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Interactions between Titanium Dioxide Nanoparticles (NPs) and mucin

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INTERACTIONS BETWEEN TITANIUM DIOXIDE NANOPARTICLES (NPS) AND MUCIN

A Dissertation Presented

by

BOIMIN

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DEDICATION

I dedicate this thesis to

My country, INDONESIA

My beloved families and friends

for their inspiration, unconditional love, and support

My respected professors

for their guidance.

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ABSTRACT

INTERACTION BETWEEN TITANIUM DIOXIDE NANOPARTICLES (NPS) AND MUCIN

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Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in many food, consumer, and industrial products. However, little is known about the overall effects of TiO_2 NPs on the environment or human health. In order to elucidate the fate, transformation, transport, and toxicological impact of TiO_2 NPs, a better understanding is needed of how the physicochemical properties of TiO_2 NPs (e.g. size, charge, curvature, hydrophobicity, and surface functionality) interact with their microenvironments (e.g. pH, temperature, bile acids, microbiome, enzymes, surface-active components, and biopolymers).

Living organisms including humans have a natural mechanism to protect themselves from physical, biological, and chemical perils by generating mucin—the main gel-forming polymers of mucus and consists of core protein domains and densely O-

linked oligosaccharide chains. However, there is a very limited study even no study examining the interaction between TiO_2 NPs and mucin comprehensively.

This thesis was divided into three parts: first, a literature review focusing on the major routes of TiO_2 NPs entered the environment and human body, and the mechanistic interactions of biomolecules on the surface of TiO_2 NPs; the second part, the effect of TiO_2 NPs-mucin interaction on the alteration of physicochemical properties of TiO_2 NPs and mucin during the formation of biomolecular corona (BMC), aggregation and accumulation of TiO_2 NPs in water; the last part, the effect of phosphate-buffered saline (PBS) and pH to the change of surface charge, the formation of BMC, and hetero aggregation.

There were several major pathways of TiO_2 NPs entered the environment and the human system. TiO_2 NPs interacted with the human system via the respiratory system, skin, and gastrointestinal (GI) tract. Interaction between TiO_2 NPs and mucin might be induced by polyvalent binding as evidenced by three or more ligands (TiO_2 NPs) that were likely to interact with one molecule receptor (mucin). TiO_2 NPs-mucin interaction (at a mass ratio of TiO_2 NPs and mucin= 0.25) was likely to cause the massive hetero aggregation since evidenced by the increase of size, the hypochromic effect and redshift of UV-Vis spectra, and the appearance of spectral peaks of TiO_2 NPs and mucin by the surface-enhanced Raman Spectroscopy (SERS). The formation of unstable hetero aggregation in PBS required a higher concentration of TiO_2 NPs than in DIW. In deionized water (DIW), cationic BMC was potentially developed in acidic conditions; while in PBS there was, no cationic BMC formed. It might be caused by the effect of buffers and pH on physicochemical properties of TiO_2 NPs, particularly the surface

charge. Surface charges of TiO_2 NPs in DIW were changed from positive to negative with the increase of pH, while surface charges of TiO_2 NPs in PBS were all negative in various pH. Albeit both TiO_2 NPs and mucin in PBS had a negative surface charge, SERS exhibited that TiO_2 NPs-mucin interaction still occurred. In conclusion, the interaction between TiO_2 NPs and mucin is potentially polyvalent binding and thereby induces irreversible hetero aggregation through various interactions. Further studies to know the types of interaction, reaction products, and possible consequences are required.

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CHAPTER I

1. INTRODUCTION

The estimated global production of TiO_2 NPs was approximately 2000 metric tons worth $70 million in 2005 [1] and had increased to 5000 tons by 2010. This trend is expected to increase until 2025 continuously [2]. TiO_2 NPs are widely used in aquaculture, agriculture, and are listed as the top five nanoparticles (NPs) found in consumer products including food [3]. These facts have raised concerns related to ecological and human health [4-15]. But, determining the fate, transformation, transport, and toxicity of TiO_2 NPs remains challenging. It profoundly depends on the chemical reactivity and physicochemical property of TiO_2 NPs in a complex environment. Taking all these facts together, studies to understand the bioactivity of TiO_2 NPs through investigating the alteration of their chemical reactivity and physicochemical property are a necessity.

The reactivity and physicochemical property of NPs in the aquatic environment is strongly influenced by water properties (such as pH, water hardness, ionic composition, and temperature) [16-22] suspended particulate matter (SPM) in water [23-28] and the presence of living organisms [17, 29-32]. Water is also called the “universal solvent” [33] because of the capability of water to dissolve more substance than any other solvent, and thereby it is pivotal for living ting on earth including for human, particularly as a great solvent for nutrients in the gastrointestinal (GI) tract. Interactions of water with surrounding substances and smooth alterations in water chemistry may significantly modify the surface properties of nanoparticles (NPs). In summary, the chemical reactivity

and physicochemical properties of TiO_2 NPs in the aquatic environment and human GI tract may be influenced by water properties.

Natural organic matter (NOM) in water is expected to provide a profound impact on the surface properties of NPs through coating and surface charge alterations [25, 27, 34-35]. Mucin is well known as NOM that protects the host from biological, physical, and chemical hazards [36-39]. Mucin is the main gel-forming polymers of mucus and consists of core protein domains and densely O-linked oligosaccharide chains; hence, it confers negative charge to the mucin through carboxyl and sulfate groups [36]. Interactions between TiO_2 NPs and mucin and other NOM cause aggregations and accumulations of NPs in fish [40] and invertebrates [41]. Yin, C. et al. (2019), for instance, suggested that the accumulation of TiO_2 NPs may pass through aquatic organisms to the higher trophic levels (particularly to human) via seafood [42]. Subsequently, this food may be digested in the human gastrointestinal (GI) tract. Therefore, TiO_2 NPs trapped in the food matrix are released and absorbed by the human gut [43]. In general, TiO_2 NPs-mucin interaction may lead to hetero aggregation and accumulation of TiO_2 NPs in aquatic organisms and humans.

Although the absorption of TiO_2 NPs in the human GI tract is considered at low levels [44], it may be toxic enough in cells. Bare TiO_2 NPs generated more free radicals (hydrogen peroxide (H_2O_2)) in phosphate-buffered saline (PBS) than in pure water [45]. As PBS is well known as a cell buffer, it indicates that bare TiO_2 NPs may be toxic in cells. However, TiO_2 NPs contained in digested food models (1.5 % w/w) did not significantly perturbed cellular proteome due to their interaction with food matrix [46] and mucin (mucus) [47]. Mucin excludes foreign or hazardous molecules from the host,

and permit the useful one such as nutrients [36, 48]. It can be noticed that buffer and mucin may play an important role in determining the toxicity of TiO_2 NPs in cells.

Disputes associated with the accumulation of TiO_2 NPs in tissue have been caused by the increase in organ levels was not always detected [44] due to very low absorptions and concentration-dependent. Although the absorption of TiO_2 NPs is low, they could be approved by visual detection in organs [49]. So, the detection of TiO_2 NPs accumulation may depend on the absorption and concentration of TiO_2 NPs.

The interaction between TiO_2 NPs and mucin occurs in the GI tract at various pH. TiO_2 NPs are poorly dissolved in the model gastric and intestinal environment [44]. TiO_2 NPs exposed with juices mimicking the gastric and intestinal compartment (pH= 2 & 7) tend to alter their size and surface charge due to agglomeration and protein adsorptions on their surface [50, 51]. However, cationic nanoparticles/biomolecules are reported to have the capability to cross the epithelial barrier [52]. *In vitro* studies on Caco-2 intestinal cells [53-56] and TR146 buccal cells [57] have shown the translocation of NPs through the epithelial barrier. Studies associated with the penetration of cell barrier by NPs have been conducted; but, the condition was not very representative of the real gut environment, particularly they did not consider the presence of mucin (Mercier-Bonin et al., 2018). It is interesting to be noted that pH may affect the formation of cationic TiO_2 NPs/BMC, the change of physicochemical properties, and the penetration of the epithelial barrier.

Taking all these together, the long-term goal of this study is to simplify the development of theory in dealing with the fate, transformation, transport, and toxicity of TiO_2 NPs by understanding the mechanistic interaction between TiO_2 NPs and a

complex environment. To achieve this goal, the overall objective of this study is to develop a basic theory about the mechanistic interaction between TiO_2 NPs and mucin by examining the effect of pH and buffer on the change of physicochemical properties of TiO_2 NPs/BMC. The central hypothesis of this study is that the interaction between TiO_2 NPs and mucin may alter the physicochemical properties of TiO_2 NPs (particularly surface charge and size). Additionally, pH and buffer may play a crucial role in terms of the physicochemical alteration of TiO_2 NPs/BMC properties, the formation of hetero aggregation, and cationic TiO_2 NPs/BMC.

In order to test the central hypothesis and achieve the objective, some specific aims are pursued:

1. ***Determination of the surface charge of TiO_2 NPs and mucin in deionized water (DIW).*** The surface charge of TiO_2 NPs and mucin will be determined by using dynamic light scattering (DLS) in various pH to evaluate pH values near the zero points of charge (pH_{pzc}) and the formation of cationic TiO_2 NPs. It is expected to observe a homo aggregation of TiO_2 NPs at pH_{pzc}, while dispersion at cationic TiO_2 NPs.
2. ***Determine the surface charge, size, and turbidity of BMC and characterize the TiO_2 NPs-mucin interaction in DIW.*** DLS will be utilized to determine the surface charge of BMC at a different pH, while the size will be measured by using Mastersizer. UV-Vis spectrophotometry will be performed to determine the turbidity (O.D. 600) and the maximum absorption of BMC at different concentrations of TiO_2 NPs. Surface-enhanced Raman spectroscopy (SERS) will be employed to characterize TiO_2 NPs-mucin interaction. It is an

expectation to observe the formation of a cationic BMC and a hetero aggregation at a certain mass ratio of TiO_2 NPs and mucin.

3. ***Determination of the type of binding and the change of energy during TiO_2 NPs-mucin interaction in DIW.*** Isothermal titration calorimetry (ITC) will be used to examine the change of energy during the formation of BMC and to determine the type of binding between TiO_2 NPs and mucin. This experiment anticipates observing exothermic energy and polyvalent binding.
4. ***Determination of the surface charge of TiO_2 NP, mucin, and BMC, and characterize the TiO_2 NPs- mucin interaction in PBS.*** The surface charge of TiO_2 NPs and BMC will be examined by using DLS. It is expected to observe an interaction between TiO_2 NPs and mucin, although the surface charge of both TiO_2 NPs and mucin is the same.

CHAPTER II

2. LITERATURE REVIEW

Introduction

Nanotechnology has been widely applied in consumer products, environmental protection, agriculture, aquaculture, and medicine. A nanoparticle is described as a particle of matter that is between 1 and 100 nanometers (nm) in at least one dimension [58]. TiO_2 NPs, also known as nanocrystalline titanium dioxide, ultrafine titanium dioxide, or microcrystalline titanium dioxide, are one of the most widely used nanoparticles in the world [59]. Consequently, TiO_2 NPs are listed as the top five NPs found in consumer products [3].

TiO_2 NPs are commonly used as a colorant because of their brightness and opacifying strength. TiO_2 NPs consist of three different types of crystal polymorphs: anatase, brookite, and rutile. They are resistant to chemical disruptions and exhibit excellent thermal stability. When exposed to temperatures above 800 ^{o}C, brookite and anatase can transform into rutile, a more stable TiO_2 NP. Most importantly, TiO_2 NPs can absorb and scatter ultraviolet light due to their high refractive index. TiO_2 NPs have photoactive properties which are characterized by the different band gaps in their electron structure. Anastase is the most photoactive due to its highest bandgap among all crystal forms of TiO_2 NPs [60-62].

Due to widespread use, the potentially detrimental effects of TiO_2 NPs have raised environmental and health concerns, and the cause of those effects is mostly still debatable. In particular, the formation of biological corona (BMC), the adsorption of

biomolecules on the surface of TiO_2 NPs, has been a critical determinant of the bio-fate of the nanoparticles (NPs) [63]. Bare TiO_2 NPs tend to aggregate in aqueous media; consequently, these aggregates reduce the cell viability and cause expression of stress-related genes, for instance, those encoding interleukin-6 (IL-6) and heat shock protein 70B'[64]. Although TiO_2 NPs are likely to induce inflammatory and heat shock response, the conjugation of TiO_2 NPs with PEG, for instance, can eliminate aggregation due to steric hindrance and reduce their toxicity [64]. There is a growing interest in studying BMC due to its possible association with certain essential phenomena in biological systems [65]. BMC research stem faces specific challenges as the mechanism of TiO_2 NPs-biomolecules interactions is poorly understood [66]. As illustrated in the conceptual framework on potential TiO_2 NPs-biomolecule interactions, investigations on the formation of BMC in conditions relevant to environmental and/or physiological situations represent a research challenge and a gap in the literature **(Fig. 1)**.

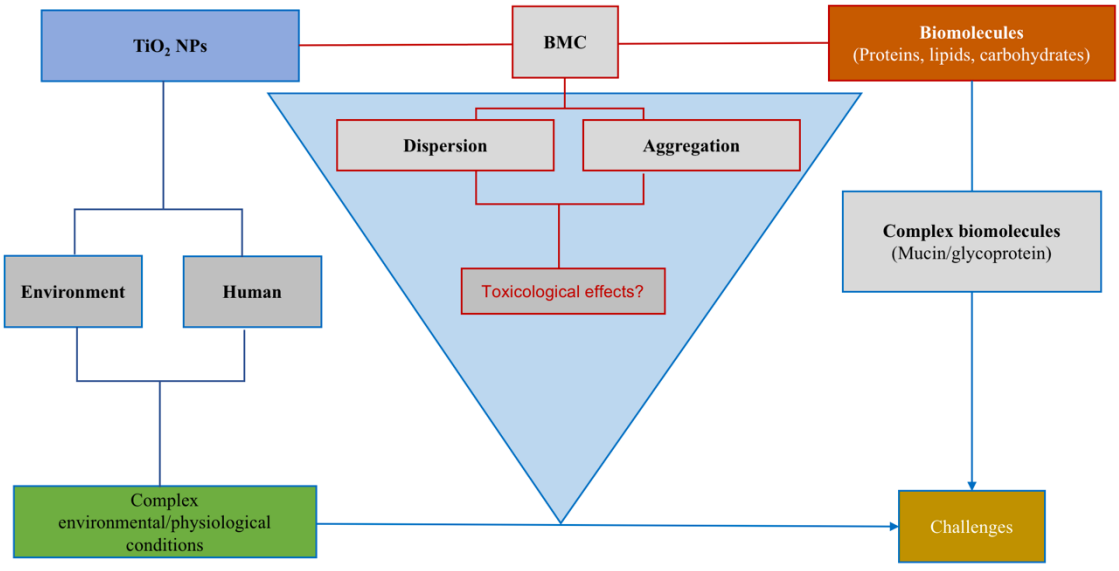


Figure 1: Conceptual framework of potential TiO2 NPs-biomolecule interactions in environmental and human physiological conditions.

The application of TiO_2 NPs across multiple industries increases their release into the environment with emerging concerns regarding the adverse impacts on aquatic and terrestrial organisms [12]. TiO_2 NPs in water can interact with biomolecules or natural organic matter (NOM) such as humic acid or fulvic acid [67]. These organic substances are adsorbed by TiO_2 NPs, covering the surface of TiO_2 NPs, and forming biomolecular corona (BMC). The formation of BMC can induce either dispersion or aggregation which influence the cellular/tissue response of TiO_2 NPs.

Furthermore, TiO_2 NPs can enter terrestrial organisms via three primary routes: the respiratory system, gastrointestinal tract (GI), and skin. The GI tract route has gained the attention of researchers due to its significance to the immune system and digestive system. Studies report that when TiO_2 NPs interact with the outer cell membrane of organisms, they have various forms, such as micelles, droplets, vesicles and the pristine one. Once TiO_2 NPs enter the cell, they can interact with cytoplasmic molecules and organelle cells, and therefore generate radical oxygen species (ROS) and induce toxicity [11, 68].

The purpose of this systematic literature review is to provide a mechanistic understanding of the interaction between TiO_2 NPs and biomolecules (particularly mucin). This review examines the fate, conformation, transport, and toxicity of TiO_2 NPs and focuses on the change of physicochemical properties of TiO_2 NPs.

Materials and Methods

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols (PRISMA-P) guidelines were applied to this systematic review.

Eligibility Criteria

Inclusion criteria were studies focusing on TiO_2 NPs pathways into the environment; ingestion and absorption pathways of TiO_2 NPs by humans; and interactions between TiO_2 NPs and various biomolecules. This review considered research articles, books and documents, clinical trials, meta-analyses, randomized controlled trials, review papers, and systematic reviews. Research papers not written in English were excluded from the study.

Search Strategy

An extensive bibliographic search using PubMed (core collection), Web of Science, and Agricola databases was conducted to screen articles written in English and published with no limitations on the year of publication. Keywords were employed as search terms in all selected databases. Search terms included but were not limited to TiO_2 NPs and aquatic environment, TiO_2 NPs and gastrointestinal tract, interactions TiO_2 NPs and biomolecules, BMC formation, TiO_2 NPs and toxicity, TiO_2 NPs and dispersion, TiO_2 NPs and aggregation, TiO_2 NPs and mucin, etc. (Table 1).

Data Management, Screening, and Selection

Titles and abstracts of articles were initially screened for further review. After duplicate and irrelevant articles were excluded, a careful review of full-length articles

was conducted. Articles were categorized under key search terms and systematically recorded in an excel spreadsheet (Office Excel software, Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399, USA) (Table 1).

Table 1: Literature review based on PRISMA guidelines: Web of Science (highly cited), Agricola, and PubMed (including books, documents, clinical trials, meta-analyses, randomized controlled trials, reviews, systematic reviews)

No	Subject	WoS	Agricola	Pub Med	Articles with duplicates	Close duplicates	Articles found	Selected articles
1	TiO2 NPs in environments	301	1,782	175	2,258	158	2,178	
2	TiO2 NPs in human	22	93	136	251	12	245	311
3	Interactions TiO2 NPs and biomolecules	35	467	96	598	22	587	
4	TiO2 NPS (dispersion / aggregation)	107	887	83	1,077	60	1,047	
5	NPs and mucin	28	217	122	367	48	342	
6	TiO2 NPs and mucin	2	3	1	6	2	5	777
	Total	495	3,449	613	4,557	302	4,404	1,088

Results and Discussions

Search Results

The initial search identified 4,557 articles comprising 603 titles from PubMed, 495 from Web of Science, and 3,449 from Agricola. After duplicates had been removed, 4,404 articles remained. The further selection was conducted and selected 1,088 articles, consisting of 311 articles related to the major route of TiO_2 NPs into environment and human, and 777 articles associated with the interaction between TiO_2 NPs and biomolecules. 301 articles had their titles and abstracts screened, and 120 were excluded

for not meeting inclusion criteria. The full-text screening did not exclude any studies, resulting in the inclusion of 181 articles. More specifically, this literature review included five (5) articles addressing the research on TiO_2 NPs and mucin:

1. Mercier-Bonin, M., Despax, B., Raynaud, P., Houdeau, E., & Thomas, M. (2018). Mucus and microbiota as emerging players in gut nanotoxicology: The example of dietary silver and titanium dioxide nanoparticles. *Critical reviews in food science and nutrition*, *58*(6), 1023–1032. https://doi.org/10.1080/10408398.2016.1243088 (PubMed & Agricola) [47].
2. Brun, E., Barreau, F., Veronesi, G., Fayard, B., Sorieul, S., Chaneac, C., . . . Carriere, M. (2014}). Titanium dioxide nanoparticle impact and translocation through ex vivo, in vivo and in vitro gut epithelia. *Particle and Fibre Toxicology}, 11}* doi:{10.1186/1743-8977-11-13 (Web of Science (Highly cited)) [50].
3. Hajirezaee, S., Mohammadi, G., & Naserabad, S. S. (2020}). The protective effects of vitamin C on common carp (cyprinus carpio) exposed to titanium oxide nanoparticles (TiO2-NPs). *Aquaculture}, 518}* doi:{10.1016/j.aquaculture.2019.734734 (Web of Science (Highly cited)) [40].
4. Bourgeault, A., Veronique Legros, Gonnet, F., Daniel, R., Aurelie Paquirissamy, Clemence Benatar, . . . Pin, S. (2017). Interaction of TiO2 nanoparticles with proteins from aquatic organisms: The case of gill mucus from blue mussel. *Environmental Science and Poll* (Agricola) [69].

5. Tsai, S., Duran-Robles, E., Goshia, T., Mesina, M., Garcia, C., Young, J., . . . Chin, W. (2018). CeO2 nanoparticles attenuate airway mucus secretion induced by TiO2 nanoparticles. *Science of the Total Environment, 631*, 262-269. doi:10.1016/j.scitotenv.2018.03.001 (Agricola) [70].

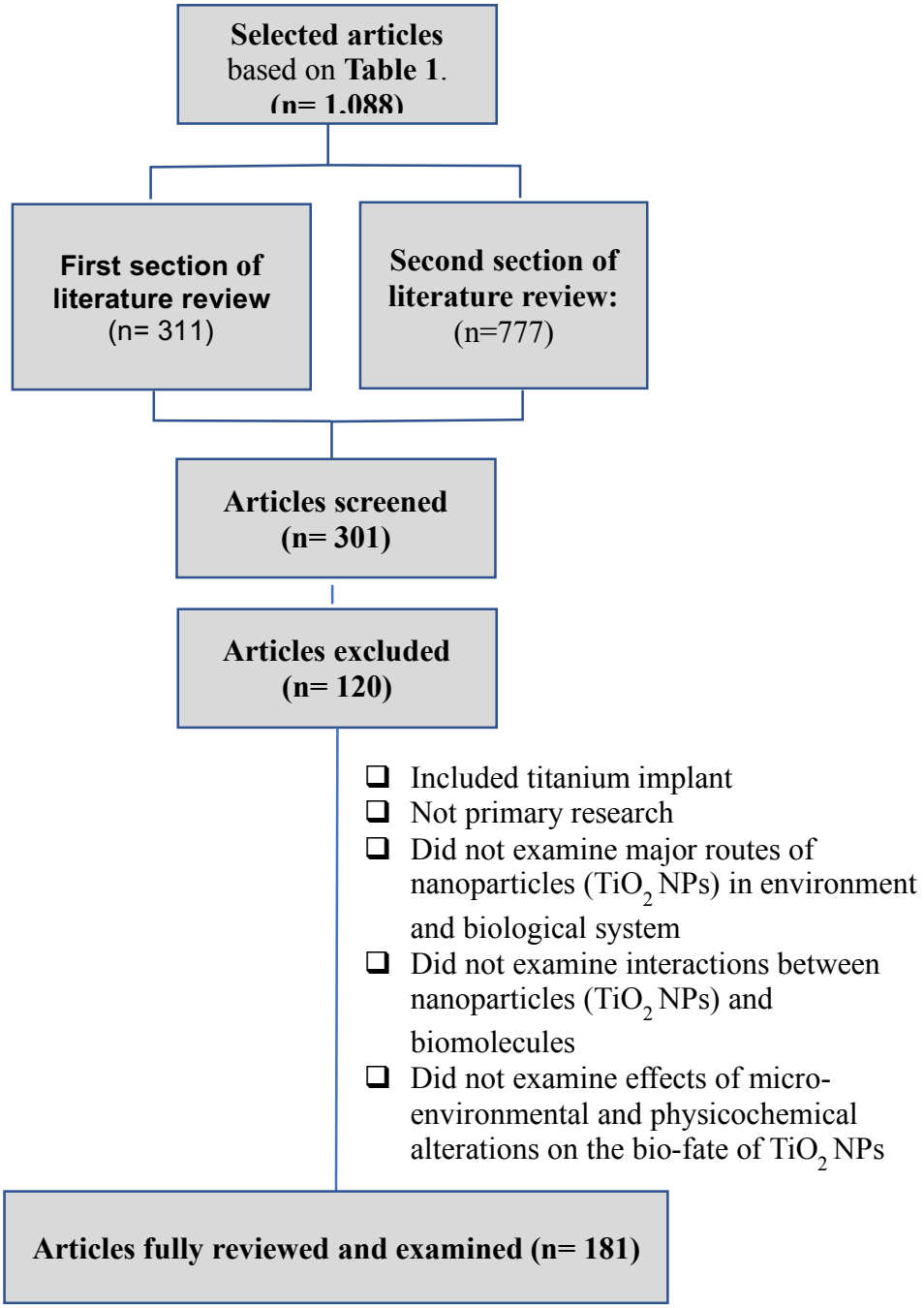


Figure 2: Flow chart for selection of articles adapted from PRISMA.

