Materials Horizons

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Integrating thermoresponsive copolymer with host-guest interactions for fabricating molecular recognition surfaces

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

A platform capable of integrating variable molecular recognition moieties, tunable function and regenerable/reusable ability has been developed to build bio-functional surfaces. More specifically, mannose and biotin-modified β-CD were incorporated into poly(*N*-isopropylacrylamide-*co*-1-adamantan-1-ylmethyl acrylate) [poly(NIPAAm-*co*-Ada)] surfaces by host-guest interaction to investigate their specific interaction with ConA and avidin, respectively. The surfaces have showed a thermoresponsively tunable recognition for specific proteins, while keeping resistant to nonspecific protein adsorption. By varying Ada content, the regulation of specific protein adsorption in different temperature range could be achieved. The poly(NIPAAm-*co*-Ada) surfaces can be easily regenerated and reused for bio-funtionalization.

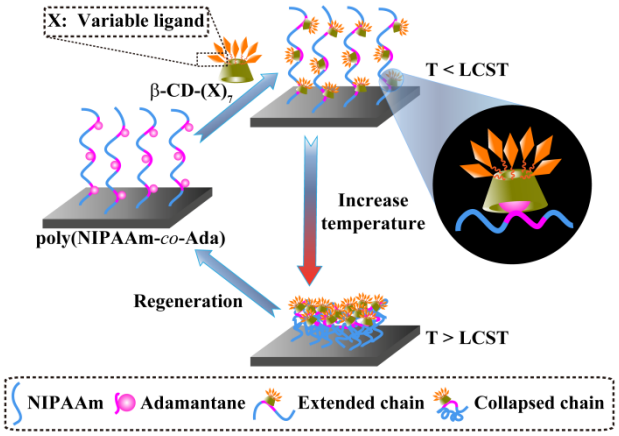
Introduction

Molecular recognition, which plays an important role in biological systems and is observed in between sugar-lectin, antigen-antibody, DNA-protein, RNA-ribosome, *etc*., is essential in life. Therefore, they have been widely used in biosensor, bioseparation, bioanalysis, microfluidic devices and biomaterials for human health.[1-7] To improve the selectivity, accuracy, controllability, and reproducibility of devices, it's very important to understand, control and utilize the specific and nonspecific interactions between molecules in recognition.[7-13] The decrease of nonspecific protein adsorption imparts biocompatibility to blood contacting materials, and reduces false signals in biosensors; the selective and tunable protein adsorption enhances the function of materials and the localized controllability. Poly(*N*-isopropylacrylamide) (PNIPAAm) is a classical thermoresponsive polymer for controlling protein adsorption and cell adhesion corresponding to its swelling/shrinking status at temperatures below and above the lower critical solution temperature (LCST).[14,15] The previous work of our group has found that PNIPAAm surfaces with high grafting density exhibit good protein-resistant properties in a certain thin range (< 15 nm) regardless of the temperature (above or below its LCST).[16,17] So, we believe that the introduction of functional groups capable of molecular recognition into PNIPAAm chains can not only provide the ability to recognize specific proteins and repel nonspecific proteins, thereby improving the signal-to-noise ratio of bio-analytical devices, but also the ability to switch on/off specific protein adsorption, which can be potentially applied in smart switching.[18-20]

There is generally two ways for incorporation of functional groups: covalent and non-covalent binding method. The covalent method is relatively strong and enhances the stability of biomaterials, whilst non-covalent methods allow more flexibility, such as good reversibility, adaptivity, facile fabrication, *etc*.[21-23] Host-guest interactions have been recently utilized as a new versatile and robust post-modification methodology for integrating functional moieties into biomaterials. The host-guest interactions based on β-cyclodextrin (β-CD) have the ability to tune ligand valency, type, orientation and location, or even allowing for reversible binding.[24-31] Amongst different host-guest pairs, β-CD and adamantane (Ada) are a strong pair and have been widely used as linkers for integration of biomolecules to construct functionalized bio-substrates.[32-37] They can also be dissociated for the regeneration of biomaterials and sensor

Conceptual insights

This work presented the design of a platform capable of integrating variable molecular recognition moieties, tunable function and regenerable/reusable ability. These features are important in the applications of bio-related devices, considering their sophisticated fabrication and complex surface modification. The key of the design is PNIPAAm and host-guest interaction. The antifouling property and smart switching function of PNIPAAm was simultaneously introduced to improve signal-to-noise ratio, and host-guest interaction was adopted to reversiblely integrate variable molecular-recognition moieties. Two model surfaces were fabricated utilizing the aforementioned approach and they displayed excellent reusability and thermoresponsively tunable recognition for specific proteins, while keeping resistant to nonspecific protein. The versatile approach emphasizes the possibilities of its introduction in biomedical and biotechnological applications.



