International Journal of Pharmaceutics 367 (2009) 73–78

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Concentration dependent aggregation properties of chlorhexidine salts

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ARTICLE INFO

Article history:
Received 28 April 2008
Received in revised form 3 July 2008
Accepted 17 September 2008
Available online 25 September 2008

Keywords:
Chlorhexidine
Chlorhexidine digluconate
Chlorhexidine diacetate
Chlorhexidine dichloride
Micelle
Solubility product
Light scattering
NMR spectroscopy

ABSTRACT

Purpose: Chlorhexidine (CHX), a chemical antiseptic, is known to bind to dentin and has been shown to be effective in treating bacterial infections caused by microbes. The solubility and aggregation properties of CHX salts were determined to guide the development of a sustained release formulation for long-term disinfection.
Methods: The amount of CHX in solution was determined as a function of counterion concentration (chloride, acetate (Ac) or gluconate (G)) by UV spectrophotometry at 255 nm. The weight average molecular weight was determined from the angular dependence of the scattered light. Proton NMR spectroscopy was used to investigate the dependence of the peak intensity and chemical shift on solution concentration and diffusion measurements were performed by Fourier-transform pulsed-field gradient spin-echo (PFG-SE) ^{1}H NMR.
Results: The observed CHX concentration was highly dependent on the type and concentration of salt present in solution with the greatest CHX concentration achieved with gluconate, moderate to low with diacetate, and very low with dichloride solutions. Addition of sodium gluconate enhanced the amount of CHX-Ac_2 in solution; however, only low concentrations of chlorhexidine can be achieved in the presence of chloride ions. For solutions of CHX-G_2, the aggregate number appeared to range from a dimer at 40 mM to perhaps a pentamer at 150 mM. In contrast, no aggregation of CHX-Cl_2 or CHX-Ac_2 was detected, which was corroborated by diffusion NMR results. The change in chemical shift of protons is consistent with association of the phenyl group of one CHX with the hexamethylene chain of a second CHX. Based on the analysis of NMR peak intensities of CHX, gluconate, and acetate in saturated solutions, it appears that solubilization of the diacetate species occurs within digluconate aggregates, since the solubility product of chlorhexidine diacetate is such that the concentration of CHX will exceed the critical micelle concentration (CMC). However, no solubilization of CHX-Cl_2 occurs because the solubility product falls below the CMC.
Conclusions: The low concentration of CHX that can be achieved in physiological concentrations of chloride in the oral cavity may be problematic for dental and slow release formulations. Achieving a high concentration of CHX appears to require that the monomer be present at a concentration greater than that required to produce self-association.

1. Introduction

Dental infections remain a concern with all orthodontic procedures. One specific example is the re-emergence of bacteria following a root canal procedure, which involves drilling a small hole into the affected tooth (Wu et al., 2006; Zehnder, 2006). This hole is permanently sealed after 2–4 weeks, during which time microorganisms can invade the tooth. Chlorhexidine (CHX, Fig. 1) is an antimicrobial agent that is used in endodontic therapy, and a 2% chlorhexidine aqueous solution has been shown to be effective in treating root canal infections (Dolby et al., 1972; Nerurkar et al., 1995). However, development of a delivery system that maintains the concentration of chlorhexidine for the entire 4-week period would further reduce the incidence of dental infections.

A number of controlled release formulations have been developed to provide for the slow release of CHX (Lee et al., 2005; Leung et al., 2005). While potentially effective, approaches that rely on the release of CHX from the formulation into the pulp chamber may not achieve a sufficiently high concentration for antimicrobial activity due to the interaction of CHX with physiological ions in the oral cavity. Specifically, a saturated solution of chlorhexidine diacetate (CHX-Ac_2) yields a concentration of 2%, but the corresponding dihydrochloride (CHX-Cl_2) has a concentration of only 0.2%. The release rate of CHX from polymeric formulations was also found to

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doi:10.1016/j.ijpharm.2008.09.031

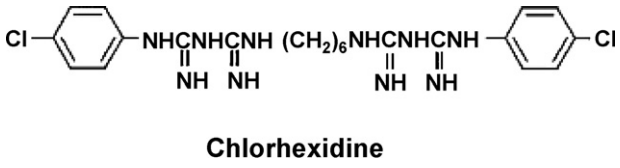

Fig. 1. Chemical structure of chlorhexidine.

be dependent on chloride ion concentration in the medium (Leach, 1979). CHX-Cl_2 has been observed to precipitate in the oral cavity when a solution of sodium hypochloride (NaOCl) is administered following CHX-G_2 treatment (Basrani et al., 2007). Thus, not only is the release rate affected by the chloride ion but released CHX can also be expected to precipitate rapidly in the oral cavity in manner consistent with the low solubility product.

