Do Crosslinking and Vitamin E Stabilization Influence Microbial Adhesions on UHMWPE-based Biomaterials?

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Abstract

Background Microorganism adhesion on polyethylene for total joint arthroplasty is a concern. Many studies have focused on vitamin E-stabilized ultrahigh-molecular-weight polyethylene (UHMWPE), whereas first-generation, highly crosslinked UHMWPE, which is the most commonly used in clinical practice, has been scarcely evaluated.

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The in vitro adhesion assays were performed at the Department of Public Health and Pediatrics, University of Torino, Turin, Italy; the physicochemical biomaterial characterization was performed at the Department of Chemistry, University of Torino, Turin, Italy.

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Questions/purposes We aimed (1) to compare the adherence of *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with virgin (untreated) UHMWPE (PE) and crosslinked UHMWPE (XLPE); (2) to correlate the results with the biomaterial surface properties; and (3) to determine whether the decreased adhesion on vitamin E-stabilized UHMWPE (VE-PE) previously recorded for bacteria can also be confirmed for *C. albicans*.

Methods Microbial adhesion of biofilm-producing American Type Culture Collection (ATCC) and clinical strains on XLPE and VE-PE were compared with PE at 3, 7, 24, and 48 hours of incubation and quantified, as colony forming units (CFU)/mL, using a sonication protocol. Sample surfaces were characterized by scanning electron microscopy, roughness and contact angle measurements, attenuated total reflection-Fourier transform infrared spectroscopy, and x-ray photoelectron spectroscopy (XPS) to reveal qualitative differences in surface composition and topography that could influence the microbial adhesion. The results were analyzed by descriptive statistics and tested by unpaired t-tests.

Results All microorganisms, both ATCC and clinical strains, showed lower adhesion ($p < 0.05$) on XLPE with adhesion percentages ranging from 18% to 25%, compared with PE with adhesion percentages ranging from 51% to 55%, after 48 hours. Only the ATCC *S. epidermidis* showed a reduced adhesion profile even at 3 hours (adhesion ratio of 14% on XLPE versus 50% on PE) and 24 hours (19% on XLPE versus 55% on PE) of incubation. ATCC and clinical *C. albicans* were less adherent to XLPE than to PE ($p < 0.05$) showing even at the earlier incubation time points adhesion values always of 10^3 CFU/mL and 10^4 CFU/mL, respectively. Roughness and contact angle were $0.8 \pm 0.2 \mu\text{m}$ and $92^\circ \pm 3^\circ$, respectively, with no

differences among samples. Qualitative differences in the surface chemical composition were revealed by XPS only. A confirmation of the decreased adhesion on VE-PE respect to PE was also registered here for *C. albicans* strains ($p < 0.05$).

Conclusions Vitamin E stabilization and crosslinking of UHMWPE are capable of reducing microbial adhesion. Further studies are needed to fully elucidate the mechanisms of modulation of microbial adhesion to medical-grade UHMWPE.

Clinical Relevance Our results suggest that VE-PE and XLPE may have an added benefit of being more resistant to bacterial adhesion, even fungal strains.

Introduction

Ultrahigh molecular-weight polyethylene (UHMWPE) components, articulating against a metallic or ceramic counterface, are the most commonly used bearing biomaterials in total joint arthroplasty. To address the problems of wear and failures associated with oxidative degradation [10, 12, 29, 33], the original UHMWPE has been improved through crosslinking for improved wear resistance and thermal treatments or addition of antioxidants to address oxidation with excellent clinical results, both long- and short-term [5, 6, 8, 17, 18, 21, 26]. Crosslinked and thermally treated UHMWPE is now the most clinically used UHMWPE, whereas antioxidant (for example, vitamin E), crosslinked UHMWPEs have been introduced into clinical practice over the last 7 years [16].

Septic failure of prosthetic implants causes patient morbidity and mortality and imposes a large economic burden on society. Microorganism attachment to prosthetic surfaces has been identified as the first step in biomaterial-associated infection pathogenesis [13, 32]. Microbial adhesion on biomaterial implant surfaces depends on the physicochemical interactions between substratum and microorganisms as well as the physical properties of the biomaterial surface (roughness, hydrophobicity, surface energy, electrostatic charge, and coating), which can strongly influence the adhesion process [4, 20, 27, 30, 34]. Accordingly, the adhesive behavior of microorganisms on UHMWPE recently has been studied. Several studies have focused on the effects of the antioxidant vitamin E and showed that vitamin E may have the potential to reduce bacterial adhesion. Furthermore, some differences related to specific bacteria behaviors that affect their potential adhesion have been reported [1, 2, 13, 15, 23].

Substantial effort is being made by biomaterial scientists to improve both in vivo performance and biomaterial resistance to microbial adhesion of crosslinked UHMWPE materials, but data about microbial adhesion on either crosslinked UHMWPE or vitamin E-added UHMWPE

compared with conventional (gamma sterilized) or virgin (untreated) UHMWPE are scarce. In addition, the majority of studies have only evaluated the most commonly involved bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*) and have not considered other relevant microorganisms that are potential sources of infection [14, 22].

