The Effect of Electrostatic Charge Interactions on Release Rates of Gentamicin from Collagen Matrices

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Purpose. This work studied the effect of changes in the magnitude of electrostatic charge interactions on the release kinetics of gentamicin from collagen matrices. ***Methods.*** The charge distribution on collagen was altered by specific charge chemistries to yield net negative charges which exhibited binding interactions with positively charged gentamicin. The adsorption isotherms were measured to characterize binding interactions and release of gentamicin from modified matrices were measured. The release rates were compared to a mathematical model based on an instantaneous desorption coupled with diffusion mechanism. ***Results.*** Ninety percent of the gentamicin loaded was released from native collagen matrices in 2.5 days (one-sided slab geometry in-vitro). Succinylated collagen matrices released 70% in 2.5 days and phosphonylated collagen matrices released 50% in 2.5 days. Excellent agreement between model predictions and experiment results were obtained. ***Conclusions.*** Modified collagen can be much more effective in antibiotic therapy in sustaining release rates compared to native collagen for charged antibiotics like gentamicin.

KEY WORDS: diffusion; desorption; collagen; gentamicin; electrostatic; binding; release rates.

INTRODUCTION

There has been great interest in developing delivery systems for aminoglycoside antibiotics like gentamicin. Aminoglycoside antibiotics are therapeutically important for the treatment of severe infections caused by gram-negative bacilli. An intensive gentamicin therapy is required for the treatment of bacterial keratitis, sepsis in new born, corneal infections, osteomyellitus and other post-surgical procedures (1-3). Collagen shields, collagen sponges, implantable collagen, high viscosity bone cement, and poly(L-Lactic acid), have recently been investigated for use in delivering gentamicin therapeutically. Collagen, because of its biocompatibility is especially attractive. A mechanistic understanding of the effect of electrostatic binding interactions on release kinetics of gentamicin from collagen matrices is limited and here we attempt to fill this gap.

Diffusion is a common mechanism used to modulate drug delivery in collagen matrices. However due to small sizes (and hence large diffusion coefficients) of antibiotics a prolonged release can not be obtained by simple Fickian diffusion. In this study the viability of adsorption—desorption as a release mechanism for gentamicin is evaluated. In a recent paper (4) we have mathematically modeled the release behavior of drugs mediated by a diffusion coupled with desorption process. We were able to demonstrate that by altering the binding strength, binding capacity, and the rates of adsorption and desorption it was possible to modulate the release over extended time periods. Further we have established (5) the mechanisms governing release of poly-L-lysine (a charged polypeptide) from collagen matrices were instantaneous desorption coupled with diffusion. Here, the release rates of gentamicin from collagen matrices in the instantaneous desorption limit were measured and compared with model predictions based on adsorption isotherm measurements.

The collagen triple helix contains about 17% charged amino acid residues which are uniformly distributed over its entire length. Collagen has a net neutral charge at physiological conditions. The charge distribution on the collagen molecule can be modified by specific chemical reactions to yield a net negative or a net positive charge. These electrostatic charges enhance the retention of the oppositely charged drugs by binding interactions. Modification of the surface charge chemistry can therefore be used to modulate the binding interactions between the collagen matrices and charged drug molecules. The effect of two such charge modifications, succinylation and phosphonylation, on the release rates of gentamicin are reported here.

THEORETICAL: MATHEMATICAL MODEL

A detailed analysis of the governing equations and the mathematical solution corresponding to the release experiments is available (4). Briefly, the drug delivery matrix is treated as a slab of thickness L, which comprises a drug homogeneously dispersed in a polymer. Previous experiments have established that the mechanisms governing polylysine release from collagen matrices are instantaneous desorption coupled with diffusion resulting from non-specific electrostatic binding interactions between collagen matrices and polylysine (5). Since the binding interactions between the collagen matrices and gentamicin studied here are also primarily non-specific electrostatic interactions desorption rates are expected to be much faster than diffusion rates (instantaneous desorption limit). In this limit the concentration profile of drug (gentamicin in the matrix is governed by:

$$\partial C_A/\partial t = \frac{D(1 + KC_A)^2}{((1 + KC_A)^2 + C_{matrix}M_A^{max}K)} \partial^2 C_A/\partial x^2 \quad (1)$$

where, C_A is the free drug concentration (mass/volume), x is spatial position, t is time, D is the drug diffusivity (length^2/time) in the matrix, C_{matrix} is the concentration of collagen in the matrix (mass/volume), M_A^{max} is the maximum mass of drug bound/ mass of collagen and K ((mass/volume)$^{-1}$) is the binding constant (Langmuir adsorption constant). For the case when amount of drug loaded is much smaller than the

