**Surface Complexation Modeling of Terbium Biosorption onto *E. coli* Bacterial Surfaces with Lanthanide Binding Tags**

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**Abstract**

Lanthanide binding tags (LBTs) have been engineered onto native *Escherichia coli* (*E. coli)* bacterial surfaces to enhance extraction and recovery of rare earth elements (REEs). Three strains of *E. coli* were studied: (1) the native *E. coli* surface, (2) a mutant *E. coli* surface with hindered, non-binding lanthanide binding tags, and (3) an LBT *E. coli* surface with fully functioning lanthanide binding tags. A three discrete site, constant capacitance surface complexation modeling approach was taken in studying these strains with an ultimate goal of comparing site type affinities to the model rare earth, Terbium. Our results show a possible increase in native carboxyl functional groups when the LBTs are overexpressed on the cell surface. LBTs are confirmed to have a higher stability constant with Terbium than that of the native functional groups. Incorporation of LBTs into the *E. coli* cell wall poses two major benefits: (1) the presence of a high-affinity, low-capacity LBT site for selective Terbium binding at low metal loading regions, and (2) a lower-affinity carboxyl site that increases the sorption capacity of the native bacterial surface during sorption at higher metal loading regions.

**Introduction**

 Conventional rare earth element (REE) extraction methods can be energetically and chemically intensive, harming the environment with waste products and inefficient use of energy for the mining of low concentration metals. Scientists have begun implementing lanthanide binding tags (LBTs), short peptides with high affinity and selectivity for REEs, to bind trace rare earth metals to bacterial surfaces [1,2]. LBT affinity for REEs has been tested under rapid luminescence detection [3] and NMR spectroscopy [4], both supporting that LBTs have high selectivity for REEs. This current study advances the claims of experimental literature through surface complexation modeling (SCM). Because SCMs account for important solution conditions such as pH through the incorporation of protonation reactions and their affiliated equilibrium constants, an SCM approach has the significant advantage compared to experiments of predicting sorption behavior as a function of parameters such as pH and ionic strength. Our objective was to develop an SCM for Terbium (Tb) biosorption onto *E. coli* bacteria with and without LBTs. Centering our modeling around *E. coli* - Tb adsorption capacity experiments with and without LBTs (Figure 1), we implement a bottom-up approach by first creating a native *E. coli* SCM and then introducing additional site types in other SCMs that build upon the first model. We aim to create discrete native *E. coli* and LBT site types in order to compare their affinities to Tb.

**Methods**

*Acid/base titration of native (UN) E. coli cells*

 Potentiometric titrations were conducted on native *E. coli* cells at mid-exponential phase to determine pKa values and discrete site concentrations on the cell surface. The solution was initially acidified with 0.1 M HCl to a pH of 4.0. The bacterial suspension was purged with N2 gas for thirty minutes after which the pH readings remained constant at 4.75. A base titration using 0.1 M NaOH titrant was conducted at room temperature using a Titronic 300 automatic titrator. A monotonic equivalence point titration method was used to add the same volume of titrant at each step. This study focuses on a pH range of 4.75 – 10.26. pH below 3.5 have been shown to irreversibly damage bacteria by displacing structurally bound Mg and Ca [5,6]. Although bacterial surfaces have been cited to have substantial buffering capacity below our studied pH range [7], our current titration avoids lower pH values in order to help determine pKa’s and site densities of healthy, undamaged bacterial cell surfaces.

*Native E. coli (UN) surface complexation model*

Our surface complexation models were implemented under a temperature of 24 °C, pH 6, and 10 mM NaCl ionic strength. We initially created a model for Tb sorption onto the native *E. coli* bacterial surface. Bacteria cell walls are complex and contain multiple types of functional groups, ranging from carboxyl, hydroxyl, and phosphoryl groups [7,8] to sulfhydryl [9,10] and amine groups [11]. While Fein [12] suggests that a one-site carboxyl model can be appropriate to characterize some types of metal cation-bacteria adsorption, other works [6,7,8,13,14] attempt to use two-, three-, and four-site SCMs to better describe experimental sorption data. Rare earth element biosorption onto bacterial surfaces has been shown to be predominantly controlled by carboxyl and phosphoryl functional groups [14,15, 16,17]. Thus, a two-site model of carboxyl and phosphoryl sites was first tested before also attempting three-site and four-site discrete pKa models.

We assumed the native carboxyl and phosphoryl sites to be monodentate in binding with Tb3+ cations [12]. However, we note that it has been suggested that multidentate binding could be possible [16]. Assuming a rod-shaped cell of 5.0 $μ$m long and 1.0 $μ$m wide and the 1010 cells/mL in a 2.5 g/L suspension approximation from Fein (1997) [7], the native *E. coli* cells were modeled to have 140 m2/dry gram surface area.

