Analysis of Lipid Metabolites

= Lipidomics

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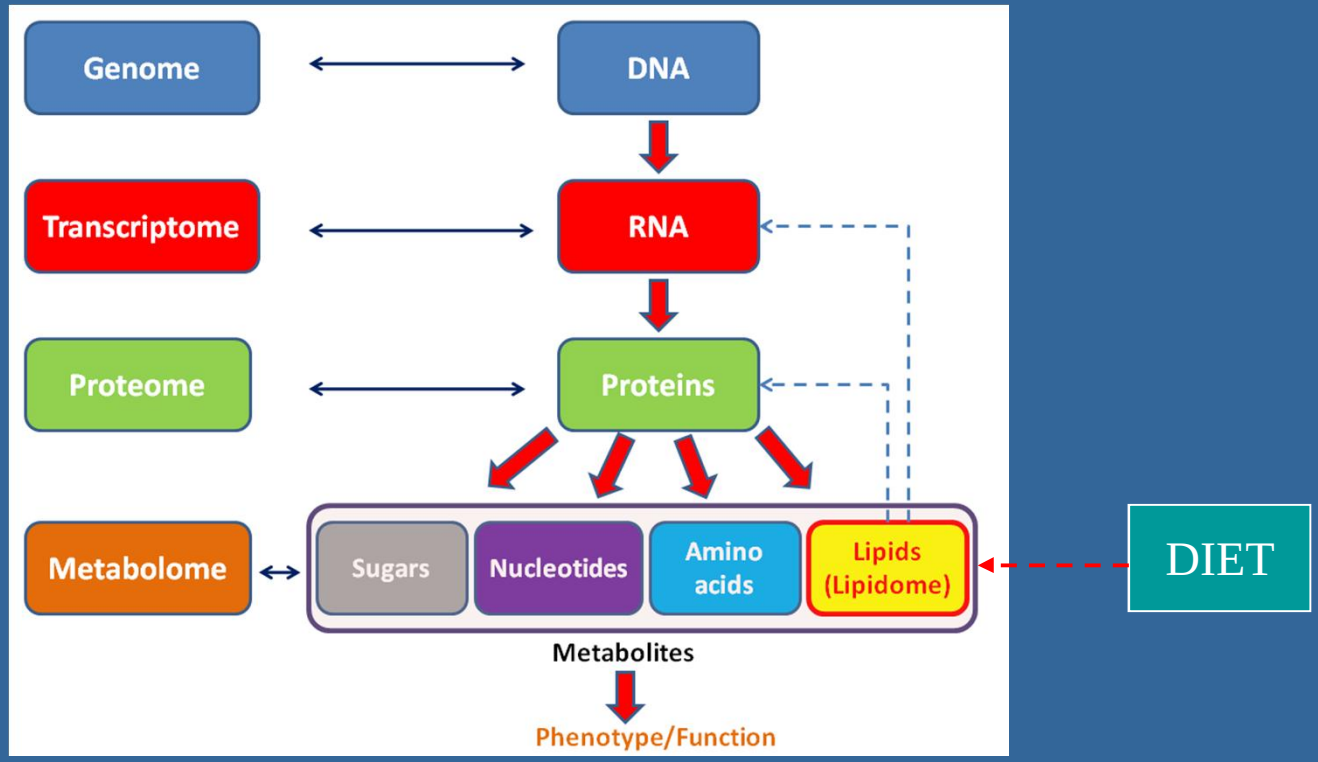
Outline of the talk

- What is Lipidomics?
- Where is lipidomics needed?
- Lipid classes and their functions
- Why mass-spectrometric analysis?
- Targeted or nontargeted analysis?
- How to improve selectivity of detection?
- Data analysis and interpretation
- Dynamic lipidomics (study on lipid metabolism)
- Glycerophospholipid homeostasis
  - Regulation of synthesis
  - Regulation of degradation
  - Coordination by superlattice formation?

Lipidome is part of the metabolome



Functional Lipidomics = How other molecules affect the lipidome and *vice versa*

Where is lipidomics needed?

Biology

- Functions of lipids?
- Regulation lipid composition of membranes?

Clinics

- Search of diagnostic/predictive markers
- Search of drugs targeting lipid disorders

Industry

- Modification of fats and oils
- Quality control

Significance of lipidomic data?

- Very similar changes in lipidome when P53 or ApoE is knocked out

  =>Changes in lipidome can be nonspecific!

- ”False biomarkers” also when the number of lipids analyzed exceeds the number of samples (patients)

- Many confounding factors: diet, gut microbiota, physical activity, age, genetic background etc

Targeted analysis

- When you know what you are looking for (e.g. studies on lipid homeostasis)
- Selective detection (MS/MS or LC-SRM)

Nontargeted analysis

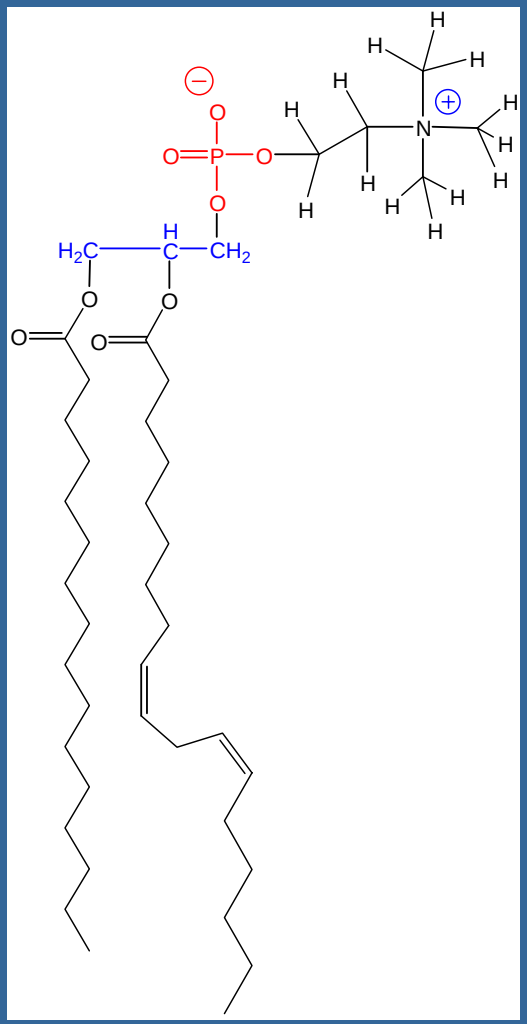
- When you do not know…

(search for disease markers/biomarkers)

- Nonselective detection (LC-MS)

Mammalian Lipid classes and their main Functions

Glycerophospholipids



Phosphatidylcholine

>10 classes (PC, PE, PS, PI, PA etc)

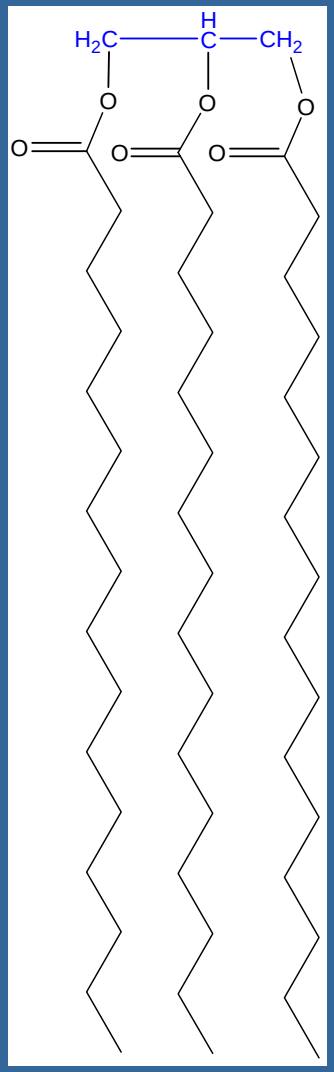
- Each class consists of numerous species due to different fatty acid combinations

=> *Thousands of different species possible!*

Functions:

- Main structural components of membranes
- Second messengers in signal transduction
- Regulators of membrane trafficking
- etc

Apolar (neutral) lipids



Fatty acids

- Structural componets of other lipids
- Energy source/storage
- Precursors of eicosanoids etc

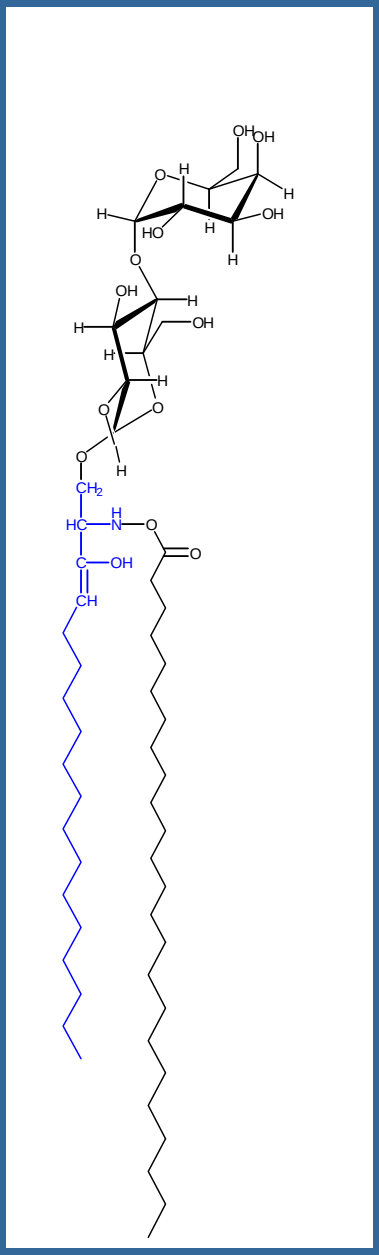
Acylglycerols (TG etc)

- Fatty acid storage and transport
- Hundreads of species possible

Cholesteryl esters

- Storage forms of cholesterol

Glygosphingolipids



Lactosylceramide

- Tens of different classes (head groups)
- Many different fatty acid

  => Hundreads of possible species

Functions

- Structural component of membranes
- Cell-cell regognition
- Signal transduction

Other lipid classes

Sterols (cholesterol etc)

- Structural components of membranes
- Precursor or steroid hormones

Eicosanoids (prostagandins etc)

- Signaling

Prenol lipids

- Membrane anchors in some proteins

A mammalian cell may contain thousands of differerent lipid species!

The biological challenge: Why?

- Each lipid species has a specific function?
- No..most lipids act in an ensemble!

The Analytical challenge

How to quantify so many species with so different properties and present at so different concentrations?

....with Mass Spectrometry!

Advantages of mass-spectrometry

Conventional analysis (PL)

1. Lipid extraction
2. Separation of lipid classes by TLC or HPLC
3. Separation of molecular species by HPLC
4. Treatment by phospholipase A2
5. Analysis of fatty acids by GC
6. Data processing

- Slow (several days)
- Low sensitivity
- Plenty of manual work

MS-analysis

1. Lipid extraction
2. MS/MS or LC-MS analysis
3. Data processing

- Fast (even less than 1 hour)
- Very high sensitivity
- Can be automated

Which ionization mode?

Electrospray (ESI)

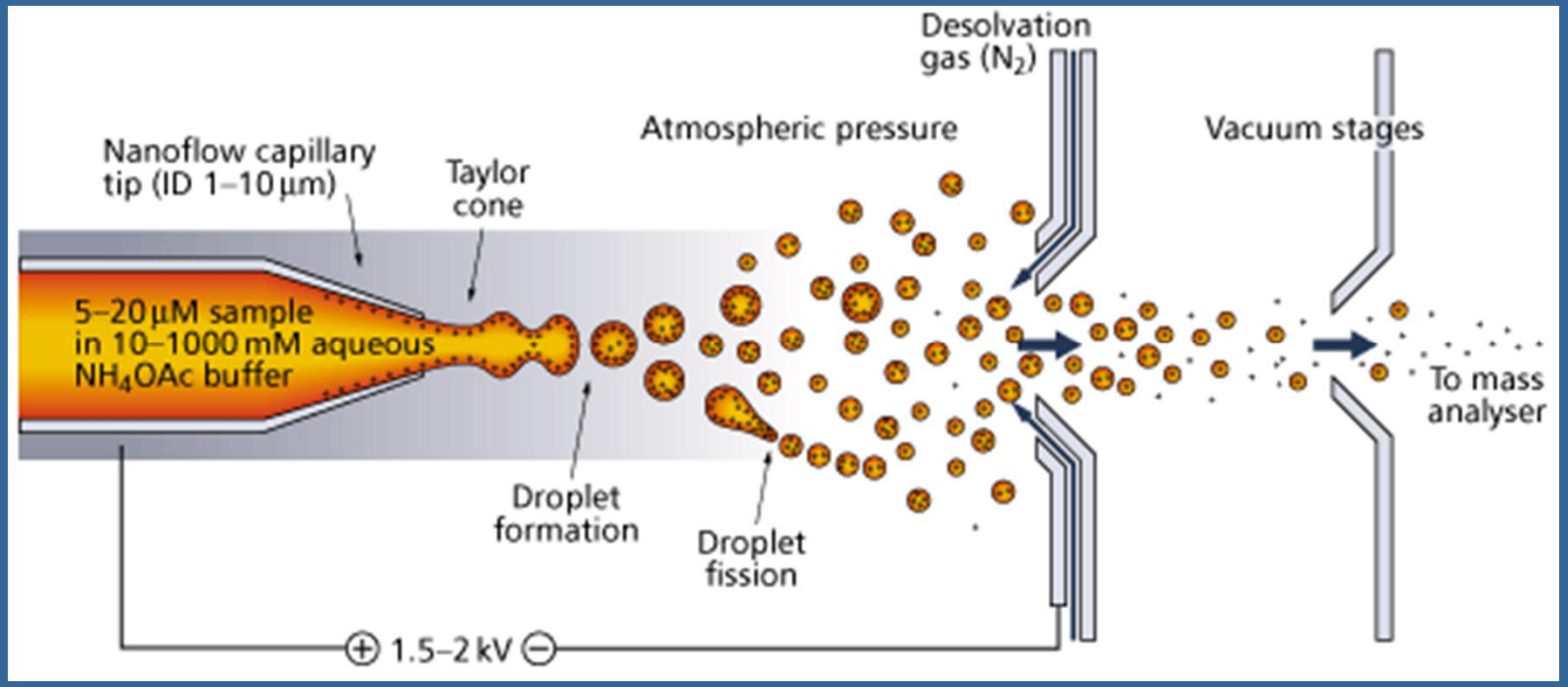
- Does not cause fragmentation
- Compatible with on-line LC

Matrix-assisted laser desorption (MALDI)

- Used less due to e.g. suppression effects

  => All lipids not detected

Electrospray ionization



Competition for charge => Suppression effects!

Which mass analyser?

