The Synthesis and Testing of Anti-Cancer Therapeutic Nanodevices

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Abstract. Nanotechnology provides the sized materials that can be synthesized and function in the same general size range and Biologic structures. We have attempted to develop forms of anticancer therapeutics based on nanomaterials. Our project seeks to develop dendritic polymer nanodevices that serve as a means for the detection of cancer cells, the identification of cancer signatures, and the targeted delivery of anti-cancer therapeutics (cis-platin, methotrexate, and taxol) and contrast agents to tumor cells. Initial studies documented the synthesis and function of a targeting module, several drug delivery components, and two imaging/contrast agents. Analytical techniques have been developed and used to confirm the structure of the device. Progress has been made on the specifically triggered release of the therapeutic agent within a tumor using high-energy lasers. The work to date has demonstrated the feasibility of the nano-device concept in actual cancer cells *in vitro*.

Key Words. cancer, dendritic polymers, nanotechnology, drug delivery, imaging

Introduction

Bioengineering nanomaterials

Nanomaterials are complex synthetic molecules that are hundreds of times smaller than the cells of our bodies. The processes to generate, manipulate and deploy these substances, called nanotechnology, represents an area holding significant promise for health care and biotechnology in the next century.

The field of nanotechnology was first predicted by Professor Richard P. Feynman in 1959 (Nobel Laureate in Physics, 1965) with his famous Cal Tech lecture entitled, ''There's Plenty of Room at the Bottom.'' The name nanotechnology comes from the size of these materials, as these compounds are only nanometers (1/1,000,000,000 of a meter) in diameter. Consequently, nanostructures are similar in size to biologic molecules such as proteins. These materials can be made either from polymers, carbohydrates (sugars) or lipids, leading to a great variety of functional and physical characteristics. This structural versatility allows for the development of new industrial applications and artificial assemblies (such as lipid vesicles, dendritic polymers, DNA aggregates and nano rods or tubes) that are miniaturized to a previously inaccessible size.

This area of technology is now viewed as one of the last major technological frontiers remaining to be explored. Considering the impact this technology could have on communications, information storage and the medical sciences, it will undoubtedly have a significance comparable to that of the ''nuclear revolution'' of the 1930s or attaining speeds beyond the ''sound barrier'' in the 1940s. These non-biologic applications offer limitless opportunities for miniaturization (i.e., for information storage, communications, etc). At the same time, understanding the principles of nanotechnology is certain to provide insights into critical biologic nanostructures related to disease control, correction of genetic disorders and longevity.

The study of biomolecular applications of nanotechnology will be important to the future of science. Medical applications of nanomaterials will revolutionize health care in much the same way that materials science changed medicine 30 years ago with the introduction of synthetic heart valves, nylon arteries and artificial joints. The correction of genetic defects that cause diseases such as muscular dystrophy, heart failure or cystic fibrosis are only a few of the possible medical advances from these compounds.

Recent improvements in materials science technology have enabled researchers to create ''custom designed'' nanomaterials to suit specific biomedical purposes and to produce them in massive quantities. These materials are hypothesized to be able to repair or replace normal biologic components of cells. A remarkable range of human afflictions could be addressed by new therapies developed with these materials. Nanomaterials could protect the body from infections and accelerate wound healing. These synthetic substances could also carry drugs and genes into cells, ameliorating the defects that
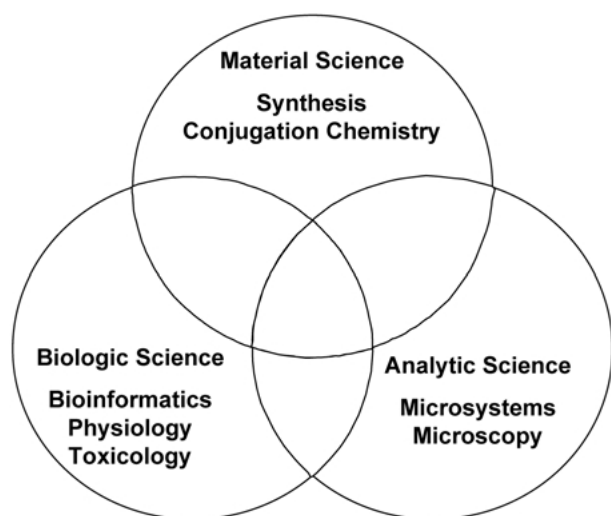
Fig. 1. *The interaction of multiple disciplines supporting antitumor nanotherapeutics. Biologic science is fully integrated with material science and analytical science to produce unique solutions for clinical problems. This integration occurs early in the development process so that issues such as toxicology of the materials do not become problems.*

cause diabetes, high blood pressure and atherosclerosis (hardening of the arteries).

