New Developments in Capillary Electrophoresis with focus on Bioanalysis

Lecture 6

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Lecture today

- Two-dimensional separation
- CE-MS
- Micro total analysis systems
- Biomarker analysis by CE
- Molecular Imprinted Polymers

Diagonal CE

- Two-dimensional CE
- Identical separation modes
- Enzyme-based microreactor
- Analytes that are unaffected by the microreactor will be detected at the diagonal in the 2D electropherogram

Diagonal CE

- Alkaline phosphatase
  - Remove phosphate groups
  - Most effective at high pH
  - Present in the periplasmic space in bacterias
  - Function?
    - Generate phosphate group for uptake and use?
    - Regulate uptake into cell? Phosphate groups usually prevents organic molecules to pass through the membrane

Diagonal CE

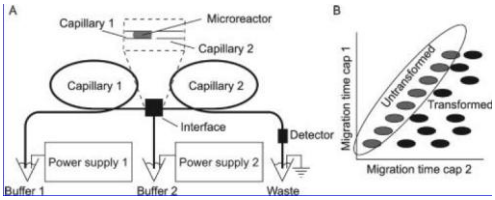
- Use of alkaline phosphatase to monitor phophorylation of a mixture of peptides
- Could be used also for other post translational modification

Diagonal CE

- Previous use:
  - Paper chromatography to characterize disulfid bonds
  - Diagonal LC to characterize phosphorylation, glycosylation and acetylation
- Use of diagonal CE:
  - Easier to automate
  - Faster

Diagonal CE

- One fraction is separated in the second dimension while the following fraction is in the microreactor



Diagonal CE

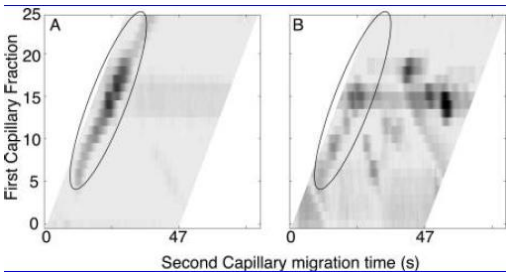
- Microreactor
  - Alkaline phosphatase immobilized on magnetic nanoparticles
  - Microreactor kept at right position by magnets
  - 1.0 or 2.8 µm nanoparticles
  - Replaced between each analysis
  - Microreactor: 1-3 mm plug

Diagonal CE

- A fraction present for 45 s in the microreactor
- Several fractions present in the second capillary simultaneously
  - Possible as long as the fastest moving component do not overtake the slowest moving component of previous fraction
- Laser induced fluorescence for detection
  - Peptides fluorescent labeled

Diagonal CE

- Separation of tryptic digest of casein



Proteomics

- Liquid chromatography routinely used for bottom up proteomics to detect peptides from tryptic digests
- Very long gradient elution separations often produce extraordinary peak capacity and resolution

Proteomics

- Desireable to have a complementary technique to reversed phase chromatography
- CE have so far mostly been used for analysis of standard peptides or tryptic digest of a few standard proteins
- However, analysis of complex proteomic samples are now developed

CZE-MS/MS for proteomics

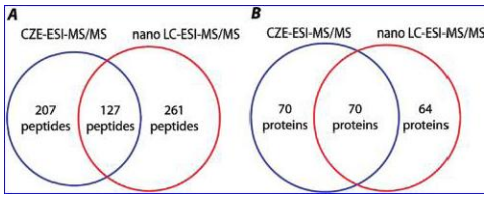
- Advantages
  - Separation mechanism
  - Faster
  - Higher efficiency
- Disadvantages
  - Speed and efficiency must be handled by the mass spectrometer. The acquisition rate can be limiting
  - Loading capacity is lower

Proteomics

- CZE-MS/MS as an alternative to UPLC-MS/MS for proteomics studies
- 11 fractions were first separated by reversed-phase LC
- Each fraction was then separated by CZE-ESI-MS/MS
- 250 ng of sample and 165 min of MS time was used

Proteomics

- CZE similar to UPLC and the techniques were complementary
- CZE favor basic, hydrophilic, small peptides



CE-MS for Proteomics

- Analysis of whole proteome
  - Time
  - Amount of sample
  - Labor
- LC-MS usually employed
- Detection of 10 000 proteins?
- Large dynamic range of proteome
  - 7 order of magnitude
- CE-MS?

