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Expert Review

Encapsulation of Nucleic Acids and Opportunities for Cancer Treatment

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Abstract. The development of nucleic acid drugs for the treatment of various cancers has shown great promise in recent years. However, efficient delivery of these drugs to target cells remains a significant challenge towards the successful development of such therapies. This review provides a comprehensive overview of encapsulation technologies being developed for the delivery of nucleic acid-based anticancer agents. Both micro and nanoparticles systems are discussed along with their use in delivering plasmid DNA as well as oligonucleotides. The majority of the systems discussed have used DNA immunotherapy as the potential mode of anticancer therapy, which requires targeting to antigen presenting cells. Other applications, including those with oligonucleotides, focus on targeting tumor cells directly. The results obtained so far show the excellent promise of encapsulation as an efficient means of delivering therapeutic nucleic acids.

KEY WORDS: encapsulation; cancer; DNA; gene; particles.

INTRODUCTION

One of the continuing problems related to cancer chemotherapy (be it in the adjuvant or metastatic setting) has been the relatively high *toxicity* of treatments combined with the relatively low *specificity* of drug therapy, which collectively limit the overall antitumor efficacy. Over one-third of those diagnosed with cancer will die from it, and their treatment may be long, painful and with side effects that seriously alter their quality of life. There is a great deal of discussion as to whether or not some cancers originate with genetic errors, either hereditary or environmentally triggered. Often cells can create their own short interfering RNA (siRNA) that will ultimately silence or destroy mutated genes (1). Recognition of genetic changes in cancer cells will yield opportunities to identify, repair or destroy those cells as desired. Repair of errors in oncogenes (mutations and rearrangements), tumor suppressor genes (correcting inactivating mutations) or DNA pathway repair genes (inactivating mutations) are ideal challenges for delivery systems incorporating nucleic acids (2).

This review will address many recent advances in encapsulation of nucleic acids, primarily in synthetic polymer micro- and nanoparticles, and look at opportunities for the use of these systems in the treatment of cancer. Some of the work described has already evaluated the particle systems developed for their utility in cancer therapy, but some have not yet advanced to that state and still others have not yet taken that step to move to the cancer arena. However, it is hoped that they will soon or that others can also see the opportunities evident in this review. However, before assuming that all particle-based systems may easily be used in cancer therapy, it must be realized that there are a number of barriers to transport within tumors. As has been extensively reviewed by Jain and others, transport of particles into solid tumors involves passage through the microvascular wall and also through the interstitial compartment (3–7). However, due to the enhanced permeability and retention effect and other physiologic factors, nanoparticles will accumulate within tumors, delivering up to four times the amount of chemotherapeutic drug, compared with systemic administration, to tumors based on *in vivo* studies, including human trials (8–11).

IMPROVING GENE TRANSFER TECHNOLOGY

The field of gene therapy has developed as the molecular basis of many diseases became evident and the ability to manipulate genetic material in the laboratory improved. This ever-broadening field is generally defined as the transfer of genetic material with therapeutic intent. Since the first human *in vivo* gene transfer study in 1989 (12), over 900 clinical trials involving gene transfer have been approved by regulatory bodies worldwide.

The most obvious application of gene therapy is correction of inherited genetic diseases, such as the hemoglobinopathies, immunodeficiency syndromes, and metabolic disorders. Indeed, gene therapy clinical trials are ongoing for patients with adenosine deaminase deficiency ("bubble baby syndrome"), cystic fibrosis, hemophilia, chronic granuloma-

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tous disease, and other genetic diseases. Over the past two decades, investigators have realized that gene transfer technology can provide novel approaches to a large variety of illnesses not traditionally thought of as genetic disease.

In fact, over two thirds of approved clinical gene therapy trials are for cancer. A wide variety of anti-cancer strategies are being evaluated (13). A few examples include oligonucleotides, ribozymes and now siRNA to disrupt or alter gene expression of oncogenes. Vectors expressing drug resistance genes have been explored as a means of increasing the ability of marrow to tolerate chemotherapy. Tumor vaccines use gene transfer technology to express antigens or cytokines in order to elicit an immune response against autologous tumor cells. More recently, T cell receptors engineered to recognize tumor antigens have been incorporated into vectors and used to re-program T cells to now recognize autologous tumor.

