Liposomes

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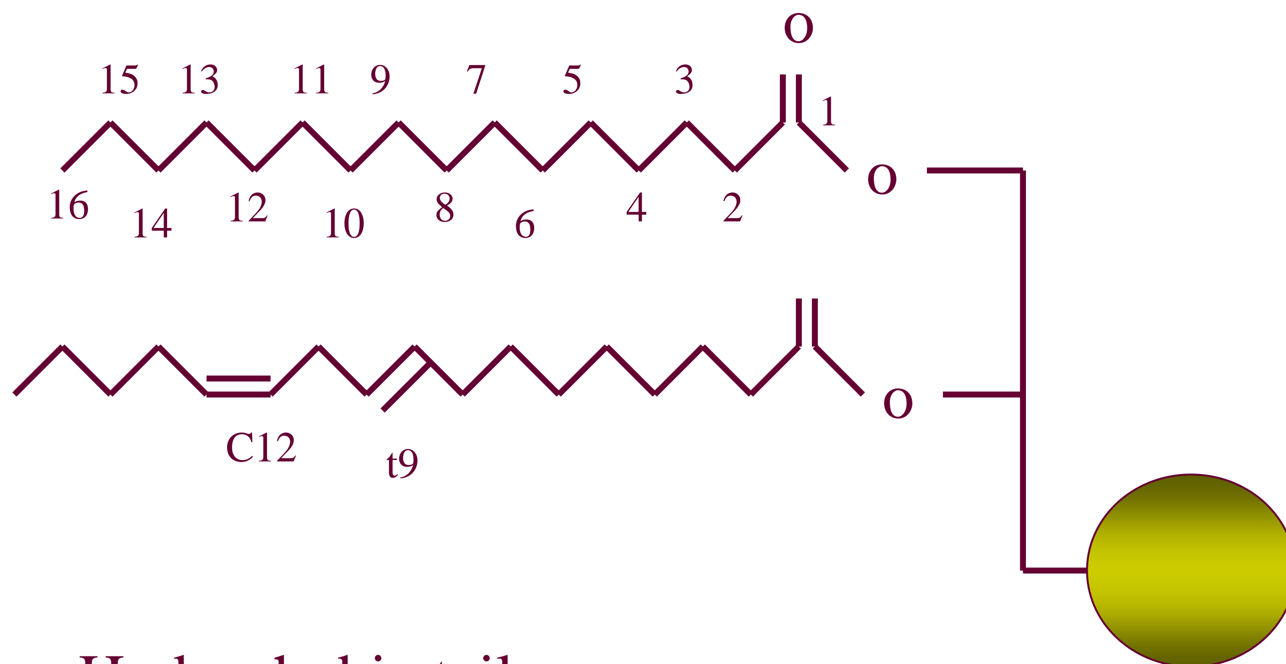
Presentation Outlines

- Liposome – definition
- Phospholipids
- Surfactant properties
- Liposome types and their characteristics
- Applications of liposomes
  - Controlled release of proteinases in cheese
  - Liposomes with whey-based protein ingredients

Liposomes

- Liposomes are vesicles in which aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids)
- Suitable for microencapsulation of predominantly water but also oil-soluble active components
- Liposomes are formed spontaneously when the lipids in an appropriate state are dispersed in aqueous media

General structure of phospholipids



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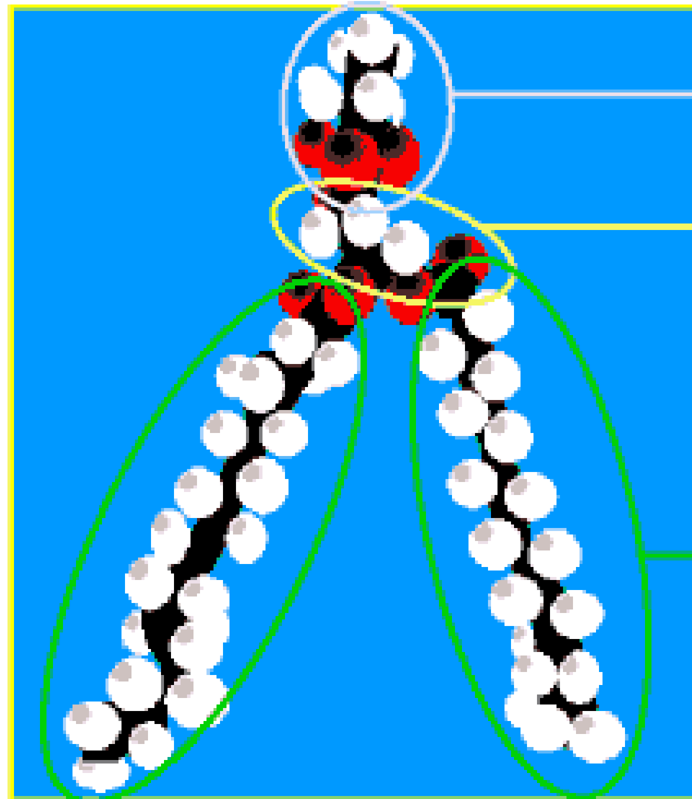
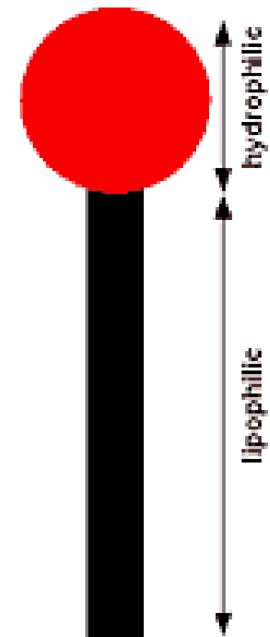
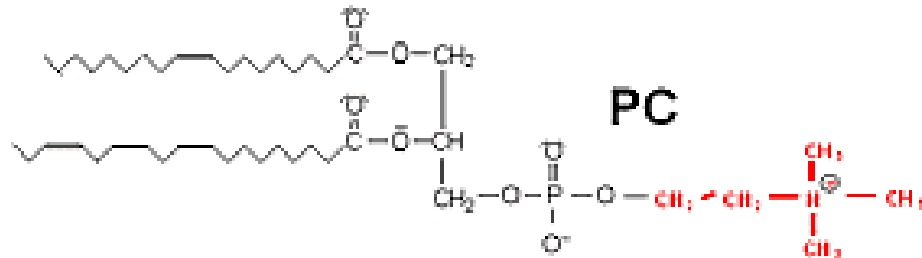
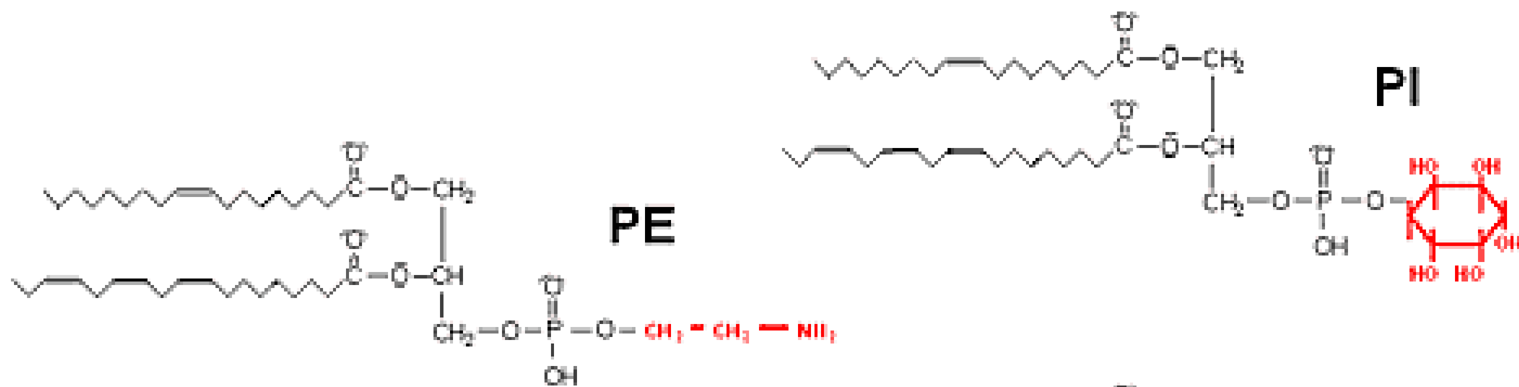
Phospholipids

