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Nanostructures and Mechanics of Living Exosomes Probed by Atomic Force Microscopy*

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Abstract Exosomes play an important role in the fulfillment of cellular physiological activities and are strongly involved in the pathological processes of numerous diseases. Investigating the behaviors of exosomes is therefore of critical significance for revealing the underlying mechanisms guiding life mysteries and diseases. Nevertheless, due to the lack of adequate tools, the detailed structures and mechanics of living exosomes in their native states are still not fully understood. In this work, atomic force microscopy (AFM), a powerful multifunctional tool for characterizing native biological samples without pretreatments under aqueous conditions, was utilized to probe the nanostructures and mechanics of single living exosomes prepared from clinical cancer patients. Firstly, by attaching exosomes isolated from the bone marrow of lymphoma patients onto the substrates with electrostatic adsorption, single living exosomes were clearly visualized by AFM *in situ* imaging in liquids. The morphological differences of exosomes in liquids and in air were revealed. Secondly, the mechanical properties of single living exosomes were quantitatively and visually studied by AFM indentation assays and AFM multiparametric imaging, respectively. Finally, structural and mechanical changes of exosomes after the treatment of chemical fixation were revealed by AFM. The research benefits investigating the structures and properties of living exosomes at the nanoscale for comprehensively understanding the behaviors of exosomes, which will have potential impacts on the studies of exosomes.

Key words atomic force microscopy, exosome, nanostructure, mechanics, force curve
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Exosomes play an important role in the life activities. All cells, including prokaryotes and eukaryotes, release extracellular vesicles (EVs) as part of their normal physiology and during acquired abnormalities[1]. Exosomes are EVs with a size range of ~40 to 160 nm (average ~100 nm) in diameter[1]. Exosomes have been shown to contain proteins, RNA transcripts, microRNAs and even DNA that can be transferred to other cells and thereby trigger a broad range of cellular activities and biological responses[2]. As such, exosomes are appreciated as essential mediators of cell-cell communication[3]. Studies have shown that exosomes are strongly involved in the metastasis of tumors[4]. Tumor-derived exosomes carry a pro-EMT(epithelial-mesenchymal transition)

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program including transforming growth factor beta (TGF-β), caveolin-1, hypoxia-inducible factor 1 alpha (HIF-1α), and β-catenin that enhances the invasive and migratory capabilities of recipient cells, and contributes to stromal remodeling and premetastatic niche formation[5]. For example, studies have shown that tumor-derived exosomes induce the activation of cancer-associated fibroblasts (CAFs) and the activated CAFs promote cancer progression by secreting pro-inflammatory cytokines[6]. Studies have also shown that exosomes help the metastatic circulating tumor cells escape from immunologic surveillance, survive in the blood circulation and proliferate in host organs[7]. Consequently, investigating the behaviors of exosomes significantly benefits understanding the underlying mechanisms guiding life processes and diseases.

The invention of atomic force microscopy (AFM) provides a novel powerful tool for the studies of exosomes. So far, knowledge about exosomes is commonly acquired by traditional biochemical assays, including mass spectroscopy, protein quantification, Western blot, immunoblot, fluorescence imaging analysis, electron microscopy, and so on[8-11], which require a lot of complex pretreatments on exosomes and the results obtained with these methods only reflect the biochemical properties of exosomes. With the use of AFM, exosomes without pretreatments can be directly probed and researchers have applied AFM to visualize the structures and measure the properties of individual exosomes[12-14]. Nevertheless, it should be noted that current studies about utilizing AFM to investigate exosomes are commonly performed in air, and air-drying could inevitably result in the structural and mechanical alterations of exosomes, causing that the results obtained in air cannot completely reflect the exosome behaviors in real situations and so far the detailed nanostructures and mechanics of living exosomes are still not fully understood. Here, in this work, based on poly-L-lysine electrostatic adsorption, we utilized AFM imaging and force spectroscopy in liquids to successfully image the nanostructures and characterize the mechanical properties of single living exosomes. The study improves our understanding of exosomes and offers a novel approach for investigating the structures and mechanics of single living exosomes at the nanoscale.

1 Materials and methods

1.1 Isolation of exosomes

Exosomes were isolated from the bone marrow of clinical lymphoma patients based on centrifugation. The bone marrow aspiration samples (I in Figure 1a) were prepared by the medical personnel from the Liaoning Cancer Hospital (Shenyang, China). The procedures of isolating exosomes from the bone marrow biopsies are following: (1) Add 1 ml bone marrow (previously treated by EDTA for anticoagulation) to a fresh centrifuge tube and centrifuge at 2 000 *g* for 20 min at room temperature (18℃); (2) After centrifugation, the supernatant was transferred to a new centrifuge tube without disturbing; (3) Centrifuge the new tube at 10 000 *g* for 20 min at room temperature; (4) After centrifugation, the supernatant was transferred to a new centrifuge tube and 0.4 ml phosphate buffered saline (PBS) (Hyclone Laboratories, Logan, UT, USA) was also added to the tube; (5) Add 0.24 ml exosome precipitation reagent (Life Technologies, Thermo Fisher Scientific Inc., Waltham, USA) to the centrifuge tube and incubate the sample at room temperature for 10 min; (6) After incubation, the sample was centrifuged at 10 000 *g* for 5 min at room temperature; (7) After centrifugation, the supernatant was removed and exosomes were contained in the pellet at the bottom of the tube (II in Figure 1a); (8) Add 0.4 ml PBS to the tube to resuspend the exosomes; (9) The obtained exosomes were stored at −20℃.

