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Polymeric Chemistry

Metronidazole-Loaded Pseudo-Protein Microspheres for Intravaginal Drug Delivery: Evaluation of Drug Encapsulation Efficiency and Drug Release

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Intravaginal drug delivery for treating various diseases related to the female reproductive system still is a challenge in gynecology. One promising way of overcoming this problem is to use the special micronized biodegradable drug carriers, which can safely deliver drugs directly to the locus of disease and can maintain a proper therapeutic concentration of drug for a prolonged time. The purpose of the present study is to elaborate a novel formulation – gelling suspension of drug (metronidazole, MET) loaded pseudo-protein microspheres (MSs), and to study the MET encapsulation and the kinetics of its release from the MSs. The gelling suspension of MET-loaded pseudo-protein MSs (MET-MSs) was successfully obtained using the W/O/W double emulsion-solvent evaporation method. MET incorporation into the MSs was studied using the UV-spectrophotometric method. The drug release kinetics was studied using the dialysis method under sink conditions in PBS at 37°C. To make the suspension gelling at body temperature, Poloxamer 407 was dissolved in it at a 20% w/v concentration. The gelling suspension of MET-MSs was prepared and characterized. The size range of the MET-MSs was from 0.44 μm to 2.27 μm with the average diameter of 1.17 μm. The encapsulation efficiency (EE%) of MET into the pseudo-protein MSs was 18.2%. The release kinetics of MET from the MET-MSs showed a biphasic release pattern - an initial burst release and a much more continuous slow release. The size characteristics of the MET-MSs were suitable for intravaginal (topical) drug administration. The drug MET was successfully encapsulated into the pseudo-protein MSs with an average efficiency of 18.2%. The kinetics of MET release from the MET-MSs was typical of biodegradable micronized particles with a biphasic release pattern. The obtained gelling suspension of MET-MSs is promising as an intravaginal drug delivery formulation. *© 2021 Bull. Georg. Natl. Acad. Sci.*

Pseudo-protein, microspheres, metronidazole, drug loading, drug release

The development of novel products for female health represents an important issue for modern gynecology. Since intravaginal drug delivery remains a challenge for certain drugs, the elaboration of high-performance intravaginal drug delivery systems is still topical [1]. Despite the progress of current medicine, pathologies of cervix remain one of the important problems of

gynecology, which is diagnosed in 15% of the reproductive aged women and represents a risk factor for precancerous diseases and cervix cancer [1]. In the case of local treatment, it is challenging to design delivery systems providing high drug concentrations in the vagina for a prolonged period, whereas in the case of systemic treatment the major challenge is to gain high drug bioavailability [2].

One promising way of overcoming the problems related to the intravaginal drug delivery is to use special micronized biodegradable drug carriers which can safely deliver drugs directly to the locus of disease (vagina, cervix) and can maintain a proper therapeutic concentration of drug for a prolonged time. The main goal of this research was the elaboration of novel and effective micronized intravaginal drug carriers on the basis of pseudo-proteins – poly(ester amide)s (PEAs), and the investigation of drug incorporation/drug release processes. As a polymer matrix for preparing intravaginal drug carriers – microspheres (MSs) we have chosen a relatively new class of amino acid based biodegradable polymers - pseudo-proteins [3, 4]. Namely, we have selected the pseudo-protein labeled as 8L6 which is composed of sebacic acid (8), L-leucine (L), and 1,6-hexanediol (**6**). We have found this polymer as an optimal for fabricating stable MSs previously [5]. As a drug for incorporating in the pseudo-protein MSs, we selected metronidazole (MET) – an antibiotic and antiprotozoal agent, which is frequently used to treat cervicitis and pelvic inflammatory disease.

