CHEMICAL APPLICATIONS IN TECHNIQUES OF EMERGING SIGNIFICANCE:
NANOPARTICLE TRANSFORMATION IN MITOCHONDRIA AND RELATIVE
TAUTOMER POPULATIONS IN CELLULAR AUTOMATA

A thesis submitted in partial fulfillment of the
requirements for the degree of

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Gregory Arland Bowers ENTITLED Chemical Applications in Techniques of Emerging Significance: Nanoparticle Transformation in Mitochondria and Relative Tautomer Populations in Cellular Automata BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT


Two techniques of emerging importance were used to study different biochemical phenomena: the fate of silver nanoparticles in Vero 76 African green monkey kidney cell mitochondria, and the effect of pH on tautomer ratios using cellular automata. In the first area of research Vero 76 Africa green monkey kidney cells were incubated with silver nanoparticles (AgNP) and ionic silver (Ag⁺). After 24 hours the cell mitochondria were harvested, then processed with CPE-TFF and ICP-OES. The initial AgNP incubation formed 52.9% ± 15.5% AgNP and 29.9% ± 4.0% Ag⁺, while the ionic silver incubation formed 9.5% ± 0.9% AgNP and 60.6% ± 6.3% Ag⁺. In addition to this, relative proportions of tautomers in solutions of differing pH were examined using cellular automata. The rule-based, bottom-up design of cellular automata can cause complexity to arise as an emergent property rather than being a programmed variable. The tautomeric equilibrium of 9-anthrone, 9-anthrol, and their common ion was studied. pH values of 4, 7, and 10, along with the tautomeric equilibrium equation and Henderson-Hasselbalch equation, and accompanying pKₑ and pKₐ values were used to determine simulation parameters. At pH values of 4 or 7, 9-anthrone was found to comprise over 99% of the total population, while at pH 10 both 9-anthrone and the common anion (deprotonated 9-anthrol) contributed 49.8% of the total population. In all cases 9-anthrol contribution was
so small as to be considered inconsequential. In all cases results were found to agree with those obtained from differential equations, with less time and derivation involved while accounting for such realities as stochasticity and a system of non-continuous components.
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THESIS INTRODUCTION

The work for this Master’s Degree was done with two different research advisors, which two very different areas of focus.

Nanoparticles and Mitochondria

This work examined the interaction of silver nanoparticles with mitochondria. Biological cells were incubated with the nanoparticles for a set period of time. Then, the mitochondria of those cells were isolated. Through Cloud Point Extraction coupled with Tangential Flow Filtration (CPE-TFF), along with Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), the state of nanoparticles on or in the mitochondria was probed.

Cellular Automata and Tautomers

This work focused on the use of a cellular automaton model to examine tautomer equilibria. Tautomers are a fundamental part of chemistry, which involve the movement of protons around a structure based on various “environmental” factors such as pH. This particular study examined the tautomer equilibrium in 9-anthrone and 9-anthrol at multiple pH values. By examining different pH values, different environments (such as a biological one) could be emulated, and the molecule(s) of study’s behavior in that environment could be better understood.
SILVER CONTENT AND SPECIATION DETERMINATION IN AFRICAN GREEN MONKEY KIDNEY CELL MITOCHONDRIA USING CPE-TFF AND ICP-OES

Acknowledgement. This project was a joint venture between Ali Akbar and the writer, with both doing various aspects of the work and thus reporting joint results.

Introduction

Humans interact with a wide variety of substances every day. One of these is nanosilver, which has seen extensive personal, commercial, and medical use.

Personal use of silver is recorded from many millennia ago. The use of a “silver cup” is recorded in the book of Genesis. The historical age of this book is of course contentious, but the assembling of the Torah (the first five books of the Bible) is estimated, at latest, as the 5th century BC\textsuperscript{1}. Alexander the Great (335 B.C.) was known to use silver for water storage as well\textsuperscript{2}. In more recent times, the term “silverware” is an obvious sign of the use of silver in daily life, though actual silver is rarely encountered anymore.

Medical use of silver extends back many years as well. Silver has been used to treat burn wounds for over 200 years\textsuperscript{2}. In the 1880s, a silver nitrate solution was found to reduce the incidence of neonatal eye infections\textsuperscript{2}. More recently, it has been used for wound dressings, catheters, and water purification\textsuperscript{2}.

Silver has also seen commercial application in more recent years. Products include water filters\textsuperscript{3}, disinfectants\textsuperscript{3}, and silver-coated fabrics such as sports socks\textsuperscript{4}. The purpose behind
all of this silver use may already be clear: Silver has been shown to have antimicrobial properties\textsuperscript{5-8}. Studies with both gram-positive (\textit{S. aureus}) and gram-negative (\textit{P. aeruginosa}) bacteria have shown the inhibitory and bactericidal (at concentrations ranging from 3150-8985 µg mL\textsuperscript{-1}) effect of silver\textsuperscript{9-11}.

The reason for this antimicrobial effect is not fully known, and is currently under intensive study. However, while specific mechanisms have not been completely identified, the primary silver source of the effect is fairly well-known: ionic silver. Out of bulk, nanoparticle, and ionic silver, ionic has been shown to have strong antimicrobial effects\textsuperscript{12-13}. Possible causes for the effect (spanning all types of silver) may include inhibition of cell wall synthesis\textsuperscript{9}, production of radical species such as reactive oxygen species (ROS)\textsuperscript{7}, dephosphorylation of some proteins necessary for bacterial growth\textsuperscript{14}, and DNA damage upon interaction with the silver\textsuperscript{15}.

However, the antibacterial nature of silver necessitates a further concern: potential cytotoxicity of silver. The same mechanisms which make silver toxic to bacterial cells may also make it toxic to animal or plant cells. This has been shown to be the case, with ionic silver having a cytotoxic effect at concentrations as low as 5 µg mL\textsuperscript{-1} for both non-human\textsuperscript{16-17} and human cells\textsuperscript{18-19}.

At this point, the strengths of silver nanoparticles become important. A silver nanoparticle is a conglomeration of silver atoms arranged in some shape, with at least one dimension on the nano scale. The size and shape of the particle affects its properties\textsuperscript{20-21}. It is typically suspended in a liquid as a “colloid,” with some type of capping agent
protecting the outer layer of silver and giving the particle a charge as well as preventing aggregation.

Though silver nanoparticles are capped with some chemical moiety, they have been shown to release silver ions over time\textsuperscript{22-24}. The rate of release depends on various factors such as the pH of the solution\textsuperscript{24}, capping agent\textsuperscript{23}, surface area to size ratio\textsuperscript{23}, oxidation from oxygen\textsuperscript{24}, and likely other factors. It should be noted that silver ions can also form silver nanoparticles, demonstrated in one case by interaction with humic acid\textsuperscript{22}. Given that even moderate concentrations of silver ions have been shown to have cytotoxic effects, one might conclude that the use of silver nanoparticles allows one to add silver to a solution and then release silver ions over time, in concentrations low enough to avoid the cytotoxic effect while maintaining the antibacterial effect.

Previous studies have examined the interaction between silver nanoparticles and mammalian or human cells themselves. However, to better understand the effect of the silver on the cells, a more in-depth study is necessary, looking within the cell for specific organelles with implications in cell growth and death. One such organelle is the mitochondrion.

The mitochondrion has been described as “the powerhouse of the cell.” It is the main site of ATP production, the source of energy for a cell. It is an organelle with its own genome, which encodes several proteins specific to mitochondrial use\textsuperscript{25}. That genome is circular, like the genome of prokaryotes, meaning that the mitochondria may have originated from prokaryotes before being brought to eukaryotes. It has an outer and inner
membrane, with an intermembrane space between them, and a matrix located within the inner membrane. The outer membrane has channels allowing the diffusion of molecules or particles smaller than 1000 Daltons\textsuperscript{25}. The name “mitochondrion” is somewhat inadequate for description—mitochondria are dynamic, constantly fusing and separating in a cell. It plays a fundamental role in apoptosis: the actions of certain proteins can cause the formation of pores on the mitochondrial outer membrane, causing the release of cytochrome $c$ (a component of the electron transport chain, used in the synthesis of ATP) and the activation of caspace-9 (an enzyme that cleaves proteins), leading to cell death\textsuperscript{25}. Given these factors, mitochondria are uniquely suited to study with regards to silver nanoparticle and ion interaction.