Triple quadrupole

- "Workhorse"of lipidomics
- Allows precursor and neutral-loss scanning
  = Lipid class-specific detection
- Modestly (?) priced

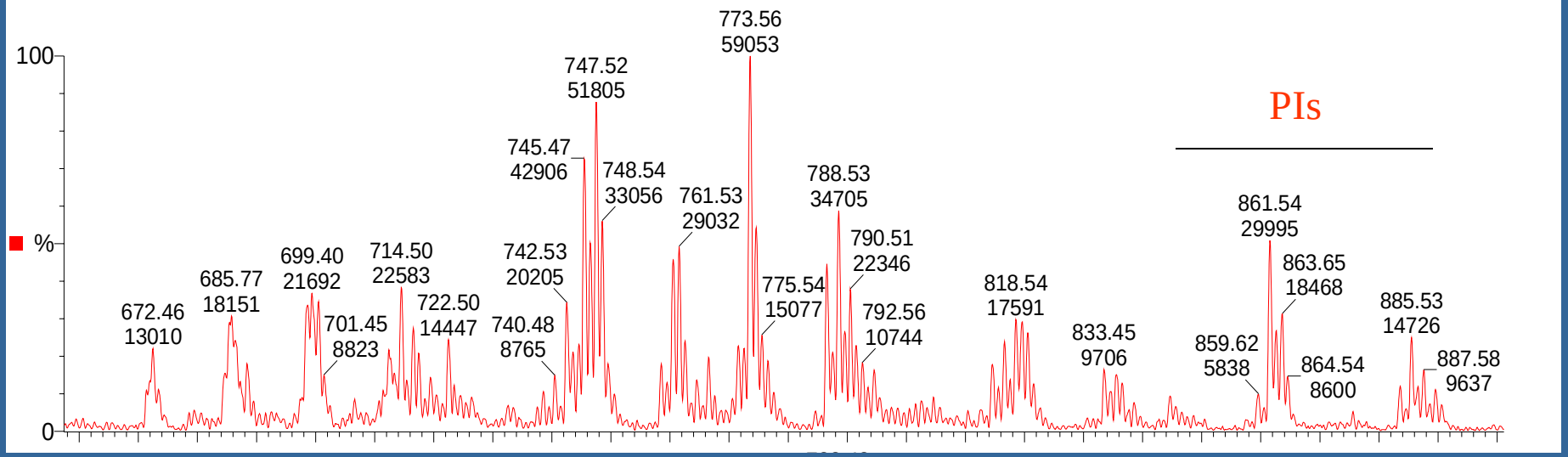
Fourier transform or Orbitrap

- Very high mass resolution and accuracy
- Allow detailed analysis of lipid structure
- Very expensive!

Direct MS scanning (triple quadrupole)

MS1 Collision cell MS2 Detector

MS- spectrum of HeLa cell lipid extract



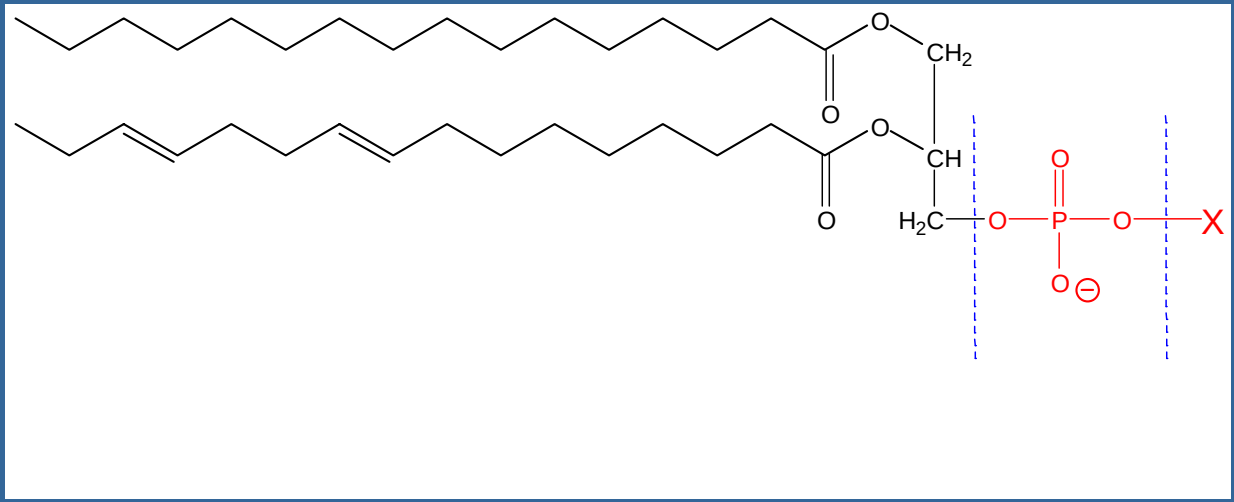
> More resolution/selectivity needed!

Means to improve resolution

- MS/MS (tandem MS)
- LC-MS (with SRM)

Lipid class -specific scanning

Phospholipid class consist of species with the same polar head-group but different fatty acid combination



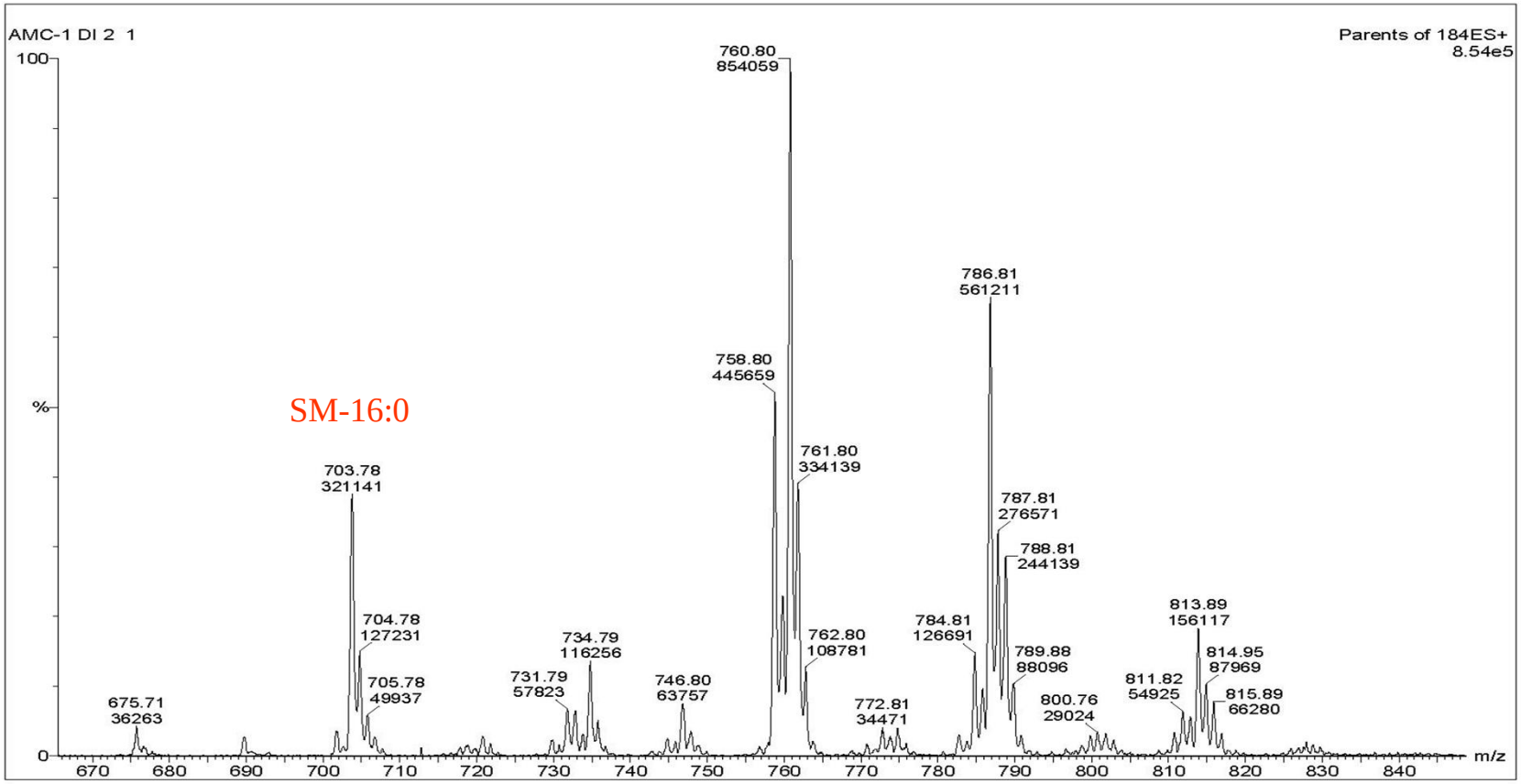
Phospholipid class	**Specific scan**	
Phosphatidylcholines	Precursors of	+184
Phosphatidylinositols	Precursors of	-241
Phosphatidylethanolamines	Neutral-loss of	141
Phosphatidylserines	Neutral-loss of	87

Precursor ion scanning

- Requires a characteristic, charged product ion

PC => Diglyceride + phosphocholine (+184)

