Staphylococcus epidermidis RP62A adhesion to chemically modified cellulose derivatives

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The adhesion of coagulase negative *Staphylococcus epidermidis* expressing capsular polysaccharide/adhesin (PS/A) to cellulose diacetate (CDA), as well as to primary reference low-density polyethylene, was assessed *in vitro*. Attached bacteria were released by gentle sonication and quantified as colony forming units. Surface free energy of cells and materials and the free energy of interaction between cells, each type of material and water molecules was calculated through contact angle measurement, also enabling the determination of materials surface hydrophobicity. The influence of CDA surface modification by deacetylation and phosphorylation on bacterial adhesion was studied. Chemical modifications of CDA by deacetylation and by phosphorylation were effective in lowering bacterial adhesion.
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1. Introduction

Infection at the site of implantation of a biomaterial remains a major complication hindering the long-term use of implanted materials [1, 2]. The exact mechanism by which these foreign body infections occur remains unknown. Moreover, it is known that the presence of a foreign body greatly decreases the number of organisms required to establish an infection [3–5]. The bacteria frequently associated with infections involving materials for blood contact or orthopedic applications are the coagulase negative staphylococci, most notably *Staphylococcus epidermidis* expressing capsular polysaccharide/adhesin (PS/A) and slime [6, 7].

The sources of bacteria that cause the vast majority of implant-associated infections include perioperative contamination, exit site contamination for percutaneous devices and hematogenous spread out from locations distal to the implant area [8].

From an overall physicochemical viewpoint, microbial adhesion can be mediated by nonspecific interaction forces, with a long-range character, including Lifshitz–van der Waals forces, electrostatic forces, acid-base interactions, and Brownian motion forces [9, 10]. Specific interactions are forces acting in highly localized regions of the interacting surfaces, over distances smaller than 5 nm [10]. As soon as a surface is reached, microorganisms will be attracted or repelled by it, depending on the resultant of the different nonspecific interaction forces. Most organisms are negatively charged and consequently a negatively charged surface exerts a repulsive electrostatic force [11]. Controlling the charge and hydrophobic properties of substratum surfaces is likewise a way to influence bacterial interaction with the surface and must be taken into account when novel anti-infective biomaterials are to be developed [12, 13].

Cellulose is a well-known polymer which has found extensive use in biomedical applications [14], due to its excellent properties, like nontoxicity and stability. Cellulose dialysis membranes are among the most widely used polymeric medical devices. Phosphorylated cotton incorporating silver ions was reported as an adequate substrate to inhibit bacterial adhesion [15]. However, the effect of phosphate functionalities alone on bacterial adhesion has not been investigated yet. The negative charge of phosphate functionalities would promote an electrostatic repulsive effect towards negatively charged bacterial cell wall. In addition, phosphate groups are hydrophilic, which would further increase its anti-adhesive properties.

The aims of this investigation are the evaluation of *S. epidermidis* adhesion to cellulose diacetate sheets chemically modified by deacetylation and by phosphorylation, and the study of the relationship between surface energetic parameters and bacteria–synthetic surface interactions.

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2. Materials and methods

2.1. Bacteria storage and growth

The bacterial strain used in this study was the reference type culture *Staphylococccus epidermidis* RP62A (ATCC 35984), PS/A positive.

Overnight broth cultures of the RP62A strain were grown in tryptic soy broth (TSB) (Difco, USA) in a rotary shaker (New Brunswick Scientific, USA), at 150 rpm and 37 °C, for at least 18 h, to reach the stationary growth phase, in order to minimize growth phase-dependent variations in bacterial surface properties [16, 17].

