Nanosecond pulsed Atmospheric Pressure Plasma
for Fabrication of Hybrid Nanostructures and
Decontamination of Milk

by

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Master of Technology (M.Tech)

Submitted in fulfilment of the requirements for the degree of

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Deakin University
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I am the author of the thesis entitled

**Nanosecond pulsed Atmospheric Pressure Plasma for Fabrication of Hybrid Nanostructures and Decontamination of Milk**

submitted for the degree of Doctor of Philosophy

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List of Publications

Journals


Conference proceedings

Presented an **oral presentation** on ‘Plasma fabrication of hybrid nanostructures for biomedical applications’ in the 55th Gaseous Electronic Conference at Princeton, New Jersey, USA, 2013.

Presented a **poster presentation** on ‘Sterilization of milk using nanosecond pulsed atmospheric pressure plasma’ in the 22nd International Symposium on Plasma Chemistry at University of Antwerp, Belgium, 2015.

Presenting an **oral presentation** on ‘Investigation of bacterial safety and nutritional quality in plasma treated cow’s milk’ in the 19th Gaseous Electronic meeting at Deakin University, Geelong Waterfront Campus, Australia, 2016.

**Other achievements**

Won the **Three Minute Thesis (3MT) competition** at Institute for Frontier Materials and participated as a finalist at Burwood Campus, Deakin University, 2014.

The research based on ‘Decontamination of Cow milk using nanosecond pulsed atmospheric pressure plasma’ was **published in Australian Broadcasting (ABC) News**, 2015.

Won the **student bursary award**, to present ‘Sterilization of milk using nanosecond pulsed atmospheric pressure plasma’ in the 22nd International Symposium on Plasma Chemistry at University of Antwerp, Belgium, 2015.

Won the **IFM travel award**, to establish a potential collaboration between IFM, Deakin University and Ruhr University Bochum, Germany, 2015.
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Abstract

The potential of using nanosecond pulsed atmospheric pressure plasma in contact with liquid has been investigated 1) to fabricate an improved hybrid nanostructures, and 2) to decontaminate milk with extended shelf life whilst maintaining nutrition. The electrical discharges in contact with liquid provide a combination of unique physical and chemical environment, enabling to achieve these complex processes in a simple and efficient way.

Two experimental set ups were used to generate plasma, (1) plasma above the de-ionized water surface (set up I) and (2) plasma in gas bubbles immersed in milk (set up II).

In the fabrication of hybrid nanostructures, using set up I, argon plasma treatment shortened the length and functionalized boron nitride nanotubes (BNNTs) in one step. Gold nanoparticles (GNPs) were successively assembled onto the functionalized short nanotubes. The reactive species from the plasma along with the nanosecond pulsed electric field seem to play a role in the fabrication of BNNT-GNP hybrids. The potential for targeted drug delivery was tested using doxorubicin-loaded plasma treated nanotubes which were effective at killing ~99% of prostate cancer cells.

In the treatment of raw milk, using set up II, argon plasma treatment at 90 and 120 seconds effectively reduced the bacterial load and significantly increased the shelf life to at least six weeks compared to pasteurization, which showed reduced bacterial load but started to deteriorate after two weeks. β-lactoglobulin (β-LG) was significantly reduced in 90 s plasma treated milk compared to raw and pasteurized milk. β-LG, α-lactalbumin and immunoglobulins were significantly reduced in 120 seconds plasma treated milk compared to raw and pasteurized milk. Lactoferrin was significantly reduced in pasteurized milk compared to 90 and 120s plasma treated milk and raw milk. Casein proteins were significantly increased in 90 and 120 s plasma treated milk whereas it was significantly reduced in pasteurized milk compared to raw
milk. The total lipids, saturated fatty acids and carbohydrates were significantly reduced in pasteurized milk compared to raw and plasma treated milk (both 90 and 120 s). Plasma treated milk had significantly increased miRNA concentration compared to pasteurized milk. Raw milk and 90 s plasma treated milk did not effect the viability of human fetal intestinal cells whereas pasteurized and 120 s plasma treated milk significantly reduced viability compared to untreated cells. Annexin V staining showed this loss of viability was due to apoptosis.

These results demonstrated that plasma treatment as a superior method to decontaminate and increase shelf life of milk compared to pasteurization. This plasma approach could be potentially extended to treat human breast milk and used in other dairy applications for manufacturing of cheese, butter and yoghurt to retain nutrition.
In this PhD thesis, the potential of nanosecond pulsed atmospheric pressure plasma in contact with liquid has been investigated to fabricate hybrid nanostructure without the use of toxic chemicals and to decontaminate bovine milk, prolong shelf life without damaging the nutrition. In Chapter 1, a comprehensive literature review is presented and focused on plasma in contact with liquids 1) to fabricate hybrid nanostructure for biological applications and 2) to decontaminate and extend shelf life of bovine milk whilst maintaining nutrition. The research challenges are identified, summarized and a strategy to address the research challenges have been discussed.

The chapter 2 presents the investigation of argon plasma to shorten and functionalize BNNTs and assemble GNPs onto them using set up I, i.e. plasma in gas phase above the liquid surface. The pH, conductivity and reactive species in plasma treated water were measured to understand the plasma mediated fabrication of hybrid nanostructure. The drug loading, delivery and anti-cancer potential of plasma treated BNNTs were studied in prostate cancer cells.

In Chapter 3, a prelude has been given to summarize the key challenges faced during the generation of plasma in gas phase above the liquid surface and the development of gas bubble-in-liquid method (set up II), and the research motivation behind in investigating the potential of set up II to decontaminate milk. The plasma was generated in bubbles immersed in water using set up II to optimize the plasma conditions before experimenting with milk. The plasma frequency and treatment time in water was varied to study the changes in pH, temperature and hydrogen peroxide. After optimization, plasma was generated in milk using set up II to investigate its ability to kill bacteria and increase shelf life. The results of plasma treated milk have been compared with commercially pasteurized milk and raw milk. DNA sequencing was performed to analyze the bacteria survived after plasma treatment.
In Chapter 4, the effects of argon plasma treatment on proteins, lipids, carbohydrates and miRNA present in bovine milk compared to commercially pasteurized milk and raw milk is presented. The total protein, lipid and carbohydrate measurements in milk have been presented. The changes in casein and whey proteins were studied using methods that include high performance liquid chromatography (HPLC), Western Blot and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The changes in fatty acids, total and micro RNA were studied using gas chromatography (GC), NanoDrop spectrophotometer and Bioanalyzer. The cytotoxic and apoptotic potential of plasma and pasteurized milk have been studied in human intestinal cells. The main contents of this chapter are abstract, introduction, detailed experimental section, results and discussion and conclusion.

The chapter 5 presents the general discussions of this study and potential future works.
Chapter 1 Background

1.1 Plasma Introduction

Although the history of plasma dating back to eighteenth century, Sir William Crookes (1832-1919) was the one who correctly identified “radiating matter” as “the fourth state of matter” in discharge tubes.\(^1\) Later Irving Langmuir, observed oscillations in an ionized gas through the measurements of its electron density and mass and then termed it as ‘plasma oscillations’.\(^2\) The supply of sufficient energy to gas, breaks the bond between the atoms and ionizes the atoms to create ions and electrons. The interactions between these electrically charged particles and the neutral gas, ionizes the significant number of atoms present in the gas. This results in the electrically neutral medium composed of equal positive and negative charged particles, free radicals, ions and reactive species, which constitutes plasma.\(^3\)

Generally, plasmas are distinguished as equilibrium and non-equilibrium plasmas. In equilibrium plasma, the temperature of the electron (\(T_e\)) and ion (\(T_i\)) are equivalent to the temperature of gas (\(T_g\)). The collision process is generally high in equilibrium plasma, which govern the transitions and chemical reactions between the species. The electrons in the plasma, loses energy favourably toward ions to let other particles reach thermal equilibrium. These type of plasmas have higher ionization percentages (~100%) and average temperatures ranges between 10\(^3\) and 10\(^4\) K (called thermal plasmas). In contrast to equilibrium plasma, the electron temperature is greater than the gas temperature (\(T_e>>T_g, T_i\)) in non-equilibrium plasma. In this case, the electrons possess high temperature that ranges from 10\(^4\) and 10\(^5\) K, whereas the ions and gas temperature are at ambient temperature. These are called as non-thermal plasmas and have very low percentages of ionization.

The application of energy in the form of heat, electrical or electromagnetic radiation to a gas that is filled in between two electrodes generates plasma. The properties of plasma changes
with respect to its electron density and temperature. Plasmas can also be classified according to the type of input energy and the transfer of energy to the plasma (Figure 1.1).\textsuperscript{4}

![Figure 1.1. Classification of plasmas using electron density and temperature.\textsuperscript{4, 5}](image)

The plasmas can be generated at low or atmospheric pressure. Atmospheric pressure plasmas are extensively studied in both gas and liquid phases for various applications.\textsuperscript{5} This thesis mainly focuses on plasma in and in contact with liquids. Depending upon the mode of plasma generation, electrode configurations and power sources, the electrical discharges can be categorized as follows:

1) Discharges directly in liquids (in which both the electrodes are immersed)
2) Discharges in gas phase when two or one electrode above the liquid surface
3) Discharges in bubbles that are externally introduced into liquids

**1.2 Discharges directly in liquids**
The plasmas that can be directly generated in liquids are highly dynamic and transient. Earlier, the electrical breakdown in liquid was considered based on either electron multiplication theory or bubble mechanism theory. Later, few researchers explained that the application of high voltage in liquid leads to the formation of discharge through the pre-existing bubbles or formed bubbles in liquid, low density regions and joule heating. However, till date, there is no single theory that completely addresses all the aspects of electrical breakdown in liquid. Liquids are denser and have a short mean free path of electrons which demands higher electric strength in the order of MV/cm to breakdown, compared to gases (kV/cm). Moreover, the presence of impurities could also play a significant role in the breakdown process. These factors make it challenging to understand the plasma induced chemistry in liquids. Pulsed corona and arc or spark discharges in liquids were commonly reported discharge patterns. The power sources applied to generate direct discharge in liquid can be direct current (DC), alternating current (AC), high frequency (HF) or microwave (MW). Tendero et al. have documented the advantages and disadvantages of these power resources in generating plasmas. The electrode configurations used for these types of discharges are pin-plate electrode, pin-pin and diaphragm or capillary discharge. An example of direct discharge in liquid using pin-plate electrode configuration is shown in Figure 1.2. Based on the type of discharges, the intense physical processes such as UV, shock waves, cavitation and active chemical species like hydroxyl radicals (OH\(^-\)), oxygen (O\(_2\)), ozone (O\(_3\)), hydrogen (H\(_2\)) and hydrogen peroxide (H\(_2\)O\(_2\)) are produced. The electrical discharges directly in liquids have been employed in applications like removal of organic compounds, micro-organisms in water and breaking kidney stones. The higher electric field requirements and the erosion of electrodes during operation are the main bottlenecks of direct discharge in liquids.
1.3 Discharges in gas phase above the liquid surface

Unlike the direct discharge in liquids, the discharges can be generated in the gas phase while keeping one or two electrode above the liquid surface. The discharges in gas phase have been studied extensively by researchers.\textsuperscript{21,22} Plasma jets, corona and dielectric barrier discharges are the common types of plasma that have been studied so far.\textsuperscript{23-25} An example of discharge in gas phase above the liquid surface using pin-plate electrode configuration is shown in Figure 1.3. The gas mixture, electrode material, field polarity and the properties of liquids such as pH, conductivity and temperature could play an important role in determining the composition or properties of plasma.\textsuperscript{11,17,26} Depending upon the feed gas, input power and type of plasma, the reactive species like OH, O$_3$, H$_2$O$_2$, nitrite (NO$_2$), peroxynitrite (ONOO$^-$) and nitrate (NO$_3$) can be formed.\textsuperscript{27-29} The gas phase discharges have been widely used for applications such as removal of organic compounds, micro-organisms in liquid and solid surface and biomedicine.\textsuperscript{30-33} Although the gas phase discharges were quite easy to operate and less complex in understanding the interaction between the reactive species, the difficulty in the selectivity of gas phase species, interference from atmospheric air and formation of unwanted by-products hindering further developments of this approach.\textsuperscript{34,35}
1.4 Discharges in bubbles that are externally introduced into liquids

As mentioned earlier that the direct discharge in liquid requires higher electric field, results in faster electrode erosion and thereby reduces the life time of electrodes, whereas the discharge in gas phase facing issues of less selectivity in reactive species, interference from atmospheric air and formation of unwanted by-products. To overcome the afore-mentioned challenges, several researchers developed the concept of introducing gas into liquids to produce plasma in bubbles. The discharge in bubbles that are externally introduced into liquids can be two types 1) using capillary tube and 2) using hollow needle (in which needle acts as one electrode). An example of the above two types are shown in Figure 1.4. This approach needs lesser electrical strength to generate discharge in bubbles that are externally injected into liquids compared to the electrical strength requirement for liquids. The other advantages in this method includes high surface to volume ratio and clearly defined bubble boundaries i.e, plasma-liquid interface which all provides the opportunity to manoeuvre the chemical reactions between species and liquid with chosen feed gas. It was reported that the discharge pattern inside the bubble could be glow to spark or streamer to spark or develop in the form of streamers according to the power sources. In this case, the gas temperatures, electron densities and physical processes mainly depend on the composition of gas in the bubble (and its size), conductivity of the liquid and input power. These factors affect the local chemistry
through decomposition and charged species acceleration induced by electric field. It was found that the formation of discharge and reactive species mainly occur at the bubble (gas) and gas-liquid interface. Plasma generation in bubbles, immersed in liquids have been applied to synthesize nanomaterials, produce synthetic fuel, and improve plants growth.

Figure 1.4. Schematic of discharge in bubbles that are externally a) introduced into liquids using capillary tubes and b) introduced into liquids using a hollow needle, which acts as one electrode while plate acts as another electrode.

1.5 Advantages of plasma technology compared to conventional methods

The chemical methods such as chlorination, ozonation and hydrogen peroxide and physical methods such as UV radiation, and ultrasound have been studied as separate or in combination and reported elsewhere for water treatment applications. Yang et al. have discussed the advantages of plasma in and in contact with liquids compared to conventional technologies like chlorination, use of in-line filters, pulsed electric field, UV radiation and ozonation in water treatment and applications.

Plasma in and in contact with liquids is an exciting new approach to initiate both chemical and physical effects in liquid and has limitless potential to act in applications such as fabrication of nanomaterials, decontamination, plasma medicine and agriculture. It has been reported...
that the formation of chemical species during plasma treatment mainly depends upon the type of gas used. The chemical reactive species such as OH\(^-\), H\(_2\)O\(_2\), H\(_2\), O\(_2\), O\(_3\), atomic oxygen (O\(_\cdot\)) and superoxide anion (O\(_2\cdot\)) were produced while using argon and oxygen gases whereas the use of air produced reactive species like NO\(_2\), ONOO\(^-\) and NO\(_3\).\(^{17, 27-29, 34}\) Plasma treatment includes the physical processes such as electric field, ultraviolet (UV) radiation, shock waves and cavitation which are based on the intensity of the input power and the conductivity of liquid.\(^{17}\) Compared to conventional methods, plasma requires lower energies to deliver the afore-mentioned physical and chemical process in a single step to effectively remove the organic pollutants and micro-organisms in liquids.\(^{32, 53, 59-62}\) This could make plasma technology as a potential and viable alternative in achieving the applications with higher efficiency compared to the individual physical and chemical counterparts.

In this thesis, the plasma was produced in contact with the liquid using two set ups 1) in gas phase above the liquid surface (set up I, Figure 1.7) to fabricate hybrid nanostructure and 2) in bubbles that are externally introduced in milk (set up II, Figure 1.8) to decontaminate and extend shelf life while maintaining nutrition. A comprehensive literature review for the applications 1) and 2) have been discussed in the following context.

**1.6 Fabrication of boron nitride nanotube-gold nanoparticle hybrids**

The use of toxic chemicals, longer preparation time and damage to structure of materials has constantly demanded alternative methods to synthesize nanomaterials. In case of hybrid nanomaterials fabrication, the above challenges could be even worse. Here, plasma was produced in gas phase above the liquid surface to fabricate BNNT-GNP hybrid nanostructure. The choice of material (nanotubes and nanoparticles), their properties, synthesis and functionalization methods, applications, the research challenges and our approach to addresses the research challenges have been discussed in detail in the following sections.
1.6.1 Hybrid nanostructure

Hybrid nanostructures possess the advantage of delivering multifunctional properties when two different classes of nanomaterials are incorporated. The fabrication of hybrid nanostructures helps to integrate the useful properties of two different materials to overcome each of their drawbacks and also to benefit from the synergies of their physical and chemical properties. In this work, nanotubes and nanoparticles were used to fabricate hybrid materials using plasma in contact with liquid approach.

1.6.2 Choice of material

Inorganic nanomaterials such as nanocrystals, nanotubes and nanowires have advantageous physical, chemical and biological properties for biological applications. In particular, nanotubes have a number of advantages because they possess 1) a larger inner volume which enables them to encapsulate more drug molecules, 2) their volume is readily accessible, 3) the end caps could be removed easily to deliver drugs and 4) their inner and outer surfaces could be functionalized for targeted delivery.

Nanotubes have a hexagonal network of atoms connected together in a rolled fashion. Since their discovery, carbon nanotubes (CNTs) have been studied in detail. Recently, their potential of CNTs in biological application has become uncertain due to cyto-compatibility issues. Boron nitride nanotubes (BNNTs) are a structural analogue of carbon nanotubes with promising properties compared to CNTs. A brief comparison of the properties of CNTs and BNNTs is given in Table 1.1.

Table 1.1. Comparison of BNNT and CNT properties

<table>
<thead>
<tr>
<th>Properties</th>
<th>BNNTs</th>
<th>CNTs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

31
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical (Young’s Modulus)</td>
<td>~1.18 TPa</td>
<td>~1.25 TPa</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>~350 W/mK</td>
<td>~300 W/mK and &gt;3000 W/mK (varies based on the diameter of tube)</td>
</tr>
<tr>
<td>Chemical stability</td>
<td>stable up to 1100 °C in air</td>
<td>stable up to ~500 °C in air</td>
</tr>
<tr>
<td>Electrical properties</td>
<td>constant wide band gap (~5.5 eV)</td>
<td>based on the diameter, chirality and the number of walls, it could be semimetallic or semiconducting</td>
</tr>
<tr>
<td>Optical properties</td>
<td>applicable in the deep-UV regime</td>
<td>applicable in the near-IR regime</td>
</tr>
</tbody>
</table>

1.6.3 Structure of BNNTs

Boron nitride (BN) is isoelectronic and is similar to carbon in terms of structure and properties (Figure 1.5), and also to other compounds such as gallium nitride. Based on its preparation, BN crystallizes into a tetra-hedrally linked structure, like graphite and diamond. The similarities between BN and carbon could be understood from their isoelectronic structure. BN exists in four different polymorphic forms with different properties, namely hexagonal BN (h-BN), rhombohedral BN (r-BN), cubic BN (c-BN) and wurtzite BN (w-BN).

It was first synthesized from a reaction between molten boric oxide and potassium cyanide. The first product obtained was amorphous BN powder, which was converted into crystalline h-BN by annealing in nitrogen flow at > 1500 °C. Later, several methods were reported for producing other polymorphic forms of BN. Nanotubes of BN are quite similar to CNTs in which alternating B and N atoms replace C atoms. BNNTs could be either multi-walled or...
single-walled. The latter are difficult to produce, because single-layer BN does not appear to be as stable as multi-layers. The ionic character of BN may stabilize multi-walled BNNTs because of strong “lip-lip” interactions between the adjacent layers.\textsuperscript{75}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic of boron nitride nanotube (left) and carbon nanotube (right)}
\end{figure}

1.6.4 Properties of BNNTs

1.6.4.1 Mechanical properties

Min Feng Yu \textit{et al.} studied the elastic modulus and the resonance behaviour of BNNTs under the electric field induced resonance inside a transmission electron microscope (TEM) and showed that the elastic modulus of BNNTs was 722 GPa comparable to the theoretical estimate of \( \sim 850 \) GPa.\textsuperscript{76} The Young’s moduli of both the CNTs (1.25 TPa) and BNNTs (1.18 TPa) are similar which could be the reason both BNNTs and CNTs are used for various mechanical applications.\textsuperscript{77, 78}

1.6.4.2 Thermal and Chemical properties

There is a big demand for nanomaterials with high thermal conductivity to handle high radiation generating electronic devices. The thermal performance of BNNTs has been studied using theoretical methods like tight binding, density functional theory and the valence shell model.\textsuperscript{79-81} The experimental studies showed that BNNTs possess a higher low-temperature thermal conductivity compared to CNTs.\textsuperscript{82} Another important thermal property of BNNTs is their pronounced resistance to oxidation. Han \textit{et al.} obtained BCN nanotubes from BN
nanotubes under oxidation and claimed that they were more stable than CNTs.\textsuperscript{83}

1.6.4.3 Electronic properties

Theoretical calculations showed that BNNTs are expected to have a uniform electronic band gap (\textasciitilde5.5 eV), independent of the diameter and chirality of the nanotube.\textsuperscript{69, 84} This exhibited the uniform electrical behavior of BNNTs, unlike CNTs which are either metallic or semiconducting. However, zigzag BNNTs (n, 0) possess a direct band gap whereas armchair BNNTs (n, n) have an indirect band gap.\textsuperscript{84} The band gap of BNNTs could be tuned by several factors including doping with carbon, applying a transverse electric field and radial deformation.\textsuperscript{85-88}

1.6.4.4 Piezoelectric properties

Mele \textit{et al.} studied the piezoelectric properties of BNNTs using a model electronic Hamiltonian and suggested that BNNTs are piezoelectric and pyroelectric depending upon the direction of the spontaneous electric field that changes with the index of the tubes.\textsuperscript{89} Golberg \textit{et al.} found that elastic deformation of BNNTs occurred only when extending the bending angles beyond 30-40°, which was continued until the generation of consecutive momentary kinks.\textsuperscript{90} This suggested that BNNTs are promising piezoelectric systems whose mechanical and electrical properties could be tuned under defined conditions to achieve greater efficiency in nanoscale sensors, actuators and advanced nanoelectromechanical systems.

1.6.4.5 Optical properties

Cathode luminescence of multi-walled BNNTs showed a strong and weak absorption band at \textasciitilde3.9 eV and \textasciitilde5.3 eV.\textsuperscript{91} Chen \textit{et al.} found the band gap of multi-walled BNNTs as 5.75 eV using photoluminescence and optical absorption measurements.\textsuperscript{92} These studies suggest that BNNTs emit light in the deep ultraviolet region (around 200 nm) and might be useful in applications like information storage technology, medicine and environmental protection.\textsuperscript{93}
1.6.5 Synthesis of BNNTs

BNNTs could be synthesized by a range of methods including arc discharge, laser ablation, substitution reactions from CNTs, chemical vapour deposition (CVD) and ball milling and have been discussed in the following sections.

1.6.5.1 Arc discharge

The arc discharge method was the first successful method to synthesize BNNTs and was apparently similar to the method for growing CNTs. In this method, a rod of hexagonal BN was inserted into a tungsten electrode which acts as an anode with a rapidly cooled pure copper electrode as cathode. A constant potential drop and environmental helium gas were maintained at optimum conditions during the arc discharge. This led to the deposition of dark grey soot on the copper cathode which contained some spattered BNNTs at the end.

1.6.5.2 Laser ablation

Yu et al. have demonstrated that BNNTs could be fabricated by means of excimer laser ablation at high temperature. The factors such as wavelength, energy density of the laser beam, pressure, flow rate, temperature and the nature of target materials could be used to control growth, thereby a superior quality crystalline structure of one to several atomic layers and high yield could be obtained using laser ablation. In another report, Lee et al. have elaborated the bulk synthesis of BNNTs without the application of catalyst particles using continuous CO₂ laser ablation.

1.6.5.3 Substitution reactions from CNTs

Due to the structural similarity of CNTs and BNNTs, the synthesis methods of CNTs were used as a guide for producing BNNTs. This helped to extend the synthesis routes of BNNTs. According to the reaction mentioned below, Han et al. introduced a template substitution
method to incorporate boron and nitrogen atoms instead of carbon atoms in CNTs leading to the formation of BNNTs.\textsuperscript{97, 98} In this method, boron oxide powder was heated with carbon nanotubes in the presence of nitrogen at 1500 °C for 30 minutes. The analysis indicated the traces of carbon in BNNTs which could be purified by means of oxidation at 550 °C.

\[
\text{B}_2\text{O}_3 + 3\text{C(CNTs)} + \text{N}_2 \rightarrow 2\text{BN (BNNTs)} + 3\text{CO}
\] (1)

1.6.5.4 Chemical vapour deposition

Chemical vapour deposition (CVD) is one of the successful methods for growing CNTs.\textsuperscript{99} Most of the adopted CVD methods produced poor crystalline structures and structures with traces of carbon in BNNTs.\textsuperscript{100, 101}

1.6.5.5 Ball milling method

Unlike the previous methods explained above, where BNNTs are formed either by physical deposition or chemical reactions from a boron vapour phase, Chen \textit{et al.} reported that BNNTs could be prepared by reactive ball milling at room temperature.\textsuperscript{102, 103} Ball milling is quite different from the other methods and the key feature is the grinding of BN into nanomaterial, from which the ultimate BNNTs are formed.

In this process, elemental boron powder was used instead of BN and was ball milled in the presence of ammonia gas and then later annealed in nitrogen up to 1400 °C. The resultant BNNTs were predominantly bamboo-like structures rather than cylindrical tubes, capped by particles of Fe which acted as a catalyst for the growth of nanotubes.

1.6.6 Applications of BNNTs

1.6.6.1 Biomedical applications

The use of BNNTs in biomedicine gained popularity when it was reported that BNNTs has no or less toxicity in cells.\textsuperscript{104} Ciofani \textit{et al.} was the first to propose BNNTs as a potential drug
delivery system because of its much improved chemical stability, dispersibility and biocompatibility.\textsuperscript{105} In addition, filling BNNTs with magnetic nanoparticles has produced magnetic hybrid systems which could be used as contrasting agents for magnetic resonance imaging.\textsuperscript{106} The surface functionalization of BNNTs with biomolecules such as proteins, DNA and RNA could be used for bio-sensing and bio-imaging applications.\textsuperscript{107}

1.6.6.2 BNNT composites

Due to their high strength and other useful properties, BNNTs could reinforce novel composite materials. The addition of 4 wt.% of BNNTs in barium calcium alumosilicate glass has increased the fracture strength by 90% and fracture toughness by 35%.\textsuperscript{108} Lahiri \textit{et al.} proposed BNNT reinforced hydroxyapatite (HA) as a novel composite material for orthopaedic implants.\textsuperscript{109} This composite offered a 120% increase in elastic modulus, 129% higher hardness, 86% increase in fracture toughness and 75% improvement in wear resistance.

