STUDENT BACKGROUND FOR EXPERIMENT A:

NATURAL NANOMATERIALS

We have many **natural nanomaterials around us** and in this experiment you will learn that two very common materials, milk and gelatine, are indeed two of these. The properties of these materials are directly connected to their molecular supra-organisation, which includes nanostructures.

In this experiment you will:

1. Prepare gelatine and test it with a laser pen to confirm its colloidal nature.
2. Confirm that milk is a colloid and treat it with acid to induce its aggregation. This experiment will give you practical evidence of the link between structure and function, and how manipulation of the molecular organisation of a material, like milk, leads to materials with different colour, odour and taste!

BACKGROUND 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 with our eyes**. These nanostructures arise from the supra-molecular organisation of the material: tens to hundreds of molecules which are arranged into shapes and forms in the nanoscale range. 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.

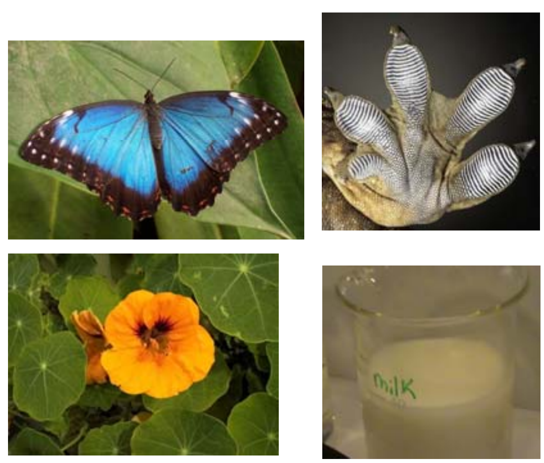

Figure 1. Examples of natural nanomaterials. From top left corner clockwise: a butterfly, the foot of a gecko, nasturtium leaves, milk. (Images credit: Top left, Wiki Commons, Creative Commons Attribute ShareAlike 3; top right: A. Dhinojwala, University of Akron, NISE network, reprinted under NISE network terms and conditions; bottom, left: Wiki Commons, Creative Commons Attribute ShareAlike 3; bottom right: iNANO, University of Aarhus, Creative Commons Attribution ShareAlike 3.0)

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In Nature we encounter some outstanding solutions to complex problems in the form of fine nanostructures with which precise functions are associated.
In this experiment the natural nanomaterials that you will analyse are **gelatine and milk**. Both are types of **colloids**. A *colloid* is a type of chemical 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*). This occurs because the particles in a colloid are larger than in a solution. Generally speaking, a **colloid is composed of particles in the range of 10-300nm**. 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).

A simple way to test if a mixture is a solution or a colloid is to **shine a laser beam though the mixture**: the light will be scattered only by the colloid. **WARNING**: never shine a laser beam near the eyes nor look straight into the beam!

In this experiment you will realise that **without these nanostructures common materials like milk lose their appearance and function**.

Gelatine

Gelatine is a tasteless solid substance, derived from the collagen inside animals' skin and bones. It is 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 by **partial hydrolysis of collagen** found in the bones, connective tissues, organs and some intestines of **mammals** such as pigs. However gelatine from **fish** is also becoming a common source.

Gelatine is often found in the form of powder. When mixed with water it forms a solution of high viscosity, which sets to a gel on cooling, forming a **colloid gel**. Gelatine gel melts to a liquid when heated and solidifies when cooled again. Therefore its existence as a gel is limited to a **specific temperature window**.

HOW IS IT "NANO"? Recent studies with the **Atomic Force Microscope (AFM)** have shown that gelatine is indeed formed by numerous **nanostructures** which have various shapes depending on the type of gelatine analysed. For instance an AFM analysis of gelatine extracted from catfish (*Ictalurus punctatus*) skin has revealed the presence of **annular pores** with diameters averaging 118 nm and **spherical nano-**

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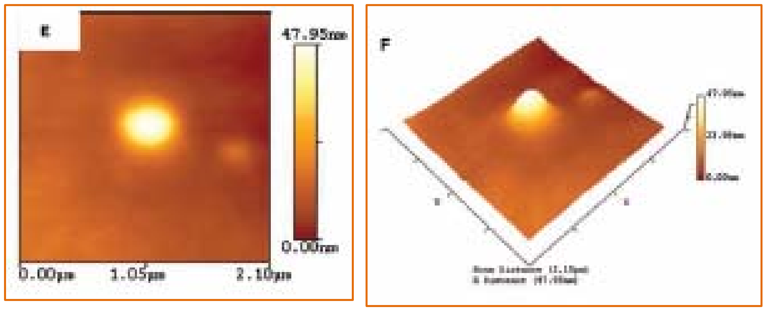
aggregates with diameter around 260 nm. **The presence of these nanostructures proves that gelatine is a colloid and explains its light-scattering behaviour**.

Figure 2. AFM images of gelatine extracted from catfish revealing the presence of spherical nanostructures. (Image credit: reprinted by permission of Wiley-Blackwell Publishing Ltd from Yang et al., Journal of Food Science (2006), 72(8), pp c430-c440, copyright (2006) Wiley-Blackwell Publishing Ltd.).



