System control-mediated drug delivery towards complex systems via nanodiamond carriers

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Cells represent the basic units of life and contain an intertwined network of signaling and regulatory circuitries that drive the processes of life. These processes include the mediation of mechano-sensation, onset of and protection against disease, inflammation, and others. In the network of bio-complex systems, each pathway interacts nonlinearly with others through different molecular intermediates. As a result, specific functionalities cannot be simply linked to the cellular molecules in isolation. A complex system generally possesses very rich information content that can be characterized by the following features: (i) they contain a large number of building blocks; (ii) their interactions among building blocks and with their environment; (iii) they display organization without an external organizing principle being applied; and (iv) they exhibit adaptability and robustness. These properties account for the innate intelligence of biology and its ability to regulate its homeostatic behavior. However, this same complexity underlies, in cancer for example, challenges towards disease management, as the addressing of singular pathways with therapeutic compounds is not sufficient. Therefore, combinatorial therapy often serves as a key strategy towards tumor suppression. Because iterative searching for optimized therapeutic combinations is indeed a daunting, if not preclusive task, we introduce the feedback system control (FSC) scheme, which may serve as a clinically relevant approach, provided a translational approach towards drug delivery is employed. Due to their innate biocompatibility, which has been comprehensively observed, as well as their ability to delivery virtually any type of therapeutic in a sustained fashion due to their unique surface properties, nanodiamonds may serve as a foundation for nano-enabled combinatorial therapy.

Keywords: bio-complex system; feedback system control; drug delivery, nanodiamond combinatorial medicine; signaling network; nanomedicine

1. Complex systems

Systems that consist of a large number of interacting components and display self-organization capability are commonly regarded as complex systems [1]. Due to the large number of possible configurations within the systems, complex systems usually exhibit adaptive and robust characteristics [2], as well as emergent properties (i.e. the systemic collective
behaviors cannot be directly deduced from that of the individual constituent) [3]. Attempts to understand a complex system using the conventional reductionist approach are difficult to provide complete physical insights for the underlying system dynamics. As a result, to understand and eventually engineer a complex system requires new concepts and generalizations other than that of the bottom-up method.

At present, little is known about how the hierarchical architectures within different complex systems emerge from their respective interacting constituents. For example, the actual processes of how biological cells are integrated into tissues [4,5], and tissues into organs remain largely unknown (Figure 1). This could be due to the fact that the system information (e.g. the key molecules dictating morphogenesis) governing the biological processes are only partially known [6]. By using an integrated approach of evolutionary computing and the theory of dynamical systems, we may be able to engineer/direct complex biological systems to a desired outcome (e.g. disease management) by extracting the key parameters (e.g. constituents) from the systems, and revealing the corresponding dynamics between the interacting constituents.

2. The bottom-up and top-down approaches to study bio-complex systems

2.1. The bottom-up approach

Under the influences of atomic/molecular forces, bio-molecules such as DNA, RNA and proteins, form structures with specific recognition capabilities [9]. Reactions only happen in molecules of selected types, e.g. antigen and antibody, and complimentary base pairing in DNA. Reactions of these molecules take place as they are brought close to each other by stochastic diffusion processes. The chain reactions of these molecules become deterministic due to the specific recognition capability and serve as the basis of signaling/ regulatory pathways. Exploring the types of molecular structures and mapping out the network of pathways, in fact, help to build a vast information data base (Figure 2). However, we should note that cellular networks are not like hardwired electronic circuitry networks. In fact, biological networks dynamically connect and disconnect on a very frequent basis. In addition, the network response of a diseased cell can be profoundly different from that in a homeostatic state. When drugs are applied to the diseased cell, the cellular network response to the external stimulations will be very different from that of a health cell. It is evident that the buildup of such an information rich data base is extremely laborious and time consuming. A reductionist approach is important and absolutely necessary for laying the fundamental foundation for scientific understanding. However, emergent cellular properties are derived from self-organizing interactions among numerous pathways and are a “discontinuity” (Figure 1) which remain a main challenge of the bottom-up approach for transcending from nanoscale bio-molecules to larger scale complex systems (cell, organ and body) [11].
2.2. The top-down system approach

The rich research findings developed in the control field [12] have offered many pathways to direct electrical, mechanical, fluid, or thermal systems toward required performances. The feedback control scheme is a powerful tool to accomplish such a task. A feedback system control scheme based on integrative system responses has been broadly applied in engineering research for optimizing designs of electronic circuit and mechanical systems [13,14].

