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Assessing aggregation of peptide conjugate of doxorubicin using quasi-elastic light scattering and 600 MHz NMR

Shyam B. Karki*, Drazen Ostovic

Pharmaceutical Research and Development, Merck Research Laboratories, Merck & Co., Inc., P.O. Box 4, West Point, PA 19486, USA

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Abstract

The use of doxorubicin in treating prostate cancer is limited by its systemic toxicities especially cardiotoxicity and immunosuppression. Prodrugs that reduce the systemic exposure of doxorubicin are believed to provide a safety advantage. A prodrug of doxorubicin which contains a peptide sequence that can be recognized by prostate-specific antigen (PSA) and cleaved in the prostate was formulated for clinical use. The IV formulation and manufacture of this peptide conjugate posed several challenges. The main issue of the IV formulation were chemical and physical stability. The physical stability challenges posed during formulation and manufacture of his peptide conjugate is described herein. A heptapeptide conjugate of doxorubicin was found to aggregate in solution forming large ill defined aggregates (60–1300 nm). In contrast to doxorubicin, the average hydrodynamic diameter measured for this compound by dynamic laser light scattering technique is very large. Increasing concentration of the drug and lowering pH promoted aggregation. We rationalize the difference in the effective hydrodynamic diameter due to hydrogen bonding of the peptide which allows for the formation of large particle sizes relative to doxorubicin. We have also used 600 MHz ^{1}H NMR to assess the aggregation of this compound.

Keywords: Aggregation; Doxorubicin; Hydrodynamic diameter

1. Introduction

The biological rationale for the selection of PSA cleavable peptide linked to doxorubicin has been described previously by DeFeo-Jones et al. (2000). The synthesis and medicinal chemistry attempts to prepare PSA selective peptide conjugates of doxorubicin has also been recently published (Garsky et al., 2001). Prostate cancer is one of the leading causes of cancer mortality in men (Landis et al., 1999). The current therapy has limited effectiveness in prolonging life for men with androgen refractory prostate cancer (Husain et al., 1996). The current therapy for prostate cancer includes a combination of mitoxantrone and prednisone and provides limited (20–30%) response (Beedassy and Cardi, 1999). Doxorubicin peptide conjugate (Fig. 1) is a cytotoxic agent targeted for the treatment of refractory prostate cancer. This peptide conjugate is a prostate targeted prodrug that is itself non-toxic until activated by proteolytic cleavage to doxorubicin. The prostate-specific antigen (PSA) is the protease that cleaves the molecule and releases doxorubicin at the local site. The cytotoxic agent doxorubicin is a well known anthracycline antibiotic which has been extensively reviewed previously (Arcamone, 1978).

* Corresponding author. Tel.: +1-215-652-2443; fax: +1-215-993-3730.
E-mail address: shyam_karki@merck.com (S.B. Karki).

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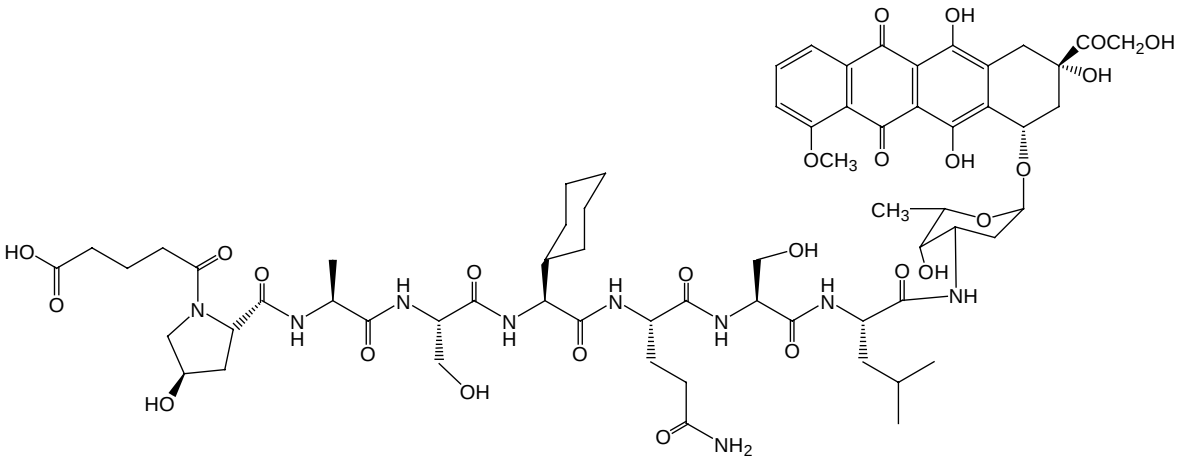

