Nanomedicine – nanoparticles, molecular biosensors and targeted gene/drug delivery for combined single-cell diagnostics and therapeutics

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ABSTRACT

Next generation nanomedicine technologies are being developed to provide for continuous and linked molecular diagnostics and therapeutics. Research is being performed to develop "sentinel nanoparticles" which will seek out diseased (e.g. cancerous) cells, enter those living cells, and either perform repairs or induce those cells to die through apoptosis. These nanoparticles are envisioned as multifunctional "smart drug delivery systems".

The nanosystems are being developed as multilayered nanoparticles (nanocrystals, nanocapsules) containing cell targeting molecules, intracellular re-targeting molecules, molecular biosensor molecules, and drugs/enzymes/gene therapy. These "nanomedicine systems" are being constructed to be autonomous, much like present-day vaccines, but will have sophisticated targeting, sensing, and feedback control systems – much more sophisticated than conventional antibody-based therapies. The fundamental concept of nanomedicine is to not to just kill all aberrant cells by surgery, radiation therapy, or chemotherapy. Rather it is to fix cells, when appropriate, one cell-at-a-time, to preserve and re-build organ systems. When cells should not be fixed, such as in cases where an improperly repaired cell might give rise to cancer cells, the nanomedical therapy would be to induce apoptosis in those cells to eliminate them without the damaging bystander effects of the inflammatory immune response system reacting to necrotic cells or those which have died from trauma or injury.

The ultimate aim of nanomedicine is to combine diagnostics and therapeutics into "real-time medicine", using where possible in-vivo cytometry techniques for diagnostics and therapeutics. A number of individual components of these multi-component nanoparticles are already working in in-vitro and ex-vivo cell and tissue systems. Work has begun on construction of integrated nanomedical systems.

Keywords: nanomedicine; nanoparticles, molecular biosensors, drug delivery, gene therapy; flow cytometry; confocal microscopy; molecular diagnostics

1. INTRODUCTION

1.1 What is nanomedicine? Nanomedicine is a fundamentally different paradigm for medicine. It is "nano" not only because it uses nanometer scaled tools, but also because it employs a cell-by-cell regenerative and repair philosophy working at the single cell level rather than at the organ level. Medicine has progressed from primitive surgery to detailed microsurgery on small regions of organs.

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The next step is nanomedicine which performs "nanosurgery" inside living single cells to either repair those cells or to induce un-repairable cells to die by natural programmed cell death, avoiding the effects of a inflammatory response by the body's immune system which in many cases can be more severe than the original disease itself.

1.2 Some goals for nanomedicine: A goal of modern medicine is to provide earlier diagnostics so that diseases can be treated when they are most treatable. Dramatic results have been achieved in a number of diseases. However, diagnostics and therapeutics have not yet been combined in a continuous system to not only diagnose, but also to treat, at the earliest possible stage – perhaps before actual symptoms appear. The three conventional treatments for cancer are (1) surgical removal of the tumor, (2) radiation therapy, and (3) chemotherapy. Nanomedicine attempts to make smart decisions to either remove specific cells by induced apoptosis or repair them one cell-at-a-time. Single cell treatments will be based on molecular biosensor information that controls subsequent drug delivery to that single cell. Such a system would also need to be semi-autonomous, with pre-determined decisions points for when a diagnosed condition warrants treatment. Clinical decisions are usually much delayed by the negative potential side effects of that treatment, particularly if it turns out to be a misdiagnosis. If both the accuracy of treatment and the minimization of unfavorable side effects and bystander effects could be brought to acceptable levels, the consequences of faster autonomous treatments in continuous response to in-vivo molecular diagnostics with slightly higher probabilities of misdiagnosis could be tolerated. In this paper we discuss work still in the early stages of such a nanomedical system.

Conventional medicine is not readily available to much of humanity because it is labor-intensive. Medical labor is sophisticated and expensive. Nanomedicine will be much more preventive, combining very early diagnostics with initial therapeutics. In some ways it could resemble a smart, multifunctional vaccine. It might also be less expensive because it will minimize use of expense human experts for early diagnostics, and potentially could be mass produced and distributed.

1.3 Potential pitfalls of nanomedicine: We believe that it is important to discuss some of the potential pitfalls, as well as the promise, in nanomedical systems for future drug-gene delivery. We believe that the technology, while still in its infancy, has great promise to achieve these objectives. But a realistic examination of the tradeoffs must also be assessed. For example, some nanosystems very good for in-vitro work may have attributes that may preclude or limit their use in-vivo. Among these are the potentially long-term cytotoxic effects of some nanomaterials. The cytotoxicity of some nanomaterials in atom-by-atom or molecule-by-molecule nano self-assembly may prove to be very different from those same materials in elemental form. Also, cytotoxicity is a far more complex effect than simple cell killing, or even induction of apoptosis (programmed cell death). If the gene expression pattern is disturbed, those cells may not function, proliferate, or differentiate properly. There is currently very little "cytotoxicity" data for nanomaterials in biological systems.

