10-8 Chemical Complexations Mentor Meeting

Attendees:
Sarwat Khatak
Gaurav Chauhan
Neil McCracken
Jian Wu
Kevin C

Questions/Comments:

- *Is there a preference for the Fe/EDTA speciation?* You probably don't have a choice… speciation is mostly controlled by pH. Higher pH = higher Fe(OH)EDTA. Your only choice would be to replace EDTA with another chelator (CDTA, etc.)
- *How well can you reliably measure [H2O2]?* Might be more challenging in cell culture media, but it is done thoroughly in seawater systems with probe molecules. If dissolved O2 and [Fe] are high enough, you will definitely have H2O2
- *Can you comment on the bioavailability of these metals?* Cell uptake is proportional to the free metal concentration, so equilibrium will release more free metal. The challenge is determining the rate at which this happens.
- *In industry we focus on "practical approaches" and not fundamental exploration.* We would love that info on what you guys do
- *Most interesting part (for some) is considering both right when you make the media and long term storage*
- *Questions P1, P2, P3, C1 are important to mentors for both short and long term storage*
- *In cases where cloudiness or precipitation is observed, we usually just toss the media and start over.* You could observe these "cloudy" issues via TEM (but won't work well with organic compounds)
- [H2O2] and redox questions (R1, R3) are important but secondary to "P" questions
- Feed strategy: 1-5% feed daily/every other day. Based on consumption rate using amino acid analysis/trace metal analysis
- Most important questions are ones that directly effect cell growth and protein production. Others are interesting but are not as high priority
- What temperature ranges are relevant? *Obviously 25, 37, but also higher temps like 50-60 C for feed formulation*

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Industry/University Cooperative Research Center (I/UCRC)
Advanced Mammalian Biomanufacturing Innovation Center (AMBIC)

Characterizing Chemical Complexation and Speciation in Order to Improve Medium and Feed Formulations

Alan T. Stone, Marc Donohue, Mike Betenbaugh
Johns Hopkins University

Mentor's Meeting 9

October 8, 2018 (Monday at 3:00 pm, EST)

A National Science Foundation-facilitated
academic – industry – government consortia
to advance precompetitive knowledge in biomanufacturing

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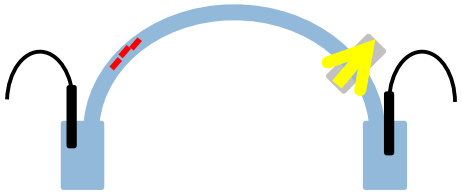
Goals & Objectives

Medium Chemistry Issues

- Insufficient nutrient bioavailability (including elements)
- Precipitation (phase separation) - Need for pH, metal ion control
- Chemical transformations (e.g. irreversible oxidation)
- Toxicity (e.g. excess bioavailability, metabolite buildup)



Computer-Based Modeling

Equilibrium Speciation Modeling using *Visual Minteq*

- Add concs. of all AMBIC components.
- Flag those likely to degrade.
- Import logKs, from CRITICAL (NIST, 2004) and literature.
- Fix pH, partial pressures, calculate complex speciation, precipitation.
- To calculate pH, must log protons and counterions added with each component.
- ***SCIENTIST*** program for kinetics.

Lab Experiments - Capillary Electrophoresis (CE)

- Identify/quantify components and their break-down products (e.g. cysteine to cysteine, RSR, R(S=O)R, RSO_3^{2-}, etc.)
- Identify/quantify distinct metal ion-chelating agent complexes, i.e. $Fe^{III}(OH)(citrate)^-$, $Fe^{III}EDTA^-$) Free metal ion activity by ion selective electrodes, colorimetric methods, etc.)
- Explore pathways/rates, e.g. EDTA capture of Fe^{III} from citrate complex; Mn^{II}, Fe^{II}, Cu^{II}-catalyzed autoxidation; kinetic retardation by Mg^{II}, Ca^{II}.

Provide a framework for innovations in medium composition.