Bacteria were then harvested by centrifugation at 5000 rpm for 10 min and subsequently washed three times with phosphate-buffered saline (PBS) (pH 7.4; Sigma), resuspended in PBS, and maintained at room temperature. In order to remove bacteria clusters, suspensions were filtered through a membrane filter (Type SC, Milipore, USA), with a pore size of 5 μm. Bacterial cell concentration was adjusted to about 1×10^8 colony forming Units (CFU)/ml using a calibration graph correlating optical density – OD_{600} with the number of viable cells. In each experiment CFU counting of the initial suspension confirmed this correlation and showed that no significant bacterial lysis occurred within 12 h of suspension in PBS. Experiments were conducted within 5 h after resuspension in PBS.

2.2. Materials

Discs with 10 mm diameter where prepared from sheets of the following materials, which were generous gifts: low-density polyethylene (LDPE; Abiomed, USA) primary reference from the National, Heart. Lung and Blood Institute (NHLBI); cellulose diacetate (CDA; Clarifoil P20/P27 from Courtaulds Chemicals, UK). Materials were then sterilized with ethylene oxide, for 2 h at 52 °C, before use [1, 2].

2.3. Chemical modification of cellulose diacetate

All chemicals were of research grade purity, and used without further purification.

Deacetylation of CDA films was adapted from Ishizu *et al.* [18], through immersion in hydrochloric acid solution (10% v/v), during 48 h at room temperature. Afterwards, the disks were washed with bidistilled water during 10 min.

Chemical modification by phosphorylation was carried out by the $H_3PO_4/P_2O_5/Et_3PO_4$ hexanol method [14], with minor modifications. Reaction was performed in a four-necked round-bottomed flask, equipped with a nitrogen inlet, a condenser, a $CaCl_2$ guard tube, a thermometer and a mechanical stirrer. CDA disks were suspended in hexanol directly in the flask. Phosphorous pentaoxide was dissolved in triethylphosphate and orthophosphoric acid and the mixture added to the suspension. The reaction was allowed to proceed under constant stirring, for 48 h at 30 °C. After this period of time the disks were washed consecutively in hexanol and ethanol and then rinsed repeatedly in water, in order to wash out the excess H_3PO_4 [14].

2.4. Surface characterization

2.4.1. Contact angle measurements

Contact angle measurements were performed on lawns of cells as well as on the materials surfaces, using the sessile drop technique, in a standard contact angle apparatus (Kruss, Germany). The measurements were performed automatically, with the aid of an image analysis system (Kruss, Germany). The images were received by a video camera connected to a personal computer, with an automatic measuring system (G2/G40) installed. All measurements were done at room temperature and three different liquids were used: water, di-iodomethane and glycerol, with known surface tension components [19, 20].

The contact angle values were used to calculate the surface free energy of cells and materials, considering the relative contributions of Lifshitz–van der Waals and electron acceptor and electron donor components. Those values enabled the calculation of the free energy of interaction between each type of surface and water (ΔG_{iwi}^{TOT}), i.e., surface hydrophobicity, and the surface free energy of interaction between the bacteria and each type of material (ΔG_{bws}^{TOT}). All the calculations were performed using the approach of Van Oss [9, 10]

To prepare the bacterial lawns, the cells were harvested by centrifugation, washed once with deionized water, then with increasing concentrations of ethanol (10%, 25%, 70% and 90%), and finally resuspended in pure ethanol to a concentration of 10^8 cell/ml [19]. This procedure, using increasing ethanol concentrations, was used to avoid changes in cell wall structure due to dehydration. The cell suspension was then immersed for 5 min in a sonication bath, for better homogenization. Meanwhile, a solution of 20 g/l agar and 10% glycerol was cast in glass petri dishes and cooled at room temperature. Finally, the agar was cut into small squares of $1.5 \times 1.5\,cm^2$. A cell suspension aliquot of 250 μl was spreadout over each agar square. After drying the first layer of cells, two more layers were added, completely covering the agar square [19].

2.4.2. Spectroscopic analysis

Attenuated total reflectance-Fourier transformed infrared (ATR-FTIR) spectra were recorded in a System 2000 Nir FT Ramam spectrometer (Perkin Elmer). Zinc selenide (ZnSe) was used as the element for internal reflection.

