

# **Desenvolvimento de fases de afinidade *para a separação de glicoproteína e análise***

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**Brasil**  
**May 2013**



**Dr. Brendan O'Connor**  
*Biotechnology & Protein Biochemistry*

**Dr. Dermot Walls**  
*Biotechnology & Virology ?*

**Dr. John Gallagher**  
*Chemical Sciences & Crystallography ?*

# Equipe multidisciplinária para promover colaboração entre universidades brasileiras e irlandesas ;

- *a colaboração na investigação*
- *intercâmbio de **estudantes***
- *intercâmbio de **pessoal docente***
  - *3 ou 6 meses*
  - *1, 2, 3 anos*

**‘Ao longo dos próximos cinco anos, **sete** dos **dez** medicamentos comercializados em todo o mundo será biológicos’**

**Randall Hyer**, Global Head, **Sandoz** Agosto 2012

**Estes agentes biológicos são principalmente **glicoproteínas****



# Farmacologia na Irlanda

- **13 de 15** mundos principais empresas farmacêuticas
- Representa **52%** exportações merchandise da Irlanda (**2012**)
- Prioridade número 1 Nacional
- Prioridade para Educação e pesquisa

**Johnson & Johnson**

**Eli Lilly**

**Merck, Sharp and Dohme**

**Pfizer-Wyeth**

**Schering-Plough**

**Bristol-Myers-Squibb**

**Amgen**

**Allergan**

**Glaxo Smith Kline**

**Aventis , Novartis**

# Farmacologia na Brasil

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## Brazilian Biosimilars Market

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[Atrisa](#) [Bionovis](#)  
[Brazil](#) [Datamark](#)  
[Novartis](#)  
[Omnitrope](#)  
[Orygen](#) [Sandoz](#)

According to a news release by Datamark, a [market research firm](#) which was founded in 1982, Sandoz wants to expand its presence in Brazilian Market.

World leader in biosimilar medicines, Sandoz, the [generics](#) arm of pharma giant Novartis, already sells the growth hormone Omnitrope since June last year in Brazil and has two other products approved for [cancer treatment](#) in European and American markets, in addition to a pipeline with eight to ten products under development in this segment.

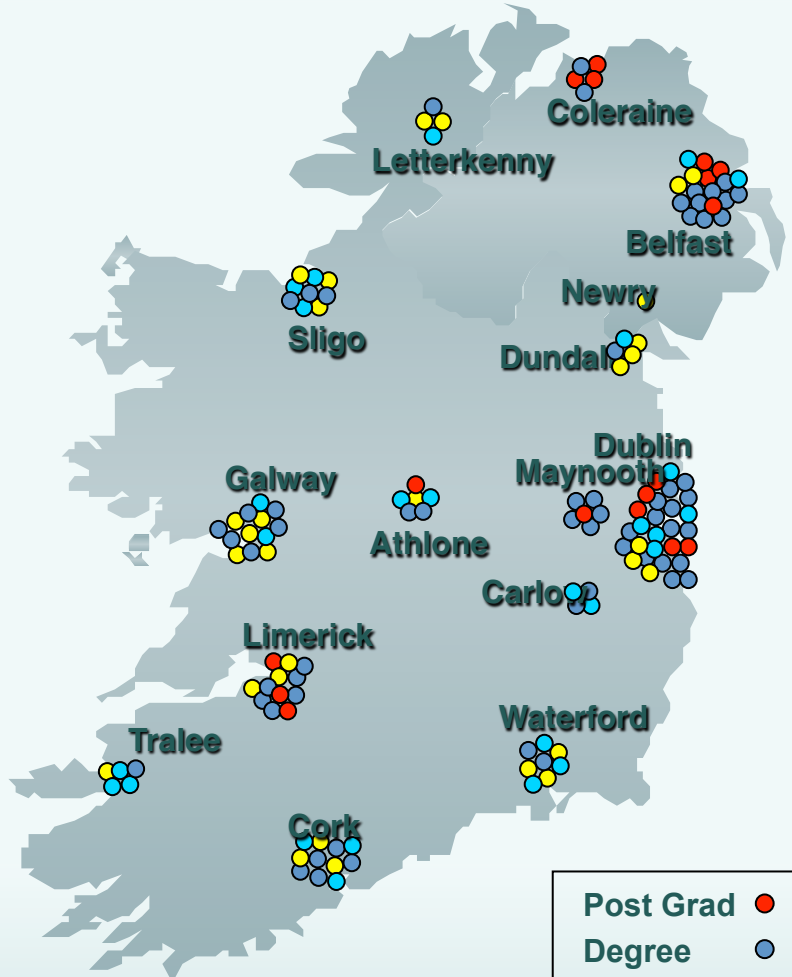
In an interview with Randall Hyer, Global Medical Affairs Head at Sandoz Biopharmaceuticals – said that biosimilar drugs have an important role to increase access [of the population](#) to treat serious illnesses, like cancer and rheumatoid arthritis, for example. "The medicines [biological] are still very expensive. The biosimilar drugs have a solution to this problem, providing the same high biological quality and greater access."

No Brasil, o governo está apoiando duas empresas recém-criadas, a **Bionovis** (Aché, EMS, Hypermarchas e União Química) e **Orygen** (Biolab, Eurofarma, Cristália e Libbs), querendo ser as primeiras empresas nacionais a desenvolver **biofarmacos**.

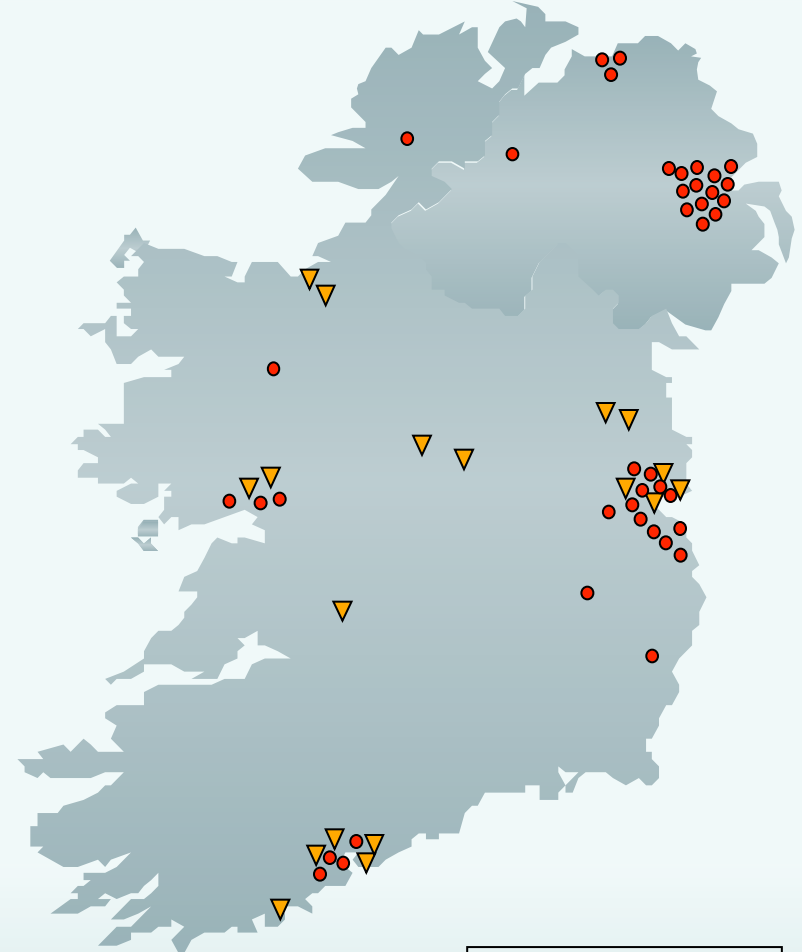
- **A Irlanda é um dos maiores exportadores de produtos farmacêuticos em todo o mundo**
- **Employment alta qualidade**
- **50%** dos funcionários são graduados
- **30%** dos funcionários são licenciados em ciências

# Biotecnologia em Irlanda

## Biotecnologia - academia



## Biotecnologia - indústria



|                              |   |
|------------------------------|---|
| <b>Johnson &amp; Johnson</b> | <b>Procrit<sup>®</sup> Remicade<sup>®</sup></b> |
| <b>Pfizer</b>                | <b>Viagra<sup>®</sup></b>                       |
| <b>Schering-Plough</b>       | <b>Intron A<sup>®</sup></b>                     |
| <b>Amgen</b>                 | <b>Neupogen<sup>®</sup></b>                     |
| <b>Biogen</b>                | <b>Avonex<sup>®</sup></b>                       |
| <b>Genentech</b>             | <b>Rituxan<sup>®</sup></b>                      |
| <b>Wyeth / Amgen</b>         | <b>Enbrel<sup>®</sup></b>                       |
| <b>Wyeth</b>                 | <b>Prevenar<sup>®</sup></b>                     |
| <b>Eli Lilly</b>             | <b>Humulin R<sup>®</sup></b>                    |
| <b>Allergan</b>              | <b>Botox<sup>®</sup></b>                        |
| <b>Genzyme</b>               | <b>Renagel<sup>®</sup></b>                      |

Produzido na Irlanda

# Aumento da demanda por novas ferramentas de análise-glico

## ❑ Indústria bio-farmacêutica

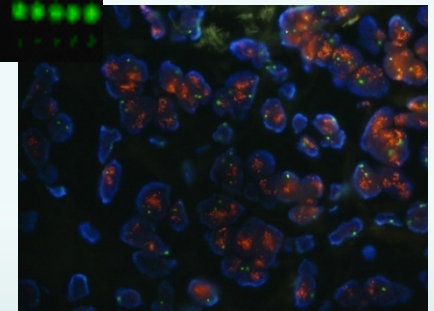
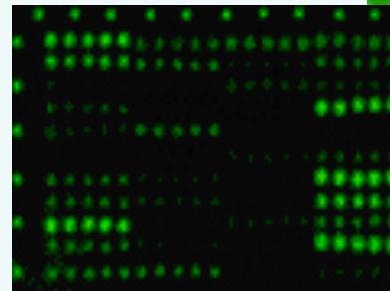
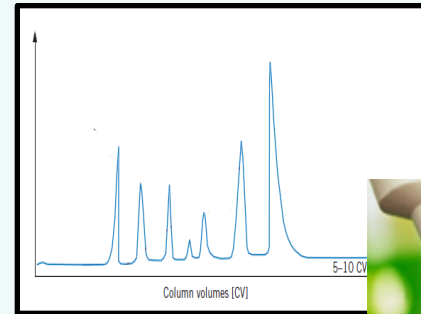
muitas novas terapêuticas são glico-proteínas

## ❑ Diagnósticos médicos

muitas alterações precoces do câncer são glico relacionado

## ❑ Ferramentas de investigação

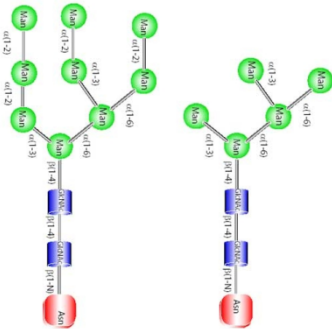
significado biológico da glico-proteína é de grande importância para a pesquisa básica



