Vitamin E blended UHMWPE has the potential to reduce bacterial adhesive ability [*G.Banche and P. Bracco contributed equally to this work; ** AM Cuffini is the corresponding author]

Vitamin e blended uhmwpe has the potential to reduce bacterial adhesive ability?

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Running title: Vitamin E UHMWPE and bacterial adhesion

Abstract

Biomaterial-associated infection (BAI), a clinical significant problem often resulting in the implant septic failure, is initiated by the bacterial adhesion, mainly by *Staphylococcus epidermidis*. Ultra High Molecular Weight Polyethylene (UHMWPE) has been the material of choice in total joint replacement for many years; reducing the adhesion of *S. epidermidis* to the polymer could be a means to decrease infection. This interdisciplinary study examined the adhesion of 2 ATCC and one clinical strain of *S. epidermidis* to standard Polyethylene (PE), Vitamin E blended UHMWPE (VE-PE) and oxidised UHMWPE (OX-PE) after different incubation times: a significant ($p<0.01$) decrease in the adhered staphylococci on VE-PE and a significantly highest incidence of the dislodged biofilm bacteria on OX-PE was observed compared with that registered on PE. At ATR-FTIR spectroscopy before and after suspension in bacterial medium for 48 h, new absorptions were observed mainly in OX-PE, indicating adsorption of protein-like substances on the polymer surface. We hypothesized that the different hydrophilicity of surfaces with different chemical characteristics can influence protein adsorption and bacterial adhesion.

These results may have clinical implications concerning the prevention of septic loosening: the VE-PE could have the potential to reduce *S. epidermidis* adhesive ability if the preliminary data observed in these selected strains is further confirmed, as diversity among clinical strains is well known.

Keywords

UHMWPE, vitamin E, oxidation, bacterial adhesion, septic loosening

Introduction

The increasing use of biomaterials for the prosthetic substitution of tissues and organs led to the development of new diseases related to the biomaterials themselves.[1] Biomaterial-

associated infection (BAI) is a clinical significant problem: in total joint replacement (TJR) it causes a decrease in the success rate of the implant, often resulting in the septic failure, with the consequent need of highly expensive and health threatening revision surgery. BAI is initiated by the adhesion and subsequent growth of microorganisms to the implant surface.[2-4] The microorganisms frequently associated with infections involving synthetic blood contact devices are the coagulase negative staphylococci, most notably *Staphylococcus epidermidis.*[5] *S. epidermidis*, a major component of the normal bacterial flora of human skin and mucous membranes, is the leading cause of TJR infections, resulting in substantial morbidity and costs.[6,7] Bacterial adhesion to biomaterial surface is the first step in colonization and infection of an implant device; only the subsequent growth of adherent bacteria leads to the formation of biofilm. In addition once a monolayer of bacteria is formed on an implant surface, the performance of the device may be compromised. Therefore, the pathogenesis of biomaterial-centered infections is critically dependent on the initial bacterial adhesion to and early growth on a surface.[8] The initial adherence of bacteria to biomaterials is a process related to several factors. It involves both non-specific and reversible interactions, influenced by such factors as bacterial surface charge and hydrophobicity, and specific interactions mediated by various bacterial adhesins and host extracellular matrix proteins (e.g. fibrinogen, fibronectin and vitronectin).[7,9,10] In particular, bacterial adhesion entails different steps involving both physical and chemical forces for instance short-range forces, e.g. hydrophobic/hydrophilic interactions, and interactions between strongly localized functional groups on bacterial cells and material surfaces.[11] Following adhesion, some bacteria, i.e. *S. epidermidis*, can produce a structured community enclosed in an exopolysaccharide matrix resulting in a stable, mature biofilm, a survival mechanism for microorganisms that represents a significant virulence factor in medical-device-centred infection.[4,9]

It is well known that an interdisciplinary complex strategy is required in order to efficaciously deal with the BAI.[12] The best aseptic techniques cannot totally remove the risk of contamination, and perioperative antibiotic prophylaxis is not sufficient: reducing the adhesion of bacteria to the biomaterial could be an attractive means to reduce BAI.

In orthopaedics, prosthetic Ultra High Molecular Weight Polyethylene (UHMWPE), hereafter also polyethylene, is already a very important component of TJR; in order to improve its properties as bearing and articulating surface and eliminate the *in vivo* failure due to oxidation, [13-15] new vitamin E-containing UHMWPE has been developed.[16,17]

In this interdisciplinary study, we hypothesized that the adhesion of 2 ATCC *S. epidermidis*, one biofilm producing and one non-producing strain, and one clinical biofilm producing strain, could be different on polyethylenes characterized by various chemical surface features, such as standard UHMWPE, vitamin E blended UHMWPE and oxidised UHMWPE. To gain this goal, ATR-FTIR spectroscopy techniques, to characterise the chemical structure, and *in vitro* bacterial adhesion assays were used.