To begin to address the lack of knowledge, the main purposes of our interdisciplinary study were (1) to compare the adherence of *S. epidermidis*, *S. aureus*, *Escherichia coli*, and *Candida albicans* with virgin (untreated) UHMWPE (PE) and crosslinked UHMWPE (XLPE); (2) to correlate the results with the biomaterial surface properties; and (3) to determine whether the decreased adhesion on vitamin E-stabilized UHMWPE (VE-PE) previously recorded for bacteria can also be confirmed for *C. albicans*. We tested these microorganisms because they are known to show a certain capacity to adhere and easily form biofilm on a great deal of biomaterials and to be involved in biomaterial-associated infection [1, 2, 32].

Materials and Methods

Biomaterials

Compression-molded sheets of virgin (untreated) GUR 1020 UHMWPE (PE) and of UHMWPE blended with 0.1% w/w vitamin E (VE-PE) were provided by Mediatech/Quadrant (Fort Wayne, IN, USA). Half of the virgin PE samples were e-beam irradiated at 75 kGy and then remelted at 150° C to obtain highly XLPE. Cylindrical specimens (height, 14 mm; diameter, 5 mm) for the adhesion assays were punched out from the sheets by means of a die cutter. All specimens were subjected to identical preparation steps to ensure comparable surface roughness. To preserve the materials' chemical and physical integrity, sterilization was achieved through 70% ethanol immersion followed by washing in sterile, demineralized water.

Surface Roughness and Water Contact Angle Measurements

Surface roughness (R_a) and water contact angle were determined as reported previously [1, 2]. Briefly, surface roughness was measured by using a roughness tester (Form Talysurf 50; Taylor-Hobson, Leicester, UK). Six independent measurements per sample were taken on three samples per group and then averaged. Static contact angle (CA) measurements were performed using a DSA 100 (KRÜSS, Hamburg, Germany) apparatus and distilled water. CA was

measured at four independent locations per sample on three samples per group and then averaged.

Scanning Electron Microscopy

To ensure reasonably homogeneous surface topographies, the samples were also qualitatively observed using a Stereoscan 420 scanning electron microscope (SEM) (Leica Cambridge Instruments, Cambridge, UK). Samples were sputtercoated with gold and the microscope was operated at 15 kV with magnifications from $\times 200$ to $\times 1000$. At least four independent images were taken from each sample.

Fourier Transform Infrared Spectroscopy

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) was used to assess the initial chemical characteristics of the sample surfaces and to monitor possible changes that occurred during the adhesion assays. The spectra (32 scans/spectrum, 4 cm^{-1} resolution) were collected before and after the adhesion assays using a FTIR microscope (Spectrum Spotlight; Perkin-Elmer, Waltham, MA, USA) equipped with an ATR objective (Germanium, incidence angle of the IR beam, 45° ; $100 \times 100\text{-}\mu\text{m}^2$ nominal surface area). The average IR beam penetration is on the order of 1 to $2\text{ }\mu\text{m}$. To evaluate possible modifications of surface chemical properties exerted by the culture medium (ie, absorbed proteins), one sample per group was kept in bacterial-free Tryptone Soya Broth (TSB; Merck KGaA, Darmstadt, Germany) or fungal-free Sabouraud dextrose broth (SAB; Biolife Italiana Srl, Milan, Italy), as appropriate, at 37°C for 48 hours and was used as control. An average of 10 spectra per sample was collected to check for homogeneity.

X-ray Photoelectron Spectroscopy

The results obtained from ATR-FTIR spectroscopy, whose penetration into the sample is on the order of microns, may be not fully representative of the surface chemical characteristics, whereas these characteristics are likely to influence the ability of binding species (proteins, for example), which in turn can facilitate biofilm formation and/or bacterial/fungal attachment. With this in mind, x-ray photoelectron spectroscopy (XPS) measurements were performed to obtain a detailed chemical characterization of the surface of the original samples.

A PHI 5000 Versaprobe II scanning XPS (Physical Electronics, Chanhassen, MN, USA), equipped with a

monochromatic Al K-alpha x-ray source (1486.6 eV energy, 15 kV voltage, and 1-mA anode current), together with a double-beam neutralization system, made up of an ion gun (Ar^+) combined with an electron gun, dedicated to reduce the samples' surface charging effect during the analyses, was used. A spot size of $100\text{ }\mu\text{m}$ was chosen to collect the photoelectron signal for both the high resolution (HR) and the survey spectra. Different pass energy values were exploited: 187.85 eV for survey spectra and 23.5 eV for HR peaks. Before each HR scan, at least two survey scans were performed in different areas of the samples to check the uniformity.

In Vitro Microbial Adhesion Assays

Biofilm-producing microorganisms obtained by American Type Culture Collection (ATCC) and by isolation from orthopaedic implant infections were carefully selected as representative pathogens of implant infection. In particular, *S epidermidis* (ATCC 35984), *S aureus* (ATCC 29213), *E coli* (ATCC 25922) and *C albicans* (ATCC 10231) were used for in vitro adhesion assays.

Each bacterial strain was cultured on Tryptic Soy Agar (TSA; Merck KGaA, Darmstadt, Germany) and the yeast on SAB agar (Biolife Italiana Srl, Milan, Italy), as previously described in detail [3, 31]. Young colonies were inoculated into cryovials and kept at -80°C for extended storage, according to the manufacturer's recommendations (Pro-Lab Diagnostics UK, Neston, South Wirral, UK).

