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Adhesion force of *staphylococcus aureus* on various biomaterial surfaces

Fahad Alam, Kantesh Balani*

Biomaterials Processing and Characterization Laboratory, Materials Science and Engineering, Indian Institute of Technology Kanpur, UP, India

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ABSTRACT

Staphylococcus comprises of more than half of all pathogens in orthopedic implant infections and they can cause major bone infection which can result in destruction of joint and bone. In the current study, adhesion force of bacteria on the surface of various biomaterial surfaces is measured using atomic force microscope (AFM). *Staphylococcus aureus* was immobilized on an AFM tipless cantilever as a force probe to measure the adhesion force between bacteria and biomaterials (viz. ultra-high molecular weight poly ethylene (UHMWPE), stainless steel (SS), Ti–6Al–4V alloy, hydroxyapatite (HA)). At the contact time of 10 s, UHMWPE shows weak adhesion force (~4 nN) whereas SS showed strong adhesion force (~15 nN) due to their surface energy and surface roughness. Bacterial retention and viability experiment (3M™ petrifilm test, agar plate) dictates that hydroxyapatite shows the lowest vaibility of bacteria, whereas lowest bacterial retention is observed on UHMWPE surface. Similar results were obtained from live/dead staining test, where HA shows 65% viability, whereas on UHMWPE, SS and Ti–6Al–4V, the bacterial viability is 78%, 94% and 97%, respectively. Lower adhesion forces, constrained pull-off distance (of bacterial) and high antibacterial resistance of bioactive-HA makes it a potential biomaterial for bone-replacement arthroplasty.

1. Introduction

It is known that *Staphylococcus aureus (S. aureus)* biofilm plays a significant role in biomaterial associated infection and can cause the failure of implants (Bisno, 2000; Lamagni et al., 2015; Zimmerli et al., 2004). Establishment of infection starts from initial adhesion and leads to biofilm formation (Bos et al., 1999; Drake et al., 1999; Gottenbos et al., 2000). A biofilm consists of sessile communities of bacteria that are protected by a self-produced, hydrated polysaccharide containing matrix (Busscher and van der Mei, 2012; Nejadnik et al., 2008b). The biofilm leads to a major physico-chemical hurdle for anti-microbial therapies due to the reduced diffusion of antibiotics into the polymeric matrix (Cochis et al., 2015; Dastgheyb et al., 2014; Wu et al., 2013). Gradual changes in the microenvironment (e.g. oxygen, nutrients, pH) within the biofilm matrix and the existence of persisted bacterial cells further complicate the treatment of biofilm (Anwar and Costerton, 1992; Parra-Ruiz et al., 2012). Microbial cell development (Wang et al., 2009), metabolic activity (Ribeiro et al., 2012), viability (Terada et al., 2006) and biofilm formation (Nickzad and Déziel, 2014) on the surface of biomaterials are strongly dependent on the bacterial adhesion (An and Friedman, 1998). Therefore, it is critically important to study the bacterial adhesion on different available biomaterials' surfaces.

Till date there are different techniques in use for the measurement of the adhesion strength: such as plate-and-wash assay (Klebe et al., 1977), spinning–disc assay (García et al., 1997), centrifugation assay (Reyes and García, 2003), step pressure technique (Salánki et al., 2014), flow chamber (Nejadnik et al., 2008a), optical tweezers (Zhang and Liu, 2008), atomic force microscopy (AFM) (Gilbert et al., 2007) and scratch based technique i.e. nanoindentation (Lahiri et al., 2011). Out of these techniques, most of them are non-quantitative and report only approximation of the bacterial adhesion strength to a surface, whereas AFM is one of the most effective and quantitative techniques applicable in wide range of force quantification right from molecular interaction of cell ligands to the cellular adhesion (Engel et al., 1999; Müller and Dufrêne, 2011; Thewes et al., 2014; Toyoda et al., 2011).

