Cellular imaging using Nanomaterials

ID 603: Introduction to Nanoscience and Nanotechnology
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Introduction: Implementation of nanoparticle use in cell biology has been one of the most exciting developments in this field in the past 5 years. Understanding of the interactions between nanoparticles and cells is the first step toward mechanistic understanding of the relationship between organisms and nanomaterials. Cellular studies provide a preliminary step for nanoparticle use in *in vivo* therapeutic or imaging purposes. Although cytotoxicity and the effects of cell loading by nanoparticles are of little consequence in fixed cells, nanoparticle biocompatibility and cellular uptake mechanisms are particularly relevant to live cell studies. Studies of the effects of nanoparticles on cellular proliferation and viability have shown that in most cases toxicity/biocompatibility of nanoparticles depends on their concentration. Surface modifications are often used to increase the functionality of nanoconjugates. In work with cells, surface modifiers serve to:

- increase cellular uptake of nanoconjugates,
- increase the specificity of cellular uptake, and
- increase the efficiency of intracellular targeting or retention of nanoconjugates.

These nanoparticle modifiers/ conjugants include various antibodies and peptides which improve cell type and subcellular compartment targeting, while nucleic acids (and their mimics) have been demonstrated to modify sub cellular retention of nanoconjugates.

Nanoparticle Chemistry: Nanoparticles are mesostructures with some unique properties compared to bulk materials on one hand and atomic or molecular structures on the other. Compared to the bulk materials with constant physical and chemical properties regardless of their sizes (until it reaches the nano-regime), the nanoparticles have size-dependent properties.

Compared to other types of nanoparticles, metal nanoparticles, particularly the noble metal nanoparticles, easily form various stable nanostructures, are non-toxic and able to bind different targeting molecules. In particular, gold nanoparticles are easily modified with alkanethiols forming a chemical bond between gold and sulfur, while silver nanoparticles react with amino-compounds due to the formation of silver–nitrogen bond. This surface chemistry provides diverse ways for functionalizing through conjugation of nucleic acids (DNA, RNA, and synthetic nucleic acids such as locked nucleic acids [LNAs], peptide nucleic acids [PNAs] etc.),

(poly)peptides or cellular ligands; e.g. thiocitic acid–polyethylene glycol–folate gold conjugates developed for targeting of cells with folate receptors. The noble metal nanoparticles such as gold and silver have strong, size-dependent and shape-dependent optical properties with an absorbance of surface plasmon resonance.

Thus different colors of the nanoparticles can be prepared from the same bulk metal by making nanoparticles of different sizes or shapes.

In summary, many nanoparticles have unique properties when compared to bulk materials of the same chemical composition. These unique chemical properties can be exploited for use in a variety of different applications including cellular imaging and delivery. The major types of nanoparticles that have been used for cellular imaging include polymer/ biomacromolecular nanoparticles, semiconductor nanoparticles, and metal nanoparticles. Each of these types of nanoparticles has different properties that permit binding of proteins and nucleic acids that can be used for cellular and intracellular targeting.

New developments in nanoparticle technology in recent years have offered numerous improvements to the study of fixed cells. Compared to traditional fluorescent dyes and proteins, modified quantum dots and gold nanoparticles possess alternative properties that enhance their imaging capabilities in cells that are fixed before imaging. Additionally, these nanoparticles enable multi-functional analyses of single samples using different forms of detection. Disadvantages of using nanoparticles are relatively minor and are increasingly being circumvented as technologies improve. For example, when conjugated to an antibody and used as a fluorescent tag, a quantum dot may be transformed into a non-fluorescent state upon initial illumination (termed blinking). This blinking may result in a false negative fluorescence reading during shorter periods of illumination. This problem may be avoided by increasing the length of time that quantum dots are illuminated prior to recording of fluorescence intensity. Future nanoparticles will likely have an increased number and diversity of properties which will widen the scope of their use as cellular biomarkers.

Two-photon excitation microscopy: Two-photon excitation microscopy is a fluorescence imaging technique that allows imaging living tissue up to a depth of one millimeter. The two-photon excitation microscope is a special variant of the multiphoton fluorescence microscope. Two-photon excitation can be a superior alternative to confocal microscopy due to its deeper tissue penetration, efficient light detection and reduced phototoxicity.

Two photon absorption (TPA) is the simultaneous absorption of two photons of identical or different frequencies in order to excite a molecule from one state (usually the ground state) to a higher energy electronic state. The energy

difference between the involved lower and upper states of the molecule is equal to the sum of the energies of the two photons. Two-photon absorption is many orders of magnitude weaker than linear absorption and is therefore not an everyday phenomenon. It differs from linear absorption in that the strength of absorption depends on the square of the light intensity, thus it is a nonlinear optical process.

