NEW IMAGING AGENTS

XIV. INTERNATIONAL SYMPOSIUM ON RADIONUCLIDES IN NEPHROUROLOGY
Mikulov Czech Republic

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MOLECULAR IMAGING DISCIPLINE

• One of the most rapidly growing areas of science.

• Visualize, characterize, and measure processes on molecular and cellular levels

• Perform experiments noninvasively in living systems.

• The key points to be considered:
  • the molecular imaging agent (probe or tracer)
  • the localisation of the target, which can be intracellular or cell surface proteins
  • the performances of detection system
IMAGING DEVICES

• A growing demand for imaging tools for biomedical research and medicine.
• Each technique has advantages and limitations, thus making them complementary.
  ➢ increasing use for biodistribution studies
  ➢ a significant reduction in the number of required animals
  ➢ an increase in the experimental data harvested for each animal in longitudinal studies.
• However, the high cost of several of these techniques and a number of technological barriers impair their widespread use.
Non invasive imaging approaches

- Nuclear imaging
- Optical imaging
- Magnetic resonance imaging
- Computed tomography,
- Ultrasound.

All these techniques require the accumulation of a signal delivered by a probe at the target site.
May 13, 2010 Mikulov

- micro-PET
- micro-SPECT
- micro-CT
- optical reflectance fluorescence (GFP) imaging
- optical bioluminescence (luciferase) imaging
- micro-MRI
ROLE OF CHEMISTRY

• Since 1970, the refined analytical techniques developed have allowed chemists to understand the true structure of a lot of materials at the atomic and molecular scales.
• Soft chemistry processes are well suited for the construction of new materials with original structures and shapes.
• Currently, chemical processes enable the controlled design of new materials for applications in imaging such as:
  • Nanotubes of carbons
  • Inorganic Quantum dots
  • Organic nanoparticles
TARGETS OF MOLECULAR IMAGING

Proteins  - membrane receptors
- cellular metabolism
- cell functions

RNA (antisens imaging)

DNA (reporter gene/reporter probe)

adapted from R.Weissleder - U.Mahmood - Radiology, 2001 ; 219 :316-333
RENAL TUBULAR CELLS

Cellular surface proteins
➢ Antigens
➢ Receptors

Membrane transporters: OAT, OCT, ….

Enzyme CAIX overexpressed in clear cell renal carcinoma

adapted from Z. Szabo, 2006
TARGETS OF MOLECULAR IMAGING

The number of targets varies by its type. It is very low for genes and may represent 1 million of potential targets for a protein.

<table>
<thead>
<tr>
<th>Target</th>
<th>Number/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene (DNA)</td>
<td>2</td>
</tr>
<tr>
<td>Message</td>
<td>50-1,000</td>
</tr>
<tr>
<td>Protein</td>
<td>100 - 1,000,000</td>
</tr>
<tr>
<td>Function</td>
<td>massive</td>
</tr>
</tbody>
</table>

adapted de R.Weissleder - U.Mahmood - Radiology, 2001 ; 219 :316-333
MCQ 1: what is the best sensitivity with optical imaging

1. The sensitivity is about $10^{-3} - 10^{-5}$ mol/L
2. The sensitivity is about $10^{-6} - 10^{-9}$ mol/L
3. The sensitivity is about $10^{-9} - 10^{-12}$ mol/L
4. The sensitivity is about $10^{-12} - 10^{-14}$ mol/L
5. The sensitivity is about $10^{-15} - 10^{-17}$ mol/L
<table>
<thead>
<tr>
<th>MODALITY</th>
<th>Resolution</th>
<th>Sensitivity (mole/L)</th>
<th>Amount required</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>1-2 mm</td>
<td>$10^{-11}$-$10^{-12}$</td>
<td>ng</td>
</tr>
<tr>
<td>SPECT</td>
<td>1-2 mm</td>
<td>$10^{-10}$-$10^{-11}$</td>
<td>ng</td>
</tr>
<tr>
<td>Optics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bioluminescence</td>
<td>3-5 mm</td>
<td>$10^{-15}$-$10^{-17}$</td>
<td>ng - pg</td>
</tr>
<tr>
<td>fluorescence</td>
<td>2-3 mm</td>
<td>$10^{-9}$-$10^{-12}$</td>
<td>ng</td>
</tr>
<tr>
<td>MRI</td>
<td>25-100 µm</td>
<td>$10^{-3}$-$10^{-5}$</td>
<td>mcg - mg</td>
</tr>
<tr>
<td>TDM</td>
<td>50-100 µm</td>
<td>not applicable</td>
<td>not applicable</td>
</tr>
<tr>
<td>US</td>
<td>50-500 µm</td>
<td>not applicable</td>
<td>mcg - mg</td>
</tr>
</tbody>
</table>

