Glucose-Triggered Drug Delivery from Borate Mediated Layer-by-Layer Self-Assembly

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ABSTRACT In this study, we report a novel approach for glucose-triggered anticancer drug delivery from the self-assembly of neutral poly(vinyl alcohol) (PVA) and chitosan. In the present study, we have fabricated multilayer thin film of PVA-borate and chitosan on colloidal particle (MF particle) and monitored the layer-by-layer growth using Zeta potential measurements. Formation of multilayer membrane on MF particle has been further characterized with transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). Subsequently, disintegration of multilayer thin film and microcapsules was observed in presence of glucose. We investigated the disassembly of PVA-borate and chitosan self-assembly under CLSM and atomic force microscopy. These results suggest that this multilayer thin film is very efficient for encapsulation and release of DOX molecules above certain concentration of glucose (25 mM). This glucose-sensitive self-assembly is relevant for the application of anticancer therapeutic drug delivery.

KEYWORDS: PVA-borate • chitosan • glucose • disintegration • layer-by-layer

INTRODUCTION

The layer-by-layer (LbL) method has emerged as one of the most promising technique for the preparation of a thin film at molecular level (1, 2). LbL films are formed through the sequential adsorption of oppositely charged polyelectrolytes through electrostatic interaction (3). In the recent past, various other nonelectrostatic interactions have also been reported to build a multilayer thin film such as charge-transfer halogen interactions, Van der Walls force, and covalent and hydrogen bonding (4−8). The build-up is facilitated by an overcompensation of the surface charge/functional group in each adsorption step (9−11). The unique advantage of this technique has been demonstrated by their simplicity and applicability to a wide range of substrates (12−14). Moreover, the LbL thin films have an ability to encapsulate functional biomaterials such as therapeutic drugs, DNA, and colloidal particles (15−24). These applications of polyelectrolyte membrane (PEM) found monumental utility in the area of drug delivery. Another major advantage of LbL thin film is that the internal structure of self-assembly can be tailored by modifying the charge density, molecular weight and functional group of polyelectrolytes. Such control over the LbL thin film at nanoscale is particularly important for delivery as well encapsulation of therapeutics. In past two decades, different kinds of materials have been used by several groups to form external and internal stimuli responsive multilayer membranes (25−29). Thus, this approach has been extensively explored for control and sustained drug delivery. Salt- and pH-triggered drug delivery is a very common approach to disassemble LbL membrane (30).

In the human body system, other than salt and pH, concentration of glucose is also a very decisive factor. In different parts of our body, the concentration of glucose is variable. For a healthy person average extra-cellular (blood) concentration of glucose is ∼5 mM (31, 32). Mean intracellular glucose concentration of muscle is within the range of 0.07−0.32 mM depending on the conditions (33). On the other hand, cancer cells metabolize differently than normal cells and accumulate glucose faster than normal cells (34). Concentration of glucose varies with different types of cancer cells as well as rate of growth of cancer cells. The ratio of cellular uptake of glucose analogue 18fluoro-2-deoxy-D-glucose (F-18-FDG) in 12 patients for tumor-to-contralateral lung was found to be 6.6. On the other hand, accumulation of F-18-FDG is 3.3−4.7 times greater for tumor than normal liver (35, 36).

In cancer cells, researchers believe that glucose concentration has a strong influence on the tumor hexokinase type II promoter. Maximal activity of this promoter has been found at 25 mM concentration (37). Thus, consumption of glucose increases in cancer cell and this high glucose concentration can be utilized for targeting drug delivery in infected cells. Many researchers have explored this approach, for example, Gleb & co-workers (38) introduced a path to form glucose sensing multilayer thin film, but this assembly is more suitable for sensing different carbohydrates rather than drug-delivery application, as this membrane disintegrates rapidly in presence of glucose molecules even at very low concentration. In 2006, Geest et al. (39) introduced another glucose response multilayer assembly based on LbL electrostatic adsorption of a polyanion (PSS) and a phenylboronic acid containing polycation. In the presence of glucose, this multilayer assembly gets over charged due to induction of negative charge on boron atom and that leads to rapid disassembly of whole membrane

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within 5 min. So, this multilayer assembly is more applicable for fast release application rather than control drug delivery application.

In our previous paper (40), we have shown the fabrication protocol for borate mediated LbL self-assembly of neutral PVA polymer and chitosan. In the present work, we have investigated the effect of glucose concentration on similar assembly and also studied the disintegration of this material in presence of glucose molecules. PVA-borate complex undergoes a strong interaction with glucose molecules and destructs the physically (hydrogen bonded) (41) cross-linked PVA−borate complex. Based on this principle, we have developed our present study in context of glucose triggered control release application. Disintegration of this multilayer membrane has been confirmed with CLSM study at higher concentration of glucose. We have also examined the effect of glucose on release behavior of anticancer drug doxorubicin encapsulated in PVA−borate/chitosan multilayer. This methodology nurtures one of the promising new drug delivery strategies for targeting and treating cancer cells.