Scheme 1 Schematic illustration of the preparation of bio-functional and thermoresponsive surfaces by host-guest interaction between poly(NIPAAm-*co*-Ada) and β-CD-$(X)_7$.

chips.[37,38] Herein, poly(*N*-isopropylacrylamide-*co*-1-adamantan-1-ylmethyl acrylate) [poly(NIPAAm-*co*-Ada)] was used as a platform for attaching molecular recognition functionalities onto silicon surface via host-guest interactions between adamantane and ligands bearing β-CD (CD(X)) (**Scheme 1**). A series of thermoresponsive and bio-functional surfaces can be built just by changing ligands on the β-CD ring. Therefore, the platform capable of integrating variable molecular recognition moieties, tunable function and regenerable/reusable ability offers more potential for biomedical and biotechnological applications.

Results and discussion

The surface-initiated process, grafting density and surface composition of poly(NIPAAm-*co*-Ada) on silicon surface have been described in our previous work.[39] Surfaces with Ada content less than 5% were used in this work, as at higher Ada content, CD(mannose) is hard to complex with poly(NIPAAm-*co*-Ada) layer due to steric hindrance.[39] The thickness of all polymer layers was 10 - 12 nm, where the antifouling property of PNIPAAm can be retained. In a-proof-of-concept study, mannose-modified β-CDs were incorporated onto poly(NIPAAm-*co*-Ada) layers to investigate their specific recognition with ConA. The discussions about the successful complexation of CD(mannose) and poly(NIPAAm-*co*-Ada) surfaces with different Ada feed ratios were described in **Supporting Information**. As Concanavalin A (ConA, 104 - 112 kDa, pI = 4.5 - 5.5) binds to α-D-mannosides specifically, while human serum albumin (HSA, 66.5 kDa, pI = 4.9) does not and has a comparable molecular weight and pI to ConA, they were chosen as a model to study the specific recognition of ConA and nonspecific protein adsorption on α-D-mannose-decorated thermoresponsive surfaces. The ^{125}I-radiolabeling method was adopted in this work as it is very sensitive and accurate to quantify the adsorbed protein on surfaces, and has been successfully used for labeling ConA.[40,41] We checked the dynamic adsorption of ConA on CD(mannose)-modified surfaces with incubation time, and its adsorption reached an equilibrium after about two hours **(Fig. S5)**, so in this research three-hour adsorption was adopted.

In order to investigate the effect of the sugar content on protein adsorption at temperatures above and below LCST, 37 and 4 °C were chosen as the LCST of all mannose-decorated surfaces obtained were within the temperature range **(Fig. S2)**. At 37 °C, above the LCST, although PNIPAAm is in a collapsed conformation, the adsorption of ConA and HSA for Si-PNIPAAm were 31.2 and 27.4 ng cm^{-2}, respectively **(Fig. 1A and 1B),** which are still in a very low level, indicating the antifouling property of PNIPAAm. With the increase of sugar content, the specific ConA adsorption increased to 334 ng cm^{-2}, while the nonspecific HSA adsorption decreased to 9.0 ng cm^{-2}, due to the nonspecific protein-repelling property of glycopolymers.[42-44] In addition, the sugar density regulated by Ada content had a great influence on protein adsorption. For sugar surfaces with 1.64% and 4.76% Ada feed ratio, ConA adsorption increased 5.5 and 9.7 times of that on PNIPAAm surface **(Fig. 1A)**; while HSA adsorption decreased 54% and 67% compared with PNIPAAm surface **(Fig. 1B)**. As cyclodextrin may attach to surfaces by interacting with alkyl side chains,[45] we compared the ConA adsorption on PNIPAAm surface without Ada moieties before and after immersion with CD(mannose) solution. The surfaces

