Expert Opinion

Nanostructured hyaluronic acid-based materials for active delivery to cancer

Dmitri A Ossipov
Uppsala University, Polymer Chemistry, Material Chemistry Department, S-75121 Uppsala, Sweden

Importance of the field: Active targeting of bioactive molecules by physicochemical association with hyaluronic acid (HA) is an attractive approach in current nanomedicine because HA is biocompatible, non-toxic and non-inflammatory.

Areas covered in this review: This review focuses on synthesis, physicochemical characterization and biological properties of different nanoparticulate delivery systems that include HA in their structures. Chemically based approaches to the delivery of small molecule drugs, proteins and nucleic acids in which they become chemically or physically bound to hyaluronic acid are reviewed, including the use of molecular HA conjugates and nanocarriers. The systems are considered in terms of intracellular delivery to different cultured cells that express HA-specific receptors (hyaladherines) differently. The *in vivo* biodistribution and therapeutic effect of these systems are discussed.

What the reader will gain: Different synthetic methodologies for preparations of HA-based nanoparticles are presented extensively. HA nanoparticulate systems of various structures can be compared with respect to their *in vitro* assays and *in vivo* biodistribution.

Take home message: To make HA useful as an intravenous targeting carrier, strategies have to be devised to: reduce HA clearance from the blood; suppress the HA uptake by liver and spleen; and provide tumor-triggered mechanisms of release of an active drug from the HA carrier.

Keywords: cancer, drug delivery, hyaluronic acid, nanoparticles

Expert Opin. Drug Deliv. (2010) 7(6):681-703

1. Introduction

One hundred years ago Paul Ehrlich first postulated the term 'magic bullet', or more specifically a chemotherapeutic agent that seeks out subtle changes between disease-causing cells and the other 200 or so types of healthy cell found in the body. By distinguishing the transformed cells from normal ones, a sufficiently toxic dose of the drug is thus provided to the cancer cells to kill them, whereas the healthy cells remain unaffected. By contrast, traditional chemotherapy relies on cancer cells proliferating more rapidly and therefore their chances of being killed by cytotoxic agent are higher. The vast majority of clinically used traditional drugs are low-molecular-mass compounds that penetrate both tumor and normal cells by diffusion, which does not completely exclude the systemic toxicity; this means severe side effects such as nephrotoxicity, bone marrow toxicity, neurotoxicity, cardiotoxicity and gastrointestinal toxicity. As a result, anticancer agents are typically given with the inclusion of treatment-free periods to allow the recovery of normal cells. On the other hand, the half-life of the most low-molecular-mass drugs (<500 Da) is short in the bloodstream, forcing them to be used at their maximum tolerated dose. Consequently, several creative drug delivery systems have been devised

informa
healthcare

10.1517/17425241003730399 © 2010 Informa UK Ltd ISSN 1742-5247

Article highlights.

- The major benefits of using HA as a drug delivery vehicle are that it is: biocompatible, non-toxic and non-inflammatory; biodegradable; can efficiently function as a 'homing device' because HA receptor CD44 is overexpressed in many types of tumor cell; can provide protection to its 'cargo'; and imparts solubility to hydrophobic drugs.
- The shortcomings of using HA, however, are that it is rapidly cleared from the blood circulation by means of: recognition by HA receptors of reticuloendothelial system organs (liver, spleen); and degradation by hyaluronidases.
- Chemical modifications or ionic complexation of HA allows its reorganization into nanoparticles, which reduces HA degradation and prolongs its circulation.
- HA chemical modifications performed include conjugation to drug molecules, grafting hydrophobic or cationic side chains, as well as crosslinking of HA chains into nanogels.
- *In vitro* studies revealed that HA nanoparticles can effectively recognize CD44-overexpresing cells and thus specifically deliver associated with the particles bioactive molecules by means of the receptor-mediated endocytosis.
- *In vivo* studies with HA nanoparticles are still quite limited. They have shown that HA nanoparticles can reach liver and tumor sites by means of blood circulation. However, to improve targeting tumor sites further and increase tumor/normal ratio for drug-HA carrier distribution there is a need to design new, more sophisticated intelligent HA-based vehicles.

This box summarizes key points contained in the article.

to improve the therapeutic index of traditional drugs through regulation of biodistribution and tuning the release kinetics [1].

Among the various concepts proposed for drug delivery, nanoparticles-based systems have shown significant potential to impart site-specificity to bioactive agents as well as to provide their time-controlled delivery. The popularity of these systems is a result in part to the advantages they provide for delivery of their drug payload. First of all, they can be injected directly into systemic circulation without the risk of blocking blood vessels. Following systemic administration, particles with diameters ranging from 10 to 70 nm can travel through even very small capillaries [2], whereas particles with diameters ranging from 70 to 200 nm demonstrate the most prolonged circulation time [3]. These particles can circulate much longer than free drug, whose half-life is typically < 5 min [4]. The prolonged circulation time of such nanoparticles (> 1 h) is also to avoid clearance by the reticuloendothelial system (RES), whereas larger particles (> 200 nm) can be cleared and removed from the body [5]. At the same time, leaky tumor vessels and poor lymphatic drainage in the tumor tissues allow accumulation of long-circulating nanoparticles in tumors. The enhanced permeability and retention (EPR) effect provides the most probable mechanism for passive tumor localization [6]. Importantly, particles < 100 nm can be enclosed within endocytic vesicles [7]. With respect to size and surface properties, nanoparticulate drug delivery vehicles often mimic viruses that infect specific cells within host organisms [8]. Hence, nanoparticulate delivery systems are capable of reaching widely spread niches throughout the body and eradicating the metastasized cancer cells.

By a general definition, nanostructured carriers can include solid organic or inorganic dense particles, soft aggregates, gels, and actually even single macromolecules with spatial dimensions on the order of a few nanometers up to a micrometer. **Figure 1** summarizes diverse classes of nanomaterials that have received increasing attention during the last decade. Nanoparticles can be inorganic, such as magnetic iron oxides [9,10], gold nanorods [11] and nanoshells [12], semiconductor quantum dots [13], titanium dioxide [14], hydroxyapatites [15] and carbon nanotubes [16], and can be built up from organic compounds. The rationale for organic polymer carriers was formulated in 1975 by Ringsdorf [17] after the discovery of the ability of macromolecules to localize to lysosomes. Later, polymeric drug delivery was expanded to encompass not only linear homopolymers, but also dendritic macromolecules [18] as well as amphiphilic block copolymers that can spontaneously assemble into spherical (micelles), cylindrical (filomicelles) or vesicular (polymersomes) shapes [19]. Polymeric chains were also physically or chemically crosslinked, forming hydrogels of submicrometer size [20-22]. Other types of organic compounds in nanotechnology are solid lipid nanoparticles (SLNs) [23] and phospholipids that in aqueous media can form closed bilayered structures, that is, liposomes [24]. Generally, all these nano-objects can act as carriers for cytotoxic agents, or they can be made cytotoxic themselves in response to stimuli such as light, heat or pH (**Figure 1**). Functioning as drug delivery vehicles, nanoparticulate carriers normally physically incorporate drugs in their interior or adsorb the cytotoxic agents on their surface. Alternatively, low-molecular-mass drug can be covalently conjugated to a nanocarrier through a releasable linker, albeit this option has been presented mainly by drug-polymer conjugates [25]. Intelligent nanoparticles have also been designed to be responsive to a stimulus that converts them into a cytotoxic state or produces cytotoxic species. For example, the ability of gold nanoparticles to absorb near-infrared light (NIR) and convert it into heat on a picosecond timescale allows them to kill cancer cells after endocytic uptake and exposure to NIR light (photothermal cancer therapy) [26,27]. Photosensitizer nanoparticles such as quantum dots, dendrimers, polymer micelles, liposomes and hydrogel nanoparticles were used for photogeneration of cytotoxic reactive oxygen species that destroy tumor cells in the course of photodynamic cancer therapy [28,29]. Super-expandable nanogels that undergo nano- to micro-scale volume transition on brief cold-shock treatment have recently been developed to induce traumatic death of cells. At 37°C the collapsed

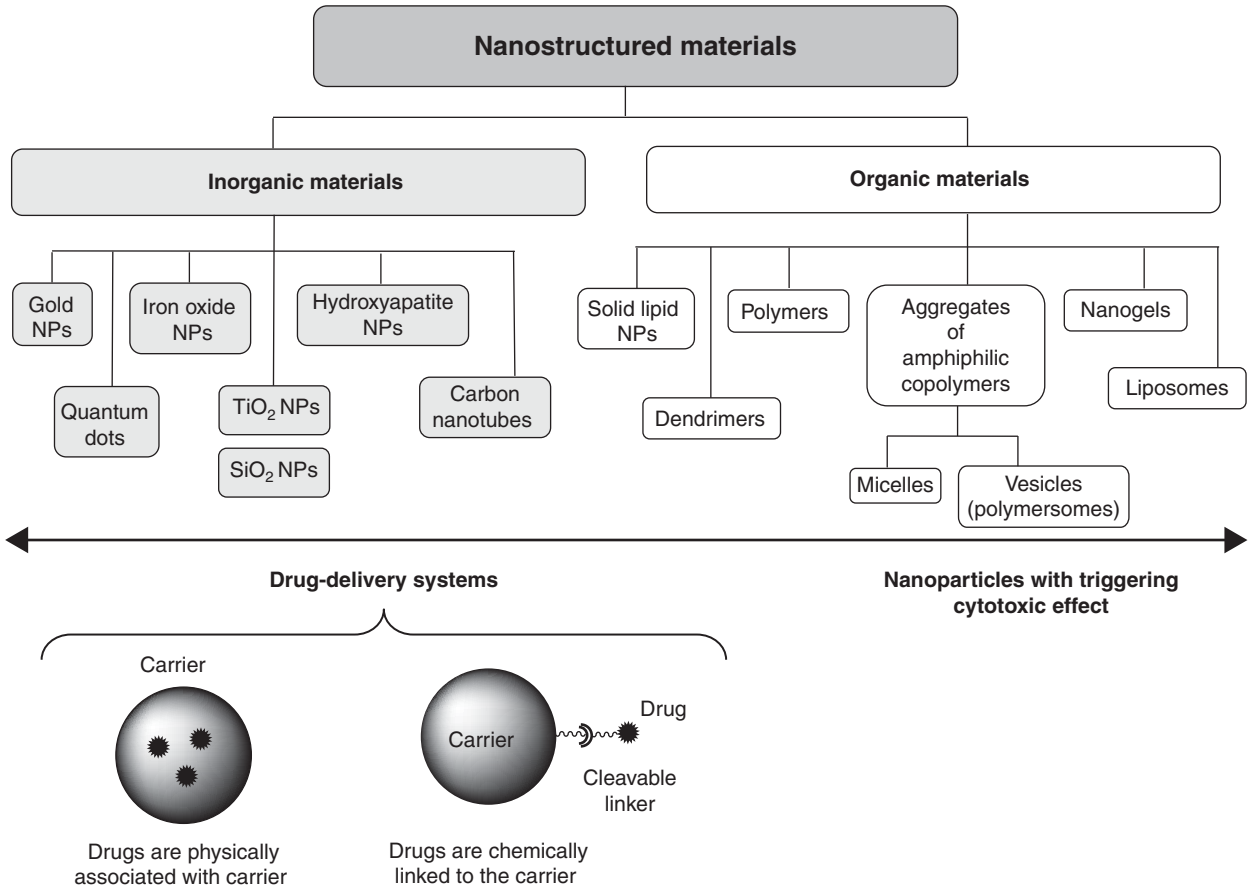


Figure 1. The types of nanostructured material.

nanogels were endocytosed within human cervical cancer cells followed by cooling to 15°C, which was likely to result in physical rupture of the cells' plasma membrane [30].

From the very beginning of cancer nanotechnology, the size of nanoparticles was fundamental, but in a 'passive' sense – that is, only particles of the right size can permeate the gaps in the tumor's blood vessels. However, more recent studies have shown that polymeric drug carriers encounter difficulties in interacting with target cells after extravasation [31]. This is because macromolecules or other nanocarriers are internalized by endocytosis, which is a relatively slow process requiring high concentration of the nanocarriers in extracellular space. To improve targeting efficiency of nanoparticles, the concept of 'active' targeting, that is, the use of biomolecular recognition molecules, has been introduced to provide a greater degree of specificity [32]. By analogy with the 'prodrug' system, in which a tumor recognition moiety is directly connected to a cytotoxic warhead, the surface of nanoparticles can be decorated with biological targeting moieties.

Hyaluronan (HA), a non-sulfated glycosaminoglycan polymer found as a main component of the extracellular matrix (ECM) in mammalian bone marrow and loose connective tissues, regulates diverse cellular responses, including proliferation, differentiation, motility, adhesion and gene expression [33]. HA establishes multivalent interactions with proteoglycans and other extracellular macromolecules that determine the ECM porosity and permeability. It is well known that cells divide and migrate within an extracellular matrix that is rich in hyaluronan. HA-rich matrices create hydrated pathways that facilitate penetration by the invading tumor cells [34]. Various types of tumor, for example, epithelial, ovarian, colon, stomach and acute leukemia, overproduce HA such that elevated HA contents are prognostic for malignant progression [35]. Cellular HA receptors CD44 and RHAMM are overexpressed in many types of cancer cell, demonstrating enhanced binding and internalization of HA [36,37]. Consequently, to increase the concentration of anticancer agent at the target site and decrease it elsewhere in the body, two platforms have been exploited so far: i) direct conjugation of cytotoxic drugs to low-molecular-mass HA, when HA itself functions as a carrier and a 'homing device'; and ii) physical incorporation of a drug within a nanoparticulate carrier to which HA is attached as a targeting coating (**Figure 2**). From a structural aspect, hyaluronic acid nanogels encapsulating bioactive compounds present a delivery system that is intermediate between the above two

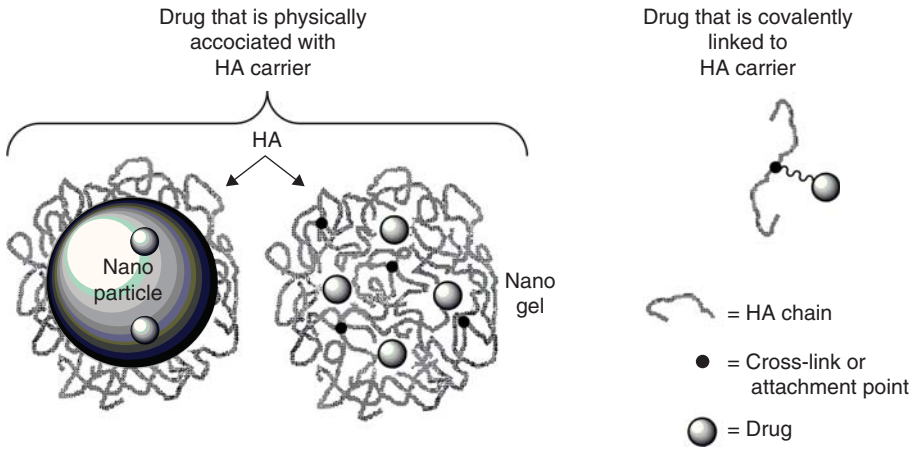


Figure 2. The types of HA-based nanostructured material.