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binding capacity (weak binding strength), the binding isotherm is linear ($M_A = KM_A^{max} C_A$) and Eq. 1 reduces to:

$$\partial C_A/\partial t = \frac{D}{(1 + C_{matrix}M_A^{max}K)} \quad (2)$$

which is simple Fickian diffusion with an effective diffusivity (D_{eff}):

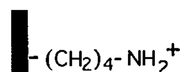
$$D_{eff} = D/(1 + K\ M_A^{max}\ C_{matrix}) \quad (3)$$

This indicates that for the limiting cases of instantaneous desorption and a linear adsorption isotherm, the release rates only depend on the diffusion coefficient (D) and the slope of the adsorption isotherm ($K\ M_A^{max}$). Consequently, only the product $K\ M_A^{max}$ needs to be measured in adsorption experiments and not K and M_A^{max} separately.

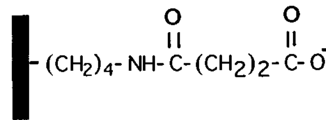
EXPERIMENTAL: MATERIALS AND METHODS

Materials

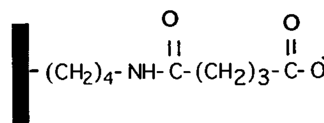
Purified Bovine type I collagen was supplied by Collagen Corp. (Palo Alto, CA). Gentamicin sulfate and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Preparation of succinylated collagen and native collagen has been described previously (6, 7). Glutarylation of collagen is similar to the succinylation reaction. Glutarylation substitutes a negative charge at positively charged lysine groups. The difference between these two modifications is in the length of the spacer arm (number of carbon atoms between the collagen backbone and the charge). Glutarylation was carried out under similar conditions to succinylation (6, 7) substituting glutaric anhydride for succinic anhydride. Glutarylated collagen precipitated at pH 4.3. Collagen was phosphonylated using the bifunctional crosslinking reagent glutaraldehyde which reacts with the primary amines of collagen and 2-amino ethyl phosphonic acid (AEPA). AEPA has been reported to have three pk_a values (2.6, 6.2 and 10.5) such that at neutral pH both the OH groups are ionized (8). Phosphonylated collagen was separated from the unreacted glutaraldehyde and AEPA by centrifuging through 30,000 molecular weight cut off Centriprep tubes (Amicon, MA). Phosphonylated collagen was dialyzed against deionized water to remove any remaining unreacted glutaraldehyde and AEPA. The phosphonylated collagen was centrifuged to concentrate it and was equilibrated with PBS (20 mM Sodium Phosphate, 130 mM Sodium Chloride adjusted to pH 7.2). Figure 1 shows the structures of the different side chains of the derivatized collagen molecules.



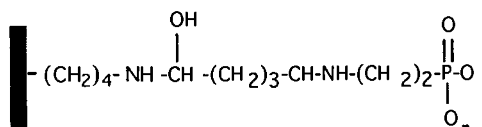
Lysine group of collagen



Succinic acid group of succinylated collagen



glutaric acid group of glutarylated collagen



Phosphonic acid group of phosphonylated collagen

Fig. 1. Structures of side chains of derivatized collagens and native collagen molecules. Native collagen has a net neutral charge at physiological conditions. Derivatized collagen molecules have a net negative charge at physiological conditions.