Materials and Methods

Materials. The surfactants poly (vinyl alcohol) (PVA) (MW 84-89 kDa of 86.7%–88.7% hydrolyzed) and Poloxamer 407 (PEG-PPG-PEG triblock copolymer, MW≈12.5 kDa), the organic solvent dichloromethane (DCM), and the drug metronidazole (MET) (2-Methyl-5-nitroimidazole-1-ethanol) were purchased from Sigma-Aldrich (*Saint Louis*, MO, USA). All the chemicals were used as received. The dialysis bag (MWCO 25 kDa) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). The pseudo-protein 8L6, selected for the present study, was originally synthesized *via* the interfacial polycondensation as reported previously [3, 4].

Preparation of MET-loaded MSs. MET-loaded 8L6 MSs (MET-MSs) were prepared according to the water-in-oil-in-water (W/O/W) double emulsion-solvent evaporation method [6]. Following this method we prepared the organic and inorganic (aqueous) phases separately. Briefly, the organic phase was prepared by adding 8 g of the polymer 8L6 to 160 mL of DCM). For preparing the aqueous phase 4 g (1% w/v) of PVA and 4 g (1% w/v) of MET were dissolved in 400 mL of distilled water. The primary "water-in-oil" (W/O) emulsion was prepared by adding 15 mL of the aqueous phase into the organic phase (160 mL) and by homogenizing for 30 sec at 10,000 rpm using a high-speed homogenizer (High shear strength disperse homogenizing machine C25, Shanghai HC Mechanical Equipment Co., LTD, Shanghai, China). Then, the primary emulsion was immediately added to 385 mL of aqueous phase and was homogenized for 3 min at 16,000 rpm. The obtained "water-in-oil-in-water" (W/O/W) emulsion was transferred to a magnetic stirrer and was stirred for 24 h at 400 rpm to evaporate DCM. After complete evaporation of DCM, 400 mL of milky suspension of MET-MSs was obtained. In order to make the suspension gelling at body temperature (36-37°C), 80 g (20% w/v) of Poloxamer 407 was dissolved in 400 mL of the prepared suspension under low temperature using an ice bath (Poloxamer 407 has the negative temperature coefficient and dissolved in water at 5-10°C. The experiment was repeated for preparing three independent batches.

MSs' size, size distribution and morphology. The obtained MET-MSs were characterized by mean

diameter, lower limit diameter, upper limit diameter and particle size distribution, which were determined using a laser particle analyzer (Laser particle sizer LS-C(III); Omec Instruments Co., LTD, Guangdong, China). Particle size distribution (R) was determined using the following ratio:

$$R=\frac{\text{Upper limit diameter-Lower limit diameter}}{\text{Mean diameter}}$$

The R>1.5 corresponds to a wide particle distribution, and R<1.5 to a narrow particle distribution. The sizes and particle size distribution characteristics are presented as an average of 5 measurements±standard deviation (SD). The morphological examination of the MSs was performed using the XSG series biological microscope.

Determination of drug incorporation. MET incorporation into the 8L6 MSs was studied using a spectrophotometric method as reported elsewhere [7]. Two main characteristics of drug incorporation process–encapsulation efficiency in percentage (EE%) and actual drug loading in percentage (DL%) were determined using the following procedure: freshly prepared suspensions of MET-MSs were centrifuged at 6,000rpm for 30min using LCEN-101 clinical centrifuge. Then, the obtained supernatant was transferred to the pure tube and the concentration of MET was determined by measuring the absorbance of the solution at λ=325nm and comparing it with a calibration curve. The experiments were performed in triplicate and the results were indicated as the mean±SD. EE% and DL% were calculated using the following equations:

$$EE\%=\frac{\text{Weight of drug encapsulated into the MSs}}{\text{Weight of drug initially added in solvent}}\text{x}100\%$$

$$DL\%=\frac{\text{Weight of drug encapsulated into the MSs}}{\text{Weight of MSs containing drug}}\text{x}100\%$$

In vitro drug release study. The in vitro release behavior of MET from the MET-MSs was analyzed under sink conditions using the dialysis method as described by Krishnan et al. [8]. Briefly, 10 mL of freshly prepared and centrifuged suspension (free of supernatant) of MET-MSs containing about 18.2mg MET was loaded into dialysis bag with MWCO 25kDa. The bag was subsequently immersed in the release media (200mL phosphate-buffered saline, PBS with pH=7.0) under gentle stirring (300rpm) at 37°C. At predetermined time points, 4mL of the release media was extracted and analyzed using UV-spectrophotometer to determine the amount of the released drug. Afterwards, 4mL of the analyzed release media was returned to the experimental release media to maintain constant volume. The experiments were performed in triplicate and the results were indicated as the mean±SD. Cumulative release profile was calculated by dividing the amount of drug released in one specific measurement time by the total mass initially loaded (i.e. 18.2mg).