1.2 Sample preparation

Living exosomes were immobilized to glass slides based on poly-L-lysine electrostatic adsorption. Studies have shown that the surface (lipid bilayer) of exosomes is negatively charged[15-16], and thus we used positively charged macromolecules[17] to immobilize exosomes for living exosome probing (Figure 1b). Poly-L-lysine is a type of positively charged macromolecules and is commercially available and thus we used poly-L-lysine for the studies. The purchased poly-L-lysine solution (1 g/L) (Solarbio Life Sciences, Beijing, China) was diluted 10 times with pure water (Milli-Q, Merck KGaA Company, Darmstadt, Germany). The diluted poly-L-

lysine solution was dropped to the surface of fresh glass slides which were stored overnight for air-drying. Exosome solution was dropped to the poly-L-lysine-coated glass slides and incubated for 2 min for establishing firm electrostatic adsorption between exosomes and glass slides. The glass slides were then placed in petri dishes containing PBS and then the living exosomes immobilized on glass slides were probed by AFM in PBS.

1.3 Atomic force microscopy

AFM imaging and force spectroscopy experiments were performed with the use of a commercial AFM called Dimension Icon AFM (Bruker, Santa Barbara, CA, USA). The type of the AFM probe used for the studies is ScanAsyst-Fluid (Bruker, Santa Barbara, CA, USA). The AFM and probe used in this study are shown in Figure 1c. The material of the probe is silicon nitride. The nominal spring constant of the cantilever is 0.7 N/m and the nominal radius of the tip is 20 nm. There is a reflective gold layer on the back side of the cantilever. AFM experiments were performed in PBS at room temperature. For comparing the results obtained in PBS and the results obtained in air, AFM images of exosomes were also obtained in air. Under the guidance of AFM's optical microscope, AFM probe was moved to the different areas on the substrates for scanning the living exosomes attached on the substrates. Before performing AFM imaging, force curves were obtained on the bare areas of substrates to calibrate the deflection sensitivity of the cantilever, which was subsequently used for calculating the exact spring constant of the cantilever by using AFM's thermal noise module.

AFM peak force tapping (PFT) mode[18] was utilized to image living exosomes here. In the PFT mode, the vibrating AFM tip is controlled to perform approach-retract cycles in a pixel-by-pixel manner on the samples for obtaining force curves[19]. The driving frequency of the probe in PFT mode is much less than the resonant frequency of the cantilever while the driving frequency of the probe in conventional tapping mode is near the resonant frequency of the cantilever, and thus the tapping forces exerted by the probe in PFT mode are much less than the forces exerted by the probe in conventional tapping mode, causing that PFT mode is particularly suited for probing the fragile biological specimens and specimens which attach loosely to the substrates[20]. Besides, by analyzing the recorded force curves, different mechanical properties (such as elasticity, adhesion, deformation) of the samples can be visualized simultaneously with the topographic image of samples in PFT mode, facilitating understanding the structures and properties of specimens. The experimental parameters of AFM imaging of exosomes in PFT mode are following. The driving frequency of the probe was 2 kHz, the scan rate was 1 Hz, the number of scan line was 512 and the number of sampling points for each scan line was 512.

In order to quantitatively characterize the mechanical properties of exosomes, AFM indentation assays were performed on single exosomes in PBS. We know that the mechanics of specimens measured by AFM is significantly dependent on the experimental parameters, such as the approaching velocities of AFM probe[21-22]. Despite PFT imaging is able to qualitatively visualize the mechanics of specimens, it is challenging to exactly define the approaching velocities of the vibrating probe for the force curves recorded during PFT imaging, which potentially influences the standardization and comparability of the experimental results. Hence, we performed AFM indentation assays for quantitatively measuring the mechanics of exosomes here, in which the approaching velocities of AFM probe are known. After visualizing single exosomes by AFM imaging, AFM tip was controlled to move to the exosomes and then force curves were recorded on the exosomes. Fitting the approach curves with Sneddon-modified Hertz model yields the Young's modulus of exosomes[23]:

$$F = \frac{2E\delta^2 \tan\theta}{\pi(1-\upsilon^2)} \tag{1}$$

$$F = kx \tag{2}$$

where υ is the Poisson ratio of exosomes (υ=0.5 here), F is the loading force exerted by AFM probe, δ is the indentation depth, E is the Young's modulus of exosomes, θ is the half-opening angle of conical tip, k is the spring constant of the cantilever, and x is the deflection of cantilever.

