Synthesis and biopharmaceutical characterization of a new poly hydroxyethyl-aspartic acid copolymer as potential genital tract mucosal drug delivery

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ABSTRACT

In the present study poly aspartic acid-co-glutamic acid (PAG) was prepared by acid-catalyzed polycondensation of aspartic acid and glutamic acid in mixed solvents. Metronidazole(MTZ) was attached to PAG via an ester bond to form copolymeric nano-carrier prodrug(PAG-MTZ). The obtained PAG-MTZ was then characterized. The results showed that the PAG-MTZ prodrug was spherically shaped with an average molecular weight of 14281 $g \cdot mol^{-1}$. The diameter of the particle was 198.9 nm with a narrow size distribution, the drug loadings was about 12% and the water regain percentage was exceeded 20%. In vitro and vivo drug release properties of the MTZ loading in the PAG-MTZ nano-carrier prodrug were showed all to be sustained obviously. It is concluded that PAG, as a delivery system, could increased solubility and prolonged drug release, and be potentially useful for treatment of Trichomonas vaginitis, one of the most prevalent non-viral sexually transmitted disease.

Keywords: poly aspartic acid, glutamic acid, copolymer, nano-carrier prodrug, drug release

1 INTRODUCTION

In the past few years, considerable interest has been devoted towards the design of new drug delivery systems with the aim to overcome the serious undesirable side effects of conventional therapeutic systems[1]. A fascinating approach to advanced drug delivery is to couple a biologically active compound to a soluble natural or synthetic polymeric carrier via a cleavable bond. Consequently, such macromolecular prodrugs (polymer–drug conjugates) may offer many advantages, such as increased drug solubility, prolonged drug release, reduced adverse effects, more convenient drug regimen, and increased stability and targetability[2].

To date, several biodegradable and biocompatible polymers, especially poly(amino acids), have been widely studied and developed as prodrug carriers. Among them polyaspartic acid, including its derivatives, is one of the most promising drug carriers since it is a non-toxic, non-antigenic, non-teratogenic, and environment friendly, water-soluble bioorganic polymer with a protein-like structure. Other benefits include multipoint drug attachment, excellent biocompatibility and low cost[3]. In vitro and in vivo studies, performed with different therapeutic molecules, demonstrated that the conjugation to polyaspartic acid can improve their therapeutic potential allowing for a sustained drug release[4-6]. However, little work was focused on the mucosal drug delivery. Furthermore, polyasparamide is degradable rather slowly in living body due to existence of amide linkage. One strategy for improvement is to modify the polymeric backbone structure by means of multi-amino acids copolymerization.

Metronidazole(MTZ) is an antibacterial agent that is effective against anaerobian and is the main drug of choice for treating trichomoniasis, such as Trichomonal vaginitis, a non-viral sexually transmitted disease (STD). Oral administration of this drug, however, may cause severe gastrointestinal tract reaction, or even developmental abnormalities of the fetus[7]. When delivered intravaginally, it should be dosed once or twice daily and the leakage gives little time for drug action after dosing. Therefore the course of treating is long, and the compliance of patients is low.

In order to explore the possibility of harnessing polyaspartic acid as a potential drug carrier, novel copolypeptide consisting of L-aspartic acid and glutamic acid(1:1 mol/mol) was synthesized in our laboratory. Metronidazole was attached to the copolymer via an ester bond. The effects of preparation condition and external parameters of the nano-prodrug was studied in detail. Furthermore, the preliminary release properties of the prodrug-loaded metronidazole have been studied in vitro and vivo.

2 MATERIALS and METHODS

2.1 Materials

L-aspartic acid and glutamic acid were purchased from

Zhangjagang Chemical Co., China. Metronidazole(MTZ, 99%) was purchased from Shanxi Jingxin Shuanghe Pharmaceutical Co., China.Ethanolamine and dicyclohexylcarbodiimide (DCC) were purchased from Shanghai Chemical Reagent Co., China. *N*,*N*dimethylformamide (DMF) (Shanghai Chemical Reagent Co.) was dried using P_2O_5 and distilled under reduced pressure before use. All other chemicals were reagent grade.

