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Effect of Health-Promoting Agents on Exclusion-Zone Size

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Abstract

It is now well-confirmed that hydrophilic surfaces including those within the cell generate structural changes in water. This interfacial water is ordered and acquires features different from the bulk. Amongst those features is the exclusion of colloidal and molecular solutes from extensive regions next to the hydrophilic surface, thereby earning it the label of "exclusion zone" (EZ) water. The transition of ordered EZ water to bulk serves as an important trigger of many cellular physiological functions, and in turn cellular health. We tested physiological doses of half a dozen agents generally identified to restore or build health on the extent to which they build EZs. All agents known to enhance biological function resulted in EZ expansion. On the other hand, the weed killer, glyphosate, considerably diminished EZ size. While the expansion effect of the health-promoting agents was observed over a wide range of concentrations, excessive doses ultimately reduced EZ size. We hypothesize that EZ buildup may be a mechanistic feature underlying many health-promoting agents, while agents that impair health may act by diminishing the amount of EZ water.

Keywords

exclusion zone water, hydrophilic surface, dose dependent, health-promoting agents, nutraceuticals, dose response

Introduction

Earlier studies have revealed the extensive presence of ordered, interfacial water inside cells.[1,2] Subsequent reports[3,4] have detailed the properties of this interfacial water, which lies next to hydrophilic surfaces including those inside the cell. Interfacial, ordered water has been given the name "exclusion zone" (EZ) or "fourth phase" water.[5]

The central role of water in cell function has been known since the time of Szent-Gyorgyi, the father of modern biochemistry. Among Szent-Gyorgyi's famous quotes, a relevant one is "Life is water dancing to the tune of solids." This aphorism was given additional substance with evidence that the transition from ordered (now called "EZ") water to bulk water was an important trigger of many physiological actions, and that without that water, the cell could not properly function.[2]

The critical role of EZ water in cell function has led to the hypothesis that any deficiency in EZ water will impair cellular function and therefore impair health. Conversely, rebuilding of EZ water in EZ-deficient situations ought to return health toward normal.

We tested that hypothesis by using agents generally acknowledged to promote or restore health. Physiological doses of nutraceuticals such as tulsi (holy basil), Culturelle (Cromwell, CT) kids probiotics, turmeric, and coconut water as well as commonly used pain-relieving medications such as aspirin and Tylenol were tested for their ability to build or diminish EZ water. A weed killer, Roundup that contains glyphosate as its main ingredient and is well-known to adversely affect health, was also tested to see if its effects are opposite.

To test for the quantity of EZ water, we used a preparation that has become standard.[4] It involves the polymer, Nafion, which nucleates a sizeable EZ, the extent of which is defined by the exclusion of microspheres. By varying the concentration of the respective agents in question, we could assess the extent to which the agent increased or decreased EZ size.