AMBIC 1.1 - Visual Minteq -

TOTX

HEPES^{-}
(Citrate)$^{3-}$ ❶
(edta)$^{4-}$ ❷

	{M^{n+}}
Mn^{II}	**$1.04x10^{-10}$ M**
Fe^{III}	**$6.54x10^{-16}$** ❸
Cu^{II}	**$1.57x10^{-32}$**
Zn^{II}	**$5.77x10^{-16}$**

❶ Citrate

54.0 % $Fe(OH)(cit)^{-}$
27.6 % $Mg(cit)^{-}$
14.5 % $(cit)^{3-}$
1.4 % $H(cit)^{2-}$
1.4 % $Na(cit)^{2-}$

❷ EDTA

78.7% $(Fe(edta)^{-}$
21.3 % $Fe(OH)(edta)^{2-}$

❸ Iron(III)

99.5 % $Fe(OH)(cit)^{-}$
0.39 % $Fe(edta)^{-}$
0.11 % $Fe(OH)(edta)^{2-}$
0.019 % $Fe(OH)_2^{+}$

Compare with DMEM/F12 (D0547)

Mn^{II} is 5-times higher ***Cu^{II} is 50-times higher***

Fe^{III} is 280-times higher ***Zn^{II} is 10-times higher***

(no added Ni^{II})

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Media Chemistry *from a user's perspective*

(M1) Is TOTM in my system high enough?

(M2) Is $\{M^{n+}\}$ within the range that I need?

(M3) How does $\{M^{n+}\}$ change as cells grow and feed is added?

(R1) What is the oxidation state of an element component?
(Organic compounds that can exist in two or more redox forms, e.g. cysteine/cystine.)

(R2) What is $[O_2]$?

(R3) Above and beyond $[O_2]$, what other factors affect redox status of particular elements/components?
(Might be useful and important to measure $[H_2O_2]$)

(P1) I have observed cloudiness or a precipitate. How do I find out what it is?

(P2) Is there anything "after the fact" that I can do?

(P3) What steps can I take to proactively prevent precipitates from forming?

(C1) How do I know that a particular component has degraded?
(Cloudiness. Color change. Separation techniques/detection. Problems with cell growth or protein production)

(W1) What pairs of components require special scrutiny?
(Which run risk of precipitate formation, component breakdown?)

(W2) Is $\{M^{n+}\}$ under equilibrium or kinetic control?
Scenarios: - Two bottles are mixed. - Major shifts in pH - Cell growth is occurring.
- Dried medium is added to water. - Feed solution is added.

(W3) Management of impurities . . . . Transition metal catalysis
(Can't just use Milli-Q water. Must depress free metal ion activity using a strong chelating agent, i.e. EDTA.)

What analytical instrumentation is available (am I comfortable with)?

- pH meter/ion selective electrodes
- colorimetric methods
- UV-visible spectrophotometry
- ion chromatography
- HPLC-UV, HPLC_ESI-MS, GC-MS
- ICP-OES, ICP-MS