Results and Discussion

Selection of a pseudo-protein. Pseudo-protein 8L6 composed of L-leucine (L), 1,6-hexanediol (**6**), and sebacic acid (**8**) was selected as a basic matrix for preparing MSs. In our previous reports [5, 9] we found this polymer as an optimal for fabricating resorbable MSs in terms of size characteristics, stability upon storage and cell compatibility. Another reason for selecting 8L6 as a basic polymer was the simplicity of its synthesis via the interfacial polycondensation and the cheapness and availability of the building blocks required for its synthesis. The structure of the selected pseudo-protein is given in Fig. 1.

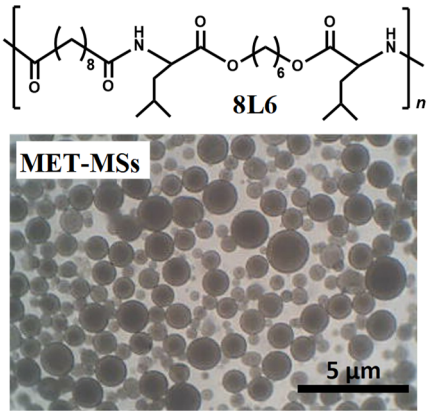

Fig. 1. The structure of the pseudo-protein 8L6 and the microscope image of MET-MSs (Magnification 200X).

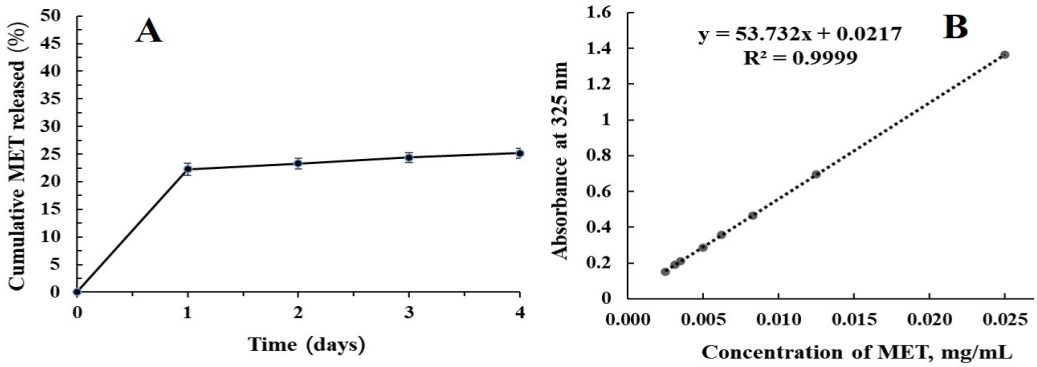

Fig. 2. In vitro release profiles for MET from the MET-MSs in PBS at 37°C (**A**) and the calibration curve of MET (**B**).
Note: Data are presented as averages of three independent experiments ± SD.

Preparation of MET-MSs, their characteristics and morphology. The MET-MSs were prepared using the relatively simple and cost-effective W/O/W double emulsion – solvent evaporation method. As an organic solvent we chose DCM, since it is easily evaporable and less toxic than chloroform which is frequently used as a volatile organic solvent for preparing MSs by the said method. It should be noted that the drug MET was included in the aqueous phase at its highest available concentration (10 mg/mL) to reach the maximum incorporation to the MSs. It should also be noted that the incorporation of drug into the MSs (microencapsulation) occurred during the MSs' formation process (and not after formation of MSs). As it was described in the Materials and Methods at the last step of preparing the formulation the surfactant Poloxamer 407 was dissolved in the MSs' suspension (at a concentration 20% w/v) upon cooling in order to make it gelling at body temperature. As a result, we obtained the formulation of MET-MSs, which is liquid at low temperature (refrigerated) and gelled at room temperature and above. We designed the MET-MSs as a gelling suspension in order to ease delivering and fixing the formulation in, and to prevent leaking of the formulation from the locus of disease (vagina).

