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The effect of Hofmeister series counterions on the colloidal and antimicrobial properties of triple-headed Cationic amphiphiles

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The Effect of Hofmeister Series Counterions on the Colloidal and Antimicrobial Properties of Triple-Headed Cationic Amphiphiles

An Honors Program Project Presented to the Faculty of the Undergraduate College of Science and Mathematics James Madison University

by Kirstie Ann Thompson

May 2016

Accepted by the faculty of the Department of Chemistry and Biochemistry, James Madison University, in partial fulfillment of the requirements for the Honors Program.

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PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at James Madison University Department of Chemistry and Biochemistry Spring Symposium on April 14th 2016.
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Acknowledgements

I would like to thank Dr. Kevin Caran for his constant support and guidance throughout this entire process, as well as throughout my time at James Madison University. I would not be where I am today without everything he has taught me. I would also like to thank my readers Dr. Yanjie Zhang and Dr. Kyle Seifert, for reviewing my thesis and giving me invaluable feedback. I also thank the Jeffrey E. Tickle '90 Endowment, NSF-REU (CHE-1461175), a Multi-Investigator Cottrell College Science Award from the Research Corporation for Scientific Advancement (MICCSA 10709), the James Madison University College of Science and Mathematics and the Center for Materials Science as well as the Departments of Chemistry and Biochemistry, and Biology at James Madison University for providing the funding and support that made this project possible.
Abstract

Antibacterial resistance is becoming increasingly prominent, causing a great need for novel antibacterial products. This work focuses on developing an understanding of the relationship between counterion identity on colloidal and antibacterial activity for cationic amphiphiles. In an amphiphile series with three hydrophilic quaternary ammonium bromide head groups and one hydrophobic tail, the amphiphile with a linear hydrocarbon chain with 18 carbons is more antibacterial than those with longer or shorter tails. Counterion exchange of the bromide counterions with various Hofmeister series anions significantly affects both antibacterial and colloidal activity. The logarithm of critical aggregation concentration [log(CAC)] was found to have a linear correlation with Gibbs free energy of hydration ($\Delta G_{\text{hydr}}$), enthalpy of hydration ($\Delta H_{\text{hydr}}$), partial molar volume ($V_i^0$), and surface tension ($\sigma$) of counterions present. These trends were found to correlate with an inverse Hofmeister effect, which is common for cationic species in the presence of low anion concentration. At this point no specific trend between the Hofmeister series and amphiphile antibacterial potency have been observed, although significant changes in potency have been seen with varying ions. Chloride and iodide counterions improve the potency of both $M-1,1,18$ and $M-1,12,12$ (both the best of there corresponding series) significantly, especially when combating Gram-negative bacteria. This is significant, as it shows the antibacterial correlations for one series ($M-1,1,18$) are applicable to at least one other series ($M-1,12,12$).
Introduction

The Hofmeister series was first introduced in 1888 by Franz Hofmeister, as a series of ions ordered by their ability to precipitate proteins out of solution.\textsuperscript{1-5} In recent years attempts have been made to generalize the Hofmeister effect to a diverse set of properties. The Hofmeister series has been correlated to water solubility\textsuperscript{6}, Kraft temperature and melting temperature\textsuperscript{7}, micellization\textsuperscript{8}, surface tension\textsuperscript{9}, pH\textsuperscript{10}, bacterial growth\textsuperscript{11}, etc. While correlations to these and other properties have been observed with both cationic and anionic Hofmeister series, those of the latter are generally more pronounced.\textsuperscript{6,11} The typical Hofmeister anion series is below (Figure 1). The ions studied in this work are bolded.

\[
\text{CO}_3^{2-} > \text{SO}_4^{2-} > \text{PO}_4^{3-} > \text{C}_2\text{H}_3\text{O}_2^- > \text{Cl}^- > \text{Br}^- \approx \text{NO}_3^- > \text{ClO}_4^- > \text{I}^- > \text{SCN}^- \\
\text{kosmotropes} \quad \text{chaotropes}
\]

Figure 1. Hofmeister series anions. Bolded anions are those studied in this work.

Ions in the series are broken into two distinct groups, where chloride is generally defined as the dividing ion. Those on the left of the series are referred to as kosmotropes while those on the right are referred to as chaotropes. These terms stem from the original theory that changes in bulk water structure were responsible for the mechanism of the Hofmeister effect; kosmotropes were believed to be strongly hydrated, water structure makers that have a salting out (aggregate forming) effect; chaotropes were believed to be weakly hydrated, water structure breakers that have a salting in (solubilizing) effect.\textsuperscript{2,12,13} Current proposed mechanisms of the Hofmeister
series focus more on direct ion-macromolecule (or ion-aggregate) interactions between ions and other solutes (ionic or non-ionic) as opposed to ion-water interactions. In recent years a rapid increase in antibiotic-resistant bacteria has been observed. As the prevalence of antibacterial resistant bacteria continues to increase it is becoming more important to minimize bacterial transference. Amphiphiles have a number of applications in the medicinal and industrial fields in which they assist in controlling bacteria transference due to their bactericidal activity. Many different amphiphiles have been synthesized in an attempt to improve effectiveness and specificity of antimicrobial action.