Nonetheless, it should be kept in mind that one of the main barriers remaining in modern medicine is the effective and controlled delivery of drugs or genes to the targeted cells, with minimal disturbance of non-targeted cells. The development of highly efficient and well-controlled delivery of drugs or genes to cells would represent a significant advance to modern medicine. That fact should be kept in mind in these still early days of nanomedical systems development. While there are risks in the new systems, there are many problems of the existing old systems, too readily accepted, because there have been no available alternatives. The proper development of nanomedicine will require the balancing of risks with opportunities.

1.4 Need for a nanoparticle gene delivery system: Existing gene delivery systems have a variety of limitations (De Smedt, Demeester, and Hennink 2000). Liposome based gene transfer methods have 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, Demeester, and Hennink 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 which has minimal side effects but high affectivity and efficiency. One such system could be that of the self-assembled nanoparticles coated with targeting biomolecules (Lvov and Caruso 2001).

1.5 Application of nanomedicine to radiation damage in astronauts: In addition to describing the general concepts of nanomedicine, we show a particular application being developed for NASA as part of their nanomedicine for astronauts program. New paradigms of any technology, including nanomedicine, are often first developed for extreme environments and/or extreme circumstances. Longer voyage space exploration qualifies on both fronts. For example, on a manned roundtrip voyage to Mars and exploration on the surface of that planet, astronauts will encounter levels of radiation that are impossible to shield. There will also be no hospitals with either diagnostic or therapeutic treatments. The signal delays in Earth-Mars communications represents a major challenge to telemedicine, and largely precludes procedures requiring real-time Earth control. Any systems brought along must be small, low weight, intelligent, and autonomous. While there may be large radiation damage effects, the most likely scenario is a series of fractionated radiation doses, each of which is not necessarily a pivotal event, but whose accumulation can lead to further downstream events such as organ injury or cancer. The strategy of nanomedicine would be to try to repair the radiation damage on a continuous basis using DNA repair enzymes in nanoparticle systems targeted to cells likely to have experienced radiation as a counter-measure to more serious radiation injury at the organ level.

2. MATERIALS AND METHODS

In the early developmental stages of this project we have used a variety of experimental model systems which are very reproducible and testable, as described below. A basic strategy for a nanomedical system applied to the problem of repairing radiation damage in astronauts on a continuous basis is shown in the concept diagram of **Figure 1**.

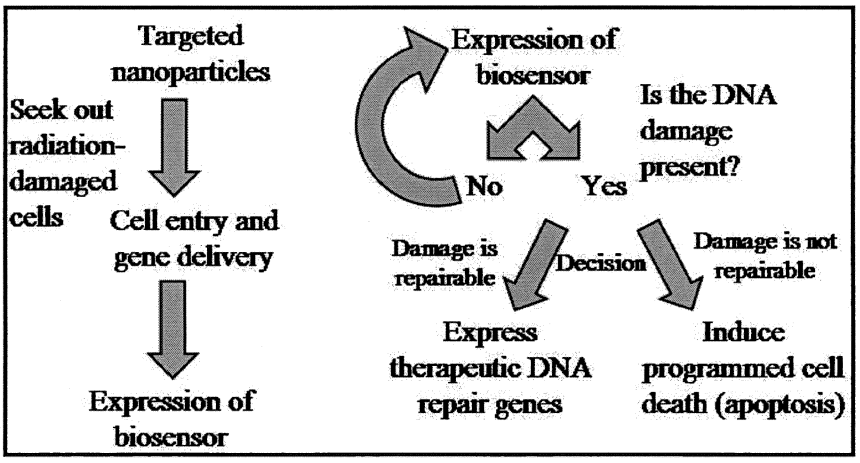


Figure 1: A nanomedicine concept applied to the problem of continuous repair of radiation damage in astronauts. A terrestrial application would be targeted repair of radiation-damaged normal tissue injured during radiation treatment of cancer patients.

2.1 Nanoparticles: There are two general categories of nanoparticles (with many variations) currently being used for applications in in-vitro and in-vivo nanomedicine: (1) nanoparticle cores with single or multilayered coatings, (2) hollow nanoparticle capsules without cores.

Nanoparticle cores materials vary greatly, the most common being made from gold, silica, or semiconductor materials. Some are made from magnetic materials which can be useful for recovery of gene products within cells (Prow et al., 2004a). Many are now commercially available. Most of these nanoparticles must be coated for two general purposes. First, some of them are not water-soluble. To exist and to function in an in-vivo aqueous environment, some of these hydrophobic materials must be marked with a layer of hydrophilic molecules. Second, some of these materials are cytotoxic to cells and tissues. Typically these are covered with lipophilic or other organic molecules to provide a barrier between the cell and the core nanoparticle materials.

