Potencial analítico de los polímeros de impronta molecular en el análisis de residuos de antibióticos empleados en medicina humana y veterinaria

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Outline

1. Introduction
2. MIPs application in analytical separations
3. MIPs application in optical sensing: fluorescence based sensors
4. Final remarks

La ONU planta cara a la resistencia a los antibióticos

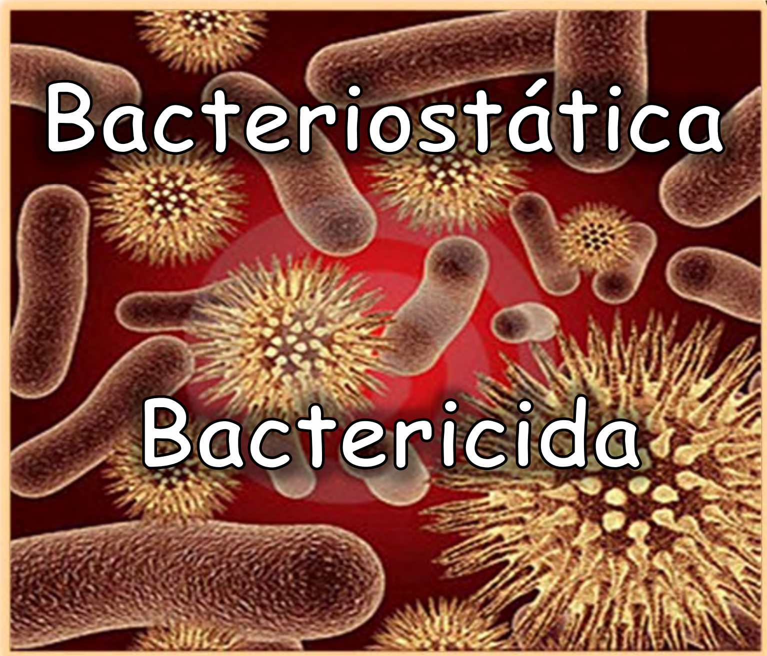
El Mundo 21/09/2016

- Los 193 países miembros de la ONU han firmado un **acuerdo global para hacer frente a la resistencia a los antibióticos**.
- Es tan solo **la cuarta vez en su historia** que la Asamblea General de las Naciones Unidas da protagonismo a un tema de salud. Únicamente el VIH, las enfermedades no transmisibles y el ébola merecieron la misma atención.
- El acuerdo recoge **tres compromisos fundamentales que deberían cumplirse en un plazo de dos años:**
  - Se insta al **desarrollo de sistemas regulatorios y de vigilancia** para el uso de estos fármacos en humanos y animales.
  - Se fomenta el **desarrollo de nuevos productos** .
  - Se pretende **mejorar la formación** tanto de **profesionales sanitarios** como de la **población en general**.

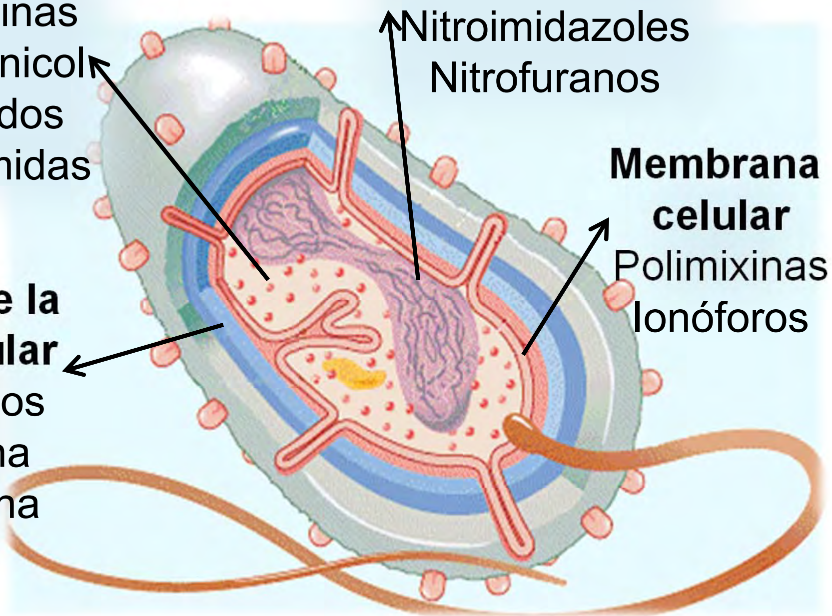
ANTIBIOTICO

Sustancia que se emplea en el tratamiento de infecciones causadas por microorganismos.

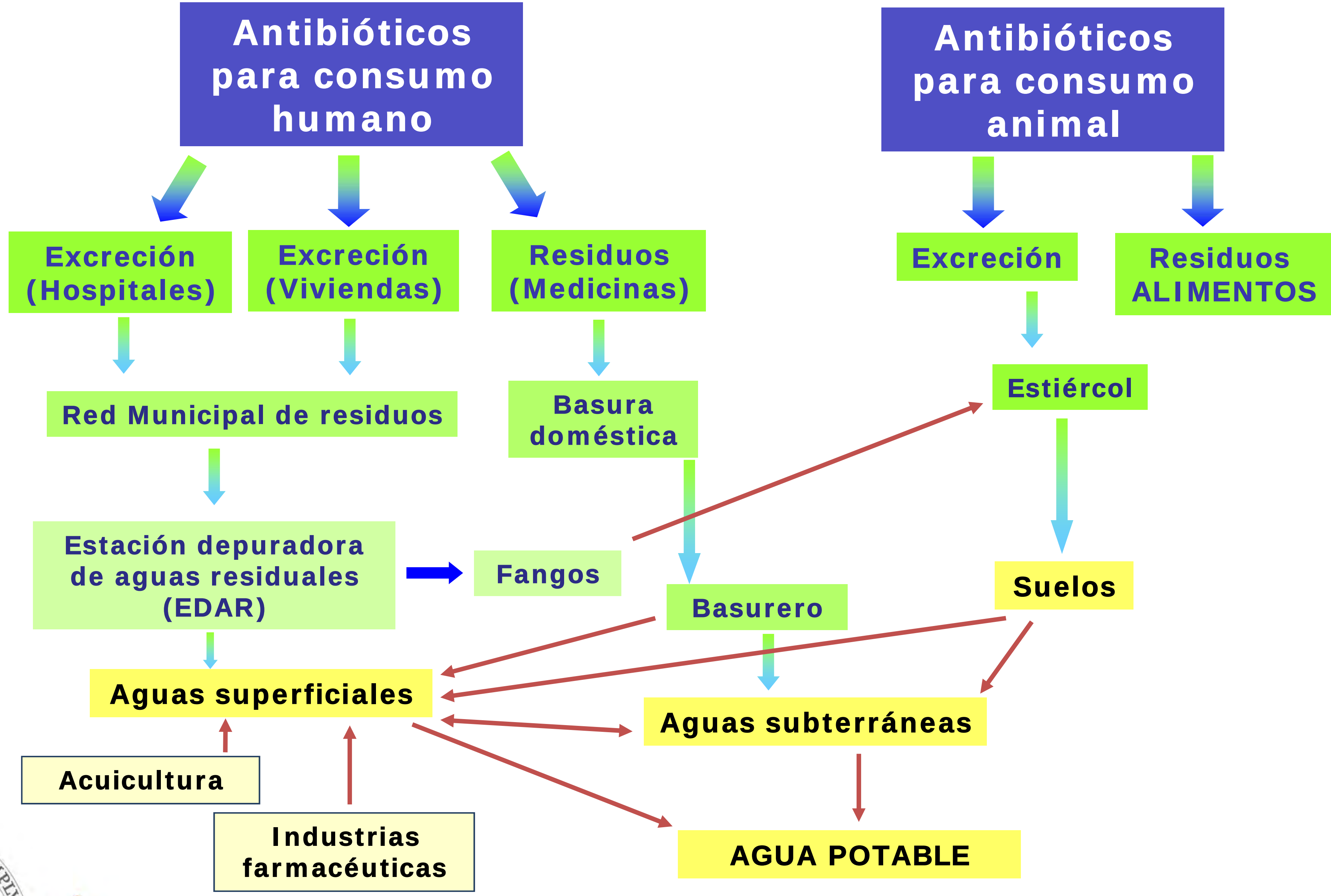
Mecanismo de acción



Clasificación



Fuentes de contaminación



Molecular Biorecognition

Antibodies
Enzymes
Hormonal receptors

- Affinity separations
- Biosensors
- Biochips
- Immunoanalysis
- Drug targeting
- Catalysis
- Directed synthesis

√ Highly specific molecular recognition

- Limited stability
- Sometimes difficult to obtain
- High production costs
- Difficult to integrate into industrial processes

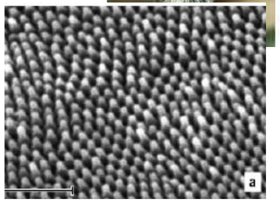
Biomimetic Nanomaterials

O.H. Schmitt (1969) from the Greek: *bios* (*life*) and *mimesis* (*imitate*)

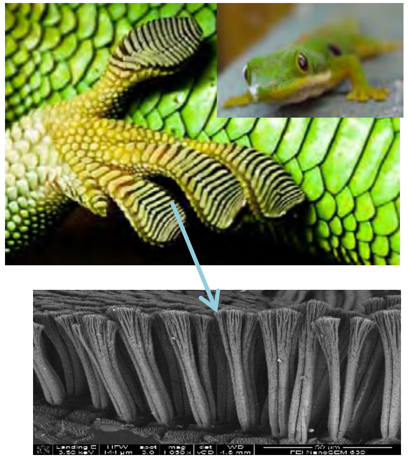
Applications

- Anti-reflective coatings
- Diffraction pigments
- Self-cleaning surfaces
- Elastomers
- Adhesives
- High strength composites
- Membranes
- Fuel cells
- Anti-corrosion coatings

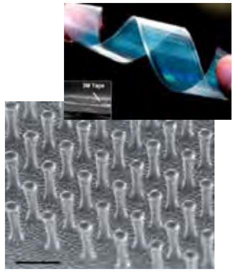
Cicada Cryptotypana aquila



Antireflective



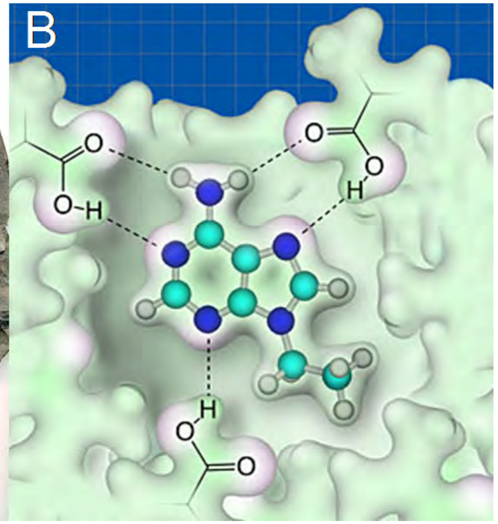
Self-cleaning



Gecko tape®

Molecularly Imprinted Polymers

FOSSIL: **"the remains or impression of a prehistoric plant or animal embedded in rock and preserved in petrified form."**

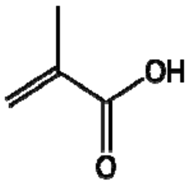


A) IMPRINT of *Trilobites arthropods*

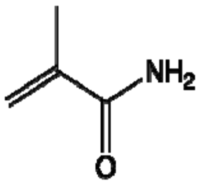
B) TEMPLATE-MIP

"Synthetic polymer with cavities of pre-determined selectivity that can be tailored to mimic the molecular recognition ability of certain species such as antibodies, enzymes, receptors, etc."

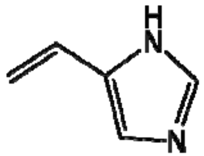
Artificial vs. natural proteins



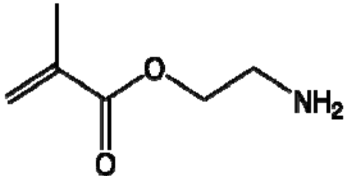
Methacrylic acid
(Glu, Asp)



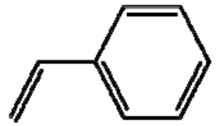
Methacrylamide
(Gln, Asn)



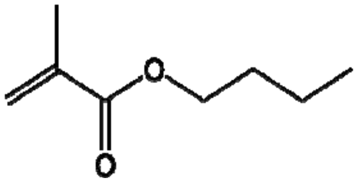
4-Vinylimidazole
(His)



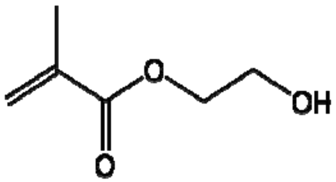
Aminoethyl methacrylate
(Lys)



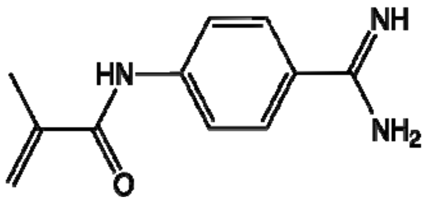
Styrene
(Phe)



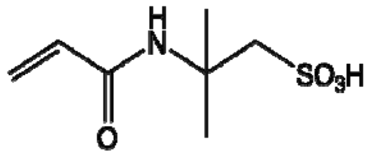
Butyl methacrylate
(Leu)



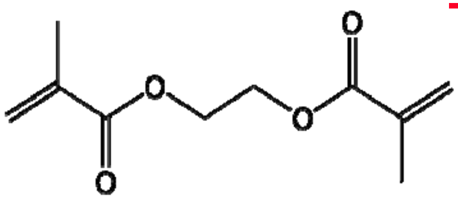
Hydroxyethyl methacrylate
(Ser)



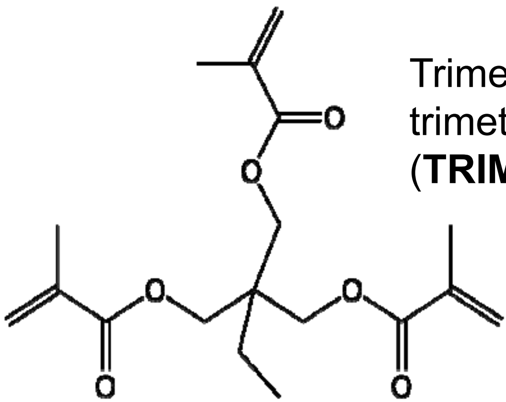
Methacrylamidobenzamidine
(Arg)



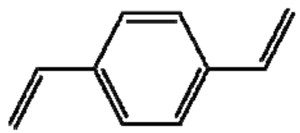
2-Acrylamido-2-methyl-1-propanesulfonic acid



Ethyleneglycol dimethacrylate
(**EDMA**)



Trimethylolpropane trimethacrylate
(**TRIM**)



Divinylbenzene
(**DVB**)

Courtesy of Prof. K. Haupt

Artificial vs. natural proteins

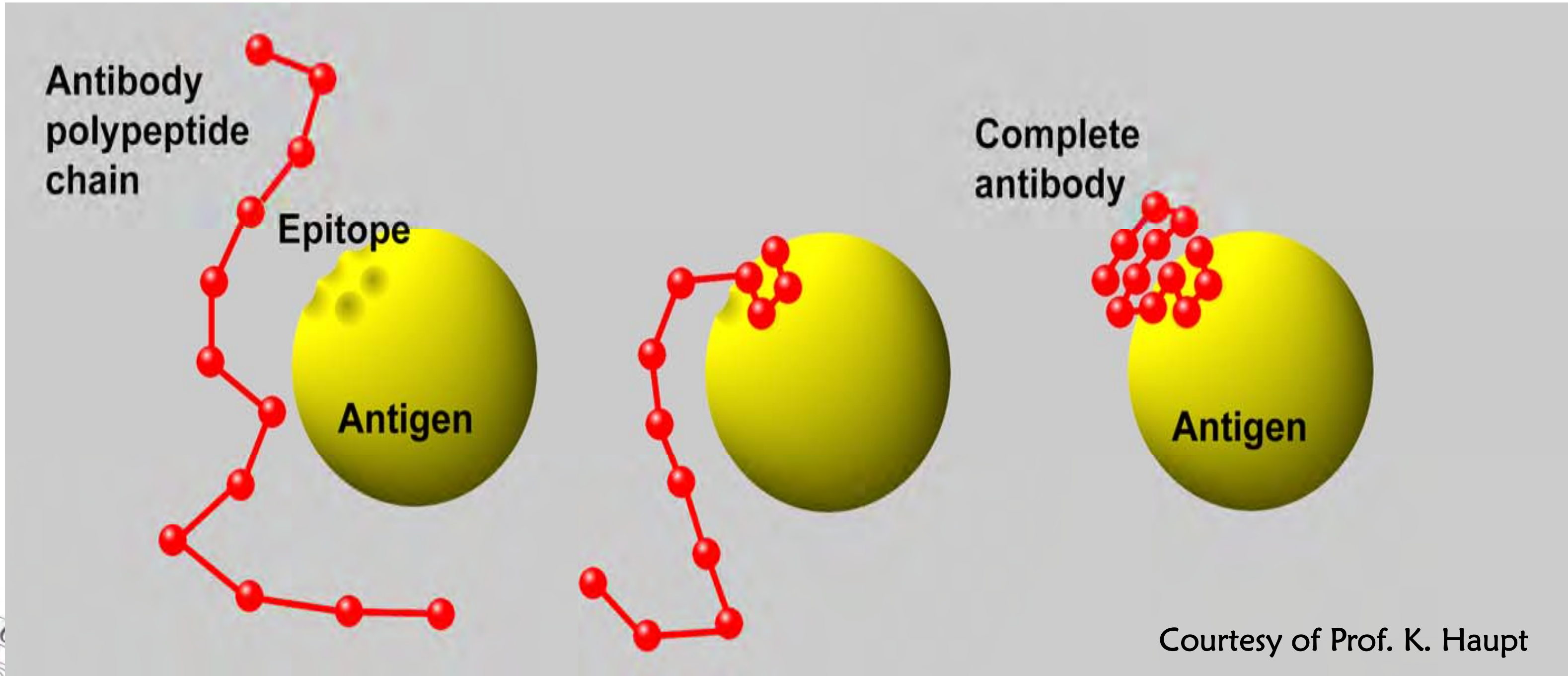
- Polymer (polyamide)
  - Polymer (polymethacrylate)
- Variety of monomers (amino acid functional groups)
  - Variety of monomers (functional groups)
- Defined sequence
  - Random sequence
- Defined spatial arrangement of monomers
  - Defined spatial arrangement of monomers by templating
- Stabilised by weak bonds
  - Stabilised by chemical cross-links (more stable)
- Molecular recognition
  - Molecular recognition (binding site created by templating)
- Optimized through millions of years of evolution
  - Molecular modelling, combinatorial libraries, chemometrics...

