APPROACH

1. Background

Most Water in the Cell is Interfacial. The cell is so crowded with macromolecules that most water molecules reside near one surface or another. My 2001 book, *Cells, Gels and the Engines of Life*, describes this situation in detail: The measured distance between macromolecules averages the span of approximately seven water molecules; hence, even if interfacial ordering adjacent to each macromolecule extends to only two or three water molecular layers — the commonly accepted view — then most cellular water is ordered. That book goes on to present experimental evidence confirming that kind of ordering. It then demonstrates that acknowledging a role for that ordered, interfacial water helps explain a variety of fundamental intracellular mechanisms.

An Experimental Model Has Revealed Much More about Interfacial Water. In the cell, the extent of interfacial ordering generally projects to only a handful of molecular layers next to each molecular surface. Interfacial water is therefore difficult to study. In 2003, we found an experimental model in which ordering could extend out to much greater distances. This large ordered zone was seen in pure water, when salt was absent. It diminished substantially with the addition of salt: in concentrations similar to those found in cells, the ordered zone diminished to sizes comparable to those seen in the cell. Nevertheless, the ability to use that macroscopically ordered model to probe physical chemical properties has yielded a rich harvest of information on the nature of the interfacial zone. Our transformative award has enormously aided this pursuit, with 37 papers published or submitted and one book published (Pollack 2013) over the four-year period.

Among the rich harvest of experimental observations, we found that water orders next to numerous hydrophilic surfaces, but not at all next to hydrophobic surfaces. The ordered zone could extend sometimes even to hundreds of micrometers in salt-free water, but diminished to the nanometer scale in high-salt conditions similar to that of the cell (Zheng and Pollack, 2003). This water excluded particles and solutes in a manner similar to ice. This profound exclusion led us to refer to this interfacial water as the "exclusion zone" or more simply, the "EZ."

Secondly, we found that the EZ generally has negative charge (Zheng et al., 2006). This charge builds as water molecules split into negative and positive constituents, OH^- and positive protons. The negative components build the EZ; they create an ordered liquid-crystalline zone adjacent to the respective hydrophilic surface. The positively charged components exist in the form of hydronium ions (water plus proton), dispersed in the bulk water beyond the EZ (Chai et al., 2009). Because of this charge separation, this system has battery-like features, implying energy content.

The EZ's negative charge arises from the "excess" oxygen atoms lying in each of the EZ's honeycomb layers (see Fig. 1). While the ratio of oxygen to hydrogen in bulk water is 1:2, in the EZ it is 2:3 (Pollack, 2013). With relatively more electronegative oxygen atoms than neutral bulk water, the overall EZ bears net negative charge. One might say that the EZ is not truly "water" but another entity analogous to the hydronium ion. Hydronium ions and EZ water are both variants of H_2O.

Third, the density of EZ water is higher than that of bulk water. This comes about because charges in one honeycomb plane attract out-of-register opposite charges in the adjacent honeycomb plane, leading to a particularly dense structure. Based on refractive index measurements by other groups, EZ water density is approximately 1.1 times that of bulk water (Tychinsky, 2011; Bunkin et al., 2013). This feature is relevant because increased pressure is expected to shift the equilibrium from bulk water to the denser EZ water.

The results above show the EZ as a dense, negatively charged structure that lies immediately adjacent to hydrophilic or charged structures such as macromolecules, while positively charged hydronium ions lie in the bulk water beyond the EZ.

Incident Radiant Energy (light) Drives Interfacial Water Buildup. Separated positive and negative charges will ordinarily recombine over time to annihilate one another. In the EZ system, however, electrical measurements show that this separation is maintained. The reason may be incident radiant energy: absorbed electromagnetic energy (light) sustains and expands the EZ, building order and separating charge (Chai et al., 2009). We found that all wavelengths, ranging from ultraviolet, to visible, to mid-infrared, increase EZ size, but most effective were the IR wavelengths that are strongly absorbed by water. Modest intensities, weak enough to cause less than 1° temperature increase in the experimental chamber, could greatly expand the EZ; hence, this energy-driven expansion appears as a non-thermal effect (Chai et al. 2009).

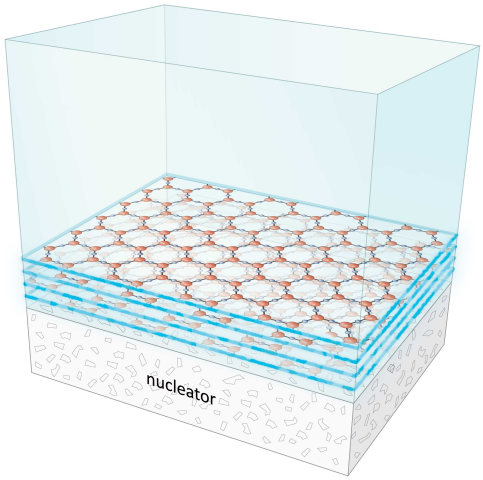

Figure 1. EZ structure. "Nucleator" represents any generic hydrophilic surface. Oxygen atoms (red) lie at hexagonal vertices; hydrogen atoms lie in between oxygens. Excess oxygen atoms relative to water yield net negative charge. (after Pollack, 2013).

