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Nanoparticles, molecular biosensors, and multispectral confocal microscopy

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Summary

Complex, multilayered nanoparticles hold great promise for more sophisticated drug/gene delivery systems to single cells. Outermost layers can include cell targeting and cell-entry facilitating molecules. The next layer can include intracellular targeting molecules for precise delivery of the nanoparticle complex inside the cell of interest. Molecular biosensors can be used to confirm the presence of expected molecules (for example, reactive oxygen species (ROS) as a surrogate molecule for signs of infection, or for activation in radiation damage, etc.) prior to delivery of counter-measure molecules such as drugs or gene therapy. They can also be used as a feedback control mechanism to control the proper amount of drug/gene delivery for each cell. Importantly, the full nanoparticle system can be used to prevent any cells from encountering the drug unless that cell is specifically targeted. Thus, if a cell is initially non-specifically targeted, a secondary check for other molecular targets which must also be present inside the target cell of interest can be used to catch initial targeting mistakes and prevent subsequent delivery of treatment molecules to the wrong cells. The precise intracellular location of nanoparticles within specific regions of a cell can be confirmed by 3D multispectral confocal microscopy. These single cell molecular morphology measurements can be extended from individual cells, to other cells in a tissue in tissue monolayers or tissue sections.

Introduction

Existing gene delivery systems have a variety of limitations (De Smedt *et al.* 2000):

(1) these systems are designed to eliminate an infection by transferring a therapeutic gene to host cells, however, they have been largely unsuccessful since only low doses of genetic material can reach the target specific cell types that are infected;
(2) increased side effects are also due to non-specific targeting of non-infected cells with genes;
(3) host cells reaction to the carrier molecules used associated with their gene delivery.

To date, the majority of gene therapy systems are based on viral vectors delivered by injection to the sites where the therapeutic effect is desired. Other systems in which naked DNA, RNA or modified nucleic acids have been injected directly into the blood stream, all of which produce many undesirable side effects which can compromise the treatment of patients. Viral vectors can have potentially dangerous side effects due to unintended integration of the viral DNA into the host genome which include incorporation of the virus into the hosts immune system and hence, have been less successful than originally hoped (De Smedt *et al.* 2000). Liposome based gene transfer has relatively low transfection rates, are difficult to produce in a specific size range, can be unstable in the blood stream, and are difficult to target to specific tissues (De Smedt *et al.* 2000). Injection of naked DNA, RNA, and modified RNA directly into the blood stream leads to clearance of the injected nucleic acids with minimal beneficial outcome (Sandberg *et al.* 2000). As such, there is currently a need for a gene delivery system that has minimal side effects but high potency and efficiency. One such system could be that of the self-assembled nanoparticles coated with targeting biomolecules (Lvov & Caruso 2001).

Bionanoparticle complexes, while still in their infancy as a new bionanotechnology, hold great promise for more sophisticated targeting and controlling drug/gene delivery to specific cells. Delivery of the drug/gene to a cell surface by conventional targeting does not insure

that it is delivered to the site of required action within the cell. The nanosystems can contain intracellular targeting molecules that re-direct the nanomedical system to the correct intracellular location for specific molecular and biochemical actions. For example, the interior of a 10 micron diameter cell is approximately a billion times larger than the volume of an individual 10 nm diameter nanoparticle. Prior studies using confocal microscopy have been used to verify that the drug/gene is targeted to the correct location within single cells (White *et al.* 1999).

Molecular biosensors can also confirm the correct targeting by sensing the environment and to provide an initial control of drug/gene delivery. In addition, when the drug/gene treatment has resulted in the elimination of the pathogen, there will be a subsequent elimination of the indicator molecules; this decrease in indicator molecules will then shut off expression of the therapeutic gene due to increased biosensor activity. These secondary checks for correct targeted and control of drug/gene delivery can be used to help minimize 'bystander' effects now common as 'side effects' in conventional drug administrations to illustrate the use of nanoparticles and molecular biosensors in molecular morphology.

Two major biological models were chosen as vehicles for this publication: (1) the Hepatitis C virus (HCV) infection of single cells; and (2) cellular radiation damage to single cells as is likely to occur during long term/deep space missions by astronauts. In the case of the HCV infection model, one expression product of these constructs, the biosensor, is targeted to the same sub-cellular location where the HCV proteins are synthesized. Only when the viral protease is detected, would the expression of an anti-HCV gene product be triggered. For the DNA repair illustration, the aim was to develop a gene therapy technique that would provide increased *in vivo* protection against radiation damage to the blood and bone marrow of astronauts who experience long term/deep space missions. The primary goal was to develop an *in vivo* intra-cellular DNA repair system for astronauts to repair radiation damaged cells that had suffered radiation damage before they progress to radiation induced leukemia. This system would therefore use a promoter based sensor which could detect the presence of reactive oxygen species (ROS). When ROS is present, the sensor would trigger the transcription of foreign DNA repair enzymes, which may be more efficient and effective than our innate DNA repair system in repairing the increased level of radiation induced DNA damage.

The approaches described in this paper are advantageous for the following reasons: (1) these techniques have the ability to deliver genes using targeted particles that cannot replicate and are biodegradable; (2) the therapeutic gene generated can be silenced at any time by the addition of tetracycline; (3) the amount and duration of the gene therapy developed can be controlled without having to treat the individual with extremely high non-physiological doses of harmful drugs, as is currently being done with ribavirin/interferon therapies and chemotherapy for cancer; (4) the gene therapy products are only specifically expressed when the pathogen or radiation damage is present in the cell; and (5) the anti-HCV treatment is genuinely specific for HCV, thereby minimizing side effects.

