Molecular switches for sensing and theranostics in cancer

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MOLECULAR SWITCHES

They are short nucleic acid molecules capable of turning on or modifying their light emission after interaction with well-defined molecular targets.

Oligonucleotide optical switches are among the most promising optical sensors proposed in recent years.
Molecular beacon

Aptamer beacon
25-90 bases ssDNA/RNA

Proteins or smaller molecules (ATP)

Hybrid molecular probes or Binary Probes

Nanoflares and Sticky-flares

Giannetti A. et al., Anal Bioanal Chem., 2013, 405, 6181-6196
Briley WE. et al., PNAS, 2015, 112, 9591-9595
Proteins or smaller molecules (ATP)

Molecular beacon

Aptamer beacon
25-90 bases ssDNA/RNA

ssDNA

"OFF"

+ TARGET

mRNA/DNA

"ON"

Hybrid molecular probes or Binary Probes

Nanoflares and Sticky-flares

Giannetti A. et al., Anal Bioanal Chem., 2013, 405, 6181-6196

Briley WE. et al., PNAS, 2015, 112, 9591-9595
MAPPING THE RNAs INSIDE THE CELL:
SIGNIFICANCE in ONCOLOGY

The ability to IMAGE specific RNAs in living cells in real-time provides information on RNA localization and transport.

This information should offer new opportunities in:

- development of clinical diagnostic procedure for the early detection of cancer
- monitoring therapy
- follow-up after surgery or chemotherapeutic treatment

Molecular Switches act as nano-probes for sensing in cells

Santangelo PJ, WIREs Nanomed Nanobiotechnol, 2010, 2, 11–19
Thakor AS. and Gambhir SS., CA Cancer J Clin, 2013, 63, 395-418
FUTURE MEDICINE: MOLECULAR SWITCHES AS THERANOSTIC AGENTS

THERANOSTICS: a new concept which involves a combination of diagnosis and therapy

Eo J.S. et al., World J. Gastroenterol., 2014, 20(18):5375-5388

Theranostics 2015, Vol. 5, Issue 1

Review

Nucleic Acid Aptamer-Guided Cancer Therapeutics and Diagnostics: the Next Generation of Cancer Medicine

Dongxi Xiang1, Sarah Shigdar2, Greg Qiao3, Tao Wang1, Abbas Z. Kouzani1, Shu-Feng Zhou1, Lingxue Kong4, Yong Li1, Chunwen Pu1, and Wei Du4

See also: http://www.molecular-beacons.org
### Intracellular Delivery of Oligonucleotide Switches

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<th>Delivery method</th>
<th>Target</th>
<th>Cell line</th>
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<td>MB for β-actin mRNA</td>
<td>Kangaroo rat PK2 cells</td>
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<tr>
<td>Microinjection</td>
<td>LNA MB for β-actin and MsSOD mRNA</td>
<td>MDA-MB-231 breast carcinoma cells</td>
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<tr>
<td>Microinjection</td>
<td>Hybrid molecular probe for β-actin and MsSOD mRNA</td>
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<td>TAT peptide</td>
<td>MB for GAPDH and survivin mRNA</td>
<td>Primary human dermal fibroblast cells</td>
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<tr>
<td>Steptolysin O</td>
<td>MB for bone morphogenetic protein 4 mRNA</td>
<td>the pancreatic cancer cell line MiaPaca-2</td>
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<tr>
<td>Steptolysin O</td>
<td>MB for octamer-binding transcription factor-4 mRNA</td>
<td>Human dermal fibroblast cells</td>
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<td>Steptolysin O</td>
<td>HIV-1 reverse transcriptase aptamer beacon</td>
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<td>Lipofectamine</td>
<td>Glowing LNA MB for Pdx mRNA</td>
<td>Mouse embryonic fibroblast cells (3 T3-L1)</td>
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<tr>
<td>Lipofectamine</td>
<td>MB for miR-206 and miR-26a</td>
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<td>Lipofectamine</td>
<td>MB for poly(A)-RNA</td>
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<td>Polyethyleneimine</td>
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<td>Commercial lipid-based</td>
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<td>Human embryonic kidney 293 cells</td>
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<td>transfection agent</td>
<td>Light-activatable MB for GAPDH mRNA</td>
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<td>Patch pipette</td>
<td>Diacyclipid-MB conjugate for e-NFL-1 mRNA</td>
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<td>Micelle</td>
<td>MB for miR-34a</td>
<td>MCF-7 and MDA-MB-231 breast cancer cell lines, A549 cell line, MCF-10A (normal epithelial cell line)</td>
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<tr>
<td>PEGylated lipidic</td>
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<td>nanocarriers</td>
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</table>