The development of synthetic nanomaterials for biologic applications is by necessity, a multidisciplinary endeavor. This process is a fusion of material science, analytical sciences and biological sciences in a collaborative effort to apply new technology to biologic and medical applications. This occurs in our center (Figure 1), with each discipline playing a crucial role. While it is easy to focus on the material science, without adequate analytic techniques to specifically define the structure and consistency of nanomaterials no finite application can be achieved. More importantly, biological issues such as toxicology may over ride any potential advantage a nanomaterial makes for a specific application. Thus the early and continuous interaction of all three disciplines accelerates discovery and raises the likelihood of success.

Cancer: Current status of treatment approaches

New initiatives in chemotherapeutics and radiopharmaceutics have improved the survival of patients with many forms of neoplasm, and several cancers now have five-year survival rates greater than 80% [1]. Despite these successes, many problems still exist concerning cancer therapy. Many common neoplasms, such as colon cancer, are poorly responsive to therapy [2] and in tumors that are responsive to current methods, only a fraction of cancers respond well to therapy [3]. In addition, despite the improvements in therapy for many cancers, most currently used therapeutic agents have severe side effects

causing morbidity and even mortality for the patient [3]. These side effects often limit the usefulness of chemotherapeutic agents and result in a significant portion of cancer patients having no therapeutic options. It has been hypothesized that other types of therapeutic initiatives, such as gene therapy or immunotherapy, might be more specific and have fewer side effects than chemotherapy. However, while showing some progress in a few clinical trials the practical use of these approaches remains limited [4,5]. In contrast to these limited, yet significant, advances in therapy, the growth in understanding of the underlying biology of neoplastic cells has been truly remarkable. The cellular events involved in neoplastic transformation and altered cell growth are now identified and the multiple steps in carcinogenesis of several human tumors have been documented [6]. Oncogenes that cause unregulated cell growth have been identified and characterized as to genetic origin and function [7]. Specific pathways that regulate the cell replication cycle have been characterized in detail and the proteins involved in this regulation have been cloned and characterized. Also, molecules that mediate apoptosis and negatively regulate cell growth have been clarified in detail [8]. It has now been demonstrated that manipulation of these cell regulatory pathways has been able to stop growth and induce apoptosis in neoplastic cells [9,10]. Clearly, the metabolic pathways that control cell growth and replication in neoplastic cells are important therapeutic targets.

Characteristics of an idealized anti-cancer therapeutic

Despite these impressive accomplishments, important obstacles exist to the use of therapies that address specific metabolic alterations in cancer cells *in vivo*. This type of therapy requires multiple analyses (Table 1). First is the identification of specific pathophysiologic changes in an individual's particular tumor cells. This currently means the mechanical invasion (biopsy) of a tumor and subsequent delays in diagnosis necessitated by *in vitro* cell culture and testing. An analysis of tumor phenotype has to then allow the rapid selection of therapeutic agent(s) that can be efficiently delivered to specifically

Table 1. *Technical requirements for a smart nano-device therapeutic to diagnose and treat cancer*

Targets to tumor
Imaging capability to document presence in tumor
Senses tumor cells for pathophysiologic defects
Selects therapeutic agents based on tumor characteristics
Non-invasive: External trigger releases therapeutics
Documents response to therapeutic
Identifies residual tumor cells

kill tumor cells without collateral damage. In this regard, it would also be of value to have a ''fail-safe'' mechanism that would prevent the activation of a therapeutic agent in a normal cell because the most agents that are ''specific'' for tumors are only *relatively* specific. These agents will stop cell growth or could be detrimental to cell survival if inadvertently activated in normal cells [11]. Tumor targeting is not an adequate solution to specificity in itself since most tumors do not have unique antigens and targeting antigens are not always unique to tumors. Therapeutic agents should have several, different mechanisms of action that work in parallel to prevent the selection of resistant neoplasms, and be released by an operator after verification of the location of the therapeutic. Finally, it would be of value for the therapeutic agent to identify residual or minimal disease before and immediately after treatment, and to monitor the response to therapy. This is crucial as even a few remaining cells can result in re-growth, or worse lead to a tumor that is resistant to therapy. Identifying residual disease at the end of therapy (rather than after tumor re-growth) would facilitate eradication of the few remaining tumor cells. Thus, an idealized therapeutic must have the ability to target a tumor, image the extent of the tumor and identify the presence of the therapeutic in tumor cells. It must also determine why cells transformed to a neoplasm, select therapeutic molecules based on the pathophysiologic abnormalities in the tumor cells, activate the therapeutic agents only in abnormal cells, document the response to the therapy and identify residual disease. A therapeutic with all these capabilities still needs to be small enough to readily enter an individual cell.

Smart, nanomolecular therapeutics

Given the requirements for multiple, smart tasks in a molecular therapeutic, the only currently available technology that serves these purposes is a nano-device. These are designed, synthetic materials with structures less than 10 to 1,000 Å in size. Useful devices are limited to a few hundred nanometers in diameter since this is the largest sized particle that most cells will readily internalize. Unlike ''nano machines'' based on fictional mechanical machines that have been ''shrunken'' to nanometer dimensions, several true molecular nano-structures have now been synthesized and used for drug delivery, gene transfer, antimicrobial therapeutics and immunodiagnostics.

