Targeted delivery of ECM based biopolymer-drug conjugate for cancer therapy

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List of content

Abstract .......................................................................................................................... 2

Introduction .................................................................................................................. 3

- Heparin .................................................................................................................. 4

- Chondroitin sulfate ................................................................................................. 5

- Hyaluronic acid ....................................................................................................... 6

Objective ..................................................................................................................... 6

Results ......................................................................................................................... 7

Discussion .................................................................................................................... 17

Conclusion ................................................................................................................... 20

Materials and Methods ............................................................................................... 20

Acknowledgement ....................................................................................................... 24

References ................................................................................................................... 24
Abstract

Since extracellular matrix (ECM) based biopolymers such as hyaluronic acid (HA), heparin (HP) and chondroitin sulfate (CS) are biocompatible, biodegradable and non-toxic, suitable modification of these polymers could make them ideal drug delivery system. Due to their high affinity to bind to specific receptors such as CD44 and heparin sulfate receptor, which are over-expressed on the surface of cancer cells, they could be used as nanocarriers for targeted delivery of anticancer drugs to cancer cells. Such targeted delivery of chemotherapeutic drugs will enhance the efficacy of the drug and will also reduce side effects. In this study, internalization of HA, HP and CS based nanocarriers in human colorectal cancer (HCT 116) cells, which is known in over expressing CD44 receptors was investigated. To trace the cellular uptake of these carriers, they were labeled with fluorescein-5-thiosemicarbazide (FTSC) probe using standard carbodiimide coupling reaction. Localization of labeled polymers in the nucleus of cancer cells was confirmed by confocal microscopy. Dynamic light scattering studies showed that the hydrophobic FTSC-labeled polymers are nanoparticles with sizes varying from 100-500 nm. These particles were loaded with a well known clinically approved drug, namely Doxorubicin. In this work initial cellular cytotoxic studies were carried out with hyaluronan-doxorubicin particles and their IC\textsubscript{50} value were determined. IC\textsubscript{50} values in the presence of chloroquine (CQ) as a lysosomotropic agent proved that some amount of drug remain entrapped within the endosome. Further evaluation of drug efficacy of these potential drug delivery candidates is an ongoing project.
Introduction

In recent years, Extra Cellular Matrix (ECM) based biopolymer-drug conjugates are used as an efficient delivery system for targeting various diseases. They are also used as hydrogels, scaffolds for tissue engineering, 3D matrix for cell culture, biosensors, diagnostics etc (Vulic and Shoichet, 2012; Kraehenbuehl et al, 2011; Peppas et al, 2006) Among them, hyaluronic acid, collagen, fibrinogen etc plays a pivotal role in cell and tissue integrity, proliferation, wound repair, inflammation, morphogenesis and intracellular signaling etc (Burdick et al, 2004a, 2004b). They are however attractive carrier for delivering drug payload as they are taken up by cells by receptor mediated endocytosis. Another advantage of using these natural biopolymers for drug delivery is that it enhances the solubility of hydrophobic chemotherapeutic drugs thereby increasing the pharmacokinetics and efficacy of the drug (Li and Wallac, 2008).

To increase the efficiency of drug delivery system different types of nanomedicines have been designed (Danlier et al, 2010). Figure 1 shows types of nanomedicines.

Figure 1. Types of nanomedicines designed for drug delivery system. (A) Various nanocarriers which are used in clinical applications; (B) Schematic of PEGylation and liganded PEGylation (Danlier et al, 2010).
Among different polysaccharides derived biopolymers heparin, Hyaluronic acid (HA) and chondroitin sulfate (CS) based glycosaminoglycan (GAG) has been extensively used in drug delivery system. HA is the only non-sulfated polysaccharide known in the ECM, while HP and CS are highly sulfated. They offer significant advantage over synthetic polymers as they are biocompatible, biodegradable and prevent complement activation. They are widely used in drug delivery, gene delivery and controlled release studies etc (Reddy et al, 2011; Liu et al, 2008). These GAG polysaccharides are internalized into cells by specific binding to the cell surface receptors. Since these receptors are over-expressed in many solid tumors they offer new avenues for designing drug delivery systems which can target specifically to cancer cells without affecting normal cells. These polysaccharides polymers possess long hydrophilic sugar chains with different functional groups. Chemical modification of these sugar chains modifies the structure of polymer and consequently its physiochemical and biological properties (Liu et al, 2008; Hassani et al 2012). Introducing hydrophobic units to the polysaccharide chains makes it amphiphilic which self-assembles to form nanoparticles and micelles in water (Hassani et al 2012). Although there are several reports in literature where these GAGs are used as carrier molecules for active targeting, there is no substantial evidence on their comparative internalization and nuclear localization ability. This motivated us to explore these three different GAGs as a drug delivery system against cancer cell. In the present work we will focus on these three GAGs as a drug carrier for targeting cancer cells.

