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Dynamic adsorption properties of n-alkyl glucopyranosides determine their ability to inhibit cytolysis mediated by acoustic cavitation

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Abstract

Suspensions of human leukemia (HL-60) cells readily undergo cytolysis when exposed ultrasound above the acoustic cavitation threshold. However, n-alkyl glucopyranosides (hexyl-, heptyl- and octyl-) completely inhibit ultrasound-induced (1057 kHz) cytolysis (Sostaric, et al., *Free Radic. Biol. Med.* **2005**, 39, 1539–1548). The efficacy of protection from ultrasound-induced cytolysis was determined by the n-alkyl chain length of the glucopyranosides, indicating that protection efficacy depended on adsorption of n-alkyl glucopyranosides to the gas/solution interface of cavitation bubbles and/or the lipid membrane of cells. The current study tests the hypothesis that “sonoprotection” (i.e., protection of cells from ultrasound-induced cytolysis) *in vitro* depends on the adsorption of glucopyranosides at the gas/solution interface of cavitation bubbles. To test this hypothesis, the effect of ultrasound frequency (from 42 kHz to 1 MHz) on the ability of a homologous series of n-alkyl glucopyranosides to protect cells from ultrasound-induced cytolysis was investigated. It is expected that ultrasound frequency will affect sonoprotection ability, since the nature of the cavitation bubble field will change. This will affect the relative importance of the possible mechanisms for ultrasound-induced cytolysis. Additionally, ultrasound frequency will affect the lifetime and the rate of change of the surface area of cavitation bubbles, hence the dynamically controlled adsorption of glucopyranosides to their surface. The data support the hypothesis that sonoprotection efficiency depends on the ability of glucopyranosides to adsorb at the gas/solution interface of cavitation bubbles.

Keywords

Dynamic surface tension; gas/solution interface; surfactants; sonochemistry; sonolysis; sonoprotection; acoustic cavitation; cytolysis; sonodynamic therapy; sonoporation; sonophoresis; HIFU; ultrasound

Introduction

When ultrasound is passed through an *in vitro* suspension of cells at constant temperature (i.e., no thermal effects) and at intensities above the acoustic cavitation threshold, a percentage of

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the cell population undergoes a very rapid cytolysis (herein referred to as “sudden cytolysis”). In the absence of any specifically introduced gas-bubbles or contrast agents typically used in ultrasound therapy^{1–3} and imaging⁴ studies, sudden cytolysis only occurs in the presence of acoustic cavitation.^{5–7} It is widely accepted that sudden cytolysis is exclusively due to the physical effects of acoustic cavitation, for example microstreaming of liquid around cavitation bubbles leading to shear forces that act to disrupt the lipid membrane.^{8,9} As a result, experimental studies in the field of ultrasound therapy almost universally rely on numerical modeling of the cavitation bubble field and/or a detailed physical description of the ultrasonic wave.

Studies of acoustic cavitation in the field of sonochemistry, however, do not rely on complete characterization of the ultrasonic field in the apparatus. Rather, these studies are performed from the perspective of observations of how various parameters affect the observed chemical, physical or biological effects of acoustic cavitation, under a given set of ultrasound exposure conditions. This approach stems from the observation that the chemical, physical and biological effects of ultrasound depend not only on parameters associated with the ultrasonic wave, but also on those associated with the solution/medium under investigation (for e.g., solution temperature, viscosity, exposure geometry and so on).^{10,11} We have extended the sonochemical approach to studies on the effects of acoustic cavitation on cells using relatively low concentrations (i.e., mM to μ M) of non-toxic surfactants.^{12,13}

Surfactants have been used widely in aqueous sonochemistry to study the nature of acoustic cavitation bubbles in multibubble^{14–21} and single bubble^{22,23} experiments. However, scant attention has been given to the effect of the surfactant properties of solutes when cells are exposed to ultrasound.

Cells exposed to ultrasound (1 MHz) in the presence of one of a homologous series of surface-active n-alkyl β -D-glucopyranosides (i.e., n-octyl, -heptyl and -hexyl) were completely protected from ultrasound-induced cytolysis¹² (herein “sonoprotection”), an important finding given that a number of compounds with known radical scavenging or antioxidant properties were essentially ineffective at protecting cells.²⁴ Sonoprotection (1-MHz) ability of the glucopyranosides increased with increasing n-alkyl chain length, i.e., $C_8 > C_7 > C_6$ and was not observed when methyl β -D-glucopyranoside was added to the cell suspension.¹² Based on these observations, the two initial steps in the mechanism of sonoprotection involve adsorption of glucopyranosides at (1) the gas/solution interface of cavitation bubbles or (2) the lipid membrane of cells. The following step in the mechanism would involve (a) quenching of cytotoxic radicals and/or their precursors or (b) suppression of the physical effects of acoustic cavitation. Sostaric et al.¹² proposed a radical quenching mechanism for sonoprotection (1-MHz) at the gas/solution interface of cavitation bubbles, i.e., 1(a), which has some merit since von Sonntag and coworkers have shown that $\cdot$ OH radical attack on various sugar compounds results in the formation of byproducts that would have no cytotoxic properties.^{25–27}

