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Determination of Provisional K_{sp} for Calcium Phosphosilicate Nanoparticles Used for Targeted Drug Delivery

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ABSTRACT

Despite significant advances in treatment options for all types of cancer, cancer is still the second leading cause of death in the world. Recently, nanomedicine and targeted strategies have emerged as promising candidates for the new age of cancer treatment. Among current research efforts, calcium phosphosilicate nanoparticles (CPSNPs) are some of the most promising due to their pH dependent solubility. CPSNPs can be surface bioconjugated with a wide variety of aptamers to target specific cells and cancers and have shown the ability to encapsulate an extensive array of chemotherapeutics and imaging agents. These nanoparticles have shown success in knocking down metastatic tumors in murine models as well. However, little is known of their quantitative solubility.

This study expands upon the work of previous studies done to determine the chemical solubility of calcium phosphosilicate nanoparticles. Previous studies determined a provisional solubility product (K_{sp}) for incorrect calcium phosphosilicate formulations. In this paper, the formulations are corrected. Calcium ion selective electrodes and inductively coupled plasma – optical emission spectroscopy (ICP-OES) are used in conjunction with the electrolyte thermodynamic simulation program from OLI Systems to develop a quantitative solubility of CPSNPs. In addition to a provisional K_{sp} for the empty CPSNPs, a provisional K_{sp} was also attempted for CPSNPs encapsulating the florescent dye Rhodamine WT and CPSNPs encapsulating Indocyanine Green dye. Quantitative knowledge of the solubility of CPSNPs will allow further tailoring of the synthesis to design a nanoparticle that dissolves in highly specific physiological environments to treat a variety of cancers on-demand.

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Chapter 1

Introduction to CPSNPs and Background on Former Work Done

1.1 Nanoparticles for Cancer Treatment

Chemotherapeutics have long been the most immediate and viable treatment option for cancer that cannot be removed through surgery or radiation. This treatment method often involves high doses of highly cytotoxic material. One of the main issues with the material is the lack of ability to distinguish and target cancer cells directly. Therefore, continuously dividing healthy cells such as skin, hair and intestinal lining cells are usually the first to be affected, resulting in a multitude of side effects such as nausea, loss of hair, and vomiting. In addition, many of the therapeutic agents are not water soluble, meaning that they must be dissolved in an organic solvent which is even more toxic.

In recent years, alternatives to traditional chemotherapy for treating cancer have arisen. These alternatives include immunotherapy, personalized medicines, and targeted drug therapy[1] to minimize adverse effects on healthy cells. Targeted therapy aims to deliver the drug right to the tumor mass. Among the most promising of targeted therapies are nanoparticle-based delivery methods, materials defined by that National Science Foundation as having at least one dimension less than 100 nm. Some different kinds of nanoparticles include polymeric biodegradable nanoparticles, metallic nanoparticles, ceramic nanoparticles, dendrimers, and liposomes[2].

One of the main advantages of using nanoparticles is that they can penetrate across small capillaries to reach individual cells and enhance drug accumulation at specific targets[2]. Due to their wide variety of differing shapes, sizes, and materials, nanoparticles can be targeted towards

specific cells[2], and they can also undergo surface bioconjugation to increase specificity. Nanoparticles have the ability to protect the drug from degradation, a large loading capacity, and a large surface area to bioconjugate molecules for biological targeting.[3]

1.2 Advantages of CPSNPs

Figure 1 shows the schematic of a calcium phosphosilicate nanoparticle (CPSNPs). The active drug or imaging agent is embedded within an amorphous calcium phosphosilicate matrix. The surface around this matrix can be conjugated with a wide variety of tethering molecules, and an aptamer can then be attached to these molecules for specific targeting.

