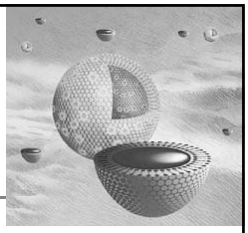
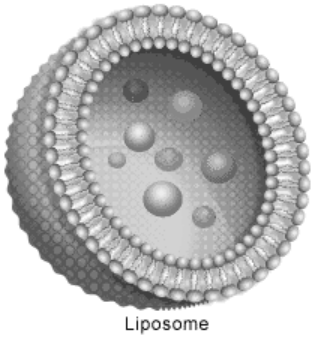


Applications of Liposomal Micro/Nanovesicles on Detection of Biomolecules

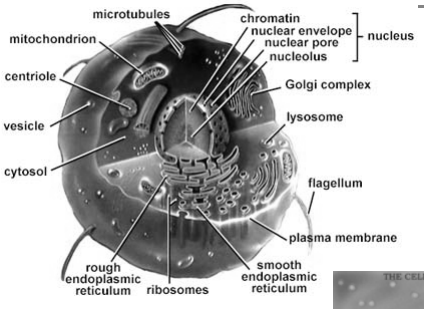
Dr. Wen Hsiao-Wei

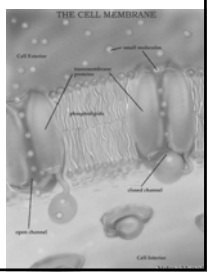
2007/10/11

Liposomes v.s. Cells



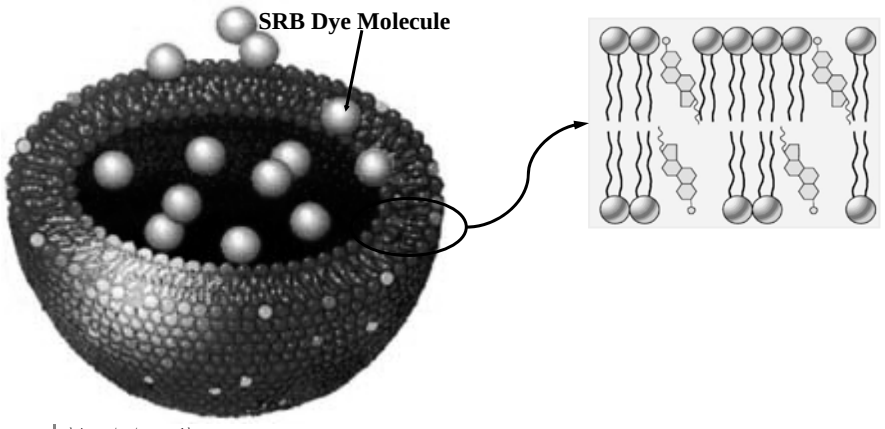
Liposome





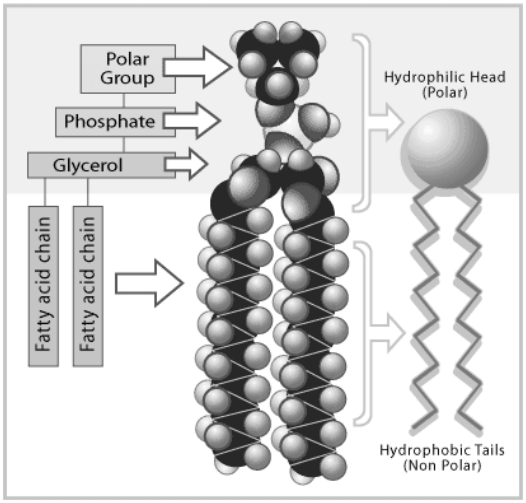
Liposomal Nanovesicles Structure

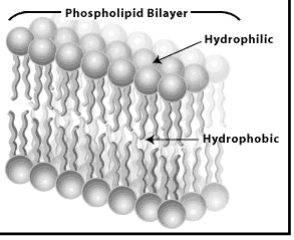
Bilayer Membrane **Cholesterol + Phospholipid**



www.transtechnics.com/ eng/conceptos1.htm

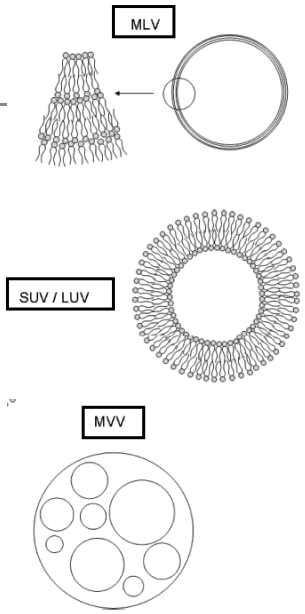
Phospholipids





Liposomal Nanovesicles Structure

Liposome	Formulation methods	Characteristics
MLV	Thin-film dehydration/rehydration.	Low energy input required for formation. Useful for larger batch formulations. Good entrapment efficiency. No significant technical training required to form and low cost of reagents and equipment. Maximum storage stability.
LUV	Membrane extrusion of MLVs; may budd-off from MLVs in suspension. Can also be formed from detergent depletion (dialysis) methods.	High energy input not required for formation. Good encapsulation efficiency. Longer storage stability vs. SUV liposomes due to reduced curve stress.
SUV	Membrane extrusion of MLVs or LUVs; Ultrasonication (probe or bath) of MLVs or LUVs; dialysis of MLVs; high pressure homogenization.	Increased homogeneity of liposomes. Increased circulation times/half-life.



Manufacture of Liposomal Nanovesicles

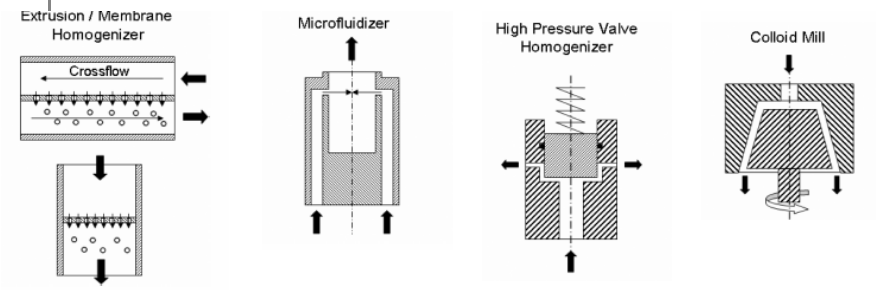
A. Chemical Methods

Methods	Strengths	Weaknesses
Thin-Film Rehydration	Rapidly formed; low energy input to form. Highest stability and transition cooperativity.	Highly heterogeneous dispersion; lower encapsulation efficiency vs. LUVs. Nearly impossible to determine mass encapsulated/vesicle and surface area.
Freeze-Dried Rehydration Vesicles	High entrapment efficiency. Improved homogeneity in mixing of lipid species/liposome. Useful for forming antigen-entrapping vesicles.	Dehydration best controlled by freeze-drying; long time to process.
Reverse-Phase Evaporation	Higher entrapment efficiency reported for variety of molecules then MLVs (proteins, nucleic acids, etc.).	Heterogeneous distribution of MLVs and unilamellar vesicles; additional homogenization required. Solvent exposure may inhibit protein activity. Possible incomplete removal of organic solvent. Entrapment above 50% very difficult.
Detergent-Depletion	No solvent used; protein activity retained. No mechanical energy input. Homogenous distribution of liposomes. Useful for multiple lipids and molecules for encapsulation. Best for entrapping membrane-associated proteins.	Limited number of useful detergents. Dialysis is very slow process and some detergent is likely to remain in the sample. Detergent may negatively interact with molecule of interest.

