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New multifunctional textile biomaterials for the treatment of leg venous insufficiency

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Abstract Acryloyl monomers have been synthesized by reaction of β-cyclodextrin and troxerutin with acryloyl chloride and grafted on a knitted material obtained from polyamide 6.6 and polyurethane fibers. Polyamide grafted with β-cyclodextrin derivative binds troxerutin as a physical complex. The obtained biomaterials have been tested in vivo on rats for their ability to deliver troxerutin (a flebotonic drug) to different skin areas (epidermis, dermis and vascular wall). The results showed that the new synthesized materials can act as multifunctional bioactive textiles that can display both compression (given by the textile material) and sustained venotonic and haemostatic properties (given by the troxerutin delivered from biomaterial to the skin).

1 Introduction

The use of textiles in medicine has a long tradition, starting with bandages and wound dressings. Nowadays, multifunctional textiles have been designed and produced for a controlled release of active species to the skin, in order to help and accelerate the healing process of diseases, pains or wounds [1–3]. Much progress has been obtained especially in the field of antimicrobial textiles, used both for prevention and healing purposes [1].

Textile application for the treatment of leg chronic venous insufficiency is mainly based on elastic compression (stockings or bandages), sometimes associated with simple bandages for the direct contact with wounds, having antibacterial or reparatory properties [4, 5]. Flebotonic drugs are orally or topically administrated as an independent treatment of venous insufficiency.

Troxerutin (Trox) (2-[3,4-bis(2-hydroxyethoxy)phenyl]-5-hydroxy-7-(2-hydroxyethoxy)-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3S,4S,5S,6R)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one) exhibits erythrocyte anti-aggregant and viscosity reducing activities on blood, and has a protective effect on vein walls against the development of varicose veins. The primary effect of Trox is the reduction of microvascular permeability, therefore the target organ for Trox is miacrovascular endothelium. The therapeutic indications of commercially-available products containing troxerutin include: venous insufficiency, hemorrhoids and capillary fragility [6]. There is little known about the pharmacokinetics and pharmacodynamics of Trox in humans. Its chemical similarity to quercetin and rutin [7], two flavonoid drugs used in cardiovascular diseases, could indicate polyphenolic moiety as the bioactive part of the drug, and di-glycosidic group as a carrier moiety. Trox can be i.v., orally or topically administered [8]. After oral or i.v., administration, it has a very short half-life in blood [9], consequently the doses are high (up to 1 g/day). On the other hand, topical treatment of skin related diseases is very attractive, since systemic load of active pharmaceutics and systemic side effects are reduced as compared to i.v.,

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or oral drug administration [10]. Recent findings indicate that changes appear within seconds in the skin microcirculation of the distal leg after local application of Trox gel [11], showing a quick therapeutic activity and a more efficient localization of the drug at the target site (varicous veins). The efficiency of drug uptake from conventional drug carrier systems, such as creams and ointments (containing 1–3% active compound), is low [10], resulting in repeated application of topical treatment. Therefore, a sustained topical administration in case of chronic varicose veins would give, besides the overcome of side-effects associated with systemic or oral administration, a more efficient uptake of the drug at the target site.

We present here the synthesis and evaluation of new multifunctional bioactive textile materials (biomaterials) containing chemically or physically bound troxerutin. A knitted material from polyamide and polyurethane fibers was used as a support in order to obtain a bioactive material that can display both compression and sustained venotonic and haemostatic properties. These multifunctional biomaterials are intended for use as medicinal stockings providing simultaneous benefits of elastic compression and topical varicose edema treatment. Two procedures were used for Trox durable fixation on textile fabric: (i) cyclodextrin binding followed by its complexation with Trox, based on host-guest principle; (ii) direct fixation of Trox to textile material by appropriate drug chemical modification. Biomaterials obtained by these procedures were evaluated in vitro and in vivo according to the criteria for a material to be used as drug delivery system: high drug loading, constant and sustained release, evidence of the drug uptake by the target site. Confocal microscopy was used for the first time to assess the uptake and localization of Trox applied on skin either as ointments or as drug incorporated in a textile material.