Fig. 1. Doxorubicin peptide conjugate.

In the parenteral (IV) formulation development of this doxorubicin peptide conjugates it was observed that the compound formed large aggregates in solution. Our primary interest is to understand the self-association properties of the peptide conjugate so improvements in the formulation can be made to limit the problems associated with physical instability. We have used quasi-elastic light scattering measurements (QELS) to measure the effective hydrodynamic diameter of aggregates formed in solution under a variety of conditions. Large aggregates (60–1300 nm) were observed by QELS when the doxorubicin peptide conjugate is solubilized in water. The size of these aggregates depends on the concentration of the drug, pH and salts present in the formulation. Since ^{1}H NMR has previously been used to study the aggregation of doxorubicin we have also used this technique to gain insight into the nature of these aggregates formed in water. A downfield shift of the aromatic proton resonances is observed as the concentration of doxorubicin peptide conjugate is increased. In the case of doxorubicin, the downfield shift observed for the aromatic proton resonances has been used to support a mechanism of aggregation involving π stacking of the anthracycline rings. Although we observe a downfield shift of the proton resonances of the aromatic region of doxorubicin by ^{1}H NMR, the particle sizes measured by QELS for these aggregates are too large to be simple π stacked dimers or trimers. The large aggregates in addition to π, π stacking of the anthracycline rings may include extended hydrogen bonded peptide aggregates.

2. Experimental section

2.1. Quasi-elastic light scattering procedure

QELS experiments were performed with a BI 200 SM Goniometer (Brookhaven Instrument) equipped with a 10 mW HeNe laser from Melles Griot. The laser aperture was 400 μm and the detection angle was varied depending on the inherent scattering intensity of the samples. The instrumental description and experimental set-up has been previously described (Feng and Schelly, 1995). Solutions of the doxorubicin peptide conjugates were filtered through a 0.22 μm filter after preparation and allowed to equilibrate at room temperature for 2 h prior to collecting data.

The QELS data were analyzed by cumulants method. The translational diffusion coefficients were obtained from the intensity auto-correlation function, and the hydrodynamic diameter (D_h) of the aggregates was calculated through the Stokes–Einstein equation. Since diffusing particulates interact with each other at elevated solute concentrations, interpreting light scattering spectra of concentrated solutions involves substantial complications. Therefore, the hydrodynamic diameter obtained from QELS can only be considered as the apparent particle size

(Feng and Schelly, 1995; Phillies, 1990; Filella et al., 1997).

2.2. Using ^1H NMR to assess aggregation

^1H NMR experiments (600 MHz) were conducted to assess the aggregation of doxorubicin peptide conjugate in water. The proton chemical shift of the aromatic region of the peptide conjugate was used to assess the degree of aggregation. The use of chemical shift of the aromatic protons has been used to postulate π stacking of daunomycin (Chaires et al., 1982).

3. Results and discussion

3.1. Measurement of hydrodynamic diameter by QELS

The IV formulation of this doxorubicin peptide conjugate posed many challenges. One of the primary issue was the formation of large aggregates which made sterile filtration impossible. Due to the chemical instability of this doxorubicin conjugate, it was unlikely that a formulation could be terminally sterilized. It was therefore imperative that we understand and minimize aggregation of this molecule to facilitate sterilization by filtration. Uncontrolled particle size growth of our IV product during manufacture would hinder our ability to adequately sterilize via sterile filtration. Since this is a derivative of a cytotoxic agent any leaks during filtration endangers the workers involved in manufacturing this formulation. The large aggregates of this peptide conjugate is trapped by the filter and causes the lines to rupture placing operators at risk of being exposed. This challenge was made worse by the need to adequately protect the compound from chemical instability as well.