*LBT-deactivated E. coli (MUT) surface complexation model*

In modeling the bacterial mutant strain that has its LBTs hindered from chelating with rare earths, we started with our UN SCM, but also introduced another monodentate site type called X-LBTs. We classified X-LBT sites as additional carboxyl functional groups that were created during dLBTx8 overexpression. Overexpression of membrane proteins have been associated with physiological changes to the cell surface [18]. In this case, we hypothesize that the cell surface physiological change is a compositional one that introduces additional functional groups. The X-LBT sites likely consist of a range of different site types such as carboxyl and phosphoryl sites, but no current study has confirmed specific functional groups or site densities. To simplify our model, we assumed all X-LBT sites to be carboxyl groups with the same protonation constants and Tb stability constants as that of the native *E. coli* carboxyl groups. We allowed X-LBT site density to be optimized during parameter fitting, but kept the underlying native *E. coli* SCM site densities and equilibrium constants fixed. In this way, we conduct a bottom-up approach in building an SCM that captures Tb sorption behavior consistent with both UN and MUT experimental bacterial strains.

*LBT-present E. coli (LBT) surface complexation model*

In building upon the MUT SCM, we allowed LBT site density to be an optimizable parameter because of uncertainties in explicitly calculating LBT site concentration. We also solved for the LBT-Tb stability constant in order to compare this value with the native functional group-Tb equilibrium constants. Although Tb3+-LBT complexes are eight-fold coordinated [19], we consolidated the polydentate Tb binding into a single monodentate Tb binding constant. Similar to the MUT model, the LBT model used a surface area of 140 m2/gram dry cell weight. Using the three described SCMs from this method, we aim to compare Tb binding constants with native *E. coli* and LBT site types to determine whether the engineered *E. coli* strains that contain dLBTx8 expression are more selective for Tb.

*Treatment of electrostatic potential of bacterial surfaces*

 The presence of charged complexes and adsorbed ions on surfaces generates surface charge, which impacts the thermodynamic affinity of surface complexation reactions. Previous studies have implemented a constant capacitance model approach, allowing the capacitance (C) parameter to be optimized in order to create a better model fit with experimental sorption data [13]. This approach is limited because it relates surface charge to surface potential using a C value that is unconstrained in optimization and relies on only the curve-fitting procedure without external and physiological validation. Some studies approach electrostatics as playing a much smaller role in bacterial sorption behavior compared to other parameters such as site density, surface area, and functional group pKa’s [6]. Despite the limitation of a constant capacitance model (CCM) to have physical validation in its capacitance value, a CCM approach is still used in this study to importantly account for the high valence of lanthanides when sorbed to the bacterial surface. Not accounting for electrostatics using any charge model (non-electrostatic model) would pose an even more troubling assumption that charge build-up from lanthanide biosorption is negligible. In this study the capacitance value in the CCM was applied as an optimizable parameter in order to fit the titration data, similar to the procedure conducted in Fein (1997) [7].

**Results and Discussion**

*Titration fitting of UN E. coli cells*

Acid/base titrations were fit using a PHREEQC-compatible optimization program, PhreePlot based on the following deprotonation reactions:

(1) R-COOH $\leftrightarrow $ R-COO- + H+

(2) R-POH $\leftrightarrow $ R-PO- + H+

(3) R-SOH $\leftrightarrow $ R-SO- + H+

(4) R-OH $\leftrightarrow $ R-O- + H+

where R- is the bacterium the respective functional groups are attached to. Reaction (1) represents carboxyl deprotonation with a constrained optimization range of pKa 2 – 6 [8], reaction (2) and (3) represent phosphoryl deprotonation with a range of 5.65 – 7.20 and 0.2 – 2.91 respectively, and reaction (4) represents hydroxyl deprotonation with a range of 9.6 – 10.8. pKa ranges were used as constraints for optimization based off Hong et al (2006) [8] study on pKa ranges of *E. coli* and *B. brevis* under various growth phases and conditions. In this manner, a more informed optimization was conducted. Associated mass action equations for the given deprotonation reactions are as follows:

(5) K1 = $\frac{\left[R-COO^{-}\right]a\_{H}^{+}}{[R-COOH]}$

(6) K2 = $\frac{\left[R-PO^{-}\right]a\_{H}^{+}}{[R-POH]}$

(7) K3 = $\frac{\left[R-SO^{-}\right]a\_{H}^{+}}{[R-SOH]}$

(8) K4 = $\frac{\left[R-O^{-}\right]a\_{H}^{+}}{[R-OH]}$

where K is the equilibrium constant for each given reaction and aH+ is the proton activity.

