

# Targeted Stimuli-Responsive Dextran Conjugates for Doxorubicin Delivery to Hepatocytes

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**Abstract** – A targeted, stimuli-responsive, polymeric drug delivery vehicle has been developed to help alleviate the severe side-effects caused by narrow therapeutic window drugs. Doxorubicin, a commonly used chemotherapeutic agent has been conjugated to dextran by two different techniques. In the first method, doxorubicin and hepatocyte-targeting galactosamine were attached to dextran through amine bonds. Conjugation efficiency based on the amount loaded of each reactant varied from 1% to 50% for doxorubicin and from 2% to 20% for galactosamine, depending on various synthesis parameters. For the second conjugate, doxorubicin was attached to dextran through an acid-labile hydrazone bond. Fluorescence quenching indicated that all our conjugates can bind to DNA. The degree of binding was improved with increasing polymer molecular weight and substitution of doxorubicin, and also with hydrazone-bonded conjugate. In cell culture experiments, we have found that the uptake of conjugates was much lower than that of free doxorubicin. Lower uptake of conjugates decreased the toxicity of doxorubicin. Also, the uptake of non-galactosylated conjugate was lower than that of the galactosylated conjugate. Microscopy studies indicated that doxorubicin was localized almost exclusively at the nucleus, whereas the amine-bonded conjugates were present throughout the cell. Targeted amine-linked conjugates and hydrazone-bonded conjugates achieved greatly improved cytotoxicity. Following uptake, the doxorubicin was dissociated from the hydrazone conjugate in an endosomal compartment and diffused to the nucleus. The LC<sub>50</sub> values of non-targeted amine-linked, targeted amine-linked, and hydrazone-linked doxorubicin were 19.81 µg/mL, 7.33 µg/mL and 4.39 µg/mL, respectively. The amine-linked conjugates were also tested on a multidrug-resistant cell line; the LC<sub>50</sub> values of doxorubicin and the non-targeted amine-linked conjugate were 8.60 µg/mL and 36.02 µg/mL, respectively.

**Index Terms** – Dextran conjugates, doxorubicin, drug delivery, galactose-targeting, hepatocytes

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## I. INTRODUCTION

Cancer is one of the leading causes of death. Current treatment involves various combinations of surgery, radiation therapy and chemotherapy. Chemotherapeutic agents are cytotoxic drugs, which affect any cells in the body that are actively dividing. Therefore, in addition to cancerous cells, they affect bone marrow, causing a suppression of the immune system, loss of hair and skin cells, etc. Furthermore, they can cause long-term damage to major organs in the body. To avoid these serious side-effects, conventional chemotherapeutic agents must be administered at a suboptimal dose, which is insufficient to treat the cancer satisfactorily in one dosage. Physicians have attempted to treat cancer using these lower doses over a longer period of time, however, this has also proven ineffective due to the development of drug resistance by the cancerous cells [1].

These challenges can be tackled by targeted drug delivery, which increases the range in which a drug is both safe and effective. Targeted delivery can be achieved by two approaches: *site-specific transport* and *site-specific activation*. Site-specific transport is attained when an active drug is preferentially concentrated at the target site, such as can be achieved with an implant or transdermal drug delivery. Site-specific activation or stimuli-responsive delivery is accomplished when a drug is activated by a controlled mechanism at or near the target site [2].

The least invasive and most promising method of targeted drug delivery is protein targeted delivery. Protein targeting, specifically, lectin (carbohydrate receptor) mediated targeting, holds potential due to high specificity and affinity, rapid internalization by receptor-mediated endocytosis, and relative ease of processing [2–4].

Much research has been performed in the area of protein targeted delivery in the last two decades. Crews *et al.* have used a number of different antibodies to target human breast cancer cell lines to improve cytotoxicity of the drugs [5]. Monsigny *et al.* have shown that murine leukemia cells express a high concentration of L-fucose (a modified monosaccharide) receptors [6], and are studying the use of glycoconjugates for gene delivery. Yamazaki *et al.* are using glycoprotein-liposome conjugates for drug delivery [7]. In spite of the significant progress in this field, more work is needed in improving target recognition, minimizing premature release, and preserving drug efficacy regardless of chemical alteration [8–10].