**Heparin (HP)**

Both heparin and heparan-sulphate glycosaminoglycans (HSGAGs) are sulfated polysaccharides. Their structure is similar, but the proportion of glucoronic acid and iduronic acid in their structure is different and heparin is more sulfated, so it is more electronegative. HSGAGs bind to proteins and mostly are found on the surface of vascular endothelium cells and leukocytes to make them negatively charged (Lever et al, 2002). They possess several roles in physiochemical reactions in the body such as anticoagulation, angiogenesis, cell differentiation, cell-cell interaction and cell-ECM interaction (Zacharski et al, 1998; Lever et al, 2002). HSGAGs possess several main roles in metastasis. Exogenous heparin which is similar in structure and function to
HSGAGs is able to compete with HSGAGs to prevent its function in metastasis. The mechanisms of metastasis are as follows:

1) On the surface of cancer cells, HSGAGs acts as a ligand for p-selectin and as a co-receptor for integrins (I) to bind to the platelets/endothelial cells of blood vessels. This is one way of transferring cancer to other parts of the body (metastasis).

2) HSGAGs is cleaved by heparinase and by releasing growth factor induces metastasis.

3) HSGAGs by binding to thrombin could create a local coagulated fibrin layer to keep tumor protected against natural immune system.

4) HSGAGs interact with growth factors to induce proliferation and migration signals in cancer cells (Lever et al, 2002).

Heparin as a known anticoagulant drug has many other applications in drug delivery area. This polysulfated polymer is also used to deliver various proteins and growth factors as it effectively binds to the sulfate groups. HP nanoparticles are also designed for various applications as improved anticoagulant drugs, designing new anticancer drugs, biosensors and tissue engineering (Kemp et al, 2010). Amphiphilic HP conjugate are also designed by conjugating with Deoxycholic acid to form HP-DOCA binds to chemotherapeutic drugs such as Doxorubicin to increase their efficacy. It has dual action in killing cancer cells, first by specific delivering of conjugated anticancer drug (Dox) to the nucleus and second by internalized HP which inhibits cell proliferation (Park et al, 2004). Langer et al showed that by conjugating poly (β-amino ester)s with HP, the conjugate will be internalized to the cancer cells and consequently by promoting apoptosis, the cells will be killed (Berry et al, 2004). Recently researcher by employing microfluidic devices could increase the degree of substitution in heparin chain and this way they could enhance the loading capacity of heparin based drugs for an efficient targeted drug delivery (Tran et al, 2012).

*Chondroitin sulfate (CS)*
CS is one of the major ECM glycosaminoglycans in animal tissues which are consisting of N-acetylgalactosamine and glucuronic acid (GalNAc-GlcA) units. The disaccharide units join to each other to produce different types of CS. Normally the medium size of CS which is found in animal tissues is around 20 kDa. Studies showed that plenty of CS is found in some kinds of cancerous cells such as prostate cancer (Ricciardelli, 1999) and melanomas (Smetsers et al, 2003, 2004). Sugahara et al showed that in ECM of some tumors, the amount of low molecular weight CS type E (CSE) bound to cleaved CD44 receptors is in a high level. It proves that interaction between CSE and CD44 and followed by endocytosis of CSE, activates a signaling pathway to stimulate production of chondroitinases and proteases. Proteases could cleave CD44 and consequently cancer cells could migrate in blood circulation to create metastasis (Saguhara, 2008). In some recent works, researchers have tried to use CS for gene delivery and gene therapy. Hamada et al employed DNA/PEI which had been coated with low molecular weight CS (10 kDa) for transfection. They observed that transfection of plasmid in ovarian cells was 6 times better than that of in normal one (Hamada et al, 2012). Several cancer cells over-express CD44 receptor which could internalize CS. Thus low molecular weight CS to form CS-Drug conjugate is also used for targeting cancer cells and delivering drug payload to control the tumors growth.