Information on the mechanism of sonoprotection, be it physical- or radical-quenching in nature, can be gained from consideration of the process of adsorption of n-alkyl glucopyranosides at the gas/solution interface of cavitation bubbles compared to that in the lipid membrane of cells. Adsorption of glucopyranosides from the bulk solution to the lipid membrane would reach equilibrium over the course of an experiment and is therefore governed by the thermodynamic adsorption properties of these surfactants. However, adsorption at the rapidly expanding and contracting gas/solution interface of cavitation bubbles will be limited by their dynamic adsorption properties.^{18,21,28}

In the current study, the effect of ultrasound frequency on the relative ability of n-alkyl glucopyranosides to protect cells from ultrasound-induced cytolysis was investigated, since

ultrasound frequency is known to affect the rate of change of surface area of cavitation bubbles and their lifetime.^{18,21} This is compared to earlier studies on the relative ability of n-alkyl anionic surfactants to accumulate at the gas/solution interface of cavitation bubbles in aqueous surfactant solutions.¹⁸ In light of these findings the mechanism of sonoprotection of *in vitro* cell suspensions by glucopyranosides is discussed.

Experimental methods

Chemicals

Dulbecco's phosphate buffered saline (DPBS, pH = 7.4) was obtained from Biofluids. Methyl β -D-Glucopyranoside (MGP) was obtained from Sigma-Aldrich, hexyl β -D-Glucopyranoside (HGP, $\geq 98\%$), heptyl β -D-Glucopyranoside (HepGP, $\geq 98\%$) and octyl β -D-Glucopyranoside (OGP, $\geq 99\%$) were obtained from Fluka.

Cells

HL-60 myeloid leukemia cells (American Type Culture Collection) were grown in a suspension of RPMI 1640 medium (GIBCO, Gaithersburg, MD) containing 10% calf serum. The population of HL-60 cells doubled every 23 ± 1 hr (mean $\pm$ SD, where $n = 6$) when incubated at 37°C in a CO_2 (5 %) containing atmosphere. Cells were harvested, re-suspended in fresh RPMI medium and kept at 25°C until the start of the experiment (< 1 hr). The cell concentration was $\approx 5 \times 10^5$ cells/ml in all experiments because of the possible effect of cell concentration on ultrasound-induced cell lysis.²⁹ The fraction of intact cells before and after exposure to ultrasound was determined using a Coulter Multisizer (model IIe) connected to a sampling stand (model IIa). Cytolysis% was determined by subtracting the number of intact cells following sonolysis from the number of intact cells before sonolysis. This value was divided by the number of intact cells before sonolysis and multiplied by 100. This method correlated well with the percentage cytolysis measured using the Trypan blue exclusion assay immediately following sonolysis, as explained in detail elsewhere.¹³

Ultrasound Exposure

Cell suspensions (1 ml) were placed in an exposure vessel (13×100 mm disposable, autoclaved Pyrex tubes, Corning Inc., Corning, NY) which were reproducibly fixed in the center of a bath which was part of a commercially available ultrasonic exposure apparatus (Figure 1). Continuous wave ultrasound at a number of frequencies was delivered through the bottom of the glass exposure vessel as described in detail elsewhere.¹⁸ In brief, one of two stainless steel flat plate (diameter ≈ 5.6 cm) bench-top ultrasonic transducers (L3 Communications, ELAC-Nautik GmbH) operating at frequencies of 1057 or 354 kHz (model USW 51–52) or 614 kHz (model USW 51–51) were powered at various ultrasound intensities by a generator ("Cesar"; model 7500 18003; ELAC-Nautik GmbH), as shown in Figure 1. A cylindrical glass vessel was clamped to the top of the housing of the transducer unit (Figure 1). The glass cylinder was filled with 300 ml of distilled and degassed water prior to sonolysis. The temperature of the 300 ml coupling water was kept constant ($25 \pm 1^\circ\text{C}$) by passing cooling water through the water jacket surrounding the cylindrical cell with a self-regulating water circulator (model DC50) and cooling bath (model K20) supplied by Haake, USA. The glass exposure vessel was positioned in the center of the coupling water at a reproducible height (13.8 cm) above the flat plate using a clamp.

At a frequency of 42 kHz, sonolysis was conducted using a Branson ultrasound bath (model number 1510) and a rheostat was used to decrease the power of the bath to 50% of its original value. The bath was filled with water according to the manufacturer's specifications and the exposure tube was positioned in the center of the bath using a clamping system built in house.