 As Ngwenya et al (2010) [14] use a two-site constant capacitance model to study lanthanide sorption onto the gram-negative bacteria, *P. agglomerans*, we started our titration fitting with a two-site CCM as well (results not shown here). However, the upper pH curve did not fit well and so a three-site and four-site model were tested. A four-site model produced equally good fit compared to a three-site model, so for the rest of this study, a three-site CCM approach (Figure 1) was taken to model Tb sorption data.

The 3-site CCM fit to the base titration data output a carboxyl pKa of **4.2**, a phosphoryl pKa of **6.1**, and a hydroxyl pKa of **9.6** with site densities of **7.53**$ ×$ **10-5** moles/dry gram, **2.32**$ ×$ **10-4** moles/dry gram, and **2.06**$ ×$ **10-4** moles/dry gram, respectively. A total site concentration of **5.13**$ ×$ **10-4** moles/dry gram was thus calculated. In converting from moles/wet grams to moles/dry grams, a dry to wet cell weight ratio of 1:8 was used [6]. The total site concentration we computed falls within an appropriate range of total moles of sites/dry gram values found in literature (Table 1) and is notably similar to Martinez (2014)’s [17] multisite Langmuir model that uses a linear programming regression method (LPM) to solve for site densities based off REE sorption data. In addition to fitting pKa values and site concentrations, a constant capacitance value of **8.09** posed the best fit and was used as a fixed value in all surface complexation models moving forward in this study.

Figure 1. Native *E. coli* 3-site CCM acid/base titration fitting. Red dots indicate base titration data points and the black line refers to modeling results. A 4-site CCM (not shown here) produced equally good fit to the titration data as this 3-site model.

Table 1. Total site densities and pKa’s of various models describing bacterial sorption of REEs

|  |  |  |  |
| --- | --- | --- | --- |
| **Model Type** | **Total mol sites/dry gram** $×$ **10-4** | **pKa** | **Source** |
| multisite Langmuir | 5.49 | N/A | [17] |
| 4-site non-electrostatic | 25.6 | 3.1, 4.7, 6.6, 9.0 | [6] |
| 3-site constant capacitance | 3.25 | 4.4, 6.9, 11.2 | [20] |
| 3-site constant capacitance | 5.13 | 4.2, 6.1, 9.6 | [This work] |
| 2-site constant capacitance | 7.70 | 4.3, 6.9 | [14] |

*Surface complexation modeling of Tb sorption isotherms*

 Using the 3-site CCM generated from the native UN *E. coli* titration data, we initially solved for carboxyl-, phosphoryl-, and hydroxyl-Tb stability constants to fit the UN Tb sorption isotherm (Figure 2). The first four Tb sorption isotherm data points were used during the variable fitting in order to prioritize the lower Tb concentration ranges for realistic geothermal fluids [2]. The resulting carboxyl-, phosphoryl-, and hydroxyl-Tb logK values were **5.88**, **-5.73**, and **-2.94**, respectively (Table 2). These stability constants inform us that the main and dominant mechanism of Tb sorption is likely with the carboxyl functional group. It is encouraging that Ngwenya et al (2010) [14] calculate a similar carboxyl-Tb stability constant of 5.37. Yet, their study poses a phosphoryl-Tb stability constant of 8.59 while our model computes a value of -5.73. While Ngwenya et al (2010) [14], which obtains its values from a sorption envelope, suggests that carboxyl and phosphoryl groups are both large contributors in macroscopic adsorption of rare earths, this study, which focuses on a pH of 6, shows a carboxyl-dominated mechanism. From the results of this paper, we suspect that a 1:1 Tb:R-COO- stoichiometry is responsible for the majority of Tb biosorption onto the native *E. coli* surface at a pH of 6.

 In fitting the MUT sorption isotherm, an X-LBT site concentration of **4.43**$×$**10-5** mol/dry gram was computed. This means that the induction and overexpression of LBTs on the cell surface increases the native carboxyl functional group concentration by 1.5 times. We pose a caveat here that despite good model fit, surface complexation modeling many times poses non-unique solutions. Although our model with X-LBTs fits nicely to the MUT sorption isotherm data, there may likely be other ways of generating surface complexation models that equally fit this data. In this study, we pose the hypothesis that overexpression of LBTs on the cell surface causes functional group compositional changes as a sign of physiological changes to the *E. coli* surface. Wagner et al (2008) discuss physiological responses of *E. coli* to the overexpression of membrane proteins; one of these responses is a change and hindrance in growth and yield [21]. Hong et al (2006) informs us that there are variations in pKa values and site concentrations as a function of growth phase [8]. Thus, when modeling the MUT and LBT strains that overexpress lanthanide binding tags, it is a reasonable approach to allow site concentrations to vary for X-LBT and LBT site types.