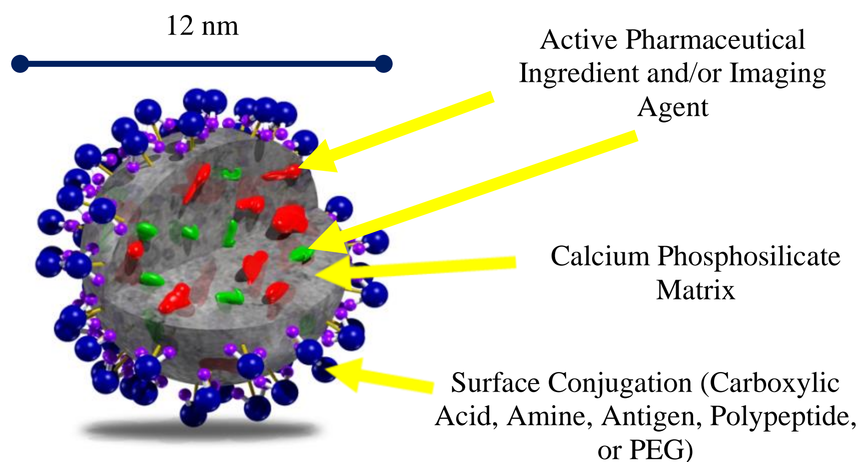

Figure 1. Structure of Calcium Phosphosilicate Nanoparticles

CPSNPs have several advantages over other nanoparticle systems. One of the main advantages is their biocompatibility and low toxicity. Calcium and phosphate are both elements that are already present in human plasma as well as in human bone and teeth in the form of hydroxyapatite[4]. The CPSNPs dissolve into ionic constituents, which prevents nanoparticle

accumulation in cells as seen with inorganic or metallic nanoparticles. These ionic constituents can then be reused by the body in processes such as bone ossification or muscle contractions. In the case that the CPSNPs fail to reach the targeted cell, they are cleared via the hepatobiliary system. In vivo mice studies have shown that polyethylene glycol (PEGylated) CPSNPs that accumulated in a tumor or that failed to reach the tumor were both excreted as fecal matter[5]. This removes the fear of bioaccumulation.

Another advantage of CPSNPs is that their solubility varies based off pH, meaning that their synthesis can be tuned to be useful at different pHs. At pH 7.4, most calcium phosphates are sparingly insoluble, and they become more soluble below a pH of 6.20[4]. Therefore, the particles will remain intact at physiological pH and then dissolve when being transported by lower pH endosomes into the cell. Tumors also have slightly lower pH, increasing the solubility of the CPSNPs[4].

A third advantage of CPSNPs is their ability to encapsulate a wide variety of imaging and biological agents as well as the ability to functionalize their exterior surface with macromolecules that target tumors. The encapsulation of drugs or imaging agents inside the amorphous matrix protects the cargo from degradation in the bloodstream[4]. The Adair group has been able to encapsulate organic dyes, oligonucleotides, and biologically active molecules such as insulin, docetaxel, and cisplatin.[4] CPSNPs have been surface bioconjugated with human holotransferrin, anti-CD71 antibody, and short gastrin peptides[5]. The bioconjugation of the human holotransferrin and anti-CD71 antibody specifically targets breast cancer cells, while the bioconjugation of gastrin peptides targets pancreatic cancer. Images of the nanoparticle targeting to transplanted mice tumors have been successfully captured.[5]

Finally, the nanoparticles have colloidal stability at physiological conditions. The synthesis yields well-defined particles sizes and morphologies[5]. The citrate capped CPSNPs are also negatively charged, which is advantageous to prevent interaction with the negatively charged and phosphate rich cell membranes.[5] The neutrally charged methoxy-polyethylene glycol (mPEG) capped CPSNPs also do not interact with the cell membrane[5], allowing for long enough dispersion to reach the target tissue.

1.3 Previous CPSNP Solubility Work

The importance of pH dependent CPSNP solubility has been discussed above. The input ratios of each of the elements is known. However, the exact chemical composition of the final CPSNPs and the stoichiometric ratios remains unknown.

Previous work has been done in determining a provisional solubility constant for CPSNPs. A calcium ion selective electrode (ISE) was used in combination with ICP - OES and the thermodynamic simulation software OLI Systems to determine the K_{sp}[6]. However, these experiments and calculations were done with incorrect formulations of CPSNPs. Due to the unknown issue of endotoxin in the water used to prepare CPSNPs, the concentration of citrate was increased to compensate for the adverse effects. Therefore, the incorrect formulations had an 3:1 Ca:Citrate concentration ratio instead of a 89:1 Ca:Citrate ratio. The excess concentration of citrate pulled some of the calcium off the nanoparticle, leading to a calcium depleted sample. Figure 2 shows the ICP – OES elemental analysis that was done on previous formulations. These results show that the stoichiometric ratios do not match synthetic parameters for any sample except JMM 2-17, in which the correct formulation without excess citrate was used. All other samples also appear to be depleted of calcium.

