Lectin-bearing Polymerized Liposomes as Potential Oral Vaccine Carriers

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Purpose. The potential of using lectin-modified polymerized liposomes as Peyer's patch targeted oral delivery vehicles was examined.
Methods. Two types of lectins, Ulex Europaeus Agglutinin I (UEA I) and Wheat Germ Agglutinin (WGA), were modified with a hydrophobic anchor N-glutaryl-phosphotidylethanolamine (NGPE). The modified lectins were incorporated into liposome bilayers and the liposomes were subsequently stabilized through polymerization. The presence of the lectins on the liposome surfaces was first confirmed with X-ray photoelectron spectroscopy. Surface-immobilized lectins were then shown to retain their carbohydrate binding activities as well as specificities based on an *in vitro* aggregation assay. Finally, delivery efficiencies of lectin-bearing liposomes were determined in mice.
Results. About 10.5% UEA I liposomes and 5.8% WGA liposomes were taken up from the gastrointestinal tract. These numbers are significantly higher than the 3.2% observed in the case of lectin-free liposomes. At the same time, UEA I liposomes exhibited the most effective Peyer's patch targeting among the three, which directly correlated with the highest delivery efficiency observed.
Conclusions. This establishes that lectin modification of liposomes can promote binding to Peyer's patches, which will give improved efficiency for Peyer's patch targeted delivery. All these point to the potential for these lectin-modified liposomes as novel vehicles for oral vaccination.

KEY WORDS: lectin; polymerized liposomes; Peyer's patches; surface modification; targeted delivery.

INTRODUCTION

Novel oral vaccine formulations protect antigens against degradation by encapsulating them into carrier systems. While various systems have been studied for potential application in oral vaccine delivery, liposomes have been shown to have some advantages as vaccine carriers (1, 2). Susceptibility of conventional liposomes to bile salt dissolution in the gastrointestinal tract, however, has limited their utilization in oral delivery (1, 3, 4). Disruption of liposomal bilayers leads to exposure of encapsulated vaccines and therefore the loss of protective function. As a potential solution, polymerized liposomes have been proposed (3). By creating a crosslinked network in liposomal membranes, we have been able to significantly improve liposome stability in animal gastrointestinal tracts compared to conventional liposomes (5).

Peyer's patches are a collection of organized lymphoid tissues lining the intestinal tract. They are the most important structural units of gut-associated lymphoid tissue. Peyer's patches contain a large number of IgA-committed cells which can be stimulated by antigens absorbed through M cells, specialized "sampling" cells which can be found in the epithelium overlying the patches (6, 7). As a result, Peyer's patches are the primary induction sites for mucosal immunity and the target sites for oral vaccine delivery (6).

Development of a successful oral vaccine formulation involves not only the encapsulation and protection of antigens, but delivering them into the Peyer's patches as well. It is now generally accepted that a small percentage of orally administered particles can be taken up by M cells and transported into Peyer's patches (8). This was confirmed by our previous observation that a small amount of polymerized liposomes can indeed be taken up by mouse Peyer's patches after oral administration, but only with limited efficiency (5). In 1987, Neutra *et al.* reported that particles capable of adhering to M cell surfaces are taken up more effectively (9). In attempts to optimize the delivery efficiency for our polymerized liposomes, we modified the liposome surfaces with molecules which can promote liposome binding to M cells.

Lectins are a heterogeneous group of proteins or glycoproteins that recognize carbohydrate residues on cell surface glycoconjugates with a high degree of specificity. Lectins have been applied as useful reagents for the cytochemical detection of cell surface glycoconjugates on the mammalian intestinal epithelium. Recently, it was reported that certain lectins such as Ulex Europaeus Agglutinin I (UEA I), when exposed to mouse Peyer's patches, are almost exclusively M cell specific (10, 11). This suggests the presence of unique fucosylated glycoconjugates on mouse M cell surfaces. Other lectins with affinity to sialic acids such as Wheat Germ Agglutinin (WGA) fail to specifically label M cells, but instead bind to all epithelial cells with equal specificity.

In this paper, lectin Ulex Europaeus Agglutinin I was used to modify the liposome surfaces for M cell targeted liposome delivery. Wheat Germ Agglutinin was also used in order to study the effect of binding specificity on the extent of liposome uptake.

