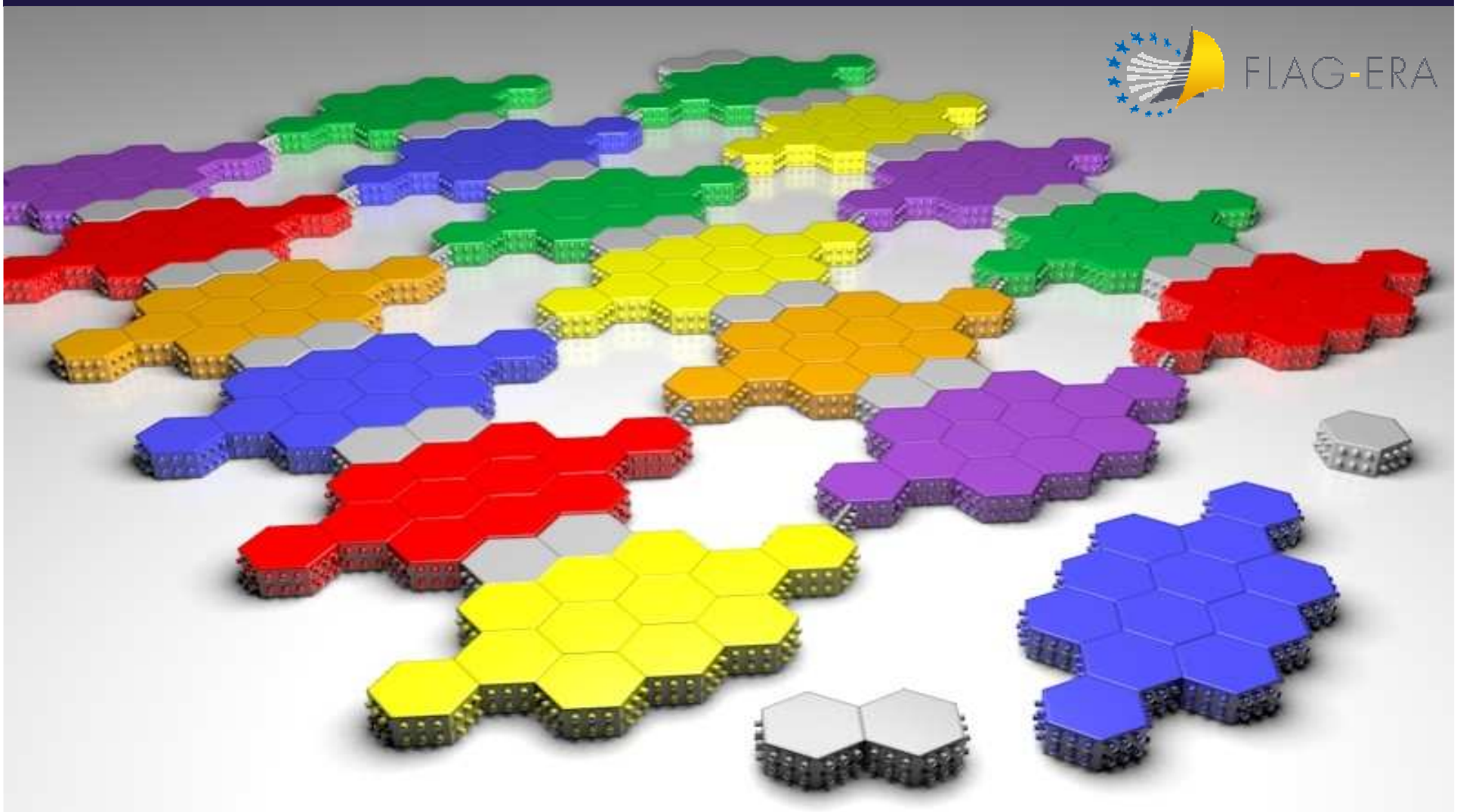


Dr. César Moreno (PI-Coord.)

Universidad de Cantabria, Santander | Spain

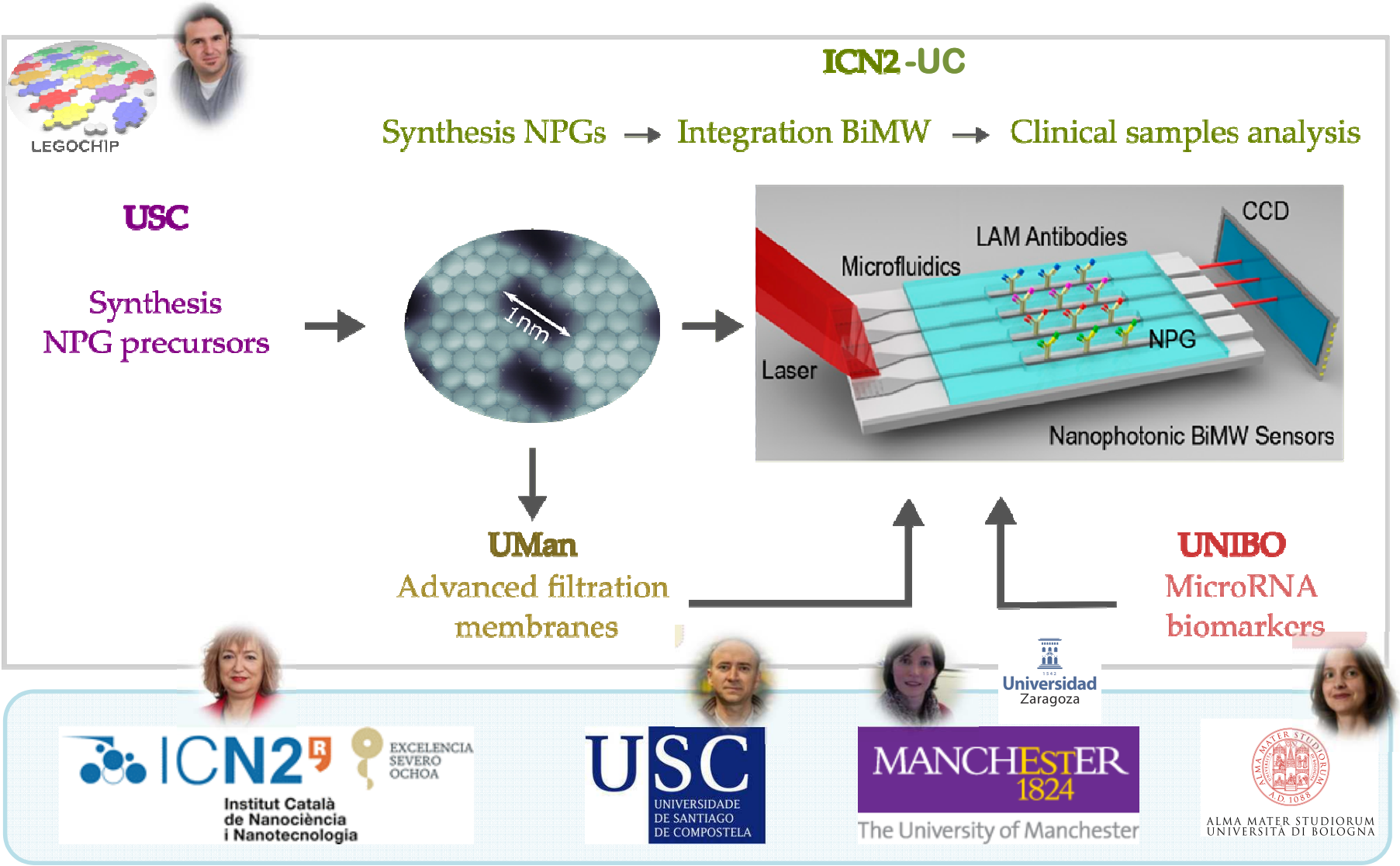
Catalan Institute of Nanoscience and Nanotechnology (ICN2), Barcelona |Spain

FLAG-ERA 2022 Project Workshop

21-22 March 2022

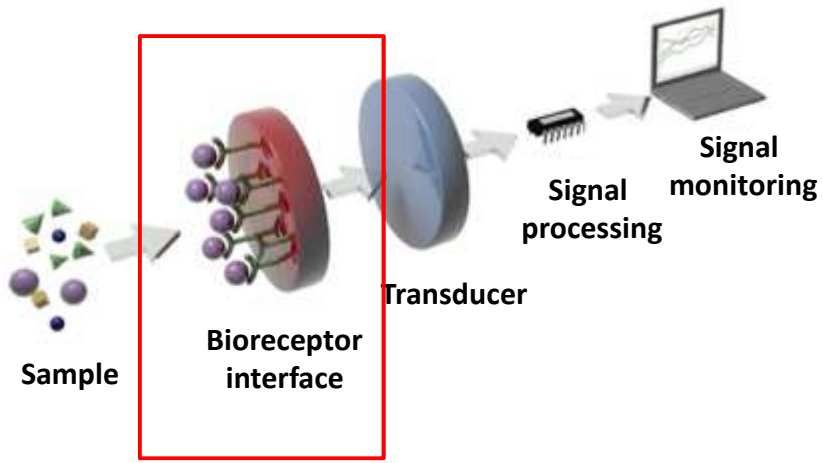
LEGOCHIP:

Multifunctional Nanoporous Graphene Integration in Operational Nanophotonic Biosensor Devices



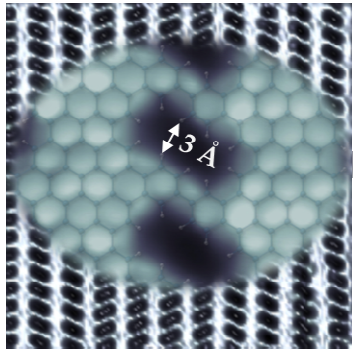
The aim

Surface Biofunctionalization

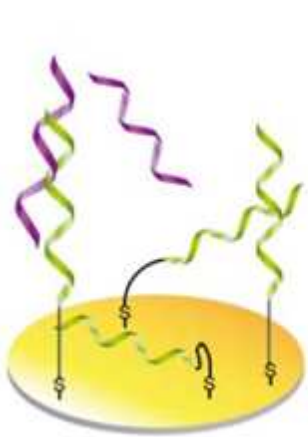


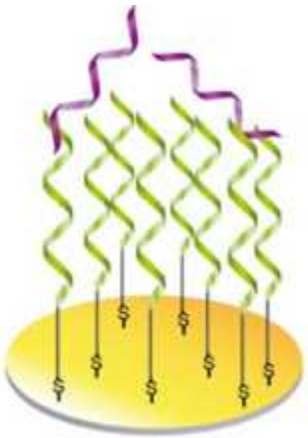
Key factors

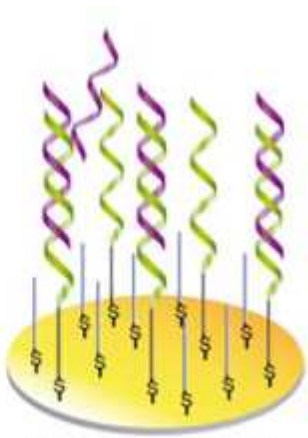
- Stable attachment
- Density control
- Orientation control
- Reproducibility
- Anti-fouling



Importance of Density Control


Non-specific adsorption


Steric Hindrance


Optimum accessibility

Strategy

DNA hybridization

DNA inmobilization

Amine-terminated groups

Amino groups (NH2)

Hydroxil-terminated groups

Silanol groups (SiOH)

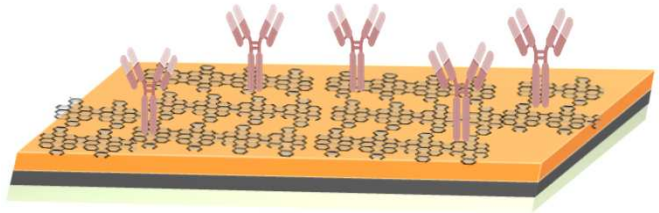
Silicon-based sensor

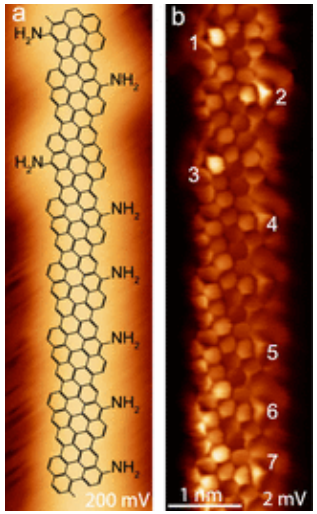
Key factors

- Stable attachment
- Density control
- Orientation control
- Reproducibility
- Anti-fouling

LEGOCHIP approach

Graphene nanoarchitectures as precise template for controlled biofunctionalization





Silicon-based sensor

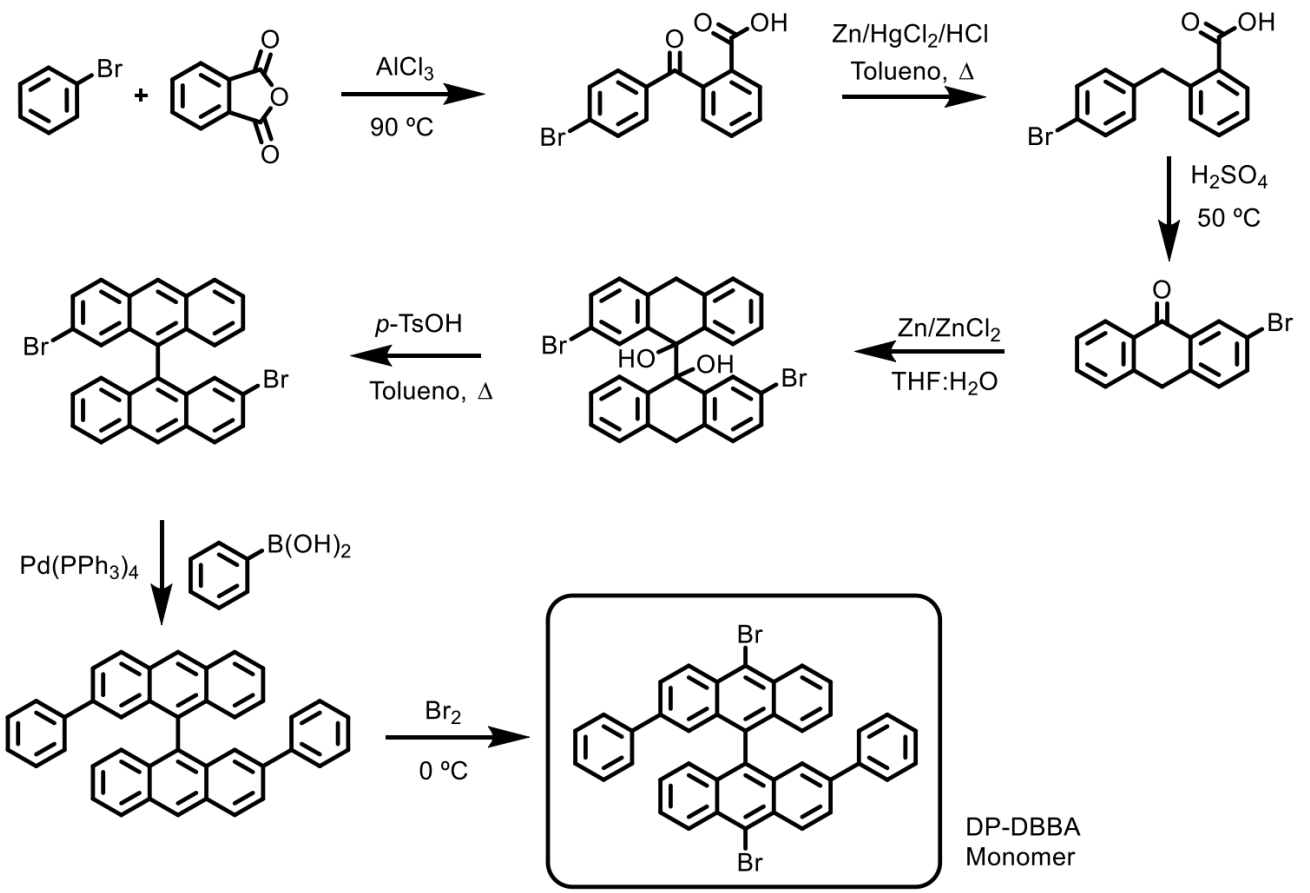
1. **Synthesis of graphene nanoarchitectures**
   - Intrinsic functionalization
   - Strategies to improve order

2. **Integration of graphene nanoarchitectures into biosensors**
   - NPG/GNR transfer to BiMW sensors
     - Effect on light transmission
     - Effect on sensing performance
   - Graphene biofunctionalization
     - Selection of crosslinkers
     - DNA immobilization optimization
   - microRNA detection

