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Glycyrrhizic acid delivery system Chitosan-coated liposome as an adhesive anti-inflammation

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ABSTRACT

Nowadays, medicinal plants are used to overcome the side effects of prescription drugs in modern medicine. Glycyrrhizic acid (GA) derived from the root of the licorice plant is one of the plant compounds whose effectiveness has been confirmed in the treatment of inflammatory bowel disorders (IBD). Liposome thin film hydration method was used to synthesize chitosan-coated liposomes containing GA. In the present study, chitosan-coated liposome was characterized by dynamic light scattering (DLS), zeta potential, scanning electron microscope (SEM) and Fourier transforms infrared spectroscopy (FTIR). The FTIR spectrum confirmed the coating of liposomes by chitosan polymer. Liposome coating leads to an increase in the size and values of zeta potential. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay of chitosan-coated liposomes containing GA confirmed that it has no cytotoxicity toward fibroblasts cell line, therefore confirming their cytocompatibility. Overall, drug loading, release and cytotoxicity were evaluated and it was found that chitosan decreased the release rate of GA. It seems; chitosan-coated liposomes may be a suitable system for delivering liposomal GA in the treatment of IBD.

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Introduction

Inflammation is the most important defense mechanism of the body, which plays an important role in host defense against infectious agents, reactive oxygen species (ROS), metabolic stress and maintaining tissue homeostasis (1). In some conditions, a chronic inflammatory response develops and may persist for a long time, which can lead to tissue damage or chronic diseases such as inflammatory bowel disease (IBD) (2). IBD is a chronic disease that includes Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (3). Genetic, host and environmental factors are considered the main causes of the disease (4). Genetic components are one of the important causes of this disease, the risk of IBD in relatives of affected patients is high. Studies have shown that IBD is maybe to be a polygenic process (5, 6). The host immune response is another factor that plays a role in the development of IBD. T cell-mediated responses are augmented in IBD and lead to the secretion of pro-inflammatory cytokines interleukin (IL)-17, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) which leads to perpetuating the cycle of disease (5). The T-cell function can be affected by the genetic loci related to IBD (7). Though genetic susceptibility is an important factor, environmental factors can play a role in the development of IBD (8). Today, steroidal or non-steroidal anti-inflammatory drugs, anti-leukotrienes and pro-inflammatory cytokine inhibitors such as anti-TNF alpha or anti-IL-1 monoclonal antibodies are used as therapeutic agents. However, these treatments are not sufficiently favorable due to side effects and drug limitations such as nonspecific bio-distribution, low bioavailability and short half-life (9). To overcome the limitations of treatment, use of the natural phytochemicals such as polyphenols, terpeniods, flavonoids and alkaloids can act as a therapeutic factor for the treatment of chronic diseases such as IBD. Studies have shown that phytochemicals play an important role in reducing oxidative stress, suppressing inflammation and cell proliferation, modulating signal transmission pathways, and inducing apoptosis (10). Glycyrrhizic acid (GA) is a triterpene extracted from the root of the licorice plant and has a wide range of medicinal properties such as anti-inflammatory, anti-diabetic, antioxidant, anti-tumor, antimicrobial and antiviral properties (11). However, in order to obtain the desired clinical application and pharmacological activity, the use of nanoparticles (NPs) as the optimal drug delivery system has been considered to overcome the obstacles (12). Today, various types of NPs are being designed with good encapsulated efficiency and increased efficacy of therapeutic agents. Liposomal NPs are lipid bilayer structures with appropriate attributes for drug delivery (13-15). In the structure of liposomes, lipophilic drugs are inserted in to the lipid bilayer and hydrophilic drugs are encapsulated in the interior of the carrier. Therefore, liposomes are used for the simultaneous delivery of several drugs with dif-

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ferent pharmacokinetic properties. In addition, liposomes have other properties such as easiness of synthesis and the ability to improve drug bio-distribution (16-18). In a drug delivery system, the size of the NPs is a critical factor, so liposomal carriers of the appropriate size can be used to prevent the encapsulated drug from entering healthy cells (19, 20). In addition, the surface coating of liposomes has been done for their stability and to prevent the leak of compounds at the target site (21). Chitosan is a natural biopolymer that is used in the form of compression coating the NPs in drug delivery. Chitosan increases the therapeutic effects of drugs due to their physicochemical properties, such as biocompatibility, biodegradability, low toxicity, mucoadhesive property, and increase absorption (22, 23).

