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Review

Review: doxorubicin delivery systems based on chitosan for cancer therapy

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Abstract

Objectives This review sheds insight into an increasingly popular polymer that has been widely explored as a potential drug delivery system. The abundant, biodegradable and biocompatible polysaccharide chitosan, with many other favourable properties, has been favoured as a drug delivery system for the purposes of encapsulating and delivery of doxorubicin with reduced side-effects.

Key findings Doxorubicin is frequently used as a frontline chemotherapeutic agent against a variety of cancers. It has largely been able to demonstrate anti-tumour effects, though there are major shortfalls of doxorubicin, which include serious side-effects such as cardiomyopathy and myelosuppression, and also an ever-present danger of extravasation during drug administration. In view of this, drug delivery systems are currently being explored as alternative methods of drug delivery in a bid to more effectively direct doxorubicin to the specific lesion site and reduce its systemic side-effects. Liposomes and dendrimers have been tested as potential carriers for doxorubicin; however they are not the focus of this review.

Summary Recent advancements in doxorubicin and chitosan technology have shown some preliminary though promising results for cancer therapy.

Keywords cancer therapy; chitosan; doxorubicin; drug delivery systems

Doxorubicin

Doxorubicin is a member of the cytotoxic anthracycline antibiotics, consisting of an amino-sugar daunosamine, linked through a glycosidic bond to the C7 of a tetracyclic aglycone, doxorubicinone.[1] The chemical structure of doxorubicin is shown in Figure 1.[2] This cytotoxic agent interacts with the DNA double helix to interfere with nucleic acid synthesis, producing a marked cytotoxic effect on cells in the S phase. This is because intercalation inhibits nucleotide replication and the actions of DNA and RNA polymerases. The second mechanism by which doxorubicin affects the cell is through enzyme inhibition. Doxorubicin binds and inhibits topoisomerase II and prevents transcription. The ring phenolic groups of doxorubicin contribute acidic functions, while the sugar amino group adds to a basic function, making the molecule amphotheric.[3]

In a clinical setting, doxorubicin has been widely used for a variety of cancers, successfully producing regression in acute leukaemia, lymphomas, soft-tissue and osteogenic sarcomas, paediatric malignancies and adult solid tumours, in particular breast and lung carcinomas.[1] Doxorubicin is commonly used in combination regimens with other cytotoxic drugs such as methotrexate, cisplatin, ifosfamide, vincristine and etoposide.

Although effective, doxorubicin therapy also carries an inherent risk. The drug clears rapidly from the plasma, but terminal clearance is slow. The drug is subsequently metabolised in the liver, and about 40% of the drug and its metabolites are excreted.[4] Hence, only a small amount of drug actually reaches and acts on the tumour target site. Doxorubicin-induced cardiac toxicity has also been well documented by several groups.[5–8] In some patients, symptomatic cardiomyopathy started within 1 year of doxorubicin administration, while for others it occurred 15 years after the end of chemotherapy.[9] A study involving 755 patients with localised osteosarcoma of the extremities noted that about 1.7% of patients developed clinically symptomatic cardiac toxicity and that contributory risk factors were cumulative dose and dose intensity.[8] Research is now focusing on ways to protect myocardial cells from damage during treatment.

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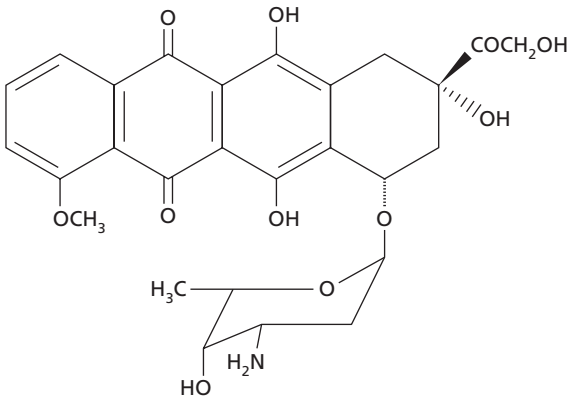

Figure 1 The structure of doxorubicin.

In a bid to improve administration and reduce the toxicity problems of doxorubicin, there have been attempts at various drug delivery systems (DDSs) looking to deliver doxorubicin in a controlled and localised manner at a clinically relevant concentration. Liposomal doxorubicin, which is currently marketed as Doxil, consists of doxorubicin encapsulated in small unilamellar vesicles.[10] The bilayer comprises hydrogenated soy phosphatidylcholine, cholesterol and N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, which prevents opsonisation by plasma proteins and decreases uptake by the reticuloendothelial system (RES).[11] Liposomal doxorubicin was noted for its ability to protect patients from some side-effects of doxorubicin, especially cardiomyopathy,[12] although its efficacy is still in question as partial response rates by patients in randomised clinical trials done by the company were less than 50%.[10] In addition, recent data have shown that a toxic dermatological reaction, palmar–plantar erythrodysesthesia (hand–foot syndrome), occurred in about 50% of all Doxil-treated patients.[13]

Another approach would be to entrap doxorubicin into a positively charged carrier, which would favour cell adhesion and cellular uptake because of its attraction to negatively charged cell membranes.

Ideally, treatment regimens for cancer should not only target the disease but also minimise any side-effects that may bring additional trauma to the patients. Improved management options, which may include better DDSs, may therefore alleviate some of the concerns associated with doxorubicin toxicity.

Materials commonly used to formulate these carriers or nanoparticles for use as DDSs include lipids,[14] chitosan,[15] proteins such as human serum albumin,[16] carbohydrates,[17] and other synthetic materials such as gold.[18] These particles have individual characteristics and differ 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. Of these, chitosan has been largely favoured as a nanoparticle carrier for various agents, including doxorubicin, because of its favourable properties, listed in Table 1. Nanoparticle technology belongs to a burgeoning field of nanotechnology which 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.[74]

Features of chitosan

Chitosan is found in the natural environment as β(1-4) 2-amino-2-deoxy-D-glucose (Figure 2). The polysaccharide is harvested from the exoskeleton of crustaceans and insects as chitin and goes through alkaline deacetylation, resulting in chitosan. The positively charged polysaccharide is biodegradable and is formed from a copolymer of N-acetyl-D-glucosamine and D-glucosamine.[19] Varying the conditions used to harvest chitosan results in differences in molecular weight (50 kDa–2 MDa) and degree of N-acetylation (40–98%).[75] Chitosan nanoparticles are prepared by some of the following techniques: chemical cross-linking, the emulsification–solvent diffusion method, complex coercavation and ionotropic gelation; each have their advantages and disadvantages.

