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# Effects of Cryogenic Sample Analysis on Molecular Depth Profiles with TOF-Secondary Ion Mass Spectrometry

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Although the benefits of decreased sample temperature for the molecular profiling of organic materials with time-of-flight secondary ion mass spectrometry (TOF-SIMS) have been established, the mechanism behind spectral changes observed at low temperature, particularly increased protonated molecular ion ( $M + H$ )<sup>+</sup> yields, have not been examined in detail. We have developed a procedure to investigate these effects by monitoring secondary ion yields under sustained primary ion bombardment as the sample temperature is cooled from room temperature down to 80 K. Examination of biomaterials such as an amino acid (arginine), a polypeptide (Gly-Gly-Tyr-Arg), a lipid (1,2 dipalmitoyl-sn-glycero-3 phosphatidylcholine), and a drug molecule (cyclosporine A) each provide evidence of ion yield enhancement at 80 K under either 20 keV C<sub>60</sub><sup>+</sup> or 20 keV Au<sub>3</sub><sup>+</sup> bombardment. For example, arginine shows a 2-fold increase in the steady-state intensity for the ( $M + H$ )<sup>+</sup> ion at 80 K compared to the steady state at 300 K. It is shown that there is a correlation between the yield enhancement and a reduction in the damage cross section, which for arginine under 20 keV Au<sub>3</sub><sup>+</sup> bombardment decreases from  $5.0 \pm 0.4 \times 10^{-14} \text{ cm}^2$  at 300 K to  $2.0 \pm 0.3 \times 10^{-14} \text{ cm}^2$  at 80 K. The role of water as the facilitator for this reduction is explored through the use of H<sub>2</sub>O and D<sub>2</sub>O dosing experiments at 80 K.

Molecular depth profiling of biological materials using secondary ion mass spectrometry (SIMS) is a developing field that is evolving to meet the needs of the biological community. The chemical sensitivity and high spatial resolution offered by SIMS endows the technique with the ability to be a powerful tool for the imaging of cells and tissue.<sup>1</sup> Advances in instrumentation including cluster and polyatomic primary ion sources, such as SF<sub>5</sub><sup>+</sup> and C<sub>60</sub><sup>+</sup>, not only increase ion yields but also offer the advantage of efficiently removing accumulating chemical damage that occurs at high ion fluences, thereby allowing molecular information to be acquired in depth as the sample material is

eroded.<sup>2–5</sup> The development of new mass spectrometer configurations<sup>6,7</sup> to exploit the capabilities of these ion beams have made the prospect of rapid 3-D molecular imaging of cells or tissue a reality.<sup>8–14</sup>

In SIMS, the high energy primary ion beam that sputters the information rich secondary ions from the surface may also cause significant subsurface chemical damage which accumulates with continuous bombardment. As a consequence, useful molecular depth profiling of organic materials using SIMS was challenging until quite recently. Molecular profiling with polyatomic primary ions has radically changed the capabilities for SIMS by providing an efficient means of removing the accumulating chemical damage at higher fluences such that molecular ion signals persist into the bulk of the sample. Despite these encouraging developments, there are still several issues that hinder the technique, such as low ion yields and damage accumulation, which must be addressed before molecular profiling of biological samples can be considered routinely reliable.

Low temperature experiments have been used for the imaging of cells and tissue to preserve the sample integrity by maintaining a hydrated state and preventing damaging effects from the vacuum environment, which is required for SIMS analysis.<sup>15–19</sup> Interest-

- (2) Gillen, G.; Fahey, A. *Appl. Surf. Sci.* **2003**, *203*, 209.
- (3) Weibel, D.; Wong, S.; Lockyer, N. P.; Blenkinsopp, P.; Hill, R.; Vickerman, J. C. *Anal. Chem.* **2003**, *75*, 1754.
- (4) Xu, J.; Ostrowski, S. G.; Szakal, C.; Ewing, A. G.; Winograd, N. *Appl. Surf. Sci.* **2004**, *231*, 159.
- (5) Hill, R.; Blenkinsopp, P. W. M. *Appl. Surf. Sci.* **2004**, *231*, 936.
- (6) Fletcher, J. S.; Rabbani, S.; Henderson, A.; Blenkinsopp, P.; Thompson, S. P.; Lockyer, N. P.; Vickerman, J. C. *Anal. Chem.* **2008**, *80*, 9058.
- (7) Carado, A.; Passarelli, M. K.; Kozole, J.; Wingate, J. E.; Winograd, N.; Loboda, A. V. *Anal. Chem.* **2008**, *80*, 7921.
- (8) Winograd, N. *Anal. Chem.* **2005**, *77*, 142A.
- (9) Delcorte, A. *Appl. Surf. Sci.* **2008**, *255*, 954.
- (10) Jones, E. A.; Lockyer, N. P.; Vickerman, J. C. *Anal. Chem.* **2009**, *80*, 2125.
- (11) Wucher, A.; Cheng, J.; Zheng, L.; Winograd, N. *Anal. Bioanal. Chem.* **2009**, *393*, 1835.
- (12) Vickerman, J. C. *Surf. Sci.* **2009**, *603*, 1926.
- (13) Fletcher, J. S.; Lockyer, N. P.; Vickerman, J. C. *Mass Spectrom. Rev.* **2010**, DOI: 10.1002/mas.20275.
- (14) Fletcher, J. S.; Vickerman, J. C. *Anal. Bioanal. Chem.* **2010**, *396*, 85.
- (15) Chandra, S.; Morrison, G. H. *Biol. Cell* **1992**, *74*, 31.
- (16) Colliver, T. L.; Brummel, C. L.; Pacholoski, M. L.; Swanek, F. D.; Ewing, A. G.; Winograd, N. *Anal. Chem.* **1997**, *69*, 2225.
- (17) Cliff, B.; Lockyer, N. P.; Jungnickel, H.; Stephens, G.; Vickerman, J. C. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2163.
- (18) Mony, M. C.; Larras-Regard, E. *Biol. Cell* **1997**, *89*, 199.
- (19) Nygren, H.; Johansson, B. R.; Malmber, P. *Microsc. Res. Tech.* **2004**, *65*, 282.

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(1) Vickerman, J. C.; Briggs, D. *TOF-SIMS: Surface Analysis by Mass Spectrometry*; Surface Spectra and IM Publications: Chichester, 2001.

ingly, earlier studies at cryogenic temperatures showed a correlation between the presence of a frozen water matrix and an enhancement in molecular ion signal from phosphatidylcholine lipids that were attributed to proton transfer from the water matrix to the lipid molecules.<sup>20</sup> This observation was supported in a study by Conlan et al in which it was observed that the yield of protonated species from amino acids and a nucleic base also benefited from the presence of matrix water, especially when polyatomic sources were used.<sup>21</sup> Previous work by Piwowar et al. also showed an improvement in protonated ion signal from the amino acid arginine when it was examined in a frozen-hydrated environment or as a cooled sample.<sup>22,23</sup> These observations suggest that cryogenic sample analysis with SIMS may be able help deal with low ion yields.

