Ultramicroscopy 137 (2014) 30–39

Contents lists available at ScienceDirect

Ultramicroscopy

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

Immobilizing live *Escherichia coli* for AFM studies of surface dynamics

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ARTICLE INFO

Article history:
Received 9 April 2013
Received in revised form
23 October 2013
Accepted 25 October 2013
Available online 12 November 2013

Keywords:
Atomic Force Microscopy (AFM)
Live cell imaging
Bacteria
Immobilization
PLL
Outer membrane vesicle (OMV)

ABSTRACT

Atomic force microscopy (AFM) is a probe-based technique that permits high resolution imaging of live bacterial cells. However, stably immobilizing cells to withstand the probe-based lateral forces remains an obstacle in AFM mediated studies, especially those of live, rod shaped bacteria in nutrient media. Consequently, AFM has been under-utilized in the research of bacterial surface dynamics. The aim of the current study was to immobilize a less adherent *Escherichia coli* strain in a method that both facilitates AFM imaging in nutrient broth and preserves overall cell viability. Immobilization reagents and buffers were systematically evaluated and the cell membrane integrity was monitored in all sample preparations. As expected, the biocompatible gelatin coated surfaces facilitated stable cell attachment in lower ionic strength buffers, yet poorly immobilized cells in higher ionic strength buffers. In comparison, poly-L-lysine surfaces bound cells in both low and high ionic strength buffers. The benefit of the poly-L-lysine binding capacity was offset by the compromised membrane integrity exhibited by cells on poly-L-lysine surfaces. However, the addition of divalent cations and glucose to the immobilization buffer was found to mitigate this unfavorable effect. Ultimately, immobilization of *E. coli* cells on poly-L-lysine surfaces in a lower ionic strength buffer supplemented with Mg^{2+} and Ca^{2+} was determined to provide optimal cell attachment without compromising the overall cell viability. Cells immobilized in this method were stably imaged in media through multiple division cycles. Furthermore, permeability assays indicated that *E. coli* cells recover from the hypoosmotic stress caused by immobilization in low ionic strength buffers. Taken together, this data suggests that stable immobilization of viable cells on poly-L-lysine surfaces can be accomplished in lower ionic strength buffers that are supplemented with divalent cations for membrane stabilization while minimizing binding interference. The data also indicates that monitoring cell viability as a function of sample preparation is important and should be an integral part of the work flow for determining immobilization parameters. A method for immobilizing a less adherent *E. coli* mutant for AFM imaging in nutrient broth is presented here in addition to a proposed work flow for developing and optimizing immobilization strategies.

1. Introduction

As a scanning probe technique, AFM is ideally suited for investigating the surface properties of bacteria, including topography, composition, and adhesion [1]. The capacity of AFM to capture high resolution images, a capability once exclusive to electron microscopy, facilitates quality imaging of nanoscale structures. Importantly, in contrast to electron microscopy, AFM sample preparation does not require fixation and dehydration, which allows for imaging of live cells in a native state. Despite these advantages, AFM has been slow to become a routine technique for researching membrane dynamics. This is in part due to the significant obstacle of immobilizing cells for imaging in physiological conditions.

For successful AFM imaging, cells must be firmly adhered to a substrate in order to prevent displacement by lateral forces exerted by the AFM probe. Since the bacterial surface is the binding interface, and given the inherent differences in bacterial surface properties, the methodology of sample preparation must be determined for each specimen. This process includes selecting the form of immobilization (i.e. physical vs. chemical) [2], and in live cell imaging, identifying physiologically compatible immobilization and imaging buffers [3]. Both aspects must be carefully chosen based on the experimental goals and the data to be collected. Specifically, the strategy of immobilization must not alter the cell properties being studied, and the cells must be oriented in such a fashion that interactions between the probe and the region of interest are unimpeded. The development of immobilization methods, and subsequent improvement of those methods, has continued over the last 20 years for which there is a

Abbreviations: AFM, Atomic Force Microscopy; OMVs, outer membrane vesicles; PLL, α-Poly-L-lysine; LB, Luria Bertani; MM, minimal media; MM+, minimal media with 0.05% protein hydrolysate amicase; PBS-S, supplemented PBS; LPS, lipopolysaccharide

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http://dx.doi.org/10.1016/j.ultramic.2013.10.017

correlating increase in the application of AFM in microbial surface studies [4–6]. Immobilization methods vary, and include physical immobilizations, such as filter or molten agar entrapment, or chemical immobilizations, using glutaraldehyde cross-linking, gelatin, or poly-L-lysine [7–11]. In addition, a recent chemical immobilization study by Meyer et al. using Cell-Tak™ coated surfaces demonstrated successful imaging of Gram negative and Gram positive bacteria in nutrient media [12]. Each method has both advantages and disadvantages, and can be thusly chosen based on suitability, convenience, and expense [13]. For example, the tools of physical entrapment are often inert but may result in both unpredictable obstructions of the cell surface and the exertion of non-native forces on the cell, which may hinder spatial and elasticity investigations, respectively. In contrast, chemical immobilization influences only the binding interface of the cell, yet chemical reagents may have adverse effects on cell physiology. Regardless of method type, the evolution of immobilization protocols has not completely addressed a major aspect of sample preparation for live cell imaging, which is the necessity to monitor cell viability within immobilizing parameters. An immobilization strategy must be optimized to balance the efficiency and stability of cell entrapment/attachment with adequate cell viability. The aim of this study was to outline a systematic approach to optimally immobilize live bacterial cells for AFM imaging in nutrient media.