Polymeric drug delivery vehicles can enhance the performance of a drug in several ways. They increase the circulation time in the body, protect the drug from various enzymes and decrease non-specific toxicity [8]. Also, chemical modification of polymers allows addition of functionalities, such as receptor targeting and stimuli-responsive activation of the drug. Another advantage is that polymer-bound drugs are less likely to be expelled from multidrug-resistant cells [9]. Conversely, conjugation to a polymer results in reduced therapeutic effect, compared to the free drug. Hence, release of the drug after delivery to the target or *site-specific activation* is desirable. A number of intracellular vesicles such as endosomes and lysosomes are maintained at low pH, and incorporating an acid-labile bond between the polymer and drug would allow for activation after delivery.

The purpose of our project is to synthesize a targeted, pH-sensitive, polymeric drug delivery vehicle to deliver doxorubicin for cancer chemotherapy to hepatocytes. Doxorubicin is most commonly used in the treatment of lymphoma, osteosarcoma and other sarcomas, carcinomas, and melanoma. In this paper, we describe the synthesis of two dextran-doxorubicin conjugates. The amine-linked dextran-doxorubicin-galactose (DDG) conjugate expresses galactose, and preferentially transports doxorubicin to hepatocytes, which express a high surface density of galactose receptors. The dextran-hydrazide-doxorubicin (DHD) conjugate contains a pH-sensitive hydrazide bond between dextran and doxorubicin, and releases the drug in the endosome at a low pH.

## II. EXPERIMENTAL

### A. Synthesis of Amine-Linked Dextran-Doxorubicin-Galactose Conjugate

The hydroxyl groups on dextran were activated by stirring 1 g of dextran in 100 mL of 0.03 M NaIO<sub>4</sub> overnight [11]. The activated polyaldehyde dextran (PAD) was purified by dialysis against deionized water for three days, and recovered by lyophilization. PAD reacted with the primary amines on doxorubicin and galactosamine to form an unstable imine bond, which could be reduced with sodium borohydride to form a stable amine bond. PAD (100 mg) was dissolved in 5 mL of phosphate buffer solution (PBS) (pH 7.4), reacted with doxorubicin and galactosamine, and subsequently reduced with NaBH<sub>4</sub> at 37°C for 2 h. The resulting DDG conjugates were dialyzed against deionized water and lyophilized. The loadings of doxorubicin and galactose on the conjugates were determined by absorbance at 485 nm and elemental analysis, respectively.

### B. Synthesis of Acid-Labile Dextran-Hydrazide-Doxorubicin Conjugate

The synthesis of acid-labile DHD conjugates was adapted from work by Ramirez [12] and Etrych [13]. All chemicals in this synthesis were dried under vacuum overnight; the reaction was also carried out under vacuum. One gram of

dextran was dissolved in 100 mL of a solution of 20 g/L of LiCl in dimethyl formamide at 90°C. The mixture was then transferred to an ice bath, and 1.5 mL of pyridine and 3.7 g of 4-nitrophenylchloroformate were added. The reaction mixture was kept in an ice bath for 4 h, and the resulting nitrophenyl carbonate-dextran (Dex-ONP) precipitated in a 4:1 mixture of ethanol and diethyl ether. Dex-ONP was centrifuged, washed two more times with ethanol/diethyl ether, and dried under vacuum. The presence of the nitrophenyl group on dextran was confirmed by nuclear magnetic resonance (NMR) and photoacoustic Fourier-transform infrared (PA-FTIR) spectroscopies.

One gram of the synthesized Dex-ONP was dissolved in 25 mL of dimethyl sulfoxide, and reacted with a ten-fold excess of hydrazine. The reaction was allowed to proceed for 3 h, and then dialyzed against deionized water for 2 days. The product, Dex-hydrazide was recovered by lyophilization.

In the final step, 100 mg of Dex-hydrazide was dissolved in 5 mL of PBS at a pH of 7.4. Doxorubicin (8 mg) and 1 drop of glacial acetic acid were added, and allowed to react for 48 h. The final polymer conjugate was dialyzed against PBS (pH 7.4) and freeze dried. The degree of substitution of doxorubicin was determined using absorbance of doxorubicin at 485 nm.

