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ARTICLE TYPE

Synthesis of end-functionalized boronic acid containing (co)polymers and their bioconjugates with rodlike viruses for multiple responsive hydrogels

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When smart or responsive polymers are conjugated to biomolecules such as peptides or proteins, bioconjugates are resulted which combine both bioactivities of the biomolecules and the responsive properties of the synthetic polymers. Among the responsive polymers, these containing boronic acid or derivatives are unique due to their affinity to diol-containing compounds and pH dependent amphiphilicity. However, boronic acid containing polymers based bioconjugates are rare, probably due to the challenges faced by the preparation of such bioconjugates. In this work, we report the synthesis of boronic acid containing polymers with an *N*-hydroxysccinidic ester end functional group that can react with amino groups of a protein or peptide. Using a natural protein assembly-rodlike M13 virus as a model, we demonstrate the preparation of boronic acid containing polymer-protein bioconjugates. Such virus-polymer bioconjugates can reversibly form hydrogels and the gelation behavior can be regulated by temperature, pH or diol containing compounds such as glucose. Bioactive species can be loaded inside such hydrogel and the glucose regulated insulin release is demonstrated under physiological conditions.

Introduction

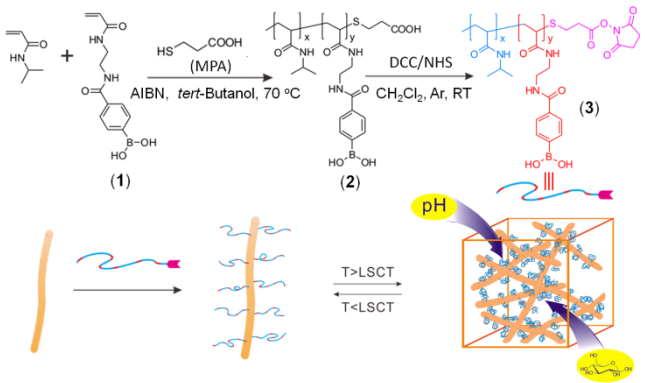
Polymer–protein and polymer–peptide bioconjugates refer to proteins or peptides covalently grafted with one or more synthetic polymers.[1-3] If prepared appropriately, bioconjugates are believed to combine both bioactivities of the biomolecules and the inherent properties of the synthetic polymers. Such bioconjugates have attracted much interest in the past decades due to increasing applications of peptides or proteins as therapeutic medicines and components for various kinds of biomaterials such as hydrogels.[4, 5] It is no doubt that the most famous bioconjugates are those grafted with poly(ethylene glycol) (PEG) or its derivatives. Conjugating of PEG to biomolecules has been demonstrated to increase blood circulation time, biocompatibility and solubility of the biomolecules.[6] PEG mainly functions in a passive way since it is a neutral hydrophilic polymer. Many efforts have recently been dedicated to designing bioconjugates with responsive or smart polymers, which can change their physical properties under certain environmental stimuli.[7, 8] Conjugating of such kinds of polymers to biomolecules may confer intriguing properties to the end hybrid products and results in new functional materials. Stimuli responsive affinity purification, ligand-substrate recognition, and enzyme activity, etc, have been elegantly demonstrated. [9-12]

With the development of controlled/living polymerization (CLP) methods and advanced chemical modification techniques such as click chemistry, many other smart polymers have been prepared.[13-16] Among these responsive polymers, polymers containing boronic acid or derivatives (referred to as boronic acid containing polymers) are unique due to their affinity to diol-containing compounds and pH responsiveness of the boronic acid moieties.[17] Boronic acid has a pH sensitive amphiphilicity: it is in the neutral trigonal hydrophobic state at pH < pKa while turns into the ionic tetragonal state above pKa. Furthermore, boronic acid can form reversible, dynamic chemical bonds with 1,2- or 1,3-*cis*-diol containing species such as saccharides, glycoproteins, ribonucleic acids and catechols.[18] This latter property has been widely exploited in biomaterials.[17] For instance, polymeric micelles self-assembled from boronic acid containing block copolymers and crosslinked polymeric (micro)hydrogels containing boronic acid moieties have been harnessed for controlled insulin delivery and release, targeted drug delivery, and so forth.[19-25] In these applications, boronic acid containing polymers have been obtained by conventional free radical polymerization,[19, 26, 27] or post-modification of pre-formed polymers with boronic acid moieties.[20, 28-30] Recently, controlled/living polymerization methods, such as ATRP, RAFT, and nitroxide mediated polymerization (NMP), have been used to design boronic acid containing homo or block copolymers with well controlled molecular weights and architectures.[31-39]

