STUDENT LABORATORY WORKSHEET

EXPERIMENT A: NATURAL NANOMATERIALS

Student name:……………………

Date:…………………………………..

In this experiment you will study the relations between nanostructures and properties of natural nanomaterials. In this worksheet we provide you with some background information as well as instructions for preparing the experiments.

Aims:

- Be aware of the existence of natural nanomaterials (for example, gelatine and milk)
- Have indirect evidence of the presence of nanoparticles in natural nanomaterials through light interaction with colloids
- Understand the link between nanoscale structure and function in the observable world
- Understand how the reorganisation of molecules of a material, like milk, leads to different products (cheese, yogurt etc) that have distinct macroscopic properties (taste, smell, etc.)

MATERIALS:

- 1 hotplate
- 2 beakers 50 mL
- 1 beaker 200 mL
- 2 beakers 500 mL
- 0.5 g gelatine powder (Sigma-Aldrich product number G1890)
- 1 tablespoon
- 800 mL of skim milk
- 4 tablespoons of white vinegar

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- Thermometer
- 1 spatula
- Latex gloves
- Safety glasses
- Magnifying glass

This experiment has two parts:

Part A: Test natural nanomaterials to have indirect evidence of their nanostructure;

Part : Test natural nanomaterials to understand the relation between nanostructure and macroscopic properties.

DISCLAIMER: The experiments described in the following training kit use chemicals which need to be used accordingly to MSDS specifications and according to specific school safety rules. Personal protection must be used as indicated. As with all chemicals, use precautions. Solids should not be inhaled and contact with skin, eyes, or clothing should be avoided. Wash hands thoroughly after handling. Dispose as indicated. All experiments must be conducted in the presence of an educator trained for science teaching. All experiments will be carried out at your own risk. Aarhus University (iNANO) and the entire NANOYOU consortium assume no liability for damage or consequential losses sustained as a result of the carrying out of the experiments described.

General information - Natural nanomaterials

Many materials that **belong to the natural world** (animal and mineral) have properties which are the result of **inherent nanostructures**.

The interaction of light, water and other materials with these nanostructures gives the natural materials **remarkable properties which we can see**

Figure 1. Examples of natural nanomaterials. From top left corner clockwise: a butterfly, the foot of a gecko, nasturtium leaves, milk. (Images credit: Top left, Wiki Commons, Creative Commons Attribute ShareAlike 3; top right: A. Dhinojwala, University of Akron, NISE network, reprinted under NISE network terms and conditions; bottom, left: Wiki Commons, Creative Commons Attribute ShareAlike 3).

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with our eyes. We have **hundreds of examples of nanoscience before our eyes daily**, from geckos that walk upside down on a ceiling, apparently against gravity, to butterflies with iridescent colours, to fireflies that glow at night.

Part A: Test natural nanomaterials to have indirect evidence of their nanostructure

In this experiment you will learn a method that gives you indirect evidence of the presence of particles that are too small to see even with the help of an optical microscope.

The natural nanomaterials that you will analyze are **gelatine and milk**.

Gelatine

Gelatine is a tasteless solid substance, derived from the collagen inside animals' skin and bones. It is a well known substance used in many kitchens to prepare deserts. It is also used as a gelling agent in food products (cakes, etc.), in pharmaceuticals (e.g. gelatine capsules), in cosmetic products and in photography.
Gelatine is a **protein** produced from **collagen** found in the bones, connective tissues, organs and some intestines of **mammals** such as pigs. However gelatine from **fish** and algae is also becoming a common source.

Milk

Bovine milk contains minerals and a number of biomolecules, such as lipids and proteins, which are dispersed in water. As you certainly know**,** bovine milk is produced naturally within the cow, but usually undergoes several processes such as pasteurizing and homogenizing before reaching our table. These processes do not affect the nanostructure that we will observe in this experiment.

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The teacher will prepare a 10 mg/mL gelatine sample (by mixing each 0.5 mg of gelatine powder with 50 mL of cold water, and bringing it close to boil) .

You will now need the following materials:

A cool sample of gelatine,

A beaker of plain water

A beaker of milk

A beaker of diluted milk: Take 150 mL of distilled water in a beaker or glass, and add 1-2 droplets of milk (using a pipette). Mix well and let stand for a couple of minutes (you don't want air bubbles). The liquid will look pale grey.