In order to minimize processing and chemical stability issues there was a need to understand the nature of the aggregates being formed. The first question that was addressed was the apparent size of the aggregates that were being formed. QELS experiments were conducted to measure the hydrodynamic diameter of the aggregates under various conditions. Table 1 shows the hydrodynamic diameter (D_h) of aggregates of the peptide conjugate measured by QELS at a fixed pH while increasing the concentration of the conjugate.

Table 1
The effect of concentration on hydrodynamic diameter (D_h)

Concentration (mg/ml)	pH	D_h (nm)
5	4.3	61.1
10	4.3	140
20	4.3	219
40	4.3	767
60	4.3	855

Note. All pH adjustments were done with 0.1N NaOH.

The data indicates that the apparent hydrodynamic diameter of the aggregates increases as the concentration of the doxorubicin peptide conjugate increases. The pH of the solution was adjusted by sodium hydroxide solution due to the fact that the doxorubicin peptide conjugate has a terminal carboxylic acid. Due to day-to-day variability all the solutions were prepared for this study, filtered to remove dust and other insolubles and allowed to equilibrate for 2 h prior to determination of D_h by QELS.

The hydrodynamic diameters measured for this doxorubicin peptide conjugate is very large (60–1300 nm). The apparent particle size is too large to be a simple dimer or a trimer. The self-association behavior of anthracycline antibiotics such as doxorubicin has been reported previously in the literature (Eksborg, 1978; Chaires et al., 1982; Menozzi et al., 1984; McLennan et al., 1985; Hayakawa et al., 1991). Chaires pointed out that the ^1H NMR data for daunomycin fit an infinite association model better than a simple dimerization model. The apparent particle sizes being measured here for the peptide conjugate is more in line with an infinite association model. We do observe the apparent particle size grow with an increase in concentration of the peptide conjugate at a fixed concentration.

The apparent size of the aggregates were also found to depend on the pH of the solution. Table 2 lists the hydrodynamic diameter as a function of pH for a fixed concentration of doxorubicin peptide conjugate.

Table 2
The effect of pH on hydrodynamic diameter (D_h)

Concentration (mg/ml)	pH	D_h (nm)
20	3.45	1386
20	5.04	323.5
20	5.73	61.3
20	6.30	44.5

Note. All pH adjustments were done with 0.1N NaOH.

Table 3
The effect of ionic strength on D_h

Concentration (mg/ml)	Sodium chloride (M)	pH	D_h (nm)
40	0.05	5.6	4.9
40	0.15	5.6	7.3
40	0.30	5.6	7.7
40	0.50	5.6	9.1

Note. All pH adjustments were done with 0.1N NaOH.

The data suggests that lowering the pH of the solution (i.e., acidifying the solution) allows the aggregates to get larger. One explanation consistent with this data is that the ionized form does not form large extended aggregates. The repulsive forces of the ionized drug can be one reason for the particles size to be reduced at pH greater then the pK_a of this compound. The pK_a of this compound was determined titrimetrically to be 4.8. Protonating the compound allows for extended H-bonding to take place and aggregates through the peptide portion of the molecule. Since pH is being adjusted with NaOH there is some differences in salt concentrations in the solutions so it was important to check for the effect of ionic strength on the size of aggregates. Table 3 shows the hydrodynamic diameter of a 40 mg/ml solution of the doxorubicin peptide conjugate at a fixed pH with varying amounts of sodium chloride added to the formulation. The data in Table 3 shows that the particle size does not increase significantly as the ionic strength is increased. It is worth noting that particle size is significantly reduced in the presence of added ions as can be seen from row 1/Table 3 when compared to row 3/Table 2. In the absence of sodium chloride the particle size is larger even at a lower concentration of drug. The salts probably breakup the hydrogen bonding interactions between the peptide chains of the doxorubicin peptide conjugate and reduce the overall particle size of the aggregates.