In this paper, nanoparticles capable of delivering DNA payloads were developed using different core components. Biosensor platform technology was developed in a way that facilitates the rapid development of biosensors for other applications. *In vitro* experiments were designed to test the feasibility of a protease activated biosensor.

Materials and methods

Overview of methods

This work is part of a general program for development of nanomedical systems for both diagnostics and therapeutics. Multilayered nanoparticle systems are usually, but not always, built on a nanoparticle core of polystyrene, silica, gold or other material. Drug or genes, molecular biosensors, and extracellular (as well as intracellular) targeting molecules can be added to the nanoparticles to construct a 'nanomedical system' (Prow 2004).

Nanoparticles

The details of nanomaterials science are beyond the scope of this paper. But briefly, multilayered nanoparticle systems are usually, but not always, built on a nanoparticle core of polystyrene, silica, gold or other material (De Smedt *et al.* 2000, Prow 2004). These nanoparticles are self-assembled atom-by-atom or molecular layer-by-layer (LBL). Additional layers can be added containing drugs or genes to be delivered, molecular biosensors, and targeting molecules (including both extracellular and intracellular). Two types of nanoparticles were used in this work – nanocrystals and nanocapsules. Nanocrystals, self-assembled atom-by-atom and made of semiconductor materials such as CdTe from the laboratory of Dr. Kotov (Tang *et al.* 2002, Westenhoff & Kotov 2002), were used to provide very small (7–10 nm diameter) nanoparticle delivery systems. They also have advantages in terms of brightness of fluorescence and absence of photobleaching during confocal microscopic analyses for *ex vivo* analyses. Nanocapsules, typically self-assembled LBL using alternately charged polymers from the laboratory of Dr. Lvov (Ai *et al.* 2003), are typically much larger – on the order of 100 nm in diameter. These nanocapsules can be with or without solid cores and have a larger capacity

for holding drugs or genes in their interior. The polymers can also be made from biodegradable polymers, some of which already have US Food and Drug Administration approval for *in vivo* human use. LBL nanoparticles are formed around a core particle (Lvov *et al.* 2001, Lvov & Caruso 2001). The layers are held together by the charge of the individual molecules, thus they are composed of alternating positive and negative charged species (Lvov *et al.* 2001, Lvov & Caruso 2001). One benefit of using this type of particle is that, once constructed, the particle core can be dissolved and then the particle can be made porous by changing solvents without damaging the layers. Incubating these porous nanocapsules with dissolved chemicals, one can load the nanocapsules through diffusion. Once loaded, the nanocapsules can be made non-porous by changing the solvent, thereby encapsulating the compound in the core of the particle chemical of choice. Through this technique one can encapsulate fluorescent dyes and possibly other molecules (Lvov *et al.* 2001, Lvov & Caruso 2001). Reporter genes may also be used as an interior layer because of the inherently negative charge of DNA. Additional layers of targeting molecules may then be added to help direct the particle to the correct cell types (Lvov *et al.* 2001, Lvov & Caruso 2001).

Molecular biosensors

A multicomponent HCV biosensor was constructed. This protease activated biosensor is a triple fusion protein consisting of a transactivator, cleavage, and localization domains that should target the protein to the perinuclear region. The transactivator region functions to activate transcription when released from the localized biosensor that is anchored to the targeted endoplasmic reticulum (ER). This anchored protein cannot move to the nucleus and initiate transcription due to the cytoplasmic localization, thus the transactivator is restrained such that it cannot move to the nucleus and bind to DNA. The cleavage domain separates the transactivator from the anchor region and is designed to the enzymatic activity of a specific protease. If the appropriate protease is present and cleaves the substrate, the transactivator released from the anchor domain end is then free to induce transcription within the nucleus.

For detection of ROS in chemically or radiation-damaged cells, Zhu & Fahl (2000) used an ROS molecular biosensor constructed based on previously reported sequences. These authors describe the characterization and utilization of promoter based biosensors for the detection of oxidative stress. The ROS biosensor is being used to identify and help induce the expression of DNA repair enzymes during times of increased oxidative stress. The antioxidant response element was coupled to an enhanced green fluorescent protein (EGFP) reporter. Zhu & Fahl (2000) first adapted this promoter for use as an *in vitro* assay for cells experiencing oxidative stress. The most sensitive of the constructs contained four repeats of the antioxidant response element followed by the EGFP coding sequence of the gene and a poly A tail and was a kind gift from Dr. Zhu, Arizona Cancer Center (Zhu & Fahl, 2000), and was put to use in our laboratory.

