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European Journal of Pharmaceutics and Biopharmaceutics 54 (2002) 125–133

European Journal of Pharmaceutics and Biopharmaceutics

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Research paper

Microparticles derived from marine sponge collagen (SCMPs): preparation, characterization and suitability for dermal delivery of all-*trans* retinol

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Received 19 October 2001; accepted in revised form 17 April 2002

Abstract

Collagen microparticles were prepared using marine sponge collagen. For this purpose a previous method by Rössler et al. (J. Microencapsul. 12 (1995) 49) of emulsification and cross-linking of native calf collagen was modified. The modified method for sponge collagen microparticles (SCMPs) achieved a yield of 10%. Scanning electromicroscopic photographs showed spherical particles with a diameter of 120–300 nm and photon correlation spectroscopic measurements indicated particle size range from 126 (±2.9) to 2179 (±342) nm. This broad size distribution was caused by some agglomerates that could not be destroyed by ultrasonication. The surface charge was measured as a function of pH. At pH 2.8 the particles were nearly uncharged, at pH 9.0 the particles showed a strong negative charge of about −60 mV. The preformed SCMPs were loaded by adsorption of all-*trans* retinol. A loading of up to 8% was obtained. Retinol-loaded SCMPs were incorporated into hydrogels and drug stability was investigated. The in vitro penetration of retinol into hairless mice skin in this formulation was compared to retinol formulations without microparticles. The SCMPs had no influence on the chemical stability of retinol in the hydrogel. The dermal penetration of retinol into the skin increased significantly by approximately two-fold.

Keywords: Sponge *Chondrosia reniformis*; Marine sponge collagen; Sponge collagen microparticles; Dermal drug delivery; All-*trans* retinol

1. Introduction

Particulate drug-carrier systems were intensively studied over the past years with the objective to achieve drug-targeting, sustained release or to improve oral or ocular bioavailability [2–5]. Furthermore, these systems have also been employed for dermal drug delivery [6–8]. Some years ago Rössler et al. demonstrated that the delivery of retinol to the skin could be enhanced by binding to calf collagen microspheres [7]. The employment of this material has to be reconsidered because of the risk of bovine spongiform encephalopathy (BSE). Therefore, the objective of the present paper was the investigation of the suitability of sponge collagen for the production of microspheres for dermal drug delivery.

Collagen, as a biomaterial, has the advantage of biodegradability, low toxicity and immunogenicity [9,10]. Microparticles based on calf or bovine collagen were previously prepared by emulsifying and cross-linking [1], spray-drying [11] or by coacervation techniques [11]. However, biomaterials from bovine origin bear the risk of BSE. This disease, which became abundant in some countries during the mid-1980s [12], is increasingly discussed to be transmittable to humans [13,14].

Therefore, collagen from marine sources offer a very attractive alternative, for instance jelly fish [15,16], fish skin [17], or sponges [18]. Accordingly, collagen from the marine sponge *Chondrosia reniformis* Nardo recently was investigated yielding about 30% collagen [19]. This sponge can be found ubiquitously in the Mediterranean Sea. Furthermore, it is the only eatable sponge [20] as confirmed by native inhabitants. This fact indicates its non-toxicity.

Fine structure and physicochemical characteristics of *Chondrosia*-collagen have already been investigated [21]. Isolated fibrils are 19.6 nm in diameter and display a distinct 66 nm periodicity and two intraperiod bands. Biochemical analysis, wide angle X-ray diffraction and infrared spectrum

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0939-6411/02/$ - see front matter
PII: S0939-6411(02)00046-2

confirmed classical collagen features: triple helical configuration of the molecules, presence of hydroxyproline and hydroxylysine, and an amino acid composition in the same range of known data for collagen. Furthermore, the transition temperature values of isolated *Chondrosia* fibrils are comparable to calf skin collagen [21]. Some special characteristics, however, are found, such as the presence of arabinose [22] and the very high insolubility of this collagen [23].

The objective of the present study was the preparation and characterization of sponge collagen microparticles (SCMPs) and the investigation of their effects on drug-stability and on dermal drug delivery.

2. Materials and methods

2.1. Materials

The sponge *Chondrosia reniformis* Nardo (Demospongiae: Hadromerida: Chondrosiidae) was collected from the Aegean Sea and stored in 50% (v/v) ethanol until used.

A 25% (v/v) aqueous glutardialdehyde solution, hydrogen peroxide 30% (v/v) in water and the emulsifier Span® 20 were obtained from Merck AG (Darmstadt, Germany). Liquid paraffin was purchased from BUFA B.V. Pharmaceutical Products (Uitgeest, Netherlands).

In addition, the following materials were used: all-*trans* retinol and α-tocopherol (Sigma, Deisenhofen, Germany), Ethanol-Uvasol®, sodium chloride, sodium hydroxide, hydrochloric acid and ascorbic acid (Merck, Darmstadt, Germany), sodium citrate H_2O (Roth, Karlsruhe, Germany), Tylose® H300P (Hoechst AG, Hoechst, Germany), ethanol 96% (v/v) (Bundesmonopolverwaltung für Branntwein, Frankfurt, Germany), glycerol (Vaseline Fabrik Wasserfuhr, Bonn, Germany), [15-^{3}H(N)] vitamin A (free alcohol) (Biotrend, Köln, Germany), tissue solubilizer BTS-450, scintillation cocktails Ready Organic® and Ready Value® (Beckmann, Munich, Germany). Other reagents used in this study were of analytical or high-performance liquid chromatography (HPLC) grade.