The ultrasound exposure apparatus described herein are typical of those used in sonochemistry, but are not typical of those used in ultrasound therapy studies, in which the ultrasound apparatus is such that the acoustical waves can be characterized in terms of established physical parameters, such as acoustical intensities or pressure amplitudes (negative and/or positive) expressed in variations of spatial and temporal peak or average values.³⁰ Nevertheless, we attempted to characterize the ultrasound field at frequencies of 354, 614 and 1057 kHz by measuring the pressure amplitude in the exposure tube using a 1.0 mm-diameter PVDF needle hydrophone (Precision Acoustics Ltd, model HPM1/1) and a digital oscilloscope (Tektronix, model TDS5032B).

As expected, the acoustical pressure amplitude inside of the exposure vessel fluctuated due to the complex acoustical field in the ultrasound exposure apparatus. For a comparison of the acoustical pressure amplitude experienced by cells in the exposure vessel, the pressure amplitudes were measured under the same conditions as during the cell experiments. Using a micropositioner (Newport, model MS-500-XYZ), the needle hydrophone was placed near the bottom of the exposure vessel and the peak-to-peak pressure amplitude was measured for 5 seconds of sonication within each setting of the power generator. The accumulated waveforms were averaged by the digital oscilloscope. The generator power indicated on the generator display (P) and the averaged peak-to-peak pressure amplitudes (PA; mean $\pm$ SD, where $n = 6$) determined following 5 seconds of sonolysis at each frequency were as follows: $P_{354 \text{ kHz}} = 15 \text{ W}$, $PA_{354 \text{ kHz}} = 0.13 \pm 0.02 \text{ MPa}$. $P_{614 \text{ kHz}} = 20 \text{ W}$, $PA_{614 \text{ kHz}} = 0.21 \pm 0.08 \text{ MPa}$. $P_{1057 \text{ kHz}} = 10 \text{ W}$, $PA_{1057 \text{ kHz}} = 0.18 \pm 0.08 \text{ MPa}$. Therefore, the effect of the addition of glucopyranosides on cytolysis was determined at a constant generator power supplied to the transducers at a given frequency.

It is not surprising that the standard deviations of these pressure amplitude measurements are high due to the complex interactions of traveling and standing waves inside the exposure vessel since this ultrasound apparatus lacks the appropriate conditions of a well established acoustical methodology, such as the use of an acoustical absorber or transducers that have been characterized or calibrated for the specific purpose. Nevertheless, as in the field of sonochemistry, a completely characterized ultrasound field from the physical acoustics perspective is not a prerequisite to drawing conclusions on the relative effects of various surfactants on the acoustic cavitation process under a constant set of ultrasound exposure parameters. The acoustical measurements above have no bearing on the outcome of the current study and should only be viewed as an estimate of the order of magnitude of acoustical pressure amplitudes experienced in this particular experimental setup.

Cell experiments were completed within 5 to 10 minutes of adding the glucopyranosides to the cell suspensions, and each data point represents the average $\pm$ SD, where $n = 5$ to 8. It was found that 15 minute exposures of HL-60 cells to MGP, HGP, HepGP and OGP at the highest concentrations used in this study had no detrimental effect on the reproduction rate of the cells over the course of 120 hours. OGP has been used for the non-cytolytic extraction of membrane proteins,^{31,32} but as described elsewhere¹² it would not be effective at extracting a significant amount of membrane proteins under the conditions in the current study.

Results

Sonolysis of cell suspensions at all frequencies and under the conditions described resulted in sudden cytolysis of a certain percentage of the cell population. This is represented in Figure 2, Figure 3, Figure 4, and Figure 5 at a concentration of zero. It has been shown¹² that the addition of HGP, HepGP or OGP to the cell suspensions just prior to sonolysis at an ultrasound frequency of 1 MHz resulted in a concentration dependent decrease in the cytolysis percentage, as shown in Figure 2. It is interesting to note that the concentration of glucopyranoside required

to completely protect cells followed the order of the n-alkyl chain length of the glucopyranosides, i.e., OGP (2 mM) < HepGP (3 mM) < HGP (5 mM) (Figure 2).¹² Furthermore, MGP, the hydrophilic derivative had no effect on cytolysis% at 1-MHz, even up to a concentration of 30 mM. Thus, protection of cells by glucopyranosides from 1-MHz ultrasound is not only dependent on the concentration of glucopyranosides, but also on the surfactant properties of these solutes.

When the frequency of sonolysis was decreased from 1 MHz to 42 kHz (see Figure 2 to Figure 5) there is a transition for OGP, HepGP and HGP from protection of HL-60 cells (at 1 MHz) to no protection of HL-60 cells at 42 kHz. In order to gain an appreciation for the effect of ultrasound frequency on the ability of each glucopyranoside to protect HL-60 cells from ultrasound, cytolysis% was normalized to a value of 1 at a concentration of zero and graphed as a function of concentration for each glucopyranoside, at the four different frequencies (Figure 6 a – 6d). Normalization of cytolysis% was accomplished by dividing cytolysis% observed at all concentrations of a particular glucopyranoside by cytolysis% observed at a concentration of zero.