MATERIALS AND METHODS

Lectin Modification

UEA I and WGA (both purchased from Sigma) were derivatized with N-glutaryl-phosphotidylethanolamine (NGPE, Avanti Polar Lipids) using 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC, Pierce) and N-hydroxysulfosuccinimide (NHS, Pierce) (12). Briefly, 1.2 mg of dried NGPE was dissolved in 2 mL MES buffer (50 mM, pH 5.5) containing 5 mg/mL n-octylglucoside (Sigma). 48 mg EDC and 60 mg NHS were then added and the mixture incubated at room temperature for 5 minutes before 4 mL of HEPES buffer (pH 7.5) containing 2 mg/mL UEA I or WGA was added. The pH of the reaction mixture was adjusted to 7.6 and the mixture was incubated overnight at 4°C under constant gentle agitation. The product was dialyzed in a Spectrum® dialysis tube (MWCO 1,000) overnight against PBS (20 mM, pH7.6) in the presence of 50 mg Biobeads® (Biorad) to facilitate the removal of the detergent

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and other excess reagents. The final solution was stored at 4°C until use.

Determination of the Degree of Lectin Modification

The degree of modification was determined with 2,4,6-trinitro-benzenesulphonic acid (TNBS) assay. 700 μL of aqueous solution containing UEA I, WGA, NGPE-modified UEA I, or NGPE-modified WGA (all diluted to 0.5 mg of protein/mL) was added to 700 μL of 0.1 M sodium borate buffer (pH 9.2). 350 μL of TNBS aqueous solution (1.65 mg/mL) was added and the solution was rapidly mixed. After incubation at 40°C for 45 minutes, the reaction was stopped by adding 350 μL of 0.1 M NaH_2PO_4 containing 1.5 mM Na_2SO_3 and absorption at 420 nm was determined on a UV/VIS spectrometer (Perkin-Elmer 553).

Formation of Lectin-Bearing Polymerized Liposomes

30 mg of 1,2-Di(2,4-octadecadienoyl)-sn-glycerol-3-phosphorylcholine (DODPC, Kawahara Yuka Co.) was dissolved in chloroform and ethyl ether (volume ratio 1:10). 3 μL of 1,2-Dipalmitoyl-L-3-phosphatidyl[N-methyl-^{3}H]choline ([^{3}H]DPPC, Amersham Life Science, Inc.) (1mCi/mL) was added as a lipid marker. The mixture was rotary-evaporated into a thin lipid film. The dried lipid was hydrated with 3 mL of the modified UEA I or WGA solution obtained as described in the last section. PBS was used instead when preparing lectin-free liposomes. The liposomes obtained were freeze-thawed and extruded through stacked 0.1 μm polycarbonate membranes (Poretics). Finally, polymerization was carried out overnight at room temperature with redox initiators $Na_2S_2O_5$ and $K_2S_2O_8$ (10 mol% of DODPC, $Na_2S_2O_5$: $K_2S_2O_8$ = 1:1). The resulting polymerized liposomes were purified on a Biogel A15M column (1.5cm×12cm) with PBS as eluting buffer. Fractions containing liposomes were identified by their turbid appearance and were collected. The final lipid concentration was adjusted to ~ 10 mg/mL.

The stability of lectin modified polymerized liposomes against detergent dissolution was tested with Quasi-elastic Laser light Scattering (QELS) (Model BI-90, Brookheaven) using TritonX-100 [3]. Briefly, a change in scattered light intensity was recorded after addition of TritonX-100 to a diluted liposome suspension (lipid concentration ~ 1 mg/ML). Both polymerized as well as unpolymerized liposomes were examined for comparison.

XPS Analysis of Lectin-Bearing Liposomes

The presence of lectins on the liposome surface was confirmed using X-ray Photoelectron Spectroscopy (XPS). Lectin-bearing liposomes were prepared as described above. Droplets of the liposome suspensions were applied on a clean aluminum substrate. Once the water evaporated, a thin layer of liposomes was formed on the substrate. The surface chemical composition of the liposome layer was then examined using a Surface Science SSX-100 spectrometer.

In Vitro Aggregation Assay for Immobilized Lectins

The carbohydrate binding activities and specificities of the immobilized lectins were examined *in vitro* using an aggregation assay. The substrate for UEA I or for WGA (see Figure 3 legend) was dissolved in PBS buffer with different concentrations and 100 μL was added to suspensions of UEA I or WGA-modified liposomes (lipid concentration adjusted to ~ 1 mg/mL). The total volume was 1.5 mL. The mixtures were vortexed and incubated at 25°C for 20 minutes and then transferred to the UV/VIS spectrometer. Liposome aggregation was followed by the turbidity increase (A450) of the suspensions. Carbohydrate specificity of the immobilized lectins was tested by incubating the lectin-bearing liposomes with 100 μmol of the inhibitors (see Figure 3 legend) before the substrates were added.

