Research Article

การศึกษาปัจจัยเบื้องต้นสำหรับเตรียมระบบนำส่งยาเมทโทเทรกเสท ด้วยรีเวิร์สไมเซลล์สำหรับการรักษาโรคสะเก็ดเงิน

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บทคัดย่อ

การศึกษาปัจจัยเบื้องต้นสำหรับเตรียมระบบนำส่งยาเมทโทเทรกเสทด้วยรีเวิร์สไมเซลล์สำหรับการรักษาโรคสะเก็ดเงิน
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ยาเมทโทรเทรกเสท (MTX) เป็นยาตัวหนึ่งที่ใช้ในการรักษาโรคสะเก็ดเงินขั้นรุนแรง แต่ผลข้างเคียงของยาที่ไม่พึงประสงค์ เช่น เป็นพิษต่อตับ และกดการทำงานของไขกระดูกยังเป็นข้อจำกัดในการใช้ยา ดังนั้นเพื่อหลีกเลี่ยงผลข้างเคียงที่ไม่พึงประสงค์ ระบบนำส่งยา MTX เฉพาะที่ทางผิวหนังด้วยรีเวิร์สไมเซลล์ที่ประกอบด้วยสารลดแรงตึงผิว sodium bis (ethyl hexyl) sulfosuccinate (AOT), ตัวทำละลายอินทรีย์เฮกเซน (Hexane), ตัวเชื่อมโยงโมเลกุลกลูตารัลดีไฮด์ (GTA) และไคโตซาน (CS) เป็นเปลือกหุ้ม **วิธีดำเนินการวิจัย:** ในการศึกษานี้มีจุดประสงค์เพื่อศึกษาผลความแตกต่างของน้ำหนักโมเลกุลของไคโตซาน ที่มีต่อขนาดอนุภาคเฉลี่ย ค่าประจุที่ผิวสัมผัส และประสิทธิภาพในการกักเก็บ MTX (%EE) โดยศึกษาคุณสมบัติทางกายภาพของรีเวิร์สไมเซลล์โดยเทคนิคการกระเจิงแสงและ UV-Vis spectrophotometry **ผลการวิจัย:** ขนาดอนุภาคเฉลี่ยที่เตรียมจากน้ำหนักโมเลกุลของไคโตซานที่แตกต่างกันจะอยู่ในช่วงประมาณ 400 ถึง 900 นาโนเมตร ค่าประจุที่ผิวสัมผัสของอนุภาคประมาณ -60 ถึง -30 mV และร้อยละประสิทธิภาพในการกักเก็บยา MTX ประมาณร้อยละ 90 ในการศึกษานี้พบว่าปัจจัยหลักที่มีผลต่อขนาดอนุภาคเฉลี่ยคือ เมื่อน้ำหนักโมเลกุลของไคโตซานเพิ่มขึ้น ขนาดอนุภาคเฉลี่ยก็จะมีค่าเพิ่มขึ้น แต่ค่า %EE ของรีเวิร์สไมเซลล์ที่เตรียมจากไคโตซานที่น้ำหนักโมเลกุลต่ำมีค่าไม่แตกต่างกัน **สรุปผลการวิจัย:** ค่าร้อยละประสิทธิภาพการกักเก็บยา MTX ที่สูงของอนุภาครีเวิร์สไมเซลล์นั้นสามารถนำไปพัฒนาเป็นตัวนำส่งยาที่มีประสิทธิภาพทางผิวหนัง เพื่อช่วยลดผลข้างเคียงของ MTX และปรับปรุงประสิทธิภาพในการรักษาโรคสะเก็ดเงินได้

คำสำคัญ: รีเวิร์สไมเซลล์, เมโทรเทรกเสท, ไคโตซาน, โรคสะเก็ดเงิน, ระบบนำส่งยาทางผิวหนัง

The Preliminary Study of Methotrexate Encapsulated Reverse Micelles preparation for Psoriasis Treatment

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Abstract

The Preliminary Study of Methotrexate Encapsulated Reverse Micelles preparation for Psoriasis Treatment