The challenge is whether we can apply the same concept to control a biological complex system. Exploring and controlling the self-organized network of a bio-complex system encounters unprecedented challenges, whereas well-defined physical principles are absent. Rather than measuring and understanding all the molecule–molecule interactions, the alternative but very different top-down approach is to treat the system as a black box and then to identify the system response under input stimulations [15]. With enough information, the system identification technique can enable us to “predict” the output of the system at the given input conditions without unraveling the details inside the system.

In addition to studying the input–output relation of a system, this top-down approach provides us with a possibility to guide complex systems toward a desired destiny. The feedback control scheme is a powerful tool for accomplishing this task. The difference between the desired and actual output of a system under stimulations will be fed into a search algorithm, which serves as a multi-parametric search/optimization guideline to determine
the needed stimuli and then iteratively drive the complex system into a certain systematic fate. As such, the feedback control scheme can achieve the goal without requiring a potentially prohibitive laborious understanding of the entire interconnectivity between individual molecular components in advance.

The search algorithm, such as genetic algorithms [16] or differential evolution [17], appear to be appropriate candidates to perform effective searching in the large parameter space. The development of these algorithms was inspired by the biological mechanisms of evolution, where natural selection processes (e.g. mutation and selection) are executed within the algorithms to allow for rapid convergence of the optimal solutions [16]. Very recently, the evolutionary computing schemes have been successfully implemented to identify potent drug combinations in a variety of biological systems towards desired phenotypic and genotypic destinies [18,19].

3. Direct a complex cellular system to a desired phenotype by the feedback system control (FSC) technology

3.1. Combinatorial drug treatment

Treatment of a disease is a typical problem of controlling a biological complex system. Among numerous examples of applying drugs to cure a disease, the introduction of combinatorial drugs for treating HIV is a striking success. The death rate of AIDS increased linearly with time until 1995. With the introduction of combinatorial drugs [20], the death rate dropped 60% in 1997 and has remained at the same level until now (Figure 3). Currently, many cancer chemotherapies also include combinations of many drugs. By simultaneously manipulating several pathways of a complex cellular system, it is expected be more effective than single drug therapy.

The full understanding of how multiple drugs stimulating the cellular network and attaining synergetic interactions is almost unreachable. The top-down feedback system control (FSC) approach, on the other hand, can provide a quantitative and systematic method to derive the optimized drug cocktails to reach the best therapeutic outcome [18,19].

![Figure 3](https://example.com/figure3.png)

Figure 3. The effectiveness of combinatorial drug on combating AIDS (AIDS surveillance trends CDC.gov).
The FSC scheme uses close loop feedback control concept (Figure 4) and combines experimental exploration with the search algorithm to reach the goal. Instead of laboriously mapping out all the detailed activities of cellular components under the initial multiple drug stimulations, only the endpoint response is used to determine the next drug combinations through the search software. In other words, the biological system will do all the hard work, we only need, based on the most important information, the cell system endpoint response to make the next move.

3.2. Inhibition of vesicular stomatitis virus (VSV) infection by FAS technology

A specific example is to inhibit infection of vesicular stomatitis virus (VSV) of host cell, 3T3 fibroblast. VSV is a virus that can infect mammals, and its genome is a single molecule of negative-sense RNA. Viruses grow very rapidly and mutate rapidly as well. Because of that, a virus can become resistant to a particular drug. This is why it is so important to be able to use a combination of more than one drug. If the virus mutates to become resistant to one drug, it still can be sensitive to the other drugs.

Five drugs, ribavarin, puromycin, IFNα, IFNβ and IFNγ, were used in the experiment [18]. Three of these drugs were interferons (IFN), which are naturally produced proteins that are highly effective in organisms to block virus infections by producing an antiviral state. In addition, we used ribavarin, a broad spectrum antiviral drug that is well characterized to stop RNA viruses like VSV. Also, we used puromycin, a non-selective terminator of protein synthesis that at low doses can stop the rapid growth of viruses.

Viruses replicate by infecting a host cell and using the host cell’s molecular machinery to create new progeny virions. Consequently, many of the molecular components of the virus and the host cell are similar. Therapeutics that target virus replication thus represent a double edged sword in that, though they can efficaciously block viral replication, they may also interfere with normal host cellular metabolism. Indeed, many antiviral therapies that work effectively in vitro suffer in the clinical setting by toxic side effects. A central question in the effective clinical management of viral infection thus remains: is there an optimal method to treat a patient by inhibiting viral replication and still limit or abolish the toxic side effects of the drugs? To limit the side effects of high dose antiviral therapies, treatments have entailed combinatorial drug use where multiple antiviral drugs work in concert to limit the replication and spread of virus. We used five drugs with 10 concentrations, which resulted in $10^5$ possible combinations. To find the most potent combination becomes like finding a needle in a haystack. In other words, the task of combating viral infection by drug cocktail (Figure 5) faces two challenges, a large parameter space and manipulating complex systems under multiple stimulations.