The "nonlinear" in the description of this process means that the strength of the interaction increases faster than linearly with the electric field of the light. In fact, under ideal conditions the rate of TPA is proportional to the square of the field intensity. This dependence can be derived quantum mechanically, but is intuitively obvious when one considers that it requires two photons to coincide in time and space. This requirement for high light intensity means that lasers are required to study TPA phenomena. Further, in order to understand the TPA spectrum, monochromatic light is also desired in order to measure the TPA cross section at different wavelengths. Hence, tunable pulsed lasers (such as frequency-doubled Nd:YAG-pumped OPOs and OPAs) are the choice of excitation.

The Beer's law for OPA:

$$I(x) = I_0 e^{-\alpha\, c\, x}$$

changes to

$$I(x) = \frac{I_0}{1 + \beta c x I_0}$$

for TPA with light intensity as a function of path length or cross section x as a function of concentration c and the initial light intensity I_0. The absorption coefficient α now becomes the TPA cross section β. (Note that there is some confusion over the term β in nonlinear optics, since it is sometimes used to describe the second-order polarizability, and occasionally for the molecular two-photon cross-section. More often however, is it used to describe the bulk 2-photon optical density of a sample. The letter δ or σ is more often used to denote the molecular two-photon cross-section.)

Second-harmonic generation: Second harmonic generation (SHG; also called frequency doubling) is a nonlinear optical process, in which photons interacting with a nonlinear material are effectively "combined" to form new photons with twice the energy, and therefore twice the frequency and half the wavelength of the initial photons. It is a special case of Sum frequency generation.

Sum-frequency generation (SFG) is an example of a second order non-linear optical process. This phenomenon is based on the annihilation of two input photons at frequencies ω_1 and ω_2 while, simultaneously, one photon at frequency ω_3 is generated. In order that the sum-frequency generation takes place it is necessary that the following two conditions happen:

1. Energy conservation: $\hbar\omega_3 = \hbar\omega_1 + \hbar\omega_2$

2. Momentum conservation: $\hbar k_3 = \hbar k_1 + \hbar k_2$

The first condition shows the relationship of the frequencies between the input and output photons. It is possible to notice that the sum-frequency generation is a generalization of second harmonic generation. In the latter, $\omega_1 = \omega_2$, both of which can be provided by a single light source

In biological and medical science, the effect of second harmonic generation is used for high-resolution optical microscopy. Due to the phase-matching condition only non-centrosymmetric structures are capable of emitting SHG light. One such structure is collagen, which is found in most load-bearing tissues. Using a short-pulse laser such as a femtosecond laser and a set of appropriate filters the excitation light can be easily separated from the emitted, frequency-doubled SHG signal. This allows for very high axial and lateral resolution comparable to that of Confocal microscopy without having to use pinholes. SHG microscopy has been used for extensive studies of the Cornea and Lamina cribrosa sclerae, both of which consist primarily of collagen.

- Unlike fluorescence, the process of SHG only involves virtual electron energy transition without nonradiative energy loss.
- Such probes do not bleach over time and emit a stable, nonsaturating signal with a femtosecond-scale response time.
- This also results in the flexibility of tuning the wavelength of the SHG signal by changing the excitation wavelength accordingly.
- The coherent nature of the SHG signal is also a main advantage, providing a possibility to detect the second harmonic signal with interferometric optical techniques

This report primarily focuses on gold nanoparticles and SHG using $BaTiO_3$ crystals. Recently published papers in these fields are analysed in a later section.

Case Study # 1: Gold Nanoparticles in Biology: Beyond Toxicity to Cellular Imaging, Murphy, Gole et al. Accounts of Chemical Research Vol 41, No 12, December 2008 (1721-1730)

Before any discussion on the paper, the term Plasmon is introduced.

Plasmon: In physics, a plasmon is a quantum of plasma oscillation. The plasmon is a quasiparticle resulting from the quantization of plasma oscillations just as photons and phonons are quantizations of light and sound waves, respectively.

Plasmons can be described in the classical picture as an oscillation of free electron density against the fixed positive ions in a metal. To visualize a plasma oscillation, imagine a cube of metal is placed in an external electric field to the right. Electrons move to the left side (uncovering positive ions on the right side)

until they cancel the field inside the metal. The electric field is now switched off, and the electrons move to the right, repelled by each other and attracted to the positive ions left bare on the right side. They oscillate back and forth at the plasma frequency until the energy is lost in some kind of resistance or damping. Plasmons are a quantization of this kind of oscillation.

