Polymer Chemistry

Cite this: *Polym. Chem.*, 2012, **3**, 3180

www.rsc.org/polymers

PAPER

Triggered insulin release studies of triply responsive supramolecular micelles†

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Received 31st May 2012, Accepted 12th July 2012
DOI: 10.1039/c2py20380d

The synthesis of a supramolecular double hydrophilic glucose responsive block copolymer (DHBC) held together by cucurbit[8]uril (CB[8]) ternary complexation and its subsequent self-assembly into micelles is described. The supramolecular block copolymer assembly consists of poly(*N*-isopropylacrylamide) (PNIPAAm) and poly(acrylamidophenyl boronic acid) (PAAPBA) as temperature and glucose responsive blocks, respectively, and poly(dimethylacrylamide) (PDMAAm) as a hydrophilic block. Drug release studies of insulin-loaded micelles using three external triggers were studied with release of insulin achieved by changing temperature, glucose concentration or by adding a competitive guest for CB[8]. This system offers good control over the release of insulin under physiological conditions (pH 7.4, 37 °C). These exciting results suggest that this system could be a model for a clinically relevant drug delivery vehicle for diabetic treatment.

1 Introduction

Diabetes mellitus (DM) is a metabolic disease whereby a person does not produce sufficient insulin or does not respond well to insulin produced in the body on the account of elevated blood glucose levels[1–3] leading to conditions, such as polyuria, polydipsia and polyphagia. Type I DM results from the body's failure to produce insulin as a result patients require frequent injections of the hormone. It remains a challenge to develop systems that are able to release insulin in a triggered manner, in order to allay patient discomfort and the inconvenience of multiple injections every day. Glucose responsive polymers are highly attractive candidates for use in insulin delivery to patients as these polymers are able to release insulin in response to the blood glucose concentrations. Phenylboronic acid derivatives have been widely used as a sensing moiety for glucose.[4] Hydrophobic phenylboronic acid derivatives (**A**) produce water-soluble species (**B**) when complexed with hydroxyl ions below their pK_a as illustrated in Fig. 1.[5] The addition of glucose favours the formation of the stable water-soluble phenyl borate (**C**) between the phenyl boronic acid derivatives and glucose.

When phenylboronic acid moieties are included in the main polymer chain, the polymer is endowed with glucose sensitivity which has potential applications in drug delivery. Recently, a variety of glucose-responsive hydrogels have been reported.[6–8] Kataoka *et al.* have embarked on a series of experiments to utilise phenylboronic acid derivatives at the physiological pH of 7.4, for potential glucose triggered release of insulin.[9–11] Several systems relying on micron or sub-micron particulate matter have been reported. For example, glucose and temperature sensitive microgels have been utilised for the development of self-regulated insulin delivery systems.[12,13] These systems release insulin when they are induced to swell under the application of either a glucose or temperature stimuli. Insulin can also be loaded into mesoporous silica particles for controlled delivery. These silica particles serve as an insulin reservoir, and enzyme multilayers cross-linked with glutaraldehyde act as a valve to control the release of insulin in response to the external glucose level.[14] Insulin loaded nanoparticles derived from a copolymer of phenylboronic acid and sugar-based side chains were shown to be non-toxic and useful for glucose sensitive delivery. These nanoparticles self-assemble by forming cross-links between the pendant boronic acid groups and the free hydroxyl groups of the sugar, with insulin incorporated between the chains. When free glucose is added, it competitively binds with the boronic acid moieties, breaking the cross-links and swelling the nanoparticle, thus releasing an insulin payload.[15] Micelles are a highly desirable system for the encapsulation and delivery of hydrophobic compounds.[16–18] Temperature sensitive micelles can be fabricated by chemically conjugating

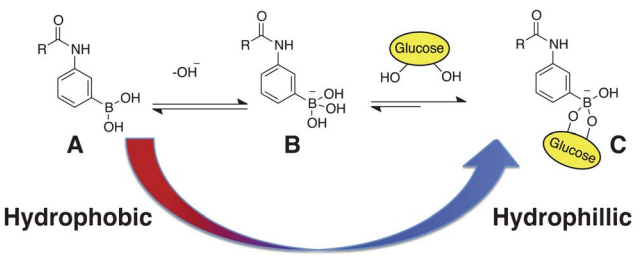

Fig. 1 Formation of the boronic acid–glucose complex.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2py20380d

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poly(*N*-isopropylacrylamide) (PNIPAAm) to other hydrophilic block copolymers. These polymers have extremely good stability in solution and have high drug encapsulation efficiency.[16–18]

Preparation of these block copolymers by typical chemical synthetic methods, however, renders this technology beyond the reach of most non-chemists. Therefore, a need exists to develop a non-chemical facile preparation method for the synthesis of block copolymers that can be utilised as drug delivery carriers for hydrophobic drugs and proteins. It would be ideal to modify the properties of block copolymers in a non-covalent manner by selecting a desired stimuli–responsive material from a catalog of polymers through simple mixing with other functionalised non-responsive polymers. Such a non-covalent polymeric construct would require the use of supramolecular techniques to prepare the structures from basic building blocks. Cucurbit[8]uril (CB[8]) is an attractive choice for building stable modular supramolecular structures in an aqueous environment. CB[8] is a water-soluble macrocyclic host that has a cavity volume of 479 $Å^3$ and stabilises ternary complexes consisting of electron-deficient and electron-rich aromatic motifs.[19–22]