Integrated nanoparticle system

Multilayered nanoparticle systems can be constructed which combine cell targeting molecules (e.g. antibodies), membrane entry facilitating molecules, intracellular targeting molecules (e.g. amino acid sequences that target to intracellular organelles), molecular biosensors, and drugs or genes for therapy (Leary & Prow, US Patent pending). Such an integrated sequence of events constitutes an integrated system which produces a sequence of molecular events Figure 1a. In operation, the nanosystem unfolds LBL to expose the appropriate attached or embedded molecules to

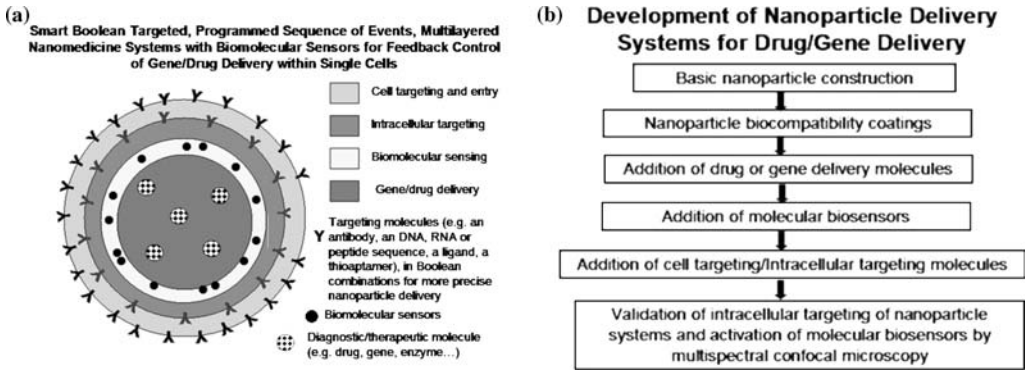

Figure 1. A multilayered nanoparticle system can contain targeting, biosensing and drug delivery molecules that are released a layer at a time. (a) shows a schematic of a smart nanoparticle system that can produce an ordered series of events for drug/gene delivery. Biosensing molecules allow the controlled release of drugs, or expression of specific gene sequences, at the individual cell level. (b) is a flow diagram of the development of such a nanoparticle system. This diagram shows the basic steps necessary to develop a biocompatible nanoparticle based system for therapeutic applications.

accomplish the next step in the sequence for a 'nanomedicine' system, as shown in Figure 1b.

Confocal microscopy

Multispectral confocal microscopy was used as a validation tool for both nanoparticle/ biosensor targeting and intracellular localization. Since multiple fluorescent colors were used to label biosensors, cell membranes, ER and nuclei, it became necessary to use a multispectral confocal microscope that could separate the color optical overlaps. The method of spectral deconvolution was developed at JPL/Cal Tech (Pasadena, CA) and this invention, as described subsequently in a publication (Dickinson *et al.* 2001), was implemented in a new generation of multispectral confocal microscope (Model 510 META, Zeiss, Inc.). The basics of the method are shown in Figure 2. The 'emission fingerprinting' algorithm (Dickinson *et al.* 2001) works by fitting the spectral components over low or non-overlapping portions of the combined spectrum of a multicolor image. The components are appropriately weighted so that the combination of color components matches the overall spectrum from the image pixel-by-pixel in each image plane.

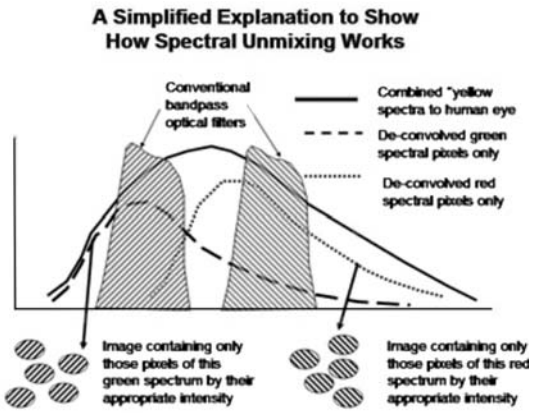

Figure 2. An illustration of the spectral unmixing algorithm implemented on the Zeiss 510 META multispectral confocal microscope used for this work.

Results

Nanoparticle targeting

Initial experiments were conducted to compare the behavior of conventional antibody staining and nanoparticle labeling. In Figure 3a, live human BJAB cells were directly labeled with FITC-conjugated anti-CD95 IgG whereas in Figure 3b, live BJAB cells were indirectly labeled with unconjugated anti-CD95 IgG, and then incubated with a secondary reagent constructed of FITC-conjugated 500 nm polystyrene nanoparticles coated with a secondary goat anti-mouse antibody against IgG. The nanoparticle labeling system showed similar results to that of the conventional antibody labeling system. These very large 500 nm nanoparticles were used in order to permit direct optical visualization by fluorescence microscopy.

To test the targeting accuracy and efficiency of the nanoparticle system, we first labeled non-targeted, CD95-negative, MOLT-4 cells with CellTracker^{TM} Blue CMAC (7-amino-4-chloromethylcoumarin), a fluorescence tracking dye (Molecular Probes, Eugene, Oregon). Green fluorescent nanoparticles were added to a mixture of CD95 positive BJAB cells, previously labeled with unconjugated anti-CD95 antibody (non-fluorescent), and MOLT-4 cells, previously labeled with CMAC, was made. Green fluorescent nanoparticles (as described in Figure 3) were then added to the cell mixture. Figure 4a shows a 10× objective phase-