Surface plasmons are those plasmons that are confined to surfaces and that interact strongly with light resulting in a polariton. They occur at the interface of a vacuum or material with a positive dielectric constant, and a negative dielectric constant (usually a metal or doped dielectric). They play a role in Surface Enhanced Raman Spectroscopy and in explaining anomalies in diffraction from metal gratings (Wood's anomaly), among other things. Surface plasmon resonance is used by biochemists to study the mechanisms and kinetics of ligands binding to receptors (i.e. a substrate binding to an enzyme).

More recently surface plasmons have been used to control colours of materials. This is possible since controlling the material's surface shape controls the types of surface plasmons that can couple to it and propagate across it. This in turn controls the interaction of light with the surface. These effects have been engineered for both visible light and microwav radiation. Much research goes on first in the microwave range because at this wavelength material surfaces can be produced mechanically as the patterns tend to be of the order a few centimeters. To produce optical range surface plasmon effects involves producing surfaces which have features <400 nm. This is much more difficult and has only recently become possible to do in any reliable or available way.

What gives nano-particulate Gold a colour which is different from the colour shown by Bulk Gold?: In the range of ∼5-200 nm in diameter, gold nanoparticles are large enough to support a conduction band, are comparable to the mean free path of electrons in the metal at room temperature (∼100 nm), but are rather small compared with the wavelengths of visible light (∼400-750 nm). Irradiation with light at certain frequencies results in a collective oscillation of electrons known as “plasma oscillations” or “plasmons” that are generally pictured as washing over the surface of the particle (“surface plasmons” or “localized surface plasmon resonance”, LSPR). The optical properties of small metal nanoparticles are dominated by such collective oscillations that are in resonance with the incident electromagnetic radiation.

For gold, it happens that the resonance frequency of this oscillation, governed by its bulk dielectric constant, lies in the visible region of the electromagnetic spectrum. Because nanoparticles have a high surface area to volume ratio, the plasmon frequency is exquisitely sensitive to the dielectric (refractive index) nature of its interface with the local medium. Any changes to the surroundings of these particles (surface modification,

aggregation, medium refractive index, etc.) leads to colorimetric changes of the dispersions Particle aggregation leads to the coupling of plasmons, with a concomitant shifting of plasmon frequencies, resulting in a surface sensitivity that has been widely used for chemical sensing, assuming that particle aggregation is controlled by surface chemistry. Not only is light strongly absorbed by plasmons, it is also Rayleigh (elastically) scattered by them, and as the particle gets larger, a larger proportion of the outgoing light is scattered, compared with that absorbed. Because the light scattered from gold nanoparticles is in the visible portion of the electromagnetic spectrum in accord with their plasmon bands, it is possible to optically track the position of individual nanoparticles, paving the way for imaging applications.

In the past decade or so, numerous advances in the chemical synthesis of gold nanoparticles that are *not* spherical, especially anisotropic shapes such as nanorods, have opened up even more possibilities for sensing and imaging applications, for several different reasons. First, gold nanorods typically display two plasmon bands (one in the visible and another either in visible or in near-infrared, NIR) that are tunable depending on the dimensions of the nanorod; these two bands correspond to short axis (transverse) and long-axis (longitudinal) plasmon modes. Thus, if one wanted gold nanoparticles to absorb at a certain wavelength or frequency of light in the visible or NIR, one could synthesize particles of appropriate shape.

Second, anisotropic nanoparticles may have different chemical reactivity for different crystal faces. This property may lead to new assembly strategies or chemical sensing strategies; for instance, the longitudinal plasmon band, but not the transverse, will red-shift upon end-to-end aggregation of gold nanorods.

Toxicity of gold particles: The paper examined the uptake and potential toxicity of a series of gold nanoparticles in human leukemia cells. The nanoparticle library consisted of gold nanospheres that varied in both size (4, 12, and 18 nm diameter) and surface modifier. The surface modifiers included a range of anionic, neutral, and cationic groups: citrate, cysteine, glucose, biotin (aka vitamin B7 or H), and cetyltrimethylammonium bromide (CTAB). CTAB is the structure-directing agent that is used to control gold nanorod shape, and it appears to form a tightly bound cationic bilayer on gold nanoparticles, with the cationic trimethylammonium headgroup exposed to the solvent. The K562 leukemia cell line was exposed to the nanoparticles for three days, at which time the cell viability was determined using a colorimetric MTT assay, which measures mitochondrial activity in viable cells. The data suggested that none of the spherical gold nanoparticles were toxic to the human leukemia cells up to $\sim$100 μM in gold atom concentration, even though they were being taken up into the cells (confirmed by transmission electron microscopy of cell slices). Similar viability studies with immune system cells also

showed that gold nanoparticles were not cytotoxic and that they reduced the amount of potentially harmful reactive oxygen species in the cells.