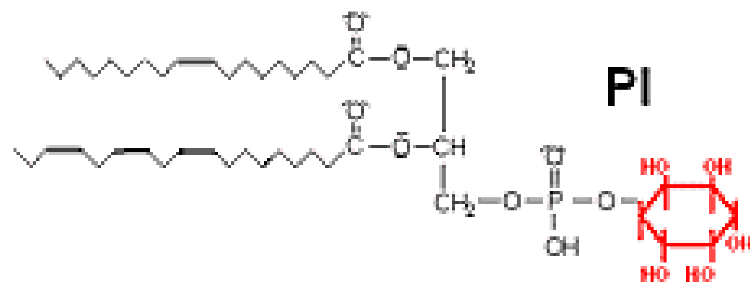


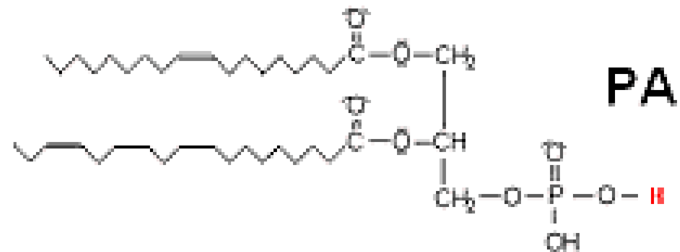


Main phospholipids





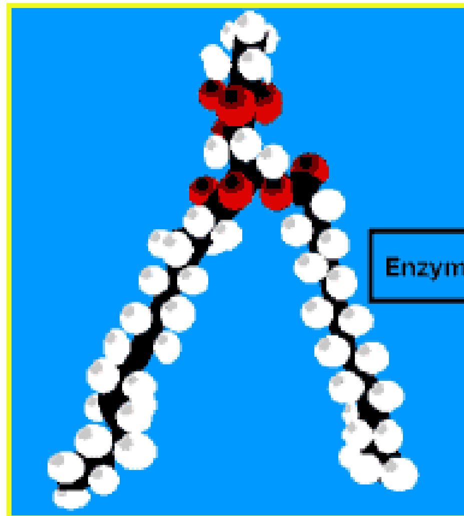


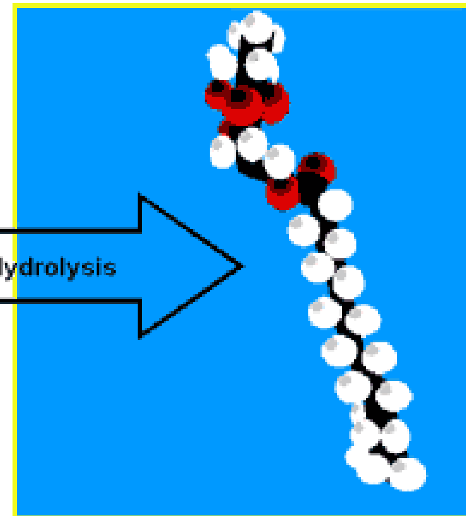


Hydrolyzed lecithin

Regular Phospholipid...

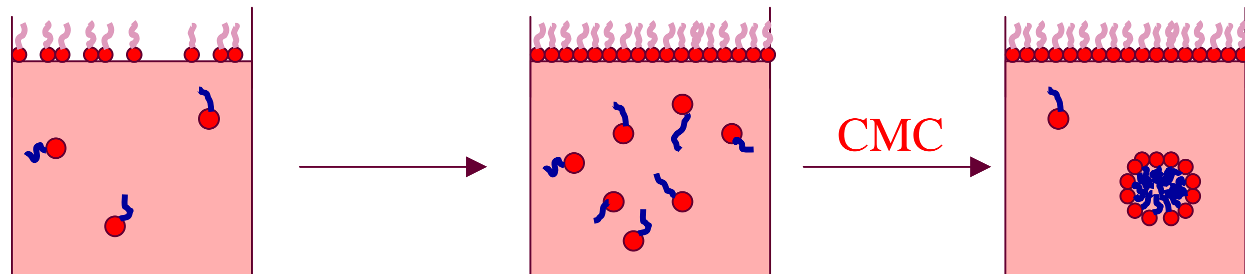
...enzymatically hydrolized Phospholipid

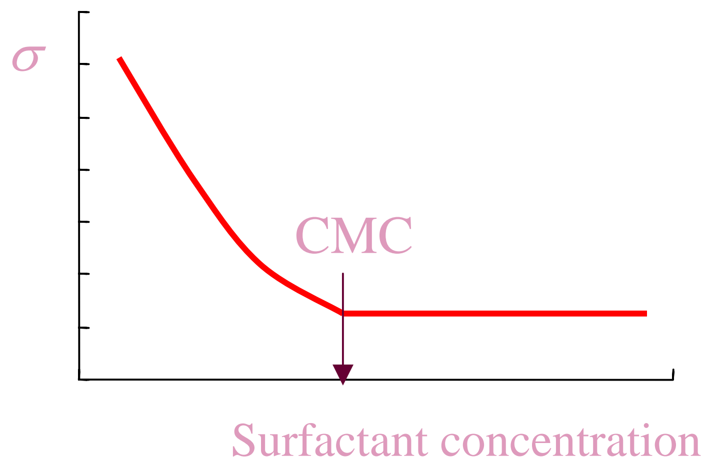




From: http://www.texturant-systems.com/skw_texturant/html/e/r_d/lecith.htm

Critical Micelle Concentration





- Below CMC only unimers are present
- Above CMC there are micelles in equilibrium with unimers

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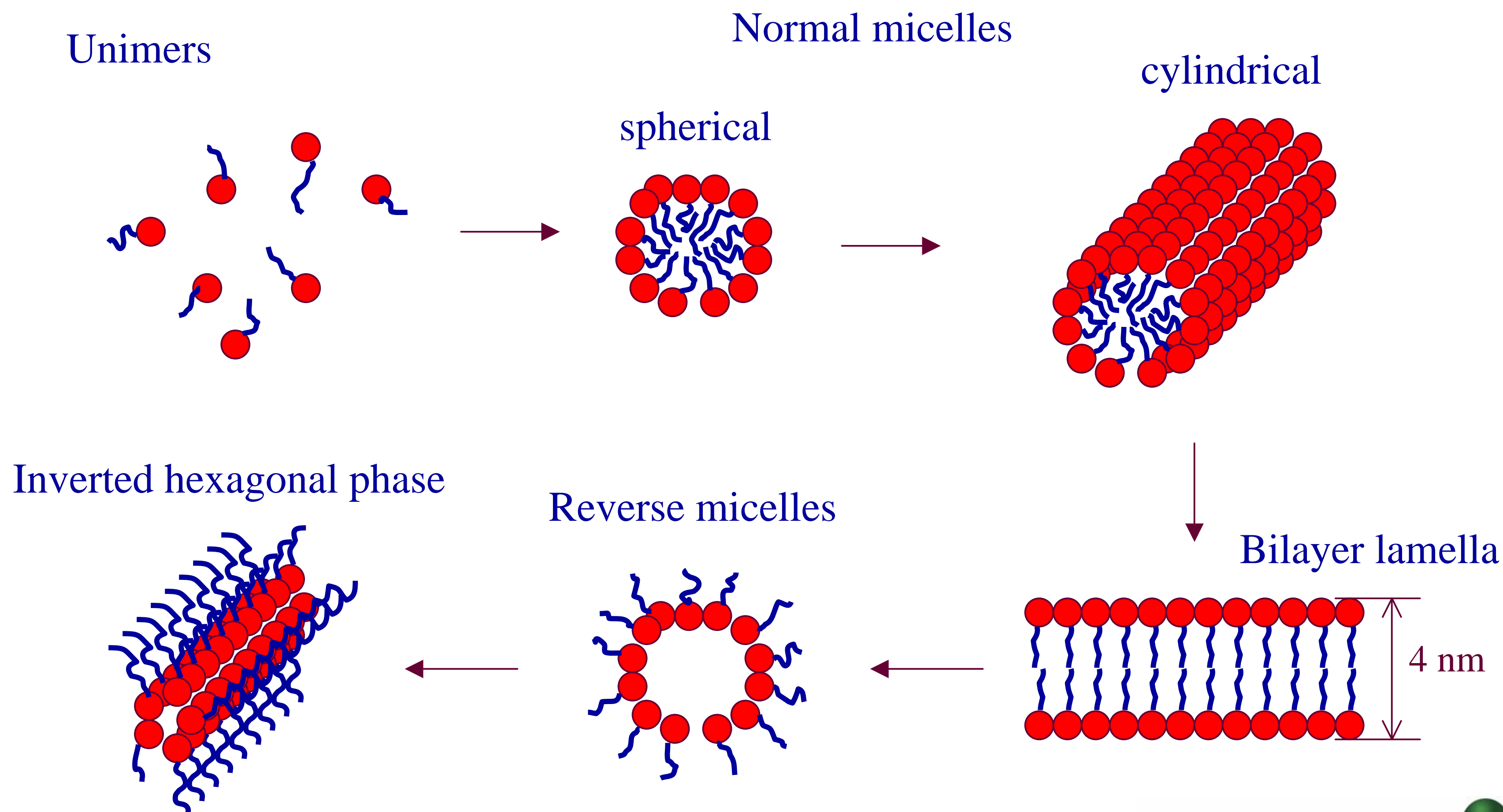
Surfactant Aggregates



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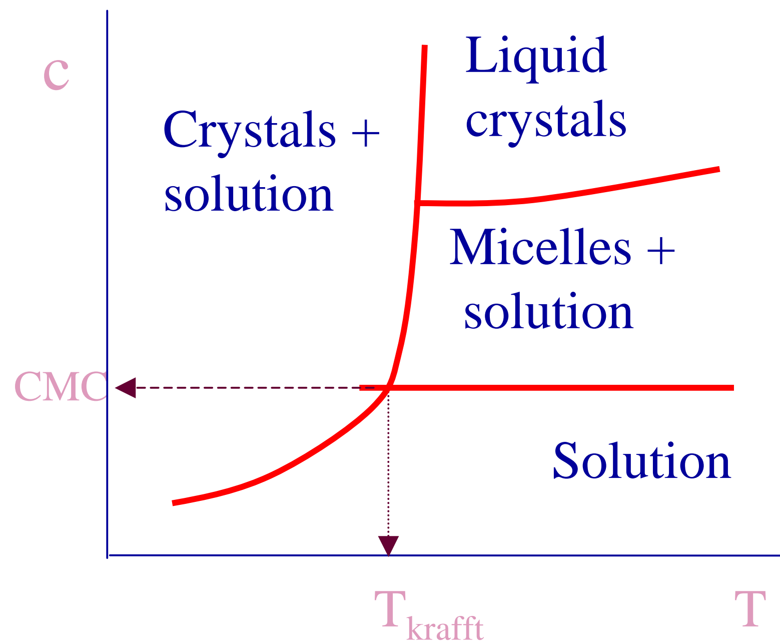
Krafft Point



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The Krafft temperature of surfactants

- The Krafft temperature, or Krafft point, of a surfactant determines it's operating range. At the Krafft point the solubility of the surfactant is equal to its cmc and micelles can form. Below this temperature the behaviour is unremarkable and the surfactant behaves like a simple salt.

