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Bioadhesion and enhanced bioavailability by wheat germ agglutinin-grafted lipid nanoparticles for oral delivery of poorly water-soluble drug bufalin

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ABSTRACT

Wheat germ agglutinin (WGA)-grafted lipid nanoparticles has been prepared and its *in vitro* association with Caco-2 cells has been studied previously. The purpose of this study was to further investigate the potential of WGA-grafted lipid nanoparticles for oral delivery of bufalin, a poorly water soluble drug, by evaluating its *ex vivo* bioadhesion with intestinal mucosal segments and *in vivo* bioavailability. A significant higher adhesion between WGA-grafted lipid nanoparticles and intestinal mucosa was found compared with that of WGA-free lipid nanoparticles ($p < 0.05$). The *in vivo* pharmacodynamic studies were performed by oral administration of WGA-grafted lipid nanoparticles and suspensions to fasted rats. Compared with suspensions, WGA-grafted lipid nanoparticles showed much larger AUC and C_{max}, and a 2.7-fold improvement in oral bioavailability. These results illustrate the potential utility of WGA-grafted lipid nanoparticles for oral delivery of a poorly water-soluble drug such as bufalin.

1. Introduction

Improved intestinal absorption and high bioavailability are desired for oral delivery of a poorly water-soluble drug. There are several factors that contribute to the efficiency and usefulness of pharmaceutical preparations as carriers for oral delivery of poorly water-soluble drugs. These include the capacity of drugs for dissolving, the speed at which the drugs travel through the gastrointestinal tract, and the fate and uptake of drugs by intestinal mucosa after oral administration. In recent years, various drug delivery systems have been developed and investigated for oral delivery of poorly water-soluble drugs, such as solid dispersions, polymer nanoparticles, lipid nanoparticles, micelles, and microemulsions. Among them, lipid nanoparticles have shown a promising effect as carriers of poorly water-soluble drugs and raised many hopes. In addition to the common contribution of nanoparticles for improving the dissolution rate and bioavailability of poorly water-soluble drugs caused by increased surface area resulting in a high dissolution rate, lipid nanoparticles have the capacity to protect the drug from degradation; improve intestinal absorption by surfactants (Luo et al., 2006); and produce non-specific adhesion to the mucosal surface due to small particle size (Lim et al., 2004).

While lipid nanoparticles are one of the important strategies to enhance the bioavailability of a poorly water-soluble drug, extensive research has been conducted to further improve the existing deficiencies. Specific bioadhesive nanoparticle systems may utilize the ligand grafted to the particles for recognition and attachment to the intestinal mucosal surface (Ponchel and Irache, 1998). Therefore, it is proposed that the use of lipid nanoparticles with specific bioadhesive properties may be one of the strategies to further improve intestinal drug absorption and bioavailability.

It has been reported that some plant lectins such as tomato lectin (Lehr and Lee, 1993), wheat germ agglutinin (WGA) (Irache et al., 1994), and peanut agglutinin (Cai and Zhang, 2005), carbohydrate-binding proteins or glycoprotein, may specifically recognize and bind to glycosylated membrane components on the intestinal mucosal surface, resulting in oral specific bioadhesion. The potential of WGA for oral drug delivery has been proved by *in vitro* experiments with Caco-2 cells (Wirth et al., 2002). In addition, WGA is of low toxicity and is resistant to proteolytic degradation (Gabor et al., 1997). Therefore, it has been suggested that WGA may be a desirable ligand for providing oral specific bioadhesion (Weissenböck et al., 2004; Yin et al., 2006).

In our previous studies, WGA was used as a bioadhesive ligand to prepare WGA-grafted lipid nanoparticles for oral delivery of bufalin (Liu et al., 2010a). Bufalin is an active component extracted from toad venom, and has shown significant antitumor effects. However, its low and variable oral absorption due to poor water solubility has limited its clinical application. The results of *in vitro* association of WGA-grafted lipid nanoparticles with Caco-2 cells showed that

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WGA enhanced the binding and cellular uptake of lipid nanoparticles, and sugar specific interaction may have contributed to its enhanced association. These studies suggested that WGA could be useful as a specific bioadhesive ligand for lipid nanoparticles intended for oral administration. Based on these investigations, the bioadhesive properties and the improvement in oral bioavailability need to be clear to determine its efficiency as an oral bioadhesive drug delivery system. Therefore, the objective of this study was to evaluate the bioadhesion potential of WGA-grafted lipid nanoparticles, and evaluate their bioavailability. The *ex vivo* bioadhesion studies were performed with isolated intestinal mucosal segments, and the pharmacokinetics of WGA-grafted lipid nanoparticles were investigated by oral administration of bufalin preparations to rats.