The methods for the in vitro adhesion assays were used in a previous paper of ours [1, 2]. Briefly, after an overnight culture at 37°C in TSB (for bacteria) or SAB broth (for yeasts), microorganisms were concentrated 10^7 colony forming units (CFU)/mL. Two milliliters of these microbial suspensions were incubated, for 3, 7, 24, and 48 hours at 37°C together with the sterile biomaterials. Microbial growth controls were also performed. At each incubation time, quantitative in vitro analysis of bacterial or fungal adhesion was performed by using a sonication protocol to dislodge adherent microorganisms [1, 2, 24]. The adhesion experiments were assayed in triplicate and repeated a minimum of three times.

Statistical Analysis

The adhesion assay results (CFU/mL) were analyzed by descriptive statistics (mean $\pm$ SD). The adhesion ratio (%) of microorganisms, at each incubation time, was calculated by dividing the CFU/mL of the adhered bacteria or yeast to a single biomaterial by the total CFU/mL of the adhered bacteria or yeast to all biomaterial assayed $\times 100$. The

results were tested by unpaired t-tests to highlight significant differences ($p \leq 0.05$; 95% confidence interval) between the biomaterials, using Graphpad Prism, Version 6 for Windows (Graphpad Software, San Diego, CA, USA).

Results

Adhesion results on XLPE and PE were different among the ATCC bacteria, although a comparable trend was recorded (Table 1). For both *S aureus* and *E coli* a similar adhesion pattern was registered within 24 hours with no differences between PE and XLPE. For *S epidermidis* a reduced adhesion profile, on the same biomaterials, was obtained after 3 and 24 hours of incubation. However, after 48 hours, all collection bacteria showed lower adhesion on XLPE compared with PE ($p < 0.05$). At all incubation times, ATCC *C albicans* was less adherent to XLPE than to PE ($p < 0.05$; Table 2). The microbiologic findings obtained with biofilm-producing strains recently isolated from orthopaedic implant infections were concordant with those registered with ATCC strains (Tables 3, 4). At 48 hours, *S epidermidis*, *S aureus*, and *E coli* adhered to XLPE much less than to PE. *C albicans* had diminished adherence on both XLPE and VE-PE compared with PE ($p < 0.05$).

There were no differences in surface topography (Fig. 1), R_a , or CA between material groups. The overall mean R_a was $0.8 \pm 0.2 \mu\text{m}$ ($n = 18$); CA was $92^\circ \pm 3^\circ$ ($n = 12$) for all samples. No differences were found among the ATR-FTIR spectra of the three samples before the adhesion assay (data not shown); however, after TSB (Fig. 2) or SAB broth suspension, only barely distinguishable traces of proteins adsorbed on the surface were observed in the form of very weak absorption bands at 1645, 1578, and 1542 cm^{-1} [2]. The XPS survey spectra showed the characteristic C1s peak at 284.5 eV usually observed for PE. A noticeable O1s peak at 533 eV was also present, indicating a surface oxygen level of approximately 12 to 13 atomic % for PE and XLPE, which rose to 16% for VE-PE. The high-resolution C1s peak of the three samples, however, did show some differences (Fig. 3). A very low shoulder toward higher binding energy was observed in the spectrum of PE (Fig. 3A); deconvolution of the peak envelope showed the presence of two weak chemically shifted peaks at 286.5 eV and 288 eV attributed to alcohols/ethers (C-OH/C-OR) and to carbonyl groups (C=O), respectively [19, 28]. The same peaks were identified in the XLPE spectrum (Fig. 3B), but with a more intense 286.5-eV alcohols/ethers signal. The high-resolution VE-PE C1s peak (Fig. 3C) showed a highly asymmetrical shape and four peaks were fitted within the envelope; in addition to the peaks at 286.5 and 288 eV (with intensities comparable

to that observed in PE), an intense peak appeared at 283.5 eV. Precise attribution of this signal is still under investigation but we tentatively correlate it with the presence of vitamin E.

Lower adhesion of ATCC *S epidermidis*, *S aureus*, and *E coli* on XLPE compared with PE after 48 hours overlapped that on VE-PE (Table 1). Similarly, adhesion of ATCC *C albicans* on polyethylenes was also strongly limited ($p < 0.05$) by vitamin E addition within 48 hours with respect to PE (Table 2).

Discussion

UHMWPE is a particularly important polymer as a bearing surface in total joint arthroplasty as a result of its physical, chemical, and mechanical properties as well as its good biocompatibility [7, 15]. However, UHMWPE is susceptible to wear-related problems and mechanical degradation after long-term postirradiation oxidation [13]. Hence, many efforts have been made to improve UHMWPE in vivo performance—first by irradiation, to obtain crosslinked UHMWPE, and second, by adding antioxidant compounds such as vitamin E. Despite these improvements, microbial adhesion on these polymers and therefore the eventual ability to develop a biomaterial-related infection has not been well studied. In this context, the aim of our study was to compare *S epidermidis*, *S aureus*, *E coli*, and *C albicans* adhesion on XLPE and virgin PE, and, if possible, to correlate the findings with the biomaterial surface properties. In addition, we wanted to verify if the reduced adhesion on VE-PE highlighted by the bacteria [3, 4] could be obtained with *C albicans* as well. Our results do indicate that XLPE showed a reduced microbial adherence, compared with PE, but we were not able to correlate this evidence to the biomaterial surface properties. The reduced adhesion on VE-PE previously observed with bacteria was confirmed also with *C albicans*.