Precursors of +184 => PC + SM

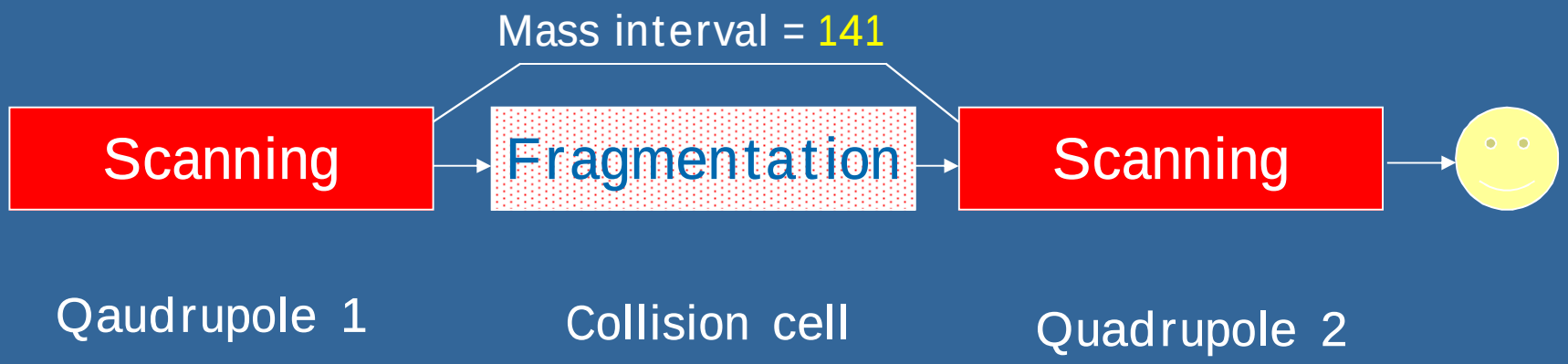


-Alkaline hydrolysis can be used to remove PCs

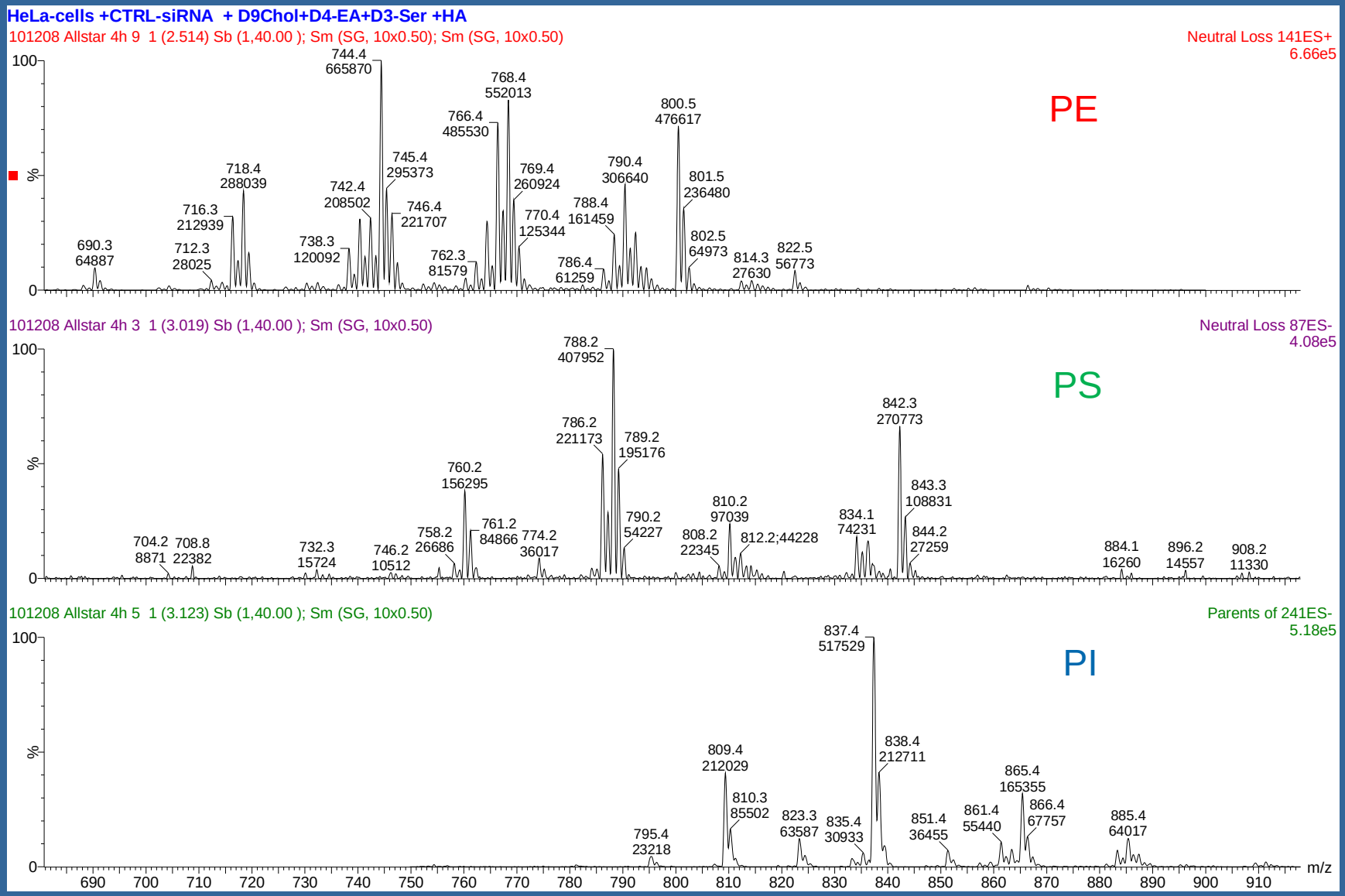
Neutral-loss scanning

..when the characteristic fragment is uncharged

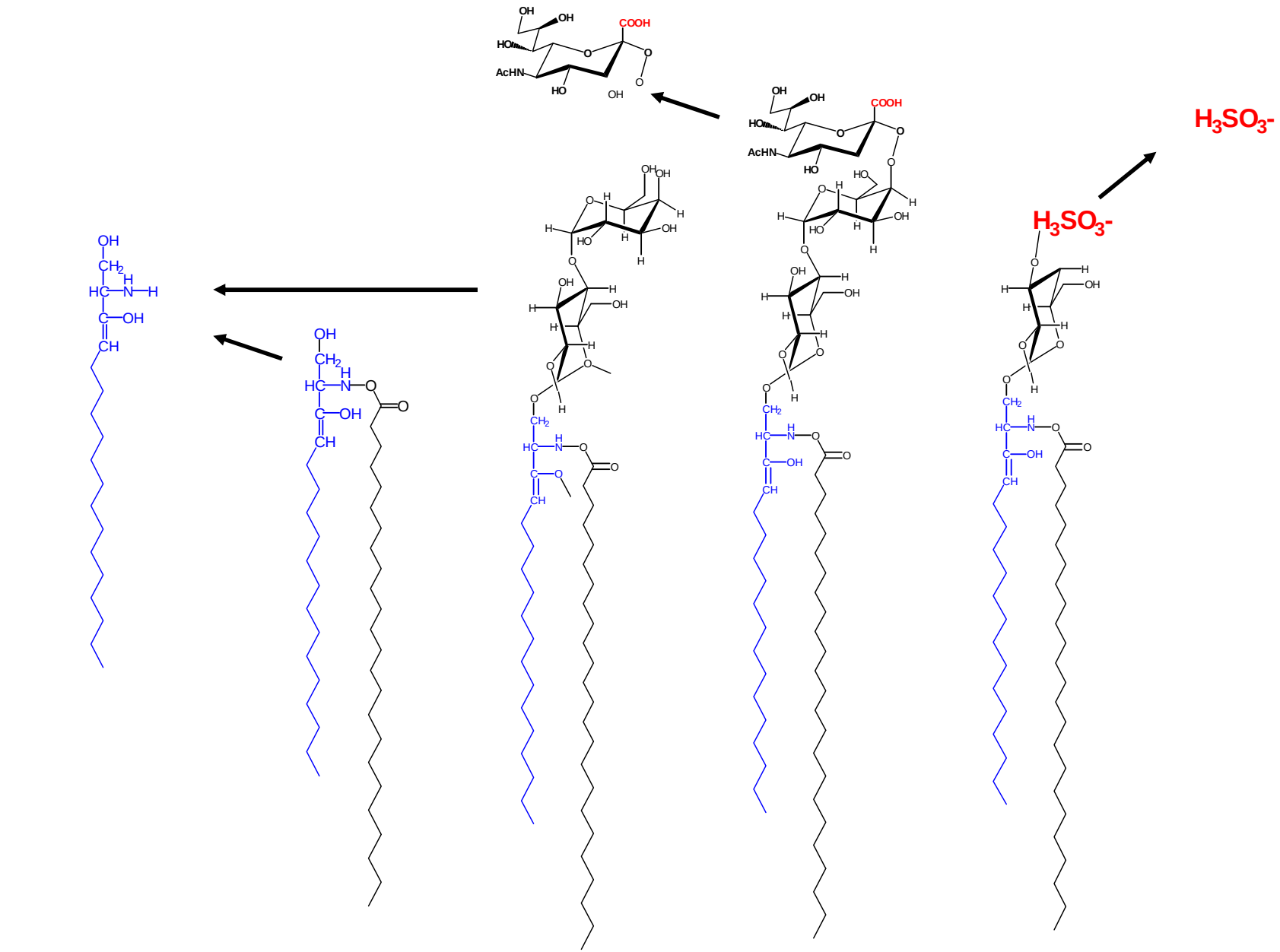
PE => Diglyceride (+) + phosphoethanolamine (141)



Selective detection of PE, PS and PI



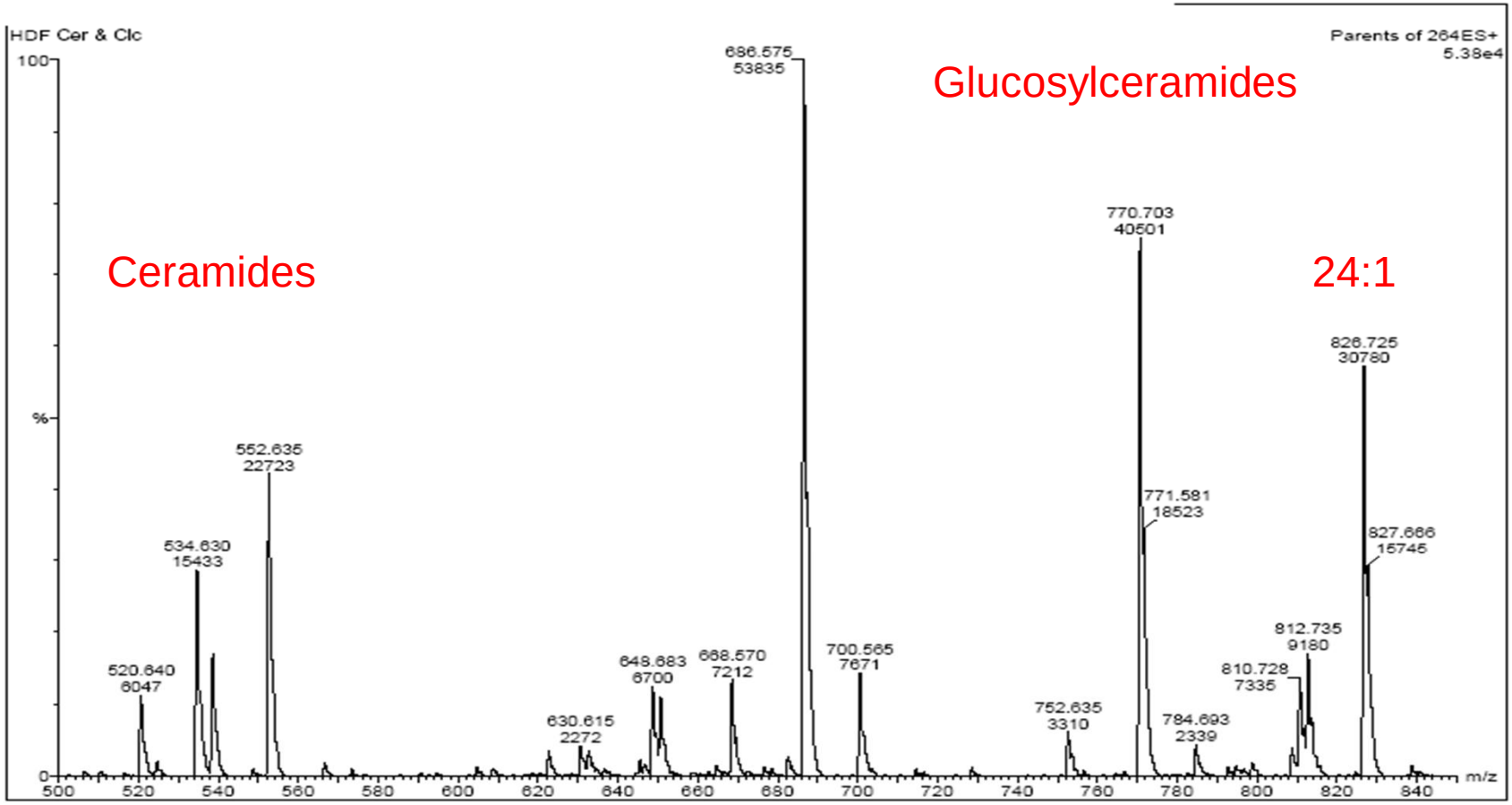
Analysis of Sphingolipids



Sphingosine ***Ceramide*** ***Lactosylceramide*** ***Ganglioside*** ***Sulfatide***

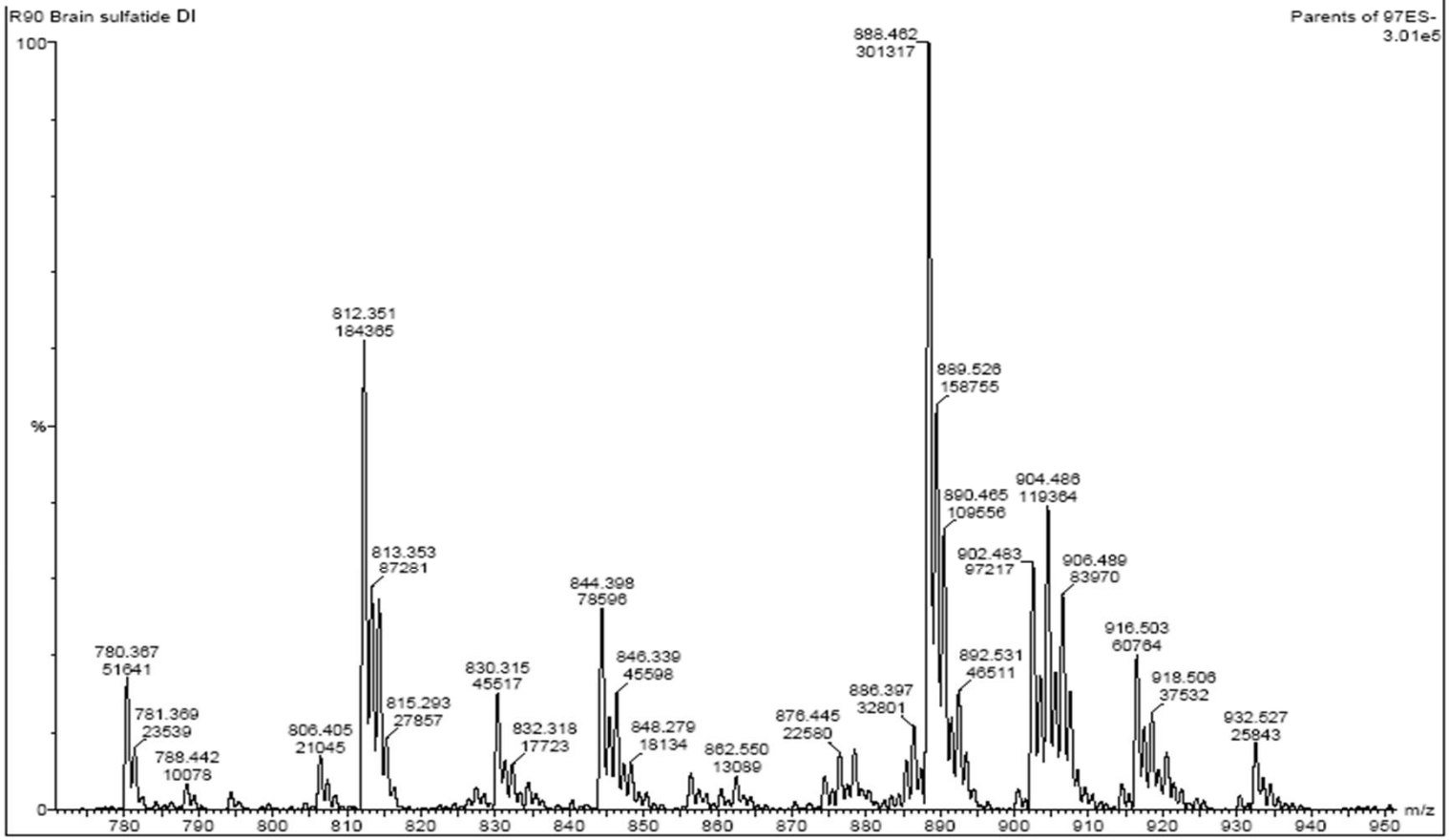
Ceramide and Neutral Glycosphingolipids

- Precursors of sphingosine (m/z +264)



Sulfatides

- Precursors of sulfate (m/z -97)



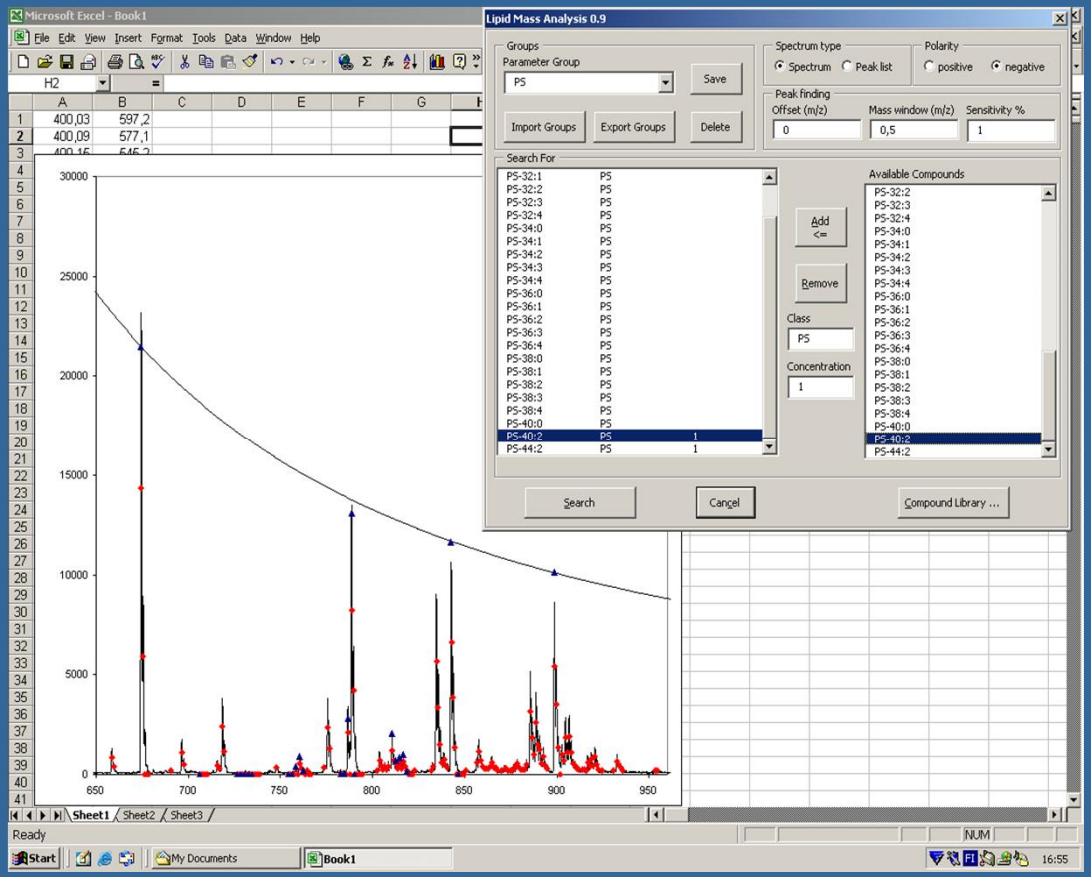
Quantification is not simple because intensity depends on:

- Lipid head-group structure
- Acyl chain length
- Acyl chain unsaturation
- Ions present (adduct formation)
- Detergent and other impurities (suppression)
- Solvent composition and instrument settings

=> Internal standards necessary!

