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Review Article

Cancer, chitosan nanoparticles and catalytic nucleic acids

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Abstract

Objectives The aim of this review was to examine gene therapy involving DNAzyme and siRNA encapsulation into chitosan nanoparticles, discussing the current and future status of this drug delivery system in enhancing drug delivery and cancer therapy.

Key findings Cancer is a disease state in which the cells in our body undergo mutations at the genetic level and are transformed, acquiring the ability to replicate limitlessly. Conventional cancer treatment involves the use of surgery and cytotoxic chemotherapy and/or radiotherapy, which have the potential of harming normal, otherwise healthy, non-neoplastic cells. Newer forms of therapy such as immunotherapy and gene therapy have shown initial promise, but still require better ways to limit exposure to cancerous lesions in the body. As a result drug delivery systems have been developed in attempts to deliver therapeutics specifically to the target lesion site. One recent drug delivery system has revolved around the use of chitosan nanoparticle technology, where therapeutics are encapsulated into nanoparticles and targeted to tumours.

Summary Though few, attempts at encapsulating therapeutics such as deoxyribozymes and small or short interfering RNA have been optimistic and encouraging.

Keywords cancer; chitosan; drug delivery systems; gene therapy; nanoparticles

Basic facts on cancer

Cancer is a disease where cells in our body become transformed, obtain the potential for limitless replication and start proliferating aggressively into a mass of cells.[1] The growth is insensitive to anti-proliferative signals and instead supplies its own growth signals allowing growth not responsive to normal restraint. Cells invade and destroy neighbouring tissues and also metastasise to other anatomical parts of the body via the lymphatic or circulatory system. Cancer cells are also self-sustaining by angiogenesis to ensure their survival.[2] The mutations and transformation in the DNA of cells occur when normal, healthy cells come into contact with cancer causing agents (carcinogens). These agents include physical (UV and ionising radiation), chemical (asbestos, aniline dyes and alcohol) and biological carcinogens (viruses, mycotoxin and fungi). Carcinogens cause genetic alterations giving rise to transformation and mutations that escape and prevent normal cell growth and repair.

Tumours can be classified into benign or malignant, with benign being slow growing and noninvasive. Benign tumours are usually also encapsulated in a fibrous connective tissue capsule, preventing the cells from detaching and allowing easier surgical dissection. Malignant tumours lead to cancer due to their fast growing, nonencapsulated and invasive nature.[3] Most tumours, in general, are leaky due to basement membrane abnormalities and a decreased number of pericytes lining the rapidly proliferating endothelial cells.[4]

Current cancer therapies

Therapy options for cancer management depend largely on the tumour stage, the tumour response to adjuvant therapy, patient's age, gender, general condition, life expectancy which diminishes with increasing age, and quality of life. These factors have to be considered carefully as operative risks differ between individuals.[5] Major treatment options include surgery, chemotherapy and radiotherapy.[6] New cancer management methods, which are currently still being explored, include hormonal therapy.[7] Gene therapy and immunotherapy are also increasingly favoured; however, they are restricted or made less efficient by the cancer type, human heterogeneity and therapy applications.[5,8,9]

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Surgery

For those solid tumours that can be accessed via surgical incision, which includes a majority of cancers, surgery remains the mainstay for management of patients. The advances in surgical techniques in the past decade have dramatically improved the outcome of a good proportion of sufferers. Such practices as endoscopic surgery have made recovery from surgery quicker and decreased the extent of scarring left by the procedure. Other practices such as tissue- or limb-salvage procedures are improving the quality of life patients may have post-surgery.[10,11] As cancers are often plagued with relapses, surgery is often applied together with adjuvant therapy pre- and post-operatively in an attempt to destroy primary tumour cells. This leads to a decreased size and shrinkage of tumour and counters micro-metastatic spread concurrently. The major class of such therapies falls within chemotherapy.

Chemotherapy

Chemotherapy involves effective usage of cytotoxic drugs to kill cancerous cells. Nonetheless, problems of toxicity exist as cytotoxic drugs are highly toxic but poorly specific; the drugs are not able to differentiate between normal or cancer cells, leading to destruction of cancerous as well as healthy cells, especially rapidly dividing cells of the endothelial surfaces, which further affects the patients. This is due to the mechanism of some drugs such as anthracyclines, which intercalate with DNA to form free-radicals and induce DNA damage, resulting in DNA replication and translation failure.[12] Chemotherapy drugs are usually given in carefully controlled administration regimens, tailor-made to suit the patient's age, physical health, and other current medications. Combinational therapy acts to produce maximal cell kill while bearing in mind tolerance of toxicity in the host and also preventing the opportunity for development of tumour resistance.[12] Chemotherapy drugs can be used alone or together with other treatment regimes such as surgery. Neo-adjuvant chemotherapy occurs in the pre-operative state, and aims are to eradicate micrometastasis, destroy primary tumour cells to reduce tumour burden and to evaluate the histological response to pre-operative chemotherapy.[13] Avella *et al.*[14] have reported on adjuvant therapy used after surgery to eradicate micrometastasis that had already spread at the time of diagnosis and to improve the survival rate of patients with localised disease.

