



Short communication

## Chitosan nanoparticles for targeting and sustaining minoxidil sulphate delivery to hair follicles



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## ABSTRACT

This work developed minoxidil sulphate-loaded chitosan nanoparticles (MXS-NP) for targeted delivery to hair follicles, which could sustain drug release and improve the topical treatment of alopecia. Chitosan nanoparticles were obtained using low-molecular weight chitosan and tripolyphosphate as crosslink agent. MXS-NP presented a monomodal distribution with hydrodynamic diameter of  $235.5 \pm 99.9$  nm (PDI of  $0.31 \pm 0.01$ ) and positive zeta potential ( $+38.6 \pm 6.0$  mV). SEM analysis confirmed nanoparticles average size and spherical shape. A drug loading efficiency of  $73.0 \pm 0.3\%$  was obtained with polymer:drug ratio of 1:1 (w/w). Drug release through cellulose acetate membranes from MXS-NP was sustained in about 5 times in comparison to the diffusion rate of MXS from the solution ( $188.9 \pm 6.0$   $\mu\text{g}/\text{cm}^2/\text{h}$  and  $35.4 \pm 1.8$   $\mu\text{g}/\text{cm}^2/\text{h}$ ). Drug permeation studies through the skin *in vitro*, followed by selective recovery of MXS from the hair follicles, showed that MXS-NP application resulted in a two-fold MXS increase into hair follicles after 6 h in comparison to the control solution ( $5.9 \pm 0.6$   $\mu\text{g}/\text{cm}^2$  and  $2.9 \pm 0.8$   $\mu\text{g}/\text{cm}^2$ ). MXS-loading in nanoparticles appears as a promising and easy strategy to target and sustain drug delivery to hair follicles, which may improve the topical treatment of alopecia.

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### 1. Introduction

Minoxidil (MX) is the most used drug for the topical treatment of androgenic alopecia, a genetic disease, that is the mainly cause of hair loss in men and women. Although exact mechanism by which MX stimulates hair growth is not yet clearly understood, there are reports suggesting that it extends the anagen phase by activating beta-catenin activity in the hair follicle dermal papilla cells, which represent the main target site for MX [1].

As MX is poorly water soluble, the majority of marketed formulations are solutions with high percentage of ethyl alcohol and/or propylene glycol, which may lead to severe adverse effects following repeated applications (e.g., scalp dryness, irritation, burning, redness, allergic contact dermatitis). To reduce these adverse effects and improve the therapeutic efficiency, current research has been focusing on development of novel non-irritating formulations that could target the active compound to the superficial skin layers, and, ideally, into hair follicles, sustaining drug release, as recently revised [2]. For this, several nano-sized drug delivery systems have been proposed: *i.e.* solid lipid nanoparticles, which were less corrosive than commercial tested formulations [3]; penetration

enhancer-containing vesicles, which promoted drug deposition in the skin [4]; and, more recently, “squarticles,” nanoparticles formed from sebum-derived lipids increased by 7 times the follicular uptake of MX in comparison with control [5]. Nonetheless, further clinical work is needed to establish the administration protocol. Furthermore, for the success of a topical drug delivery system, other parameters than the increment in penetration have to be considered, as stability of the systems and ability to sustain the drug delivery, reducing administration intervals and increasing patient compliance.

Our research group has recently shown that chitosan microparticles loaded with minoxidil sulfate (MXS), an active, hydrophilic metabolite of MX, were able to sustain about three times the release rate of MXS, resulting in a prolonged effect of the microparticles formulation in comparison to conventional MX formulations [6]. However, it has been demonstrated that penetration of nanosystems into hair follicles opening is quite dependent of their diameters and nanoparticles may have higher accumulation than microparticles [7,8]. Chitosan is a natural-sourced, cationic, biocompatible polymer, commonly used as a permeation enhancer. The use of chitosan to prepare a nano-system could result in a non-irritant formulation, which could enhance and target MXS to hair follicle.

In this way, the purpose of this study was to prepare and characterise MXS loaded chitosan nanoparticles (MXS-NP) in a suitable

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**Table 1**  
Chitosan and MXS contents per 20 mL of solution for the preparation of MXS-NP1, MXS-NP2, MXS-NP3 and control nanoparticles (NP Control).

System	Chitosan (mg)	MXS (mg)	Chitosan:MXS proportion (w/w)
MXS-NP1	35.0	35.0	1:1
MXS-NP2	35.0	17.5	2:1
MXS-NP3	35.0	8.75	4:1
NP Control	35.0	0.0	0:0

size range ideal for hair follicle targeting and capable of sustaining drug release.