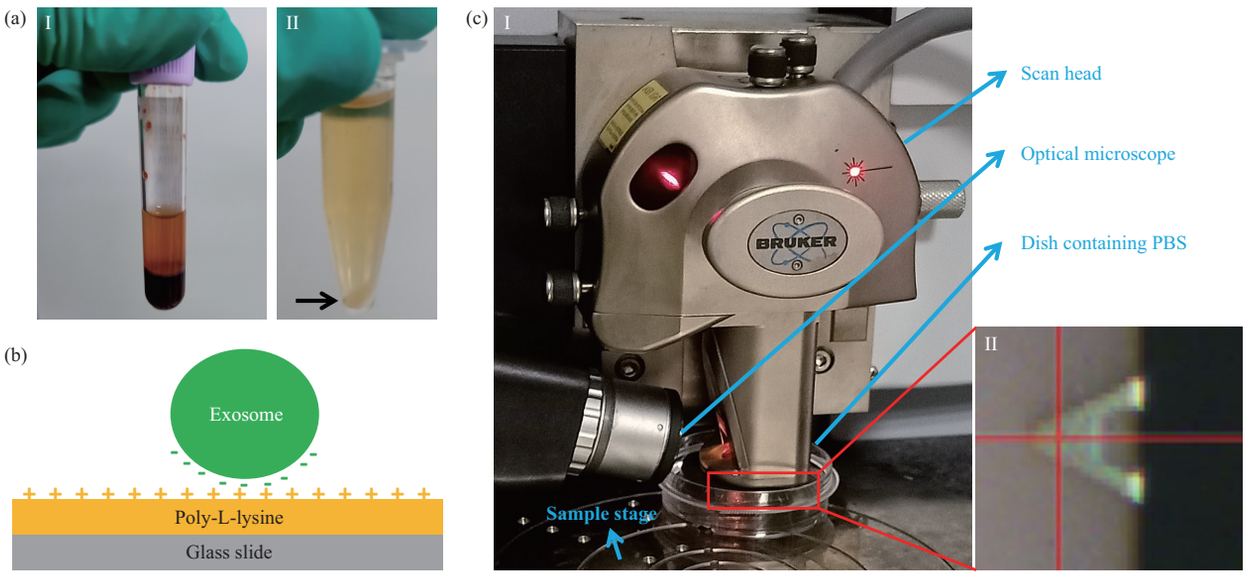

Fig. 1　Sample preparation and instrumental platform for probing living exosomes by AFM

(a) Exosomes isolated from the bone marrow of clinical lymphoma patients. I: Bone marrow sample. II: Exosomes isolated from the bone marrow sample by centrifugation method. Exosomes deposit at the bottom of the centrifuge tube, as denoted by the black arrow. (b) Schematic of immobilizing living exosomes onto the substrate *via* electrostatic adsorption. Negatively charged exosomes are attached to the surface of substrates coated by a layer of positively charged poly-L-lysine molecules. (c) Photographs of AFM (I) and probe (II) used here. Substrates with living exosomes are placed in a dish containing PBS and the dish is placed on the sample stage. AFM probe is immersed in the solution to probe the living exosomes immobilized on the substrates.

2　Results and discussion

AFM imaging in liquids at PFT mode visualized single living exosomes based on the immobilization of electrostatic adsorption. From the AFM images (Figure 2a, b), we can clearly see the round and balled living exosomes which are discretely distributed on the substrate. The sizes of living exosomes are quite heterogenous, including large exosomes (typically denoted by white arrows in Figure 2a, b) and smaller exosomes (typically denoted by green arrows in Figure 2a, b). Figure 2c is the AFM height image of a single living exosome and Figure 2d is the corresponding three-dimensional image, distinctly showing the detailed situations of the living exosome immobilized on the substrate. Section curves (Figure 2e) taken along the AFM height image show that the height of the exosome is about 35 nm. Notably, the diameter of living exosomes visualized from the section curves (Figure 2e) is much larger than the height of the exosomes. This may be related to the electrostatic adsorption between living exosomes and poly-L-lysine-coated substrates, which can cause the attachment and spread of living exosomes on the substrates. After analyzing the heights of fifty exosomes, the statistical results show that the height of the exosomes is about (31.76±7.74) nm. Researchers have widely used AFM to image the topography of exosomes[24-28], but these studies commonly required the air-drying treatment of exosomes and so far utilizing AFM to image living exosomes is still scarce. The prerequisite for imaging living exosomes by AFM is immobilizing exosomes onto the substrates. Here, with the use of poly-L-lysine electrostatic adsorption, the living exosomes isolated from the bone marrow of clinical lymphoma patients were attached to the substrates in aqueous solutions. In order to examine the effects of air-drying on AFM imaging of exosomes, we also obtained AFM images of exosomes in air (Figure 3). From the section curve (Figure 3c), we can clearly see that the height of exosomes (11 nm) visualized by AFM in air is much less than the height of exosomes (35 nm) visualized by AFM in aqueous conditions (Figure 2e), showing that air-drying can significantly cause the shrinking of exosomes. Besides, the advent of PFT imaging provides a novel powerful AFM imaging

mode for resolving the fine structures of fragile biological specimens, for example the microvilli on living cells[29]. Here, our experimental results (Figure 2, 3) demonstrate that combining poly-L-lysine electrostatic adsorption with PFT imaging mode allows high-quality AFM imaging of living exosomes, providing a novel idea to investigate living exosomes for better characterizing the behaviors of exosomes in their native states.