Manufacture of Liposomal Nanovesicles

B. Mechanical Methods

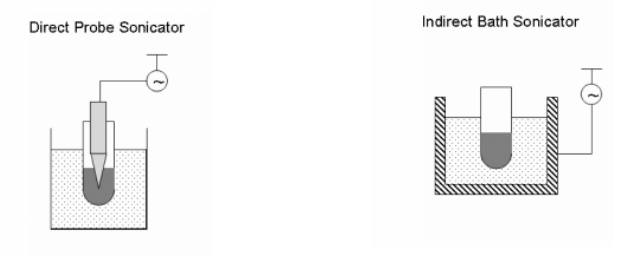
Methods	Strengths	Weaknesses
Membrane Extrusion	Homogenization of liposomes improved over other methods; rapid preparation of unilamellar vesicles from MLVs. Equipment for small-large batches is readily available.	Liposomes must be held above T_M to facilitate extrusion. Manual extruders handle only small volumes (1 ml). Solute leakage can occur during extrusion. High salt concentration (>150 mM) can preclude liposome formation.
High-Pressure Homogenization, Microfluidization	Can process high lipid concentration (~150 mg/ml). Lab data is easily inferred to processing plant. High reproducibility. Most useful for large-scale production of liposomes.	Solvent ionic strength must be carefully controlled to control liposome size. Complete homogenization is time consuming in microfluidizer.



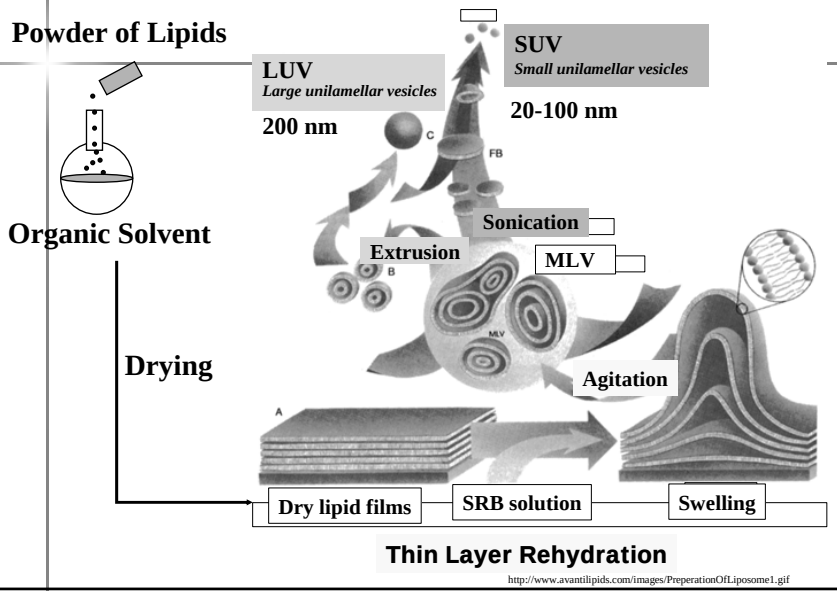
Manufacture of Liposomal Nanovesicles

B. Mechanical Methods

Methods	Strengths	Weaknesses
Ultrasonication: Probe	Can be performed directly on hydrated MLVs. Preferred method to form SUVs. Excellent for reduction of large MLVs to more homogenous dispersion of SUVs.	Probe sonication can degrade sample/localized overheating. Requires constant cooling. Liposomes are metastable (low storage-stability). Low volume required for effective treatment.
Ultrasonication: Bath	Less destructive to liposomes vs. probe method. Greater reproducibility and more homogenous product vs. probe sonicator. Increased sample volume capability. Increased control over sample temperature.	Requires extensive sonication to obtain minimum size limit of SUV; not always possible. Non-homogenous product; requires removal of larger vesicles via chromatographic or centrifugal methods.



Preparation of Liposomes



http://www.avantilipids.com/images/PreperationOfLiposome1.gif

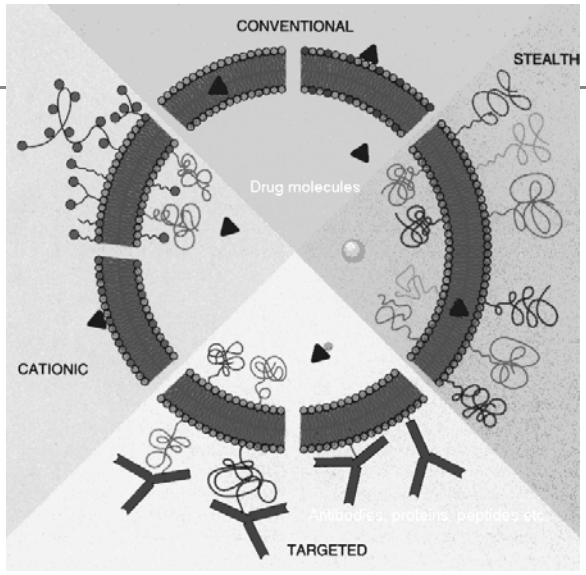
Tagged Liposome

Ligands:
Ganglioside

Receptors

Antibody

Antigen



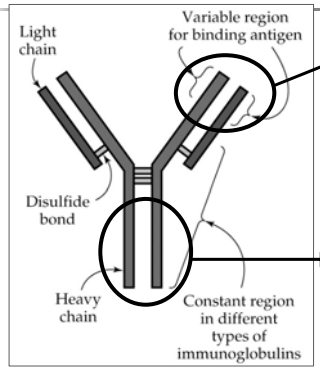
DNA

Liposme-Based Bioanalytical Assays

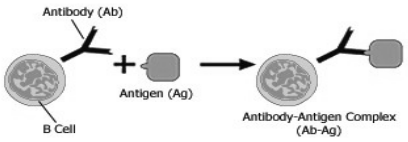
RECOGNITION	**Antigen-Antibody Interaction** **Nucleic Acid Hybridization** **Ligand-Receptor interaction**
SIGNAL ENHANCEMENT	**Liposomes** **NASBA/PCR**
DETECTION	**Optical**
FORMATS	**Flow Injection Immunoliposome assay** **Lateral Flow Assay (LFA)** **Magnetic Bead Immunoliposome Assay**