Comparing the effect of each surfactant on ultrasound-induced cytolysis at the different frequencies shows that: Figure 6a; OGP fully protects cells from cytolysis at 1-MHz, however at 614-kHz it can only protect 50% of the cell population. When the frequency is decreased to 354 kHz or 42 kHz, OGP acts as a weak sonosensitizer, thereby increasing the cytolysis%. Figure 6b; HepGP fully protects cells from cytolysis at 1-MHz and 614-kHz (no data is available for 354-and 42-kHz). Figure 6c; HGP fully protects cells at 1-MHz, 614-kHz and 354-kHz, but not at 42-kHz. Figure 6d; within the error of the experiment, MGP has no effect on ultrasound-induced cytolysis at any ultrasound frequency.

There are two noticeable trends in the effect of the surface active glucopyranosides (i.e., OGP, HepGP and HGP) on cytolysis% as the frequency is decreased from 1 MHz to 42 kHz. First, all of the surface active glucopyranosides become less effective at protecting cells from ultrasound-induced cytolysis. Secondly, the ability of the longest n-alkyl chain possessing glucopyranoside (OGP) to protect cells from ultrasound-induced cytolysis is most affected by a decrease in the frequency of sonolysis, compared to HepGP and HGP.

Discussion

The trends observed for sonoprotection efficiency at 1-MHz (Figure 2; OGP>HepGP>HGP; MGP no sonoprotection) and as the ultrasound frequency was decreased (Figure 2 to Figure 5; OGP<HepGP<HGP, MGP no sonoprotection) clearly depend on the n-alkyl chain length of the glucopyranosides.³³ As described below, the latter trend supports a dynamic-controlled adsorption mechanism and therefore the *a priori* hypothesis that sonoprotection depends on adsorption of glucopyranosides at the gas/solution interface of cavitation bubbles and not in the lipid membrane of cells.

Evidence for dynamic-controlled adsorption of glucopyranosides

Sostaric and coworkers^{18,21,28,34,35} have shown that the frequency of ultrasound affects the dynamic ability³⁶ of certain surfactants to adsorb at the gas/solution interface of cavitation bubbles, a conclusion that is supported by Sunartio, et al.³⁷ As the frequency of ultrasound is decreased, the ability of surfactants to adsorb to the gas/solution interface of cavitation bubbles decreases, due to an increase in the rate of change of surface area of the bubbles and/or a decrease in their lifetime.¹⁸ Therefore, surfactants that possess a relatively long n-alkyl chain within a homologous series cannot adsorb as readily to the gas/solution interface of cavitation bubbles (especially at lower ultrasound frequencies),^{18,21,28,34} since a longer time is required for these surfactants to attain the equilibrium surface excess.³⁸ This dynamic adsorption effect

is described in Figure 7, which compares the effect of ultrasound frequency on sonoprotection efficacy of glucopyranosides (Figure 6) to that of the relative ability of n-alkyl anionic surfactants to adsorb to the gas/solution interface of cavitation bubbles (based on sonochemical radical yields).¹⁸

The $\text{CH}_{\text{SDS}}:\text{CH}_{\text{SPSo}}$ ratio (Figure 7) was calculated from the maximum plateau yield of $\cdot\text{CH}$ -radicals for sodium dodecyl sulfate (SDS) to that of sodium pentane sulfonate (SPSo) shown in Figure 9 of reference¹⁸ and is a measure of the limiting, dynamic-adsorption controlled ability of SDS to adsorb at the gas/solution interface of cavitation bubbles, compared to that of SPSo. The “OGP:HGP protection ratio” was calculated by dividing the maximum percentage of cells that could be protected by OGP, to that protected by HGP at the same ultrasound frequency. The observation of a similar trend between the $\text{CH}_{\text{SDS}}:\text{CH}_{\text{SPSo}}$ ratio and the OGP:HGP protection ratio with ultrasound frequency (Figure 7) supports the *a priori* hypothesis that sonoprotection depends on adsorption of glucopyranosides to the gas/solution interface of cavitation bubbles.

However, to be valid the current hypothesis should also explain the effect of ultrasound frequency on the sonoprotection effect (Figure 6) in terms of generally accepted hypotheses on the nature of acoustic cavitation bubbles and their effects on cells at different ultrasound frequencies, as described below.