Even with such attracting properties and the rapid advancement of the preparation methods, boronic acid containing polymer based polymer-protein bioconjugates are rare. This might be due to the fact that the potential applications of such bioconjugates have not been recognized yet but might also be due to the challenges faced by the preparation of boronic acid containing polymer based bioconjugates. Normally, polymer-protein

bioconjugates can be prepared through either "graft from" or "graft to" methods, both with its own pros and cons. The "graft from" method mainly relies on the controlled/living polymerization (CLP) methods such as ATRP or RAFT, during which elaborately designed initiators or chain transfer agents are pre-installed onto the specific site of the targeted proteins or peptides.[40-44] CLP is then initiated under conditions that are biocompatible with the stability of the proteins or peptides, i.e. in buffered aqueous media with only limited amounts of organic solvents involved.[45] However, polymerization of monomers containing boronic acid moieties has often been performed in organic solvents.[31-39] Furthermore, the protection of the two hydroxyl groups of the boronic acid is often needed and deprotection to release them involves harsh conditions such as strong acids.[34, 36, 37] Therefore, even though successful grafting such kinds of polymers to certain inorganic solid substrates has been realized by in situ "graft from" strategies in organic solvent,[46, 47] it is unpractical to construct boronic acid containing polymer-protein bioconjugates through the "graft from" strategies at this stage. Instead, such goal can be approached through the "graft to" method, during which end-functionalized boronic acid containing polymers are pre-designed and then coupled to proteins or peptides.[48-51] To the best of our knowledge, no example exits for this latter strategy either.

Herein, by using conventional chain transfer free radical polymerization, we synthesize boronic acid containing polymers with an *N*-hydroxysccinidic ester end functional group that can react with amino groups of a protein or peptide (Scheme 1). It is obvious that CLPs such as ATRP or RAFT should be the top choice to synthesize α,ω-functioned polymers with unprecedented levels of control on the composition, molecular shape, and chain length.[48-51] However, the post-modifications of the polymers obtained by CLPs to produce useful functional end groups normally involve conditions which are not compatible with the boronic acid moieties.[31-39] In contrast, although with the disadvantages of less controllability of the molecular weight and distribution, conventional chain transfer free radical polymerization has been demonstrated as a robust method to synthesize semitelechelic oligomeric polymers with various kinds of end functional groups by the proper choice of the chain transfer agents (CTAs).[9, 52-54] In addition, this method often produces relatively clean end-functioned polymers devoid of the thiocarbonylthio groups from RAFT and the contaminated metal ions from ATRP, both of which may have certain toxicity. Furthermore, we shall demonstrate that this method can be used to directly prepare end-functionalized boronic acid containing polymers with no need to protect the sensitive boronic acid moieties. For our purpose, a monomer of phenylboronic acid (PBA) with a pKa of ca. 7.8 is copolymerized with NIPAM, resulting in boronic acid containing polymers with temperature, pH, and glucose responsiveness (Scheme 1). Using a natural protein assembly-rodlike M13 virus, which consists of many copies of identical coat proteins, as a model, the preparation of boronic acid containing polymer-protein bioconjugates will be demonstrated. The multi-responsive gelation behavior of the resulting bioconjugates is investigated and glucose regulated release of insulin is demonstrated under physiological conditions.



Scheme 1 Schematic representation of the synthesis of end-functionalized phenylboronic acid containing PNIPAM random copolymers and grafting of such polymers to the rodlike M13 virus.

Experimental

Materials

All of the reagents used in the synthesis were purchased from J&K Scientific (Beijing, China) and used without further purification unless otherwise stated. Solvents in the highest purity were supplied by local suppliers. Ultrapure water from a Milli-Q UltraPure system (18.2 mΩ·cm) (Millipore) was always used. The M13 virus was grown and purified following the standard protocol using the ER2738 strain of *E. coli* as the host bacteria.[55]

Chain transfer free radical copolymerization of NIPAM and DDOPBA

The synthesis of phenylboronic acid containing monomer, 4-(1,6-dioxo-2,5-diaza-7-oxamyl) phenylboronic acid (DDOPBA, **1** in Scheme 1) , was based on the procedure devised by Kataoka (see ESI for the detailed synthesis procedure†).[26] 3-Mercaptopropionic acid (MPA) was used as the chain transfer agent (CTA) for the copolymerization of NIPAM and DDOPBA in order to introduce a carboxyl group at one end of the polymer for further post modifications. Briefly, DDOPBA (0.26 g, 1 mmol), NIPAM (1.02 g, 9 mmol), AIBN (4.10 mg, 0.025 mmol) and MPA (17.20 μL, 0.2 mmol) were dissolved in 10 mL DMF. Degassing was performed through three cycles of free-thaw processes. After this, the polymerization was carried out at 70℃ for 24h. The polymerization solution was added to cold diethyl ether (10x) and the white precipices were collected by vacuum filtration and dried under vacuum. The dried product was dissolved in THF, precipitated in cold diethyl ether and dried in vacuum for twice, resulting in the polymer as a white solid, which will be referred to as poly(NIPAM-*co*-PBA)-COOH. The polymer was characterized by ^{1}H NMR (400MHz, DMSO-d_6) (^{1}H NMR shown in Fig. S2†). (δ, ppm): the methine proton on the isopropyl group NIPAM (-CH(CH_3)$_2$, δ=3.8 ppm) and the aromatic protons DDOPBA (COC_6H_4, δ=7.8-7.9 ppm).

