RETRACTED ARTICLE: LDH hybrid thermosensitive hydrogel for intravaginal delivery of anti-HIV drugs

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ABSTRACT

Microbicides based on hydrogel have become an effective way to prevent the HIV replication and transmission because of their convenience and prolonging drug release. In this study, a hybrid thermosensitive hydrogel constituted by nanosized layered double hydroxides and poloxamer 407 (P407) was constructed and co-loaded with both hydrophobic and hydrophilic drug. The LDH-P407 hydrogel could achieve sol–gel transition at body temperature. The in vivo experiment showed that LDH-P407 hydrogel can achieve controlled release of theaflavin and Nile red (hydrophobic drug models) into blood by vaginal drug delivery, meanwhile the hydrogel showed barely mucosal irritation. In addition, ex vivo experiment showed that the nifeviroc-loaded LDH-P407 hydrogel was able to specifically bind co-receptor CCR5 of DCs cells. Therefore, the LDH-P407 hydrogel would be a promising carrier for intravaginal delivery of anti-HIV drugs.

Introduction

According to the UNAIDS survey data, there are 36.9 million people living with HIV and 1.8 million people newly infected with HIV worldwide in 2016 [1]. The number of AIDS patients is large and the newly infected patients increase fast, AIDS prevention and control tasks remain arduous. Among all the transmission routes of AIDS, sexual transmission is still the most important way of HIV infection [2]. To find a safe and effective prophylactic drug, to reduce the sexual transmission of AIDS has become a hot spot in the field of AIDS prevention [3]. The combination antiretroviral therapy (cART) for HIV pre-exposure prophylaxis has led to its universal recommendation [4,5]. However, most oral antiretroviral drugs have low bioavailability company with side effects and high cost of treatment, which makes the prophylactic effect greatly reduced [6]. Therefore, it is urgent to find new drug delivery system to reduce the side effects and improve the bioavailability of drugs.

The vaginal and rectal mucous have large surface and abundant blood vessels. The absorption of drugs through vaginal or rectal mucosa can avoid the liver first-pass effect and the peak and valley phenomena caused by multiple dosing [7,8]. Microbicide is a kind of agent containing drug such as reverse-transcriptase inhibitors, which can prevent the mucosal adhesion of HIV virus or invade the target cells in the mucous membrane and inhibit the replication of the virus in the cells, thus playing a role in preventing sexually transmitted diseases such as AIDS [9]. Microbicides based on hydrogel hold a promising application in the vaginal/rectal drug delivery system due to its convenient administration and good controlled release effect [3,10,11]. The thermosensitive hydrogel prepared by hydrophobically modified methylcellulose was reported to show great controlled release of the hydrophilic anti-HIV drug tenofovir for 10 h without burst release [12]. PLGA nanoparticles were reported to load the hydrophobic anti-HIV drug rilpivirine and vaginal topical administration with poloxamer thermosensitive gel, demonstrating that thermo-sensitive gel can significantly reduce the probability of BLT humanized mice infected with HIV-1 [13]. Besides traditional reverse-transcriptase inhibitors, some HIV entry inhibitors were also in research. Theaflavin was reported as an hydrophilic inhibitor against the formation of HIV-1 gp41 six-helix core structure, which becomes an ideal candidate for microbicides [14,15]. Nifeviroc is a hydrophobic small molecule antagonist against CCR5, which can specifically bind with CCR5 receptor on the surface of HIV-1 target cells to inhibit HIV infection into cells. However, due to its poor solubility, nifeviroc showed low bioavailability via oral administration [16].

