Application of *Ulex europaeus* Agglutinin I-Modified Liposomes for Oral Vaccine: *Ex Vivo* Bioadhesion and *in Vivo* Immunity

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The conjugation of *Ulex europaeus* agglutinin I (UEAI) onto surface of liposomes has been demonstrated to effectively improve the intestinal absorption of antigen, subsequently induced strong mucosal and systemic immune responses. In this context, we prepared bovine serum albumin (BSA)-encapsulating UEAI-modified liposomes (UEAI-LIP) and unmodified ones (LIP). The specific bioadhesion on mice gastro-intestinal mucosa was studied *ex vivo*. An important increase of interaction between UEAI-conjugated liposomes and the intestinal segments with Peyer's Patches (PPs) was observed compared with the unconjugated one ($p<0.01$). However, under the presence of α-L-fucose, which is the reported specific sugar for UEAI, specifically inhibited the activity of these conjugates. The immune-stimulating activity *in vivo* was studied by measuring immunoglobulin G (IgG) levels in serum and immunoglobulin A (IgA) levels in intestinal mucosal secretions following oral administration of BSA solution, LIP and UEAI-LIP in mice. Results indicate that antigen encapsulated in liposomes, especially the UEAI-modified ones, was favorable for inducing immune response. At 42 d after the first immunization, the highest IgG and IgA antibody levels produced by UEAI-LIP occurred, respectively showing 4.4-fold and 5-fold higher levels compared to those of the groups receiving BSA alone. This data demonstrated high potential of UEAI-modified liposomes for their use as carrier for oral vaccines.

Key words liposome; oral immunization; *Ulex europaeus* agglutinin I; systemic immune response; mucosal immune response

Vaccine is the best preventive strategy against infectious diseases. Traditionally, vaccine research has mainly focused on the induction of systemic immunity through the intramuscular or the subcutaneous route, usually by means of an injection using a needle and a syringe.[1,2] It not only requires trained persons to administer them but also has a higher cost.[3] However, most pathogens and allergens naturally access their hosts through the mucosal membranes.[4] Therefore, the development of new antigen delivery systems for mucosal vaccination would be an interesting goal. Oral vaccines have the advantages of low risk of contamination, self-administration, and a reduced price, furthermore it induce systemic and mucosal immune responses simultaneously.

Nevertheless, oral immunization has a problem of low bioavailability, antigens are degraded by gastric acidity and proteolytic enzymes in the intestinal lumen and only extremely large doses are required to achieve an adequate immune response.[5] Encapsulation of the vaccine in particulate systems became widely employed to solve this problem.[6] It has been demonstrated that oral immunization with antigen-loaded microparticles induces mucosal IgA and systemic IgG antibodies responses, providing a complete immune response.[7,8] Besides protecting the antigen against the harsh environment of the gastro-intestinal tract, microparticles are efficiently taken up by M cells, key players of the mucosal immunity induction. Among all candidates for oral vaccine carriers, liposomes can increase the drug's absorption because of the lipid-bilayer membrane similar to enterocyte.[9,10] But the susceptibility of conventional liposomes to bile salt dissolution and enzymatic degradation in the gastrointestinal tract has remained as the main barrier for their utilization in oral delivery. As a possible way, lectin modified liposomes have been suggested as potential carriers for oral vaccination.[11] *Ulex europaeus* agglutinin I (UEAI), a lectin specific for α-L-fucose residues, which is a glycoprotein with a molecular weight (Mw) of 63000 and binds almost exclusively to the apical surface of mouse Peyer's patch M cells and enhances subsequent macromolecule absorption across the intestinal epithelial barrier.[12] In addition, lectin-modified microparticles allow a sustained release of the antigen, increasing the duration of the contact between antigen and immune cells thus favoring an effective immune response.

In the present study, we have selected UEAI as bio-adhesive and targeted material, bovine serum albumin (BSA) as model antigen to prepare UEAI-modified liposomes and the aims of this work were to investigate their potential as carries of oral vaccine delivery system.

