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Poly (D,L) Lactic Acid Blending with Vitamin E Increases Polymer Hemocompatibility: An Hydrophilic Effect

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ABSTRACT: Biocompatible and resorbable polylactic acid (PLA) derived polymers are used for clinical applications. Antioxidant vitamin E (Vit.E) and vitamin E acetate (Vit.E Ac), a Vit.E commercial analog, were added to PLA during films preparation. Wettability, protein adsorption analysis, platelet adhesion and whole blood coagulation experiments highlighted that Vit.E incorporation improved PLA hemocompatibility. NMR analysis showed that Vit.E and Vit.E Ac blending differentially altered PLA polymer organization with Vit.E leading to the formation of microcavities, as observed by SEM analysis, while reducing surface hydrophobicity.
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INTRODUCTION

Biodegradable polymers are used for a large amount of clinical applications and in particular as drug delivery systems. A very interesting field of application for those polymers is the metallic stents coating where they act to reduce stents thrombogenicity and neointima hyperplasia and consequent vascular stenosis by delivering drugs or other agents (e.g. nucleic acids).[1] One of the major side effects of stent application, which still arise in a 10–50% of the treated cases, is the pathobiological process known as in-stent restenosis[2]: for this reason many efforts to reduce this relevant clinical problem are necessary. In this research field the study of the interactions occurring between the polymer used to coat the drug eluting stents and the complex blood environment has a pivotal role. It has been demonstrated that biodegradable polymers, such as polyglycolic acid/polylactic acid or polycaprolactone, which are considered as good candidates for this kind of application on the basis of *in vitro* tests, after implantation induce a marked inflammation with subsequent neointimal thickening.[3] In the last years many other polymers used for drug eluting stents coating have been described to be biologically inert and stable for at least 6 months,[4,5] so the current focus of the ongoing research is the use of biomimetic substances not interfering with the re-endothelization and neointimal formation, such as phosphorylcoline.[6] Among biodegradable polymers poly(lactid acid) (PLA) [both in the form of poly(L-lactid acid) (P(L)LA) and poly(D,L-lactid acid) (P(D,L)LA)] appears as an interesting candidate for stent coating thanks to its biocompatibility, in fact in physiological conditions it undergoes biodegradation to lactic acid with L-lactic acid as a natural intermediate in carbohydrate metabolism.[7,8] In particular, P(D,L)LA has emerged as the material of choice in the field of drug-eluting stents coating as it is degraded faster than P(L)LA. Nevertheless P(D,L)LA coating has also some drawbacks, in fact, it is known to activate a local inflammatory response characterized by both platelet[9] and granulocyte[10] activation. In the last years P(D,L)LA coating has been used for paclitaxel-eluting coronary stents, showing good results in inhibiting restenosis in an animal model, even if the unloaded polymer induced a long-lasting local inflammatory response that probably caused an underestimation of paclitaxel effects.[11] In our laboratory we produced P(D,L)LA films enriched with vitamin E (Vit.E) [α-tocopherol, 10–30% (w/w)], showing an increased wettability and hemocompatibility.[12,13] Vit.E [Figure 1 (A)] is a natural biological agent with both anti-oxidant and anti-inflammatory[14–16] properties which have been extensively used to improve different biomaterials biocompatibility[17] and hemodialysis cellulose membrane hemocompatibility.[18–20] Tocopheryl acetate, also known as vitamin E acetate (Vit.E Ac) [Figure 1 (B)], is the acetic acid ester of tocopherol and it is often used in different dermatological products. This

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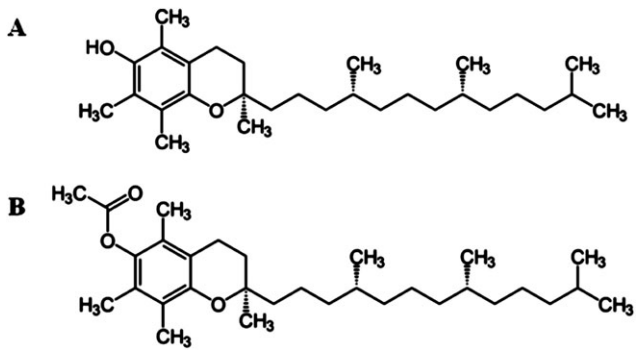

Figure 1. Chemical structures: chemical structure of Vitmain E (A) and vitamin E acetate (B).

compound is commonly used as an alternative to tocopherol itself because its phenolic hydroxyl group is blocked, resulting in a less acidic product. It is believed that this molecule, when absorbed at the skin level, undergoes hydrolysis, regenerating α-tocopherol.[21] In this article, we investigated the effects of P(D,L)LA blending with Vit.E or Vit.E Ac [40% (w/w)] on polymer wettability, human plasma protein adsorption and hemocompatibility, characterized in terms of clotting time and platelet adhesion. Moreover the effects of Vit.E and Vit.E Ac blending on the original polymer surface characteristics were evaluated by both microscopic (SEM) and solid state NMR analysis.