As mentioned before, previous work has examined the interaction between a cell and silver nanoparticles. Some studies have examined the effect of silver nanoparticles on mitochondria\textsuperscript{26-28}. A few effects noticed were decreased metabolic activity\textsuperscript{27}, ROS generation\textsuperscript{27}, ATPase activity inhibition (implying increased membrane permeability)\textsuperscript{28}, mitochondrial membrane potential depolarization\textsuperscript{26}, and caspace-3 activation\textsuperscript{26}. However, no studies to date have examined the AgNP/Ag$^+$ relative amounts, arguably a critical component of nanosilver’s toxic effects. Thus, to better understand the effect of the silver nanoparticles and silver ions on the cells, a more in-depth study is necessary, examining this partitioning of the two.
Previous work has been done with a variety of cells. Research within the group was done with Vero 76 African green monkey kidney cells. This was replicated for the current study, with additional processing to examine isolated mitochondria.

The importance of both silver nanoparticles and ionic silver in potential cytotoxicity has already been discussed. Given this, a method to probe the status of each within the cell was needed. This was accomplished through a method called Cloud Point Extraction (CPE), which can delineate between charged and non-charged species: by adjusting the pH of a mixture with silver nanoparticles and silver ions, one can neutralize the charge of one of the two, separating both out into two phases. Further separation is done through tangential flow filtration (TFF). Once the two have been separated, quantification is performed through Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

The entire process is thus defined: synthesis of nanoparticles, culturing of cells, incubation of cells with nanoparticles, harvesting of cells and isolation of mitochondria, separation of silver moieties in the cell and mitochondria through CPE-TFF, and finally quantification of the amounts of both through ICP-OES.

The first step of this process, the synthesis of nanoparticles, follows procedures well established by this group and others\textsuperscript{29-31}. It involves the mixing of silver nitrate and sodium borohydride in a cooled solution, causing the formation of silver nanoparticles, followed by filtration through TFF.
In the TFF portion of the synthesis, a colloidal suspension is passed through a recirculating loop that includes a peristaltic pump, a filter, and several collection containers to achieve size selection, concentration, and purification of colloidal AgNPs. As liquid enters a membrane filter of a pore size in the 0.65 – 1000 kD range, it flows across (or tangential to) the filter rather than directly against it. Pressure from the pump pushes particles smaller than the filter pore size through the filter, thereby size-separating the particles in the liquid. Particles which have passed through the pores are deposited in a secondary container as the **permeate**. In addition to this, a user-determined amount of liquid is passed into the permeate. The **retentate** or colloid that has not passed through the pores can be recirculated as many times as desired for further filtration, and concentration (through addition of particles to a constant liquid volume).

Before use and after synthesis, the silver synthesized is characterized. This involved UV-Visible Light absorption spectrophotometry, Raman spectroscopy, and ICP-OES.

Some nanometals, including silver, undergo surface plasmon resonance with excitation by light. This is caused by oscillation of electrons on the surface of the metal, and results in absorption of light at certain wavelengths (governed by the size and shape of the nanoparticle). This can be seen with a UV-Vis absorption spectrophotometer, which gives a peak in the 390-430 nanometer (nm) range for silver nanoparticles as evidence of this effect. The width of the peak can be correlated to the dispersity of particle size in the sample.
In addition, Raman spectroscopy can be performed to validate the purity of the sample. Raman spectroscopy involves light (typically laser) excitation energizing vibrational and rotational modes of chemical bonds. An impure sample would have spectroscopic peaks corresponding to those unwanted chemicals and their bonds.

Finally, ICP-OES is performed on the colloid to determine the final concentration. ICP-OES uses a plasma vaporizer to ignite a sample, which causes it to emit radiation at element-specific wavelengths. Each metal of interest has specific emission wavelengths. With the use of a calibration curve, the concentration of the metal of interest in the sample can be determined. It should be noted that in the preparation of a silver nanoparticle sample for ICP-OES analysis, silver nanoparticles are “digested” with nitric acid (HNO₃) and oxidized to silver ions, Ag⁺. Thus ICP-OES determines the total silver content of silver ions, which is presumed to be the same as silver nanoparticles in the original sample.

Following the culturing, incubation, and harvesting of cells and mitochondria, CPE-TFF and ICP-OES were performed.

CPE involves the separation of phases of a heterogeneous mixture. In this technique, a surfactant is added to the mixture in order to form micelles (surfactant phase), which can encapsulate the “hydrophobic” or nonpolar portion of the mixture when heated to the “cloud point” temperature (the temperature at which the micelles form a separate, surfactant-rich layer). The aqueous phase then contains any hydrophilic or polar
components of the mixture. These two phases can then be separated through centrifugation followed by filtration (TFF in this specific instance).

This CPE-TFF technique is very useful for the separation of silver nanoparticles (AgNPs) and ionic silver (Ag⁺). Although the silver nanoparticles used in this experiment have a negative charge, by adjusting the pH of the mixture one can reach the point of zero charge, leaving the AgNP uncharged (or nonpolar) and the silver ions charged. Thus, by employing this method one can discover the relative amounts of either species in a sample.

The primary motivation for this study is the lack of knowledge on the fate of silver, ionic or nanoparticle, in the cell, and more specifically the mitochondrion. Given the implications of only one of those species (ionic silver) being the main mechanism for toxicity, the relative amounts of each is important. The mitochondrion is chosen because of its vital importance in both cell growth and death, as well as its similarities to prokaryotes, or the domain Bacteria.

The Main Goal of this study is to quantify the AgNP/Ag⁺ relative amounts present within the whole cell and mitochondria, through the use of a number of chemical techniques. They include CPE-TFF and ICP-OES.

In order to determine expectations for the results of the study, a bit more understanding of mitochondria is necessary. As previously mentioned mitochondria have an outer and inner membrane surrounding them. Because of the electrochemical activities of mitochondria (partly in ATP synthesis), there is a mitochondria membrane potential
(MMP) and a pH gradient\(^{25}\). The membrane potential is known to be negative (-120 mV\(^{32}\)), while the pH of the interior is slightly elevated at 8 from a standard cytosolic pH of ~7\(^{25}\).

The basic impetus for this study is the belief that mitochondria will interact significantly with silver in any form. The justification for this claim is simply the known toxic effects of silver, as described before, and how these toxic effects relate to mitochondria. One simple example is the proposed ROS generation of silver ions\(^7\): ROS is known to be generated in significant amounts from mitochondria\(^{33}\).

Separate from and beyond this initial statement, both the negative membrane potential and the slightly basic interior environment of the mitochondria support the supposition that a mixture of initially negatively charged AgNPs and Ag\(^+\) in the mitochondria will have a higher proportion of Ag\(^+\), compared to the proportion found in the cell itself.

In order to achieve the main goal and examine the hypothesis, the following specific aims were completed.

**Specific Aim 1**: Culture and incubate cells with silver nanoparticles and ionic silver, harvesting and isolating mitochondria.

**Specific Aim 2**: Perform Cloud Point Extraction combined with Tangential Flow Filtration to separate AgNPs and Ag\(^+\).

**Specific Aim 3**: Quantify total silver content and relative amounts of AgNPs and Ag\(^+\) with Inductively Coupled Plasma Optical Emission Spectroscopy.
Through understanding of the relative amounts of these silver species in the mitochondrion, one can gain insight into the impact of silver nanoparticle usage on the mitochondrion.