2. Materials and methods

2.1. Materials

Bufalin was purchased from Jiangxi Herbfine Hi-Tech Company Limited (Nanchang, China) with a purity of >98%. Wheat germ agglutinin (WGA) and 6-coumarin were provided by Sigma–Aldrich (St. Louis, MO, USA). Tyrode's solution (1000 mL) contained 8 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2{\cdot}6H_2O$, 1.0 g $NaHCO_3$, 0.05 g NaH_2PO_4, 0.1 g $MgCl_2{\cdot}6H_2O$ and 1.0 g glucose (self-prepared). Other reagents were of analytical grade.

2.2. Preparation of WGA-grafted lipid nanoparticles

Lipid nanoparticles and WGA-grafted lipid nanoparticles were prepared as reported in our previous studies (Liu et al., 2010a).

2.3. Transmission electron microscopy (TEM)

The morphologies of lipid nanoparticles and WGA-grafted lipid nanoparticles were viewed using a transmission electron microscope (JEM 1230; JEOL, Tokyo, Japan). After dilution with double distilled water, one drop of sample was placed on the copper grid. Then, it was stained with 2% solution of uranyl acetate for 15 min followed by staining with lead nitrate for observation.

2.4. Particle size and zeta potential

After dilution with distilled water, the samples (fluorescent 6-coumarin-loaded lipid nanoparticles and 6-coumarin-loaded WGA-grafted lipid nanoparticles) were analyzed for droplet size using a Nicomp™ 380 ZLS Zeta Potential/Particle sizer (PSS Nicomp, Santa Barbara, CA, USA) at a fixed angle of 90° at a temperature of 23 °C. Zeta potential was measured for the above samples at the same temperature.

2.5. Ex vivo bioadhesion

Ex vivo bioadhesion studies were performed using the previously reported methods with slight modification (Yin et al., 2007). *Ex vivo* bioadhesion experiment was conducted as follows: Male Sprague-Dawley rats weighing between 200 g and 240 g were provided by the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine. All experimental protocols in this study were approved by the institutional animal ethical committee. The rats were bred in the Laboratory Animal Center of our university under standard and controlled conditions (five animals per breed cage; constant temperature of 23 ± 3°C and relative humidity of approximately 50%; free access to standard rat chow and water).

Rats were starved for at least 12 h prior to the experiment, but free access to water was allowed. The animals were sacrificed with an overdose of ether, and the intestinal segments were quickly isolated and the luminal content was carefully washed out with Tyrode's solution (37 °C). The intestinal mucosa segments with Peyer's patches (with one Peyer's patches each in the center of the segment) or without Peyer's patches were used. They were from jejunum and ileum, 3 segments in each section and 3 cm in length for each segment. 1 mL of 6-coumarin-loaded preparations was dropped into each intestinal segment to assure that amount of preparations adhered in the intestinal segment can be detected. Then, the intestinal segments were ligated closely and subsequently immersed in Tyrode's solution at 37 °C with oxygen supply. The intestinal segments were taken out after incubation for 2 h. The contents in the intestinal segments were fully withdrawn. Then, the surface was rinsed with PBS (4 °C) three times and blotted dry. All experiments were carried out in the dark. The fluorescence intensity of the sample was determined according to the following methods (Salman et al., 2005; Yin et al., 2007). The same experimental procedure was performed except that the 6-coumarin-loaded preparations were replaced with PBS, which was used as control.

