Biosensor-Controlled Gene Therapy/Drug Delivery with Nanoparticles for Nanomedicine

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ABSTRACT

Nanomedicine involves cell-by-cell regenerative medicine, either repairing cells one at a time or triggering apoptotic pathways in cells that are not repairable. Multilayered nanoparticle systems are being constructed for the targeted delivery of gene therapy to single cells. Cleavable shells containing targeting, biosensing, and gene therapeutic molecules are being constructed to direct nanoparticles to desired intracellular targets. Therapeutic gene sequences are controlled by biosensor-activated control switches to provide the proper amount of gene therapy on a single cell basis. The central idea is to set up gene therapy "nanofactories" inside single living cells. Molecular biosensors linked to these genes control their expression. Gene delivery is started in response to a biosensor detected problem; gene delivery is halted when the cell response indicates that more gene therapy is not needed.

Cell targeting of nanoparticles, both nanocrystals and nanocapsules, has been tested by a combination of fluorescent tracking dyes, fluorescence microscopy and flow cytometry. Intracellular targeting has been tested by confocal microscopy. Successful gene delivery has been visualized by use of GFP reporter sequences. DNA tethering techniques were used to increase the level of expression of these genes. Integrated nanomedical systems are being designed, constructed, and tested in-vitro, ex-vivo, and in small animals.

While still in its infancy, nanomedicine represents a paradigm shift in thinking – from destruction of injured cells by surgery, radiation, chemotherapy to cell-by-cell repair within an organ and destruction of non-repairable cells by natural apoptosis.

1. INTRODUCTION

In earlier work we have described the general principles of nanomedicine and demonstrated feasibility of the overall concept. In this paper we discuss three specific areas: (1) how to construct multilayered nanoparticles that can then perform in a controlled sequence of steps to accomplish nanomedicine, (2) the importance of zeta potentials for controlling the initial charge interactions of these multilayered nanoparticles and targeted cells, and (3) the concept of "nanofactories" whereby a gene template is delivered to a living cells and therapeutic genes are manufactured "in-situ" within a living cell using local materials.

When one realizes that nanoparticles must perform a complex sequence of tasks, it becomes obvious that one way to accomplish this is to contain each step in a single layer of a multilayered nanoparticle [4]. Nanomaterials are inherently self-assembled atom-by-atom, or layer-by-layer (LBL). A way to do this is to use alternately charged polymer layers [5-7] that also contain antibodies, or other targeting molecules such as aptamers, intracellular localization amino acid sequences, molecular biosensors, and therapeutic genes.

Each of these charged layers contributes to the overall "zeta potential" (an oversimplification but essentially the "net" charge of the nanoparticle as seen at a distance) of the nanoparticle which must interact with a charged living cell. A living cell typically has a fairly highly negatively charged cell surface layer of molecules. If the nanoparticle were positively charged it would stick non-specifically to cells and destroy any specificity of targeting. The zeta potential of

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both nanoparticles and cells changes according to environmental factors such as pH and ionic strength of the surrounding fluid. Since the composition of the cells is what it is, the nanoparticle zeta potential, and the zeta potential of the remaining NP as each layer is stripped away during the multi-step process, must be adjusted to prevent non-specific interactions.

Lastly, if the purpose of nanomedicine is to deliver the precise amount of therapy to each cell, there must be several components of the therapeutic gene delivery system. First, the NP must be delivered near the site required for therapeutic efficacy. The volume of NP is roughly one millionth the volume of a human cell. Since chemical reactions are driven by local concentrations and diffusion mechanisms, the NP must be intracellularly "localized" (targeted) with appropriate secondary targeting molecules. Often this is done with amino acid sequences which tend to chaperone other molecules to the nucleus, mitochondria, endoplasmic reticulum, etc. To deliver the correct amount of therapy on a cell-by-cell basis, ultimately the most powerful feature of nanomedicine, there must be molecular biosensors present to detect molecules which indicate whether the cell is responding appropriately to therapy. If these biosensor molecules are "upstream" of the promoter sequences and the therapeutic gene, the biosensor can be used to turn on (or off) the production of copies of the therapeutic gene [8]. Lastly, the therapeutic gene can be "manufactured" in-situ (i.e. inside the cell) using these gene templates and local ingredients already within a cell. This is the concept of "nanofactories" manufacturing therapeutic genes to order on a cell-by-cell basis to deliver exactly the correct amount of nanomedicine to each cell – neither more nor less than what the cell optimally needs. This form of nanomedicine represents a new and exciting way to practice regenerative medicine, not just killing diseased cells, but actually altering the development of the disease in its early stages (e.g. treatment of pre-malignant cancer cells to try to restore them to a state of normalcy). This sense of nanomedicine "repair" on a cell-by-cell basis will be even more important in treatment of infectious diseases. If a cell cannot be repaired we would like it to be eliminated in the way that nature designed cells to be eliminated – through apoptosis (programmed cell death). As a cell undergoes apoptosis it does not trigger the immune system into an immune response, which may prove worse than the original disease. Rather, it neatly and cleanly degrades cells into their constituent molecules and effectively recycles them for further use.