In this study, a hybrid thermosensitive hydrogel was constructed by nanosized layered double hydroxides (LDHs) and poloxamer. LDHs ([Mg₆Al(OH)₂n+2]·nH₂O, where A = chloridion or nitrate ion, n = 2–3, m = ~2) are clay-based nanoparticles which can intercalate hydrophobic drug molecules into their brucite-like layers, thus exhibiting advanced biocompatibility and enhanced drug delivery efficiency [17,18]. Here, we reference the idea of “cocktail therapy” to co-load hydrophilic theaflavin and hydrophobic nifeviroc into
this hybrid thermosensitive hydrogel, by dispersing theaflavin into poloxamer solution and intercalating nifeviroc into LDH, thus forming a complex hydrogel-based microbicide candidate for blocking HIV entry into cells. In vivo experiment results showed that this LDH hybrid hydrogel could achieve rapid sol–gel transformation at body temperature, and exhibited barely mucosal irritation and sustained controlled release of both hydrophilic and hydrophobic drugs into blood. Moreover, it should be noted that the local concentration of both hydrophobic drug and hydrophilic drug in vaginal tissue was hundreds times as much as its in blood, which benefits its application as a pre-exposure prophylaxis microbicide during sex behaviour.

Materials and methods

Materials

Poloxamer 407 (P407) was purchased from Solarbio (Beijing, China). Mg(NO₃)₂·6H₂O, Al(NO₃)₃·9H₂O, NaOH, NaCl, carbinol were analytical grade. Nile Red was purchased from Macklin (Shanghai, China). Nifeviroc and theaflavin were purchased from Ziqibio Ltd. (Shanghai, China). All the materials used in the cell culture study were obtained from Solarbio (Beijing, China). Recombinant human TNF-α was purchased from Pepro Tech (Rocky Hill, NJ). New Zealand White rabbits were from Qingdao Experimental Animals and Animals Centre (Qingdao, China).

Synthesis and characterization of LDH

A solution containing 1 mmol (0.375 g) Al(NO₃)₃·9H₂O and 3 mmol (0.76 g) Mg(NO₃)₂·6H₂O in 10 ml ultrapure water was poured quickly into a solution containing 6 mmol NaOH. The suspension was vigorous stirred for 10 min under argon atmosphere, after which the solid precipitate was collected by centrifuged and washed thoroughly with 40 ml ultrapure water several times. Then, 40 ml suspension was placed in a hydrothermal kettle and reacted at 150 °C for 16 h under high pressure. The product was homogeneous nanoparticles dispersion which was freeze-dried to obtain white powder. The morphology of LDH was observed by transmission electron microscope (TEM). The particle size, particle distribution index and zeta potential were evaluated by Malvern Zetasizer (Malvern, UK).

Preparation of LDH hybrid thermo-sensitive hydrogel

The LDH hybrid thermo-sensitive hydrogel was formed by dispersing 0.1 g LDH into P407 solution (containing 2.0 g P407 and 8 ml ultrapure water) at 4 °C under stirring [19,20]. For in vivo pharmacokinetics evaluation, Nile red was chosen as a hydrophobic drug model to be intercalated into LDH by dispersing LDH in Nile red methanol solution then dialysis against ultrapure water, after that theaflavin and P407 were dissolved in this drug loaded LDH dispersion with final concentration of P407 at 20% (wt%).

Rheological measurements

The rheological properties of P407 gel and LDH-P407 gel were measured by rheometer (Anton Paar MCR 301, Graz, Austria). The rheological parameters of these two gels were measured under the coaxial cylindrical scanning dynamic oscillation mode. The strain amplitude was 1%, the frequency was 1 Hz, the heating rate is 1 °C/min, the scanning temperature was 15–40 °C. The storage modulus (G′), loss modulus (G″) and complex viscosity (η) were used as the observation index.

Mucosal irritation assessment

New Zealand white rabbits were used as animal models to observe the irritation of the LDH-P407 gel to vaginal mucosa. The mature female New Zealand white rabbits (weight 2.5 ± 0.5 kg) were maintained under standardized laboratory (temperature 20 ± 2 °C, relative humidity 45 ± 10%). Forage and water were qualified. Normal saline, P407 gel, LDH-P407 gel and nonoxinol gel were injected into rabbits with 10 Fr catheter continuously for five days. The rabbits were sacrificed on the sixth day and the vaginal tissues were dissected. The vaginal tissues were immobilized in 4% paraformaldehyde for 48 h. After ethanol dehydration, paraffin embedded, HE staining, the remaining tissues were used to detect the levels of TNF-α and IL-6 by immunohistochemistry.