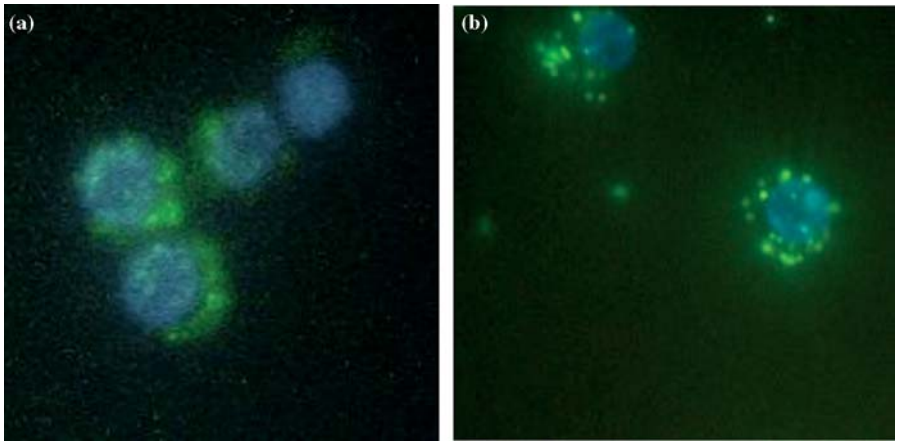

Figure 3. Nanoparticle targeting of CD95 positive BJAB cells. Live BJAB cells were direct labeled with anti-CD95 FITC (a). BJAB cells labeled with anti-CD95 with no FITC as the primary antibody and then exposed to FITC-conjugated polystyrene nanoparticles coated with secondary antibodies specific for the Fc portion of anti-human CD95 antibodies (b). Nuclei were counterstained with Hoechst 33342 and visualized on an inverted fluorescent microscope.

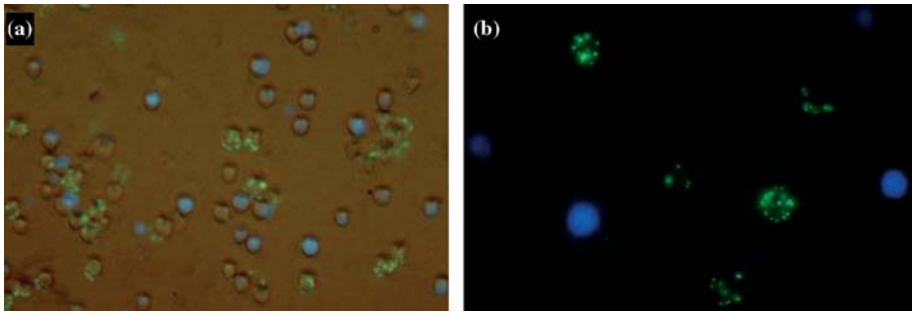

Figure 4. Nanoparticles were used to target CD95-positive cells in a 1 : 1 cell mixture of these cells and CD95-negative MOLT4 cells which were stained blue with a cell tracking dye (CMAC, Molecular Probes, Inc., Eugene, Oregon). As shown in both panels, none of the CD95-negative blue MOLT4 cells were targeted by the green nanoparticles. Only the BJAB (unstained) cells, that are CD95-positive, were bound to the 500 nm green nanoparticles. (a) is a photomicrograph with a 10× objective the cell mixture in combined phase-contrast and fluorescence microscopy; (b) shows the cell mixture with a 40× objective in fluorescence only.

fluorescence photomicrograph of the combined mixture of cells and nanoparticles. Figure 4b shows a 40× two color fluorescence only photomicrograph. While not all of the CMAC-negative BJAB cells were labeled at this ratio of nanoparticles and cells (Figure 4A), none of the CMAC-positive, CD95-negative MOLT-4 cells bound nanoparticles (Figure 4b). The MOLT4 cells were used as a negative control because they do not normally express CD95 on their surface whereas BJAB cells were used as a positive control because most, but not all, BJAB cells constitutively express CD95 on their surface. Only the BJAB (CMAC negative) cells, that are CD95 positive, were bound to the green nanoparticles.

Biocompatibility of nanoparticles

While molecular membrane transport facilitating sequences were being developed, the biocompatibility of CdTe semiconductor material nanocrystals to living T24 human cells was tested by direct microinjection of nanocrystals into living cells using a Narashige microinjection system mounted on to an inverted phase-fluorescence microscope (Nikon DiaPhot) as shown in Figure 5. Initial results showed considerable cytotoxicity in the absence of biocoatings being applied to the nanoparticle surface. Coating the nanocrystals with either galactosamine or Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA) was found to reduce this