1.6.6.3 Electronics

BNNTs could be used to electrically insulate nanocables with embedded metallic or semiconducting nanowires. This kind of cable could be employed in very small electrical devices and complex multi-cable circuits.\textsuperscript{110} Kenji \textit{et al.} claimed that they produced pure h-BN crystals under high pressure and temperature, which showed a dominant luminescence peak and a series of s-like exciton absorption bands around 215 nm, showing it to be a direct bandgap material.\textsuperscript{111} The latter property made BNNT as a promising material for ultraviolet lasers and light emitting diodes.
1.6.7 Shortening of BNNTs

It has been reported that the long CNTs length makes it difficult to tailor them for applications in molecular electronic devices, solar cells. In composites, avoiding agglomeration is important to reduce stress concentration and obtain maximum mechanical performance. In biological applications, length causes poor solubility, difficulty in cell uptake and unwanted toxic issues in the cells.

These suggested that next to synthesis of BNNTs, reducing the length is essential in improving the process-ability of BNNTs to extend its use in bio and other applications. Zhi et al. first reported the shortening of BNNTs using oxidation and sonication to improve its processability. Later, Chee et al. demonstrated cutting of functionalized BNNTs in water using sonication.

The physical approaches such as ultra-sonication, ball milling and cryogenic crushing used for shortening of CNTs could cause damage to the nanotube structure while shortening the length and are time consuming methods. The chemical methods that utilized oxidizing solutions in shortening the CNTs are toxic and violent, hence it is hard to control the oxidation process in order to obtain the desired structure. Zhi et al. used very high temperature to oxidize the BNNTs and then sonicated them in alcohol for 5 hours to shorten BNNTs whereas Chee et al. functionalized the BNNTs using PEGylated phospholipids and sonicated the suspension for 30 hours to obtain short BNNTs.

1.6.8 Functionalization of BNNTs

Surface modification is another approach to increase the solubility, target a specific site and to attach nanoparticles. Covalent bonding and weak interactions have been reported as effective approaches to modifying the surface of BNNTs.

1.6.8.1 Covalent bonding
Covalent modification of BNNTs has been developed to improve its solubility. The bonding could be done either at B or N sites. The convenient methods for B site modification are generally lacking. Zhi et al. shown that B sites could be activated by oxidation of BNNTs using H₂O₂ under high pressure and temperature.¹³² The N site modification has been solely focused based on amino group chemistry.¹³³ The growth of BNNTs mostly used NH₃ as a reactant and this left occasional N-H bonds on its surface. The chemical reaction between the COCl group of stearoyl chloride and N-H bonds of BNNTs resulted the increase in solubility when N, N-dimethylacetamide (DMF) was used as an organic solvent.¹³⁴

1.6.8.2 Weak interactions

The weak interactions are more appropriate than covalent bonding to conserve nanotube properties. Although BNNTs are insulators because of the π electronic structure, this property might be used to attach molecules through π stacking interactions.¹³⁵, ¹³⁶ The first such interactions were observed when a conjugated polymer poly [m-phenylenevinylene-co-(2,5-dioctoxy-p-phenylenevinylene)] (PmPV) was attached to BNNTs, which then showed better solubility in solvents such as DMF and chloroform but not in water and ethanol.¹³⁶ Cation-π interactions and some non-specific interactions have also been used to modify BNNTs.¹³³, ¹³⁷

From the above reports which have focused on the functionalization of BNNTs, it is evident that a simple and effective method of functionalization is still required to overcome the problems in treatment instabilities, formation of functional groups in low density, damage to the tube structure, poor dispersion and solubility. Physical adsorption methods (hydrophobic or van der Waals interactions) of BNNT modification has resulted in reversible and unstable functionalization.¹³⁶, ¹³⁸ Chemical functionalization of BNNTs has given higher stability and reactivity which opened up the door for many applications.¹³³, ¹³⁴, ¹³⁹ However, the convenient methods for B site modification are generally lacking. Zhi et al. shown that B sites could be activated by oxidation of BNNTs using H₂O₂ under high pressure
and temperature. Ciofani et al. demonstrated that oxidation of BNNTs using concentrated nitric acid solution (65% w/w) and sonication for 6 hours, introduced -OH functional groups at B site. These two B site modification methods utilized high temperature, pressure, concentrated chemicals, and longer treatment times to achieve the modification of BNNTs at B site. The N site modification has been solely focused based on amino group chemistry. Again, a low density of functional groups and damage to the tube structure are reported as major problems while modifying N site of BNNTs. Moreover, the low chemical reactivity and poor wetting properties of untreated BNNTs does not allow them to easily solubilize in many organic solvents which could hamper their application prospects in biomedicine, energy storage and electronics.

1.6.9 Attachment of nanoparticles onto BNNTs

There have been a few attempts to modify BNNTs with nanoparticles such as SnO₂, Au and Fe with the help of non-specific interactions. SnO₂ nanoparticles are strongly attracted to BNNTs due to the change in lattice distances of both BNNTs and SnO₂ through Sn-N bond formation or electrostatic interactions. The other nanoparticles Au and Fe, did not entirely cover BNNTs. However, Sainsbury et al. showed that Au nanoparticles could fully cover BNNTs using S-Au chemistry. In their work, the BNNTs were functionalized with a thiol group containing compound called 3-mercaptopropionic acid (MPA) before attachment of GNPs. Huang et al. synthesized Fe decorated BNNTs using an ethanol thermal process and they studied the magnetic properties of prepared nanocomposites. Yu et al. developed a strategy to decorate BNNTs with Pd nanoparticles using sodium dodecyl sulphate. Recently, Volkmann et al. demonstrated the direct attachment of colloidal semiconducting nanoparticles such as PbSe, CdSe and ZnO onto BNNTs and studied its fluorescence properties.

The difficulties in positioning the nanoparticles at a particular location, controlling the structure, morphology and damage to the nanotube structure are still remaining as major
1.6.10 Research challenges, objectives and our approach

The major challenges that handicap BNNTs for further application are 1) length and surface modification of nanotubes and 2) assembly of nanoparticles onto modified nanotubes. The highlights of the challenges associated in this research are listed in Figure 1.6.

![Figure 1.6. Schematic of major challenges in synthesis, functionalization of BNNTS and assembling of nanoparticles onto them](image)

Shortening and functionalization of BNNTs

As mentioned earlier, the previous methods used to shorten BNNTs are hazardous, not easy to control, time consuming and could cause damage to the tube structure. The use of short nanotubes and their advantages such as improved dispersibility, process-ability,
biocompatibility and easy cellular uptake for bio applications has been discussed earlier. Zhi et al. observed two main advantages of shortened BNNTs that possess 1) a hole-rich structure and more ends which increased the possibilities for molecules or cargoes to enter into the tubes instead of solely locating on the surface and 2) better dispersibility which enhanced the chances of BNNT-molecule interactions. These advantages suggest that shortened BNNTs might be useful for efficient delivery systems.\textsuperscript{118} In addition to shortening BNNTs, producing boron nitride nanocups (BNNCs) might be also a useful approach to achieve high dispersibility. BNNCs, a kind of hollow BN nanomaterials, possess cup like structures which was produced by the combined treatment of ball milling and followed by ultra-sonication in BNNTs.\textsuperscript{147} It maintains the intrinsic properties of BNNTs and also possesses other advantages such as high surface area and low effective densities.

Vandsburger et al. have claimed that oxidation induced by gaseous oxygen plasma discharge could play a key role in reducing the length of CNTs.\textsuperscript{148} Zhi et al. have shown that oxidization of BNNTs at high temperature leads to shortening of the nanotubes.\textsuperscript{118} The above reports have shown that oxidation plays a key role in reducing the length of BNNTs. However, there are no reports on the use of atmospheric pressure plasma in contact with liquids to shorten nanotubes from bamboo like BNNTs.

Next to shortening, the key challenges in modifying the surface of BNNTs are instability, low density of functional groups, maintaining the tube structure, poor dispersion and solubility. Zhi et al. have demonstrated the use of H\textsubscript{2}O\textsubscript{2} under high pressure and temperature to oxidize and modify the BNNTs at B site.\textsuperscript{132} In another method, Ciofani et al. have used concentrated nitric acid solution and sonication to oxidize BNNTs and then introduce -OH functional groups at B site.\textsuperscript{140} Gas plasmas possess several advantages for BNNT surface modification. Point defects were created in BNNTs which provided nitrogen vacancies for oxygen substitution, as a result, a high density of functional groups was achieved in a well-controlled manner.\textsuperscript{149-151} Dai et al.
have successfully achieved the controlled functionalization of BNNTs by gentle plasma treatment while maintaining the intrinsic tube structure.\textsuperscript{152} The previous studies by others have shown that use of high temperature and pressure, hydrogen peroxide and nitric acid oxidizes the BNNTs which facilitate the shortening and functionalizing the nanotubes.\textsuperscript{118, 132, 140, 148} However, there are no reports on the functionalization of BNNTs using atmospheric pressure plasma in contact with liquids.

Assembly of GNPs onto the functionalized short BNNTs to fabricate hybrid nanostructures

The attachment of nanoparticles with BNNTs could broaden the range of nanotube properties and uses, while the BNNTs could act as a support for the nanoparticles. Several reports have demonstrated that nanoparticles attached nanotubes could be used for applications in single electron transistors, plasmon waveguides and also for creating novel nanostructures.\textsuperscript{153-155} However, as mentioned earlier, most of the approaches reported for fabrication of hybrid nanostructures have faced issues such as use of toxic chemicals, longer treatment times, positioning the nanoparticles at a particular location, controlling the structure, morphology and damage to the nanotubes. So far there are no studies related to the attachment of nanoparticles onto BNNTs using atmospheric pressure plasma in contact with liquids.

Atmospheric pressure plasma-aided nanofabrication is one of the alternative approaches to produce nanostructures and uniformly tailor the surface of nanomaterials in a well-controlled manner.\textsuperscript{156-159} It was demonstrated that gas-liquid interfacial discharge plasma could be used to attach nanoparticles onto CNTs using ionic liquids.\textsuperscript{160-162} As mentioned earlier, there are no reports which have investigated the use of atmospheric pressure plasma in contact with liquids to shorten, functionalize BNNTs and attach nanoparticles onto them. This is the first study which aimed to fabricate functionalized short BNNTs-GNPs hybrid nanostructures using nanosecond pulsed atmospheric pressure plasma in gas phase above the liquid surface (set up I is shown in Figure 1.7), to address the afore-mentioned challenges. Gold nanoparticles
(GNPs) have been widely used and accepted due to their well-established unique physical, chemical and biological properties.\textsuperscript{163, 164} Hence GNPs were chosen for this study. This approach includes the following steps.

- Shortening and functionalization of BNNTs
- Assembly of GNPs onto the functionalized short BNNTs to fabricate hybrid nanostructures

The development of this kind of hybrid nanostructures has potential for applications in drug delivery, bio-imaging and biosensors.

\textbf{Figure 1.7.} Schematic view of nanosecond pulse atmospheric pressure plasma system, using argon gas. The top electrode was above the liquid and bottom electrode was immersed in liquid.

\textbf{1.7 Decontamination of milk to extend shelf life while maintaining nutrition}

Milk is a liquid food, that is enriched with nutrients and growth factors, that provide many health benefits to humans.\textsuperscript{165} The nutrients in milk also serves as an ideal substrate for the growth of micro-organisms.\textsuperscript{166} The consumption of raw or ineffectively processed milk causes milk-borne diseases in humans.\textsuperscript{167-170} Thermal pasteurization is the widely accepted technique
to remove bacteria in milk but this damages the nutrients in milk by several modifications.\textsuperscript{171, 172} An alternative to pasteurization is the potential to use plasma produced in bubbles that could be externally introduced into milk to potentially decontaminate and extend the shelf life of milk. This technique may be gentler on milk constituents and therefore may maintain milk nutrition compared to pasteurization.

The composition of milk that include proteins, lipids, carbohydrates, miRNA and other trace elements, micro-organisms, milk-borne diseases, thermal and non-thermal methods to process milk, research challenges and our strategy to address the challenges have been discussed in detail here.

1.7.1 Composition of milk

Milk is a nutritious liquid food secreted from the udder of mammalian species like bovine, goat, sheep and buffalos for the growth and development of its offspring. Milk and milk products have been consumed by human since early times and contributes to a significant health benefit in humans.\textsuperscript{173} The average composition of bovine milk constitutes 87 % water, 3.4 % protein, 3.6 % fat, 4.9 % lactose, 0.7 % minerals and other trace elements. In cows the composition of milk can considerably vary depending upon the breed, genetic variation, feed, season, lactation stage and udder health.\textsuperscript{174, 175} Milk is a complex liquid, in which fat is present in the form of globules surrounded by a membrane, whereas proteins, carbohydrates and other substances are dispersed and solubilized in skim milk (without fat).\textsuperscript{174} The nutritional compounds present in milk have been discussed in the following sections.

1.7.2 Proteins

Proteins in milk are broadly divided into two types, namely casein and whey proteins. These two proteins chemically and physically differ from each other and they exist in different milk phases. Casein proteins are present in casein micelles which are dispersed in the skim phase of
milk (i.e. milk minus fat) while whey proteins are solubilized in the skim phase of milk. Dalgleish et al. found that casein proteins tend to precipitate at pH 4.6 at 20 °C, while whey proteins stay in solution under the same conditions. This showed that both proteins characteristically differ in terms of stability. Milk is composed of 80% casein and 20% whey proteins. The casein (CN) proteins are majorly classified into alpha (αs1-CN, αs2-CN), beta (β-CN) and kappa casein (κ-CN) according to the homology of their primary structure. In bovine milk the whey proteins mainly consist of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulins (Ig), while human milk is devoid of β-LG.

1.7.2.1 Casein proteins and micelles

Caseins are the major proteins in milk and they are distinguished as αs1-CN, αs2-CN, β-CN and κ-CN, approximately present in the ratio about 11:3:10:4. Each of them has its unique amino acid composition, genetic variations, and functional properties. Each components of casein contain high amounts of ester bound phosphate, proline residues with no or less disulphide bonds. Due to this, CN proteins have low levels of secondary structure and they lack tertiary structure. This keeps the caseins in an open structure and expose its hydrophobic residues which provide stability against denaturation by heat because there is very less structure to unfold. This open structure also makes caseins susceptible to gastrointestinal proteolytic digestion which results in the release of bioactive peptides that might contribute to a lower risk against type-1 diabetes, cardiovascular disease, schizophrenia, dental caries and autism.

The hydrophobic nature of CN proteins allow them to react or associate with each other to make them soluble in water. These self-associated CN proteins together with calcium phosphate (CCP) constitutes the casein micelles. The casein micelle is made up of 94% casein protein and 6% CCP. The CCP plays a key role in the formation and stability of the CN micelles. The surface of the CN micelle is mainly composed of κ-CN, which behaves as...
an emulsifier by keeping the hydrophilic C-terminal region in the soluble phase and the hydrophobic region inside the micelle. The size of the CN micelles varies from <50 nm to >500 nm based on the content and genotype of κ-CN. These micelles play a key role in providing fluidity to casein molecules and solubilize phosphate and calcium. Moreover, it has been suggested that the casein-micelle complex with calcium phosphate helps in preventing the calcification of the mammary glands.

1.7.2.1.1 Alpha casein

The common alpha caseins identified so far are αs1 and αs2-CN. αs1-CN has a high net negative charge and a high phosphate content (i.e. 7-9 per molecule). Hydrophobic interactions are also involved in the association of this protein. It has five to nine variants and are reported as αs1-CN A, B, C, D, E, F, G, H and I. αs1-CN B is the common variant and accounts for about 34% in milk. This protein contains 199 amino acid residues and has a molecular weight of 23.614 kDa. αs1-CN is sensitive to calcium and it can be precipitated by calcium at very low levels.

The αs2-CN have four variants such as αs2-CN A, B, C and D and they vary based on the number of ester phosphate groups (i.e. 10-14 per molecule). This protein consists of 207 residues and the molecular weight was calculated as 24.350 kDa. It contains two cysteines, resulting in intermolecular disulphide (–S–S–) bridges (no carbohydrate groups) and exists as either a monomer or dimer. αs2-CN is the most hydrophilic CN and contributes to about 8% of the proteins in milk. This protein can also be precipitated at very low levels of calcium.

1.7.2.1.2 Beta casein

β-Casein (β-CN) is the most hydrophobic casein (5 phosphate groups) and constitutes 25% of the proteins in milk. 13 genetic variants of β-CN are identified so far and listed as β-CN A1, A2, A3, B, B2, C, D, E, F, G, H1, H2 and I. The most common variant is A2 with 209 amino
acid residues and it has a molecular weight of 23.983 kDa. The charge is unevenly distributed in \( \beta \)-CN and so it is referred as amphiphilic protein. Unlike alpha CN, \( \beta \)-CN is less sensitive to calcium precipitation.

1.7.2.1.3 Kappa casein

Kappa casein (\( \kappa \)-CN) is considerably different from \( \alpha s1 \)-CN and \( \beta \)-CN. Like \( \alpha s2 \)-CN, \( \kappa \)-CN contain two cysteine residues (forming an –S–S– bridge) but it also possess a carbohydrate group. To date, 12 different \( \kappa \)-CN protein variants (A, B, C, D, E, F1, F2, G1, G2, H, I, and J) and one synonymous variant (AI) have been identified. The common variant B contains 169 amino acid residues and has a molecular weight of 19.023 kDa. \( \kappa \)-CN is positioned on the outside of the casein micelle and it about 9% of the proteins in milk. \( \kappa \)-CN is very resistant to calcium precipitation which helps to provide stability to other types of caseins. The cleavage of \( \kappa \)-CN using rennet enzyme makes it to lose the stabilizing ability which results in the coagulation of milk through aggregation of CN micelles.

1.7.2.2 Whey proteins

Unlike the limited number of CN proteins, the whey fraction contains a significant number of proteins. The whey proteins are compact, globular proteins that have a high level of secondary, tertiary and quaternary structure and contain intermolecular disulphide bonds that stabilize their structure. The key whey proteins in milk are \( \alpha \)-LA, \( \beta \)-LG, BSA, Ig and Lf. They account for about 20% of the total protein content in milk.

1.7.2.2.1 Beta lactoglobulin

Beta-lactoglobulin (\( \beta \)-LG) is the major protein in bovine whey and constitutes about 50% of the whey fraction in milk. There are 11 known genetic variants of \( \beta \)-LG identified in bovine milk. \( \beta \)-LG is a globular protein and naturally exists as a dimer in raw milk, each subunit has a molecular weight of 18.3 kDa with 162 amino acids, two disulphide bridges and a free thiol group. In \( \beta \)-LG, each \( \beta \) sheet has one hydrophobic side and a hydrophilic side. The
hydrophobic sides of β-LG subunits are tightly bound to each other through hydrophobic interactions. The function of β-LG is not completely identified, however studies showed that the three dimensional structure of β-LG has homology to the lipocalin family, and is considered to bind and transfer fatty acids and Vitamin A. The members of this family generally have high allergenic potential and present well-conserved sequence homologies in their N-terminus moiety where tryptophan at position 19 is always present.

1.7.2.2.2 Alpha lactalbumin

Alpha lactalbumin (α-LA) constitutes about 2% of the whey fraction of milk. α-LA known to have three different variants is reported as α-LA A, B and C. α-LA has a molecular weight of 14.186 kDa with 123 amino acids and eight cysteine residues. Native α-LA contains two domains, a large α-helical and small β-sheet domain, which are held together by cysteine residues, forming calcium binding loop. α-LA has a characteristic property of hydrophilic and hydrophobic based on the presence and absence of calcium ions. The biological function of α-LA is that it plays an important role in the formation of lactose from glucose and galactose. Dimeric α-LA has been implicated in initiation of apoptosis, and it is thought that this dimer is formed at weaning and initiates involution via apoptosis of mammary epithelial cells (sharp et al, 2016). This results in restructuring of the mammary gland back to a virgin-like state.

1.7.2.2.3 Bovine serum albumin

Bovine serum albumin (BSA) is a minor whey protein which contains 582 amino acids, 17 disulphide bridges and one thiol-group, with a molecular weight of 66.267 kDa. BSA has the biological function of binding and delivering free fatty acids, other lipids as well as flavour compounds.

1.7.2.2.4 Immunoglobulins
Immunoglobulins (Igs) are majorly classified into IgG, IgM and IgA in bovine milk and they are made up of 90% of protein and 10% of carbohydrate. IgG is the common type, consists of four polypeptide chains; two identical light chains (κ and λ, 23 kDa) and two identical heavy chains (53 kDa). The complete Ig or antibody molecule possesses stereo-chemically a Y-shaped structure and has a molecular weight of about 150-160 kDa. The light chains have 211 to 217 amino acids in length and differ in their chain structure. Whereas the heavy chains possess five types γ, δ, α, μ and ε. The α and γ heavy chains contain about 450 amino acids, while μ and ε have approximately 550 amino acids. The light chains are joined to the heavy chains by disulphide bonds and the two heavy chains are held together by disulphide bonds near a hinge region, which it gives the molecule the flexibility needed in antibody-antigen interactions. The stem of the Y shaped, called Fc fragment, which consists of two heavy chains depending on the class of the antibody. Igs are an important component of immunological activity in milk and are delivered to the young to protect them from infection at a stage when they might be immunologically naive. Igs have multiple biological functions such as activation of complement proteins, bacterial opsonization, cell lysis and agglutination.

1.7.2.2.5 Lactoferrin

Lactoferrin (Lf) is a monomeric globular, iron binding glycoprotein composed of approximately 680 amino acids with a molecular weight of ~80 kDa. Lf belongs to the transferrin family, which contain two main domains. Each main domain consists of two α-helical/β-sheet domains separated by a cleft that includes an iron binding site. Lf has multiple biological functions like antimicrobial, anti-viral and anti-parasitic activities.

1.7.3 Lipids

Milk fat is an important source of energy, fat soluble nutrients and bio-active lipids. Milk fat is mainly composed of triacylglycerols (98%), di and monoacylglycerols (2%), cholesterol (>0.5%), phospholipids (~1%) and free fatty acids (FFA) (~0.1), averagely. The other trace
elements include ether lipids, hydrocarbons, fat-soluble vitamins and flavour compounds which is available based on the diet of the cow.\textsuperscript{210} The chemical composition of lipids in milk is also influenced by breed, lactation stage and cycle and season.\textsuperscript{211,212} It has been reported that lipids in milk possess biological functions such as anticancer, anti-inflammatory, antimicrobial and immunosuppression properties.\textsuperscript{213}

1.7.3.1 Milk fat globule membrane

Lipids in bovine milk are in the form of globules, as an oil-in-water emulsion.\textsuperscript{214,215} The diameter of fat globules is 0.1–10 μm, but averages in the range of 1–5 μm.\textsuperscript{174} The membrane surrounding the lipid globules in milk is called the milk-fat-globule membrane (MFGM), which is assembled and secreted by the epithelial cells of the mammary gland. The MFGM enables the fat to remain dispersed throughout the aqueous phase of milk. The composition of MFGM commonly consists of a mixture of proteins, glycoproteins, enzymes, neutral lipids and phospholipids.\textsuperscript{216} In MFGM, proteins and enzymes are the major constituents (70%) and the remaining fraction compose lipids such as phospholipids (25%), cerebrosides (3%) and cholesterol (2%).\textsuperscript{217}

The common proteins identified in MFGM are xanthine oxidoreductase, butyrophilin, PAS 6 and PAS 7.\textsuperscript{214} The other enzymes in MFGM are namely acetylcholine esterase, alkaline and acidic phosphatase.\textsuperscript{174} It is generally accepted that the role of MFGM is generally to prevent lipolysis in milk. Hence, if the milking and storage methods are properly done, then raw milk can be kept for several days without the development of a rancid off-flavor. However, during homogenization milk is forced through small passages under pressure which reduces the diameter of the globules according to the pressure applied. This causes the casein proteins to come in contact with the milk lipids. A very active lipoprotein lipase present in the casein micelles, bind to the fat globules and hydrolyzes the triacylglycerols within a few minutes. This
demands the pasteurization technique to inactivate lipoprotein lipase before homogenizing the milk.174

1.7.3.1.1 Triacylglycerols

Triacylglycerols are synthesised in the mammary gland through enzymatic reactions, which exert some selectivity over the esterification of different fatty acids at each position of the sn-glycerol moiety.218 Triacylglycerols, composed of three fatty acids covalently bound to a glycerol molecule by ester bonds, form the major composition of milk lipids (~98%) which largely decides the properties of milk fat.214, 219 The main properties for example are, hydrophobicity, density and melting characteristics. Triacylglycerols are very non-polar and not surface active. In the liquid state, they could be used as a solvent for other non-polar substances such as sterols, carotenoids, and tocopherol. These properties vary with the fatty acid composition. These triacylglycerols are a complex mixture, and vary considerably in molecular weight and degree of unsaturation. This complexity could be due to the presence of a wide variety and large number of fatty acids, which constitute the composition of triacylglycerols.179

The fatty acids in milk lipids consists of a straight chain of a number of carbon atoms, surrounded with hydrogen atoms and a carboxyl group at one end. In the carbon-to-carbon bond chain, if all the bonds are single, they are referred to as saturated fatty acid; the unsaturated fatty acid contains double bonds at any position in the length of the chain. The fatty acids differ by carbon length, level (example mono) and position (example \textit{cis}9) of unsaturation, and branching. The differences in the above characteristics and the composition of fatty acids in milk lipids determines the flavour and texture (for example, spread-ability of butter).214 The fatty acids in milk lipids could be beneficial for human health while certain others are considered to have negative effects on human health.213

1.7.3.1.2 Di and monoacylglycerols
Lok reported that fresh milk contains only less percentage of diacylglycerols, monoacylglycerols and free fatty acids.\textsuperscript{220} Lok identified that diacylglycerols composed mainly of \textit{sn}-1,2 diacylglycerols and hence they may be the intermediates in the biosynthesis of triacylglycerols instead of a by-product from lipolysis.\textsuperscript{220} Later reports claimed that di and monoacylglycerols have been found in higher quantities after lipolysis. Diacylglycerols are largely non-polar and similar to triacylglycerols in properties. Monoacylglycerols are somewhat polar, surface active and accumulate mainly at an oil-water interface.\textsuperscript{179} Some of the free fatty acids are present in fresh milk and lipolysis could increase their quantities. Free fatty acids consist of both longer and shorter length fatty acids. The longer ones are surface active and like to accumulate at an oil-water interface. The shorter fatty acids could be responsible for the development of rancid flavour after lipolysis.\textsuperscript{179}

