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Healthy fats and exclusion-zone size

Abha Sharma*, Gerald H. Pollack

Department of Bioengineering, University of Washington, Seattle 98195, WA, United States

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ABSTRACT

A fourth phase of water, labeled exclusion-zone or "EZ," extends from hydrophilic surfaces. Salient features include exclusion of colloidal and molecular solutes, and characteristic light absorbance at 270 nm. In cell systems, EZ water interfaces with membranes, macromolecules, and organelles, and its buildup appears to be vital for function. For years thought to build health, fats have gained a negative reputation over the last few decades. While their exact role in health remains unclear, now they have become more accepted. We tested several fats for their capacity to generate EZ water. Large EZs formed next to ghee, coconut oil, lard, organic clarified butter, and 'Brain Octane®' oil. Cold ghee surfaces produced especially large EZs. Thus, EZ growth, confirmed by microsphere exclusion and UV–VIS absorbance spectroscopy of samples flanking the fat, may be an important factor in cellular hydration and might well underlie the health-promoting function of fats.

1. Introduction

Water is the most abundant molecule in the cell, accounting for some 70% of the total cell mass. The cell also contains organelles and various macromolecules, such as nucleic acids, proteins, lipids, and carbohydrates, packaged tightly within the cell membrane. Because of this tight packing, most intracellular water molecules lie within several nanometers' proximity of hydrophilic surfaces. Cell water is, therefore, mainly interfacial, a feature of potentially major significance for cell function (Pollack, 2001). We have termed this water "exclusion zone" (EZ) water, or "fourth phase" water (Pollack, 2013). Well characterized as building extensively next to hydrophilic surfaces, such as gels and biological surfaces, and to exclude solutes, EZ water has been shown to be vital for cellular health and function (Pollack, 2001). Shifts of EZ to bulk water, on the other hand, have been shown to be a key factor underlying malfunction of many cellular and physiological actions (Pollack, 2013).

Since EZ water is critical to cell function, we hypothesized that substances known to promote health could plausibly mediate their effects by building EZ water, thereby hydrating the cell, normalizing cell function, and restoring health. By this mechanism, such substances would be deemed health promoting.

Once considered anathema to health, fats are now recognized widely to be important for health (Sserunjogi, Abrahamsen, & Narvhus, 1998; Zevenbergen et al., 2009). Amongst different fats, ghee is traditionally considered to be a food, medicine, and the healthiest source of edible fat with many beneficial properties (Sharma, Zhang, & Dwivedi, 2010; Sserunjogi et al., 1998; Sud & Kateriya, 2015; Tirtha, 1998). Ghee is also the common Indian name for clarified butter (Sserunjogi et al., 1998). Similarly, while coconut oil is becoming increasingly popular as a saturated fat (Gopala Krishna, Raj, Bhatnagar, Prasanth Kumar, & Chandrashekar, 2010; Marina, Che Man, & Nazimah, 2009), lard has been used widely in the past (Li et al., 2017).

We, therefore, wanted to determine whether these and related fats including caprylic acid triglycerides from highly refined coconut oil (Brain Octane®) build EZ water. If so, this would support the hypothesis that, as a class, health-building substances (Sharma et al., 2018) could mediate their effects through the buildup of EZ water.

We used a standard approach for measuring EZ size (Sharma et al., 2018; Zheng, Chin, Khijniak, Khijniak, & Pollack, 2006). This included an EZ-nucleating surface, such as Nafion, immersed in an aqueous microsphere suspension. EZ is defined as the zone adjacent to the Nafion from which suspended microspheres are excluded. In the current experiments, we replaced Nafion with a cylinder made of one or other of the fats and examined the size of the exclusion zone around the cylinder. To our knowledge, this is the first study demonstrating the presence of EZ against the surfaces of fats.

2. Materials and methods

2.1. Microsphere suspension

The suspension used for determining EZ size contained polycarboxylate-coated 2 μm microspheres (Polysciences Inc; # 18327;

* Corresponding author.
E-mail address: abha6@u.washington.edu (A. Sharma).

