Tool-box for chemical biology

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I - Molecular tool-box synthesis for functional biology and proteomics

Strasbourg University, France
Laboratory of Functionnal Chemo-System

II - Developement of imaging agents for in vivo detection of oncogenic target

Massachusetts General Hospital, Harvard Medical School, USA
Center for Systems Biology

III - Developement of probes to detect and quantify bacteria with nanoparticles
What is proteomics?

Study of all the proteins of an organism (proteome) at a given time.

1 organism = 1 genome = thousands of different proteins

The proteome differs from cell to cell and from time to time

Proteomic analysis allows:

- Identification
- Quantification of protein expression
- Define Protein/protein interactions
- Characterize biological processes
- Decode mechanisms of control of genes expression
- Disease diagnostic or prognostic for personalized treatment
Comparative proteomic

Global analysis of proteins abundance variations by comparison of proteome

- 2-DE gel
- Separation
- Visualization

1) Fluorescents reagents
2) Separation
3) Visualization

Proteomes

Isotopes tagged chemicals reagents
Tagged proteome

Digestion
Isolation
Separation by LC

Identification by MS

Identification by MS

Excision
Digestion

Quantification
Functional proteomic: ABPP approach

Activity-Based Protein Profiling (ABPP):

- Use specifically designed chemical probes targeting the active site of a protein class
- Differentiate active proteins from their inactive forms
- Quantification of protein expression level by in-gel fluorescence
- Extraction and identification by mass spectrometry (pull-down)

Two-steps reaction allows minimum structural modification of the probe and minimize interference with affinity, selectivity, cell-permeability or bioavailability.

ABPP probes are based on a covalent approach: need RG

Analysis are in denaturating conditions: loosing of structural informations
Objectives

Development of non-denaturing and non-covalent technique for fluorescent labeling and protein pull-down by bioorthogonal reaction

**Advantage**: Live cell imaging, labeling of protein complex and identification of protein complex

**Challenges**: Use non-denaturing bioorthogonal reaction, label protein with small molecule non-covalently attached, develop mild cleavable linker to release protein complexes
Bioorthogonal reactions

Reactions selective between two chemical functionalities absent of natural biological media and unreactive towards biomolecules.

Reactions between carbonyl groups and nucleophilic amines


Staudinger ligation


Copper catalyzed 1,3-dipolar Huisgen cycloaddition (“Click-Chemistry”)


2-cyanobenzothiazole and Cysteine


Strain-promoted copper-free click-chemistry


Cycloaddition with Alkenes


I - Molecular tool-box synthesis for functional biology and proteomics
Bioorthogonal fluorescent tools

Synthesis

1. $\text{NaNO}_2, \text{HCl, H}_2\text{O}$
   2. $\text{KI, H}_2\text{O}$
   1h, RT, 65%

1. $\text{Pd(OAc)}_2 (5\%), \text{TEA, Toluene}$
   2. $\text{N-Hydroxysuccinimide}$
   3. $\text{EDC, DCM}$
   4. $\text{RT, 16h, 96%}$


1 - Molecular tool-box synthesis for functional biology and proteomics

Monday, November 26, 2012
Validation on Biological systems: Gyrase

- Bacterial enzyme essential for chromosome replication
- Relieves DNA strain in an ATP-independent manner
- Removes knot by breaking DNA double helix in an ATP-dependent manner
- Gyrase inhibitor: Novobiocine (antibiotic) ATP competitor

Supercoiled DNA

Relaxed DNA

![Supercoiled DNA](image1)

![Relaxed DNA](image2)

Novose | Coumarine | Benzamide
---|---|---
\[
\begin{align*}
\text{Novose:} & \quad \text{H}_2\text{N} - \text{O} - \text{OH} \\
\text{Coumarine:} & \quad \text{OH} - \text{N} - \text{O} - \text{OH} \\
\text{Benzamide:} & \quad \text{N}_3
\end{align*}
\]