In addition to the complex ionic equilibria, CHX was shown to undergo self-association by surface tension and dye solubilization measurements (Heard and Ashworth, 1968). Aside from estimating the concentration for the onset of association at 7 and 11 mM for the diacetate and digluconate solutions, respectively, little is known about the properties of the aggregate. The self-association of digluconate CHX no doubt contributes to the extremely high concentrations (water solubility > 70%, w/v) that can be achieved, where an aqueous solution with a concentration of 20% (w/v) is commercially available. A better understanding of the ionic equilibria as well as the self-association behavior would be helpful in designing sustained release delivery systems for the prevention of dental infections.

Previously, the amount of chlorhexidine in solution as dichloride and diacetate mixtures with chlorhexidine digluconate was determined (Zeng et al., 2008). The limitation of these studies was that there was limited assessment of the extent of association. In addition, only the concentration of CHX was determined, and the concentrations of acetate and gluconate that remained in solution were not measured. In this study, light scattering and NMR spectroscopy were used to characterize the concentration dependent aggregation process. Measurements were also made of both the pulsed-field gradient diffusion and the change in chemical shift as a function of CHX and counterion concentration. The results from assessing the aggregation and solubilization process provide a quantitative basis for the development of appropriate sustained release formulations of CHX.

2. Theory

Chlorhexidine, 1,1′-hexamethylene-bis-5-(4-chlorophenyl)biguanidide, is a symmetric molecule with two ionizable guanidide moieties. The pK_a values are 2.2 and 10.3, thus making it dicationic over the entire range of physiological pH values (Lee et al., 2005). For the three commonly used salts, digluconate (diG), diacetate (diAc), and dihydrochloride, the solubility products may be written as

$$K_{sp}(\text{CHX-G}_2) = K_1K_2 = [\text{CHX}^{2+}][\text{G}^-]^2 \quad (1)$$

$$K_{sp}(\text{CHX-Ac}_2) = K_3K_4 = [\text{CHX}^{2+}][\text{Ac}^-]^2 \quad (2)$$

$$K_{sp}(\text{CHX-Cl}_2) = K_5K_6 = [\text{CHX}^{2+}][\text{Cl}^-]^2 \quad (3)$$

where the n and $n+1$ equilibrium constants represent the dissolution of the first and second ion. The reported K_{sp} of the dihydrochloride salt (2.1×10^{-8} M) is about 20-fold lower than the diacetate (2×10^{-4} M) at 37 °C (Lee et al., 2005).

It has been reported that chlorhexidine also forms aggregates in solution similar to the self-association of planar dyes (Heard and Ashworth, 1968). In this type of aggregation, a mass action model often is used to describe the distribution of aggregates where X refers to the monovalent anion

$$2\text{CHX-X}_2 \Leftrightarrow (\text{CHX-X}_2)_2 \quad (4)$$

$$\text{CHX-X}_2 + (\text{CHX-X}_2)_2 \Leftrightarrow (\text{CHX-X}_2)_3 \quad (5)$$

which can be written in general for the nth aggregate combining with CHX to form the $n+1$ aggregate as follows:

$$\text{CHX-X}_2 + (\text{CHX-X}_2)_n \Leftrightarrow (\text{CHX-X}_2)_{n+1} \quad (6)$$

each expression has an equilibrium association constant, K_{assoc}

$$K_{assoc} = \frac{[(\text{CHX-X}_2)_{n+1}]}{[\text{CHX-X}_2][(\text{CHX-X}_2)_n]} \quad (7)$$

In the isodesmic model of self-association, the association constants are assumed to be equal, and the distribution function, $F(n)$, of aggregates can be expressed as

$$F(n) = \frac{[(\text{CHX-X}_2)_n]}{\sum_i [(\text{CHX-X}_2)_i]} \quad (8)$$

3. Materials and methods

Analytical/reagent grade chlorhexidine dihydrochloride, chlorhexidine digluconate (20%, w/v), sodium gluconate and calcium gluconate were purchased from Sigma Chemical (St. Louis, MO) and used as-received. Chlorhexidine diacetate hydrate was purchased from Acros Organics and sodium acetate was purchased from Fisher Biosciences.

