Concept validation of theranostics imaging of tumours labelled with Gold NanoParticles

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The «dwarfs»...team & institutions

✓ This project sits at the interface between engineering, life sciences and physical science.

✓ The results presented in this contribution stem out a collaborative interdisciplinary effort aimed at identifying the most suitable theranostic imaging techniques to map the distribution of functionalized nanoparticles acting as biological markers taken up by specific cellular receptors in healthy and pathological biological tissues: tissue engineering 3D in vitro cancer models; manufacturing gold nanoparticles targeted to cancer for imaging; development of sensitive x-ray diagnostic methods for imaging biomarkers; and assessing the radiobiological impact during radiotherapy treatments.
Theranostics is a treatment strategy that combines diagnostics and therapeutics. Originally used to describe the process of diagnostic therapy for individual patients for drug testing, nowadays it is a concept referring to the integration of imaging and therapy. As an evolving new field, it is related to but different from traditional imaging and therapeutics. It embraces multiple techniques to arrive at a comprehensive diagnostic, in vivo molecular images and an individualized treatment regimen. More recently, there is a trend of tangling these efforts with emerging materials and nanotechnologies, in an attempt to develop novel platforms and methodologies to tackle practical issues in clinics.

- almost 4 decades since the “war on cancer” was declared.
- now generally believed that personalized medicine is the future for cancer patient management.

Outline

✓ Introduction
✓ Use of GNPs
✓ 3D tumour model
✓ X-ray spectrometer design and performance
✓ X-ray imaging of the tumour
✓ A step beyond...
✓ Conclusions
Introduction

- Tumours are heterogeneous tissues that encompass a variety of cell types of differing radiosensitivities.
- Distribution of cells is also heterogeneous throughout the tumour volume.
- Infiltrating cells may lie outside the treatment volume and survive therapy, thereby reducing treatment efficacy.

- Tumour microenvironment also dictates treatment success.
- Modern intensity modulated radiotherapy techniques used to deliver heterogeneous dose distributions across the tumour, boosting the dose to less radiosensitive regions.

Need for a functional imaging technique sensitive to tumour characteristics providing radiosensitivity information (such as oxygen concentration, proliferative ability and angiogenesis [1]).

Use of GNPs in nanomedicine

✓ GNPs are increasingly used in *in vivo* cancer models to act as a contrast agent for X-ray based imaging techniques.

**Passive targeting**
- tumour microenvironment: leaky vasculature - pores up to 400 nm - and dysfunctional lymphatic drainage system increasing retention of particles

- uptake of nanoparticles by cancer cells *could also enable detection of small clusters of infiltrating cancer cells* currently missed by commonly used imaging modalities.

✓ GNPs are *biocompatible* (Au biologically inert and stable), and have readily *modifiable surfaces to increase solubility* (in order to travel through the bloodstream) and enhance cell-specific uptake

✓ GNPs are an *ideal contrast medium for use in X-ray imaging techniques*:
  - high atomic number (Au:79) \(\rightarrow\) more effective than iodinated (I:53) contrast techniques
  - molecular imaging possible due to the greater number of atoms delivered to the receptor site (~250 Au atoms per 1.9 nm nanoparticle compared with 3 (monomer) or 6 (dimer) I atoms per molecule)
  - as an imaging contrast agent for radiotherapy *enhance local dose deposition for both X-ray radiotherapy and proton radiotherapy*

**Active targeting**
- antibodies attached to GNPs taken up by specific cellular receptors \(\rightarrow\) markers for bio-parameters providing information about cell radiosensitivity

Use of GNPs
3D tumour model: why?

2D *in vitro* cell culture

- reproducible, dependable and robust techniques and models
- seeding established cancer cell lines into culture grade plasticware,
- cells attach to the bottom plastic surface and grow to form a confluent monolayer, in the presence of liquid nutrients
- however, in the human body cells reside in 3D configurations often surrounded by ECM

Animal models

- most commonly using the laboratory mouse (*Mus musculus*), used to address the limitations of 2D culture systems
- human tumour xenografts (implanting human cancer cells in organs / tissues of the mouse) potential to mimic in vivo human tumours and associated microenvironment
- mice immuno-compromised do not reject human cells
- more realistic 3D model of disease (cancer cells closer to the phenotype found in native human cancers) but the mouse as a tumour host does not necessarily respond as the human body.
3D tumour model: why?