Nanoparticles act as sophisticated delivery vehicles for drugs and genes, protecting them during the process of targeting to cells and guiding them to the specific cells of interest to minimize bystander cell injuries due to drug/gene toxicity. Targeted drug or gene delivery can be used to kill or eliminate diseased cells as in cancer therapies. IN this paper we show preliminary results from a nanomedicine approach designed to "repair" cells undergoing DNA damage to prevent their becoming cancer cells. Cancer is a progressive disease. The early steps of cancer transformation might be reversible. But a far more sophisticated strategy than simply killing diseased cells can be used to repair tissues and organs at the single cell level using targeted nanoparticles. Hence the most profound use of nanomedicine may eventually be in the areas of regenerative medicine for treatment of heart disease and infectious diseases. We show some very preliminary data in regenerative medicine involving hepatitis virus infected cells, a new approach to fighting virus inside living single cells.

2. METHODS

The overall conceptual approach to nanomedicine has been previously described [4, 9]. In this paper we will concentrate on three important steps in the overall process. First, we will overview the construction of multilayered nanoparticles for drug/gene deliver using layer-by-layer (LBL) assembly methods. Second, we will provide a brief discussion of the importance of the interacting zeta potentials of nanoparticles and cells for initial targeting. Third, we will describe a new conceptual approach for the delivery of therapy to single cells, namely the delivery of "nanofactories", templates which allow for the manufacture of therapeutic genes in-situ under the control of a molecular biosensor.

2.1 Construction of multilayered nanoparticles for nanomedicine:

Multilayered nanoparticles containing cell targeting molecules, membrane entry promoting molecules, intracellular targeting molecules, molecular biosensors and therapeutic genes have been previously described [4]. The concept is illustrated in Figure 1. Essentially layer-by-layer (LBL) assembly of molecules are built on a core which may itself allow for single or multi-modal in-vivo imaging. The first layer on the core would represent the last step on the programmed sequence of nanomedical events. The next layer, typically of opposite charge, would then be added, building up in layer-by-layer fashion [5].

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2.2 The importance of coordinating zeta potentials of multilayered nanoparticles with cells:

This LBL assembly of the nanoparticle results in a net charge as seen at a distance – the "zeta potential". The charged nanoparticles essentially order the charged and polarized molecules in the surrounding aqueous medium as show schematically in **Figure 2A**. This complex, multilayered nanoparticle must then interact in a directed targeted to selected single cells which themselves have a complex charge structure and a cellular zeta potential as shown in **Figure 2B**. The trick is to design alternating inner layers of the multilayered NP which will have the desired charge structure when they are in the particular pH and ionic environment inside the cell.

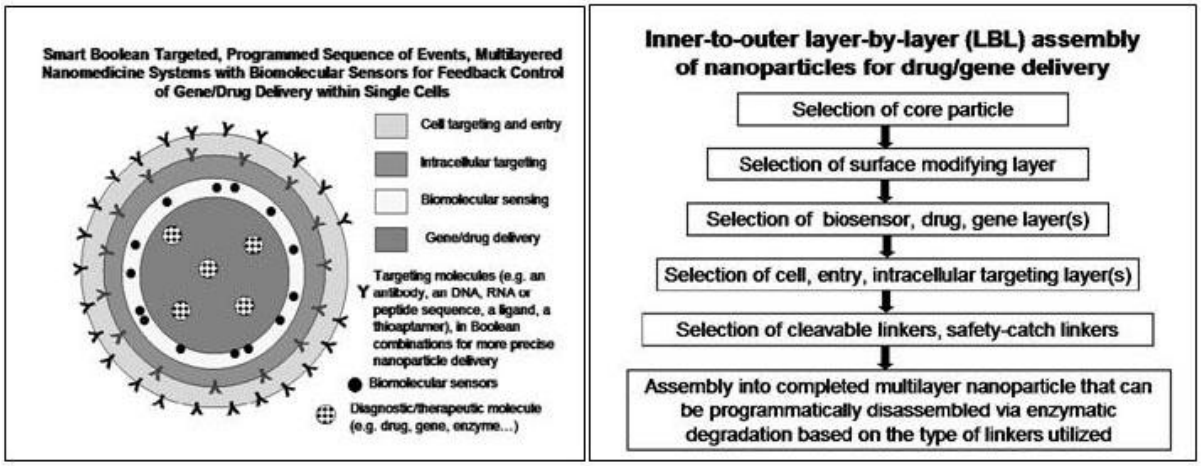

Figure 1: Schematic of process of building a multilayered and multifunctional nanoparticle for nanomedicine. Multilayered and multifunctional nanoparticles are assembled via cleavable and/or safety-catch linkers. First the surface modifying layer is attached to the core with linker molecules. Next the functional layer is added and attached with linker molecules. Finally any targeting or entry layers are added and attached with linker molecules. This motif can be repeated for delivery of multiple functional layers to various different subcellular locations. The principle of layering is to assemble molecules to be used from first use (outer layers) to last use (inner layer). This allows for "molecular programming of the sequence of events (Leary and Prow, 2004). The inner core material can be chosen to allow optical, magnetic, X-ray or MRI contrast agents to be used for simultaneous in-vivo imaging.