(**Figure 2**). HA nanogels are composed of several HA polymeric chains that are associated into three-dimensional soft nanomatrices. With their high water content, these HA nanogels are similar to macroscopic bulk hydrogel materials and thus belong to soft drug delivery systems. It should be noted, however, that covalent linking of some hydrophobic drugs to hydrophilic HA molecules results in amphiphilic HA-drug conjugates that may potentially associate, forming particles of nanometer size, that is, nanogels. Therefore, the boundary between HA-drug conjugates and HA nanogels cannot be drawn sharply.

A confounding difficulty for HA as a targeting carrier is that HA is cleared from circulation by the liver [38]. For HA to be a useful intravenous targeting carrier, strategies have been devised to reduce HA clearance from the blood. Degradation of HA by hyaluronidases can be reduced through chemical modifications such as attachment of HA to a nanocarrier/drug or crosslinking of individual HA polymer chains with the formation of nanogel particles. The constraints applied to individual HA chains by their crosslinking or binding to the surface of more dense nanoparticles are believed to alter HA pharmacokinetic properties. It has been shown recently that HA nanoparticles can efficiently accumulate in tumors and be retained there for at least 2 days, whereas native HA is quickly cleared from the body within 1 h post-injection [39]. The authors pointed out that this might result from the increased circulation time and EPR effect as well as tumor selective binding of HA to CD44 receptor. Overall, the production of nanostructured materials from hyaluronic acid can tailor many properties of nano-scale drug delivery systems for specific applications: solubility (inherent hydrophilicity of HA), biodistribution (targeting to cancer cells), biocompatibility (electrical charge), biodegradability (binding/crosslinking density) and drug release (HA-drug spacer or physical interaction between a drug and a carrier). This review focuses on synthesis, physicochemical characterization and biological properties of different nanoparticulate delivery systems that include hyaluronic acid in their structures. Chemically based approaches to the delivery of small molecule drugs, peptides, proteins and nucleic acids in which they become chemically or physically bound to hyaluronic acid are reviewed and analyzed, including use of molecular HA conjugates and nanocarriers. The reviewed HA-based nanoparticulate delivery systems are considered in terms of intracellular delivery to different cultured cells that express HA-specific receptors (hyaladherines) differently. The *in vivo* biodistribution and therapeutic effect of these systems are discussed.

2. Hyaluronic acid-drug conjugates

For targeting of the anticancer drugs to the tumors, there should be some form of prolonged association between the HA and drug/s. The idea was realized in the early 1990s by chemical linking of cytotoxic agents to HA. Covalent linking of a given drug molecule to HA macromolecular chain converts this drug to its prodrug derivative in which the chemical bond between the HA carrier and the drug molecule is designed taking into account the following important principles: i) a cleavable bond should be sufficiently stable extracellularly to increase the prodrug's *in vivo* half-life, while being rapidly broken after cell entry; ii) cleavage of the bond that is triggered by intracellular environment should preferably release drug molecule intact, that is, without altering its chemical structure; and iii) generally, the carrier molecule itself should also be stable enough in the blood circulation. As the carrier, however, native HA molecule is the subject of many clearance mechanisms, the most efficient of which is elimination by a HARE receptor present in the liver and spleen [38,40]. The short half-life of high-molecular-mass HA in the blood [41] might be the reason that HA-drug conjugates show effective cytotoxicity at a cellular level, but *in vivo* studies did not use intravenous dosing, opting for intratumoral, subcutaneous or intraperitoneal injections.

The two most common sites used for chemical modification of HA as well as for conjugation to drugs and other ligands are the carboxylic acid and hydroxyl functionalities (**Figure 3**). As carboxylate and hydroxyl groups are multiply present along the HA backbone, the modifications that involve these groups are polylithic. Hence, they can be utilized for further purposes such as, for example, crosslinking. The scope of reactions that have been exploited for multiple attachments to HA include amidation and esterification of the HA carboxylates, as well as the reactions of primary hydroxyl groups to give ester and ether linkages. Contrary to the carboxylate and hydroxyl groups, there is only one reducing end in polysaccharides that is essentially an aldehyde group. It is therefore monolithic, that is, it provides only one attachment point for the HA molecule. Reductive amination at the reducing end was used to prepare terminally modified low-molecular-mass HA-ligand conjugates, as well as to graft HA oligomers to another polymer carrying amino groups.

The earliest reports on HA-drug conjugates were performed for HA-anthracyclines by Cera *et al.*, who linked amino groups of the drugs by means of not releasable carbamate linkages to HA hydroxyls (**Table 1**, (**1**)) [42]. The conjugates obtained were shown to be, in principle, prevented from entering the cells, showing, however, efficient binding to the cell surfaces. Some partial and slow drug incorporation into cells was still observed as a function of time. The conjugates' penetration was probably a result of nonspecific endocytosis, as qualitatively similar results were obtained using the HA conjugates with a molecular mass of 120 kDa or carboxymethylcellulose as the drug carriers. Cell growth inhibition was found to be less with high-molecular-mass HA conjugates (700 kDa) than with low-molecular-mass HA conjugates (120 kDa).

Indeed, targeted delivery of doxorubicin to several cancer cell lines (human breast HBL-100, human ovarian SK-OV-3 and human colon HCT-116) has been demonstrated *in vitro* by *N*-(2-hydroxypropyl)-methacrylamide (HPMA) polymer (M_r = 14,500) grafted with both doxorubicin (DOX) and low-molecular-mass HA (M_r = 3880) by means of lysosomally degradable GFLG-tetrapeptide spacer (**Table 1**, (**2**)) [43]. Despite the reduced toxicity of the HPMA-HA-DOX conjugates relative to the free drug, the HPMA-HA-DOX conjugates were much more potent than HPMA-DOX conjugates lacking the HA targeting moiety. The role of the degradable tetrapeptide linker in the HPMA-HA-DOX was shown to be crucial, as the conjugation of DOX directly to HA by means of a poorly hydrolyzable hydrazide linkage resulted in the HA-DOX conjugates (**Table 1**, (**3**)), which were even less toxic than the HPMA-DOX conjugate. More importantly, however, both HPMA-HA-DOX and HA-DOX conjugates demonstrated selective toxicity against cancer cells overexpressing HA receptors, whereas free doxorubicin, as well as HPMA-DOX conjugate, were indiscriminately toxic to both cancer cells and to NIH 3T3 fibroblasts. The cytotoxicity of HPMA-HA-DOX conjugates was corroborated with their rapid uptake by cancer cells compared with non-targeted HPMA-DOX.

Another potent anticancer agent, paclitaxel (Taxol), was linked at 2′-OH by means of a succinate ester spacer to low-molecular-mass HA (M_r = 3880) (**Table 1**, (**4**)) [44,45]. The advantage of such linking was obvious because the parent drug is poorly soluble in aqueous media. Also, targeting the drug to human cancer cell lines (HBL-100, SK-OV-3 and HCT-116) was provided *in vitro*. Only CD44 overexpressing cells internalized the HA-Taxol conjugate after 20 min of incubation, whereas non-transformed NIH-3T3 cells did not show membrane binding and rapid uptake as judged from confocal microscopy and laser flow-activated cell sorting (FACS) experiments. The relative stability of the ester linkage between Taxol and HA in the media with cells, complemented with the rapid drug release in the presence of esterase, allowed the conclusion that the HA-Taxol conjugate is first internalized by receptor-mediated endocytosis followed by esterase-catalyzed drug release in the lysosomal compartment. In accord with specific uptake of the conjugate and hydrolytic stability of the drug-carrier linkage, human cancer cells were selectively killed whereas NIH-3T3 cells were unaffected. The HA receptor-mediated mechanism of cytotoxicity was strengthened further by competition experiments, in which preincubation with HA protected susceptible cancer cells from the action of HA-Taxol conjugates. It is noteworthy that the most effective HA-Taxol had a lower IC_{50} value relative to free Taxol. The presented HA-Taxol conjugates also showed the elevated drug release in the human plasma; they might therefore break down *in vivo* before reaching the target.

Butyric acid, the smallest histone deacetylase inhibitor and a potent inducer of cell growth arrest, was esterified with primary hydroxyl groups of HA in order to investigate biological activity of the obtained HA-But conjugates (**Table 1**, (**5**)) against human hormone-dependent breast cancer cell line (MCF7) [46]. Coradini *et al.* showed that the HA-But derivatives exert their inhibitory effect through binding to CD44 *in vitro*. The fluorescein-labelled HA-But (M_r = 85,000) was almost completely internalized by MCF7 cells after 2 h whereas pretreatment of the cells with anti-CD44 MAb J173 antibody led to inhibition of the HA-But binding to the receptor. The optimal degree of esterification was 19 mol% per disaccharide unit, resulting in a threefold decrease in IC_{50} compared with free butyric acid. The presence of butyrate residues at higher degree of substitution, however, did not correspond to an increase in the prodrug activity, which was explained by steric hindrance from the butyrate residues impairing HA affinity to the receptor. It should be noted, however, that the IC_{50} of free sodium butyrate (0.5 mM) is within the range where the hyaluronic acid part of the HA-But conjugate becomes toxic itself. Further *in vitro* studies revealed, for example, that native HA was not toxic towards CD44-poor HepG2 cells at the highest

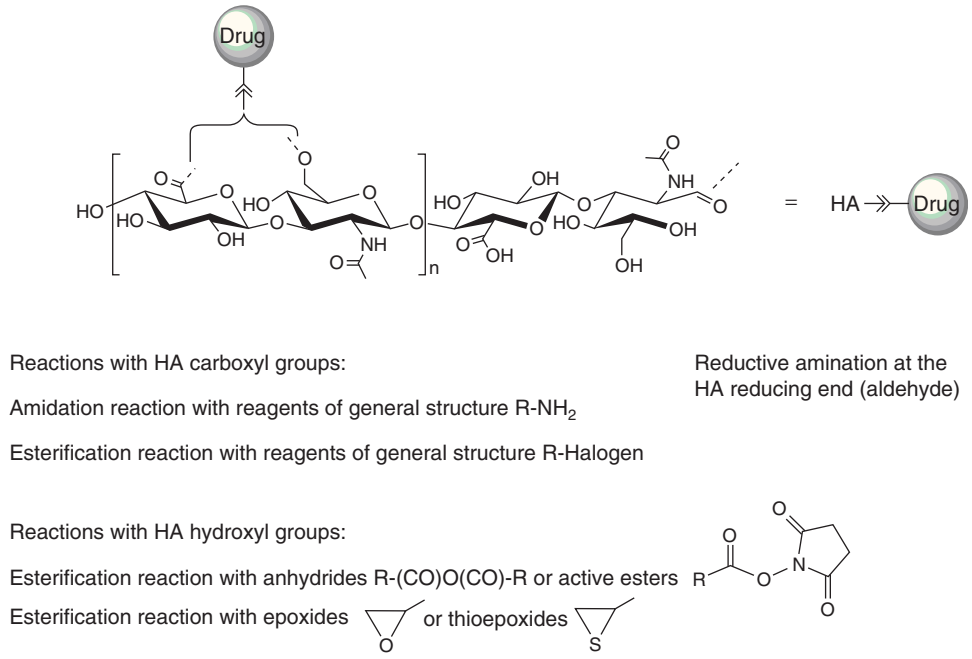


Figure 3. General structure of HA-drug conjugates showing three major attachment points to the HA backbone with the corresponding reactions that have been used for these covalent attachments.

concentration evaluated (4 mg/ml), whereas incubation of the CD44-rich HepB3 cells with HA at 4 mg/ml showed almost 100% inhibition of the cells growth [47]. At this concentration, however, the HA-But conjugate induced 90 and 60% inhibition of the HepB3 and HepG2 cells growth, respectively, after incubation for 6 days. This was explained by very rapid turnover of the CD44 receptor after HA-But uptake. The authors also investigated the distribution of ^{99m}Tc-labelled HA-But conjugate in accord with the route of administration *in vivo*. These studies revealed that intravenous injection led to very fast and substantial accumulation of the conjugate in the liver and spleen. Conversely, the intraperitoneal (i.p.) and subcutaneous (s.c.) treatments resulted in relatively slow distribution with marked persistence of the drug at the site of injection.

A carborane polyhedron containing 10 boron atoms has been conjugated to HA (M_r = 200,000) by means of several linkers with the aim of developing selective tumor-targeted boron neutron capture therapy (BNCT) [48,49]. BNCT is a potential cancer therapeutic modality, based on the capacity of ^{10}B to capture thermal neutrons and to disintegrate, releasing high energy α particles, thus leading to ablation of malignant cells. The HA-carborane conjugates (**Table 1**, (**6 – 8**)) were shown to have the same ability to bind to CD44 receptor as the native HA, even in the case of 30% substitution with carboranes (conjugate HApCB, **Table 1**, (**6**)). Cellular uptake of the conjugates was subsequently studied *in vitro* by counting boron atoms inside the cells by atomic emission spectrometry. However, the cells that express 10-fold fewer CD44 receptors acquired almost the same content of boron atoms as CD44-positive cells after 1 h of incubation with HA-carborane conjugates. Moreover, the amount of incorporated boron atoms was dependent on the structure of a linker between carborane cages and the HA backbone. Specifically, the ester-linked HApCB (**6**) was 10-fold more effective in carborane delivery than the amide/triazole-linked HAAACB (**7**) and HApACB (**8**). Unfortunately, the authors did not reveal comparative stability of the HA-carborane conjugates in the cell media in order to exclude the possibility of the mechanism by which the carborane cages are first cleaved from the HA carrier in the extracellular fluid and then cross the membrane as low-molecular-mass species.

Recently, the author's group has been developing crosslinkable hyaluronic acid derivative with covalently attached bisphosphonate groups [50]. Bisphosphonates (BP) were shown to inhibit osteoclast differentiation, thus preventing osteoclastic bone resorption activity [51], and direct antitumor action of BPs was demonstrated *in vitro* [52]. High-molecular-mass HA (1.5 MDa) was dually functionalized with two chemically orthogonal groups, such as hydrazide and thiol, and acrylated aminomethylene bisphosphonate was linked to the thiol groups by conjugate addition reaction (**Table 1**, (**9**)). A new prodrug concept was devised that relies on high binding affinity of high-molecular-mass HA to CD44-overexpressing cells, but it is not able to internalize further owing to steric hindrances [53]. Internalization is, however, initiated by means of action of the HA degrading enzyme hyaluronidase (Hase), which cleaves the cell-bound HA to a smaller size that allows uptake. The

Table 1. Chemical structures of HA-drug conjugates used for cancer treatment *in vitro* or *in vivo*.