Developing a strategy to stably image actively growing bacteria is the first step towards investigating membrane dynamics, particularly outer membrane vesicle (OMV) production, in real time with AFM. To date, research findings on the spatial and temporal production of OMVs are based on electron microscopy and biochemical analyses. AFM mediated investigations would allow visualization of real-time vesiculation events, and consequently, permit both spatial and temporal data collection under native conditions. Since physical entrapment methods often result in partially obstructed surfaces, and because the most commonly used method, filter entrapment, is not applicable to rod shaped bacteria, chemical immobilization methods were explored. Furthermore, chemical immobilization maximizes the cell surface area that is accessible to the AFM probe, thereby allowing full exploration of the spatial pattern of OMV production along the cell axes. Electrostatic mediated binding was explored with three gelatins of varying bloom strengths and α-poly-L-lysine (PLL) chosen as immobilization reagents. Non-covalent attachment was used to achieve a binding equilibrium (cycles of release and re-binding) that would accommodate the cellular dynamics expected to occur in nutrient media, including both changes in overall cell dimension and at localized regions of the outer membrane.

Both gelatin and PLL coated surfaces are widely used in microbial and mammalian cell culture, readily available, inexpensive, and easy to prepare. Gelatin facilitated immobilization has been well established as an effective means to stably image Gram-negative and Gram-positive bacteria in aqueous conditions [10]. Furthermore, gelatin is a naturally derived, non-cytotoxic molecule that is routinely used in microbial diagnostic assays. Alternatively, PLL has been shown to have antimicrobial properties, which have been characterized for both α-PLL and ϵ-PLL with ϵ-PLL having the most severe effect [14–16]. Regardless, PLL has been used as an immobilizing reagent in recent studies for imaging bacteria in aqueous conditions, including growth media [11,17–22].

The outer membrane is the binding interface of Gram-negative bacteria. Consequently, changes in the outer membrane composition can affect cell adhesion. For example, *E. coli nlpI* (outer membrane lipoprotein) mutants have demonstrated a lower incidence of adherence to host cells [23,24]. In this study, an *E. coli nlpI* mutant, previously characterized as a hypervesiculating strain, was used to develop an immobilization strategy that is likely applicable to other Gram-negative bacteria [25]. The immobilization of both mutant and wild type cells on gelatin or PLL substrates was assessed in various buffers. Most importantly, the membrane integrities of immobilized cells were monitored throughout the sample preparation to evaluate cell viability.

2. Materials and methods

2.1. Bacterial strains and reagents

E. coli DH5α wild type strain 221 and *nlpI* mutant strain MK8A44 were kindly provided by Dr. Meta Kuehn, Duke University Medical Center. Mutant and wild type strains were grown in Luria Bertani (LB) broth with or without 50 μg/ml kanamycin, respectively, at 37 °C.

2.2. Immobilization and permeability assays

Glass slides were coated with 0.5% high (G2500, Sigma-Aldrich), medium (G2625, Sigma-Aldrich), or low (G6144, Sigma-Aldrich) bloom gelatin solutions and allowed to dry on end at room temperature, as previously described by Doktycz et al. [10]. Once dried, a 0.75 in. square area was demarcated on the back of each gelatin coated slide. PLL slides were prepared by spreading 20 μl of PLL (P4707, Sigma-Aldrich) within a 0.75 in. square area on each glass slide and allowed to dry at room temperature.

Cultures of 221 and MK8A44 were inoculated from overnight cultures and allowed to grow 2.5 h at 37 °C to reach log phase (MK8A44 OD_{600} 0.015–0.03; 221 OD_{600} 0.03–0.06). Cells were collected at 9391*g* for 2 min at room temperature, washed in 0.01 × PBS, 0.1 × PBS, 1 × PBS, minimal media (MM) (1 × M9 Solution (M6030; Sigma-Aldrich), 20 mM glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$), or MM supplemented with 0.05% protein hydrolysate amicase (82514, Fluka) (MM+), and centrifuged at the same settings. Cell pellets were resuspended in the respective immobilization buffers and a 100 μl of each suspension was applied to the demarcated regions on each coated slide. Cells were allowed to immobilize on the surfaces in a humid chamber for 30 min at room temperature. The unbound cells were removed by copiously rinsing with the respective immobilization buffers. For immobilization assays, MK8A44 samples were imaged on an Olympus IX81 inverted light microscope. Immobilized cells within five random fields were counted using MetaMorph software (Molecular Devices, Sunnyvale, CA). In permeability assays, immobilized MK8A44 and 221 cells were treated with 30 μM propidium iodide (PI) and 5 μM Syto9 fluorescent, nucleic acid probes (Live/Dead-®BacLight™, L7012, Invitrogen) in the respective immobilization buffers. The slides were rinsed with equal volumes of the respective immobilization buffers and fluorescently imaged on the Olympus IX81. The numbers of green and red fluorescent cells were tallied within 3–5 random fields. Cells fluorescing green with Syto9 were considered to have normal permeability, whereas cells fluorescing red with PI at any percentage were interpreted to have abnormal permeability and reduced membrane integrity.

2.3. Protection and rescue assays

Log growth cultures of MK8A44 and 221, as described above, were washed and resuspended in 0.01 × PBS supplemented with 20 mM glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ (PBS-S) or 0.01 × PBS alone, applied to PLL surfaces, and allowed to immobilize for 30 min at room temperature. Following incubation, surfaces were rinsed with the respective immobilization buffer and cells were either recovered in MM or incubated in fresh immobilization buffer for 30 min. Permeability assays were performed on each sample to evaluate the level of protection provided by the addition