Description of the acoustic cavitation bubble field:³⁹

Transient cavitation bubbles^{40a} grow to a maximum bubble radius (R_{max}) within a few acoustic cycles and then collapse almost adiabatically to a minimum size (R_{min}), producing sonochemistry, sonoluminescence and possibly shock waves. *Stable cavitation bubbles*^{40b} pulsate around the equilibrium radius (R_0) and persist for hundreds of acoustic cycles. Stable bubbles can grow through coalescence with other bubbles^{37,41} or through a process known as rectified diffusion.⁴² There are two sub-categories:⁴³ (a) High energy stable (HE-stable) bubbles produce similar high energy phenomena associated with the collapse of transient cavitation bubbles and (b) non-inertial stable (NI-stable) cavitation bubbles dissolve away due to the effects of Laplace pressure or experience a low energy, fragmentary or shear collapse^{40b} that does not produce chemistry or sonoluminescence.

The definitions described above are distinct from those used in ultrasound therapy research, where transient and HE-stable bubbles are referred to as “inertial” and all other bubbles as “non-inertial”. These definitions are insufficient since they do not distinguish between the different mechanisms of bioeffects produced by either transient or HE-stable bubbles (described in the following Section). Furthermore, the lifetime of transient cavitation bubbles (only 3 to 60 μs at 1057-kHz to 42-kHz, respectively)¹⁸ is much shorter than that of HE-stable bubbles, which persist for tens to hundreds of milliseconds.⁴⁴ Given that linear, n-alkyl anionic surfactants such as SDS adsorb at equilibrium concentrations to the gas/solution interface on a millisecond time scale,⁴⁵ no significant adsorption of glucopyranosides occurs at the gas/solution interface of transient bubbles. This conclusion is supported by the observations of Sostaric and Riesz¹⁸ and Sunartio et al.³⁷ It follows from the comparisons made in Figure 7 that sonoprotection depends only on adsorption of glucopyranosides at the gas/solution interface of either HE-stable or NI-stable cavitation bubbles and not at the gas/solution interface of transient cavitation bubbles.

Mechanisms of ultrasound-induced sudden cytolysis

Transient cavitation bubbles cause sudden cytolysis through both collapse near a cell (resulting in shear stress)⁴⁶ and asymmetric collapse⁹ on the surface of the cell. In addition to these physical mechanisms for cavitation-induced sudden cytolysis, there is a possibility that free

radicals may play a role through lipid peroxidation reactions in the lipid membrane.⁴⁷ Free radicals could first destabilize the lipid membrane in this way, thereby allowing the physical effects described above to take effect.¹²

Similar physical and chemical mechanisms also occur during the collapse of HE-stable cavitation bubbles. HE- and NI-stable bubbles may also produce sudden cytolysis through long term pulsations leading to microstreaming of liquid around the bubble surface and shear stress on the lipid membrane.⁹ Additionally, radiation forces (such as primary Bjerknes forces) can cause stable bubbles to move rapidly towards pressure nodes or antinodes (depending on their size),^{9,40} resulting in high speed bubble-cell collisions. Given the current data and the above description of the cavitation bubble field and its effects on cells, a mechanism of sonoprotection is proposed.

Mechanism of sonoprotection by glucopyranosides

The conclusion that glucopyranosides can not protect cells from cytolysis induced by transient cavitation bubbles (*vide infra*) is highly relevant. At 1-MHz the estimated transient bubble lifetime (3 μ s) is of the order of the collapse time of a bubble. Therefore, by definition the majority of bubbles at 1-MHz are HE- or NI-stable bubbles, whereas transient bubbles are more likely to persist at frequencies below 1 MHz. Indeed, it is generally accepted that the ratio of transient to stable bubbles is higher at lower ultrasound frequencies.^{11,18,41,44,48,49} Therefore, this discussion is consistent with the observation that sonoprotection is most effective at the highest ultrasound frequency. Given this, it can be concluded that sonoprotection by glucopyranosides at 1-MHz involves suppression of the physical and/or chemical effects produced by stable cavitation bubbles.

That OGP had the best sonoprotection properties at 1-MHz (Figure 2) is a result of its greater thermodynamic adsorption properties. Note that the observation of 100% cell survival (Figure 2) at a given bulk concentration of glucopyranoside during 1-MHz ultrasound exposure can only be interpreted as enough glucopyranoside adsorbing at the gas/solution interface of stable bubbles to produce a 100% protection effect, and yields no information on the limiting, dynamic ability of the glucopyranosides to adsorb to the gas/solution interface of cavitation bubbles. In essence, at relatively low bulk solution concentrations at 1-MHz, the surface excess has a greater dependence on the thermodynamic adsorption properties of the surfactant. A similar effect is also evident from the observations at a frequency of 614 kHz (Figure 3). Note that although OGP can only produce a maximum of 50% sonoprotection (due to the dynamic controlled limit in adsorption), at bulk concentrations ≤ 1 mM, OGP is slightly more efficient at protecting cells compared to HGP or HepGP due to its greater thermodynamic adsorption properties (Figure 3).