Experimental

Materials Crystallized bovine serum albumin (BSA, The Fourth Military Medical University, China), phosphatidyl choline (PC, Shang hai Taiwei Medicine Co., Ltd., China), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC, GL Biochem, Shanghai, Ltd., China), *N*-hydroxysuccinimide (NHS, GL Biochem, Shanghai, Ltd., China), cholesterol (CH, Tianjin Bodi Chemical Co., China), *U. europaeus* agglutinin I (UEAI, Sigma), succine anhydride (Tianjin Bodi Chemical Co., China), 1-palmitoyl-2-[1,2-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine powder (NBD-PE, Sigma), sepharose 4B (GE Healthcare).

Preparation of Conventional Liposomes (LIP) and UEAI-Conjugated Liposomes (UEAI-LIP) BSA-loaded large unilamellar vesicles (LUV) composed of 200 mg PC and 100 mg CH (obtained LIP) or 200 mg PC, 50 mg CH and 50 mg cholesterol succinate (CHS, obtained CHS-LIP) was prepared by conventional reverse-phase evaporation technique[13]: the lipids dissolved in ethyl ether were slowly evaporated until thin film was formed. The film was redissolved by 15 ml dichloromethane (oil phase), and 5 ml aqueous phase containing 10 mg BSA was added to form water-in-oil emulsion by water-bath sonication. And the emulsion was rotary-evaporated entirely and thoroughly hydrated to form 10 ml liposomes suspension. UEAI was covalently bound to liposomes by an appropriate modification of the two-stage carbodiimide method.[14,15] In brief, the carboxylic groups on the CHS-LIP surface were activated in phosphate buffered saline (PBS) by addi-

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tion of a variable amount of EDC and NHS at 4 °C for 12 h.[16] After removing the excess carbodiimide reagent, 125 μg UEAI solution was added to 1 ml liposomes and coupling was carried out by overnight incubation at room temperature to obtain UEAI modified liposomes (UEAI-LIP). Additionally, for the purpose of fluorimetric characterization, 40 μg of the fluorescence marker NBD-PE was incorporated in above LIP and UEAI-LIP to obtained NBD-LIP and NBD-UEAI-LIP.

Characterization of Liposomes The mean diameter and particle size distribution were determined using a laser light scattering instrument (LS 320; Beckman, U.S.A.). Size was expressed as volume diameter. The zeta potential was measured using a zeta potential analyzer (Delsa 440SX; Beckman, U.S.A.). Encapsulation efficiency (EE%) was detected by Bradford method (n=3). Conjugation efficiency (CE%) was quantified using gel permeation chromatography on Sepharose 4B column (n=3). Placebo liposomes formulation was used as control.

$$EE\% = [(\text{total amount of BSA added} - \text{amount of BSA detected in supernatants})/\text{total amount of BSA added}] \times 100\% \quad (1)$$

$$CE\% = [(\text{total amount of UEAI added} - \text{amount of free UEAI})/\text{total amount of UEAI added}] \times 100\% \quad (2)$$

Integrity Analysis of BSA in Liposomes Integrity of BSA before and after encapsulated into liposomes was analyzed by vertical, discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Activity Analysis of UEAI after Bounding to Surface of Liposomes Lectin was specific protein capable of binding to or agglutinating human red blood cells. In order to verify biologic activity of UEAI after linking to surface of liposomes, we did agglutination test through 5% standard human red blood cells suspension (RBCS). UEAI modified liposomes (collected by Sepharose 4B column), equivalent UEAI solution as positive control, isoosmotic PBS as negative control were all added into diluted RBCS respectively, at different time in 3 h, we detected absorbance at 620 nm and draw the sedimentation curve of RBCS.

***In Vitro* Release Studies** *In vitro* release experiments of BSA from LIP and UEAI-LIP were performed at 37±0.5 °C using the dynamic dialysis method. The liposomes were incubated for 2 h in 0.1 M HCl (pH 1.0, 250 ml), as the average gastric emptying time was about 2 h.[17] Then the dissolution medium was replaced with 250 ml of pH 6.8 PBS buffer and tested for drug release for 3 h, as the average small intestinal transit time was about 3 h. Next, 250 ml of pH 7.4 PBS buffer was used to test for drug release for 19 h. Dialysis bags (100000 Mw cut off) containing 5±0.1 ml liposomes were introduced into the system. Each 2 ml sample was withdrawn at designated time points, then filtered and assayed for drug released.