Giannetti A. et al., Anal Bioanal Chem., 2013, 405, 6181-6196

Delivery through coupling to other carriers

- Metal-NPs
- Quantum dots
- Carbon-based nanostructures
- Polymeric-NPs

B. Adinolfi
ONE SPECIFIC APPLICATION
Survivin mRNA detection and silencing in human living cells

Carpi S., Adinolfi B. et al., PlosOne, 2014, 9(12): e114588
WORK LAYOUT

Choice of the intracellular target (survivin mRNA)

Choice, design and characterization of the sensing element (Molecular Beacon, MB)

Choice and characterization of the intracellular carrier (PMMA nanoparticles)

Characterization of the sensing element coupled to the carrier

in buffer, in cell culture medium or in cells
Survivin belongs to a family of proteins, known as inhibitors of apoptosis protein (IAP), which plays a key role in the regulation of apoptosis and cell division.

Survivin expression is very high in most cancer cells. It is scarcely present in healthy tissues.
**MB: CHARACTERIZATION IN SOLUTION**

5’(ATTO647N)-CGACGGAGAAAGGCTGCA CGXCG(BBQ)-3’ X=C₆-dT Thio

ATTO 647N, the fluorescence (λex 635 nm, λem 670 nm)

BBQ (BlackBerry Quencher 650) (quenching wavelength band 550-750 nm)

**Target** 5’-CCCCTGCCTGGCAGCCCTTCTCAGGACC-3’

Nitin et al., Nucleic Acids Res., 2004, 32(6), e58

NanoItaly 2015, September 22, Rome, Italy
MB (100nM) IN CELLS (CLASSICAL TRANSFECTION REAGENT, LIPOFECTAMINE)

**Fluorescence ➔ specific binding** between the MB and survivin mRNA

Carpi S., Adinolfi B. et al., Plos One, 2014, 9(12), e114588
**MB (100nM) in cancer cells:**

**EFFECTS ON SURVIVIN mRNA and PROTEIN LEVELS**

Real-time PCR and Western Blotting experiments demonstrated a time-dependent reduction of survivin mRNA and protein after 100nM-MB treatment, respectively.

**MB induces apoptosis in cancer cells → therapeutic agent**

Carpi S., Adinolfi B. et al., Plos One, 2014, 9(12), e114588
Nanoparticles consist of a core of PMMA, surrounded by a shell bearing cationic groups and amine groups. Fluoresceine is covalently immobilized inside the nanoparticles.

Dimensions: $57.2 \pm 1.3$ nm (PCS)
Zeta potential: $74.4 \pm 14.1$ mV

Fluorescence spectra ($\lambda_{ex} = 488$ nm, pH=8, int. time 1 sec)
(Fluoresceine)

Duchi S. et al., Journal of Controlled Release, 2013, 168 (2), 225-237
**PMMA NANOPARTICLES: CELL VIABILITY STUDIES**

**WST-1 assay** on human A549 cancer cells in presence of fluorescent-PMMA nanoparticles

**Experimental conditions:**
- RPMI 1640, 10% FBS
- time exp: 1h/3h

PMMA–NPs (0.5-50 µg/mL) are not toxic for human A549 cancer cells
PMMA nanoparticles (10 µg/mL) were tested on A549 cells at different times (10’-1h30’) in RPMI 1640 10% FBS.