Commonly used in preparation of nanoparticles for drug delivery, chitosan base is insoluble at neutral and alkaline pH solutions because of the D-glucosamine residue, providing a pK_a value of 6.2–7.0.[76] Conversely, it is soluble in organic acids such as acetic acid, citric acid and glutamic acid. Less is known about chitosan salts; however, depending on the degree of deacetylation, they are largely soluble in water. Generally, chitosan salts with lower degrees of deacetylation ($\leq$ 40%) are soluble up to pH 9.0 while those with higher degrees of deacetylation ($\geq$ 85%) are soluble only up to pH 6.5.

Focusing on particulate formulation of doxorubicin with chitosan, this review aims to give an overview and some updates regarding chitosan technology with doxorubicin. The following section introduces the various groups geared towards research in this area and their recent endeavours. A literature search using the keywords 'chitosan' and 'doxorubicin' was conducted on PubMed to reveal the groups who have experimented with chitosan technology as a DDS for doxorubicin.

Doxorubicin–chitosan nanoparticles

Dextran sulfate–chitosan nanoparticles

A common problem encountered during doxorubicin–chitosan encapsulation can be attributed to the cationic, hydrophilic nature of doxorubicin. The polyanion dextran sulfate (DEX) has been used to mask the positive charge of doxorubicin for encapsulation into positively charged chitosan.[77] Using ionotropic gelation with sodium tripolyphosphate, Janes *et al.* prepared doxorubicin-loaded chitosan nanoparticles incorporating DEX. The addition of DEX yielded about 20% doxorubicin encapsulation efficiency (EE) into chitosan. The dense, spherical nanoparticles were 260 nm in diameter and had a positive ζ-potential of 30 mV. In-vitro release studies of nanoparticles incorporating DEX (in acetate buffer at pH 4) showed a burst release of 17% at 2 h, followed by an additional release of 4.5% over the next 48 h. Based on their results, the

Table 1 Basic structures and materials used in nanoparticle formulations

Description	Potential therapeutics	Advantages	Disadvantages
Chitosan			
• Polysaccharide modified from chitin • Chitin is converted to chitosan by alkaline deacetylation • Co-polymer of N-acetyl D-glucosamine and D-glucosamine[19] • Found naturally in the exoskeleton of crustaceans	• Plasmid DNA[20–22] • DNAzyme[23–26] • Antisense oligonucleotides[27] • Peptides[28] • siRNA[29]	• Abundant natural supply, making it easier and cheaper to manipulate compared with other DDSs[20,22] • Safe, non-toxic, biocompatible and biodegradable[30] • Mucoadhesive polymer that is able to rearrange tight junction proteins, allowing a carried drug molecule increased interaction with membrane epithelium, thus creating more efficient uptake[31,32] • Taken up by endosomes, allowing encapsulated therapeutics to overcome permeability barrier posed by epithelium[32,33] • Provides protection against enzymatic degradation, ensuring that encapsulated therapeutics can be delivered with minimal degradation[20,34,35] • Control the release of encapsulated therapeutics in a controlled, sustained manner[15,35,36] • Shown to enter the nuclear membrane and deliver the therapeutics directly into the nucleus[15,22] • Chitosan *per se* demonstrates growth inhibitory effects on cancer cells and demonstrated apoptosis of bladder tumour cells via caspase-3 activation and other mechanisms[37,38]	• 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
Dendrimers (vastly different in composition due to complex make-up consisting of polymers and carbohydrates)			
• Macromolecules regularly arranged in a three-dimensional branch format with structures consisting of a multifunctional central core molecule • Synthetic	• Peptides (e.g. BH3)[39] • DNAzymes[40–44] • DNA[45]	• Outer branches provide a large number of functional groups on the surface as attachment sites for carrier molecules[46] • Inner branches provide dendritic channels that entrap carrier molecules • Able to host both hydrophobic and hydrophilic molecules[17] • Enhances solubility of drugs[47]	• Have not been investigated for their potential effect relating to biocompatibility or therapy[48] due to lack of clinical studies • May generate immune response
Gold			
• Biocompatible and inert, non-toxic metal nanoparticle[49] • Synthesis of gold nanoparticles involving Turkevitch process of reduction of $Au(III)Cl_3$ with trisodium citrate[50]	• Insulin[51] • Tumour necrosis factor[52] • Methotrexate[53]	• Binds readily to amino acids, proteins/enzymes and DNA via exposure of large surface areas for their immobilisation[54] • 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[51] • More studies have to be done on the excretion, accumulation and toxicity of gold nanoparticles following chronic use • Expensive if bulk quantity required
Human serum albumin			
• Produced in the liver and present in human blood plasma • Abundant protein in the blood	• Plamid DNA[55] • Antisense oligonucleotides[56] • Doxorubicin[16]	• Highly tolerable by the human body • Good drug loading efficiencies[16] • Able to carry functional groups that are amenable to surface modifications[56] • Passive tumour targeting possible due to EPR effect[57]	• Needs to be modified and stabilised before usage[56] • More studies need to be conducted to determine effects relating to cancer therapy
Liposomes (composed mainly of phospholipids or surfactants)			
• Sub-micron-sized vesicles made up of lipid layers • Classified as unilamellar or multilamellar depending on number of lipid bilayers[58] • Synthetic	• Doxorubicin[10] • Oligodeoxynucleotides[59] • Plasmid DNA[60–67] • Camptothecin[68,69]	• Unilamellar systems entrap water-soluble drugs due to aqueous core • Multilamellar systems encapsulate lipid-soluble drugs • Able to extravasate into defective, leaky vasculated tumours and be retained[11] • Maximises amount of drugs reaching tumour sites while minimising systemic toxicity[70,71] • pH-sensitive formulations provide sensitivity to lowered pH; allows for degradation in areas of tumour hypoxia[72]	• Requires stearic stabilisation, through the coating of inert polymers, due to electrostatic, hydrophobic and van der Waals forces affecting and disintegrating liposomes[14,73]