Methods

Biomaterials

Cylindrical specimens (height 14 mm, diameter 5 mm), suitable to fit in the vials and tubes, were punched out from compression molded sheets of standard GUR 1020 UHMWPE (PE) and of UHMWPE blended with 0.1% w/w vitamin E (VE-$PE_{0.1}$) or 0.5% w/w vitamin E (VE-$PE_{0.5}$) (MediTECH/Quadrant, Fort Wayne, IN, USA). Half of the standard PE samples were exposed to accelerated ageing in a ventilated oven at 95°C for 210 h, in order to obtain a third group of samples: oxidised UHMWPE (OX-PE).

In order to preserve the chemical and physical properties of the biomaterials, sterilization was achieved through 70% ethanol immersion followed by washing in sterile demineralised water.

The same manufacturing steps were used for all specimens, with the aim to obtain comparable surface roughnesses.

Surface roughness and water contact angle measurements

The surface roughness R_a of samples has been determined by using a roughness tester (Form Talysurf 50, Taylor-Hobson, Leicester, England). Six measurements were made for each sample and then averaged.

Static contact angle (CA) measurements were carried out by the sessile drop method using a KRÜSS DSA 100 (KRÜSS, Hamburg, Germany) apparatus and distilled water. The volume of the individual water droplet used for the static CA measurements was 5 µl. The water contact angle has been measured at four different locations on each sample to ensure homogeneity.

Scanning Electron Microscopy

The surface topography of the UHMWPE samples were imaged using a Leica Stereoscan 420 (Leica Cambridge Instruments, Cambridge, UK) scanning electron microscope (SEM). The samples were sputter-coated with approximately 30 nm of gold and directly observed in the microscope, operating at 15 kV, with magnifications ranging from 200 to 1000X. At least four independent images were taken from each sample.

FTIR spectroscopy

Attenuated Total Reflectance (ATR) - FTIR spectra of the sample surfaces before and after the adhesion assays were collected by using a FTIR Microscope (Spectrum Spotlight, Perkin-Elmer) equipped with an ATR objective (Germanium, incidence angle of the IR beam 45°, 100x100 εm^2 nominal surface area). The average penetration of the IR beam in the present conditions is in the order of 1 εm. Each spectrum was collected using 32 scans at a 4 cm^{-1} resolution.

The ATR spectra were corrected for the wavelength dependence of the beam penetration by the Atrcorr algorithm of Grams AI/8.0 (Thermo Electron corporation). One sample per group was kept in bacteria-free Tryptone Soya Broth (TSB; Basingstoke, Hampshire, England) at 37°C for 48 h and was used as a FTIR control to evaluate possible modifications on the biomaterial surface chemical properties, exerted by cultural medium components, that may influence bacterial adhesion. An average of 10 spectra was collected per each sample and the most representative are reported.

Bacterial cultures

Three *Staphylococcus epidermidis* strains were used for adhesion assays as representative pathogens of implant infection: *S. epidermidis* ATCC 35984, a well-characterized biofilm producing strain, selected because it causes most of infections related to orthopaedic implants and tested as more adherent and pathogenic bacterium; *S. epidermidis* ATCC 12228, a well-characterized biofilm non-producing strain tested as less adherent and pathogenic bacterium; a clinical biofilm-producing *S. epidermidis*, isolated from orthopaedic infection, selected because it has a priory demonstrated ability to establish clinically relevant biofilm infection on joint prothesis.

Each strain was cultured on Tryptic Soy Agar (TSA; Merck KGaA, Darmstadt, Germany); young colonies (18-24 h) were picked to approximately 3ï 4 McFarland standard and inoculated into cryovials containing both cryopreservative fluid and porous beads to allow bacteria to adhere (Microbank, Biomérieux; Rome, Italy). After inoculation, cryovials were kept at -80°C for extended storage.[18]