ImageJ software: MEDNUC Ortvew
PMMA NANOPARTICLES COUPLED WITH MB IN CELLS

NPs 10µg/mL

MB 100nM

Merge

PMMA-MB are inside the cells where they exhibit quite low colocalization

Software: 3D Volume viewer 2.1

NPs 10µg/mL

MB 100nM

Software: 3D Volume viewer 2.1

time exp.=1h30’ RPMI 1640 10%FBS

MB is inside the cells

B. Adinolfi
HEALTHY CONTROL: the Human Dermal Fibroblasts (HDFa) at high cell density

HDFa at high cell density

**Experimental conditions:**
- PMMA-NP: 25 µg/mL
- MB: 100 nM
- time exp: 6h

PMMA-NP are inside the cells and extremely low red fluorescent signals are visible

**PMMA-NPs CELLULAR UP-TAKE:**

ENDOCYTIC PATHWAY INVOLVEMENT

**time exp: 30’**

- **Endocytic vesicles dimension range:** $0.1 \mu m^2 - \infty$

- **Vesicle density (Vs. Area/Cell Area)**
  - DXT 1.64%
  - DXT NP 4.49%

Mann-Whitney test
Mean ± SE
**CONCLUSIONS**

- The **MB** is **specific** for **survivin mRNA** and it acts as a **silencing agent** which induces **apoptosis**
- The **PMMA-NPs** **promote endocytosis** in A549 cells
- The **PMMA nanoparticles** promote the **MB internalization**
- The **HDFa** cells at **high cell density** can be used as a **healthy control** for PMMA-survivin MB cellular up-take studies

Next experimental work

- Further investigation will be carried out to get insight into the **NPs** and **MB intracellular localization** by using specific dyes for Endosomes, ER, Golgi..
Possible strategies for a correct targeting of cancer cells:
- magnetic field driving
- nanocarriers
- surface modifications

Long-term effects??
Toxicity??
ACKNOWLEDGMENTS

• Work supported by the national flagship project NANOMAX

Email address: b.adinolfi@ifac.cnr.it
OUTLINE

- Molecular Switches
- Molecular Switches as Sensors and Theranostic Agents in Cancer
- Molecular Switches Intracellular Delivery Strategies

- One specific application: a Survivin-Molecular Beacon as theranostic agent in Human cancer cells

- Conclusions and Open issues
**MOLECULAR SWITCHES IN CELL: DISADVANTAGES**

✧ false-positive signal due to the degradation of the oligo-switch by means of endogenous nucleases

✧ presence of a single fluorophore giving low signal intensity

✧ high background signal due to incomplete quenching of the quencher

Mechanism of action of nucleases

DNA

RNA

Giannetti A. et al., Anal Bioanal Chem., 2013, 405, 6181-6196
MOLECULAR SWITCHES IN CELL: DISADVANTAGES

✧ false-positive signal due to the degradation of the oligo-switch by means of endogenous nucleases

MBs have been synthesized with **several modifications of the structure**

2'-O-methylation

Locked Nucleic Acids (LNA)

Peptide Nucleic Acids (PNA)

Giannetti A. *et al.*, Anal Bioanal Chem., 2013, 405, 6181-6196
 presença de um único fluoróforo resultando em baixa intensidade de sinal

Conjugated Polymers (CPs), conhecidos por exibir **photoluminescence** com alta eficiência de quantum.