Substitution by a bromoacetamide
Bioorthogonal affinity probe synthesis

Synthesis of Novobiocine-N₃

5 steps
20% global yield
IC₅₀ (NovoN₃) = 1.7 uM
IC₅₀ (Novobiocine) = 0.5 uM
Non-covalent and non-denaturing fluorescent labeling

1 : Proteine + NovoN$_3$
2 : Proteine + NovoN$_3$ + Phos-Rh
3 : Proteine + NovoN$_3$ + Alkyne-Rh + reagents

Staudinger ligation reaction compatible with non-denaturing conditions
**Labeling specificity on cell lysate**

- Selectivity and sensitivity of the Staudinger ligation in a cell lysate
- Conditions: NovoN$_3$ = 15 μM, Phos-Rh = 150 μM, RT, 1h

<table>
<thead>
<tr>
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<th>4</th>
<th>5</th>
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<td>1</td>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
<td>1000 ng of Gyrase B + 100X Novobioicine</td>
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</table>

- Sensitivity of the method
- Staudinger ligation specific of the azide
- Specific interaction

A GyrB/NovoN$_3$ complex was conjugated by the Staudinger ligation with a fluorescent phosphine without losing the non-covalent interaction

The formed complex was analyzed by native gel allowing visualization of an intact protein

Development of a non-denaturing protein pull-down procedure

Isolation and identification of an intact and functional multiprotein complex
Cleavable Purification tag for protein pull-down

Design of cleavable linker

**Goal:** develop a rapid and biocompatible cleavable linker

Known cleavable linker

<table>
<thead>
<tr>
<th>Cleavable linker</th>
<th>Cleavage reagent</th>
<th>Cleavable linker</th>
<th>Cleavage reagent</th>
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</thead>
<tbody>
<tr>
<td>(\text{S-S})</td>
<td>DTT</td>
<td>(\text{O-N=CH}_2)</td>
<td>Acylhydrazine</td>
</tr>
<tr>
<td>(\text{O-CON=NC}_2)</td>
<td>TFA</td>
<td>(\text{S})</td>
<td>Thiol</td>
</tr>
<tr>
<td>(\text{O-CON=NC}_2)</td>
<td>(\text{hv})</td>
<td>(\text{N}^+\text{N-N=CH}_2)</td>
<td>Dithionite</td>
</tr>
</tbody>
</table>

Azobenzene cleavable linker

Cleavage conditions: dithionite **25 mM, 3X 15 min**

Conditions non compatible for non-denaturing condition

Optimization of cleavable linker

- Structure activity relationship study

A Electron poor arene
B Electron rich arene

Synthesis of a library of 30 compounds

Solution Dithionite 1mM
$\frac{t_1}{2} < 1\text{s}$
total cleavage = 15s

COOH have to stay free
Phenol in para position have to stay free

- Pull-down cleavable linker design

Bi-functionalizable linker with two orthogonal groups Fmoc et Boc
A tetraethylene glycol chain has been introduced to increase water-solubility

Synthesis of cleavable linker

\[ \text{I} \quad \text{COOH} \quad \text{Benzyl chloroformate} \quad 0.1 \text{ mol\% \( \gamma \)-CD, } H_2O, \text{RT, 16h} \quad 71\% \]

\[ \text{I} \quad \text{COOH} \quad \text{Dimethyl sulfate, } K_2CO_3 \quad \text{acetone, 40°C, 20 min} \quad \text{MW, 63\%} \]

\[ \text{I} \quad \text{COOMe} \quad \text{N-Fmoc-propargylamine 39} \quad \text{PdCl}_2(\text{PPh}_3)_2, \text{Cul, TEA, DMF, RT, 16h} \quad 60\% \]

\[ \text{FmocHN} \quad \text{49} \quad \text{H}_2/\text{Pd/C} \quad \text{DMF/EtOAc, RT, 3h} \quad 65\% \]

\[ \text{FmocHN} \quad \text{48} \]
Synthesis of cleavable linker

B

A

I - Molecular tool-box synthesis for functional biology and proteomics

Synthesis of cleavable linker

1) Piperidine, DCM, RT, 3h
2) 5-hexynoic acid-NHS, TEA, DMF, RT, 16h, 78% over two steps

