European Journal of Pharmaceutics and Biopharmaceutics 75 (2010) 277–283

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

A novel method for modifying AFM probe to investigate the interaction between biomaterial polymers (Chitosan-coated PLGA) and mucin film

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ARTICLE INFO

Article history:
Received 29 May 2009
Accepted in revised form 22 February 2010
Available online 25 February 2010

Keywords:
Chitosan
PLGA
Mucin film
Atomic force microscopy
Repulsive force
Attractive force
Probe modification

ABSTRACT

A new method for modifying atomic force microscope (AFM) probe with polymer was proposed to estimate the interaction between Chitosan (CS)-coated poly (lactic-co-glycolic acid) (PLGA) polymer and mucin film. In this method, the mixture of polymers and its suitable volatile dissolvent were deposited on the AFM probe tip through a micropipette controlled by micromanipulator. After being dried, the polymer film forms on the probe tip. Several evaluation experiments show that using the new method, the AFM probe tip can be smoothly and uniformly coated with PLGA and CS with approximately same curvature radius as that of probe tip less than 500 nm. As a preliminary application of the proposed method, the interaction force between PLGA/CS and mucin film in air was investigated. It was revealed that when a PLGA probe is retracting from the mucin film, a repulsive force appeared; however, after the PLGA probe was further overcoated with CS, the force became attractive if the amount of CS was enough, such as at CS concentrations of 0.2% W/V. The observed force can be interpreted by the surface hydrophilic/hydrophobic characteristics of PLGA/CS and mucin film.

1. Introduction

The atomic force microscope (AFM), an attractive tool for studying surface properties of material and also interaction between materials in the molecular scales [1–5] has been recently applied in pharmaceutical fields to measure adhesive forces between particle-to-inhaler walls [6–12]. In order to measure such forces, AFM probe tip has to be modified. A popular way to modify AFM probe is to attach the particle directly on the probe tip with glue [6,13], forming a so-called colloid probe. It was reported that a particle with a size as smaller as 1.5 μm has been successfully mounted on the probe tip with this method [11]. However, one should note that with this method not only the possibility of contaminating the particle with glue is high, especially when the particle is smaller than 3 μm, but there is also some difficulty to control precisely the position of the particle. Furthermore, during the interaction measurements, the particle position could easily vary if the particle is not attached firmly. Any variation of particle position could cause considerable difference in interaction measurements. On the other hand, since the interaction force was reported to be dependent of tailored surface roughness [11,14], a given roughness of surface may be very important in studying the physical property of surface by using AFM from the viewpoint of experimental reliability. With conventional methods, it is very difficult to control the surface roughness of a particle. To overcome all these problems, we have developed a method for modifying AFM probe tip with polymers. Our experiments demonstrated that the AFM probe tip can be coated not only more smoothly but also have a much smaller curvature radius than the particle attached and thus such probe tip could be considered as a nanosphere. Our modification method should be more reliable and meaningful in studying the physical and chemical property of nanoparticles. The objective of this paper is to describe our method and some of its fundamental applications in studying the interaction between PLGA, a material widely used in drug delivery system [15], Chitosan, a bio-adhesive polymer proposed for oral mucosal drug delivery [15–18], and mucin film, fundamental issue for understanding the mucoadhesive mechanisms of Chitosan.

2. Materials and method

2.1. Materials

Poly (lactic-co-glycolic acid) (PLGA-7520, Mw 20,000, Wako Chemical Inc.), mucin (from stomach, Wako Chemical Inc.), 6-Coumarin (ICN Biomedicals Inc.), fluorescein isothiocyanate (FICT, Sigma) used as fluorescence to label PLGA and Chitosan were used as

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doi:10.1016/j.ejpb.2010.02.013

received. Pure water was prepared using reverse osmosis apparatus (Milli-Q, Millipore Co.), and Chitosan (CS, Mw ~150,000 and the degree of deacetylation 85%, respectively.) was a gift from Katakurachikkarin, Japan. The grade of CS is that used for dry powder aerosols for sustained drug delivery by pulmonary administration in our study.

The mica used was a freshly cleaved mica plate (for atomic scale calibration, Veeco) with its surface being smooth at the atomic level. The mucin film used was prepared by ourselves and described in detail in the following.