Q1. Can you see any small particles by looking at the different samples? _________ Can you see any small particles by looking at the different samples with a magnifying glass?

____

Now shine a laser beam through the four samples, while holding a white page on the other side.

WARNING: never shine a laser beam near the eyes nor look straight into the beam!! You must wear **safety glasses** when doing this test.

Q2. Look from above and describe what you can see. Record how the laser beam behaves as it penetrates each sample.

Gelatine: ____

Water:____

Milk: ____

Diluted milk: ____

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Q3. Did all the samples behave the same way? Can you guess what disrupted the laser's path? (Notice that if you can see the laser's path, then something disrupted its path, otherwise its light wouldn't reach your eyes.)

____

____

Q4. So what happened here? Please fill in the missing words

Nanoscale particles are suspended in the sample, and when the laser light hits them, it changes its direction. This is seen as a path of light in the sample, as can be seen in the _______ sample and the ________sample. The laser forms a spot of scattered light in the sample if the particles are so dense that the light can't pass through, as seen in the _______ sample. In the _______the light passes through without hitting anything, and so we cannot see the laser light.

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A mixture containing nanoparticles

A ***colloid*** is a type of mixture in which one substance is dispersed evenly throughout another but **the particles of the dispersed substance are only suspended in the mixture**, they are not completely dissolved in it (unlike a *solution*). Generally speaking, a **colloid is composed of particles in the range of 5-1000 nm**. These particles cannot be seen by the naked eye, and most cannot be seen even using an optical microscope. They are small enough to be dispersed evenly and maintain a homogenous appearance, but large enough to **scatter light**. The particles in a colloid can be so well dispersed that they have the appearance of a solution (e.g. transparent). Therefore the laser test gives indirect evidence of the presence of nanoparticles in the colloid. **This is what happens in gelatine and milk.**

HOW IS GELATINE A COLLOID? Recent studies using a type of microscope called the **Atomic Force Microscope (AFM)**, which can detect structures on the nano scale, have shown that gelatine is indeed formed by numerous **nanostructures** that are evenly dispersed. For instance, an AFM analysis of gelatine extracted from catfish (*Ictalurus punctatus*) skin has revealed the presence of **pores with a diameter of about** 100 nm and **spherical nano-aggregates** with diameter around 260 nm. **The presence of these nanostructures proves that gelatine is a colloid (the suspended nanoparticles here are both the pores, which are essentially nano air bubbles, and the aggregates)**.

HOW IS MILK A COLLOID The amount of proteins in bovine milk is between 2.5 and 3.5%, depending on the animal breed, of which about 80% are **caseins** (the rest being whey or serum proteins). Caseins are naturally found in milk in the form of spheres of tangled proteins which are in the **range 50-300 nm** and are called **casein micelles**. The micelles contain the caseins combined with calcium, phosphate and a small amount of citrate. As such, **milk is a colloid, with these micelles as the suspended nanoparticles**. The presence of these micelles determines the **white colour of milk** due to their light scattering.

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Q5. Milk owes its properties to the existence of casein micelles, which are spherical nanostructures with a diameter of about 50-300 nm. Select from the images provided at the end of this worksheet the AFM image that you think corresponds to casein micelles (**take into account that 1000 nm = 1 μm**). Paste the image here.

Part B: Test natural nanomaterials to understand the relation between nanostructure and properties.

In this experiment you will treat milk with acid to disrupt its nanostructure in order to create a new product (different structure with different properties).

pH acid indicators are used to determine the pH level of common materials such as vinegar, lemon juice, battery acid and even yeast infections.

- A pH of 7 indicates neutral solutions
- A pH above 7 indicates alkaline solution
- A pH below 7 indicates acidic solution

You should wear safety glasses and gloves during the entire experiment.

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STEP 1

- Use the same beaker containing milk that you used in part 1. With the use of a pH paper (which checks for the level of acidity) record the acidity (pH) of skim milk (fill table provided on next page).

STEP 2

- Take a clean beaker and add another sample of 400 mL of cold milk. Place the beaker containing milk on a hotplate, turn the hotplate on and warm the milk to about 60°C. If a hotplate is not available, the milk can be heated using water that has been boiled separately and poured into a water bath container. Your teacher will instruct you on how to warm up the milk. When the milk is warm, check the acidity with a new piece of pH paper, and record the result in the table below. Observe the milk and stir it with a spoon, and record the way it looks, smells and feels in the table on the next page.

