Inhibition of vitamin C oxidation by DNA aptamers

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ABSTRACT

Vitamin C (ascorbic acid) is not only commercially available as a nutritionally important dietary supplement, but is also used as a stabilizer in a wide range of other products, including beverages, cosmetics and therapeutics, because of its antioxidant properties. However, vitamin C itself undergoes oxidation upon storage, which decreases its efficacy as a stabilizer of other materials and also significantly reduces the shelf-life of products containing it as an active ingredient. Protecting such formulations and products against oxidation-induced degradation would thus be valuable. Here, we report the development of a new DNA aptamer that decreases the rate of oxidation of vitamin C in aqueous solution. We found that the presence of this aptamer reduced the rate of CuSO4-stimulated oxidation of vitamin C by more than 50% in aqueous solution. Furthermore, we also found that the aptamer increased the half-life of vitamin C by up to 1.7-fold in certain commercially available vitamin water formulations. This is the first description of aptamer-based stabilization of a commercially important substance and has significant implications for multiple industries.

KEYWORDS: SELEX, Vitamin C, Aptamer, Oxidation, Ascorbic acid, dehydroascorbic acid

INTRODUCTION

Vitamin C (also known as ascorbic acid) is widely used in the therapeutic, cosmetic, nutritional supplement, food, and beverage industries as a dietary supplement as well as a preservative. Maintaining physiological levels of vitamin C has been shown to aid in preventing or treating diseases ranging from neurodegenerative conditions such as Alzheimer’s Disease (Mehta et al, 2018), to cancer (Ravi et al, 2018). The use of vitamin C in food and beverage industries is primarily because of its antioxdizing properties, which are the result of vitamin C’s ability to neutralize free oxygen radicals, through a process called radical scavenging (Padh 1991; Nimse and Palb 2015). However, because of these same antioxidant properties, the molecule itself is inherently susceptible to degradation via oxidation. Key factors affecting its oxidation include temperature, pH, oxygen, metal ion concentrations, light and the presence of certain enzymes (Zerdin et al, 2003). In the commercial space, degradation has long been recognized as a fundamental limitation of vitamin C because the phenomenon decreases both the effectiveness (e.g., active ingredient dose) and shelf life of products containing the molecule (Spagnol et al, 2017). Despite in-depth studies relating to the oxidation of vitamin C and its oxidized product, dehydroascorbic acid (DHA), which have been performed since the mid-1900s, protecting vitamin C from oxidative degradation continues to be an unmet need as evidenced by elaborate engineering solutions being investigated as means to reduce vitamin C degradation that occurs during processing of beverages (Escajeda et al, 2018). The development of an aptamer that conveys oxidative protection to reduce the degradation of vitamin C, and could be easily implemented into current processes simply as an additional ingredient, has the potential to have a significant economic impact for a wide range of industries.
Aptamers are single-stranded DNA- or RNA-based oligonucleotides capable of selectively binding a wide range of molecules. Aptamers are commonly identified by an in vitro method of selection referred to as Systematic Evolution of Ligands by Exponential enrichment or “SELEX.” The process begins with a highly diverse pool of randomized oligonucleotides, which is generally narrowed to a small number of selectively binding aptamers for any given molecular target (Tuerk and Gold 1990; Ellington and Szostak 1992). Once multiple (typically 10–15) rounds of SELEX are completed, the DNA sequences in the enriched library are identified by sequencing. Aptamers have been developed as ligands to peptides and proteins, rivaling antibodies in both affinity and specificity (Ruckman et al, 1998; Jayasena 1999; Srisawat and Engelke 2001). Aptamers have been developed to bind small organic molecules, including vitamins and cellular toxins (Hirao et al, 1996; Babendure et al, 2003; Lee et al, 2004; Chu et al, 2006; Liu and Lu, 2006), viruses (Gopinath et al, 2006), and even targets as small as heavy metal ions (Chang et al, 2005; Wrzesinski and Ciesiolka 2005).