We have recently exploited the ability of CB[8] to simultaneously bind two guest molecules conjugated to the chain ends of polymers. This work was utilised in the construction of the first supramolecular doubly hydrophilic block copolymers (DHBCs) through a dynamic CB[8] "handcuff" as illustrated in Fig. 2.[23] Hierarchical self-assembled structures in the aqueous environment can be prepared from CB[8] ternary complexes. This system offers strong binding of two complementary motifs (binding constants $K_{eq} = 10^{12}$ M^{-2}) in water and can be used to prepare a variety of stable structures *via* a non-covalent route.[24–26] Our recent work on fast modulation of temperature sensitive behaviour of methylviologen (MV)-PNIPAAm exploits CB[8] as a supramolecular switch to alter the hydrophobic and hydrophilic properties of a polymer.[27]

The supramolecular approach offers versatility and simplicity in the preparation of DHBCs as compared to conventional covalent techniques for polymer synthesis as well as augmented function on account of its stimuli responsive nature. Significant advantages are provided to end users where preparation of drug formulations require only simple mixing without the need for complicated chemical synthesis. In addition to conventional stimuli, such as temperature and glucose, the use of CB[8] offers a new dimension as supramolecularly linked copolymers can be unlocked by addition of a chemical stimuli such as adamantane amine.[23,27] Herein we synthesised a supramolecular DHBC held together by CB[8] ternary complexation, which self-assembles into micelles at physiological temperature and can be used to encapsulate and release insulin. The sensitivity of this system to temperature, glucose and a competitive guest allows for the regulated release of insulin at different rates depending on the user requirements.

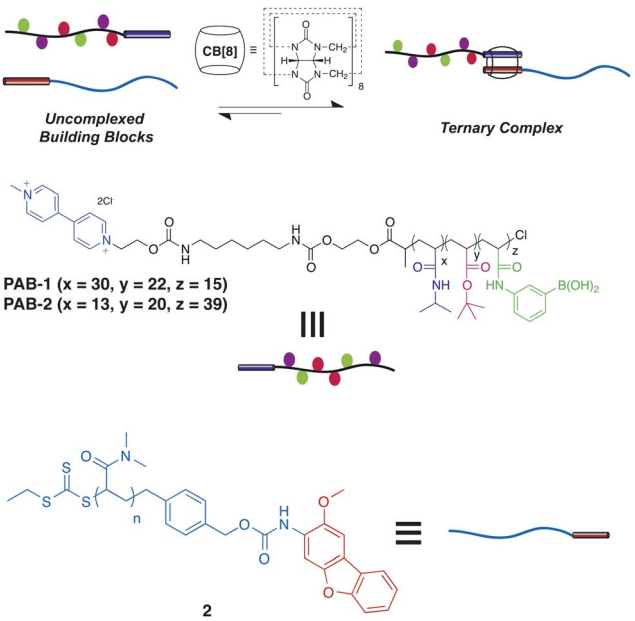

Fig. 2 Formation of polymeric CB[8] ternary complex.

2 Experimental section

2.1 Materials

All starting materials were purchased from Alfa Aesar or Sigma Aldrich and were used as received unless stated otherwise. *N*-Isopropylacrylamide (NIPAAm) was recrystallised twice from hexane. CB[8] was prepared as documented previously.[19]

2.2 Instrumentation

^{1}H NMR (500 MHz) and ^{11}B NMR (160 MHz) spectra were recorded on a Bruker Advance BB-ATM 500 MHz machine. ^{1}H NMR chemical shifts are reported in ppm from tetramethylsilane with the solvent resonances as the internal standards (d_6-DMSO = 2.54 ppm). $BF_3 \cdot OEt_2$ is used as the external reference in $CDCl_3$ for ^{11}B NMR. UV/vis spectra were recorded on a Varian Cary 100 Bio UV/vis spectrophotometer equipped with a temperature controller. Dynamic light scattering (DLS) measurements were performed on Malvern Zetasizer NS90 instrument.

2.3 Synthesis of methylviologen terminated poly(*N*-isopropylacrylamide)-*r*-poly(*t*-butylacrylate), PNIPAAm-PtBA-MV (P-1 and P-2)

In brief, 25 mg of the initiator,[28] 0.466 g of NIPAAm and 0.3 g of *t*-butylacrylate were dissolved in 5 mL of ethanol–water mixture (1 : 1 v/v). The solution was purged by bubbling with nitrogen gas for 30 min. Following that, a solution containing 11 μL of Me_6TREN and 4 mg of CuCl (100 μL) was added to the polymer solution to initiate the polymerisation.[29] The polymerisation was carried out overnight for 12 hours. After the polymerisation, the solution was dried by adding $MgSO_4$. The solution was filtered and the filtrate was diluted with THF. The copper was removed by running the polymer solution through a pad of aluminium oxide. The polymer was collected by precipitating in hexane and the final yield was above 80% after isolation. ^{1}H (500 MHz, d_6-DMSO) δ 9.34 (d, $J = 6.8$ Hz, 2H, CH_3), 9.29 (d, $J = 6.8$ Hz, 2H), 8.79 (d, $J = 6.8$ Hz, 2H), 8.25 (d, $J = 6.8$ Hz, 2H), 3.72–3.96 (br, 30H, PNIPAAm methylidyne protons), 1.78–2.16 (br, 90 H, PNIPAAm C–C backbone), 1.4 (br, 198H, *t*Bu protons), 0.90–1.70 (br, 180H, PNIPAAm methyl protons). Details of the polymer characteristics can be found in Table 1.