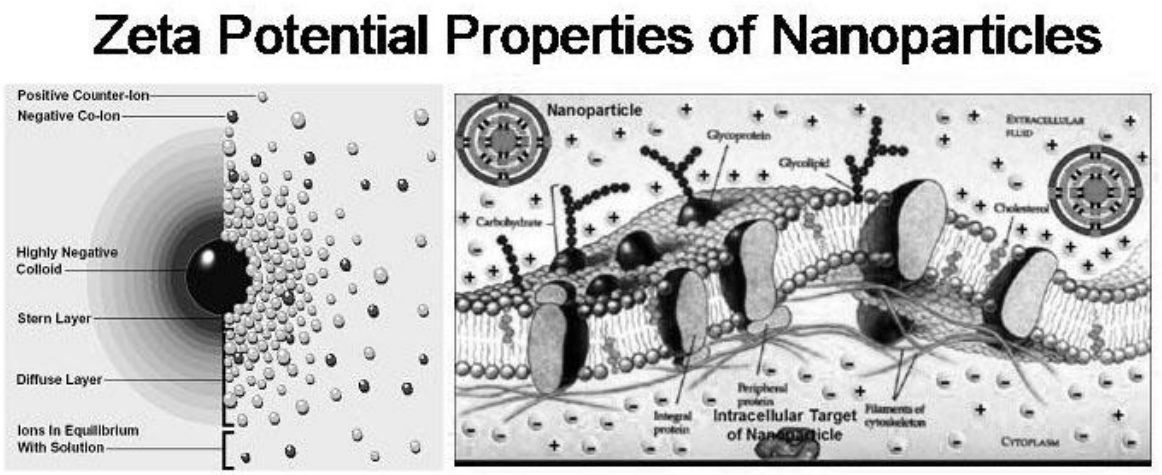

Figure 2: Interaction of charged bodies is a function of their zeta potentials. In an aqueous environment, cells and nanoparticles not only have their own intrinsic charge, but also organize the ions in the surrounding fluid. This zeta potential is a function of many variables including pH and ionic strength. The zeta potential (left-hand-side)(adapted from [1])is important for the stability of the nanoparticles in the various solutions encountered in vivo and it is also important for determining how the nanoparticle will react electrically with the surface of the cell (adapted from [3]).

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2.3 Concept of "nanofactories" to deliver appropriate therapy to each cell

There are several problems in nanoparticle drug delivery. First, since a nanoparticle has a very low volume (roughly one millionth) compared to a human cell volume, it is difficult to deliver enough drug to be therapeutic unless that drug is extremely potent. For example, a single molecule of Ricin A, one it gets inside a human cell, is enough to kill that cell – as is now done with targeted antibody therapies. So we don't care whether we more than kill the cell as long as we kill it for example killing a tumor cell in cancer. However, if we are trying to repair, and not just kill a cell, we need to deliver enough, but not too much drug. That is much more difficult and requires precise control. Also, while it is possible to target cells down to frequencies of one cell in a million, the probability of getting a particular number of nanoparticles to bind to a cell is very difficult. The NP drug delivery is "quantized" in discrete units and it is difficult to target just the correct number of NP to each desired cell. A far easier way to approach the problem is to use therapeutic genes which can be manufactured in any desired number and controlled with an upstream biosensor as shown in **Figure 3**.

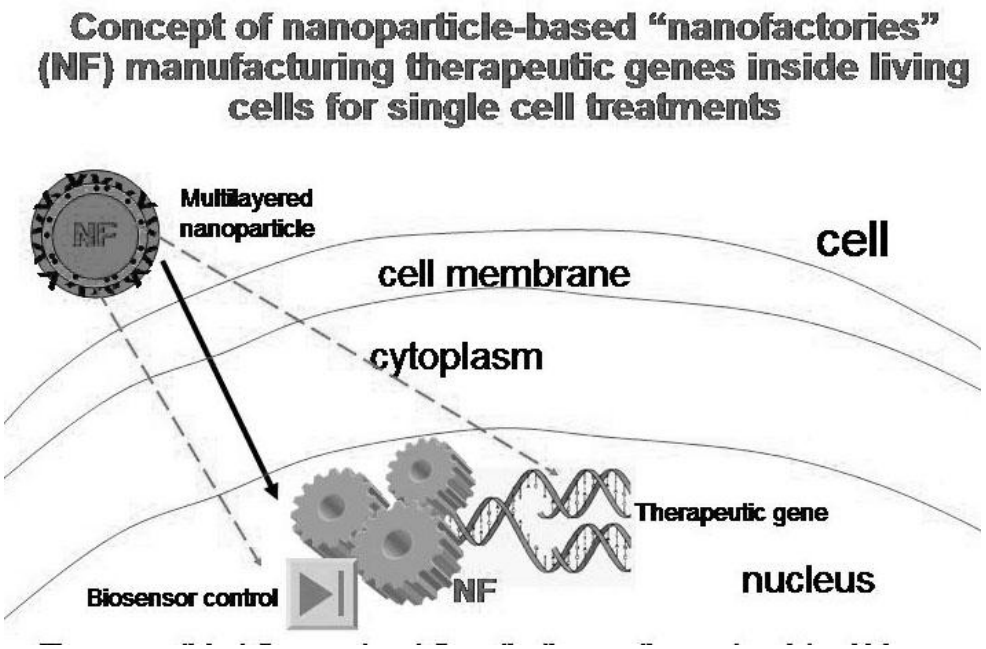

Figure 3: The nanoparticle delivery system delivers the therapeutic gene template which uses the host cell machinery and local materials to manufacture therapeutic gene sequences that are expressed under biosensor-controlled delivery.