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2.5% solids-latex, Warrington, PA, US) or polystyrene-coated 2 μm microspheres (Polysciences Inc; # 19814; 2.5% solids-latex, Warrington, PA, US) respectively. These were suspended in deionized (DI) water obtained from a Barnstead D3750 Nanopure Diamond purification system (type 1 HPLC grade, 18.2 MΩ, ThermoFisher Scientific, Waltham, MA, USA). The volume ratio of microspheres to DI water was kept constant (1:300) to eliminate any effects that might arise from concentration differences.

2.2. Fats

We studied four different fats that are solid at room temperature (RT), namely: Bulletproof (BP) ghee (grass-fed) (Seattle, WA, USA); Dr. Bronner's Magic "All-One" Fair Trade, and Organic Virgin Coconut Oil (VCO- fresh-pressed, unrefined, and "whole kernel") (Victoria, Australia); Tendergrass Farms organic lard (Floyd, VA, USA); and *O* Organics® sweet cream butter (unsalted) from Albertsons Companies' (Boise, Idaho, USA). Additionally, we also investigated Bulletproof "Brain Octane®" oil (Bellevue, WA, USA) (caprylic acid triglycerides from highly refined coconut oil) which is liquid at room temperature.

2.3. Fat cylinders

A small amount of each fat (ghee, coconut oil, or lard) was scooped out of its container with a clean spatula and placed in a 15 mL polypropylene test tube. The tube was placed in a boiling-water bath for 5 to 10 min, after which the liquid fat was poured into a 1.0 mL syringe and allowed to solidify for 20 to 30 min in a − 20 °C freezer. A small cylinder (3 to 8 mm long and about 1 mm diameter) was fashioned by carefully ejecting the contents of the syringe out through the opening (no needle) onto a 35 mm petri dish and allowed to set for 20 to 30 min in a refrigerator (4 °C). These samples were termed "cold fat cylinders". On the other hand, cylinders fashioned from fats solidified (about an hour or less) in the 1.0 mL syringe at room temperature were termed as "room temperature (RT) cylinders".

2.4. Organic butter

A small amount of butter was melted in a 15 mL polypropylene test tube placed in a boiling-water bath. Like the fat cylinders, a portion of the liquid fat was poured into a 1.0 mL syringe and allowed to solidify for 20 to 30 min in the freezer. This was labelled as organic butter (Org B). A second portion of the liquid organic butter was centrifuged at 570 × g for 30 min. The homogenous mixture separated into two parts. The lower portion containing the white milk solids was discarded. The supernatant, constituting the clarified butter or organic ghee, was pipetted out into a fresh polypropylene tube and labelled as "organic clarified butter" (Org CB). Cylinders were fashioned from Org CB in the same way as described above.

2.5. Bulletproof Brain Octane® oil

Brain Octane oil (BO) was impregnated into strips (1 mm wide and 4 to 5 mm long) cut from nitrocellulose (NC) membranes (Bio-rad; 0.45 μm pore size) or Whatman Grade 42 ashless filter paper, (2.5 μm pore size) and placed in a covered petri dish for 4 or 2 h, respectively. Similar-sized strips were impregnated with DI water (controls) in a covered petri dish. An impregnated strip (BO or DI) was gently blotted onto a Kimwipe, placed in 3.0 mL of a polycarboxylate-coated 2 μm microsphere suspension in a petri dish and used for EZ measurements, as described below for a fat cylinder.

2.6. Setup for measurements of EZ

A fat cylinder was submerged in 3 to 4 mL of the prepared microsphere suspension in a 35 mm × 10 mm Falcon® polystyrene petri dish. The dish was covered and placed on the stage of a Zeiss Axiovert 35 microscope equipped with a 2.5x objective lens. Amscope software, downloaded onto the computer was set up on auto-image to capture an image of the EZ forming against the fat cylinder. All measurements of EZ were taken at intervals of one minute for duration of 20 min. The graph tool from Image J (https://imagej.nih.gov/nih-image/) was used to make three measurements, on one side of the cylinder, from which the mean EZ size was obtained.