2.2. AFM system

Fig. 1 shows schematically the AFM system (Nanoscope □a, Digital Instruments) used in the present study. By detecting the deflection of the cantilever through the combination of the laser and photodiode, the interaction force between the cantilever tip and the sample at molecular level can be obtained with a known spring constant of cantilever using Hookes law. By scanning a sample surface with the probe tip at a constant interaction by controlling the piezo scanner, a 3D sample surface can be imaged. A detailed description related to force measurement by AFM has been given elsewhere [10]. Both the 3D imaging and the interaction force measurement are performed in the present study by using contact mode in atmosphere (room temperature: 25 °C; relative humidity: 25%). The probes used were oxide-sharpened silicon nitride probes (Si_3N_4, NPS) of nominal spring constant 0.38 nN/m and nominal tip radius of curvature ~20 nm and tip height 2.5–3.5 μm (Veeco Nanoprobe TM tips).

2.3. Mucin film preparation

Mucin is a high molecular weight glycoprotein, major constituent of saliva, gastric juice, intestinal juice, and has been used to evaluate the mucoadhesive property of polymer [19]. In this paper, we prepared a mucin film on a glass sheet and then fabricated a sample. The detailed procedures are as follows:

(1) The given mucin was dissolved in an ammonia solution and then was stirred with a touch mixer (MT-31, Yamato) for a few minutes to prepare initial 1% mucin suspension.
(2) In order to make the mucin particle micronized, the mucin suspension was sonicated with a probe ultrasonic disruptor (UR-200P, Tomyseiko Co., Ltd., Japan) for a few minutes in an ice bath.
(3) Procedure (2) was repeated until the size of the particles attained an equilibrium value of about 160 nm. The size of particles in suspension was measured with a Zeta meter (Zeta sizer, Malvern).
(4) The suspension was filtrated via a 0.45 μm filter so that a suspension with a uniform particle size of 160 nm was obtained.

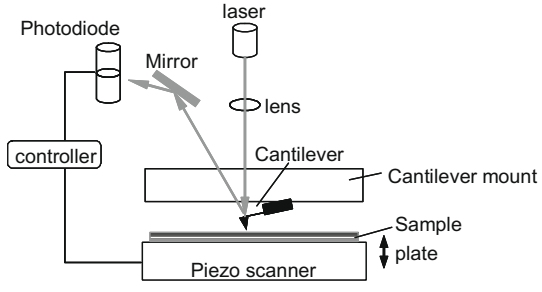

Fig. 1. Schematic of the AFM used in this study.

(5) Fifty microliters of the suspension was put on a clean treated glass sheet (Φ = 15 mm, thickness: 0.12–0.17 mm), which served as a substrate. The suspension diffused uniformly soon and then was put in a desiccator with silica gel 1–2 days for drying. A mucin film formed after above processes.

To evaluate the surface roughness of the prepared mucin film, 3D images of the glass sheet and the prepared mucin film were taken by using AFM. Fig. 2a shows a 3D image example of the glass sheet and Fig. 2b is for the mucin film. Fig. 2c is the magnified image of Fig. 2b. For typical areas (1 μm^2) in Fig. 2c, the standard deviation from mean height was 5.8 nm and the highest asperity was 13.9 nm. We have also performed the section analysis by using AFM and the result is shown in Fig. 2d. Although the prepared mucin film showed some roughness at a large scale as seen in Fig. 2b, the mucin film was enough smooth at a smaller scale as seen in Fig. 2c and d for studying the interacting force with AFM. The thickness of the prepared mucin film is shown in Fig. 3. In image, the hollowed section is the glass sheet plane and the rest section is the mucin film. The film thickness was obtained by averaging the values measured at four different points to be of (1.3 ± 0.03) μm.

Three mucin film samples have been chosen to measure their interaction forces with a NPS tip. For each sample, the forces were measured at four different points of each mucin film. Fig. 4 shows the adhesion force appeared when the probe and the mucin films were separated. Apparently, all the forces were nearly the same and this implies that the mucin films prepared by us were of high quality and suitable for studying their characteristics with AFM.