Here we report the identification and initial characterization of a new DNA aptamer that reduces the rate of vitamin C oxidation in aqueous solutions. To identify aptamers possessing this capability, after SELEX and bioinformatics analysis, candidate aptamers were screened using CuSO₄ as an oxidizing agent, in a fluorescence-based microplate assay. The best aptamer was then further scrutinized for its ability to reduce oxidation induced by additional biologically relevant oxidizing agents, including 4-hydroxy-TEMPO (TEMPOL), NaIO₄ and H₂O₂. CuSO₄ is highly soluble in water, prevalent in the environment, and can cause a wide array of mild to severe disease symptoms if ingested in sufficient quantities (Georgopoulos 2001). TEMPOL is an industrial oxidizing agent that has been implicated in neurological and pancreatic disease conditions (Dohare et al, 2014). NaIO₄ is a potent oxidizer that can influence the cytopathic effect of influenza virus on mammalian cells (Paulpandi et al, 2011). H₂O₂ is an oxidizing agent that is present in almost all biological systems (Devasagayam et al, 2004). Finally, the best candidate aptamer was analyzed for its ability to protect vitamin C in a commercially available vitaminwater® formulation from oxidation, using quantitative liquid chromatography/mass spectrometry.

**MATERIALS AND METHODS**

**Preparation of treated H₂O**

To ensure that the vitamin C was not oxidized prior to its use in SELEX and oxidation protection screening studies, all buffers and solutions were prepared using molecular biology grade water (Phenix Research) that was stripped of adventitious metals by gentle shaking in the presence of Chelex® 100 resin (BioRad) for 1hr, filtered through a 0.2μm filter (Sarstedt) to remove all resin, and then purged with N₂ gas (Praxair) for 10min (Buettner 1988), to remove as much dissolved oxygen gas from the water as possible.

**DNA aptamer selection and sequencing analysis**

Nine rounds of SELEX were performed using a natural DNA library (Base Pair Biotechnologies) consisting of approximately 10⁴⁵ unique oligonucleotides each with a 32-base random sequence region flanked by 19-base primer sites. To partition the library during each round of SELEX, the library was incubated with an immobilized vitamin C-bovine serum albumin (BSA) conjugate (MyBioSource) for thirty minutes, in 50mM sodium acetate pH 5.5, 1mM MgCl₂, 0.05% (v/v) Tween 20, 1% (w/v) bovine serum albumin (BSA), and 1μM–1μM glutathione (Sigma, all components), to permit binding to take place. The resulting complexes were then subjected to five washes using the same solution, to remove unbound library members and weak binders, all of which were discarded. A freshly prepared solution of 1mM vitamin C (Sigma, cat # A7506), was then used to elute vitamin C-binding aptamers from the conjugate. The aptamer pool was then amplified using PCR, the second strand removed using λ-exonuclease (New England Biolabs) according to published methods (Little 1981), and the resulting enriched pool of single-stranded aptamers was allowed to bind to the conjugate again. SELEX stringency was increased in successive rounds by increasing the number of washes from five to eight, decreasing the binding time of the aptamer library to the conjugate from thirty to five minutes, and decreasing the vitamin C concentration used for elution from 1mM to 10nM.

Following SELEX, the aptamer candidate pool was sequenced using an Ion Torrent™ Personal Genome Machine (ThermoFisher). The resulting sequences were then analysed to identify sequence and secondary structure motifs, determine the frequency at which each such motif arose in the converged pool, and identify the candidates with the greatest predicted secondary structure stability at 25°C, according ViennaRNA (Lorenz et al, 2011).

