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RESEARCH ARTICLE

Preparation and characterization of marine sponge collagen nanoparticles and employment for the transdermal delivery of 17β-estradiol-hemihydrate

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Abstract

Background: Transdermal administration of estradiol offers advantages over oral estrogens for hormone replacement therapy regarding side effects by bypassing the hepatic presystemic metabolism. *Aim:* The objective of this study was to develop nanoparticles of *Chondrosia reniformis* sponge collagen as penetration enhancers for the transdermal drug delivery of 17β-estradiol-hemihydrate in hormone replacement therapy. *Method*: Collagen nanoparticles were prepared by controlled alkaline hydrolysis and characterized using atomic force microscopy and photon correlation spectroscopy. Estradiol-hemihydrate was loaded to the nanoparticles by adsorption to their surface, whereupon a drug loading up to 13.1% of sponge collagen particle mass was found. After incorporation of drug-loaded nanoparticles in a hydrogel, the estradiol transdermal delivery from the gel was compared with that from a commercial gel that did not contain nanoparticles. *Results*: Saliva samples in postmenopausal patients showed significantly higher estradiol levels after application of the gel with nanoparticles. The area under the curve (AUC) for estradiol time–concentration curves over 24 hours was 2.3- to 3.4-fold higher and estradiol levels 24 hours after administration of estradiol were at least twofold higher with the nanoparticle gel. *Conclusions*: The hydrogel with estradiol-loaded collagen nanoparticles enabled a prolonged estradiol release compared to a commercial gel and yielded a considerably enhanced estradiol absorption. Consequently, sponge collagen nanoparticles represent promising carriers for transdermal drug delivery.

Key words: *Estradiol-hemlhydrate; hormone replacement therapy; marine sponge collagen; nanoparticles; skin penetration enchancers*

Introduction

Nanoparticulate drug delivery systems offer many advantages compared to conventional dose forms such as sustained release, improved bioavailability, reduced side effects, and drug protection against enzymatic and chemical degradation[1–4]. Up to now collagen, a biodegradable biomaterial, often has been used for drug delivery[5], as shields in ophthalmology[6], injectable dispersions for local tumor treatment, as a scaffold[7,8], or for transdermal drug delivery[9,10]. Collagen from the marine sponge *Chondrosia reniformis* Nardo does not bear the risk of bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE). Therefore, it was used in this study for the preparation of nanoparticles that were loaded with 17β-estradiol-hemihydrate for transdermal delivery. The collagen of this sponge was isolated and characterized previously[11,12] and was the subject of physicochemical and ultrastructural studies[13,14].

Transdermal administration of estradiol has become increasingly popular because of certain advantages over oral formulations with respect to adverse effects such as increasing risk for coronary heart disease and venous thromboembolism in postmenopausal women[15–18]. The findings in the past with collagen from the marine

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sponge *Chondrosia reniformis* in dermal delivery of all-*trans* retinol[10] suggest that sponge collagen nanoparticles (SCNPs) are a promising drug carrier system for poorly water-soluble estradiol-hemihydrate to improve bioavailability and to achieve a prolonged drug release. In this study, the first in vivo data on the marine sponge as a drug carrier are presented.

Materials and methods

Materials

The marine sponge *Chondrosia reniformis* Nardo (Demospongiae: Hadromerida: Chondrosiidae) was harvested by diving in the culture site of the Greek island of Kalymnos in the south eastern Aegean Sea and stored in 50% (v/v) ethanol until used. Estradiol-hemihydrate was purchased from Fagron GmbH & Co. KG (Barsbüttel, Germany). All other chemicals and reagents were obtained as follows: sodium hydroxide from Caesar & Loretz GmbH (Hilden, Germany), potassium dihydrogen phosphate from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), carbomer 50,000 mPas and propylene glycol from Euro OTC Pharma GmbH (Bönen, Germany), ethanol 96% Ph. Eur. from Laborchemie Apolda GmbH (Apolda, Germany), and ethanol 96% from Bundesmonopolverwaltung für Branntwein (Offenbach am Main, Germany).