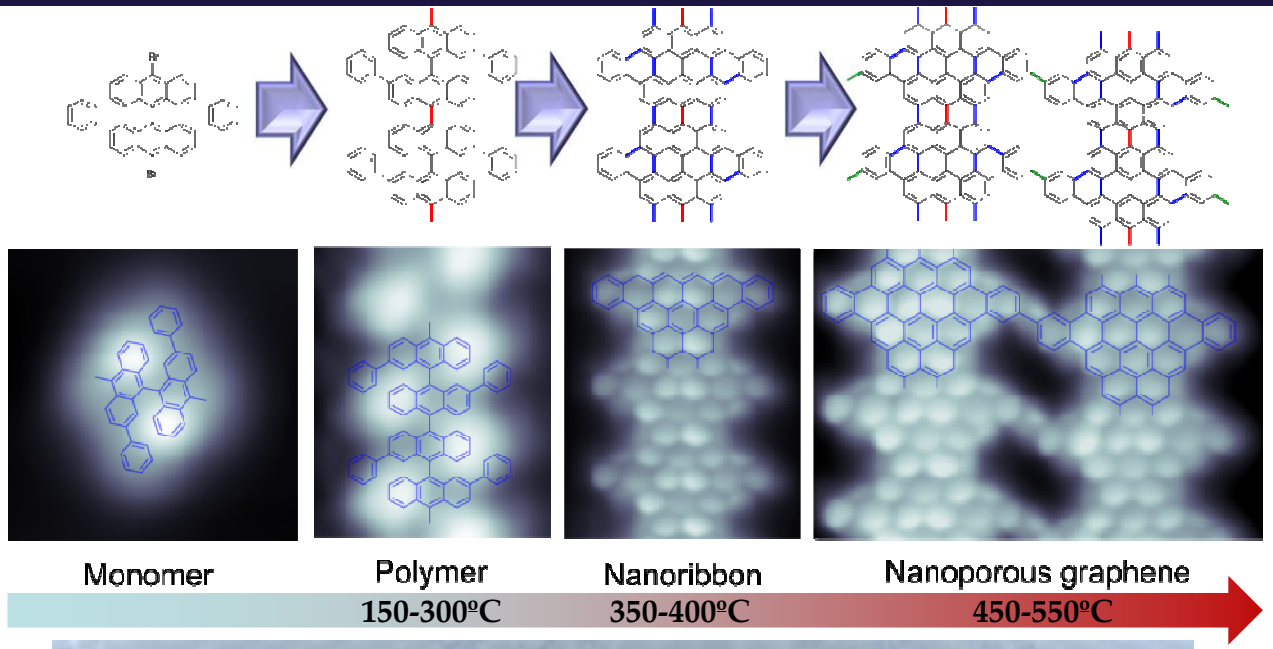
In-solution synthesis

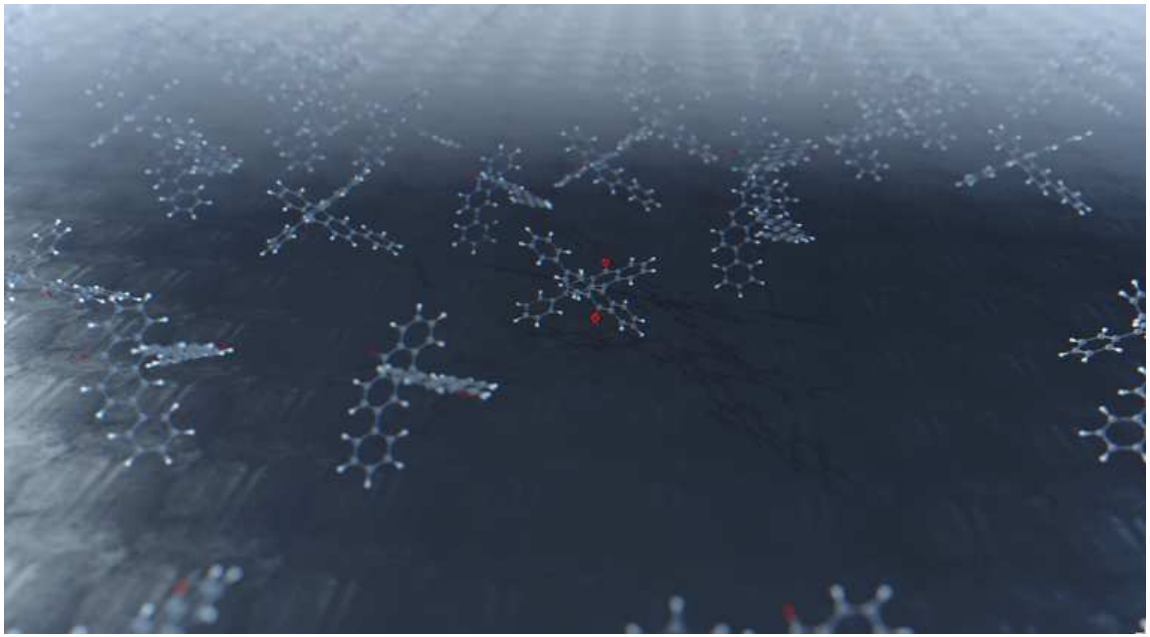
Diego Peña
(USC-CiQUS)

in-solution synthesis



On-surface synthesis





C. Moreno et al.
Science, 360-6385
(**2018**)

On-surface synthesis

LEGOCHIP
Nanoporous Graphene Integration in Operational Nanophotonic Biosensors

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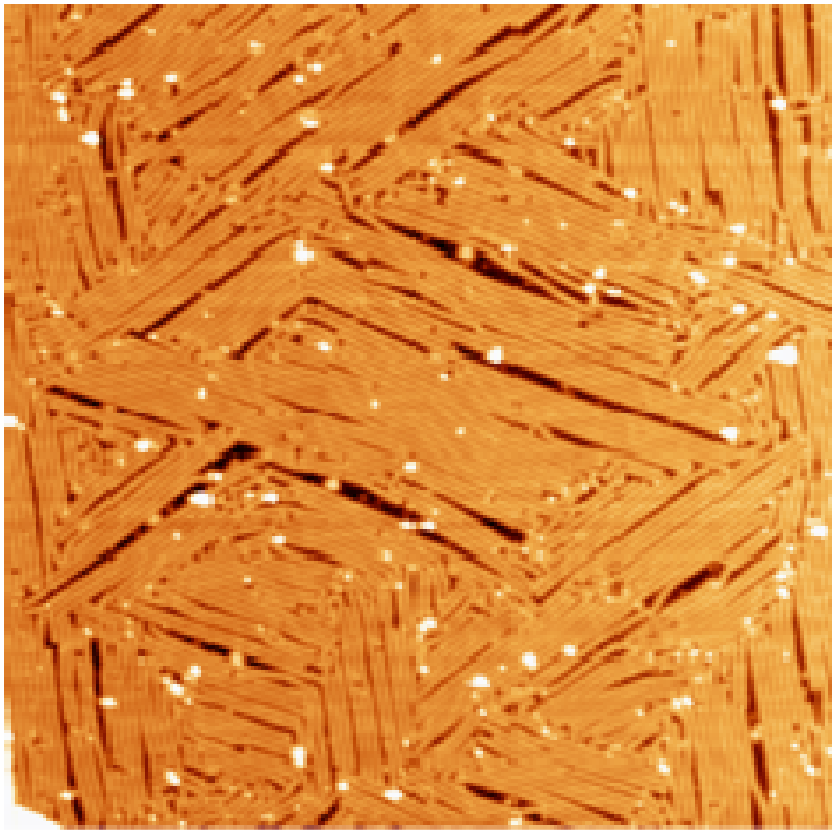
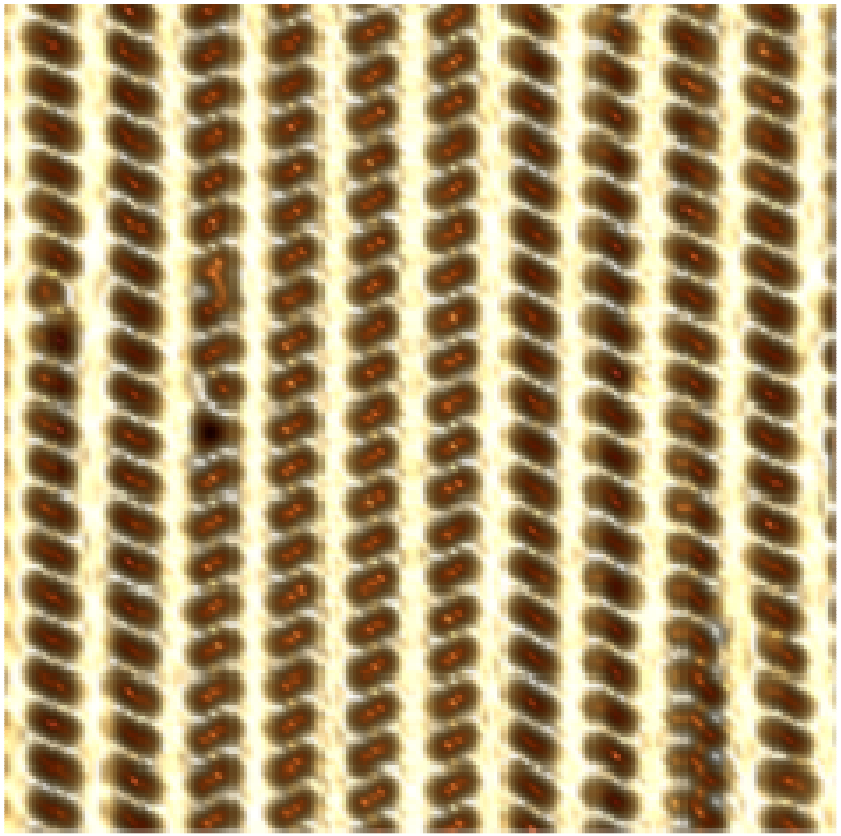
Density of 0.5 10^6 pore/μm^2





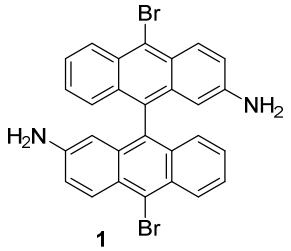
C. Moreno et al.
Science, 360-6385
(**2018**)

Chemical functionalization for DNA anchoring

LEGOCHIP
Nanoporous Graphene Integration in Operational Nanophotonic Biosensors

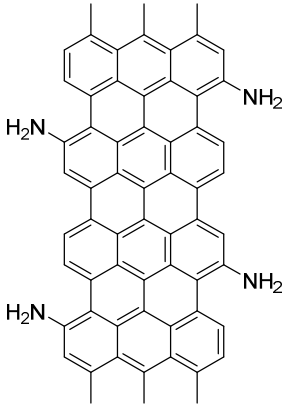
ICN2

1



1

GNR-1



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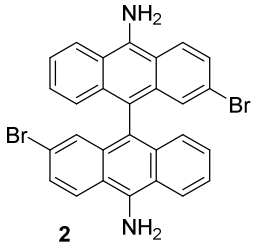
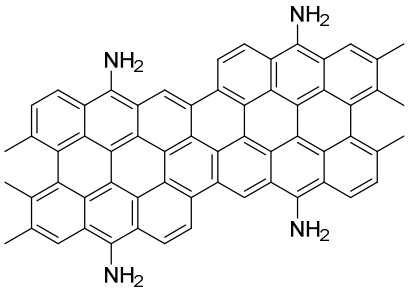


2

GNR-2



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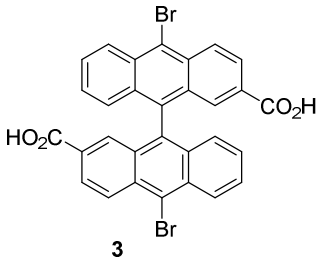

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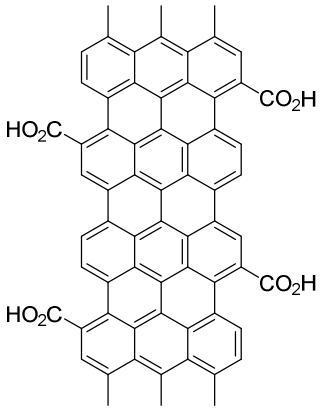
GNR-3



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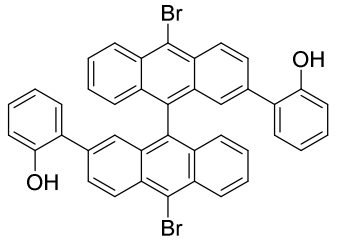
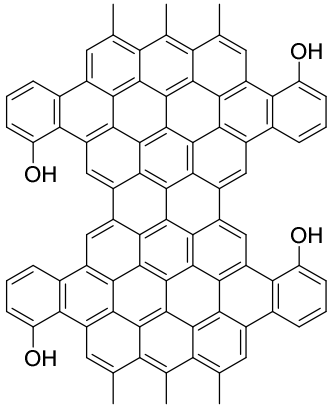
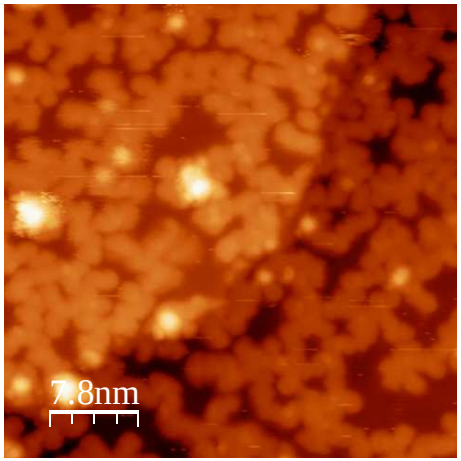
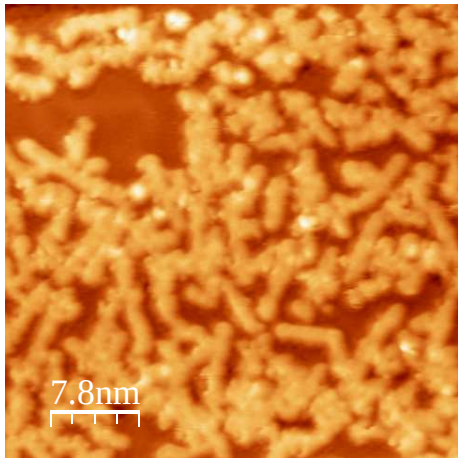


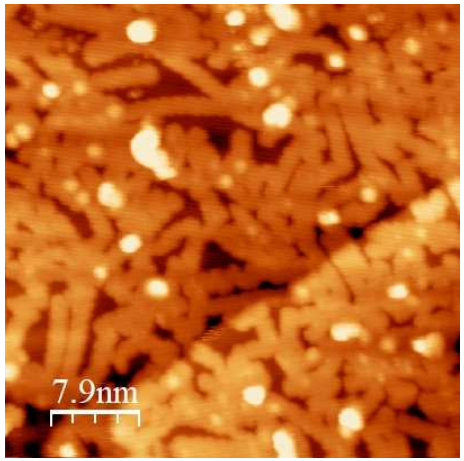
4

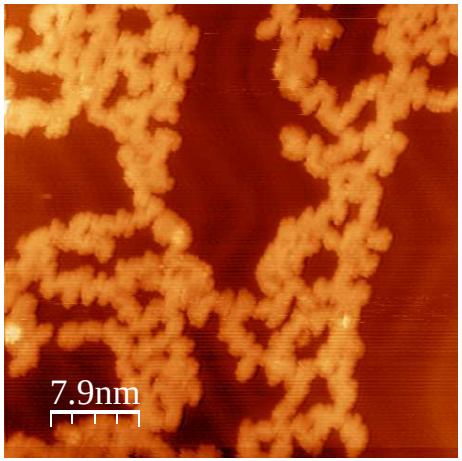
GNR-4











Increasing order

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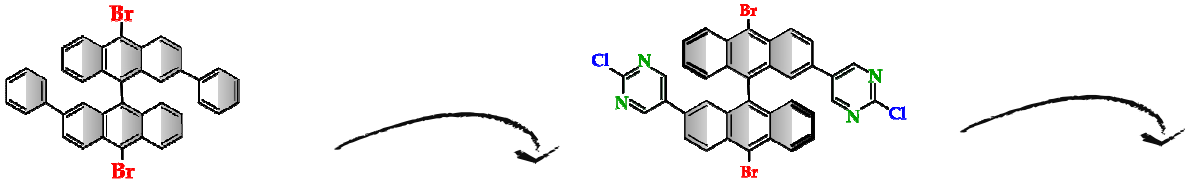
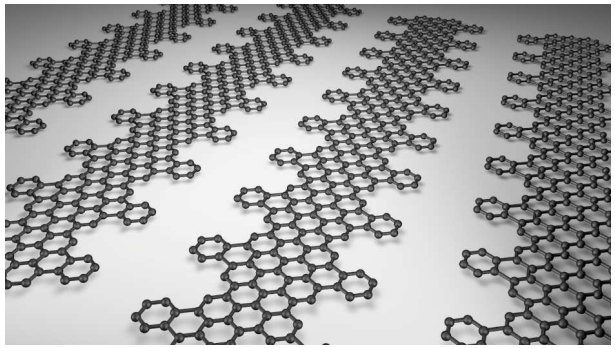
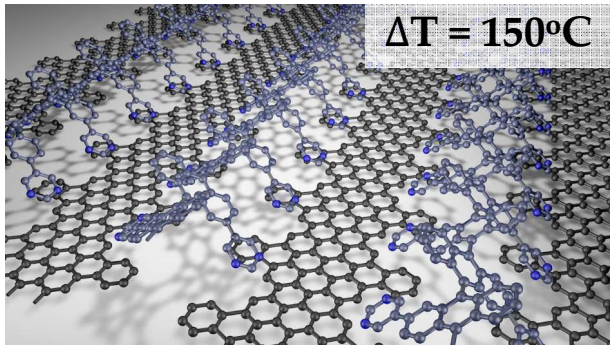
Nanoporous Graphene Integration in Operational Nanophotonic Biosensors