3.1. Static light scattering

The weight average molecular weight was determined by measuring the angular dependence of the scattered light using a Dawn EOS particle light system (Wyatt Technology Corp.). The instrument was calibrated with polystyrene in toluene and by dextran in water. A series of solutions of CHX-Ac_2 and CHX-G_2 were prepared and repeatedly filtered through a 0.02-μm filter until stable light scattering intensities were obtained. Following measurement of the scattered light, the concentration was determined by measuring the absorbance of the solution at 255 nm. The angular dependence of the scattered light was analyzed with the accompanying software, which used the Debye model of a randomly coiled polymer to infer the weight average molecular weight.

3.2. UV and NMR spectroscopies

Chlorhexidine digluconate was initially frozen and then dried over night under high vacuum. It was then reconstituted with D_2O to yield one series of concentrations. In addition, a second series of solutions were prepared by placing excess solid CHX-Ac_2 in D_2O and adding variable volumes of a sodium gluconate solution. In a similar manner, a third series was prepared by dissolving the dried CHX-G_2 in D_2O and adding variable volumes of a sodium acetate solution. Following equilibration, the latter two series consisting of suspensions were centrifuged, and the supernatant was transferred to thin-wall 5-mm NMR tubes. In addition, aliquots were also taken, and the absorbance at 255 and 231 nm was measured from which the concentration of CHX in solution was determined from appropriate standard curves.

The ^{1}H spectra were acquired with a Varian 600 MHz spectrometer following a single 90° pulse using a 2-s relaxation delay. The chemical shift was measured, using the residue HDO peak as reference (4.76 ppm), and the peak area was determined by integrating

the area under the curve. The Fourier-transform pulsed-field gradient spin-echo (PFG-SE) ^{1}H NMR diffusion measurements were performed on non-spinning samples. The sample temperature was controlled at 25 ± 0.2 °C. A stimulated spin echo pulse sequence was used, and the transformed intensity was analyzed by the following equation:

$$A(\tau_1 + \tau_2) = \left(\frac{1}{2}\right) A(0) \ \exp\left(\frac{-2\tau_1}{T_2}\right) \exp\left[\frac{-(\tau_2 - \tau_1)}{T_2}\right] \quad (9)$$

where $A(\tau_1 + \tau_2)$ is the peak intensity at time, $\tau_1 + \tau_2$, $A(0)$ is the peak intensity at time 0, T_2 is the spin–spin relaxation time, T_1 is the spin–lattice relaxation time, γ is the gyromagnetic ratio, G is the strength of field gradient, δ is the duration of field gradient, D is the diffusion coefficient and Δ is the time interval between the first and second gradient pulses. The diffusion experiments were performed at constant δ and Δ values, using a series of ten G values. To obtain absolute values for the self-diffusion coefficients, the field gradient strength was calibrated from measurements of reference 1% and 10% H_2O in D_2O.

The results were analyzed using a two-state model for the observed diffusion coefficient of chlorhexidine, D_{obs},

$$D_{obs} = f_i D_i + f_m D_m \quad (10)$$

where f_i and D_i are the fraction and diffusion coefficients of chlorhexidine as monomer, and f_m and D_m are the fraction and diffusion coefficients of chlorhexidine in the aggregated form. The fractions may be written in terms of the total chlorhexidine concentration, $[CHX]_t$, and monomer concentration, cmc, as follows:

$$D_{obs} = \left(\frac{cmc}{[CHX]_t}\right) D_i + \left(1 - \left(\frac{cmc}{[CHX]_t}\right)\right) D_m \quad (11)$$

This may be rearranged as

$$D_{obs}[CHX]_t = cmc(D_i - D_m) + D_m[CHX]_t \quad (12)$$

so that a plot of the product of the observed diffusion coefficient and total chlorhexidine concentration as a function of the total chlorhexidine concentration will yield a straight line with a slope equal to the diffusion coefficient of the aggregate. A viscosity, η, of 1 cP was assumed to allow calculation of the hydrodynamic radius, R_h, from the inferred diffusion coefficients of the aggregates using the Stokes–Einstein equation

$$R_h = \frac{kT}{6\pi\eta D_m} \quad (13)$$

where k is Boltzmann's constant, and T is the absolute temperature (K).