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Methotrexate (MTX) is commonly used in severe psoriasis treatment but major side effects such as hepatoxicity and bone marrow suppression are mainly limited its use. To avoid these problems, localized topical delivery has been initiated to prepare using a reverse micelle system (RMs) that consisted of sodium bis (ethyl hexyl) sulfosuccinate (AOT) as a surfactant, n-hexane as a solvent, glutaraldehyde (GTA) as a crosslinking agent, and chitosan (CS) as core-shell. **Methods:** The aim of the study is to investigate the effect of different molecular weights (MWs) of CS on the physical properties; mean diameter, zeta potential, and MTX encapsulation efficiency (%EE). All RMs formulations were physically characterized by dynamic light scattering (DLS) and UV-Vis spectroscopy. **Results:** Results reveal that the mean diameter of the prepared RMs with different MWs of CS was in averaged range of 400-900 nm. The zeta potential and %EE were approximately -60 to -30 mV and 90%, respectively. In view of the experiment, the MWs of CS is a major parameter affecting to the mean diameter of RMs, but at low molecular weight of chitosan in reverse micelles preparation showed no difference in %EE. **Conclusion:** High encapsulation of MTX in RMs can suggest that further develop to be a potential carrier in topical delivery system and MTX loaded RMs for topical delivery might reduce its side effects and improves its therapeutic efficacy in the treatment of psoriasis.

Keywords: reverse micelles; methotrexate; chitosan; psoriasis; topical drug delivery systems.

Introduction

Psoriasis is a chronic, potentially psychological and physical immune-mediated inflammatory disease of the skin with a prevalence of 1.5-2.0 % in Thai population (Institute of Dermatology, Thailand). To date, its etiology evidence suggests that unregulated cutaneous immune response, characterized by tumor necrosis factor- alfa (TNF- α) dependence and exaggerated helper T cell 1 (T_H1) and T- helper 17 (T_H17) activation, occurs in genetically susceptible individuals (Elder *et al.*, 2010). Approximately 25% of individual psoriatic patients develop progressively with painful and arthritis (Tsoi *et al.*, 2012). Moreover, their quality of life (QOL) are also decreased. Typically, the lesion of psoriasis is a plaque of red and silvery scales covering the top layer of upper skin or joints. Additionally, the diagnosis consists of excess keratinocyte proliferation with abnormal differentiation, and inflammation in the epidermis and dermis (Linden and Weinstein,1999, Bayliffe *et al.*, 2004). The approval medical treatments for the disease include cyclosporine, methotrexate (MTX), acitretin, ultraviolet B (UVB), and ultraviolet A with psoralen (PUVA). Methotrexate (MTX), a well- known antifolate drug, is specifically applied for treating severe psoriasis with more than 20% of affected lesion or unresponsive to other treatments. The administration of MTX is generally given by oral, parenteral, and injection routes (Frank, 2004). However, MTX is not recommended for regular use for a long term because of their serious side effects in many organs such as bone marrow suppression, hepatic fibrosis, and cirrhosis (Lebwohl and Ali, 2001). To expect for high clinical efficacy and minimize the number of those serious side effects, the topical administration of MTX is preferably route. (Srisuk et al, 2012). Due to topical MTX delivering to the psoriasis lesions, different methods and preparations have been introduced. Wong *et al.*, (2005) reported the topical delivery of MTX by electroporation and its relative total MTX penetration profile was found 50% in the epidermis and dermis (Wong *et al.*, 2005). Another studied on electroporation in combination with the pretreated with low-fluence erbium: yttrium-aluminum-garnet (Er:YAG) laser showed the enhancement of MTX permeation through the psoriatic lesion significantly (Lee *et al.*, 2008). The efficacy and tolerability of 1% topical MTX revealed no severe side effects while histopathology study was improved significantly when compared to the placebo group (Eskicirak *et al.*, 2006). The synthesis of nanogel based on co- polymerized N- isopropylacrylamide (NIPAM) and butylacrylate (BA) loaded with MTX and aqueous sodium carbonate (Na_2CO_3) were characterized. The obtained results demonstrated that the penetration was increased by inducing de- swelling, temperature system changes, and expulsion mechanism (Singka *et al.*, 2010). However, the structure of human skin, stratum corneum (SC) is the main limitation as well as a barrier of the skin function for delivering MTX, and the diffusion rate through SC have been considered as the rate limiting factor (Kumar *et al.*, 2004). To circumvent from this limited permeability of MTX through SC, the series of various controlled release carriers and nanoparticulate systems have been extensively demonstrated. Particularly, liposomes and micelles play a pivotal role as drug carriers for localized topical delivering biomolecules or drugs in different purposes (Müller- Goymann, 2004). Deformable liposomes which containing dipotassium glycyrrhizinate (KG) showed 3 to 4 fold higher amount of MTX through pig skin than conventional liposomes (Trotta *et al.*, 2004). The skin permeability of MTX encapsulated oleic acid containing deformable liposomes was higher concentration and flux accumulated in the epidermis and dermis layers of porcine skin (Srisuk *et al.*, 2012). Even though liposomes can be concluded that they are able to enhance the drug skin permeability, but the high cost of phospholipids and their liposomes instability are disadvantageous. Reverse micelles (RMs) are alternate promising drug carriers with low cost and ease of synthesis for delivering bioactive molecules. The dispersion of RMs is spherical aggregates of surfactant molecules that solubilized in the organic solvent. The shape of RMs typically form aggregates with the single tail of