**Hyaluronic acid/hyaluronan (HA)**

Hyaluronic acid (HA) is a natural, biocompatible and non-sulfated GAG polymer which is made of alternating units of D-glucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc). HA as a main component of ECM is involved in many biological processes, such as proliferation, differentiation, gene expression, adhesion, and motility (Lee, 2000). After specific interaction between HA and cell surface receptors, of which CD44 is well known, the biological processes are activated (Misra, 2011). Studied showed that cancerous cells such as colon, breast, ovarian and epithelial are over-expressed with CD44. High level of HA production in cancerous cells promotes metastasis and specific by binding of HA to CD44 receptors, prognosis of cancer will be difficult (Hua, 1993). There are two major types of hyaluronidases (HyalS) in the body. Hayal-1 which is over-expressed in tumors is found in lysosomes and cleaves HA polymers to
smaller fragments in acidic pH. Hyal-2 is near to the CD44 receptors and cleaves HA polymers to 50-unit fragments to help with internalization of HA by endocytosis (Stern, 2008).

Selectivity of HA to use as a drug carrier for targeted delivery to tumors makes it a good option for chemotherapeutic drug carrier (Kim, 2008). HA is biocompatible, biodegradable, non-immunogenic, and with its ability to effectively target CD44, it is used in cancer research (Gaffney et al, 2010; Xian, 2012). By using different functional groups, like carboxylate and hydroxyl groups, various chemotherapeutic drugs can be linked chemically to HA polymer. Releasing of HA by the reticulum-endothelial system (RES) in the livers cells and also degradation of them by Hyals, are two major problems with the using of HA as a drug carrier. Slightly chemical modification of HA will effect on its degradation and release (Ossipov, 2010; Fraser et al, 1983; Erickson et al, 2012).

Objective: In the present study we intend to undertake a detailed investigation of the cellular uptake capability of HA, HP and CS. For this purpose human colorectal cancer, HCT 116 cell line was used as it is known to over-express the CD44 receptor. To do this, we synthesized fluorescein labeled nanoparticles with HA, HP and CS and used it to study its cellular uptake and nuclear trafficking ability. Specifically, we labeled the polymers with Fluorescein-5-thiosemicarbazide (FTSC) which is a fluorescent probe and localization of the polymers was studied using confocal microscopy technique. Using HA, we have also synthesized Doxorubicin conjugates and evaluated its efficacy as an anti-cancer drug against HCT-116.

Results

Labeling the polymers (HP, CS and HA) with FTSC

We first synthesized fluorescein labeled nanoparticles of HA, HP and CS following standard EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) coupling protocol in presence of N-hydroxybenzotriazole (HO-Bt). These were purified by extensive dialysis and characterized by NMR. Figure 2 shows the mechanism of the reaction. In this reaction, N-hydroxybenzotriazole HO-Bt is a rescue agent which could avoid formation of urea.
Figure 2. Mechanism of Labeling the polymers (HP, CS and HA) with FTSC. (1) acid, (2) O-acylisourea, (3 and 4) desired amide, (5) acid anhydride, (6) N-acylurea (Nakajima and Ikada, 1995; Wikipedia, 2012)

Determination of degree of substitution in FTSC-labeled polymers by UV/VIS spectroscopy

The calculated degree of substitution (wt%) according to the explained method for HA, HP and CS respectively are 2.8%, 0.63% and 1.72. Figure 3 shows (a) the standard curve of free FTSC and (b) concentration of FTSC vs. labeled-polymer equivalent concentration. The degree of
substitution in our labeled polymers are different, so using the standard curve, equal amount of FTSC was loaded in *in vitro* assays.

