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Research paper

Targeted transfollicular delivery of artocarpin extract from *Artocarpus incisus* by means of microparticles

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Abstract

Artocarpin (Ar), an extract of heartwood of *Artocarpus incisus*, possesses potent 5α reductase inhibitory effect. The penetration of Ar into the deeper layers of the skin where androgen receptors are present is limited. Therefore, this study was aimed to prepare alginate/chitosan (ACS) microparticles for targeted transfollicular delivery. It was found that a suitable particle size ranging from 2 to 6 μm can be prepared using the ionotropic gelation technique. Entrapment efficiency of Ar in ACS microparticles was 18.7 ± 1.7%. The release of Ar from the ACS microparticles over 6 h was 0.7% of the loading dose suitable for a long-term release of Ar in the follicular ducts. The optimal growth suppression of the hamster flank organs could be achieved by topical application of Ar-ACS microparticles with a content of 0.1 mg in 5 mg microparticles to one hamster flank while the other flank (intraspecies control) showed the normal growth of the flank organs and Ar at the same concentration in solution form could not suppress the growth of the flank organs to the same extent. The efficiency of Ar 0.1 mg loaded in ACS microparticles was shown to be comparable to a dose of 1 mg Ar applied as solution. However, Ar formulated in microparticles did not show significant systemic action compared to the dermal application of an Ar solution and a flutamide preparation (1 mg) as positive control.

Keywords: Alginate; Chitosan; Microparticles; Artocarpin; *Artocarpus incisus*; 5α-Reductase inhibitor; Targeted follicular delivery; Pigmented macules

1. Introduction

Artocarpin (Ar) (Fig. 1) is a naturally occurring compound which can be isolated from a diethyl ether extract of heartwood (*Artocarpus incisus*, Family Moraceae). Ar has been reported to have potent 5α-reductase inhibitory effects [1] resulting in the inhibition of the conversion of testosterone into 5α-dihydro-testosterone. The 5α-reductase inhibitor acts on androgen receptors which are found in both preputial skin and nongenital skin [2]. In the preputial skin, an androgen receptor is expressed in the epidermal cells as well as in fibroblasts, smooth muscle cells and endothelial cells of blood vessels in the dermal tissue. In nongenital skin, the androgen receptor is expressed in the basal cells and glandular cells of the sebaceous glands, in the outer root sheath of hair follicles and in eccrine sweat glands. Therefore, Ar may be useful in selective treatment of androgen-dependent disorders such as male pattern alopecia and acne. To treat these disorders, the compound must be delivered through skin. However, skin is generally known as a physical barrier, which prevents water loss as well as the entry of micro-organisms. Hence, the delivery of Ar as 5α-reductase inhibitors into the skin may be limited.

Polymeric colloidal particles including nanoparticles and microparticles are defined as particles with a size in nanometer and micrometer size range, respectively [3,4]. They are generally made of biodegradable or non-biodegradable polymers such as chitosan, alginate and other suitable polymers. Depending on the particle size, polymeric colloidal particles may enable drug penetration

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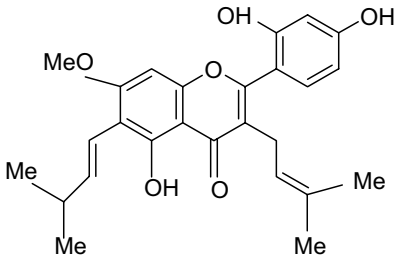

Fig. 1. Chemical structure of artocarpin.

through stratum corneum [5–8]. Small particles having a size less than 1 μm are known to enhance penetration of a substance along the transepidermal route whereas larger particles having size of 3–10 μm are found to be able to be targeted as carriers for the transfollicular route [8]. Particles having size larger than 10 μm are reported to remain on top of the skin [8]. The structures of the stratum corneum of the skin may enable drug substances to permeate across the stratum corneum via two main routes: the transepidermal and transfollicular routes [6,9]. Using the transepidermal route a substrate permeates through either the intercellular lipid domain (intercellular or paracellular transport) or through the corneocytes (transcellular or transcorneocytic transport) [6,9,10]. When using the appendical route a substrate permeates through either hair follicles or sweat glands or through both appendages [11].