3. Results and discussion

3.1. Modification of AFM probes with PLGA and CS

The system used to modify the AFM probes is schematically shown in Fig. 5, which consists of an optical microscope (Olympus, Japan), a micromanipulator (Narishige, Co., Ltd., Japan) and a micropipette. With this system, we modified the AFM probe tip with polymers of poly (lactic-co-glycolic acid) (PLGA) and Chitosan (CS). The modification procedures are shown in Fig. 6 and described as follows:

(1) Fixing a blank NPS on a micro slide glass and setting it under the optical microscope.
(2) Sucking 2.5% PLGA acetone mixture labeled with 6-Coumarin that is used as a fluorescence marker, into the micropipette about 3 mm from its tip.
(3) Setting the micropipette loaded with PLGA mixture on the micromanipulator arm, and then touching and dropping the mixture from the micropipette on the tip. As a result, the mixture covers uniformly the probe tip and form a thin film.
(4) Store the probe in a desiccator with silica gel and keep it in vacuum over night for drying. Following these procedures, a tip modified with PLGA was obtained (P-NPS). Just before using it for AFM measurement, nitrogen was slowly introduced to the desiccator to release the vacuum and take the probe out.

To have the probe being modified with CS, a modified PLGA probe (P-NPS) was fixed on a glass sheet and then put it under the microscope. 0.4% CS labeled with FITC acetate buffer (0.1 N) and ethanol mixture was drawn into the micropipette, where FITC is used as a fluorescence marker. The procedures (2), (3) and (4) described above were repeated so that a tip modified with CS/PLGA

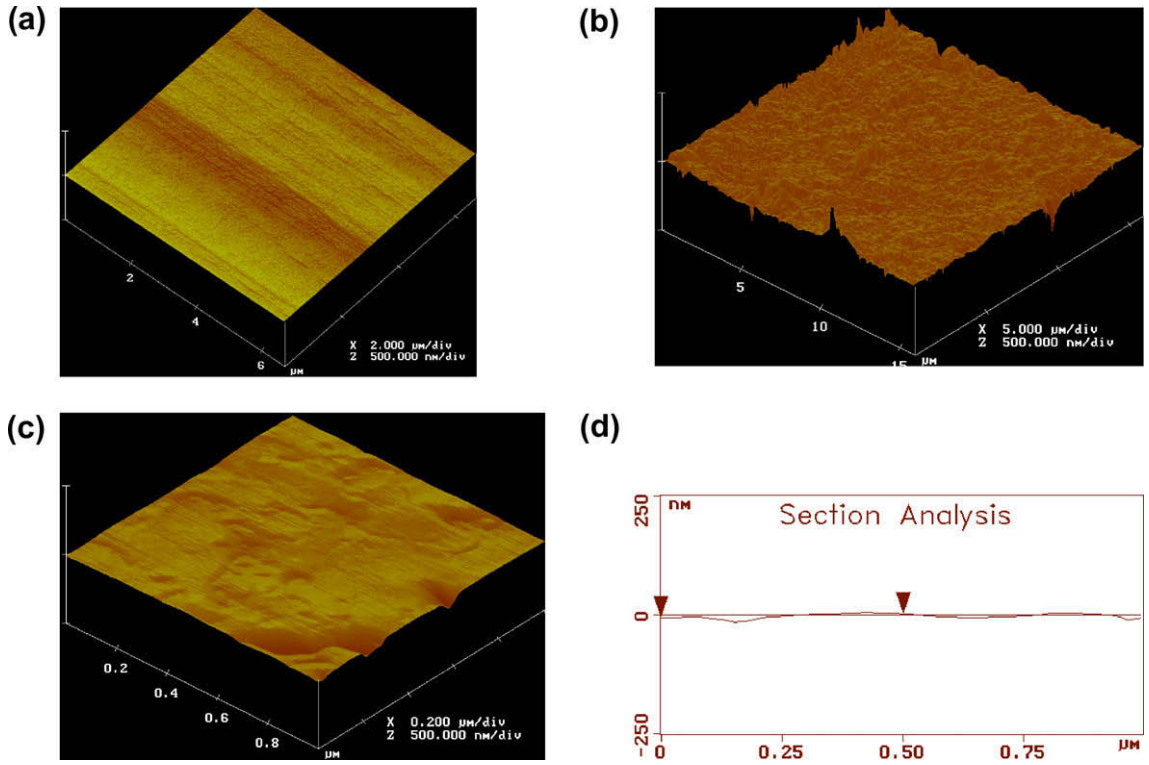

Fig. 2. 3D images of glass sheet and mucin film obtained by AFM: (a) glass sheet; (b) mucin film; (c) mucin film at an expanded size of (b); and (d) section analysis of mucin surface performed for (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