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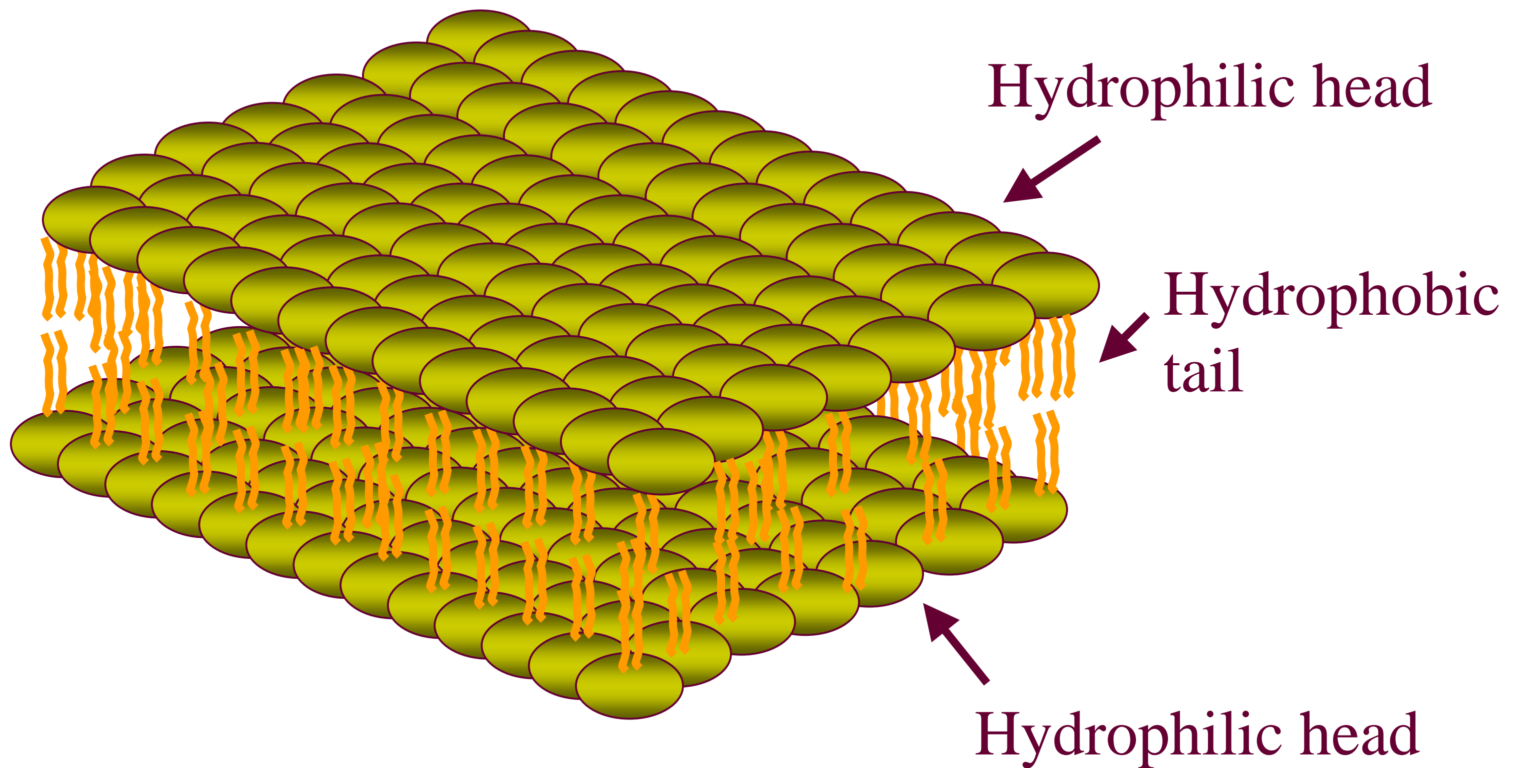
Lipid bilayer matrix in aqueous environment



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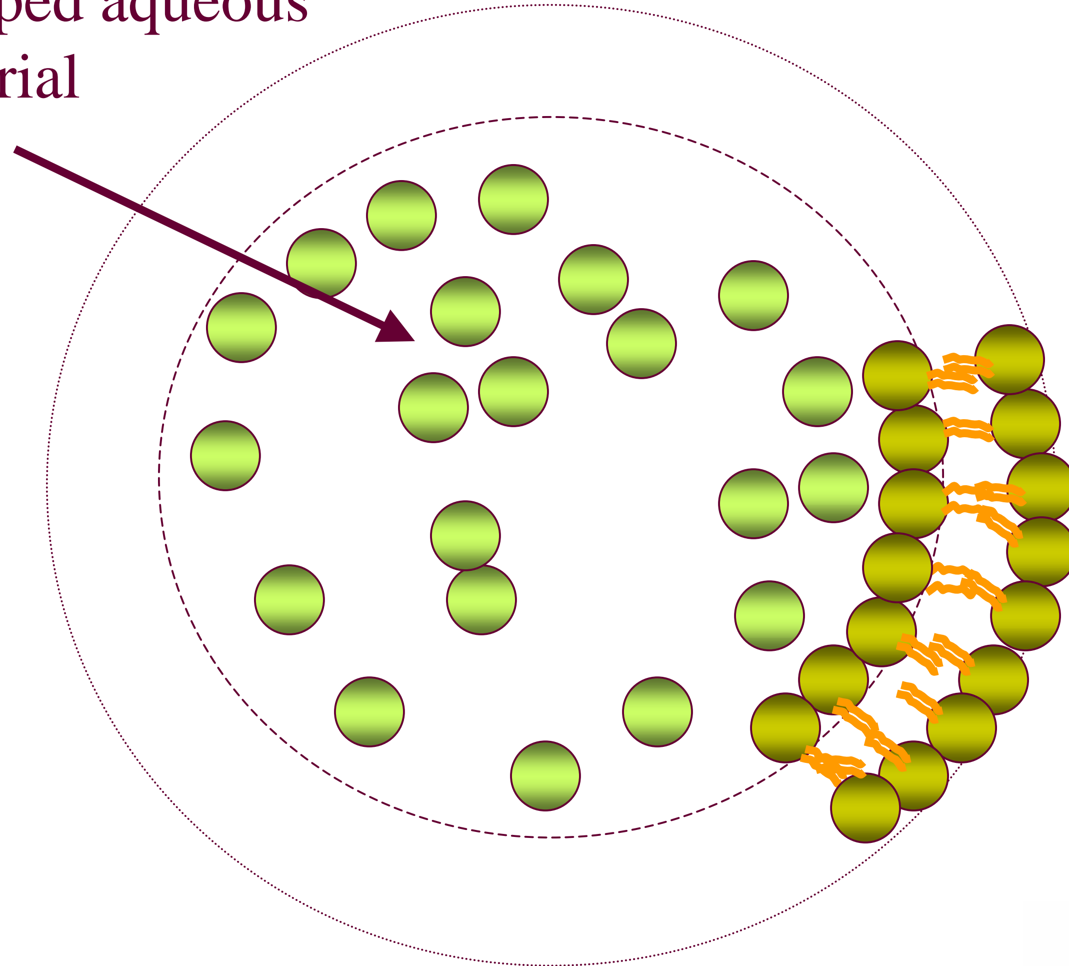
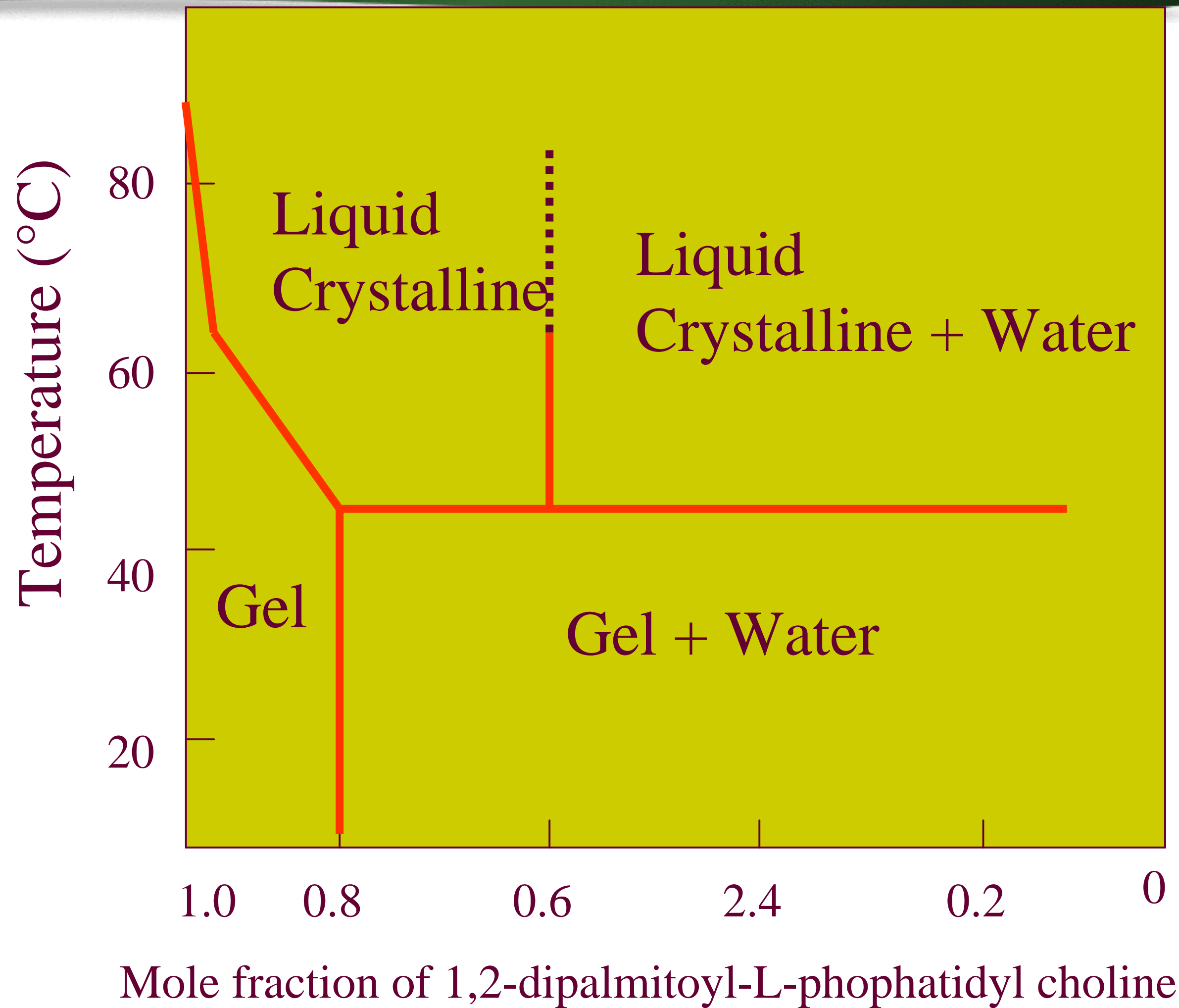
Liposome formed by a closed bilayer