### C. Cell-Free Efficacy of Polymer Conjugates

Doxorubicin intercalates with DNA in the nuclei of cells. This interferes with cell division, and eventually leads to cell death. Binding with DNA causes fluorescence quenching of doxorubicin, and allows a fluorescence-based cell-free assay to evaluate the efficacy of the polymer conjugates [14]. Fluorescence was measured (ex. 488 nm/em. 590 nm) for a uniform amount of doxorubicin or conjugate in the presence of increasing concentrations of calf thymus (ct)-DNA.

### D. Cell-Free Release from Acid-Labile Conjugates

The release of doxorubicin from the acid-labile DHD conjugate was tested in 0.1 M of sodium acetate buffer with 0.05 M of sodium chloride at pH 7.4 and 5.0 [13]. The DHD conjugate was dissolved in PBS (pH 7.4), transferred to a Macro Fast DispoDialyzer cassette (Harvard Apparatus) and immersed in 5 mL of buffer at each pH. At each time point, 0.75 mL of sample was removed for analysis, and replaced with fresh buffer. Release was observed over a 24-h period. The concentration of doxorubicin in the buffer was determined by absorbance at 485 nm.

### E. In Vitro Efficacy of Polymer Conjugates

Hepatocytic cell lines were used to test uptake of galactosylated conjugates since hepatocytes are known to express a high surface concentration of galactose binding sites. The BNL CL.2 cell line (murine hepatocytes) was purchased from ATCC, and cultured in Dulbecco's

Minimum Essential Medium supplemented with 10% FBS and 1% Penicillin-Streptomycin (ATCC) at 37°C in a 5% CO<sub>2</sub> atmosphere.

For cytotoxicity studies, the cells were plated at 400/mm<sup>2</sup> in six-well plates, and allowed to adhere for 12–18 h. The medium was then replaced with complete medium containing doxorubicin or conjugates, and incubated for up to 24 h. All experiments were performed in triplicates. Cells were harvested with 0.25% Trypsin/0.53 mM of ethylenediaminetetraacetic acid (ATCC), and the relative uptake of doxorubicin or conjugate was evaluated using flow cytometry (Becton Dickinson FACScan, FL-2). To determine the killing efficiency, harvested cells were replated in 96-well plates (n = 6), and allowed to adhere overnight. The medium was replaced with 100 μL of fresh medium and 10 μL of the MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and the plate was incubated for 2 h. The MTT reagent reacted in the mitochondria of living cells to produce a purple precipitate. One hundred microliters of the detergent were then added, and the cells were incubated for another 3 h. The absorbance at 570 nm was measured when all the precipitate had dissolved.

Hep G2, a human hepatocyte cell line was also used to test the amine-linked conjugates. Hep G2 cells contain the multidrug-resistant protein, which is a membrane transporter that expels a wide range of exogenous toxins from the cell [15]. Due to this, Hep G2 cells have a resistance to several chemotherapeutic agents including doxorubicin. However, it is more difficult for the cells to expel a drug if it is polymer-bound instead of free. Hep G2 cells were grown in Eagles' Minimum Essential Medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. Cell culture and the cytotoxicity experiments were carried out as described for the BNL CL2 cell line.

The concentration at which 50% cell death occurred (LC<sub>50</sub>) was determined from the slope and intercept of the straight line obtained by inverting the dose vs. cell death data from the cytotoxicity studies.

Microscopy studies were conducted in chamber slides using 2 μg/mL of nominal doxorubicin concentration at 4, 8 and 24 h. Cells were fixed with 1% paraformaldehyde solution in PBS for 1 h, followed by 2% paraformaldehyde for 30 min. In some experiments, the nuclei of cells were counterstained with SYBR-Green (Molecular Probes) for 5 min after fixing the cells. Images were taken with a Zeiss LSM confocal microscope.