In the present study, GA was encapsulated into the liposome to reduce inflammatory disorders bowel, and chitosan was used as a coat on the surface of the liposome due to its mucoadhesive properties. Finally, chitosan-coated liposomes containing GA have been studied as an anti-inflammatory agent of mucosal adhesion.

Materials and Methods

Chitosan, Soylethitin, and Glycyrrhizic acid were purchased from Sigma-Aldrich, USA. Ethanol, Chloroform, Acetic acid, NaOH and HCL were obtained by Merck, Germany. 3- (4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT reagent), Phosphate-Buffered Saline (PBS), Fetal Bovine Serum (FBS), and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium, were attained from Sigma-Aldrich, USA.

Preparation of liposomes containing GA and chitosan-coated liposomes

Liposomes were prepared according to the thin film hydration method. In brief, soy lecithin (20 mg) was dissolved in chloroform (1 mL) in a round bottom flask. The organic solvent was evaporated in a rotary vacuum evaporator (HB 10 S099 instrument, IKA, Germany) under lipid transition temperature, and traces of solvent was removed from the deposited lipid films. GA (7 mg) was dissolved in 50 mL of distilled water (33 ˚C), then added to the obtained lipid film and hand shaken at room temperature. The suspension solution contained multilayered liposomes. Therefore, the size of liposomes was reduced with an ultrasound probe (UW2070, BANDELIN electronic, Germany) at 85 W, in five cycles of 5 min with a resting period of 5 min, then the solution was placed in a bath sonicator (D-78224, Elma, Germany) for 55 min. For the preparation of chitosan-coated liposomes, chitosan of various concentrations (0.3%, 0.7% and 0.9% (W/V)) in acetic acid (1%) was prepared. Liposome suspension (2 ml) with chitosan solution (100 µl) was mixed and shaken, then the solution was centrifuged at 120,000 rpm. (Fig. 1)

Characterization of liposomes containing GA and chitosan-coated liposomes

The hydrodynamic diameters and polydispersity index (PDI) of the liposomes were performed by dynamic light scattering (DLS) (Malvern Instruments Ltd, Germany) and the zeta potentials were analyzed with the same instrument. The vesicle shape of liposome formulations was evaluated by scanning electron microscope (SEM) (Fei Instruments, Quanta450, USA). The selected samples were spread on an aluminum foil and evaluated by SEM after complete drying at room temperature. The Fourier transforms infrared (FTIR) spectroscopy method was used to investigate the interactions between GA and compounds used in the synthesis of liposomes and chitosan-coated liposomes. The compounds used in synthesis were mixed with predried KBr. Finally, FTIR spectrums were analyzed in the range of 400–4000 cm^{-1} by an FTIR spectrometer.

Encapsulation efficiency and drug loading

The amount of unentrapped drug in the supernatant obtained by centrifuging the liposome, at 12,000 rpm for 30 min was assayed by UV-spectrophotometry at 258 nm. Therefore, the amount of trapped drug was accounted via subtracting the unencapsulated amount from the total amount of GA added to the formulation. The amount of drug encapsulated in hydrated liposomes is considered as the loading capacity (24). The drug loading (DL) and entrapment efficiency (EE) were calculated using the following equations:

$$\text{Encapsulation\%} = \frac{\text{Total drug} - \text{Free drug Total drug}}{\text{Total drug}} \times 100$$

$$\text{Drug loading\%} = \frac{\text{Total drug} - \text{Free drug}}{\text{Loaded drug} + \textit{Carrier weight}} \times 100$$

In vitro drug release

In order to investigate liposome function and GA release, the dialysis method was used in a buffer and simulated the natural physiological environment (pH 7.4) (25, 26). Briefly, the dialysis bag (MW cutoff 12,000Da) was pretreated in the usual method. 10 mg of NPs were dissolved in 1 ml of buffer and placed into a dialysis bag. Then the dialysis bags were immersed in 50 ml of phosphate buffer (pH 7.4) to maintain the sink condition. At determined time intervals of 1, 2, 4, 6, 8, 16 and 24 hours, samples (1 ml) were withdrawn, and replaced with the same volume. After sampling, samples were assayed at 258 nm using UV-spectrophotometer. In this study, all results were the average of triplicate measurements (n=3).

