J Mater Sci: Mater Med (2011) 22:1701–1706
DOI 10.1007/s10856-011-4340-5

Bacterial adherence on UHMWPE with vitamin E: an in vitro study

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Received: 24 November 2010 / Accepted: 7 May 2011 / Published online: 15 May 2011

Abstract Orthopaedic materials may improve its capacity to resist bacterial adherence, and subsequent infection. Our aim was to test the bacterial adherence to alpha-tocopherol (frequently named vitamin E, VE) doped or blended UHMWPE with *S. aureus* and *S. epidermidis*, compared to virgin material. Collection strains and clinical strains isolated from patients with orthopaedic infections were used, with the biofilm-developing ability as a covariable. While collection strains showed significantly less adherence to VE-UHMWPE, some clinical strains failed to confirm this effect, leading to the conclusion that VE doped or blended UHMWPE affects the adherence of some *S. epidermidis* and *S. aureus* strains, independently of the concentration in use, but the results showed important intraspecies differences and cannot be generalized.

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1 Introduction

Bacterial adherence is the initial step in biofilm development. However, limited information is available regarding the adherence of microorganisms that cause orthopaedic infection to biomaterials currently used in orthopaedic implants. Furthermore, the different adherence of these microorganisms to metals and polymers has been rarely studied [1]. Yet it is important to understand the differences between orthopaedic biomaterials to bacterial adherence, and to devise new methods for protecting these materials from such phenomenon.

Of particular interest is ultra-high molecular weight polyethylene (UHMWPE). Up to 80% of total hip replacements in the Canadian registry [2] incorporate components of UHMWPE, and the vast majority of total knee replacement systems use it. The incidence of infection in total joint replacements averages 2%, only in the USA a total of 12,000 new total joint infections per year have been projected [3]. The necessity of understanding and alleviating this problem becomes evident.

Alpha-tocopherol (currently named vitamin E) has been recently incorporated to UHMWPE to decrease oxidation and subsequent material degradation by mixing with the grade before consolidation [4–6] or by diffusion into the conformed material [7, 8]. In both, the resistance to oxidation was confirmed and vitamin E (VE) remains within the material [9]. As the innovation is widespread, an appealing supplementary clinical benefit would appear if the susceptibility of microorganism adherence to

polyethylene, previously studied by our group [10], was altered by the addition of alpha-tocopherol. Particularly, when potential effects of alpha-tocopherol on chronic infection are currently being investigated, favoring the antibiotic action through cellular immunity enhancement [11] and cellular redox state [12]. The use of alpha-tocopherol in UHMWPE as a method to minimize material oxidization may also change the surface of the substratum and the bacterial adhesion process, thus limiting the extent of subsequent infection. With this hypothesis, we set the aims of the present study in the investigation of the adherence of the most frequently isolated bacterial species from orthopaedic infections (*Staphylococcus aureus* and *Staphylococcus epidermidis*) in alpha-tocopherol doped or blended UHMWPE, compared to virgin material. For this, we investigated different alpha-tocopherol concentrations with both collection and also with strains isolated from orthopaedic infections.

2 Methods

2.1 UHMWPE

The raw material used in the first part of the study was a compression molded sheet of GUR 1050 UHMWPE (Orthoplastic Ltd., Lancashire UK), from which 3 mm thick and 20 mm diameter discs were machined. All discs were grounded and polished using SiC papers to a surface roughness of $R_a = 0.80 \pm 0.05$ μm, measured by using a confocal microscope Sensofar PLm 230D (Sensofar, Barcelona, Spain). Vitamin E was introduced into UHMWPE by diffusion, soaking the disc in a bath of VE (α-tocopherol, Sigma-Aldrich Chemicals, USA) at 120°C in nitrogen gas atmosphere. After the diffusion process, the vitamin E was homogenized at 120°C during 24 h. Two alpha-tocopherol concentrations were prepared, and gravimetric changes confirmed a VE content of 3 and 0.4 wt% in the discs. Vitamin E was also detected in FTIR spectra of infused UHMWPE sections by means of Perkin-Elmer model 1600 spectrometer (range: 4,000–400 cm^{-1}; 64 repeat scans per sample location; resolution 2 cm^{-1}), as the vibrational band centered at 1,262 cm^{-1}. All discs were sterilized with gas-plasma 10 days before the experimentation.