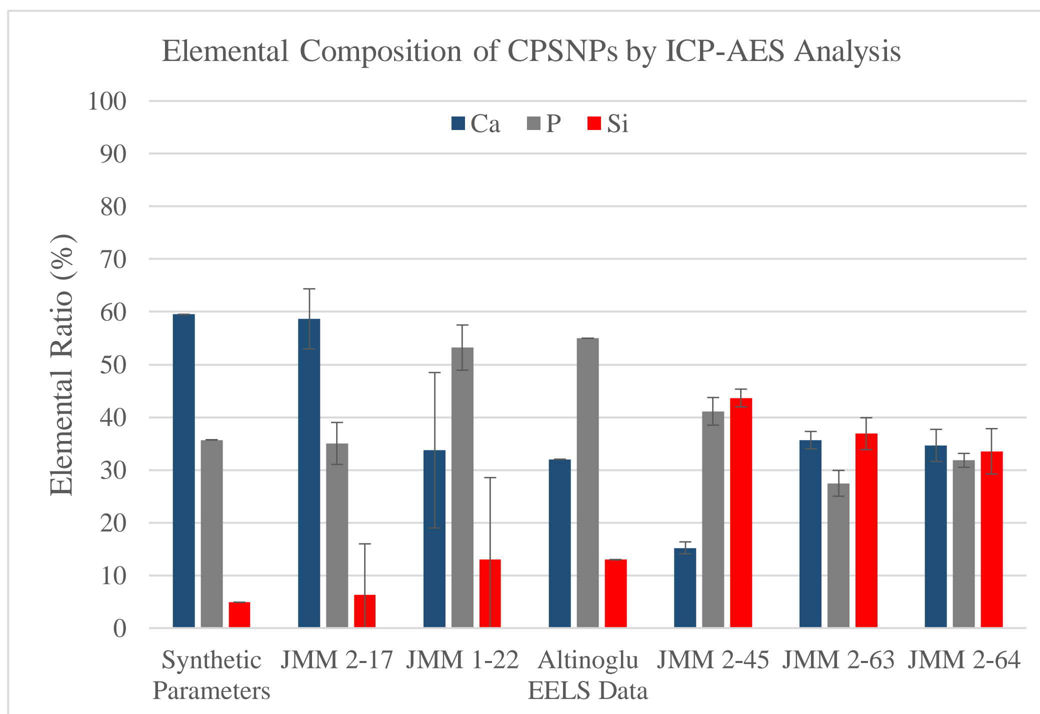

Figure 2. Comparison of different elemental ratios presented in cit-CPSNP formulations. Reprinted from reference 6.

The goal of this study is to determine a provisional K_{sp} for the correct CPSNP formulations and experimentally determine every variable in the equation below:

$$k_{sp} = [Ca^{2+}]_x[H_yPO_4^{3-y}]_z[SiO_2]_w[OH^-]$$

Chapter 2

Synthesis and Drying Down of CPSNPs

Four CPSNP samples each with different encapsulants were prepared by Bernadette Adair (Penn State, Materials Science). One formulation was done encapsulating Rhodamine WT (RWT), another formulation was done encapsulating indocyanine green (ICG) and two more formulations were empty. All four of these formulations also had citrate on the surface.

Table 1. CPSNP Samples Prepared

Sample	Encapsulant	Surface Conjugation
BMA 18-1 C2	RWT	Citrate
BMA 18-2 C2	none	Citrate
BMA 18-37 C1	ICG	Citrate
BMA 18-42 C1	none	Citrate

2.1 Materials

The CPSNPs were prepared using a microemulsion technique. Cyclohexane (C_6H_{12}, Sigma-Aldrich), Igepal® CO-520 ($4\text{-}(C_9H_{19})C_6H_4(OCH_2CH_2O)_nOH$, Sigma-Aldrich), and deionized H_2O (Millipore Filtration System) were used to prepare the two separate microemulsions. Calcium chloride ($CaCl_2{\cdot}2H_2O$, Sigma-Aldrich), disodium hydrogen phosphate (Na_2HPO_4, Sigma Aldrich), and sodium metasilicate (Na_2SiO_3, Sigma-Aldrich) were used as

reagent grade precursors to the nanoparticles. RWT ($C_{29}H_{29}ClN_2Na_2O_5$, Dyechem) and ICG ($C_{43}H_{47}N_2NaO_6S_2$, Akorn) were used as the encapsulate for in the dye-encapsulating particles. Disodium hydrogen citrate dihydrate ($HOC(COOH)(CH_2COONa)_2 \cdot 2H_2O$, Sigma-Aldrich) was used to treat the surface and cease particle growth to the desired size. Neat ethanol was obtained from Pharmco. All water used was deionized, distilled, and degassed with Argon for at least 20 minutes prior to utilization.