Discussions

Impact of NP Properties on Biomolecular Corona (BMC) Formation

There are two important terms in the formation of BMC, biological identity and synthetic identity. Adsorbed layers of biomolecule are assigned as the biological identity of NP, while physicochemical properties of NPs are defined as synthetic identity. The depiction of the biological and synthetic identity may reveal the existing gap in knowledge in the formation of BMC [71]. Biological identity is a derived property of an NP and depends on the myriad of physiological biomolecules. The first protein interacting with NPs has the highest abundance in the BMC due to the "memory" effect, which affects the subsequent protein-protein interaction during BMC formation [72]. Almost all the BMC research focuses on protein-NPs interaction. However, the biological identity of TiO_2 NPs which interact with lipids, carbohydrates, and complex molecules (such as glycoproteins) is mostly unknown.

The nano-bio interface study, initiated by Dawson and co-workers [63, 73-74] delivered the new field, which might be categorized as the safe version of NPs. Nevertheless, understanding the Nano-bio interface is very challenging due to its complexity. The Nano-bio interaction is highly associated with a dynamic physiological environment and presents difficulty in finding appropriate methods to analyze the interaction [66]. Here, we systematically present existing evidence in the literature and explain the current gap of knowledge on decoding the interaction between TiO_2 NPs and various types of biomolecules.

Size, Shape, Curvature, and Charge

The surface properties of TiO_2 NPs are considered a fundamental determinant of BMC bioactivity. A slight change in the physicochemical properties of TiO_2 NPs may alter the biological effect of BMC, and thereby give rise to the unpredictability of the toxicity and bio-fate. Studies on BMC reveal the unpredictable behavior of TiO_2 NPs.

Studies report that the particle size is a determinant of NPs delivery and toxicity [75-76]. Particle size affects the qualitative and quantitative composition of BMC [77-78]. Additionally, current studies show that other surface properties of NPs, such as shape, curvature, hydrophobicity, and charge, also influence the binding of NPs with biomolecules. The higher curved surface and the smaller size of NPs results in less protein-protein interactions and oligomerizations of adsorbed proteins [79-80]. However, smaller particle size can lead to higher surface area and hydrophobicity, and thus faster BMC formation [81].

The formation of BMC may be driven by the size as well as other surface properties of TiO_2 NPs. The charge of both NPs and biomolecules play important role in the formation of BMC. The negative charge of NPs has an affinity towards protein with an isoelectric point (PI)>5.5, whereas the positive charge of NPs exhibits affinity towards protein with PI<5.5 [82]. However, the isoelectric point of TiO_2 NPs with protein and other biomolecules, particularly complex biomolecules such as glycoproteins, are not fully known.

More than 130 prior experiments showed that the different methods, proteins, and NPs systems, can cause a notable difference in the estimation of the dissociation

coefficient [83]. Studies have observed that positively charged NPs tend to have smaller dissociation coefficients than negatively charged NPs [83]. In addition, Calatayud et al. (2014) reported that the charge density of surface modified magnetic NPs in cell culture mediums exhibits a fivefold escalation in the particle size within a few minutes of incubation [84]. The final charge and size of BMC is frequently different from the initial charge and size of NPs. These findings suggest that the characterization of the final properties of TiO_2 NPs in environmentally and physiologically relevant bio-fluids is needed.

The shape of NPs matter [85]. Few studies observed the effect of shape on the formation of BMC [86]. NPs with more irregular shapes, for example, are reported to have less uptake in the human macrophage cell line [85]. Moreover, NPs with the same shape and mass concentration but different species can provide different toxicity [85]. For example, rod-shaped silver NPs are toxic, while rod-shaped NPs with the same concentration were found safe on the human lung epithelial cell [85]. More investigations are needed on the shape of TiO_2 NPs and the effect of shape on uptake and toxicity in cells.

Surface Chemistry

Protein adsorption may be adjusted by adding different functional groups on the NP surface. The application of polyethylene glycol (PEG) to gain "stealth" behavior, for example, is the gold standard for controlling surface properties and maintaining corona-free conditions [66]. Some studies have reported the application of PEG on TiO_2 NPs to assess its potential to reduce the cytotoxicity of this NP [64]. Manipulation of surface

functional groups of NPs and their density exhibit a direct association with the model of protein adsorption [87.]The adsorption of proteins on the surface of colloidal alumina NPs, for instance, has a linear association with the NPs functionality and the composition of amino acids on adsorbed proteins [88].

In addition, previous studies have reported that surface chemistry influences the surface charge of NPs and plays an important role in the composition and evolution of BMC [66]. The different composition of BMC can translate to different biological and physicochemical properties of NPs [66]. For example, the BMC composition of a native SiO_2 NPs is different from the amine (--NH2) and carboxyl (--COO(-)) modified surface of SiO_2 NPs owing to the different surface functionality of SiO_2 NPs [66]. Carboxyl-modified NPs show more stability (i.e. the size of aggregates and agglomeration rate) and less toxic effects on cells than native and amine-modified ones [89].

Another key regulator in the formation of BMC is exhibited by the arrangement of lipid functional groups in liposome-protein interactions [66]. Small alterations in lipid composition may substantially influence the formation of BMC [90], and thereby may contribute to changes in the biological impact of BMC. Studies suggest that each surface chemistry is specific and has its own potential bioactivity [66]. Limited data have reported the surface chemistry of TiO_2 NPs; therefore, further studies about the surface chemistry of TiO_2 NPs in association with the formation of BMC are required.

Hydrophobicity

Modifying surface hydrophobicity may prevent the exposed surface of NPs from adsorbed protein; so, their biological properties are more likely to be governed by

physicochemical properties of NPs than adsorbed protein [66]. For example, zwitterion NPs may not form hard coronas at physiological serum concentrations [91] and the hydrophobic NPs adsorb albumin more readily than hydrophilic NPs [92]. Besides hydrophobicity, topography and surface curvature may affect the denaturation of adsorbed proteins; and the effect may be multiplied by the surface chemistry [93]

The smaller size of NPs may lead to more hydrophobic proteins being adsorbed [92]. Hydrophobicity tends to result in curvature increases and shields NPs from hydrophilic/aqueous environments. Hydrophobic interactions are like electrostatic interactions, playing an essential role in the qualitative and quantitative composition of BMC, and having wide toxicological effects [66]. Understanding the interplay of different physicochemical properties of TiO_2 NPs may provide a more comprehensive overview of BMC formation. Furthermore, exposing TiO_2 NPs with different surface chemistry to various biomolecules may provide a better understanding of the formation of BMC. However, studies to examine the effect of different physicochemical properties and surface chemistry of TiO_2 NPs on the formation of BMC are very limited, even unknown. Figure 6 illustrates findings from several studies on different types of NPs, including TiO_2 NPs. It is important to note that various NPs may lead to differential results).

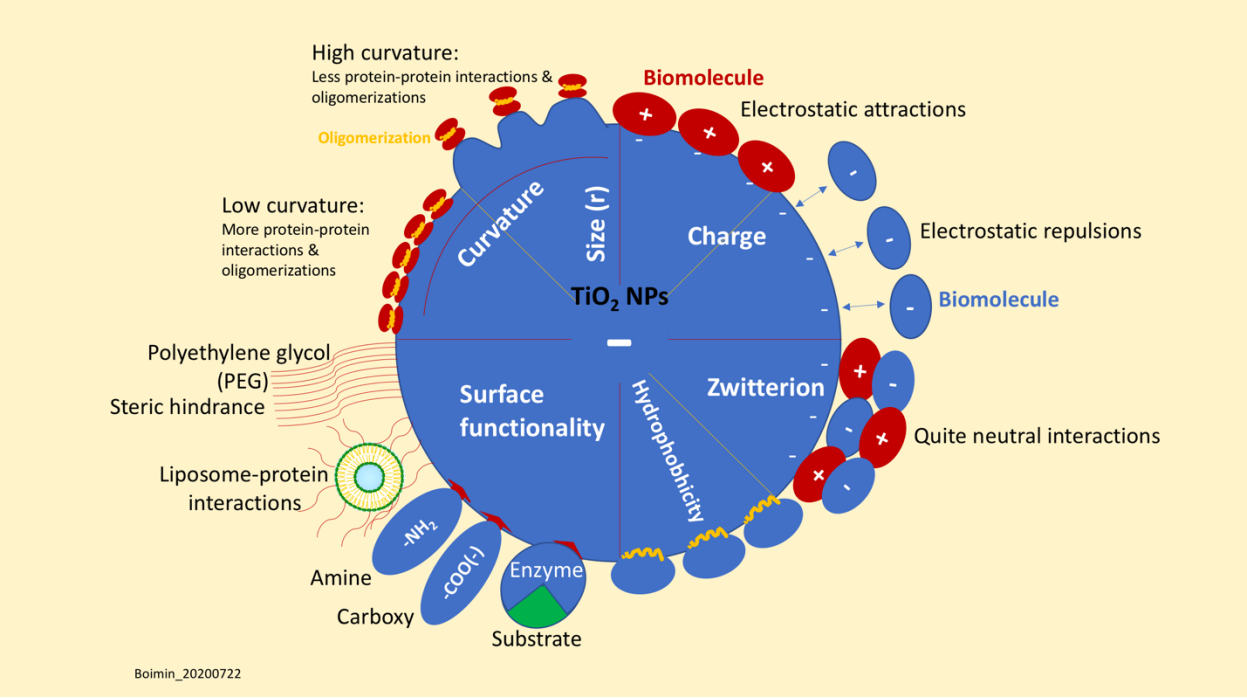


Figure 3: Illustrations based on the literatures [66; 75-92]: possible surface chemistry and/or physicochemical properties of NPs, including TiO2 NPs. Different results may arise depending on the type and properties of NPs.

Interaction between TiO_2 NPs with Different Biomolecules and Potential Host Impact

Once TiO_2 NPs enter the environment, they may interact with organic substances (biomolecules), form BMC, and transform their physicochemical properties and biological fate. The main components of biomolecules are proteins, lipids, and carbohydrates. However, some constituents, such as surfactants, colorants, minerals, preservatives, nutraceuticals, bases, acids, and buffers, are frequently included in the interactions between NPs and biomolecules [94-95], which cause complexities.

McClements, Xiao, and Demokritou (2017) argued that since TiO_2 NPs have different physicochemical properties compared to TiO_2 fine particles, bio-safety assessment for negative health and environmental outcomes is essential. Properties of

TiO_2 NPs may be altered by various components of industrial processing, the food matrix, or other elements, thus affecting dissolution, precipitation, adsorption, de-adsorption, release, entrapment, association (aggregation), and dissociation (dispersion) [43]. Zhang, D. et al. (2020) explained the main types of nanoparticle dispersions in liquids such as electrostatic repulsion, reversed micelle wrapping, and steric hindrance. The higher dispersion may indicate greater toxicity caused by TiO_2 NPs [10].

Klaine et al. (2008) described three possible models of aggregation between inorganic particles and biomolecules in liquids, based on Buffle et al. (1998) [96]. Sugars, amino acids, and other very fine biological substances may interact with inorganic particles and formed gels; while the larger biological substances such as polysaccharide fibrils may create small aggregation (stable suspension) and large aggregation (unstable suspension) [97]. **Fig.** 4 illustrates the potential effects on TiO_2 NPs when they are dispersed into liquids containing various biomolecules [10, 43, 96].

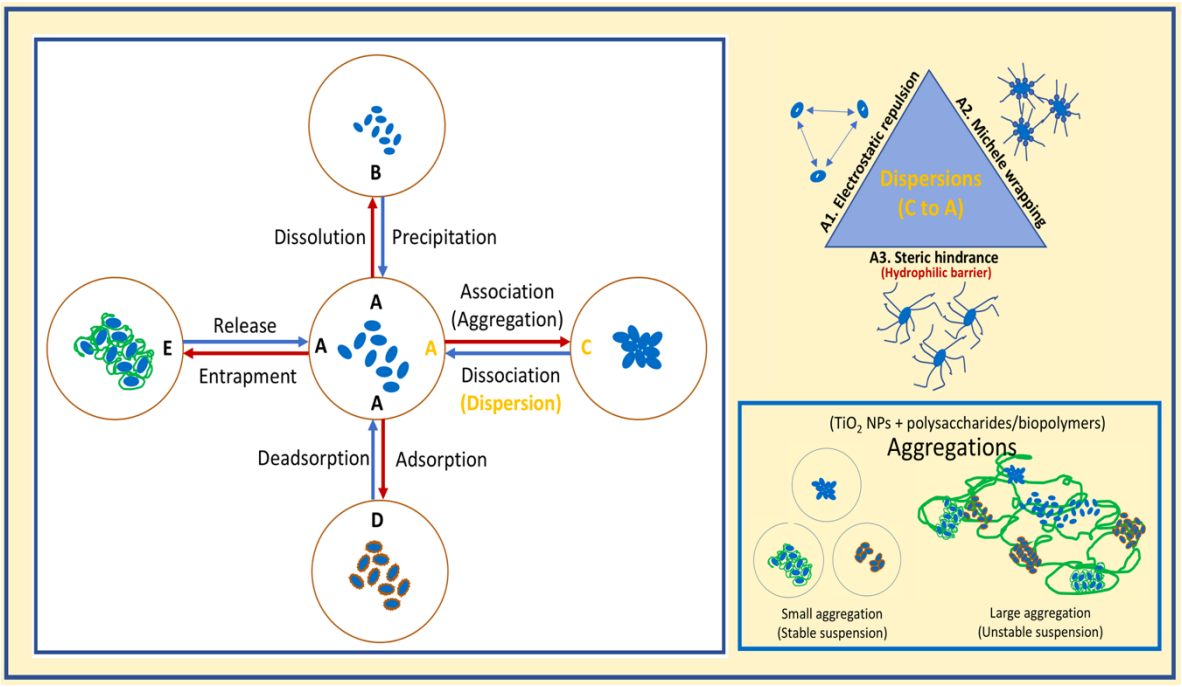


Figure 4: Potential effects of TiO2 NPs when exposed to liquids which contain various biomolecules (this illustration is inspired and reflects findings by Buffle et al. (1998), Klaine et al. (2008), McClements, Xiao, & Demokritou (2017), and Zhang, D. et al. (2020).

The mechanisms by which exposure to TiO_2 NPs potentially leads to adverse environmental or human outcomes are not fully understood. The potential pathways of the interaction between TiO_2 NPs and cells are illustrated in **Fig.** 5. Initially, TiO_2 NPs - having some configurations such as pristine form, micelles, droplets, and vesicles - are dispersed in a liquid and adsorbed by the outer membrane of a cell before entering the cell [11]. Hypothetically, TiO_2 NPs interact with cytoplasmic molecules, organelle cells (particularly mitochondria), and generate radical oxygen species (ROS), which in turn likely induces toxicity [11].

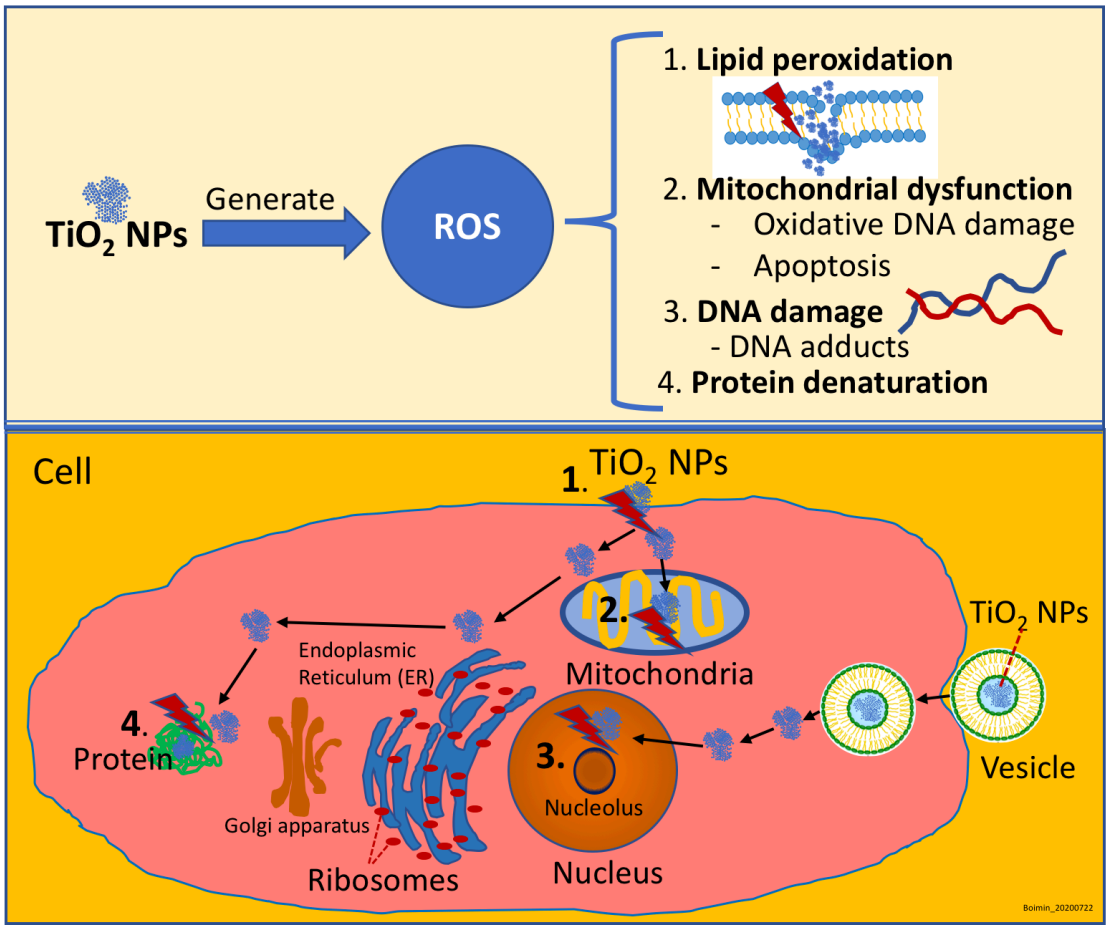


Figure 5: Possible effects of ROS generated by TiO2 NPs on cell.

Interaction of TiO_2 NPs and Proteins

Interaction between protein and inorganic nanoparticles has been extensively researched [98]. McClements, Xiao and Demokritou (2017) elucidate inorganic nanomaterials within the food matrix and their implications for gastrointestinal fate [43]. They also report the main properties of various food proteins (Table 2).

Table 2:Summary of molecular characteristics of some common food-grade proteins [43].

Name	Main source	Molecular weight (kDa)	Main structural type	pI	~T_m (^oC)	Solubility
ß-lactoglobulin	Milk	18.4	Globular	~5.0	~75	Water
Casein	Milk	19.0-25.2	Flexible	~4.6	~125-140	Water
Bovine serum albumin (BSA)	Milk/blood	66.5	Globular	~4.7	~180	Water
Lactoferin	Milk	80	Globular	~8.0	~60-90	Water
Ovalbumin	Egg white	45	Globular	~4.6	~74; 82^s	Water
Lysozyme	Egg white	14.3	Globular	~11.0	~74	Water
Phosvitin	Egg yolk	36-40	Globular	~4.0	~80	Water
Gelatin	Animal collagen	Varies	Flexible	~8^A	~5 (fish)	Water
				~8^B	~40 (animal)	Water
Soy Glycinin	Soybean	360	Globular	~5.0	~67^{7s}; 87^{11s}	Water
Zein	Corn	18-26	Globular	~6	~90	Organic solvent
Gliladin	Wheat	28-55	Globular	~6	~90	Organic solvent

Proteins can potentially have five different physicochemical mechanisms by which they can change the GI tract fate of nanoparticles [43]. Entrapment is where proteins may build a food matrix that traps NPs, and thus breaking the matrix is required prior to releasing NPs [99]. Surface active adsorption is when proteins can be adsorbed by NPs; hence, the interfacial characteristics of NPs are altered [100-101]. Proteins are good thickening and gelling agents and can modify the rheology and transport of NPs in the GI tract [102-103]. Proteins may lead to NPs aggregation due to their potential in bridging molecules and depleting flocculation [104]. And finally, proteins are effective buffering agents and in a sufficient amount, they can modulate the pH of GI tract fluids

[102].

Cao, X. and co-workers (2019) examined the interaction between TiO_2 NPs and casein (milk proteins), using a simulated GI tract model. Findings suggest that the aggregation takes place due to the bridging effect and reduction of electrostatic repulsion. This study reports that the gastric digestion of caseins which interact with TiO_2 NPs takes longer and their rate of digestion is lower than just caseins. Hence, the formation of BMC may inhibit pepsin to interact with peptide bonds [105].

Adsorption effects of TiO_2 NPs and temperature on the structure of plasmatic proteins such as bovine serum albumin (BSA) and fibrinogen (Fib) have been studied. The study found that the structure of adsorbed BSA on the surface of TiO_2 NPs differs from the solution one; while adsorbed Fib is no different from the Fib solution. Increasing the temperature causes the structure of adsorbed BSA does not change, but BSA solution is changed or denatured; meanwhile, both adsorbed Fib and Fib solution are denatured [106].

Nikpasand et al. (2019) reported that TiO_2 NPs could potentially accelerate wound healing in the early stages of injury due to the fibroblast proliferation and the rearrangement of tissue and collagen fibers granulation [107]. Although the TiO_2 NPs-protein interactions have been well-studied, there is limited data on the potential risks of TiO_2 NPs to human health.

Interaction between TiO_2 NPs and Lipids

Lipids, generally consist of either neutral lipids (e.g. triacylglycerols (TAGs), diacylglycerol (DAGs), monoacylglycerols (MAGs), terpenes and hydrocarbons) or polar

lipids (e.g. free fatty acids (FFA), surfactants, and phospholipids) [108]. TAGs, DAGs, and phospholipids usually own a hydrolyzable ester bond which can induce the release of free FFAs and MAGs. These simple lipids can be both integrated into mixed micelles and adsorbed by epithelium cells [109].

TiO_2 NPs may damage lipid membranes via lipid peroxidation and lipid removal. NPs interact with lipid membranes in various structures, compositions, and dimensions, with each structure having its own potential bioactivity [110]. However, lipid peroxidation can be minimized through adsorption or layering the surface of TiO_2 NPs with serum protein [111]. TiO_2 NPs are scavengers of Ca^{2+} ions, which may weaken the interaction between the membrane and support membrane; therefore, the addition of TiO_2 NPs can potentially remove the lipid membrane [112]. The removal of the lipid membrane can be prevented or minimized by neutralizing the membrane charge, particularly in the hydrophilic area. Previous research found that bilayers of membrane containing more zwitterion (relatively neutral regions) are more resistant to TiO_2 NPs perturbation than bilayers of membrane populated by anionic lipids [113]. These interesting findings raise questions about the overall impact of TiO_2 NPs on lipid membranes co-exposed to other chemicals contained in various skincare products.

Interaction of TiO_2 NPs and Carbohydrates

Carbohydrates are typically categorized into two main groups, digestible and indigestible polysaccharides such as starch, cellulose pectin, alginate, carrageenan, and xanthan [110]. While some starches are easily hydrolyzed by amylases in the mouth and small intestine and transform into oligosaccharides and glucose, dietary fibers,

carbohydrate polymers with >10 monomeric units, are not hydrolyzed by digestive enzymes [114-115].

Carbohydrates have physicochemical mechanisms that may change the GI fate of TiO_2 NPs [110]. First, TiO_2 NPs are trapped into a solid matrix, a necessary step to release the nanoparticles [4, 116]. Second, some types of polysaccharides are good thickening and gelling agents that can change the rheological properties of the GI fluids and thereby alter the transport of TiO_2 NPs in the GI tract [117-119]. Third, some carbohydrates have electrostatic and hydrophobic interactions with TiO_2 NPs due to their nonpolar properties and charge, and thus lead to the adsorption [120-121]. Fourth, polysaccharides have mechanisms, such as bridging processes and depleted flocculation, that can promote aggregation. Starches are easily hydrolyzed in the mouth, small intestine, and stomach [122], and hence the interaction between starches and TiO_2 NPs in the GI tract is insignificant. Given that dietary fibers are persistently intact in the upper GI tract [123], they may interact and provide a significant impact on the physicochemical properties of TiO_2 NPs.

Although dietary fibers cannot be digested in the upper GI tract, the human gut microbiome can facilitate fermentation and produce metabolites like short-chain fatty acids. These in turn can impact metabolic regulation or become a substrate for microbial uptake [124]. Some studies have reported a closed relationship between dietary fibers and the microbiome, but no studies have documented the interactions between dietary fibers and the microbiome in the presence of TiO_2 NPs (Mercier-Bonin et al., 2018).