Our study is subjected to at least two major limitations. First, although most vitamin E-stabilized UHMWPE in clinical use is radiation crosslinked, we chose not to irradiate our vitamin E-containing sample and this can be regarded as a limitation affecting the clinical relevance of our findings. Nevertheless, we have shown in previous studies that oxidation of UHMWPE has a strong effect on bacterial adhesion and proliferation [2] and that irradiation, even under an inert atmosphere, can induce oxidation to various extent [9, 11]. Thus, we chose to focus on the effect of the addition of vitamin E alone, without the “confounding” factor of irradiation. In light of our finding of an unexpected, positive effect of crosslinking on bacterial/fungal adhesion, it might be expected that the combination of vitamin E stabilization and crosslinking can provide further advantages in terms of

Table 1. Adherence (CFU/mL) of ATCC biofilm-producing bacteria on XLPE and VE-PE versus PE

Incubation time for bacteria (hours)	Biomaterials		p value ^a ; 95% CI
	PE, mean $\pm$ SD (%) [*]	XLPE, mean $\pm$ SD (%)	VE-PE, mean $\pm$ SD (%)
<i>S. epidermidis</i> 3 hours	<i>Staphylococcus epidermidis</i> ATCC 35984 3.74 $\times$ 10 ⁵ $\pm$ 2.84 $\times$ 10 ⁴ (50%)	1.02 $\times$ 10 ⁵ $\pm$ 4.21 $\times$ 10 ³ (14%)	PE versus XLPE p < 0.0001; -3.32 $\times$ 10 ⁵ to -2.12 $\times$ 10 ⁵
			PE versus VE-PE p = 0.0348; -2.08 $\times$ 10 ⁵ to -1.03 $\times$ 10 ⁴
7 hours	1.56 $\times$ 10 ⁶ $\pm$ 8.98 $\times$ 10 ⁵ (35%)	1.59 $\times$ 10 ⁶ $\pm$ 2.44 $\times$ 10 ⁵ (36%)	PE versus XLPE p = 0.9129; -4.85 $\times$ 10 ⁵ to 5.36 $\times$ 10 ⁵
			PE versus VE-PE p = 0.2161; -6.73 $\times$ 10 ⁵ to 1.72 $\times$ 10 ⁵
24 hours	3.08 $\times$ 10 ⁷ $\pm$ 6.8 $\times$ 10 ⁶ (55%)	1.06 $\times$ 10 ⁷ $\pm$ 4.43 $\times$ 10 ⁶ (19%)	PE versus XLPE p = 0.0361; -3.87 $\times$ 10 ⁷ to -1.57 $\times$ 10 ⁶
			PE versus VE-PE p = 0.0109; -2.87 $\times$ 10 ⁷ to -4.39 $\times$ 10 ⁶
48 hours	3.26 $\times$ 10 ⁷ $\pm$ 3.89 $\times$ 10 ⁶ (55%)	1.05 $\times$ 10 ⁷ $\pm$ 3.24 $\times$ 10 ⁵ (18%)	PE versus XLPE p = 0.0040; -3.45 $\times$ 10 ⁷ to -9.68 $\times$ 10 ⁶
			PE versus VE-PE p = 0.0041; -2.67 $\times$ 10 ⁷ to -7.11 $\times$ 10 ⁶
<i>S. aureus</i> 3 hours	<i>Staphylococcus aureus</i> ATCC 29213 4.12 $\times$ 10 ⁶ $\pm$ 2.52 $\times$ 10 ⁵ (34%)	4.16 $\times$ 10 ⁶ $\pm$ 2.40 $\times$ 10 ⁵ (34%)	PE versus XLPE p = 0.9768; -3.33 $\times$ 10 ⁶ to 3.4 $\times$ 10 ⁶
			PE versus VE-PE p = 0.926; -3.53 $\times$ 10 ⁶ to 3.25 $\times$ 10 ⁶
7 hours	1.31 $\times$ 10 ⁷ $\pm$ 7.08 $\times$ 10 ⁶ (35%)	1.07 $\times$ 10 ⁷ $\pm$ 1.14 $\times$ 10 ⁶ (29%)	PE versus XLPE p = 0.5256; -1.09 $\times$ 10 ⁷ to 3.05 $\times$ 10 ⁶
			PE versus VE-PE p = 0.946; -7.72 $\times$ 10 ⁶ to 8.21 $\times$ 10 ⁶
24 hours	1.61 $\times$ 10 ⁷ $\pm$ 4.93 $\times$ 10 ⁶ (38%)	1.34 $\times$ 10 ⁷ $\pm$ 1.66 $\times$ 10 ⁶ (32%)	PE versus XLPE p = 0.4361; -1.03 $\times$ 10 ⁷ to 4.79 $\times$ 10 ⁶
			PE versus VE-PE p = 0.429; -1.24 $\times$ 10 ⁷ to 5.89 $\times$ 10 ⁶
48 hours	3.18 $\times$ 10 ⁷ $\pm$ 9.75 $\times$ 10 ⁶ (54%)	1.45 $\times$ 10 ⁷ $\pm$ 1.99 $\times$ 10 ⁶ (24%)	PE versus XLPE p = 0.0263; -3.18 $\times$ 10 ⁷ to -2.62 $\times$ 10 ⁶
			PE versus VE-PE p = 0.024; -3.42 $\times$ 10 ⁷ to -3.41 $\times$ 10 ⁶