The term gene therapy vector refers to a system designed to transfer genetic material into a target cell. The simplest systems are vectors composed of naked DNA, usually in the form of plasmid DNA. Plasmids are designed to contain the gene of interest and regulatory elements that enhance gene expression (14). Plasmid vectors are limited by low gene transfer efficiency and are not well suited to systemic administration as the DNA may be degraded before sufficient material is exposed to the target tissue. To address these limitations, investigators have engineered a diverse number of viruses to transport genetic material, each with their own advantages and disadvantages. For example, retroviral and lentiviral vectors integrate into target cells so are ideal for target cells that will multiply many times since the vector will be passed to all daughter cells. Adenoviral and adeno-associated viral vectors can be made at high titer and can express proteins at very high levels. Unfortunately, the vector generally does not integrate and is usually lost as the cell divides. As viral vectors have moved into the clinic, a number of limitations have been recognized. First, immunologic recognition of viral proteins by the innate immune system can limit gene transfer and has been associated with severe adverse reactions, including death (15). Insertional mutagenesis of integrating vectors leading to leukemia has now been reported, although the vector transgene in this situation is believe to contribute to the pathogenesis of the malignancy (16,17). Furthermore, immune reactions to vector administration are likely thereby limiting the potential for repeat administration. Given these limitations there has been renewed enthusiasm for plasmid vector based on their favorable safety profile, if novel approaches aimed at improving delivery can be found.

Various non-viral delivery methods have been developed throughout the years to help mediate nucleic acid transport into cells. Liposomal delivery of nucleic acids has been used for several years, and has been previously reviewed (18). The recent developments of cationic lipids have provided a new tool for delivery through cell endocytosis. Although effective at transfecting into the cell, these lipoplexes, as they are called, do not effectively protect nucleic acids from degradation within endosomes once in a cell. Combinations of cationic liposomes with lipids such as DOPE, which can facilitate release of the complex from the endosome, have demonstrated improved possibilities for delivery. However, drawbacks of lipid-based delivery include low-modifiability, difficulty targeting, and little control of degradation rate.

Natural polymers have also been studied as possible vehicles for gene transfer and include materials such as chitosan, cellulose, and gelatin. The main advantage of natural polymers is improved biocompatibility, however it is often difficult to modify natural polymers as well as limitations on large-scale production. Modification of natural polymers can also be quite difficult. Some promise has been shown in the development of PEG-modified gelatin nanoparticles for the tumor-targeted delivery of plasmid DNA. Particles were produced averaging 200 nm in diameter with nearly 100% encapsulation efficiency. An *in vitro* transfection efficiency of 61% was achieved in NIH-3T3 cells, while *in vivo* studies with i.v. and i.t. delivery showed increases of nearly 42% and 56%, respectively, in delivery (19). Other natural polymers such as chitosan will be discussed later in this review.

PARTICULATE DELIVERY SYSTEMS

An excellent review by Hoffman *et al.* describes the challenges that are faced with delivering genetic material to cells of interest as well as those polymers that are being studied to achieve this (20). While microparticles have been prepared using PLA and PLGA for many years, nanoparticles of these materials are fairly new and are the result of modifications of existing preparation techniques and the realization that sub-micron particles could find utility in particular drug targeting applications (21). Our research group is one of the few to have published work on optimization of such preparation techniques (22) and has scaled up production of such nanoparticles from 100 mg per batch to 100 g per batch. It has been found that unmodified PLA nanoparticles injected intravenously are taken up by cells of the mononuclear phagocyte system, mainly the Kuppfer cells (23). This may naturally concentrate these particles close to liver parenchymal cells and facilitate biliary clearance and enterohepatic circulation. In general, such nanoparticles, without surface modification, are rapidly cleared from the blood and are concentrated in the liver, spleen and bone marrow. Unmodified nanospheres of PLGA (75/25) can be prepared especially for site-specific delivery based on their size (24). Biodistribution of injected colloidal carriers is highly dependent upon their size and their surface properties. For example, for targeted administration to the lung, particles should be several microns in diameter. Modification of the surfaces of colloidal particles with PEG will modify the uptake of particles and reduce immediate liver sequestration. Simply because of their size and the vascular structure surrounding tumors, some particles will usually concentrate in tumor vasculature. This has become known as the enhanced permeability and retention effect (EPR) (25). However, many groups, including our own, are attempting to develop more specific targeting to cancer tissue to avoid even more side effects due to active agents going to healthy tissue.

As technology and knowledge has advanced into the nano-scale, more research groups have addressed targeted drug delivery, especially for cancer treatment. A number of researchers are evaluating using vitamin B12 and folate (26)

which have shown a four-fold increase in targeting of HPMA nanoparticles to hydbridoma cells in mice (27). Biotin-conjugated particles of biotinylated pullulan acetate can preferentially target to hepatic carcinoma cells over fibroblast cells (28). The permeability of tumors to particle uptake appears to be dependent not only on the specific targeting agents, but also on the particle surface charge. An analysis of liposome uptake has shown that the adenocarcinoma tumors and melanoma tumors studied preferentially took up cationic liposomes over anionic and neutral liposomes (29). Opportunities with targeted delivery using ligands and including steric coatings have only recently been explored and have still not shown their full potential (30).