Cryogenic temperatures may also greatly improve the quality of profiles. Mahoney et al. discussed the benefits of lowered sample temperature for depth profiling of polymer films showing increased secondary ion stability, narrower interface widths, and a reduction in sputter-induced topography for poly (methyl methacrylate) (PMMA) when compared to room temperature studies.<sup>24,25</sup> Zheng et al. also showed sustained high mass signal and stable sputtering of Langmuir–Blodgett multilayer films with analysis at liquid nitrogen temperature compared to room temperature.<sup>26</sup> These trends of consistent sputtering yield and the maintenance of secondary ion yield with cooling of the sample were confirmed by Sjövall et al.<sup>27</sup>

An understanding of the effect of temperature on molecular depth profiling of biological materials is necessary to determine if decreased sample temperature can provide a further means of enhancing the quality of information collected from complex biological systems such as cells and tissue. In this study, we subjected several biologically relevant materials to sustained sputtering and monitored the secondary ion yields as the temperature was reduced from 300 to 80 K. Our results show that lowered sample temperature produces several changes to the spectra including increases in intensity for the protonated molecular ion for each of the samples examined. We observed a reduction in the amount of accumulated damage with ion bombardment for the arginine system, indicating that reduced sample temperature decreases the damage cross section, which may apply to other biological materials. Changes to the protonated molecular ion for arginine also are correlated to the presence of adsorbed surface water and possibly to physical changes within the sample which increase intermolecular hydrogen bond strengths.

## EXPERIMENTAL SECTION

**Material and Sample Preparation.** L-Arginine and Gly-Gly-Tyr-Arg were dissolved in Chromasolv HPLC grade water (50 and 135 mg/mL, respectively) while 1,2-dipalmitoyl-sn-glycero-3 phosphocholine and cyclosporin A were dissolved as 5 mg/mL in chloroform and 160 mg/mL in ethanol, respectively; all of which were purchased from Sigma-Aldrich, U.K. Solutions were pipetted onto a cleaned  $0.5 \times 0.5 \text{ cm}^2$  silicon shard (Agar Scientific, Essex, U.K.) and left to dry within a fume hood for approximately 30 min before introduction into the sample analysis chamber. For the sputter yield measurements, spin-cast (Laurell Technologies Corporation) films of arginine were prepared at 6700 rpm using two 7  $\mu\text{L}$  droplets of the 50 mg/mL solution. The film thickness was estimated to be between 150 and 190 nm based on the interference color shown in reflected light.

**Instrumentation.** Mass spectra were acquired using a Bio-TOF SIMS instrument; the design of which has been described previously.<sup>28</sup> The instrument is equipped with two ion beam columns: 20 kV  $\text{C}_{60}^+$  primary ion source and a 25 kV gold/germanium liquid metal ion source, both supplied by Ionoptika Ltd. Generally, analysis with 20 keV  $\text{C}_{60}^+$  was performed using 50 ns pulses with dc beam currents of 150–200 pA, with precise conditions varying slightly dependent on the experiment. Analysis with 20 keV  $\text{Au}_3^+$  was performed using 20 ns pulses with dc beam currents of 300 pA. Spectral data were collected over areas of  $200 \times 200 \mu\text{m}^2$  within etched areas of  $600 \times 600 \mu\text{m}^2$  using a spectral fluence below  $2.0 \times 10^{10}$  ions/ $\text{cm}^2$  (typically at 100 000 shots per acquisition) and an etch fluence of  $1.0 \times 10^{13}$  ion/ $\text{cm}^2$ . Sputter yield and damage cross section data were acquired using spectral fluences of  $9.5 \times 10^9$  ions/ $\text{cm}^2$ . Low energy (25 eV) electrons were flooded onto the sample between primary ion pulses to limit any effects from sample charging. The sample stage was held at ground during ion impact, and the secondary ions were directed into a two-stage reflectron by applying a delayed extraction pulse of 2.5 kV to the stage. Ions were postaccelerated to 20 keV and detected using a dual microchannel plate assembly. Mass spectra were acquired between room temperature (300–295 K) and the base temperature of the stage which is in the range of 80–90 K. Where specified, cooling experiments began at room temperature and slowly declined to cryogenic temperature. Analysis did not cease during the cooling procedure. Cooling of the sample was accomplished by introducing liquid nitrogen cooled nitrogen gas through a cooling line into the sample stage. Because the sample stage is raised to high potential during analysis, direct temperature measurement of the sample stage was not possible during SIMS acquisition. Temperature measurements were collected using a thermocouple reading from the stage in separate experiments and compared to the SIMS data as a function of time. Multiple investigations confirmed the reproducibility of the cooling procedure. Partial pressures of species in the gas phase within the analysis chamber were monitored with a residual gas analyzer (Hiden Analytical, UK). The measured base pressure of the instrument during these experiments at room temperature was  $2.5 \times 10^{-8}$  mbar with a partial pressure from water ranging from  $1.5$  to  $2.0 \times 10^{-8}$  mbar.

(20) Roddy, T. P.; Cannon, D. M., Jr.; Ostrowski, S. G.; Ewing, A. G.; Winograd, N. *Anal. Chem.* **2003**, *75*, 4087.

(21) Conlan, X. A.; Lockyer, N. P.; Vickerman, J. C. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1327.

(22) Piwowar, A. M.; Lockyer, N. P.; Vickerman, J. C. *Anal. Chem.* **2009**, *81*, 1040.

(23) Piwowar, A. M.; Fletcher, J. S.; Lockyer, N. P.; Vickerman, J. C. *Surf. Interface Anal.* **2010**, DOI: 10.1002/sia.3461.

