Microbicidal male contraceptive—Risug induced morphostructural damage in *E. coli*  

Shivani Sharma⁵, P. Sen⁶, S.N. Mukhopadhyay⁷, S.K. Guha⁸,⁎

⁵ Centre for Biomedical Engineering, Indian Institute of Technology, New Delhi 110 016, India  
⁶ School of Physical Sciences, Jawaharlal Nehru University, New Delhi 110 067, India  
⁷ Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, New Delhi 110 016, India  
⁸ School of Medical Science and Technology, Indian Institute of Technology, Kharagpur 721 302, India

Received 12 December 2002; accepted 5 May 2003

Abstract

Risug, a new polymeric drug with antimicrobial properties has been shown to alter the cell morphology and structure of *Escherichia coli*. Topographic images, traces of surface topography and analysis of surface roughness were performed, using tapping mode atomic force microscopy in air, for Risug treated as well as untreated *E. coli* cells. Surface roughness was quantified in terms of root mean square average of the height deviation. The treated cells indicate adhesion of Risug particles to the cell surface, particularly at the terminal ends. Damaged cells indicate flattening of the middle region and bulging at the apical ends. Marked alteration in surface roughness and cell fusion at some regions was observed.

Keywords: Risug; AFM; *E. coli*; Cell morphology; Antibacterial action

1. Introduction

There have been a number of studies on bacterial cell structures, investigating morphological alterations after exposure to various chemicals such as antibiotics, antimicrobial peptides and polyether agents. Since there are variations in the mechanism of action of such antimicrobials, a variety of morphological alterations and types of damage can occur in bacterial cells.

β-Lactam antibiotics, at both supra and sub-MICs can induce morphostructural alterations in Gram-negative bacteria resulting in the formation of bulges, filamentation and spheroplasts [1]. When exposed to antibiotics, Gram positive bacteria can enlarge in size, change their shape and undergo cluster formation [2]. Filamentation in bacterial cells has been shown to be induced by nalidixic acid as revealed by electron microscopy [3]. Bicyclomycin exposure leads to high undulation and blebs of the outer membrane leading to cell lysis in *Escherichia coli* [4]. While Indolicidin causes bacterial filamentation without lysing bacteria. Antimicrobial peptides such as cecropins,
magnamins, seminalplasmins and defensins have been reported to form ion channels and aqueous pores in outer and cytoplasmic membranes leading to cell lysis [5].

A detailed knowledge of the effects of these agents on bacterial cell structure is important in the evaluation of novel molecules as antibacterial agents. However, so far the studies relating to the cellular morphology of bacteria and their subsequent disruption of integrity as a result of certain chemicals have been performed using optical or scanning electron microscopes [6].

Recently, a new family of instruments called scanning probe microscopes (SPMs) has been introduced to study biological specimens. Based on the concept of ‘near field microscopy’, SPMs overcome the problem of the limited diffraction-related resolution inherent in conventional microscopes, because the probe, located in the immediate vicinity of the sample itself (usually within a few nanometers), can be scanned with extreme precision, providing the topography of the sample surface with unprecedented lateral resolution [6].

Atomic force microscope (AFM) is one such SPM and is an ideal tool for determining changes in cellular morphology [7]. AFM imaging can be performed in contact or tapping mode. In contact mode, the tip is dragged across the sample surface and maps of surface topography can be constructed by monitoring tip deflection. The tip–sample atoms react through repulsive interatomic forces.

In tapping mode, the tip does not make contact with the sample as the tip is oscillated near its resonance frequency in the attractive region of the tip–sample force distance curve. Thus the advantage in tapping mode is the decreased possibility of sample damage by the tip and marked reduction in lateral forces.