EXPERIMENTAL SECTION

Preparation of P(D,L)LA Films

P(D,L)LA (100% D,L, average molecular mass 75–120 kDa), Vit.E (α-tocopherol) and Vit.E Ac were purchased from Sigma-Aldrich (Milwaukee, WI). P(D,L)LA films were prepared by casting 0.05 g/mL P(D,L)LA solution in chloroform with or without Vit.E and Vit.E Ac in 150 mm glass dishes. After 5 min shaking 40% (w/w) Vit.E or Vit.E Ac, both diluted 1 : 1 in chloroform, were added to P(D,L)LA solution and the solvent was evaporated at room temperature for 24 h under vacuum in the dark. Film sheets (approx. 1 mm thick) were then cut under sterile conditions into square samples (1 cm^2) and stored at 4°C for no more than 1 week.

Wettability Tests

Contact angle measurements were carried out in order to evaluate the wettability of the vitamin-E or Vit.E Ac-enriched P(D,L)LA films. An equal volume of distilled water (100 μL) was dropped onto each dry sample by means of a micropipette. Photos were taken through lenses [Leitz IIA optical stage microscope equipped with a Leica DFC320 (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) video-camera]. Measure of the contact angle was performed by analyzing drop images (3 for each samples) using Scion Image software for Windows.

Plasma and Platelet Separation

Human peripheral venous blood (20 mL) was obtained from 10 healthy donors (20–36 years) using EDTA as anticoagulant. All blood samples were used within 3 h from sampling. Blood (10 mL) was centrifuged at 200 × g for 10 min to obtain the platelet-rich plasma (PRP) fraction. This enriched fraction was then centrifuged at 1600 × g for 10 min to separate platelets.[22] Platelets were then resuspended in RPMI 1640 medium (Euroclone, Milan, Italy) containing penicillin (100 U/mL), streptomycin (100 mg/mL), and L-glutamine (2 m*M*) (Euroclone) and supplemented with 10% heat-inactivated fetal calf serum (Euroclone) while the remaining plasma was stored at −20°C until use.

Protein Adsorption Assay

Protein adsorption assays were performed in triplicate using human plasma (pooled from 10 healthy donors). P(D,L)LA and Vit.E or Vit.E Ac P(D,L)LA film specimen were covered with 200 μL of undiluted human plasma and incubated for 1 h at 37°C. At the end of the incubation time plasma was removed and specimen were washed three times with phosphate buffer (PBS, pH = 7.4). Adsorbed proteins were collected by incubating control and Vit.E or Vit.E Ac enriched P(D,L)LA films with 1 mL of 2% sodium dodecyl sulphate (SDS) solution in PBS for 4 h at room temperature under vigorous shaking. The amount of adsorbed proteins was measured in triplicate using a commercial protein quantification kit (BCA, Pierce, Rockford, IL). Samples optical density was read at 562 nm and the results were expressed as μg total protein adsorbed/cm^2 ± standard deviation (S.D.). Adsorbed proteins were also analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver staining (Silver Staining Kit, Fermentas).

Platelets Adhesion

For platelets adhesion assays platelets obtained from three different donors were used to perform triplicate experiments. Aliquots of platelets suspension (200 μL) were dropped onto P(D,L)LA, P(D,L)LA/Vit.E and P(D,L)LA/Vit. E Ac films and incubated for 30 min in a humidified atmosphere containing 5% CO_2 at 37°C. At the end of the incubation time platelets adhered onto P(D,L)LA films were gently washed three times with PBS (pH = 7.4) and fixed at room temperature in formaldehyde 3.7%, sucrose 3% solution. Platelets were then stained with Giemsa staining solution and microphotographs were taken using a Leica DM2500 microscope. Platelets adhesion was evaluated as surface coverage (% area coverage ± S.D.). Area coverage was measured using Leica QWin software.

Clotting Time

The tromboresistence properties of the P(D,L)LA and Vit.E or Vit.E Ac enriched P(D,L)LA films were evaluated using fresh human blood by the kinetic clotting method.[23] Briefly, 100 μL of fresh blood were taken directly from the plastic syringe used for the blood collection and immediately dropped onto the film specimen. After a predetermined contact time (10, 30, 60 min) specimen were transferred to plastic tubes containing 20 mL distilled water and incubated for 5 min. Specimen ability to induce blood clotting was deduced by the quantity of free hemoglobin measured at each time point. In these experimental conditions, red blood cells not entrapped in a thrombus were hemolysed and free hemoglobin concentration was measured by monitoring specimen absorbance at 540 nm.