Synthesis of NHS terminated random copolymers of NIPAM and DDOPBA

The end carboxyl group of the above synthesized poly(NIPAM-*co*-PBA)-COOH was transferred into *N*-hydroxysccinidic ester by standard carbodiimide chemistry. Briefly, poly(NIPAM-co-PBA)-COOH (0.8 g) and *N*-hydroxysuccinimide (37 mg, 0.32 mmol) were dissolved in 20 mL THF and cooled in an ice-water

bath. The solution of *N*,*N'*-dicyclohexylcarbodiimide (DCC, 0.32 mmol dissolved in 1 mL THF) was added into the polymer solution, and kept stirring for 2 hrs. After this, the mixture was brought to 25 ^{o}C and kept at this temperature for 12h. The mixture was filtered to remove the precipitates. The filtrate was added into cold diethyl ether (×10) and the precipitated product was collected by vacuum filtration. The same procedure was repeated twice, resulting in the polymer as 0.7g white solids, referred to as poly(NIPAM-*co*-PBA)-NHS.

Grafting M13 viruses with poly(NIPAM-*co*-PBA)

The M13 virus was suspended in PBS buffer (100 mM, pH = 8.0) to obtain a 10 mL suspension with a concentration of 2 mg mL^{-1}, which was cooled in an ice-water bath. Poly(NIPAM-*co*-PBA)-NHS (0.8 g) was dissolved in 200 μL anhydrous DMF and the resulted solution was then added slowly into the cooled M13 suspension under strong stirring. The final mixture was kept in the ice-water bath for another 2hrs and then kept at 12 ^{o}C for 24hrs. The remained polymers was removed by several rounds of ultracentrifugation at 10000g at 4 ^{o}C. The final virus grafted with poly(NIPAM-*co*-PBA) was suspended in PBS buffer and stored at 4 ^{o}C.

Determination of the cloud point of the free polymer

In order to determine the cloud point of poly(NIPAM-*co*-PBA)-COOH, the optical transmittance of copolymer aqueous solutions (0.1 wt%, in 100 mM phosphate buffer) versus temperature at various pH was measured at 500 nm using a UV/visible spectrophotometer (UV-Vis 2550 UV-Vis spectrometer, Shimadzu). The temperature of the sample cells was controlled by an HAAKE A28 water bath installed with an HAAKE SC 100 controller (Thermo scientific) and with an accuracy of 0.01 ^{o}C. Rates of heating/cooling for the sample cell were 1 ^{o}C/5min. The cloud point was defined as the temperature where 50% optical transmittance of the copolymer aqueous solutions was observed. To investigate the influence of glucose on the cloud point, glucose was added to the copolymer aqueous solution to a concentration of 25 mM and the optical transmittance of the solution was then determined as described.

Turbidimetric analysis of the sol-gel transition of the poly(NIPAM-*co*-PBA) grafted M13 virus

The polymer grafted M13 virus was suspended in the phosphate buffer (100 mM, pH=7.4) at T = 4 ^{o}C. In order to determine the the sol-gel transition, the optical transmittance of the polymer grafted M13 virus suspension (3 mg mL^{-1}) versus temperature at various pH was measured in the same way as the determination of the cloud point of the free polymer. To investigate the influence of glucose, glucose was added to the polymer grafted M13 virus suspension to a concentration of 25 mM and the optical transmittance of the solution was then determined as described.

Rheological Analysis.

Oscillatory shear experiments in the linear regime was performed on a TA Instruments AR-G2 rheometer equipped with a Peltier plate. A plate-and-plate geometry (20 mm) was used for the measurements. The storage modulus (G′) and loss modulus (G″) as a function of temperature were determined from temperature sweeps which were conducted at a fixed strain of 0.8% using a frequency of 1 Hz. The concentration of the polymer grafted virus is 7 mg mL^{-1}. The heating/cooling rate was 4 ^{o}C min^{-1}.