Appropriate nanomaterials: Dendrimers

Selection of an appropriate nanomaterial to use for this application is predicated on the unique requirements of a nano-molecular anticancer therapeutic. While there are several nanomaterials currently synthesized, we feel there is only one that is developed to a point that it is applicable for this complex device. That nanomaterial is the dendritic polymer or dendrimer. These polymers are synthesized as defined spherical structures ranging from 10 to 200 Å in diameter (Figures 2 and 3). Several generations of polyamidoamine (β-alanine subunit) dendrimers are depicted on the right. Molecular weight and the number of terminal groups increase exponentially as a function of generation (the number of layers) of the polymer. Different types of dendrimers can be synthesized based on the core structure that initiates the polymerization process. These core structures dictate several characteristics of the molecule such as the overall

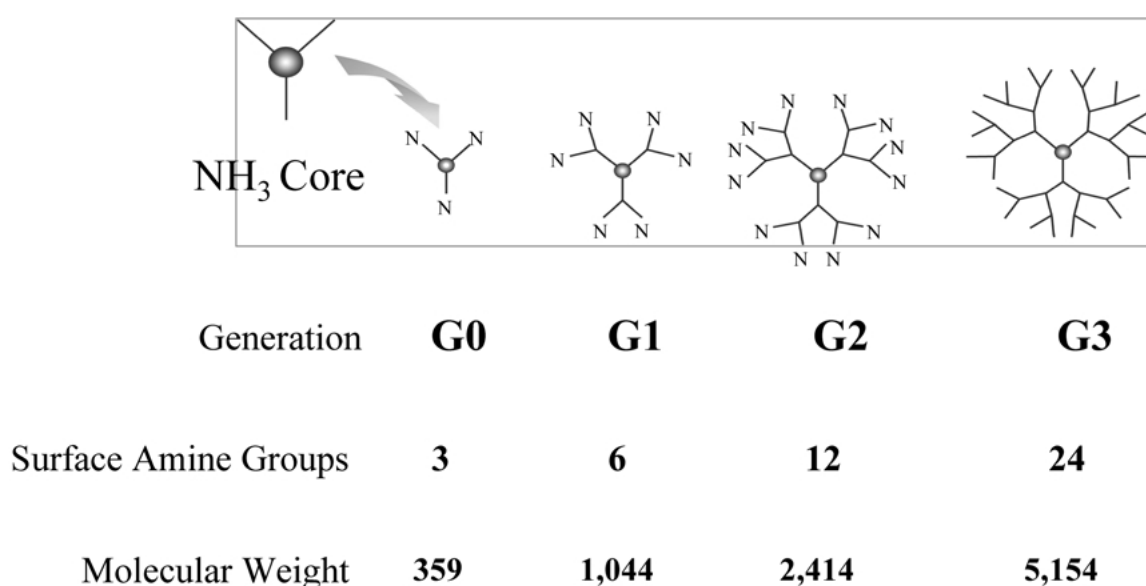| Generation	G0	G1	G2	G3
Surface Amine Groups	3	6	12	24
Molecular Weight	359	1,044	2,414	5,154

Fig. 2. Structure and synthesis of PAMAM dendrimers. Each generation of dendrimer molecule increases the size of the molecule and the number of terminal amino groups as well as the molecular weight. Generations beyond generation 3 increase in an analogous manner.
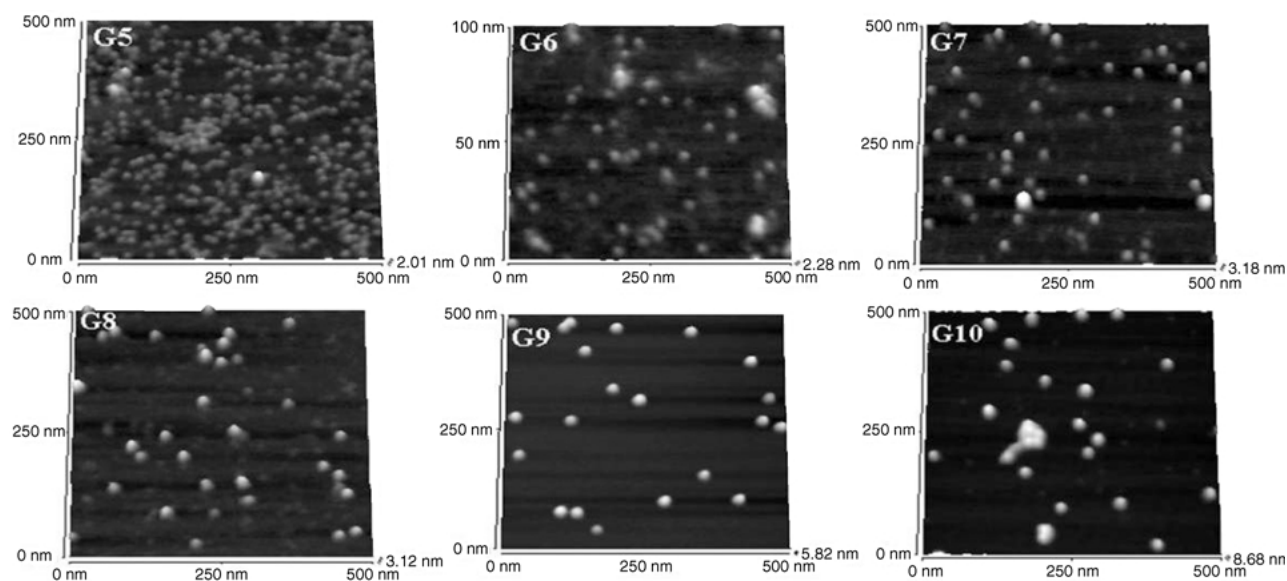
Fig. 3. *Atomic force microscopy analysis of PAMAM dendrimers. Six generations of PAMAM dendrimers are shown ranging from generation 5 to generation 10. Note the uniformity in the structures and the progression in size with each generation.*