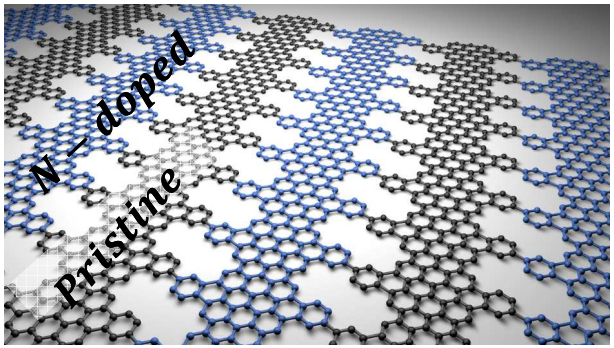
ICN2

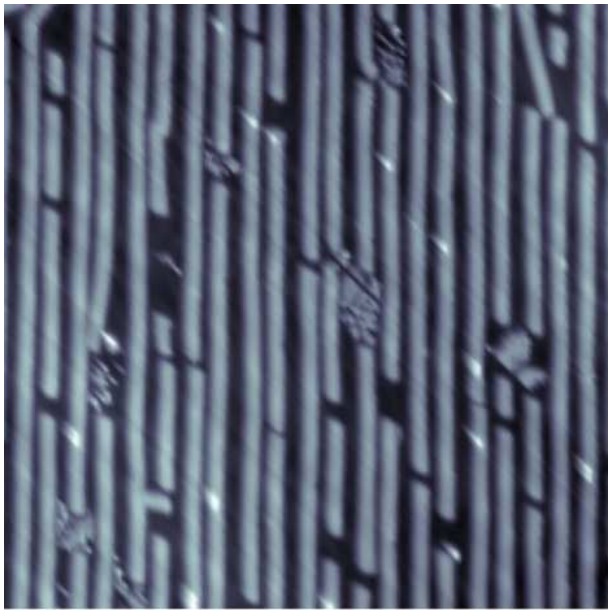


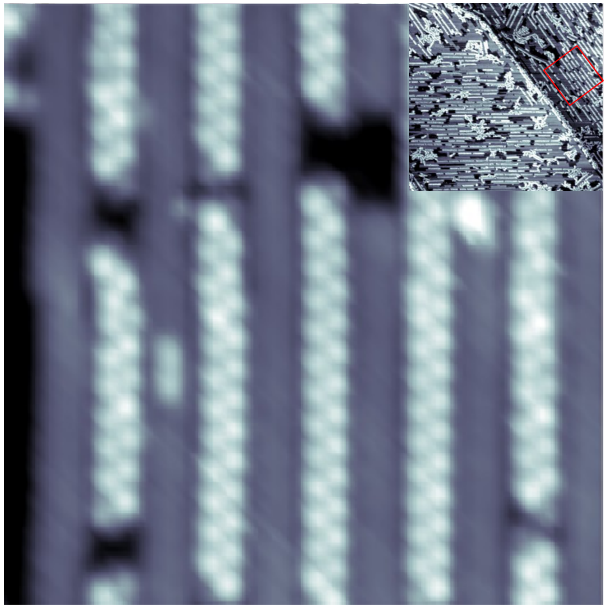
ΔT = 300ºC

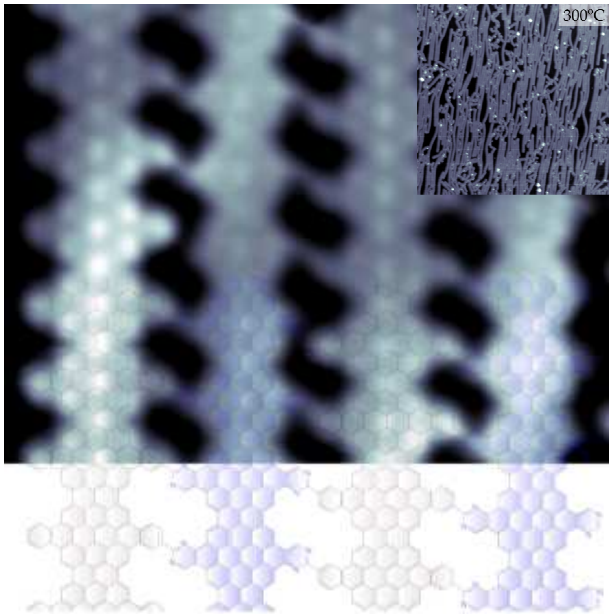












Increasing order

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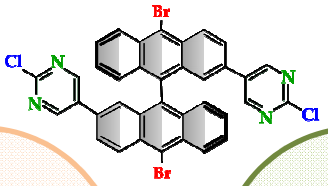
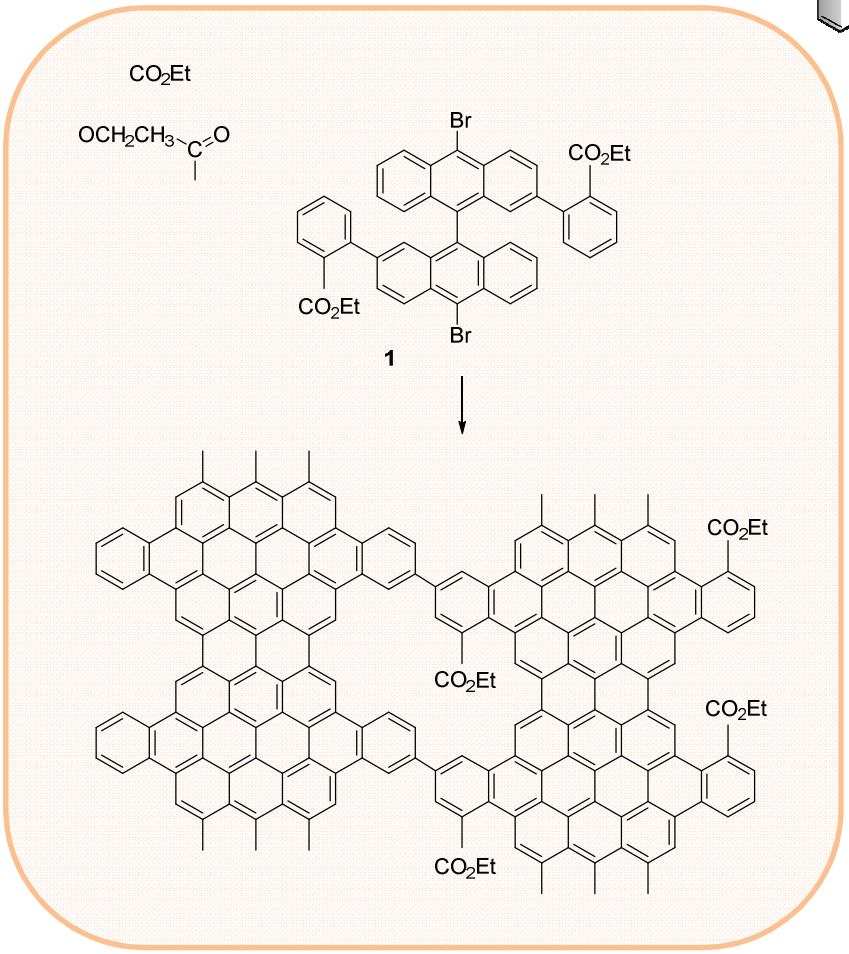
Nanoporous Graphene Integration in Operational Nanophotonic Biosensors

ICN2

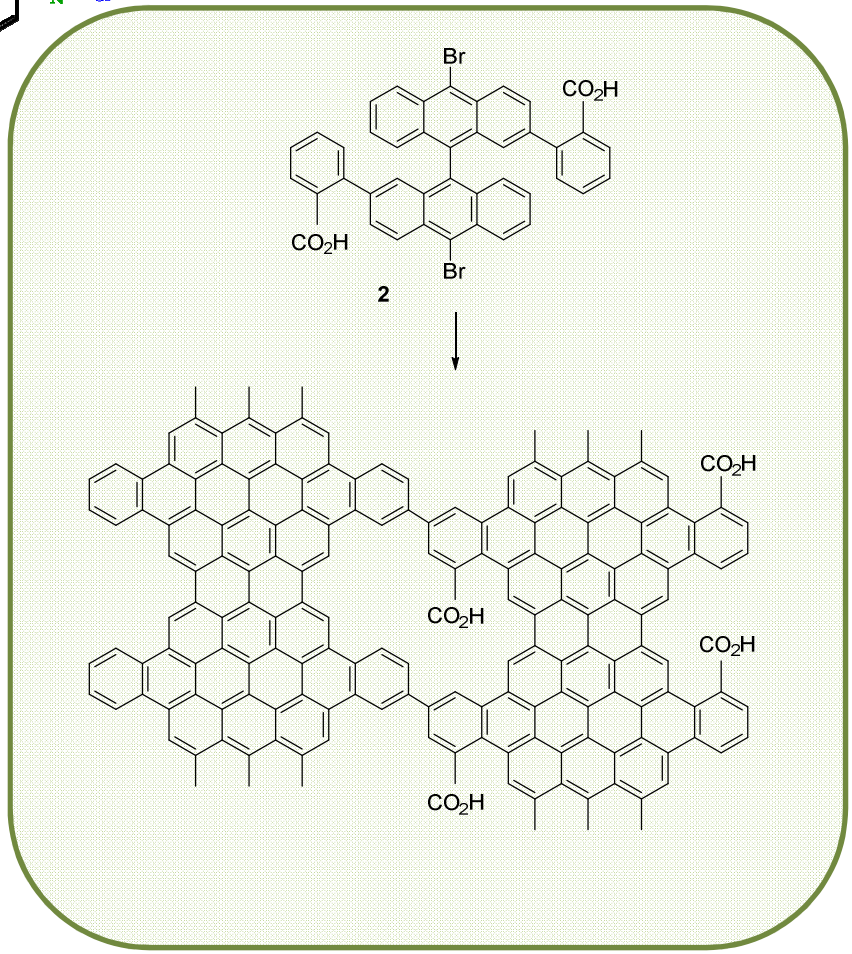
Precursor synthetized → ... in progress



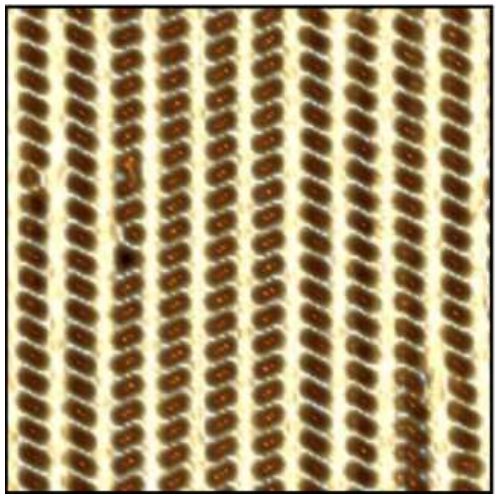
ethoxycarbonyl



carboxyl

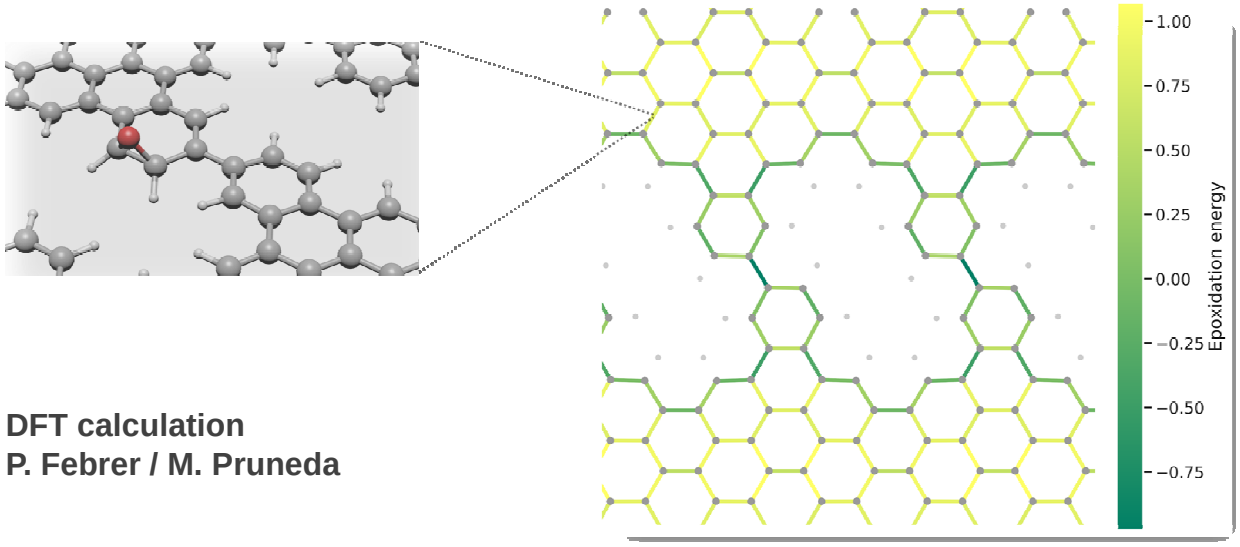


Synthesis of NPG



Ex-situ functionalization

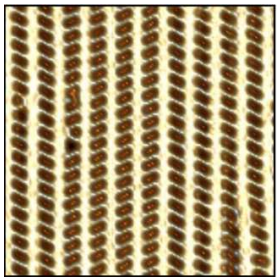
Risk description	Likelihood	Impact	Mitigation plan
Difficulties to synthetize functionalized NPG	Low	Low	Oxidation of NPG by using a UHV oxygen craker as a source of atomic oxygen. Ongoing DFT calculation demonstrate highly selective oxidation of the pores in the NPG structure. Other extrinsic methods, such as dosing triasine or borazine to chemically functionalize the nanopores.



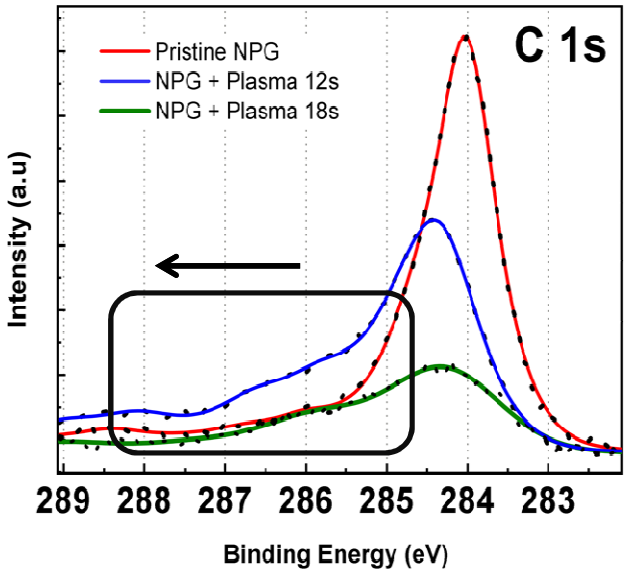
DFT calculation
P. Febrer / M. Pruneda

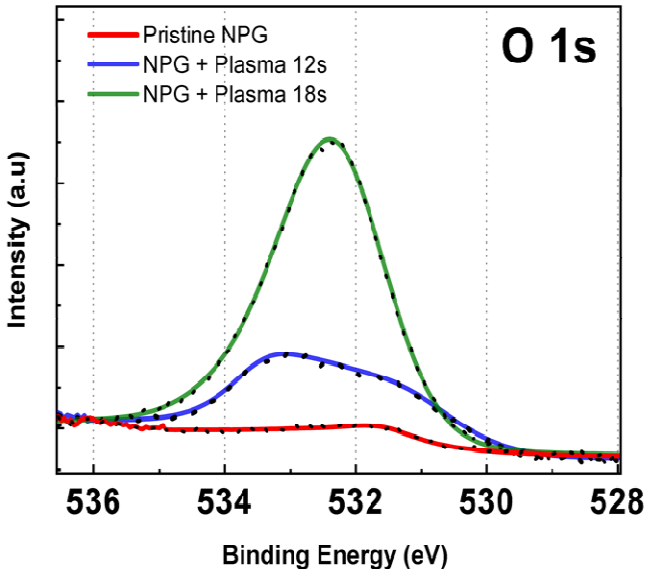
Epoxidation of the C-C double bonds is very selective at the pore edges.

Synthesis of NPG



Ex-situ oxidation





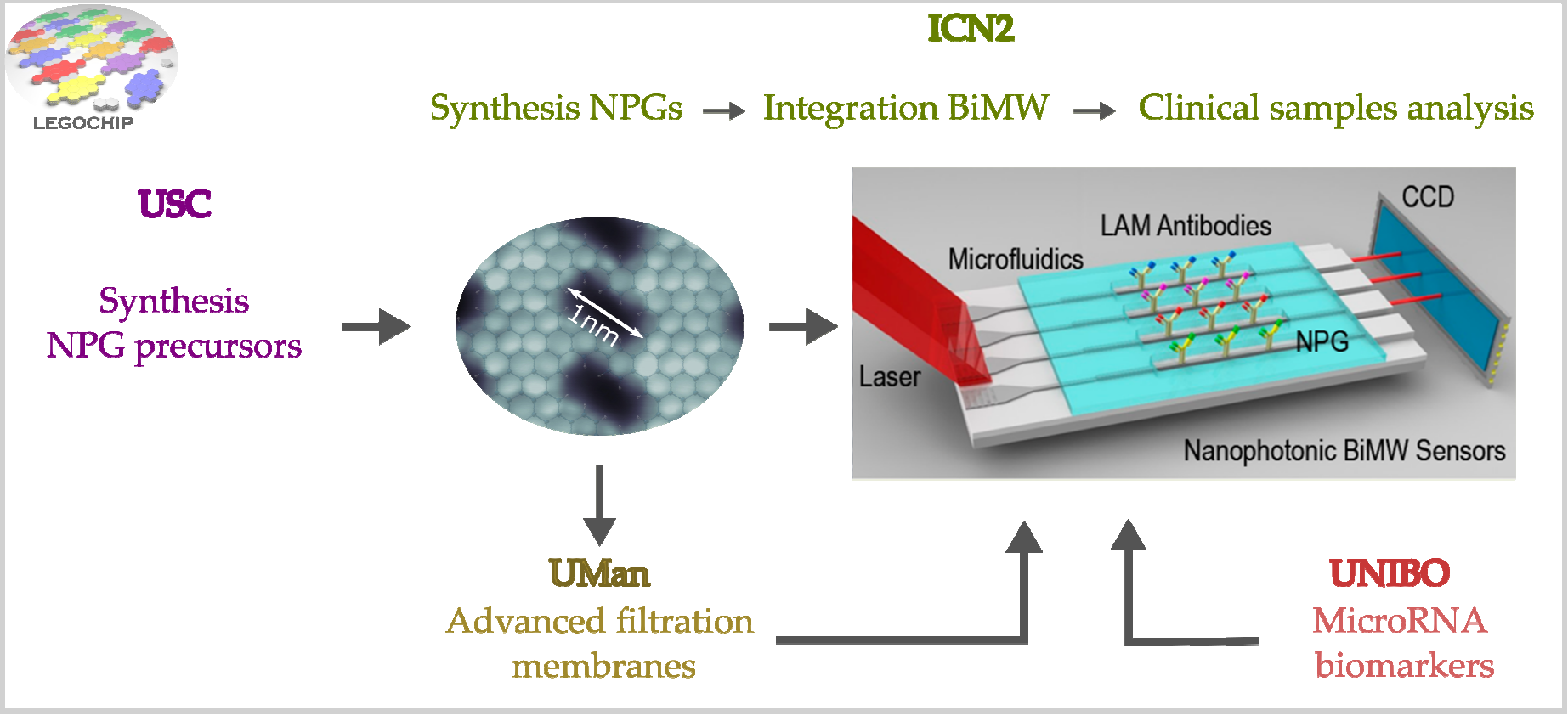
- Hydroxyl C-OH
- epoxy C-O-C
- carbonyl C=O
- carboxyl -COOH