Fluorescence measurement

All of the fluorescence measurements were performed on an F-4600 fluorescence spectrophotometer (Hitachi High-Technologies). For the Alizarin Red S (ARS) fluorescence,[18] the polymer grafted M13 virus was suspended in phosphate buffer (100 mM, pH = 7.4) at T = 4 ^{o}C to make sure the grafted polymer in the soluble state. To 200 μL 3 mg mL^{-1} such suspension, 1 μL ARS solution of various concentration in the same buffer was added. The final concentration of ARS was in the range of 0 ~ 65 μM. After incubation in dark at T = 4 ^{o}C for 15 min, the fluorescence spectra were recorded. The excitation wavelength is 469 nm and scanning range is in the 500-700 nm.

For the competitive glucose binding in the presence of ARS, a 200 μL aqueous mixture in phosphate buffer (100 mM, pH = 7.4) was prepared at T = 4 ^{o}C, containing 3 mg mL^{-1} the polymer grafted M13 virus and 65 μM ARS. To such mixture, glucose in the same buffer was added and the end concentration of glucose was varied. After incubation for 15 mins at T= 4 ^{o}C, the fluorescence spectra were recorded at T= 4 ^{o}C with an excitation wavelength of 469 nm and scanning range of 500-700 nm.

Atomic force microscopy of polymer grafted M13 viruses

Atomic force microscopic images were obtained with a Nanoscope IV atomic force microscopy (Veeco) operating in the tapping mode under ambient conditions using an etched silicon cantilever tip. A drop of polymer grafted virus suspension with a concentration of 10^{-4} mg mL^{-1} was added to the smooth surface of a freshly cut mica. The mica was dried for two days under vacuum and at room temperature before AFM measurement.

Insulin release behaviour of the viral hydrogel

In order to monitor the insulin release behavior of the viral hydrogel under physiological conditions, insulin labelled with FITC (insulin-FITC) was used which can be monitored by fluorescent measurements. The polymer grafted M13 virus was mixed with insulin-FITC in PBS buffer (100 mM, pH=7.4) at T = 4 ^{o}C, resulting in a homogenous mixture with a 6 mg mL^{-1} virus and 50 μM insulin-FITC. To a glass vial, 100 μL of such mixture was added and incubated at 37 ^{o}C for 10 min in dark to bring the sol into a gel. To the surface of the gel, 500 μL PBS buffer, which was pre-warmed to 37 ^{o}C, was added as a buffering media into which insulin can be released. To determine the amount of the released insulin in the buffering media, 200 μL of the liquid above the gel was taken at each interval and the fluorescent intensity due to the insulin-FTIC was measured with an excitation at 495 nm and an emission at 516 nm. A calibration curve was constructed under the same conditions to determine the amount of insulin-FITC. The volume of the buffered media above the gel was kept constant by adding the same amount of pre-warmed buffer after taking away aliquots for the insulin concentration determination.

Instrumental

^{1}H NMR spectra were recorded on an AVANCE III 400MHz spectrometer (Bruker). The concentration of the virus was

determined with a UV-Vis 2550 UV-Vis spectrometer (Shimadzu). Centrifugation and ultracentrifugation were performed on a benchtop Allegra X15R centrifuge and Optima L-90K ultracentrifuge (Beckman Coulter), respectively. Molecular weight (M_w) and its distributions were determined by gel permeation chromatography (GPC) (Waters 1525) using a series of two linear Waters Styragel columns HT2, HT3, HT4, and an oven temperature of 40 °C. Waters 1525 pump and Waters 2414 differential refractive index detector (set at 40 °C) were used. The eluent was THF at a flow rate of 1.0 mL/min. A series of eight polystyrene standards with molecular weights ranging from 1500 to 100,000 g mol^{-1} were used for calibration. Poly(NIPAM-*co*-PBA)-COOH was dissolved in THF to a solution of 25mg mL^{-1} and filtrated with 0.22μm filter before GPC.