2.4 Using "Biomimicry" and "nanofactories" to combat viral infections at the single cell level:

Does such an approach actually work? The answer comes from both "historical" and experimental evidence. First, Nature has done exactly this using viruses for millions of years. The problem is that the virus produces what is beneficial to the virus, not necessarily the host cell. Our approach is essentially "Biomimicry" that copies the example provided by Nature. Beyond this we have some very preliminary evidence that a simpler system involving coordinated molecular biosensors and therapeutic genes does indeed work. We connected a viral biosensor that recognized a hepatitis C viral protein NS3/4 and a ribozyme that cuts the IRES region of the hepatitis C virus (HCV), effectively inactivating it. In **Figure 4** we show how a nanoparticle (NP) coated with a surface protein of HCV can be used to mimic the virus. In **Figure 5** we show the molecular mechanism of action of a ribozyme molecule which targets the critical IRES region of HCV, acting as "molecular scissors" to cut the critical region and inactivate the virus.

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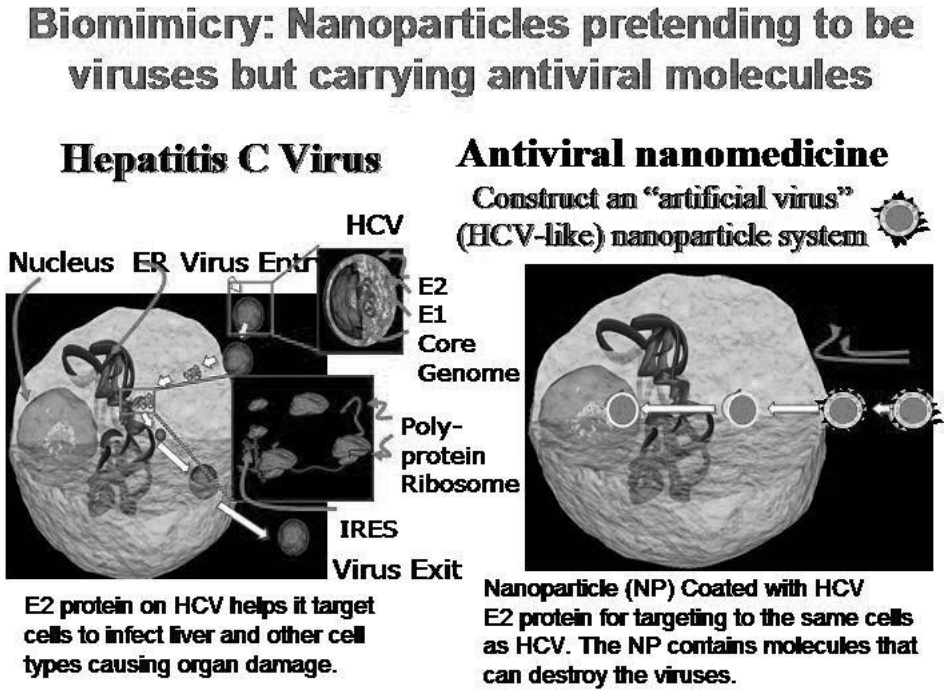

Figure 4: Using the principle of "Biomimicry" we can mimic Nature by making a nanoparticle look, at least at first encounter, like a virus. But the nanoparticle can contain antiviral molecules inside to combat viral infection at he single cell level.

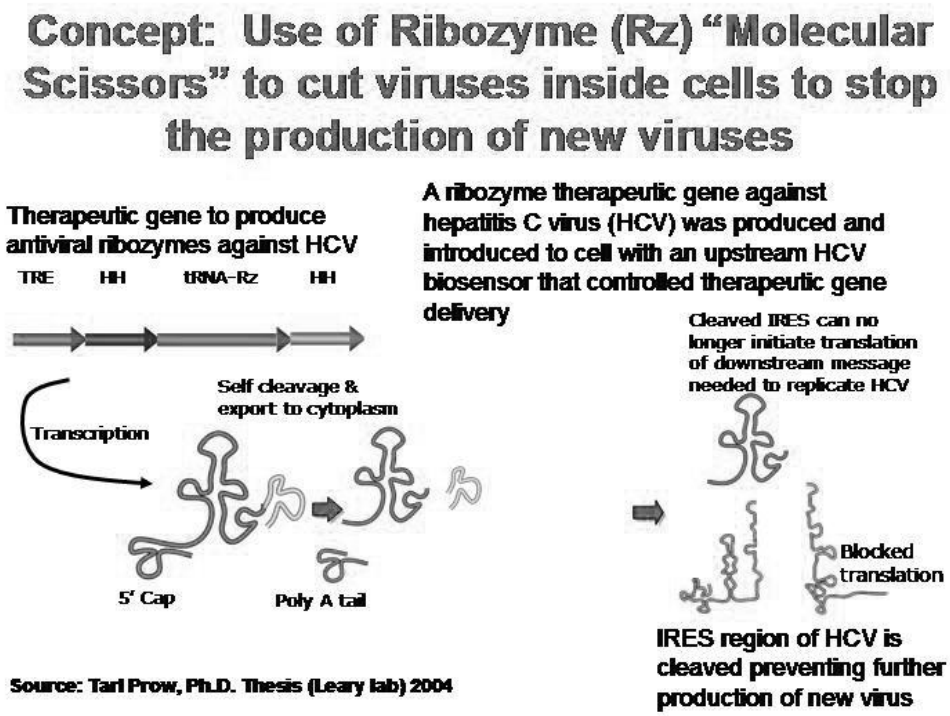

Figure 5: Ribozymes can provide nanomedical therapy at the single cell. These molecules function as "molecular scissors" that can cut recognized viral sequences to inactivate viruses inside single cells.