**Methods**

**AgNP Synthesis**
Silver nanoparticles were synthesized following previously reported Creighton procedures\(^{29-30}\). A solution of 1.0 mM silver nitrate (AgNO\(_3\)) and a solution of 2.0 mM sodium borohydride (NaBH\(_4\)) were first made in high quality water (18 MΩ). Three hundred (300) mL of the NaBH\(_4\) solution was placed in a 500 mL Erlenmeyer flask, which was kept on ice with a magnetic stir bar within. Next, 50 mL of the AgNO\(_3\) solution was titrated into the flask at a drop rate of 1 drop per second, while stirring strongly with the stir bar. After the mixture was complete, evidenced by a light yellow color, the stir bars were removed from the colloidal suspension. Six independently prepared batches were collected into one large container for filtration and characterization.

**AgNP Filtration**
Filtration was achieved through Tangential Flow Filtration (TFF) following well-established protocols\(^{29}\). In this work, a two-step TFF process was employed with a KrosFlo Research 2i, using modified polyether sulfone filters (Midi Kros). In the first step, the colloid was pumped through a 500 kD filter (surface area of 790 cm\(^2\), pore size...
of 50 nm), keeping the permeate. This was performed again on the permeate with a 10 kD filter (surface area of 790 cm$^2$, pore size of 5 nm), keeping the retentate instead this time. This served to size-select the nanoparticles to a small distribution, while also ridding the colloid of silver ions and other excess reagents and byproducts. This concentrated sample was then subjected to a battery of characterization techniques.

The TFF system as well as the filters were chemically cleaned before and after use. Cleaning before use was performed by passing 2% nitric acid (HNO$_3$) and then water through the system for several minutes. Cleaning afterward consisted of 4% HNO$_3$ and water rinsing the system.

**Silver Characterization**

To characterize the silver nanoparticles, all three of the previously discussed characterization steps were performed. The colloid was examined with a Varian Cary 50 Bio UV-Vis spectrophotometer, Horiba LabRam HR800 Raman spectrometer, and Varian 710-ES ICP-OES.

*UV-Vis Absorption Spectrophotometry*

UV-Vis analysis was done on the EM spectrum from 200-800 nm at a scan rate of 600 nm/min and a spatial resolution of 1 nm.
Raman Spectroscopy
Raman analysis was performed with a 532 nm Nd:YAG green laser from 100-4000 cm\(^{-1}\), using a confocal hole of 300 µm and a grating of 600 grooves per mm. An accumulation time of 3 seconds was used, with 3 cycles (meaning the spectrum was collected thrice, and averaged).
**Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)**

ICP-OES analysis began with chemical digestion of the samples of interest before using the instrument. The sample was first digested by adding 2 mL of 70% trace metal grade HNO₃ (Fisher Scientific) and leaving it for 15 minutes at room temperature (“cold” digestion). This was then heated to 225 °C and boiled, evaporating off the liquid until approximately 200 µL remained (“hot” digestion). After cooling back to room temperature, this was diluted to 10 mL with 2% HNO₃. Both the sample and standards were digested this way. Standards were prepared using a stock solution of 1000 µg mL⁻¹ silver nanoparticles (SPEX CertiPrep). At this point, the sample and silver calibration standards were analyzed using ICP-OES.

The analysis was performed at silver emission wavelengths of 328.068 nm and 338.289 nm. HNO₃ blanks (2%) were added between each sample, and the instrument used a replicate read time of 15 seconds, internal stabilization delay of 45 seconds, sample uptake delay of 40 seconds, rinse time of 15 seconds, nebulizer pressure of 200 kPa, plasma flow of 15.0 L min⁻¹, auxiliary flow of 1.50 L min⁻¹, and radio frequency power of 1.2 kW.
Cell Growth
This experiment used adherent Vero 76 African green monkey kidney cells (ATCC CRL # 1567), which were grown on 100 mm petri dishes in a growth media made up of 1% penicillin streptomycin (HyClone), 10% fetal bovine serum (Corning), and 89% Dulbecco’s Modified Eagle’s Medium (DMEM) (+L-Glutamine,) (HyClone). They were maintained in an incubator at 37 °C with 5% CO₂. The cells were grown to roughly 75% confluency (percentage of plate covered), and were then incubated with silver.

Silver Incubation
Cells were incubated with silver nanoparticles (AgNP) made using the previously described method. Before incubation, all cell media was removed from the plate and discarded. Next, 9 mL of media were added. Finally, 1 mL of a 200 µg/mL (ppm) colloidal solution of silver nanoparticles was added. The plate was swirled several times
to disperse the silver nanoparticles through the media freely. This was placed in the incubator for 24 hours before harvesting the cells.

Cells were also incubated with silver ions (Ag\(^+\)) following the same procedure, using an initial concentration of 10 µL/mL (ppm) silver nitrate (AgNO\(_3\)).

**Cell Harvesting**
The process of harvesting cells began by first removing the media + AgNP mix. The plate was washed once with 5 mL of 10X Phosphate Buffered Saline (PBS) (ThermoFisher). Both solutions were analyzed by ICP-OES to determine the silver content left unabsorbed by the cells.

Approximately 2 mL of 0.05% trypsin (Hyclone) was then added to the cell plate, which was left in the incubator for 4 minutes, causing the cells to lift from the plate into the solution. After this, 8 mL of media was added to stop the action of trypsin. The solution was mixed by pipetting in and out 10 times, then placed in a 15 mL centrifuge tube. After further mixing, 1 mL of the solution was taken for ICP-OES analysis and cell counting. The remaining 9 mL was centrifuged at 1000 g for 10 minutes to pellet out the cells. The supernatant was set aside for ICP-OES analysis, and the pelleted cells were used for mitochondrial isolation.

**Mitochondrial Isolation**
The mitochondrial isolation was done with the Mitochondrial Isolation Kit for Cultured Cells from Thermofisher. The kit uses proprietary reagents and differential centrifugation to lyse cells and separate out mitochondria.
In the procedure, a suspension of cells in PBS was centrifuged at 900 g for 2 minutes to pellet out the cells. The PBS supernatant was removed, and then 800 µL of Reagent A (from the kit) was added to the tube. This was vortexed at 1500 rpm for 5 seconds, then placed in ice for 2 minutes. After this, 10 µL of Reagent B was added to the tube. This was vortexed at 3000 rpm for 5 seconds, then placed on ice for 1 minute. This process (5 seconds of vortexing, followed by 1 minute on ice) was repeated four times, for a total of five times.

At this point, 800 µL of Reagent C was added to the tube. This was gently mixed by inverting the tube several times, before centrifuging at 700 g for 10 minutes. Once this was finished, the supernatant was transferred to a new tube. That supernatant was centrifuged at 3000 g for 15 minutes. The supernatant from this step was removed from the tube, leaving a pellet. To this pellet was added 500 µL of Reagent C, before gentle mixing by inverting the tube. This was then centrifuged at 12,000 g for five minutes and then the supernatant was discarded, leaving an isolated mitochondrial pellet.

Once the mitochondria were isolated, they were redispersed in 2 mL PBS and mixed several times (including vortexing), before being split into 2 aliquots of 1 mL each, with 1 mL for ICP-OES analysis and the other for CPE+TFF analysis.