Cellular Imaging Using the Plasmon Bands of Gold Nanorods: The plasmons of metal nanoparticles enable one to image individual particle location with optical microscopy. Two main imaging modalities have been demonstrated: dark-field optical microscopy and two-photon luminescence microscopy. Two-photon luminescence (TPL), which is thought to arise from coupling of weak electronic transitions in the metal to the plasmons in a nanoparticle, relies on the NIR properties of metallic nanorods. Implemented as a microscopy experiment, which requires femtosecond laser pulses, TPL has been used to image the location of individual gold nanorods as they flow in blood *in vivo* and as they target cancer cells by virtue of the proper surface chemistry. In dark-field optical microscopy, transmitted steady-state white light is blocked so that only steady-state scattered light is detected. Because the scattering propagates as a cone, the spot size is much larger than the nanoparticle itself. Spatial resolution of ~200 nm is achievable, and single particles can be imaged. Gold nanorods, compared with nanospheres, absorb intensely in the visible and NIR as a function of nanorod dimension

The group uses this technique as the basis for measuring local deformations in optically transparent materials. The paper uses the movement of gold nanorods to observe material response under loading. The response measured is displacement, with a resolution of ~200 nm; strain, which is a gradient of displacement, can then be numerically approximated from the displacement field.

Gold nanorods were added to thin collagen films and subsequently plated with neonatal cardiac fibroblasts, the cells responsible for depositing and modifying the ECM. Cells were stained with a fluorescent dye and could be imaged nearly simultaneously with the scattering from gold nanorods in a combination fluorescence-dark-field optical microscope. The positional displacements of the gold nanorods, generated by the traction forces applied by the cells through their attachments to the ECM, were tracked, and strain fields were calculated from the displacements.

Prospects for the future: Gold nanorods are an attractive alternative to traditional organic fluorescent dyes in that they do not photobleach, they can absorb throughout the visible and NIR, and they can be nontoxic under certain experimental conditions. In parallel and for each study, toxicity and side effects need to be thoroughly examined in the broadest possible context, as a function of nanoparticle size, shape, and surface coating, beyond simple cell lines, before human subjects can be exposed to these materials.

Case Study # 2: Two-Photon Luminescence Imaging of Cancer Cells using Molecularly Targeted Gold Nanorods; Durr, Larson et al *Nano Lett. 2007 April ; 7(4): 941–945*

The paper demonstrated the use of gold nanorods as bright contrast agents for two-photon luminescence (TPL) imaging of cancer cells in a three dimensional tissue phantom down to 75 µm deep.The TPL intensity from gold nanorod labeled cancer cells is three orders of magnitude brighter than the two-photon autofluorescence (TPAF) emission intensity from unlabeled cancer cells at 760 nm excitation light. Their strong signal, resistance to photobleaching, chemical stability, ease of synthesis, simplicity of conjugation chemistry, and biocompatibility make gold nanorods an attractive contrast agent for two-photon imaging of epithelial cancer.

Gold nanorods exhibit highly efficient single- and two-photon induced luminescence, which may be due to their ability to sustain resonating surface plasmons with minimal damping. Furthermore, their longitudinal plasmonic resonance can be tuned to the near infrared wavelengths, where biological tissue exhibit relatively small extinction coefficients. The TPL emission spectra shows that much of the emission light is also in the NIR region. These optical properties make gold nanorods an attractive contrast agent for biomedical imaging of highly scattered tissue.

By embedding cells in a collagen matrix, the performance of nanorods as contrast agents for deep-tissue imaging was explored. We found that TPL imaging of labelled cancer cells could be performed using less than 60 times the laser excitation power needed for two-photon autofluorescence (TPAF) imaging from unlabeled cancer cells, corresponding to a three order-of-magnitude increase in emitted signal for equal excitation intensity.

To functionalize the gold nanorods, the positive surface potential was converted to a negative surface potential by coating the CTAB with polystyrene sulfonate (PSS). Anti-EGFR antibody solution and nanorods were mixed at 1:1 volume ratio and allowed to interact to form fully functionalized nanorods. For the control, a non-specific antibody was used in place of the anti-EGFR antibody. Three-dimensional tissue phantoms were prepared using EGFR-over-expressing A431 skin cancer cells.