Table 1. continued

Incubation time for bacteria (hours)	Biomaterials		p value [†] ; 95% CI	
	PE, mean $\pm$ SD (%) [*]	XLPE, mean $\pm$ SD (%)	VE-PE, mean $\pm$ SD (%)	
<i>E. coli</i>	<i>Escherichia coli</i> ATCC 25922			
3 hours	1.01 $\times 10^7 \pm 3.31 \times 10^6$ (30%)	1.22 $\times 10^7 \pm 2.83 \times 10^6$ (37%)	1.09 $\times 10^7 \pm 4.71 \times 10^6$ (33%)	PE versus XLPE p = 0.3618; -2.95×10^6 to 7.21×10^6 PE versus VE-PE
7 hours	1.18 $\times 10^7 \pm 8.92 \times 10^6$ (36%)	1.10 $\times 10^7 \pm 1.41 \times 10^6$ (34%)	1.00 $\times 10^7 \pm 4.17 \times 10^6$ (30%)	p = 0.730; -4.31×10^6 to 5.94×10^6 PE versus XLPE p = 0.9066; -1.65×10^7 to 1.49×10^7 PE versus VE-PE
24 hours	1.65 $\times 10^7 \pm 4.64 \times 10^6$ (34%)	1.59 $\times 10^7 \pm 1.76 \times 10^6$ (32%)	1.66 $\times 10^7 \pm 6.83 \times 10^6$ (34%)	p = 0.684; -1.15×10^6 to 7.83×10^6 PE versus XLPE p = 0.8234; 9.25×10^6 to 2.54×10^7 PE versus VE-PE
48 hours	1.15 $\times 10^7 \pm 3.22 \times 10^6$ (52%)	4.89 $\times 10^6 \pm 9.70 \times 10^4$ (22%)	5.83 $\times 10^6 \pm 9.13 \times 10^5$ (26%)	p = 0.977; -7.41×10^6 to 7.61×10^6 PE versus XLPE p = 0.0035; -1.04×10^7 to -2.80×10^6 PE versus VE-PE p = 0.0081; -9.47×10^6 to -1.88×10^6

^{*} Percentage of microbial adhesion ratio; [†] p < 0.05 = significant; CFU = colony-forming units; ATCC = American Type Culture Collection; XLPE = crosslinked UHMWPE; VE-PE = vitamin E-stabilized UHMWPE; PE = virgin UHMWPE; CI = confidence interval.

Table 2. Adherence (CFU/mL) of biofilm-producing *Candida albicans* on XLPE and VE-PE versus PE

Incubation times (hours)	Biomaterials		Significance	
	PE, mean $\pm$ SD (%) [*]	XLPE, mean $\pm$ SD (%)	VE-PE, mean $\pm$ SD (%)	p value [†] ; 95% CI
3 hours	1.52 $\times$ 10 ⁴ $\pm$ 3.55 $\times$ 10 ³ (61%)	5.22 $\times$ 10 ³ $\pm$ 3.49 $\times$ 10 ² (21%)	4.39 $\times$ 10 ³ $\pm$ 1.08 $\times$ 10 ² (18%)	PE versus XLPE p = 0.0040; -1.56 $\times$ 10 ⁴ to -4.39 $\times$ 10 ³ PE versus VE-PE
7 hours	1.65 $\times$ 10 ⁴ $\pm$ 3.16 $\times$ 10 ³ (66%)	4.58 $\times$ 10 ³ $\pm$ 3.08 $\times$ 10 ² (18%)	3.98 $\times$ 10 ³ $\pm$ 3.53 $\times$ 10 ² (16%)	p = 0.0202; -1.92 $\times$ 10 ⁴ to -2.54 $\times$ 10 ³ PE versus XLPE p < 0.0001; -1.57 $\times$ 10 ⁴ to -8.11 $\times$ 10 ³ PE versus VE-PE
24 hours	1.47 $\times$ 10 ⁴ $\pm$ 2.65 $\times$ 10 ³ (61%)	4.45 $\times$ 10 ³ $\pm$ 6.41 $\times$ 10 ² (18%)	5.22 $\times$ 10 ³ $\pm$ 1.09 $\times$ 10 ² (21%)	p = 0.0011; -1.18 $\times$ 10 ⁴ to -6.67 $\times$ 10 ³ PE versus XLPE p = 0.0010; -1.44 $\times$ 10 ⁴ to -6.01 $\times$ 10 ³ PE versus VE-PE
48 hours	1.45 $\times$ 10 ⁴ $\pm$ 4.50 $\times$ 10 ³ (72%)	2.81 $\times$ 10 ³ $\pm$ 1.25 $\times$ 10 ² (14%)	2.95 $\times$ 10 ³ $\pm$ 3.13 $\times$ 10 ² (14%)	p = 0.0176; -1.57 $\times$ 10 ⁴ to -3.13 $\times$ 10 ³ PE versus XLPE p = 0.0272; -2.16 $\times$ 10 ⁴ to -1.76 $\times$ 10 ³ PE versus VE-PE p = 0.0226; -2.09 $\times$ 10 ⁴ to -2.17 $\times$ 10 ³

^{*} Percentage of microbial adherence ratio; [†] p < 0.05 = significant; CFU = colony-forming units; ATCC = American Type Culture Collection; XLPE = crosslinked UHMWPE; VE-PE = vitamin E-stabilized UHMWPE; PE = virgin UHMWPE; CI = confidence interval.