For quantification of coumarin in intestinal segments, first, a fluorescence assay method was set up for sample fluorescence intensity measurement. Fresh intestinal segments were isolated from the jejunum and ileum. Then the lumenal contents attached to the surface of the intestinal segments were carefully removed with Tyrode's solution. Then it was blotted dry, well homogenized and digested in 1 M NaOH. After centrifugation at 14,000 rpm for 10 min, the supernatant was aspirated and kept for use. Increasing amounts of 6-coumarin-loaded lipid nanoparticles or 6-coumarin-loaded WGA-grafted lipid nanoparticles were introduced to the supernatant obtained above. Its fluorescence intensity (FI_{sample}) was measured using a Synergy 2 microplate reader (BioTek Instruments, Winooski, USA) at 485 nm. The fluorescence intensity of supernatant ($FI_{control}$) was also measured at 485 nm and used as a control. Calibration curves were prepared by linear regression analysis of the plot of fluorescence intensity ($FI_{sample} - FI_{control}$) against the amount of coumarin (Q).

For the intestinal segments obtained after the bioadhesion experiment, the samples for fluorescence intensity determination were prepared and the fluorescence intensity was measured as described above. The resulted value of ($FI_{sample} - FI_{control}$) was substituted into the calibration curves, and $Q_{adhered}$ was obtained. The adhesion percentage was calculated according to the following equation:

$$AD\% = \frac{Q_{adhered}}{Q_{initial}} \times 100\%$$

where AD represents the adhesion percentage; $Q_{initial}$ and $Q_{adhered}$ represent the amount of fluorescence before and after *ex vivo* bioadhesion experiments, respectively.

2.6. Bioavailability studies

In this study, the bioavailability of bufalin loaded WGA-grafted nanoparticles was compared with that of bufalin loaded nanoparticles and bufalin suspension. Bufalin suspension was prepared using the previously reported method (Liu et al., 2010b). Bufalin loaded nanoparticles and bufalin loaded WGA-grafted nanoparticles were prepared according to the methods described previously. Male Sprague-Dawley rats (250 ± 20 g) were used in this study. The experimental procedures were approved by the institutional animal ethical committee of Shanghai University of Traditional Chinese Medicine. They were bred and cared under the same condition mentioned above. The rats were fasted for 12 h prior to dosing, but with free access to water. The rats were divided into three groups consisting of 5 animals in each group. Three groups of rats were administered bufalin suspension, bufalin loaded nanoparticles and

Table 1
Results of particles size and zeta potential analysis.

Formulation	Particle size (nm)	Zeta-potential (mV)
6-coumarin-loaded Lipid nanoparticles	118.3 ± 4.5	−16.2 ± 1.6
6-coumarin-loaded WGA-grafted lipid nanoparticles	170.5 ± 9.8	−9.4 ± 2.3

bufalin loaded WGA-grafted nanoparticles, respectively, by oral gavage (dose of 2.4 mg/kg). Approximately 0.5 mL blood sample was collected from the retro-orbital plexus into heparinized tubes before administration and at 0, 30, 45, 60, 90, 120, 150, 240, 360, 480 and 720 min after administration. The blood samples were centrifuged at 8,000 rpm for 10 min, and the supernatant plasma was obtained and transferred to a centrifuge tube. The plasma samples were kept at −20 °C prior to analysis by HPLC. HPLC Assay of bufalin plasma concentration was performed as previously reported (Liu et al., 2010b).

Non-compartment pharmacokinetic analysis was carried out on the data of bufalin plasma concentration versus time using DAS 2.0 software, and the pharmacokinetic parameters such as C_{max}, T_{max}, AUC were calculated.

2.7. Statistical analysis

The results were expressed as mean ± S.D. The statistical significance for the bioadhesion data and the data between different formulations were tested *via* one-way analysis of variance (ANOVA). *P*-value <0.05 was considered significant.

3. Results and discussion

3.1. Particle size and zeta potential analysis

The preparation of WGA-grafted lipid nanoparticles and *in vitro* evaluation of the association with Caco-2 cells have been reported recently (Liu et al., 2010a). In this study, to investigate its bioadhesion capacity with intestinal mucosa, fluorescent 6-coumarin-loaded lipid nanoparticles and 6-coumarin-loaded WGA-grafted lipid nanoparticles were prepared and the particle size as well as zeta potential were determined (Table 1). Both nanoparticles had a size of less than 200 nm. The zeta potentials of the nanoparticles were negative with values of −16.19 mV and −9.43 mV, respectively.