Results and Discussion

Preparation of NHS end functionalized boronic acid-NIPAM copolymers

The monomer containing phenylboronic acid (PBA) (DDOPBA**, 1**) was synthesized following ref. 24 (see ESI for the detailed synthesis procedure†). The feature property of the PBA moiety in this monomer is that it has a pKa of pH 7.8, close to the physiological pH value and has many potential applications.[35] Chain transfer free radical polymerization was chosen here since it is a well-established method to synthesize various kinds of end-functionalized oligomeric polymers by choosing appropriate chain transfer agents (CTAs). The polymerization can be performed under conditions that compatible with many monomers. Especially, we showed here that boronic acid containing monomers can be directly polymerized without protecting the hydroxyl groups of the boronic acid moieties. When 3-mercaptopropionic acid (MPA) was used as the CTA, a carboxyl group was installed at one terminal of the polymer, resulting in poly(NIPAM-*co*-PBA)-COOH (**2** in Scheme 1. See Fig. S2 for ^{1}H NMR†), which can be further transformed into a NHS ester by carbodiimide chemistry. Poly(NIPAM-*co*-PBA)-NHS (**3** in Scheme 1) was characterized by NMR and GPC (Fig. 1). In the ^{1}H NMR spectrum, besides the signal at 3.8 ppm which is attributed to the -$CH(CH_2)_2$ of the NIPAM monomer units, these observed at 7.7 and 3.6 ppm are due to the aromatic protons (C_4H_4-) and –CH_2-CH_2- protons of the DDOPBA monomeric units, respectively. The NHS end groups can be confirmed by the signal at 2.7 ppm. The molar ratio of PBA to NIPAM in the polymer was estimated as 10:1 from the peak intensity ratio between the methane proton of the isopropyl group of PNIPAM and the aromatic protons of the DDOPBA. This value is comparable to the feeding ratio of the two monomers (NIPAM: DDOPBA = 9:1), suggesting similar monomer reactivity ratios since both the two monomers belong to *N*-alkyl-substituted acrylamides.[56] The number- and weight-average molecular mass (M_n, M_w) was estimated by GPC as 4295 and 5905 g mol^{-1}, respectively, resulting in a PDI of 1.8. This high PDI, compared to CLP, is probably due to many possibilities of chain terminations, as expected for the conventional free radical polymerization.

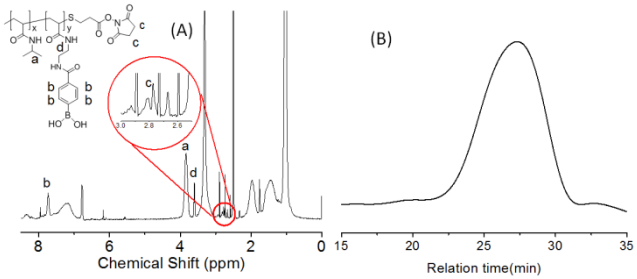


Fig. 1 ^{1}H NMR spectra (A) and GPC trace (B) of the end-functionalized phenylboronic acid containing PNIPAM random copolymers.

Solution behavior of poly(NIPAM-*co*-PBA)-COOH

The PNIPAM is well known for its thermoresponsive phase transition behavior while PBA has pH dependent amphiphilicity (Fig. 2C). Therefore, introduction of PBA moieties into the backbone of PNIPAM is expected to endow the copolymer with pH dependent thermo-responsiveness. The cloud point determined by the classic transmittance versus temperature confirmed such behavior (Fig. 2A). For instance, the 10% PBA containing poly(NIPAM-*co*-PBA)-COOH has a cloud point of 25 °C at pH < pKa where the PBA moieties are in the hydrophobic trigonal state, lower than ca. 32 °C of the normal PNIPAM. At pH > pKa, where most of the PBA moieties in the polymers are in the hydrophilic charged tetragonal state, the cloud point shifts to 31 °C. By monitoring the pH dependence of the cloud point of the poly(NIPAM-*co*-PBA)-COOH, the pKa of the PBA in the polymers can be estimated as 8.1. In addition, the pKa of the PBA moieties was also estimated by titration and a value of 8.5 was obtained (Fig. S4 in ESI†). Both values are higher than 7.8 of the monomer (Fig. 2C). This is consist with the observation by Kataoka and Sumerlin who think such phenomena are due to the electrostatic repulsions of neighboring PBA units which hinder further ionization of the PBA moieties.[26, 35] Compared to pure PNIPAM with similar molecular weight which normally has a broad hysteresis in the cooling process,[57-59] poly(NIPAM-*co*-PBA)-COOH exhibits a much more uniform thermal profile with comparable heating and cooling cycles (Fig. 2). This behavior is independent of pH (See Fig.S3 for the heating and cooling cycles of the polymer at all of pH in the presence or absence of glucose†).

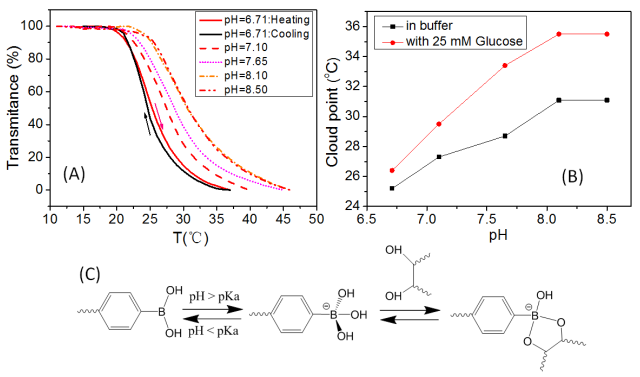


Fig. 2 (A) Transmittance versus temperature of end-functionalized phenylboronic acid containing PNIPAM random copolymers in the absence of glucose. (B) pH dependence of the cloud point of the same polymer extracted from Figure S3 in the ESI. (C) Schematic illustration of ionization and diol binding equilibria of phenylboronic acid in aqueous solution.