2.2. Preparation of collagen microparticles

Collagen was isolated from the marine sponge *Chondrosia reniforms* Nardo by a previously described method [19]. SCMPs were prepared by emulsifying and cross-linking of collagen using a modified method introduced by Rössler et al. [1]. A 0.75% (w/v) aqueous sponge collagen dispersion (pH 9.5; potassium carbonate) was prepared using a rotor/stator type homogenizer (Ultraturrax T50, IKA-Werk, Staufen, Germany). Eighty five milliliters of this dispersion were added to a mixture of 10 g Span® 20 and 250 g liquid paraffin. The emulsion was homogenized with a Ultraturrax at 6000 rpm for 10 min. Then, the emulsion was stirred with a blade stirrer (Cito-UNGUATOR®-B,GAKO Konietzko GmbH, Bamberg) at about 500 rpm, and 12 ml of the glutardialdehyde solution was added. The cross-linking of the collagen chains was terminated after 12 min by addition of 16 ml of the H_2O_2 solution. After 15 min stirring with a magnetic stirrer, 100 ml 2-propanol was added.

Purification of the SCMPs was performed by centrifugation (10,000 × *g*, 30 min, 2° C, Sorvall® RC-5B, Du Pont Instruments, Bad Nauheim, Germany). The pellet was resuspended in 100 ml of 2-propanol 50% (v/v) and centrifuged again. This procedure was repeated and 200 ml of a 4% (w/v) ascorbic acid solution was added. The solution was heated in a 75°C water bath for 30 min and stirred for 24 h at room temperature. Final purification was performed by centrifugation (10,000 × *g*, 30 min, 2°C) and washing the pellet twice with water. The SCMPs were lyophilized for preservation.

2.3. Characterization of SCMPs

2.3.1. Scanning electromicroscopy

SCMPs were examined by scanning electron microscopy (SEM) (SE 4500 Hitachi S) for their size, shape and surface characteristics. The samples were platin sputtered (MCS 010, BAL-TEC, Balzers, Liechtenstein), having a platin film thickness of about 4 nm.

2.3.2. Photon correlation spectroscopy

Particle size determination was performed by photon correlation spectroscopy (PCS) using a BI-200 SM Goniometer Version 2 (Brookhaven Instruments Corp., Holtsville, N.Y. USA). Freeze-dried SCMPs were resuspended in double-distilled and filtered water (pH 9.8 adjusted with K_2CO_3) and ultrasonicated for 5 min (Transsonic Digital, Elma®).

2.3.3. Surface charge

The surface charge of SCMPs was determined by measuring the electrophoretic mobility. A Lazer Zee Meter™ Model 501 (Penkem, Bedford Hills, NY) was used. To investigate the pH dependency of the zeta potential, SCMPS were suspended in sodium chloride solutions (0.09% w/v) in the pH range from 2.0 to 10.0. The pH was adjusted using appropriate amounts of hydrochloric acid and sodium hydroxide solution, respectively. The measured values were corrected to a standard reference temperature of 20°C.

2.3.4. Determination of the adsorption isotherm of all-trans retinol microparticles

All-*trans* retinol was adsorbed onto the surface of previously formed microparticles as follows. Seven hundred microlitres of aqueous SCMP suspensions were incubated with 300 μl of ethanolic retinol solutions in micro test tubes (Roth, Karlsruhe). The final retinol concentrations ranged from 0.5 to 1.5 mg/ml, the final concentration of SCMPs always was 2.5 mg/ml. The mixtures were allowed to equilibrate for 10 min at 20°C by shaking with a thermomixer (Model 5437, Eppendorf, Hamburg, Germany). After drug-

adsorption, the suspensions were centrifuged (10,600 × *g*, 30 min). The loaded SCMPs were washed twice with MQ-water to separate from unbound retinol (10,600 × *g*, 30 min). Surface-bound retinol was extracted three times from the SCMPs with 1 ml Ethanol-Uvasol® each. The extracts were collected and diluted with Ethanol-Uvasol® to 5.0 ml. The absorption at $\lambda = 324.8$ nm was measured by UV-spectrophotometry (U-3000-spectrophotometer, Hitachi, Berkshire, UK) and the absolute loading was calculated and plotted in form of a loading isotherm. Each experiment was run three times. Because of the low stability of retinol, it was necessary to protect it from light during all steps of the investigation.

2.4. Stability of SCMP-bound retinol in hydrogel-formulations

Retinol-loaded SCMPs were incorporated into hydrogel-formulations according to Rössler et al. [7].

