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Article

Efficacy and Mechanisms of Copper Ion-Catalyzed Inactivation of Human Norovirus

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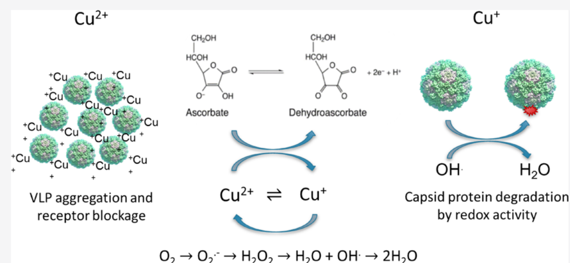
ABSTRACT: The antinoroviral effect of copper ions is well known, yet most of this work has previously been conducted in copper and copper alloy surfaces, not copper ions in solution. In this work, we characterized the effects that Cu ions have on human norovirus capsids' and surrogates' integrity to explain empirical data, indicating virus inactivation by copper alloy surfaces, and as means of developing novel metal ion-based virucides. Comparatively high concentrations of Cu(II) ions (>10 mM) had little effect on the infectivity of human norovirus surrogates, so we used sodium ascorbate as a reducing agent to generate unstable Cu(I) ions from solutions of copper bromide. We found that significantly lower concentrations of monovalent copper ions (~0.1 mM) compared to divalent copper ions cause capsid protein damage that prevents human norovirus capsids from binding to cell receptors *in vitro* and induce a greater than 4-log reduction in infectivity of Tulane virus, a human norovirus surrogate. Further, these Cu(I) solutions caused reduction of GII.4 norovirus from stool in suspension, producing about a 2-log reduction of virus as measured by a reverse transcriptase-quantitative polymerase chain reaction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) data indicate substantial major capsid protein cleavage of both GI.7 and GII.4 norovirus capsids, and TEM images show the complete loss of capsid integrity of GI.7 norovirus. GII.4 virus-like particles (VLPs) were less susceptible to inactivation by copper ion treatments than GI.7 VLPs based upon receptor binding and SDS-PAGE analysis of viral capsids. The combined data demonstrate that stabilized Cu(I) ion solutions show promise as highly effective noroviral disinfectants in solution that can potentially be utilized at low concentrations for inactivation of human noroviruses.



KEYWORDS: *norovirus, viral inactivation, antiviral, copper, Tulane virus, disinfection*

Human norovirus is estimated to cause 685 million illnesses and over 200,000 deaths globally annually.[1] Noroviruses are particularly difficult to inactivate owing to their highly stable protein capsids, which are resistant to heat, pH, and drying.[2] Commonly used disinfection agents such as ethanol, quaternary ammonium compounds, and peroxides have limitations for inactivation of noroviruses,[3] with these viruses having the potential to environmentally persist and spread under typical cleaning protocols. Bleach remains the most widely accepted inactivation agent against norovirus, but it is also too corrosive, noxious, and aggressive for many applications.[4]

Metallic copper in the form of vessels and kitchenware has long been known empirically as a potent antimicrobial agent.[5,6] Recently, numerous groups have reported that solid copper surfaces are able to trigger norovirus inactivation likely via production of reactive oxygen species, with viral load reduced by up to 4 logs when measured by RT-qPCR and up to 5 logs when measured by a plaque assay of the human norovirus surrogate murine norovirus (MNV)[7,8] and similar inactivation via a plaque assay with another norovirus surrogate, Tulane virus.[9] The ability of copper-containing alloys to inactivate noroviruses has been found to depend on the alloy composition, with the copper fraction directly correlated to the degree and rate of virus inactivation.[7,8,10] Copper surfaces were among the first materials to be recognized by the US Environmental Protection Agency (US EPA) as having antimicrobial properties.[11] Numerous groups have generated copper nanoparticles for their biocidal action[12] and embedded them into materials such as thin-film composite membranes to prevent biofouling.[13]

The use of copper ions instead of copper alloys and nanoparticles has a number of advantages, including using and releasing of significantly lower metal amounts into the environment and avoiding the release of potentially hazardous nanomaterials. Cu(II) salts and mixtures have demonstrated biocidal activity[14] and have been loaded onto material matrices such as gels[15] and polymer fibers.[16] Cu(I) ions have been

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identified as contributors to the innate immune response against bacterial pathogens. Upon phagocytosis of a pathogen by a macrophage, the phagolysosome develops a myriad of antimicrobial actions, including increased uptake of Cu(I) ions that can cause oxidative damage to proteins, lipids, and DNA via Fenton chemistry.[17] Many bacterial pathogens have developed resistance to the toxic effects of copper ions using ion-specific pumps to remove elevated ion levels,[18,19] but viruses do not have the ability to acquire this kind of defense mechanism. Copper iodide nanoparticles that release Cu(I) ions have demonstrated high efficacy against a number of human norovirus surrogates.[8,9,20]

The biocidal effects of Cu(I) ions appear to be a result of the oxidative effects from copper's redox activity.[21] Copper is a redox-active transition metal that can be present in its monovalent or divalent form in solution. Cu(I) ions are unstable and either react with dissolved oxygen to form Cu(II) or disproportionate into Cu(s) and Cu(II).[22] The required concentration of Cu(I) ions in solution can be attained by the addition of a reducing agent to solutions of Cu(II). Many reactions, such as those carried out in click chemistry,[23] commonly use the ascorbate ion as a reducing agent for copper. Solid iron surfaces have also been used as reducing agents for copper ions to generate a biocidal environment.[24]

Noroviruses are the leading cause of foodborne illnesses globally[1] and cause considerable economic losses, estimated at $65 billion (US).[25] Noroviruses are highly transmissible and result in the emergence of new pandemic strains every few years, as the virus mutates in response to herd immunity.[26,27] Norovirus strains are placed into genotypes and genogroups based on sequence similarities of the major capsid protein, VP1, and strains within a genogroup have at least 60% sequence homology, while strains within a genotype are more closely related.[28] Of the six norovirus genogroups, GI, GII, and GIV contain strains that cause human illness.[28,29] Histo-blood group antigens (HBGAs) are present on the surface of intestinal cells and are important in facilitating infection, although their role in infection is not fully resolved.[30,31] Human norovirus binding to HBGAs is strain-dependent, allowing certain strains to infect individuals with specific blood types and not others.[28,32] The capsids of new norovirus strains appear to antigenically evade the immune response while still allowing differential binding to HBGAs.[33−35] These capsid changes may also contribute to differences in strain responses to disinfection methods.