Beside pH, 1,2 and 1,3 diol containing compounds such as glucose are also expected to have influence on the thermo-responsiveness of the copolymer. In the presence of glucose (Fig.S3 in the ESI†), the thermoresponsive transition of the poly(NIPAM-*co*-PBA)-COOH shifts to higher values at each pH than that without glucose (Fig. 2B). The degree of difference increases upon the pH approaching the pKa. These behavior is clearly due to the fact that glucose forms hydrophilic boronates with the pendant PBA moieties of polymers, which shifts the ionization and diol binding equilibrium of phenylboronic acid to the right side and makes the copolymer more hydrophilic (Fig. 2C).

Bioconjugation of poly(NIPAM-*co*-PBA)-NHS with a model protein nanoassembly.

Using a natural protein assembly-M13 virus-as a model, we demonstrated here the conjugation of the poly(NIPAM-*co*-PBA)-NHS to proteins to form functional bioconjugates. Viruses have recently been recognized as natural biomaterials and found wide applications in many fields, due to their nanoscale size, genetic and chemical modifications.[60, 61] The M13 virus consists of 2700 identical coat proteins arranging into a rodlike protein capsid with a length of 880 nm and a diameter of 6.6 nm. There exists a large amount of functional groups arranging in a nanoscale precision on the surface of the virus, making the virus a natural nanoplatform for chemical modifications that have been well-established.[62-64] Wang and coworkers has confirmed that the preferred reaction location of the NHS containing compounds is the *N*-terminal amino group of the coat protein.[62] For the polymer grafting, excess amount of poly(NIPAM-*co*-PBA)-NHS was added to the M13 virus in PBS buffer at low temperature where the polymer is in the soluble state. Excess polymers were removed by ultracentrifuge to spin down the hybrid viruses. To confirm the grafting of the PBA containing polymer, SDS-PAGE was performed with the denatured virus via which the migration of the coat proteins can be monitored (Fig. 3B). Compared to the native M13 virus, a weak band with a slower diffusion velocity appeared which is assigned to these coat proteins grafted with the polymers. By comparing the density of the two bands, there are around 400 poly(PNIAM-*co*-PBA) per virus. AFM reveals the rodlike shape after polymer grafting, indicating the structure integrity of the virus (Fig. 3A). Most of the polymer grafted viruses have a length of 900nm.

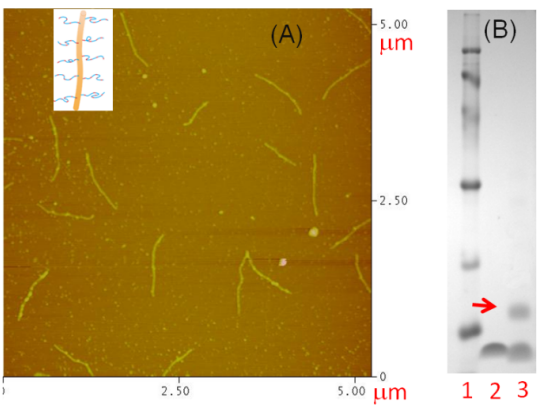


Fig. 3 Characterization of the polymer grafted M13 virus. (A) AFM photo of M13 viruses after grafting with end-functionalized phenylboronic acid containing PNIPAM random copolymers. (B) SDS-PAGE of the denaturized coat proteins of the M13 virus or those grafted with polymers. Lane 1: molecular weight markers (From bottom to top: 14, 20, 30, 48, 67, 94 kDa); Lane 2: M13 virus; Lane 3: M13 virus grafted with poly(PNIPAM-*co*-PBA).