Another shortfall encountered during chemotherapy can be attributed to less than ideal drug release characteristics. Most of the drug content is released upon administration, causing a sudden increase in drug levels in the body followed by a rapid decline, resulting in undesirable side effects at the peak and insufficient therapy at the troughs.[15] In the case of the anthracycline antibiotic doxorubicin, the drug rapidly clears from the plasma, but there is slow terminal clearance of doxorubicin. The drug is subsequently metabolised in the liver and approximately 40% of the drug and its metabolites are excreted.[16] Hence, only a small amount of drug actually reaches and acts on the tumour target site. This calls for improved management options where the use of controlled and localised release technology could work as a better drug delivery system.

Radiotherapy

Other treatment options for cancer include radiotherapy, however, it is not used as widely as chemotherapy. One reason for this is that it is most amenable to non-deep-seated tumours in the body. Delivered in stepwise and scheduled amounts, radiation nonspecifically breaks nucleic strands and causes cells to die. Radiotherapy is often not the primary line of treatment for patients and it is more commonly used in patients who have refused definitive surgery, require palliation or have lesions in axial locations.[5] Current radiotherapy application is a multi-disciplinary approach involving surgery, radiotherapy and chemotherapy.[17] This way, radiotherapy can be employed as a neo-adjuvant (pre-operative), adjuvant (post-operative) or primary local therapy.

Immunotherapy

Immunotherapy involves therapeutic antibodies that have been designed to be more cytotoxic, to enhance effector functions, and to be fused with enzymes for pro-drug and cancer therapies.[18] Antibodies also form the driving force behind targeted therapy, which blocks the proliferation of cancer cells by interfering with specific molecules required for tumour development and growth as opposed to traditional chemotherapy, which works through the inhibition of cell division of cells in general.

Receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) family participate in several steps of tumour formation including proliferation and metastasis. Several known RTKs are upregulated in different forms of cancer and have become targets of a more tailored therapy. Current preclinical data suggest a potential benefit of their use, especially in combination with 'conventional' cytostatic therapy (reviewed by Yee).[19] We see this in the case of the chimaeric antibody, cetuximab, used in the treatment of colorectal cancer.[8] Cetuximab targets the EGFR, which is responsible for cell proliferation, invasion and migration, and blocks ligand binding to the receptor.[20] This results in a disruption of cellular processes that rely on EGFR signal transduction, namely, apoptosis, mitosis and angiogenesis.[21]

Another example is the humanised anti-VEGF (vascular endothelial growth factor) monoclonal antibody, bevacizumab, which limits cancer growth by preventing angiogenesis.[22] This process prevents the growth of tumours 2–3 mm beyond the existing vasculature and somewhat stabilizes the vasculature within the tumour, thus improving delivery of other chemotherapeutic drugs.[23,24] Bevacizumab has now been approved for treatment of nonsmall cell lung cancer and colorectal cancer; it is marketed under the name of Avastin.[25] A third example is the humanised anti-HER2/neu monoclonal antibody Herceptin, which has been approved for breast cancer therapy. Immunotherapy with Herceptin has been beneficial as the functional duality of the Fab region (blocks interactions between the targeted receptor and its ligands) and the Fc region (induces effector functions following interactions with the complement component) has increased its therapeutic potential.[26]

Gene therapy

Genetic material (DNA and RNA) has been explored for use as a treatment of genetic abnormalities or deficiencies. This

method, termed gene therapy, works by transferring healthy genetic material or nucleic acid constructs, such as ribozymes, antisense molecules, decoy oligodeoxynucleotides (ODNs), DNAzymes and siRNA, into diseased cells in an attempt to achieve a therapeutic effect that results in restoration of protein production, that was absent or deficient due to the genetic disorder. Gene therapy offers a solution to controlled, targeted and specific delivery of genes and short nucleic acid sequences to target cells; however, the disadvantage is that the vector systems cannot always be regulated or controlled, leading to unfortunate events such as side effects that are yet unknown, and patient death.[27,28]