Pharmacokinetics evaluation and local drug concentration

The experimental conditions were the same as the mucosal irritation assessment of gel. Then 1 ml normal saline and the drug loaded LDH-P407 gel into rabbit vagina were injected with 10 Fr catheter. Blood was collected at a fixed point and centrifuged for 10 min (4 °C, 3000 rpm). The vaginal tissues were taken after 24 h and stored at –20 °C. The vaginal tissue was put into the 1.5 ml EP tube with 1 ml methanol/ultrapure water. Then, grinding the tissue completely with a grinder and centrifuging it at 3000 rpm for 10 min. Theaflavin was determined by high performance liquid chromatography (HPLC) with a Agilent SB-C18 column (250 mm × 4.6 mm, 5 μm) at a flow rate of 0.5 ml/min and 25 °C. The mobile phases were A (2% glacial acetic acid solution):B (acetonitrile) = 82:18. Quantification of the samples was performed with the absorbance at 280 nm with authentic samples as standards [21]. The fluorescence spectrophotometer was used for the determination of Nile red in plasma and vaginal tissues (EX: 543 nm, EM: 598 nm). The concentration of Mg²⁺ and Al³⁺ in plasma was detected by the inductively coupled plasma.

The blocking effect of drug loaded gel on CCR5

Human dendritic cells (DCs) in logarithmic growth phase were seeded in six-well plates at 1 × 10⁵ cells per well. After culturing for 24 h, 2 ml TNF-α (10 μg/ml) was added to stimulate the differentiation and maturation of DCs for 24 h. One millilitre LDH-P407 hydrogel loaded with nifeviroc (10...
mg/ml) same intercalation method with Nile red was taken into wells and other groups were added cell culture medium. After 2 h, the medium in each cell was replaced by 1 ml fresh medium containing FITC-labelled CCR5 antibody and incubated for another 40 min. The cells were digested with trypsin and washed by cold PBS for three times. The FITC fluorescence intensity of each group was detected by flow cytometer (Ex: 488 nm, Em: 528 nm).

Results and discussion

Characterization of LDH

The morphology of LDH was observed by TEM (Figure 1(b)). As can be seen, LDH showed a homogeneous hexagonal structure, complete structure and good morphology. The dynamic light scattering technique revealed that the average particle size of the LDH nanoparticles was about 60 nm with the PDI of 0.17, and the zeta potential was about 32 mV.

Rheological properties

The rheological properties of 20% P407 and LDH-P407 hydrogel were evaluated. The rheological properties of gels depend on temperature [22]. As shown in Figure 2, the storage modulus ($G'$) and the loss modulus ($G''$) increase gradually with the increase of temperature, and reach the gelation temperature at 25 °C. The gelation temperature ensured that the gel can transform into gel state quickly after entering human body. The results also show that in this case, the addition of LDH nanoparticles will not affect the gelation temperature of the P407 gel.

Irritation assessment of the gel to cervicovaginal mucosa

The irritation of LDH-P407 gel to rabbit vagina mucosa was studied compared with 0.9% normal saline, P407 gel and nonoxinol gel (Figure 3). After HE staining, the mucosal structure remained intact in the saline group, at the same time, in the LDH-P407 group, P407 group and nonoxinol group, the vaginal epithelium was little loose but almost no oedema, bleeding or neutrophil infiltration occurred [12]. The immunohistochemical results showed that there were less inflammatory cytokines of TNF-α and IL-6 in the saline group, P407 group and LDH-P407 gel group than in the nonoxinol gel group. Nonoxinol gel has long been used in clinic as a short-term contraceptive for female and P407 has also been used as a pharmaceutical excipient in clinical practice for many years; therefore, the biosafety of LDH-P407 gel could be trusted. The irritation assessment results revealed that LDH-P407 gel could be used as a safe carrier for intravaginal administration.