Chitosan is a natural cationic polysaccharide that has been used extensively in pharmaceutical and biomedical fields because of its biodegradable, biocompatible and bioactive characteristics [12]. It is derived from chitin, a polysaccharide found in the exoskeleton of shellfishs like shrimps or crabs. Alginate is obtained from an extract of seaweed [13]. It is an anionic polysaccharide that is known to be biodegradable and biocompatible. Calcium alginate microparticles, prepared by dispersing a solution of sodium alginate into a solution of a polycation such as calcium chloride solution, are known to be porous bioerodible particles [14–17]. Coating of these microparticles with cationic polymers, including chitosan, has been shown to improve the stability of alginate microparticles during storage and in biological fluids and to also achieve controlled release characteristics [14–17]. Additionally those microparticles consisting of an alginate core and a chitosan shell are further stabilized by interpolymer ionic gelation. Those particles with a suitable size and delivery profile are therefore hypothesized to render better efficiency in the treatment of androgen-dependent disorders.

Hamster flank organs are the pigmented macules, oval in shape which are located in the costovertebral region. They consist of dermal melanocytes, sebaceous glands and hair follicles [18]. Their growth is known to be regulated by intracellular active androgen 5α-dihydrotestosterone (DHT). Therefore, this animal model has been used in this study to test if the Ar known as 5α-reductase inhibitor can inhibit the conversion of testosterone to DHT and consequently inhibit the growth of the flank organs [18]. For treatment of some dermatological and androgen-dependent disorders such as androgenic alopecia and acne, a 5α-reductase inhibitor that is active only locally in the skin after topical application without causing systemic side effects would be ideal. Hamster flank organs are therefore a good model and can be used as indication if a test compound has systemic effects by applying a test compound topically to one of the flank organs and leaving the other untreated. If the test compound in combination with the delivery system shows systemic effects, both flank organs will be affected.

As a result of these considerations, this study was aimed to prepare ACS microparticles with particle size in the range of 3–10 μm and a slow release profile and to investigate their targeting possibilities to the follicular ducts of the skin resulting in an enhanced delivery of Ar extract from *A. incisus* and decreased area of pigmented macules in the hamster flank.

2. Materials and methods

2.1. Materials

Diethyl ether (AR grade) and methanol (HPLC grade) were obtained from Labscan, Asia Co. Ltd, Thailand. Polysorbate 80 (Crillet 4®) was purchased from Cinnamon Ltd, Thailand. Capric/caprylic triglyceride (Crodamol GTCC®) was kindly provided by Croda, Singapore Pvt. Ltd., Singapore. Ethanol (AR grade), acetic acid and calcium chloride dihydrate were obtained from Merck, Darmstadt, Germany. Sodium alginate (viscosity of a 2% solution at 25 °C: 250 cps) was obtained from Sigma, St. Louis, USA. Chitoclear® (chitosan having degree of deacetylation of 94.5%) was obtained from Primex, Norway. Nembutal®, Pentobarbitone sodium was purchased from Sanofi (Thailand) Ltd., Bangkok, Thailand. Artocarpin was kindly provided by the Department of Forest Products, Faculty of Agriculture, Kyushu University, Fukuoka, Japan. Flutamide was obtained from Sigma, MO, USA.

2.2. Methods

2.2.1. Preparation of artocarpin

Ar was isolated from diethyl ether extracts of heartwood of *A. incisus* according to a modified method from Shimizu et al. [1].

2.2.1.1. Extraction of artocarpin from heartwood of A. incisus. The heartwood portion of *A. incisus* was chipped and dried at 50 °C until dryness. Then dried chipped heartwood was milled and was extracted by a maceration technique. Maceration was performed for 3 cycles (1 day/cycle) with sufficient diethyl ether (ca 100 ml more) to fully cover the heartwood chips. Percolates collected from each cycle

were pooled and concentrated to dryness using a Rotavapor (Büchi, Switzerland) equipped with water bath (RM6, Lauda, Germany) and a condenser with controlled temperature at 33 and 5 °C, respectively. Pressure applied was 560 mbar.

2.2.1.2. Purification. The dried extract from all extraction steps was re-dissolved in diethyl ether and recrystallized by the addition of hexane (half of the diethyl ether volume). This process was performed twice and the dried powder was kept in a desiccator to remove diethyl ether residue and moisture. Then the extract was re-dissolved in methanol and recrystallized by the addition of purified water (half of the volume of the methanol volume). The purified extract obtained in this step was re-dissolved in diethyl ether and evaporated to dryness using the Rotavapor. The dried, yellow crystals obtained were collected and kept in a desiccator for further studies.