CZE-ESI-MS/MS for Proteomics

- 50 min separation
- Identification of 1250 peptides in *E Coli*
- 1-100 ng protein digest
- Electrokinetically pumped nanospray interface
- Coated capillary
- Stacking conditions for injection

CZE-ESI-MS/MS for Proteomics

- Electrophoretically pumped sheath flow
- Very low flow rate
- Less dilution of sample

CZE-ESI-MS/MS for Proteomics

- Comparsion to UPLC-ESI-MS/MS
  - With 100 ng digest UPLC was able to identify more pepides
  - With 1 ng digest CZE was able to identify many more peptides
- The methods were complementary

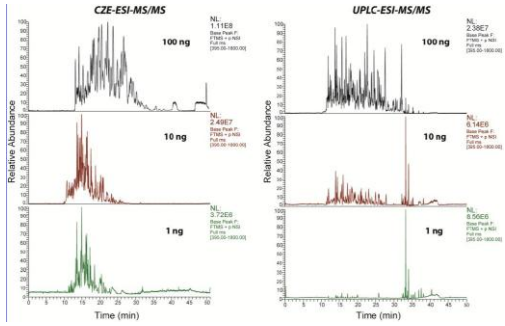
CZE-ESI-MS/MS for Proteomics

E. coli digests loading amount (ng)	single shot CZE–ESI-MS/MS (triplicate runs)		single shot UPLC–ESI-MS/MS (triplicate runs)	
	protein groups	peptides	protein groups	peptides
100	312 ± 29[a]	1,377 ± 128	395 ± 3	1,875 ± 32
10	212 ± 19	997 ± 54	280 ± 9	1,108 ± 104
1	142 ± 10	627 ± 38	140 ± 25	342 ± 113

CZE-ESI-MS/MS for Proteomics

- Coated capillary (Linear polyacrylamide)
  - Prevent capillary wall adsorption
  - Reduce EOF / Increase separation time
- Sample buffer modified
  - Facilitate sample stacking
  - Injection of a larger amount of sample

CZE-ESI-MS/MS for Proteomics



CZE-ESI-MS/MS for Proteomics

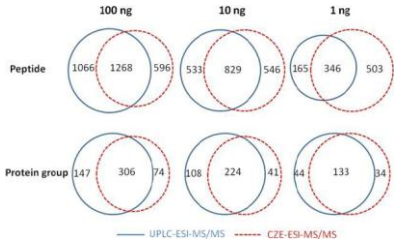
- Sample stacking
  - Varying amount of formic acid in the sample buffer
    - Either 0.05% or 0.1% formic acid
  - Better sample stacking with 0.05%
  - Reduce the effect of a larger sample plug
  - Improve sample capacity
  - More peptides identified
    - 1132 instead of 815 peptides

CZE-ESI-MS/MS for Proteomics

- Different capillary length
  - 40 or 60 cm
- Longer capillary and gave identification of more peptides
  - 1520 vs 1184
  - Longer separation time
  - More mass spectra generated

CZE-ESI-MS/MS for Proteomics

- Complementary techniques
- Can improve coverage of proteins



CZE-ESI-MS/MS for proteomics

- CZE as a competitor to UPLC for proteomics
- Improvements by further optimizing
  - Sample preparation
  - Separation conditions
  - Electrospray conditions
  - MS parameters

CZE-ESI-MS/MS

- Analysis tryptic digests from cellular homogenate
- 700 pg (volume of ~10 eukaryote cells) was analyzed
- 10 proteins could be detected

Micro Total Analysis Systems for Cell Biology and Biochemistry

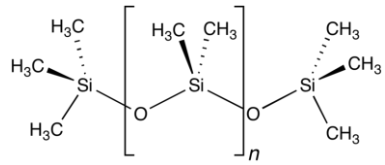
- Rapidly maturing
- Still improvements of fabrication
- More focus on biological applications

Micro Total Analysis Systems for Cell Biology and Biochemistry

- Fabrication
  - Cost
  - Robustness
  - Surface Chemistry
  - Optical properties
  - Biocompatibility
  - Ease of fabrication and integration
  - Suitability for large scale production