Physical properties of liposomes



(Kim & Baianu, 1991)

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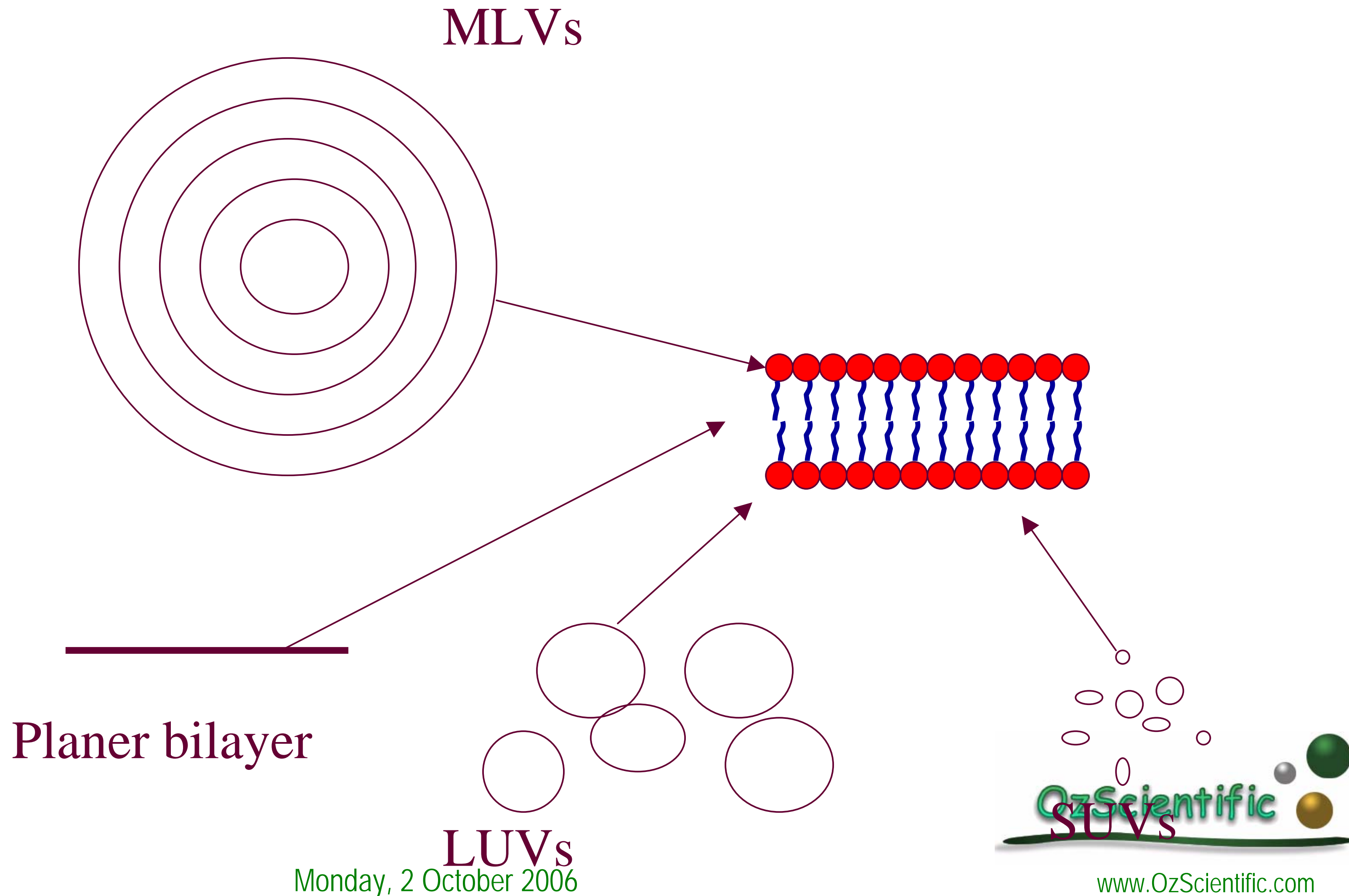
Liposome classifications

- Multilamellar vesicles (MLVs)
- Small Unilamellar Vesicles (SUVs)
- Large Unilamellar Vesicles (LUVs)

Two-dimensional presentation of the lipid bilayer



Multilamellar vesicles (MLVs)

- Large number of concentric lipid bilayers separated by water layers
- Characteristic onion-like arrangement of 5-50 um
- Often form spontaneously when anhydrous membrane lipid takes up 30% of water (by weight) at a temperature above the lipid phase transition temperature

Preparation of MLVs

PLs + Chloroform

Evaporation to form thin film

Hydrate with aqueous solution
To be incorporated

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MLVs – Advantages and disadvantages

- Advantages
  - Easy to prepare
  - Lipids and aqueous solution to be incorporated are not subjected to harsh treatments, such as exposure to organic solvents
- Disadvantages
  - Heterogeneous size distribution
  - Low efficiency of encapsulation (5-14%)

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Small Unilamellar Vesicles (SUVs)

- Methods of preparation
  - Sonication of MLVs. High intensity ultrasound results in MLVs of much smaller size (25-50 nm)
  - Injection of an ethanol solution of lipids into desired aqueous phase; the diameters of resulting vesicles are in the range 30-110 nm
  - Pumping MLVs through a French pressure cell to produce liposomes with diameter in the range 30-50 nm

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SUVs – advantages and disadvantages

- Advantage
  - Fairly easy to prepare
- Disadvantages
  - Small diameter and as a consequence their low capture volume
  - Sonication method can potentially cause contamination of vesicle suspension with metal tip of the probe
  - Long sonication can lead to disintegration of membrane lipid
  - Poor reproducibility of size

Large unilamellar vesicles (LUVs)

- Often most useful liposomes (sizes >100 nm)
- Methods of preparation
  - Carefully controlled hydration of a thin layer of PLs; large number of thin-walled vesicles of 0.5-10 um are formed
  - LUVs appear after controlled removal of detergent from a detergent-lipid mixture
  - Controlled injection of ethanol-lipid mixture or an ether-lipid mixture into an aqueous solution. The vesicles are formed by dissolution or evaporation of the ethanol or ether solvent respectively, leaving the amphiphilic lipids to aggregate into a bilayer
  - Reverse-phase evaporation – based on the phase reversal of a water-in-oil emulsion induced by the removal of the organic phase under reduced pressure
  - Calcium-induced fusion of small unilamellar vesicles (SUVs)