Two-photon images of a single layer of labelled and unlabeled cells at 760 nm excitation wavelength were taken. The images show successful labelling, cellular distribution of EGFR, and relative brightness of nanorods. The unlabeled cells show a relatively uniform distribution of TPAF signal throughout cellular cytoplasm. No signal is associated with the nuclei, which do not have significant concentrations of endogenous fluorophores that can be excited in the visible region. In labelled cells, bright rings can be easily seen; this is a characteristic

pattern of EGFR labelling and has been also reported in confocal reflectance imaging of EGFR using spherical gold nanoparticles. The discrete bright spots in the cytoplasm of nanorod labeled cells are indicative of endosomal uptake of EGF receptors labelled with nanorods inside cells

TPL images of cells treated with non-specifically conjugated nanorods show agglomeration of contrast agent and little attachment to cell membranes By tuning the wavelength from 710 nm to 910 nm, it was found that 760 nm excitation wavelength yielded the brightest TPL signal from the nanorods, as well as the brightest TPAF signal from the cancer cells. This wavelength corresponds to the longitudinal Plasmon resonance frequency of the nanorods. For the cancer cells, this finding is consistent with other reports that show that the biological molecules that are primarily responsible for the signal in TPAF imaging (NADH and flavins), have two-photon cross sections that increase with decreasing excitation wavelength from 1,000 nm to 750 nm, and level off around 750 nm To test the imaging ability of TPL deep into tissue, we imaged cancer cells embedded in a collagen matrix that mimics the epithelium tissue. To maintain a constant detected intensity throughout the phantoms, a 26% power increase was required for each 20 μm increase in imaging depth. The excitation power ratio of 26 between two imaging modalities indicates 675 times brighter TPL than TPAF.

Conclusions: This paper demonstrated the effective use of gold nanorods as bright contrast agents for TPL imaging. By labeling cancer cells with anti-EGFR nanorod conjugates, molecular specific imaging could be carried out with very high signal-to-noise ratios deep into a tissue phantom.

In cases where imaging depths will be limited by the maximum available power that can be delivered to the imaging plane without causing damage to tissue, gold nanorods might provide sufficient brightness to extend the maximum depth of imaging. Additionally, it was shown that the use of gold nanorods can expand the capabilities of TPI to allow non-invasive three dimensional imaging of a variety of new molecular signatures.

Case Study # 3: Three-dimensional harmonic holographic microcopy using nanoparticles as probes for cell imaging; Hsieh, Grange et al. 16 February 2009 / Vol. 17, No. 4 / OPTICS EXPRESS 2880

The paper describes a new type of marker using second harmonic generation (SHG) from noncentrosymmetric $BaTiO_3$ nanocrystals. These nanoparticles are attractive due to their stable, non-saturating and coherent signal with a femtosecond-scale response time and broad flexibility in the choice of excitation wavelength. A new type of marker was developed using the SHG from noncentrosymmetric nanocrystals which was referred to as “Second Harmonic Radiation IMaging Probes (SHRIMPs)”. Due to the intrinsic properties of SHG, SHRIMPs emit a stable coherent signal

which is suitable for long-term observations. These types of observations are usually complicated when using the fluorescent signal because of photobleaching and luminescence blinking.

Stabilization of the BaTiO3 SHRIMPs in a colloidal suspension: The authors obtained monodispersed BaTiO3 nanoparticles in colloidal suspensions by coating the particle surface with amine groups measured the 90-nm particle size distribution in suspension by dynamic light scattering (Brookhaven Instruments Corp. 90Plus) showing that most of the particles were well dispersed while less than 10% of the particles are clusters of double the size. The scanning electron microscope image confirms the size of the particles which were dried on a conducting substrate.

Optical Characterization of the BaTiO3 SHRIMPs: The authors measured the SHG efficiency by directly imaging BaTiO3 nanoparticles. The excitation light source consisted of 150 fs laser pulses centered at 800 nm wavelength. SHG optical spectrum centered at 400.6 nm with a full width half maximum of 5 nm. The power dependency and the optical spectrum was measured to confirm the SHG signal (Fig. 2). The SHG signal was observed for an hour with less than 5 % decrease under continuous excitation.

Single SHRIMP detection with the H2 microscope: The H2 microscope was sensitive enough to image an isolated BaTiO3 nanocrystal. Since the particle size is smaller than the optical diffraction limit at the SHG wavelength, the image of a single particle reflects the spatial resolution of the H2 microscope.