Courtesy of Prof. K. Haupt

The basis of molecular imprinting

"I assume…that all antibody molecules contain the same polypeptide chains as normal globulin, and differ from normal globulin only in the configuration of the chain."

LINUS PAULING
(1901-1994)



Courtesy of Prof. K. Haupt

The basis of molecular imprinting

F.H. Dickey (1949)

- Preparation of **silica gel** with specific affinities for predetermined substances.
- Same **mechanism** as that proposed by Pauling for the formation of **antibodies** using **antigens** as **template** molecules.

PROCEEDINGS
OF THE
NATIONAL ACADEMY OF SCIENCES
Volume 35 May 15, 1949 Number 5

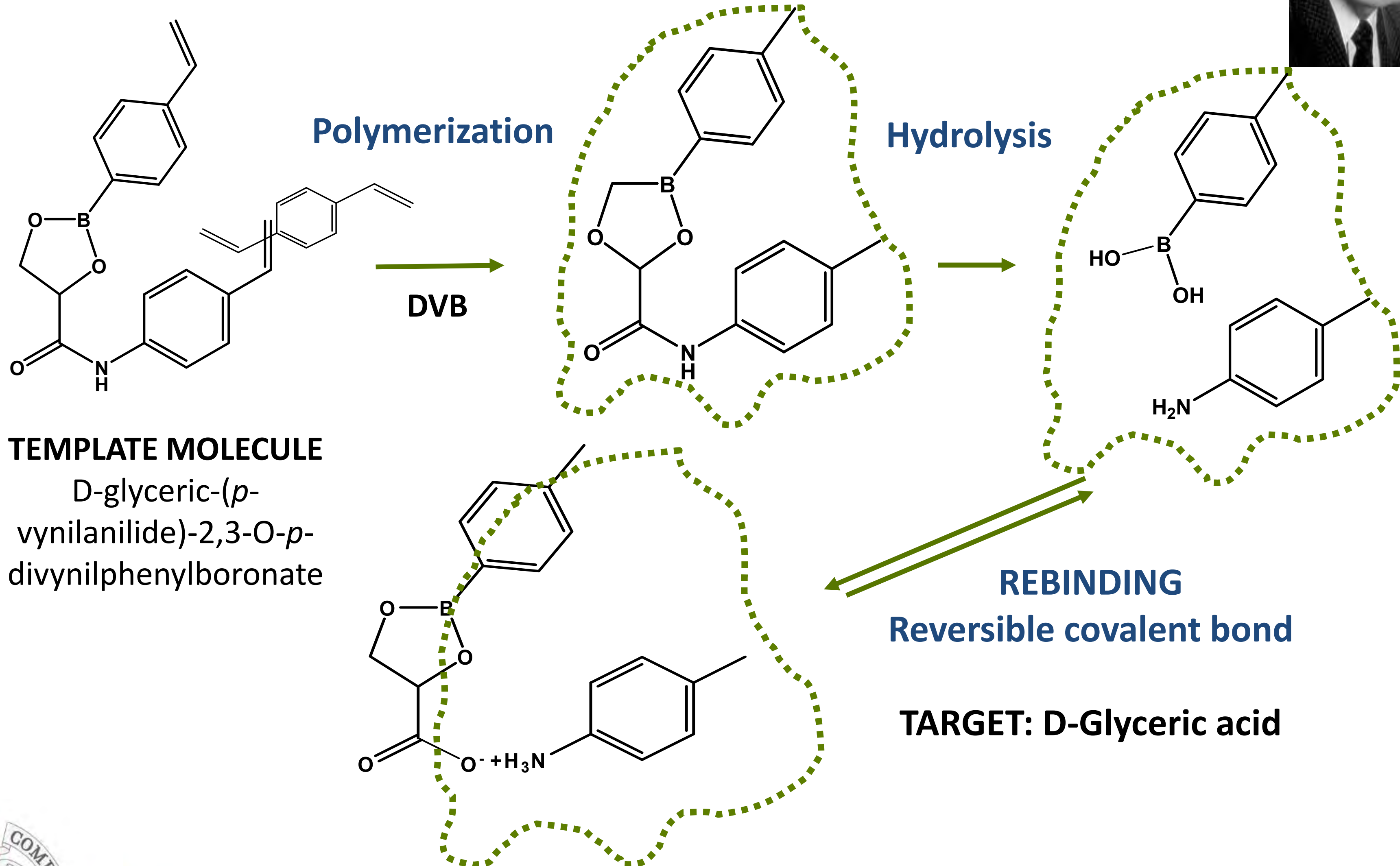
THE PREPARATION OF SPECIFIC ADSORBENTS
BY FRANK H. DICKEY*

Proc. Nat. Ac. Sci. ***(1949)*** 35:227

TEMPLATE	RELATIVE ADSORPTION POWER			
	Methyl Orange	**Ethyl Orange**	**Propyl Orange**	**Butyl Orange**
Methyl Orange	**3.5**	1.6	1.1	1.1
Ethyl Orange	2,5	**9**	2.1	2.2
Propyl Orange	2.3	5	**20**	6
Butyl Orange	1.5	2.8	5	**15**

Molecular imprinting with organic polymers

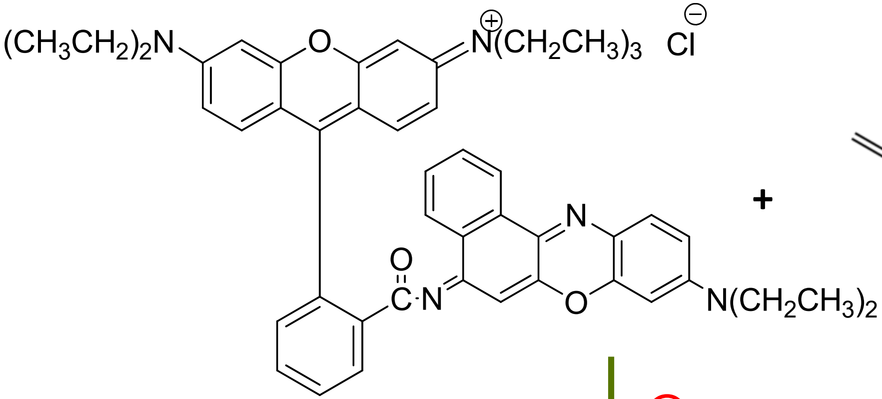
Wulf and Sarhan, COVALENT IMPRINTING, 1972



Wulf, G., Sarhan, A., (**1972**) Angew. Chem. 84:364.

Molecular imprinting with organic polymers

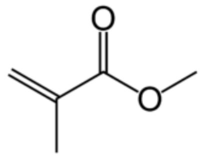
Mosbach and Arshady, NON-COVALENT IMPRINTING, 1981



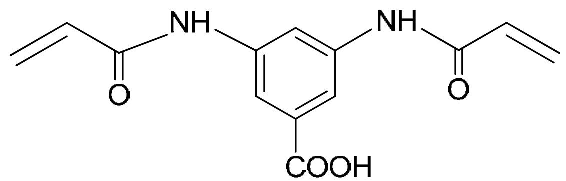
TEMPLATE MOLECULE
Rhodaline Blue

\+

MMA

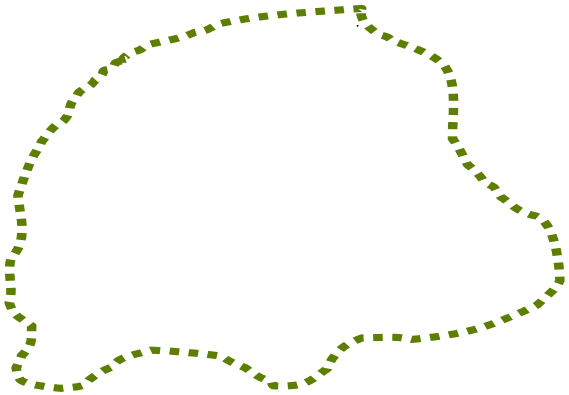


\+

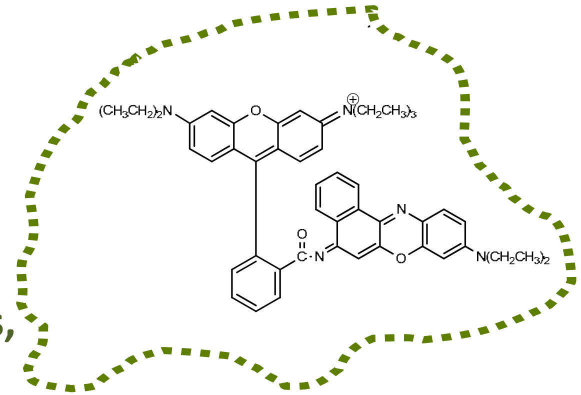


3,5-bis(acryloylamido)benzoic acid

① Polymerization
② Template extraction



REBINDING



Non-Covalent:
Ionic, π-π,
hydrophobic interactions,
hydrogen bonding

Arshady, R., Mosbach, K., (1981) Makromol. Chem. 182:687.

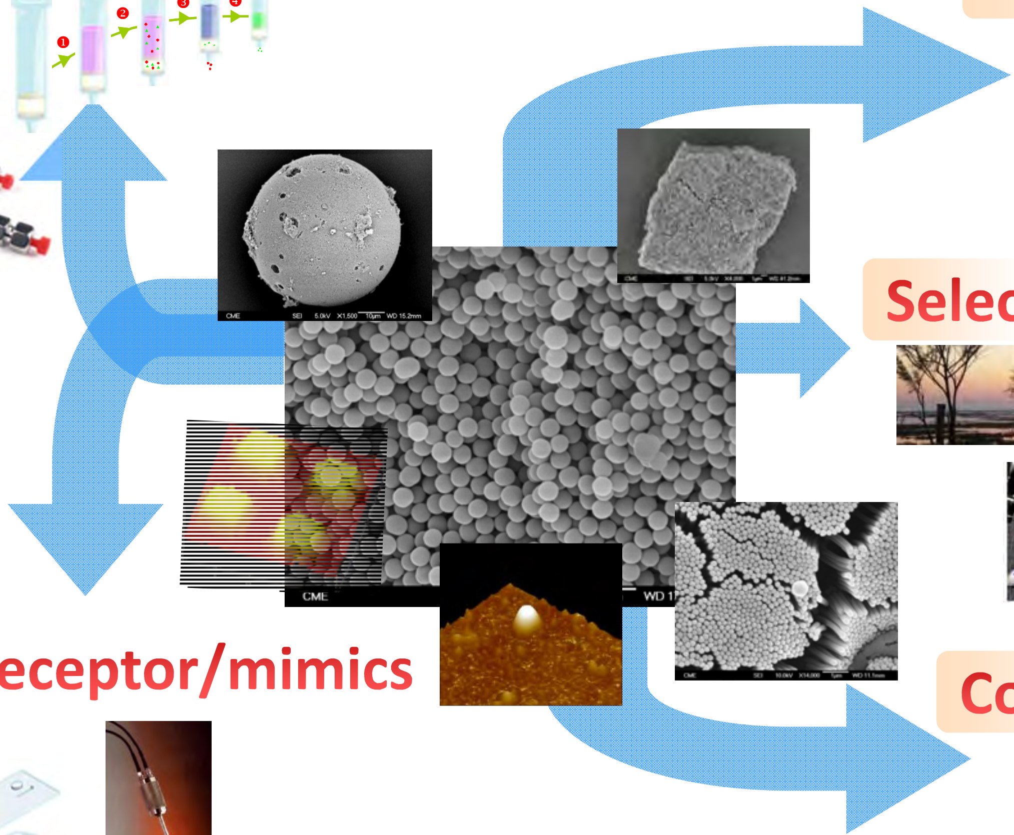
MIP applications

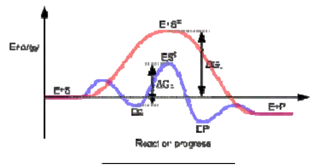
Analytical Separations

SPE

HPLC

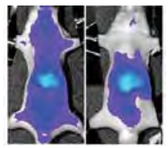
Catalysis



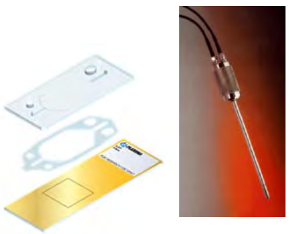


Selective removal

Contaminants
Drugs
Toxins…



Sensors
Antibody receptor/mimics



Optical
Electrochemical
Piezoelectric…

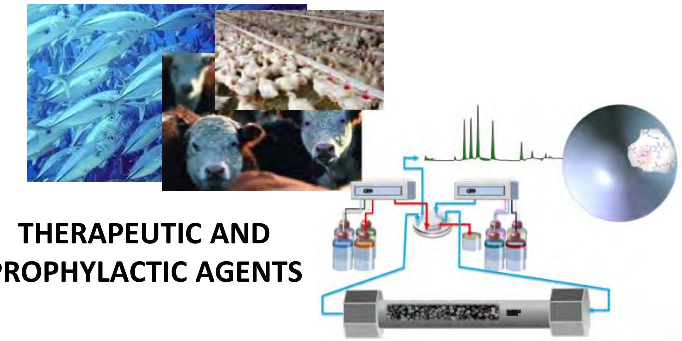
Controlled release

Drugs
Antioxidants…

U. Dortmund

1 MIPs as selective solid phase extraction sorbents (MISPE)

E. Benito-Peña, *J. Chrom A*, 1208, **2008**, 62
E. Benito-Peña, *Anal. Bioanal. Chem. 393,* **2009**, *235*
E. Rodriguez, *Anal. Chem.* 3, **2011**, 2046
M.D. Luaces et al., Microch. J. 110 , **2013**, 458
J. Urraca et al. , *J. Chrom A*, 1343, **2014**, 1
E. Benito-Peña, *ACS Appl. Mat. Interf.* 7, **2015**, 10966