## 2. Materials and methods

### 2.1. Material

Minoxidil sulphate (99%) was kindly provided by Galena Química e Farmacêutica Ltda. (Campinas, Brazil). Low molecular weight chitosan (75–85% of deacetylation) and sodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich (Steinheim, Germany). Cellulose acetate membranes (MW 12,000–14,000) were purchased from Fisher (Pittsburg, KS, USA). Scotch Book Tape No. 845 (3M, St Paul, MN, USA) was used for tape stripping and cyanoacrylate superglue (Henkel Loctite, Dublin, Ireland) was used for follicles biopsies. The solvents used for extraction and chromatographic analysis were all of HPLC grade: methanol and acetonitrile were purchased from Tedia (Rio de Janeiro, Brazil) and acetic acid was purchased from Sigma–Aldrich (Steinheim, Germany). The water used in all preparations was of Milli-Q grade (Millipore, Illkirch–Graffenstaden, France).

### 2.2. Skin

Porcine ear skin was used in all *in vitro* experiments. The ears were obtained from a local abattoir (Bonasa Alimentos, São Sebastião, Brazil) less than 2 h post-sacrifice of the animal. The whole skin was removed from the outer region of the ear, separated from its underlying layer, and used “full-thickness” to guarantee the intactness of the hair follicles. The skin was stored frozen at  $-20^{\circ}\text{C}$  for a maximum of 1 month before use.

### 2.3. Preparation of chitosan nanoparticles

MXS-NP were prepared by inducing the gelatin of chitosan solutions with a crosslink agent (TPP). Solutions containing determined amounts of chitosan and MXS (Table 1) were obtained by dissolving both components in water acidified with 1% (v/v) of acetic acid (pH 4.7). After 4 h of magnetic stirring (2000 rpm) at  $30^{\circ}\text{C}$ , 2 mL of a TPP aqueous solution (1 mg/mL) was dropped over 5 mL of the chitosan:MXS solution. Nanoparticles formation was visualised by the light turbidity of the initial solution.

### 2.4. Nanoparticles characterisation

The hydrodynamic diameter, polydispersity index (PDI) and the zeta potential were determined using a “Zetasizer Nano ZS” equipment (Malvern, Worcestershire, UK). Direct analysis of 1 mL of nanoparticles aqueous suspension samples were performed for each measurement of size and PDI, whereas 3 mM NaCl was added to nanoparticles samples before the zeta potential measurements.

The nanoparticles morphology and size distribution were also verified in a scanning electron microscope (SEM). Samples of chitosan nanoparticles were coated with gold and taken to the SEM (JEOL, JMS-7000 IF, Tokyo, Japan), which was operated at magnifications from 4000 to 45,000 times.

The loading efficiency was determined assessing indirectly the amount of drug associated to nanoparticles. MXS-NP suspensions were centrifuged at 4000 rpm for 1 h in a “Centri Bio” centrifuge (TDL 80-2B, Belo Horizonte, Brazil). The supernatant was afterwards filtered and analysed following a HPLC validated method. The loading efficiency (LE%) was calculated using Eq. (1):

$$\text{LE\%} = \left[ \frac{(Q_{\text{theoretical}} - Q_{\text{obtained}})}{Q_{\text{theoretical}}} \right] \times 100 \quad (1)$$

where  $Q_{\text{theoretical}}$  is the amount of MXS initially added to prepare the chitosan nanoparticles and  $Q_{\text{obtained}}$  is the quantified amount of MXS.

### 2.5. Release studies

The release rate of MXS from 1 mL of MXS-NP suspension containing the equivalent to 1.25 mg/mL, pH 5.5, was determined *in vitro* using a modified Franz-type diffusion cell (diffusion area =  $1.77\text{ cm}^2$ ) and hydrophilic cellulose membranes over a period of 6 h. The MXS release rate from the formulation was compared to MXS diffusion from an aqueous solution of the drug at the same concentration and pH (control formulation).