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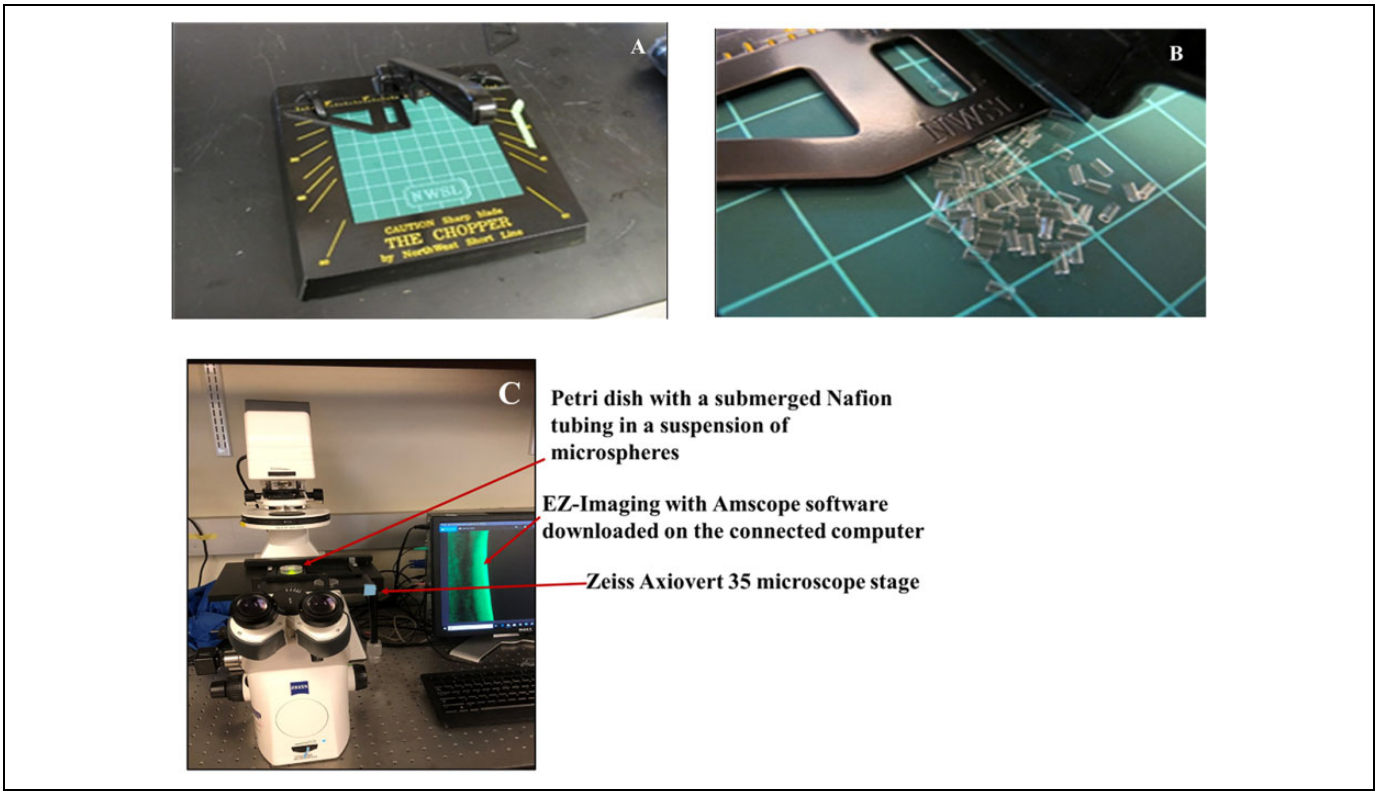

Figure 1. Nafion tubing was cut with the chopper (A) into smooth-edged equal-length 2 to 3 mm pieces. (B) A cut piece of Nafion was then manipulated with tweezers to be well-submerged in a suspension of microspheres contained in a covered petri dish. The tubing was oriented normal to the optical axis and placed on the stage of a Zeiss Axiovert 35 microscope equipped with a 2.5× objective lens. (C) Amscope software downloaded onto the computer was set on auto-image to capture an image of the exclusion zone (EZ) forming against the Nafion tubing.

Materials and Methods

Exclusion Zone Nucleating Surface

Nafion tubing was obtained from PermaPure LLC (Lakewood, NJ). Two models, TT-020 (internal diameter 0.36 mm) and TT-0030 Nafion (internal diameter 0.53 mm) were used in these studies. Care was taken while handling the tubing by wearing sterile gloves. The Nafion tubing was cut with a commercial instrument, "The Chopper" (NorthWest Short Line), into smooth-edged, equal length pieces (Figure 1 A and B).

Microsphere Suspension

The suspension used for determining EZ size consisted of either of 2 kinds of microspheres, polycarboxylate-coated 0.5, 1, or 2 µm (Polysciences Inc; # 18327; 2.5% solids-latex), and polystyrene-coated 1 µm (Polysciences Inc; # 19814; 2.5% solids-latex, Warrington, PA). The microspheres were suspended in deionized (DI) water obtained from a Barnstead D3750 Nanopure Diamond purification system, Dubuque, IA) (type 1 high-performance liquid chromatography grade, 18.2 MΩ). The volume ratio of microsphere to DI water was between 1:100 and 1:1000 depending on the kind of experiment performed, and kept constant during each experimental series to eliminate any effects that might arise from concentration differences.