2.7. Hydrophilicity of ghee

To determine the degree of hydrophilicity, we measured the contact-angle (θ °) between a drop of water on the ghee surface, under standard conditions of laboratory light and temperature of 22–23 °C.

Liquid ghee was layered thinly (0.2 mm thick) and allowed to solidify on a 35 mm × 10 mm Falcon® polystyrene petri dish before a drop of DI water (drop volume-50 μl) was pipetted onto the set surface. The water droplet was allowed to stabilize for 5 min to reach its final static state. The contact angle (Kowk-Yee & Zhao, 2015) between the water droplet and solid ghee surface was observed as a three-dimensional visual through the dual objective lenses (0.7–4.5x magnification) of a dissecting microscope, suitably adjusted to focus along the horizontal axis of the droplet atop the ghee surface.

Additional illumination was provided by two easily manipulated fiber-optic gooseneck light cables attached to the fiber optical microscope illuminator (Amscope cold-light source haloid lamp 150 W, Irvine, CA, USA). An integrated video camera captured the magnified image that was displayed and saved on the attached high-resolution monitor for drop profile/angle analysis.

2.8. UV–Vis spectroscopy of water surrounding the ghee cylinders

The UV–VIS absorption spectrum of exclusion-zone (EZ) water is defined specifically by its signature absorption peak at 270 nm. To test whether the microsphere-free region around the ghee was indeed EZ water, we extracted fluid from the apparent EZ and tested it spectroscopically. To do so, we placed a cylinder of ghee in 3 mL of DI water (containing no microspheres) in a 35 mm × 10 mm Falcon® polystyrene petri dish. The dish was covered and placed on the stage of a Zeiss Axiovert 35 microscope equipped with a 2.5x objective lens. The microscope lamp light was set in the "on" position for 10 to 20 min.

EZ water in the immediate vicinity of the ghee cylinder was drawn off after 10 min, using a 1 mL tuberculin syringe and 27G × 1/2″ BD PrecisionGlide needle, and deposited into ready-labelled tubes for UV–Visible absorbance spectroscopy.

Due to the difficulty of extracting EZ water from adjacent to the cylinder, we also collected interfacial EZ water using a slightly modified version of the same technique. Liquid ghee was layered as a 3 to 4 cm diameter pellet-circle on a 65 mm petri-dish and allowed to set for 20 to 30 min in a refrigerator (4 °C). DI water was added carefully, so it was 0.8 to 1 mm above the ghee pellet. After 10 min, the overlaid water was aspirated carefully with a pipette tip for spectroscopic measurement in the UV–Visible wavelength range between 200 and 350 nm.

2.9. Statistical analysis

Data are presented as mean ± SD of N (number of experiments). Statistical significance between different groups was determined using Students *t* test. A p value of < 0.05 was considered significant.

3. Results

3.1. EZ formation around cylinders of different fats

Fig. 1 shows representative images of EZs formed against the cylinders of cold ghee, coconut oil, lard, and organic clarified butter (Org