***Ex Vivo* Bioadhesion on Mice Gastro-Intestinal Mucosa.**

***Ex Vivo* Experiments** Female KM mice (SPF, 18—22 g, provided by Shenyang Pharmaceutical University Animals Center) were used for *ex vivo* studies. All animals were treated complied with the guidelines of the National Act on the use of experimental animals (People's Republic of China). On the day before experiment, mice were starved overnight but free access to water. Fresh stomach, 20 cm of small intestines (six peyer's patches (PPs) in all), small intestine (without PPs) and colon of sacrificed mice were quickly excised and rinsed with warm (37 °C) Tyrode to remove any contents. Each stomach and intestinal segment was ligaturated, filled with 1 ml suspension of NBD-LIP or NBD-UEAI-LIP and immediately incubated in a bath containing Tyrode gassed with oxygen. All experiments were carried out in the dark at 37 °C. After 2 h, these segments were recovered and the liposomes suspension was removed. The segments were washed three times in ice-cold PBS to remove residual liposomes and blotted dry and weighed precisely. Control stomach and intestinal segment were performed incubated under the same conditions with PBS only.

Quantitative Analysis The bioadhesion effect to different segments of NBD-LIP or NBD-UEAI-LIP was expressed by quantitative amount of PC adhered and determined by the fluorescence measurement. Each biological sample was homogenized using a tissue homogenizer with 2 ml pH 7.4 PBS. After centrifugation (10000 rpm·min^{-1}, 10 min), the fluorescence intensity of the supernatant was measured using the fluorometer. The fluorescence intensity of each empty stomach and intestinal segment was determined and used as control. Fluorescence intensity of other segments was measured and deducted the control value respectively. Therefore, the calibration curves were set up. The interaction was defined as the amount of bioadhered PC per gram of biological samples.

Competition Studies Each stomach and enter-segment was ligaturated, filled with 1 ml suspension of NBD-LIP or NBD-UEAI-LIP simultaneously with a competing sugar, α-L-fucose present in no less than 10-fold concentration excess of UEAI. These liposomes suspensions were then incubated with biological sample for 2 h, and the binding amount of PC was determined as before.

***In Vivo* Immune Research by Oral Immunization.**

Immunization Protocol Female KM mice (SPF, 18—22 g) were used throughout all experiments. Animals were housed in groups of eighteen with free access to food and water. Three groups of mice were immunized with the same quantity of BSA, respectively received BSA solution, LIP and UEAI-LIP. Before oral immunizations, mice were deprived of food for 12 h and then administered 0.5 ml different formulations by intragastrically (i.g.) with a urethral polyvinyl catheter calibrated to reach the stomach. All animals received four immunization doses at 0, 7, 14 and 21 d and samples were collected after 7, 14, 21, 28, 35 and 42 d of primary immunization.

Sample Collection Every 7 d after administration, three mice of each group were bled by retro-orbital puncture and serum was collected after coagulation for 30 min and centrifugation at 1000 ***g*** for 15 min, and then stored at −20 °C until used for assessing the IgG antibody activity. The proximal intestine (from the top of the duodenum to 20 cm below the top of it) was excised with scissors to open the intestinal lumen.[18] Intestinal mucus was collected from the intestine by gentle scraping with spatula and transferred to a tube. After addition to 5 ml PBS, washed samples were vortexed for 1 min, and centrifuged at 10000 rpm for 20 min, and supernatants was collected and stored at −80 °C until used for assessing IgA antibody in the intestine.