Solid State NMR Analysis

All solid state NMR (SS-NMR) spectra were acquired on a Bruker Avance III 500 spectrometer and a wide bore 11.7 Tesla

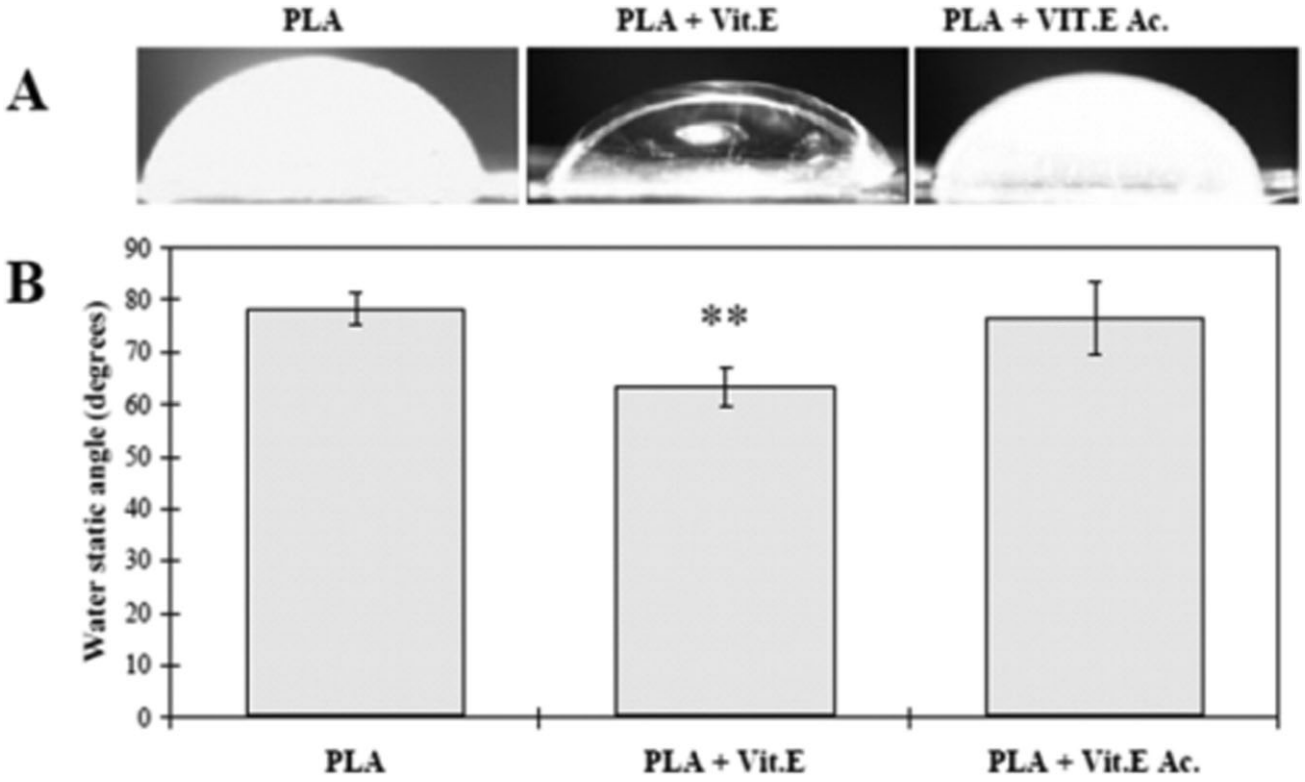

Figure 2. Wettability: (A) Representative image of the water drop formed onto P(D,L)LA, P(D,L)LA/Vit.E, and P(D,L)LA/Vit.E Ac specimen. (B) Water drop static contact angle measurements (**$P < 0.001$).

magnet with operational frequencies for 1H, and 13C of 500.13, and 125.77 MHz, respectively. A 4 mm triple resonance probe with MAS was employed in all the experiments. The samples were packed on a Zirconia rotor and spun at a MAS rate between 5 and 15 kHz. The relaxation delays, d1, between accumulations were between 5 and 30 s for 1H and 13C MAS NMR, respectively. For the 13C{1H} CPMAS experiments, the magnetic fields υrfH of 55 and 28 kHz were used for initial excitation and decoupling, respectively. During the CP period the 1H RF field υrfH was ramped using 100 increments, whereas the 13C RF field υrfC was maintained at a constant level. During the acquisition, the protons are decoupled from the carbons by using a TPPM decoupling scheme. A moderate ramped RF field υrfH of 62 kHz was used for spin locking, while the carbon RF field υrfC was matched to obtain optimal signal and the CP contact time of 1 ms was used. All chemical shifts are reported using δ scale and are externally referenced to adamantane at 38.48 ppm.