shape, density and surface charge [12]. Spherical dendrimers have ammonia as a trivalent initiator core or ethylenediamine (EDA) as a tetravalent initiator core. Recently described rod-shaped dendrimers [13] use polyethyleneimine linear cores of varying lengths; the longer the core, the longer the rod. Dendritic macromolecules are available commercially in kilogram quantities and produced under current good manufacturing processes (GMP) for biotechnology applications. PAMAM dendrimers are characterized by electrospray-ionization mass spectroscopy, ^{13}C nuclear magnetic resonance spectroscopy, high performance liquid chromatography, size exclusion chromatography with multi-angle laser light scattering, capillary electrophoresis, gel electrophoresis, and gas chromatography. These tests assure the uniformity of the polymer population and are essential in the quality control of dendrimer manufacture in GMP applications for *in vivo* usage. Importantly, extensive work has been done with dendrimers examining them *in vivo* and showing no evidence of toxicity when administered intravenously [14,15].

Remarkably, dendrimers have been used for almost every specific component required for the nano-device anti-cancer therapeutic. PAMAM dendrimers have been used as a backbone for the attachment of several types of biologic materials. The resulting conjugates have applications in a number of different areas of biology and medicine. This work has focused on the preparation of dendrimer-antibody conjugates for use in *in vitro* diagnostic applications [16], for the production of dendrimer-chelant-antibody constructs, and for the

development of boronated dendrimer-antibody conjugates (for neutron capture therapy); each of these latter compounds are used as cancer therapeutics [17–20]. Some of these conjugates have also been employed in the magnetic resonance imaging of tumors [17,18].

Results from this work have documented that, when administered *in vivo*, antibodies can direct dendrimer-associated therapeutic agents to antigen-bearing tumors. Dendrimers also have been shown to specifically enter cells and carry either chemotherapeutic agents or genetic therapeutics. In particular, current studies show that cisplatin encapsulated in dendrimer polymers has increased efficacy and is less toxic [21]. Dendrimers have also been conjugated to fluorochromes and shown to enter cells and can be detected within the cell in a manner compatible with sensing apparatus for evaluation of physiologic changes within cells [22]. Finally, dendrimers have been constructed as block co-polymers where the outer portions of the molecule digest with either enzyme or light-induced catalysis [23]. This would allow the controlled degradation of the polymer to releases therapeutics, and could provide a mechanism for an external trigger to release of the therapeutic agents.

Materials, Methods and Results

Modular designs for anti-cancer nanotherapeutics
Several technological barriers must be addressed to make a therapeutic based on this technology viable. The most obvious barrier is to produce a single device that has all

of the different functions necessary for an active sensing, imaging therapeutic. It is possible that each of the different conjugates could be conjugated to a single dendritic polymer, given that 8 to 128 (or more, dependent on the generation) primary amines are present on the surface of that polymer (Figure 4, Plate A). This would allow attached molecules such as sensing units, MRI contrast agents, triggering devices and targeting molecules to be present on the same molecule. In this situation, the interior space would still be available to carry specific therapeutic molecules. However, this is an enormous technical endeavor given the multiple conjugation steps that would be necessary to produce such a multifunction agent. Another approach, which we favor, would be to construct cluster molecules (Figure 5, Plate B). This would involve making separate conjugates for each of the different activities, i.e., one dendrimer conjugate for sensing, one for targeting and another for therapeutic carrier. These different polymers can then be clustered together and covalently linked in a manner that will yield a single therapeutic device. In this approach, one dendrimer will act as a core around which other dendrimers will be covalently attached. The core molecule will be an amine-terminated dendrimer that has been reacted with methyl acrylate. This reaction allows for the covalent attachment of other dendrimers to the core. A highly concentrated mix of amino-terminated dendrimers with different functional groups of the same