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4. DATA/RESULTS

4.1 Construction of multilayered nanoparticles:

The construction of nanoparticles using LBL assembly as described by Lvov et al., (ref; and Prow 2004) using charged polymers (**Figure 6A** results in, as expected, a gradual increase in mean nanoparticle diameter (**Figure 6B**)). The zeta potential swings from positive to negative depending on the charge of the particular layer (**Figure 6C**).

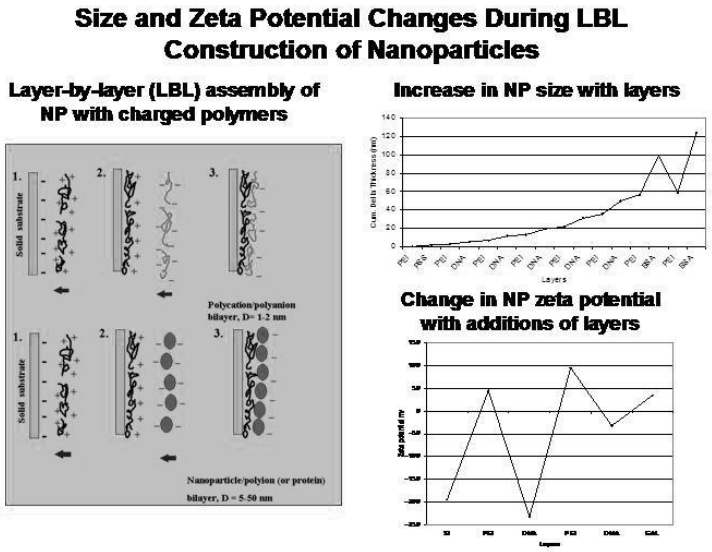
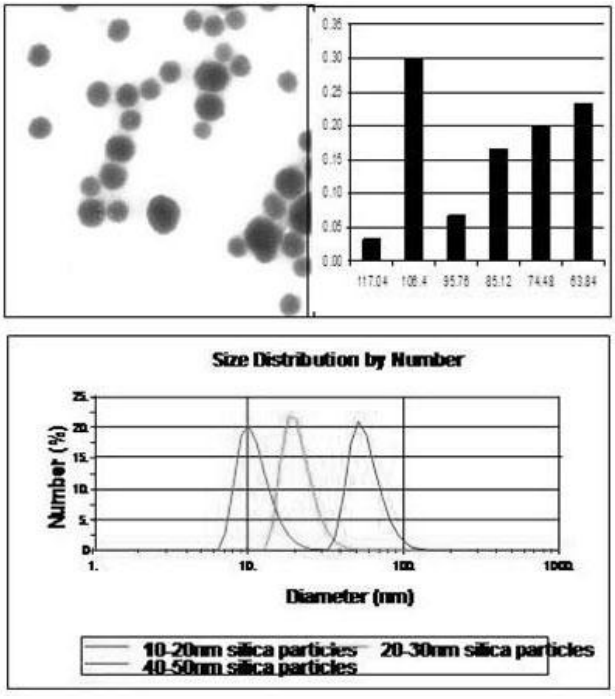


Figure 6: Layer by layer assembly. The core nanoparticle (NP) is composed of silica and is negatively charged (**Panel A1.**). The addition of a positive charged moiety causes the layer to pack tightly onto the surface of the particle (**Panel A2.**). Alternate layers are thereby deposited onto the surface of the particle (**Panels A1. to A3.**). The NP increases in size (**Panel B**), as expected, with the addition of each layer, as measured by AFM (atomic force microscopy). The zeta potential increases and decreases with the addition of charged layers.

4.2 Problems in nanoparticle uniformity of size and charge:



However, in practice, there can be a wide distribution of particle sizes and charges due to incomplete assemblies. In **Figure 7A** we see a distribution of nanoparticles visualized by electron microscopy (EM). In **Figure 7B** we see a distribution of NP diameters as measured from EM photographs. This is a very tedious process. We can rapidly obtain a measure of the distribution of NP sizes using differential light scattering techniques, but the individual variations are lost. This result means that for LBL assembled NPs to be practical and useful the NPs must be purified by either size or charge or both. We have not yet solved this problem but are exploring ways to purify NPs using particle electrophoresis methods.

Figure 7: Structural analysis of nanoparticles. **Panel A** shows layer-by-layer particles constructed with DNA layers and a BSA outer coat analyzed by TEM. **Panel B** shows a bar graph containing a histogram showing nanoparticle diameter data based on the TEM data. **Panel C** shows sizing of silica NP by differential light scattering.

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4.3 Effects of pH and dilution on NP zeta potential:

As described earlier in this paper, the zeta potential of the NPs varies according to the pH. In experiments measuring the zeta potential of NPs (**Figure 8A**) we also showed that at least these NPs can be kept stable and unclumped for periods of at least 5 days after preparation. Since NPs will be greatly diluted in-vivo it is important to see the effects of dilution, particularly since the NPs are typically manufactured under conditions of relatively higher salt concentrations. Since zeta potential is also caused by the ordering of ions in the solution around the NP, there is an obvious effect of dilution on the zeta potential of the NP.