Measurements of EZ

The cut piece of Nafion tubing was typically held by a pair of tweezers, while a prepared microsphere solution in a syringe was injected inside the tube until no air bubbles were present. The Nafion tubing was then manipulated with the tweezers so that it was well-submerged in a 35 mm × 10 mm Falcon polystyrene petri dish containing 3 to 4 mL of the prepared microsphere suspension. Occasionally, the cut piece of Nafion tubing was hydrated by submerging it first in a 35 mm × 10 mm Falcon polystyrene petri dish containing 3 to 4 mL DI water for 15 to 30 minutes before starting the experiment. For control EZ measurements, the hydrated tubing was removed and placed in a fresh petri dish with 3 to 4 mL DI water containing microspheres. For experimental measurements, another segment of Nafion tubing was placed in 3 to 4 mL of the suspension to be tested (experimental). The petri dish with the submerged Nafion tubing oriented normal to the optical axis was covered and placed on the stage of a Zeiss (Thornwood, NY) Axiovert 35 microscope equipped with a 2.5× objective lens (Figure 1 C). Amscope software (Version: x64, 3.1.615), downloaded onto the computer, was set on auto-image to

capture an image of the EZ forming against the Nafion tubing. All measurements of EZ were taken when a steady-state EZ size was attained, ie, $\sim$15 minutes after tubing and suspension were combined. In some cases, the time period was extended to 30 minutes. The graph tool from the Image-J (version, 1.49v) program was used to make 2 to 6 measurements of EZ size on each side of the Nafion tubing, from which the mean EZ size was obtained.

Measurements of EZ in the Presence of Functional Agents

We tested diverse functional agents, for example, holy basil, turmeric, probiotics, and coconut water for their effects on EZ size. These agents are time-honored and well-established to promote health as established by several conventional biochemical and other methods. Aspirin and Tylenol, analgesic substances in effective use for pharmaceutical purposes were also added to the list. We expected each of these agents to enhance EZ formation, while Roundup was expected to diminish EZ. Each agent was tested at various concentrations ranging above and below the anticipated concentrations in the human body; the latter based on substance weight or volume and the approximate weight/volume of water inside a human body, about 40 to 45 L.[6] Exclusion zone measured in DI water in the absence of any agent served as control as described above.

Tulsi (Holy Basil)

We obtained Tulsi extract from Herb Pharm (Williams, Oregon). A stock solution of the liquid extract (5 mL in 50 mL of DI water) was used to formulate a range of diluted solutions (up to 1×10^{-7} times diluted) that were tested independently for EZ measurements against Nafion.

Culturelle Kids Probiotic

The water-soluble probiotic powder contained 5 billion colony-forming units of the clinically well-known *Lactobacillus* GG per packet. All probiotic trials were from fresh packets opened just before the experimentation, in line with the recommendations of the manufacturer (Culturelle). The standard probiotic concentration was 1 mg/40 mL (0.00125 g/100 mL). We tested the probiotic for a range of concentrations extending up to 100 mg/40 mL (0.25 g/100 mL) of DI water.

Coconut Water

One hundred percent pure coconut water (Vita Coco.com) was diluted to make a range (0.005%-10%) of concentrations with DI water.

Turmeric

Turmeric (*Curcuma longa)* was obtained from Nature Made (Mission Hills, California). It is insoluble in water. Therefore, we used dimethyl sulfoxide (DMSO) to help dissolve the turmeric. After mixing the weighed amounts of turmeric in DI water containing DMSO (final concentration of DMSO was 1% in DI water), the solutions were centrifuged for 10 minutes to separate the clear solution from the rest. Supernatants were decanted and used to make experimental solutions with the usual carboxylate microspheres. A range of turmeric solutions ranging from 0.02 to 1.0 g/L were prepared in this manner.

Aspirin

A typical aspirin tablet (Premier Value, Gouverneur, NY) contains 325 mg (1.8 mM) of acetylsalicylic acid and was used to prepare a wide range of convenient concentrations (0.45-45 mM) in DI water.

Tylenol

We used "over-the-counter" Tylenol (acetaminophen) from a local drugstore (labelled as 160 mg per teaspoon, or 5 mL) for a range of formulations with DI water that ranged from 0.09 to 14 mM. In these experiments, the microsphere concentration was 1 drop per 5 mL DI water.