4. Results and discussion

The results from the light scattering measurements are shown in Fig. 2 where the molecular weight is given as a function of CHX-G_2 concentration. It appears that there is a continual increase in the molecular weight of the aggregate as the concentration is increased from about 40 mM to nearly 150 mM. Given that the molecular weight of CHX-G_2 is 675 Da, the aggregation size ranges from apparently a dimer at low concentration to perhaps a pentamer at 150 mM. This relatively small aggregation number and its change with concentration are consistent with the earlier suggestion that CHX associates like planar dyes (Heard and Ashworth, 1968).

Because of the low solubility, the concentration of CHX-Ac_2 was limited to less than 30 mM. In this case, no aggregation was detected. This differs from the surface tension and dye solubilization studies of Heard and Ashworth, who indicated that CHX-Ac_2 undergoes self-association (Heard and Ashworth, 1968). The use

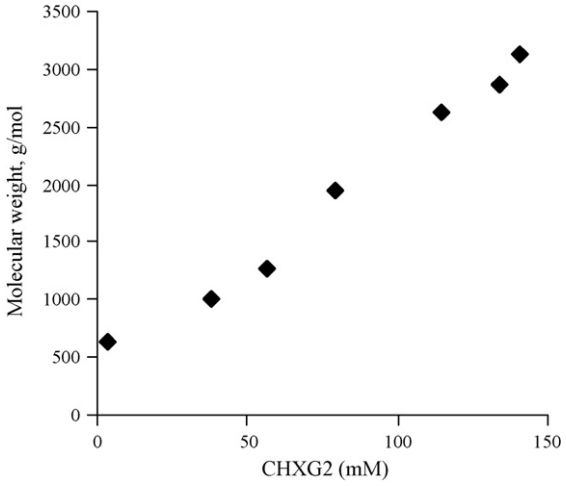

Fig. 2. Weight average molecular weight of chlorhexidine digluconate as a function of concentration.

of dye solubilization can induce aggregation that would not occur with a pure compound as used in these light scattering measurements. However, surface tension measurements are free from this potential misinterpretation, and thus at this point, the discrepancy remains unresolved.

NMR spectroscopy was used to investigate the aggregation process of the gluconate solutions. Because of the symmetric nature of the molecule, there are only five unique peaks in the reported proton spectrum of CHX (Kalachandra et al., 2005). Two peaks near 7 ppm arise from the four protons on the para-chlorophenyl group, and the three remaining peaks at about 2.8, 1.3, and 1.1 ppm from the 12 protons on the symmetric hexamethylene chain in the center of the molecule. The protons associated with the biguanidine group exchange with the D_2O and are not observed.

In Fig. 3, the observed decrease in the chemical shift of CHX is given as a function of concentration. It should be noted that no change in the chemical shift in the gluconate resonances was observed. The decrease in chemical shift is expected to arise from self-association that brings the protons into close proximity with the phenyl ring, which reduces the effective field causing an upfield

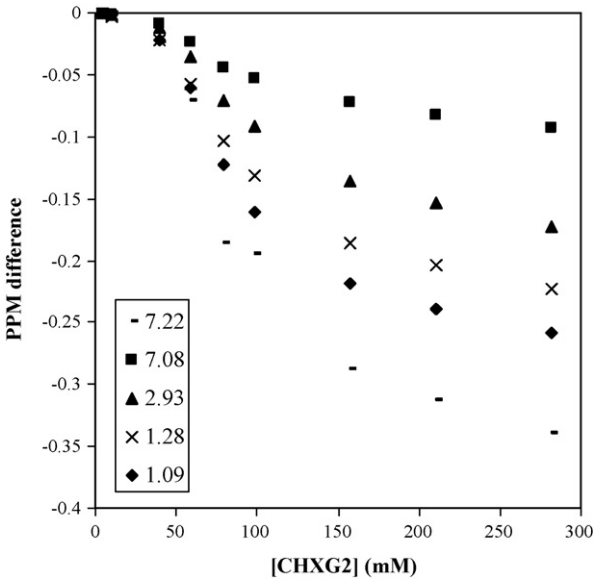

Fig. 3. The change in chemical shift of CHX-G_2 given as a function of concentration.