### III. RESULTS AND DISCUSSION

#### A. Synthesis and Characterization of Polymer Conjugates

##### 1) Amine-Linked DDG Conjugate

The degree of substitution of doxorubicin varied from 1% to 5%, and that of galactose varied from 5% to 15%. Fig. 1 shows the degree of substitution of galactosamine and doxorubicin for a number of different conjugates. The mass of doxorubicin was kept constant, while increasing the mass of galactosamine. Conjugation efficiency based

on the amount loaded of each reactant varied from 3% to 20% for doxorubicin and from 2% to 20% for galactosamine.

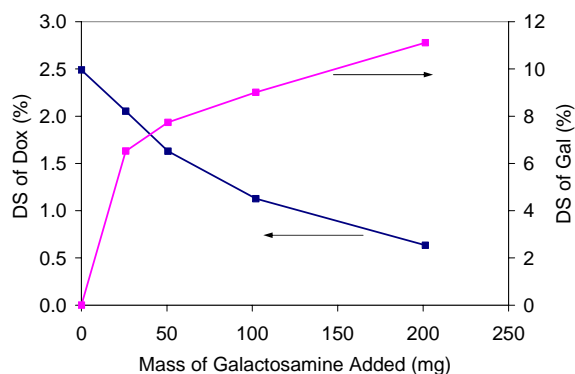


Fig. 1. Effect of varying the mass of galactosamine added on the degrees of substitution (DS) of doxorubicin and galactose for the DDG conjugate. Mass of doxorubicin was kept constant at 20 mg.

As shown in Fig. 1, the degree of substitution of galactosamine could be controlled by varying the amount of galactosamine loaded into the reaction vessel. However, the degree of substitution of doxorubicin decreased with an increased loading of galactosamine. Both doxorubicin and galactosamine reacted with PAD by the same mechanism; therefore, adding excess galactosamine would lower the degree of substitution of doxorubicin. Increasing the mass of doxorubicin would help improve the degree of substitution of doxorubicin (see Fig. 2). The reaction was also carried out over two days: PAD was first reacted with doxorubicin and subsequently with galactosamine. This increased the substitution efficiency significantly.

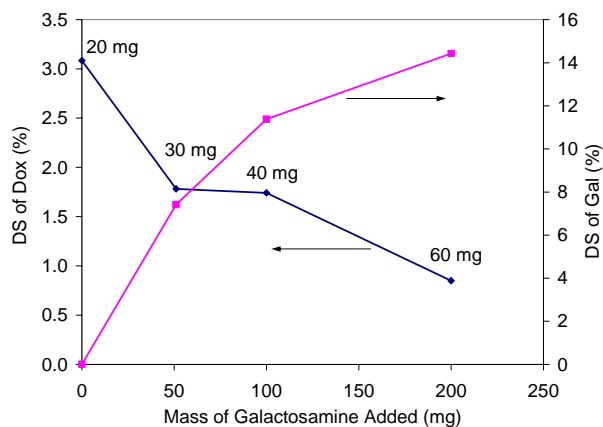


Fig. 2. Effect of varying the masses of galactosamine and doxorubicin added on the degrees of substitution (DS) of doxorubicin and galactose for the DDG conjugate. The mass of doxorubicin added was marked for each point.

##### 2) Acid-Labile DHD Conjugate

The structure of Dex-ONP was confirmed by <sup>1</sup>H NMR and PA-FTIR (Figs. 3 and 4). The glucosidic protons appeared on the NMR spectrum in several bands between

3.5 and 5.5 ppm. The peaks at 7.5 and 8.3 ppm corresponded to the phenyl protons on the 4-nitrophenyl carbonate group. The acyclic carbonate on Dex-ONP showed a characteristic infrared peak at  $1770\text{ cm}^{-1}$ , which shifted to a lower wavenumber for Dex-hydrazide.