Measurement of Specific IgG and IgA Response Serum IgG and mucosal IgA levels in mice plasma and intestinal washes were determined by ELISA with commercially available solid-phase enzyme-linked immunoassay kit (n=6, Shanghai Shen-xiong Biology Co., China). Each well of 96-well culture plate was coated with 100 μl per well of a 10 μg/ml BSA solution in 0.05 M sodium carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. After being washed three times with PBS containing 0.05% Tween-20 (PBST), the wells were blocked with 100 μl 2% skim milk in PBST for 1 h at room temperature to avoid non-specific adsorption of the antibodies. One hundred microliters samples were added into ninety-six-well polystyrene plates, after 1 h at 37 °C, plates were washed three times by PBST, then incubated with 100 μl horse radish peroxidase (HRP) conjugated goat anti-mouse IgA for 1 h or IgG for 30 min at 37 °C. IgG antibody present in serum were detected using 1/5 dilution. IgA antibodies present in mucosal samples were analyzed using the first dilution of the sample. After washing, 100 μl of *o*-phenylenediamine dichloride (OPD) in phosphate citrate buffer was added and acted for 10 min. Color development was stopped *via* the addition of 50 μl of 1 N H_2SO_4 and the absorbance was measured at 492 nm.

Statistical Analysis Statistical analysis was performed on the data obtained in the *in vitro* and *in vivo* studies using a Student's *t*-test or analysis of variance (ANOVA). All the experiments were carried out in triplicate (n=3) and the differences between the results were judged using ANOVA parametric test at a significance level of 0.05 (*i.e.*, $p<0.05$).

Results and Discussion

Characteristics of Liposomes The main physicochemical characteristics of LIP, NBD-LIP, UEAI-LIP and NBD-UEAI-LIP were listed in Table 1. The mean diameters of all performed liposomes were about 5 μm. And almost 90% of the liposomes produced ranged in size between 1 to 10 μm, what was regarded as the most appropriate size distribution to induce systemic and mucosal immune response. These

Table 1. Characteristics of LIP, NBD-LIP, UEAI-LIP and NBD-UEAI-LIP (Mean±S.D, n=3)

Characteristics	LIP	NBD-LIP	UEAI-LIP	NBD-UEAI-LIP
Mean diameter (μm)	4.0±0.4	4.6±1.1	5.1±1.7	5.6±0.8
Zeta potential (mV)	−15.4±2.6	−16.3±4.1	−24.4±4.7	−21.6±3.5
EE%	69.7±3.4	64.2±6.1	72.4±4.1	68.5±6.1
CE%	—	—	65.4±3.8	61.3±4.1

LIP: conventional liposomes. NBD-LIP: NBD-PE was incorporated into LIP. UEAI-LIP: UEAI-conjugated liposomes. NBD-UEAI-LIP: NBD-PE was incorporated into UEAI-LIP.

liposomes were negatively charged and the Zeta potential ranged from −15.4±2.6 to −24.4±4.7 mV. The results of drug entrapment efficiency revealed about 64.2±6.1 to 72.4±4.1% of drugs having been incorporated into the liposomes. The amount of UEAI conjugated onto the surface of liposomes was calculated through Sepharose 4B column and the CE% was 61.3±4.1 to 65.4±3.8%. Moreover, the haemagglutination test confirmed UEAI still retained their specific carbohydrates binding activity on the surface of liposomes. These fluorescent labeled liposomes, NBD-LIP and NBD-UEAI-LIP, were used throughout the present study to estimate the *ex vivo* bioadhesion studies. And liposomes with or without UEAI modification, LIP and UEAI-LIP, were used to evaluate the immune studies.

As was known, UEAI was the targeted and bio-adhesive material modified on the surface of liposomes, so its' stability of conjugating bond between UEAI and liposomes was also considered, we continuously determined the CE% of UEAI-LIP once for one month after storage for six months at 4 °C, the RSD% was 6.29%. The data proved that the conjugating bond was stable and the targeted effect of UEAI did not change. Stability studies confirmed UEAI-LIP on storage at 25±1 °C, around 10.6% of antigen leakage was detected from the system whereas at 4±1 °C only 6.3% of antigen leaked after six months. Meanwhile, no change in unilamellarity and insignificant difference in vesicular size (data were not shown) were observed after storage of UEAI-LIP for six months.

Integrity Confirmation of BSA in Liposomes Integrality of BSA before and after release from liposomes was analyzed by vertical, discontinuous SDS-PAGE. From Fig. 1, we could see that electrophoresis band of protein release from liposomes was the same location as that of unencapsulated BSA, which suggested that the method was mild and the structural integrity of BSA was not significantly destroyed by the entrapment procedure.