THERAPEUTIC AND PROPHYLACTIC AGENTS

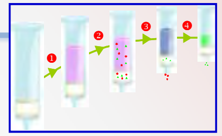
TARGET ANTIBIOTICS

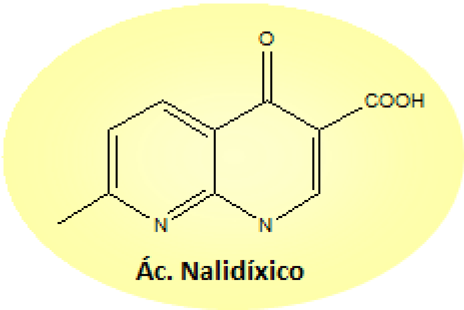
FLUOROQUINOLONES

PENICILLINS

CEPHALOSPORINS

Synthesis of fluoroquinolone selective MIPs

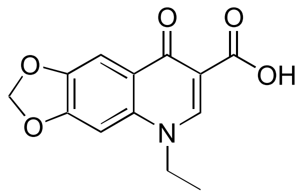




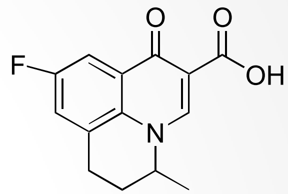
✓ Bactericidal agents

First Generation

Oxonilic acid

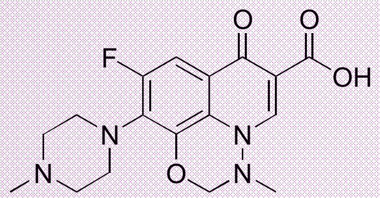


Flumequine

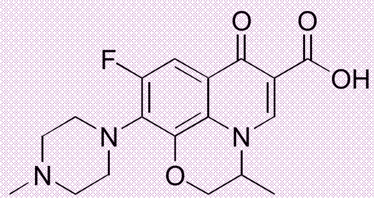


Second Generation

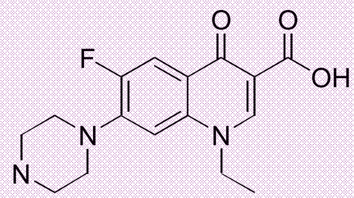
Marbofloxacin



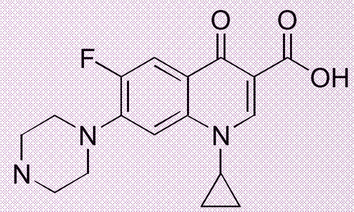
Ofloxacin



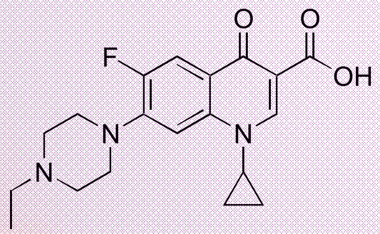
Norfloxacin



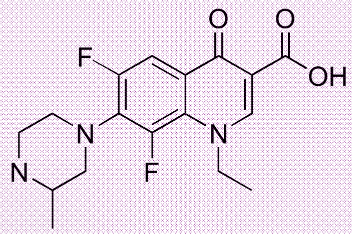
Ciprofloxacin



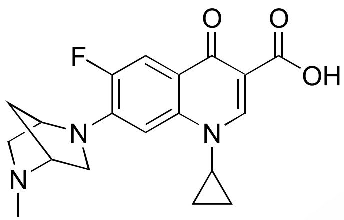
Enrofloxacin



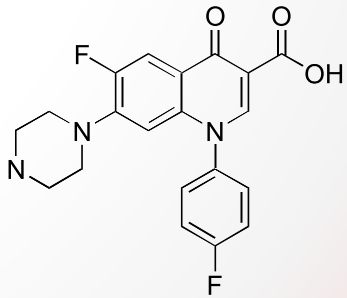
Lomefloxacin



Danofloxacin

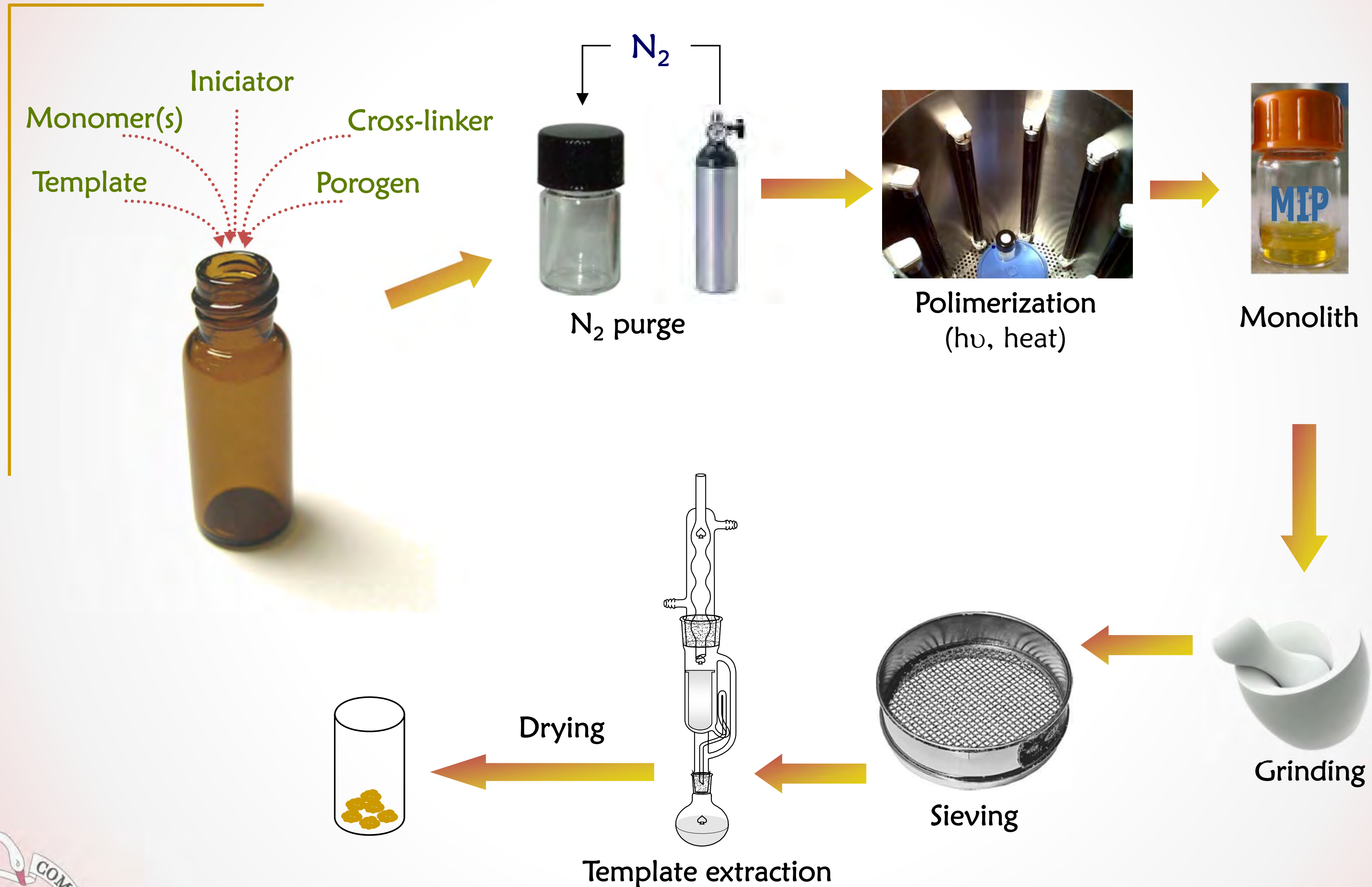


Sarafloxacin

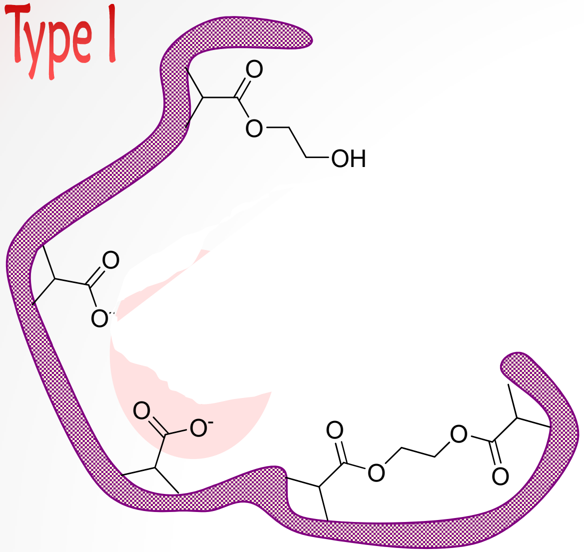


Third Generation

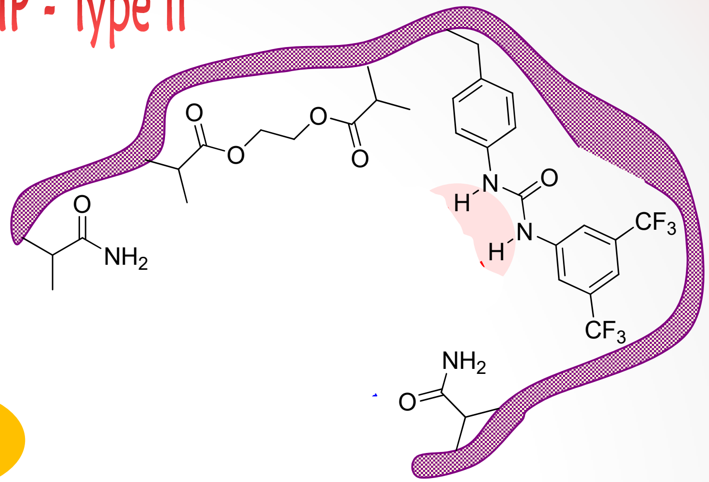
Bulk polymerization



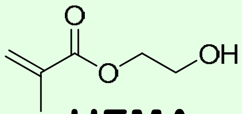
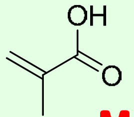
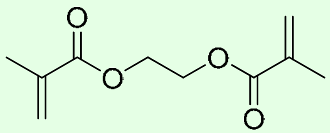
MIP - Type I

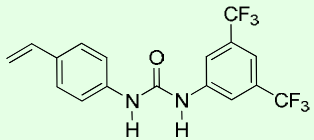
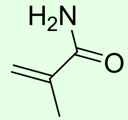
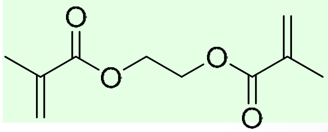


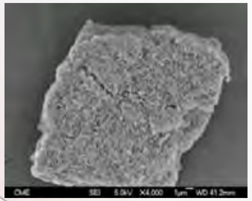
MIP - Type II



NIP

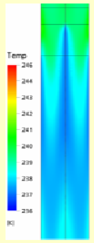
Monomers	 **HEMA**	 **MAA**
Crosslinker	 **EDMA**	

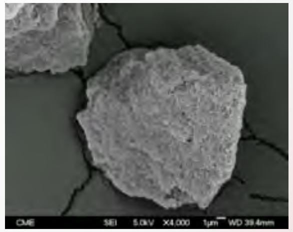
Monomers	 **UREA MONOMER**	 **MAM**
Crosslinker	 **EDMA**	



Porogen:
Acetonitrile

Initiator:
ABDV
60 °C





E. Benito-Peña et al., (2008) *J. Chrom. A*, 1208:62
E. Benito-Peña et al. (2009) *Anal. Bioanal. Chem.*, 393:235

MIP - Type I

2. CHROMATOGRAPHIC CHARACTERIZATION

1. SLURRY PACKING OF THE MIP/NIP IN LC COLUMNS

Mobile phase* ACN/H_2O (v:v)	k_{MIP}	k_{NIP}	IF
100:0	n.e.	33.3	-
75:25	24.0	1.2	20.8
60:40	37.5	3.0	12.4
50:50	**52.1**	**1.5**	**34.3**
40:60	n.e.**	3.08	-
25:75	n.e.	5.70	-
15:85	n.e.	n.e.	-
10:90	n.e.	n.e.	-
0:100	n.e.	n.e.	-

*ACN:buffer (HEPES 0.1 M, pH 7.5)
[ENROFLOXACIN] = 3 mM, Flow rate = 1 mL min^{-1}.
** n.e.: not eluted in 140 min

Retention factor:

$$k = \frac{t_R - t_0}{t_0}$$

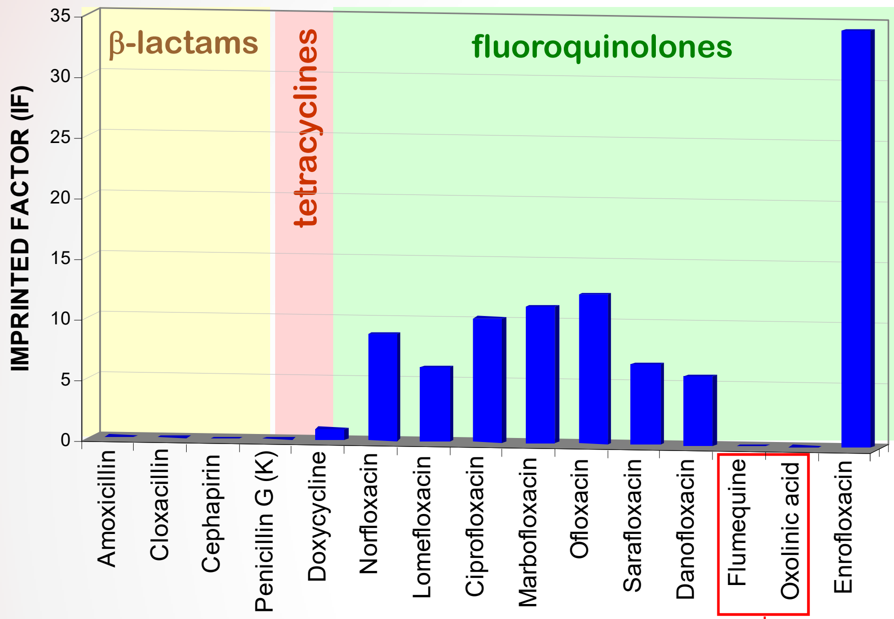
Imprinting effect:

$$IF = \frac{k_{MIP}}{k_{NIP}}$$

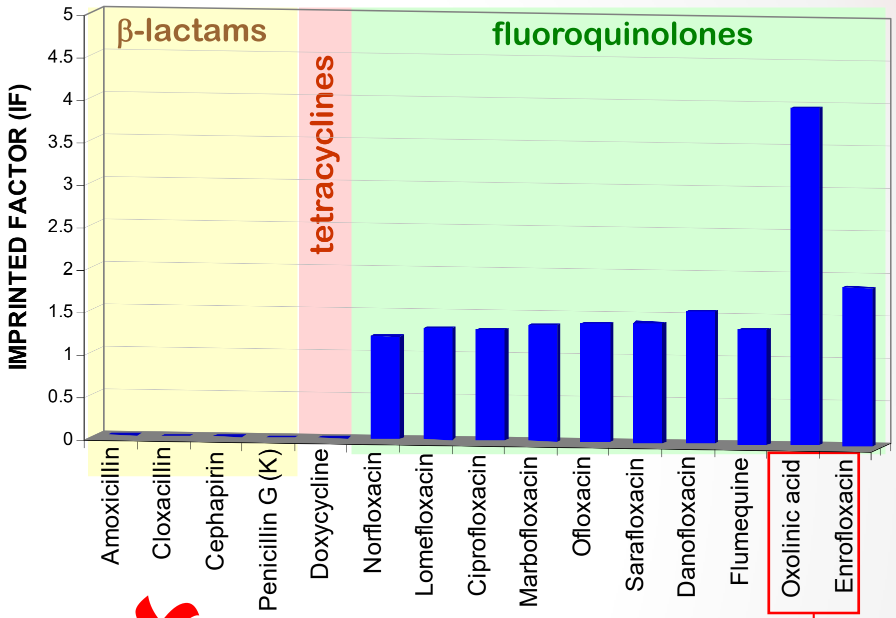
SELECTIVE RECOGNITION: Washing solvent

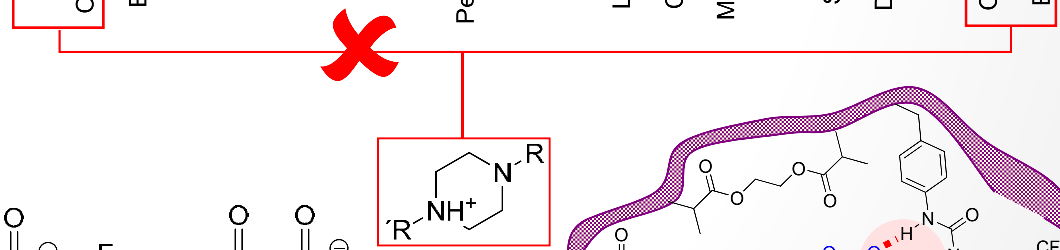
MIP selectivity towards different antibiotics

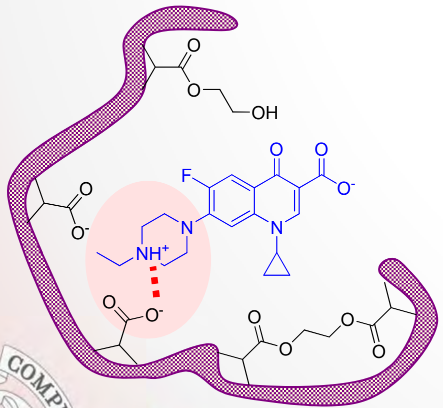
MIP - Type I

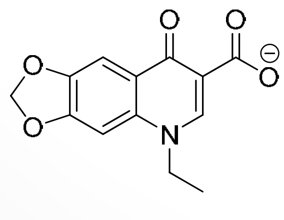


MIP - Type II

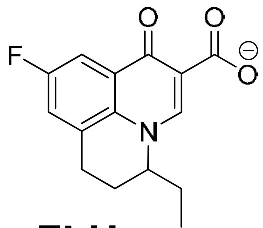




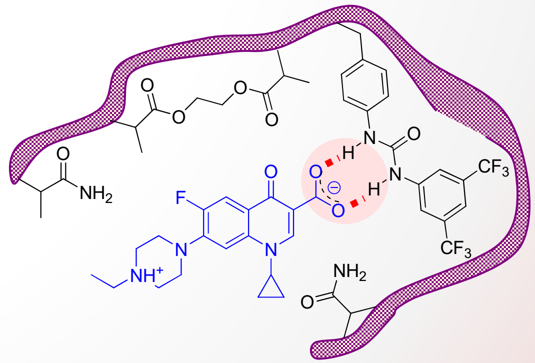




OXO



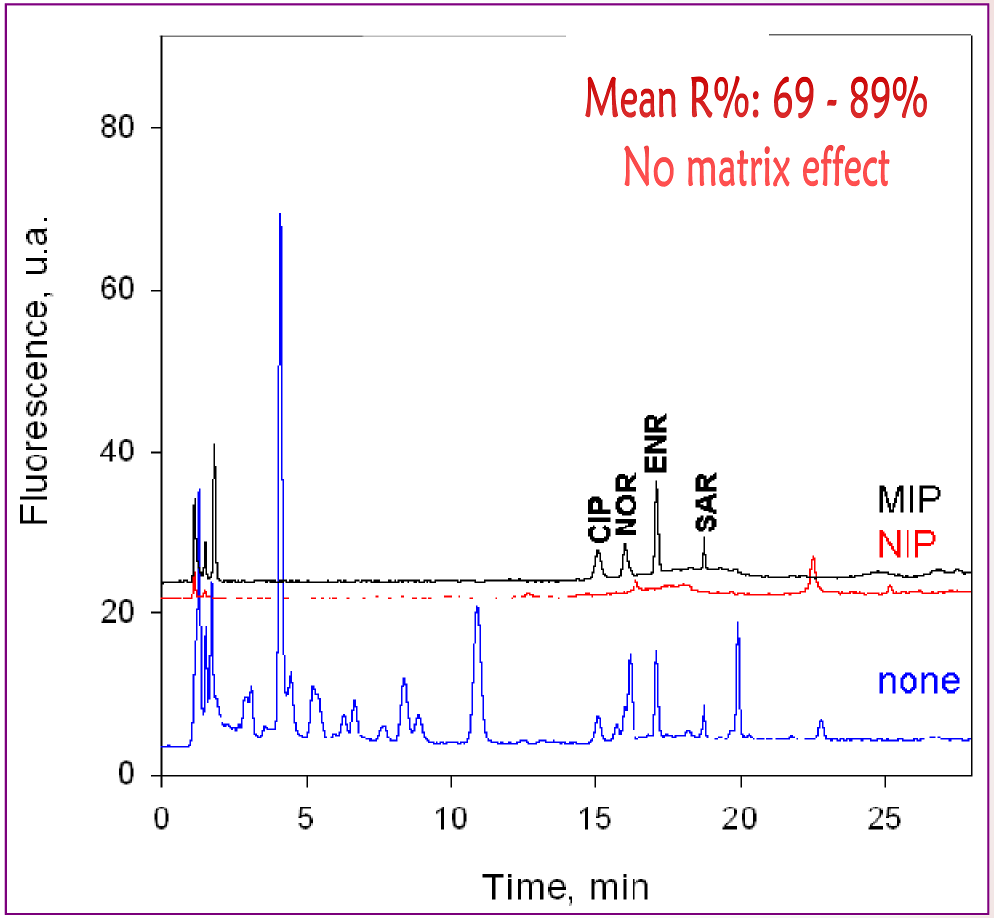
FLU



MISPE APPLICATION

MIP - Type I

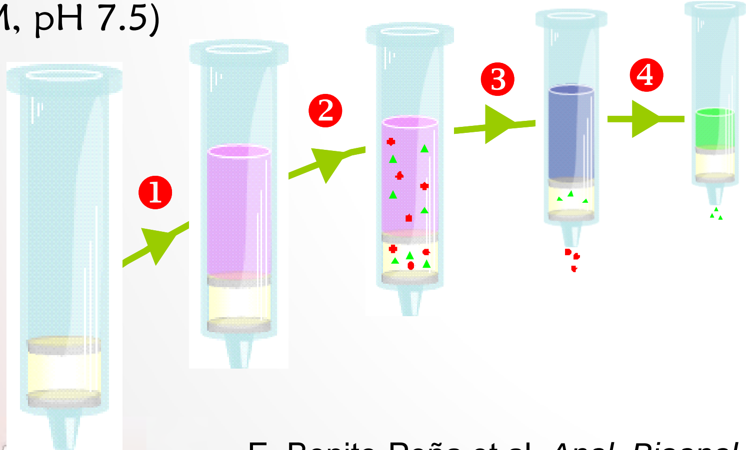
Urine samples



SAMPLE
4 mL urine HEPES (1 M, pH 7.5)

WASHING STEP
ACN/H_2O, 50:50 (0.1 M HEPES, pH 7.5)