The transfer of genes can occur through two main groups of drug delivery systems: viral-mediated vectors or nonviral-mediated vehicles. Viral-vector mediated gene therapy utilises genetically modified viruses which carry genes of therapeutic interest into a host cell. The applicable vectors used include retroviruses, adenoviruses, lentiviruses and herpes viruses. Viral vectors have been used widely and have been reported to be highly effective for transfer of genes such as p53.[29,30]

Viral vectors, however, can be disadvantageous in certain ways. Firstly, viruses are not endogenous to the human body, and their introduction often results in immunorecognition of the viral capsid proteins. An excellent review by Hartman *et al.*[31] documents the various immune responses generated upon adenovirus-mediated gene therapy. Secondly, viruses are able to be incorporated into host genomes, though not always accurately, and subsequently replicate autonomously. A case of de-novo T-cell leukaemia three years after retroviral gene therapy was commenced revealed that the retrovirus vector integration occurred in proximity to the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2.[32] The death of a young adult patient receiving adenoviral-mediated vector treatment for ornithine transcarboxylase deficiency (OTCD) caused a further setback to gene therapy advances. Incidents such as those resulted in a push for more intensive clinical trials in animal models but more importantly, a better, safer way by which therapeutics could be delivered or targeted to the defective cell.

On the other hand, nonviral vectors are seen to be increasingly attractive as most are safe enough to be used repeatedly with minimal risk to the host, are stable during storage and can be easily scaled up for production and characterisation.[33,34] Nonviral vectors range from liposomes to nanoparticles made up of various materials including ceramics, polymers and metals. Although nonviral vectors are generally more advantageous in terms of safety, ease of preparation, flexibility for vector to be tailor-made and cost-effective, their use is not without complications.[35–37] As nonviral vectors can be synthesised using a variety of materials ranging from biomaterials to even gold, multiple dose administrations of nucleic acid-complexed vectors may heighten immunostimulation. It is also possible that the complex chemistry employed to make these vectors could cause unknown and possible cytotoxic side effects when the studies progress from cell culture testing to in-vivo usage and evaluation. A major challenge that still remains for vector delivery is the ability to target tumour cells only, and spare healthy cells. Thus, it is essential that the therapeutic gene or downregulation agent can be delivered by the vector close to the primary tumour or its secondary growth.

Despite some initial optimism, many other cases of gene therapy that followed have mostly resulted in nonresponsive or even negative outcomes, requiring more rigorous and comprehensive animal trials before any clinical trials are considered.[38] Partly responsible for this failure is the obvious lack of availability of good drug delivery systems for cancer gene therapy. One currently hotly contested field of cancer drug delivery systems is nanotechnology.

Cancer therapy and nanotechnology

Nanotechnology has been defined by the Royal Society and Royal Academy of Engineering as the design, characterisation, production and application of structures, devices and systems by controlling the shape and size at nanometre scale. Nanotechnology involves the use of nanomaterials, which are described as materials with one or more components that have at least one dimension in the range 1–100 nm, inclusive of nanoparticles. Nanoparticles are defined as single particles with a diameter of less than 100 nm, varying in terms of size, shape and materials.[39]

Materials commonly used to formulate nanoparticles currently used in drug and gene delivery include lipids, chitosan, proteins such as human serum albumin, dendrimers and other synthetic materials such as gold.[40–45] These particles have individual characteristics and each differs in their particle and drug stability, drug loading capacity, drug release rates and targeted delivery ability. A short description together with the advantages and disadvantages of each material is given in Table 1.[46–71]

Features of chitosan

Chitosan occurs naturally as β(1–4)2-amino-2-deoxy-D-glucose and it is obtained through alkaline deacetylation of a polysaccharide found in the exoskeleton of crustaceans of marine anthropods and insects, called chitin. The biodegradable polysaccharide has a positive charge and is formed from a copolymer of N-acetyl-D-glucosamine and D-glucosamine.[62] Varying the conditions used to harvest chitosan results in differences in the molecular weight (50 00–2000 000 Da) and degree of N-acetylation (40–98%).[72] Chitosan nanoparticles are usually prepared by some of the following techniques: chemical cross-linking, the emulsification solvent diffusion method, complex coacervation and ionotropic gelation; each has their advantages and disadvantages. A summary of the various methods is shown in Table 2.[73–79]