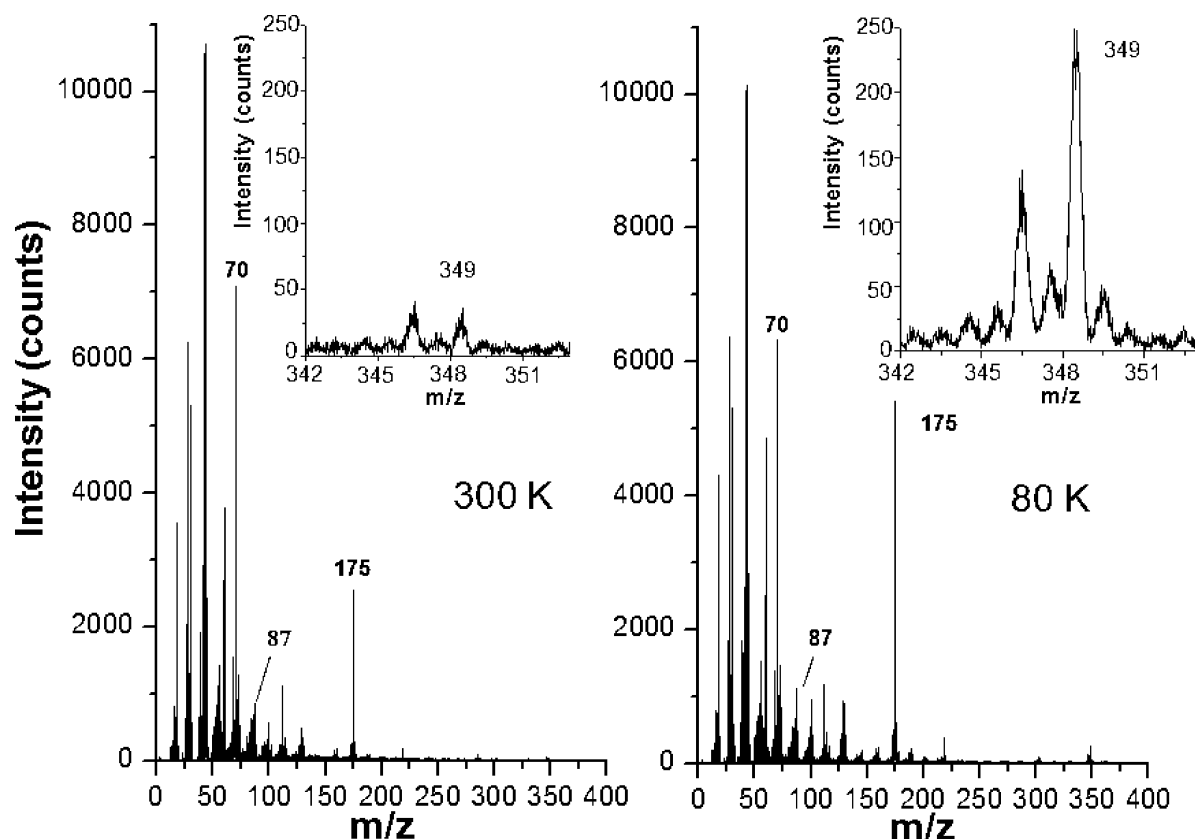
(24) Mahoney, C. M.; Fahey, A. J.; Gillen, G.; Xu, C.; Batteas, J. D. *Appl. Surf. Sci.* **2006**, *19*, 6502–6505.

(25) Mahoney, C. M.; Fahey, A. J.; Gillen, G.; Xu, C.; Batteas, J. D. *Anal. Chem.* **2007**, *79*, 837.

(26) Zheng, L.; Wucher, A.; Winograd, N. *Anal. Chem.* **2008**, *80*, 7363.

(27) Sjövall, P.; Rading, D.; Ray, S.; Yang, L.; Shard, A. G. *J. Phys. Chem. B* **2010**, *114*, 769.

(28) Braun, R. M.; Blenkinsopp, P.; Mullock, S. J.; Corlett, C.; Willey, K. F.; Vickerman, J. C.; Winograd, N. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1246.



**Figure 1.** Spectral comparison of arginine bombarded by 20 keV  $C_{60}^+$  ions at 300 K (etch dose  $2.0 \times 10^{14}$  ions/cm<sup>2</sup>; spectral acquisition dose  $1.0 \times 10^{10}$  ions/cm<sup>2</sup>; left) and 80 K (etch dose  $4.5 \times 10^{14}$  ions/cm<sup>2</sup>; spectral acquisition dose  $1.0 \times 10^{10}$  ions/cm<sup>2</sup>; right) from the steady-state region shown in Figure 2. Since the spectra were collected under similar conditions (identical fluence), the intensities are directly comparable. The main species are  $m/z$  70 ( $C_4H_6N^+$ ),  $m/z$  87 ( $C_4H_7N_2^+$ ), and  $m/z$  175 ( $M + H^+ - C_6H_{15}O_2N_4^+$ ). Inset,  $m/z$  349 (protonated dimer –  $C_{12}H_{29}O_4N_8^+$ ).

Assuming a sticking coefficient of unity, at base pressure, a monolayer of water will form on a clean sample surface in about 100 s. To avoid multilayer ice formation, acquisition time per spectrum was approximately 55 s followed by an etch cycle, ensuring submonolayer ice coverage. Multilayer ice formation blocks sampling of the sample surface, producing spectra dominated by  $(H_2O)_nH^+$  and  $(H_2O)_n^+$  ions. Deuterium oxide (99.99%; Sigma-Aldrich, U.K.) was admitted to the instrument using a leak valve connected to polytetrafluoroethylene (PTFE) tubing inside the analysis chamber. The end of the tube was positioned approximately 2 to 3 cm from the sample analysis stage.

## RESULTS AND DISCUSSION

Because it is the principal focus of this study, the SIMS spectrum of arginine is described first. Diagnostic ions for arginine observed under 20 keV  $C_{60}^+$  bombardment are shown in Figure 1. The protonated molecular ion at  $m/z$  175 is believed to arise from the protonation of the guanidino group, as it is the most basic part of the acid.<sup>29</sup> Investigations using ESI (CID) and FAB (CID) have resulted in a proposed fragmentation mechanism for the formation of the major fragment ion at  $m/z$  70 from the loss of  $H_2O$  and  $CO$  from a  $[M + H - \text{guanidine}]$  fragment (see Supporting Information Scheme 1).<sup>30,31</sup> The protonated dimer observed at  $m/z$  349 is thought to be made up of a neutral

zwitterionic arginine combined with a protonated ion assembled in a head to tail configuration with the charged guanidine group of the protonated molecule interacting with the carboxyl group of the neutral molecule.<sup>29</sup> Another common fragment from arginine,  $m/z$  87, may arise from the loss of carbodiimide from the immonium ion.<sup>32</sup>

**Secondary Ion Yields as a Function of Temperature from Arginine Using 20 keV  $C_{60}^+$ .** In general, with decreased sample temperature, several characteristic ions generated from arginine undergo changes in intensity. A typical plot of intensity as a function of  $C_{60}^+$  fluence for an arginine film as the temperature declines from 300 K to about 80 K is shown in Figure 2. The plot results from a continuous acquisition of data, with sustained ion bombardment as the sample temperature falls at approximately 9 K per minute. In a separate experiment to mimic the fast freezing scenario associated with biological cells<sup>33,34</sup> (data not shown), arginine samples were rapidly cooled with mass spectra recorded only at 80 K. These spectra were identical to

(29) Geller, O.; Lifshitz, C. J. *Phys. Chem. A* **2003**, *107*, 5654.

(30) Rogalewicz, F.; Hoppilliard, Y.; Ohanessian, G. *Int. J. Mass Spectrom.* **2000**, *195/196*, 565.