EXPERIMENTAL SECTION

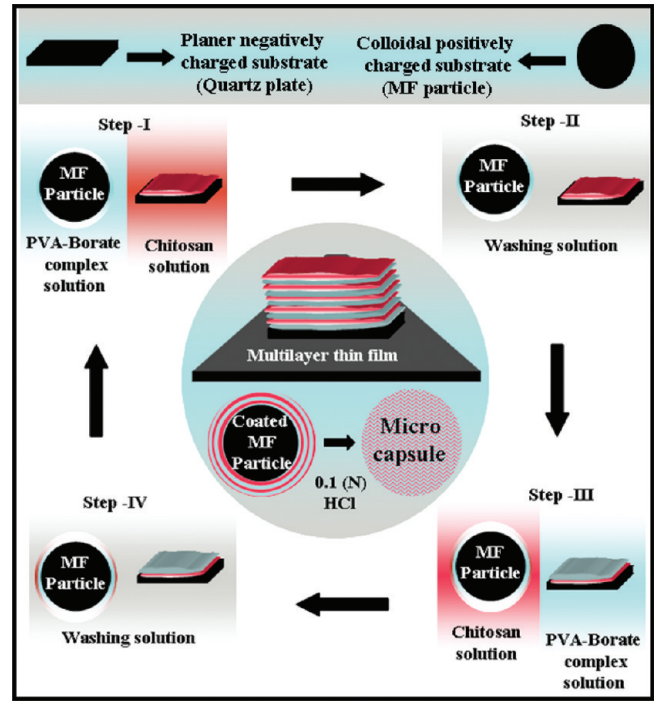
Materials and Method. Melamine formaldehyde (MF) (hydrochloric acid, HCl, soluble) particles 3.3−3.5 μm in size were procured from Microparticles GmbH, Berlin, Germany, and used as a colloidal template. Chitosan ($M_w \approx 200\,000$), poly(vinyl alcohol) ($M_w \approx 14\,000$) (from Aldrich), HCl, AcOH and Glucose were used as received without any purification. Ultrapure water (Millipore) with a specific resistance of 18 MΩ cm was used. All the experiments have been performed at room temperature.

Preparation of PVA Gel. We have prepared PVA hydrogel by following our earlier experimental protocol by adding 0.5 mL of aqueous borax (40 mg/mL) solution into the 50 mL of PVA solution (concentration of 23 mg/mL) (40).

Preparation of Free-Standing Thin Film. The Quartz substrates cleaned in piranha (7:3 v/v H_2SO_4/H_2O_2) solution, rinsed with Millipore water for several times and dried under nitrogen flow. Subsequently, placed into the chitosan (1 mg/mL, pH 4.8) solution for 2 h for adsorption, carefully rinsed with water to remove unadsorbed chitosan and dried under nitrogen gas flow. The chitosan adsorbed quartz plate was placed into the PVA−borate solution (pH 7.2) for adsorption followed by three washing steps with water and dried under nitrogen flow before placing into the chitosan solution for coating of a successive layer. By repeating this cycle we have fabricated multilayer thin film of PVA−-borate/chitosan polymer on quartz plate as shown in Scheme 1. We have dropcasted PVA polymer solution (10%) on this multilayer thin film (11 bilayers); it was allowed to air dry for 2 days and then peeled off the film and placed into the Millipore water for 72 h to dissolve the PVA.

Capsule Preparation. Chitosan and PVA−borate gel layers were adsorbed onto MF particles by using LbL approach. The positively charged MF particles (0.1 mL of 10 wt % dispersion) have been placed in PVA-borate gel for 2 h for adsorption of PVA-borate on bare MF particles. The suspension of these MF particles was centrifuged, followed by four washings steps with water in order to remove nonadsorbed hydrogel on MF particles. The MF particles were then incubated in chitosan solution (concentration of 1 mg/mL) and allowed to adsorb for 20 min, and followed by three washing steps with water before coating the next layer of PVA−borate gel. Alternately, hydrogel and chitosan steps have been repeated to fabricate multilayer membrane on MF particles. By dissolving MF particles within multilayer coated membrane in 0.1N HCl solution for 10 min, the hollow microcapsules have been fabricated and then washed three times with water and centrifuged as shown in Scheme 1.

Scheme 1. Protocol for the Fabrication of Multilayer Thin Film and Microcapsules Using LbLApproach[a]



[a] Colloidal (MF) particles and planar substrate (quartz) are used for the fabrication of microcapsules and thin film. Step I, electrostatic interaction between substrate (planar/colloidal) and polyelectrolyte (chitosan/PVA-borate); step II, washing of unabsorbed polyelectrolyte; steps III and IV, adsorption of oppositely charged polyelectrolyte and washing of excess polymer.