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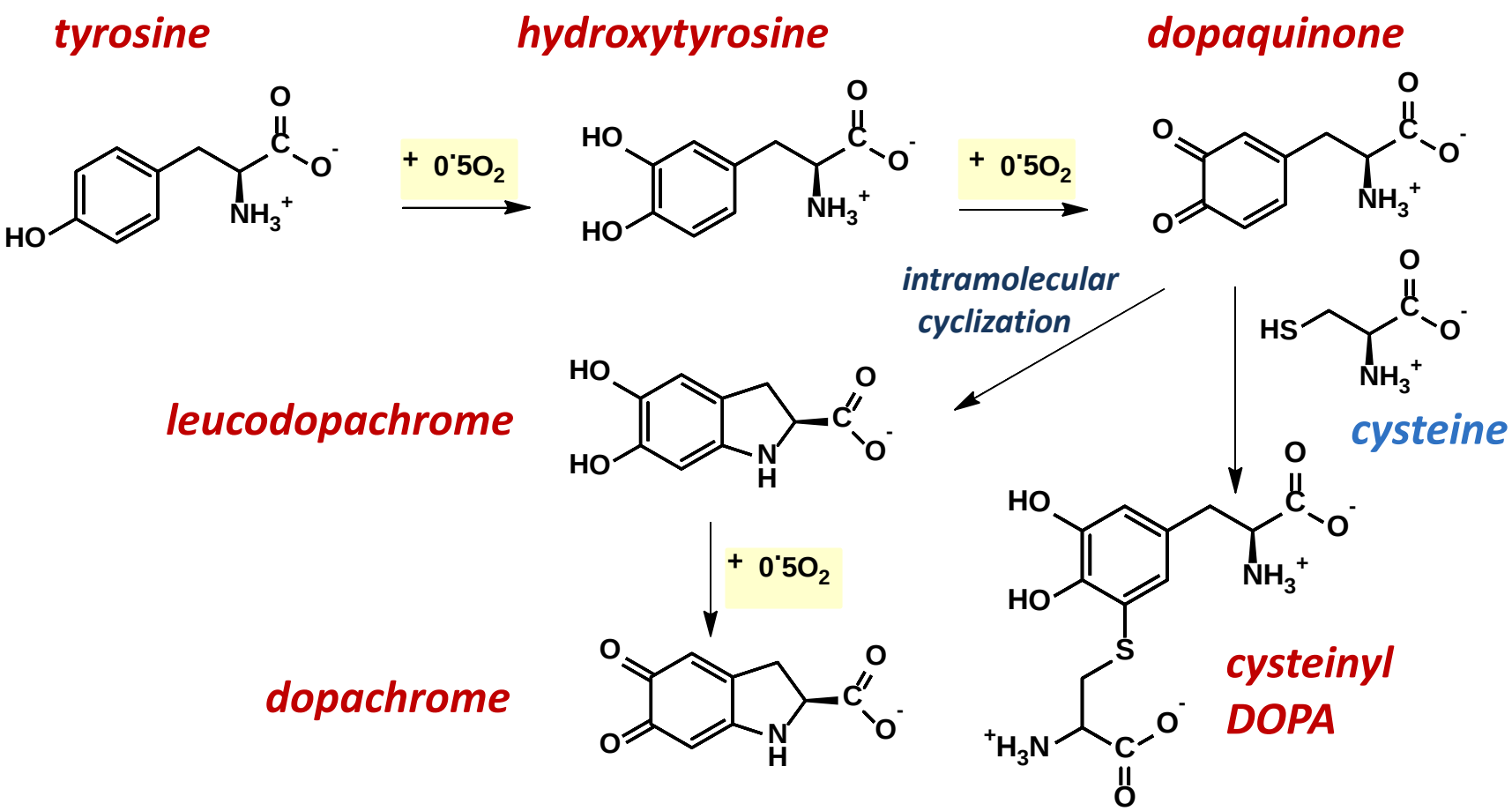
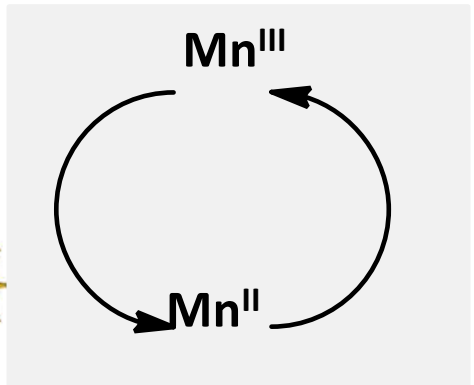
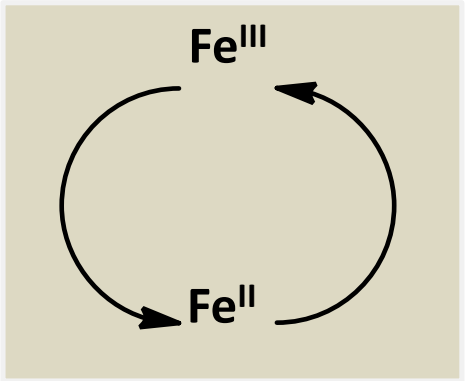
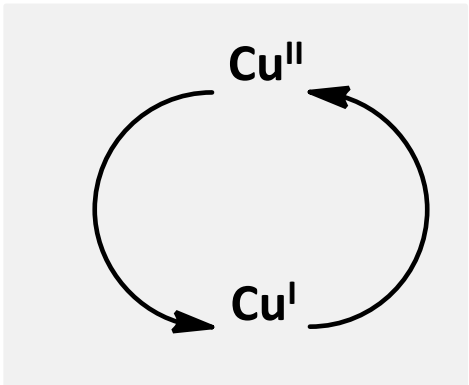


