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Biomedical applications and colloidal properties of amphiphilically modified chitosan hybrids

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ABSTRACT

Chitosan is among the most abundant biopolymers on earth and has been either used or exhibited potential in a wide variety of industrial and biomedical applications. With the advancement of materials technologies, chitosan has been chemically modified to self-assemble into nanoarchitectures that are usable in advanced biomedical applications, such as drug nanocarriers, macroscopic injectables, tissue-engineering scaffolds, and nanoimaging agents. Colloidal amphiphilically modified chitosan (AMC) is a relatively recent material receiving increased attention with numerous publications addressing the medical advantages of specific systems. To date, many reviews have focused on the synthesis and biomedical properties of chitosan-based biomaterials, but a comprehensive study focusing on the colloidal properties of AMC in relation to biomedical performance appears to be lacking. This review provides a survey of the field, critically reviewing the colloidal properties and biomedical performance of AMC systems, such as nanoparticle drug delivery systems and macroscopic medical devices. Finally, the future development, market potential, and clinical implications of these promising colloidal-structured biomaterials are summarised.

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1. Introduction

Recent advancements in medicine combined with improvements in understanding fundamental cellular and molecular biological processes have led to an increased demand for materials that can fulfil specific functions to improve therapeutic effects. In particular, nanoparticles and nano-microstructured materials can allow for functionalities that are not achievable through either the administration of free drugs or the use of materials that lack critical structural characteristics. Nanotechnology can use engineered molecules and/or supra-molecular entities to form assemblies on the scale of individual cells, organelles, or even smaller components [1]. Polymeric nanoparticles and structures have been heavily investigated and shown great potential in numerous medical applications [2–6]. The synthesis of "smart" molecules and the control over molecular self-organisation into well-defined nanostructures are current areas of research, as the colloidal properties of these materials can have dramatic effects on both properties and performance in various applications. For medical applications, increased efforts have been made to prepare biodegradable and non-toxic polymeric amphiphiles based on natural polymers, such as polysaccharides.

Chitosan is a polysaccharide that has been extensively investigated for biomedical applications, with many reports on modified chitosan exhibiting new functionalities [7–15]. Multiple reports on the modification of chitosan and promise for utilisation in pharmaceutics and biomedicine are available in the literature. Amphiphilic modifications that promote self-assembly into nanoparticles and/or provide increased interactions with lipid structures, such as cell membranes, are of particular interest. As colloidal properties are important for therapeutic effects, as often discussed in individual papers, a comprehensive review focusing on the colloidal behaviour of AMC nanoparticles, the assembly of these entities into gels and films, and their effectiveness in biomedical applications is needed. This review provides a condensed source of new insights and an improved understanding on the role and prospects of colloidal AMC in biomedical applications, critically important for advancing current promising research into clinical practices. This review begins with a brief overview on the synthesis of AMC followed by a critical examination of the literature on AMC in biomedical applications. Reviewing multiple applications, the examined materials ranged from nanoparticles to macroscopic assemblies, including the delivery of conventional drugs, proteins/peptides and genes, as well as the use of carriers for contrast agents, and nanostructured materials for wound dressings and cell scaffolds. Amphiphilic chitosan-inorganic hybrids are also included, as this newly developed material class has exhibited potential for applications involving sensing, imaging, diagnosis, and multifunctional drug delivery processes. This review summarises the most important papers in the field of this highly versatile polymer class and reflects over their relevance in potential applications to facilitate the translation of these research initiatives into clinical benefits.

2. Amphiphilically modified chitosan

2.1. General information on chitosan

Chitin, the precursor of chitosan, is a biopolymer composed of N-acetylglucosamine units linked by β-1,4 linkages and is among the most abundant biopolymers, clearly outranked only by cellulose. Chitosan is formed by extensive deacetylation of chitin, forming amino groups at the C2 carbon (Fig. 1). These groups accept protons, dramatically increasing the solubility of chitosan even at slightly acidic pH, compared to chitin. The positive charge of chitosan has generated great interest in biomedical research for the possible interactions of this polymer with negatively charged biomolecules and therapeutic substances.

Numerous reports on the promising uses of chitosan in medical applications, both as macroscopic materials and as nanocarriers, are available in the literature, suggesting that chitosan has antibacterial, biodegradable, biocompatible, and mucoadhesive properties with reasonable biocompatibility for biomedical uses [8,11,12,14,16,17]. Chitosan has also been shown to transiently open the tight junctions between cells [18,19]. However, complications have been reported for chitosan based materials. In a study by Skogh and Engstrand, a heparin-chitosan sponge used as a carrier for recombinant human BMP-2 in cranial repair generated inflammatory reactions [20]. Clinically relevant biodegradation may vary with chitosan properties and the site of degradation. The chitosan prepared from chitin has varying degrees of deacetylation. Together with molecular weight (Mw), this degree of deacetylation determines the properties, colloidal behaviours, and biological performances [11,16,17,21–23], which in turn will affect biomedical performance. Control over these two parameters is critical. One approach to overcome variations in properties associated with the degree of deacetylation (or at least make the formulations more robust) could be to attach groups with

Nomenclature

AMC	Amphiphilically modified chitosan (chitosan modified to enhance the amphiphilic characteristics of the polymer)
BSCB	Blood-spinal cord barrier
CAC	Critical aggregation concentration
CHC	Carboxymethyl-hexanoyl chitosan
CPT	Camptothecin
DCMC	Deoxycholic acid modified carboxymethylated chitosan
DOX	Doxorubicin
ECPs	Extracellular products
EPR	The enhanced permeability and retention effect
G-CS-DCA	Glycidol-chitosan-deoxycholic acid
Gene delivery	Used for the delivery of genetic materials, including DNA, RNA and modified analogues
GP	Glycerophosphate
HAp	Hydroxyapatite
HGC	Hydrophobically modified glycol chitosan
hm-chitosan	Hydrophobically modified chitosan
HTCC	N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride
ICPTES	3-isocyanatopropyltriethoxysilane
iPSCs	Induced pluripotent stem cells
LA	Linoleic acid
LC	Loading capacity
LCC	N-lauryl-carboxymethyl chitosan
LE	Loading efficiency
LMC	Linoleic acid/poly(β-malic acid) double grafted chitosan
LSC	Lauroyl sulfated chitosan
MDR	Multidrug resistance
MPC	Methylpyrrolidone chitosan
MRI	Magnetic resonance imaging
Mw	Molecular weight
Nanocarrier	Nanoparticles loaded with cargo
Nanoparticle	Any nano-sized object (<1000 nm), irrespective of actual structure
NIR	Near-infrared
NMPCS	N-methylene phosphonic chitosan
NOSC	N-octyl-O-sulfate chitosan
N/P	The ratio of chitosan nitrogen to DNA phosphorus
NSC	N-succinyl-chitosan
OCMCS	Oleoyl-carboxymethyl chitosan
OcCMC	N-octyl-N,O-carboxymethyl chitosan
OQLCS	Octadecyl-quaternised lysine modified chitosan
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PMLA	Poly(β-malic acid)
PTX	Paclitaxel
PVP	Polyvinylpyrrolidone
SCI	Spinal cord injury
SOC	N-succinyl-N′-octyl chitosan
SPIONs	Superparamagnetic iron oxide nanocrystals
TEOS	Tetraethyl orthosilicate
ZrP	α-zirconium hydrogen phosphate hydrate

dominating characteristics, such as strong hydrophilicity and/or hydrophobicity, depending on the desired results.

2.2. Preparation of amphiphilically modified chitosan

Chitosan can be readily modified using the C2 amino groups as well as the C3 and C6 hydroxyl groups, with the reactivity of C6 > C3 for polysaccharide reactions [24]. Extensive work has been conducted on the modifications of chitosan, as summarised in several excellent reviews covering this topic [12,14,21].

Modifications of chitosan with both hydrophobic and hydrophilic groups were first reported in 1995 by Yoshioka et al. [25]. Hydrophobic alkyl chains of various lengths were attached to the amino groups and hydrophilic sulfate groups were attached to the hydroxyl groups, producing a modified chitosan that formed polymeric micelles. Miwa et al. synthesised N-lauryl-carboxymethyl-chitosan (LCC) by attaching hydrophobic lauryl groups to the amino groups and hydrophilic carboxymethyl groups to the hydroxyl groups [26], also forming polymeric micelles. Based on these findings, preparations of AMC are often performed with a hydrophilic modification by carboxymethylation to increase water solubility [10] and with additional modifications using hydrophobic groups, such as cholesterol [27], lauryl aldehyde [26], linoleic acid (LA) [28,29], deoxycholic acid [30], and hexanoyl anhydride [31–33]. With an extensive number of possible configurations, the general modification strategies typically include reactions with hydrophilic groups to increase water solubility and with hydrophobic groups to facilitate the self-assembly into nanostructures as well as to improve the interactions with bio-relevant lipid structures, such as cell membranes. Modifications of chitosan can dramatically change the physiochemical characteristics and the degradation products of these polymers, suggesting that there is great need to characterise both the physiochemical properties and the medical safety of these new chitosan derivatives.