**Figure 3.** UV/VIS measurement of wt% FTSC in HA-FTSC, HP-FTSC and CS-FTSC at 456 nm. (a) FTSC UV/VIS standard curve at 456 nm. (b) Absorbance converted to amount of FTSC in mg per mg of labeled polymer (HA-FTSC, HP-FTSC and CS-FTSC). (c) Summary of calculated wt% of FTSC for 100 mg of labeled polymers.

**Determination of particle size using dynamic light scattering (DLS) machine**

FTSC is a hydrophobic agent and after binding to hydrophilic polymers make them amphiphilic nanoparticles which are appropriate for drug loading. **Figure 4** shows the particle size for HA-FTSC, HP-FTSC and CS-FTSC.
**Figure 4. Particle size determined by DLS: (A) HA-FTSC, (B) HP-FTSC, (C) CS-FTSC**

These results show that after labeling of the polymers and dissolving in PBS pH 7.4 they produce nano-particle which could be uptaken by CD44 over expressed cell lines. Nano-particle which was produced by HA, HP and CS polymers respectively are 505.5 nm, 95.1 nm and 146.1 nm. The size of the nanoparticles are in a right size for efficient endocytosis.

**Confocal microscopy and image analysis of cellular localization of labeled-polymers.**

Confocal images taken after 4h incubation of three polymers (HA-FTSC, HP-FTSC and CS-FTSC) with HCT 116 cells according to the explained method (in material and method part) shows various fluorescent intensities (**Figure 5 to 7**).
Figure 5. Confocal images of uptaken HA-FTSC by HCT 116 cells. (A1) plasma membrane, stained with Deep Red plasma membrane stain; (A2) HA-FTSC which is green; (A3) overly of A1 and A2. (B1) nucleus of the cell stained with DAPI which is blue; (B2) bright field image of the cell; (B3) internalized green HA-FTSC; (B4) overlay of three images.
Figure 6. Confocal images of uptaken HP-FTSC by HCT 116 cells. (A1) internalized green color HP-FTSC; (A2) bright field image of the cells; (A3) overlay image of A1 and A2. (B) four different images including nucleus of the cell stained with DAPI which is blue; bright field image of the cell; internalized green HP-FTSC and overlay of three images on the right bottom corner. (C1) plasma membrane, stained with Deep Red plasma membrane stain; (C2) uptaken HP-FTSC which is green; (C3) overly of C2 and C3.
Figure 7. Confocal images of uptaken CS-FTSC. (A1) internalized green color CS-FTSC; (A2) bright field image of the cells; (A3) overlay image of A1 and A2. (B) four different images including nucleus of the cell stained with DAPI which is blue; bright field image of the cell; internalized green CS-FTSC and overlay of three images on the right bottom corner. (C1) plasma membrane, stained with Deep Red plasma membrane stain; (C2) uptaken CS-FTSC which is green; (C3) overlay of C2 and C3.
Among the three polysaccharide polymers, HA is extensively studied for drug delivery. This offers enormous opportunity to evaluate other candidates like HP and CS which can equally perform as targeting ligand. Further work to load drugs to these nanoparticles is in progress. In another ongoing project, we developed doxorubicin conjugated HA and evaluated its efficacy.

*Cellular toxicity (determination of IC<sub>50</sub> value for Doxorubicin)*

Figure 8 shows the cytotoxicity of Dox and its derivatives on HCT 116 cell line. The results have been summarized in Table 1.