1) TFA, DCM, RT, 16h
2) Biotine, HBTU, TEA, DMF, RT, 16h, 78% over two steps

LiOH, MeOH/H₂O 4:1, 40°C, 16h, 61%

12 steps
3% global yield
Cleavage in 10s with 6 mM dithionite in cell lysate

Cleavable Pull-down probe

**NovoN₃**

\[ \text{CuSO}_4 \text{ Sodium ascorbate tBuOH/H}_2\text{O} \]

38%

**Alkyne-azo-biotin**

Total cleavage in 20 s with a 6 mM dithionite solution

Total cleavage in 60 s with a 1 mM dithionite solution

**Novo-azo-biotin**
Pull-down on spiked cell lysate

Optimization of time, conc, number of elutions

- E.coli cell lysate + recombinant GyrB
- Novo-azo-biotin 2.5 uM, 1h
- Magnetic streptavidin beads, 1h
- Dithionite 5 mM, 5 min
- Loading on SDS-PAGE gel
- Protein band recovery and LC-MS/MS

Coomassie stained SDS-PAGE gel

Dithionite cleavage (5mM) is specific and fast (5 min)
A single elution is needed
Cleavage yield = 95%

Elution of a protein complex with a non-covalent small-molecule

Pull-down on endogenous cell lysate

**Goal**: identification of GyrB protein partners and secondary targets of Novobiocine

**Validation in progress**

<table>
<thead>
<tr>
<th>Band</th>
<th>Proteine</th>
<th>Sequence coverage (number of identified peptides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ADN GyrA</td>
<td>52% (41)</td>
</tr>
<tr>
<td>2</td>
<td>ADN GyrB</td>
<td>69% (50)</td>
</tr>
<tr>
<td>3</td>
<td>Dehydrogenase aceF</td>
<td>40% (21)</td>
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<tr>
<td>4</td>
<td>ATP synthetase alpha atpA</td>
<td>48% (18)</td>
</tr>
<tr>
<td>5</td>
<td>Facteur d’élongation tuf1</td>
<td>50% (14)</td>
</tr>
<tr>
<td>6</td>
<td>Proteine chaperonne dnaJ</td>
<td>47% (13)</td>
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<tr>
<td>7</td>
<td>Proteine de transport ompF</td>
<td>43% (11)</td>
</tr>
<tr>
<td>8</td>
<td>Proteine ribosomal rplA</td>
<td>53% (11)</td>
</tr>
<tr>
<td>9</td>
<td>Methyltransferase yibK</td>
<td>89% (13)</td>
</tr>
</tbody>
</table>

Identification of 51 proteins with at least 3 unique peptides

Validation in progress

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I - Molecular tool-box synthesis for functional biology and proteomics

Monday, November 26, 2012
**Functionnal assay**

**Goal**: Check the non-denaturing process of the elution

Gyrase: DNA relaxation activity preserved without ATP

![BET stained agarose gel](image)

Non-denaturing elution conditions
Intact and functionnal protein complex

Synthesis of a tool-box for proteomic

A fluorescent labeling technique was developed in native conditions thanks to an ABPP probe non-covalently attached to the target

A pull-down and elution technique was developed in native conditions and allowed to isolate a functional protein complex
II- Development of imaging agents for in vivo detection of oncogenic targets

The challenge of tracking small-molecule drugs

- Direct fluorescent labeling of small molecules cumbersome (size, charge, polarity are challenges)
  - affects binding affinity
  - limited cellular uptake/distribution
  - affects pharmaco-kinetics
- Recent advances in “click-chemistry”
  - 2-step reactions:
    - chemically modified drugs (“tags”) first bind to target
    - fluorophore is “clicked” in vivo to the bound drug

- Big picture: where do drugs really go?
  - How do drugs localize in different cells/tumors?
  - What targets/off-targets do drugs bind to in vivo?
  - Can we track these drugs and relate to drug resistance?
II- Development of imaging agents for in vivo detection of oncogenic targets