Giannetti A. et al., Anal Bioanal Chem., 2013, 405, 6181-6196
MOLECULAR SWITCHES IN CELL: DISADVANTAGES

✧ high background signal due to incomplete quenching of the quencher

Giannetti A. et al., Anal Bioanal Chem., 2013, 405, 6181-6196

Engineered oligonucleotides

Increased number of quenchers

Caged molecular beacons
Cell tracking

10^{-1} 1 10 10^2 10^3 10^4 10^5 10^6 10^7 10^8 nanometers

- H₂O
- glucose
- antibody
- virus
- bacterium
- cancer cell
- pencil tip
- tennis ball

- oligonucleotide
- nanotubes
- nanoparticles
- peptide
The interaction within two molecules (D=donor and A=acceptor) without photon emission. The Förster energy transfer is the phenomenon that an excited donor transfers energy (not an electron) to an acceptor group through a non-radiative process.

**FRET**

Förster Resonance Energy Transfer
Aptamer Beacon

The SELEX System

INITIAL LIBRARY

POOL GENERATION

TARGET

EVALUATION

REGENERATION

BINDING

AMPLIFICATION

WASH

ELUTION
The **fluorescence imaging** is a fast, easy, noninvasive, selective and sensitive *diagnostic tool* in clinical practice.

Sieron A. *et al.*, *OncoTargets and Therapy*, 2013, 6, 977-982
IN CELLS: **SURVIVIN mRNA DETECTION** by RT-PCR

Survivin mRNA is expressed at **high levels in cancer** A375 cells and **it is not expressed** in human **monocytes**

Carpi S., Adinolfi B. *et al.*, Plos One, 2014, 9(12), e114588
**MB: CHARACTERIZATION IN SOLUTION**

Fluorescence spectra of the MB solution without and with target 100 nM at zero and 90 min of incubation in RPMI 1640 medium supplemented with 10% FBS ($\lambda_{ex}$ 635 nm).

Fluorescence spectra of the MB solution without and with target 100 nM at zero and 90 min of incubation in RPMI 1640 medium supplemented with 10% FBS in presence of PMMA nanoparticles (NP) at a concentration of 10 µg/mL ($\lambda_{ex}$ 635 nm).
**LINEAR PROBE (100nM) IN HUMAN MONOCYTES**

*(CLASSICAL TRANSFECTION REAGENT, LIPOFECTAMINE)*

Linear probe: $5'(\text{ATTO647N)-GAGAAAGGCTGCCA-3'}$ Thio

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**Monocytes**

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>DIC</th>
<th>Merge</th>
</tr>
</thead>
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The **human monocytes** are **trasfectable** and represent a **real negative control** for **survivin mRNA expression**

Carpi S., Adinolfi B. *et al.*, Plos One, 2014, 9(12), e114588
**Results**

**MB AND A549 CELLS**  
(*WITHOUT LIPOFECTAMINE/NANOCARRIER*)

**Experimental conditions:**

- RPMI 1640, 10% FBS
- MB: 100 nM
- time exp: 1h 30’

→ **MB is closed**
**MB (100nM) in cancer cells: INDUCTION OF APOPTOSIS**

Mitochondrial membrane Potential (DYm) assay

- **t exp:48h**
- **CTRL**
- **Depolarized**
- **Dead**

**ELISA assay**

- **Living**
- **Depolarized**
- **Dead**

**DAPI staining:** nuclear morphology changes

**MB induces apoptosis in cancer cells → therapeutic agent**
Experimental conditions:

RPMI 1640, 10% FBS

PMMA-NP: 10 µg/mL

Plasma membrane labelled with

Vybrant DiI (λexc. 543nm - λem. 590nm)
QUESTIONS

➢ What happens in non-cancer cells?

➢ Is there any kind of healthy cell that is able to internalize PMMA-NPs AND not express survivin mRNA?

Healthy Control: are the Human Dermal Fibroblasts (HDFa) the «right» healthy control for PMMA-MB cellular up-take studies?
Healthy Control: are the Human Dermal Fibroblasts (HDFa) the «right» healthy control for PMMA-MB cellular up-take studies?