Oxidative degradation of components - possible transition metal catalysis







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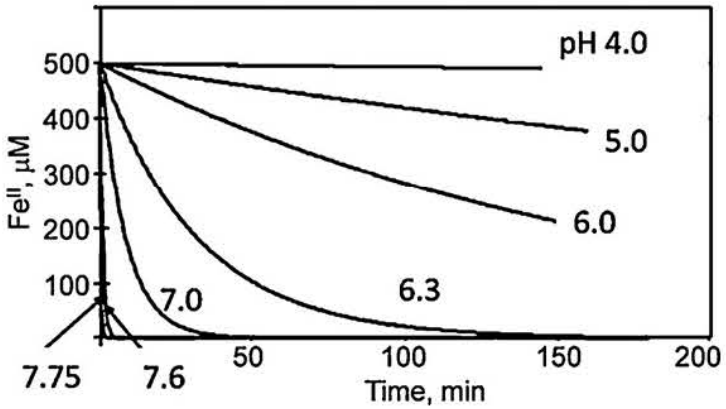
Kosman, D.J. (2013) Coord. Chem. Rev. 257: 210-217. "Iron metabolism in aerobes: managing ferric iron hydrolysis and ferrous iron autoxidation"



50 μM O_2 (human blood)

Fig. 4. Autoxidation of Fe^{II}. $Fe^{II} \rightarrow Fe^{III}$ conversion is quantified by consumption of O_2 using a Clark electrode; the electron stoichiometry was 3.8:1 at all pH values. Overlapping buffers used were 100 mM acetate, MES and MOPS.
Chen and Kosman (unpublished).

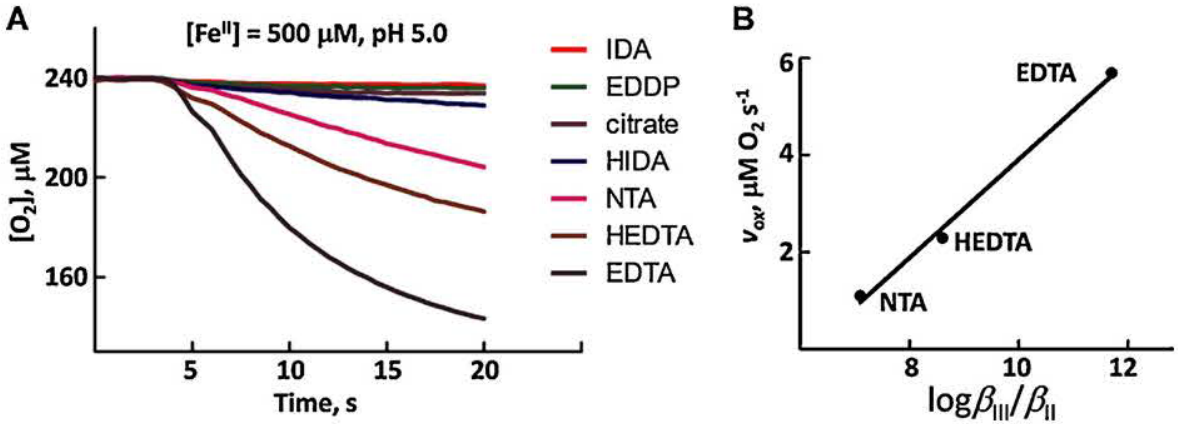


Fig. 5. 'Catalysis' of Fe^{II} autoxidation by chelation. (A) Autoxidation of Fe^{II} in the presence of oxygenous chelators of increasing ligand stability. (B) Rate equilibrium free energy relationship between relative stability of Fe^{III} complex and autoxidation rate. The buffer was 50 mM MES with [chelator] = 1 mM. In addition to citrate and EDTA, the chelators were: IDA, iminodiacetic acid; EDDP, ethylenediamine-N,N′-dipropionic acid; HIDA, N-(2-hydroxyethyl)iminodiacetic acid; NTA, nitrilotriacetic acid; and HEDTA, N-(2-hydroxyethyl)ethylenediamine-N,N′,N′-triacetic acid.
Chen and Kosman (unpublished).

