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Analysis method and characterization of the antioxidant capacity of vitamin E-interactive polysulfone hemodialyzers

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Abstract

The lipophilic antioxidant vitamin E was used as a surface modifier (or coating agent) of hollow-fiber hemodialyzer membranes with the aim of increasing their biocompatibility and preventing oxidative stress, which are the main clinical drawbacks in hemodialysis (HD) therapy. At present, the redox chemistry of vitamin E-modified dialyzers is not well characterized and there is no standard method to assess the antioxidant capacity of these biomembranes under conditions that simulate those observed during HD therapy. With this study, we developed an original online method to determine the antioxidant capacity of vitamin E-modified dialyzer membranes during circulation experiments. This method is based on a spectrophotometric assay known as the ferric reducing/antioxidant power assay (FRAP). The principle of FRAP and its application to the qualitative and quantitative assessment of miniaturized polysulfone (PS)-based vitamin E-modified dialyzers (PS-VE) were verified by the accurate in vitro analysis of the iron-catalyzed oxidation of vitamin E. The antioxidant capacity of miniaturized PS-VE samples assessed in this study was of 14.5 μM Fe^{2+}, which corresponded to the transformation of nearly one-third of the vitamin E bound to the hollow-fiber membrane to its oxidation end product α-tocopherol quinone. This method shows good reproducibility and intra- and inter-assay precision, and can be easily adapted to determine the redox activity of every type of vitamin E-modified dialyzers during technological investigation, manufacturing control and clinical research.

Keywords: Vitamin E; Tocopherol; Tocopheryl quinone; Antioxidants; Iron

1. Introduction

In the early 1990s, vitamin E (α-tocopherol) was first introduced in the field of hemodialysis (HD) biomaterials to produce vitamin E-modified dialyzer membranes (Fig. 1A). This vitamin, which is well known for being a lipophilic antioxidant of cell membranes and lipoproteins [1], was used as a modifier (or coating agent) on the blood surface of cellulosic hollow-fiber dialyzer membranes; the ultimate goal of using this vitamin was to increase the biocompatibility and to prevent the oxidative stress that is related to leukocyte activation and other inflammation-related effects that may result from the interaction between blood constituents and cellulosic fibers [2].

In the last few years, several small clinical trials have indicated the positive effect of these membranes on surrogate markers of oxidative stress and inflammation in HD patients (reviewed in Refs. [2,3]). A recent meta-analysis confirmed the effect of protection against oxidative stress as measured by low-density lipoprotein oxidation during HD performed with cellulose-based hemodialyzer membranes [4]. Various multicenter studies have suggested that these membranes may play a more effective role in the management of uremic anemia [5], which is of particular relevance in clinical practice [3].

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Recently, the natural evolution of HD technology toward more biocompatible biomaterials has stimulated industries to produce synthetic polysulfone (PS)-based membranes modified with vitamin E (PS-VE) that will substitute the cellulosic ancestor. This new generation of dialyzer membranes has been introduced in the clinical practice in Japan and Europe, and early clinical evidence of improved biocompatibility and oxidative stress indices was reported in a controlled trial performed with a previous version of these modified membranes [6]. Further studies of PS-VE currently available for clinical use are in progress and the preliminary data indicate that these membranes can ameliorates anemia management by an improved erythropoietin responsiveness in maintenance HD patients [7].

The available clinical evidence is based on the implicit claim that these biomembranes can be used in the antioxi-