It is important to note that many of the studies reviewed in this paper focus on *in vitro* studies of the delivery systems. The use of *in vitro* studies has long been a tool to determine the potential of delivery mechanisms for drug therapies, and this is also the case with systems for gene transfer. The stability of nucleic acids is of key importance within cellular environments, and thus can be studied through the use of agarose or polyacrylamide gel electrophoresis. Loading studies are also necessary to determine how effectively nucleic acids can be loaded onto the particles, and is a determining factor in feasibility of a particulate system long term. Sizing and zeta-potential studies are also key in helping to understand mechanisms that will be in play during *in vivo* transfection, and also for possible side effects that may occur. In vitro cell studies are also often important to determine the types of cells that the system can penetrate and deliver the nucleic acids to efficiently, as many cell types differ in transfection characteristics. Use of data obtained from *in vitro* studies can provide guides for developing *in vivo* animal studies.

PLASMID ENCAPSULATION

Microparticle Encapsulation of Plasmids

Most gene delivery systems utilizing polymers that have been studied are those where a polymer-DNA complex, liposomes, or other lipid-based systems are formed (31–38). A key point in many of these systems in achieving DNA stabilization and complexation is having a cationic polymer. A recent analysis of polyethylenimines (PEI), which are often studied as a complexing agent for gene delivery because of the high positive charge density, has failed to elucidate, based on physico-chemical data, their predicted performance as transfection agents (31). Transfection has been shown to be up to 200% of that of the DNA alone (33). In evaluating the ability to condense the DNA for enhanced stability, polymethacrylates have also been studied and it was found that those polymers containing only tertiary amine functional groups could perform similarly to PEI, but the presence of pyridine groups, acid functions and imidazole groups were all detrimental to stabilizing and encapsulating DNA (39).

Even more exciting is the fact that particles prepared from cationic lipids and PEG have shown an order of magnitude greater DNA expression *in vivo* than similar DNA complexes (35). Research with biodegradable particles for DNA delivery began with microparticles and has advanced to the point where long-term *in vivo* delivery and processing stability have been studied (40–42). Encapsulated DNA from PLGA microparticles that have been administered to mice either intravenously or subcutaneously can be detected up to 100 days post-injection (41). The potency of two different plasmids was analyzed using three different techniques (cell transfection assay, *in vitro* transcription/translation system and bacterial transformation assay) and it was found that the encapsulation procedure did not affect the potency of the encapsulated DNA. In addition, the lyophilized formulations could be stored at refrigerator or freezer temperatures for at least 90 days with no loss of DNA activity (40).

To counter the barriers experienced by plasmid DNA, polymeric micro- and nanoparticles have emerged as viable options to serve as non-viral delivery mechanisms. PLGA microspheres have been shown to provide prolonged transgene expression through the use of pDNA encapsulated within the microspheres. PLGA-DNA microparticles produced with diameters less than 5 μm and encapsulation efficiencies of over 50% showed burst release rate followed by a slow sustained release. Sustained release of the PLGA microparticles is attainable compared to largely declining release rates seen with lipid delivery despite the significantly higher initial transfection rates. Use of PLGA has been shown to show a sustainable release of pDNA in a stable and bioactive form (43). Poly-β amino-ester (PBAE) and PLGA combinations provide a modified pH sensitive and degradable microparticle that increased transfection efficiency of pDNA three to five-fold compared to plain PLGA and 25-fold compared to liposomal delivery along with encapsulation efficiencies as high as 78%, while also providing activation of dendritic cells (44).

An important aspect of delivery of DNA vaccines is the need to direct the plasmid DNA to antigen-presenting cells (APC) to provide improved immunogenic response. Microparticles provide an ideal non-viral vector for delivery based on targeting ability, degradability, facile modification, and lack of an immune response to the polymers. Previous studies have shown the use of poly lactic-co-glycolic acid (PLGA) as a viable degradable delivery system, however slow release rates of greater than 10 days, or the lifetime of the dendritic cell, after uptake into targeted cell (45). Lack of endosomal escape of PLGA microparticles (46) and changes in local pH due to degradation of the microparticles (47) has also been demonstrated and reduces bioavailability and bioactivity of the plasmid DNA. Therefore, a need for novel microparticles and modifications has become necessary to provide improved efficacy for their use as a delivery vehicle for plasmid DNA vaccines. Improved efficacy of DNA vaccines has also been established through the use of PLGA microparticles demonstrating the ability to immunize organisms for specific tumor cell lines (48).