2 Experimental section

2.1 Materials

β-Cyclodextrin (Cyd) and acryloyl chloride were from Aldrich and were used as received. Troxerutin (Trox) was a kind gift from Antibiotice S.A Iasi, Romania. All the other chemicals were of reagent grade and purified and dried by appropriate procedures. A knitted material obtained from 75% polyamide 6.6 fibers (44 dtex) and 25% polyurethane fibers (156 dtex) was used as textile support (PA) over the entire study. The material was cut from an elastic compression stocking which provides a constant pressure of about 17 mmHg.

2.2 Methods

Fourier Transformed Infrared (FTIR) spectra were recorded on a Vertex 7 spectrophotometer (Bruker, Austria). H^1-NMR spectra were obtained on a Varian EM390 type spectrometer, at 90 MHz. Scanning electron microscopy (SEM) was performed with a TESLA BS 3001 type instrument. Ultraviolet spectra were recorded with a UV-Vis spectrophotometer (Specord 200, Analytic Jena, Jena, Germany). Laser scanning confocal microscopy was performed with a system (Microradiance, Biorad/Zeiss) containing Nikon Eclipse TE300 inverted microscope with Ar or/and Kr/Ar laser and excitation wavelength fixed at 488 nm. The fluorescence emitted light was recorded by using specific filters located in the scanning end of setup and Nikon 10×, 40× and 60× objectives, with large aperture.

2.3 Synthesis of acryloyl derivatives

Acryloyl derivatives of β-cyclodextrin (A-Cyd) and troxerutin (A-Trox) were prepared by the reaction of corresponding compounds with acryloyl chloride. The synthesis of A-Cyd is given here as a typical example: Cyd (1.134 g, 1 mmol) was dissolved in 5 ml *N*,*N*-dimethyl formamide (DMF) under heating and stirring. The resulting clear solution was cooled to r.t., mixed with triethylamine (TEA; 1.46 ml, 10.5 mmol), then acryloyl chloride (0.85 ml, 10.5 mmol) in 6 ml DMF was added drop-wise to the mixture cooled at −5 to 0°C (ice bath). The reaction mixture was stirred at 0–5°C for 1 h and at 40°C for 2 h, then filtered for triethylammonium chloride removal, and the filtered solution precipitated in acetone. The precipitate was extensively washed with acetone and chloroform, and dried under vacuum at r.t. The resulting white powder (0.95 g, 80% yield) was analyzed by TLC (eluent: n-butanol/ethanol/water = 5/4/3 v/v/v, $R_f = 0.48$ comparing with $R_f = 0.33$ for β-cyclodextrin), FTIR (KBr) and H^1-NMR (D_2O).

Synthesis of A-Trox was carried out under conditions similar to those used for A-Cyd, but the reaction mixture was stirred at r.t. for 24 h. After filtration, the final product was recovered by precipitation in diethyl ether, followed by repeated trituration with diethyl ether for the complete DMF removal. The yield was 89%. TLC analysis (eluent: n-butanol/ethanol/water = 5/4/3 v/v/v) gave a $R_f = 0.73$ (0.6 for unmodified Trox). The content in acryloyl groups was determined by a method recommended for double bond quantitative analysis [12, 13], which implies the treatment with the mixture $KBr/KBrO_3/H_2SO_4$, followed by specific titration of Br_2 in excess.

2.4 Synthesis of polyamide grafted with acryloyl derivatives (PA-CyD and PA-Trox)

PA knitted fabric (0.5 g) was washed for 30 min with a nonionic detergent aqueous solution (2 g/l) at 45°C, and then dried under air stream at r.t. The dried PA fabric was immersed in an aqueous solution of ammonium persulfate (APS, 3%, w/v) for 20 min at r.t. The fabric was squeezed, thoroughly washed with water, and the excess water was removed with a filter paper. Application of acryloyl derivatives on PA fabrics was performed by two different procedures:

(a) Spraying. PA material tightly fixed on a rectangular surface (7 cm × 2.5 cm) was sprayed on one face of the fabric with a solution of acryloyl derivative (1.5 ml, 40% w/v) containing three drops of nonionic detergent as an emulsifier.
(b) Immersion. A fabric sample (0.5 g) was immersed into a flask containing 5 ml of a solution of monomer (24%, w/v) and a nonionic detergent.