1.7.3.1.3 Phospholipids

Phospholipids are polar, amphiphilic and account for only 0.8\% of milk lipids. Most of them are found in MFGM (65\%), and remaining in the aqueous phase. Phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin are the major phospholipids in milk. Sterols are present in lower quantities (0.3\% of the fat). In sterols, cholesterol is the major component.\textsuperscript{214} The fatty acid pattern of phospholipids varies considerably in chain length and number of double bonds compared to triacylglycerols. For example, sphingolipids average chain length is longer, and phosphatidyl ethanolamine has a high proportion of polyunsaturates (double bonds). Due to this, the phospholipids are highly susceptible to oxidation.\textsuperscript{179}

1.7.4 Carbohydrates

The major component of carbohydrate in milk is reducing disaccharide lactose. Lactose is composed of glucose and galactose linked by \textit{β} 1-4 glycosidic bond.\textsuperscript{206} Both glucose and galactose are largely available in mammalian metabolism, lactose is synthesized only in the Golgi vesicles of the lactating cells, which is dependent on the presence of \textit{α}-LA protein. \textit{α}-LA
alters the action of the enzyme galactosyl-transferase to catalyze the formation of lactose from uridine-diphosphate-galactose and glucose.\textsuperscript{179} It is the major osmolar factor in milk and its synthesis is responsible for drawing water into the milk as it is being formed in the mammary gland.\textsuperscript{212} Lactose is also responsible for the increased absorption of calcium.\textsuperscript{221} In addition, glucidic compounds such as hexosamines and N-acetyl neuraminic acid are present in milk, however many of them are covalently bonded to membrane proteins, or in cerebrosides.\textsuperscript{179}

1.7.5 RNA and micro RNA

Micro RNAs (miRNAs) are a new class of non-coding RNAs ranging from of 18 to 26 nucleotides in length which play an important role in gene regulation by controlling translation and stability of mRNAs.\textsuperscript{222} Recent research has revealed that miRNAs are involved in important biological functions such as in gene regulation, signal transduction, cellular communication, and human health.\textsuperscript{223-226} Micro RNAs (miRNAs) have been identified in bovine, human, rat and other species milk and serve as an effective biomarker for milk quality and udder function.\textsuperscript{227-229} Izumi \textit{et al.} reported the presence of miRNAs and mRNA in bovine milk exosomes, which were absorbed by human macrophages, this suggest that bovine milk exosomes might have biological effects in humans.\textsuperscript{229} These milk miRNAs are encapsulated in exosomes which gives them stability and the ability to withstand harsh conditions such as the acid environment of the stomach.\textsuperscript{228-230} Baier \textit{et al.} demonstrated that humans absorb biologically meaningful amounts of miRNAs from cow milk, this intake could affect human gene expression \textit{in vivo} and in cell cultures, and the endogenous synthesis of miRNAs might not be sufficient for dietary miRNA deficiency in mice.\textsuperscript{231} Chen \textit{et al.} identified seven miRNAs (miR-26a, miR-26b, miR-200c, miR-21, miR-30d, miR-99a, and miR-148a) which were consistently found in both colostrum and raw cow milk. They proposed that these milk specific miRNAs, could be potential biomarkers for the quality control of raw milk and other milk-related products.\textsuperscript{228}
1.7.5 Bioactive peptides/Enzymes/Vitamins/Minerals

Milk contains bioactive peptides, enzymes, vitamins and minerals in very small quantities which may be beneficial to human health. Milk proteins form the major source of biologically active peptides.\textsuperscript{232} The proteolytic digestion of these proteins in the gastrointestinal tract or processing releases the bioactive peptides.\textsuperscript{233} Milk contains the naturally available bioactive substances such as oligosaccharides, growth factors, hormones, gangliosides, mucin and endogenous peptides.\textsuperscript{234} Several studies have shown that milk peptides have antihypertensive effects, both by inhibiting angiotensin-converting enzyme, having opioid-like activities, antithrombotic properties and by binding minerals.\textsuperscript{235, 236}

Milk contains a great number of enzymes which are not only useful to evaluate the quality of processed or heat-treated milk but could also have functional values.\textsuperscript{174} Xanthine oxidase, plasmin and lactoperoxidase are the common enzymes. The other enzymes include amylases, lipases, esterases, proteinases and phosphatases. The applications of these milk enzymes have been documented elsewhere.

Milk contains many minerals, vitamins and antioxidants. Vitamin A, Vitamin E, Vitamin B12 and folate are the common vitamins available in milk. Calcium, selenium, magnesium, iodine and zinc are the commonly available minerals in milk.\textsuperscript{173, 237-240} Several studies have documented the presence and uses of various minerals, vitamins and antioxidants present in milk.\textsuperscript{173, 237-240}

1.7.6 Microorganisms in milk and milk borne diseases

The presence of large amount of nutrients with high water content and neutral pH (6.4–6.8) makes milk an ideal medium for the growth of many organisms.\textsuperscript{241, 242} Microorganisms in milk can be divided into three main groups, probiotics, spoilage and pathogenic. Probiotic microorganisms are those which provide beneficial effects for the host or humans by providing microbial balance in the gut.\textsuperscript{243} The common probiotics identified in milk are \textit{Lactobacillus},
*Bifidobacterium and Enterococcus species*. These probiotics can also be classified into the spoilage micro-organisms category because of their ability to produce lactic acid which can sour milk.

Spoilage organisms have the potential (using enzymes such as protease, peptidase, lipase, esterase, oxidase, polymerase, β-galactosidase) to hydrolyze protein, fat and lactose present in milk to produce substances which are appropriate for their growth. This kind of hydrolysis lead to spoilage, off-flavours and odours, modification of appearance and texture in milk. They can be classified in to two types, gram positive and negative bacteria. The gram negative bacteria are the psychrotrophs such as *Pseudomonas spp, Enterobacteriaceae, Flavobacterium, Aeromonas* and the gram positive bacteria are spore-forming bacteria which include *Geobacillus stearothermophilus, B. licheniformis, B. cereus, B. subtilis and Clostridium spp.*

Several pathogenic organisms have been identified in raw milk which varies according to the geographical area, season, farm size, hygiene and management practices. They are namely toxin-producing *Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni, Salmonella spp. and Yersinia enterocolitica* which cause outbreaks of illness in humans through milk-borne diseases. The outbreaks related to milk-borne diseases are always a serious concern in Australia and at the global level. The list of pathogenic micro-organisms and its associated milk-borne diseases are provided in below table 1.2.

**Table 1.2 List of pathogenic micro-organisms and its associated milk-borne diseases**

<table>
<thead>
<tr>
<th>Micro-organisms</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Campylobacteriosis&lt;sup&gt;253&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Brucella spp</em></td>
<td>Brucellosis&lt;sup&gt;254&lt;/sup&gt;</td>
</tr>
<tr>
<td>Microorganism</td>
<td>Disease</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>Mastitis²⁵⁵</td>
</tr>
<tr>
<td><em>Staphylococcal enterotoxins</em></td>
<td>Food poisoning²⁵⁶</td>
</tr>
<tr>
<td><em>Salmonella species</em></td>
<td>Salmonellosis²⁵⁷</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Tuberculosis²⁵⁸</td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> 0157:H7</td>
<td>Haemorrhagic colitis and</td>
</tr>
<tr>
<td></td>
<td>haemolytic uraemic syndrome²⁵⁹</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Listeriosis²⁶⁰</td>
</tr>
</tbody>
</table>

1.7.7 Processing technologies in milk

Thermal methods (pasteurization), are in practice, to decontaminate and sterilize milk to reduce the risk associated with the afore-mentioned harmful micro-organisms. In addition to thermal methods, non-thermal methods are also emerging in recent years to improve microbial safety and quality of milk. A detailed review, advantages and disadvantages of these milk processing technologies have been discussed here.

1.7.7.1 Pasteurization

Pasteurization is the process of applying heat to milk to destroy microorganisms and make it safe for human consumption. The history of pasteurization starts with William Dewes (40 years before Pasteur’s experiment) who suggested heating milk, before feeding to infants after observing that heat treatment reduces milk spoilage.²⁶¹ Later Pasteur, discovered that heating liquids at ~60 °C improved the shelf life of wines. Germany was the first country to make a pasteurizer commercially in the year 1882. Denmark and Sweden adopted pasteurisation as a
common practice in mid-1880s. The first commercial milk pasteuriser was installed in Bloonwille, New York in the year 1883.\textsuperscript{262}

To date, pasteurization is the only accepted technique available for processing milk. The common pasteurization types are, low temperature long time (LTLT), high temperature short time (HTST) and ultra-high temperature (UHT).\textsuperscript{263} LTLT is generally regarded as a batch or holder pasteurization. In LTLT, the milk is heated at 63 °C for 30 min to decontaminate milk. HTST is a continuous process method in which milk gets heated at 72-75 °C for 15-30 seconds. HTST is the commonly followed method which has advantages such as shorter treatment time and less changes in milk quality compared to other methods.\textsuperscript{263} UHT is the process of sterilizing the milk at 135-140 °C for few seconds to increase the shelf life for several months. In all pasteurization methods, cooling of milk immediately followed by heating is an important step to prevent heat induced changes in milk.

Milk is mainly pasteurized to destroy the pathogenic and vegetative microorganisms present in the milk.\textsuperscript{264,265} This helps not only to increase and keep the quality of milk but also allows the milk to be used for manufacturing in other milk-related products such as cheese, butter, cream, yoghurt and milk powder.\textsuperscript{266} Despite these advantages, pasteurization affects the organoleptic and nutritional properties of milk through several modifications.\textsuperscript{171,172,267-269}

The components in milk start to change at 60 °C and the extent of damage gets intensified with the increase in temperature. Proteins such as Lf, Igs, BSA, β-LG, α-LA and caseins are modified in the 65-80 °C temperature range with the given treatment time.\textsuperscript{270} LTLT also results in damage of vitamin C, folacin, and B6.\textsuperscript{268} On the other hand, it has been reported that HTST causes less chemical changes when compared to LTLT.\textsuperscript{263} Siciliano \textit{et al.} observed that heating milk at high temperatures (70-120 °C) damages and structurally modifies the proteins, thereby it showed noticeable changes in sensory qualities.\textsuperscript{269} Heating milk >70 °C denatures proteins
and damages the phospholipids in MFGM. The denaturation of proteins causes the release of 
H$_2$S and results in the development of off-flavours. Furthermore, heat induces the association 
of whey proteins with MFGM through disulphide exchange reactions.$^{271}$

Martinez et al. reported that heating the milk above 100 °C results in lactose degradation and 
calcium phosphate precipitation that causes the formation of organic acids which finally results 
in drop of pH value.$^{272}$ During UHT pasteurization, the milk components (lactose and lysine) 
react with each other through non-enzymatic browning reactions called Maillard reactions. 
This result in off-flavours, change in colour, and loss of amino acid lysine which has high 
nutritional benefits.$^{273}$

Although, pasteurization eliminates the vegetative and pathogenic bacteria in milk, heat has 
limited effect in spore-forming bacteria (example, *B. Cereus* spores). These bacterial spores 
can survive pasteurization processes and might contaminate milk again, thus limiting the shelf 
stability of the product.$^{274}$

Nada et al. have extensively studied and reported that the manufacturing and distribution 
dynamics in the milk industry are constantly affected while improving the shelf-life and 
safety.$^{275}$ Moreover, the shelf-life of unopened, pasteurized milk is 8-14 days based on the 
intensity of the treatment. This utilizes a significant amount of energy to produce a short 
shelved product and hence the pasteurization process are neither effectively robust nor cost 
effective.$^{268,276}$

1.7.7.2 High hydrostatic pressure

High hydrostatic pressure (HHP) is extensively studied and considered as one of the emerging 
non-thermal methods in processing milk and to produce modified functional foods. It is the 
process of applying high pressure in the range 50-1000 MPa according to the type of food and 
the micro-organisms present. In 1899, Hite studied the effect of HHP on microbial inactivation 
in milk and found that HHP treatment at 680 MPa for 10 min delayed the spoilage of milk.$^{277}$
The commercially available HHP treated products include juices, yoghurts, jellies etc.\textsuperscript{278-280} Rastogi \textit{et al.} found that HHP can be successfully applied to inactivate enzymes with minimal effects on nutritional and sensory components compared to heat treatment\textsuperscript{281}

Several authors have reported that the vegetative bacteria in foods can be destroyed using HHP. The common proposed mechanism of inactivation is that HHP permeabilize the membrane to cause leakage of the contents of bacterial cells and also alters the proteins, enzymes and nucleic acids in them.\textsuperscript{282-284} The effect of HHP on microbial inactivation is dependent on the type and state of growth, the pressure and time of application, and the physico-chemical characteristics of food. HHP produces a restricted effect on bacterial spores compared to vegetative cells. Due to this, several authors used the combined treatment of HHP with heat and/or additives (for example, sorbic and benzoic acid, lysozyme, chitosan) to effectively inactivate microorganisms in foods.\textsuperscript{285, 286} The use of temperature in combination with HHP complements each other in inactivating spores and it is suggested that the increase in temperature affects the cells and its germination properties.\textsuperscript{287, 288} The effects of HHP on enzymes such as alkaline phosphatase, lactoperoxidase, $\gamma$-glutamyltransferase and phosphohexoseisomerase which are naturally present in milk, have been studied and commonly found that this technology can only partially inactivate or denature the enzymes.\textsuperscript{289, 290}

The effect of HHP on milk and milk products has been studied by several authors and studies suggest that this technology inactivates infectious food-borne pathogenic microorganisms, modifies functional properties and could be applied to the manufacture cheese, yoghurt and other dairy related products.\textsuperscript{291-297}

Several reports suggested that HHP alone has fewer effect on enzymes compared to thermal methods.\textsuperscript{289, 290, 298, 299} The effect of HHP on milk components mainly depends upon pH, treatment temperature and time. HHP treatment induces changes in milk appearance and casein particle size, denaturation of whey proteins such as $\beta$-LG, $\alpha$-LA and Lf and induces the
interaction of whey proteins with casein micelles and MFGM through sulphydryl-disulphide exchange with the thiol group from β-LG or from MFGM proteins.\textsuperscript{292-295}

Despite HHP treatment of milk being studied extensively, the commercialization of this technique is far behind due to the extent of changes that it induces to milk components, less effect on enzymes and the requirement of heat to accomplish the complete microbial inactivation. Moreover, HHP treatment of milk requires high capital cost and energy which therefore could increase the cost of processing and affect the affordability to consumers.\textsuperscript{300}

1.7.7.3 Pulsed electric field

Pulsed electric fields (PEF) is emerging as one of the interesting alternatives to conventional thermal processing methods in liquid foods. In the early 1960s, PEF was introduced to inactivate microbes in foods.\textsuperscript{263} PEF is the process of applying electric field (approximately 20-80 kV/cm) in foods to inactive microorganisms, enzymes, and/or modifying the product. The application of PEF causes temporal destabilization and perforation of the lipid bilayer and proteins of bacteria, and they re-seal once the electric field is stopped or removed. Whereas high intensity electric fields cause irreversible damage to microorganism through leakage of cellular contents or lysis of the cell wall preventing repair.\textsuperscript{301} The key factors that determine the efficiency of PEF in microbial inactivation are electric field strength, pulse length, pulse shape, number of pulses and initial temperature.\textsuperscript{302,303}

PEF has been used in processing of liquid foods such as juices, milk, yoghurt, soups and liquid eggs. PEF was shown to inactivate various spoilage and pathogenic microorganisms with a marginal increase in temperature. Moreover, PEF has the advantage of causing lower fouling compared to thermal methods.\textsuperscript{304} Sampedro \textit{et al.} did not observe a significant influence of PEF on the physical, chemical or sensorial properties of milk and taste was similar compared to pasteurised milk.\textsuperscript{305} Bendicho \textit{et al.} observed a small reduction in the vitamin content of milk after intense PEF treatment with a large number of pulses.\textsuperscript{302}
Shamsi et al. studied PEF treatments (25-37 kV cm\(^{-1}\) and up to 50 kV cm\(^{-1}\)) plus temperature ranges of 30 °C to 75 °C in skim milk. They found that combining PEF treatment with heat increased the inactivation level of all enzymes and showed an increasing trend with the increasing field intensity and temperature. The size of casein micelles and fat globules was not affected by PEF treatment while severe heating of milk at 97 °C for 10 min decreased both micelle and fat globule sizes marginally.\(^{306}\)

Fernandez-Molina et al. found that PEF treatment is more effective in combination with high temperature. The high temperature and PEF enhanced the microbial inactivation, especially the temperature effects are dominated on the cell membrane properties, and reduced the thickness of the lipid bilayer to cause cell damage. They have shown that treated milk using this method has potential storage for up to 30 days.\(^{307}\)

Several authors found that PEF has limited effect on bacterial spore and enzyme inactivation. The recent investigations have focused on the use of combining PEF and heat to increase the inactivation efficiency in milk.\(^{305,308}\) Wouters et al. reported that both microbial and enzyme inactivation by PEF are largely dependent on liquid composition, conductivity, ionic strength and pH.\(^{309}\) The presence of high fat and/or protein content seem to have a protective effect for bacteria during PEF treatment in milk, whereas PEF is more effective in liquid foods with low conductivity, i.e. low salt or ionic strength, however, other studies did not notice any influence of the above-mentioned parameters.\(^{302}\)

Bendicho et al. investigated the inactivation kinetics of enzymes such as alkaline phosphatase, plasmin, lipase and peroxidase present in milk using PEF.\(^{302}\) The authors found varying effects on enzyme activity during PEF treatment. In their other work on enzymes of bacterial origin, they found only 13% inactivation of lipase from \textit{P. fluorescens}.\(^{310}\) In contrast, it was found that PEF has effectively inactivated protease enzyme from \textit{Bacillus}.\(^{303}\) Floury et al. found that PEF also affected proteins, especially casein micelles, decrease in viscosity and enhanced
coagulation properties of PEF-treated milk.\textsuperscript{311} Xiang \textit{et al.} observed an increase in the effective volume of the fat globule due to interaction with denatured skim milk serum proteins.\textsuperscript{312} The electric field intensity, number of pulses, and concentration of proteins in their study contributed to the alteration of whey proteins.\textsuperscript{312} Belloso found that the whey protein denatured in the order of $\alpha$-LA $>$ BSA $>$ $\beta$-LG after PEF treatment.\textsuperscript{313}

1.7.7.4 Ultraviolet light

Ultraviolet (UV) irradiation or light is also considered as one of the potential non-thermal methods. UV light is the process of applying light energy to foods to decontaminate and extend the product shelf life. The common three types of UV light are, UV-A (315-400nm), UV-B (280-315nm) and UV-C (200-280nm).\textsuperscript{314, 315} The UV-C (200-280nm) possess antimicrobial activity and is effective to kill pathogens in this wavelength range, however beyond 300 nm this potential is limited. It was shown that this wavelength is efficient to kill micro-organisms such as bacteria, algae, yeasts, moulds, protozoa and viruses.\textsuperscript{316-318} The exposure of micro-organism to UV-C light results in damage of DNA molecule due to thymine dimer formation which prevents the genetic processes such as transcription and replication that eventually leads to cell death.\textsuperscript{316-318} The key factors such as resistance of micro-organisms based on their type to UV light, and absorption properties of medium in which micro-organisms are suspended, and the UV dose applied to the medium determines the efficacy of UV light treatment.\textsuperscript{319, 320} UV treatment have certain advantages such as low installation, production and maintenance costs, emits less carbon when compared to traditional decontamination methods.\textsuperscript{321, 322} UV light is generally regarded as suitable to reduce the microbial load in pumpable fruit and vegetable products. Moreover, since 2000, United States Food and Drug Administration (USFDA) has approved the use of UV radiation to treat fruit juices.\textsuperscript{323}

UV treatment received considerable interest as an alternative non-thermal method to treat milk.\textsuperscript{324} Several authors have reported the use of UV-C to decontaminate raw cow and goat
milk with different fat amounts and at different temperatures.\textsuperscript{325-327} Choudhary et al. used two coiled tube reactors and investigated the use of UV-C to inactivate \textit{Escherichia coli} W1485 and \textit{Bacillus cereus} endospores in non-skimmed and skimmed raw cow milk at room temperature. They found that the microbial inactivation was higher in skimmed compared to non-skimmed cow milk.\textsuperscript{328} Krishnamurthy et al. has reported that pulsed UV has a higher penetration depth than continuous mode and has effectively reduced bacteria in milk.\textsuperscript{329} The key elements such as UV wavelength, intensity, thickness of the radiation path and flow turbulence determines the UV effects in milk. Werrrs et al. showed that the combination of \textit{H}_{2}\textit{O}_{2} and UV radiation with or without heat produces a greater lethal effect on bacterial spores suspended in phosphate buffer solution compared to their individual counterpart effects.\textsuperscript{330} They explained that the UV irradiation breaks down the \textit{H}_{2}\textit{O}_{2} into OH radicals which then enter into the bacterial spore to achieve the bactericidal effect and the presence of heat enhanced this effect.

Matak et al. reported on sensory properties of goat’s milk treated with UV technology. The extent of lipid oxidation and hydrolytic rancidity was measured by TBARS and acid degree values. However, sensory studies indicated that the increase in free fatty acids was not enough to cause detectable off-odors in the milk. The formation of pentanal, hexanal, and heptanal was identified and they were increasing significantly with the increase in UV dose. The chemical analyses supported the findings from the olfactory studies showed that UV irradiation at the wavelength 254 nm was detrimental to certain chemical properties of fluid milk.\textsuperscript{331} Although UV treatment possess certain advantages in terms of capital and operating costs, it suffers in maintaining the sensory and nutritional quality of milk. The limitations in inactivating the microbes, loss of nutrients, lipid oxidation and formation of off-flavors in milk after UV treatment still remain as major challenges.\textsuperscript{324, 327, 331, 332}
1.7.7.5 Ultrasound technology

Ultrasound or ultrasonication is the process of generating waves with a frequency higher than 20 kHz, to travel through gas, liquid and solid materials.\(^ {333}\) Although, it was reported in the late 1920’s that ultrasound can be used to destroy microorganisms, it was not considered as a sterilization method until further improvement in the ultrasound technology had been made.\(^ {334}\) When ultrasound waves transmit into liquid medium, alternating compressions and expansion cycles are produced. Ultrasound waves create small bubbles which grow in the liquid medium during the expansion cycle, these small bubbles implode violently once they reach a stage when they can no longer absorb more energy. During this implosion, very high temperatures (~5500°C) and pressures (~50 MPa) are exerted inside these bubbles.\(^ {335, 336}\) This violent collapse breaks the cell walls of micro-organisms which eventually leads to cell death.\(^ {337}\) The effects of cavitation on micro-organisms are diffusion of microbial clumps, puncturing of cell wall, alternation in cellular activity, and increased heat sensitivity.\(^ {338}\) Reports claimed that these could be the reasons for ultrasound mediated bacterial inactivation.\(^ {339}\) However, the efficiency of ultrasonication depends on what type of micro-organism are tested, the suspending medium, cell size, and electrical power unit.\(^ {337}\)

Ultrasound can be classified into two types based on the operating frequencies and power input.\(^ {340}\) Low intensity ultrasound is operated in the regime of high frequencies (0.1–20 MHz) and low power levels (≤1Wcm\(^{-2}\)). This method generally leaves the system unaltered and could be used as a tool to measuring food properties. High intensity ultrasound is often operated in the low frequency (≤0.1 MHz) range and high power (10-1000 W cm\(^{-2}\)) which could alter physico-chemical properties of products.\(^ {340}\)

Ultrasonication in milk depends on factors such as frequency, power, and stimulation time.\(^ {341}\) Ultrasonication is mainly used for the reduction of fat globules present in milk which efficiently homogenizes the milk. This technique reduces the particle size of MFGM and uniformly
distributes the smaller milk fat globules.\textsuperscript{342, 343} Also, this causes changes in the composition and structure of the fat globule membrane that improves the homogenization in casein gel when compared to other methods.\textsuperscript{344} Ultrasonication treatment causes changes in milk protein secondary structure, protein particle aggregation, and denaturation which could help in the processing of several dairy foods.\textsuperscript{345} Many researches have demonstrated that the high power ultrasound technique improves the cheese or yogurt making process, by destroying bacteria and inactivating enzymes.\textsuperscript{346, 347}

The use of ultrasound in milk and milk products is generally to inactivate bacteria and enzymes, homogenize the fat globules, extract enzymes and hydrolyze lactose.\textsuperscript{348} Zenker \textit{et al.} reported that ultrasound aided thermal treatments required lower processing temperatures to decontaminate milk compared to conventional thermal methods.\textsuperscript{349} Mano-thermo-sonication (MTS) is the method of combining ultrasound, heat and pressure to process liquid foods. Vercet \textit{et al.} found that the use of MTS did not significantly affect the nutrient content of milk, whereas higher non-enzymatic browning was observed under MTS than under heat treatment.\textsuperscript{350} The same author, in other work, assessed the effect of MTS in milk for the production of yoghurt and found that MTS yoghurts had stronger structures which could be due to the increased viscosity and whey protein denaturation.\textsuperscript{351}

Annandarajah studied the feasibility of integrating ultrasound at short durations (\(\leq 60\) s) to inactivate plasmin enzyme in milk as an adjunct to pasteurization, thereby extend shelf life. The author did not find any significant difference in reducing plasmin activity between control pasteurized and psychrosonicated samples followed by pasteurization, thus confirming that the major reduction of plasmin activity is a result of pasteurization.\textsuperscript{352}

Several authors studied the effect of high intensity ultrasound and heat treatment on fat, proteins and native enzymes in milk. They commonly found little effect on enzymes when ultrasound alone was used. On the other hand, the size of the fat globule was significantly
reduced and distributed evenly, whey proteins such as α-LA and β-LG and native enzymes such as alkaline phosphatase, lactoperoxidase and γ-glutamyltranspeptidase were highly denatured when ultrasound and heat was used combinedly.\textsuperscript{343, 353} It was reported that the ultrasonication hydrolyzed lactose into glucose and galactose by releasing the enzyme β-galactosidase.\textsuperscript{354}

Ultrasound related studies in milk showed that this technique could produce only a slight effect on microbes and enzymes and could work better only in combination with heat and/or pressure treatments.\textsuperscript{349, 351} This requires higher energy consumption and increases the cost of processing. Although it has not been well studied, ultrasonication treatment might induce changes in some physiochemical properties, off-flavours of food products, degradation of some components and changes in physical properties.\textsuperscript{355} Moreover, this might be difficult to scale up and bring the technology to commercial application.

1.7.8 Research challenges, objectives and our approach

As discussed current pasteurization techniques are known to induce changes in the physicochemical characteristics of milk and milk products. These changes are protein denaturation, fat agglomeration, loss of vitamins, lactose degradation, browning reactions, and flavor.\textsuperscript{263, 268-271, 273}

Non-thermal technologies such as high hydrostatic pressure, ultrasound, pulsed electric field and UV radiation suffer from lower efficiencies, extensive treatment times, specialized equipment requirements and the requirement of additional heat to achieve effective inactivation, energy consumption, capital and processing costs. These significant challenges make the emerging non-thermal technologies difficult to scale up to an industrial level.