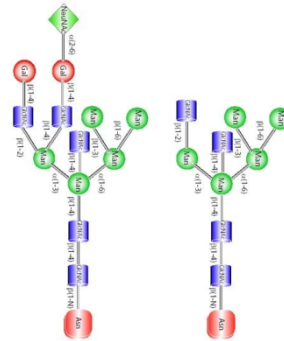
# Glicosilação - essencial para a vida

## □ Diversidade estrutural e biológica

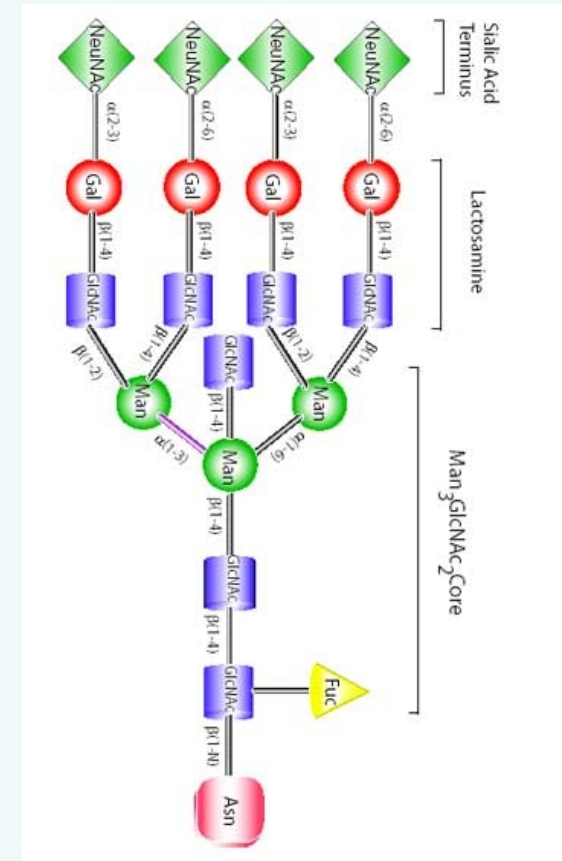
- Glicosilação - adição de açúcares
- N-ligados e O-ligados açúcares



N-ligados manose      Galactose



Glicanos complexos





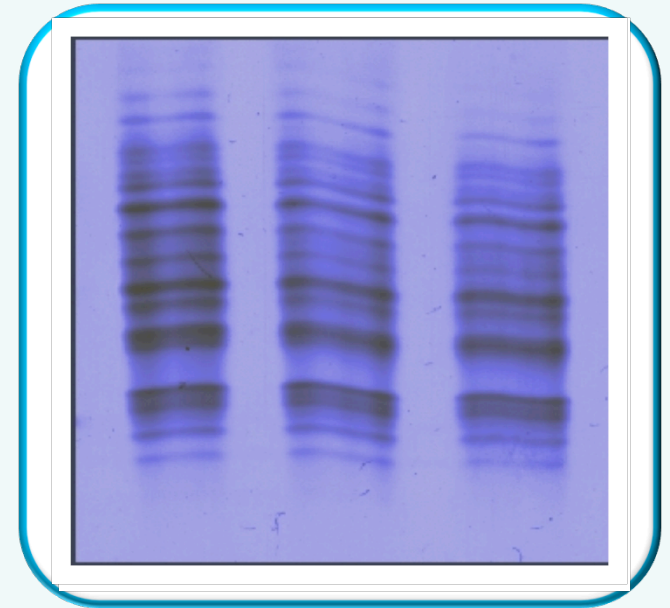
# Glico-formas - problema para a indústria !!

## ❑ Glico-formas - difícil separar

**P.A.G.E. Análise v I.E.F. Análise**

**Glico-formas –**

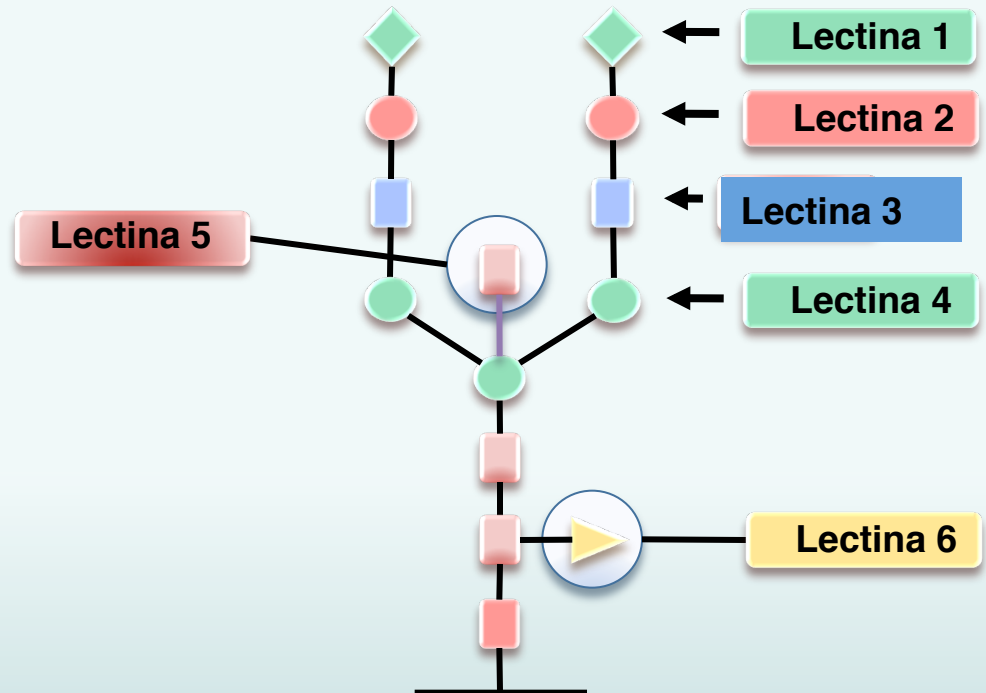
- **Força terapêutica diferente**
- **Diferente semi-vida**
- **Antigenicidade diferente**



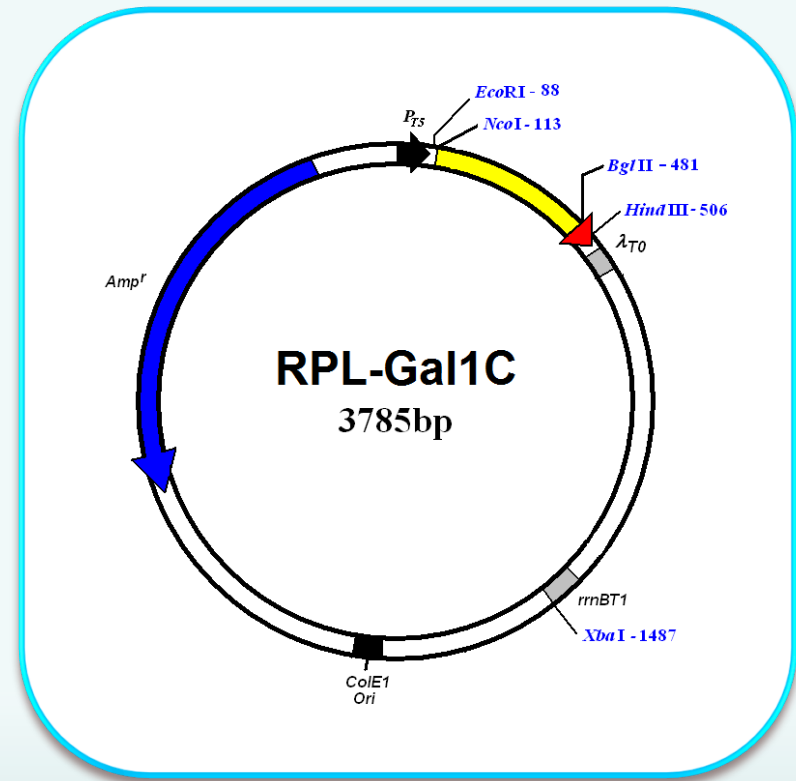
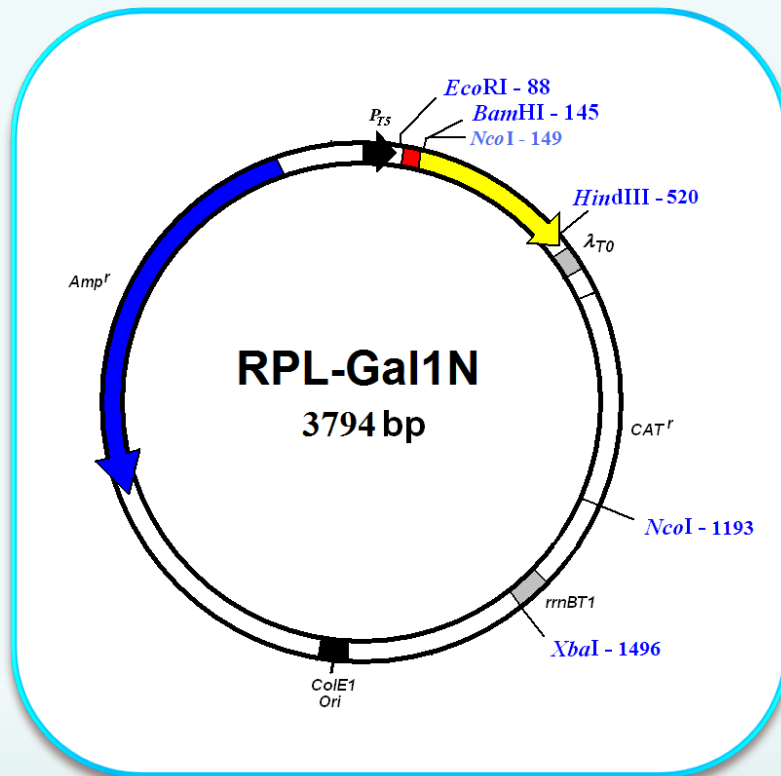
**Necessita de ser capaz de separá-las**

- ❑ **Lectinas** - proteínas que se ligam glicanos
- ❑ Usado para criar **superfícies de afinidade** para Glico-proteína de separação e análise