Micro Total Analysis Systems for Cell Biology and Biochemistry

- Polymers for production of devices
- Polymethylsiloxane (PDMS) extensively used



PDMS

- Easy to fabricate
- Low cost
- Established procedures

- Absorption of hydrophobic molecules
- Swelling in organic solvent

Alternatives to PDMS

- Poly(methyl methacrylate)
- Polystyrene
- Polycarbonate
- Cyclic olefin copolymer

- Relatively complex fabrication
  - Less suitable for prototyping at academic labs
- More suitable for industrial mass production

Glass and Silicon

- More complicated fabrication
- Glass
  - Chemical inert, optical transparent, thermal stability
- Silicon
  - Derived from eletronics industry

Sample preparation

- A challenging step for biological samples
  - Often complex mixtures of compounds
- Extensive off chip sample preparation reduce the utility of a micro total analysis system
  - Especially important for samples of low amount and volume

Biology and µTAS

- Single cell analysis
  - Large heterogenity within a population of a certain cell type
  - Understand cell variation

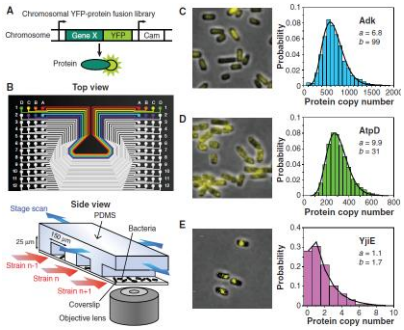
Analysis of intact cells

- Imaging cytometry
  - Labeling specific cell components
  - Microfluidics for high throughput sample preparation
- Number of certain protein or mRNA differ from cell to cell within the same population
- However, hard to detect low abundant compounds in single cells

Analysis of intact cells

- Yellow fluorescent protein (YFP) fusion library
- Particular genes tagged with YFP
- YFP can be detected with single molecule sinsitivity in living cells.
- High throughput analysis of the different stains on microchip

Analysis of intact cells



Single enzyme detection

- Fluorescence-based
- A microchip with an array of wells
- Dilute the sample so there will be a maximum of 1 enzyme molecule per well.
- Reporter system
  - Fluorescent reactant
  - Fluorescent product

CE for biomarker analysis

- Proteins and peptides as biomarkers
- From biological fluids
- Handling of real samples

- NIH: Biomarker = a characteristic that is objectively measured and evaluated as an indicatorof normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic invention

CE for biomarker analysis

- Small sample volume
- High efficiency and resolution
- High sensitivity with LIF or MS detection

CE for biomarker analysis

- Difficulties
  - Complex sample matrix
  - Low concentration
    - Low abundant substances may interact with other molecules in the sample
  - Often a sample preparation step necessary
    - Remove non-interesting compounds
    - Concentrate biomarker

Biomarkers in tears without sample pretreatment

- Advantage: Relatively pure
- Used for detecting lysozyme and lactoferrin by CE-UV
- Sjögrens syndrome, an automimmune disease
- Acidic buffer and cationic coating

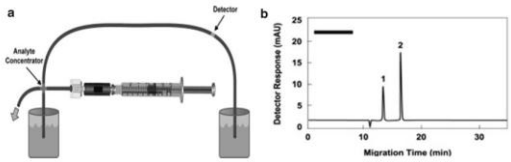
Basis of pretreatment methods

- Based on affinity for solid phase
- Based on immunoaffinity supports with antibodies
- Membrane-based techniques: dialysis or filtration
- Liquid-Liquid-based techniques: centrifugation or precipitation

- Advantage if the technique can be implemented on column

Detection of neuropeptides in urine

- Detection of angiotensin II and neurotensin
- Fab fragments from polyclonal antibodies immobilized on glass beads at an microreactor



Capillary coating

- Avoid adsorption and tune EOF

- Preparation of capillary coating is often time consuming and the stability is limited
- Often physically adsorbed neutral och possitively charged polymers are used
  - Possible to regernerate

Capillary coating

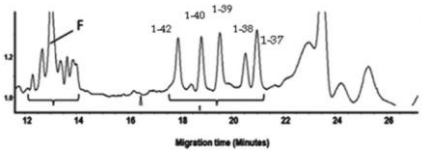
- Successive multiple ionic polymer layer (SMIL)
- Layers of polyelectrolyte are attached by succesive rinsing steps with cationic and anionic polymers