RECOGNITION

- **Antigen-Antibody Interaction**



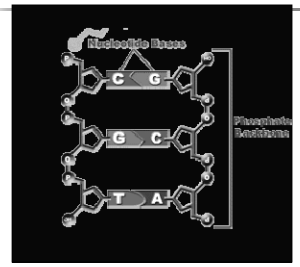
Antibody Structure



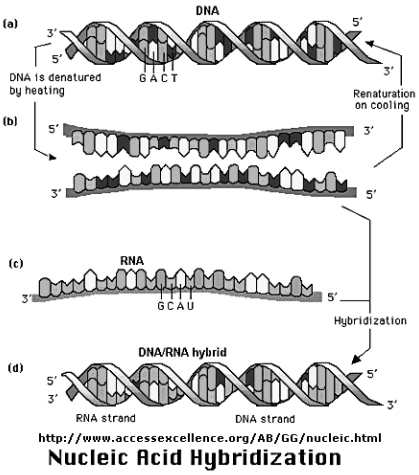
Immunoassay

RECOGNITION

- **Nucleic Acid Hybridization**

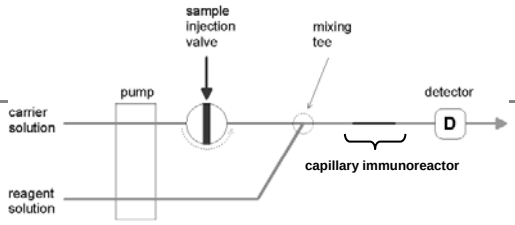


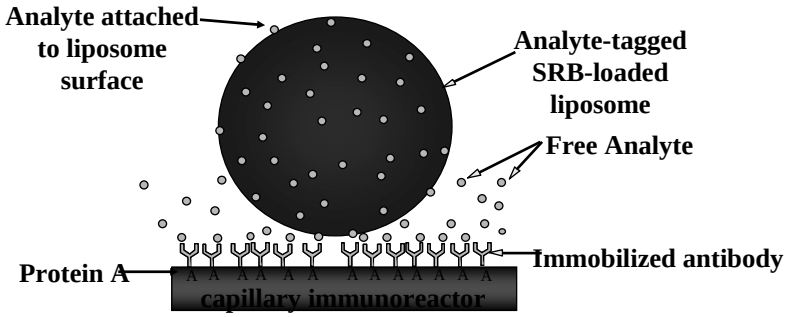
DNA-DNA: A=T, G≡C
DNA-RNA: A=U, G≡C



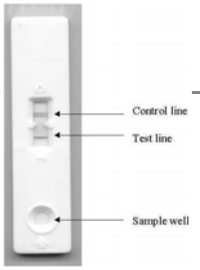
http://www.accessexcellence.org/AB/GG/nucleic.html
Nucleic Acid Hybridization

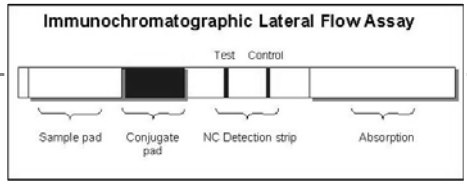
Detection System 1: Flow Injection Assay

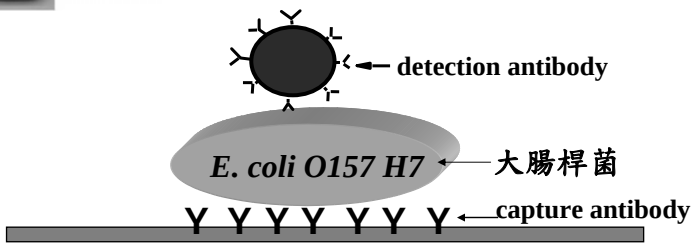




Detection System 2: Lateral Flow Assay

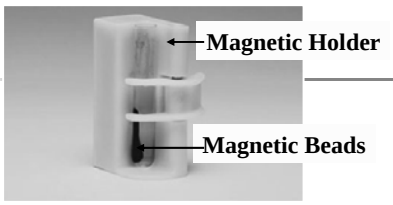


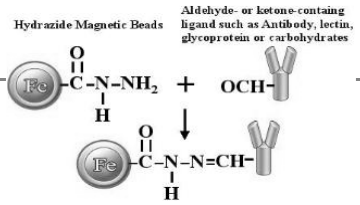


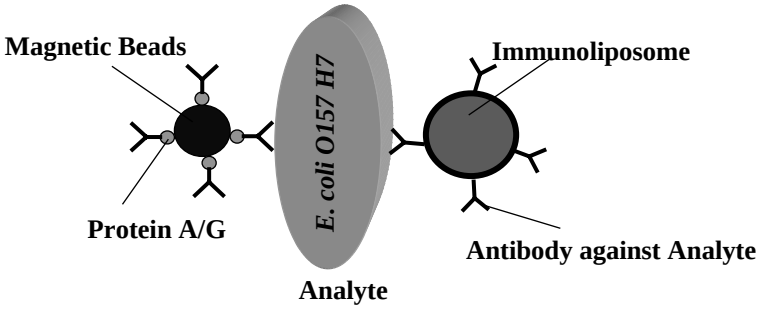


Test Line
Solid phase (NC membrane)

Detection System 3: Magnetic Beads Assay





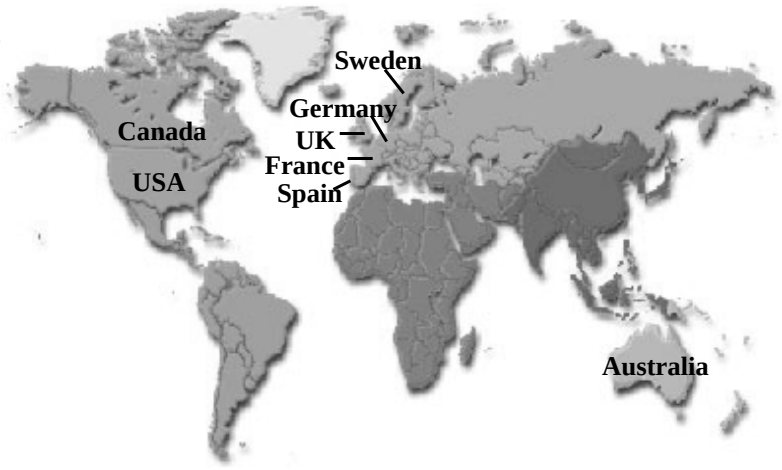


Development of A Rapid Assay for Detecting Peanut Allergens in Chocolate

Adverse Reactions to Foods

- **Food Aversion**
- **Food Intolerance**
  - Enzyme Deficiencies
  - pharmacological reactions
- **Food Allergy**
  - IgE-mediated
  - Non-IgE mediated
- **Food Poisoning**
  - Bacteria
  - Virus
  - Molds
  - Chemicals
  - Toxins