Regarding other forms of carbohydrates, Chen, Z., et al. (2015) observed the interactions between TiO_2 NPs and glucose. In this study, the toxicological effect of

varying doses of orally administered TiO_2 NPs and glucose to young animal models was monitored. Findings suggested that oral exposure to both TiO_2 NPs and TiO_2 NPs plus glucose caused liver, kidney, and heart injuries, as well as altered the number of white and red blood cells in a time, dose, and gender-dependent manner [125]. However, this study was not able to delineate the causal effects of TiO_2 NPs or glucose on observed injuries.

Another study by Chen and co-workers (2015) investigated the effect of TiO_2 NPs, glucose, and TiO_2 NPs plus glucose in animal models. Results indicated that the toxic effect of high-dose glucose was greater than both TiO_2 NPs and the combination of TiO_2 NPs with glucose. They suggested that it might be more essential to control the uptake of sugar than TiO_2 NPs [125]. Based on these findings, TiO_2 NPs seemed less harmful in animal models than glucose, but the unstable characteristics of TiO_2 NPs should be carefully considered prior to clinical trials.

Implications: Environmental and Human Pathways and Potential Risks

TiO2 NPs & Their Major Routes into Environment

Disposal and domestic wastewater, containing NPs, are inevitably released into the aquatic environment [1, 126-129] posing concerns about their environmental behavior and associated ecological risks. Previous studies have reported that NPs cause significant toxicity by inhibiting protein expression [130] and generating oxidative stress [131]. NPs exposed to water will generally either aggregate or disperse, resulting in the expression of different physicochemical properties and varying levels of toxicity. Dispersed NPs are likely to exhibit higher toxicity than the aggregated ones [10]; however, the aggregation

of NPs causes their accumulation in aquatic organisms [41]. Accumulation of TiO_2 NPs to higher trophic levels, particularly to humans due to the consumption of fish and seafood [42], is another concern. The toxicity of TiO_2 NPs in aquatic organisms is debated by scholars fueled by limited data on interactions between TiO_2 NPs and biomolecules in water.

Aquatic organisms can be susceptible to the toxic effects of TiO_2 NPs. Articles have described the environmental transformation of TiO_2 NPs in aquatics system and their accumulation in fish, bivalve mollusk, algae, some surimi products, as well as their possible toxic effects [9, 40, 42, 69, 132-133]. Shi, W., et al., (2019) observed the effect of ocean acidification (OA) on the bioaccumulation of TiO_2 NPs in three different bivalves and investigated the realistic amount of TiO_2 NPs that impacted the bivalves' health. This study reported that the accumulation of TiO_2 NPs in bivalves alleviates significantly due to OA, and thereby may increase the health risk of seafood consumers [133].

Studies showed that the oxidative stress and toxicity of fish and bivalve mollusks are altered due to TiO_2 NPs [11, 69]. Bivalve mollusks and TiO_2 NPs which are co-exposed to different organic and inorganic particles exhibit various effects including (1) enhanced accumulation and toxicity (TCDD, Cu, Pb, PCPs, Phenanthrene (Phe), As, Cd, Cu, Pb) [134-140], (2) decreased accumulation and increased toxicity (benzoa(a)pyrene) [141], (3) decreased accumulation and toxicity (Cd^{+2}) [142-143], (4) no change in accumulation and toxicity (Cd^{+2}, TCDD, PBDEs) [140, 144-146].

Evaluation of TiO_2 NPs concentration in white-colored seafood and surimi-based food products found that TiO_2 NPs concentrations are relatively high [42]. These kinds of

food are considered an important route of TiO_2 NPs uptake, particularly among younger generations, aged 20-20 years, of the human population [42]. Lee and An (2013) investigated the eco-toxicity effects of TiO_2 NPs on the green algae *(Pseudokirchneriella subcapitata)* under the irradiation of visible UVA and UVB light. Their results showed that the increase of TiO_2 NPs concentration inhibits alga growth due to the destabilization of cell membranes and inhibition of alga growth was not alleviated by UV pre-irradiation conditions [132]. Indeed, the aquatic environment is complex due to the diversity of organisms, pH variations in fresh and sea water, and potential interactions with biomolecules in the aquatic environment. Further research is needed to understand the interactions of TiO_2 NPs and potential toxicity in aquatic environments.

Soil and terrestrial plants are also affected by the widespread distribution of TiO_2 NPs in the environment [147]. Tan, W. et al. (2018) reviewed studies on the interactions of TiO_2 NPs with soil and plants [8]. This review article described the change of physicochemical properties of TiO_2 NPs during the interactions. TiO_2 NPs are released into the environment via three primary routes: groundwater (18.5 %), soil (13.8%), and air (2.2%) [8]. TiO_2 NPs are then absorbed and translocated from the soil into leaves and fruits. As a result, plants such as cucumber fruits have increased phosphorus and potassium content [148]; barley and rice experience a decrease in kernels and grain yield [149]; an alteration of nutritional elements occurs in lettuce roots and shoots [150], tomato stems and leaves [151], and soybean roots [152]; and a change occurs in the antioxidant system of pinto bean plants sprayed with TiO_2 NPs at different stages of growth [153]. In a review article, Liu, Y., et al. (2019) explained that NPs enhance plant photosynthesis [154]. Many challenges and questions remain in association with TiO_2

NPs-plants interactions, such as the specific effect of TiO_2 NPs on plant proteins, the transgenerational effect of TiO_2 NPs on plants, and the impact of other substances in soil on these interactions. **Fig.** 6 illustrates the pathways of environmental exposure to TiO_2 NPs as described by studies in this systematic review.

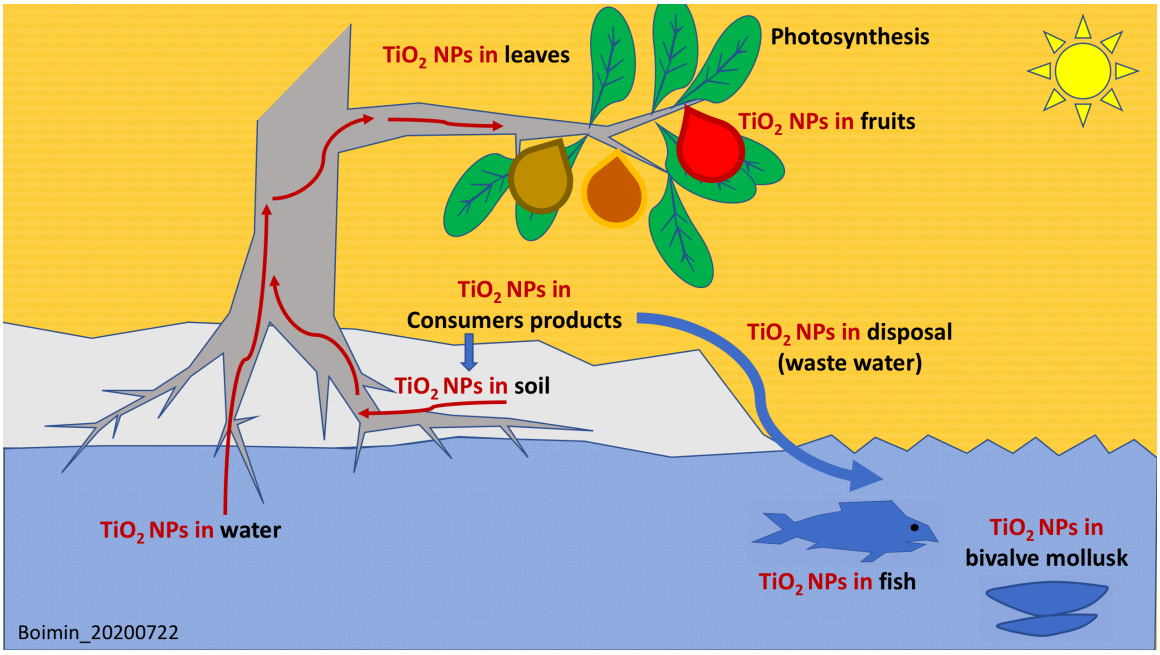


Figure 6: The major routes of TiO2 NPs into the environment.

TiO_2 NPs, Pathways into the Human Body, and Health Risks

The International Agency for Research on Cancer has categorized TiO_2 NPs as a possible carcinogen in humans [60] and the U.S. Institute of Occupational Safety and Health suggests TiO_2 NPs exposure be limited to 2.4 mg/m^3 [55]. TiO_2 NPs are widely used in toothpaste, sunscreen, cosmetics, paints, plastics, self-cleaning devices, pharmaceuticals, and food additives, as well as in industrial and medical applications, because of their strong photocatalytic activity [4, 156-158]. Investigations on the cytotoxicity and genotoxicity potential of TiO_2 NPs have been conducted in both in vitro

and in vivo studies, however, toxicological data are disputable [159]. Scientific evidence suggests that TiO2 NPs cause cytotoxic, genotoxic, and oxidative effects through oxidant generation, inflammation, and apoptosis [15, 157-158, 160-162]. Other studies have found that TiO2 NPs induced low or no toxic effects [163-164].

There are three major pathways for human exposure to TiO2 NPs – through the skin, respiratory system, and via ingestion to the gastrointestinal tract (Figure 4).

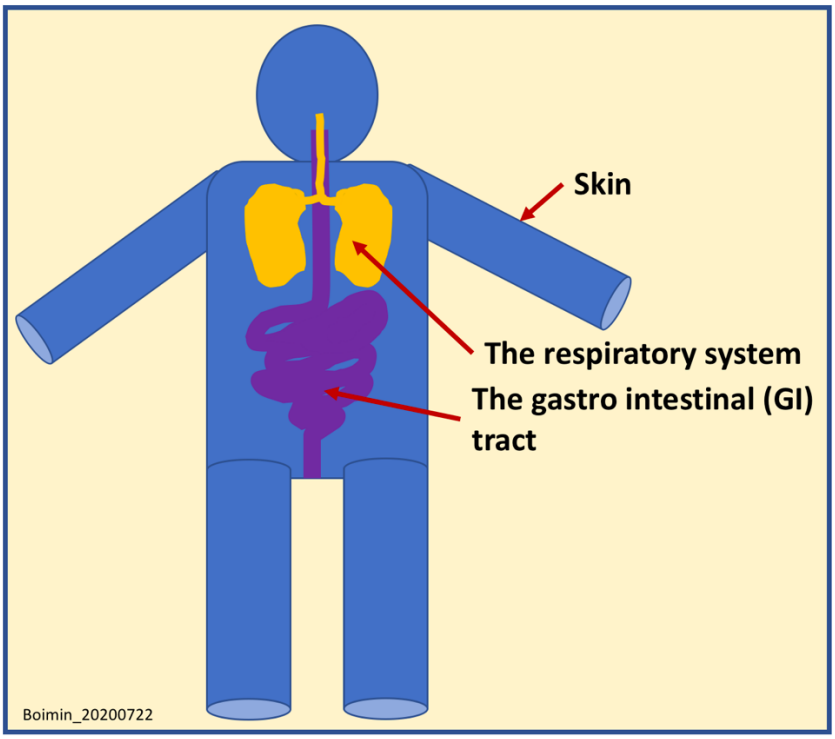


Figure 7: The major pathways of TiO2 NPs into the human body.

The Skin Pathway

Geppert et al. (2020) reviewed the interaction of TiO_2 NPs with ingredients from modern lifestyle products and their effect on human skin cells [165]. They found that the outer layer of human skin was firm, inducing a limited penetration of inorganic TiO_2 NPs [166]; however, the penetration of TiO_2 NPs on the skin is possible [167-168]. Although most studies reported that no penetration of TiO_2 NPs on the skin [169-170], Wu, J. et al. (2009) observed that TiO_2 NPs penetrated the hairless skin of mice without causing breakage. Furthermore, higher doses given to the mice resulted in a higher number of TiO_2 NPs found in organs such as the heart, brain, spleen, and liver. Liver exposure to TiO_2 NPs in mice showed the alteration of malondialdehyde and superoxide dismutase levels [171]. A recent systematic review article reported that using a 3D skin model has exhibited to be promising to evaluate the toxicity of TiO2NPs [172].

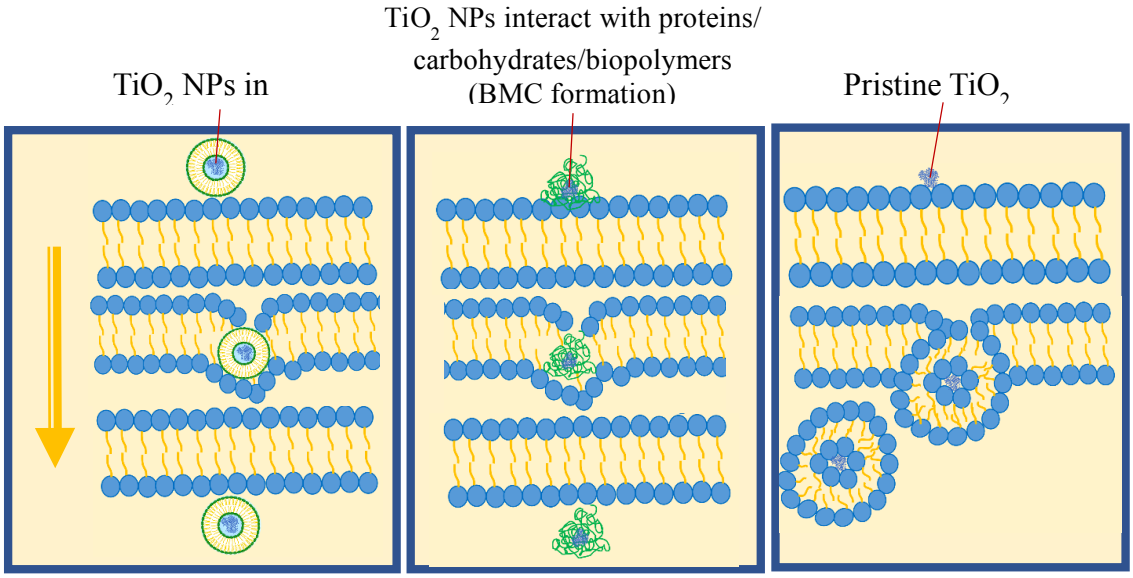


Figure 8: Interactions of TiO2 NPs (in various forms) with bilayers membrane.

Evidence indicates that dermal exposure to TiO_2 NPs does not pose a significant risk to human health, however, there is a considerable number of studies reporting a toxic effect of TiO_2 NPs in animal models [15]. Effects of TiO_2 NPs on the skin, which are co-exposed with an external stressor such as UV light and some chemicals, have also been studied [61-173]. Photo-toxicity to human skin keratinocytes with UV irradiation of TiO_2 NPs with four different sizes (<25 nm, 31 nm, <100 nm, and 325 nm) and two different crystal forms (i.e. rutile and anatase) was assessed [159-162]. Results suggested that all types of TiO_2 NPs generate ROS, and the smaller size TiO_2 NPs produce higher levels of photo-toxicity than larger size TiO_2 NPs [159-162].

Pelclova et al. (2019) reported that TiO_2 NPs are present in samples of human plasma and urine after 6-48 hours of applying sunscreen, indicating penetration of NPs to the protective layer of human skin cells [174]. Wright et al. (2017) proposed that all sizes of TiO_2 NPs tend to have a dose-dependent intensification of caspase 8 and 9 activity, superoxide production, and apoptosis on human keratinocyte cells (HaCaT) [175]. Crosera et al. (2015) corroborated the study findings presented by Wright et al. (2017) by demonstrating that TiO_2 NPs cause cytotoxic effects on HaCaT cells (EC_{50} 10^{-4}-10^{-5} mol/L) [169]. In addition, human dermal fibroblast exposure to TiO_2 NPs results in a decrease in the cell area, mobility, proliferation, and a contractive ability of collagen [176]. Given these findings, the mechanistic interactions between TiO_2 NPs and skin cells, particularly the lipid membrane, needs to be investigated further.

The Respiratory System Pathway

The major route by which ambient particles enter the human body is the respiratory tract, specifically through the nasal epithelium, the trachea-bronchiolar region, and the alveolar interstitium, i.e. respiratory bronchiole and pulmonary alveoli [177]. These particles can be retained in the lung, be cleared through the airways, and translocated and distributed within the body. Understanding the deposition of inhaled nanoparticles in the respiratory tract is crucial to evaluating air contamination, drug delivery, and health risks [178].

Since the first observation of particle deposition in the respiratory tract in 1881[179], there have been extensive investigations on particles entering the human body. Early studies on the deposition of aerosol particles in the respiratory system, involving in allergens, occupational and atmosphere dust, clinical aerosol, cigarette smoke, radioactive particles, and consumer aerosol products have been conducted [180]. The particle deposition in the respiratory tract depended on the properties of specific particles, including size, charge, density, as well as the breathing pattern of an individual [181].

It appears that smaller particles tend to be stored in the more distal region of human respiratory tract [181]. Particles with sizes >10 μm, >2.5 μm, <2.5 μm, and <0.1 μm tend to rest in the nose and upper airway (by filtration), the tracheobronchial region (by impaction and deposition), the pulmonary region (by sedimentation and diffusion), and the alveolar region (by penetration of alveolar epithelium to the bloodstream) [181-184].

Scientists have indicated that airborne NPs are deposited in three regions of the human respiratory tract. Airborne NPs with a size of 1 nm, for instance, can be found in the nasopharyngeal (90 %), tracheobronchial (10 %), and (3) alveolar regions of the body [185]. These nanoparticles can be found in the same regions in line with proportion. For example, 5 nm airborne NPs have a 30 %, 30 %, 30% distribution, and 20 nm particles have a 15%, 15%, 50% distribution to the nasopharyngeal, tracheobronchial, and alveolar regions, respectively. In addition, small nanoparticles exhibit penetration to cells (transcytosis) and can circulate in the circulatory and lymphatic systems [186].

Besides particle size, individual breathing patterns may affect particle deposition; and several studies reported that the deposition of particles in nasal breathing was higher than in oral breathing for both hydrophobic and hygroscopic aerosols [187]. For ethical reasons, there are currently no human studies on the deposition of TiO_2 NPs in the human respiratory tract due to their high toxic possibility.

Current meta-analysis studies in correlation with NPs and the respiratory system have been reported, such as transcriptional profiling to identify physicochemical properties of NPs that are determinants of the in vivo pulmonary response [188], occupational TiO_2 exposure, and lung cancer mortality [189], and transcriptomic responses to identify pulmonary disease outcomes for engineered NPs [190].

Gastrointestinal (GI) Tract Pathway

NPs enter the human body and gastrointestinal (GI) tract through the ingestion of foods, water, drugs, and via topical application of some cosmetic products [185]. Payne et al. (1960) and Sanders & Ashworth (1961) presented key studies on the uptake and

absorption of particles in the GI tract. Payne et al. (1960) reported uptake of small resin by the alimentary canal of the calf [191]. Sanders & Ashworth (1961) observed the intestinal absorption of particulates and hepatocellular uptakes of PS latex particles [192]. These findings provide the fundamental knowledge base for researchers worldwide on uptake and absorption of particles in the GI tract [193-198].

Travel of NPs through the environment of the human GI tract is complex [199]. NPs firstly are exposed to digestive elements in the mouth (neutral pH, mucin, amylase, and electrolytes), then pass through to the esophagus and into the stomach where they are exposed to a pH of 2-3, lipase, pepsin, and electrolytes. NPs then move through the small intestine with exposure to a pH of 5-7, mucin, bile salts, phospholipids, pancreatic lipase, proteases, amylase, and electrolytes. Finally, NPs reach the colon with exposure to a pH of 6-7, gut microbiota, and undigested food substances [110]. Through the digestion process, NPs trapped within the food can be released into GI tract fluids due to the disruption of the food matrix [109, 200-202]. Moreover, the region where NPs are released in the GI tract depends on the structure and composition of the food [110].

McClements and Xiao (2017) explained that the riches of fluids, which have certain properties in the GI tract, may alter NPs properties such as 1) pH and ionic strength: these parameters could specify the surface potential and electrostatic interactions that affect the aggregation state and interactions of NPs with other components; and 2) surface-active components contained in gastrointestinal fluid: these components, which consist of surfactants, proteins, bile salts, phospholipids, and free fatty acids (FFAs), can be adsorbed by NPs and change their biological fate. The interfacial properties of inorganic NPs, for instance, is altered when they enter the GI

tract [110]. As a consequence, the cellular and tissue response to the NPs is also changed [108, 203]. Furthermore, their biological fate can be modified as well [204-207]. Notwithstanding pH and ionic strength effect to the alteration of the physicochemical property of TiO_2 NPs, the TiO_2 NPs-food component interactions and associated biological fate in the human GI tract are poorly understood.

Enzyme activity and enzymatic digestion can alter NPs properties [110]. NPs carrying carbohydrates, protein, or lipids, for example, can be digested by amylases, proteases, and lipases. As a result, the properties of NPs exhibited in certain regions of the GI tract may be different from the initial properties of NPs [110]. Biopolymers adsorbed on the surface of NPs can change the NPs interfacial properties. Adsorbing polymers may promote bridging flocculation while non-absorbing biopolymers may oppose flocculation [110].

The mucus layer in the surface of the GI tract may provide new insights into the inhibition of entry of NPs beyond the GI tract to other organs. The human GI tract can be analogized as a series of tubes and chambers with specific surface morphologies. The surface of the GI tract consists of villi and microvilli and a thin layer of mucus that serves as a gut barrier between NPs and epithelial cells [208]. The gut microbiome is primarily in the large intestine. The microbiome can ferment foods and generate metabolites that may change the NPs properties; reciprocally, ingested NPs may alter the properties of the gut microbiome [209]. Studies report that many types of NPs can disturb the balance of gut microbiome (dysbiosis) and cause adverse effects [209-210].

There are some subsequent mechanical forces that occur during NPs digestion in the GI tract, such as mastication in the mouth, peristaltic movements in the esophagus

and small intestine, and churning in the stomach. These forces may alter NPs properties, particularly their aggregation state [110]. Studies associated with the interaction between TiO_2 NPs and mucus or biopolymer in the presence of gut microbiome in the human GI tract are very limited due to complex biomolecules and microenvironments [47].

Besides the digestive process and the riches of fluids in the GI tract, some studies have reported and effects of NPs ex vivo, in vitro, and in vivo gut cell [5, 50, 211-223]. Studies have described the production of reactive oxygen species (ROS) which can induce toxicity [224-226]. The impact and translocation of TiO_2 NPs through ex vivo, in vivo, and in vitro gut epithelia have been observed by Brun et al., (2014). TiO_2 NPs were likely to translocate via both Peyer's patches and the regular epithelium lining, would cause epithelium disruption, then would be stuck out in intestinal cells and induce chronic damage [50].

Conclusions and Perspectives

The formation of BMC, which constitutes the adsorption of biomolecules on the TiO_2 NPs surface, is influenced by many types of interactions, particularly electrostatic and hydrophobic interactions. These interactions may alter the physicochemical properties and toxicological effect of TiO_2 NPs. The applications of TiO_2 NPs have raised concerns because of their possible toxicity. Some detrimental effects induced by TiO_2 NPs on cells, such as lipid peroxidation, leads to disruption of the bilayers membrane, protein denaturation that induces protein oxidation and enzyme dysfunction, and mitochondria dysfunctions that cause oxidative DNA damage and apoptosis. The production of ROS is considered a primary cause of cell toxicity.

The BMC formations are complicated due to the riches of fluids, such as varying pH and isoelectric point (PI), mechanical forces, surface active components, biopolymers, microbiome, enzymes, and mucus layers. Although some studies have revealed the interaction between TiO_2 NPs and proteins, lipids, and carbohydrates, more comprehensive studies are required, particularly on the mechanistic interactions of TiO_2 NPs with complex molecules such as mucus. Mucus as epithelial barrier provides pivotal protection to living organisms, especially in protecting the respiratory system, GI tract, eyes, and reproductive organs from physical, chemical, and biological hazards.

The physicochemical mechanisms of biomolecules that may affect the GI fate of TiO_2 NPs are entrapment, thickening, and gelling process, adsorption, aggregation, and pH buffer which is specifically owned by proteins. Interactions of NPs with proteins have been studied more rigorously than other biomolecules such as lipids and carbohydrates. Recent studies have examined the interactions between TiO_2 NPs and gut microbiomes, as well as dietary fibers and microbiomes. However, no studies have investigated the interactions between dietary fibers and the microbiome in the presence of TiO_2 NPs. Applied research examining the potential toxicity and associated environment and human health risks of TiO_2 NPs are essential. Research is also needed on the mechanistic interactions between titanium dioxide nanoparticles (TiO_2 NPs) and biomolecules, with a focus on mechanisms that prevent or minimize entry of TiO_2 NPs into the cells or organisms.