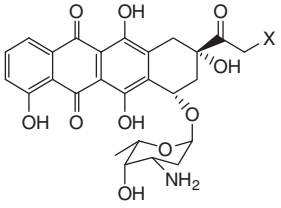
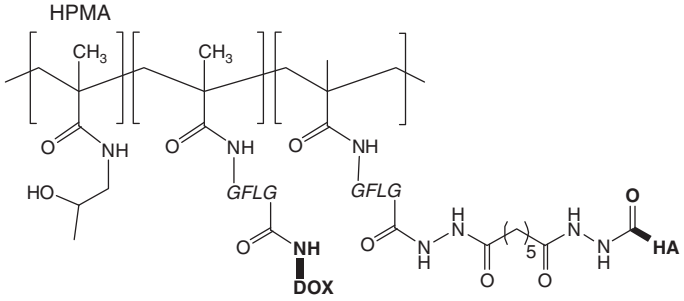
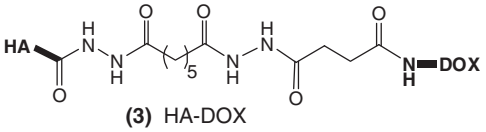
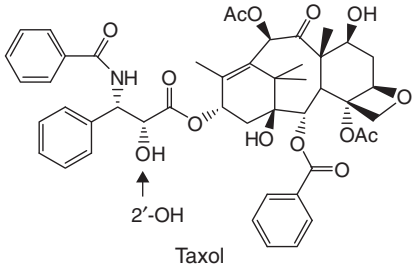
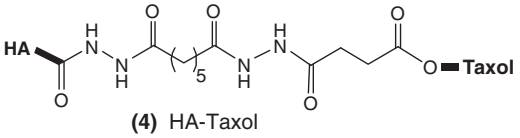
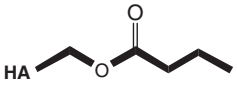
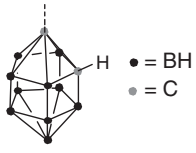
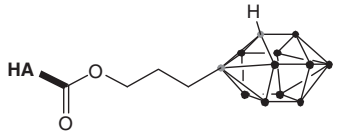
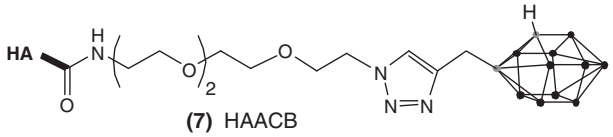
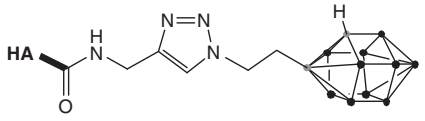
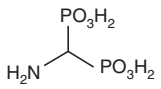
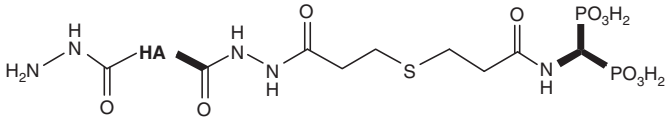
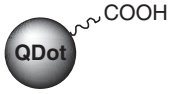
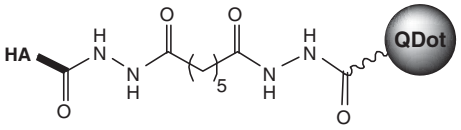
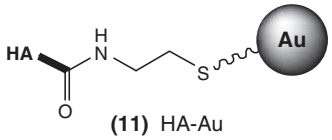
Drug/bioactive molecule	HA-drug conjugate	Drug-spacer linkage
 Doxorubicin (DOX), X = OH Daunorubicin (DNR), X = H	 **(1)** HPMA	Carbamate
	 **(2)** HPMA-HA-DOX	GFLG tetrapeptide
	 **(3)** HA-DOX	Amide
 Taxol	 **(4)** HA-Taxol	Ester
$CH_3CH_2CH_2COOH$ Butyric acid	 **(5)** HA-But	Ester
 Carborane	 **(6)** HApCB	Ester
	 **(7)** HAACB	Amide
	 **(8)** HApACB	Amide

Table 1. Chemical structures of HA-drug conjugates used for cancer treatment *in vitro* or *in vivo* (continued).

Drug/bioactive molecule	HA-drug conjugate	Drug-spacer linkage
 Aminomethylene bisphosphonate	 **(9)** HA-BP/hydrazide	Amide
 Quantum dot	 **(10)** HA-QDot	Amide
 Gold nanoparticle	 **(11)** HA-Au	

increased amount of Hase is characteristic for the tumor environment [54], providing a rationale for the mechanism of action of high-molecular-mass HA-drug conjugates as prodrugs. The concept was also expanded to HA hydrogel materials as local depots of drugs that are covalently linked to the hydrogel matrix and can be released later as low-molecular-mass HA-drug conjugates in the course of hydrogel degradation.

The effect of chemical modification of HA on its cellular uptake and *in vivo* distribution was investigated by conjugating quantum dots (QDot) to HA backbone, which resulted in a multiple QDot system [55]. HA (M_r = 132,300) was first modified with adipic acid dihydrazide (ADH) and the HA-ADH obtained was coupled to QDots with carboxyl terminal ligands through the formation of amide linkage (**Table 1**, (**10**)) [56]. The HA-QDot conjugates had a morphology in which several QDots aligned possibly along the HA molecules. The same type of morphology was also observed when thiolated HA was used as a template for one-dimensional array of gold nanoparticles (**Table 1**, (**11**)) [57]. Real-time bio-imaging of HA-QDot conjugates was performed by injecting them either subcutaneously or in the tail vein of mice. Owing to near-infrared emission of QDots at 800 nm, which has high penetration depth, the fluorescence of HA-QDot conjugates could be detected for up to 2 months at the site of subcutaneous injection. Interestingly, the distribution of HA-QDot conjugates after intravenous injection appeared to be dependent on the degree of ADH modification. HA-QDot conjugates with 35 mol% ADH content maintaining enough binding sites for HA receptors accumulated mainly in the liver, the organ with highest specificity to HA molecules. HA-QDot conjugates with 68 mol% ADH content presented as a conjugate that has lost many of its HA binding characteristics. As a result, it was distributed evenly to the tissues in the body [56]. The same trend was also observed on a cellular level when B16F1 cells were incubated *in vitro* with various HA-QDot conjugates [58]. After 2 h HA-QDot conjugates with a degree of modification <25 mol% appeared to be taken up more efficiently by cells than QDots alone. Increasing the degree of modification led to a decrease of the fluorescence intensity in accordance with the fact that at least three carboxyl groups (hexasaccharide) in the HA molecule are required for binding to HA receptors [59]. The HA receptor-mediated mechanism of internalization of HA-QDot conjugates was confirmed further by preincubation of the cells with intact HA, which caused a drop in the fluorescence intensity of the endocytosed HA-QDot conjugates.

3. Hyaluronic acid nanogels

Colloidally stable particles made from hydrogels, also referred to as micro- or nanogels, can be classified by the type of crosslinks in the same way as their macrogel counterparts: physically crosslinked and chemically crosslinked nanogels. The crosslinks in physically crosslinked nanogels arise from non-covalent attractive forces between the polymer chains, namely hydrophobic interactions, hydrogen bonding, or ionic interactions, whereas in chemically crosslinked nanogels the polymer chains are linked with covalent bonds. This section surveys the nanogel particles in which HA polymer chains take a leading role in the particles' core formation.

3.1 Soft hyaluronic acid nanogel carriers made by chemical crosslinking

Intravenous injections of HA-drug conjugates usually show short residence time in the body as a result of their rapid degradation in the blood and efficient uptake by the liver endothelial cells. The normal/tumor ratio HA distribution was demonstrated to be decreased by pretreating the animals with chondroitin sulfate, which binds to HARE receptors of the liver cells but not to CD44 receptors [60]. To increase further the plasma half-life of HA, fabrication of chemically crosslinked HA nanogel particles was suggested. Chemically crosslinked hydrogels are usually more stable than the physically crosslinked analogues.

To prepare chemically crosslinked HA nanogel particles, one has to spatially localize the HA molecules and crosslinkers within the nano-sized compartments, that is, to provide a high concentration of reacting species only in very small volumes and not apart of them. The synthetic strategies that can achieve this are normally based on phase separation, such as the inverse water-in-oil (w/o) microemulsion method (**Figure 4**). The first report on the preparation of chemically crosslinked HA nanoparticles was published in 2004 [61]. Hyaluronan microspheres were prepared by homogenizing the aqueous solution of high-molecular-mass HA ($1.6 - 3.3 \times 10^6$ Da) and ADH crosslinker in mineral oil and stabilizing the obtained microemulsion with surfactant (**Table 2**, entry 1). Chemical crosslinking of the HA chains with ADH was subsequently initialized by adding water-soluble amide coupling reagent ethyl-3-[3-dimethylamino] propyl carbodiimide (EDC) to the emulsion. By using this technology, the plasmid DNA encoding a β-galactosidase reporter was encapsulated in the microspheres before the chemical crosslinking with EDC. The diameter of the microspheres obtained ranged from 5 to 15 μm and they released DNA that was structurally intact. Furthermore, HA-DNA microspheres injected into the hind limb of rats showed positive transfection of DNA, which is not native to the rat genome.

A new anticancer drug delivery system was proposed based on nanogel particles that were made by amidation crosslinking between HA carboxylates and acylhydrazide groups of polyaspartylhydrazide (PAHy) (**Table 2**, entry 2) [62]. A reversed-phase microemulsion was first prepared by adding an aqueous solution of HA (M_r = 174,000), PAHy and EDC to the propylene carbonate-ethylacetate organic phase, followed by stirring the obtained mixture in the presence of surfactant. The speed of activation of HA with EDC in the aqueous nanodroplets was slowed down by maintaining pH at 7.5, which prevented network formation before phase separation. *N*-hydroxysulfosuccinimide (NHSS) was then added to the established microemulsion in order to convert the unstable *N*-acylurea derivative into the more stable *N*-hydroxysulfosuccinimide ester and thus to ensure crosslinking coupling with hydrazide groups. The nanogel particles obtained had diameters ranging from 200 to 300 nm, they were resistant to degradation even at pH 1.0, and while in the presence of hyaluronidase a partial degradation occurred. The particles themselves were not toxic to cells and were able to encapsulate a water-soluble model drug, 5-fluorouracil, although the release of the 5-fluorouracil was almost completed in 1 h.

Amide crosslinking in an oil-in-water type emulsion was used to make HA nanoparticles of diameter ranging from 80 to 160 nm for subsequent electrostatic hybridization with Fe_2O_3 nanoparticles (**Table 2**, entry 3) [63]. The resulting hybrid HA-Fe_2O_3 particles were characterized by a corral-like structure in which Fe_2O_3 core is surrounded by smaller HA particles. The potential of both HA and HA-Fe_2O_3 hybrid particles to deliver atrial natriuretic peptide (ANP) to the CD44-expressing cells was investigated *in vitro* by encapsulating fluorescein isothiocyanate (FITC)-labelled ANP in the HA particles and examining the cells under a fluorescence microscope at 24, 48, and 72 h after incubation. Fluorescence has been detected in the cells, suggesting that peptides were delivered to almost all cells. No studies, however, were performed on peptide release from the HA nanogels or on peptide uptake itself to show clearly the transferring role of the HA nanogels.

Another crosslinking reaction apart of amide formation that has been used for the preparation of HA-based soft hydrogel particles is the conjugate addition of HA hydroxyls to divinyl sulfone (DVS) crosslinker under basic conditions (**Table 2**, entry 4) [64,65]. The crosslinking reaction was performed by the addition of DVS to the water-in-isooctane microemulsion stabilized with dioctyl sulfosuccinate sodium salt. The aqueous droplets containing HA molecules (490 kDa) served as nanoreactors where the crosslinking reaction between the HA hydroxyls and the added DVS occurred.

A considerable potential of HA nanogel particles for targeted delivery of drugs was clearly demonstrated *in vitro* by Lee *et al.* [66]. Thiolated HA (M_r = 132,000) was disulfide crosslinked in aqueous nanodroplets of water-in-oil emulsion (**Table 2**, entry 5). Green fluorescence protein (GFP) siRNA was physically entrapped within the HA nanogels during the emulsion/crosslinking procedure. No extra crosslinker or coupling reagent was used in this case because thiolated HA can be slowly crosslinked itself by exposure to air [67]. The HA nanogels had a mean diameter ~ 200 nm. The disulfide linkages in the prepared nanogels can be cleaved on treatment with glutathione (GSH). The rhodamine-labelled HA nanogels were already visualized in the early endosomes of the CD44-overexpressing HCT-116 cells after 10 min incubation. The nanogels were subsequently traced in the cytosol at 2 h time point. Conversely, CD44-deficient NIH-3T3 cells did not readily take up the HA nanogels, suggesting a specific CD44-mediated HA nanogel transport mechanism. The GFP gene silencing effect of the GFP siRNA-loaded HA nanogels in GFP overexpressing HCT-116 cells reached the level of GFP inhibition of expression by the standard siRNA/PEI complex. At the same time, HA nanogels did not show any

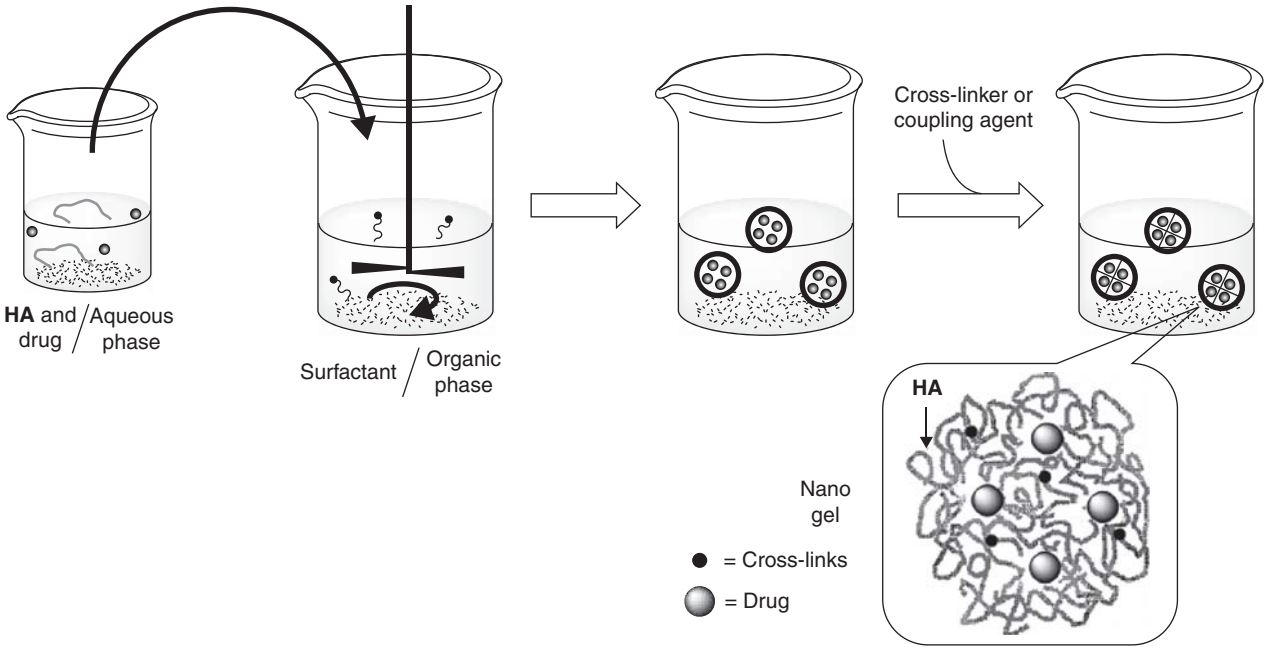


Figure 4. Reverse-phase water-in-oil microemulsion method of preparation of chemically crosslinked HA nanogels.

cytotoxicity up to 1 mg/ml concentration, whereas polyethyleneimine (PEI) was 100% detrimental for cells at concentration < 0.1 mg/ml. Unfortunately, the GFP gene silencing was not performed with NIH-3T3 cells to demonstrate that the gene silencing effect of siRNA can be selectively directed to specific cells by HA nanogels carriers.