Table 1
The observed supernatant concentrations of CHX, Ac, and G determined by NMR peak integration or UV spectroscopy for a series of $CHXAc_2$ suspensions to which NaG was added (series 2).

NMR	UV	NMR	
[CHX] (mM)	[CHX] (mM)	[Acetate] (mM)	[Gluconate] (mM)
30.4	24.9	49.8	0
32.7	37.2	54.6	19.5
34.9	42.6	65.0	82.5
37.8	45.9	74.2	121
40.8	66.3	82.3	157
44.3	80.5	91.1	200

shift in the observed chemical shift. One may have expected that the phenyl groups on CHX would stack as seen with dyes, and thus these protons would be most greatly affected. However, two of the protons on the phenyl group exhibited the maximum and two exhibited the minimum change in the chemical shift.

CHX has a linear structure with a hydrophobic ring at each end of the molecule, and the two relatively hydrophilic biguanidide groups separate the central hydrophobic hexamethylene chain from the phenyl rings. As such, simple stacking of the CHX to bring the phenyl groups into contact may not minimize the exposure of the hydrophobic groups with water and optimize the intermolecular association. Thus, the aggregation may involve association of the phenyl group of one CHX with the hexamethylene chain of a second CHX and thereby form of a staggered chain. Such an arrangement would be consistent with the larger changes in the chemical shifts observed with the methylene chain. For the polar, negatively charged gluconate molecules, these can be expected to be near the positively charged biguanidide groups and freely exchange with the water such that no change in chemical shift is noted.

To examine the effect of acetate and gluconate concentrations on the concentration of CHX in solution, three series of mixtures were combined. The first solution consisted of CHX-G_2 that ranged in concentration from 10 to 300 mM. In the second series, the initial suspension concentration of CHX-Ac_2 was 100 mM, and sodium gluconate was added to achieve a final total concentration of NaG that ranged between 0 and 200 mM. In the third series, the initial concentration of CHX-G_2 was constant at 100 mM, and a solution of sodium acetate was added to yield a final total concentration of NaAc that ranged between 0 and 200 mM. All of the samples in the second and third series were suspensions at equilibrium, presumably due to the precipitation of CHX-Ac_2.

The concentration of CHX in the supernatant was determined by UV and NMR spectroscopies in the supernatant of the second and third series, and the results are given in Table 1. As can be seen from the data from the second series, the concentration of CHX in solution increased with increasing gluconate concentration. The concentration of acetate also increased and was numerically twice as great (based NMR measurements) as CHX consistent with the dissolution of CHX-Ac_2. There is a discrepancy between the concentration of CHX determined by UV and NMR spectroscopies. The lack of correspondence may arise from an underestimation of the peak area due to peak broadening from relaxation/diffusion effects (Wiedmann et al., 2001). This type of problem would require that the acetate to be associated with a motional, slower species, such as a micelle. In addition, there may be changes in the absorptivity of CHX secondary to aggregation/counterion effects.

For the third series, the addition of acetate led to a reduction in the CHX concentration in solution. The concentration of gluconate remained relatively constant indicating that the reduction in CHX was the result of CHX-Ac_2 precipitation (Table 2). With this series, the agreement between the UV- and NMR-determined concentrations of CHX was within the expected variability of the measurement. However, the lack of correspondence cited above for series 2 occurred at higher concentrations than those used in this series.