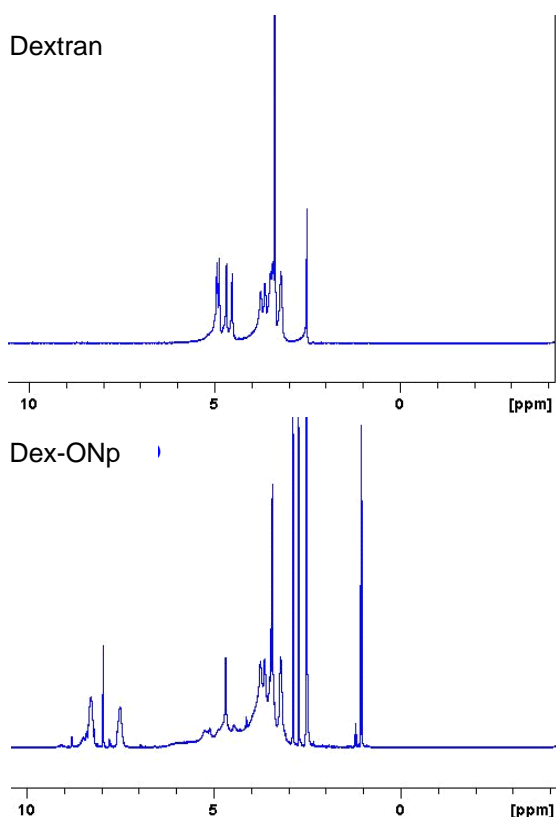


Fig. 3.  $^1\text{H}$  NMR spectra of dextran and Dex-ONP.

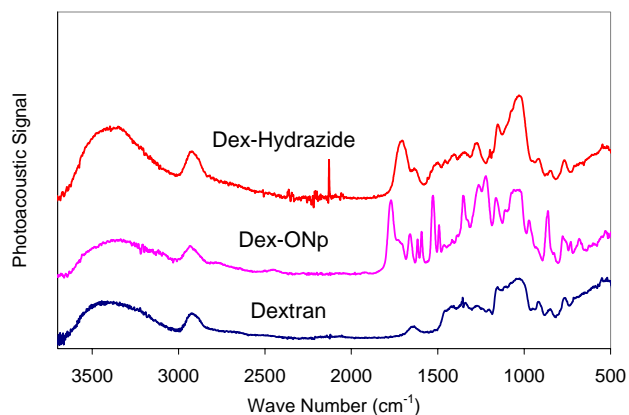


Fig. 4. PA-FTIR spectra of dextran, Dex-ONP and Dex-Hydrazide. Carbonate peak at  $1770\text{ cm}^{-1}$  was highlighted.

The effect of various synthesis parameters on the degree of substitution of doxorubicin was studied. The amounts of 4-nitrophenyl chloroformate, hydrazine and doxorubicin, as well as the molecular weight of the drug, were varied. The optimum values of the three reactants that gave the maximum degree of substitution for doxorubicin were

given in the synthesis protocol. The amount of doxorubicin added was found to be the most important factor. The degree of substitution also increased when polymers with higher molecular weights were used.

## B. Cell-Free Testing

### 1) DNA Binding

Fluorescence quenching indicated that all our conjugates were bound to DNA. As shown in Fig. 5, amine-linked conjugates of various molecular weights and substitutions exhibited this effect, though not to the same extent as free doxorubicin. The degree of binding increased with polymer molecular weight. 170kDa dextran-doxorubicin (DD) and DDG underwent almost complete quenching, whereas 10kDa and 40kDa samples only showed  $\sim 50\%$  quenching. A greater degree of substitution of doxorubicin also improved quenching (170kDa DD quenched slightly more than 170kDa DDG).

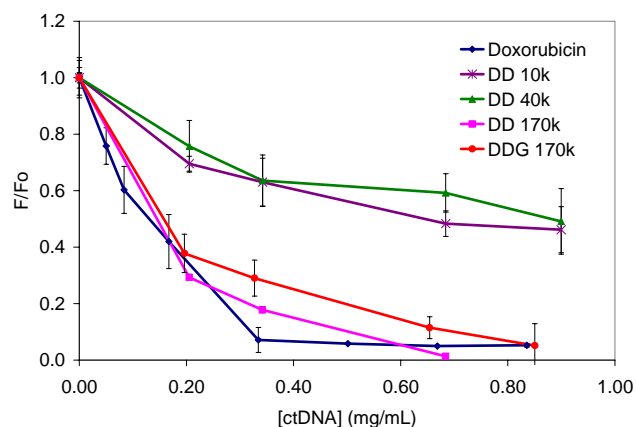


Fig. 5. Fluorescence quenching of amine-linked conjugates. Molecular weights of polymers were indicated.