Some reports suggest that human norovirus disinfection by a variety of methods is strain-dependent. Specifically, GI strains have been found more susceptible than GII strains to inactivation by alcohols[36,37] and heat treatment.[38] A recent report by Recker and Li[9] also presents a similar trend with GII.4 Sydney being less susceptible to inactivation than GI.3B Potsdam norovirus when exposed to copper surfaces evaluated with HBGA-binding prior to RT-qPCR. However, many disinfection studies tend to use GII.4 strains of human norovirus, as it is the most prevalent genotype, and the genotype for which stool-containing human noroviruses is generally the most available, in addition to related cultivable surrogate viruses. Demonstration of the loss of HBGA binding is useful in evaluating inactivation of noroviruses on the basis of the fact that HBGAs are necessary co-receptors/attachment factors for many norovirus strains; thus, if a viral capsid is unable to bind a co-receptor necessary for infection, it is unlikely to be infectious.[39,40] We compare the effects of copper-mediated oxidative damage on GI.7 and GII.4 VLPs to further elucidate differences in susceptibility to disinfectant treatments between human norovirus genotypes.

Different inactivation techniques target non-enveloped viruses in distinct ways. For example, in disinfection of an MS2 bacteriophage, free chlorine causes capsid protein cleavage that inhibits genome injection in addition to direct genome damage that inhibits replication, while singlet oxygen mainly targets genome replication.[41] Multiple mechanisms by which microorganisms are inactivated by contact with metallic copper have been suggested, including damage to nucleic acid; damage to the plasma membrane of cells; obstruction of enzyme activity; and indirect oxidation of proteins, lipids, and nucleic acids by formation of reactive oxygen species (ROS).[42] Because of their lack of a membrane and enzymes, non-enveloped viruses are only susceptible to the effects of nucleic acid damage and oxidative damage by generation of ROS. Both of these mechanisms have been implicated in the contact killing of human norovirus and its surrogates on copper alloys.[7,8,10] In copper ion-mediated disinfection, copper ions in multiple oxidation states, reduction products of dissolved oxygen,[43] and reducing agents could all be present and active. Oxidative nucleic acid damage has been recognized and studied extensively due to its implications in carcinogenesis and other age-related diseases.[44−46] The generation of ROS during copper ion redox produces more potent oxidative conditions than *in vivo* metabolism because of the absence of many antioxidants,[47] so nucleic acid damage is expected to be a significant contributor to the loss of virus infectivity in copper ion-mediated virucides. Indeed, Manuel et al. demonstrated a 4-log reduction in human norovirus RNA copy number after incubation on copper alloy surfaces.[10]

Although some research has been done on the effects of copper surfaces and nanoparticles, the antiviral efficacy on human noroviruses of ionic copper in solution with a reducing agent has not been thoroughly investigated. We report the mechanisms of action of alternative copper ion-based disinfectants on human norovirus while investigating potential differences caused by genotype susceptibilities. Tulane virus, another virus in the *Caliciviridae* family, is a commonly utilized human norovirus surrogate with similar structural properties to noroviruses and exhibits relatively comparable pH, heat, ethanol, and chlorine susceptibility to noroviruses and other surrogates.[48,49] After treatment, we estimate the resulting loss of human norovirus infectivity and capsid integrity using numerous techniques, including Tulane virus (TV) plaque assays, histo-blood group antigen (HBGA) binding assays of human norovirus virus-like particles (VLPs), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of VLPs, and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) of infectious GII.4 human norovirus from stool.

■ RESULTS AND DISCUSSION

We used plaque assays of a human norovirus surrogate, Tulane virus (TV), in combination with HBGA binding assays of human norovirus VLPs to assess the effects of different copper ion formulations on human norovirus infectivity. We selected copper bromide as a source of divalent copper ions and added sodium ascorbate in at least 10-fold excess to reduce Cu(II) and create a redox active environment. For plaque assay experiments, we treated TV stocks with $CuBr_2$ solutions with concentrations ranging from 0.01 to 1 mM for 30 min to

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evaluate the effects of Cu(II) ions alone. We also treated TV stocks with $CuBr_2$ at concentrations ranging from 0.001 to 1 mM in combination with 10× excess ascorbate for 30 min. After determining a formulation that induces greater than 4-log reduction in TV infectivity using a 30 min incubation, we treated the virus stocks for shorter times to find out the minimum time it takes to achieve substantial inactivation.

The efficacy of the viral inactivation is presented in Figure 1. $CuBr_2$ alone at 1 mM induced only 1-log reduction in virus titer and yields even lower reduction at a lower concentration (Figure 1a). The addition of ascorbate significantly enhanced the observed inactivation, causing greater than a 4-log reduction in virus titer with as low as 0.1 mM concentration of copper and approximately 1-log reduction at 0.01 mM copper. Based on these results, we evaluated the efficacy of a solution containing 0.1 mM $CuBr_2$ and 10 mM ascorbate at different treatment times. As seen in Figure 1b, greater than 4-log reduction of virus titer was observed in as short as 1 min, indicating rapid and effective inactivation at these solution conditions.