Detection sensitivity

- High sensitivity necessary for early diagnostics
- Use of flurescence (labeling often necessary) or MS
- Preconcentration

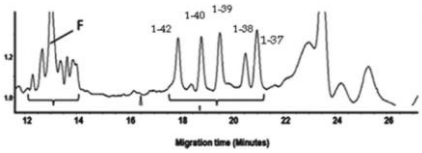
Example – CE-LIF of amyloid peptides

- Alzheimer disease
- Fluorescent labeled peptides
- From cerebrospinal fluid
- Offline immuno-capture were used prior to the CE-LIF analysis

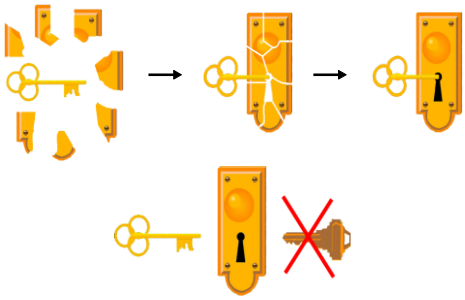


Example – CE-LIF of amyloid peptides

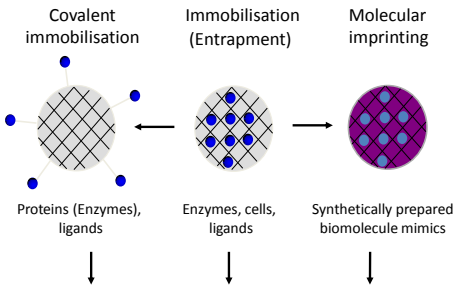
- Magnetic nanoparticles coated with monoclonal antibodies was used pretreatment



Molecular Imprinting
Making a lock to a molecular key...



HISTORY



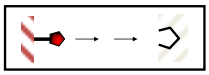
For separation, catalysis, assays, sensors

Improvement of MIPs

By combining MIPs with a support material, namely silica,

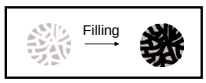
I) target molecules were immobilized to solid silica supports

better binding sites

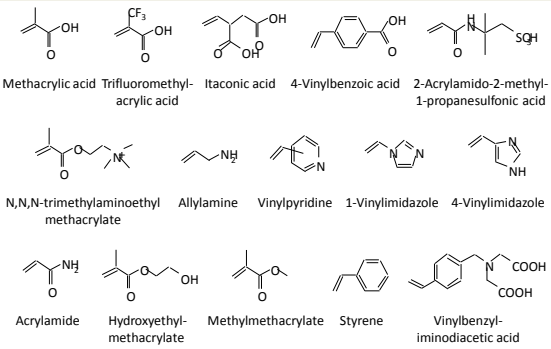


II) porous silica beads were filled with imprinted polymer

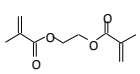
better particle shapes



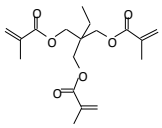
FUNCTIONAL MONOMERS



CROSS-LINKERS

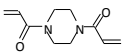


Ethyleneglycol dimethacrylate



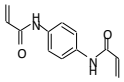
Trimethylolpropane trimethacrylate

Divinylbenzene



3,5-Bis(acryloylpiperazine)

N,N'-Methylene diacrylamide



N,N'-1,4-Phenylene diacrylamide

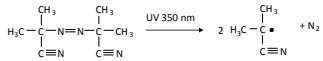
POROGENIC SOLVENTS

- Solubilise all components of the imprinting mixture
- Create porous structure
- Inert
- Provide an favourable environment for non-covalent interactions

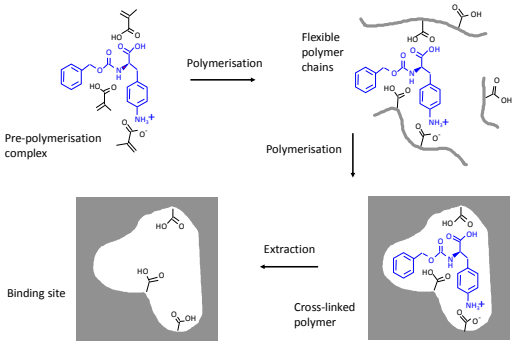
Chloroform	$\varepsilon_r = 5$
Dichloromethane	$\varepsilon_r = 9$
Toluene	$\varepsilon_r = 2$
Tetrahydrofurane	$\varepsilon_r = 8$
Acetonitrile	$\varepsilon_r = 36$
Water ?	$\varepsilon_r = 80$