In the current study, different class of widely used biomaterials {viz. ultra-high molecular weight poly ethylene (UHMWPE), stainless steel (SS), Ti–6Al–4V and hydroxyapatite (HA)} (Geetha et al., 2009; Hryniewicz et al., 2009; Mucalo, 2015; Musib, 2011) are selected and adhesion force of *S. aureus* was measured. Further, viability test of *S. aureus* was carried out on these samples by using 3M™ petri film test (Neut et al., 2011), tryptone soya broth (TSB) agar plates and live/dead staining tests. AFM study (Younes et al., 2012) provides adhesion in a very short duration (0, 5 and 10 s), 3M™ and TSB test elucidates the bacterial viability whereas live/dead staining quantifies the number of adhering bacteria along with the viability. The whole study will help to select the implant material so that bacterial associated infection can be reduced.

* Corresponding author.
E-mail address: kbalani@iitk.ac.in (K. Balani).

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2. Materials and methods

2.1. Materials

Medical grade UHMWPE powder (GUR™ 1020) with a density of 0.93 g/cm^3, molecular weight 2.7×10^6 g/mol was supplied by Ticona GmbH (WerkRuhrchemie) Germany with particle size of 10–300 µm. The sheets were processed using compression molding (SCM-30 M/s Santec Automation, Delhi) at a pressure of 7.5 MPa and 200 °C with a dwell time of 1 h, followed by cooling of the die to room temperature in air. Commercially available SS 316L and Ti–6Al–4V were cleaned by ultra-sonication in the presence of ethanol. HA powders were processed by wet chemical method (Mucalo, 2015) and the pellets were prepared by conventional sintering (1100 °C for 3 h with a heating and cooling rate of 5 °C/ min) in air. All the prepared samples were autoclaved to sterilize them before testing.

2.2. Surface energy and surface roughness measurement

Contact angle (CA) of all samples with water were measured by sessile drop method in static mode using contact angle goniometer (Dataphysics Contact Angle System OCA). A single drop of water was placed on the surface and the angle made between the drop and the material on the either side was captured using the imaging software. The tangent method was employed to measure the contact angle. Average CA (repeated nine times) for each sample is reported after performing measurements three times on three different coupons of the sample. The surface free energy of the samples was calculated by performing sessile drop (nine times with each liquid) using one polar (water) and three non-polar (chloroform, xylene, and toluene) liquids and then the polar and dispersion components were calculated using Owens–Wendt–Rabel–Kaelble geometric mean equations, whose detail is provided in supplementary information (**SI**) equations 1–5 (Bos et al., 1999; Yuan and Lee, 2013).

AFM was used to observe the microstructure of the selected biomaterials using "V" shaped AFM cantilever (Bruker, NP-10, Camarillo, CA, USA). Micrographs were taken at three randomly selected spots of each surface. The surface roughness was analyzed by Nanoscope Analysis (Bruker, version 1.40) software. Three readings with a scan area of 50 µm× 50 µm on each sample were taken to obtain an average roughness (Ra) of the sample.

2.3. Bacterial strain and culture condition

S. aureus strain (ATCC25923) was grown in 10 ml tryptone soya broth (TSB, Oxoid, Basingstoke, UK) overnight and transferred in fresh 200 ml TSB and incubated overnight at 37 °C. The bacteria were harvested by centrifugation for 5 min at 5000 g (five thousand times the acceleration due to gravity) at 10 °C, washed thrice with phosphate buffered saline (PBS: 150 mM NaCl. 10 mM potassium phosphate; pH 7.0) and suspended in 10 ml of the same buffer. Number of bacteria was counted, using a Bürker-Türk counting chamber, and their number density was maintained at 10^6 bacteria/ml (for preparing AFM probe) towards use in adhesion-force measurements.