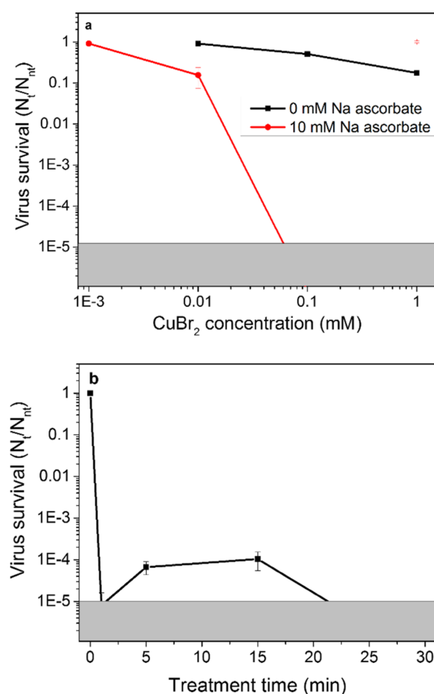

Figure 1. Addition of sodium ascorbate dramatically reduces Tulane virus infectivity via the plaque assay. Results of the TV plaque assay after treatment with (a) varying concentrations of $CuBr_2$ with and without 10 mM sodium ascorbate as a reducing agent and (b) 0.1 mM $CuBr_2$ with 10 mM sodium ascorbate at varying time points. Virus survival is the number of plaque forming units (pfu) after each treatment (N_t) normalized to the number of pfu without treatment (N_{nt}) determined during the same set of experiments. The areas outside the limit of detection of the assay are marked in gray. Error bars represent the average of three replicates at each condition.

Taken together, these results show the promise of Cu(I) as a potential inactivation agent against Tulane virus, a human norovirus surrogate. The ability of 0.1 mM Cu(I) in 1 min at room temperature is a notably higher amount of inactivation observed for a human norovirus surrogate compared to many solution-based inactivation agent formulations on norovirus surrogates.[50−52] For example, sodium hypochlorite is widely regarded as one of the most effective inactivation agents for noroviruses and their surrogates. Hirneisen and Kniel observed about a 3- and 5-log_{10} reduction of Tulane virus treated with 200 and 2000 ppm chlorine after 5 min of exposure at room temperature, respectively.[51] Comparatively, 3- and 4-log_{10} reductions for Tulane virus were reported by Tian et al.[53] after 10 min of treatment with 300 and 500 ppm chlorine at room temperature, respectively. Arthur and Gibson[48] observed the less efficacy of chlorine against Tulane virus dried on a surface, observing a less than 2.5 log_{10} reduction in Tulane virus treated with 1000 ppm chlorine at room temperature. However, it should be noted that this work was performed in suspension rather than on a surface as performed by Arthur and Gibson;[48] and none of these studies evaluate the effect of organic load on inactivation of virus. As a reference, application of 1000−5000 and 200 ppm chlorine on non-food contact and food contact surfaces are the concentrations recommended by the U.S. CDC and FDA, respectively.[54] In sum, this example demonstrates the potential for Cu(I) in solution to serve as an inactivation agent for noroviruses based on its ability to inactivate Tulane virus, a commonly used human norovirus surrogate.

To better ascertain the effects of copper solution treatment on the viral capsid, we evaluated the ability of the virus capsid to bind histo-blood group antigens (HBGAs), a carbohydrate cell marker and putative viral receptor/co-factor,[28,32] using VLPs of the GI.7 and GII.4 Sydney strains of human norovirus. The degree of VLP binding to HBGAs is an indication of capsid integrity, as loss in binding is generally correlated with a loss of infectivity. These assays are colorimetric, and each absorbance data point was normalized to a positive VLP control that was not treated. GI.7 VLPs were treated for 30 min with solutions of $CuBr_2$ ranging in concentrations from 0.001 to 100 mM, both with and without ascorbate. Ascorbate (1 mM) was used because higher concentrations interfered with assay results. Concentrations of $CuBr_2$ ranging from 0.0001 to 0.1 mM $CuBr_2$ in combination with ascorbate were applied to the VLPs at shorter times to determine the time required for the loss of capsid integrity at each concentration.

As seen in Figure 2a, $CuBr_2$ alone only slightly reduces GI.7 VLP binding to HBGA at 10 mM copper, while near-complete suppression of binding required 100 mM copper. Complete reduction in binding occurred after treatment with $CuBr_2$ at 10^5-fold lower concentration in combination with ascorbate. At 100 mM Cu(II) + ascorbate, a higher VLP-HBGA binding was observed than with Cu(II) alone. We believe that this may be due to the fact that ascorbate is only in excess of Cu(II) ions below 1 mM $CuBr_2$, and it is possible that the mixture of Cu(I) and Cu(II) ions actually had a counteractive effect compared to the predominantly one type of ion alone. Thus, we used only copper concentrations below 1 mM to evaluate the efficacy of $CuBr_2$/ascorbate mixtures to reduce VLP-HBGA binding at shorter treatment times to ensure excess ascorbate. Further work investigating this possibility and the potential influence of mixtures of copper ions on antiviral efficacy should be conducted in the future. As seen in Figure 2b, we observed very strong antiviral activity at low Cu ion concentrations. Only 1 min was required to damage capsid integrity with as low as 0.01 mM $CuBr_2$ combined with ascorbate. Even 0.001 mM $CuBr_2$ with ascorbate was found to eliminate VLP-HBGA binding in 15 min.

We observed notable differences between the susceptibility of GI.7 and GII.4 VLPs to inactivation by Cu(I) solutions.