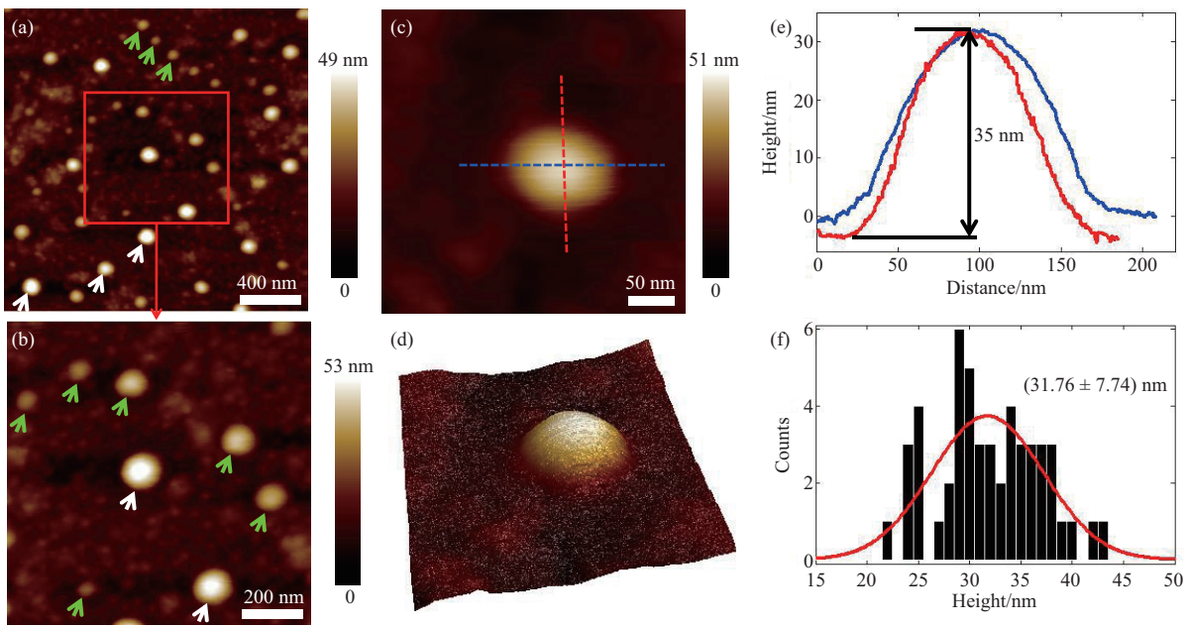

Fig. 2　AFM *in situ* imaging visualizing individual living exosomes immobilized on substrates

(a,b) Large-size scan (a) and small-size scan (b) (denoted by the red square area in (a)) AFM height images. Exosomes with different sizes are clearly distinguishable from the AFM images, as typically denoted by the white and green arrows. (c, d) AFM height image (c) and corresponding three-dimensional image (d) of a single exosome. AFM images were recorded in PBS. (e) Section profile curves taken along the red and blue dashed lines in (c) respectively. (f) Statistical histogram of the height of living exosomes (N=50).

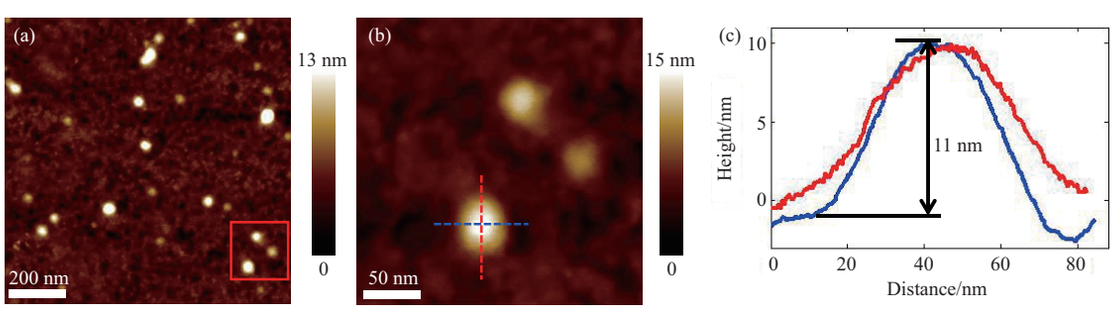

Fig. 3　AFM images of exosomes recorded in air

(a,b) Large-size scan (a) and small-size scan (b) (denoted by the red square area in (a)) AFM height images. (c) Section profile curves taken along the red and blue dashed lines in (b) respectively.