análise bio

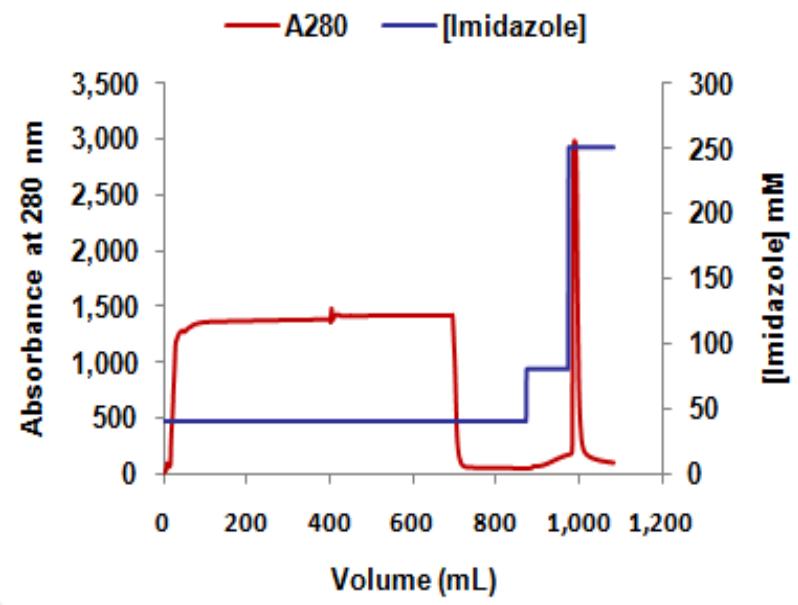


## Cloning em *Escherichia coli* .....

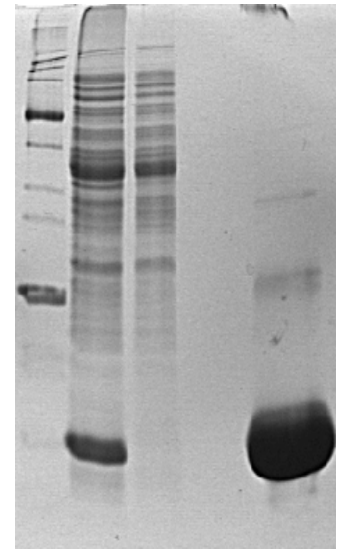


## Expressa e purificar lectinas

### IMAC

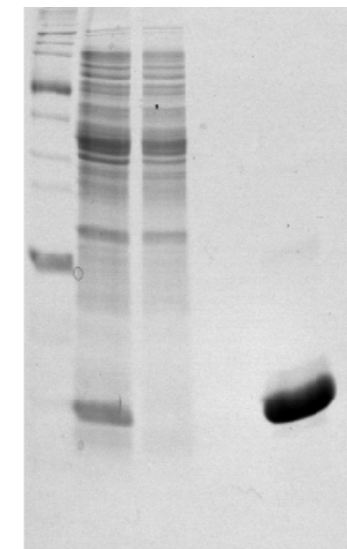


### RPL-Gal1N



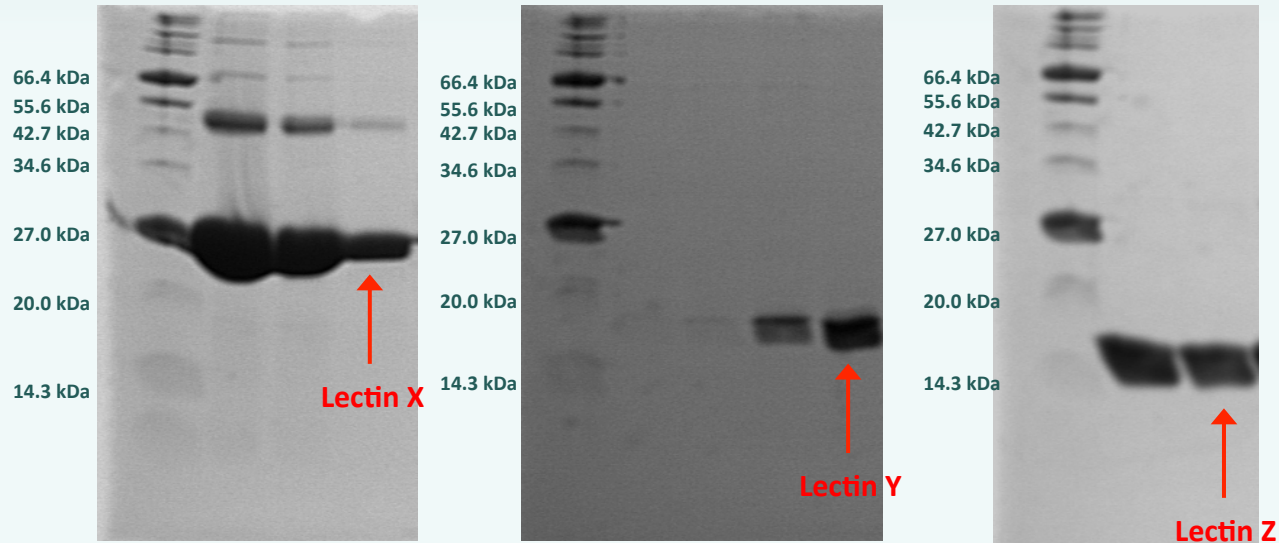
Yield = 150 mg L<sup>-1</sup>

### RPL-Gal1C



Yield = 100 mg L<sup>-1</sup>

# Clonado e expressa Lectinas



- Clone , expressa e purificar lectinas
- fontes microbianas

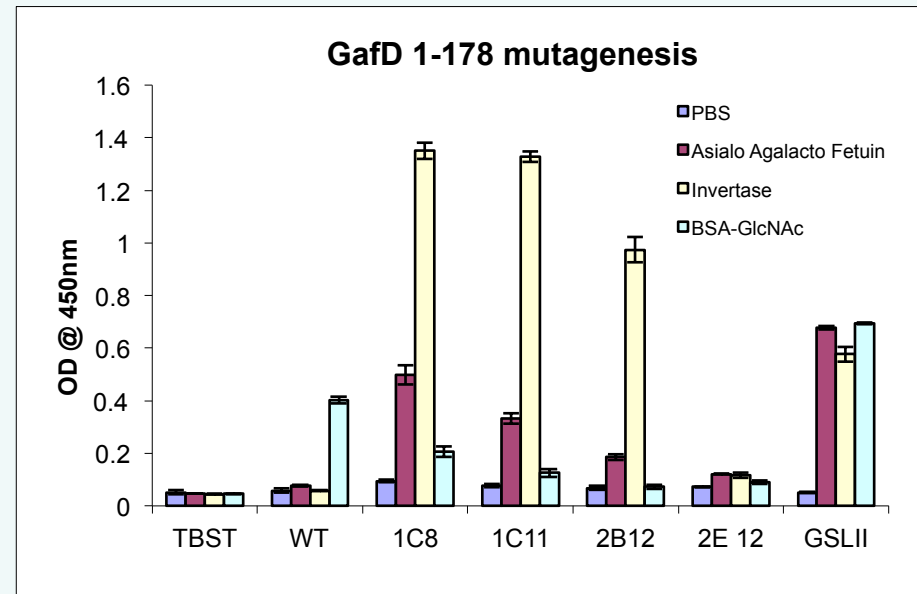
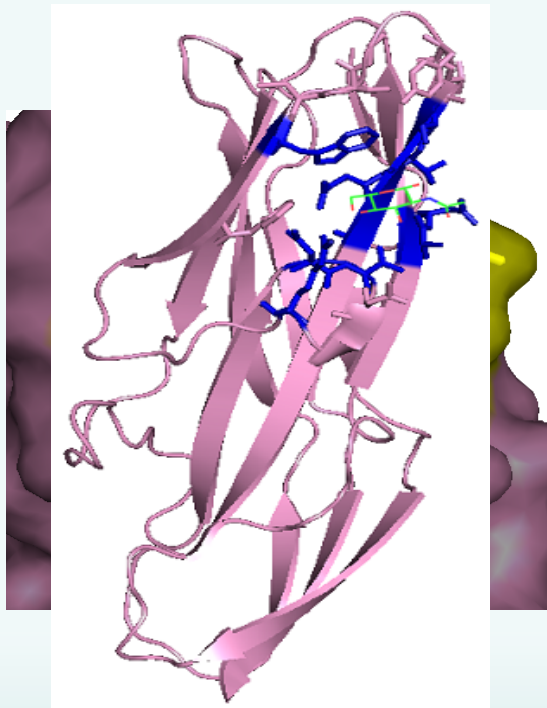
Roisin Thompson, Ruth Larragy, Damian Keogh, Brendan O'Connor, Michael O'Connell and Paul Clarke. 'Exploring the specificity determinants of the *Pseudomonas aeruginosa* PA-IL lectin through site specific mutagenesis'. (2013) *Biochem. Biophys. Acta* , In Press

Damian Keogh,,Ruth Larragy, Ken McMahon, Michael O'Connell, Brendan O'Connor, Roisin Thompson and Paul Clarke. 'Generating Novel Recombinant Prokaryotic Lectins with Altered Carbohydrate Binding Specificities and Affinities through Mutagenesis of the PA-IL Protein from *Pseudomonas aeruginosa*'. (2013) *Biochemistry J* , Submitted

- Clone , expressa e purificar lectinas
- Glicano de ligação para.....**Manose, Fucose, GlcNAc ,GalNAc e Sialyic acid**
- Clonado em *E. Coli* os sistemas de expressão
- Expansão da biblioteca lectina - **mutagénesse dirigida**  
*afinidades e especificidades alteradas*

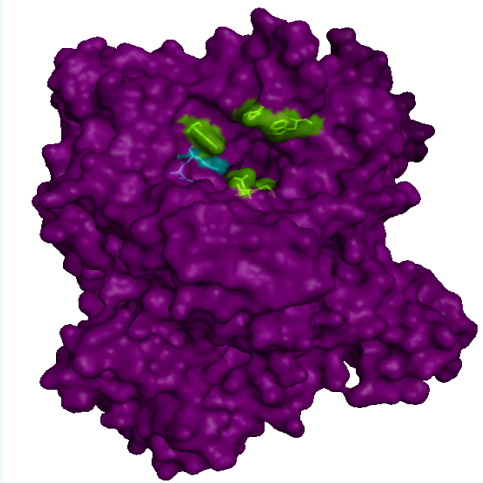
# Lectina-X — se liga GlcNAc

- mutagénesse dirigida - *afinidades alteradas*

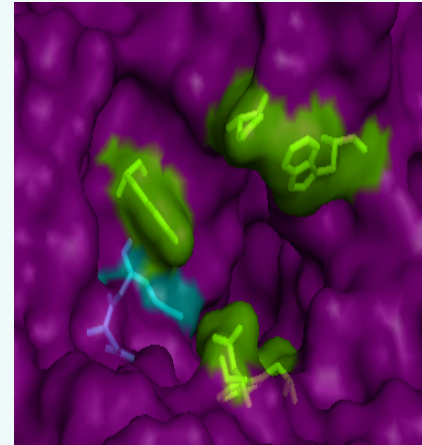


## Mutagénese dirigida de uma enzima

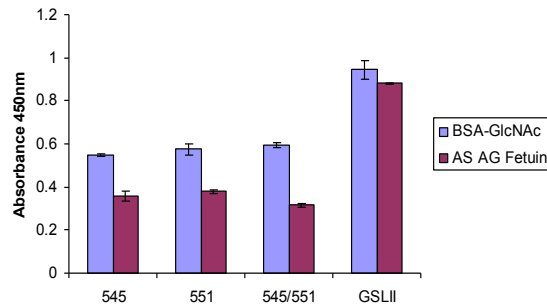
a)



b)

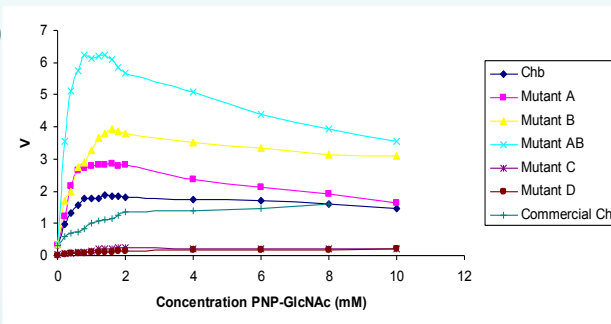


a)



ligação retida

b)



atividade catalizador abolida



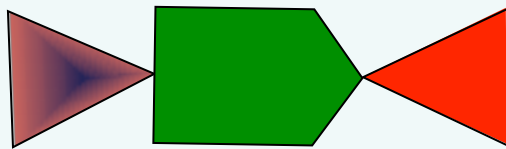
## Toxinas bacterianas – Cry

- Clonado toxinas Cry
- liga-se a **GalNAc** & Tn antigen
- domínio de ligação apenas