As the frequency of sonolysis is decreased from 1 MHz to 42 kHz (Figure 2 to Figure 5), the rate of change of the surface area of stable bubbles increases and their lifetime decreases.¹⁸ Therefore OGP, having the lowest dynamic adsorption properties in the series, cannot adsorb at high enough concentrations to the more rapidly changing surface area of stable bubbles at the lower ultrasound frequencies,¹⁸ compared to HepGP and HGP (Figure 6b and c, respectively).

HGP, with the shortest n-alkyl chain, is the most dynamic surfactant in the series and is able to protect cells from ultrasound-induced cytolysis even at 354 kHz (Figure 6c). However, at 42 kHz, the rate of change of surface area of HE- and NI-stable bubbles increases to the point where none of the glucopyranosides can adsorb at the gas/solution interface of stable cavitation bubbles to concentrations required to create a sonoprotection effect. A secondary effect could be that the proportionately higher population of transient cavitation bubbles and also the relatively large magnitude of physical effects of acoustic cavitation bubbles at 42 kHz negate

glucopyranosides from producing a protection effect, irrespective of the amount that can adsorb to the gas/solution interface of stable cavitation bubbles.

The magnitude of physical effects experienced by cells due to cavitation definitely increases at lower ultrasound frequencies because the resonance radius, amplitude of bubble oscillations and the compression ratios ($R_{\max}/R_{\min}$) on collapse will all be of a larger magnitude.⁴⁰ This is supported by a number of observations made in studies on sonoluminescence,^{41,50} sonoporation^{51–53} and sonomechanical shear stress on macromolecules.^{54,55}

On a final note, elucidation of the mechanism of sonosensitization (i.e., enhanced ultrasound-induced cytotoxicity by glucopyranosides) observed at 354-kHz for OGP and relatively low concentrations of HGP (Figure 4), and for all glucopyranosides at 42 kHz (Figure 5) requires further investigation. With this in mind, we propose the following: The observation that sonosensitization arises at the two low frequencies in the study suggests that the phenomenon has to do with the higher magnitude of physical effects of cavitation bubbles at low frequencies.

At lower frequencies, it is possible that sonoporation of a certain population of cells is more prevalent, thereby exposing the cytoplasm to glucopyranosides that may have toxic effects therein, resulting in a mechanism for sudden cytotoxicity that is in addition to that of the direct physical and/or chemical effects of acoustic cavitation. This hypothesis is supported by the observation that all glucopyranosides result in similar levels of sonosensitization at 42 kHz, suggesting that the effect is due to glucopyranoside from the bulk medium. This cannot be the sole mechanism at play at 354-kHz (Figure 4), since MGP has no sonosensitization properties at this frequency, suggesting a surface chemical component for the sonosensitization effect at 354 kHz. There is a possibility that at relatively low concentrations of HGP, a larger population of stable bubbles exists due to prevention of bubble coalescence.^{37,49} This results in an increase in the active bubble population, therefore enhancing sudden cytotoxicity. As the concentration is increased, HGP can accumulate at the surface of stable bubbles to high enough concentrations to create the sonoprotection effect, however OGP cannot (due to its relatively low dynamic surface adsorption rate) so that the sonosensitization effect prevails at all OGP concentrations (Figure 4).

Conclusions

The current study shows that sonoprotection by glucopyranosides depends on their accumulation at the gas/solution interface of cavitation bubbles. On the basis of this, and on current understanding of the biological effects of acoustic cavitation, we propose that sonoprotection arises following adsorption of glucopyranosides only at the gas/solution interface of HE- or NI-stable cavitation bubbles. Glucopyranosides appear ineffective at protecting cells from transient cavitation bubbles and are especially ineffective at low ultrasound frequencies, possibly due to a combination of factors including; high transient to stable bubble ratios, a larger magnitude of physical effects and an inability to adsorb dynamically at the more rapidly changing surface area of stable cavitation bubbles at low ultrasound frequency. The sonoprotection effect described herein is unlikely to be important for low frequency applications such as sonophoresis, but has promising potential for protecting cells from cytotoxicity in higher frequency applications, for example sonoporation for gene and drug delivery to cells.