Chitosan can exist as a base or a salt. Commonly used in preparation of nanoparticles for drug delivery, chitosan base is a weak base due to the D-glucosamine residue with a pKa value of 6.2–7.0, making it insoluble at neutral and alkaline pH solutions.[80] However, it is soluble in organic acids such as acetic acid, citric acid and glutamic acid. Little is known about chitosan salts except that they are largely soluble in water, depending on the degree of deacetylation. An advantage of chitosan salts is their higher protein encapsulation efficiency and release profiles; however, these are largely

Table 1 Common materials used for making nanoparticles, their associated properties, and the advantages and disadvantages

Material	Description	Availability	Advantages	Disadvantages
Liposomes	Sub-micron sized vesicles made up of lipid layers Classified as unilamellar or multilamellar depending on number of lipid bilayers[46]	Synthetic	Unilamellar systems entrap water soluble drugs due to its aqueous core Multilamellar systems encapsulate lipid soluble drugs Able to extravasate into defective, leaky vasculated tumours and be retained[47] Maximises amount of drugs reaching tumour sites while minimising systemic toxicity[48,49] pH-sensitive formulations provide sensitivity to lowered pH; allows for degradation in areas of tumour hypoxia[50]	Requires steric stabilisation, through the coating of inert polymers, due to electrostatic, hydrophobic and van der Waals forces affecting and disintegrating liposomes[40,51]
Dendrimers	Macromolecules regularly arranged in a 3-D branch format with structures consisting of a multifunctional central core molecule	Synthetic	Outer branches provide a large number of functional groups on the surface as attachment sites for carrier molecules[52] Inner branches provide dendritic channels that entrap carrier molecules Able to host both hydrophobic and hydrophilic molecules[44] Enhances solubility of drugs[53]	Have not been investigated for its potential effect relating to biocompatibility or therapy due to lack of clinical studies[54] May generate immune response
Human serum albumin	Produced in the liver and present in human blood plasma	Abundant protein in the blood	Highly tolerable by the human body Good drug loading efficiencies[43] Able to carry functional groups which are amenable to surface modifications[55] Passive tumour targeting possible due to enhanced permeability and retention (EPR) effect[56]	Needs to be modified and stabilised before usage[57] More studies need to be conducted to determine effects relating to cancer therapy
Gold	Biocompatible and inert, non-toxic metal nanoparticle[58]	Synthesis of gold nanoparticles involving Turkevitch process of reduction of $Au(III)Cl_3$ with trisodium citrate[59]	Binds readily to amino acids, proteins/enzymes and DNA via exposure of large surface areas for their immobilisation[60] Surface chemistry of gold nanoparticles can be modulated to bind suitable ligands	Requires stabilisation by a reducing agent Requires penetration enhancer for proteins and vaccines administered across the mucosal routes[61] More studies have to be done on the excretion, accumulation and toxicity of gold nanoparticles following chronic use Expensive if bulk quantity required
Chitosan	Polysaccharide modified from chitin Chitin is converted to chitosan by alkaline deacetylation Co-polymer of N-acetyl D-glucosamine and D-glucosamine[62]	Found naturally in the exoskeleton of crustaceans	Abundant natural supply Safe, non-toxic and biocompatible and biodegradable in normal biological environment[63] Mucoadhesive properties allowing a carried drug molecule increased interaction with membrane epithelium, thus creating more efficient uptake[64–66] Able to overcome permeability barrier posed by epithelium[66,67] Protects against enzymatic degradation[68] Control the release of genes to a certain extent[69] Shown to enter the nucleus and deliver drugs/genes directly[70] Possesses apoptotic properties on its own[71]	Positively-charged surface prevents interaction with other positively-charged molecules More studies have to be conducted regarding its drug/gene release profiles in view of gene therapy

Table 2 Methods used by different research groups to formulate chitosan nanoparticles