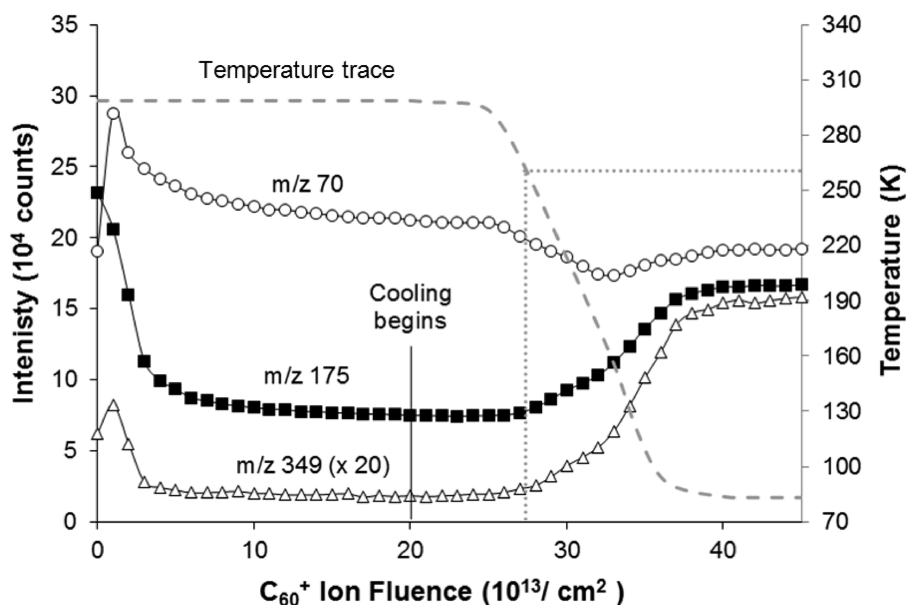
(31) Dookeran, N. N.; Yalcia, T.; Harrison, A. G. *J. Mass Spectrom.* **1996**, *31*, 500.

(32) Gehrig, P. M.; Hunziker, P. E.; Zaharieva, S.; Pongor, S. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 142.

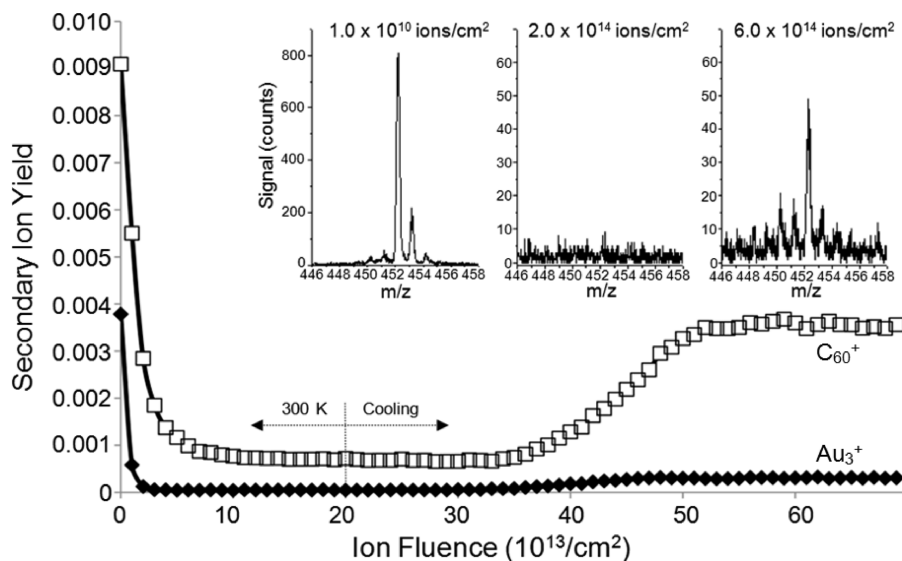
(33) Stolinski, C.; Breathnach, A. S. *Freeze-Fracture Replication of Biological Tissues: Techniques, Interpretation and Applications*; Academic Press: London, 1975.

(34) Robards, A. W.; Slevtr, U. B. *Low Temperature Methods in Biological Electron Microscopy*; Elsevier Science Publishers B.V. (Biomedical Division): Amsterdam, 1985.





**Figure 2.** Sample cooling plot of secondary ion intensity as a function of 20 keV  $C_{60}^+$  ion fluence for various arginine species. Cooling is initiated at a fluence of  $2.00 \times 10^{14}$  ions/cm<sup>2</sup> from an initial temperature of 300 K. From this point, the sample temperature decreases to 80 K over several minutes. The start of ion intensity changes is identified by the gray dot line.



**Figure 3.** Plot of secondary ion yield (number of secondary ions detected per primary ion) as a function of fluence for the  $[M + H]^+$  ( $m/z$  452) of Gly-Gly-Tyr-Arg with a 20 keV  $Au_3^+$  etch and analysis beam (filled black diamonds ( $\blacklozenge$ )) and a  $C_{60}^+$  etch and analysis beam (open white squares ( $\square$ )). The  $[M + H]^+$  ion intensity at  $m/z$  452 is shown in the insets for various  $Au_3^+$  ion fluences.

the 80 K spectral data reported in Figure 2. Hence, both slow and rapid cooling of the arginine film produces virtually identical spectra.

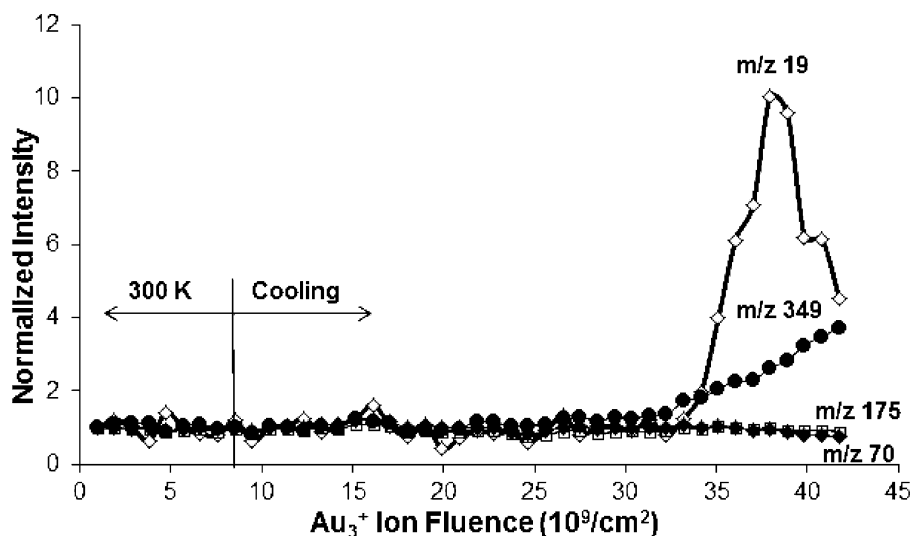
The ion yield changes begin to occur in the temperature range 250–230 K, as identified by the dashed gray line within Figure 2. The  $(M + H)^+$  ( $m/z$  175) and dimer ions ( $m/z$  349) increase by a factor of 2.2 and 6.8, respectively, whereas the  $m/z$  70 fragment declines slightly. It can also be seen that the highest yield for the  $(M + H)^+$  ion is reached at the lowest temperature attained (80 K), suggesting that lower sample temperatures may lead to even greater increases in intensity.