Glutarylated collagen showed similar solubility properties to succinylated collagen as a function of pH. Its isoelectric point (minimum solubility) was observed in the range 4.0 - 4.5 which is similar to succinylated collagen (4.2 - 4.6) (7). This indicates succinylated and glutarylated collagen have similar charge densities. The protein content was measured by the BCA assay (9) and the degree of charge modification was quantified by measuring unmodified lysine content using trinitrobenzene sulfonate (TNBS) (10). Glutarylated collagen showed an 85% conversion and succinylated collagen showed an 83% conversion based on the TNBS assay. This indicates that succinylated and glutarylated collagen matrices have similar charge densities. Phosphonylated collagen was 22% modified based on the TNBS assay. The low degree of modification of phosphonylated collagen results from the use of low concentrations of glutaraldehyde in the reaction mixture to avoid crosslinking the collagen. The degree of chemical modification was also characterized by titration of derivatized collagen with NaOH as described elsewhere (11). Good agreement was obtained between the two techniques in quantifying the extent of charge modification.

Methods

Adsorption Isotherm Measurements

A simple approach using centrifree ultrafiltration tubes (Amicon, MA) was utilized to measure the concentration of bound and free gentamicin. The adsorption isotherms were measured in different salt concentrations at pH 7.4. Collagen (5-10 mg/ml in phosphate buffer with desired NaCl concentration at pH 7.4) was mixed with different concentrations of gentamicin (2-15 mg/ml in phosphate buffer with desired NaCl concentration at pH 7.4) in a 1.5 ml centrifuge tube. The adsorption mixture was allowed to mix and equilibrate overnight. This mixture was added to a centrifree tube of molecular weight cut off 30,000 daltons. The centrifree tubes containing the sample were centrifuged at 3000 rpm for 1

hour. Free gentamicin passes through the membrane and is collected at the bottom as filtrate whereas bound gentamicin can not pass through the membrane because of small pore size of the filtration membrane (30,000 daltons cut off). The free gentamicin concentration was analyzed by a TNBS (10) assay which detects primary amines. Gentamicin concentrations as low as 20 μg/ml could be measured reliably (Standard deviation < 5%) using this assay. Adsorption isotherm experiments were performed in duplicate and from each experiment three samples were analyzed to measure the concentration of free gentamicin. The standard deviation for the measured free concentration value was less than 5%.

Release Rate Measurements

Collagen release matrices were adjusted to a concentration of 20 - 25 mg/ml in phosphate buffer of desired salt concentration at pH 7.4. All the collagen matrices were equilibrated in the same medium with which the release experiments were performed. Gentamicin (1 - 10.5 mg/ml) was loaded by a syringe to syringe mixing. One ml of collagen - gentamicin admixed matrix was placed in a 4 ml cylindrical vial. The interface was flattened and trapped air bubbles removed by mild centrifugation. Each matrix was equilibrated for 2 days prior to measuring release profiles. In vitro release experiments were performed by placing 1 ml of phosphate buffer (20 mM sodium phosphate, pH 7.4) with desired salt concentration on top of 1 ml of matrix at ambient temperature. The buffer was removed every 6 - 24 hours and replaced with fresh buffer. All release experiments were performed in triplicate. Gentamicin released at each time point was assayed by the TNBS assay, however, collagen present in the release buffer was removed by centrifuging through centrifree tubes (molecular weight cut off 30,000) prior to performing the TNBS assay.

Measurement of Diffusion Coefficients

The free solution diffusivity for gentamicin was computed by application of Fick's second law to the release profile obtained for gentamicin loaded into succinylated collagen (SC) matrices with a 0.5 M salt concentration. Based on the partition measurements described above no adsorption was indicated for gentamicin in the SC matrix with 0.5 M salt concentration. Fick's second law for long times gives the following proportionality between log (fraction of loaded gentamicin remaining in the matrix) and time (6):

$$\ln(\text{fraction remaining}) \sim -D\pi^2 t / 4L^2 \quad (4)$$

In Eq. 4, D is the diffusion coefficient, t is release time and L is the diffusional path length (here L = 1 cm). The diffusion coefficient was calculated from the slope of the linear plot of ln (fraction remaining) versus time (graph not shown). The gentamicin release rates were measured using 0.5 M NaCl in 20 mM phosphate buffer at pH 7.4. The calculated diffusion coefficient is 46 x 10^{-7} cm^2/sec. This value is reasonable for a 562 daltons molecular weight molecule (12) (sucrose, 344 daltons, for example has D = 53 x 10^{-7} cm^2/sec).