In Vivo Uptake of Liposomes

Balb/C mice of female sex (19–21 grams) were purchased from Charles River Lab. Upon arrival, they were housed under normal conditions with free access to food and water.

Four groups of four mice each were used to study the *in vivo* uptake of UEA I liposomes, WGA liposomes, or lectin-free liposomes. All mice were fasted for 12 hours before the experiment but allowed free access to water. Mice from groups one to three were gavaged with 200 μL suspensions of UEA I liposomes, WGA liposomes, or lectin-free liposomes each using a 24 gauge ball-tipped gavage needle (Harvard Apparatus). The fourth group were given PBS only. They were used as a control for the experiment. Food was restored immediately after administration. At 2 hours post administration, mice were anaesthetized with ether inhalation and blood was drawn from the Ophthalmic Venous Plexus using Pasteur pipettes as described elsewhere (13). The mice were then sacrificed with carbon dioxide. Incisions were made in the abdomen (13) and the small intestines were removed first. Spleen, mesentery, liver, kidney, heart, and lung were collected. The excised intestines were then cut open along their mesenteric edges and the lumen unfolded with the mucosal surfaces facing upwards. The lumenal sides of the small intestines were thoroughly rinsed with PBS containing 1 mM dithiothreitol to clear away any residual lumenal contents. Peyer's patches were visually identified with their unique appearance as white opaque nodules and then excised. A patch-free intestinal segment of ~ 2 cm in length was cleaned and excised as well. All tissue samples were patted dry before their weights were recorded individually. Samples obtained were treated with the tissue solubilizer Soluene-350® (Packard Instruments, CT) and incubated at 50°C. Periodic vortexing was applied to facilitate the complete sample digestion. 1 mL of each digestate was taken and mixed with 10 mL of Hionic-Fluor® liquid scintillation cocktail (Packard Instruments, CT) and counted for ^{3}H activity using a Liquid Scintillation Counter (Tricarb 2000CA, Packard Instrument). Total activity administered to each mouse was quantified by counting 200 μL of the original liposomes suspension.

RESULTS

Formation of Lectin-Bearing Polymerized Liposomes

TNBS reacts with the free amino groups in proteins and gives absorption at 420 nm (14). Since the NGPE modification also takes place on the amino groups in the lectins, the TNBS assay is used to quantify the reduction in the number of amino

groups during lectin modification. The relative decrease in A_{420} measured for the same lectin before and after the modification reaction can be used to calculate the extent of lectin modification. A typical degree of modification obtained using this assay was between 40–70%.

The size distribution of lectin-bearing liposomes was determined using Quasi-elastic Laser light Scattering. The liposomes had an average diameter of about 100 nm. This was also confirmed by transmission electron microscopy (data not shown). These polymerized liposomes remained intact in the presence of the detergent Triton X-100 (Figure 1). On the other hand, the unpolymerized liposomes were completely dissolved by the addition of Triton X-100. This indicates that incorporation of modified lectin molecules does not interfere with the polymerization process, and the polymerized liposomes are stable against detergent dissolution.

Table 1. Chemical Compositions of Liposome Surfaces from XPS Survey Analysis[a]

Compound	UEA I-bearing Liposomes (%)	WGA-bearing Liposomes (%)	Lectin-free Liposomes (%)
C	77.52	73.09	74.43
O	17.33	19.82	20.10
P	1.76	1.89	2.77
N	3.39	4.11	2.70
S	n.d.[b]	1.08	0
Total	100	100	100
N : P ratio	1.93	2.17	0.98

[a] The results shown in each column of this table were collected from a single liposome preparation. No significant variation was observed among different liposome preparations (data not shown).
[b] Level below detection limit.