ELUTION
3 mL MeOH/ TFA 3%

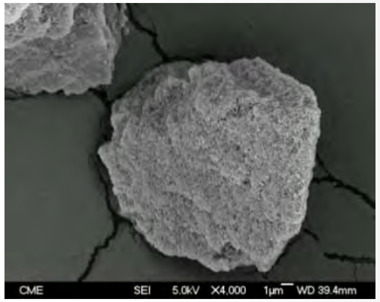


E. Benito-Peña et al. *Anal. Bioanal. Chem.*, 393, **2009**, 235

BULK POLYMERIZATION, LIMITATIONS…

- IRREGULAR PARTICLE SIZE AND SHAPE

- Low packing efficiency in SPE cartridges
- Low resolution



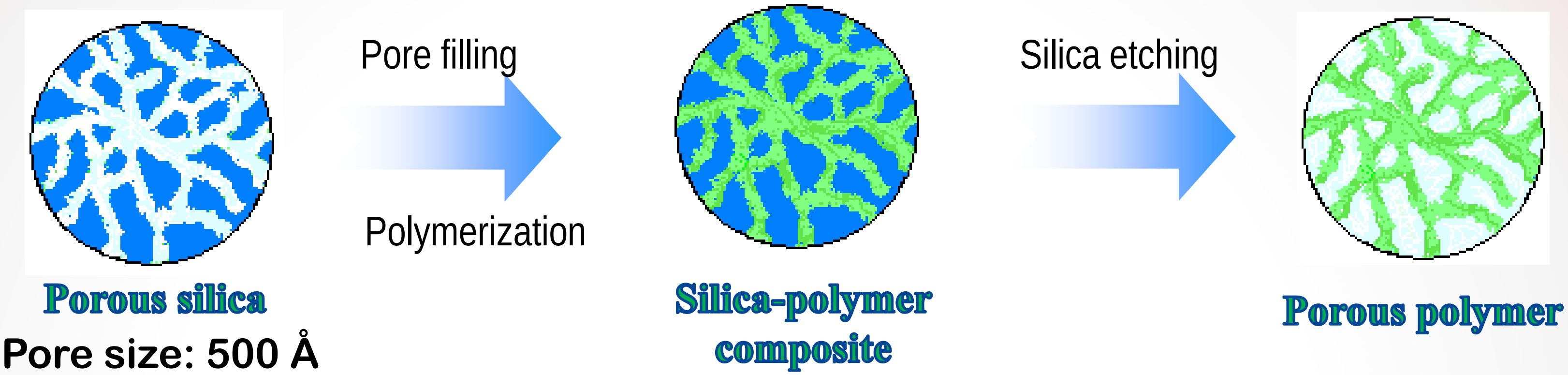
- MONOLITH GRINDING

- Partial destruction of binding sites

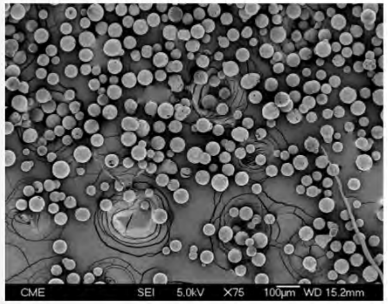
SYNTHESIS OF SPHERICAL MIP BEADS OF TUNNABLE SIZE:

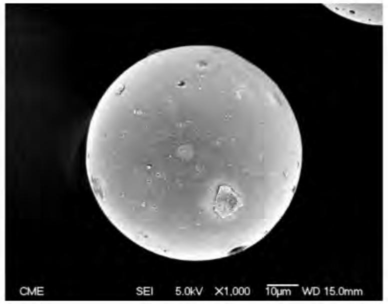
- SPE (40 – 75 μm)
- On-line SPE-HPLC: 2 – 10 μm

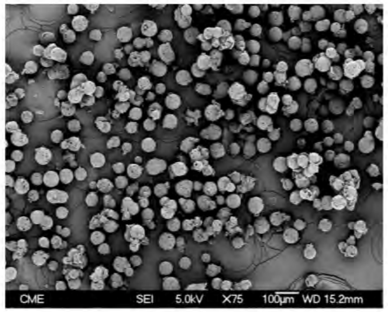
Synthesis of MIP beads: Sacrificial molds

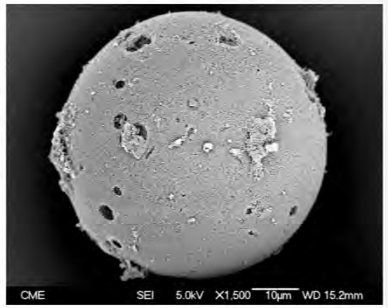


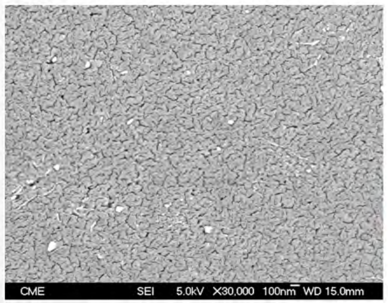
Pore size: 500 Å
Particle size: <u>40-75 µm</u>





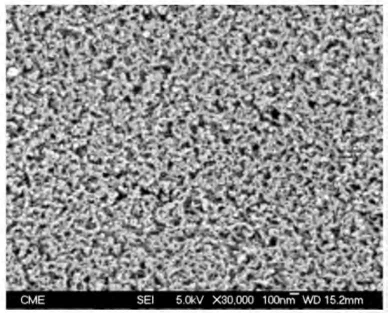






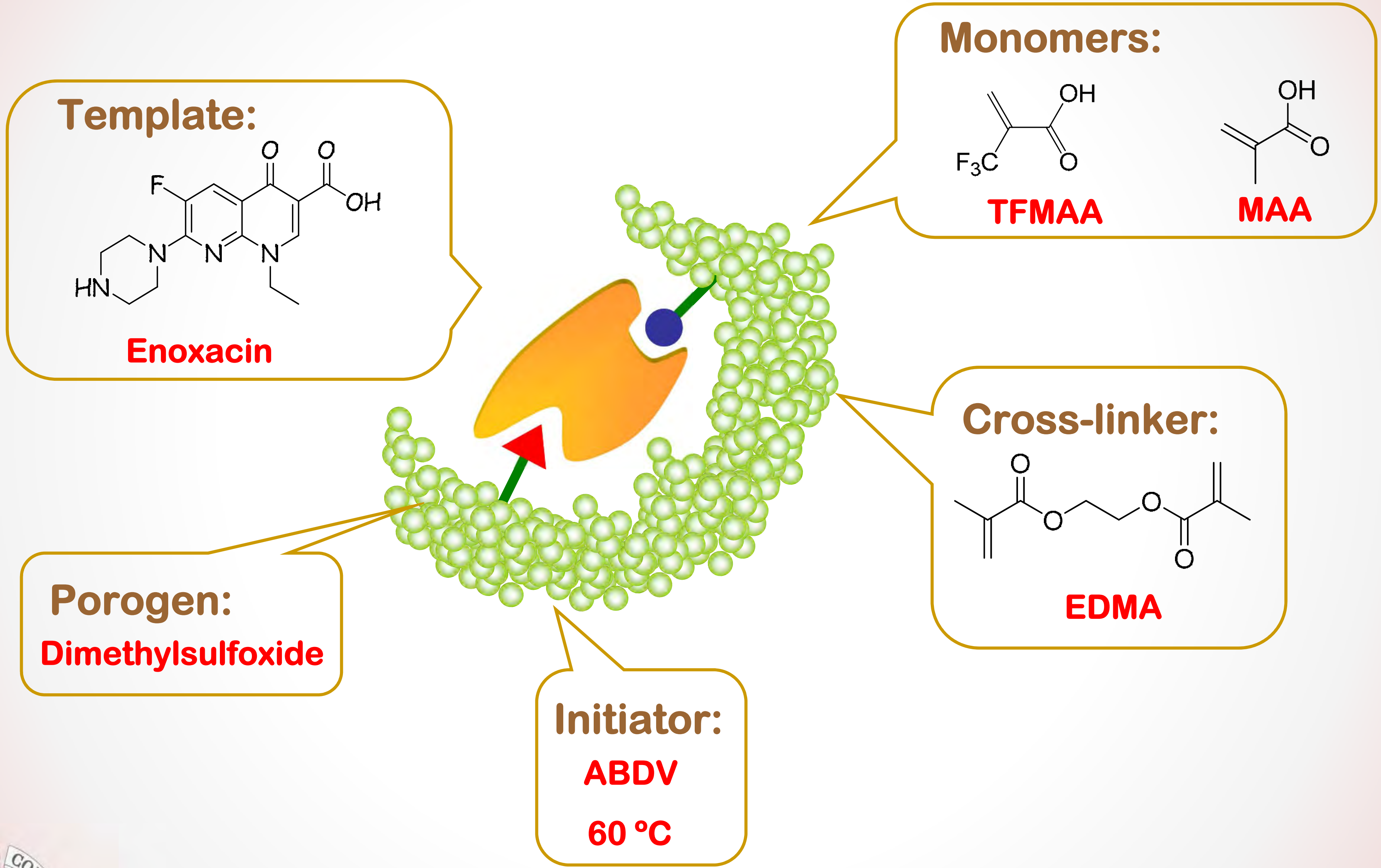
Composite

Polymer

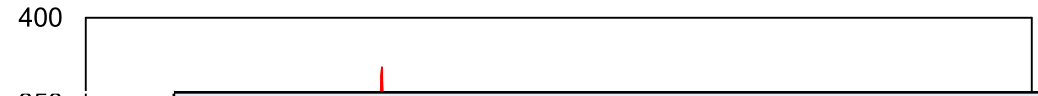


J. Urraca et al. , J. Chrom A, 1343, **2014**, 1

Synthesis of enrofloxacin imprinted microspheres



Food analysis



	Chicken muscle samples					
	NOR	CIPRO	LOME	DANO	ENRO	SARA
MRL (µg Kg^{-1})	--	50	--	200	50	30[a]
LOD (µg Kg^{-1})	**0.2**	**1.7**	**1.7**	**2.7**	**0.8**	**0.7**
Reproducibility (interday) (HPLC-MS/MS)						
Spiked level (µg Kg^{-1})	10-30	25-75	20-60	100-300	25-75	15-45
Recovery (%)	**92-100**	**93-96**	**74-87**	**88-101**	**93-96**	**92-102**
RSD (%) (n = 18)	9-11	6-12	4-12	14-27	4-7	6-13

(a) Reference MRL value set in salmonidae muscle

HPLC–FLD chromatograms of: a) A chicken blank extract after MISPE (—); b) A chicken extract spiked with the six FQs (each one at the corresponding MRL level) without MISPE (—); c) A chicken extract spiked with the six FQs (each one at the corresponding MRL level) after MISPE (—).
(1) NOR; (2) CIPRO; (3) LOME; (4) DANO; (5) ENRO; (6) SARA.

Controlled size MIP beads

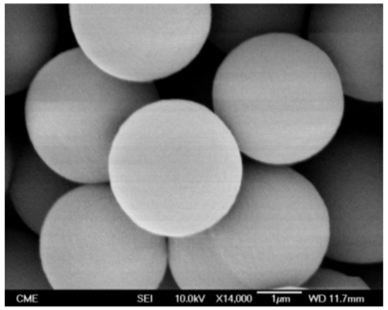
➤ Precipitation polymerization

Low monomer concentration

< 5%

Stirring

Enrofloxacin-imprinted MIPs



Monodisperse spherical beads

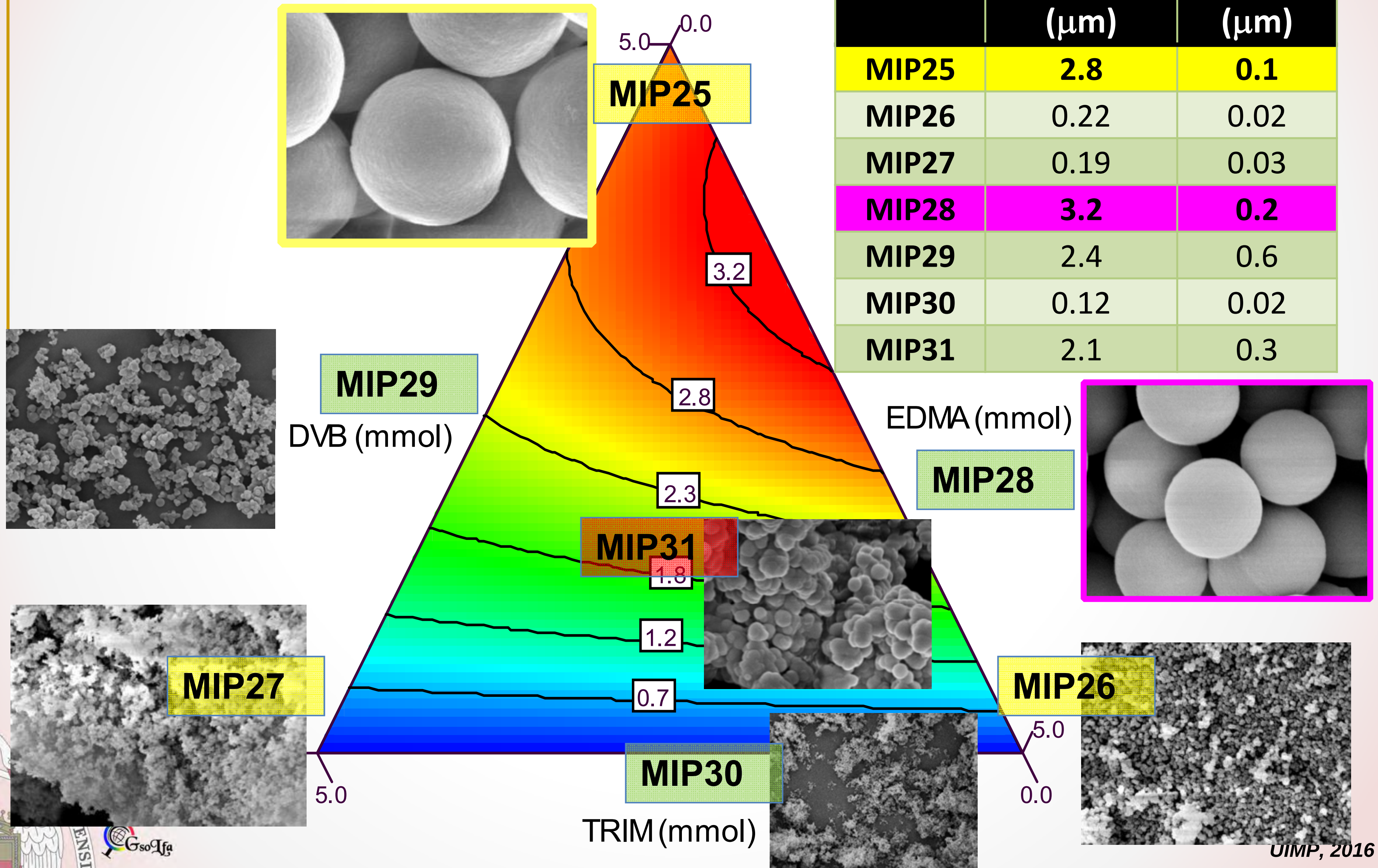
Size: nm to μm

Moreno-Bondi et al. (2015) *ACS Appl. Mat. Interf.* 7:10966-76

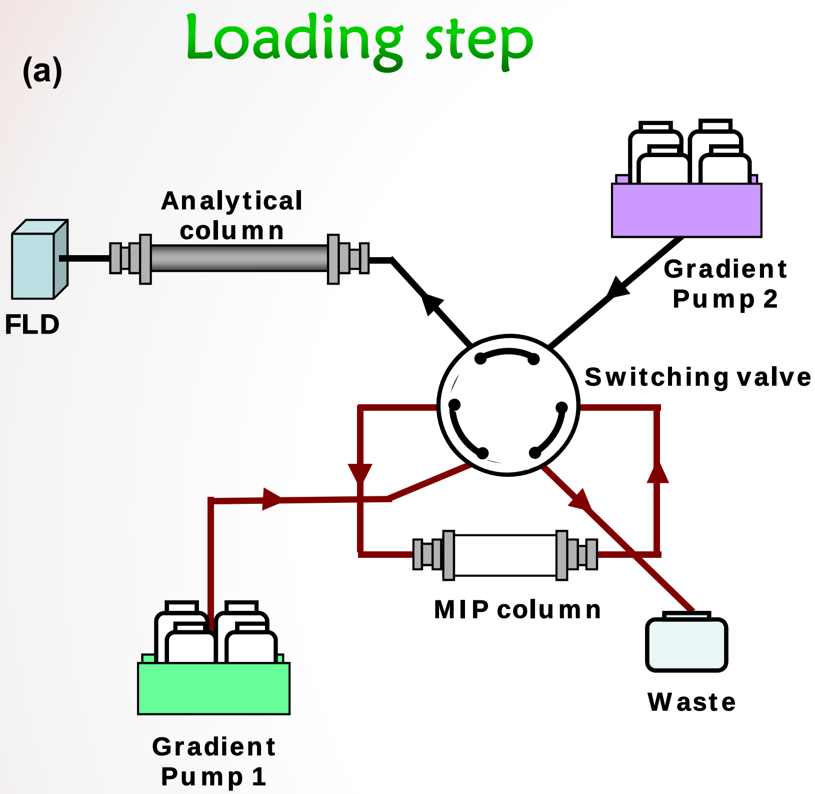
Cross-linker selection

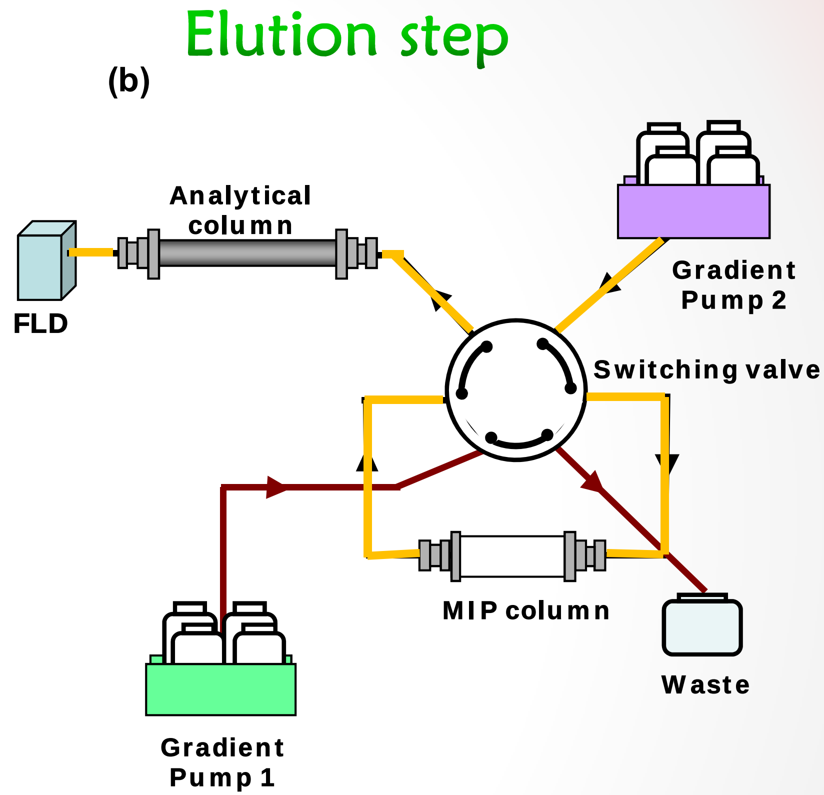
Experimental design: SIMPLEX-LATTICE DESIGN