Atmospheric pressure plasma is one of the emerging potential non-thermal technique which has been demonstrated to kill bacteria either in solid or liquid food matrices without a
significant increase in temperature during food processing. Plasma, in and in contact with liquid, produces both chemical (short lived species mainly O, OH radicals and long lived species mainly H₂O₂, NO₃) and physical effects (PEF and UV radiation). These effects were reported to involve etching of the cell surface or membrane, leakage of contents, DNA damage and volatilization of compounds.

However, there are very few studies examining plasma treatment on microbial quantity, shelf life and physicochemical characteristics in milk. Gurol et al demonstrated that corona discharge can significantly reduce *E. coli* colony forming units without having any negative effects on pH and color measurements in milk. Kim et al achieved aerobic bacterial reduction using dielectric barrier discharge and noticed non-significant changes in the fatty acid composition of milk. Rahman et al investigated pulsed high voltage discharges and proposed shock waves might play a critical role to kill bacteria in milk. The previous plasma related milk studies have produced plasma (such as corona and dielectric barrier discharge) above the liquid (milk) surface where it might be difficult to achieve high production of controlled and selective reactive species. This could be due to the interference from the atmospheric air and recombination of reactive species in the air before diffusing into milk. Therefore, a technology which improves the microbial safety and extend shelf life while maintaining nutritional qualities of milk is still lacking.

As mentioned earlier, the direct discharge in liquid requires higher electric field, resulting in faster electrode erosion and thereby reducing the life time of electrodes, whereas the discharge in gas phase faces issues of less selectivity in reactive species, interference from atmospheric air and formation of unwanted by-products. Several researchers have developed a method of generating plasma in bubbles that are externally injected into liquids to reduce the energy consumption, electrode erosion, control and increase the gas-liquid interface reactions, thereby improving the productivity of physical and chemical processes in liquid.
These advantages can help extend the application of plasma technology to treating thermally sensitive materials, especially liquid foods.

Several authors reported that the discharge in bubbles that are externally injected into liquids (water) produce chemical and physical effects based on the composition of gas in the bubble (and its size), conductivity of the liquid and input power. In other work, we have shown that the discharge at higher frequency created more fragmentation in gas bubbles which decreased the size and increased the number of bubbles in DI water. This increased the interface reactions between the gas-plasma and liquid. The increase in the interface reactions, along with the movement of bubbles, enabled higher production of selective reactive species based on the chosen gas.

The major requirements of the dairy industry are the use of low temperature and fewer chemicals to reduce the bacterial load, increase shelf life with minimal or no changes in the nutritional quality of milk. To address these challenges associated with thermal and non-thermal decontamination technologies, our approach aimed to develop a technique using nanosecond pulsed atmospheric pressure plasma in bubbles that are externally introduced in milk (set up II is shown in Figure 1.8) to decontaminate, extend shelf life whilst protecting nutrition. This approach includes the following steps.

- Decontamination of milk to extend shelf life
- Assessing the effect of plasma on proteins, lipids, carbohydrates and micro RNA present in milk

The development of this kind of technology has potential applications in the dairy industry, including in cheese making, butter, cream and yoghurt production. This technology may also be used to treat human breast milk and other types of milk (for example buffalo, goat and sheep etc.).
Figure 1.8. Schematic view of plasma in bubbles that are externally introduced using a hollow needle electrode (top) and mesh electrode (bottom) immersed in liquid (milk or water)
Chapter 2 Fabrication of Boron Nitride Nanotube-Gold Nanoparticle Hybrids using Pulsed Plasma in Liquid

This work was published in Langmuir Journal. In this work, as a PhD student, I have contributed more than 50% and all the co-authors in this publication has agreed and signed the % contribution declaration form set by Deakin University Higher Degrees by Research for Thesis by Publication. Herewith, I have attached the same declaration form.

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### Abstract

Plasma, generated in liquid at atmospheric pressure by a nanosecond pulsed voltage, was used to fabricate hybrid structures from boron nitride nanotubes and gold nanoparticles in de-ionized water. The pH was greatly reduced, conductivity was significantly increased and concentration of reactive oxygen and nitrogen species in the water were increased by the plasma treatment. The treatment reduced the length of the nanotubes, giving more individual cup-like structures, and introduced functional groups onto the surface. Gold nanoparticles were successively assembled onto the functionalized surfaces. The reactive species from the liquid plasma along with the nanosecond pulsed electric field seem to play a role in the shortening and functionalization of the nanotubes and the assembly of gold nanoparticles. The potential for targeted drug delivery was tested in a preliminary investigation using doxorubicin-loaded plasma-treated nanotubes which were effective at killing ~99% of prostate cancer cells.

**Graphical Abstract**
Plasma in liquid was used to shorten and functionalize boron nitride nanotubes yielding short tubes and nanocups with attached gold nanoparticles

Note: In this chapter, the term ‘liquid plasma’ refers to the plasma in gas phase generated above the liquid surface.

2.1 Introduction

Hybrid nanostructures using assemblies of nanotubes and nanoparticles have been investigated for hetero-junctions, hydrogen storage and gas sensors.\textsuperscript{129, 364, 365} The hybrid structure can help to integrate the useful properties of the nanotubes and nanoparticles and also benefit from synergies of their physical and chemical properties. These hybrid nanostructures have been used in biosensors but also have other bio-applications such as drug delivery and anti-cancer treatment.\textsuperscript{366, 367} The aim here was to fabricate a hybrid structure of boron nitride nanotubes (BNNTs) with gold nanoparticle using the new technique of generating plasma in liquids and evaluation of their potential for drug delivery. The fabrication necessitated shortening and functionalization of the BNNTs and assembly of gold nanoparticles onto the functionalized surface.

Carbon nanotubes (CNTs) have been predominantly been used in hybrids, so far, but possible CNT cytotoxicity remains an issue.\textsuperscript{368, 369} Boron nitride nanotubes are structural analogues of CNTs with promising mechanical, thermal and chemical properties compared to CNTs, which
makes them suitable for potential bio-applications.\textsuperscript{70, 71, 78, 370-372} However, improving the process-ability, i.e. shortening, functionalization and attachment of nanoparticles onto BNNTs, is the main challenge. Especially in biological applications, the long nanotubes may behave like an asbestos fibre with difficulty in cell uptake and cell toxicity.\textsuperscript{116, 373, 374} Recently, the use of short nanotubes and their advantages of improved biocompatibility and easy cellular uptake for bio-applications have been discussed.\textsuperscript{375} Vandsburger \textit{et al.} have claimed that oxidation induced by gaseous oxygen plasma discharge can play a key role in reducing the length of CNTs.\textsuperscript{148} The production of boron nitride nanocups (BNNCs), a kind of hollow BN nanomaterial, might also be an additional advantage.\textsuperscript{376}

Shortening can improve the performance of BNNTs to a certain level, however functionalization of BNNTs is essential for specific interactions (for example in targeted drug delivery) and also for the attachment of nanoparticles. A simple and effective method of functionalization is still required to overcome the problems of instability of the treatment, low density of functional groups and damage to the nanotube structure.\textsuperscript{136, 138, 139} It has been reported that the controlled surface functionalization of BNNTs can be achieved by gentle low pressure gas plasma treatment while maintaining the intrinsic nanotube structure.\textsuperscript{152, 377}

There have been a few attempts to modify BNNTs with nanoparticles such as tin dioxide (SnO\textsubscript{2}), gold (Au) and iron (Fe) with the help of non-specific interactions.\textsuperscript{127, 129, 143} Except for the SnO\textsubscript{2} nanoparticles, either Au or Fe nanoparticles did not entirely cover the BNNTs. However, Sainsbury \textit{et al.} showed that Au nanoparticles can fully cover BNNTs using S-Au chemistry.\textsuperscript{128}

Plasma-aided nanofabrication is an alternative approach that can be applied both to produce nanostructures and uniformly tailor the surface of nanomaterials in a well-controlled manner. It has been claimed that oxidation induced by a low pressure gaseous oxygen plasma discharge
can be used to shorten nanotubes. It has also been shown that low pressure gas plasma can be used to functionalize nanotubes. The use of atmospheric pressure plasma in liquids is a new approach which has been investigated for the surface modification of different nanomaterials and substrates.

In this work, a new “liquid plasma” method was developed to fabricate boron nitride nanotube-gold nanoparticle hybrid nanostructures using a nanosecond pulse atmospheric pressure plasma system (NPAPP) in a two-step process. The first step was to shorten and functionalize the BNNTs and the second step was to assemble the gold nanoparticles onto the functionalized short BNNTs, both using liquid plasma. In order to understand the chemistry, the changes in pH, conductivity and the concentration of reactive oxygen and nitrogen species were recorded after the plasma treatment in water. The plasma treated materials were characterized by transmission electron microscopy and X-ray photo electron spectroscopy. The potential of the plasma treated nanotubes to deliver drugs was tested in a preliminary investigation in which prostate cancer cells were exposed to the untreated and plasma-treated BNNTs at different volumes, with and without the model drug doxorubicin.

2.2 Experimental Section

2.2.1 Plasma system description

Liquid plasma is a system that consists of a nanosecond pulse generator, two output channel electrodes and the reaction chamber with 10 mL of DI water. The two electrodes, with one electrode inside the liquid (bottom) and the other electrode 3 mm above the liquid (top) are shown in scheme 2.1. The plasma is generated between the top electrode and the bottom electrode using argon gas with a flow rate of 0.083 L/min. The pulse parameters were 10 nanosecond pulse width, 10 kHz frequency and ±15 kV voltage.
Scheme 2.1. Schematic view of nanosecond pulse atmospheric pressure plasma system, using argon gas

2.2.2 Synthesis of BNNTs

Ball milling was used to produce the BNNTs. Initially, a few grams of B powder and 10 wt% Fe (NO₃)₃.9H₂O were sealed in a rotating steel-milling jar with four hardened steel balls where the ball to powder ratio was 132:1. Prior to the milling treatment, anhydrous ammonia (NH₃) as the reaction gas was aerated into the jar with a static pressure of 300 kPa. The milling process lasted for 150 hours to produce nanosized B particles with metastable structures. Finally, the milled sample was placed in a crucible which was positioned in the middle of a horizontal tube furnace and heated to 1100 °C in N₂ + 15% H₂ atmosphere to produce BNNTs. The BNNTs had a length of 12-15 µm.

2.2.3 Synthesis of GNPs

An equal volume of aqueous hydrogen tetrachloroaurate (III) solution (HAuCl₄·3H₂O) (5 ml, 10 mM, 37 °C) was mixed with bovine serum albumin (BSA, Bovogen Biologicals) solution (5 ml, 50 mg/ml, 37 °C), under vigorous stirring. After two minutes, sodium hydroxide (NaOH) solution (0.5 ml, 1 M) was added to the mixture which was incubated at 37 °C for a maximum of 12 hr. After this addition, the colour of the solution changed from light yellow to light brown
and then to dark brown. The final product was purified by centrifugation at 7000 rpm for 15 minutes and then filtered with a 0.22 µm syringe filter. Prior to use, all glassware was washed with Aqua Regia (hydrogen chloride (HCl): nitric acid (HNO₃), volume ratio – 3:1) and rinsed with ethanol and Millipore water.

2.2.4 Hybrid nanostructure

Liquid plasma was used to 1) produce short BNNTs and BNNCs from long bamboo-like BNNTs and to functionalize the surface of short BNNTs and BNNCs and then 2) to assemble GNP onto the functionalized short BNNTs and BNNCs, as shown in scheme 2.2.

The as-synthesized BNNTs were added to 10 ml of de-ionized water and magnetically stirred at a constant 1000 rpm for 5 minutes to make a uniform suspension. These uniformly suspended BNNTs were then treated by the liquid plasma for 15 minutes with an argon gas flow rate of 0.083 L/min.

For comparison, pure GNP were allowed to assemble onto the liquid plasma treated BNNTs separately under two conditions i) in liquid plasma for 15 minutes and ii) undisturbed for 12 hours at room temperature. 1.4 mL of purified gold nanoparticles (1:10 dilution) were added to the plasma treated BNNTs, and magnetically stirred at a constant 1000 rpm for 5 minutes to make a uniform suspension. This solution was equally divided into two parts.

1) One part of this solution was then treated by the liquid plasma for 15 minutes with an argon gas flow rate of 0.083 L/min.

2) The other part of the solution was left undisturbed for 12 hours.
Scheme 2.2. Schematic view of shortening, surface functionalization of short BNNTs and BNNCs and attachment of GNP

2.2.5 pH and Conductivity

pH (TPS, Australia) and Conductivity (TPS, Australia) probes were used to measure the pH and conductivity of the plasma treated de-ionized water.

2.2.6 Reactive oxygen and nitrogen species measurement

2.2.6.1 Hydrogen peroxide

Hydrogen peroxide (H$_2$O$_2$) concentration was measured using the potassium permanganate method. In a titration, plasma treated water which contains H$_2$O$_2$, reduces the potassium permanganate to a colourless product.

2.2.6.2 Nitrite and Nitrate

Nitrite and Nitrate concentration was measured using a flow injection analysis method (at Eurofins laboratory, Melbourne)

2.2.7 Drug loading
Doxorubicin (Sigma Aldrich) model drug (0.6 mg/mL) was mixed with untreated and plasma treated BNNTs. The absorbance values were measured at 490 nm, immediately and after 16 hours to estimate the amount of drug loaded onto the untreated and plasma treated BNNTs. The reduction in the absorbance values of doxorubicin at 490 nm indicated the adsorption and loading of the drug on to the BNNTs. Loading capacity of the BNNTs were calculated as follows

\[
\% \text{ Drug loading capacity} = \left( \frac{\text{Absorbance of loaded samples}}{\text{Absorbance of pure doxorubicin}} \right) \times 100
\]

2.2.8 Cell Lines and cell culture conditions

Human prostate adenocarcinoma cells (DU145) were obtained from American type culture collection (ATCC), Manassas, VA, USA. Minimum Essential Media with Earle’s salts (EMEM) was purchased from Gibco, Australia. The growth factor, fetal bovine serum (FBS) was purchased from Bovogen, Australia. The antimycotic-antibiotic and penicillin/streptomycin were purchased from Gibco, Australia. The 0.25% Trypsin/EDTA was purchased from Gibco, Australia. Cells were cultured at 37 °C in EMEM containing 10% FBS in an incubator with 5% CO\textsubscript{2}. Media was changed every 3 days and once the cells reached a confluency level of 80-90% in the culture flasks, they were trypsinized.

2.2.9 Analysis of cell proliferation by CyQuant assay

In order to observe the effect of untreated and plasma treated BNNTs on the proliferation of prostate cancer cells (DU145), a CyQuant assay was performed which has been accepted as an optimum quantitative measure of cell proliferation.\textsuperscript{381} 2 x10\textsuperscript{3} cells were seeded in 96 well plates and once confluent they were exposed for 24 hours to the untreated and plasma treated BNNTs at different volumes (30, 60 and 90 µL) with and without doxorubicin drug (0.6 mg/mL). Untreated cells cultured in complete growth media were used as the positive control and the cells treated with 1% Triton-X-100 were used as the negative control. After media
removal, the plates were wrapped in foil and stored at -80 °C for a little over 24 hours. The CyQuant reagents were added to cells according to manufacturer’s instructions (Invitrogen, Australia). In short, 20 μL of CyQuant GR dye was diluted 400 fold in 1X lysis buffer which was prepared by diluting 400 μL of component B into 7.6 mL sterile water and 200 μL of this solution was added per well in the dark. Fluorescence readings were taken at 480/520 (absorbance/emission) and percentage cell proliferation was calculated relative to the controls. Also, the changes in the cell morphology caused by the cytotoxicity of these BNNTs were observed using an inverted light microscope.

2.3 Results and Discussion

2.3.1 Shortening of BNNTS

Long, bamboo-like BNNTs were produced by a ball milling and annealing method. The diameter of the long, bamboo-like BNNTs was in the range ~40-60 nm with lengths up to ~12-15 µm (Figure 2.1a). The as-synthesized long BNNTs were treated by the liquid plasma for 15 minutes which resulted not only in shortening of the BNNTs and separation into BNNCs (Figure 2.1) but also functionalized the short BNNTs and BNNCs in a single step (Figure 2.2).

After liquid plasma treatment for 15 minutes, the length of the BNNTs was reduced from ~12-15 µm to ~1-2 µm (see Fig. 2.1a and 2.1b-d) and the observed BNNCs were ~100-200 nm (Figure 2.1e inset). The comparison of peak length distribution of untreated (~12-15 µm) and plasma treated BNNTs (~1-2 µm) is shown in Figure S2.1 of the supporting information. It has been reported that the exposure of atmospheric pressure plasma to a liquid creates an acidic environment that consists of reactive oxygen (hydroxyl radicals, ozone, hydrogen peroxide, superoxide anions, singlet oxygen) and nitrogen species (nitric oxide, nitrite, peroxynitrite and nitrate).
Figure 2.1. TEM images of a) untreated and b) to d) liquid plasma treated BNNTs on a carbon coated grid. e) Inset showed the magnified image of individual BNNCs

In order to understand the chemistry of the plasma treated aqueous solution, de-ionized water was separately treated by liquid plasma at 5, 10 and 15 minutes. The changes in pH, conductivity and the concentration of reactive oxygen and nitrogen species were recorded after plasma treatment in water. It was found that the pH values were drastically reduced and the conductivity values were increased with respect to increase in plasma treatment time (Table S2.1, supporting information). This suggests the generation of reactive oxygen and nitrogen species from the plasma which has also been observed by others. The importance was particularly paid to measure the increase in the concentration of long lived species like hydrogen peroxide (Table S2.1, supporting information) and nitrate (25 and 49 mg/L at 5 and 10 minutes respectively) which may play a significant role in the shortening of BNNTs.
In X-ray photoelectron spectroscopy (XPS), it was also observed that the atomic percentage of oxygen on the BNNT surface was increased from 8 to 27.4% (Figure S2.2, supporting information) after plasma treatment, which suggests that the produced reactive oxygen species like hydrogen peroxide contributed for the increase in the percentage of atomic oxygen. The shortening of BNNTs might be attributed to the reactive oxygen species like hydrogen peroxide from the plasma which oxidize the nanotube ends and cup junctions, thus promoting the shortening of nanotubes and/or separation of the cups (Figure 2.1). This hypothesis appears also to be supported by reports which utilized chemical methods (using hydrogen peroxide) to shorten BNNTs by oxidation. The shortening may depend upon the level of reactive oxygen species formed in liquid or the plasma treatment time. It may also be due to physical processes such as the strong nanosecond pulsed electric field from the plasma, which may attack defective sites of nanotubes. As a result of shortening, an increase in the dispersibility of the BNNTs (clear suspension) was observed. Golberg et al. have also observed that the shortening of BNNTs increases their dispersibility and drug loading potential.

4.3.2 Functionalization of shortened BNNTs and BNNCs

The X-ray Photoelectron Spectroscopy (XPS), N1s peak for the liquid plasma treated BNNTs is shown in Fig. 4.2a and the peak for the untreated sample is shown in the inset. The treated sample showed an additional peak at 406.8 eV (N-O) for the N1s peak (Figure 2.2a), which was only observed after liquid plasma treatment and was not observed in the gas plasma treatment. One possible mechanism is that the reactive oxygen species including hydrogen peroxide (H2O2) from the liquid plasma may oxidize the nitrogen sites of BNNTs. Studies of the oxidation of BNNTs by other methods have reported that the oxidation affects only the ‘B’ site. In this work, no change in the B1s, XPS spectrum was observed after plasma treatment (Figure S2.3, supporting information). Another mechanism is that some NOx groups (nitrate-NO3− or nitrite-NO2−) formed at the gas-liquid interface, diffuse into the liquid and then
attach to the surface of the BNNTs and BNNCs. However, in this work, argon was used as the working gas (free of air) and so the amount of reactive nitrogen species formed should be less than that from plasma generated using air.\textsuperscript{389} This suggests that the possibility of reactive nitrogen species playing a role is very limited.

It was found that the N-O component, as a fraction of the total N1s peak area, was increased by prolonged plasma treatment time (Figure S2.4, supporting information for more details). The other peaks at 397.9 eV and 399.8 eV in Fig 2.2a, correspond to N-B and N-H functionalities. The C1s peak for the liquid plasma treated BNNTs is shown in Figure 2.2b and the peak for the untreated sample is shown in the inset. The treated sample showed two additional peaks at 286.4 eV and 288.3 eV, indicating that OH and OOH functional groups\textsuperscript{390} were successfully introduced onto the surface of shortened BNNTs/BNNCs. This functionalization facilitated the easy attachment of gold nanoparticles onto the surface of BNNTs and BNNCs.

Figure 2.2. XPS spectra of liquid plasma treated BNNTs a) N1s and b) C1s. Inset shows the N1s and C1s spectra of untreated BNNTs

4.3.3 Assembly of GNPs onto functionalized short BNNTS and BNNCS
Gold nanoparticles were produced using the bovine serum albumin (BSA) method (see experimental section). GNPs were chosen particularly because of their well-established unique physical, chemical and biological properties for applications in bio-imaging and biosensors. The synthesized GNPs were characterized by transmission electron spectroscopy (TEM) and XPS. TEM images (Figure S2.5, supporting information) showed that the average diameter of GNPs was ~5±2 nm. XPS showed a single doublet with a principal peak at 83.5 eV which corresponds to (metallic) Au4f7/2 (Figure S2.6, supporting information).

For comparison, pure GNPs were allowed to assemble onto the functionalized short BNNTs separately under two different conditions: in liquid plasma for 15 minutes and undisturbed for 12 hours at room temperature.

A comparison of Figures 2.3a and 2.3b indicates that the GNPs were more effectively assembled onto the BNNTs in the liquid plasma than when left undisturbed for 12 hours. Notably, the GNPs were partially aligned and assembled especially at the centre and cup joints/necks of BNNTs (see Figure S2.7, supporting information). This can be possibly explained by the influence of reactive oxygen species which leads to localized oxidation at the center and cup joints of BNNTs, and as a result of functionalization (OH and OOH groups) the GNPs were assembled there. It may also be due to the increased conductivity of the liquid by adding GNPs, together with the defective sites of BNNTs/BNNCs, result in a redistribution of the electric field. This redistributed electric field might contribute the assembly of the GNPs only at the center and cup joints of BNNTs. Interestingly, in some instances, the GNPs assembled around the tip of a short BNNT (inset Figure 2.3c) which further suggests that liquid plasma effect is stronger at the defective regions.

It should be noted that the efficiency of hybrid nanostructure fabrication may depend upon the level of reactive species in water. The reactive species like ozone in the liquid may disappear
quickly due to their short lifetimes at atmospheric conditions and the long lived species like hydrogen peroxide may play a key role in the effective fabrication of hybrid nanostructures.

![Image](image.png)

**Figure 2.3.** TEM images of the functionalized BNNTs with GNPs under a) undisturbed for 12 hours and b) liquid plasma for 15 minutes. Inset c) shows the assembly of GNPs around the tip of a short BNNT. Black arrows indicates the GNPs attached to BNNTs

2.3.4 Chemistry involved in the assembly

A proposed mechanism is that the OH and OOH functional groups at the surface of liquid plasma treated BNNTs and BNNCs have a strong affinity to the sulphur group from the 35 cysteine residues of BSA covered GNPs as shown in scheme 2.3. The Sulphur (S2p) XPS spectra of (a) synthesized GNPs, (b) functionalized BNNTs with GNPs left undisturbed for 12 hours and (c) functionalized BNNTs with GNPs in liquid plasma for 15 minutes (c) are shown in Figure 2.4. This comparison (Figure 2.4) can be used to explain the changes in chemistry involved in the attachment of GNPs to the surface of BNNTs/BNNCs. The XPS scans of sulphur showed two peaks i) sulphur (right) and ii) oxidized sulphur (left). After the liquid plasma treatment, the intensity of the sulphur peaks was greatly reduced and the intensity of
the oxidized sulphur peaks was significantly increased in (b) and (c). In addition, the position of the sulphur peaks was unchanged but the oxidized sulphur peaks were slightly shifted in (b) and (c) after the plasma treatment (brown dotted lines). This peak shift resulted from the conversion of the sulphur (S) to sulphones (-S(=O)2-) when attaching to OH or OOH functional groups on the BNNTs (as described in Scheme 2.3). These changes suggest that the BSA (which contains sulphur residues) covered GNPs were more oxidized in (b) and (c) compared to (a) due to the presence of reactive oxygen species from the plasma. It was observed that the intensity of the oxidized sulphur peak in (b) was also equivalent to (c). This could be due to the prolonged incubation (12 hours) of GNPs in the plasma treated BNNTs, but the equivalent intensity of oxidized sulphur peak was achieved in (c) when liquid plasma was used for 15 minutes. This effective assembly of GNPs to BNNTs shows that pulsed liquid plasma has the capability to produce hybrid nanostructures in a short time.
Figure 2.4. XPS, Sulphur (S2p) spectrum of synthesized GNPs (a), functionalized BNNTs with GNPs left undisturbed for 12 hours (b) and functionalized BNNTs with GNPs in liquid plasma for 15 minutes (c)

Scheme 2.3. Schematic of chemistry involved in the attachment of GNPs to the BNNTs by the liquid plasma treatment

2.3.5 Potential of plasma-treated BNNTS as an anti-cancer agent

In a preliminary trial to understand the biological interaction of BNNTs, the ability to hold more drug molecules (loading capacity) and cytotoxicity of untreated and plasma treated BNNTs were assessed. The decrease in the doxorubicin absorbance indicated that the drug loading on to the BNNTs was efficient. It was observed that the plasma treated BNNTs can hold more drug molecules (~36%) than the untreated BNNTs (Figure 2.5). Golberg et al. also observed that the BNNTs shortened by chemical methods can hold more drug molecules. They demonstrated that shortened BNNTs possess a hole-rich (i.e. porous) structure and more ends which might provide i) increased possibilities for molecules to enter the tubes instead of
solely locating on the surface and ii) better dispersibility which enhances the chance of BNNT-molecule interaction. These advantages suggest that shortened BNNTs might be useful for efficient drug delivery systems.

Figure 2.5. Doxorubicin model drug (0.6 mg/mL) was mixed with untreated and plasma treated BNNTs. The absorbance values were measured at 490 nm, immediately and after 16 hours. Results are expressed as mean ± standard deviation of triplicate determinations.