Adhesion assays

All *S. epidermidis* strains stored at -80°C were cultured over night at 37°C in TSB. After incubation, bacteria were re-suspended in 100 µl of TSB, harvested by 10 min centrifugation at 4,000 rpm and then diluted in TSB to 10^7 colony-forming-unit (CFU)/ml, as confirmed by

colony counts on TSA. The sterile biomaterials were placed in 2 ml of bacterial suspension and incubated by shaking for different incubation times (3, 7, 24, 48 h) at 37°C to allow *in vitro* bacterial adhesion, biofilm formation and its maturation. Controls represented by bacteria incubated in TSB in the absence of the biomaterial were also performed. The number of strongly bound bacteria remaining on the cylinders after incubation, was quantified after sonication (40 kHZ) for 7 min at 22°C in 1.5 ml of sterile NaCl 0.9% (Bieffe Medital S.p.A., Grosotto SO, Italy). The number of CFU in each sonication product was quantified by serial plate counts into TSA. This sonication protocol is ideal for dislodging biofilm bacteria without affecting bacterial viability.[19,20]

All the experiments were performed simultaneously for each biomaterial. The adherence experiments were assayed in triplicate and repeated a minimum of three times.

Statistical analysis

The adhesion assay results, expressed as CFU/ml, were analyzed by descriptive statistics (mean values and standard deviations) and tested by unpaired T-Student test, in order to highlight significant differences ($p<0.05$) between the different biomaterials. PE was used as control material.

Results

The average surface roughness R_a of the UHMWPE samples was 0.8 ± 0.2 μm. No significant difference of the surface roughness was found among the different samples: SEM micrographs representative of the investigated surfaces are shown in Fig. 1, and water contact angles measured on the three PE samples were 93±2, 91±2 and 84±2 degrees for PE, VE-$PE_{0.1}$ and OX-PE, respectively.

The ATR-FTIR spectra representative of the different polyethylene groups (PE, VE-$PE_{0.1}$ and OX-PE) before the adhesion assays are shown in Fig. 2. The only significant difference was observed in the spectrum of the aged group, OX-PE, where the multiple absorption centered

at 1718 cm^{-1}confirmed the presence of abundant oxidised products. In particular, the concentration of ketones can be estimated to be in the order of 1 mol/l.[13]

Adhesion assays were performed by using both biofilm producing and biofilm non-producing *S. epidermidis*. As shown in Fig. 3, after 3 h of incubation the initial bacterial adhesion of the biofilm producing ATCC *S. epidermidis,* on all the different assayed biomaterials occurred with rates of adhesion ranging from $2.95x10^5$ to $5.50x10^5$ CFU/ml. Even after 7 h of incubation for all three assayed biomaterials the staphylococcal adhesion rates were similar (from $1.44x10^6$ to $1.80x10^6$ CFU/ml) with no statistically relevant differences. In contrast, after 24 and 48 h of incubation a significantly different number of dislodged biofilm bacteria was achieved on different biomaterials, highlighting a bacterial adhesion significantly lower on VE-$PE_{0.1}$ compared with that obtained on both PE and OX-PE (Fig.3). In fact, a significant (p<0.01) decrease in the number of adhered staphylococci was observed on VE-$PE_{0.1}$ ($1.47x10^7$ CFU/ml at 24 h and $2.00x10^7$ CFU/ml at 48 h), compared with that registered on PE ($2.67x10^7$ CFU/ml at 24 h and $4.21x10^7$ CFU/ml at 48 h). In contrast, in the same experimental conditions, a considerable enhancement of adhered bacteria was seen for the OX-PE: a significantly highest incidence of bacterial adhesion was registered on OX-PE both at 24 h (p<0.05) and 48 h (p<0.01) with dislodged biofilm bacteria of $3.69x10^7$ CFU/ml and $7.25x10^7$ CFU/ml, respectively, compared with that observed on standard PE (Fig. 3).

A similar trend of adhesion was observed with the biofilm non-producing *S. epidermidis* and with the clinical biofilm-producing strain (data not shown): a significantly lower incidence of bacterial adhesion was detected on VE-$PE_{0.1}$ during the 48 h of incubation, even if the level of adherent bacteria remained at lower rates ($1.00x10^6$ and $2.00x10^5$ CFU/ml respectively; p<0.01) showing that initial adhesion on inert surfaces, which is considered to be manifestation of one of the major virulence factors of *S. epidermidis*, is strain-dependent.

All the adhesion tests carried out even on samples of VE-$PE_{0.5}$ indicated no differences compared to those obtained with VE-$PE_{0.1}$ (data not shown).

Each polyethylene group was further analyzed by ATR-FTIR, after suspension in TSB for 48 h. An average of 10 spectra was collected in different areas of each sample, showing a good reproducibility. No detectable differences were observed between VE-$PE_{0.1}$ and VE-$PE_{0.5}$. One spectra representative of each group is shown in Fig. 4. New absorptions centred at 3394, 1646, 1630, 1578, 1542 cm^{-1} were observed in the spectra of OX-PE. Only traces of these absorptions were found in the spectra of the standard UHMWPE and VE-$PE_{0.1}$.