**Fluorescence-based microplate assays for vitamin C oxidation**

Oxidation of vitamin C was measured by detecting the oxidized product dehydroascorbate (DHA) using a modified version of the method described by (Vislisel et al, 2007). In this method DHA is detected by reaction with o-phenylenediamine (OPDA) to form the fluorescent condensation product 3-(dihydroxyethyl)fluoro[3,4-b]quinolino-1-one. The assay was performed as follows in black 384-well plates (Greiner Bio-One). Aptamers were first dissolved in phosphate-buffered saline, pH 7.2, containing 1mM MgCl₂, at a concentration of 200μM then folded by heating to 95°C and allowing to cool slowly to room temperature for 15min. The folded aptamers were then diluted 1:1 (v:v) into a freshly prepared solution of 50μM vitamin C in assay buffer [50mM sodium acetate, 1% (w/v) BSA, 0.05% (v/v) Tween 20, 1mM MgCl₂ (Sigma, all components) adjusted to pH 5.5], and the mixture was incubated for 30min at 22°C, to allow aptamers to bind prior to addition of oxidizer. Oxidizers were then added to the vitamin C/ aptamer solutions at final concentrations of 10.3μM for CuSO₄ (EM Science) and H₂O₂ (Sigma) and 103.3μM for TEMPO (Sigma) and NaIO₄ (Sigma). All oxidizers were pre-diluted to their working concentrations in assay buffer. Samples were then incubated at 22°C for 10min before the addition of OPDA (Sigma) at a final concentration of 954.6μM in assay buffer. When reagents had been added, the final concentrations of aptamers and vitamin C were 41.3μM and 10.3μM, respectively. Immediately following the addition of OPDA, fluorescence of the samples at 425nm was determined using a SpectraMax® i3X plate reader (Molecular Devices) with excitation at 435nm, over a 45-min time course with measurements.
made every 60 seconds. Control experiments were performed to determine whether certain aptamer candidates interfered with the DHA detection assay, the assay was performed by comparing DHA alone (at a final concentration of 10.3 μM) to DHA in the presence of aptamer (at a final concentration of 41.3 μM) with OPDA at a final concentration of 954.6 μM in assay buffer. Each sample was assayed in triplicate. To determine the degree of protection offered by the test aptamers in the accelerated oxidation assays, the data were normalized such that the negative control (vitamin C only) was used as a blank (0% oxidation) and the positive control (vitamin C + oxidizer) was set to 100% oxidation. All test samples were applied to this scale so that, for example, a sample that fell exactly halfway between the controls would be interpreted as providing a 50% reduction in the rate of oxidation in the presence of the oxidant/oxidation accelerator. Endpoint fluorescence readings were made at 30 min following the addition of oxidizer. Values are presented as means ±/− one standard error of the mean of the triplicate measurements. For the control experiments, time course plots of the detection of DHA with and without the presence of aptamers were overlaid; any deviation of the aptamer treated sample from the DHA only sample was deemed to be assay interference. All sample and reagent containing vessels were wrapped in foil to protect them from light during all incubations performed for the fluorescence assays.

**Aptamer 12 dose-response study**

Aptamer 12 was dissolved, folded and diluted into Assay Buffer as described above. 10 μl of a dilution series of Aptamer 12, at final concentrations ranging from 103 μM to 1.03 μM in Assay Buffer, was combined with an equal volume of a 50 μM solution of vitamin C, to reach a final concentration of 10.3 μM vitamin C. The mixtures were incubated at 22°C for 30 min to allow aptamers to bind, as above, and then CuSO4 was added to a final concentration of 10.3 μM. Samples were then incubated at room temperature for 10 min before the addition of OPDA at a final concentration of 954.6 μM, to measure the conversion of vitamin C to DHA. Fluorescence was measured at 425 nm, following excitation at 345 nm. Measurements were made every 60 sec for 45 min. Each sample was assayed in triplicate.

**Mass spectrometry**

**Sample preparation**

Aptamer 12 was dissolved and folded at 200 μM as described above. Folded Aptamer 12 was then diluted to a final concentration of 41.3 μM into freshly prepared 50 mM sodium acetate, pH 5.5 containing a final concentration of 10.3 μM vitamin C. Samples were incubated at room temperature and 10 μl of each was collected at 0, 1, 2, 4, and 8 hr. Vitamin C (Cerilliant) and DHA (Sigma) controls were injected before and after each time point to monitor the stability of the starting material and the anticipated final reaction product during liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. To each sample and control, a vitamin C internal standard solution labelled with C13 (vitamin C-C13; Toronto Research Chemicals) was added, to a final concentration of 200 ng/ml in H2O. Stock solutions of vitamin C (Cerilliant mg/ml in acetonitrile:H2O, 50:50) and vitamin C-C13 were diluted to 10 μg/ml and 1000 ng/ml respectively in H2O. To prepare a vitamin C control solution 10 μl of 10 μg/ml vitamin C was diluted into 90 μl of 1000 ng/ml vitamin C-C13 to obtain a final concentration of 1000 ng/ml vitamin C and 900 ng/ml vitamin C-C13. The DHA control was similarly prepared at a final concentration of 1000 ng/ml in 900 ng/ml vitamin C-C13. 10 μl samples were obtained at each time point and diluted 1:200 into the same internal standard solution and analysed by LC-MS/MS.