Preparation of marine sponge collagen nanoparticles

SCNPs were prepared by alkaline hydrolysis of Chondrosia collagen, which was isolated according to a previously described method and upscaled to industrial production dimensions[19]. Hydrolysis was carried out in 1.5 M NaOH under constant stirring (500 rpm) at room temperature for different time periods with subsequent dialysis (Nadir®, 25–30 Å; Carl Roth GmbH & Co. KG) against deionized water. Dialysis was undertaken after 18 hours, 48 hours, and 7 days of hydrolysis and in all three cases terminated at a pH of between 6 and 7 of the collagen dispersion. Collected batches were filtered through 0.22 µm pore size filters (Stericup®; Millipore Corporation, Billerica, MA, USA).

Characterization of empty nanoparticles: atomic force microscopy

Atomic force microscopy (AFM) images were obtained by using a Nanoscope IIIa Bioscope (Digital Instruments/Veeco, Santa Barbara, CA, USA) operating in tapping mode®. Fifty microliters of the collagen suspensions were placed on the surface of titanium-sputtered glass discs. After adsorption for 30 minutes, the samples were nitrogen-dried. The tips for AFM were fabricated out of silicon with a reflecting aluminium film deposited on the backside of the cantilever (force constant 40 N/m). All AFM images were taken at scan rates of 1.2 Hz, scanning 512 lines. Deflection and height images were taken simultaneously. AFM results were used to estimate grain sizes assisted by WSxM software[20].

Characterization of empty nanoparticles: photon correlation spectroscopy and zeta potential

The average particle size was determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer $3000HS_A$ (Malvern Instruments Ltd., Malvern, UK) at a temperature of 25°C and a scattering angle of 90°. For measurement, the 0.75% (w/v) nanoparticle suspensions were diluted 1:30 with Ampuwa®. In addition, samples were freeze-dried by means of a Virtis Ultra 35 LE (Virtis Co., Gardiner, NY, USA), redispersed in Ampuwa®, and measured after 10 minutes of ultrasonication (Sonorex; Bandelin Electronic GmbH & Co. KG, Berlin, Germany). For this purpose, a 1% (w/v) dispersion was diluted 1:40. The surface charge of all samples was determined by measuring the electrophoretic mobility by laser Doppler microelectrophoresis using the same instrument (Malvern Instruments Ltd.) and the same concentration of samples. The pH was adjusted to 7.4 using 0.1 M phosphate buffer. Particle size and zeta potential of nanoparticles were determined in triplicate, and the average values were calculated.

Adsorption isotherm of estradiol-hemihydrate to collagen nanoparticles

To determine the adsorption isotherm of estradiol-hemihydrate to empty suspended SCNPs, a stock solution of estradiol-hemihydrate of 5 mg/mL with ethanol 96% as solvent was prepared, and volumes between 500 and 2000 µL (adjusted with ethanol 96% to 2.0 mL if required) were added to 10 mg freeze-dried nanoparticles in 2-mL test tubes (Carl Roth GmbH & Co. KG). As estradiol-hemihydrate is practically insoluble in water but sparingly soluble in alcohol, ethanol 96% (v/v) was used as solvent. After incubation under constant shaking (1400 rpm, Thermomixer comfort; Eppendorf AG, Hamburg, Germany) for 2 hours at 20°C, the samples were centrifuged (16,100 × *g*, 15 minutes, Centrifuge 5415D, Eppendorf AG), and the concentration of unbound estradiol in the supernatants was measured by UV spectrophotometry (U-3000-spectrophotometer, Hitachi Ltd., Tokyo, Japan) after appropriate dilution to 10 mL with ethanol 96% and following dilution of 5 mL thereof to 50 mL with 0.1 M sodium hydroxide. Absorbance of the solution was measured at the maximum at

238 nm according to the European Pharmacopoeia (Ph. Eur., 5th edition, monograph 0821)[21]. The fraction of estradiol-hemihydrate adsorbed to SCNPs was calculated and a sorption isotherm was plotted. Furthermore, samples of 10 mg freeze-dried SCNPs without estradiol-hemihydrate were treated equally to confirm that SCNPs were precipitated completely after centrifugation and no absorbance took place at 238 nm by collagen. To assure that no estradiol-hemihydrate was precipitating during the experiments, a supersaturated drug suspension in ethanol 96% was prepared prior to determination of the adsorption isotherm, and after 30 minutes stirring at 20°C (650 rpm, Thermomixer comfort; Eppendorf AG) the suspension was centrifuged through a Microcon® Centrifugal Filter Device (Ultracel YM-30, size exclusion 30 kDa; Millipore). The estradiol concentration in the filtrates was determined by UV spectrophotometry after dilution (100 µL to 10 mL) with ethanol 96% and 5 mL thereof to 50 mL with 0.1 M sodium hydroxide). Samples in the investigated drug concentration range without SCNPs were treated equally and drug recovery was measured. Each experiment was run in triplicate.