For series 2 and 3, the diffusion coefficients of CHX, Ac and G were estimated in each sample. The observed values relative to the diffusion coefficient of the monomers were plotted as a function of CHX concentration in solution (Fig. 4a–c). To calculate the relative values, the measured diffusion coefficients of CHX, gluconate, and acetate as solutions of the gluconate or sodium salts were used, which were 4.29×10^{-6}, 6.99×10^{-6}, and 1.361×10^{-5} cm^2/s, respectively. The diffusivity of gluconate as a salt of CHX was slightly lower than that as a sodium salt with a value of 6.0×10^{-6} cm^2/s. This suggests that CHX is not completely dissociated from gluconate at a low concentration of 10 mM. The diffusion coefficients were also measured for a 0.5%, 1% and 2% CHX-Ac_2 solution, and the values were 5.55×10^{-6}, 5.36×10^{-6}, and 5.21×10^{-6} cm^2/s. These falling values with concentration may indicate some degree of aggregation.

In Fig. 4a–c, the relative diffusion coefficients of each species, CHX, Ac, and G, decreased as the concentration of CHX increased. At a concentration near 300 mM, the diffusivity of CHX fell to a value just 30% of that observed at low concentration. The reduction in diffusivity of acetate was even more dramatic, falling to 10% of that observed for NaAc at a concentration of CHX of 100 mM. Finally, the diffusion coefficient of G also fell, but to only about 50% of that of NaG at a 100-mM CHX concentration.

The diffusivity data were then plotted as the product of the diffusion coefficient and concentration of CHX as a function of CHX. The results are given in Fig. 5. For low concentrations of CHX, the data could be fitted by a straight line, although the scatter precluded ruling out the possibility of a non-linear relationship. According to equation (12), the slope of the line is equal to the diffusion coefficient of the aggregate and the intercept is the product of the diffusion coefficient and CMC. This assumes a two state model with a discrete critical micelle concentration and a constant micelle size. For the low concentrations, the estimated diffusivity of the aggregates from series 1, 2, and 3 were 2.9×10^{-6}, 3.0×10^{-6}, and 2.9×10^{-6} cm^2/s, respectively. The corresponding CMC estimated from the intercepts were 11, 14, and 13 mM.

The high concentration data from series 1 was fitted to a separate line, despite the observation that the non-random residuals are consistent with a non-linear relationship that has negative curvature. The non-linearity indicates that the model is not valid, since the size of the aggregate and/or effective CMC changes with CHX concentration. Nevertheless, the slope and intercept were used to provide an estimate of the effective CMC and diffusivity of the larger aggregate, which were 50 mM and 4.79×10^{-7} cm^2/s.

Additional insight into nature of the aggregation can be obtained by combining the results of the static light scattering and PFG-NMR spectroscopy. Specifically, static light scattering provides a measure of the weight averaged molecular weight and is very sensitive to the presence of large aggregates, whereas PFG-NMR provides a

Table 2
The observed supernatant concentrations of CHX, Ac, and G determined by NMR peak integration or UV spectroscopy for a series of CHX-G_2 solutions to which NaAc was added resulting in a suspension (series 3).

NMR	UV	NMR	
[CHX] (mM)	[CHX] (mM)	[Acetate] (mM)	[Gluconate] (mM)
46.7	46.8	–	94
43.0	47.1	97.9	99
20.5	22.9	104	98
9.24	13.5	129	98
3.12	5.7	162	96

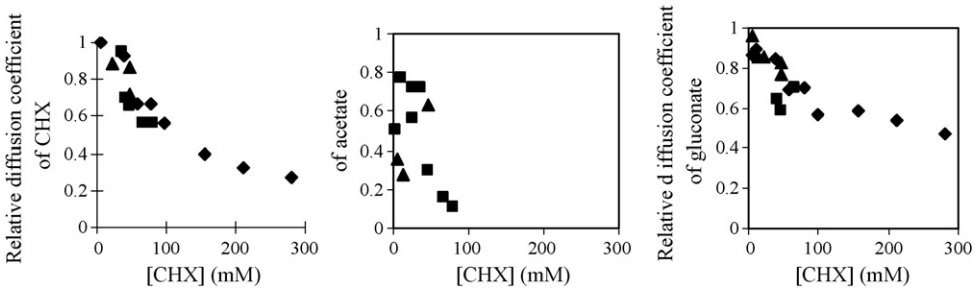

Fig. 4. Relative diffusion coefficients of (a) CHX, (b) Ac, and (c) G given as a function of CHX concentration for series (♦) 1, (▲) 2, and (■) 3.

measure of the number averaged molecular weight. NMR is also less sensitive to large-sized aggregates, but can be used at very high concentrations. As indicated earlier, no aggregation was evident with CHX-Ac_2 by light scattering. The estimated decrease in the diffusion coefficient was only about 10% for CHX-Ac_2. Using a critical aggregation concentration of 11 mM, the aggregation number is at most 2. No data above 50 mM was obtained for solutions containing acetate due to the imposed constraint of the CHX-Ac_2 solubility product.