Our group has synthesized several series of amphiphilic molecules with non-conventional structures. Within these series, structure was altered by changing the type and number of hydrophilic head groups as well as the length and relative number of hydrophobic tail regions. Colloidal properties such as critical aggregation concentration (CAC), the concentration at which amphiphiles begin to aggregate in solution, are profoundly affected by amphiphile structure. Increasing the hydrophobicity of an amphiphile, for example, by increasing the length or number of tails decreases water solubility, thus decreasing the CAC. Likewise, increasing the number or hydrophobicity of polar head groups increase water solubility thus increasing the CAC. An amphiphile’s structure also affects its antimicrobial potency. One measure of potency is minimum inhibitory concentration (MIC), which is the minimum concentration at which a substance inhibits bacterial growth. Previous studies, by our group and others, have shown that MIC decreases (corresponding to an increase in potency) as
tail length increases up to an optimal point, above which MIC increases with further increases in tail length. 22-24,28-33

One series of amphiphiles previously reported by our group contains three hydrophilic head groups and one hydrophobic tail. 36 Within this series the compound with an 18-carbon long tail \((M-1,1,18+3Br^-) (1)\) has antimicrobial activity higher than those with shorter or longer tails. (Figure 2)

\[
\text{M-1,1,18 (1-6)} \quad \text{M-1,12,12 (7-10)}
\]

Previous to this report, all compounds within this series contained three bromide counterions. It was hypothesized that replacing the bromides with other counterions would affect CAC and MIC values. Various studies have been reported on the effect of added salts 37,38 and varying counterion identity 39-41 on the colloidal properties of different surfactants. The only work studying the Hofmeister effect on bacterial growth with which we are familiar, have evaluated the effect of added salt. 11 To our knowledge, this is the first comprehensive study on the effect of Hofmeister series counterions of surfactants on both colloidal, and antimicrobial properties.

\[
\text{Figure 2.} \quad M-1,1,18 (1-6) \quad \text{and} \quad M-1,12,12 (7-10) \text{ with various Hofmeister series counterions.}
\]
In this work, we report CAC and MIC data for $M-1,1,18$ with 6 different Hofmeister series counterions. Colloidal results suggest a reverse Hofmeister effect, were CAC decreases steadily with the presence of increasingly chaotropic counterions. While no obvious trend with MIC was observed, as a large combination of factors are believed to be at play within each biological system, notable differences in activity were observed. For example, antibacterial potency was found to increase for $M-1,1,18$ with $\text{Cl}^-$ and $\text{I}^-$ counterions, particularly against Gram-negative bacteria. Antibacterial testing was also completed on the most potent compound of a series containing three hydrophilic head groups and two hydrocarbon tails, $M-1,12,12$, to determine if trends observed in counterion effects are applicable to other amphiphile series or vary between series. In almost every case $\text{Cl}^-$ and $\text{I}^-$ compounds improved upon the original $\text{Br}^-$ derivative. This is significant, as it shows that ion effects observed in one series are at least applicable to one other series, and can improve antimicrobial potency.

Table 1. Hofmeister series counterions exchanged within the $M-1,1,18$ series

<table>
<thead>
<tr>
<th>Counterion</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>$\text{(NO}_3^-)$</td>
</tr>
<tr>
<td>Acetate</td>
<td>$\text{(C}_2\text{H}_3\text{O}_2^-)$</td>
</tr>
<tr>
<td>Chloride</td>
<td>$\text{Cl}^-$</td>
</tr>
<tr>
<td>Bromide</td>
<td>$\text{Br}^-$</td>
</tr>
<tr>
<td>Carbonate</td>
<td>$\text{(CO}_3^{2-})$</td>
</tr>
<tr>
<td>Iodide</td>
<td>$\text{I}^-$</td>
</tr>
</tbody>
</table>

[Diagram showing structures of different counterions]
Experimental

Sample Preparation. Amphiphiles with bromide counterions (1, 7) were prepared as described previously. \(^{36}\) Counterion exchanges were completed following a procedure adapted from Manet et al, as described below. \(^{42}\)

General Protocol A: Preparation of amphiphiles with nitrate, acetate, or carbonate counterions via ion exchange

To prepare amphiphiles with the counterions nitrate (NO\(_3\)\(^-\)), acetate (C\(_2\)H\(_3\)O\(_2\)\(^-\)), or carbonate (CO\(_3\)\(^{2-}\)), exchange of the bromide ions is performed by preparing a solution of the tris-bromide amphiphile in methanol and adding the corresponding silver salt (AgNO\(_3\), AgC\(_2\)H\(_3\)O\(_2\), or AgCO\(_3\)) in excess. The exchange is driven by the insolubility of AgBr, and allowed to run overnight in the absence of light to minimize potential for degradation of the silver salt. After the exchange a light grey precipitate is present indicating the formation of AgBr. The solution is filtered through Celite, 545 filter aide, to remove the majority of the AgBr. The resulting filtrate is concentrated \textit{in vacuo} using rotary evaporation and centrifuged, using a Fisher Scientific accuSpin Micro 17 at a rate of 13.3 min\(^{-1}\)g for 2 minutes, to remove any remaining AgBr. A small aliquot of the supernatant is removed, combined with AgNO\(_3\) and subjected to powder x-ray diffraction (PXRD) under copper K\(\alpha\) radiation to confirm the complete absence of AgBr (thus verifying the removal of bromide ions from the sample). Once confirmed, excess methanol is removed by rotary evaporation and the product is dried under vacuum over P\(_2\)O\(_5\). Successful ion exchange is confirmed through electrospray ionization time of
flight mass spectrometry (ESI-TOFMS), using conditions as described in supporting information. The C$_2$H$_3$O$_2$\(^{-}\) counterion exchange was further confirmed using $^1$H NMR by the presence of a peak at 1.55 ppm integrating for 9 protons.

Figure 3. Preparation of amphiphiles with nitrate, acetate, or carbonate counterions via ion exchange.

**General Protocol B: Preparation of amphiphiles with chloride and iodide counterions.**

The preparation of amphiphiles with chloride (Cl\(^-\)) or iodide (I\(^-\)) counterions is accomplished by addition of the corresponding conjugate acid (HCl or HI) to an aqueous solution of the acetate amphiphile. The low pK\(_a\) of these acids drives the protonation of the acetate to produce acetic acid (which is removed *in vacuo*), leaving the corresponding conjugate bases as counterions. The acetate salt is cooled on a
water/ice bath in aqueous solution and the corresponding acid HX is added dropwise in slight excess. The solution is subsequently lyophilized to remove the water and acetic acid formed. The exchange is confirmed through $^1$H NMR by the disappearance of the acetate peak as well as through ESI-TOFMS.