XPS Analysis

Both survey and high resolution XPS scans were used to confirm the presence of lectins on the liposome surfaces. An XPS survey scan gave a summary of the chemical compositions on the polymerized liposome surfaces. A typical summary is shown in Table 1. Carbon (C), oxygen (O), nitrogen (N), and phosphorous (P) were detected on the lectin-free liposomes, with the nitrogen to phosphorous atom ratio (N:P) very close to unity. In comparison, both UEA I liposomes and WGA liposomes showed an increased N:P ratio, implying the presence of some nitrogen-rich molecules (i.e., lectins). In WGA-bearing liposomes, about 1.08 % sulfur was also detected besides carbon, oxygen, nitrogen, and phosphorous. Since the only possible source of sulfur is the methionine or cysteine residues in the lectins, the sulfur detected proves the existence of WGA molecules on these liposome surfaces. Sulfur was not detected on UEA I liposomes, however, due to the low sulfur content (only one cysteine and one methionine out of 244 amino acids) in this lectin. To confirm the lectin presence on the UEA I modified liposomes, a high resolution scan for nitrogen atom was conducted. For the lectin-free liposomes (Figure 2A), a single peak with binding energy of 397 ev was detected. UEA I liposomes, however, exhibited an extra peak with slightly lower peak binding energy (364 ev) (Figure 2B). This indicates the presence of a new type of nitrogen on the UEA I-modified liposomes. This new type of nitrogen has a slightly different chemical environment than that of the original nitrogen in the phospholipids, which can only represent the nitrogen content from the UEA I molecules. A similar pattern was also seen for WGA liposomes as shown in Figure 2C.

In Vitro Aggregation

Turbidity changes of the liposome suspensions in the presence of their corresponding substrates are plotted in Figure 3.

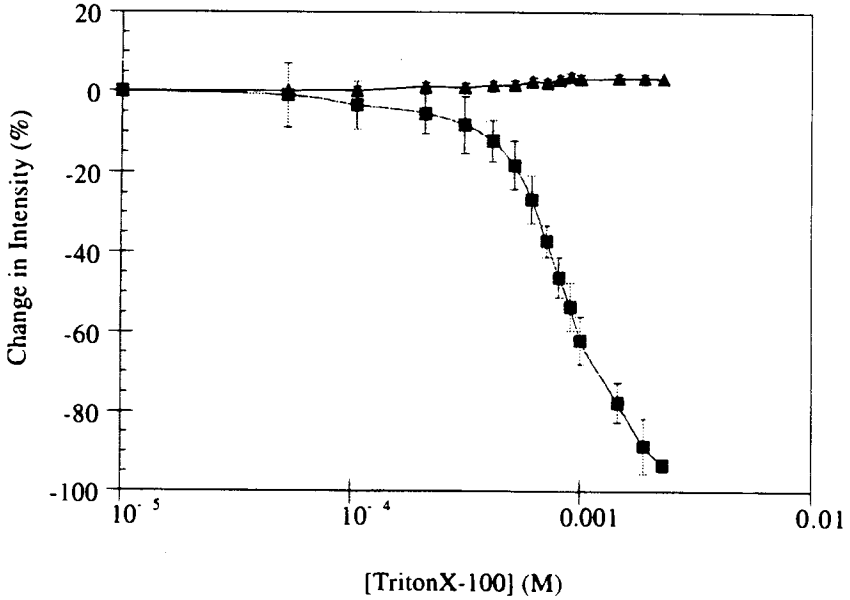

Fig. 1. *In vitro* stability of lectin-bearing liposomes against TritonX-100 dissolution, measured with Quasi-elastic Laser light Scattering (QELS) technique: comparison between unpolymerized (■) and polymerized (▲) lectin-bearing liposomes.