The use of cross-linked microparticles has also been studied as a possible non-viral delivery mechanism for plasmid DNA vaccines. Recently, polyacrylamide microspheres were produced with sizes ranging from 0.2 to 1 μm in diameter, which is an appropriate size for targeting to APCs. These cross-linked microspheres were prepared via modified reverse microemulsion polymerization. Encapsulation efficiencies ranged from 44 to 54% and pDNA was shown to remain stable and mostly in super coiled form. DNA transcription activity was shown to be similar whether

transfected with the particles or lipofectamine 2,000, thus demonstrating effective delivery of active plasmid and protection from degradation. Control of release by pH was shown with minimal release of DNA at pH 7.4, while at pH 5.0 there was complete release within 2 days. Through the use of bacterial DNA plasmids, ELISA studies showed that immunostimulation 40-fold higher than seen with naked DNA was achieved through the use of these particles. These particles have demonstrated promise as delivery mechanisms for plasmid DNA and triggering the appropriate mechanisms for effective use (49).

Surface complexation of plasmids rather than encapsulation has provided some interesting results for delivery. Branched polyethylenimine (PEI) conjugated to the surface of PLGA microparticles for delivery of a plasmid has been shown to direct APCs. Surface conjugation of the cationic polyethylenimine improves transfection and endosomal escape of active molecules in cells. This combination has been demonstrated to provide effective and biodegradable delivery of not only pDNA, but also simultaneous delivery of immunomodulatory agents that can improve targeting and transfection. Surface conjugation of the PEI to the PLGA microparticles provided a mean zeta-potential of approximately 35 mV as compared to surface adsorbed PEI particles, which have zeta-potentials ranging from 10 to 24 mV. PEI-conjugated PLGA microparticles also served to improve pH buffering, which through use of the proton sponge characteristics of PEI, allowed for early endosomal escape. Cytotoxicity of the combination particles also showed reduction when compared to pure PEI up to concentrations of 1 mg/ml of particles (pDNA doses up to 2 μg/ml). Plasmid DNA loading efficiencies ranged from 70 to 90% for 70 kDa PEI and 50 to 80% for 25 kDa PEI. Transfection efficiencies of pDNA to APCs demonstrated an 8-fold increase in comparison to PLGA encapsulated plasmids (50).

A summary of the results from those publications that provided critical formulation information such as particle size, encapsulation efficiency and release performance is shown in Table I.

Nanoparticle Encapsulation of Plasmids

Specific work with nanoparticles for gene delivery is more limited but also quite promising. Some of the work uses adsorption on to nanoparticlesas seen with oligonucleotides on polyalkylcyanoacrylates nanoparticles (51). Several studies for for adsorption of DNA to nanoparticles include combinations of either PEG and poly(L-lysine) (PLL) that can target HepG2 cells *in vitro* and PLA with polysaccharide-graft-PLL polymers that can be made as small as 60 nm in diameter (52). A number of these studies discuss difficulties that are confronted when encapsulating the DNA, which has lead to concentration on adsorbing the DNA to the particles. For preparing polymeric gene delivery carriers, the polymer of choice is polyethylenimine (PEI), however it is not biodegradable. This polymer and similar ones will electrostatically associate with plasmid DNA due to their cationic nature (53–55).

However, in many applications, the plasmid must be encapsulated within the nanoparticles. Biodegradable nanoparticles based on PLGA have been studied for delivery and targeted therapy for conventional drugs, proteins, peptides as well as DNA (56). As DNA can have a diameter or 100 nm when relaxed, it should be encapsulated in the supercoiled state. In the past few years the amount of work published on DNA encapsulation in PLGA microparticles and nanoparticles has increased dramatically (57–61). Encapsulation can be achieved through judicious use of methods to protect the DNA during particle preparation and to enhance the encapsulation efficiency. Nanoparticles of 300–700 nm diameter utilizing human serum albumin as well as PEI showed low toxicity and transfection efficiencies for human epithelial kidney cells approximately the same as that of Superfect™ and DOTAP™ (62). Some useful analyses of encapsulation methods have shown that higher molecular weight PLGA will encapsulate DNA effectively (up to 3 wt.%) and that particles with lower amounts of surface-associated PVA (from the preparation of the nanoparticles) will then show a higher transfection (63). Cellular analyses of DNA delivery

Table I. Summary of Formulation Variables and Performance Characteristics for Plasmid Microparticles