The mechanical properties of single living exosomes were measured by AFM. Biological systems are now broadly appreciated to be mechanical as well as biochemical systems[30]. As an important method of charactering the mechanics of biological specimens, AFM indentation technique has been widely used to reveal the mechanical cues involved in life activities[31], contributing much to the communities of cell biology[32]. Notably, so far the mechanics of exosomes remains poorly understood, and here we used AFM indentation to analyze the mechanical properties of exosomes. After locating

single exosomes by AFM imaging, AFM tip is moved to the exosome and perform indentation on the exosome in the vertical direction (Figure 4a). Force curves are recorded during the AFM indentation, which enables obtaining the Young's modulus of exosome (Figure 4b). The force curve is composed of two portions, including approach curve and retract curve (Figure 4c), which reflect the approach-retract movement of AFM tip during indentation. There are many theoretical models for extracting Young's modulus from force curves, and Hertz-Sneddon model is the most widely used one for practical reasons[33]. Hertz model is suited for spherical tip, while Sneddon model is suited for conical tip. The tip shape was conical in this study, and thus Sneddon model was used here. The approach curve was firstly converted to the indentation curve according to the contact point in the approach curve (Figure 4d). Subsequently, fitting the indentation curve with Sneddon model yields the Young's modulus of exosomes (Figure 4e). The statistical results measured on tens of exosomes show that the Young's modulus of living exosomes is about (9.67±0.95) MPa (Figure 4f). Notably, the theoretical fitting curve in Figure 4e is not fully consistent with the experimental data, particularly for the indentation range of 0−10 nm. During the indentation process, AFM tip successively touches different parts of exosomes[34], including lipid bilayers and various biological molecules inside exosomes. The mechanical properties of these different parts are diverse and fitting different sections of the indentation curve gives the mechanics of different parts of exosomes[35]. Hence, fitting the indentation curve only approximately gives the Young's modulus of the whole exosomes. Researchers have also used adhesive interactions between AFM tip and sample surface to measure the elastic modulus of soft samples[36], which can significantly eliminate the influence of substrate on the measurements. Besides, Hertz-Sneddon model is based on several assumptions, including homogeneity, isotropicity, linear elastic material properties, axisymmetry, infinitesimal deformation of the sample, infinite sample thickness and a smooth sample surface[37]. Despite these assumptions are not met for the case of exosomes, for example, the size of exosomes is comparable to that of AFM tip, Hertz-Sneddon fitting generally matches the experimental indentation curve and thus benefits obtaining the approximate Young's modulus of exosomes. In fact, researchers have used Hertz-Sneddon model to measure the Young's modulus of even smaller samples such as nanofibrils[38]. Hence, we used Hertz-Sneddon here to obtain the Young's modulus of exosomes.

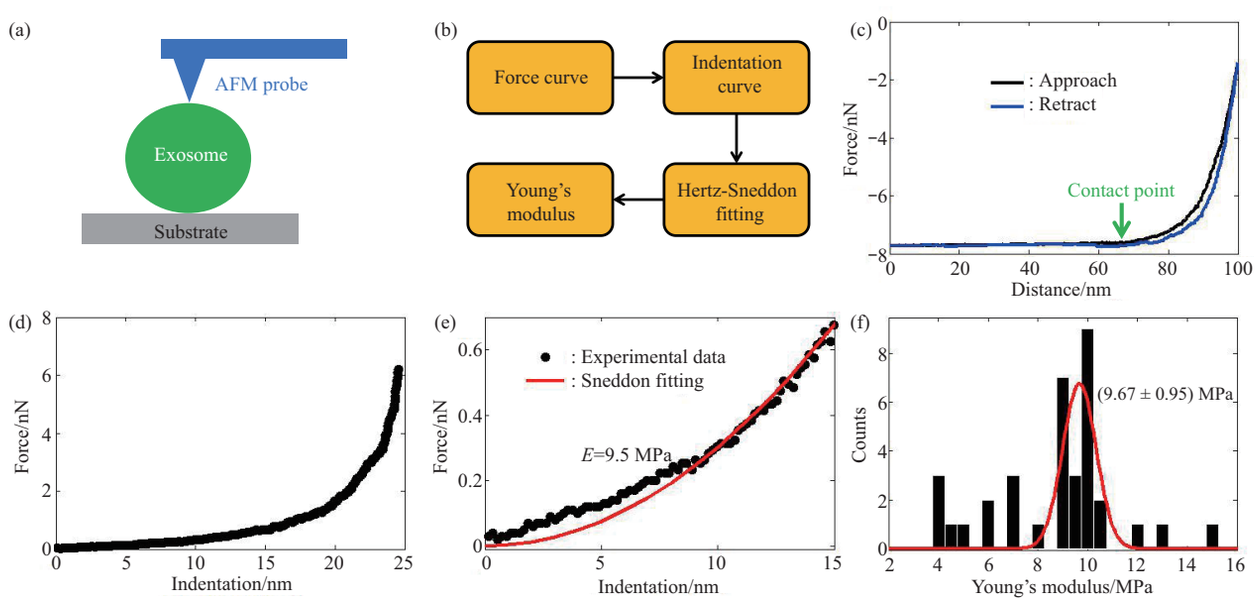

Fig. 4　Measuring the Young's modulus of single living exosomes by AFM indentation assays

(a) Schematic of AFM indenting on single exosome. AFM tip vertically performs approach-retract movement on the exosome. (b) Process of measuring the mechanics of exosomes by AFM indentation. (c) A typical force curve recorded on a living exosome. (d) The indentation curve converted from the approach curve in (c). (e) Fitting the indentation curve with Sneddon model yields the Young's modulus of living exosomes. (f) Statistical histogram of the Young's modulus of living exosomes (N=35).