Roundup

Roundup (Monsanto, St Louis, Missouri) is a commonly used herbicide with the active ingredient, glyphosate (2% of total ingredients). A 1% solution (1.2×10^{-3} M) of Roundup in DI water was subsequently diluted to give a range of concentrations down to 1.2×10^{-14} M.

Data Collection and Analysis

Exclusion zone size was commonly averaged from 2 up to 6 measurements along one or both sides of the Nafion tube, measured in the control and each experimental sample, as described above. Data were normalized with respect to the control, the latter sometimes averaged over several runs on the day the experimental sample was evaluated. The resulting quotient value was then plotted against the concentrations of the material being tested. A third order polynomial regression was used to fit the data points.

Results

Tulsi

Results (Figure 2) indicate that at the low concentrations of Tulsi extracts (from -8 to -4 on the log v/v axis), EZ size initially increased in a concentration-dependent manner. Exclusion zone size diminished at the higher concentrations (from -3 to -1 on the log v/v axis). These results show that at low doses, Tulsi extract significantly expands the EZ and may therefore have significant health benefits.

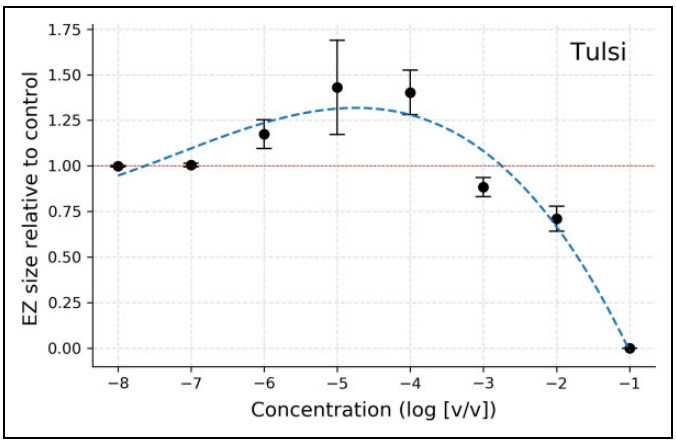

Figure 2. Effect of Tulsi on exclusion zone (EZ) size. Each point represents a mean with standard deviation (n; number of experiments = 5).

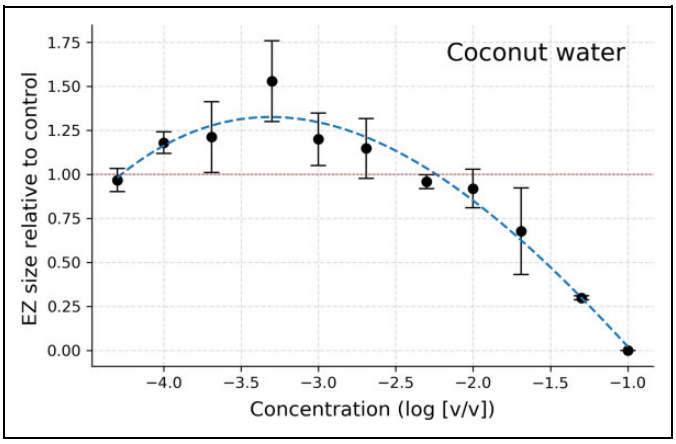

Figure 4. Effect of coconut water on exclusion zone (EZ) size. Each point represents a mean with standard deviation (n; number of experiments = 3-7).

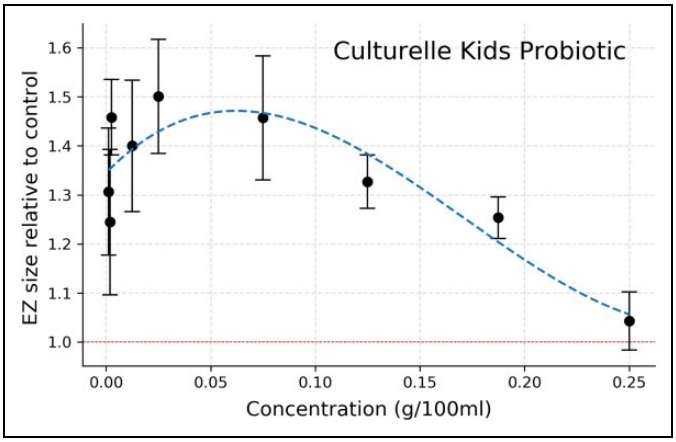

Figure 3. Effect of Culturelle Kids Probiotic on exclusion zone (EZ) size. Each point represents a mean with standard deviation (n; number of experiments = 3).