To completely evaluate all the formulation components the effect of buffer concentration on the particle size was also evaluated. The ionic strength was kept high at 0.5 M to minimize the effect of increased buffer concentrations on the ionic effect. At 0.5 M NaCl concentration no change in effective particle size was noted by QELS. The data in Table 4 show that at constant ionic strength (0.5 M NaCl), no reduction is observed in particle size as the buffer concentration is increased.

Table 4
The effect of buffer concentration at constant ionic strength (0.5 M NaCl)

Concentration (mg/ml)	Citrate (mM)	pH	D_h (nm)
40	10	5.6	9.4
40	25	5.6	9.8
40	50	5.6	10.7
40	100	5.6	11.9

Note. All pH adjustments were performed with 0.1N NaOH.

3.2. ^1H NMR studies

A second experimental technique was used to substantiate the experimental observations with QELS. 600 MHz ^{1}H NMR experiments were conducted to assess the aggregation of the doxorubicin peptide conjugate in water. The chemical shift of the aromatic proton region of doxorubicin conjugate was used to assess the degree of aggregation. It has been postulated that a change in chemical shift of doxorubicin aromatic proton resonances is due to aggregation of doxorubicin through π stacking of the aromatic region (Chaires et al., 1982; McLennan et al., 1985). We have also observed a downfield chemical shift change for the aromatic proton resonances of the doxorubicin peptide conjugate both as a function of concentration and pH. Even at dilute doxorubicin peptide conjugate concentrations (ca. 0.17 mg/ml) the molecule is aggregated in water based on the linewidth of the proton resonance in D_2O. From a formulation standpoint the fact that the particle size depends on the pH of the solution for a given concentration is important because this gives a formulator handle in reducing particles.

Fig. 2 shows the aromatic region of the ^{1}H NMR of the doxorubicin peptide conjugate. As the concentration of the drug increases the proton chemical shifts of the aromatic region shifts downfield. This kind of shifts have been observed for daunomycin (Chaires et al., 1982). Another noteworthy observation is the significant broadening of the linewidths of the proton resonances as the concentration of the drug increases. This provides further evidence for the aggregation of this molecule (and the interaction of anthracycline rings in the aggregates formed) (McLennan, 1985). Couple of the downfield resonances coalesce into one peak as the concentration is increased. The downfield shift in the proton resonance of H_2 proton versus log of concentration gives a straight line (Fig. 3). The data