The second part of the study was performed in a commercially available GUR 1020 UHMWPE with vitamin E, at a concentration of 1,000 ppm (0.1%) obtained by blending (Meditech, Fort Wayne, Indiana, USA). Specimens of 228 ± 13 μm thick and of 1 cm^2 (1×1 cm) were cut from a sheet. The average roughness measured by a confocal microscope was 0.42 ± 0.15 μm. The vitamin E concentration was also detected by ultraviolet UV spectroscopy using an Aligent 8453 Diode Array spectrophotometer at a range of 1,100–190 nm. Spectra of vitamin-E infused UHMWPE sections revealed the presence of a noticeable peak at 290 nm. GUR 1020 UHMWPE sheets without vitamin E from the same manufacturer were used as controls.

Contact angle (CA) measurements were repeatedly performed (7 measurements per 2 material samples) with a CAM100 optical system (KSV Instruments, Helsinki, Finland) using deionized water and methylene iodide as polar and non polar liquids, respectively. Surface free energy and their polar and dispersive components were obtained using the method described by Owens et al. [13]. Identification of the functional groups at the surface of the UHMWPE samples before and after sterilization processes was performed by X-ray Photoelectron Spectroscopy (XPS). Measurements were performed using a PHOIBOS100-5MCD (SPECS GmbH, Berlin, Germany) electron spectrometer with unmonochromatised MgKα radiation. Pass energies of 20 or 90 eV were used for high resolution or survey spectra acquisition, respectively.

2.2 Bacterial strains

Collection strains of biofilm-producing *S. aureus* 15981, provided by Dr. Lasa [14], and *S. epidermidis* ATCC 35984 were used in the first set of experiments with different VE concentrations.

Clinical strains of both species were also tested in the second set of experiments with the blended VE UHMWPE. Five *S. aureus* clinical strains and four *S. epidermidis* strains, isolated from prosthetic joint infections using a sonication protocol previously described [15], were used apart from the collection strains. These clinical strains were identified using conventional microbiological techniques. Quantification of the biofilm-forming ability of the strains was tested and graded from 0 to 3 using the Stepanovic method [16]. All the strains were frozen in skim milk at −80°C until the experiments were performed.

2.3 Adherence study

Five samples of each experimental (3 and 0.4% doped) and 0.1% blended VE UHMWPE were tested for each bacterial strain. After overnight culture in Tryptic-soy broth, bacteria were harvested by 20 min centrifugation at $3{,}500 \times g$, and washed twice with sterile Phosphate Buffered Saline (PBS). Bacteria were then suspended and diluted in PBS to a concentration of 10^8 colony-forming units (CFU)/ml. The biomaterial samples were placed in this bacterial suspension and incubated for 90 min at +37°C. After the incubation, specimens were rinsed twice with PBS, and sonicated during 5 min in equal volume of PBS. The number of adhered bacteria was quantified by 1:10 serial plate counts.

2.4 Statistics

ANOVA was used to compare the results of CFU quantification for *S. aureus* and for *S. epidermidis*, with post-hoc test of Bonferroni. When studying clinical strains, paired t-test was performed to independently compare the adherence of *S. aureus* and *S. epidermidis* on virgin and VE UHMWPE where Kolmogorov test confirmed a normal distribution of the data, and Wilcoxon when not. Mann–Whitney test was used to compare the adherence of each particular strain in virgin versus VE UHMWPE. The Stepanovic grading was investigated as a grouping variable through the Kruskall-Wallis test for *S. aureus* and *S. epidermidis*, and as a covariable through one factor ANOVA and Bonferroni. SPSS 17.0 was used as statistical package (SPSS Inc., Chicago, IL).

3 Results

The incorporation of vitamin E significantly increased the surface hydrophobicity in GUR1050 VE doped UHMWPE (Table 1). In GUR1020 UHMWPE samples (with the lowest VE concentration in the study, 0.1%), water CA showed no significant differences between samples with and without VE (Table 1). The CA using diiodomethane in raw GUR1050 UHMWPE samples slightly increased when the polyethylene was doped with VE, but in GUR1020 samples, the incorporation of VE decreased the diiodomethane CA (Table 1). From these data, the total surface free energy, γ, was lower with more VE in GUR1050. In all cases, the polar component, γ_p, was very low (below 2 mN/m). Therefore, only the dispersive component γ_d contributed to the total surface energy observed in the samples, as summarized in Table 1.