In both cases, the fabric samples were maintained at 60°C for 2–5 h for grafting, then washed with warm and cold distilled water, and dried for 24 h under vacuum, at 40°C.

The amount of grafted product was determined from the relationship:

$$\%\,\text{Grafted} = 100(G - G_0)/G_0$$

where G and G_0 are the weight (g) of the fabric after and before grafting, respectively.

Grafted materials were characterized by FTIR (reflection absorption) and SEM.

2.5 Preparation of inclusion complexes of PA-CyD

Complexes between benzoic acid (BA) or Trox and grafted cyclodextrin were obtained as follows: a dried and weighed PA-Cyd sample was immersed in 7.5 ml water (BA) or methanol/water 6/1 v/v (Trox) solution containing 1% ligand, and kept under stirring at r.t. for 48 h. The concentration of the ligand in supernatant was determined by measuring the UV absorbance at 235 nm (BA) or 350 nm (Trox) and using calibration curves for absorbance versus ligand concentration. The amount of drug retained by biomaterial was calculated as the difference between the ligand concentration of the supernatant containing grafted and non-grafted PA fabric.

2.6 In vitro release of troxerutin from biomaterials

Release tests were performed in a medium simulating the pH and salt content of human sweat at the calf region (pH 5.35, 0.73 meq $NaHCO_3$/l, 2.41 meq KCl/l, 30.60 meq NaCl/l) [14], at 37°C. The amount of released Trox was quantified by UV, and the released drug was characterized by UV, NMR and FTIR.