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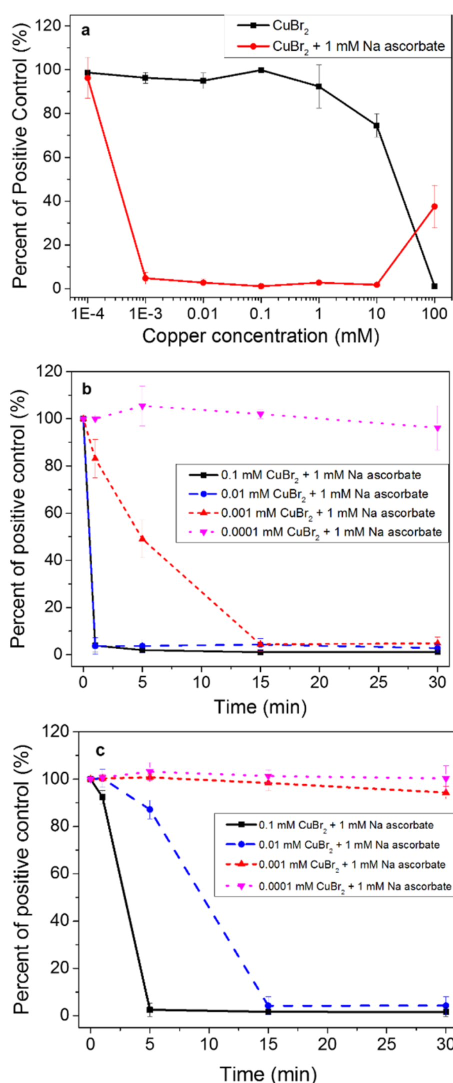

Figure 2. Copper(I) solutions dramatically reduce the ability of norovirus capsids to bind HBGAs. Results of the VLP binding assay with HBGA after (a) GI.7 VLP exposure to $CuBr_2$ at varying concentrations for 30 min with and without sodium ascorbate and (b) GI.7 VLP and (c) GII.4 Sydney VLP exposure to $CuBr_2$ at varying concentrations with 1 mM sodium ascorbate over time. Error bars represent the standard error of three replicate samples.

GII.4 Sydney VLPs appeared to be more resistant to ionic copper, requiring about an order of magnitude higher concentration to induce the same reduction in HBGA binding. As seen in Figure 2c, even 0.1 mM $CuBr_2$ combined with ascorbate took 5 min to reduce GII.4 Sydney VLP binding to HBGA, and 0.01 mM $CuBr_2$ with ascorbate took 15 min for complete binding reduction. No reduction in binding was observed for treatments of 0.001 and 0.0001 mM $CuBr_2$ with ascorbate. The apparent difference in susceptibility to Cu(I) solution treatment between GI.7 and GII.4 Sydney VLPs indicates that the sequence and morphological differences between human norovirus genotypes can influence virus resistance to inactivation. However, it should be noted that the possibility of different amounts of residual organic material in the different VLP preparations could influence these results. Multiple lots of the VLPs were utilized to obtain these results, but it cannot be completely dismissed that residual organic materials in the VLP preparation influenced the observed difference. Similarly, there is a possibility that susceptibility of VLPs versus infectious viral particles could be different based on the lack of VP2 and genomic RNA. These results support additional work suggesting that it cannot be assumed that new emerging norovirus strains will be inactivated by the same treatments with the same efficiency that they have shown against previous strains. This higher susceptibility of GI than GII has also been reported for different alcohols.[36]

Both TV plaque assays and VLP-HBGA binding assays indicate that Cu(II) ions alone are relatively ineffective in triggering norovirus inactivation except at very high concentrations. The addition of a reducing agent such as ascorbate is required to generate Cu(I) ions and introduce an oxidative environment that damages the viruses enough to reduce infectivity. The resultant inactivation occurs at very short treatment times, making these solutions both rapid and highly effective. This is accompanied by the loss of capsid integrity observed in TEM images of VLPs treated with $CuBr_2$/ascorbate mixtures for varying times. TEM allows direct facile observation of the integrity of VLPs and virus envelope shells.[55] Intact VLPs that had not been treated with any copper solution can be seen in Figure 3a. VLPs treated with 0.1 mM

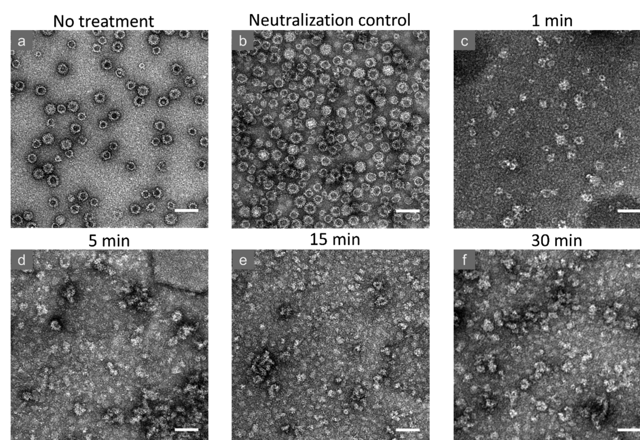

Figure 3. Degradation of norovirus capsids by ionic copper(I) via electron microscopy. Representative TEM images of GI.7 VLPs (a) without treatment; (b) after treatment with 0.1 mM $CuBr_2$ and 10 mM sodium ascorbate in the presence of 10 mM EDTA (to bind copper ions) for 30 min; and after treatment with 0.1 mM $CuBr_2$ and 10 mM sodium ascorbate for (c) 1 min, (d) 5 min, (e) 15 min, and (f) 30 min. Significant capsid degradation is seen within minutes, leaving mostly capsid protein aggregates and a few recognizable capsid structures. Scale bars represent 100 nm.

$CuBr_2$ and 10 mM ascorbate for as short as 1 min lost most of their structure, with only capsid protein aggregates and a few damaged capsids remaining, as seen in Figure 3c−f. These images correlate with the inactivation data obtained from TV plaque assays and VLP-HBGA binding assays, which indicate the loss of infectivity and binding at the same copper concentration in combination with ascorbate. However, there are a number of inherent difficulties and limitations related to what can be inferred from VLP HBGA binding assays (and microscopy below), as previous evidence suggests that these VLPs are not as stable as infectious particles, the assays do not account for particles with fatal genomic mutations, and disruption of capsid functionality and integrity (via binding or visual observation of disruption) does not necessarily correlate 1:1 with inactivation as evaluated by the plaque or

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$TCID_{50}$ assay.[40] Further, evidence for some norovirus strains suggests that there are other potential molecules involved in norovirus infection. Regardless, the data from these assays suggest and confirm that ionic copper disrupts norovirus capsid stability and functionality.