CHAPTER III

3. INTERACTIONS OF TiO_2 NPs AND MUCIN INDUCE THE ALTERATION OF PHYSICOCHEMICAL PROPERTIES, AGGREGATION, AND ACCUMULATION OF TiO_2 NPs IN WATER

Introduction

Domestic and industrial water effluent contained TiO_2 NPs are released inevitably in aquatic environments such as inland surface water, seas, and ground water. Concerning for nanoparticles (NPs) release, fate and effects on ecosystems and human health are emerging [31, 227-231]. Some studies have reported the assessment of TiO_2 NPs toxicity in aquatic organisms, including the rainbow trout [232-233] and bivalve mollusks [234-237]. TiO_2 NPs can generate radical oxygen species (ROS), and hence to cause toxicity [11, 238]. However, determining the fate, transport, transformation, and toxicity of TiO_2 NPs in the aquatic environment remains challenging and depends on the chemical reactivity and physicochemical property of TiO_2 NPs.

The reactivity and physicochemical property of NPs are strongly influenced by water properties (such as pH, water hardness, ionic composition, and temperature) [16-22], the present organism living in water [17, 29-32], and suspended particulate matter (SPM) [23-28]. Smooth alterations in water chemistry and interactions with surrounding substances can significantly modify NPs surface properties and cause either dispersion or aggregation [17, 239-240]. The presence of NOM is expected to provide a profound impact on the surface properties of NPs (such as coating and surface charge alterations) and the behavior of hetero-aggregation of NPs [25, 27, 34-35].

Naturally, aquatic organisms produce NOM, named mucus (mucin), to protect the hosts from biological, physical and chemical perils [39]. Mucin is the main gel-forming polymers of mucus and consists of core protein domains and densely O-linked oligosaccharide chains; hence, it confers negative charge to the mucin through carboxyl and sulfate groups [36]. Meanwhile, mucus is a complex mixture of water, lipids, salts, nucleic acids, and various proteins, including protein [241-245]. A recent review article has reported the significant effect of electrostatic, steric, and hydrophobic interaction on selective permeability of mucin [37]. Studies, in relation with biological and ecological roles of mucin in aquatic metazoans, have been conducted such as: Atlantic salmon [246-255].

NOM-TiO_2 NPs interaction in water consecutively leads to the formation of biomolecular corona (BMC), hetero-aggregation of BMC that can disrupt to BMC stability in water [34], and dispersion of BMC due to steric hindrances [67, 256]. BMC aggregation is potentially to facilitate the ingestion and accumulation of TiO_2 NPs in the aquatic organisms such as fish and invertebrates. Yin, C., et al. (2019), for instance, suggested that the accumulation of TiO_2 NPs may pass through to the higher trophic levels (particularly to human) via seafood. However, no studies have reported the interaction between TiO_2 NPs and mucin—one of NOMs produced by aquatic animals. Thus, the purpose of this study is to know TiO_2 NPs-mucin interaction, emphasizing on their physicochemical alterations during the formation of BMC in water.

Materials and Methods

Materials

TiO_2 NPs (anastase; 5-15 nm) was purchased from US Research Nanomaterials (TX, US) and mucin (Type II, porcine gastric mucin) was purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The following products were purchased from Life Technologies (Thermo Fisher Scientific, Agawam, MA, USA): hydrochloric acid, sodium hydroxide, sodium chloride, calcium chloride, dimethyl sulfoxide. Silver (Ag) dendrites were prepared based on He, Lin, Li, & Kim (2010) [257]. Deionized water (DIW) was applied for preparation of all solutions.

Methods

The change of physicochemical properties is considered as the main factors affecting the formation of aquatic biomolecular corona (BMC). The formation of BMC was identified by observing the change of physicochemical properties of TiO2 NPs and mucus (e.g. charge and size) in the function of pH. These alterations may lead to a specific interaction (e.g. electrostatic interaction or hydrophobic interaction) and induce a new formation (e.g. dispersion or aggregation), and thereby change the bioactivity of BMC. Some experiments that have been done to measure these alterations: *first*, observing the appearance of TiO2 NPs and mucin in the various pH; *second,* examining zeta potential (ζ) of TiO2 NPs and mucin in the different pH using a dynamic light scattering (DLS); *third*, measuring the change of turbidity (O.D. 600) and aggregation of BMC by utilizing UV-Vis. (wave length 200 nm-750 nm); *fourth*, observing the charge

alteration of BMC by employing dynamic light scattering (DLS); *fifth*, measuring the change of size through by utilizing a static light scattering (Master sizer) and optical microscopy; *sixth*, investigating the change of energy during the formation of BMC by using isothermal titration calorimetry (ITC); *seven*, characterizing the interaction occurs between TiO2 NPs and mucus through employing surface-enhanced Raman spectroscopy (SERS). In the aquatic environment, water is considered as an important buffer, especially in the formation of biomolecular corona (BMC). Thus, this study used the deionized water (DIW) as the buffer.

Alterations of BMC Appearance & the Basic Experiment (BE)

0.1% v/v of TiO2 NPs were dispersed in DIW. 5 mg/ml mucin was stirred into DIW overnight and then centrifuged. Before experiments were conducted, pH of TiO2 NPs and mucin should have been adjusted. The change of BMC appearance was observed every time a certain amount of TiO2 NPs was added. The experiment was started with observing the appearance of control (mucin) by adding 1 mL mucin (5mg/mL) into a glass reaction tube and add 9 mL DIW. While treated groups, the amount of TiO2 NPs (0.1% v/v) was increased 0.5 mL and the amount of DIW was reduced simultaneously; therefore, the total volume of sample was 10 mL. Vortex was applied for each tube (10 s), and eventually the alteration of appearance was observed, particularly to identify the possibility of aggregation during TiO2 NPs-mucin reaction. This experiment was basic experiment (BE) and should be understood before doing other experiments.

Particle Size and Charge Characterization

The particle size of BMC was measured by a static light-scattering instrument (Master sizer 2000, Malvern Instruments, Worcestershire, U.K.), while zeta potential (ζ) was measured by Zetasizer nano ZS series, Malvern Instruments Ltd, Worcesterhire, UK).

UV-Vis Spectrophotometry

TiO_2 NPs and mucin were mixed and prepared as the same to BE. The absorption spectrum was determined using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Isothermal Titration Calorimetry (ITC)

The change of energy (ΔH), when TiO_2 NPs were titrated into either mucin solution or buffer solution (DIW), was measured by an ITC instrument (Microcalorimeter VP-ITC, MicroCal Inc., Northampton, MA, USA). Twenty-nine 10 μL aliquot of TiO_2 NPs suspension were injected sequentially into a 1,450 μL cell (mucin) initially. The mass ratio of total samples (TiO_2 NPs: mucin) is 0.25.

Surfaced-Enhanced Raman Spectroscopy (SERS)

Mucin and TiO_2 NPs were dispersed and stirred in buffer overnight. Three different categories of solution (mucin, TiO_2 NPs, and the mixture of TiO_2 NPs and mucin with the mass ratio= 0.25) were prepared. These solutions were homogenized for 10 seconds and added Ag dendrites. These mixtures were homogenized for 10 seconds and

sedimented. These sediments were centrifuged and rinsed with buffer three times. Each sediment (2 μL) was deposited on a glass slide covered with aluminum foil as a sample. The sample was air-dried before observing it under the Raman laser. A DXR Raman Microscope (Thermo Scientific, Madison, WI) equipped with a 785 nm-excitation laser and a 50x objective was employed. Spectra were collected with a 5.0 mW laser power and a 50 μm slit aperture for 2 seconds scanning time. All SERS experiments were done minimally twice. There were 7-9 spots per sample were characterized by SERS. The elicited spectra were analyzed using TQ analyst software, version 8.0 (Thermo Fisher Scientific).

Statistical Analysis

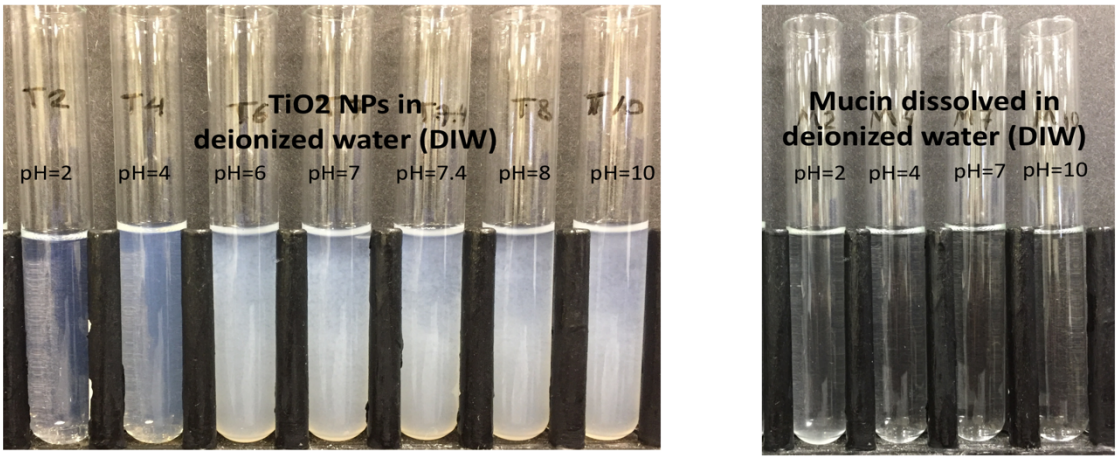
All experiments were conducted at least twice and reported as the results of means and deviations.

Results and Discussions

The change of Physicochemical Properties of TiO_2 NPs & Mucin

The alteration of physicochemical properties is essential in determine the fate, transformation, transport and toxicity of TiO_2 NPs [258-262]. The physicochemical property of TiO_2 NPs was strongly affected by the pH, as evidenced by the change of their appearance. They were opaquer, when exposed to the more basic microenvironment (**Fig**. 9 (A)). The appearance of TiO_2 NPs at acidic condition (pH 2 to pH 4) were relatively clear and changed gradually. It was becoming cloudier with the increase of pH; while in the neutral to alkaline conditions (pH 6 to pH 10), the appearance of TiO_2 NPs

was all opaque. Knowing the charge is essential to determine the possible interactions caused by the ionic strength such as electrostatic repulsion or attraction. The surface charge data showed that in acidic conditions, TiO_2 NPs were potentially positive charge; while in the quite neutral to the alkaline condition they had negative charge (**Fig**. 10 (A)). Since the electrostatic attraction at pH= >7 is not the case, the change of TiO_2 NPs appearance in DIW may be induced by aggregation. The addition of hydrochloric acid (HCl) was required to reduce pH, while sodium hydroxide (NaOH) was also required to increase pH. The pH adjustment from acidic to alkaline condition could cause the interaction between HCl and NaCl, and thereby develop sodium chloride (NaCl). It could be concluded that the cloudiness can be caused the formation of NaCl, although it is not significant.

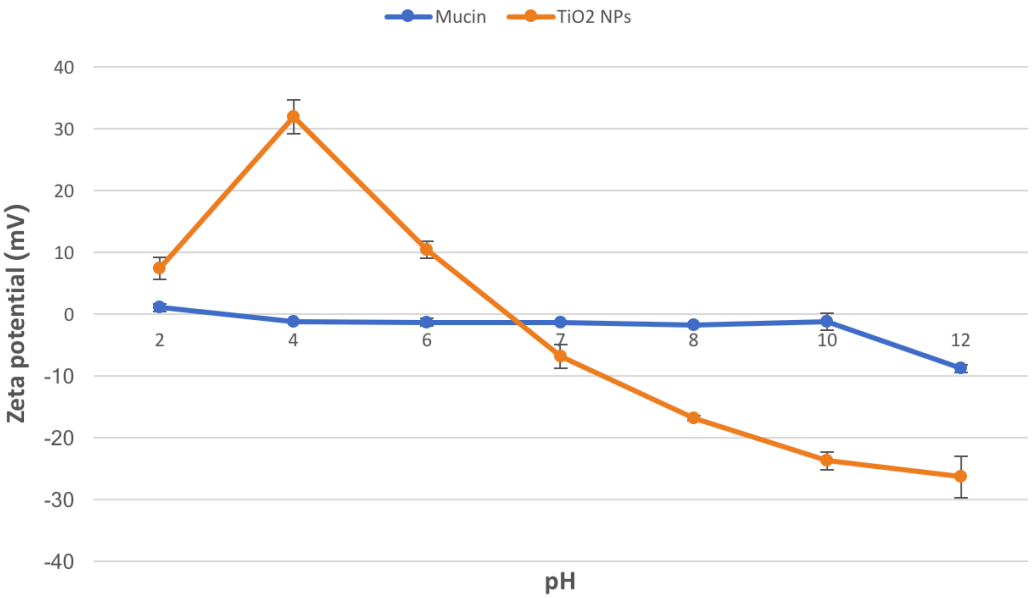


(A) (B)

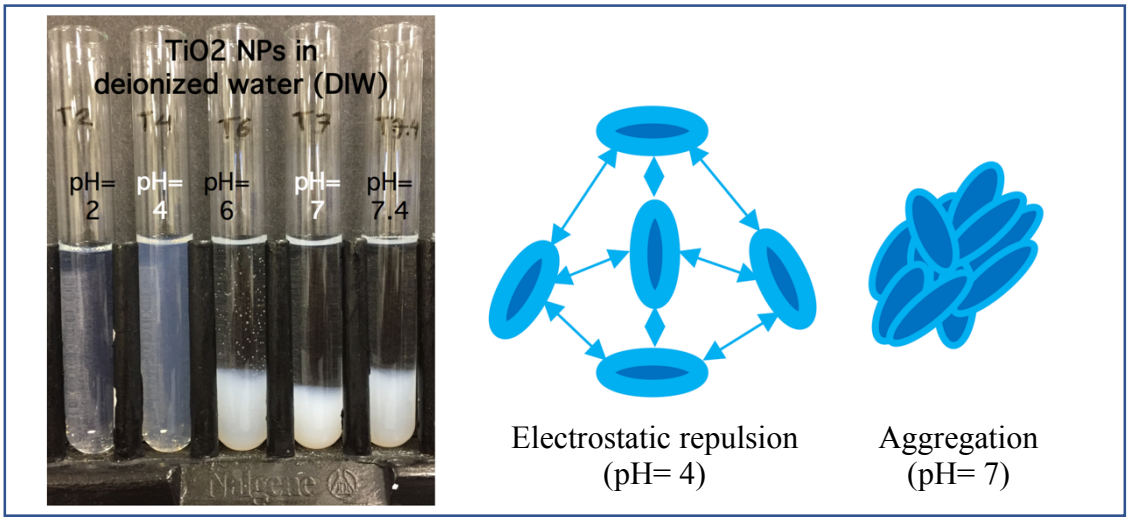
Figure 9: The appearance of TiO2 NPs (A) and mucin (B) in deionized water (DIW) at various pH

In aqueous media, TiO_2 NPs tend to aggregate [64]. The average size of TiO_2 NPs was much bigger (from 5-15 nm to 30 nm) when they were exposed in deionized water [263]. In theory, TiO_2 NPs are supposed to aggregate for pH value near the zero points of charge (pH_{pzc}) [67]. The data showed that the charge of TiO_2 NPs at pH=6.0 to pH=7.4

was low or close to zero (<10). This means that pH_{pz} of TiO_2 NPs in water is more likely 6.0-7.4 and potentially causes aggregation. Whereas, Domingos and co-workers reported that pH_{pz} for TiO_2 NPs in water was pH range of 4.5-5.2 [67]. This difference is possible and may be induced by the different size range, producer, and preparation of TiO_2 NPs.



10 (A)



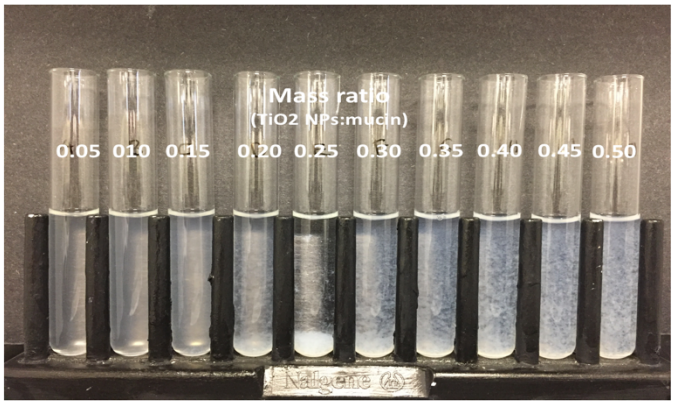
10 (B)

Figure 10: (A) Zeta potential of TiO2 NPs and mucin in the different pH; (B) The illustration of possible interactions between TiO2 NPs at pH=4 (electrostatic repulsion) & pH=7 (aggregation).

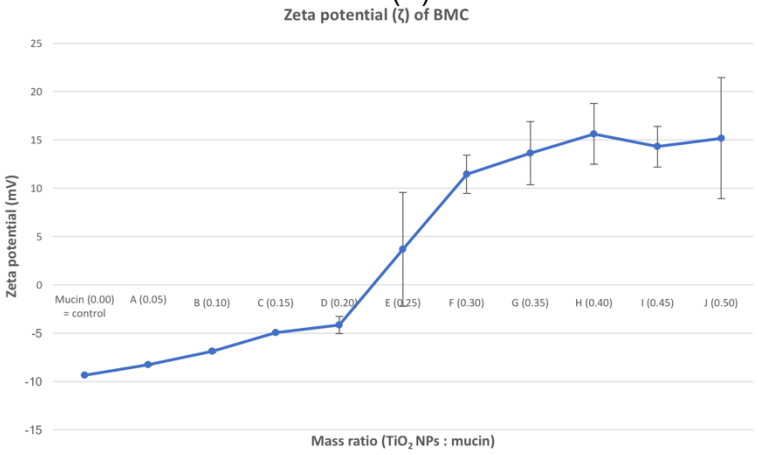
Formations of BMC & Hetero Aggregation

The addition of TiO_2 NPs into mucin induced the formation of BMC. The different concentration of TiO_2 NPs resulted the different level of turbidity. The clarity of control (mucin) was the same to DIW; while the TiO_2 NPs treated groups, the turbidity was gradually increased depending on the amount of TiO_2 NPs added (**Fig**. 11 (A) & (C)). Therefore, it concluded that the more TiO_2 NPs added is the higher turbidity resulted. Interestingly, a massive aggregation was occurred in the mass ratio 0.25; and since that point, the aggregate was relatively stable with the addition of TiO_2 NPs (**Fig**. 11 (B)).

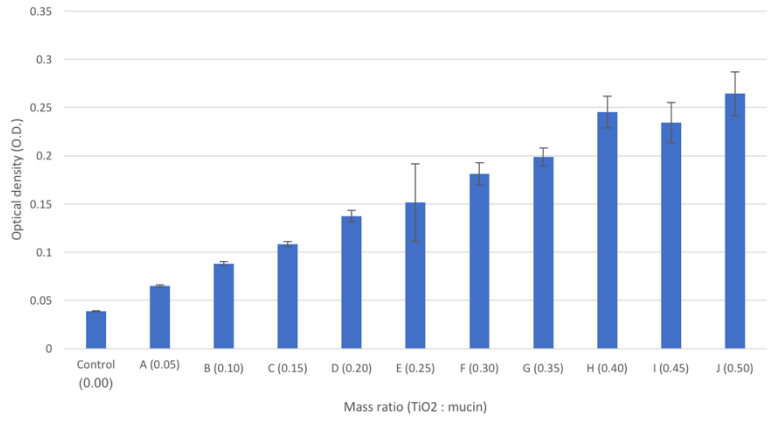
Also, the hydrophobic interaction might affect significantly on TiO_2 NPs-mucin interactions. Zeta potentials of BMC treated with low concentrations of TiO_2 NPs (mass ratio 0.05 to 0.20) were positively to neutrally charged. Meanwhile, zeta potentials were changed from neutral to positive charge, since at mass ratio (TiO_2 NPs: mucin) = 0.25 (**Fig**. 11 (B)). Generally, the formation of BMC at pH=4 is likely to be induced by hydrophobic interactions, and affected by the amount of TiO_2 NPs added.



11 (A)



11 (B)



11 (C)

Figure 11: Formations of BMC at pH=4: (A) The massive aggregation may occur in the mass ratio (TiO2 NPs: mucin) = 0.25; (B) zeta potential alterations of BMC (from negative to positive charge) are affected by the amount of TiO2 NPs added into the interaction; (C)

The significant increase of BMC size might indicate a massive hetero aggregation. Hetero aggregation can be described as the aggregation of dissimilar particles, and can render significant effect on NPs fate, uptake, transport, bioavailability, and eco-toxicity in environment [231, 264-265]. The size augmentation of aggregate was observed through observing under optical microscopy (**Fig**. 12 (A)) and applying Master sizer depicted in **Fig.** 12 (B). **Fig.** 12 (B) showed that aggregations were started at C (TiO_2 NPs: mucin=0.15), and massively developed since E (TiO_2 NPs: mucin=0.25). Smaller aggregates were identified at J (TiO_2 NPs: mucin=0.5), and might be caused by separation phase. Further observation to identify separation phase using TEM might be required.

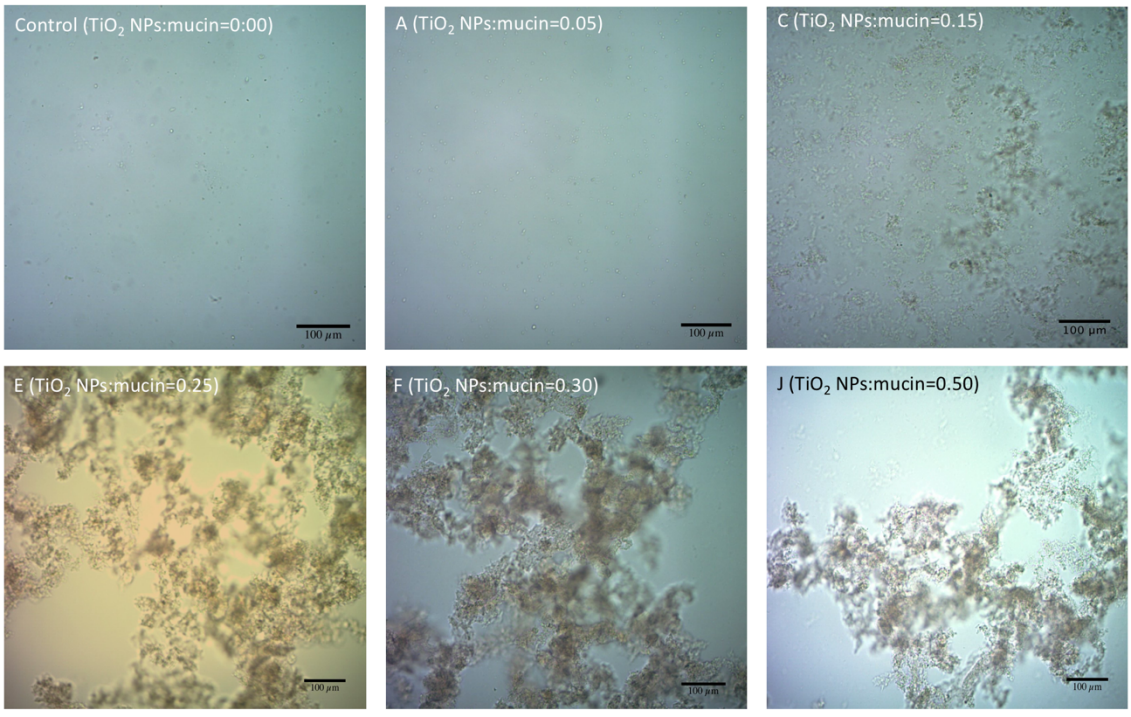
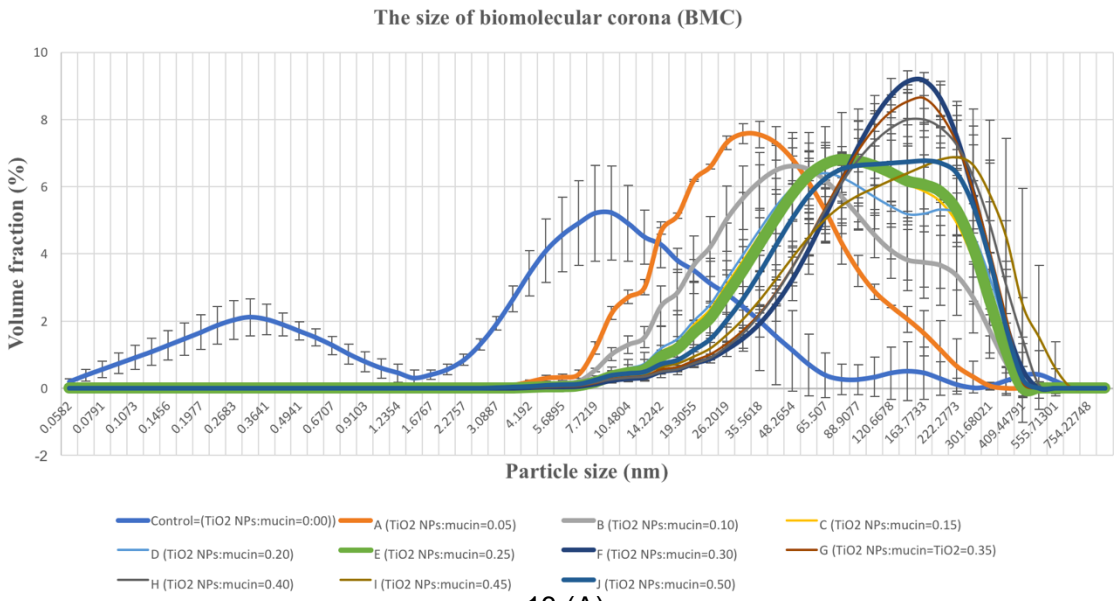
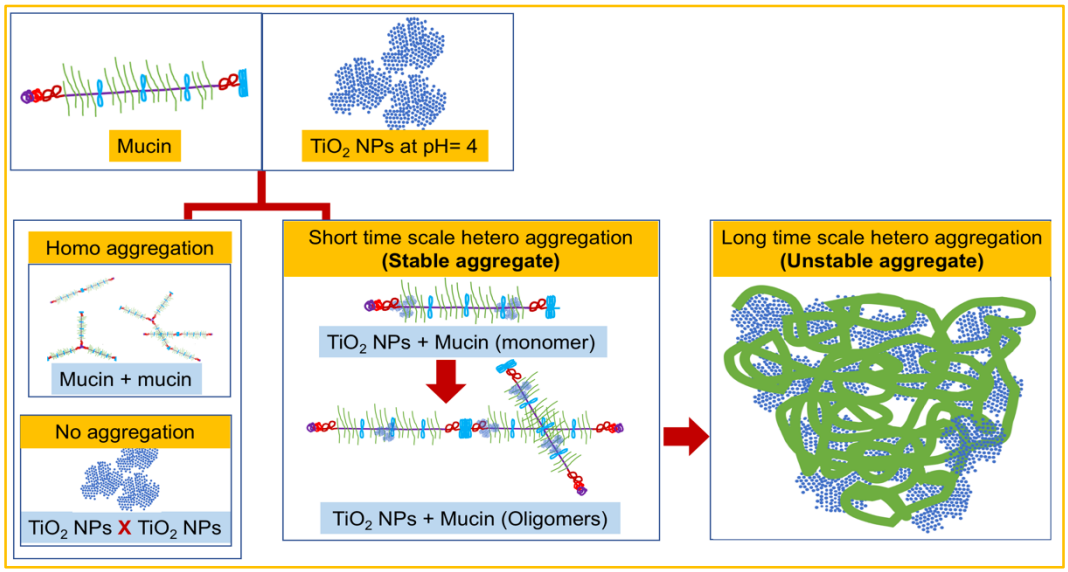


Figure 12: Observations of BMC under optical microscopy. The massive aggregation occurs at mass ratio (TiO2 NPs: mucin) 0.25.