2.2.2. Preparation of microparticles

2.2.2.1. Empty alginate/chitosan microparticles. Empty ACS microparticles were prepared as follows. Alginate solutions were prepared by dispersing various amounts of sodium alginate in distilled water and stirred overnight. Chitosan solution (0.5% w/v) was prepared by dissolving chitosan in 1% acetic acid and stirred overnight. The oil phase containing polysorbate 80 (50 mg), capric/caprylic triglyceride (Crodamol GTCC®, 100 mg) and ethanol (300 mg) was mixed with 2 g of the aqueous alginate solution by a hand homogenizer until an o/w emulsion was obtained. The o/w emulsion was then dropped into 200 ml of the chitosan solution containing 0.5% w/v calcium chloride by a spray gun (Badger 150, Tru-International Co. Ltd., Bangkok, Thailand). The pressure applied on the spray gun was 30 lb/in^2. The system was left overnight under magnetic stirring. Then, the particles formed were separated by centrifugation (Ultracentrifuge, Beckman, USA, equipped with rotor JA 20.0) at 18,000 rpm for 30 min at 25 °C and were washed twice with distilled water. After each washing process, the particles were separated from the distilled water by centrifugation at 18,000 rpm for 30 min at 25 °C. Following the final wash the particles were redispersed in distilled water and kept in the refrigerator for further investigations. The redispersion of the particles after each step was performed using a sonicator bath (Transsonic 820/H, Elma, Singen, Germany). The empty ACS microparticles were freeze-dried (Dura Dry, FTS System Inc., NY, USA).

2.2.2.2. Alginate/chitosan microparticles containing artocarpin. The ACS microparticles containing Ar (ACS-Ar) were prepared in the same way as the preparation of the empty ACS microparticles whereby Ar was dissolved in the oil phase containing polysorbate 80, capric/caprylic triglyceride and ethanol before mixing with alginate solution. The Ar loaded ACS microparticles were then freeze-dried.

2.2.3. Morphological characterization

The freeze-dried particles were coated with gold palladium and their surface morphology was visualized by scanning electron microscope (SEM, Leo 1455 VP, USA).

2.2.4. Determination of entrapment efficiency

Percentage of entrapment of Ar within the ACS particles was determined as follows: ACS-Ar particles were redispersed after the second washing process in 8 ml distilled water. To 2 ml of the particle suspension 5 ml ethanol (HPLC grade) was added. The ethanolic dispersion was stirred overnight at 37 °C. Then, the dispersion was subjected to centrifugation at 1300 rpm for 10 min at room temperature. The amount of Ar in clear supernatant was quantified by HPLC (Consta Metric 3200, USA). Shortly, 60 μl aliquots of the clear supernatant were injected into the HPLC system, equipped with a packed C18 column (Appollo SC-04, 5 μm, 250 × 4.6 mm). Flow rate was 1.0 ml/min. The mobile phase consisted of methanol and purified water at a ratio of 90:10 (v/v). The detection wavelength was 282 nm. The entrapment efficiency was expressed as a percentage of the amount of Ar in the primary solution and the amount isolated from the microparticles.

$$\text{Entrapment efficiency } (\%) = \frac{C_I - C_{MP}}{C_I} \times 100$$

where C_I is the total amount of Ar initially incorporated in the system and C_{MP} is the amount of Ar obtained after incubating the microparticles in ethanol for overnight.

2.2.5. Release studies

The ACS-Ar microparticles were dispersed in phosphate buffer solution, pH 7.4, which was used as the release medium to yield a final concentration of 2.5 mg microparticles/1 ml dissolution medium. Five milliliters of the particle suspension was transferred to a double-walled beaker with controlled temperature of 37 °C. The dispersion was continuously stirred using a magnetic flea at a rate of 200 rpm. At 0, 0.25, 0.5, 1, 3, 6 and 24 h, 300 μl of the release medium was withdrawn and the volume of each sample was replaced by the same volume of fresh medium. The sample (300 μl) was subjected to centrifugation at 1300 rpm for 10 min at room temperature. The clear supernatant was analyzed for the amount of Ar released using HPLC.

2.2.6. Growth suppression of hamster flank organs

2.2.6.1. Animals. Fifty-one male golden hamsters, aged 8 weeks, were obtained from National Laboratory Animal Centre, Mahidol University, Nakorn Pathom, Thailand. The hamsters were housed individually on sawdust and had free access to food and tap water. They were acclimatized to the laboratory conditions for at least 1 week prior to the experiments.