Name of technique	Methodology	Advantages	Disadvantages
Chemical cross-linking	Surfactant, AOT (sodium bis(ethylhexyl) sulfosuccinate), is dissolved in n-hexane, followed by addition of chitosan solution, Tris-HCl buffer, ammonia and glutaraldehyde solution. The mixture solvent is evaporated off and treated to a multi-step precipitation and centrifugation procedure before the final chitosan solution is dialysed and lyophilised to dry powder[73]	Produces nanoparticles ranging from 30–75 nm[73] Able to evade reticuloendothelial system (RES) and circulate in the blood for a few hours[73]	Long (> 12 h) and tedious process Involves hazardous chemicals (AOT and n-hexane) Requires specialised equipment
Emulsification–diffusion–evaporation	PLGA 70 : 30 is dissolved in ethyl acetate to form the organic phase while the aqueous phase is prepared by dissolving PVA in milliQ water followed by the addition of chitosan chloride. The organic phase is then added to an equal volume of the aqueous phase drop-wise. The emulsion is homogenised and nanoprecipitation performed by adding milliQ water drop-wise and stirred continuously to remove traces of organic solvent, resulting in chitosan-coated PLGA nanoparticles[74]	Varying the amount of PVA used allows the formulation of different sized nanoparticles Different types of PLGA result in nanoparticles of different sizes[75]	Laborious (involves many steps) Time consuming process (> 12 h) Produces nanoparticles that are large in size, ~148 nm[76]
Vortex-assisted complex coacervation	Chitosan in a sodium acetate–acetic acid buffer is added to sodium sulfate while the samples are vortexed at high speeds[77]	Simple and quick procedure Does not require toxic solvents Economical	Only small formulations volume trialled to date
Ionotropic gelation	0.1% w/v TPP (sodium tripolyphosphate) is added drop-wise into chitosan solution while stirring[78]	Relatively simple method requiring only a few steps for encapsulation of drugs/genes within the nanoparticles Spontaneous complex formation due to positively-charged chitosan and negatively-charged TPP ions[79]	TPP is potentially harmful to the lung

The method would depend on its applications. Each method possesses its advantages and disadvantages. PLGA, poly(lactide-co-glycolide); PVA, polyvinyl alcohol.

dependent also on the molecular weight.[81] Generally chitosan salts with lower degrees of deacetylation (≤ 40%) have solubility up to pH 9.0 while higher degrees of deacetylation (≥ 85%) are soluble only up to pH 6.5. The degree of deacetylation of chitosan also affects the degradation rate, stability of particle size and efficiency of gene carrier. Kiang *et al.*[82] reported that a higher degree of deacetylation increased DNA binding; this was due to a larger number of positively-charged amino groups on chitosan forming tight complexes with the negatively-charged phosphate groups on DNA. The formulation of chitosan also affects its encapsulation efficacy. A high molecular weight chitosan with a high deacetylation degree had a higher plasmid DNA encapsulation efficiency.[83] The same groups also reported that chitosan encapsulation of plasmid DNA conferred DNase I protection. In addition, nanoparticle-loaded antisense oligonucleotides were capable of entering lung cancer cells.[75]

Favourable properties of chitosan

Chitosan has been largely favoured as a potential nanoparticle carrier due to some of its favourable properties. It is a mucoadhesive polymer that has the ability to enhance drug absorption by re-arranging the tight junction proteins.[64,66] Chitosan nanoparticles are taken up by the endosomes allowing the drug to overcome the permeability barrier posed by the epithelia.[66,67] Chitosan nanoparticles provide protection against enzymatic degradation, ensuring that encapsulated drugs or genes can be delivered.[34,84] Chitosan nanoparticles have been shown to be able to control the release of genes or drugs in a controlled, sustained manner.[42,69,84,85] Chitosan as a raw material is abundant and it is easier and cheaper to manipulate as compared with other drug delivery systems such as cationic liposomes or formulations made from other materials.[34,70] Chitosan nanoparticles were able to enter the nuclear membrane and deliver the treatment agent directly into the nucleus.[42,70] Chitosan on its own was able to demonstrate growth inhibitory effects on cancer cells and to a certain extent apoptosis of bladder tumour cells via caspase-3 activation or other yet unknown mechanisms.[71,86]

Thus, chitosan is a useful biomaterial due to the above positive attributes. The ability of the material itself to inhibit cancer cells gives it an added advantage over other types of materials used for formulating drug delivery systems. Such drug delivery systems include those falling within the nanotechnology category. We shall now look at potential therapeutic deoxyribozymes (DNAzymes) and small or short interfering RNA (siRNAs) that have been encapsulated

with nanoparticle technology, focusing on chitosan platform technology, against a variety of cancers.