The surface chemical analysis obtained from the XPS measurements showed identical surface composition before and after gas-plasma sterilization process in all samples, confirming that the sterilization process did not incorporate functional groups to the surface. Figure 1 shows typical spectra (survey, C 1s, and O 1s regions) obtained from samples with and without VE. As a general trend, the spectra showed signals from the carbon and the oxygen atoms at the sample surface. The main C 1s carbon peak was set at 285.0 eV for binding energy calibration. Under these conditions, the O 1s oxygen peak was detected at 532.2 eV. This is consistent with the presence of the hydroxyl groups (–OH) at the surface. The [O]/[C] atomic ratio (with [O] and [C] the oxygen and carbon atomic concentrations, respectively) was 0.06 ± 0.01 in VE doped samples, disregarding the doping level. In case of the UHMWPE virgin material, $[O]/[C] = 0.02 \pm 0.01$. Besides, the shape of the O 1s survey spectra (inelastic background in the lower kinetic energy side of the spectra) points to an homogeneous in-depth distribution of the oxygen species incorporated to the VE doped samples [17], while in the case of the undoped samples, these –OH groups are mainly located at the topmost ($\sim$2 nm) surface.

In the experiments with the collection strains on GUR1050 with or without doping with VE, no significant differences were observed in the adherence of *S. aureus* (ANOVA, $P = 0.561$). Mann–Whitney test showed non-significant differences in the adherence of *S. aureus* on UHMWPE with 3% VE ($P = 0.222$) or with 0.4% VE ($P = 0.421$), versus UHMWPE without VE. When the experiment was performed with the ATCC collection strain of *S. epidermidis*, significant differences in the adherence among series was detected with ANOVA ($P = 0.001$), and post-hoc tests confirmed differences between control and VE doped GUR1050 UHMWPE. The Mann–Whitney test showed that the difference between GUR1050 UHMWPE without and with 3% VE concentration was significant ($P = 0.008$), and that the difference stood when the VE concentration was lowered to 0.4% ($P = 0.008$), as seen in Fig. 2.

When collection and clinically isolated strains adherence was studied on the 0.1% VE blended GUR1020 UHMWPE, no significant differences were obtained when

Table 1 Water and diiodomethane contact angles, total surface energy γ, and their polar γp and dispersive γd components corresponding to virgin and VE doped/blended UHMWPE samples

UHMWPE samples	Water CA (°)	CH_2I_2 CA (°)	γ (mN/m)	γ_p (mN/m)	γ_d (mN/m)
GUR1020 Virgin	90 ± 5	61 ± 2	28 ± 1	3 ± 2	25 ± 2
GUR1020 0.1% VE	88 ± 3	54 ± 5	32 ± 2	3 ± 2	29 ± 4
GUR1050 Virgin	100 ± 6	51 ± 3	35 ± 4	0 ± 1	35 ± 3
GUR1050 0.4% VE	114 ± 7	51 ± 3	10 ± 4	1 ± 1	9 ± 5
GUR1050 3% VE	115 ± 3	58 ± 2	11 ± 5	1 ± 1	11 ± 6

Mean and standard deviation of the reported values

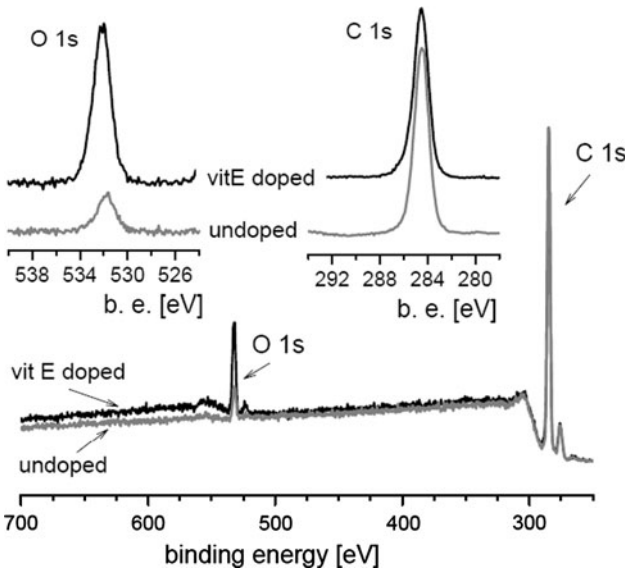

Fig. 1 Typical XPS survey spectra, and high resolution O 1s and C 1s signals corresponding to undoped (*grey lines*) and VE doped (*black lines*) UHMWPE samples