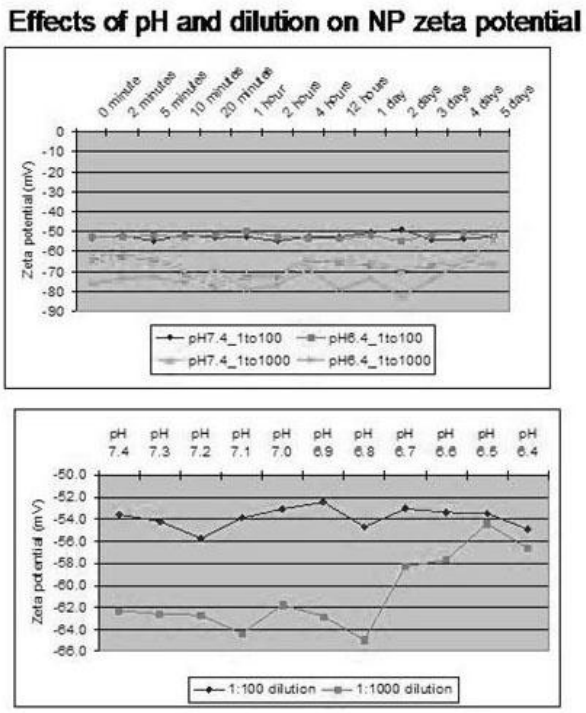

Figure 8: Zeta potential testing of SnowTex© particles. SnowTex© 40-50nm silica nanoparticles where diluted to two different concentrations using distilled/deionized water and utilizing HCl the solutions were adjusted to two different pH concentrations. **Panel A** shows that these tested solutions are stable for at least 5 days when stored at room temperature, which is important for the future storage of the complete nanoparticle solutions. **Panel B** shows zeta potential testing of SnowTex© particles. SnowTex© 40-50nm silica nanoparticles where diluted to two different concentrations using distilled/deionized water and utilizing HCl the solutions were adjusted to different pH concentrations. This figure shows that the pH of the solution and the dilution of the nanoparticles in the solution are important for controlling the zeta potential of the nanoparticle solution.

4.4 Use of tethering to increase therapeutic gene expression

Therapeutic genes can not simple be allowed to adhere or coat a NP. In those instances, probably owing to problems of steric hindrance and accessibility, there is a huge loss of gene expression. To deal with this problem we have been experimenting with gene tethering methods (**Figure 9**) whereby the gene is attached to the NP in a way such that its expression is relatively unhindered. The concept of tethered gene expression was first demonstrated in our laboratory with magnetic NPs which also had the advantage of being easily recoverable [8].

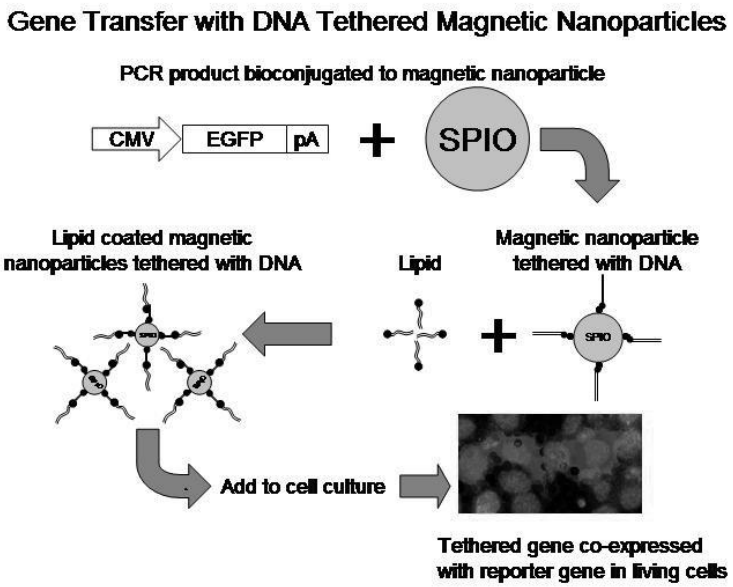

Figure 9: Schematic of tethered gene delivery with magnetic nanoparticles. Biotin labeled primers are used to generate labeled PCR products encoding EGFP that are mixed with streptavidin coated magnetic nanoparticles. These DNA coated particles are washed and then coated with lipid. Lipid coated particles are then added to cell culture.

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In the absence of such gene tethering, the gene expression levels are reduced by more than two thirds as shown in **Figure 10**. However, therapeutic genes tethered to NPs express at levels which are not statistically different from those of free vectors.