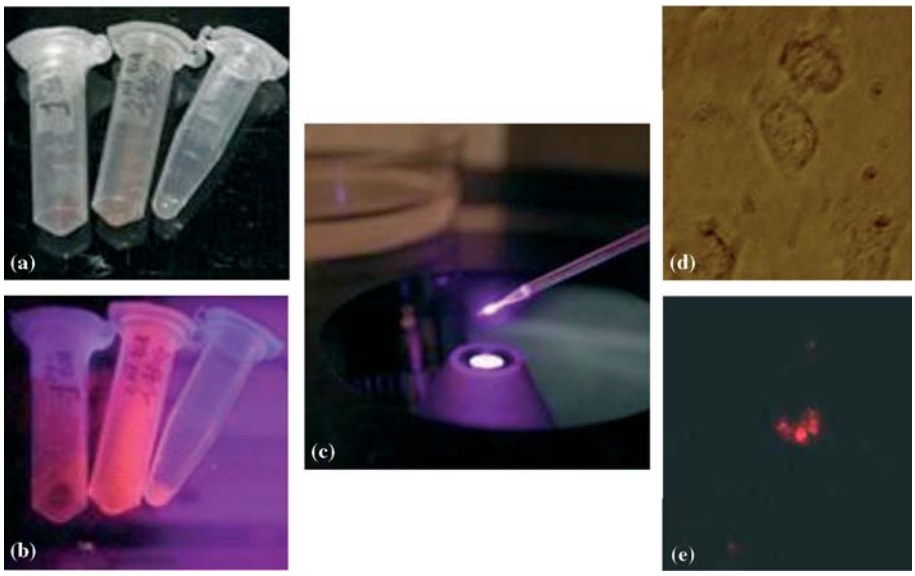

Figure 5. Cells microinjected with CdTe nanocrystals. (a) CdTe nanocrystals in plastic tubes under room light, (b) the same nanocrystals under UV light. Tube contents (from left to right): Dry CdTe nanocrystals (red) coated with BSA/Avidin, CdTe nanocrystals coated with BSA/Avidin in PBS (pH 7.4), and BSA/Avidin coated nanocrystals diluted 1 : 10 in PBS. (c) CdTe nanocrystals loaded into micropipette prior to microinjection and illuminated on an inverted microscope, (d) CdTe nanocrystals coated with BSA/Avidin in PBS being microinjected into T-24 cells (bright field), (e) fluorescence image of T-24 cell micro-injected with CdTe nanocrystals.

cytotoxicity to control levels for the time span of these experiments.

Molecular biosensor design

The successful design and construction of a triple-fusion protein molecular biosensor that locates its target cell or cellular compartment, detects the presence of the target molecule based on the specific reaction between it and the biosensor, and release of a signaling molecule that in turn generates a reporter molecule, is shown in Figure 6. The overall biosensor construct must contain a sub-cellular domain localization sequence, a cleavable segment that is activated upon contact with the intracellular target sequences (an enzyme), and a signaling molecule which is then released to interact with the nucleus to produce a gene product under the action of promoter sequences.

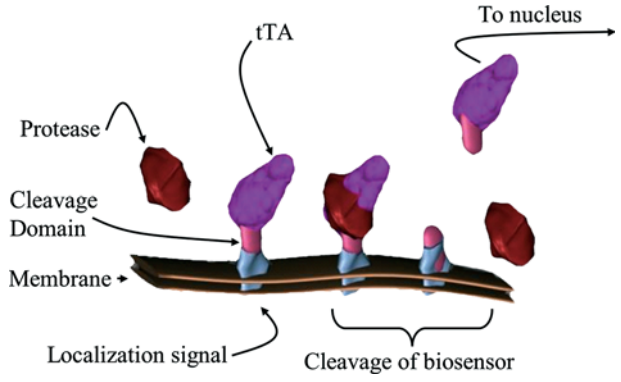

Figure 6. Overview of a protease activated biosensor for HCV. The biosensor protein is a triple-fusion protein containing a tetracycline inactivated transactivator (tTA), a protease specific cleavage domain, and a localization domain. The activated protease cleaves the cleavage domain, releasing the tTA. The tTA then localizes to the nucleus and activates transcription of a predetermined gene.

The data obtained by use of this construct is shown in Figure 7. Measurements were made of the intracellular localization of molecular biosensors BS-1, BS-2, and BS-3 (three different sequences) targeted molecular biosensors in BT7H cells. Confocal images from BT7H cells transfected with molecular biosensors BS-1 (Panel 7a), BS-2 (Panel 7b), or BS-3 (Panel 7c) were stained for biosensor (red) and the DNA counterstained with DAPI (blue). The results show successful targeting to the nucleus, to the ER, and to the plasma membrane.

Intracellular localization of molecular biosensors

A typical biosensor molecule will require at least 3 domains: (1) a homing sequence that will guide the protein to the compartment in the cell where detection needs to occur; (2) the detector or cleavage domain, i.e. a peptide sequence that is recognized by a specific protease; and (3) an activator element that will induce transcription of a target gene upon cleavage of the detector sequence. We have constructed such a triple fusion protein consisting of a membrane anchor, a HCV NS3 protease cleavage domain and a tetracycline transactivator protein (Figure 7). Three color multispectral confocal intracellular co-localization of molecular biosensors and NS3-specific flavivirus protein in Huh7 cells data obtained from this construct are shown in Figure 8. Protease containing Huh7 cells were transfected with nothing (8a), or molecular biosensors BS-2 (8b, e), BS-3 (8c, f), and pTet-Off (8d). Panels 8b, c show what appears to be inactivated biosensor (BS) proteins that seem to localize within the cytoplasm. Panels 8d–f show cells with tTA, BS-2, or BS-3 throughout the entire cell, which indicates activated BS proteins. The cells shown in panel 8d were transfected with only the transactivator portion of the BS protein and therefore serve as a positive control for the acti-