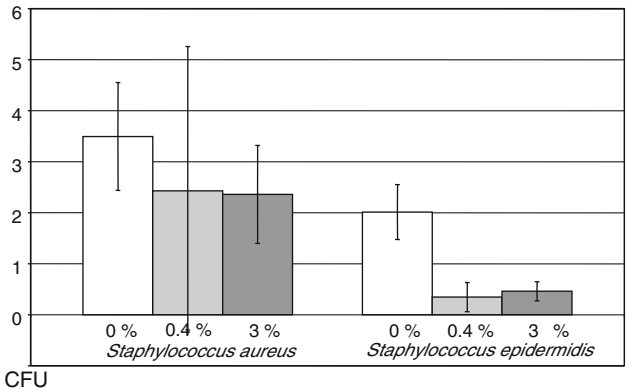

Fig. 2 CFU (mean, *error bars* for SD) quantified after adherence on UHMWPE without VE, with 0.4 and 3% VE, for collection *S. aureus* and collection *S. epidermidis* ($n = 5$)

comparing in a paired t-test the culture counts of all *S. aureus* strains ($P = 0.107$) or those of *S. epidermidis* strains ($P = 0.252$) on virgin versus VE-UHMWPE. However, the results were highly variable among individual strains. One factor ANOVA and Kruskal–Wallis tests, used in view of the $n = 5$ repetitions of the adherence experiment per strain, showed that culture counts significantly differed among strains of *S. aureus* on virgin ($P = 0.010$) and VE ($P = 0.014$), while in the case of *S. epidermidis*, differences were significant among strains on VE ($P = 0.003$). When each strain was investigated on GUR1020 UHMWPE without VE or blended with 0.1% VE, Mann–Whitney test showed that *S. aureus* collection strain significantly decreased its adherence in the presence of VE (Table 2), but not in the case of clinical strains. *S. epidermidis* collection strain did not significantly modify its UHMWPE adherence in the presence of VE in this material, but it did in two clinical strains (one increasing, one decreasing) of the four under investigation (Table 2).

The investigation on the covariable of biofilm-forming ability (Stepanovic grading) showed that this grade did not influence changes in adherence of *S. aureus* strains on VE UHMWPE or virgin material, but did in the case of *S. epidermidis* (Tables 3, 4). When the ANOVA test with Bonferroni post hoc was used to clarify the effect of multiple comparisons of culture counts among Stepanovic graded strains, significant differences were found in the adherence on virgin UHMWPE and VE UHMWPE as shown in Tables 3 and 4.

4 Discussion

Significant efforts, such as VE doping or blending, have been placed in the control of UHWMPE oxidation to decrease material and implant failure in total joint replacements. But the burden of total joint replacement failure is markedly related to infection, particularly with biofilm formation after bacterial colonisation of the biomaterial. In this study, VE affected the adherence of *S. epidermidis* and *S. aureus* on UHMWPE, although in a variable way in the different species and strains. This finding stood with different VE concentrations in the case of *S. epidermidis*, and there was no apparent direct relationship to VE dosage in GUR1050.

The mechanism by which VE may affect the bacterial adherence to UHMWPE is currently unknown. Pure VE does not influence bacterial growth, and the interest is placed in the material surface. Microstructural changes affecting surface energy and OH– content may influence strain differences in the adherence, although this is hypothetical. Besides, blending and diffusion temperature differences may also slightly modify oxidation differently, although XPS do not show significant manifestations. Floating bacteria are highly adaptive to colonize the free biomaterial surface with an initial phase of non-specific, reversible physical contact, with long-range interactions distance (more than 150 nm) based on Brownian motion, Van der Waals intermolecular attraction forces, gravitation, and surface electrostatic and hydrophobic forces. Secondarily, interactions occur at short-range distance (less than 3 nm), based on hydrogen bonding, ionic, dipole, and hydrophobic interaction [18, 19]. In this study, we correlated surface free energy and bacterial adherence, as the lowest bacterial adherences were obtained with surfaces showing lowest surface energy (i.e., the more hydrophobic), as seen in Table 1 and Fig. 2. Our results were consistent to the trends reported by Hallab et al. [20] for

Table 2 Comparison of adherence per strain, GUR1020 UHMWPE without VE versus 0.1% VE blended to GUR1020 UHMWPE (Mann–Whitney test, $P > 0.05$)