Discussion

The role of UHMWPE wear in determining the failure of a TJR is well documented; in particular, it has been demonstrated that oxidation, induced by high energy radiation sterilization, was the main cause of the dramatic implant failures occurred in the last years.[13-15,21-23] As a consequence, many efforts have been made to improve the quality and the performance of UHMWPE, for example through crosslinking of the polymer, and, in particular, through the reduction or elimination of the oxidation by means of adequate sterilisation methods. The newest frontier is the addition of antioxidants, such as the alpha-tocopherol (Vitamin E).[24-28] Nevertheless, together with these new generations of polyethylenes, ñhystoricalò, oxidised material can still be found in clinical use.[29]

In this interdisciplinary study, the adherence of 2 ATCC and one clinical strain of *S. epidermidis* to polyethylenes with different chemical surface properties was tested. Besides the chemical and physical characteristics, the roughness of the material surface is also known to have the potential to influence the bacterial adhesion.[30] This is not the case of the samples tested in our experiments: thanks to the same manufacturing steps for all biomaterial groups, the measured surface roughness was similar for all samples and therefore it must have the same influence on the adhesion (Fig. 1).

The pathogenesis of prosthetic device infections is a complex process involving interactions between the pathogen, the biomaterial, and the host. The results of the adhesion assays on PE, VE-$PE_{0.1}$ and OX-PE samples, by using both biofilm producing and biofilm non-producing *S. epidermidis* strains, with different adhesion capacity, lead to the observation that the surface oxidation facilitates the bacterial adhesion. In fact, a significant highest ($p<0.01$) incidence of ATCC 35984 biofilm producing *S. epidermidis* adhesion was detected on OX-PE, whereas a significant decrease ($p<0.01$) in the number of dislodged biofilm staphylococci was registered on VE-$PE_{0.1}$, in comparison with that observed on PE, within both 24 h and 48 h of observation (Fig. 3). A similar trend was observed either for the ATCC 12228 biofilm non-producing *S. epidermidis*, tested as less adherent and pathogenic strain, or for the clinical biofilm-producing *S. epidermidis* strain, recently isolated from an orthopaedic infection, tested to compare its behavior with that of two reference *S. epidermidis* ATCC strains. These obtained results indicate that initial adhesion on inert surfaces, which is considered to be manifestation of one of the major virulence factors of *S. epidermidis*, is strain-dependent.

The clinical implication of these results has to be emphasized if we consider that orthopaedic implants that become infected are exposed to relatively small numbers of bacteria at the time of implantation, not the very large inoculum used in our *in vitro* assay and that our *in vitro* assays do not account or host defense and other *in vivo* factors. In fact host factors associated with susceptibility to infection make a major contribution to the outcome of an infectious process, and this cannot be determined by measuring bacterial load. The count of dislodged biofilm staphylococci at 24 h and 48 h has been performed taking into account that bacterial cells causing orthopaedic implant infection, at different growth phase, keep on expressing alternative adhesions or other virulence determinants.[7]

Moreover it has to be underlined that the effect of vitamin E does not depend on its concentration, since no significant differences in the bacterial adhesion between VE-$PE_{0.1}$ and

VE-$PE_{0.5}$ have been detected. These results are in agreement with those obtained by Adames et al:[31] the authors showed a reduced bacterial adhesion on VE-PE, compared to that on standard PE, and did not find any apparent correlation by testing the effect of three different Vitamin E concentrations.

Since it is generally accepted that the outermost cell surface plays a crucial role in bacterial binding to surfaces, as it interacts directly with the substratum surface,[6] we can assume that the different chemical surface properties of these different polyethylenes can play an active role in their behaviour. The bands observed in the ATR-FTIR spectra of control samples suspended in TSB cultural medium (Fig. 4) can be attributed to the absorptions of the amide I and amide II groups,[32-34] indicating adsorption of protein-like substances on the polymer surface, most likely coming from cultural medium, which contains proteins, lipids, and oligosaccharides. The extent of the proteins adsorption was maximized in OX-PE, whereas it was barely detectable in the other polyethylenes tested.

Oxidation of polyethylene is known to increase the surface hydrophilicity through the incorporation of polar, oxygen-containing, functional groups.[35,36] Accordingly, a decrease in contact angle was observed in our OX-PE group.

It must be pointed out that protein adsorption is often observed on retrieved prostheses.[37] Unfortunately, the adsorbed layer can easily be altered or partially removed by the multiple manipulation steps occurring in the operating theatre during revision surgery, making quite difficult a reliable quantification of the adsorbed species and a statistically significant correlation to the surface oxidation level. Moreover, diffusion of cholesteryl esters coming from the synovial fluid, which has been observed into polyethylene components after *in vivo* service,[37,38] may be another factor concurring to modify the surface polarity.