2.3. Colloidal properties of amphiphilically modified chitosan

Self-assembled colloids have shown great potential in numerous applications, with an ease of production, regularity in both size and shape, and the presence of a core, a bi-layer, or nano-domains with polar properties that are opposite those of the solvent. Particle size can also be important, as this characteristic influences both cell internalisation and clearance from circulation [34–36]. Literature guidance often states that nanoparticles should be sized smaller than the sinusoid in the spleen and the fenestra of the liver (150–200 nm) to avoid clearance and that passive tumour targeting through the enhanced permeation and retention effect (EPR) occurs through gaps

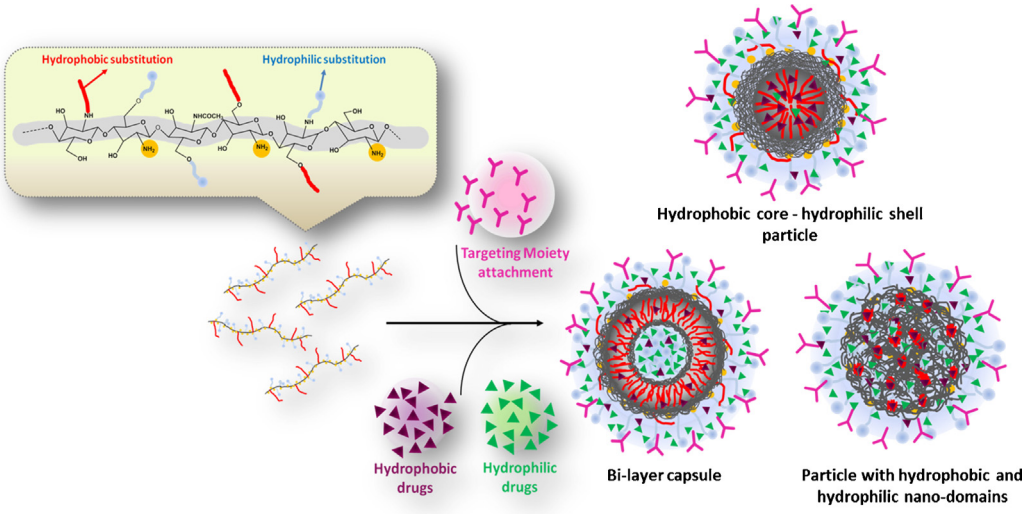

Fig. 1. Schematic drawing illustrating the structure of AMC, with possible sites for hydrophilic and hydrophobic modifications. AMC can self-assemble into nanoparticles. Conceptual nanoparticle structures are presented, including a hydrophobic core - hydrophilic shell particle, a bi-layer capsule and a particle with hydrophobic and hydrophilic nano-domains. The nanoparticles can be loaded with drugs, which will distribute to minimise the chemical potential. The nanoparticles can carry surface modifications for targeting. The hydrophobic and hydrophilic modifications can also be groups that induce functionality, such as targeting or imaging capability. For visual clarity, the components in the figure are not depicted at scale.

of 100–600 nm [37]. However, the authors want to suggest that polymeric nanoparticles commonly are deformable, so increased sizes may pass through pore sizes that might nominally exclude the nanoparticles, as reported by Chen and Weiss for red blood cells in the spleen [38]. The surface of nanoparticles is often characterised in terms of the zeta potential, with an absolute value >25 mV suggesting good stability in aqueous solutions. *In vivo*, charged particles interact with biomolecules and cells [35,36,39], thus the zeta-potential is a relevant property. However, these electrostatic interactions are complex, making the *in vivo* stability difficult to predict from the zeta-potential values.

The nanostructure of self-assembled colloids allows for the incorporation of molecules into regions leading to reduced chemical potentials, surrounded by a barrier with different polar properties. The nanoparticle surface exposed to the surroundings can be modified for specific needs, including targeting applications [40–42], the avoidance of protein adsorption, phagocyte uptake, particle aggregation and blood clotting in the blood stream, and a prolongation of the circulation time [43–45] (see Fig. 1).

Liposomes and low-molecular micelles have been widely studied for pharmaceutical and biotechnological applications, such as drug delivery, gene therapy, protein delivery, and cell targeting [46]. These nanostructures have often been found to be structurally unstable under relevant conditions [25], and a current challenge is to overcome the fast elimination of these nanoparticles through the reticulo-endothelial system [47].

Self-assembled polymeric micelles of AMC have been proposed to be more stable than low Mw surfactant based micelles [25], producing an improvement in a desired property for many applications. Following the reporting of these results, colloidal properties and applications of AMC have generated increased attention in the research community. Typically, new molecules are characterised with regard to physiochemical properties, such as critical aggregation concentration (CAC), particle sizes, zeta-potentials, and (if possible) nano-microstructural characteristics, with the properties depending on both the nature and degree of substitution. The polarity map and steric properties change upon modification, and electrostatic contributions can also be introduced. In particular, acidic substituents allow for pH dependent interactions with the amino groups. The range and complexity of the structure-property relationships in various AMCs make simplifications difficult, but several noteworthy comments can be made.

Commonly, AMCs and other self-associating polymers are assumed to have a hydrophobic core-hydrophilic shell structure. Hydrophobic domains may also be interspersed through the particle, especially for short hydrophobic substituting groups. Korchagina and Philippova [48] proposed this type of structure for nanoparticles from chitosan modified with n-dodecyl side chains, suggesting a nanogel structure with hydrophobic domains at the crosslinks. With regards to nanocapsules, Liu et al. [33,49] and Li et al. [50] reported AMCs that self-assembled into either compact core-shell or hollow bi-layered nanoparticles, depending on the specific molecular characteristics (see schematic drawing in Fig. 1). For the system investigated by Liu et al., alkyl chains constituted the hydrophobic groups, and the transition from a compact core to hollow capsules occurred with increasing hydrophobicity. In contrast, Li et al., reported that increasing the grafting of the hydrophobic group poly(L-lactic acid) produced a decrease in the fraction of the hollow capsules. There should be cases when particles with compact core, particles with interspersed nano-domains and hollow capsules behave differently and offer structure specific opportunities, such as loading of trans-membrane structures and loading of hydrophilic substances. However, since the distinction between different structures is seldom reported in

biomedical studies using AMC, such distinction will not be made in the general discussions of this review.

The self-assembly of AMC is driven by the orientation of hydrophobic groups to form hydrophobic domains with the hydrophilic groups orientated towards the aqueous environment. Generally, CAC decreases with increasing degree of hydrophobic substitution and size of the hydrophobic groups, as reported in the literature [29,33,50–53].

A broad range of sizes has been reported for AMC particles, ranging from tens of nanometres [54,55] up to the micrometre scale [50], with the majority of reports presenting values in the interval of 100–500 nm. The size of the formed nanoparticles can depend on the polymer backbone length, the nature of the attached groups and the degree of substitution. Preparation conditions can also greatly influence the self-aggregation results, especially because polymer aggregate systems can form kinetically trapped structures. For AMC very different dependences of nanoparticle size on the different parameters have been reported. For example, Kim et al. reported that for nanoparticles of deoxycholic-acid modified chitosan, an increase of Mw from 5 to 200 kDa led to an increase in nanoparticle sizes from 130 nm to 300 nm [56]. Park et al. reported that for nanoparticles of glycol chitosan modified with cholanic acid, the size after self-aggregation only increased slightly with Mw values ranging from 20 to 250 kDa [57]. Similarly, Korchagina and Philippova reported that the aggregate sizes in dilute solution of both chitosan and hydrophobically modified chitosan were independent of Mw in the range 55–150 kDa, suggesting that the number of associating groups was the determining factor [48]. The extent of broadening of the interval without affecting the size of the aggregates would be of interest, as well as a determination of the effects of more bio-relevant solutions.

For hydrophobic modifications there have been multiple reports on how the length and degree of substitution influence the nanoparticle size. With increasing hydrophobic modifications, both increased [48,58,59] and decreased [51–53,60,61] sizes, as well little effect [33] and more complicated dependences [55] have been reported in the literature. The incorporation of drug substances can also influence nanoparticle size.

For zeta-potentials, the effects are somewhat less complicated. Typically, the characteristics of AMC can be associated with two situations: (i) the native amino groups will be the dominating charge contributors, suggesting that the zeta-potential is expected to be positive and to decrease with the neutral or acidic substitutions that are either attached to the amino nitrogen or electrostatically interacting with the charged amino groups; (ii) the dominating charge contributors are the substituting groups, which can be either positive or negative, with the corresponding zeta-potential increasing with increasing degree of substitution. With the pH dependence of the amino groups and the other substituents, electrostatic interactions with the loaded molecules and the structural orientation may increase the complexity of the zeta-potential behaviour.

In summary, for a given AMC system, both particle size and surface properties can be tuned by changing the type and degree of chemical modification. The dependences of the polymer characteristics with nanoparticle size and surface properties are complicated, suggesting that each system is unique.

3. Colloidal amphiphilically modified chitosan in biomedical applications

The investigation of AMC for biomedical applications has increased in the last two decades, with intensification of efforts in recent years. The literature on AMC nanoparticles for drug, protein, gene delivery and imaging, as well as macroscopic nano-microstructured AMC for biomedical applications is reviewed in this work. An informative list of multiple AMC, colloidal structures and biomedical applications is given in Table 1.

3.1. Nanoparticles

3.1.1. Delivery of conventional drugs

Commonly, hydrophobic domains of amphiphilic self-assembled nanoparticles can provide an effective loading space for hydrophobic moieties, such as drugs, fluorescent probes, and contrast agents, with the hydrophilic surface allowing for suspension stability in aqueous solutions [57,62]. The loading of hydrophilic moieties is also possible (see conceptual drawings in Fig. 1). Nanocarriers have applications in a number of medical applications, such as cancer therapy. One current challenge relates to the low water solubility of many drugs, such as Paclitaxel (PTX) [63], Camptothecin (CPT) [64], and Doxorubicin (DOX) [65]. In addition to improved solubility, the loading of drug into nanocarriers can offer a range of benefits, including increased time in circulation, passive targeting through the EPR, protection of the drug from degradation, reduced drug related side effects, and the active targeting by surface modifications. The nanocarriers can also be designed to respond to external stimuli, such as pH, light, magnetic fields, etc. [4–6,21,66–71]. To summarise, an ideal polymeric nanoparticle matrix for drug delivery would exhibit the following characteristics: (i) allow for the incorporation of the drug into the nanoparticles; (ii) provide protection of the drug from enzyme degradation; (iii) facilitate cellular uptake in target cells; (iv) release drug at the site of action (i.e., to increase the local drug concentration and prolong the duration of drug activity); (v) decrease drug toxicities; and (vi) provide low manufacturing costs. To achieve these properties, chitosan has been modified to produce nanoparticles and widely investigated for use as a drug carrier [2,12–14,72].

One promising class of materials that has been developed is AMC. Numerous studies have demonstrated the self-assembly of this material into nanoparticles with excellent drug loading capacities, modified release properties, and good colloidal stability under physiological conditions, as discussed below. With excellent colloidal stability for well-encapsulated therapeutic substances, AMC formulations are highly promising for practical drug delivery uses, especially for modified polymers that retain the biocompatibility of chitosan.

Miwa et al. reported in 1998 that PTX loaded nanocarriers of chitosan derivatives with both hydrophobic and

Table 1
List of selected AMCs with colloidal properties and biomedical applications.