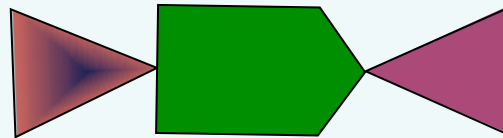


# Imobilização das lectinas

- Combinação de ; tecnologia de ADN recombinante  
imobilização química



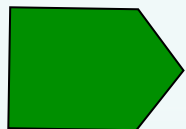
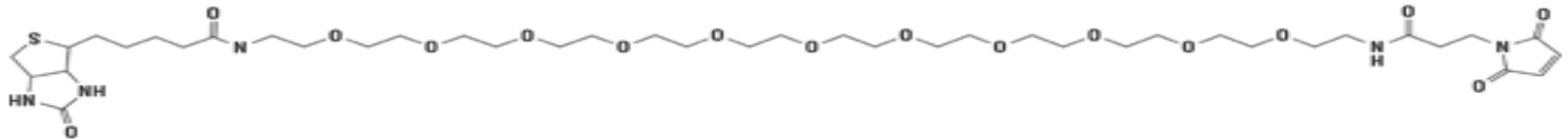
VOS3



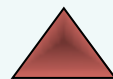
VOS4



VOS12



Lectina



6x Histidine



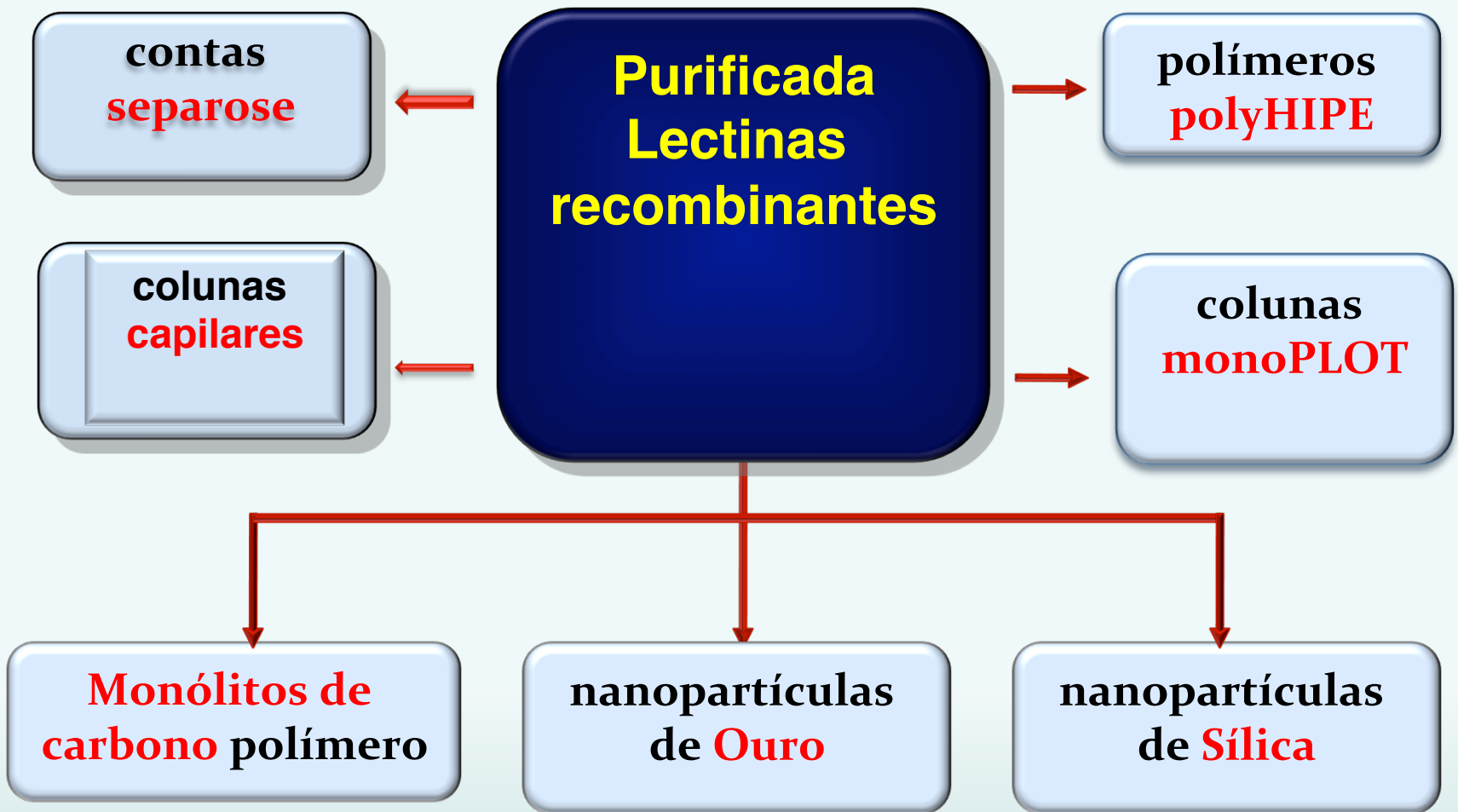
1 Cysteine



6x Lysine

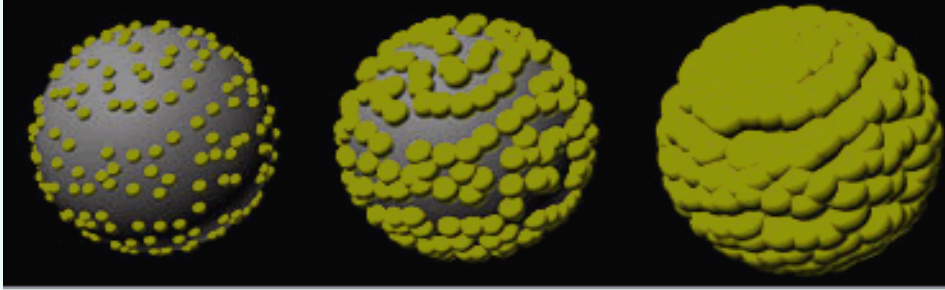


6x Cysteine



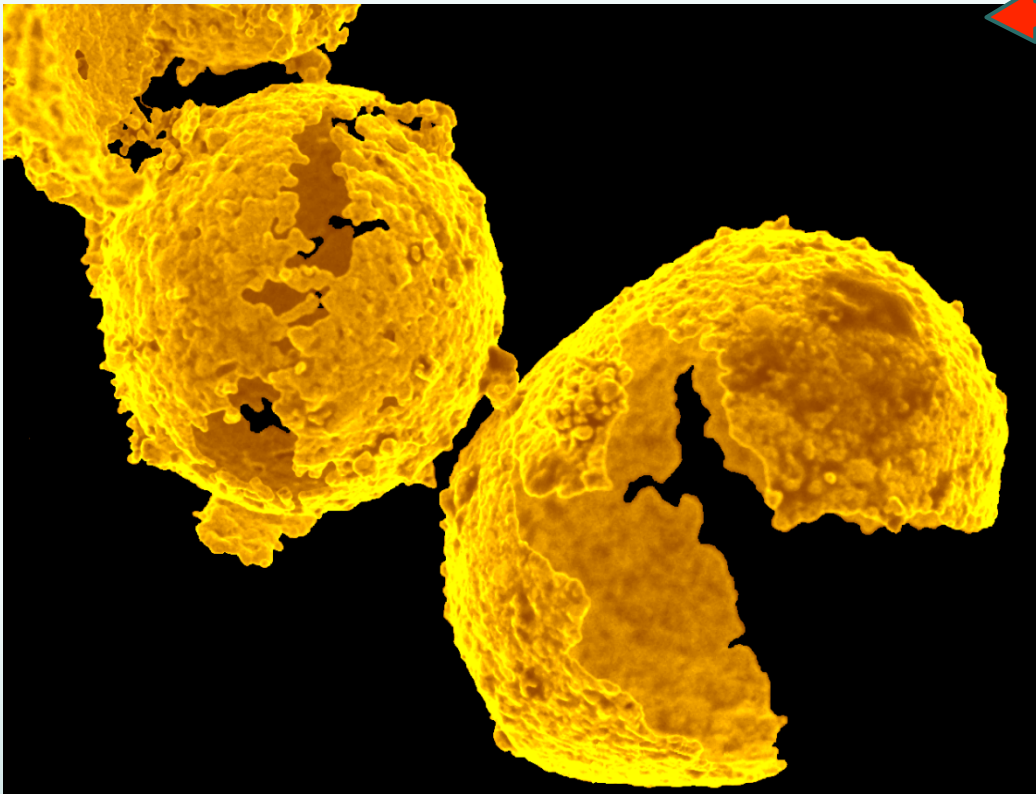


# Nanopartículas de **Ouro** (AuNP)



- microesferas de ouro feita em centro de pesquisa DCU

- muito bom para a imobilização de proteínas

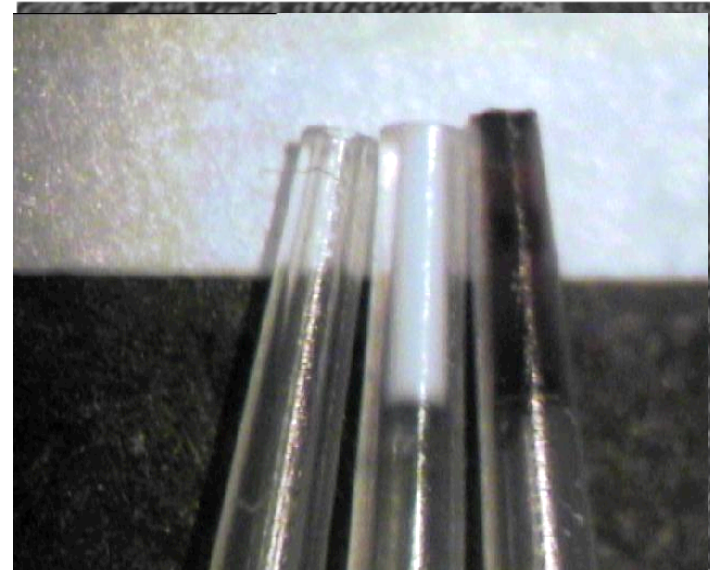
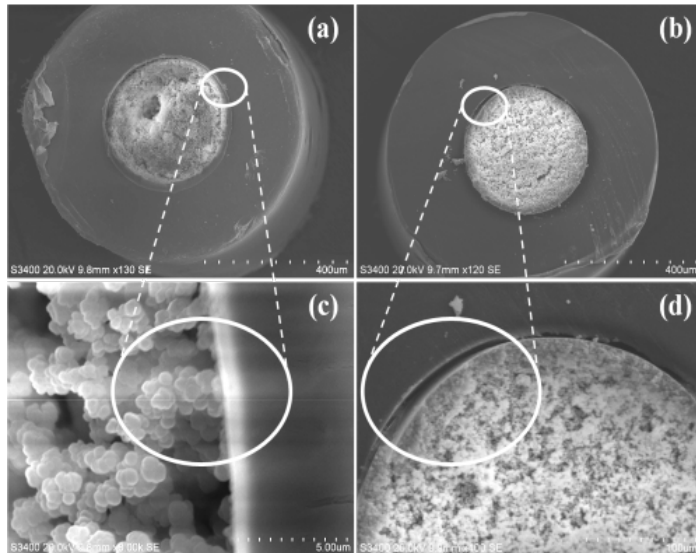
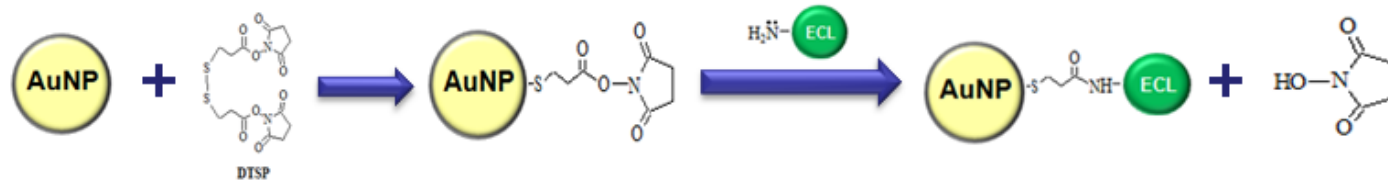