Figure 4. Feedback system control (FSC) of bio-complex systems.
Since the best dosages of the drugs are unknown, the first combination started with arbitrarily determined drug concentrations. The VSV was engineered. While the infection of the host cell took place, the 3T3 expressed green fluorescent protein (GFP). The percentage of cells not expressing GFP after incubation with VSV for 13 h was considered as the systemic outputs for the optimization of the antiviral activity in the host cells.

The experimentally measured infection percentage was fed into a search algorithm, Gur game [21] in this case, to determine the next combination of drugs which many led toward the most potent drug cocktail. The major unknown is how many iterations like this are needed for converging to the desired endpoint outcome, i.e. high level of inhibiting viral infection.

In the experiment, three sets of independent tests were carried out (Figure 6b). It is truly surprising that all rapidly converged to the optimal conditions, 100% inhibition, in tens of iterations (Figure 6). These results showed that the system output is insensitive to the initial guesses of the drug dosages. In addition, the total inhibition of the virus occurred at much lower drug doses than would be needed if drugs were used singly. In fact, the concentrations of the drugs were only about 10% of that required for individual drugs [18]. This will definitely lead to an overall goal of creating a drug combination that is far less toxic while still as effective as high dose combinations.

Based on recent unpublished data, the fast convergence, tens of iterations, also holds for controlling two other types of viral infections and eradications of cancer cells by combinatorial drugs. The pleasant surprise of the top-down FSC technique actually roots in one of the fundamental features of the complex system. After millions of years of evolution of the bio complex system, the landscape of the cellular responses to the drugs will be very smooth due to the robustness and adaptiveness of the complex system. The smooth search space dictates

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Figure 5. Inhibition of VSV infection by FSV scheme.

Figure 6. Inhibition of VSV infection with (a) no drug treatment, (b) fast converge to 100% inhibition with three different initial conditions, (c) optimal drug cocktail [18].
that only small numbers of iterations can lead toward success of directing cellular systems toward desired phenotype.

Integrating the FSC technique and the bottom-up approach can form a powerful modality to study cellular functions. The bottom-up model seeks to identify all the players of a regulatory or signaling pathway to decipher how to block or augment the pathway. The FSC approach model overlooks these details and lets the system determine what works best for itself. FSC technique can provide a bird’s-eye view first to identify the important molecular activities and then integrate with the bottom-up model to home in on the detailed interactions at the molecular level.

4. Drug delivery – an enabling technique to crossover the discontinuity of hierarchical complex system

Translating drugs that are developed at the cellular level to clinical applications is a challenging endeavor. For example, the discontinuities emerging at the hierarchical complex system that spans the cell, tissues, organs and body can preclude the direct application of optimized combinatorial medicine. Drug delivery is thus a requisite technique needed to overcome this challenge.

Many strategies to efficiently deliver a broad range of therapeutics, particularly those enabled via nanoparticles, have previously been explored [22–28]. In this review, we will focus on the newly developed nanodiamond (ND) platform given its promising characteristics, which unite many of the requisite properties for translationally-significant therapy.

4.1. Nanodiamond drug delivery

Nanodiamonds have been successfully explored as agents for a number of applications, including imaging and therapeutic delivery [29–33]. This review will focus primarily on its foreseen relevance as a drug delivery agent. The requisite properties of a favorable platform for multi-therapeutic delivery include: (i) the ability to carry nearly any type of therapeutic, such as a hydrophilic or hydrophobic small molecule; (ii) scalable production with high yield; (iii) biocompatibility and ability for efficient clearance; (iv) sustained drug release properties so as to avoid toxicity associated with burst elution; (v) applicability as both a systemic and localized release modality. Recent studies have shown that nanodiamonds are capable of delivering a broad array of therapeutic compounds towards the comprehensive addressing of the aforementioned conditions.

4.2. ND–Dox nanoparticles for chemotherapeutic delivery

This work was among the first to demonstrate the application of NDs as drug delivery systems. Specifically, ND-drug particles were developed for the induced apoptosis (programmed cell death) of HT-29 human colon cancer cell lines [34]. This work showed the feasibility of high-throughput ND-drug conjugation. Furthermore, we demonstrated the ability for the NDs to switch Dox activity on/off depending on drug desorption/adsorption.