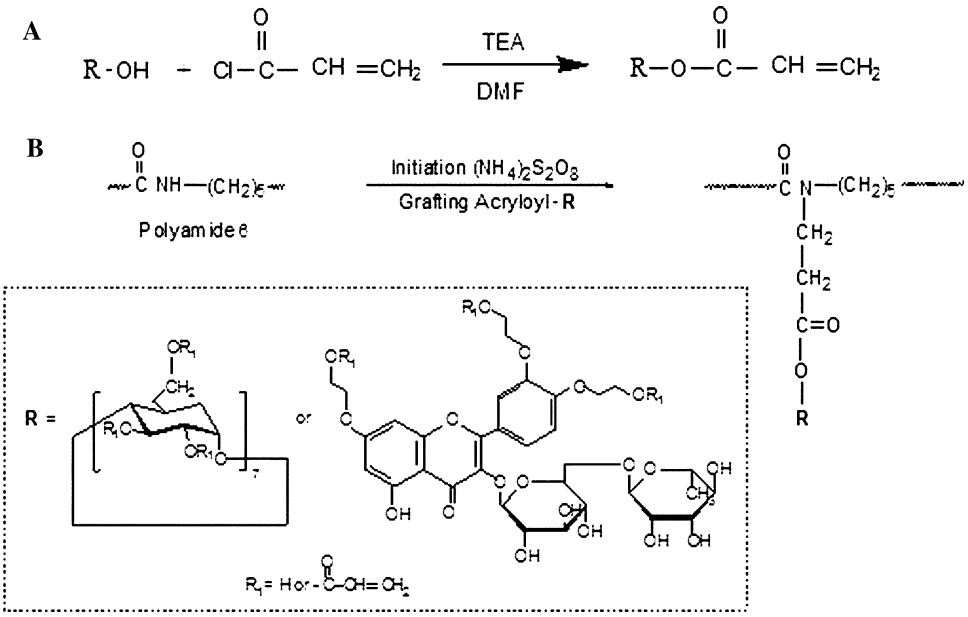
2.7 In vivo tests of biomaterials

Wistar adult male rats with 200–300 g weight were used for in vivo experiments. Before experiments, a 10 cm^2 surface of their back was epilated with a special cream and the rats were let to rest for 24 h. Several groups received different treatments: group I (control) received no treatment, group II was treated with free drug (100 mg/g pharmaceutical ointment) as a thin layer, and group III was treated with the biomaterial which was tighten on the skin (0.5 cm^2) with the help of an adhesive. At given time intervals after beginning of the experiments, 5 rats of each group were anesthetized with Thiopental, and sacrificed. After that, a skin fragment from the treated area was taken from each animal, kept in formaldehyde 37% solution for 24 h, then cut in a cryotom into sections of about 30 μm perpendicular to the skin thickness. Within 2–3 h after that, the fluorescence intensity of different areas of skin sections was quantified by laser scanning confocal microscopy. It was converted to electric signals by photomultipliers. Up to 1,024 × 1,024 pixels could be recorded, meaning that the distance between pixels corresponds to 1 μm in the specimen. The light intensity of each pixel was recorded as 8 bits, giving values in the range 0–255 light intensity units. Fluorescence intensity was followed at the epidermis, dermis and vascular wall subregions. Statistical analysis was carried out using Student-T test and one-way ANOVA test. Values were taken as significant when $P < 0.05$.

3 Results and discussion

3.1 Synthesis of acryloyl derivatives

The extent of acryloyl derivative grafting depends mainly on the number of double bonds per derivative molecule. Our goal was the synthesis of acryloyl derivatives (Scheme 1a) with 2–3 double bond groups per substrate molecules in order to realize a better fixation (by grafting and crosslinking) of the derivative on the textile material. Both CyD and Trox were reacted with a high excess of acryloyl chloride (Table 1). Due to the high number of substrate reactive OH groups, the resulting derivatives are mixtures of 1, 2 and 3 substituted Cyd or Trox, but the composition inhomogeneity was significantly reduced with increasing substitution degree (as seen by TLC analysis). The presence of more than 2 acryloyl groups/mol substrate determines a decrease in water solubility of the derivative,



Scheme 1 General reaction schemes for biomaterial synthesis. **a** Acryloyl derivative synthesis, **b** Grafting of acryloyl derivatives on polyamide chains

especially in the case of more hydrophobic Trox (Table 1). The average degree of Cyd substitution was determined by ^{1}H-NMR analysis as the ratio of the area under the peaks assigned to the three vinyl protons [6.83, 6.73 ppm (=CH_2) and 6.55 ppm (=C*H*)] and that of the peak assigned to the C^1-H of the cyclodextrin molecule (5.56 ppm) (Fig. 1). This procedure could not be applied to A-Trox due to the overlapping of several signals, therefore the degree of substitution was evaluated by a specific titration procedure for double bonds. The qualitative proof for derivatization of both substrates was obtained from FTIR spectra, where new bands at 1,726 cm^{-1} and 1,201 cm^{-1} (C=O and C–O of ester groups, respectively) and at 810 cm^{-1} (double bond) were observed, as shown in Fig. 2 for Cyd derivative.

3.2 Grafting of acryloyl derivatives to PA fabrics

Grafting takes place according to a mechanism reported for polyamide materials [15, 16] and depicted in Scheme 1b. Two procedures were used for application of acryloyl derivatives on the knitted fabric, as described in Experimental part. Influence of different grafting conditions (application procedure, double bond content of acrylic monomer, type and concentration of initiator, time, and temperature) was evaluated by the grafting yield and is summarized in Table 2. The best results were obtained by immersion, in the presence of APS as initiator, without any benefit of using $CuSO_4$. The reaction time was 2–6 h and optimal temperature was 60°C. As expected, grafting yield was higher for higher double bond content in monomer.