SEM Analysis

SEM images were recorded on a Quanta 200 FEI Scanning Electron Microscope equipped with EDS attachment, using a tungsten filament as electron source. A conductive gold coating of 20 nm by low-pressure plasma was deposited to avoid that the insulating materials were electronically charged under the electron beam.

The mean dimension of the pores present on the Vit. E P(D,L)LA sample surface was evaluated by count a statistical relevant number (>1000) of pore using a specific software (Particule).

Statistical Analysis

Unpaired Student's *t*-tests were done for statistical analysis. Probability values of $P < 0.05$ were considered statistically significant. Data were expressed as mean values ± standard deviation (S.D.).

RESULTS AND DISCUSSION

Wettability and Protein Adsorption

Vit.E addition to P(D,L)LA films significantly increased their wettability as indicated by the water drop contact angle measurements [Figure 2 (A,B)]. In fact, the static contact angle expressed as degree ± S.D. was 78° ± 3° for normal P(D,L)LA films while the addition of the hydrophobic Vit.E decreased the static contact angle to 63° ± 4° ($P < 0.001$). On the other hand Vit.E Ac, a commercial analog of Vit.E displaying just an additional acetate group (that replace the [oxygen bond]–OH group) on the first aromatic ring compared to Vit.E addition did not modified the hydrophobic behavior of P(D,L)LA (static contact angle = 76° ± 7°). According to wettability data Vit.E blending also modified P(D,L)LA protein adsorption from human plasma. In fact, both the qualitative silver staining analysis [Figure 3 (A)] and the quantitative BCA assay for the adsorbed proteins [Figure 3 (B)] showed that Vit.E presence strongly increased protein adsorption onto P(D,L)LA films. In fact, normal P(D,L)LA films adsorbed 90 ± 31 μg prot/mL, while Vit.E P(D,L)LA films adsorbed 586 ± 110 μg prot/mL ($P < 0.001$ compared to control samples). As

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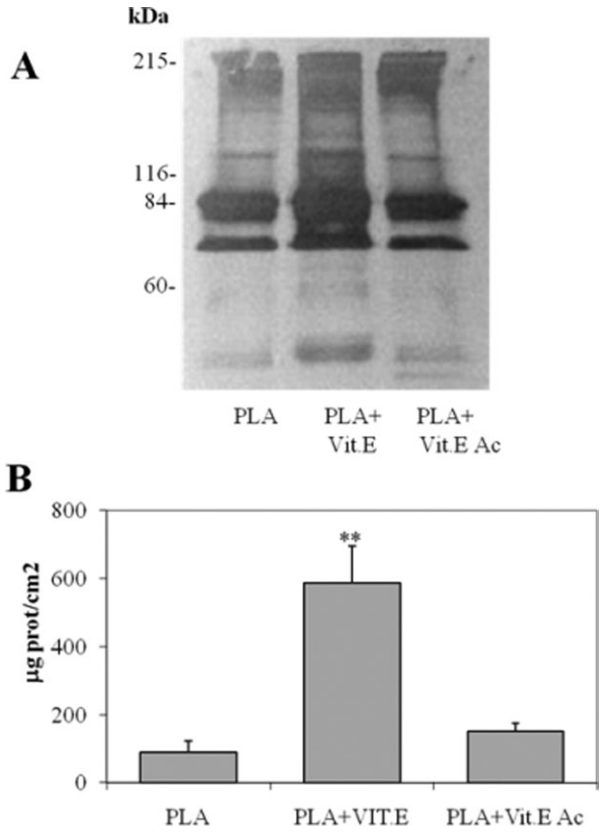

Figure 3. Protein adsorption: (A) representative SDS-PAGE separation and silver staining of the plasma proteins adsorbed onto P(D,L)LA, P(D,L)LA/Vit.E, and P(D,L)LA/Vit.E Ac specimen. (B) Quantification of protein adsorption onto P(D,L)LA, P(D,L)LA/Vit.E, and P(D,L)LA/Vit.E Ac specimen by commercial BCA assay (**$P < 0.001$).

expected Vit.E Ac had no significant effects on protein adsorption (150 ± 34 µg prot/mL).