Clinical strain	Microorganism	Culture from sonicated material after adherence to virgin UHMWPE (mean ± SD)	Culture from sonicated material after adherence to VE UHMWPE (mean ± SD)	Significance
1	*S. aureus*	4.33 ± 4,58	1.23 ± 0.39	0.114
2	*S. aureus*	0.75 ± 0.40	0.74 ± 0.27	0.690
4	*S. aureus*	3.59 ± 4.25	1.32 ± 0.51	0.200
61	*S. aureus*	1.39 ± 0.81	1.28 ± 0.81	0.886
95	*S. aureus*	0.08 ± 0.04	0.27 ± 0.38	0.343
Collection strain	*S. aureus*	1.11 ± 0.34	0.12 ± 0.10	0.036*
53	*S. epidermidis*	0.38 ± 0.36	3.20 ± 1.38	0.008*
55	*S. epidermidis*	1.21 ± 0.68	5.61 ± 6.67	0.886
74	*S. epidermidis*	1.31 ± 1.07	1.58 ± 0.74	0.686
101	*S. epidermidis*	2.65 ± 1.51	0.07 ± 0.05	0.008*
Collection strain	*S. epidermidis*	0.64 ± 0.17	0.75 ± 0.56	0.841

Table 3 Descriptive culture counts of *S. epidermidis* clinical strains grouped by Stepanovic grade (number of cultures *n*, mean culture counts M, standard deviation SD)

UHMWPE	Stepanovic grade	*n*	M	SD
Virgin	0	5	2.64700	1.515188
	1	8	1.26375	0.835394
	2	5	0.38480	0.357527
VE	0	5	0.0700	0.04899
	1	8	1.8771	1.29404
	2	5	3.2020	1.37905

Table 4 Comparison of *S. epidermidis* culture counts of strains with different Stepanovic grading for the two studied materials (ANOVA with Bonferroni post hoc test, significance with $P > 0.0125$)

Polyethylene	Compared Stepanovic grades	Bonferroni significance ($P > 0.0125$)
Virgin UHMWPE	0 vs. 1	$P = 0.079$
	1 vs. 2	$P = 0.416$
	0 vs. 2	$P = 0.007$*
VE UHMWPE	0 vs. 1	$P = 0.041$
	1 vs. 2	$P = 0.106$
	0 vs. 2	$P = 0.002$*

fibroblast adhesion. This correlation was more pronounced for *S. epidermidis*. Adhered *S. epidermidis* are more resistant to cephalosporins, thus associating adherence and antibiotic resistance [21]. Then, adherence may be a discriminant factor in the risk of infection by certain *S. epidermidis* strains.

As the relative adherence to a material may be thus associated to strain differences, we tested several clinical strains randomly chosen from the bank of isolates from patients with orthopaedic infection. With significant variability among these clinical strains, the decreased adherence of the collection *S. epidermidis* to the VE doped UHMWPE was not confirmed with the 0.1% blended VE UHMWPE, and was only confirmed in one of the four clinical strains used to further test this material. On the opposite, the decreased adherence of the collection *S. aureus* observed in the 0.1% blended VE polyethylene was not confirmed in the studied five clinical strains. As corroborated in this study, the evaluation of clinical strains is mandatory to ascertain the consistency of adherence studies results, although material surfaces may also alter this adherence capability.

The ability to form biofilm in these strains per Stepanovic grading showed a paradoxal effect on UHMWPE adherence, being lower in strains with higher Stepanovic grades. Other studies of biofilm formation on UHMWPE doped with vitamin E are on the way to follow this issue.

Potential effects of VE on clinical infection under investigation include immunomodulation in chronic diseases [11] and cellular redox state changes [12]. Surface redox states modulate adherence and may affect certain microorganism strains, yet unclear, as we previously confirmed that UHMWPE surface changes affect bacterial adherence [10]. Thus, VE modifying the surface properties of UHMWPE may affect bacterial adherence. Following this hypothesis, we could only confirm that VE significantly affected the adherence of some strains, but not of others. Nevertheless, VE incorporates a biological surplus in the modified material that was effective in reducing the adherence of some strains of *S. aureus* (collection strain in our study) and *S. epidermidis* (collection strain in VE-doped 1050GUR, one out of five clinical strain in 0.1%

VE-blended 1020 GUR) despite the absence of specific antibacterial effect of vitamin E tested by microdilution (data not shown).

This finding could introduce an added value to vitamin E doped UHMWPE. The use of clinical strains is mandatory in an in vitro study on the very early stage of the colonization process, because of intrinsic intra and interspecies variability among different bacterial genera, being a study limitation the number of clinical strains to be tested.

Acknowledgments The work is funded by the Spanish Ministry of Education (grant MAT2006-12603-CO2-O2), Comunidad de Madrid (grants S-0505/MAT0324, S2009/MAT-1472), and Ministry of Science (grant CONSOLIDER-INGENIO 2010 CSD 2008-0023 FUNCOAT). DMM was granted by Fundación Conchita Rábago de Jiménez Díaz. The authors thank Dr. J.J. Granizo, epidemiologist, for his help with statistical analysis.

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