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LUVs – advantages and disadvantages

- Advantage
  - Large single-shelled vesicles are able to entrap a large fraction of the aqueous solution in which they are formed
- Disadvantages
  - Relatively difficult to prepare
  - Heterogeneous size distribution
  - Poor stability when prepared from saturated lipids; tend to form MLVs spontaneously

Microfluidisation

- Liposomes are formed by processing mixtures of phospholipids and water through microfluidiser that creates cavitation forces. These forces compel the bilayer membranes to assume a spherical configuration, with water filling the core volume.
- The liposome sizes are a function of membrane composition and the intensity of applied force
- Active molecules can be added during the formulating process

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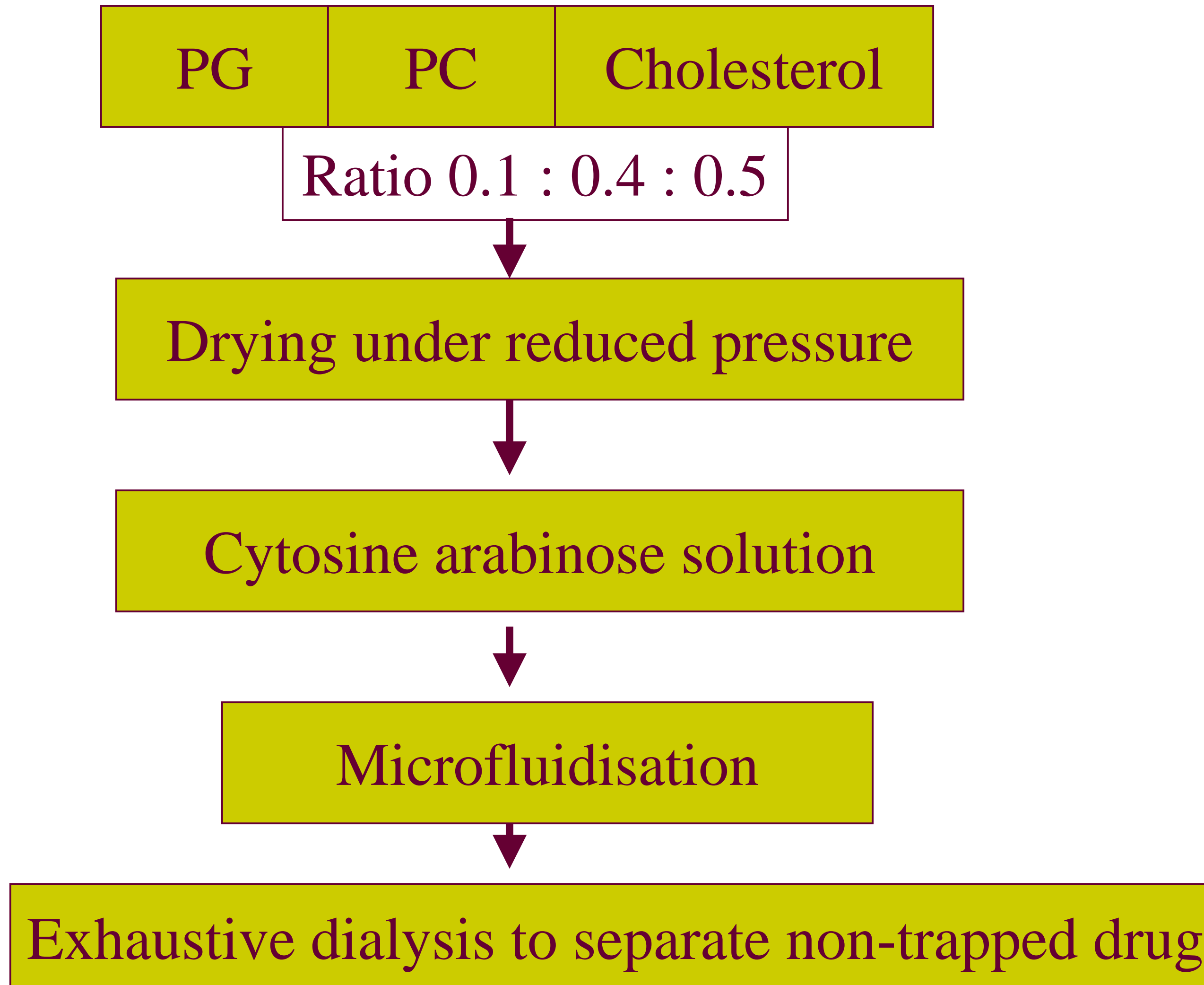
Application of microfluidiser – Mayhew *et al.* (1984), BBA 775 (1984) 169-174



Application of microfluidiser – Mayhew *et al.* (1984) - Analysis

Liposomes analysed for

- Permeability
- Drug capture
- Electron microscopy – freeze fracture

Application of microfluidiser – Mayhew *et al.* (1984) - results

Type of liposome	Initial lipid conc (µmol/mL)	Recycling time (min)	% Capture	Litre aqueous/mol lipid
MEL	60	2	6.3	1.03
	60	10	5.0	0.83
	180	2	17.4	0.97
	180	10	16.0	0.89
	300	10	78.0	0.73
	300	30	73.9	0.69
	300	60	74.6	0.69
SUV	60	-	3.2	0.54
	180	-	8.4	0.47
MLV	60	Unextruded*	10.7	1.79
	60	0.2 µm	9.0	1.50
	180	Unextruded	26.9	1.49
	180	0.2 µm	24.9	1.38
REV	60	Unextruded	45.7	7.6
	60	0.1 µm	9.6	1.6
	180	Unextruded	50.0	2.8
	180	0.1 µm	19.2	1.1

MEL – Microfluidised, SUV – small unilamellar, MLV – multilamellar,
REV – reverse phase evaporation

Application of microfluidiser – Mayhew *et al.* (1984) - Conclusions

- MF liposomes have properties similar to small extruded MLVs
- Uniform size distribution
- No clogging as noticed for extrusion process
- MF can work at considerably higher conc. of lipids – reduced loss of entrapped material and higher final concentration of drug material
- Unlike reverse phase evaporation, MF does not require expensive organic solvents
- Continuous processing with MF
- Permeability results suggest that MF liposomes are at least as stable as MLVs – permeability increases with decrease in the size

Characterisation of liposome molecules

- Size distribution
  - Difficult to analyse whole size range
- Analytical gel filtration on agarose gels
  - Suitable for SUVs; LUVs are eluted in the excluded volume
- Analytical ultracentrifuge
- Electron microscopy

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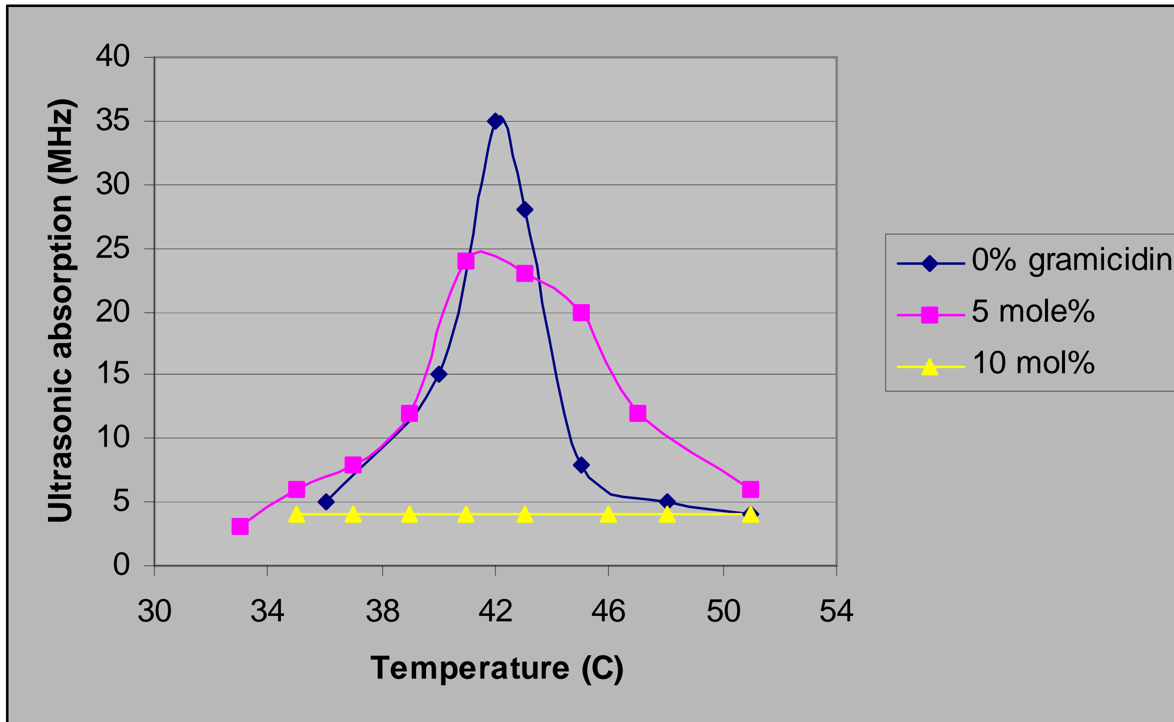
Characterisation of liposome molecules -contd