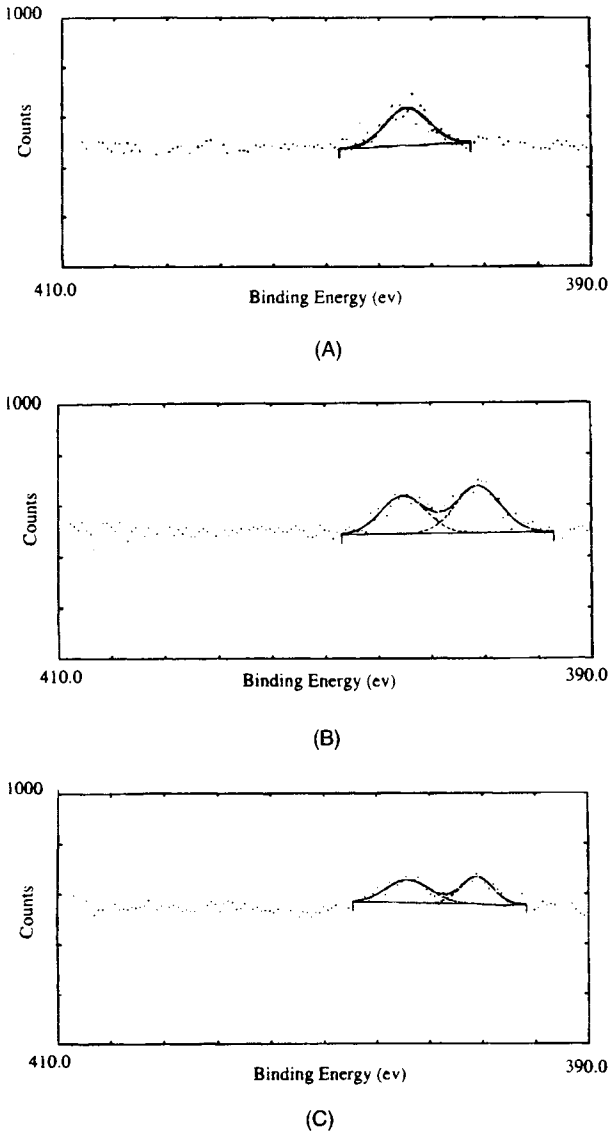

Fig. 2. High resolution X-ray photoelectron spectra on nitrogen atom for liposomes of different compositions: (A) lectin-free liposomes; (B) UEA I liposomes; and (C) WGA liposomes.

When substrates were added to lectin-bearing liposomes, the turbidity of the suspensions increased with increasing substrate concentration, indicating increasing aggregation of the lectin-modified liposomes (Figure 3). This establishes that the lectins maintained their binding activity after immobilization. In the presence of the inhibitors, however, virtually no change in turbidity was observed even when the same substrates were added (Figure 3). The liposome aggregation caused by the binding substrates was effectively inhibited by the binding inhibitors (Figure 3). This indicates that the carbohydrate specificity of these lectins remained unaltered after immobilization.

In Vivo Uptake of Liposomes

No radioactivity was detected in the tissues when the mice were administered with PBS. The amounts of radioactivity retained in all other tissue samples were measured and were divided by the total activity administered to give the percent-distribution of the radiolabel in each tissue. These percentages were then normalized with individual tissue sample weight to compensate for animal-to-animal variations. The normalized results are summarized in Figure 4. Liposomes gave a significantly higher level of binding ($p < 0.001$) to Peyer's patches when their surfaces were modified with UEA I, without increasing binding to the regular intestinal epithelium (Figure 4). WGA liposomes gave slightly stronger binding to the Peyer's patches than lectin-free liposomes (statistically significant, $p < 0.01$). However it also led to a higher binding level to the regular intestinal epithelium (Figure 4). When lectin containing liposomes were administered, a greater amount of radioactivity was detected in organs such as liver and kidney. UEA I liposomes showed ten times and WGA liposomes three times as much radioactivity in liver when compared to liposomes that were not modified with any lectins. This implies an improved level of liposomal uptake from the gastrointestinal tract. If we take an average weight for each tissue, an overall bioavailability of the liposomes can be estimated (Table 2). The tissue weights used for the bioavailability estimation were calculated by averaging the tissue weight measurements obtained from the twelve mice used. The errors presented in the table were the standard deviations calculated. Only slight variation was observed for each tissue among the animals (Table 2) since they were of the same age and sex. Of the total amount of liposomes administered, ~ 3.2 % lectin-free liposomes were taken up. This is consistent with our previous observations (15). Compared to lectin-free liposomes, about twice as much (~ 5.8 %) WGA liposomes and three times as much (~ 10.5 %) UEA I liposomes were transported from the animal gastrointestinal tract into the circulation after a single dose oral administration. These latter two values are significantly higher than conventional lectin-free polymerized liposomes when they are examined using statistical (ANOVA) tests ($p < 0.001$).

DISCUSSION

Lectins are natural components of the daily diet. They bind to particular sugar structures specifically with affinities similar to those of monoclonal antibodies. In contrast to antibodies, lectins are generally stable in the gastrointestinal tract. When the liposomes are modified with lectins, the lectin-sugar specific interactions may allow the differentiation among intestinal epithelial cells and therefore facilitate targeted delivery of the liposomes.