2.4 Synthesis of methylviologen terminated poly(*N*-isopropylacrylamide)-*r*-poly(*t*-butylacrylate)-*r*-poly(acrylic acid), PNIPAAm-PtBA-PAA-MV (PA-1 and PA-2)

Methylviologen terminated poly(*N*-isopropylacrylamide)-*r*-poly(*t*-butylacrylate), PNIPAAm-PtBA-MV (0.5 g) in DCM (5 mL) was treated with 0.15 g of CF_3COOH at room temperature in CH_2Cl_2 for 24 h. The polymer was collected by precipitating in hexane and the final yield was above 85% after isolation. This reaction cleaves the tertiary butyl group to form PAA. ^{1}H (500 MHz, d_6-DMSO) δ 12 (br, 15H, carboxylic acid protons), 9.34 (d, $J = 6.8$ Hz, 2H, CH_3), 9.29 (d, $J = 6.8$ Hz, 2H), 8.79 (d, $J = 6.8$ Hz, 2H), 8.25 (d, $J = 6.8$ Hz, 2H), 3.72–3.96 (br, 30H, PNIPAAm methylidyne protons), 1.78–2.16 (br, 90H, PNIPAAm C–C backbone), 1.4 (br, 198H, *t*Bu protons), 0.90–1.70 (br, 180H, PNIPAAm methyl protons). In our reaction, the PtBA was only partly cleaved to maintain the hydrophobic characteristics of the MV-terminated polymer for the better encapsulation of insulin.

2.5 Synthesis of methylviologen terminated poly(*N*-isopropylacrylamide)-*r*-poly(*t*-butylacrylate)-*r*-poly(*m*-amidophenylboronic acid), PNIPAAm-PtBA-PAPBA-MV (PAB-1 and PAB-2)

Methylviologen terminated poly(*N*-isopropylacrylamide)-*r*-poly(*t*-butylacrylate)-*r*-poly(acrylic acid) (25 mg, 5.14×10^{-2} mmol carboxylic acid) in 1 mL THF was added a solution of 42.5 mg (0.310 mmol) *meta*-amino phenyl boronic acid (10 mL H_2O). The reaction mixture was then cooled down to 0 °C and followed by dropwise addition of EDC (49.3 mg, 0.257 mmol) in 2 mL H_2O. After 17 hours, the reaction solution was then dialysed against deionised water in a dialysis bag with a molecular cutoff of 2000 g mol^{-1} for 3 days to afford the resulted polymer in 30%. ^{1}H (500 MHz, d_6-DMSO) δ 9.34 (d, $J = 6.8$ Hz, 2H, CH_3), 9.29 (d, $J = 6.8$ Hz, 2H), 8.79 (d, $J = 6.8$ Hz, 2H), 8.25 (d, $J = 6.8$ Hz, 2H), 7–8 (m, br, 60H, aromatic protons on *m*-amido phenyl boronic acid), 3.72–3.96 (br, 30H, PNIPAAm methylidyne protons), 1.78–2.16 (br, 90H, PNIPAAm C–C backbone), 1.4 (br, 333H, *t*Bu protons), 0.90–1.70 (br, 180H, PNIPAAm methyl protons); ^{11}B (160 MHz) δ 28 ppm (br).

Table 1 Molecular characteristics of the MV-terminated polymers used in this work

		Chemical composition (no. of monomer repeat unit)			
Sample	M_n (g mol^{-1})	PNIPAAm[a]	PtBA[a]	PAA[a]	PABA[a]
MV-PNIPAAm	6300	50	—	—	—
P-1	8100	30	37	—	—
P-2	9000	13	59	—	—
PA-1	7290	30	22	15	—
PA-2	6840	13	20	39	—
PAB-1	9340	30	22	—	15
PAB-2	12 180	13	20	—	39

[a] Numbers of the repeating units are determined by ^{1}H NMR.

2.6 Preparation of insulin-loaded micelles

The preparation of the insulin-loaded micelles was adapted from the procedure of Wang *et al.*[30] In brief, the ternary complexes (1 mg mL^{-1}) was dissolved at 15 °C. Insulin solution (5 mg mL^{-1}), prepared at pH = 2 was added into the polymer solution dropwise under magnetic stirring, and the solution was adjusted to pH = 6. The encapsulation of insulin was accomplished by heating the solution to 37 °C. The insulin-loaded micelle solution was dialyzed against deionized water at pH = 6 in a dialysis bag with a molecular weight cutoff of 6 kDa to remove the unloaded insulin. The final yield of the micelles was 54% after drying. The amount of insulin was determined by measuring the solution at 15 °C. The amount of insulin in the micelle was 320 μg mg^{-1}.

2.7 Release study of insulin-loaded micelles

The release profile of insulin from insulin-loaded micelles was determined by dialysis. Insulin-micelle (1 mg mL^{-1} insulin-micelle containing 320 μg insulin) was loaded into MWCO 100 000 g mol^{-1} dialysis tubing and dialysed against 7 mL of phosphate buffered saline (PBS, pH = 7.4) in the dark at 37 °C. At specified time points, 1 mL of the dialysis buffer was collected and replaced with equal volume of fresh PBS. The concentrations of insulin present in the dialysate were determined by measuring UV absorbance at 273 nm. The concentration of insulin released from the micelles was expressed as a percentage of the total insulin and plotted as a function of time.

2.8 Triggered release experiments

The triggered release studies was performed as follows: for the temperature trigger, the "ON" state whereby the trigger was effected was triggered by placing the dialysis setup in 15 °C. After incubation for a period of time, the dialysis setup was removed and placed in 37 °C. This was carried out for several more cycles to get the "ON" and "OFF" profile. For the glucose trigger, the "ON" state was triggered by placing the dialysis setup in a solution with a certain glucose concentration (1, 10 or 30 mg mL^{-1}) at 37 °C. After incubation for a period of time, the dialysis setup was removed and placed in a buffer solution without glucose at 37 °C. This was repeated for several cycles. For the adamantane amine trigger, the "ON" state was triggered by placing the dialysis setup in a solution with an adamantane amine concentration of 3 mM at 37 °C. After incubation for a period of time, the dialysis setup was removed and placed in a buffer solution without adamantane amine at 37 °C. This was repeated for several cycles.