**Lectinas**

Enzimas

Antocorpos

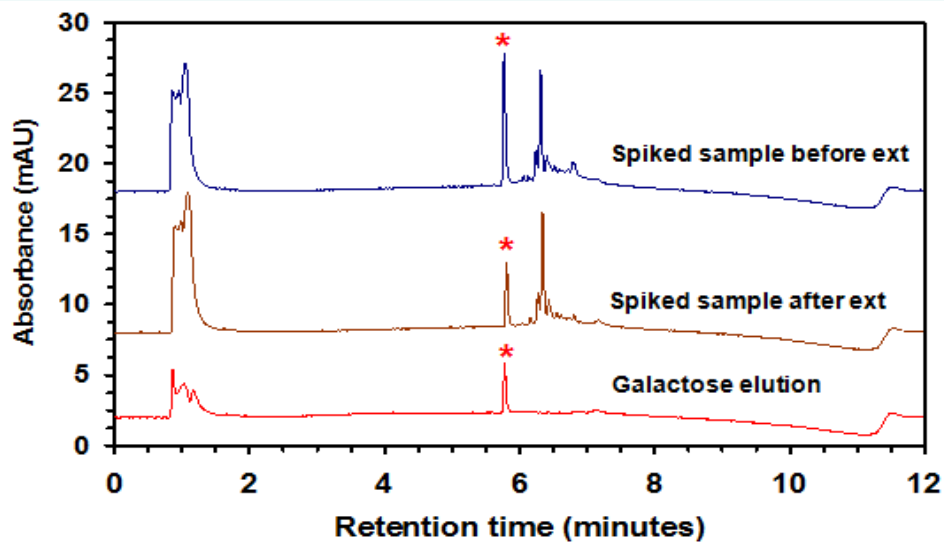
# Lectinas immobilizada em nanopartículas de ouro em monólitos



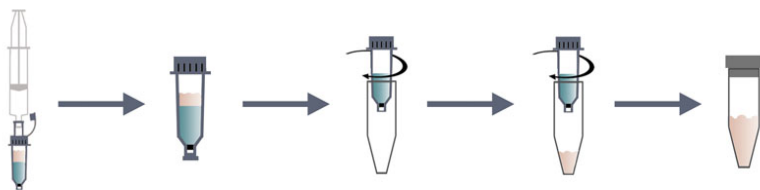
Hassan Alwael , Damian Connolly , Paul Clarke , Roisin Thompson, Brendan O'Connor and Brett Paull. 'Pipette-tip selective extraction of glycoproteins with lectin modified gold nano-particles on a polymer monolithic phase'. (2011) *The Analyst* , 136 , 2619-2628.

## Extracção de glicoproteínas de amostras 'reais'

Galactosylated Glycoproteins from *E. coli* Lysate



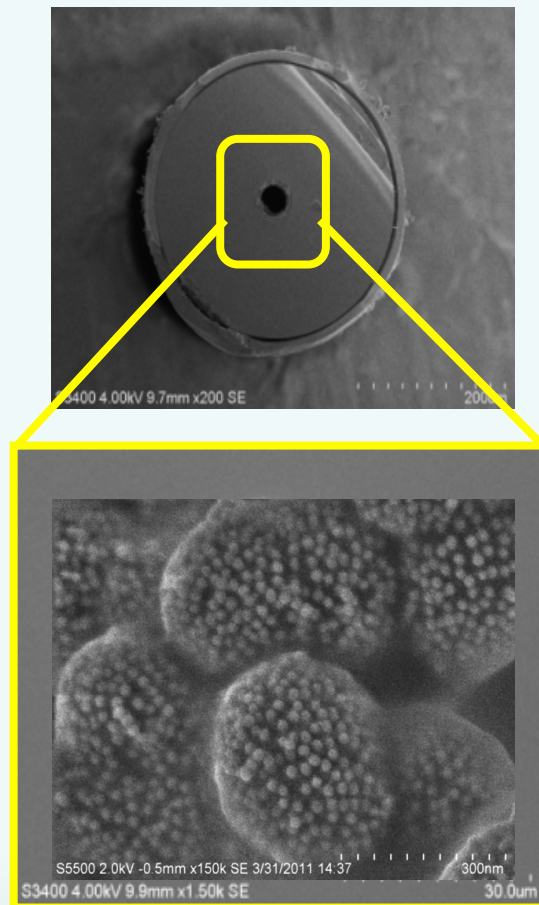
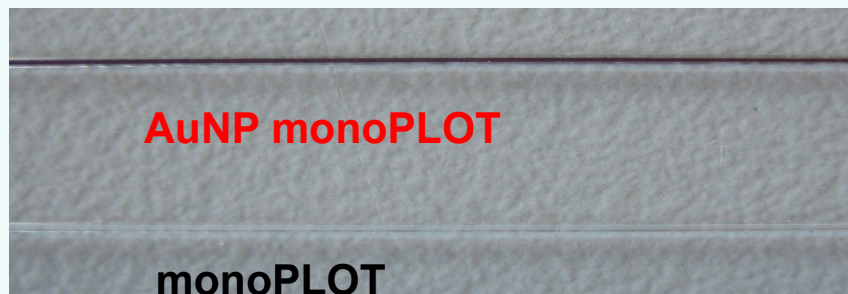
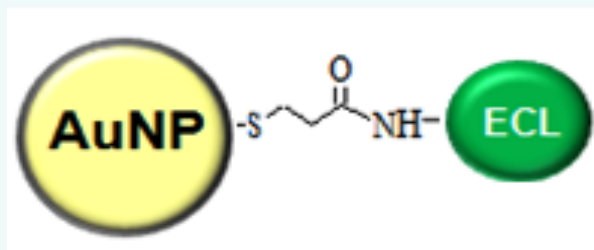
monólito rotação coluna & disco sim





# Lectinas immobilizada em nanopartículas de ouro em monóPLOTS

## Lectina



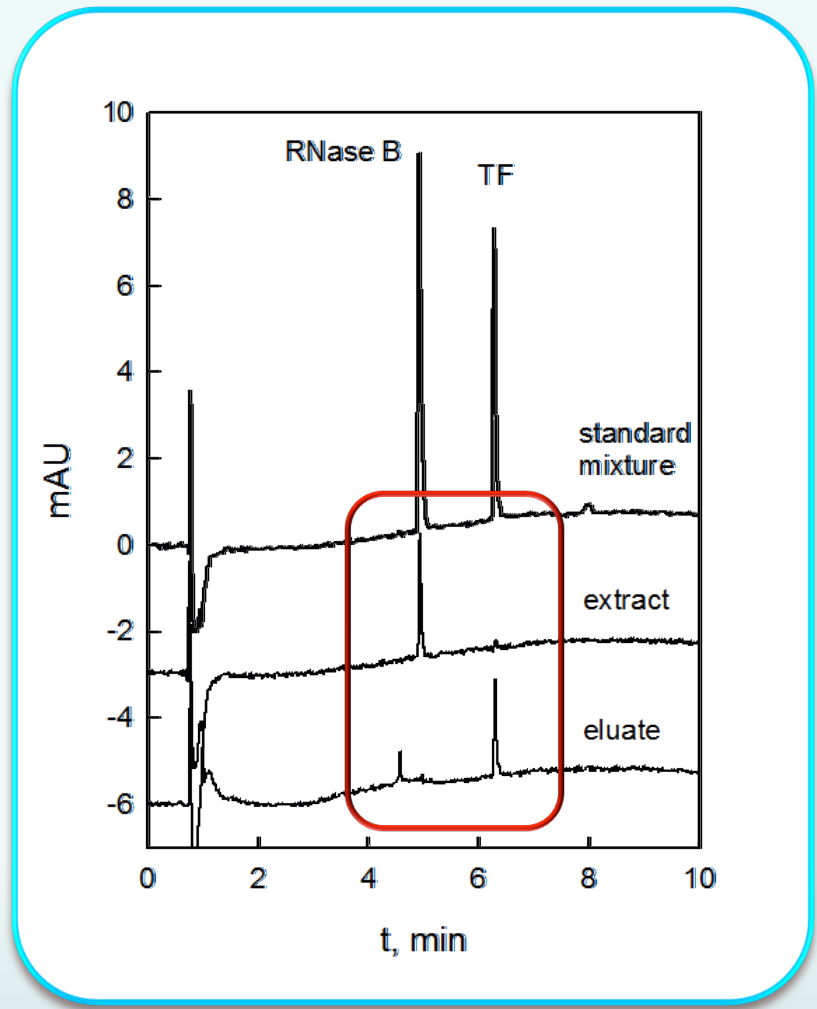
Damian Connolly , Vincent O'Shea , Paul Clarke , Brendan O'Connor and Brett Paull.  
'Evaluation of Photografted Charged Sites Within Polymer Monoliths in Capillary Columns  
using Contactless Conductivity Detection'. (2007) *Journal of Separation Science* , 30 , 17 , 3060-3068.