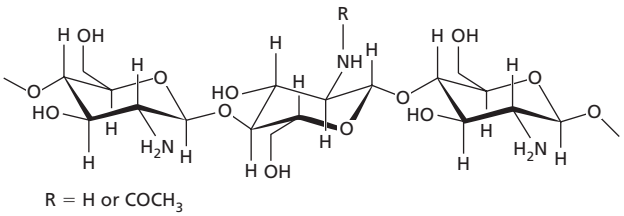

Figure 2 The structure of chitosan.

authors also speculated that the initial phase of doxorubicin release could be attributed to doxorubicin located at the surface of the particles, while the remainder of the unreleased doxorubicin was assumed to be entrapped within the chitosan nanoparticles. The degradation of chitosan would therefore be essential for accomplishing doxorubicin release. DEX also played an important role in doxorubicin release, as nanoparticles without DEX showed over twice the burst effect after 2 h under the same conditions. Employing this understanding of doxorubicin release, in-vitro cytostasis studies for doxorubicin bioactivity on C26 murine colorectal carcinoma cells and A375 human melanoma cells demonstrated the ability of these nanoparticles to slow tumour cell proliferation at a released doxorubicin concentration one-fifth of the control doxorubicin solution. This indicated that doxorubicin bioactivity was not impeded, but was retained within the nanoparticle. Similar experiments carried out on chitosan–doxorubicin complexes (213 nm in diameter) yielded less impressive results for doxorubicin release and lesser cytostasis, suggesting the presence of strong chemical bonding that might prevent doxorubicin release or cause doxorubicin damage. The authors also hypothesised that the cytotoxic activity displayed by these nanoparticles was due to endocytosis, as confocal microscopy results suggested that nanoparticles were internalised by cells and degraded intracellularly to release the drug. As the seminal study describing chitosan nanoparticles as carriers for the delivery of the cationic anthracycline drug doxorubicin, the authors were successful in encapsulating appreciable quantities of doxorubicin by incorporating the polyanion DEX, circumventing the inherent polymer–drug charge repulsion. The nanoparticles also demonstrated minimal burst release, which is favourable for a controlled, sustained release mechanism for drug delivery. Although promising, most of the published results were preliminary findings that will require more in-vitro studies with doxorubicin-resistant cell lines and in-vivo studies to clarify the effect of doxorubicin–chitosan nanoparticles before recommending it as a potential viable DDS. Although no updates have been published since the first report,[77] the authors have contributed greatly to the knowledge and experimental proceedings involved in doxorubicin–chitosan nanoparticles, providing a platform for subsequent researchers to build on.

Dextran sulphate–chitosan hydrogel nanoparticles

A similar study that utilised DEX to first couple doxorubicin into a conjugate before encapsulating it into chitosan hydrogel nanoparticles through a reverse microemulsion method with surfactant reported the potential of doxorubicin–DEX chitosan nanoparticles *in vivo*.[78] These 100 nm spherical nanoparticles were administered to mice intravenously via the tail vein at a dose of 15 mg/kg body weight weekly for 1 month and evaluated through studies looking at the regression of tumour size and survival of mice over a period of 70 days. As expected, untreated tumour-bearing control mice showed a progressive increase in tumour size over a period of 40 days, after which the mice died as a result of the tumour volume. A similar observation of increased tumour volumes was seen in a group receiving chitosan alone. Mice receiving treatment with doxorubicin alone (8 mg/kg body weight) showed a slow gradual increase in tumour size after day 45. Comparatively, the group of mice treated with doxorubicin–DEX conjugate (15 mg/kg body weight) or doxorubicin–DEX chitosan hydrogel nanoparticles demonstrated initial increases in tumour volume up to 45 days, followed by gradual regression of the mean tumour volume. Doxorubicin–DEX chitosan nanoparticles resulted in faster regression in tumour volume compared with doxorubicin–DEX conjugates, clearly showing that encapsulation of doxorubicin–DEX in chitosan nanoparticles has greater effects in terms of tumour regression than the doxorubicin–DEX conjugate itself. In addition, there were significant differences in survival rates between the conjugate (25% over 90 days) and nanoparticle system (50% over 90 days). This potentially beneficial effect of doxorubicin–DEX nanoparticles may be attributed to tumour-selective accumulation through the enhanced permeability and retention effect (ERP) of macromolecules in solid tumours. A higher accumulation of nanoparticles in tumour tissues and gradual release of doxorubicin aided by lysosomal degradation at the tumour site could also have contributed to better performance of the nanoparticle, a view also supported by Janes *et al.*[77] Interestingly, the authors boldly concluded that this new nanoparticle formulation was able to effectively reduce the toxicity of doxorubicin without first observing the target organs of the animals for possible side-effects. Nonetheless, the work of Mitra *et al.*[78] has been helpful in establishing the working potential of doxorubicin–DEX nanoparticles *in vivo*, previously not determined by Janes *et al.*,[77] using a similar nanoparticle formulation. Despite initial positive results, the authors seemed to have discontinued work on these doxorubicin–DEX chitosan hydrogel nanoparticles, as no reports have been published since.

Glycol–chitosan nanoaggregates

In a novel in-vivo study reporting the tumour-targeting effect of glycol–chitosan as a carrier material, fluorescein isothiocynate (FITC) was conjugated and used to track the accumulation of glycol–chitosan nanoaggregates (FITC–GC) at the tumour site.[79] Similar nanoaggregates consisting of doxorubicin attached to glycol–chitosan (GC–doxorubicin) via a pH-sensitive linker (N-*cis*-aconitic anhydride) allowed observation of intratumour release of doxorubicin from the nanoaggregate carrier. Results revealed that the FITC–GC and GC–doxorubicin nanoaggregates were similarly sized, around 250–300 nm diameter, and the GC–doxorubicin nanoaggregates were reported to be near spherical. The authors also determined that physical entrapment of doxorubicin into GC–doxorubicin nanoaggregates yielded about