Freeze-dried SCMPs (500 mg) were resuspended in 140 ml MQ-water by ultrasonication (Transsonic digital, Elma, Singen, Germany). The SCMP suspension was added to 25 ml of an ethanolic retinol solution (10 mg/ml). After 10 min stirring, the suspension was centrifuged (10,600 × *g*, 30 min, Optima L-80, Beckmann, Munich, Germany). The supernatant was discarded, while the SCMPs were separated from unbound retinol by two times washing with MQ-water and centrifugation (10,600 × *g*, 30 min).

The loaded SCMPs were resuspended in 30 ml of an aqueous buffer (0.125 g ascorbic acid and 0.275 g sodium citrate). The suspension was added to a mixture of 2 g Tylose® H300P, 6 g glycerol, and 7 g of an ethanolic solution of 20 mg α-tocopherol. MQ-water was added to reach a final weight of 30 g and then stirred slowly until the hydrogel was totally swollen. The hydrogels were stored in a vacuum drying cabinet (Heraeus, Hanau, Germany) to remove incorporated air and afterwards filled into reaction tubes. After short centrifugation, the reaction tubes were completely filled with hydrogel and closed. For comparison, a second hydrogel containing freshly precipitated retinol instead of retinol-loaded SCMPs was prepared analogously.

The reaction tubes were stored at temperatures of 4, 21, 31and 41°C. One tube of each gel was sampled in intervals of about 2–4 weeks and their content was analyzed. Samples of freshly prepared hydrogels also were investigated and used as references. The retinol content was determined after repeated extractions with Ethanol-Uvasol® by UV spectroscopy ($\lambda = 324.8$ nm, U-3000 spectrophotometer, Hitachi, Berkshire, UK). Again, retinol was protected from light during all steps of the investigation.

2.5. In vitro penetration study through hairless mouse skin

2.5.1. Radioactive retinol solution

The tritium-labelled retinol (37 MBq) was dissolved in 50 ml EtOH 96% (v/v) and diluted with 150 mg cold all-*trans* retinol and 50 mg α-tocopherol to protect it from oxidization. The resulting solution had a specific activity of 0.74 MBq/ml.

2.5.2. Hydrogel preparation containing precipitated retinol

Six milliliters of the above described radiolabelled retinol solution and 21 ml of a buffer solution (0.36 g ascorbic acid and 0.79 g sodium citrate in 100 ml H_2O) were mixed with 1.2 g Tylose H300P and 3.6 g glycerol. The mixture was kept at 4°C until the hydrogel structure was obtained and stirred slowly three times for 1 min. The content of the 30.56 g hydrogel was 0.06% (w/v) retinol and the radioactivity was 0.1453 MBq/g.

2.5.3. Hydrogel preparation containing retinol-loaded SCMPs

Freeze-dried (315 mg) SCMPs were resuspended in MQ-water by ultrasonication and added to 25 ml of the above described retinol solution (0.74 MBq/ml). The suspension was stirred for 10 min. Beside adsorption of retinol onto the SCMPs a partial precipitation of retinol took place. The loaded SCMPs were seperated from the residual amount of retinol by centrifugation (10,600 × *g*, 30 min, Ultracentrifuge Optima L-80, Beckmann, Munich, Germany) whereupon the precipitated part of the retinol floated and was discarded. The SCMPs were purified by centrifugation and washing twice with H_2O.

Afterwards the SCMPs were resuspended in 21 ml of the above described buffer solution by ultrasonication and mixed with 6 ml ethanol 96% (v/v), 1.2 g Tylose H300P and 3.6 g glycerol as described for the preparation of the hydrogel containing precipitated retinol. The final radioactivity was 0.1377 MBq/g.

2.5.4. Hairless mouse skin

The 18–22 weeks old female hairless mice (HsdOla:MF1-hr, Harlan-Winkelmann, Borchen) were sacrificed with CO_2. Both, abdominal and dorsal skin were carefully excised and cleaned from subcutaneous tissue. The full-thickness skin was then used for the study.

2.5.5. In vitro penetration

The freshly excised hairless mouse skin was mounted in Franz diffusion cells (Burdich Labortechnik, Limburg, Germany). The acceptor chamber was filled with 21 ml of isotonic sodium chloride solution (Fresenius, Bad Homburg, Germany), kept at 37°C. Hydrogel (1 g) was applied on the penetration area of 3.14 cm^2 of the diffusion cells, so that a film of about 3.18 mm thickness was obtained. The donor chamber was covered with a perforated piece of Parafilm® to prevent the preparations from total dehydration.