Pham, A.N.; Waite, T.D. (2008) J. Phys. Chem. A 112: 643-651. "Oxygenation of Fe(II) in the presence of citrate in aqueous solutions at pH 6.0-8.0 and 25°C: interpretation from an Fe(II)/citrate speciation perspective"

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Minotti, G.; Aust, S.D. (1987) Free Rad. Biol. Med. 3: 379-387. "An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation"

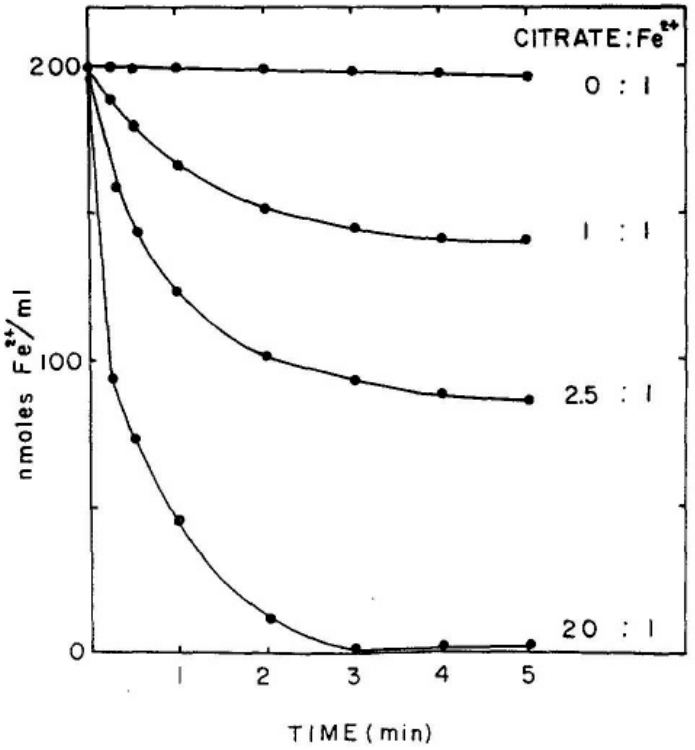
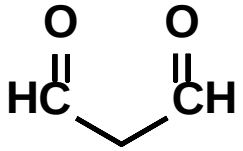


Fig. 2. The autoxidation of Fe^{2+} at different citrate–Fe^{2+} molar ratios. Incubations contained 0.2 mM $FeCl_2$ in 50 mM NaCl, pH 7.0, at 37°C. Where indicated, citrate was also added to achieve the specified citrate–Fe^{2+} molar ratios. Fe^{2+} autoxidation was monitored as described in Materials and Methods.



Malondialdehyde

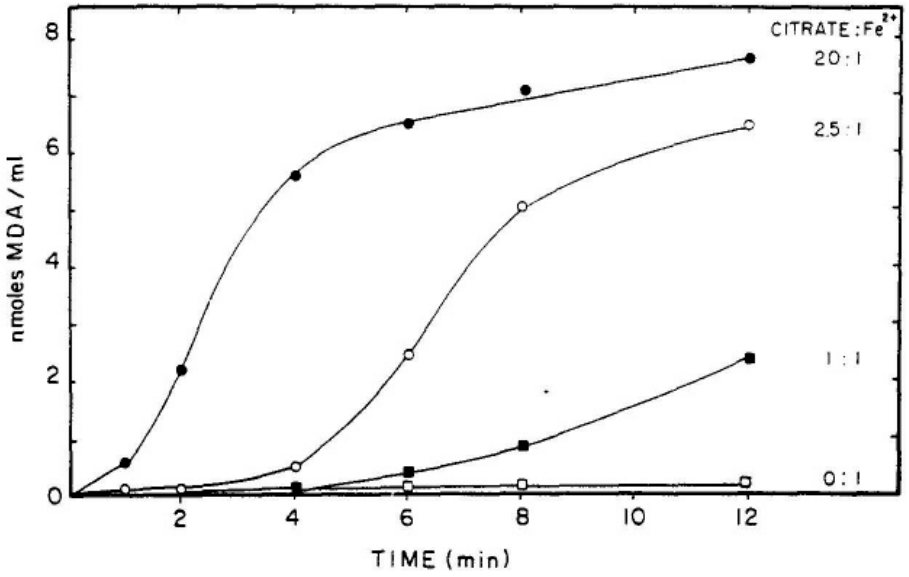


Fig. 1 The initiation of lipid peroxidation by citrate–Fe^{2+}. Incubations contained phospholipid lipsomes (1 μmole lipid phosphate/ml) in 50 mM NaCl, pH 7.0, at 37°C. Reactions were started by the addition of citrate and $FeCl_2$ at the specified citrate–Fe^{2+} molar ratios. The concentration of $FeCl_2$ was 0.2 mM. MDA formation was monitored as described in Materials and Methods.