eight-fold greater entrapment of doxorubicin compared with chemically conjugated doxorubicin (39% vs 5% weight, respectively). This resulted in an impressive doxorubicin loading efficiency of 97%. FITC–GC nanoaggregates were injected via the tail vein into rats bearing human mesothelioma tumours, at predetermined time points. When the study was concluded, urine and blood were collected and various tissues such as the liver, heart, lung, kidney, spleen and tumour were excised. Results from fluorescence microscopy imaging revealed that FITC–GC nanoaggregates were sustained at high levels in the blood and increasingly detected in the tumour tissues, levels peaking 8 days after injection, when the animals were sacrificed. On the other hand, initial distribution of FITC–GC to the kidney and liver decreased over 8 days, suggesting that the increased distribution of FITC–GC nanoaggregates in the tumour could be attributed to its long residence in blood and limited distribution to other tissues. This also indicated that the FITC–GC nanoaggregates should accumulate passively in the tumour tissue, probably via the EPR effect, and escape the RES. These observations were supported by a subsequent study,[80] which reported biodistribution in the liver (~4% of dose), spleen (~1% of dose), kidney (~35% of dose) and tumour (> 20% of dose). This finding also supported the previous study of Son *et al.*,[79] which reported that the amount of nanoaggregates in the tumour gradually increased as blood circulation time increased. FITC–GC nanoaggregates demonstrated circulation in the blood (14% of dose) and were minimally detected in the heart and lungs, which is promising for prevention of doxorubicin-associated cardiac toxicity.

Upon intravenous injection of the chemically conjugated doxorubicin nanoaggregates (doxorubicin/GC–doxorubicin) to tumour-bearing rats (10 mg of equivalent doxorubicin/kg), tumour growth was suppressed over 10 days while constantly maintaining body weight, compared with decreasing body weight in rats receiving doxorubicin solution alone (10 mg/kg). These data supported results from fluorescence microscopy indicating that doxorubicin/GC–doxorubicin mainly accumulated in tumour sites while free doxorubicin was delivered to tumour as well as normal tissues. With this, Park *et al.*[80] demonstrated that doxorubicin/GC–doxorubicin exhibited lower systemic toxicity but comparable in-vivo anti-tumour activity. A drawback to glycol–chitosan nanoaggregates prepared by Son and colleagues[79] and Park and colleagues[80] were the particularly long and tedious processes of nanoparticle formation, which required up to 3 days of preparation using complex procedures and specialised equipment. In addition, the authors speculated and inferred from results of fluorescence microscopy that GC–doxorubicin nanoaggregates would similarly localise to tumour sites like FITC–GC, although they did not actually demonstrate true localisation. Thus, a possible alternative could be to use fluorescently tagged GC–doxorubicin nanoaggregates for future studies. Nevertheless, it is evident from both studies that nanoaggregates based on hydrophobically modified chitosan have promising potential as carriers for hydrophobic anti-tumour agents.

Oleoyl-chitosan nanoparticles

Another approach adopted involves hydrophobically modified chitosan as a carrier for hydrophobic doxorubicin.[81] It was observed that longer hydrophobic chains and bigger hydrophobic groups helped to stabilise micelle structure and protect drug compounds from the environment. Thus, oleic acid was chosen to prepare oleoyl-chitosan (OCH) to form self-assembled nanoparticles. Doxorubicin–OCH nanoparticles were prepared using a complex oil/water emulsification technique, resulting in nanoparticles with a mean diameter of about 315 nm. Doxorubicin (10 mg) was efficiently loaded into OCH nanoparticles with an EE% of 52.6%, and the drugs could later be released at various rates depending on the pH of the solution. A pH of 3.8 allowed doxorubicin to be rapidly and completely released from OCH nanoparticles, whereas pH 7.4 showed sustained release followed by burst release. Doxorubicin–OCH nanoparticles also outperformed free doxorubicin in terms of growth inhibitory rates in four human cancer cell lines (A549, Bel-7402, HeLa and SGC-7901 cells) at all concentrations ranging from 0.05 to 10 μg/ml equivalent doxorubicin, showing better inhibition of cancer cells than doxorubicin. OCH nanoparticles maintained the pharmacological activity of doxorubicin. OCH nanoparticles alone also displayed less damaging effects on erythrocyte membranes in a haemolysis test, and almost no cytotoxicity was detected in the four cell lines, displaying great potential as new drug carriers. The authors have embarked on further in-vivo studies.

Doxorubicin–chitosan microspheres

Microspheres or nanospheres, as shown in Figure 3, are spherical structures which consist of a matrix system where drug is entrapped, attached or encapsulated. The surface of the sphere is amenable to modifications by addition of polymers and biological materials such as ligands or antibodies for targeting purposes.[82]

Doxorubicin-loaded chitosan microcapsules in transcatheter arterial chemoembolisation

A major potential for chitosan DDSs lies in the treatment of hepatocellular carcinoma (HCC), in which the tumour is fed by the hepatic arteries while the normal tissue receives its blood supply from the portal vein. Doxorubicin-loaded chitosan microcapsules are currently being explored as therapy options for HCC via transcatheter arterial chemoembolisation (TACE),[83] which serves as an embolic material enabling tumour ischaemia and necrosis while preserving functional liver tissue. Kim *et al.*[83] prepared doxorubicin hydrochloride (DHCl)-containing chitosan microspheres (CM) using a complex expanding–loading–shrinking process (10 mg/100 mg CM) to increase drug loading rate and decrease drug

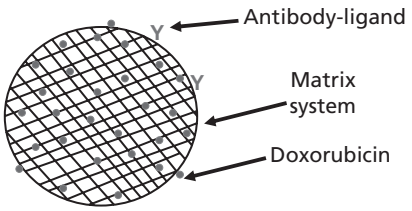

Figure 3 Structure of doxorubicin-loaded microsphere.

loss in solution. The sizes and charge potential of these particles were unknown, although the authors showed a red, evenly sized, smooth-textured, spherical particle, whose round shape would be important to allow effective ischaemic effect of TACE in HCC. In-vitro drug release studies conducted over a period of 7 days reported that 22.6% of DHCl was released, while the remaining 77.3% was thought to be retained within the network of the CMs. Kim *et al.*[83] postulated that, when extrapolated to an in-vivo setting, the release of drug will be sustained over a long time because of the physical trapping of doxorubicin. On the basis of these findings, Kim *et al.* conducted TACE with DHCl–CM in rabbits transplanted with hepatic tumours simulating HCC. Seven days post-TACE showed excellent results of total infarctions of the tumours due to necrosis in five of the seven rabbits and an overall size reduction (about 15%) of tumours. In this study, DHCl was not observed to be directly involved in chemoembolisation; rather, the infarction developed because of the embolisation of CMs, as observed by necrosis caused by lack of nutrition and oxygen. These DHCl–CMs present a potential candidate both as chemoembolisation materials and for drug encapsulation, banking on the simple, effective, time-saving method that actively loads drug and avoids excess drug loss. With further research, it is also possible that the expanding–loading–shrinking method of microsphere formation can be extended to a diverse range of drugs.