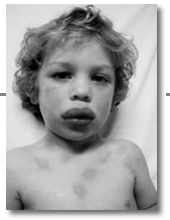
Peanut Allergy in the World



Peanut Allergy Population

A major allergic food in USA

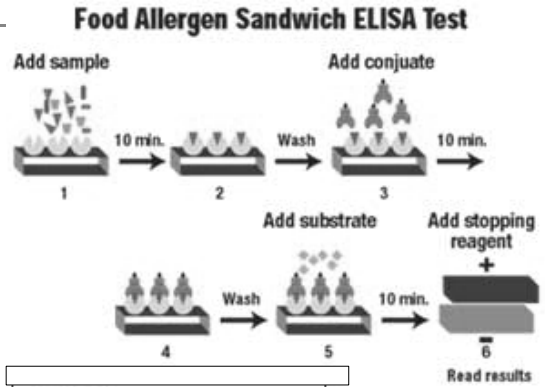
Food	Children	Adults
Milk	2.5%	0.3%
Egg	1.3%	0.2%
Peanut	0.8%	0.6%
Tree nuts	0.2%	0.5%
Fish	0.1%	0.4%
Shellfish	0.1%	2.0%
Overall	6.0 %	3.7%



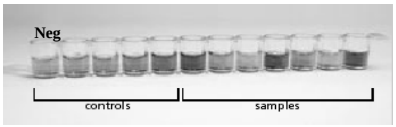
We have a No Peanut Butter Policy, due to the severe allergic reaction experienced by some children

Protein-Based Methods

Sandwich ELISA



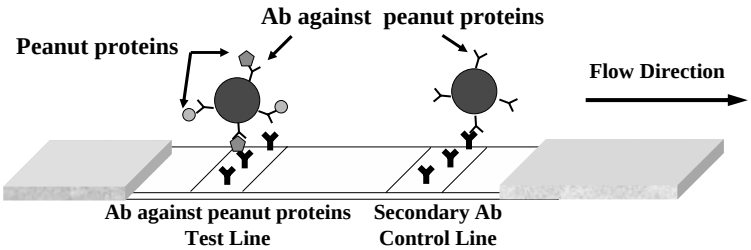
http://www.dairyfoods.com/CDA/ArticleInformation/features/BNP__Features__Item/0,6775,109279,00.html

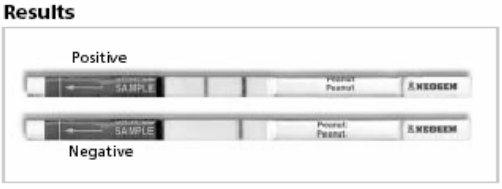


http://www.neogen.com/pdf/FS_CatalogPages/VeratoxPeanut.pdf

Protein-based Methods

Sandwich Lateral Flow Assay (LFA)





http://www.neogen.com/pdf/FS_CatalogPages/RevealPeanut.pdf

Comparison of Methods

	PCR	ELISA	LFA
Analyte	DNA	peanut allergen peanut proteins	peanut allergen peanut proteins
LOD	10-50 ppm	<10 ppm	~10 ppm
Sample Preparation	laborious	easy	easy
Time Requirement	2-6 h	0.5-2 h	10-30 min
Handling	training in DNA skills	simple	very simple

LOD: limit of detection

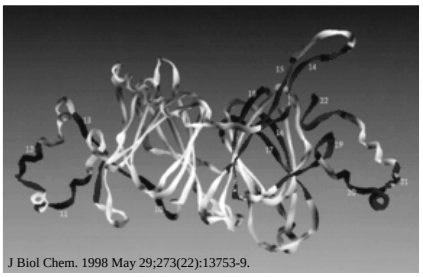
Peanut Allergens

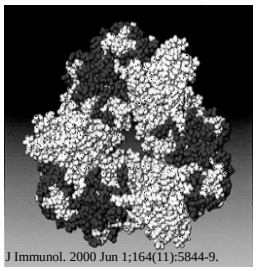
- **Peanut Proteins (not fat or carbohydrate)**
- **Heat Resistant & Acid Stable**
- **Resistant to Proteases in the GI Tract**

	Allergen	MW	Sensitization	% Total Protein
Major Allergen	Ara h 1	63.5 kDa	65-100%	12-16%
	Ara h 2	17 kDa	71-100%	5.9-9.3%
Minor Allergen	Ara h 3/4	57 kDa	44-53%	
	Ara h 5	14 kDa	13%	
	Ara h 6	14.5 kDa	38%	
	Ara h 7	15.8 kDa	43%	
	Ara h 8	16.9 kDa		

Ara h1

- **Glycoprotein (63.5 kDa)**
- **23 linear IgE-binding epitopes**
  - ***Tertiary structure → clustered into two regions***

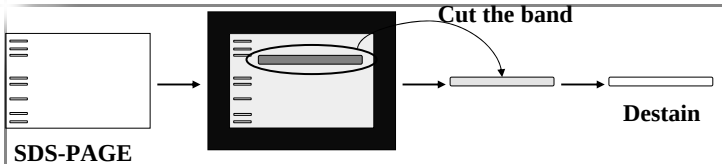




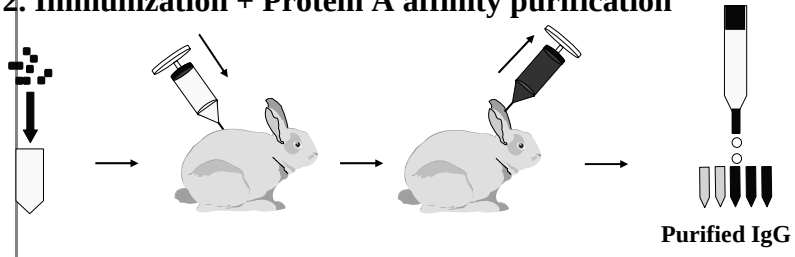
N-terminal Region **C-terminal Region**

Production of α-Ara h1 Abs

1. Preparation of immunogens



2. Immunization + Protein A affinity purification



Production of α-Peptide Abs

Protein Analysis Data

- antigenicity
- hydrophilicity
- surface probability
- secondary structure

AA603-615

AA493-507 (epitope 17)