2.2 Synthesis of CPSNPs

To prepare the CPSNPs, two different microemulsions were combined. One microemulsion contained calcium chloride and the other microemulsion contained disodium hydrogen phosphate and sodium metasilicate either with water or with an encapsulant. After the two microemulsions were combined, disodium citrate was added to stop particle growth. KOH was then added to disrupt the micelles, leaving well-dispersed CPSNPs. The particles were then laundered to remove the excess surfactant on the surface.

2.3 Drying Down of CPSNPs

Initially, 1 mL of prepared CPSNPs in ethanol/water was taken out of each formulated 5 mL sample and diluted with 9 mL of degassed, distilled, and deionized (D^3) water. 0.1 mL of Corning Dulbecco's phosphate-buffered saline (PBS, 1X without calcium and magnesium, Mediatech, Manassas,VA) was added to this dilution. Upon drying the sample down with Argon gas, it was apparent that the samples should have been diluted in 70:30 ethanol/water instead. The dilution in pure D^3 H_2O transformed the metastable CPSNPs to thermodynamically stable

hydroxyapatite. Therefore, using these samples in ISE measurements would not have yielded activity for calcium phosphosilicate nanoparticles, but for hydroxyapatite. Therefore, 4 mL of each 5 mL sample was lost.

0.5 mL was then taken out of the remaining samples still in ethanol/water. 0.05 mL of PBS was added to each 0.5 mL sample and the mixture was dried down using Argon gas for a few hours until there was no more visible liquid. Samples were weighed at every step using a precision balance (Mettler Toledo) to determine the mass of the final dried calcium phosphosilicate nanoparticles. These masses are shown in Table 2. Flow charts for all the different drying procedure are shown in Appendix A.

Sample	Weight of Tube (g)	Weight of Tube + Sample (g)	Weight of Tube + Sample + PBS (g)	Weight of Tube + Dried Sample (g)	Weight of Dried Sample (g)
BMA 18-1 C2	7.04424	7.44773	7.48822	7.05216	0.0079167
BMA 18-2 C2	7.03531	7.44926667	7.4936	7.04246333	0.0071533
BMA 18-37 C1	7.05799	7.45631	7.49687	7.05434	-0.0036533
BMA 18-42 C1	7.05431	7.48792	7.53230	7.08652	0.03221

Table 2. Weighed Masses of CPSNP Samples through Drying Protocol

The determined final dried mass for BMA 18-37 C1 is negative. Masses on the scale kept fluctuating downward for several seconds after a sample was placed on the scale. It is possible that when initially measuring the mass of the tube, the tube was not left on the scale long enough for the scale to equilibrate to an accurate measurement. This issue could have also affected BMA 18-42 C1, where the weight of the dried sample is larger.

After drying all samples down, 10 mL of D^3 H_2O was added to BMA 18-1 C2. 2 mL was then taken out of the 10 mL of 18-1 C2 and used in ISE measurements to see whether the concentration of Ca was above ISE detection limits.

Chapter 3

Determination of Calcium Activity Using Ion Selective Electrode

3.1 Calibration Procedures

A calcium ion selective electrode (ISE) with replaceable membrane was obtained from Cole-Parmer and calibrated immediately before each sample measurement. A 10 ppm Ca^{2+} stock solution and 100 ppm Ca^{2+} stock solution (100 ml) were prepared by diluting a 1000 ppm Ca^{2+} standard (Cole-Parmer) with degassed, deionized water. A 1 vol% PBS solution (100 mL) was prepared by dilution with degassed, deionized water. The reference chamber of the ISE was filled with 4M KCl reference solution (Cole-Parmer). The assembled electrode was swung in a downward motion to remove trapped air bubbles within the apparatus and the membrane was conditioned in a 1% by volume PBS solution for 1 hour prior to measurements. The calcium ISE was calibrated by adding specific volumes of 10 ppm Ca^{2+} stock solution stepwise to 50 mL of 1% PBS and measuring the potential on a Corning Pinnacle 530 Meter.