2.2 Preparation of poly α,β-hydroxyethyl-aspartic acid-co-glutamic acid-metronidazole (PAG-MTZ)

Poly α,β-hydroxyethyl-aspartic acid-co-glutamic acid (PAG) was prepared by acid-catalyzed polycondensation of aspartic acid and glutamic acid in mixed solvents. Briefly, 8.0g L-aspartic acid and 8.83g glutamic acid were dissolved in strong phosphoric acid(85%), 1,3,5-trimethyl benzene/ sulfolane 16:6 mixed solvent by thermal polycondensation (160℃, N_2). The solution was precipitated with propanol and dried in 45℃ vacuum to produce PAG. Then 5.9g PAG, 5.4g MTZ were taken and dissolved into 25ml DMF, 10ml of DCC water solution was dropped into above solution and left in oil bath at 60℃ for agitating 24 hr. Afterwards, 10ml ethanolamine solution was dropped into above filtrate stirring with a magnetic stirrer on ice. This mixture was further reacted for 3-4h under room temperature, the copolymer nano-carrier prodrug was precipitated, filtered and dried in vacuum.

2.3 Characterization of PAG-MTZ prodrug

The PAG-MTZ copolymer prodrug was characterized by FT-IR(Bruker IFS66V, Germany), the ^{1}H-NMR spectra were recorded on Avavce 300 MHz (Bruker DPX-300, Germany). The molecular weight was determined by GPC (Shimadzu, Japan). The morphological examination was performed using TEM (JEM 1010, Japan) following negative staining with sodium phosphotungstate solution. The particle size was measured by a laser scattering particle size analyzer(Malvern Zetasizer 3000 HAS, UK) at 20℃, 90°test angle.

The water regain percentage was measured after incubating the prodrug with a KCl saturated solution at 37 °C for 10d. The water regain percentage was calculated as follows:

water regain percentage = $(W_w - W_d)/W_d \times 100\%$

being W_w and W_d the weights of the later wet and initial dry prodrugs, respectively.

2.4 Determination of loading capacity

To determine the drug-loading content, the PAG-MTZ prodrug was dissolved in 0.05mol/L NaOH. The MTZ concentration in the solution was then determined by an ultraviolet-visible spectrophotometer (Shimadzu UV-3100, Japan) at the wavelength of 320 nm.

2.5 In Vitro Drug Release Study

350 mg of PAG-MTZ prodrug were suspended in 10 ml of PBS (0.1M, pH 5.8, simulated vaginal fluids) and then transferred into a dialysis bag. The dialysis bag was sealed and immersed into 240 ml of PBS. The system was shaken in a shaking water bath at 37℃. At predetermined intervals, 1 ml of PBS solution was taken out and replaced by fresh PBS. The drug concentration in each harvesting was determined by UV spectroscopy. The cumulative releases were calculated.

2.6 In vivo Drug release Study

One hundred and twenty female ICR mice (Certificate: SCXK (Su)2002-0013) weighing 30±2g were used. This study was conducted in compliance with the principles of laboratory animal care of Nanjing Medical University. The animals were randomly divided into two groups. Each group was injected intravaginal with free MTZ 120mg/kg or PAG-MTZ prodrug at an equivalent drug dose to MTZ group. Before injection and 0.083, 0.167, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 24.0 and 48.0 h later, 6 mice were killed by dislocation of the cervical vertebra and cervix, vagina were removed, washed, weighed, and homogenized using a tissue blender in 0.9% NaCl according to 1:3.3(W/V). After extraction, concentrations in tissues were detected by HPLC.

HPLC analyses were performed by using a Waters TM High Performance Liquid Chromatograph (USA). The detective wavelength was set at 317 nm. HPLC analysis of samples was performed using a discovery C_{18} column (4.6 · 250 mm, 5μm, Japan). The column temperature was 30℃. The mobile phase was a mixture of methanol:acetonitrile:0.075mol/L NaH_2PO_4 (5:25:75, v/v). The flow rate was maintained at 1.0 ml/min. The peak areas corresponding to MTZ were elaborated according to standard curve previously obtained by analysis of tissues containing known a mounts of free MTZ. Each sample was repeatedly measured 3 times.