Table 3. Adherence (CFU/mL) of clinical biofilm-producing bacteria on XLPE and VE-PE versus PE

Incubation time for bacteria (hours)	Biomaterials		p value [†] ; 95% CI	
	PE, mean $\pm$ SD (%) [*]	XLPE, mean $\pm$ SD (%)	VE-PE, mean $\pm$ SD (%)	
<i>S. epidermidis</i> 3 hours	<i>Staphylococcus epidermidis</i> 3.82 $\times$ 10 ⁵ $\pm$ 3.20 $\times$ 10 ⁴ (37%)	3.13 $\times$ 10 ⁵ $\pm$ 1.24 $\times$ 10 ⁴ (31%)	3.25 $\times$ 10 ⁵ $\pm$ 1.28 $\times$ 10 ⁴ (32%)	PE versus XLPE
				p = 0.0639; -1.43 $\times$ 10 ⁵ to -5.23 $\times$ 10 ³
				PE versus VE-PE
				p = 0.01105; -1.32 $\times$ 10 ⁵ to 1.70 $\times$ 10 ⁴
7 hours	1.54 $\times$ 10 ⁶ $\pm$ 1.05 $\times$ 10 ⁵ (34%)	1.54 $\times$ 10 ⁶ $\pm$ 1.97 $\times$ 10 ⁵ (34%)	1.46 $\times$ 10 ⁶ $\pm$ 1.94 $\times$ 10 ⁵ (32%)	PE versus XLPE
				p = 0.9892; -4.62 $\times$ 10 ⁵ to 4.57 $\times$ 10 ⁵
				PE versus VE-PE
				p = 0.6869; -5.40 $\times$ 10 ⁵ to 3.71 $\times$ 10 ⁵
24 hours	3.11 $\times$ 10 ⁷ $\pm$ 5.75 $\times$ 10 ⁶ (40%)	2.25 $\times$ 10 ⁷ $\pm$ 4.26 $\times$ 10 ⁶ (29%)	2.43 $\times$ 10 ⁷ $\pm$ 1.63 $\times$ 10 ⁶ (31%)	PE versus XLPE
				p = 0.2704; -2.48 $\times$ 10 ⁷ to -7.67 $\times$ 10 ⁶
				PE versus VE-PE
				p = 0.2042; -1.78 $\times$ 10 ⁷ to 4.13 $\times$ 10 ⁶
48 hours	3.41 $\times$ 10 ⁷ $\pm$ 3.89 $\times$ 10 ⁶ (53%)	1.38 $\times$ 10 ⁷ $\pm$ 2.24 $\times$ 10 ⁶ (22%)	1.58 $\times$ 10 ⁷ $\pm$ 1.44 $\times$ 10 ⁶ (25%)	PE versus XLPE
				p = 0.0039; -3.18 $\times$ 10 ⁷ to -3.95 $\times$ 10 ⁶
				PE versus VE-PE
				p = 0.0022; -2.79 $\times$ 10 ⁷ to -8.78 $\times$ 10 ⁶
<i>S. aureus</i> 3 hours	<i>Staphylococcus aureus</i> 4.01 $\times$ 10 ⁶ $\pm$ 9.48 $\times$ 10 ⁵ (34%)	3.87 $\times$ 10 ⁶ $\pm$ 6.84 $\times$ 10 ⁵ (33%)	3.91 $\times$ 10 ⁶ $\pm$ 7.62 $\times$ 10 ⁵ (33%)	PE versus XLPE
				p = 0.9101; -2.89 $\times$ 10 ⁶ to 2.61 $\times$ 10 ⁶
				PE versus VE-PE
				p = 0.9424; -3.17 $\times$ 10 ⁶ to 2.97 $\times$ 10 ⁶
7 hours	1.43 $\times$ 10 ⁷ $\pm$ 1.88 $\times$ 10 ⁶ (35%)	1.29 $\times$ 10 ⁷ $\pm$ 1.23 $\times$ 10 ⁶ (32%)	1.34 $\times$ 10 ⁷ $\pm$ 1.98 $\times$ 10 ⁶ (33%)	PE versus XLPE
				p = 0.5250; -6.37 $\times$ 10 ⁷ to 3.49 $\times$ 10 ⁶
				PE versus VE-PE
				p = 0.7336; -7.25 $\times$ 10 ⁶ to 5.30 $\times$ 10 ⁶
24 hours	1.45 $\times$ 10 ⁷ $\pm$ 1.75 $\times$ 10 ⁶ (34%)	1.33 $\times$ 10 ⁷ $\pm$ 2.75 $\times$ 10 ⁶ (31%)	1.48 $\times$ 10 ⁷ $\pm$ 2.86 $\times$ 10 ⁶ (35%)	PE versus XLPE
				p = 0.7305; -8.42 $\times$ 10 ⁶ to 6.11 $\times$ 10 ⁶
				PE versus VE-PE
				p = 0.9174; -7.17 $\times$ 10 ⁷ to 7.85 $\times$ 10 ⁶
48 hours	3.13 $\times$ 10 ⁷ $\pm$ 3.04 $\times$ 10 ⁶ (51%)	1.54 $\times$ 10 ⁷ $\pm$ 4.13 $\times$ 10 ⁶ (25%)	1.41 $\times$ 10 ⁷ $\pm$ 3.57 $\times$ 10 ⁶ (23%)	PE versus XLPE
				p = 0.0236; -2.90 $\times$ 10 ⁷ to -2.75 $\times$ 10 ⁶
				PE versus VE-PE
				p = 0.0106; -2.86 $\times$ 10 ⁷ to -5.66 $\times$ 10 ⁶