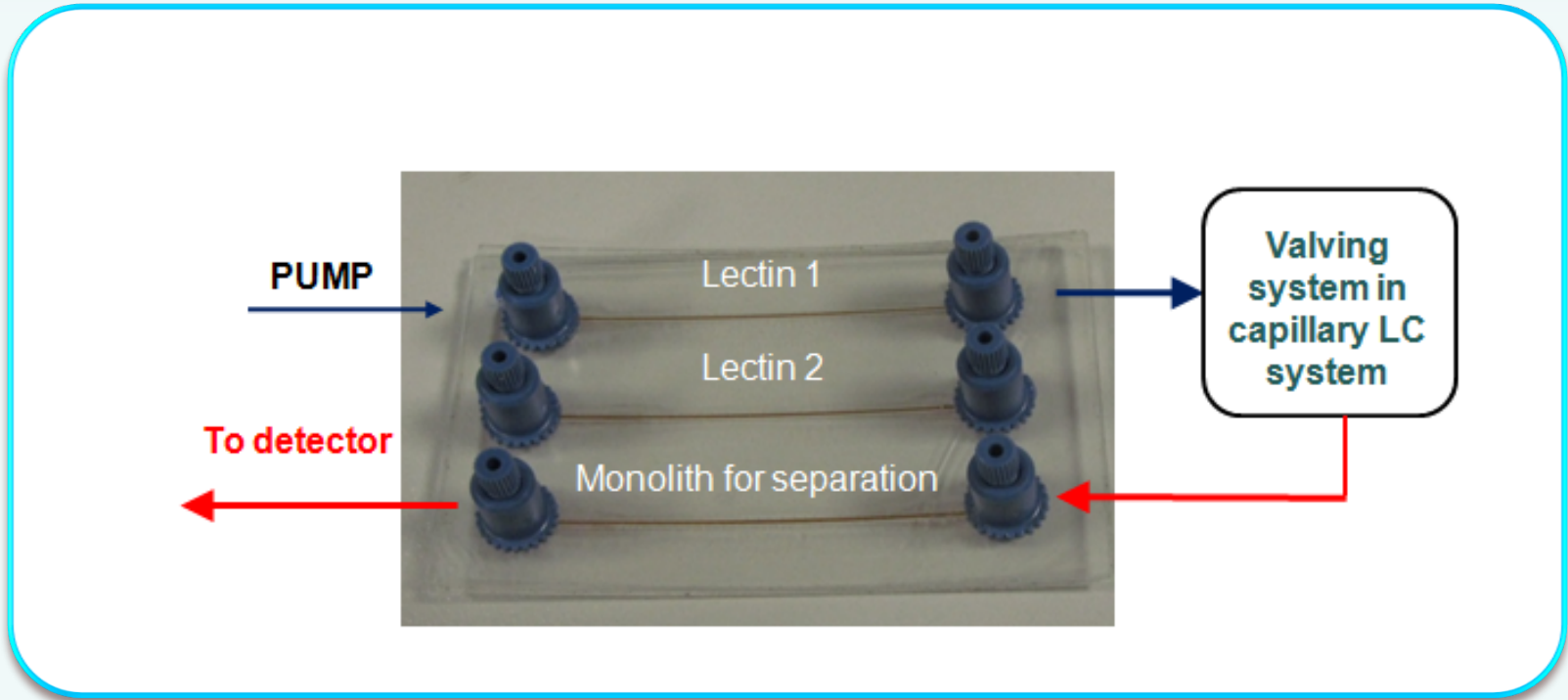


# Lectinas imobilizada em nanopartículas de ouro em **monóPLOTS**

## monoPLOT – AuNP - Lectina

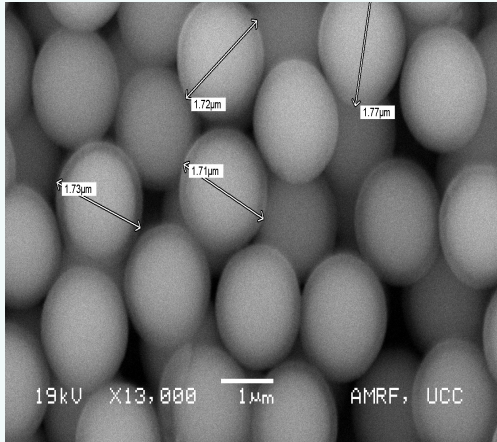
- ❑ separação de glicoproteínas afins
- ❑ liberar por gradiente sem açúcar



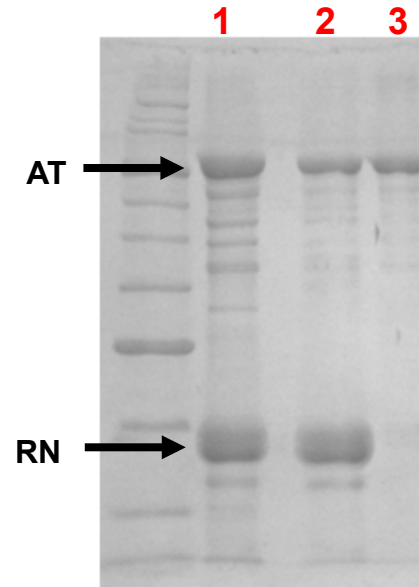


- **multi-lectinas** de separação por afinidade da terapêutica glicoproteína

# Lectinas imobilizada em nanopartículas de sílica



**Fluorescência**  
**lectinas** marcadas  
imobilizada em  
sílica  
nano-partículas



- 1 amostra de teste
- 2 não ligado
- 3 eluição com açúcar livre

# Lectina immobilizada em polyHIPES

## □ HIPE: High Internal Phase Emulsion

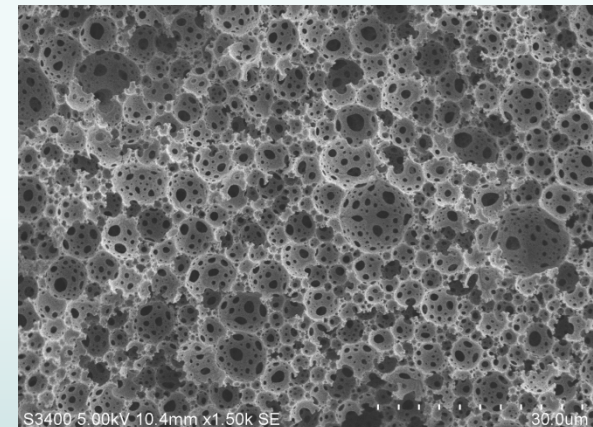
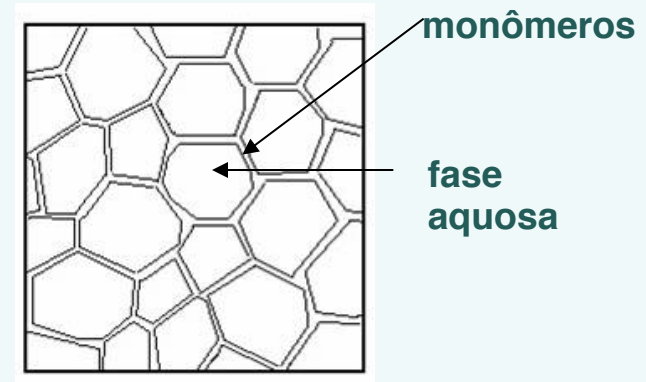
*Emulsão de alta fase interna*

a estrutura de células abertas ;

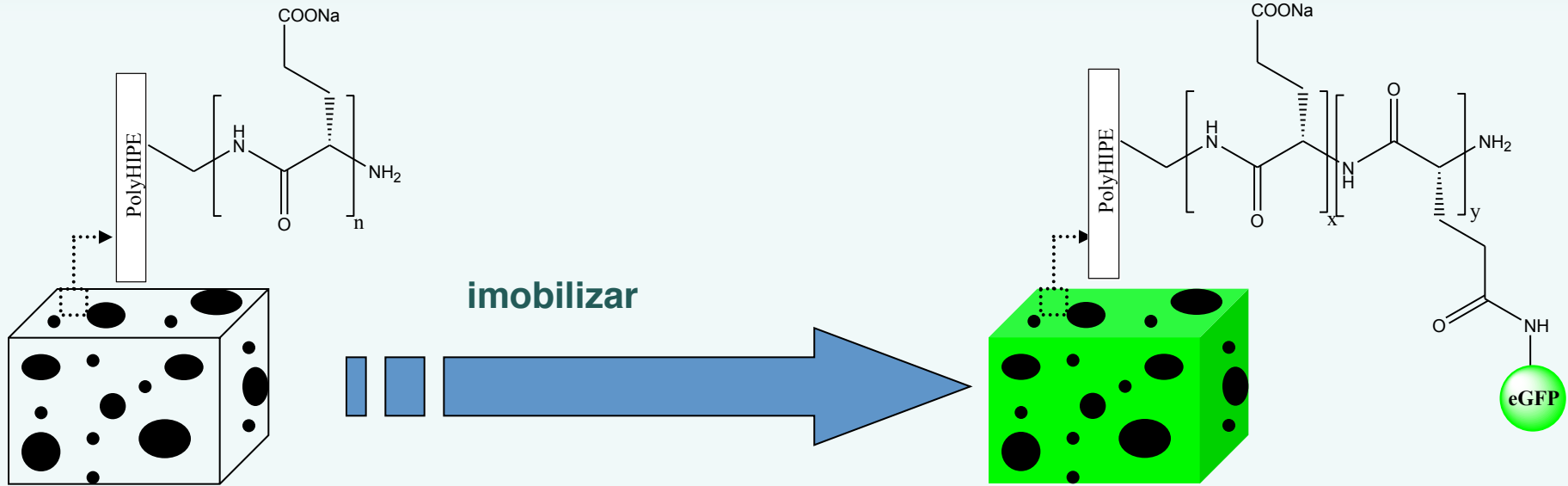
- Macropores: **> 50 nm**
- Mesopores: **2 nm - 50 nm**
- Micropores: **< 2 nm**

□ área de superfície elevada

□ baixo custo



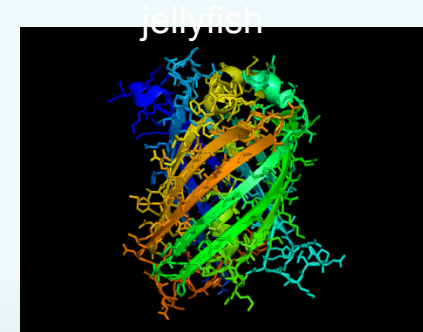
# Lectina imobilizada em polyHIPES



polyHIPE

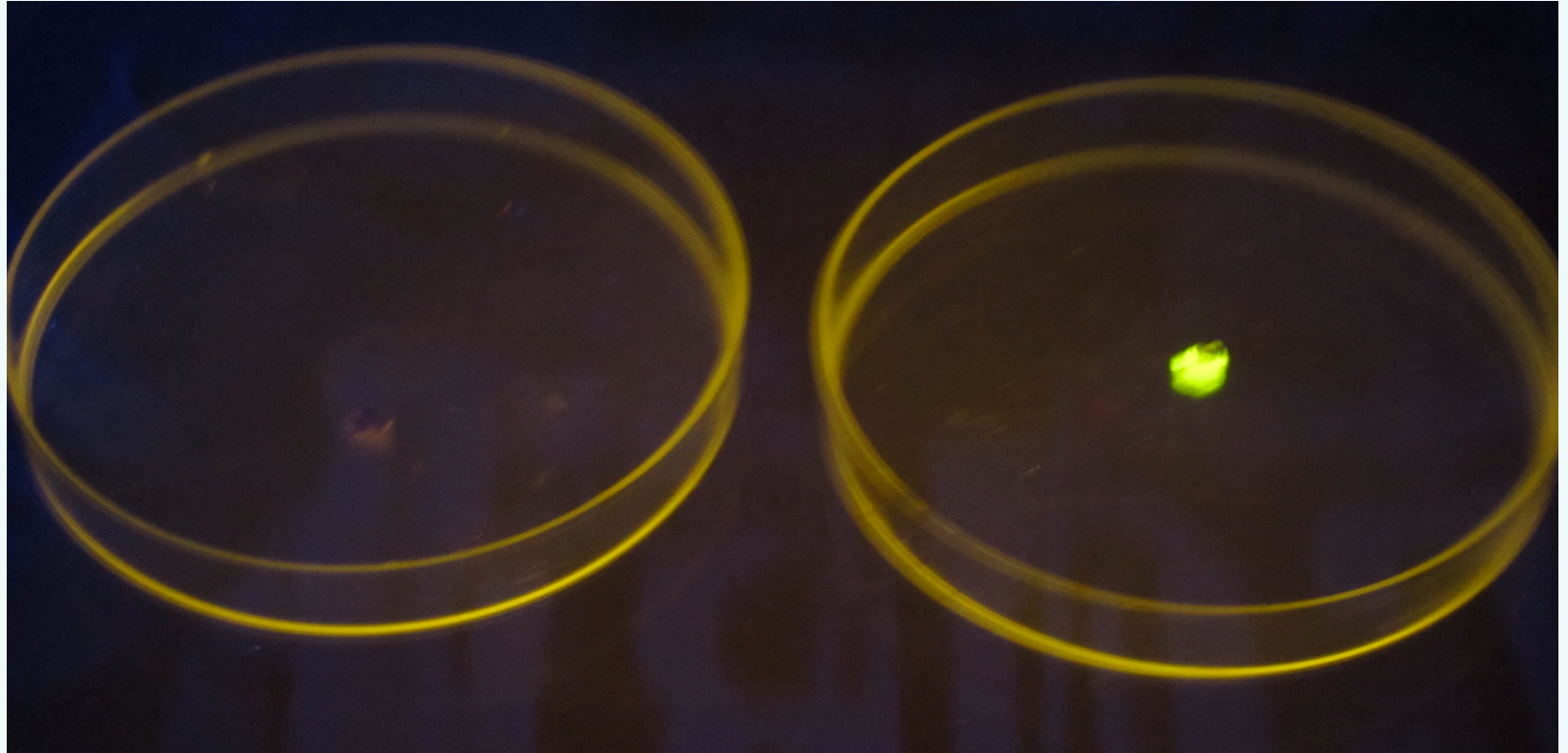


=



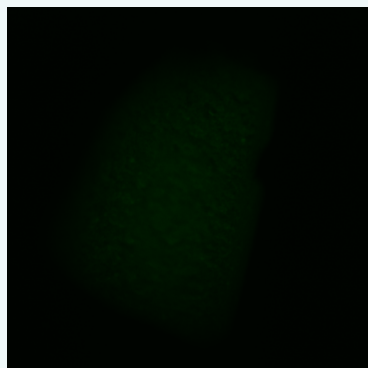
eGFP = fusão GFP lectina (fluorescente)