AMC	Colloidal properties	Biomedical applications	References
Acylated chitosan	Nanoparticles, spun nanofibres, micro porous sponge, forming AC-cell gel network.	Drug delivery, gene delivery, cell scaffold, wound dressings	[95,131,190,191,199]
Acylated carboxymethyl chitosan	Nanoparticles	Drug delivery	[79]
Alkyl chain-modified succinyl chitosan	Nanoparticles	Drug delivery	[96]
Benzene-n-octadecyl-chitosan	Micro porous sponge, forming benzene-n-octadecyl-chitosan-cell gel network	Wound dressing	[200]
Carboxymethyl chitosan-6-mercaptopurine	pH dependent nanoparticle formation	Drug delivery	[72]
Carboxymethyl-hexanoyl chitosan	Nanocapsules, nanoparticles, shell constituent in core-shell nanoparticles, nano-microstructured macroscopic gels	Drug delivery, injectable depot gels, wound dressings, injectable cell scaffolds	[29,31–33,97,169,192,202]
Carboxymethyl-hexanoyl chitosan –Silica	Multilayered nanoparticles	Drug delivery	[223]
Chitosan-silica hybrids	Silica core-chitosan shell nanoparticles, nanostructured membrane	Biomaterials, bone regeneration and separation membranes	[219–221]
Deoxycholic acid modified carboxymethylated chitosan	Nanoparticles	Drug delivery	[30]
Deoxycholic acid modified chitosan	Nanoparticles	Gene delivery	[56]
Deoxycholic acid-N, O-hydroxyethyl chitosan	Nanoparticles	Drug delivery	[88]
DOX-conjugated stearic acid-g-chitosan	Nanoparticles	Drug delivery	[89]
Folate-decorated succinylchitosan	Nanoparticles	Drug delivery	[54]
Glycidol-chitosan-deoxycholic acid	Nanoparticle	Drug delivery	[58]
Hydrophobically modified glycol chitosan	Nanoparticles	Drug delivery, gene delivery, imaging and diagnosis	[52,53,57,74–78,92,134,156]
Hydrophobically modified glycol chitosan-Cy5.5-RGD_peptides	Nanoparticles	Protein delivery, Imaging and diagnosis system	[154,155]
Hydroxypropyl chitosan	Micro-nanostructured organic inorganic hybrid network together with ethylene glycol functionalised nanohydroxyapatite	Cell scaffold	[210]
Lauroyl sulfated chitosan	Nanoparticles	Drug delivery, protein delivery	[100,101]
Linoleic acid-carboxymethyl chitosan	Nanoparticles	Drug delivery	[28,29]
Linoleic acid grafted chitosan oligosaccharides	Nanoparticles	Drug delivery	[85]
Linoleic acid/poly(β-malic acid) double grafted chitosan	Nanoparticles	Drug delivery	[61]
Methylpyrrolidone chitosan	Gel forming freeze dried sponge	Wound healing	[201]
N-alkyl-N-trimethyl chitosan	Nanoparticles	Drug delivery	[55]
N-alkyl-O-sulfate chitosan	Nanoparticles	Drug delivery	[91]
N-lauryl-carboxymethyl chitosan	Nanoparticles	Drug delivery	[26]
N-methylen phosphonic chitosan	Nanoparticles	Gene delivery	[136]
N-octyl-O-sulfate chitosan	Nanoparticles	Drug delivery	[80,81,90,98]
O-carboxymethyl-chitosan-methotrexate	Nanoparticles	Drug delivery	[227]
Octadecyl-quaternised lysine modified chitosan	Nanoparticles together with cholesterol, multi-lamellar structure, could have folate-PEG coating	Drug delivery	[42]
Oleoyl-carboxymethyl chitosan	Nanoparticles	Protein delivery	[99]
PEGylated amphiphilic octadecyl quaternised carboxymethyl chitosan	Nanoparticles	Protein delivery	[42]
SPIONs-loaded water soluble chitosan-linoleic acid nanoparticles	Nanoparticles	Imaging and diagnosis	[144]

hydrophilic modification (i.e., LCC) inhibited human epidermoid carcinoma cell growth better than the free PTX [26]. The nanoparticles had sizes less than 100 nm, zeta-potentials between −25.5 and −20 mV, and low haemolytic effects. The carriers have been effective at the passive targeting of tumours. Following this discovery, numerous reports on AMC as a drug nanocarrier have been made. In 2003, Kwon et al. reported on self-assembled nanoparticles of hydrophobically modified glycol chitosan (HGC) prepared by the attachment of 5β-cholanic acid to glycol chitosan [60]. The nanoparticles sizes decreased from 850 to 210 nm with increasing hydrophobic modifications, which was explained by the formation of a more compact core. The HGC exhibited significantly lower CAC than low Mw surfactants, indicating improved stability. In subsequent studies, HGC was effectively loaded with cisplatin [73], docetaxel [74], DOX [75,76], PTX [76], and CPT [77]. The nanocarriers provided a sustained release of the drug substance for times exceeding one week and protected the drug from degradation. Antitumor efficiency was improved in animal models, with a corresponding reduction in toxicity. The improved properties were attributed mainly to the sustained release and the passive targeting through the EPR-effect. Huo et al. compared the PTX-loaded octyl modified HGC to the pharmaceutically approved Taxol® formulation, which is based on PTX in Cremophore EL® solubiliser. The HGC nanocarriers had sizes of 190–230 nm, high positive zeta-potentials of +24.6 to +30.2 mV and were reported to have better safety and toxicity properties than Taxol® [78]. Similar results have been reported for low Mw N-octyl-N,O-carboxymethyl chitosan (OcCMC) that was used as a PTX carrier. Blank nanoparticles demonstrated lower cytotoxicity towards HepG2 cells than Cremophor EL®, while PTX loaded formulations had comparable cytotoxicities [79]. Both studies clearly demonstrate the potential safety benefits of AMC.

Zhao et al. developed nanoparticles of biodegradable LA/poly(β-malic acid) (PMLA) double grafted chitosan (LMC), with diameters ranging from 190 to 329 nm and zeta-potentials ranging from −17 to −11 mV [61]. The PTX loaded nanocarriers inhibited tumour growth better than free PTX in S-180 mice. Recently, Zhou et al. synthesised glycidol-chitosan-deoxycholic acid (G-CS-DCA) and prepared nanoparticles with sizes of 160–210 nm, with these sizes increasing with the degree of hydrophobic substitution, in contrast with the reports on HGC discussed above. The formed nanoparticles were reported to be stable for up to three months in PBS at pH 7.4, despite having a zeta-potential value that was close to zero. Finally, the DOX loaded nanocarriers could be efficiently internalised into MCF-7 cells *in vitro*, with good anti-tumour efficiency (cytotoxicity), even though the efficacy was less than free DOX [58]. N-octyl-O-sulfate chitosan (NOSC) has also shown promise for use in cancer therapy. Zhang et al. performed safety studies on NOSC in mice with excellent results and investigated the anticancer efficiency of PTX-loaded NOSC for multiple tumour types in mouse models. PTX-loaded NOSC had similar anti-tumour effects as the free drug, with decreased toxicity and increased bioavailability [80,81].

Recently, succinyl chitosan modified with folic acid derivatives prepared using a simple carbodiimide reaction was reported for the effects of the polymer as a targeted cancer therapy [54]. The nanoparticles were 60–80 nm in size, exhibiting non-toxic *in vitro* effects with a capacity for high drug LE. The particles also exhibited pH dependent drug release characteristics and were capable of tumour cell targeting. Similarly, folate-PEG coated nanoparticles with multi-lamellar structure were prepared from octadecyl-quaternized lysine modified chitosan (OQLCS), folate conjugated OQLCS, and PEGylated OQLCS [42]. The nanoparticles had small particle sizes (approximately 160 nm), increased uptake in MCF-7 cells and a calcein release rate of approximately one tenth of that from traditional phosphatidylcholine/cholesterol liposomes.

A highly relevant research question is if AMC nanoparticles can help overcome multidrug resistance (MDR). For example by allowing loaded drug to escape the P-glycoprotein efflux pump in tumor cells and other multidrug-resistant proteins (MRP) or processes which can cause exocytosis, degradation, or inactivation of drugs [82,83]. Promising results have been reported for drug nanocarriers and, of particular interest for this review, for native chitosan nanocarriers [84]. To the best of our knowledge, AMC has not been widely investigated for the effects on MDR. These studies would be of great interest and value, given the urgency of overcoming MDR. The versatility of modified chitosan should allow for an intelligent design of a possible therapy to overcome MDR, while maintaining both the biocompatibility and bioactivity of chitosan. For example, hydrophobic modifications using cholesterol [27], LA [29,61,85], deoxycholic acid [30,58,86–88] or stearic acid functionalisation [89] could lead to the disruption of membrane structure and fluidity, possibly enabling drug vehicles to be incorporated into the lipid bilayer of the cells. As the intracellular-extracellular pH gradient is reversed in many solid tumours, a modification with proton donating groups, such as carboxymethyl [27,29,87], sulfate [80,81,90,91] and folic acid [87], or groups with strongly pH-dependent hydrophilicity, such as acetyl histidine [92], in combination with the pH sensitivity of the amino groups of chitosan (pKa of approximately 6.5), could allow for improved targeted nanoparticle accumulation and/or drug release in the extracellular tumour space as well as in/from early endosomes. These properties could be highly beneficial in cancer therapy, as discussed in a recent review by Lee et al. [93]. On this topic, Jin et al. prepared pH-sensitive self-aggregated deoxycholic acid modified carboxymethylated chitosan (DCMC) nanoparticles [30]. The nanoparticles escaped the endosomal entrapment of MCF-7 cells and exhibited acidic pH-induced aggregation and deformation, accelerating the drug release at endosomal-relevant pH values. DOX-loaded DCMC was superior to free DOX in suppressing drug resistant MCF-7/Adr cells, which was explained by increased cellular uptake and retention. Hu et al prepared DOX-conjugated stearic acid-g-chitosan nanoparticles having highly positive zeta-potentials and sizes smaller than 100 nm [89]. They also reported better performance of the DOX-nanoparticles than of free DOX for repressing MCF-7/Adr cells, and that the cytotoxicity

of the DOX-nanoparticles decreased with increasing DOX content.