For CHX-G_2, static light scattering indicated that dimers formed at low concentration and progressively larger aggregates were formed about a concentration of 50 mM. From the observed diffusivity and the Stokes–Einstein equation, the hydrodynamic radius of CHX-G_2 at low concentration (monomeric) was near 5 Å whereas the diffusion coefficients derived from the fitted lines of Fig. 4 resulted in a hydrodynamic radius between 7.2 and 7.6 Å. This corresponds to an increase in volume by a factor of 2.7, which would be consistent with dimer or perhaps some trimer formation.

At high concentration, there was a more dramatic decrease in the diffusivity of CHX-G_2 indicating the formation of a much larger aggregate, qualitatively similar to the light scattering results. However based on the fitted slope, the hydrodynamic radius was estimated to be 46 Å. This is much larger than that expected for a pentamer aggregate that was suggested by the light scattering data. While hydration has a greater effect on the measured size with NMR spectroscopy in comparison to light scattering, there may also be additional relaxation or exchange effects that lead to an underestimate of the diffusion coefficient (Wiedmann et al., 2001).

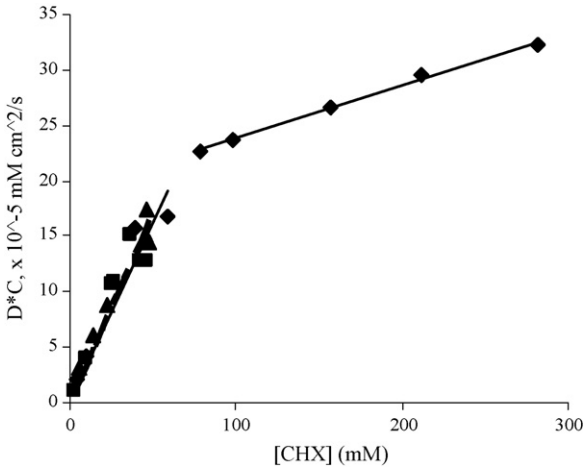

Fig. 5. Product of the diffusion coefficient of CHX and CHX concentration ($\times 10^{-5}$ mM cm^2/s) given as a function of CHX concentration for series (♦) 1, (▲) 2, and (■) 3. The solid lines represent best fits from linear regression where series 1 has been fitted by two separate lines because of the apparent break in the curve.

These fundamental studies provide a means to design a sustained release delivery system for root canals (Riggs et al., 2000; Okino et al., 2004; Farkas et al., 2007; Gong et al., 2007). Although chloride concentration has been shown to affect the release rate, quantitative information concerning the effect of all counterions is necessary to maintain the CHX concentration at its minimum effective concentration under physiological conditions. Thus, a better approach than polymeric systems that involve the slow release of CHX for preventing root canal infection would be to fill the entire pulp chamber with a formulation that maintains CHX at an effective concentration. That is, CHX-G_2/CHX-Ac_2 mixtures can be introduced that will control the concentration of CHX, such that additional CHX can undergo dissolution should chloride ions diffusive into the chamber. This compensatory dissolution will negate the loss of CHX due to precipitation of CHX-Cl_2.

5. Conclusions

In consideration of both the solubility data and size information, it appears that high concentrations of CHX obtained with gluconate ions are a result of the formation of relative large aggregates. There may be limited aggregation in the presence of acetate, and acetate appears to associate more extensively with the aggregates, but large aggregates and high concentrations were only formed with gluconate ions. In addition, NMR chemical shift changes are consistent with an aggregate where ring-hexamethylene chain interacts possibly revealing the packing arrangement. Finally, with proper controls, NMR spectroscopy can be used to quantify the concentration of each organic salt species, which would prove useful to characterize the salt effects on solubility products.

Acknowledgement

The financial support of the University Of Minnesota Academic Health Center is gratefully appreciated.

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