STEP 3

- To the cool milk add 2 tablespoons of white vinegar (an acidic solution) and stir well as you do so. What happens (look, smell, feel)? Record your observations in the table provided. Record the **pH of the liquid** (in the table on the next page). **Safety note**: You should not taste aggregated acid-milk!

STEP 4

- Repeat the test, adding the same amount of vinegar (2 tablespoons) but to the **warm milk (60°C)**. Stir, and let it sit for a minute or two. Remember that the beaker will be hot! What happens? (Remember, do not taste!) Record your observations in the table provided. Record the **pH of the liquid** (fill the table provided below):

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Step:	**Sample**	**Temperature**	**pH**	**state of the milk (appearance, colour, odour, viscosity)**
TEST 1	**Milk**	cold		*Smooth white liquid, with macroscopic aggregates*
TEST 2	Milk	60^0C		
Test 3	Milk + **2 tablespoons Vinegar**	cold		
Test 4	Milk + **2 tablespoons Vinegar**	60^0C		

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Q6. Was there a clear difference in the milk state and pH ***that took place just by heating the milk, without adding vinegar*** (Test 1 & 2)? If yes, describe the difference.

____

____

Q7. Was there a clear difference in the milk state and pH ***when adding vinegar*** to warm milk or cold milk (Test 3 & 4)? If yes, describe the difference.

____

____

Q8. Based on the results of the test of adding vinegar to warm milk or to cold milk, do you think the reaction that takes place is based only on the acidity (pH)? Explain.

____

____

IS IT JUST ACIDITY, OR IS IT SOMETHING ELSE?

First, let's understand what happens in the casein micelles at the molecular level:

The micelles are composed of a tangle of casein molecules, but scientists have found that there is more than one kind of casein molecule (see figure 2). All types of casein molecules are long and thin, and all possess a hydrophobic region (i.e. repelled by water) and a region which has an effective electrostatic charge. The different casein types differ in the placing of these regions. As a result of these regions, all caseins, except for one, called k-casein, possess the ability to bind to a calcium ion, Ca^{2+}

Now, let's understand how these molecules link together:

There can be two types of linkages between caseins in the casein micelles:

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- The first linkage is **hydrophobic**, where two or more hydrophobic regions from different molecules (α-caseins and β caseins) form a cluster (similar to oil forming droplets in water). These are indicated as a **rectangular bar** in **Figure 2**. These links are sensitive to temperature.

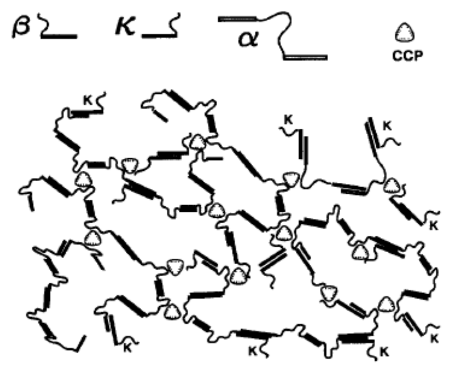

Figure 2. Dual bonding model in casein micelles, with a, b and k-casein depicted as indicated. Reprinted from: Horne D.S., Inter. Dairy Journal (1998), 8 (3), 171-177, with permission from Elsevier.

- The second linkage is of **hydrophilic ("water loving") charged regions** which bind to small nanocrystals termed calcium phosphate nanoclusters or colloidal calcium phosphate nanoclusters (indicated as CCP in Figure 2). These links are sensitive to acidity (pH).

Now we can understand how the micelles are built:

The k-caseins cannot link with the calcium nanoclusters; therefore they interact with the other molecules only through hydrophobic interactions, and because of this the micelles cannot grow further beyond the k-caseins, which therefore act as an outer layer in the micelle. The **role of k-caseins is to stabilize the casein micelles**, preventing excessive growth and micellar aggregation (where the micelles stick to each other), which would otherwise lead to precipitation (the micelles sinking to the bottom).

So we can see that all these different repelling, sticking and binding properties cause the molecules to stick together, in a self-assembly process, to form casein micelles that contain inorganic calcium phosphate. This ensures that the calcium will be dispersed evenly within the milk (as the micelles are dispersed within the milk), and that the calf will receive both proteins and minerals with every gulp.