The observed increase in P(D,L)LA/Vit.E wettability could be ascribed to an increased amount of the [oxygen group]–OH groups introduced by the presence of Vit.E.[12] Surface modifications leading to an increased wettability and protein adsorption negatively modulated platelets adhesion and activation.[24] Such effect has also been described for hydrophilic modification of P(D,L)LA surface with 2-hydroxyethyl-methacrylate (HEMA).[9]

Physical parameters of biomaterial surfaces, such as wettability, solubility, and roughness strongly influence the composition of the adsorbed protein layer. In particular, fibrinogen, albumin, and immunoglobulin G (IgG) are the main proteins adsorbed from plasma and are involved in regulating both granulocyte and platelets adhesion and activation.[25–28] In this study, the specific composition of the adsorbed protein layer was not investigated in detail, but in a previous article[12] our group demonstrated that the main plasma protein adsorbed onto P(D,L)LA samples enriched with 10% Vit.E was albumin, which is known to be involved in the transport and delivery of a wide variety of metabolites, drugs, anionic ligands, and cations. Albumin adsorbs mainly onto hydrophilic surfaces, so we can speculate that the same phenomenon observed for 10% Vit.E enriched P(D,L)LA surfaces might occur also onto the more hydrophilic surface of 40% Vit.E enriched P(D,L)LA samples.

Hemocompatibility

In vitro platelets adhesion testing was performed to study the quantity of adherent platelets onto control P(D,L)LA and both Vit.E and Vit.E Ac enriched P(D,L)LA films. As shown in Figure 4 P(D,L)LA blending with Vit.E dramatically reduced platelets adhesion onto the film surface compared to unmodified P(D,L)LA samples (% of area coverage = 21.27 ± 1.8 and 9.32 ± 0.32 respectively, $P < 0.05$). Also in this case Vit.E Ac blending showed no significant effects (% of area coverage = 22.23 ± 0.53).

In Figure 5 the blood clotting profile for control and both Vit.E and Vit.E Ac enriched P(D,L)LA films are shown. The absorbance of the hemolyzed hemoglobin solution is time dependent and it is known that to an high absorbance correspond a better thromboresistance. The clotting time has been defined as the time at which the hemolyzed hemoglobin absorbance equals 0.2. Unmodified P(D,L)LA samples were completely coagulated after about 50 min , while Vit.E blending significantly slowed the coagulation process ($P < 0.001$). As expected, also in this case, Vit.E Ac blending did not significantly alter the coagulation process.

Platelets aggregation, and the subsequent thrombi and thromboemboli formation, is due to their rapid adhesion and activation: for this reason the reduced platelets adhesion observed in P(D,L)LA/Vit.E samples could explain its thromboresistence monitored using the clotting time for whole blood. When a

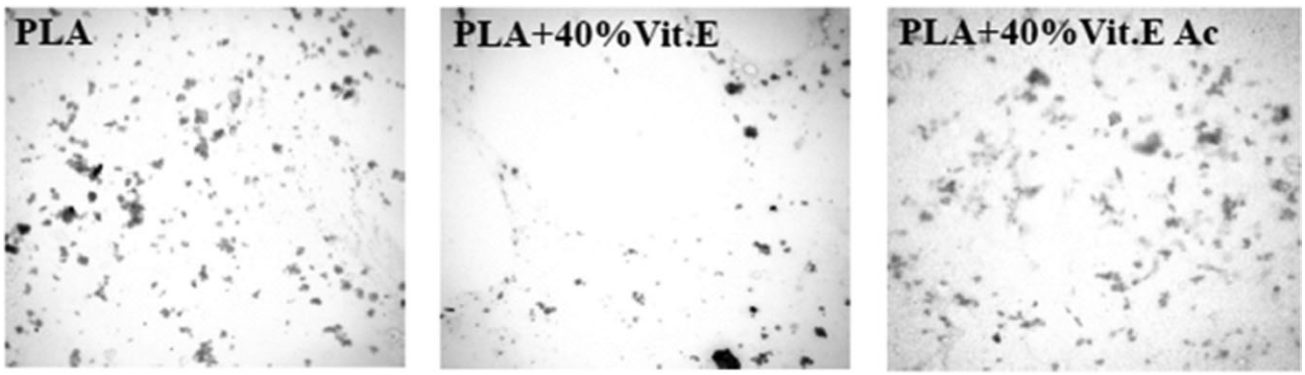

Figure 4. Platelet adhesion: quantification of platelets adhesion onto P(D,L)LA, P(D,L)LA/Vit.E, and P(D,L)LA/Vit.E Ac specimen. Magnification 10×.