Preliminary work was done to figure out the lower detection range on the ISE.
An example calibration curve is shown in Appendix B. From this curve, it was determined that the minimum amount of Ca detected by the ISE is 0.05988 ppm. When activity of the 2 mL sample BMA 18-1 C2 was measured, the correlation coefficient value on the curve was only 0.96. The reason for this lower value could have been because the probe had not been used for months prior to the measurement and had not been conditioned in deionized water prior to the procedure.

The volumes added, resulting concentrations, and potential measurements are shown in Table 3. The calibration curve is shown in Figure 3.

Table 3. Sample calcium ion selective electrode calibration steps, volumes added, concentrations, and potential measurements. Measurements were performed on a pre-conditioned Cole-Parmer calcium ion selective combination electrode and a Corning Pinnacle 530 Meter.

Step	Volume of 10 ppm Ca^{2+} Added (mL)	Ca^{2+} (M)	Ca^{2+} ppm	Average Activity (mV)
1	0.2	9.940×10^{-7}	0.039840637	-46
2	0.2	1.988×10^{-6}	0.079681275	-42
3	0.4	2.9743×10^{-6}	0.119205717	-36
4	1	6.873×10^{-6}	0.275443511	-27
5	1	9.785×10^{-6}	0.392156863	-19

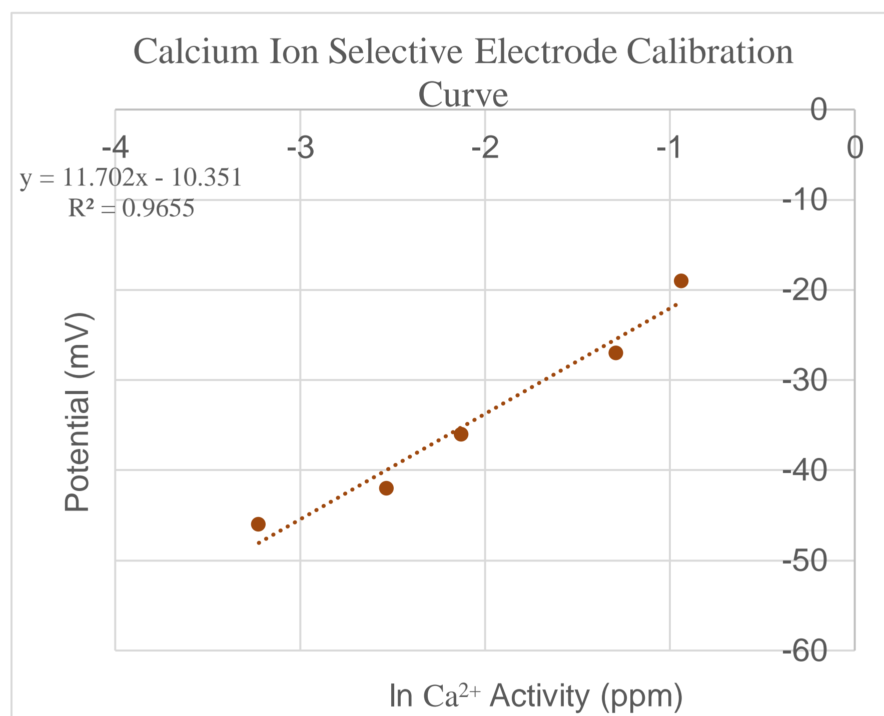

Figure 3. Sample calcium ion selective electrode calibration curve corresponding to the measurements in Table 3. A large slope such as the one in the figure is indicative of good electrode performance. However, the low R^2 value is not precise enough for determination.

3.2 CPSNP Measurement Methods and Results

The 2 mL sample of BMA 18-1 C2 was put in a 25.0 °C water bath for one hour. The temperature of the sample was measured as 25.0 °C at the time of ISE measurement, and the calcium activity measured was -40 mV, corresponding to 1.89×10^{-6} ppm Ca on the curve above. The pH of the sample was also measured at this time, and the pH was 6.24. This pH was lower than the expected pH of 7.4. The lower pH may be due to carbon dioxide dissolving from the air into the solution or the lower pH of the D^3 water the particles were suspended in.