Data analysis software is essential

LIMSA



LIMSA does:

- Peak picking and fitting
- Peak overlap correction
- Peak assignment (database of >3000 lipids)
- Quantification using internal standards

PAUSE

Dynamic Lipidomics: Analysis of lipid Metabolism

- Biosynthesis
- Degradation

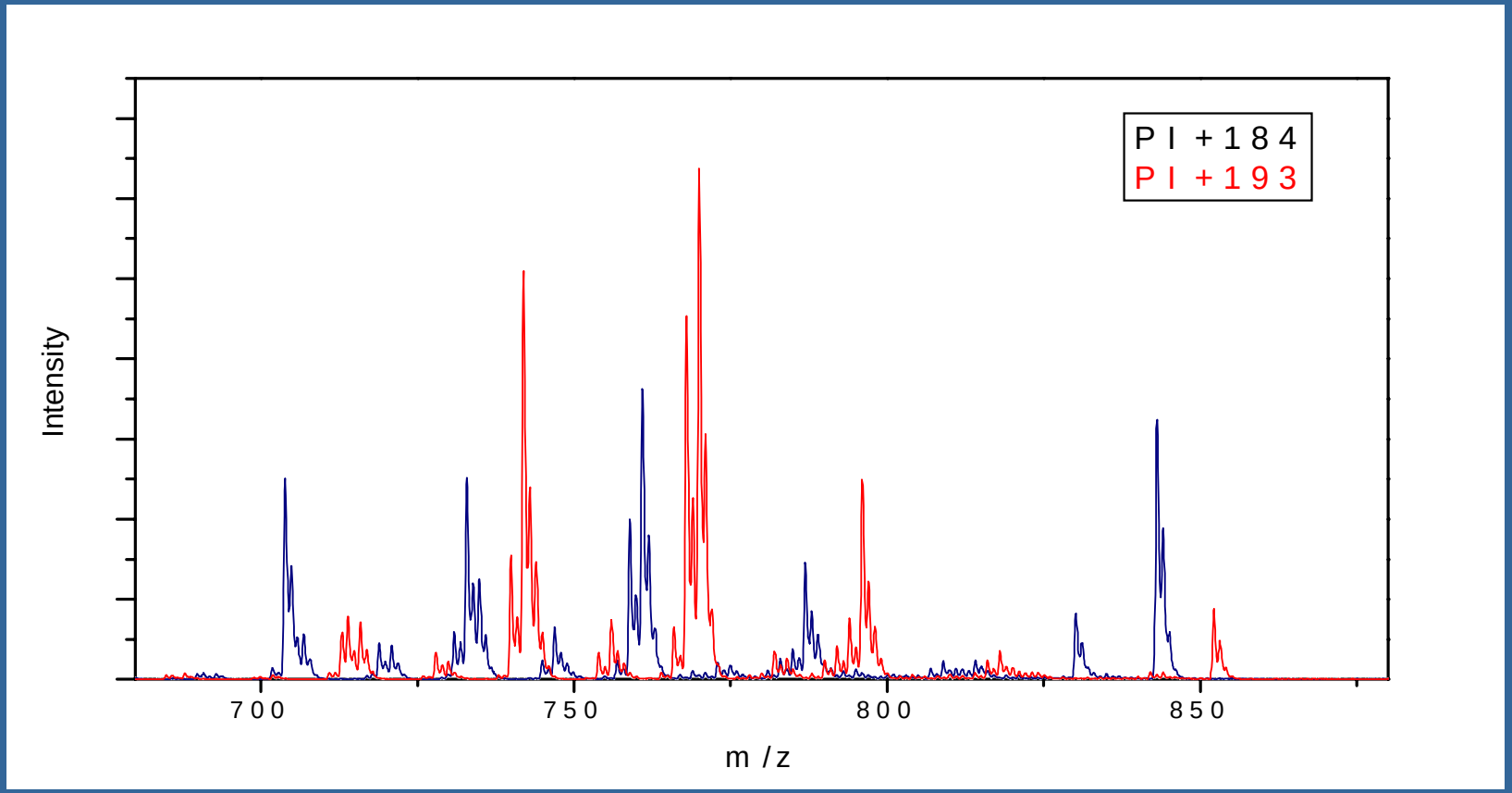
Precursors:

- Choline, ethanolamine, glycerol, fatty acids,
- Sphingosine, monosaccharides etc

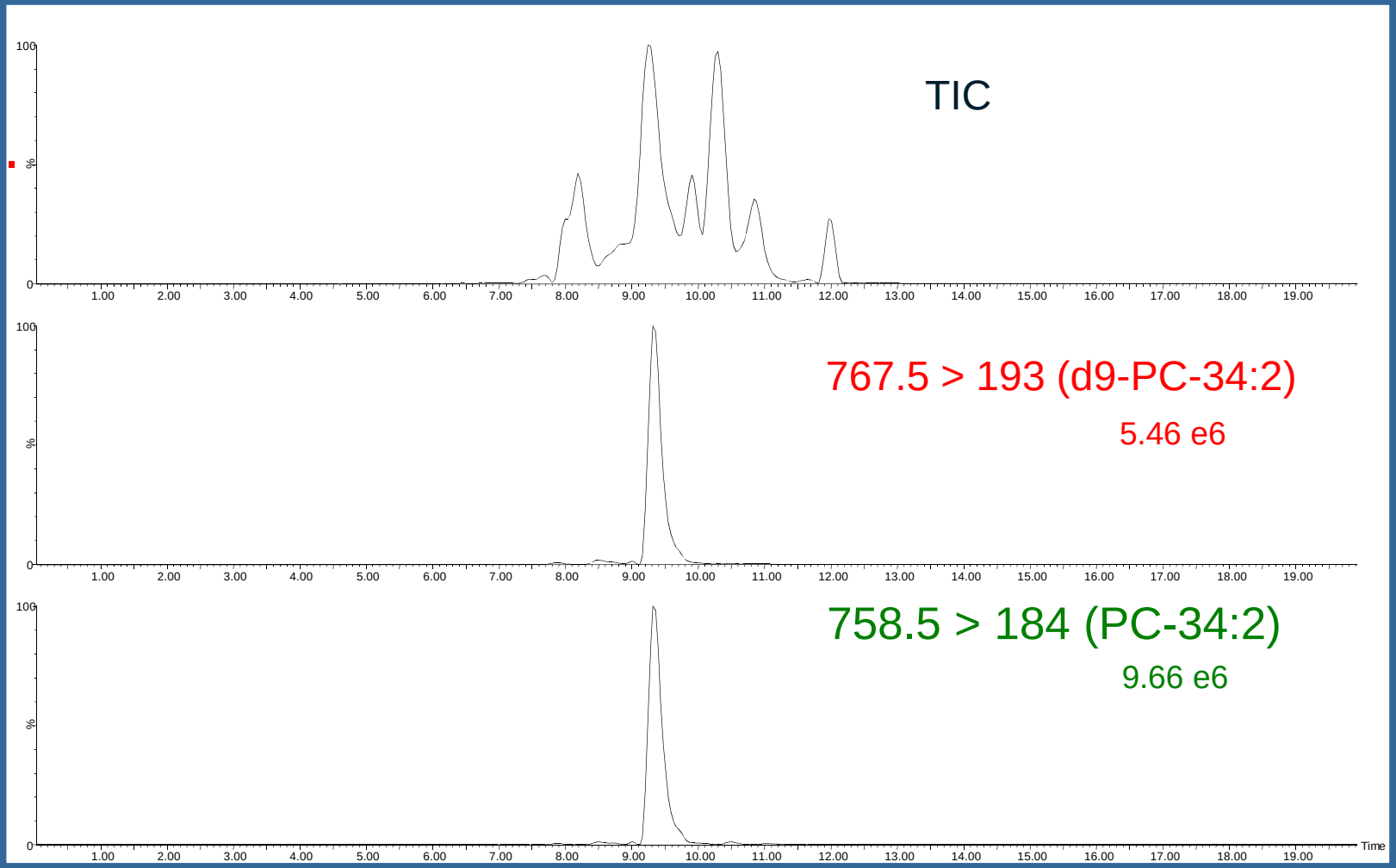
- ^{2}H or ^{13}C -labeled

Selective detection of headgroup-labeled PCs

D_9-PC > Diglyceride + D_9-Phosphocholine (+193)



LC-MS/MS with selective reaction monitoring



Selective detection of other labeled GPLs

D_6-PI = Precursors of -247

D_4-PE = Neutral loss of 145

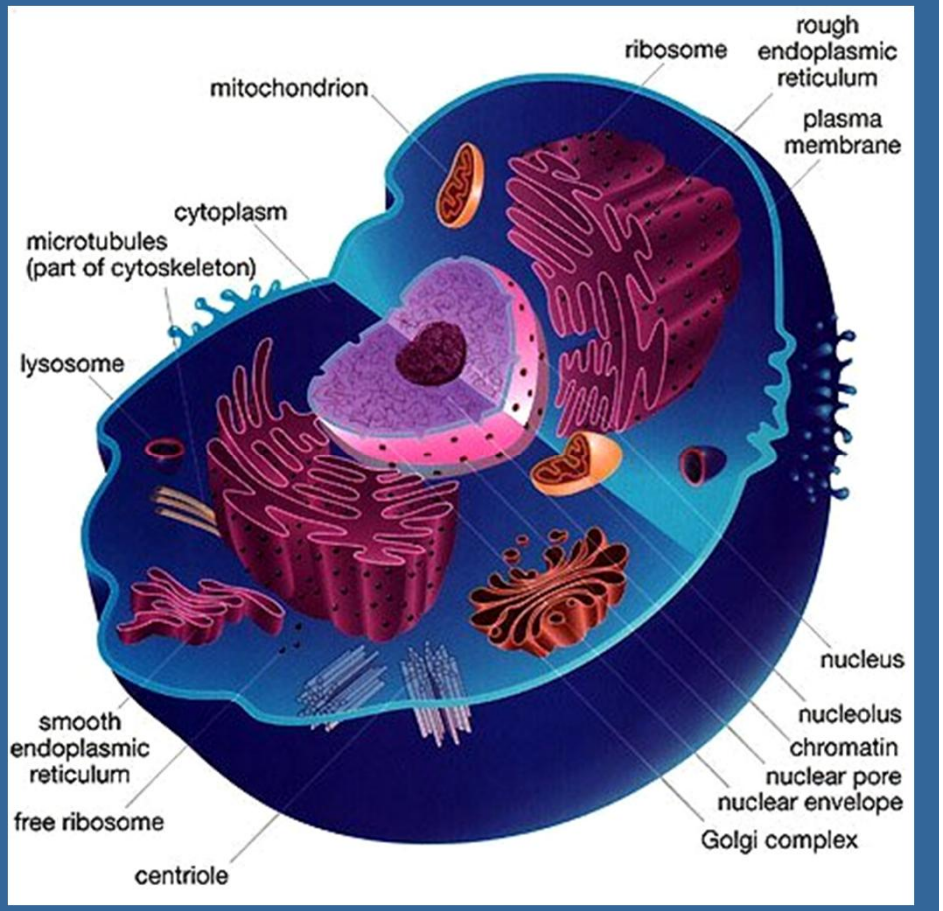
D_3-PS = Neutral loss of 90

- Specific labeling is easy to determine
- All precursors can be present simultaneously!

How a cell maintains the phospholipid homeostatis of its membranes?

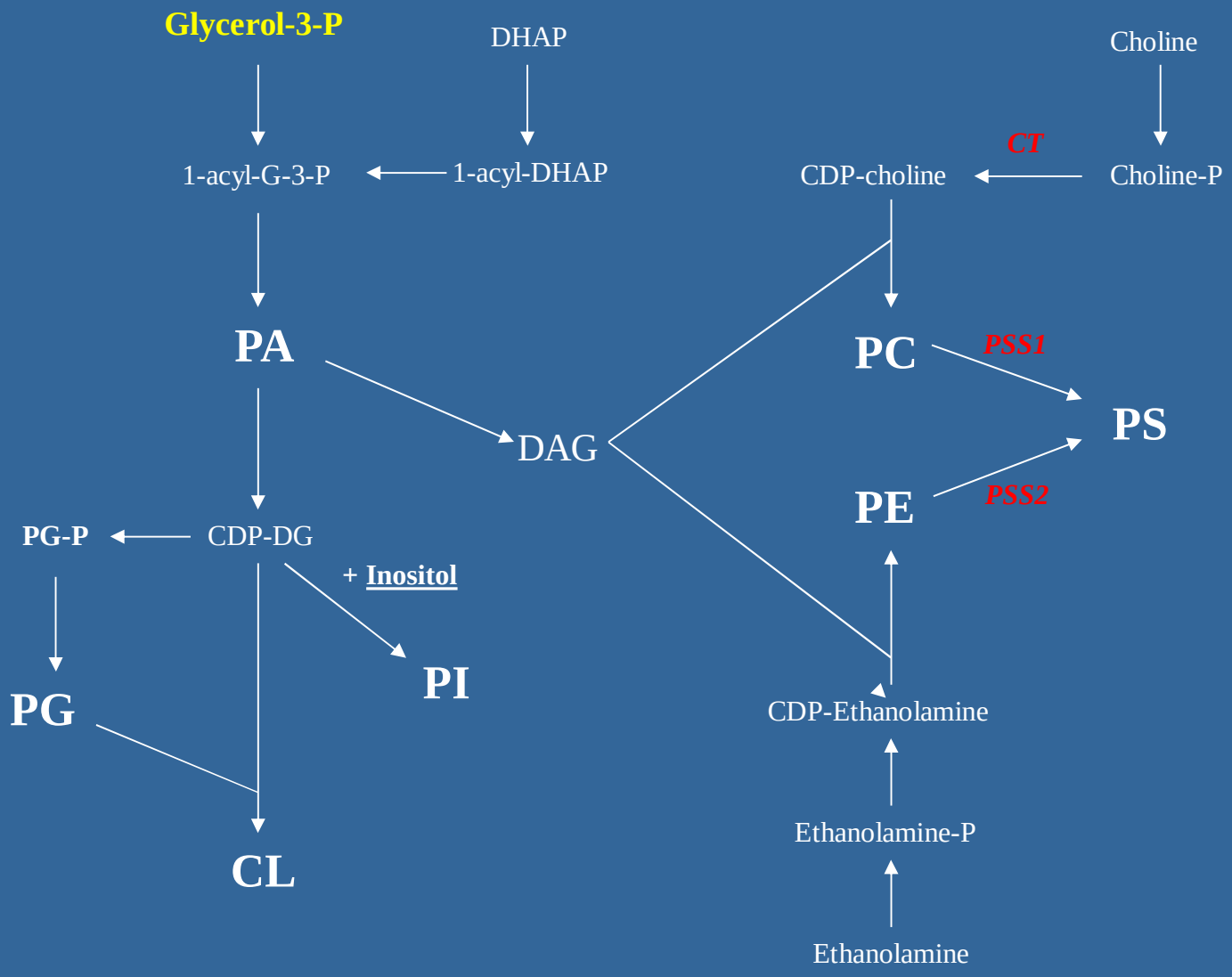
- Biosynthesis
- Remodeling
- Degradation
- Trafficking

How are these coordinated?