13 (A)



13 (B)

Figure 13: (A) Size measurements using Master sizer show that the size of BMC tends to be bigger with the more addition of TiO2 NPs; (B) Illustrations of hetero aggregation between TiO2 NPs and mucin at pH= 4, which is inspired by Clavier, Praetorius, & Stoll, (2019).

Respective of the size, the control or mucin was poly-dispersed molecule, as evidenced by the presence of some peaks with the wide range of size (Fig. 13 (A)). It confirmed previous theory that the size of mucin is varies (200 KDa-200 MDa) [266]. While the treated groups (mucin + TiO_2 NPs) showed that the addition of different amount TiO_2 NPs induced the various size of BMC. Experiment A, for example, showed although the amount of TiO_2 NPs added was very little, but it exhibited the peak shift. It means that the size is much bigger than the control. Fig. 13 (A) also exhibited the shifting peak was persistent unless the mass ratio of TiO_2 NPs and mucin reached 0.25. Once their mass ratio reached 0.25 (E), not only the shifting peak but also the formation of two peaks occurred. It is interesting to note that these phenomena may represent aggregations. The maximum size gained by experiment F; afterwards, the size and volume fraction of BMC were decreased. These reductions might be caused by a phase separation, and resulted an insoluble aggregate. In general, the size of BMC became bigger, when the higher amount of TiO_2 NPs added into mucin, and the mass ratio 0.25 to 0.30 were a critical point, where the maximum size may be achieved (**Fig**. 12 & **Fig.** 13 (A)).

Fig. 13 (B) illustrated the development of hetero aggregation between TiO_2 NPs and mucin. This illustration is inspired by **Fig.** 12 & **Fig.** 13 (A) and **Fig.** 11 (A) showed enormous aggregates and a massive precipitation. Interactions of some similar molecules/particles are an example of mono aggregation such as dimer and trimer; while hetero aggregation is categorized into two types of aggregation: stable hetero aggregation (which is formed by dimer/trimer aggregate) and unstable hetero aggregation (which is

developed by large and complex aggregate) [34]. Therefore, it can be noticed that the interaction between TiO_2 NPs and mucin is likely to generate unstable hetero aggregation.

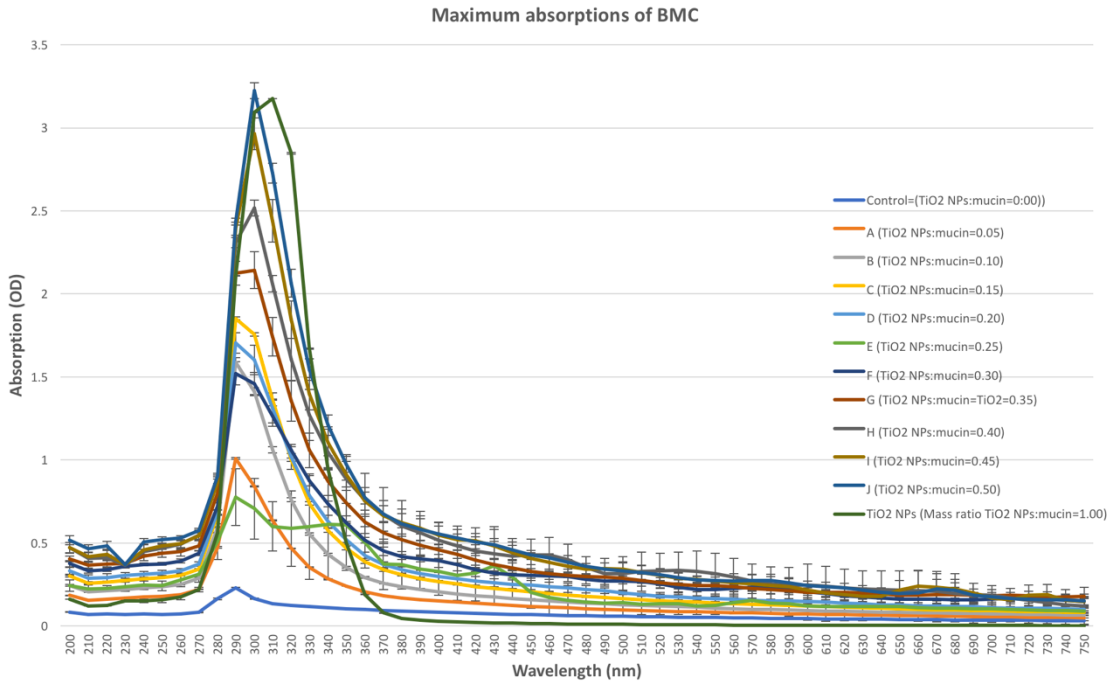


Figure 14: UV-Vis absorbance spectra of BMC with different concentrations of TiO2 NPs (wavelength 200-750 nm). At E (TiO2 NPs: mucin= 0.25), there are multiple peaks, hypochromic effect, and redshift which may indicate the massive aggregation.

Furthermore, UV-Vis spectroscopy was employed to examine the formation of hetero aggregates by scanning the maximum absorption of BMC with the range of wave length 200-750 nm. **Fig.** 14 exhibited that the more TiO_2 NPs added was likely to create more bounds between TiO_2 NPs and mucin, then led to aggregation. This was evidenced by the increase of BMC absorbance. The spectrum absorbance of control groups did not show any absorbance at wave length longer than 400 nm (mucin) and 390 nm (TiO_2 NPs); meanwhile the spectrum of mucin exposed with TiO_2 NPs showed a vivid absorbance at 400 nm even at >600 nm, depending on the mass ratio of TiO_2 NPs to

mucin. In general, the treated groups resulted in the red shift of their absorbance spectra. It suggests that the molecular interaction and direct binding may occur. It is an interesting note that the red shift was persistent to all treated groups.

However, there was an exception shown by the experiment E (the mass ratio of TiO_2 NPs to mucin= 0.25): a hypochromic effect was significantly appeared, and there were multiple peaks found when the mass ratio reached 0.25 (**Fig.** 14). The red shit and hypochromic effect accompanied by multiple peaks may an indication that the unstable hetero aggregation massively takes place at mass ratio (TiO_2 NPs: mucin) = 0.25. In theory, UV-Vis spectrophotometry is commonly applied to detect aggregation due to electron transfer from an absorbed molecule to empty conduction band on TiO_2 which induce the decrease of ionization energy (IE) of electron donor [267]. Aggregation can be identified through a bathochromic sift of UV-vis spectra (the red shift) [268]. Therefore, it was concluded that the interaction between TiO_2 NPs and mucin leads to hetero aggregation.

TiO_2 NPs-Mucin Interaction: Polyvalent Binding and Irreversible Aggregate

Although mucin is mostly constituted by carbohydrate, mucin's backbone consists of apomucin—the apoprotein of mucin [36]. Although extensive studies have observed protein-NPs interactions [98], the mechanistic interaction between TiO_2 NPs and mucin limited information have been unclear. TiO_2 NPs may interact with apomucin, and induce the alteration of mucin structure. Studies revealed that the mucin aggregate could be constituted by five-, six-, and seven-sided ring structure; where these ring structures were composed by multiple mucin trimers [269]. Mucin trimers (**Fig.** 15) were formed in low

and high pH conditions [269]. Mucin can build net-like structure through hydrophobic cross linking, and interact with other biomolecules or particles via some possible interactions such as hydrophobic interactions, hydrogen binding and electrostatic interactions (**Fig.** 15) [36, 270-271].

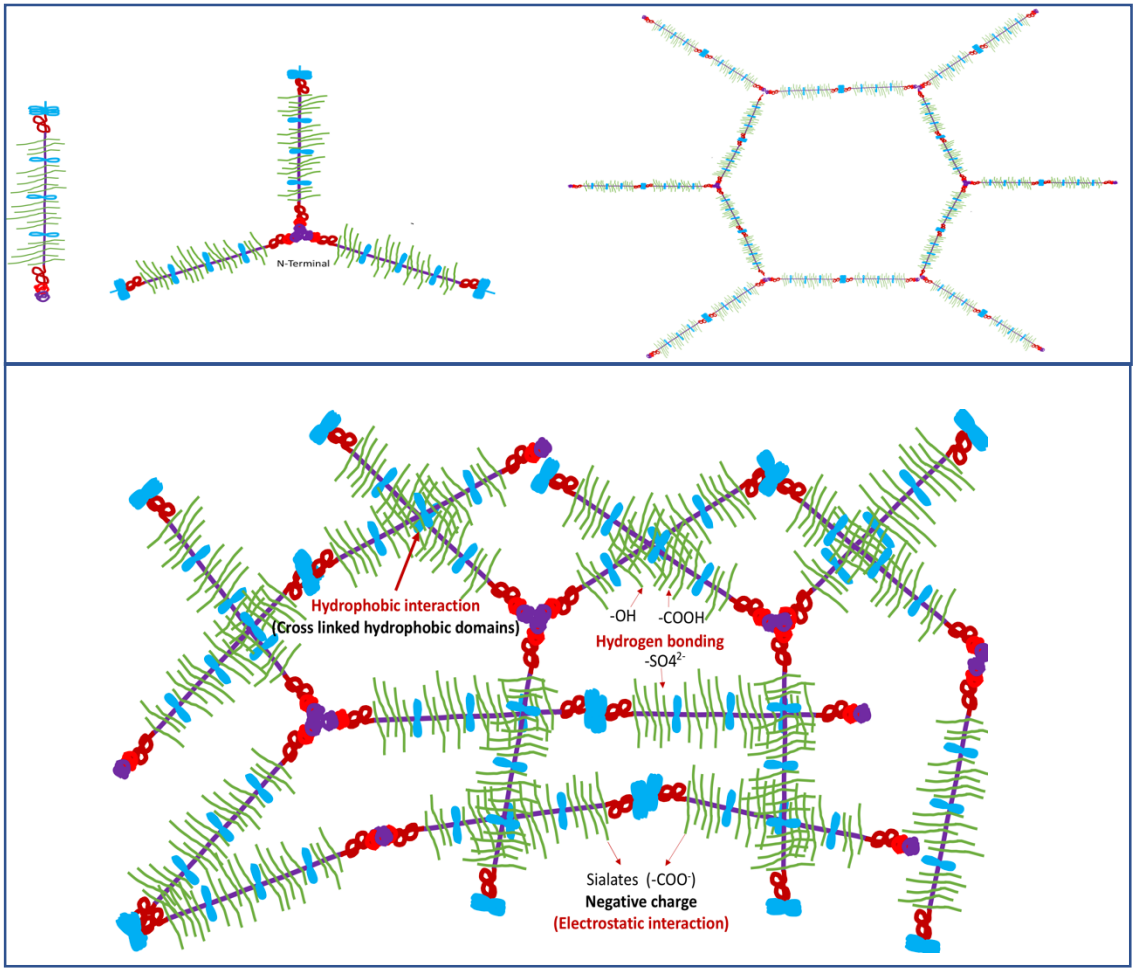


Figure 15: Illustrations: The structure of mucin which is like a tube brush (inspired by Bansil, & Turner (2006)), and mucin trimer and six sided-ring structure which are inspired by Ambort et al., (2012). Possible interactions of mucin with biomolecules or inorganic particles which are inspired by Wagner et al., (2017); Yang et al., (2012); & Bansil & Turner, (2006).

Some studies have reported their findings related to interactions between TiO_2 NPs and natural organic matter (NOM) in aquatic environment such as humic acid/fulvic acid [67, 255]. They explained that once TiO_2 NPs are exposed into water, their stability is reduced, and hence to lead to agglomeration. Besides, TiO_2 NPs will adsorb NOM existing in aquatic environment, form BMC, increase their stability owing to steric hindrance, and cause dispersion. The addition of electrolytes lead to disrupt the stability of BMC due to charge neutralization, depletion, and bridging effect [67, 255]. Mucin is another essential NOM generated by aquatic organisms to elicit protection from physical, biological, chemical hazards; however, mechanistic interactions between TiO_2 NPs and mucin in aquatic environment are still questioned.

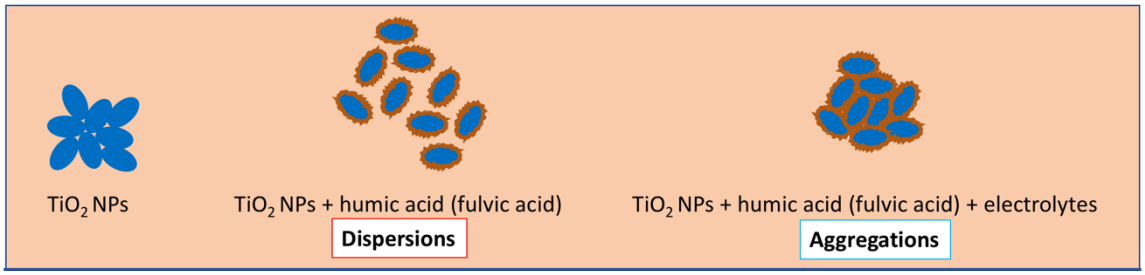


Figure 16: Illustrations of the interactions between TiO2 NPs and NOM (humic acid/fulvic acid) in the aquatic environment, which is based on findings by Domingos, Tufenkji, & Wilkinson, (2009) and Wang et al., (2019).

Witten and co-workers (2018) reviewed the biochemical mechanisms emphasizing on mucin penetration and binding, particularly the significance of electrostatic, steric, and hydrophobic interactions [37]. Impact induced by mucin-particle interactions depends on not only the specific biochemistry but also the number of binding sites on the particle which can interact with mucin [272]. The illustration of polyvalent binding between TiO_2 NPs and mucin was showed by **Fig.** 17 (A) & (B). The size of mucin was not actual size. It was just the estimated size based on the Mastersizer data

(±8500 nm), while the size of TiO_2 NPs was varies depending on pH (aggregation). **Fig.** 17 tried to provide a simple illustration; therefore, the polyvalent binding between TiO_2 NPs and mucin and the cross linking of mucin were more understandable.

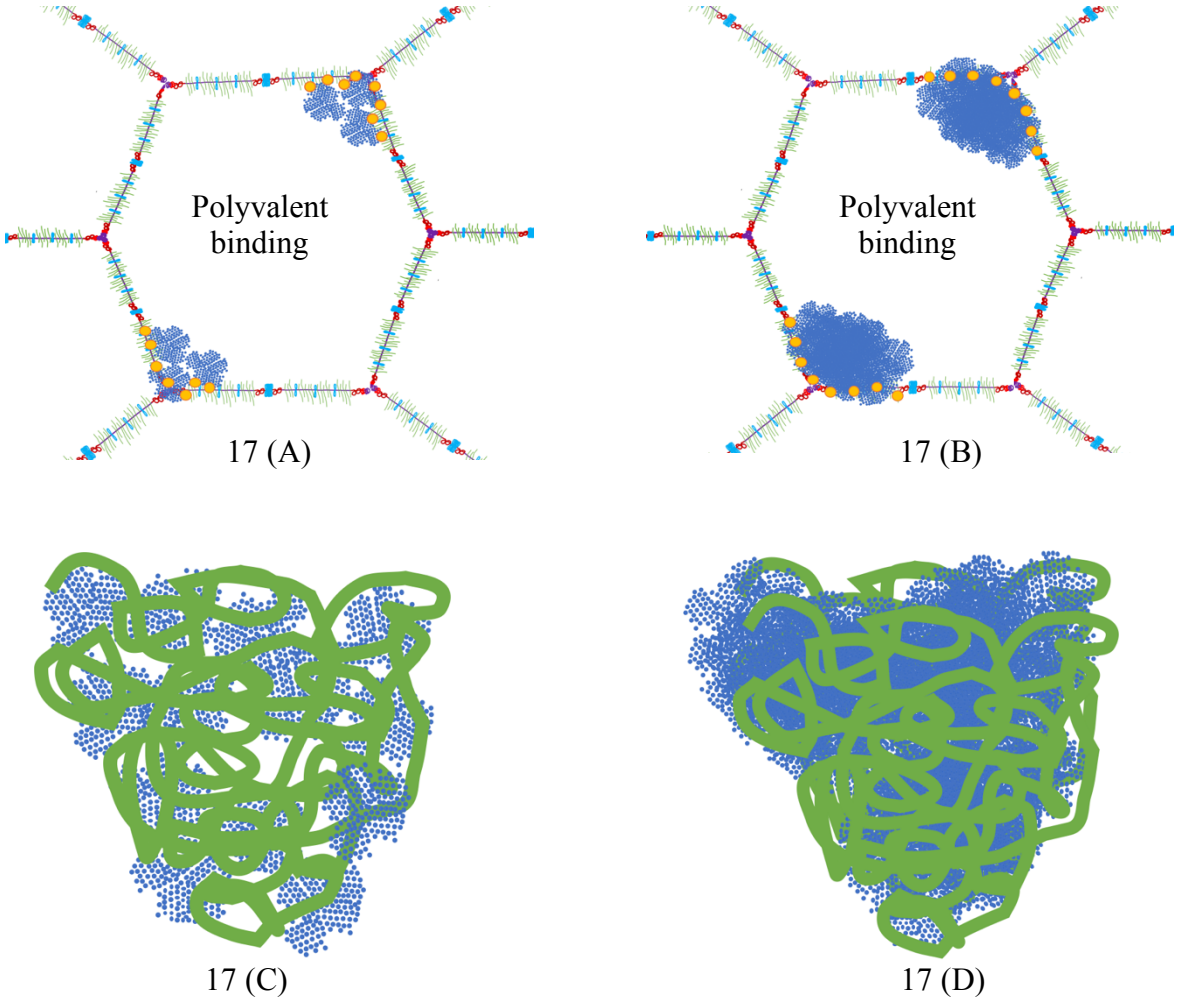


Figure 17: Illustrations of polyvalent binding between TiO2 NPs and mucin at pH=4 (A) & pH=7 (B) (inspired by Cone, R.A., (2009)). Effect of polyvalent binding and hydrophobic cross-linking on the formation of irreversible aggregate at pH=4 (C) & pH=7 (D).

Based on the measurement of turbidity (**Fig.** 11 (A) & (B)), size (**Fig**. 12 & **Fig.** 13 (A)), UV-Vis maximum absorbance (**Fig**. 14 (A) & (B)), and exothermic energy (**Fig**. 18), interactions between TiO_2 NPs and mucin caused irreversible aggregates. It can be

noticed that polyvalent binding may play important role in the formation of irreversible aggregate (**Fig**. 17 (A) & (B)). In theory, almost all NPs own multi biding sites; therefore, mucin can reduce the diffusivity of NPs more than small particles. Although each binding is weak; the total effect of polyvalent binding is likely to induce near-irreversible binding [37, 272]. Multiple bindings presented by NPs are termed polyvalent binding [272]. Besides, the hydrophobic cross linking that form net-like mucin structure [36, 271] is potentially to facilitate the entrapment of TiO_2 NPs.

One of the common approaches to determine the type of energy change and binding during the interaction between biomolecule and ligand is ITC. The combination of thermodynamic data and structural biomolecule has enhanced the understanding of macromolecular interactions in solutions (particularly protein), and thereby allowing predictions in terms of size, thermodynamic properties of binding interface, protein solvation, and conformational changes of protein [273-274]. It was difficult to determine the number of binding sites from **Fig**. (18). Based on the theory **Fig**. (18) showed a multiple binding [275]. It means that three or even more ligand molecules (TiO_2 NPs) bind a receptor molecule (mucin). The highest affinity binding site has the largest exothermic enthalpy change, while the next two or more binding processes have overlapping the equilibrium constant *(K)* values, and thereby they indistinguishable [275]. It may be induced by a complex sequence of modifications that are not being fully understandable when TiO_2 NPs interact with mucin.

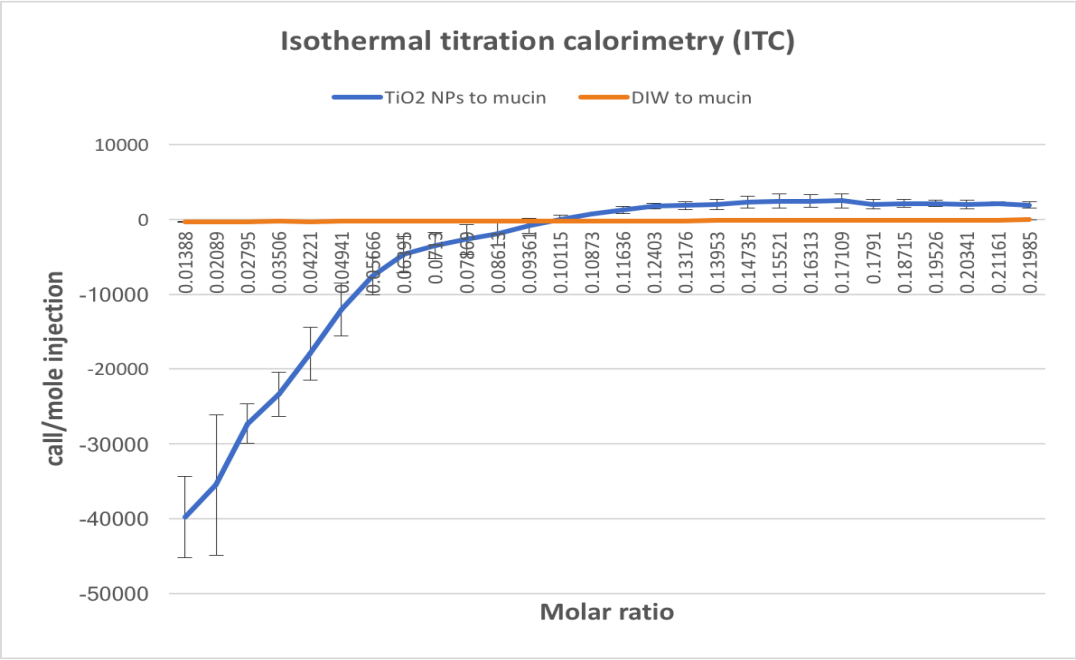


Figure 18: Isothermal titration calorimetry (ITC) shows that exothermic energy. It indicates that bonds are formed and energy is released.