ICP-OES analysis proceeds as detailed above, and the CPE procedure is outlined below. In addition to these methods, separate analysis through a bicinchoninic acid (BCA) assay was performed at this point to determine the mass content of mitochondria per $10^4$ cells.
Cloud Point Extraction-Tangential Flow Filtration (CPE-TFF) Analysis
Cloud point extraction began by diluting each sample with water to 10.5 mL. Next, 1 mL of the solution was taken for ICP-OES analysis, with the remaining 9.5 mL used in the CPE procedure. The pH of that 9.5 mL solution was adjusted to 3.5 by adding 2% HNO$_3$. Following the addition of CPE reagents (0.2 mL of 1 M Na$_2$S$_2$O$_3$ and 0.2 mL of 10% TX-114), the solution was vortexed and heated for 30 minutes at 40 °C to reach the cloud point temperature, then centrifuged at room temperature and 1500 g for 5 minutes. After centrifugation, the supernatant was set aside for TFF, and the pellet (containing the AgNPs) was analyzed with ICP-OES for total silver content, using previously shown procedures. TFF was much similar to previously described procedures, using a single 1 kD filter (surface area of 60 cm$^2$, pore size of ~1 nm). The retentate, presumed to be comprised of larger-than-1-nm (and thus containing AgNPs) particles, and the permeate, made up of smaller particles (thus containing Ag$^+$), were both analyzed with ICP-OES for total silver content.

BCA Assay
The bicinchoninic acid (BCA) assay uses bicinchoninic acid to bind to Bovine Serum Albumin (BSA)-like proteins, which produces a color change in the sample based on the total number of proteins. This method was performed with a BCA Assay Kit from Pierce (ThermoFisher). In this method, a set of standards is first created using Bovine Serum Albumin, to produce a calibration curve. These are added to a 96-well plate. The samples of interest are also added to the well plate. Next, 200 mL of a proprietary reagent mix are
added to each well, which begins the assay and colorimetric change. After some variable incubation time, the light absorbance is measured with a UV-Vis spectrometer. This step was performed with a Varian Cary 50 MPR microplate reader extension for the previously mentioned Varian Cary 50 Bio UV-Vis spectrophotometer.

**Results and Discussion**

**AgNP Characterization**

Synthesized silver nanoparticles were characterized with UV-Vis spectroscopy, ICP-OES, and Raman spectroscopy. A UV-Vis spectrum is below.

![UV-Visible spectrum of colloidal silver](image.png)

*Figure 4. UV-Visible spectrum of colloidal silver*
As can be seen, there is a strong and fairly narrow peak at 397 nm, which is characteristic of silver.

A spectrum of the Raman spectroscopy analysis for the nanoparticles is below.

The three peaks in the spectrum at 1648 cm\(^{-1}\), \(\sim 3253 \text{ cm}^{-1}\), and 3402 cm\(^{-1}\) can all be attributed to water\(^{29}\). As mentioned before, this characterization method is primarily used to analyze silver nanoparticles for any impurities or remaining reactants in the suspension. The lack of any other peaks in the spectrum confirms that neither of these exist in the sample.

**Silver Quantification**

Analysis with CPE-TFF and ICP-OES revealed that whole cells absorbed 14% and 13.3% of the AgNP and Ag\(^+\) available in the media, respectively. Mitochondria were
isolated to determine silver amounts within that specific organelle. To normalize the amounts reported, the mitochondria mass relative to whole cells was determined as 4.4 mg per $10^4$ Vero 76 cells. The mitochondria were also found to absorb $36.2 \pm 18.1\%$ and $48.1 \pm 25.0\%$ of the AgNP and Ag$^+$ available from cells, respectively, which is quite significant: the mitochondria A) interacts with a significant amount of the total absorbed silver, and B) absorbs a greater amount of Ag$^+$ compared to AgNPs (validating the hypothesis).

For both cells and the isolated mitochondria, total silver counts were first found by taking a sample to be quantified by ICP-OES. Then, on a separate sample, CPE-TFF was performed to determine AgNP and Ag$^+$ amounts. Given the total silver amount calculated previously, a percentage of the total for each species (AgNP and Ag$^+$) was reported.

Following tables show these results.

<table>
<thead>
<tr>
<th>Separation process</th>
<th>AgNPs extracted (% of total)</th>
<th>Ag$^+$ extracted (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells + AgNPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPE</td>
<td>53.2 $\pm$ 16.3</td>
<td>47.0 $\pm$ 16.9</td>
</tr>
<tr>
<td>CPE + TFF</td>
<td>66.0 $\pm$ 11.6</td>
<td>24.1 $\pm$ 8.7</td>
</tr>
<tr>
<td><strong>Cells + AgNO$_3$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPE</td>
<td>5.5 $\pm$ 2.1</td>
<td>82.6 $\pm$ 11.7</td>
</tr>
<tr>
<td>CPE + TFF</td>
<td>9.1 $\pm$ 0.8</td>
<td>74.5 $\pm$ 13.6</td>
</tr>
</tbody>
</table>

As can be seen above, the TFF procedure increased recovery of AgNPs. The initial “CPE” reported amount of Ag$^+$ extracted includes some AgNPs. The single TFF step further recovered some AgNPs ($12.8\% \pm 20.0\%$ in the AgNP treatment and $3.6\% \pm 2.3\%$...
in the Ag⁺ exposure from Table 1) from the Ag⁺ fraction (CPE aqueous phase), thereby increasing the AgNP fraction (CPE-TFF retentate) and lowering the Ag⁺ fraction (CPE-TFF permeate). The fact that the silver ion results show significant silver nanoparticle amounts (9.1% ± 0.8%) implies that some silver ions are forming silver nanoparticles within the cellular matrix. Similarly, the silver nanoparticle treatment produces silver ions (24.1% ± 8.7%) as shown. Reasons for this may include the production of ROS, which some studies show can produce silver ions from silver nanoparticles. The significant amounts of the non-incubated silver species (Ag⁺ in the case of the AgNP exposure, and AgNPs in the case of the Ag⁺ exposure) in each case implies that the two are in a sort of equilibrium, with the alternate silver species to the one added forming an eventually stable proportion of the total. The uncertainties of these results should also be noted. Most of them are moderately high at ± 10% or more (with some dramatic increases in the derived values). Thus, any conclusions drawn should be broad rather than specific.

Two more points worth mentioning are the disproportionate decreases in Ag⁺ along with the overall recovery rate. For the AgNP treatment, the Ag⁺ percentage decreased from 47% to 24.1%, equaling a 22.9% ± 19.0% decrease through the addition of TFF to CPE (in Table 1). At the same time, AgNP amounts only increased from 53.2% to 66.0%, equaling a 12.8% ± 20.0% increase during this the separation process. The Ag⁺ treatment shows much the same results, with an 8.1% ± 17.9% Ag⁺ decrease while only giving a 3.6% ± 2.3% increase in AgNPs through the addition of TFF. Additionally, the total percent recovery for AgNP treatment is high (100.2% ± 23.5%) in CPE alone but
decreased to 90.1% ± 14.5% through CPE-TFF. The combined percentage for Ag⁺ is somewhat smaller (88.1% ± 11.9%) for CPE alone, yet decreases by a lesser amount (to 83.6% ± 13.6%) in CPE-TFF. Both of these issues point to the loss of silver in the TFF step, which may be a consideration for further use: additional AgNPs are extracted, increasing experimental accuracy, while some amount of total silver is lost, thereby decreasing experimental accuracy. A tradeoff exists which must be evaluated on a case-by-case basis.

One evaluation one might make is to examine the percentage of AgNPs gained through the TFF step and compare that to the percentage of silver loss through the TFF step. If the difference between the two is not significant, one might argue that the TFF step adds accuracy without unduly lower total silver recovery rates. Attempting this evaluation on the above results (a 12.8% ± 20.0% increase in AgNPs, compared to a (100.2% ± 23.5% – 90.1% ± 14.5% =) 10.1% ± 27.6% decrease in total silver recovered) reveals that the difference between the two does not seem significant, implying that the TFF method is entirely valid in this case.