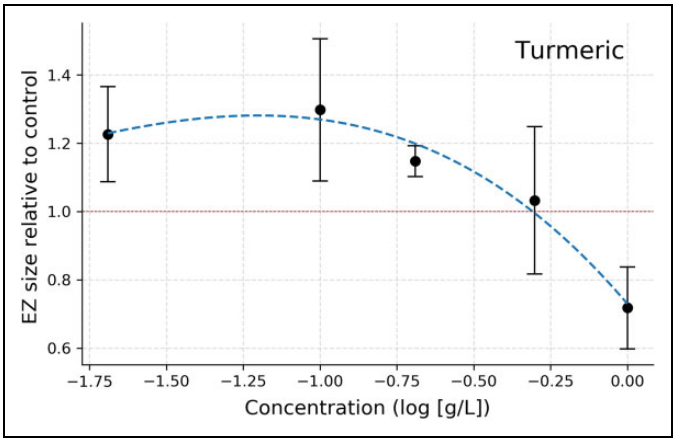

Figure 5. Effect of turmeric on exclusion zone (EZ). Each point represents a mean with standard deviation (n; number of experiments = 7-10).

Culturelle Kids Probiotic

We observed a steady growth of EZ size relative to control up to about 0.075 g/100 mL, beyond which EZ diminished (Figure 3).

Coconut Water

As before, data were normalized with respect to the control values measured on the day the experimental sample was tested and plotted against concentration. Our results indicated that EZ size increased with increasing concentration of coconut water in DI water, remaining above the control value over an unusually extensive range of concentration (up to 0.5%-1%) after which it diminished rapidly (Figure 4).

Turmeric

Data were normalized with respect to controls and plotted against concentration (Figure 5). While concentrations lower than 0.02 g/L of turmeric formulations were not tested, we observed that EZ size increased up to 0.1 g/L and diminished at increasing concentrations thereafter (0.2-1 g/L).

Aspirin

The resulting microsphere-free zone (EZ) size relative to the control in the vicinity adjacent to Nafion was plotted against the log of aspirin concentrations. Our results indicate that at a low concentration of aspirin (from 0.45-4.5 mM), EZ size exceeded control values, declining below controls at high concentrations (Figure 6).

Tylenol

Data presented in Figure 7 show a similar trend of increased EZ size at relatively low Tylenol concentrations (0.09-4.54 mM), finally diminishing in size at high enough concentrations.

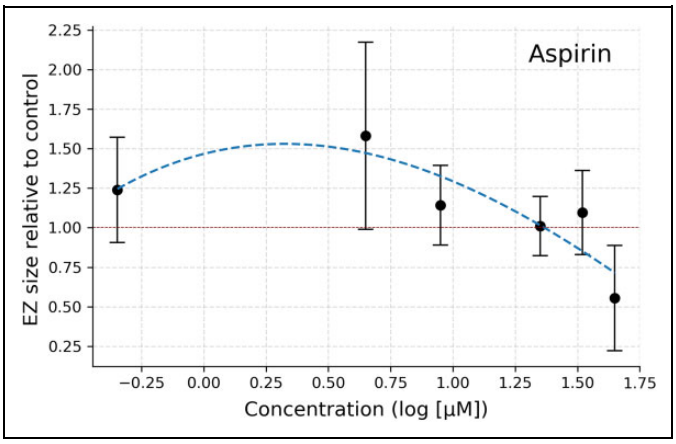

Figure 6. Effect of aspirin on exclusion zone (EZ) size. Each point represents a mean with standard deviation (n; number of experiments = 5).