*Figure 8. Cytotoxicity of Dox and derivatives on HCT 116 cells* (A) Standard chloroquine (CQ). According to the MTS results, 100 µM CQ in which 92% cells are alive will be used as a fixed amount for *in vitro* experiments. (B) Dox standard curve which was used to take equal amount of Dox in conjugates. (C) Determination of IC<sub>50</sub> value for Dox, Dox=HA and Dox+HA by MTS assay (D) Determination of IC<sub>50</sub> values in the presence of 100 µM CQ by MTS assay.
**Table 1: IC₅₀ value for Dox and its derivatives**

<table>
<thead>
<tr>
<th></th>
<th>%Dox</th>
<th>IC₅₀*</th>
<th>IC₅₀ with CQ**</th>
<th>Fold increase in cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dox</td>
<td>100%</td>
<td>1.2 µM</td>
<td>0.7 µM</td>
<td>1.7x(0.5 µM)</td>
</tr>
<tr>
<td>Dox=HA</td>
<td>0.2%</td>
<td>0.7 µM</td>
<td>0.3 µM</td>
<td>2.3x(0.4 µM)</td>
</tr>
<tr>
<td>Dox+HA</td>
<td>0.5%</td>
<td>0.9 µM</td>
<td>0.2 µM</td>
<td>4.5x(0.7 µM)</td>
</tr>
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*, ** Mean value of triplicates which was tested in two sets of 96 well plates

**Competitive assay:**

To prove if cellular uptake of HA is via CD44-receptor-mediated endocytosis, a competitive assay was done (Figure 9).
Competitive assay

Figure 9. Competitive Assay. Blue bars are alive% in the absence and red one is in the presence of 7.5 kD HA. Cells incubated with only 7.5 kD HA were taken as a control.

Release experiment

Figure 10 shows the release results of Dox in different derivatives of that.

Figure 10. Release of Dox, conjugates and Dox+HA at pH 7 and 5
**DNA binding**

Degree of DNA binding in derivatives of Dox added to calf thymus (ctDNA) was determined by fluorescence quenching test (**Figure 11**).

![DNA binding graph](image)

**Figure 11. DNA binding after 1h incubation in room temperature**

**Discussion:**

Confocal images showed that in all the cases polymers were internalized into the HCT 116 cells, but the intensity of green fluorescent FTSC is different. In the case of HP and CS, the intensity is higher (**Figure 5 to 7**). Considering that the concentration of FTSC is the same for all the polymers, it seems that cellular internalization in the case of HP and CS is more efficient. It may be because of smaller sizes of nanoparticles of HP and CS compared to HA. Some recent research works show that low molecular weight CS coated on the surface of DNA/PEI for gene delivery in CD44 over-expressed cancer cells worked more efficiently than that of low molecular HA (Hamada et al, 2012). Images confirm that the polymers localize mostly in the nucleus of HCT 116 cells, so they are applicable to deliver anticancer drugs specifically to cancer cells.
which are over-expressed in CD44 receptors. According to the previous studies, cellular uptake and nuclear binding of labeled HA, HP and CS with biotinylated probes, are cell type specific and also type of probe is important in nuclear binding (Chovanec et al, 2004).

To evaluate if the cellular uptake of the fluorescent biopolymers or their conjugates with drugs is via CD44-mediated endocytosis, we selected HA out of these three biopolymers and we did competitive assay for its conjugates. The evaluation for two other biopolymers is ongoing. As we see in the Figure 9, in all the cases when we add the drug to the cells pre-incubated with 7.5KDa HA, or co-cultured with HA, cytotoxicity diminishes and viability increases. In the case of Dox-HA and Dox=HA, decrease in cytotoxicity is because of competition to occupy the CD44 receptors. In the case of free Dox, it is comparable with Dox+HA case. Even if electrostatically mixing of HA and Dox increases its efficacy, but here CD44 receptors already have been occupied and increasing the efficacy via receptor mediated internalization is not possible, so viability increases.