The acid-labile DHD conjugates showed a much greater degree of binding with ctDNA than the DD conjugate of similar molecular weights (Fig. 6). The doxorubicin on the DDG conjugates was bound through the amine sugar group of doxorubicin, whereas for the acid-labile conjugate, the acetyl group was involved in the binding. Frederick *et al.* have reported that though the major binding occurs at the chromophore site of doxorubicin, the amino sugar also extends into the minor groove of DNA to form a hydrogen bond [16]. Since the amine-linked conjugates were bound through the sugar group, the binding with DNA was weaker in nature. This explained the significant difference in binding seen in Fig. 6.

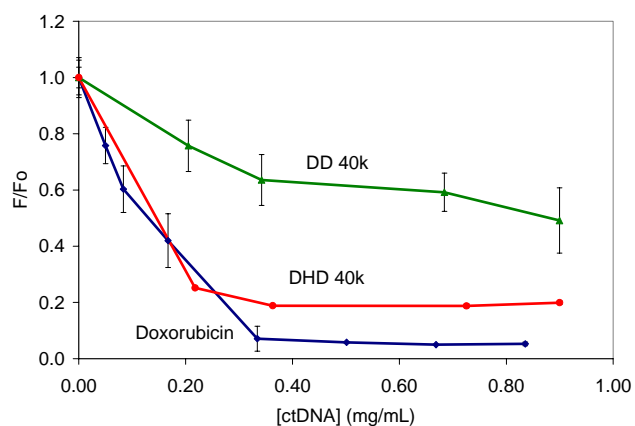


Fig. 6. Fluorescence quenching of acid-labile conjugate vs. amine-linked conjugate.

### 2) pH-Responsive Release of Doxorubicin

Fig. 7 shows the pH-sensitive release of doxorubicin from DHD conjugates. At the endosomal pH of 5, the rate of release was much greater than that at a physiological pH of 7.4, indicating that this material would release the active drug inside the endosome. There was some release from the conjugate at pH 7.4 as well, though the percentage released was significantly less than that at pH 5.0.

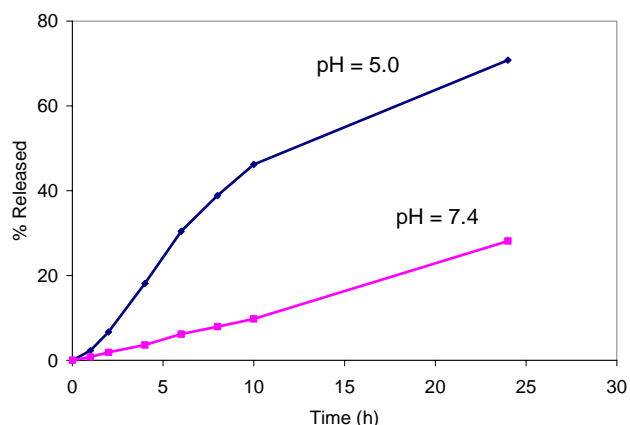


Fig. 7. Release of doxorubicin from DHD conjugate (MW 10,000) in buffers of different pH's.

## C. Cell Culture Studies

### 1) Doxorubicin Uptake

All cells have a basal level of fluorescence associated with them. For the cytotoxicity studies, flow cytometry was used to determine the excess fluorescence of cells incubated with either doxorubicin or conjugates. Fig. 8 shows the ratio of the fluorescence of cells incubated with doxorubicin or conjugates to the fluorescence of control cells. BNL CL.2 cells were incubated in increasing concentrations of various conjugates. Figure 8 shows that the uptake of free doxorubicin was much higher than the uptake of the conjugates, which reached a plateau at a relatively low ratio. Therefore, we would expect the conjugates to be less toxic than doxorubicin. The DDG conjugate, which was targeted to hepatocytes by galactose,

was taken up more than the non-targeted DD conjugate. The acid-labile DHD conjugate also experienced a higher uptake than the DD conjugate.