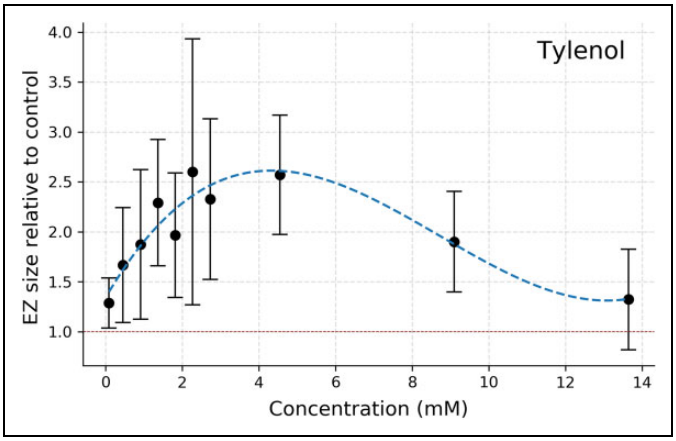

Figure 7. Effect of Tylenol on exclusion zone (EZ) size. Each point represents a mean with standard deviation (n; number of experiments = 10-20).

Roundup

Roundup was an exception to the general trend. With increasing concentration, we observed a progressive decline in EZ size (Figure 8). At no concentration did EZ size exceed the control value.

Discussion

The aim of this study was to test whether agents known to promote health also enhance the buildup of EZ water. Since EZ water builds next to hydrophilic surfaces, including those of the dense matrix within the cell, most intracellular water is structured, or EZ.[2,5] Hence, a possible explanation for the holistic effects of various health-promoting agents could lie in buildup of EZ water within the cells, thus influencing general health, for water is everywhere. The results show that this is indeed the case with a selection of nutraceuticals and pain-relieving medications undertaken for the study that build EZ water over a sizable range of concentrations. By contrast, the

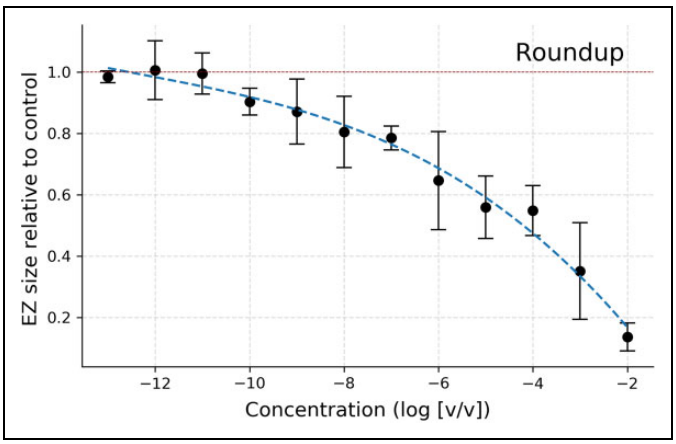

Figure 8. Effect of Roundup on exclusion zone (EZ). Each point represents a mean with standard deviation (n; number of experiments = 7-10).

weed killer, Roundup, whose active ingredient is glyphosate, failed to show any EZ expansion; instead, EZ diminished steadily with increasing concentration.

With regard to the proposed hypothesis, several questions arise. One is whether the substances tested are necessarily health-promoting. Another is whether the concentrations over which the EZ expands correspond to the concentrations that one might anticipate in vivo. Once the substance is ingested, concentrations begin building; hence, the smallest concentrations must be relevant. Evaluating the extent to which higher concentrations are relevant is less simple, for substances are absorbed, metabolized, nonuniformly distributed, excreted, and so on. The entire field of pharmacokinetics addresses these questions, and not all answers are secure.[7,8] Nevertheless, some rough estimates can be made.

Holy basil, also known as Tulsi, comes from the herb known as *Ocimum Tenuiflorum* or *Ocimum Sanctum.* It is an Ayurvedic medicinal plant, traditionally used for general health and a long life.[9-11] We used the liquid extract of Tulsi, prepared from the leaves of *O tenuiflorum* and *Ocimum gratissimum* plants with a suggested serving size of 0.7 mL to 2 ozs (about 60 mL) of water. This would roughly translate to 0.5 μL in a typical human body with 40 L of water and is equal to the area between the concentrations of −4 and −5 (log [v/v]) in Figure 2. Exclusion zone was observed to be the highest at these concentrations.