Light-induced buildup of energy may seem unexpected; however, it occurs in plants all the time. Plants absorb light and use it to split water adjacent to light-absorbing chromophores, forming a proton or pH gradient. That process is the analog of what we found for the EZ next to hydrophilic surfaces. In some sense, then, the light driven buildup process under consideration here is not unusual or anomalous; it shares elements of the energy transduction process common to plants.

Page constraints limit this presentation to a brief capsule of the full body of evidence. The material and its logical consequences are presented much more extensively in Pollack (2013) and in many published papers.

Interfacial Water Relates to Function. This body of evidence provided much new information about the interfacial water that surrounds each macromolecule, well beyond Pollack (2001). We now refer to this interfacial water as EZ water. And we now know that EZ water is ordered and negatively charged; and that negative charge is maintained/restored through absorption of electromagnetic energy.

Pollack (2001) argues that each macromolecule and contiguous interfacial water make up a functional unit, one that cannot be separated. Cellular action occurs through the collective action of protein and water: as proteins fold, the interfacial water becomes transiently disordered, while subsequent unfolding brings reordering. If EZ water is central for folding, then long-term maintenance of EZ water must be critical for proper folding and function. Hence, any protein/EZ unit compromised by dysfunction or pathology requires EZ restoration to become functional. That is the central hypothesis on which this proposal builds.

2. Hypothesis

It is known that without water, most macromolecules would collapse and precipitate; they could not function properly. This scientific premise underlies our hypothesis.

Many diseases have been recognized as dysfunctions of proteins or protein regulatory systems (Dobson, 2002; Wiggins et al., 2009). In this protein-centered framework, creating the dysfunction may involve two components: the protein, or the intimate EZ water that envelops the protein. Thus, a dysfunctional mitotic apparatus that endlessly drives cell division (cancer) may involve dysfunction not only in microtubules

and motor proteins, but also of the surrounding EZ water. The state of interfacial water can be part of the problem — one that has received much less attention than the protein.

Reversing pathologies is a daunting task. Many approaches are palliative; i.e., drugs are given to reverse the *effects* of the pathology but not necessarily the cause. Depending on whether the cause lies with the protein or with the interfacial water, molecular hydration might alleviate the effects or perhaps even get to the root of the problem.

Stemming from our previous work aimed at characterizing EZ water, we recently began exploring a possible correlation between EZ water and biological function. In studies of muscle myofibrils using IR spectroscopy at the synchrotron beam line at UC Berkeley, we found that relaxed muscle contained mainly EZ (ice-like) water, while in contracting muscle the water transitioned to mainly bulk water (Yoo and Pollack, submitted). Thus, we could identify a change of state of water associated with functional state.

We also recently explored anesthetics — which compromise nerve function (and also unicellular organism function). Local and general anesthetics diminished EZ size. Lidocaine reversibly reduced EZ size in a dose-dependent manner; the dose required for achieving half-maximal size was ~0.6 mM, which is in the range of tissue concentrations for clinical application. Bupivacaine showed a 50% reduction of EZ size at ~0.2 mM. Similar reduction was seen with Isoflurane, a general inhalation anesthetic (Kundacina et al., submitted).

We also recently began studying agents that *promote* function (Kim and Pollack, in preparation). Aspirin promotes function by reducing fever, relieving pain, diminishing inflammation, etc. We found that aspirin substantially increased EZ size. The increase was as much as 300%.

(An aspirin sidelight: Aspirin reduces fever. Fever is associated with elevated temperature, which means increased generation of infrared energy. Infrared energy builds EZs. Therefore, by building EZs, fever may be one of nature's internal ways of reversing dysfunction.)

All three types of experiments above show correlation between EZ size and biological function, and hence the possible biological relevance of EZ water. These preliminary results triggered the hypothesis that restoration of function might be brought about by manipulations of the water around malfunctioning proteins. One might call it a functional repair through EZ restoration. This hypothesis is warranted not only by the exploratory studies mentioned above, but also by the fact that many simple, traditional therapies include factors that were found to increase EZ size.

To wit, consider heat (infrared) and light — two forms of electromagnetic energy. Both build EZs (Chai et al., 2009); and both are used for therapeutic purposes. Heat therapy has broadly recognized therapeutic value for pain relief from arthritis, muscle spasms, and migraines; heat also combats edema, bursitis, fibromyalgia, and even some cancers. Light helps reverse depression and sleep disorders; and it is often used to treat dementia, bulimia, acne, juvenile jaundice, psoriasis, pre-menstrual pains (PMS), attention deficit disorder, bipolar disorders, high blood pressure, circadian disorders, pain, hair loss, reproductive disorders, Parkinson's disease, and wounds (for a representative review on wound healing, see Peplow et al., 2012). Regarding depression, an interesting report is by Lam et al., (2006), a multi-center, double blind randomized controlled trial that found bright-light therapy to be equally effective as Prozac, with faster results and fewer side effects.