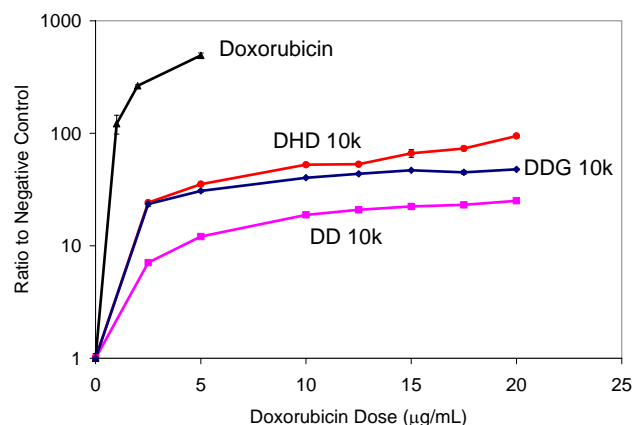


Fig. 8. Doxorubicin-associated fluorescence of hepatocytes incubated with doxorubicin and conjugates.

### 2) Cytotoxicity Studies on the BNL CL.2 Cell Line

Fig. 9 shows the dose response curve of doxorubicin and two conjugates. The  $LC_{50}$  of several conjugates are listed in Table 1. The amine-linked, non-targeted conjugate (DD) required 20 times the dose of free doxorubicin to achieve 50% cell death ( $LC_{50}$  of 19.81  $\mu\text{g/mL}$  compared to 0.97  $\mu\text{g/mL}$ ). It was much less toxic than free doxorubicin for a number of reasons. There was less uptake of doxorubicin in the polymer-bound form as shown in Fig. 8. Furthermore, the polymer-conjugated doxorubicin that was taken up did not bind as strongly to DNA as free doxorubicin (Fig. 5).

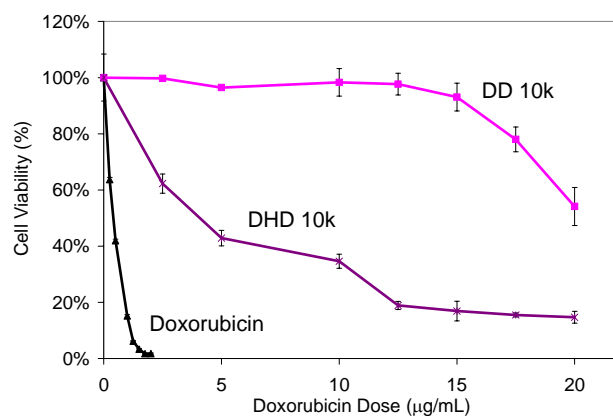


Fig. 9. Dose response curve of doxorubicin and polymer conjugates in BNL CL.2 cells.

Conjugates that were targeted to hepatocytes with galactose were significantly more toxic than the non-targeted DD conjugate. The  $LC_{50}$  values of two different types of targeted conjugates were shown in Table 1. The toxicity of the DDG conjugates increased with an increase in the degree of galactose substitution from 3.69% to 5.62%.



TABLE 1  
LC<sub>50</sub> of Doxorubicin and Various Conjugates.

Cell Line	Conjugate	LC <sub>50</sub> (μg/mL)
BNL CL.2	Doxorubicin	0.97
	DD	19.81
	DDG 3.69*	7.33
	DDG 5.62*	5.86
	DHD	4.39
Hep G2	Doxorubicin	8.60
	DD	36.02

\*Degree of substitution of galactose.

Fig. 10 shows the fluorescence micrographs of hepatocytes incubated with doxorubicin (left) and DDG 5.62 (right) for 8 h. The free doxorubicin was localized almost exclusively in the nuclei of cells. In contrast, the polymer conjugate was found in the entire cell, with localization occurring mostly outside the nucleus. This was another reason that the polymer conjugates were less toxic than the free doxorubicin.

For the pH-sensitive DHD conjugate, the LC<sub>50</sub> was 4.39 μg/mL, which was over 75% lower than that of the DD conjugate (Table 1), and comparable to the DDG conjugates.