Incorporation of SCNP-bound estradiol-hemihydrate in a hydrogel formulation

Fifty milliliters of estradiol-hemihydrate (5 mg/mL) was added to 250 mg freeze-dried SCNPs. The mixtures were stirred for 2 hours (1400 rpm) at 20°C to achieve an adsorption equilibrium of estradiol-hemihydrate on the particle surface. The suspension was centrifuged (4000 rpm, 60 minutes, Kontron® Centrifuge Hermle Z364; Hermle Labortechnik GmbH, Wehingen, Germany), and the concentration of free estradiol-hemihydrate was analysed in the supernatant by UV spectrophotometry after suitable dilution. The supernatant was discarded, and the nanoparticles were washed twice with 5 mL purified water followed by subsequent centrifugation (4000 rpm, 60 minutes). Apart from incorporation in a hydrogel formulation, the nanoparticles were analysed for estradiol content. Estradiol-hemihydrate was extracted three times from the SCNPs with 10 mL ethanol 96% each step whereupon extracts were collected and diluted as previously described for quantitative analysis. To produce a hydrogel concentrate, 250 mg loaded SCNPs were resuspended in 4.75 g Ampuwa® and mixed slowly with 40 g of a freshly prepared hydrogel consisting of 1.0% crosslinked polymethacrylate (Carbomer 50000 mPas), 0.3% sodium hydroxide, 10.0% propylene glycol, and 88.7% deionized water using a TopiTec® Automatic (300 rpm; WEPA Apothekenbedarf GmbH & Co. KG). The preparations were stored in an exsiccator (Nalgene®, Rochester, MN, USA) connected to a suction pump to remove air bubbles that were formed during blending. The estradiol content in the hydrogels was determined in a small aliquot after three times extraction with ethanol 96%. After the determination of the estradiol content, additional hydrogel was added to the estradiol gel concentrate to obtain a final estradiol concentration of 0.06% in the gel.

Long-time stability of aqueous dispersions of SCNP-bound estradiol-hemihydrate

To determine the long-time stability of SCNP-bound estradiol-hemihydrate, 15 samples [10 mg SCNP, 2 mL of estradiol-hemihydrate (5 mg/mL)] were prepared as described above. The supernatants were discarded, and the SCNPs were separated from unbound estradiol-hemihydrate by washing two times with Ampuwa® followed by centrifugation (16,100 × *g*, 15 minutes). After discarding the supernatant, loaded nanoparticles were analysed for estradiol content (three independent repetitions) as described above. The residual 12 samples were resuspended in 2 mL Ampuwa® each, stored at 2°C–6°C for storage times of 1, 7, 30, and 180 days and analysed for estradiol after redispersing by shaking for 15 seconds (lab dancer; VWR International GmbH, Darmstadt, Germany), subsequent centrifugation and discarding of the supernatant as described above. The drug loss during storage was calculated.

Transdermal absorption of estradiol from nanoparticle gels and gels without nanoparticles

Five postmenopausal women, who had not been on estrogen or progestogen therapy before (patients 1–5), obtained one application of 0.75 mg estradiol either in form of a conventional gel formulation (Gynokadin® 0.06% gel; Dr. Kade GmbH, Berlin, Germany) or as the nanoparticle preparation 0.06% (KliniPharm GmbH, Frankfurt, Germany) with a wash-out of at least 1 week between the applications. The sequence of application of the two preparations was selected randomly. Saliva samples for a diurnale profile were taken immediately after waking up directly before estradiol gel application and 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, and 24 hours after the dose of Gynokadin® and nanoparticle preparation. Before starting hormone treatment, three samples were taken at intervals of 0.5 hour to determine the base estradiol values.