For every preparation, 18 penetration experiments were carried out, with different penetration times (2, 4, 8, 16, 24, and 30 h); each run for each time point was performed in triplicate. The penetration was terminated after the above

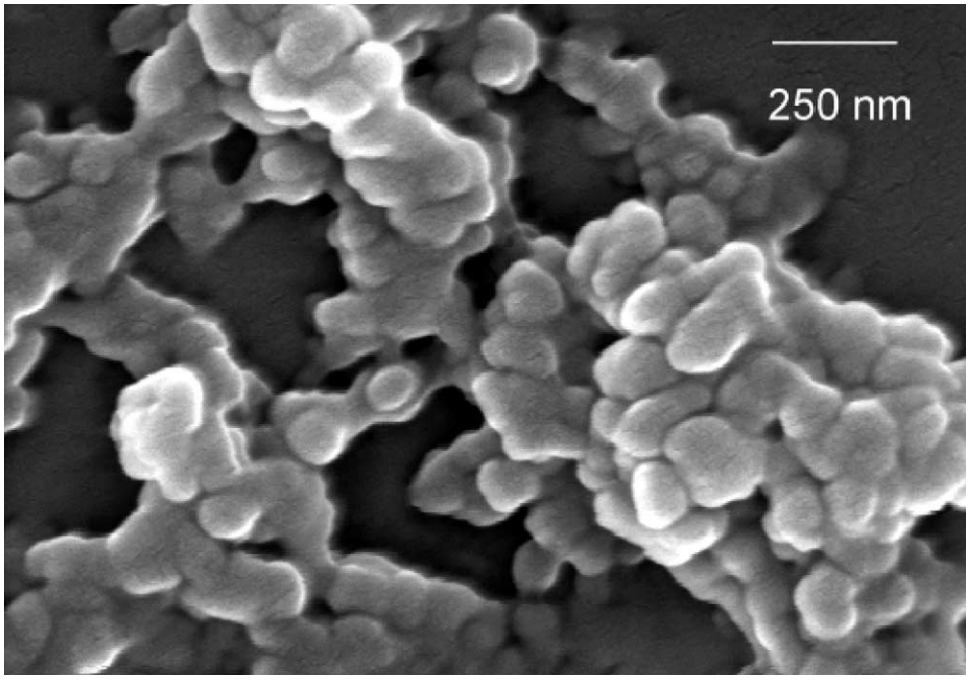

Fig. 1. SEM photograph of SCMPs (courtesy of Mr Schewe and Mr Ruppel).

mentioned time periods, and 100 μl of the acceptor solution was sampled and diluted with 15 ml scintillation cocktail Ready Value® for measurement. The skin then was removed from the diffusion cell and carefully cleaned with H_2O. Afterwards, the stratum corneum was excised by stripping with Tesa® kristallklar (Beiersdorf, Hamburg, Germany) eight times. The strips were collected in two vials, and to each vial 10 ml scintillation cocktail Ready Organic® was added. The residual skin was dissolved in 3 ml tissue solubilizer BTS 450® at 50°C. The solution was decolorized with 100 μl hydrogen peroxide 30% (v/v) and diluted with 10 ml Ready Organic® after addition of 70 μl acetic acid.

The radioactivity was measured in a scintillation counter LS 6500 (Beckman, Munich, Germany).

2.5. Statistics

All data are presented as arithmetic mean values ± standard deviation ($x \pm$ SD). Significance of differences was analyzed using *F*-, and student's *t*-tests and $P \leq 0.05$ was considered as significant.

3. Results and discussion

3.1. Preparation and characterization of SCMPs

The method of emulsifying and cross-linking of native calf collagen for the preparation of CMPs was described before [1,24,25]. In the present study, this method was modified and applied to collagen, which was isolated from the marine sponge *Chondrosia reniformis* Nardo according to Swatschek et al. [19].

A 10% yield (freeze-dried SCMPs in relation to totally used freeze-dried sponge collagen) was achieved for the resulting SCMPs. SEM-photographs show spherical particles with a smooth surface and a diameter of 120–300 nm (Fig. 1). These particles were found to be agglomerated (in the dry state) to secondary particles of a size of up to 2.2 μm. PCS studies were performed to investigate the influence of resuspension, ultrasonication, and swelling on the particle size of three SCMP batches. Resuspension of SCMPs in water was improved by adjusting the pH of the samples to 9.8 (K_2CO_3) and ultrasonication. The results are given in Table 1. The lower particle size limit of 126 (±2.9) nm correlated well with the minimum size of the

Table 1
Particle size of three SCMP-batches (SCMP 1–3) by photon correlation spectroscopy (PCS)

	Effective diameter (nm)	Polydispersity	Size range (nm)
SCMP 1	582	0.3358	123–2486
SCMP 2	611	0.3514	128–2239
SCMP 3	537	0.3599	128–1811
Mean (±SD)	577 (±37.3)	0.3490 (±0.01)	126 (±2.9)–2179 (± 342)