The measurements performed by laser particle sizer showed that the mean diameter of MET-MSs is 1.17±0.10 µm, the upper limit diameter is 2.27±0.48 µm, and the lower limit diameter is 0.44±0.06 µm (Table). The particle size distribution in the MET-MSs' suspension was wide

(R=1.56). Morphological examination of the MET-MSs showed that the particles have spherical shape (Fig. 1). The microscope images also reflect the wide particle size distribution in the suspension.

Table. The main size and drug incorporation characteristics of MET-MSs

Batch #	Average diameter (μm)±SD	Lower limit diameter (μm)±SD	Upper limit diameter (μm)±SD	Particle size distribution (R)	EE%	DL%
1	1.35	0.47	2.32	wide (1.64)	17.9	7.8
2	1.02	0.44	2.26	wide (1.52)	20.1	9.0
3	1.16	0.41	2.25	wide (1.53)	16.5	7.4
Averages ±SD	**1.17 ± 0.10**	**0.44 ± 0.06**	**2.27 ± 0.48**	**wide (1.56±0.1)**	**18.2 ± 2.1**	**8.1 ± 0.8**
Note: in the last row the data are presented as the averages of three independent experiments ± SD.						

Drug incorporation and in vitro drug release studies. As noted above, in order to reach maximum drug loading, MET was dissolved in the aqueous phase at its highest concentration (10 mg/mL). The results of drug incorporation studies showed that MET was successfully entrapped in 8L6MSs with EE% and DL% of 18.2±2.1% and 8.1±0.8%, respectively (Table 1). In vitro release of MET from the MET-MSs was evaluated by incubating the samples in PBS (pH=7.0) under sink conditions at 37°C for 4 days. Results of drug release study are given in Fig. 2. The in vitro release profiles for MET from MET-MSs showed a biphasic release pattern, viz an initial burst release and a much more continuous slow release. The initial burst release showed that 22.3±1.1% of the initially accumulated MET was released from the MET-MSs at the first 24 hours. From day 2 to day 4, MET release profile has a sustained and slow character. Thus, during 3 days only 2.9% of the accumulated DEX was released with an average rate of 1.0% per day. During the whole experimental period (4 days) only 25.2±0.9% of the initially accumulated MET was released. The initial burst release is normally attributable to release the drug that is adsorbed or close to the surface of the MSs permeable to water [10]. In the second stage, the release of MET from MET-MSs was sustained and slow due to the relatively low rate of degradation of the pseudo-protein 8L6 by hydrolysis (because of having CO-NH amide groups in the backbone apart from the easily hydrolysable ester bonds).

Preparation of gelling formulation. Poloxamer 407 was added to the suspension with the purpose of gelling it at 36-37°C in order to facilitate its delivery and fixing in and preventing the leak of the formulation from the locus of disease (vagina).

Conclusion

The suspension of MET-loaded pseudo-protein MSs was successfully obtained using W/O/W double emulsion-solvent evaporation method. The size characteristics of the MET-MSs were suitable for intravaginal (topical) drug administration since the size range was from 0.44 μm to 2.27 μm with the average diameter of 1.17 μm. The drug MET was successfully encapsulated into the pseudo-protein MSs with the EE% of 18.2%. The release kinetic of MET from the MET-MSs showed a biphasic release pattern - an initial burst release and a much more continuous slow release. The suspension rendered gelling by dissolving Poloxamer 407 at a concentration of 20% w/v. The obtained gelling suspension of MET-MSs is promising as an intravaginal drug delivery formulation.