Cell culture

The Fibroblast cell line was got from the Pasteur Institute of Iran (IPI). Fibroblast cell line cells were full-grown in cell culture flasks (Eppendorf® Cell Culture Flask T-25 polystyrene flask) in DMEM/F12 medium, supplemented with 10% FBS, 0.25 µg/ml of amphotericin,100 µg/ml of streptomycin, 100 U/ml of penicillin incubated under 5%

Figure 1. Preparation of Chitosan coated liposome containing GA.

CO_2 at 37 °C. The culture media was refreshed every two days until achieved more than 80% confluence. Then they were trypsinized and seeded in 96 well plates to make a dilution of 2×10^3 cells/well.

In vitro cytotoxicity test

The sensitivity of liposomes containing GA was evaluated employing 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-based cytotoxicity assay. The cytotoxicity of the chitosan-coated liposome containing GA was studied by MTT assay on fibroblast cells. First, cells were exposed to three groups: G1: control (fibroblast cell line), and G2: CS-Lipo, for 24h. After this time, media from the control and experimental group were collected and washed with sterile PBS to exclude any constituents of test composites in the culture. Then, 50 µl of MTT color (5 mg/ ml PBS) was added to each well and incubated for 4 h at 37 °C in a humidified atmosphere of 95 air% 5 CO_2. After the incubation period, redundant non-replied color was discarded and added 200µl of DMSO was to dissolve the colored formazan formed in each well. The absorbance was measured by a plate reader spectrophotometer (Lonza BioTek ELx808 Absorbance Plate Read) at 570 nm and the cell viability chance was calculated using the formula below:

$$\text{Cell survival\%} = \frac{\textit{Absorption related to treated samples}}{\textit{Absorbance of control samples}} \times 100$$

Statistical analysis of data

The data obtained from each group was defined as Mean ± SD Then, the comparison between the groups via statistical analysis was conducted using analyses of variance (ONE WAY ANOVA), with $P < 0.05$ considered to be statistically significant.

Results

Characterization of liposomes containing GA and chitosan-coated liposomes

The hydrodynamic diameter of the liposome containing GA determined by DLS was 108 nm (Fig 2A), after coating the liposome with chitosan, the size increased to 168 nm (Fig 2B). The PDI for liposome containing GA and chitosan-coated liposome containing GA formulation were smaller than 0.3, it has shown relative homogeneous dispersion.

Zeta potential was measured for liposome (F1), Liposome containing GA (F2), chitosan-coated liposome with a concentration of 0.3% (w/v) (F3), chitosan-coated liposome with a concentration of 0.7% (w/v) (F4), chitosan-coated liposome with a concentration of 0.9% (w/v) (F5) respectively are -36.69 mV, -36.03 mV, -31.3 mV, -21.61 mV and -8.88 mV (Fig. 3).

Morphological characterization of the chitosan-coated liposome containing GA was performed by SEM and structures are shown in Fig 4, which displayed spherical morphology. There is a difference in the hydrodynamic diameter and those of the particle itself, which leads to some differences between the SEM and DLS results.