While all of this has been known for some time, our studies raise the new question whether heat and light exert their therapeutic effects through water: through the buildup of EZs. This is the hypothesis that we propose to investigate. Do heat, light, and several other agents affect biological function through a common mechanism of building EZ water? And, is EZ water buildup not merely correlative, but causal? Those are the questions we wish to answer.

And, if the answers turn out to be "yes," then this research would lead naturally to the question of *what is* the detailed mechanism underlying this causal effect of EZ water buildup on function.

3. Strategic Considerations

The experimental strategy is to test the proposed hypothesis that EZ restoration leads to functional restoration. We will test *various* agents' ability to expand EZs in biological models, as well as those agents' ability to reverse pathologies and restore normal organ function.

Only after such a link is clearly established at several levels of complexity can we proceed to address details of the underlying mechanism: Is the effect direct, or merely correlative? How does EZ water buildup lead to functional restoration? This pursuit could involve questions of protein folding, protein synthesis, reactive oxygen species and other toxins, etc. — all of which clearly lie beyond the scope of the current proposal.

With a scientific basis in place, then predictions and systematic improvements of traditional therapies becomes feasible. For example, one can ascertain which particular wavelengths of electromagnetic energy (heat, light) are most effective for increasing EZ size in biological models; once ascertained, it becomes possible check whether those wavelengths are equally effective for ameliorating a particular pathology. Any such correlation would strengthen the hypothesis that EZ buildup leads to functional restoration. It would also lead to improved efficacy of the respective treatments.

The experimental strategy outlined above needs to be realistic in terms of what can be accomplished in a five-year period. A sensible strategy is to focus on a small number of pathologies. Later, if the results are positive, subsequent studies can pick up on additional pathologies.

The approach also needs to take account of the laboratory's history and focus. Though well equipped to deal with studies of water and experiments involving *in vitro* biological preparations (more than 30 years of experiments on muscle contraction and cardiac electrophysiology), carrying out extensive animal experiments and/or clinical studies is beyond the laboratory's natural scope; hence, collaborations will be necessary. We anticipate that animal studies will be undertaken in collaborators' laboratories — almost contract style — under our close supervision to ensure protocol consistency.

If the results are promising, then dissemination will be another strategic objective. The scientific community and the public are justifiably skeptical of cheap, simple therapies. This skepticism may resolve if a proper scientific basis can be found for these simpler therapies — but only if the public is made aware. Hence, dissemination may be important for this project, and the PI's visibility can be of some advantage in that regard.

4. Procedure

The procedure involves four phases. We first describe the development of biological model systems for testing the impact of various biological agents on EZ buildup (***a***). We then detail the specific therapeutic agents under consideration ***(b)***. Third, we outline procedures designed to test whether the therapeutic efficacy of these agents arises from EZ buildup ***(c)***. Finally we deal with the mechanism by which EZ buildup may lead to functional restoration (***d***).

a. Model Systems

Previous work has used biological and non-biological models to establish the presence of exclusion zones (Pollack, 2013); however, most of the work was carried out on non-biological models such as Nafion and polyacrylic-acid gels because of their relative simplicity. A working assumption has been that the EZs in biological and non-biological models are similar because the raw material is always water; however, that assumption needs to be tested.

Hence, an initial phase of the experimental plan will identify suitable models to test this assumption. Biological gel models are promising since they have been partially tested. A gel model built of actin or collagen or some combination thereof is especially suited since these are common proteins representing intracellular and extracellular tissues. Making those gels is routine; we have already built actin gels in preliminary work carried out early on. The choice among those options will depend on which models prove to build the most stable and reliable, with easily visible EZ.

Once suitable models have been identified, the most critical experiments already carried out on non-biological models such as Nafion will be repeated on these biological models. Of particular interest will be the effects of various biological agents such as aspirin, antioxidants, and other agents known to enhance function; and also agents known to impair function, such as anesthetics and various toxic substances. The goal is to test whether these agents' effects on biological EZs are indeed similar to their effects found earlier on non-biological EZs. Will function-impairing agents diminish EZs? And, will function-enhancing agents expand EZs?

The second biological experiment will focus on function. We will directly compare enzyme activity in a bulk water environment vs. an EZ water environment. In the former, the enzyme will be suspended in the washing solution; in the latter it will be included within the above gels or on the gel surfaces. A simple color test will allow qualitative and quantitative detection of any difference of enzyme activity between the two. An interesting variant will test the effect of increasing amounts of enzyme inhibitors on enzyme activity in EZ versus bulk water environments. Also with this system we will repeat the treatments known to change the EZ size next to Nafion, and test whether they have the ability to restore the activity of partially inhibited enzymes. Several enzymes will be tested, starting with amylase, whose activity can be conveniently tested through the release of blue color from starch azure particles (Sigma) suspended in the solution around the gels. By adopting suitable microscopic techniques, other enzymes such as muscle ATPase will be tested as well.