Hollow nanocapsules without cores come in a variety of sizes and materials. The simplest ones consist of single or multiple layered liposomes, which are designed to fuse with the lipophilic molecules of a cell membrane and then to spill the contents of the liposome into the interior of the cell. More complex, layer-by-layer assembly nanocapsules are being made by some research groups. These nanocapsules are self-assembling by alternating charged layers of polymers and similar materials. In other work we have begun to develop nanosystems based on this concept (Prow et al., 2004b). These nanocapsules are potentially biodegradable and may be less cytotoxic to biological systems, although more detailed studies need to be conducted.

2.2 Nanoparticle targeting: Nanoparticle targeting can be accomplished in a variety of ways. But the two most common, as shown in this paper, are use of antibodies (e.g. anti-CD95 antibody) bound to the nanoparticle outer surface, or coating of the outer surface of the nanoparticles with molecules that are the ligands for cellular receptors (e.g. mannose to target nanoparticles to liver cells which have mannose receptors). While antibody targeting is very common for in-vitro applications, their use in-vivo can be problematical since some of these targeting antibodies can illicit an immune response from the human or animal. The mannose represents less of a problem in this regard because the body already recognizes mannose and does not tend to mount an immune response against it.

In the particular application of nanomedicine for astronauts we are using up-regulation and transport of the CD95 molecule to the radiation (or oxidative stress) damaged cell. Amounts of cell surface CD95 vary in roughly a dose dependent manner with radiation exposure (Sheard, 2001). So CD95 serves as the initial surrogate biomarker for radiation damage. We also have modeled radiation dosed cells with two cell lines, one of which expresses no CD95 (human MOLT-4 monocyte cell line) and another cell line (BJAB) which expresses high quantities of cell surface CD95. Once inside, the nanoparticle system performs a secondary check for oxidative stress which is highly correlated to radiation exposure using a biosensor sensitive to the presence of reactive oxygen species molecules. Since exact radiation exposure is difficult to control, we have used, in initial studies, a chemical which produces the same oxidative stress as radiation but in an easily dosed manner.

The other significant difference between in-vitro and in-vivo targeting is the great difference in specificity required. Cells are usually not rare in-vitro, while targeted cells are almost always rare in-vivo. Rare cell targeting presents considerable challenges in terms of specificity. Considering the number of possible interactions in-vivo, the specificity of the overall targeting system must, in most cases, be better than a million to one. No antibodies alone have this degree of specificity. To solve this problem, Boolean combinations of antibodies must be chosen (for review, see Leary, 1994). The good news is that these levels of specificity can be reached with antibody combinations of two

positive biomarkers and one negative biomarker. The correct repertoire of these three biomarkers (or cocktails of biomarkers as are frequently used for detection of rare stem cells)

2.3 Cell entry facilitation: Most people do not realize that targeting of nanoparticles to the cells of interest for nanomedicine represents only the first part of a long and complex journey. A nanoparticle is roughly one billionth the volume of a cell. So the interior of the cell represents another new universe to the nanoparticle. The nanoparticle and/or its contents must get successfully from the outside to the inside of a cell. How it enters the cell can control its subsequent fate. And simply dumping the drug or gene contents of the nanoparticle into the interior of the cell, while perhaps superior to dumping the drug or gene into the body for general circulation in the bloodstream, still does not guarantee that the drug or gene gets to the intracellular site where it can have therapeutic action. A further step of intracellular targeting is really required to be effective in this process. We have used three entry facilitation methods: (1) arginine-repeat peptides, (2) Lipofectamine^{TM} coatings to promote fusion of nanoparticles with the cell membrane, and (3) artificial tat-specific sequences, the entry and nuclear targeting molecule used by HIV-1.

2.4 Intracellular targeting: We have used a variety of specific amino acid localization sequences to deliver and anchor delivery of molecules to three intracellular regions of the cell: (1) the endoplasmic reticulum (ER), (2) the mitochondria, and (3) the nucleus. An important technology to visualize and study the proper localization of nanoparticles to their intracellular targets is confocal microscopy. Since to allow for improved detection of nanoparticles we used a Zeiss 510 META confocal microscope with multispectral imaging capabilities which correct for color overlaps on a pixel-by-pixel basis within each optical section. This spectral unmixing algorithm (Bearman et al., 2002), referred to as "emission fingerprinting" provides for improved color deconvolution compared to use of conventional optical bandpass filters as shown in **Figure 2**.