The FTIR spectra of GA, liposome containing GA were shown in Fig 4. The obtained spectrum of GA includes the bands 3363.86 cm^{-1} (OH), 2927.94 cm^{-1} (C-CH3(, 1718.58 cm^{-1} (C=O), 1649.14 cm^{-1} (C=O), 1454.33 cm^{-1} (CH_2), 1390.68 cm^{-1} (CH_3), 1213.23 cm^{-1} (acetate), 1168.86 cm^{-1} (format), 1043.49 cm^{-1} (secondary ring alcohol). The spectra collected from chitosan-coated liposome containing GA including main bands 3342 cm^{-1} (O-H bond between water and lecithin molecule), 2924 cm^{-1} (CH_2 asymmetric stretching vibration), 2854 cm^{-1} (CH_2 symmetric stretching vibration), 1739 cm^{-1} (C=O symmetric tensile vibration). (Fig 5A). Two bands 1577 cm^{-1} and 1654 cm^{-1} are observed in the spectrum of chitosan polymer. The spectra collected from chitosan-coated liposome containing GA include the main bands 3342 cm^{-1} (O-H bond between water and lecithin molecule), 2924 cm^{-1} (CH_2 asymmetric stretching vibration), 2854 cm^{-1} (CH_2 symmetric stretching vibration) and 1739 cm^{-1} (C=O symmetric tensile vibration) (Fig 5B). According to the results of FTIR spectroscopy, it was

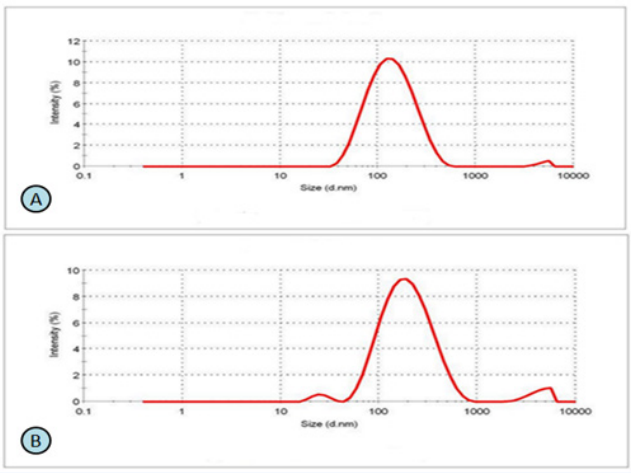

Figure 2. Glycyrrhizic acid delivery system Chitosan-coated liposome) A(Hydrodynamic diameter of liposome containing GA) B(Hydrodynamic diameter of chitosan-coated liposome containing GA.

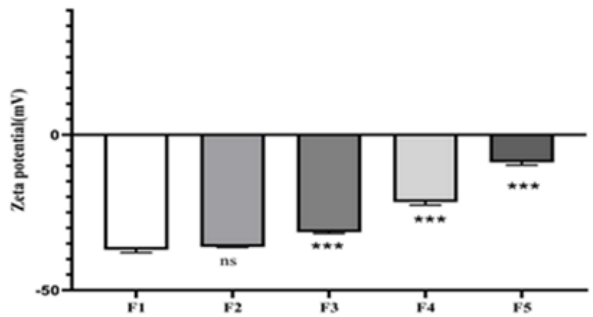

Figure 3. Chart of zeta potential values of formulations F1, F2, F3, F4 and F5.

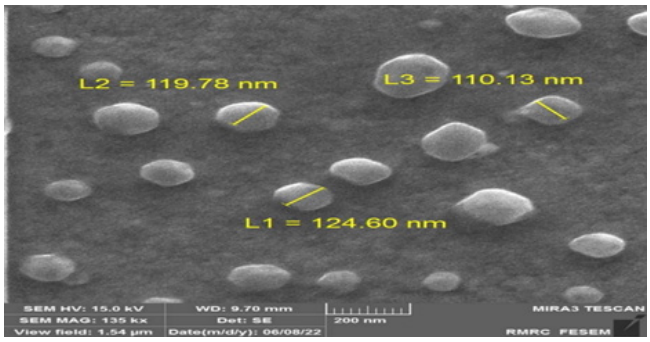

Figure 4. SEM imaging of chitosan-coated liposome containing GA.