- **3D in vitro tumour models**
  - 3D culture systems range in complexity from cancer cell spheroids to more complex tissue engineered models
  - attempt to address limitations of 2D culture
  - aim to provide viable, but presently limited, alternatives to animal models

- **Multi-cellular sferoids (MCSs)**
  - MCSs formed by culturing cells in spinner flasks or agar coated culture plates
  - cells form spherical clusters up to some mm in size able to survive for weeks
  - offer a reproducible model that more closely resembles in vivo tissue
  - however, MCS do not fully emulate native tumour tissue in that they do not include a complex ECM

- **3D matrices (Scaffolds)**
  - porous structures supporting cell growth, organisation and differentiation
  - engineered to mimic the ECM - in human tissue composed of proteins, proteoglycans and polysaccharides
  - engineering the scaffold enables control of matrix characteristics such as density and porosity.
  - may be used as an animal-model replacement.
3D tumour model: GNP uptake

- previously developed technique that embeds cells in a 3D collagen matrix [1] extended to create novel 3D phantoms consisting of cells incorporating GNPs.
- cellular phantoms designed with the ability to control parameters such as cell type, cell density, GNP concentration and GNP distribution.
- rate of transport of GNPs into cells governed by:
  - NP size
  - NP shape
  - NP surface chemistry
  - NP charge
  - properties of the cell membrane dependent on cell type

Use of GNPs 3D tumour model: GNP uptake

- TEM images of GNP-filled vesicles in HT29 cells incubated at different GNP concentrations

1.9 nm GNPs uptake

- 3T3
- HT29

GNP conc. per million cells (mgAu/ml / M cells)


- tumour masses *in vivo* will passively take-up a greater amount of GNPs than healthy tissues/cells. This is due to a combination of leaky vasculature and poor lymphatic drainage around tumour sites (EPR, enhanced permeability and retention effect)

- preferential tumour uptake can be further improved through functionalising the GNPs with ligands that are actively taken up by proteins present on tumour cell membranes.
3D tumour model: Artificial Cancer Mass

- Previously developed technique that embeds cells in a 3D collagen matrix [1] extended to create novel 3D phantoms consisting of cells incorporating GNPs.
- Cellular phantoms designed with the ability to control parameters such as cell type, cell density, GNP concentration and GNP distribution.

**Cells 2D culturing**

Mouse embryonic fibroblast 3T3 [2] and colon adenocarcinoma HT29 [2] cell lines grow in flasks, adhering to a plastic surface, cultured in DMEM.

**GNP solution**

Prepared with Aurovist 1.9nm GNP at a concentration of 5 mgAu/ml for the HT29 cells and 2 mgAu/ml for the 3T3 cells.

**Type 1 collagen gel preparation**

Neutralizing NaOH

**Incubation with GNPs**

Cells incubated for 24 hours with the GNPs solution.

**Cells ready**

Black colour of pellet indicates successful uptake of the GNPs.

Type 1 neutralized collagen gel + GNP incubated Cells

- Black colour of pellet indicates successful uptake of the GNP.

Addition of GNP loaded cells to the collagen gel

HT29 gel plastic compression

- HT29 cells populated gel poured into a cuboidal mould and allowed to set. Afterwards gel compressed under a load.

Artificial Cancer Mass
dense ACM, populated with GNP loaded tumour cells, obtained

3D tumour model: Artificial Cancer Mass

**Artificial Cancer Mass**
- dense ACM cut in pieces, ready to be inserted in the model.

**non-dense collagen gel populated by 3T3 fibroblasts (healthy cells).**

Compressed high density artificial cancer mass of HT29 cells with GNP inclusions

Low density collagen matrix, populated with 3T3 (healthy) cells with GNP inclusions

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X-ray fluorescence imaging

- Energy of XRF X-ray is characteristic of element present in sample.
- Intensity of XRF signal is related to concentration of element in sample.
- XRF is non-destructive.