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References and Notes

- Postema M, Gilja OH. *Curr. Pharm. Biotechnol* 2007;8:355–361. [PubMed: 18289044]
- Postema M, Schmitz G. *Ultrason. Sonochem* 2007;14:438–444. [PubMed: 17218145]
- Postema M, Van Wamel A, Lancee CT, De Jong N. *Ultrasound Med. Biol* 2004;30:827–840. [PubMed: 15219962]
- Postema M, Schmitz G. *Expert Rev. Mol. Diagn* 2006;6:493–502. [PubMed: 16706749]
- Morton KI, ter Haar GR, Stratford IJ, Hill CR. *Ultrasound Med. Biol* 1983;9:629–633. [PubMed: 6670148]
- Miller DL, Bao SP. *J. Acoust. Soc. Am* 1998;103:1183–1189. [PubMed: 9479770]
- Schlicher RK, Radhakrishna H, Tolentino TP, Apkarian RP, Zarnitsyn V, Prausnitz MR. *Ultrasound Med. Biol* 2006;32:915–924. [PubMed: 16785013]
- Marmottant P, Hilgenfeldt S. *Nature* 2003;423:153–156. [PubMed: 12736680]
- Anonymous. *Ultrasound Med. Biol* 1998;24:S11–S21. [PubMed: 9841460]
- Suslick, KS., editor. *Ultrasound: Its Chemical, Physical and Biological Effects*. New York: VCH; 1988.
- Mason, TJ.; Lorimer, JP. *Sonochemistry: Theory, applications and uses of Ultrasound in Chemistry*. West Sussex: Ellis Horwood Limited; 1988.
- Sostaric JZ, Miyoshi N, Riesz P, DeGraff WG, Mitchell JB. *Free Radic. Biol. Med* 2005;39:1539–1548. [PubMed: 16298679]
- Miyoshi N, Sostaric JZ, Riesz P. *Free Radic. Biol. Med* 2003;34:710–719. [PubMed: 12633748]
- Alegria AE, Lion Y, Kondo T, Riesz P. *J. Phys. Chem* 1989;93:4908–4913.
- Sostaric JZ, Mulvaney P, Grieser F. *J. Chem. Soc.-Faraday Trans* 1995;91:2843–2846.
- Grieser, F.; Ashokkumar, M.; Sostaric, JZ. *Sonochemistry and sonoluminescence in colloidal systems*. In: Crum, LA.; Mason, TJ.; Reisse, JL.; Suslick, KS., editors. *Sonochemistry and Sonoluminescence*. Vol. Vol. 524. Kluwer Academic Publishers: Dordrecht; 1999. p. 345–362.
- Sostaric JZ, Pandian RP, Weavers LK, Kuppusamy P. *Chem. Mat* 2006;18:4183–4189.
- Sostaric JZ, Riesz P. *J. Phys. Chem. B* 2002;106:12537–12548.
- Grieser F, Ashokkumar M. *Adv. Colloid Interface Sci* 2001;89:423–438. [PubMed: 11215808]
- Ashokkumar M, Lee J, Kentish S, Grieser F. *Ultrason. Sonochem* 2007;14:470–475. [PubMed: 17234444]
- Sostaric JZ. *J. Am. Chem. Soc* 2008;130:3248–3249. [PubMed: 18302375]
- Yasui K. *Phys. Rev. E* 1998;58:4560–4567.
- Ashokkumar M, Guan JF, Tronson R, Matula TJ, Nuske JW, Grieser F. *Phys. Rev. E* 2002;65:046310/046311–046310/046314.
- See the discussion section of reference 12 for a review of these studies
- Schuchmann MN, Sonntag CV. *J. Chem. Soc.-Perkin Trans* 1977;2:1958–1963.
- Bothe E, Schultefrohlinde D, Sonntag CV. *J. Chem. Soc.-Perkin Trans* 1978;2:416–420.
- Schuchmann MN, Sonntag CV. *Z. Naturforsch.(B)* 1978;33:329–331.
- Sostaric JZ, Riesz P. *J. Am. Chem. Soc* 2001;123:11010–11019. [PubMed: 11686706]
- Brayman AA, Church CC, Miller MW. *Ultrasound Med. Biol* 1996;22:497–514. [PubMed: 8795177]
- Kinsler, LE.; Frey, AR.; Coppens, AB.; Sanders, JV. *Fundamentals of Acoustics*. Vol. 4th ed.. John Wiley and Sons; 2000.
- Lazo JS, Quinn DE. *Anal. Biochem* 1980;102:68–71. [PubMed: 6243887]
- Legrue SJ, Macek CM, Kahan BD. *J. Natl. Cancer Inst* 1982;69:131–136. [PubMed: 6954305]
- Note that the observations for HepGP at the two frequencies studied (i.e., 1 MHz and 614 kHz) support the trends observed as a function of n-alkyl chain length. For clarity, the current discussion is generally limited to discussing these trends for OGP, HGP and MGP
- Yang L, Sostaric JZ, Rathman JF, Kuppusamy P, Weavers LK. *J. Phys. Chem. B* 2007;111:1361–1367. [PubMed: 17249713]