### 2.6. Skin permeation

The diffusion cell was assembled with the skin of the porcine's ear separating the donor to the receptor compartment. The receptor compartment was filled with 15 mL of 0.01 M phosphate isotonic buffer, pH 7.4. In the donor compartment it was added 1 mL of (i) MXS-NP formulation, pH 5.5 or (ii) control formulation, pH 5.5. By any means was the donor formulation in direct contact with the receptor solution. The experiments were conducted for 3 h, 6 h, 9 h and 12 h. At the end of each series of experiment, receptor solution was withdrawn from the diffusion cells and analysed for MXS content in HPLC. The skin was removed from the diffusion cell and placed onto a flat surface with the stratum corneum (SC) facing up. The skin was cleaned with a water-soaked gauze pad and tape-stripped 15 times, using Scotch Book tapes. MXS content in the tapes was determined as described below after exhaustive extraction of the drug with methanol over a 24 h period. A drop of cyanoacrylate superglue was applied to the stripped skin area and covered with a further tape-strip using light pressure. After total polymerisation of the glue ( $\sim 5$  min), this tape-strip was then removed and the skin surface biopsy obtained in this way contained follicular casts, from which MXS was extracted with methanol and quantified. The efficiency of recovery of MXS from the SC and the follicular material was determined, with MXS recoveries from SC and follicular material of  $92.4 \pm 6.7\%$  and  $91.6 \pm 4.7\%$ , respectively.

### 2.7. HPLC analysis

The quantification of MXS was performed by reversed-phase HPLC method as follows: 50  $\mu\text{L}$  aliquots of the samples were injected into an LC (model LC-20AD, Shimadzu, Kyoto, Japan). A reverse-phase  $\text{C}_{18}$  column (Shimadzu, 4.6 mm  $\times$  15 mm, 5  $\mu\text{m}$ ) was used. The mobile phase consisted of a mixture of water/acetonitrile (80:20 v/v) and flow rate of 1.0 mL/min. UV detection was performed at 285 nm. This method was validated in accordance to FDA guidelines, and was selective and linear ( $r=0.9997$ ). The intra- and inter-day precision and accuracy of the method showed coefficient of variation (%CV) and relative error (%E) values not greater than 2.8% and 4.4%, respectively.

**Table 2**

Average hydrodynamic size (nm), polydispersity index (PDI), zeta potential (mV), and loading efficiency (%) of control nanoparticles (Control NP) and nanoparticles loaded with MXS (MXS-NP).

System	Average diameter (nm)	PDI	Zeta potential (+mV)	Loading efficiency (%)
MXS-NP1	235.5 ± 99.9	0.310 ± 0.008	38.6 ± 6.0	73.0 ± 0.3
MXS-NP2	271.0 ± 46.1	0.307 ± 0.019	32.3 ± 5.3	28.8 ± 0.2
MXS-NP3	304.7 ± 40.1	0.323 ± 0.042	35.4 ± 5.9	26.2 ± 0.1
Control NP	243.3 ± 77.0	0.295 ± 0.033	47.7 ± 0.6	–

### 2.8. Statistical analysis

The results are presented as means ± standard deviation of at least three replicates. The data were analysed by ANOVA, followed by Tukey test. The statically significance was fixed at  $P < 0.05$ .

## 3. Results and discussion

This work aimed to develop and characterise a nanoparticulated MXS formulation and evaluate its ability to (i) sustain drug delivery, thus prolonging pharmacological effect of MXS, and (ii) target drug delivery to the hair follicles in order to guarantee higher MXS concentrations in hair bulb than in other parts of skin.

### 3.1. Nanoparticles characterisation

Table 2 shows the average size, PDI, zeta potential and loading efficiency of the NP-MXS prepared with three different proportions of chitosan:MXS. The highest loading efficiency was obtained with