Short oxygen plasma allow functionalization avoiding NPG damage

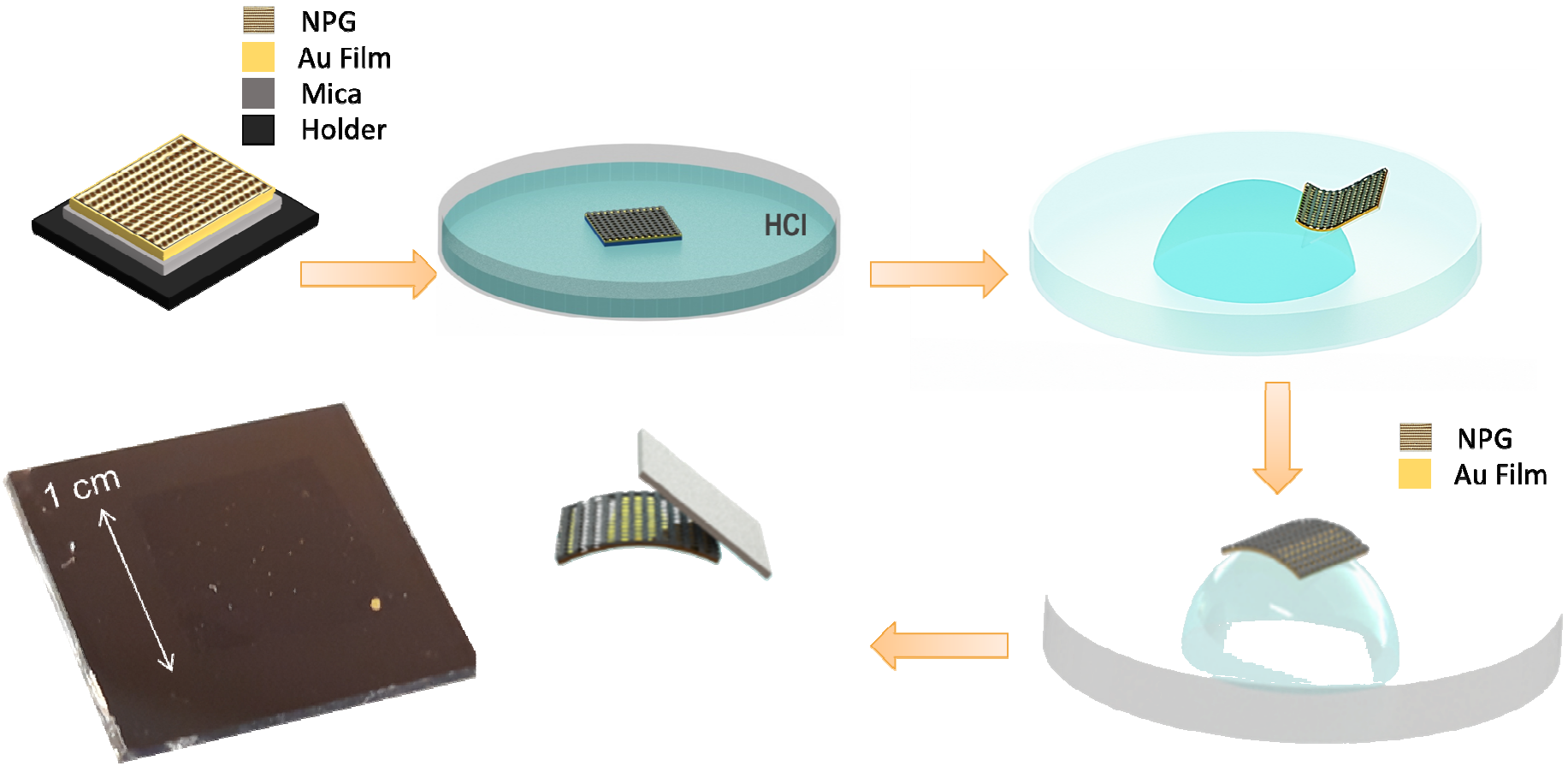
At Conc %	C/Au	O/Au	O\C
Pristine	43,0	1.6	3.7
Plasma 12s	41,0	11.5	28.0
Plasma 18s	20.0	12.5	62.5

Long oxygen plasma starts to damage NPG

Integration into BiMW



Integration of graphene nanoarchitectures: transfer



Wet and polymer-free transfer route of samples until 1x1 cm^2 size by gold etching

Transferring onto desired substrates

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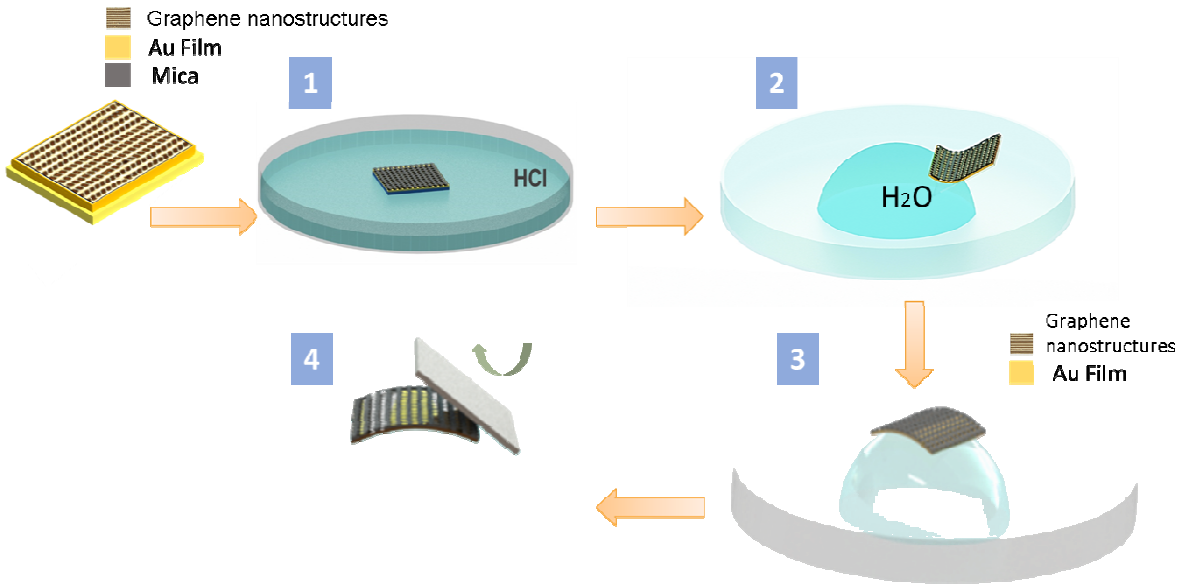
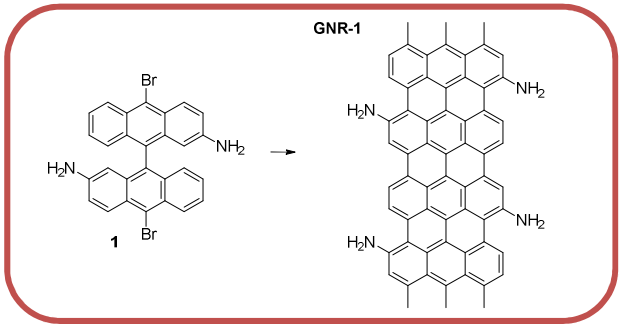
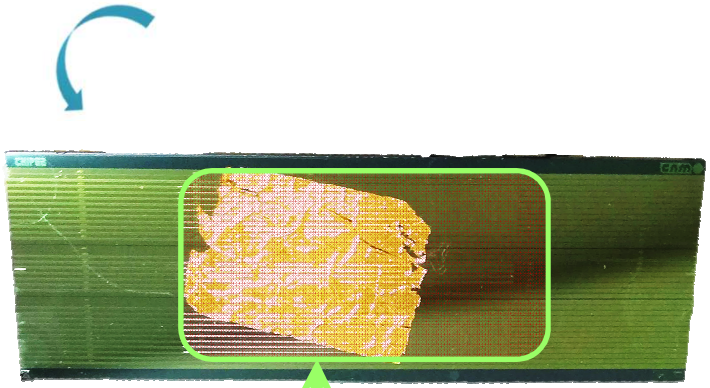
ICN2





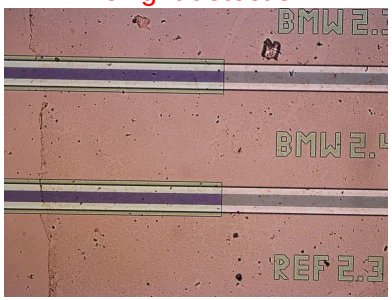


SENSING AREA

Sonication in MiliQ water as effective route to clean gold residues

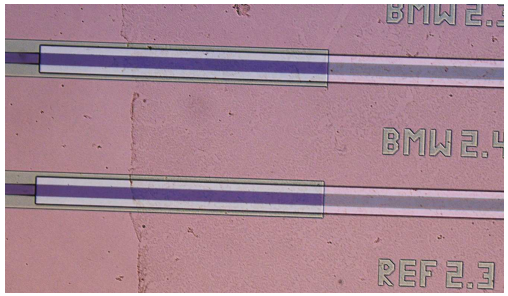
BEFORE sonication

No light detection

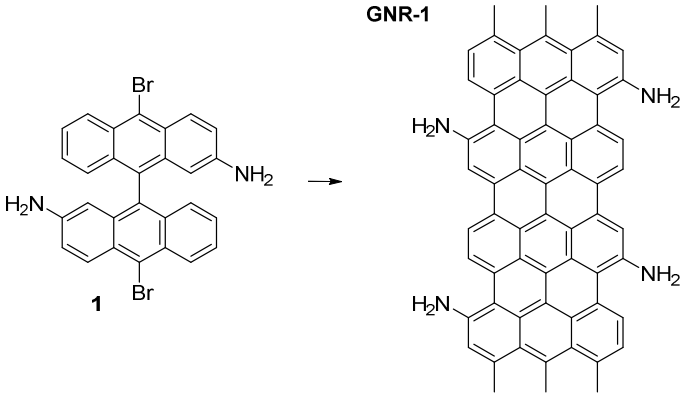


AFTER sonication in miliQ water

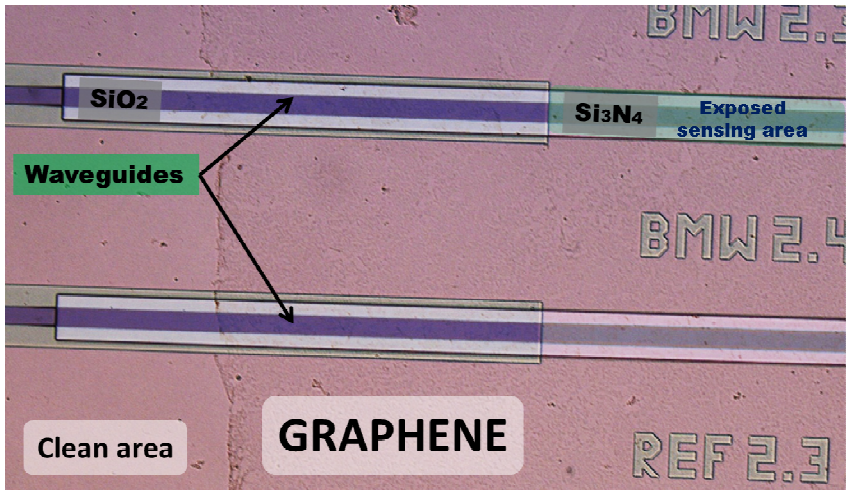
Light detection



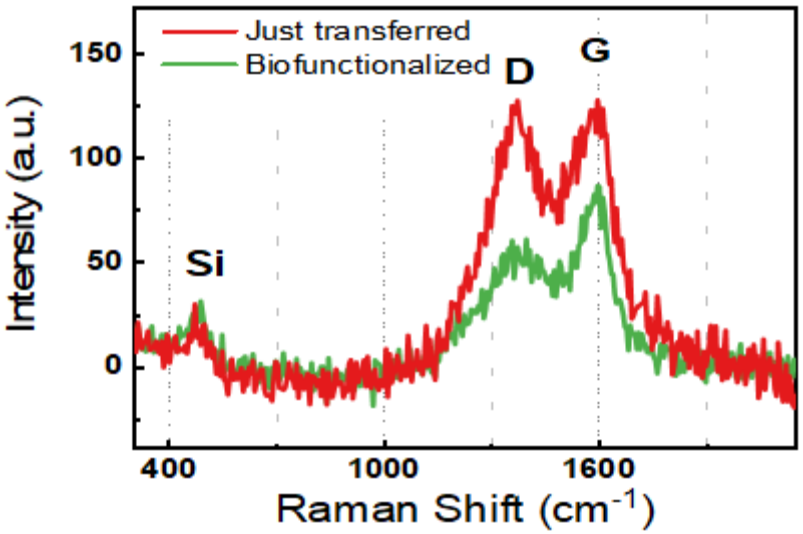
Structural integrity



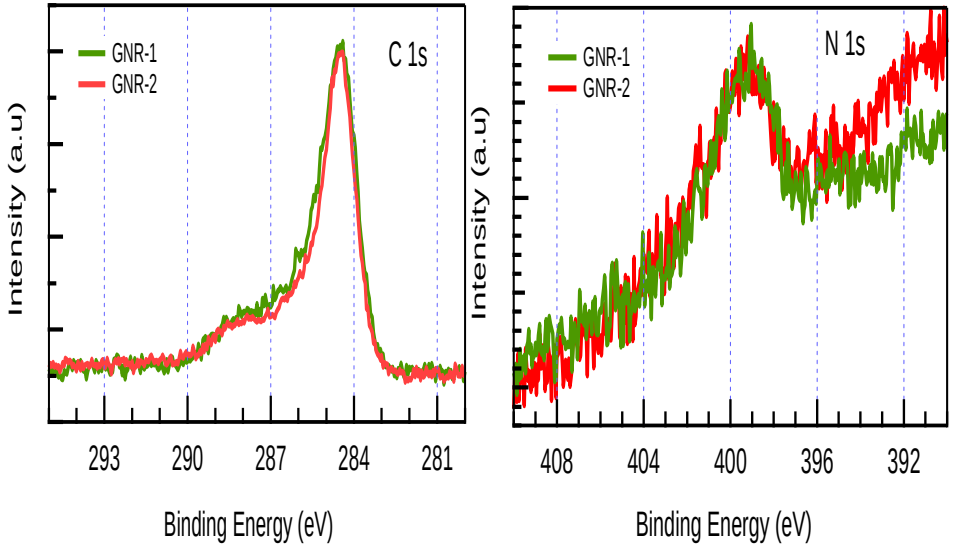
1.- Integrity check by optical microscopy



2.- Integrity check by Raman

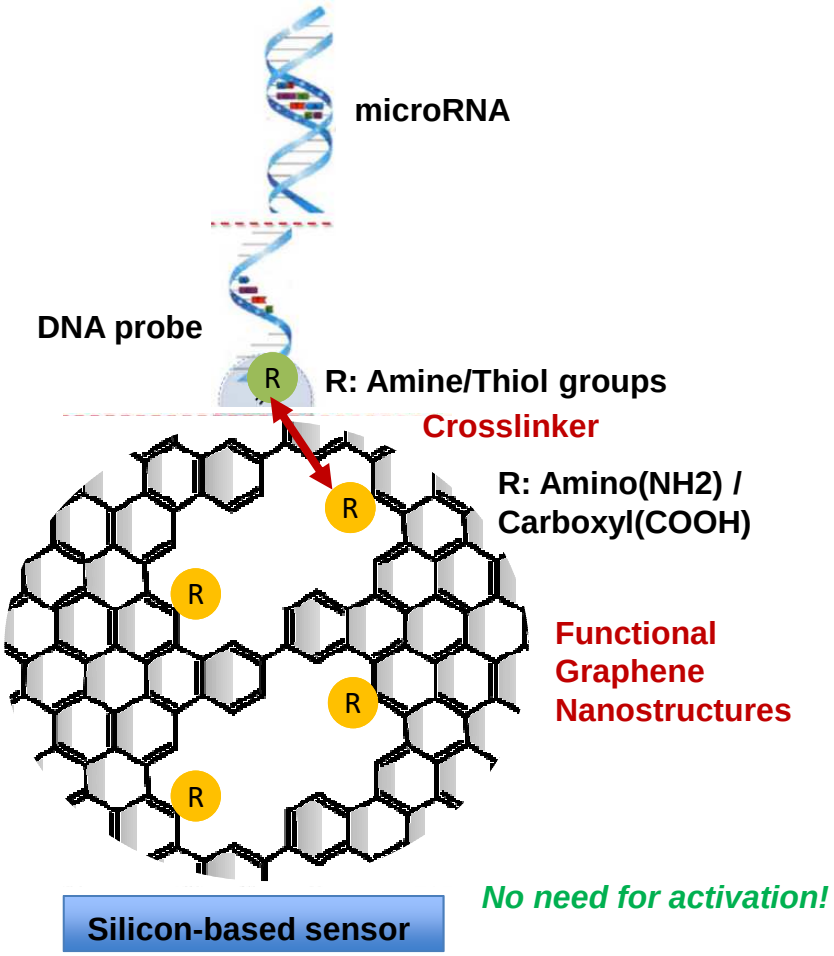


3.- Functional groups by XPS



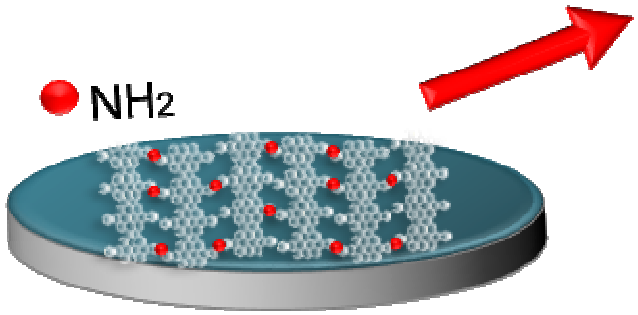
Selection of crosslinkers

Novel graphene-based scheme:

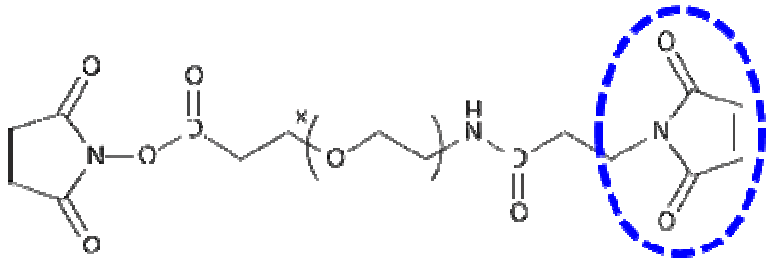


Graphene Biofunctionalization

Cross-linking decision

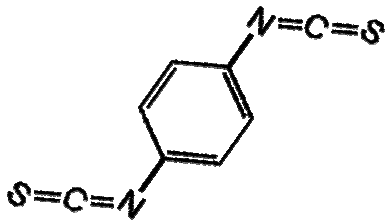


NHS-PEG-Maleimide



Free to bind!