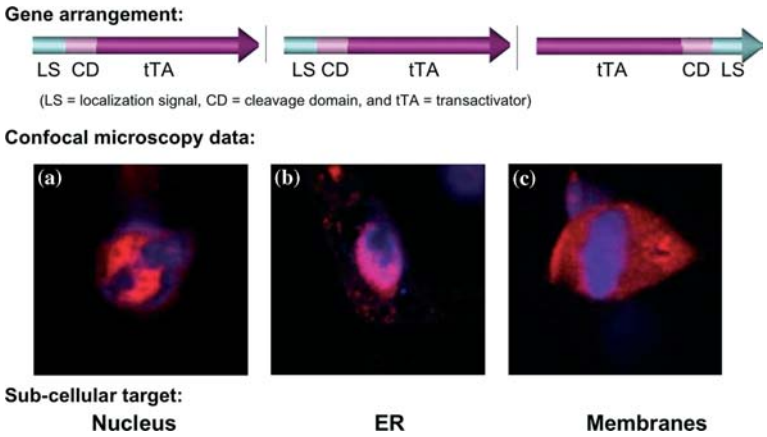

Figure 7. Intracellular localization of three biosensors (BS-1, BS-2, and BS-3) within BT7H cells. The gene arrangement of the three biosensors is shown with the three major components: the localization signal (LS), cleavage domain (CD), and the tetracycline inactivated transactivator (tTA). Confocal images from BT7H cells transfected with BS-1 (a), BS-2 (b), or BS-3 (c) were stained for biosensor (red) and the DNA counterstained with DAPI (blue).

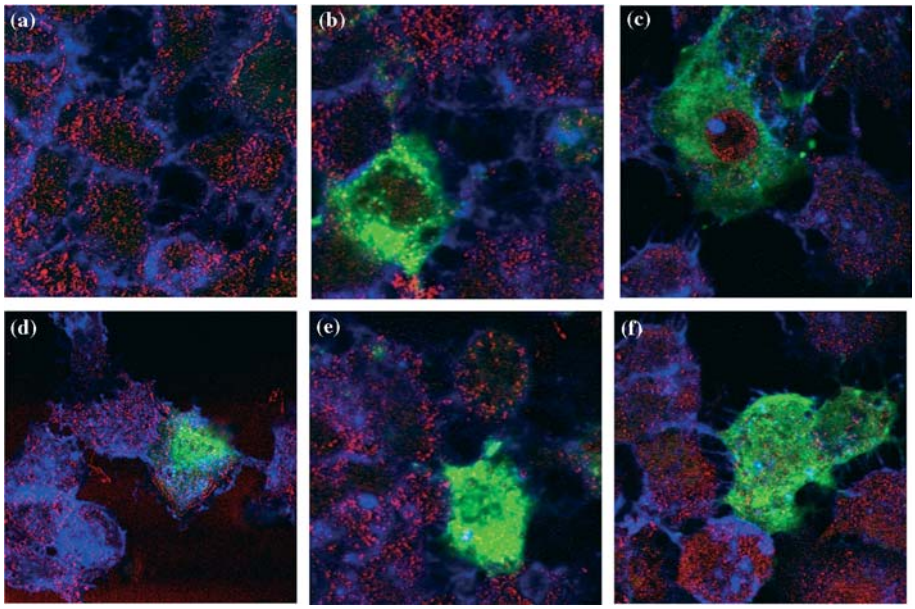

Figure 8. Three color multi-spectral confocal intracellular colocalization of molecular biosensors (green) and NS3 (red), a specific flavivirus protease in Huh7 cells. Protease containing Huh7 cells were transfected with nothing (a), BS-2 (b, e), BS-3 (c, f), and pTet-Off (d). Panels b and c show what appears be inactivated BS proteins that seem to localize within the cytoplasm. Panels d, e, and f show cells with tTA, BS-2, or BS-3 throughout the entire cell, which indicates activated BS proteins. Actin staining was used to counterstain the cells (blue).

vated BS protein from either BS construct. Panels 8d–f were found to have BS throughout the *z* axis, as opposed to panels 8b, c that were found to have large regions without protease or BS proteins. Because previous experiments described the localization of the BS proteins to the nucleus, all cells were counterstained with a fluorescent phallotoxin (actin stain, blue) to visualize the localization of the BS proteins (green) with respect to the entire cell. The NS3 protease was also immunostained and is shown in red.

In a second application area, involving the detection of oxidative stress and DNA damage caused by either chemical agents or radiation, a ROS biosensor was used. This biosensor is promoter based biosensor (Fahl & Zhu 2000) and is composed of three elements: a series of response elements (EpRE), minimal thymidine kinase promoter (TK), and a reporter gene (green fluorescent protein, GFP). Cells were transiently transfected at ~60% confluence with either ARE-GFP or TK-GFP and at 24 h post transfection the cells were treated with 90 μm tert-butylhydroquinone (tBHQ), an ROS inducing agent. The cells were examined every 12 h post treatment. Weak GFP fluorescence was present at 48 h after treatment, and stronger GFP fluorescence was observed after 60 h and photographs were taken (Figure 9). As expected at this concentration of tBHQ a fraction of cells showed signs of oxidative stress.

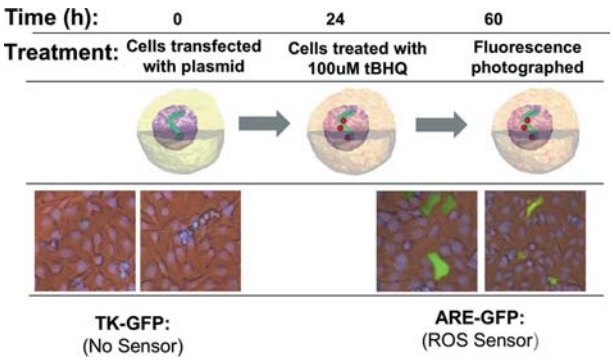

Figure 9. ROS biosensor. This promoter based biosensor (Fahl & Zhu, 2000) is composed of three elements: a series of response elements (ARE), minimal thymidine kinase promoter (TK), and a reporter gene (EGFP, green). T-24 cells transfected with this construct respond to oxidative stress by expressing EGFP. Cells were counterstained with Hoechst 33342 (blue). The images are a combination of phase contrast and fluorescent microscopy.