35. Yang L, Sostaric JZ, Rathman JF, Weavers LK. J. Phys. Chem. B 2008;112:852–858. [PubMed: 18085771]
36. Eastoe J, Dalton JS. Adv. Colloid Interface Sci 2000;85:103–144. [PubMed: 10768479]
37. Sunartio D, Ashokkumar M, Grieser F. J. Am. Chem. Soc 2007;129:6031–6036. [PubMed: 17439213]
38. Ferri JK, Stebe KJ. Adv. Colloid Interface Sci 2000;85:61–97. [PubMed: 10696449]
39. It should be noted here that the current study is conducted in the absence of any "stabilized gas bubbles" that might typically be used for *in vitro* studies in ultrasound therapy (for example, ultrasound contrast agents). The terms "bubbles", "stable cavitation" and "stable cavitation bubbles" should not be confused with "stabilized gas bubbles", which are not relevant to this study.
40. Leighton, TG. The Acoustic Bubble. London: Academic Press; 1994. (a) pp. 332–335. (b) pp. 424–427.
41. Price GJ, Ashokkumar M, Grieser F. J. Am. Chem. Soc 2004;126:2755–2762. [PubMed: 14995192]
42. Crum LA. J. Acoust. Soc. Am 1980;68:203–211.
43. Leighton described three main sub-categories of stable cavitation bubbles, i.e., high energy, irregular and low energy. For the current study, there is effectively no distinction between irregular and low energy stable cavitation bubbles. Therefore, these bubbles are grouped in a separate category; non-inertial stable bubbles.
44. Price GJ, Ashokkumar M, Hodnett M, Zequiri B, Grieser F. J. Phys. Chem. B 2005;109:17799–17801. [PubMed: 16853282]
45. Fainerman VB, Makievski AV, Miller R. Colloid Surf. A 1994;87:61–75.
46. Miller MW. Ultrasound Med. Biol 2004;30:1263–1267. [PubMed: 15582225]
47. Misik V, Riesz P. Ann. N.Y. Acad. Sci 2000;899:335–348. [PubMed: 10863551]
48. Beckett MA, Hua I. J. Phys. Chem. A 2001;105:3796–3802.
49. Ashokkumar M, Hodnett M, Zeqiri B, Grieser F, Price GJ. J. Am. Chem. Soc 2007;129:2250–2258. [PubMed: 17279753]
50. Price GJ, Ashokkumar M, Cowan TD, Grieser F. Chem. Commun 2002:1740–1741.
51. Alvarez-Roman R, Merino G, Kalia YN, Naik A, Guy RH. J. Pharm. Sci 2003;92:1138–1146. [PubMed: 12761803]
52. Tezel A, Sens A, Mitragotri S. J. Pharm. Sci 2002;91:444–453. [PubMed: 11835204]
53. Tang H, Wang CCJ, Blankschtein D, Langer R. Pharm. Res 2002;19:1160–1169. [PubMed: 12240942]
54. Henglein A, Gutierrez M. J. Phys. Chem 1990;94:5169–5172.
55. Portenlanger G, Heusinger H. Ultrason. Sonochem 1997;4:127–130. [PubMed: 11237029]

Figure 1. Ultrasound exposure apparatus. Ultrasound passes through a stainless steel flat plate, is transmitted through a bath of degassed water and the wall of the glass exposure vessel before entering the cell sample. The system was kept at constant temperature (25°C) by cooled water circulating through an outer water jacket surrounding the bath. Interchangeable transducers operated at 1057 kHz, 614 kHz and 354 kHz.

Figure 2.

The effect of glucopyranoside concentration (0 – 30 mM) on cytolysis %. Conditions: 1057 kHz ultrasound, air exposed, 298 K, power = 10 W, sonolysis time = 5s, cytolysis % $\pm$ SD (n = 5 to 8). (Reproduced from figure 2 in reference¹²).

Figure 3.

The effect of glucopyranoside concentration (0 – 10 mM) on cytolysis %. Conditions: 614 kHz ultrasound, air exposed, 298 K, power = 20 W, sonolysis time = 5s, cytolysis % $\pm$ SD (n = 5 to 8). MGP data was gathered at half the ultrasound power, i.e., 10 W.

Figure 4.

The effect of glucopyranoside concentration (0 – 30 mM) on cytotoxicity %. Conditions: 354 kHz ultrasound, air exposed, 298 K, power = 15 W, sonolysis time = 5s, cytotoxicity % $\pm$ SD (n = 5 to 8).

Figure 5.

The effect of glucopyranoside concentration (0 – 30 mM) on cytolysis %. Conditions: 42 kHz ultrasound, air exposed, 298 K, power = 50% reduction of the baths natural operating power, sonolysis time = 5s, cytolysis % $\pm$ SD (n = 5 to 8).

Figure 6.

Effect of ultrasound frequency on the sonoprotecting properties of glucopyranosides. The data from Figure 2 to Figure 5 has been normalized at zero concentration for all glucopyranosides: (a) OGP; (b) HepGP; (c) HGP and (d) MGP.

Figure 7.

Comparison of the effect of ultrasound frequency on OGP:HGP protection ratio and $\text{CH}_{\text{SDS}}:\text{CH}_{\text{SDS}}$ ratio. The $\text{CH}_{\text{SDS}}:\text{CH}_{\text{SPSo}}$ ratio was calculated from the normalized plateau CH radical yields in reference²⁸, Figure 9.