The substantially lower recovery rates for Ag⁺ may be the result of many different factors. One that immediately springs to mind is the lower concentration of Ag⁺ used (1 µg mL⁻¹ versus 20 µg mL⁻¹), which may cause equal losses to impact the Ag⁺ amounts more strongly. Similarly, the TFF filter used is made of modified polyether sulfone, which is known to have electrostatic interactions with various chemical moieties. Similar data for isolated mitochondria is reported below.
Table 2. AgNP and Ag+ Relative Amounts Present in 4.4 mg Mitochondria Isolated from 10⁴ Whole Cells

<table>
<thead>
<tr>
<th>Separation process</th>
<th>AgNPs extracted (% of total)</th>
<th>Ag+ extracted (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria + AgNPs</td>
<td>CPE</td>
<td>23.6 ± 33.2</td>
</tr>
<tr>
<td></td>
<td>CPE + TFF</td>
<td>52.9 ± 15.5</td>
</tr>
<tr>
<td>Mitochondria + AgNO₃</td>
<td>CPE</td>
<td>9.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>CPE + TFF</td>
<td>9.5 ± 0.9</td>
</tr>
</tbody>
</table>

Observed results are much the same as before, though in this case no increase is seen in the AgNPs coming from AgNO₃. AgNP extraction increased 29.3% ± 36.6% while Ag⁺ extraction decreased 43.6% ± 33.8% in the AgNP treatment. A 27.7% ± 10.2% decrease in Ag⁺ extraction is seen for the Ag⁺ treatment, with no corresponding AgNP extraction increase. A much higher loss of Ag⁺ compared to AgNP gain is thus observed. Along similar lines, total recovery rates show a lower and broader distribution, with the AgNP values moving from 97.1% ± 47.2% in CPE alone to 82.8% ± 16.0% in CPE-TFF, and the Ag⁺ results moving from 97.8% ± 8.1% in CPE alone to 70.1% ± 6.4% in CPE-TFF. One area which must immediately be addressed is the (in some cases) enormous uncertainties. Once again these preclude any exact conclusions, though allowing for broad and general patterns to be observed.

One explanation for the generally poorer results is the lower amount of silver involved. As was previously shown, the mitochondria uptakes 36.2% and 48.1% (for AgNP and Ag⁺, respectively) of the silver within the cell (which is itself only 14% and 13.3% of the total AgNP and Ag⁺ amounts the cell was incubated in). Much like what was proposed
for Ag$^+$ in the cell-specific results, this lower amount may cause greater error in the results.

Along those lines, one area of interest is the recovery rate of Ag$^+$, which with CPE alone is at 97.8% ± 8.1%, significantly higher than the results for whole cells (88.1% ± 11.9%). And yet, applying TFF brings the total down to 70.1% ± 6.4%, which is significantly lower than the total for whole cells (83.6% ± 13.6%). This seems to lend further credence to the supposition that Ag$^+$ are interacting with the TFF filtration system.

The final CPE+TFF results in each category can be averaged, arriving at single values for both cells and mitochondria. This is reported in the table below.

<table>
<thead>
<tr>
<th>Silver source</th>
<th>AgNPs extracted (% of total)</th>
<th>Ag$^+$ extracted (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNP</td>
<td>59.5 ± 9.7</td>
<td>27.0 ± 4.8</td>
</tr>
<tr>
<td>AgNO$_3$</td>
<td>9.3 ± 0.6</td>
<td>67.6 ± 7.5</td>
</tr>
</tbody>
</table>

Table 3. Averaged AgNP and Ag$^+$ Relative Amounts for Whole Cells and Mitochondria

While some care should be taken in accepting the averaged results, given some of the high uncertainties in the initial results, the similarities are quite clear. They imply consistent AgNP and Ag$^+$ behavior (in shedding of silver ions, or formation of silver nanoparticles) in both environments despite differences.

As mentioned before, silver ions seem to be primary source of biological toxicity. Given this, the main focus will be on the possible interactions of silver ions and mitochondria.

The “electron transport chain” and ATP production of a cell and mitochondria were previously mentioned. In this process, a number of enzymatic complexes transfer protons
from the mitochondrial matrix to its intermembrane space located between the outer and inner membrane. In this process, the pH of the matrix shifts from 7 to 8. Later, these protons re-enter the matrix through another complex, which catalyzes the production of ATP. The re-entry of the protons is mediated by both a concentration gradient and an electric potential gradient\(^\text{25}\). A proton is a positively charged ion. Its similarity to a silver ion, \(\text{Ag}^+\), is obvious and suggests that this may be a possible entry point for silver ions.

One chemical species observed in mitochondria is an aldehyde\(^\text{35}\). A test called the “Tollon’s test” involving an aldehyde, silver ion, and water creates a silver coating and turns the aldehyde into a carboxylic acid. This may alter the intended activity of these aldehydes in the mitochondria.

One area related to silver nanoparticles is possible interaction with thiol groups. The interaction of silver nanoparticles with thiols is known\(^\text{36-37}\), as is the existence of thiols in the mitochondria\(^\text{38}\). This is yet another possible source of interference in the mitochondria from silver.

Another is the interaction of proteins and silver. This phenomenon is known\(^\text{39}\), and has possible implications in mitochondrial interaction as well. While the mitochondrion has its own genome, the majority of proteins it uses are translated in the cell and later enter the mitochondrion. Some of these proteins may interact with silver nanoparticles and allow their entry into the mitochondrion.

These various areas addressed are only a subset of the possible interactions silver may have with mitochondria. However, the elucidation of silver nanoparticle and silver ion
relative amounts allows for increased understanding of the impact of silver upon a biological cell.

**Conclusion**
The main goal in this study was properly achieved: through cellular incubation with AgNPs and Ag⁺, CPE-TFF, and ICP-OES, relative amounts of AgNPs and Ag⁺ were determined in both whole cells and mitochondria. The two initial hypotheses are supported by the total silver amounts which arrive at the mitochondria from the whole cell: AgNP-derived silver (36.2 ± 18.1%) comprises a significant portion of the silver added to the whole cell, and Ag⁺ derived silver (48.1 ± 25.0%) does the same, while also being significantly higher than the AgNP absorption. However, it must be noted that the situation is more nuanced than it appears on the surface: this difference seems to disappear when the two incubated species form their “equilibrium,” leaving largely the same proportions of AgNPs and Ag⁺ in the AgNP incubation, and in the Ag⁺ incubation.
WHAT IS A CELLULAR AUTOMATON?

The concept of a cellular automaton was first developed by John von Neumann and Stanislaw Ulam in the 1950s. It is an example of a so-called “agent-based model” since the individual agents, or ingredients, effectively act independently under a given set of rules. Proponents of cellular automata models include Stephan Wolfram who published “A New Kind Of Science” in 2002, arguing that cellular automata offer a new, more realistic approach to many scientific problems. A cellular automaton is a computer-, agent-, and rule-based model where higher-level complexity arises emergently from lower-level simplicity.

A cellular automaton begins with a grid of cells. These cells can be occupied by agents. Depending on the system of interest, these agents have a variety of actions available to them. The two most typical actions are state change—changing from one type of agent to another—and movement. These actions are governed by simple, straightforward rules, which are applied to all agents of a given type. A standard rule is the current state of any neighboring cells (for a given neighborhood type). Based on the state of the cells vertically and horizontally connected (a von Neumann neighborhood), and possibly including the cells diagonally connected as well (a Moore neighborhood), the central cell might undergo a state change of its own. A simple probability of the change occurring can also be applied—these rules can be probabilistic or deterministic.
Time moves in iterations, meaning that space, time, and state are all discrete. When the rules for each agent type’s possible actions have been declared, the simulation can then begin. It steps forward in time, with every agent evaluating its ruleset each iteration. How this is performed varies between models: some pick each successive agent randomly, to prevent any spatial “domino” effects, while others store a copy of the grid and then have each agent change from that, finally updating the grid after all agents have been iterated through.