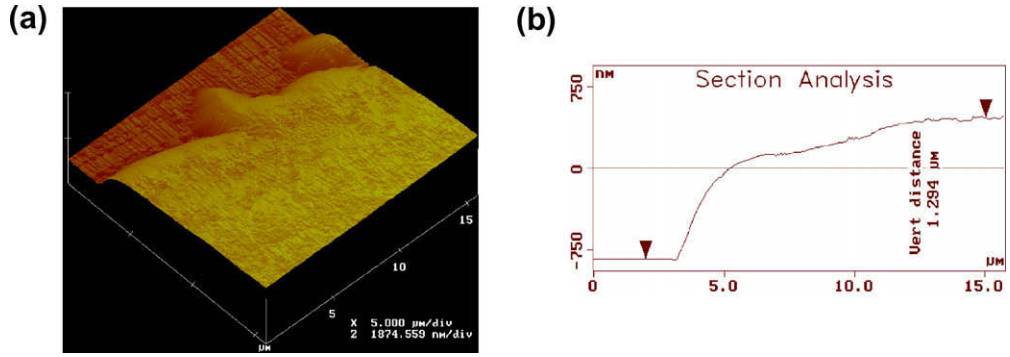

Fig. 3. Analysis of mucin film thickness: (a) surface image of glass sheet with mucin film and (b) section height analysis of the mucin film. The glass sheet is assumed as zero height. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was obtained (CS–P-NPS). It was required at least two days to dry before use.

To have the tip being properly modified, the diameter of the micropipette has to be optimized. If the diameter was too small, the mixture solution could not come out, while if the diameter was too large, the probe could be covered with too much material and be contaminated. In this study, the micropipette was made by extending a glass tube (1×90 mm) with a heater (PC-10, Narlshige). By adjusting the heater stage levels, the micropipette can be made with different diameters. After a number of experimental trials, we found that a micropipette with a tip diameter of around 2 µm is the best.

3.2. Evaluation of the modified probes

To evaluate the performance of our new modification methods, three different approaches as described below were used to evaluate the modified probe tips.

3.2.1. CLSM method

With a confocal laser scanning microscopy (CLSM), the photos of the probes before and after modification were taken and the results are shown in Fig. 7. Fig. 7a is the tip without modification and Fig. 7b–d are the tips modified, respectively, by Coumarin-PLGA, PLGA and FITC–CS–PLGA. The green marker appeared on the tips of Fig. 7b and d corresponds to Coumarin and FITC. As shown in these photos, all the probes were properly modified.

3.2.2. SEM method

To further characterize the modified probe tips, these tips are observed with a scanning electron microphotograph (SEM, JSM-T 330A, JEOL). Fig. 8a shows the NPS tip without modification. Fig. 8b and c are the tips modified respectively with PLGA and CS/PLGA. As seen in these figures, compared with the NPS in Fig. 8a, the tips in Fig. 8b and c are smoothly modified with a tip curvature radius of less than 500 nm.

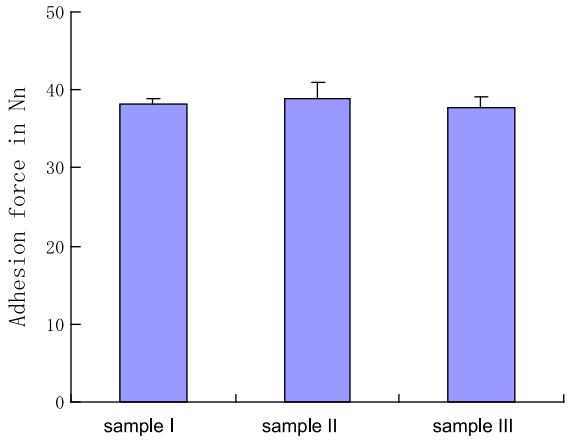

Fig. 4. Adhesion force magnitudes when NPS is separated from the mucin films ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

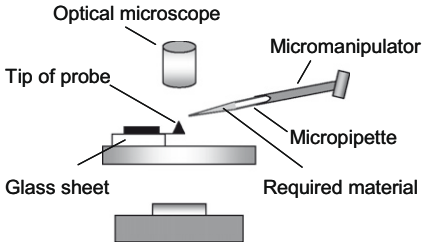

Fig. 5. A schematic drawing of the system used to modify AFM probes.