Table 1 Synthesis conditions and characteristics of acryloyl derivatives

Cod sample	Substrate (S)	Reaction conditions		Product characteristics	
		Mol ratio AcrCl/S	Temp. 0°C/time, h	Mol ratio C = C/S	Solubility
A-Cyd1	Cyclodextrin	6	0/1 40/2	1.5[a]	Water
A-Cyd2	Cyclodextrin	10	0/1 40/2	2.5[a]	Acetone, EtOH/water 1/1
A-Trox1	Troxerutin	6	0/1 20/24	1.5[b]	Water
A-Trox2	Troxerutin	10	0/1 20/24	2.5[b]	Chloroform, EtOH/water 2/1

[a] Determined from ^{1}H-NMR spectra

[b] Determined by double bond titration

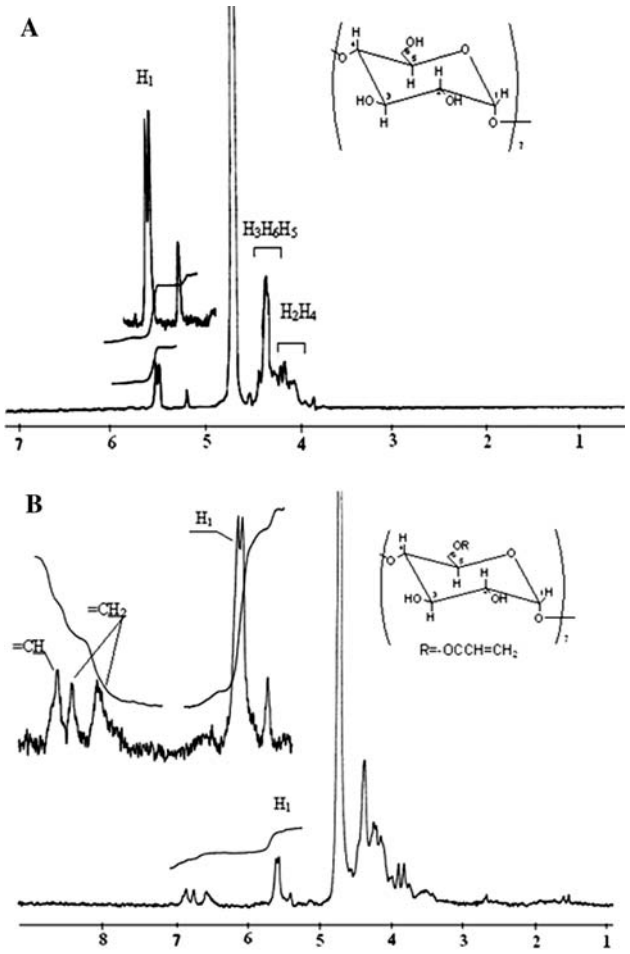


Fig. 1 ^{1}H-NMR spectra in D_2O of **a** β-cyclodextrin and **b** A-Cyd2

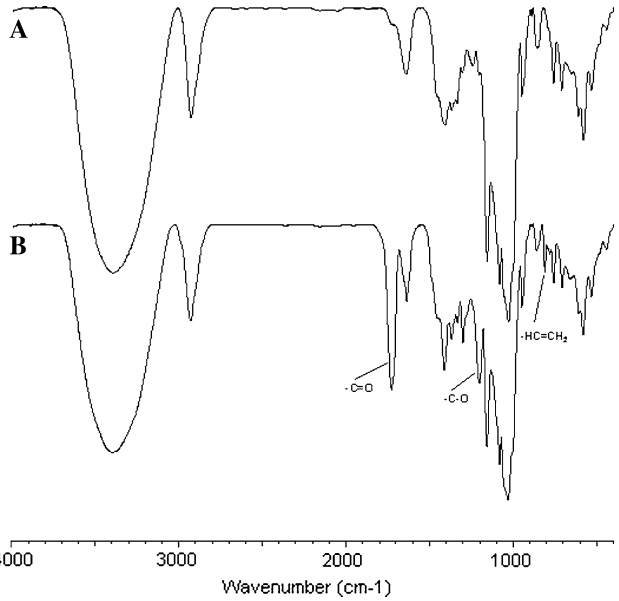


Fig. 2 FTIR spectra of **a** β-cyclodextrin and **b** A-Cyd2

FTIR spectra of grafted materials account for chemical modification of PA due to the presence of specific bands for grafted compound, for example the band at 1,726 cm^{-1} assigned to ester carbonyl group (Fig. 3).