- Synthesis of chemical probes targeting specific proteins in cancer cells
- Bioorthogonal reaction with an imaging reporter (fluorescent dye or PET agent)
- Strained Trans-cyclooctene (TCO) and Tetrazine (Tz), react via an inverse Diels-Alder [4+2]
- Reaction highly specific and fast reaction kinetic in vitro and in vivo
- Turn-on probes: fluorescent signal increases upon tetrazine/TCO reaction

Cell surface and plasma membrane targets
(Growth factor, G-protein, cytokine, chemokine receptors)

Nuclear targets (DNA repair, histone modification, gene regulation, transcription factors)

Cytosolic targets (signal transduction, survival, death, metabolic pathways)
Experimental Plan

1. Strategy: start with cell-based feasibility assays
   - Synthesis and characterization of TCO-modified drugs
     - Does TCO modification affect affinity?
     - Determine IC$_{50}$, $K_d$, and on/off rates (in vitro assays, Biacore)
   - Where do drugs localize in cells?
     - Co-localize TCO-modified drugs with fluorescent genetic reporters or immunostaining

2. Live cell imaging questions
   - Can we image the live cell dynamics of TCO-modified drugs at single cell resolution?
     - High-throughput imaging experiments probing dose and time
   - Do the TCO-modified drugs distribute differently in divergent cancer cell types?
     - Image drug distribution/cellular uptake (over time) in a variety of cancer cell lines
     - Determine if any cancer cell lines are resistant to these drugs and, if yes, include them in imaging experiments

3. In vivo imaging questions
   - Do the TCO-modified drugs localize to the targets in tumors?
   - What are the in vivo dynamics of drug distribution and uptake?
     - Determine uptake and how drugs distribute in different tumor types
Nuclear target: Polo-like Kinase 1

Challenge: imaging nuclear target with a two-steps labeling reaction

Serine/Threonine kinase
Mediator of the cell cycle regulating mitotic progression
Overexpression associated with neoplastic cells including cancer

**BI2536** active clinical trial drug

A

![Chemical structure of BI2536](image)

B

![3D model of BI2536 interaction with a protein](image)
Nuclear target: Polo-like Kinase 1

Synthesis

1) MeOH, SOCl$_2$
2) NaOAc, DCM
3) Na$_2$CO$_3$, Acetone
4) BOP, DCM, DIPEA
5) TCO-NHS, DIPEA, DMF

1. $\text{HOOC-}NH_2$
2. $\text{MeOH, SOCl}_2$
3. NaOAc, DCM
4. Na$_2$CO$_3$, Acetone
5. BOP, DCM, DIPEA
6. TCO-NHS, DIPEA, DMF

Bl2536-TCO
Nuclear target: Polo-like Kinase 1

Drug characterization

Click-chemistry

Affinity

<table>
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<tr>
<th>Compound</th>
<th>Compound</th>
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<th>-Error</th>
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<td>0.61</td>
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<td>9.35 nM</td>
<td>2.48</td>
<td>1.96</td>
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<td>BI 2536-TCO/Texas Red-Tz</td>
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<td>103 nM</td>
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<td>Texas Red-Tz</td>
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<td>&gt;&gt;1 μM</td>
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</table>
Nuclear target: Polo-like Kinase 1

Cell imaging: localization and quantification

Polo-like Kinase 1 (PLK1)
Mediator of mitosis
Nuclear target

Target molecule

BI 2536
IC₅₀ = 4.3 nM

BI 2536-TCO
8 steps
IC₅₀ = 9.4 nM

TexasRed-Tz

PANC1 cell: A) Ab/PLK1, B) BI 2536-TCO/Texas Red-Tz, C) Hoechst staining, D) Merge


II - Development of imaging agents for in vivo detection of oncogenic target

Monday, November 26, 2012
Nuclear target: Aurora Kinase A

Challenge: imaging nuclear target with a two-steps labeling reaction in live cell