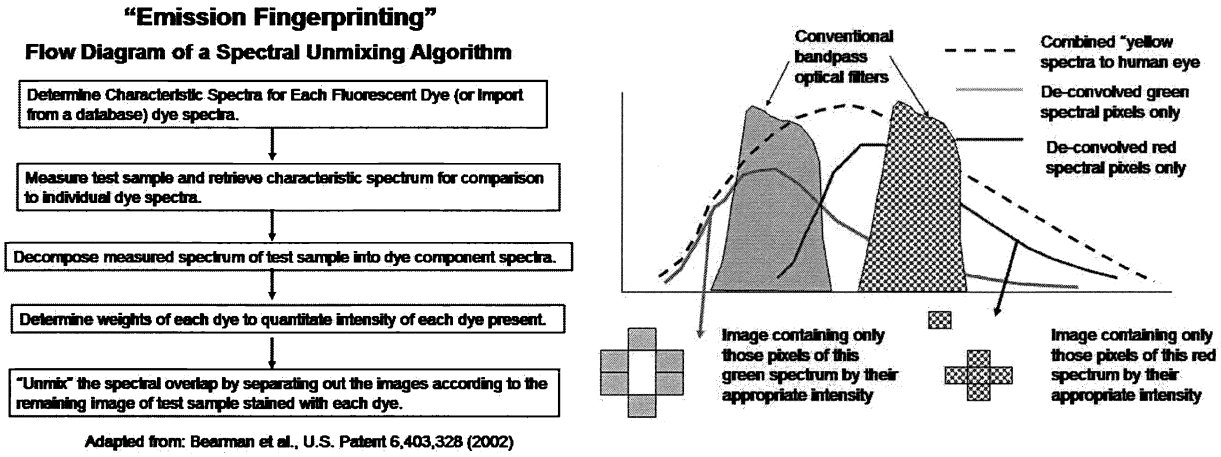


Figure 2: (**A**) The basic concept of emission fingerprinting is shown in schematic form. By knowing directly or indirectly, all of the spectral contributions of each dye or probe plus the autofluorescence spectrum of a cell, the colors can be "unmixed" on a pixel-by-pixel basis on each plane of a multi-plane confocal image. (**B**) The algorithm essentially fits the overall color curve using regions of each dye or component spectrum that are less contaminated with the overlap of other colors. The resulting emission fingerprinting technique can be superior to the use of conventional optical filters which still leave considerable optical overlap.

2.5 Molecular biosensing of the intracellular environment: Thus far we have explored both viral biosensors and reactive oxygen species (ROS) biosensors. This paper will describe use of ROS biosensors (Zhu and Fahl, 2000) coupled to an eGFP reporter gene which fluoresces green when activated. Many biosensors share certain characteristics as shown in **Figure 3**.

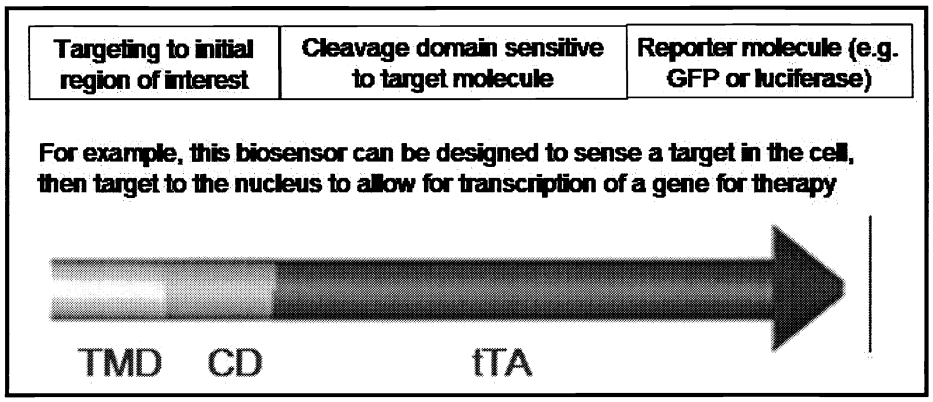


Figure 3: Many molecular biosensors used for diagnostics have targeting regions, a cleavage domain that is sensitive to enzymes or proteases, and a fluorescent reporter molecule.

2.6 Controlled drug-gene delivery: One of the ways we have tried to provide controlled drug/gene delivery to living single cells, is to attach the gene therapeutic to the biosensor. That way the therapeutic gene is only produced as long as the biosensor sees its target molecule. In this case we are developing a transient gene therapy for DNA repair of radiation-damaged DNA based on the biosensing of reactive oxygen species which has high correlation to radiation exposure.

An alternative treatment to repair is to accelerate the cell's natural "programmed cell death" or apoptosis. This is particularly important in the case of trying to repair radiation damaged cells in astronauts. If the cell is not repairable, we would prefer to have it die in a way that does not trigger inflammatory responses of the immune system which can, in many diseases and injuries, be of greater danger to the person than the disease or injury itself. In this application it is important to not allow cancerous cells to arise from mutations produced by radiation damage.

3. DATA/RESULTS

3.1 Comparison of molecular immunochemical and nanoparticle targeting: While the two processes are indeed quite different, fluorescent nanoparticle labeling can yield similar results to those of conventional fluorescent antibody labeling techniques as shown in **Figure 4**. In this case we used very large nanoparticles (approximately 500 nm diameter) in order to visualize the nanoparticle labeling pattern. Improved concordance of nanoparticle labeling with molecular labeling is obtained when nanoparticles are 100 nm or less diameters (data not shown, Prow thesis, 2004).