![Reaction Scheme]

Figure 4. Ion exchange method for preparation of amphiphiles with chloride and iodide counterions.

**Critical Aggregation Concentration (CAC).** Measurement of CAC was performed by measuring the conductivity of aqueous amphiphile samples over a range of concentrations. Conductivity measurements were recorded using a Vernier conductivity probe and Logger Pro software (version 3.8.5.1) for 10 seconds, from which the average value was recorded. Stock solutions of amphiphile in nanopure H₂O were prepared at concentrations above the predicted CAC and equilibrated at 37°C, consistent with the temperature of the biological studies. Samples were prepared from a serial dilution of the stock solution. With each dilution the sample was thoroughly mixed and conductivity was recorded. Conductivity ($\kappa$) is graphed against concentration. CAC is determined by the intersection of the linear regions above and below the CAC, where there is a discernable change in slope. Aggregate degree of ionization ($\alpha$) was determined from the ratio of the linear regressions above and below the CAC.
Minimum Inhibitory Concentration (MIC). The MIC value was determined for two Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and five Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus anthracis* Sterne, and *Streptococcus agalactiae*) as previously described. Compounds were serially diluted and 100 µL of each dilution were added to the wells of a 96-well flat-bottomed microtiter plate in triplicate. After adding 100 µL of the bacterial cell suspension (5x10^6 CFU/mL), the plates were incubated at 37° C for 72 hours. The MIC of the compound was defined as the minimum concentration that resulted in visible inhibition of bacterial growth.
Results & Discussion

CAC and Ion properties. CAC for M-1,1,18 with 6 different Hofmeister series counterions (1-6) are reported in Table 2. The results were found to follow the inverse Hofmeister series, where CAC decreases with increasingly chaotropic counterions.

Table 2. Experimental colloidal data for M-1,1,18 with various Hofmeister series counterions and compiled physical ion properties

<table>
<thead>
<tr>
<th>Compound (Counterion)</th>
<th>CAC (mM)</th>
<th>α (Å³)</th>
<th>σ (mN L m⁻¹ mol⁻¹)</th>
<th>Vᵢ⁰ (cm³ mol⁻¹)</th>
<th>ΔSₜₜₜ (J K⁻¹ mol⁻¹)</th>
<th>ΔHₜₜₜ (kJ mol⁻¹)</th>
<th>ΔGₜₜₜ (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 (CO₃²⁻)</td>
<td>4.54</td>
<td>2.6</td>
<td>6.7</td>
<td>-245</td>
<td>-1397</td>
<td>-479</td>
<td></td>
</tr>
<tr>
<td>(2) C₂H₃O₂⁻</td>
<td>10.10</td>
<td>0.472</td>
<td>5.50</td>
<td>1.59</td>
<td>46.2</td>
<td>-170</td>
<td>-425</td>
</tr>
<tr>
<td>(5) Cl⁻</td>
<td>5.96</td>
<td>0.237</td>
<td>3.42</td>
<td>1.6</td>
<td>23.3</td>
<td>-75</td>
<td>-367</td>
</tr>
<tr>
<td>(1) Br⁻</td>
<td>3.13</td>
<td>0.170</td>
<td>4.85</td>
<td>1.3</td>
<td>30.2</td>
<td>-59</td>
<td>-336</td>
</tr>
<tr>
<td>(3) NO₃⁻</td>
<td>2.07</td>
<td>0.403</td>
<td>4.13</td>
<td>1.1</td>
<td>34.5</td>
<td>-76</td>
<td>-312</td>
</tr>
<tr>
<td>(6) I⁻</td>
<td>1.03</td>
<td>0.192</td>
<td>7.51</td>
<td>1.0</td>
<td>41.7</td>
<td>-36</td>
<td>-291</td>
</tr>
</tbody>
</table>

CAC (mM) and aggregate degree of ionization (α) values for M-1,1,18 with the specified counterion (compound number listed to the left of specific ion) measured at 37°C, CO₃²⁻ data was not collected. Physical properties of select Hofmeister series anions: polarizability (α), surface tension increments (σ), partial molar volume (Vᵢ⁰), ΔGₜₜₜ, ΔHₜₜₜ, and ΔSₜₜₜ. Values for α (calculated from molar refractivity), νₛ, ΔSₜₜₜ, ΔHₜₜₜ, ΔGₜₜₜ taken from ref 44; ⁴⁴ values for σ taken from ref 6, ⁶ except for C₂H₃O₂⁻ taken from ref 45 as the middle value of a given range. ⁴⁵

In order to determine the specific physical ion properties contributing to the trends in CAC observed, the log(CAC) values of M-1,1,18 with various counterions were
compared to 6 different physical ion properties (Figure 5). Of the properties compared, \( \Delta G_{\text{hydr}} \), \( \Delta H_{\text{hydr}} \), partial molar volume, and surface tension had significant linear trends, whereas correlations to \( \Delta S_{\text{hydr}} \) and polarizability were less evident. The observation of a linear correlation suggests that a particular property plays a role in the CAC trends observed. Aggregate degree of ionization (\( \alpha \)) was also compared to ion physical properties but no significant correlation was observed (Figure S1).