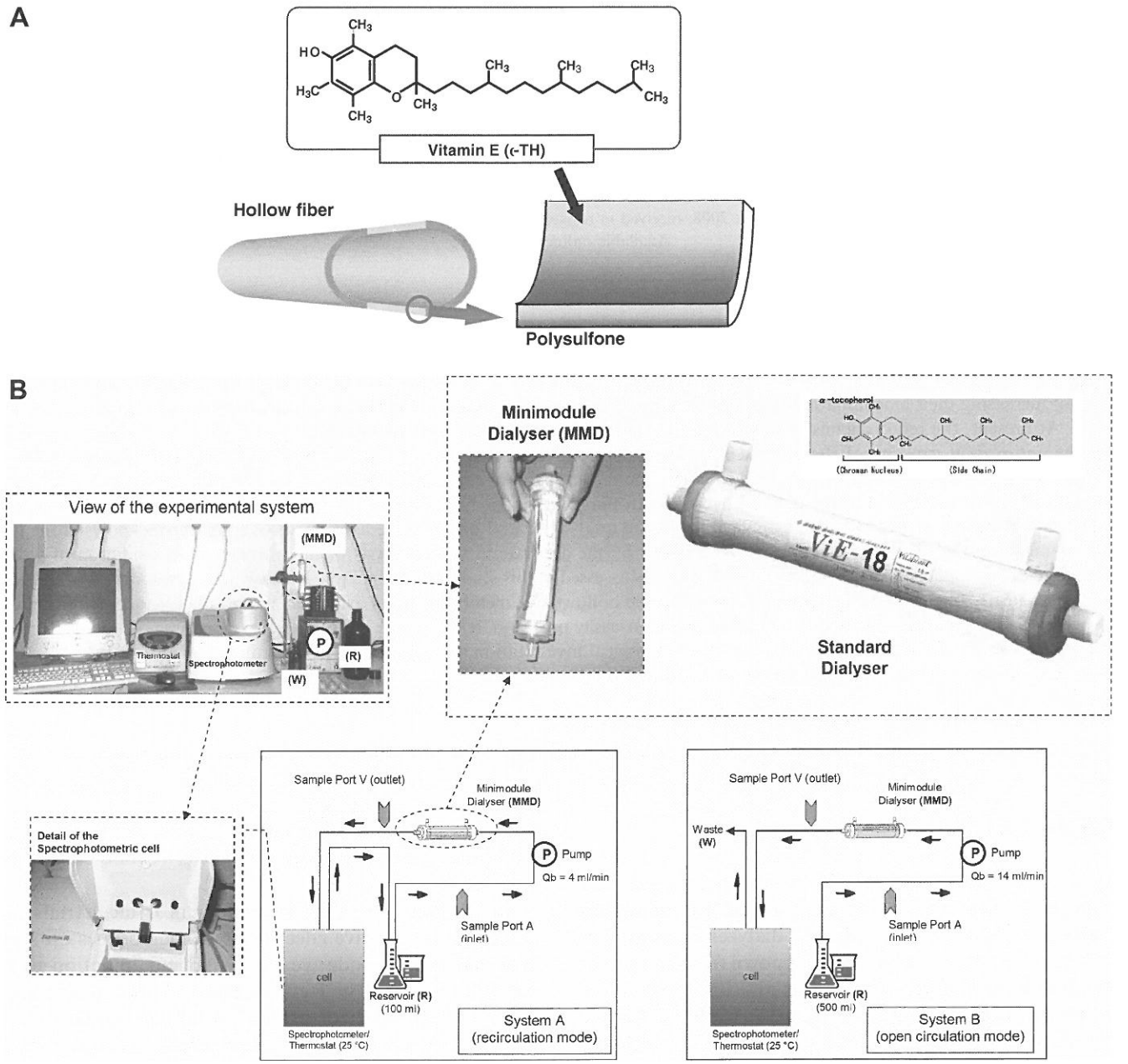

Fig. 1. Circulation systems and dialyzer samples used in this study to evaluate the antioxidant capacity of vitamin E-modified dialyzers. (A) Schematic representation of a hollow fiber modified with vitamin E in a vitamin E-interactive polysulfone membrane. (B) View of the in vitro circulation system with the online spectrophotometric detection apparatus (upper left) and hollow-fiber dialyzers (upper right insert); a scheme of the two circulation modes employed in the study is also reported and includes a closed-loop circuit (system A, bottom left insert) and an open circuit (system B, bottom right insert). (C) Scanning electron microscopy analysis of PS and PS-VE samples (Table 1). The photographs are cross-sections of PS 2 (a) and PS-VE 2 (b), the internal surface of PS 2 (c) and PS-VE 2 (d), and the outer surface of PS 2 (e) and PS-VE 2 (f), respectively. This analysis was representative of the entire set of experiments performed to assess both series 1 and 2 of the VE-modified and unmodified MMDs investigated in this study. The magnitudes of the view were indicated in each photograph.

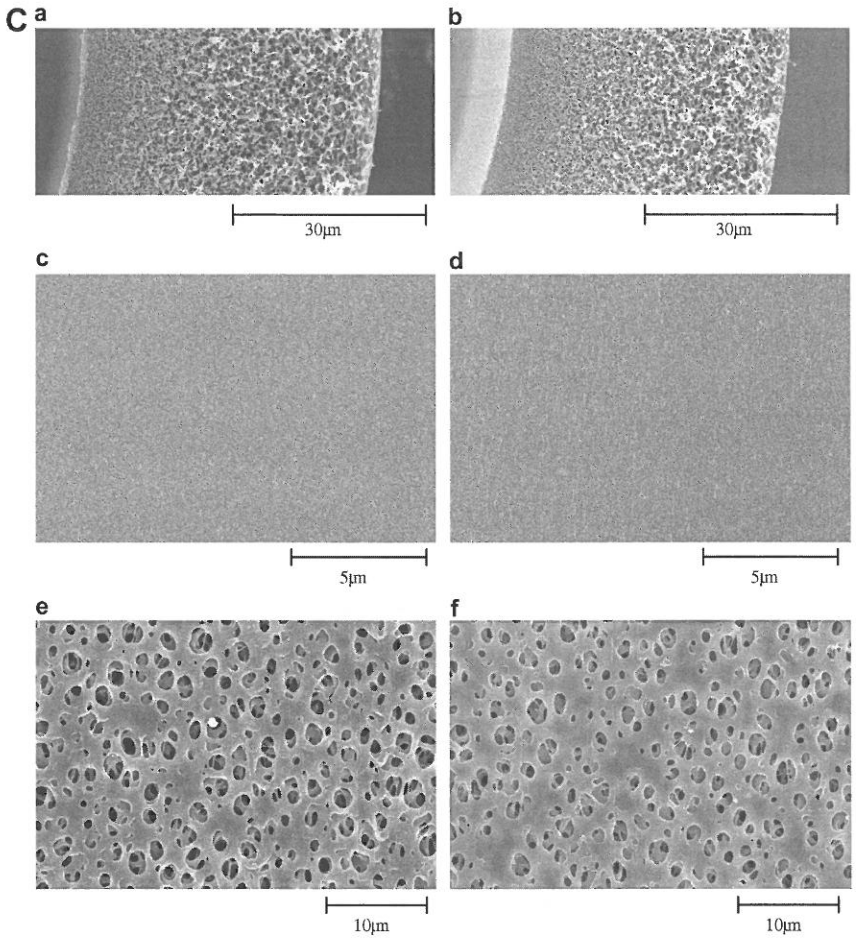

Fig. 1 (*continued*)

dant therapy of HD patients [2–4], but the antioxidant properties of such new vitamin E-modified hemodialyzers have yet to be defined with regard to their biochemical mechanisms and levels of activity. This lack of knowledge is essentially due to the lack of well-defined assay procedures that can measure the redox chemistry of vitamin E membranes under conditions as similar as possible to those observed during HD therapy.