Chitosan–poly(acrylic acid) hollow nanospheres

The observation of sustained drug release using chitosan DDSs is supported in another study by Hu *et al.*,[84] where chitosan–poly(acrylic acid) (CS–PAA) hollow nanospheres (118 nm in diameter) demonstrated a continuous release of entrapped doxorubicin (4.3% drug loading content) up to 10 days *in vitro* and comparable cytotoxicity against HepG2 cells compared with free doxorubicin. In this study, which aimed to identify localisation of doxorubicin nanospheres following in-vivo doxorubicin delivery (4.3 mg/kg doxorubicin equivalent) revealed a gradual decrease of doxorubicin nanospheres in the plasma compared with a rapid decrease of free doxorubicin (4.3 mg/kg body weight) over a period of 24 h. This phenomenon, which has also previously been seen,[78–80] was indicative of a prolonged circulation of doxorubicin nanospheres in the blood, resulting in considerably higher doxorubicin concentration in blood than with free doxorubicin. Hu *et al.*[84] observed high initial concentrations of doxorubicin nanospheres, which decayed rapidly in the kidney, spleen and lung, showing some correlation to the findings by Park *et al.*,[80] who reported considerable decreases in doxorubicin in the kidney, blood and liver 3 days after injection. In addition, an interesting observation was the discovery of FITC–tagged CS–PAA hollow nanospheres delivered to the brain, which has always been a hurdle for treatment because of the blood–brain barrier (BBB). The authors believe that the good mucoadhesive properties of both chitosan and PAA might have enhanced the interaction between CS–PAA nanospheres and brain microvessel endothelial cells, resulting in the transfer of CS–PAA nanospheres to the brain. The main criticism of this report is the lack of studies in a relevant tumour-bearing animal model, as the presence of a tumour would be likely to affect the biodistribution of CS–PAA nanoparticles and result in a different understanding of nanosphere deposition in a cancerous setting. Nonetheless, the authors have helped identify some potential target organs (e.g. brain, blood, liver) that might benefit from the CS–PAA DDS systems. Hu and colleagues are now embarking on further studies looking at the transfer ability of CS–PAA hollow nanospheres across the BBB.

Doxorubicin–chitosan microcapsules

Microcapsules or nanocapsules (Figure 4) are vesicular systems with a central cavity or core where a drug is confined. The core, either hydrophobic or hydrophilic, is surrounded by an outer shell polymeric membrane which allows the attachment of surface-bound targeting ligands or antibodies.[84]

Chitosan–alginate microcapsules

Chitosan–alginate microcapsules were formulated as a drug carrier/delivery system for doxorubicin.[85,86] The formulation of these microcapsules was achieved in two different ways. Firstly, a simple and easily replicable emulsification–gelation technique was used to formulate alginate–chitosan microcapsules, resulting in particles that were about 77 μm in diameter.[85] Although the resultant particles were fairly large, this gentle formulation method has an advantage as its weak electrostatic attraction means that the structure of the loaded drug is not destroyed, compared with other tougher methods used such as chemical cross-linking and heating solidification. Another method formulated chitosan multilayer microcapsules using a laborious method that required deposition of oppositely charged chitosan and alginate onto carboxylmethyl cellulose-doped $CaCO_3$ colloidal particles in a layer-by-layer fashion.[86] This was then completed by cross-linking with glutaraldehyde and decomposition of the cores by disodium ethylenediaminetetraacetic acid, producing significantly smaller microcapsules of 5 μm in diameter. Both microsphere formulations achieved good doxorubicin-loading capacities into the alginate–chitosan microcapsules. Li *et al.* reported 11% doxorubicin loading and increased EE% from 67% to 80% with the enhancement of drug/carrier ratio from 1 mg/ml to 2 mg/ml.[85] Although Zhao *et al.* did not report doxorubicin loading or EE%, the authors noticed that increased drug feeding concentration resulted in linear and non-linear increases of drug concentrations in the bulk and in the capsule interiors, respectively.[86] Subsequently, in-vitro testing of the doxorubicin-loaded microcapsules revealed a striking

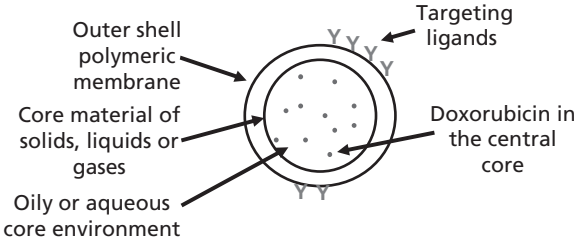

Figure 4 Structure of doxorubicin-loaded microcapsule.

inhibitory effect on the growth of three cancer cell lines (human gastric carcinoma BGC-823, liver cancer Bel-7402, cervical carcinoma HeLa) that became more distinct and significant after 48 h.[85] In addition, phase-contrast microscopy of HepG2 cells after incubation with doxorubicin-loaded microcapsules over 48 h revealed condensed chromatin and fragmented nuclei – signs of apoptosis.[86] This substantiated observations made by transmission electron microscopy of dark centres in doxorubicin-loaded microcapsules with strong fluorescent emissions from doxorubicin molecules, and also confirmed doxorubicin release data, which suggested initial rapid doxorubicin release followed by plateaued release after 6 h.[86] Similar to results reported by Kim *et al.*,[83] chemoembolisation in rabbits successfully delivered doxorubicin microcapsules to the renal artery and caused kidney embolism, with serious ischaemic necrosis after 4 weeks of embolisation.[86] This demonstrated the effectiveness of microcapsules to carry chemotherapeutic drugs for chemoembolisation and for embolisation in cancer treatment. Doxorubicin-loaded microcapsules (2 mg/kg per week for 3 weeks) injected directly into hepatic tumours was also more effective at tumour inhibition than free doxorubicin (40.3 vs 30.6%) loaded at similar concentrations.[86]

This is a ground-breaking study, as it demonstrates for the first time in animal studies, the applicability of extensively investigated multilayer microcapsules in the field of drug delivery and cancer treatment. Unfortunately, the adopted procedure for microcapsule formulation is painfully complex and time consuming, and requires some specialised equipment. Thus, this poises the emulsification–gelation method[85] as a better alternative to microcapsule formulation, unless particle size becomes an important factor in its application.