The single particle behaved as a point source at the SHG frequency. The radiated spherical waves interfered with the reference plane waves and a set of interference rings (Fresnel zone plates) were formed and recorded on the detector plane. By digital propagation, the field can be reconstructed at any plane from the recorded Fresnel zone plates.

H2 microscopic 3D imaging: A 3D distribution of SHRIMPs was prepared by randomly embedding 30-nm BaTiO3 nanoparticles in a ~100-µm thick polydimethylsiloxane (PDMS) film These four clusters were then imaged by the H2 microscope. The focus was changed by digitally reconstructing the images at different planes.

The H2 microscopy would have difficulty imaging a high density of SHRIMPs. This difficulty results from the intrinsic speckle noise and it is a fundamental limit of storing 3D information with a 2D image. However, when the density of SHRIMPs is low, the H2 microscopy with SHRIMPs makes scan-free 3D imaging possible.

3D imaging of the SHRIMPs in HeLa cells: HeLa cells were incubated for 24 hours at 37 ^{o}C with 30- nm BaTiO3 particles that were stabilized with aminomethylphosphonic acid. During the incubation, the amine group present on the SHRIMPs surface encouraged the cells to uptake them through endocytosis.

SHRIMPs would be engulfed non-specifically into vesicles and packed as clusters randomly by the cells. In this way a 3D distribution of SHRIMPs was formed inside the cells. Cross section views of a cell imaged by a confocal scanning microscope (Leica, SP5) confirmed that the SHRIMPs entered the cells by endocytosis. After 24-hour incubation, the HeLa cells were still alive. H2 microscopy was performed by focusing and reconstruction at different depths. Since the cells uptook the SHRIMPs randomly, the size of the SHRIMP clusters, and therefore the SHG intensity, varied in a wide range. Since the cells uptook the SHRIMPs randomly, the size of the SHRIMP clusters, and therefore the SHG intensity, varied in a wide range.

Conclusion: H2 microscopy was performed with $BaTiO_3$ nanoparticles as SHRIMPs for high resolution 3D imaging. The coherent SHG signal from SHRIMPs allows us to capture the 3D distribution of these markers in specimens by a H2 microscope without scanning.

The amine group present on the particle surface also offers the general crosslink for further conjugation to proteins or antibodies for specific labeling. Taking advantage of the coherent signal of SHRIMPs, the use of the H2 microscope as a 3D imaging technique was demonstrated. For the first time, the 3D distributions of SHRIMPs in biological specimens were imaged by the H2 microscope with diffraction limited spatial resolution.

New Frontiers: In conclusion, both techniques, SHG and TPF are pretty useful to image cells and this is helped by the fact they share many advantages over traditional fluorescence imaging techniques. In conclusion, imaging of live cells is important in assessing biological function, and nanotechnology offers many new approaches for such studies. whole cell imaging and manipulation by nanoparticles are at this time gathering momentum. The cell is the best starting point for development of new therapeutics and new cellular molecular biology techniques, because the whole cell as a biological entity has always been the first target en route to mechanistic understanding of both intracellular and whole organism pathways and processes.

Other Avenues in Nanoparticle-assisted imaging:

MRI Contrast agents: MRI depends on the fact that water and fat account for approximately 63 per cent of the body's hydrogen atoms. The proton in each hydrogen atom's nucleus has a tiny magnetic moment which is forced to line up with or against an MRI scanner's powerful magnetic field. Radio pulses of just the right frequency cause some protons to flip their spins, which can then relax back to their original state between pulses - the change in distribution of these magnetic moments forms the MRI signal. Contrast agents, injected into patients undergoing MRI, help to speed up this relaxation process. This in turn boosts the intensity of the MRI signal and helps to form much sharper images of tissue.

Conventional contrast agents include paramagnetic transition metal ions (such as Mn2+ and Fe3+) or rare-earth chelates (such as Gd3+). But there are drawbacks to using these metals. Free manganese ions substitute for calcium, causing cardio-vascular toxicity. Gadolinium chelates, used for central nervous system imaging, show breakages in the blood-brain barrier following strokes, but can cause kidney damage especially in patients already suffering renal impairment.

A recent solution by a Korean team (Hyeaon et al.) was to prepare insoluble manganese oxide (MnO) nanoparticles and then encapsulating them in a biocompatible polyethyleneglycol-phospholipid shell. Their size could be controlled during preparation by adjusting the solvent or the reaction time, with smaller nanoparticles - around 25 nanometres across - giving brighter contrast.