2.4.3. Roughness

Surface roughness of polymers was determined by a laser rugosimeter (Perthometer S3P, Perthen, Germany). Ten successive distances were examined and the mean value of roughness was taken as the final result [13].

2.5. Bacterial adhesion experiments and quantification of adhered bacteria

The disks were placed in sterile borosilicate glass tubes with 5 ml of the bacterial suspension, and incubated at

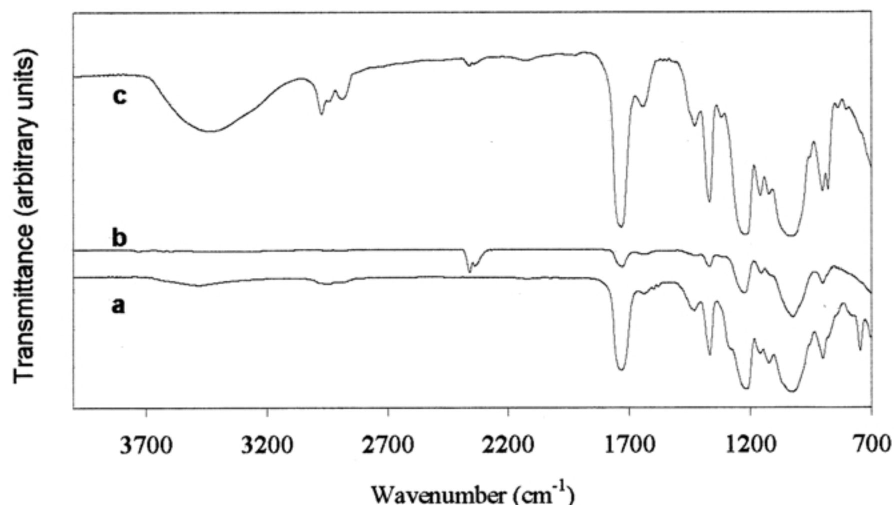

Figure 1 ATR-FTIR spectra of (a) CDA, (b) deacetylated CDA and (c) phosphorylated CDA.

37 °C, with gentle shaking for 1 h [21, 22]. Three replicates were used for each experiment.

After incubation, non-adherent or loosely adherent bacteria were removed from each specimen according to a technique previously described [1, 23, 24]. Then each specimen was placed into a tube with 5 ml of sterile PBS containing 0.1% (v/v) Tween 80 [25]. All the tubes were sonicated for 45 min in an ultrasonic cleaner (Branson, USA, 45 kHz) [26]. Then, 10-fold serial dilutions of the sonicated solutions were plated onto culture plates of Mannitol Salt (Difco, USA), and after 48-h incubation at 37 °C [23], the number of adherent bacteria colonies were counted.

2.6. Statistical Analysis

Statistical analysis between the mean values of all pertinent groups for a given assay were performed using a one-way analysis of variance (ANOVA), with Fisher's protected least significant difference (PLSD) determining 95% confidence limits. Linear trends were determined by completing a least-squares fitted simple linear regression analysis on the data. All statistical analysis was done using Statistica 3.0 software (Statsoft, USA).

3. Results

In this investigation of *S. epidermidis* RP62A adhesion, standard biomedical reference materials (NHLBI LDPE), cellulose diacetate (CDA), and chemically modified CDA were studied.

The viability of *S. epidermidis* cells after the sonication treatment was examined and no bacterial death was observed. Bacterial concentration during the 1 h adhesion experiments did not change.

3.1. Preparation and characterization of surfaces

In comparison with CDA (Fig. 1(a)), ATR-FTIR spectrum of deacetylated CDA (Fig. 1(b)) showed a reduction of the peak at 1720 cm^{-1}, generally assigned to carbonyl functionalities in acetate groups [27]. The spectrum of phosphorylated CDA (Fig. 1(c)) gave evidence of phosphates groups since additional peaks were found at 1300 and 870 cm^{-1}, which can be attributed to P=O and P-O-C groups, respectively [28]. The OH group band became more pronounced at 3100–3650 cm^{-1}.