Activity Confirmation of UEAI after Bounding to Surface of Liposomes UEAI, a lectin specific for α-L-fucose residues, binds almost exclusively to M cells on the apical surface of mice Peyer's patches and conveys signals to cells in order to trigger subsequent macromolecule absorption, and then induces systemic and mucosal immune response. So the activity of UEAI should be validated after its amino groups were conjugated with activated carboxylic groups on the surface of liposomes. Figure 2 showed UEAI whether free or linked to surface of liposomes could get similar sedimentation effect to RBCS, and the fitting correlation coefficient was 0.9937. So, the haemagglutination test confirmed UEAI still remained their specific binding activity even if conjugated on the surface of liposomes.

***In Vitro* Release Studies** The effect of UEAI on surface of liposomes on release of encapsulated bioactive BSA was investigated by studying release of BSA from UEAI-LIP and compared with that of LIP. Figure 3 indicated that there was significant difference in the release rates, encapsulated bioactive macromolecule released for prolonged period of time from UEAI-LIP as compared to LIP over 24 h in the simulated gastrointestinal pH conditions ($p<0.05$). At first 2 h, 31.62±1.03% of BSA released from LIP, suggesting there existed a obvious burst release effect, which was due to the amount of protein present on surface of liposomes, and the more drug released before they reached small intestine, the more antigen would be degraded by digestive enzyme and strong acid. As for UEAI-LIP, the initial release in 2 h was only 16.71±1.75%, which meant more antigens would be transported to small intestine. Furthermore, in whole release pattern, UEAI-LIP depicted gradual release of protein, and the accumulative release was 53.86±2.32% at 12 h and 76.22±3.28% at 24 h. One reason was that UEAI on the surface of liposomes could suppress 'burst effect' to some extent by space block of UEAI, moreover, even if UEAI incubating with abnormal high amounts of pepsin, trypsin and pancreatin, no degradation products were observed,[7] that

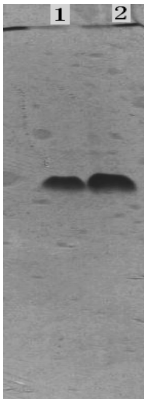

Fig. 1. SDS-PAGE Analysis of Native BSA

Lane 1: encapsulated BSA released from BSA-loaded liposomes; lane 2: plain BSA antigen (control).

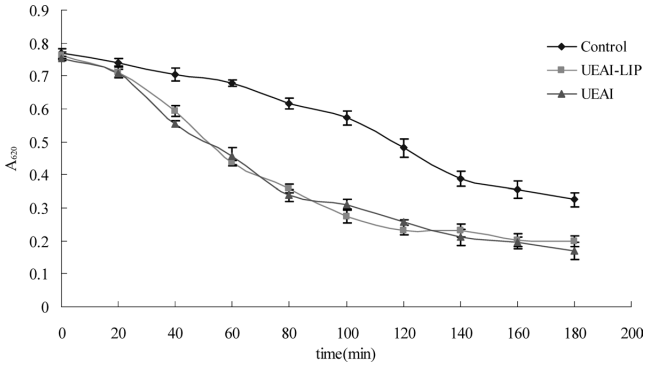

Fig. 2. The Sedimentation Curve of Diluted RBCS Influenced by Isoosmotic PBS Solution (Control), UEAI Solution (UEAI) and UEAI-Modified Liposomes (UEAI-LIP)

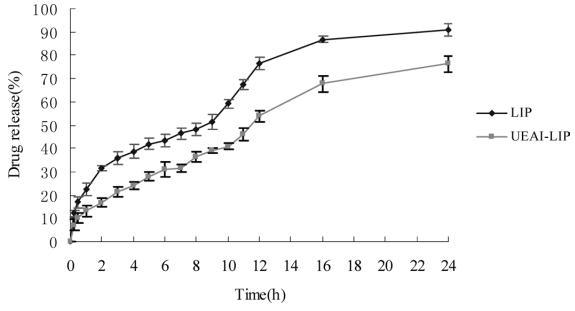

Fig. 3. Cumulative Release of BSA from LIP (Conventional Liposomes) and UEAI-LIP (UEAI-Modified Liposomes) in the Simulated Gastrointestinal pH Conditions (Mean±S.D, $n=6$)

was to say, this protection effect of UEAI to liposomes would maintain constantly. And the other, under pH 7.4, BSA and UEAI both had negative charge, so the rejection between molecules prevented release of BSA. This could not only guarantee enough antigens were transported to M cells, but also warranted longer release time strongly stimulated the immune system to induce mucosal and systemic immune response. And the release data obtained of UEAI-LIP was fitted with the equations of first-order, Higuchi and Weibull,[19] respectively, in which Weibull equation fitting results was the best ($y=1.5134x+3.0709$, $r=0.9710$).