An inverse emulsion crosslinking technique was also applied to prepare hydrazone crosslinked HA microgels [68]. For this purpose, the aqueous solution of the hydrazide-modified HA-ADH was homogenized in mineral oil containing surfactant, followed by addition of the aqueous aldehyde crosslinker that was either poly(ethylene glycol)-dialdehyde or HA-polyaldehyde (**Table 2**, entry 6). The aldehyde-hydrazide ratio was above unity to have the residual aldehyde groups in the resulting hydrazone crosslinked microgels. These aldehyde functionalities were used for subsequent crosslinking of the microgels with HA-ADH to give doubly crosslinked bulk networks. Thus, unlike other HA nano/microgel particles that are chemically inert, the synthesized HA microgels allowed for covalent conjugation of therapeutic agents by means of the residual aldehyde groups.

3.2 Soft hyaluronic acid nanogel carriers made by ionic gelation

The microemulsion method requires high energy sources, that is, high-speed mechanical stirring or ultrasonication, and use of organic solvents and surfactants, which represents a limitation for the entrapment of delicate molecules such as peptides and proteins. Moreover, chemical crosslinking that exploits non-chemoselective reactions could compromise the biological activity of proteins, nucleic acids, and even low-molecular-mass agents. This limitation does not exist, however, in physically crosslinked HA nanogel particles because physical gelation is much milder in terms of affecting complex structures of the corresponding cargo molecules. Nanoparticles in which HA chains are interconnected into an interpenetrated network can be established by means of, for example, ionic interaction with oppositely charged polymers.

Methods of ionic self-assembling of HA molecules into nanogels exploit the same principle that has been used in cationic vectors for nucleic acids, in which anionic plasmid DNA, siRNA or single-stranded antisense oligonucleotides are bound to cationic carriers forming an ionic complex [69]. As HA is a polyanionic polysaccharide, it provides multivalent charges for interaction with polycations. Depending on the nature of the cargo molecule, it can be pre-mixed in either HA solution or the solution of cationic polymer (**Figure 5A**). The ionotropic gelation technique has been explored recently to prepare fine hyaluronan-chitosan (HA/CS) nanoparticles (**Figure 5**, (**1**)) [70]. From a physicochemical point of view, the formation of nanoparticles resulted from strong ionic interaction between positively charged chitosan amino groups and negatively charged HA carboxylic acid groups. The ratio of the low-molecular-mass chitosan (low-molecular-mass CS, 5 kDa) to HA and HA molecular masses (17, 35 and 64 kDa) were varied such that the particles in the 100 – 200 nm range were obtained by mixing low-molecular-mass CS with 64 kDa HA in a weight ratio of 4:1. The zeta-potential of these particles was + 32 mV, indicating that the particles were covered with an excess of

Table 2. Summary of HA micro/nanogels prepared by covalent crosslinking in inverse microemulsion.

Entry	Crosslinkage	Aqueous phase (HA, crosslinker, 'drug')	Organic phase (solvent, surfactant)	Trigger	Ref.
1	Amide	HA, ADH, **'pDNA'**	Mineral oil, Span 80	EDC (coupling agent)	[61]
2		HA, PAHy, EDC	EtOAc-PC, Span 85	NHSS	[62]
3		HA, dihydrazide	Not specified	EDC (?)	[63]
4	Ether	HA, NaOH	Isooctane-*n*-heptanol, AOT	DVS	[64,65]
5	Disulfide	Thiolated HA, **'siRNA'**	Hexane (Span 65 + Span 80 + Tween 80)	-	[68]
6	Hydrazone	HA-ADH	Mineral oil, Span 80	PEG-dialdehyde or HA-aldehyde	[72]

DVS: Divinyl sulfone; EDC: Ethyl-3-[3-dimethylamino]propyl carbodiimide; NHSS: *N*-hydroxysulfosuccinimide.

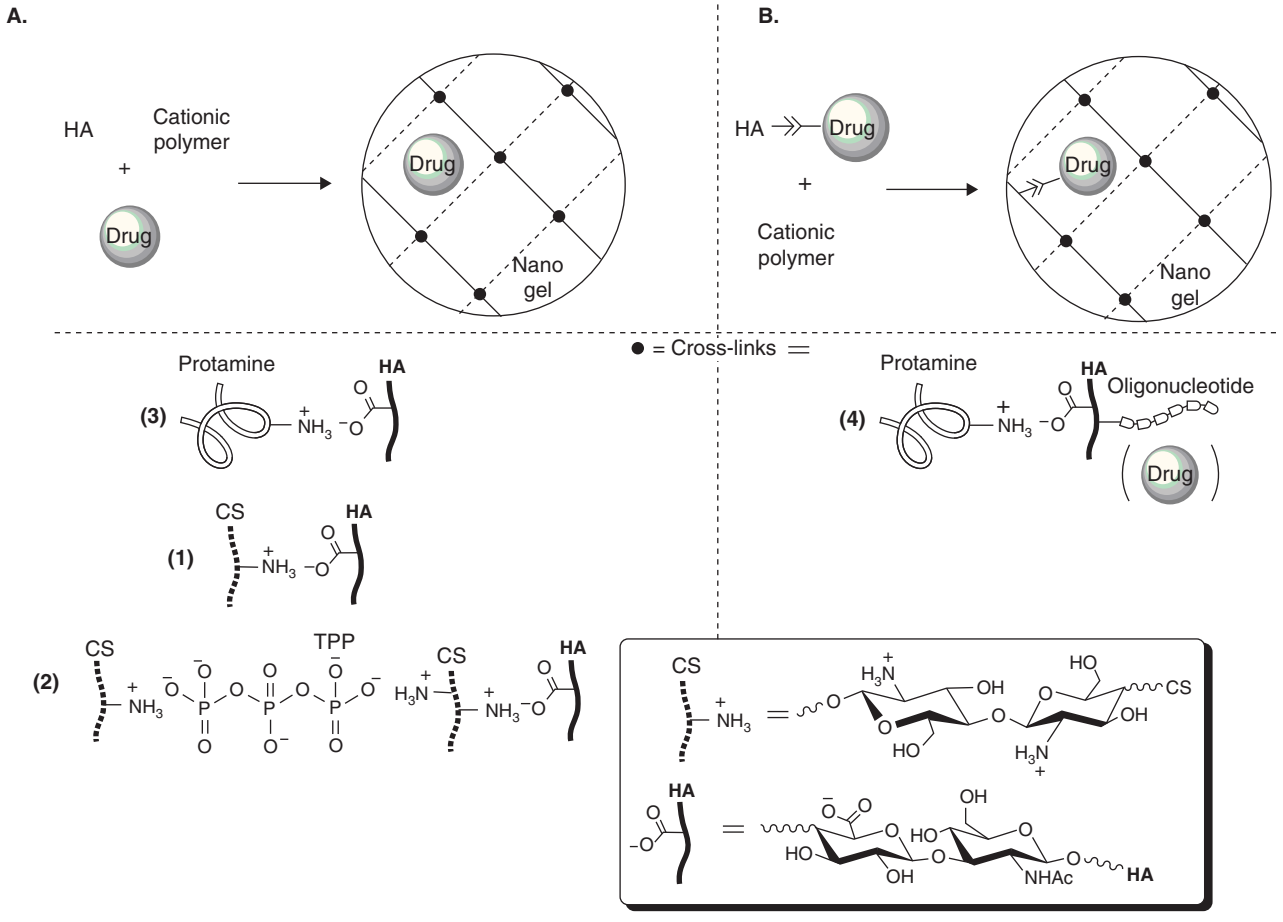


Figure 5. Physical (A) and chemical (B) encapsulation of drug molecules in the HA nanoparticles formed by ionotropic gelation.

CS molecules. This permitted efficient incorporation of GFP encoding DNA plasmid within the CS/HA particles. The DNA/CS/HA complexes obtained were able to transfect DNA 35 times more efficiently compared with DNA/CS vectors under the same optimized transfection conditions *in vitro*. This was attributed to the fact that the release of DNA from DNA/CS complex could be inhibited or delayed by strong electrostatic interaction between CS and DNA. Conversely, interaction between DNA and CS is weaker owing to the competing polyanionic HA, which facilitates the release of DNA without loosening the CS/HA complex.

In another variant, hyaluronic acid (170 kDa) was co-admixed with sodium tripolyphosphate (TPP, $Na_5P_3O_{10}$), which is known to interact with chitosan thus triggering its

liquid-to-gel transition [71-73]. These new nanoparticles composed of HA, TPP and CS (**Figure 5**, (**2**)) were mainly prepared with excess of CS and characterized by positive zeta-potential. The zeta-potential of particles as well as their size can be controlled by adjusting the HA and TPP content [72]. The particles were shown to associate with a significant amount of plasmid DNA, and showeda a high level of the genetic material transfection *in vitro* [71,73]. The action of the pDNA-loaded particles was through their active internalization into cells. This was proved by monitoring cell fluorescence after incubation with HA/TPP/CS nanoparticles carrying fluorescently labelled pDNA. The encapsulated fluorescently labelled pDNA was efficiently transported at 1 h post-incubation, whereas the naked pDNA was not able to enter the cells. Fifteen hours after internalization green spots associated with the labelled pDNA were localized in the cytoplasm and the nuclei. Also, the HA/TPP/CS nanoparticles showed excellent capacity for the entrapment of proteins, such as bovine serum albumin, and the hydrophobic polypeptide cyclosporine A when these compounds were included in the HA/TPP solution.

It should be noted that the above HA nanoparticles were positively charged, containing HA molecules inside the particles and the cationic polymer molecules on the exterior of the particles. The positive charge of the particles can be inverted, however, on increasing amounts of HA [68]. Negatively charged complexes were also formed between HA and protamine, the non-toxic, cationic and naturally occurring polypeptide with arginine-rich sequence (**Figure 5**, (**3**)) [74]. Thus, a mixture of siRNA and HA was condensed with protamine in 1:1 weight ratio, which gave negatively charged particles with a size of 150 nm [75]. These particles, however, were not tested for their transfection ability and silencing of protein expression. This has been done with another HA-protamine nanocomplex in which antisense oligonucleotide (ODN) was covalently conjugated to HA by means of a reducible disulfide bond (**Figure 5**, (**4**)) [76]. This nanocomplex exemplifies the only type of HA soft particles elaborated so far in which the bioactive molecule is covalently linked to the nanoparticulate HA carrier (**Figure 5B**). The protamine/HA-ODN complexes were 166 nm in diameter at protamine: HA-ODN weight ratio of 0.5. They were fivefold more stable against decomplexation by competing polyanion heparin than the protamine/ODN analogues. Finally, protamine/HA-ODN complexes were internalized by cells, whereas the protamine/ODN counterparts did not show any detectable uptake. The authors noticed that the protamine/HA-ODN complexes could be internalized by cells via HA receptor-mediated endocytosis.

In place of a cationic polymer, as a complexing agent for ionic association of HA chains, metal cations or positively charged metal complexes can be used for HA nanocomplexation. HA has been shown to have metal-chelating properties because HA is partially or totally co-precipitated with Ni^{2+}, Co^{2+} and Cu^{2+} when phosphate salts are added [77]. This mechanism of nanoprecipitation was probably in action when the known anticancer agent cisplatin [78] was mixed with HA (M_r = 100,000) in different proportions to yield cisplatin-incorporated nanoparticles with size ranging from 80 to 160 nm [79]. In this case an actual drug molecule was functioning as a particle-forming agent so that the release of the drug should lead to the particles' disintegration. The release profiles showed dependence on the amount of cisplatin used (the release was faster with less cisplatin in the particle formulation) and the presence of hyaluronidase, which facilitated the cisplatin release. Generally, the almost complete release was observed in 100 h if no HAse was present in the release medium.

3.3 Soft hyaluronic acid nanogel carriers made by hydrophobic association

Another method of preparation of hydrophilic polysaccharide-based nanoparticles is the polymeric micelle method, in which hydrophilic polysaccharide chains are grafted with hydrophobic segments. The hydrophobically modified polysaccharides can therefore self-aggregate into unique nanoparticles with hydrophobic core and a hydrophilic shell (**Figure 6**). These nanoparticles are considered to be hydrogels in which the crosslinks are provided by the associated hydrophobic groups. Supramolecular assembly of hydrophobized HA was demonstrated when hydrophobic tetradecylamine [80], PLGA [81], poly(ethylene glycol)-poly (lactide-*co*-glycolide) (PEG-PLGA) [82], poly(ethylene glycol)-polycaprolactone (PEG-PCL) [83] and 5β-cholanic acid [39] were chemically anchored to the HA backbone. Subsequently, hydrophobized HAs were self-aggregated in the presence of doxorubicin such that the drug molecules were localized in either the PLGA [81,82] or PCL [83] core of the formed core/shell type micellar aggregates.

