J. Biomater. Sci. Polymer Edn, Vol. 17, No. 1–2, pp. 239–246 (2006)

Also available online - www.vsppub.com

Short communication

Staphylococcus epidermidis adhesion on modified urea/urethane elastomers

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Received 14 March 2005; accepted 7 October 2005

Abstract—Block urea/urethane co-polymer films present elastomeric properties with the possible tuning of their surface properties within a wide range and are therefore considered relevant surfaces for possible medical applications. In particular, thin free standing films of urea/urethane elastomers with two soft segments, polypropylene oxide and more hydrophobic polybutadiene, develop multi-stable states with surface topography features with remarkable regularity. Moreover, complex surface structures may be obtained by UV radiation treatment followed by suitable mechanical action and also by extraction of the elastomer with a suitable solvent. In the present work, different modified elastomer samples were assayed for *Staphylococcus epidermidis* adhesion during 2 h and the extent of bacterial adhesion was evaluated by automatic cell enumeration. Bacterial adhesion assays demonstrate that the typical trend relating the increase in the number of adhered bacteria with the increase of the surface roughness does not hold for all materials. Results may be interpreted taking into account both the surface topography and the different types of micro-phase segregation of hydrophobic and hydrophilic parts of the elastomer.

Key words: Elastomer; co-polymer; urea; urethane; adhesion; *Staphylococcus epidermidis*.

INTRODUCTION

One of the major problems associated with biomedical materials used in implants or indwelling devices concerns the existence of biomaterial-centered infections from coagulase-negative staphylococci [1], in particular *Staphylococcus epidermidis*, noted as responsible for nearly 30% of the overall infections [2]. Biomaterial-

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centered infections start from bacterial adhesion [3] that occurs due to specific interactions between cell-surface structures and specific molecular groups of the biomaterial (e.g., lock-and-key), and by intermolecular forces [4], namely van der Waals forces, electrostatic forces, hydrophobic/hydrophilic interactions and steric interactions. After the initial adhesion, a second process of bacterial cell–cell adhesion results in a biofilm that is very difficult to eliminate, since bacteria inside the biofilm are protected from phagocytosis and antibiotics [5].

Block urea/urethane co-polymer films present elastomeric properties with the possible alteration of their surface properties within a wide range [6]. These materials consist of alternating soft and hard segments that exhibit micro-phase separation. In particular, urea/urethane elastomers develop multi-stable states with surface topography features with remarkable regularity [7]. Moreover, complex surface structures may be obtained by suitable mechanical action, UV radiation treatment and also by extraction of the elastomer with a selected solvent. The aim of this work was to study the ability of each type of modified urea/urethane elastomer to be colonized by *S. epidermidis*, in order to evaluate their possible application in the manufacture of medical indwelling devices.

MATERIALS AND METHODS

Bacteria and growth conditions

The strain used in this work was *Staphylococcus epidermidis* 9142, a clinical and biofilm-positive strain provided by Gerald B. Pier (Harvard Medical School, Boston, MA, USA). Trypticase soy broth (TSB) and trypticase soy agar (TSA) plates were prepared according to the manufacturer's instructions. The strain was grown for 24 ± 2 h at 37°C in a shaker rotating at 130 rpm in 15 ml TSB using as inocula bacteria grown on TSA plates not older than 2 days. Then, 50 μl of each cell suspension was transferred to 30 ml fresh TSB, which was incubated for 18 ± 2 h at 37°C and 130 rpm. After being harvested by centrifugation (for 5 min at $9000 \times g$ and 4°C), cells were washed twice and resuspended in saline solution (0.9% NaCl prepared in distilled water) at a concentration of approximately 1×10^9 cells/ml, determined by the optical density at 640 nm. These cell suspensions were used in the subsequent adhesion assays.

Elastomers

Urea/Urethane elastomer samples were prepared from polypropylene oxide based isocyanate terminated triol prepolymer (PU) and polybutadienediol (PBDO) with the proportion of 40 wt% PBDO, according to the synthesis procedure described previously [8]: the pre-polymers were dissolved in toluene, under appropriate conditions with a solid content of 40 wt% and the reaction, under nitrogen atmosphere, was allowed for at least 30 min, the mixture was then cast and sheared

by moving a casting knife at a controlled shear rate ($v_1 = 5$ mm/s). The PBDO was supplied by Aldrich and the PU was acquired from Portuguese Petrochemical Industry (CPB). These elastomer films, with a typical thickness of 60–100 μm, present a smooth surface at the nanometer scale with a mean square roughness $R_a = 0.59$ nm [9].