Biosynthesis

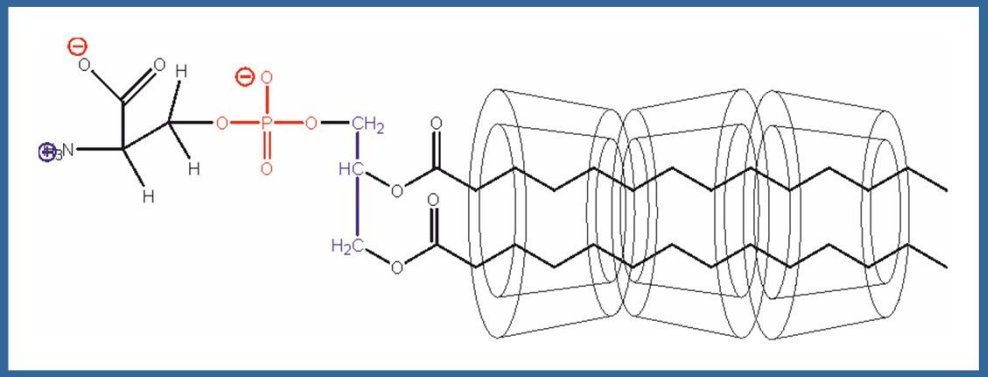
Biosynthesis of Glycerophospholipids



Our studies on GPL biosynthesis

- PROTOCOL
- Label cellular GPLs using a mix of D_9-choline, D_4-ethanolamine, D_3-serine and D_6-inositol
- Load a GPL to cells using mβ-CD
- Incubate and extract lipids
- Quantify the labeled and unlabeled GPLs by MS using HG-specific scans

Introduction of GPLs to cells using Cyclodextrin

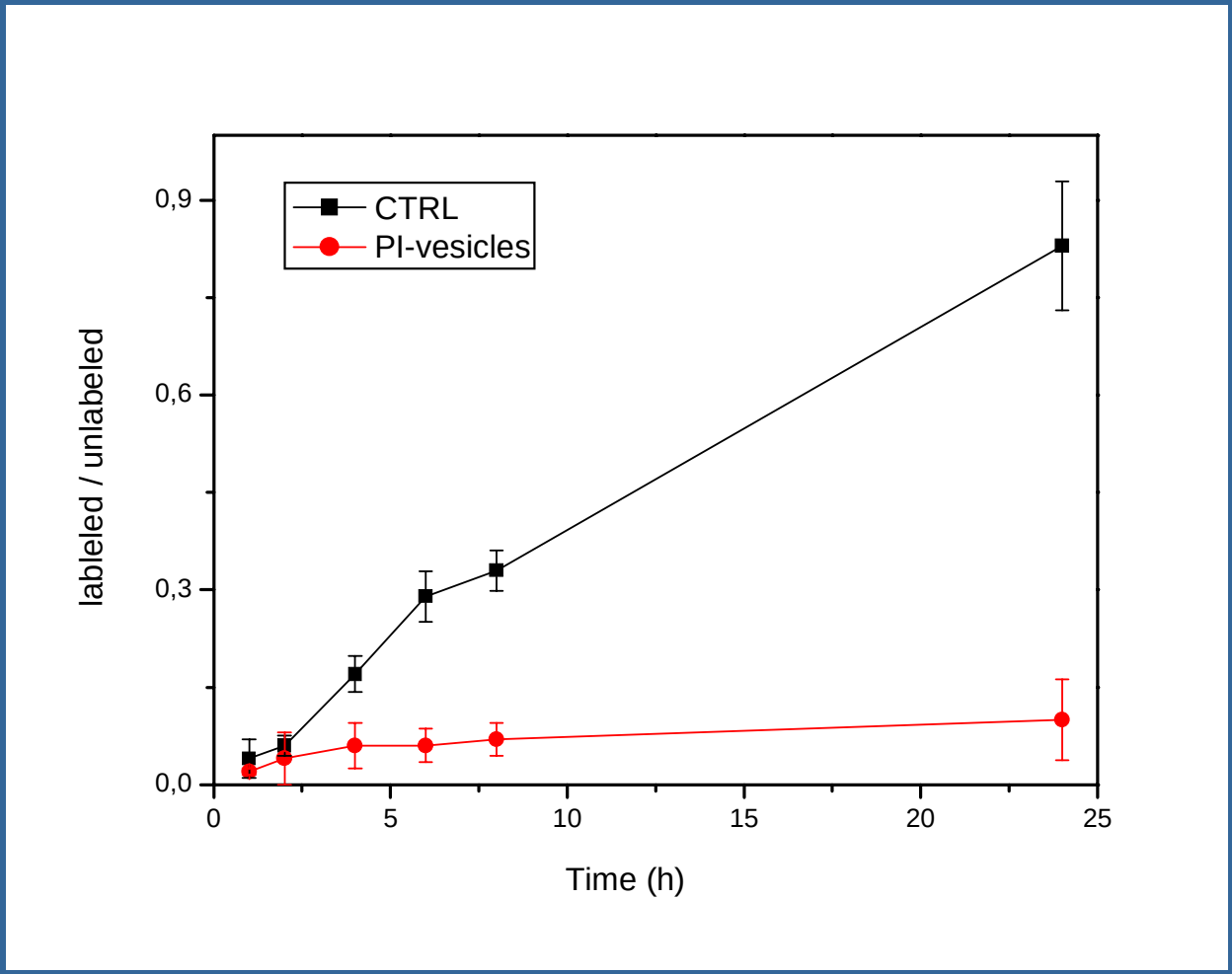


Purpose:

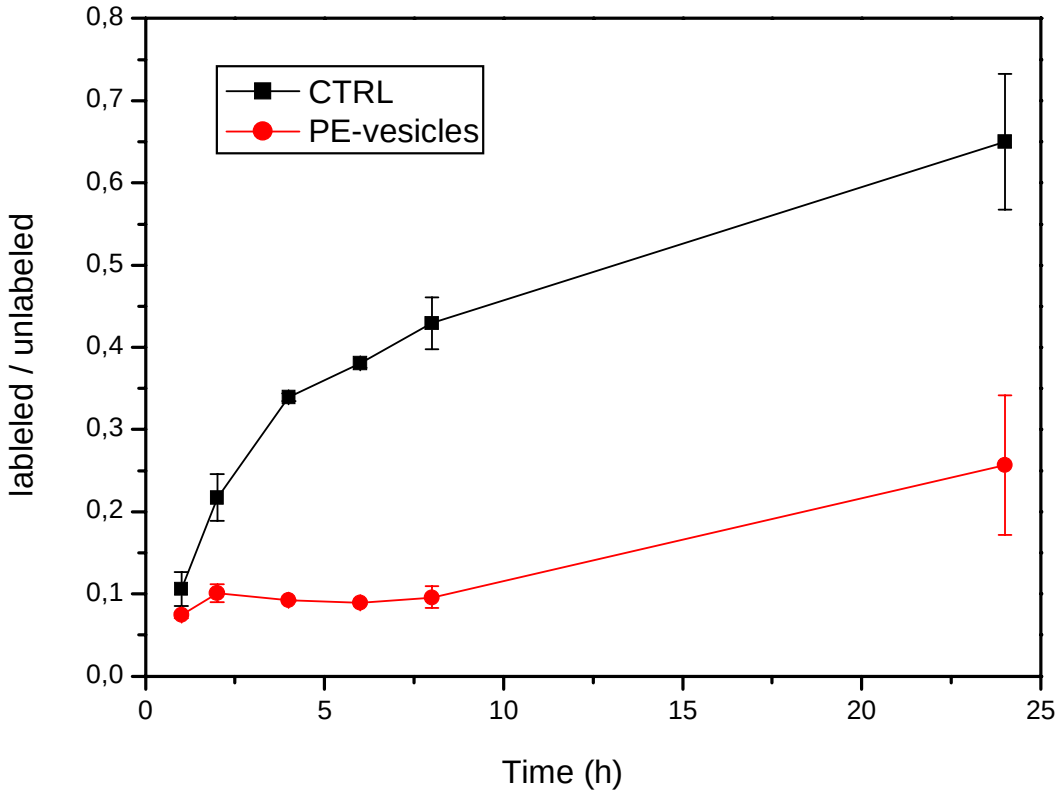
1. Introduction of exogenous labeled GPLs to cells
2. Perturbation of GPL homeostasis

=> Concentration of a GPL can be encreased by 30 - 400%
without compromizing cell viability (Kainu et al. J. Lipid Res. 2010)

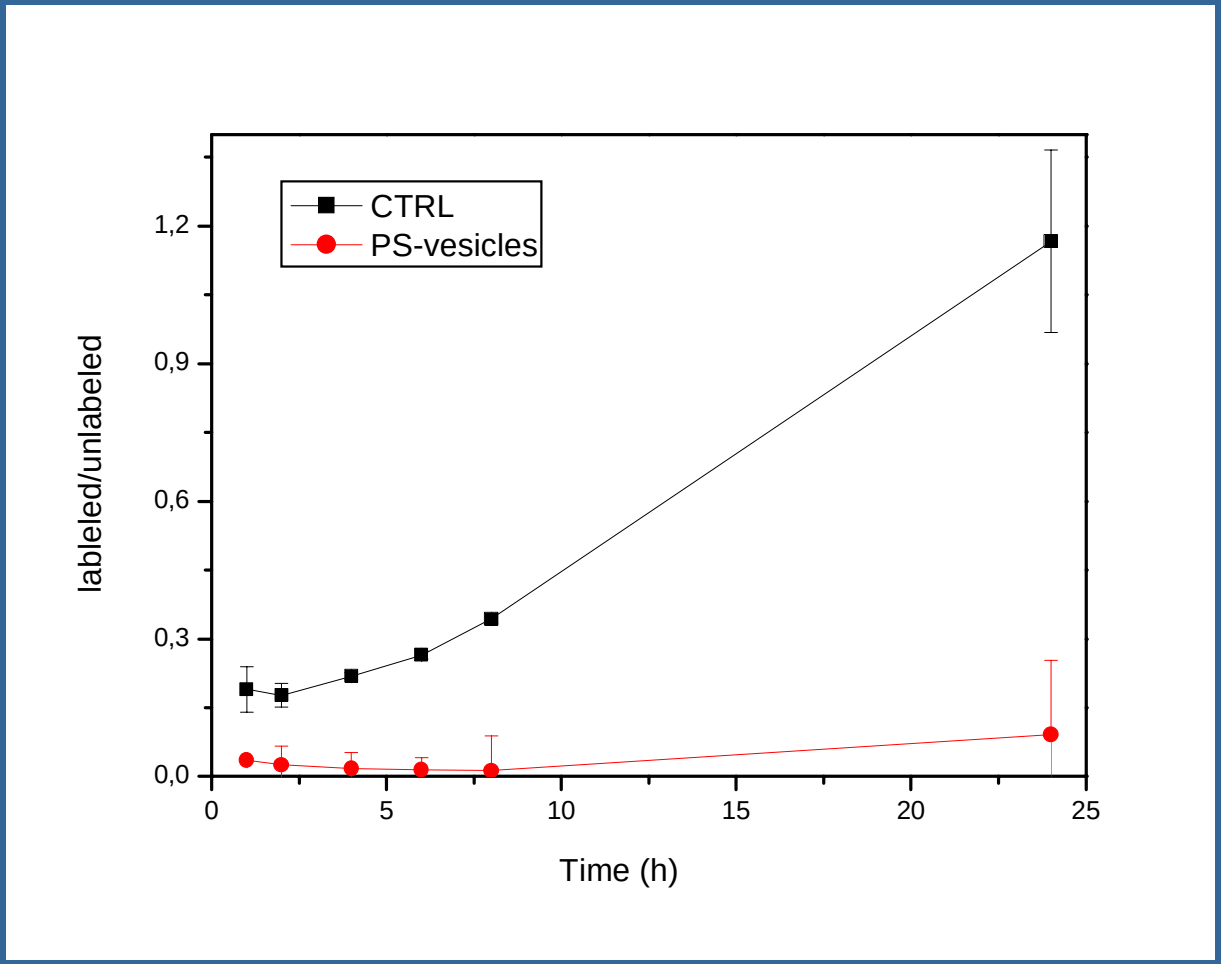
PI loading inhibits PI synthesis



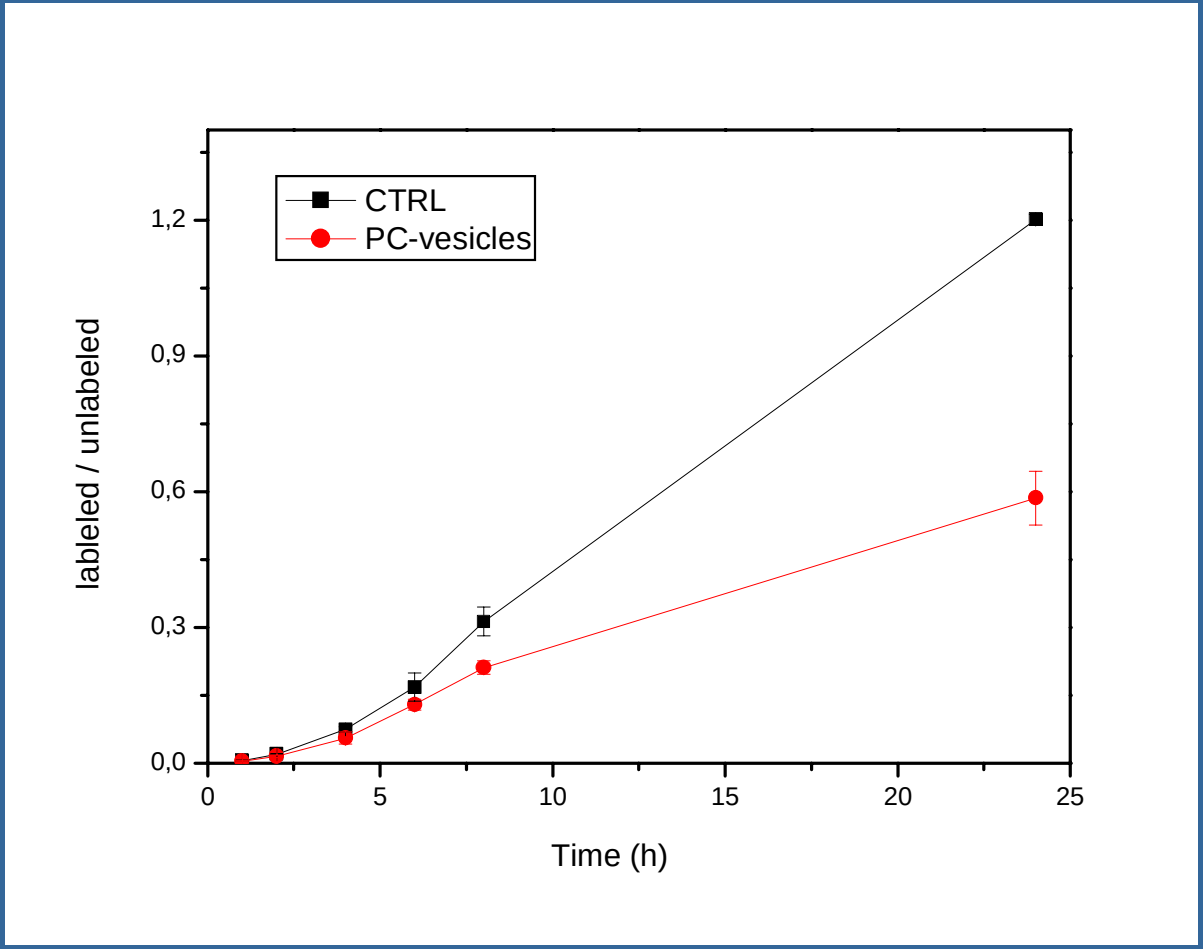
PE loading inhibits PE synthesis



PS loading blocks PS synthesis



PC loading inhibits PC synthesis



Product inhibition is specific!

But the details of the mechanism remains unknown..

Reversal on biosynthesis??

Degradation

A-type phospholipases (PLAs) are important players in GPL homeostasis because:

- Boosting of the biosynthesis of a phospholipid class does not increase its cellular content

-E.g. over-expression of cytidyl transferase in HeLa cells did not increase tha amount of PC significantly (Baburina & Jackowski 1999)

…but the concentration of the deacylation product (glycerophospholine) was greatly increased

-Analogous data have been obtained for PE and PS

Which PLAs are involved?