2.9 Toxicity studies

Cells and media NIH 3T3 cells were cultivated in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were grown as a monolayer and were passaged upon confluence using trypsin (0.5%, w/v in PBS). The cells were harvested from culture by incubating in trypsin solution for 10 min. The cells were centrifuged and the supernatant was discarded. Serum-supplemented DMEM (3 mL) was added to neutralise any residual trypsin. The cells were resuspended in serum-supplemented DMEM at a concentration of 2×10^4 cells

per mL. Cells were cultivated at 37 °C and 5% CO_2. The toxicities of the polymers were assessed by determining their ability to affect the proliferation and viability of 3T3 cells cultured in DMEM. The polymers were incubated in 24-well multiplates at 1×10^4 cells per well for 24 h at 37 °C in 500 μL of medium. The different cell viabilities were evaluated using the MTT assay on the 3T3 cell lines. Here, 10 mL of sterile filtered MTT stock solution in PBS (5 mg mL^{-1}) was added to each well, reaching a final MTT concentration of 0.5 mg mL^{-1}. After 5 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in DMSO (100 μL per well), and the absorbance was measured using a microplate reader at a wavelength of 570 nm. Cell viability (%) = $[A]_{test}/[A]_{control} \times 100\%$, where $[A]_{test}$ is the absorbance of the wells with polymers and $[A]_{control}$ is the absorbance of the control wells. All experiments were conducted with six repetitions and averaged. The control group consists of cells incubated without polymers and cultured in DMEM.

3 Results and discussions

3.1 Polymer design and characterisation

In order to create a supramolecular nano-carrier for insulin, we planned to conjugate a temperature and glucose responsive terpolymer to a hydrophilic polymer chain *via* supramolecular methods. Previously, PNIPAAm has been conjugated to poly(2-(diethylamino)ethyl methacrylate) using cyclodextrin–adamantane host–guest interactions for the fabrication of a supramolecular DHBC.[31] In our recent report, we noted that the formation of supramolecular DHBCs is most effective when both block copolymers remain fully water-soluble.[23] Therefore, in the current design of the two functionalised polymers, important solubility issues have to be taken into account based on end group properties. The PNIPAAm-containing terpolymer functions as the hydrophobic block and forms the core of the micelle when the temperature is raised above the lower critical solution temperature (LCST). In order to stabilise the nanoparticle construct after liberation of the hydrophilic corona (see Section 3.3), we decided to functionalise the end terminus of the hydrophobic PNIPAAm-containing terpolymer with the doubly charged hydrophilic MV. This confers a certain degree of water solubility to the system and readily allows for the formation of the supramolecular constructs.

The second guest used in the formation of the CB[8] ternary complexes is usually a hydrophobic moiety such as naphthalene or dibenzofuran (DBF). A DBF-terminated PDMAAm is used as the second guest containing block homopolymer. The molecular weight of the polymer was 6300 g mol^{-1}, and the conjugation of DBF to PDMAAm allows the DBF moiety to be solvated as a result of the hydrophilic PDMAAm. The molecular weight of the polymers were kept below 10 000 g mol^{-1} to allow for easy excretion of the polymers from the body.[32]

MV-terminated random terpolymers consisting of PNIPAAm, PtBA and PAAPBA were prepared by a previously reported procedure using ATRP.[29] Our first attempt at the direct synthesis of the terpolymer using ATRP with the boronic acid containing monomers was not successful. We observed that NIPAAm remained unreacted and the boronic acid monomer decomposed under the CuCl/Me_6TREN condition. It is very likely that the boronic acid monomer suffered from decomposition due to the Cu(I) insertion into carbon–boron bond.[33–35] We therefore decided to synthesise the terpolymer using a post-functionalisation approach, as illustrated in Fig. 3. A copolymer of PtBA and PNIPAAm was synthesised (Fig. 3, **PA-*x***). The tertiary butyl groups on PtBA were partially hydrolysed using trifluroacetic acid (TFA) in dichloromethane (DCM). A partial rather than complete hydrolysis of the tertiary butyl groups was done in order to render a degree of hydrophobicity to the micelle core component. We then attached boronic acid moieties to the polymer backbone **PA-*x*** using EDC coupling with quantitative conversion leading to **PAB-*x*** and the boronic acid moieties will be utilised for direct glucose response. We thought that with an increase in the stability of the core, and incorporation with the boronic acid moieties, the long term and stimulated release of the encapsulated insulin can be achieved.

The composition of the polymers was determined from ^{1}H NMR spectroscopy and the details are summarised in Table 1. The peak at δ 1.4 ppm is attributed to the *t*Bu of the PtBA block. As the polymer was subjected to hydrolysis, this peak decreased in intensity. The MV-PtBA-PAA-PNIPAAm terpolymer (**PA-1** and **PA-2**) was then reacted with *meta*-amino phenyl boronic acid to afford the MV-PtBA-PAAPBA-PNIPAAm polymer (**PAB-1** and **PAB-2**). The addition of *meta*-amino phenyl boronic acid results in peaks at δ 7.8 ppm due to the phenyl protons in the PAAPBA groups in the terpolymer. We also observed a broad peak at δ 27 ppm in ^{11}B NMR corresponding to boronic acid group, which confirms the successful incorporation of the boronic acid moiety into polymer **1**.