The cytotoxicity of untreated and plasma treated BNNTs in these cells (DU145) was quantified by their ability to inhibit cell proliferation by CyQuant assay which measures the cellular DNA content to quantify the amount of cell proliferation. The results indicate that plasma treated BNNTs were more efficient in arresting cell proliferation when compared to the untreated BNNTs. An average of 36% and 80% of prostate cancer cells exposed to untreated BNNTs without and with doxorubicin at different concentrations were killed (Figure 2.6) respectively. Whereas, an average of 78% and 97% of prostate cancer cells exposed to plasma treated BNNTs without and with doxorubicin at different concentrations were killed (Figure 2.6). This result also suggests that the plasma treated BNNTs have a greater ability to deliver the cytotoxic drug to the cells in comparison to the untreated BNNTs. The morphology of the cells was also
altered upon treatment (Figure 2.7(ii)) and the integrity of cellular adherent monolayer was compromised resulting in the increasing number of floating cells (Figure 2.7(iv)). It was observed that the cells were unhealthy with the presence of several membrane blebs and apoptotic bodies indicating the process of apoptosis leading to cell death (Figure 2.7(iii)) and at a higher concentration almost all the cells were detached and were floating on the top surface which confirmed the cell death (Figure 2.7(v)). The increased number of dead cells in plasma treated BNNTs is presumably because they were shorter, easier for the cells to uptake, held more drug and had higher specificity because of the presence of functional groups. The increased cytotoxicity confirms that pulsed liquid plasma has the potential to produce hybrid nanostructures with significant advantages.

![Cell viability graph](image)

**Figure 2.6.** Cell viability assay of DU145 Prostate cancer cells, treated with different volumes (30 µL and 90 µL) of i) control, ii) untreated BNNTs, iii) plasma treated BNNTs with iv) and without v) doxorubicin drug (0.6mg/mL). 1% Triton X was used as a negative control. Cell viability results are expressed as mean ± standard deviation of triplicate determinations.
Figure 2.7. Cell culture images of DU145 Prostate cancer cells, exposed with different volumes (30 µL (top) and 90 µL (bottom)) of i) control, ii) untreated BNNTs, iii) plasma treated BNNTs without doxorubicin drug, iv) untreated BNNTs and v) plasma treated BNNTs with doxorubicin (0.6mg/mL). Cells were analyzed at different spots and then all images were taken at 10X magnification.

2.4 Conclusion

Liquid plasma, produced by nanosecond pulses, provided an efficient and simple way to shorten and functionalize BNNTs and also to fabricate a hybrid nanostructure. The chemical reactive oxygen and nitrogen species in synergy with physical processes such as nanosecond pulsed electric field from plasma, may have significantly contributed to the reduced length of the BNNTs, and formation of nanocups, to the surface functionalization and to the more efficient fabrication of hybrid nanostructures. The shortening enhanced the dispersibility and drug loading potential of the BNNTs and the functionalization facilitated the effective assembly of gold nanoparticles onto the BNNTs. The plasma treated BNNTs possess the ability to carry more drug molecules and contain desired functional groups on their surface for easier uptake by cells. These factors played a significant role in increasing cytotoxicity in prostate cancer cells. Deeper studies on the role of reactive species and plasma parameters in the
shortening and functionalization of nanotubes and assembly of gold nanoparticles are underway. The efficacy of an optimized nanotubes and hybrid structures for targeted drug delivery could then be assessed.

Acknowledgments

We thank RMIT University for access to XPS and TEM facilities, Rosey Van Driel and Prabhukumar Sellamuthu of Deakin University and Phil Francis of RMIT University for assistance in microscopic observations. We thank Peter Lamb, David Rubin de Celis Leal, Robert Lovett, Steve Atkinson, Ladge Kviz, Marion Wright and Ajay Ashok for technical and editorial support.

Supporting Information

Characterization

The physical structure of the surface of BNNTs and GNPs was analyzed by a JEOL 2010 TEM with EDX and Gatan image filter at an acceleration voltage of 200 kV. The chemical structure of the surface of BNNTs and GNPs was characterized by X-ray photoelectron spectroscopy (XPS). The fabricated hybrid nanostructures were studied by TEM and XPS.

XPS results

The XPS spectra (Figure S2.2) show an increase in the atomic percentage of oxygen (O 1s peak area) in plasma treated BNNTs compared to untreated BNNTs. There was no change in the boron (B 1s) spectrum of both untreated and liquid plasma treated BNNTs (Figure S2.3). There is an increase in the percentage of N–O with increased liquid plasma treatment time.
Figure S2.1. Length distribution of plasma treated (left) and untreated BNNTs (right). Total number of nanotubes, plasma treated = 180, untreated = 189

Table S2.1. pH, conductivity and hydrogen peroxide concentration measurements in deionized water at different plasma treatment times

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<th>15 min</th>
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<td>Hydrogen peroxide (mg/L)</td>
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</table>
Figure S2.2. XPS, Oxygen (O 1s) spectrum showed an increase in O 1s peak area for untreated (8%) and plasma treated BNNTs (27.4%)

Figure S2.3. XPS, Boron (B 1s) spectrum showing no significant shift (dotted lines) between (a) untreated and (b) liquid plasma treated BNNTs
Figure S2.4. XPS, Nitrogen (N 1s) spectrum showing an increase in oxidation of nitrogen (N–O peak area—black arrows) with increased plasma treatment time. Liquid plasma functionalized BNNTs with GNPs a) undisturbed for 12 h (includes functionalization of BNNTs (15 min)) and b) liquid plasma for 15 min (includes functionalization of BNNTs (15 min) + assembly of GNPs (15 min))

Figure S2.5. TEM image of synthesized GNPs. Inset shows a magnified image of the GNPs (scale bar 20 nm)
Figure S2.6. XPS spectrum of synthesized GNPs showing a single doublet with principal peak at 83.5 eV corresponding to Au4f7/2 (metallic)

Figure S2.7. TEM images of the functionalized short boron nitride nanotubes with randomly assembled GNPs using liquid plasma for 15 minutes
Chapter 3 Argon Gas Plasma to Decontaminate and extend Shelf Life of Milk

3.1 Prelude

The previous chapter (2) focused on the generation of a plasma discharge in gas phase above the liquid surface (set up I). The key challenges faced during the generation of plasma above the liquid surface were the difficulty in selecting the desired reactive species and achieving high production of the same. The selectivity of required reactive species was hindered mainly due to the presence of atmospheric air present in between the top and bottom electrode (liquid) which interfered during the recombination of gas phase reactive species before diffusing into the liquid, thus limiting the productivity of required reactive species.

To address the above challenges, Dai et al. had developed a plasma gas bubble-in-liquid method, in which the chosen gas was introduced into the liquid through a hollow needle to produce bubbles. The plasma was generated in bubbles present in between the hollow needle and mesh electrode (see below scheme). This work was published in Plasma Processes and Polymers Journal and I contributed 10% of this work.

Citation:

Schematic of plasma generation in bubbles immersed in liquid. The positive (stainless steel mesh) and negative electrodes (hollow needle) were immersed in liquid. Argon plasma produced a high density of hydrogen peroxide and air plasma produced a high density of nitrate and nitrite.

It was reported that the generation of plasma directly in liquid requires an electric field of $>10^5$ V/cm.$^{10,11}$ Moreover, this high electrical energy created strong shock waves and intense UV radiation in liquid which resulted in the faster erosion of electrodes and leads to contamination and hence makes it less feasible for treating sensitive materials.$^{19,20}$ The generation of a discharge in bubbles that are externally introduced into liquids requires much less power compared to direct plasma generation in liquid due to the large difference in dielectric constants of liquid and gas and this method also helps to avoid or minimize electrode erosion.$^{34,38}$

A point-to-plate electrode assembly was used to produce discharge in bubbles immersed in liquid with chosen gas (see above scheme).$^{34}$ This method resulted in high production of approximately 100% of selected reactive species with the consumption of less energy. Using this set up, a high concentration of $\text{H}_2\text{O}_2$ was produced when using argon gas whereas the air plasma produced high concentrations of $\text{NO}_2$ and $\text{NO}_3$ in deionized water.$^{34}$ The discharge decreased the size and increased the number of bubbles in the liquid. This resulted in the increased interface reactions between the gas-plasma and liquid. The increase in the interface
reactions, along with the movement of bubbles, enabled higher production of selective reactive species based on chosen gas.\textsuperscript{34}

The capability to control and selectively produce a high concentration of \( \text{H}_2\text{O}_2 \) while using argon gas have opened up possibilities to utilize this approach mainly but not limited to decontamination of foods at ambient temperature. Luck \textit{et al.} had documented the use of hydrogen peroxide as a bactericide and preservative in milk and milk products.\textsuperscript{391} This motivated me to use this already developed method\textsuperscript{34} i.e. using Ar as the chosen gas with slight modifications (defined as set up II) to investigate its potential in decontamination of milk.

\textbf{3.2 Argon Gas Plasma to Decontaminate and extend Shelf Life of Milk}

The bubble-in-liquid method using Ar gas was applied to the treatment of milk, and this work was published in Plasma Processes and Polymers Journal.\textsuperscript{392} In this work, as a PhD student, I have contributed more than 50\% and all the co-authors in this publication have agreed and signed the \% contribution declaration form set by Deakin University Higher Degrees by Research for Thesis by Publication. Herewith, I have attached the same declaration form.

\begin{center}
\begin{tabular}{|l|l|}
\hline
\textbf{Candidate name} & Sri balaji Ponraj \\
\hline
\end{tabular}
\end{center}
| **Thesis title** | Nanosecond pulsed atmospheric pressure plasma for fabrication of hybrid nanostructure and for decontamination of milk |
| **Degree** | Doctor of Philosophy |
| **School** | Institute for Frontier Materials |
| **Date** | 19-06-2017 |

We confirm that the following publication is 50% or more of the above student’s contribution.

| **Publication title** | Argon Gas Plasma to Decontaminate and extend Shelf Life of Milk |
| **Journal** | Plasma Processes and Polymers |
| **Authors Signature** | (Julie Sharp) | **Date** | 20-06-2017 |
| **Authors Signature** | (Jagat Kanwar) | **Date** | 21-06-2017 |
| **Authors Signature** | (Andrew Sinclair) | **Date** | 20-06-2017 |
| **Authors Signature** | (Ladge Kviz) | **Date** | 20-06-2017 |
| **Authors Signature** | (Daniel Fabijanic, Head of School Delegate, for Kevin) | **Date** | 24-06-2017 |
Abstract

Treatment of milk with argon gas plasma was investigated for its ability to kill bacteria and increase shelf life and compared with commercially pasteurized milk. The discharge was generated by a nanosecond pulse generator using the needle point-to-plate electrode. The total bacterial count of 6.8 log CFU/mL in raw milk was reduced to 1.9 log CFU/mL after two minutes of plasma treatment and 2.2 log CFU/mL after thirty minutes of pasteurization. The plasma treated milk stored at 4 °C showed no significant changes in the bacterial count for six weeks while the bacterial counts in pasteurized milk were significantly increased from 2.2 to 4.2 log CFU/mL after two weeks. These results demonstrated plasma treatment as an alternative way to decontaminate and increase shelf life of milk.

Graphical Abstract

*Argon gas plasma to reduce the bacterial count and prolong the shelf life in bovine milk.*
3.3 Introduction

Bovine milk is a natural food, rich in nutrients and other bioactive substances, that provide many health benefits to humans.\textsuperscript{165} Milk is also an ideal medium that supplies nutrients for the growth of micro-organisms such as \textit{Escherichia coli}, \textit{Staphylococcus aureus}, \textit{Listeria monocytogenes} and \textit{Campylobacter sp.}\textsuperscript{166} The consumption of raw or ineffectively processed milk leads to dairy-related outbreaks such as brucellosis, listeriosis, and tuberculosis in humans.\textsuperscript{167-170} The removal of bacteria from milk, without altering its nutritional properties, has long been a challenge.\textsuperscript{393} The current pasteurization and ultra-high pasteurization technique is known to effectively eliminate microbial populations but it induces undesirable changes in the nutritional properties of milk by several modifications.\textsuperscript{171, 172} This necessitates the development of non-thermal technology to improve the microbial safety, increase shelf life while maintaining the quality of milk. Non-thermal technologies such as high hydrostatic pressure, pulsed electric field, ultrasonication and UV radiation has been developed to replace conventional thermal processing in milk.\textsuperscript{291, 394-396} However, the lower efficiencies, extensive treatment times, the specialized equipment requirement and processing cost present significant challenges to scale up non-thermal technologies to an industrial level.

The plasma technology has been widely used and investigated in applications such as fabrication of nanostructures, improving the efficiency of battery and solar cells and plasma medicine.\textsuperscript{363, 397-399} Atmospheric pressure plasma has been demonstrated to kill bacteria either in solid or liquid food matrices without a significant increase in temperature and therefore makes it a potential non-thermal technique for food processing.\textsuperscript{356, 357} However, there are very few studies examining plasma treatment on microbial quantity, shelf life and physicochemical characteristics of milk.\textsuperscript{360, 361} Gurol et.al demonstrated that corona discharge can significantly reduce \textit{E. coli} colony forming units without having any negative effects on pH and color measurements in milk.\textsuperscript{360} Kim et.al achieved aerobic bacterial reduction using dielectric barrier
discharge and noticed non-significant changes in the fatty acid composition of milk.\textsuperscript{361} Rahman \textit{et.al} investigated pulsed high voltage discharges and proposed shock waves might play a critical role to kill bacteria in milk.\textsuperscript{362} The previous plasma related milk studies have produced the plasmas (such as corona and dielectric barrier discharge) above the liquid (milk) surface where it might be difficult to achieve high production of controlled and selective reactive species. This could be due to the interference from the atmospheric air and recombination of reactive species in the air before diffusing into the liquid (milk).

To address these challenges we developed a method of gas bubble discharge (plasma) in liquid that enables efficient and selective production of the required reactive species.\textsuperscript{34} Using this approach, a high selective production of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and nitrate (NO\textsubscript{3}) were obtained by argon and air gas respectively. The H\textsubscript{2}O\textsubscript{2} is well known for its bactericidal and preservative effects in milk.\textsuperscript{391} The United States Food Drug and Administration (USFDA) approved the use of H\textsubscript{2}O\textsubscript{2} in milk for cheese making and prescribed that the H\textsubscript{2}O\textsubscript{2} weight should not exceed 0.05\% of the weight of milk.\textsuperscript{400} The World Food and Agriculture Organisation permitted the use of H\textsubscript{2}O\textsubscript{2} (0.05 - 0.25\%) in milk for processing on the basis that it should be converted into oxygen and water by catalase before human consumption.\textsuperscript{401}

The major requirements of the dairy industry are the use of low temperature and less chemicals to reduce the bacterial load, increase the shelf life with minimal or no changes in the nutritional quality of milk. This work aims to achieve these criteria by argon gas bubble discharge in milk which produce H\textsubscript{2}O\textsubscript{2} concentration lower than 0.05\% (approved level of H\textsubscript{2}O\textsubscript{2} by FDA) at near ambient temperature. The total bacterial reduction in plasma treated milk was significantly greater than pasteurization. More excitingly, the plasma treated milk stored at 4 °C showed no significant changes in the bacterial count for six weeks, whereas in pasteurized milk, the bacterial counts were significantly increased after two weeks.

\textbf{3.4 Experimental Section}
3.4.1 Plasma system description

The experimental setup consisted of a needle to plate electrode configuration to generate plasma (Scheme 3.1). The positive and negative polarities have been used in this work to generate plasma. A hypodermic stainless steel needle (0.6 mm diameter and 32 mm in length) was used as a positive (high voltage) electrode through which argon gas (25 sccm) was fed into the system to produce bubbles. A flat 316 stainless steel mesh (10 mm × 20 mm) was used as a negative electrode (high voltage). Both electrodes were immersed in liquid (both de-ionized water and milk) and spaced at a distance of 1 cm.

Scheme 3.1. Schematic view of bubble discharge between needle electrode (top) and mesh electrode (bottom) immersed in liquid (milk or water). Positive high voltage polarity from nanosecond pulse generator was treated as channel 1 and negative high voltage polarity was treated as channel 2.
3.4.2 Plasma treatment in de-ionized water

De-ionized (DI) water was used as an initial model to study and optimize plasma properties before experimenting with milk. Plasma was generated inside the bubbles immersed in DI water by a nanosecond pulse generator FPM 15-10MC2 (FID GmbH, Germany). The plasma parameters such as applied voltage at ±9 kV, 2 ns rise time and 10 ns pulse width were always kept constant for all experiments. The experiments in DI water were performed at least twice and analyzed in duplicate. The pH, temperature and hydrogen peroxide concentration in plasma treated DI water were optimized by varying the plasma frequency and treatment time. The plasma frequency was optimized by varying the plasma frequency (2, 3 and 4 kHz) at a constant two minutes treatment time with and without a cooling jacket. Subsequently, the treatment time was varied from 30 to 120 s at a constant 4 kHz plasma frequency with the cooling jacket.

3.4.3 Measurement of pH, temperature and hydrogen peroxide in DI water

The concentration of hydrogen ions (pH) in a solution is very important in assessing the system’s decontamination capabilities. A pH probe (TPS, Australia) was used to measure the pH in DI water before and after plasma treatment. A thermometer was used to immediately measure the temperature in all samples. A titanium sulphate colorimetric method was used to measure the hydrogen peroxide concentration in plasma treated DI water. The concentration of ozone, nitrate and nitrite in DI water were also measured by commercially available photometric assay kits (Spectroquant NOVA 60, Merck).

3.4.4 Plasma treatment in raw milk

The same batch of raw and full cream pasteurized (63.3 °C for 30 minutes) bovine milk samples were purchased from a local farm in Geelong, Australia and were stored at 4 °C. The same batch raw milk was immediately aliquoted for plasma treatment. The plasma treatment time in milk was varied from 30 to 120 seconds by keeping the plasma frequency as constant at 4 kHz.
The experiments in milk were repeated at least three times (each condition) and all samples were analyzed in triplicate. The untreated (both milk and DI water) samples (control) were left in the treatment chamber for two minutes with bubbling argon.

3.4.5 Measurement of pH and temperature in plasma treated milk

The pH and temperature in milk samples were recorded (only after a stable reading has reached) before and after plasma treatment. The effect of plasma treatment and pasteurisation in milk was compared with the raw milk samples.

3.4.6 Measurement of bacterial count and shelf life in plasma treated milk

Samples (100 μL) of serially diluted untreated (control), plasma treated (30 and 60 seconds) milk were plated onto plate count agar (PCA) media and incubated at 37 °C for 48 hours. Samples (100 μL) of plasma treated (90 and 120 seconds) and pasteurized milk were plated onto PCA without dilution and incubated at 37 °C for 48 hours.

Milk shelf life was assessed for up to six weeks by repeating the same procedure at 7 day intervals for both the plasma treated and pasteurized milk. The bacterial colonies were counted using standard plate count method and the results were expressed in log CFU per mL.

3.4.7 DNA sequencing of survived bacteria after plasma treatment

The bacteria in milk that survived after highest plasma dose (4 kHz for 120 seconds) were randomly chosen for bacterial identification using DNA sequence analysis. One colony from each plasma experiment (three colonies from three individual plasma experiments) was chosen, based on different morphology to isolate different bacterial strains. The chosen colonies were streaked onto fresh plates to obtain pure stocks. One colony from each of these purified colonies was chosen at random for DNA sequence analysis. The fast MicroSeq®500 16S rDNA bacterial sequencing method was used for the identification of bacterial species at Microgenetix Pty Ltd Company, Melbourne, Australia. Sequences were obtained that spanned approx. 197 -
495 base pairs in both the forward and reverse directions. Chromatograms were further analyzed using Chromas software and both strands were aligned to form a consensus sequence for each colony obtained. Consensus sequences were blasted against a nucleotide database using national center for biological information.

3.4.8 Statistical analysis

A student’s t-test was performed to obtain the statistical significance of differences between treatments of the milk samples.

3.5 Results and Discussion

3.5.1 pH and temperature of plasma treated DI water

DI water was treated by argon gas bubble discharge at different frequencies (2, 3 and 4 kHz) for two minutes without and with a cooling jacket (Figure 3.1). By increasing the frequency, the pH of plasma treated DI water was decreased from 5.1 (untreated) to 3.3 and 3.5 at 4 kHz without and with the cooling jacket, respectively. The pH reduction was slightly more without a cooling jacket. This could be due to the temperature differences between the treatments. The temperature of plasma treated DI water was found to be increased with the increase in plasma dose (Figure 3.2). After the highest plasma dose (4 kHz for two minutes), a final temperature of 65°C and 34 °C in DI water was recorded without and with cooling jacket, respectively. The temperature increase in the liquid was most likely due to the joule heating that occurred during the plasma treatment.403
Figure 3.1. pH of plasma treated de-ionized water without and with cooling jacket at different plasma frequencies (kHz) for two minutes. The untreated DI water (control) samples were left in the treatment chamber for two minutes with bubbling argon. The error bars indicate the mean ± standard error of the mean (SEM) of duplicate measurements and the statistical significance (* indicate $P \leq 0.05$) was calculated between each plasma frequencies without and with cooling jacket samples.
Figure 3.2. Temperature of plasma treated de-ionized water without and with cooling jacket at different plasma frequencies (kHz) for two minutes. The untreated DI water (control) samples were left in the treatment chamber for two minutes with bubbling argon. The error bars indicate the mean and SEM of samples and the statistical significance (* indicate $P \leq 0.05$) was calculated between each plasma frequencies without and with cooling jacket samples.

3.5.2 Hydrogen peroxide in plasma treated DI water

The concentration of $\text{H}_2\text{O}_2$ in DI water was measured without and with a cooling jacket and at different frequencies (2, 3 and 4 kHz) for two minutes to optimize the plasma frequency and temperature (Figure 3.3). The $\text{H}_2\text{O}_2$ concentration was increased with the increase in plasma input energy and found significantly higher in treatments without cooling compared to with cooling jacket. The highest plasma dose (4 kHz for two minutes) produced a maximum concentration of 119 ppm (0.0119%) and 75 ppm (0.0075%) $\text{H}_2\text{O}_2$ at 65 and 34 °C for without and with a cooling jacket respectively. The higher frequency created more fragmentation in gas bubbles which leads to the decrease in size and increase in number of bubbles in the liquid. These latter effects along with the movement of bubbles greatly increased the interface
reactions between the gas-plasma and water, enabling higher production of selected species based on chosen gas. Using argon gas, the most reactive species in the plasma (energetic electrons, excited Ar atoms and ions, UV radiation) directly interact with water, which results in decomposition of the water and generation of OH radicals. H₂O₂ was then formed by the recombination of OH radicals either in gas phase or in water. A high density of H₂O₂ species in Ar plasma treated DI water was attributed by the transfer of OH and H₂O₂ species from bubbles to bulk liquid as well as the direct recombination of OH radicals in water.

Figure 3.3. Hydrogen peroxide concentration plasma treated de-ionized water without and with cooling jacket at different plasma frequencies (kHz) for two minutes. The hydrogen peroxide values in plasma treated DI water were calculated by subtracting the untreated sample (control) values with plasma treated samples. The error bars indicate the mean ± SEM of samples and the statistical significance (** and *** indicate P ≤ 0.01 and ≤ 0.001) was calculated between each plasma frequencies without and with cooling jacket samples.

After optimizing the plasma frequency and temperature, the plasma treatment time (at 4 kHz) was varied from 30 to 120 s in DI water with the presence of cooling jacket (Figure 3.4). The
concentration of $\text{H}_2\text{O}_2$ in DI water was significantly increased to 38 (0.0038%), 62 (0.0062%) and 75 ppm (0.0075%) with the increase in plasma treatment time (60, 90 and 120 seconds).

Figure 3.4. Hydrogen peroxide concentrations in plasma treated DI water at varying plasma treatment time (seconds) in the presence of cooling jacket. The hydrogen peroxide values in plasma treated DI water were calculated by subtracting the untreated (control) sample values with plasma treated samples. The error bars indicate the mean ± SEM of samples and the statistical significance (* indicate $P \leq 0.05$) was calculated between each treatment time.

The highest plasma dose condition (4 kHz for two minutes) in DI water produced a maximum concentration of 75 ppm (0.0075%) $\text{H}_2\text{O}_2$ at 34 °C in the presence of the cooling jacket. The latter plasma condition was chosen for further optimization and investigation in milk, because an addition of 75 ppm $\text{H}_2\text{O}_2$ in milk at 34 °C for two minutes is not sufficient to effect bacterial reduction. Luck *et al.* reported that an incubation of pure 800 ppm (0.08%) $\text{H}_2\text{O}_2$ in milk at 49-51 °C for 30 minutes is needed for milk decontamination.\(^{391}\) Also, pasteurization, a current thermal method which requires 63.3 °C for 30 minutes to kill bacteria in milk.
3.5.3 pH and temperature of plasma treated milk

Argon gas bubble discharge at 4 kHz was employed for 30, 60, 90 and 120 seconds in milk to investigate its potential to kill bacteria and increase shelf life. The pH was slightly reduced from 6.76 (untreated) to 6.67 (120 s) in milk samples (Table 3.1). On the other hand, the pH of plasma treated DI water reduced from 5.1 to 3.5 at 4 kHz for 120 s in presence of cooling jacket (Figure 3.1). These stark differences in pH changes between plasma treated milk and water showed that the plasma-milk interaction differs from the plasma-water interaction. The presence of phosphate systems and casein proteins in milk exhibits buffering capacity and helps to resist pH changes.\textsuperscript{404} This could be the reason for the small pH change in plasma treated milk compared to plasma treated DI water. Moreover, the temperature difference in all plasma treated milk samples could also be a factor for a slight pH change (Figure 3.5). Kim et.al also observed changes in pH (from 6.90 to 6.60) of milk after dielectric barrier air discharge treatment.\textsuperscript{361} On the contrary, Gurok et.al mentioned that no negative effects on pH (6.7 ± 0.05) was observed in milk after atmospheric air corona discharge.\textsuperscript{360} These differences in results could be due to the plasma energy input and working gas. The temperature of milk was found to be increased with the increase in plasma dose (Figure 3.5). The final temperature 42 ± 2 °C was recorded at the highest plasma treatment (4 kHz for two minutes) in milk. This temperature was higher than the final temperature (34 °C) of the plasma treated DI water.

Table 3.1. Shows the pH measurement of all milk (control, plasma treated and pasteurized) samples. The plasma treatment was performed at 4 kHz with different treatment times from 30 to 120 seconds in the presence of cooling jacket.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average pH of milk samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.76</td>
</tr>
</tbody>
</table>
Note: The untreated milk (control) samples were left in the treatment chamber for two minutes with bubbling argon. The statistical significance (** indicate $P \leq 0.01$) was calculated between the control, plasma treated and pasteurized milk samples.