RADICAL INITIATORS

2,2'-Azobis-isobutyronitrile (AIBN) UV 350 nm, or 65 °C
2,2'-Azobis-(2,4-dimethyl valeronitrile) (ABDV) 45 °C
2,2-Dimethoxy-2-phenyl acetophenone (DMPAP) UV 350 nm

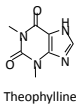
AIBN 

NON-COVALENT IMPRINTING



IMPRINTING EFFECT

Template (T)



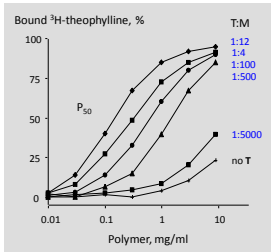
Theophylline

Functional monomer (M)

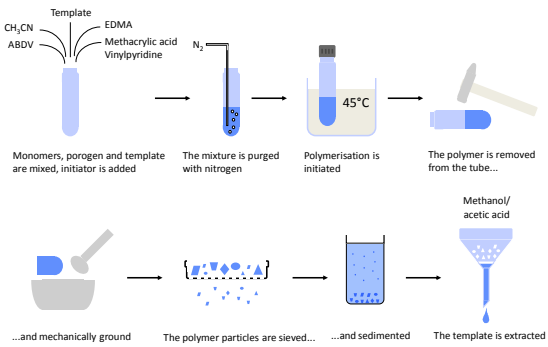
Trifluoromethyl acrylic acid

Cross-linker

Divinylbenzene

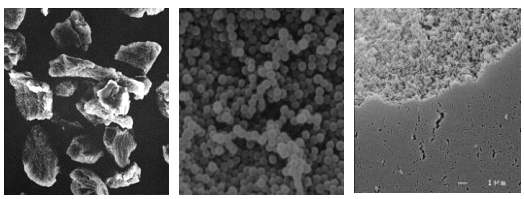


TYPICAL PROCEDURE



CONFIGURATIONS

- Fragmented polymer monoliths
- Polymer beads prepared by suspension, emulsion or precipitation polymerisation
- Composite polymer beads
- *In situ*-prepared polymer rods
- Polymer particles bound in thin layers
- Thin polymer membranes or films
- Surface-imprinted polymer substrates



CHARACTERISTICS

Feature	Characteristics
Physical Stability	Resistant against mechanical stress, high pressures and elevated temperatures
Chemical Stability	Resistant against acids, bases, various organic solvents and metal ions
Storage Endurance	> 1 year without loss of performance
Imprint Memory	Repeated use >100 times without reduction
Recovery of imprint molecule	> 95%
Capacity	≈ 10 mg imprint molecule/g polymer for baseline separation of racemic mixtures

TEMPLATE MOLECULES

Substance class	Examples
Amino acids	Free and derivatised amino acids
Peptides	Enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH)
Steroids	Cholesterol, cortisol, testosterone
Carbohydrates	Derivatised sugars, glycosides
Nucleotides	NAD^+
Nucleotide bases	Adenine
Dyes	Safranine O, rhodanile blue
Pesticides	Atrazine, 2,4-D
Metal ions	Ca^{2+}, Cu^{2+}, Eu^{3+}
Drugs	Propranolol, theophylline, morphine, nicotine, penicillin
Proteins	Transferrin, RNase A, urease, HGH, myoglobin, IgG
Bacteria cells	*Staphylococcus aureus, Listeria monocytogenes*
Crystals	Calcite

APPLICATIONS

- Antibody / receptor binding site mimics
  - Immunoassays
  - Drug development / screening
- Biomimetic sensors
- Tailor-made separation materials
- Facilitated synthesis / Catalysis (enzyme mimics)
- Biomedical
  - Slow release matrices
  - In-situ or extracorporeal removal of unwanted molecules