To determine saturation concentrations, two more postmenopausal women (patients 6 and 7) received one of the two preparations of 0.6 mg estradiol daily for 28 days followed by treatment with the other preparation for another 28 days where one of the patients started with the nanoparticles and the other with the

reference gel. Saliva samples were obtained after 28 and 56 days (23–24 hours after last application).

All patients applied the preparations on the same area of the same arm and shoulder. The formulations were supplied in round glass bottles with dispensers (R. Gerschon GmbH, Königstein, Germany), which provide 0.125 g gel (0.075 mg estradiol and 0.0775 mg estradiol-hemihydrate, respectively) per lift. Patients did not eat, drink, chew gums, brush teeth, or use mouthwash for 30 minutes before sampling of 1–2 mL of saliva in collection vials, and they were instructed not to donate samples when any microinjury occurred in the oral cavity, as blood in saliva affects estradiol levels[22]. All samples were controlled for blood contamination by using hemoglobin test kits (Combur 3 Test E; Roche Diagnostics GmbH, Mannheim, Germany), and samples with detected hemoglobin were excluded to avoid inaccurate estradiol concentrations.

Determination of estradiol in the saliva samples

Saliva samples were assayed for estradiol using an enzyme immunoassay with a limit of sensitivity of 0.3 pg/mL (Estradiol Saliva LUM IA; IBL Gesellschaft für Immunchemie und Immunologie mbH, Hamburg, Germany) for a quantitative determination of free, nonprotein-bound 17β-estradiol as apparently accurate reflection of the concentrations of physiologically active estradiol in the circulation[23–26]. The method was validated with regard to linearity, recovery, and precision. The mean linearity was 107% ± 8.0% and the mean recovery of estradiol was 94% ± 9.5%. The intra-assay coefficient of variation ranged from 3.7% to 7.9% and inter-assay coefficient from 5.9% to 13.9%. Determination of highly concentrated samples was conducted by dilution with zero standard, and no relevant deviation of the expected linearity was observed. All samples were assayed in duplicate for patients 1–5, the concentration–time curve was plotted, and the AUC was calculated using the linear trapezoidal method.

Results and discussion

Preparation and characterization of SCNPs

In previous studies, the calf collagen microparticles or sponge collagen microparticles (SCMPs) were prepared using a method of emulsification followed by cross-linking with glutaraldehyde[10,27,28]. In this study, sponge collagen nanoparticles were prepared by alkaline hydrolysis (cut-off size of 220 nm by sterile filtration) yielding 10.2 ± 0.7% (mean ± SD, n = 3) freeze-dried SCNPs in relation to freeze-dried isolated collagen raw material.

High-resolution AFM imaging showed uniform spherical nanoparticles that decreased in size with increasing periods of time of hydrolysis (Figures 1–3). According to height images, the diameter of all particles was clearly below 100 nm, whereas PCS showed a size of 123 ± 5.5 nm (n = 3, polydispersity ≤ 0.18) with particles that were dialysed after 18 hours. The particle size was increased after redispersion of freeze-dried particles to 168 ± 9.1 nm (n = 3, polydispersity ≤ 0.22). The average zetapotential was –38.7 ± 3.1, suggesting the electrokinetic stability of the formulations. The discrepancy in the size of SCNPs between AFM and PCS may be explained by taking into account that the AFM images give a topographical view of the samples which may not represent the true height and diameter of the particles, because of shrinkage during drying. On the other hand, the hydrodynamic diameter measured by PCS possibly was higher caused by the swelling of the particles in the aqueous media. The larger mean size after redispersion of the freeze-dried samples might be caused by agglomeration of smaller particles. Similar phenomena were