2.2.6.2. Treatment protocol. The animal treatment protocol was approved by the Ethical Committee of Naresuan

University. The hamsters were randomly divided into six groups each group comprising nine hamsters except the control group consisting of six hamsters. At the beginning of experiments, the area of the flank organ of each side of all hamsters was shaved with an electric hair clipper. The hamsters were anesthetized by an intraperitoneal injection of pentobarbitone (40 mg/ml) 300 μl per animal before applying the formulations. Each group was treated with one of the following formulations:

(a) Propylene glycol:ethanol (20:80, w/w) (negative control),
(b) Ar 0.1 mg/20 μl ,
(c) Ar 0.5 mg/20 μl,
(d) Ar 1.0 mg/20 μl,
(e) ACS microparticles containing Ar equivalent to 0.1 mg (ACS-Ar microparticles, dispersed in distilled water),
(f) Flutamide 1 mg/20 μl (positive control).

For groups b, c, d and f, Ar and flutamide were dissolved in 20 μl of propylene glycol:ethanol (20:80, w/w). For group e, ACS-Ar particles were prepared as described in Section 2.2.2.2. After the second washing, the known amount of particles containing Ar equivalent to 0.1 mg was redispersed in 20 μl distilled water.

One hindleg flank of each hamster was treated with the respective formulation while the other flank was used for intra-animal control. The formulations (20 μl) were applied topically to the flank area once daily using a pipette and smoothly and evenly distributed with the help of a propylene disposable tip. The application sites of the hamster's flanks were covered with a type of small "cotton shirt" (without any occlusion effect of the flanks) in order to avoid the possibility of oral absorption of the test substances by licking of the hamster. The experiment was performed for 5 days a week for 4 weeks. The surface of the flank was wiped with an alcohol pad to remove residual compounds of the last treatment before a new treatment. The area of the pigmented macule of flank organ of the treated and untreated sides was observed every week by measuring the length of the long axis and the short axis of the pigmented macule using a Vernier caliper (Mitutoyo Corp., Japan). The surface area (length of long axis times length of short axis) was calculated.

2.2.7. Statistical analysis

The data are expressed as means ± SD. Statistical analysis was measured according to unpaired *t*-test. The level of significance was taken at p-value < 0.05.

3. Results and discussion

3.1. Morphological characterization

Fig. 2 shows a representative scanning electron micrograph of dried ACS particles which indicates that the shape of the particles formed is round shaped. The approximate particle size was determined ranging from 2 to 6 μm.

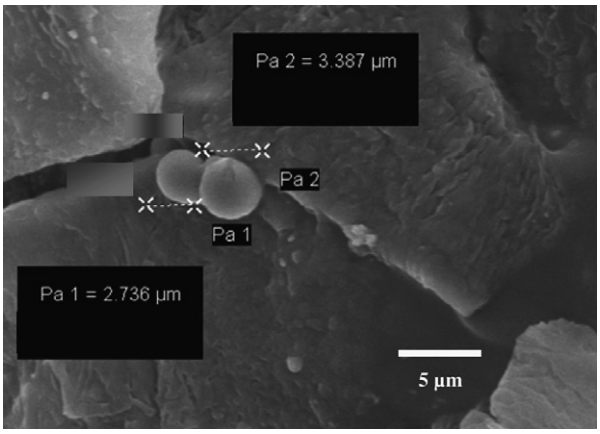

Fig. 2. Scanning electron micrograph of ACS particles.

3.2. Determination of entrapment efficiency

The amount of Ar in ACS particles was determined by dissolving ACS-Ar particles in ethanol overnight and was expressed as entrapment efficiency. Entrapment efficiency of Ar was found to be 18.7 ± 1.7% ($n = 3$).