Chapter 4

Stoichiometry of CPSNPs using ICP-OES

To determine the provisional solubility without calcium ISE data, it was necessary to determine the stoichiometric ratios of each principal element in the calcium phosphosilicate nanoparticles. This data was then put into OLI Systems to determine the provisional K_{sp}. Previously established elemental ratios of CPSNPs were not stoichiometric due to the excess citrate used in the formulations. The synthetic input ratios are 59:36:5 for Ca:P:Si, and it was hypothesized that the stoichiometric ratios measured in this study would be similar.

4.1 Background on ICP – OES

ICP – OES stands for Inductively Coupled Plasma Optical Emission Spectroscopy, and it has been widely regarded as one of the most sophisticated multi-element analysis techniques. Due to its low detection limits and high precision,[7] the technique has been used in official testing of drinking water, proteins, food, medicine, and more.[8]

The Inductively Coupled Plasma part of the instrument involves a nebulizer, pump, and a torch tube. Argon gas is supplied to the torch tube, and an electromagnetic field is generated by a coil and positioned towards the torch tube. The electromagnetic field ionizes the gas, forming plasma with high electron density and at high temperature (10,000 K). The digested liquid sample is then introduced into the tube using a pump into the nebulizer. The nebulizer turns the liquid sample into tiny, mist-like droplets. The smaller droplets are directed into the tube, where the plasma destroys the chemical bonds in the sample and the resulting atoms are excited to a

higher state. When the atoms return to a lower energy state, emission rays corresponding to the specific atom are released.[8]

These emissions are transferred to the Optical Emission Spectroscopy part of the instrument. An induction coil within the plasma tube allows for the wavelengths emitted from the different elements to be separated and measured. A detector quantifies the light intensity for each wavelength. Using analytical software, these intensities are quantified into concentration units for the specific element.[8] Detection limits for elements of interest inside calcium phosphosilicate nanoparticles are shown in Table 4.

Table 4. Detection limits of calcium, phosphorus and silicon, the active components of calcium phosphosilicate nanoparticles, in parts per billion (ppb).

Element	**Detection Limit (ppb)**[9]
Ca	0.1-1.0
P	50-100
Si	10-50

4.2 ICP – OES Methods

Because the presence of organic solvents can impact ICP – OES, samples in ethanol water underwent another drying procedure. To 0.5 mL of CPSNPs, in 70:30 ethanol/water, 0.005 mL of PBS was added. The samples were then dried under a stream of Argon gas and resuspended in 0.5 mL of D^3 H_2O.

Due to issues in obtaining tubes for the ultracentrifuge, this work is still pending. However, the protocol for sample prep for ICP – OES was fully developed for the future. The dried sample resuspended in D^3 H_2O would be ultracentrifuged. The supernatant would be pipetted out and the

remaining pellet will be suspended in 10 mL of 0.3 M HNO_3 at 43.0 °C for 16 hours. This solution would then be analyzed against EPA standards for calcium, phosphorous, and silicon.

Chapter 5

Future Work

In this study, limited amounts of sample were prepared, and much of this work focuses on refining a protocol to calculate the provisional K_{sp} for the correct formulations of CPSNPs. In future studies, larger amounts of sample and more batches should be prepared and tested to verify that a similar provisional K_{sp} is calculated based off experimental data each time. Statistical analysis using software like ANOVA should also be used to determine if differences in K_{sp} values are statistically significant.

The calcium ISE would also give more reliable results with greater sample concentration. The calibration curve for the ISE should be graphed and an R^2 value larger than 0.99 should be verified before measuring the activity of any sample.

CPSNPs should be prepared with different encapsulants and with different aptamers attached. The physical characteristics and provisional K_{sp} of each of these would be determined experimentally. There would then be an accumulated database of physical characteristics for each CPSNPs to reference as desired for different diseases.

Appendix A

Flow Diagrams for Drying Protocols

1st Drying Protocol for Calcium ISE

Start with 5 mL of CPSNPs in 70:30 ethanol/water.

Take out 1 mL of CPSNPs in in 70:30 ethanol/water and put them in a 15 mL falcon tube.

Add 9 mL of D^3 H_2O to the falcon tube.

Add 0.1 mL of PBS to the falcon tube.

Dry under a stream of Argon gas overnight.