primary particles by SEM. The broad size distribution from 126 (±2.9) to 2179 (±342) nm probably was caused by agglomerates that were not destroyed by ultrasonication. Secondary particles could partly be desagglomerated. Consequently, the present procedure using sponge collagen resulted in particles that were relatively small size compared to CMPs prepared by different other procedures and with different collagen. For example, CMPs from native calf collagen which were also prepared by a similar emulsifying and cross-linking method were found to range from 3 to 40 μm [24]. Smaller CMPs in the size-range of 250 nm to 3 μm only could be prepared after thermal denaturation of collagen (18 h, 43°C) [1]. CMPs prepared by spray-drying ranged from 3 to 9 μm [11]. The zeta potential is of importance because of dispersion stability [26], drug loading efficiancy [25], and electrostatic interactions between particles and biological surfaces in vivo [25,27]. In this study, the surface charge was determined in relation to the pH (Fig. 2). At pH 2.0 the zeta potential had a positive charge of about 15 mV. Between pH 2.5 and 3.0, the surface charge was zero (P_0), which goes along with a low physical stability of the system. Compared to the isoelectric point of sponge collagen between pH 6.5 and 8.5 [19], the P_0 was shifted to a lower pH. This effect was already described for CMPs from calf collagen and was explained with the reaction of the cross-linking agent, glutaraldehyde, with the amino groups of collagen, which reduces the number of protonizable amino groups. Furthermore, an incomplete reaction of the bifunctional reagent could lead to free aldehyde groups which are oxidized to the corresponding acid in the course of CMP preparation [25]. By increasing the pH up to 9.0, the surface charge decreases to about −60 mV. Instead of reaching a plateau after a complete dissociation of all functional groups as described in literature [28], the zeta potential then increases to about −47 mV at pH 10. This effect possibly results from hydrolysis reactions at higher pH. The fact that the system shows a strongly negative surface

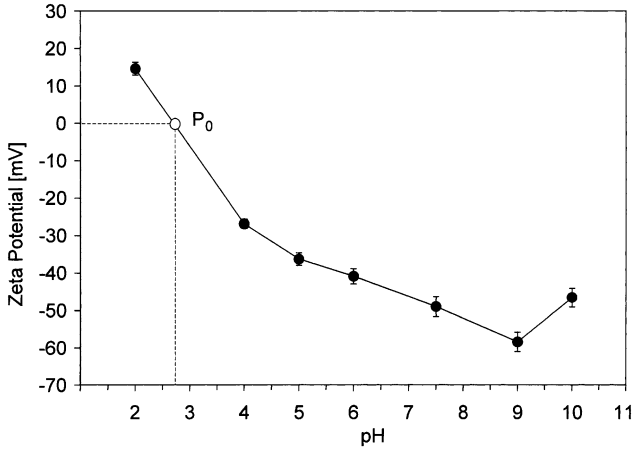

Fig. 2. Zeta potential of SCMPs as a function of pH (mean ± SD; $n = 3$); P_0 = point of zero charge of SCMPs.

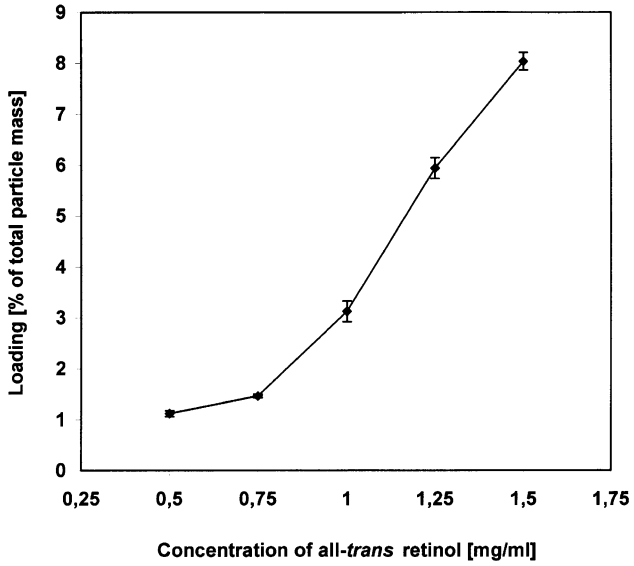

Fig. 3. Loading isotherme of all-*trans* retinol (mean ± SD; $n = 3$).

charge at higher pH values explains the improved resuspendibililty of SCMPs as described above.

Loading of CMPs can be achieved by adsorption of drugs onto the particle surface [1,25]. In this study, all-*trans* retinol was used and the loading isotherm was determined. As previously described, a partial precipitation of all-*trans* retinol took place during adsorption [1]. The precipitated and unbound fraction of the drug flotated after centrifugation and consequently could be removed. Above a retinol concentration of 0.75 mg/ml, an almost linear increase of drug adsorption was observed (Fig. 3). Drug loading of 1–8% corresponding to particle mass (w/w) was obtained which is in good agreement with the adsorption experiments of retinol onto CMPs with native calf collagen [1].

3.2. Stability of SCMP-bound retinol in hydrogel formulations

The effect of SCMPs on the chemical stability of all-*trans* retinol was investigated in comparison to hydrogel formulations with freshly precipitated all-*trans* retinol. Figs. 4–7 show that retinol was not essentially protected from oxidization by adsorption onto SCMPs. In this respect, SCMPs and CMPs from calf collagen show the same characteristics [7].