The attachment of hydrophobic side chains has been performed mainly by means of amidation of HA carboxylates with amino-terminated reagents using EDC as a coupling agent in water [80,82] or DMF [39]. In another case, the HA carboxylates were esterified with PLGA hydroxyls using dicyclohexylcarbidiimide and a base in dimethyl sulfoxide (DMSO) as a solvent [81]. HA-*g*-PEG-PCL was obtained by polymerization of ε-caprolactone in the presence of stannous octanoate and HA-*g*-PEG-NH_2 as an initiator under anhydrous conditions and 110°C [83]. The obtained hydrophobized HA derivatives demonstrated amphiphilic nature; therefore, they formed aggregates of nanometer size on interaction with water. There are several ways to bring the hydrophobically modified HA in contact with water and thus conduct the process of molecular association into nanogel particles. In one way, the solid material is sonicated in water and the obtained solution is filtered through a syringe type membrane filter. In this way, the HA of molecular mass of 200 – 300 kDa and modified with degrees of substitution (DS) from 5 to 30% was processed [39,81]. In the co-dialysis method, the solid hydrophobized HA is dissolved in polar

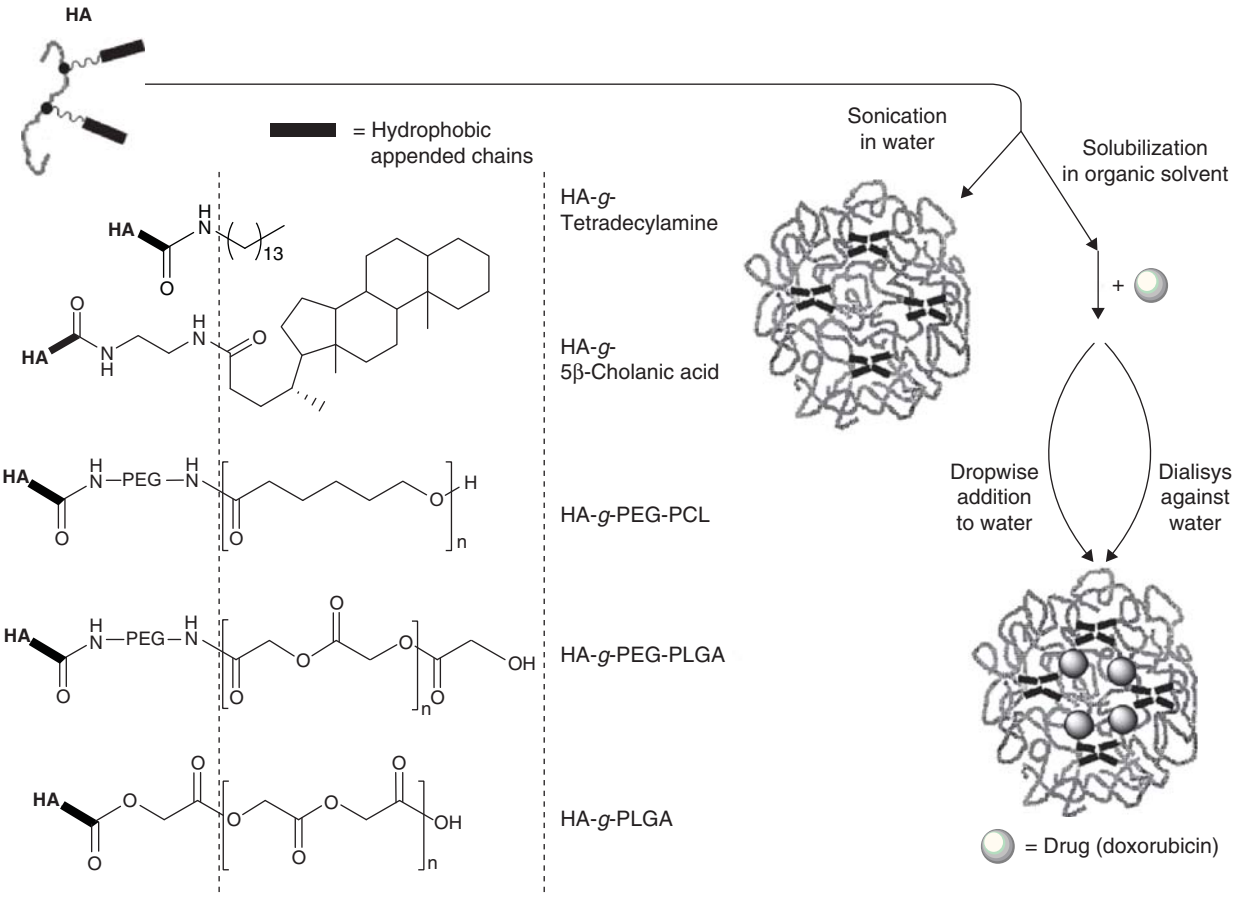


Figure 6. Examples of hydrophobically modified hyaluronic acid derivatives and methods that provide self-assembly of the hydrophobized HA molecules into nanogel particles.

organic solvent such as DMSO and the solution obtained is then dialyzed against water. In the course of solvent exchange the amphiphilic molecules come into contact with water molecules, thus inducing their hydrophobic association. The co-dialysis method was used for HA (M_r = 17,000 and 64,000) grafted with PLGA with DS ranging from 5.4 to 26.7% [81]. Finally, the nanoprecipitation method was used for very short HA derivatives (M_r = 5740) grafted with PEG-PLGA [82] or PEG-PCL [83]. According to this method, the solid HA-*g*-PEG-PLGA or HA-*g*-PEG-PCL was dissolved in acetone and the solutions obtained were precipitated by dropwise addition to water.

In vitro cellular studies were performed with HA-*g*-PLGA particles loaded with doxorubicin [81] as well as with HA-cholanic acid conjugates labelled with near-infrared fluorescence dye Cy5.5 [39]. Thus, DOX-loaded HA-*g*-PLGA particles were visualized within endosomal compartments of the CD44-overexpressing HCT-116 cells after 30 min of the cells' incubation with the particles. Free DOX was predominantly localized in the nuclei, suggesting passive diffusion of DOX into the cells followed by rapid binding to chromosomal DNA. The difference in mechanism of cellular uptake of the free DOX and DOX-loaded HA-*g*-PLGA particles was confirmed further by performing the experiments at 4°C and adding free HA in the incubation media to block HA receptors on the surface of HCT-116 cells. Almost no fluorescence from DOX moieties was observed in the cells treated with DOX-loaded HA-*g*-PLGA particles at 4°C, whereas free DOX uptake was still observed at the reduced temperature. This indicated that HA-*g*-PLGA particles were internalized by means of an endocytic pathway because it is energy dependent. Blocking the HA receptors led to a substantial reduction of the cellular uptake of the particles as judged from the flow cytometry (FACS) analysis, suggesting receptor-mediated endocytosis of the nanoparticles. As a result of the tumor cell-specific delivery of the DOX-loaded HA-*g*-PLGA nanoparticles, they showed even higher cytotoxicity (IC_{50} = 0.67 μg/ml) than free DOX (IC_{50} = 3.48 μg/ml). Similarly, squamous cell carcinoma (SCC7) cells internalized Cy5.5-labelled HA-cholanic acid conjugates in the cytoplasm after incubation for 90 min. Interestingly, the pure Cy5.5-labelled HA polymer showed an intense fluorescence signal along the cell membrane, but not in the cytoplasm. This should indicate a strong binding affinity of high-molecular-mass HA

molecules to CD44 receptors on the surface of SCC7 cells. On the other hand, it was demonstrated that HA cellular uptake is very size-dependent because internalization of linear HA molecules of higher molecular mass is sterically hindered [53]. This might be the reason why non-particulate high-molecular-mass HA did not show internalization into cytoplasm of the cells.

In vivo biodistribution of HA-*g*-PEG-PLGA [82], HA-*g*-PEG-PCL [83] and HA-*g*-5β-cholanic acid [39] nanoparticles was studied, allowing the following important conclusions to be drawn.

1) Nanoparticulate delivery of the drug was more specific than the free drug distribution. Thus, DOX-loaded HA-*g*-PEG-PLGA and HA-*g*-PEG-PCL particles accumulated mainly in the liver and kidney, with still quite substantial amounts localized in the tumor, whereas the free DOX was almost evenly distributed in the blood, heart, lung, liver, spleen and kidney.
2) HA played the role of a tumor-targeting component in the particles. In particular, HA was substituted to methyl poly(ethylene glycol) (MPEG), giving MPEG-PLGA [82] or MPEG-PCL [83] particles, which delivered DOX to the tumor tissue much less efficiently than the HA-based particles.
3) Nanoparticulate structure provides stealth character to HA molecules, thus leading to longer circulation in the blood and better chances to accumulate in tumor. This was shown when the biodistribution of pure HA polymer was compared with that of the HA-*g*-5β-cholanic acid: the strongest intensity of Cy5.5-labelled HA particles was observed at the tumor site and the intensity was approximately fourfold higher than that of the Cy5.5-labelled HA molecules [39].

It was mentioned in the Introduction that covalent linking of hydrophobic drugs to hydrophilic HA molecules should also result in amphiphilic HA-drug conjugates. Despite the fact that previously described HA-drug conjugates such as HA-DOX [42,43], HA-Taxol [44,45], HA-butyrate [46,47] and HA-carborane (see **Table 1** for structure) [48,49] may potentially associate with the formation of particles of nanometer size, there was only one study in which the micellization of the HA-drug conjugate into nanogel particles was clearly presented [84]. In this study, HA carboxylates were partially esterified with Taxol in anhydrous DMSO using a new HA solubilization technique. This technique is based on the formation of HA/PEG nanocomplexes that are soluble in DMSO. After the reaction was complete, the DMSO was exchanged to water by dialysis. As Taxol was conjugated to HA by means of acid-cleavable ester linkage, the release of the drug in a free form is expected to be facilitated in the slightly acidic environment of extracellular solid tumors and intracellular endosomal vesicles. Such covalent conjugation of a drug into HA chains that self-assemble into nanoparticles provides the target-triggered release profile, whereas physical entrapment of the drug within the particles gives an initial burst release. The HA-Taxol particles showed more cytotoxicity for HA receptor overexpressing cancer cells *in vitro*, but reduced cytotoxicity for HA receptor-deficient cells, as compared with the commercially available Taxol formulation.

4. Hyaluronic acid-decorated nanoparticles

Polysaccharides in general [85] and HA in particular have been used as coating materials of different colloidal drug carriers or solid inorganic nanoparticles. Overall, the idea behind this coating has been to protect nanoparticles, prevent their protein adsorption and subsequent phagocytosis, and mainly to regulate the circulation time and biodistribution of the coated nanoparticles though the targeting function of HA. The HA-decorated nanoparticles contain HA molecules only in the outer shell of the particles, which is in contrast to HA nanogels, where HA chains constitute the core and/or the surface of the particles. As amphiphilic HA derivatives have micellar structure with hydrophobic side chains located in the core of the particles and the HA backbones exposed to the outer aqueous phase, they can be considered as HA-coated nanocarriers as well. Generally, the core of such particles can be presented by any type of inorganic or organic nano-sized materials, which are summarized in the **Figure 1**. In the case of HA-decorated nanoparticles, soft nanogels [86], liposomes [87], as well as solid polyester [88], iron oxide [89], or gold nanoparticles [90] were used as core materials (**Figure 7**). To be coated on nanoparticles, HA macromolecules should contain the appropriate functionalities that will provide the attachment of the HA chains to the nanoparticulate core either by means of covalent bonds or by exploiting secondary forces.

4.1 Hyaluronic acid-coated nanogels

Chitosan nanogels prepared by ionotropic gelation with TPP were subsequently coated with HA outer shell by means of either ionic interactions [91] or covalent bonds [86]. In contrast to the above-mentioned HA/TPP/CS nanogels (**Figure 5**), HA was not used in the step of ionotropic gelation of chitosan with TPP, but it was added after the formation of the chitosan nanogel particles to obtain the nanogel-coated type system. Thus, the adsorption of HA polyanion of two molecular masses (15 and 360 kDa) on positively charged chitosan nanoparticles (240 nm) has been studied by varying such parameters as CS nanoparticle concentration and HA concentration (**Figure 7**, (**1**)) [91]. Using macrophages as a sensitive cell model, it appeared that HA-coated CS nanoparticles were less toxic than their uncoated positively charged CS analogues.

Covalent coupling between HA shell and CS core was also performed in order to prepare more stable CS nanoparticles (NPs) (**Figure 7**, (**2**)) for colon delivery of anticancer drug [86]. For this purpose, the TPP solution containing

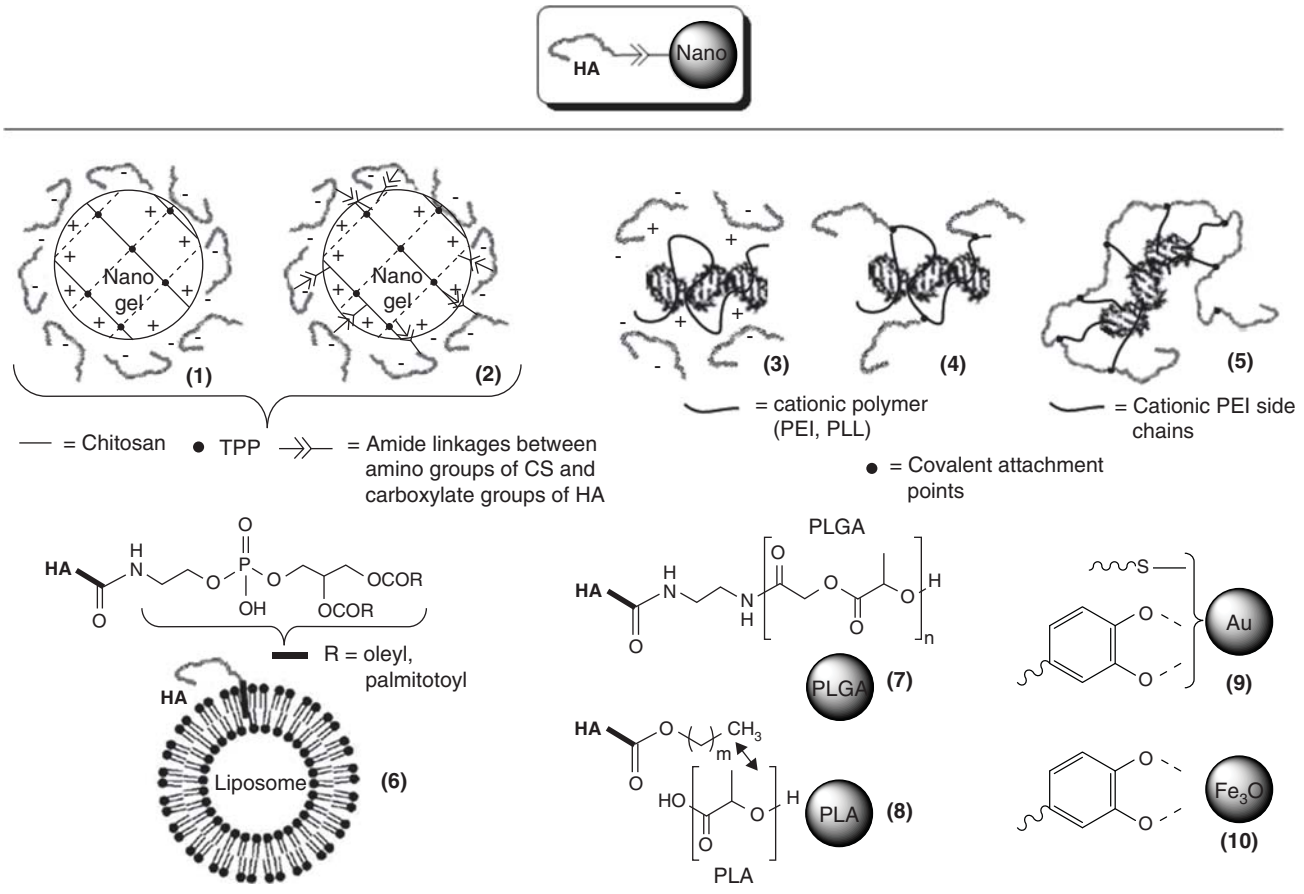


Figure 7. Different classes of HA-decorated nanoparticles/nanocomplexes.