- Radioactive tacers
  - 1-b-arabinofuranosylcytosine (a water soluble tracer)
- Fluorescence quenching
  - Carboxyfluorescein self-quenching
- Ultrasonic absorption (acoustic resonance absorption)
  - Hydrophobic peptide (gramicidin-A)
- Electronic spin resonancy (ESR)
  - Water-soluble, electron paramagnetic resonance probe, trimethy-4-amino-2,2,6,6-tetramethyl-1-oxy-piperidine (CAT_1)
- Nuclear magnetic resonance (NMR)

Temperature dependence on ultrasonic absorption by liposome



Magin & Niesman, 1984

Instability of liposomes during storage

- Chemical stability of lipid
- Change in vesicle size with time
- Vesicle structure
- Leakage of core material
- Environmental damage to integrity and permeability
- Destabilisation in presence of oil in food system
- Destabilisation in presence of hydrophobic proteins such as milk protein b-basein

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Factors affecting stability

- Liposome type
  - ML>LUV>SUV
- Temperature
  - 40C>25C>37C
- Lipid composition
  - Saturated PLs>Saturated PLs plus cholesterol>Unsaturated PLs+cholesterol>unsaturated PLs

Mayhew & Papahadjopoulos, 1983

Liposome extraction methods

- Bligh-Dyer two-phase extraction
- Sep-Pak minicolumn extraction
- Ultrasonic disruption

Applications of liposomes

- Pharmaceutical industry
  - Targeted drug delivery
  - Immune modulation
- Cosmetics industry
  - Efficient delivery of moisturizing ingredients (including water)
  - Delivery of oxygen to the skin to retard ageing??
- Food industry

Applications in food industry

- Controlled release of proteinases to enhance flavour development of cheese (Kheadre etal 2000)
- Fortification of cheese with Vitamin D (Banville et al., 2000)
- Decrease in vapour pressure (modulation of water activity)
- Controlled release of amino acids from whey-based protein ingredients (US 6,019,999)
- Infant milk formula containing liposome encapsulated nutrients (AU9913074A)
- Liposomal powdered beverage (DE29704822)
- Whey-derived fat substitute (US5413804-A)
- Encapsulated ferrous iron (US5534268-A)
- Food additive as antioxidant (JP08154598-A)

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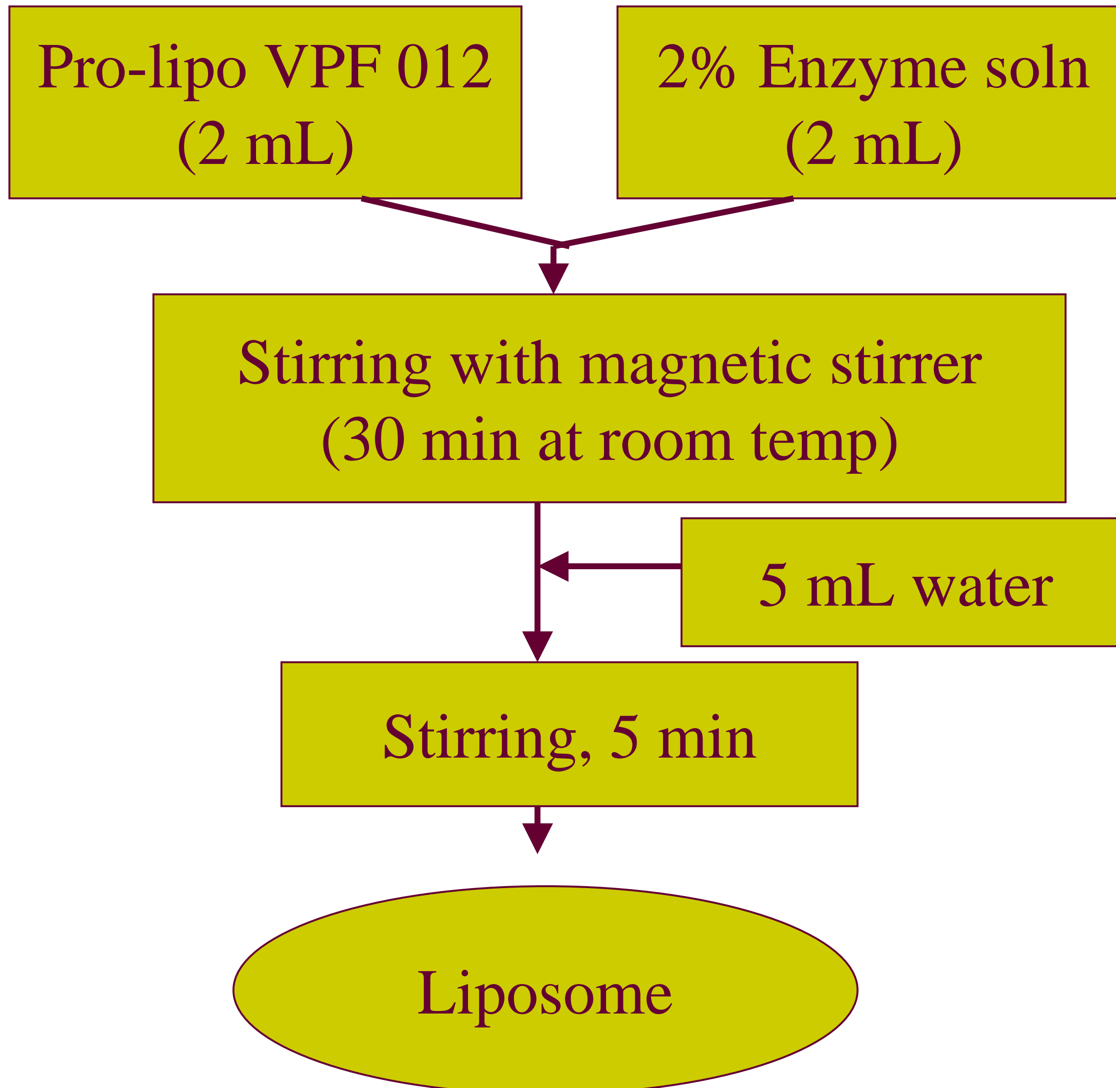
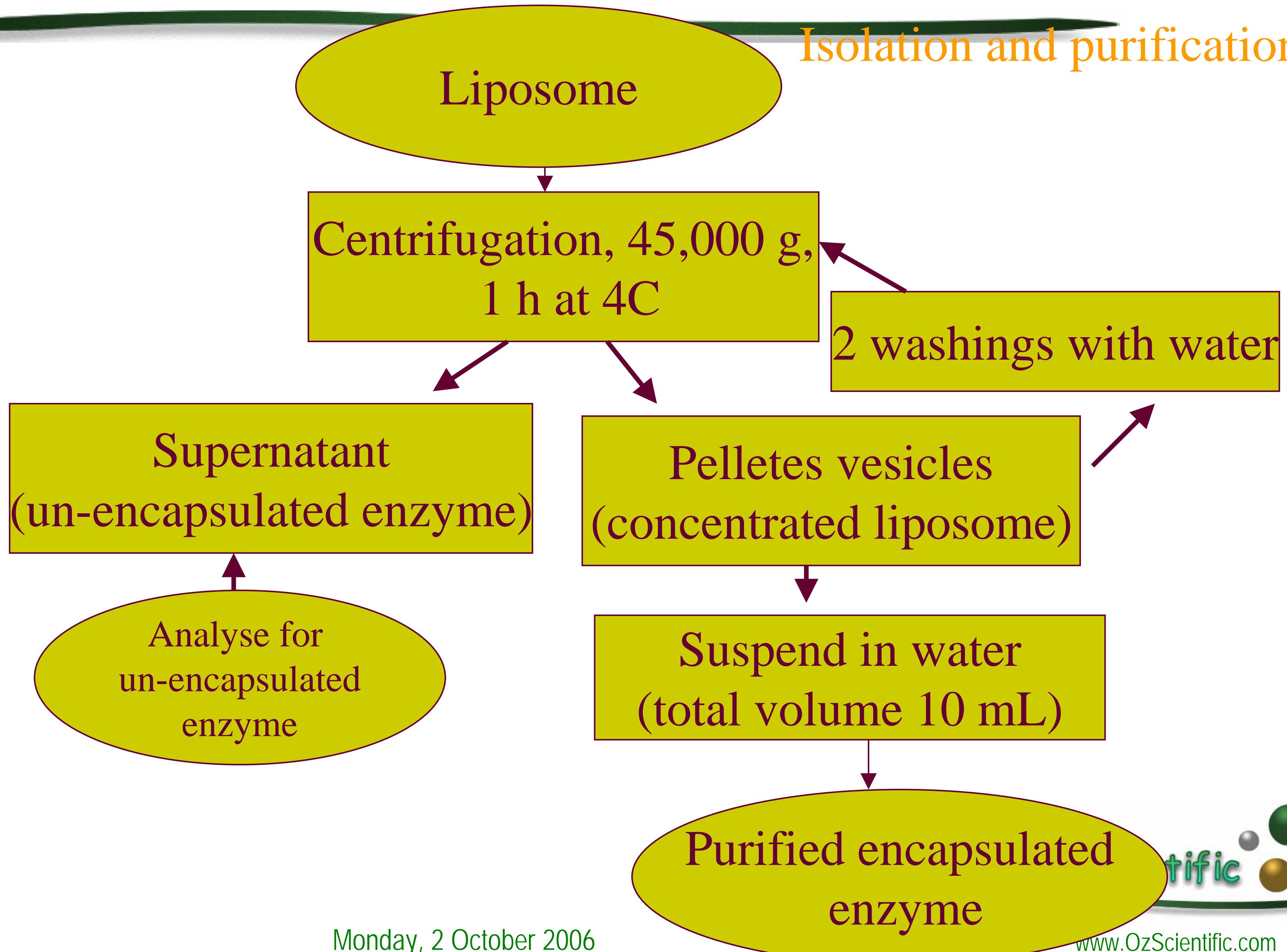
Cheese ripening (Kheadre etal 2000)