The cytotoxic effect of Dox conjugates was examined on HCT 116 cell line which is known to over-express the CD44 receptors for HA. This cell line is an appropriate model to study targeted delivery of Dox conjugates by receptor mediated endocytosis uptake. In this study, it was hypothesized that short covalent amide linkage has potential cytotoxicity, in spite of previous publication about covalent linkage to drug-carriers (Luo et al, 2002; Santra et al, 2010). The idea comes from here that some amount of drug is trapped in endosomal pathway. Chloroquine (CQ) is an antimalaria drug which is slightly basic and as a lysosomotropic agent is able to rupture the integrity of the membrane. CQ by buffering proton from lumen to cytosol disturbs the proton gradient through the membrane and subsequently changes the pH of lysosomes. The drug will release in cytoplasm to go to nucleus (He et al, 2011). The optimum concentration of CQ was determined by MTS cytotoxicity assay according to the explained protocol in the material and method section. In 100 µM reported concentration of CQ (Cheng et al, 2006; Erbacher et al, 1996), 92% of HCT 116 cells in our experiment was alive and this concentration was used for other MTS assays. MTS cytotoxicity assay for Dox, HA=Dox and HA+Dox examined on HCT 116 cell line in the presence and absence of CQ (Figure 8C and 8D). CQ by releasing the
trapped drug in lysosomes, increases the IC$_{50}$ value for conjugates. Table 1 shows the IC$_{50}$ value and degree of substitution for Dox and conjugates.

Comparing the IC$_{50}$ values in the presence and absence of CQ for free Dox shows that even if Dox is a small hydrophobic molecule to diffuse through cell membrane, but some amount of that can be trapped in lysosomal pathway. By adding CQ, the trapped amount is released and the cytotoxicity increases.

In the case of Dox=HA, cleavable hydrazone conjugate, previous studies shows increased cytotoxicity of the conjugate (Cai et al, 2010). In our study, lower IC50 value respect to free Dox, confirms the efficacy of the cleavable hydrazone linkage. Decreasing in IC$_{50}$ value in the presence of CQ, shows the entrapment of conjugate in the endosomal pathway. Even though the hydrazone linkage in Dox=HA is pH sensitive and should be cleaved in the acidic pH to release free Dox.

Results of IC$_{50}$ values shows lower value for Dox+HA (0.9 µM) in comparison with free Dox (1.2 µM). This is probably is because of increase in endosomal uptake of electrostatically bound Dox and HA. By adding CQ, trapped amount of Dox+HA releases and the IC$_{50}$ value decreases to 0.2 µM a greater effect then observed for HA=Dox. This is may be because of weak bound between electrostatically bound Dox and HA compared to Hydrazone linkage in Dox=HA. After lysosomal effect of CQ, released Dox from Dox+HA into the neutral pH of cytoplasm can more easily translocate to the nucleus. So we came to this conclusion that electrostatically mixed HA with Dox can increase its cytotoxicity respect to free Dox in the absence of CQ and increase its cytotoxicity respect to Dox=HA in the presence of CQ.

The pH 7.4 and 5 resemble the pH of the blood system and lysosomal agent medium respectively. At both pH (5 and 7.4), Dox-HA showed the lowest Dox release, of which some amount is due to sticking of conjugate to dialysis membrane (in all cases) (Figure 10). We observed that the release of Dox=HA is more than Dox-HA and this is because of weaker bound of hydrazone compared to covalent bound. The release in the case of Dox+HA is less than free Dox, may be because of electrostatic interaction between Dox and HA which keep it in the mixed form with HA and it shows less release in comparison with free Dox. Release in all the
cases in pH 5 is more than pH 7.4 and this is in accordance with previous studies for some other derivatives of Dox (Ulbrich et al, 2003)

Fluorescence quenching results showed that Dox and derivatives were bound to the ctDNA. The lowest degree of binding is related to non-cleavable Dox-HA and that of the highest is for free Dox and Dox+HA (Figure 11). Degree of binding in the cleavable Dox=HA compared to the non-cleavable Dox-HA is higher. This is due to the difference in the type of linkage in two conjugates. Dox=HA which is an acid-labile component, uses hydrazone linkage to bind to Dox, however in Dox-HA, amine group is the linkage between Dox and HA. According to the previously reported results, amino sugar part of the Dox-HA conjugate could create a hydrogen bound with minor groove of DNA, but this bound is weaker than that of Dox=HA which has a free amine group to extend a stronger hydrogen bounds with DNA (Fredrick et al, 1990; Zaman et al, 2006).