Polymer	Diameter (μm)	s (μm)
MIP25	**2.8**	**0.1**
MIP26	0.22	0.02
MIP27	0.19	0.03
MIP28	**3.2**	**0.2**
MIP29	2.4	0.6
MIP30	0.12	0.02
MIP31	2.1	0.3



On-line MISPE set up





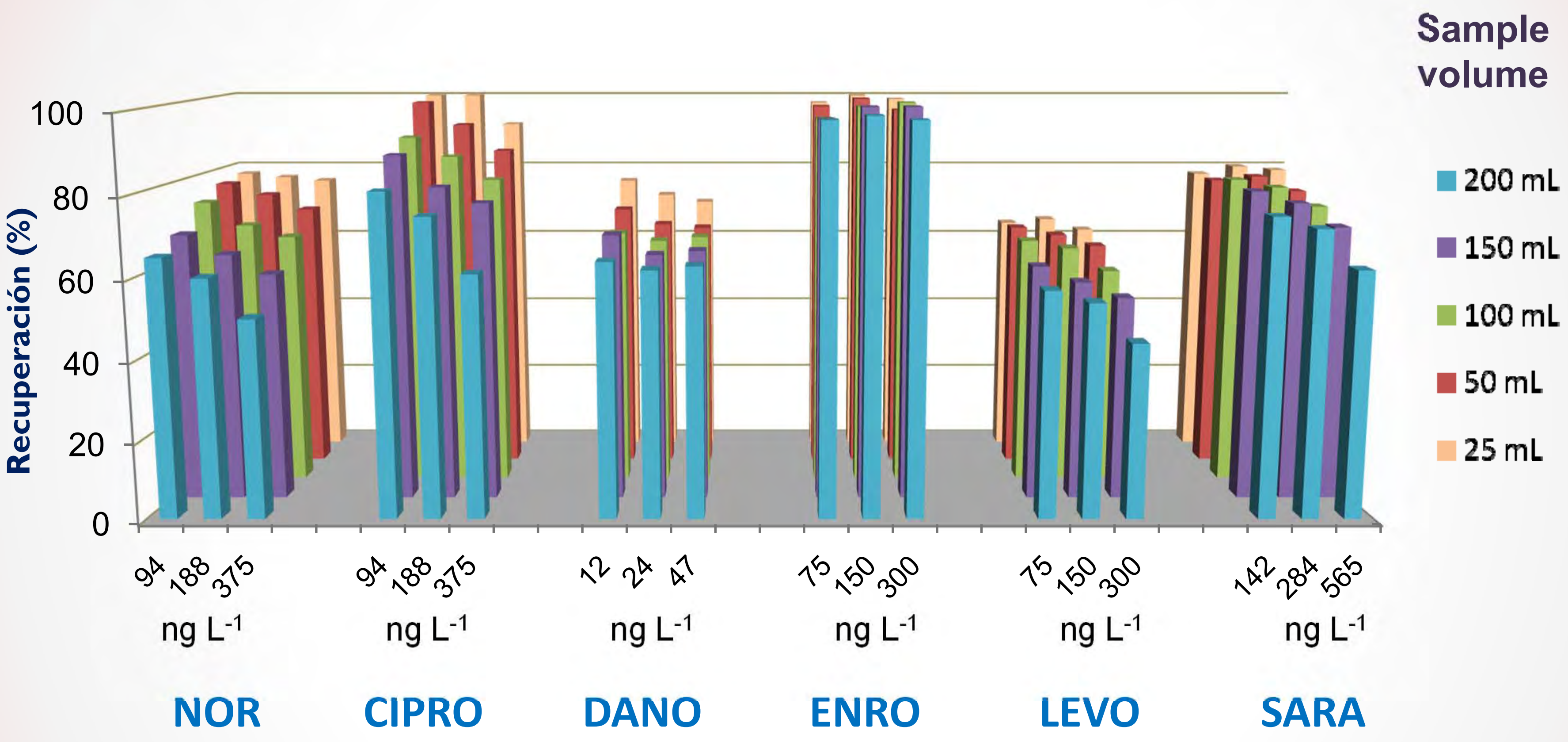
MISPE

Conditioning: 10 mL of HEPES (25 mM, pH 7.5)
Loading: sample in HEPES buffer (25 mM, pH 7.5)
Washing: ACN/HEPES (25 mM, pH 7.5) (25:75, *v/v*)
Flow rate: 1 mL min^{-1}

Elution: Back-flush with 2.5 mL H_2O/ACN (74:26, *v/v)* with 0.5% TFA
Flow rate: 0.5 mL min^{-1}

Chromatographic separation

Breakthrough volume



Washing: **5 mL** HEPES (25 mM, pH 7.5)/ACN (**75:25**, v/v, 1 mL min^{-1})

Elution: "Back-flush" with 2.5 mL H_2O/ACN (**74:26**, v/v) both with 0.5% TFA

Water analysis

25 mL water sample in HEPES buffer (25 mM, pH 7.5)

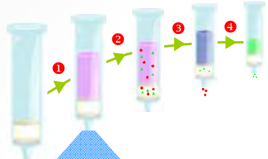
	Spiked level (ng L^{-1})	Drinking water			Fish farm water		
		Recovery (%)	RSD (%) (n = 4)	LOD (ng L^{-1})	Recovery (%)	RSD (%) (n = 4)	LOD (ng L^{-1})
NOR	**47; 94; 188**	95 - 97	2 - 4	**8**	97 - 100	3 - 4	**12**
CIPRO	**47; 94; 188**	95 - 97	3 - 4	**5**	94 - 95	1 - 4	**5**
DANO	**6; 12; 24**	93 - 101	2 - 3	**1**	91 - 94	2 - 4	**1**
ENRO	**38; 75; 150**	97 - 101	1 - 5	**3**	93 - 101	1 - 3	**4**
SARA	**71; 142; 284**	97 - 101	1 - 3	**11**	94 - 108	1 - 4	**8**
LEVO	**38 - 150**	97 - 102	3 - 4	**7**	94 - 102	3	**5**

Rodriguez et al. (2011) *Anal. Chem.* 3:2046
Benito-Peña et al. (2015) ACS Appl. Mat. Interf. 7:10966-76

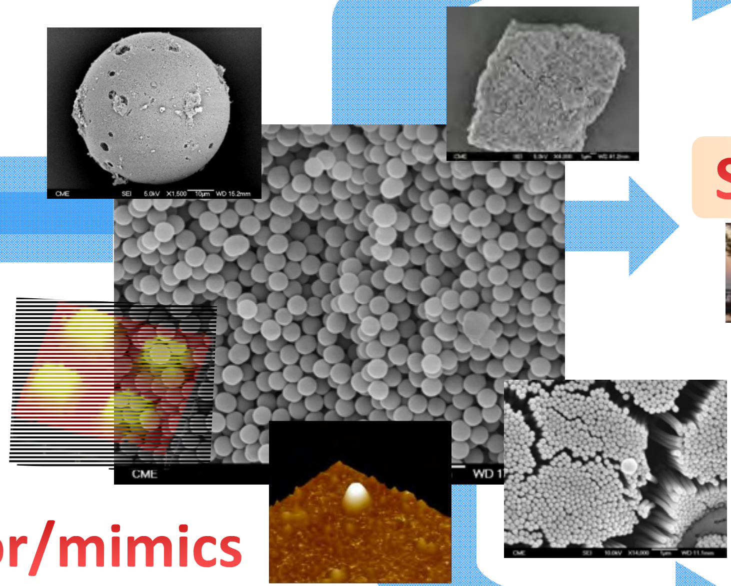
MIP applications

Analytical Separations

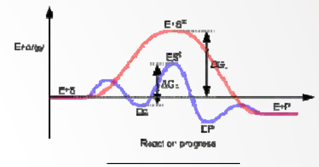
SPE



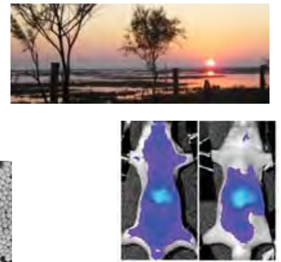
HPLC



Catalysis

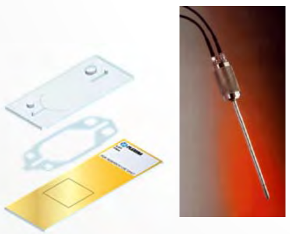


Selective removal



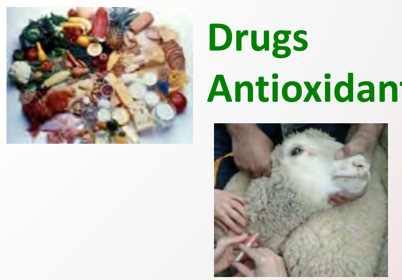
Contaminants
Drugs
Toxins…

Sensors
Antibody receptor/mimics



Optical
Electrochemical
Piezoelectrical…

Controlled release



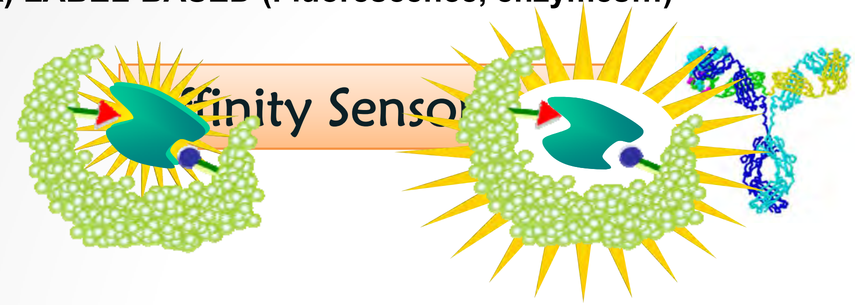
Drugs
Antioxidants…

Biomimetic sensors

Direct assays

Immuno-like sensor

A) LABEL-BASED (Fluorescence, enzymes…)



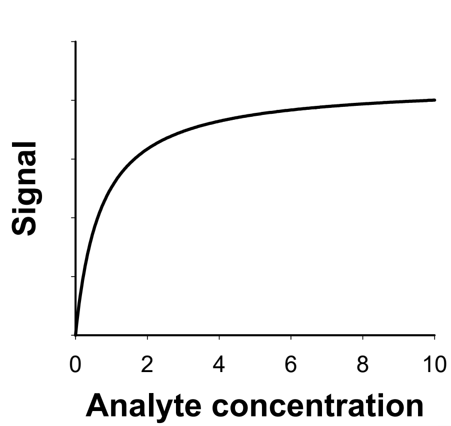
finity Senso

LABELLED … MIP

Catalytic Sensors

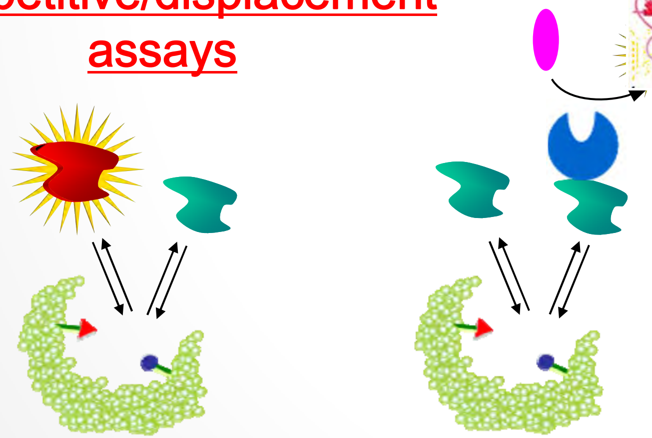
B) LABE…)

Signal response



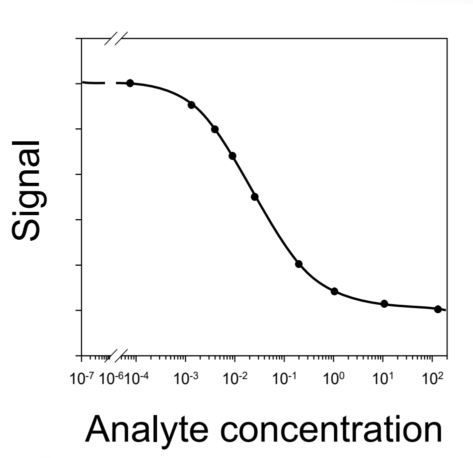
Competitive/displacement assays

Immuno-like sensor



TARGET + LABELLED TARGET ANALOGUE

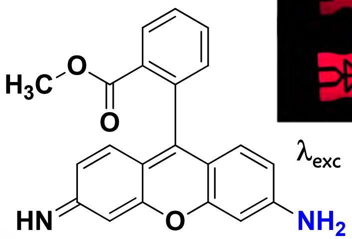
Signal response

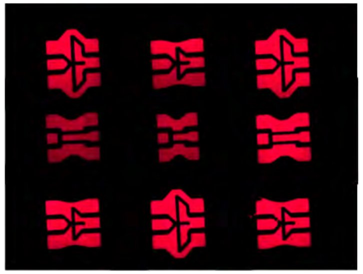


2 MIP nanopatterns on silicon substrates prepared by electron beam lithography (EBL) direct writing

S. Carrasco, V. Canalejas-Tejero, F. Navarro-Villoslada, C. A. Barrios, M. C. Moreno-Bondi

J. Mater. Chem. C, 2, **2014**, 1400
Spanish Patent: P201330947

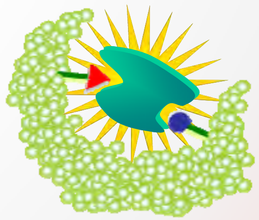




λ_{exc} = 508 nm (methanol)

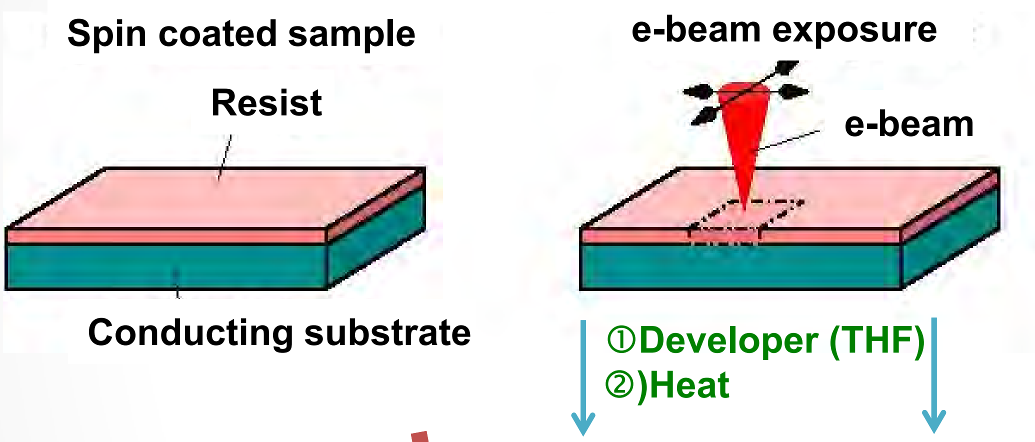
MODEL TARGET

RHODAMINE 123

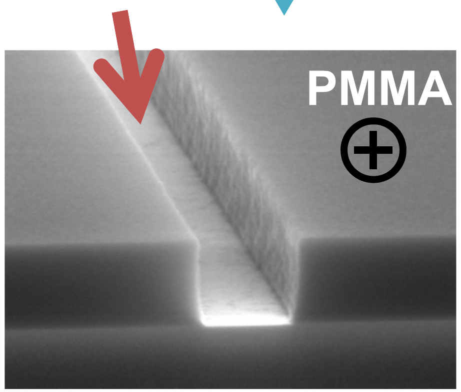


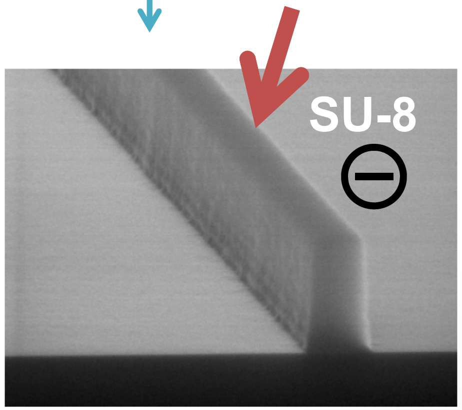
MIP nanopatterning

- **EBL** can generate patterns with **nanometer** resolution without the need for **moulds** or contact masks, **avoiding contamination** of the surface to be patterned



Positive
tone resist
(i.e. **irradiated** regions are **removed** after development)