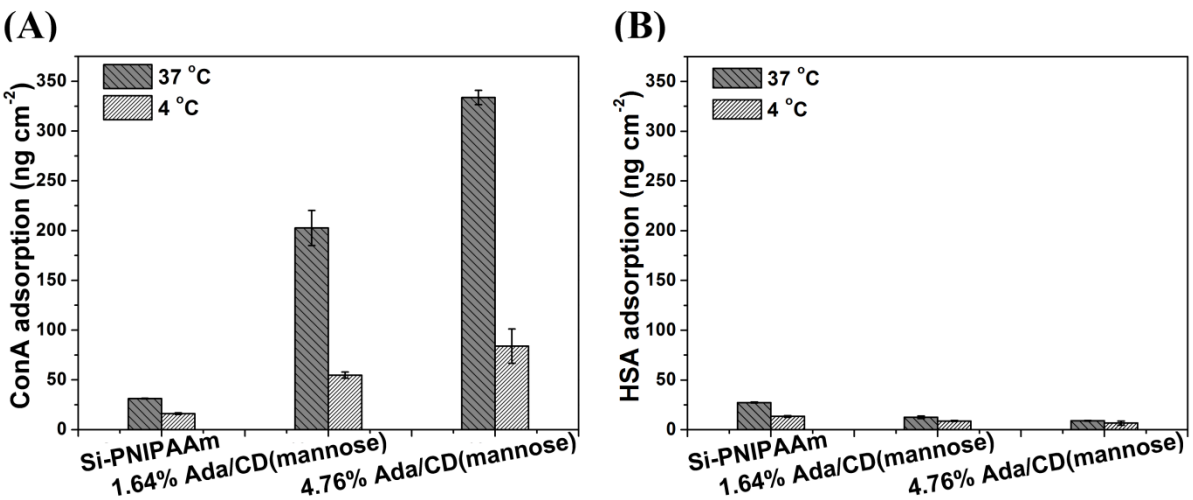


Fig. 1 Specific and nonspecific protein adsorption on Si-PNIPAAm, Si-1.64% Ada/CD(mannose) and Si-4.76% Ada/CD(mannose) surfaces at 37 and 4 °C. (A) ConA in PBS (10 mM, pH 7.4, containing 1 mM Ca^{2+} and 1 mM Mn^{2+}), and (B) HSA in PBS. Data are means ± standard error (n = 3).

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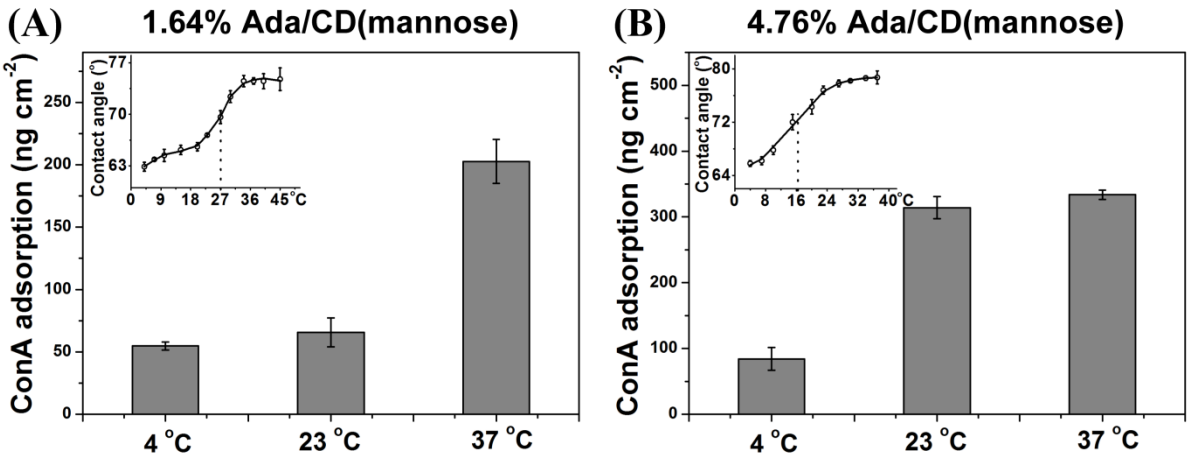

Fig. 2 Regulation of ConA adsorption in different temperature ranges. ConA adsorption on poly(NIPAAm-*co*-Ada)/CD(mannose) surfaces with (A)1.64% Ada feed ratio and (B) 4.76% Ada feed ratio at 4, 23 and 37 °C. The inserted graph is the thermoresponsive wettability transition of the relative surface (The dashed vertical line indicates the LCST.). Data are means ± standard error (n = 3).