Group	Polymer	Particle Size (Diameter)	Encapsulation Efficiency	Zeta Potential (mV)	Sustained Release (Time)	Degradable	Targeted Cancer
Tinsley-Bown *et al.* (42)	PLGA	Mode 2.9–3.2 μm	19–54%	N/A	>35 days	Yes	None
Lunsford *et al.* (41)	PLGA	Mean 5 μm	33–50%	N/A	Up to 100 days post injection	Yes	Cervical (via HPV)
Stern *et al.* (43)	PLG	80%<5.2 μm	50.7±2.3%	N/A	>21 days (Gene Expression)	Yes	No: Cystic Fibrosis
Little *et al.* (44)	PBAE	Mean 5.53 to 6.01 μm	68–78%	−0.8-60.46 mV	No data given	Yes	SIY-expressing Tumors
Goh *et al.* (49)	Polyacrylamide	0.2–1.0 μm	44–54%	No data given	>24 h	Yes	No, but applicable to DNA Vaccines
Kasturi *et al.* (50)	PEI-PLGA	85%<10 μm	Surface Loaded >25-fold compared to PLGA	+6.0– +7.5 mV	>21 days	Partially	B Lymphoma

from these nanoparticles (400–700 nm) showed release for at least 3 days.

Some research groups, including some of the best-known drug delivery researchers in the US, have been working to encapsulate DNA in PLGA and PLGA-co-PEG recently. They have been able to prepare nanoparticles less than 150 nm in diameter with 15–50% plasmid incorporation. The final formulation is dependent upon the initial plasmid concentration and the type of polymer used. In order to encapsulate the DNA in the coiled state, cationic lipids were utilized as excipients and condensing agents during the nanoparticle preparation. The nanoparticles showed *in vitro* release for as long as one month with no initial burst (64). However, their final conclusions were that addition of condensing agents was not necessary if the organic solvent used was a 1:1 ratio of ethyl acetate:methylene chloride. Another group arrived to the same conclusion, while also adding optimized amounts of Span 80 and Tween 80 as surfactants (65). Other studies have prepared nanoparticles of poly(lactic acid)-co-poly(ethylene glycol) smaller than 300 nm in diameter where plasmid DNA was either encapsulated alone or co-encapsulated it with poly(vinyl alcohol) or poly(vinylpyrrolidone). These techniques have achieved up to 90% encapsulation efficiency. Their analyses included characterization of the particle size, zeta potential, DNA loading and *in vitro* release. Release could be detected for up to 28 days and did have an initial burst of release. The most important parameters in achieving longer release and minimizing the initial burst were the plasmid loading and the type of emulsion used for the nanoparticle preparation (66).

As is often the case in preparing controlled release systems, several groups have found that high loading efficiencies of up to 66% can be achieved but only with a final plasmid loading of 1–2 wt.% and the use of either methylene chloride or ethyl acetate as the organic phase solvent (42,67). Use of cryopreparation and carbohydrate stabilization has achieved over 85% loading efficiency of plasmid DNA into microparticles of PLGA (68). Also, utilizing triblock copolymers of PLGA-PEG-PLGA to encapsulate PEI-DNA complexes resulted in increases in transfection efficiency more than 10-fold over that of the PEI-DNA complexes alone (69).

Another material that has attracted considerable interest in the formulation of DNA is chitosan. Chitosan is a natural, non-toxic polysaccharide that is biocompatible and biodegradable and will complex well with DNA and protect it from nuclease degradation. For chitosan, parameters such as molecular weight and degree of deacetylation can have an effect on encapsulation efficiency and ultimate performance of chitosan-based formulations (70–72). Bozkir and Saka recently performed several studies in which they found that nanoparticles prepared using a chitosan with a high deacetylation degree could yield 90% or better encapsulation efficiency (73). Release profiles showed *in vitro* delivery for 24 h for particles prepared using complex coacervation and 96 h for those prepared using evaporative techniques. The opportunities with PLGA and chitosan have also spurred researchers to include both materials in the same formulation. Lehr and associates have prepared PLGA nanoparticles using a PVA-chitosan complex to stabilize the nanoparticles (74). Depending on the method used, the resulting particles could be 200, 400 or nearly 900 nm in diameter. In this case, DNA was then adsorbed onto the particles instead of being incorporated within the nanoparticles.

As was presented for microparticles, a summary of the results from those publications that provided critical formulation information such as particle size, encapsulation efficiency and release performance for nanoparticle systems is shown in Table II.