The most clinically studied probiotic, Culturelle kids probiotic, is free from dairy, gluten, and sugar, and helps to restore the balance of good and not-so-good bacteria in children's digestive systems.[12] Probiotic packets are recommended to be taken in servings of 1 gram. In an average human body with 40 L of water, this translates to 1 mg/40 mL (0.0025 g/100 mL). Exclusion zone increased steadily in the presence of this probiotic and plateaued out at 0.125 g/100 mL. Higher concentrations decreased EZ; however, even the highest concentration of 100 times more than the recommended amount (1 mg/40 mL) elicited a larger EZ than the control (Figure 3). This suggests

that even very large concentrations of probiotics can have an EZ-amplifying effect.

Coconut water is very rich in potassium and contains sodium, chloride, and carbohydrate.[13] Used as a rehydration fluid,[14] it is also reported to have antioxidant properties.[15] The usual amount of coconut water consumed by a human being at one time is about 11 ounces (325 mL). This would amount to 0.8% when calculated based on a typical human with 40 L of water. We obtained a good-sized EZ at concentrations of coconut water lower than 0.5% to 1% (−2.3 to −2 on the log concentration axis; Figure 4).

We also studied turmeric, which is a bright yellow "golden spice for all seasons" used in daily cooking in most Indian households. Derived from the rhizome of *C longa*, it is also valued as an herbal medicinal product and is a potential candidate for the prevention and/or treatment of many diseases due to its antioxidant, anti-inflammatory activities.[16] Normal dosage lies within the 0.5 g range, which translates to 0.0125 g/L in a typical human body containing 40 L of water. While data presented in Figure 5 do not represent the effect of turmeric at concentrations lower than 0.02 g/L, it does indicate that concentrations as high as 0.1 g/L may be safely used to elicit a good-sized EZ.

Prompted by the popular saying, "An aspirin a day, keeps the doctor away," we studied one of the most widely used drugs worldwide over the past 2 centuries—acetylsalicylic acid,[17] for testing the growth of EZ in its presence. Touted for its many health benefits, aspirin can reduce pain, fever, swelling, and inflammation, and prevent heart attack, stroke, and even certain cancers.[18] There is also evidence to show that aspirin may play a role in fighting neurodegenerative diseases such as Parkinson, Alzheimer, Huntington disease, and mood disorders.[19,20] We carried out experiments with a typical aspirin tablet that contained 325 mg of acetylsalicylic acid. Thus, in a typical human body with 40 L of water, we estimated a typical dosage of 45 μM aspirin, which was comparable to data obtained in earlier reports with minor modifications in experimental details.[21,22] The other concentrations tested were based off this typical dosage. Our data show that concentrations 10 times higher than 45 μM (ie, 0.45 mM, equivalent to −0.35 on the log concentration axis), even extending up to 100 times (4.5 mM, equivalent to 0.65 on the log concentration axis) elicited a reasonably sized EZ (0.45-4.5 mM), before falling off at higher doses (Figure 6).

Another widely used and prescribed drug that is generally considered to be effective and safe in therapeutic doses is Tylenol.[23] We used "over-the-counter" Tylenol from a local drugstore, so the maximum concentration that we could obtain was limited to the concentration in which the Tylenol was sold (160 mg per teaspoon, or 5 mL). Given that the standard dosage of liquid Tylenol is 15 mL, each dose delivers 480 mg of Tylenol into the body. Again, since the average human body has 40 L of water, this translates to 0.078 mM Tylenol. Our data indicate a steady increase of EZ size at the tested Tylenol concentrations up to 2 mM (Figure 7), after which there is a rapid decline at the higher concentrations (between 4.5 and 13 mM).

By contrast, glyphosate, marketed by Monsanto as Roundup, is the second most popular weed killer and has been getting a lot of attention lately. It is a broad-spectrum, nonselective, systemic herbicide, which is now well-associated with the endemic kidney disease epidemic.[24-27] The Environmental Protection Agency (EPA) has set an enforceable regulation for glyphosate, called a maximum contaminant level (MCL), at 0.7 mg/L, which translates to 4.14 μM glyphosate with respect to water present in a human body. This value lies in between −4 (1.21 μM) and −5 (0.121 μM) on the log concentration axis of our tested solutions, indicating that while the EPA-quoted "permissible MCL level" diminished the size of EZ, glyphosate concentrations both below and those above MCL levels diminished EZ by 5 times (Figure 8).