Surface morphology of grafted materials was examined by SEM (Fig. 4). The deposition of grafted material is more uniform when the monomer was applied by immersion (Fig. 4c) than by spraying (Fig. 4b), and higher amounts of grafted material result in a multilayered coverage connecting several fibers together (Fig. 4d).

Polyurethane fibers have not been affected by the grafting process (no change in material contention was observed).

3.3 Complexation capacity of PA-Cyd

The availability of grafted Cyd for complex formation was evaluated by its capacity to bind BA, as it is well known that β-cyclodextrin and BA form a 1:1 inclusion complex ($Ka = 10^3\ M^{-1}$) [17]. The experiments revealed a decrease in availability of grafted CyD cavity in comparison with free Cyd, and the difference becomes higher with increasing grafting degree (2/3 of Cyd molecules were available in case of PA-Cyd3, but only 1/3 for PA-Cyd7) (material samples as given in Table 2).

Complexation of Trox by Cyd was already described in literature [18] and was proved by our own fluorescence experiments (data not shown) performed with mixtures Cyd-Trox which gave a complexation constant of 100 M^{-1}. The amount of Trox complexed by grafted CD was only 1/3 of that found for BA, meaning that only 20% of grafted Cyd molecules were able to form complex with Trox.

3.4 In vitro tests

In order to establish if the bound Trox can be released from the biomaterials, in vitro tests were performed in a medium simulating the human sweat. The physically bound Trox was released very fast (90% in 1 h), the chemically bound drug was released very slow (15% in 24 h). The UV, NMR and FTIR analysis of released drug indicated that its chemical structure was not altered, irrespective of the binding form.

Consequently, the biomaterials might be able to release the free drug in contact with skin either by diffusion from PA-Cyd-Trox, or by a chemical or enzymatic hydrolysis of the ester groups of PA-Trox.

3.5 In vivo tests

Trox autofluorescence (which is a property of the polyphenolic core) has been already used as a marker for localization of the drug in the venous wall, after oral or i.v.

Table 2 Conditions for grafting of acryloyl monomers to polyamide material

Sample	Grafting procedure*	Monomer solution			APS conc. (%, w/v)	$CuSO_4$ conc. (%, w/v)	Reaction time (h)	Grafting yield (%, w/w)
		Monomer	Solvent	Conc. (% w/v)				
PA-Cyd1	(a)	A-Cyd2	Water	40	3	0.3	6	1.1
PA-Cyd2	(a)	A-Cyd2	Water	40	5	–	2.5	3.4
PA-Cyd3	(a)	A-Cyd2	Water	40	5	–	5	4.2
PA-Cyd4	(a)	A-Cyd2	Water	24	5	–	5	7.1
PA-Cyd5	(a)	A-Cyd1	Water	40	5	–	5	
PA-Cyd6	(b)	A-Cyd2	Water	18	3	0.15	6	2.3
PA-Cyd7	(b)	A-Cyd2	Water	20	5	–	5	30.5
PA-Trox1	(b)	A-Trox1	Water	20	3	–	6	9.0
PA-Trox2	(b)	A-Trox2	Water/EtOH 2/1 v/v	20	3	–	2	25.0