5-fluorouracil (5FU) was added into CS solution to make CS nanogel particles by ionotropic gelation. Afterwards, the purified CS particles were chemically coated with HA molecules by addition of the water-soluble amide coupling reagent (EDC) into the suspension containing both CS NPs and HA. During the conjugation process, HA carboxylates were amide-linked to the CS amino groups. The *in vitro* drug release was investigated by exposing the nanoparticles to different simulated gastrointestinal tract fluids of varied pH. The decreased drug release from HA-coated particles was observed in comparison with uncoated analogues. The 5FU-loaded HA-coated CS particles showed significant cytotoxic effect against HT-29 cancer cell line after 48 h incubation (IC_{50} = 2.31 μg/ml) as compared with non-targeted CS particles (IC_{50} = 5.05 μg/ml) and a free drug (IC_{50} = 6.02 μg/ml). The blank nanoparticles (without drug) showed low cytotoxicity in the cell line tested. The improved cytotoxicity arises from the enhanced uptake of the HA-coated CS particles by HT-29 colon cancer cells relative to the uncoated CS particles, which were shown by examining cells fluorescence intensity after 4 h incubation with calcein fluorescent marker-loaded particles of both types. The fluorescence seen in the cells was caused by NPs and not by the free dye because the authors revealed that the dye did not leach from the NPs during the experimental time frame. When the cellular uptake experiments were performed in the presence of free HA, an 11.4-fold decrease in the uptake of the HA-coated CS NPs occurred and only a 1.9-fold decrease in the uptake of the uncoated CS counterparts. On the basis of those results, it can be concluded that the HA-coated CS nanogel particles are internalized through the HA receptor-mediated pathway.

4.2 Hyaluronic acid-coated nucleic acid/polycation complexes

HA was utilized for coating of polymeric gene/oligonucleotide delivery vectors. These vectors were formed as complexes between polyanionic nucleotide therapeutics and polycationic polymers in a ratio to have positive overall charge. Although the mechanism of intracellular delivery with cationic vectors remains under debate, the cationic cell-penetrating peptides appear to use an energy-dependent endocytic pathway [92]. By designing the complexes to include the HA molecules in the outer shell, HA targeting properties can therefore be conferred to the resulting complexes. Moreover, negative charges of HA can effectively shield the positive charges of the

conventional vectors, which leads to a decrease of the toxicity that is normally associated with positively charged surface characteristics [93]. Analogously to the HA-coated ionic nanogels with positive zeta-potential, the nucleic acid/polycation complexes can be linked to the HA molecules simply through ionic interactions between the polycation and HA carboxylates, and the polycation can be covalently conjugated to the HA molecules.

Non-covalent DNA/PEI/HA ternary complexes have been made either by pre-addition of HA to DNA and then mixing with PEI solution [94] or by post-addition of HA to the already formed DNA/PEI complex [94,95]. In both cases the ternary DNA complexes were surrounded by anionic HA segments on their surfaces (**Figure** 7, (**3**)) with only slight difference in surface charge, whereas the size of the ternary complexes was much altered by the order of mixing [94]. Post-addition of HA resulted in rather larger particles than those formed by pre-addition of HA. The transfection efficiency of the complexes made by pre-addition of HA was fivefold higher than that of the DNA/PEI reference, whereas post-addition of HA enhanced the transfection efficiency little, as examined on B16 cells *in vitro*. The transfection mechanism was demonstrated to include the receptor-mediated binding of the particles, as pretreatment of the B16 cells with free HA inhibited such binding. Moreover, the *in vivo* distribution of expression of either luciferase or GFP induced by the corresponding ternary DNA/PEI/HA complexes was different from that induced by the binary DNA/PEI complexes after either intratumoral or intravenous injections of the complexes. More enhanced protein expression in tumor was observed in the case of the ternary complexes than in the case of the binary complexes. Another function of HA in the ternary complexes is loosening of the plasmid/polycation complex, which is essential for dissociation of the complex after endosomal internalization. Interestingly, the ampholytic HA-spermine conjugate with similar numbers of amino to carboxyl groups was found to be more effective in loosening of the DNA/PEI complex than normal HA, leading to overall higher transcription efficiency [95].

Polycations such as poly(L-lysine) (PLL) [96,97] and PEI [98] have been covalently grafted with short HA fragments for subsequent complexation with DNA (**Figure** 7, (**4**)) for delivering DNA to the liver sinusoidal endothelial cells (SECs) *in vivo* [96,97] or human mesenchymal stem cells (hMSCs) *in vitro* [98]. A reductive amination reaction was used to couple amino groups of PLL or PEI to the reducing end of the HA molecules, resulting in polycation-*g*-HA ampholytes in which HA molecules have only one attachment point to the polycation backbones. The $[\text{carboxyl group}]_{HA}/[\text{amino group}]_{PLL(PEI)}$ ratio was <1, ensuring excess of positive charge in the resulting copolymers. It should be noted that comb-type copolymers such as PEI-*g*-HA or PLL-*g*-HA can have inter- and intra-molecular ionic bonding because of opposite charges that can induce association and even aggregation/precipitation of the vectors. Such association is dependent on the ionic strength and pH. The improved DNA transfection efficiency of the comb-type copolymers versus pure polycations has also been achieved at the optimized salt concentration and pH. Specific targeting of the DNA/PLL-*g*-HA complex to the liver was achieved (>93% after 1 h of injection into a tail vein of rats), whereas DNA/PLL complex was distributed mostly in the lung [97].

In another study, HA of much higher molecular mass (M_r = 130,000) was used as a backbone molecule to which many PEI (25,000 kDa) molecules were grafted by means of EDC-mediated amidation coupling [58,99]. On complexation with siRNA, the zeta-potential was inverted from positive for the HA-*g*-PEI to negative, reflecting the core/shell structure of the HA-*g*-PEI/RNA complex with HA backbone exposed in the shell and the PEI grafted chains complexed with RNA molecules in the core (**Figure** 7, (**5**)). The HA-*g*-PEI/RNA complex was specifically taken up by B16F1 cells that overexpress LYVE-1 receptor, but not by HEK-293 cells without HA receptors. According to the cellular uptake results, the PGL3-Luc gene silencing was more effective in B16F1 cells than in HEK-293 cells, whereas the control Lipofectamine complex did not discriminate much between different cell lines. The HA-*g*-PEI/RNA complex was mainly distributed *in vivo* in the tissues with HA receptors such as liver, kidney and tumor, whereas PEI/RNA complex was found in the lung. Finally, anti-VEGF siRNA complex with HA-*g*-PEI suppressed effectively tumor growth when injected intratumorally as compared with PEI/RNA complex. Notably, the scrambled anti-VEGF RNA complex with HA-*g*-PEI was also much less effective, proving that the tumor growth inhibition was indeed through silencing of VEGF production, which is responsible for tumor angiogenesis.

4.3 Hyaluronic acid-coated liposomes

Liposomes (nano-sized phospholipid vesicles) with surface-bound HA were prepared (**Figure** 7, (**6**)) that showed advantageous properties, such as stability against aggregation, long circulation and high affinity to recognition sites that are overexpressed in tumors. HA has been covalently attached to the surface of liposomes in two ways. In one way, HA was conjugated to phosphatidylethanolamine in preformed liposomes through amidation reaction between HA carboxylates and the amino groups on the liposome surface [100,101]. In another way, HA was first linked to either many phosphatidylethanolamine molecules using EDC-mediated amidation reaction [102] or only one phosphatidylethanolamine molecule by reductive amination at the reducing end of HA [87]. The synthesized HA-phosphatidylethanolamine conjugates were then used in a mixture with other lipids in the lipid film hydration step of the liposomes' preparation. The HA-coated liposomes were 75 nm [101] or 110 – 140 nm [87] in diameter and characterized by negative zeta-potential, thus confirming surface modification by the negatively charged HA. HA was also attached to cationic liposomes [102] and in this case the liposomes displayed a net positive charge.

Interaction of the HA-immobilized liposomes with cells having receptors for HA was examined *in vitro* either by confocal microscopy in the case of fluorescein-loaded or fluorescein-HA-labelled liposomes [101], or by radiography in the case of ^{125}I-labelled liposomes [91]. In the first case [101] only effective cell surface binding was observed, whereas in the second case (liposomes decorated with HA oligomers) [87] it was shown that after cell surface binding, the liposomes were internalized into the targeted cells by a temperature-dependent process. Specific binding and uptake of the liposomes decorated with HA oligomers has been proved by several assays: i) by comparing binding to high and low CD44-expressing cells; ii) by comparing the amount of the surface-bound and internalized liposomes at two temperatures, 4 and 37°C; and iii) by inhibiting the binding-uptake by pretreating the cells with either free HA or monoclonal antibodies directed against the CD44 receptor.

To exploit the tumor cell specificity of the HA-decorated liposomes in cancer cure, the liposomes were either loaded with drugs such as DOX [87,100] and mitomycin C (MMC) [101] or the cationic liposomes were complexed with plasmid DNA pCMV-luc [102]. Drug efflux from the liposomes was studied showing that the halves of the entrapped DOX [100] and MMC [101] were released after 139 and 53 h, respectively. *In vitro* cytotoxicity of the drug-loaded liposomes [101] was examined after incubation with both CD44-positive and CD44-negative cell lines and compared with the non-targeted liposomes as well as with the free drugs. The following observations were noteworthy: i) cells' viability was unaffected by the 'empty' liposomes, both hyaluronan receptor targeted and non-targeted; ii) both free drug and the drug-loaded non-targeted liposomes were comparably toxic to the cells that overexpress HA receptors as well as to the cells that underexpress HA receptors; iii) loading the drug to the non-targeted liposomes led to a slight reduction in cell cytotoxicity; and iv) loading the drug to the HA-coated liposomes resulted in a substantial increase of toxicity against the hyaladherines-overexpressing cells, whereas the toxicity against the hyaladherines-underexpressing cells was reduced as compared with the free drug. These results clearly demonstrate that the HA-coated liposomes can function as tumor-targeted carriers for the cytotoxic agents.

The positive *in vitro* results obtained with the HA-coated liposomes made it feasible to proceed to the tumor-bearing animals [100,101]. Indeed, encapsulation of DOX and MMC in the HA-coated liposomes resulted in longer circulation of the encapsulated drugs compared with circulation of the free drugs. Drug accumulation in the lung tumors was significantly affected by carrier mediation. The sequence of drug accumulation in the tumor was: HA-coated liposomes > non-targeted liposomes >free drug. This sequence was reversed (free drug >non-targeted liposomes >HA-coated liposomes) for the uptake of drugs by tumor-free organs. Moreover, the drug biodistribution dictated by the HA-coated liposomes was tumor-specific rather than organ-specific. This targeting ability of the HA-coated liposomes had a positive impact on therapeutic responses in different mice tumor models, such as in reducing lung metastatic burden, in slowing down progression of solid tumors, and in bringing about the complete regression of the tumor xenografts.

4.4 Hyaluronic acid-coated polyester nanoparticles

Aliphatic polyesters such as polylactide (PLA), polyglycolide (PGA) and copolymers (PLGA) are biocompatible and biodegradable polymers that are FDA-approved for use as biomaterials, including preparation of nanoparticles [103,104]. Subsequently, coating of polyester nanocarriers with hyaluronic acid was envisaged to delegate the tumor-targeting responsibility to the carrier. Both covalent coupling of HA chains to the PLGA core (**Figure** 7, (7)) [105] and non-covalent hydrophobic association of the amphiphilic derivatives of HA on the surface of PLA particles (**Figure** 7, (**8**)) [88] were undertaken to prepare HA-covered polyester nanoparticles. In the case of covalent attachment, amine-modified HA (1.2 MDa) was amide-coupled to the *N*-hydroxysuccinimide ester pre-activated PLGA nanoparticles obtained by nanoprecipitation. The PLGA particles covalently coated with HA were spherical and moderately uniform sized (~ 80 nm), characterized by negative zeta-potential, and were fairly stable. High affinity of the particles to the CD44-overexpressing human breast cancer cells was shown by FACS analysis and confocal microscopy comparing two cell lines (CD44-positive and CD44-deficient) as well as two types of fluorescently-labelled nanoparticle, the non-targeted PLGA particles and the HA-coated PLGA particles. Doxorubicin was loaded into the HA-coated PLGA particles in the course of their preparation, that is, by co-dissolving DOX and PLGA in acetone followed by nanoprecipitation in water. Sustained release of DOX from the particles was observed with half the amount of the encapsulated drug released after 6 days of incubation in PBS buffer. Encapsulation of DOX in the HA-coated PLGA particles was advantageous, as the toxicity of the DOX-loaded particles against the cells not presenting the tumor marker (CD44) was 3.5 – 4 times lower than the toxicity of the free DOX, whereas cytotoxic efficiencies of both DOX-loaded particles and free DOX were equal against CD44-overexpressing cells. However, the role of HA in the CD44 targeting mechanism was not verified in this study by comparing with the analogous non-coated particles.

The CD44 receptor is also expressed at the surface of chondrocytes, the cartilage cells. This was exploited for delivering chondroitin sulfate and HA to the chondrocytes because both glucosaminoglycan (GAG) macromolecules are known for their positive effect on cartilage repair [106,107]. For this purpose, an internal aqueous phase containing GAGs was dispersed by sonication in the organic phase containing PLA, giving a primary water-in-oil emulsion. This primary emulsion was poured into a second aqueous phase (external aqueous phase) containing amphiphilic derivatives of HA, the HA molecules grafted with alkyl side chains (hexyl or

dodecyl) [88]. The double emulsion was then obtained by sonication and the HA-coated solid PLA nanospheres were collected by evaporation of organic solvent followed by centrifugation. It should be noted that in these particles HA molecules were the cargo entrapped in the interior of PLA particles as well as external HA molecules served as the targeting coat. The uptake of the HA-coated PLA nanoparticles by articular cells was shown to be partly mediated by the CD44 receptor. A confocal microscopy analysis of the dextran-FITC-loaded HA-covered particles showed that they were captured by articular cells, whereas the particles covered with poly(vinyl alcohol) were internalized far lower. Second, preincubation of the articular cells with monoclonal anti-CD44 antibody resulted in a decrease of the amount of internalized HA-covered particles.