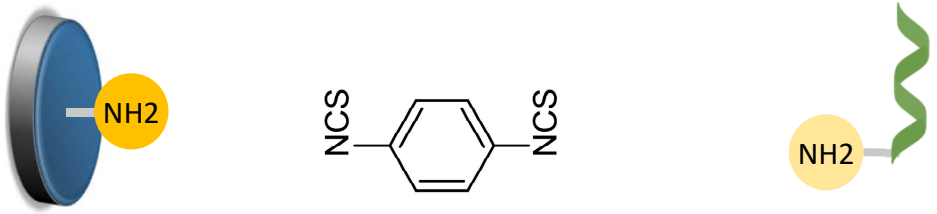
PDITC



Selection of crosslinkers

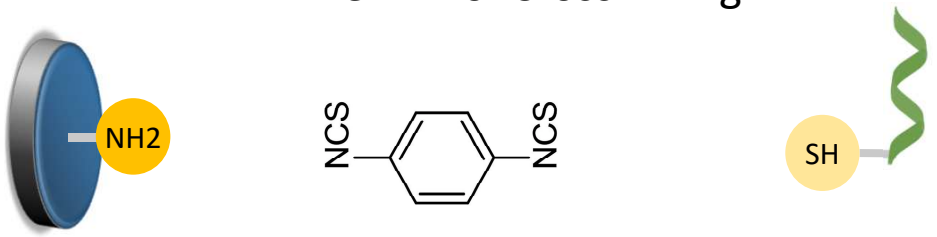
cross-linker **bioreceptor**

Amine - Amine Crosslinking:



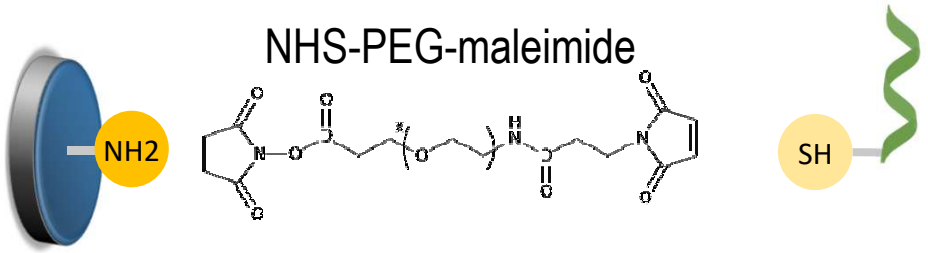
- **PDITC:** no water-soluble

Amine - Thiol Crosslinking:



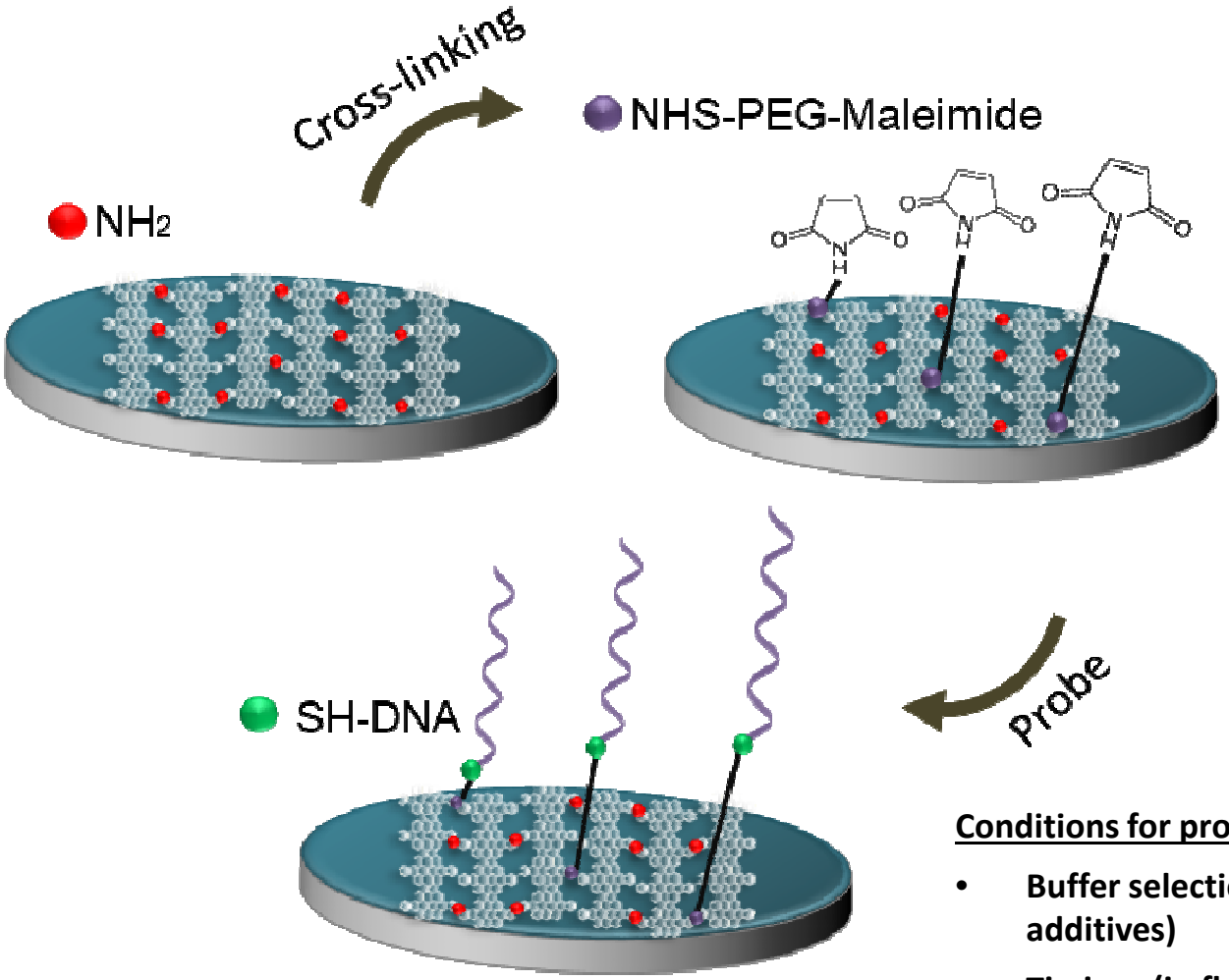
- **PDITC:** no water-soluble

NHS-PEG-maleimide



- **NHS-PEG-MAL:** water-soluble

Protocol of immobilization

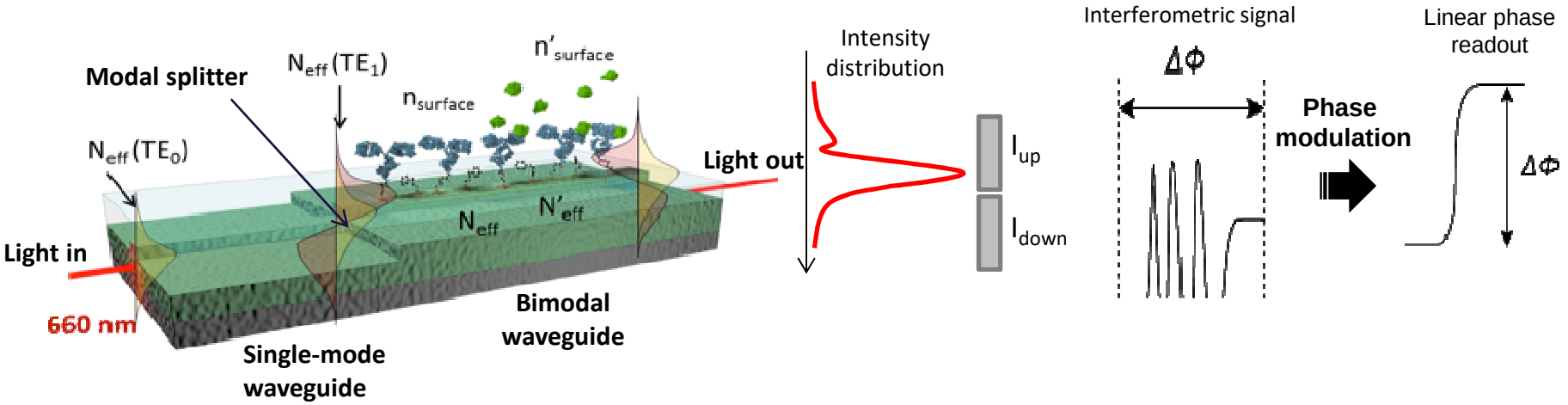


<u>Conditions for probe covalent binding</u>:

- **Buffer selection (pH, salts concentration, additives)**
- **Timings (in flow, ex-situ incubation)**

Biosensors

BiMW interferometer

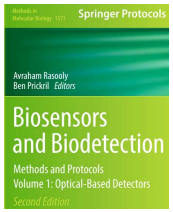


Working principle

- Single channel waveguide interferometer
- Operated on interference of two light modes (fundamental and first order) of the same polarization
- No need anymore of Y-shape splitters (as in MZI or Young Interferometer)
- The modes propagate with different velocities and create an interference pattern at the exit, which intensity distribution depends on the refractive index of the cladding layer through the interaction with the evanescent field.

$\Delta n_{min} = 10^{-7} – 10^{-8}$ RIU
LOD protein: pM – fM

Biosensors



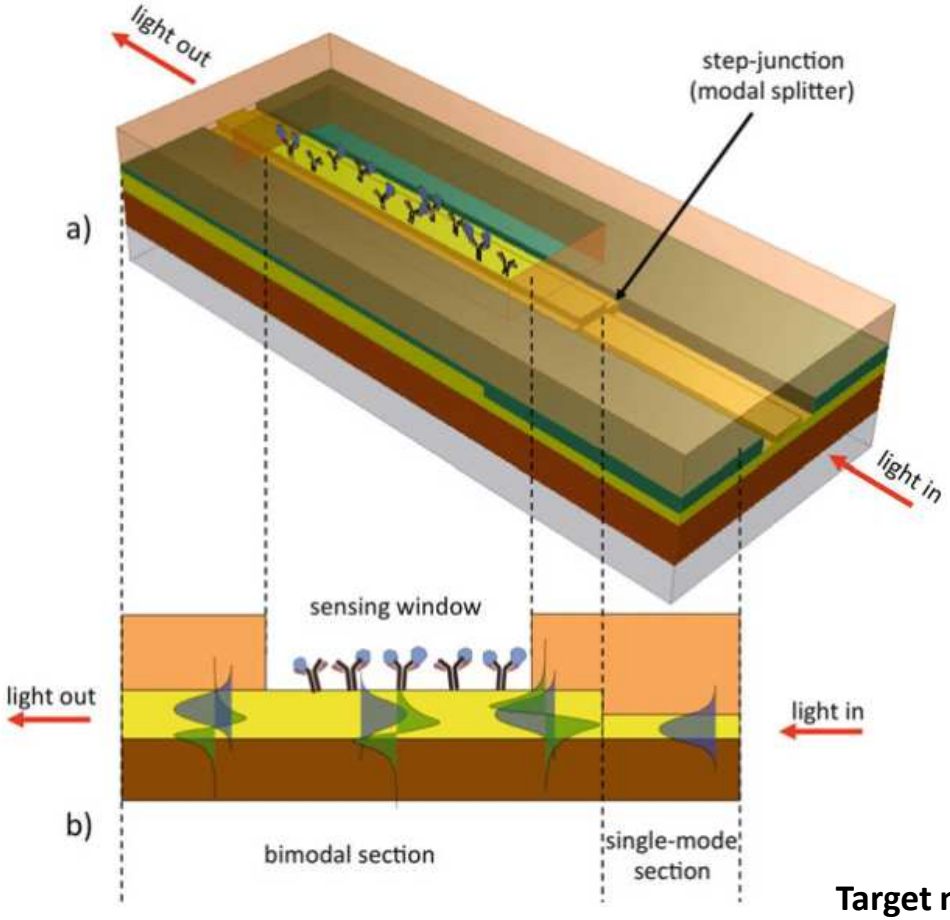
Label-Free Biosensors Based on Bimodal Waveguide (BiMW) Interferometers

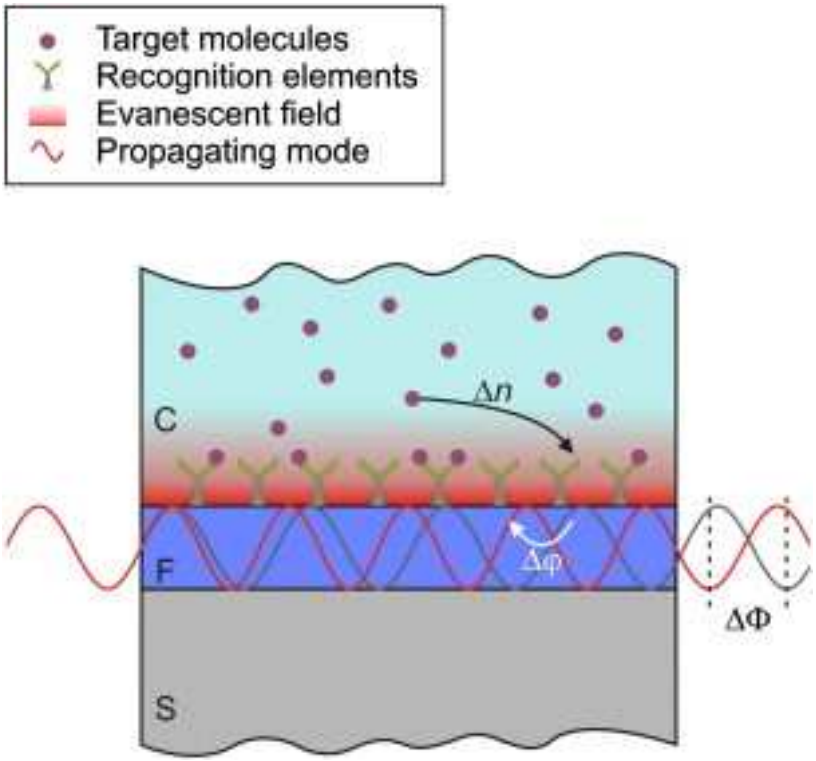
Sonia Herranz*, Adrián Fernández Gavela*, and Laura M. Lechuga

Integrated planar optical waveguide interferometer biosensors: A comparative review

Peter Kozma [a,*,1], Florian Kehl [b], Eva Ehrentreich-Förster [a], Christoph Stamm [c], Frank F. Bier [a]

Biosensors and Bioelectronics 58 (2014) 287–307





Target molecules→ change refraction index→ phase change

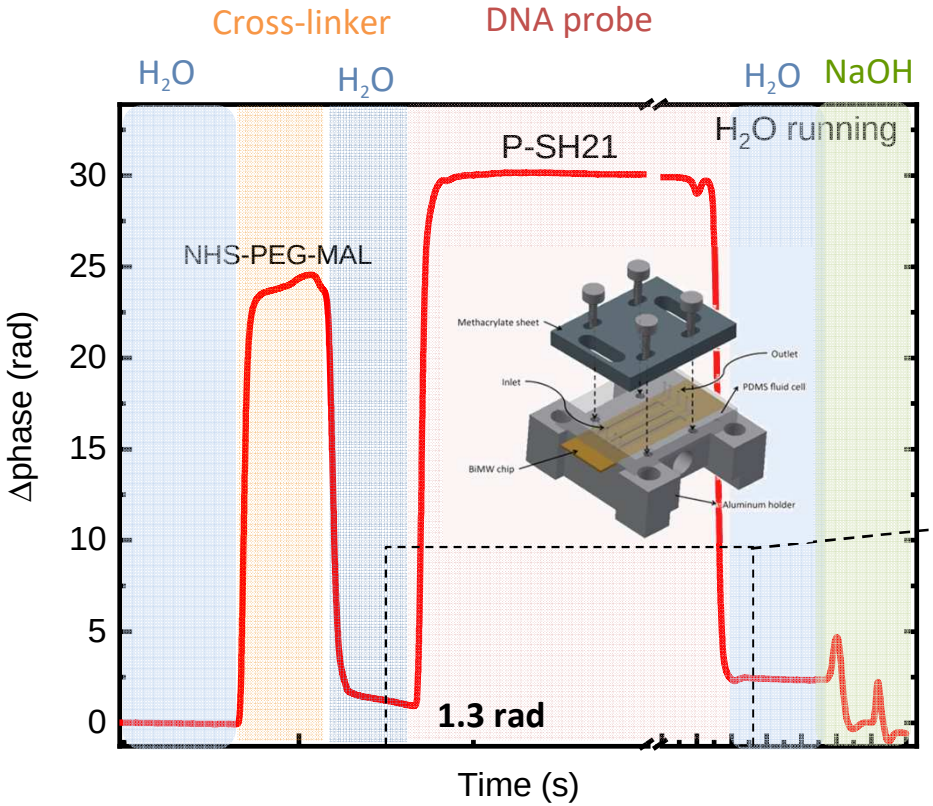
DNA immobilization

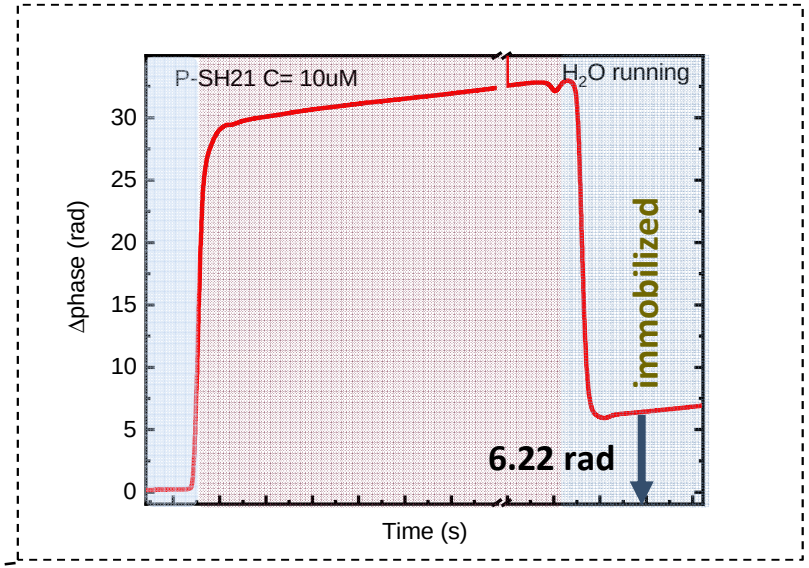
- Graphene: NH_2-7AGNR
- Crosslinker: NHS-PEG-MAL
- Probe: SH-DNA

$$\Delta n_{\min} = \frac{\Delta\varphi_{\min}}{S_{\text{bulk}}} = \frac{\Delta S_{\text{R,min}}}{S_{\text{bulk}}}\frac{\pi}{V} = \frac{3\cdot\sigma_{S_{\text{R}}}}{S_{\text{bulk}}}\frac{\pi}{V}$$