Properties that have trends inverse to the typical Hofmeister series have been reported for positively charged systems with low salt concentrations.\(^{46}\) Previous studies have suggested that the influence of charge pairing interactions is relevant at concentrations below a few hundred mM, after which saturation occurs, and the trend reverts to the typical Hofmeister series order.\(^{46}\) As our amphiphile solutions are never above 50 mM, and the ratio between cationic amphiphile and counterion remains constant in all of our experiments, charge-charge interactions are likely the predominant forces. When charge-charge interactions predominate anionic counterions are expected to interact very closely with the positively charged quaternary ammonium head groups of the amphiphiles. This close interaction between the negatively charged counterion and positively charged head group effectively neutralizes the ionic character of the head group region, thus attenuating charge repulsions between the head groups in an aggregate. This decrease of charge repulsion allows amphiphile aggregation to occur more easily, lowering CAC. Counterions that allow for more charge interaction will therefore allow for lower CAC values.
Figure 5. Properties A. partial molar volume \((V_i^0)\), \(^{44}\) B. free energy of hydration \((\Delta G_{\text{hydr}})\), \(^{44}\) C. enthalpy of hydration \((\Delta H_{\text{hydr}})\), \(^{44}\) D. entropy of hydration \((\Delta S_{\text{hydr}})\), \(^{44}\) E. polarizability

[^44]: References or notes for the data sources or calculations.
(σ), 44 and F. surface tension (σ) 6,45 as a function of log(CAC). Trend lines indicate correlations between the two properties. For A. and E. separate series containing only chaotropic ions show specific chaotropic property correlations.

An inverse trend between partial molar volume, which is defined as the size of a hydrated ion, and log(CAC) was observed (Figure 5A). 47 Partial molar volume is directly correlated to the Gibbs free energy of hydration (∆G_{hydr}), which is a measure of the energy required to remove waters of hydration from a species. In previous studies it was found that larger anions (chaotropes), which characteristically have fewer waters of hydration and expel those present more easily (less negative ∆G_{hydr}), were more effective at associating with positively charged species. 5,46,48 Increased binding affinity between cationic headgroup and anionic counterions causes more efficient screening of electrostatic repulsions, fostering salting-out behavior. In turn, increased salting out behavior would be expected to decrease CAC values, as observed. 46 Acetate was an outlier from this trend, which may be due to its kosmotropic nature, as it is not uncommon for chaotropes and kosmotropes to correlate with different properties. 6,49

The weak hydration of chaotropic ions is consistent with their characterization as “soft ions,” where as the strong hydration associated with kosmotropes makes them “hard ions.” 5 Ionic head groups commonly found in surfactants can also be characterized as “hard ions” or “soft ions.” Accordingly, hard cationic head groups prefer to associate with hard anions and visa versa. 5 Alkyl ammonium head groups are characterized as “soft ions.” 50 Therefore, the soft nature of quaternary ammonium head groups present in our amphiphile structures suggests that they will more readily associate with “soft” chaotropic anions. This increased association, as previously
discussed, more effectively screens electrostatic repulsions between head group regions allowing aggregation to more readily occur. This further supports the inverse Hofmeister series observed. 5,50

An inverse trend was also observed with enthalpy of hydration ($\Delta H_{\text{hydr}}$) (Figure 5C). At the modest temperatures of our studies (37°C), enthalpy has a more significant contribution to the Gibbs free energy of a system than entropy, where $\Delta G = \Delta H - T\Delta S$. It is believed that due to this more significant contribution, enthalpy is the main driving force for inverse Hofmeister series environments where charge-charge interaction predominate, and therefore has a correlation with CAC. No significant trend was observed with $\Delta S_{\text{hydr}}$ in this specific study, where chaotropes dominate the counterions studied. It has been reported that $\Delta S_{\text{hydr}}$ often correlates with the behavior of kosmotropic ions. 6,49

MIC. MIC values of 1-6 were determined for five Gram-positive strains ($\text{Enterococcus faecalis}$, $\text{Staphylococcus aureus}$, $\text{Bacillus subtilis}$, $\text{Streptococcus agalactiae}$, and $\text{Bacillus anthracis}$) and two Gram-Negative strains ($\text{Escherichia coli}$ and $\text{Pseudomonas aeruginosa}$) (Table 3). Gram-positive (G+) and Gram-negative (G-) bacteria differ in the structure of their cell membrane and cell wall. Gram-negative bacteria possess an outer plasma membrane separating their cell wall from the environment. The presence of this outer membrane protects the cell against dessication and chemical stressors such as antibiotics and detergents and therefore, makes infections of these types of organisms inherently more difficult to treat. Gram-positive bacteria lack this outer membrane, and instead have a thicker cell membrane that adds rigidity. 51 Previous studies by both our group and others have found it to be
more difficult to kill Gram-negative bacteria, likely due to the additional outer membrane.

Table 3. MIC values for M-1,1,18 (1-6) and M-1,12,12 (7-10) counterion exchanged compounds against 5 Gram positive (G+) and 2 Gram negative (G-) bacterial strain

<table>
<thead>
<tr>
<th>MIC</th>
<th>P. aeruginosa (G-)</th>
<th>E. coli (G-)</th>
<th>E. faecalis (G+)</th>
<th>S. aureus (G+)</th>
<th>B. subtilis (G+)</th>
<th>S. agalactiae (G+)</th>
<th>B. anthracis (G+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₃²⁻</td>
<td>250</td>
<td>31</td>
<td>31</td>
<td>31</td>
<td>16</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>C₂H₃O₂⁻</td>
<td>250</td>
<td>2.4</td>
<td>63</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td>63</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>8</td>
<td>2</td>
<td>16</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Br⁻</td>
<td>125</td>
<td>16</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>4.4</td>
<td>2</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>250</td>
<td>63</td>
<td>16</td>
<td>31</td>
<td>16</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>I⁻</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>4.8</td>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

No significant correlation between MIC values for counterions across the Hofmeister series was observed (Figure 6). It is believed that the high level of complexity and variation present at the biological interface causes many factors to be involved. Plots of log(MIC) for each bacterial strain against the ionic properties described above did not reveal any apparent correlations (Figure S2-S7). Though no Hofmeister-specific trend was observed, significant variations in MIC between the different counterions was seen.
Figure 6. Comparison of log(MIC) values for each $M-1,1,18$ counterion (1-6) against all bacterial strains tested.