ANTISENSE ENCAPSULATION

Another method for the use of nucleic acids as a pathway to fight cancer is through antisense technologies, which includes the use of both DNA oligonucleotides (ODNs) and short interfering RNA (siRNA). These technologies focus on the suppression of gene expression, rather than initiating expression of a gene. Short nucleic acids prevent gene expression in a cell by blocking translation of mRNA for a specific gene (75). This can be achieved very effectively through the use of oligodeoxynucleotides (76–80) and siRNA (81). Several issues that arise in delivery and targeting of antisense oligonucleotides include degradation of oligonucleotides by nuclease and other enzymes, inadequate bioactivity within cells, and targeting to appropriate cells (82). Despite modifications such as phosphorothioate backbones to improve stability within cells (83), targeting and improved bioactivity require delivery through the use of vectors such as microparticles or nanoparticles. Viral vectors offer high transfection rates for antisense oligonucleotides, but as previously stated are oncogenic and immunogenic and thus not effective for multiple treatments. The need for polymeric delivery vehicles has therefore become a very important aspect of delivery for antisense therapies.

The use of conventional polymers such as PLGA has been shown as potential vehicles for delivery of oligonucleotides. In 2005, Kilic *et al.* demonstrated the use of PLGA nanospheres to deliver antisense oligonucleotides to the

Table II. Summary of Formulation Variables and Performance Characteristics for Plasmid Nanoparticles with Potential for Use in Cancer Therapy

Group	Polymer	Particle Size (Diameter)	Encapsulation Efficiency	Zeta Potential (mV)	Sustained Release (Time)	Degradable	Targeted Cancer
Hirosue *et al.* (64)	PLGA	<150 nm	15–50%	N/A	>30 days	Yes	No
Perez *et al.* (66)	PLA-PEG	Range 130–300 nm	>80%	−19–−33 mV	>28 days	Partially	No
Bozkir and Saka (73)	Chitosan	Range 450–820 nm	85–96%	+9–+34 mV	>4 days	Partially	No
Kumar *et al.* (74)	PLGA & Chitosan−PA	Mean 180 nm	No data given	+10 mV	No data given	Yes	No

receptors specifically in the brain, demonstrating the ability to target delivery simply using a biocompatible and biodegradable polymer like PLGA. The homogeneous nanospheres had a mean diameter of 250–300 nm depending on type of PLGA and stabilizer concentration. Encapsulation efficiencies ranged from 13 to 43%, however zeta potential was not determined. Release characteristics of the optimized nanospheres and loading protocol led to a near linear release of the antisense oligonucleotide with a small burst effect (<3%) and a cumulative 60% release after 21 days, and gel electrophoresis demonstrated that oligonucleotides remained stable throughout the synthesis and release process (84). The potential of PLGA nanospheres for delivery increases were based on the available sites for modification (such as attachment of targeting antibodies) along the surface of the uncapped PLGA nanoparticles.

Polyalkylcyanoacrylate nanocapsules and nanospheres have also recently been developed as delivery vectors for antisense oligonucleotides. Nanocapsules were prepared using a modified water-in-oil emulsion and interfacial polymerization to allow for effective production of particles. Nanoparticles produced ranged in size from 20 to 50 nm or 150 to 350 nm with a mean zeta potential of −40 mV. Loading efficiencies were compared to nanospheres with ODN adsorption which had a limit based on saturation of the surface. Nanocapsule encapsulation efficiency was largely dependent on initial loading levels. Degradation of oligonucleotides was reduced by 5-fold in nanocapsules providing more control through release kinetics and reducing the required dosage of ODN by as much as 35-fold. Tailored delivery of cancer therapy antisense ODNs can be effectively delivered through the use of such nanocapsules (85).

In 2004, Hollins *et al.* published their study on the use of poly(propylenimine) dendrimers as a vehicle for delivery of antisense oligonucleotides. Their studies showed that the use of this polymer allowed for effective transfection of antisense oligonucleotides into cells that was comparable to that of cationic polymeric delivery vehicles. They also demonstrated that lower toxicity could be achieved with this polymer due the efficacy of delivery for lower generations as compared to poly(amidoamine) dendrimers (86). Use of poly(propylenimine) dendrimers was also established with cancerous cell lines by Santhakumaran *et al.* in 2004. In this study, hydrodynamic radius of the dendrimers ranged from near 130 nm to over 280 nm based on the generation (from 1 to 5) and concentration. Uptake of the polymer/ODNs varied based on the number of terminal amino groups (which determines positive surface charge) and molecular weight of the complex. Almost no cell death was noted for any of the concentrations and generations of dendrimer studied by this group, showing a very high biocompatibility with individual cells. Optimal efficiency was achieved with fourth generation dendrimers (87,88).