To summarize, we have seen that the structure of milk, on the level of the micelles, depends both on the temperature and the acidity (pH) of the milk.

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MILK PROCESSING

Maintenance of micellar integrity is a balancing act and numerous methods exist to disrupt this balance. These methods are widely used in the dairy industry to make cheese and fermented products like yogurt, etc. Yogurt is a fermented milk product obtained by the controlled growth of specific microorganisms, mainly bacteria that convert lactose (milk sugar) into lactic acid. By lowering the pH of milk, its consistency and taste change. In cheese making, enzymes are used to induce the aggregation and precipitation of caseins. In all milk-processing methods **the molecular organisation of caseins is altered**, which leads to thickening, precipitation and other effects. The appearance, taste and other "macro" properties of milk are deeply connected to its supra-molecular (nano) structure.

The main processes that milk undergoes are:

- **Decreasing pH to 4.6** by adding an acid induces dissociation of the casein micelles. The reason for this is that calcium micelles only exist because of the presence of calcium phosphate; therefore its dissolution necessarily causes changes in the stability of the micelles. If an acid (a proton-donor) is added to milk, the charged regions are no longer capable of interacting electrostatically with calcium phosphate nanoclusters, and these are released from the micelles. It should be noted that this does not necessarily cause the caseins to dissociate from the micelles. At temperatures below 25°C, increasing dissociation occurs, but otherwise the caseins remain in the micelles. The reason lies in the fact that the stability of casein micelles is not exclusively connected to electrostatic interaction, but also to hydrophobic interactions. The latter are extremely temperature-dependent: hydrophobic interactions are stronger at higher temperatures. **Therefore hydrophobic interactions maintain the stability of casein micelles in cold milk even when its pH has been lowered.** On the other hand, **if acidification occurs after milk has been warmed (to about 60°C), micelles are dissociated (calcium phosphate is released from the micelle) and will**

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aggregate due to increased electrostatic forces *and* increased hydrophobic interaction (just as mixed oil and water eventually separate).

- **Attack by chymosin leads to micelle precipitation and formation of a curd**. This process is employed in **cheese manufacturing**. Chymosin is an enzyme which is the active principle in rennet, the extract of calf's stomach used in cheese making. Chymosin specifically attacks a single bond in the k-casein. As mentioned before, the presence of k-caseins is fundamental for the overall stability of the casein micelle; therefore its disruption causes the micelle to lose stability, aggregate and eventually form a curd.

- The controlled addition of **lactic acid bacteria** (bacteria that produce lactic acid, such as Lactobacillus, Lactococcus, and Leuconostoc) under specific processing conditions leads to fermented milk products such as yogurt. This process differs from simple acidification as the milk is heat-treated and whey proteins are also incorporated. The coagulation is induced by the acidification but does not lead to the formation of a curd but to a product which is more viscous than plain milk.

Q9. Which of the processes explained above did you perform on the milk in this experiment?

Q10. Use the information you have just read to explain the difference between the result of the cold milk experiment and the warm milk experiment.

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Q11. Did the protein molecules change to new ones in the experiment? If not, what did change?

Q12. Using the information you have just read, can you describe in your own words what happens to the micelles in the process that result

WHAT CAN THIS EXPERIMENT TEACH ABOUT NANOTECHNOLOGY?

- Nanostructure means physical properties (colour, odour, viscosity etc.): materials in the "real" natural world, like milk, appear as they do because of fine nanostructures they posses. Milk is white because it contains colloidal nanoparticles (micelles). If we alter the structure of these micelles, we alter some "macro" properties of milk like **colour, odour and viscosity.**

- Nanostructure means function: natural materials have very specific functions which are dictated by the fine supra-organisation of their molecules (nanostructures). If we alter these, we can obtain a material with a new function. In cheese production, altering the casein micelles through specific processes (e.g., chymosin treatment or lactic acid bacteria fermentation) leads to different products (cheese, yogurt etc.). **This is exactly one of the key concepts in nanotechnologies**: to engineer new materials with new functions from the manipulation of their molecular organisation.

Notice that in all the processes we have performed in this experiment, we didn't change the molecules. We only changed the links between the molecules, and the supra-molecule structure on the nano-scale.