2.4. Preparation of bacterial AFM probe for adhesion force measurement

A micromanipulator (Narishige International, Tokyo, Japan) was used to attach bacteria to a "V" shaped tipless AFM cantilever (Bruker, NP-O10, Camarillo, CA, USA) under a microscope (Leica, DMIL, Wetzlar, Germany) (Younes et al., 2012). On a glass slide, a very small droplet (30 µl) of poly-L-lysine (PLL) solution was placed and the pointed edge of the cantilever was dipped in the PLL droplet for 2 min. After 2 min in PLL, the cantilever was air dried for 2 min, then a very small droplet of bacterial suspension was placed on the glass slide and the PLL coated cantilever was dipped for 2 min in the bacterial suspension. To avoid any contamination and drying of the attached bacteria the AFM probe was kept in a box with wet paper till it was fitted in AFM. Fig. 1 explains the working protocol of the AFM setup that was used to measure the adhesion forces. The description of attachment of bacteria to the tipless AFM cantilever (to prepare AFM probe) is described in Fig. 1a**.** The fluorescent image the of bacteria attached to a cantilever with live/dead staining is shown (Fig. 1a**. (iii)**) after obtaining force-distance curve (Fig. 1b).

The prepared bacterial AFM probe was used immediately for adhesion force measurement. All AFM measurements were carried out at room temperature in sterile PBS (pH 6.0) using an optical lever microscope (Nanoscope V, Digital Instruments, Woodbury, NY, USA) with z-scan rates of 1.0 Hz under a maximal loading force of 5 nN. All cantilevers were calibrated using AFM Tune-it Version 2 software to get the spring constant of the cantilever. Force–Distance curves were measured at different contact time (0, 5 and 10 s). All force curves were analyzed using Nanoscope Analysis (Bruker, version 1.40) software. For every test a freshly prepared probe was used, and on each sample, force-distance curves were recorded thrice at three random spots and averaged. Fig. 1b presents a typical interaction of the AFM tip with attached bacteria and corresponding force-distance curve.

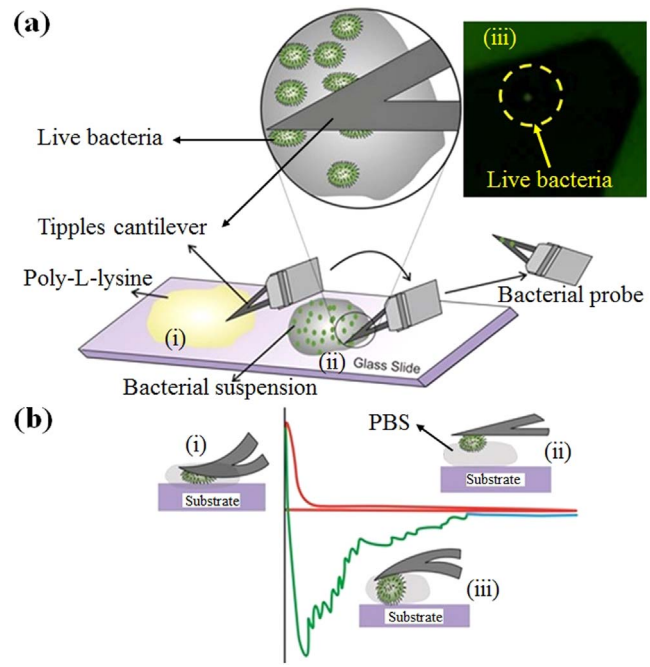

Fig. 1. (a) Preparation of AFM probe; (i) add glue to the AFM cantilever by dipping into PLL, (ii) attaching bacteria to the PLL coated AFM cantilever by dipping into the bacterial suspension, (iii) Fluorescent imaging of the AFM probe after performing test shows to confirm if the cell is still alive or has become dead, (b) typical AFM force-distance curve, (i) Approaching AFM probe towards the sample, (ii) AFM probe is in contact with surface of the sample and (ii) retracting the AFM probe after providing a controlled dwell time (0–10 s).

Table 1
Water contact angle surface energy, polar and dispersion component of all the samples.

Sample	Water contact angle (degrees)*	Surface energy (mJ/m^2)*	Polar part (mJ/m^2)	Dispersion part (mJ/m^2)
UHMWPE	81.9 ± 2.3	29.86 ± 0.50	3.06	26.80
SS	48.7 ± 1.9	32.43 ± 0.58	13.21	19.22
Ti–6Al–4V	68.8 ± 5.6	30.13 ± 0.39	8.56	21.56
HA	94.9 ± 1.2	26.15 ± 0.68	1.03	25.12

* Confidence level > 95% ($p < 0.05$).

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