Dedicated animals systems
INTRINSIC SENSITIVITY OF THE DIFFERENT MODALITIES

«MRI requires concentrations of paramagnetic substances of about $10^{-6}$ molar to change relaxivity, while iodinated contrast agents require about $10^{-2}$ molar to achieve opacification with CT»

«…, radiotracers are useful to evaluate high affinity, low abundance systems, … will not cause a pharmacological effect»

«… biological systems that are particularly sensitive to small quantities of material, such as many receptor systems and intra-cellular processes, can be readily evaluated with radiotracers»

Blankenberg FG, Strauss HW.
CHOICE OF MODALITIES

- Although PET is more sensitive than SPECT, the latter offers advantages, such as broad availability and lower costs of radioisotopes and detection instruments.
- In addition, since SPECT radionuclides emit photons with different energies, several biomarkers can potentially be distinguished at the same time, as opposed to PET radionuclides, which all emit the same energy photons (511 keV).
- MRI is a powerful imaging modality as regards to its high spatial resolution (100 µm) and tomographic capabilities, but the low signal sensitivity has been a major limitation for molecular imaging.
PET radiopharmaceuticals

• Advantages:
  ➢ High molecular specificity and selectivity
  ➢ High detecting sensitivity and resolution
  ➢ Nearly perfect quantification
    ❖ High signal to background ratio
    ❖ High signal to noise ratio
    ❖ 4D image acquisition (x,y,z,t)

• Disadvantages:
  ➢ Costs
  ➢ Long time for the RP development
  ➢ Difficult approval process by authorities
OPTICAL IMAGING APPROACHES

• Attractive low-cost alternatives to nuclear medicine imaging approaches.
  ➢ very high sensitivity
  ➢ $10^{-15} - 10^{-17}$ mol/L for bioluminescence,
  ➢ $10^{-9} - 10^{-12}$ mol/L for fluorescence
  ➢ realtime imaging of small animal models

• Major disadvantage when using visible light is poor tissue penetration (1 cm) due to scatter and absorption of light.

• Bioluminescence has some advantages over fluorescence, since signal is produced in the presence of substrate (no excitation light source is needed), and there is no background autofluorescence.

• Tomographic imaging remains a challenge.
MAIN FIELDS OF APPLICATION

• Analysis of perfusion, Flux and transit time
• Kinetics
• Metabolism
• Binding to membrane receptors
• Neurotransmission
• Monoclonal antibodies
• Aptamers
• Apoptosis
• Gene expression
A NEW MODEL FOR THE OPTIMIZATION OF IMAGING AGENTS

Antibody based vectors
MCQ 2: about antibodies

1. The blood clearance of humanized mAbs is higher than that of murine mAbs
2. The molecular weight of IgG is about 250,000 dalton
3. The clearance rate of a fragment $F_{\text{ab}}$ is lower than that of mAb
4. Fragment $F_{\text{ab}}$ is monovalent.
MONOCLONAL ANTIBODIES

• Attractive candidates for targeted therapy and diagnostics
• Advantages:
  ➢ Monoclonal antibodies are high affinity molecules
  ➢ Highly specific targeting ability
  ➢ High signal delivery to cell surface molecules.

• But slow progress and a lot of disappointments during the development of mAbs
  ➢ poor performance of rodent mAbs in human beings
  ➢ human anti-mouse antibody responses,
  ➢ short half-lives, and inability to trigger human effector functions).
MONOCLONAL ANTIBODIES

• Today’s antibody engineering technologies enable routine production of novel fully human or humanized mAbs.
• Despite the development of nonimmunogenic antibodies, only HumaSPECT® is a human mAb among the diagnostic mAbs/Fab.
• A major disadvantage of using intact antibodies as imaging probes is that they circulate in the blood for several days, making them unsuitable as imaging probes.

\[
\text{Clearance rate of } F_{\text{ab}} \text{ or } F_{\text{ab}'} > F_{(ab')2} > \text{IgG.}
\]
MONOCLONAL ANTIBODIES

- Efforts to improve antibodies pharmacokinetics without compromising affinity and specificity have been made through protein engineering.