Disintegration of Multilayer Thin Film. We have prepared 10 bilayer thin film on quartz plate (cleaned with piranha solution) as described before and placed into 25 mM glucose solution for different time scale. With regular interval, film was collected from glucose solution and gently washed with Millipore water 3−4 times and dried the film before observing the disintegration of the thin film under AFM.

Disintegration of Microcapsule. Similar experiments were performed for 5 bilayers hollow microcapsules loaded with Rhodamine-B. We have incubated 25 μL of microcapsule solution in 1 mL of 25 mM glucose solution for different duration. The samples were then centrifuged and washed with Millipore water twice and then the sample was prepared for microscopy measurement to study the degradation behavior of microcapsules.

Glucose-Triggered Drug Delivery. We have encapsulated doxorubicin hydrochloride within this multilayer film by placing this multilayer (10 bilayers) thin film into the 10 mL of 0.2 mg/mL DOX solution overnight. The DOX-loaded multilayer film was washed with Millipore water 3−4 times to remove unloaded DOX molecules from thin film surface. Subsequently, the loaded thin film was placed into 25 mM glucose solution at neutral pH and desorption of DOX from the thin film was followed by UV−visible spectroscopy. Similar experiments have been carried out with 5 and 0.5 mM glucose solution.

Characterization. The UV−vis spectra of doxorubicin for loading and release study were obtained with Perkin-Elmer (Lambda 35) spectrometer.

Specific Viscosity. The specific viscosity of PVA and PVA−borate gel was determined by using Ubbelohde viscometer at room temperature with a flow time of about 1 min 46 s. for 10 mL of

Millipore water. The average flow times were calculated for four consecutive measurements before being converted to specific viscosities.

Microscopy. ***Transmission Electron Microscopy (TEM).*** Dilute solution of coated MF particle suspension was drop-casted on the carbon-coated copper grid. The measurements were carried out with Technai F-30. The instrument was operated at 100 kV.

Field-Emission Scanning Electron Microscopy (FESEM). FESEM samples were prepared from water solution drop-casted on silicon wafer and dried overnight at room temperature. The experiment was performed using SIRION scanning electron microscope at 5 kV after gold coating.

Atomic Force Microscopy (AFM). AFM measurements were carried out using a Digital, Nanoscope IVA AFM, Veeco Instruments, USA, in tapping mode. Thin films were prepared on silicon wafer (cleaned with piranha solution) from PVA-borate gel and chitosan solution and after gentle drying under nitrogen flow, examined under AFM, where the outermost layer is PVA−borate gel or chitosan.

Confocal Laser Scanning Microscopy (CLSM). CLSM image were recorded with Zeiss Confocal microscope using 60× objective. The excitation wavelength used in this experiment is 554 nm. Experiment was carried out in the solution state by placing a drop of dilute solution of sample suspension on microscopic glass slide.

Microelectrophoresis. The LbL growth of PVA hydrogel/chitosan on MF particles was followed by measuring the electrophoretic mobility of the coated particles using a Malvern Zetasizer with DTS version 4.2 software. The mobilities (u) were converted to the electrophoretic potential (ζ) using the Smoluchowski relation $\zeta = u\eta/\varepsilon$ where η is the viscosity and ε is the permittivity of the solution, respectively. All measurements were performed at room temperature in Millipore water having specific resistance around 18 MΩ cm.

RESULTS AND DISCUSSION

Multilayer Thin Film. To account for the glucose triggered drug delivery from borate mediated multilayer thin film, we have prepared PVA−borate/chitosan membrane using earlier described protocal (40). The self-assembly of PVA−borate/chitosan was formed by repeated adsorption of acidified chitosan followed by PVA−borate complex and the growth of thin film was followed by QCM (quartz crystal microbalance) analysis (not shown here). To demonstrate the stability of the thin film, we attempted to peel off by casting high concentration of native PVA (water-soluble) film on top of multilayer thin film as reported in literature (42).

We were able to peel off PVA−borate/chitosan multilayer assembly from quartz substrate and placed into water solution. The native PVA dissolved in water and we were able to fabricate stable multilayer free-standing thin film of PVA−borate/chitosan as shown in Figure 1.

Layer-by-Layer Coating on MF Particles. Similar LbL experimental protocol has been used to self-assemble PVA−borate/chitosan on MF particles.

Layer-by-layer electrostatic assembly of PVA-borate followed by acidified chitosan polymer on colloidal MF particle has been examined with zeta potential measurement as shown in Figure 2. A ζ-potential was calculated from the mobility measured after deposition of each layer. Figure 2 shows that alternate layer of PVA-borate/chitosan coating on MF particles induces a sign reversal. A ζ-potential of ca. −24 mV was obtained when PVA−borate hydrogel was the outermost layer on MF particles whereas ca. +30 mV was noted when chitosan was outermost layer. But a slightly lower value was observed as compared to previously reported value for hyaluronic/chitosan polyelectrolyte on cell membrane (43).