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td>30 s</td>
<td>6.74</td>
</tr>
<tr>
<td>60 s</td>
<td>6.72**</td>
</tr>
<tr>
<td>90 s</td>
<td>6.69**</td>
</tr>
<tr>
<td>120 s</td>
<td>6.67**</td>
</tr>
<tr>
<td>Pasteurized</td>
<td>6.74**</td>
</tr>
</tbody>
</table>

Figure 3.5. Temperature measurement in plasma treated milk samples at 4 kHz and at different plasma treatment times (seconds) in the presence of cooling jacket. The untreated milk (control) samples were left in the treatment chamber for two minutes with bubbling argon. The error bars indicate the mean ± SEM of samples and the statistical significance (**, *** and **** indicate $P \leq 0.01$).
**** indicate $P \leq 0.01$, $\leq 0.001$ and $\leq 0.0001$) was calculated between the control and other milk samples.

3.5.4 Bacterial reduction in plasma treated milk

The total bacterial population was found to be 6.8 log CFU/mL in untreated (control) milk. The number of CFUs were decreased with the increase in plasma dose (Figure 3.6). The 90 (at 34°C) and 120 seconds plasma treatment (at 42 °C) achieved a 4.6 and 4.9 log CFU/mL bacterial reduction in milk. The pasteurization (63.3 °C for 30 minutes) achieved a 4.6 log CFU/mL bacterial reduction in milk. This showed that the 120 seconds plasma treatment significantly achieved a higher bacterial reduction (0.3 log CFU/mL) than pasteurisation. Interestingly, the 90 seconds plasma treatment and pasteurization achieved the same amount of bacterial reduction in milk. The 90 and 120 seconds plasma treatment has achieved an industrial standard bacterial reduction (4.3 log bacterial reduction) in milk according to the USFDA pasteurized milk ordinance 2011.405,406
Figure 3.6. Bacterial count in milk following plasma treatment at 4 kHz and at different plasma treatment times (seconds) in the presence of the cooling jacket. The untreated milk (control) samples were left in the treatment chamber for two minutes with bubbling argon. The error bars indicate the mean ± SEM of samples and the statistical significance (*, **, *** and **** indicate $P \leq 0.05$, $P \leq 0.01$, $\leq 0.001$ and $\leq 0.0001$) was calculated between the control, plasma treated and pasteurized milk.

Although, the plasma interaction in milk and water are different, the H$_2$O$_2$ concentration produced in 90 (62 ppm at 24 °C) and 120 s (75 ppm at 34 °C) plasma treated DI water (Figure 3.4 and 3.2) and the bacterial reduction achieved at 90 (4.6 log) and 120 s (4.9 log) plasma treated milk (Figure 3.6) was correlated to see the role of H$_2$O$_2$ and temperature in plasma-mediated bacterial reduction in milk. This correlation showed that the plasma treatment requires a lower temperature and ten times lesser H$_2$O$_2$ concentration to effect milk decontamination. As mentioned earlier, an incubation of pure 800 ppm H$_2$O$_2$ concentration in milk at 49-51 °C for 30 minutes is necessary to cause decontamination. This implied that, in addition to H$_2$O$_2$, the other plasma components such as nanosecond pulsed electric field (PEF), UV radiation, short lived species (OH radicals) and joule heating might have played a role in reducing the bacterial population in milk. Moreover, the interface reactions between the Ar gas-plasma (including UV radiation and reactive species, such as OH and H$_2$O$_2$) and milk were greatly increased through the bubbles (size, number and movement), enabled more efficient reaction.

It has also been reported that the electrical discharges in gas bubbles immersed in liquid produces both physical (PEF and UV radiation) and chemical processes (short lived species mainly O, OH radicals and long lived species mainly H$_2$O$_2$, NO$_3$). Craven et al. showed that high intensity PEF has the capability to effect 1-5 log bacterial reductions in milk based on the energy input and treatment temperature. Krishnamurthy et al. has reported that pulsed
UV has a higher penetration depth than continuous mode and has effectively reduced bacteria in milk.\textsuperscript{329} The key elements such as UV wavelength, intensity, thickness of the radiation path and flow turbulence determines the UV effects in milk. Werrrs \textit{et al.} showed that the combination of H\textsubscript{2}O\textsubscript{2} and UV radiation with or without heat produces a greater lethal effect on bacterial spores (in phosphate buffer solution) compared to their individual counterpart effects.\textsuperscript{330} They explained that the UV irradiation breaks down the H\textsubscript{2}O\textsubscript{2} into OH radicals which then enter into the bacterial spore to achieve the bactericidal effect and the presence of heat enhanced this effect.

All these above reports suggest that the plasma components such as nanosecond PEF, UV radiation, short lived species (OH radicals) and joule heating might have an additive effect to effect milk decontamination. However, the measurement of H\textsubscript{2}O\textsubscript{2} in milk after plasma treatment will be necessary to understand the bacterial reduction process.

3.5.5 Shelf life of plasma treated milk

The plasma treated milk samples were stored at 4 °C and assayed for bacterial count each week to assess shelf life of milk for a period of 6 weeks (Figure 3.7). There were no significant changes in the bacterial count for up to six weeks in plasma treated milk samples (in both 90 and 120 s) while the pasteurized sample showed bacterial counts were significantly increased from \(~2.2\) to \(4.2\) log CFU/mL at 2 weeks. This confirms that the plasma treatment reduced the viable bacteria load at 90 and 120 s and the treatment continued to protect the milk for a further 6 weeks. Gurol \textit{et al.} also observed an immediate \textit{E.coli} in milk and found the milk could be stored for six weeks after atmospheric corona discharge.\textsuperscript{360} The immediate reduction of bacteria could be due to the plasma reactive species which can directly target the bacterial cell wall.\textsuperscript{408} They also observed the further reduction of bacterial load during the first week of storage, suggesting the presence of a post plasma effect. Similarly, Song \textit{et.al} showed that the number of \textit{Listeria monocytogenes} were decreased during storage after plasma treatment.\textsuperscript{409} In our
work, the argon bubble discharge immediately reduced the bacteria and demonstrated a continued effect in the milk to maintain the same bacterial load during storage by restricting bacterial growth. This suggests that plasma treatment has the potential to reduce the bacteria and also to arrest its growth during the treatment time. It is well known that pasteurized milk stored at $\leq 4 \, ^\circ C$ can only be consumed for up to 14 days after processing and this was in good agreement with our results.\textsuperscript{410} The bacteria that survived after pasteurization has the potential to proliferate after two weeks which limits the shelf stability of the pasteurized milk. Thus in comparison to pasteurization, plasma treatment in this study demonstrated an advantage of increasing shelf life by limiting further bacterial growth in milk.

![Shelf life comparison](image)

*Figure 3.7. Shelf life comparison (up to 6 weeks) of plasma treated and pasteurized milk. The error bars indicate the mean $\pm$ SEM of samples and the statistical significance (* and ** indicate $P \leq 0.05$ and $\leq 0.01$) was calculated between 90 s, 120 s and pasteurized milk samples.*
3.5.6 DNA sequencing of survived bacteria after plasma treatment

DNA sequencing was performed for the bacteria that survived after highest plasma dose (4 kHz for 120 seconds). The DNA sequencing results showed that *B. licheniformis* was predominantly identified as the surviving bacterial species after plasma treatment (Table 3.2). A detailed report on the DNA sequencing results were provided in the supplementary information (Table S3.1 and Figure S3.1).

*Table 3.2. Shows the identified bacterial species that survived after highest plasma dose (4 kHz for 120 seconds)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Identified species</th>
<th>Z Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>colony 1</td>
<td><em>B. licheniformis</em> strain B26_13</td>
<td>424 (229) bits</td>
</tr>
<tr>
<td>colony 2</td>
<td><em>Bacillus</em> sp. B4.5</td>
<td>776 (420) bits</td>
</tr>
<tr>
<td>colony 3</td>
<td><em>Bacillus</em> sp. B4.5</td>
<td>793 (429) bits</td>
</tr>
</tbody>
</table>

*B. licheniformis* is a common aerobic spore forming bacteria that exists in all stages of dairy processing in Australian milk. However, there are arguments related to *B. licheniformis* strains acting both as probiotics and also as a disease-causing agent through foods. Unlike the vegetative cells, the bacterial spores are highly resistant to a number of stress factors such as heat, UV radiation, chemicals and high pressure. This could be the reason for *B. licheniformis* survival after plasma treatment. However, in plasma-treated milk no increase in CFUs was observed during the shelf life assessment (Figure 3.7). The effect of plasma treatment on major nutritional components such as protein, lipids and carbohydrates needs to be studied in detail to ensure food safety and quality before extending this plasma approach for practical applications.
3.6 Conclusion

We have shown that the argon gas bubble discharge in milk significantly reduced the bacterial concentration than pasteurization. The plasma treatment also significantly increased the storage life of milk for up to six weeks compared to pasteurization. The plasma treatment caused only a small change in pH and a moderate increase in temperature of all milk samples. This demonstrated that the argon gas plasma treatment could be a promising alternative method to decontaminate and increase shelf life of milk.

Acknowledgements

We thank David Rubin de Celis Leal, Robert Lovett, Alex Orokity, Magnolia Beer, Marion Wright, Leanne Farago and Steve Atkinson for technical assistance; Daniel Fabijanic for 316 stainless steel mesh supply; C. Anderson and M. Pavlovich of the University of California, Berkeley, for the measurement procedure for hydrogen peroxide; Yakov. E. Krasik and Peter Lamb for valuable discussions and editorial support.

Supporting Information

The gene sequence of identified bacterial species B. Licheniformis

Keywords: argon plasma; bacteria; hydrogen peroxide; milk; shelf life

1) Table S3.1. Top 10 Blastn hits for each colony sequenced

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<th>Total score</th>
<th>Query cover</th>
<th>E value</th>
<th>Ident</th>
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2) Nucleotide sequence comparison of colonies

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<td>TAACTCCCGGAAACC GGGCTTAAATACCGGATGCTGTTGATTGAACCAGCATGTTCCAAATCAT</td>
</tr>
</tbody>
</table>
COLONY3  TAACTCCGGGAAACC GGCTAT AACC GGATGCTTGAT TGAACCGC ATGTTTC-AATCAT

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COLONY1  AAAAGGTGGCTTTTAGCTACCACTTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGA
COLONY2  AAAAGGTGGCTTTTAGCTACCACTTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGA
COLONY3  AAAAGGTGGCTTTTAGCTACCACTTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGA

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COLONY1  GGTAACGGCTCACCAAGGCACGCTGCTAGCCTGAGAGGGTGATCGGCCACACTG
COLONY2  GGTAACGGCTCACCAAGGCACGCTGCTAGCCTGAGAGGGTGATCGGCCACACTG
COLONY3  GGTAACGGCTCACCAAGGCACGCTGCTAGCCTGAGAGGGTGATCGGCCACACTG

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COLONY1  GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
COLONY2  GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
COLONY3  GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA

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COLONY1  CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTG
COLONY2  CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTG
COLONY3  CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTG

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COLONY1  TTGTTAGGGAAGAA AAGT ACCGTTGCGGTAATAGGCGGGCACCCTTGACGGT ACCCAA CAG
COLONY2  TTGTTAGGGAAGAA AAGT ACCGTTGCGGTAATAGGCGGGCACCCTTGACGGT ACCCAA CAG

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Figure S3.1. Nucleotide sequence comparison of colonies that survived after highest plasma dose (4 kHz for 120 seconds). Identical base pairs between all 3 sequences are indicated by an asterisk while differences are highlighted in yellow.
Chapter 4 Plasma Treatment- A potential alternative to minimize damage in nutritional bio-actives of bovine milk


Abstract

A first study to address the effects of argon plasma treatment on proteins, lipids, carbohydrates and miRNA present in bovine milk compared to commercially pasteurized milk and raw milk. High performance liquid chromatography analysis showed that α, β and κ-casein proteins were significantly increased in 90 and 120 s plasma treated milk whereas in pasteurized milk, the α and β-casein proteins were significantly reduced and κ-casein was significantly increased compared to raw milk. Beta-lactoglobulin (β-LG) was significantly reduced in 120 s plasma treated milk compared to pasteurized milk, and it was also significantly reduced in 90 and 120 s plasma treated and pasteurized milk compared to raw milk. SDS-PAGE analysis of whey and skim fractions of milk showed that α-lactalbumin (α-LA) protein was significantly reduced in 120 s plasma treatment compared to raw milk. Among the minor whey proteins like lactoferrin (Lf), immunoglobulins (Ig) and bovine serum albumin (BSA), the 120 s plasma treatment significantly reduced Ig compared to raw milk. This showed that, next to β-LG, Ig were sensitive to higher plasma treatment time. Western blotting and SDS-PAGE analysis of whey showed that Lf was significantly reduced in pasteurized milk compared to 90 s plasma treated and raw milk. The total lipids and the saturated fatty acids, and carbohydrates were significantly reduced in pasteurized milk compared to both 90 and 120 s plasma treated and raw milk. No major significant change in fatty acids were observed in both 90 and 120 plasma treated milk compared to raw milk. The miRNA concentration in 90 s plasma treated milk was significantly increased compared to pasteurized milk. Overall, the least changes in the
nutritional components were observed in 90 s plasma treated milk compared to pasteurized and 120 s plasma treated milk. The changes in the composition of milk samples was reflected in the cell viability of human fetal intestinal cells (FHs 74 Int) and human colon adenocarcinoma cells (Caco-2). The raw and 90 s plasma treated milk did not effect the cell viability of FHs 74 Int whereas the pasteurized and 120 s plasma treated milk significantly reduced viability compared to untreated cells. The cell viability of Caco-2 cells was significantly reduced by all milk samples compared to untreated cells. Our results demonstrated that 90 s plasma treatment has the potential to maintain or minimally alter the nutritional components in milk compared to pasteurization.

**Graphical Abstract**
The plasma reactive components in the gas phase (bubble) and at the gas-milk interface, diffuse to interact with components in milk. The proposed order of higher interaction are labelled from 1 to 4 based on the results obtained. The plasma reactive components might effectively interact with micro-organisms, whey proteins, carbohydrates, enzymes, salts, and water soluble vitamins present in the water phase of milk. Whilst, the interaction with casein micelle and milk fat globule membrane might be less effective compared to water phase components in milk. 

Note: Some of the elements in this figure are inspired from Pankaj et al. and re-drawn according to the results obtained.

4.1 Introduction

Milk is a nutritious food enriched with protein, lipids, carbohydrates, vitamins, salts and bioactive substances like growth factors, enzymes and peptides. However, milk can also contains harmful microorganisms such as Escherichia coli, Staphylococcus aureus, Streptococcus sp, Micrococcus sp, spores and etc. The presence of these type of microorganisms adversely affect the quality of milk and human health through dairy borne diseases such as listeriosis, brucellosis, salmonellosis and tuberculosis. Pasteurization and ultra-high pasteurization are the current techniques to kill bacteria and extend the shelf life of milk. But these processes damage the immunological properties, bioactive proteins, peptides and vitamins present in milk. A non-thermal technology is therefore required to ensure the microbial safety whilst reducing the nutritional damage in milk, to fulfil the increasing consumer demands for fresh-like, nutritious milk and milk products.

Alternative non-thermal technologies such as high hydrostatic pressure (HHP), pulsed electric field (PEF), UV radiation and ultrasound have been developed to replace conventional thermal methods in milk decontamination and nutrition preservation. HHP treatment induces changes in milk appearance and casein particle size, denaturation of whey proteins.
(particularly β-lactoglobulin (β-LG) and lactoferrin (Lf)) and interaction of whey proteins with casein micelles. The effect of HHP on milk components mainly depends upon pH, treatment temperature and time. Limited number of papers reported the effect of PEF treatment on nutritional compounds which caused aggregation of β-LG in whey protein solution, increase in peroxide value in oleic acid models and reduction of vitamin C. Floury et al. found that PEF also affected proteins, especially casein micelles, decrease in viscosity and enhanced coagulation properties of PEF-treated milk. Xiang et al. observed an increase in the effective volume of the fat globule due to interaction with denatured skim milk serum proteins. The electric field intensity, number of pulses, and concentration of proteins in their study contributed to the alteration of whey proteins. Belloso found that the whey protein denatured in the order of α-LA > BSA > β-LG after PEF treatment.

Several authors have reported the use of UV-C to decontaminate raw cow and goat milk with different fat amounts and at different temperatures. Choudhary et al. demonstrated that the microbial inactivation (Escherichia coli W1485 and Bacillus cereus endospores) of UV-C was higher in skimmed compared to non-skimmed cow milk. Matak et al. showed that ultraviolet irradiation (at 254 nm) induced sensory and chemical changes in goat milk. The limitations in inactivating the microbes, loss of nutrients, lipid oxidation and formation of off-flavors in milk after UV treatment still remain as major challenges. The effect of high intensity ultrasound and heat treatment on fat, proteins and native enzymes in milk has been reported in numerous studies. It was commonly found that ultrasound have a limited effect on enzymes when used alone. On the other hand, the size of the fat globule was significantly reduced and distributed evenly, whey proteins such as α-LA and β-LG and native enzymes such as alkaline phosphatase, lactoperoxidase and γ-glutamyltranspeptidase in whole and skim milk were highly denatured when ultrasound and heat was used combinedly.

Although each non-thermal technology discussed above have certain advantages in processing
milk, they suffer from lower efficiencies, extensive treatment times, specialized equipment requirements and the requirement of additional heat to achieve effective inactivation, energy consumption, capital and processing costs. These significant challenges make the emerging non-thermal technologies difficult to scale up to an industrial level.

Plasma can be defined as a quasi-neutral system composed of gaseous and fluid-like mixtures of free electrons, ions, neutral particles, heat, UV light, electric field and free radicals like reactive oxygen and nitrogen species. Plasma technology has been widely investigated in applications such as fabrication of nanostructures, textiles, waste water treatment, batteries, solar cells and medicine. Plasma technology is an environmentally friendly approach and it has the potential to reduce viable bacteria on various surfaces including solid and liquid foods with lower temperatures, and could be of great interest to food processing industries. Although the decontamination capability of plasma has been demonstrated in medical devices, dentistry, dermatology, agriculture and foods, only very few reports are available in processing milk. El Aragi and Rahman studied pulsed high voltage arc discharges (AD) in milk and explained that the production of shock waves might have played a key role in bacterial death. Gurol et al. reported that the corona discharge (CD) significantly reduced E. coli in whole, semi skimmed and skimmed milk without any negative effects on pH and color measurements. Kim et al. demonstrated that the dielectric barrier discharge (DBD) treatment in milk improved the microbial quality with slight changes in the fatty acid composition. Similarly, Korachi et al. also observed a slight but non-significant changes in milk fatty acid composition after corona discharge. However, the effect of plasma on milk constituents such as proteins, lipids and carbohydrates was not largely explored, despite fatty acid analyses in two of the above studies. In our previous study, argon bubble discharge (plasma) was generated in milk which achieved 4.6 and 4.9 bacterial log reductions in 90 and 120 seconds of treatment and increased the shelf life to at least six weeks compared to pasteurization. Micro RNAs
miRNAs have also been identified in bovine, human, rat and other species milk and serve as an effective biomarker for milk quality and udder function. These milk miRNAs are a new class of non-coding RNAs ranging from of 18 to 26 nucleotides in length which play an important role in gene regulation. Milk miRNAs are encapsulated in exosomes which gives them stability and the ability to withstand harsh conditions such as the acid environment of the stomach. To the best of our knowledge, this is the first study which assess the effect of argon plasma on proteins, lipids, carbohydrates, total RNA and miRNA content compared to pasteurized and raw milk. These are all useful parameters in addressing the milk quality and the impact each treatment has on milk integrity.

4.2 Experimental section

Raw and full cream pasteurized (63.3 °C for 30 minutes) bovine milk samples from the same batch were purchased from a local farm in Geelong, Australia and were stored at 4 °C. The raw milk was immediately aliquoted for plasma treatment. A needle to plate electrode configuration was used to generate plasma (Scheme 3.1, see previous chapter). The hypodermic stainless steel needle (0.6 mm diameter and 32 mm in length) was used as a positive (high voltage) electrode through which argon gas (25 sccm) was fed into the system to produce bubbles. The flat 316 stainless steel mesh (10 mm × 20 mm) was used as a negative electrode. Both electrodes were immersed in milk and spaced at a distance of 1 cm. Plasma was generated inside the bubbles immersed in milk by a nanosecond pulse generator FPM 15-10MC2 (FID GmbH, Germany). The plasma treatment time was varied from 30 to 120 s in milk while keeping the other plasma parameters such as applied voltage at ±9 kV, frequency 4 kHz, rise time 2 ns and pulse width 10 ns as constant for all experiments. The plasma treated milk samples were compared with the same batch pasteurized milk samples to assess the impact of plasma and pasteurization on nutritional components such as protein, lipid, carbohydrate and
RNA. The untreated milk samples left in the treatment chamber for two minutes with bubbling argon were treated as control.

4.2.1 Measurement of total proteins

The total protein content in the diluted (1:1000) milk samples was measured using the bicinchoninic acid (BCA) micro protein assay kit (Thermo Scientific, USA). Known concentration of bovine serum albumin (BSA) standard solutions were prepared. 150µL of either standard, water (as blank) or diluted milk samples was pipetted into each microplate well. 150µL of working reagent (composed of Micro BCA reagents A, B and C in the ratio of 25:24:1) was added to each well and the plate was shaken for 30 seconds on a plate shaker. The plate was incubated at 37 °C for 2 hours. After cooling the plate to room temperature, the absorbance was measured at 562nm using a plate reader. A standard curve was established using BSA protein standards to calculate the total protein content in all milk samples.

4.2.2 High performance liquid chromatography (HPLC)

HPLC was used to study the changes in individual milk proteins after plasma treatment and pasteurization. Skim milk was prepared by centrifuging the milk samples twice at 1000g for 5 minutes at 4 °C to remove the fat layer. Skim milk was diluted by mixing 100 µL skim milk and 900 µL of 50 mM phosphate buffer (pH 6.8). This diluted milk sample was filtered by 0.22 µm syringe filter to remove any particulate matter and then subjected to HPLC analysis.

Chromatographic analysis was performed with an Agilent 1260 Infinity 2D-HPLC system (Agilent Technologies, Mulgrave, Victoria, Australia) comprised of a binary capillary pump with solvent degasser; an auto-sampler; a 1290 Infinity thermostated column compartment and DAD UV detector that monitored absorbance a 214 nm. All injection volumes were 20 µL and all separations were completed at 40 °C thermally equilibrated for 1 hour prior to the first analysis. A Kinetex C18 column (100 mm × 4.6 mm i.d., 2.6 µm, 100 Å, Phenomenex, Lane
Cove, New South Wales (NSW), Australia) was used for all separations. A gradient mobile phase was used for all separations where Solvent A consisted of deionised water (Continental Water Systems, Victoria, Australia), which was filtered through a 0.45 µm filter (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) prior to use, and Solvent B was HPLC grade acetonitrile (Ajax Finechem Pty. Ltd., Taren Point, NSW, Australia); 0.1% v/v trifluoroacetic acid (TFA, Reagent Plus 99%, Sigma-Aldrich, Castle Hill, NSW, Australia) was added to all solvents prior to analysis. The mobile phase increased from 20% to 65% Solvent B over 25 minutes before returning to the initial composition whereby the column was re-equilibrated for 5 minutes. The flow rate was 1 mL per minute.

The whey protein peaks were identified by running pure standards of β-LG, α-LA, Lf and bovine serum albumin (BSA) whereas the casein protein (α-CN, β-CN and κ-CN) peaks were identified based on its retention time described in Yuksel and Erdem work. The fold changes in each protein were calculated by dividing the protein peak area of plasma treated and pasteurized samples by area of the control samples. The protein peak areas were summed for proteins which exist in more than one form. For example, β-LG exists in three forms and, the three peak areas were summed for the treated samples (plasma treated and pasteurized) and divided by the three peak areas of untreated (control) samples.

4.2.3 Sodium dodecyl sulphate – Poly acrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for separating proteins on the basis of their ability to move within an applied electrical current, which is directly proportional to the length of their polypeptide chains and their molecular weight. The SDS imparts a negative charge to the protein by coating them and removes secondary and tertiary structure forms to maintain the proteins as polypeptides. Thus, all peptides resent in the milk samples ranging from 250 kDa to 10 kDa could be visualized.
Skim milk was prepared as mentioned above and the whey fraction was prepared by centrifuging the skim milk samples at 10,000xg for 10 minutes at 4 °C. The whey fraction (supernatant) and the casein pellet were separated. The protein concentration of skim and whey fractions was measured in all samples using Micro BCA protein assay kit. A 1.5 mm, 10 % gel was casted using 40% acrylamide (Sigma Aldrich, Australia) and 50 µg of skim and whey milk samples were loaded and ran at 200 volts. The gel was stained using coomassie blue stain for a period of 30 minutes and kept for de-staining overnight. The gel was imaged using Chemi-doc with XRS camera (Bio-Rad). The skim milk and whey fraction samples were ran twice and densitometry analysis were performed on all gels using microarray plugin tool in ImageJ software. The average fold changes in proteins were calculated by dividing the band density of plasma treated and pasteurized samples by band density of untreated control samples.

4.2.4 Western Blot

1.5 mm gels (10%) were casted using 40% acrylamide and 50 µg of milk samples were loaded and ran at 200 volts for approximately 45 minutes. A polyvinylidene difluoride (PVDF) membrane (GE healthcare) of size 6 X 8.4 cm was cut and placed in 100% methanol for 30-60 seconds. The membrane was then transferred into Milli-Q water and kept completely immersed for 8 minutes. The membrane was then placed into the 5X transfer buffer for 15-20 minutes in order to equilibrate it. The proteins were then transferred from the gel onto a PVDF membrane using a Turbo-transfer system (Bio-Rad). In order to do so the membrane gel sandwich was assembled as follows: within the turbo transfer box- filter pads-PVDF membrane-gel-filter pads. The transfer was run according to the Bio-Rad guidelines.

After the transfer, the PVDF membrane was washed using tris buffer saline (TBS) twice and then incubated in blocking buffer (1% skim milk in TBS) for a period of 1h. The membrane was then washed twice with TBS and later incubated in primary antibody (rabbit anti-alpha lactoglobin (Abcam), rabbit anti-beta lactoglobin (Abcam), goat anti-lactoferrin (Bethyl
Laboratories) for 1 h at 37 °C. The membrane was washed thrice using Tris buffer containing 0.1% Tween-20 (TBS-T) followed by another three washes with TBS. The membrane was then incubated with secondary antibody (anti rabbit HRP and anti-goat HRP- R&D systems, Australia) for 1 h at 37 °C. The washing steps were repeated and the membrane was developed using the HRP substrates Detection reagent 1 (500µl) and Detection reagent 2 (500µl), (Thermo Scientific, Australia) and membrane was imaged using a Chemi-doc with XRS camera (Bio-Rad, Australia). Densitometric analysis and fold changes were performed as mentioned previously.