The third and most complex model experiment is the use of muscle tissue down to the level of myofibrils. This laboratory has long experience working with muscle tissues. Recent work has confirmed the presence of EZ water both inside and immediately adjacent to myofibril bundles and single myofibrils (Yoo and Pollack, submitted). With this muscle model we will repeat the treatments used to manipulate EZ size next to Nafion and test whether the changes are identical or show differences. We will also compare the results with the preceding results on actin/collagen gels. This will permit a comparison of the action of all agents over a wide scale: from the level of non-biological systems, to protein gels, and finally to intracellular organelles. This will allow us to identify any differences or specific features at each level of organization.

The models outlined above are also useful tools for examining the six therapeutic agents described in the section below. Our primary goal is to test whether the agents' actions on biological EZs are indeed similar to, or different from, their known effects on non-biological EZs.

These experiments set the stage for answering the main question: do common, simple, therapeutic agents work by building EZ water?

b. Therapeutic Agents

The six therapeutic agents under consideration are detailed here. How these agents will be tested is considered in Section ***c***.

(i and ii) Light and heat. Light and heat are widely used as therapeutic agents (see above). The presumed mechanism is an increase of circulation through tissue heating. While circulation may be enhanced, these radiant electromagnetic energies have also been demonstrated to build EZs (Chai et al., 2009). All wavelengths studied thus far, including ultraviolet, visible, and infrared, are effective EZ builders. Some infrared wavelengths appear to be particularly effective: at 3-μm wavelength, for example, five minutes of exposure at intensities weak enough to induce less than 1°C heating produced a threefold EZ expansion.

A practical limitation in applying these therapies is depth of penetration. Some infrared wavelengths can barely penetrate beyond the skin and superficial capillaries. On the other hand, other wavelengths, which are almost as effective therapeutically (e.g., 0.8 μm), can penetrate far enough to permit trans-cranial scans deep into brain tissue. Hence, energy is certainly available to the body for building EZ water. The goal is to test whether EZ buildup is responsible for the known therapeutic effects of heat and light. Is the increase of function proportional to the increase of EZ water?

(iii) Drinking Water. Throughout history, various waters have been considered therapeutic. Those waters include so-called healing waters from sites such as Lourdes, Ganges, and Hunza (the latter studied extensively by Nobel Laureate Henri Coanda). Despite claims of long, healthy lives among populations who drink these waters, reports have seemed apocryphal and non-scientific.

Recently, various "therapeutic" waters have become available worldwide, especially in Asia and Europe. Many claims of therapeutic efficacy seem suspicious because of profit motives. Other claims are backed by published studies. For alkaline ionized water, for example, see Kim and Yokoyama (1997); also, see review by Rubik (2011). For so-called "IE" water, or "double helix" water see Ye et al. (2008), Li et al. (2010), and Park et al. (2011). In Japan, "electrolyzed" waters have become standard treatment for hospitalized GI patients (S. Shirohata, personal communication). And Taiwanese studies have shown improved dialysis results when the dialysate water has been electrolyzed (Huang et al., 2010).

These published studies support the hypothesis that the type of drinking water does matter. Mineral content may help explain the efficacy of some of those waters; however, the fact that non-mineralized liquid water contains not just H_2O, but also variable quantities of H_3O_2 (EZ water) offers a clue as to the difference of health efficacy among the various waters. Possibly, those waters rich with EZ water have higher health benefit. Some natural waters may fit this paradigm: high mountain water contains glacial melt: melt water contains high concentrations of EZ water (So et al, 2012). Also deep spring water is naturally pressurized from above: because EZ water is denser than bulk water, that pressure should convert bulk water into EZ water, and that prediction is confirmed (Ypma and Pollack, in preparation). And vortexed water, continually in motion, shows EZ content (Pollack, 2013). Thus, reasonable rationale exists for understanding why some waters may be distinctly different from others in terms of restoring health. Water is not simply H_2O.

A question is whether EZ water can survive the stomach's acid environment. At typical stomach pH of 4, we found that more than 60% of the EZ was retained (Park and Pollack, unpublished). Hence, the EZ should largely make its way through the stomach environment, and on to the tissues.

Another question is hydration. Cells take up water as needed. EZ water is extremely dipolar — highly negative EZ abutting positive protons. Envision such a dipole situated near a cell with negative electrical potential. The positive end of the dipole will orient toward the negatively charged cell. The resulting attractive force will pull the dipole toward the cell. Strongly dipolar EZ water should therefore hydrate cells faster and more extensively than bulk water, with weak dipoles. We already have some evidence that charge plays a role: water with charge and therefore higher dipole moment passes more readily through semi-permeable membranes than neutral water (Zhao et al., 2009).

Thus, the hydrating ability of exogenous water may be variable. Water containing little or no EZ might hydrate reasonably well. Hence, advocating "plenty of water" (but not excessive amounts) should be an effective health strategy. Natural healing waters and other waters with high EZ fraction might hydrate tissues better — perhaps much better if the dipole moment is as strong as we have found — yielding appreciable functional restoration. We want to objectively test the comparative health restorative effects of various types of water, as detailed below.