Conclusion:

The results confirmed the internalization of these polymers in HCT 116 which is known to over-expressed in CD44 receptor. FTSC-Labeled polymers are hydrophobic nanoparticles with the size ranging from 100 nm to 500 nm which are appropriate for anticancer drug loading and specific delivery to HCT 116 cells. In this study we used HA as a carrier for Dox. Synthesized hydrazone-linked Dox=HA showed a good drug potency for cancer treatment. Optimized concentration of lysosomotropic agent CQ (100 µM) by releasing the endosomal pathway entrapped drug could enhance its cytotoxicity and consequently the efficacy.

Materials & Methods

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H tetrazolium (inner salt) and PMS (phenazine methosulfate, an electron coupling reagent, ), CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay (MTS) reagents were purchased from
Promega. The Slide-A-Lyzer MINI Dialysis Devices, 3.5K MWCO was purchased from Thermo Fisher Scientific. Calf Thymus DNA (ctDNA) and all other chemicals were purchased from sigma. The concentration of ctDNA was measured by UV/VIS spectroscopy (260 nm).

The conjugates used in this study, were synthesized and characterized by O.P. Oommen in polymer chemistry group, department of Materials Chemistry at Uppsala University. To avoid missing some amount of the hydrophobic drug, all conjugates were dissolved in low-binding Eppendorf tubes and after filtration with 0.8µm filters under sterile conditions, one part of that was used for UV measurement and second part was used for invitro experiments.

Labeling the polymers (HP, CS and HA) with FTSC
To 0.5 mmol polymer (Hp, CS and HA) dissolved in 40ml DI water, 0.05 mmol FTSC dissolved in 5ml DMSO was added followed by 0.5 mmol N-hydroxy- benzotriazole (HO-Bt). pH was adjusted to 5 and then 0.1 mmol (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to the mixture and stirred overnight. After 48h dialysis agaist HCl (pH 3) including NaCl 0.1 M in a dialysis membrane (Mw cutoff=3500 g/mol), it was dialysed against HCl (pH3) in the absence of NaCl for 24h more. The sample was lyophilized and kept in freezer. Due to sensitivity of FTSC to light the vessel was covered with aluminium foil.

Determination of degree of substitution in FTSC-labeled polymers by UV/VIS spectroscopy
Degree of substitution in (DOS) FTSC-labeled polymers conjugates was determined using UV/VIS spectroscopy at 456 nm (Lambda 35 UV/VIS spectrometer, Perkin Elmer). 2 mg of free FTSC was dissolved in 1 ml DMSO:H2O (50:50) and using this stock solution, the standard curve was plotted for various concentration of FTSC. 2 mg of labeled polymers was dissolved in 1 ml of DMSO:H2O (50:50) and the FTSC concentration of them was calculated by comparing their absorbance at 456 nm with that of standard curve. By using the standard curve equal loading of FTSC concentration was used for in vitro experiments. DOS in a fixed amount of labeled polymers was calculated using the following formula:

\[
\text{DOS} = \left( \frac{\text{amount of FTSC in gr or mmol}}{\text{amount of labeled polymer in gr or mmol}} \right) \times 100
\]
In vitro cell culture

Human colorectal cancer cell line, HCT 116 (American Type Culture Collection (ATCC)-LGC standards, Sweden) was cultured in a mixture of Dulbecco’s Modified Eagle Medium (DMEM), F-12 Ham (1:1), 10% fetal bovine serum (FBS Hyclone, Perbio Scientific, Sweden) and 1% antibiotics (10.000U penicillin and 10 mg/mL streptomycin). Cells were incubated in a humidified incubator at 37°C and 5% CO₂.

Determination of particle size using dynamic light scattering (DLS) machine

1 mg from each labeled polymer was dissolved in 1ml of PBS pH 7.4 and the particle size was measured by zeta-sizer.

Confocal microscopy and image analysis of cellular localization of labeled-polymers.