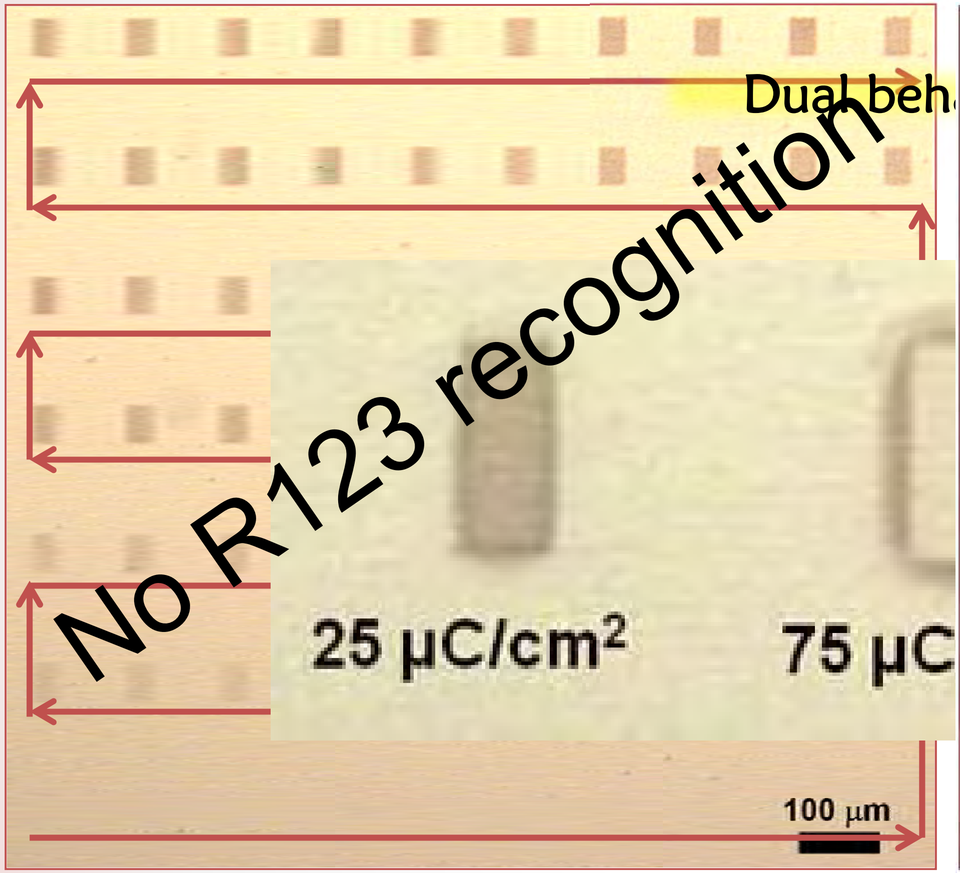
Negative
tone resist
(i.e. **irradiated** regions **remain** after development)

MIP synthesis: electron beam lithography (EBL)

Negative-tone resist → Positive-tone resist

For e^- doses D < 100 µC/cm^2

For e^- doses D > 100 µC/cm^2

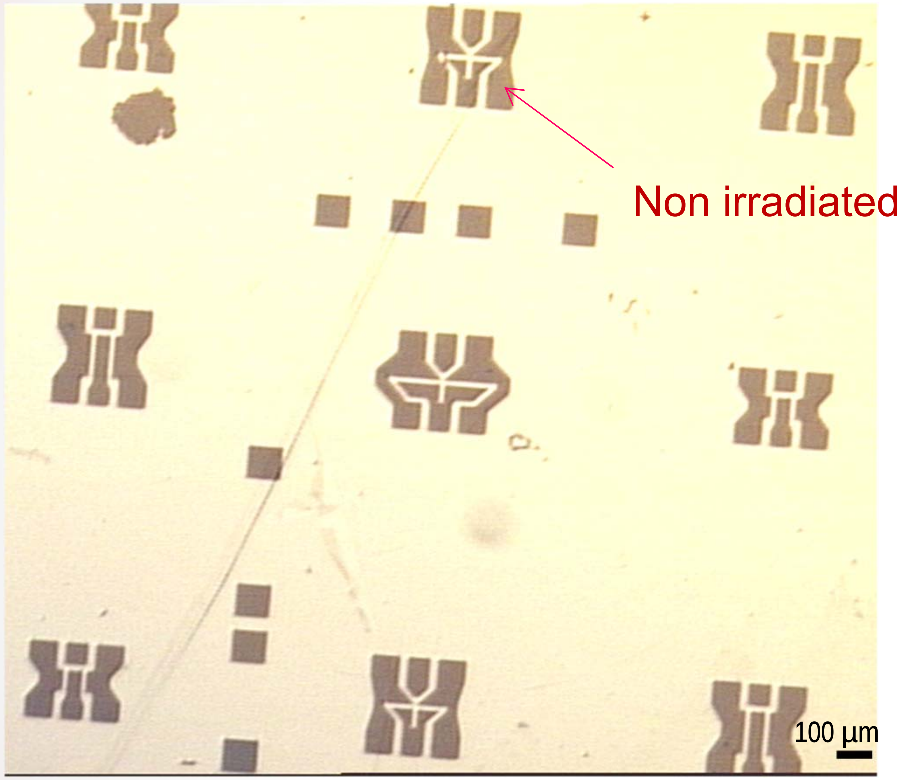


(60 x 40 µm) Acceleration voltage: 50 keV.
Electron beam current: 10 pA

(0.3 x 0.1 µm) 750 µC/cm^2
Developer: THF 1 min
MIP polymerization: Heat, 30 min

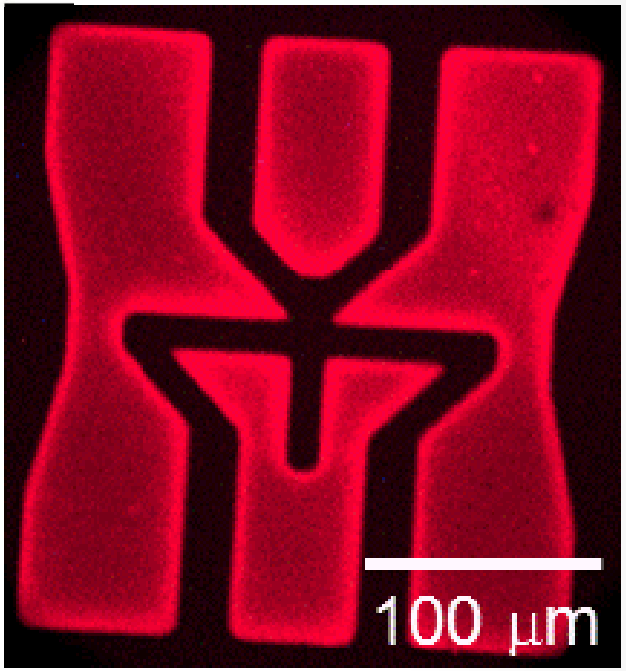
MIP behaviour

Positive-tone resist: DUV photolitography



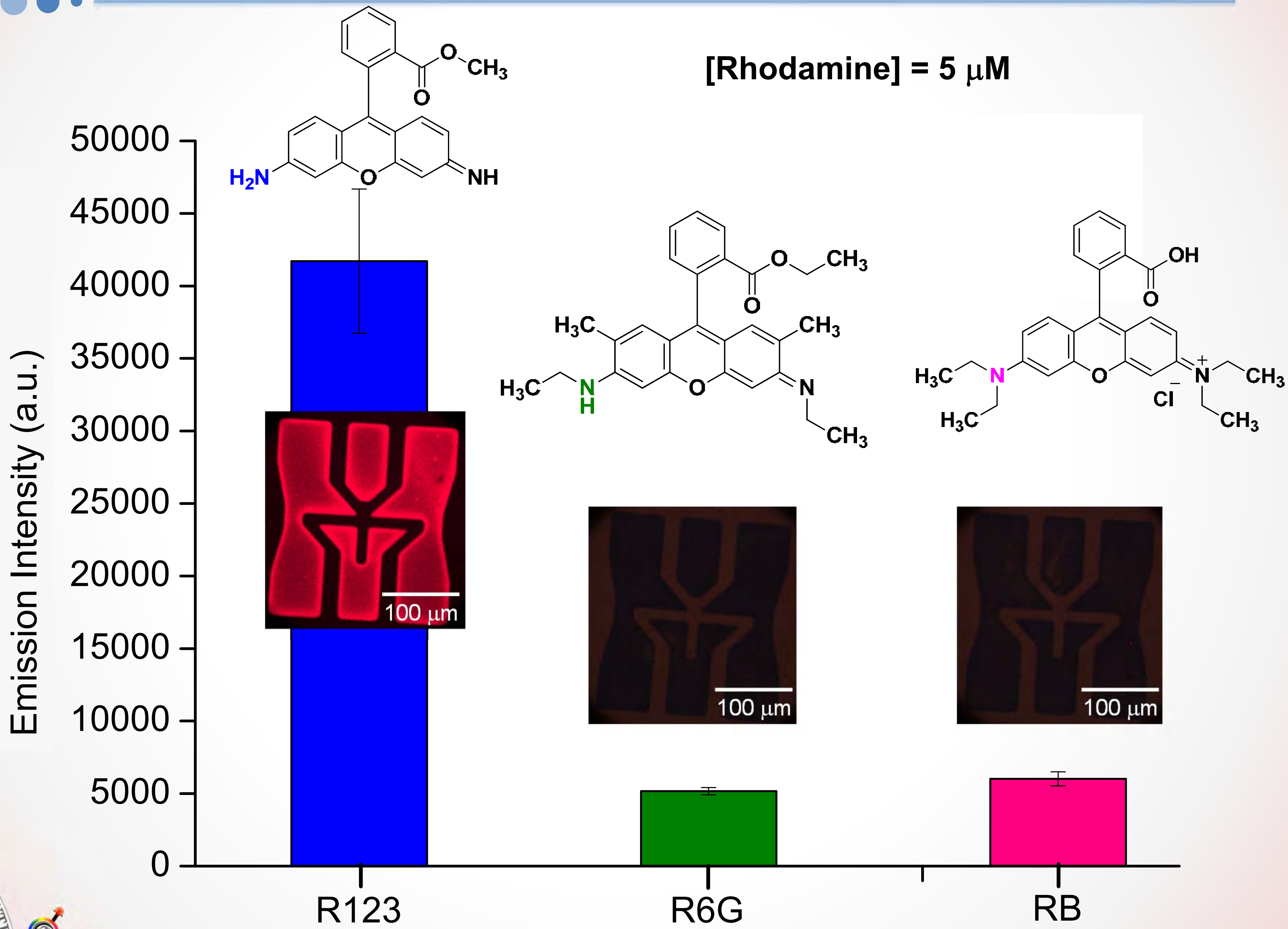
Photolithography: λ = 255 nm, 650 W, 30 min
Developer: THF 1 min
MIP polymerization: Heat, 30 min

[R123] = 5 µM



Fluorescence microcopy image
Excitation path: 488 nm interference filter.
Emission path: 600 nm dichroic mirror, 590 nm cut-off filter

Analytical characterization



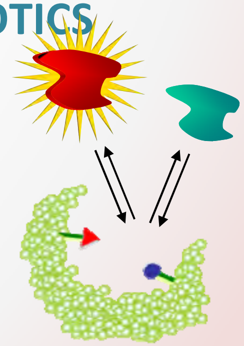
3 Fiber optic microarray platforms by random self-assembly of MIP beads into a fiber optic microwell array

S. Carrasco, E. Benito-Peña, D. Walt, M.C. Moreno-Bondi
Chem. Sci., 6, **2015**, 3139

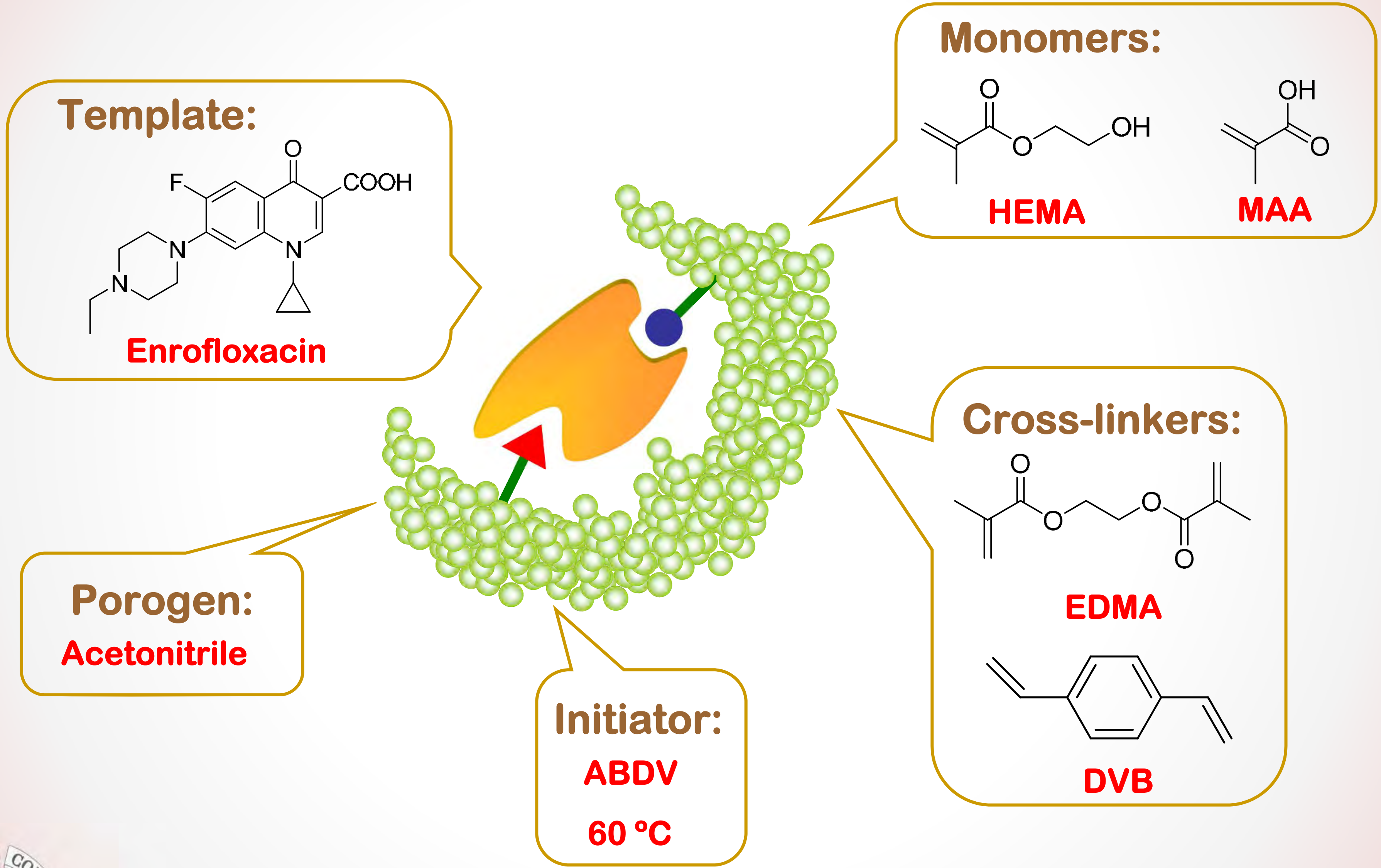
THERAPEUTIC AND PROPHYLACTIC AGENTS

TARGET

FLUOROQUINOLONE ANTIBIOTICS



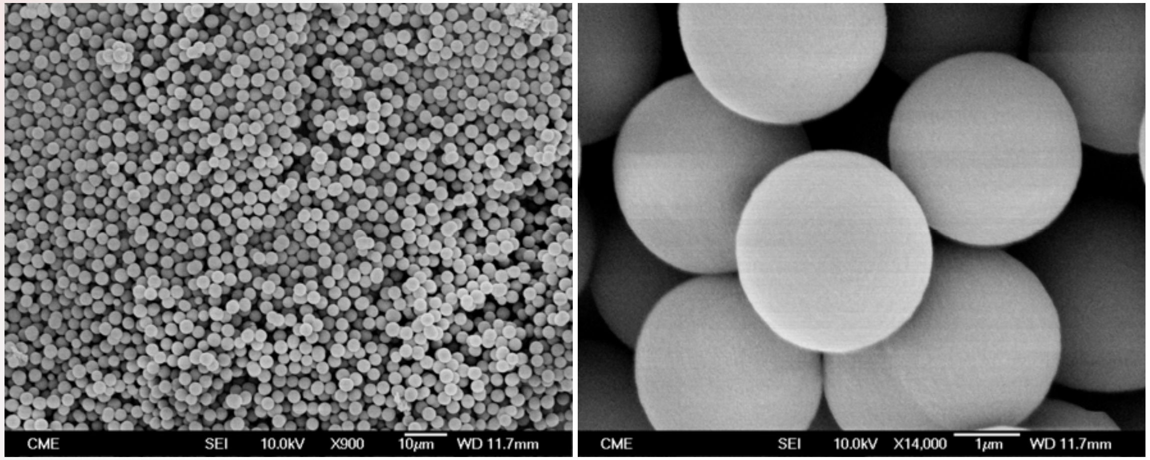
Synthesis of enrofloxacin imprinted microspheres



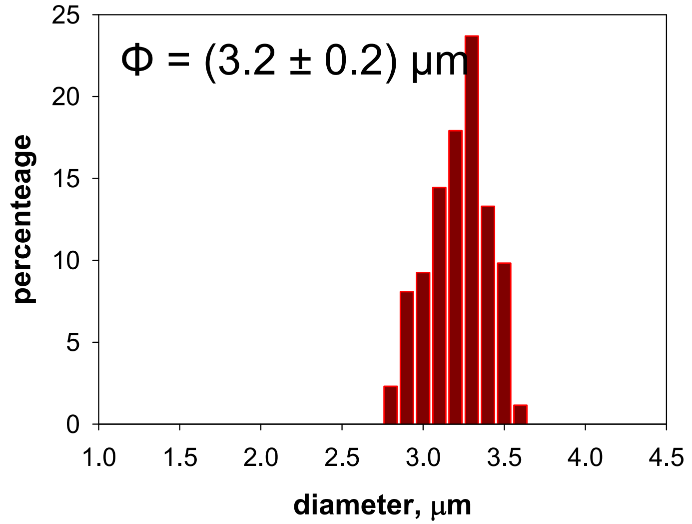
E. Rodríguez et al., *Anal. Chem*. 83, **2011**, 2046
Benito-Peña et al. Anal. Bioanal. Chem., 393, **2009**, 235