$M-1,1,18$ with chloride, bromide, and iodide counterions exhibited similar MIC values that were significantly lower than those of other counterions against Gram-positive strains. Against several strains chloride ($\textit{Staphylococcus aureus}$, $\textit{Bacillus subtilis}$, and $\textit{Bacillus anthracis}$) and iodide ($\textit{Staphylococcus aureus}$) improved upon the potency of the parent bromide derivative. In previous studies we $^{33,36}$ and others $^{22,23,25,26}$ have found that almost all amphiphiles are considerably better at combatting Gram positive strains than Gram negative strains, with $\textit{Pseudomonas aeruginosa}$ typically having the highest MIC of all strains tested. It was observed that chloride and iodide counterions greatly improved the antimicrobial effectiveness of $M-1,1,18$ against both of the Gram negative strains tested ($\textit{Escherichia coli}$ and $\textit{Pseudomonas aeruginosa}$). The MIC values of 1 ($M-1,1,18+3Br^-$) against $\textit{Pseudomonas aeruginosa}$ and $\textit{Escherichia coli}$ (125 μM and 16 μM, respectively) were improved to 8 μM and 16 μM for the chloride (5) and 16 μM and 8 μM for the iodide (6).
To determine whether specific counterion effects on MIC observed with $M-1,1,18$ apply to other amphiphiles, ion exchange was completed on $M-1,12,12$, the most potent compound within its series. Again in almost every case $\text{Cl}^-$ and $\text{I}^-$ compounds improved upon the original $\text{Br}^-$ derivative (Table 3). Of particular significance is $M-1,12,12$ with $\text{I}^-$. In every case the amphiphile with $\text{I}^-$ was the most potent (in some cases with the equivalent potency of another ion). Importantly, this work demonstrates that the antibacterial correlations from one of our amphiphile series (e.g. increased potency of iodide and chloride counterions) is more broadly applicable to at least one other series.
Conclusions

In this study we provide a comprehensive report on the effects of Hofmeister series counterions on the colloidal and antimicrobial of the tris-cationic amphiphile. Colloidal studies suggest an inverse Hofmeister series trend, consistent with the relatively low anion concentration, as described. Chaotropes characteristically have low levels of hydration allowing these anions to more closely interact with positively charged head groups, minimizing charge repulsions, thus leading to CAC values. The specific quaternary ammonium head group also allows for increased charge screening in the presence of chaotropes due to the “soft-soft” associations. Of ion properties compared, $\Delta G_{\text{hydr}}$, $\Delta H_{\text{hydr}}$, partial molar volume, and surface tension had significant correlations to CAC. Though no clear trends were observed with $\Delta S_{\text{hydr}}$ and polarizability, a more in depth study that included a larger number of kosmotropic counterions may lead to elucidation of a correlation with anions present.

MIC data indicate no direct correlation with the Hofmeister series. This is likely due to the complex nature of the biological systems. Although no trend was observed, significant variations in potency were seen between counterions. Of distinction, $\text{I}^-$ and $\text{Cl}^-$ counterions greatly improved the potency of $M-1,1,18$ and $M-1,12,12$, each the best of its respective series, against Gram negative bacterial strains. This is significant as Gram negative infections are notoriously harder to combat than their Gram-positive counterparts, and it indicates that ion effects for one amphiphile series are applicable to atleast one other series.
Supplemental Information Available

Synthesis, α vs. ion property graphs, MIC vs. ion property graphs, \(^1\)H NMR spectra, \(^{13}\)C NMR spectra
References


Appendix 1: Supplemental Information

Supplemental Information for:

The Effect of Hofmeister Series Counterions on the Colloidal and Antimicrobial Properties of a Triple-Headed Cationic Amphiphile

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Synthesis of M-1,1,18+3Br⁻, 1

Ion Exchange of M-1,1,18, 2-6

Synthesis of M-1,12,12+3Br⁻, 7

Ion Exchange M-1,12,12, 8-10

Figure S1 Degree of Ionization (α) vs. physical properties of ions

Figures S2-S7 MIC vs. Physical properties of ions

Figures S8-S27 NMR Spectra

Synthesis and analysis. All solvents and reagents were used as received from the indicated chemical supplier unless otherwise specified. Melting points for solids were measured using an OptiMelt MPA100 Automated Melting Point System (Stanford Research Systems, Inc.). Nuclear magnetic resonance spectra were collected using a Bruker-Spectrospin 400 (¹H: 400 MHz, ¹³C: 100 MHz). NMR Spectra were analyzed using Bruker TopSpin software, version 3.2. The solvent residual peak was used as a reference. Coupling constants are estimated to be correct within ±0.1Hz. Exact mass measurements were obtained in flow injection experiments on a 6224 time of flight mass spectrometer (TOF-MS) (Agilent Technologies, Santa Clara, CA). Compounds were ionized by positive ion electrospray (ESI) under the following conditions: capillary voltage, +2500V, nozzle voltage, 500 V; fragmentor voltage, 175 V; drying gas temperature, 325 °C; drying gas flow, 5 L/min; nebulizer, 40 psi. MS data was collected in full scan mode (500 ms/scan) over the range of 100-1700m/z. Mass errors were less than 5 ppm for all observed compounds. Mass resolving power, m/Δm, was ~19,000 at 922 m/z. Mass Hunter software version B.04 was used for all data acquisition and analysis.
The product was synthesized via the procedure detailed in the submitted manuscript by Gallagher, T et al. Intermediate M-1,1 (500 mg, 1.05 mmol) was dissolved in ethanol (60 mL) and reacted with N,N-dimethyoctylamine (TCI, 85%, 0.437 mg, 1.26 mmol). Reaction yielded 457 mg (43% yield) of a white solid, mp = 224.3 – 229.5 °C (dec). ¹H NMR (DMSO-d₆, 400MHz, 25 °C) δ: 7.90 (2H, ArH); 7.86 (1H, ArH); 4.68 (4H, ArCH₂N+Me₃); 4.64 (2H, ArCH₂N+Me₂R); ~3.4 (N+CH₂CH₂, partially obscured by H₂O/HDO signal); 3.15 (18H, N+(CH₃)₃); 3.06 (6H, N⁺(CH₃)₂R); 1.80 (2H, N⁺CH₂CH₂); 1.15–1.43 (30H); 0.85 (3H, CH₂CH₃). ¹³C NMR (DMSO-d₆, 100 MHz, 25 °C) δ: 138.83, 138.58, 129.74, 129.54, 66.82, 65.28, 63.57, 51.83, 49.11, 31.27, 29.05, 29.03, 28.99, 28.92, 28.68, 28.59, 25.93, 22.08, 21.85, 13.95. TOF-HRMS calculated for [M-Br]⁺: 690.39364, 691.397, 692.3916, 693.39496, 694.38955, 695.39291; observed (ppm error): 690.3938 (0.23), 691.3960 (-1.45), 692.3937 (3.03), 693.3958 (1.21), 694.3922 (3.82), 695.3930 (0.13).