3.2 Ternary complex formation

The ternary complex was formed by mixing **1**, **2** and CB[8] in an equimolar (1 : 1 : 1) ratio in an aqueous solution (Fig. 2). Upon mixing the components, the original colourless polymer solutions (without CB[8]) instantaneously became pink, indicative of the formation of a charge transfer complex between the MV and DBF moieties bound in the CB[8] cavity. The ease of formation of the ternary complex illustrates the potential wide applicability of this technology in areas where synthetic chemistry is not readily accessible. The LCST of the block copolymer **PAB-2** was determined to be 28 °C. In the absence of CB[8], an aqueous

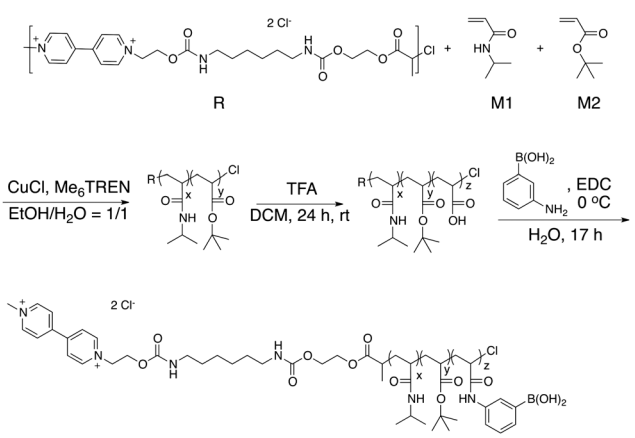

Fig. 3 Synthesis of polymer **1** (**PAB-*x***, $x = 1$ or 2).

mixture of **1** and **2** exhibits a typical phase transition of PNIPAAm above its lower critical solution temperature (LCST). When CB[8] is added to such a solution, it remains transparent, even as the temperature is raised above the LCST of PNIPAAm. The particle size observed by DLS at temperatures below the LCST is <10 nm, corresponding to unimers. On the another hand, above the LCST, the particle size increased to 85 nm, indicating the self-assembly of compartmentalised superstructures, as shown in Fig. 4. Lowering the temperature of the micelle results in the reformation of a double-hydrophilic ternary complex and the micelle superstructure collapses to form the unimeric structure as shown in Fig. 4(b). These findings corroborate with our previous reports on the formation of hierarchical CB[8] polymeric ternary complexes.[23,36]

3.3 Hierarchical self-assembly at physiological temperature

We proceeded to investigate the self-assembly behaviour of the supramolecular micelles by light scattering techniques. The critical micelle concentration (CMC) in water was determined by investigating the scattering intensity of light as a function of the supramolecular DHBC concentration (ESI Fig. S1†). An abrupt increase in the scattering intensity was observed when the polymer concentration was above 30 μM, which corresponds to the CMC.[37] This is consistent with the previously reported value for supramolecular DHBCs derived from PDMAEMA and PNIPAAm.[23]

When the micelle is treated with a competitive guest for the CB[8] cavity, such as adamantane amine, the particle size decreased to roughly 30 nm (Fig. 4(c)). The addition of adamantane amine removes the hydrophilic PDMAAm segment from the micelle, resulting in the formation of PNIPAAm nanoparticles (see Section 3.1). As the PNIPAAm nanoparticles are stabilised by the hydrophilic MV periphery, 30 nm sized particles remain in solution. When the micelles were titrated with glucose solution (10 mg mL^{-1}), the size of the particles increased to about 300 nm (Fig. 4(d)). This can be explained in terms of the swelling of the micelle core when the boronic acid derivatives binds to the hydrophilic glucose molecule.[38] The attachment of glucose to the boronic acid moieties makes the core more hydrophilic. As the core becomes more hydrophilic, the uptake of water by the core results in the increased size. These DLS measurements corroborate with the images obtained using transmission electron microscopy (Fig. 5). As can be seen in Fig. 5(a), the insulin-loaded micelles have a size of about 80–100 nm. When the micelles are dissociated, it leads to the formation of particles <15 nm in size are observed.

3.4 Triggered release of insulin from hierarchical micelles

Insulin was incorporated into the micelles by a standard solvent exchange and dialysis protocol.[30] In this work, we have prepared the insulin-loaded micelles at pH 6 which is very close to the isoelectric point (IEP) of insulin (IEP 5.3). At the IEP, it is expected that there is no net charge on the surface of the molecule, making the insulin molecule hydrophobic entity. This allows the protein to be encapsulated within the hydrophobic micelle core *via* hydrophobic interactions as previously reported by different researchers.[30,39,40]

Glucose triggered release was attempted using **PAB-1** and **PAB-2** (Fig. 6). However, we found that even at a high glucose concentration of 30 mg mL^{-1}, the insulin release rate of **PAB-1** was only increased by a factor of 3 compared to that of the non-triggered system. As it was difficult to detect any reasonable response from the **PAB-1** system, **PAB-2** was chosen as the system for further investigation. Indeed the rate of insulin release was affected by glucose, however, the concentrations of 10 mg mL^{-1} and 30 mg mL^{-1} used in this case were much higher than that observed in hyperglycemic conditions. The drug release profiles of

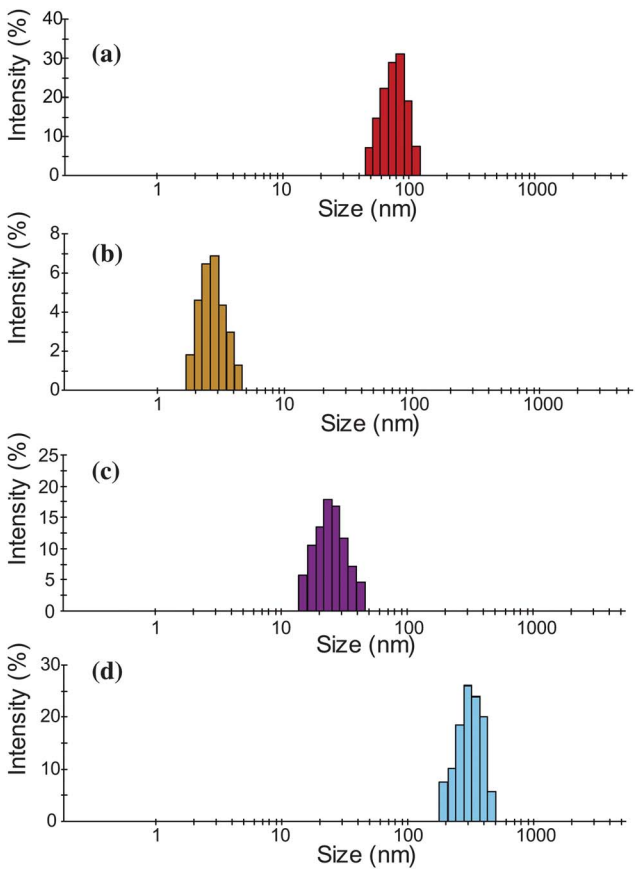

Fig. 4 Particle size variation of **PAB-2** under different condition. (a) without trigger; (b) temperature (15 °C) as the trigger; (c) adamantane amine as the trigger; (d) glucose as the trigger (10 mg mL^{-1}).