Polymer characterization

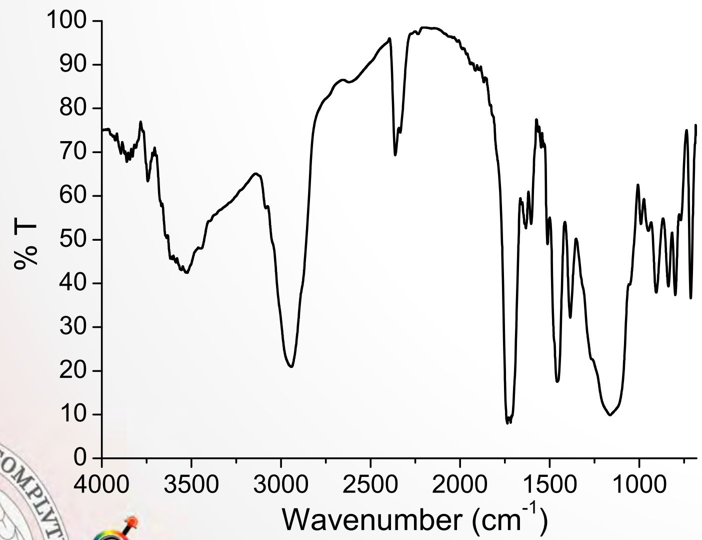
SEM



Particle size



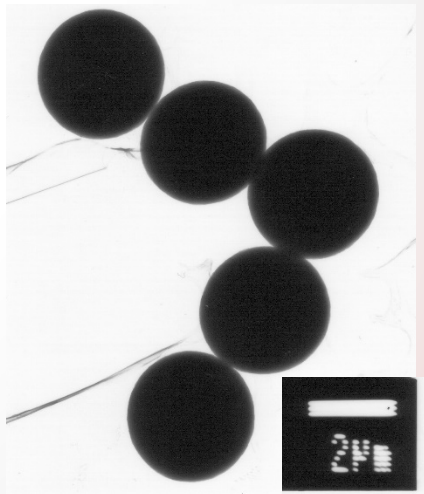
FTIR



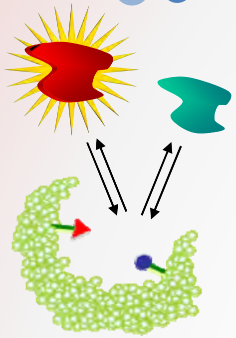
N_2 Porosimetry

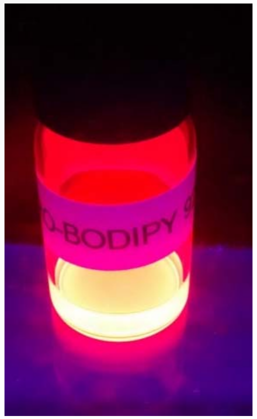
BET Surface Area
1.2 m²/g

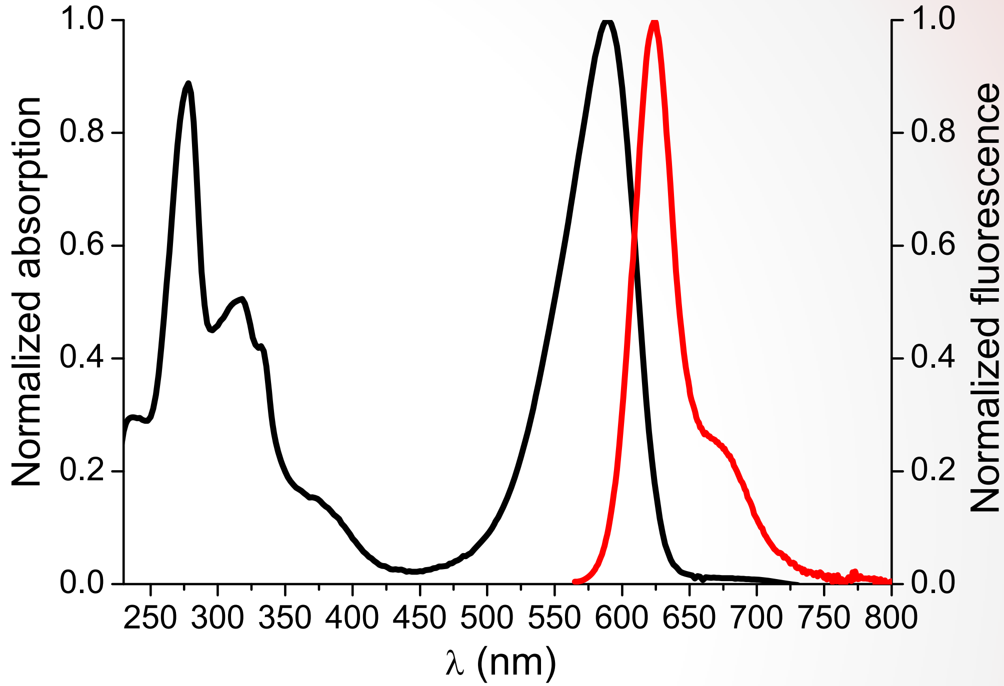
TEM

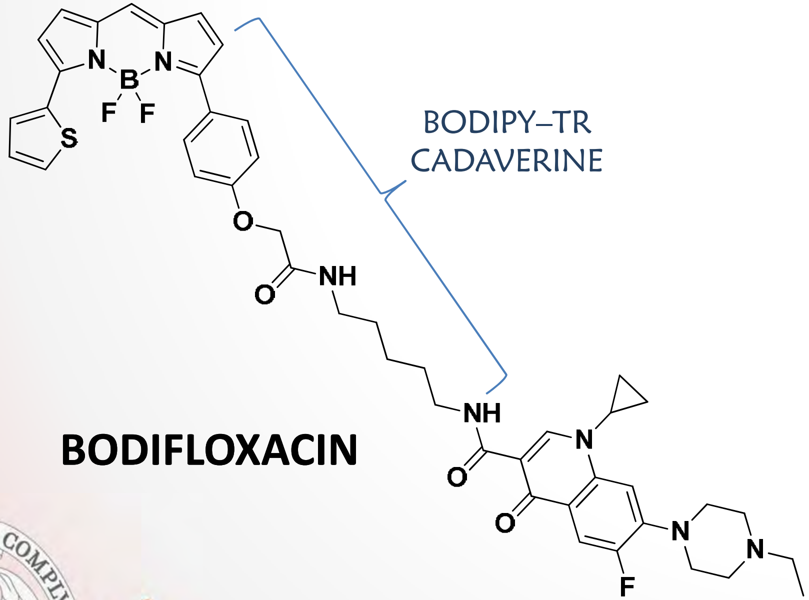


Characterization of Bodipy-labelled ENROFLOXACIN









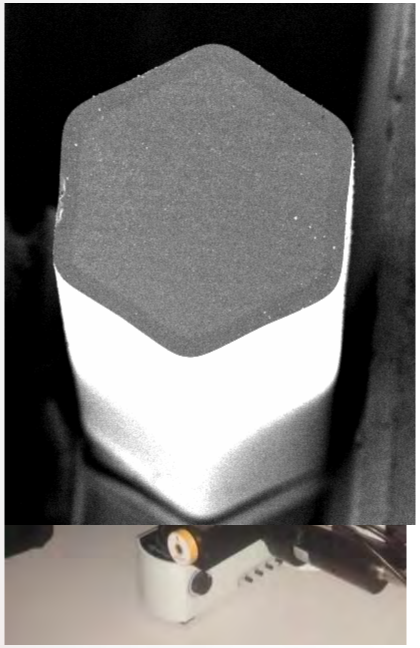
BODIFLOXACIN

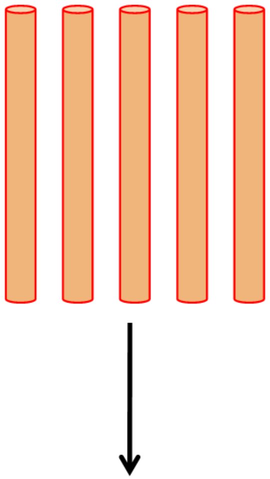
Parameter	Value (in MeCN)
λ_{abs}^{max}	588 nm
λ_{em}^{max}	624 nm
Φ_f	0.73 ± 0.02
ε (λ = 588 nm)	(12750 ± 130) $M^{-1}\,cm^{-1}$

Bead-based microarrays using fiber optic bundles

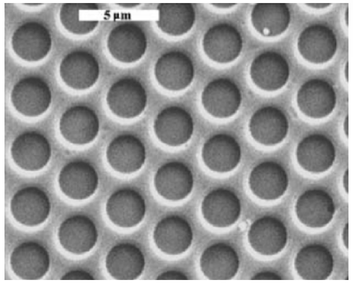
MIP/NIP beads **randomly loaded** into the etched wells of an optical fiber bundle

Epi-fluorescence microscopy with CCD

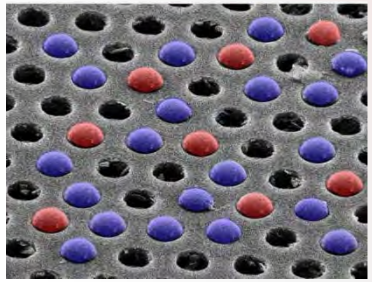




50.000 individually clad waveguides

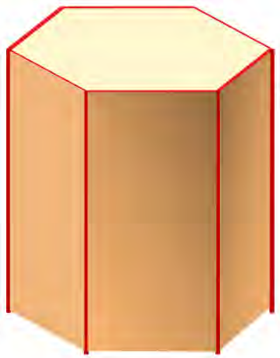


Etched wells
$\phi = 3.1\ \mu m$



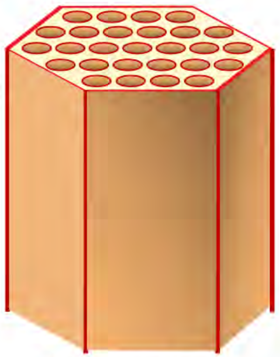
Beads in wells

Silica Jacket



Polished Fiber

Selectively etch fiber core



Fiber-Optic microwell array

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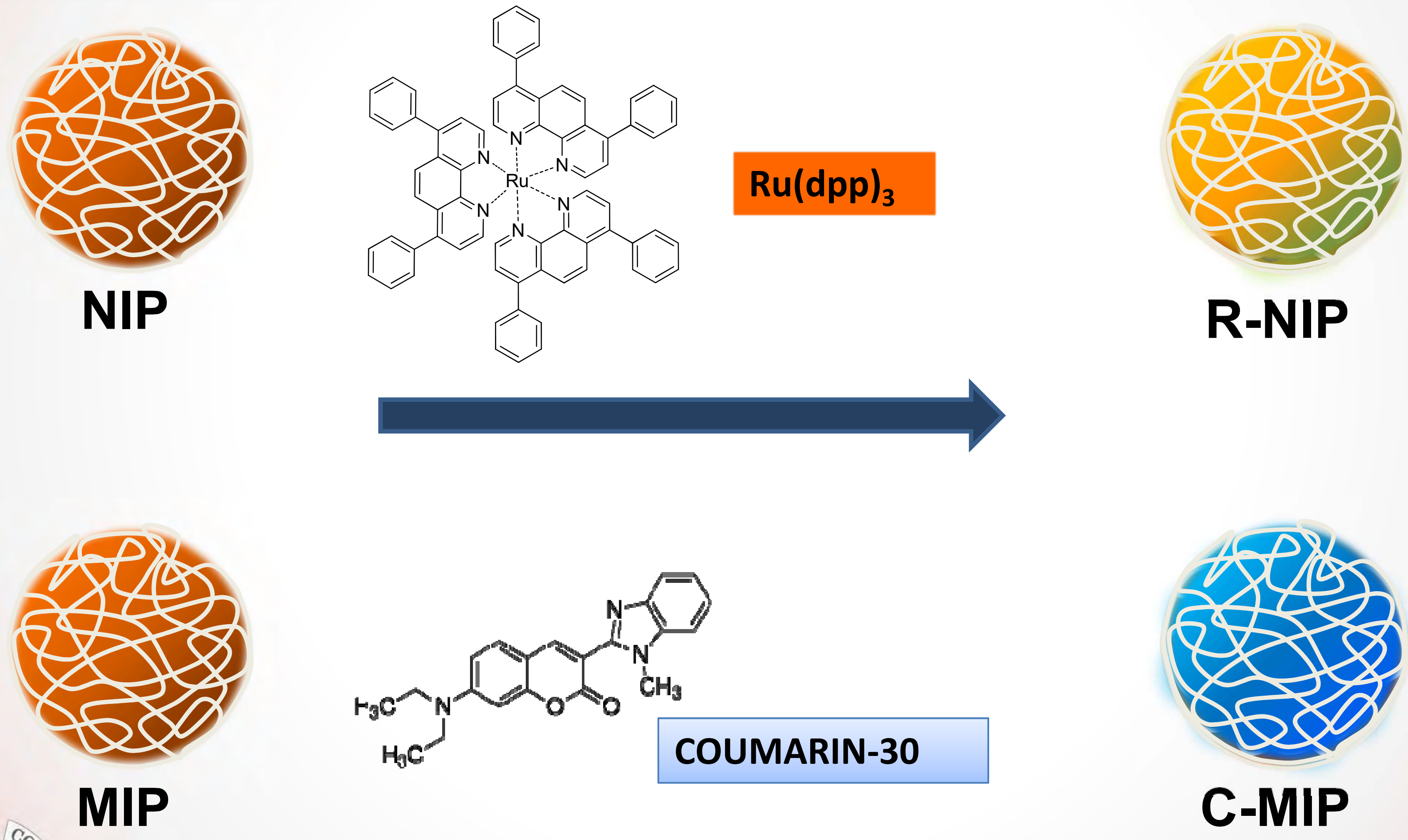
P. Pantano et al., *Chem. Mater.* (1996), 8, 283
J.R. Epstein et al., *Chem. Soc. Rev.* (2003), 32, 203