SEPARATION

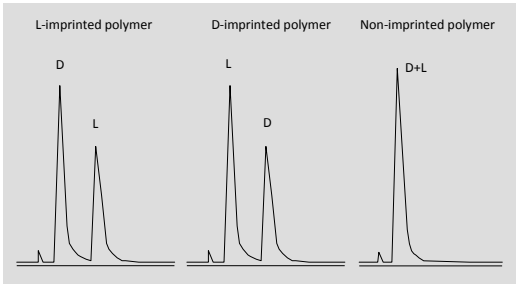
Tailor-made affinity separation materials

Chromatography
Solid-phase extraction
Capillary electrophoresis
Thin layer chromatography
Membrane-based separation

- **Chiral separation**
- **Separation of closely related compounds**

CHIRAL CHROMATOGRAPHY

Pre-determined elution order

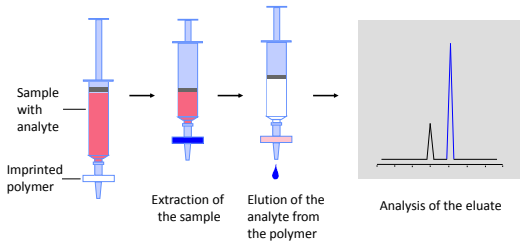


SOLID-PHASE EXTRACTION

Sample clean-up
Analyte preconcentration

Possible alternative for

- Solvent extraction
- Extraction with non-specific matrices
- Immunoextraction



SCREENING OF COMBINATORIAL LIBRARIES

Selection of
- Ligands
- Potential drugs
- Enzyme inhibitors

from
- Chemical combinatorial libraries
- Biological combinatorial libraries

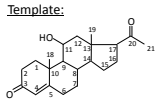
Imprinting of a polymer with a target compound

Screening of a combinatorial library for binding to the imprinted artificial receptor

Imprinted polymers could be useful
- Because of their high selectivity
- Because of their robustness
- If natural receptor does not exist or is poorly characterised
- If natural receptor is difficult to purify

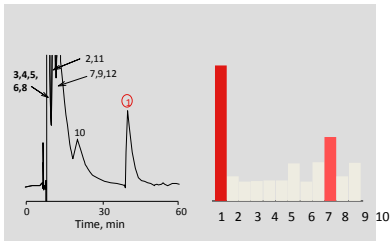
COMBINATORIAL STEROID LIBRARY

Template:



1 11-α-Hydroxyprogesterone
2 11-β-Hydroxyprogesterone
3 17-α-Hydroxyprogesterone
4 Progesterone
5 4-Androsten-3,17-dione
6 1,4-Androstadiene-3,17-dione
7 Corticosterone
8 Cortexone
9 11-Deoxycortisol
10 Cortisone
11 Cortisone-21-acetate
12 Cortisol-21-acetate

Screening on imprinted polymer



Ramström, Ye, Krook, Mosbach (1998) *Anal. Comm*. 35, 9-11.

ANTIBODY MIMICS

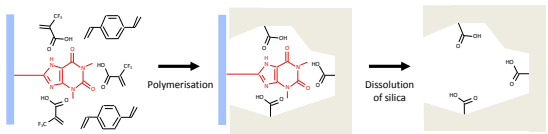
Imprinted polymers — Rigid, Insoluble, large structures

Biological antibodies — Flexible, Soluble, small in size

Specific binding of a target molecule

Possible alternative or complement
- If biological antibodies are difficult to obtain:
  Small and non-immunogenic molecules (no conjugation required!)
- Use in difficult environments (organic solvents, high temperatures…)

IMMOBILISED TEMPLATES



Silica
Immobilised template
Functional monomers
Crosslinker

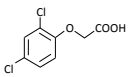
Polymer with binding site

Advantages
- Reduced tumbling rate of template
- Homogeneous orientation of binding sites
- Better accessibility of binding sites
- Tagging of analyte possible

Yilmaz, Haupt, Mosbach (2000) *Angew. Chem. Int. Ed.* 39, 2115-2118.