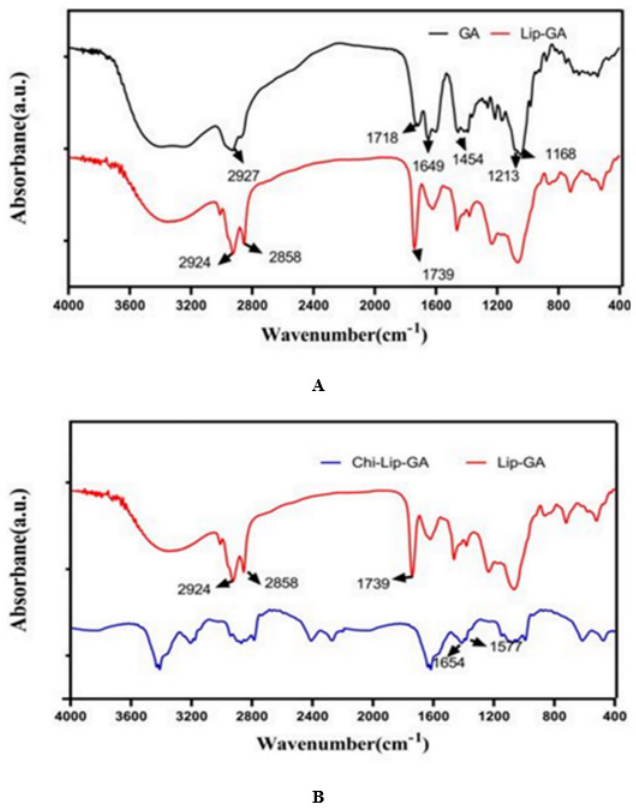

Figure 5. FTIR spectra. A: FTIR spectra of GA and chitosan-coated liposome containing GA. B: FTIR spectra of liposome containing GA and chitosan-coated liposome containing GA.

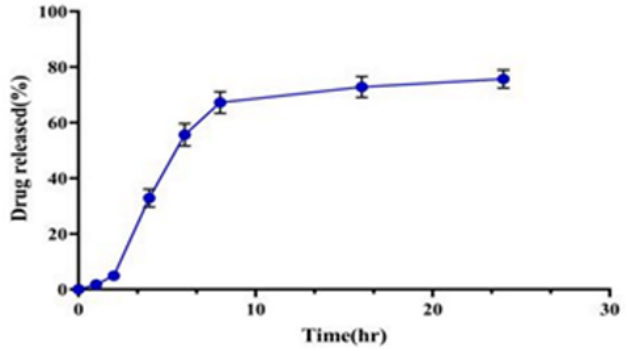

Figure 6. Release of liposome containing GA without chitosan coating.

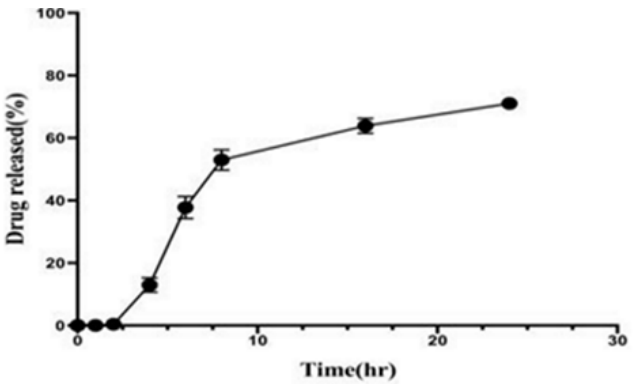

Figure 7. Release of GA from chitosan-coated liposome.

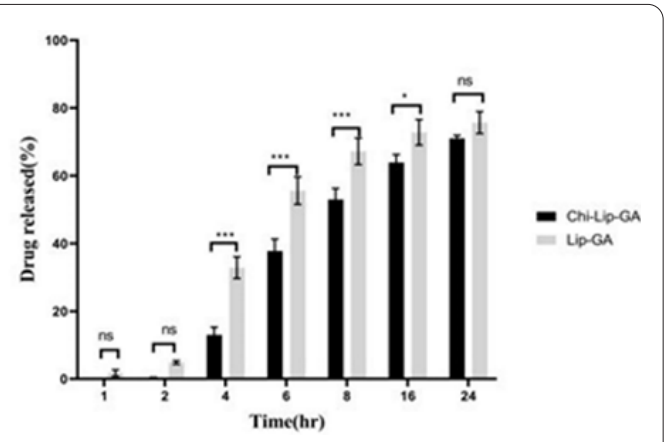

Figure 8. Comparison of GA release from the chitosan-coated liposome and uncoated liposome.