We used RT-qPCR of GII.4 norovirus-infected stool to evaluate the loss of genomic copy numbers, as the reported human norovirus cell culture systems were not available to us for direct *in vitro* infectivity studies.[56,57] The RT-qPCR method allowed us to determine the effects of Cu(I) solutions on human norovirus, but it should be noted that such analysis also reflects the signal due to non-infectious viral RNA and often underestimates the degree to which infectious virus is reduced. Future work evaluating the effects of these treatments on human norovirus inactivation using additional *in vitro* methods to remove some of the non-infectious viral particles, such as PMAxx,[58−60] and binding pre-treatment should be conducted.[9,40] In fact, Recker and Li[9] utilized porcine gastric mucin binding prior to RT-qPCR for noroviruses subjected to copper alloy surfaces. Because we have plaque assay data for TV, we also measured the loss of genomic copy number within TV samples after treatment with Cu(I) solutions. The comparison of TV plaque assay and RT-qPCR data further supports previous observations that RT-qPCR often underestimates reduction of infectious virus. Further, the possibility that human noroviruses are inherently less susceptible to these treatments can also not be dismissed, but future work utilizing human norovirus cultivation techniques should investigate this. As shown in Figure 4, only 2-log reduction in GII.4 norovirus genomic copy number (in stool medium) occurred after treatment with 0.1 mM $CuBr_2$ in combination with 100 mM sodium ascorbate. At this copper concentration, we observed about a 4-log reduction in TV genomic copy number. Similarly, at 1 mM $CuBr_2$ with 10 mM sodium ascorbate, a 3-log difference exists in the loss in genomic copy numbers between TV and GII.4 norovirus. This 3-log difference in inactivation between GII.4 norovirus and TV may potentially suggest differences in the nature of susceptibility to copper between the two viruses. However, given the previously reported relative hardiness of TV to other oxidative disinfectants,[48−50] this seems less likely. It may be possible that the presence of stool material (organic load) in the human norovirus samples had a more pronounced quenching effect on the ionic copper than the cell culture buffer, as doping feline calicivirus into stool has been reported to increase its resistance to heat.[61] As has been observed in multiple previous studies,[26,40] RNase treatment followed by RT-qPCR overestimated the number of infectious Tulane virus particles in solution at the various treatments (Figure 4), thus indicating lower reductions than were observed with the plaque assay. This is likely due to inactivation of particles by more subtle damage to intact capsids' higher order protein structure, which is needed to bind receptor(s).[39]

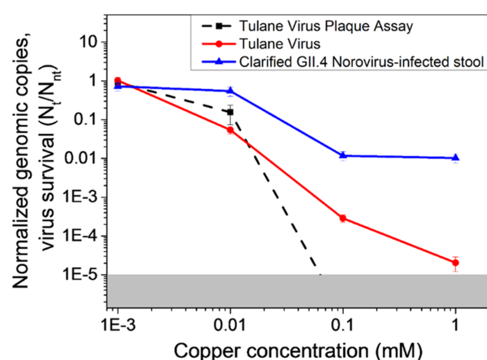

Figure 4. Ionic copper(I) solutions inactivate Tulane virus and infectious human norovirus in solution. Loss of genomic copy number of Tulane virus and clarified stool containing GII.4 norovirus after treatment with 10 mM sodium ascorbate and various copper concentrations. After copper treatments, the samples were digested with RNase. Error bars represent the standard error of three replicate samples.

HBGA-VLP binding assays demonstrated that GII.4 VLPs completely lost their ability to bind HBGA after treatment with 0.01 and 0.1 mM $CuBr_2$ with ascorbate for 30 min, whereas RT-qPCR data showed 1-log reduction or less in genomic copy number at these conditions. These data indicate that Cu(I) solutions may inactivate human norovirus by disrupting binding to host cells instead of destroying the virus particle completely. We therefore investigated the effects of Cu(I) solution treatments on human norovirus capsid proteins using SDS-PAGE of GI.7 and GII.4 Sydney VLPs. As seen in Figure 5a, the major capsid protein band of GI.7 VLPs was reduced to less than 40% of the value of an untreated control after only 5 min of treatment and was reduced to less than 20% of the control after 30 min of treatment with 0.1 mM $CuBr_2$ and ascorbate. This loss of band intensity indicates that significant

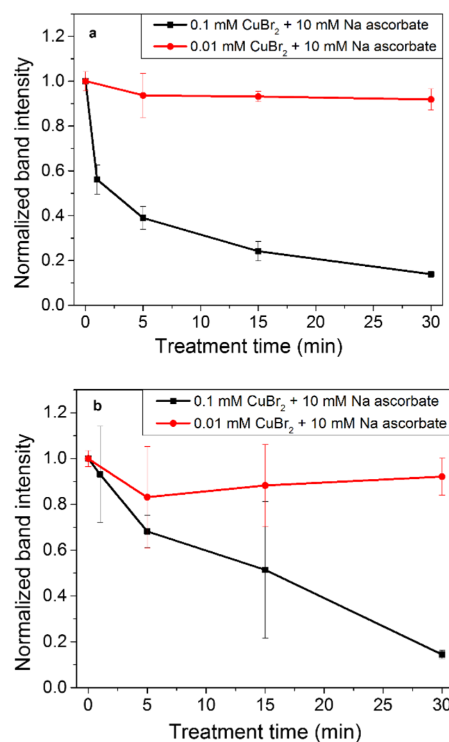

Figure 5. Ionic copper(I) treatment likely degrades norovirus capsid protein. SDS-PAGE data demonstrating capsid protein cleavage of (a) GI.7 VLPs and (b) GII.4 Sydney VLPs after treatment with 10 mM sodium ascorbate and 0.1 or 0.01 mM copper bromide. Normalized intensity represents the intensity of the major capsid protein band adjusted to the background of the gel and normalized to a control sample that was not treated. The GI.7 capsid protein is degraded more rapidly by copper ion treatment than GII.4 Sydney VLPs at 0.1 mM ion concentration. Error bars represent the standard error of three replicate samples.