**Comparison of Ion Yield Enhancement with 20 keV  $C_{60}^+$  and 20 keV  $Au_3^+$  for Gly-Gly-Tyr-Arg as a Function of Temperature.** It is important to compare the temperature behavior observed using  $C_{60}^+$  to that of  $Au_3^+$ , a cluster of about

the same mass as  $C_{60}$  but with many fewer atoms and which produces much greater sputter induced damage.<sup>35,36</sup> In this case, we report the observations using Gly-Gly-Tyr-Arg, but the trends observed are reproduced in all the samples. A comparison of the ion intensities for Gly-Gly-Tyr-Arg under both 20 keV  $Au_3^+$  and 20 keV  $C_{60}^+$  bombardment as a function of fluence and temperature are shown in Figure 3. With  $Au_3^+$  at 300 K, the protonated molecular ion signal ( $M + H^+$ ,  $m/z$  452) quickly drops in intensity with increased fluence until it is almost completely absent (see inset in Figure 3). Under  $C_{60}^+$  bombardment, secondary ion intensity also falls but a steady state is

(35) Weibel, D.; Wong, S.; Lockyer, N.; Blenkinsopp, P.; Hill, R.; Vickerman, J. C. *Anal. Chem.* **2003**, *75*, 1754.

(36) Kersting, R.; Hagenhoff, B.; Kollmer, F.; Möllers, R.; Niehuis, E. *Appl. Surf. Sci.* **2004**, *231–232*, 261.



**Figure 4.** Intensity normalized to specific molecular intensity at the first acquisition, as a function of 20 keV  $\text{Au}_3^+$  ion fluence under low dose conditions for a film of arginine at 300 K and under cooling conditions. Black filled-circles (●) correspond to the  $m/z$  349 (dimer), black-filled diamonds (◆) correspond to  $m/z$  70, open squares (□) correspond to  $m/z$  175, and open diamonds (◇) correspond to  $m/z$  19 ( $\text{H}_3\text{O}^+$ ) reflecting surface adsorbed water.

reached at  $15\times$  greater intensity than that with  $\text{Au}_3^+$ . Acquisition from the same area after cooling to 80 K results in an intensity increase for the  $(\text{M} + \text{H})^+$  signal under both ion beams as shown in Figure 3, with the inset showing the recovery of signal for the film bombarded with  $\text{Au}_3^+$ . This figure demonstrates that enhancements in intensity with decreased sample temperature are qualitatively independent of primary ion beam. Because of the similarities observed, in this article, we have examined the cooling phenomenon using both ion beams. Other bio-organics such as a phospholipid (1,2-dipalmitoyl-sn-glycero-3 phosphocholine) and an immunosuppressant drug (cyclosporin A) were examined with  $\text{C}_{60}^+$  at 300K and at 80 K and were found to exhibit similar ion yield changes under cryogenic conditions (see Supporting Information Table 1).

**Secondary Ion Yields as a Function of Temperature from Arginine under Low Ion Dose Conditions.** The improved response of the samples to cooling discussed above may arise either from a change in the sputtering yield with temperature or by a change in the sputtering mechanism, resulting in lowered damage accumulation. To separate these effects, we have monitored the secondary ion intensities as a function of temperature under low dose conditions where damage accumulation would not be observable, even with the greater sputter induced damage from the  $\text{Au}_3^+$  beam. Thus, a series of analyses were obtained using 20 keV  $\text{Au}_3^+$  as the temperature declined such that the total fluence was  $<4.5 \times 10^{11}$  ions  $\text{cm}^{-2}$ , well below the static limit. Signal intensity normalized to the first spectrum shown in Figure 4 demonstrates that the  $(\text{M} + \text{H})^+$  ion and the fragment  $m/z$  70 ion do not change significantly as a function of temperature, whereas the protonated dimer,  $m/z$  349, increases quite dramatically. Thus, the doubling of the  $(\text{M} + \text{H})^+$  ion yield reported earlier in the article requires both cooling and sustained sputtering, whereas the  $>4$  times increase in dimer ion yield occurs with cooling alone, although it is also observed under sustained sputtering. Hence, there may be at least two mechanisms at work in the steady-state experiment. Sputter yields have been observed to change when the analysis

temperature of a sample is lowered.<sup>26,27</sup> For arginine, the fluence required under  $\text{Au}_3^+$  bombardment to reach the substrate interface for a uniform spin-cast film was found to be slightly less at 80 K (a 12% decrease) than at 300 K. This decrease is insufficient to account for the 2 times increase in the  $(\text{M} + \text{H})^+$  yield at the steady state, nor the  $>4$  times increase in the dimer yield. The fact that for all the samples discussed here ions at different masses are not equally affected by the change in temperature also does not fit with a uniform change in sputtering yield altering the ion yields. This is not to imply that sputter yield does not change for samples analyzed at lowered temperature but rather that a change in sputter yield is not the principal cause of the increases in steady-state ion yield observed for this system.

**Steady State at High Fluence.** Because the  $(\text{M} + \text{H})^+$  ion increased in yield when cooled under sustained sputtering, we have employed an analytical model describing the trends of the steady-state intensity for profiled materials produced by Cheng et al.<sup>37</sup>

According to the semiquantitative predictive model, the signal intensity at steady state ( $S_{ss}$ ) is related to the total sputtering yield ( $Y^{\text{tot}}$ ) and primary ion beam induced damage:

$$S_{ss} = S_0 \frac{Y^{\text{tot}}}{Y^{\text{tot}} + nd\sigma_D}$$

where  $S_0$  is the signal intensity at zero fluence,  $n$  is the density of molecules in the sample,  $d$  is the altered layer thickness, and  $\sigma_D$  is the damage cross section across the entire altered layer. We focus on the  $\text{Au}_3^+$  data for this discussion. The sputtering yield of arginine changes little with temperature, so  $Y^{\text{tot}}$  is expected to remain constant at low temperature. The investigation under static conditions showed that the signal intensity at very low fluence ( $S_0$ ) does not change from 300 to 80 K (see Figure 4). The density of molecules in this single component system will not change dramatically during the cooling

(37) Cheng, J.; Wucher, A.; Winograd, N. *J. Phys. Chem. B* **2006**, *110*, 8329.

process. The altered layer thickness is related to the depth of ion-induced damage, which at 20 keV is likely to remain constant with cooling. The remaining term,  $\sigma_D$ , was examined at 300 and 80 K by monitoring the change in  $(M + H)^+$  intensity as function of primary ion fluence up to  $\sim 2.0 \times 10^{13}$  ions  $\text{cm}^{-2}$ . At 300 K, this value was determined to be  $5.0 \pm 0.4 \times 10^{-14}$   $\text{cm}^2$ , while at 80 K a value of  $2.0 \pm 0.3 \times 10^{-14}$   $\text{cm}^2$  was measured. It is instructive to explore the effect of this reduction in  $\sigma_D$  on the steady-state signal if physically reasonable numbers are utilized in the erosion equation. Using  $Y_{\text{tot}}$  for arginine as  $\sim 200$  per impact,  $n \sim 2 \text{ nm}^{-3}$ ,  $d$  at 20 keV as  $\sim 50 \text{ nm}$ ,<sup>37</sup> and the values of  $\sigma_D$  discussed above gives a ratio of  $S_{\text{ss}}(80 \text{ K})/S_{\text{ss}}(300 \text{ K})$  of 1.8. Thus, the lowered damage cross section seems to explain the significant increase in the  $(M + H)^+$  steady state yield observed at low temperatures. A reduction in the damage cross section may also explain why lower mass fragments, such as  $m/z$  70 for arginine, decrease or remain of constant intensity with cooling.