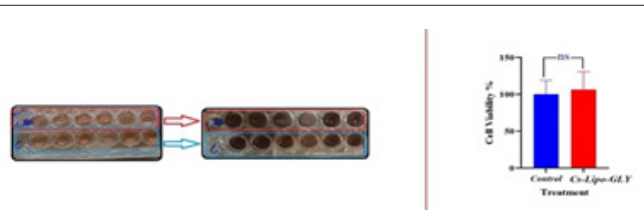

Figure 9. Cell viability percentage graph of fibroblast cell line after treatment with optimal concentrations of chitosan-coated GA liposome, the error barres in all columns are reported as mean ± SEM ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively).

found that chitosan was successfully modified in chitosan-coated liposomes containing GA.

Encapsulation efficiency (EE) and drug loading (DL)

The measured values for drug loading (DL) and encapsulation efficiency (EE) are 23.11±0.9 and 70±1.15. Chitosan-coating liposomal did not affect drug loading.

***In vitro* drug release**

Drug release of different GA formulations was performed in laboratory conditions in phosphate-buffered saline (PBS, pH 7.4). The release of GA from the chitosan-coated liposome and the release from liposome without chitosan coating was investigated. The release rate of GA from uncoated liposomes is%75.69 ± 2.63 (Fig 6). Then, under the same conditions, the release of GA from the chitosan-coated liposome was measured and it was observed that the release rate after 24 hours was%71.03 ± 0.7 (Fig 7). In the following, the comparison of release from both formulations was investigated and, it was found that there is no significant difference at 1, 2 and 24 hours, but there is a significant difference at times 4, 6, 8 and 16, and a reduction and slow release from chitosan-coated liposome was observed (Fig 8).

Cytotoxicity of chitosan-coated liposome containing GA on fibroblast cells

The cytotoxicity of chitosan-coated liposome containing GA optimal concentrations on fibroblast cell lines was investigated by MTT assay for 24 h (Fig 9). Our study showed that chitosan-coated liposome containing GA enhanced the cell viability percentage in the fibroblast cell line compared with the control group (106% vs 100%), although this enhancement was not significant. In addition, chitosan-coated liposome containing GA is cytocompatible, supporting its safety for in vivo biomedical applications.

Discussion

The increase in size is due to the placement of a hydrophilic shell on the surface of the liposome that leads to electrostatic interactions between chitosan (positive charge) and phosphate (negative charge). This hydrophilic layer can also increase the stability of liposomes (27, 28). In this study, there is no statistically significant difference between F1 and F2 groups. But there is a significant difference between the F1 group and F3, F4 and F5 groups. Zeta potential is a finding index of the surface electrical charge of the particles that influence liposomal function (29). Chitosan as a cationic polymer was coated on the surface of liposomes which lead to an increase in the density of the positive charges and the zeta potential of the liposome (30). This study investigated the coating mechanism via possible interactions between chitosan and liposome bilayers. FTIR is one of the effective methods for the investigation of molecular structure and wave number of the vibrational modes of the different functional groups (31). In the drug release properties, a biphasic template was shown an initial fast phase due to drug release from the liposomal surface and then a slow sustained phase due to release from the inner layer (32).

In this study, Chitosan-coated liposome was synthesized to encapsulate GA. The chitosan-coating was confirmed through the characterization of the hydrodynamic diameter, zeta potential, and the obtained FTIR spectrum and the successful encapsulation of GA was also observed. The modification on liposomes increased the density of the positive charges and their functional potential, the chitosan-coated liposome also exhibited slow and controlled release of GA. Although the entrapment efficiencies of the GA in liposomes were relatively low, the optimized efficiency of the encapsulated drug can lead to the development of effective clinical products and novel pharmaceutical formulations in order to improve effectiveness. Overall, this modified spherical nanostructure with the drug-targeted transport leads to reduce the drug dosage and side effects.

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Conflict of interest

The authors declare that they have no conflict of interest.

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