They tested the nanoparticles by rapidly injecting them into a mouse's tail, and subjecting it to an MRI scan 72 hours later. The images they saw were as clear as those usually obtained from dissection and histological examination. And by conjugating the MnO nanoparticles to a tumour-specific antibody, the Korean team could selectively target and image breast cancer cells in a metastatic brain tumour that had been implanted into another mouse.

Nanoparticle-CT Scan: Use of nanomaterials for one of the most common imaging techniques, CT, has remained unexplored. Current CT contrast agents are based on small iodinated molecules. They are effective in absorbing X-rays, but non-specific distribution and rapid pharmacokinetics have rather limited their microvascular and targeting performance. While most of the nanoparticles are designed to be used in conjunction with MRI, bismuth sulfide (Bi2S3) nanoparticles naturally accumulate in lymph nodes containing metastases and show up as bright white spots in CT images. A polymer-coated Bi2S3 nanoparticle preparation has been proposed as an injectable CT imaging agent. This preparation demonstrates excellent stability at high concentrations, high X-ray absorption (five-fold better than iodine), very long circulation times (>2 h) in vivo, and an efficacy/safety profile comparable to or better than iodinated imaging agents. The utility of these polymer-coated Bi2S3 nanoparticles for enhanced in vivo imaging of the vasculature, the liver and lymph nodes has been demonstrated in mice. These nanoparticles and their bioconjugates are expected to become an important adjunct to in vivo imaging of molecular targets and pathological conditions. Tumor-targeting agents are now being added to the surfaces of these polymer-coated Bi2S3 nanoparticles.

Combination of Diagnostics and Therapeutics for Cancer: Biomimetic Nanoparticles Targeted to Tumors: Nanoparticle-based diagnostics and therapeutics hold great promise because multiple functions can be built into the

particles. One such function is an ability to home to specific sites in the body. Biomimetic particles that not only home to tumors, but also amplify their own homing, have been described. The system is based on a peptide that recognizes clotted plasma proteins and selectively homes to tumors, where it binds to vessel walls and tumor stroma. Iron oxide nanoparticles and liposomes coated with this tumor-homing peptide accumulate in tumor vessels, where they induce additional local clotting, thereby producing new binding sites for more particles. The system mimics platelets, which also circulate freely but accumulate at a diseased site and amplify their own accumulation at that site. The self-amplifying homing is a novel function for nanoparticles. The clotting-based amplification greatly enhances tumor imaging, and the addition of a drug carrier function to the particles is envisioned.

Gold Nanorods for Diagnosis Plus Photothermal Therapy of Cancer: In vitro studies have demonstrated that gold nanorods are novel contrast agents for both molecular imaging and photothermal cancer therapy. Nanorods are synthesized and conjugated to anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies (MAbs) and incubated in cancer cell cultures. The anti-EGFR antibody-conjugated nanorods bind specifically to the surface of the malignant-type cells with a much higher affinity due to the overexpressed EGFR on the cytoplasmic membrane of the malignant cells. As a result of the strongly scattered red light from gold nanorods in dark field, observed using a laboratory microscope, the malignant cells are clearly visualized and diagnosed from the nonmalignant cells. It is found that, after exposure to continuous red laser at 800 nm, malignant cells require about half the laser energy to be photothermally destroyed than the nonmalignant cells. Thus, both efficient cancer cell diagnostics and selective photothermal therapy are realized at the same time. To ensure accumulation of nanoparticles in neoplastic tissue, targeting ligands, such as antibodies and targeted gene therapy vectors, are being incorporated into the nanoparticles, which act as thermal scalpels upon laser irradiation and destroy tumor tissue.

pHLIP Nanotechnology for Detection and Targeted Therapy of Cancer: The pH-selective insertion and folding of a membrane peptide, pHLIP (pH low insertion peptide), can be used to target acidic tissue in vivo, including acidic foci in tumors. pHLIP nanotechnology is considered to be a promising approach for mapping areas of elevated acidity in the body. The peptide has three states: soluble in water, bound to the surface of a membrane, and inserted across the membrane. At physiological pH, the equilibrium is toward water, which explains its low affinity for cells in healthy tissue; at acidic pH, the equilibrium shifts toward membrane insertion and tissue accumulation. This peptide acts like a nanosyringe to deliver tags or therapy to cells. Tumors can be detected by labeling pHLIP peptide with Cy5.5 and imaging by

use of near-infrared (NIR) fluorescence with wavelengths in the range of 700-900 nm. In a mouse breast adenocarcinoma model, fluorescently labelled pHLIP detects solid acidic tumors with high accuracy and accumulates in them even at a very early stage of tumor development. The fluorescence signal is stable and is approximately five times higher in tumors than in healthy counterpart tissue. Tumor targeting is based on the fact that most tumors, even very small ones, are acidic as a result of the way they grow, known as the Warburg effect (awarded Nobel Prize in 1931). Tumors may be treated by attaching and delivering anticancer agents with pHLIP. The advantage of this approach is that hypoxia and acidosis are uniformly present biomarkers of cancer whereas gene signatures are variable.