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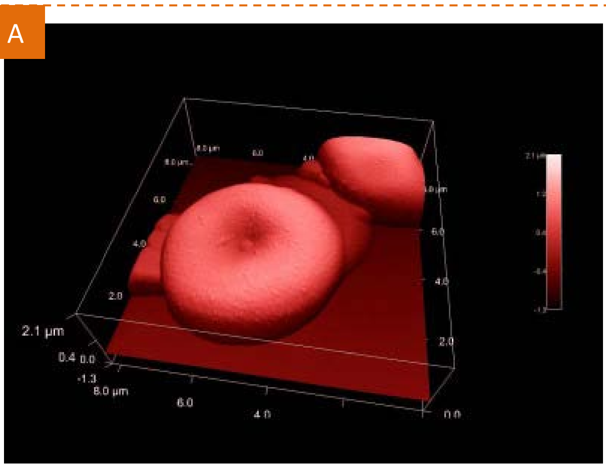
Images for question Q5: Cut out the image you think is the right one and paste it in the space provided in Q4.

A



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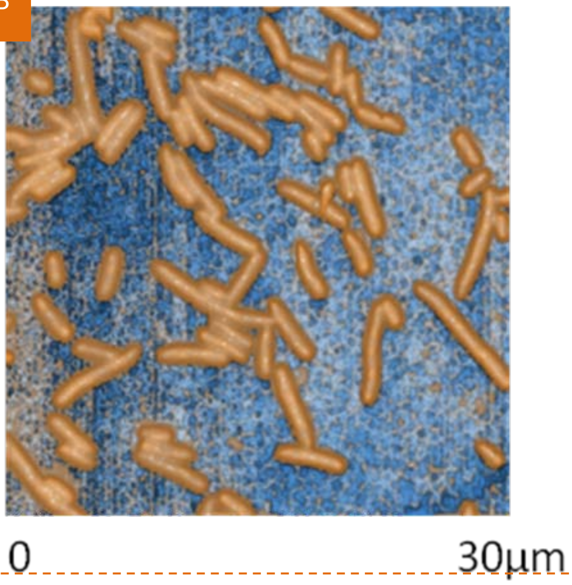

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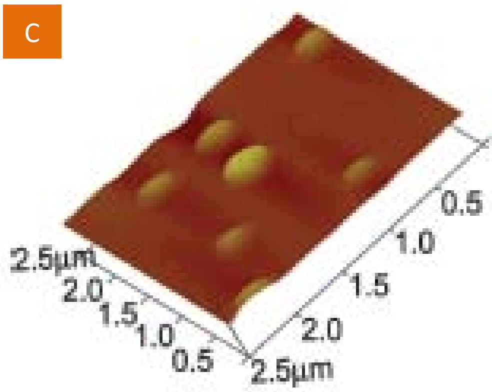

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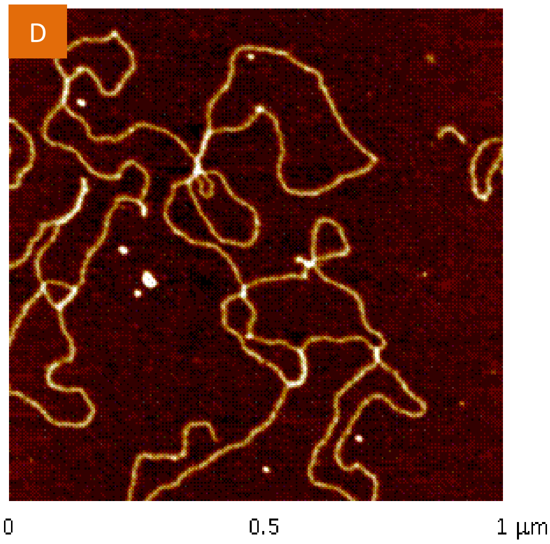

IMAGES CREDIT: Images were kindly provided by: (A) Christoph Gösselsberger, image is "taken from O. Hekele, C.G. Goesselsbergerand I.C. Gebeshuber: Nanodiagnostics performed on human red blood cells with atomic force microscopy"; (B): AFM image of bacteria cells collected at iNANO, Aarhus University, image courtesy of Park Systems XE-Bio; (C) Reprinted with permission from: Shekar et al., PNAS (May 23, 2006), vol. 103, no. 21, pp 8000-8005. Copyright 2006 National Academy of Sciences, U.S.A. (D): AFM image of DNA double strands on mica surface, image courtesy of JPK Instruments AG. No further use of these images is allowed without written approval of copyright holders.

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