1. Incident X-ray
2. Ejected electron
3. Electron falls into vacant space
4. Characteristic fluorescence X-ray

Usually technique performed by a 2D positional scan of a sample against a collimated beam with an energy dispersive detector.
X-ray spectrometer design and performance
detection module

- Custom vacuum-tight assembly with Peltier cooling,
- Silicon Drift Detector 10 mm² active area, 450 μm thick (provided by PNSensor GmbH, Munich, Germany)
- No collimator on the detector.
- Custom Pulsed-Reset Charge Amplifier configuration with on-chip JFET as first transistor and a parasitic capacitance between anode and a neighbor p⁺ ring as feedback capacitor.

⇒ very high resolution and peak stability, nearly insensitive to count rate variations
⇒ 97% efficiency at Au Lα line
X-ray spectrometer design and performance
84MLS11 X-ray polycapillary mini-lens

Specifically designed lens realised by IfG GmbH (Berlin, Germany), optimised for a few hundred microns source size, with the following parameters:

<table>
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<th>Parameter</th>
<th>Description</th>
<th>Value</th>
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<td>( f_1 ), mm</td>
<td>Focal distance - sample side</td>
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</tr>
<tr>
<td>( f_2 ), mm</td>
<td>Focal distance - detector side</td>
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<td>( L ), mm</td>
<td>Lens length</td>
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<td>( D_{\text{in}} ), mm</td>
<td>Lens diameter - sample side</td>
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<td>( D_{\text{max}} ), mm</td>
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<tr>
<td>( \phi ), rad</td>
<td>Capture angle</td>
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<td>( R_{\text{curv}} ), mm</td>
<td>Lens curvature radius</td>
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<tr>
<td>( D_{\text{capill}} ), ( \mu )m (center)</td>
<td>Capillary diameter - centre of the lens</td>
<td>30</td>
</tr>
</tbody>
</table>

X-ray spectrometer design and performance

Use of GNP3D tumour model

X-ray imaging of 2D collection profiles [1], measured with scanning \( \mu \)-beam PIXE [2]


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X-ray spectrometer design and performance

X-ray spectrometer performance - energy resolution

**Measured energy spectrum with a $^{55}\text{Fe}$ radioactive source in the lab (134 eV FWHM at the Mn $K_\alpha$ line at 3 $\mu$s shaping time).** The detector is Peltier-cooled to -30°C.

Energy resolution (and corresponding ENC) at the Mn $K_\alpha$ line measured with a $^{55}\text{Fe}$ radioactive source in the lab vs. shaping time constant. The detector is Peltier-cooled at -30°C.

X-ray spectrometer design and performance
Use of GNP's 3D tumour model

**X-ray spectrometer design and performance**

**X-ray spectrometer performance - sensitivity curve**

Energy spectra in the region of Au Lα and Au Lβ lines after subtraction from the pure water reference spectrum. Measurement time was set to 900 s for all spectra.

- DIAMOND, B16
- detection module mounted with a 90° geometry
- CCD camera behind the sample
- X-Y-Z micrometric stage system
- X-Y slit system and ionization chamber
- primary beam energy: 15 keV

Fluorescence yield vs GNP's concentration in distilled water. The straight line is the linear least square fit.
Use of GNP

3D model of tumour

X-ray imaging of the tumour

X-ray spectrometer design and performance

X-ray spectrometer performance – position resolution

holes: 9mm diameter, 8mm spacing
hole edge from X-rays entrance side of Perspex slab: 1mm

Au fluorescence yield vs scan coordinate

Phantom

Highest conc.
Decreasing conc.
Water

X-rays entrance side

8 mg/ml

0.5 mg/ml

0.1 mg/ml

2 mg/ml

8 mg/ml

Measure spectra as a function of the scan coordinate in the case of 8 mg/ml concentration

meas. time = 60 s

exp. data

best fit

Au fluorescence yield

(counts/s)

8 mg/ml

Au La + Lβ fluorescence yield

(counts/s)

(x-mm)

Au fluorescence yield

(counts/s)

x sample, scan coordinate (mm)

8 mg/ml

2 mg/ml

0.5 mg/ml

0.1 mg/ml

8 mg/ml

Measurement spectra as a function of the scan coordinate in the case of 8 mg/ml concentration.

Use of GNP's

3D tumour model

X-ray imaging of the tumour

X-ray spectrometer design and performance

A step beyond...
Use of GNP's solution filled hole.