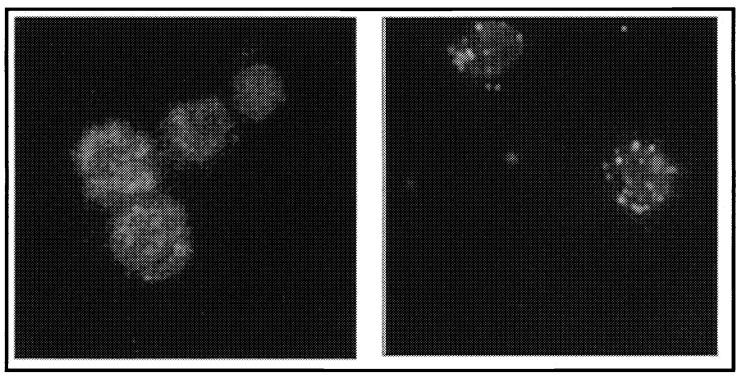


Figure 4: Nanoparticles with targeting molecules can label cells in a manner that gives similar results to conventional labeling with antibodies. However, as the nanoparticles get larger, they tend to bind in a "quantized" manner that leads to increasing discordance of results with increasing nanoparticle size.

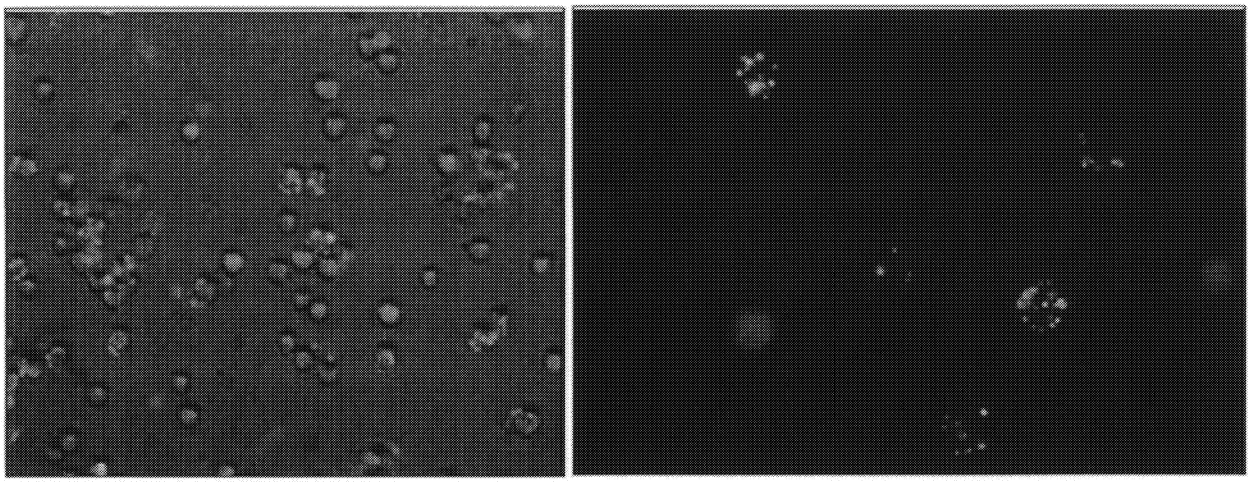


Figure 5: (**A**) BJAB and MOLT-4 cell mixtures were labeled with 500 nm diameter nanoparticles (small white spheres) and viewed by simultaneous phase contrast/fluorescence microscopy using a Nikon Optiphot microscope. (**B**) To improve assay accuracy we pre-labeled contaminating cell types (CD95 negative MOLT-4 cells) with CMAC (Molecular Probes, Inc.) a blue fluorescence tracking dye (shown in this grey scale image as diffuse grey). Nanoparticle (small white spheres in this grey level digital image) labeling showed highly specific labeling of the CD95-positive BJAB cells with undetectable labeling of CD95-negative MOLT-4 cells.

3.2 Measures of nanoparticle targeting efficiency and accuracy: To study nanoparticle binding efficiency we performed phase contrast/fluorescence to allow viewing of cells that labeled or did not label with nanoparticles (**Figure 5A**). Since this did not always allow for accurate identification of the specific cells of interest, we used tracking dye labeling of the larger contaminating cells, not of interest, to allow correct identification of all non-specific cell types in in-vitro experiments that allowed modeling of rare in-vivo targeting (**Figure 5B**).

3.3 Targeted delivery of nanocrystals: Streptavidin labeled nanocrystals were targeted to cells labeled with biotinylated anti-CD95. The purpose of these experiments was to explore the entry mechanisms of semiconductor nanocrystal nanoparticles. "Naked" nanoparticles (with no biocoatings) did enter some cells non-specifically, showing that biocoatings were not only necessary to target nanoparticles to the cells of interest but also necessary to prevent non-specific uptake. Interestingly, Streptavidin coated nanocrystals when bound to the surface of cells labeled with biotinylated anti-CD 95 monoclonal antibodies, not only targeted those cells in a highly efficient manner, but also tended to internalize and track to the nuclei of those cells. HIV tat sequences proved highly efficient cell entry and nuclear localization molecules. Arginine rich amino acid repeat peptides did not prove useful for nuclear localization, at least in our hands.