or higher generation will then be added to a core dendrimer. A cluster then forms through amide bonds between the terminal methyl ester groups of the core and the remaining free terminal amino groups of the functional outer dendrimers. A limited number of bonds can form between the core dendrimer and each outer-layer dendrimer because of steric interactions, and the molar excess of the outer-layer dendrimer biases the reaction so that each outer core dendrimer only reacts with a single core molecule. Ultra-filtration will be used to isolate the dendrimer clusters and multi-angle light scattering coupled with size exclusion chromatography will determine the molecular weight and the number of dendrimers in the cluster. This is a much easier approach because only single conjugations steps are required. Given the uniform nature of the dendritic molecules, clusters made of each of these units could be expected to have independent function regardless of their conjugation to the other polymers. A final potential approach would be just using the different polymers together in a non-conjugated mixture. Each of the molecules would have to be targeted but could have a different secondary function such as sensing, analyzing, imaging, etc. and the mixture would be readily imported into the cell given its small size.

A therapeutic nanodevice will truly facilitate the concept of non-intrusive sensing, signaling, and intervention for cancer. Since specific protocols of molecular
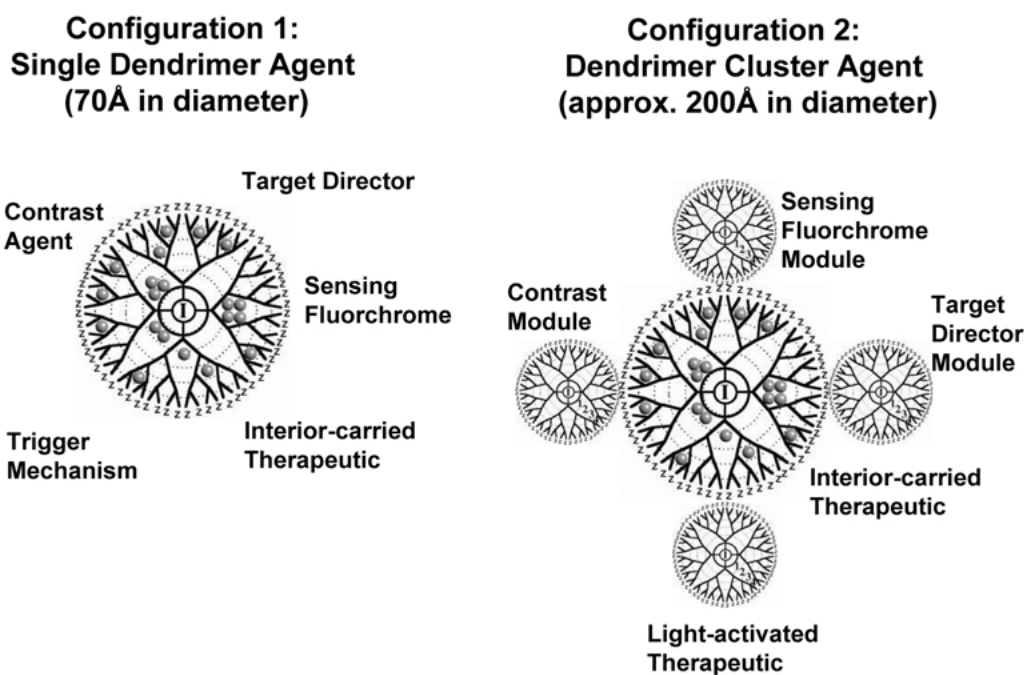