The influence of surface hydrophilicity on the ability of both binding proteins and directing the interaction between the adsorbed proteins and microorganisms has been widely studied. A

correlation has been established between increased surface hydrophilicity and enhanced capability of binding proteins through hydrogen bonding interactions.[36,39] Moreover, protein adsorption has been postulated to increase cellular adhesion playing a significant role in the attachment, proliferation, differentiation, and longer term stability of some cell types.[40] Several studies demonstrated that the initial bacterial adhesion to a biomaterial is modulated by non-specific interactions, including short-range forces, such as hydrophobic interactions. It is widely recognized that biomaterials exhibiting hydrophobic surfaces generally show the greatest bacterial adhesion,[5,6,9,41] but the presence of proteins has been shown to strongly affect this phenomenon. In particular, it has been observed that the presence of plasma proteins adsorbed on the biomaterial surface leads to a general reduction in bacterial adhesion,[5] but there are some other indications that, in these conditions, polar, hydrophilic surfaces may result in an increased bacterial adhesion and aggregation,[9] which is consistent with our observations.

However, in apparent contrast to this explanation, no significant differences in the oxidation level nor in the proteins adsorption were detected between PE and VE-$PE_{0.1}$, whereas a significantly lower staphylococcal adhesion rate was found on the latter.

Polyethylene is known to be extremely sensitive to oxidation: the surface, in particular, is exposed to processing stresses and to continuous contact with oxygen and it is certainly more affected. Poulsson et al.[36] found some oxygen contamination even on the surface of standard UHMWPE, when analyzed by X-ray Photoelectron Spectroscopy (XPS). Conversely, vitamin E, which is widely known to be an effective antioxidant for UHMWPE,[25-28] can inhibit both bulk and surface oxidation, resulting in a lower surface hydrophilicity. Hence, we first hypothesized that the difference observed in bacterial adhesion between PE and VE-PE might be influenced by the presence of a thin oxidised layer on the surface of PE, which cannot be detected by ATR-FTIR. In contrast to such a hypothesis, contact angle measurements showed

no significant variations in hydrophilicity, between these two groups suggesting that other, concurrent factors must be involved in the different staphylococcal adhesion. For example, a direct effect of vitamin E itself, even if present in very low concentration in the polyethylene matrix, on the bacterial adhesive ability cannot be ruled out.

Since it is widely known that BAI in TJR causes a decrease in the success rate of the implant with consequent enormous burden on the patients and high cost to the healthcare system, the results obtained in this interdisciplinary study may have important clinical implications concerning one aspect of the multifactorial septic loosening. We have postulated a correlation among polymer surface chemical characteristics, proteins adsorption and bacterial adhesion. Although adhesion is the first step in biomaterial colonization, it is the subsequent growth of adherent bacteria that leads to the formation of biofilms: within the limitations of the study, our *in vitro* results, regarding three different *S. epidermidis* strains within 48 h, underline that the VE-PE could have the potential to reduce bacterial adhesive ability and its antioxidant properties, due to the vitamin E addition, regardless of its concentration, may be one of the key point, even if some other factors may be involved.

It may be concluded that these data should be considered tentative pending results from planned additional studies that could provide a more thorough understanding of the whole process. Assuming that all microorganisms can potentially colonize an implant system, including Gram positive, Gram negative bacteria and mycetes, other isolates from orthopaedic infections such as *Staphylococcus aureus*, *Staphyloccosus aureus* methycillin resistant (MRSA) and *Candida albicans* will be mandatorily tested in order to drive a conclusion about the real clinical value. There is also a need for further investigation to increase our knowledge of this item, i.e., different types of crosslinked PE, different degrees of PE oxidation, direct effect of vitamin E on microorganisms.

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Figure legends

Fig. 1. SEM micrographs of a) PE, b) OX-PE, c) VE-$PE_{0.1}$ (original magnification 500X).

Fig. 2. ATR-FTIR spectra of the three polyethylene groups (——— PE, ·····VE-$PE_{0.1}$, ------ OX-PE) before the adhesion assays. In the spectrum of the aged oxidised group, the absorption centered at 1718 cm^{-1} confirmed the presence of abundant oxidised products.

Fig. 3. Differences in adhered *S. epidermidis* ATCC 35984 (CFU/mL) to the different assayed biomaterials (PE, VE-$PE_{0.1}$, OX-PE) within 48 h of incubation. Bacterial adhesion is significantly lower on VE-$PE_{0.1}$ compared with that obtained on both PE and OX-PE at 24 and 48 h.