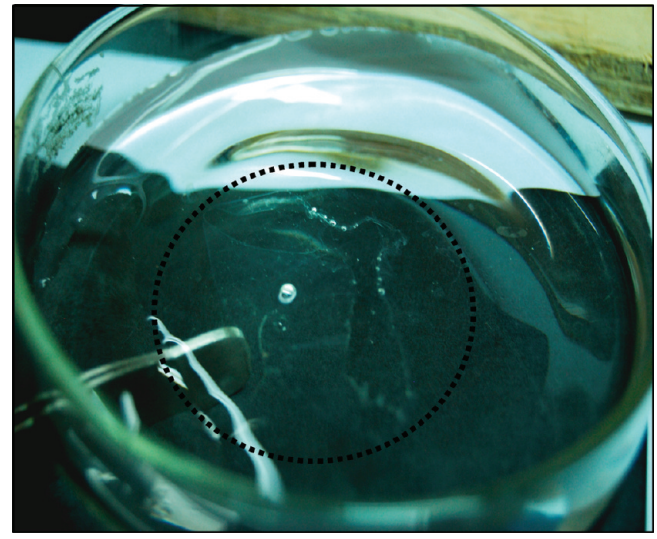


FIGURE 1. Free-standing multilayer (11 bilayers) thin fim of PVA-borate and chitosan.

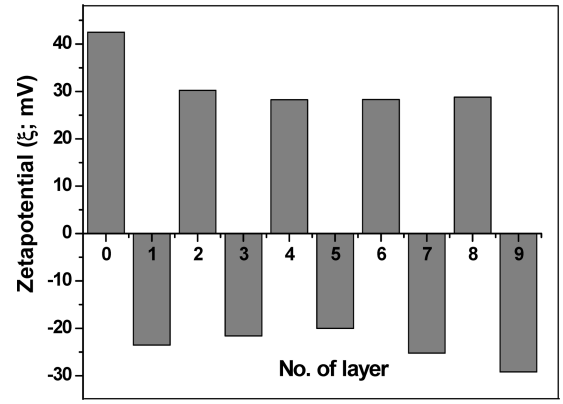


FIGURE 2. ζ potential as a function of layer numbers for the LbL multilayers of PVA hydrogel and chitosan on MF particles. The odd layers (1, 3, 5, 7, and 9) correspond to PVA hydrogel deposition and the even layers (2, 4, 6, and 8) correspond to chitosan adsorption and 0th layer signifies bare MF particles.

This could be due to less charge density on PVA−borate complex than hyaluronic acid polymer and better interpenetration of one layer into another layer in case of chitosan and PVA-borate complex (44). Nevertheless, positive followed by negative value of ζ potential accounts for LbL growth of hydrogel (PVA−Borax) followed by chitosan on MF particles. The PVA−borate/Chitosan multilayer growth on MF particle was characterized both by TEM and CLSM. Figure 3a shows the TEM images of coated MF particles, which indicates the homogeneous coating of PVA−borate/chitosan on MF particle. Each bilayer thickness on an average corresponds to ∼19 nm, which has been determined from the TEM image (Figure 3a). This bilayer thickness value is greater than those attributed to coating of conventional linear polyelectroytes (45). This could be the

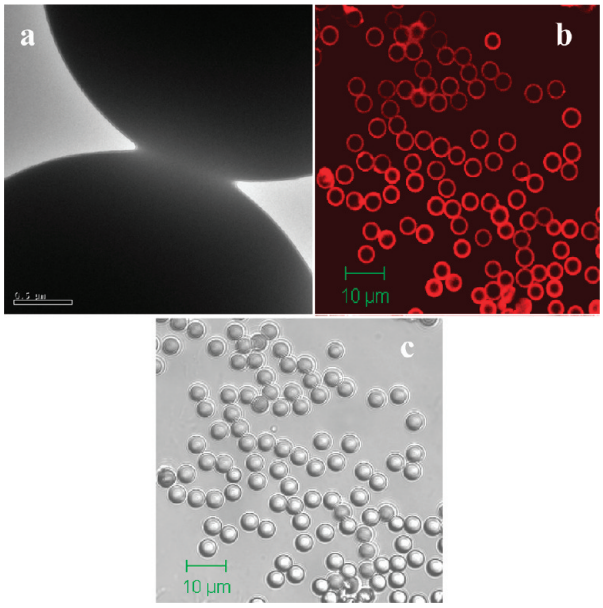


FIGURE 3. (a, b) TEM and CLSM image of MF coated (5 bilayers) particles and (c) bright-field image of coated particle.

effect of variable charge density, different interaction in between PVA−borate hydrogel and chitosan unlike linear polyelectrolytes. PVA−Borax hydrogel has strong extended H-bonding network system; it may further interact with active functional group of chitosan by H-bonding. The PVA−borate/chitosan coated particles were subsequently incubated overnight with Rh-B solution. The resulting particles were washed several times with water. CLSM images were recorded for dye-containing particles and it clearly shows a ringlike structure as shown in Figure 3b. The center of the ring is dark because of presence of MF core. Figure 3c is the corresponding bright-field image.