10^{-7}–10^{-8} RIU

LOD at low ng–pg/L level



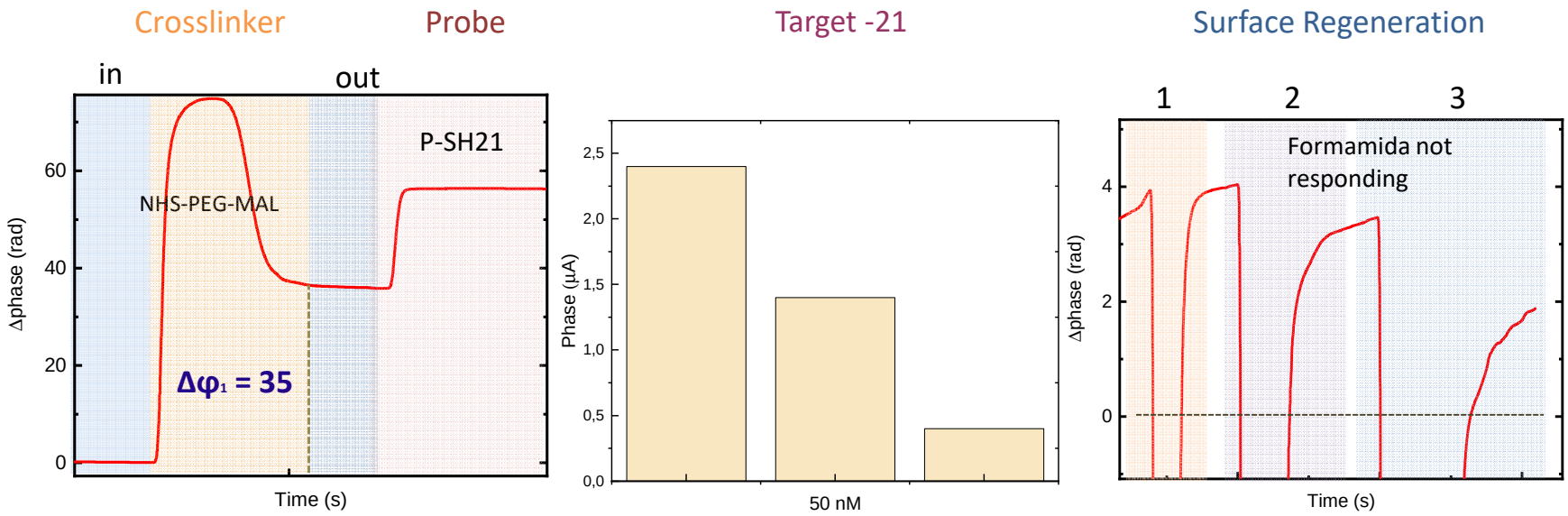


Next Steps:

- **Optimize Buffers and Reaction times**
- **Test target detection**

DNA immobilization

- Graphene: NH_2-7AGNR
- Crosslinker: NHS-PEG-MAL
- Probe: SH-DNA



Problems:

- **Reproducibility with target**
- **Unspecific bindings**

Next Steps:

- Study different crosslinkers for amine-amine coupling
- Minimize non-specific adsorption issues

→ To test directly with DNA probes and target microRNA detection

→ To probe other microRNA biomarkers in gold to advance tests related to the assay conditions

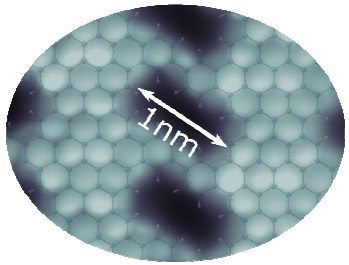
microRNA biomarkers

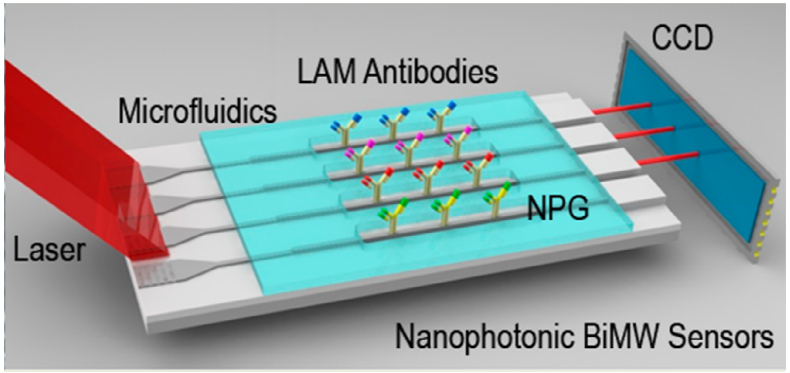
ICN2

Synthesis NPGs → Integration BiMW → Clinical samples analysis

USC

Synthesis
NPG precursors





UMan
Advanced filtration membranes

UNIBO
MicroRNA biomarkers

Manuela Ferracin
(U. Bologna, IP)

- ✓ **Selection of novel target microRNA biomarkers for melanoma diagnostics**
- ✓ **Collection of clinical sample in oncological patients.**

microRNA biomarkers

To date we have enrolled 57 melanoma patients and collected 172 blood samples.

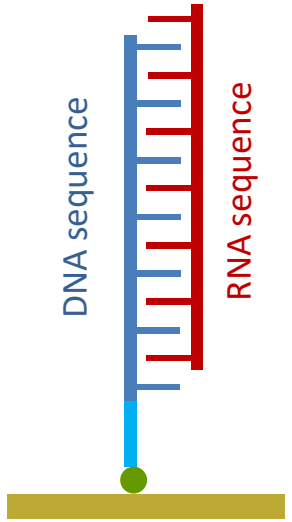
28 Stage III patients

24 Stage IV patients

12 Stage IV patients treated with immunotherapy

microRNAs	DNA probes
miR-21-5p	**probe-21**
UAG CUU AUC AGA CUG AUG UUG A	SH-$(polyT)_{15}$ - ATC GAA TAG TCT GAC TAC AAC T
miR-150-5p	**probe-150**
UCU CCC AAC CCU UGU ACC AGU G	SH-$(polyT)_{15}$ - AGA GGG TTG GGA ACA TGG TCA C
miR-155-5p	**probe-155**
UUAAUGCUAAUCGUGAUAGGGGUU	SH-$(polyT)_{15}$ - AAT TAC GAT TAG CAC TAT CCC CAA
miR-221-3p	**probe-221**
AGC UAC AUU GUC UGC UGG GUU UC	SH-$(polyT)_{15}$ - TCG ATG TAA CAG ACG ACC CAA AG
miR-320a-3p	**probe-320a**
AAA AGC UGG GUU GAG AGG GCG A	SH-$(polyT)_{15}$ - TTT TCG ACC CAA CTC TCC CGC T
miR-424-5p	**probe-424**
CAG CAG CAA UUC AUG UUU UGA A	SH-$(polyT)_{15}$ - GTC GTC GTT AAG TAC AAA ACT T

Based on previous and current clinical studies for early melanoma diagnostics and patients monitoring, we have selected an initial panel of circulating microRNA biomarkers



From these microRNA, we have designed the oligonucleotide sequences corresponding to complementary DNA probes

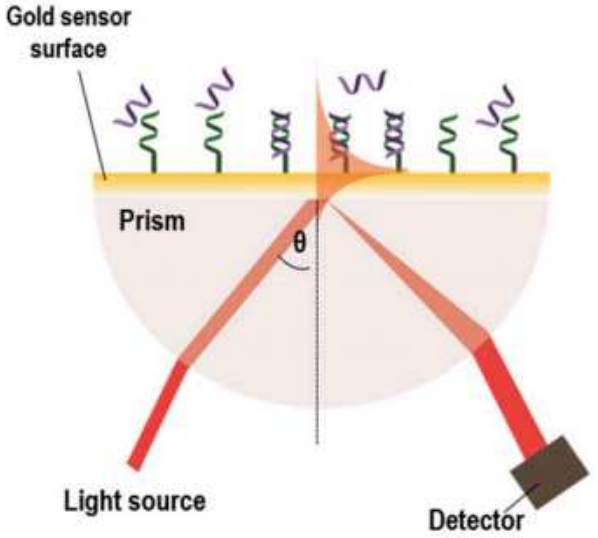
NanoB2A

Preliminary optimization & evaluation of microRNA analysis with SPR biosensor

- **Analytical parameters for microRNA detection**
- **Anti-fouling tests**

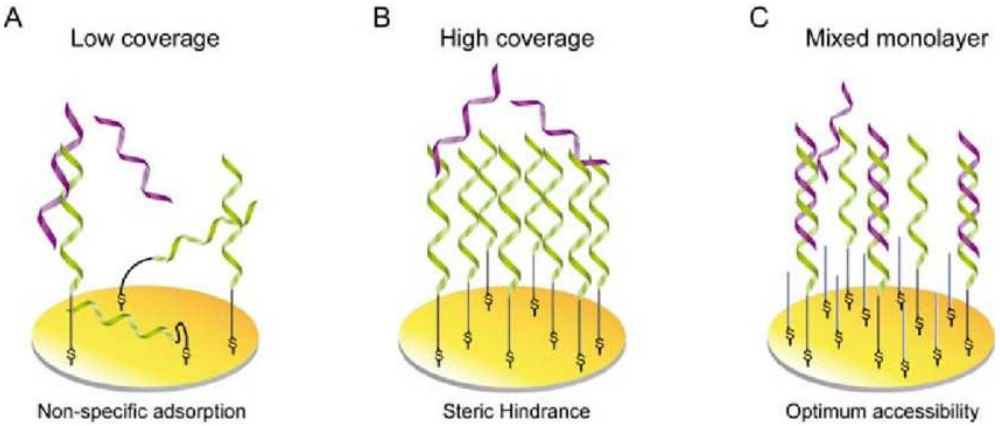
1. Probe density
2. DNA vs RNA analysis
3. Plasma fouling
4. Towards real samples

Surface Plasmon Resonance (SPR) Biosensor

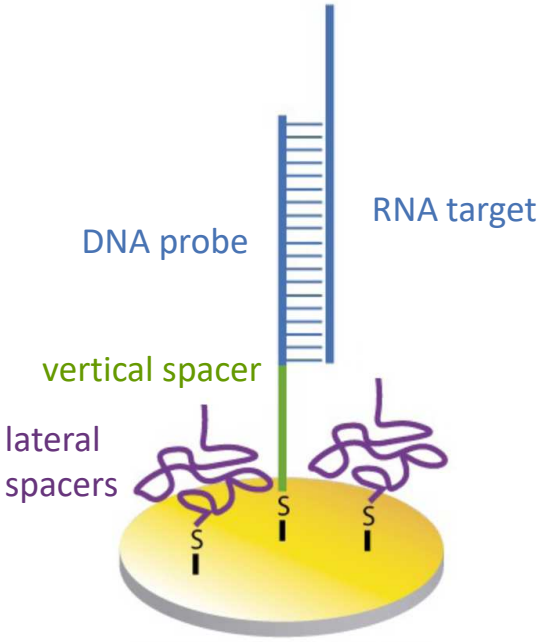


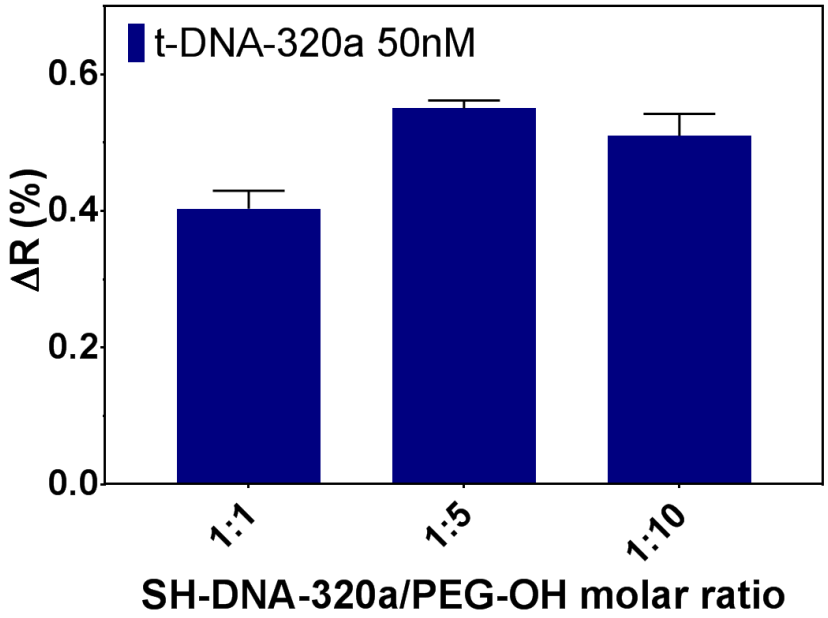
- ✓ Label-free and real-time analysis
- ✓ Sensitivity: 10^{-6} RIU (nM-pM range)
- ✓ User-friendly operation
- ✓ Gold surface chemistry

Probe Density

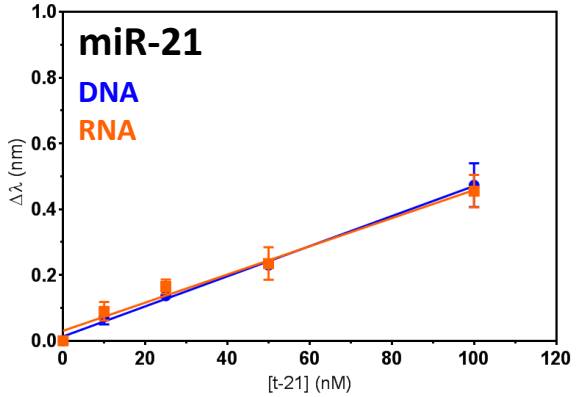


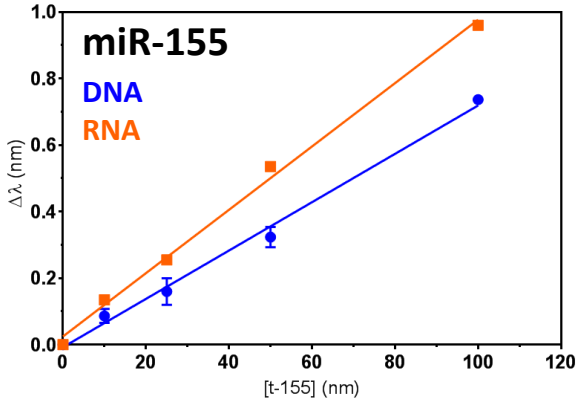
It is important to optimize the probe density to ensure maximum detection efficiency

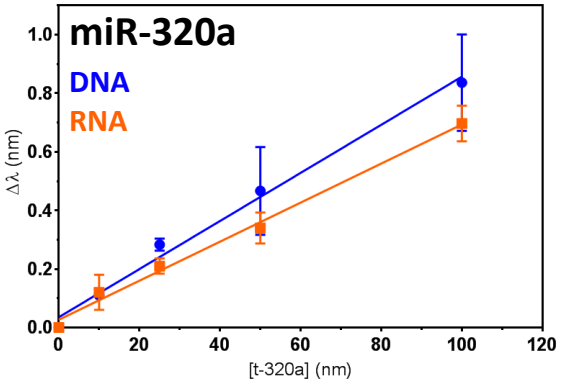


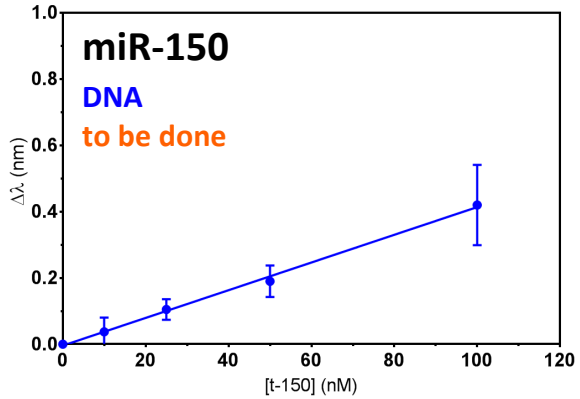


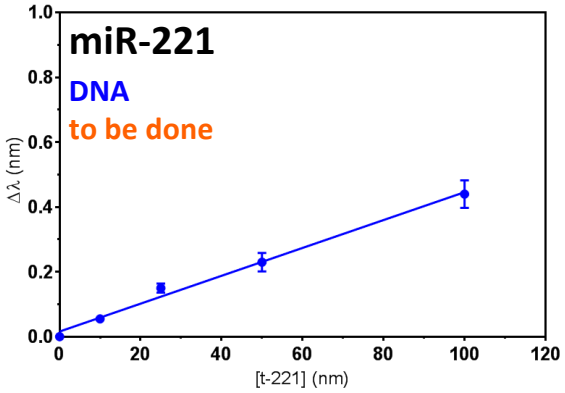
miR detection in buffer

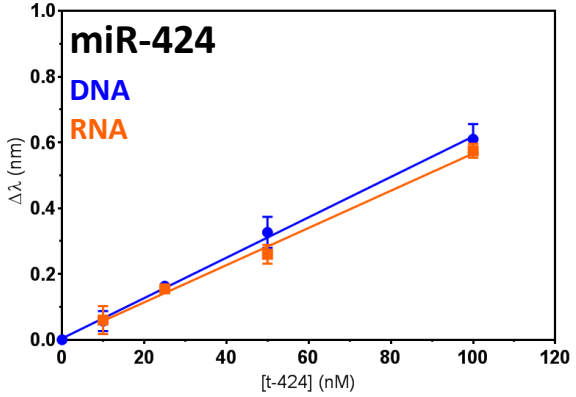












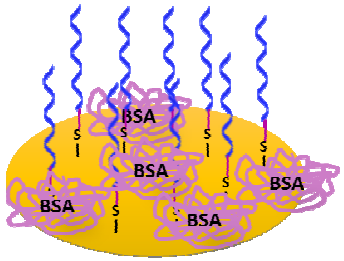
target	LOD (nM)	LOQ (nM)
tDNA-21	2.34	9.78
tDNA-155	1.56	5.20
tDNA-320a	2.02	6.73
tDNA-150	1.13	12.4
tDNA-221	1.16	3.88
tDNA-424	2.72	9.05

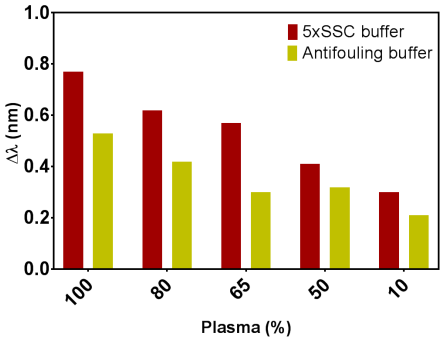
target	LOD (nM)	LOQ (nM)
miR-21	1.89	23.0
miR-155	5.11	22.5
miR-320a	1.03	12.7
miR-424	2.92	10.2
miR-221	-	-
miR-150	-	-

- DNA and RNA behave similar
- **LOD in the nM range**

miR detection in plasma

Surface bloking: **BSA** 30 mg/mL
Antifouling buffer: SSC 2.5x + **CHAPS** 10 mM and **Tween 20** 0.05%