Similar to DNA plasmids, both oligonucleotides and siRNA are anionic allowing for the formation of complexes with cationic polymers such as PEI, chitosan, polyamidoamine (PAMAM) dendrimers, polyphosphoesters, and many more. A recent study by Weyermann *et al.* determined the efficiency of cationic acrylic nanoparticles and protimine based nanoparticles for the delivery of antisense oligonucleotides. Polyalkylcyanoacrylate nanoparticles were produced with zeta potentials ranging from 21.7 to 28.1 mV, mean particle diameter of 313–327 nm, and mean loading efficiencies over 96%. Although efficient transfection was noted through this vehicle, cytotoxicity was a grave concern at effective concentrations (88). Studies on albumin-protimine-oligonucleotide nanoparticles produced particles with a mean diameter of 285 nm, a mean zeta potential of −12.3 mV, and a mean loading efficiency of over 96%. These protimine based nanoparticles showed comparable efficacy to the liposomal delivery, but had minimal cytotoxic effects (89).

The cationic polymer polyethylenimine (PEI) has been heavily studied for its application as a delivery vector for nucleic acids, and not only has cationic properties to form stable complexes with DNA easily. Together with highly positive zeta potential due to surface amine groups and high buffering capacity, PEI allows for relatively high transfection and protection of the nucleic acids during the process. The characteristic "proton sponge" effect that is found in PEI also offers a mechanism for release of the ODN under certain conditions (pH controlled release). In 2006, Seong *et al.* demonstrated the use of linear PEI-oligonucleotides complexes in order to deliver anti-IL-4 antisense oligonucleotides. Spherical complexes were produced with a mean diameter of 98 nm, and showed resistance to degradation of ODNs by DNase I. Complexes demonstrated a 30% reduction in IL-4 expression compared to less than 1% seen with naked oligonucleotide along with significant reduction in IgE

Table III. Summary of Formulation Variables and Performance Characteristics for Antisense Oligonucleotide Microparticles and Nanoparticles with Potential for Use in Cancer Therapy

Group	Polymer	Particle Size (Diameter)	Encapsulation/ Loading Efficiency	Zeta Potential (mV)	Sustained Release (Time)	Degradable	Targeted Cancer
Kilic *et al.* (84)	PLGA	Range 250–310 nm	13–43%	No data given	>21 days	Yes	No
Lambert *et al.* (85)	Polyalkylcyano-acrylate	Range 150–350 nm	81±8%	No data given	3 h	Yes	Sarcoma
Santhakumaran *et al.* (87)	Poly(propylene-imine) dendrimers	Range 130–280 nm	No data given	No data given	>48 h	No	Breast Cancer
Weyermann *et al.* 2005 (89)	Polyalkylcyano-acrylate	Mean 313–327 nm	>96%	21.7–21.8 mV	No data given	Yes	No
Seong *et al.* (90)	PEI	Mean 98 nm	No data given	No data given	No data given	No	No
Gao *et al.* (91)	Chitosan	Mean 80 nm	No data given	+13.7 mV	No data given	Partially	No

levels. Despite the high transfection efficiency and low immune response, toxicity due to use of PEI is still a serious issue along with non-degradablibilty (90).

Other cationic polymers, such as the polysaccharide chitosan, have shown to provide an effective vehicle for delivery of antisense oligonucleotides. A recent study by the Gao group demonstrated the use of chitosan to efficiently deliver antisense oligonucleotides to hepatic cells, illustrating the versatility of the chitosan with its availability of modification sites. The nanoparticles were homogenous and had a mean diameter of 80 nm based on AFM, a mean effective radius 253.8 nm, and a mean zeta potential of 13.7 mV. Transfection efficiencies were lower than lipofectin, but significantly higher than naked DNA. Cytotoxicity of the modified chitosan was minimal even at concentrations of 1,400 μg /mL as compared to the severe cytotoxicity of lipofectin at concentrations above 250 μg/mL (91).

A summary of important formulation and performance parameters are given in Table III for antisense oligonucleotide-containing particle systems.