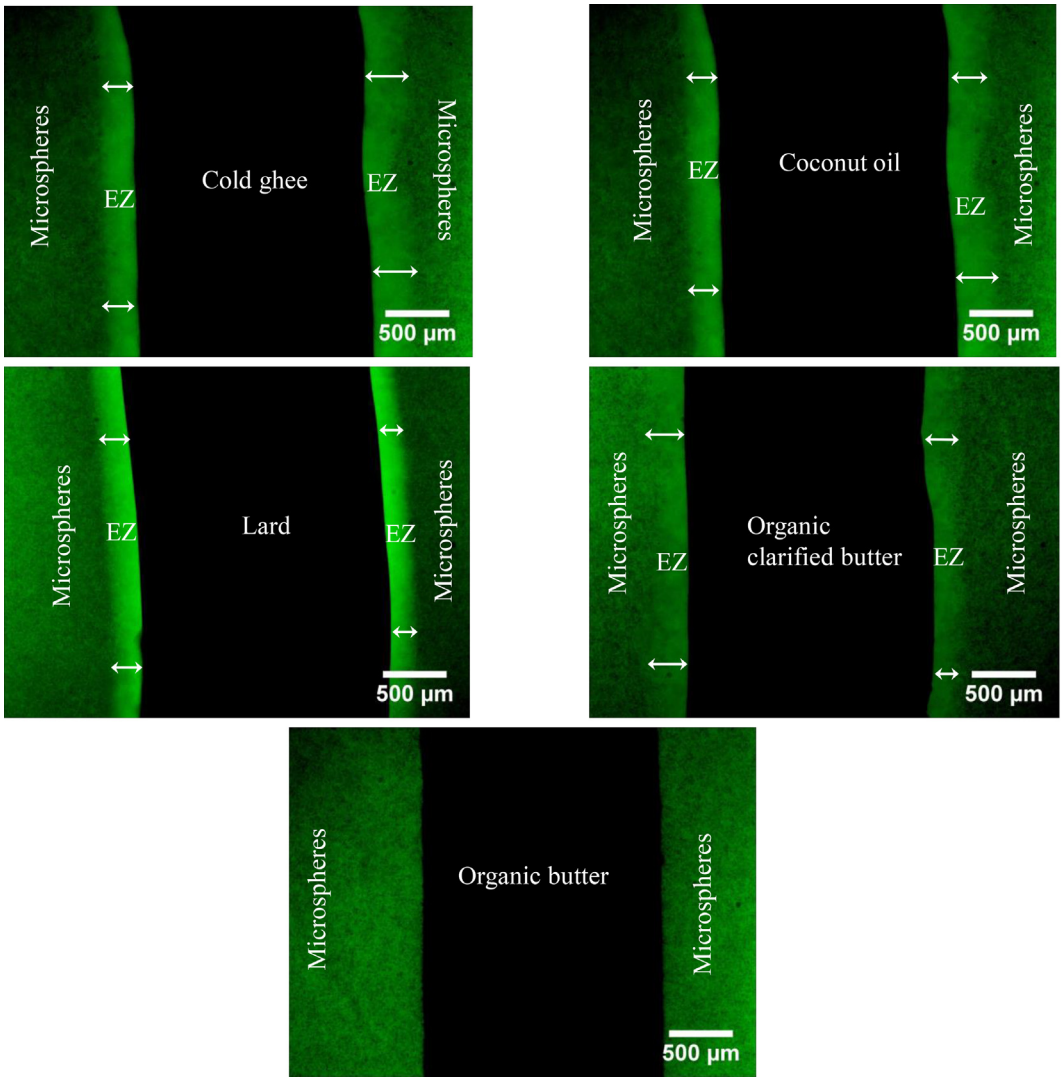

Fig. 1. Exclusion zone (EZ) water formation around cylinders of different fats. Representative EZs, marked by double-headed arrows, formed against fat cylinders in an aqueous suspension of 2-µm polycarboxylate microspheres.

CB). Microsphere-free zones are marked with double-headed arrows and show EZs of various sizes around the different cylinders; no EZ is visible against a cylinder made from organic butter (Org B).

We found a steady build-up of EZ against fat cylinders (cold ghee, coconut oil, and lard) over the initial time-period, followed by a slight diminution after 10 min. These findings are similar to those observed with Nafion (Pollack, 2013), where EZ size increased significantly in the first 10 min (Fig. 2). At 10 min, EZ was the greatest with cold ghee (789 ± 220 µm) and coconut oil (764 ± 230 µm) and was 20–25% larger than that formed with lard (585 ± 36 µm) (p value ≤ 0.05). On the other hand, EZ next to a cylinder made from Org B was only 55–100 µm (and observed only at some places), while that next to a cylinder of clarified butter (Org CB) obtained from centrifuging Org B was almost six-times larger at 10 min (p value ≤ 0.01).