Therefore, the aim of this study is to set up an in vitro assay procedure to reliably determine the antioxidant activity of these dialyzer membranes during circulation experiments. The ferric reducing/antioxidant power assay (FRAP), which is widely used to assess the antioxidant capacity of several biological matrices and individual compounds, was found to be suitable for developing an online spectrophotometric method that can be used successfully to study the antioxidant capacity of miniaturized and standard dialyzer membranes.

2. Materials and methods

2.1. Chemicals

2,4,6-Tripyridyl-*s*-triazine (TPTZ), (*R*)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (*R*)-α-tocopherol (α-TH), ascorbic acid, pyrogallol red fluorescein, Fe salts as Fe^{3+} ($FeCl_3 \cdot 6H_2O$) and Fe^{2+} ($FeSO_4 \cdot 7H_2O$), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium acetate trihydrate, glacial acetic acid, and other unspecified reagents and solvents were purchased from Sigma Chemicals (Milan, Italy) and were of the highest purity available. D-α-Tocopherylquinone (α-TQ) was obtained from Acros Organics by Carlo Erba Reagenti, Milan, Italy, or was prepared by the iron-catalyzed oxidation of α-TH. In brief, 2.5 ml of a solution made by dissolving 1.2 g of $FeCl_3$ in 15 ml of methanol/water (50/50 vol.%), was added to a solution of tocopherol in diethyl ether (1 g in 10 ml). After agitation for 30 min, the mixture was centrifuged and the lower phase was removed. This procedure was repeated thrice with thorough washing of the ether phase using water (8–10 times). The ether phase was then evaporated and the residue was dissolved in ethanol or ether. The exact concentration of the α-TQ solution was determined at 260 nm using a molar extinction coefficient of 19,500, and its purity was verified by high-performance liquid chromatography (HPLC) analysis of α-TH and α-TQ (see below).

To prepare the FRAP reagent, TPTZ was dissolved in 1 N HCl and then mixed with the other components as

Table 1
Some of the main characteristics of the four types of MMD used in this study.

MMD type	PS 1	PS-VE 1	PS 2	PS-VE 2
Corresponding device	APS-U	ViE	REXEED™-L	ViE-L
Hollow fiber material*	PS	PS-VE	PS	PS-VE
Internal diameter of the hollow iber (μm)	200		185	
Shape of the hollow fiber	Straight		Waved	
Number of hollow fibers	800[a]		860[a]	
Wall thickness of the hollow fiber (μm)	45			
Effective hollow fiber length (mm)	100[a]			
Effective surface area (cm^2)	500[a]			
Priming volume (ml)	4[a]			
Potting marerial	Urethane			
Housing and header material	Polycarbonate			

* PS, polysulfone; PS-VE, vitamin E-interactive PS.
[a] Approximate measure.

described below. Trolox and α-T were dissolved in HPLC-grade methanol.

2.2. Miniaturized dialyzer samples and the in vitro circulation system

The antioxidant activity of PS-VE was investigated using miniaturized (or mini-module) hollow-fiber dialyzer membranes (MMD) connected to the in vitro circulation system shown in Fig. 1B. The MMD samples have the same gross structure as the standard dialyzer membranes used in HD therapy (Fig. 1, upper-left inset). Four types of MMD were prepared for use in this study and were designated as PS1, PS-VE1, PS2 and PS-VE2. These MMD samples correspond to the commercially available products APS-U™, ViE™, REXEED™-L and ViE™-L, respectively (Table 1). These samples were prepared as follows: a bundle of hollow fibers was introduced into a cylindrical housing made of polycarbonate. Both ends of the bundle were then potted with liquid urethane and cured to make solid urethane. The solid urethane ends were cut to make flat surfaces and the hollow fiber ends were opened. The headers were then attached to make blood ports. The assembled module was then filled with pure water, packed into a nylon sealing bag and sterilized by gamma-irradiation. The main physical properties of the entire set of samples prepared for this study were investigated to verify their ultrastructural homology by scanning electron microscopy and the fiber strength of matching dialyzers (PS-VE vs. PS).

The circulation system was developed using a peristaltic pump and Tygon® lines, and was connected to a spectrophotometric apparatus equipped with a thermostat and a cell module for continuous ultraviolet/visible (UV/Vis) light monitoring (Thermo Fisher, Milan, Italy). This circuit system was set to operate either in the closed-loop recirculation mode or open circulation mode, and could easily be adapted for the use of other detection equipment, such as fluorimetric or electrochemical detectors for HPLC, which may provide a high sensitivity. These detectors were preliminary tested to evaluate the most suitable antioxidant test to be used in the evaluation of PS-VE during circulation experiments.