Modified elastomers

The original elastomer films were then modified into different materials by UV irradiation followed by mechanical shear stress in different directions and solvent immersion (in this particular case using toluene). The UV irradiation of the elastomer is known to promote interlinking of the polybutadiene diol chains and amine linkages, enhancing the orientational order and enabling instabilities to appear [10]. Mechanical shear stress, applied with unidirectional stretching cycles, favours micro-phase segregation of the soft and hard parts of the co-polymer and results in a bi-stable behaviour of the material topography [7]. The immersion of the elastomer films in toluene leads to its volume expansion. The removal of the solvent gives rise to the development of special surface topography features that promote a strong increase in surface roughness. The combination of these modification approaches led to 5 different types of modified elastomers with stable surface structures: non-modified urea/urethane elastomer (material 1); toluene-modified elastomer (material 2); UV–toluene-modified elastomer (material 3); UV-stretching along the shear direction–toluene-modified elastomer (material 4); UV-sequential stretching parallel and perpendicular to the shear direction–toluene-modified elastomer (material 5). Other intermediate states prior to toluene immersion do not present stable surfaces and were not considered for the present study.

The resulting modified elastomers present typical and unique surface characteristics in terms of surface topography, previously observed by means of atomic force microscopy [9]. Samples of material 2 still present a smooth surface with no visible change on the average surface roughness ($R_a = 0.59$ nm). The UV irradiation used in the modification process of the other materials (materials 3–5) led to corrugated sample surfaces with features at the micrometer scale in all directions, resulting in a clear increase of the average surface roughness ($R_a \approx 220$ nm).

Initial bacterial adhesion

Squares of urea/urethane elastomer films were placed in 6 well tissue-culture plates containing 3.5 ml of a suspension of 2×10^9 cells/ml in saline solution. Initial adhesion to each substratum was allowed to occur for 2 h at 37°C in a shaker rotating at 130 rpm. Negative controls were obtained by placing urea/urethane copolymer films squares in a saline solution without bacterial cells. The squares were then gently transferred to 100-ml glass beakers containing distilled water, and were allowed to rest there for approximately 10 s. Afterwards, a new transfer was made to a different 100-ml glass beaker containing distilled water, followed by a third

transfer 10 s later. These washing steps were carefully performed in order to remove only the cells that were suspended in the liquid interface formed along the surface and to minimize cell detachment from the surface [11]. The substrate squares with adhered cells were dried at 37°C. All experiments were done in triplicate with 4 repeats.

Image analysis

Before image observation and enumeration of adhered cells, the substrate squares were stained with a 0.2% safranin solution or with a 0.01% DAPI (4′-6-diamidino-2-phenylindole) solution, for better image contrast. Direct bacterial counts were done using an epifluorescence microscope coupled to a 3 CCD video camera that acquires images with 820×580 pixels resolution and at a magnification of 1000×. With this magnification 1 cm^2 is equivalent to 1.198×10^4 captured images (as determined by a Neubauer chamber). Cells were counted using automated enumeration software.

SEM analysis

Dried samples of materials with adhered bacteria were stuck on metal holders with double-sided adhesive tape and coated with gold in a vacuum evaporator. Observations were performed at 10–15 kV with a Leica S360 scanning electron microscope and observations were documented through the acquisition of representative microphotographs.

RESULTS AND DISCUSSION

SEM images of the surface topographies of elastomer films with adherent bacteria (Fig. 1) confirm the roughness parameter results previously reported [7]. SEM images of periodic modulated substrates (materials 3–5) show that *S. epidermidis* adhered preferentially to the surface valleys (e.g., Fig. 1d). This fact confirms expectations, since these surface valleys may act as microscopic niches protecting adhered cells from shear forces, increasing the probability of irreversible attachment [12].