Protocol to identify the homeostatic PLAs

1. Determine which PLAs expressed in HeLa cells

Ca2+-independent PLAs

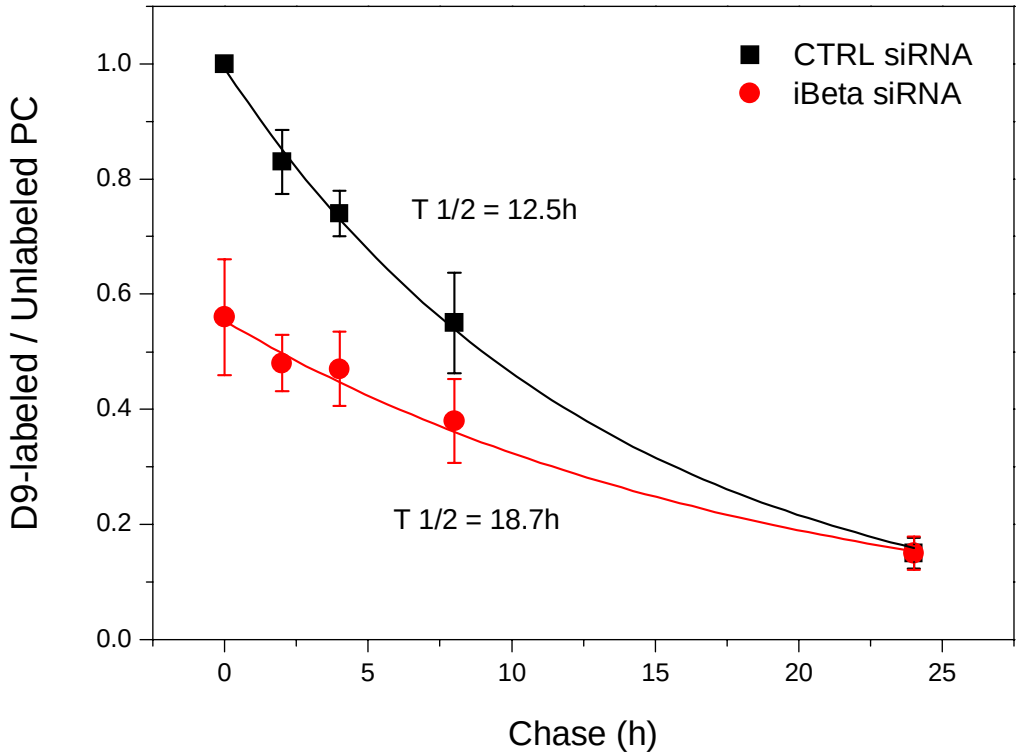
iPLA-*beta*
iPLA-*gamma*
iPLA$_2$-*delta*
iPLA$_2$-*epsilon*
iPLA$_2$-*zeta*
iPLA$_2$-*eta*
+ several cPLAs and sPLAs (not considered homeostatic)

2. Knock-down each iPLA in turn using RNAi

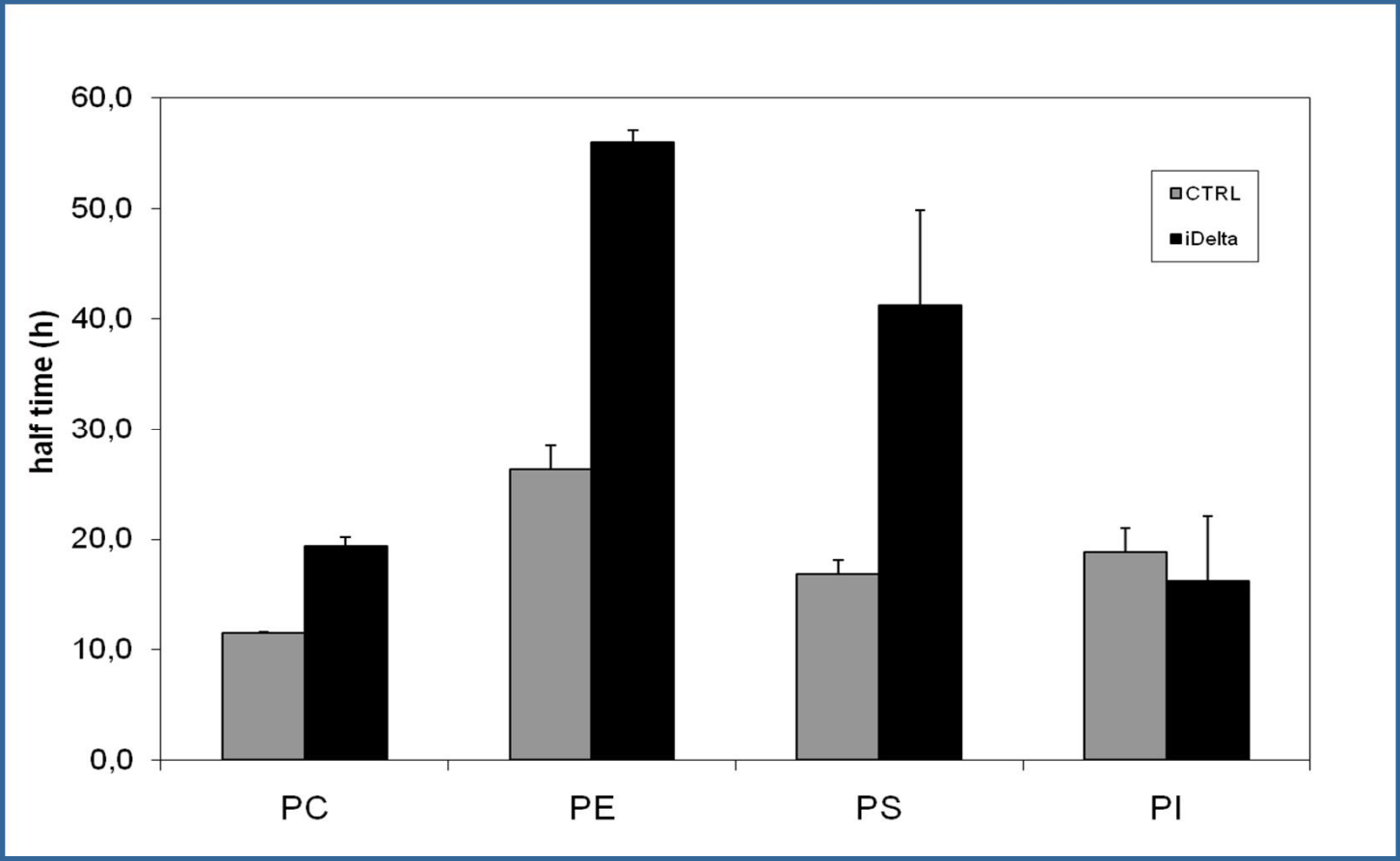
3. Determine effects on phospholipid turnover using labeled precursors and MS-analysis

4. Purify the implicated iPLAs and determine specificity and regulation in vitro (and in vivo..)

PC turnover is 50% slower in iPLAβ knock-down cells



Also PE and PS turnover is decreased in iPLAβ -knock down cells

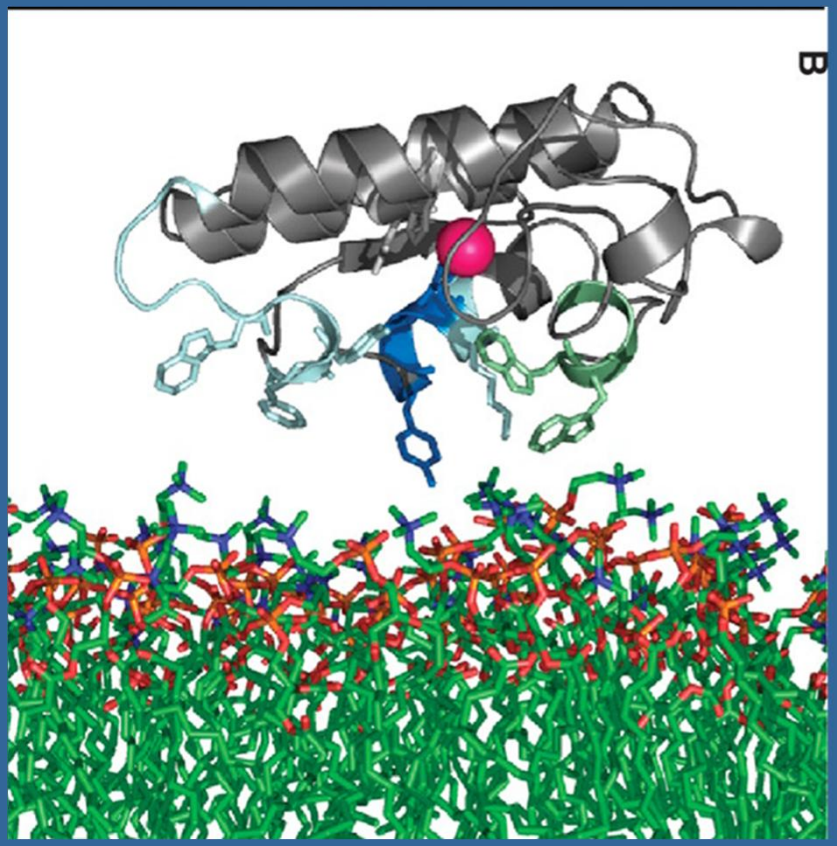


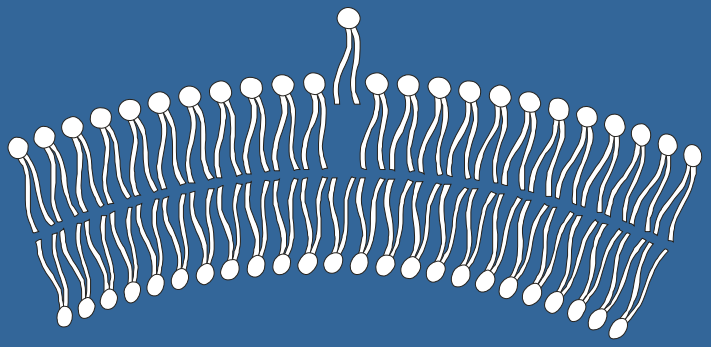
Similar results for iPLA-delta and -gamma

Open questions:

- How do the homeostatic PLAs sense the ”proper” composition, i.e. how they are regulated?
- How biosynthesis and degardation are coordinated?

Does iPLA-β activity depend on substrate efflux propensity?





Burke JE , Dennis EA (2009) J. Lipid Res. 50:S237-S242

MS-based assay of PLA specificity

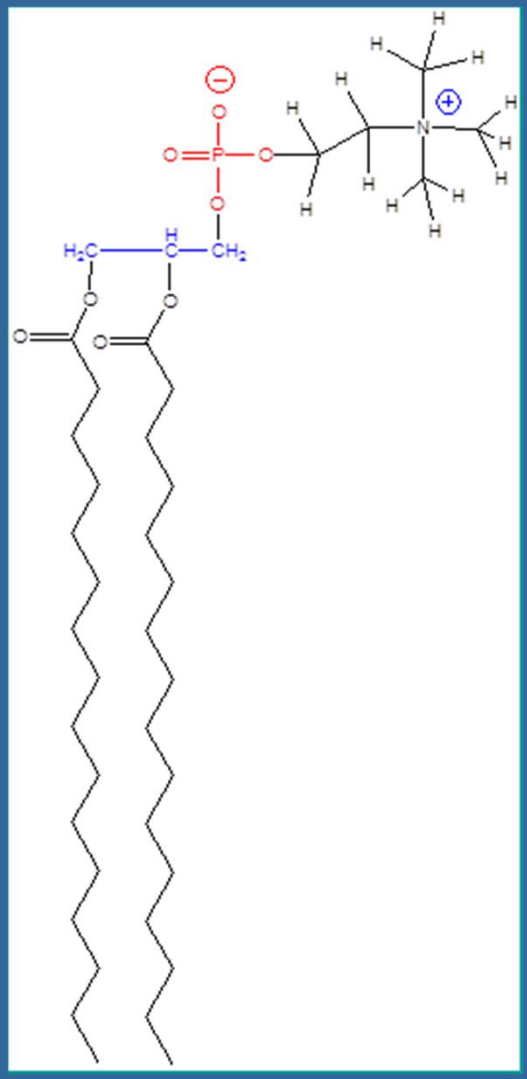
=> High throughput

=> No matrix ambiquiety!

Protocol

- Mix the phospholipids (>100 different allowed)
- Make vesicles
- Add a phospholipase
- Incubate and take samples at intervals
- Extract lipids and analyze by MS

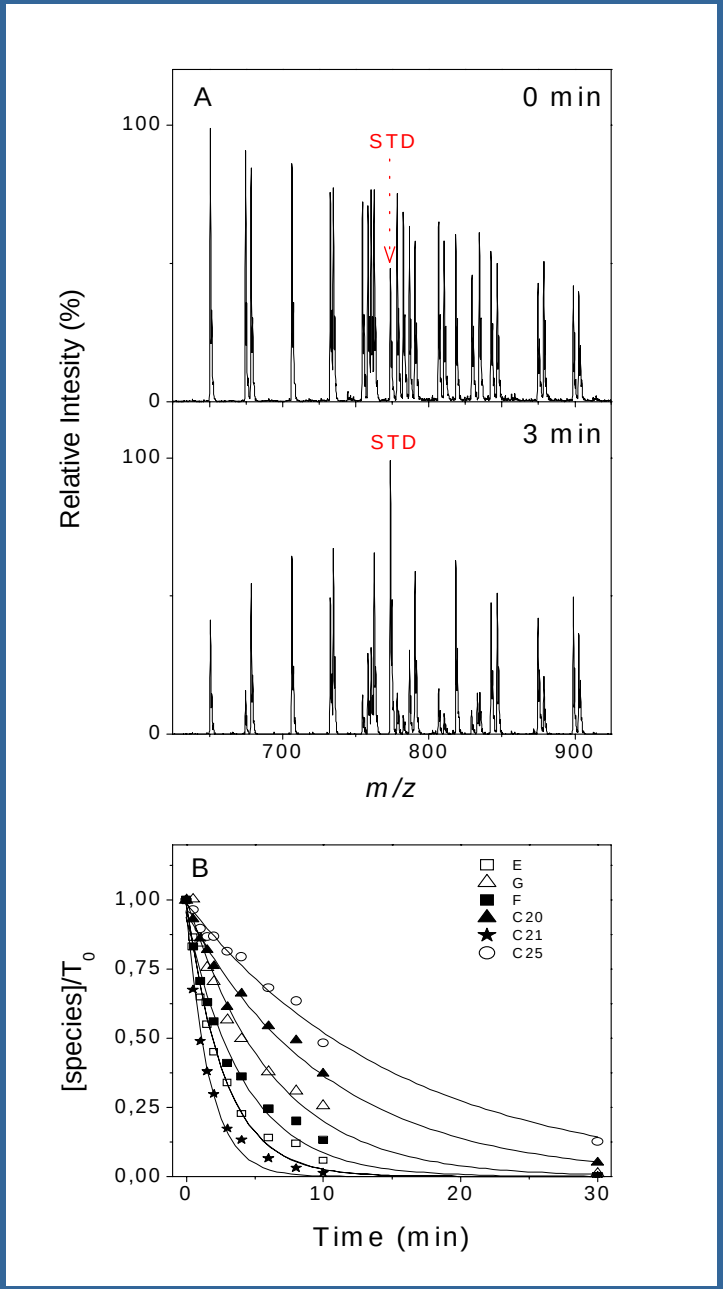
Effects of acyl chain length and unsaturation on hydrolysis by PLA



Mixture of 27 PC species:

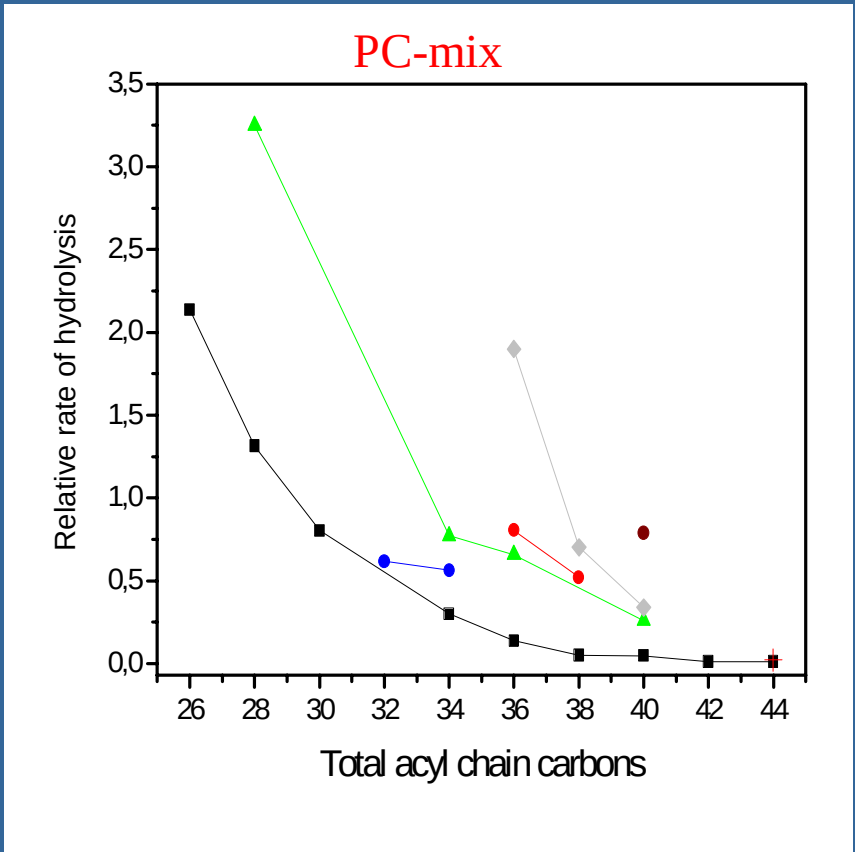
- Acyl Chain lenght = 6 - 22 carbons

- Double bonds = 0 - 12 per molecule



STD = Shingomyelin

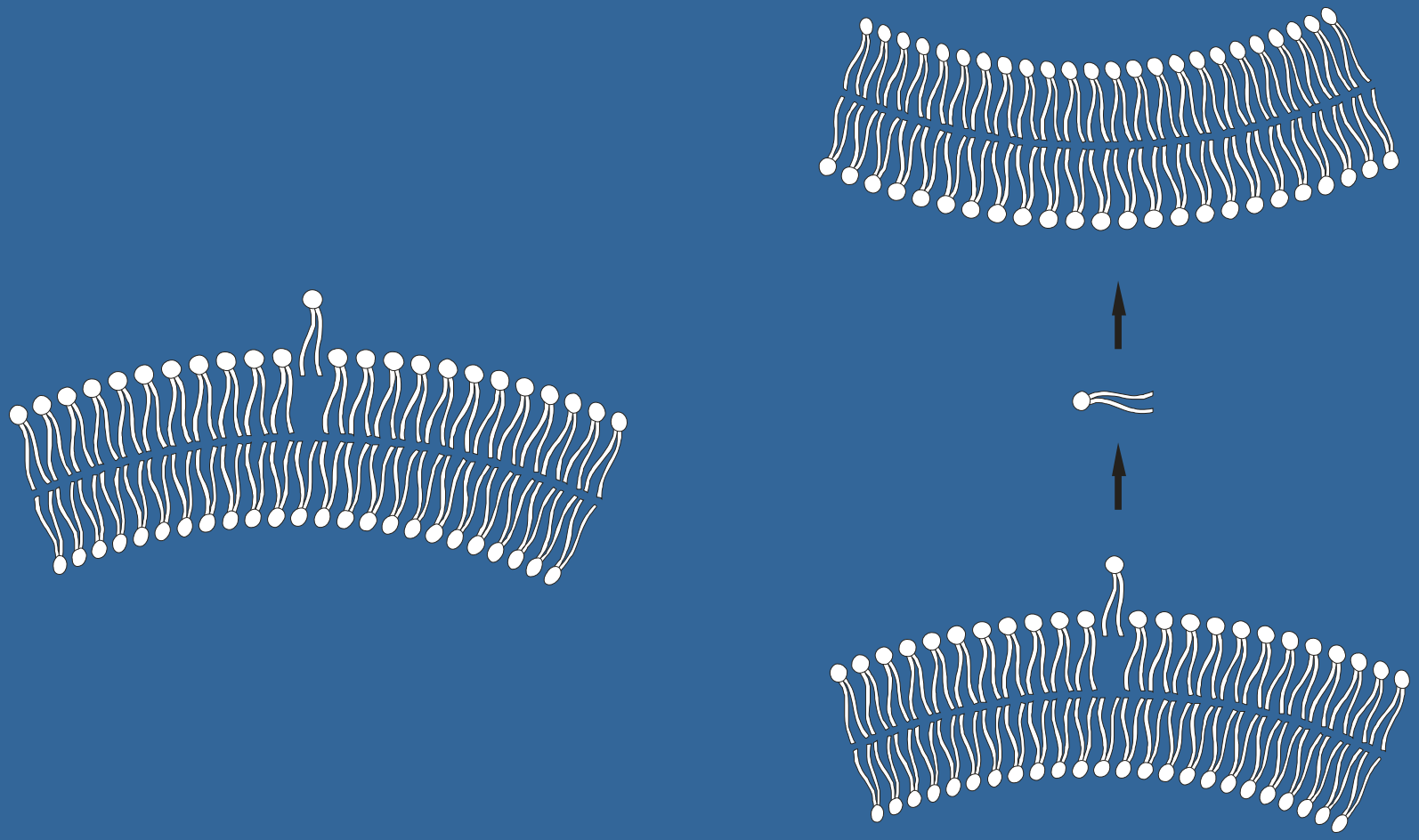
Activity of iPLA-β deceases with increasing substrate hydrophobicity

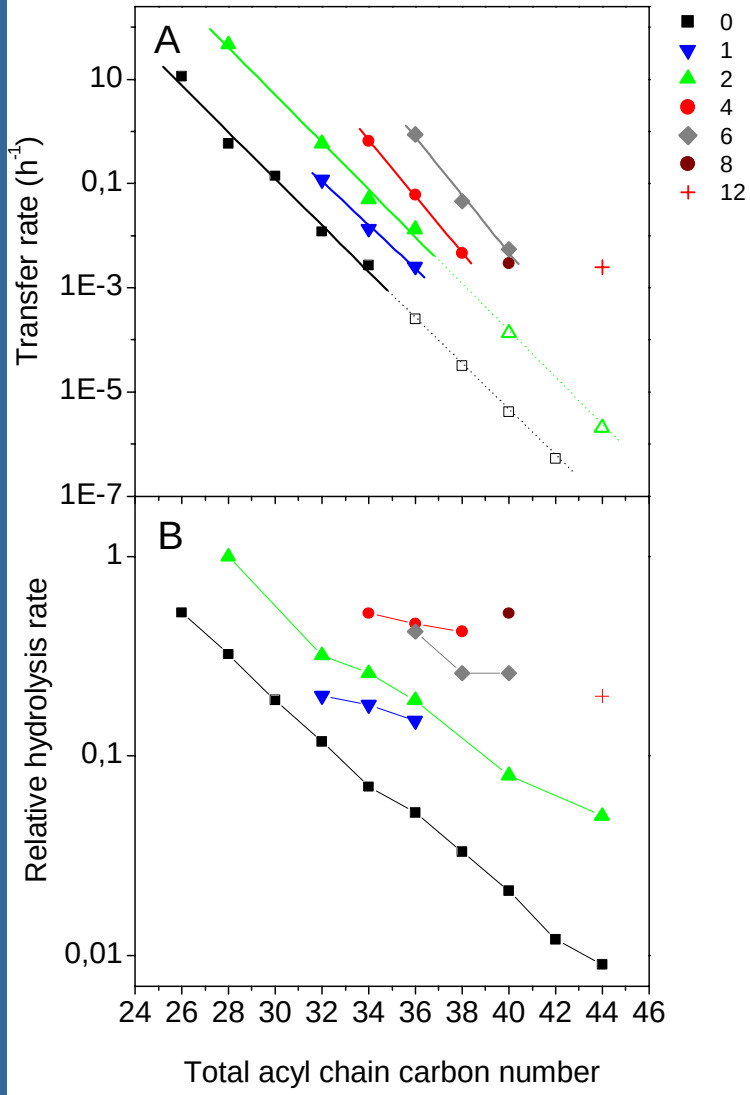


- Hydrolysis decreases strongly with acyl chain length
- Hydrolysis increases with increasing unsaturation

⇒ Substarate efflux is rate-limiting?

Efflux propensity can be determined from the rate of interbilayer translocation

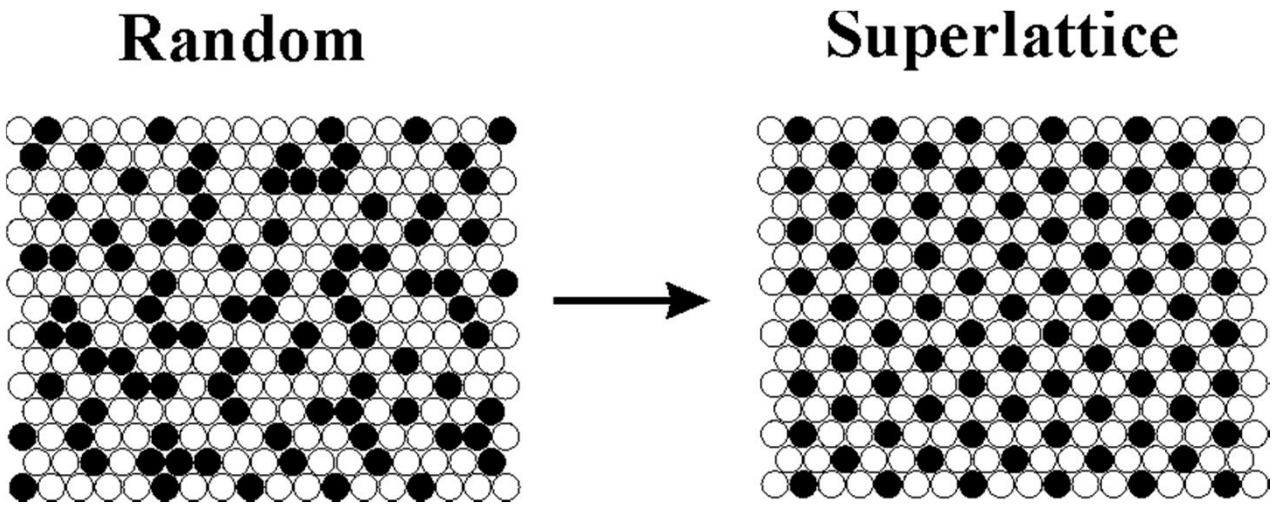



A
B
Transfer rate (h-1)
Relative hydrolysis rate
10
0,1
1E-3
1E-5
1E-7
1
0,1
0,01
24 26 28 30 32 34 36 38 40 42 44 46
Total acyl chain carbon number
0
1
2
4
6
8
12

How could efflux regulate homeostatic PLAs in vivo?

Superlattice Model predics that efflux propensity (chemical activity) increases abruptly at ”critical” compositions

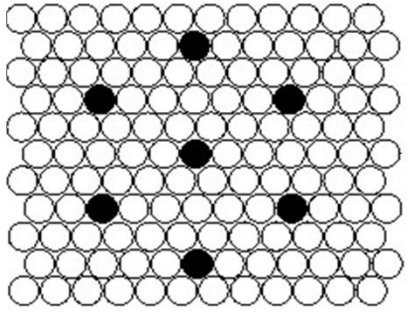
Superlattice model



=> Only a limited number of
“allowed” compositions !

Two-component bilyers

$$X_{guest} = 1/(a^2 + a * b + b^2)$$



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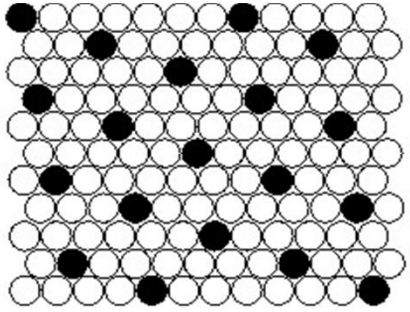

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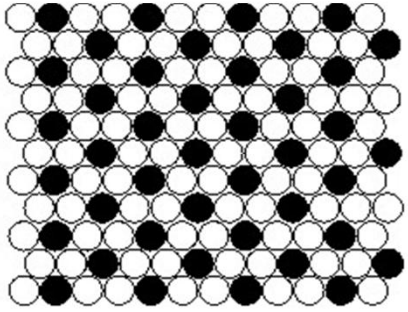
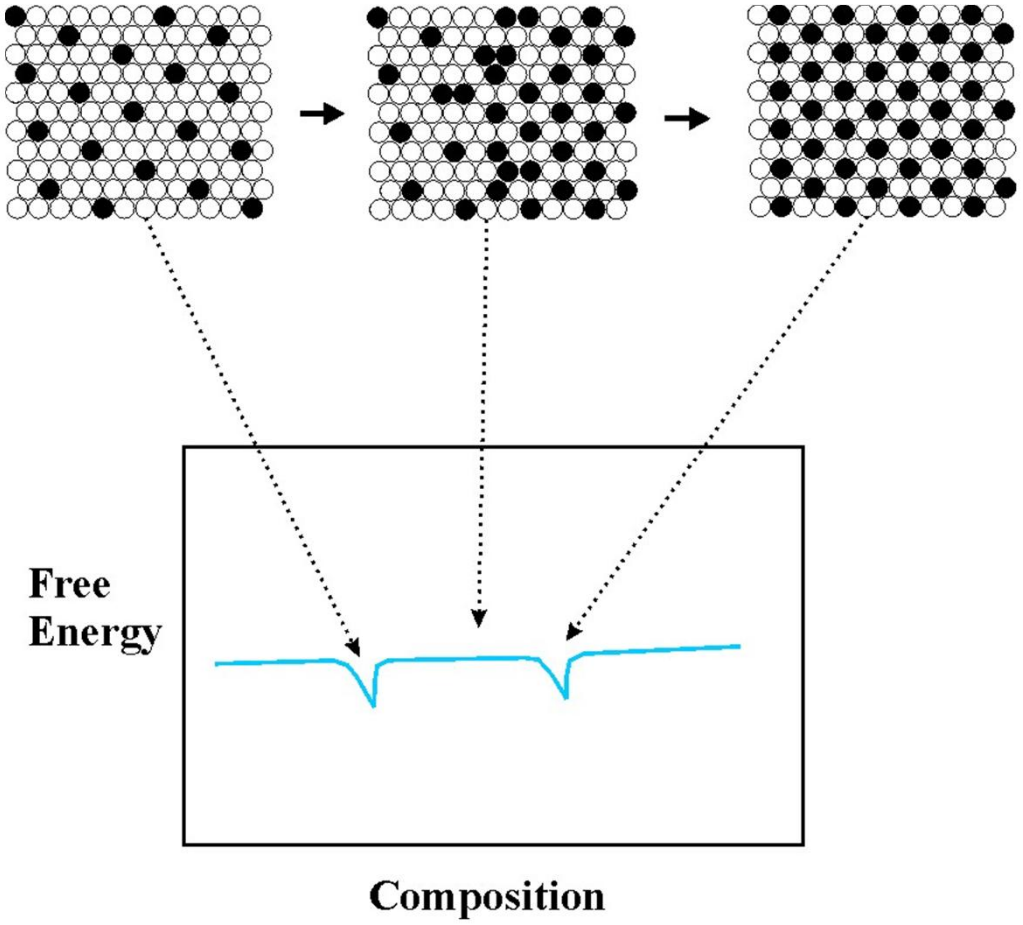