Fig. 4. *Two alternative designs for a dendritic nanodevice cancer therapeutic. Configuration 1 is a therapeutic agent built around a single dendrimer molecule. Configuration 2 is a cluster reagent where multiple dendrimers are clustered together, each providing a different functional unit. The advantage to this approach is that functional units can be mixed and matched in a combinatory method to address almost any type of cancer.*
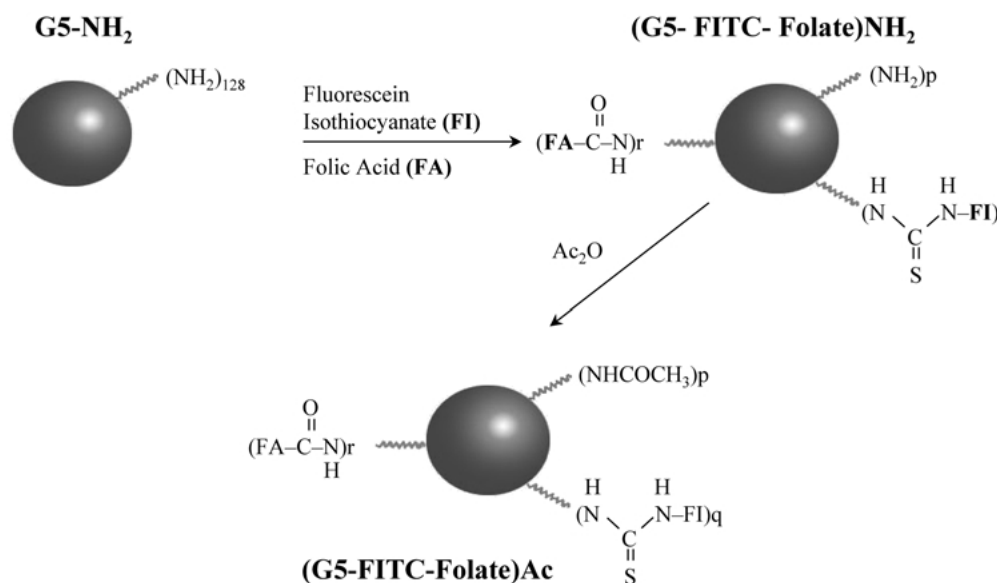
Fig. 5. *The attachment of fluorescein and folic acid to the surface of G5 dendrimers. This synthesis pathway yields molecules that are highly uniform and have about 20% fluorescein and 5% folic acid molecules substituted on their surface. The polymers then have remaining surface amines neutralized to the addition of acetamide groups.*

alterations in cancer cells would be identified using this technique, non-intrusive sensing through the dendritic molecules could be achieved and then employed automatically phenotype tumors. If the polymer cluster approach is employed, the targeting, sensing, and therapeutic conjugates can be interchanged to address varied tumor types or different pathophysiologic alterations. Thus, the cluster approach allows for common, interchangeable therapeutic platforms in a combinatorial manner that transcends any single type of tumor or cellular abnormality.

Synthesis and performance of a prototype target director module of an anti-tumor nanodevice

As an initial step in the development of a dendrimer-based therapeutic platform, we prepared molecules that had targeting (folate) and imaging (fluorescein) agents attached to the surface of amino terminated generation 5 PAMAM dendrimers (Figure 5).

To test the ability of the platform nanodevice to target drugs to cells, three cell lines used The first KB, a human epidermoid carcinoma, has high expression of folate receptors while NIH3T3, a murine fibroblast exhibiting minimal expression of these receptors. The third line was um/ssc38, a human squamous cell carcinoma line developed at the University of Michigan that lacks folate receptors. The KB cells were grown in folic acid deficient RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (final free folic acid

concentration 2 nM), and the other two cell lines in DMEM supplemented with 10% FA at a final free folic acid concentration 10 μM). About 100,000 cells/well were seeded in 12 well/plates the day before the experiments and one hour before start them, the cells were washed three times with PBS and 1ml of fresh medium with low folic acid (2 nM) was added to each well. The cells were incubated for 30 minutes (except for the time-course experiments) after the addition (10 μl/well) of known concentrations of dendrimer conjugates. After washing two times with cold PBS, the cells were harvested using trypsin EDTA (except in the experiments performed at 4°C, where the cells were harvested by scraping). After centrifugation at 4°C, they were finally suspended in 0.1% bovine albumin in PBS for flow cytometry analysis or confocal microscopy observation (the characteristics of instruments and methods were described in the previous report). In the antagonism experiments, free folic acid was added to the medium 15 minutes before the conjugates (or immediately after the incubation period). For the study of the evolution of the uptake process, after the incubation with the conjugates the cells were washed and fresh medium with low folic acid was added for an additional period (30 minutes to 24 hours).

Initial studies and characterization the G5-dendrimer conjugated with folic acid for the targeting of cells expressing the high affinity folate receptors were thwarted by non-specific uptake of the device, potentially
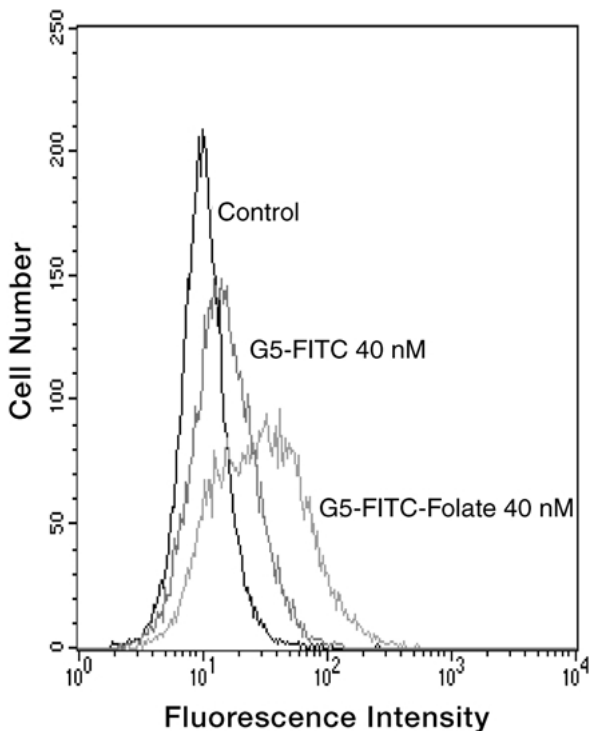
Fig. 6. *Uptake of amine surface dendrimers substituted with either fluorescein or fluorescein and folate. Note that there is increased uptake in the fluorescein and folic acid conjugates suggesting a receptor-mediated activity. However, there is also substantial non-specific uptake of the amine-terminated material, though the non-specific uptake required four hours to occur.*