3.3. In vitro-penetration study through hairless mouse skin

Three layers of the skin act as a series of diffusional resistances: the stratum corneum, the living cellular epidermis, and a region of the dermis which lies above the locally active vasculature [29]. Lipophilic substances are very sparsely released from lipophilic ointments. As demonstrated already by Rössler et al. [7], the release of retinol can be considerably improved by incorporation into hydrogels. The release can be further improved by binding to collagen

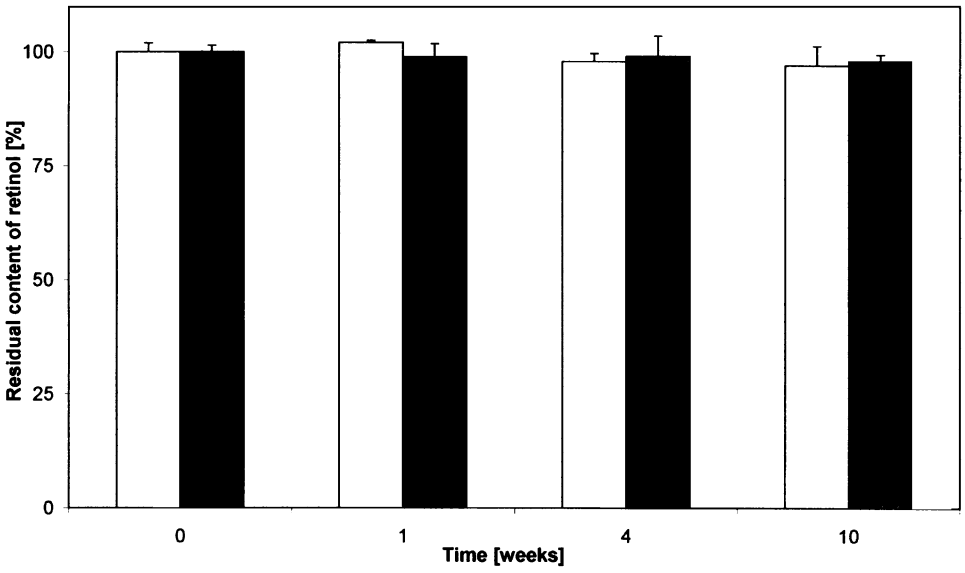

Fig. 4. Retinol content as a function of time at a storage temperature of 4°C (mean ± SD; $n = 3$). Hydrogel containing freshly precipitated retinol (□). Hydrogel containing retinol adsorbed onto SCMPs (■).

microspheres prior to incorporation into the hydrogel. Additionally, this enhances the physical stability of retinol [1,7].

Fig. 8 shows the amount of retinol in the upper skin-layers, especially the stratum corneum. This upper layer was removed from the residual skin after the experiments by Tesa®-strippings according to Refs. [30,31,7]. Formulation A (hydrogel with SCMP-bound drug) transported about two times more retinol into the stratum corneum. The values for retinol after 4 and 30 h were significantly higher ($P < 0.01$ and $P < 0.05$) than those found with formulation B (hydrogel with retinol freshly precipitated).

The amount of retinol in the residual skin after Tesa®-strippings as a function of penetration time is represented in Fig. 9. Again, formulation A was superior to formulation B. After 30 h, the amount of retinol in the residual skin was 1.7 times higher with formulation A.

Fig. 10 shows the amount of retinol detected in the acceptor mediums after penetration of the full skin. In contrast to the above mentioned findings, no significant differences were found between the investigated formulations. Furthermore, the amount of retinol found in the acceptor chambers was much lower, reaching 15 μg in 20 ml. Although the exact solubility is not known (practically insoluble, i.e. below one part in 10^4 parts [32]), the observed concentration

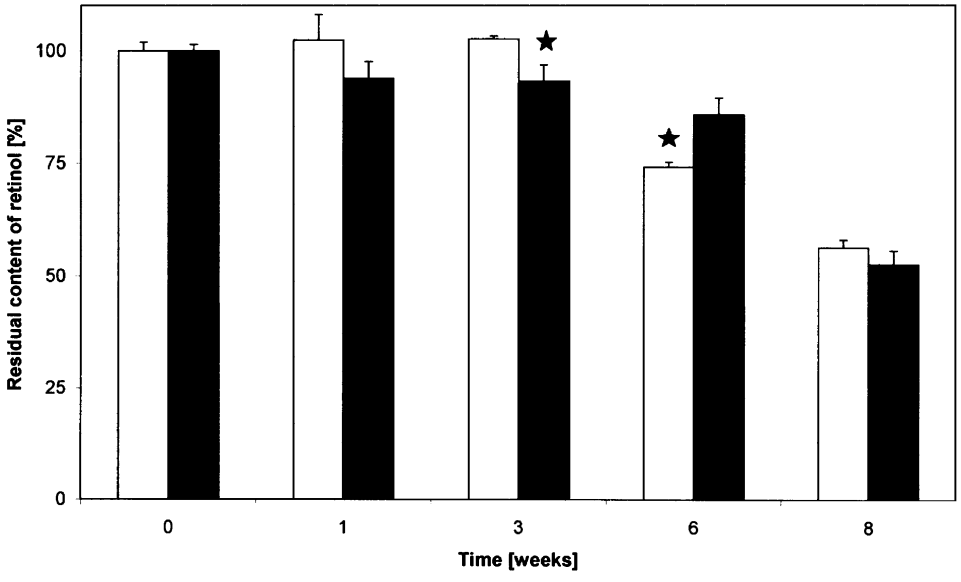

Fig. 5. Retinol content as a function of time at a storage temperature of 21°C (mean ± SD; $n = 3$). Hydrogel containing freshly precipitated retinol (□). Hydrogel containing retinol adsorbed onto SCMPs (■) Significant differences ($P < 0.05$) are marked with asterisks (★).