0.50

Superlattices are dynamic minimum-energy structures

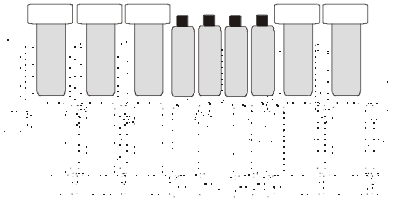


Regular distribution represent the lowest free energy state of the bilayer because it:

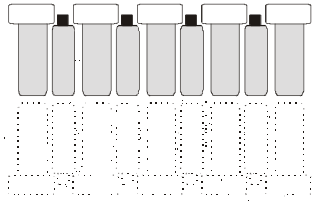
1. Allows optimal packing of different lipids in the bilayer

2. Minimizes the proximity of charged lipids

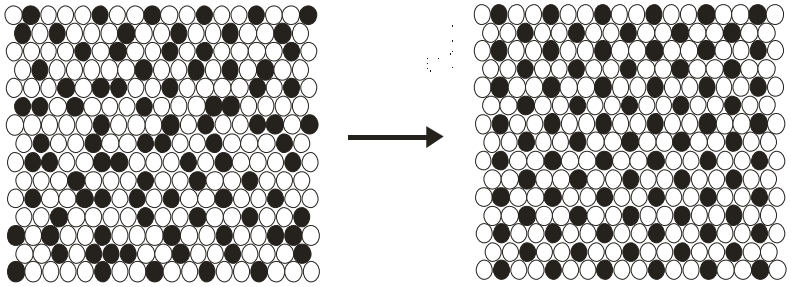
Optimimal packing of lipids with complementary shapes



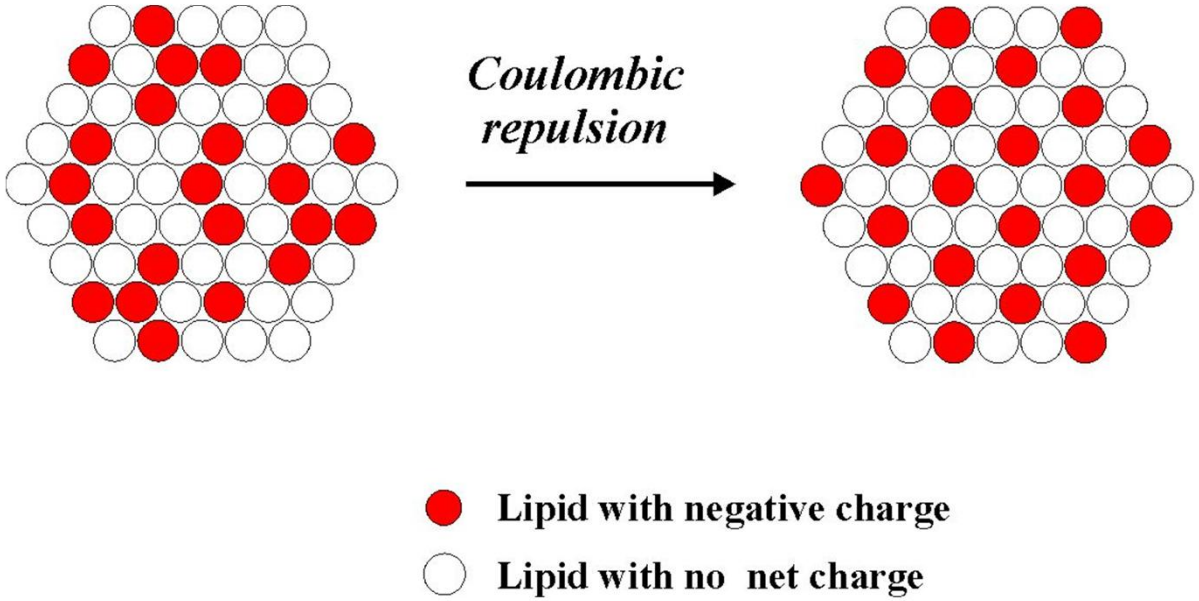
Suboptimal packing



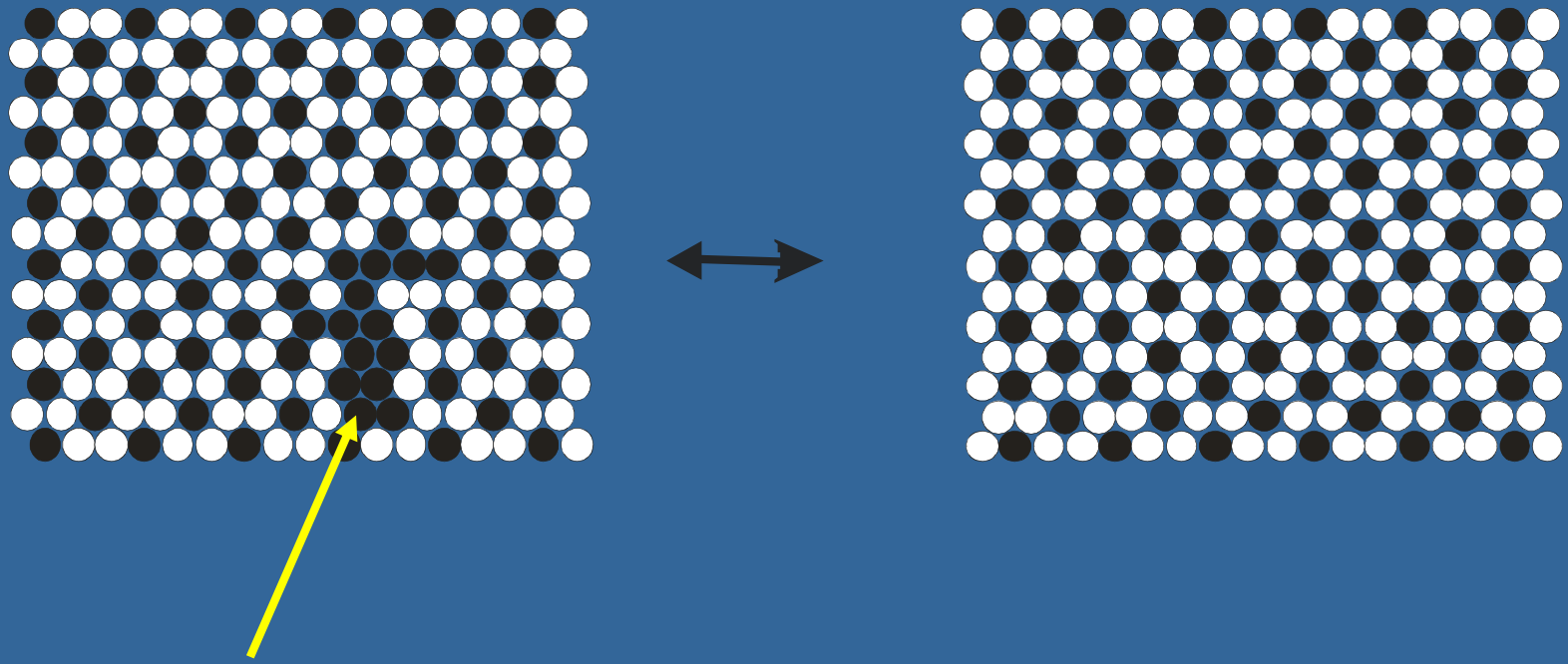
Optimal packing



Minimal charge-charge repulsion



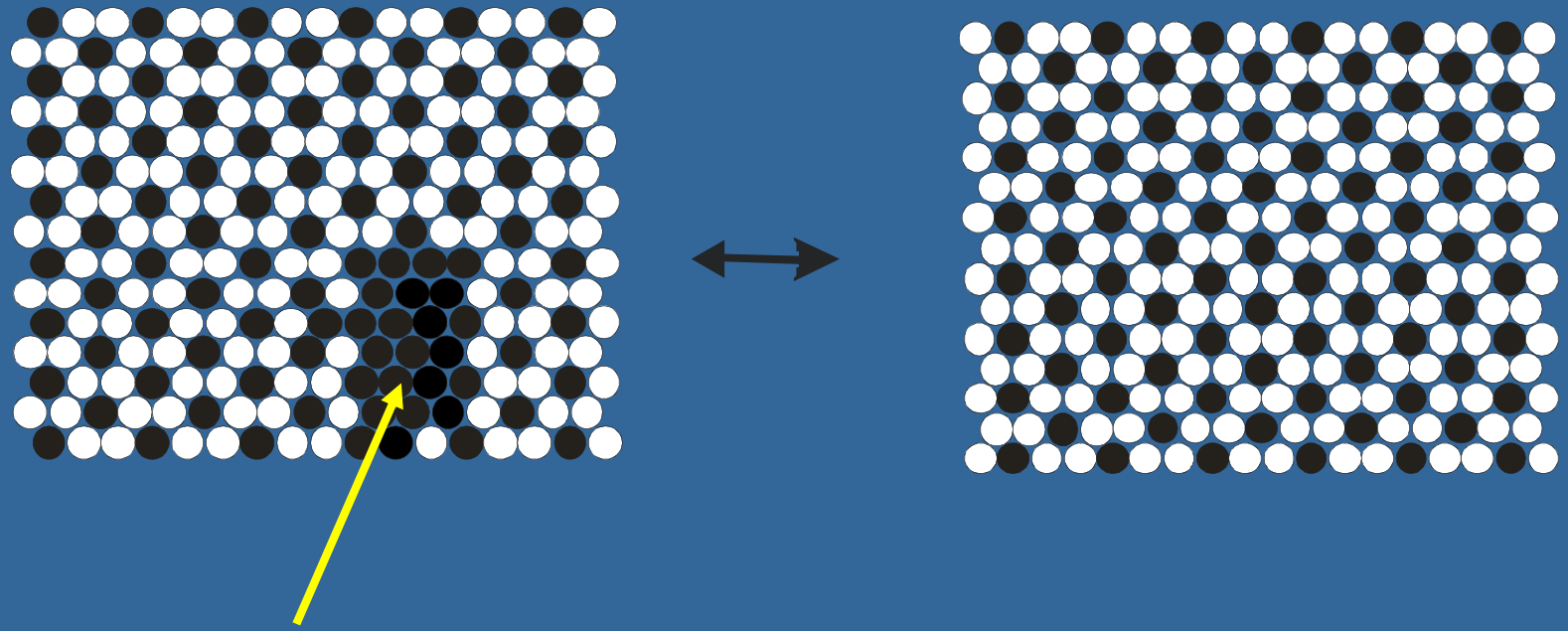
Regulation of homeostatic phospholipases by Superlattice formation?



Excess of lipid A => Enhanced efflux (fugality)

=> Hydrolysis of by a homeostatic PLA

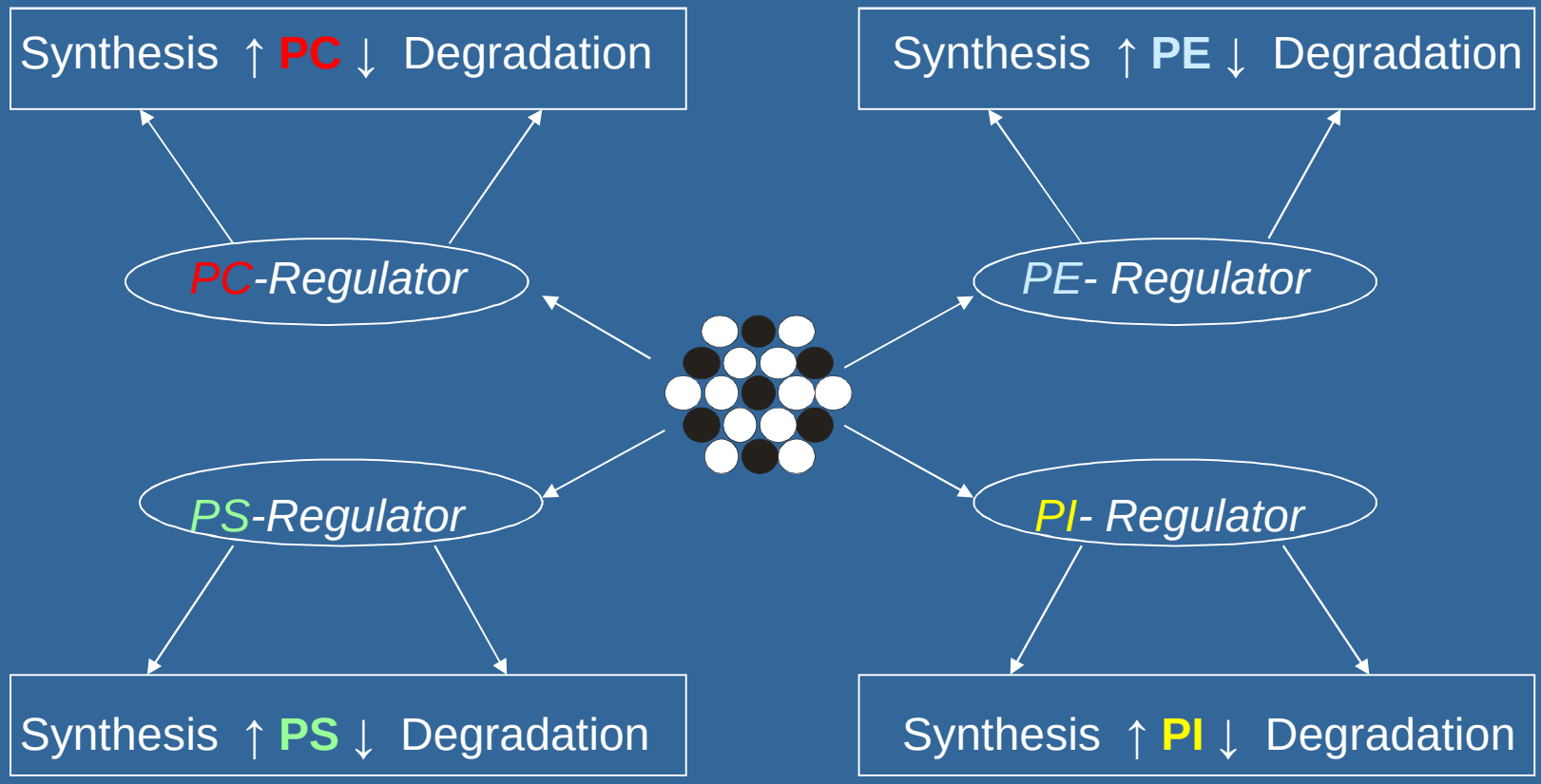
Regulation of biosynthetic enzymes by Superlattice formation



Excess of a phospholipid A => Increased chemical activity of A

=> Increased feed-back inhibition of the synthetic enzyme

SL-based regulation of synthesis and degradation



- Simple mechanism that minimises compositional fluctuations

Conclusions

- Heavy-isotope –labeled precursors combined with Mass spectrometry is a superior tool to study GPL metabolism
- Feed-back -inhibition by the end product seems to regulate the biosynthesis of major GPLs
- Substrate efflux propensity could regulate the activity of homeostatic PLAs
- Superlattice formation could coordinate synthesis and degradation by modulating the chemical activity/efflux propensity of GPLs