A number of cells have been examined in tapping mode. Grantham and Dove [8] used tapping mode for imaging bacterial cells in air. Butt et al. [9] imaged Halobacterium halobium in air and were able to resolve features as small as 10 nm, although they did not explicitly identify these features. Gunning et al. [10] examined Pseudomonas putida biofilms in air to determine their morphology. Kasas et al. [11] studied the morphology of E. coli and Bacillus subtilis in air after cells were exposed to penicillin. Johansen and Grill [12] examined the effect of protamine on air dried bacteria to determine how this chemical affected the cell morphology. Braga and Ricci [13] studied morphological alterations of living bacteria exposed to the β-lactam antibiotic cefodizime. Shetty et al. [14] studied the effect of superoxidised water, Sterilox on E. coli cells and on sulphate-reducing bacterium Desulphovibrio indonesiensis. Sokolov et al. [15] imaged Lactobacillus helveticus cells, prior and after exposure to LiCl. Umeda et al. [16] reported differences between Gram-negative and Gram-positive bacteria related to their specific surface structures.

Risug (an acronym for reversible inhibition of sperm under guidance) is an injectable intravasal contraceptive for use by a male possessing antimicrobial properties, currently undergoing phase III clinical trial. The new polymeric drug consists of a specific combination of copolymer of styrene maleic anhydride (SMA) and dimethylsulfoxide (DMSO) [17]. The polyelectrolytic nature of the polymer generates charge disturbances in the acrosomal membrane of the spermatozoon resulting in membrane damage and leakage of acrosin and hyaluronidase from the spermatozoon rendering it incapable of fertilization [18].

The aim of the present study, is to perform a higher resolution investigation on the surface and morphological alterations induced in E. coli cells after exposure to this new antimicrobial polymeric drug molecule ‘Risug’. It is expected that employing scanning electron microscopy (SEM), transmission electron microscopy (TEM) and AFM with the attendant resolution of the latter for biological specimens would help to elucidate the possible mechanism of action of this drug against bacterial cells.

2. Experimental methods

2.1. Preparation of bacteria

The most famous and extensively studied rod-shaped Gram-negative bacterium E. coli was chosen for the experiments. E. coli B37, obtained
from the Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology New Delhi, was grown from overnight cultures at 37 °C with mild shaking at 120 rpm in Luria broth medium (tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹, NaCl 5 g l⁻¹) to mid exponential growth phase (optical density at 600 nm = 0.2). Cells were harvested by centrifugation at 8000 rpm for 8–10 min. After washing, the pellet was incubated with 100 µg ml⁻¹ of Risug in phosphate-buffer saline (NaH₂PO₄·H₂O 4.25 g l⁻¹, Na₂HPO₄·2H₂O 3.45 g l⁻¹ pH 7.2) for 4–5 h at 37 °C. Cells were washed thrice with PBS and used for microscopy. The cells unexposed to Risug served as control.

2.2. Preparation of Risug

The new polymeric injectable male contraceptive possessing antimicrobial properties Risug, consisting of a specific preparation of the copolymer SMA dissolved in DMSO, undergoing Phase III clinical trials was obtained from the Risug pilot plant facility, Indian Institute of Technology, New Delhi.

One hundred micrograms of Risug suspended in 1 ml PBS (pH 7.2) and vortexed for 10 min formed a uniform suspension of the drug, which was then used to treat E. coli cells.

2.3. Electron microscopy

The cells were fixed with 2.5% glutaraldehyde in phosphate buffer for 30 min and washed three times in PBS. For SEM a thin film of cells was smeared on a SEM stub with silver paint, alcohol dried, sputter coated with gold, and observed under a Philips (Eindhoven, The Netherlands) scanning electron microscope (501 B).

For TEM, the pellet was post fixed in 1% OsO₄ for 30 min, washed, dehydrated in acetone, embedded in low viscosity spurr media and polymerized at 60 °C for 48 h. The ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Philips transmission electron microscope (CM-10).

2.4. AFM operating conditions

The AFM pictures were taken employing the CP Research model of ThermoMicroscopes, Sunnyvale, CA, USA operating in the non-contact mode. In this mode of operation, a silicon cantilever with a force constant of 17.2 N/m was vibrated near its resonance frequency of about 320 kHz. The deviation of the vibration frequency from resonance depends on the tip–sample separation and forms the dataset providing the topography of the bacterial cells.