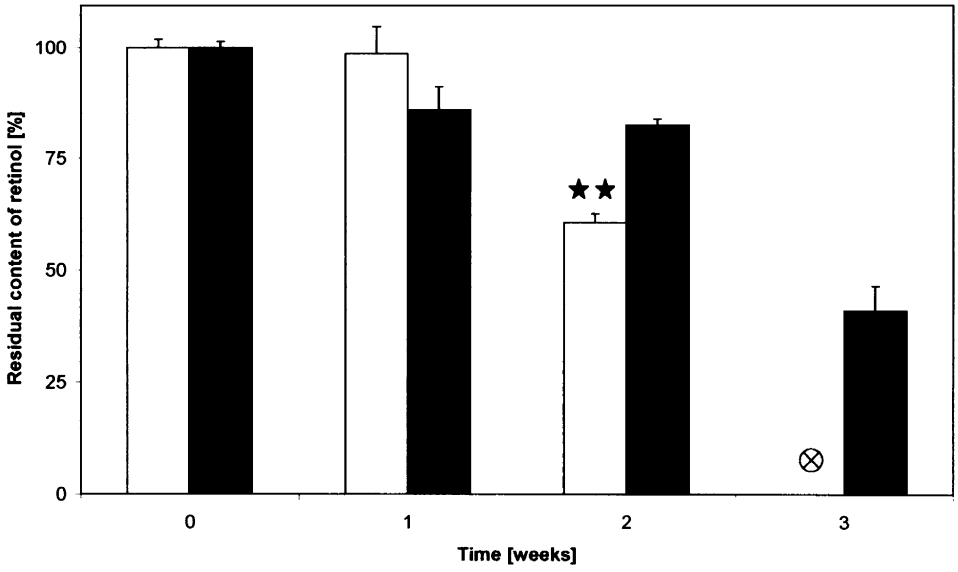

Fig. 6. Retinol content as a function of time at a storage temperature of 31°C (mean ± SD; $n = 3$). Hydrogel containing freshly precipitated retinol (□). Hydrogel containing retinol adsorbed onto SCMPs (■). Significant differences ($P < 0.01$) are marked with two asterisks (★★). Retinol content undetectable (⊗).

is close to one part in 10^6 parts. The linearity of the appearance slope of retinol in the acceptor chamber indicates that the observed concentration does not reach equilibrium.

Both formulations, A and B, were suitable for the local therapy of the skin: retinol, as a lipophilic drug, accumulates in the upper skin, which forms a hydrophilic–lipophilic multilayered structure [33]. Formulation A exhibits two essential advantages over formulation B. Firstly, the amount of retinol that penetrated into the stratum corneum and the residual skin was increased by the SCMPs. Secondly, the drug transport into the stratum corneum was accelerated, especially during the first 4 h. This latter effect is of practical importance, as part of the formulation may rapidly be removed from the skin surface either mechanically or by washing during the first hours after a topical application.

Therefore, this investigation demonstrates, in analogy to earlier studies with particulate delivery systems, e.g. microparticles from calf collagen [7] or solid lipid nanoparticles (SLN) [8], that dermal drug delivery can also be enhanced by collagen microspheres. Sponge collagen microspheres were slightly superior to calf collagen microspheres [7] in

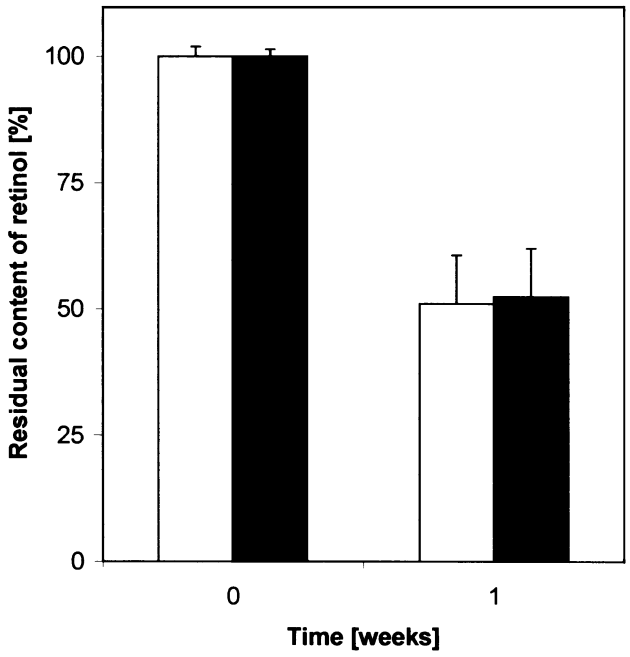

Fig. 7. Retinol content as a function of time at a storage temperature of 41°C (mean ± SD; $n = 3$). Hydrogel containing freshly precipitated retinol (□). Hydrogel containing retinol adsorbed onto SCMPs (■).