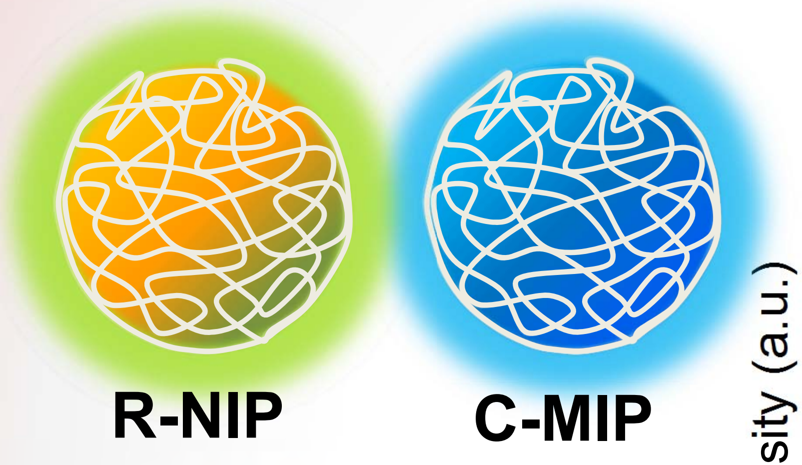
Microsphere encoding

Simultaneous monitoring of MIP/NIP microspheres

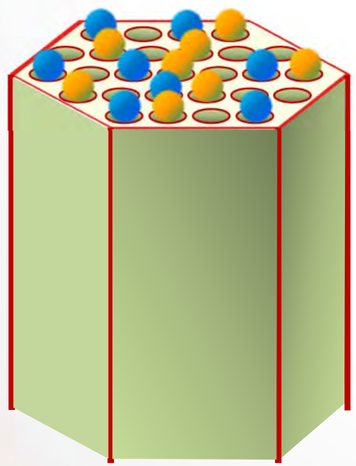


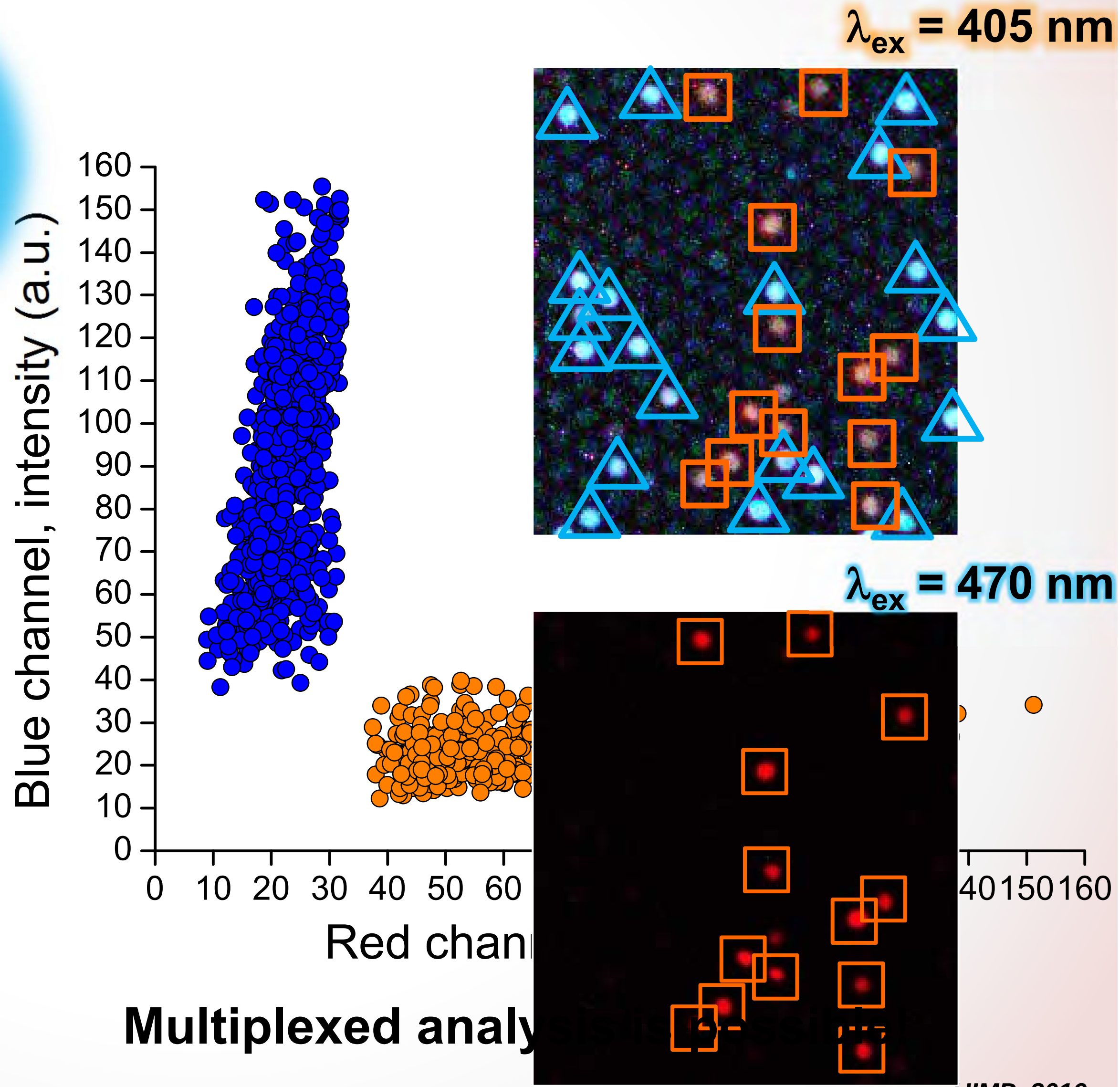
Microsphere encoding

Image acquisition



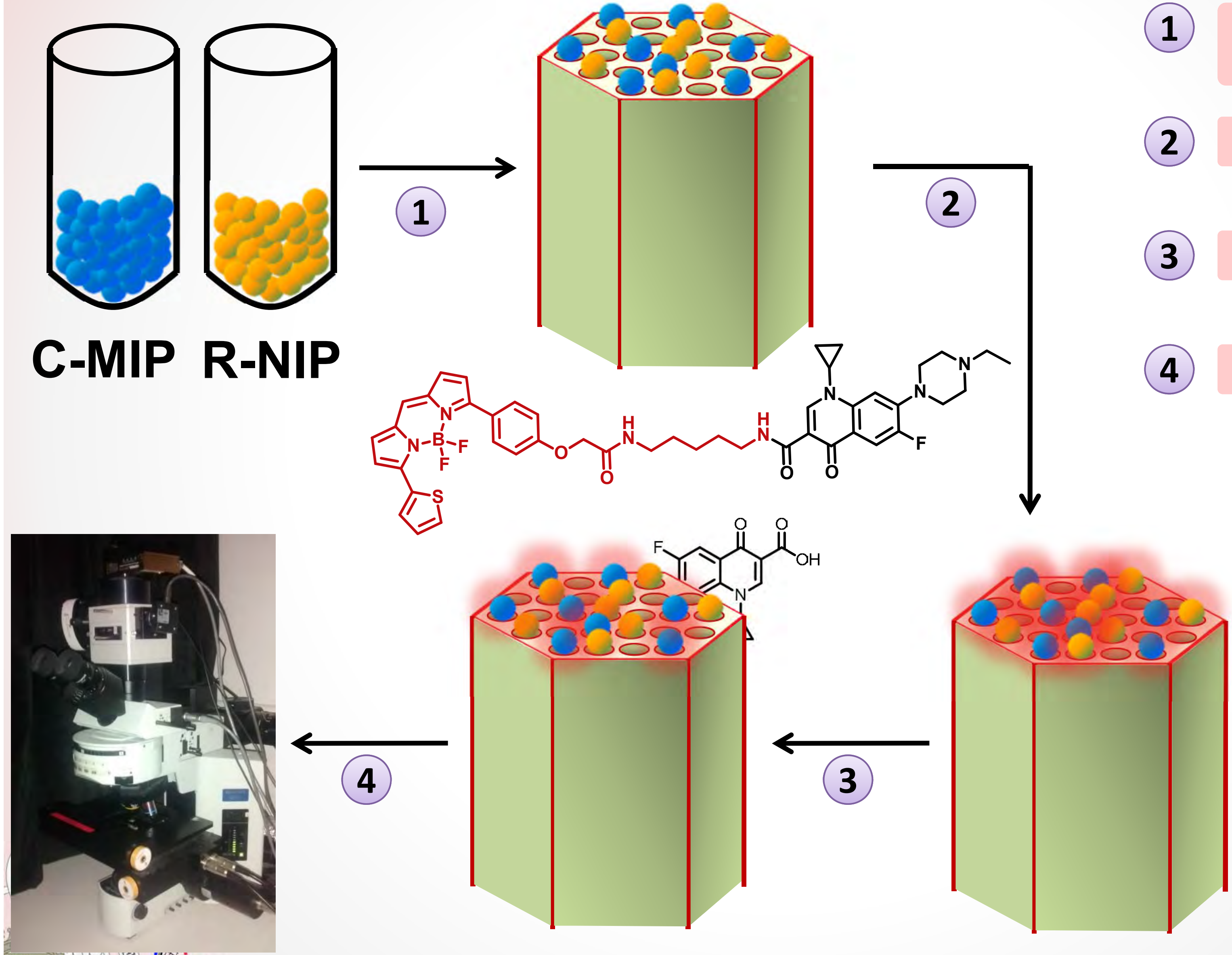
λ_{ex} = 405 nm





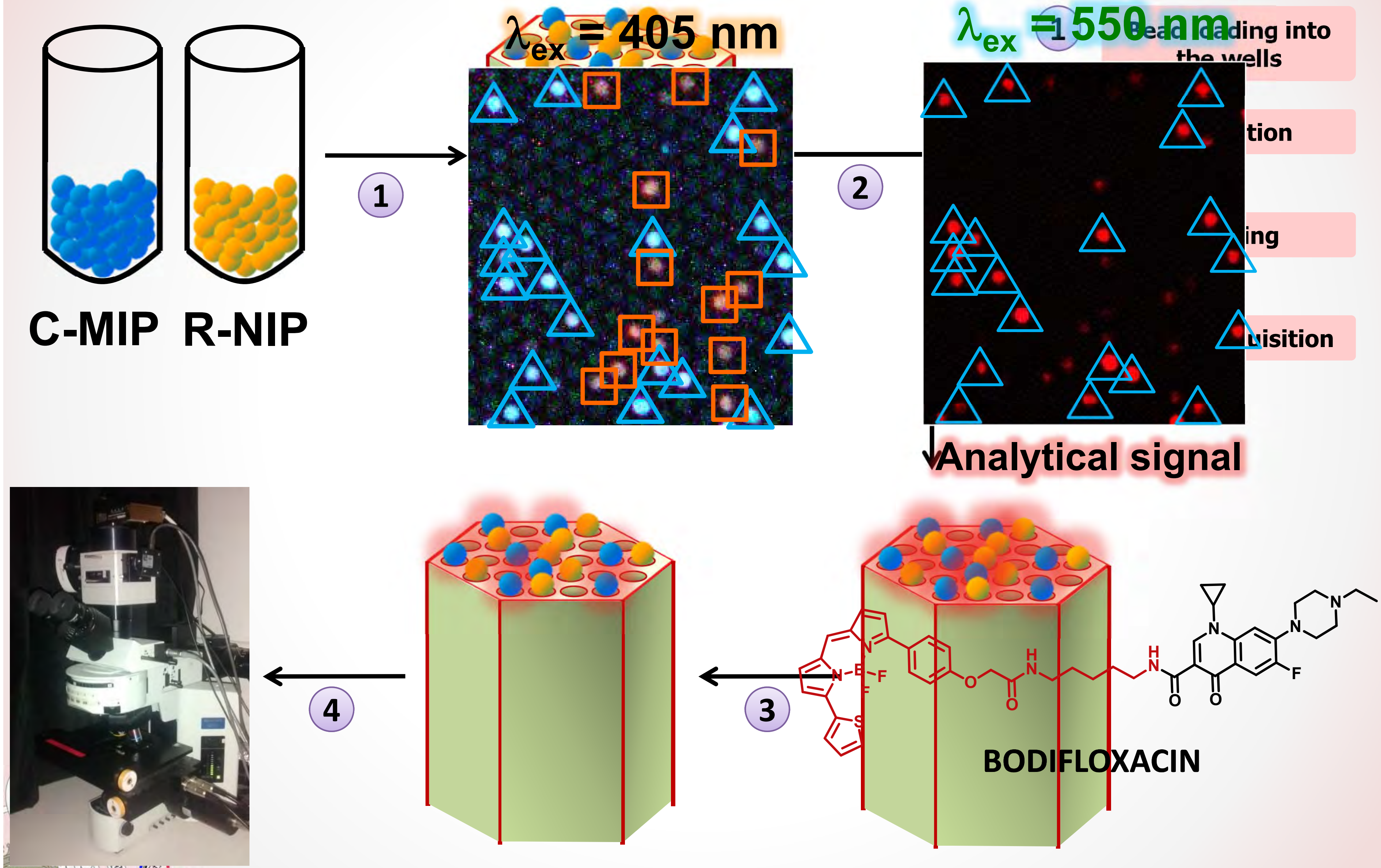
Multiplexed analy

Assay protocol

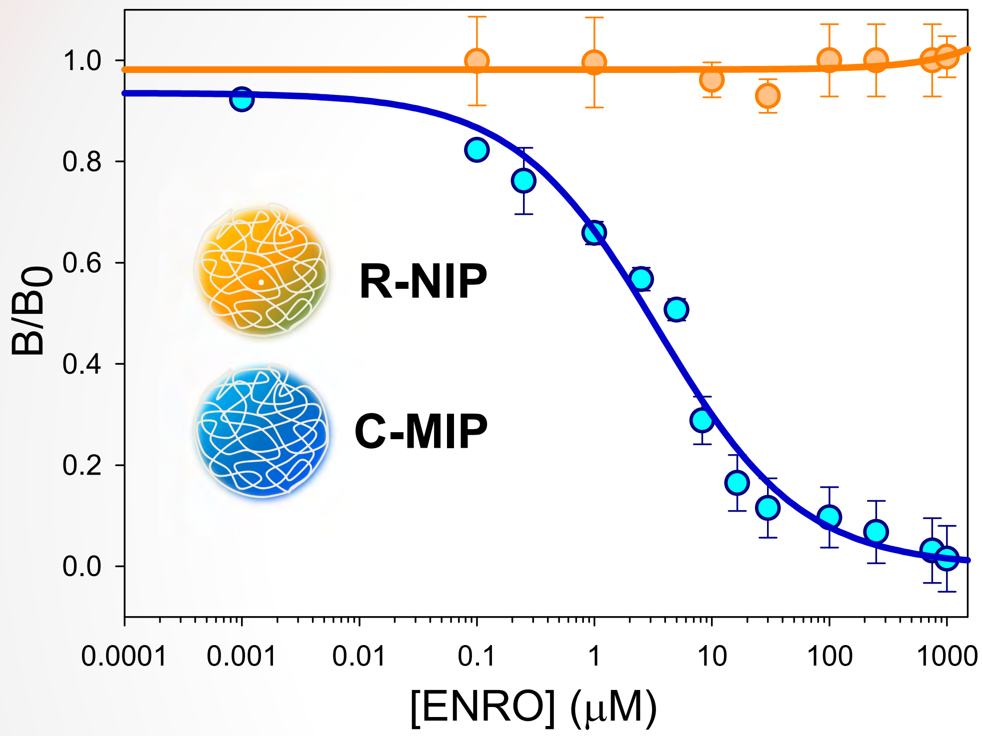


1. Beads loaded into the wells
2. Incubation
3. Washing
4. Image acquisition

Assay protocol



Analytical characterization



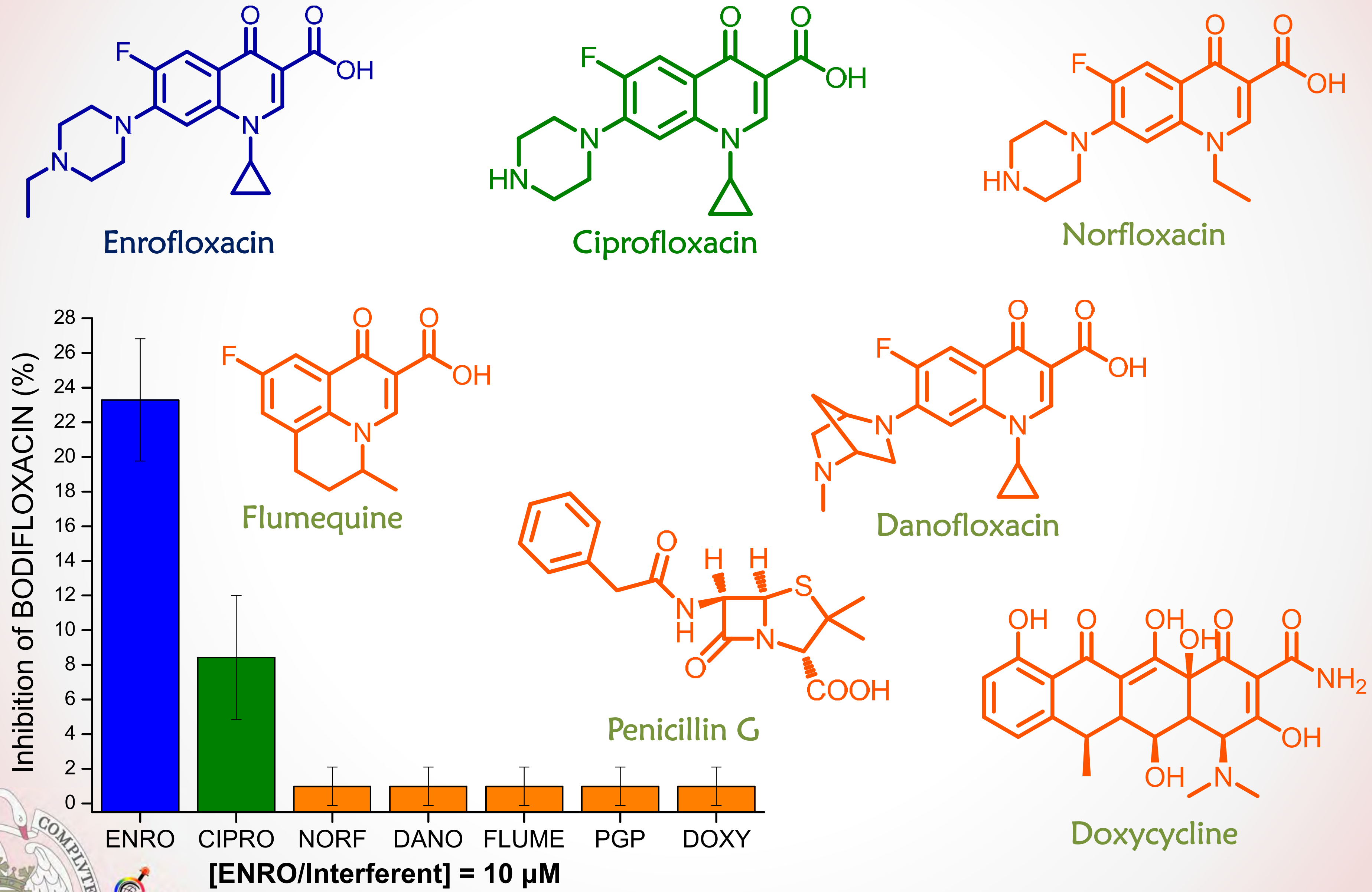
[microspheres] = 4 µg/mL; Incubation 60 min
Solvent MeCN:HEPES (50:50, *v/v*)

$$Normalized\ signal\ (Y) = \frac{B_{max} - B_{min}}{1 + \left(\frac{[ENRO]}{EC_{50}}\right)^{b}} + B_{min}$$

Parameter	Value (µM)
LOD (10%)	0.04
LOQ (20%)	0.29
DR (20-80%)	0.29 - 21.54
EC50 (50%)	3.48

S. Carrasco et al., *Chem. Sci.*, 6, **2015**, 3139

Analytical characterization

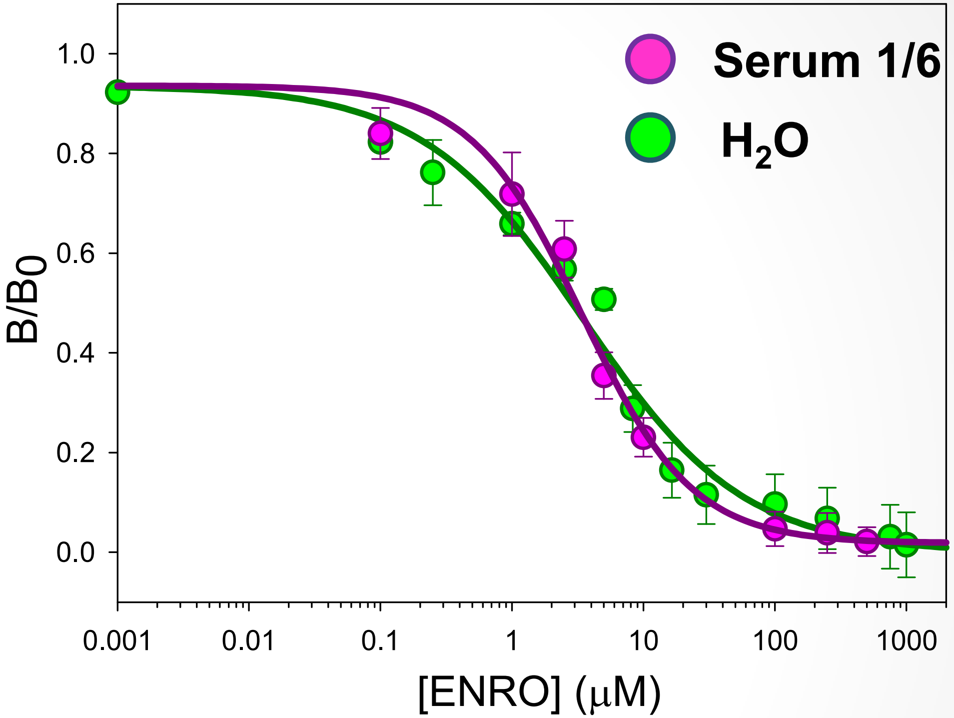


Sample analysis

- Intravenous administration: Baytril (Bayer, 10% ENRO)
- Lactating sheep of ~70 Kg
- Blood extraction: 5 min after administration

✓ No matrix effect

✓ Sample treatment with MeCN precipitates proteins

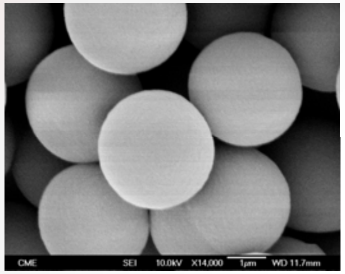


	HPLC-FLD (μM)	Fiber-Optic microarray (μM)
Sample	19.9 ± 0.2	20 ± 5

± ts/√n (Confidence limit 95%)

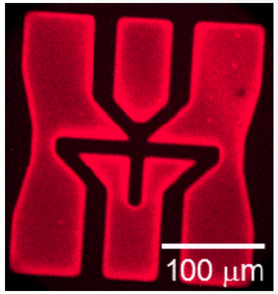
A. Rahal et al., *J. Vet. Pharmacol. Therap*. (2006), 29, 321

Summary

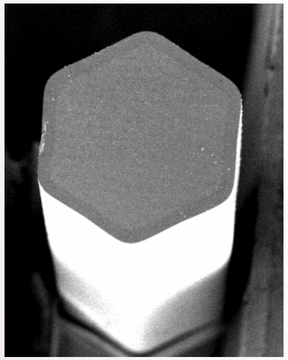


MIP microbeads have excellent performance as SPE sorbents

MIP micro/nanostructuring improves the binding kinetics

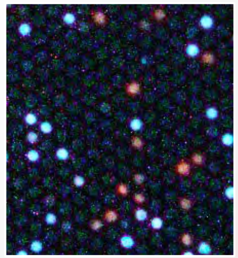


Potential for preparation of cost-effective MIP chips on planar substrates (e.g. Si wafers) by using mass-production microfabrication techniques



The use of image fiber optic bundles allows preparation of MIP microarrays and facilitates coupling to the transducer

MIP encoding is a feasible alternative for multiplexed detection



Acknowledgements

U. Dortmund

Prof. Sellergren

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Prof. D. Walt

ISOM

POLITÉCNICA

Prof. C.A. Barrios

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