Nanomaterials – A Thermal Aspect:

Yet another application of nanomaterials is seen in Gannon et al. where the role of gold nanoparticles (GNPs) in human hepatocellular and pancreatic cancer cells was evaluated to determine: 1) absence of intrinsic cytotoxicity of the GNPs and 2) external radiofrequency (RF) field-induced heating of intracellular GNPs to produce thermal destruction of malignant cells.

Radiofrequency ablation (RFA) has been used in clinical practice to treat some malignant tumors, yet it suffers from serious limitations. It is highly invasive requiring insertion of needle electrodes directly into the tumor(s) to be treated.

In theory, non-invasive RF treatment of malignant tumors at any site in the body should be possible, but such a treatment would require the presence of intracellular or intratumoral agents that release heat under the influence of the RF field. For such a novel RF treatment approach to be effective, it will require identification of agents that have little or no intrinsic cellular or tissue toxicity that can also be targeted or directed to malignant cells while sparing normal cells.

Gobin et al. in a previous paper showed that that gold-silica nanoshells release significant heat when exposed to near-infrared (NIR) light (650–950 nm) and have been used to produce thermal cytotoxicity *in vitro.* However, this treatment approach is mechanistically limited to use in superficial malignant tumors because of the minimal tissue penetration (< 2–3 cm depth) by NIR wavelength light.

Gannon et al. showed that cancer cells containing GNPs exposed to a focused, non-invasive RF field would develop lethal, thermal-induced injury. In order to produce thermally-induced cancer cell death in response to the RF field *in vivo*, intracellular or intra-tumoral resonant or metallic heat-producing molecules are required. GNPs are excellent conductors of electrical and thermal energy and in this system provide non-specific RF targeting to human gastrointestinal cancer cells *in vitro*.

Thus, nanomaterials can also be used indirectly to kill targeted cells using the

concept of their thermal conductivity. This, if proved successful in vivo, can be another way of targeting the problem that is cancer.

Nanomaterials in Agriculture:

The great potential of using nanodevices as delivery systems to specific targets in living organisms was first explored for medical uses. In plants, the same principles can be applied for a broad range of uses, in particular to tackle infections. Nanoparticles tagged to agrochemicals or other substances could reduce the damage to other plant tissues and the amount of chemicals released into the environment.

Gene transfer by bombardment of DNA-absorbed gold particles has been successfully used to generate transgenic plants in a species-independent manner (Christou et al., 1988). Recently, Torney et al. (2007) reported the efficient delivery of DNA and chemicals through silica nanoparticles internalized in plant cells, without the requirement of specialized equipment. But the work was limited to isolated plant cells and intact leaves.

Gonza´lez-Melendi et al. in 2007 showed the first known case of penetration and transport of nanoparticles inside whole plants. They showed how magnetic nanoparticles (Carbon coated nanoparticles) can penetrate into the plant and the bioferrofluid travels through the vascular system to other parts of the plant, and how this bioferrofluid can be concentrated in the desired areas by using magnets.

Many things must be addressed prior to utilization of nanoparticles in agriculture. First, those concerning the plant physiology and growth in mid- and long-term, i.e. do the nanoparticles significantly affect the plant and are they phytotoxic?

It is possible that nanoparticles produce some local damage in cells, as has been reported for in vitro treatments (Pavel et al., 1999; Cotae and Creanga, 2005; Pavel and Creanga, 2005), but it does not mean that the whole plant would be affected. The main advantage of using magnetic nanoparticles is in laboratory and research applications because they allow a very precise localization of the particles to unload their charge, which is of great interest in the study of local treatments in whole living plants. Nevertheless, nanoparticles lacking magnetic properties could be used in large-scale situations with extensive crops, designed with other systems which allow their accumulation and/or guidance into specific areas. In that case, field applications could be done through the leaves and/or root system.

More recently, Corredor et. al have worked on Nanoparticle penetration and transport in living pumpkin plants using the same concept of Carbon coated nanoparticles as in the above (Gonza´lez-Melendi et al.)

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