Lectins are non-membrane hydrophilic molecules. They can be incorporated into the membrane by modifying them with a hydrophobic anchor, NGPE. During liposome formation, increased lipophilicity will force the hydrophobic tail region to partition into the membrane phase and expose the lectins to the water phase (12).

During the modification, structural changes were introduced in the lectins. Later on, the lectins were also exposed to the redox initiators during liposome polymerization. It is therefore crucial to verify lectin binding activity as well as carbohydrate specificity after the modification and polymerization processes. This is done using the *in vitro* aggregation assay. When the substrates (molecules that contain multiple copies of the specific carbohydrate residues recognized by UEA I or WGA) are added into the lectin-bearing liposome suspension, interaction between the carbohydrate residues and the surface-

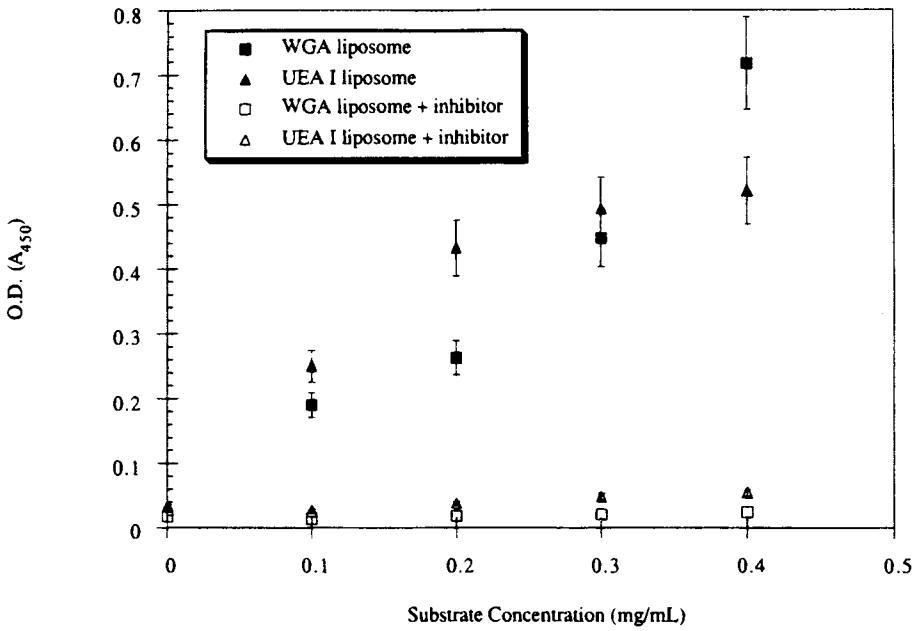

Fig. 3. *In vitro* aggregation of WGA and UEA I liposomes in the presence of their substrates: glycophorine (Sigma) for WGA and 2′-fucosyllactosamine HSA conjugate (Accurate Chemical & Scientific Corp.) for UEA I; or in the presence of their substrates as well as inhibitors : N-acetyl-glucosamine (Sigma) for WGA and L-fucose (Sigma) for UEA I.

immobilized lectins should lead to 'bridge' formations among the liposomes, if the lectins remain biologically active. This results in liposome aggregation and can be estimated by following the increase in the turbidity of liposome suspensions. When inhibitors (small sugar molecules containing a single copy of the carbohydrate residues) are incubated with the lectin-modified liposomes first, they should compete with the substrates for lectin binding, if the lectins still recognize the same carbohydrate residues. As a result, the bridges can no longer form and aggregation should disappear. This assay can therefore provide insight as to whether the immobilized lectins still retain their carbohydrate binding activities as well as their sugar specificities.

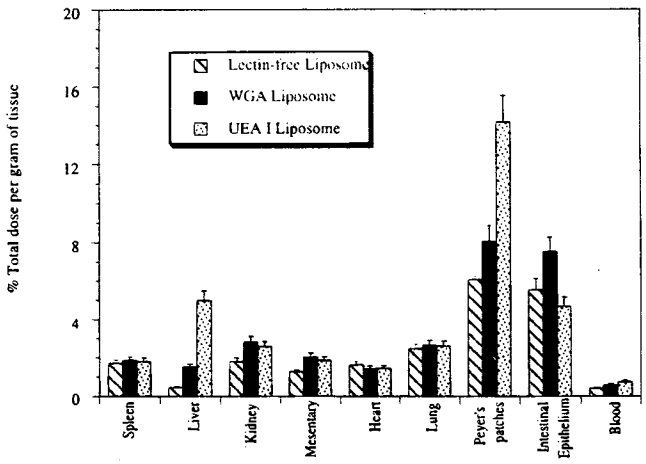

Fig. 4. *In vivo* distribution of lectin-free liposomes, WGA liposomes, and UEA I liposomes in mouse tissues after a single dose oral administration. Each column represents the average of data collected from four mice. The errors presented are standard deviations.