In either case, these agents act on the smallest scale possible—themselves. Applied across an entire grid, however, this can create meaningful patterns which can model many types of systems. Cellular automata have been used to model automobile traffic\textsuperscript{42} and pedestrian flow\textsuperscript{43}, natural flow phenomena\textsuperscript{44}, urban\textsuperscript{45} land development, cryptography\textsuperscript{46}, recrystallization\textsuperscript{47}, vegetation dynamics\textsuperscript{48}, chemical phenomena\textsuperscript{49-51}, and many other systems. These many different applications of cellular automata show their incredible and unexhausted potential.

Cellular automata offer extensive customization within a fairly simply framework. In a situation where one adjusts the probability of a state change of a central cell based on the
states of the neighbors, current systems allow a user to define completely different probabilities for successively greater numbers of neighbors of a given state, producing various effects (such as a crystallization effect). One can also abandon this more complex setup to focus solely on state change of a central cell regardless of any neighbors, and through this produce meaningful models of simpler phenomena.

The reality is that cellular automata are uniquely suited to modeling any system which acts in a “bottom-up” manner, where individual components act separately (though quite possibly interdependently) and form patterns not seen in those individual components. This includes a significant portion of the natural world, as well as a moderate portion of manmade systems.
Through the following work the yet-untapped potential of cellular automata is presented in the hope that it can become a stronger player in the world of computational chemistry.

*Figure 7. Image of a first-order CA model with three cell types. Contains 10,000 cells.*
A STOCHASTIC CELLULAR AUTOMATA MODEL OF TAUTOMER EQUILIBRIA

Introduction

A large number of compounds, including many pharmaceuticals, biomolecules, and environmental pollutants, appear in nature not in a single form, but rather as a collection of different forms—tautomers, or alternatively, enantiomers, conformers, or neutral and ionic forms--each with its particular physical, chemical, and biological properties. The overall effect of the collection will depend on the collection's composition and the properties of its components. Tautomers in particular play key roles in a wide range of chemical and biochemical processes\textsuperscript{52-56}, an example being found in the tautomers of the nucleobases in DNA and RNA, where minor tautomers have been suggested as possible agents responsible for mutations and cancer\textsuperscript{57-58}. Proper understanding of tautomer and analogous equilibria and activities is now recognized as essential in such areas as drug design\textsuperscript{59-62} and chemical database management\textsuperscript{63-64}, but extends also to areas such as protein-ligand interactions, chemical reactivity, and environmental chemistry.

The most common approach to the study of chemical equilibria is to employ a set of coupled differential equations, often solved by numerical methods. This normally yields a deterministic solution in the sense that if the initial concentrations are specified the species concentrations at later times are fully determined by the equations representing the assumed transition mechanisms. Despite the widespread presentation of this approach
in textbooks, the shortcomings of the deterministic coupled-differential-equation approach to dynamic systems are well known and have been discussed elsewhere\textsuperscript{50, 65-66}. As Steinfeld et al. have noted in their widely-used kinetics text, the differential equation approach "has no theoretical justification, and its use is based solely on empiricism."\textsuperscript{65}

For example, the species concentrations are necessarily treated as continuous functions, and accordingly this approach cannot be applied to systems with small numbers of reactive components. Moreover, the deterministic method ignores the inherent fluctuations that play important roles in many natural processes. Although stochastic extensions of the differential equation approach can be constructed\textsuperscript{65-68} the necessary calculations frequently become exceedingly complicated, and in some cases are "mathematically intractable."\textsuperscript{65}

Given the importance of tautomer and similar equilibria, the development of practical alternative models for the study of these equilibria is especially desirable. We show here that \textit{cellular automata} (CA) models provide a highly efficient and fundamentally sound means for studying tautomer and similar equilibria. These models are an example of "agent-based" models, which trace their origins to ideas of the mathematician/physicist J. von Neumann and the mathematician S. Ulam in the 1950s\textsuperscript{40, 69-70}. In contrast to the more familiar differential equation-based formulations normally employed, CA models are rule-based, typically employing rather simple rules which nonetheless can often generate highly complex behaviors. The CA simulations take place on a one-, two-, or three-dimensional grids, with time advancing in discrete time-steps (iterations). The rules of a
cellular automaton can be deterministic or probabilistic (stochastic), the latter implying only a certain specified probability of an action, such as a transformation from one species to another, taking place. In an asynchronous, stochastic CA the probabilistic rules are applied in random order to all the ingredients on the grid in each iteration. Since each run is an independent trial, the important fluctuations found in natural systems appear naturally in the collective output from repeated trials. The deterministic solutions emerge, as they do in the real phenomena, as averages from many trials or the results from trials with a large number of ingredients.

CA models have been applied to a wide variety of systems ranging from the physical and biological sciences\textsuperscript{71-76} to the social sciences\textsuperscript{77-78}. In the following sections we shall first describe the form of a general CA model of tautomer equilibria, and then describe a practical application in which a stochastic cellular automaton is used to examine the aqueous equilibrium between 9-anthrone, 9-anthrol, and their common anion at different pH.
**Methods**

Cellular automata simulations were carried out using the program CASim of Cheng, Kier, and Seybold\textsuperscript{79}. (A copy of this program is available on a CD in the back sleeve of reference \textsuperscript{75}. Example 7-3 from Chapter 7 was used for the set-ups.) General reviews and applications of the CASim CA model can be found elsewhere\textsuperscript{50,74-75}. A survey of first-order kinetic applications of the CASim program, the type of approach used in the present work, is given in Ref. \textsuperscript{66}.

**General Model for Tautomer Equilibria**

In general one can picture the tautomeric equilibria of a compound and its charged species as some variant of the diagram shown in **Figure 7** for the simplified case of two neutral tautomers and two associated anionic tautomers. (The actual relevant species will depend on the specific compounds involved and the pH region of interest. A completely analogous figure would hold for a situation in which neutral and cationic tautomers are of interest. Only in unusual circumstances would the pH range of interest be extensive enough to include cationic, neutral, and anionic tautomers, although this could be treated as an obvious extension.)
In order to model a specific system certain information is required. In the most ideal case it would be desirable to have the rate constants for each of the transitions, but such values are almost never available. Fortunately, because performance of the CA models depends almost entirely on the relative rates of the competing transitions and within most circumstances is insensitive to the specific rate values, only the equilibrium constants for the transitions are needed. In principle, the relative stabilities of competing species can be computed accurately for gas-phase species, although less accurately for species in solution due to the limitations of present solvent models. In some cases experimental values for the tautomer ratios are available in specific solvents. In the (unfortunately rare) case where pK\textsubscript{a} values are available for the tautomers the Henderson-Hasselbalch equation

\[ \text{Figure 8. Image of generic tautomer system, with separate dissociated anions.} \]
\[ \text{pH} = \text{pK}_a + \log_{10}(\text{[A}^-]/\text{[HA]}) \]

can be used to determine the ratio of the anion to neutral (or neutral to cation) species concentrations at a given pH.

Because our CA simulations are stochastic and use probabilistic rules for the transitions and cell interrogations, each run is an independent experiment. It is therefore convenient to employ a series of runs, which yield an average result after a number of iterations (enough to establish a suitable, if nonetheless dynamic, steady state) plus a standard deviation which accounts for the fluctuations present in real systems of finite size. We have found it convenient to employ a two-dimensional 100 x 100 grid of cells and 10 runs, although larger or smaller grids and run numbers can be used. For these systems a steady state is generally achieved within about 100 iterations, regardless of the starting concentrations used for the ingredients.