4.2.5 Measurement of total lipids

The total lipid content in all milk samples was determined using colorimetric method. Milk samples without dilution were used for this assay. Triolein standards were prepared using chloroform (Chemsupply, South Australia) and methanol (Chemsupply, South Australia) in 2:1 mixture. 2.5 µL of blank (chloroform: methanol: 2:1), standards and milk samples were transferred to microfuge tubes. 600 µL of ethanol (Chemsupply, South Australia) was added to each tube followed by the addition of 100 µL of 2 M hydroxylamine hydrochloride (Sigma, Sydney, Australia). The contents of the tube were then vortexed. 100 µL of 3.5M Sodium hydroxide (NaOH, Merck, Victoria, Australia) was added to each tube, vortexed and the tubes were incubated at room temperature for 20 minutes. 100 µL of 4 M hydrochloric acid (HCl, Merck, Victoria, Australia) was added to the tubes followed by the addition of 100 µL of 0.37 M ferric chloride (Sigma, Sydney, Australia) made up with 0.1 M HCl. The tubes were vortexed and 250 µL of the contents were transferred to a 96 well plate. The absorbance was measured at 540 nm using a plate reader (Biorad xMarkTM microplate absorbance spectrophotometer). A standard curve was established using triolein standards to calculate the total lipid content in all milk samples.

4.2.6 Gas chromatography (GC)
Prior to analysis by GC, fatty acids in milk were converted to methyl esters following the method of Christie and Han with some modifications. All milk samples (1 mL) were stored at -80 °C and subjected to freeze drying. The weight of the freeze dried milk samples was recorded and the samples dissolved in 1 mL toluene, and 200 μL of internal standard (5 mg/mL methyl nonadecanoate (Sigma–Aldrich) in toluene) and 200 μL of antioxidant (1 mg/mL 2, 6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene; BHT, Sigma–Aldrich) in toluene) were added. To this, 2 mL of acidic methanol reagent (prepared by adding 1 mL of acetyl chloride (Sigma–Aldrich) drop wise to 10 mL methanol on ice and stirring for at least an hour) was added, the solutions mixed and left overnight at 50 °C in a sealed tube. The solution was cooled and 5 mL sodium chloride solution (5% m/v) was added. The fatty acid methyl esters were extracted twice with heptane (5 mL) and the heptane layer washed with 5 mL potassium bicarbonate solution (2% m/v). The heptane layer was dried over sodium sulphate, and subjected to analysis by GC with flame ionisation detector (FID).

Samples were analysed using an Agilent 6890 GC-FID, equipped with a BPX70 capillary column (SGE) (30 m, 0.25 mm i.d., 0.25 μm film thickness). The oven was programmed from 140 °C (5 minutes hold) to 220 °C (5 minutes hold) at a rate of 4 °C/minute for a total run time of 30 minutes. A volume of 1 μL of solution was injected with a split ratio of 50:1 (injector temperature, 250 °C). Helium was used as the carrier gas (1.5 mL/minute, constant flow). Detector gases were 30 mL/minute hydrogen, 300 mL/minute air and 30 mL/minute nitrogen. Peak areas were integrated by ChemStation software and corrected using theoretical relative FID response factors. The percentage composition of fatty acids in milk samples were calculated by dividing the area of particular fatty acid by the sum of the area of all fatty acids. The fold changes in fatty acids were calculated by dividing the fatty acid % value of plasma treated and pasteurized samples by fatty acid % value of untreated control samples.

4.2.7 Measurement of total carbohydrates
The total carbohydrate content in all diluted (1:1000) milk samples was determined using the phenol-sulphuric acid method in micro-plate format. A set of various known concentrations of glucose (Ajax Finechem, Victoria, Australia) standards were prepared for the assay. 50 μL of glucose standards, blank (water) and diluted milk samples were placed in microfuge tubes. 150 μL of concentrated sulphuric acid (Merck, Victoria, Australia) and 30 μL of 5% phenol (Sigma, Sydney, Australia) were added. The tubes were incubated at 90 °C for 5 minutes. The tubes were cooled to room temperature for 5 minutes and the contents were transferred to a 96 well plate, and absorbance was measured at 490 nm using a plate reader (Biorad xMarkTM microplate absorbance spectrophotometer). A standard curve was established using glucose standards to calculate the total carbohydrate content in all milk samples.

4.2.8 RNA extraction and estimation

1 mL of milk was centrifuged at 5000 xg for 10 minutes at 4 °C to remove the fat and cells. This skim milk was removed and centrifuged again at 17,000 xg for 20 minute to remove any remaining cells and fat. Micro-RNA (miRNA) was isolated from skim milk using a commercially available miRNA isolation Kit (mirVana™ miRNA Isolation Kit, Ambion) and the isolation was performed as instructed in the manufacturer’s manual. The miRNA was eluted with 100 μl of elution solution provided in the kit. The samples were stored at -80 °C until further analysis. The total RNA concentration in eluted samples was measured using NanoDrop1000 spectrophotometer (Thermo Scientific). The total small RNA and miRNA quantity and integrity in milk was estimated using an Agilent 2100 Bioanalyzer (Agilent Technologies).

4.2.9 Cell lines and cell culture

The colon adenocarcinoma cells (Caco-2) and the normal intestinal cells (FHs-74-Int) were obtained from American type culture collection (ATCC, USA). The culture media Dulbecco’s
modified eagles medium (DMEM) was obtained from Sigma-Aldrich; (cat.no. D5671), supplemented with 10% heat-inactivated newborn calf serum (ThermoFischer Scientific; cat.no. A15-043), 2% L-glutamine (ThermoFischer Scientific), penicillin (100 IU/mL) and streptomycin (100 μL/mL) (ThermoFischer Scientific). The cells were cultured in incubators with 5% CO₂ at 37°C and trypsinized using 0.25% Trypsin/EDTA (Invitrogen) once they attained 75% confluence. All the experiments were conducted with cells between passages 13-22.

4.2.10 Cell viability using trypan blue assay

The cell viability was calculated by trypan blue exclusion assay. 1 x 10⁶ colon adenocarcinoma cell line (Caco-2) was grown in 6 well plates in DMEM until they reached 80% confluence. They were then treated with 3.2 mg/mL protein of filtered whole milk samples for 24 hours, an untreated well was maintained as a control. The study was also performed in 1 x 10⁶ normal intestinal cells (FHs 74 Int). Following treatment the cells were washed with phosphate buffer solution (PBS) twice and trypsinized using trypsin/EDTA (Life Technologies). The cells were then pelleted by centrifugation at 1500 rpm and resuspended in sterile 1xPBS. 10 μL of cell suspension from each sample was mixed with same quantity of trypan blue stain (Sigma Aldrich, Australia) and the live cells were counted under the microscope using a haemocytometer. The imaging was performed using invested microscope (Prism optical).

4.2.11 Annexin V assay

The annexin-V assay was performed in order to confirm the apoptosis induced by milk samples in Caco-2 cells and in order to compare the apoptotic effects in non-cancerous (FHs 74 Int) cells. 1 x 10⁶ cells were seeded in 6 well plates and once confluent were treated with milk samples for 24 hours. The cells were then washed and stained with annexin-V staining solution
provided in the annexin-V staining kit (Invitrogen, Australia). The cells were further analysed using BD FACS canto II (BD Biosciences).

4.2.12 Statistical analysis

The plasma experiments in milk were repeated at least three times and each sample was analyzed in triplicate. The data were analyzed using GraphPad Prism software (version 7.03). The statistical significance (P < 0.05) between the samples were calculated using the Holm-Sidak method in GraphPad Prism software.

4.3 Results and Discussion

4.3.1 Analysis of total protein, lipid and carbohydrate in milk

The chemical composition of milk (protein, lipids and carbohydrates) undergoes changes during processing by different methods and under different storage conditions. The extent of changes in chemical composition of milk mainly depends upon the intensity of the treatment technique and the time. In this work, the changes in protein, lipids, carbohydrates and miRNA content of milk after plasma treatment and pasteurization have been studied. The total protein, lipid and carbohydrate content in raw milk samples were 3.69, 4.02 and 4.81 g/100 mL, respectively (Figure 4.1). The total protein content was significantly reduced to 3.54 and 3.57 g/100 mL in 90 s plasma treated and pasteurized milk compared to control milk samples. In 120 s plasma treated milk, the protein content remained as 3.65 g/100 mL. The total lipid content was significantly reduced in pasteurized milk (2.73 g/100 mL) compared to 90 and 120 s (3.88 and 3.87 g/100 mL) plasma treated milk. The total carbohydrate content was significantly reduced in plasma treated and pasteurized milk compared to control samples. The carbohydrates were significantly reduced in pasteurized milk (3.86 g/100 mL) compared to 90 and 120 s (4.15 and 4.12 g/100 mL) plasma treated milk. The lactose in milk reacts with proteins and salts through Maillard reactions due to heating. Suyama et al. demonstrated that
the concentration of lactose ureide (a by-product formed through Maillard reaction) increased with the increase in temperature and treatment time compared to raw milk. They found that, the lactose ureide concentration in raw milk was increased from 0.3 to 1.2 and 1.6 mg/L after pasteurization (65 °C for 30 min) and UHT. In our previous work, it was shown that the plasma setup used in this study mainly produced hydroxyl (OH) radicals and hydrogen peroxide (H₂O₂) in de-ionized water, while using argon gas. Luck et al. reported that H₂O₂ at high concentrations could reduce the lactose concentration in milk. The H₂O₂ and other plasma components (nanosecond pulsed electric field and UV radiation) might have contributed to the carbohydrate reduction in plasma treated milk samples. The measurements of any by-products formed from the interaction of plasma components with carbohydrates (also proteins and lipids) are important to understand the extent of damage and their effects in milk quality. The changes in individual protein and fatty acids of lipid in both plasma treated and pasteurized milk have been measured and discussed in the below section.

Figure 4.1. The concentration of total protein, lipid and carbohydrate content in raw (control), pasteurized and plasma treated milk samples. The control (raw milk) samples were left in the treatment chamber for two minutes with bubbling argon gas. The error bars represent the
standard error of the mean (SEM) of samples. Statistical significance (a - \( P \leq 0.01 \) and b - \( P \leq 0.0001 \)) was shown between the raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.

4.3.2 Analysis of skim fraction by HPLC

Skim milk was prepared according to the method in experimental section and subjected to high performance liquid chromatography (HPLC) analysis to study the changes induced by plasma treatment and pasteurization on individual proteins (Figure 4.2). In the chromatographic profiles of all milk samples, a stark difference in peak 5 (three forms a, b and c were identified as \( \beta \)-LG) was found after plasma treatment compared to other peaks. The \( \beta \)-LG (5a+5b+5c) whey protein was significantly reduced to 0.74 and 0.41 fold in 90 and 120 s plasma treated milk compared to control milk whereas the pasteurization reduced to 0.87 fold. \( \beta \)-LG is a major whey protein present in water phase of milk. The plasma reactive species (particularly OH radicals and H\(_2\)O\(_2\)) might have easily attacked the free sulfhydryl groups (SH) present in \( \beta \)-LG. It was also observed that addition of pure H\(_2\)O\(_2\) in milk oxidized the SH groups of \( \beta \)-LG which in turn reduced the concentration of \( \beta \)-LG.\(^{391}\) Creamer et al. showed that at 65 °C, the tertiary structure of \( \beta \)-LG undergoes reversible changes to initiate a chain reactions involving SH or disulphide (S-S) exchange.\(^{434}\)

The casein peaks in Figure 4.2 were identified using the literature work.\(^{425}\) The kappa casein (\( \kappa \)-CN) and alpha casein (\( \alpha_{\alpha2} \)-CN) are generally present in almost equal concentration, whereas alpha casein (\( \alpha_{\alpha1} \)-CN) is the most abundant protein in bovine milk.\(^{179, 191}\) The peak 1, 2 and 3 in Figure 4.2 at retention times 11.98, 13.39 and 14.02 min were likely to be identified as \( \kappa \)-CN, \( \alpha_{\alpha2} \)-CN and \( \alpha_{\alpha1} \)-CN. Beta casein (\( \beta \)-CN) exists in one or more variants based on the genetic breed varieties and its largely available next to \( \alpha \)-CN.\(^{435}\) The peak 4 (three forms a, b and c) was considered to be \( \beta \)-CN. The \( \kappa \)-CN, \( \alpha_{\alpha1} \)-CN, \( \beta \)-CN (4a+4b+4c) proteins were significantly increased to 1.25, 1.09 and 1.18 fold after 90 s plasma whereas the 120 s plasma treatment
further increased them to 1.38, 1.13 and 1.21 fold compared to control. The decrease in β-LG with respect to increase in plasma treatment time might contribute to a higher increase in casein proteins compared to 90 s plasma treatment. In pasteurized milk, the κ-CN was non-significantly increased to 1.39 fold and α_{s1}-CN was significantly reduced to 0.92 fold and β-CN was non-significantly reduced to 0.95. Anema et.al also found higher levels of κ-CN, lower levels of α-CN and nearly same level of β-CN when milk was heated up to 70 °C. The dissociated α-CN in pasteurized milk might have moved to serum phase while the denatured whey proteins could have associated with κ-CN to increase its concentration.

The α_{s2}-CN protein was significantly reduced to 0.76 fold in 120 s plasma while it remain unaltered in 90 s plasma treatment (0.98 fold) and non-significantly reduced to 0.85 fold in pasteurized milk compared to control. Patel et al. found that α_{s2}-CN protein is unlikely to bind with whey proteins at temperatures <100 °C or at pressure treatment <150 MPa. The increase in plasma treatment time (120 s) might attack the disulphide bonds present in α_{s2}-CN and thus leads to its reduction. The decrease in β-LG protein and increase in casein proteins in both plasma treated (90 and 120 s) and pasteurized milk suggests the possible interaction between casein and whey proteins. Considine et al. explained that non-covalent and/or disulphide bonds (via thiol-disulphide exchange) exchange are the common interaction pathways involved between casein and whey proteins. A possible interaction between casein and whey proteins in milk after 90 and 120 s plasma treatment is shown in Figure 4.6. However, further investigations are required to validate this proposed mechanism.

The peaks at retention time 13.98 and 14.31 min were unidentified. The component at 13.98 min was significantly increased to 1.11 and 1.08 fold in 90 and 120 s plasma treated milk while it was significantly reduced to 0.85 fold in pasteurized milk compared to control. The other component at 14.31 min remains unaltered after 90 s plasma whereas it was significantly decreased to 0.89 fold after 120 s plasma treatment. In pasteurized milk, the latter component
was significantly increased to 1.06 fold. From HPLC analysis, it was inferred that 120 s plasma treatment caused the most significant changes in milk proteins compared to 90 s plasma and pasteurized milk.

![HPLC analysis of individual proteins in raw (control), pasteurized and plasma treated milk samples. Protein peak areas 1, 2, 3 and 4 (a, b and c) are kappa, alpha-s1, alpha-s2 and beta casein proteins, respectively while 5 (a, b and c) are β-LG whey proteins, respectively. The error bars represent the SEM of samples. Statistical significance (a - P ≤ 0.05, b - P ≤ 0.01, c - P ≤ 0.001 and d - P ≤ 0.0001) was shown between the raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.](image)

4.3.3 Analysis of whey proteins using Western blotting

Western blot analysis followed by densitometry confirmed the reduction of Lf, β-LG proteins in both the plasma treated and pasteurized milk samples, while α-LA remained unchanged between treatments is shown in Figure 4.3. The Lf band density was significantly reduced to 0.96, 0.93 and 0.90 fold in 90 and 120 s plasma treated and pasteurized milk compared to control. The Lf degradation in pasteurized milk is significant compared to 90 s plasma
treatment. Lf is a minor whey protein which can be easily degraded at 65 °C temperature or less.\textsuperscript{437}

Similar to HPLC, the β-LG was reduced with the increase in plasma treatment time. The band densities were significantly reduced to 0.93 and 0.88 fold in 90 and 120 s plasma treated milk compared to control. Pasteurization non-significantly reduced the β-LG to 0.94 fold compared to control. No substantial change in α-LA protein fold was observed in both plasma treated (90 and 120 s - 1.01 and 0.99 fold), and pasteurized milk (0.99 fold) compared to control. α-LA is a relatively stable protein and it does not have any SH group like β-LG.\textsuperscript{438} In pasteurized milk, β-LG and α-LA, both undergoes smaller changes due to their better heat stability compared to Lf protein. Fox \textit{et al.} showed that the significant changes in β-LG and α-LA occurs only at high temperatures (70–75 °C).\textsuperscript{437}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure}
\caption{Western blotting of whey proteins following treatments with raw (control),}
\end{figure}
pasteurized and plasma treated milk samples. Western blotting of Lf, β-LG and α-LA (top) and the corresponding band density fold changes (bottom) in control (A), pasteurized (B) and plasma treated (C-30 s, D-60 s, E-90 s and F-120 s) whey fractions in milk samples were shown. Statistical significance (a - P ≤ 0.05 and b - P ≤ 0.01) was shown between the raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.

4.3.4 Analysis of whey proteins using SDS-PAGE

Whey portions of all milk types were ran in gels to study the changes in electrophoretic mobility of proteins using SDS-PAGE. The whey fraction SDS-PAGE and corresponding band densitometry analysis of treated milks are shown in Figure 4.4. The Lf protein was significantly reduced to 0.97 fold in pasteurized milk compared to control. The Lf degradation is significantly different compared to 90 s plasma treatment. No significant differences in Lf were found in plasma treated (90 and 120 s) milks compared to control. It appears that the BSA fold densities remained largely unaltered in both plasma treated (90 and 120 s) and pasteurized milk compared to control samples. The immunoglobulins (Ig) were not significantly reduced in 90 s plasma treated (0.97 fold) and pasteurized milk (0.98 fold) whereas the 120 s plasma treatment (0.93 fold) significantly reduced it compared to control. This showed that Ig was sensitive to plasma treatment next to β-LG protein in milk. The β-LG was non-significantly reduced to 0.89 and 0.95 fold in 90 s plasma treated and pasteurized milk compared to control. The 120 s plasma treatment (0.79 fold) significantly reduced β-LG compared to control. No significant change in α-LA protein fold was observed in 90 s plasma treated (0.99) and pasteurized milk (1.00) compared to control samples. The 120 s plasma treatment (0.96 fold) significantly reduced α-LA compared to control. The significant changes in Ig, β-LG and α-LA proteins were observed only in 120 s plasma treated milk compared to pasteurized milk (Figure 4.4).
4.3.5 Analysis of skim proteins using SDS-PAGE

SDS-PAGE was performed for skim milk proteins and the corresponding band densitometry analysis is shown in Figure 4.5. The Lf protein was significantly reduced in both plasma treated (90 and 120 s - 0.90 and 0.91 fold) and pasteurized skim milk (0.87 fold) compared to control. BSA was found significantly reduced to 0.88 and 0.85 fold in both 90 and 120 s plasma treated...
skim milk compared to control. Similarly, pasteurization also significantly reduced the BSA to 0.85 fold compared to control samples. No significant differences in BSA were found between plasma treated (90 and 120 s) and pasteurized skim milk. The 120 s plasma significantly reduced the Ig to 0.84 fold while the 90 s plasma treated and pasteurized skim milk were non-significantly reduced to 0.85, and 0.87 fold compared to control. The 90 and 120 s plasma treatment significantly reduced the β-LG band density to 0.85 and 0.81 fold whereas the pasteurization reduced it to 0.88 fold compared to control. No significant change in α-LA protein was observed between plasma treated (90 and 120 s - 0.86 and 0.84 fold) and pasteurized (0.85 fold) skim milk. The casein proteins in skim milk were non-significantly reduced to 0.98, 0.88 and 0.91 fold after 90 s, 120 s plasma treatment and pasteurization compared to control samples. In HPLC, the κ-CN, αs1-CN, β-CN were significantly increased in 120 s plasma compared to 90 s plasma treatment and pasteurization (Figure 4.2). However, the 120 s plasma treatment (significantly) and pasteurization (non-significantly) reduced αs2-CN compared to control which did not occur in 90 s plasma treatment. As mentioned earlier that the αs2-CN protein might be dissociated from casein micelles and moved to serum phase of milk (Figure 4.6), which could be the reason the casein fold were non-significantly reduced in skim milk SDS-PAGE.

Overall, the 90 s plasma treatment caused the least changes in milk proteins compared to pasteurization and 120 s plasma treatment. The 90 s plasma mainly reduced β-LG (more than pasteurization) and Lf (lesser than pasteurization) proteins. Pasteurization significantly reduced casein, Lf and β-LG proteins. The 120 s plasma treatment was the most effective to reduce whey and αs2-CN proteins. Based on the results obtained, we propose that the sensitivity of proteins to plasma treatment were in the order β-LG > Ig > Lf > BSA or α-LA. The sensitivity of proteins to heat were in the order Lf > Ig > BSA > β-LG > α-LA. Patel et al. found a similar trend in sensitivity of proteins to heat treatment.294
Figure 4.5. SDS-PAGE of skim milk proteins following treatments with raw (control), pasteurized and plasma treated milk samples. SDS-PAGE of proteins (top) and the corresponding band density fold changes (bottom) in control (A), pasteurized (B) and plasma treated (C-30 s, D-60 s, E-90 s and F-120 s) skim milk samples were shown. Statistical significance (a - $P \leq 0.05$) was shown between the raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.
Figure 4.6. Schematic representation of proposed changes of whey and casein proteins in milk after 90 and 120 s plasma treatment. The 90 s plasma treatment (left) in milk keep both whey (except β-LG association with κ-CN) and casein proteins intact compared to control (top) while the 120 s plasma treatment (right) in milk effectively altered the whey (β-LG and α-LA) which then associated with disordered α, β and κ-CN proteins through SH or disulphide (S-S)
exchange. This figure was inspired from Patel et al.\textsuperscript{294} and re-drawn according to the results obtained.

4.3.6 Fatty acid analysis by GC

Milk fat predominantly contains 98% triacylglycerols and 2% other type of lipids (mono and diacylglycerols, phospholipids, cholesterol, glycolipids and free fatty acids).\textsuperscript{439} GC results showed the changes in fatty acid content of all milk samples (Table 4.1). The 90 and 120 s plasma treatment did not induce any major changes in the fatty acid content of milk samples compared to control. This showed that plasma treatment in this study had no negative effects on milk fatty acids. The raw milk (control) samples used for plasma treatment in this study were not homogenized, which leaves the milk fat globule membrane (MFGM) intact, and could be the possible reason for no negative effects on milk fatty acids. It would be interesting to see the effects of plasma treatment in milk before and after homogenization. Kim and Korachi \textit{et al.} observed non-significant changes on fatty acids in milk after plasma treatment using DBD and CD, respectively.\textsuperscript{361,423}

It was reported that the plasma reactive species were effective in oxidizing the polar phospholipids present in the bacterial cell wall to cause cell injury.\textsuperscript{440} The phospholipids are present in the MFGM and account for less than 1% by weight of total milk fat.\textsuperscript{439} The separation of phospholipids from other lipid components (tri, di, and monoacylglycerols and others) in plasma treated milk and subjecting to analysis will help to understand any plasma mediated effects in each lipid components. However, the GC results showed that no major change in fatty acid content of plasma treated milk samples compared to control. It is also important to mention that the degraded whey proteins might associate with either casein proteins and/or MFGM which was observed in heated milks.\textsuperscript{441} The consequences of this kind of association and any by-products such as aldehydes, ketones and acids formed as a result of plasma species interaction with fatty acids need to be evaluated.
In pasteurized milk, the saturated fatty acids (SFAs) such as caproic acid (0.94 fold), capric acid (0.97 fold), lauric acid (0.98 fold), myristic acid (0.99 fold) and palmitic acid (0.94 fold) were significantly reduced (Table 4.1). This observed decrease also matches with the decrease in the total lipids in pasteurized milk (Figure 4.1). The significant decrease in the percentage composition of SFAs in pasteurized milk leads to a significant increase in other fatty acids like pentadecanoic acid (1.04 fold), heptadecanoic acid (1.06 fold), stearic acid (1.04 fold), elaidic acid (1.22 fold), oleic acid (1.07 fold), linoelaidic acid (1.23 fold), linoleic acid (1.23 fold), α-linolenic acid (1.05 fold) and arachidic acid (1.06 fold). The measurement of fatty acids concentration in milk after pasteurization will show the heat induced changes in unsaturated fatty acids. Greenbank and Pallansch found that phospholipid content in milk were reduced after heat treatment.\textsuperscript{442} In contrary, Houlihan \textit{et al.} reported that the heat treatment reduced the triacylglycerols and not phospholipids in milk.\textsuperscript{443} This showed that heat treatment alters the lipid content in milk based on the different treatment conditions. Moreover, it is known that homogenization reduces the MFGM size in milk and helps to expose all lipid components to heat. Heat treatment in milk before homogenization caused the association of whey proteins (particularly β-LG) with MFGM in smaller proportion compared to heat treatment in milk after homogenization.\textsuperscript{444} Notably, the trans fatty acids like elaidic acid (1.22 fold) and linoelaidic acid (1.23 fold) and a cis fatty acid, linoleic acid (1.23 fold) were significantly increased compared to any other fatty acids in pasteurized milk and also compared to plasma treated and raw milk samples. This larger increase in the trans and cis fatty acids might be a direct result of the pasteurization in milk. Herzallah \textit{et al.} observed a significant 1.20 fold increase in trans isomer content (elaidic acid) in the milk heated at 63 ± 1.0 °C for 30 min.\textsuperscript{445} The increase in the in-take of trans fatty acids can cause adverse effects on human health.\textsuperscript{446} Jing \textit{et al.} reported that linoelaidic induced a stronger lesion effect in human umbilical vein endothelial cells through lipid rafts (a region rich in cholesterols and sphingolipids as functional microdomain
in membrane) compared to elaidic acid.\textsuperscript{447} It was observed that, linoleic acid also plays a key role in clustering of proteins in cholesterol-dependent microdomains.\textsuperscript{448}