Immunodominant epitope

+

Selected Peptide: AA 493-507

↓

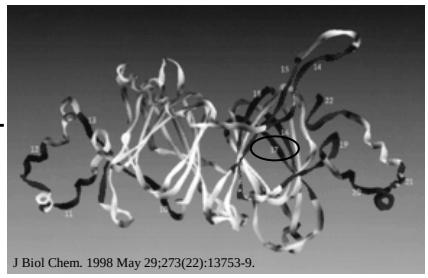
KLH-peptide

↓

Injected to rabbits

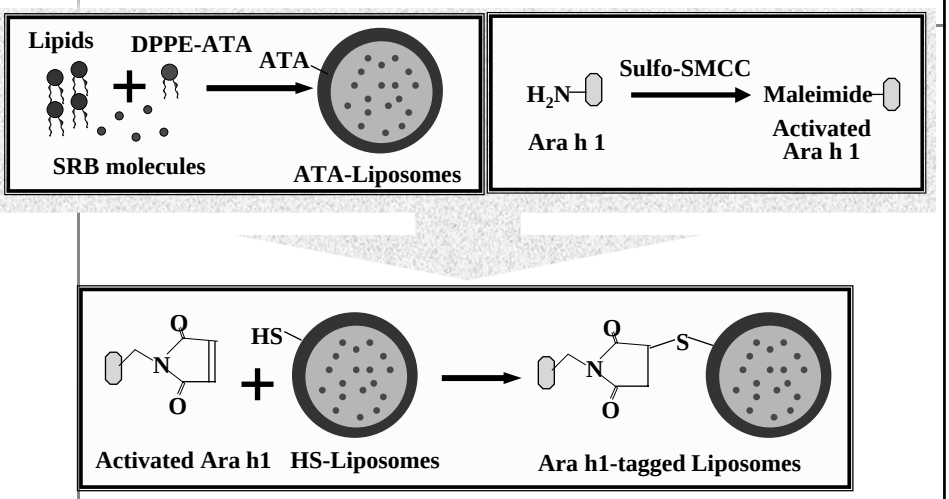
↓

Purified in Protein A column

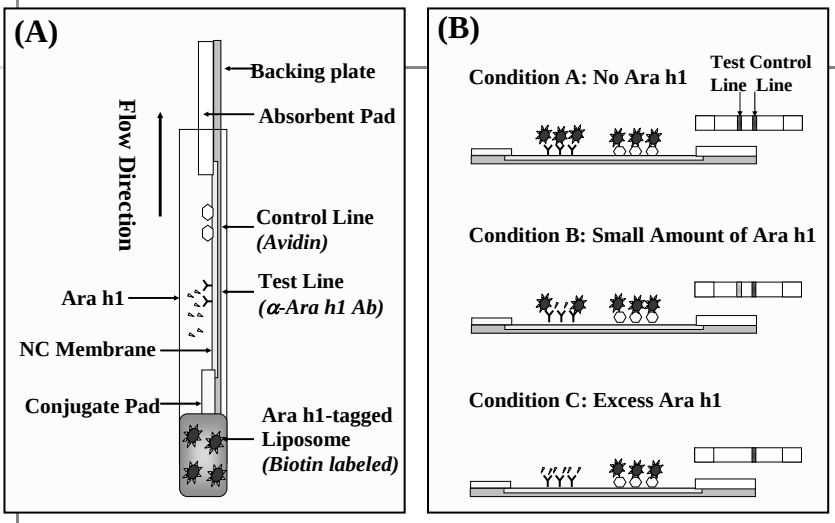


J Biol Chem. 1998 May 29;273(22):13753-9.

Ara h1-tagged Liposome



Assay Format



Procedures for Assay Development

Develop a Lateral Flow Assay for Detecting Peanut Allergens in Chocolate

1. Purification & Identification of Ara h1 from Peanuts

↓

2. Production & Purification of Antibodies against Ara h1

↓

3. Competitive Lateral Flow Assay Development

↓

4. Optimization for Peanut Detection in Chocolate

1. Purification & Identification of Ara h1 from Peanuts

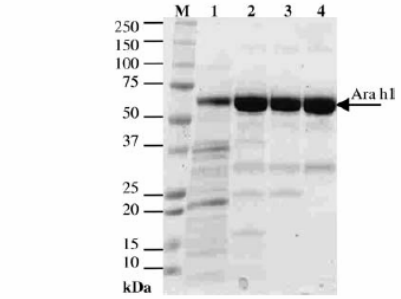


Fig. 2 SDS-PAGE of each step of the overall purification process of Ara h1. The purification steps were the extraction of total peanut proteins (*lane 1*), 70–100% AS fractionation (*lane 2*), ion-exchange chromatography on High Q column (*lane 3*), and hydrophobic interaction chromatography on HIC column (*lane 4*). *Lane M* contained pre-stained protein standards. The gel was stained with Coomassie brilliant blue G 250

```
1  mrgrvsplmlllgilvlasvsathakssspy
31 qkkkteenpcaqrclqscqqqepddlkqkaces
61 rctkleydprcvydprghtgttnqRSPPGE
91 RTrgrqpgdyddddrrqprreeggrwgpagp
121 rererreedwrqpredwrrpshqqprkirpe
151 gregeqewgtpgshvreetsrnnpfyfpst
181 rfstrygnqngrirvlqrfdqrsrqfqnlq
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331 eirrvlleenaggeqeergqrrwstrssen
361 negvivkvskehveeltkhaksvskkgsee
391 egditnpinlregepdlsnnfgklfevkpd
421 kknpqlqdldmmltcveikegalmlphfns
451 kamvivvvnkgtgnlelvavrkeqqqrgrr
481 ecccdedeccccegsnrevrrytarlkegdvf
511 impaahpvainasselhllgfginaennhr
541 iflagdkdnvidqiekqakdlafpgsgeqv
571 ekliknqkeshfvsarpqsqsqspsspeke
```
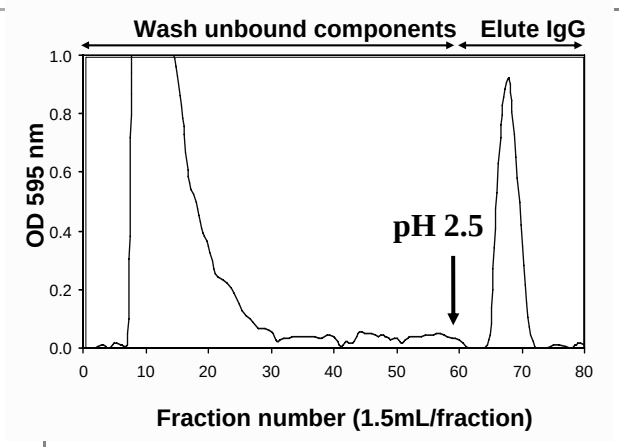
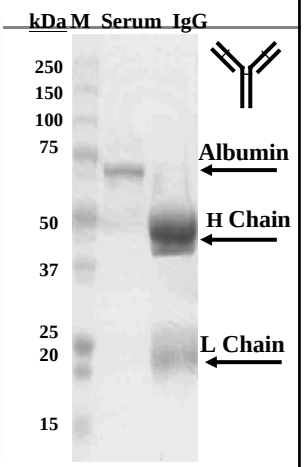
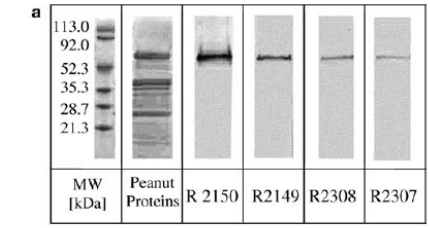

- **N-terminal Sequence: SPPGERT**
- **Peptide Mapping: 33 peptides 43% AA of P41B clone**