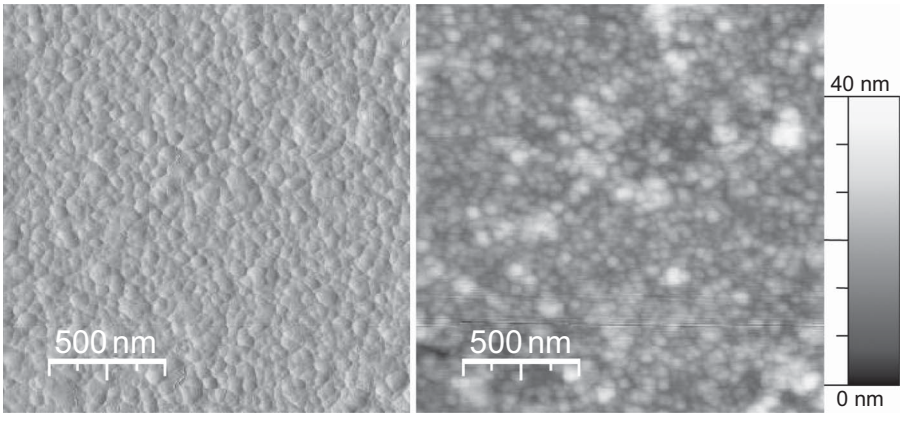

Figure 1. High-resolution AFM imaging of collagen (deflection and height image) after alkaline hydrolysis for 18 hours followed by dialysis.

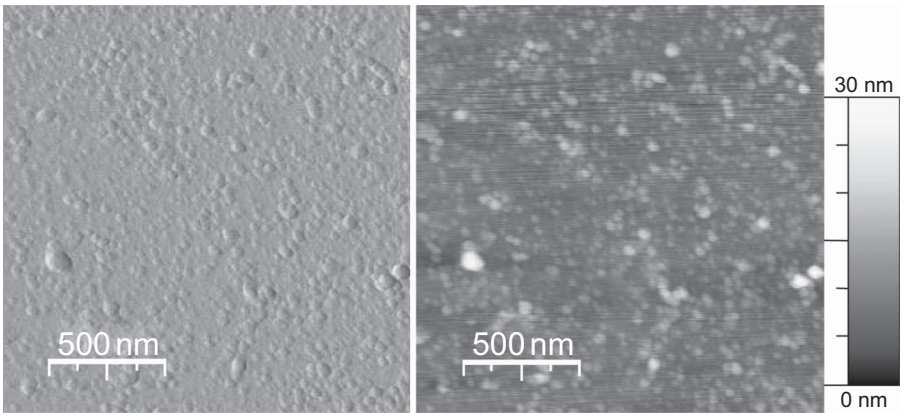

Figure 2. High-resolution AFM imaging of collagen (deflection and height image) after alkaline hydrolysis for 48 hours followed by dialysis.

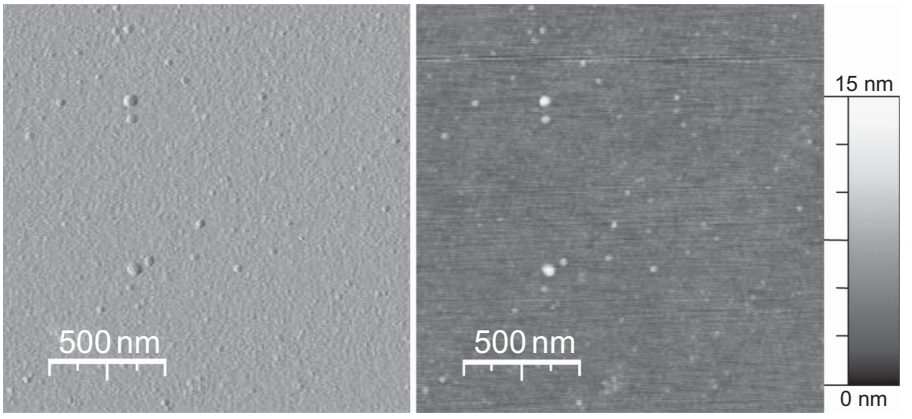

Figure 3. High-resolution AFM imaging of collagen (deflection and height image) after alkaline hydrolysis for 7 days followed by dialysis.

observed by Swatschek[10]. The SCNPs made by the novel process, alkaline hydrolysis, are more consistent in size compared with previously marine sponge-derived particles[10] used for dermal drug delivery which ranged in size from 126 (±2.9) to 2179 nm (±342) and exhibited a higher polydispersity of 0.3490 (±0.01) compared to the SCNPs.