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capsid protein cleavage likely occurred during the treatment. Thus, redox activity involving ascorbate as a reducing agent and copper as a catalyst to generate damaging ROS is the likely mechanism behind the substantial covalent destruction of the major capsid protein. Measurement of Cu(I) generated from the reaction was confirmed with bathocuproinedisulfonic acid, though we did not directly measure ROS or ROS species generated from the work. Future work deciphering and directly measuring ROS levels, potential ROS products, and their direct effect on norovirus infectivity would be valuable. Minimal protein cleavage occurred after treatment with 0.01 mM $CuBr_2$ and ascorbate, with less than 10% loss of band intensity regardless of treatment time. As seen in Figure 5b, the major capsid band of GII.4 Sydney VLPs was reduced to about 15% of the untreated control after 30 min of treatment with 0.1 mM $CuBr_2$ and ascorbate. Minimal loss of capsid protein band intensity was observed after treatment with 0.01 mM copper and ascorbate, regardless of treatment time. These results would be expected given the lower reduction observed in RT-qPCR data, as capsid degradation was not severe for GII.4 at this concentration. At treatment intervals shorter than 30 min, the loss in band intensity after copper ion treatment was about 20−30% less for GII.4 Sydney VLPs than for GI.7 VLPs, indicating that the GII.4 major capsid protein may have greater stability and resistance to oxidative treatments. However, it should be noted that the loss in band intensity could also be due to the residual protein content from VLPs that was co-purified in gradient ultracentrifugation, which could also contribute to these observed losses of band intensity. Although Western blot analysis was not conducted, previous observations of capsid degradation via SDS-PAGE and Western blot were observed after norovirus exposure to copper alloys with VLPs.[10] Although Western blot analysis conducted here to confirm the loss of band intensity was solely attributable to norovirus capsid degradation, similar results to what was observed by Manuel et al. could be expected, though future work should confirm this observation. The representative gels for GI.7 and GII.4 VLPs are presented in Figure S1.

Both GI.7 and GII.4 Sydney VLPs exhibit a distinct difference between the effects of copper at 0.01 and 0.1 mM concentrations as evaluated by SDS-PAGE. These data correlate well with TV plaque assay data, which show a decrease of 4-log in virus survival after increasing the copper concentration from 0.01 to 0.1 mM. This increase in copper concentration could be a threshold for raising ROS to a level where free radical initiation and propagation reactions significantly exceed termination reactions and therefore cause widespread protein damage. HBGA-VLP assay data showed the loss of binding after treatments with <0.01 mM Cu(I), but such loss of binding could occur with capsid conformational changes induced by less potent oxidizing conditions. Both HBGA-VLP binding assays and SDS-PAGE data indicated that GII.4 Sydney VLPs are less susceptible to damage by Cu(I) solutions than GI.7 VLPs.

The effects of copper solutions containing ions in the +2 or +1 oxidation states on human norovirus and its surrogates are summarized in Figure 6. Stable Cu(II) ions in the absence of a reducing agent bind onto the surface of the virus capsid and cause VLP aggregation. At high concentrations, the bound ions may have the potential to block HBGA receptor binding, as demonstrated by HBGA-VLP binding assays, and cause 1-log loss of virus titer, as demonstrated by the TV plaque assay. In the presence of ascorbate as a reducing agent, copper ions

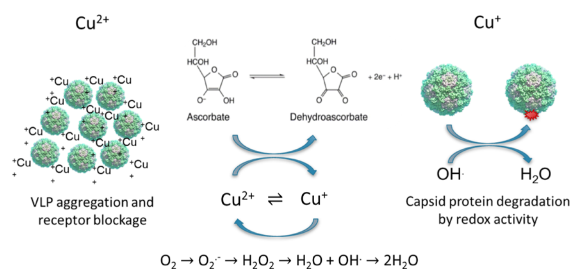

Figure 6. Schematic showing a summary of the effects of Cu(II) ions and Cu-ion catalyzed ROS generation on virus particle stability and integrity. Cu(II) ions aggregate viruses and cause some inactivation at a high concentration. When a reducing agent such as ascorbate is added, Cu(II) acts as a catalyst to generate ROS that, in addition to the unstable Cu(I) ion, can cause denaturation and cleavage of the norovirus capsid protein.

cycle between the +2 and +1 oxidation state. Ascorbate oxidizes to dehydroascorbate as it reduces Cu(II) to Cu(I), and Cu(I) oxidizes back to Cu(II) by either dissolved oxygen and its reduction products or by reacting directly with the protein capsid. Cu(I) may reduce disulfide bonds within the major capsid protein and cause protein unfolding that inhibits VLP binding to HBGAs, which has been reported in binding assays to rely heavily on maintenance of a higher order capsid protein structure.[39,62] ROS are generated as oxygen is sequentially reduced to water, and these species cause covalent damage to the viral capsids, as demonstrated by SDS-PAGE. This ascorbate and copper system relies on a fresh supply of ascorbate and treatment of virus or VLPs immediately after mixing, as ascorbate is rapidly depleted in the presence of copper and dissolved oxygen. Thus, we have a system of coupled redox reactions that is very efficient in inactivating the norovirus or its surrogates but requires a precise balance of the components in order to operate efficiently.