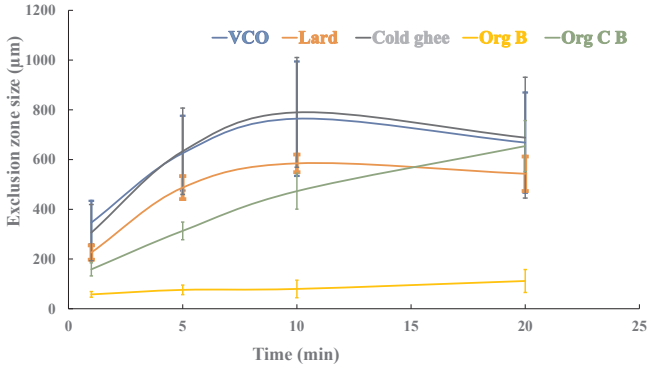

Fig. 2. Time course of EZ formed against cylinders of coconut oil (VCO), lard, ghee (Cold ghee), organic butter (Org B) and organic clarified butter (Org CB). Each point represents a mean with standard deviation N (number of experiments): 10–12 each.

3.2. EZ formation around ghee cylinders formulated under different conditions

EZ formation depended on conditions under which the ghee cylinders were formed. Overall, colder environments (cold ghee cylinder) elicited larger EZs than warmer one (Fig. 3). Similar results were obtained irrespective of whether we used 2 µm polystyrene-coated microspheres or 2 µm polycarboxylate-coated microspheres. At 10 min, EZ measured against cold ghee cylinder was almost two-fold greater than that formed against a ghee cylinder formulated at room temperature (p value ≤ 0.01).

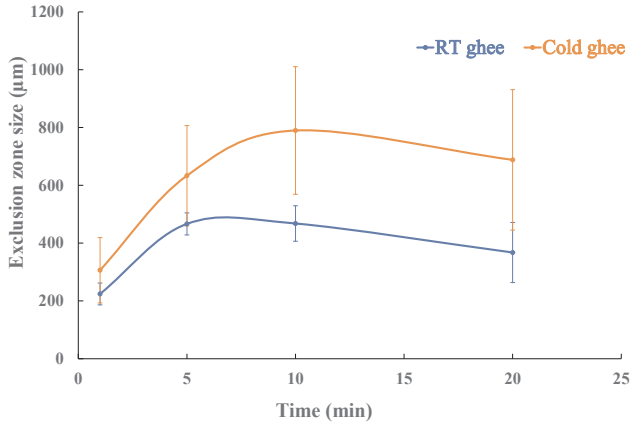

Fig. 3. Time course of EZ formed against ghee cylinders formulated under room temperature (RT ghee) and Cold ghee. Each point represents a mean with standard deviation N (number of experiments): 12 each for room temperature (RT) and Cold ghee, respectively.

3.3. EZ formation against strips impregnated with Brain oil®

'Brain oil' is liquid at room temperature (RT), so cylinders could not be formed. To test for the presence of EZ, we impregnated strips of nitrocellulose membrane (NC) or Whatman Grade 42 (W42) paper with Brain oil, using DI water for the control. A time-course study indicated that EZ was present in both cases (Fig. 4a and b) and that, in each case, it was substantially larger in those strips impregnated with 'Brain oil' compared with the DI water control.

3.4. Hydrophilicity of ghee

Ghee displayed a hydrophilic nature ((Fig. 5: $\theta < 90^{\circ}$); the contact angle formed by the water droplet was initially $68^{\circ} \pm 6^{\circ}$, but gradually diminished, by spreading to $50^{\circ} \pm 6^{\circ}$ after 5 min (N (number of experiments): 4).

3.5. UV–Vis spectroscopy of EZ water

A broad absorbance peak between 260 and 300 nm was observed in samples drawn from the immediate vicinity of ghee cylinders after 10 min of development (Fig. 6a). More strikingly, interfacial EZ water samples taken after the same time period from the top of the ghee pellet displayed a sharp peak around 280 nm (Fig. 6b).

4. Discussion

Fats make headline news almost daily. These calorie-dense nutrients are an indispensable but controversial part of our food supply. Questions arise, such as whether fats are good for health, which ones are healthful and which are not, and whether all saturated fats are bad for health.