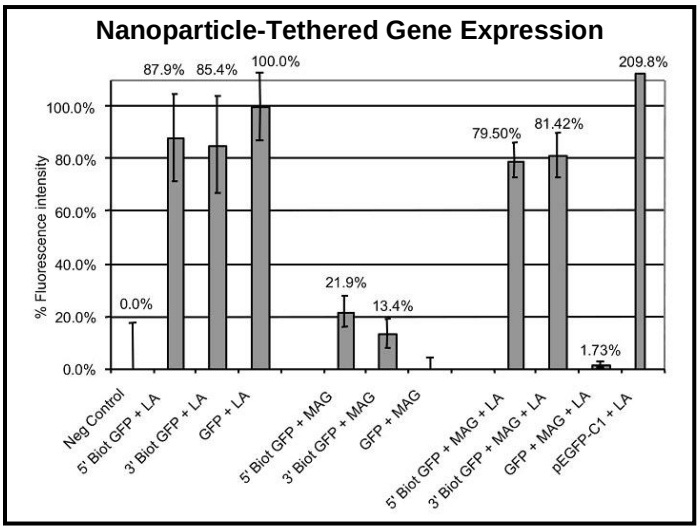

Figure 10: Relative gene expression levels comparing tethered, untethered, and free gene sequences linked to fluorescent GFP reporter sequences. If genes are not tethered properly to the nanoparticles, there is a significant drop in gene expression level as compared to free genes.

4.5 Example 1: A nanomedicine approach to antiviral therapy at the single cell level

To demonstrate the feasibility of combating viral infection at the single cell level using nanomedicine, we applied ribozyme therapy to HCV infected single cells. In a model cell system in-vitro, we constructed a nanomedical system with a ribozyme directed against the IRES version of the hepatitis C virus (HCV)[8]. The cells shown in **Figure 11** are both infected with HCV, but only cell # 2 has been treated with the ribozyme. In Panel A, cell #1 (untreated) and cell # 2 (treated) are stained for their DNA. In Panel B, the cells are stained with a molecular biosensor directed against the ribozyme. Cell # 1 is untreated, and cell # 2 is ribozyme-treated as seen by the ribozyme biosensor present in cell # 2 but absent in cell # 1. The therapeutic response of the ribozyme is shown in Panel C which shows a continued presence of NSE protein in untreated cell # 1 but its reduction in ribozyme-treated cell # 2.

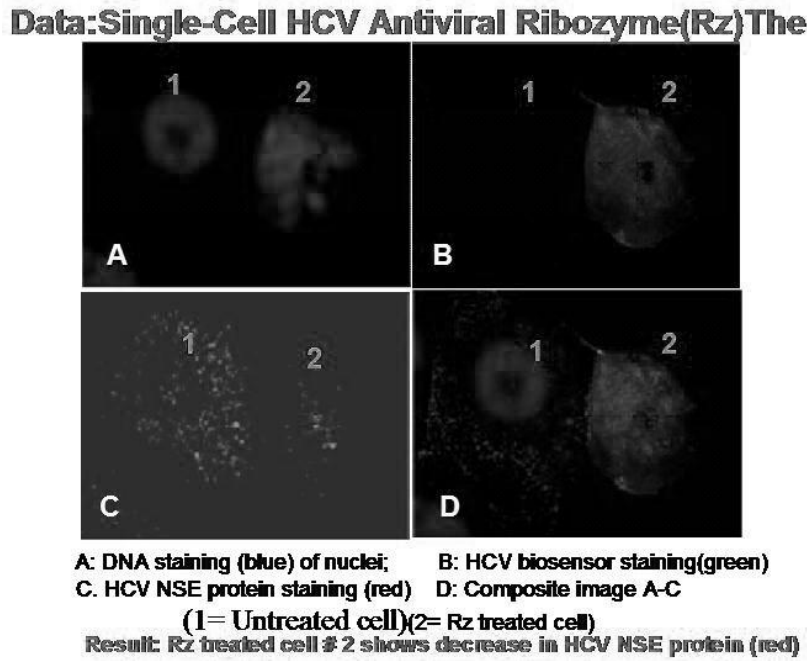

Figure 11: Example of single cell ribozyme therapy. The progression of Panels A-D show the reduction of NSE HCV viral protein under the action of ribozyme therapy.

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4.6 Example 2: A nanomedicine approach to preventing progression to cancer

A problem of some interest and concern to NASA is the health of astronauts during the planned voyage to Mars. Both during the over 18 month journey and during time on surface of Mars where there is little shielding of radiation, astronauts will encounter a significant amount of radiation-induced cell injury. Rather than trying to shield astronauts, which is not currently possible with present-day technology, one nanomedicine approach is to use nanoparticles with DNA repair enzymes to continuously repair the radiation-induced damage in the astronauts to prevent organ injury or cancer. We have already demonstrated general feasibility of the steps necessary in the overall process. What remains to be done, an not a trivial task, is to produce an integrated nanomedical system consisting of a multilayered nanoparticle system containing targeting, localization, biosensing, and therapeutic genes (in this case, DNA repair genes).

A radiation damaged cell undergoes oxidative stress, producing reactive oxygen species (ROS). These ROS molecules can be detected with an anti-oxidant reactive element (ARE) molecular biosensor, previously described [2] and used in our laboratory. If the ARE biosensor is put upstream of a DNA repair enzyme, the binding of the ROS molecules to the ARE biosensor can be used to control the production levels of DNA repair enzymes until there are no more ROS molecules to drive the production. Such a nanomedical system described previously in more detail [9] and shown in **Figure 12** is being developed to be potentially used to continuously repair radiation induced DNA damage in astronauts.