2.3. Antioxidant capacity tests

To determine the antioxidant activity of PS-VE, several redox-active probes were preliminarily evaluated for use in spectrophotometry, fluorimetry and electrochemical detection. These included ascorbate anions, pyrogallol red, fluorescein and ferric ions (Fe^{3+}). Ascorbate anions spontaneously auto-oxidize in solution to form dehydroascorbate, and this reaction was used to detect transition metals in solution [8]. Pyrogallol red and fluorescein change their spectroscopic properties when incubated in the presence of peroxyl radicals, and were thus used to measure antioxidant compounds with a principle that derives from that of the oxygen radical absorbance capacity (ORAC) assay [9]. In the FRAP method [10], ferric ions are used as electron acceptors, which are reduced to ferrous ions (Fe^{2+}) in acetate buffer by antioxidant compounds. The ferrous ions formed by this redox reaction bind to the chromogen TPTZ, which increases its absorbance at 600 nm in a concentration-dependent way. Further details and the changes made to adapt these tests to the in vitro analysis system of this study are reported in Section 3.

2.4. Analysis of vitamin E

The amount of vitamin E in PS-VE was measured after elution with a solution of 5% tetrahydrofuran (THF) in HPLC-grade methanol carried out using the same system as used for antioxidant activity tests (Fig. 1, system B) with solvent resistant lines. Vitamin E, like α-T and α-TQ, was measured by either HPLC or gas chromatography–mass spectrometry (GC-MS) analysis using the procedures previously published in Ref. [11]. Briefly, RP-HPLC analysis was carried out on a Prostar HPLC system (Varian, Milan, Italy) consisting of two model 210 solvent delivery modules with a high-pressure gradient mixer, an in-line degasser and a model 410 autosampler. This HPLC system was connected with a model 320 UV/Vis detector mounted in series

with an electrochemical detector EG&G PARC model 400. The EC detector was set to the coulometric detection mode with an oxidation potential of 750 mV and a sensitivity of 10 μA, while the UV detector was set at 262 nm for 5 min and then switched to 292 nm for the remaining part of the analysis. Chromatographic separation was performed under isocratic conditions (flow rate = 1 ml min^{-1}) by using a Synergy C12 RP-max column (150 × 4.6 mm, 4 μm; Phenomenex, Milan, Italy) and a mobile phase composed of methanol/THF/H_2O 93/5/1 (vol.%) containing 10 mM $LiClO_4$. The injection volume was 20 μl. Peak identification and analysis calibration were performed using external standards (concentration range: 0–1.6 mM for α-T and 0–1 mM for α-TQ), and tocol was used as an internal standard when required.

To confirm the identity of α-T and α-TQ, GC-MS analyses were carried out using a GC Varian CP3800 coupled with a Saturn 2000 MS-equipped ion trap mass detector (Varian, Milan, Italy). The carrier gas was helium, with a constant flow of 1 ml min^{-1}. The injector temperature was 260 °C. The capillary column used a Factor Four VF-5 MS with low polarity (Varian, Milan, Italy), 30 m × 0.25 mm and $d_f = 0.25$ μm. The column temperature was set as follows: 150 °C for 2 min, 20 °C min^{-1} to 240 °C, 2 min, 25 °C min^{-1} to 285 °C, 3 min, 22 °C min^{-1} to 300 °C, 8 min. The detector settings were: emission current, 10 μA; temperatures: trap, 210 °C; transfer line, 170 °C; manifold, 100 °C; and mass range, *m/z* 200–550. Compound quantification was carried out by selected ion monitoring (SIM) using the following ions: for TMS-α-T: *m/z* 236, *m/z* 237, *m/z* 277 and *m/z* 502; for TMS-α-TQ: *m/z* 293, *m/z* 342, *m/z* 429 and *m/z* 504.

Samples for GC-MS analysis were obtained in the manner described above for HPLC analysis and then dried under a stream of N_2; thus pyridine (100 μl) and BSTFA with 1% TMCSC (100 μl) were added and the mixture was heated at 70 °C for 15 min and then evaporated under N_2. The residue was dissolved in 200 μl of hexane and vortexed for 30 s; 1 μl of this sample was then injected into the GC system.

3. Results and discussion

3.1. Scanning electron microscopy of hollow-fiber dialyzers

Ultrastructural morphology examination of matching hollow-fiber dialyzers (PS-VE vs. PS, see also Table 1) revealed that these types of samples showed close structural homology (Fig. 1C). Hollow-fiber strength, flow dynamics and hydraulic properties were also equivalent between matching dialyzer samples (data not shown).

3.2. Antioxidant capacity test setup

In preliminary experiments, the spontaneous autoxidation of ascorbate anions [8] and the AAPH-induced oxidation of pyrogallol red or fluorescein [9] were assessed as reactions useful for measuring the antioxidant capacity of PS-VE. Both these methods were found to be inappropriate to accurately determine the redox activity of vitamin E-modified membranes (not shown). The main limiting factor of these assay methods was the poor efficacy of electron transferring (i.e. the difference in reduction potential) between the redox probes and the reducing agent vitamin E, which is bound to PS-VE fibers. This was the main aspect that constrained the sensitivity and precision of the assay methods, together with the limited versatility of the HPLC detection systems employed to maximize the output of these assays (see Section 2).