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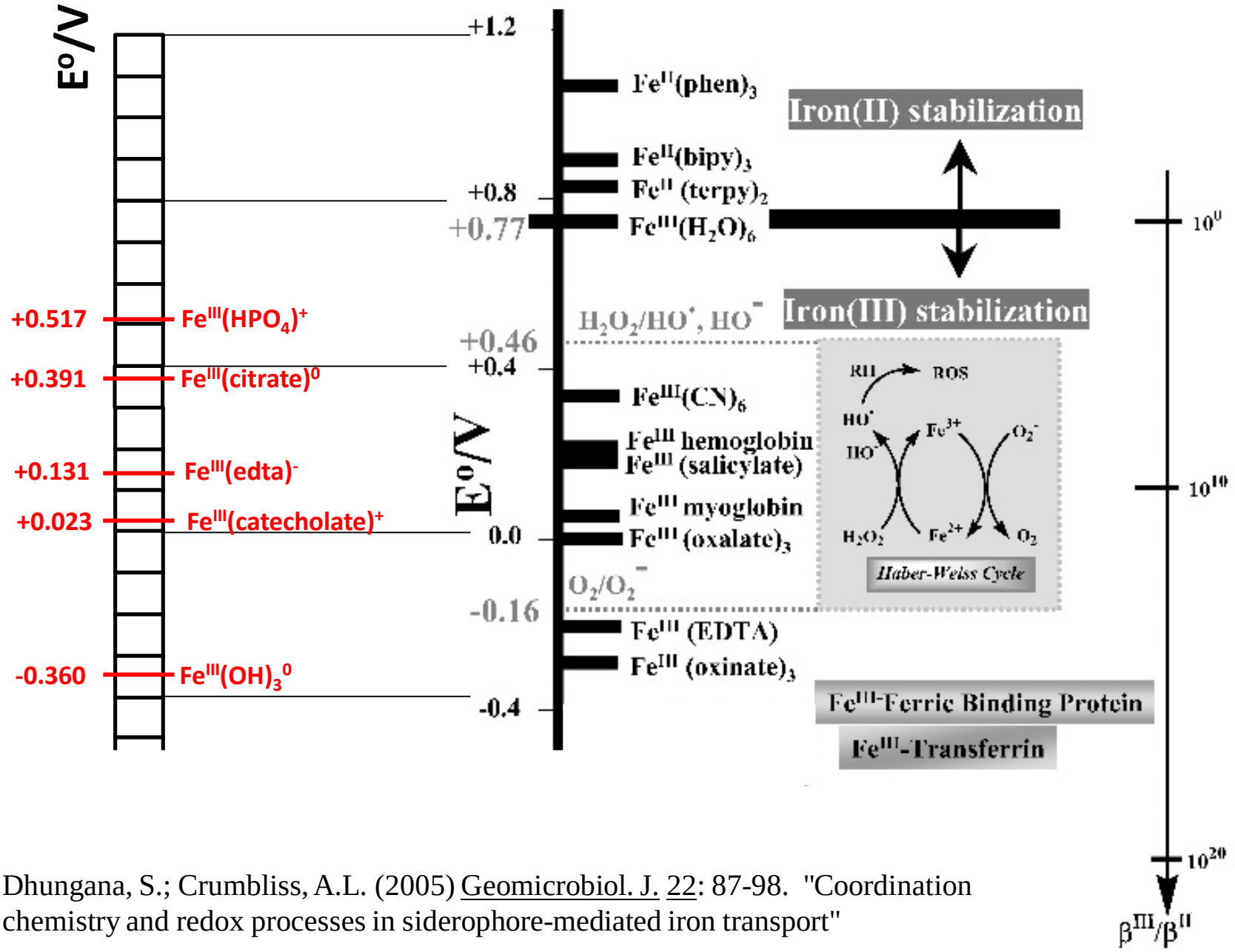