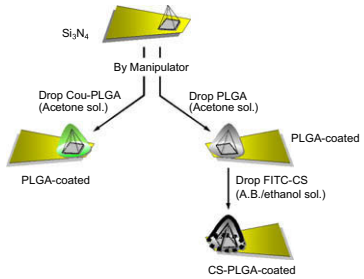

Fig. 6. Procedures of modifying NPS with PLGA and CS/PLGA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The solution of PLGA used for modifying has good affinity to probe (Fig. 8b). Plentiful silanol groups (SiOH) exist in silicon nitride and hydrogen bonds could form between such silanol groups and —COOH group in a terminal position on the PLGA. For CS-modified P-NPS, similar hydrogen bonds could also form between —COOH on PLGA and —NH_2 on CS as shown in Fig. 9. All these reasons may explain why the modified probe is smooth and stable.

Ability of modifying a probe with a curvature radius of less than 500 nm is a significant advantage of the proposed method because it allows us to perform force measurement for nanoparticles.

3.2.3. AFM method

Although CLSM and SEM methods can be used to check the modified probe tips as seen above, since both of them would contaminate the probe tips, they cannot be used prior to AFM experiments. Therefore, the modified probes can be checked with these two methods only after AFM experiments. This means that although some probes may not be properly modified for AFM experiments, they cannot be noted in advance of AFM experiments. This influences the efficiency of experiments. For this reason, we have developed an AFM method that allows us to check the probes soon after modification and before real AFM experiments. In this method, the interaction between the probes and a mica plate that was freshly cleaved before use was measured with the AFM. Fig. 10a shows a force curve between the surface of mica and NPS in air. By first approaching (extending) the mica plate to the tip, a force curve (A → B → C) was obtained. By separating (retracting) the mica plate, another force curve (C → D → E) was found indicating an attraction force appeared (D → E). Fig. 10b shows the force curve between the surface of mica and a probe modified with PLGA (P-NPS). The repulsive forces appeared either approaching or separating (A → B → C and C → D → E). Fig. 10c shows a force curve between the surface of mica and a probe modified with CS/PLGA (CS–P-NPS) when the mica plate is approaching (A → B → C) and separating (C → D → E) from the tip. It is very clear to see that an attractive force appeared when they separated.

In air, it is assumed that mica surface is adsorbed with water molecules layer due to the surface composition of silanol groups [20]. On the other hand, for a NPS probe, its surface also exhibits same characteristics [21]. An adsorbed water molecules layer is expected on the surface either. Two layers of water molecules contacted should attract each other during their separation by the capillary force due to the water bridge between two surfaces. We think that this may be the reason why a regularly adhesive force was observed when the probe separates from mica surface as shown in Fig. 10a, which is very larger than the adhesive from the classical DLVO theory. On the other hand, when the NPS was modified with PLGA, the probe becomes hydrophobic and no water layer would form on the probe as aforesaid. When the probe separates from water layer that was formed on the mica surface, the

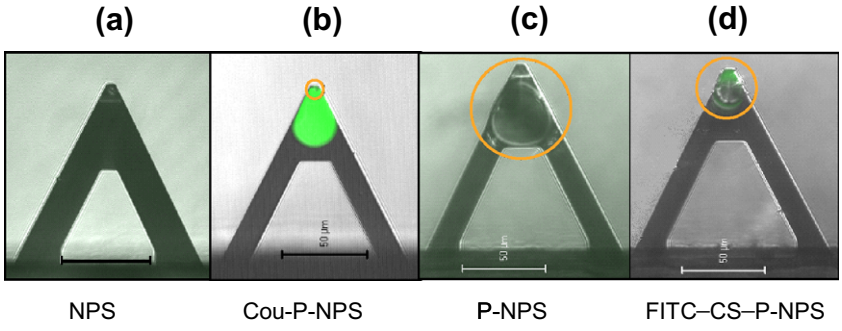

Fig. 7. CLSM photos of probe tips: (a) NPS, non-modification; (b) Cou–P-NPS, modified with Coumarin–PLGA mixture to NPS; (c) P-NPS, modified with PLGA to NPS; and (d) FITC–CS–P-NPS, modified with FITC–CS–PLGA mixture to NPS.