In contrast with these early negative results, the Fe^{3+}–TPTZ reagent system, also known as FRAP reagent [10], was found to offer the most suitable operative conditions to develop an online method to measure the antioxidant activity of vitamin E either in a solution (as α-tocopherol or Trolox as test molecules) or bound as a modifier of PS-VE fibers. The Fe^{3+} and TPTZ concentrations used to prepare the typical FRAP solution (2.46 g l^{-1} sodium acetate, 15 ml l^{-1} glacial acetic acid, 100 mg l^{-1} $FeCl_3 \cdot 6H_2O$ and 60 mg l^{-1} TPTZ) [10] were adjusted to obtain optical density values below 0.8 arbitrary units during the recirculation experiments. In a typical experiment, 100 ml of a solution of $FeCl_3$ and TPTZ (both 5 mg ml^{-1}) was flushed in the recirculation mode (Fig. 1A, system A) at a flow rate of 4 ml min^{-1} throughout the four types of MMD described in Table 1, and the presence of vitamin E in the MMD fibers (i.e. PS-VE test samples) produced a characteristic

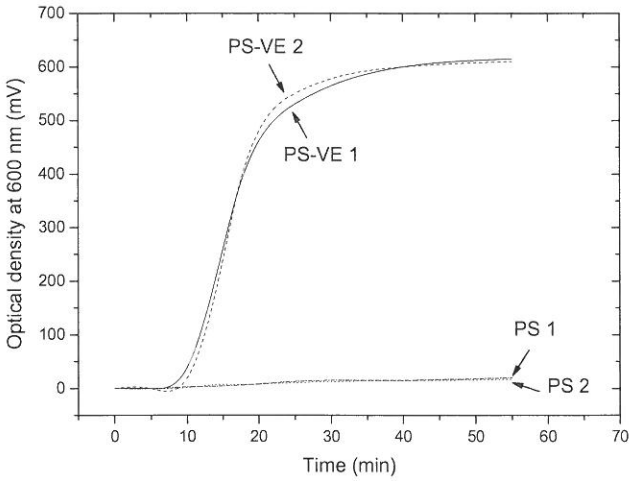

Fig. 2. Fe^{3+}–Fe^{2+} reduction curves produced during the online FRAP assay performed in recirculation mode on PS-VE and unmodified PS samples. These tests were carried out using the PS-VE (vitamin E-modified) and PS samples described in Table 1. These MMD samples were mounted on the apparatus shown in Fig. 1B, system A; 100 ml of a modified FRAP solution containing 5 mg ml^{-1} of both $FeCl_3$ and TPTZ was recirculated in the system at a flow rate of 4 ml min^{-1} for a time sufficient to complete the colour development that was monitored in the continuous mode by spectrophotometric recording at 600 nm. Further details are reported in the text.

increase in the curve of absorbance monitored at 600 nm, while MMD without vitamin E did not significantly affect the absorbance values (Fig. 2). The reduction curve produced by PS-VE reached the plateau after approximately 30 min of recirculation with a maximum absorbance of approximately 0.65 Abs units. The spectrophotometric detection method adopted to perform this FRAP assay was found to be robust and reproducible. Intra- and interassay CV values for this method were determined in three separate experiments run in triplicate and were found to be less than 8% in both cases.

Moreover, this test can easily be used to evaluate standard dialyzer membranes that have a considerably bigger surface area and require flow rates up to or above 250 ml min^{-1} (see below), provided that the colour of the FRAP solution is adjusted by dilution to obtain a suitable absorbance at 600 nm after the reduction response is produced by the dialyzer membrane. Alternatively, to avoid excessive colour formation in this type of experiments, the FRAP test can be carried out in the offline mode. In this case, $FeCl_3$ solution is circulated throughout the dialyzer membrane (i.e. without the TPTZ reagent) and the antioxidant capacity of PS-VE dialyzers is determined by the regular sampling of the Fe^{3+}–Fe^{2+} mixture formed by the reaction with vitamin E. These samples are mixed with the TPTZ reagent and measured by spectrophotometric analyses against an appropriate Fe^{2+}–TPTZ calibration curve. When applied to MMD samples, this offline procedure was demonstrated to provide the same results in terms of antioxidant capacity values then in the case of online circulation experiments (not shown).

3.3. Determination of the actual antioxidant capacity of PS-VE

The optimal conditions to measure the actual antioxidant capacity of PS-VE were determined using the open circulation mode shown in Fig. 1B (system B), and these conditions were applied to the MMD samples in Table 1 named PS2 and PS-VE2. The FRAP solution described in Ref. [10] and in Section 2 above was circulated through these MMD samples at a flow rate of 10 ml min^{-1} (this simulates the flow rate/MMD surface ratio applied to standard dialyzer membranes during HD therapy) and the Fe^{3+}–Fe^{2+} reduction profile was determined by recording, in continuous mode at 600 nm, the development of colour that resulted from the formation of the Fe^{2+}–TPTZ complex. Fig. 3 shows a series of reduction curves ($n = 3$) obtained during a typical one-day session of experiments. These curves were calculated using the Fe^{2+}–TPTZ response equation shown in the inset of Fig. 3. This curve was obtained by mixing 1 vol of FRAP solution (2×) with 1 vol of the solution of $FeSO_4$ ($7H_2O$) that was diluted in water to achieve the required final concentrations of Fe^{2+}. The average curve obtained from all the experiments on PS-VE2 performed in this study ($n = 10$) was characterized by a reduction peak that corresponded to 0.56 ± 0.09 μM Fe^{2+} produced at 6.8 ± 0.3 min. Thus, Fe^{2+} formation progressively returned to baseline values (peak half-life: 52 min) with a kinetics that was completed after 253 ± 10 min.