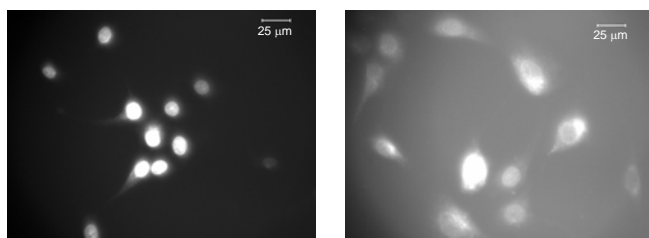


Fig. 10. BNL CL.2 cells incubated with doxorubicin (left) and DDG 5.62 (MW 10,000) (right) for 8 h at a doxorubicin concentration of 2 μg/mL.

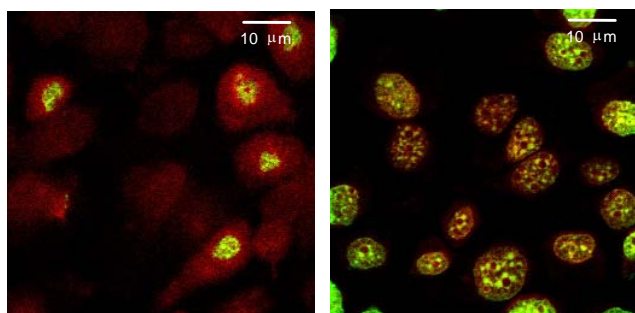


Fig. 11. BNL CL.2 cells incubated with DD (MW 10,000) (left) and DHD (MW 10,000) (right) for 4 h at a doxorubicin concentration of 2 μg/mL. The nuclei were counterstained with SYBR Green.

Fig. 11 shows the confocal laser scanning microscopy images of BNL CL.2 cells incubated with DD (left) and DHD (right) with the nuclei counterstained with SYBR Green. Yellow indicated the co-localization of doxorubicin (red) and nucleus (green). This micrograph confirmed our previous observation that the amine-linked conjugates did not enter the nucleus. It also showed that the doxorubicin on the DHD conjugates entered the nuclei efficiently,

indicating that doxorubicin was released from the polymer in an intracellular vesicle.

### 3) Cytotoxicity Studies on the Hep G2 Cell Line

Cytotoxicity studies were also carried out on Hep G2 cells (Fig. 12), which are resistant to several drugs including doxorubicin. The LC<sub>50</sub> values for doxorubicin and a non-targeted amine-linked conjugate (DD) were 8.60 μg/mL and 36.02 μg/mL, respectively. In this case, only four times the loading of doxorubicin was required by DD for the same effect, compared to the 20 times higher doxorubicin loading required by DD for the non-resistant cell line (BNL CL.2).

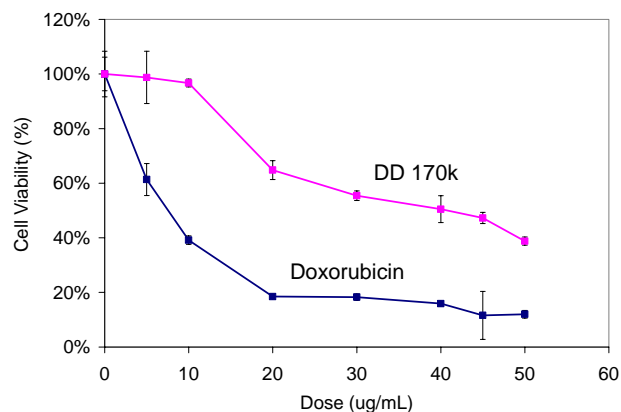


Fig. 12. Dose response curve of doxorubicin and amine-linked conjugate (MW 170,000) in Hep G2 cells.

## IV. CONCLUSIONS

We have synthesized two conjugates with two different functionalities. The dextran-doxorubicin-galactose (DDG) conjugate can successfully target hepatocytes. The targeted conjugates are significantly more effective at inducing cell death than the control non-targeted conjugates. The dextran-hydrazide-doxorubicin (DHD) conjugates released doxorubicin in the low pH endosomal compartments when taken up by the cells, and are also effective at inducing cell death. We are currently working on developing materials that would combine the two functionalities to further improve the drug delivery system.

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