Doxorubicin–chitosan microshells

Microshells are conventionally constructed by a layer-by-layer self-assembly of oppositely charged polyelectrolytes onto dissolvable colloidal particles.[87,88] The constructed hollow shells (Figure 5) have been used for nanoscale encapsulation of drugs, proteins, dyes and nanomaterials.[89,90] Encapsulation of anti-cancer drugs in the microshells can provide a means of concentrating and protecting drug molecules as well as decreasing toxic side-effects. Furthermore, the ability to customise the microshells with tailored wall thickness, composition, size and shape presents an advantage for microshells as an ideal drug delivery vehicle.

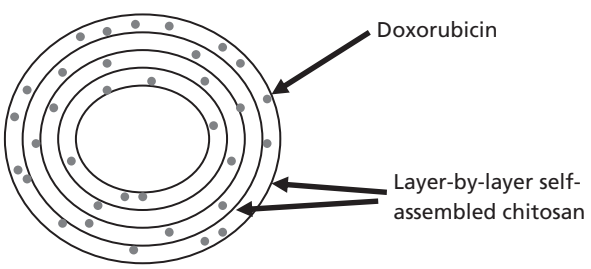

Figure 5 Structure of doxorubicin-loaded microshell.

Alginate–chitosan microshells

In a technique closely resembling formulation of microcapsules,[86] alginate sodium (ALG) and chitosan were used in an electrostatic layer-by-layer self-assembly technique to fabricate bio-polyelectrolyte microshells.[91] Tao and colleagues were able to easily and effectively load doxorubicin (2 mg/ml) into the interior of the shells in an uncomplexed manner, verified by confocal laser scanning microscopy, which yielded a high fluorescence intensity of fluorescent doxorubicin in the shells. Doxorubicin-loaded microshells were about 8 μm; the size depended largely on the number of layers of shells assembled. In addition, the group was able to successfully load another anti-cancer drug, peptide BH_3, into the ALG–chitosan (ALG–CS) shells using this same method, extending the application of ALG–CS microshells as a potential therapeutic delivery system for peptides. The results of in-vitro tests of the biological activity of doxorubicin-loaded microshells also suggested that, with the same concentration of doxorubicin, a consistently higher percent of cell kill was observed in both chemo-sensitive (H460 cell line) and chemo-resistant (A549) lung cancer cells, while no cytotoxic activity was observed with the control microshells alone. Thus, the binding of the doxorubicin-loaded microshells to the cells would effectively result in a higher local concentration of doxorubicin in the direct vicinity of the cells and account for the greater cytotoxicity observed. Some future studies could involve looking at timed release of doxorubicin from the microshells, as this DDS could potentially work to achieve sustained release of doxorubicin over extended periods of time through the degradation of each layer of the microshell, increasing circulation time for doxorubicin-loaded microshells and organ accumulation.

Doxorubicin–chitosan micelles

Micelles are spherical, globular structures consisting of hydrophobic and hydrophilic regions (Figure 6). Constituent molecules with a hydrophobic end clump together to form the central core of the sphere in a liquid environment, while hydrophilic ends of the molecules are in contact with the surrounding liquid environment, forming a mantle.[92] The hydrophobic central cores of micelles are traditionally used for delivery of water-insoluble drugs in DDSs, whereas modification of the hydrophilic shell affects pharmacokinetic

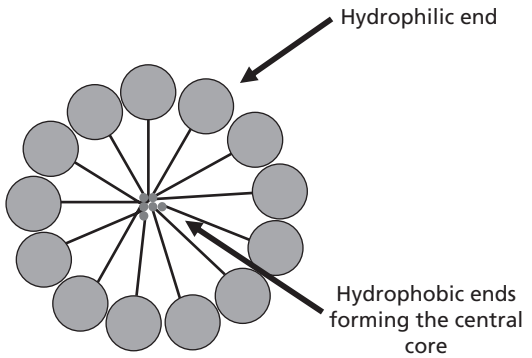

Figure 6 Structure of doxorubicin-loaded micelle.

behaviour.[93,94] Polymeric micelles are advantageous as DDSs as they exhibit prolonged circulation, tumour localisation by the EPR effect and possibly controlled drug release.[57,95,96]

Chitosan moieties were modified in two ways: addition of a long-chain alkyl group, -N-succinyl-N′-octyl,[97] and chemical conjugation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC),[98,99] to allow self-aggregation for the formation of chitosan micelles.

N-succinyl-N′-octyl chitosan micelles

^{1}H NMR spectra analysis and thermograph results of N-succinyl-N′-octyl chitosan (SOC) showed that amino groups of chitosan were substituted by octyl and succinyl groups.[97] EE% and doxorubicin loading were dependent on the drug-to-carrier ratio, achieving 76.9% EE and 36.4% doxorubicin loading at the optimum drug : carrier ratio of 0.8 : 1. Doxorubicin loading was also significantly affected by the octyl amount: the higher the amount of octyl chain, the higher the EE% and doxorubicin loading, as well as an increase in particle size (from 130 nm to 170 nm in diameter). The authors also observed sustained release of doxorubicin over a period of 16 days, with higher octyl-content micelles displaying a decreased release rate. Observations from flow cytometry showed that doxorubicin-loaded micelles all emitted higher fluorescent intensity in contrast with free doxorubicin, suggesting that doxorubicin-loaded micelles were taken up by cells to a much greater extent than free doxorubicin. This observation was supported by in-vitro cytotoxicity tests of SOC on four different cancer cell lines (HepG2, A549, BGC, K562), which established that the 50% inhibitory concentration of doxorubicin-loaded micelles displayed a three- (HepG2 and A549) to five-fold (BGC and K562) increase in cytotoxicity in these cancer cell lines compared with free doxorubicin. Xu *et al.* also postulated that doxorubicin-loaded micelles could become more cytotoxic against cancer cells when they were incubated with cancer cells over a longer period (> 3 days).[97]