Table I lists the contact angles obtained using the three liquids tested, which enabled the quantification of bacterial and material surface hydrophobicity (ΔG_{iwi}^{TOT}). LDPE showed negative values of ΔG_{iwi}^{TOT}, which means that they may be classified as hydrophobic, while CDA, presenting a positive value, is considered hydrophilic. Chemical modification of cellulose diacetate by de-acetylation and phosphorylation promoted a slight increase in hydrophilicity.

TABLE I Contact angles, apolar and polar surface tension parameters, and hydrophobicity (ΔG_{iwi}^{TOT})

	Contact angles (°)			Surface tension (mJ/m^2)					Free energy (mJ/m^2)
	θ_{water}	$\theta_{glycerol}$	$\theta_{diiodomethane}$	γ^{Lw}	γ^{+}	γ^{-}	γ^{AB}	γ^{TOT}	ΔG_{iwi}^{TOT}
Bacteria									
RP62A	23.1 ± 2.1	24.2 ± 2.8	64.5 ± 2.4	26.0	5.7	45.3	32.2	58.2	17.5
Material									
CDA	40.5 ± 2.0	41.2 ± 2.2	23.4 ± 1.9	46.0	0.35	31.0	6.5	52.6	0.3
CDA-D	39.6 ± 2.3	41.4 ± 1.8	32.4 ± 2.4	42.0	0.50	33.0	8.1	50.1	5.5
CDA-P	39.6 ± 3.1	42.0 ± 2.0	36.0 ± 1.7	40.0	0.55	34.0	8.6	48.6	7.9
LDPE	80.0 ± 3.5	63.9 ± 2.9	54.0 ± 3.4	32.0	1.34	4.7	5.0	37.0	− 46.9

CDA-D – Deacetylated cellulose diacetate.
CDA-P – Phosphorylated cellulose diacetate.

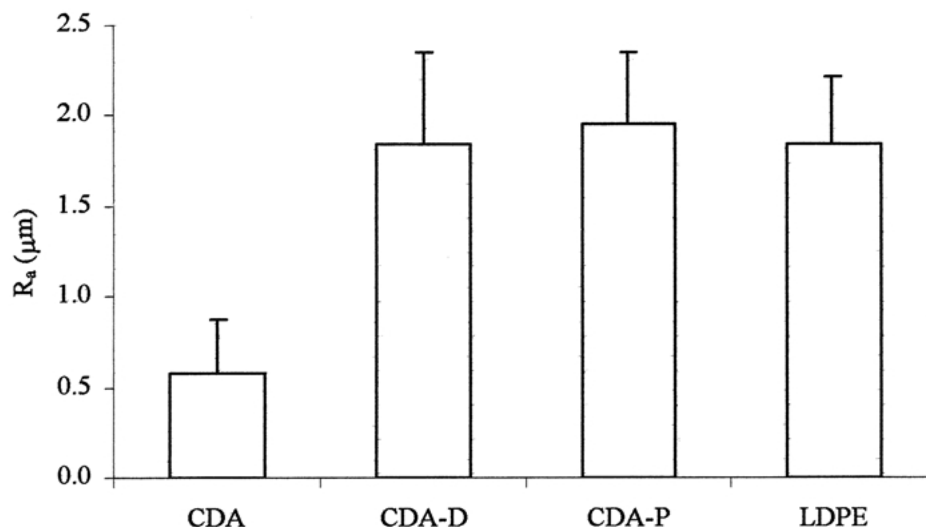

Figure 2 Average roughness (R_a) of treated and untreated polymers.

TABLE II Free energy of interaction between *S. epidermidis* (RP62A) and materials surface immersed in water

Material	ΔG_{bws}^{TOT}
CDA	15.92
CDA-D	16.73
CDA-P	17.21
LDPE	− 3.11

Fig. 2 shows that the CDA was the polymer, which presented the lowest average roughness. Chemical modification of cellulose diacetate by deacetylation and phosphorylation promoted an increase in surface roughness, which was similar to LDPE.