3.4 Application of nanomedical strategies to repair of radiation-damaged cells: To test the feasibility of these approaches we conducted a series of in-vitro experiments. A antioxidants-sensitive biosensor (Zhu and Fahl, 2000) was attached to DNA repair enzymes previously designed and prepared in the lab of co-author RSL. When a cell undergoes oxidative stress it produces a series

of molecules which will attach to the ARE biosensor which then can be used to drive the synthesis of special DNA repair enzymes MutY/Fpg designed by RSL (**Figure 6**).

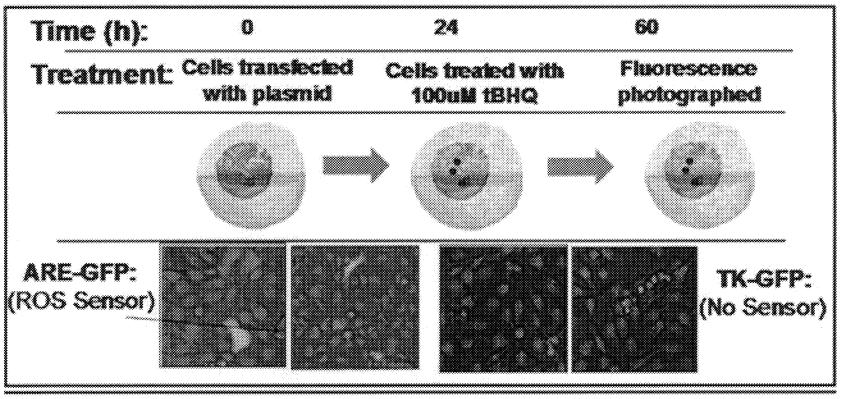


Figure 6: During oxidative stress a cell will produce molecules which will binds to the anti-oxidant response element (ARE). The binding of these molecules can be used as a molecular biosensor to drive the process of DNA repair enzymes downstream in the gene sequence construct.

This ARE biosensor, attached to a green fluorescent protein reporter sequences (eGFP), was tested in cultures of T24 cells subjected to tert-butylhydroquinone (tBHQ), a chemical which produces oxidative stress in cells in a manner similar to UV radiation, but which is more easily dosed and controlled. The cell detects ROS and activates transcription factors which bind to specific promoter regions. Transcription downstream of these response elements is then activated. The construct used was constructed by Zhu and Fahl, 2000. This construct is composed of a number of antioxidant response element (ARE) repeats followed by a minimal thymidine kinase promoter, ahead of a EGFP reporter gene. The sensitivity of the biosensor is dramatically affected by the number of ARE repeats, with four repeats giving optimal sensitivity (Zhu and Fahl, 2000). The activity of this biosensor can be seen in T24 cells through the addition of 100 μM tert-butylhydroquinone, an inducer of ROS (Sigma-Aldrich Chemical, Inc., St. Louis, MO). T24 cells are a human cell line derived from a transitional cell carcinoma that constitutively expresses CD95 on the surface (Mizutani et al. 1997) (American Type Culture Collection, Manassas, VA). Cells showing signs of oxidative stress were detected as green fluorescent positive cells as shown in **Figure 7**.

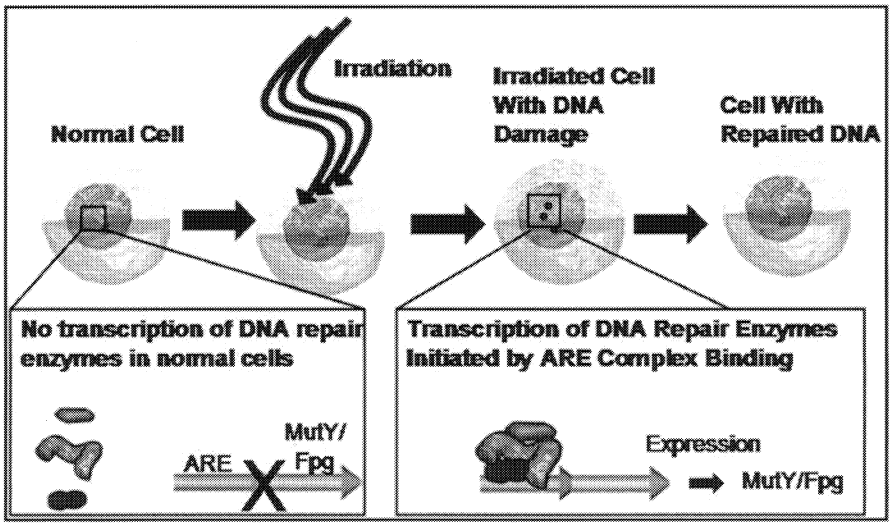


Figure 7: Cells were transiently transfected at ~60% confluence with either ARE-GFP or TK-GFP. 24 hours later the cells were treated with tert-butylhydroquinone (tBHQ), an ROS inducing agent, to simulate the ROS effects of radiation doses The cells were examined every 12 hours post treatment. Weak fluorescence (ARE-GFP) shown in white was present at hour 48 and photographs were taken at hour 60.