DNAzymes

DNAzymes comprise a cation-dependent catalytic core of approximately 15 deoxyribonucleotides which bind to and cleave target RNA in an RNase-independent manner.[87] DNAzymes bind to and cleave the target RNA between an unpaired purine and paired pyrimidine through a de-esterification process. This cleavage is supported by the large availability of such sites in the secondary structures of the target RNA's own intramolecular base pairing.[88]

The first reported use of DNAzymes against a target in cancer cells was by Wu *et al*.[89]; they designed three DNAzymes against the two variants of the p210 *bcr-abl* gene and the p190 variant. Mutated DNAzymes with an alteration of one critical base did not cleave their targets while the unmutated cleaving DNAzymes specifically inhibited p210bcr-abl protein expression in chronic myeloid leukaemia K562 cells by approximately 40% and inhibited cell growth by more than 50% over a five-day assay period. A variety of in-vitro studies have also suggested that different cancer gene targets could be successfully downregulated by the use of appropriate DNAzymes.[90–93] Some of the effects could lead to anticancer activity as judged by the phenotypic changes in cells. DNAzyme technology can be improved through chemical modification to provide more resistance against nuclease degradation, such as a common integration of a 3′–3′ inverted nucleotide at the 3′-end of the DNAzyme to prevent exonuclease degradation and to increase the stability of the molecule.[94,95] There are no clinical studies to investigate the potential of DNAzymes as antineoplastic agents thus far; however, a few animal studies have shed light on the in-vivo potential of DNAzymes as sequence-specific molecular tools.

DNAzymes against different cancer target genes such as *c-myc*, *bcr-abl*, *c-Jun*, *Egr-1*, *VEGF* have been designed and tested for either cell proliferation or cell death in relevant in-vitro models.[94] A few such as *c-Jun*, *Egr-1* and *VEGF* were examined using in-vivo studies. In one study looking at the RNA-cleaving ability of early growth response (Egr-1) DNAzymes, it was observed that Egr-1 DNAzymes blocked angiogenesis in subcutaneous Matrigel plugs in mice and inhibited MCF-7 human breast carcinoma growth in nude mice. Egr-1 DNAzymes also inhibited endothelial expression of downstream pro-angiogenic fibroblast growth factor (FGF)-2, but not that of anti-angiogenic vascular endothelial growth factor (VEGF), allowing for suppressed tumour growth without influencing other factors such as body weight, wound healing, haemostasis or reproduction.[96] A similar study involving breast cancer cells and Egr-1 DNAzymes demonstrated an inhibition of breast carcinoma cell migration and chemoinvasion in microchemotaxis chambers and additionally solid tumour growth in nude mice.[97] A DNAzyme (Dz13) designed to target c-Jun mRNA specifically cleaved c-Jun mRNA and blocked c-Jun protein expression in vascular smooth muscle cells, resulting in inhibition of vascular smooth muscle cell proliferation.[98] Similarly, Dz13 DNAzymes transfected into human microvascular endothelial cells resulted in a decrease in c-Jun protein expression followed by an inhibition of endothelial cell proliferation, migration, chemoinvasion, and tubule formation. Dz13 DNAzymes injected subcutaneously into solid melanomas in mice decreased tumour volume and vascular density significantly.[99]

Although DNAzymes were shown to be effective in both in-vitro and in-vivo conditions, there were issues regarding their stability and degradability. Attempts to improve stability and degradability were countered with the incorporation of modified nucleotides, however concerns arose about decreased catalytic activity and mild cellular toxicity.[100,101] Thus, drug delivery systems were devised to aid in the protection and delivery of DNAzymes until the target area was reached. However, a literature search revealed very few reports outlining the encapsulation of DNAzymes into a drug delivery system.

Recently, in a first study of its kind, we demonstrated the utility of the c-Jun DNAzyme, Dz13, incorporated into a nanoparticle formulated from chitosan via the complex coacervation method.[102,103] The biocompatible Dz13-chitosan nanoparticle was tested in SaOS-2 osteosarcoma cells, inducing apoptotic cell death and inhibiting osteosarcoma cell growth via c-Jun knockdown. Dz13-chitosan nanoparticles enhanced apoptosis rather than hindered, as previously expected. The nanoparticles were of a median diameter (350 nm), had a high positive surface charge and high encapsulation efficiency allowing for a reasonable loading capacity. The particles were amenable and notably quite efficient at intracellular delivery as transmission electron microscopy (TEM) revealed that nanoparticles were intracellular after 48 h within vesicles resembling endosomes. The formulation was stable in serum for a week and at room temperature for a month. Injection of nanoparticles (equivalent to 500 ng Dz13) into the muscle and into the tibial lumen did not cause any adverse effects *in vivo*. We have since found this formulation to be effective in various orthotopic models of osteosarcoma, liposarcoma and bone metastasis (unpublished data). This initial study was very useful in establishing that chitosan nanoparticles enhanced rather than hindered, the effect of Dz13 on cells.