For mounting the bacterial cells, glass, mica and single crystalline silicon (100) substrates were employed. The glass substrates were found to be best suitable for this study. Ten microlitres of the bacterial suspension was mounted on a glass substrate. After air-drying the cells were imaged in air with AFM in tapping mode.

3. Results

3.1. SEM of E. coli before and after Risug treatment

The SEM results of the E. coli bacteria are shown in Fig. 1. The normal morphology of E. coli grown in the absence of Risug shows the common appearance of the bacillus ultrastructure with dispersed cytoplasm and cell walls with well-defined shape. This is shown in Fig. 1(A). The SEM micrographs for untreated E. coli shows normal smooth surface of these cells. Clustering of cells is observed together with a few scattered individual cells.

In Fig. 1(B) we show the E. coli cells treated with Risug. The pictures are no longer sharp indicating changes in morphology. Details of the morphology of individual cells are not possible due to lack of resolution. Clustering is seen here as well, with possible cell fusion.

3.2. TEM of E. coli before and after Risug treatment

Prior to Risug treatment, we show the details of a well-formed cell. The cell walls are uniformly
smooth and the cytoplasm is intact as shown in Fig. 2(A). Detailed structure of the cell walls are seen but signs of bulging of the cell is not observed. This would indicate uniform pressure applied by the contents of the cell (turgor pressure) when the cells were prepared for the TEM observation.

After Risug treatment, the TEM pictures reveal dramatic changes in the nature of the contents of the cell as well as in the structure of the cell walls. This is shown in Fig. 2(B). The cytoplasm is no longer uniform following vacuolation, but remains piled up in selected regions of the cell forming patches interspersed with empty spaces. The cell walls appear intact but the cytoplasm, in places, has left the cell walls. Emptying of the contents of the cell in the form of whiskers is almost not evident.

3.3. AFM of E. coli before and after Risug treatment

The AFM image of the untreated E. coli shows evidence of smooth surfaces although some variation in surface topography is seen over the length of the bacteria. We show in Fig. 3(A) a cluster of bacteria, about 4 in number. The clustering is similar to what has been observed with SEM. As AFM allows the recording of information relating to the height of the bacteria sitting on the glass substrate, we show in Fig. 3(B), a line analysis of the height of the bacteria along the length of the body for an isolated cell at the bottom left corner of this picture. In general the surface is smooth and the maximum variation in roughness is not greater than 5% near the middle of the cell. An
The overall variation here is over 50%. This is shown in Fig. 4. An inset to the figure shows the bacterial cell employed for this study. A cluster of particles, almost uniform in size and shape seen surrounding the bacteria are believed to be that of the polymeric drug Risug. This is confirmed later in detail. Emptying of cell contents reported earlier employing AFM is normally in the form of whiskers [13]. We have never observed whisker formation in our experiments.

The above observation showing preferential variation in the cell structure in the middle prompted us to take a closer look at the interaction of Risug with the bacteria. We show in Fig. 5, a relatively large cell under attack by Risug. The preparation method and the time of drying the sample allow the observation of the bacteria in presence of Risug particles. As expected, the cell shows large variation in topography along its body. An interesting observation here is the mode in which the Risug particles have attached to the cell wall. In Fig. 5a, we show an AFM picture of the specimen where scanning was performed in the $x$-direction. The particles at the terminal ends are of Risug. A natural shadowing is obtained when scanning large objects in AFM as a result the center of the cell is largely not clear.

Fig. 3. (A) AFM pictures from a cluster of *E. coli* bacterial cells on a glass substrate prior to Risug treatment; (B) height of a cell (from the glass substrate) along its body length for a bacterial cell in the bottom left portion of the cluster. A line indicates the arc along which the height has been measured starting at the white arrow.

The steep fall of the height plot in Fig. 3(B) around 2.3 μm is due to termination at the edge of the bacteria. The situation is considerably altered following Risug treatment. Although the cell walls are intact, the surface of the bacteria is now extremely rough. A typical line analysis of the bacterial surface along the body length of the cell body shows the reduction in the height of the cell in the middle.