Fluorescence microscopy images of adhered bacteria (Fig. 2) show a higher extent of bacterial adhesion on mechanical-modified elastomers (materials 4 and 5) in comparison to non-modified and toluene extracted (materials 1 and 2, respectively). Figure 3 shows the average values and the corresponding associated error of *S. epidermidis* cells adhered to the different elastomer substrates. These results were obtained from several adhesion experiments and the high dispersion level of the results is due to the natural variability of the bacterial response among different samples of the same type of material. Moreover, materials with higher surface non-uniformity induce higher dispersion on the number of cells adhered, resulting in higher relative measurement errors. This fact is particularly evident in the case of materials obtained from mechanical stretching cycles (materials 4 and 5). It

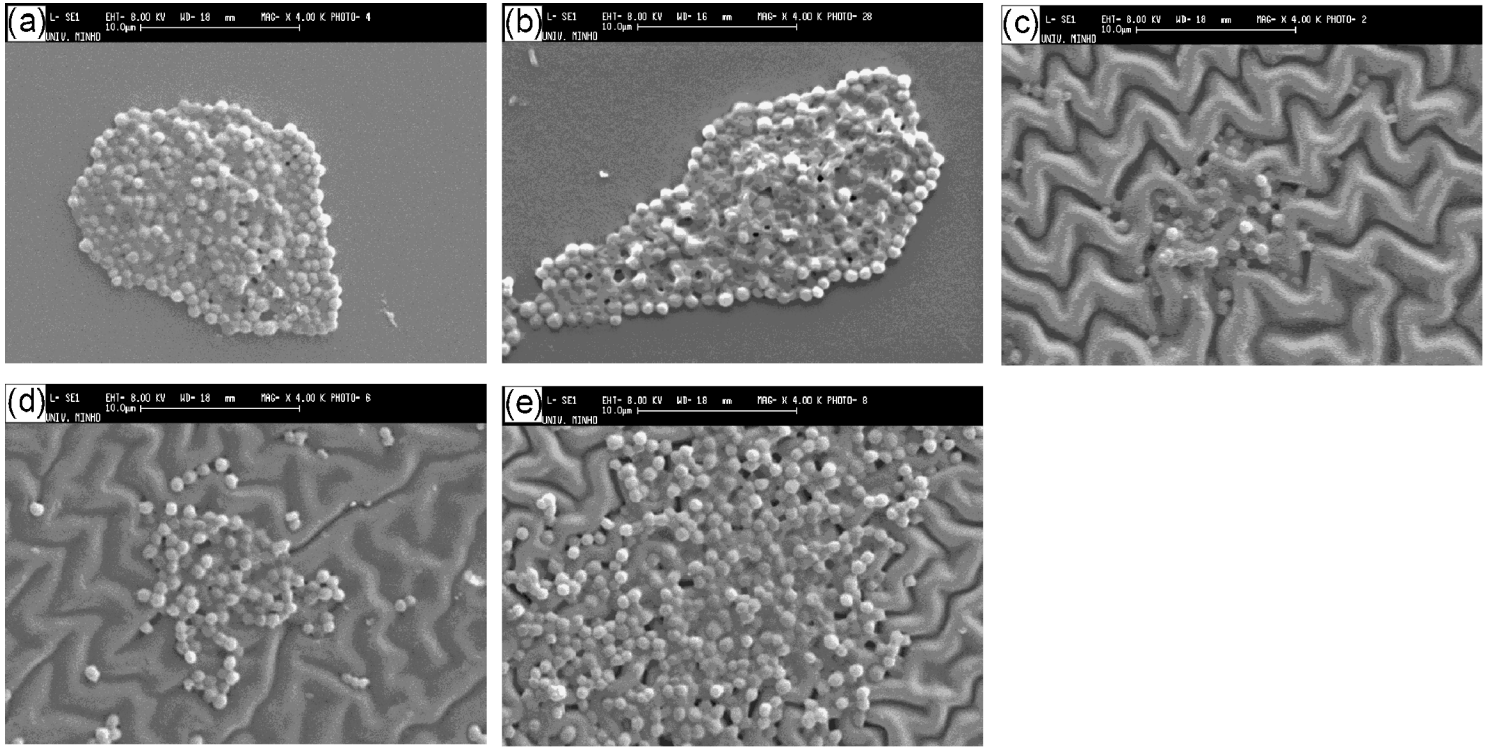

Figure 1. SEM images of *S. epidermidis* adhered to modified elastomer surfaces after 2 h culturing. (a) Standard, non-modified urea/urethane elastomer type 1; (b) extracted with toluene, elastomer type 2; (c) UV-exposed and extracted with toluene, elastomer type 3; (d) UV-exposed, stretched along the shear direction and finally extracted with toluene, elastomer type 4; (e) UV-exposed, sequentially stretched parallel and perpendicular to the shear direction and finally extracted with toluene, elastomer type 5.