Antibody Purification





Production & Purification of Antibodies against Ara h1



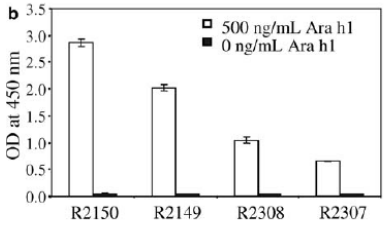


Fig. 4 Western blot (**a**) and indirect-ELISA (**b**) of peanut proteins incubated with four batches of antibodies against Ara h1. Western Blot was performed with total peanut proteins (0.5 μg/lane), and ELISA with the purified Arah1 (50 ng/well). (In *panel A*, *first column* contained pre-stained protein standards; *second column* contained total peanut proteins stained with Coomassie brilliant blue G 250)

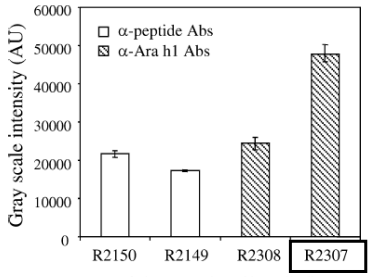
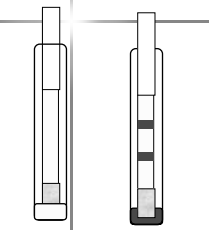


Fig. 5 Immunoassay of the two anti-peptide antibodies (R2150 and R2149) and two anti-Ara h1 antibodies (R2038 and R2037) with Ara h1-tagged liposomes as the detector reagent on the nitrocellulose membrane test strip. Antibody (4 μg cm^{-1}) was coated in the test line on the strip, and the signal intensity of the immunoassay was determined by gray scale intensity (GSI) with a computer-scanning program

Competitive Lateral Flow Assay Development



Spiked Liposomes Sample

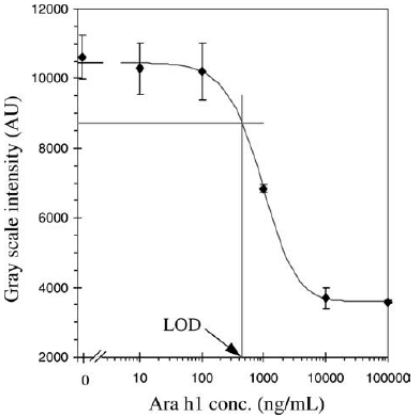


Fig. 7 A competitive lateral flow assay with a serial dilution of Ara h1 (0, 1×10^1; 1×10^2; 1×10^3; 1×10^4; 1×10^5 ng mL^{-1}). Dose–response curve for Ara h1 samples generated from the test strip assay by gray scale intensity (GSI) measurement. A 5-parameter sigmoidal function (R^2 = 0.9991) was calculated from this dose–response curve

$$y = 3592 + \frac{6865.5865}{\left\{1 + e^{-\left[\frac{x-3.147}{-0.3156}\right]}\right\}^{1.535}}$$

R^2 value as 0.9991

- **LOD**
  450 ng/mL Ara h1
- **Dynamic Range**
  10^2 ~10^4 ng/mL Ara h1

Ideas

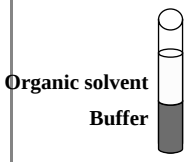
1. Why Chocolate?

- Frequently recalled item for peanut contamination

2. Higher extraction efficiency

- higher [Ara h1] in a sample, higher sensitivity
- remove factors lowering extraction efficiency

a) Lipids **b) Phenolic compounds**



Organic Solvent

Insoluble PVPP
poly(vinylpolypyrrolidone)

Optimization for Peanut Detection in Chocolate

chocolate/peanut

↓ **Grinding with L.N_2**

0.5 g chocolate + spiked peanut

1. Buffer Selection → **Extraction** ← **2. Organic Solvent Selection**

1 mL buffer + 4 mL organic solvent

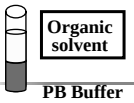
↓ **Centrifugation** → **organic solvent**

3. Extraction Time & Temp → **PVPP treatment**

↓ **Centrifugation**

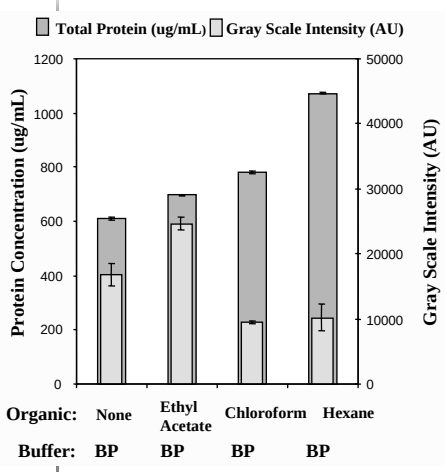
Extracted sample solution ← **4. Determine LOD**

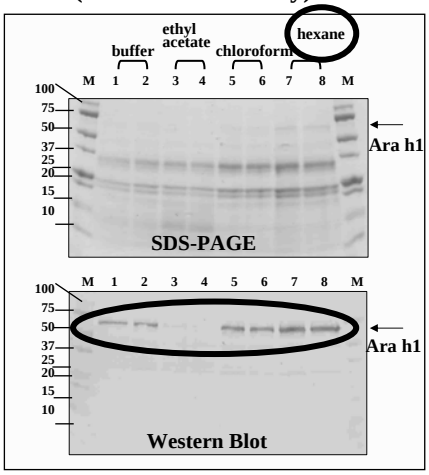
Organic Solvent Selection



Extraction Efficiency:

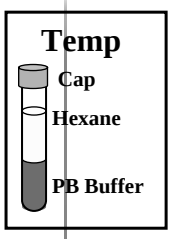
1. **Total Protein Concentration**
2. **Ara h1 concentration (Lateral Flow Assay)**





Extraction Time & Temp.

Temperature: 25, 35, 45, 55°C
Time: 15, 30, 45, 60 minutes



Incubator
(with shaking)

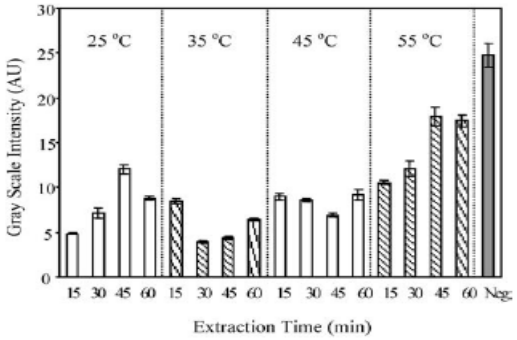


Fig. 6 Effects of temperature and time on the signal intensity in the competitive lateral flow assay for peanut-spiked chocolate samples. Samples were extracted at 25, 35, 45 and 55 °C for 15, 30, 45 and 60 min using the PBS and hexane mixture.