Hollow Microcapsule. The capsules were prepared by exposing PVA−Borax hydrogel/chitosan multilayer (4 bilayers) coated MF particles to 0.1 M HCl solution. In our previous report (40), we have shown that these microcapsules with PVA−borate complex as an outermost layer has a stable morphology in aqueous media but it undergoes significant shrinking upon air drying. We followed the change in morphology with chitosan as the outermost layer. Interestingly, the shrinking of microcapsules was controlled by simply keeping chitosan polymer as the outermost layer as shown in Figure 4a. These results suggest that PVA−borate hydrogel was protected by the outermost chitosan layer, whichs retard the fast removal of entrapped water molecules from inner hydrogel layer, and thus shrinking of capsules was limited upon air drying. Similarly, the spherical shape of microcapsule was further confirmed by CLSM after encapsulation of positively charged dye Rh-B (Rhodamine-B) molecules as shown in Figure 4b.

Disassembly of Microcapsule. We incubated these microcapsules and thin films in a glucose solution to understand the response of self-assembly. Subsequently, this unique nature of self-assembly can be exploited for glucose-triggered drug delivery application. We followed disassembly in the presence of different concentrations of glucose.

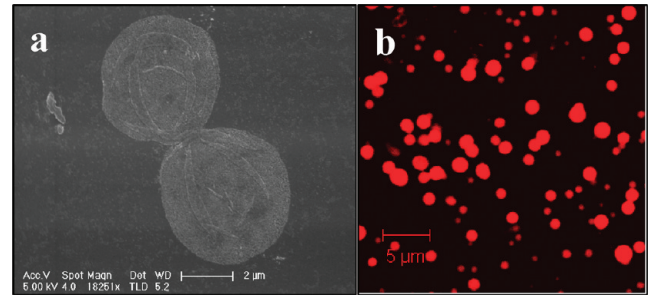


FIGURE 4. (a) FESEM image of hollow multilayer (4 bilayers) microcapsules of PVA-borate/chitosan self-assembly and (b) CLSM image of Rh-B loaded PVA-borate/Chitosan multilayer microcapsule.

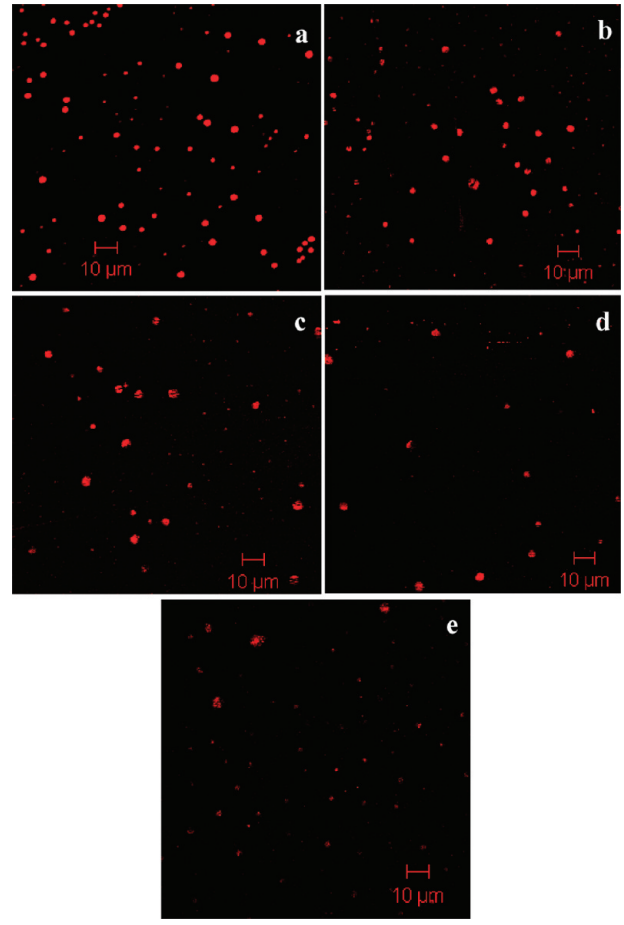


FIGURE 5. Degradation of multilayer capsule membrane in glucose (25 mM) solution. (a) Rh-B (probe molecules)-loaded capsule image before incubation in glucose solution. (b−e) Capsule morphology after incubation in glucose solution for 1, 2, 3, 6, and 9 h, respectively.