Ball-milled nanodiamond powders were acid treated to yield negatively charged surfaces that could be easily integrated with positively charged drugs such as doxorubicin (Dox), which is a potent chemotherapeutic that causes DNA fragmentation (cutting), which induces cell death (apoptosis). Drug–ND interactions were confirmed through TEM imagery and FTIR analysis. A particularly significant component of this study demonstrated the ability to selectively control drug release based upon a salt (NaCl)-mediated mechanism. A specific
point of note is that Dox is normally systemically/non-specifically active, meaning it induces cell death in both healthy and unhealthy cells. This study showed that Dox activity could be switched off and on based upon the adsorption and desorption, respectively, of the drug from the ND surface in a reversible fashion. Furthermore, the NDs were capable of moving in and out of cells quickly which is a hallmark property of an optimized drug delivery platform with optimized biocompatibility. Dox–ND composites were further shown to be highly efficient towards causing cell death in multiple cell lines including human colon cancer cell lines. Fundamental cell-material interaction studies were also conducted. For example, the expression of multiple inflammatory and apoptosis regulating genes was examined to determine the innate biocompatibility/lack of toxicity of the ND material. Whereas the Bcl-x apoptosis gene examined the impact of NDs upon cellular toxicity, inflammatory gene expression examined the presence of upregulated inflammation markers which has been shown to promote tumor spreading, and even counteract the core function of several cancer drugs. Following quantitative real-time PCR interrogation of IL-6, iNOS, and TNFα inflammation marker levels, no significant increase in gene expression was observed which provided the comprehensive and quantitative insight into innate ND biocompatibility. The lack of increased expression confirms that the NDs themselves are bio-amenable materials for scalable drug delivery device fabrication. This study thus provided the first demonstration of ND-mediated drug delivery which serves as a cornerstone of nanocarbon-based applications in biology.

4.3. ND dispersal of water insoluble therapeutics

In this previous study, water-insoluble purvalanol A and 4-hydroxytamoxifen (4-OHT), promising drugs for liver and breast cancer, respectively, and the anti-inflammatory dexamethasone were complexed with ND clusters, greatly improving water dispersion (Figure 7) [35].

Purvalanol A and 4-OHT are typically solubilized in DMSO or ethanol, respectively, solvents normally unsuitable or unfavorable for injection. Prior to ND-complexing, purvalanol A and 4-OHT aggregated into particles on the order of ~100 μm. Subsequent binding

Figure 7. Nanodiamond-mediated insoluble drug delivery: (left) suspended NDs; (center): ND–drug; (right): insoluble drug.
with NDs resulted in a three orders of magnitude decrease in particle size on the order of \(\sim 100\ \text{nm}\). In addition, the zeta potential of the resulting drug–ND complexes emerged more positive, augmenting the surrounding of hydration shells, ultimately increasing water dispersibility [20]. Accordingly, NDs have been proposed as a probable candidate for breast cancer pharmacotherapy due to ND retention in cytoplasmic vesicles within estrogen receptor positive breast cancer cells (MCF-7). These effects, in addition to the biocompatibility, scalability and versatility of NDs lend themselves well as important precursors for emerging water-insoluble therapeutic delivery.

4.4. A new method for nanodiamond and cell response characterization

Towards the translation of utilizing NDs for feedback-mediated delivery, the mechanisms of cellular uptake and intracellular trafficking must be investigated. So far, it is known that pH and salts within buffers have a considerable effect on ND aggregation and stability, which ultimately effects cell internalization behavior [36].

Fluorescently tagged NDs were injected into a variety of cell lines via capillary forces (Figure 8). Since physical mechanisms were used to introduce the nanoparticles into the cell, no chemical modifications on the probe or ND surface were required during the introduction process.

The interplay between cellular response and size has shown that particle properties have a crucial role in mediating cellular response. As the nanofountain probes (NFPs) are operated with an AFM, real-time control over spatial fidelity and measurement of forces can be made on the order of nm and nN, respectively. Investigations comparing the kinetics of transmembrane transported and intracellular injected NDs within cells are currently underway. The

Figure 8. Nanofountain probe (NFP)-patterned nanodiamond–drug hybrids.
resulting findings could provide a basis in determining optimal dosages, and timing sequence work elucidates a relevant system in which to test for drug delivery efficacy, time scales and dosages for novel nanoparticle delivery mechanisms.

4.5 **ND–parylene devices with sustained functionality**

This work first confirmed the enormous functional potential of ND devices. Unprecedented slow drug release in this preliminary system was demonstrated on the order of 30 ces in more complex situations.