hydrophobic portions in the contact with head of hydrophilic portions and sequestering in the center of the micellar system (Kafshgari *et al.*, 2012). They have much attention to delivering therapeutic MTX because they have all the advantages over liposomes. RMs have a long shelf life and higher drug entrapment. Besides, RMs may possess their versatility in terms of thermodynamically stability, inverse structure, hydrophilic core, and flexibility (Kreilgaard, 2002, Jones *et al.*, 2008, Onoue *et al.*, 2014). Therefore, the preliminary study of MTX encapsulated RMs formulations is necessary to develop with the increasing hydrophilic drug affinity (Tran Nguyen *et al.*, 2015). Chitosan (CS) is a positively charged, biodegradable, biocompatible, nontoxic, and mucoadhesive biopolymer (Sonia and Sharma, 2011). CS has a special role in many applications in the targeted delivery system including their availability of free amine groups for cross-linking with a variety of biocompatible polyanionic substances (ST Lim, 2001).

Over the past decades, for drug-based nanoparticles, many methods and different applications have been developed. However, a very little research for MTX approaches to localized topical treatment in psoriasis conditions were carried out. Instead of using liposome preparations, CS-RMs might have been developed as potential candidate for enhancing MTX permeability across through targeted skin. The main purpose of this study is to prepare chitosan based on reverse micelles and investigate the effect of the variation of chitosan molecular weights on MTX encapsulated in the RMs system, and subsequently evaluated physical characterization, and MTX encapsulation efficiency.

Materials and methods

Materials

Hydrophilic methotrexate (MTX) and sodium bis (ethyl hexyl) sulfosuccinate (AOT) were purchased from Sigma-Aldrich Co., Ltd., Singapore. Low molecular weight chitosan (MWs ~ 50,000-190,000, DD ~ 75%), medium molecular weight chitosan (MWs~190,000-300,000, DD~75-85%) and high molecular weight chitosan (MWs~310,000-375,000 DD~80%) were purchased from Sigma-Aldrich Co., Ltd., Singapore. Other chemicals were of analytical grade unless where otherwise stated.

Preparation of Reverse micelles (RMs)

In this study, the specific procedure of reverse micelles (RMs) preparation was modified from elsewhere (Banerjee *et al.*, 2002) and depicted in **Figure 1**. The addition of surfactant, 0.3 M to 1.2 M of sodium bis (ethyl hexyl) sulfosuccinate (AOT), to n-hexane (0.015 to 0.06 M) was a spontaneous reaction to form RMs. The dispersion of 20 mL of AOT in n-hexane, 100 µL of 0.1% chitosan (CS) (low, middle, and high molecular weight) in 1.0 % glacial acetic acid containing various amounts of MTX solution (0.50, 0.75, and 1.00 %w/v) were vigorously mixed under constant stirring in order to avoid the turbidity. A cross-linking agent, 10 µL of 0.01 % glutaraldehyde (GTA) and 10 µL of liquor ammonium were added into the dispersion and continuously stirring at room temperature for 6 h until the dispersion became homogeneous and optically transparent. The organic solvent (n-hexane) was then evaporated to obtain a thin film by rotary evaporator. Subsequently, this thin film was resuspended in 5 mL of phosphate buffer solution (pH 7.4) under sonication at 45°C for approximately 2 h. One milliliter of $CaCl_2$ solution (0.3, 3, 10, 20, and 30 %w/v) was dropwise to precipitate the excess surfactant out of calcium salts. In this step, the dispersion without MTX was centrifuged at $6x10^3$ rpm, at 4°C for 40 min but the determination of the percentage of MTX encapsulation efficiency (EE %), was centrifuged at $12x10^3$ rpm, 4°C for 1 h.

