibition zones.

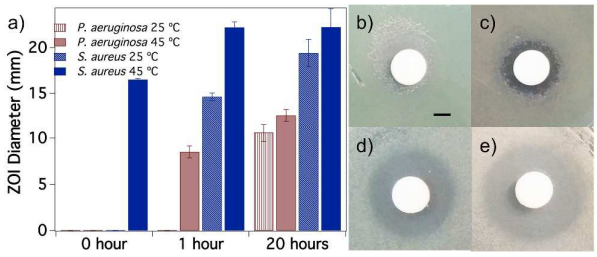


Fig. 3 a) Measured ZOI for *P. aeruginosa* and *S. aureus* using aliquots of LVX release solutions collected at the time points mentioned. 0 hour corresponds to samples taken immediately after mixing the diatoms and buffer. Representative images of the ZOIs formed using the 1 hour LVX release solution collected at b) 25 ˚C and c) 45 ˚C with *P aeruginosa* and at d) 25 ˚C and e) 45 ˚C with *S. aureus* (scalebar = 5 mm).

Conclusion

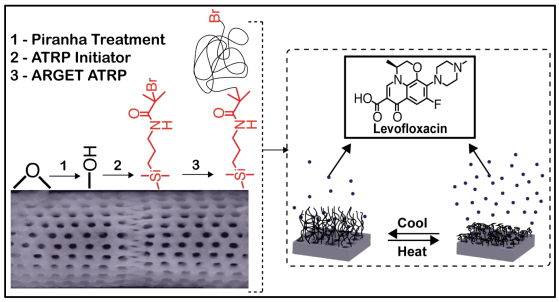
In summary, we have demonstrated a controlled drug delivery vehicle fabricated by grafting thermo-responsive oligo(ethylene glycol) methacrylate copolymers from the surface of diatom biosilica microcapsules using SI-ATRP. Drug release experiments from the copolymer modified microcapsules showed strong temperature dependence of drug release when comparing release kinetics below and above the LCST of the grafted copolymer. The antimicrobial action of the released drug was confirmed against two common wound pathogens. The composites described here offer an inexpensive source for the scalable production of drug carriers facilitating controlled delivery of therapeutics.

Acknowledgements

This research was conducted and funded by the Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology (project number CE140100036) and the Wound Management Innovation Co-operative Research Centre.