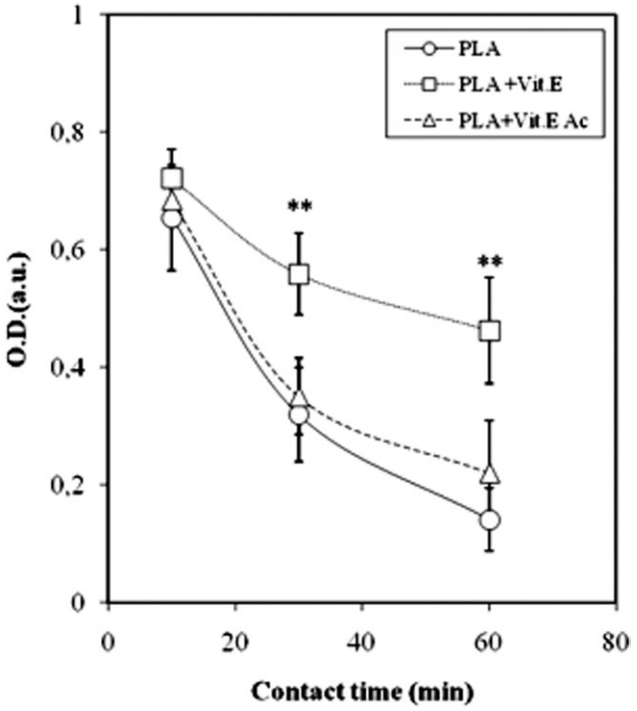

Figure 5. Blood clotting profile: absorbance of hemolyzed hemoglobin solution obtained after contact with P(D,L)LA, P(D,L)LA/Vit.E, and P(D,L)LA/Vit.E Ac specimen (**$P < 0.001$).

biomaterial interacts with blood, the first events occurring at its surface are protein adsorption and platelets adhesion, which contribute, in addition to leukocytes activation, to the acute inflammatory response occurring at the site of biomaterial grafting.[29]

SEM and Solid State NMR Analysis

In order to investigate Vit.E induced modifications in P(D,L)LA morphology and structure, SEM, and solid state NMR analysis were performed.

SEM analysis showed that Vit.E incorporation into P(D,L)LA films dramatically altered their surface characteristics, resulting in a very porous structure (average pore diameter = 0.334 ± 0.08 μm), while Vit.E Ac incorporation only slightly affects PLA surface morphology (Figure 6).

This effect can be related at the change in interfacial tension during the process of casting, in fact is know[30,31] that the presence of amphiphylic substances change the polymer/solvent interface that evolve in a different organization in the final films.

Furthermore, the ^{1}H MAS NMR spectra of the composite samples showed a very good resolution for a solid sample. It presented broad lines characteristic of a rigid solid as well as sharp lines characteristic of mobile species. In the pure P(D,L)LA spectrum [Figure 7(A — a)], the most intense signals were in the range of 1–3 ppm and 5–7 ppm and were assigned to methyl and methine protons. The broadness was due to the overlapping of resonances arising from different stereoisomers and polymer chains.[32,33] The methyl resonances comprised two sharp peaks in addition to broad closely spaced resonances which arose from the crystallographically in-equivalent sites of rigid and mobile domains. Surprisingly, a resonance at 8.1 ppm was visible in the spectrum of pure P(D,L)LA and was attributed to the entrapped chloroform protons. The spectra of composite samples consisted of resonances from P(D,L)LA as well as Vit.E/Vit.E Ac [Figure 7(A — b, c)]. The existence of multiple resonances at different chemical shifts in the range of 1–3 ppm, suggested that the methyl protons of PLA and Vit.E/Vit.E Ac exist in a number of different chemical environments within the mobile/rigid interfaces. This effect might arise from the differences in the proximity of PLA and Vit.E/Vit.E Ac molecules or from difference in the packing order. The peaks from Vit.E Ac were well resolved compared to that of Vit.E and they were associated to higher mobility. The absence of 8.1 ppm chloroform proton resonance in P(D,L)LA/Vit.E composite was striking. However, the chloroform peak was present in the P(D,L)LA/Vit.E Ac composite, in addition to a resonance at 7.6 ppm. These two