Stearic acid-grafted chitosan oligosaccharide micelles

Hu *et al.*[98] and Ye *et al.*,[99] both from the same group, investigated the potential of stearic acid-grafted chitosan oligosaccharide (CSO–SA) as a potential DDS for doxorubicin. Glutaraldehyde cross-linked CSO–SA micelles loaded with doxorubicin (30 nm in diameter) were postulated to reduce initial drug burst release from micelles and improved the existing characteristics of micelles. Although cross-linking did not produce much effect on the micelle size and EE%, it was effective in decreasing the drug burst (31% unmodified micelles vs 22% crosslinked micelles at time 0) and promoting sustained drug release over a period of 24 h. Still, both cross-linked and unmodified doxorubicin-loaded CSO–SA micelles obtained 93% EE, a positive ζ-potential (51.8 vs 69.1%) but a less impressive 1.67% doxorubicin loading. Nonetheless, it was shown that doxorubicin-loaded micelles (across a concentration range of 0–4 μg/ml doxorubicin micelles) were more effective at inducing cell death than free doxorubicin (across a concentration range of 0–4 μg/ml doxorubicin) in three different cells lines (A549, Lewis lung carcinoma LCC and human ovarian cancer cells SKOV3). Here, doxorubicin-loaded glutaraldehyde cross-linked CSO–SA significantly improved the cytotoxicity in the chemoresistant SKOV3 cell line, indicating the improved uptake of doxorubicin into the cells. This could be attributed to the spatial structure of the multi-hydrophobic core, which allows the CSO–SA micelles to be rapidly internalised into tumour cells, providing an enhanced delivery of doxorubicin with decreased toxicity. If further verified, this finding would potentially be useful in treating chemoresistant tumours that no longer respond to conventional chemotherapy.

Using a similar formulation method to Hu *et al.*,[98] further characterisation was conducted to study the effect of incorporation of stearic acid (SA) into CSO–SA micelles using doxorubicin as a model drug.[99] As a result of increased amount of SA in the formulation (8 mg in Hu *et al.*[98] vs 0.2 g in Ye *et al.*[99]), micelle size dropped by more than 10 nm for both doxorubicin-loaded and empty CSO–SA micelles. However, further characterisation, which involved varying the ratio of SA to CSO–SA, showed that increasing SA ratios resulted in larger micelles, up from 20 nm to 80 nm, accompanied by a decrease in ζ-potential. Doxorubicin-loaded micelles were also reported to be smaller than empty micelles,[99] which was in contrast to previous observations;[98] the authors attributed this effect to van der Waals interactions between doxorubicin and CSO–SA molecules. Varying the pH from 7.2 to 2.7 also yielded an increase in micelle size from 30 nm to 250 nm. The effect of varying the SA : CSO–SA ratio had no significant effect on EE% and drug loading, as all formulations maintained consistent EE% (about 50%) and drug loading (5%). Drug release profiles of doxorubicin-loaded CSO–SA micelles were similar to an earlier report,[100] supporting observations that drug release rates reduced with increasing pH, where the fastest release occurred at pH 2.7 (70% in 8 hours) and the slowest drug release at pH 7.2 (30% in 8 hours). This release sustained over a period of time is again a desirable feature for DDSs, as it will allow consistent drug release at target organs instead of immediate dumping of drugs, which might cause toxicity.

Chitosan sheets/films

In their seminal study involving chitosan sheets, Saito *et al.*[101] used a simple agglutination technique to develop pure flat, flexible chitosan sheets in order to explore the feasibility of chitosan as a drug carrier in topical lesions. Under phase-contrast microscopy, the sheet appeared as a knitted stitch with fine fibrous structure. The presence of uneven apertures also suggested the possibility of air and water permeation. The smooth and flexible texture of the chitosan sheet is likened to a sponge or gauze that would be easily handled in medical applications. Thus, a doxorubicin-containing chitosan sheet (1 mg/ml) was inserted into the peritoneal cavity of a mouse to study its degradation. Scanning electron microscopy showed initial degradation of the doxorubicin-containing sheet after 24 h; the sheets gradually degraded into lumps of particulates after several months. Fluorescence microscopy also confirmed doxorubicin adsorption by chitosan. Drug release studies confirmed that doxorubicin was released from the sheet, as doxorubicin