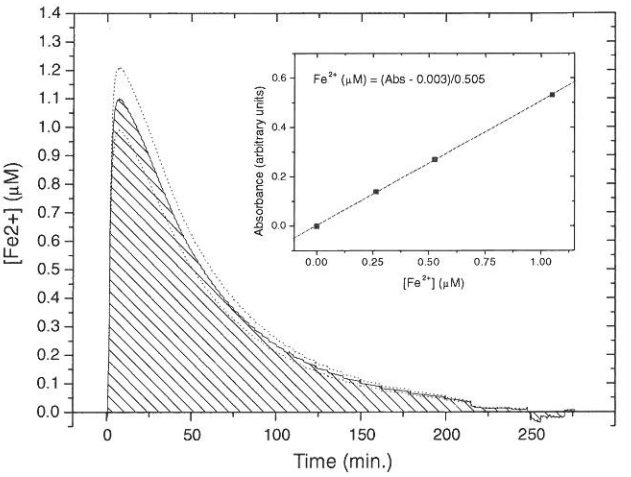

Fig. 3. Average Fe^{3+} reduction curve produced during the circulation of FRAP solution throughout miniaturized PS-VE2. The average and 1SD curves (straight and dotted lines, respectively) were calculated as best fit of absorbance data recorded at 600 nm in 10 separate experiments in which the FRAP solution (2.46 g l^{-1} sodium acetate, 15 ml l^{-1} glacial acetic acid, 100 mg l^{-1} $FeCl_3 \cdot 6H_2O$ and 60 mg l^{-1} TPTZ), was circulated through PS-VE 2 mounted using the open circuit described in Fig. 1C. Fe^{2+}–TPTZ data were derived from the response curve obtained by the analysis of colour developed by mixing 1 vol of FRAP reagent (2×) with 1 vol of $Fe(SO_4)$ that was adjusted by serial dilutions to the desired final concentrations (inset).

The area under the iron reduction curve (AUC) normalized by the Fe^{2+}–TPTZ response equation above (Fig. 3, insert), allowed in calculating a total antioxidant (reduction) capacity of 72.6 ± 6.5 μM Fe^{2+} for PS-VE2, while PS2 did not exhibit any significant antioxidant activity (not shown).

Once normalized by the volume of the FRAP solution circulated into the PS-VE (approximately 2.5 l), and assuming that a two-to-one electron reduction process is required to explain the mechanism of the metal-catalyzed oxidation of vitamin E [12–15], the average AUC value obtained in our experiments corresponds to the 14.5 μmol equivalents of α-TH (i.e. 6.25 mg) that was oxidized during the reaction shown in equation (a). This corresponds to approx. 29 nmol of α-TH per cm^2 of dialyzer membrane surface.

As a preliminary finding, this assay was applied to the analysis of the antioxidant capacity of a standard vitamin E-modified dialyzer membrane of the series ViE™-15 (surface area: 1.5 m^2; priming volume: 86 ml), which corresponds to the MMD sample PS-VE1 in Table 1. The test involved the circulation of 10 l of a 5 mg ml^{-1} solution of $FeCl_3$ (flow rate of 210 ml min^{-1}) through the dialyzer membrane. Thus, at the time points shown in Fig. 4, an aliquot of this solution was collected and measured offline by

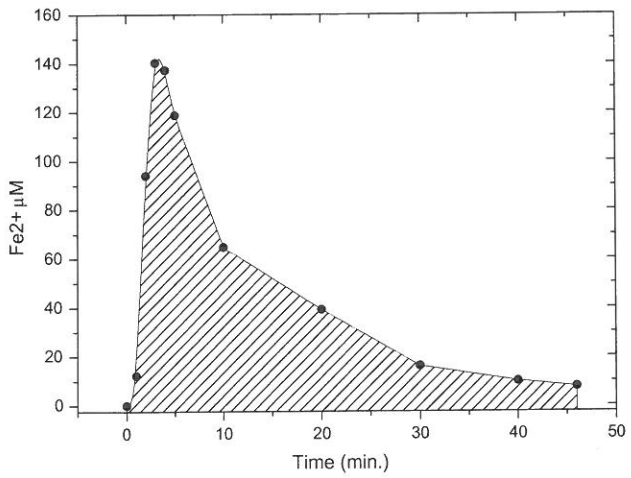

Fig. 4. Fe^{3+} reduction curves produced by the circulation of the FRAP solution throughout a standard vitamin E-modified dialyzer membrane of the series ViE™-15. This dialyzer membrane with a surface area of 1.5 m^2 and a priming volume of 86 ml corresponds to PS-VE1 in Table 1. Further details on this experiment are reported in the text.

the FRAP analysis, as described in Ref. [10]. The reduction curve obtained from this analysis showed a maximum response after 3 min with a value of 140 µM Fe^{2+}, and the AUC calculation showed an antioxidant capacity of 188 µmol equivalents (corresponding to 81 mg) of redox-active vitamin E. From these early data, a relative amount of 12.5 nmol of vitamin E per cm^2 is calculated for this standard dialyzer membrane. Assuming that the experimental settings of this test and the tests performed with MMD are completely equivalent (see above), this result suggests the possibility that the amount of vitamin E accessible to redox reactions in this PS-VE1 could be lower (by approx. 60%) than in PS-VE2, which is based on the recently developed technology Rexeed™. Further studies are in progress to ashertain whether hollow-fiber geometry might influence the antioxidant chemistry of vitamin E-interactive membranes.