Besides quantitatively measuring the mechanical properties of living exosomes, AFM can also qualitatively visualize the mechanics of exosomes at PFT multiparametric imaging mode (Figure 5). At the PFT multiparametric imaging mode, multiple types of mechanical images (*e. g.* deformation, adhesion, Young's modulus) of the specimen are generated simultaneously with the topography of the specimen. For more detailed descriptions of PFT multiparametric imaging mode, readers are referred to the references [19-20, 39-40]. Figure 5a shows the large-size PFT multiparametric imaging results of living exosomes. Besides topographical image (I in Figure 5a), exosomes are also distinguishable from the mechanical images (II-IV in Figure 5a). We can clearly see that on the whole exosomes have larger deformation (II in Figure 5a), smaller adhesion force (III in Figure 5a), and smaller Young's modulus (IV in Figure 5a) than the stiff substrates. On the whole large exosomes (typically denoted by red arrows in Figure 5a) exhibit larger deformation than smaller exosomes (typically denoted by green arrows in Figure 5a). Figure 5b shows the small-size PFT multiparametric imaging results of a single living exosome, presenting the detailed and heterogeneous mechanics across the exosome and visually establishing the correlation between exosome topography and exosome mechanics. The experimental results (Figure 4, 5) remarkably show the outstanding capabilities of AFM in characterizing the mechanics of individual living exosomes, which benefit understanding the structures and mechanics of exosomes and will have active impacts on the studies of exosomes.

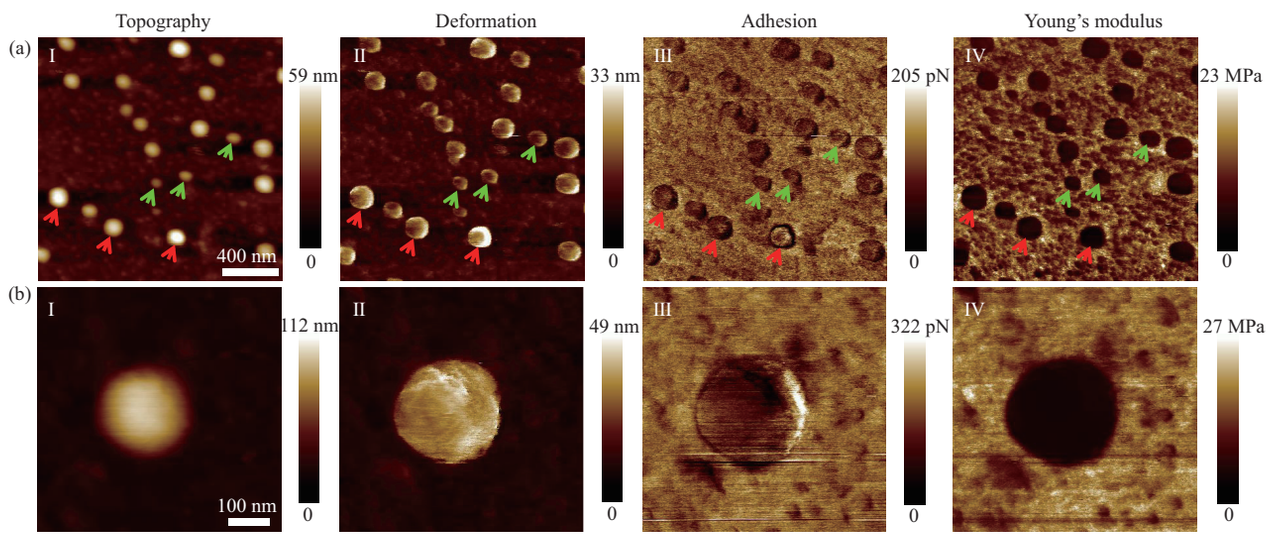

Fig. 5 AFM PFT multiparametric imaging of living exosomes visually correlating exosome mechanics with exosome topography

(a) Large-size scan of multiparametric imaging. (b) A single living exosome. Topography image (I) and corresponding deformation image (II), adhesion image (III), and Young's modulus image (IV) of exosomes. Red arrows typically denote the large exosomes and green arrows typically denote the smaller exosomes.