Milk

Bovine milk contains a number of biomolecules, such as lipids and proteins, which are dispersed in water. The amount of proteins 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). Four proteins comprise the casein group and are: α_{s1}-casein, α_{s2}-casein, β-casein and k-casein. The caseins are characterised by the fact that they are **phosphoroproteins** that precipitate at pH 4.6 (isoelectric point), at which pH whey proteins remain soluble. Another property of caseins is their existence as **casein micelles** which are in the **range 50-300 nm**. Micelles contain the caseins combined with calcium, phosphate and small amount of citrate. As such, **milk is a colloid** (a mixture of nanoparticles evenly dispersed but only suspended in a liquid medium). The presence of these micelles determines the **white colour of milk** due to their light scattering.

FROM STRUCTURE TO FUNCTION

The fine molecular self-organisation of proteins and minerals in milk is fundamental for realising its natural function of transporting calcium from the mother to the offspring. Numerous studies have revealed that this organisation results in nanostructures which have precise functions (**casein micelles**). In the next section we will describe how this organisation is determined by electrostatic interactions but also hydrophobic interactions between the proteins that constitute milk and some minerals that are associated with the proteins. Without this fine organisation calcium would not be "trapped" inside the milk micelles and the biological function of milk would not be realised.

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MILK PROCESSING

Processing of milk with various treatments is widely used in the dairy industry. For instance 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. As will be discussed in the next section, 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.

In this experiment you will use vinegar (a source of acid) and heat to alter the properties of milk.

CASEINS

Casein in milk (which has a pH near to neutrality, about 6.7) is negatively charged (I.P. is 4.6). All caseins, except k-casein, possess the ability to **bind to Ca^{2+} which occurs mainly through their phosphate residues**. The binding of Ca^{2+} is fundamental for milk to fulfil its function, that is, to transport calcium (and other nutrients) from the mother to the offspring. Each casein is composed of a different peptide sequence and therefore has a different secondary and tertiary structure.

CASEIN MICELLES: STRUCTURE AND FUNCTION

Caseins in milk are believed to exist as **casein micelles** in the range **50-300 nm**. Micelles contain the **caseins combined with calcium, phosphate and small amount of citrate.** The structure of casein micelles (like that of caseins itself) is still a matter of debate and intense research. Since all caseins possess a hydrophobic region and a polar region, it is believed that hydrophobic interactions as well as electrostatic interactions play a role in the self-association of caseins to form casein micelles. Casein micelles differ from the polymers of the individual caseins in one crucial aspect: they contain inorganic calcium phosphate, which exists in the form of small microcrystalline inclusions termed **calcium nanoclusters**. The fact that the stability of casein micelles is not due only to electrostatic interaction has been demonstrated by the fact that **casein micelles can be dissociated using urea**, which is an agent that does not rupture the calcium phosphate linkages.

Two types of linkages between caseins in the casein micelles have been postulated:

- The first linkage is **hydrophobic**, where two or more hydrophobic regions from different molecules (α-caseins and β caseins) form a bonded cluster. These are indicated as a **rectangular bar** in **Figure 3**.

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- The second linkage is of **hydrophilic charged regions** containing phosphoserine clusters which bind to colloidal calcium phosphate nanoclusters (indicated as CCP in Figure 3).

The k-caseins do not have the phospheroserine group to link with the calcium nanoclusters; therefore their association is only possible through hydrophobic interactions. Also, 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 stabilise the casein micelles**, preventing excessive growth and micellar aggregation which would otherwise lead to precipitation.

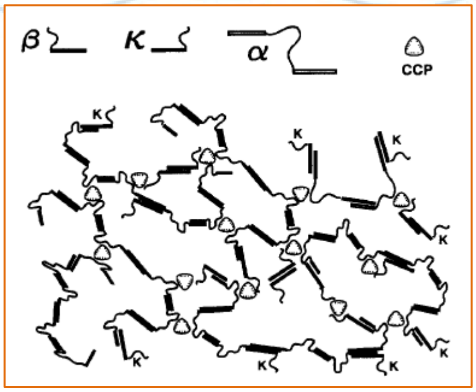

Figure 3. Dual bonding model in casein micelles, with a, b and k-casein depicted as indicated. Reprinted from: Horne D.S., Inter. Dairy Journal (1998), 8 (3), 171-177, with permission from Elsevier.

CASEIN MICELLE DISSOCIATION AND AGGREGATION

As outlined above, casein micelles are believed to have an intricate structure which is an interplay of hydrophobic and electrostatic interactions. 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.

- **increasing pH (to about 8)** leads to casein **micelle dissociation**, and the effect is that heated milk becomes more translucent. The reason for this is that increasing the pH from the natural neutrality converts the phosphoseryl groups from singly to doubly charged units which are no longer capable of linking the calcium phosphate nanoclusters. The increased negative charge of the micelles induces electrostatic repulsion, and the micelles dissociate.

- **decreasing pH to the isoelectric point (4.6)** 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 phosophoseryl and carboxyl groups in the proteins become protonated, so they 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. This effect will be tested in this experiment by adding vinegar (a source of acid) to cold milk. The reason lies in the fact that the stability of casein micelles is not

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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 to the isoelectric point.** 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 aggregate due to increased electrostatic forces *and* increased hydrophobic interaction.** This will be tested in this exercise by adding vinegar to warm or cold milk.

- **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.

WHAT CAN THIS EXPERIMENT TEACH ABOUT NANOTECHNOLOGY? Through this exercise you will learn two fundamental concepts:

- **Structure means physical properties (colour, odour 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** and **odour.**

- Structure 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 the concept of nanotechnologies**: to engineer new materials with new functions from the manipulation of their molecular organisation.

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