IN VIVO ANALYSES

Few studies are found in the literature which have progressed to the point of *in vivo* evaluation for the types of systems described in this review. Based on the work to date, this should be changing rapidly. Several *in vivo* studies have been performed to determine the efficacy of polymeric delivery vehicles for DNA vaccines to help fight cancerous tumors. Kasturi *et al.* demonstrated that the use of PEI-conjugated PLGA microspheres for delivery of the MCP3-sFv20 pDNA vaccine through both intradermal and intramuscular injection were able to immunize mice at significantly higher rates compared naked vaccine, as well as comparable to gene gun when injected intramuscularly (92). This trend was also seen with polymeric delivery vehicles such as PBAE-PLGA particles encapsulating pCMV- SIY pDNA vaccine, which were injected intradermally, significantly reduced tumor growth rate as compared to plain PLGA microparticles and PBS controls (44). Other than vaccines, several other areas of nucleic acid delivery

Table IV. Summary of Particles for Potential Nucleic Acid Delivery in Cancer According to Physicochemical Nature of Carrier

Particles for Potential Nucleic Acid Cancer Vaccine Delivery (Target Antigen Presenting Cells)				
Microparticles for Encapsulation of Plasmids				
Group	Polymer	Particle size	Biodegradable	Encapsulation Efficiency
Tinsley-Bown *et al.* (42)	PLGA	Mode 2.9–3.2 μm	Yes	19–54%
Lunsford *et al.* (41)	PLGA	Mean 5 μm	Yes	33–50%
Stern *et al.* (43)	PLG	80%<5.2 μm	Yes	50.7±2.3%
Little *et al.* (44)	PBAE	Mean 5.53–6.01 μm	Yes	68–78%
Goh *et al.* (49)	Polyacrylamide	0.2–1.0 μm	Yes	44–54%
Combinatorial Encapsulation and Complexation of Plasmids				
Group	Polymer	Particle size	Biodegradable	Loading Efficiency
Kasturi *et al.* (50)	PEI-PLGA	85%<10 μm	Partially	Surface Loaded >25-fold compared to PLGA
Particles for Potential Nucleic Acid Delivery to Tumoral Cells				
Nanoparticles for Encapsulation of Plasmids				
Group	Polymer	Particle size	Biodegradable	Encapsulation Efficiency
Hirosue *et al.* (64)	PLGA	<150 nm	Yes	15–50%
Perez *et al.* (66)	PLA-PEG	Range 130–300 nm	Partially	>80%
Nanoparticles for Delivery of Oligonucleotides				
Group	Polymer	Particle size	Biodegradable	Encapsulation/Loading Efficiency
Kilic *et al.* (84)	PLGA	Range 250–310 nm	Yes	13–43%
Lambert *et al.* (85)	Polyalkylcyano-acrylate	Range 150–350 nm	Yes	81±8%
Weyermann *et al.* 2005 (89)	Polyalkylcyano-acrylate	Mean 313–327 nm	Yes	>96%
Nanoplexes				
Group	Polymer	Particle size	Biodegradable	Loading Efficiency
Seong *et al.* (90)	PEI	Mean 98 nm	No	No data given
Gao *et al.* (91)	Chitosan	Mean 80 nm	Patially	No data given
Santhakumaran *et al.* (87)	Poly(propylene-imine) dendrimers	Range 130–280 nm	No	No data given
Kumar *et al.* (74)	PLGA & Chitosan-PA	Mean 180 nm	Yes	No data given
Bozkir and Saka (73)	Chitosan	Range 450–820 nm	Partially	85–96%

have also been studied *in vivo* to determine efficacy and combating cancerous tumors. Novel development of poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) was demonstrated to deliver plasmid DNA (mIL-12) encoding for the IL-12 into CT-26 colon adenocarcinoma tumor challenged Balb/c mice showed improved suppression of tumor growth and prevention of metastasis when compared to naked mIL-12 (93). *In vivo* studies have not been limited to plasmid DNA delivery, and have also focused on delivery of siRNA to combat tumor cells. PEI pegylated with RGD, which was used to deliver both pLuc and anti-pLuc siRNA through injection intravenously into the tail vein, showed significant improvement in targeting to N2A tumor cells and increased suppression of luciferase activity when compared to plain PEI nanoparticles in female nude mice (94). Overall, *in vivo* studies have shown promising results in the development of non-viral vectors for gene therapy of cancer, and provide further notice on the possibilities that either micro- or nanoparticles created from polymers are a key component to improved treatment of cancer.

CONCLUSIONS

Although achievement of targeted delivery of gene therapy is a significant challenge, many research groups are making great strides towards this goal and by building on the successes of these groups we can look forward to seeing effective and efficient formulations to treat conditions using DNA therapy. The critical factor of course is maintaining the stability and activity of the nucleic acids until they can reach their desired site of action in the body. Particulate systems, especially biodegradable ones, show the greatest promise because their *in vivo* mobility and targetability allow the most precise placement of the nucleic acid delivery systems Table IV. The merging of targeted formulations and nucleic acid incorporation will yield therapies to correct not only inherited genetic disorders but also the genetic errors caused by environmental and unknown means that can become cancer.

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