2nd Drying Protocol for Calcium ISE

Start with 1 mL of CPSNPs in 70:30 ethanol/water.

Take out 0.5 mL of CPSNPs in 70:30 ethanol water and put them in a 15 mL falcon tube.

Add 0.05 mL of PBS to the falcon tube.

Dry under a stream of Argon gas for 3 hours.

Add 10 mL of D^3 H_2O to BMA 18-1 C2.

3rd Drying Protocol for ICP - OES

Start with 1 mL of CPSNPs in 70:30 ethanol/water.

Take out 0.5 mL of CPSNPs in 70:30 ethanol water and put them in a 15 mL falcon tube.

Add 0.005 mL of PBS to the falcon tube.

Dry under a stream of Argon gas for 2 hours.

Add 0.5 mL of D^3 H_2O to each sample.

Put samples in appropriate centrifuge tubes and place in an ultracentrifuge at 120,000 rpm.

Pipette out supernatant liquid, leaving solid pellet.

Add 10 mL of 0.3 M HNO_3 to supernatant. Let sit for 16 hours at 43.0 °C.

Appendix B

Determination of Calcium ISE Lower Detection Limit

Table 5. Bolded points are within the detection limit of the ISE. To determine the lower detection limit, multiple calibration lines were graphed excluding earlier steps. The first step included in the curve with the highest R^2 value was determined as the lower limit.

Step	Volume of 10 ppm Ca^{2+} Added (mL)	Ca^{2+} (M)	Ca^{2+} ppm	Average Activity for 4 Trials (mV)
1	0.1	4.98×10^{-7}	0.01996008	-25.25
2	0.1	9.96×10^{-7}	0.03992016	-23.75
3	**0.1**	$\mathbf{1.49\times10^{-6}}$	**0.05988024**	**-22**
4	**0.2**	$\mathbf{2.49\times10^{-6}}$	**0.09972088**	**-18**
5	**1**	$\mathbf{7.38\times10^{-6}}$	**0.29579931**	**-7.5**
6	**1**	$\mathbf{1.23\times10^{-5}}$	**0.49187774**	**-1.5**
7	**1**	$\mathbf{1.72\times10^{-5}}$	**0.68795617**	**2.5**
8	**1**	$\mathbf{2.21\times10^{-5}}$	**0.8840346**	**6**
9	**1**	$\mathbf{2.70\times10^{-5}}$	**1.08011303**	**8.5**
10	**1**	$\mathbf{3.18\times10^{-5}}$	**1.27619147**	**10.25**

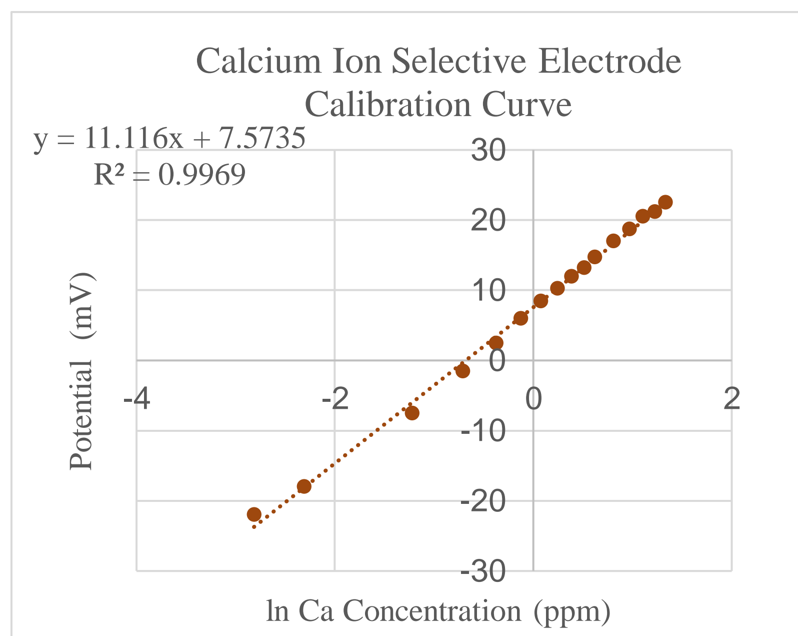

Figure 4. Sample Calibration Curve for Calcium ISE. Bolded values in the table above are graphed.

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