**LC-MS/MS Parameters, multiple reaction monitoring (MRM)**

Acquisition was performed with an ABSciex QTRAP 6500 instrument (ABSciex) equipped with an electrospray interface with a 50 μm inner diameter capillary, and coupled to an Eksigent μHPLC instrument (Eksigent). Analyst 1.6.3 software was used to control the instrument in "low mass hardware configuration" and for data processing and acquisition. Optimized MRM parameters were used to monitor vitamin C, vitamin C-C13 and DHA. MS/MS parameters are listed in Table 1. Samples were injected by loop overfilling into a 2 μl loop and analysed by LC-MS/MS. Separation was performed using a 50 mm × 0.5 mm Gemini C18 column from Phenomenex, which was maintained at 45°C. For the 3.5 min LC gradient, the mobile phase consisted of 10 mM tert-butyl alcohol (TBA) and 15 mM acetic acid in water (solvent A) and 10 mM TBA and 15 mM acetic acid in methanol (solvent B) at a flow rate of 30 μl/min. The gradient started at 100:0 solvent A:solvent B. TBA was used as an ion pairing agent throughout. All samples were analysed in triplicate. Values are presented as mean ±/− one standard error.

**Degradation of vitamin C in vitaminwater**

Mass spectrometry was performed exactly as above, using kiwi-strawberry vitaminwater® (GLACÉAU) in the place of Assay Buffer. The amount of vitamin C in the vitaminwater®

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**Table 1. MS/MS run parameters.**

<table>
<thead>
<tr>
<th>MS/MS Parameters</th>
<th>Q1</th>
<th>Q3</th>
<th>Declustering potential</th>
<th>Collision energy</th>
<th>Curtain gas</th>
<th>Ion spray voltage</th>
<th>Source temperature</th>
<th>Ion source gas 1</th>
<th>Ion source gas 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>174.893</td>
<td>114.900</td>
<td></td>
<td>−60</td>
<td>−16</td>
<td>20</td>
<td>−4500</td>
<td>200</td>
<td>15</td>
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<tr>
<td>DHA</td>
<td>172.908</td>
<td>112.700</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ascorbic acid-C13</td>
<td>180.800</td>
<td>119.040</td>
<td></td>
<td></td>
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<tr>
<td>DHA-C13</td>
<td>178.800</td>
<td>116.700</td>
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was measured over 168hr with and without the addition of a final concentration of 50μM Aptamer 12.

Equilibrium binding studies
Microscale thermophoresis (MST) was performed as described by (Jerabek-Willemsen et al, 2011). Briefly, a 50μM solution of Aptamer 12, labelled at its 5’ end with Cy5 dye was prepared and folded as described above, then diluted into 50mM Sodium acetate, 0.05% (v/v) Tween-20 buffer to a final concentration of 10nM. Folded conjugates were then combined with sixteen serial dilutions of either vitamin C or DHA, at final concentrations ranging from 50μM to 1.53nM. Mixtures were incubated for 15min at room temperature, and then 5μl of each was loaded into a scanning capillary. The fluorescence of each sample was measured during exposure to a thermal gradient using a Monolith NT.115 MST instrument (NanoTemper Technologies) to generate binding curves. Each assay was performed in triplicate.