were found to exhibit almost the same low level of ConA adsorption. In addition, control experiments for ConA adsorption on copolymer surfaces of poly(NIPAAm-*co*-Ada) at 37 °C also showed a very low level of ConA adsorption, below 37 ng cm^{-2}, which was comparable to that on PNIPAAm surface **(Fig. S4)**.Theses results demonstrated that it was the host-guest interaction between cylodextrin and Ada that integrated CD(mannose) onto copolymer surface to form a glycopolymer surface, thereby increasing the specific protein adsorption and decreasing the nonspecific protein adsorption. Moreover, the mannose content on surfaces can be tuned by varying Ada feed ratios. At 4 °C, below the LCST, the adsorption of HSA for all the surfaces was very low, slightly lower than the protein adsorption above the LCST **(Fig. 1B)**; For specific ConA adsorption, the biggest reduction was observed for 4.76% Ada/CD(mannose) surface, which reduced ~75% compared with that at 37 °C **(Fig. 1A)**, indicating a notable thermoresponsive protein adsorption. The significant difference of recognizing ConA at different temperatures will be beneficial for preparing responsive biomaterials/devices. The temperature-induced tunable adsorption could be explained by conformational change of polymer chains, indicated by the variation of surface wettability **(Fig. S6)**. Above the LCST, PNIPAAm chains collapsed, exposing the bulky and hydrophilic CD(mannose) and forming many glycoclusters, which can enhance the affinity with lectins as a result of "cluster glycoside effect" (mainly chelating effect and statistical effect).[46-49] Therefore, a large amount of ConA was adsorbed on surfaces above LCST. Whilst below the LCST, the hydrated and extended chains can not only prevent the approach of CD(mannose) to ConA, but also weaken the binding constant by decreasing the sugar density on the surface. So ConA were repelled by the hydrated PNIPAAm chains.

The thermoresponsive molecular recognition ability can be easily tuned at different temperature ranges by changing Ada content. For example, the LCST of 1.64% Ada/CD(mannose) is ~27 °C, **(Fig. 2A, insert)**, therefore the ConA adsorption can be regulated between 37 and 23 °C. As shown in **Fig. 2A**, ConA adsorption on 1.64% Ada/CD(mannose) surface reduced 68% from 37 °C to 23 °C. For comparison, ConA adsorption on 4.76% Ada/CD(mannose) surfaces showed almost no change at the two temperatures, as it has a lower LCST of ~16.5 °C **(Fig. 2B, insert)**. Actually, 4.76% Ada/CD(mannose) surface showed a reduction of 73% for ConA adsorption from 23 °C to 4 °C (**Fig. 2B**). Generally speaking, the temperature rise is probable to increase protein adsorption itself, but unlikely to be in a significant way. This is because the observed changes in ConA adsorption were minor when 1.64% Ada/CD(mannose) and 4.76% Ada/CD(mannose) surfaces were changed from 4 °C to 23 °C and from 23 °C to 37 °C, respectively. Therefore, it is the conformational changes of thermoresponsive polymers triggered by temperature that mainly controls the arrangement of CD(mannose) and therefore regulates protein adsorption. And by simply adjusting Ada content to acquire thermoresponsive glycopolymer surfaces with different LCSTs, the regulation of specific protein adsorption in different temperature ranges can be achieved.

In order to prove the versatility of the poly(NIPAAm-*co*-Ada) platform, biotin-modified β-CD ring was also introduced onto the surface, forming a biotin-functionalized thermoresponsive surface. The specific recognition ability of the biotin-modified surface was investigated using fluorescein-labeled avidin and BSA. β-CD-$(biotin)_7$ (CD(biotin)) was synthesized and the conjugation ratio of biotin to β-CD was calculated as 6.39 according to the integral values obtained from the H protons of β-CD at C1 and of 1,2,3-triazole group in ^{1}H NMR spectra (**Fig. S7**). CD(biotin) was introduced onto 1.64% Ada surfaces via host-guest interaction to form CD(biotin)-complexed surfaces, which also showed a tunable avidin-recognition ability. The results of FITC-labeled

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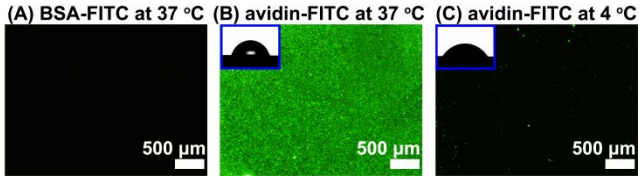