The observation of a reduction in damage cross section for the arginine system is in contrast to results detailed by Mahoney et al. for polymer films, including PMMA.<sup>24,25</sup> From those studies, it was concluded that the benefits of low-temperature depth profiling for PMMA (increased secondary ion stability, reduced interface widths, etc) were not associated with decreased chemical damage, which actually appeared to increase at low temperature, but are instead likely to be a consequence of changing polymer properties at low temperatures resulting in improved interand intrachain coupling, producing more uniform sputtering from the films. However, ion formation and damage accumulation mechanisms for polymers are likely to be different from those for small biomolecules. Secondary species from high molecular weight polymers are fragment ions, which are produced by more than one bond scission and/or rearrangement of the polymer backbone. Damage accumulation for polymer samples are due to physio-chemical modifications including branching and cross-linking leading to a decrease in signal intensity and not due to the "disappearance" of the molecules.<sup>38</sup> For the biomolecules examined in this study, the generation of intact ionized species are observed and high fluence damage derives from the degradation of the parent molecules via chemically diagnostic fragmentations, sputtering, and/or complete destruction.<sup>38</sup> As will be discussed later in the article, the presence of a surface layer of water also appears to play a role in the apparent damage reduction for the biosystems and will have a greater effect on  $[M + H]^+$  generation, a situation which will be less effective for polymeric systems.

**Possible Mechanisms for Ion Yield Changes.** It appears that a reduction in damage cross section is a significant contributor to the enhanced steady-state signal of the  $(M + H)^+$  ion at low temperatures. The crucial question is why lowering the analysis temperature decreases the damage cross section. In addition to changes in sputter yield mentioned above, we have investigated the following: decreased internal energy of the sputtered molecules, physical changes within the sample, and the deposition of species (e.g., water) from the gas phase onto the sample surface. While it is possible that more than one

contributes to the ion yield changes, we are interested in determining which has the greatest impact and whether any can be further manipulated to provide continued enhancements of ion yields.

A decrease in molecular internal energy would contribute to increased molecular stability and decreased fragmentation. The reduction in sample temperature of about 220 K translates to a change of approximately 0.02 eV. This energy shift accounts for a less than 1% change in intramolecular bond strength when compared to single C–C, C–H, or C–N bonds. Although this energy effect may influence ion formation characteristics, its effect will be slight and has, therefore, been ruled out as a strong contributor to the observed changes. We, therefore, focus on the two remaining parameters: the possible influence adsorbed water from the vacuum and changes in intermolecular attractive forces (hydrogen bonding).

**Gas Phase Water Deposition.** Earlier studies have suggested that water in the vicinity of an analyte may enhance protonated ion yields.<sup>20–23</sup> Essentially, water molecules when bombarded with high energy primary ions are believed to be a source of excess protons for ionization events.<sup>21,39</sup> This present work focuses on the deposition of water onto a sample surface from the vacuum and not to samples in a frozen-hydrated matrix. An earlier study has suggested that the deposition of water onto sample surfaces may enhance ion yield ratios for lipids and cholesterol samples.<sup>40</sup>

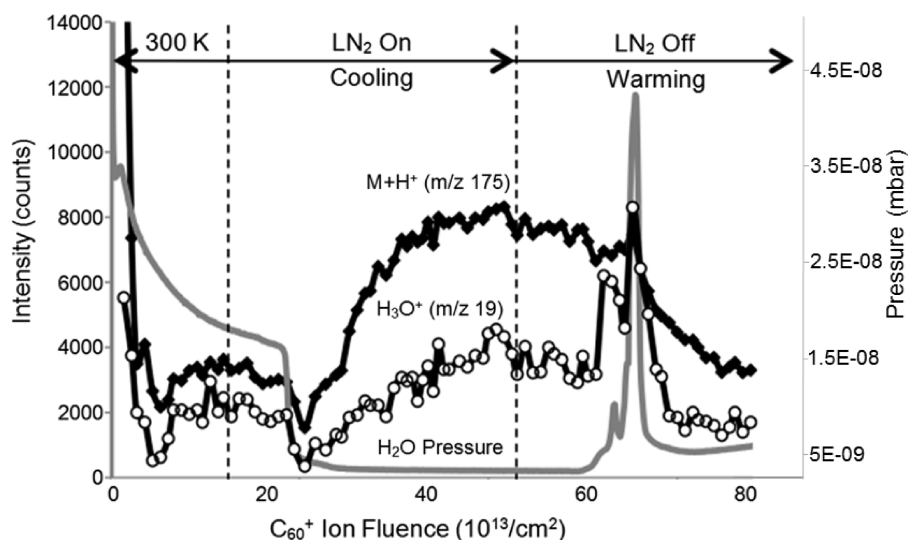
An extended sample cooling plot for the arginine molecular ion under 20 keV  $\text{C}_{60}^+$  bombardment starting at 300 K, decreasing to about 80 K, and then warming back to 300 K is shown in Figure 5. Within this plot, an overlay of the partial pressure of water in the analysis chamber, measured with a residual gas analyzer during spectral acquisition, shows a sharp decrease shortly after cooling of the analysis stage began due to water vapor from the residual gas depositing onto the liquid nitrogen cooled areas of the stage (sample analysis stage, cooling lines, sample surface, etc). After the 2-fold increase for the arginine  $(M + H)^+$  was reached at 80 K, and liquid nitrogen cooling ceased, allowing the system to warm up slowly. Several minutes later, a large pressure spike corresponding to the sublimation of deposited water into the gas phase was observed at approximately 165 K.<sup>41</sup> Following the pressure spike, the  $(M + H)^+$  signal begins to decrease, reaching near the steady state observed at room temperature prior to cooling. The variation in surface  $\text{H}_3\text{O}^+$  ( $m/z$  19) associated with these surface and gas phase changes is plotted, and it can be seen that there is broad correlation between the variation in surface  $\text{H}_3\text{O}^+$  and the yield of  $(M + H)^+$ , although there is a temperature lag in the cooling stage. These observations demonstrate that ion changes due to sample cooling are reversible, indicating that no permanent sample modification takes place during cooling and that the presence and subsequent absence of the surface adsorbed water appears to be correlated with the ion yield changes.