3.2. Morphology

Fig. 1 shows the TEM images of lipid nanoparticles and WGA-grafted lipid nanoparticles. WGA-grafted lipid nanoparticles were close to spherical in shape. They were much larger in size compared with WGA-free lipid nanoparticles.

3.3. Ex vivo bioadhesion study

To find out the potential and efficiency of lectin grafted microparticles or nanoparticles as an oral bioadhesion drug delivery system, it is necessary to study the interactions of lectin-grafted carriers with gastrointestinal mucosa. During the past decades, a lot of experimental approaches have been established and used in bioadhesion assays. They are divided into *in vitro*, *ex vivo* and *in vivo* models. *In vitro* studies are generally carried out by incubation of sample with mucin, followed by centrifugation and adhesive amount determination (Ezpeleta et al., 1999; Kim et al., 2005; Yoncheva et al., 2005). In addition, the bioadhesive action *in vitro*

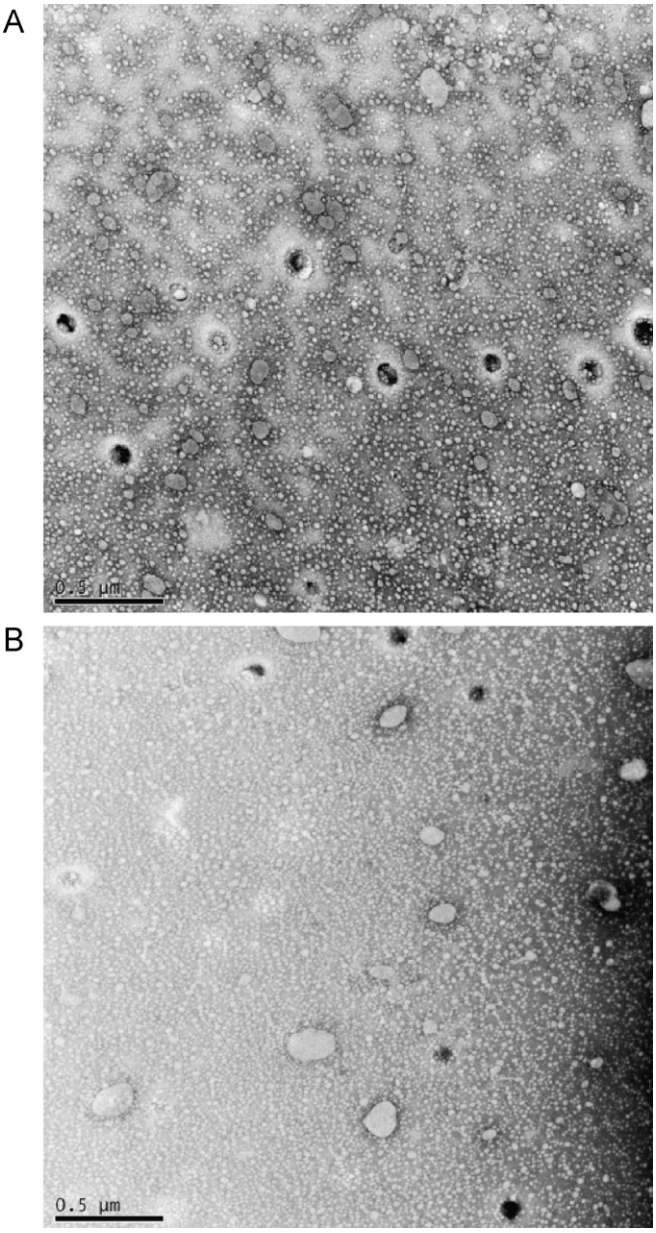

Fig. 1. Transmission electron micrograph of (A) lipid nanoparticles and (B) WGA-grafted lipid nanoparticles (30,000×).