In addition, the Dz13 nanoparticle caused regression of growth and metastasis of pre-established bone tumours, especially when used in combination with a frontline therapeutic agent for osteosarcoma, doxorubicin.[104] This was also the first study of its kind demonstrating DNAzyme-mediated gene modulation in a clinically relevant metastasising model of neoplasia. Dz13-chitosan nanoparticles were formulated via the technique previously mentioned and administered (250 ng Dz13) into the proximal tibial region 21 days after tumour seeding. Doxorubicin (3 mg/kg per injection) was also administered intraperitoneally at the same time. As the treatments were administered three weeks after tumour cell inoculation, this served as a valid test for the ability of Dz13-chitosan nanoparticles as a therapy that hindered established tumour growth in the exponential and aggressive growth phase. The authors were aware that it was during this period that patients presented to the clinic with initial complaints and underwent initial diagnosis. Results affirmed the efficacy of Dz13-chitosan nanoparticles for tumour regression against pre-established tumour growth, especially in combination

with doxorubicin, causing less bone damage as well. Furthermore, Dz13-chitosan nanoparticles inhibited establishment of metastasis in the lungs of mice. This extended to total inhibition when complemented with doxorubicin therapy. Thus, chitosan appeared to be an efficient drug delivery system for controlled delivery of DNAzymes, which would otherwise have difficulty in entering cells effectively and would create problems of cytotoxicity if complexed to cationic lipofection reagents.

siRNA

siRNA is made up of 21–23 nucleotides with a two nucleotide overhand at each 3′ end; it is processed by an RNase III family member Dicer before incorporation into the RNA-induced silencing complex (RISC). The sense strand of the double-stranded RNA is cleaved during RISC complex formation.[105] RNA helicases unwind the double-stranded siRNA and the antisense strand guides the RISC to the complementary target mRNA to be cleaved by the RISC. The next step was to look at delivering siRNA *in vivo* for selective silencing of genes promoting cancer. However, siRNA is susceptible to nuclease destruction and cannot penetrate the cell membrane due to its highly-charged backbone. Most of the delivered siRNA tends to be taken up and accumulated by the reticuloendothelial system (RES) in the liver and the spleen.[106]

Nonetheless, siRNA has been used commonly to silence genes *in vitro*, and *in vivo* with successful outcomes in animal models where siRNA was delivered locally and systemically against target genes.[107–109] To overcome the major problems of siRNA tissue-specific delivery, researchers have experimented with encapsulation techniques to preserve siRNA integrity and aid delivery to the tumour site.

In a seminal study, three methods of siRNA association with chitosan nanoparticles were reported.[110] The three methods, simple complexation, ionic gelation by siRNA entrapment into sodium tripolyphosphate (TPP)–chitosan nanoparticles and adsorption of siRNA onto the surface of preformed TPP–chitosan nanoparticles were examined using four different types of chitosan: 270 and 110 kDa chitosan hydrochloride, 470 and 160 kDa chitosan glutamate. Through the study, the authors aimed to develop, characterise and investigate the biological effects of siRNA-chitosan nanoparticles so as to develop an effective system which could protect and transport siRNA to the cytoplasm of the target cells. Results from particle sizing revealed that the smallest particle was 276 nm while the largest was 709 nm, and particle sizes generally followed the trend where a smaller mean particle size of chitosan nanoparticles was obtained when a lower molecular weight chitosan was used. The nanoparticles were all slightly positive charged, which was desirable for electrostatic interaction with the overall negative charge of the cell membrane and also prevented particle aggregation. In addition, the authors reported an impressive siRNA loading efficiency of up to 90% using siRNA TPP–chitosan nanoparticles and full protection of siRNA from nuclease digestion for up to 24 h. All the siRNA-chitosan nanoparticle preparations (4 pmol/well) displayed comparable gene silencing effects compared with Lipofectamine 2000 transfections. Of the nanoparticles, chitosan glutamate at 470 kDa showed the highest gene silencing effect at 24-h post-transfection either by simple complexation (51% gene knockdown) or ionic gelation (82% and 63% gene knockdown for siRNA entrapment and siRNA adsorption, respectively). However, in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cytotoxicity, only 18–40% loss of cell viability was observed for siRNA associated with chitosan–TPP nanoparticles, which was neither significant nor expected based on results of gene knockdown. The increase in cell viability at 48-h post-incubation also indicated some recovery of cells within this period, this being unhelpful for inhibition of tumour growth. Overall, the authors demonstrated the utility of chitosan as a drug delivery system for siRNA, with chitosan glutamate as the best candidate. Furthermore, in-vitro studies of the mechanism of siRNA-chitosan nanoparticle gene silencing were unclear due to the conflicting information about siRNA chitosan nanoparticle cytotoxicity in Chinese hamster ovary cells. Therefore, further investigations are necessary to verify the potential of siRNA-chitosan nanoparticles as a potential therapeutic, especially in terms of inducing cell death and tumour growth inhibition, in relevant in-vivo studies. This study, however, was very useful and informative about the characterisation of different forms of chitosan and formulation methods, serving as a guide and platform for other researchers looking into chitosan drug delivery systems.