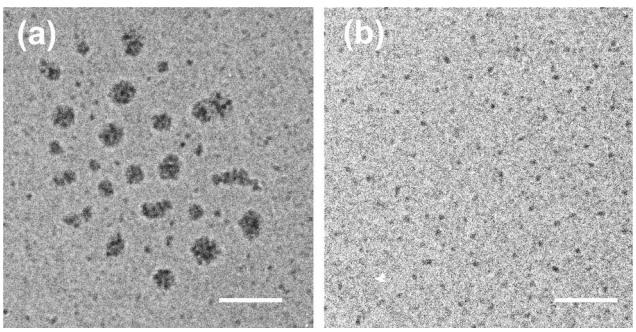

Fig. 5 TEM micrographs of micelles after being exposed to stimuli: (a) no trigger, (b) temperature trigger (scale bar denotes 200 nm).

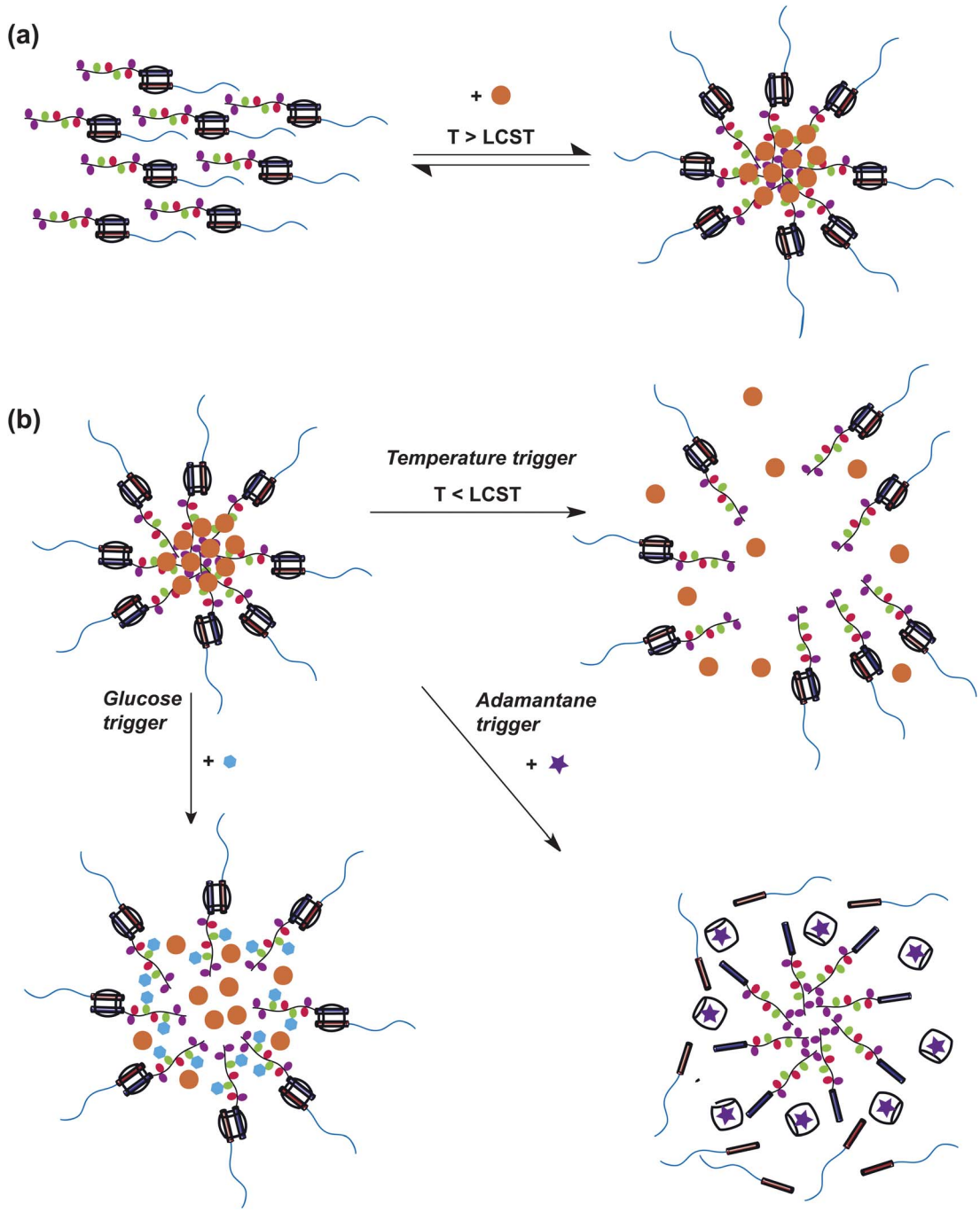

Fig. 6 Schematics showing the micelle formation under the influence of different stimuli. (a) The formation of the micelles by raising the temperature of the system above the LCST, (b) the effect of different triggers on the micelle morphology.

the supramolecular micelles are shown in Fig. 7. The micelles without the influence of the any triggers was able to sustain the release of insulin for about 72 hours. In order to trigger drug release from the micelle, three different triggers were used. First, when the temperature was lowered from 37 to 15 °C, the hydrophobic PNIPAAm core solubilises and releases insulin from the core (Fig. 7(a)). The resultant "burst" releases a large payload of insulin within a few hours. There have been previous reports on site-specific delivery, which requires the use of a remote temperature trigger to tune the rupture of the micelles in real time.[41] Thus allowing the drug to be released at both the right time and place. Localised thermal therapy at the target site can be achieved using microwave, ultrasound, or infrared irradiation.