3.4. Method verification: evaluation of FRAP-induced vitamin E oxidation of PS-VE

The fact that the non-extensive oxidation of vitamin E by transition metals indicated by the equation (a) can be assumed to produce equimolar amounts of Fe^{2+} ions and α-TQ as the main oxidation end products [13] provides a means to confirm the antioxidant capacity of PS-VE through the analysis of the amount of α-TQ formed during the online FRAP test developed in this study.

Vitamin E was eluted from untreated PS-VE2 samples ($n = 5$) using 5 vol.% THF in methanol according to the procedure described in Section 2. The elution profile was monitored at 292 nm to ensure the complete recovery of vitamin E (Fig. 5A). These eluates were further characterized by the analysis of their UV/Vis spectra between 200 and 500 nm and the HPLC-UV profile at 292 nm

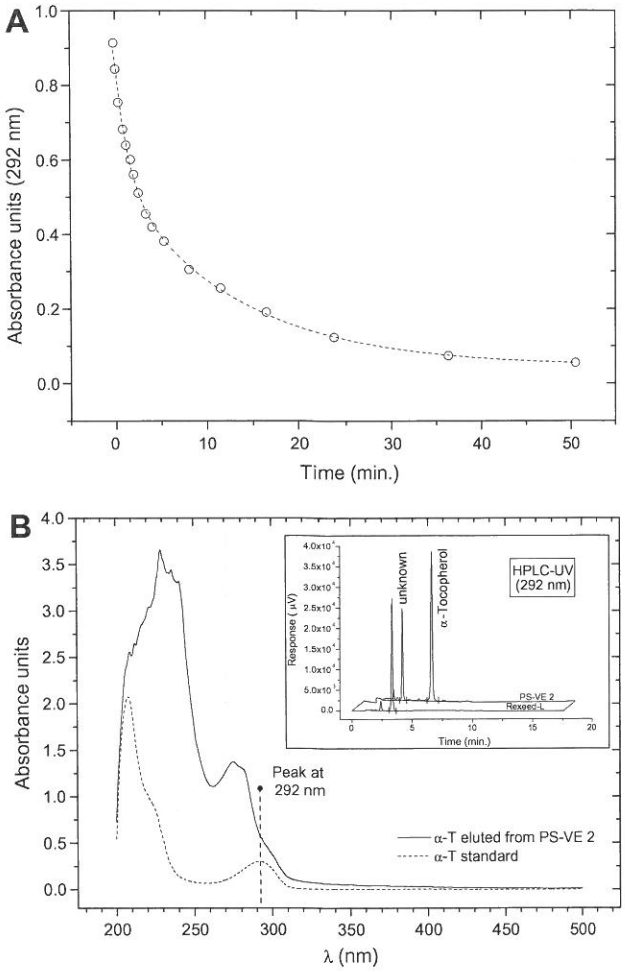

Fig. 5. Elution profile and chemical characterization of vitamin E in PS-VE2 samples. (A) The complete elution of vitamin E from PS-VE2 was achieved by the circulation (10 ml min^{-1}) of 500 ml of methanol/THF 95/5 vol.%. (B) The spectroscopic characteristics of these eluates were thus investigated in the region between 200 and 500 nm, and the presence of vitamin E was further verified by HPLC-UV analysis at 292 nm (insert).

Table 2
HPLC-UV analysis of vitamin E eluted from PS-VE 2.

	PS-VE 2 (untreated)	PS-VE 2 (after FRAP)	Difference (%)*
α-TH	23.7 ± 1.3	17.3 ± 3.5	−6.4 (−27%)
α-TQ	0.9 ± 0.5	6.7 ± 1.5	+5.8 (+644%)
α-TH + α-TQ	24.6 ± 1.4	24.0 ± 2.8	−0.6 (2%)

The data (in mg) were mean ± SD of five separate determinations.
* Calculated between mean values. Percentage data refer to untreated PS-VE 2 data.

(Fig. 5B, insert) was determined to confirm the presence of vitamin E. Other undefined substances with absorbances lower than α-TH (i.e. 292 nm) were eluted by this procedure. Further, the HPLC-UV analysis showed that these substances include only a minor fraction of α-TQ Table 2(Fig. 6, trace No. 3), which has a maximum absorbance

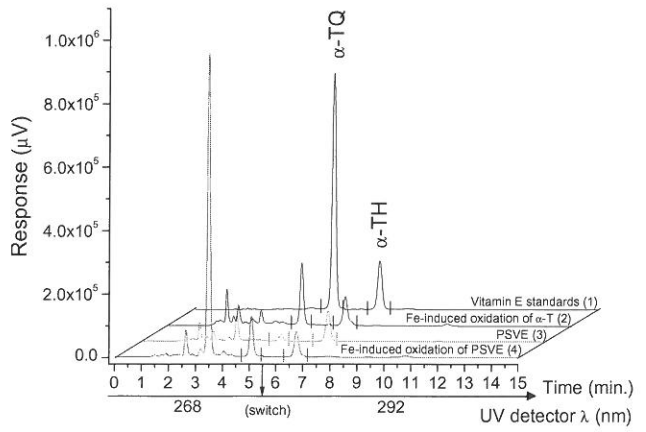

Fig. 6. HPLC-UV analysis of vitamin E eluted from PS-VE2 after FRAP-mediated oxidation. Reported chromatograms were as follows: (1) authentic standards of α-TQ and α-TH; (2) oxidation profile of methanolic solution of vitamin E (as α-TH) obtained by the elution of PS-VE2 carried out as described in Fig. 4 and incubated for 48 h with 2-mg $FeCl_3$ in acetate buffer (pH 3.8); (3) chromatographic profile of vitamin E eluted from PS-VE2; (4) chromatographic profile of vitamin E eluted from PS-VE2 after the circulation of the FRAP solution carried out as described in Fig. 3. The chromatograms are representative of all the experiments carried out in this study.

at 262 nm (Fig. 6, trace No. 1) and is formed as the main iron-induced oxidation product of vitamin E (trace No. 2) [12,13].