XPS is a commonly used surface analysis technique. It uses X-rays to bombard a surface and a detector which measures the binding energies of electrons released. The binding energies of the electrons knocked out of the sample surface are unique to the elements from which they are released. As a result, XPS can be used to identify and determine these elements. The relative intensity of the signals detected reflect the relative concentration of the elements present on the surface. For example, the close-to-unity N:P ratio seen for lectin-free liposomes in Table I is consistent with the 1:1 stoichiometric ratio between the two elements in the phospholipids. In comparison, the same ratio increased to ~ 2.0 for both UEA I and WGA liposomes, suggesting a change in the relative concentration of the two elements. However, normal XPS analysis can only yield detectable information on elements that are present on the surface at a level no less than 1% (16). This is determined by the instrument sensitivity. As a result, the low sulfur content in UEA I was below the detection limit to show up on the survey scan.

Small but measurable changes in electron binding energy exist with the changing chemical environment of the atom. In other words, core electron binding energies exhibit chemical shifts when a range of compounds containing the same element are compared. Variations in the elemental binding energies arise from differences in the chemical potential and polarizability of compounds. These can usually be seen with a detailed high resolution scan. For instance, a high resolution scan reveals that both WGA and UEA I liposomes exhibit a new nitrogen form which is different from the original one present in the phospholipids (Figure 2).

The amount of liposome uptake *in vivo* was calculated from the amount of radioactivity found in the tissues. This was

Table 2. Distribution of Radiolabel in Mouse Tissues

Tissue	Tissue weight[a] (g)	% uptake per tissue[b]		
		UEA I liposome	WGA Liposome	Lectin-free liposome
Peyer's patches	0.0100 ± 0.0005	0.14 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
Spleen	0.08 ± 0.02	0.14 ± 0.07	0.15 ± 0.08	0.14 ± 0.01
Liver	1.3 ± 0.1	6.5 ± 1.0	2.0 ± 0.3	0.6 ± 0.1
Kidney	0.33 ± 0.03	0.86 ± 0.16	0.93 ± 0.03	0.41 ± 0.07
Heart	0.12 ± 0.01	0.17 ± 0.03	0.17 ± 0.03	0.20 ± 0.03
Lung	0.165 ± 0.005	0.43 ± 0.03	0.46 ± 0.04	0.40 ± 0.03
Mesentery	0.42 ± 0.07	0.79 ± 0.26	0.86 ± 0.29	0.53 ± 0.18
Blood (100 μL sampled)	0.105 ± 0.005	0.08 ± 0.01[c]	0.06 ± 0.01[c]	0.044 ± 0.004[c]
Total		10.6 ± 1.1[d]	5.8 ± 0.4[d]	3.2 ± 0.2[d]

[a] Each number is the average of data collected from twelve mice. Errors presented are standard deviations.
[b] % uptake per tissue = % Total dose per gram of tissue (See Figure 4) × Tissue weight.
[c] Calculation done based on the assumption that the total blood volume per mouse is about 2 mL.
[d] These numbers are statistically different base on an ANOVA test ($p < 0.001$).

based on previous observations that polymerized liposomes remain intact in mouse gastrointestinal tract, and the radioactive membrane markers stay tightly associated with the liposomes (5). As a result, the amount of radioactivity detected in the tissues can effectively represent the amount of intact liposomes taken up from the gastrointestinal tract (5).

The observed binding patterns of UEA I liposomes and WGA liposomes to intestinal epithelium are consistent with those for UEA I and WGA molecules reported previously (10, 11). UEA I is specific for M cells and is therefore not binding to the rest of the intestinal epithelium with a high intensity. WGA, on the other hand, recognizes glycoconjugates present on both M cells and regular intestinal absorptive cells. Both types of lectin modified liposomes gave a higher level of uptake due to their increased Peyer's patch binding when compared with lectin-free liposomes. UEA I liposomes, however, gave the best delivery efficiency due to its specific binding to Peyer's patch M cells.

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