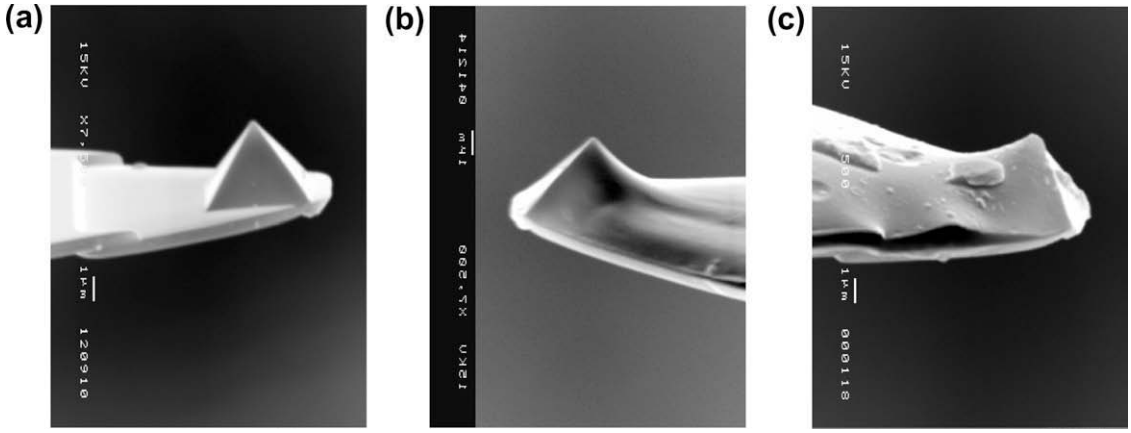

Fig. 8. SEM photos of AFM probes: (a) NPS, non-modification; (b) P-NPS, modified with PLGA to NPS; and (c) CS–P-NPS, modified with CS to P-NPS.

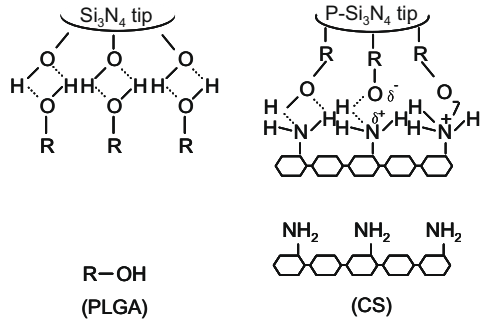

Fig. 9. Schematic illustration of the hydrogen bonds formed on the probe tip modified with PLGA and CS.

hydrophobic repulsive force dominates and this probably is the reason why a stronger repulsive force that is very larger than electrostatic repulsive was observed as shown in Fig. 10b. With the modification of CS, the probe surface characteristics will be dominated by CS and the probe becoming hydrophilic. This may explain why after the P-NPS was modified with CS, a larger attractive force was observed as shown in Fig. 10c.

The differences between Figs. 10a–c are so apparent and they can be used to check whether the probe was properly modified with PLGA and further with CS. It was also noted in Fig. 10b that the force curves of approaching and separating were overlapping very well. This was additional evidence, which indicates that the tip was smoothly modified without any distortion. So in our experiments, we chose only these types of P-NPS for further modification with CS.

3.3. Interaction between PLGA/CS and mucin film

As a preliminary application of the proposed method, the interaction between PLGA/CS and mucin film was investigated. To perform the force measurement, the prepared mucin film was fixed onto a stainless steel substrate (Φ = 15 mm, thickness: 0.7 mm) used for AFM. All the experiments were carried out in room temperature (25 °C) and relative humidity (25%).