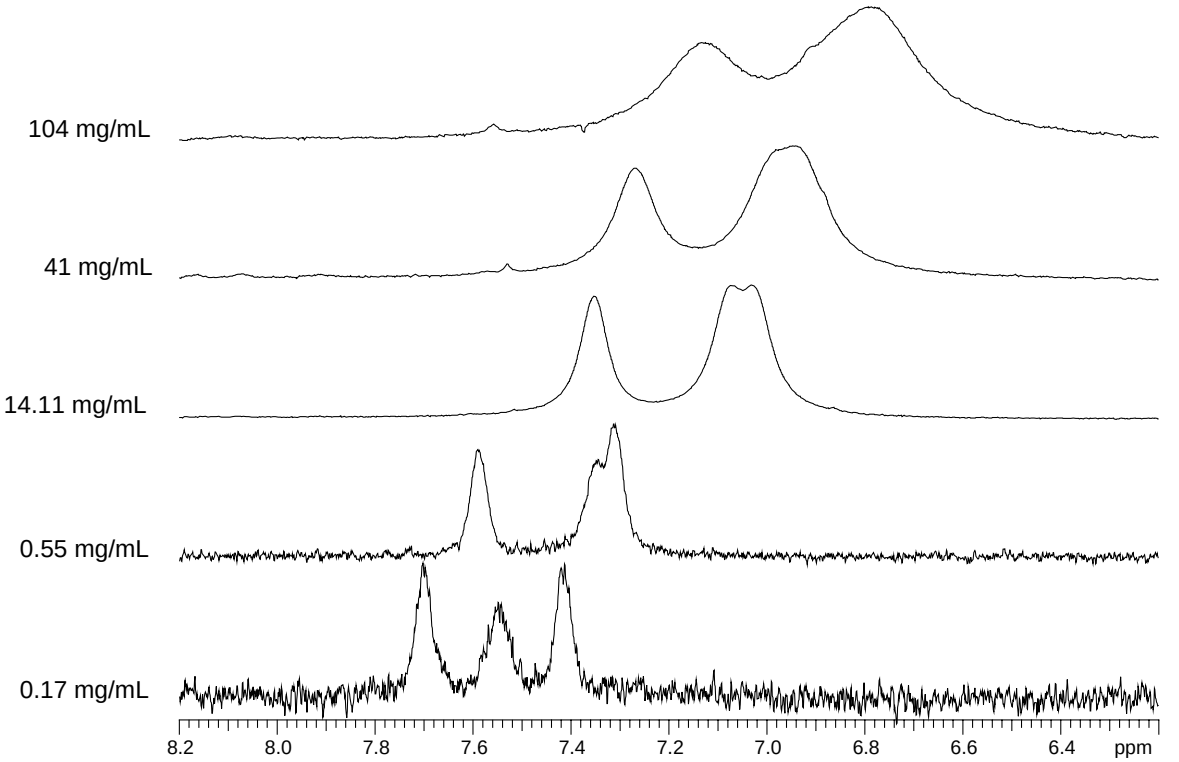

Fig. 2. Chemical shift of the aromatic protons of doxorubicin peptide conjugate vs. concentration in D_2O (600 MHz).

show no break in the line over a wide concentration range (0.17 to over 100 mg/ml) further providing support that even at low concentration of the peptide conjugate some aggregation is occurring and the particle size grows as the concentration is increased.

^1H NMR was also used to assess the effect of pH on a fixed concentration of doxorubicin peptide conjugate. Fig. 4 shows the aromatic proton resonances of the doxorubicin peptide conjugate as pH is changed. Consistent with the QELS data a downfield shift and

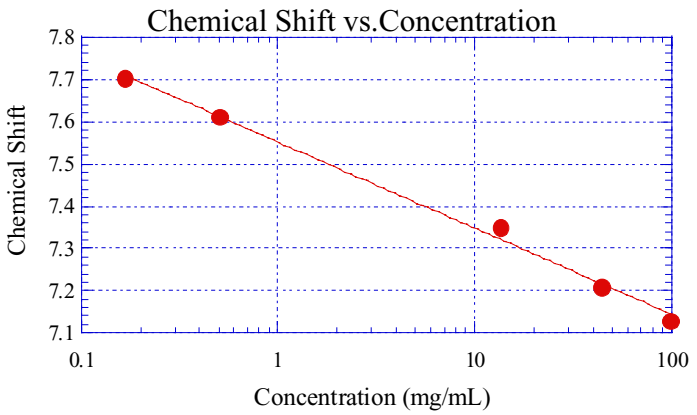

Fig. 3. Plot of chemical shift of H_2 proton vs. log of concentration of doxorubicin peptide conjugate in D_2O (600 MHz).