With regard to administration routes, the majority of delivery systems on the market are intended for peroral administration, as this route has comparably high patient compliance and is non-invasive. AMC can be designed for peroral drug delivery [88,90,94,95]. Through the proper molecular modification, the drug can be protected from degradation by the metabolic system and the aggressive acid environment of the stomach. The intestinal attachment site as the drug delivery site should be tunable by using the correct chemistry to exploit the variability in pH within the intestinal system. Shelma et al. investigated the mucoadhesivity and release of hydrophobic curcumin and hydrophilic insulin under stomach (pH 1.2) and intestinal (pH 7.4) conditions for chitosan and hydrophobic derivatives prepared by N-acylation with hexanoyl, lauroyl, and oleoyl chlorides [95]. Increased mucoadhesion with the hydrophobic modifications and sustained release of curcumin were reported, with the slowest release for the oleoyl substituted chitosan at pH 7.4. For the insulin formulations, significant burst release was observed, followed by very slow release characteristics. This system was reported to have significant potential for the oral administration of drug substances, in particular hydrophobic drugs. As these results were interesting, stability studies of the drugs loaded in the nanocarriers should be conducted at stomach pH, as drug degradation could also potentially occur during the retention within the carriers.

The *in vitro* release kinetics from AMC can vary greatly. Some formulations release close to all of the drug in a few days, while other types can provide sustained release for weeks (Fig. 2). *In vitro* release characteristics can vary greatly with experimental setups. In particular, the commonly used dialysis method may generate results that suggest too slow release characteristics, resulting from the diffusion barrier of the membrane and a high local concentration of the nanocarriers in the dialysis tube that may lead to that drug remaining within the nanocarriers. Nanocarriers for hydrophobic drugs commonly can be loaded through simple mixing processes, with the low chemical potential of the drug in hydrophobic domains promoting both the loading and retention. Nonetheless, these commonly used *in vitro* methods are good indications of formulation performance, which will be further evaluated in subsequent stages of development using more sophisticated *in vivo* assays.

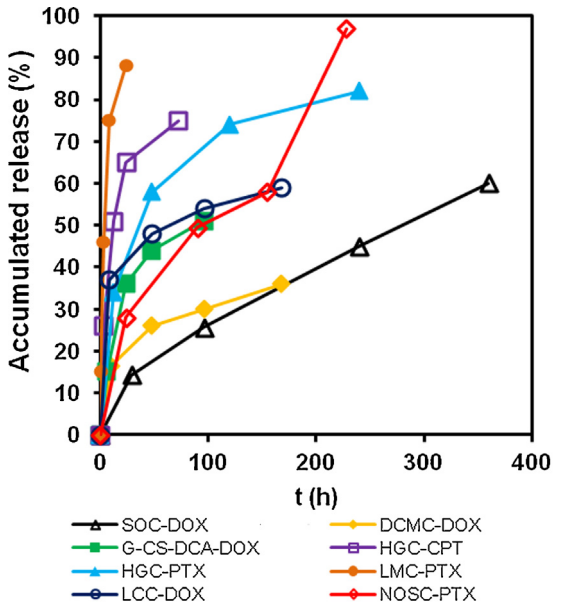

Fig. 2. Release profiles for selected amphiphilically modified chitosan and drugs. Data-points were estimated from the original figures in the cited references: SOC-DOX [96], G-CS-DCA-DOX [58], HGC-PTX [76], LCC-DOX [29], DCMC-DOX [30], HGC-CPT [77], LMC-PTX [61], NOSC-PTX [98]. All release studies were reported to have been conducted at pH 7.4 and 37 °C.

The relationships between the amphiphilic modifications of chitosan with the drug loading and release properties have also been examined. For N-succinyl-N′-octyl chitosan (SOC) [96] and G-CS-DCA [58] loaded with DOX, the reported results suggested that increasing hydrophobic modifications can lead to improved LC and LE with reductions in the release rates. Similarly, Liu et al. investigated the self-assembly behaviour and DOX LC of acylated carboxymethyl chitosan, reporting that LE was enhanced both by an increased acyl chain-length and the degree of acyl substitution [49]. Carboxymethyl-hexanoyl chitosan (CHC) has been effectively used to encapsulate drugs and modified the *in vitro* release characteristics for these drugs, such as vancomycin and naproxen [97], DOX [33], magnolol [31] and demethoxycurcumin [32]. For LMC, increasing the degree of substitution with LA and increasing the length of the PMLA chains led to decreased release rates of PTX, as explained by the increased LA-drug interactions and the increased hydrophilic shell thicknesses [61]. For low Mw amphiphilic OcCMC loaded with PTX, a chitosan precursor with Mw of 10 kDa resulted in higher LC and LE (LC = 37%, LE = 99%) than the chitosan precursors with Mw of 5 kDa (LC = 32%, LE 81%). A small decrease in both LE and LC was reported for Mw values increased to 20 kDa, indicating a complex dependence [79].

In addition to molecular characteristics, the drug loading environment may significantly influence the drug loading performance and the formed colloidal structures, as observed in the paper by Zhang et al. [98] investigating the NOSC self-assembly and PTX loading in multiple solvents. The optimal system was water-ethanol, and the PTX inside the NOSC core existed as colloidal particles rather than in a molecular dispersed state. The additional step of dissolving the colloidal PTX may have produced a slower sustained release compared to the molecular dispersion.

In summary, AMC nanocarriers appear very promising for cancer therapy and other indications. The versatility of modifications and properties allows for the design to specific needs, such as the protection of drug from degradation, mucoadhesion, overcoming MDR, and targeted delivery. Important characteristics, such as the drug release rates and physiochemical properties, are often tunable for a given system. Worth to mention is that Park et al. reported increased tumour accumulation with increasing Mw of the polymer chains, without changes in the *in vitro* physiochemical properties of the nanocarriers [28]. The increased accumulation may have resulted from the enhanced *in vivo*

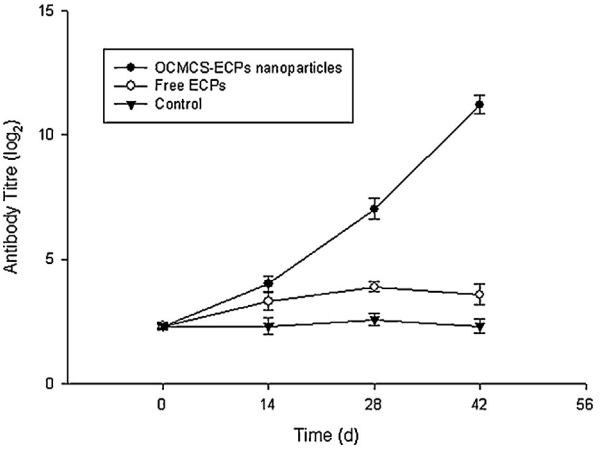

Fig. 3. Serum antibody titers in carps immunized with OCMCS-ECPs nanoparticles and ECPs alone. Data represented the mean ± SD. ($n = 4$). $^{*}P < 0.05$, as compared with control [99]. Copyright 2012. Reproduced with permission from Springer Science and Business Media.

stability and blood circulation times. These findings suggest that Mw may be an important, but often overlooked factor, in the characterisation of polymeric nanocarriers.

3.1.2. Delivery of proteins and peptides

AMC has been examined for use in the protection of loaded proteins and the controlled delivery at target sites. In particular, formulations for oral administrations (i.e., to protect the loaded protein from degradation in the stomach and promote intestinal transmucosal delivery) have generated significant interest. Recently, oleoyl-carboxymethyl chitosan (OCMCS) was prepared by chemical modification with oleic acid and monochloroacetic acid. Nanoparticles prepared by ionic-gelation using sodium tripolyphosphate were effectively loaded with extracellular products (ECPs). The nanoparticles had tunable sizes ranging from 160 to 400 nm without ECPs, and slightly larger when loaded with ECP. The nanoparticles had uniform sizes, high LE values, abilities for the sustained release of the ECPs, enhancements to mucosal delivery, and greatly improved antibody responses compared with the free ECPs, as observed in Fig. 3 [99]. Consistent with these results, Shelma and Chandra prepared lauroyl sulfated chitosan (LSC) by modification with hydrophilic sulfo groups and hydrophobic lauroyl groups, forming nanoparticles using tripolyphosphate [100]. A thorough characterisation of the formed LSC was performed, and the resulting products had excellent blood compatibility characteristics. Subsequently, LSC was investigated for use as drug and peptide nanocarriers, with the results suggesting that LSC had higher mucoadhesion than chitosan and no cytotoxicity. Furthermore, LSC increased the permeability of caco-2 cell layers, inhibited the enzymatic degradation of insulin and displayed pH-dependent release of insulin with dramatically increased release rates at high pH values. In summary, LSC seems very promising as an oral peptide-protein delivery system [101]. Loaded peptides or proteins should be retained in the particles and protected from degradation during the migration through the stomach, with the subsequent release of the drug product occurring in the intestine with the increased pH values. Although confirmational studies are still needed, this design approach appears to hold potential for the oral delivery of insulin and other proteins.

In contrast with the generally applicable oral delivery route, a specific and challenging indication for peptide or protein deliveries is spinal cord injury (SCI) repair. In clinical practices, effective treatments of SCI involve local injections of high-dose drugs [102,103]. Unfortunately, high-dose drugs are commonly associated with multiple side effects. Improved treatments could potentially be achieved by the targeted delivery of proteins that stimulate healing at the site of injury. Nanocarriers may be useful in achieving this goal by using a suitable design to deliver therapeutic macromolecular agents across the blood-spinal cord barrier (BSCB) [104].

One approach to direct nanoparticles to a target area is through the inclusion of magnetic components and the use of an external magnetic field for guidance [105,106]. Functionalisation with tat-peptides, which contain transmembrane and nuclear localisation signals, can increase the cellular internalisation of particles and peptides [106,107]. Using this approach, Wang et al. elegantly developed tat-modified, iron oxide containing nanoparticles from PEGylated octadecyl quaternised carboxymethyl chitosan and cholesterol [108]. The nanoparticles effectively crossed the BSCB and penetrated into nerve cells in a SCI-rat model using an external magnetic field for guidance. These data suggest that the nanoparticles are promising for the delivery of macromolecules, such as proteins, across the BSCB to SCI repair sites.