The addition of glucose results in the formation of a glucose–borate complex. The physical transition from a hydrophobic boronic acid to a hydrophilic glucose–borate complex will cause the micelle core to swell as discussed previously. The micelle core is not expected to fully dissolve in the solution, owing to the relatively high hydrophobic content. The swelling of the core will lead to a greater diffusion of insulin out of the micelle core and complete release of insulin occurred just under 24 hours (Fig. 7(b)).

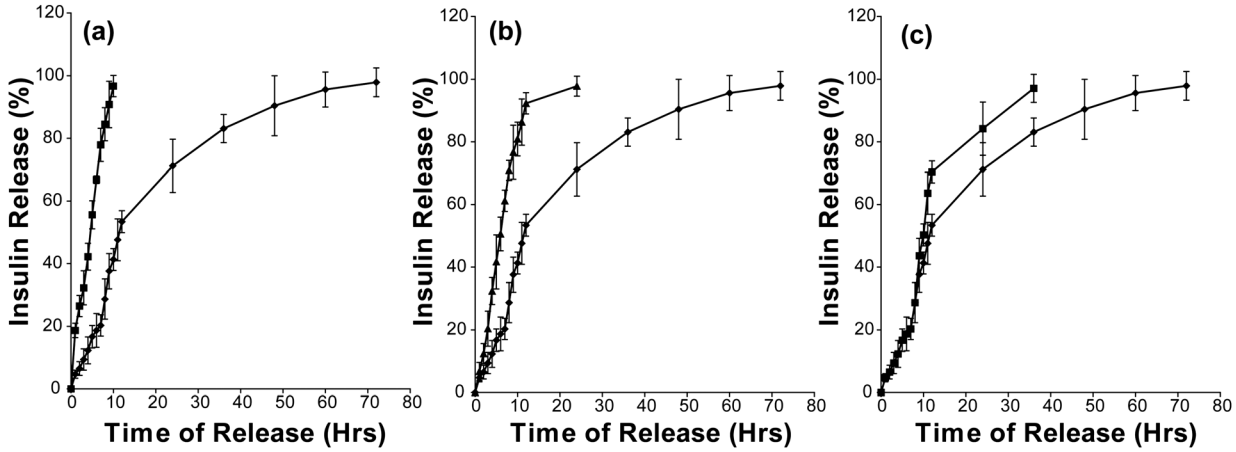

Fig. 7 Insulin release profile under various conditions: (a) temperature triggered release profile. (b) Glucose triggered release profile. (c) Adamantane amine triggered release profile.

The addition of a competitive guest such as adamantane amine disrupts the ternary complex that is formed and causes rupture of micelle superstructures, leaving the hydrophobic PNIPAAm-based core nanoparticles. The use of adamantane amine has been used by our group and others in displacement assays to competitively complex with cucurbiturils to liberate the first and second guests in solution.[23,27,42] In terms of application, a solution of adamantane amine or proteins functionalised with adamantane amine can be applied directly to trigger the disruption of the micelle solution. The complete release of insulin with the adamantane trigger takes places in 40 hours (Fig. 7(c)). As other stimuli-responsive micelles based on PNIPAAm do not have such multi-stimuli capabilities,[43] this system offers flexibility in terms of the rates of release as well as the choice of triggers.

3.5 Pulsated release of insulin-loaded micelles

Pulsated release of drugs is an emerging concept in the area of "on-demand" release.[44] In terms of triggered release, we have achieved "ON" and "OFF" release of insulin, which has not been previously presented in other insulin containing micelle systems (Fig. 8). The value of this work lies in the simplicity in application for *in vivo* applications compared to other crosslinked hydrogel systems.[45] Crosslinked hydrogel systems need to be surgically implanted and require the use of potentially toxic crosslinking agents.[46–48] Other systems reporting a glucose sensitive release also have performed their release at glucose concentrations that are much higher than those observed in physiological hyperglycemic conditions.[30,43]

In order to demonstrate the full extent of control that we have over this system, a pulsated insulin delivery was attempted with the micelles (Fig. 8). This experiment involved the "ON" and "OFF" switching of the triggers to control the insulin release. For example, using a temperature trigger in the "ON" state, the micelle solution was incubated at 15 °C to solvate a portion of the micellar core, leading to the opening of the micelles and the subsequent release of insulin. When the micelle solution was then incubated at 37 °C, the micelle core reverted to a fully hydrophobic state and returned to a closed "OFF" state. This principle was applied to the other two triggers of glucose and adamantane amine with varying enhancement of the release rates (ESI Fig. S2 and S3†). In each case where an external trigger was applied, the rate of release was accelerated as clearly indicated in Fig. 9. When glucose was used as a trigger, different rates of release can be achieved with the exposure to different glucose concentrations. For example, when glucose was added as a trigger at a concentration of 30 mg mL^{-1}, the rate of insulin release was enhanced 6-fold.