mediated by interactions between positively charged surface amines on the dendrimer and negatively charged lipids on the cell membrane (Figure 6). To prevent this problem, neutral ''capping'' units employing acetamide or bis(2,3-hydroxypropyl)amine) (Figure 5) were substituted for the amines on the dendrimer surface. Using the G5 dendrimer conjugated with folic acid, which occupies 5% of the amino surface groups, with a 20% of these groups linked to FITC for detection, and the remaining 75% of the amino groups capped either by acetamide or dihydroxyl groups. A control G5 dendrimer with 20% of amino groups linked to FITC and the remaining 80% capped as indicated have also been used in our study.

Figure 7 illustrates the results of the concentration/uptake experiments, showing the progressive increases in cell fluorescence after 30 minutes incubation with increasing concentrations of the acetamide conjugate. Flow cytometric analysis (Figure 7, Plate B) demonstrates that the extent of cellular uptake of folate-conjugated dendrimers is dose dependent. Noteworthy, after incubation with 300 nM concentration of the control dendrimer, without folic acid on the surface, there was

only a slight increase in cell fluorescence, similar to or lower than the produced by 3 nM acetamide folic acid conjugate. Antagonism experiments in which free folic acid was added to the medium before the conjugates, revealed that free folate abolished uptake of the conjugates (data not shown). The relative rapidity of uptake, its characteristic dose-dependence and the fact that uptake is antagonized by free folate show that uptake of the folate-conjugated dendrimers was mediated by folate receptors of the KB cells. Confocal microscopy corroborates and extends these findings, by showing that even at high concentrations, control dendrimers (lacking folate on their surfaces) were not taken up by KB cells during the 30 minutes incubation. Interestingly, when G5-folate acetamide dendrimers were used most of the flourescence was in the vicinity of the cell membrane, a significant fraction of the flourescent signal seems to be inside the KB cells (Figure 7, Plate B), suggesting that the conjugates have been translocated across the cell membrane.

In another set of experiments we evaluated the time-course of the uptake process. As it is known, the potocytosis process, as the endocytosis through folate receptors, is relatively fast and it appeared that the receptors can recycle and bind new molecules of folic acid. In addition, confocal microscopy has shown a progressive internalization of the conjugates.

Moreover, the binding and uptake of the conjugates can be clearly and almost completely inhibited by a high concentration of free folic acid in the medium. As shown in Figure 7, the addition of 3 μM free folic acid, 15 minutes prior to the acetamide conjugate, prevented the increase of fluorescence observed with 10, 30 and 100 nM of this conjugate in a low folic acid medium. In contrast, as can be seen in the lower part of the figure, only a small reduction was observed when the free folic acid is added at the end of the incubation period. The confocal microscopy images, on the right part, are in complete concordance with the flow cytometry data: only the normal basal fluorescence of the KB cells can be seen when free folic acid was added prior to the conjugate, whereas, only a slight lower intensity is observed, when added after. This suggests that a substantial proportion of the acetamide conjugate has already internalized at the end of the incubation period, in agreement with the microscopic images.