Figure 5a−e shows the change in morphology of microcapsules upon exposure to the glucose solution as a function of time. On placing the microcapsules into 25 mM glucose solution, we have found significant change in morphology of microcapsule. Initially, change in morphology of the capsules was not so prominent. After 6 h of incubation in glucose solution (25 mM), these capsules membranes are affected significantly and they appeared as highly distorted morphology as shown in Figure 5d. We have then extended the incubation time in glucose to 9 h and they appeared as a collection of some tiny pieces of multilayer self-assembly. After 12 h of incubation, it was hard to find any materials

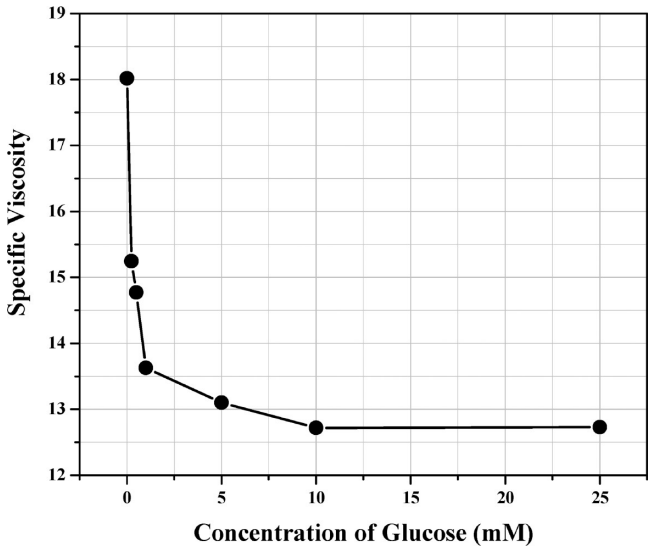


FIGURE 6. Decrement of specific viscosity value upon addition of glucose into the PVA–borate complex.

on whole sample specimen. These results clearly indicated that the self-assembly of PVA–Borax hydrogel/chitosan is strongly responding to glucose solution as a function of time. Similar experiments were carried out with 5 mM glucose concentration. We did not find significant change as that appeared for 25 mM glucose. The integrity of this multilayer assembly at 5 mM glucose concentration remains unaffected.

Disintegration of PVA–Borate Complex. To understand the mechanism of glucose triggered multilayer membrane degradation more in detail, viscosity mesurement of PVA–borate complex alone and in presence of glucose were performed. Figure 6 shows the specific viscosity of PVA–borate complex as a function of glucose concentration. Without glucose, the specific viscosity of this PVA–borate complex is around 18. With increasing the concentration of glucose the specific viscosity of the complex gradually decreased to 12.73 at 25 mM glucose and remain constant. This trend is similar to that observed in disintegration of microcapsules and can be explained on the basis of the proposed reaction pathways shown in Scheme 2. Viscosity measurement suggests that the cross-linking points are affected by addition of glucose in the solution and it revealed that on addition of glucose in this medium, borax molecules prefer to complex with glucose rather than PVA hydroxyl groups and thus physically cross-linked gel loses its gelation behavior. Thus, glucose disintegrates the physically cross-linked PVA–borate complex as shown in Scheme 2. Destruction of PVA–borate complex in presence of glucose is well-known phenomena in literature (46). In the presence of glucose, borate ions prefer to form a complex with glucose molecules rather than physical cross-linking with the PVA polymer through hydrogen bonding. In Scheme 2, we have shown two different possibilities of physically cross-linked complexes (**complex I** & **complex II**) involved between borate ion and glucose molecules. **Complex I** is simply a product of 1:1 borate:glucose ratio, whereas complex II corresponds to a 1:2 borate:glucose ratio. We believed that initially at lower glucose concentration, **complex I** will form and at higher glucose concentration **complex I** is converted to **complex II**. **Complex I** has a pair of hydroxyl group and that may help to bind further with hydroxyl groups of PVA polymer through H-bonding and then the whole thing can act as a negatively charged polyelectrolyte, which is sufficient to hold the entire multilayer thin film; as a result, the initial structure of self-assembly remains intact.