RESULTS & DISCUSSION
A rigorous approach to buffer preparation was required to successfully perform SELEX with an unstable target
Although there are many methods to select for aptamers that bind to small molecules, including those that involve immobilization of the library rather than the target, for this project, because a BSA conjugate of vitamin C was commercially available, we chose to adopt a conventional approach in which the protein-conjugated target was immobilized on a solid support and the library combined with the immobilized, conjugated target to perform SELEX. Additionally, because vitamin C is usually present in millimolar concentrations in commercially available products (Zerdin et al, 2003; Spagnol et al, 2017), we used relatively high (micromolar) concentrations of the target during selection. Because of the known instability of the target, our greatest challenge was avoiding oxidizing either the free or immobilized, conjugated target during selection, potentially resulting in a pool of aptamers that either the free or immobilized, conjugated target during selection. We additionally included glutathione in binding, wash and elution buffers, to provide an additional level of protection, by neutralizing any oxygen free radicals that may have arisen during the numerous steps in the SELEX process. We think, based on our observations, that these same precautions should be applicable for generating aptamers that bind other unstable targets and anticipate using them in the future for this purpose. Sequencing and bioinformatics analysis following selection yielded 26,562 unique aptamer candidates, of which the best twenty (patent pending) were chosen for further study, based on the frequency at which their primary sequences and predicted conformational/secondary structure motifs arose in the final selected pool, and the predicted stability of those secondary structures at 25°C.

Fluorescence-based microplate assays identified several aptamer candidates that significantly reduced the rate of vitamin C oxidation
A fluorescence-based microplate assay was then used to screen these twenty aptamer candidates, to identify any that could inhibit the rate of vitamin C oxidation in solution. In these experiments, CuSO₄ was used to accelerate the rate of oxidation, to not only reduce screening time, but also to ensure that any successfully identified aptamers would not only inhibit the intrinsic rate of vitamin C oxidation in aqueous solutions, but would also inhibit oxidation stimulated by exogenous oxidants. A 4-fold molar excess of aptamer over vitamin C was used for these experiments, to maximize the likelihood that such inhibition could be detected. Figure 1 shows that samples containing three candidates, Aptamers 12, 14 and 18 exhibited a visible decrease in the rate of oxidation compared with controls, as indicated by a reduced rate of DHA production. Aptamer 12 showed a 35 ± 13% decreased rate, Aptamer 14 showed a 43 ± 9% decreased rate, and Aptamer 18 showed a 25 ± 8% decreased rate. Aptamers 2, 5, 10, 11 and 13 displayed a minimal level of protection (slight deviation from the positive control) and twelve failed to provide any detectable protection under these conditions.

Aptamers 12, 14 and 18 were then tested to confirm that their presence did not interfere with the OPDA assay itself. Figure 2 shows that Aptamer 12 (indicated by a black border) did not affect the assay, but Aptamers 14 and 18 appeared to slightly alter fluorescence signals relative to the control, suggesting potential interference with the assay, through an unknown mechanism, which could, for example, involve competing with OPDA for DHA binding. Thus, Aptamer 12 was used for all further studies.

Aptamer 12 protects vitamin C from three different oxidizing agents
To determine whether the oxidation protection effect was specific for CuSO₄ or was more general, three additional oxidizers, NaIO₄, H₂O₂ and TEMPOL, were tested. A dilution series of each oxidizer was first combined with vitamin C in the OPDA assay, in the absence of the aptamer, to determine the lowest oxidizer concentration that would generate a suitable test window (data not shown). We found that Aptamer 12 failed to protect against oxidation in the presence of NaIO₄ however it reduced the relative amount of oxidation of vitamin C by 54 ± 5%, 19 ± 6% and 51 ± 7% in the presence of CuSO₄, H₂O₂ and TEMPOL respectively (data not shown). Thus Aptamer 12 can not only reduce the rate of intrinsic oxidation in the presence of dissolved oxygen gas, but also in the presence of oxidation accelerants such as might be found in the environment. It is proposed that the oxidative protection of vitamin C by Aptamer 12 is the result of the aptamer binding to and forming an aptamer/vitamin C (Aptamin®) complex that prevents or inhibits the interaction of certain oxidizers with vitamin C.