has also been investigated using Caco-2 cells (Weissenböck et al., 2004). For *ex vivo* studies, fresh isolated rat intestinal segments are often used to mimic *in vivo* conditions (Arangoa et al., 2000; Irache et al., 1996; Yin et al., 2007). The lectin-grafted microparticle or nanoparticle suspensions contact directly with mucosal surfaces and the amount of particles attached to the mucosa are evaluated. As to *in vivo* study, the amount of radiolabeled or fluorescence labeled lectin which was associated with intestinal tissues after oral administration has been evaluated (Arbós et al., 2002; Salman et al., 2005; Yoncheva et al., 2005). *Ex vivo* study may more efficiently mimic *in vivo* conditions compared with *in vitro* study. It also may be used to exclude the effect of gastrointestinal tract transit on bioadhesion. Therefore, based on the considerations mentioned above, an *ex vivo* bioadhesion study was carried out in this study.

In vitro release of coumarin from nanoparticles was determined. Less than 1% of 6-coumarin was released in the first 2 h, which assured the fluorescence intensity determined in intestinal segments stemmed from the 6-coumarin-loaded nanoparticles. Fig. 2

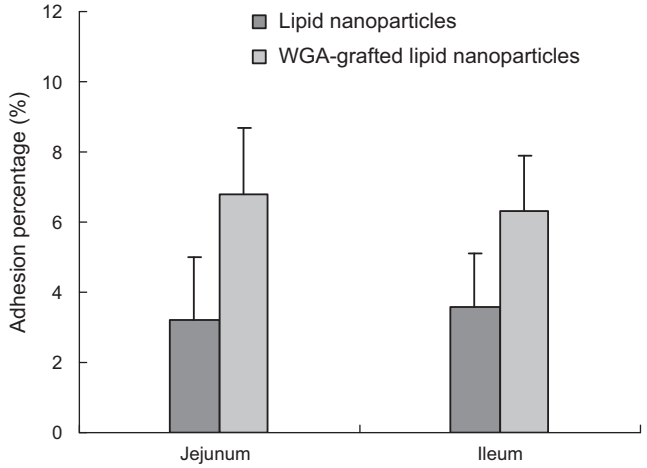

Fig. 2. Adhesive interaction of preparations with the intestinal mucosa samples without Peyer's patches.

shows the interaction of WGA-grafted lipid nanoparticles with the intestinal mucosa segments without Peyer's patches (PP). A decreased interaction was found for ileum compared with jejunum. However, there was no significant difference in adhering percentage between ileum and jejunum ($p > 0.05$), indicating that the extent of interaction was not markedly influenced by the section of intestinal mucosa. The adhering percentage for the lipid nanoparticles without WGA modification was not significantly influenced by the section of intestine ($p > 0.05$). Compared with lipid nanoparticles without WGA modification, WGA-grafted lipid nanoparticles showed much higher (1.7–2.1 fold) interaction with both jejunum and ileum. In addition, a significant increase of adhesion between WGA-grafted lipid nanoparticles with jejunum was found compared with that of lipid nanoparticles without WGA modification ($p < 0.05$). These results suggest the superior adhesive potential of WGA-grafted lipid nanoparticles with the intestines. It appears that WGA-grafted lipid nanoparticles would be beneficial for further improving the oral bioavailability of drugs.

The adhesive behavior of WGA-grafted lipid nanoparticles with the intestinal mucosa samples with PP was also studied (Fig. 3). Adhesive activity in tissue with PP was similar to that in tissue with no PP. WGA-grafted lipid nanoparticles showed a slight increase in adhering percentage for mucosa samples with PP, but this was not significantly different to that of tissue with no PP ($p > 0.05$).

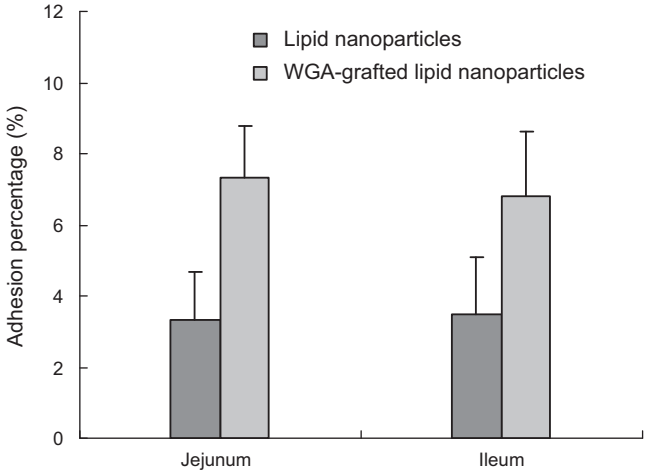

Fig. 3. Adhesive interaction of preparations with the intestinal mucosa samples with Peyer's patches.