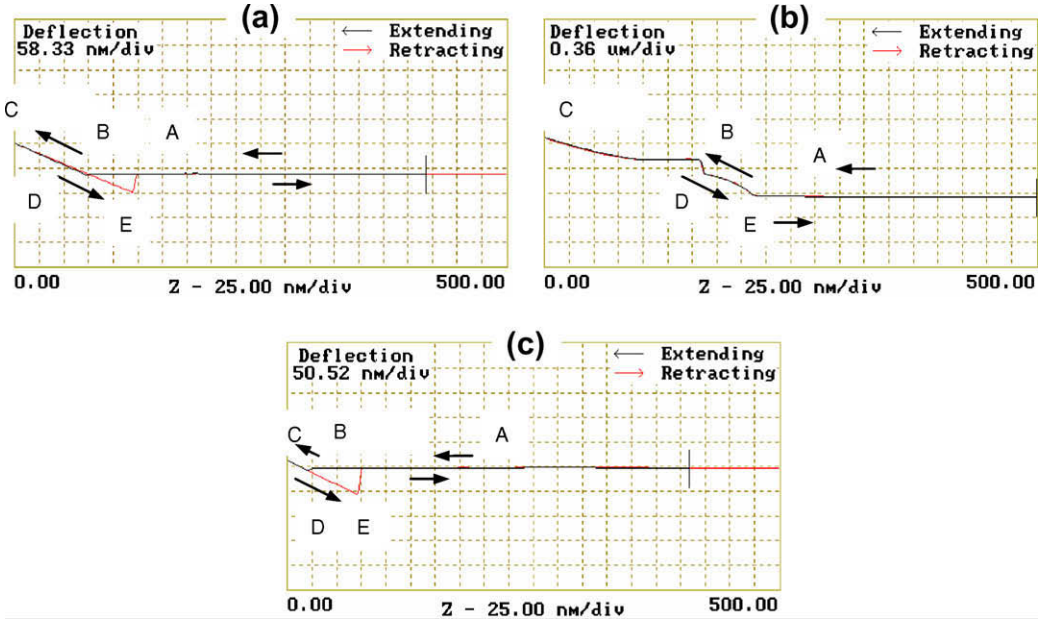

Fig. 10. Interaction force curves between the probe and mica plate: (a) NPS and mica; (b) P-NPS and mica; (c) CS–P-NPS and mica. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 11 shows a force curve between a NPS and the mucin film. As seen in this figure, when the mucin film was retracting from the probe (→), an attractive force appeared. Fig. 12 shows the force curve between the P-NPS and mucin film, where a repulsive force appeared when the mucin film was retracting (→). Fig. 13 presents the force curves between the mucin film and the CS–P-NPS at three different concentrations of CS. When the mucin film was retracting from the probe (→), a repulsive force appeared (Fig. 13a) at the concentration of 0.05%. With the increase of CS concentration to 0.1%, although the interaction force was still repulsive, its magnitude became smaller. When the CS concentration became enough higher at 0.2%, the interaction force became attractive.

The Zeta potentials of CS-coated PLGA-nanosphere measured at concentrations of 0.05, 0.1 and 0.2 (%, W/V) CS are 10.6 ± 1.3, 13.4 ± 1.2 and 21.7 ± 0.6 (mV ± SD), respectively. The higher Zeta potential at bigger concentrations of CS corresponds to larger positive charge. The results shown in Fig. 13 are consistent with the measured Zeta potentials for surplus charge would cause stronger attractive force between CS and mucin film.

Similar to mica plate, mucin film also exhibits some hydrophilic characteristics for its molecular constitution contains —COOH groups. We assumed that a layer of water molecule forms on the mucin surface. Thereby, an attractive force between NPS and mucin film (Fig. 11) and a repulsive force between P-NPS and mucin film (Fig. 12) during separation are understandable as discussed above. With the modification of CS, a small amount of CS may not influence much the hydrophobic characteristics of P-NPS, but if CS becomes considerably high, the CS characteristics become to dominate and cause the probe being hydrophilic. This may explain why when CS concentration is small, a repulsive force was observed as seen in Fig. 13a and b and when CS concentration becomes high, an attractive force was observed as shown in Fig. 13c.

As seen from these experiments, not only the surface characteristics can be controlled easily with our proposed method, but also the amount of the target material modified on the probe tip has been reflected in the magnitude of the force. Apparently, our method can be used to modify AFM probe tips with any polymer that has affinity to probe tips. It is expected that the operative mechanism of nanoparticles in vivo can be studied with the modified probe.

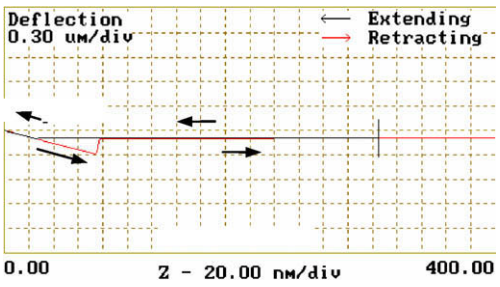

Fig. 11. Interaction force curve between a NPS and mucin film. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