The degree of vitamin C protection is dose-dependent
The OPDA assay was performed using a dilution series of Aptamer 12 at 10x, 5x, 2x, 1x, 0.5x, 0.25x and 0.1x the molar concentration of vitamin C, in the presence of CuSO₄.
Figure 1. Fluorescence-based microplate assays to screen for candidates that inhibit vitamin C oxidation in the presence of CuSO₄. Plots show the rate of oxidation of vitamin C in the presence of 10.3 μM CuSO₄ for 20 candidate aptamers. Each panel represents data obtained in the presence of an individual aptamer. Plots indicate a 45-min time course of the oxidation of vitamin C relative to “no aptamer” controls with and without CuSO₄. The intrinsic rate of vitamin C oxidation under these conditions is shown in green, blue shows the rate of vitamin C oxidation in the presence of the accelerant, and red shows the rate in the presence of the accelerant plus the aptamer candidate. Black borders indicate the panels containing aptamers 12, 14 and 18, which exhibit a reduced rate of fluorescence increase compared with controls.

Figure 2. Potential aptamer-induced interference with OPDA assay. Time courses of DHA detection, as indicated by an increase in OPDA fluorescence. Results for samples that contained the aptamer candidate and DHA are shown in red and results for control samples that only contained DHA are shown in blue. The presence of Aptamers 14 and 18 caused a slight deviation in the rate of signal generation from control samples, indicating potential interference. However, Aptamer 12 (identified by a black border) does not appear to interfere with the assay.
to determine whether a dose-dependent relationship could be established for oxidation protection. Figure 3 shows that the protection is indeed dose-dependent. At the two lowest aptamer:vitamin C molar ratios tested, (0.1x and 0.25x) no oxidative protection was observed. Protection increased as the ratio of aptamer to vitamin C increased, but did not plateau in the tested range. Therefore, the maximal oxidative protection of vitamin C by Aptamer 12 in Assay Buffer, under these specific conditions, occurs at a concentration greater than 10 times the molar concentration of vitamin C. In addition, for the majority of the aptamer concentrations tested the treated sample eventually converges with the untreated sample; however, for the 10x test the treated sample remains clearly distinct from the control over the entire time period. Thus, under these conditions, a 10-fold molar excess of the aptamer is sufficient to maintain vitamin C in the oxidation protected state for prolonged time periods, even in the presence of oxidation accelerants. From a practical perspective, this result suggests that a large amount of aptamer would be required to elicit a commercially useful effect, but this may not be the case, since this particular oxidation accelerant is unlikely to be present in most consumer product formulations and the structure of Aptamer 12 has not yet been optimized through either truncation or sequence optimization studies.

**Mass spectrometric analysis confirmed oxidation protection results**

As an independent method to confirm our fluorescence microplate assay results, we used mass spectrometry. Over an 8hr time period at room temperature, in 50mM sodium acetate (pH 5.5), without any added oxidation accelerant, the half-life of vitamin C was determined to be 0.60 ± 0.02hr in the absence of Aptamer 12 and 2.08 ± 0.4hr in the presence of Aptamer 12 (Figure 4 A), indicating that Aptamer 12 increased the half-life of vitamin C by about 3.5-fold, under these conditions. Thus, mass spectrometry confirmed that this aptamer is able to reduce the rate of oxidation of vitamin C.

**Oxidation inhibition occurs in vitaminwater® as well as artificially formulated buffers**

Since the ultimate goal of this project was to determine if it would be possible to identify an aptamer that could not only reduce the rate of oxidation of vitamin C in the laboratory, but also that could do so in an application of interest to a consumer market, we used mass spectrometry to determine whether Aptamer 12’s effect could be seen in commercially available vitaminwater®. Figure 4B shows that, with a 1.7-fold increase in vitamin C half-life in such formulations, this effect was indeed observed. Although the fold increase in half-life observed here was lower than that observed in 50mM sodium acetate, quantification of the starting amount of vitamin C present in the vitaminwater® (data not shown) revealed that at commencement of this assay there was a 33-fold molar excess of vitamin C to Aptamer 12, meaning that the relative amount of Aptamer 12 to vitamin C was 132 times lower here than in the previous assay. As the dose response data revealed that minimal oxidative protection of vitamin C by Aptamer 12 was observed at an equimolar ratio in Assay Buffer, the capability of Aptamer 12 to protect vitamin C from oxidation ...