(iv) Grounding (Earthing). Grounding is widely considered a connection to an infinite source of zero potential, or neutrality. However, that is not the case. The earth's surface bears substantial net negative charge, a fact often forgotten but long known (see Feynman Lectures, 1964). Therefore, connecting to

ground is equivalent to connecting to a vast source of negative charge. Grounding devices have become popular for their touted health benefits (Chevalier et al., 2006); they are considered functionally equivalent to walking barefoot on the Earth. If those grounding devices are as effective as claimed, then their efficacy might come from supplying negative charge to the body, and therefore building EZs: our experiments have shown that supplying negative charge greatly promotes EZ growth (Ovchinnikova and Pollack, 2009).

Establishing a link between grounding-based health improvement and grounding-based EZ buildup could affirm the value of this ultra-simple therapeutic approach — advancing it from the seemingly mystical feel-good experience like walking barefoot on the beach, to something resting on sound physical principles.

(v) Anti-oxidants. Often used as dietary supplements, anti-oxidants combat free radicals, whose high reactivity can prove harmful. Anti-oxidants are considered effective in areas ranging from stroke and coronary artery disease, to gastrointestinal cancer, neurodegenerative disease, schizophrenia, and even aging. Their mechanism of action has proved enigmatic, with numerous pharmacological studies failing to concur on any unique mechanism (Yoon et al. 2011; Gaziev, 2013, Zuo et al., 2013).

EZ dynamics provide a possible unifying mechanism. Negatively charged EZ water fills cells. Maintaining this negativity should be essential for function; but oxidants, by definition, reduce negativity by removing electrons. By combatting electron removal, anti-oxidants promote negativity, and should therefore sustain or build EZs. Maintaining the full complement of EZ water could thus be a unifying mechanism of anti-oxidant action. Preliminary experiments carried with the free radical scavenger SOD (superoxide dismutase), which is found in every cell, has shown substantial EZ expansion.

(vi) Pressure and oxygen. Hyperbaric oxygen — oxygen applied under high pressure — has become a common therapeutic tool for treating a diverse array of pathologies. Originally developed to heal wounds that would not otherwise heal, this therapy was later expanded to treat diverse pathologies including diabetic neuropathies, anemia, radiation injury, and burns. Manufacturers are seeking FDA approval for treating still other pathologies (X. Figueroa, Restorix Research Institute, personal communication).

Linkage of hyperbaric oxygen to EZ growth seems plausible. First, EZ water has higher density than bulk water (Tychinsky, 2011; Bunkin et al., 2013; Pollack, 2013). Exerting pressure should therefore push the equilibrium toward the denser EZ water. Since cells contain a mixture of EZ and bulk water, the relatively higher pressure should build EZ water in the cell. Second, hyperbaric oxygen applies high oxygen. The high oxygen should promote EZ buildup because EZ water contains relatively more oxygen than bulk water. The higher oxygen content is discernible from counting oxygen and hydrogen molecules in the unit cell of each honeycomb layer (see Fig. 1). The combination of oxygen and pressure should therefore be powerfully effective at building EZs. Our experiments have confirmed that pressure and oxygen both build EZs (Ypma and Pollack, in preparation).

In sum, experiments to date have shown that diverse agents build EZs; these agents include light, heat, anti-oxidants and oxygen/pressure. Correspondingly, all of those agents and two others yet to be explored appear to restore function: Each agent seems to impact multiple disorders, and many of those disorders seem impacted by multiple agents. Hence, the effects appear general. Generality is anticipated since water pervades all cells. Thus, water may lie at the root of health.

c. Testing therapeutic efficacy of EZ-building agents

The goal is to systematically test the therapeutic efficacy of the agents listed in the previous section, and compare those effects to the agents' EZ-building capacity. A proportional linkage would be the most positive result that could be expected. The experimental approach will employ up to five pathological models — crucial for testing generality — although it is understood that time and budgetary constraints

will limit what can be done during the grant period. For exploring each one of those pathologies we will do the following:

(i) test the therapeutic effects of the various agents on cultured cells bearing that pathology;
(ii) test the therapeutic effects of those same agents on animal models bearing that pathology;
(iii) test the effects of those agents on EZ buildup in the specimens under consideration to determine whether therapeutic efficacy is proportional to EZ content.

Space limitations preclude detailed discussion of all pathologies under consideration; therefore, a representative one will be discussed with the understanding that the others will follow the same general approach. The representative example: cancer.

Pathology 1: Cancer. The strategy is to use a model in culture, e.g., HeLa cells, along with tumor-bearing mice. An expedient strategy is to use ongoing models in other laboratories; this capitalizes on experience, avoids the pitfalls of developing new models, and minimizes the costs by marginally incrementing ongoing costs instead of beginning from scratch. In the case of cancer, Dr. Chris Kemp, an experienced investigator at Seattle's Fred Hutchinson Cancer Research Center, is keen on exploring the hypothesis and eager to collaborate. Kemp routinely uses those models for his studies.