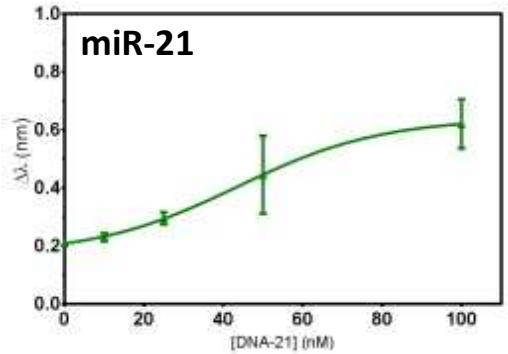


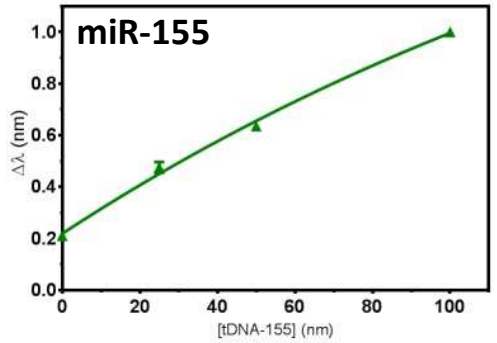


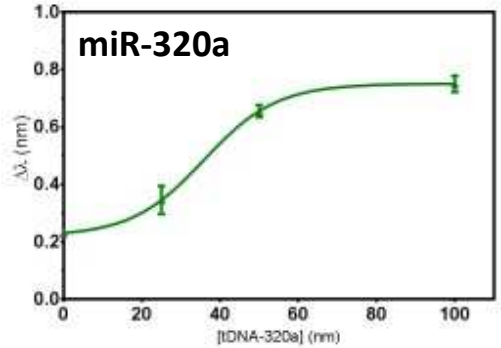
target	LOD (nM)	LOQ (nM)
tDNA-21	17.4	34.7
tDNA-155	6.05	23.2
tDNA-320a	4.88	12.9
tDNA-150	7.01	17.2
tDNA-221	3.29	10.9
tDNA-424	11.1	25.2

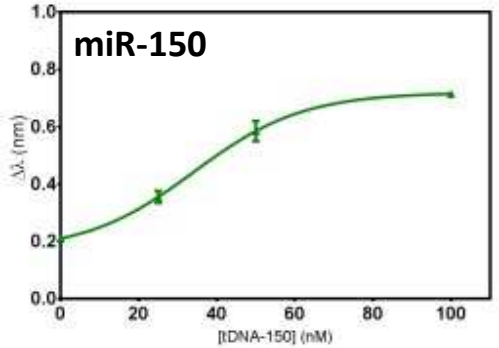
- Reduce but not eliminate fouling
- **Plasma matrix slightly affects hybridization**

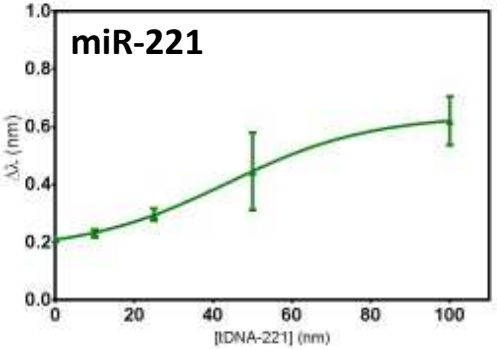
tDNA in 10% plasma

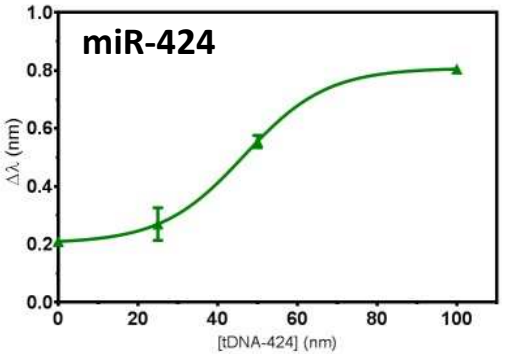












Towards real samples...

PCR results:

microRNA	Average (ct)	Minimum (ct)	Maximum (ct)
miR-155-5p	98546,9	13781,3	393750
miR-320a	3168327,7	144375	10828125
miR-424-5p	903260,1	56875	5228125

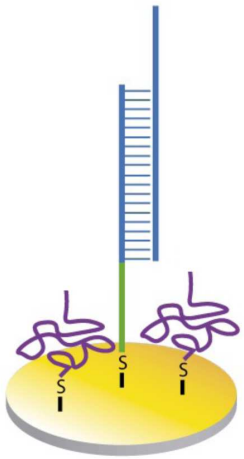
results from UNIBO!

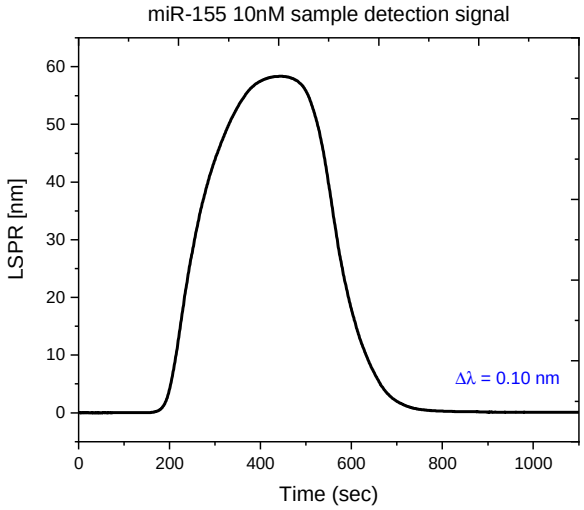
to molarity....

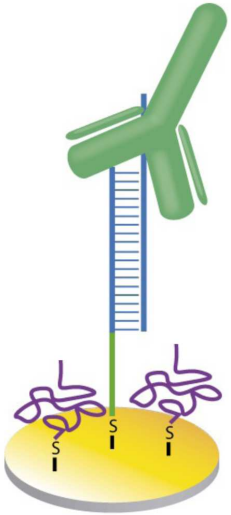
microRNA	Average (aM)	Minimum (aM)	Maximum (aM)
miR-155-5p	818,22	114,42	3269,26
miR-320a	26306,27	1198,73	89904,72
miR-424-5p	7499,67	472,23	43408,54

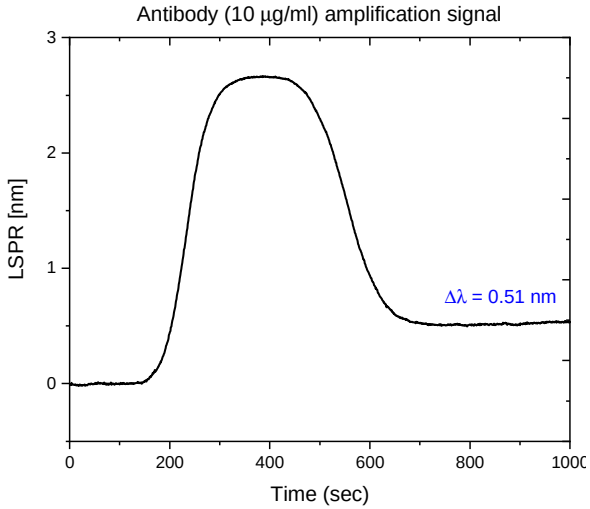
microRNAs in real samples are in the aM-fM range of concentration

Signal Amplification: second step with anti-DNA/RNA Hybrid antibody









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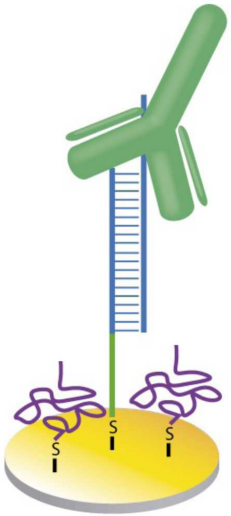
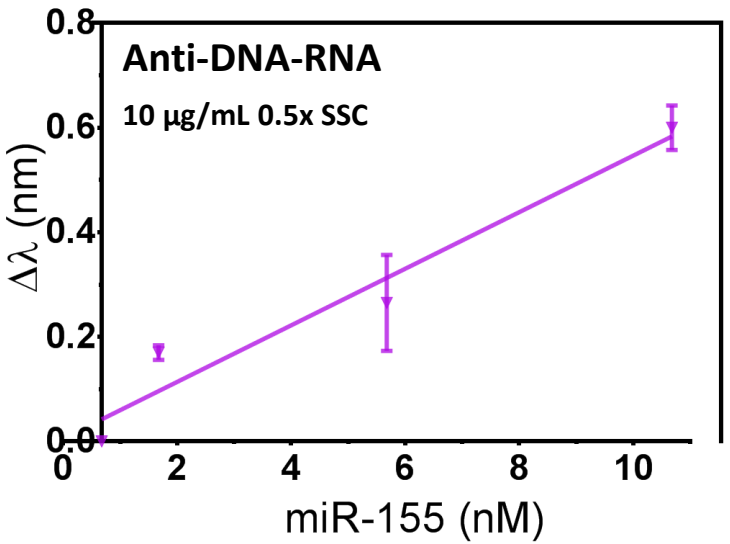




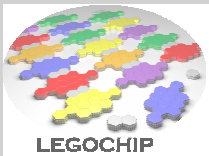
LOD in the pM-nM range!

- other amplification strategies
- transfer to BiMW biosensor

Biological filtration membranes

LEGOCHIP
Nanoporous Graphene Integration in Operational Nanophotonic Biosensors

FLAG-ERA

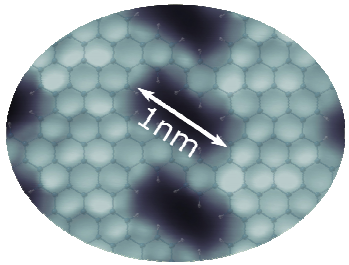


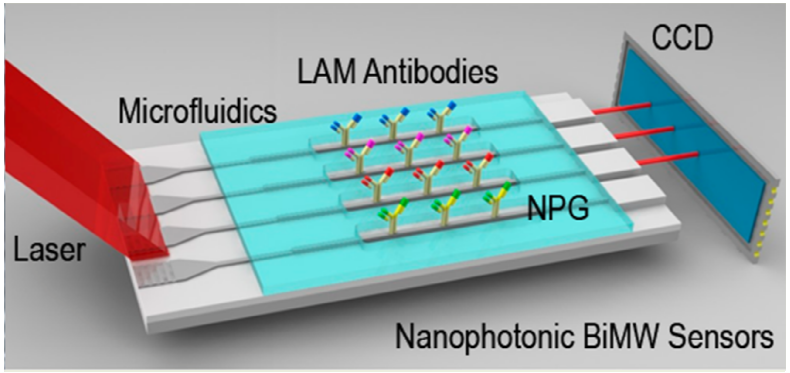
ICN2

Synthesis NPGs → Integration BiMW → Clinical samples analysis

USC

Synthesis
NPG precursors





UMan
Advanced filtration membranes

UNIBO
MicroRNA biomarkers

Patricia Gorgojo
(U. Manchester, IP)

MANCHESTER 1824
The University of Manchester

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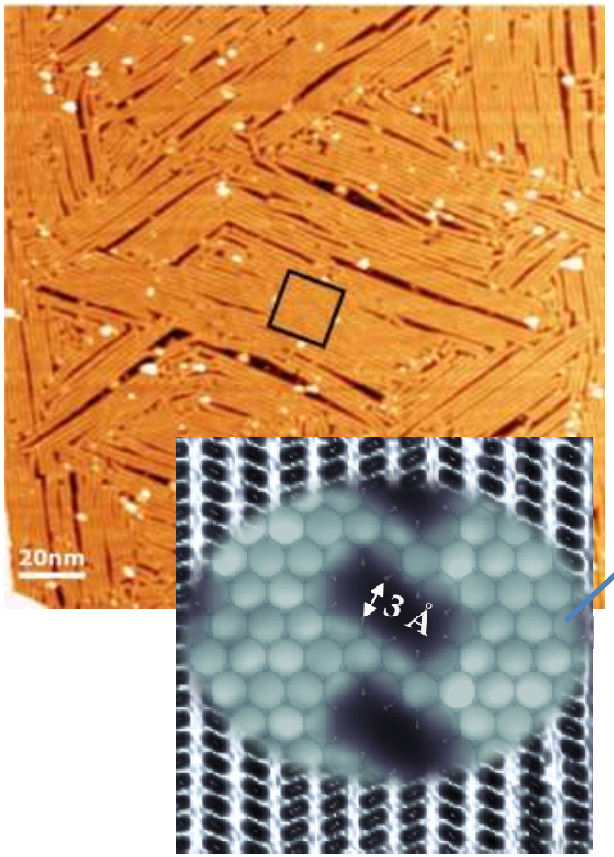
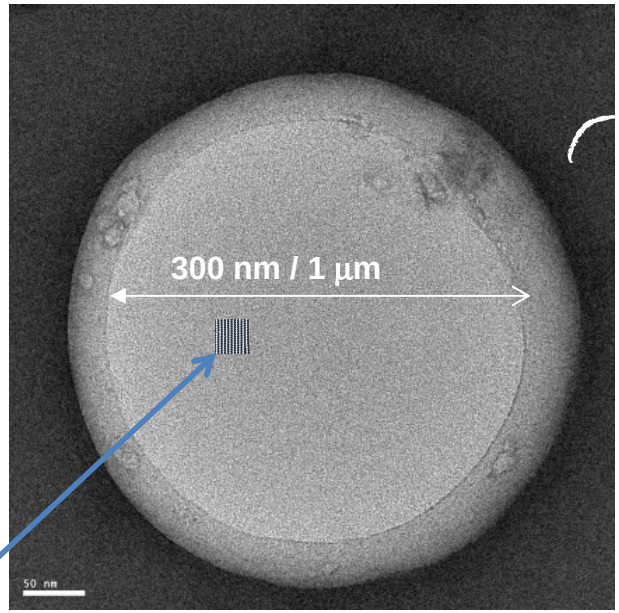
Suspended NPG characterization

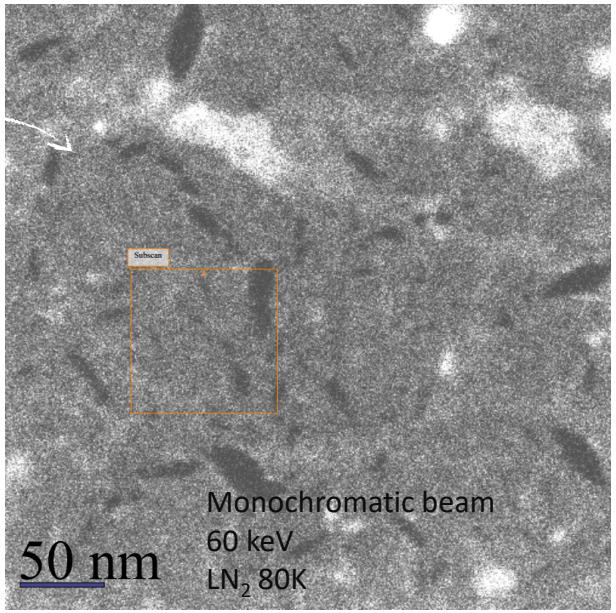
STM – NPG/Au

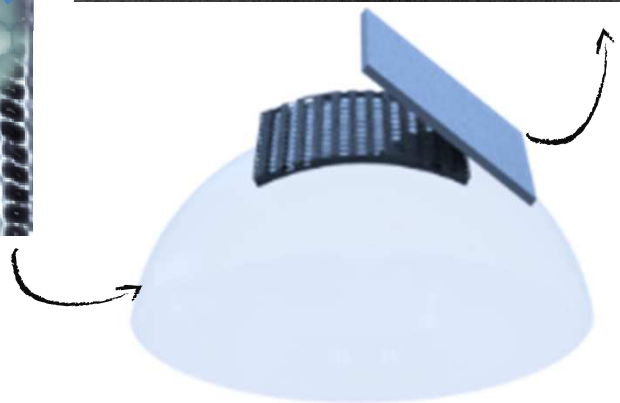


TEM – Suspended NPG/SiN membranes



Cs-TEM

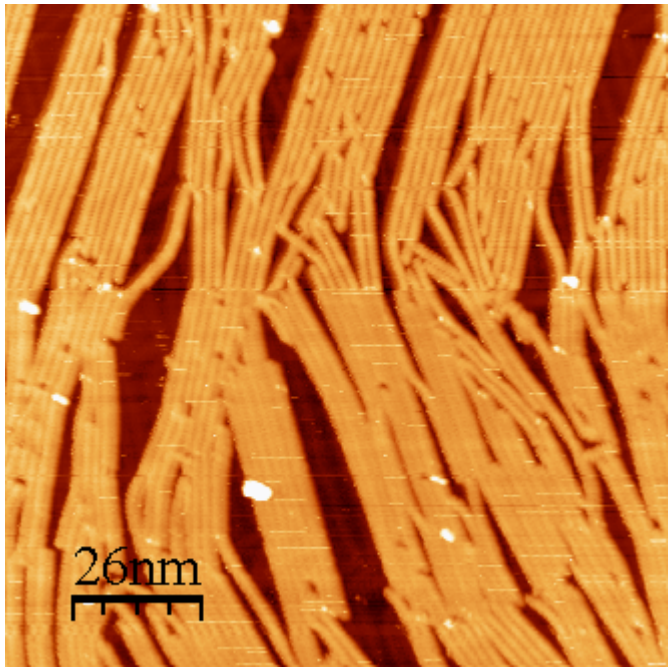




Suspended NPG:

1. **Mechanical integrity**
2. **Clean**

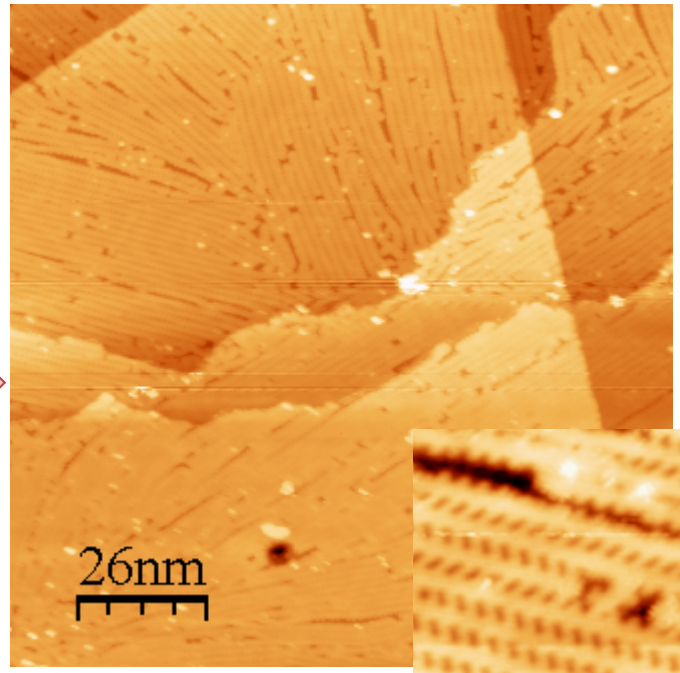
Sealing defects in NPG

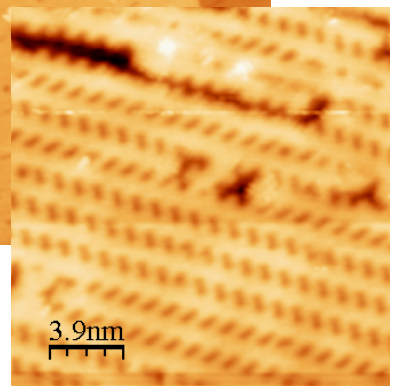


3-5 nm defect size

Same sample

2nd evaporation + reaction to seal defects





~1nm defect size

Biological filtration membranes

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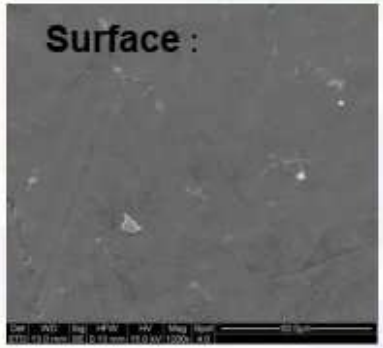
Polyethersulfone (PES) nanofiltration membranes prepared via phase inversión onto nonwoven supports