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*Figure 3. Dose response of Aptamer 12 mediated inhibition of vitamin C oxidation by CuSO4. The intrinsic rate of vitamin C oxidation in Assay Buffer is shown in green, the rate of vitamin C oxidation in the presence of CuSO4 without any aptamer is shown in blue, and the rate of oxidation in the presence of the CuSO4 and Aptamer 12 is shown in red, at the Aptamer 12:vitamin C molar ratios indicated in the panel headers. Oxidative protection of vitamin C by Aptamer 12 occurs in a dose-dependent manner.*
is orders of magnitude more potent in vitaminwater®. This implies that oxidative protection of vitamin C can be achieved without the addition of excessively high aptamer concentrations and highlights its potential utility in similar formulations.

While the data presented here might suggest that there would be little to no vitamin C remaining in commercial products by the time they reach the consumer, it should be noted that the vitaminwater® used in this study was purchased directly off the shelf and clearly contained enough vitamin C to perform the testing presented. It was not our intent to speculate on the formulations of commercialized products but rather to suggest that Aptamer 12 has the potential to extend the shelf-life (however long that may be) of products that already exist in the marketplace. Furthermore, although not discussed in detail in this manuscript, the degradation of vitamin C in beverages has been a known problem for many decades and has been extensively studied. It is known that the rate of oxidation of vitamin C in beverages is greatly reduced in factory sealed packages relative to exposure to open air. It should be noted that the results presented in this study were generated under much greater oxidizing conditions than those present in sealed pre-sale packages.

**Affinity measurement**

The equilibrium binding affinity of a 5’ Cy5-modified version of aptamer 12 to vitamin C and DHA was measured via MST. These experiments determined that Aptamer 12 binds AA with a $K_D$ of 987.9nM (95% confidence interval: 545.5–1762.7nM) (data not shown). Interestingly, the aptamer also bound to DHA with a $K_D$ of 189.7nM (95% confidence interval: 138.7–256nM). This could be because despite the measures taken to prevent extensive oxidation of vitamin C to DHA it is likely that there would have been some DHA present during the partitioning phase of the SELEX.

**CONCLUSIONS**

Vitamin C is a commercially important but unstable molecule that not only acts as an oxygen free-radical scavenger to preserve the stability of multiple products, but also is sold as an active ingredient in foods, beverages and dietary supplements. Improving its stability is thus of interest to several market sectors, each of which would need to investigate the relevant financial and regulatory hurdles that would be involved in adoption of this technology. Our work demonstrates that certain DNA aptamers have the potential to extend the shelf-life and potentially the efficacy of such products by inhibiting the rate of oxidation of vitamin C in solution. While further application-specific testing is needed to validate and fully understand the potential value of its use in any particular product formulation, this is, to the best of our knowledge, the first time that any DNA aptamer has been shown to protect a redox-sensitive molecule from degradation, suggesting, if the effect is generalizable to other types of oxidative damage, a new and potentially valuable area in which aptamers could provide solutions to economically important problems.

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**COMPETING INTERESTS**

Authors Alexander S Chiu, Vinoth Sankarapani, Rafal Drabek, George W Jackson and Robert H Batchelor are employees of Base Pair Biotechnologies where the aptamer develop-
ment and validation was performed. Yoon seong Kim is a technical consultant of Nexmos Co Ltd, which funded this project.

LIST OF ABBREVIATIONS

DHA: dehydroascorbic acid
SELEX: Systematic Evolution of Ligands by EXponential enrichment
TEMPOL: 4-hydroxy-TEMPO
BSA: bovine serum albumin
OPDA: o-phenylenediamine
MST: microscale thermophoresis
LC-MS/MS: liquid chromatography-tandem mass spectrometry
MRM: multiple reaction monitoring
TBA: tert-butyl alcohol

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