Using those experimental models, we will test the six agents listed above. All of these agents are expected to build EZ water, based on previous results. Here, systematic studies will test each of these agents to judge their anti-tumor efficacy. At the same time, laboratory tests in the PI's laboratory will be carried out to confirm and detail the effects of these agents on EZ expansion in biological models and also in the treated cells themselves to determine whether indeed therapeutic efficacy is proportional to EZ buildup.

To illustrate a representative protocol, consider one of the six agents: drinking water. It is emphasized that this is only one of six different agents to be tested, and that the others will be given due attention.

Only waters whose production methods are fully revealed will be considered for the proposed studies. Samples will be obtained from the suppliers themselves in order to avoid the risk of improper production. The receiving agent will code the samples to assure that experimenters do not know which water is being studied; nor will analysts know. Therefore, studies will be double blind. Controls will consist of local tap waters taken from several different taps, as well as distilled, de-ionized water taken from a laboratory supply. It is understood that, like the tap waters, these waters will contain some minerals, which can be analyzed by atomic absorption spectrometry, along with EZ content.

Experiments executed in the PI's laboratory will determine the relative EZ content of each water type: those with higher EZ content are hypothesized to have more therapeutic impact. For this determination we will use the 270-nm absorption magnitude, which has been established as a reliable measure of EZ content (Chai et al., 2008)**.** To complement this method we will also explore a fluorescence approach used successfully as a measure of high-energy electron content (V. Voeikov, Moscow University, personal communication), as well as a birefringence technique we have used as a measure of water ordering. These can produce a more complete characterization of the various waters.

Cultures will be grown in media made from the various waters, filtered for bacterial removal. (Sterilization by heating may destroy EZ water.) EZ water content will be measured after filtration. The cultures will be tracked for periods of several weeks and various parameters will be measured longitudinally, including cell-proliferation, metabolism, migration, differentiation, and viability. Experiments on each water type will be replicated at least five times to assure statistical significance, more if necessary. The effectiveness of a certain type of water will be judged based on its significant impact on one or several of the measured parameters relative to control.

Parallel tests will be carried out on mouse tumor models. Animals will be fed standard diets, and allowed to drink water *ad libitum*. Quantities will be tracked; and EZ content will be assessed before drinking. Tumor size will be measured as a function of time for each of the waters, repeated in at least ten animals

per water type depending on preliminary results. Additional repeats might be necessary. The objective is to determine whether different waters are more or less efficacious in reducing tumor size. Metastases will be tracked as well. These will be studied in selected animals with more advanced cancers. Animals will be sacrificed at various stages, and routine pathological examinations will be carried out to determine metastatic progress. These latter experiments should be particularly meaningful in that the metastatic cancers are the ones that are ultimately lethal.

Several water types have already been shown to contain strong 270-nm absorption peaks. Representative examples are shown in Figures 2 and 3. They show long-term persistence of EZ water. This persistence is consistent with published reports (e.g., Lo et al., 2009). For each water type, measurements will be made on multiple samples, and also on samples stored for each of various durations in different types of container prior to use. We are also able to produce our own water with varying EZ content using a filtration method we developed earlier (Klyuzhin et al., 2008). Relatively pure EZ water can be combined with distilled water in varying amounts to give the desired fraction, which can serve as a standard. Waters with highest EZ content are hypothesized to be the most effective in tumor reversal.

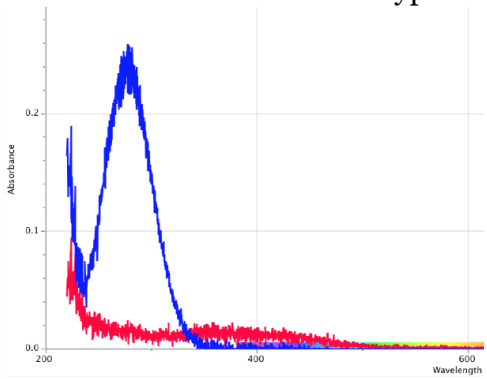

Figure 2. UV-VIS spectra of mountain well water (blue) vs. distilled water (red), courtesy Dr. James deMeo. Strong absorption peak at ~270 nm.

The final issue (iii) is whether indeed the restoration comes about *because* of EZ water restoration. To test this, we plan to measure the relative amounts of EZ water prior to and following treatment with the respective waters. We plan two approaches, one relatively certain but time intensive, the other simpler but less certain.

The first approach involves infrared spectroscopy. Infrared spectroscopy permits quantitative measurements of the amount of bulk and EZ (ice-like) water. We have used this technique in experiments on muscle water at the infrared beamline at Lawrence Berkeley National Laboratories (Yoo and Pollack, submitted). For these experiments, *in vitro* preparations seem most suitable. Cultured cells will be examined at various stages of therapeutic exposure to various waters. We will determine whether the ones that are more functionally viable contain relatively more EZ water.