At the same time, we also considered another important question. As was known to all, BSA was a protein drug composed by many amino acids, and the amids of unloaded BSA in outer water-phase and BSA exposed on the liposome surface might also react with activated succinate during the process of 12 h incubation. In order to verify the extent of conjugation reaction, we firstly prepared UEAI-modified blank liposomes (BSA excluded) and determined the UEAI content of unlinked to the surface of liposomes through Sepharose 4B columns (W_{UEAI}). Then, we prepared the BSA-loaded liposomes and detected the unloaded BSA content in outer water-phase (W_{BSA}). At the same time, according to the same formulation, we also prepared UEAI-modified BSA-loaded liposomes and detected the total content of protein in outer water-phase (W_{total}). W_{added} was defined as BSA weight primary added used to prepare the BSA-loaded liposomes. The content of conjugated BSA with activated succinate was calculated as the formula below:

$$\text{conjugated BSA}\,\% = [W_{BSA} - (W_{total} - W_{UEAI})]/W_{added} \quad (3)$$

Therefore, we measured the coupling content of free BSA onto the surface of liposomes for three times and the data was 8.58±4.03% ($n=3$). The data told us the conjugation process existed, and the coupling of BSA onto the surface of liposomes maybe one reason for suppressing "burst effect" compared to LIP.

***Ex Vivo* Bioadhesion** In order to evaluate the potential of UEAI as an intestinal bioadhesive and targeted material for oral vaccine delivery system, the binding of UEAI-conjugate fluorescent liposomes to mice stomach and enter-segments was compared with the binding of control liposomes without UEAI conjugation. Analysis of the biological segments extracted fluorescence revealed the amount of liposomes bioadhered on the mucosa and the results were displayed in Fig. 4. The bioadhesion of conventional liposomes on different structures of stomach and enter-segments was shown to be similar. But to UEAI-conjugated liposomes, significant increases of interaction between liposomes and the intestinal mucosa (with PPs) could be observed compared to the unconjugated one ($p<0.01$), but still no difference in the extent of interaction was found between stomach, small intestine (without PPs) and colon ($p>0.05$). These studies demonstrated, binding sites of UEAI to the whole gastrointestinal tract are mainly located in specialized structures called PPs on intestinal mucosa, but seldom existed in stomach and colon. So it was likely that the modification of UEAI did not increase the adhesive amount in other tissues. The higher binding efficiency of UEAI-modified liposomes (almost 4 times compared with conventional liposomes) proved UEAI could specifically target mouse M cells on PPs, which could enhance significantly adhesive amount and time of liposomes. This result has been extended to demonstrate effective M cell targeting of UEAI-conjugated liposomes could be as candidate vaccine delivery vehicles.

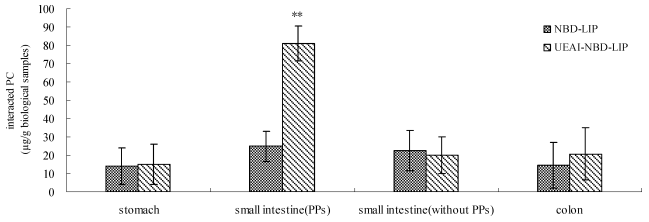

Fig. 4. Interaction of NBD-PE Incorporated BSA-Loaded Liposomes (NBD-LIP) and UEAI-Conjugated Liposomes (NBD-UEAI-LIP) with Mice Stomach, Small Intestine (PPs), Small Intestine (without PPs) and Colon Segments (mean±S.D, $n=3$)

Statistical difference between two groups was reported as: ** $p<0.01$ *vs.* NBD-LIP group (control). Liposomes were incubated with biological segments at 37 °C for 2 h.