- Antibody variants that differ in antigen binding sites and size have been generated and evaluated as imaging probes to target tissues of interest.
  - production of $F_{ab}$ fragments by proteolytic reaction
  - disadvantage of $F_{ab}$ is that it has only 1 antigen-binding site
  - decrease in the overall functional affinity of the antibody
ANTIBODY BASED VECTORS

• Single-chain variable fragment scFv:
  - variable light and heavy domains
  - joined by a flexible peptide linker
• Fast clearing fragments (scFv; 25 kDa), with 1 antigen-binding site (monovalent)
• Low accumulation in tumors because of the low exposure time to the target.
Larger fragments exhibit usually better tumor penetration, and excellent tumor to blood ratios.

Figure 1: Schematic presentation of an intact antibody and engineered antibody fragments derived from it, including a single variable domain fragment (Fv), single chain Fv (scFv), diabody, minibody, and scFv-Fc. Molecular weights are indicated below each fragment. ($V_L$, variable light [light green]; $V_H$, variable heavy [dark green]; $C_L$, constant light [purple]; $C_H$, constant heavy [blue]).

From Olafsen and Wu, Semin Nucl Med - 2010 ; 40:167-181
Once the optimal antibody-based vector has been generated, they can be tagged with:

- radionuclides (PET, SPECT)
- magnetic nanoparticles (MRI). As an example, the recent work evaluating nanoprobes of iron oxide nanoprobes conjugated to Herceptin may make Ab fragments attractive MRI imaging agents
- or optical probes. Several studies have investigated antibody fragments conjugated to fluorescence (near infrared dyes), bioluminescence (luciferases) or quantum dots.

that enable a variety of imaging modalities.
(PET) of anti-CEA T84.66 diabody in LS174T xenograft-bearing mice. (A) Coronal PET images of mice injected with diabody after radiolabeling with different positron emitting radionuclides. Specific localization to the positive tumor (arrow) and no localization to the negative C6 rat glioma tumors (arrowhead) are seen in all the images.

From Olafsen and Wu, Semin Nucl Med - 2010 ; 40:167-181
ANTIBODY BASED VECTORS

- In fact, numerous studies indicate that there are several factors that influence successful targeting and imaging.
- These include stability of the antibody fragment, the labeling chemistry (direct or indirect), whether critical residues are modified, the number of antigens expressed on the cell, and whether the target has a rapid recycling rate or internalizes upon binding.
- The preclinical data available are compelling, and it is obvious that antibody-based vectors will play an important future role in the diagnosis and management of cancer and other diseases.
MCQ 3: about aptamers (only 1 yes)

1. It is a new class of antibodies
2. It is a new family of membrane receptors
3. Aptamers are scaffold structures for building quantum dots.
4. They can be compared to antibodies.
5. Aptamers are heterodimers of proteins.
A NEW MODEL OF PRODUCTION OF IMAGING AGENTS

APTAMERS
COMBINATORIAL CHEMISTRY

Banks of candidate radiopharmaceuticals
peptides, antibodies, aptamers, oligonucleotide antisens, ..

Screening \textit{in vitro}

- Molecular Characteristics
  - Affinity and selectivity \textit{in vitro}
  - Molecular weight and size
  - Lipophilicity

- Unknown ...
  - Non specific binding \textit{in vivo} ?
  - Metabolism \textit{in vivo} ?
  - Transfer through BHE ?
SCREENING OF LIBRARIES

in vivo

Direct approach in living animals

Selection of candidate tracers directly in their future conditions of use.

- Parallel chemical synthesis
- Radiolabelling of the bank (\(^{11}\)C ou \(^{18}\)F)
- Pharmacokinetic study by PET imaging in rats
- Study of metabolic pathways in vivo
- Evaluation of the transfer through the BHE
APTAMERS - DEFINITION

• Aptamers are biochemical tools that can be used in biotechnology diagnostic or therapeutic applications.

• It depends on the target against which they are directed.

• As regards to their selectivity and binding properties to ligands, aptamers are often compared to antibodies.
APTAMERS - DEFINITION

- An aptamer is a synthetic oligonucleotide, usually an RNA which is capable of binding a specific ligand and sometimes catalyze a chemical reaction on the ligand.

- Aptamers are usually synthetic compounds, isolated in vitro from combinatorial libraries of many compounds by a random sequence selection iterative method called **SELEX**.
THE SELEX METHOD (1)

• The SELEX requires a library of oligonucleotides whose sequence to the 5′ end and 3′ end is known and whose core sequence is random.
• This collection of oligonucleotides is prepared according to the same principle of synthetic oligonucleotides.
• The difference is that, in steps of the elongation of nucleotides of the central region, the four bases are used to react instead of only put a single base.
• Thus, this method provides a collection of oligonucleotides with different central sequences.
THE SELEX METHOD (2)

The principle of SELEX method is to select the oligonucleotides that bind with higher affinity with a target.