The product was synthesized via general protocol A. Compound 1 (250 mg, 0.324 mmol) was dissolved in methanol (45 mL) and reacted with AgC₂H₃O₂⁻ (194mg, 1.16 mmol) Reaction yielded 143 mg (62.2% yield) of a white solid, mp = 169.5 – 174.5 °C. ¹H NMR (DMSO-d₆, 400MHz, 25 °C) δ: 8.097 ( 3H, ArH); 4.721 (6H, ArCH₂N⁺Me₃ & ArCH₂N⁺Me₂R ); ~3.35 (2H, N⁺CH₂CH₂); 3.14 (18H, N⁺(CH₃)₃); 3.05 (6H, N⁺(CH₃)₂R); 1.80 (2H, N⁺CH₂CH₂); 1.57 (9H, C₂H₃O₂⁻) 1.36–1.15 (30H); 0.85 (3H, CH₂CH₃). ¹³C NMR (DMSO-d₆, 400 MHz, 25 °C) δ: 173.59, 139.60, 139.33, 130.23, 129.98, 67.10, 65.77, 64.00, 52.08, 49.30, 31.75, 29.51, 29.47, 29.16, 26.61, 22.55, 14.41. TOF-HRMS calculated for [M-Br]⁺: 650.58360, 651.58696, 652.59031; observed (ppm error): 650.5854 (2.77), 651.58696 (1.75), 652.5921 (2.74).

The product was synthesized via general protocol A. Compound 1 (100 mg, 0.129 mmol) was dissolved in methanol (15 mL) and reacted with AgC₂H₃O₂⁻ (79.2 mg, 0.466 mmol) Reaction yielded 58.4 mg (62.8% yield) of a white solid, mp = 219.6 – 235.6 °C (dec). ¹H NMR (DMSO-d₆, 400MHz, 25 °C) δ: 7.80 (3H, ArH); 4.60 (6H, ArCH₂N⁺Me₃ & ArCH₂N⁺Me₂R ); ~3.3 (N⁺CH₂CH₂, partially obscured by H₂O/HDO signal); 3.08 (18H, N⁺(CH₃)₃); 3.00 (6H, N⁺(CH₃)₂R); 1.80 (2H, N⁺CH₂CH₂); 1.06–1.40 (30H); 0.85 (3H, CH₂CH₃). ¹³C NMR (DMSO-d₆, 400 MHz, 25 °C) δ: 139.28,139.04, 130.18, 130.00, 67.55, 65.92, 64.26, 52.25, 49.48, 31.75, 29.54, 29.52, 29.51, 29.46,29.38,29.16,29.06, 26.41, 22.55,22.31, 14.42. TOF-HRMS calculated for [M-Br]⁺: 656.53264, 657.5360, 658.53935; observed (ppm error): 656.5341 (2.22), 657.538 (3.04), 658.935 (0.23).
(4) M-1,1,18 + 1.5CO$_3^{2-}$

The product was synthesized via general protocol A. Compound 1 (500 mg, 0.647 mmol) was dissolved in methanol (90 mL) and reacted with Ag$_2$CO$_3$ (321 mg, 1.16 mol). Reaction yielded 176 mg (40.3% yield) of a white solid, mp = 173.2 – 189.0°C (dec). $^1$H NMR (D$_2$O, 400MHz, 25°C) δ: 7.96 (3H, ArH); 4.66 (6H, ArCH$_2$N$^+$Me$_3$ & ArCH$_2$N$^+$Me$_2$R); 3.49 (2H, N$^+$CH$_2$CH$_2$); 3.16 (18H, N$^+$CH$_3$); 3.03 (6H, N$^+$CH$_3$R); 1.93 (2H, N$^+$CH$_2$CH$_2$); 1.15–1.63 (30H); 0.95 (t, 3H, CH$_2$CH$_3$). $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) δ: 162.31, 140.00, 130.44, 130.24, 68.82, 67.58, 67.37, 53.24, 49.50, 49.36, 32.65, 30.58, 30.55, 30.45, 30.31, 30.19, 30.14, 29.70, 26.80, 23.34, 23.05, 14.62. TOF-HRMS to be completed.