Notes and references

1. R. Gordon, D. Losic, M. A. Tiffany, S. S. Nagy and F. A. S. Sterrenburg, *Trends Biotechnol.*, 2009, **27**, 116-127.
2. D. Losic, J. G. Mitchell and N. H. Voelcker, *Adv. Mater.*, 2009, **21**, 2947-2958.
3. J. E. N. Dolatabadi and M. de la Guardia, *TrAC, Trends Anal. Chem.*, 2011, **30**, 1538-1548.
4. C. S. Gaddis and K. H. Sandhage, *J. Mater. Res.*, 2004, **19**, 2541-2545.
5. M. S. Aw, M. Bariana, Y. Yu, J. Addai-Mensah and D. Losic, *J. Biomater. Appl.*, 2013, **28**, 163-174.
6. M. S. Aw, S. Simovic, J. Addai-Mensah and D. Losic, *Nanomedicine*, 2011, **6**, 1159-1173.
7. T. Kumeria, M. Bariana, T. Altalhi, M. Kurkuri, C. T. Gibson, W. Yang and D. Losic, *Journal of Materials Chemistry B*, 2013, **1**, 6302-6311.
8. M. Bariana, M. S. Aw, M. Kurkuri and D. Losic, *Int. J. Pharm.*, 2013, **443**, 230-241.
9. I. Ruggiero, M. Terracciano, N. Martucci, L. De Stefano, N. Migliaccio, R. Tatè, I. Rendina, P. Arcari, A. Lamberti and I. Rea, *Nanoscale Research Letters*, 2014, **9**, 1-7.
10. I. Rea, N. M. Martucci, L. De Stefano, I. Ruggiero, M. Terracciano, P. Dardano, N. Migliaccio, P. Arcari, R. Taté, I. Rendina and A. Lamberti, *Biochimica et Biophysica Acta (BBA) - General Subjects*, 2014, **1840**, 3393-3403.
11. R. B. Vasani, S. J. P. McInnes, M. A. Cole, A. M. M. Jani, A. V. Ellis and N. H. Voelcker, *Langmuir*, 2011, **27**, 7843-7853.
12. S. J. P. McInnes, E. J. Szili, S. A. Al-Bataineh, J. J. Xu, M. E. Alf, K. K. Gleason, R. D. Short and N. H. Voelcker, *ACS Appl. Mater. Interfaces*, 2012, **4**, 3566-3574.
13. L. Hu, L.-Y. Chu, M. Yang, J. Yu and H.-D. Wang, *Chemical Engineering & Technology*, 2007, **30**, 523-529.
14. F. Teixeira, A. M. Popa, S. Guimond, D. Hegemann and R. M. Rossi, *J. Appl. Polym. Sci.*, 2013, **129**, 636-643.
15. I. Zhuk, F. Jariwala, A. B. Attygalle, Y. Wu, M. R. Libera and S. A. Sukhishvili, *ACS Nano*, 2014, **8**, 7733-7745.
16. M. D. Kurkuri, M. R. Nussio, A. Deslandes and N. H. Voelcker, *Langmuir*, 2008, **24**, 4238-4244.
17. R. B. Vasani, M. A. Cole, A. V. Ellis and N. H. Voelcker, in *Nanomaterials for Life Sciences: Polymeric Nanomaterials*, ed. C. S. S. R. Kumar, Wiley-VCH, Weinheim, Germany, 2011, vol. 10, pp. 59 - 103.
18. Y. Li and J. Shi, *Adv. Mater.*, 2014, **26**, 3176-3205.
19. Y. Zhu, J. Shi, W. Shen, X. Dong, J. Feng, M. Ruan and Y. Li, *Angew. Chem. Int. Ed.*, 2005, **44**, 5083-5087.
20. J.-F. Lutz, Ö. Akdemir and A. Hoth, *J. Am. Chem. Soc.*, 2006, **128**, 13046-13047.
21. J.-F. Lutz, *J. Polym. Sci., Part A: Polym. Chem.*, 2008, **46**, 3459-3470.
22. C. R. Becer, S. Hahn, M. W. M. Fijten, H. M. L. Thijs, R. Hoogenboom and U. S. Schubert, *J. Polym. Sci., Part A: Polym. Chem.*, 2008, **46**, 7138-7147.
23. Z. Hu, T. Cai and C. Chi, *Soft Matter*, 2010, **6**, 2115-2123.
24. J.-F. Lutz, K. Weichenhan, Ö. Akdemir and A. Hoth, *Macromolecules*, 2007, **40**, 2503-2508.
25. R. Hernandez, *Dermatologic Therapy*, 2006, **19**, 326-337.
26. R. D. Wolcott and S. E. Dowd, *Journal of Wound Care*, 2008, **17**, 513 - 516.
27. Y. Yu, J. Addai-Mensah and D. Losic, *Langmuir*, 2010, **26**, 14068-14072.
28. N. L. Rosi, C. S. Thaxton and C. A. Mirkin, *Angew. Chem. Int. Ed.*, 2004, **43**, 5500-5503.
29. Y. Tsujii, K. Ohno, S. Yamamoto, A. Goto and T. Fukuda, in *Surface-Initiated Polymerization I*, ed. R. Jordan, Springer Berlin Heidelberg, 2006, vol. 197, pp. 1-45.
30. G. Sheng, J. Hu and X. Wang, *Appl. Radiat. Isot.*, 2008, **66**, 1313-1320.
31. J. E. Raynor, T. A. Petrie, A. J. García and D. M. Collard, *Adv. Mater.*, 2007, **19**, 1724-1728.
32. B. Kundu, A. Lemos, C. Soundrapandian, P. S. Sen, S. Datta, J. M. F. Ferreira and D. Basu, *J Mater Sci: Mater Med*, 2010, **21**, 2955-2969.
33. M. P. Montanari, M. Mingoia and P. E. Varaldo, *Antimicrob. Agents Chemother.*, 2001, **45**, 3616-3622.
34. J. M. Andrews, *J. Antimicrob. Chemother.*, 2001, **48**, 5-16.



Thermo-responsive drug release from diatom biosilica microcapsules is demonstrated for the first time using microcapsules modified with copolymers of oligoehtylene glycol methacrylates.