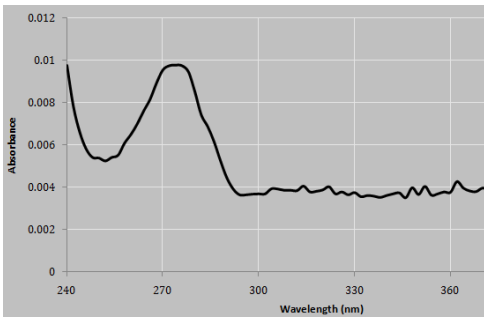

Figure 3. UV-VIS spectrum of microwave-treated water, produced by Dr. S. Sedlmayr and measured in the PI's laboratory. This spectrum was obtained two months after production. Persistence of 270-nm peak implies persistence of EZ water.

The second approach is electrophysiological: it involves measurements of cellular electrical potential. This lab has extensive experience measuring electrical potentials using microelectrodes — earlier in muscle and pacemaker cells, and more recently in EZ water. There is reason to believe that the cell's electrical potential is related to the amount of EZ water present in the cell: both are negatively charged; hence, more EZ water should bring more negativity. If this approach proves robust — if this method and the one above give consistently similar results — then we may be positioned to use this simpler electrical method as a routine tool for assessing EZ content.

The "best case" scenario from all the experiments above is waters with relatively higher EZ content yield the highest therapeutic efficacy, and that higher therapeutic efficacy is associated with greatest restoration of EZ water. If so, then EZ water (often contained in spring waters) may prove be a natural "drug" of the future for cancer treatment.

Parallel experiments will be carried out using the other five therapeutic agents. Positive results in all would lead us to conclude that EZ water buildup is an important mechanistic basis for treating cancer. Follow-up studies can then be considered for human experimentation on cancer patients, either by us or by others. Those studies would be deeply informed by the results obtained here.

Additional pathological models. Other disease models will be pursued in sequence. In addition to the collaboration with Dr. Chris Kemp (above), collaborative arrangements have been made with three prominent scientists: (1) Dr. Charles Alpers, Vice-Chair UW Department of Pathology, for studies on mouse diabetic models. Alpers's laboratory has considerable experience, especially with mouse models that develop diabetic neuropathies and glomerulopathies. Diabetic models exposed to EZ-building agents will be tested for blood sugar levels, response to insulin, and neuropathies. (2) Dr. Leanna Standish is medical director of the oncology research center at Bastyr University (adjunct appointment at UW), with extensive clinical experience. She will collaborate, using her center's cancer immunology *in vitro* and *in vivo* assays to study the effect of EZ content on immune function. (3) Dr. Jonathan Himmelfarb, who is director of the UW Kidney Research Center, with numerous ongoing projects. Himmelfarb first approached me because of his deep interest in the role of water in kidney function. The many ongoing models in his center provide a basis for testing the role of EZ water in kidney function.

From the results of preliminary studies, decisions will be made on which of those pathologies / collaborations will be most fruitful to pursue systematically. Also under consideration are heart and liver pathology models as well as aging, although budgetary limitations will restrict what can be reasonably achieved during the five-year grant period.

d. Testing the Mechanism

Should the results support the hypothesis, the question of molecular mechanism will need to be addressed. While the results above may show correlation between EZ water restoration and therapeutic efficacy, correlation cannot prove that more EZ water *drives* the observed therapeutic efficacy. Establishing proof is always a daunting task. However, a step toward that goal is establishing mechanistic rationale for how EZ water might promote function.

Toward that end, several questions will be addressed. One question is specific to imbibing EZ water. Does EZ water hydrate better than tap water? To answer that question, we will expose the biological model system to waters with varying EZ content. We will determine whether the water with higher EZ content results in faster and more extensive EZ buildup. The same can be tested with *in vitro* models such as muscle. A positive result would support the concept that drinking waters with high EZ content promote health by enhancing hydration.

Another question is *how* increased hydration, whether achieved from drinking or from applying any of the five other approaches, improves function. Since function depends on protein folding action, a suitable approach is to test whether proteins function better with higher EZ levels. This can be done in a protein system with which we have extensive experience: muscle. Muscle specimens will be treated with each of the six listed agents. Attributes of protein function such as tension development and maximum shortening velocity can be measured by methods standard in our laboratory. EZ water content will be measured using IR beamline spectroscopy. Improved function with higher EZ content will support the hypothesis that protein function depends on proper hydration.

These are examples of the kinds of tests that can probe the underlying mechanism. Others approaches exist. For example, a question is whether hydration enhances protein synthesis; another is whether hydration enhances enzyme function (as described earlier). Extensive discussion of protocols seems premature, as these tests will be carried out only if the results are strongly positive, i.e., if a strong correlation is found between EZ buildup and therapeutic efficacy.