Finally, insignificant increases of fluorescence were observed in NIH 3T3 cells after incubation with the acetamide conjugate, except for the highest, 300 nM concentration (data not shown). The small increase observed with 300 nM folic acid conjugate was higher than that of the control dendrimer and might correspond to a very low population of folate receptors, as has been described in pulmonary fibroblasts.
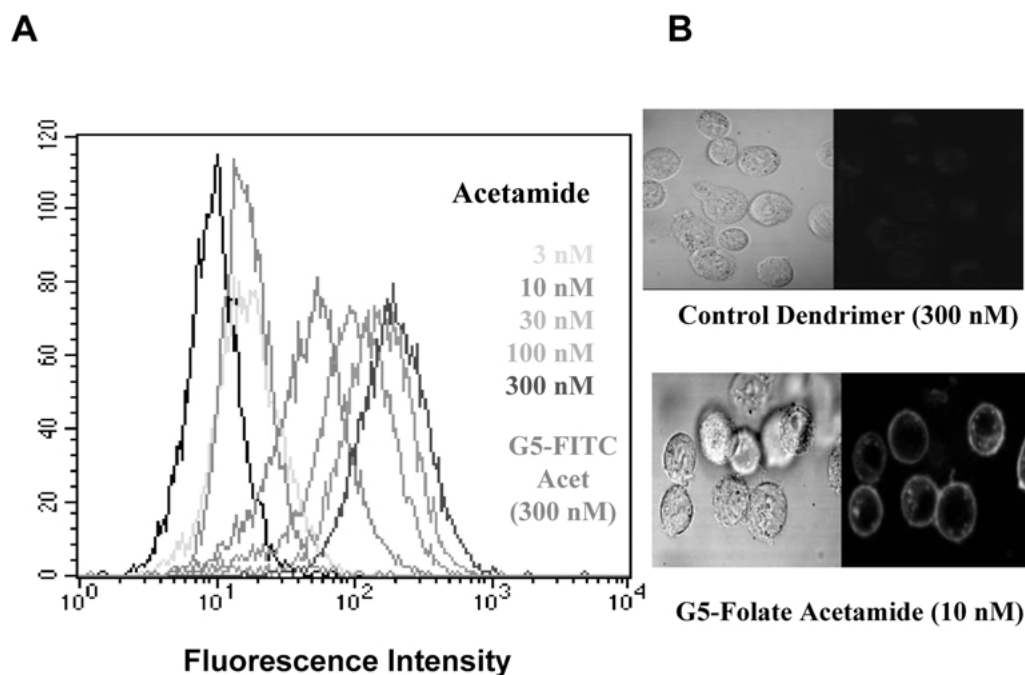
Fig. 7. *Specific, dose-dependent uptake of fluorescent, folate derivitized dendrimers by cells with folate receptors on their surfaces. Plate A: Flow cytometry revealed substantial and specific uptake of the polymer by KB cells. Not only can this uptake be increased by adding additional amounts of polymer, but it can be completely inhibited by pre-incubation of the cells with folic acid. This suggests that the uptake is entirely receptor mediated. The rate of uptake (complete within 30 minutes, as opposed to the four hours required for nonspecific uptake, as seen in Figure 6) is also consistent with a receptor-mediated process. Plate B: Confocal microscopy confirms that uptake requires by KB cells requires folate molecules linked to the dendrimer surface, again implicating receptor mediated uptake of the targeting module. Over the 30 minutes time course, the targeting modules (with folate on their surfaces) clearly label the membrane of KB cells, while control dendrimers (lacking folate on their surfaces) do not.*

Discussion

Nanotechnology offers a significant opportunity to enhance human health in novel ways. The therapeutic constructs made by nanotechnological expedients are potentially vastly more powerful therapeutic approaches than current treatment modalities, though, as illustrated here, nanotechnological drug entities themselves may also be substantially more complex than existing small molecule and protein therapeutics. We expect that burdens of cost associated with the relative complexity and sophistication of nanotherapeutics will be justified by their enhanced precision of therapeutic action, the previously unobtainable physiological information they provide to their clinician operators, and the enhanced patient outcomes their use will allow. Presumably, they will mitigate the patient morbidity and mortality associated with conventional therapeutics. Still, the argument for nanotechnological therapeutics is strongest in areas, such as oncology, where there is significant unmet patient need.

Modular approaches to nanodevice construction are (arguably) the most expiditious route to viable therapeutics in the near term. In this context, dendritic polymers are currently the structural nanomaterial of choice. Their ''tunable'' size, surface chemistry, charge, monodispersity, self-assembly properties and biocompatibility all augur well for their use in nanotherapeutics. Here we describe the use of dendritic polymers in the synthesis of a prototypic targeting module of an anticancer nanodevice. Clearly, the module is preferentially taken up and internalized by cell populations with folate receptor (a model tumor associated antigen used in our *in vitro* cell culture test system). Presumably, the intact nanodevice, were it covalently linked to the targeting module, would also localize to target cells and deliver its therapeutic and imaging payload with similar precision. The observation of perinuclear localization of the targeting module following uptake is provocative, and suggests the possibility that similar targeting modules might deliver some cytotoxic therapeutics directly to their nuclear site of action.

Although we have illustrated modular nanodevice construction in the context of an oncology application, the general strategy should be broadly applicable to construction of nanoscale devices. Furthermore, synthesis of the targeting module, and for that matter, the entire anti-tumor nanodevice can be accomplished by

standard synthetic and polymer chemistry methods. This bodes well for therapeutic nanotechnology: These methodologies allow large scale synthesis, in batch, which will be essential for any commercially-produced drug entity. Similar economies of synthesis will be essential for nanodevices that perform non-therapeutic tasks (computing, chemical synthesis, environmental remediation, etc.). We believe synthesis of nanodevices from modules synthesized using wet chemical methods is a viable route to powerful nanotechnology in the near future.

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