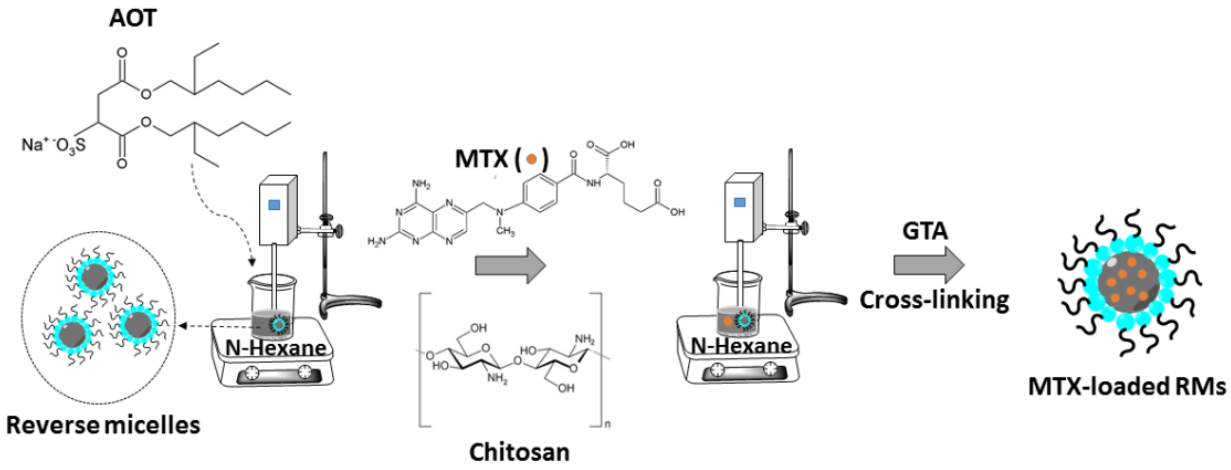

Figure 1. Schematic Methotrexate-reverse micelles preparation

MTX encapsulated reverse micelles characterization

1) Size and zeta potential

The average hydrodynamic particle size, the polydispersity index (PDI), and zeta potential (ζ) of the MTX loaded RMs were determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). Each measurement was performed using at least three independent samples at 25 ± 0.5°C and the arithmetic mean value of the three was adopted.

2) Encapsulation efficiency (%EE)

The MTX encapsulation efficiency was determined using centrifugation for separating the non-encapsulated MTX from the RMs. Five milliliters of MTX-loaded RMS were carefully transferred to 10 mL centrifugation tube. RMs and the excess of surfactant were separated from the solution by centrifugation at $12x10^3$ rpm, 4°C for 1 h. Supernatant from the centrifugation was carefully decanted and the MTX content was then measured using UV spectrophotometer (UV-1201, Shimadzu, Japan) at 302 nm. Triplicate samples were independently analyzed per experimental group at each time interval, MTX entrapment efficiency (%EE) was calculated in Equation (1)

$$\%EE = \frac{(\text{Total amount MTX} - \text{Free amount MTX in supernatant})}{\text{Total amount MTX}} \times 100 \qquad \textbf{Equation (1)}$$