**Table 4.1. The percentage fatty acid composition in raw (control), pasteurized and plasma treated milk samples.**

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Control</th>
<th>Pasteurized</th>
<th>30 s plasma</th>
<th>60 s plasma</th>
<th>90 s plasma</th>
<th>120 s plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caproic</td>
<td>2.76 ± 0.05</td>
<td>2.59 ± 0.02\textsuperscript{b}</td>
<td>2.75 ± 0.02</td>
<td>2.71 ± 0.01</td>
<td>2.74 ± 0.01</td>
<td>2.75 ± 0.01</td>
</tr>
<tr>
<td>Caprylic</td>
<td>1.64 ± 0.01</td>
<td>1.60 ± 0.04</td>
<td>1.66 ± 0.01</td>
<td>1.64 ± 0.01</td>
<td>1.64 ± 0.01</td>
<td>1.65 ± 0.01</td>
</tr>
<tr>
<td>Capric</td>
<td>3.65 ± 0.01</td>
<td>3.55 ± 0.01\textsuperscript{d}</td>
<td>3.62 ± 0.01</td>
<td>3.63 ± 0.01</td>
<td>3.63 ± 0.01</td>
<td>3.63 ± 0.01</td>
</tr>
<tr>
<td>Lauric</td>
<td>4.21 ± 0.01</td>
<td>4.14 ± 0.01\textsuperscript{d}</td>
<td>4.19 ± 0.01</td>
<td>4.20 ± 0.01</td>
<td>4.19 ± 0.01\textsuperscript{a}</td>
<td>4.19 ± 0.01\textsuperscript{a}</td>
</tr>
<tr>
<td>Myristic</td>
<td>13.18 ± 0.03</td>
<td>13.08 ± 0.01\textsuperscript{b}</td>
<td>13.11 ± 0.01</td>
<td>13.13 ± 0.01</td>
<td>13.11 ± 0.01\textsuperscript{a}</td>
<td>13.15 ± 0.01</td>
</tr>
<tr>
<td>Myristoleic</td>
<td>1.29 ± 0.01</td>
<td>1.29 ± 0.01</td>
<td>1.28 ± 0.01</td>
<td>1.28 ± 0.01</td>
<td>1.27 ± 0.01\textsuperscript{a}</td>
<td>1.28 ± 0.01</td>
</tr>
<tr>
<td>Pentadecanoic</td>
<td>1.34 ± 0.02</td>
<td>1.40 ± 0.01\textsuperscript{c}</td>
<td>1.32 ± 0.01</td>
<td>1.33 ± 0.01</td>
<td>1.33 ± 0.01</td>
<td>1.33 ± 0.01</td>
</tr>
<tr>
<td>Palmitic</td>
<td>36.55 ± 0.04</td>
<td>34.40 ± 0.05\textsuperscript{c}</td>
<td>36.66 ± 0.05</td>
<td>36.59 ± 0.03</td>
<td>36.55 ± 0.04</td>
<td>36.65 ± 0.06</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>1.93 ± 0.01</td>
<td>1.91 ± 0.01</td>
<td>1.99 ± 0.03</td>
<td>1.92 ± 0.01</td>
<td>1.95 ± 0.02</td>
<td>1.93 ± 0.01</td>
</tr>
<tr>
<td>Heptadecanoic</td>
<td>0.70 ± 0.01</td>
<td>0.74 ± 0.01\textsuperscript{d}</td>
<td>0.70 ± 0.01</td>
<td>0.70 ± 0.01</td>
<td>0.69 ± 0.01</td>
<td>0.69 ± 0.01</td>
</tr>
<tr>
<td>Stearic</td>
<td>9.95 ± 0.03</td>
<td>10.45 ± 0.01\textsuperscript{d}</td>
<td>10.01 ± 0.01</td>
<td>10.01 ± 0.01</td>
<td>10.03 ± 0.02\textsuperscript{a}</td>
<td>9.99 ± 0.01</td>
</tr>
<tr>
<td>Elaidic</td>
<td>0.35 ± 0.01</td>
<td>0.43 ± 0.01\textsuperscript{d}</td>
<td>0.36 ± 0.01</td>
<td>0.36 ± 0.01</td>
<td>0.37 ± 0.01</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>Oleic</td>
<td>19.13 ± 0.06</td>
<td>20.53 ± 0.03\textsuperscript{d}</td>
<td>19.06 ± 0.06</td>
<td>19.20 ± 0.02</td>
<td>19.19 ± 0.02</td>
<td>19.15 ± 0.03</td>
</tr>
<tr>
<td>Linolelaaidic</td>
<td>0.38 ± 0.01</td>
<td>0.47 ± 0.01\textsuperscript{d}</td>
<td>0.37 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Linoleic</td>
<td>1.65 ± 0.01</td>
<td>2.04 ± 0.01\textsuperscript{d}</td>
<td>1.63 ± 0.01</td>
<td>1.65 ± 0.01</td>
<td>1.65 ± 0.01</td>
<td>1.64 ± 0.01</td>
</tr>
<tr>
<td>α-Linolenic</td>
<td>0.61 ± 0.01</td>
<td>0.65 ± 0.01\textsuperscript{d}</td>
<td>0.60 ± 0.01</td>
<td>0.60 ± 0.01</td>
<td>0.60 ± 0.01</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.61 ± 0.01</td>
<td>0.65 ± 0.01\textsuperscript{b}</td>
<td>0.62 ± 0.02</td>
<td>0.58 ± 0.01</td>
<td>0.62 ± 0.01</td>
<td>0.62 ± 0.02</td>
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</table>

Note: Mean ± SEM were calculated and the statistical significance (letters in superscript indicate \( a \) - \( P \leq 0.05 \), \( b \) - \( P \leq 0.01 \), \( c \) - \( P \leq 0.001 \) and \( d \) - \( P \leq 0.0001 \)) was shown between the raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.

4.3.7 RNA and miRNA analysis

In our study, the total RNA content in raw milk was measured as 5.4 ng/μL (Figure 4.7). The 90 s plasma treatment non-significantly reduced the RNA content to 4.87 ng/μL compared to raw milk. The 120 s plasma treatment and pasteurization significantly reduced the RNA content to 4.05 and 4.22 ng/μL compared to control respectively. The RNA content in 120 s plasma treated milk was significantly reduced compared to 90 s plasma treatment whereas it was non-significant compared to pasteurized milk.

![Figure 4.7. Total RNA concentration measurement in raw (control), pasteurized and plasma treated milk samples. The error bars represent the SEM of samples. Statistical significance (a](image)

Figure 4.7. Total RNA concentration measurement in raw (control), pasteurized and plasma treated milk samples. The error bars represent the SEM of samples. Statistical significance (a
- $P \leq 0.001$ and $b - P \leq 0.0001$) was shown between the raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.

miRNAs are a novel class of bioactive compounds which play numerous beneficial roles in human health. The concentration of small RNA, miRNA and their ratio (miRNA/small RNA) was measured in control, 90 s plasma treated and pasteurized milk samples (Figure 4.8). The 120 s plasma treated milk sample was not used for this small and miRNA measurement, as it had significantly reduced the total RNA content. The small RNA and miRNA content in raw milk was measured as 125.36 and 82.46 pg/μL, respectively. Both the small RNA and miRNA was non-significantly reduced to 81.9 and 51.03 pg/μL in pasteurized milk. Katherine et al. reported that pasteurization and homogenization leads to a significant loss of miRNAs in bovine milk. Interestingly, the small RNA and miRNA concentration was non-significantly increased to 241.93 and 138.56 pg/μL in 90 s plasma treated milk compared to raw milk. This increased concentration of small RNA and miRNA might be due to the release of miRNA from milk exosomes and RNA from MFG after plasma treatment. Maningat et al. shown that RNA is also available in MFG. The miRNA/small RNA ratio was not significantly different between raw, pasteurized and 90 s plasma treated milk, respectively. The ratio of miRNA to small RNA remained the same suggesting that the mechanism or source of miRNA and small RNA release may be the same. The conservation of miRNA and RNA integrity suggests that 90 s plasma treated milk represents good milk quality.
Figure 4.8. Small RNA and miRNA concentration measurement in raw (control), pasteurized and plasma treated milk samples. The error bars represent the SEM of samples. Statistical significance (a - P ≤ 0.05) was shown between the raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.

4.3.8 Response of intestinal cells upon exposure to milk samples

Raw, plasma treated (90 and 120 s) and pasteurized milk were tested in the presence of human fetal small intestinal (FHs 74 Int) cells and human colon adenocarcinoma (Caco-2) cell line to determine effects of milk treatments on cell viability (Figure 4.9). Cells were incubated for 24 hours with milk samples prior to determination of cell number. The changes in composition of milk samples reflected in the cell number of FHs 74 Int and Caco-2 cells (Figure 4.9). The unaltered composition in raw milk samples caused minimal changes in cell number of both FHs 74 Int (90.80%) and Caco-2 cells (83.92%, significant) compared to untreated cells. The presence of 90 s plasma treated milk non-significantly reduced the cell number to 88.51% in FHs 74 Int cells while it significantly reduced the cell viability to 65.80% in Caco-2 cells compared to untreated cells. No significant differences were found between raw (control) and 90 s plasma treated milk in altering FHs 74 Int cell viability whereas the 90 s plasma treated
milk significantly reduced the Caco-2 cells compared to raw (control) milk. With the increase in plasma treatment time (120 s) in milk, the cell viability was significantly further reduced to 62.27 and 64.64% in FHs Int and Caco-2 cells compared to untreated cells. The FHs 74 Int and Caco-2 cell viability was significantly reduced to 60.74 and 64.82% in pasteurized milk respectively. The 120 s plasma treated and pasteurized milk significantly reduced the FHs 74 Int cell viability compared to 90 s plasma treated milk. Hongsprabhas et al. demonstrated that heat treatment in lactose containing whey protein hydrolysate, increased the Maillard reaction products which caused cytotoxicity in FHs 74 Int and Caco-2 cells. In this work, the 120 s plasma treatment and pasteurization reduced a higher concentration of lactose and proteins compared to 90 s plasma treatment (Figure 4.1-4.6). This might be the reason that the cell viability of FHs 74 Int were not effectively reduced in 90 s plasma compared to 120 s plasma treatment and pasteurization. Interestingly, the 90 s plasma treatment reduced the cell viability of Caco-2 cells equivalent to 120 s plasma and pasteurization suggesting that these two cell lines display different degrees of sensitivity to cytotoxic reagents. The comparative analysis of milk from various sources and their properties has also shown that milk components have the capability to specifically induce cell death in cancer cells. Unlike plasma treated milk, a reverse effect was observed in pasteurized milk, in which the FHs 74 Int cells were slightly reduced compared to Caco-2 cells. It is also important to consider the role of lipids and miRNA in influencing the cell viability of both FHs 74 Int and Caco-2 cells.
Figure 4.9. The relative percentage viability of FHs 74 Int cells and caco-2 after culturing with raw (control), pasteurized and plasma treated (90 and 120 s) milk samples for 24 hours as determined trypan blue assay. The error bars represent the SEM of samples. Statistical significance (a - $P \leq 0.05$, b - $P \leq 0.01$ and c - $P \leq 0.0001$) was shown between the untreated cells, raw (control), plasma treated (90 and 120 s) and pasteurized milk samples.

The cell morphology of FHs 74 Int and Caco-2 cells upon exposure to raw and plasma (90 and 120 s) treated milk samples are shown in Figure 4.10. The changes in the cell number of FHs 74 Int and Caco-2 cells after treating with milk samples do not effect overall morphology of both cells after 24 hours.
Figure 4.10. The response of FHS 74 Int cells (upper panel) and caco-2 cells (lower panel) cultured with raw (control), pasteurized and plasma treated (90 and 120 s) milk samples for 24 hours. The cell images were captured at 20x magnification.

Annexin-V assay is a standard approach in order to detect the vent of apoptosis in cell culture. Annexin V staining was performed to determine the percentage of apoptotic cells following exposure to treated milk (Figure 4.11). Cells were exposed to milk samples for 24 hours, stained with annexin V and analyzed by flow cytometry. The percentage of apoptosis in all quadrants (Q1+Q2+Q4) except Q3 (healthy cells) were added and have been used to discuss here. The raw, 90 and 120 s plasma treated milk samples led to 2.13, 1.13 and 1.56% apoptosis in FHs 74 Int cells, respectively. Pasteurized milk was the most effective to cause higher apoptosis (3.53%) in FHs 74 Int cells compared to other samples. The same analysis was performed with Caco-2 cells to measure apoptosis after treating with all milk samples. Interestingly, pasteurized milk caused the least apoptosis (6.70%) in Caco-2 cells compared to other milk samples. On the other hand, the raw, 90 and 120 s plasma treated milk samples caused higher apoptosis 7.90, 11.76 and 10.56% in Caco-2 cells compared to pasteurized milk. The apoptosis caused by raw, 90 and 120 s plasma treated milk in Caco-2 cells were higher compared to FHs 74 Int cells. The results showed that the FHs 74 Int cells and Caco-2 cells exhibited different behaviour upon exposure to all milk samples. The apoptotic and cytotoxic
functions of the milk samples was studied in order to determine the functional efficacy of the milk proteins after plasma treatment and pasteurization. Milk contains various anticancer peptides that are considered as potential candidates for the development of anticancer agents. The casein and whey proteins in milk have been evaluated for their anticancer and apoptotic potential especially in case of colon tumours suggesting the presence of apoptotic factors in milk.453-455

Dimeric α-LA is a potent Ca^{2+}-elevating and apoptosis-inducing agent with broad activity.456 It was reported that α-LA dimerizes and induces apoptosis in a variety of tumor cell lines and primary cells.456 This dimerization of α-LA in our case needs to be studied and may be stimulated by the plasma treatment. Lf is another well-known anti-cancer protein that induces apoptosis in cancer cells.453 Lf significantly modulates the molecules involved in apoptosis including p53, Bcl-2 family proteins, IAP members and their inhibitors. bLf could also directly inhibit the expression of survivin protein (key IAP) and lead to induction of apoptosis.457 In this study, Lf protein was effectively reduced in pasteurized milk compared to 90 and 120 s plasma treated milk (Figure 4.3 and 4.4). This could be one possible reason that the plasma treated milk caused more apoptosis in Caco-2 cells compared to pasteurized milk. Although the presence of above mentioned proteins in plasma treated milk likely to be the reason for causing apoptosis in cancer cells, further investigation is required to understand the apoptosis mechanism.
Figure 4.11. Annexin V assay in FHS 74 Int cells (upper panel) and caco-2 cells (lower panel) after culturing with raw (control), pasteurized and plasma treated (90 and 120 s) milk samples for 24 hours. Inset values represents the percentage of necrotic cells. The quadrant Q1 contains the early apoptotic cells, Q2 contains both early and late apoptotic cells, Q3 contains the viable cells and Q4 contains the late apoptotic cells.

4.4 Conclusion

We have demonstrated that the plasma treatment in this study has the potential to preserve nutritional components in milk compared to pasteurization. The increase in plasma treatment time (120 s) clearly altered the milk components compared to 90 s plasma treatment and pasteurization. However, the results showed that 90s plasma treatment caused the least changes and possess the advantages to retain the nutritional benefits in milk compared to pasteurization. This suggested that tuning the plasma parameters (input power, gas flow rate and mode of operation) will help to gain control in influencing the changes in milk components. The direct comparison between plasma treatment and pasteurization in milk with defined quality indices will be key before considering this technology for scale up and commercialization.

Acknowledgements

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Daniel Fabijanic for 316 stainless steel mesh supply; Yakov. E. Krasik and Peter Lamb for valuable discussions and editorial support.
Chapter 5 General discussions and future work

Several researchers have developed the method of generating plasma in bubbles that are externally injected into liquids to reduce the energy consumption, electrode erosion, control and increase the gas-liquid interface reactions, thereby improving the productivity of physical and chemical processes in liquid.\textsuperscript{19, 20, 34-38} These advantages have helped to extend the application of plasma technology in treating sensitive materials, especially liquid foods.

In this study, plasma was generated in two ways

1) In gas phase above the liquid surface to fabricate hybrid nanostructure and

2) In bubbles that are externally injected in milk to decontaminate, extend shelf life whilst maintaining nutrition.

The general discussions of this study and potential future works have been discussed here.

5.1 Fabrication of boron nitride nanotube-gold nanoparticle hybrids

5.1.1 General discussions

The fabrication of hybrid nanostructures has faced issues such as use of toxic chemicals, longer treatment times and damaging the structure of nanomaterials.\textsuperscript{118-128, 136, 138, 139, 143} To address this, in our approach, a simple two-step plasma method was developed to fabricate BNNTs-GNPs hybrid nanostructure. In the first step, plasma treatment reduced the length and functionalized the BNNTs in deionized water. In the second step, GNPs were assembled onto the functionalized short BNNTs to fabricate a hybrid nanomaterial. The total fabrication process was completed in 30 minutes compared to the other chemical methods which requires several hours.

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Various reports have shown that use of high temperature and pressure, hydrogen peroxide and nitric acid can oxidize the BNNTs which help in shortening and then functionalizing the nanotubes.\textsuperscript{118, 132, 140, 148}

In our case, this was achieved without the use of toxic chemicals or longer treatment times and altering the nanotube structure. It was proposed that the reactive oxygen and nitrogen species from plasma and the presence of physical effects such as UV radiation and nanosecond pulsed electric field might have facilitated the oxidation of long BNNTs leads to shortening and subsequently introduced the functional groups onto the surface of short BNNTs at the same time.

The assembly of GNPs onto the functionalized short BNNTs was achieved by adding GNPs to the functionalized short nanotubes suspension. The addition of GNPs could change the conductivity of the liquid, which results in the re-distribution of electrical field during plasma treatment. This re-distributed electrical field along with the reactive species and UV radiation might have facilitated the attachment of GNPs onto the functionalized short nanotubes.

Due to shortening, the treated nanotubes carried a higher percentage of drug molecules compare to the untreated nanotubes. Similarly, Zhi \textit{et al.} showed that shortening of BNNTs could create hole-rich structure and more ends, which helps in encapsulating more cargoes and displaying improved dispersibility.\textsuperscript{118} These advantages suggest that shortened BNNTs might be useful for efficient drug delivery systems. The shortened nanotubes carried more drug molecules and effectively killed the human prostate cancer cells.

5.1.2 Future work

This new approach, successfully reduced the length and introduced functional groups onto BNNTs and assembled GNPs onto them. However, the yield of functionalized short BNNTs
and the covering of GNPs onto them need to be increased for applying this hybrid nanomaterial for biological applications.

To address the above challenges, the plasma set up II can be used as one approach, to produce a high concentration of selective reactive species with the chosen gas. The use of this method on BNNTs suspension could give the option not only to increase the yield of functionalized short BNNTs but also has the potential to introduce selective functional groups with the chosen gas (for example argon and air to form oxygen (OH) and amine (NH$_2$) functional groups). The increased selective functional groups will also help to assemble more GNPs onto them.

The use of ionic liquids could be another approach, to increase the yield of selective functionalized short BNNTs and BNNCs compared to DI water. Ionic liquids have very good physical and chemical properties which can be exploited for the preparation and stabilization of nanomaterials. The increase in the yield of functionalized short BNNTs and BNNCs possibly increase the dispersibility and drug encapsulation which all could make functionalized short BNNTs as a potential candidate for effective drug delivery.

This method will form the basis to produce varieties of hybrid nanostructures with other nanoparticles such as Fe, SnO$_2$, Pd, ZnO and CdSe while keeping the functionalized short BNNTs as a support to breed properties and utilize them for potential applications such as energy conversion and storage, catalysis and optoelectronics.

5.2 Decontamination of milk to extend shelf life while maintaining nutrition

5.2.1 General discussions

Bovine milk is a liquid food that is enriched with nutrients and growth factors but also contains micro-organisms.$^{165,166}$ The consumption of raw or ineffectively processed milk causes milk-borne diseases in humans.$^{167-170}$ Pasteurization is the current technique to kill bacteria in milk but heat treatment damages the nutrients in milk.$^{171,172}$ The other non-thermal methods such as
hydrostatic pressure, pulsed electric field, UV radiation in milk have their own advantages and
disadvantages. To address these challenges, in our approach plasma was generated in bubbles
which are externally introduced in milk to decontaminate and extend the shelf life while
maintaining nutrition and compared with commercially pasteurized milk.

The 90 and 120 seconds plasma treatment caused a minimal change in milk pH change
suggesting that the milk quality was not significantly altered. The increase in temperature of
milk was still within the ambience and hence the heat effects were limited. The 90 and 120
second plasma treatment has achieved an industrial standard bacterial reduction (4.3 log
bacterial reduction) in milk according to the USFDA pasteurized milk ordinance 2011.405, 406
Excitingly, the 90 and 120 s plasma treatment did not allow the bacteria to multiply for at least
six weeks while the pasteurized milk showed that the bacterial counts were significantly
increased after 2 weeks suggesting that deterioration of milk quality. The results confirmed that
the plasma treatment not only immediately reduced the viable bacteria but also inhibited its
proliferating potential and/or killed spores present in milk.

It has been reported that the discharge in bubbles that are externally injected into liquids (water)
produce chemical and physical effects based on the composition of gas in the bubble (and its
size), conductivity of the liquid and input power.11 In other work, we have shown that the
discharge at higher frequency created more fragmentation in gas bubbles which decreased the
size and increased the number of bubbles in DI water.34 This increased the interface reactions
between the gas-plasma and liquid. The increase in the interface reactions, along with the
movement of bubbles, enabled higher production of selective reactive species based on chosen
gas.34 This process might have occurred according to the chemistry of milk. We propose that
plasma reactive components such as OH radicals and H2O2, UV radiation, nanosecond pulsed
electric field generated at the bubble and bubble-liquid interface along with the joule heating,
might have effectively interacted with the micro-organisms that are present in the water phase of milk resulting in reduced bacterial load and proliferative potential.

Subsequent to the bacterial investigation, the effects of 90 and 120 s argon plasma treatment on proteins, lipids, carbohydrates and miRNA present in bovine milk was studied compared to commercially available pasteurized milk. The increase in plasma treatment time induced more changes in the nutritional profile of milk.

β-LG was significantly reduced in 90 and 120 s plasma treated milk compared to raw and pasteurized milk. α-LA and Igs were significantly reduced in 120 s plasma treatment compared to raw milk. Lf was significantly reduced in pasteurized milk compared to 90 s plasma treated and raw milk. Casein proteins were significantly increased in 90 and 120 s plasma treated milk whereas they were significantly reduced in pasteurized milk compared to raw milk. The decrease in whey proteins might have associated with κ-CN and thus lead to the increase in casein proteins after plasma treatment. A similar association was also observed by others in heat and/or pressure treated milks. 270, 436

The total lipids and the saturated fatty acids and carbohydrates were significantly reduced in pasteurized milk compared to raw and 90 and 120 s plasma treated milk. No major changes in fatty acids were observed in both 90 and 120 plasma treated milk compared to raw milk. The use of non-homogenized milk means the MFGM was intact and could be the possible reason for no negative effects on milk fatty acids. Kim and Korachi et al. observed non-significant changes on fatty acids in milk after dielectric barrier and corona discharge.361, 423 The RNA and miRNA concentration was effectively higher in 90 s plasma treated milk compared to pasteurized milk.

Overall, the 90 s plasma treatment caused the most minimal changes in milk nutritional composition compared to pasteurization and 120 s plasma treatment. Overwhelmingly, the raw and 90 s plasma treated milk did not effect the viability of FHs 74 Int cells whereas the
pasteurized and 120 s plasma treated milk significantly reduced viability compared to untreated cells. This again verified that the milk quality was retained after 90 s plasma treatment compared to other milks. The viability of Caco-2 cells was significantly reduced by all milk samples compared to untreated cells. Annexin V staining showed increased apoptosis of cells exposed to plasma treated milk compared to untreated cells, but apoptosis was further increased in pasteurized milk.

To the best of our knowledge, this is the first atmospheric pressure plasma study in milk that has largely addressed the bacterial reduction, shelf life and its effect on nutritional compounds. These results demonstrated plasma treatment as a promising alternative to pasteurization in processing milk.

5.2.2 A potential future of “Plasmarized milk”

Argon plasma (bubble discharge) treatment in milk successfully reduced the bacterial load, increased shelf life and maintained the nutrition compared to commercially pasteurized milk. A limitation of the current study was that the mechanism of action was not determined, however, the mechanism of interaction between plasma components (such as reactive species (OH radicals, H₂O₂ and others, nanosecond pulsed electric field and UV radiation) and milk components (protein, lipids, carbohydrates and other micro-molecular compounds) is very important in optimizing and developing the process to a pilot scale. To address this, the measurement of H₂O₂ concentration in milk after plasma treatment, and a separate application of nanosecond pulsed electric field and UV radiation in milk without plasma under the same conditions (power input) used in this study could help to understand the plasma mediated bacterial reduction in milk and decipher if H₂O₂ plays a role in this process.

A comparison of the entire bacterial and nutritional profile in plasma treated and pasteurized milk with defined quality indices established by dairy industries and FDA could help to identify new potential markets to present plasma treated milk as fresh and nutritious milk. Plasma
treatment could also be used for modified whey production which could potentially reduce the allergenicity of β-LG protein and possibly help in improving the digest-ability or bioavailability of its peptides for body-builders and athletes during its consumption. The other whey proteins like α-LA, Lf, Ig and miRNAs in milk possess important bio-functionalities that are involved in the development of human health. Our study has shown that plasma treatment retained the major immune regulatory agents like Lf, Ig and miRNAs in milk compared to pasteurization, suggesting that milk quality is superior to pasteurized milk. However, the functionality of bovine milk proteins and miRNAs versus human milk proteins and miRNAs need to be taken into consideration when addressing bioactivity in milk products. Even if plasma treatment dose prove to conserve bioactivity of bovine proteins and miRNAs, their relevance to human physiology needs to be addressed. Perhaps, plasma treatment will be best suited to preservation of milk proteins and miRNAs in human milk, for example as a treatment in human milk banks for preterm babies.

The current study showed that plasma treatment did not affect the viability of FHs 74 Int cells compared to pasteurization, but a detailed in vitro and in vivo study will be necessary to understand the immunological properties of plasma treated milk and its implications in human health. Studies may include the impact on development of the gastrointestinal tract (GI) using embryonic mouse models (explant GI cultures), or mouse models of gut disease in preterm babies such as necrotizing enterocolitis (NEC).

The low birth weight and preterm infants have immature intestine and immune systems, due to this they are at increased risk of a number of complications, especially NEC, a disease with a mortality rate of 20–40%.\(^{458, 459}\) The other complications are growth retardation, feeding intolerance and infections. Preterm infants are fed with pasteurized human donor milk when mother’s own milk is insufficient.\(^{460}\) The use of pasteurization damages the immunological properties of human milk.\(^{461}\) The pasteurized donor milk is inferior to mother’s own milk in
resisting against late-onset sepsis and NEC.\textsuperscript{462} On the other hand, the formula feed to preterm babies have shown six times increased incidence of NEC than if only fed breast milk.\textsuperscript{463} The use of plasma treatment as an alternative to pasteurization, could preserve the immunological properties of human milk, thereby it would possibly improve the immunopotency of infants and reduce associated medical costs.

The plasma treatment could be possibly applied for other dairy applications such as cheese making, butter and yoghurt production. For example, the plasma treatment in milk could possibly introduce new functionalities which might influence the firmness of curd and aging time or ripening of cheese. The fatty acids in milk were largely unaltered after plasma treatment which suggests that butter making from plasma treated milk would be of higher quality compared to butter making from pasteurized milk. This kind of technological innovation in milk processing could potentially address the increasing consumer demands of fresh-like, nutritious and shelf stable milk and milk products at lower capital and operating costs.
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