■ CONCLUSIONS

Human noroviruses can persist in the environment and are generally resistant to many common inactivation agents, which require the continued development of novel disinfection formulations. We have demonstrated that mixtures of copper bromide and sodium ascorbate rapidly and efficiently inactivate human norovirus surrogates. The research data suggests that these mixtures are promising against the human pathogen. TV plaque assays and HBGA-VLP binding assays proved that solutions of Cu(I) are substantially more effective than Cu(II) at virus inactivation. The inactivation with Cu(I) solutions occurs at low concentrations and short treatment times. The data show that solutions with copper ion concentrations as low as 0.1 mM had high efficacy. Such low concentrations are likely safe to humans and have low risk of environmental harm as, for example, the EPA limit of copper in safe drinking water is 0.02 mM. TEM images and SDS-PAGE data confirmed that Cu(I) solutions cause significant damage to viral capsids, even at short treatment times and low concentrations. HBGA-VLP binding assays and SDS-PAGE of treated major capsid protein indicate that GII.4 Sydney VLPs are much less susceptible to damage by Cu(I) solutions than GI.7 VLPs. It is therefore important to evaluate the effectiveness of current inactivation strategies on new emerging strains to confirm that they remain effective on emerging and potentially more stable virus strains. The results suggest that TV may be more susceptible than human norovirus to copper based on RT-qPCR; however,

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further study on the effects of the stool matrix on copper effectiveness should be conducted. Copper and ascorbate systems have promise for being the active ingredients in novel, rapid, safe, and effective inactivation formulations for norovirus and potentially many other viral pathogens. Future research can be directed at increasing the stability and robustness of these systems for use in practical applications.

■ METHODS

Tulane Virus Plaque Assays. Rhesus monkey kidney cells (LLC-MK2, ATCC CCL-7) were passaged in M199 media (Corning/Cellgro) containing 10% fetal bovine serum (Gibco/Life Technologies) and 1% penicillin/streptomycin (Gibco). For the assay, cells were grown to about 90% confluence on 60 mm cell culture plates (Corning). To infect the cells, 450 μL of TV sample dilutions were applied to each plate following aspiration of spent media. The plates were infected for 60 min, during which they were rotated every 15 min to ensure effective delivery of viruses to the cells. After infection, 3 mL of M199 media with 1.5% low melting temperature agarose (SeaKem) was added as an overlay. Plates were then incubated at 37 °C and under 5% CO_2 for 3 days to facilitate plaque formation. After 3 days, 2 mL of 3.7% formaldehyde (Sigma-Aldrich) in PBS was poured over each plate to fix the cells. After fixing for 3−4 h, the agarose overlay was removed, and 1.5 mL of 0.1% crystal violet in PBS was added to the plates for 15 min to stain. The crystal violet solution was then poured off, and the plates were rinsed twice with tap water to remove excess stain before counting plaques.

Before the plaque assay, TV stocks were subjected to various treatments with copper in suspension. TV stock (100 μL) was added to 900 μL of each copper solution for a 1 mL total sample volume. Copper solutions containing ascorbate were prepared using 100 μL of 10× sodium ascorbate (Sigma Aldrich) stock, 10 μL of 100× $CuBr_2$ (Sigma-Aldrich) stock, and the balance PBS. Unless otherwise specified, all incubation times were 30 min. Copper ions were quenched by addition of EDTA (Sigma Aldrich) in 10× excess. After quenching, TV samples were subjected to 10× series dilutions in PBS prior to application to culture plates.

Histo-Blood Group Antigen Binding Assays. Receptor binding assays to characterize the effects of different copper treatments have on the norovirus capsid were conducted as done previously with slight modification.[10,39] Purified VLPs containing the assembled major capsid protein (VP1) of human norovirus GI.7 and GII.4 Sydney were obtained courtesy R. Atmar (Baylor College of Medicine, Houston, TX) and kept at 4 °C in concentrated form until use. VLPs were diluted to 3 μg/mL in 1× phosphate-buffered saline (PBS) and 100 μL/well of the VLP solution applied to 96-well medium-binding EIA plates (Costar 3591). Additionally, negative control wells with no VLP were seeded. Plates were incubated at 4 °C overnight with gentle shaking and then blocked for 2 h at room temperature with 5% skim milk solids (w/v) in PBS + 0.05% (v/v) Tween 20 (PBST) and gentle shaking. The wells were then washed thrice with 200 μL/well PBST, and 100 μL/well of selected dilutions of $CuBr_2$ with or without 1 mM sodium ascorbate in 0.15 M NaCl were applied at both different time points, or for 30 min with different copper solution concentrations. After selected treatment times, wells were quenched with 100 μL/well 10 mM bathocuproinedisulfonic acid (BCSA). The quenched solutions were then removed, and the plates were washed twice with 200 μL/well PBST. Next, 100 μL/well of a solution containing 1 μg of biotinylated HBGA type A (Glycotech, #01-017, Gaithersburg, MD) in 0.25% skim milk-PBST was applied for 1 h at room temperature with shaking. The plates were then washed thrice with PBST, and 100 μL/well of 0.2 μg/mL streptavidin-horseradish peroxidase conjugate (Invitrogen, Carlsbad, CA) in PBS was applied to plates for 15 min at room temperature. Plates were washed thrice with PBST, and the 100 μL/well room-temperature 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD) was applied for 5−10 min. The reaction was then stopped with 100 μL/well TMB stop solution (KPL), and plates were read at 450 nm in a Tecan Infinite m200Pro microplate reader.

No VLP control wells to account for the residual signal from the kit reagents and wells were seeded with only PBS and no VLPs, while positive control wells included untreated VLPs and neutralization control (BCSA and the highest copper solution were premixed and applied to wells for 30 min). At least two wells per treatment per plate and three separate plate replicates were performed. For each treatment, the average absorbance of the no VLP wells was subtracted from the average absorbance of each VLP well. These adjusted absorbances were then used to calculate the value of the signal of a treatment well taken as a percentage of the neutralization (positive) control.