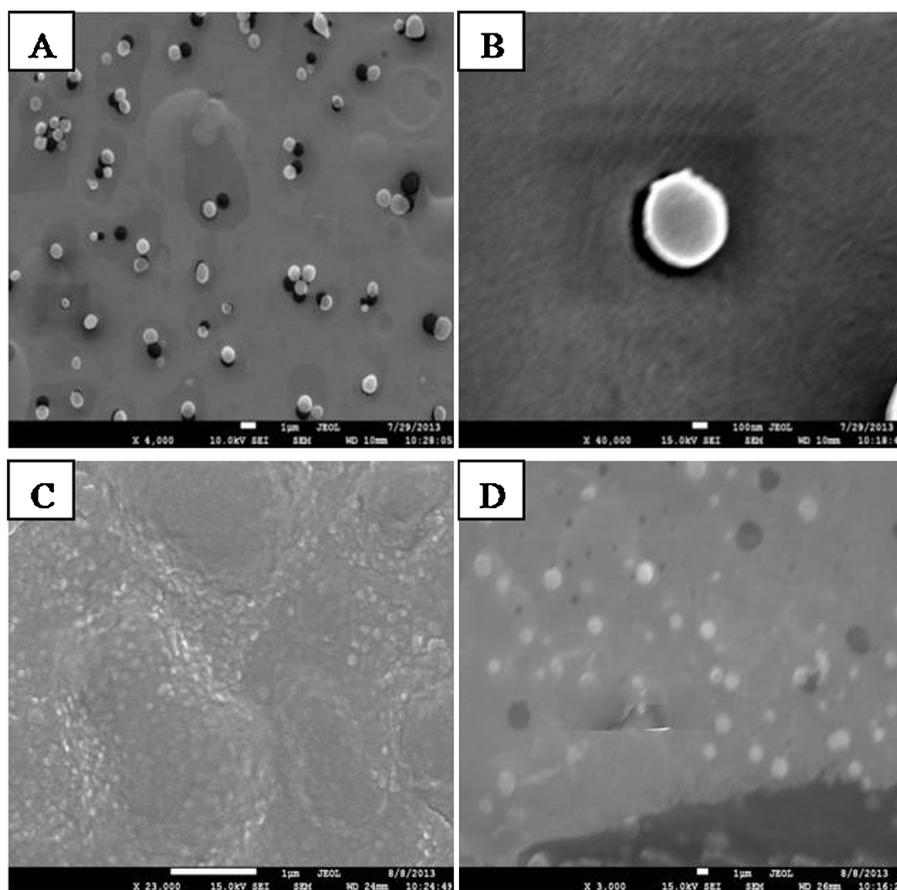
the highest proportion of MXS in relation to chitosan (MXS-NP 1). It may be explained by the fact that MXS is a weak basis ( $pK_a$  near 4.5 [10]). At pHs higher than pH 4.5, most of the molecules are in the non-ionised state, and, therefore, poorly water soluble. When MXS are added to the chitosan solution, before TPP addition, the solution pH is raised to ~5.5. At this pH approximately 90% of the drug is in its non-ionised state and a larger proportion of drug precipitates with the polymer forming nanoparticles. Hence, by increasing the MXS proportion, as with the 1:1 drug:polymer ratio, the loading efficiency is also increased.

The zeta potential values of the nanoparticles were all above +30 mV (Table 2), which ensures the stability of all the suspensions, since this surface charge prevents particles agglomeration. Although addition of MXS to the chitosan nanoparticles decreased zeta potential, no significant difference were observed among the three groups of nanoparticles prepared with different proportions of MXS. Considering that no significant differences were also observed in average size and PDI among the three particles initially prepared, the group that presented higher drug loading efficiency, i.e. MXS-NP1, was chosen for the following studies.

Control NP presented spherical shape, and formed a relatively monodisperse, monomodal population of nanoparticles. The presence of MXS (NP-MXS 1) did not result in significant alteration of particles morphology or size (Fig. 1).

### 3.2. Release studies

From the results (Fig. 2), it is evident that MXS-NP were capable of controlling drug release in comparison with control solution (release flux of  $35.4 \pm 1.8$  and  $188.9 \pm 6.0$   $\mu\text{g}/\text{cm}^2/\text{h}$ , respectively).



**Fig. 1.** Electron micrographs obtained by SEM for the morphological and size analysis of Control NP (A: 4000 $\times$ , B: 40,000 $\times$ ) and MXS-NP1 (C: 4000 $\times$ , D: 23,000 $\times$ ).

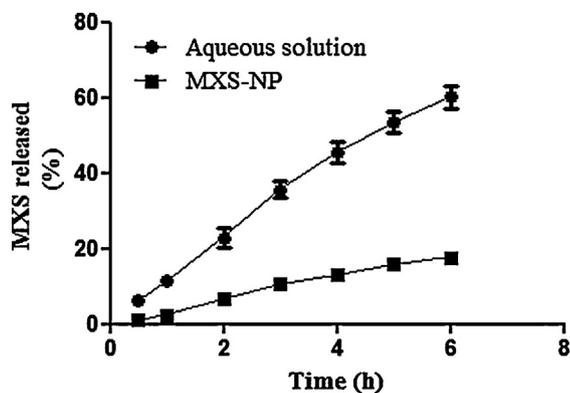


Fig. 2. Release profile of the MXS from the MXS-NP formulation compared to an aqueous solution of the drug (control formulation), both containing 1.25 mg/mL of the drug and pH 5.5.