In another study published in the same year, Howard *et al.*[111] investigated chitosan nanoparticles as a possible drug delivery system for siRNA in the context of systemic and mucosal diseases. A simple complexation method was utilised to formulate nanoparticles based on different N : P ratio (defined as the ratio of chitosan amino groups (N) to RNA phosphate groups (P)), yielding siRNA-chitosan nanoparticles ranging from 180 to 330 nm and a positive ζ potential. Stability studies revealed that intact siRNA was maintained after release from serum-incubated nanoparticles, whereas nonformulated naked RNA was degraded, again suggesting that chitosan effectively protected siRNA against nuclease breakdown. Cellular toxicity assays performed in NIH 3T3 cells demonstrated reduced cell viability of up to 39% when 50 nm/well siRNA nanoparticles were added. Next, a transgenic EGFP (enhanced green fluorescent protein) mouse model was used to investigate the ability of chitosan-based systems to mediate EGFP knockdown following nasal administration. The animals did not appear to develop adverse effects from daily nasal administration of siRNA-chitosan nanoparticles over a five-day period. Subsequent lung sections from the mice dosed with nanoparticles showed significant reduction in numbers of EGFP-expressing epithelial cells in the bronchioles (43% compared with untreated control and 37% compared with EGFP mismatch). Lastly, the therapeutic potential of chitosan nanoparticles for knockdown of disease-related proteins was demonstrated in K562 cells, which endogenously express BCR/ABL-1 protein. A single transfection treatment of siRNA-chitosan nanoparticles containing breakpoint siRNA successfully resulted in approximately 90% allele-specific knockdown in these suspension cell lines. This gives a chitosan drug

delivery system an advantage over other systems due to its amenability for efficient knockdown in both adherent and suspension cells. The nasal route utilised in this study also offered a noninvasive alternative to systemic administration of siRNA therapeutics. It provided direct access to respiratory tissue and a migration pathway to systemic sites avoiding hepatic clearance. As the application of a nebulisation method was recently found to improve chitosan/DNA nanoparticle distribution and gene expression in mouse lung, the authors are exploring the development of aerosol forms for their siRNA-chitosan system.

Thus, chitosan nanoparticles are a favourable drug delivery system for delivery of siRNA molecules, arguably the most potent gene silencing agent found to date, and lesser-known but increasingly popular DNAzymes. The combination of such a target-specific entity together with a reliable, biocompatible resource such as chitosan polymer should facilitate enhanced gene modulation in the future, with benefits seen not only against cancer, but other genetic disorders as well.

Future directions

To improve cancer therapy, nonsingular approaches combining more than one treatment agent into one delivery vehicle could be used. For example, this could be done through the co-encapsulation of siRNA with a small molecule cytotoxic drug into a chitosan formulation. However, many stringent studies have to be conducted *in vitro* and *in vivo* to examine their interaction, to prevent a reduction in activity of either agent. The use of combined agents proved useful in targeting more than one pathway leading to increased rates of tumour cell death and decreased tumour size.[104]

Given that conventional chemotherapy drugs are still highly toxic to patients, a major direction would be to find better methods for encapsulating cytotoxics for better delivery to the tumour and to avoid systemic circulation and clearance. Additional work has to be done on the drug release profiles of these cytotoxics to ensure targeted and sustained drug release over time.

Lastly, it is imperative to look into encapsulating cationic or neutral cytotoxics or compounds into the positively-charged chitosan nanoparticle so as to develop a versatile drug delivery system for a variety of treatment agents. A few groups have resorted to surface modifications of chitosan nanoparticles to allow encapsulation of hydrophilic molecules such as doxorubicin, but none have been tested using in-vivo models of cancer.[112,113] This is fast becoming a priority.

Summary

Chitosan is a naturally abundant polysaccharide that has been favoured as a nanoparticle carrier due to its many advantageous properties. We have presented a summary of current cancer treatments, the favourable properties of chitosan advocating it for use as a nanoparticle for drug delivery systems, novel treatment methods involving nanotechnology with an emphasis on chitosan nanoparticles, and brief reports about two treatment agents (DNAzyme and siRNA) that could potentially be applied together with chitosan for therapy purposes.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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