Transmission Electron Microscopy. For viewing under TEM, 100 mg mL^{-1} human norovirus GI.7 VLPs were treated with 0.1 mM copper bromide and 10 mM sodium ascorbate for varying periods of time and then quenched with 10 mM EDTA. Droplets (10 μL) of each treated VLP solution were adsorbed onto nickel grids with carbon support films (Ladd Research, Williston, VT) for 2 min. Excess liquid was then removed followed by 5−10 s of negative staining with 2% uranyl acetate. The grids were imaged by conventional TEM using a 2000FX S/TEM (JEOL, Tokyo, Japan) at 200 kV.

RT-qPCR. Samples with GII.4 Sydney infected stool kindly provided courtesy of S.R. Green (North Carolina Department of Health and Human Services, Raleigh, NC) and suspended 20% in PBS were clarified by centrifugation for 10 min at 10,000g followed by 1:1 dilution in PBS. Additionally, clarified Tulane virus cell culture lysates diluted 1:10 were also evaluated in the suspension assay. The clarified stool or Tulane cell culture stock was added 1:10 into 0.15 M sodium chloride (NaCl) solutions containing 10 mM sodium ascorbate and varying copper concentrations for a final volume of 100 μL. After 30 min of treatment at room temperature, EDTA was added to a final concentration of 0.1 M to quench the copper ions.

Sample preparation and PCR reactions closely followed the protocol used by Manuel et al.[10] and are summarized briefly here. Before RNA extraction, samples were pretreated with 1 μL of RNase ONE (Promega, Madison, WI) enzyme in 12 μL of 10× reaction buffer and 7 μL of nuclease-free H_2O for 15 min at 37 °C. The RNase reaction was stopped by placing the samples on ice for 5 min and adding 80 μL of cold PBS. The NucliSENS easyMAG system (bioMérieux, St. Louis, MO) was used for RNA extraction, and final extracted nucleic acid was collected in 40 μL of provided buffer. A CFX96 Touch real-time PCR system (Bio-rad, Hercules, CA) was used to carry out the reaction with the following protocol: (1) reverse transcription for 15 min at 50 °C, (2) denaturation for 2 min at 95 °C, and (3) 45 cycles of 15 s at 95 °C, 30 s at 54 °C, and 30 s at 72 °C (for fluorescence reading). Primers JJV2F (5′-

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CAAGAGTCAATGTTTAGGTGGATGAG-3′) and COG2R (5′-TCGACGCCATCTTCATTCACA-3′) and probe RING2-P (5′-FAM [6-carboxyfluorescein]-TGGGAGGGCGATCGCAATCT-BHQ [black hole quencher]-3′) were used for GII.4 Sydney;[57] and Tulane primers FW (5'-GAGATTGGTGTCAAAACACTCTTTG-3′), RV (5'-ATCCAGTGGCACACACAATTT-3′), and probe (5′-6-FAM-AGTTGATTGACCTGCTGTGTCA-BHQ-3′) were used. Tulane reaction cycling was performed for 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 95 °C for 15 s followed by 60 °C for 1 min.[63] The baseline threshold was set to 30 during analysis.

Serial dilutions of GII.4 Sydney infected stool were used to create the standard curve shown in Figure S2. The slope of the linear regression result was used to calculate log reductions in number of genomic copies based on the Ct value for all subsequent experiments.

SDS-PAGE. GI.7 and GII.4 Sydney VLP stocks were diluted into 0.15 M NaCl solutions containing 10 mM sodium ascorbate and varying concentrations of copper bromide for a final volume of 10 μL. Each sample contained 1 μg of VLPs. After varying treatment times at room temperature, EDTA was added to a final concentration of 0.01 M to quench the copper ions. A Laemmli buffer (10 μL) (Bio-rad, Hercules, CA) containing β-mercaptoethanol (Sigma-Aldrich) according to the manufacturer's instructions was added to each sample, bringing the total volume to 20 μL. Samples were then held at 95 °C for 5 min for protein denaturation. A total of 20 μL of each sample as well as 10 μL of a Spectra multicolor broad range protein ladder (Thermo Scientific) were loaded into separate lanes of a precast 4−15% agarose gel (Bio-rad, Hercules, CA). Gels were subjected to 200 V for 30 min until the loading dye had traveled across the entire gel. Gels were placed in PBS until 1 h staining with Acquagel (Bulldog Biolabs). The gels were then rinsed three times with PBS and de-stained in PBS overnight before imaging with a scanner (Epson, Long Beach, CA). The lasso tool within Photoshop software (Adobe, San Jose, CA) was used to outline each major capsid protein band as identified by size comparison with the standard protein ladder. The circled bands in Figure S1a,b represent the major capsid protein of GI.7 and GII.4 Sydney VLPs, respectively. The histogram analysis tool was then used to determine the average intensity of each band. Each band was outlined and analyzed three separate times to determine errors associated with this method. Each treatment condition was repeated on three separate gels. Normalized intensity was calculated by first normalizing the intensity to the background intensity of the image to obtain an optical density ratio (ODR)

$$\mathrm{ODR} = \frac{(I_{\mathrm{band}} - I_{\mathrm{background}})}{I_{\mathrm{background}}} \tag{1}$$

where I_{band} represents the average intensity of a protein band and $I_{\mathrm{background}}$ represents the average intensity of the image background. Then, the ODR of each sample was divided by the ODR of an untreated sample to obtain normalized intensity (I_{n})

$$I_{\mathrm{n}} = \frac{\mathrm{ODR}_{\mathrm{treated}}}{\mathrm{ODR}_{\mathrm{untreated}}} \tag{2}$$

where $\mathrm{ODR}_{\mathrm{treated}}$ represents the ODR of a treated sample and $\mathrm{ODR}_{\mathrm{untreated}}$ represents the ODR of an untreated control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00609.

SDS-PAGE photos used for band intensity analysis and RT-qPCR standard curve graph (PDF)

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Notes

The authors declare no competing financial interest.

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