Using the classic fluorogenic method based on Alizarin Red S (ARS),[18] the chemical accessibility and diol binding capability of the PBA moieties in the polymers grafted to the virus surface were assessed qualitatively (Fig. 4). ARS is non-fluorescent in its free form and becomes fluorescent after binding with boronic acid moieties. To a poly(NIPAM-*co*-PBA) grafted M13 virus suspension at T = 4℃ at which the virus suspension is in the well dispersed colloidal state, various amounts of ARS was added. The fluorescence intensity (I_F) due to the binding of ARS with the PBA moieties increases with increasing amount of ARS (Fig. 4A), confirming the chemical accessibility of the PBA moieties. To the virus suspension that contains ARS and showed strong fluorescence, increasing amount of glucose was added. The I_F gradually decreases with increasing glucose concentration (Fig. 4B), which is due to the competitive binding of glucose to the PBA moieties to replace the ARS.[18]

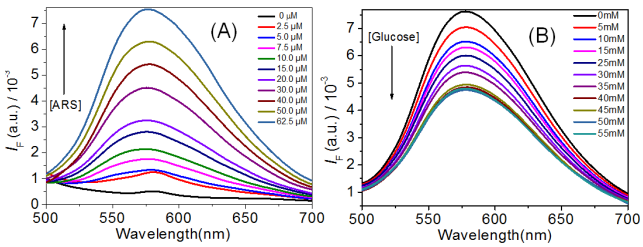


Fig. 4 Chemical accessibility and diol binding capability of the PBA moieties in the virus surface grafted polymers. (A) Fluorescence spectra of the virus suspension in the presence of various amounts of ARS. (B) Fluorescence spectra of the virus suspension at fixed amount of ARS and different amount of glucose. The virus suspension was 3 mg mL^{-1} virus in PBS buffer (100 mM, pH=7.65). The temperature was controlled at 4 °C.

Multi-responsive and reversible gelation behavior of poly(NIPAM-*co*-PBA) grafted M13 viruses

The rodlike M13 virus, with its polymer-like semiflexible conformation and high aspect ratio, is an ideal building block for nanofibrous hydrogels.[59] As confirmed so far, poly(NIPAM-*co*-PBA) will collapse into the hydrophobic state above certain temperature. Therefore, the grafted poly(NIPAM-*co*-PBA) on the virus surface in its collapsed hydrophobic state will confer attractive interactions between the viruses, driving them into a hydrogel (Insets in Fig. 5A), which can be further regulated by pH or diol containing compounds such as glucose. Similar to the cloud point of the corresponding free polymers, there exists a critical gelation temperate, T_g, which can be defined as the temperature at which the storage modulus G′ exceeds the loss moduli G″ in the rheological analysis (Fig. 5 and Fig. S3 in ESI†). At T < T_g, the poly(NIPAM-*co*-PBA) grafted virus is in the sol state (G' < G'') while turns into a hydrogel above the T_g (G' > G''). Ca. 15 °C was found for T_g at pH = 7.10 as revealed by rheological analysis (Fig. S3 in ESI†), This value is much lower than 33 °C of pure PNIPAM grafted M13 viruses under similar conditions.[59] As expected, T_g is very sensitive to pH. Increase of pH from 7.10 to 8.10 results in 6 °C of increase of the T_g (Fig. S3 in ESI†). Furthermore, in the presence of 25 mM glucose, T_g

increased at each pH (Fig. 5A and B, Fig. S3 in ESI†). The degree of increase is most pronounced at pH = 7.65, where T_g increases from ca. 18 to 23 °C (Fig. 5A and B). It is noted here that the moduli of the current gel has a magnitude of more than 100 Pa under physiological conditions (37 °C and PBS buffer), suggesting mechanical strength enough for potential applications.

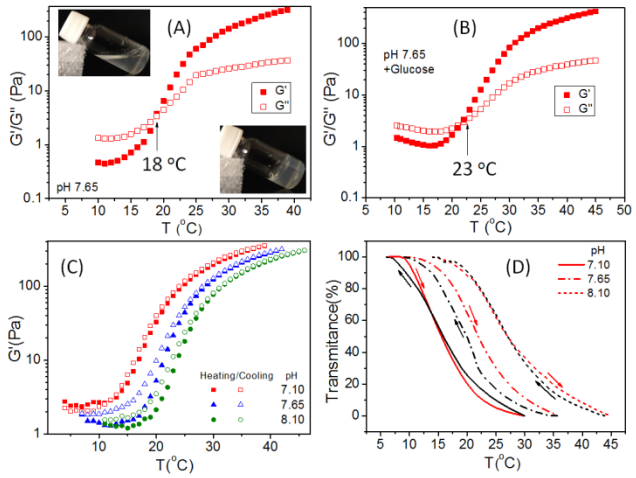


Fig. 5 Multi-responsive gelation behavior of boronic acid containing polymer-virus bioconjugates. Insets in (A): Photos of the sol and gel states. (A)~(C) Rheological measurement. Modulus G′ and G″ as a function of temperature at pH 7.65 in the absence (A) or presence (B) of glucose. (C) Storage modulus G′ as a function of temperature during heating and cooling cycles at several pH. (D) Transmittance versus temperature during heating/cooling cycles in the absence of 25 mM glucose. The concentration of virus is 3 mg mL^{-1} in PBS buffer. Red curves: heating; Black curves: cooling. The heating/cooling speed is 1 °C per 5 mins.