Multispectral confocal analysis of nanoparticles, biosensors and cells

Nanocrystals will likely be of great benefit to quantitative confocal microscopy as they show minimal photobleaching over long periods of time as shown in Figure 10. Regions of the image were sampled over almost 10 min of time by confocal imaging and the degree of photobleaching was quantitated. The nanocrystals showed a slight increase in brightness after initial excitation followed by a phase of several minutes in which the brightness remained constant. Although the degree of photobleaching varies with nanocrystal composition and methods of construction, this is a general characteristic of nanocrystals, which can be controlled for.

It is important to note that nanocrystals are actually smaller than many proteins, including Streptavidin (Figure 11). Uncoated CdTe nanocrystals were not

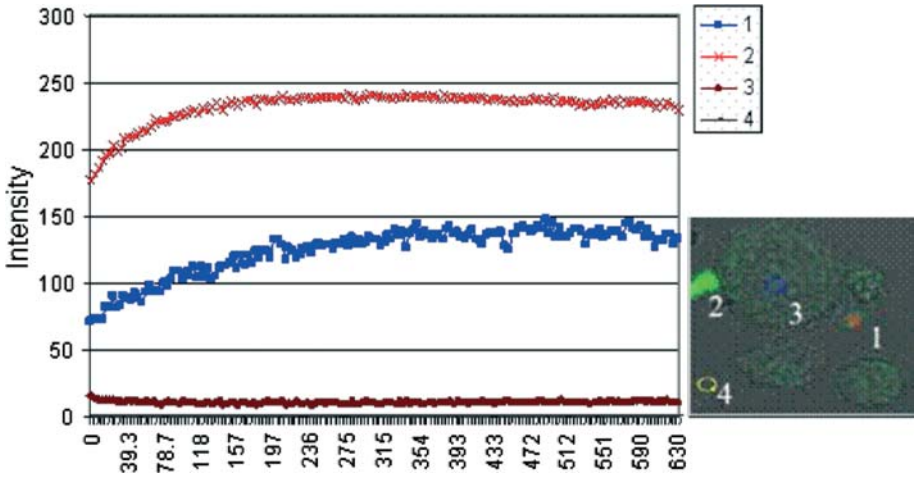

Figure 10. Nanocrystal photobleaching curves. Regions of the image were sampled over almost 10 min of time and the degree of photobleaching was determined. The nanocrystals actually become slightly brighter after initial excitation, and then remain fairly constant over a number of minutes.

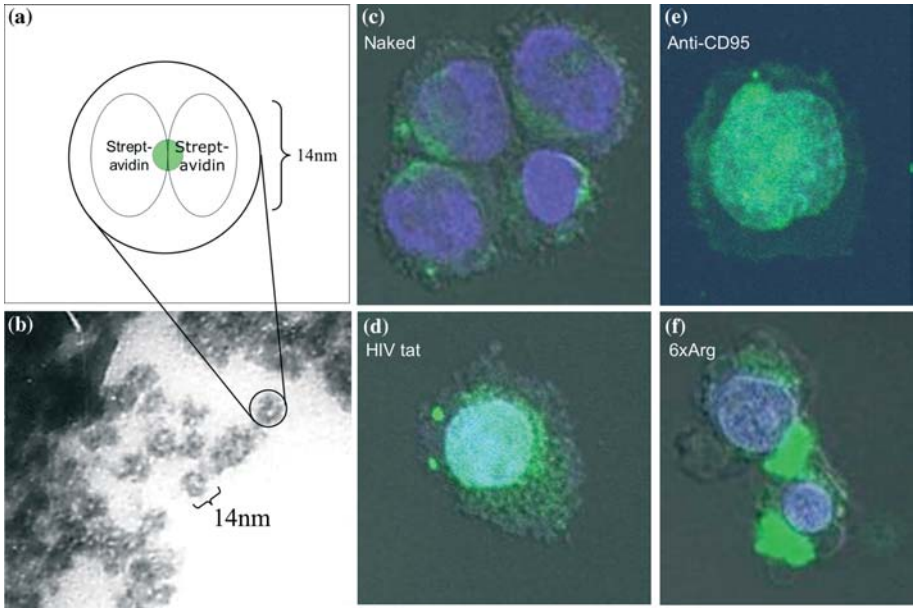

Figure 11. Nanocrystal visualization and targeted delivery to human cells. Streptavidin coated nanocrystals (a, b) were imaged by transmission EM (courtesy of Dr. Popov at UTMB). Nanocrystal coated with nothing (c, Naked), HIV tat fragment (d, HIV tat), Anti-CD95 (e), or a 6× Arginine peptide (F, 6×Arg) were incubated with live cells and imaged by multispectral confocal microscopy. Hoechst 33342, an AT base pair specific dye that enters live cells, was added as a counterstain to delineate the nuclear boundary. These are composite images of fluorescence and Nomarski contrast (left side of figure) were imaged by transmission EM (courtesy of Dr. Popov at UTMB).

only cytotoxic, but also failed to bind to, or be phagocytosed, by cells. However, once they were bound by antibody, in this case anti-CD95 antibodies to receptors on the cell surface, the nanocrystals began to enter the cells over time. HIV tat protein greatly accelerated cell entry and targeted the nanocrystals to the nucleus. A six amino acid peptide of Arginine had a similar effect.