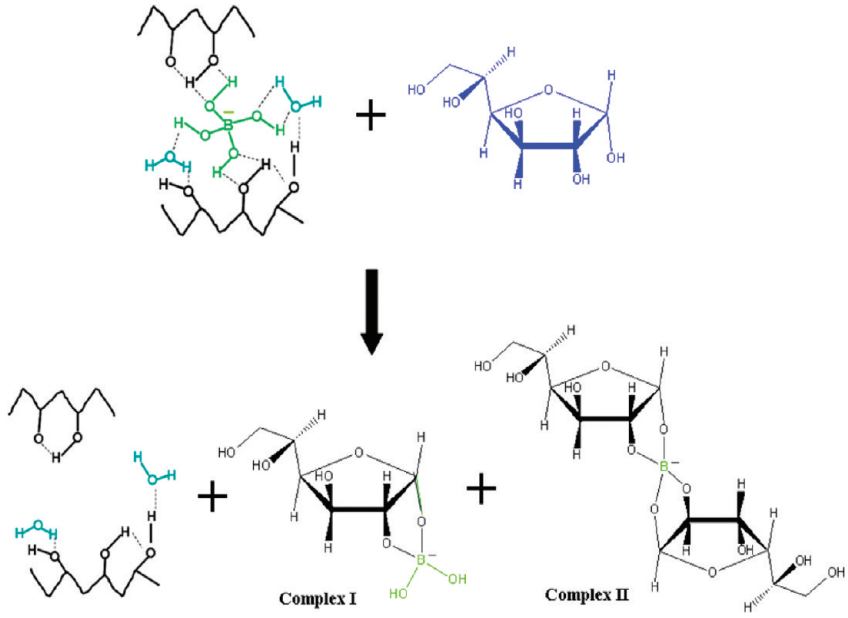
In the case of high glucose concentration, **complex II** will not allow such a possibility, as no more hydroxyl groups of borate ion are free to bind with hydroxyl groups of PVA, and furthermore, the negative charge of the borate ion will be buried within sandwich position of two glucose molecules. So, it can slowly diffuse from the multilayer assembly; the multilayer building slowly gets overly positive charged (because of chitosan) and repulsion among them leads to disassembly of the whole multilayer assembly of the PVA–borate complex and chitosan in the presence of 25 mM glucose solution.

Degradation of Thin Film. Similarly, in the case of planar substrate, we have observed very similar degradation behavior of multilayer thin films of PVA–borate complex and chitosan polymer.

Figure 7a shows the AFM images of morphology of the thin film before treatment of glucose (25 mM). Initially, within 2 h of incubation in glucose solution, we did not find any significant change in morphology. But we have observed a noticeable change after 3 h of incubation in glucose (25 mM) solution as shown in Figure 7b. From Figure 7c, we can find a clear signature of disintegration of this multilayer thin film after incubation for 6 h in glucose solution, and after 12 h of treatment, the multilayer thin film is almost vanished from the planar substrate as shown in Figure 7d. Roughness values of the thin film also reveal this disassemble phenomena. Before any glucose treatment, the roughness (rms) of the film was 8.7 nm, and after placing it in the glucose solution, the roughness of the film was decreased to 6.89, 4.89, and 1.02 nm with time 3, 6, and 12 h, respectively.

Glucose-Triggered Drug Release. The above results suggests that the self-assembly of PVA–borate/chitosan is very sensitive to glucose. We have exploited this phenomenon for glucose-triggered drug delivery. As explained earlier, the cancer cells possess high concentration of glucose, so the drug carrier responding to glucose would be highly beneficial for controlled release of anticancer therapeutics. Here, we have shown controlled release of anticancer drug (Doxorubicin) in the presence of glucose. As we have discussed the degradation behavior of PVA–borate and chitosan multilayer membrane in presence of glucose (25 mM) molecules. So, in context of drug release, this glucose-triggered release is much more effective than pH-triggered release. We have also observed that this multilayer assembly is inefficient to release DOX molecules in 0.5 mM glucose concentration. The multilayer thin film of PVA–borate/chitosan released ~20% of encapsulated DOX molecules after 10 h of treatment in lower glucose concentration (5 mM). Whereas, in higher glucose concentration (25 mM), this multilayer film released more than 80% DOX molecules

Scheme 2. Destruction of PVA–Borate Complex in the Presence of Glucose Molecules[a]



[a] **Complex I** is a 1:1 ratio of borate and glucose and **complex II** is a 1:2 ratio of borate & glucose molecules.