Adsorption of estradiol-hemihydrate to SCNPs and incorporation in a hydrogel formulation

The adsorption of estradiol-hemihydrate to preformed SCNPs was investigated in ethanol 96%. Prior to the experiments, it was assured that no estradiol-hemihydrate was precipitated and that complete precipitation of SCNPs took place before performing UV spectrophotometry. For this purpose, supersaturated suspensions of estradiol-hemihydrate were prepared. The concentrate in the filtrates of the supersaturated suspensions ranged from 23 to 25 mg/mL. Consequently, it was presumed that there was no risk for precipitation at an estradiol-hemihydrate concentration of 5 mg/mL as used for the establishment of the sorption isotherm. Recovery of estradiol in the investigated concentration range (without SCNPs) was approximately 100% (100 ± 2.8%, n = 3). No absorbance was measured in samples with SCNPs without drug. At estradiol-hemihydrate concentrations between 1.25 and 5 mg/mL, an increasing drug loading up to 13.1% of sponge collagen particle mass was found (Figure 4), while the adsorbed drug fraction decreased from 18.1% to 13.1% of the initial estradiol concentration (Figure 5). To achieve a high drug loading, the highest drug concentration of 5 mg/mL was chosen for the incorporation into the hydrogel formulation. During washing, a partial desorption of estradiol-hemihydrate took place (5.2 ± 0.8%). The stability of the estradiol-hemihydrate bound to the SCNPs in aqueous dispersions after different times of storage was examined (Figure 6). It appeared that 58% of the estradiol-hemihydrate was still bound to the SCNPs after storage for 180 days at 2°C–6°C. Consequently the drug loding of the SCNPs was between 7% and 8% vs 13.1% initially. Desorbed estradiol-hemihydrate precipitated to some extent. As this part floated after centrifugation, it could be discarded.

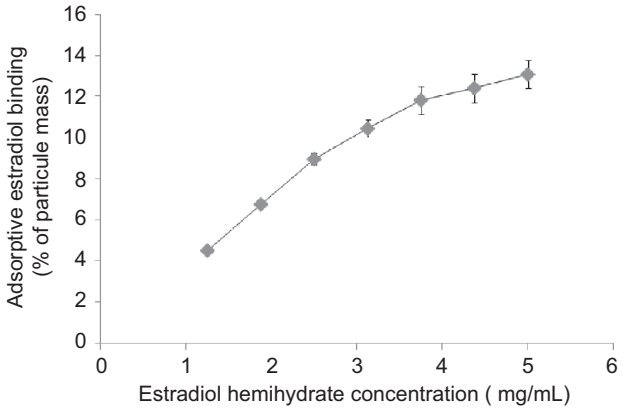

Figure 4. Adsorption isotherm (20°C) of estradiol to 10 mg SCNPs.

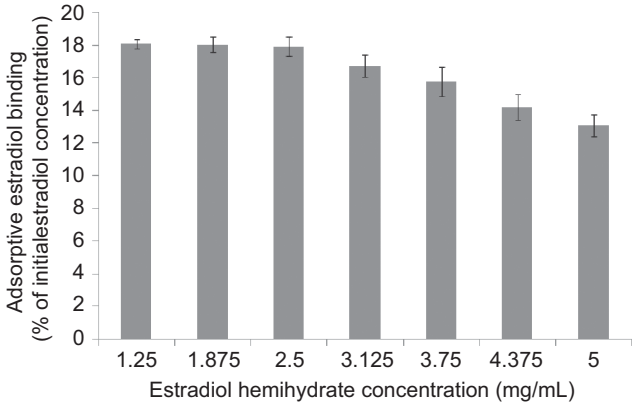

Figure 5. Fraction of estradiol (%) adsorbed to 10 mg SCNPs.

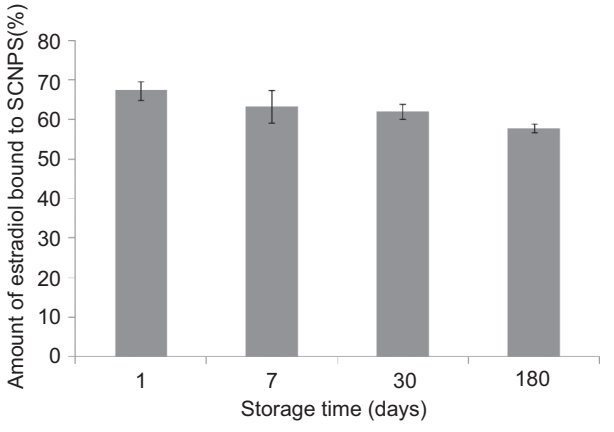

Figure 6. Amount of estradiol bound to the SCNPs after storage times of 1, 7, 30, and 180 days (2°C –6°C).