Fig. 4. Height of a cell along its body length for a typical bacterial cell, after Risug treatment. The inset shows the cell. The arrow marks the starting point of the arc along which the height is measured.
4. Discussion and conclusions

A comparative study of the antibacterial effect of the drug Risug on *E. coli* has been performed employing SEM, TEM and AFM. In general, all the techniques show clustering of cells both before and after Risug treatment with isolated individual cells. Lack of details in SEM is overcome by TEM and AFM, with the latter providing vital information on the height of the cell and details of its topography.

From TEM we observe clustering of a uniform cytoplasm inside the cell following Risug treatment. This is accompanied by sharp changes in the cell wall morphology, which shows bulging in places, both at the terminal ends as well as the body of the cell. However, TEM cannot provide height information and the information shown here is basically a section of the cell.

The information on the height and hence details of morphology can be obtained with ease in AFM. Here the cells were studied after drying on a glass slide. This procedure provides for some alteration of the cell surface as shown in Fig. 3(A). Thus the 5% roughness observed is a result of the drying process and is not related to any changes observed in the cell contents after Risug treatment.

Following Risug treatment, the roughness has increased considerably with deviation of the cell height by about 50% in the middle of the cell in some cases. This is possibly a precursor to lysing of the cell. Substantial overall variation in the smoothness of the cell is also seen in some cases as discussed below.

Such a situation is shown in Fig. 5 where a large cell is shown under attack by Risug. It is important to note that the Risug particles, which were not observed in TEM (Fig. 2), are seen here as AFM records the presence of interatomic forces, however small they may be. While TEM is basically a contrast microscopy for which the Risug particles are transparent. To really ascertain that the particles are of Risug and to confirm their size, we show in Fig. 6 an AFM height analysis of the particles near the far end of the bacteria in Fig. 5(A). The location and the arc length taken for analysis has been indicated by an arrow in the inset to this figure. The particles are overall circular in
shape with an average size of \( \sim 200 \) nm. The bacterial cell does not show any obvious sign of lysis although an increase in surface roughness is quite evident.

An assessment of the surface roughness is in order. We take the cell shown in Figs. 3 and 5 for this analysis. For each case arc lengths of \( 1.5 \) \( \mu \)m is taken from different regions of the cell and the roughness measured 10 times, and averaged. The average roughness obtained this way is \( 10.3 \) nm for the cell prior to Risug treatment, which increases to \( 27.0 \) nm after the treatment.

To compare the size and shape of the particles, we report AFM pictures of the drug Risug in Fig. 7. The average size of the polymeric particles is about \( 200 \) nm, similar to the particles seen surrounding the bacterial cells in Fig. 5 and inset to Fig. 4. Together with the particle size and the evidence of Fig. 5 that the particles are uniform allows us to confirm that the particles surrounding the *E. coli* bacteria are indeed that of the polymeric drug Risug.

Fig. 5 shows the preferential attack by Risug from the terminal ends as confirmed by the orthogonal scans. This however not only changes the morphology of the terminal ends, it changes the overall shape of the cell which is now highly structured. The overall change in the cell shape can come about due to the changes in the internal pressure that the contents of the cell asserts on the walls.

In this context we note information of the cell contents provided by TEM (Fig. 2). The clustering of the cytoplasm and appearance of voids inside the cell would then lead to collapse of the cell walls in places. We think that the AFM surface topology analysis confirms this. However, this is not a direct confirmation whether the breakdown of the cytoplasm is taking place or if it is a simple rearrangement of the cell contents. A detailed DNA analysis would help to elucidate this point.

**Acknowledgements**

The authors thank the Department of Anatomy, All India Institute of Medical Sciences, New Delhi for providing facility for obtaining the electron micrographs. We also thank the Ministry of Health & Family Welfare, Government of India and University Grants Commission, India for financial assistance.
References