3.1.3. Gene delivery

Gene therapy has generated extensive interest in the research community and holds great therapeutic promise. In the effective delivery of genes, polymeric nanocarrier-based delivery systems may be an attractive alternative to virus-based systems for which there are production and safety concerns. Generally, nanocarriers for efficient gene transfection must meet a number of requirements, including the formation of complexes condensing the DNA, the protection of genetic material from degradation, the promotion of efficient cellular uptake, an ability to enable endo-lysosomal escape and the localisation of genetic material into the nucleus as well as the release from the carriers. Cationic polymers and liposomes have been extensively investigated as gene carriers, where especially polycations enable effective DNA condensation [109,110]. Complexation with polymeric cations have been reported to protect genetic material against nuclease degradation, while facilitating both cellular uptake and endo-lysosomal escape [111].

With cationic charge characteristics, chitosan has been widely examined as a non-viral vector for transfection [3,112–118]. The effects of specific colloidal properties, including size, colloid stability, surface charge, and pH stability, on transfection efficiency have been widely considered. Several studies have been conducted on the structural optimisation of chitosan/gene complexes to enhance delivery by controlling the chitosan Mw, the degree of chitosan deacetylation, and the plasmid concentration [9,112,119,120]. The transfection efficiency

using chitosan is still low compared with the current "golden standard" polyethylenimine (PEI), most likely resulting from the poor solubility characteristics of chitosan at physiological pH and the strong interactions within the chitosan-DNA/RNA polyelectrolyte-complex preventing dissociation in the nucleus. To address these difficulties, modified chitosan has been investigated for gene delivery.

A popular choice for the hydrophilic modification of gene-nanocarriers has been PEG, allowing for multifunctionality. PEG can reduce the cytotoxicity of the carriers, increase circulation times, improve water solubility/colloidal stabilities, shield excess charges, and be used as a spacer between the carriers and targeting ligands, allowing for an improved access to targeted receptors [45,121]. With the positive results using PEI as a nanocarrier in gene delivery [122,123], chitosans modified with PEI have also been investigated [124,125]. In 2008, chitosan based gene nanocarriers modified with both PEG and PEI were examined by both Jiang et al. [40] and Wu et al. [126].

Another notable modified chitosan in the gene delivery field, galactosylated chitosan was able to improve the galactose induced hepatocyte-targeted gene delivery when hydrophilically modified with dextran, PEG, polyvinyl pyrrolidone (PVP), and PEI. The polymer-DNA self-aggregates ranged from sizes less than fifty nanometres to several hundreds of nanometres [40,127–129]. The colloidal stability in PBS of the hydrophilically modified chitosan-DNA complexes was superior to non-modified galactosylated chitosan-DNA complexes [127,128], Furthermore, a PVP modification increased stability against albumin-induced aggregation compared with conventional PEI nanocarriers [129]. The DNA-nanocarriers had improved transfection in cells presenting asialoglycoprotein receptors that allowed for interactions with the galactose groups [40,127,128], demonstrating that the hydrophilic modification did not significantly interfere with the ligand-receptor interactions. In a murine model, galacosylated chitosan nanocarriers modified with both PEG and PEI had increased circulation times and accumulation in the liver compared with PEI and PEI-chitosan nanocarriers [40]. These results demonstrate that the nanocarrier design can affect the biodistribution characteristics, both through active targeting and physiochemical properties.

Hydrophobic modifications of nanocarriers for gene delivery can allow for improved loading of the genetic material, reduced serum inhibitions, increased attachment to and translocation across cell membranes, and a weakening of the ionic interactions between the carrier and the genetic material [56,130,131], promoting transfection efficiencies. Liu et al. investigated N-alkylated chitosan as a nanocarrier for gene delivery, observing that the transfection efficiency increased with increasing chain length up to eight alkyl carbons [131]. Kim et al. prepared deoxycholic-acid modified chitosan that produced transfection efficiencies that were strongly dependent on the Mw of the precursor chitosan [56]. The optimal formulation depended on the transfection conditions (with or without serum). The transfection efficiencies were discussed primarily in terms of the DNA-polymer interaction strength and the DNA condensation, with a possible influence from both size and structure also mentioned.

An interesting approach for the enhanced delivery using thiolated chitosan-DNA complexes has been reported by Lee et al. [132]. The complexes were between 75-120 nm with a zeta potential of +2.3 to +19.7 mV and had improved stability that prevented degradation with DNase I digestion, leading to higher DNA expression in HEK293, MDCK, and Hep-2 cell lines compared with the unmodified chitosan. In addition, *in vivo* experiments in Balb/c mice demonstrated that the intranasal administration of the complexes produced gene expression for at least 14 days.

The combination of both hydrophilic and hydrophobic modifications of cationic polymers for the synergetic effects on gene delivery has led to AMC receiving increased research attention [87,133–136]. Yoo et al. prepared HGC nanocarriers from glycol chitosan, 5β-cholanic acid-modified-glycol chitosan and hydrophobised DNA. In addition to electrostatic forces, hydrophobic interactions were a driving force used to encapsulate the modified DNA [134]. *In vitro* transfection efficiencies using HGC were increased up to eight-fold higher than the experiments using chitosan. In several animal studies, HGC had significantly higher transfection efficiencies than both naked DNA and commercially available PEI (Superfect®). Both the *in vitro* and *in vivo* results suggested increased transfection efficiency with increasing HGC content, with increased HGC content corresponding with decreased nanocarrier sizes. The improved efficiency may have resulted from the decreased nanocarrier size, the improved stability of the complexes, the improved resistance to serum proteins, or a combination thereof. Similarly, Opanasopit et al. [135] prepared methylated N-(4-pyridinlymethyl) chitosan, and suggested that the permanent positive charge of the trimethylated chitosan was a key factor in condensing and protecting the DNA, while the hydrophobic N-4-pyridinylmethyl group played an important role in the transfection efficiency. Germershaus et al. [133] reported that PEG-graft-trimethyl chitosan/DNA had improved transfection efficiencies in various cell lines, including NIH/3T3, L929, and MeWo. A549 cells were also observed to be resistant to transfection, indicating the presence of cell line specific characteristics that can influence transfection. These characteristics could include the membrane charge density, different internalisation routes, the surface receptors, and other attributes.

In summary, multiple reports have been generated on the influence of both properties and formulations of amphiphilic polymers on the performance of nanocarriers for the delivery of genetic material. The delivery performance is influenced by the interactions between the polymer and the genetic material, as well as the size and the zeta-potential of the nanocarriers. Another essential parameter is the polymer nitrogen to DNA phosphorous ratio (N/P), with a clear but complicated dependence [133,136,137]. Non-electrostatic interactions appear to allow for improved dissociation of the loaded genetic material with a corresponding increase in transfection

efficiency. Establishing clear dependences between the transfection efficiency and the various parameters can be difficult as they are commonly not independent, which confounds the correlations. To the best of our knowledge, no systematic study or even discussion has been reported on the optimal ratio of the hydrophobic to hydrophilic substitutions. As a high degree of hydrophobic substitution might induce strong cytotoxicity and extensive hydrophilic substitution could lessen cellular uptake, a model system exploring *in vitro* and *in vivo* characteristics, including colloidal properties, stability, cell internalisation and transfection efficiency for the various ratios of hydrophilic/hydrophobic substitution, would be an important contribution to the field.

3.1.4. Imaging and diagnosis

In addition to use as nanocarriers for therapeutic substances, self-assembled nanoparticles can provide a potential platform for effective diagnostic imaging, possibly in combination with the delivery of substances [92,138–140]. These nanodevices may allow for selective imaging and *in vivo* monitoring of biological conditions. Nanoparticle-based optical imaging agents require several crucial properties: (i) stability; (ii) resistance to metabolic disintegration with non-toxicities; (iii) excellent absorbance and quantum yields; and (iv) adequate dispersibility in physiological environments [141]. For effective diagnostic imaging, additional requirements include excellent spatial resolution in three dimensions and an accumulation of the nanoparticles at the target sites (if desired).

Superparamagnetic iron oxide nanocrystals (SPIONs) are imaging agents that have been widely studied for magnetic resonance imaging (MRI). SPION based nanoparticles can be used both as drug nanocarriers and for *in vivo* imaging, allowing for simultaneous treatment and monitoring of effects [142]. For these applications, SPIONs should have a narrow size distribution and be stabilised in an aqueous media [143]. To achieve these properties, Lee et al. developed hepatocyte targeting self-assembled nanoparticles composed of chitosan–LA conjugates, carrying SPIONs as contrast agents [144]. LA accumulates in hepatocytes and plays a central role in cholesterol and triglyceride synthesis [145,146], suggesting that the conjugation of LA to chitosan may enhance the accumulation of the conjugate in the liver. The amphiphilic chitosan–LA carrier improved the colloidal suspension stability of the SPIONs, reduced cytotoxicity and allowed for targeted MRI of the hepatocytes without imaging the Kupffer cells. In another study, Shi et al. reported on surfaced modified SPIONs prepared by an attachment with carboxymethyl chitosan. The particles had excellent imaging properties in combination with several advantages, such as improved biocompatibility, effective uptake into cells, and the possibility of stem cell tracking [147].

Recently, nanotechnology and molecular imaging have been used to produce multifunctional nanoparticles that could facilitate simultaneous early-stage cancer diagnosis, targeted drug delivery, and real time monitoring of cancer treatment [148–150]. The combination of MRI and near-infrared fluorescence optical imaging for cancer diagnosis has several attractive benefits that have driven the development of molecular imaging using these functionalities [151–153]. On this topic, Bhattacharya et al. prepared SPIONs surface modified with carboxymethyl chitosan, adding rhodamine isothiocyanate for optical imaging and folic acid for targeting. The particles had excellent dispersion properties under physiological conditions, optical imaging capabilities, magnetic activity under external magnetic fields and increased uptake into HeLa cells, which overexpress folate receptors [41].