Fig. 6 (trace No. 4) shows that α-TQ was significantly increased in PS-VE2 after the FRAP test ($n = 5$). This chromatogram showed that the oxidation reaction catalyzed by the online FRAP test also produced other derivatives that do not appear to originate from vitamin E oxidation (Fig. 6, trace No. 4 vs. trace No. 2). The identity of α-TH and α-TQ in the eluate of the PS-VE2 treated with FRAP was further confirmed by GC-MS analysis (Fig. 7) and the data conclusively demonstrated the evidence from Fig. 6 accords with the data in the literature concerning the chemistry and analysis of vitamin E oxidation [13,14].

Using the chromatographic analyses carried out in this study, it was calculated that before the FRAP test PS-VE 2 contained 24.6 ± 1.4 mg of total vitamin E and α-TQ was less than 4 wt.%. After the FRAP test, the total amount of vitamin E (α-TH plus α-TQ) recovered by the elution of PS-VE2 was maintained to almost the same level (24.0 ± 2.8 mg), but the fraction of α-TQ increased to 6.7 ± 1.5 mg (i.e. +28%). The actual formation of α-TQ is in agreement with that expected from the moles of Fe^{2+} formed during the circulation experiments described in the previous paragraph and in the reduction curves of Fig. 3. In fact, based on the two-electron mechanism that was proposed for the oxidation of α-TH to α-TQ [12–15], we calculated that 6.4 mg of α-TQ should be produced during the online FRAP test, corresponding to approximately 96% of the actual α-TQ measured in the eluate of PS-VE2 treated with FRAP. These data confirm the reliability of the online FRAP test proposed in this study and show that approximately one-third of the vitamin E bound to PS-VE fibers is available to react with circulating redox-active substances.

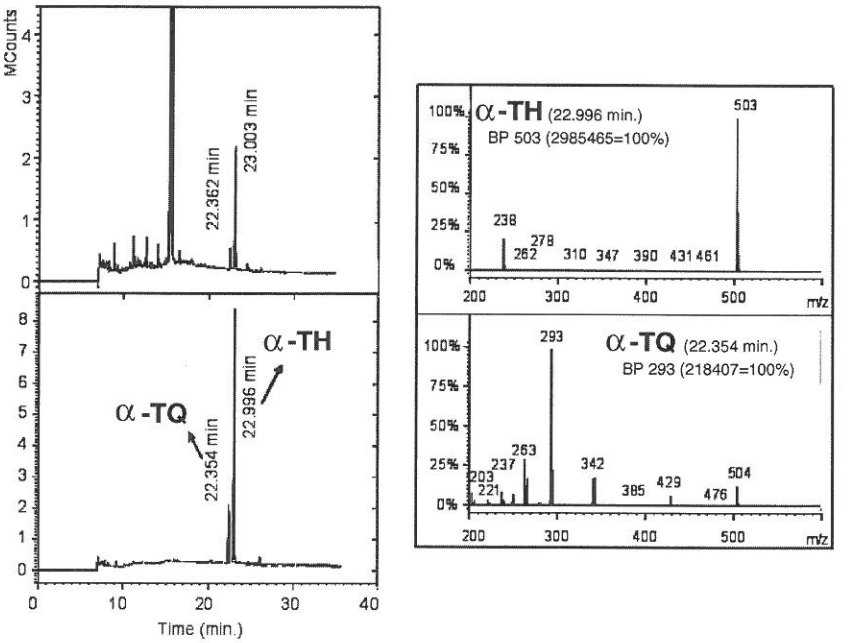

Fig. 7. GC-MS analysis of authentic standards of vitamin E (top left panel) and the eluate of PS-VE2 after the circulation of the FRAP solution (bottom left panel). The samples assessed in this experiment were the same as those assessed in the HPLC-UV analysis of Fig. 6 (trace Nos. 1 and 4). GCMS analyses were performed as described in Section 2 to confirm the molecular identities of α-TH and α-TQ. Mass scans of ion patterns are reported for the two vitamin E compounds (top right and bottom right panels) with the identification of main parental ions used for compound identification in the SIM mode.

4. Conclusion

In conclusion, this study developed an original online method to determine the in vitro antioxidant capacity of vitamin E-modified dialyzer membranes with good reproducibility and intra- and inter-assay precision. The method was verified by the accurate analysis of the iron-catalyzed oxidation chemistry of vitamin E and was successfully applied for the qualitative and quantitative assessment of the antioxidant power of miniaturized PS-VE. These data and the assay method proposed in this study will be useful to further investigate the redox properties of every type of vitamin E-modified dialyzer membrane with applications in technological investigation, manufacturing control and biological and clinical evaluations.

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