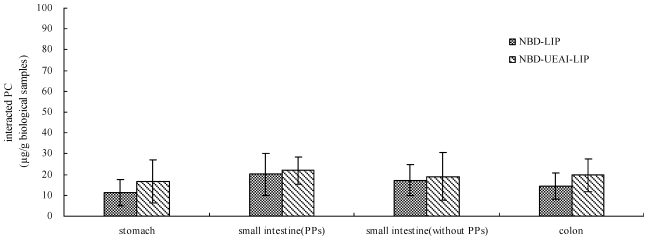

Fig. 5. Interaction of NBD-PE Incorporated BSA-Loaded Liposomes (NBD-LIP) and UEAI-Conjugated Liposomes (NBD-UEAI-LIP) with Mice Stomach, Small Intestine (PPs), Small Intestine (without PPs) and Colon Segments Competed by Excess of α-L-Fucose (Mean±S.D, $n=3$)

The liposomes suspensions with α-L-fucose were incubated with stomach and enter-segments at 37 °C for 2 h.

As in the experiments with the biological segments, liposomes were incubated simultaneously with an excess of competing sugar, α-L-fucose. The results were shown in Fig. 5. The inhibition studies with fucose confirmed the reported specific characteristics of UEAI for α-L-fucose. The competing sugar significantly reduced the binding of UEAI conjugated liposomes to different biological segments. Under these conditions, the interaction was approximately similar to that obtained with control particles ($p>0.05$). However, the interactions of conventional liposomes were not influenced by the presence of sugar. The competition studies showed α-L-fucose inhibited UEAI binding effectively, which provided further evidence for the specific nature of the binding of UEAI to intestinal α-L-fucose residues on M cells of PPs.

***In Vivo* Immune Response in Mice** Figure 6 showed the levels of BSA-specific IgG antibody in the plasma that had received oral immunization with various formulations of BSA. The antibody values recorded as optical density (OD). The effect of different formulations was statistically analyzed by using ANOVA followed by comparison tests. When immunization doses were adjusted to provide a standard amount of 500 μg BSA, the ability for production of plasma anti-BSA IgG antibody was the highest for UEAI-modified liposomes, next for unmodified ones, and the lowest for BSA solution. After 42 d, the IgG levels induced by groups of UEAI-LIP and LIP respectively represented an increase of 4.4 fold ($p<0.01$) and 1.9 fold ($p<0.05$) compared to that of group of BSA solution. The lowest specific IgG antibodies levels

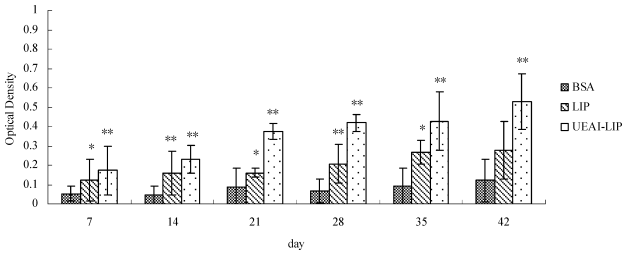

Fig. 6. Production of BSA-Specific IgG Antibody after Oral Immunization with BSA Solution (BSA), BSA-Loaded Liposomes (LIP) and UEAI Modified Liposomes (UEAI-LIP)

Mice received four immunization doses by gastric intubation at 0, 7, 14 and 21 d and samples were collected after 7, 14, 21, 28, 35 and 42 d of primary immunization and assayed for BSA-specific IgG antibody by the ELISA method. Statistical difference between two groups was reported as: $*p<0.05$, $**p<0.01$ *vs.* BSA solution group (control).