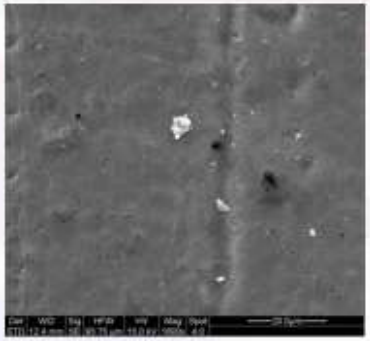
Membrane	Code	PES	APTS-GO	PVP	DMF
			g		
PES	M00	20.0	-	-	80.0
PES - PVP	M02	20.0	-	3.0	77.0
PES - APTS-GO	M20	20.0	0.1	-	79.9
PES - APTS-GO - PVP	M22	20.0	0.1	3.0	76.9

PES - PVP

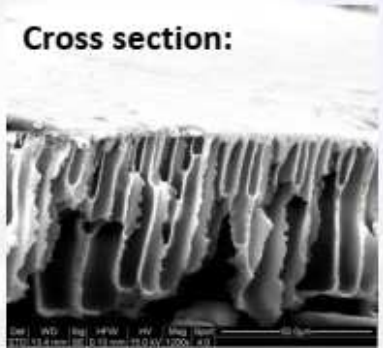
PES - APTSGO - PVP

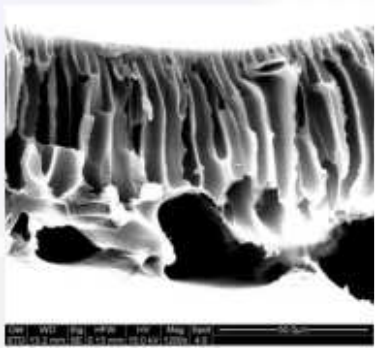
Surface :





Cross section:





Biological filtration membranes

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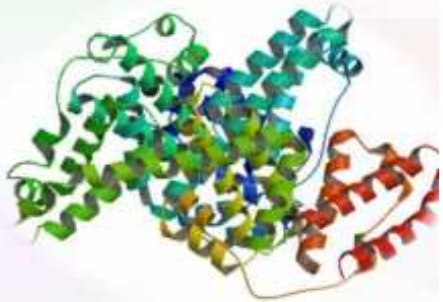
Model biological sample

- **Proteins:**

  Represented as Bovine Serum Albumin (BSA, MW ~66 kDa)
  Lyophilized powder BSA protein (Sigma-Aldrich)

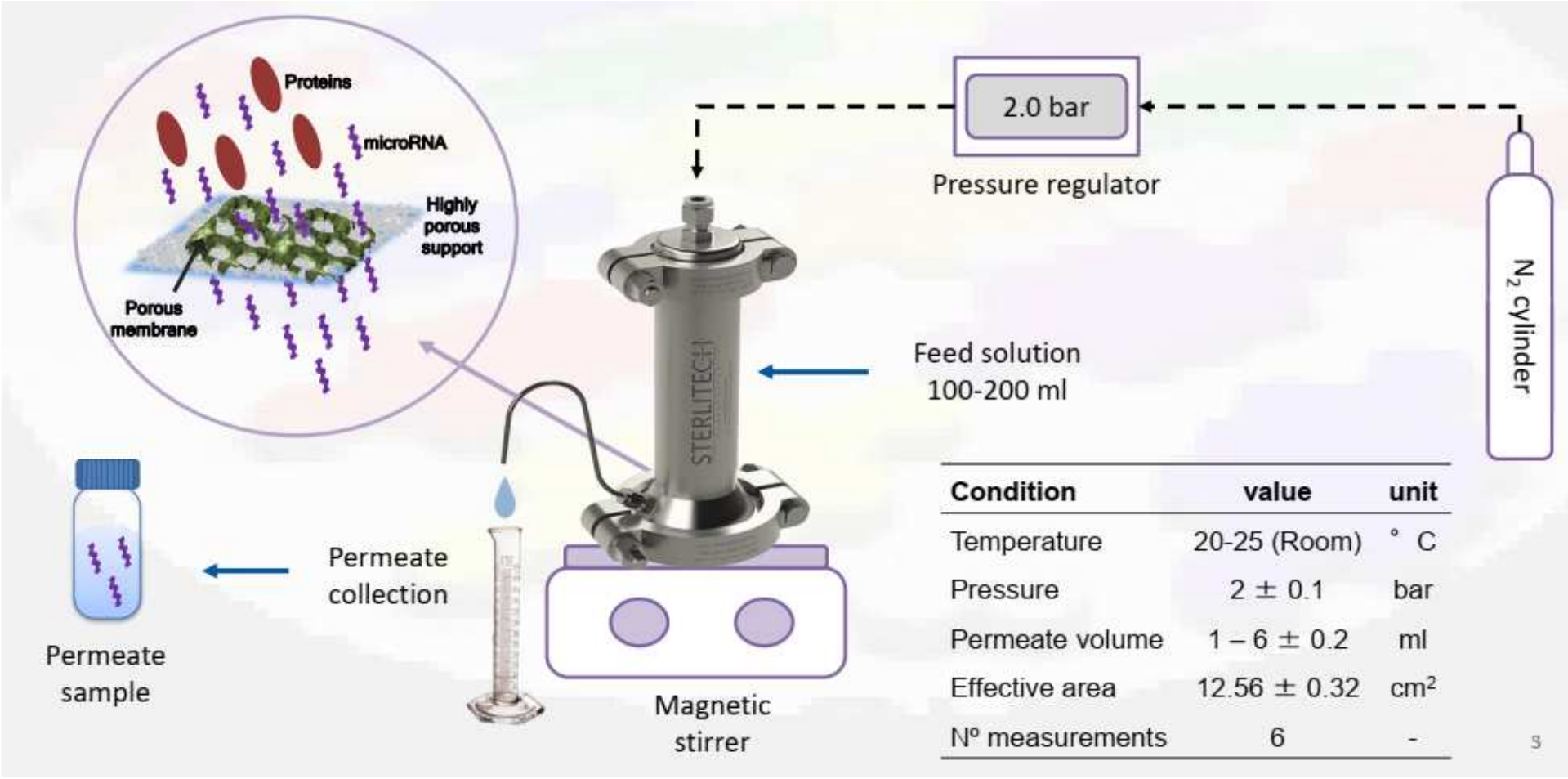


- **MicroRNA:**

  - Represented as synthetic oligos DNA of Target 21: **TAG CTT ATC AGA CTG ATG TTG A**
  - 1 µmole DNA Oligo (Integrated DNA Technologies)
  - The sample was resuspended and diluted at 100 µM
  - Aliquots of 500 µL were separately frozen for further use

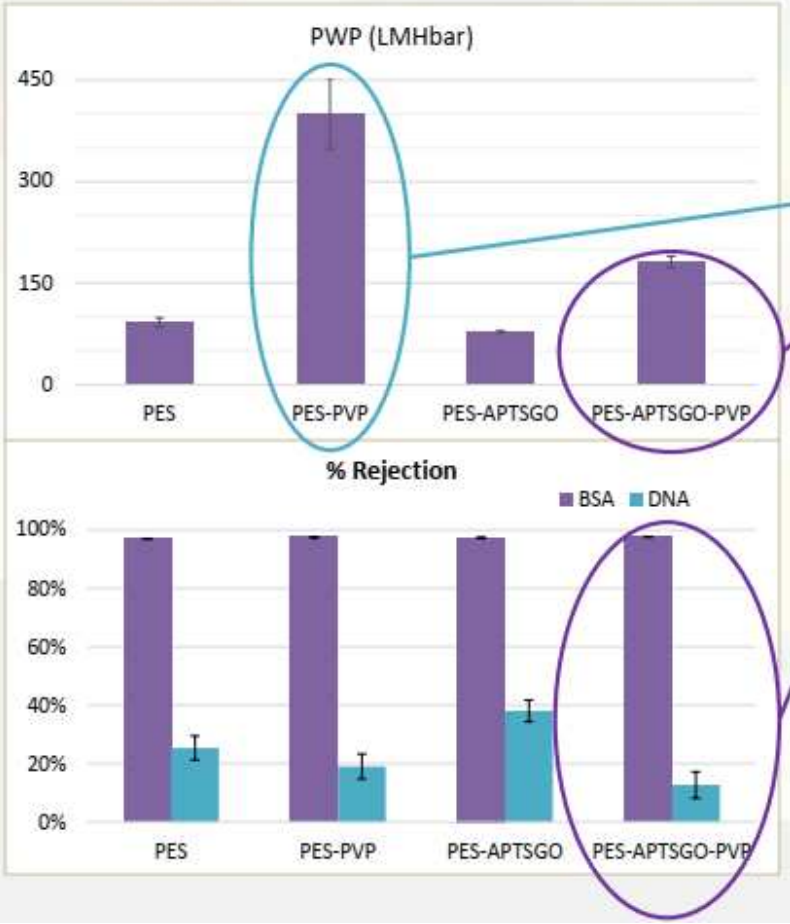
Solute	Concentration range	Unit
BSA	60 - 80	mg/ml
Oligo DNA	1 - 100	nM

Biological filtration membranes



Condition	value	unit
Temperature	20-25 (Room)	° C
Pressure	2 ± 0.1	bar
Permeate volume	1 – 6 ± 0.2	ml
Effective area	12.56 ± 0.32	cm^2
Nº measurements	6	-

Biological filtration membranes



Pure water permeance:

- Pore former based membranes show the largest permeability:
  - PES-PVP: 401 ± 52 LMHbar (L m^{-2} $h^{-1}bar^{-1}$)
  - PES—APTSGO-PVP: 181 ± 9 LMHbar

Membrane selectivity:

- All membranes show protein rejections > 97%
- PES-APTSGO-PVP membranes show best selectivity:
  - Lower DNA rejection: 12.7% ± 4.5%
  - Largest BSA rejection: 97.7% ± 0.1%

Biological filtration membranes

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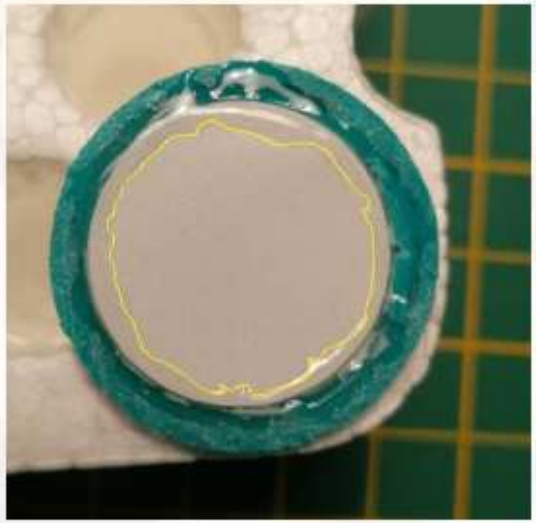
Implementation of developed membranes into commercial cartridge

Supported Membrane area:
3.9 cm^2



For ΔP of 1 bar → PES-PVP: 2.61 mL/min
PES—APTSGO-PVP: 1.18 mL/min

Next steps...

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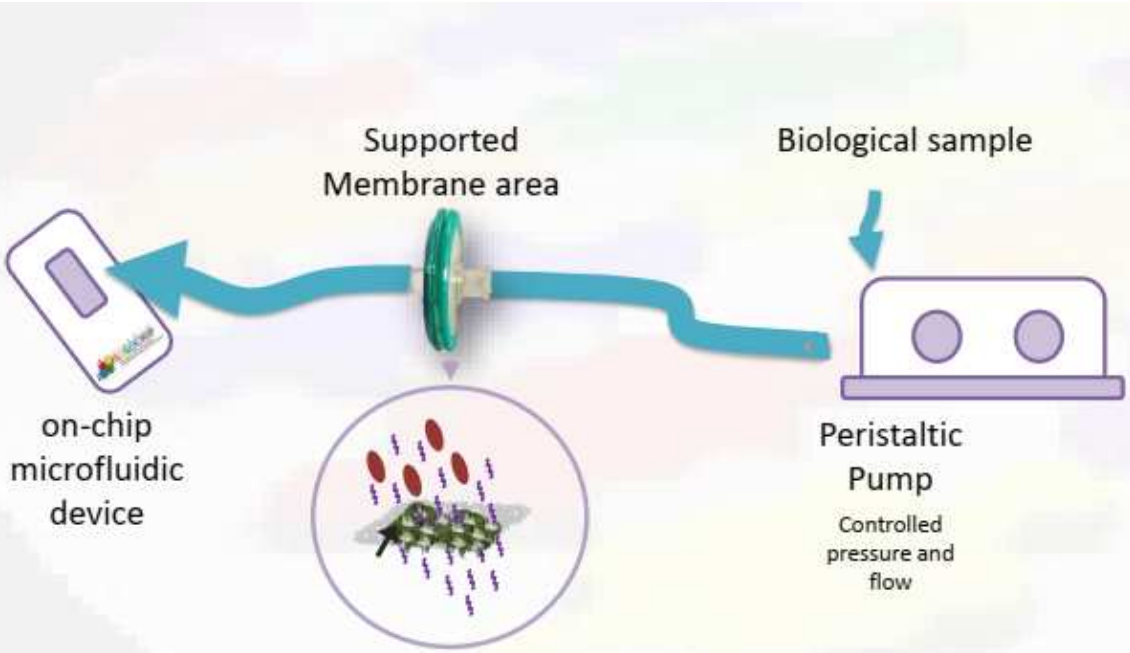
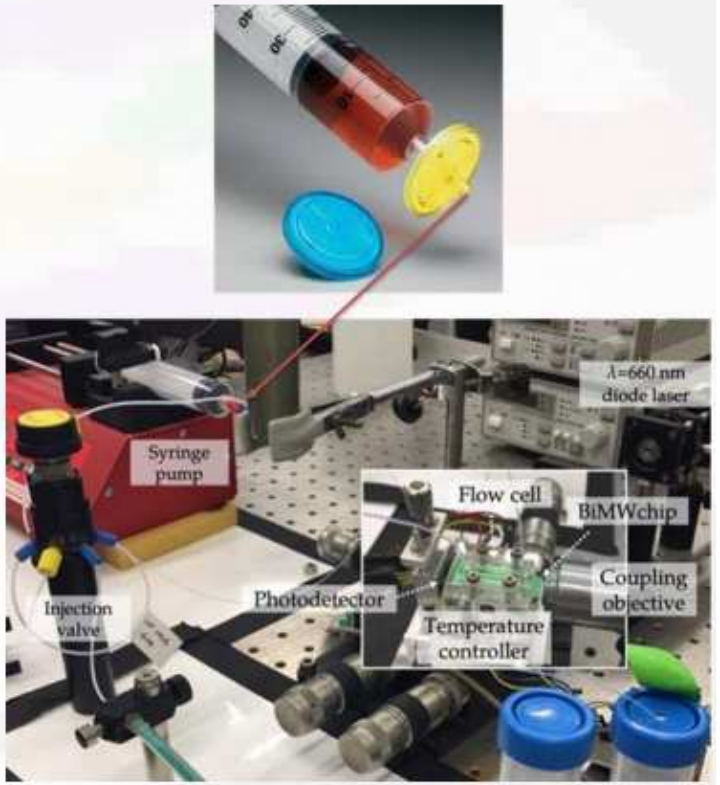
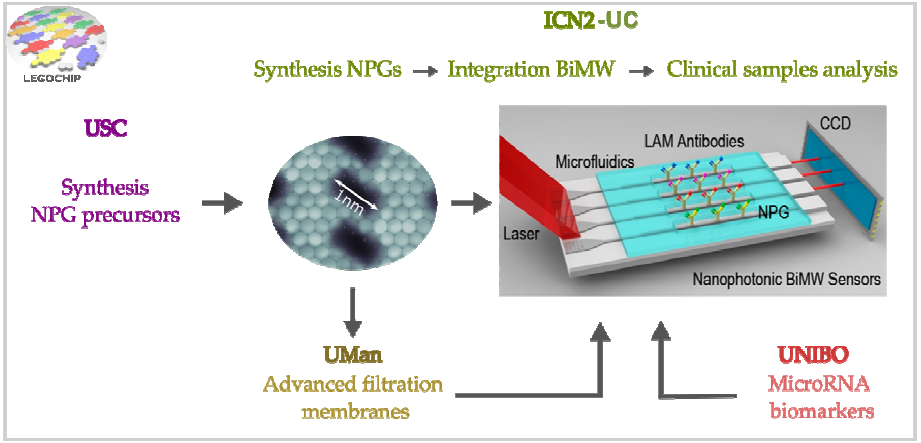
Implementation of developed cartridge into biosensing setup





Publications



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Mirco Panighel,* Sabela Quiroga, Pedro Brandimarte, Cesar Moreno, Aran Garcia-Lekue, Manuel Vilas-Varela, Dulce Rey, Guillaume Sauthier, Gustavo Ceballos, Diego Peña,* and Aitor Mugarza*

non-coding RNA 2021 MDPI

Article

MicroRNA Isoforms Contribution to Melanoma Pathogenesis

Elisabetta Broseghini [1], Emi Dika [1,2], Eric Londin [3] and Manuela Ferracin [1,*]

OL | Organic Letters 2021

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REVIEW

Circulating microRNA biomarkers in melanoma and non-melanoma skin cancer

Giorgio Durante [a], Elisabetta Broseghini [a], Francesca Comito [b], Maria Naddeo [a], Massimo Milani [a,c], Irene Salamon [a], Elena Campione [d], Emi Dika [a,e] and Manuela Ferracin [a]

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Polycyclic Aromatic Hydrocarbons Hot Paper

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An on-surface Diels–Alder reaction

Jesús Castro-Esteban[+], Florian Albrecht[+], Shadi Fatayer, Dolores Pérez, Leo Gross, and Diego Peña**