3 RESULTS and DISCUSSION

3.1 PAG-MTZ synthesis and structural characterization

In order to obtain a macromolecular nano-prodrug with appropriate drug release properties, MTZ was conjugated to PAG via an ester bond which can be hydrolyzed by both chemical and enzymatic pathways in vivo.

PAG-MTZ prodrug was synthesized by a two-step reaction: (1) synthesis of poly aspartic acid-co-glutamic acid (PAG); and (2) conjugation of MTZ to PAG followed ring-opening. The chemical structure of both PAG and PAG-MTZ were determined by FT-IR and ^{1}H-NMR.

PAG was prepared by acid-catalyzed polycondensation. The copolymerization reaction was conducted under rigorously anhydrous conditions to avoid the initiation by water. When the feed ratio of aspartic acid to glutamic acid was 1:1 and copolymerization temperature was 160℃, no reactant was detectable, indicating that the reaction was complete.

MTZ reacted with PAG in presence of DCC as a condensing agent. After purification, the synthesized PAG-MTZ FT-IR spectrum (KBr) showed the disappearance of the PAG typical band at 1792 cm^{-1} , diminished amide carbonyl absorptions at 1717 cm^{-1} (amide I) and strong amide carbonyl absorptions at 1664 (amide II) accompanied the ring-opening. Inlet of hydroxyl group made a broad band centred at 3376 cm^{-1}. The additional band at 2857cm^{-1} corresponded to the nitro group of MTZ, which indicated that MTZ was covalently bound in the synthesized copolymer-drug conjugates(fig.1).

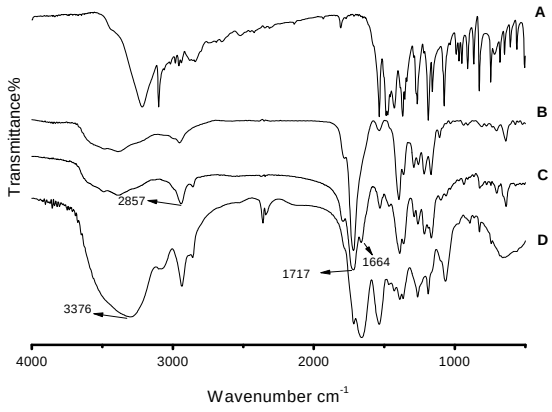

Fig.1. FT-IR spectra of: (A) MTZ; (B) PAG; (C) unring -opening PAG-MTZ; (D) the ring-opening PAG-MTZ

The ^{1}H-NMR spectra showed a shift of peak related to protons took place at 7.9 δ assignable to methylene of PAG-MTZ compared with PAG. These results demonstrated that PAG and PAG-MTZ were consistent with the predicted structures.

3.2 Physico-chemical characterisations

In order to improve polyasparamide degradation in living body and increase the solubility of MTZ, glutamic acid was selected because its high hydrophilic character[8] and the ring-opening later by ethanolamine can introduce more hydroxyl into polymeric backbone. Indeed, modifying these two factors at the same time has enabled us to increase the final dissolved MTZ levels in an aqueous solution whereas water regaining percentage of PAG-MTZ prodrug was increased by about 25 fold than free MTZ.

The morphologies of PAG-MTZ were visualized by SEM. As demonstrated in Fig.2, the prodrug synthesized in our laboratory showed spherical shapes and a narrow size distribution. The diameter of the particle was 198.9nm with an average molecular weight of 14281g·mol^{-1} and drug loadings of about 12%.

Fig.2. Transmission electron microscopy images of PAG–MTZ copolymer nano-carrier drug at 10000 magnification

3.3 In Vitro Drug Release Behaviors

The in vitro release profiles of MTZ from pure MTZ and PAG-MTZ prodrug are shown in Fig.3. As much as 57.3% of pure MTZ escaped from the dialysis bag within the first hour and the release of MTZ was almost complete by 24 h. In comparison, PAG-MTZ showed a much slower releasing dynamics: about 47.51% of MTZ was released within the first 24 hours followed by a decelerated release of MTZ until 30d. The results demonstrated that by cleavage of the ester bonds and degradation of macromolecular polymers, MTZ was released from PAG-MTZ prodrug in a more controlled manner that lasted much longer. At the beginning of the drug release process, a burst of release was observed, which may be ascribable to the dissolution of the free drug absorbed on the surface of prodrug[9].