5. Response to anticipated concerns

This application for a second transformative grant may be viewed as even riskier than the one that it will replace. Most scientists view cellular water as the background carrier of the more important molecules of life. This proposal challenges that view, putting cell water not only as a central agent for function but also as a potentially central agent for health. We are testing the hypothesis that water may be the key to a broad range of health issues that increasingly plague our society. Because this idea challenges current views, it seems worthwhile to answer questions that may inevitably arise:

The endeavor is premature. Why not wait until the role of EZ water is more universally accepted? Interest in EZ water has been building rapidly. Some two-dozen laboratories worldwide have initiated studies of EZ water. Email communications are voluminous; speaking and interview invitations come with increasing frequency. With growing interest, especially from medically oriented spheres, the time for launch seems appropriate. Healthcare is now in crisis; fresh approaches need to be pursued now, not later. This study is not premature.

Has the PI used his current transformative grant productively? The grant produced many papers in highly rated journals. All stated goals have been achieved (and more). The culmination of this work came in the recently published book, *The Fourth Phase of Water: Beyond Solid, Liquid, and Vapor*. More than a few internationally respected scientists have communicated their views that this is a landmark contribution. Recently, we have carried out varied preliminary experiments (described above) that go well beyond what was originally proposed.

Does the PI have the energy to handle a project of this magnitude? Those who know me acknowledge my unusual level of energy, which seems only to grow by the year. Enthusiasm for the proposed studies is high, as we are convinced that this approach has the potential to trigger a long-needed breakthrough in health care: if the results support the hypothesis, then simple remedies with scientific basis may begin replacing those currently dominating medical practice, with significant side effects and high cost. Hence, this project will be tackled with at least as much intensity as previous endeavors.

Does the PI have the breadth to cover all aspects? My career began in a clinical department (anesthesiology); it continued with studies of cardiac biomechanics, electrophysiology, muscle contraction, and cell biology; and of late it has been dealing with the physical chemistry of water. This unusually broad background, from fundamental basic science to clinical science, seems well suited for tackling a project of this scope. In order to assure the success of this multidisciplinary research I have assembled a strong team of collaborating experienced senior investigators.

The research is too sweeping and ambitious. The need for radical change in the practice of medicine seems increasingly urgent. Radical change is unlikely to come from an incremental, tiptoe approach. A bold approach seems warranted, and that is what is proposed here. Relative to the massive NIH investments in clinical trials, genome science, and other areas, this one seems modest in cost and sharply focused on a single hypothesis. If successful, the benefit/cost ratio could be substantial. We understand that transformative grants are intended to support ambitious, paradigm-shifting proposals.

At the same time, it is understood that all the proposed experiments cannot be finished in the projected five-year period (unless the award is increased substantially). We presented the minimally necessary components required to thoroughly test the hypothesis. Without so doing, we felt certain that the approach would be viewed as hollow.

The budget is too low for such an ambitious project. A larger budget could certainly produce more comprehensive and ultimately more impacting results. However, application is being made for a

"standard" budget, knowing the currently strapped and uncertain resources of the NIH and the stiff competition for whatever funds are available.

APPROPRIATENESS FOR TRANSFORMATIVE PROGRAM

First, the NIH Director's Transformative Research Program is intended for high risk - high reward projects, which, if successful, could transform an important paradigm in science or medicine. We believe this proposal qualifies.

A second point is that this type of application has little chance of success with standard grant mechanisms, which tend to focus on protocol details. Protocols will differ in each of the multiple experiments proposed, and will almost certainly change during the course of the experiments as we learn more. The Transformative grant mechanism recognizes that experienced and productive investigators who have succeeded in the past can figure out such details. Thus, the application space has been used mainly for describing background, potential significance, general approach, and likely impact, rather than experimental details (which applicants are instructed to omit).

Third, the strategic approach demands the flexibility that is inherent the Transformative program. For example, co-investigators are not listed. By design, co-investigators will change depending on the particular pathology under consideration, and possibly even during the course of one of those investigations if progress seems inadequate. Agility is key for successful outcome. Such flexibility is ordinarily not possible with standard granting mechanisms, but is essential if we are to achieve the maximum possible gains during the five-year period.

TIMELINE

The plan is to run three types of studies simultaneously: cell culture studies, animal studies, and corresponding studies on water. The first two of those three components will be carried out mainly in collaboration with other laboratories that have cultures and animal models going; the third will be carried out in our laboratory. If these studies go particularly well, we might move toward clinical studies before the termination of this grant, in which case permission will be sought from the appropriate authorities.

We anticipate that each year will look similar in terms of approach and timeline. In each year, we expect that we can realistically focus on one pathology. This means running experiments simultaneously on tissue culture, animals, and water, each pathological condition requiring tests on the six therapeutic variables in question. This high level of work should be feasible because it will be spread among multiple laboratories.

If studies of each pathological condition require one to two years to complete, then the five-year period will have systematically covered two or three pathologies. At the end of this period, we anticipate a comprehensive judgment of the merit of the proposed hypothesis, possibly leveraging clinical studies to test efficacy on humans and further studies on additional pathologies.