There has been considerable work towards therapeutic imaging using HGC nanoparticles, which is especially interesting given their potential for cancer therapy [53,74–77]. In one study, the near infrared (NIR) fluorophore Cy5.5 was conjugated to anti-angiogenic RGD peptides that were loaded into the HGC nanocarriers [154]. The HGC-RGD-Cy5.5 nanoparticles allowed for the *in vivo* NIR imaging of the RGD-Cy5.5 biodistribution, which at early times should correspond to particle distribution. In another investigation, the HGC was conjugated with Cy5.5 [74], allowing the imaging of the actual polymer chains constituting the particles. The HGC nanoparticles were loaded with docetaxel and had good imaging properties as well as therapeutic potential. Recently, HGC nanoparticles have been developed for dual NIR/MR imaging by modifications of the glycol chitosan-5β-cholanic acid with Cy5.5 for NIR imaging and a gadolinium (Gd(III)) chelating agent to incorporate Gd(III) for MRI. The polymer-Gd(III) complexes self-assembled into nanoparticles having average sizes of approximately 350 nm and accumulated in tumours after intravenous administration in a murine model. The nanoparticles were then used for simultaneous NIR and MR imaging [155]. By combining an improved tumour accumulation, the molecular sensitivity of NIR imaging and the three dimensional capability and spatial resolution of MRI, the Cy5.5-HGC-Gd(III) nanoparticles have the potential to be used as optical/MR dual imaging agents in cancer therapeutics and monitoring.

HGC nanoparticles can also be surface functionalised to improve targeting efficiencies. In an elegant work by Park et al., peptide-conjugated HGC nanoparticles labelled with Cy5.5 selectively bound to membranes of activated bovine aortic endothelial cells and exhibited higher binding affinity for the atherosclerotic aorta of an $Ldlr^{-/-}$ mouse than to the aorta of a normal mouse, as determined by NIR imaging [156]. These results clearly demonstrate the potential of HGC based nanodevices in atherosclerotic lesion imaging to monitor pathophysiological changes. In subsequent work, Na et al. investigated the *in vivo* imaging capability of Cy5.5 labelled HGC nanoparticles in various tumour-bearing mouse models with excellent results [157]. HGC nanodevices appear to have great flexibility with regard to imaging and targeting modifications, which could allow for the development of multifunctional nanodevices for a range of indications. Incorporating both imaging and the simultaneous release of drug (possibly with imaging-design that could allow for detailed information on the drug state to consider both the concentration and localisation within or outside of nanoparticles) would constitute a further therapeutic and imaging development that may be of significant interest.

3.2. Macroscopic assemblies with colloidal structures

3.2.1. Injectable depot gels for drug delivery

With multiple desirable biomedical properties, chitosan has been investigated for use in injectable depot drug delivery systems. In 2000, Chenite et al. developed a novel thermo-gelling hydrogel based on solutions of chitosan with a small amount of β-glycerophosphate (β-GP) [158]. The mechanism of gelation has been ascribed to chain aggregation upon the proton transfer from the chitosan to the β-GP. This transfer results from the temperature dependent pK_a of chitosan [159]. Another common explanation is that at low temperatures the β-GP assumes an entropically disfavoured arrangement surrounding the chitosan, while at higher temperatures the arrangement is broken-up, which allow the chitosan chains to aggregate. This formulation was shown to be a viable delivery system for growth factors and for living chondrocytes, and clearly has potential for the delivery of other therapeutic substances, as discussed below. Kashyap et al. used the chitosan-β-GP system as an *in situ* gelling depot for pulsatile delivery of insulin [160]. Loaded with PTX, CPT or DOX, this drug delivery system has also been investigated for the treatments of breast cancer, RIF-1 fibrosarcoma, and cervical cancer [161–163]. Even with the elegant design of the chitosan-β-GP system, improvements can be made to reduce several commonly observed disadvantages, such as the burst release of drugs, poor water-solubility and low thermo-sensitivity. To this end chitosan and formulations have been modified to increase the drug delivery depot functions for a range of applications.

Bhattarai et al. developed an injectable thermo-sensitive hydrogel based on a solution of poly(ethylene glycol)-graft chitosan (PEG-g-chitosan) using genipin for crosslinking *in situ* under physiological conditions [164,165]. The hydrogel provided close to a linear sustained release of bovine serum albumin for up to 40 days, with only a small amount of burst release. Similarly, Lu et al. used N-succinyl-chitosan (NSC) and oxidised carboxymethylcellulose as the matrix in an injectable protein delivery system [166]. The system was both pH- and thermo-sensitive, forming solid gels at physiological pH and temperature. The gelation time, equilibrium swelling and degradation could be tuned by adjusting the oxidation degree of the carboxymethylcellulose. The porous structure of the hydrogels could be tuned, providing an attractive method for controlling the diffusion rate, and thus the release of the loaded macromolecules.

Wu et al. prepared injectable hydrogels by preparing N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride (HTCC) with α-β-GP [167]. DOX release from the hydrogels had higher pH sensitivity than chitosan-α-β-GP hydrogels. Gels from both HTCC and chitosan displayed increased release rates at low pH. An additional nasal formulation intended for the delivery of macromolecular drugs was developed by combining HTCC with PEG and α-β-GP [168]. The formulation underwent a sol-gel transition at 37 °C. Loaded with insulin, the gel release profiles and initial burst release were determined to be dependent on both the gel composition and the drug loading, with decreased release rates ascribed to the formation of a tight gel network. For gels loaded with insulin and administered nasally in the rat, the HTCC-PEG-α-β-GP reduced blood glucose levels with values that were similar to subcutaneously injected insulin solution, but with a delayed response. In contrast, nasally administered insulin solutions did not have a significant effect on blood glucose levels.

The nano-microstructure of injectable hydrogels is an important parameter for controlling the release rates of the loaded substances. One method to achieve extended release with minimal burst release from injectable *in situ* gelling systems is to load the therapeutic substance into nano- or micro-carriers that are dispersed in a macroscopic gel. Hsiao et al. recently developed an injectable colloidal hydrogel composed of self-assembled nanocapsules of CHC and β-GP [169]. The CHC provided both the encapsulating functionality and the coherent gel network, making the preparation simple. The gels had extended release characteristics without burst release for the hydrophilic drug ethosuximide. Conventional injectable hydrogel drug delivery systems commonly exhibit burst release characteristics [170], with the release of small hydrophilic drugs often completed in less than 24 h [171]. Currently, the authors are investigating the release rates of colloidal CHC systems controlled by the gel microstructure, as achieved with variations in the gelation conditions and kinetic parameters.

Similarly, Ding et al. reported an injectable amphiphilic pH sensitive hydrogel composed of glycol chitosan and benzaldehyde-capped poly(ethyleneglycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (OHC-PEO-PPO-PEO-CHO) [172]. The gels formed *in situ* through the formation of covalent benzoic-imine bonds had the capacity to load both hydrophilic and hydrophobic drugs. Subsequently, a glycol chitosan/OHC-PEO-PPO-PEO-CHO hydrogel was prepared with hydrophilic DOX, mixed in the glycol chitosan solution and hydrophobic PTX, blended and solubilised in the OHC-PEO-PPO-PEO-CHO solution, before combining the two [173]. The authors described that the DOX was loaded in the aqueous regions of the gels, with the PTX located in OHC-PEO-PPO-PEO-CHO micelles. The gels had pH dependent release of both DOX and PTX, with a small decrease in pH from 7.4 resulting in an accelerated release. The gel formulations with both DOX and PTX were used for the treatment of B16F10 murine melanoma cancer in mice by intratumoural administration, resulting in improved tumour treatments that were not observed with the single drug formulations.

In conclusion, conventional injectable hydrogel systems that are composed of drug and a simple polymeric matrix are usually not ideally suited as injectable depot drug delivery systems; the medical applications of these hydrogels are limited by biodegradation, biocompatibility issues, thermo/pH sensitivity problems, cytotoxicity, and non-ideal release profiles. Multiple investigations have focused on the synthesis of new polymers or the mixing of functional molecules/polymers to produce novel injectable hydrogels that are easier to use with the improved performance profiles that are necessary for successful clinical applications. One approach to addressing these improvements is to design a complex gel structure, suggesting that

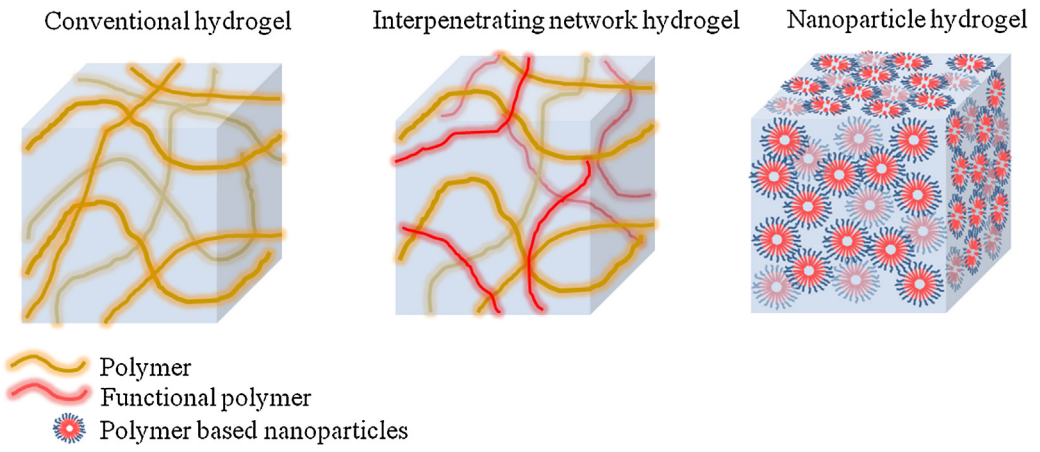

Fig. 4. Schematic drawing illustrating the simplified structures of conventional hydrogels, interpenetrating network hydrogels and polymer based nanoparticle hydrogels.

the development of these hydrogels has proceeded from single polymer systems to interpenetrating network structures and self-assembled nanoparticle gels (see schematic drawings in Fig. 4). From the discussed reports on AMC, this material class has also developed to exhibit these characteristics to become a successful candidate for injectable hydrogels with designed structure-functionality profiles.