Dietary fats are important components of cellular membranes. Not only do fats function as major metabolic fuels, but they also provide thermal/electrical insulation and mechanical protection. In addition, polyunsaturated fatty acids are involved in lipid-based cellular signaling systems and gene regulation (Rustan & Drevon, 2005). A major bone of contention in the popularity of fats arises from a lack of knowledge about differences in dietary fats and their constituent fatty acids, their chemistry (the saturated and unsaturated content), and metabolism in humans. Over time, these aspects are now being documented and play a critical role in general health, well-being and disease (Rustan & Drevon, 2005).

In recent years, however, decades of dogma that saturated fats are

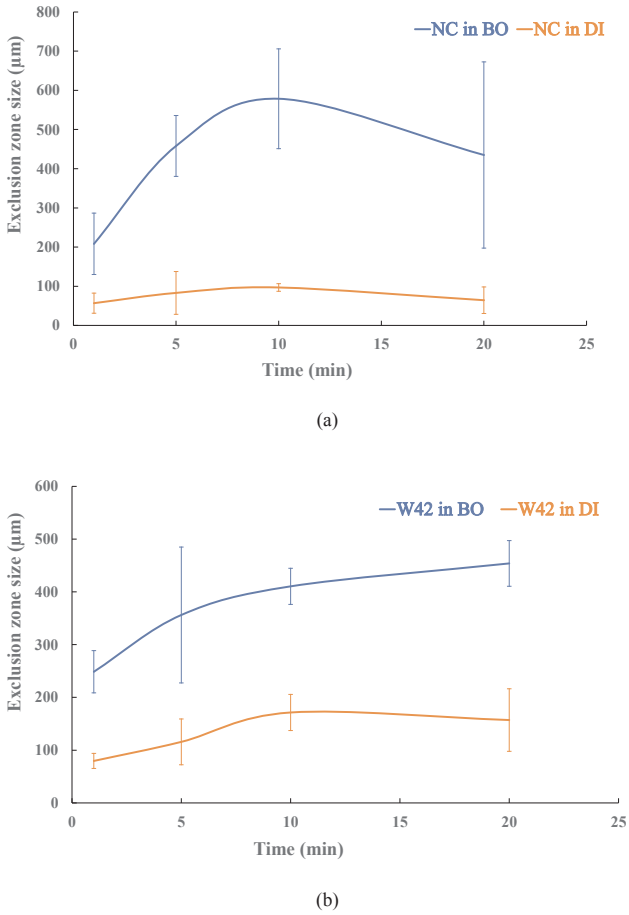

Fig. 4. Time course of EZ formed against (a) a nitrocellulose (NC) strip and (b) a Whatman Grade 42 (W42) paper strip. Strips were impregnated with Brain oil (BO) or DI (deionized water). Each point represents a mean with standard deviation N (number of experiments): 10 each respectively.

unhealthy and should be avoided is being reconsidered. In fact, several saturated fats are making a come-back. In some cultures, ghee is cited as one of the best sources of fats for human consumption (Tirtha, 1998). We wanted to test if ghee could build EZ water and, when tested, large EZs were observed. In fact, ghee EZs were larger than those formed with what has become the gold-standard material, Nafion, implying that ghee is superlative in its capacity to nucleate EZs.

We also explored other popular fats used in cooking for their ability to build EZs, such as coconut oil, lard and sweet cream butter. EZs similar to those formed by ghee were observed, substantiating our earlier findings (Sharma et al., 2018) that physiological doses of several nutraceuticals and some common pain-relieving medications that promote health may mediate their effects by building EZ water within cells, thereby enhancing function and health.

Chemically, fats are complex polycrystalline multi-component mixtures of triacylglycerols (TAGs) (Himawan, Starov, & Stapley, 2006). Each TAG has a glycerol backbone with three esterified fatty acid moieties, which may differ in chain length (ranging from 4 to 22 carbons) and degree of saturation. Generally speaking, TAGs with saturated fatty acids have higher melting points than those with triglycerides composed predominantly of shorter chain and/or unsaturated fatty acids. TAG composition can also affect the crystallization and polymorphic properties of the fats (Ramel, Co, Acevedo, & Marangoni, 2016).