Table 2 Characteristics of the various doxorubicin-loaded chitosan nanoparticles

Drug delivery system	Type of carrier	Formulation procedure	Size of particles and ζ-potential	Encapsulation efficiency (EE%)	Drug loading (%)	Reference
Nanoparticles	Chitosan–dextran sulfate nanoparticles	Ionotropic gelation with sodium tripolyphosphate and dextran sulfate	213 nm +33.7 mV	About 20%	0.43% (w/w)	Janes *et al.* 2001[77]
		Reverse microemulsion using surfactant sodium bis(ethyl hexyl) sulfosuccinate	100 nm NA	60–65%	NA	Mitra *et al.* 2001[78]
	Oleoyl-chitosan (OCH) nanoparticles	Hydrophobic modification of chitosan with oleoyl chloride followed by oil/water emulsification with doxorubicin	315.2 nm NA	52.6%	NA	Zhang *et al.* 2007[81]
Nanoaggregates	Glycol–chitosan nanoaggregates	Synthetic conjugation of doxorubicin to glycol–chitosan via *N-cis*-aconitic anhydride followed by doxorubicin entrapment by oil-in-water emulsion method	342 nm NA	97.2%	38.9%	Son *et al.* 2003, Park *et al.* 2006[79,80]
Microspheres	Doxorubicin hydrochloride-containing chitosan microspheres	Expanding–loading–shrinking method for doxorubicin hydrochloride trapping in chitosan microspheres	NA	NA	10% (w/w)	Kim *et al.* 2007[83]
Nanospheres	Doxorubicin-loaded chitosan-poly (acrylic acid) hollow nanospheres	Polymerisation of acrylic acid monomers in the presence of chitosan, followed by selective cross-linking using glutaraldehyde	118 nm +20 mV	68.9%	4.3%	Hu *et al.* 2007[84]
Microcapsules	Doxorubicin-loaded alginate–chitosan microcapsules	Emulsification–gelation method using sodium alginate	About 77 μm; NA	Increases from 67.03% to 80.46% during corresponding increase of drug/carrier ratio from 1 to 2 mg/ml	11% (w/w)	Li *et al.* 2002[85]
	Doxorubicin-loaded hollow chitosan-alginate multilayer microcapsules	Deposition of chitosan and alginate onto carboxymethyl cellulose-doped $CaCO_3$ colloidal particles in a layer-by-layer fashion, followed by cross-linking with glutaraldehyde and decomposition of the cores by sodium ethylenediaminetetraacetic acid	5.3 μm	[DOX] within microcapsules increased from 6.2 to 132 mg/ml when DOX feeding concentration increased from 50 to 2000 μg/ml	NA	Zhao *et al.* 2007[86]
Microshells	Self-assembled biopolyelectrolyte microshells	Colloidal templating layer-by-layer technique with melamine formaldehyde particles	8 μm	1.5×10^8 doxorubicin molecules in one capsule of four alginate/chitosan layers	1.5–1.8 $\times 10^{13}$ g doxorubicin per capsule	Tao *et al.* 2007[91]
Micelles	Doxorubicin-loaded amphiphilic stearic acid-grafted chitosan oligosaccharide polymer micelles	Synthesised via the reaction of carboxyl groups of stearic acid with amine groups of chitosan in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide Subsequent glutaraldehyde cross-linking for certain micelle samples	40.7 nm, +69.1 mV unmodified micelles; 30.4 nm, 51.8 mV glutaraldehyde-crosslinked micelles	93.7% unmodified micelles; 93.4% glutaraldehyde-crosslinked micelles	1.67% both unmodified and glutaraldehyde-crosslinked micelles	Hu *et al.* 2008[98]
		As above, with varying stearic acid concentrations and no glutaraldehyde cross-linking	20.4 nm, 53.1 mV	50.92–56.21%, based on stearic acid: chitosan ratio	4.86–5.51%, based on stearic acid : chitosan ratio	Ye *et al.* 2008[99]
	Doxorubicin-loaded N-succinyl-N′-octyl chitosan micelles	Chitosan moiety modified with long chain alkyl group followed by doxorubicin loading with triethylamine and DMF	170.1 nm	76.9% under 0.8 : 1.0 optimal ratio of drug/polymer (w/w)	36.4% under 0.8 : 1.0 optimal ratio of drug/polymer (w/w)	Xu *et al.* 2007[97]
Sheet/film	Chitosan sheet	Chitosan suspension subjected to acid–alkaline treatment, mixed with doxorubicin, frozen and freeze-dried	Smooth, flexible sheet with knit-like, fibrous structure	NA	NA	Saito *et al.* 2006[101]

DOX, doxorubicin; NA, not available.

was detected in the urine and liver while doxorubicin metabolites were detected in blood 2 weeks after doxorubicin-containing sheets were placed into the peritoneal cavity of mice. In addition, studies to determine residual doxorubicin in chitosan sheets after in-vivo application revealed that doxorubicin content in the sheets decreased gradually, indicating that doxorubicin was released into tissues. Doxorubicin (0.5–1 μg/g) was also detected in chitosan sheets up to 2 months after in-vivo application, suggesting the ability to maintain the pharmacological effects of absorbed doxorubicin over an extended period. Although the authors were unable to ascertain the rate of doxorubicin release and mechanism of chitosan degradation, they showed that the biodegradable chitosan sheet appeared to decompose and release doxorubicin, resulting in fragile sheets and particulates several months later. Hopefully this group will do some studies looking at how delivering doxorubicin through chitosan sheets might decrease toxicity and also further characterise their doxorubicin-containing chitosan sheet system to improve drug loading capacity and possibly delay chitosan degradation.

Conclusions and future directions

Drawing on conclusions from the various papers described above, chitosan formulated in nanoparticles, microcapsules, microspheres, micelles and films has shown great potential for encapsulating doxorubicin. The encapsulation efficiencies of doxorubicin into chitosan were reported to be about 50% in SA-grafted chitosan oligosaccharide micelles[99] but was increased to 93% when glutaraldehyde cross-linking was performed.[98] Glycol–chitosan nanoparticles have shown the best encapsulation efficiency of 97%,[79] which might be a better option as far as chitosan DDSs are concerned, compared with oleoyl-chitosan nanoparticles which only managed an EE of 52.6%. The process of doxorubicin encapsulation did not affect doxorubicin's cytotoxic properties; instead studies have shown that doxorubicin encapsulation into chitosan had improved doxorubicin's cytotoxicity both *in vitro*[81,91,97,98] and *in vitro*.[78,79,83,85,86] Conversely some groups found that doxorubicin–chitosan technology proved to be only as efficacious as doxorubicin solution alone.[77,80,84]

Administration of doxorubicin–chitosan DDSs has prevailed over doxorubicin solution in ways such as lowered systemic toxicity,[78–81,85] and sustained, controlled release of doxorubicin at the lesion site,[80,81,84,97–99,101] which counters problems of inappropriate drug loads. High concentrations of doxorubicin–chitosan nanoaggregates were also found to be in circulation for up to 8 days,[79] while a novel doxorubicin–conjugated chitosan sheet technology was shown to release doxorubicin and doxorubicin metabolites into the surrounding tissues, blood and urine for up to 2 weeks;[101] this was in comparison with doxorubicin solution which has a short half-life of several hours. Apart from distribution in the kidney, liver, lungs, blood, spleen and tumours,[79,80,101] confocal laser scanning microscopy also detected, for the first time, distribution of doxorubicin-loaded nanospheres in the brain, paving the way for future research and treatments that are currently hindered by the BBB.[84] These studies have also speculated on the potential of chitosan technology as a viable DDS for other cytotoxic drugs.

It is difficult to conclude which method might be the most effective way of doxorubicin encapsulation, as the various methods have their advantages and disadvantages. The most suitable DDS will depend on its intended use and the disease model. Table 2 summarises the different particles presented in this review to enable scientists to further explore particles that might suit their purposes. It is crucial to embark on more in-vivo studies, aiming to improve and better characterise the current technologies in order to achieve more consistent, reliable results that can potentially be translated into clinical studies.

Conflict of interest

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

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