Serine/Threonine kinase
Mediator of the cell cycle regulating mitotic progression
Overexpression in most of the epithelial cancer and cause defect in cell division
MLN8054 active clinical trial drug
Nuclear target: Aurora Kinase A

Synthesis

1) tBuLi, THF, RT to -78 °C, 0.5 h
2) -30 °C, 2.5 h, then -78 °C
3) THF, -78 °C

1) HCl, H2O, AcOH
2) NaNO2, 0 °C
3) I2, KI, RT 1h

1) CuI, PdCl2(PPh3)2 DCM, RT, 0.5h
2) Et2NH, RT, 16h

1) CuI, PdCl2(PPh3)2 DCM, RT, 0.5h
2) Et2NH, RT, 16h

TCO-NHS DIPEA, DMF, RT, 1h

13, Quant.

10 steps
5.7% overall yield
Drug characterization

Click-chemistry

Affinity

<table>
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<td>1.6 nM</td>
<td>0.087</td>
<td>0.083</td>
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<td>MLN8054\textsuperscript{7}</td>
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<td>12.21</td>
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<tr>
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<td>437 nM</td>
<td>142.42</td>
<td>107.43</td>
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<tr>
<td>Texas Red-Tz</td>
<td>\textgreater\textless 1 μM</td>
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</table>
Nuclear target: Aurora Kinase A

Cell imaging: localization and quantification

[Images of cellular imaging with different colors and labels for each cell line: HeLa, IMR-90, PANC-1, A549, SKOV-3, MDA-MB-231, U-87 MG, SW480, MIA PaCa-2, HEK-293]

Graph showing the percentage of maximal signal for AKA antibody and MLN8054-TCO 13/Texas Red-Tz across different cell lines.

Graph showing the relationship between MLN8054-TCO 13/Texas Red-Tz and AKA antibody with data points for each cell line.

[Graphs illustrating the localization and quantification of Aurora Kinase A in different cell lines]
Nuclear target: Aurora Kinase A

Live cell imaging

- GFP-AKA
- Dye-Tz
- Merge
- RFP-AKA
- Dye-Tz
- Merge

Texas Red-Tz

TAMRA-Tz

ATTO-610

Rhodamine-101-Tz

NBD-Tz

Dansyl-Tz

Oregon Green-Tz

ATTO 495-Tz

Bodipy-Tz

Developement of imaging agents for in vivo detection of oncogenic target
Nuclear target: Aurora Kinase A

Live cell imaging

FDA-Tz: activatable probe

Esterase

FDA-Tz : activatable probe

Time (min)

AUF (x10^3)

20 µg
10 µg
5 µg
2.5 µg
1 µg
0 µg

AUF (x10^3)

Quenched Fluorescein

Turn-On Fluorescein

Wavelength (nm)
## Nuclear target: Aurora Kinase A

### Live cell imaging: Cell cycle

<table>
<thead>
<tr>
<th>mApple-H2B</th>
<th>MLN8054-TCO/Fluorescein-Tz</th>
<th>Merge</th>
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<td><img src="image2" alt="Interphase" /></td>
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<td><img src="image21" alt="Cytokinesis" /></td>
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</table>


II - Development of imaging agents for in vivo detection of oncogenic target
Real-Time Imaging of MLN8054-TCO in Live Mitotic HT1080 Cells

125nM MLN8054-TCO
1 μM Fluorescein-Tz
Image every 5 min for 1 hour
Other targets

Inhibitor of B-cell lymphoma (BCL-2) regulator of apoptosis (Cytoplasmic target)  
Manuscript in preparation

Inhibitor of Vascular endothelial growth factor receptor (VEGFR2) angiogenesis (Membrane target). In vivo imaging of blood vessels

Inhibitor of RET, ALK, EGFR, PI3K/mTor, FDGFR, Her2... (Membrane target)
III- Development of probes to detect and quantify bacteria with nanoparticles