As observed above, poly(NIPAM-*co*-PBA)-COOH exhibits a much narrow hysteresis in the heating and cooling cycles, which is pH independent (Fig. 2). For the poly(NIPAM-*co*-PBA) grafted M13 virus, negligible hysteresis exists for the sol-to-gel and opposite gel-to-sol transition cycles, as revealed by the G' versus temperature during heating and cooling (Fig. 5C). However, at pH 7.65, certain hysteresis was observed in the presence or absence of glucose by turbidimetric analysis (Fig. 5D and Fig. S3F). Even so, the hysteresis in the case of poly(NIPAM-*co*-PBA) grafted M13 virus is much less than that of similar PNIPAM grafted rodlike virus.[59]

Compared to the pure PNIPAM-grafted rodlike virus,[59] the pH and glucose responsive gelation is unique to the poly(NIPAM-co-PBA) grafted virus. The pronounced responsiveness to glucose occurs at pH 7.65, which is close to the physiological pH. AFM reveals that the rodlike virus interconnected with each other inside the hydrogel with large pores (Fig. 6A). In addition, the temperature responsive gelation behavior offers a convenient way to encapsulate bioactive species homogenously inside the hydrogel with enhanced loading capacity. Bioactive species, such as insulin, can be mixed with the polymer grafted virus at 4 °C and then gelation can be initiated by quick injecting the mixture into PBS buffer at 37 °C (Inset of Fig. 6B). All together, these properties imply the current poly(NIPAM-*co*-PBA) grafted virus can be exploited in the controlled release of certain bioactive payloads. As a proof of concept, the release behavior of a model protein, insulin coupled with FTIC, was monitored under physiological conditions (pH = 7.4 and temperature = 37 °C). As shown in Fig. 6B, gradual release behavior of insulin from the virus based hydrogel was observed. Ca. 70% insulin was released during 24 hrs and before leveling off. However, in the presence of glucose, the release rate of insulin increased and approached the peak value of 90% during 10 hrs, demonstrating prestigious glucose responsive insulin release behavior, an important strategy in the regulation of diabetes.

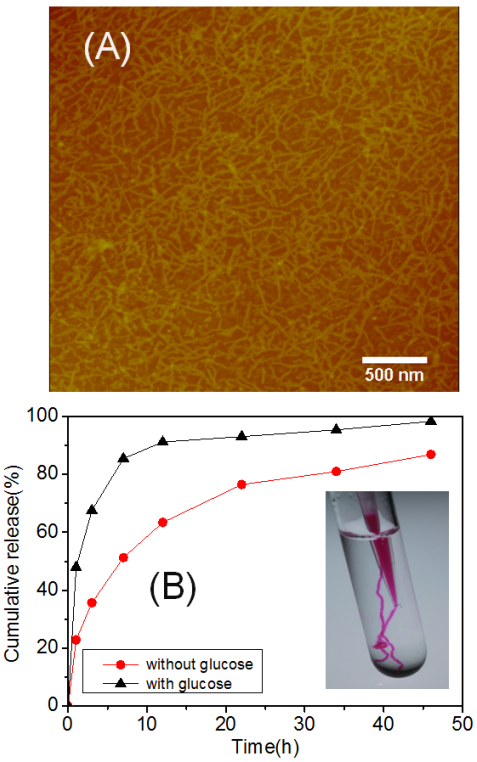


Fig. 6 Internal structure of the gel as revealed by AFM (A) and Insulin release behavior of the virus based hydrogel in the presence or absence of glucose (B). Inset in (B): Hydrogel forms instantly when the polymer grafted virus in the sol state was injected into aqueous solution at 37 °C.

Conclusions

With the chain transfer free radical polymerization method, a random copolymer of NIPAM and a phenylboronic acid (PBA) containing monomer was prepared directly without the need to protecting the sensitive boronic acid moieties. At one end of the resulted polymer, an amino-reactive NHS ester was introduced. The polymer has both pH and glucose responsiveness. By taking advantages of the convenient surface chemical modification of a well-defined rodlike virus, such polymers were grafted to the surface of M13, resulting in a hybrid virus-polymer bioconjugates. Reversible gelation behavior was observed which can be further regulated by temperature, pH, and diol containing compounds such as glucose. Bioactive species can be conveniently loaded inside the viral hydrogel. Glucose responsive insulin release behavior was demonstrated, suggesting the boronic acid containing polymer virus- bioconjugates might be used as biomaterials.

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Notes and references

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