პოლიმერული ქიმია

მეტრონიდაზოლით დატვირთული ფსევდოპროტეინული მიკროსფეროები წამლის ინტრავაგინალური მიწოდებისათვის: წამლის ინკაფსულირების ეფექტურობისა და წამლის გამოთავისუფლების შესწავლა

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წამლის ინტრავაგინალური მიწოდება, რომელიც მნიშვნელოვანია ქალთა რეპროდუქციული სისტემის მრავალი დაავადების მკურნალობისათვის, დღესდღეობით რჩება გინეკოლოგიის ერთ-ერთ ცენტრალურ პრობლემად. აღნიშნული პრობლემის გადაჭრის იმედისმომცემი გზაა მიკრონიზებული ბიოდეგრადირებადი წამლის მატარებლების გამოყენება, რომლებსაც შესწევთ უნარი მიიტანონ წამალი უშუალოდ დაავადების კერაში და შეინარჩუნონ წამლის თერაპიული კონცენტრაცია ხანგრძლივი დროის განმავლობაში. წინამდებარე ნაშრომის მთავარი მიზანი იყო ახალი პრეპარატით (მეტრონიდაზოლი) დატვირთული ფსევდოპროტეინული მიკროსფეროების (მს) ჟელირებადი სუსპენზიის მიღება და წამლის ინკაფსულირებისა და მს-დან წამლის გამოთავისუფლების პროცესების შესწავლა. მეტრონიდაზოლით დატვირთული ფსევდოპროტეინული მს-ის ჟელირებადი სუსპენზია მიღებულ იქნა ე.წ. წყალი/ცხიმი/წყალი ორმაგი ემულსიის-გამხსნელის აორთქლების მეთოდით (water-in-oil-in-water double emulsion-solvent evaporation method). მს-ებში წამლის ინკაფსულირების ეფექტურობა შევისწავლეთ სპექტროფოტომეტრული მეთოდით. მს-დან წამლის გამოთავისუფლების კინეტიკა შესწავლილ იქნა დიალიზის მეთოდით ფოსფატურ ბუფერში 37°C-ზე. იმისათვის, რომ სუსპენზია გაგვეხადა ჟელირებადი სხეულის ტემპერატურაზე, მასში გახსნილ იქნა 20% Poloxamer 407. მიღებული და დახასიათებულია მეტრონიდაზოლით დატვირთული ფსევდოპროტეინული მს-ის ჟელირებადი სუსპენზია. მს-ის ზომები მერყეობდა ზღვრებში 0,44–2,27მკმ, საშუალო დიამეტრი კი შეადგენდა 1,17მკმ. მს-ებში მეტრონიდაზოლის ინკაფსულირების (ჩართვის) ეფექტურობა შეადგენდა 18,2%-ს. მს-დან წამლის გამოთავისუფლების სიჩქარე ხასიათდებოდა ორფაზური კინეტიკით-საწყისი „მყისიერი“ გამოთავისუფლებით და შემდგომი ნელი და გახანგრძლივებული გამოთავისუფლებით. მეტრონიდაზოლით დატვირთული ფსევდოპროტეინული მს, მათი პარამეტრებიდან (ზომა, ნაწილაკების ზომების განაწილება) გამომდინარე, ოპტიმალურია როგორც ინტრავაგინალური წამლის მიმწოდებელი სისტემები. წამალი მეტრონიდაზოლი წარმატებით იქნა ინკაფსულირებული ფსევდოპროტეინულ მს-ში

საშუალო ეფექტურობით 18,2%. მს-დან წამლის გამოთავისუფლების კინეტიკა ხასიათდებოდა ბიოდეგრადირებადი მიკრონიზებული ნაწილაკებისთვის დამახასიათებელი ორფაზური მრუდით. მიღებული წამლით დატვირთული მს-ის ჟელირებადი სუსპენზია პერსპექტიულია, როგორც ინტრავაგინალური მიწოდების ახალი პრეპარატი.

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