(38) Delcorte; A. Fundamental Aspects of Organic SIMS. In *TOF-SIMS: Surface Analysis by Mass Spectrometry*; Vickerman, J. C., Briggs, D., Eds.; Surface Spectra and IM Publications: Chichester, 2001; p 161–194.

(39) Conlan, X. A.; Fletcher, J. S.; Lockyer, N. P.; Vickerman, J. C. *J. Phys. Chem. B* **2010**, *114*, 5468.

(40) Piehowski, P. D.; Kurczy, M. E.; Willingham, D.; Parry, S.; Heien, M. L.; Winograd, N.; Ewing, A. G. *Langmuir* **2008**, *24*, 7906.

(41) Cannon, D. M.; Pacholski, M. L.; Winograd, N.; Ewing, A. G. *J. Am. Chem. Soc.* **2000**, *122*, 603.



**Figure 5.** Effect of cooling followed by warming under sustained 20 keV  $C_{60}^+$  ion sputtering on the  $H_3O^+$  (○) and arginine  $[M + H]^+$  (◆) ion intensities. The relative partial pressure of  $H_2O$  as monitored with a residual gas analyzer is shown by a gray line. The lowest pressure observed with cooling was  $7.8 \times 10^{-10}$  mbar. The first part of the acquisition takes place at 300 K until an ion fluence of  $1.8 \times 10^{14}$  ions/cm<sup>2</sup> when liquid nitrogen ( $LN_2$ ) is added to the cooling dewer. At a fluence of  $5.30 \times 10^{14}$  ions/cm<sup>2</sup>, the  $LN_2$  is shut off and the sample begins a warm up cycle.

To further investigate the role of deposited water on the changes in ion yields, we examined samples while they were exposed to deuterated water vapor. Samples were examined under standard conditions, and then, after, deuterated water vapor was introduced into the analysis chamber via a leak valve at a relatively high partial pressure (the measured partial pressure for  $D_2O$  and  $H_2O$  was  $8.0 \times 10^{-8}$  and  $4.0 \times 10^{-8}$  mbar, respectively, although the pressure near the sample is likely to be higher). As mentioned earlier, partial pressures in this region will give rise to adsorbed monolayers of water in about 2 min. If the presence of water acts as an additional protonation source, these conditions should produce changes in the isotopic distribution of peaks corresponding to the molecular ion.

The addition of  $D_2O$  to the analysis system will lead to three possible scenarios for the arginine spectra. First, the presence of  $D_2O$  may have no influence on the arginine spectra, implying that water plays little or no role in the ion yield changes observed with cryogenic analysis. Second, deposited water may affect ionization, and the addition of  $D_2O$  changes the isotopic ion pattern for arginine, favoring the formation of  $M + 2$  or  $M + D^+$  ( $m/z$  176). Third, H/D exchange may take place with other hydrogen atoms in the molecule producing a distribution of ions above  $m/z$  175. It is known that proton ionization of arginine most likely occurs at the very basic guanidino group.<sup>29</sup> Once ionized, it is also known that the  $(M + H)^+$  ion then has four relatively easily exchangeable hydrogen atoms that can exchange with a basic reactant, possibly via the carboxyl group. Under the conditions of SIMS analysis, because there will be a reasonable density of reactive fragments from  $D_2O$  in the emission zone, H/D exchange can be expected to occur. Since ionization is the most likely reaction to occur first, the appearance of an ion at  $m/z$  176 accompanied by a relative decline of  $m/z$  175 strongly suggests the ionization of arginine by  $D^+$ , presumably from  $D_3O^+$ .

An overlay of the variation of three ions during the  $D_2O$  experiment is shown in Figure S1 (Supporting Information) for  $m/z$  175 ( $M + H^+$ ),  $m/z$  176 ( $M + D^+$ ), and  $m/z$  22 ( $D_3O^+$ ). For this experiment, the sample was introduced without exposure to  $D_2O$  and analyzed as a standard cooling plot. After the normal 2-fold enhancement of the  $(M + H)^+$  for arginine had been observed at 80 K, the  $D_2O$  leak valve was opened at fluence  $5.3 \times 10^{14}$  ions/cm<sup>2</sup>. There is an immediate increase in the amount of detected  $D_3O^+$  within the spectra. At the same time, the  $(M + D)^+$  increases while the  $(M + H)^+$  signal decreases although the  $(M + H)^+$  signal is not totally suppressed, which may be due to the significant partial pressure of  $H_2O$  also present. Opening and closing the valve changes these ratios in a reproducible way. As argued above, ionization and H/D exchange occurs at the same time, so it is not possible to unequivocally decouple one from the other. However, it is clear that the surface deuterium species have a significant reactivity toward the arginine molecule which lends support to the suggestion that protonated water molecules generated by sputtering are the likely source of additional  $(M + H)^+$  yield and, hence, to the apparent reduction in the damage cross section at cryogenic temperatures. MD simulations clearly show that surface species will fragment extensively in the impact region.<sup>42</sup> These fragments may then diffuse to cooler regions of the emission zone to react with undamaged molecules. It is also possible that the fragments formed from the bombardment of the adsorbed surface water may provide a means of reducing radical ion formation within the bombarded area, further contributing to lowering the observed damage accumulation.

**Dimer Ion.** We have shown that the decrease in damage cross section could contribute to the  $(M + H)^+$  yield increase and that the protonation effect outlined above may be an underlying

(42) Ryan, K. E.; Wojciechowski, I. A.; Garrison, B. J. *J. Phys. Chem. C* **2007**, *111*, 12822.



reason. However, the increase in the dimer ion is seen on cooling in the absence of sustained sputtering, as shown in Figure 4. For the dimer, cooling alone seems to have a role for an increase in ion yield. It is important to understand the effect cooling has on the dimer formation to better understand the mechanism behind the ion yield enhancements at 80 K. It can be reasonably speculated that the following factors may be involved in the increased dimer formation from cooling: a change in hydrogen bond length and interaction with an adsorbed water layer.

Analysis of amino acids and polypeptides at room temperature and under cryogenic temperatures has been reported for Raman, infrared spectroscopy, and X-ray diffraction studies.<sup>43–46</sup> One significant observation from these studies is that cooling of the sample reduces the length of molecule–molecule and molecule–water hydrogen bonds. This is shown by the absorption maxima of groups associated with hydrogen bonding (hydroxyl and amino) experiencing significant shifts to lower energy with cryogenic analysis. An L-arginine hydrochloride monohydrate single crystal studied with polarized Raman spectroscopy over the temperature range 295–10 K revealed a strong temperature dependence of the line width of  $\nu(\text{NH}_2)$  bands, which was associated with the reduction of intermolecular hydrogen bond length N–H···O, with oxygen belonging either to a water molecule or to the  $\text{COO}^-$  group of arginine.<sup>43</sup> Conformational changes within the crystal and a phase change are also proposed to occur within the arginine system with analysis at cold temperature. Although small, the decrease in hydrogen bond length will increase long-range molecular interactions between adjacent molecules and may stabilize the formation and emission of protonated dimer ions. During the sputter/ionization process, these interactions may be maintained and contribute to the increase in dimer/trimer yield as observed in our data in Figures 1, 2, 4, and 5.