To test whether DNA repair enzymes could be introduced into cells damaged by actual UV radiation to accelerate normal DNA repair mechanisms by activating a second repair pathway, not normally expressed in human cells because one enzyme, a glycosylase, is absent in normal human cells (Figure 8).

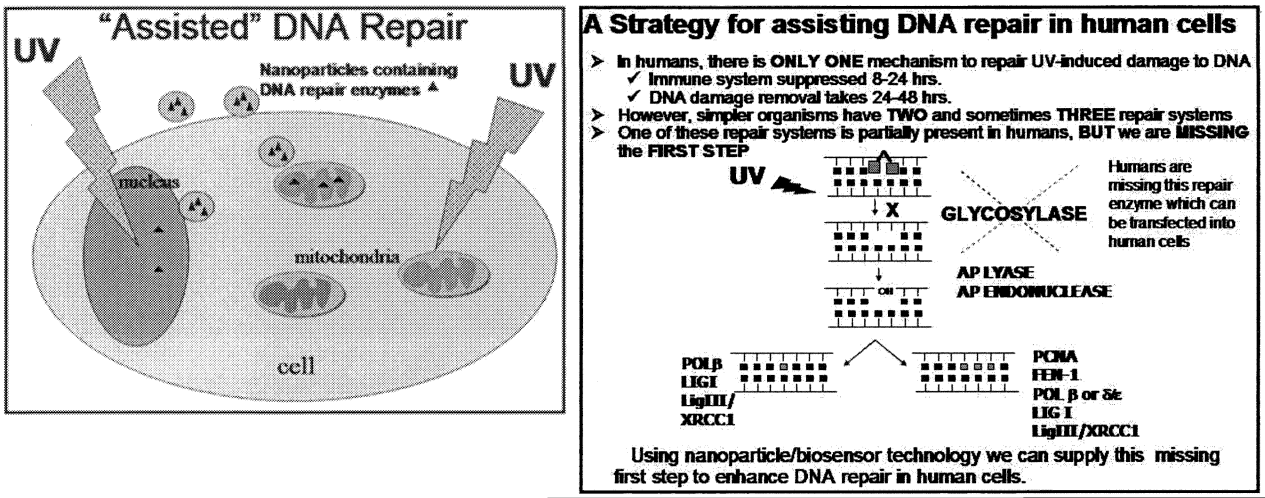
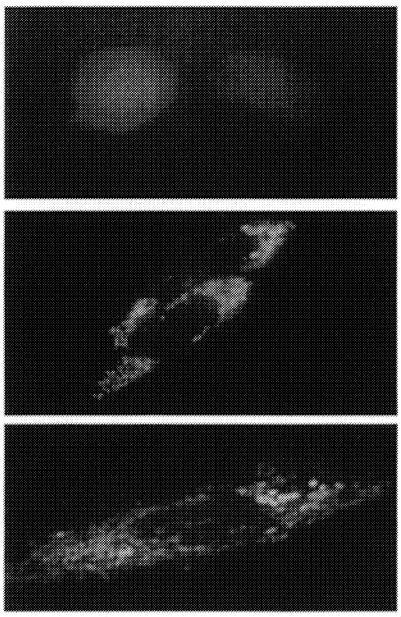


Figure 8: (A) Assisted DNA repair concept for DNA damage to both nuclear and mitochondrial DNA. (B) A specific strategy for assisting DNA repair of UV damage in human cells.

This normally absent repair mechanism was activated by transfection of a glycosylase containing gene sequence which also contained an eGFP reporter molecule. UV damage can occur in both nuclear and mitochondrial DNA. Since many molecules introduced into a cell frequently track non-specifically to the nucleus, we tested the ability of intracellular localization sequences to guide these repair molecules to the mitochondria. In the absence of localization sequences, the repair molecules did not appear to track to specific regions of the cell, as shown by the diffuse staining of **Figure 9.** However when mitochondrial localization sequences were attached to the repair enzymes, they tracked to the mitochondria as shown by confocal microscopy (**Figures 9B** and **9C**). Either transient or stable gene therapy was demonstrated.