Fig. 4. ATR-FTIR spectra of the three polyethylene groups (·····PE, ------VE-$PE_{0.1}$, ——— OX-PE) after suspension in TSB for 48 h. New absorptions centered at 3394, 1646, 1630, 1578, 1542 cm^{-1} were observed in the spectra of OX-PE; only traces of these absorptions were found in the spectra of the other two groups.

Figure 1.

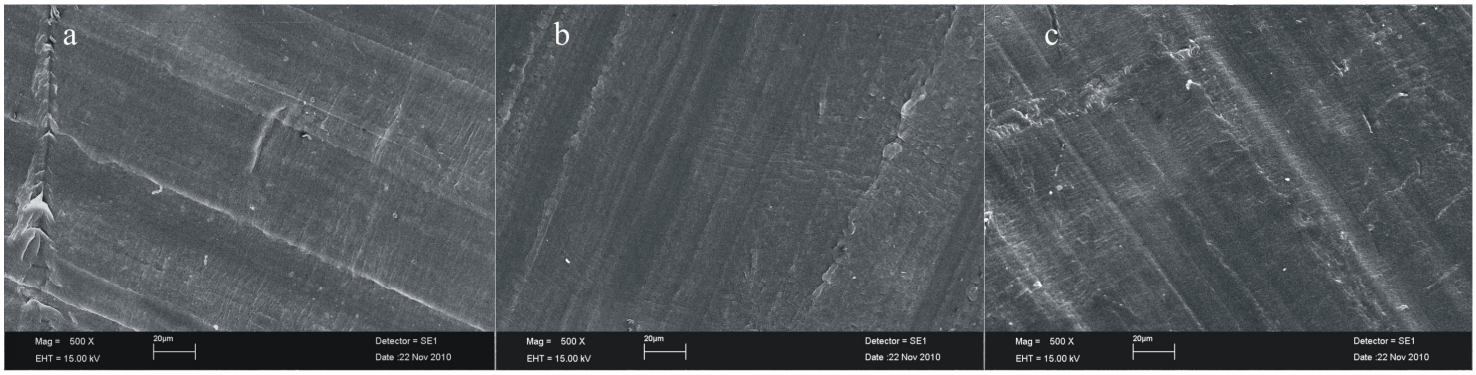

a
Mag = 500 X
EHT = 15.00 kV
20µm
Detector = SE1
Date :22 Nov 2010
b
Mag = 500 X
EHT = 15.00 kV
20µm
Detector = SE1
Date :22 Nov 2010
c
Mag = 500 X
EHT = 15.00 kV
20µm
Detector = SE1
Date :22 Nov 2010

Figure 2.