WST-1 assay on human HDFa cells in presence of fluorescent-PMMA nanoparticles

Experimental conditions:
M106 complete medium
time exp: 1h/3h/6h

PMMA–NP are not toxic at the concentrations tested in HDFa cells

Adinolfi B. et al., Biomedicine and Pharmacotherapy, 2015, 69:228-232
PMMA NPs-MB IN HDFa CELLS: INTERNALIZATION STUDIES

Experimental conditions:
- M106 complete medium
- PMMA-NP: 10 µg/mL
- MB: 100 nM
- time exp: 1h 30'

The NPs signals are very mild and mainly in correspondence of the plasma membrane and no signal of MB is detectable.
HDFa at middle cell density \textit{(in proliferation)}

**Experimental conditions:**
- PMMA-NP: 25 \( \mu \)g/mL
- MB: 100 nM
- time exp: 6h

\textit{Survivin mRNA detection by RT-PCR}

\textbf{HEALTHY CONTROL: the Human Dermal Fibroblasts (HDFa)?}


PMMA-NP are inside the cells but red fluorescent signal is visible
HDFa at very low cell density

Experimental conditions:
- M106 complete medium
- PMMA-NP: 25 µg/mL
- MB: 100 nM
- time exp: 6h

PMMA nanoparticles are inside the cells but extremely low red fluorescent signal is detectable.
**Results**

**SURVIVIN mRNA expression levels in CONFLUENT HDFa cells:**

**REAL-TIME PCR studies**

Real-time PCR experiments demonstrated a **significant reduction** of survivin mRNA expression in confluent HDFa compared to HDFa at intermediate cell density (in proliferation).

Superposed distributions of the pixel values in four regions of interest containing a single cell in dishes with low (LD), middle (MD) or high (HD) density. The BG refers to an image of an untreated cell.

Adinolfi B. et al., Biomedicine and Pharmacotherapy, 2015, 69: 228-232

- The red fluorescence is close to the BG level at both high and low cell density, while the signal is higher in the area of middle density.

- The mean of pixel values measured in two groups of cells at middle and high density are significantly different.

Survivin mRNA expression levels in HDFa at high density and in cells at middle density: REAL-TIME PCR studies

Significant reduction of survivin mRNA expression in HDFa at high density compared to HDFa at middle density.
- **System:** laser Scanning microscope Biorad RadiancePlus, on a Nikon inverted microscope Diaphot 3000

- **Objective:** 40x or 60x oil immersion

- **NA:** 1.4

- **Scanning speed:** Slow, 160 Hz

- **Pinhole:** 1.4 for 488nm laser; 2.5 for 638nm laser

- **Z stack:** 0.5 µm Z-step

- **Z stack analysis with ImageJ software with MEDNUC or 3D Volume Viewer plugins

- **Colocalization criterium:** Pearson’s index
Dextran, Alexa Fluor® 647: Fluorescence Ex/Em spectra

Dextrans are **hydrophilic polysaccharides** characterized by their moderate-to-high molecular weight, good **water solubility**, and low **toxicity**. Dextrans are **biologically inert** due to their uncommon poly-(α-D-1,6-glucose) linkages, which render them resistant to cleavage by most endogenous cellular glycosidases.

λ<sub>ex/em</sub> 660-668nm
**A549 cells: UP-TAKE STUDIES**

**Experimental conditions:**

- NPs 10 µg/mL
- MB 100 nM
- Cascade Blue Dextran (750 µg/mL PBS)
- RPMI 10% FBS
t  exp:1h 30' + 1h

Results

B. Adinolfi

NanoItaly 2015, September 22, Rome, Italy
**Experimental conditions:**

- NPs 10 µg/mL
- Cascade Blue Dextran (750µg/mL PBS)
- RPMI 10% FBS
- t exp:1h 30’ + 1h
**A549 cells: UP-TAKE STUDIES**

**Experimental conditions:**

- MB 100 nM
- Cascade Blue Dextran (750µg/mL PBS)
- RPMI 10% FBS
- t exp: 1h 30’ + 1h