Table 3. continued

Incubation time for bacteria (hours)	Biomaterials		p value [†] ; 95% CI
	PE, mean $\pm$ SD (%) [*]	VE-PE, mean $\pm$ SD (%)	
<i>E. coli</i>			
3 hours	<i>Escherichia coli</i> 1.09 $\times 10^7 \pm 1.12 \times 10^6$ (32%)	1.11 $\times 10^7 \pm 2.34 \times 10^6$ (32%)	PE versus XLPE p = 0.5275; -3.48 $\times 10^6$ to 6.28 $\times 10^6$ PE versus VE-PE
7 hours	1.27 $\times 10^7 \pm 1.84 \times 10^6$ (35%)	1.23 $\times 10^7 \pm 1.57 \times 10^6$ (34%)	p = 0.9415; -5.07 $\times 10^6$ to 5.42 $\times 10^6$ PE versus XLPE p = 0.4714; -6.09 $\times 10^6$ to 3.26 $\times 10^6$ PE versus VE-PE
24 hours	1.65 $\times 10^7 \pm 1.31 \times 10^6$ (34%)	1.59 $\times 10^7 \pm 2.49 \times 10^6$ (33%)	p = 0.8923; -6.42 $\times 10^6$ to 5.72 $\times 10^6$ PE versus XLPE p = 0.8446; -6.84 $\times 10^6$ to 5.71 $\times 10^6$ PE versus VE-PE
48 hours	1.14 $\times 10^7 \pm 9.19 \times 10^5$ (52%)	5.39 $\times 10^6 \pm 4.07 \times 10^5$ (24%)	p = 0.7384; -5.74 $\times 10^6$ to 4.21 $\times 10^6$ PE versus XLPE p = 0.0011; -8.93 $\times 10^6$ to -3.15 $\times 10^6$ PE versus VE-PE p = 0.0009; -8.91 $\times 10^6$ to -3.26 $\times 10^6$

* Percentage of microbial adhesion ratio; [†] p < 0.05 = significant; CFU = colony-forming units; ATCC = American Type Culture Collection; XLPE = crosslinked UHMWPE; VE-PE = vitamin E-stabilized UHMWPE; PE = virgin UHMWPE; CI = confidence interval.

Table 4. Adherence (CFU/mL) of clinical biofilm-producing *Candida albicans* on XLPE and VE-PE versus PE

Incubation times (hours)	Biomaterials		Significance	
	PE mean $\pm$ SD (%) [*]	XLPE mean $\pm$ SD (%)	VE-PE mean $\pm$ SD (%)	p value [†] ; CI 95%
3 hours	$1.56 \times 10^4 \pm 4.50 \times 10^3$ (62%)	$5.25 \times 10^3 \pm 2.79 \times 10^2$ (21%)	$4.18 \times 10^3 \pm 8.92 \times 10^2$ (17%)	PE versus XLPE
				p = 0.0104; -1.73×10^4 to -3.29×10^3
7 hours	$1.76 \times 10^4 \pm 2.77 \times 10^3$ (67%)	$4.27 \times 10^3 \pm 2.31 \times 10^2$ (16%)	$4.32 \times 10^3 \pm 3.19 \times 10^2$ (17%)	PE versus VE-PE
				p = 0.0332; -2.14×10^4 to -1.34×10^3
				PE versus XLPE
				p = 0.0003; -1.84×10^4 to -8.23×10^1
24 hours	$1.48 \times 10^4 \pm 2.01 \times 10^3$ (62%)	$4.19 \times 10^3 \pm 5.43 \times 10^2$ (17%)	$5.13 \times 10^3 \pm 8.7 \times 10^1$ (21%)	PE versus VE-PE
				p = 0.0003; -1.84×10^4 to -8.14×10^3
				PE versus XLPE
				p = 0.0005; -1.53×10^4 to -5.97×10^3
48 hours	$1.37 \times 10^4 \pm 2.82 \times 10^3$ (73%)	$2.47 \times 10^3 \pm 3.94 \times 10^2$ (13%)	$2.71 \times 10^3 \pm 1.61 \times 10^2$ (14%)	PE versus VE-PE
				p = 0.0135; -1.66×10^4 to -2.70×10^3
				PE versus XLPE
				p = 0.0030; -1.72×10^4 to -5.25×10^3
				PE versus VE-PE
				p = 0.0031; -1.68×10^4 to -5.08×10^3

^{*} Percentage of microbial adherence ratio; [†] p < 0.05 = significant; CFU = colony-forming units; ATCC = American Type Culture Collection; XLPE = crosslinked UHMWPE; VE-PE = vitamin E-stabilized UHMWPE; PE = virgin UHMWPE; CI = confidence interval.