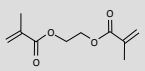
Model analyte: 2,4-dichlorophenoxyacetic acid

Template :

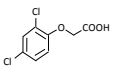


2,4-D

Polymer :

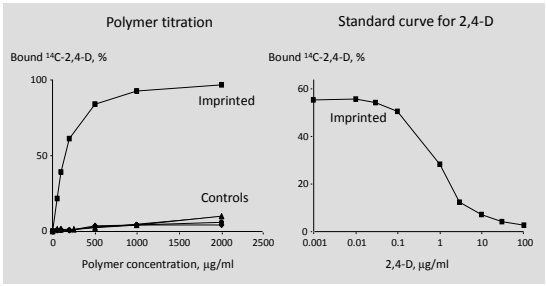
Cross-linker	Functional monomer	Porogen
 Ethyleneglycol dimethacrylate	 4-Vinylpyridine	Methanol/water 4:1

2,4-D



Radioligand displacement assay

Radiolabelled probe: ^{14}C-2,4-D

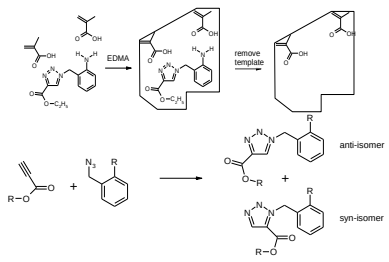


Cross-reactivities of structurally related compounds (%)

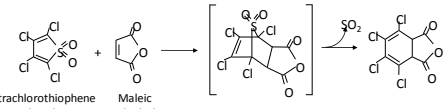
	MIP Buffer	MAb CH_3CN	Buffer
2,4-D	100	100	100
	24	50	0.5-3
	2	14	?
	<0.1	<0.1	?

	MIP Buffer	MAb CH_3CN	Buffer
	95	2	1.5-20
	7	1	30-160
	15	2	?
	10	1	?

A Molecular Imprinted Polymer as the Nanoreactor for Regioselective 1,3 – Dipolar Cycloaddition of Azides and Alkynes



Catalysis: Diels-Alder reaction

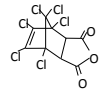


Tetrachlorothiophene dioxide

Maleic anhydride

Template:

Transition state analogue



Chlorendic anhydride

Imprinted polymer:

Methacrylic acid
Ethyleneglycoldimethacrylate
Chloroform

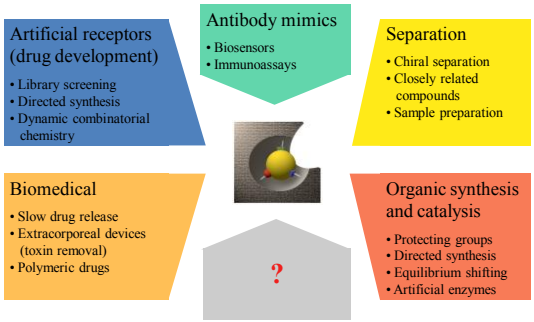
WHY USE IMPRINTED POLYMERS?

- Tailor-made receptor
- Fast preparation
- Chemical and physical stability
- Possible use in organic solvents
- Storage and reuse possible over a long period
- Easy integration in industrial fabrication process

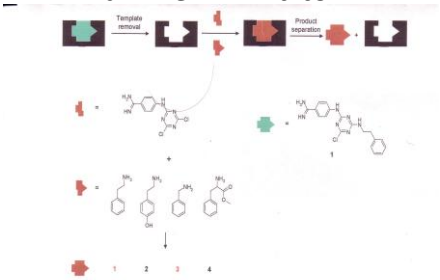
Problems to be addressed

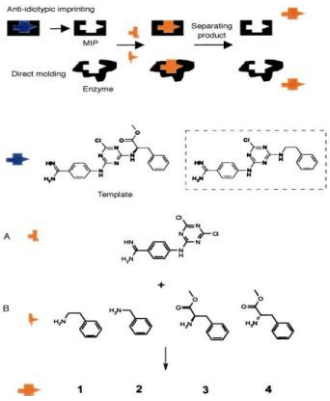
- Heterogeneity of binding sites
- Imprinting of polymers in aqueous solvents
- Imprinting of larger templates (proteins, cells...)

Application areas of Molecularly Imprinted Polymers



Antiideotypic Imprinting of a Kallikrein inhibitor





Direct Molding Imprinting

In this approach novel ligands to a receptor or enzyme can be formed directly by assembly of reactive building blocks within the binding cavity of the biological receptor, thus using the latter as a molecular scale reaction (or nano) vessel.