In the present state of gene therapy concerns about patient safety, our efforts are concentrated on the production of potentially useful transient gene therapies using nanoparticle systems. To test the actual effectiveness of these DNA repair enzymes inside living

Figure 9: (A) T4 transfected DNA repair enzyme with no localization anchoring sequence (note diffuse fluorescence) with transient expression (Wt-T4-PDG-GFP in CHO-XPG. Transient expression. 100x objective)
(B) T4 transfected DNA repair enzyme with mitochondrial localization anchoring sequence, with transient expression (MLS35-T4-PDG-GFP in CHO XPG. 100x objective)
(C) T4 transfected DNA repair enzyme with mitochondrial localization anchoring sequence, with stable expression (MLS18-T4-PDG-GFP in hXPA. 100x objective)

cells, comet assays were performed in the laboratory of RSL. The "comet" assay (Tice, 2000) shows the attempt of cell trying to repair its DNA strand breaks as a comet-like tail. When successful in DNA repair, the comet tail is eliminated. **Figure 10** clearly shows that repairable human cells can be repaired in 6 hours by this assisted DNA repair transient gene therapy approach, as compared with a required repair time of more than 24 hours using the usual DNA repair pathway functioning in normal human cells. While far from proving efficacy in-vivo, these initial results show the promise of this approach.

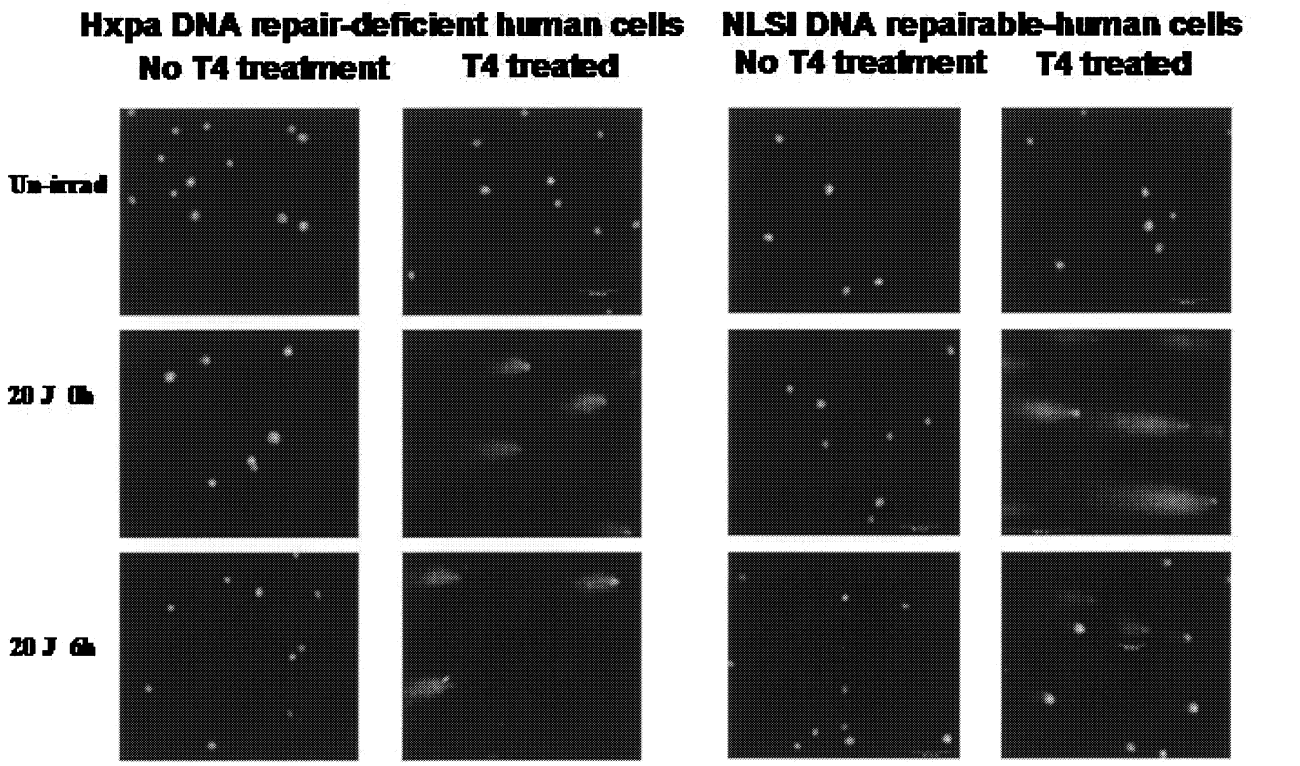


Figure 10: A comet assay shows that DNA damage is successfully repaired at an accelerated rate due to successfully activation of a second repair pathway in a transient gene therapy approach to repairing DNA damage as it occurs.

4. CONCLUSIONS

These results, while still very preliminary, reveal some of the promise of nanotechnology for the effective delivery of therapeutic genes to individual living cells. As such it presents important paradigm shifts in the delivery of medicine from a single choice of destruction of all affected cells to one of selective repair of cells whenever possible and desirable and safe destruction through apoptosis of un-repairable cells. It also shows how, in principle, autonomous nanoparticle systems can be constructed to provide continuous molecular diagnostics/therapeutics at the single cell level.

5. ACKNOWLEDGEMENTS

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