The structures and mechanics of exosomes after chemical treatments were revealed by AFM. After establishing the procedure of utilizing AFM to probe exosomes (Figure 2, 4, 5), we then observed the structural and mechanical changes of exosomes after chemical treatments. After attaching living exosomes onto the poly-L-lysine-coated substrates, 4% paraformaldehyde solution (Solarbio Life Sciences, Beijing, China) was added to chemically fix the exosomes for 30 min. After fixation, the exosomes were probed by AFM in PBS. Firstly, AFM morphological imaging of chemically fixed exosomes shows the significant changes of the structures of exosomes after chemical fixation (Figure 6a-d). We can see the significant structural changes of exosomes after fixation, for example, concave structures appeared on the exosome (Figure 6a, b) and the shape of exosomes became noncircular (Figure 6c, d). The

section curves taken along the exosomes clearly confirm the abnormal topography of the chemically fixed exosomes (II in Figure 6a-d). Subsequently, force curves were obtained on the fixed exosomes to measure the Young's modulus of fixed exosomes (Figure 6e). Compared with the stiffness of living exosomes (Figure 4e, f), we can see that exosomes significantly stiffened after the treatment chemical fixation (Figure 6f, g). AFM PFT multiparametric imaging was also utilized to visualize the mechanics of fixed exosomes, and the results are shown in Figure 7. We can see that compared with the PFT multiparametric imaging results of living exosomes (Figure 5), exosomes after chemical treatment are much indistinguishable from the mechanical images (II, III, IV in Figure 7). After fixation, exosomes significantly exhibit decreased deformation (II in Figure 7) and increased stiffness (IV in Figure 7), consistent with the results of AFM indentation measurements (Figure 6f, g). We know that engineering exosomes has been a promising approach for drug delivery[41], which requires diverse treatments and modifications of exosomes[42]. Hence, investigating the structural and mechanical dynamics of exosomes after external stimulus is of important significance for understanding the behaviors of exosomes. The experimental results (Figure 6, 7) here distinctly verify the capabilities of AFM in resolving the structural and mechanical alterations of exosomes after chemical treatment, which will potentially contribute to the studies of exosome dynamics.

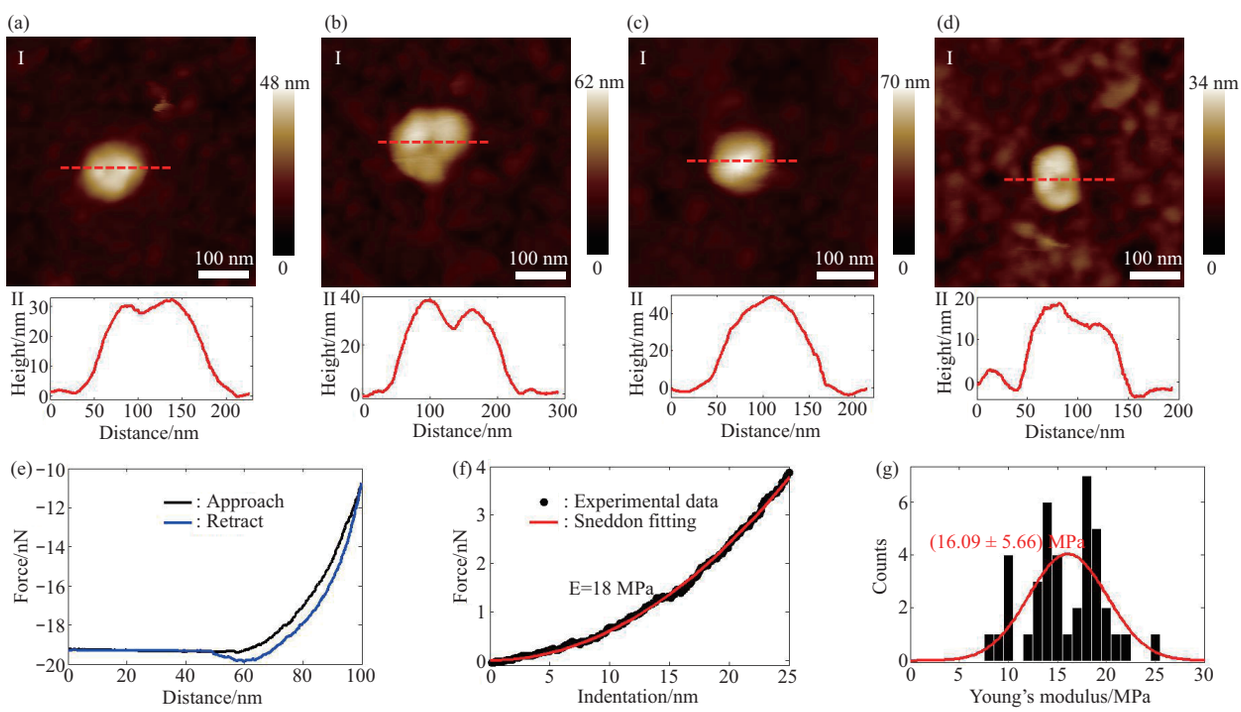

Fig. 6　AFM imaging and mechanical analysis of chemically fixed exosomes

(a−d) AFM height images of single fixed exosomes. AFM images were recorded in PBS. I: AFM images; II: Section profile curves taken along the red dashed lines. (e) A typical force curve obtained on fixed exosomes. (f) Sneddon model fitting of indentation curve for obtaining the Young's modulus of fixed exosomes. (g) Statistical histogram of the Young's modulus of fixed exosomes (N=40).