3.3. Release studies

The release of Ar from ACS particles was performed in phosphate buffer, pH 7.4. The release profiles are shown in Fig. 3. Over 6 h, approximately 0.70 ± 0.16% of the initial Ar content was released from the ACS particles. No further release was found up to 24 h. This release characteristic *in vitro* is due to low solubility of Ar in phosphate buffer but will be slightly different in the *in vivo* situation when Ar is being absorbed by the follicular tissues. As a control to demonstrate the full availability of AR to the dissolution medium the *in vitro* release of Ar from ACS particles was therefore performed again using ethanol as the release

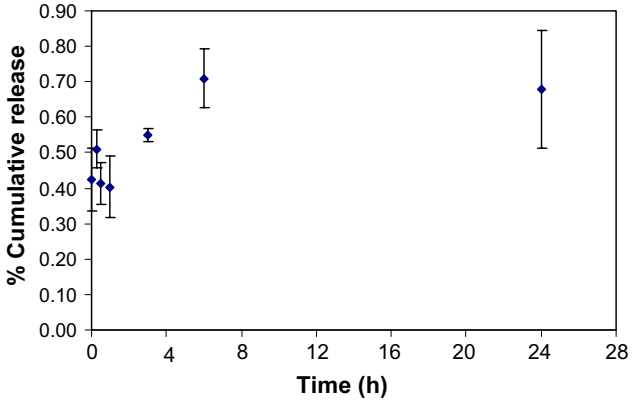

Fig. 3. Release of artocarpin from ACS particles in phosphate buffer solution, pH 7.4.

Table 1
Growth of the flank organ at week 1–5 when the organs were untreated or treated weekly with 20 μl of ethanol:propylene glycol (80:20) (negative control, group A), artocarpin solution 0.1 mg (group B), artocarpin solution 0.5 mg (group C), artocarpin solution 1 mg (group D), 5 mg ACS microparticles containing artocarpin (group E) equivalent to 0.1 mg and flutamide 1 mg (positive control, group F)

Group/ week	Untreated-side						Treated-side					
	A	B	C	D	E	F	A	B	C	D	E	F
1	31.13 (1.13)	31.10 (2.19)	30.47 (2.70)	26.22 (2.82)	28.15 (1.79)	29.03 (1.81)	29.22 (2.43)	31.16 (0.89)	33.26 (3.04)	25.19 (2.30)	28.79 (2.58)	28.05 (1.97)
2	27.64 (3.64)	33.74 (2.89)	34.20 (3.60)	24.65 (2.79)	29.52 (3.05)	32.27 (2.22)	28.97 (3.53)	35.79 (0.81)	39.20 (3.91)	27.71 (3.41)	30.54 (2.91)	31.70 (3.17)
3	33.37 (4.76)	38.04 (5.53)	40.23 (3.05)	24.69 (3.51)	32.00 (3.61)	30.18 (2.97)	32.61 (4.16)	41.39 (1.63)	35.66 (2.52)	24.34 (3.47)	24.74 (4.27)	24.47 (2.73)
4	35.41 (5.79)	34.31 (5.31)	38.16 (3.88)	21.65 (3.25)	33.93 (3.39)	23.98 (4.58)	33.16 (4.36)	42.16 (1.29)	30.74 (3.94)	22.47 (4.57)	24.60 (4.48)	18.42 (3.49)
5	36.89 (5.68)	28.17 (4.80)	33.54 (2.42)	17.49 (2.62)	29.01 (2.79)	20.94 (5.19)	36.12 (7.31)	25.43 (3.59)	26.89 (4.42)	18.32 (4.38)	19.01 (3.59)	15.02 (4.80)

Each week, the areas of the pigmented macules given in mm^2 were measured and the results are presented as means ± SEM ($n = 4$–9).

Table 2
Percentage inhibition of flank organ growth of Golden male hamsters when the organs were untreated or treated weekly with 20 μl of propylene glycol:ethanol (20:80) (negative control), artocarpin solution 0.1 mg, artocarpin solution 0.5 mg, artocarpin solution 1 mg, ACS microparticles containing artocarpin equivalent to 0.1 mg and flutamide 1 mg (positive control)

Group	Treatment	*N*	Untreated-side			Treated-side		
			Area of the flank organ (mm^2) at the end of the experiment	Inhibition (%)[a]	*p*-value[b]	Area of the flank organ (mm^2) at the end of the experiment	Inhibition (%)[a]	*p*-value[b]
A	Propylene glycol:ethanol	4	36.89 ± 5.68	–	–	36.12 ± 7.31	0	–
B	Artocarpin solution 0.1 mg/20 μl	9	28.17 ± 4.80	23.64	0.16	25.43 ± 3.59	29.60	0.08
C	Artocarpin solution 0.5 mg/20 μl	7	33.54 ± 2.42	9.08	0.27	26.89 ± 4.42	25.55	0.14
D	Artocarpin solution 1 mg/20 μl	9	17.49 ± 2.62	52.59	0.00	18.32 ± 4.38	49.28	0.03
E	ACS-Ar microparticles	8	29.01 ± 2.79	21.39	0.09	19.01 ± 3.59	47.37	0.02

[a] Inhibition (%) was calculated as follows: $\text{Inhibition}(\%) = \frac{\text{size of flank organ of control group}}{\text{size of flank organ of treatment group}} \times 1000$
[b] *p*-value was compared with control group.

medium. It was found that the complete release was achieved immediately.