NFPs were also simultaneously used for high resolution nanomanufacturing, with nanoparticle placement on the order of 100 nm. The current NFP capability reveals the potential for future studies involving single-cell dosage assays and the manufacturing of intelligent devices that require fine consistent dosage control. Therefore, we believe that the NFP can mediate drug deposition for localized drug release devices that can sustain delivery with significant drugs remaining in reserve [37]. This was a significant increase in release timescales compared to patch devices that did not contain NDs (~8 days). A significant amount of drug remained within the ND patch suggesting that sustained release can be realized on the scale of several months or greater. This work provided a foundation towards the consistent treatment of a spectrum of physiological disorders by demonstrating that the ND presence within the patch architecture significantly increased the release time-scale several-fold.

Robust devices were fabricated to thicknesses (tens of microns) that are comparable to parylene coatings that are already FDA approved for perpetual implantation. Preliminary studies revealed continuous drug elution (30 days) using only 1 ND matrix layer. Furthermore, the release profiles with ‘zero-order’ kinetics (consistent release of therapeutically significant doses), an important enhancement over the commonly observed ‘burst’ kinetics (a significant amount of drug released initially resulting in minimal drug in reserve), were realized with this device. Currently, the devices possess a significant amount of residual therapeutic in reserve within the ND matrix (Figure 9). Due to the significant surface area: volume ratio of the ND matrices coupled with their unprecedented slow release performance, a scalable multi-drug patch device with several layers (e.g. 100 layers of the NDs at

**Figure 9.** Nanodiamond–drug loaded microfilms for localized drug delivery.
5nm per layer is only 0.5 μm thick, producing a negligible addition to the device dimensions) can be engineered. This device would then be capable of drug delivery lifetimes envisioned to span well into the year-long timeframe.

**4.6. Enhanced gene delivery with optimized biocompatibility**

This work is the first to demonstrate the application of NDs as gene delivery vectors for the transfection of mammalian cells [38]. Utilized on its own, PEI800 (low molecular weight polymer for gene delivery) possesses low delivery efficiencies but favorable biocompatibility. Other forms of high molecular weight PEI can exhibit the opposite effect, where DNA delivery efficiencies are high, while the toxicity to cells is also high. Conventional approaches are thus challenged because of the difficulty of integrating both high efficiency delivery and biocompatibility into one gene delivery system. In this study, by adding PEI800 to the nanodiamonds, a 70 times enhancement in delivery efficiency over PEI800 alone was observed, and the inherent biocompatibility of PEI800 was also preserved. The HeLa cervical cancer cell line was used to test the efficiency of gene delivery. Confirmation of successful DNA insertion into the cells was demonstrated by resultant cellular fluorescence associated with the delivery of a green fluorescent protein (GFP)-encoding DNA sequence. This served as a model for broader applicability in the delivery of other specific disease fighting DNA strands. Furthermore, fluorescently labeled NDs were also shown to internalize within the HeLa cells in a comprehensive manner while bare DNA exhibited low internalization. With regards to toxicity measurements, cellular viability assays showed that low doses of the toxic high molecular PEI resulted in significant cell death, while doses of ND-PEI800 that were three times higher than that of the high molecular weight PEI revealed a highly biocompatible complex.

5. **Concluding remarks**

Complex systems are based upon rich information content and account for the innate intelligence of biology and its ability to regulate its homeostatic behavior. Challenges towards disease management are often based upon the non-hardwired behavior of the cellular signaling networks that form the foundation of these systems. Therefore, the addressing of singular pathways with therapeutic compounds is often not adequate due to cellular dynamic responsiveness. Iterative searching for optimized therapeutic combinations based upon an initial need to fully address crosstalk between every signaling pathway is clearly challenging, and as such, utilizing feedback control schemes may serve as a clinically relevant approach, provided a translational approach towards drug delivery is employed. Carbon-based nanodiamonds may serve as an applicable technology to realize the translational use of feedback control-mediated treatment, as opportunities to optimize therapeutic efficacy in alignment with clinically-significant delivery platforms will significantly enhance patient outcomes.

The FSC scheme represents an effective means of reaching desired cellular outcome goals, i.e. rapidly optimizing the drug cocktail from a very large number of possible combinations and directing the complex system to a desired destiny. By only focusing on the most important information, the endpoint system output without being distracted by the abundance of cellular signaling data that is currently being sought after is a key strategy that can realize success.

Carbon-based nanodiamonds may serve as an applicable technology to realize the translational use of feedback control-mediated treatment, as opportunities to optimize
therapeutic efficacy in alignment with clinically-significant delivery platforms will significantly enhance patient outcomes.

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