Results and Discussion

Effect of AOT concentration

As mentioned above, this experiment was focused on the variable factors which affecting to RMs formation. All those variable factors were consisted of the concentrations (0.3 to 1.2 M) of surfactant (AOT), different molecular weights (MWs) of CS (low, medium, and high), and a variety of calcium chloride; $CaCl_2$ concentrations (0.3, 3, 10, 20, and 30 %w/v). The formation of RMs occurs spontaneously by mixing of surfactant (AOT) in an organic solvent (n-hexane) (Banerjee *et al.*, 2002). After the RMs formation, we found that the thickness of the final thin film which represents the RMs formation was associated with the different of AOT concentration. More specifically, they were observed clearly that the decreasing AOT concentration was in the same tendency of the thickness of the final thin film (RMs). The mean diameter of 0.3, 0.6, and 1.2 %w/v were about 260 nm (data not shown). Meanwhile, for forming RMs, it noticed that at the lower concentration of AOT (0.3 M) showed the highest stability of RMs. From this result, it

can be explained that the concentration of AOT attributed to the influence on the thin film by affecting to the partition constant and distribution of aliphatic amines of AOT between n-hexane and RMs structure (Zingaretti *et al.*, 2005). However, the hydrogen bond interaction of aliphatic amines, hydrophobic forces, and solutes were also considered (Cambón *et al.*, 2013). To RMs dispersion, different molecular weights (MWs) of CS and MTX were added after the RMs formation. RMs based on CS with different MWs might have a role in the mean diameter, zeta potential, and MTX encapsulation efficiency (%EE), but the relative MTX paper has not been yet reported. The mean diameter of RMs with low, medium, and high MWs of CS were 438.47 ± 12.51, 895.33 ± 77.09 and 917.67 ± 177.65 nm, respectively as well as the distribution of their particle size was in a range of the quality data with a low polydispersity index (PDI ~ 0.4-0.5) as evident in Table 1. Moreover, we observed that RMs with the high MWs of CS had the largest mean diameter when compared with the lower MWs ones. It was indicated that decreasing of MWs led to decrease its mean diameter. However, we consequently explained that RMs with the high MWs of CS cannot be formed in the narrow mean diameter because of their rigidity of the backbone structure and the viscosity of large molecules or molecular mass (Li *et al.*, 2007). This result agrees well with their chains of the high MWs of CS were less flexible while low MWs of CS has more flexibility and hydrophobicity. Similar trends were also found in the other studies on CS nanoparticles by ionotropic gelation method and O/W emulsification method (Vila *et al.*, 2004, Yang and Hon, 2009, Kouchak *et al.*, 2012).

Table 1 Effect of low, medium, and high molecular weights of chitosan on the mean diameter and zeta potential of CS-RMs

Chitosan (0.1 %w/v)	GTA (%v/v)	AOT (0.3%w/v)	Mean diameter (nm)	PDI	Z (mV)
Low molecular weight	0.01	20	438.47±12.51	0.43±0.02	-60.77±6.05
Medium molecular weight	0.01	20	895.33±77.09	0.54±0.04	-55.80±6.30
High Molecular weight	0.01	20	917.67±177.65	0.45±0.13	-34.73±1.62

*GTA=Glutaraldehyde, AOT= sodium bis (ethyl hexyl) sulfosuccinate, PDI= Polydispersity index

Effect of molecular weights of chitosan

During the cross-linking process, GTA can diffuse into the chitosan and MTX droplets. We found that the electrostatic interaction which occurs between the amino group (NH_3^+) groups in CS and MTX was produced ionically cross-linked with RMs. Theoretically, Z is a scientific term for electrostatic potential which is a measurement of the electric charge near the surface of the particles and indicates the physical stability of the RMs systems. The stability of the RMs system is significantly correlated with the surface charge that provides the electrostatic repulsion between the particles. (Gallardo, Morales *et al.* 2005) In fact, the zeta potential of RMs system is typically in a range of -40 to -60 mV. From these results, with positively charged amino groups on the CS and negatively charged MTX, the zeta potential of low, medium, and high MWs are -60.77 ± 6.05, -55.80 ± 6.30, and -34.73 ± 1.62 mV, respectively (Table 1). Although, we found that high MWs of CS had the highest negative surface charge, but we believed that other parameters such as the degree of deacetylation (DD) of CS have also a greater influence on the surface charge than only specific MWs (Vila *et al.*, 2004, Yang and Hon, 2009). Another possible explanation, the positive charge from its amino group of low molecular weight of chitosan cannot appear apparently while high molecular weight of chitosan can provide highly positive charge from its amino group. This explanation describes that how low molecular weight of chitosan showed higher negative charge than high molecular weight of chitosan.