It appears, then, that all health-promoting substances tested increase the amount of EZ water in concentration ranges reasonably in accord with those anticipated in the body, whereas the health-impairing agent does the opposite.

Finally, why should an increase of EZ water promote health?

Health rests on cell function, which in turn results largely from protein folding. Depending on cell type, the folding of proteins mediates numerous functions: contraction in muscle cells, secretion in secretory cells, information transmission in nerve cells, and so on. We ordinarily consider proteins in isolation, but in fact, proteins are surrounded by interfacial (EZ) water.[5]

Hence, the normal microenvironment surrounding proteins is EZ water. Evidence indicates that the melting of this interfacial water is a necessary step for precipitating folding and that the restructuring of water, an energy-dependent event, accompanies unfolding.[2] Thus, a full complement of EZ water would seem a necessary condition for proper function. When EZ water is deficient, proteins lie outside their "normal" environment and can be expected to misfold, leading to impaired or pathological function.[28,29]

A proposal coming from that vantage point is that restoration of EZ water can restore function. Thus, agents that build EZ water should be beneficial to health, while agents that diminish EZ water should impair health. As to the latter, we found here that glyphosate, the active ingredient in the weed killer Roundup, impairs EZ buildup. In previous work, we found the same for various anesthetics.[30] Anesthetics block function, and we found that they diminish EZ buildup. By contrast, all agents studied here, which enhance biological function, resulted in EZ expansion. Hence, the results appear to support the hypothesis. A notable feature of the hypothesis is the global, or holistic, effect of many of these substances. Impact on diverse systems implies either of 2 options: multiple mechanisms of action or a single mode of action that operates widely. As for the latter, a possibility is water, which fills all cells. Thus, we hypothesize that while there may be no direct molecular interaction between the agent and water, the action

of these diverse agents may somehow facilitate the buildup of EZ water.

Water is the raw material used for the buildup of EZ honeycomb layers from the hydrophilic surface of Nafion.[5] Nafion contains oxygen atoms in its molecular structure and forms a template for the EZ water honeycomb. Each honeycomb layer builds by locking OH-units into the lattice, one at a time.[5] While the negatively charged lattice motivates positively charged protons to penetrate back into the EZ, these protons may otherwise latch onto water molecules to form hydronium ions. Ordinarily, hydronium ions cannot enter the lattice; thus, whatever EZ is formed continues to be well-stabilized. Health-promoting agents evidently impact the dynamics of these processes in a way that creates additional EZ layers. The observation that at the proper concentration, all health-promoting agents studied build EZ water fits the proposed hypothesis. It also satisfies Occam razor: the principle of simplicity.

In this context, a noteworthy feature is the expansion of EZ over large ranges of concentration—typically, several hundred or even thousand times. This implies a beneficial effect over a sizeable dose range. On the other hand, we found that excessive doses consistently diminish EZ size, a feature that may explain the negative impact of overdosing. Aspirin overdose, for example, was at one time a widely used suicidal expedient. If EZ expansion enhances health, then logically, EZ diminution impairs health.

The mechanism of high-dose diminution of EZ size is not yet clear; however, it is rather general, occurring with virtually all agents tested in our laboratory, whether health promoting, health challenging, or health neutral. Possibly, the EZ formed around each agent molecule at higher concentrations would result in an amplified production of protons. These would now be available to invade the EZ lattice, resulting in a measurable diminution of EZ size. This and other mechanistic options will require more detailed investigation.

It appears that EZ expansion may be a fundamental mechanism for health improvement, particularly in a holistic context. It will be interesting to see whether additional health-promoting agents fit within this paradigm.

Conclusion

Selected nutraceuticals and pain-relieving agents expand the EZ over a sizable range of concentrations. This result is consistent with the hypothesis that agents that promote health also build EZ. By contrast, the weed killer, Roundup, which impairs health, steadily diminished EZ with increasing concentration.

Declaration of Conflicting Interests

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