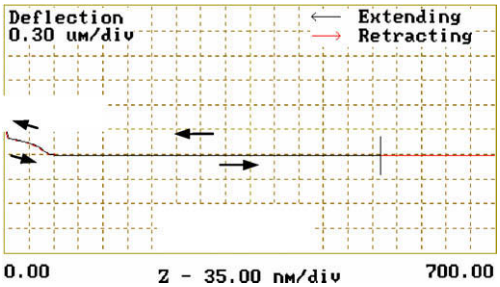

Fig. 12. Interaction force curve between a P-NPS and mucin film. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

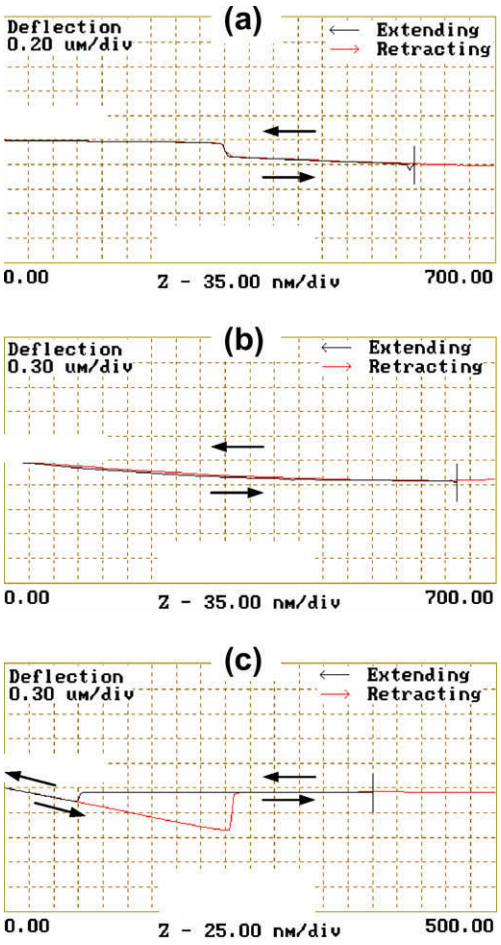

Fig. 13. Interaction force curves between a CS–P-NPS and mucin film at three different concentrations of CS: (a) 0.05%; (b) 0.1%; (c) 0.2%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To demonstrate experimentally the stability and reproducibility of the new modification method, we have measured the interaction forces between P-NPS and mucin films with eighteen different probes and three mucin films (I, II, and III). For each probe, the repulsive force between P-NPS and a mucin film is obtained by averaging the maximum values measured at six different points

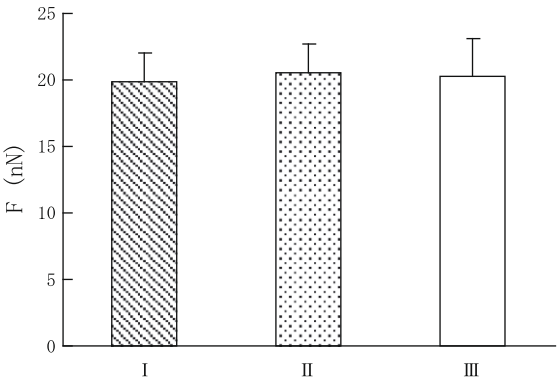

Fig. 14. The maximum repulsive forces between P-NPS and three mucin films.

of the mucin film. For each mucin film, six probes are used and we obtain the repulsive force by averaging the forces of those six probes. Fig. 14 presents the result. As evident in this figure, the average forces for all three different films are more or less the same being around 20 nN, which indicates again that the modified probe has very good stability and reproductivity. Such good stability and reproductivity may allow our modified methods to be widely applied in studying the surface characteristics of nanoparticles.

4. Conclusion

A new method for modifying AFM probes was proposed and verified by experiments. Using the new methods, we have successfully modified the AFM probes with PLGA and CS/PLGA. Moreover, the curvature radius of the modified tip can be easily controlled to be below 500 nm by choosing proper PLGA and CS concentration and coating times. As an application of the new methods, we have investigated the interaction force between PLGA/CS modified probes and mucin films in air. We found that when a P-NPS is retracting from mucin film, a repulsive force appeared, and after the P-NPS was further modified with CS, the force is still repulsive if the CS is of small amount. However, the force becomes attractive if the CS is of large amount. We suggest that the observed force can be explained simply with the surface hydrophilic/hydrophobic characteristics of probes and mucin films.

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