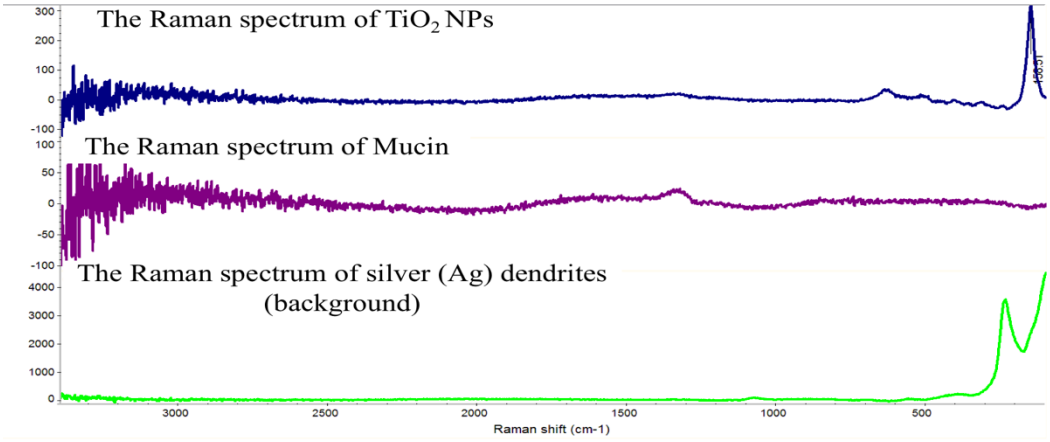
Fig. 18 showed that the reaction of TiO_2 NPs and mucin resulted exothermic energy and insignificant endothermic energy. The exothermic energy was predominantly generated from the first to thirteenth injection; afterwards, a small endothermic energy was consistently generated until the last injection. The surface properties of NPs and protein stability provide a notable effect to the alteration of protein structure and its function [274]. The disturbance of the protein structure is usually escorted by an inclusion of additional energy to the system, and thereby to shift the protein structure from the "potential hole" [274]. In thermodynamic terms, a minimal entropy is the most favorable state of the polypeptides chain. Entropy influences the intrinsic stability of protein. If the entropy of the "alternative state" is lower than the "native state", it will induce a reversible transition. However, if the entropy of the "alternative state" reaches

the critical maximum value, protein will be denatured and form insoluble aggregates or fibrils due to the irreversible transition. The reversible transition indicates the formation of "soft corona"; while the irreversible transition results "hard corona" [276]. Considering **Fig**. 11, 12, 13, 14, & 18, it can be noticed that the aggregation induced by TiO_2 NPs-mucin interaction forms "hard corona" and it is irreversible.

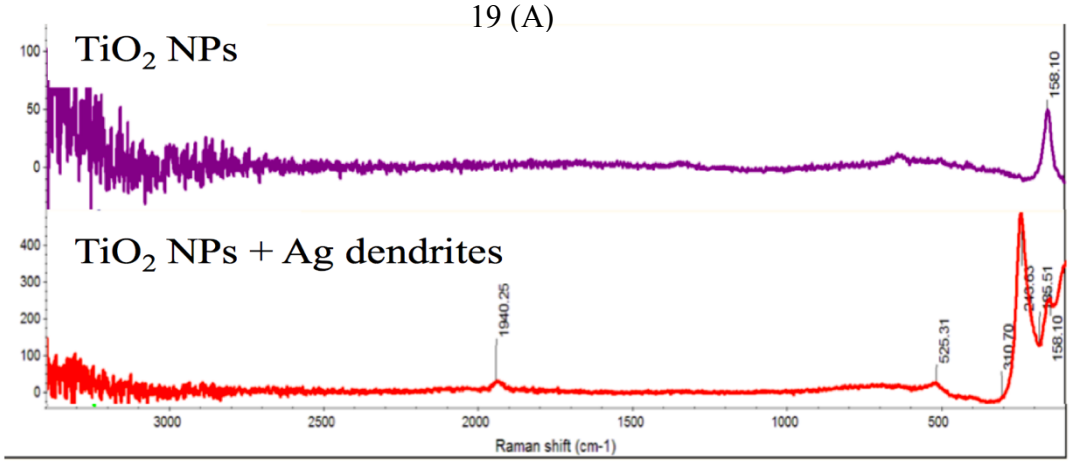
SERS and TiO_2 NPs-Mucin Interaction

Silver (Ag) Dendrites and the Enhancement of TiO_2 NPs & Mucin Spectra

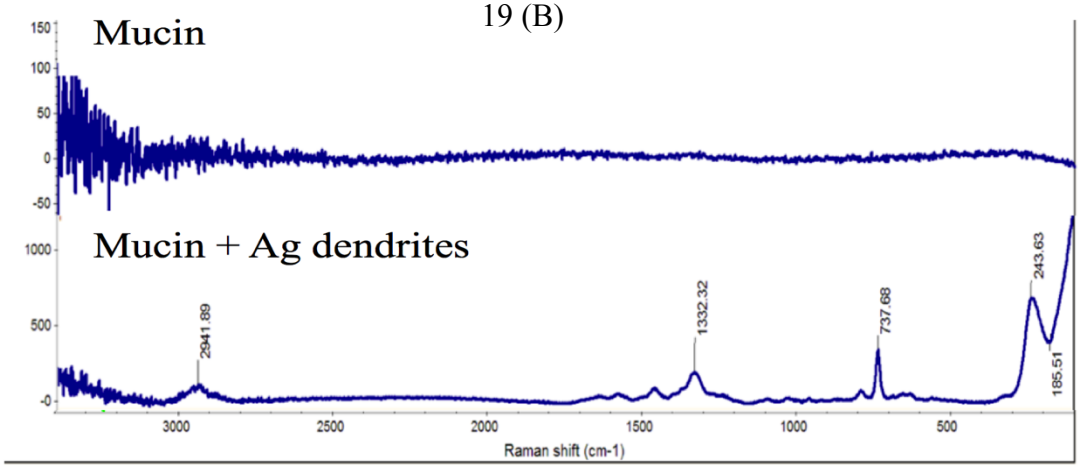
Regarding SERS enhancement mechanisms, NPs can be classified into three groups such as NPs which support electromagnetic enhancement, chemical enhancement, and neither electromagnetic nor chemical enhancement [277]. TiO_2 NPs are included in the second group, where SERS enhancement is attributed with the charge transfer (CT) between NPs and adsorbed biomolecule ligands [278]. Silver (Ag) dendrites show a high consistency and satisfactory performance with an analytical enhancement factor $\sim 10^4$ [257].



19 (A)



19 (B)



19 (C)

Figure 19: The spectral peaks of TiO2 NPs, mucin and Ag dendrites (background) (A); Ag dendrites enhance the spectral peaks of TiO2 NPs (B) and mucin (C).

The specific spectrum of every chemical substance used in the experiment is essential to be known, particularly the background (Ag dendrite). The background-result comparison will provide an idea about what happens during the experiments. TiO_2 NPs showed the spectral peak at wave number 158.51 cm^{-1}; while mucin did not exhibit a significant spectral peak. Ag dendrites represented the specific peak at wave number 243.63 cm^{-1} (**Fig.** 19 (A)).

The spectral peaks of mucin which interacted with Ag dendrite, as substrate, was significantly enhanced (**Fig**. 19 (C)). The spectral peak of TiO_2 NPs (at wave number 158.51 cm^{-1}) was also enhanced by Ag dendrites, approximately 4 times higher than TiO_2 NPs without Ag dendrites (**Fig**. 19 (B)). Several main peaks of mucin were depicted clearly when mucin interacted with Ag dendrites such as at wave number ±737.68 cm^{-1}, ±1332.32 cm^{-1}, and ±2941.89 cm^{-1} (**Fig.** 19 (C)). Assignments of Raman bands (at wave number ±737.68 cm^{-1}, ±1332.32 cm^{-1}, and ±2941.89 cm^{-1}) might be close associated with N-acetyl-D-glucosamine (GlcNAc) and D-(+)-galactosamine (GalNAc) [279-281]. GalNAc and Glc NAc are a type of amino-sugar which is secreted by mucin producing-epithelial cell in the GI tract [282]. Studies have characterized and analyzed spectral feature of biomolecules including mucin, generally, at wave number 0 to 2000 cm^{-1} [279-281, 283-285].

The main aim of SERS experiments was to determine the development of hetero aggregation by characterizing the interaction between TiO_2 NPs and mucin. This study preferred using the spectral peak of mucin at wave number ±2941.89 cm^{-1} than using the spectral peaks at wavenumber ±0-2000 cm^{-1} for two reasons. First, GlcNAc and GalNAc generally showed a higher spectrum at a wave number ±2941.89cm^{-1} than at wave

number ±1332.32 cm^{-1} or ±737.68 cm^{-1} [281]. In other words, using a higher spectral peak to determine hetero aggregation between TiO_2 NPs and mucin was more practical or efficient than the lower ones. Second, based on this study, the spectral peak of mucin at wave number ±2941.89 cm^{-1} was not only more efficient but also more consistent when TiO_2 NPs interacted with mucin in various pH and buffer (Fig. 21 (A) and 33 (A)). Therefore, the preference of this study went to the spectral peak at wave number ±2941.89 cm^{-1}.

A recent study characterized the interaction between TiO_2 NPs and mucin using SERS [285]. Their preference was to analyze the TiO_2 NPs-mucin interaction at wave number ±0-2000 cm^{-1}. The spectral peaks were characterized in their study were similar to this study with a little bit different. It is normal due to TiO_2 NPs used in the experiments might be different such as the type and size of TiO_2 NPs. They preferred using the solid TiO_2 NPs (powder) for the original sample. Meanwhile, this experiment preferred to use the liquid ones with the original size range between 5-15 nm. They only used SSF, while this experiment used DIW and PBS as a buffer.

Fig. 20 depicted two sets of experiments to examine the interaction between Ag dendrites and TiO_2 NPs or mucin: 1. (TiO_2 NPs + Ag dendrites) + mucin, 2. (mucin + Ag dendrites) + TiO_2 NPs. The results showed that these two experiments had similar results (**Fig.** 20 (A; III) & **Fig**. 20 (B; III)). The specific spectral peaks TiO_2 NPs and mucin were persistently appeared and enhanced (**Fig.** 20 (A; III) & **Fig**. 20 (B; III)); it means that Ag dendrites are a good substrate for both TiO_2 NPs and mucin. Also, the principal component analysis (PCA) of mucin and TiO_2 NPs represented a wider area when they interacted with Ag dendrites. The PC score of mucin was magnified many times

compared to mucin alone (**Fig**. 20 (C; O) & (D; O)). These findings confirmed the previous study that Ag dendrites are a good substrate [257].

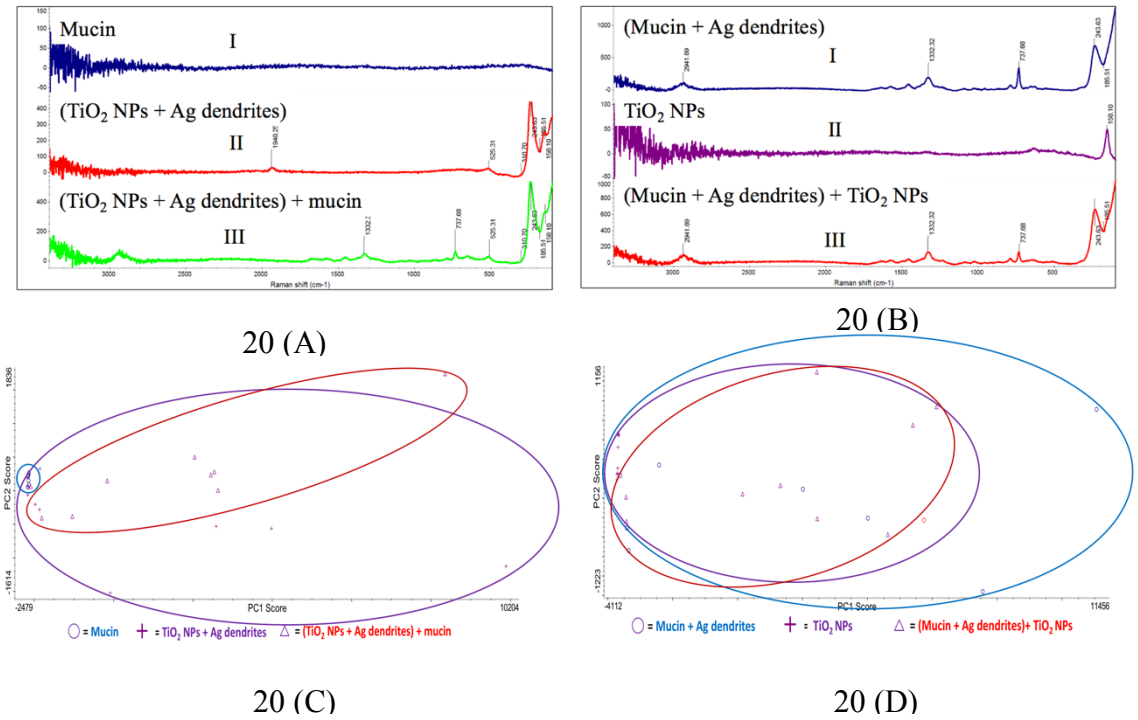
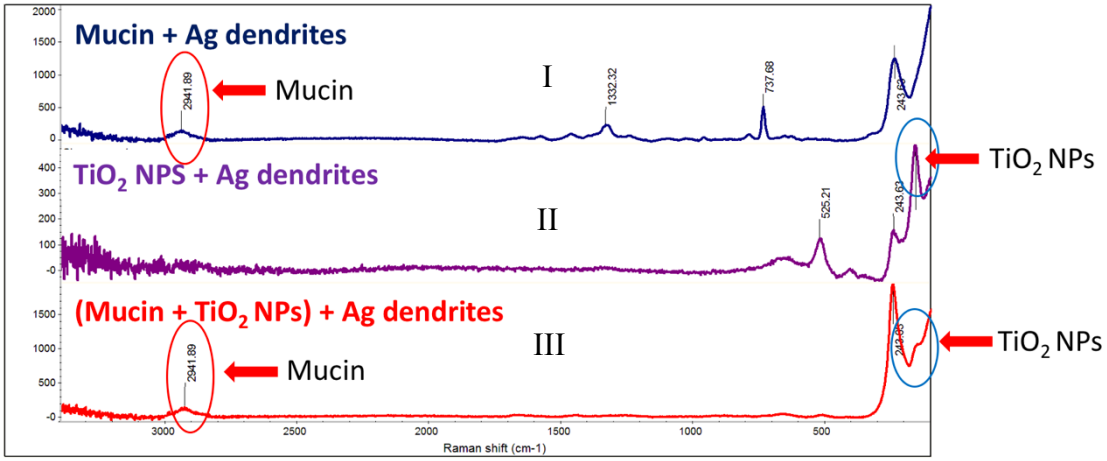


Figure 20: (A) The spectral peaks of mucin (I), TiO2 NPs + Ag dendrites (II), and (TiO2 NPs + Ag dendrites) + mucin (III); (B) The spectral peaks of mucin + Ag dendrites (I), TiO2 NPs (II), (mucin + Ag dendrites) + TiO2 NPs (III); (C) PCA of mucin (O), TiO2 NPs + Ag dendrite (+), and (TiO2 NPs + Ag dendrites) + mucin (Δ); (D) PCA of mucin + Ag dendrites (O), TiO2 NPs (+), (mucin + Ag dendrites) + TiO2 NPs (Δ).

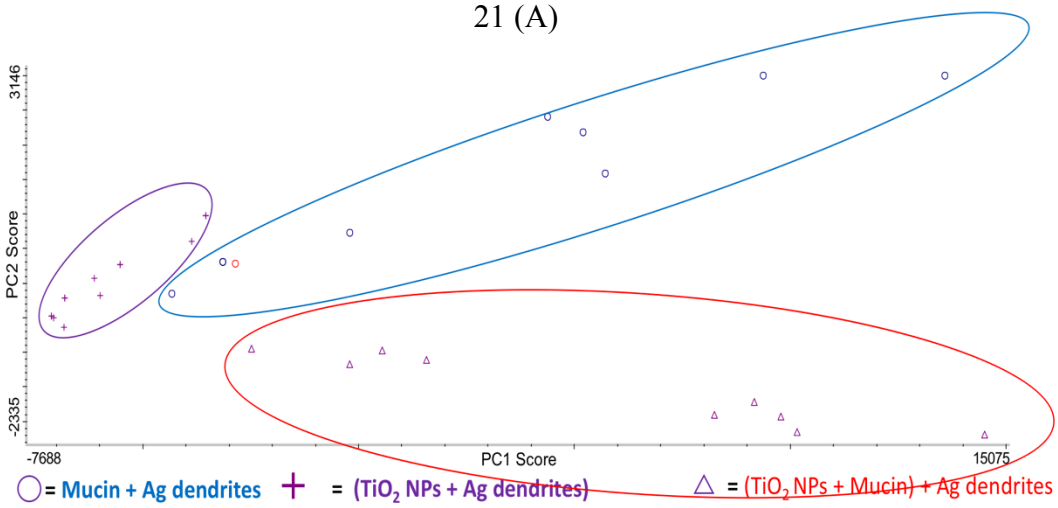
Characterizations of TiO_2 NPs-Mucin Interaction

The characterization of TiO_2 NPs-mucin interaction had some steps: First, mixing TiO_2 NPs with mucin, then adding Ag dendrites ((TiO_2 NPs + mucin) +Ag dendrites). The results exhibited that the spectral peaks of both TiO_2 NPs and mucin appeared (**Fig.** 21 (A; III). It indicates that the interaction between TiO_2 NPs and mucin occurs. Second, Analazing of PC score. The results depicted three distinctive clusters: TiO_2 NPs cluster, mucin cluster, and BMC cluster. It is interesting to note that the TiO_2 NPs-mucin

interaction may alter the synthetic identity of TiO_2 NPs and the biological identity of mucin.



21 (A)



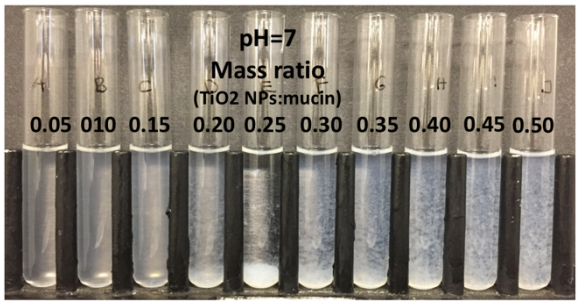
21 (B)

Figure 21: (A) the spectral peaks of mucin, TiO2 NPs, and BMC; (B) PCA of mucin, TiO2 NPs, and BMC.

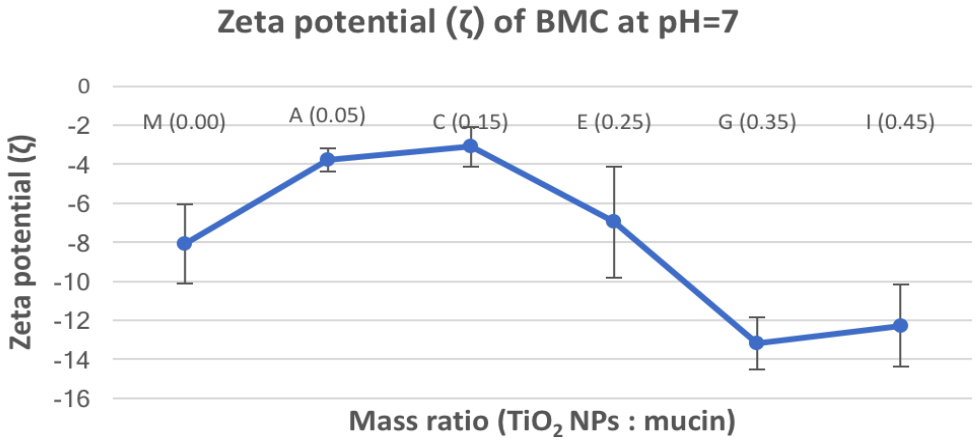
TiO_2 NPs-Mucin Interaction at Neutral Condition (pH=7)

This study also observed TiO_2 NPs-mucin interaction in neutral condition (pH= 7). The result showed that although the charge of both TiO_2 NP and mucin were negative (**Fig.** 22 (B)); aggregations were still observed (**Fig**. 22 (A)). Zeta potentials of BMC

with different concentrations of TiO_2 NPs presented negative charge, although the charge is relatively low (-2 to -15). In theory, aggregations are supposed to be occurred where molecules/particles at pH_{pzc}, which is pH where the charge is near zero [67]. It suggests that in aquatic environment, hydrophobic interactions may play significant role in the interaction between TiO_2 NPs and mucin. Therefore, the use of Nano-isothermal titration calorimetry (nITC) coupled with other methods (such as fluorescence spectroscopy and circular dichroism) to get detailed information about the interaction types, reaction products, alterations on TiO_2 NPs surface, and the possible consequences of TiO_2 NPs-mucin interaction on aquatic environment may be required.



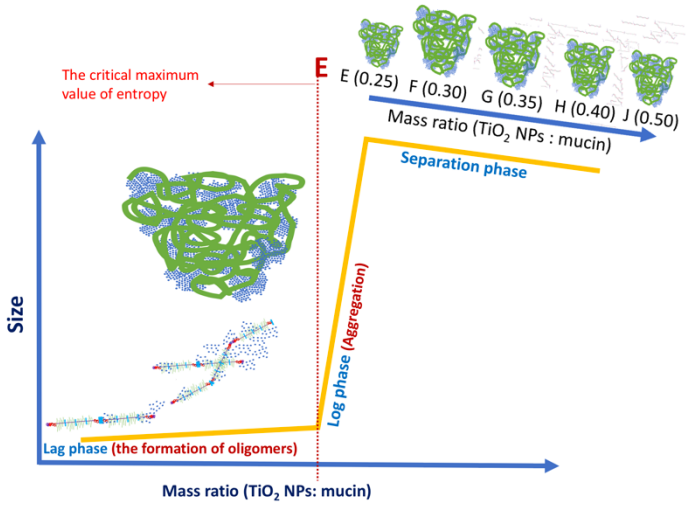
22 (A)



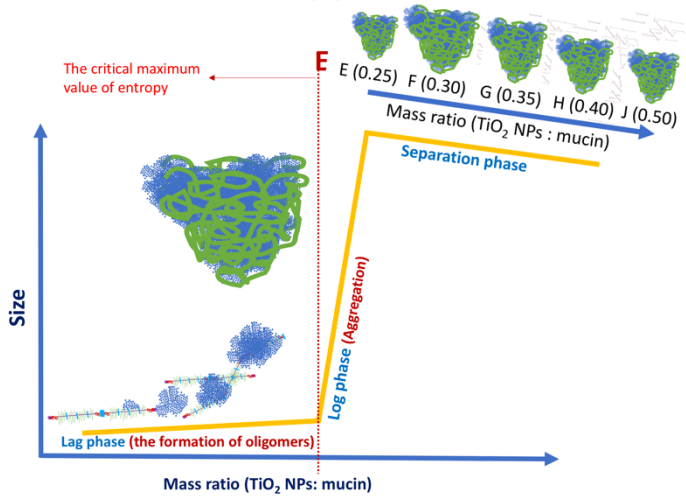
22 (B)

Figure 22: (A) Aggregations still occur; although TiO2 NPs & mucin have negatively charge; (B) Zeta potential of BMC at pH=7.

In summary, the mechanistic interactions between TiO_2 NPs and mucin in water can be illustrated by **Fig**. 23. Initially, mucin oligomers might be developed; the more addition of TiO_2 NPs in water induced the more adsorption of mucin on the surface of TiO_2 NPs via various types of interaction; small aggregates were developed unless the mass ratio (TiO_2 NPs: mucin) reach 0.25. Since this point, the massive hetero aggregation was developed. Eventually, the aggregation was followed by separation phase and resulted irreversible and insoluble aggregates.



23 (A)



23 (B)

Figure 23: Illustrations of TiO2 NPs-mucin interaction at pH= 4 (A) & pH= 7 (B).