Affinity chromatography is used to select these oligonucleotides. Then the oligonucleotides are separated from the molecules of interest and are amplified by cloning or PCR.

The stage of selection of oligonucleotides with high affinity for the target molecule and the amplification step are repeated several times to obtain oligonucleotides with the highest affinity for the target.

The oligonucleotides selected are called aptamers.
Aptamers
(target-adapted oligomers)

Systematic Evolution of Ligand by EXponential Enrichment

Kindly provided by Pr B. Tavitian  CEA-DSV
<table>
<thead>
<tr>
<th></th>
<th><strong>Aptamers</strong></th>
<th><strong>Antibodies</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight</strong></td>
<td>9-15 kDa</td>
<td>&gt;150 kDa</td>
</tr>
<tr>
<td><strong>Discrimination</strong> (Theophylline versus caffeine)</td>
<td>+++</td>
<td>+/+++</td>
</tr>
<tr>
<td><strong>Affinity</strong></td>
<td>5-100 nmol/L</td>
<td>0,1-100 nM</td>
</tr>
<tr>
<td><strong>Production</strong></td>
<td>Synthetic</td>
<td>Living cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Biotech)</td>
</tr>
<tr>
<td><strong>Validation</strong></td>
<td>in vitro</td>
<td>in vivo</td>
</tr>
</tbody>
</table>
SIMULTANEOUS PET IMAGING

4 rats

Caméra EXACT HR+ (SIEMENS)

Acquisition 60’
24 x 10s
18 x 20s
20 x 30s
40 x 60s
Screening of 26 compounds

Image 4 (~ 40 sec)
A PROMISING METHOD FOR THE CONCEPT OF PROOF

SMALL ANIMAL OPTICAL IMAGING
MCQ 4: optical imaging (only 1 yes)

1. GFP is a very useful probe in vivo because its spectrum is far from the peak of absorption of Hb
2. Autofluorescence is observed only after administration of xenobiotics.
3. Persistent luminescence can reach several hours with new generations of nanoparticles.
4. Larger quantum dots emit bluer light.
5. Fluorescence: the emitted radiation has always a larger wavelength than the excitation source.
OPTICAL IMAGING

- Photons are the information source,
- Direct applications in pharmacology, molecular and cellular biology, and diagnostics.
- Development of new and more sensitive optical sensors
  - semiconductor nanocrystals,
  - fluorescent proteins,
  - or near-infrared fluorescent molecules,
- Optical imaging can now be considered for in vivo studies.
FLUORESCENCE

- **Fluorescence** is the emission of electromagnetic radiation light by a substance that has absorbed radiation of a different wavelength.
- In most cases, absorption of light of a certain wavelength induces the emission of light with lower energy.
- However, under specified conditions, it is possible for one electron to absorb two photons (multiple photon absorption).
- It leads to the emission of radiation having a smaller wavelength than the excitation source.
GREEN FLUORESCENT PROTEIN (GFP)
FLUORESCENCE

Use of Near Infrared biomarkers

Laser excitation

Filtre

CCD


J.L.Coll - INSERM-U578
Red fluorescent protein-expressing Mia-PaCa-2 Human pancreatic cancer cells

*In vivo*

*Katz et al, J Surg Res 2003; 113, 151-60*
Evaluation of the pharmacological response
days after transpl. cell. Mia-PaCa-2 expressing RFP

Control

Irinotecan

Gemcitabine

Katz et al, Cancer Res 2003; 63, 5521-25
BIOLUMINESCENCE

Luciferin + Luciferase + ATP → Oxyluciferin + Luciferase + CO₂

O₂ → AMP

J.L. Coll
INSERM-U578
La D-luciférine is the probe of luciferase (firefly luciférase, *Fluc* gene)

Bioluminescent Reporter probe

Adapted from TF Massoud et SS Gambhir, Genes & development, 2003
BIOLUMINESCENCE

Luciferase-based system


Luciferase

Luciferin

Photons

CCD

PC
a- Fluorescence imaging in the visible light range (400-600 nm) to detect GFP.

b- Tumors expressing luciferase imaged with a photon-counting camera after ip injection of luciferin (bioluminescence)
c- Near-infrared fluorescence imaging (700-900 nm) to image deeper tumors (matrix metalloproteinase 2 enzyme levels in breast tumors – NIR fluorescence probe coupled to an MMP-2 substrate).