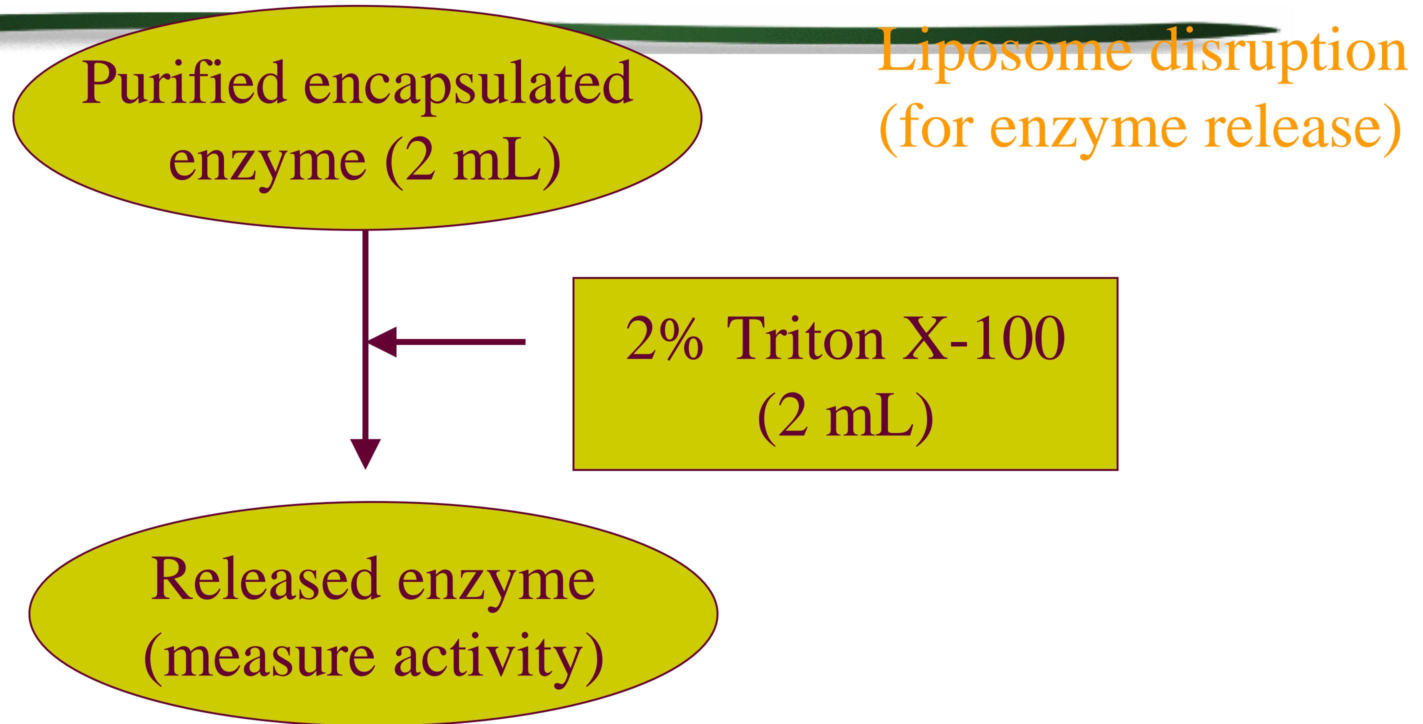
Liposome formation



Cheese ripening (Kheadre etal 2000) - contd



Cheese ripening (Kheadre etal 2000) - contd



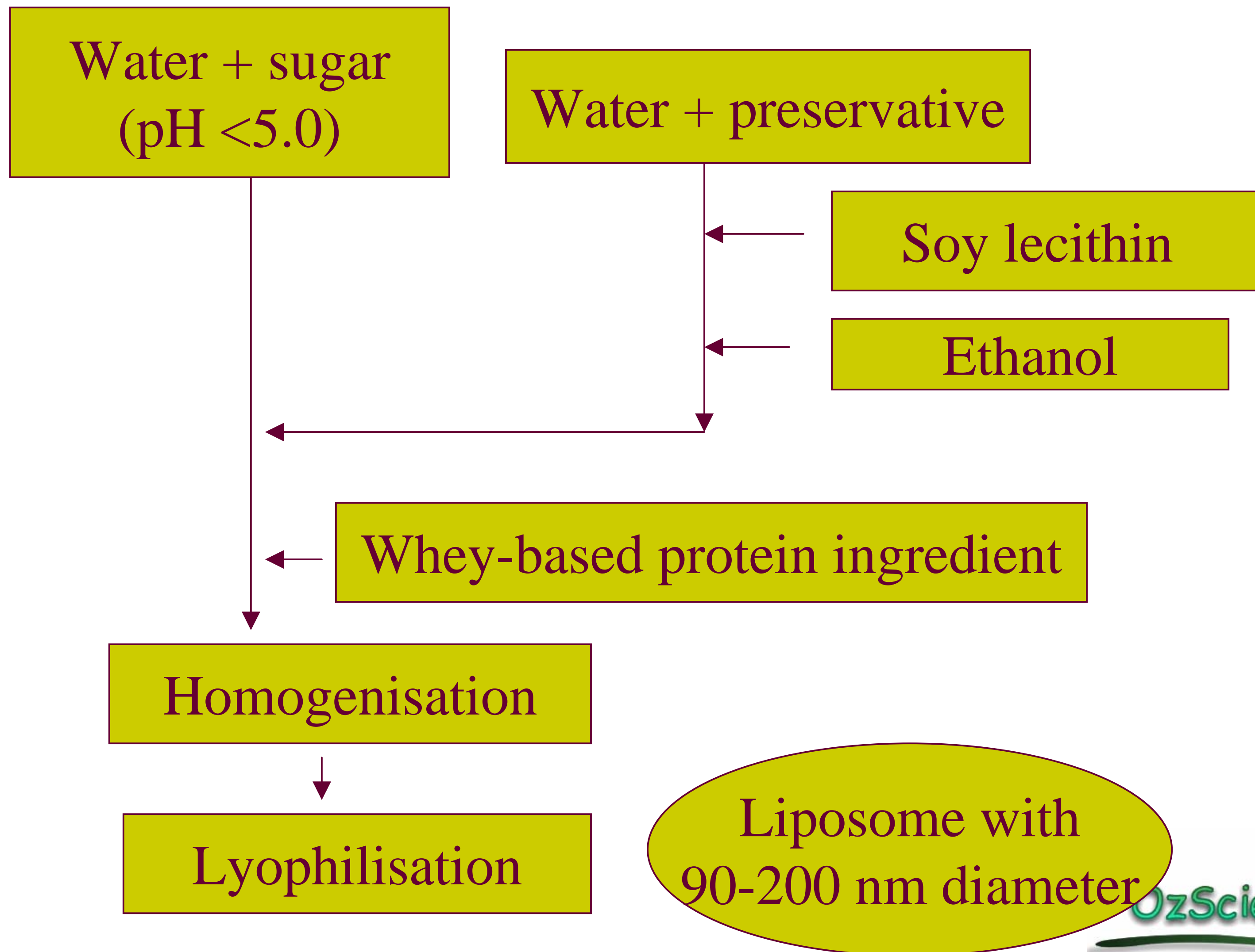
$$\text{Encapsulation efficiency (\%)} = \frac{\text{Encapsulated units}}{\text{Encapsulated units} + \text{Unencasulated units}} \times 100$$

Cheese ripening (Kheadre etal 2000) - contd

Summary of results

- Encapsulation efficiency – 32-33%
- Slight increase in cheese moisture (0.1-2%)
- Slight decrease in protein in cheese (0.1-0.8%)
- Higher proteolysis (1.1-1.5 fold) in cheese containing encapsulated proteinases in 60 days

Liposome with whey-based protein ingredients (US patent 6,019,999)