# Lectina immobilizada em polyHIPES



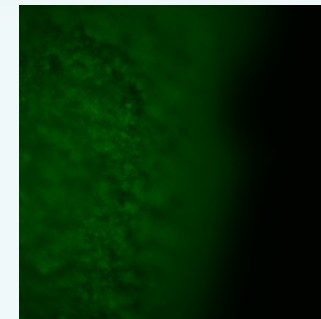
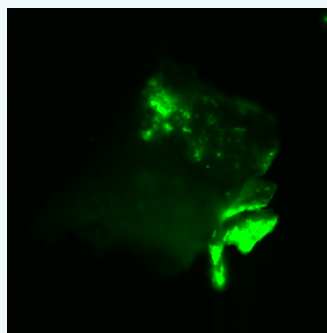


# Lectina immobilizada em polyHIPES

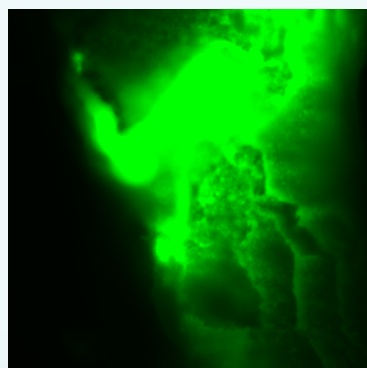


(zoom \*4)

← Ref. pHIPE-*g*-PGA →

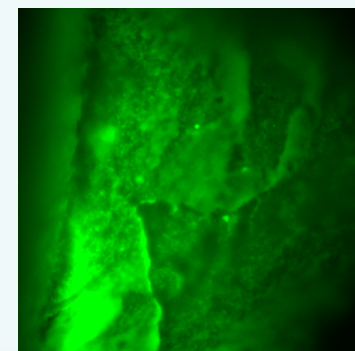


(zoom \*10)



← Phipe-eGFP2 and Phipe-eGFP1 →

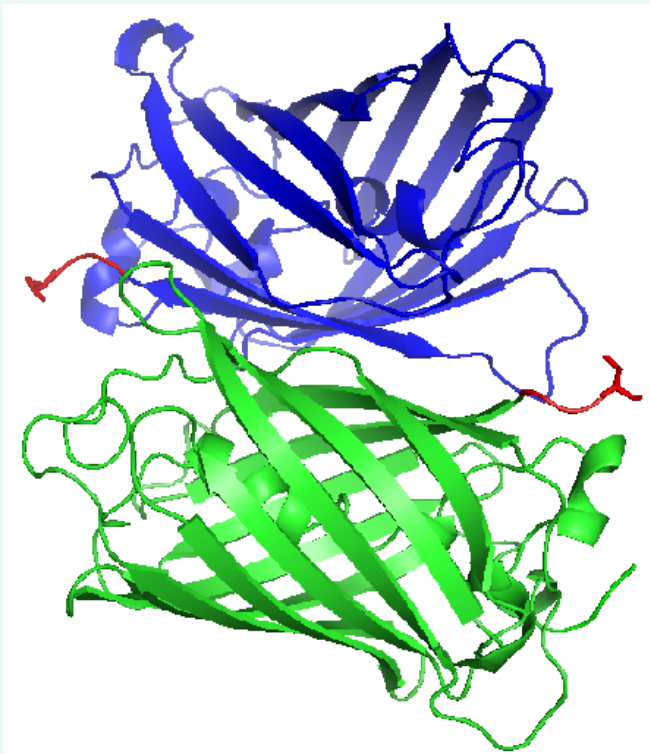
(zoom \*10)



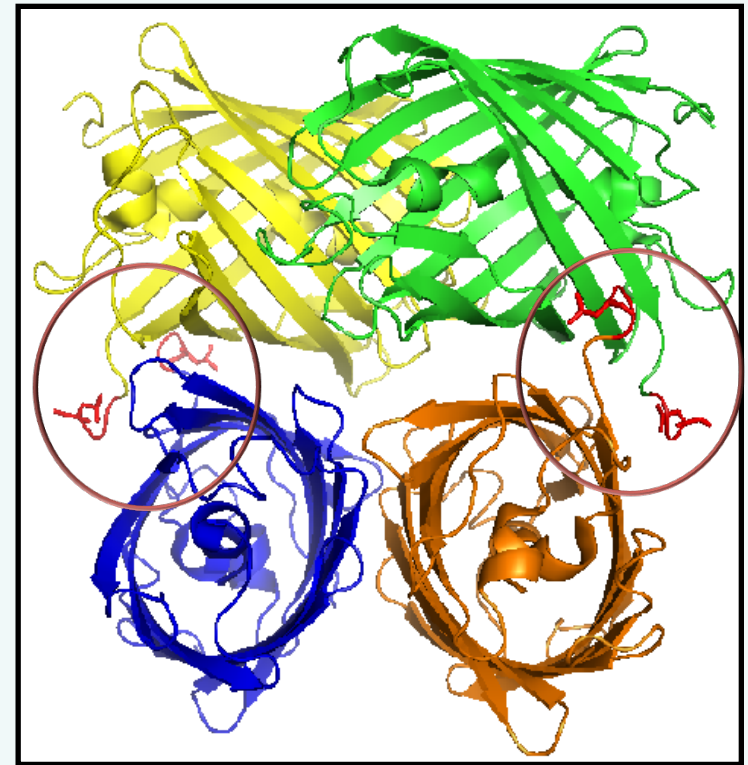
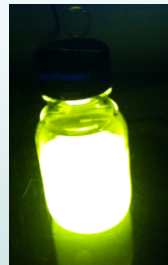
'Polypeptide grafted macroporous PolyHIPE by surface initiated N-Carboxyanhydride Polymerization as a platform for bioconjugation'. *Fabrice Audouin, Mary Fox, Ruth Larragy, Brendan O'Connor and Andreas Heise (2012) Macromolecules, 45, 6127-6135.*

Protein immobilization onto poly(acrylic acid) functional macroporous PolyHIPE via surface initiated ARGET ATRP from multi-tasking amino-polyHIPE precursor. *Fabrice Audouin, Ruth Larragy, Mary Fox, Brendan O'Connor, Andreas Heise (2012) Biomacromolecules, 13, 11, 3787-3794.*

# Ligar - ácido Sialyic SiaA



fundido com  
**GFP**



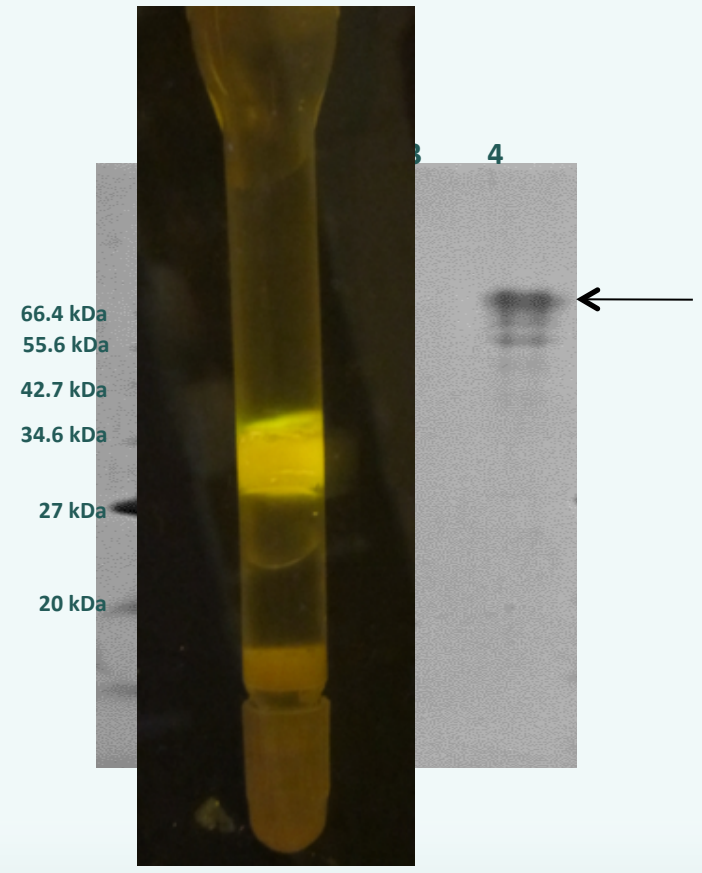
fundido com  
**DsRed**





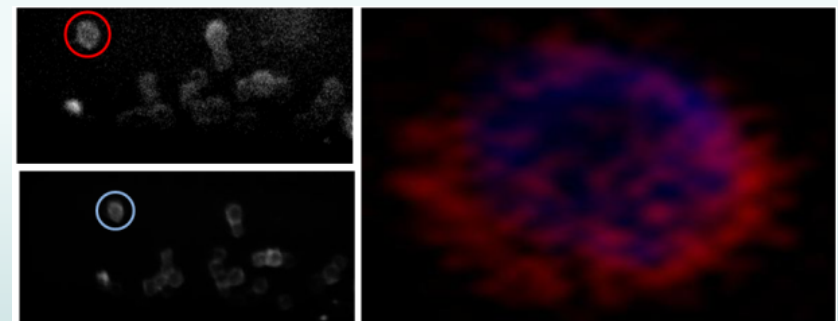
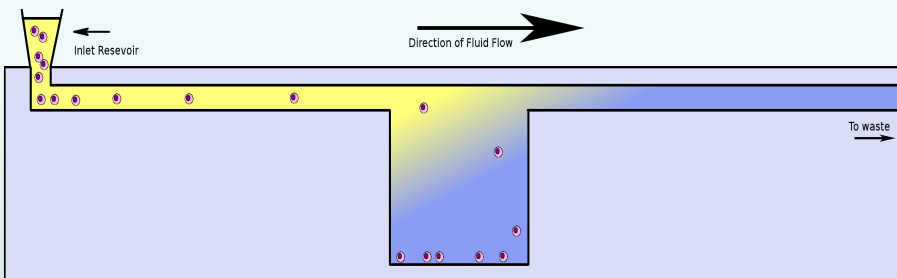
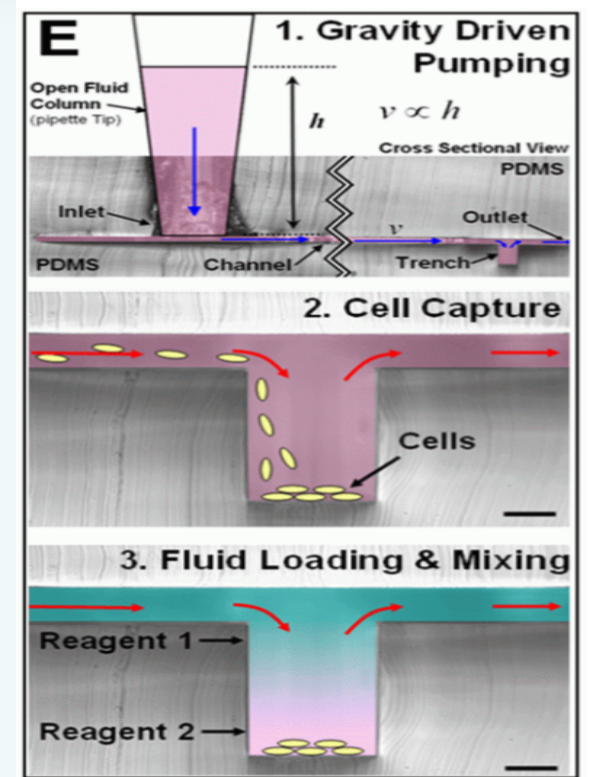
# Imobilização Sia para polyHIPES