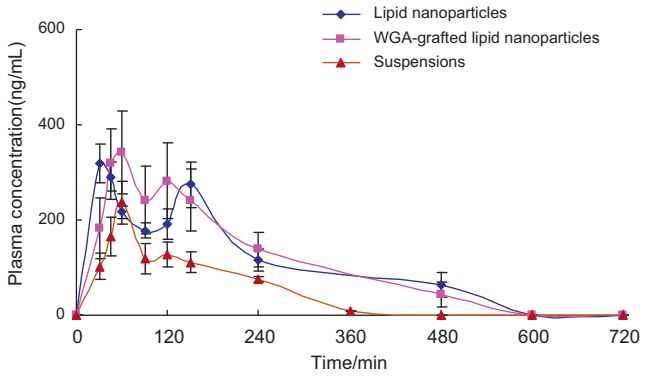

Fig. 4. Plasma concentration profile of bufalin after oral administration of lipid nanoparticles, WGA-grafted lipid nanoparticles and suspensions in rats ($n = 5$).

PP are aggregates of lymphoid follicles composed of M-cells and other cells (Irache et al., 1996) It was described in a review that WGA interacted with both enterocytes and M-cells (Gabor et al., 2004). We deduced that M-cells may contribute to the adhesion and adsorption of WGA-grafted lipid nanoparticles. From the results of this study, no marked contribution of M-cells to the adhesion was observed, and it was suggested there was no specific adhesion of WGA-grafted lipid nanoparticles to the mucosa samples with PP region.

3.4. Bioavailability studies

The *in vivo* study was carried out using Sprague-Dawley rats to quantify bufalin after oral administration of WGA-grafted lipid nanoparticles, lipid nanoparticles and suspensions, respectively. The plasma concentration versus time profiles following oral administration of the preparations above are presented in Fig. 4. Based on comparison of the plasma concentration profiles, both bufalin loaded lipid nanoparticles and WGA-grafted lipid nanoparticles showed much greater improvement in drug absorption than that of the suspensions. The pharmacokinetic parameters for the three preparations are listed in Table 2. The AUC values for the formulations of suspensions, lipid nanoparticles and WGA-grafted lipid nanoparticles were 406.70, 895.74 and 1087.73 μg/L h, respectively. The AUC values for the latter two formulations were significantly higher than that of suspensions ($p < 0.05$), and the corresponding relative bioavailability to suspensions were 220.2% and 267.5%, respectively. Therefore, it is clear that both lipid nanoparticles and WGA-grafted lipid nanoparticles significantly improved the bioavailability of bufalin by the oral route. In addition, the improvement in bioavailability for WGA-grafted lipid nanoparticles is more marked than that of lipid nanoparticles.

The possible mechanisms for common lipid nanoparticles improving the bioavailability of poorly water soluble drugs have been proposed by some researchers. The possible reasons for the bioavailability enhancement involve multiple aspects, such as small particle size, composition of surfactants as absorption enhancers promoting paracellular transport and transcellular transport, and promotion of lymphatic absorption (Bargoni et al., 1998; Luo et al., 2006). Cellular uptake of lipid nanoparticles is an important concern in bioavailability enhancement. It was proposed that adhesion of nanoparticles to the cell surface is a preresiquite for generating the interaction of cells with nanoparticles. Tranchant et al. (1997) and Yuan et al. (2008) reported that the lipid composition, as well as its melting points and length of carbon chain, contributed to the affinity between lipid material and cell membrane. After adhesion, the internalization

Table 2
Pharmacokinetic parameters of bufalin after oral administration of lipid nanoparticles, WGA-grafted lipid nanoparticles and suspensions in rats.