3.2. Adhesion of *S. epidermidis*

The results of the adhesion assays are expressed in Fig. 3. There was a statistically significant difference between bacterial adhesion to LDPE and CDA. Surface modification of CDA by deacetylation and phosphorylation was successful in reducing the level of bacterial adhesion compared to the unmodified CDA (Fig. 3). A very small number of bacteria adhered to CDA-P, which bears negatively charged chemical functional groups. This polymer showed slightly reduced bacterial adhesion when compared with CDA-D, not bearing charged functional groups. CDA-P and CDA-D presented almost the same free energy of adhesion. The difference between bacterial adhesion to deacetylated CDA and phosphorylated CDA was not significant. Table II presents the values of the free energy of adhesion (ΔG_{bws}^{TOT}) between bacteria and the polymeric materials. The corresponding value for LDPE was negative. Chemical modification of CDA by deacetylation and phosphorylation slightly increased the value of ΔG_{bws}^{TOT}.

4. Discussion

This investigation enabled the study of the effects of several material parameters such as surface chemical composition, hydrophobicity and roughness in bacterial adhesion. It should be noted that a bacterial strain of reference type was used, which is a means to avoid cell surface variability [26, 29].

ATR-FTIR spectrum of CDA-D indicated a partial deacetylation of the original CDA [18], as it was suggested by the reduction of the peak at 1720 cm^{-1}, assigned to carbonyl functionalities. CDA-P spectrum suggested that phosphorylation of cellulose diacetate occurred without deacetylation. The hydroxyl groups band became more pronounced due to the introduction of hydroxyls from phosphate groups [31].

As our results indicated, untreated CDA is more hydrophilic than LDPE and the increase in hydroxyl groups by deacetylation originated a higher hydrophilicity and lowered bacterial adhesion. These results are in agreement with the results obtained by Wiencek *et al.* [32], showing that a decrease of hydroxyl groups increased hydrophobicity and bacterial adhesion. Most surfaces, including bacterial cells, acquire a net negative charge in aqueous medium at pH near neutrality. So, a more negatively charged polymer will increase the repulsive force between the two interacting surfaces [33]. This was confirmed in the present work, using phosphorylated CDA compared to CDA. When negatively charged phosphate groups were grafted, bacterial adhesion was reduced even further, due to a higher repulsive effect against the negatively charged bacteria. Ishihara *et al.* [33] improved blood compatibility by grafting a methacrylate with a phospholipid polar group. These results enhance the relevance of phosphate groups in preventing cell adhesion.

Chemical modification of cellulose diacetate increased the average roughness to values comparable to those of the LDPE but even so this increase did not affect bacterial adhesion, which was lower in comparison to CDA. Jansen *et al.* [13] reported that a surface roughness in the range of 0.4–2.0 μm did not influence bacterial adhesion. The present results seem to be in accordance with that study.

Bacterial adhesion can be regarded, from a physicochemical point of view, as the adhesion of a particle to a solid surface in a liquid environment. Thus, adhesion is controlled by the change of the free energy of adhesion. Adhesion is thermodynamically favored if the free