3.4. Growth suppression of hamster flank organs (pigmented macules)

In this study flutamide solution was used as a positive control while a mixture of propylene glycol and ethanol at a ratio of 80 to 20 was used as a negative control. Although the pharmacological mechanism of action of flutamide is not the same as the test compound Ar, it is nevertheless a valid indicator to be used as a positive control [18,19]. Following the application of the various formulations containing Ar as active principle, the size of the hamster flank organs was gradually reduced or remained in the original size whereas the pigmented macules of the control flank increased in size (Table 1). The percentage of inhibition of the growth of flank organs of hamsters treated with an Ar solution 0.1 mg/20 μl was 29.6% ($p = 0.08$) compared with the control of the untreated flank (Table 2). The percentage of inhibition of the flank organs treated with an Ar solution 0.1 mg/20 μl was not significantly different from that one of flank organs treated with an Ar solution 0.5 mg/20 μl. However, when the dose of Ar was increased to 1.0 mg/20 μl, the size of the flank organs decreased significantly ($p = 0.03$).

ACS microparticles (ca 5 mg) containing 0.1 mg Ar also caused a significant decrease in the growth of the flank organs compared to control ($p = 0.02$) (Table 2). About similar growth inhibition of the flank organs was achieved with topically applied Flutamide in a concentration of 1 mg/20 μl. The potency of ACS-Ar microparticles to inhibit the growth of the flank organs was found to be comparable with that one of Flutamide 1 mg/20 μl ($p = 0.08$). This similar pharmacological action of the microparticles is explained by their ability to deliver Ar specifically to the follicular ducts as suggested by Rolland et al. [7]. Importantly, the dose of Ar administered in the microparticles to exert a similar inhibition of the growth of the flank organs was less than the dose of Ar in the solution form.

The size of the flank organ in the untreated control group is compared to that in the treated group in order to measure the effect of the drug and the untreated contralateral side in each hamster is used as a measure for systemic effects of the drug. The systemic effects shown by the reduction of the size of the hamster flank organs on the untreated hamster flank are shown in Tables 1 and 2. It was found that all formulations exhibited systemic action. However, ACS-Ar microparticles exhibited the lowest systemic action as can be seen from the reduction of size of flank organ (untreated control flank) which was not significantly different from that one of control hamster group ($p = 0.09$, Table 2). From these results it can be concluded that the slow release of Ar from the ACS microparticles (0.70% of the loading dose within 6 h) present in the hair follicular ducts in the hamster flank is sufficient for a local action of the 5α-reductase inhibitor after an application period of 5 weeks to achieve the size reduction of the hamster flank organs to the same extent as the liquid formulations of Ar (1 mg) and Flutamide, however without exerting the same systemic effects.

4. Conclusions

An emulsification technique for the preparation of Ar loaded ACS microparticles was developed resulting in a size range of 3–6 μm and hence suitable for the targeting to the follicular. The maximal loading degree with Ar, a 5α-reductase inhibitor, was 18.7%. 0.7% of the initial loading dose was released in vitro within 6 h. Although the release of Ar from the microparticles could not be measured transfollicularly it seems to be sufficient for a therapeutic effect. As shown in *in vivo* experiment, Ar loaded in ACS microparticles showed the same effect in the reduction of the pigmented macules in comparison to the topical application of flutamide solution (positive control) and to the strongest concentration of an Ar solution after dermal application, however showed the least systemic action. These results suggest that ACS-Ar microparticles may be a promising delivery system for the safe treatment of androgen-dependent disorders such as acne, seborrhea, hirsutism and androgenic alopecia also in humans as the structure of the hair follicles of hamster and rat is not different regarding the uptake mechanisms, i.e. interruption of the stratum corneum around the hair shaft which makes a transfollicular particle uptake possible.

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