3.2.2. *In vivo cell scaffolds*

In recent years, several significant developments in cell therapies have been made, as reported in multiple recent reviews [174–176]. Promising results using cell therapy have been reported for the treatments of metastatic melanoma [177], post-acute myocardial infarction [178], and muscular dystrophy in a murine model [179]. In addition, significant advances have been made in replacing the extensively discussed embryonic stem cells and donor derived cultured stem cells with induced pluripotent stem cells (iPSCs) that are reprogrammed from selected cells derived from the patient [180,181]. As novel therapeutic alternatives have emerged, a subsequent need for suitable delivery platforms has also evolved. For effective or improved cell therapies, a delivery platform needs to be biocompatible and provide a temporary porous well-defined 3D-environment to allow for cell proliferation and differentiation to the desired cell type [182]. In particular, cell-scaffold interactions and nano-micro architecture needs to be favourable, as demonstrated in recent publications [183–185]. To sustain viable therapeutic cellular alternatives, scaffolds also need to allow for easy and robust implantation in patients in a clinical setting. Injectable cell scaffold systems would be highly desirable, allowing for minimal invasive surgery as well as the abilities to form complex shapes, provide good contact with the surrounding tissue, and enable the homogeneous distribution of cells and signalling substances [182,186,187].

As observed in the literature, a number of promising chitosan containing [182,186,188,189] and modified chitosan based [7,188,189] injectable cell scaffolds for tissue engineering have been reported. The use of AMC in cell scaffolds is still new. Several studies have suggested that AMC may be useful as a cell scaffold matrix. Xu et al. demonstrated in rats that N,O-hexanoyl chitosan did not affect the proliferation and viability of dermal fibroblasts, had excellent biocompatibility and was degraded *in vivo*, with the degradation rate tailored by altering the degree of substitution [190]. Compared with the commonly used biodegradable polymer PLGA, a milder and lower inflammatory response was observed. In another study, Neamnark et al. used fibres of electrospun hexanoyl chitosan as scaffolds for human keratinocytes and fibroblasts [191]. Based on proliferation and cell integration results, the created fibrous mats were reported to have potential as a scaffolding material in tissue engineering. The results of Neamnark et al. and Xu et al. provide some indication that AMC may be useful in tissue engineering. In a recent study, Chien et al. demonstrated the pre-clinical use of amphiphilic CHC as a thermo gelling injectable cell scaffold carrying iPSCs for corneal repair [192], concluding that the injectable CHC gel may provide a safe injectable scaffold delivering stem cells for the treatment of corneal injury. The CHC gels were first reported by Hsiao et al. as a potential depot drug delivery system [169]. CHC nanocapsules in combination with β-GP had formed a macroscopic gel upon heating to 37 °C, with the formed gel having a micrometre scale network in which the nanocapsules remained intact, producing gels with a porous structure on the micrometre scale and a nanostructured cell-scaffold interface that might allow for an ideal scaffolding performance.

In conclusion, there are very few studies on AMC as *in vivo* cell scaffolds. The available reports clearly demonstrate significant potential for this new class of material in the field of cell-therapy and tissue engineering. In particular, the self-assembly into nanostructures, the capacity to load drugs and growth factors into nanoparticles, the ability to tune the microscale network-structure, and the ease with which chitosan can be modified may allow for the simple preparation of injectable cell scaffolds to meet new needs (see Fig. 5 for conceptual drawing).

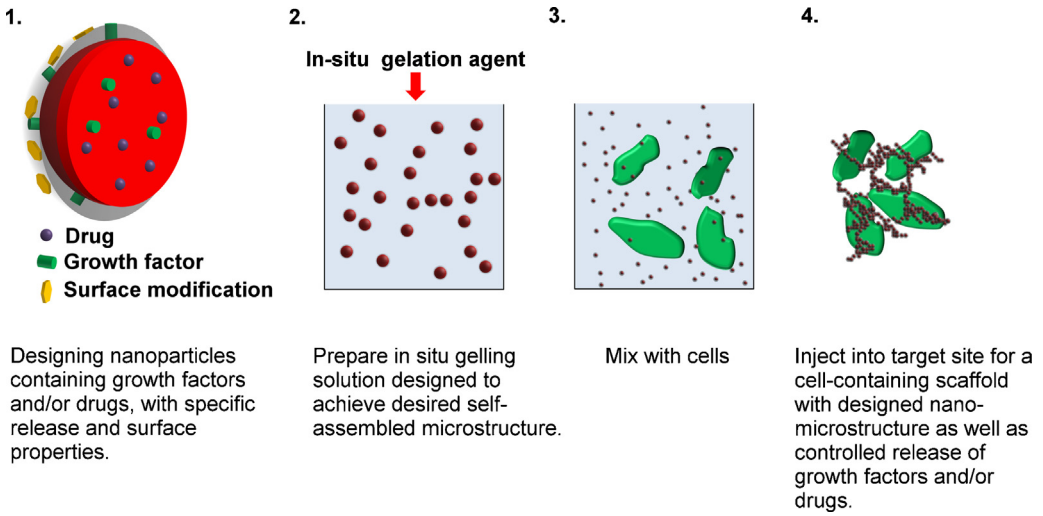

Fig. 5. Conceptual scheme illustrating the preparation of injectable nanoparticle based cell scaffolds having the controlled release of drugs and growth factors as well as a designed nano-microstructure.

3.2.3. Wound dressings

Many injuries and medical conditions involve damage to the skin barrier (i.e., wounds). These wounds can be deep or located on the surface of the skin. For small wounds in healthy areas, the body can manage the healing process unless complications, such as infections, occur. For more serious injuries, therapeutic interventions are needed. For extensive wounds with bleeding, a wound dressing is usually used to stop or reduce the bleeding. For large wound areas, as may often be the case for burn injuries, or underlying medical conditions that prevent wound healing, such as chronic ulcers, therapeutic interventions are necessary. To prevent the infection of the wound, wound dressings and antibacterial agents are commonly used. A combination of antibacterial effects with other features that can promote healing would be desirable features for the integral functions of the wound dressing. In addition to providing a microbial barrier, the dressing also needs to have proper liquid and vapour management. Excess wound liquid needs to be absorbed and/or evaporated through the dressing, maintaining a suitably moist environment for wound healing [193]. Inappropriate liquid management may lead to environments that are too dry or too wet, impairing the healing process and damaging the skin. These requirements are especially true for dressings that are to remain in place for long periods of time.

With excellent biocompatibility, biodegradability and antibacterial features, chitosan has been investigated for use in wound healing and several possibly beneficial effects have been reported [194]. The positive charge of protonated amino groups may attract the anionic glycosaminoglycans that are linked with a large number of cytokines and growth factors. Chitosan can also act as a chemo-attractant for neutrophils [195]. Chitosan has been confirmed to improve both the function and speed of migration to the area of interest for cells associated with inflammatory responses, leading to improved healing through a reduced risk for infections [8]. These features combined with the haemostatic properties of chitosan [196] and the ability to increase collagenase activity would suggest that chitosan may be suited for use in wound dressings. Indeed, multiple reports on the use of chitosan-based wound dressings, in particular on the HemCon dressing, have demonstrated efficacy. These types of wound dressings were used in Operation Iraqi Freedom and Operation Enduring Freedom [197] with excellent results, and appear safe to use with injured U.S. soldiers [198]. Clinical failures have also been reported and ascribed to poor adhesiveness as well as large batch to batch variability for the chitosan wound dressings [199], indicating a need to develop improved chitosan based dressings. Dowling et al. reported that chitosan modified with hydrophobic benzene-n-octadecyl side-chains (2.5 mole% relative to amine groups) could bind non-covalently to cells in the blood and self-assemble to form a hydrogel with the cells in the blood [200] (see Fig. 6). The hydrophobic chains may have integrated into the cell membranes so that the polymer chains and the cells formed a self-supporting network. Subsequently, a similar AMC formed by modifying chitosan with n-dodecyl side-chains (at a different mole%) was used as a wound dressing for a lethal arterial injury in swine [199]. The bandage from the hydrophobically modified chitosan was superior to both the bandage from the pristine chitosan and the standard gauze for reducing bleeding. In fact, none of the eight test animals died within the 180-min test period using the modified chitosan, while all test animals died in approximately 90 min using the pristine chitosan and in less than 10 min using standard gauze. The success of the modified chitosan was attributed to the increased tissue adhesiveness, as previously described (i.e., the hydrophobic side-chains anchor to the cell membranes). In other relevant work, Muzzarelli et al. used freeze-dried methylpyrrolidone chitosan (MPC) to augment wound healing after dental surgery [201]. MPC induced osteoconduction and angiogenesis, which combined with the biocompatibility of the material make these results very interesting.

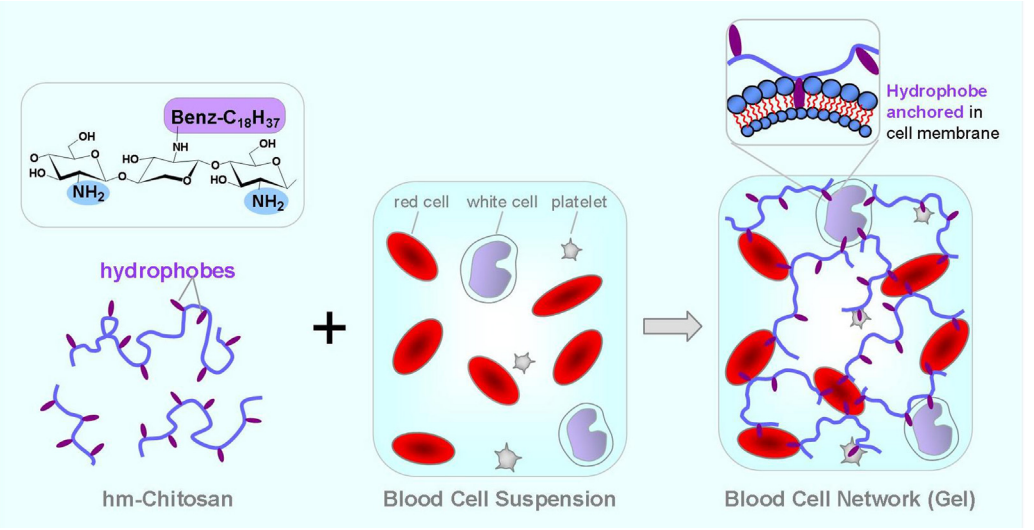

Fig. 6. Mechanism for gelation of blood by hm-chitosan. On the left the polymer is shown schematically with its hydrophilic backbone in blue and the grafted benzyloctadecyl hydrophobes in purple. When added to liquid blood, the components assemble into a three-dimensional network (gel), as shown on the right. This is driven by insertion of hydrophobes into blood cell membranes (as depicted in the top inset); thereby the polymer chains connect (bridge) the cells into a self-supporting network. [200], Copyright 2011. Reprinted with permission from Elsevier Ltd.