Formulation	C_{max} (μg/L)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (μg/L × h)	AUC_{0-t} (μg/L × h)	MRT (h)
Suspensions	237.36	1.00	2.49	725.16	406.70	1.65
Lipid nanoparticles	299.48	0.65	3.38	1363.61	895.74	2.27
WGA-grafted lipid nanoparticles	411.83	1.05	2.03	1276.04	1087.73	2.43

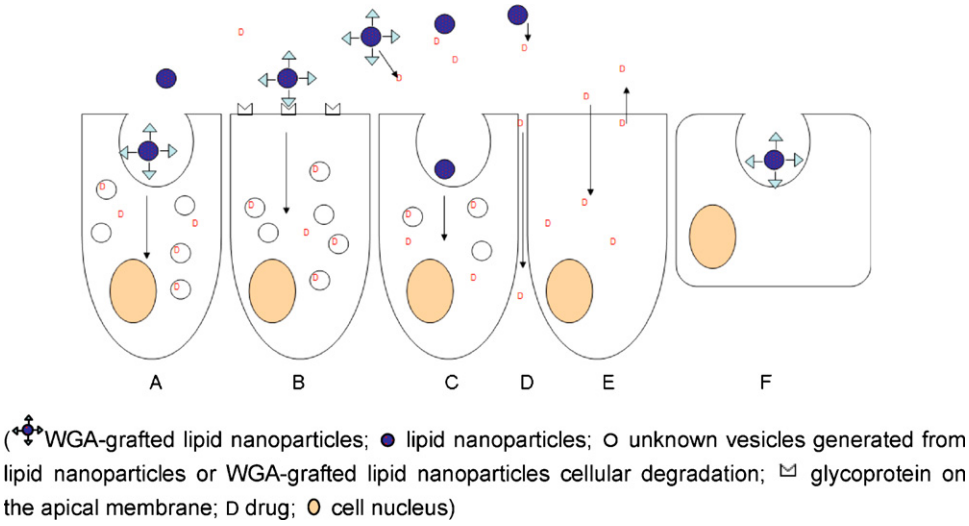

Fig. 5. Schematic diagram of intestinal drug transport from WGA-grafted lipid nanoparticles. The proposed mechanisms include: (A) and (C) fluid phase endocytosis, (B) receptor-mediated endocytosis by specific binding of WGA with glycoprotein, (D) paracellular transport, (E) transcellular transport, (F) endocytosis by M-cells ready for lymphatic absorption.

of nanoparticles in cells may occur by fluid phase endocytosis or phagocytosis (Weissleder et al., 1997). In this study, we focused much on the mechanisms used by WGA-grafted lipid nanoparticles to improve bioavailability. Receptor-mediated endocytosis is generally known as an efficient strategy to target cells and enhance the cellular uptake of nanoparticles. In our previous study, the association of WGA-grafted lipid nanoparticles with Caco-2 cells was evaluated, and the results showed that WGA as a functional ligand enhanced the cellular uptake of nanoparticles compared with WGA-free lipid nanoparticles. Therefore, it was proposed that as well as the mechanisms possessed by common lipid nanoparticles, another possible and important mechanism responsible for WGA-grafted lipid nanoparticles improving drug bioavailability is receptor-mediated endocytosis. In detail, WGA may recognize and adhere to glycosylated structures and generate bioadhesion, and may further conduct signals to cell and trigger endocytosis. On the basis of the above analysis, we produced gave the schematic diagram of intestinal drug transport for WGA-grafted lipid nanoparticles (Fig. 5).

4. Conclusion

The *ex vivo* bioadhesion with intestinal mucosal segments and *in vivo* bioavailability of WGA-grafted lipid nanoparticles for oral delivery of bufalin were evaluated. A significant higher adhesion between WGA-grafted lipid nanoparticles and intestinal mucosa was found compared with that of WGA-free lipid nanoparticles ($p < 0.05$). The WGA-grafted lipid nanoparticles also showed a 2.7-fold improvement in oral bioavailability in rats compared to suspensions. The mechanisms of intestinal drug transport for WGA-grafted lipid nanoparticles were proposed. Our results illustrate the potential utility of WGA-grafted lipid nanoparticles for oral delivery of poorly water-soluble drugs.

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