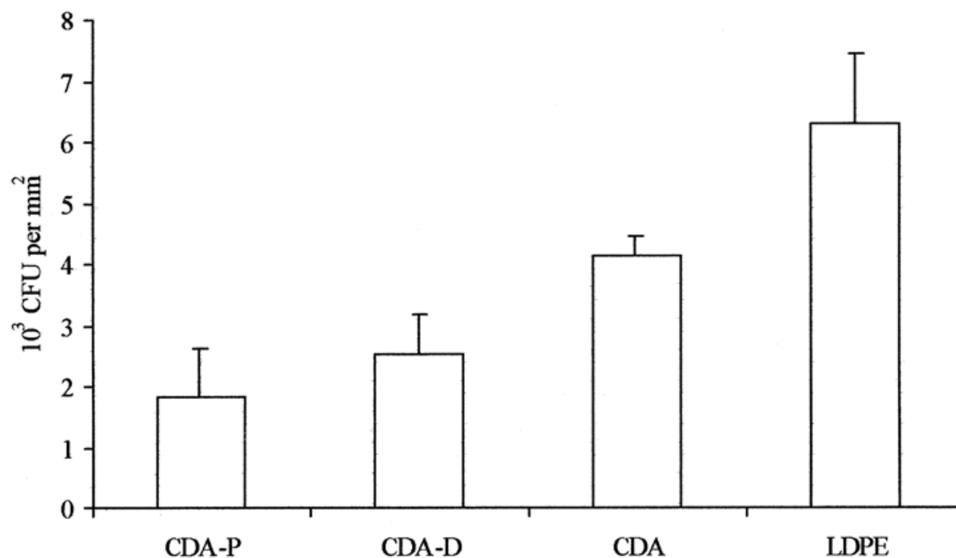

Figure 3 *S. epidermidis* ATCC35984 (RP62A) adhesion to treated and untreated polymers. Data are expressed as mean $\pm$ standard deviation of three observations.

energy of adhesion is negative and decreases with increasing free energy values [33]. This behavior was confirmed by the results obtained using LDPE, which presented high bacterial adhesion. No adhesion should occur if the free energy of adhesion is positive (bacteria need energy to adhere) [34]. In contrast to this, *S. epidermidis* RP62A adhered to treated and untreated CDA, which presented positive values of free energy of adhesion. It may be suggested that there is always a minimum number of adherent bacteria in *in vitro* experiments, even if the free energy of adhesion is positive.

5. Conclusions

Bacterial adhesion was significantly lower using the more hydrophilic cellulose diacetate, compared to a hydrophobic polymer such as LDPE. The chemical modification of CDA surface by deacetylation and phosphorylation resulted in significantly lower. *S. epidermidis* RP62A adhesion, compared to untreated CDA. These results can be explained in the framework of the Lewis acid-base theory of interfacial interaction: chemically modified CDA surfaces bear Lewis acid character to CDA surfaces and this could avoid some of the specific Lewis acid-base interactions with acidic bacterial surface. Moreover, the negative charge of phosphate groups of CDA-P can be responsible for a stronger repulsion of the negatively charged bacteria. Thus, specific interactions between chemical groups on both bacteria and biomaterials surfaces should be considered.

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Notation

ΔG_{iwi}^{AB} – Polar component of the free energy of interaction between surfaces i and water w (mJ/m^2).
ΔG_{iwi}^{LW} – Apolar component of the free energy of interaction between surfaces i and water w (mJ/m^2).
ΔG_{iwi}^{LW} – Free energy of interaction between surfaces i and water w (mJ/m^2).
ΔG_{bws}^{AB} – Apolar component of the free energy of interaction between bacterial cell b, materials' surface s and water w (mJ/m^2).
ΔG_{bws}^{TOT} – Polar component of the free energy of interaction between bacterial cell b, materials' surface s and water w (mJ/m^2).
ΔG_{bws}^{TOT} – Free energy of interaction between bacterial cell b, materials' surface s and water w (mJ/m^2).
γ^{TOT} – Surface tension (mJ/m^2).
γ^{LW} – Apolar component of surface tension (mJ/m^2).
γ^{AB} – Polar component of surface tension (mJ/m^2).
γ^{+} – Electron acceptor parameter of the polar component of surface tension (mJ/m^2).
γ^{-} – Electron donor parameter of the polar component of surface tension (mJ/m^2).
NHLBI – National Heart, Lung and Blood Institute.
CDA – Cellulose Diacetate.
CDA-P – Phosphorylated cellulose diacetate.
CDA-D – Deacetylated cellulose diacetate.
LDPE – Low-density polythylene.

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