* Grafting procedure (a) and (b) as given in experimental part

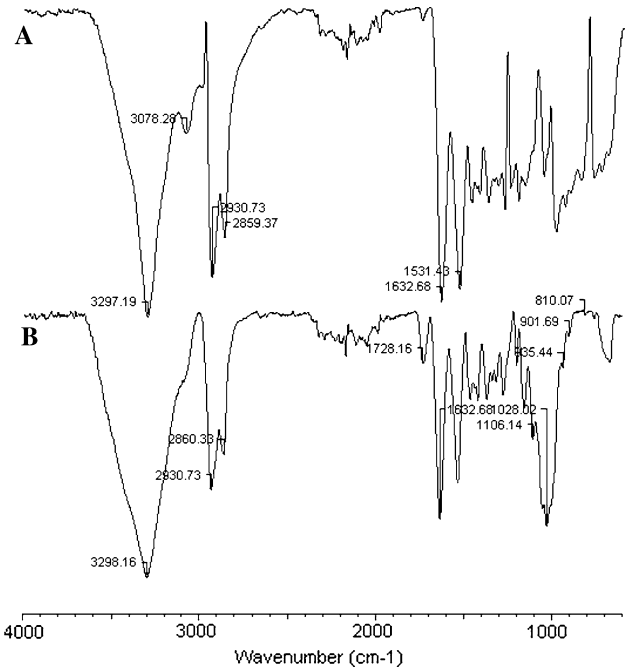


Fig. 3 FTIR spectra of **a** PA fabric and **b** PA- Cyd7

administration, by means of confocal microscopy [19, 20]. However, this is only a qualitative measurement, and it was not possible to quantify the drug in absolute values.

We used confocal microscopy to qualitatively evaluate the uptake and localization of Trox at different skin level, either from ointments or drug containing textile materials (biomaterials). To the best of our knowledge, this is the first experimental evidence of the troxerutin fate after topical application to animals.

Two biomaterial samples were used for these studies: PA-Trox2 containing Trox chemically bound to polyamide fabric, and PA-Cyd3-Trox having Trox physically entrapped in Cyd cavity.

Confocal microscopy experiments showed that all skin areas under study (epidermis, dermis and vascular wall) display a low intensity basal fluorescence of about 36–46 a.u., more obvious in vascular area. Administration of Trox determines an increase of the fluorescence of all areas, but this increase depends on Trox formulation. Administration of free Trox (as ointment) determines a fluorescence increase in all areas with a maximum at about 12 h, then the fluorescence decrease quickly to the control level.

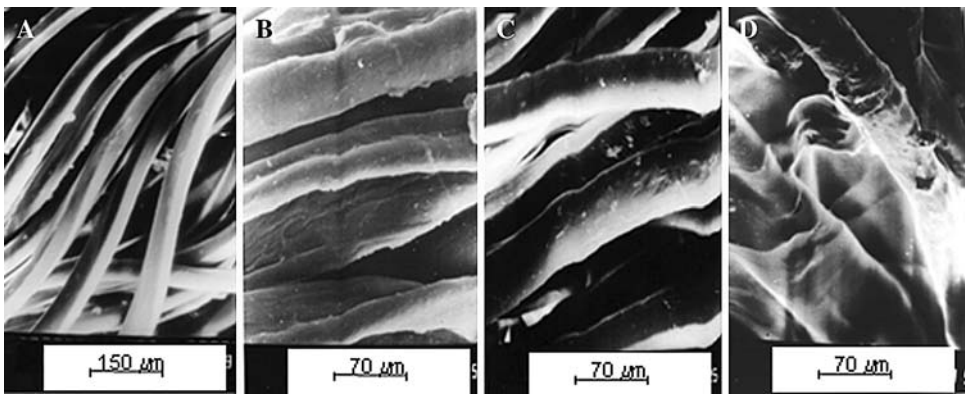


Fig. 4 Scanning electron micrographs of **a** PA fabric, **b** PA-Cyd3, **c** PA-Cyd6, **d** PA-Trox2