While consideration of these factors lies beyond the scope of the current study, we speculate that they influence the hydrophilicity of a

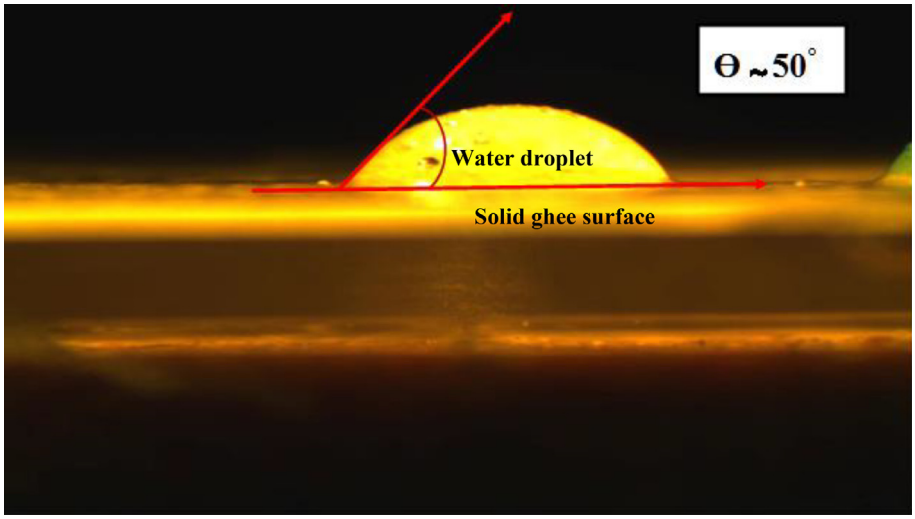

Fig. 5. A representative drop-shape profile analysis to determine the contact-angle **(θ°)** between a water droplet and the solid ghee surface.

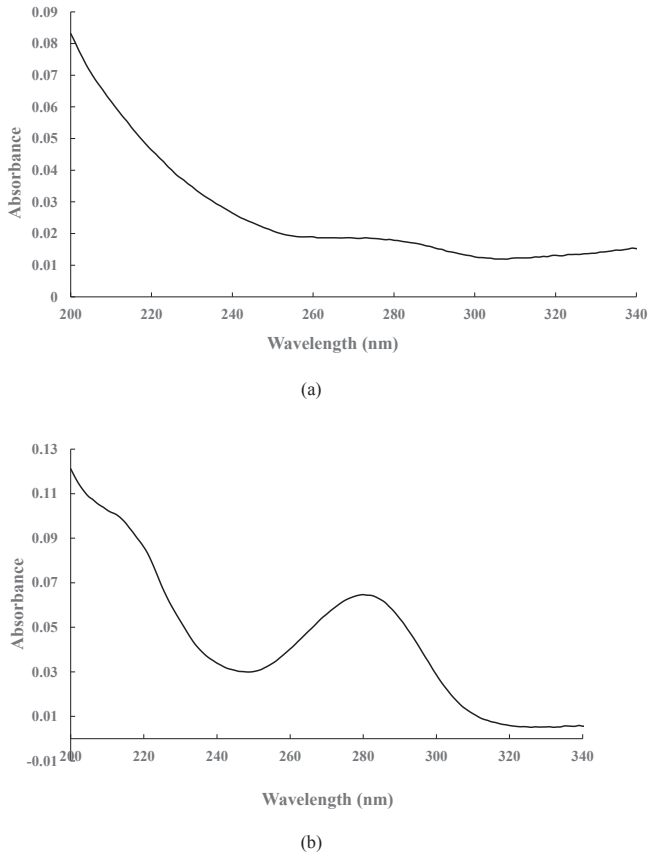

Fig. 6. A representative absorption spectrum of the DI siphoned off from (a) the immediate vicinity of the ghee cylinder (number of experiments; N: 8 independent measurements) (b) the top surface of a ghee pellet (number of experiments; N: 10 independent measurements).

fat in water. In the case of ghee, saturated fats make up almost 65% of its composition while unsaturated fats constitute 20–25% (Mehta, 2013). We observed a contact angle of 50-55° made by a water droplet atop of ghee (Fig. 5), implying a wetting surface that enabled spreading over a large area. Also, the fact that EZ continued to form against ghee cylinders that had been washed free of microspheres in successive trials, or even soaked overnight with deionized water, espoused the presence of a moisture-rich hydrophilic surface.