- Development of a miniaturized nuclear magnetic resonance system (µNMR)
- Detection based on relaxivity of magnetic nanoparticles attached to cells
- Relaxivity proportional to the number of nanoparticles
- Simple and sensitive. Used as a clinical point-of-care device
- Detection of low numbers of live mammalian cells or pathogens in complex clinical material

Detection of Gram+ bacteria by bioorthogonal reaction with magnetic nanoparticles

- Current Ab are non-specific
- Synthesis of antibiotic derivatize with TCO (vancomycin and Daptomycin, inhibit cell wall synthesis)
- Bioorthogonal reaction with fluorescent magnetic nanoparticles (MNFP) for µNMR quantification

ACS nano, 2011, 5, 8834-8841
Labeling of Staphylococcus Aureus

Fluorescent microscopy

Binding of Vanc-TCO to the bacterial cell wall of G+ in dose dependent fashion

TEM

Selective to G+

III - Development of probes to detect and quantify bacteria with nanoparticles
Labeling of Staphylococcus Aureus

Competition Assay

Viability Assay

Vanc-TCO retain its bactericidal effect

Labeling of intracellular bacteria within live macrophages
Crystal Violet used for detecting and differentiating bacteria
Bind to peptidoglycan layer of G+/G-
Retain only in G+ due to thicker peptidoglycan layer
Relies on optical microscopy
Bacteria from patient sample needs to be cultured before staining

Goal: Develop a magnetic Gram stain (more sensitive, quantification, adapted to crude sample)
A magnetic Gram staining

Synthesis

\[
\begin{align*}
\text{MW 4min, 90C} & \quad \text{H}_2, \text{Pd/C} \\
\text{H}_\text{2O} & \quad \text{THF/MeOH} \\
\text{AcOH} & \quad \text{THF/MeOH} \\
\text{NaCNBH}_3 & \quad \text{THF/MeOH} \\
\text{Cat. Aniline} & \quad \text{37%}
\end{align*}
\]

Molecular Weight: 375.4635

\[
\begin{align*}
\text{N} & \quad \text{O} \\
\text{N} & \quad \text{O} \\
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{O} \\
\text{H} & \quad \text{O}
\end{align*}
\]


\[
\begin{align*}
\text{AcOH} & \quad \text{THF/MeOH} \\
\text{NaCNBH}_3 & \quad \text{THF/MeOH} \\
\text{AcOH} & \quad \text{THF/MeOH} \\
\text{NaCNBH}_3 & \quad \text{THF/MeOH}
\end{align*}
\]

71% over 3 steps

\[
\begin{align*}
\text{TCQ, EtOAc} & \quad \text{1h, reflux} \\
\text{DIPEA, DMF} & \quad \text{1h, RT} \\
\text{TCQ, EtOAc} & \quad \text{1h, reflux} \\
\text{HCl 1N/EtOH} & \quad \text{RT, ON}
\end{align*}
\]

88%
A magnetic Gram staining

**Results**

A

- **A**
  - Gram-TCO Stain
  - Regular Gram Stain
  - Gram +
  - Gram -
  - Mixed

B

- **B**
  - GramTCO (AU) vs. Gram stain (AU)
  - S. aureus
  - E. coli

C

- **C**
  - Cellular relaxivity ($\times 10^{-6} \text{s}^{-1} \cdot \mu l$)
  - S. aureus
  - E. coli

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A magnetic Gram staining

Selectivity

III - Development of probes to detect and quantify bacteria with nanoparticles

Monday, November 26, 2012
A magnetic Gram staining


fluorescence microscopy

CV-TCO + MFNP-Tz

MFNP-Tz

-/-
Summary

- Development of chemical tools for proteomic
  Fluorescent Bioorthogonal reporter
  Bioorthogonal cleavable purification tag
  Pull-down of protein complexes
  Identification of drugs secondary targets

- Development of imaging probes for live cells/in vivo imaging via bioorthogonal reaction
  Visualization and quantification of nuclear, cytoplasmic and membrane targets

- Development of probes to detect and quantify bacteria by magnetic means
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