Conclusions

In conclusion, the physicochemical alteration of TiO_2 NPs is affected significantly by pH. In acidic condition, TiO_2 NPs are conferred by positive charge. They become opaquer and have negative charge when the pH condition is neutral to basic. While the physicochemical properties of mucin are not pH-dependence. At acidic condition (pH= 4), the interaction of TiO_2 NPs with mucin forms BMC. The amount of TiO_2 NPs exposed to mucin may play important role in the BMC formation. As the mass ratio of TiO_2 NPs to mucin has reached 0.25, it leads to the irreversible hetero aggregation, which are possibly caused by the polyvalent binding. This is evidenced by the augmentation of the size, the increase of turbidity, the hypochromic effect and red shift of the UV-Vis spectra, and the exothermic energy during TiO_2 NPs-mucin interactions. The interaction between TiO_2 NPs and mucin has been identified using SERS. The distinctive cluster of SERS spectra between TiO_2 NPs, mucin, and BMC, reveals that the exposure of TiO_2 NPs to mucin may transform TiO_2 NPs become new species with different bio-fate. It is interesting to note that although both TiO_2 NPs and mucin have negatively charge at a neutral condition (pH=7), SERS has exhibited that the interaction between TiO_2 NPs and mucin still occurs. Hence, it can be noticed that in the aquatic environment, the hydrophobic interaction may play more significant role in the formation of BMC. Therefore, further studies to observe about the interaction types, reaction products, and the possible consequences of TiO_2 NPs-mucin interaction on aquatic environment are required.

CHAPTER IV

4. INTERACTION BETWEEN TiO_2 NPs AND MUCIN: EFFECTS OF PHOSPHATE-BUFFERED SALINE (PBS) AND pH ON THE ALTERALITON OF SURFACE CHANGE, THE FORMATION OF BIOMOLECULAR CORONA (BMC) AND HETERO AGGREGATION

Introduction

Since 1966, the US FDA has approved the use of TiO_2 NPs in food up to 1% [286]. TiO_2 NPs have various forms such as rutile, brookite, and anatase [287-288]. Although anatase is more toxic [4] due to the higher photocatalytic activity than the others [287, 289], it has a higher industrial application. The massive application of TiO_2 NPs in food industries rises disputes regarding their safety. The International Agency for Research on Cancer (IARC) has categorized TiO_2 NPs as a pigment are probably carcinogenic to human (group 2B) according to mechanisms and animal experiments (particularly exposure by inhalation) [60, 290-291]. Based on the animal experiment data, the safety margin of TiO_2 NPs was 2.25 mg TiO_2 NPs/kg bw/day [292]. In 2016, the European Food Safety Authority (EFSA) reported that exposures of TiO_2 NPs (E171) to humans did not emerge concerns [292]; however, the admissible daily intake could not be determined owing to the insufficient research data.

Studies have examined oral intake of TiO_2 NPs and indicate tissue accumulations and toxic effects [156, 293-294]. The accumulation and toxic effect of TiO_2 NPs in tissue are still debatable [295]. Exposures of TiO_2 NPs at higher age resulted in a significant accumulation [296]; but, studies reported that a child is potentially to have higher accumulation in tissue. The estimation of TiO_2 consumption in a child is 2-4 times more than an adult person (TiO_2 per kg of body weight (bw) per day) because of

the higher child prefers to sweet foods (e.g. chocolate, chewing gum, and candy) containing approximately 2.5 mg TiO_2/g of food [4, 297-298]. Besides, disputes associated with TiO_2 NPs accumulation in tissue are caused by the fact that their increase in organ levels was not always detected [44] due to very low absorptions and dose-dependent. Although there were low absorptions, they could be approved by visual detection in organs [49].

The absorption of TiO_2 NPs is considered at low levels, but it may be toxic enough in cells. Experiments conducted by Degabriel (2015) exhibited that bare TiO_2 NPs generated more free radicals (hydrogen peroxide (H_2O_2)) in phosphate-buffered saline (PBS) than in pure water [45]. As PBS is well known as a cell buffer, it indicates that bare TiO_2 NPs in the cell is toxic. However, studies have shown that TiO_2 NPs contained in digested food models (1.5 % w/w) did not perturb cellular proteome significantly because of their interaction with food matrix [46] and mucus [47]. Mucus is a hydrogel, consisting of water, mucin (glycoproteins), DNA, proteins, lipids, and cell debris [243]. While mucin is densely O-glycosylated proteins which coat a wide variety of wet epithelial cell [38], exclude foreign or hazardous molecules, and permit the useful ones such as nutrients [36, 48]. It can be noticed that the interaction between TiO_2 NPs and mucin may play a significant role in cell toxicity.

The interaction between TiO_2 NPs and mucin occurs in the human gastrointestinal (GI) tract at various pH; hence, the physicochemical property and toxicity of TiO_2 NPs may be altered due to pH. Recent studies have shown that TiO_2 NPs were poorly dissolved in the model gastric and intestinal environment [44]. TiO_2 NPs exposed with juices mimicking the gastric and intestinal compartment (pH=2 & 7) tend to alter their

size and surface charge due to agglomeration and protein adsorptions on their surface [50-51]. But, limited studies have examined the basic interaction between TiO_2 NPs and mucin at different pH and its effect on physicochemical alterations. Therefore, this study observed the basic interaction between TiO_2 NPs and mucin using deionized water (DIW) as a buffer. It can be hypothesized that pH may alter TiO_2 NPs-mucin interactions, and may change the physicochemical properties of TiO_2 NPs (particularly the surface charge), and thereby alter their bio-fate.

Subsequently, BMC which was formed by TiO_2 NPs-mucin interaction in the GI tract may penetrate the cell membrane [52], then can be exposed by a cell buffer. Studies associated with the penetration of cell barrier by TiO_2 NPs have been conducted; but, the condition was not very representative of the real gut environment, particularly they did not consider the presence of mucin [47]. *In vitro* studies on Caco-2 intestinal cells [53-56] and TR146 buccal cells [57] have shown that the translocation of NPs through the epithelial barrier occurs and they stay in the cell. However, no studies have reported the interaction between TiO_2 NPs and mucin in the cell. Therefore, the main purpose of this study is to know the impact of cell buffer (PBS) on TiO_2 NPs-mucin interaction, focusing on the change of surface charge at different pH. It was hypothesized that PBS and pH may affect the alteration of surface charge, the formation of BMC and aggregates.

Materials and methods

Materials

TiO_2 NPs (anastase; 5-15 nm) were purchased from US Research Nanomaterials (TX, US) and mucin was purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The following products were purchased from Life Technologies (Thermo Fisher Scientific, Agawam, MA, USA): hydrochloric acid, sodium hydroxide, sodium chloride, calcium chloride, dimethyl sulfoxide. Silver (Ag) dendrites were prepared based on (He, Lin, Li, & Kim, 2010). This study used deionized water (DIW) and PBS as a buffer.

Methods

Initially, physicochemical properties of TiO_2 NPs and mucus in the function of pH were monitored—particularly by observing the alteration of appearance and turbidity (aggregation/size) and by investigating the change of zeta potential (surface charge). These alterations may lead to a specific interaction (e.g. electrostatic interaction), cause the formation of biomolecular corona (BMC) and aggregation, and thereby induce bio-fate alterations of TiO_2 NPs. These phenomena were measured through: ***first***, observing the change of BMC appearance due to the interaction between TiO_2 NPs and mucin at different pH, and examining the surface charge of BMC in DIW a dynamic light scattering (DLS); ***second***, comparing the appearance of TiO_2 NPs, mucin, and BMC in different buffers (DIW and PBS) and pH, then examining their zeta potential (ζ) using DLS; ***third***, measuring the change of turbidity (O.D. 600); ***fourth***, characterizing TiO_2 NPs-mucin interactions to examine the development of hetero aggregation by employing

surface-enhanced Raman spectroscopy (SERS). To know the effect of pH to the basic interaction between TiO_2 NPs and mucin, experiments used DIW as a buffer. Whereas, PBS was used when experiments observed TiO_2 NPs-mucin interactions in the cell.

Alterations of BMC Appearance & the Basic Experiment (BE)

0.15 % (v/v) of TiO2 NPs were dispersed in DIW & PBS. 5 mg/ml mucin was stirred into DIW & PBS overnight and then centrifuged. Before experiments were conducted, the pH of TiO2 NPs and mucin should have been adjusted. The change of BMC appearance was observed every time a certain amount of TiO2 NPs was added. The experiment was started by observing the appearance of control (mucin) by adding 0.5 mL mucin (5mg/mL) into a glass reaction tube and add 4.5 mL DIW. While the treated groups, the amount of TiO2 NPs (0.1%) was increased 0.25 mL and the amount of DIW was reduced simultaneously; therefore, the total volume of the sample was 5 mL. Vortex was applied for each tube (10 s), and eventually, the alteration of appearance was observed, particularly to identify the possibility of aggregation during TiO2 NPs-mucin reaction. This experiment was the primary experiment (BE) and should be understood before doing other experiments.

Surface charge Characterization

The surface charge was measured by Zetasizer nano ZS series, Malvern Instruments Ltd, Worcesterhire, UK.

UV-Vis Spectrophotometry

TiO_2 NPs and mucin were mixed and prepared as the same to BE. The absorption spectrum was determined using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Surfaced-Enhanced Raman Spectroscopy (SERS)

Mucin and TiO_2 NPs were dispersed and stirred in buffer overnight. Three different categories of solution (mucin, TiO_2 NPs, and the mixture of TiO_2 NPs and mucin with the mass ratio= 0.25) were prepared. These solutions were homogenized for 10 seconds and added Ag dendrites. These mixtures were homogenized for 10 seconds and sedimented. These sediments were centrifuged and rinsed with buffer three times. Each sediment (2 μL) was deposited on a glass slide covered with aluminum foil as a sample. The sample was air-dried before observing it under the Raman laser. A DXR Raman Microscope (Thermo Scientific, Madison, WI) equipped with a 785 nm-excitation laser and a 50x objective was employed. Spectra were collected with a 5.0 mW laser power and a 50 μm slit aperture for 2 seconds scanning time. All SERS experiments were done minimally twice. There were 7-9 spots per sample were characterized by SERS. The elicited spectra were analyzed using TQ analyst software, version 8.0 (Thermo Fisher Scientific).

Statistical Analysis

All experiments were conducted at least twice and reported as the results of means and deviations.

Results and Discussions

Basic Interaction between TiO_2 NPs & Mucin in Various pH

Studies to examine the adsorption of protein on the surface of NPs in the (GI) tract have been conducted [44, 51] in the liquid mimicking the gastric and intestinal juice (pepsin in HCl, pH=1.2 & 1.5 for gastric environment; and trypsin, KH_2PO_4 in NaOH, pH= 6.8 & 7.4 for the intestinal environment). However, the basic interaction between TiO_2 NPs and mucin which forms BMC is not well known. Therefore, the aim of this experiment was to know the basic interaction between TiO_2 NPs and mucin using DIW as a buffer, particularly the alteration of the surface charge in various pH.

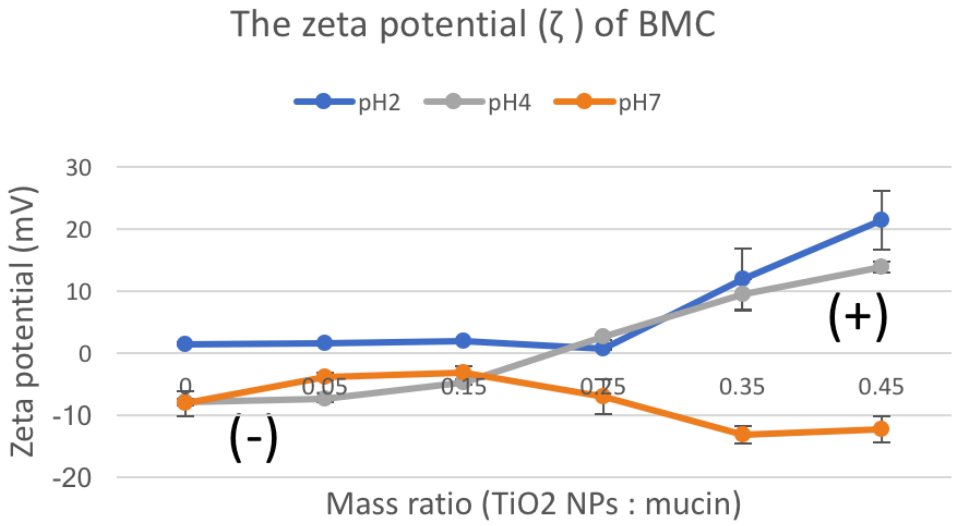
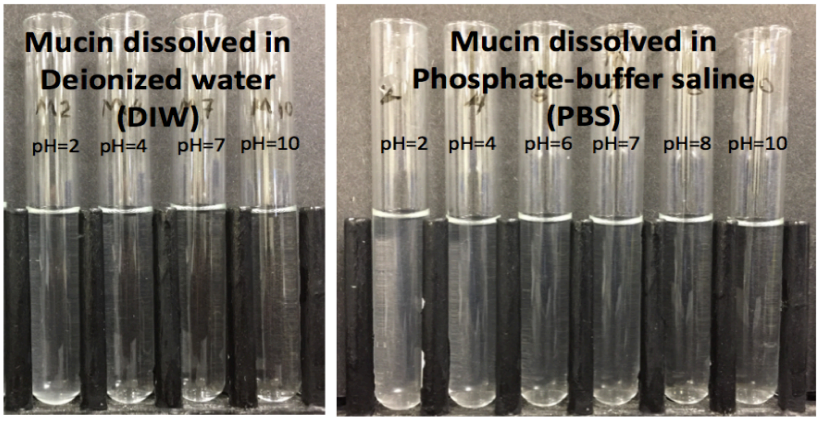


Figure 24: The surface charge of BMC in various pH.

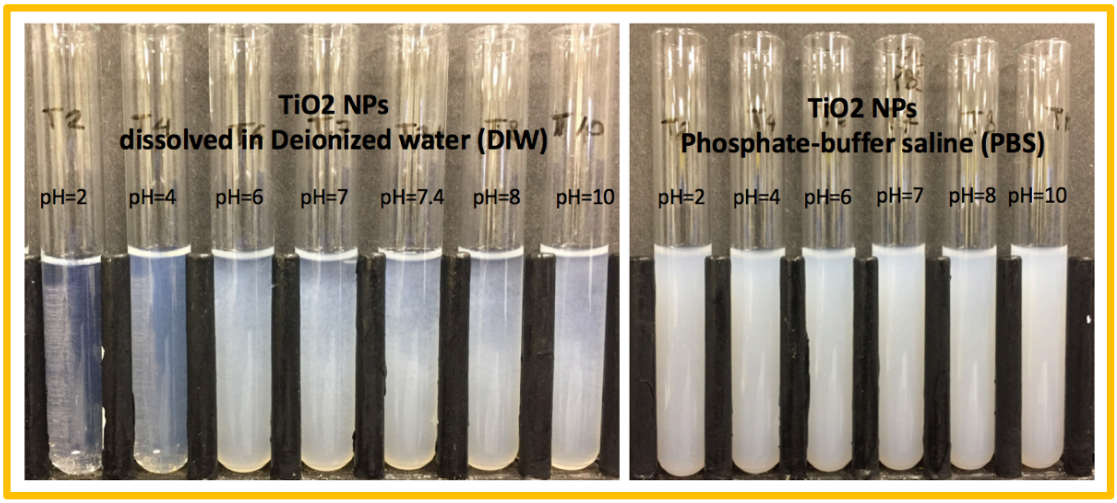
Results showed that the surface charge of BMC was various depending on pH. In acidic conditions (pH=2 & 4) the charge was positive when the mass ratio (TiO_2 NPs: mucin) reached 0.25. Meanwhile, in the neutral and alkaline conditions, the surface charge of BMC was always negative (**Fig.** 24). In theory, cationic NPs features rapid uptake by cells due to their capability to have spontaneous translocation [52]. So, it can be noticed that cationic BMC may have the same capability—the direct translocation crossing the membrane cell. This study is very basic. Therefore, studies to examine TiO_2 NPs-mucin interactions to measure the direct translocation of cationic BMC on membrane cells, using liquids mimicking the GI tract environment, are required.

TiO_2 NPs & mucin in different buffer and pH

Buffers have a significant effect on the appearance of both TiO_2 NPs and mucin. Mucin seemed very stable, the appearance was always clear, in different pH and buffers; while TiO_2 NPs exhibited, various appearances depending on pH and buffers. In DIW, the appearance of TiO_2 NPs had a gradation, the increase of pH resulted in the opaquer the appearance. Meanwhile, the appearance of TiO_2 NPs was almost the same in PBS—they were all opaque.



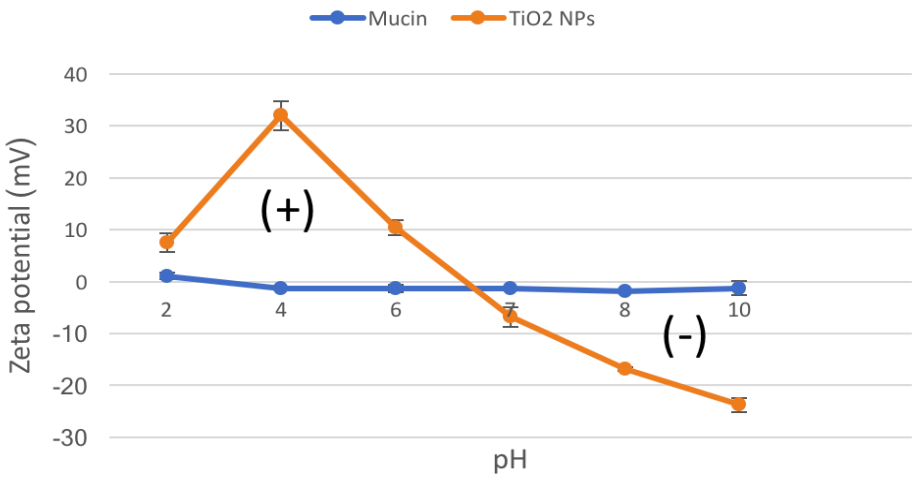
25 (A)



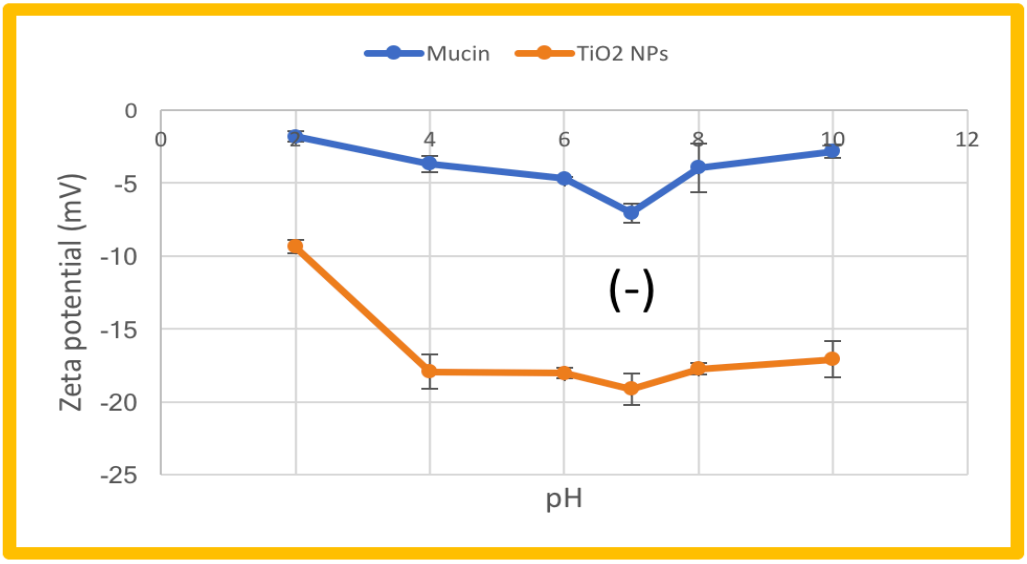
25 (B)

Figure 25: Appearance of mucin (A) and TiO2 NPs (B) in deionized water and PBS at different pH. Mucin is stable at different buffers and pH. There is a gradient of appearance when TiO2 NPs are dispersed in DIW. They tend to be opaquer with the increase of pH; while the appearance of TiO2 NPs in PBS is all the same (opaque).

Based on **Fig.** 25, it was interesting to characterizing the surface charge of TiO_2 NPs and mucin in DIW and PBS in various pH; therefore, the electrostatic interaction could be determined. If TiO_2 NPs and mucin have a different surface charge, the electrostatic attraction may occur; however, if both of them have the same charge, the electrostatic repulsion may happen.



26 (A)



26 (B)

Figure 26: Surface charge of mucin and TiO2 NPs in DIW (A) and PBS (B).

The surface charge of mucin both in DIW and PBS were stable and slightly negative; while the surface charge of TiO_2 NPs was, variable influenced by buffer and pH. In DIW and the acidic condition, TiO_2 NPs were positive charge; while in the neutral to basic condition, TiO_2 NPs were negative charge. In PBS, albeit the surface charge of

TiO_2 NPs was always negative (**Fig.** 26 (B)), the precipitation of TiO_2 NPs occurred (**Fig.** 25 (B)). It might be due to hydrophobic interaction or other interactions; thereby, studies to know the types of TiO_2 NPs-TiO_2 NPs interactions are needed.

The formation of Biomolecular Corona BMC

In PBS, all BMC exhibited negative charge. At pH= 7, the surface charge was higher than at pH= 2 and 4 (**Fig**. 27). Whereas, the surface charge of BMC in DIW buffer can be positive and negative depending on the mass ratio (TiO_2 NPs: mucin) and pH (**Fig.** 24). The higher amount of TiO_2 NPs led to becoming more positive charge of BMC. There was a notable fact, that the charge of all BMC was relatively neutral at mass ratio (TiO_2 NPs: mucin) = 0.25, then BMC was becoming either more positive or negative along with the increase of mass ratio (TiO_2 NPs: mucin), depending on the buffer. Thereby, it was concluded that the mass ratio 0.25 is the critical point in terms of defining the charge of BMC.

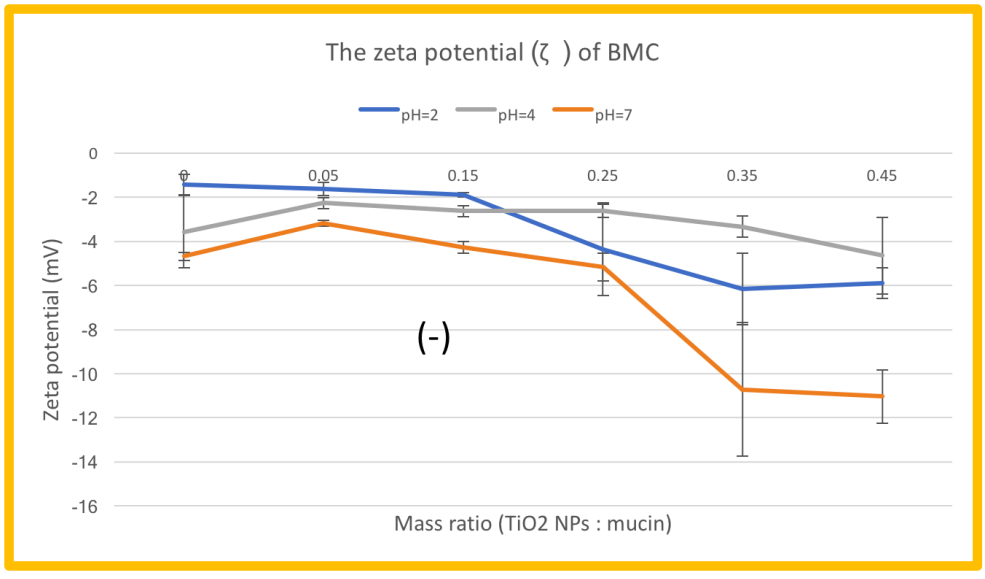
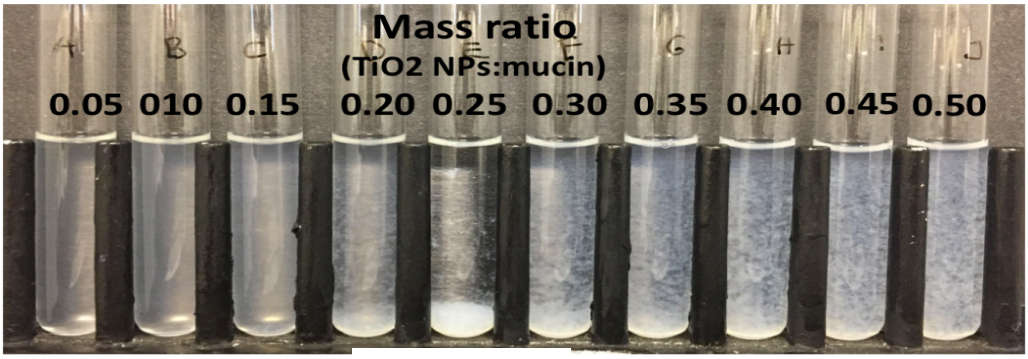
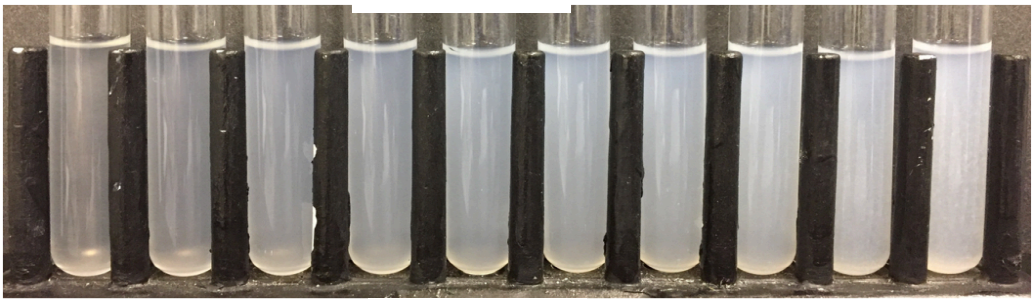


Figure 27: Zeta potentials of BMC in PBS at pH= 2, 4, and 7.