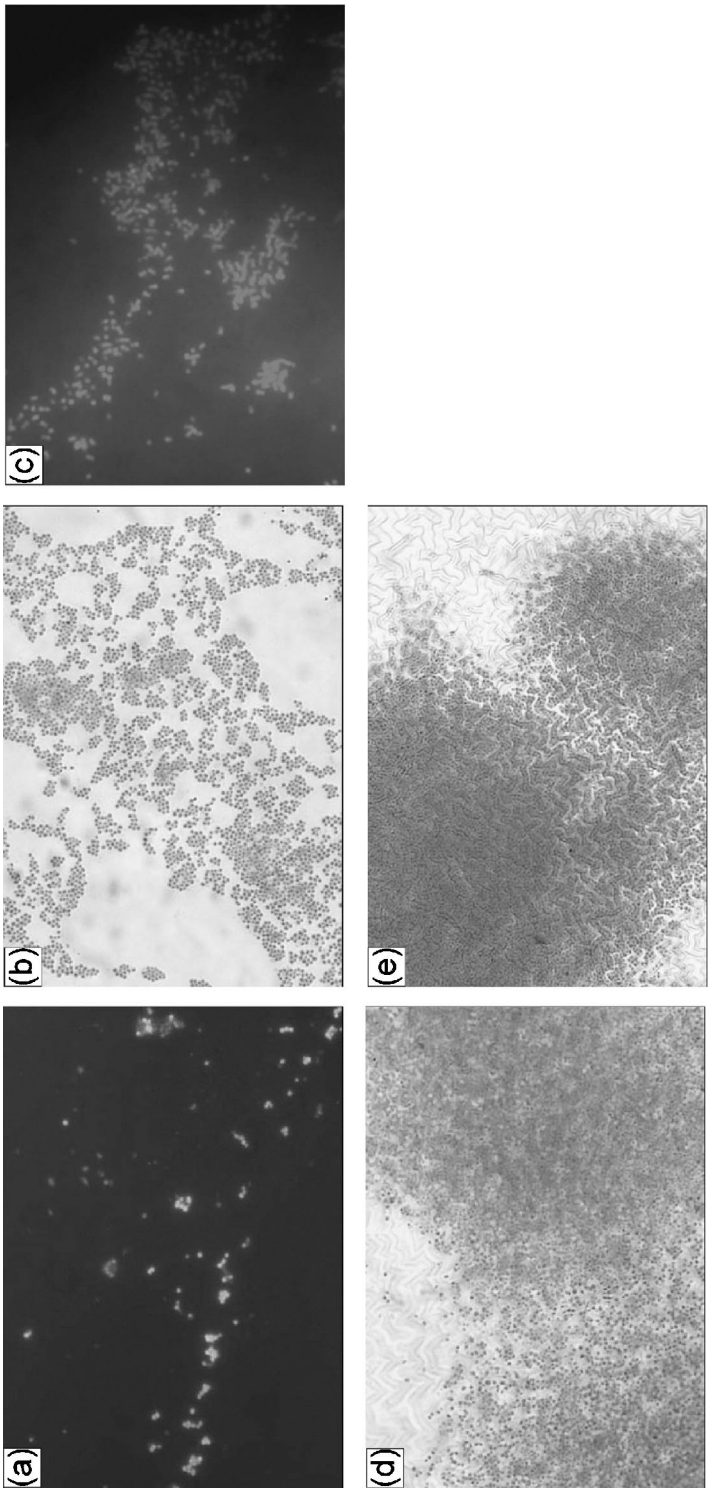

Figure 2. Optical fluorescence microscopy images of *S. epidermidis* adhered to urea/urethane elastomer surfaces. (a) Standard, non-modified urea/urethane elastomer type 1; (b) extracted with toluene, elastomer type 2; (c) UV-exposed and extracted with toluene, elastomer type 3; (d) UV-exposed, stretched along the shear direction and finally extracted with toluene, elastomer type 4; (e) UV-exposed, sequentially stretched parallel and perpendicular to the shear direction and finally extracted with toluene, elastomer type 5.