EZs have several interesting properties (Zheng et al., 2006). Apart from excluding particles and molecules, EZ water is more viscous, alkaline and denser than water (Hwang, Hong, Sharma, Pollack, & Bahng, 2018; Pollack, 2013) and ranges from several tens to several hundreds of micrometers in size. We observed extremely large EZs with ghee and coconut oil (Fig. 2). A similar finding (results not shown) was obtained with a home-made preparation of clarified ghee obtained from milk cream. Since ghee is clarified butter, we also explored formation of EZ with organic sweet cream butter that was melted and clarified to remove milk solids. The molten supernatant (clarified butter) was placed in a syringe and allowed to solidify (Org CB). As expected, EZ was formed (474 ± 71 μm) against the Org CB cylinder and was significantly larger than that formed against the Org B cylinder.

The kinetics of EZ formation against the fat cylinders (cold ghee, VCO, and lard) was similar to those observed with Nafion (Zheng et al., 2006). EZ built up steadily over the initial time-period, but this was followed by a slight diminution after 10 min (Fig. 2). Furthermore, it was almost two-fold greater when measured against a cold ghee surface compared to that formed against room-temperature ghee cylinder (Fig. 3). This difference bears similarity to our earlier observation (Pollack, 2013) where a tongue of heat-conducting material was inserted into a small chamber containing water and microspheres, and then cooled. Essentially, the cold surface of the ghee withdrew infrared energy (heat) from the adjacent water in the petri dish. In transitioning from bulk water to cold ghee, the energy passed through the exclusion zone, helping it to grow.

Brain Octane oil infused strips, cut either from nitrocellulose membrane or Whatman Grade 42, formed EZs that were at least 2–5 times larger than their respective controls, which contained DI water (Fig. 4). Thus, Brain oil may be a source of healthy fats.

While the EZ water samples from the vicinity of the ghee cylinder had a broad UV–VIS absorbance peak (Fig. 6a), spectra for samples taken from the top surface of the cold ghee pellet in water generated a sharper absorption peak between 270 and 280 nm (Fig. 6b). That peak is characteristic of EZ water (Pollack, 2013; Zheng et al., 2006). Similar findings on EZ absorbance have been reported with water samples after iterative contact with the standard Nafion® polymer membrane (Elia et al., 2013) and in supernatants obtained from QELBY® suspensions (Sharma, Toso, Kung, Bahng, & Pollack, 2017), strongly suggesting the presence of EZ water.

Thus, saturated fats, such as ghee and coconut oil, build large EZs and might well be considered healthy when observed in a holistic context. Similar conclusions are being drawn by researchers using biochemical and other analytical methodologies (Boateng, Ansong,

Owusu, & Steiner-Asiedu, 2016; Hosseini & Asgary, 2012; Lee et al., 2018; Mirzaei, Khazaei, Komaki, Amiri, & Jalili, 2018; Mohammadifard et al., 2010). In fact, the ability to form EZ may be an important criterion for selecting a healthy fat.

5. Conclusions

Large EZs (up to 800 μm) were formed against the nucleating surface of saturated fats such as ghee, coconut oil, and lard. With a formation time-course similar to that next to Nafion, the EZ formed against ghee had a characteristic absorbance signature near 270 nm. These findings provide additional support to our previously published results (Sharma et al., 2018) where we demonstrated that agents that promote health also build EZ.

CRediT authorship contribution statement

Abha Sharma: Conceptualization, Methodology, Investigation, Writing - original draft. **Gerald H. Pollack:** Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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