However, there are also other possible, perhaps more likely, contributions. It is noteworthy that other than in SIMS spectra, arginine dimer ions are only observed using electrospray MS, suggesting that the molecules have to be close together in a solvent for the dimer to be formed and possibly cooled by the loss of solvent molecules during ionization.<sup>47</sup> In the SIMS experiment, dimer ions are observed with low yield at 300 K. The molecules are close together in the solid, and although sputtering is a relatively energetic process, as they are emitted, some collisional cooling may occur in the emission zone. However, yields are increased by greater than a factor of 4 from the cooled sample (see Figure 4). It may be that the presence of adsorbed water at the surface is also a contributor to the enhanced yields.

To explore this further, a low fluence static SIMS study was carried out in which additional  $\text{H}_2\text{O}$  vapor was admitted to a sample of arginine in the same manner as the  $\text{D}_2\text{O}$  experiments. Analysis was carried out using 20 keV  $\text{C}_{60}^+$  at 300 and 170 K

(around the water sublimation temperature) without sustained bombardment (see figure S2 from Supporting Information). As in Figure 4, the  $(\text{M} + \text{H})^+$  signal scarcely increases, whereas the dimer signal increases 10-fold, roughly twice that observed in the absence of added water, indicating again the importance of water for dimer formation (note: although no water was added for the data in Figure 4, there was a significant residual pressure of water in the system). Dimer ion formation was also affected by the presence of deuterated water from the experiment summarized in Figure S1, Supporting Information. The dimer ion intensity followed a pattern similar to  $m/z$  176, providing further support to the role of water in increasing ion yields under the cooling conditions. Unfortunately, we are not able to eliminate water entirely from our vacuum system to prevent deposition on the sample at cryogenic temperatures, so we cannot unequivocally prove the role of water in this dimer formation process. However, it seems reasonable to suggest that surface water may mediate the enhanced formation of the dimer and sputtered water molecules may provide improved collisional cooling as the species are emitted. Interestingly, the immonium ion for arginine ( $m/z$  129) increases in line with the dimer. Usually, the gas phase MS of arginine does not give rise to the immonium ion; however, the protonated dimer structure has a head to tail structure with the guanidino group of one molecule interacting with the carboxyl group of the other,<sup>48</sup> and this arrangement of the dimer may encourage the stabilization of an immonium ion.

Overall, the data suggest that cooling the sample gives rise to spectral changes in the dimer region that appear to be consequent on structural and chemical changes between the molecules of the sample enhanced by the presence of surface water. When the sample is subjected to sustained sputtering under cryogenic conditions, a decrease in the damage cross section contributes to an enhanced yield in  $(\text{M} + \text{H})^+$  at steady state that seems to be largely due to adsorbed water molecules becoming effective as proton sources under ion bombardment. This correlates well with the observation that the  $\text{Au}_3^+$  and  $\text{C}_{60}^+$  are very effective in generating  $\text{H}_3\text{O}^+$  during sustained bombardment at fluences  $\geq 10^{13}$  ions  $\text{cm}^{-2}$ .<sup>39</sup>

**Negative Ion Data.** Another facet of this investigation involved the examination of negative ion data for arginine and Gly-Gly-Tyr-Arg. Our initial investigation of these samples shows that the deprotonated molecular ion  $(\text{M} - \text{H})^-$  is also influenced by the lowered sample analysis temperature. The tetrapeptide  $(\text{M} - \text{H})^-$  ion shows a 3-fold enhancement with analysis at 80 K compared to analysis at 300 K while arginine shows an increase in the  $(\text{M} - \text{H})^-$  and dimer ions by factors of 3.7 and 7, respectively, with the fragment ion at  $m/z$  131 increasing by 40%. These results mirror the work collected in positive ion mode and may also be related to the decrease in damage cross section with lowered sample temperature. Since the abstraction of a proton is involved, the causes of this change may be strongly related to those for positive ions. It will be noted that Sjövall et al. have shown that there is a reduction in intensity for all characteristic Irganox 1010 negative ions when analysis is performed at  $-80^\circ\text{C}$ , indicating that more research needs

(43) Lima, R. J. C.; Freire, P. T. C.; Sasaki, J. M.; Melo, F. E. A.; Mendes Filho, J. J. *Raman Spectrosc.* **2002**, *33*, 625.

(44) Drebushchak, T. N.; Boldyreva, E. V.; Kolesnik, E. N. *J. Struct. Chem.* **2006**, *47*, 106.

(45) Chesalov, Y. A.; Chernobay, G. B.; Boldyreva, E. V. *J. Struct. Chem.* **2008**, *49*, 1012.

(46) Chernobay, G. B.; Chesalov, Y. A.; Boldyreva, E. V. *J. Struct. Chem.* **2009**, *50*, 1059.

(47) Geller, O.; Lifshitz, C. *J. Phys. Chem. A* **2003**, *107*, 5654.

(48) Julian, R. R.; Beauchamp, J. L.; Goddard, W. A., III. *J. Phys. Chem. A* **2002**, *106*, 32.

to be conducted to better understand the cooling effects on negative ion formation.<sup>27</sup>

## CONCLUSIONS

The variations in secondary ion yields from a variety of biological molecules as a function of analysis temperature have been investigated. We have concluded from this study that: (1) Under sustained bombardment, whether by  $C_{60}^+$  or  $Au_3^+$ , following a significant decrease in yield at 300 K to a steady state, cooling the sample to 80 K during analysis increases the intensity of the protonated molecular ion up to a factor of 5 compared to analysis at room temperature. (2) Ion yields of other diagnostic ions such as fragment species and dimer/trimer species are affected to differing extents by the cooling process. Low molecular weight fragments show little or no change, while higher molecular weight species show substantial increases. (3) Under low dose static conditions, the protonated arginine molecular ion and the main fragment do not increase with cooling, suggesting that the mechanism behind the observed enhancements under sustained sputtering are related to a reduction in damage accumulation at cryogenic temperatures. This proposal is supported by the observed reduction in the 20 KeV  $Au_3^+$  damage cross section from  $5.0 \pm 0.4 \times 10^{-14} \text{ cm}^2$  at 300 K to  $2.0 \pm 0.3 \times 10^{-14} \text{ cm}^2$  at 80 K. (4) The presence of surface adsorbed water from the analysis chamber residual gas appears to be intimately linked to the

reduction in damage cross section and the enhancement process for the  $(M + H)^+$  ion under sustained sputtering at low temperatures. (5) Arginine dimer ion formation increases with cooling both in the presence and in the absence of sustained bombardment, indicating that a second enhancement mechanism is operational. The presence of adsorbed water appears to be important, possibly suggesting a structural mechanism behind the enhancements.

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## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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