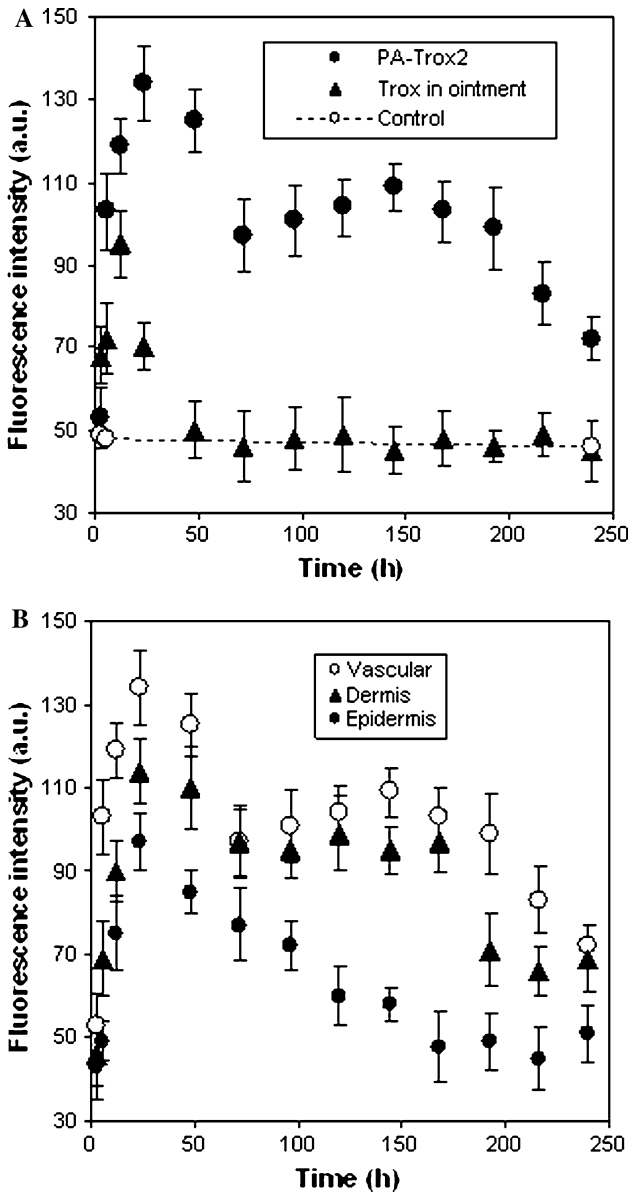


Fig. 5 Variation of fluorescence intensity with time at vascular wall level for the rat groups treated with different Troxerutin formulation (**a**) and at several skin areas for the group treated with PA-Trox2 (**b**). The values are average for $n = 5$ and the bars represent the standard deviations

However, when Trox was chemically bound to biomaterial, the increase in fluorescence in all areas is much higher with a maximum at 24–48 h after administration, then high values of fluorescence are maintained for 7–10 days (Fig. 5). Administration of Trox physically bound to biomaterial has a behavior similar to free Trox. These results are in agreement with in vitro tests. The presence of increased fluorescence in the skin areas proves the transfer of intact troxerutin from the biomaterial to the skin.

The content in residual Trox on biomaterials was determined after the longest experiment (10 days) was concluded, as the difference in weight of biomaterial before and after experiment. PA-Trox2 still retained 30% form initially bound Trox, PA-Cyd3 had no residual Trox.

4 Conclusion

New bioactive textile materials provided with both compression and localized haemostatic properties have been prepared by physical or chemical binding of troxerutin to a knitted material based on polyamide 66 fibers. Grafted acryloyl cyclodextrin was used for physical binding of the drug as a complex with Cyd, and grafting of acryloyl troxerutin on polyamide material provided a biomaterial having troxerutin chemically bound to textile support. In vitro release experiments and in vivo tests on animals (rats) indicated that the biomaterial obtained from acryloyl troxerutin can release the intact drug at the contact with the skin. By means of confocal microscopy it was shown that the drug released by the biomaterial can be taken up by skin and accumulates in different skin areas (epidermis, dermis and vascular wall). The significant accumulation of troxerutin at its target site (vascular wall) for a longer period of time than the drug administrated as an ointment, recommend this kind of biomaterial as a controlled and sustained drug delivery system able to provide an improved treatment for leg ulcers. Clinical tests on patients wearing elastic compression stockings obtained from this biomaterial are in progress.

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