Fig. 3 Specific and nonspecific protein adsorption on Si-1.64% Ada/CD(biotin) surfaces. (A) Adsorption of BSA-FITC at 37 °C. (B) Adsorption of avidin-FITC at 37 °C and (C) at 4 °C. The inserted graphs showed surface wettability of the surfaces at 37 °C (CA = 73.2 ± 1.1°) and at 4 °C (CA = 58.4 ± 0.6°).

avidin adsorption showed that 1.64% Ada/CD(biotin) surface had much higher fluorescence intensity for avidin adsorption at 37 °C (**Fig. 3B**) than that at 4 °C (**Fig. 3C**), which was attributed to the temperature-induced chain conformation change revealed by the surface wettability change (**Fig. 3B and 3C, inserts**, from 73.2 ±1.1° to 58.4 ± 0.6°). Nevertheless, Si-PNIPAAm and Si-1.64% Ada surfaces (**Fig. S8A and S8B**) exhibited negligible fluorescence intensity. These results demonstrated that avidin recognition to CD(biotin)-conjugated poly(NIPAAm-*co*-Ada) surfaces were switched on when the temperature was above the LCST and switched off when the temperature was below the LCST. With regards to the nonspecific BSA-FITC adsorption at 37 °C, there was almost no green fluorescence on 1.64 Ada%/CD(biotin) surface (**Fig. 3A**), indicating its ability of repelling nonspecific protein adsorption. The binding constant between biotin and avidin is ~10^{15} M^{-1}, which is one of the strongest known non-covalent bonds and does not break easily once formed.[50] Conversely, the desorption of avidin can be realized by dissociating the complexion between β-CD and Ada using sodium dodecylsulfate solutions (SDS).[38] As shown in **Fig. 4**, the exposure to 2% SDS resulted in almost complete desorption of avidin-FITC from poly(NIPAAm-*co*-Ada)/CD(biotin) surfaces (**Fig. 4B**). This desorption was due to the dissociation between β-CD and Ada as proved by the negligible avidin adsorption on control surfaces (**Fig. 4E**), *i.e.* the regenerated surfaces without being immersed in CD(biotin) solution. In addition, the regenerated surfaces after conjugation with CD(biotin) could further adsorb avidin-FITC with almost the same fluorescence intensity as the first time (**Fig. 4C**). Moreover, the surfaces with re-adsorbed avidin-FITC could be regenerated again upon exposure to SDS (**Fig. 4D**). During cycles, the fluorescence intensity of avidin-adsorbed surfaces reduced by almost 99.9% after being washed by SDS each time (**Fig. 4F**).