R. Weissleder, Nature Reviews Cancer, 2002
FLUORESCENCE IN VIVO AFTER ACTIVATION OF PROBES

*In vivo* imaging of tumors with protease-activated near-infrared fluorescent probes.
Matrix metalloprotease-2-sensitive fluorescent probe for in-vivo near-infrared fluorescence imaging

Synthetic peptides containing cleavable sites by MMP-2 link the fluorochromes (covalent link) to a skeleton of polylysines protected by lateral chains of méthoxy poly-éthylène glycol (MPEG)
signal preexisting to the interaction delay to increase S/B

signal created by the interaction with the target
**In vivo FLUORESCENCE**

- Fluorescent probes are used but still presents numerous disadvantages
  - Autofluorescence from tissue organic components due to constant probe illumination during signal acquisition.
  - This autofluorescence often results in poor signal-to-noise ratio.
  - Deep tissue imaging is difficult due to intrinsic tissue signal attenuation.

- The probe’s emission has thus to be tuned in the tissue transparency window (wavelength from 650 nm to the infrared), in which light attenuation is largely due to scattering rather than to absorption.
LONG LUMINESCENT NANOPARTICLES

• New technology to produce long luminescent nanoparticles emitting in the red to near-infrared range
• They can be optically excited before *in vivo* local or systemic injection.
• The long-lasting afterglow (also called persistent luminescence) can reach several hours and permits the removal of the background
• Noise is originating from *in situ* excitation.
• Thus, the significant signal-to-noise ratio improvement allows detection in rather deep organs and real-time biodistribution monitoring of active elements hours after injection.
PERSISTENT LUMINESCEENCE PROBES

• Probe is excited before the injection ⇒ no autofluorescence and tissues absorption
• Emit light in the tissue transparency window (> 650 nm) for several hours
• Nanometric size
• Functionalizable

From Le Masne de Chermont, PNAS, 2007, 104
DETECTION of the nanoparticles in vivo

- Suspension of $\text{Ca}_{0.2}\text{Zn}_{0.9}\text{Mg}_{0.9}\text{Si}_2\text{O}_6$: $\text{Eu}^{2+} \text{Dy}^{3+} \text{Mn}^{2+}$ (1 mg/mL)
- Excitation under a UV light for few minutes
- Injection to an anesthetized mice (50 µL)
- Counting of emitting photons with an ICCD camera (Biospace)

Intramuscular injection

2 minutes acquisition time

Kindly provided Cyrille RICHARD, CNRS UMR 8151, Inserm U1022
Semiconductor
QUANTUM DOTS (QDs)

• An effective approach to fluorescent nano-objects for biological imaging.
• Properties of these inorganic nanoparticles:
  - large one- and two-photon absorption cross-sections,
  - good fluorescence quantum yields,
  - broad excitation but narrow emission bands,
  - and high photostability.
• Particular interest for in vitro and in vivo imaging.
• Applications in specific labeling of cells and tissues.
Semiconductor QUANTUM DOTS (QDs)

- Photoluminescence is tunable
- because their emission spectra can be tuned by playing on their size and composition, they can be used for multicolor imaging.

*From Webb et al. Science, 2003, 300, 1434*
Semiconductor QUANTUM DOTS (QDs)

• Quantum dots are superior to traditional organic dyes:
  ➢ brightness (owing to the high extinction coefficient combined with a comparable quantum yield to fluorescent dyes)
  ➢ stability (allowing much less photobleaching)

• It has been estimated that quantum dots are 20 times brighter and 100 times more stable than traditional fluorescent reporters.

• For single-particle tracking, the irregular blinking of quantum dots is a minor drawback
QUANTUM DOTS (QDs)

• **Applications:**
  - two-photon excited fluorescence (TPEF) imaging
  - microscopic imaging, including intrinsic three-dimensional resolution and increased penetration depth in tissues.
  - acquisition of many consecutive focal-plane images that can be reconstructed into a high-resolution three-dimensional image
  - real-time tracking of molecules and cells over extended periods of time (observation of quantum dots in lymph nodes of mice for more than 4 months)
  - in vitro imaging of pre-labeled cells.

• The ability to image single-cell migration in real time is expected to be important to several research areas such as cancer, stem cell therapy and immunology.
DRAWBACKS OF INORGANIC QDs

- Biological toxicity, due in particular to the presence of heavy metals such as cadmium and blinking.
- Their surface functionalization is possible but demanding:
  - such as targeting,
  - recognition,
  - conjugation to biomolecules
  - or specific labeling is possible but not undemanding.
- These inorganic nano-objects also raise a number of questions with respect to environmental issues.
ORGANIC NANODOTS

Today, the development of new imaging agents for medical applications is highly challenging.

It is time consuming and expensive

It is worthwhile to consider carefully the best method to demonstrate the proof of concept

Thank you for your attention

and special thanks to Alain Prigent