**The 9-Anthrone-9-Anthrol Tautomer Equilibrium**

In very few cases is sufficient experimental information available for a detailed analysis of the charged and neutral species equilibria in a tautomer system, although in some cases experimental values can be found for the tautomer ratios and computational methods can then be used to estimate the tautomer pKₐs\(^8^0\). An exception is found in the independent results of McCann et al.\(^8^1\) and Freiermuth et al.\(^8^2\) on the equilibrium between the 9-anthrone and 9-anthrol tautomers, which dissociate to a common anion, as shown in Figure 8. McCann et al.\(^8^1\) determined the pKₑ = -log\(_{10}\)(Enol/Keto) to be 2.10 for the
equilibrium of the neutral tautomers, and $pK_a = 10.0$ for the anthrone, which dissociates as a carbon acid, and $pK_a = 7.9$ for the anthrol, which dissociates as a phenolic oxyacid. Freiermuth et al.\textsuperscript{82} estimated the neutral tautomer $pK_E$ to be 2.17, a value very close to that found by McCann et al.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Pattern of tautomer equilibrium for $pH = 7$ as modelled in the cellular automaton, including the transition probabilities.}
\end{figure}

The probabilities used were calculated in the following manner:

\[ pK_E = -\log K_E = -\log \frac{[B]}{[A]} \]

\[ pK_E = -\log \frac{P(A,B)}{P(B,A)} = 2.10 \]
\[
\frac{P(A, B)}{P(B, A)} = 10^{-2.1} = 0.0079 \cong 0.008
\]

if \( P(B, A) = 0.1 \)

then \( P(A, B) = 0.0008 \)

Using the pK_{E} value of McCann et al.\textsuperscript{81} yields a ratio of 126 for the keto/enol populations of the neutral tautomers. It should be noted that the anthrone-anthrol tautomer system does include a common cation in addition to the common anion. However, because the estimated pK_{a} of the cation is extremely low, however on the order of -5, this species could be ignored at the pH values considered here, and it was not included in the model.

The calculations below show the process used to determine pK_{a} related probabilities are shown below.

\[
pH = pK\alpha + \log \frac{[A^-]}{[HA]}
\]

\[
7 = 10 + \log \frac{P(A, C)}{P(C, A)}
\]

\[
\log \frac{P(A, C)}{P(C, A)} = -3
\]

\[
\frac{P(A, C)}{P(C, A)} = 10^{-3} = 0.001
\]

if \( P(C, A) = 0.1 \)

then \( P(A, C) = 0.0001 \)
Applying the Henderson-Hasselbalch equation to the pK\textsubscript{a} values at pH 7 yields a 1000/1 ratio of the anthrone to the anion and a 7.9 ratio of the anthrol to the anion. These relative values can now be applied to the respective rates of the forward and reverse transformations for these equilibria. Because as noted the CA model is dependent almost entirely on the relative rates for the equilibria and is insensitive to the specific rate values, we have for each competing equilibrium $K = k_f/k_r$ arbitrarily set the higher transformation probability/iteration to 0.1 and then set the slower transformation probability accordingly. The resulting transition probabilities per iteration at pH values of 4, 7, and 10 are given in Table 4. These transition probabilities for the three species constitute the “rules” of this specific CA model and are acted upon for each ingredient in the grid during each iteration.

**Table 4. Probability values for the species determined as explained in the text.**

<table>
<thead>
<tr>
<th></th>
<th>P(A,B)</th>
<th>P(B,A)</th>
<th>P(A,C)</th>
<th>P(C,A)</th>
<th>P(B,C)</th>
<th>P(C,B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4</td>
<td>$8 \times 10^{-4}$</td>
<td>0.1</td>
<td>$1 \times 10^{-7}$</td>
<td>0.1</td>
<td>$125 \times 10^{-7}$</td>
<td>0.1</td>
</tr>
<tr>
<td>pH 7</td>
<td>$8 \times 10^{-4}$</td>
<td>0.1</td>
<td>$1 \times 10^{-7}$</td>
<td>0.1</td>
<td>$125 \times 10^{-4}$</td>
<td>0.1</td>
</tr>
<tr>
<td>pH 10</td>
<td>$8 \times 10^{-4}$</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>$8 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**Results and Discussion**

For a 100x100 grid the collective result for the 10,000 independent ingredients is a model for the 9-anthrone-9-anthrol tautomer system. One can begin with any set of concentrations for the three species involved and the concentrations will evolve under the transition rules until a dynamic steady state is achieved. This is illustrated in Figure 9 for
a starting set of roughly equal populations (3333 anthrones, 3333 anthrols, and 3334 anions) of the species at pH = 7. It is seen that the steady state is achieved within about 50 iterations. This steady state is an emergent property of the CA model and simulation.

![Figure 10. Plot of total number of cells of each type over time for a single run at pH = 7.](image)

The steady-state population statistics achieved for $10^4$ ingredients at time = 500 iterations for 10 runs at pH values 4, 7, and 10 are given in Table 5. It is seen that at the lower pH values (pH = 4 and 7) 9-anthrone is by far the dominant species comprising over 99% of the ingredients present. The 9-anthrol, although much less stable than the anthrone and present in only quite small numbers, has a much lower pKₐ than the anthrone and therefore displays a much higher propensity to dissociate to the anion under these conditions.
conditions. At pH = 10, however, the anthrone itself is 50% dissociated and the anion concentration has increased to almost 50%, the anthrol concentration remaining low because of this species lower stability and strong tendency to dissociate. We suggest that the results demonstrate the special utility of cellular automata models for the study of tautomer and similar systems under different solution acidity conditions.

Table 5. Combined cellular automata equilibrium population results for 10 runs with 10,000 ingredients.

<table>
<thead>
<tr>
<th>Number of Cells</th>
<th>9-Anthrone</th>
<th>9-Anthrol</th>
<th>Anion</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4</td>
<td>9921 ± 7</td>
<td>79 ± 7</td>
<td>0</td>
</tr>
<tr>
<td>pH 7</td>
<td>9909 ± 11</td>
<td>80 ± 9</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>pH 10</td>
<td>4978 ± 54</td>
<td>40 ± 6</td>
<td>4982 ± 53</td>
</tr>
</tbody>
</table>

As noted above the deterministic results for the concentrations of the species emerge as averages from the cellular automata simulation trials, here for 10 trials with 10,000 ingredients, and it is of interest to compare the CA results with the results from a deterministic calculation (obtained using the Mathematica Software Suite 11.2, from Wolfram Research, Champaign, Illinois). This comparison is shown in Table 6 for the 10,000 ingredient system where the differential equation approximations should be reasonably valid. It is seen that the average results from the two approaches are for all practical purposes identical, although the CA results show these values as they in practice occur in nature—as averages to be expected from the behaviors of large numbers of interacting discrete ingredients. Accompanying the CA results are standard deviations representing the extent of the fluctuations to be expected in the results from repeated
(here 10) trials. In addition, the time courses from the starting concentrations to achievement of the equilibrium concentrations for the CA approach and the differential equation approach are identical, as seen in Figure 3.

<table>
<thead>
<tr>
<th></th>
<th>pH 4</th>
<th>pH 7</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diff. Eq. Soln.</td>
<td>CA</td>
<td>Diff. Eq. Soln.</td>
</tr>
<tr>
<td>pH 4</td>
<td>9920.35</td>
<td>9921 ± 7</td>
<td>9910.53</td>
</tr>
<tr>
<td>pH 7</td>
<td>79.641</td>
<td>79 ± 7</td>
<td>79.529</td>
</tr>
<tr>
<td>pH 10</td>
<td>0.0096</td>
<td>0 ± 0</td>
<td>9.94113</td>
</tr>
</tbody>
</table>

We assert that the CA simulation is preferable to results from differential equations for the following reasons: simplicity of the initial setup, incorporation of stochasticity, and natural emergence of a steady state equilibrium.

The CA is innately simpler to prepare for simulation. The only preparation needed is the defining of the transition probabilities, determined by equilibrium and dissociation constants, and determination of grid size (and thus initial amounts). The deterministic equations require moderately more effort in comparison.