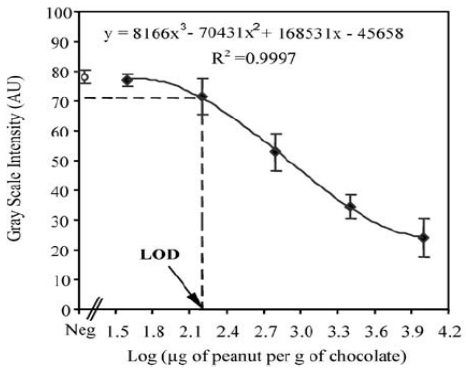
Extraction Condition: 35°C for 30 minutes

Analysis for Cross-reactivity

Line	Item	
1	Peanut	
2	Pistachio	Tree nut family
3	Cashew	
4	Almond	
5	Hazelnut	
6	Red Lentil	Legume family
7	Navy Beans	
8	Red Kidney Beans	
9	Yellow Split Peas	

α-Ara h1 Ab R2307 is highly specific to Ara h1.

Assay Sensitivity



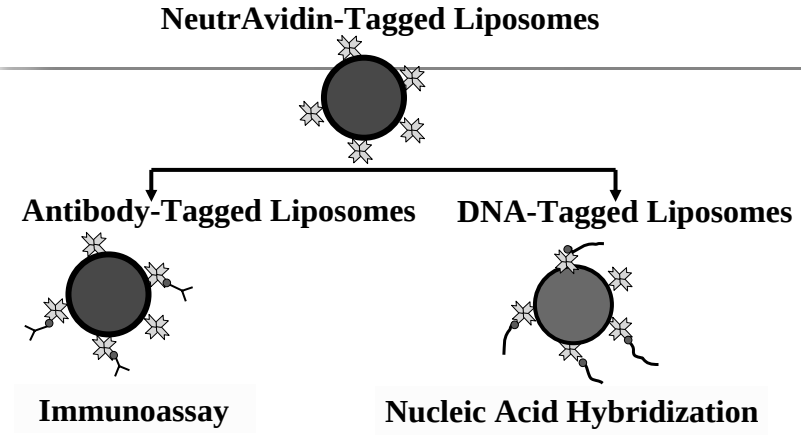
Limit of Detection
- **158 ppm of peanuts** ***(~4.0 ppm of Ara h1)***

Time Zone
- **Sample Preparation ~1.5 hr**
- **Assay ~0.5 hr**
- **Total ~2 hr**

- H. W. Wen. et al. **2005**. *A Novel Extraction Method for Peanut Allergenic Proteins in Chocolate and Detection by a Liposome-based Lateral Flow Assay.* European Food Research and Technology. 221 (3-4): 564-569. (SCI)
- H. W. Wen. et al. **2005**. *Development of a Competitive Lateral Flow Assay for the Rapid Detection of Allergenic Peanut Residues by Using Ara h1-tagged Liposomes.* Analytical and Bioanalytical Chemistry. 382 (5): 1217-1226. (SCI)

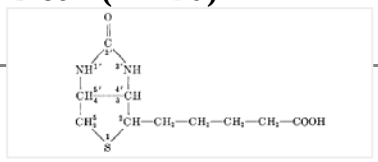
Develop NeutrAvidin-Tagged Liposomes as Universal Reagents for Preparing Detection Reagents in Bioanalytical Assays

Talanta. 2006. 68 (4): 1264-1272. (SCI)

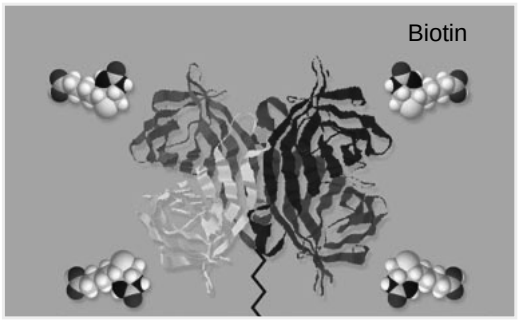


Avidin-Biotin Interaction

Biotin (224 Da)



Avidin-Biotin Complex (Ka = 10^{15} M^{-1})



Conjugate NeutrAvidin to Liposomal Surface

Attach Biotinylated Antibody to NeutrAvidin-Liposome

Competitive Lateral Flow Assay

Attach Biotinylated ssDNA to NeutrAvidin-Liposome

Sandwich Lateral Flow Assay

NeutrAvidin-Tagged Liposomes

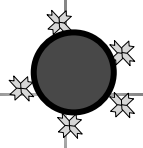
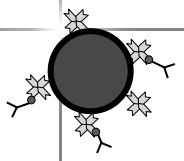


Table 1
Effect of NeutrAvidin molar percentage on its conjugation efficiency to the liposomal surface and liposome size

Starting NeutrAvidin mol%	Final NeutrAvidin mol%	Conjugation efficiency (%)	Liposome diameter (nm)
0	0	NA	241 ± 19.1
0.1	0.076	76	392 ± 39.9
0.2	0.166	83	500 ± 45.0
0.4	0.174	43	547 ± 31.7
0.8	0.365	46	741 ± 66.8

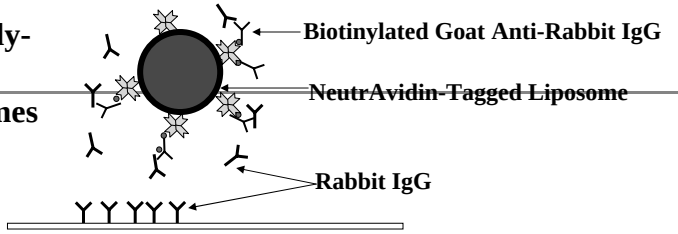
Effect of NeutrAvidin molar percentage and molar ratio of IgG/NeutrAvidin and biotin/IgG on liposome size

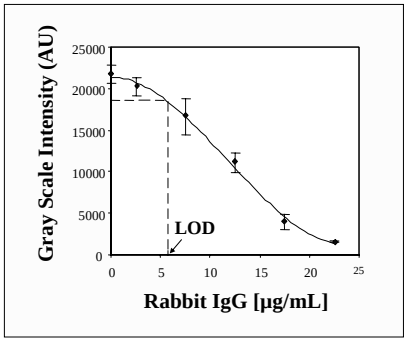


Starting NeutrAvidin mol %	Final NeutrAvidin mol %	IgG / NeutrAvidin molar ratio	Biotin / IgG molar ratio	Liposome Diameter (nm)
0.1	0.076	1/1	5	402±38.7
0.1	0.076	1/1	10	397±39.6
0.1	0.076	1/1	20	380±39.1
0.1	0.076	5/1	5	367±37.3
0.1	0.076	5/1	10	351±27.0
0.1	0.076	5/1	20	505±41.6
0.2	0.166	1/1	5	PPT*
0.2	0.166	1/1	10	PPT
0.2	0.166	1/1	20	PPT
0.2	0.166	5/1	5	433±24.7
0.2	0.166	5/1	10	423±26.6
0.2	0.166	5/1	20	364±36.7
0.4	0.174	1/1	5	PPT
0.4	0.174	1/1	10	PPT
0.4	0.174	1/1	20	PPT
0.4	0.174	5/1	5	PPT
0.4	0.174	5/1	10	PPT
0.4	0.174	5/1	20	PPT
0.8	0.365	1/1	5	PPT
0.8	0.365	1/1	10	PPT
0.8	0.365	1/1	20	PPT
0.8	0.365	5/1	5	PPT
0.8	0.365	5/1	10	PPT
0.8	0.365	5/1	20	PPT