In addition to improved haemostatic function, liquid management and healing augmentation, the controlled delivery of drugs or other therapeutic agents could represent additional wound care applications for AMC. As discussed in Section 3.1, AMC can self-assemble into nanostructures, offering extensive control over the release profiles of therapeutic substances. Recently, Lin et al. prepared all-trans retinoic acid loaded CHC nanocapsules embedded in an alginate hydrogel matrix [202]. Alginate is commonly used in wound dressings [203], and the alginate-CHC system had low cytotoxicity, as evaluated using both the MTT assay and the lack of skin irritation on rabbits. The alginate-CHC system seems to be a promising dressing material with additional control over the drug release profile. This system could be useful as a long-term therapeutic dressing with improved healing characteristics.

Based on the limited literature available, AMC appears suitable for wound healing applications. The anchoring of hydrophobic side-chains into cell membranes and the colloidal structure could provide wound dressings with both very good tissue attachment and improved control of the drug release profiles to meet various clinical demands. With both technical interest and medical importance, hydrogel dressings could be produced via a controlled self-assembly of AMC, leading to designed nanostructures and microstructures with controlled drug release profiles, evaporation rates and fluid accessibility, allowing extensive structural design for therapeutic improvements in wound healing applications.

3.3. Organic–inorganic hybrid nanostructures

Recently, organic–inorganic hybrid materials and molecules have received increased interest in the research community. The aim for creating these materials is to combine synergistically the properties of both the organic and the inorganic components, to achieve improved properties and/or functions, as compared to the individual components. Several examples of improvements could include improved mechanical properties, improved biocompatibility, introduction of responses to external stimuli, imaging capabilities, enhanced control over diffusion, etc. As chitosan has multiple desirable properties for biomedical applications, chitosan-inorganic hybrids have also been investigated, producing interesting results.

One widely investigated use of chitosan is for the preparation of composites with hydroxyapatite (HAp) nanoparticles for use in biomedical applications, typically bone replacement and/or hard tissue engineering [204–206]. The benefits of these composites can include a porous structure with tunable degradation rates, improved injectability characteristics, reduced damage from HAp nanoparticles migrating into surrounding tissues, biofunctionality improvements, improved control over the HAp nanoparticle structure, and control of the nano-microstructure scaffold [204,205,207–210].

Another hybrid material class includes chitosan coated metal/metal-oxides, such as Ag/chitosan [211,212], Iron-oxides/chitosan [41], TiO_2/chitosan [213], Au/chitosan [214,215], and α-zirconium hydrogen phosphate hydrate (ZrP)/chitosan [115,216]. The hybrids can exhibit both the properties of metals/metal-oxides and chitosan-exclusive characteristics, which allows for grafting with additional functional and improved biocompatibility.

Inorganic nanoparticles can be manufactured into multiple types of shapes. Surprisingly, this structural design approach appears to be lacking in the literature on chitosan-inorganic hybrid materials. As chitosan coatings commonly are templated onto inorganic particles, the shape and size of the inorganic core could be varied to allow for desired structural characteristics of the hybrids.

These design features could be of great interest for medical applications, as several recent reports indicate that nanoparticle structure can be an important factor in determining biodistribution, cellular uptake, circulation half-life [6,36], and targeting performance [217]. The combination of the inorganic properties with the properties of chitosan suggest that inorganic-chitosan hybrid nanoparticles and nanostructured materials may provide excellent candidates for various biomedical applications, such as biosensors [211,215], antibacterial materials [211,212], and bio-imaging contrast agents (discussed in Section 3.1.4).

Chitosan-siloxane hybrid materials have also received increased attention. In addition to mixing of siloxane and chitosan components, several studies on the development on the synthesis of novel chitosan-siloxane molecules have been made. Kweon prepared a chitosan-g-PDMS compolymer in 1998 [218]. Other types of covalently connected siloxane-chitosan hybrids have also been synthesised using different functionalised silane coupling agents, such as 3-glycidyloxy-propyltrimethoxysilane [219,220], alkyloxosilane-3-(trimethoxysilyl) propyl methacrylate [221], 3-aminopropyltriethoxy-silane [222,223], and 3-isocyanatopropyltriethoxysilane (ICPTES) [224]. Often these materials have presented interesting structures and properties for use in biomedical applications. Park et al. prepared a tetraethyl orthosilicate (TEOS)-chitosan interpenetrating network membrane with strongly pH dependent drug permeability characteristics [225]. At acidic pH, the permeability was very low, possibly explained by a swollen chitosan matrix that blocked the pores in the TEOS interpenetrating network. At neutral pH, the de-swollen chitosan allowed for fast diffusion through the open pores. The pH dependence of the system was controlled by the chitosan to TEOS ratio and was explained by the differences in the membrane structure. These membranes could be interesting materials with extended drug release characteristics, providing protection of the drugs from degradation in the stomach. Recently, Hsiao et al. developed nanoparticles composed of CHC-siloxane hybrid molecules. The nanoparticles had diameters ranging from approximately 100–600 nm with increasing siloxane modifications [223]. The Si formed structured layered networks encapsulating the core of the particles, in contrast to previously reported chitosan siloxane hybrid nanoparticles with the core composed of the siloxane network and the shell composed of chitosan [221]. The amphiphilic character of CHC allowed for the loading of the hydrophobic CPT into the core and the layered siloxane led to very slow drug release characteristics. The hybrid nanoparticles also had low cytotoxicity with effective cellular uptake characteristics, indicating a potential for cancer treatments. Quite unexpectedly, Silva et al. reported that nanostructured Chitosan-ICPTES hybrid molecules exhibited specific photoluminescence characteristics that may render them useful as optical probes for *in vivo* applications [224]. Finally, Huang et al. recently reported hydrogels formed by self-assembly of carboxymethyl chitosan-PDMS hybrid molecules into a superporous structure containing hydrophobic PDMS-microdomains [226]. These gels with hierarchical structures and properties may be useful in multiple areas of biomedicine, including bioadhesives, cell scaffolds, drug delivery, and wound-dressings.

4. Clinical implications and market potential

With the many advanced biomedical achievements and features being reported for AMC in recent decades, from bio-safety to *in vivo* therapeutic effects, a critical moment for initiating efforts toward clinical practice has been reached. Although reports from clinical studies are still lacking, the translation of these discoveries into clinical trials seems promising (and the authors believe several trials may be under development). One obvious application for AMC may be as a drug nanocarrier. Other nanocarrier systems are already present on the market, but the promising reports and properties of AMC make it likely that such products will emerge in the future. In particular, poly-cationic modifications would be suitable for transmucosal drug delivery systems. With regard to clinical and market potentials, wound dressing applications may represent attractive indications, as these fields have less complicated development requirements with improved risk/reward profiles compared with drug delivery systems (e.g. cancer treatments using drug nanocarriers). These indications may include the healing of wounds on skin or corneal surfaces, as well as cosmetic uses. A combination of an amphiphilic matrix with encapsulated drugs, peptides, growth factors, and/or stem cells may be used in these indications. These uses represent a multi-billion dollar market niche worldwide, and to the authors' recent understanding, a number of investigating trials are ongoing. Other products with good market and development potential could include injectables for various indications, such as tissue filling and the regenerations of bone defects, cosmetic skin augmentation, etc. Finally, with initial AMC products reaching the market, more sophisticated and complex applications may be explored and eventually commercialised. AMC nanoparticles acting as bio-chemosensors or imaging agents, preferably in combination with therapeutic effects, may be included in this latter category of products. Although the road towards regulatory approvals from the multiple authorities is time consuming and costly for medical-related applications, the potential market development of colloidal AMC is tremendous, suggesting that there will be a blossoming in the relevant markets in the time to come. Considering the costs and applications, the initial products to reach the market may be medical devices, followed by drug delivery systems or hybrid systems having combined functionalities. The somewhat easier market approval procedures present for medical devices suggests that the device pathways may provide advantages to commercialising these AMC products.

5. Conclusions

This review systematically and critically presents the properties and biomedical performance of the emerging material class of colloidal AMC. With extensive advances in both synthesis and characterisation within this past decade, the colloidal nature and excellent biological

performance of this material class suggest excellent therapeutic performances in a wide range of biomedical applications, from therapeutic drug nanocarriers to macroscopically assembled medical devices, such as wound dressings and injectables. In addition, more advanced uses, such as multifunctional nanodevices and inorganic–organic hybrids, also show good promise, even without extensive literature on this particular subject.

The reported molecular and colloidal structures and properties as well as the performance characteristics of different AMC materials can vary significantly, rendering simple structure-function relationships (e.g. between colloidal properties and *in vivo* behaviour) difficult. Qualitatively, the general properties derived from the amphiphilic nature of the polymer-chains, such as self-assembly, loading of hydrophobic drugs and interactions with lipid layers, are commonly shared. From the multiple promising modified chitosan that are reported for biomedical applications, several materials appear to have the characteristics that will enable use in the market. In particular, promising novel AMC materials include carboxymethyl-hexanoyl chitosan, multiple hydrophobic modifications of glycol chitosan and N-octyl-O-sulfate chitosan, all of which have been investigated thoroughly and have shown outstanding *in vitro* and *in vivo* performances.

The authors anticipate the generation of many more reports on new AMC materials with even more advanced applications. With the many promising proof of concept studies available, focusing on reaching the market, performing pre-clinical safety studies and bringing selected formulations through clinical trials into products are also needed.

To conclude, colloidal AMC is a material class with great versatility and many possible uses in multiple biomedical applications. The future of these materials is notably exciting, and the best results may yet be to come.

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