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Figure 2. Tb sorption capacity isotherm with all three *E. coli* strains. UN refers to the un-induced, native bacteria, MUT refers to the mutant strain with inhibited lanthanide binding tags, and LBT refers to the strain with fully functioning lanthanide binding tags. Surface complexation modeling results are shown as colored lines.

 The LBT sorption isotherm was fit based off an LBT site concentration of **3.93**$×$**10-5** moles/dry gram and a Tb stability constant of **7.79**. The deprotonation constant for LBT was set to -3.08 based off the ProtParam online tool for peptide pKa calculations where the dLBTx8 amino acid sequence of FIDTNNDGWIEGDELFIDTNNDGWIEGDELLA was inputted. We observe a significantly larger LBT-Tb stability constant than the native *E. coli* carboxyl-Tb stability constant, confirming the lanthanide binding tag’s high affinity for rare earths. We also note that the surface excess from the last Tb sorption isotherm data point is statistically higher than that calculated by the model. We attribute this to the potential for other simultaneous mechanisms such as micro-precipitation and extracellular bio-mineralization to be at work here beyond simple surface complexation reactions [22].

Table 2. Calculated stability constants and site concentrations from Tb sorption isotherm fitting

|  |  |  |  |
| --- | --- | --- | --- |
| **Model Type** | **UN** | **MUT** | **LBT** |
| Carboxyl-Tb logK | 5.88 | -- | -- |
| Phosphoryl-Tb logK | -5.73 | -- | -- |
| Hydroxyl-Tb logK | -2.94 | -- | -- |
| [X-LBT]/ mol/dry g |   | 4.43$×$10-5 | -- |
| LBT-Tb logK |   |   | 7.79 |
| [LBT]/ mol/dry g |   |   | 3.93$×$10-5 |

--: the previously optimized values were carried over into the current SCM

[x]: site concentration of functional group, x.

*Mechanisms of Tb biosorption*

 We confirm that LBTs have a higher Tb affinity than native *E. coli* functional groups. At current experimental concentrations of Tb up to 417.89 µM and at a pH of 6, the dominant site of biosorption is the carboxyl group. The carboxyl functional group possesses a relatively high Tb stability constant of 5.88 with a total site concentration of 1.20$×$ 10-4 moles/dry gram. We report that the incorporation of LBTs into the *E. coli* cell wall poses two major benefits: (1) a high-affinity, low-capacity LBT site for selective Tb binding at low metal loading regions, and (2) a lower-affinity carboxyl X-LBT site that increases the sorption capacity of the native bacterial surface during sorption at higher metal loading regions. Our study is consistent with findings from time-resolved fluorescence spectroscopy papers [15,20,23] suggesting that lanthanide biosorption occurs through inner-sphere carboxylate binding. Furthermore, Ngwenya et al (2009) [24] show through EXAFS that phosphoryl groups predominate at lower pH values while carboxyl groups increase in binding at higher pH values. While Takahashi et al (2005) [16] suggest sorption of the middle rare earths to have a preference for carboxyl binding, other biosorption studies [20,24,26] show that the heaviest rare earths are preferentially bound by phosphoryl groups [24]. Thus, even within the rare earth series, there seems to be differing mechanisms and dominant functional groups at work that should be continually studied in the future. In this study, we observe *E. coli* surface complexation of Tb at a pH of 6 to be dominated by a carboxylate binding mechanism with minimal hydroxyl and phosphoryl contributions. We pose a new surface complexation model for studying lanthanide binding tag sorption of Tb to *E. coli* through a constant capacitance model electrostatic treatment and monodentate binding mode simplification of LBTs. We observe that cells with lanthanide binding tags increase in carboxyl functional groups as a result of cell surface compositional changes taking place due to protein overexpression. This is consistent with work [8] showing that cells at varying growth phases have differing pKa and site concentration values and that protein overexpression hinders growth and yields for bacteria [21]. Future work includes studying lanthanide binding tags at lower than 1 µM Tb concentrations in order to compare affinities to not only native carboxyl groups but also to the newly discovered high-affinity, low-concentration sulfhydryl surface sites [9,10]. Further work should also be conducted on desorption studies in order to optimize removal of rare earths from bacterial surfaces once biosorption has occurred [22,25,26].

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