RESULTS AND DISCUSSION

Gentamicin Adsorption Behavior

Figure 2 shows the adsorption behavior of gentamicin on native collagen, succinylated collagen (83% modified based on TNBS value of conversion of lysines) and phosphonylated collagen (22% modified). Gentamicin is a hydrophilic antibiotic which has 3 - 4 primary amine groups present per molecule that ionize in solution. The net charge on gentamicin is positive at physiological conditions and gentamicin binds with negatively charged groups on an adsorbate. Figure 2 shows some binding of gentamicin to native collagen, most likely due to electrostatic interactions with the aspartic and glutamic acid (negatively charged) groups, which increase when lysines of native collagen are converted to succinic acids. A further enhancement of binding is observed for the case of phosphonylated collagen. Gentamicin has a tendency to form aggregates at higher concentrations, so the adsorption studies were restricted to the linear region of the isotherm. The legend for Figure 2 reports the values of KM_A^{max} calculated from linear adsorption isotherm. Figure 2 shows that valence of the local charges plays a very strong role on the binding interactions. Comparing the slopes of the -1 charge of succinic acid groups to the -2 charge of phosphonic acid groups demonstrates that the magnitude of the binding interaction is greater with the higher valence phosphonic acid groups. Although phosphonic acid has a smaller degree of charge modification (22%) compared to succinylated collagen (83%), the higher valence more than compensates for this and increases the KM_A^{max} value by two times.

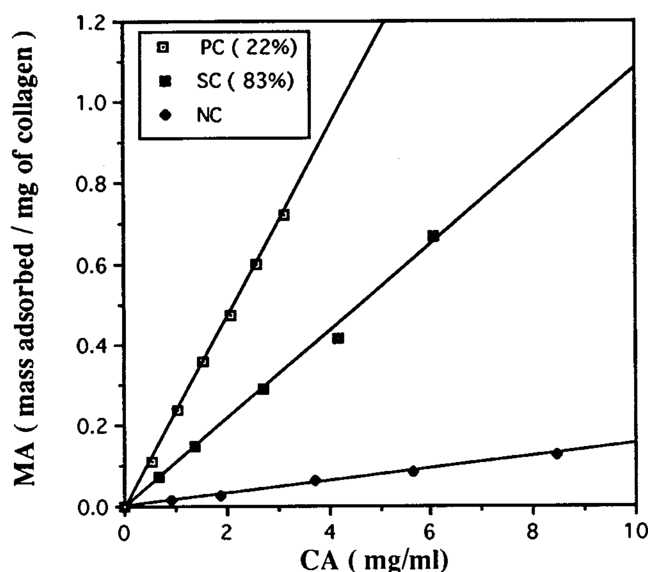


Fig. 2. Adsorption isotherm for gentamicin interacting with NC (native collagen), PC(phosphonylated collagen) and SC (succinylated collagen) in PBS (130 mM NaCl in 20 mM phosphate buffer at pH 7.4). The percentages in parenthesis indicate the degree of collagen modification based on lysines modified. Slopes of adsorption isotherm ($K M_A^{max}$) are: PC = 0.230 ± 0.012, SC = 0.107 ± 0.008, and NC = 0.015 ± 0.001. The errors represent the regression error calculated by the Marquardt algorithm for a 95% confidence interval (Ultrafit Software, Bioscience Inc., Cambridge).

Figure 3 shows the effect of ionic strength on the adsorption behavior of succinylated collagen (83% succinylated). Decreasing the salt concentration increases the binding interaction strength. At higher salt concentrations (0.5 M NaCl in 20 mM phosphate buffer) no binding is observed which indicates that the binding interaction is primarily modulated by electrostatic forces. This type of behavior is expected and consistent with binding interactions seen with other electrostatically charged matrices (13, 14). Legend reports the slopes of the adsorption isotherms ($K M_A^{max}$) calculated from Figure 3.