- Imobilizado eGFP SiaA
- Coluna polyHIPE
- separação de fetuina e asialo-fetuina
- apenas uma diferença de açúcar entre proteínas !!
- glycoforms estreitamente relacionados



## 'Lab-in-a-trench' (LiaT)

- Microfluídica
- Células capturados pela gravidade
- Roca por difusão
- **lectinas marcadas**
- Padrões de glicosilação alterada no cancro



# Obrigado !

*com desculpas para o meu terrível brasileira*

# I Coloquio de tecnologia entre Brasil e Irlanda (2012)



Obrigado por nossa ótima introdução para ... **Caipirinhas !**





*ProLegere*

## Glycoseparation Solutions For The Life Science Industries

(Funded by Enterprise Ireland Commercialisation Fund)  
**Project lead by Paul Clarke & Roisin Thompson**



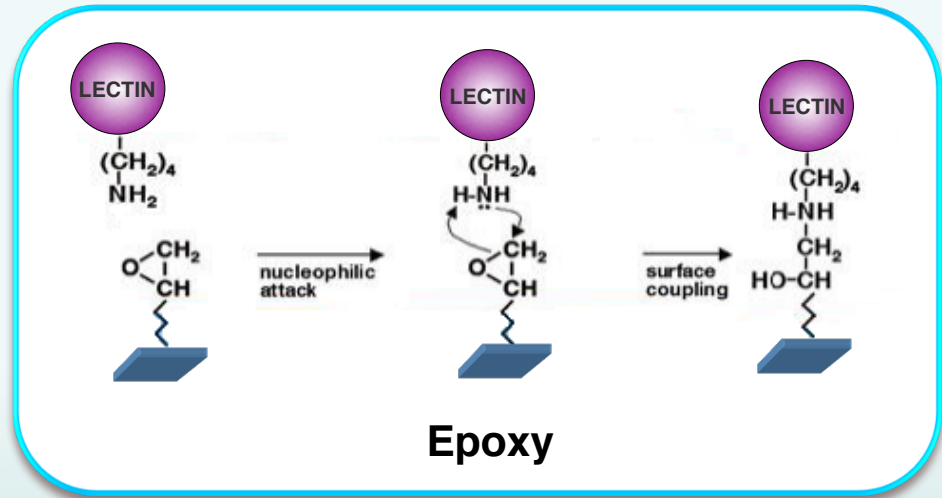
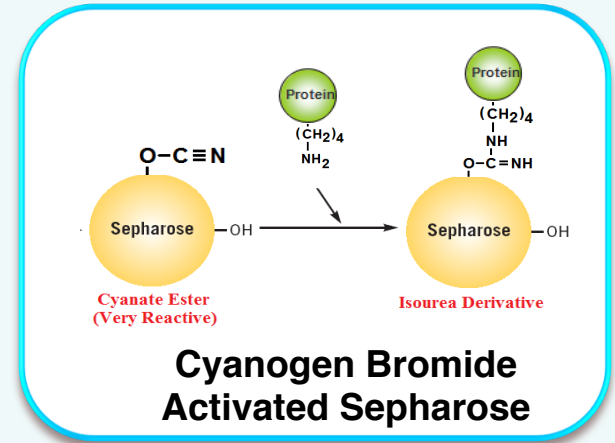
- ❑ File patent & protect IP
- ❑ Establish Large Scale RPL production in two 5 Litre Stir Tank Bioreactors
- ❑ Produce consistent batches of purified RPL protein for immobilisation onto affinity columns
- ❑ Validate the RPL -sepharose columns for consistent robust Glycoprotein separations
- ❑ Engage with instrument providers



# Affinity Chromatography ; RPL

## Lectin-Sepharose Resins

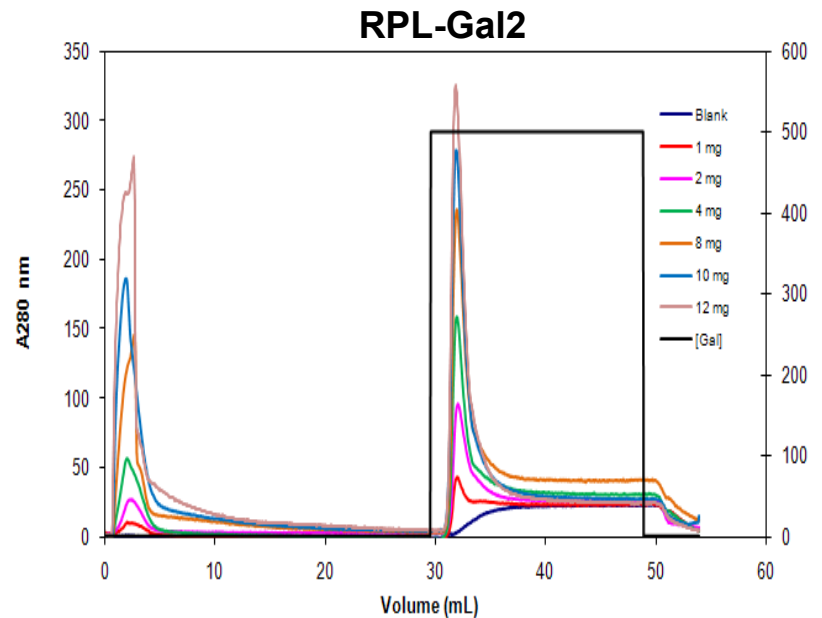
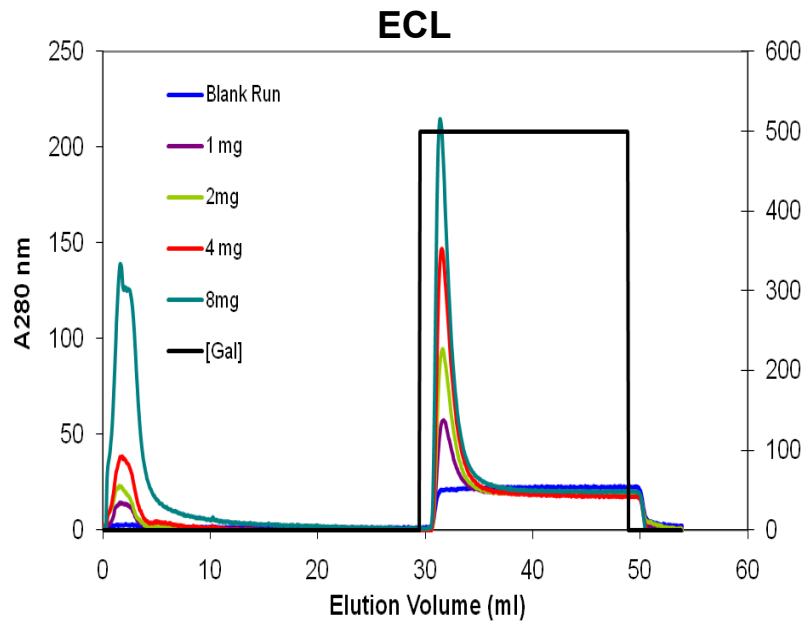
- ❑ Immobilization using well established chemistries.
- ❑ Exploits intrinsic protein amino acid residues – Lysines, Cysteines, Strept.
- ❑ Commercially available derivatised resins.



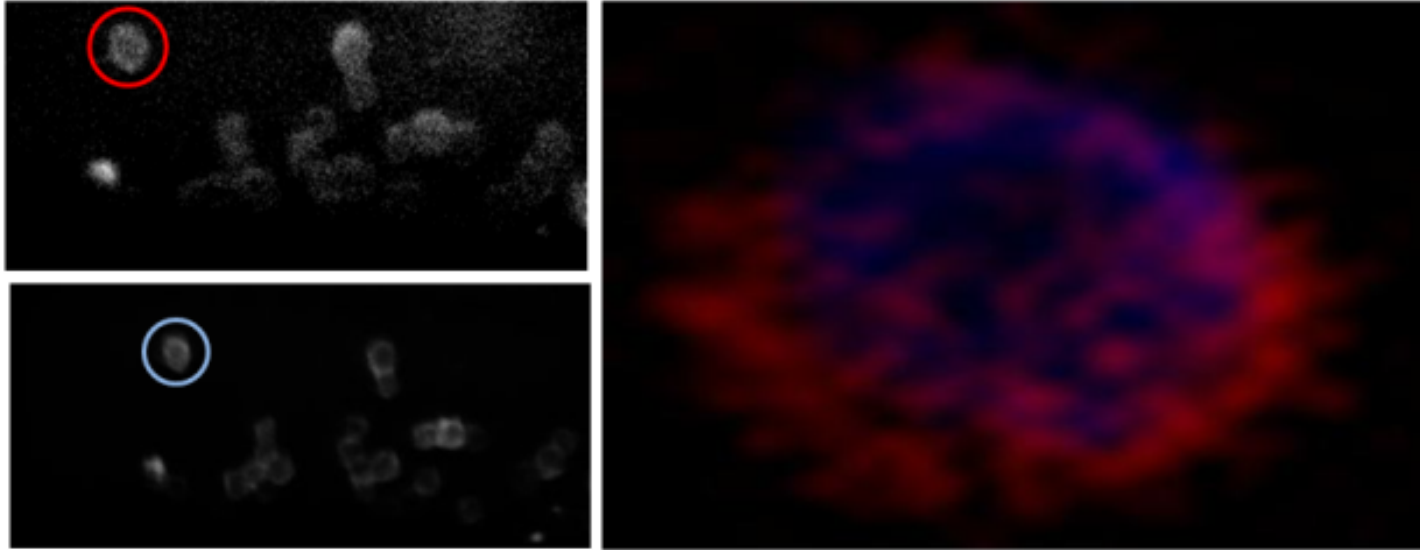
# Affinity Chromatography ; RPL Lectin-Sepharose Resins

## RPL-Gal Sepharose Resins & FPLC Columns

### Binding Capacity Experiments



# Diagnostics ; Sequential Lectin labelling *Single Live Cell Glycoprofiling*



- Red circle LCA lectin
- Blue circle ECL lectin
- Removes potential interference between lectins for multicolour imaging