4.5 Hyaluronic acid-coated inorganic nanoparticles

The review finishes with solid inorganic nanoparticles that were surface-functionalized with HA ligands. The dense solid cores of inorganic nanoparticles, such as gold, quantum dots or magnetic iron oxide, are not porous to accommodating cargo molecules for specific delivery. The only possible way to perform drug delivery in this case is from the surface of nanoparticles. For example, the use of gold nanoparticles for the delivery of the chemotherapeutic drug paclitaxel was demonstrated by covalent attachment of the drug through a flexible hexaethylene glycol linker to the surface of 2 nm gold particles [108]. The surface immobilization of HA onto inorganic nanocrystals is a promising method for targeting tumor cells, but this approach has not yet been utilized for the active delivery of drugs. At the same time, surface immobilization of HA on inorganic nanoparticles can be combined with their unique optical, magnetic and fluorescent properties to allow tumor tissue imaging. Recently, there have been a few reports on use of HA ligands as sensing molecules that recognize specific tumor-associated species. As the HA ligands are anchored to the surface of inorganic nanoparticles, the imaging of such species becomes feasible.

Surface functionalization of gold nanoparticles (AuNPs) with low-molecular-mass HA (<10 kDa) was performed either through Au–thiol bonds [109] or through Au–catechol bonds [90]. In both cases (**Figure** 7, (**9**)), HA was used as a cleavable substrate for Hase, the enzyme that degrades HA, as well as for reactive oxygen species (ROS). Enhanced local HA degradation by Hase in tumor tissue and enhanced ROS production by tumor cells [54,110] have been chosen as the mechanisms that provide tumor-associated molecular triggers for the detection of tumors. Detection has been realized through the AuNPs surface energy transfer (NSET) that offers several advantages, such as a low signal-to-noise ratio and a longer covering distance of ~ 20 nm. For this purpose, HA molecules of 15 nm length were functionalized with a fluorescence quencher and then covalently attached to the AuNPs through a single attachment point (reducing end). In this design, HA links fluorescein dye to the AuNP, thus providing efficient fluorescence quenching. Hase and/or ROS, however, can degrade HA, thus disrupting links between quenchers and AuNPs and recovering fluorescence. In live animal models of rheumatoid arthritis and metastatic tumor, local arthritic inflammation and tumor sites were clearly identified on systemic injection of the nanoprobes [109].

In a separate study, HA-dopamine conjugates were used to link HA molecules to the supermagnetic iron oxide nanoparticles through catechol functional groups (**Figure** 7, (**10**)) [89]. In the obtained magnetite nanocrystals, superparamagnetic iron oxide core served as contrast agent for magnetic resonance imaging, whereas the attached HA molecules imparted CD44 binding properties to the HA-coated iron oxide particles. The diagnostic effectiveness of the particles was evaluated on $CD44^+$ cells (HCT-116, human colon carcinoma cell line) and $CD44^-$ fibroblast cells (NIH-3T3) by measuring difference in relative relaxation rates R_2 as well as by using Prussian blue assay after 30 min incubation of the cells with the particles. HA-coated iron oxide particles were effectively internalized by HCT-116 cells but not by NIH-3T3 cells. Moreover, the uptake by HCT-116 cells was suppressed when the competing free HA molecules were present in the incubation media. These results reveal that the mechanism for intracellular delivery of the HA-anchored iron oxide nanocrystals was CD44-mediated.

5. Conclusions and outlook

Hyaluronic acid receptors have long been recognized as biomarkers of neoplastic cells. Subsequently, active targeting of cancer has been envisioned by associating anticancer agents with hyaluronic acid. Apart from targeting function, HA can also impart water solubility to hydrophobic drugs, confer long circulating properties to drugs/drug vehicles, as well as prevent complex and fragile biomolecular agents (nucleic acids, proteins, peptides) degrading. Various drug delivery systems have been designed in which the bioactive cargo molecules are either covalently attached to HA macromolecular chains or physically entrapped in particulate carriers, in which HA constitutes a part of the carrier. A third alternative that has not been yet fully elaborated is a covalent linking of a drug/bioactive molecule to the complex HA-containing particulate carrier. In all three cases we talk about HA-based architectures of submicrometer size, as only nano-objects can circulate long enough in the blood vessels without elimination by the RES, accumulate in tumor tissue by the EPR effect, and finally be internalized by cells through the endocytic pathway. Nevertheless, the current limitation of the HA targeting nanocarriers is their high accumulation in the RES organs (liver and spleen) because HA molecules expose the nanocarriers to the HA receptors identified in these organs.

6. Expert opinion

To become a successful drug delivery vehicle, some important principles should be taken into account in developing new HA-based nanostructures.

1) To be a long-acting carrier, HA should be chemically modified to become more resistant to degradation by HA-degrading enzymes, hyaluronidases. However, the degree and type of modification have to be optimized to retain the recognition properties of the native HA so that the modified HA derivatives will be specifically internalized by cancer cells.
2) Design of 'activatable' HA nanoparticles with a stealth shell that can be detached from the HA core in a tumor environment has the potential to suppress the nanoparticles' interactions with other HA receptors identified in liver and spleen cells (HA receptor for endocytosis [HARE] in liver and spleen [40] and lymphatic vessel endothelial hyaluronan receptor-1 [LYVE-1] in the lymphatic system [111]).
3) A drug payload should not rapidly leak out from a carrier to prevent systemic toxicity that is inherent to the most conventional cytotoxic agents. To achieve this, one has to exploit the intrinsic properties of a drug for establishing stable interactions between the cytotoxic agent and HA-containing carrier, such as ionic, hydrophobic, or hydrogen bonding interactions. These interactions should still be reversible, providing a mechanism for drug release. In another approach, the so-called prodrug strategy, a drug molecule is covalently linked to a carrier through a rationally designed linker that provides the release of the drug at the right place at the right time.
4) The mechanisms for endosomal escape at an early stage should be designed for bioactive cargo molecules such as nucleotide therapeutics (siRNA, DNA) to avoid their degradation by nucleases in the subsequent late endosomes.

There are two mechanistic pathways by which a drug can get into the cytoplasm from the HA-based nanocarrier. In the first pathway, the drug is cleaved/released from the carrier in the extracellular fluid when the carrier is bound to the cellular membrane. The drug then crosses the membrane by diffusion. In the second pathway, the carrier with loaded drug is internalized by HA receptor-mediated endocytosis. The drug is then split from the carrier within the lysosome and subsequently diffuses to the cytoplasm. In both ways the drug molecule should pass the phospholipid bilayer, either from extracellular space or from endosome/lysosome. Thus, the conditions found in the tumor ECM environment or in lysosome can be exploited either to induce cleavage of a drug-carrier linker or to release the drug by means of nanocarrier dissociation. The levels of some reagents, such as glutathione, the levels of enzymes, as well as pH, are specific for lysosome microenvironment, which can trigger the drug release.

Other studies revealed that the uptake of different HA derivatives by receptor-mediated endocytosis depends on their molecular mass and hydrodynamic radius, which imposes further demands for HA modifications. It has been found, for example, that high-molecular-mass HA strongly binds to the CD44-presenting cell surface without further internalization [39], which can be addressed to steric hindrance of large HA macromolecules [53,112,113]. However, modification of HA with hydrophobic side chains leads to the micelle-like structural reorganization of HA molecules, which makes them more sterically compact for cellular uptake.

Further investigations are also needed to deliver HA therapeutics to tumors *in vivo*. Thus, to decrease normal/tumor ratio for HA distribution, one should suppress the HA uptake by the liver and spleen if these organs are not the targets for the treatment. It has been shown that by choosing the route of administration, one can target the drug appropriately [47]. Combinational therapy also has the potential to improve the tumor specificity of HA therapeutics. Thus, blocking of HARE receptor by chondroitin sulfate before intravenous administration of HA nanostructured materials also allowed the treatment/diagnosis of tumors in other organs [47,109]. To improve the efficacy of HA nanoparticles *in vivo*, it would be worth also considering a strategy that relies on shielding of nanocarriers with a stealth layer that can be removed after particle extravasation, thus exposing the targeting ligands only at the tumor site [114]. In summary, more sophisticated intelligent systems based on HA nanostructured materials are still needed to navigate bioactive molecules to the target organs/tissues and cells. HA has a great potential to achieve this goal and combat tumor progression and metastasis.

Declaration of interest

The author states no conflict of interest and has received no payment in preparation of this manuscript.

Bibliography

1. Kayser O, Lemke A, Hernandez-Trejo N. The impact of nanobiotechnology on the development of new drug delivery systems. Curr Pharm Biotechnol 2005;6(1):3-5
2. Hawley AE, Davis SS, Illum L. Targeting of colloids to lymph nodes: influence of lymphatic physiology and colloidal characteristics. Adv Drug Deliv Rev 1995;17(1):129-48
3. Ishida O, Maruyama K, Sasaki K, Iwatsuru M. Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice. Int J Pharm 1999;190(1):49-56
4. Jain KK. Nanoparticles as targeting ligands. Trends Biotechnol 2006;24(4):143-5
5. Petrak K. Design and properties of particulate carriers for intravascular administration. Drugs Pharm Sci 1993;61:257-297
6. Maeda H. SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. Adv Drug Deliv Rev 2001;46(1-3):169-85
7. Ogawara K, Yoshida M, Furumoto K, et al. Uptake by hepatocytes and biliary excretion of intravenously administered polystyrene microspheres in rats. J Drug Target 1999;7(3):213-21
8. Lee ES, Kim D, Youn YS, et al. A virus-mimetic nanogel vehicle. Angew Chem Int Ed Engl 2008;47(13):2418-21
9. Corot C, Robert P, Idee JM, Port M. Recent advances in iron oxide nanocrystal technology for medical imaging. Adv Drug Deliv Rev 2006;58(14):1471-504
10. Weissleder R. Molecular imaging in cancer. Science 2006;312(5777):1168-71
11. Takahashi H, Niidome T, Nariai A, et al. Gold nanorod-sensitized cell death: microscopic observation of single living cells irradiated by pulsed near-infrared laser light in the presence of gold nanorods. Chem Lett 2006;35(5):500-1
12. Lal S, Clare SE, Halas NJ. Nanoshell-enabled photothermal cancer therapy: impending clinical impact. Acc Chem Res 2008;41(12):1842-51
13. Gao X, Cui Y, Levenson RM, et al. In vivo cancer targeting and imaging with semiconductor quantum dots. Nat Biotechnol 2004;22(8):969-76
14. Lai T-Y, Lee W-C. Killing of cancer cell line by photoexcitation of folic acid-modified titanium dioxide nanoparticles. J Photochem Photobiol A Chem 2009;204(2-3):148-53
15. Palazzo B, Iafisco M, Laforgia M, et al. Biomimetic hydroxyapatite-drug nanocrystals as potential bone substitutes with antitumor drug delivery properties. Adv Funct Mater 2007;17(13):2180-8
16. Klumpp C, Kostarelos K, Prato M, Bianco A. Functionalized carbon nanotubes as emerging nanovectors for the delivery of therapeutics. Biochim Biophys Acta 2006;1758(3):404-12
17. Ringsdorf H. Structure and properties of pharmacologically active polymers. J Polym Sci Polym Symp 1975;51:135-53
18. Sampathkumar SG, Yarema KJ. Targeting cancer cells with dendrimers. Chem Biol 2005;12(1):5-6
19. Letchford K, Burt H. A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes. Eur J Pharm Biopharm 2007;65(3):259-69
20. Hamidi M, Azadi A, Rafiei P. Hydrogel nanoparticles in drug delivery. Adv Drug Deliv Rev 2008;60(15):1638-49
21. Oh JK, Drumright R, Siegwart DJ, Matyjaszewski K. The development of microgels/nanogels for drug delivery applications. Prog Polym Sci 2008;33(4):448-77
22. Raemdonck K, Demeester J, De Smedt S. Advanced nanogel engineering for drug delivery. Soft Matter 2009;5(4):707-15
23. Muller RH, Mader K, Gohla S. Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. Eur J Pharm Biopharm 2000;50(1):161-77
24. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005;4(2):145-60
25. Haag R, Kratz F. Polymer therapeutics: concepts and applications. Angew Chem Int Ed Engl 2006;45(8):1198-215
26. Hirsch LR, Stafford RJ, Bankson JA, et al. Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance. Proc Natl Acad Sci USA 2003;100(23):13549-54
27. Nam J, Won N, Jin H, et al. pH-Induced aggregation of gold nanoparticles for photothermal cancer therapy. J Am Chem Soc 2009;131(38):13639-45
28. Chatterjee DK, Fong LS, Zhang Y. Nanoparticles in photodynamic therapy: an emerging paradigm. Adv Drug Deliv Rev 2008;60(15):1627-37
29. Oba T. Photosensitizer Nanoparticles for Photodynamic Therapy. Curr Bioactive Comp 2007;3:239-251
30. Lee Y, Park SY, Kim C, Park TG. Thermally triggered intracellular explosion of volume transition nanogels for necrotic cell death. J Control Release 2009;135(1):89-95
31. Kamb A. What's wrong with our cancer models? Nat Rev Drug Discov 2005;4(2):161-5
32. Lee RJ, Low PS. Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. J Biol Chem 1994;269(5):3198-204
33. Lee JY, Spicer AP. Hyaluronan: a multifunctional, megaDalton, stealth molecule. Curr Opin Cell Biol 2000;12(5):581-6
34. Kinzler KW, Vogelstein B. Landscaping the cancer terrain. Science 1998;280(5366):1036-7
35. Toole BP, Wight TN, Tammi MI. Hyaluronan-cell interactions in cancer and vascular disease. J Biol Chem 2002;277(7):4593-6
36. Day AJ, Prestwich GD. Hyaluronan-binding proteins: tying up the giant. J Biol Chem 2002;277(7):4585-8
37. Hua Q, Knudson CB, Knudson W. Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis. J Cell Sci 1993;106(Pt 1):365-75
38. Harris EN, Kyosseva SV, Weigel JA, Weigel PH. Expression, processing, and

glycosaminoglycan binding activity of the recombinant human 315-kDa hyaluronic acid receptor for endocytosis (HARE). J Biol Chem 2007;282(5):2785-97