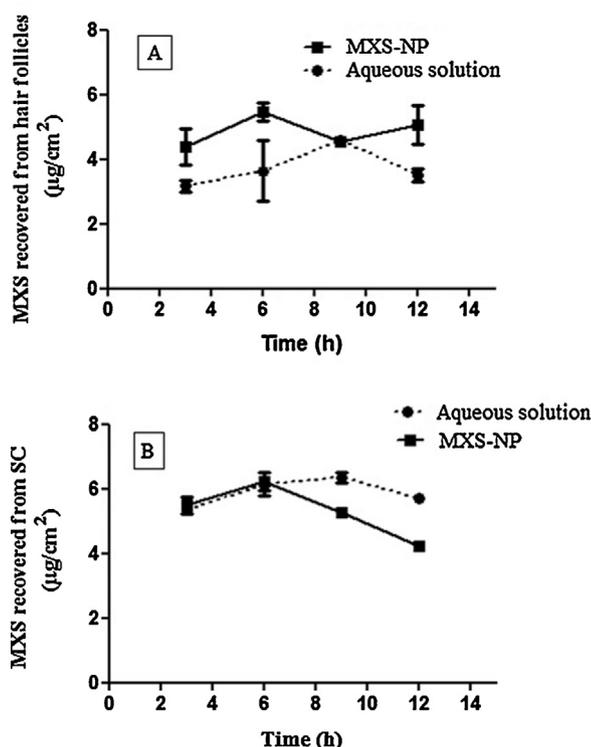


Fig. 3. Time-course of MXS recovered from (A) hair follicles and (B) SC after 3 h, 6 h, 9 h and 12 h of topical application of MXS-NP formulation in comparison to the control formulation, both containing 1.25 mg/mL MXS, at pH 5.5.

In our previous study [6], MXS-loaded chitosan microparticles of about 3 µm, *i.e.* 10 times bigger than the MXS-NP prepared in this work, were able to sustain in about 3 times the drug release. Considering that there are not any difference in composition among previously prepared microparticles and these new systems, it seems that reduction of particle size could result in a more pronounced controlling release effect.

### 3.3. MXS skin penetration

Fig. 3 shows the time-course of the *in vitro* MXS penetration in the hair follicles (Fig. 3A) and in SC (Fig. 3B) from MXS-NP in comparison to the control formulation.

After MXS-NP application on the skin, a maximum MXS accumulation of 5.9 µg/cm<sup>2</sup> into the hair follicles was achieved at 6 h of experiment. Also, MXS-NP were able to maintain, from 3 h to 12 h

of experiment, a minimum of 4.5 µg/cm<sup>2</sup> MXS in the follicles. In all time points analysed, MXS accumulation into the follicles from MXS-NP was significantly higher when comparing with the control solution, except at the 9th hour. These experiments showed for the first time that chitosan nanoparticles were able to maintain for at least 12 h high concentrations of MXS inside the hair follicle opening when compared to free drug control solution, which could mean higher efficacy in alopecia treatment, reducing the number of reapplications needed.

MX delivery has been evaluated from finite doses of ethanol solutions containing 2% of MX finding recovery rates of about 3 µg/cm<sup>2</sup> in the hair follicles after 30 min of experiment and lower MX concentrations after 5 h [9]. Similar drug amounts were found evaluating the follicular delivery of MXS from a Poloxamer<sup>®</sup> gel formulation, which recovered 2.8 ± 0.3 µg/cm<sup>2</sup> of MXS from these structures after 6 h of experiment [10]. MXS-NP formulation developed in this work, containing a 10 times lower concentration of MXS, revealed superior results in terms of follicular accumulation of MXS and also provided a prolonged release of the drugs into hair follicles.

This follicular target effect provided by the topically applied MXS-NP could be explained by the deposition of the nanoparticles in the inner part of the hair follicles.

It has been described that cuticle thickness of hair obtained from porcine skin is 320 nm and, therefore, it is speculated that an average particle size of 300 nm would have a facility to penetrate into hair follicles [11]. In fact, the quantitative results in terms of MXS follicular penetration from our 235 nm-chitosan nanoparticles show that they seem to penetrate the skin *via* hair follicles and target the release of MXS to these structures.

Fig. 3B shows that MXS penetration in SC from the MXS-NP is similar to that from the control formulation until the 6th hour of experiment. These results, associated with the fact that the drug was not detected in the receptor solution in quantifiable concentrations, corroborates the hypothesis that MXS-NP might release the MXS in a targeted way to the hair follicles, preventing the MXS permeation through the skin towards the dermis.

## 4. Conclusion

The MXS-NP formulation prepared with a proportion of chitosan/MXS equal to 1:1 w/w seems to be a promising strategy for the topical treatment of alopecia. MXS-NP were able to accumulate into the hair follicles and sustain drug release about twice more than previously prepared MXS-loaded microparticles [6], maintaining therapeutic relevant MXS concentrations in this appendages for over 12 h.

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