In summary, this study has demonstrated the excellent capabilities of AFM in investigating the structures and mechanics of individual living exosomes at the nanoscale. With the use of poly-L-lysine-based electrostatic adsorption, single living exosomes were attached to the substrate for successful AFM morphological imaging and mechanical analysis. Comparison between AFM images of living exosomes recorded in liquids and AFM images of exosomes obtained in air revealed the effects of air-drying on exosome morphology. Based on AFM indentation assays and PFT multiparametric imaging,

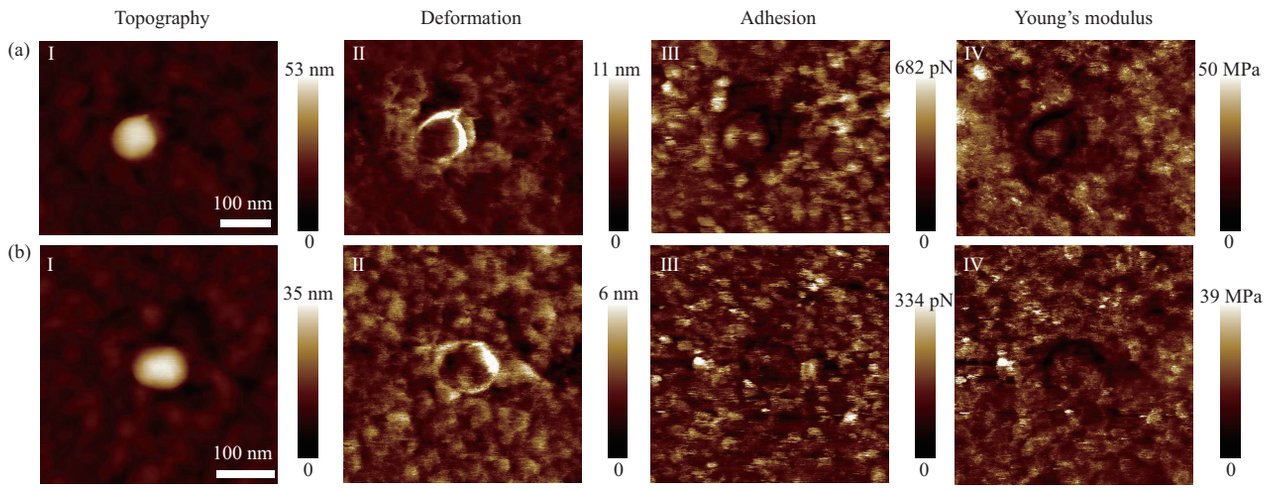

Fig. 7　AFM PFT multiparametric imaging of chemically fixed exosomes visualizing the changes of exosome mechanics after chemical fixation

Results of two fixed exosomes are shown in (a, b) respectively. Topography image (I) and corresponding deformation image (II), adhesion image (III), and Young's modulus image (IV) of fixed exosomes.

the mechanics of single living exosomes was quantitatively measured and qualitatively visualized. The structural and mechanical dynamics of living exosomes after chemical treatments were revealed, significantly showing the irregular topography and increased stiffness of exosomes after chemical fixation. The methods established here (*e.g.* exosome preparation and immobilization, AFM imaging, indentation, and multiparametric imaging) can be directly applied to other types of exosomes, which will be particularly useful for understanding the behaviors of exosomes in their native states.

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基于AFM的活体状态外泌体纳米结构及机械特性研究*

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摘要　外泌体在细胞生理病理活动过程中起着重要的调控作用，研究外泌体的行为特性对于揭示生命活动及疾病发生发展的内在机理具有重要的基础意义. 然而由于缺乏合适的观测手段及方法，目前对于活体状态下外泌体结构及特性的认知仍然很不足. 原子力显微镜（AFM）的发明为研究溶液环境下天然状态生物样本提供了强大的技术工具，已成为生物学重要研究手段. 本文利用AFM对单个活体状态外泌体的纳米结构及机械特性进行了研究. 通过多聚赖氨酸静电吸附作用将从淋巴瘤患者骨髓中分离的外泌体吸附至基底，在溶液环境下实现了对单个活体状态外泌体的高质量AFM形貌成像并通过与空气中成像结果进行对比揭示了空气干燥处理对外泌体形貌的影响. 在此基础上，分别利用AFM压痕试验和多参数成像技术实现了对单个活体状态外泌体机械特性的定量测量和可视化表征. 最后基于所建立的方法技术揭示了化学处理后外泌体结构和机械特性的动态变化. 研究结果为研究纳米尺度下活体状态外泌体的结构及特性，以更好理解天然状态外泌体的生理行为提供了新的方法和思路，对于外泌体研究具有潜在积极的意义.

关键词　原子力显微镜，外泌体，纳米结构，机械特性，力曲线
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