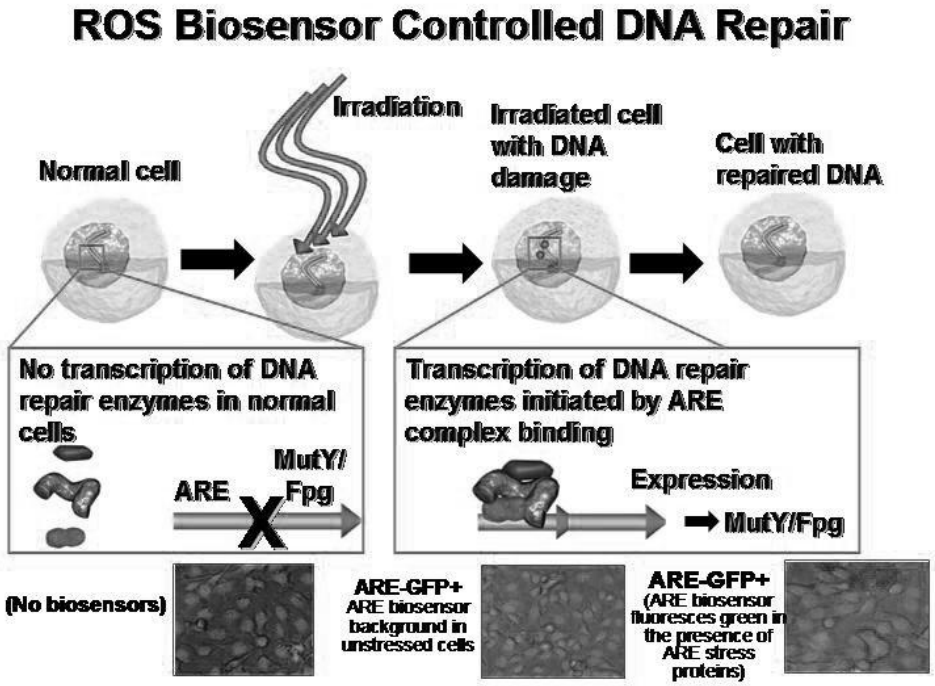

Figure 12: Cells were transfected with either ARE-GFP (stress biosensor) or TK-GFP (a control gene). 24 hours later the cells were stressed with a chemical to simulate space radiation stress. The cells were examined every 12 hours post treatment. Weak fluorescence was present at hour 48 and at hour 60 photographs were taken.

4.6. Model system for biosensor detection of radiation-induced (or a similar chemically induced) DNA damage:

A T24 cell line stably expressing the ARE-GFP biosensor, designated T24-ARE-GFP, along with a control cell line, T24–TK-GFP (expressing TK-GFP, without ARE in the promoter region) were subjected to treatment with an ROS inducer, tBHQ. Cells were plated in 6-well plates at 2X105/well and cultured in McCoy's 5a medium for 24h prior to treatment with increasing concentrations of tBHQ or DMSO (mock control). 24h post-treatment, GFP fluorescence was observed under an inverted fluorescent microscope. Images were taken using constant exposure time (1 sec). Under these conditions, no cell death was observed (**Figure 13**):

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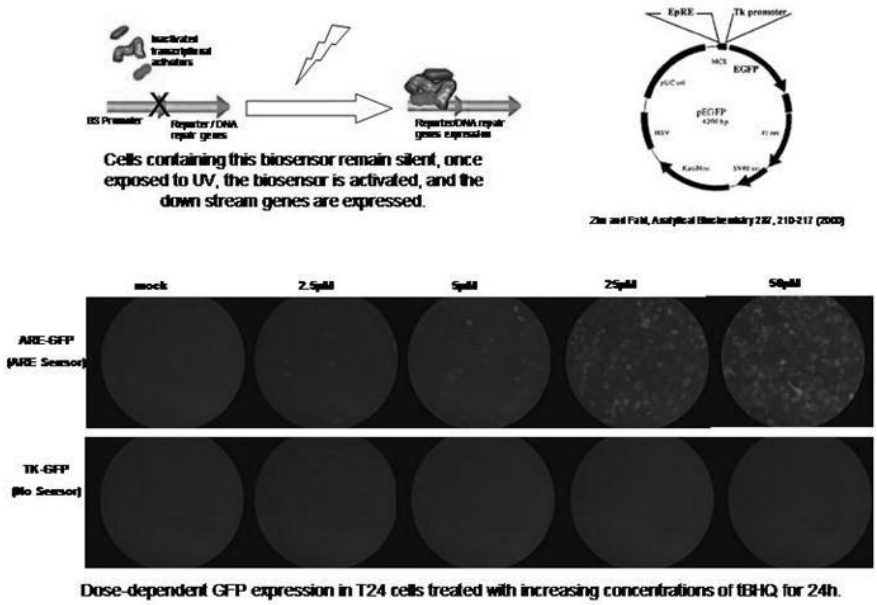

Figure 13: An ARE (Antioxidant Response Element) molecular biosensor [2] is sensitive to the production of reactive oxygen species (ROS) in stressed human T24 cells. The number of cells undergoing oxidative stress varies in a dose-dependent manner with chemical or radiation dose and can be measured using an ARE biosensor linked to an eGFP reporter gene sequence.

5. CONCLUSIONS

Multilayered and multifunctional nanoparticles can bring a new precision and control of gene delivery to single cells. Single cell dosage levels can be feedback controlled through the use of molecular biosensors. Gene therapy can actually be produced inside single cells using a gene template that uses the host cell machinery and precursor molecules already inside the living cell.

6. ACKNOWLEDGMENTS

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