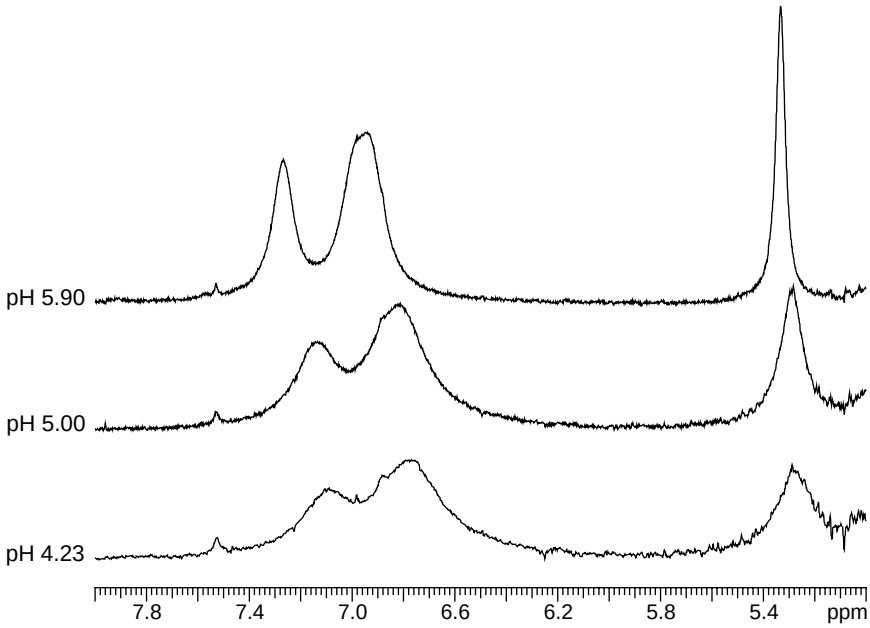

Fig. 4. Chemical shift of the aromatic protons vs. pH in D_2O.

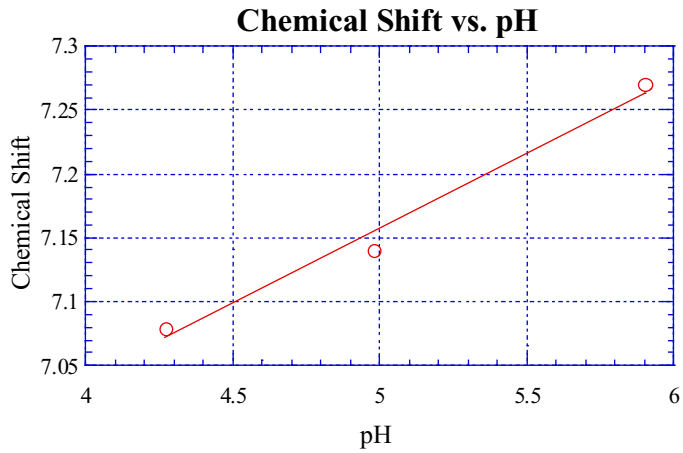

Fig. 5. Chemical shift of H_2 proton for doxorubicin peptide conjugate vs. pH at 40 mg/ml concentration.

linewidth broadening is observed as the pH is lowered from 5.9 to 4.2. Fig. 5 is a plot of the chemical chemical shift of the H_2 aromatic proton as a function of pH. A straight line indicates that the compound is aggregated at all these pH but the size depend on the ionic state of the doxorubicin peptide conjugate. The ^1H NMR data is consistent and supports the QELS data which show that the particle size can be manipulated by adjusting pH and salt concentration as well as the concentration of the doxorubicin peptide conjugate.

4. Conclusions

The QELS and ^1H NMR experiments show that doxorubicin peptide conjugate forms large aggregates

in water. The size of the aggregates are dependent on concentration, pH and presence of salts. Increasing the concentration of the doxorubicin peptide conjugate increases hydrodynamic diameter of the aggregates as does lowering the pH. Ionizing the molecule helps maintain particle size from growing. The addition of ions lowers the size of the aggregates as well. This is attributed to an increase in ionic strength. The studies have shown that large aggregates can be avoided in the formulation by increasing the native pH of the solution and adding salts to the formulation. The data suggest that the large aggregates are probably formed by H-bonding via the peptide linkages since their size is reduced substantially when ions are added (breaking H-bonding) and pH is raised to ionize the molecule. However, even at lowest concentration tested the doxorubicin peptide conjugate exists in an aggregated state. The ^{1}H NMR data clearly show that the anthracycline ring system is involved in the aggregation of this doxorubicin peptide conjugate.

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