39. Choi KY, Min KH, Na JH, et al. Self-assembled hyaluronic acid nanoparticles as a potential drug carrier for cancer therapy: synthesis, characterization, and in vivo biodistribution. J Mater Chem 2009;19(24):4102-7
40. Zhou B, Weigel JA, Fauss L, Weigel PH. Identification of the hyaluronan receptor for endocytosis (HARE). J Biol Chem 2000;275(48):37733-41
41. Sugahara S, Okuno S, Yano T, et al. Characteristics of tissue distribution of various polysaccharides as drug carriers: influences of molecular weight and anionic charge on tumor targeting. Biol Pharm Bull 2001;24(5):535-43
42. Cera C, Palumbo M, Stefanelli S, et al. Water-soluble polysaccharide-anthracycline conjugates: biological activity. Anticancer Drug Des 1992;7(2):143-51
43. Luo Y, Bernshaw NJ, Lu ZR, et al. Targeted delivery of doxorubicin by HPMA copolymer-hyaluronan bioconjugates. Pharm Res 2002;19(4):396-402
44. Luo Y, Prestwich GD. Synthesis and selective cytotoxicity of a hyaluronic acid-antitumor bioconjugate. Bioconjug Chem 1999;10(5):755-63
45. Luo Y, Ziebell MR, Prestwich GD. A hyaluronic acid-taxol antitumor bioconjugate targeted to cancer cells. Biomacromolecules 2000;1(2):208-18
46. Coradini D, Pellizzaro C, Miglierini G, et al. Hyaluronic acid as drug delivery for sodium butyrate: improvement of the anti-proliferative activity on a breast-cancer cell line. Int J Cancer 1999;81(3):411-6
47. Coradini D, Zorzet S, Rossin R, et al. Inhibition of hepatocellular carcinomas in vitro and hepatic metastases in vivo in mice by the histone deacetylase inhibitor HA-But. Clin Cancer Res 2004;10(14):4822-30
48. Di Meo C, Panza L, Campo F, et al. Novel types of carborane-carrier hyaluronan derivatives via 'click chemistry'. Macromol Biosci 2008;8(7):670-81
49. Di Meo C, Panza L, Capitani D, et al. Hyaluronan as carrier of carboranes for tumor targeting in boron neutron capture therapy. Biomacromolecules 2007;8(2):552-9
50. Varghese OP, Sun W, Hilborn J, Ossipov DA. In situ cross-linkable high molecular weight hyaluronan-bisphosphonate conjugate for localized delivery and cell-specific targeting: a hydrogel linked prodrug approach. J Am Chem Soc 2009;131(25):8781-3
51. Goldberg VM, Buckwalter JA. Hyaluronans in the treatment of osteoarthritis of the knee: evidence for disease-modifying activity. Osteoarthritis Cartilage 2005;13(3):216-24
52. Heymann D, Ory B, Gouin F, et al. Bisphosphonates: new therapeutic agents for the treatment of bone tumors. Trends Mol Med 2004;10(7):337-43
53. Knudson W, Chow G, Knudson CB. CD44-mediated uptake and degradation of hyaluronan. Matrix Biol 2002;21(1):15-23
54. Schumacker PT. Reactive oxygen species in cancer cells: live by the sword, die by the sword. Cancer Cell 2006;10(3):175-6
55. Kim J, Park K, Hahn SK. Effect of hyaluronic acid molecular weight on the morphology of quantum dot-hyaluronic acid conjugates. Int J Biol Macromol 2008;42(1):41-5
56. Kim J, Kim KS, Jiang G, et al. In vivo real-time bioimaging of hyaluronic acid derivatives using quantum dots. Biopolymers 2008;89(12):1144-53
57. Lee H, Choi SH, Park TG. Direct visualization of hyaluronic acid polymer chain by self-assembled one-dimensional array of gold nanoparticles. Macromolecules 2006;39(1):23-5
58. Jiang G, Park K, Kim J, et al. Target specific intracellular delivery of siRNA/PEI-HA complex by receptor mediated endocytosis. Mol Pharm 2009;6(3):727-37
59. Banerji S, Wright AJ, Noble M, et al. Structures of the CD44-hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. Nat Struct Mol Biol 2007;14(3):234-9
60. Mahteme H, Graf W, Larsson BS, Gustafson S. Uptake of hyaluronan in hepatic metastases after blocking of liver endothelial cell receptors. Glycoconj J 1998;15(9):935-9
61. Yun YH, Goetz DJ, Yellen P, Chen W. Hyaluronan microspheres for sustained gene delivery and site-specific targeting. Biomaterials 2004;25(1):147-57
62. Pitarresi G, Craparo EF, Palumbo FS, et al. Composite nanoparticles based on hyaluronic acid chemically cross-linked with alpha,beta-polyaspartylhydrazide. Biomacromolecules 2007;8(6):1890-8
63. Kumar A, Sahoo B, Montpetit A, et al. Development of hyaluronic acid-Fe2O3 hybrid magnetic nanoparticles for targeted delivery of peptides. Nanomedicine 2007;3(2):132-7
64. Jha AK, Hule RA, Jiao T, et al. Structural analysis and mechanical characterization of hyaluronic acid-based doubly cross-linked networks. Macromolecules 2009;42(2):537-46
65. Sahiner N, Jha AK, Nguyen D, Jia XQ. Fabrication and characterization of cross-linkable hydrogel particles based on hyaluronic acid: potential application in vocal fold regeneration. J Biomater Sci Polym Ed 2008;19(2):223-43
66. Lee H, Mok H, Lee S, et al. Target-specific intracellular delivery of siRNA using degradable hyaluronic acid nanogels. J Control Release 2007;119(2):245-52
67. Shu XZ, Liu Y, Luo Y, et al. Disulfide cross-linked hyaluronan hydrogels. Biomacromolecules 2002;3(6):1304-11
68. Jia X, Yeo Y, Clifton RJ, et al. Hyaluronic acid-based microgels and microgel networks for vocal fold regeneration. Biomacromolecules 2006;7(12):3336-44
69. Kabanov AV, Astafyeva IV, Chikindas ML, et al. DNA interpolyelectrolyte complexes as a tool for efficient cell transformation. Biopolymers 1991;31(12):1437-43
70. Duceppe N, Tabrizian M. Factors influencing the transfection efficiency of ultra low molecular weight chitosan/hyaluronic acid nanoparticles. Biomaterials 2009;30(13):2625-31
71. de la Fuente M, Seijo B, Alonso MJ. Bioadhesive hyaluronan-chitosan nanoparticles can transport genes across the ocular mucosa and transfect ocular tissue. Gene Ther 2008;15(9):668-76

72. de la Fuente M, Seijo B, Alonso MJ. Novel hyaluronan-based nanocarriers for transmucosal delivery of macromolecules. Macromol Biosci 2008;8(5):441-50

73. De la Fuente M, Seijo B, Alonso MJ. Design of novel polysaccharidic nanostructures for gene delivery. Nanotechnology 2008;19(7):1-9

74. Reynolds F, Weissleder R, Josephson L. Protamine as an efficient membrane-translocating peptide. Bioconjug Chem 2005;16(5):1240-5

75. Chono S, Li SD, Conwell CC, Huang L. An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor. J Control Release 2008;131(1):64-9

76. Mok H, Park JW, Park TG. Antisense oligodeoxynucleotide-conjugated hyaluronic acid/protamine nanocomplexes for intracellular gene inhibition. Bioconjug Chem 2007;18(5):1483-9

77. Vercruysse KP, Ziebell MR, Prestwich GD. Control of enzymatic degradation of hyaluronan by divalent cations. Carbohydr Res 1999;318(1-4):26-37

78. Rosenberg B. Platinum complexes for treatment of cancer. Interdiscip Sci Rev 1978;3(2):134-47

79. Jeong YI, Kim ST, Jin SG, et al. Cisplatin-incorporated hyaluronic acid nanoparticles based on ion-complex formation. J Pharm Sci 2008;97(3):1268-76

80. Choi KY, Lee S, Park K, et al. Preparation and characterization of hyaluronic acid-based hydrogel nanoparticles. J Phys Chem Solids 2008;69(5-6):1591-5

81. Lee H, Ahn CH, Park TG. Poly [lactic-co-(glycolic acid)]-grafted hyaluronic acid copolymer micelle nanoparticles for target-specific delivery of doxorubicin. Macromol Biosci 2009;9(4):336-42

82. Yadav AK, Mishra P, Mishra AK, et al. Development and characterization of hyaluronic acid-anchored PLGA nanoparticulate carriers of doxorubicin. Nanomedicine 2007;3(4):246-57

83. Yadav AK, Mishra P, Jain S, et al. Preparation and characterization of HA-PEG-PCL intelligent core-corona nanoparticles for delivery of doxorubicin. J Drug Target 2008;16(6):464-78

84. Lee H, Lee K, Park TG. Hyaluronic acid-paclitaxel conjugate micelles: synthesis, characterization, and antitumor activity. Bioconjug Chem 2008;19(6):1319-25

85. Lemarchand C, Gref R, Couvreur P. Polysaccharide-decorated nanoparticles. Eur J Pharm Biopharm 2004;58(2):327-41

86. Jain A, Jain SK. In vitro and cell uptake studies for targeting of ligand anchored nanoparticles for colon tumors. Eur J Pharm Sci 2008;35(5):404-16

87. Eliaz RE, Szoka FC Jr. Liposome-encapsulated doxorubicin targeted to CD44: a strategy to kill CD44-overexpressing tumor cells. Cancer Res 2001;61(6):2592-601

88. Laroui H, Grossin L, Leonard M, et al. Hyaluronate-covered nanoparticles for the therapeutic targeting of cartilage. Biomacromolecules 2007;8(12):3879-85

89. Lee BY, Kim YB, Kim J, et al. Bioinspired surface immobilization of hyaluronic acid on monodisperse magnetite nanocrystals for targeted cancer imaging. Adv Mater 2008;20(21):4154-7

90. Lee BY, Lee K, Kim I-K, Park TG. Fluorescent gold nanoprobe sensitive to intracellular reactive oxygen species. Adv Funct Mater 2009;19(12):1884-90

91. Nasti A, Zaki NM, de Leonardis P, et al. Chitosan/TPP and chitosan/TPP-hyaluronic acid nanoparticles: systematic optimisation of the preparative process and preliminary biological evaluation. Pharm Res 2009;26(8):1918-30

92. Duchardt F, Fotin-Mleczek M, Schwarz H, et al. A comprehensive model for the cellular uptake of cationic cell-penetrating peptides. Traffic 2007;8(7):848-66

93. Fischer D, Li Y, Ahlemeyer B, et al. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 2003;24(7):1121-31

94. Ito T, Iida-Tanaka N, Koyama Y. Efficient in vivo gene transfection by stable DNA/PEI complexes coated by hyaluronic acid. J Drug Target 2008;16(4):276-81

95. Ito T, Iida-Tanaka N, Niidome T, et al. Hyaluronic acid and its derivative as a multi-functional gene expression enhancer: protection from non-specific interactions, adhesion to targeted cells, and transcriptional activation. J Control Release 2006;112(3):382-8

96. Asayama S, Nogawa M, Takei Y, et al. Synthesis of novel polyampholyte comb-type copolymers consisting of a poly(L-lysine) backbone and hyaluronic acid side chains for a DNA carrier. Bioconjug Chem 1998;9(4):476-81

97. Takei Y, Maruyama A, Ferdous A, et al. Targeted gene delivery to sinusoidal endothelial cells: DNA nanoassociate bearing hyaluronan-glycocalyx. FASEB J 2004;18(6):699-701

98. Saraf A, Hacker MC, Sitharaman B, et al. Synthesis and conformational evaluation of a novel gene delivery vector for human mesenchymal stem cells. Biomacromolecules 2008;9(3):818-27

99. Jiang G, Park K, Kim J, et al. Hyaluronic acid-polyethyleneimine conjugate for target specific intracellular delivery of siRNA. Biopolymers 2008;89(7):635-42

100. Peer D, Margalit R. Tumor-targeted hyaluronan nanoliposomes increase the antitumor activity of liposomal Doxorubicin in syngeneic and human xenograft mouse tumor models. Neoplasia 2004;6(4):343-53

101. Peer D, Margalit R. Loading mitomycin C inside long circulating hyaluronan targeted nano-liposomes increases its antitumor activity in three mice tumor models. Int J Cancer 2004;108(5):780-9

102. Surace C, Arpicco S, Dufay-Wojcicki A, et al. Lipoplexes targeting the CD44 hyaluronic acid receptor for efficient transfection of breast cancer cells. Mol Pharm 2009;6(4):1062-73

103. Langer R, Folkman J. Polymers for the sustained release of proteins and other macromolecules. Nature 1976;263(5580):797-800

104. Zambaux MF, Bonneaux F, Gref R, et al. Preparation and characterization of protein C-loaded PLA nanoparticles. J Control Release 1999;60(2-3):179-88

105. Hyung W, Ko H, Park J, et al. Novel hyaluronic acid (HA) coated drug carriers (HCDCs) for human breast cancer treatment. Biotechnol Bioeng 2008;99(2):442-54

106. Bassleer CT, Combal JP, Bougaret S, Malaise M. Effects of chondroitin sulfate and interleukin-1 beta on human

articular chondrocytes cultivated in clusters. Osteoarthritis Cartilage 1998;6(3):196-204

107. Noble PW. Hyaluronan and its catabolic products in tissue injury and repair. Matrix Biol 2002;21(1):25-9

108. Gibson JD, Khanal BP, Zubarev ER. Paclitaxel-functionalized gold nanoparticles. J Am Chem Soc 2007;129(37):11653-61

109. Lee H, Lee K, Kim IK, Park TG. Synthesis, characterization, and in vivo diagnostic applications of hyaluronic acid immobilized gold nanoprobes. Biomaterials 2008;29(35):4709-18

110. Liu LZ, Hu XW, Xia C, et al. Reactive oxygen species regulate epidermal growth factor-induced vascular endothelial growth factor and hypoxia-inducible factor-1alpha expression through activation of AKT and P70S6K1 in human ovarian cancer cells. Free Radic Biol Med 2006;41(10):1521-33

111. Schledzewski K, Falkowski M, Moldenhauer G, et al. Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis. J Pathol 2006;209(1):67-77

112. Hornof M, de la Fuente M, Hallikainen M, et al. Low molecular weight hyaluronan shielding of DNA/PEI polyplexes facilitates CD44 receptor mediated uptake in human corneal epithelial cells. J Gene Med 2008;10(1):70-80

113. Lesley J, Hascall VC, Tammi M, Hyman R. Hyaluronan binding by cell surface CD44. J Biol Chem 2000;275(35):26967-75

114. Gullotti E, Yeo Y. Extracellularly activated nanocarriers: a new paradigm of tumor targeted drug delivery. Mol Pharm 2009;6(4):1041-51

Affiliation

Dmitri A Ossipov
Uppsala University,
Polymer Chemistry,
Material Chemistry Department,
S-75121 Uppsala, Sweden
Tel: +46 18 4717335; Fax: +46 18 4713477;
E-mail: dmitri.ossipov@mkem.uu.se