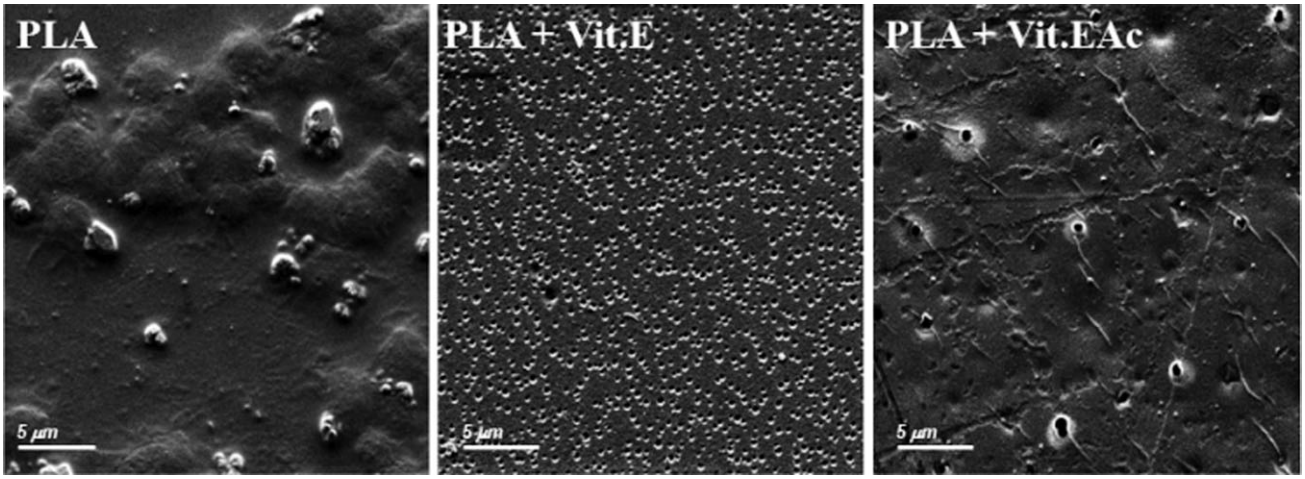

Figure 6. Polymer surface ultrastructure: SEM microphotographs of P(D,L)LA, P(D,L)LA/Vit.E, and P(D,L)LA/Vit.E Ac surfaces. Magnification 10000×.

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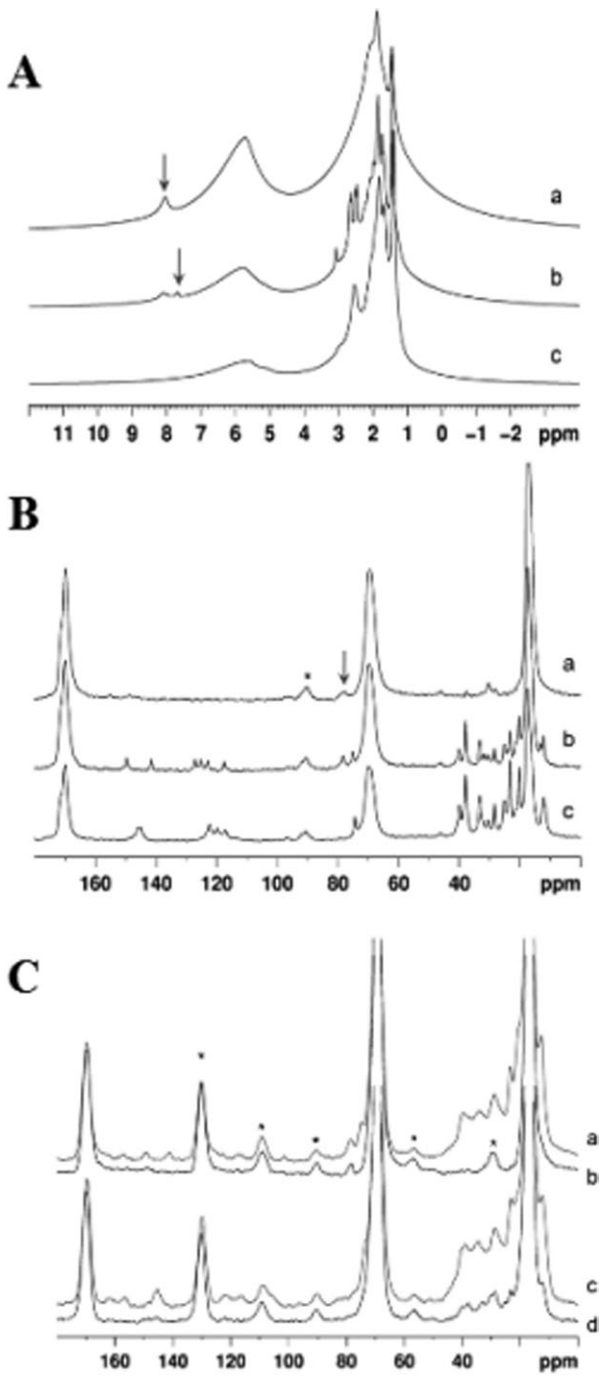

Figure 7. (A) ^{1}H MAS-NMR analysis: ^{1}H solid state MAS-NMR spectra of pure PLA (a), PLA-Vitamin E acetate (b), and PLA-Vitamin E (c). A MAS rate of 15 kHz was used in all the experiments and the arrows indicate resonance peaks at 8.1 and 7.6 ppm (see text for details). (B) ^{13}C MAS-NMR analysis: ^{13}C solid state MAS-NMR spectra of pure PLA (a), PLA-Vitamin E acetate (b), and PLA-Vitamin E (c). A MAS rate of 10 kHz was used in all the experiments and the arrow indicate resonance peak at 78.4 ppm (see text for details). * denotes spinning sidebands. (C) ^{13}C CPMAS-NMR: ^{13}C solid state CPMAS-NMR spectra of PLA-Vitamin E acetate recorded at 223 K (a) and 298 K (b) and of PLA-Vitamin E at 223 K (c) and 298 K (d). A MAS rate of 5 kHz and cross polarization contact time of 1 ms was used in all the. * denotes spinning sidebands.