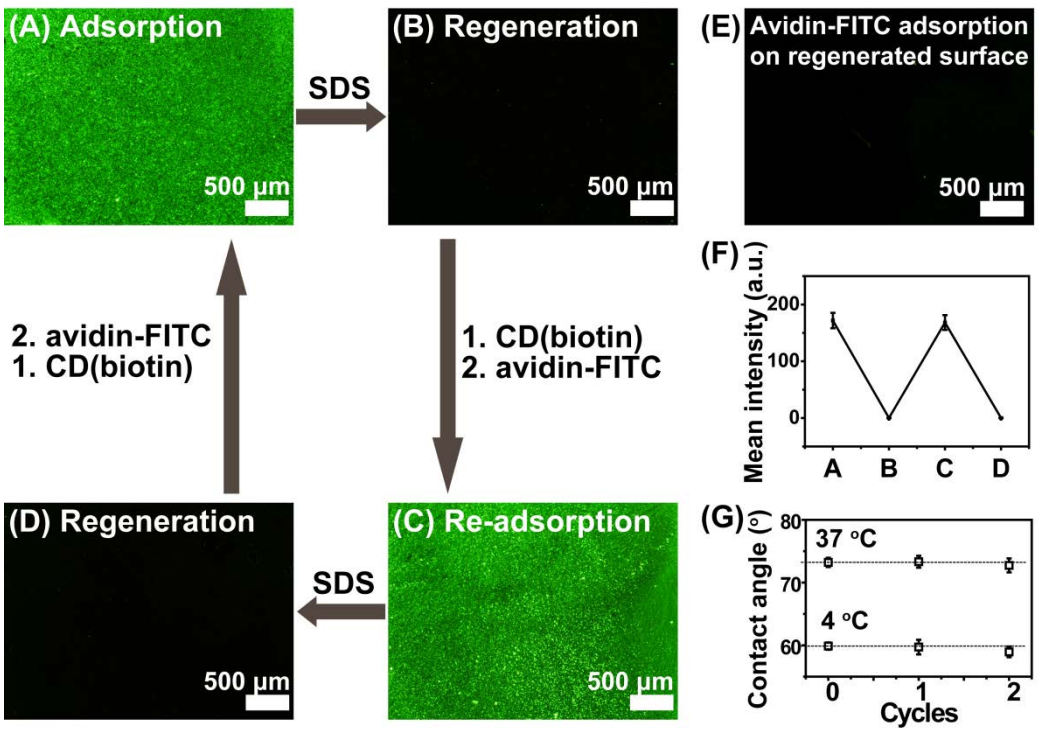

Fig. 4 Regeneration and reuse of poly(NIPAAm-*co*-Ada) surfaces. (A) Adsorption of avidin-FITC on Si-1.64% Ada/CD(biotin) surface; (B) regeneration by washing the surface with 2% SDS; (C) conjugation of CD(biotin) followed by re-adsorption of avidin-FITC; (D) regeneration with 2% SDS; (E) Avidin-FITC adsorption on the regenerated copolymer surfaces without CD(biotin) at 37 °C. (F) The cycles of regeneration/reuse of copolymer surfaces were measured by analyzing the mean fluorescence intensity of the images from surfaces during the cycles. (G) The thermoresponsivity of poly(NIPAAm-*co*-Ada) surfaces following regeneration with SDS, indicating the efficiency of regeneration and the stability of surfaces**.** Data are means ± standard error (n = 3).

Cite this: DOI: 10.1039/c0xx00000x

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We also measured the regeneration efficiency of ConA-adsorbed surfaces using radio-labelling method, and almost no proteins remained on surfaces after being washed by SDS each time (**Fig. S9**). Therefore, it is suggested that this system is able to be regenerated and thus reused. Moreover, we studied the thermoresponsivity of poly(NIPAAm-*co*-Ada) surfaces following regeneration with SDS to check the stability of the surfaces. The regenerated surfaces kept almost the same WCAs at both 37 and 4 ^{o}C as the pristine surfaces, indicating the efficiency of regeneration and the stability of surfaces (**Fig. 4G)**. Actually, other molecular recognition functionalities may be introduced onto the regenerated surfaces. Thus, the poly(NIPAAm-*co*-Ada) platform provides the feasibility of integrating functional groups and the ease of recovery via host- guest interaction. It is an important and desirable feature in the applications of bio-related devices, considering their sophisticated fabrication and complex surface modification.

Conclusions

In summary, we have developed a versatile platform of poly(NIPAAm-*co*-Ada) surfaces to integrate ligands-functionalized β-CDs, allowing for the preparation of various thermoresponsive and bio-functional surfaces. Different molecular recognition functionalities, *i.e.* mannose and biotin-modified β-CDs, were synthesized as models to investigate their specific recognition with ConA and avidin, respectively. CD(mannose)-conjugated poly(NIPAAm-*co*-Ada) surface showed a low nonspecific protein adsorption even less than that on PNIPAAm surface, and presented a thermoresponsively tunable ConA adsorption. By varying Ada content, the regulation of ConA adsorption in different temperature range could be achieved. In addition, CD(biotin)-conjugated poly(NIPAAm-*co*-Ada) surfaces also showed a selective and thermoresponsively controlled avidin adsorption. So, there are four advantages of this system: (1) well-controlled ability to recognize specific biomolecules by changing temperature; (2) easy and versatile functionalization by integrating ligands-decorated β-CD ring; (3) prevention of nonspecific protein adsorption; (4) allowing surface regeneration by simply washing with SDS. The performance of this system indicates the potential to be used in biosensor, bioanalysis, bioseparation, microfluidic devices, *etc*.

Acknowledgements

This work was supported by the National Science Fund for Distinguished Young Scholars (21125418), the National Natural Science Foundation of China (21374069, 21334004), the Project of Scientific and Technologic Infrastructure of Suzhou (SZS201207), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). D.M.H. is a Wolfson Royal Society Fellow.

Notes and references

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† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/b000000x/

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Thermoresponsive poly(NIPAAm-*co*-Ada) surfaces integrated with host-guest interactions were used as a platform for fabricating reusable and tunable molecular recognition surfaces.