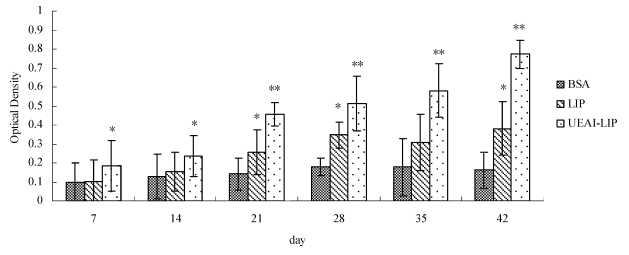

Fig. 7. Production of BSA-Specific IgA Antibody after Oral Immunization with BSA Solution (BSA), BSA-Loaded Liposomes (LIP) and UEAI Modified Liposomes (UEAI-LIP)

Mice received four immunization doses by gastric intubation at 0, 7, 14 and 21 d and samples were collected after 7, 14, 21, 28, 35 and 42 d of primary immunization and assayed for BSA-specific IgA antibody by the ELISA method. Statistical difference between two groups was reported as: $*p<0.05$, $**p<0.01$ *vs.* BSA solution group (control).

were detected in serum of animals vaccinated with plain antigen, which emphasized the utilization of this liposomal system as an adjuvant for antigens delivery *via* oral route, as they have the ability to change antigen immunogenicity. Unmodified liposome gave lower immunological response, this may be due to the comparatively less stability, quick release of encapsulated bioactive and less adherence to intestinal mucosa. These results were in accordance with the *in vitro* release study, UEAI-LIP could maintain longer time to effectively stimulate M cells to generate the higher immune response.

Next, we determined the ability of oral delivered above different formulations to elicit local immunity in the intestinal mucosa of mice. Figure 7 showed the BSA-specific IgA antibody in the intestinal wash of the individual mice. It also could be inferred from the data that surface UEAI-modified liposomes effectively elicited mucosal immune response (IgA levels) than unmodified liposomes and BSA solution. Administration of UEAI-LIP resulted in an approx. fivefold increase in the absorbance in the ELISA compared with that for the samples from the BSA solution immunized mice at day 42 ($p<0.01$). Meanwhile, after primary immunization, with the increase of immune times, the IgA levels produced by BSA solution, LIP and UEAI-LIP was found to be nearly unchanged, slowly increased and noticeably increased which showed modification of lectin could better improve absorption of antigen. The efficient priming of mucosal immune response by UEAI-LIP can be correlated to their higher cellular uptake and controlled release of entrapped protein antigen across the mucosal surface.

Above results proved through increase of intestinal bioadhesion and endocytosis, UEAI-modified liposomes successfully used to target macromolecules to mice gut loop and enhance subsequent macromolecule absorption across the intestinal epithelial barrier thereby induced more intense immune response. But to group of BSA solution, it could only induce weak systemic and mucosal immunity and unchange basically within 42 d because BSA was a kind of protein and degraded almost totally processing gastrointestinal tract by acid and digestive enzyme, moreover, its' macromolecular weight was also a barrier to absorption to oral route. On the whole, whether UEAI modified or unmodified liposomes both had some protection effect to antigen from degradation by luminal factors that normally limit absorption which meant more antigen could be transferred to intestine to induce much higher immune response compared with free BSA. Moreover, due to unmodified liposomes must be transported fast from the gastro-intestinal tract at the initial stage soon after oral administration, it only did induce an obviously less immune response when compared with UEAI-LIP which could adhere with PPs for longer time period. On the other hand, improvement of the systemic and mucosal immune response was observed with increasing immune times. Especially to group immunized by UEAI-LIP, the antibodies levels increased obviously which proved repeated booster immunization was important to oral route, because immune system should have a period of response time to irritation of outside antigen.

Conclusion

The systemic and mucosal immune responses induced after oral administration of mice were compared with free antigen, unmodified liposomes and UEAI-modified liposomes. These antigens were successfully encapsulated within various liposomal formulations without causing any damage in the protein structure. *Ex vivo* bioadhesive studies on mice confirmed an important increase of specific interaction between UEAI-conjugated liposomes and the intestinal segments with Peyer's patches, therefore we chose UEAI as a bioadhesive and targeted agent for oral vaccine. *In vivo* immune response told us that compared with unmodified liposomes and free antigen, UEAI could effectively enhance the intestinal bioadhesion and uptake of modified liposomes, subsequently induced higher mucosal IgA antibody and systemic IgG antibody response. As a consequence, UEAI-modified liposomes could be used as carries of oral vaccine delivery and could enhance the effectiveness of candidate vaccine antigens.

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