distinct resonance peaks for chloroform might arise from the different entrapped molecules in a hydrophobic cage. Furthermore evidences were available from the ^{13}C MAS NMR data shown in Figure 7 where broad chloroform carbon peaks at 78.4 ppm were visible in pure P(D,L)LA as well as P(D,L)LA/Vit.E Ac composite confirming the anisotropic broadening due to either a distribution of environments or with a reduced mobility of the molecules in the hydrophobic cage.[34] The origins of chloroform peak were further confirmed by recording NMR data (not shown) on composites synthesized using deuterated chloroform.

The key difference between P(D,L)LA/Vit.E and P(D,L)LA/Vit.E Ac composite lied in the hydrophilic/hydrophobic surface morphology and microstructural fingerprint patterning. While the ^{13}C MAS NMR peaks from P(D,L)LA component were considerably broader, the peaks from Vit.E and Vit.E Ac were surprisingly sharp [Figure 7(B)]. Such good resolution with sharp lines could be attributed either to a greater mobility of the Vit.E/Vit.E Ac molecules, or to a more regular packing, with less equivalent orientations.[34] Solid state NMR could distinguish dynamic disorder arising due to distribution of molecular motion and static disorder due to distribution of local environments. Molecular mobility associated with the amorphous phase resulted in a distribution of ^{13}C environments and a broad ^{13}C line but reduced the proton–proton dipolar coupling resulting in a narrow ^{1}H line.[35] Insights into the local motions of molecules in P(D,L)LA, P(D,L)LA/Vit.E and P(D,L)LA/Vit.E Ac have been derived from variable temperature ^{13}C cross polarized (CP) MAS NMR. Spectra were collected at room temperature (RT) and at 223K [Figure 7(C)]. The observed line shapes and chemical shifts have a different behavior at two different temperatures. Reduced mobility at low temperature enhances the ^{13}C CP response as evidenced by the appearance of Vit.E/Vit.E Ac peaks [Figure 7(C)]. However, the efficiency of CP technique is notably reduced at room temperature due to mobility of molecules. As a matter of fact, there is a better CP efficiency for Vit.E in composite at RT, probably because of superior organization of the molecules in composite. This leads to the conclusion that at RT Vit.E/Vit.E Ac molecules act like liquids while lowering temperature pilot to the solidification of molecules. It is thus demonstrates that the addition of Vit.E to P(D,L)LA polymer leads to molecular dispersion and support the formation of microstructural cavities were the organization of Vit.E molecule guides to the reduction of surface hydrophobicity of the material and probably the formation of pores.

The existence of multiple NMR signals suggested that the methyl protons of P(D,L)LA and Vit.E/Vit.E Ac exist in different chemical environments within the mobile/rigid interfaces as effects of the differences in the proximity of P(D,L)LA and Vit.E/Vit.E Ac molecules or differences in the packing order. Moreover Vit.E Ac molecules showed higher mobility compared to Vit.E molecules. These characteristics caused a main difference in the hydrophilic/hydrophobic surface morphology and microstructural fingerprint patterning of the two composites. Moreover the higher Vit.E CP efficiency in composites at RT indicates a superior organization of this molecule in the composite.

These observations lead to the conclusion that at RT Vit.E/Vit.E Ac molecules act like liquids but Vit.E "organizes" the composite structure. This study demonstrates that the addition of Vit.E to P(D,L)LA polymer leads to molecular dispersion and support the formation of microstructural cavities were the organization of Vit.E molecule guides to the reduction of surface hydrophobicity of the material and probably the formation of pores as observed by SEM and the subsequent increasing in the composite hemocompatibility.

CONCLUSIONS

The use of Vit.E for P(D,L)LA modification was suggested by its attractive anti-oxidant and anti-inflammatory properties in addition to the possibility of improving P(D,L)LA biocompatibility using a natural and biocompatible agent without side effects. The blending of P(D,L)LA with pure Vit.E, but not with its commercial analog (Vit.E Ac), has been demonstrated to dramatically alter polymer surface and biological characteristics through an "hydrophilic" effect probably due to a peculiar molecular organization occurring in the P(D,L)LA/Vit.E composite as indicated by NMR analysis. The new biological characteristics displayed by P(D,L)LA/Vit.E composite could suggest new interesting fields of application for this composite.

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