We also investigated the effect of spacer arm length, between the surface charge site and backbone of collagen, by comparing succinylated collagen (SC) and glutarylated collagen (GC) with similar degrees of charge modification. Succinylated collagen is 83% modified and has a pI (isoelectric point) in the range of 4.2 - 4.6 and glutarylated collagen is 85% and has a pI in the range of 4.0 - 4.6. This indicates both have similar charge densities. Glutarylated collagen has a 4 carbon spacer group compared to succinylated collagen which has a 3 carbon spacer group between the lysine side chain on collagen and the COOH group. The adsorption isotherm measurements were conducted in 1 mM phosphate salt solution (pH 7.4) since at low salt concentrations the electrostatic interactions are greater. The slopes of adsorption isotherms (KM_A^{max}) were 0.216 ± 0.011 for glutarylated collagen and 0.190 ± 0.011 for succinylated collagen. This indicates no significant effect on the adsorption behavior when comparing succinylated collagen with glutarylated collagen.

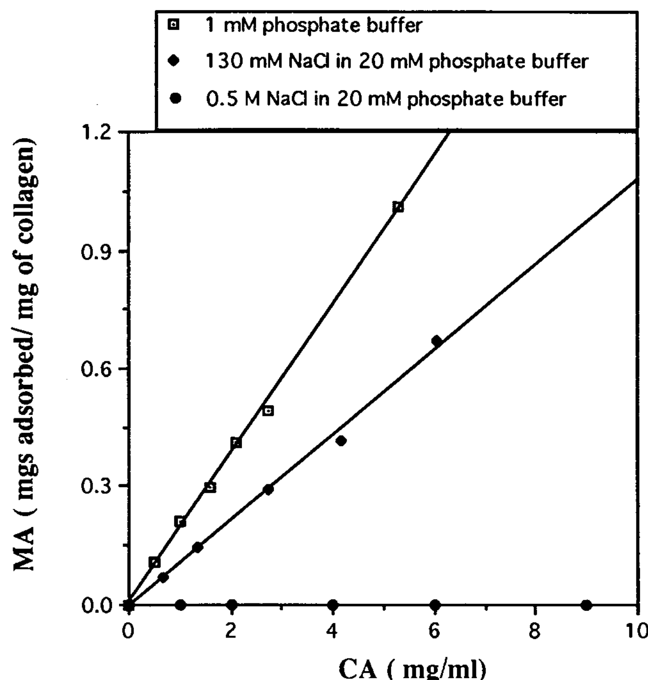


Fig. 3. The effect of changing ionic strength of adsorption buffer is shown for gentamicin binding on succinylated collagen (83% modified). Slopes of adsorption isotherm ($K M_A^{max}$) are: SC in 0.5 M NaCl with phosphate buffer = 0.0, SC in PBS = 0.107 ± 0.008, and SC in 1mM phosphate buffer = 0.190 ± 0.010. The errors represent the regression error calculated by the Marquardt algorithm for a 95% confidence interval (Ultrafit Software, Bioscience Inc., Cambridge).

Gentamicin Release Measurements and Comparison with Mathematical Modeling

Gentamicin release rates can be predicted from Equation 2 in the limit of a linear isotherm. Equation 2 was solved using the appropriate boundary conditions to calculate the release rates (6, 15). Figure 4 shows the effect of charge density on the release rates and comparisons with model predictions. The data points represent mean values for triplicate measurements (n = 3) and the standard deviation was less than 5% for a 95% confidence interval. Solid lines indicate the model predictions based on adsorption isotherm measurements. The degree of charge modification and the concentrations of collagen employed are indicated in the parenthesis. The release rates here are measured in PBS (130 mM NaCl in 20 mM phosphate buffer, pH 7.4). They show that increasing the charge density decreases the release rates. Also, increasing the valence of charges employed results in slower release rates due to enhanced binding interactions.

Figure 4 shows that 50% of the gentamicin loaded releases from native collagen in about 15 hours compared to 75 hours for the case of phosphonylated collagen. It shows that by altering the binding interactions it is possible to increase the duration of delivery from collagen matrices. The model predicts (solid lines) the release rates very accurately indicating that the release rate is controlled by an instantaneous desorption coupled with diffusion process.