Once the behavior of targeted nanoparticles without any drug or gene delivery payload was explored, experiments were initiated to compare the efficiency of expression of plasmid transfected DNA sequences with those same sequences on nanoparticles. Figure 12 shows the delivery of two different plasmids with lipid coated nanoparticles. Human Huh-7 liver cells (Panels 12a, and 12b.) were transfected either with a 1 : 1 mixture of plasmids pEGFP-C1 and pDsRed2-C1 or exposed to 100 nm LBL assembled nanocapsules containing a single layer of DNA (1 : 1 mixture of pEGFP-C1 and pDsRed2-C1) (panels 12c, d.). Although the transfection efficiency was low, there were cells expressing both EGFP (green) and

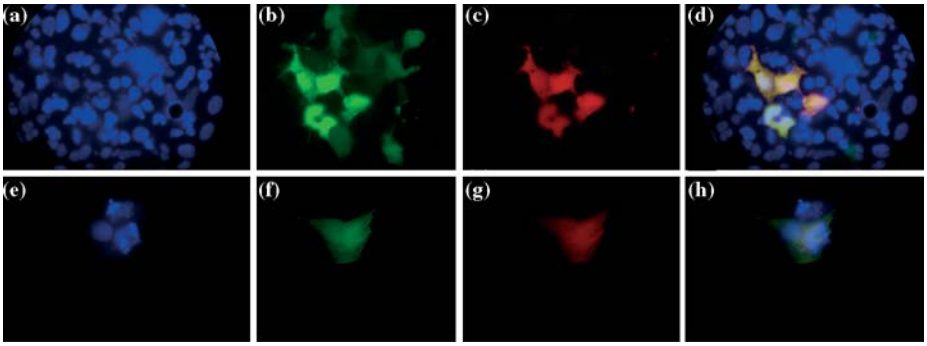

Figure 12. Delivery of 2 different plasmids with lipid coated LBL. Huh-7 cells were transfected with a 1 : 1 mixture of pEGFP-C1 and pDsRed2-C1 (top row) or exposed to ~100 nm LBL assembled nanoparticles containing a single layer of DNA (1 : 1 mixture of pEGFP-C1 and pdsRed2-C1) (bottom row). Although the transfection efficiency was low, there were cells expressing both EGFP (green; b, f) and DsRed (red; c, g) protein. All cells were counterstained with DAPI (blue; A, E). Each row is a single field, with composite images shown in d, h.

DsRed (red) protein. All cells were counterstained with DAPI (blue).

Discussion

Cells are widely known to only allowed particles within a particular size range, 30–200 nm, to pass the outer membrane (Zauner *et al.* 2001). The nuclear membrane is even more tightly guarded, only allowing specific molecules to pass into the nuclear compartment (Stewart *et al.* 2001). Passing through these barriers is paramount for the success of nanomedicine. The size of the particle delivered is therefore critical for the success of nanoparticle mediated gene delivery. The major size determinant for nanoparticles is the nature of the core particle, onto which the coats are layered.

Nanocrystals offer several unique advantages over traditional nanoparticles and lipid mediated gene/drug delivery tools. The nanocrystals have spectral properties that are especially convenient for *in vitro* use (Chan *et al.* 2002, Dubertret *et al.* 2002, Jaiswal *et al.* 2003). These particles fluoresce very brightly and do not photobleach like traditional dyes or fluorescent proteins. These nanoparticles are also small enough (<6 nm) to enter the cell without endocytotic signals (Figure 11). Additionally, the streptavidin coating allows a multitude of biological moieties to be bioconjugated to the surface of these tiny crystals (Figure 11). Even with large, biologically active molecules conjugated to the surface of these particles, they remain under 20 nm in diameter. With these characteristics in mind, streptavidin was bioconjugated to the surface of these particles to give this system the flexibility needed for development and testing. The streptavidin–biotin bridge gives the researcher the ability to bioconjugate single or even multiple targeting, internalization, and therapeutic molecules. One possible complication of this approach is that all signals are located in the same layer.

Size undoubtedly plays a role in the localization of these particles. Even with no cell entry or targeting molecules on board, the nanocrystals readily gain entry into the cellular milieu. These particles have been observed to distribute evenly throughout the cell. Because of their small size, optical properties, and structural flexibility, one can conceive of using nanocrystals as research based gene/drug delivery agents.

To utilize semiconductor based nanoparticles as gene delivery agents could be beneficial for *in vitro* use. The spectral characteristics of these particles would facilitate the development of targeting, sub-targeting, and cell entry strategies. Toxicity of the nanocrystals is a major concern because the core of these particles is composed of toxic elements. Throughout these experiments the nanocrystals have been shown to cause problems with membrane permeability, as demonstrated with the trypan blue dye exclusion assay. One reason for this may be the poisoning of the transporter for trypan blue export (data not shown). The cells do not appear to undergo necrosis or apoptosis. In fact, the cells appear to tolerate the nanocrystals quite well, allowing for multi-day studies. One concern brought to light by these observations was that incompletely coated nanocrystals may slowly release the toxic core elements into the cells/media. This can be alleviated by using a different core particle that allows for purification and/or does not contain a toxic core particle.

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