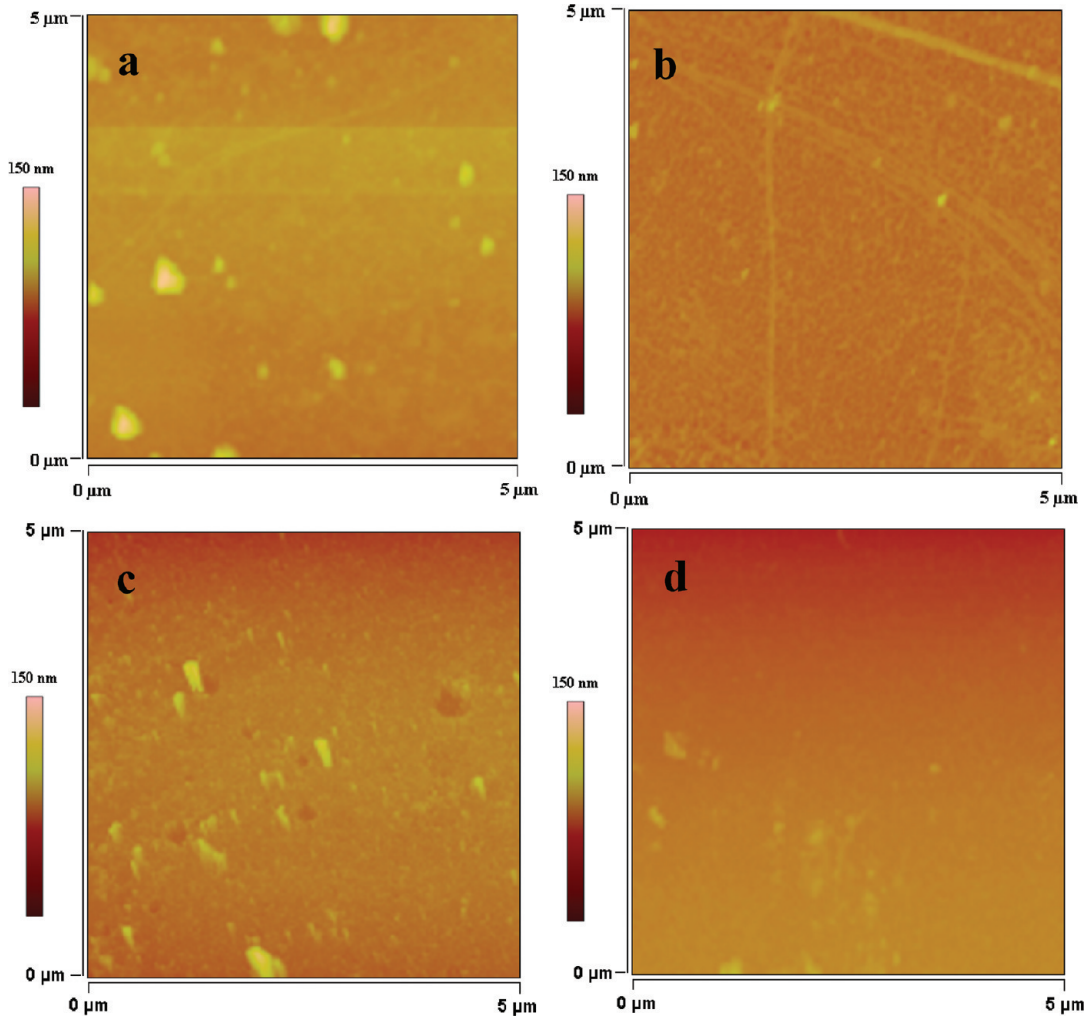


FIGURE 7. Destruction of multilayer film on planar substrate in presence of glucose. (a) The film morphology before glucose treatment. (b–d) Morphologies of multilayer thin films after incubation in glucose for 3, 6, and 12 h, respectively.

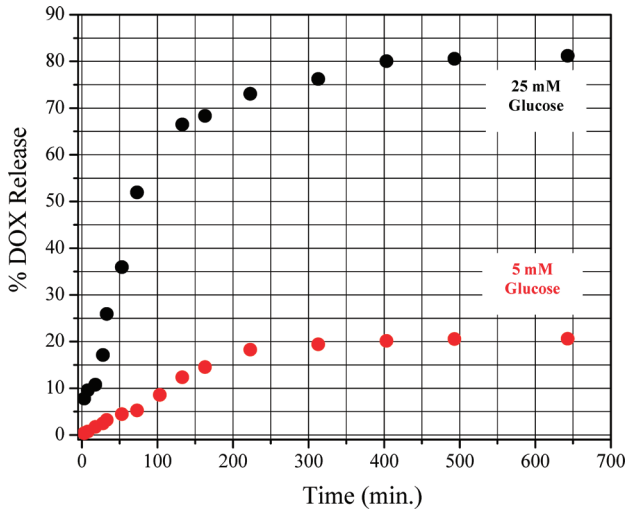


FIGURE 8. Glucose response DOX release from multilayer assembly.

over the same period of time (Figure 8). As explained earlier, a lower concentration of glucose (5 mM) leads to formation of **complex I** (Scheme 2) and characteristically delays DOX release, but at higher concentration of glucose, complete disassembly of the multilayer thin film takes place and causes higher removal of the drug. These results indicate that the self-assembly of PVA–borate/chitosan can be utilized to target drug release in cancer cells containing a higher percentage of glucose.

CONCLUSION

In conclusion, we have shown the controlled disintegration of layer-by-layer self-assembly of biodegradable and biocompatible polymers in the presence of glucose molecules. Because of the presence of physically cross-linked PVA hydrogel inside the multilayer, the capsules morphology and size can be tuned. The self-assembly is mediated by borate ion and the presence of borate in the multilayer wall provides the possibility for controlled release of anticancer drugs by means of variable glucose concentration. We believe that borate mediated self-assembly of PVA hydrogel and chitosan can find useful application in smart anticancer drug delivery systems. The vivo studies are under progress in our laboratory.

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