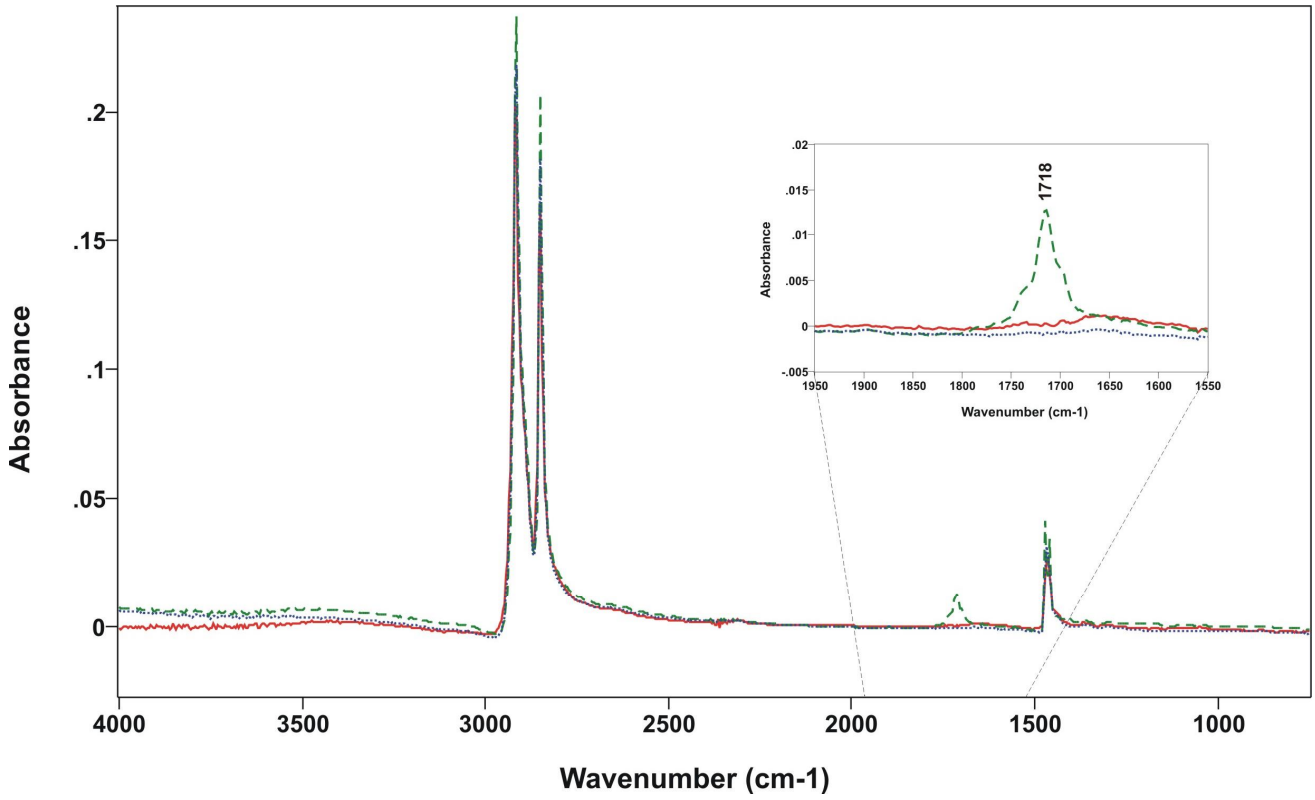
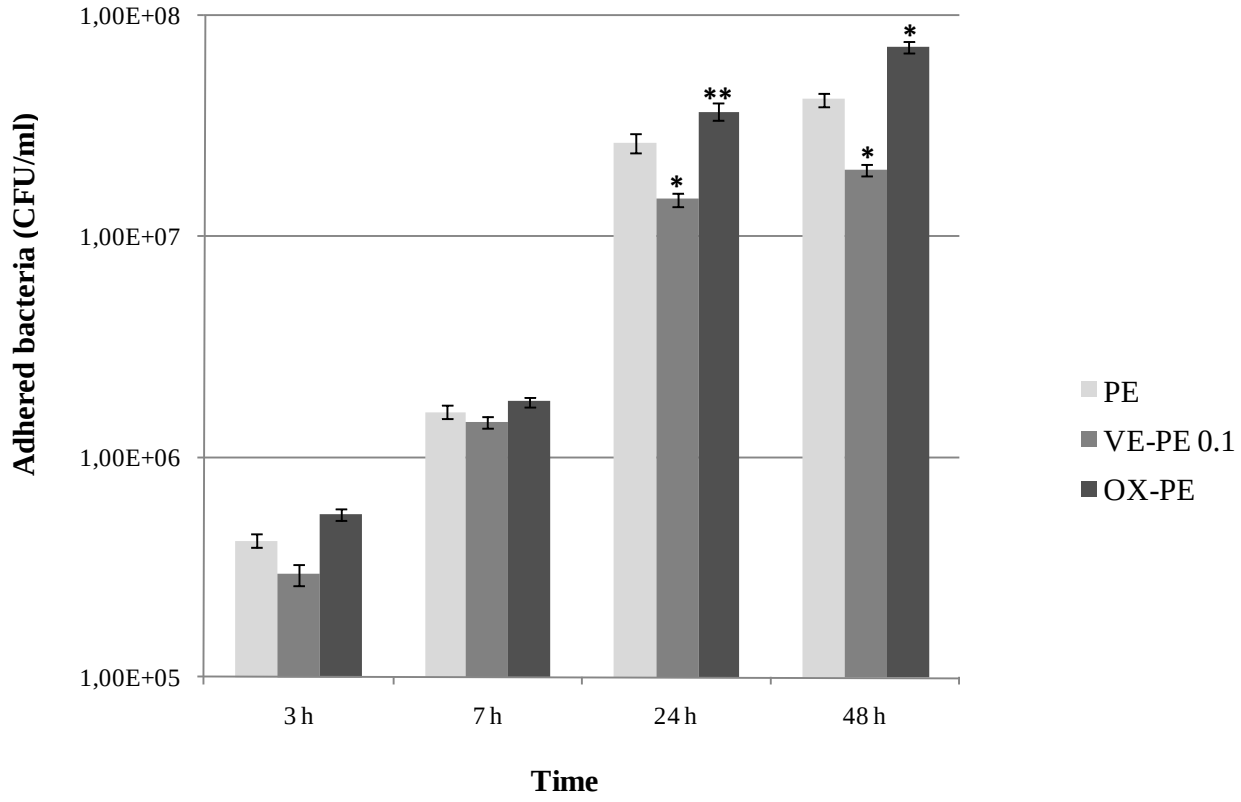
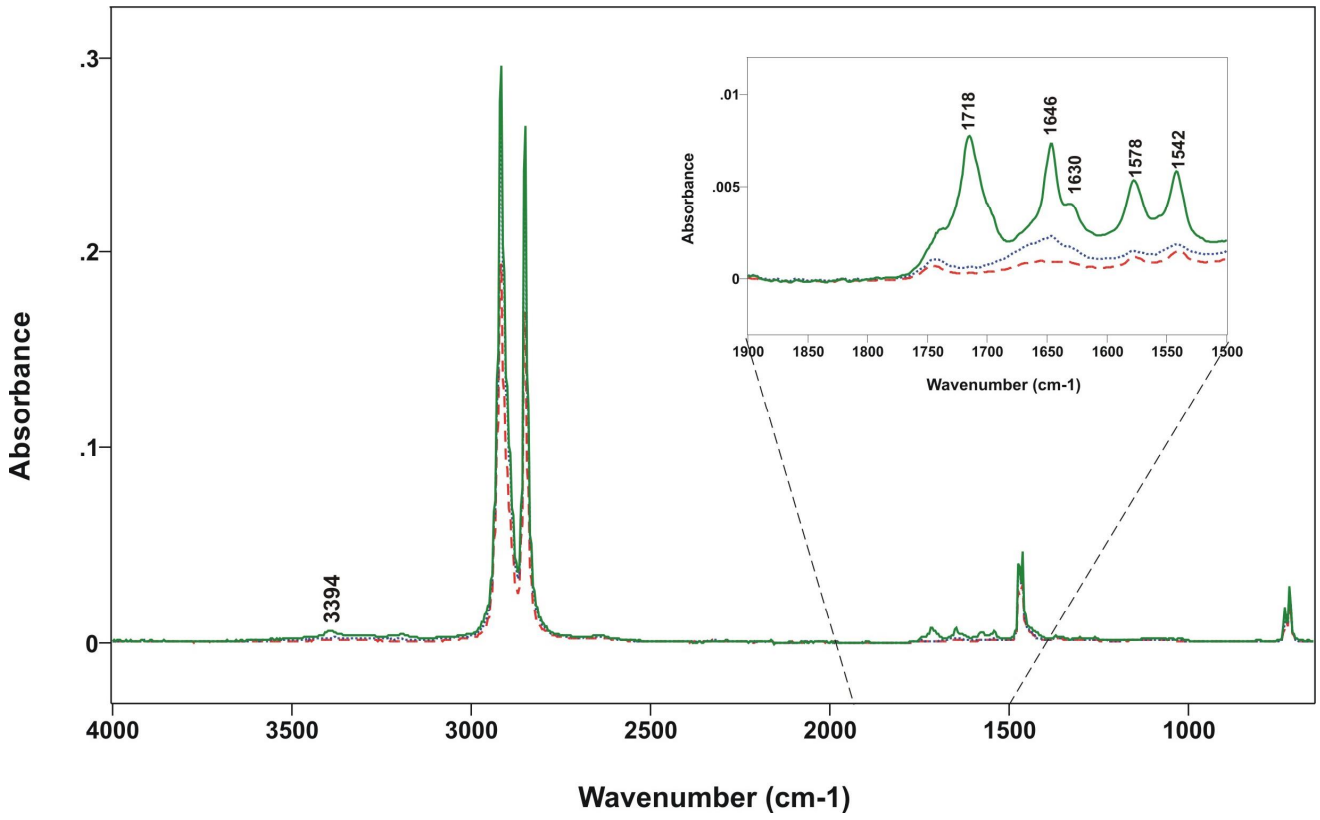

Absorbance
.2
.15
.1
.05
0
4000
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)
1718
Absorbance
.02
.015
.01
.005
0
-.005
1950
1900
1850
1800
1750
1700
1650
1600
1550
Wavenumber (cm-1)

Figure 3.



***** Significantly different from PE ($p<0.01$);
****** Significantly different from PE ($p<0.05$)

Figure 4.


Absorbance
.3
.2
.1
0
3394
4000
3500
3000
2500
2000
1500
1000
Wavenumber (cm-1)
Absorbance
.01
.005
0
1718
1646
1630
1578
1542
1900
1850
1800
1750
1700
1650
1600
1550
1500
Wavenumber (cm-1)