Table 2 Effect of calcium chloride with low molecular weight of chitosan on mean diameter and zeta potential of CS-RMs

Chitosan (0.1% w/v)	GTA (0.01%v/v)	AOT (0.3%w/v) 20 mL	Mean diameter (nm)	PDI	Z (mV)
	$CaCl_2$ (% w/v)				
LMW	0.3%		287.27±3.43	0.30±0.01	-51.50±1.39
LMW	3.0%		228.87±2.80	0.27±0.01	-54.28±3.12
LMW	10%		266.33±7.10	0.32±0.04	-98.90±5.35
LMW	20%		556.40±33.16	0.61±0.09	N.D.
LMW	30%		1652.33±645.33	0.97±0.05	N.D.

*N.D. = Not detected

Effect of $CaCl_2$ concentration

After the cross- linked process, different concentrations of $CaCl_2$ solution were added into RMs for removal of the excess AOT from the RMs system through its precipitation as a calcium salt. (Rodriguez *et al.*, 2001) We found that the mean diameter and zeta potential of the highest concentration of $CaCl_2$ (30 % wt.) cannot be measured by DLS technique. According to the DLS analysis of 30 % wt. $CaCl_2$, undetectable mean diameter, we explained as due to the RMs particles aggregation with the presence of a large amount of the excess of Ca^{+2} ion. Their particles were likely precipitated at the bottom of the system due to their relative mean diameter were quite high to maintain the stability of dispersion in the reverse micellar system (Bharali *et al.*, 2003). Moreover, in terms of zeta potential, the increasing of ionic strengths and electrostatic repulsion in the RMs dispersion that occasionally might be the main reason why they could not be able to obtain the zeta potential results from DLS at that high concentration of $CaCl_2$ (20% and 30% wt.). From Table 2, the results of the mean diameter and zeta potential were approximately 229 nm and - 54 mV. Therefore, the maximum concentration of $CaCl_2$ determination for this study requires less than 10 %w/v.

Effect of MTX concentration

MTX was successfully loaded into RMs system following their self- formation. In RMs systems, MTX molecules as water soluble drug were physically encapsulated into the aqueous core (hydrophilic region) or adsorbed onto the surface of the core- shell of RMs. The capacity of MTX was calculated using the equation (1). For low MWs of CS values which are not affected by the MTX encapsulation efficiency for RMs, as shown in **Table 3**, where the values range 90- 91%. It can be seen that the MTX encapsulation efficiency did not show any statistical different. Therefore, RMs prepared with low MWs of CS showed high MTX encapsulate capacity that represents their sufficient positively amine groups charged to interact counterion with anionic surfactant (AOT) and cross- linked by GTA.

Table 3 Effect of MTX concentration of CS-RMs on encapsulation efficiency (%EE)

Low MWs Chitosan (0.1% w/v)	**GTA (0.01%v/v)**	**AOT (0.3%w/v) 20 mL**	**MTX (%EE)**
	$CaCl_2$ (% w/v)		
0.50%w/v MTX	10%		91.65±1.29
0.75%w/v MTX	10%		90.38±0.04
1.00%w/v MTX	10%		91.24±0.02

Conclusions

MTX and chitosan can successfully synthesize in the hydrophilic core of reverse micelles with cross-linked through glutaraldehyde. We investigated the formation of MTX-loaded in reverse micelles. It was found that the mean diameter increased with increasing molecular weight of chitosan. However, the mean diameter did not increase with increasing $CaCl_2$ concentration. A narrow distribution of particle size of the synthesized MTX loaded reverse micelles at a low molecular weight of chitosan was presented. These results confirmed the conclusion that higher molecular weight had a tendency to give larger particles. Its high value of MTX encapsulated efficiency displays a remarkably possibility in topical delivery systems. Taken together, reverse micelles are expected to overcome the undesirable side effects which induced by the systemic MTX administration. However, the further permeation study of this drug carrier will be tested due to affirm their potentiality. Reverse micelles offer several advantages over conventional topical dosage form prepared from a natural polymer; chitosan, being good candidate for use in topical drug delivery as a non–toxic carrier for MTX in antipsoriatic therapy.

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