Cellular uptake and localization of FTSC- labeled polymers (modified heparin, chondroitin sulfate and HA) was analyzed by confocal microscopy to confirm that labeled-polymers are able to enter the cell. Cultured HCT 116 cells on tissue-culture treated glass chamber slides (BD Biosciences) at a concentration of 100,000 cells/4 ml was incubated overnight. For FTSC-labeled polymers ($\lambda_{ex} = 494$ nm and $\lambda_{em} = 518$ nm), the same concentration of fluorescent probe (10 μM) for all the samples was used. 4',6-diamidino-2-phenylindole (DAPI) was used for staining the nucleus of the cells ($\lambda_{ex} = 358$ nm and $\lambda_{em} = 461$ nm) and For staining the plasma membrane, CellMask™ Deep Red plasma membrane stain ($\lambda_{ex} = 649$ nm and $\lambda_{em} = 666$ nm) was used after fixing the cells. The images were taken at 63x magnification by a Zeiss LSM 510 META confocal microscope after 4h incubation on the surface of glass slides with cover slips.

Cellular toxicity (determination of IC₅₀ value for Doxorubicin)

After overnight incubation of HCT116 cells in a 96-well plate (concentration of 5000 cells/100 μL/well), the culture medium was refreshed with medium containing different concentrations of doxorubicin and doxorubicin-conjugates in the presence or absence of optimized concentration of Chloroquine (100 μM). As a control, the same concentrations of free Dox were mixed with
100 µg/mL HA (150 kD) to make electrostatically bound HA-Dox were added to the well-plate. Concentration of stock solution of free Dox and its conjugates were measure by UV/VIS spectroscopy before using. The well-plate was incubated for 48h in a humidified incubator at 37°C and 5% CO₂.

Cell viability was determined by MTS assay. MTS substrate [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H tetrazolium] can be bioreduced by mitochondria of live cells into a soluble formazan product, which is measurable in 450 nm by UV/VIS. The reagent was mixed with PMS (phenazine methosulfate, an electron coupling reagent,) and was added (20 µL/well) to incubated samples in 96 well-pate. The well-plate was incubated in the same conditions for additional 4h, and then the absorbance in 450nm was recorded using the Labsystems Multiskan MS plate reader. The IC₅₀ value was calculated using following formula:

\[
\frac{(\text{drug-treated A}_{450} - \text{mean blank})/(\text{mean untreated control A}_{450} - \text{mean blank} \times 100%)
\]

All the samples were in triplet and after calculating mean value (in the presence of MTS reagent) and standard deviation, Percentage of viability of the cells was plotted against the concentration of the drug. 50% viability on the graph shows the IC₅₀ value of the drug (relative to untreated cells in the presence of MTS reagent).

**Competitive assay**

In order to study specific cellular uptake via CD-44 mediated endocytosis in HCT 116 cell line, competitive assay was used. After overnight incubation of 5000 cells/100 µL/well in a 96-well plate, excess amount of 7.5 KD HA (20mg/ml) was dissolved in PBS buffer pH 7.4 was used to block the CD44 receptors. After 1h incubation, the medium was removed from the wells and drugs (free dox, HA-conjugates and electrostatically bound drug) were added to the wells. After 48h incubation in a humidified incubator at 37°C and 5% CO₂, IC₅₀ values were determined using MTS cytotoxicity assay.

**Release experiment**
Release of doxorubicin from doxorubicin-conjugates, electrostatically bound Dox and HA, and free Dox was determined by UV/VIS spectroscopy at 490 nm (Lambda 35 UV/VIS spectrometer, Perkin Elmer). 2 mg of each sample was dissolved in 0.8-1 ml of PBS pH 5 and 7.4 and their absorbance at 490 was measured. The solutions were poured into the Slide-A-Lyzer MINI Dialysis Devices, 3.5K MWCO. The absorbance of each sample at wavelength of 490 nm after 72 hours was measured again. Using excel software, the percentage of release was plotted against the type of the drug.

**DNA binding**

Fluorescence quenching of doxorubicin from doxorubicin-conjugates, electrostatically bound Dox and HA, and free Dox was determined by Tecan Infinite M200 Flurospectrophotometer (Tecan Group Ltd. Switzerland). Fixed amount of three samples including 2 µM of Dox was added to various concentration of ctDNA (0-0.5 µM) in a Corning 96 Flat Bottom Black Polystyren well plate ($\lambda_{ex} = 485$ nm and $\lambda_{em} = 595$ nm). Relative fluorescence of each sample was plotted against ctDNA concentration using excel software.

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