Universal Liposomes

Antibody-Tagged Liposomes



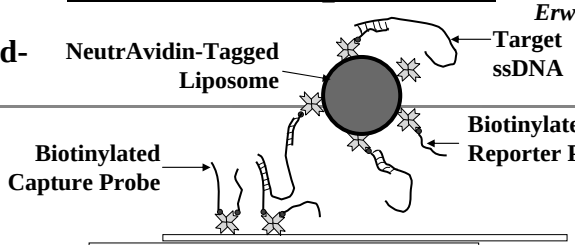


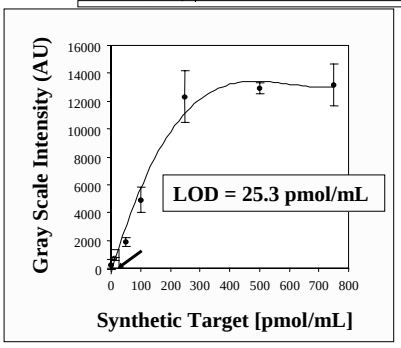
LOD = 5.72 µg/mL
= 38 pmol/mL

Universal Liposomes

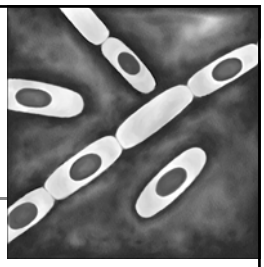
16S/23S rRNA
Erwinia amylovora

Nucleic Acid-Tagged Liposomes



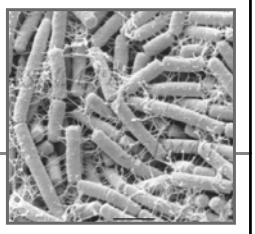


H. W. Wen. et al. **2006**. *Development of NeutrAvidin Tagged-Liposomal Nanovesicles as Universal Detection Reagents in Bioassay*. Talanta. 68 (4): 1264-1272.



Develop rapid assays for detecting *Bacillus cereus* in foods

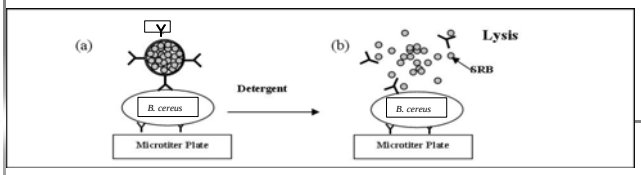
Bacillus cereus（仙人掌桿菌）



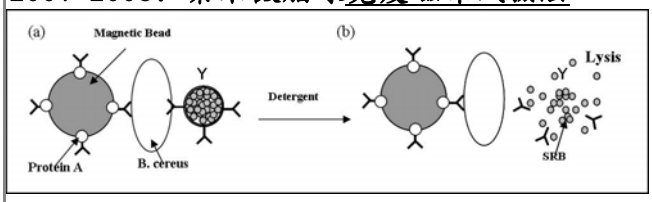
近年來台灣地區食品中毒之病因物質及其患者數之統計表

	2001	2002	2003	2004	2005
Vibrio parahaemolytius (腸炎弧菌)	1073	2014	1405	864	775
Staphylococcus aureus (金黃葡萄球菌)	162	853	114	403	138
Bacillus cereus (仙人掌桿菌)	200	160	564	166	104
Salmonella (沙門氏菌）	176	30	117	206	89
Escherichia coli (大腸桿菌）	0	0	0	0	0
Clostridium botulinum (肉毒桿菌）	0	0	0	0	0

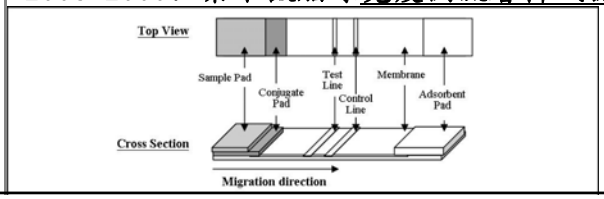
2006-2007：奈米微脂球免疫吸附試驗法



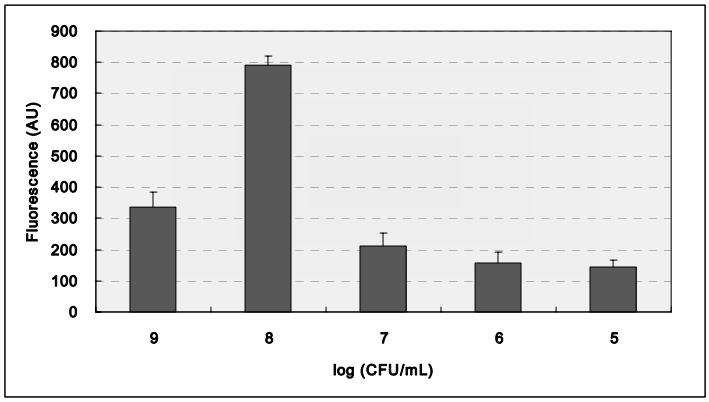
2007-2008：奈米微脂球免疫磁珠試驗法



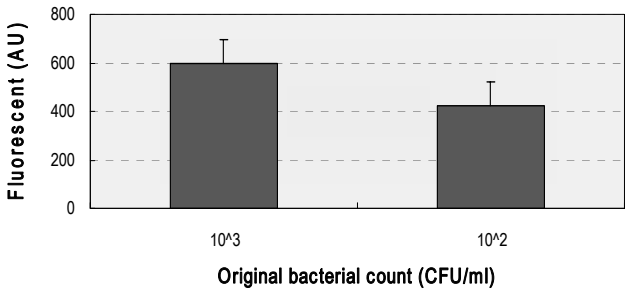
2008-2009：奈米微脂球免疫側流層析試驗法



The fluorescent signal intensity of buffered-serial dilutions of *B. cereus*



The fluorescent signal of B. cereus samples inoculated from 10^3 and 10^2 CFU/ml after an 5 hr-enrichment



Conclusions

Liposomes

1. Insertion → **Ganglioside**

- Detecting
  - Botulinum neurotoxin (BT) from *Clostridium botulinum*
  - Cholera enterotoxin (CT) from *Vibrio cholerae*

2. Covalent Binding → **Ag** / **Ab**

- Sandwich Assays
  - *E.coli* O157:H7
  - *Salmonella Enteritidis*
  - *Bacillus. cereus*
- Competitive Assays
  - Peanut Allergens

3. NeutrAvidin-Biotin → **DNA**

- Detecting
  - *Cryptosporidium parvum*
  - *Erwinia amylovora*

Thank you