Dhungana, S.; Crumbliss, A.L. (2005) Geomicrobiol. J. 22: 87-98. "Coordination chemistry and redox processes in siderophore-mediated iron transport"

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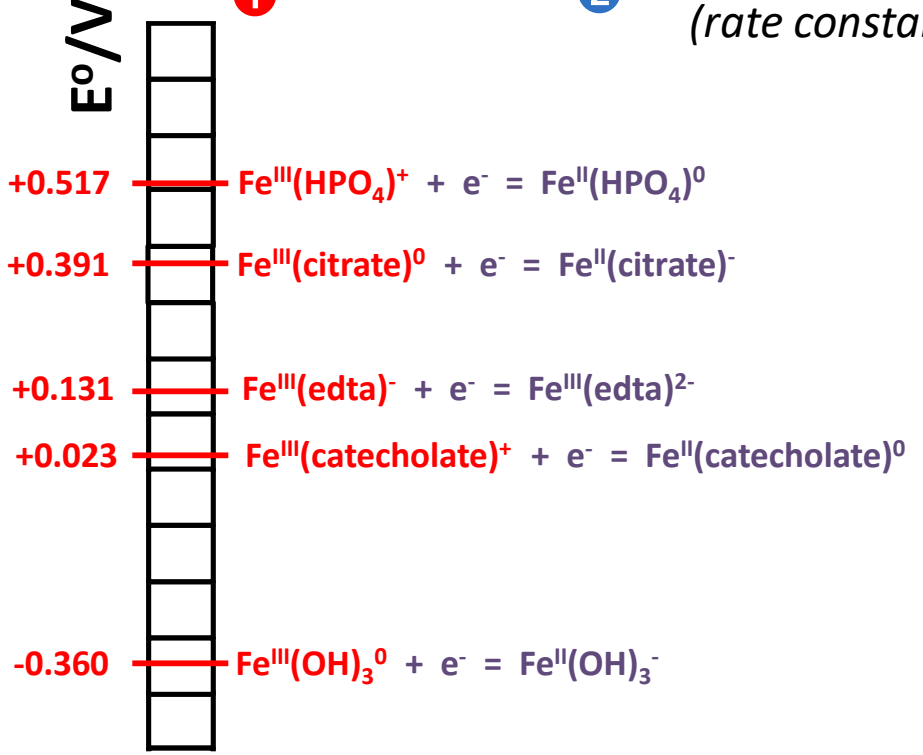
$$\frac{-d[S]}{dt} = k_1[Fe^{3+}][S] + k_2[FeOH^{2+}][S] + k_3[Fe(OH)_2^0][S] + \ldots + k_{C1}[Fe(citrate)^-][S] + k_{C2}[Fe(OH)(citrate)^-][S] + \ldots\ldots\ldots$$

❶ ❷

Relative contribution of each term given by (rate constant)x(species concentration)



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***Rate constants* are addressed using a Marcus Theory approach.**

Need half-reactions where "nothing moves" except electrons.

❷ ***Species concentrations* might be under either "pre-equilibrium" control or under kinetic control.**

$$Fe^{II}_T = [Fe^{2+}] + [FeOH^+] + [Fe(OH)_2^0] + \ldots + [Fe(citrate)^-] + [Fe(OH)(citrate)^{2-}] + \ldots$$

$$Fe^{III}_T = [Fe^{3+}] + [FeOH^{2+}] + [Fe(OH)_2^+] + \ldots + [Fe(citrate)^0] + [Fe(OH)(citrate)^-] + \ldots$$