First, the equations needed for the differential solutions must be derived. This is shown below\(^{83}\).

\[
\begin{align*}
A & \xrightleftharpoons[k_{BA}]{k_{AB}} B \\
A & \xrightleftharpoons[k_{CA}]{k_{AC}} C
\end{align*}
\]
\[
\dot{A} = \frac{dA}{dt} = -(k_{AB} + k_{AC}) \cdot A + k_{BA} \cdot B + k_{CA} \cdot C
\]

The same process is followed for the other two species. This is fairly straightforward, but nonetheless constitutes an extra step. It should also be noted that this will dramatically increase in complexity given larger systems, such as a four-member system. One might recall that this was initially a four-member system, with anthrone and anthrol sharing a common cation. One might also consider situations where the two tautomers do not have a common anion or cation, as well as systems with more than two tautomers—the complexity of the differential calculation will increase even further.

This must then be solved using a computer program, such as Stephan Wolfram’s Mathematica. It is not shown here, but the general form of the equation involved a 100+ page derivation. One can again consider that with only two tautomers and a single common dissociative anion, the simplest form of a tautomeric system is being modeled.

At this point one incorporates the transition probabilities already defined for the CA into the differential equation to arrive at a final value.

The CA also incorporates stochasticity. No physical system in reality will perform with such exactness as a differential equation predicts. By incorporating a measure of randomness in transitions, the CA better approximates a real-world system’s variance and shows the possible deviations from theory one might experience in such a system. The similarities to differential equation results arise as an average of total population amounts, much like how other real-world properties such as temperature arise as an
average of kinetic energy for all molecules (which certainly vary from single value implied in the single temperature) of a system.

The emergent nature of the CA simulation is yet another strength. The results, comparable to (in fact essentially identical to) those from the differential equations, are not defined by a series of expressions which predict the conclusion; rather, they arise as a result of a several simple interconversions acting on a single agent or species at a time, expanded across an entire system. Real-world physical systems do not act as “ordered” by a complex equation—they instead evolve on a microscopic scale, with each single species acting as governed by such simple things as its $pK_E$ or $pK_a$, and contributing to an overall population. Both a CA and a real-world system change on the scale of a single unit, with overall measurable system change arising from the combination or superposition of individual actors.

**Conclusions**
Agent-based models such as cellular automata models are ideally suited for the analysis of tautomer and other multi-component equilibria. In contrast to the traditional differential-equation based approach, which necessarily assumes populations so large that they can be justifiably approximated as continuous functions, a stochastic, rule-based CA model closely approximates the real system of discrete molecules transforming between different species. Systems ranging in size from a single molecule to more than a million molecules can be examined. In the CA simulation the sought-after steady-state condition
among the species arises naturally as an *emergent property*, along with information on the fluctuations expected to appear in such a system. In addition, the simulations require only modest computational effort.

The 9-anthrone-9-anthrol tautomeric system has been examined as a representative example. In this example the parameters ("transition rules") for the CA model were obtained from experimental reports in the literature\textsuperscript{81-82}, although in principle these parameters could be estimated (most likely with reduced accuracy) computationally. Application of the CA model to this system allowed the steady-state species populations prevailing at pH values 4, 7, and 10 to be determined directly. The deterministic values for the steady-state populations were seen to arise naturally in the Ca model as averages of repeated simulations. In our experience, in addition to (1) providing a more fundamentally correct approach to the dynamics of the tautomer system, (2) more closely representing the actual molecular phenomena in question, and (3) providing stochastic information on the system studied, the CA calculations are more simply computed than are the differential equation-based deterministic solutions.

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Capstone: A Cellular Automata Program

In the course of studying the previous systems, the value of a more versatile modelling software became apparent. Many public cellular automata programs exist, such as Golly and Mirek’s Cellebration (two more well-known examples). However, the overwhelming majority use deterministic models and allow the user little control relative to research requirements. The CASim program is multi-featured, but future research requires further features, such as the ability to “prepare” a grid with a specific design or pause a simulation and introduce new agents.

As a result of all of this, a program for stochastic immobile and mobile cellular automata modelling was developed, titled “Capstone.” The program was first designed with the Unity software tool, before moving to a “Windows Form” application designed in Microsoft Visual Studio 2017.

The basic architecture of the program loosely follows a model-view-controller design. The user inputs settings into the controller, which are then used by the model, the result of this being shown to the user in the view. Those settings include grid settings (CA type, grid type, grid dimensions, number of agent states) and state settings (color, initial amount, probabilities of transition to other states, probability of movement). The model uses the CA type to determine whether agents move or are immobile, grid type to govern edge-of-grid behavior, grid dimensions to set the grid size, and then color and initial amounts of each agent state to initialize the grid.
Then, at every iteration the model examines each agent for change based on the probabilities of transition from one state to another (this change can be spatially independent, or based on the state of the cells in a user-defined neighborhood around the agent). Movement of agents is checked for as well (for CA of a certain type – immobile CA do not have this feature). A “snapshot” of the grid taken before any change occurs allows a neighbor-dependent change to be independent of agent order—in this way, changing one agent does not affect the agent next to it during a single iteration. The result of each iteration is then shown to the user.
As previously mentioned, Capstone was first designed in the Unity engine and then moved to a “Windows Form.” As a result, all reported user interface (UI) design is based on the later version.

**USER INTERFACE**

Capstone uses one main window which is made up of a main menu, a CA menu, and an open area designed to contain the CA view. The main menu controls functions like creating or editing CA settings, saving or loading settings, some tools such as saving an
image of the grid or a count of amounts of each cell type per iteration, and some analysis functions. The CA menu controls some simulation-specific settings such as starting, pausing, and resetting the CA, as well as some looping functions.

When the user creates a new CA, a second window is opened which allows the user to enter the previously mentioned settings. After doing so they return to the main window, where they can run the CA. They can save images of the grid (in bitmap (BMP), PNG, JPEG, or TIFF file types) at any point in time, or save a cell count (recording total amounts of each agent state per iteration) to a text file.
NEW FEATURES

As previously mentioned, some new features separate Capstone from other stochastic CA programs (such as CASim, or the open-source JCASim\textsuperscript{86}).

The first of these is the ability to edit the grid. The user can pause the simulation at any time and then edit any point on it. The agent at that point moves through all possible
states incrementally. The user can also click-and-drag to edit more than one agent at a time. One possible application of this is the creation of an initial structure of some entity, such as a crystal, which then acts in a typical manner.

Another feature is the ability to change settings of the CA in real time. At any time the user can pause the grid and then change probabilities of transition, and then resume running the CA, which continues with the changed probabilities. This might be used to introduce an agent type during the simulation, by initially setting all transitions to that type to zero, and then later adjusting them to make its appearance possible. This might be used to simulate a situation where one option is introduced at a later point in time than another, with different growth rates.

Yet another feature is the enhancement of settings for the user to adjust, to allow for simulation of increasingly varied systems. Whereas previous programs were typically limited to standard von Neumann and Moore neighborhoods, with transition probabilities based solely on the number of neighbors of a given state, this program allows the user to create any neighborhood he wishes. In addition, a user enters probabilities for each specific location on a probability grid, meaning that various “types” of effects can exist within one neighbor set. For example, an inhibitory effect might be in place for horizontal growth, while vertical growth is promoted. This would result in narrow, vertical “bands” or stripes, similar to those of tigers and zebras.
It should be noted that the user is not required to use this advanced setup, but can also use simpler settings (which are sufficient in many cases), merely inputting the transition probabilities for any neighbor rather than a specific one.

The combination of these new features, combined with previous, more traditional features and the user-friendly interface, make this program quite valuable for research use. As it stands, the program has been used by the author for study of various scientific phenomena differing from the previous discussion of tautomer ratios in varying pH.

Topics include ongoing research in other areas such as crystal growth, allelopathy, fractal dimension analysis, and other areas requiring custom neighborhoods, neighbor-specific parameters, and intermediate modification of a system.

The program is under continual development to enhance its feature set. A current version is available at (https://github.com/GABowers/Capstone-Application).


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