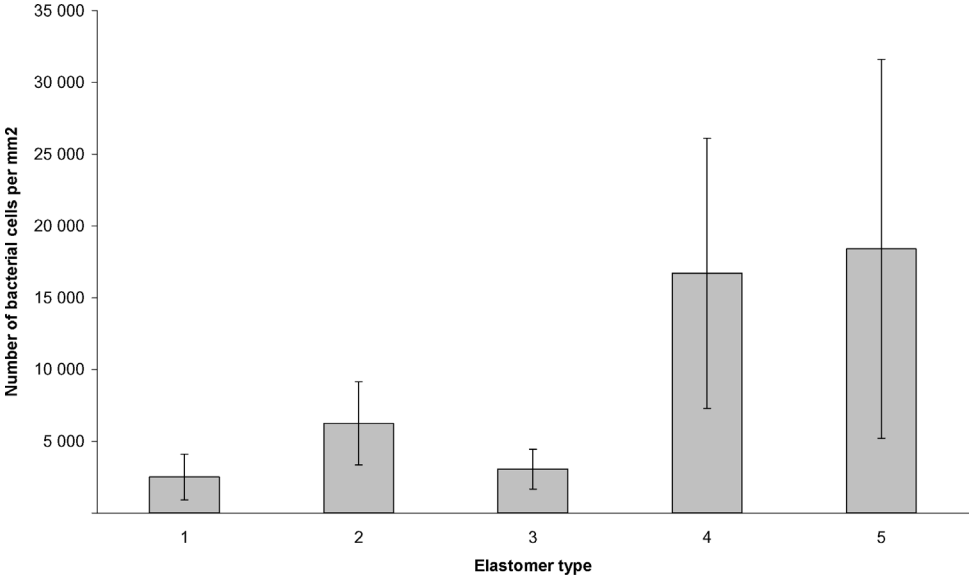

Figure 3. Adhesion rates of *S. epidermidis* on modified urea/urethane elastomers. The bar represents the standard deviation at 95% confidence level.

should also be noted that the relative high errors associated to the extent of bacterial adhesion may come from uncontrolled variations of the surface topography within each material lot.

The number of cells adhered is found to increase from smooth surfaces (materials 1 and 2) to periodically modulated surfaces obtained from mechanical stretching cycles (materials 4 and 5), which is in accordance to expectations. In contrast, the results obtained with material 3 do not follow the common pattern of an increase in the number of adhered cells with an increase in surface roughness. Actually, this material shows equal or even lower extent of adhesion of *S. epidermidis* in comparison to smooth surfaces (types 1 and 2). This fact suggests that the two surface modification mechanisms, UV irradiation and mechanical stretching, induce different micro-segregations of the soft (hydrophobic) and hard (hydrophilic) polymeric parts of the elastomer material. One possible explanation for the observed differences in the extent of bacterial adhesion is that the UV irradiation induces soft/hard segregation with the hydrophilic part in the surface valleys and the hydrophobic part on the surface peaks, whereas the mechanical stretching cycles induce an opposite segregation. It is known that hydrophilic materials are less favourable for the adhesion of bacteria [13].

Elastomers with the hydrophilic part placed on the surface valleys would reduce the bacterial extent of adhesion, despite the protective effect from shear forces. In contrast, materials with the hydrophobic part corresponding to the surface valleys would induce a coupling effect of the surface chemistry and surface topography increasing the number of adhered bacterial cells. Let us notice that the advancing

and receding angles do not present a direct correlation with the bacterial adhesion results, since measurements were performed using simply the sessile method where the effect of the corrugated surface is not precisely taken into account.

The results showed that the surface roughness is not the only significant mechanism involved in the adhesion process and suggest that the micro-phase separation, characteristic of the elastomer materials, play a significant role on bacterial adhesion. This work demonstrates the interest in using these elastomers as model surfaces in bacterial adhesion studies, mainly due to the ability to modify their surface properties by acting on simple experimental parameters. Regarding their possible biomedical applications, the modified elastomer type 3 is the most appropriated material to be used, since it presented the lowest extent of adhesion. This constitutes a challenge to future research in tuning the surface properties of the urea/urethane elastomers towards their adequacy in minimizing bacterial adhesion.

Acknowledgements

J. G. F. and P. T. thank the Portuguese Science Foundation (FCT) for the Scholarships PRAXIS XXI/BPD/5633/2001 and SFRH/BPD/11553/2002, respectively. The authors acknowledge FCT for the financial support through POCTI/CTM/45286/2002.

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