Evaluation of pharmacokinetics of gel

The concentration of theaflavin was detected by HPLC and the Nile red was detected by fluorescence spectrophotometer. As seen in Figure 4, the theaflavin and Nile red in plasma increased slowly within 24 h, and there was nearly no sudden release. After 24 h, the drug concentration remained at a high level. Meanwhile, the concentrations of Mg$^{2+}$ and Al$^{3+}$ in the plasma were increased too, which proved that the LDH nanosheets in P407 hydrogel could deliver hydrophobic drugs into the bloodstream through vaginal mucosa. The anti-HIV drugs in plasma may bind to HIV and inhibit the binding of HIV to target cell [23]. The results indicated that the gel had a good controlled release performance. Combined with the rheological results, this drug-loaded LDH-P407 gel could rapidly cover the vaginal mucosa and form a drug repository after entering the human body, which could continuously release anti-HIV drugs. It can meet the requirements of long-term controlled release vaginal drug delivery system.

Local drug concentration of vaginal tissue

The local drug concentrations in vaginal tissue were also detected after single administration at 24 h. The detection methods of drug concentration were consistent with plasmic concentration. As shown in Figure 5, the concentrations of Nile red and theaflavin in vaginal tissue were still holding...
high level. And the local concentration of Nile red and theaflavin was hundreds times of the plasmic concentration, which may benefit to the prevention of HIV infection via sexual transmission [24,25]. In addition, local drugs could keep penetrating through the mucosa into the bloodstream, preventing the HIV virus from invading the immune system.

Figure 2. The total viscoelasticity including the storage modulus ($G'$), the loss modulus ($G''$) and the viscosity ($\eta$) of (a) 20% P407 and (b) 20% LDH-P407 gel.

Figure 3. Pathological photos of rabbits’ cervicovaginal tissues after intravaginal application of normal saline, P407 gel, LDH-P407 gel and nonoxinol gel for five days.
The blocking effect of nifeviroc against CCR5

The blocking effect of the drug-loaded gel against the surface protein CCR5 of DCs cells was detected by flow cytometry. The results of Figure 6 showed that the fluorescence intensity of the drug-loaded gel group was reduced compared with the untreated group. The results demonstrate that the drug-loaded gel was capable of releasing nifeviroc to binding to the cell surface receptor CCR5. The low expression level of CCR5 in DCs cells may be the reason why the fluorescence intensity of the dosing group and the untreated group are not much different. The binding of HIV virus to CCR5 on the surface of target cells results in a conformational change of the viral surface protein gp41, which was an important step for HIV into target cells [26,27]. The nifeviroc released by LDH-P407 gel could inhibit the entry of HIV into the human body through competitive binding of CCR5.

Conclusions

We have studied a hybrid thermo-sensitive nano-hydrogel by mixing LDH nanosheets into P407 hydrogel, aiming for delivering both hydrophilic and hydrophobic anti-HIV drugs at the same time. The gelation temperature of LDH-P407 hydrogel was around 25 °C which ensured the hydrogel could quickly transform into gel station after entering human body. In the mucosal irritation assessment, LDH-P407 hydrogel showed good biocompatibility. In vivo drug release results indicated that LDH-P407 hydrogel could sustain releasing both hydrophilic theaflavin and hydrophobic Nile red into the bloodstream. The flow cytometry results showed that the drug-loaded hydrogel was able to release nifeviroc and bind...
with CCR5 to inhibit HIV entry into target cells. Therefore, we believe that LDH-P407 hydrogel holds a great promising as a carrier for multiple anti-HIV drugs via intravaginal delivery.

Disclosure statement
No potential conflict of interest was reported by the authors.

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