Adsorption isotherm studies have indicated that increasing the salt concentration in the buffer reduces the electrostatic binding interactions. Figure 5 shows the effect of ionic strength on the release rates. The release rates were measured from succinylated collagen matrices in phosphate

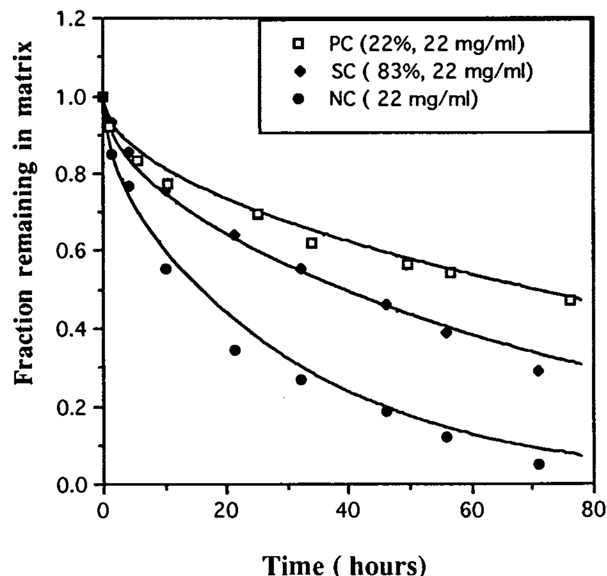


Fig. 4. Release kinetics of gentamicin from native (NC), succinylated (SC) and phosphonylated (PC) collagen matrices in PBS (130 mM NaCl in 20 mM phosphate buffer at pH 7.4). The solid lines indicate the model predictions based on adsorption isotherm measurements. The data points represent mean values for triplicate experiments and the standard deviations for a 95% confidence interval are less than 5%.

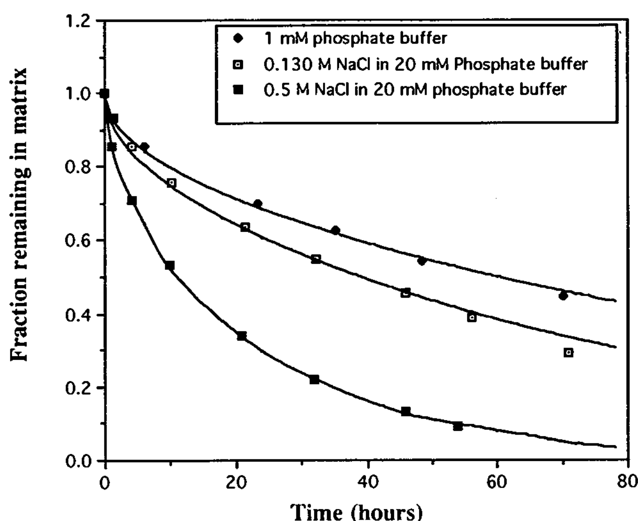


Fig. 5. Effect of ionic strength on the release kinetics of gentamicin from 83% succinylated collagen. The solid lines indicate the release rates as predicted by mathematical model based on adsorption parameters. The data points represent mean values for triplicate experiments and standard deviations for a 95% confidence interval are less than 5%.

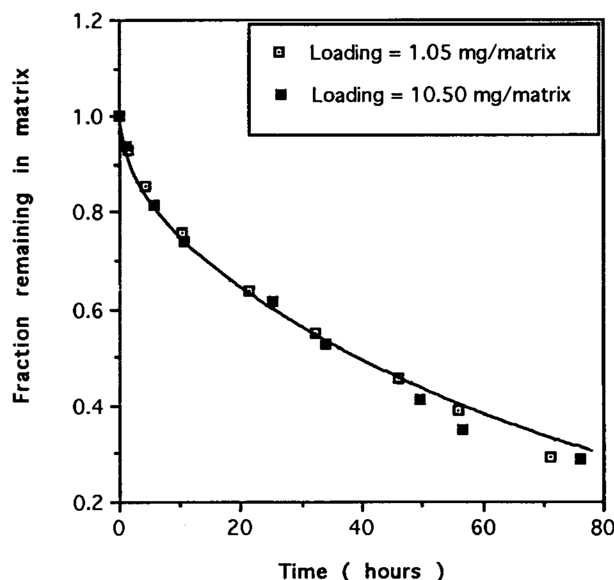


Fig. 6. Effect of gentamicin loading on release kinetics from succinylated collagen (83%) in PBS (130 mM NaCl in 20 mM phosphate buffer at pH 7.4). The solid lines indicate the release rates as predicted by mathematical model based on adsorption parameters for both loadings levels. The data points represent mean values for triplicate experiments and the standard deviations for a 95% confidence interval are less than 5%.