(5) M-1,1,18 + Cl$^-$

The product was synthesized via general protocol B. Compound 2 (200 mg, 0.282 mmol) was dissolved in nano-pure H$_2$O (8 mL) and chilled on ice for 10 min. HCl (1014μL, 1M) was added dropwise. Reaction yielded 155 mg (85.1% yield) of a white solid, mp = 212.1 – 224.5°C (dec). $^1$H NMR (DMSO-$d_6$, 400MHz, 25°C) δ: 7.83 (s, 3H, ArH); 4.63 (6H, ArCH$_2$N$^+$Me$_3$ & ArCH$_2$N$^+$Me$_2$R); ~3.3 (N$^+$CH$_2$CH$_2$, partially obscured by H$_2$O/HDO signal); 3.11 (s, 18H, N$^+$CH$_3$); 3.02 (s, 6H, N$^+$CH$_3$R); 1.79 (2H, N$^+$CH$_2$CH$_2$); 1.14–1.37 (30H); 0.85 (3H, CH$_2$CH$_3$). $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) δ: 139.35, 139.11, 130.23, 130.01, 67.25, 65.76, 64.00, 52.23, 49.49, 31.75, 29.54, 29.51, 29.46, 29.16, 26.41, 22.55, 22.33, 14.42. TOF-HRMS calculated for [M-Cl$^+$]: 602.49468, 604.49173, 603.49804, 605.49509; observed (ppm error): 602.4935 (-1.96), 604.4927 (1.60), 603.4963 (-2.88), 605.4944 (-1.14).

(6) M-1,1,18 + I$^-$

The product was synthesized via general protocol B. Compound 2 (100 mg, 0.141 mmol) was dissolved in nano-pure H$_2$O (4 mL) and chilled on ice for 10 min. HI (507μL, 1M) was added dropwise. Reaction yielded 112 mg (85.34% yield) of a yellow solid, mp = 197.3 – 221.6°C (dec). $^1$H NMR (DMSO-$d_6$, 400MHz, 25°C) δ: 7.81 (3H, ArH); 4.61 (s, 6H, ArCH$_2$N$^+$Me$_3$ & ArCH$_2$N$^+$Me$_2$R); ~3.3 (N$^+$CH$_2$CH$_2$, obscured by H$_2$O/HDO signal); 3.10 (s, 18H, N$^+$CH$_3$); 3.00 (s, 6H, N$^+$CH$_3$R); 1.80 (m, 2H, N$^+$CH$_2$CH$_2$); 1.15–1.37 (30H); 0.85 (3H, CH$_2$CH$_3$). $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) δ: 139.30, 139.06, 130.17, 129.99, 67.37, 65.73, 64.21, 52.39, 49.66, 31.75, 29.54, 29.53, 29.51, 29.46, 29.38, 29.16, 29.06, 26.40, 22.55. TOF-HRMS to be completed.
The product was synthesized following the procedure detailed by Marafino, J et al.\textsuperscript{33} Intermediate \textit{M-1} (105 mg, 0.30 mmol) was dissolved in ethanol (15 mL) and reacted with \textit{N,N}-dimethydodecylamine (MP Biomedicals, 170 mg, 0.75 mmol). Reaction yielded 160 mg (63% yield) of a white solid, \textit{mp} = 202.9 – 204.2 °C (dec). \textit{\textsuperscript{1}H NMR (DMSO-\textit{d}_6, 400MHz, 25 °C)} \(\delta\): 7.84 (1H, ArH); 7.81 (2H, ArH); 4.61 (2H, ArCH\textsubscript{2}); 4.59 (4H, ArCH\textsubscript{2}); 3.10 (9H, N(CH\textsubscript{3})\textsubscript{3}); 3.01 (12H, N(CH\textsubscript{3})\textsubscript{2}); 1.80 (4H, NCH\textsubscript{3}CH\textsubscript{2}); 1.16–1.40 (36H); 0.86 (6H, CH\textsubscript{2}CH\textsubscript{3}). \textit{\textsuperscript{13}C NMR (DMSO-\textit{d}_6, 100 MHz, 25 °C)} \(\delta\): 139.0, 138.8, 129.7, 129.5, 66.9, 65.4, 63.5, 51.8, 49.1, 31.3, 29.06, 29.02, 28.99, 28.92, 28.73, 28.62, 25.9, 22.1, 21.9, 14.0. TOF-HRMS calculated for [M-Br]+: 760.47189, 761.47525, 762.46985, 763.47321, 764.46780, 765.47116; observed (ppm error): 760.47027 (-2.13), 761.47219 (-4.02), 762.46895 (-1.18), 763.47087 (-3.06), 764.46784 (+0.05), 765.47061 (-0.72).

The product was synthesized via general protocol A. Compound \textit{7} (250 mg, 0.297 mmol) was dissolved in methanol (90 mL) and reacted with AgC\textsubscript{2}H\textsubscript{3}O\textsubscript{2} (178 mg, 1.07 mmol). Reaction yielded 99.6 mg (43.3% yield) of a white solid, \textit{mp} = undetermined. \textit{\textsuperscript{1}H NMR (DMSO, 400MHz, 25°C)} \(\delta\): 8.09 (3H, Ar-H); 4.70 (6H, Ar-CH\textsubscript{2}); 3.01 (21H, N-(CH\textsubscript{3})\textsubscript{3} & N-(CH\textsubscript{3})\textsubscript{2}); 1.80 (4H, N-CH\textsubscript{2}CH\textsubscript{2}); 1.54 (9H, C\textsubscript{2}H\textsubscript{3}O\textsubscript{2}−)1.15–1.39 (m, 36H); 0.86 (6H, -CH\textsubscript{2}CH\textsubscript{3}). \textit{\textsuperscript{13}C NMR (DMSO, 100 MHz, 25°C)} \(\delta\): 173.29, 139.74, 139.52, 130.23, 129.97, 67.26, 65.84, 63.97, 52.15, 49.39, 31.17, 29.52, 29.49, 29.46, 29.39, 29.19, 29.10, 26.74, 22.56, 14.42. TOF-HRMS to be completed.