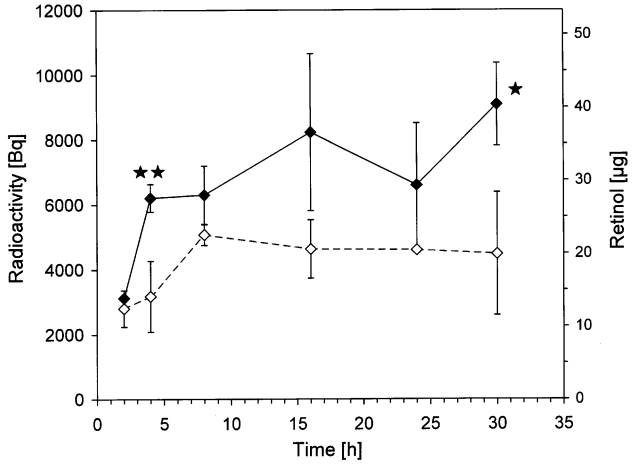

Fig. 8. Penetration of retinol into hairless mouse skin in vitro (mean ± SD; $n = 3$). Total amounts of retinol found in the stripped stratum corneum as a function of time are shown. Hydrogel A containing retinol adsorbed onto SCMPs (◆). Hydrogel B containing freshly precipitated retinol (◇). $P < 0.05$ (★); $P < 0,01$ (★★).

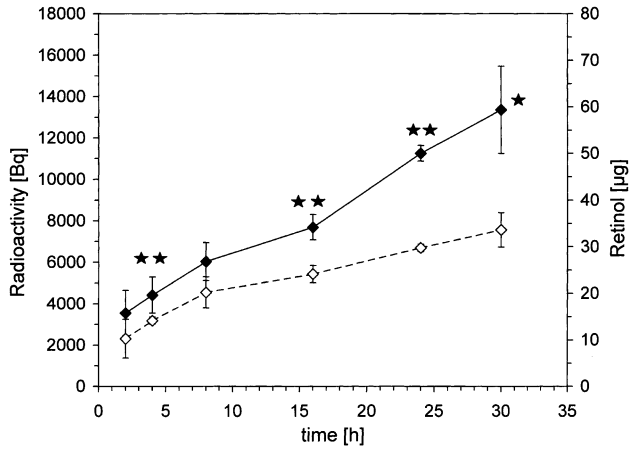

Fig. 9. Penetration of retinol into hairless mouse skin in vitro (mean ± SD; $n = 3$). Total amounts of retinol found in the residual skin after removal of the stratum corneum by stripping as a function of time are shown. Hydrogel A containing retinol adsorbed onto SCMPs (◆). Hydrogel B containing freshly precipitated retinol (◇). $P < 0.05$ (★); $P < 0.01$ (★★).

transporting all-*trans* retinol into the stratum corneum and dermis and led to an increased transport for longer periods. The largest advantage is that sponge collagen avoids the danger of BSE contamination.

Acknowledgements

This project was supported by the Bundesministerium für Bildung, Wissenschaft und Forschung (FK 0310932) and by the European Commission (QLK3-1999-00672). We would like to thank Mr Ruppel, Botanisches Institut, University of Frankfurt, Germany for taking the SEM photographs and Dr Maas, Tierversuchsanstalt, Aventis AG, Germany for the demonstration of mouse skin preparation techniques. We wish to thank Dr K. Langer, Institute of Pharmaceutical Technology, University of Frankfurt, Germany and Dr R. Löwenberg, Faculty of Pharmacy and Pharmaceutical Science, University of Alberta, Canada for numerous and valuable discussions. The authors are grateful to Beckman, Munich, for the material support with scintillation cocktails.

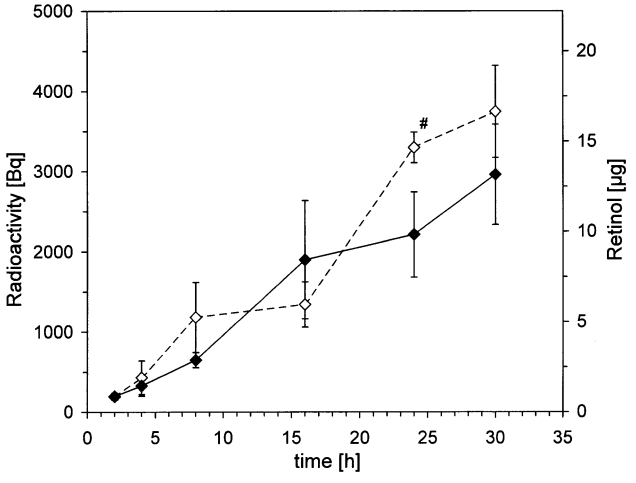

Fig. 10. Permeation of retinol through hairless mouse skin in vitro (mean ± SD; $n = 3$). Total amounts of retinol found in acceptor chambers as a function of time are shown. Hydrogel A containing retinol adsorbed onto SCMPs (◆). Hydrogel B containing freshly precipitated retinol (◇). $P < 0.1$ (#).

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