3.4 In Vivo Drug Release Behaviors

To assess the realistic picture of drug release in a biological system, concentrations of MTZ in cervical, vagina tissues were measured by HPLC. Fig.4 shows the tissue MTZ concentration–time profile after administration of MTZ and PAG-MTZ suspension. For the free MTZ, the tissue MTZ concentration rose quickly to the maximum concentration (C_{max}) of 170.59±21.59μg/mL at 0.0 83h

(t_{max}), then quickly decreased to 0.93±0.59μg/mL at 2.0 h. 24 h after the administration the MTZ level was no longer detectable. In contrast, PAG-MTZ prodrug also reached a peak at 0.0 83h. However, the peak concentration was about 3-fold lower than that in the free MTZ group and decreased slowly to basal levels at 48h. The half-life time ($t_{1/2}$) and mean residence time(MRT) were 2.18 and 3.87 times longer than those of free MTZ respectively. This suggests that PAG-MTZ also had delayed release characteristics in vivo. We consider that macromolecular prodrugs fabricated on a nanometer scale may show good bioadhesiveness and thus make its remain in the vagina long enough for a prolonged action[10].

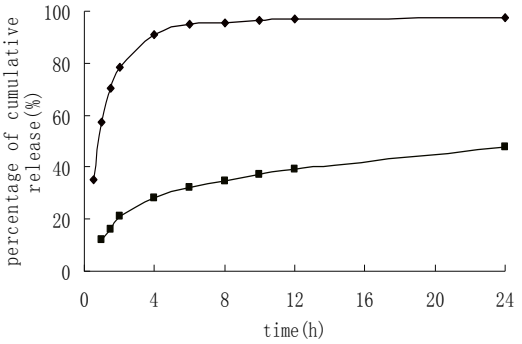

Fig. 3. In vitro release profiles of MTZ (◆) and PAG–MTZ copolymer nano-carrier drug (■) respectively in pH 5.8 phosphate buffer at 37±0.1℃

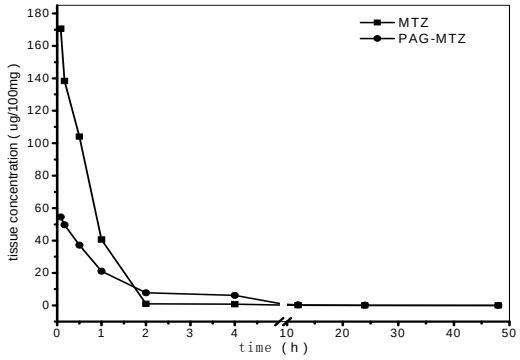

Fig. 4. The average MTZ tissue concentration-time profile after intra- vaginal administration of MTZ and PAG-MTZ(n=6)

The results also indicate that *in vivo* release of MTZ from PAG-MTZ was faster than its *in vitro* release. This may be due to the complex environment in the living body, particularly, the existence of enzyme effects[11].

4 CONCLUSIONS

The poly α,β-hydroxyethyl-aspartic acid-co-glutamic acid (PAG) was prepared by acid catalyzed polycondensation of aspartic acid and glutamic acid in mixed solvents and metronidazole(MTZ) was attached to PAG via an ester bond by using DCC to form copolymeric nano-carrier prodrug(PAG-MTZ). The reaction condition was simple, feasible and mild. The solubility of the prodrug increased considerably. The TEM images confirmed the regularly spherical shapes and narrow size distribution. In vitro and in vivo experiments of the prodrug showed PAG-MTZ nano-prodrug presented a sustained release of MTZ compared to the free MTZ solution, which is an interesting way of improving MTZ for treatment of Trichomonas vaginitis. These studies provided a reference for the synthesis of polymeric prodrugs for mucosa administration.

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