Transdermal absorption of estradiol from nanoparticle gels and gels without nanoparticles

While saliva samples for patients 1–3 were available after 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, and 24 hours after dosing, patients 4 and 5 delivered samples after 1, 2, 3, 4, 5,

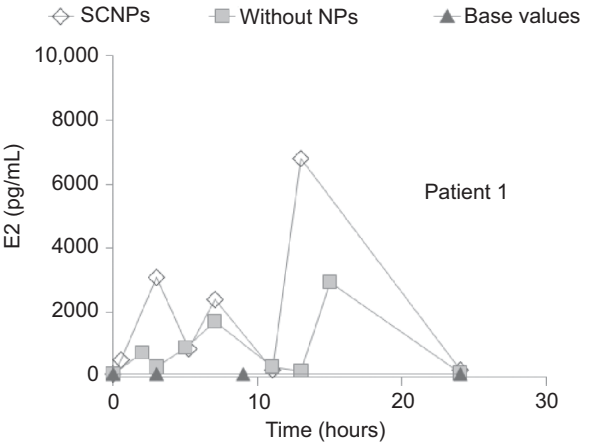

Figure 7. Estradiol saliva levels in patient 1 after application of 0.75 mg estradiol using the following preparations: ✧ a hydrogel containing nanoparticles (SCNPs) or ■ a commercial gel (Gynokadin®) (base values ▲).

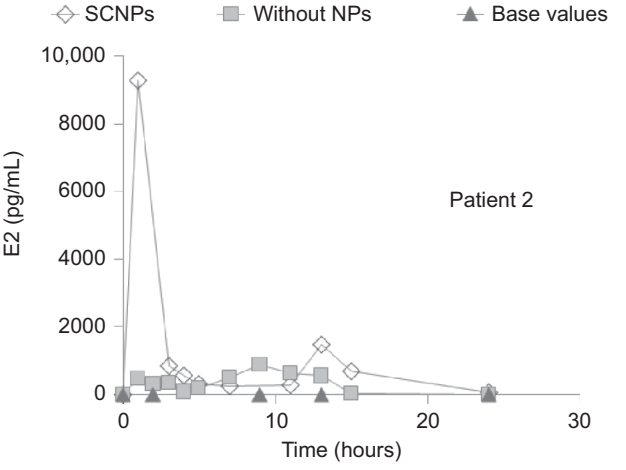

Figure 8. Estradiol saliva levels in patient 2 after application of 0.75 mg estradiol using the following preparations: ✧ a hydrogel containing nanoparticles (SCNPs) or ■ a commercial gel (Gynokadin®) (base values ▲).

6, 7, 8, 9, 10, and 24 hours. With both gels, the estradiol levels in all patients rose soon after dosing, and the first peak was achieved within 3 hours after which concentration was declining again (Figures 7–11). One or two further peaks during 24 hours were observed. The peak saliva levels as well as trough levels (except patient 1 after 11 hours) were significantly higher with the nanoparticle formulation than with the reference gel. After 24 hours of dosing, estradiol levels were still at least twofold, and in the case of patients 4 and 5 (Figures 10 and 11) even considerably higher than mean base values with the nanoparticle gel whereas the estradiol levels were about at baseline level when the gel without NPs was applied. Accordingly, the calculated AUC^{0-24} (patients 1–3) or AUC^{0-10} (patients 4 and 5) (Table 1) was between 2.3- and

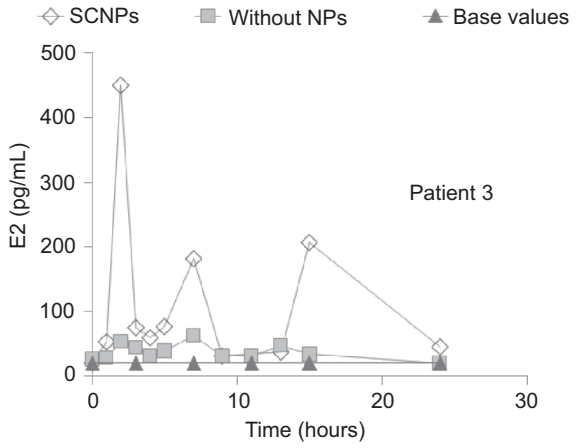

Figure 9. Estradiol saliva levels in patient 3 after application of 0.75 mg estradiol using the following preparations: ✧ a hydrogel containing nanoparticles (SCNPs) or ■ a commercial gel (Gynokadin®) (base values ▲).