Fig. 1A–C SEM micrographs representative of the surface topography of the three materials before microbial adhesion assay are shown: (A) PE, (B) XLPE, and (C) VE-PE. Each material type had a comparable surface topography.

Fig. 2 Representative ATR-FTIR spectra from each of the three materials after immersion in TSB for 24 hours are shown: PE (blue), VE-PE (red) and XLPE (green). The inset shows weak traces of proteins adsorbed on the surfaces: weak absorption bands at 1645, 1578, and 1542 cm^{-1} [2].

Fig. 3A–C Representative XPS spectra of the three materials are shown: (A) PE, (B) XLPE, and (C) VE-PE. The deconvolution of the C1s peak envelope showed the presence of chemically shifted peaks at 286.5 eV and 288 eV indicating the presence of alcohols/ethers

(C-OH/C-OR) and carbonyl groups (C=O), respectively. An additional shoulder at 283.5 eV, whose attribution is still unclear, appeared in the spectrum of VE-PE.

reduction of microbial adhesion. Although a synergistic effect of crosslinking and vitamin E remains to be proved, nothing indicates a possible adverse response of the two combined factors. Second, the in vitro closed system used in our experimental model did not account for environmental factors such as the presence of host defenses (ie, serum proteins) and the associated flow conditions that, together with microbial properties and biomaterial surface characteristics, are extremely important for in vivo infection resolution.

Microbial adherence and subsequent biofilm formation depend on multiple factors, including the causal microorganism, inoculum size, environmental physicochemical conditions, nutrient availability, hydrodynamic forces, and the nature of the accretion substrata [13, 32]. From our results, both ATCC and clinical microorganisms showed less adherence to the modified PEs; *S epidermidis*, *S aureus*, and *E coli* adhered to XLPE much less than to standard PE ($p < 0.05$) at 48 hours (Tables 1, 3). Moreover, a significant ($p < 0.05$) reduction in fungal adhesion was detected for XLPE compared with standard PE at all incubation times (Tables 2, 4). Our experimental condition was a stressed infection condition; in fact, the microbial inoculum used (10^7 CFU/mL) was bigger than 10^2 CFU, which is the microbial load usually inducing in vivo infection [25].

Biomaterials with different physicochemical surface properties are differently affected by microorganism adherence and biofilm formation [15]. We previously demonstrated [2] a preferential adsorption of proteins on the polar surface of heavily oxidized UHMWPE and we postulated that this can favor bacterial adhesion, which was in fact increased compared with that on virgin (unoxidized) PE. The samples investigated in the present study showed no differences in terms of surface topography, R_a , and hydrophilicity when assessed by SEM (Fig. 1) and CA, whereas the XPS measurements showed a low but measurable oxygen contamination on the surface of all samples. Similar levels of oxygen contamination on the surface of UHMWPE was previously reported in the literature [28] and attributed to a slight mechanical oxidation, plausibly caused by manufacturing and processing. The XPS spectra of different samples did exhibit slight differences in surface chemical composition (Fig. 3), but the importance of these changes and the mechanism by which they could affect microbial adhesion are still to be determined. However, the ATR-FTIR investigation did not reveal a preferential adsorption of protein on any particular sample, although significantly reduced microbial adhesion values were detected for VE-PE and XLPE compared with PE (Tables 1–4). Overall, we found no clear correlation between the observed differences in microbial adhesion and the investigated surface properties.

Bacterial adhesion was reduced on VE-PE compared with virgin PE, as previously reported [1, 2], and was

confirmed with *C albicans* (Tables 2, 4). The most interesting result was that *C albicans* adherence was significantly lower on VE-PE ($p < 0.05$) from as early as 3 hours incubation. The use of vitamin E in PE as a method to minimize material oxidation may also change the surface of the substratum and microbial adhesion process, thus limiting the extent of subsequent infection [15, 23]. Data on microbial adhesion reduction on VE-PE are still conflicting; although a reduction was recorded by Gómez-Barrena et al. [15], the decrease seems to be species- and strain-dependent. Our data, in contrast, highlight a similar adhesion decrease for both bacteria and fungi. The results obtained with biofilm-producing *S epidermidis*, *S aureus*, *E coli*, and *C albicans* isolated from biomaterial-associated infection were in agreement with those registered with ATCC strains (Tables 1–4). It is to be underlined that the majority of authors do not use different genera and species, but limit their in vitro studies using only staphylococcal strains as the most common bacteria isolated from biomaterial-associated infections [15, 22, 23, 25]. The encouraging results obtained by assaying ATCC strains led us to also include clinical strains of *S epidermidis*, *S aureus*, *E coli*, and *C albicans* in our study to get practical results that could be applied in a clinical setting [13].

In conclusion, within the limits of the study, our data corroborate a better antiadherent performance of VE-PE against microbial adhesion and also constitute a step forward compared with XLPE, which is similarly associated with a reduction of either bacterial or fungal adhesion. Further analysis on both physicochemical characteristics of the UHMWPE-modified surfaces and their morphology will be necessary to correlate the different patterns of microbial adhesion and the consequent biofilm formation. Our results must be considered tentative pending results from further studies that should determine: (1) the roles of vitamin E and irradiation of UHMWPE in reducing microbial adhesion, and consequently the risk of infection, in the clinical setting; and (2) the effect of in vivo wear of vitamin E and irradiated UHMWPE on microbial adhesion.

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