In order to demonstrate this under physiological conditions, a glucose concentration of 1 mg mL^{-1} was used to trigger the release of insulin from the micelles (ESI Fig. S2†). Even at such a low concentration, the micelle displayed a response and the rate of insulin release was increased by slightly more than 50% (ESI Fig. S5†). While we are still working towards achieving a better response from this system, this result shows the potential utility of this micelle for insulin release for *in vivo* applications. Finally, when adamantane amine was added to trigger the release, the rate of insulin release was also enhanced by over 100%. The advantages of pulsated release with three different types of

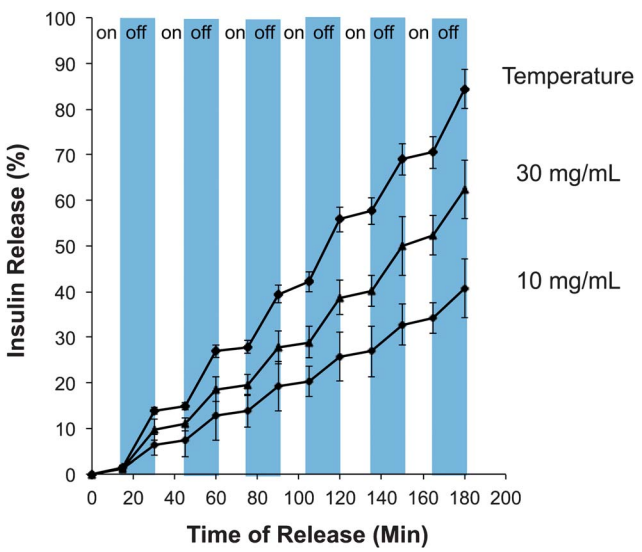

Fig. 8 "ON–OFF" glucose and temperature triggered insulin release.

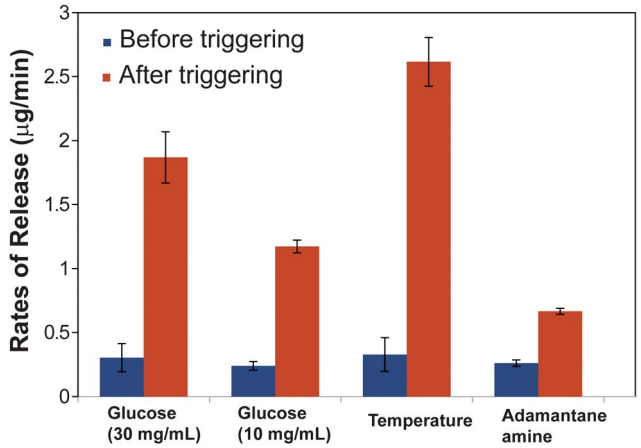

Fig. 9 Comparison of the different forms of triggered insulin release.

triggers allows the user to have full control over the release kinetics of this micelle system. Complete control over the release kinetics coupled with the simplicity of preparation for the insulin loaded micelle system based on CB[8] supramolecular technology may likely be useful in the biomedical field.

3.6 Toxicity studies of polymers

In vitro cytotoxicity studies are of major importance when considering any biomedical application for such polymeric micelles and were performed using 3T3 fibroblast cells. These studies were performed using the CB[8] ternary complexes at various concentrations. Recently, the *in vivo* and *in vitro* toxicity of cucurbit[*n*]urils has been reported,[49] demonstrating the biocompatibility and extremely low toxicity of these macrocyclic hosts. The cytotoxicity of the polymers employed for this system was tested at various concentrations ranging from 0.01 to 1 mg mL^{-1} and quantification of the cytotoxic response was carried out using an MTT assay. In general, the polymers do not show significant toxicity (Fig. 10). From these preliminary studies, the ternary complexes do not appear to be cytotoxic, highlighting that neither the viologen moiety nor the boronic acid moieties are toxic when conjugated to a polymer backbone, and the resulting micelles are expected to be safe for biomedical applications.[26]

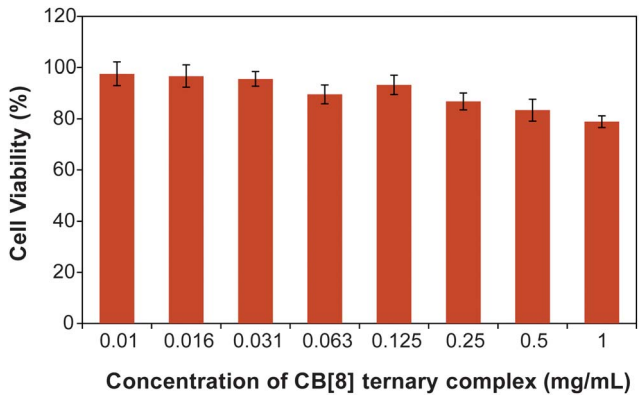

Fig. 10 Toxicity study of the ternary complexes in the presence of NIH 3T3 cells.

4 Conclusions

The system presented herein highlights several advantages over other systems designed to release insulin, which have been previously reported. For example, certain systems presented for insulin delivery in literature do not have have glucose sensitivity.[39,40] In this system, the release of insulin can be achieved by using three different triggers, namely, temperature, glucose, and a competitive guest. By an appropriate choice of trigger, different rates of insulin release can be achieved. Furthermore, we demonstrate a pulsated insulin delivery to show the full extent of control that we have over this system. While most of the glucose concentrations tested in this work are above physiological concentrations, we are currently working to optimise the system and enhance its response to low glucose concentrations. We envision that this supramolecular insulin nano-carrier will lead to a new generation of stimuli-responsive drug delivery vehicles for insulin therapies and the treatment of diabetes.

Acknowledgements

X.J.L. thanks A*STAR for a Postdoctoral Fellowship and J.d.B. is grateful for a Marie Curie Intraeuropean Fellowship. M.-H.T. thanks Cambridge Overseas Trust for the PhD scholarship. This work was also supported by an ERC Starting Investigator Grant (ASPiRe) and a Next Generation Fellowship provided by Walters-Kundert Foundation.

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