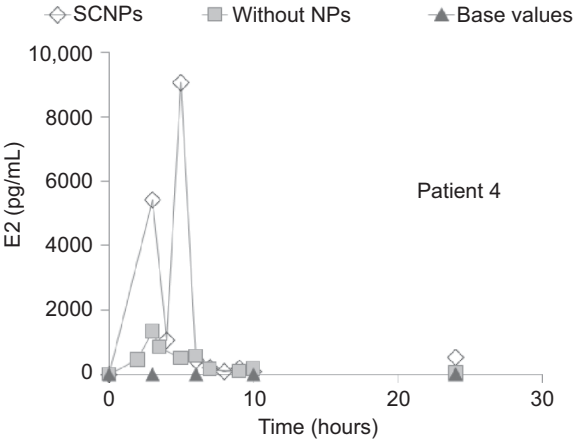

Figure 10. Estradiol saliva levels in patient 4 after application of 0.75 mg estradiol using the following preparations: ✧ a hydrogel containing nanoparticles (SCNPs) or ■ a commercial gel (Gynokadin®) (base values ▲).

3.4-fold higher with the gel containing estradiol-loaded nanoparticles. In patient 3 (Figure 9), poor absorption was observed with the reference gel but good absorption with SCNP-gel.

The morning estradiol levels in patients 6 and 7 (both <0.2 pg/mL before hormone replacement) on the 29th or 57th day, respectively, receiving daily applications were significantly higher with the nanoparticle-containing gel (patient 6, 163.7 pg/mL; patient 7, 62.5 pg/mL) versus estradiol levels with the conventional gel (patient 6, 64 pg/mL; patient 7, 3.5 pg/mL).

Despite the high interindividual variability that was already observed before with transdermal estradiol gels or patches[29,30], the release of estradiol from a dermal gel can be prolonged and the extent of estradiol absorption can be considerably enhanced by binding to SCNPs.

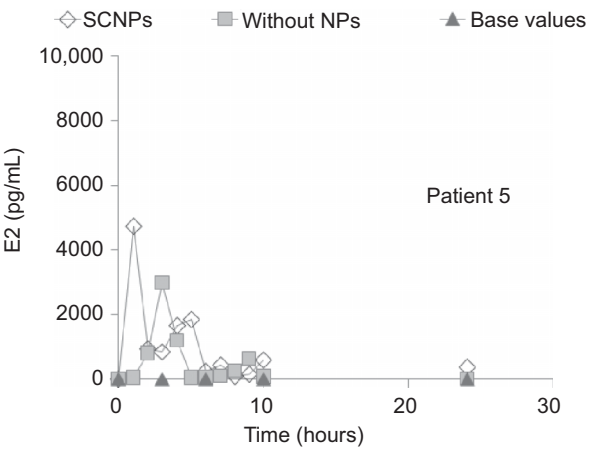

Figure 11. Estradiol saliva levels in patient 5 after application of 0.75 mg estradiol using the following preparations: ✧ a hydrogel containing nanoparticles (SCNPs) or ■ a commercial gel (Gynokadin®) (base values ▲).

Table 1. Comparison of the AUCs of patients 1–5 after single application of 0.75 mg estradiol as a hydrogel containing nanoparticles (SCNPs) or a commercial gel (Gynokadin®).

	Gel with SCNPs	Gel without NPs
AUC^{0-24} (pg × h/mL) patient 1	63120	26980
AUC^{0-24} (pg × h/mL) patient 2	25011	7292
AUC^{0-24} (pg × h/mL) patient 3	22095	4323
AUC^{0-10} (pg × h/mL) Patient 4	11405	6267
AUC^{0-10} (pg × h/mL) patient 5	2692	863

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Declaration of interest: The authors report no conflicts of interest.

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