✓ primary beam: 4mm x 4mm
✓ acq. time: 40s per point

Sample spectra in perspex and in the GNP's solution

Au L lines

X-ray spectrometer design and performance

-A step beyond...
X-ray spectrometer design and performance

X-ray spectrometer performance - 2D scans

2D step&scan image of the low and high concentration "donut" hole normalized to the image obtained in the elastic scattering ROI.
X-ray imaging of the tumour

experimental setup @B16 beamline - Diamond synchrotron light source

DIAMOND

B16 beamline

SYNCHROTRON LIGHT

POLYCAPILLARY LENS

SAMPLE

CHILLED SAMPLE HOLDER

SDD

CCD

✓ primary beam energy: 16 keV
✓ acq. time per point: 200s
✓ sample Peltier cooled at 5°C
✓ step&scan pitch: 0.25mm
Use of GNPs 3D tumour model X-ray spectrometer design and performance X-ray imaging of the tumour transmission imaging & X-ray subtraction imaging

CCD images dark-field subtracted and flat-field corrected

Imaged sample (concentration ratio 1:5)


Gold mass absorption coefficient

11.970 keV

11.870 keV

CCD images dark-field subtracted and flat-field corrected

X-ray imaging of the tumour

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X-ray imaging of the tumour
transmission imaging & X-ray subtraction imaging

X-ray subtracted image

11.870 keV
11.970 keV

Use of GNP
X-ray spectrometer
3D tumour model
X-ray imaging of the tumour

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X-ray imaging of the tumour

XRF imaging

Sample energy spectrum collected in one point

Use of GNRs
X-ray spectrometer design and performance
A step beyond...

3D tumour model

X-ray imaging of the tumour

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X-ray imaging of the tumour

XRF imaging - Au L image

Image of a X-Z slice interpolated to 0.1 mm. Gold Lα + Lβ counts in log scale

X-ray imaging of the tumour

Use of GPNs

X-ray spectrometer design and performance

A step beyond...
X-ray imaging of the tumour

Use of GNRs

3D tumour model

X-ray imaging of the tumour

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A step beyond: single-shot 2D XRF imaging

Controlled-Drift Detector

exp. setup @ SYRMEP

hor & ver slits

integrated phase

readout phase

2D single-shot image in the Au L-lines ROI.

Use of GNNs

X-ray spectrometer design and performance

3D tumour model

X-ray imaging of the tumour

Fe slab for alinement

(1.6 mg/ml)

(0.5 mg/ml)

(4 mg/ml)

(0.25 mg/ml)

hor & ver slits

ionization chamber

polycapillary collimating lens

Controlled-Drift Detector

Polycapillary collimating lens

[Image of the setup with labels for each component]

Elettra

[Image of Elettra facility]

Controlled-Drift Detector architecture

[Diagram of the detector architecture]

[Graph showing the distribution of Au L-lines ROI]

[Graph showing the distribution of Fe concentration]

(log scale)

acquisition time:

high contrast: 6519 s

low contrast: 11048 s.
A step beyond: translating to a lab system

**Mo anode XOS X-BEAM X-ray generator with collimating optics**

- Optic divergence:
  - $< 0.1^\circ$ @ Mo $K\alpha$ (FWHM)
- Mo $K\alpha$ intensity:
  - $> 5 \times 10^8$ ph/s @50kV/1mA

✓ sensitivity down to 3ppm
✓ already measured 3T3 & HT29 cells incubated with different GNPs concentrations (down to 0.5mgAu/ml)
✓ ready to acquire first images in step&scan and single-shot mode
Conclusions

- We developed a novel 3D in vitro tumour model, with gold nanoparticles inclusions, which closely mimic in vivo tumour conditions.
- As a diagnostic tool we conceived a high-resolution, high-sensitivity XRF spectrometer able to 3D-image the GNP distribution.
- For the first time we imaged a biological sample, with a challenging GNP concentration ratio of 5:1 between the tumour region and the surrounding gel, obtaining excellent results in distinguishing tiny tumour details.
- Feasibility of single-shot 2D XRF imaging, in which a wide beam excites the whole specimen and a 2D energy resolving detector detects the fluorescent X-rays with without scanning has been investigated.
- Successful translation from the synchrotron to a lab-based system with a white beam from a “conventional” X-ray generator.

Next step is to move the system in a bio-lab where 3D tumours are produced to perform real biological studies.
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Thank you for your attention!