The product was synthesized via general protocol B. Compound \textit{8} (50 mg, 0.0641 mmol) was dissolved in nano-pure H\textsubscript{2}O (2 mL) and chilled on ice for 10min. HCl (230μL, 1M) was added dropwise. Reaction yielded 36.1 mg (79.0% yield) of a white solid, \textit{mp} = 223.5 –232.8 °C (dec). \textit{\textsuperscript{1}H NMR (DMSO, 400MHz, 25°C)} \(\delta\): 7.92 (3H, Ar-H); 4.66 (6H, Ar-CH\textsubscript{2}); 3.13 (21H, N-(CH\textsubscript{3})\textsubscript{3} & N-(CH\textsubscript{3})\textsubscript{2}); 1.79 (m, 4H, N-CH\textsubscript{3}CH\textsubscript{2}); 1.16–1.40 (36H); 0.86 (6H, -CH\textsubscript{2}CH\textsubscript{3}). \textit{\textsuperscript{13}C NMR (DMSO, 100 MHz, 25°C)} \(\delta\): 139.48, 139.27, 139.52, 130.23, 130.00, 67.35, 65.88, 63.85, 52.23, 49.50, 31.77, 29.54, 29.50, 29.48, 29.41, 29.20, 29.11, 26.44, 22.57, 22.35, 14.43. TOF-HRMS to be completed.

The product was synthesized via general protocol B. Compound \textit{8} (22.8 mg, 0.0292 mmol) was dissolved in nano-pure H\textsubscript{2}O (2 mL) and chilled on ice for 10min. HI (105μL,
1M) was added dropwise. Reaction yielded 23.6 mg (82.1% yield) of a yellow solid, mp = 181.3 – 189.4°C (dec). $^1$H NMR (DMSO, 400MHz, 25°C) $\delta$: 7.86 (3H, Ar-H); 4.63 (6H, Ar-CH$_2$); 3.08 (21H, N-(CH$_3$)$_3$ & N-(CH$_3$)$_2$); 1.80 (4H, N-CH$_3$-CH$_2$); 1.15–1.40 (36H); 0.86 (6H, -CH$_2$CH$_3$). $^{13}$C NMR (DMSO, 100 MHz, 25°C) $\delta$: 138.99, 138.78, 129.66, 129.48, 66.94, 65.35, 63.62, 51.90, 49.16, 31.28, 29.04, 29.01, 28.98, 28.89, 28.71, 28.60, 25.93, 22.08, 13.95. TOF-HRMS to be completed.
Figure S1. Plots of ion properties A. partial molar volume ($V_i^0$), B. free energy of hydration ($\Delta G_{\text{hydr}}$), C. enthalpy of hydration ($\Delta H_{\text{hydr}}$), D. entropy of hydration ($\Delta S_{\text{hydr}}$), E. surface tension ($\sigma$), F. polarizability ($\alpha$) vs. degree of ionization ($\alpha$)
Figure S2. Plots of log(MIC) vs. Partial molar volume ($V_i^0$) for each bacterial strain tested
Figure S3. Plots of log(MIC) vs. free energy of hydration ($\Delta G_{\text{hydr}}$) for each bacterial strain tested.
Figure S4. Plots of log(MIC) vs. enthalpy of hydration ($\Delta H_{\text{hydr}}$) for each bacterial strain tested. Inset graphs show data with CO$_3^{2-}$ omitted.

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$P. \text{aeruginosa} \ (G-)$

$E. \text{coli} \ (G-)$

$E. \text{faecalis} \ (G+)$

$S. \text{aureus} \ (G+)$

$B. \text{subtilis} \ (G+)$

$S. \text{agalactiae} \ (G+)$

$B. \text{anthracis} \ (G+)$
Figure S5. Plots of log(MIC) vs. entropy of hydration ($\Delta S_{\text{hydr}}$) for each bacterial strain tested.
Figure S6. Plots of log(MIC) vs. Surface tension (σ) for each bacterial strain tested.
Figure S7. Plots of \( \log(\text{MIC}) \) vs. ion polarizability (\( \alpha \)) for each bacterial strain tested.
Figure S8. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound $(M-1,1,18)$ with Br$^-$ counterions
Figure S9. $^{13}$C NMR (DMSO-$d_6$, 100 MHz, 25°C) of compound ($M$-$1,1,18$) with Br$^-$ counterions.
Figure S10. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M-1,1,18$) with $C_2H_3O_2^-$ counterions
Figure S11. $^{13}$C NMR (DMSO-d$_6$, 400 MHz, 25°C) of compound (M-1,1,18) with C$_2$H$_4$O$_2$ counterions.
Figure S12. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M-1,1,18$) with NO$_3^-$ counterions
Figure S13. $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M-1,1,18$) with NO$_3^-$ counterions
Figure S14. $^1$H NMR ($D_2O$, 400 MHz, 25°C) of compound (M-1,1,18) with $CO_3^{2-}$ counterions
Figure S15. $^{13}$C NMR (D$_2$O, 400 MHz, 25°C) with added MeOH of compound (M-1,1,18) with CO$_3^{2-}$ counterions.
Figure S16. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound (M-1,1,18) with Cl$^-$ counterions
Figure S17. $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound (M-1, 1, 18) with Cl$^-$ counterions
Figure S18. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M-1,1,18$) with $I^-$ counterions
Figure S19. $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M$-1,1,18) with I$^-$ counterions.
Figure S20. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound (M-1,12,12) with Br$^-$ counterions
Figure S21. $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M-1, 12, 12$) with Br$^-$ counterions
Figure S22. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound (M-1,12,12) with C$_2$H$_3$O$_2^-$ counterions
Figure S23. $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound (M-1,12,12) with C$_2$H$_3$O$_2^-$ counterions
Figure S24. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M-1,12,12$) with Cl$^-$ counterions
Figure S25. $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound (M-1,12,12) with Cl$^-$ counterions
Figure S26. $^1$H NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound ($M$-1,12,12) with I$^-$ counterions
Figure S27. $^{13}$C NMR (DMSO-$d_6$, 400 MHz, 25°C) of compound (M-1,12,12) with I$^-$ counterions