There was another interesting finding correlated with the appearance of BMC in DIW and PBS. Using the same concentration of TiO_2 NPs (low concentration), **Fig.** 28 showed that there was no precipitation appeared in PBS, while the massive precipitation occurred in DIW. No precipitation in PBS was likely caused by either the stable homo aggregation or the stable hetero aggregation [34]. However, once the concentration of TiO_2 NPs was increased significantly (approximately three times than the initial dose), the aggregation in PBS was depicted (**Fig**. 28). That was also confirmed by the turbidity data. The more amount of TiO_2 NPs added in the experiment generated the higher turbidity (**Fig.** 29 B).

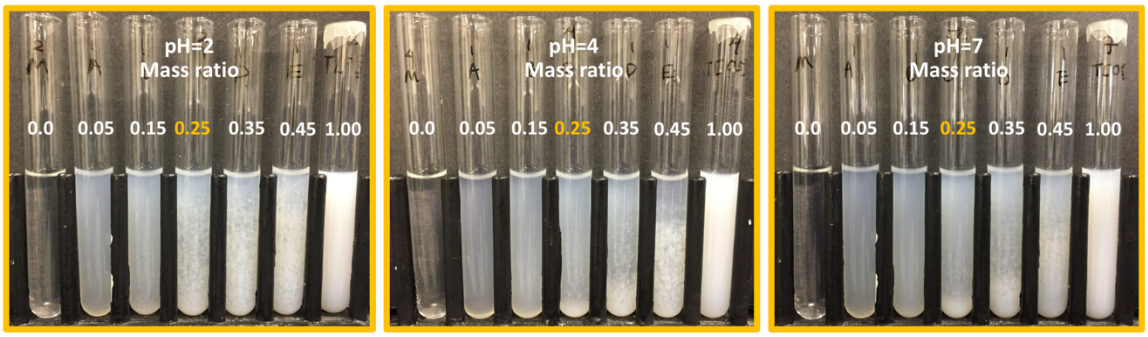


28 (A)

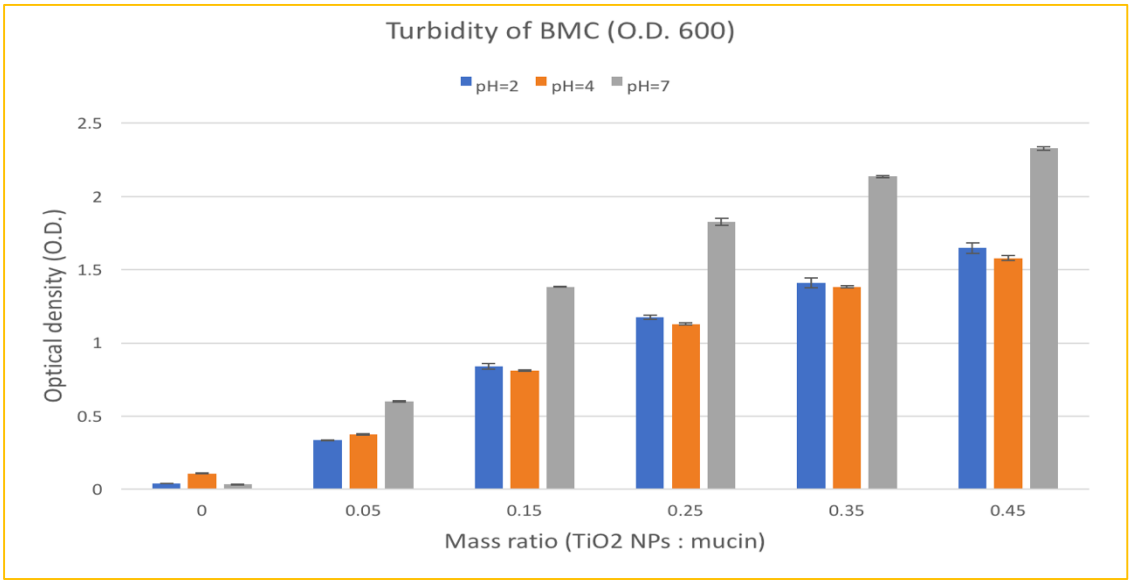


28 (B)

Figure 28: Interaction between TiO2 NPs and mucin in DIW (A) and PBS (B).



29 (A)



29 (B)

Figure 29: (A) The formation of BMC at different mass ratio (TiO2 NPs: mucin) and at pH=2, 4, and 7. (B) Turbidity (O.D. 600). Turbidity at pH=7 is significantly different from turbidity at pH=2 & 4.

Albeit the accumulation of TiO_2 NPs is not always detected in organs due to low absorptions and concentration-dependent [44], it can be visualized [49]. It could be caused by the insufficient concentration of TiO_2 NPs; therefore, unstable hetero aggregation could not be developed. It can be extrapolated that PBS buffer may require a higher concentration of TiO_2 NPs to develop unstable aggregate compared than DIW.

This experiment was still preliminary; further studies, particularly to know the interaction between PBS, TiO_2 NPs and mucin, are required.

SERS and TiO_2 NPs-Mucin Interactions: Homo/Hetero Aggregation

The interaction between TiO_2 NPs and mucin led to developing the unstable hetero aggregation (**Fig**. 28 (A) & 29 (A)). SERS was employed to determine the hetero aggregation, by characterizing the TiO_2 NPs-mucin interaction. This experiment had two steps: first, to know the impact of Ag dendrites on the spectral peaks of TiO_2 NPs and mucin; second, to characterize the interaction between TiO_2 NPs and mucin.

Silver (Ag) Dendrites and the Spectrum Enhancement of TiO_2 NPs & Mucin

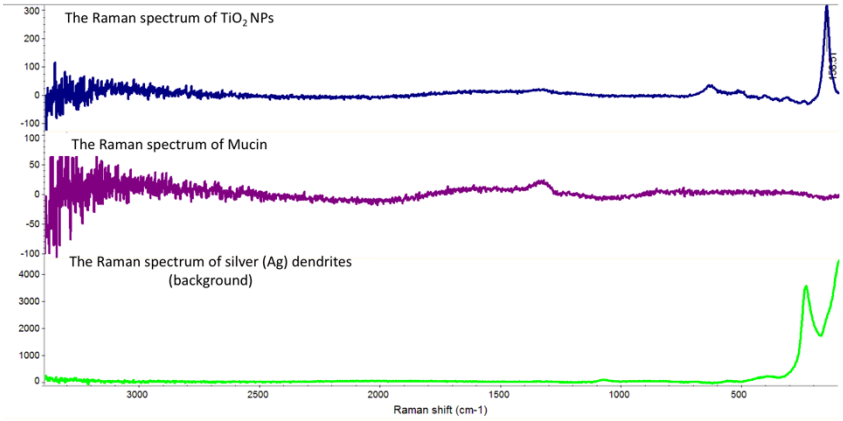
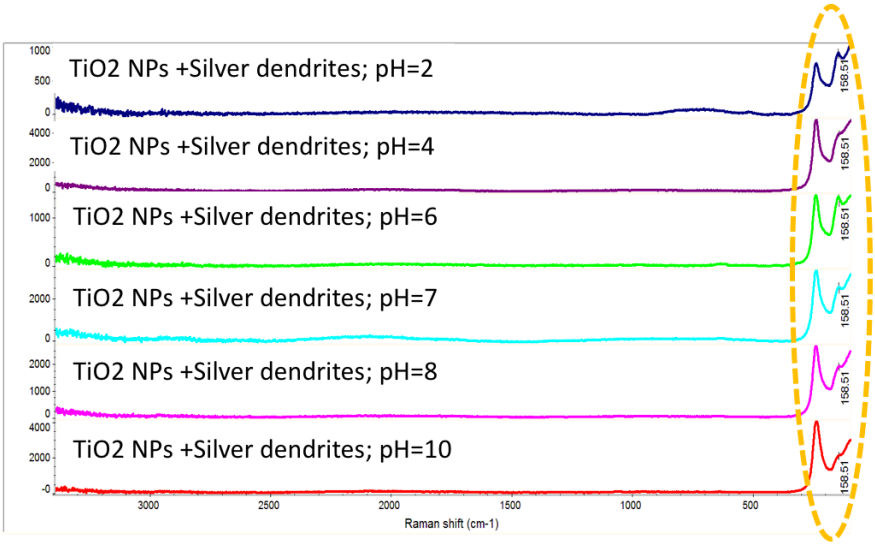


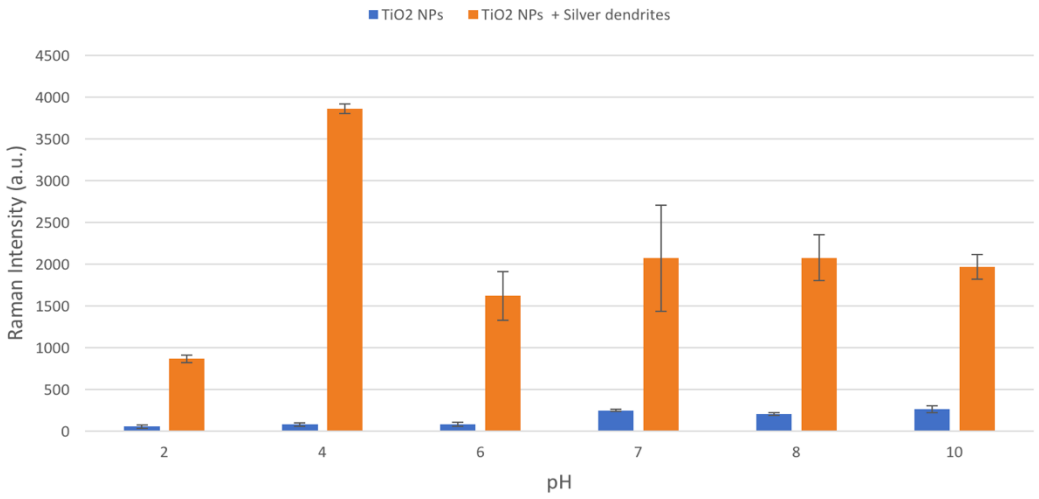
Figure 30: Spectral peaks of TiO2 NPs, mucin and Ag dendrites (background).

Knowing the original spectral peak of chemicals is pivotal. TiO_2 NPs exhibited a specific spectral peak at wave number 158.51 cm^{-1}; while mucin did not exhibit a significant spectral peak. Ag dendrites, as background, represented a spectral peak at wave number 243.63 cm^{-1} (**Fig.** 30). Ag dendrites are a good substrate [257] to enhance the spectral peak of TiO_2 NPs and mucin. There was an enormous spectral enhancement

when TiO_2 NPs interacted with Ag dendrites. TiO_2 NPs at pH=4 generated the highest spectral peak (>3700 a.u.), while at pH= 6 to 10 showed spectral peaks from >1300 to 1700 a.u., and at pH= 2 resulted in ±800 - 900 a.u. (**Fig.** 31 (A) & (B)).



31 (A)



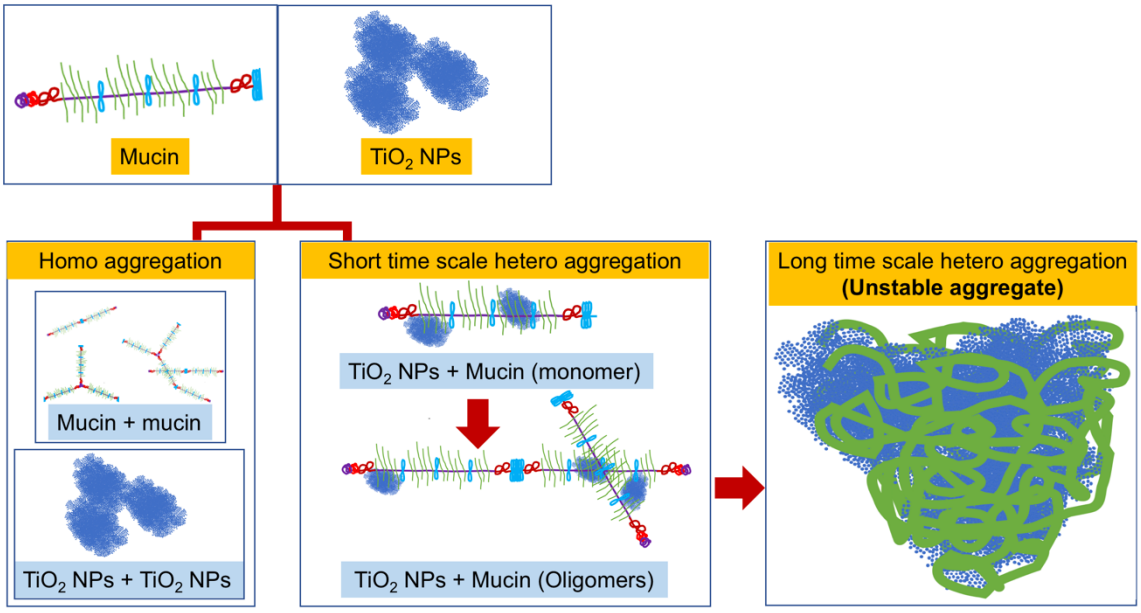
31 (B)

Figure 31: Spectral peaks of TiO2 NPs and the use of Ag dendrites as a substrate (A). The addition of Ag dendrites tremendously alleviates the spectral peak of TiO_2 NPs (B).

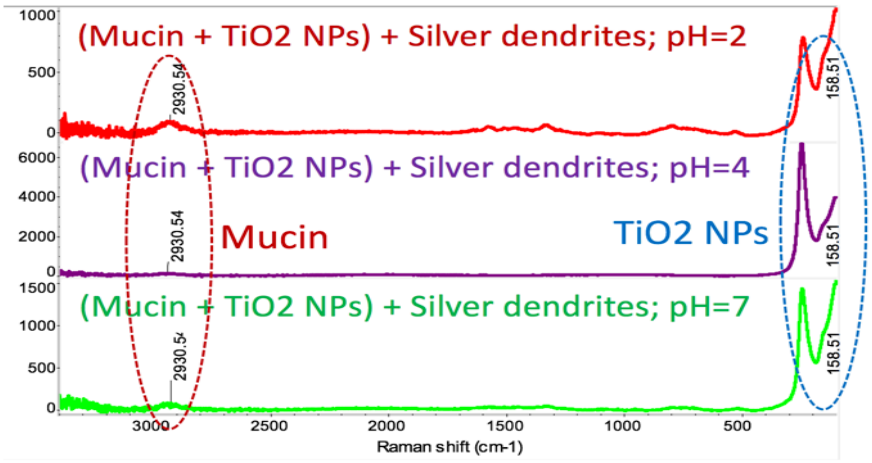
Characterizations of TiO_2 NPs-Mucin Interactions

Characterizing the interaction between TiO_2 NPs and mucin was important for determining the type of aggregations. **Fig.** 29 (A) showed that aggregates were shuttled down, but the type of aggregation was not unclear either unstable homo aggregation or hetero aggregations. Clavier, Praetorius, & Stoll, (2019) described that mono aggregation is interactions of some similar molecules/particles such as dimer and trimer. Whereas, hetero aggregations are divided into two different categories: stable hetero aggregation (which is formed by dimer/trimer aggregate) and unstable hetero aggregation (which is developed by large and complex aggregate) [34]. Based on the theory and **Fig.** 29, it can be concluded that TiO_2 NPs-mucin interactions are potentially to generate unstable hetero aggregations in PBS (**Fig.** 32).

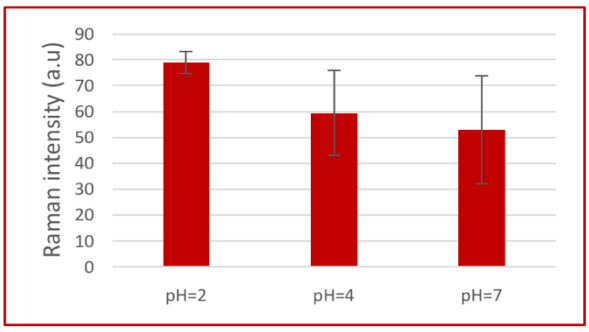
Figure 32: Illustrations of hetero aggregation between TiO2 NPs and mucin in PBS, which is inspired by Clavier, Praetorius, & Stoll, (2019).



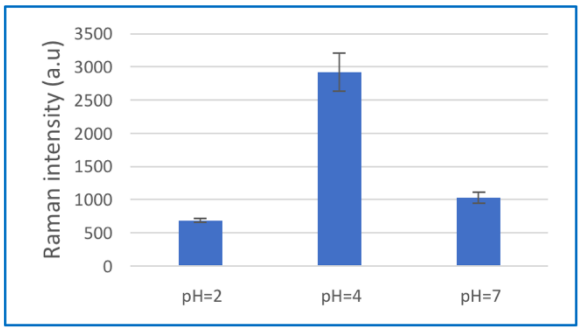
An interaction between TiO_2 NPs and mucin was found in various pH. The spectral peaks of TiO_2 NPs and mucin were appeared (**Fig**. 33 (A)). The spectral peak of TiO_2 NPs was 158.51 cm^{-1} and the spectral mucin was at wavenumber at ±2930.54 cm^{-1}. The use of mucin spectral peaks at ±2930.54 cm^{-1} have the same reason to previous experiment in DIW. The spectral peaks of mucin at various pH were relatively low, stable, and no significant difference (**Fig**. 33 (B)). However, pH influenced the spectral peak of TiO_2 NPs significantly. TiO_2 NPs had the highest spectral peak at pH=4 (**Fig**. 31). For the same reason, the principal component (PC) score of BMC at ph= 4 was the highest than pH=2 and 7 (**Fig**. 33 (C)).



33 (A)



33 (B)



33 (C)

Figure 33: Spectral peaks of BMC (TiO2 NPs-mucin interactions) at pH=2, 4 and 7 (A); Spectral peaks of mucin (B) and TiO2 NPs (C).

The principal component analysis (PCA) showed that clusters of BMC at pH=2, 4, and 7 were separated obviously (**Fig.** 36). These data may indicate that the change of pH generates a different species of BMC with new bio-fate; however, more studies are needed to prove that.

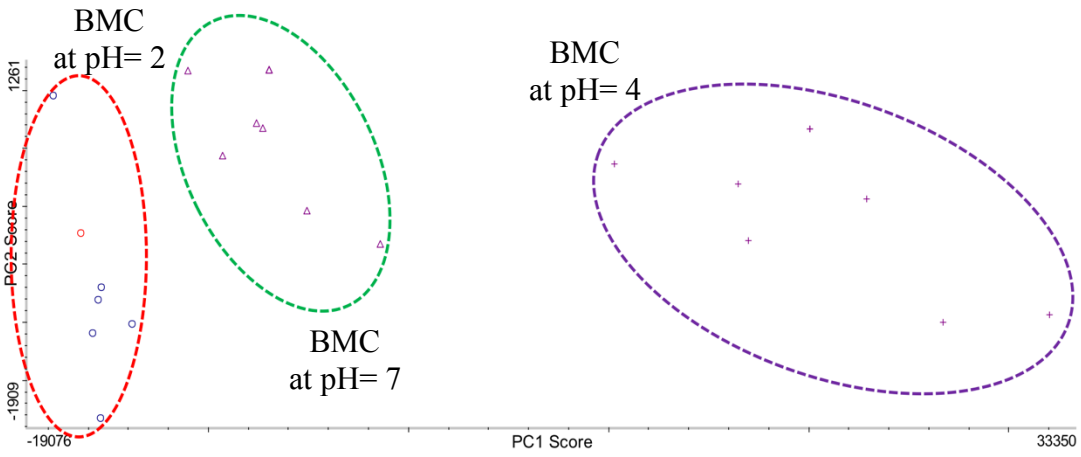


Figure 34: The principal component analysis (PCA) shows that clusters of BMC at pH= 2, 4, and 7 are separated.

Conclusions

In summary, TiO_2 NPs-mucin interactions can induce the developmet of BMC and hetero aggregation; however, the accumulation of TiO_2 NPs in cells is not always detected. PBS and pH play a pivotal role in the surface charge alteration, the formation of BMC, and hetero aggregation. Using a low concentration of TiO_2 NPs provides a different result in the formation of BMC in DIW and PBS. BMC formations in DIW tend to generate unstable aggregate due to large hetero aggregation, especially when the mass ratio (TiO_2 NPs: mucin) has reached 0.25; while in PBS, unstable aggregates are not formed. In other words, unstable aggregates are not developed in PBS when the

concentration of TiO_2 NPs is insufficient. The PCA of SERS spectra show that a different pH is possibly to develop a different species of BMC. It may be concluded that the formation of hetero aggregation in PBS is dose-dependent. The difficulty to detect the TiO_2 NPs accumulation in the cell may be caused by an insufficient concentration of TiO_2 NP. Studies to know the type of interactions, and the effect of different species BMC in cells are required.

CHAPTER V

5. CONCLUDING REMARKS

TiO_2 NPs are one of the most inorganic nanoparticles used worldwide. Estimation of the global production of TiO_2 NPs increases continuously until 2020. The potential adverse effect of TiO_2 NPs has raised ecological and health concerns. However, the fate, transformation, transport, and toxicity of TiO_2 NPs in a complex environment are not well known. Therefore, understanding the mechanistic interaction of TiO_2 NPs with mucin—well known as a host barrier from biological, physical, and chemical perils [36] —is necessary. The physicochemical properties (such as surface charge and size) may play a significant role in the TiO_2 NPs-mucin interaction.

The interaction between mucin and TiO_2 NPs occurred in both DIW and PBS. In DIW, the results exhibited that surface charges of TiO_2 NPs were changed from positive to negative with the increase of pH, while surface charges of mucin were not pH-dependent. The development of the strongest cationic TiO_2 NPs was at pH= 4. Meanwhile, pH values near the zero point of charge (pH_{pzc}) of TiO_2 NPs were between 6.0 to 7.4. Hence, TiO_2 NPs at pH= 4 was stable due to electrostatic repulsion, while TiO_2 NPs at pH_{pzc} between 6.0 to 7.4 was supposed to aggregate.

At pH=4, surface charges of BMC were changed from negative to positive along with the increase of TiO_2 NPs concentration. The interaction between TiO_2 NPs and mucin induced the formation of BMC, massive aggregations at mass ratio (TiO_2 NPs: mucin) = 0.25, and an increase in BMC size.

The interaction between TiO_2 NPs and mucin might develop polyvalent binding and result in exothermic energy at pH=4. Despite no electrostatic interaction, hydrophobic interaction, and other interactions may play a crucial role in TiO_2 NPs-mucin interactions at pH= 7.

Cationic BMC was possibly formed at acidic condition (pH= 2 & 4) at mass ratio (TiO_2 NPs: mucin) = 0.25. Based on the result and literature [52], this study proposed that cationic BMC might be able to cross the cell membrane.

In PBS, although surface charges of TiO_2 NPs were all negative, the appearance of TiO_2 NPs in PBS was opaque due to unstable aggregation that may be induced by the hydrophobic interactions or other interactions.

The development of hetero aggregation of BMC in PBS required a higher concentration of TiO_2 NPs than in DIW. Different pH might generate different species and bio-fate of BMC in PBS.

Knowing the interaction between TiO_2 NPs and mucin is required to understand the fate, transformation, transport, and toxicological effect of TiO_2 NPs in a complex environment. Interactions between TiO_2 NPs and mucin may induce polyvalent binding, formation of BMC, and hetero aggregation. In PBS, the development of unstable hetero aggregate of BMC requires a higher concentration of TiO_2 NPs than in DIW. The cationic BMC may be developed in DIW with acidic conditions; while in PBS, there is no cationic BMC formed. This study presented the primary interaction between TiO_2 NPs and mucin in DIW and PBS. Further studies to determine of the types, products, and effects of TiO_2 NPs-mucin interaction are required, particularly in the complex environment mimicking a real ecological and physiological condition.

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