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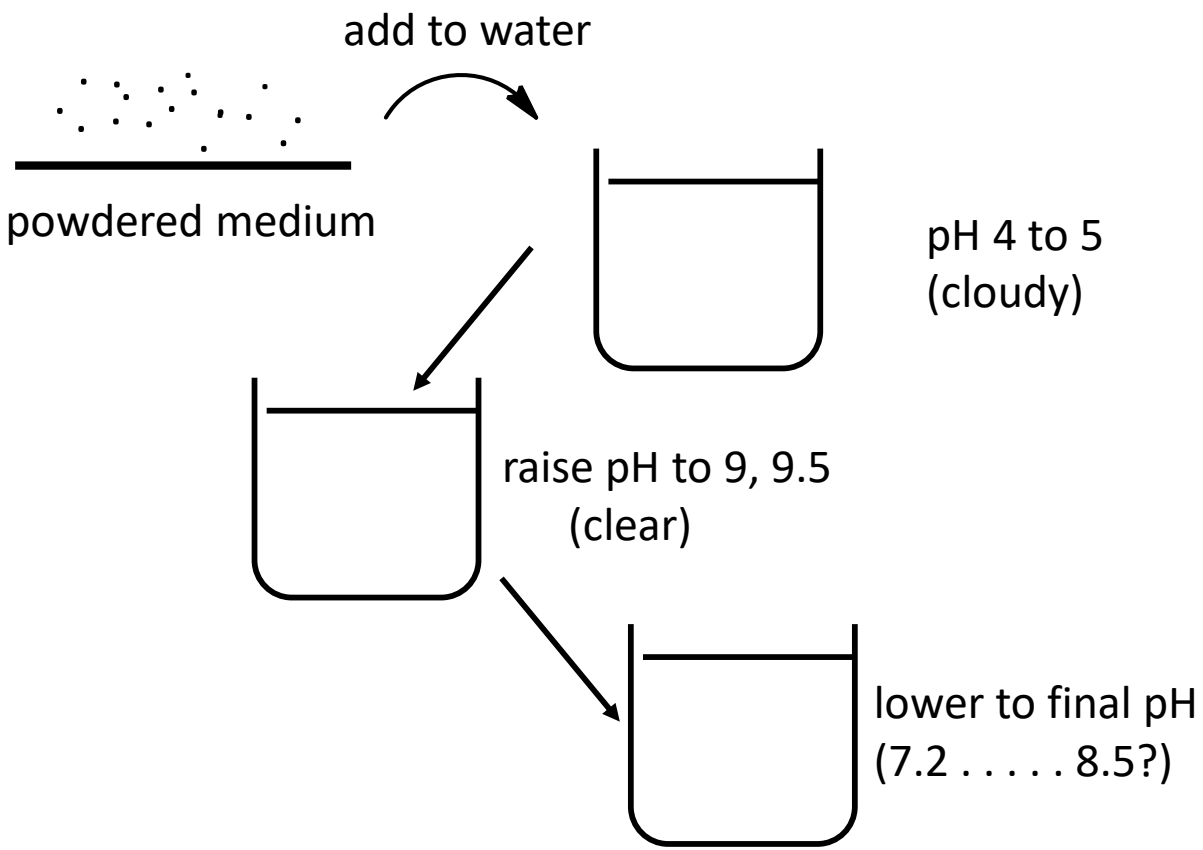

Complex formation $Zn + Y \rightarrow ZnY$

Ligand exchange $Fe^{III}L + Y \rightarrow Fe^{III}Y + L$

Metal ion exchange $Fe^{III}Y + Zn \rightarrow ZnY + Fe^{III}$

To what extent does speciation keep up with pH changes? Can "metastable" speciation yield problematic metal-chelating agent complexes?

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Immediate Plans (Next 30 days)

Visual Minteq

- Go through components list and thermodynamic data and assure they are self-consistent and robust.
- Document the steps necessary to set up, run, and interpret one Visual Minteq run.

Capillary Electrophoresis

- Make a list of all BGE (background electrolyte) attributes affecting analyses. Going down the checklist, systematically optimize the BGE.
- Use known, robust analytes (e.g. $Fe^{III}(edta)^{-}$) to test the BGE that we come up with.
- Once the new BGE has been established, perform analyses with new analytes (e.g. Zn^{II}) and with additional sample constituents (e.g. HEPES).

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Year 1 Deliverables

Visual Minteq

- Beta version of a "user's manual for AMBIC applications" that walks users through several key Visual Minteq runs.

- Solicit feedback from mentors and being compiling "frequently asked questions" and addressing them in documentation.

Capillary Electrophoresis

- Complete kinetic experiments that bracket timescales for Fe^{III} to attain equilibrium in citrate and EDTA-containing solutions.

- Establish the extent to which AMBIC 1.1 concentrations of Ca, Mg, and Zn affect timescales for Fe^{III} to attain equilibrium in citrate and EDTA-containing solutions.

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