buffer with three different salt concentrations at pH 7.4. All the experiments were performed in triplicate ($n = 3$) and data points shown in graph represent mean values. The standard deviation was less than 5% for a 95% confidence interval. Lowering the salt concentration decreases the release rates due to enhanced binding interactions. The release rates at high salt concentration are by simple Fickian diffusion with no binding interactions. This result was used to calculate the free solution diffusivity as discussed previously. Solid lines indicate model predictions based on the adsorption isotherm measurements. Good agreement between model predictions and experimental release rates were obtained.

We also evaluated the effect of spacer arm length on release rates of gentamicin by comparing the succinylated and glutarylated collagen matrices in 1 mM phosphate buffer (pH 7.4). No effect on release rates is observed between the succinylated and glutarylated collagen matrices, confirming that increasing the spacer length from 3 to 4 carbons does not enhance the binding interactions (graph not shown).

Equation 2 indicates that, for the case of a linear adsorption isotherm, the normalized release rates (fraction of drug loaded) are independent of the amount of drug loaded as long as the drug diffusion is not hindered by the higher concentration of drug. Figure 6 compares the release rates for two different loadings on the succinylated collagen (83%) matrices. The release measurements were conducted in PBS. Gentamicin loading of 1.05 mg/ml and 10.50 mg/ml were used to compare the release rates. The data points in Figure 6 represent mean values for $n = 3$ and the standard deviation was less than 5% for a 95% confidence interval. The solid lines represent model predictions for two different loadings of gentamicin. Figure 6 shows no appreciable effect of loading on the release rates.

CONCLUSIONS

Collagen matrices, sponges, and shields have been widely investigated for release of gentamicin and other antibiotics. Here, we investigated the effect of charge modification, ionic strength and spacer arm length on the release of gentamicin from collagen matrices. Increasing the charge density increases the strength of binding interactions and thereby retards release rates. Succinylated collagen (83%) showed a seven fold increase in the KM_A^{max} (from 0.015 to 0.107) value compared to native collagen. Ninety percent of gentamicin loaded released from native collagen matrix in about 2.5 days (from the 1 sided thick-slab geometry used here). Charge interactions of succinylated collagen can increase this duration to about 7 days (based on model calculations). Increasing the charge valence to -2, by using phosphonylated collagen (22%), doubled the KM_A^{max} value compared to succinylated collagen. The model calculations for phosphonylated collagen show that it can increase the duration of 90% release to 12 days. This represents a five fold increase (compared to native collagen) in the duration in which phosphonylated collagen will be effective and the profile is more closer to zero order as well. Modified collagen will presumably be more effective in antibiotic therapy in sustaining release rates compared to native collagen for charged antibiotics like gentamicin. However, there are very apparent limitations to electrostatic interactions as a mechanism for sustaining release rates in the instantaneous desorption limit. These systems can not enhance the release duration by orders of magnitude when low to moderate binding strengths and fast desorption rates prevail under physiological conditions.

NOTATION

C_A = Free concentration, C_{matrix} = Collagen concentration, D = Free solution diffusion coefficient, Deff = Effec-

tive diffusion coefficient, GC = Glutarylated collagen, K = Langmuir adsorption constant, L = Diffusional path length, M_A = amount bound/mg of collagen, M_A^{max} = Binding capacity, NC = Native collagen, PBS = 130 mM NaCl in 20 mM phosphate buffer, pH 7.4, PC = Phosphonylated collagen, SC = Succinylated collagen, t = Release time.

ACKNOWLEDGMENTS

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