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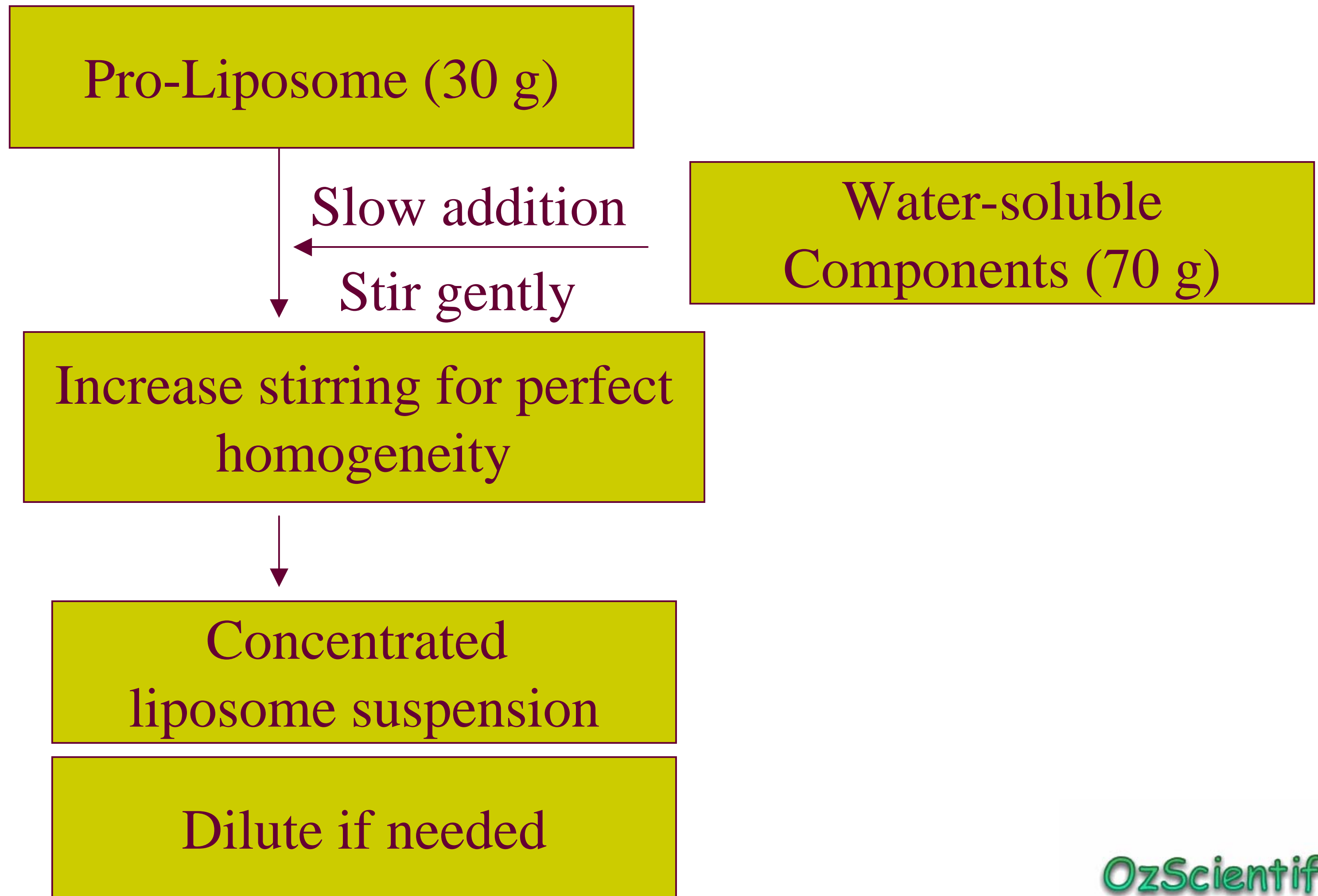
Important factors in liposome formation

- Lipid phase transition temperature
  - Degree of saturation (animal vs plant lecithin)
  - Carbon chain length of fatty acids
- Type and concentration of phospholipids
- Type and concentration of active component
- Method of liposome formation

Pro-liposome technology (Lucas Meyers)



Proposed method for liposome formation – Scheme 1 lab-scale process for large unilamellar vesicles (LUV)

Phosphatidyl Choline or hydroxylated Lecithin or dairy lecithin (4 g)

Choloform/ethanol (1:2) mixture (50 mL)

Spread at the bottom of a large flask

Evaporate solvents at room temp with circulation of nitrogen (without stirring)

Disperse the lipid bilayers in the aqueous solution of the active components

Mix with a high-speed stirrer to form liposomes

Scheme 2 – Pilot-scale formation of large unilamellar vesicles

Phosphatidyl Choline or hydroxylated Lecithin or dairy lecithin (4 g)

Choloform/ethanol (1:2) mixture (50 mL)

Spread at the bottom of a Stephan kettle (temp- and vacuum-controlled)

Evaporate solvents at room temp with circulation of nitrogen (without stirring)

Disperse the lipid bilayers in the aqueous solution of the active components

Mix with a high-speed stirrer to form liposomes

Scheme 3 – Use of microfluidiser

1. Manufacture of smaller large uni-lamellar vesicles by microfluidisation of LUV

2. Manufacture of liposomes by microfluidisation of PLs in the solution of the active ingredient

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Efficiency of encapsulation

- Use of a marker component (Fe or a marker dye)
- Monitoring HPLC profiles of major peaks in the supernatant.

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Release mechanisms

- SHEAR
- pH CHANGES
- CHEMICAL/ENZYMATIC REACTIONS
- OSMOTIC PRESSURE

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Microencapsulation of water-soluble components – liposome technology

- Pro-liposome technology
  - Pro-lipo S
  - Pro-lipo C
  - Pro-lipo duo
- Liposome technology
  - Hydroxylated lecithin
  - Phosphatidyl choline

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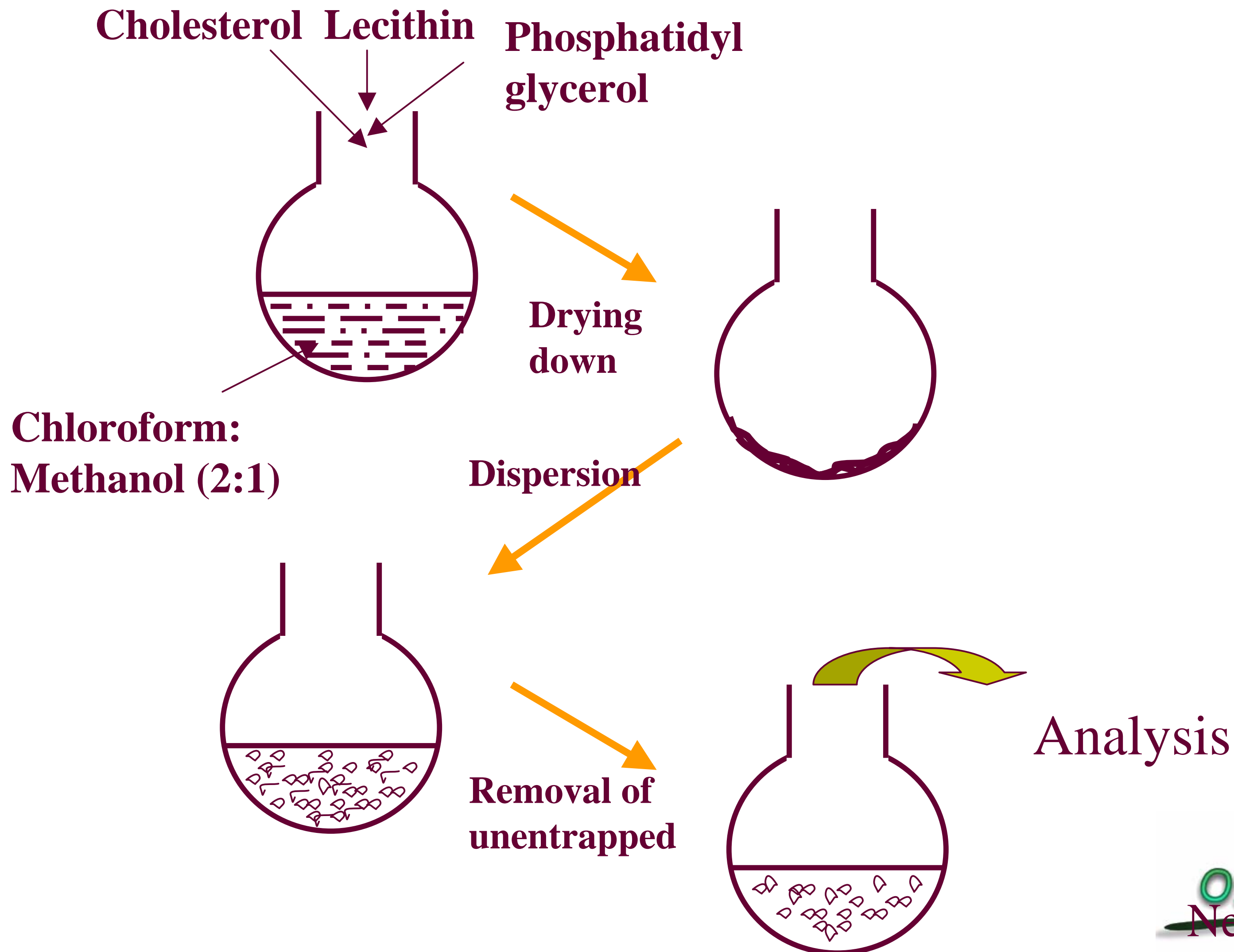
Pro-liposome - variables

- Type of Pro-liposome
  - Pro-lipo s
  - Pro-lipo C
  - Pro-lipo duo
- Temperature during liposome formation
  - 20°C
  - 60°C
- pH of water-soluble extract
  - 3
  - 7
  - 9
- Effect of shearing, e.g. microfluidisation?

Common steps in liposome preparation



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