Faculty of Resource Science and Technology

Ecotoxicity Assessment of Zinc Oxide Nanoparticles in Embryo Stages of Zebrafish (*Danio rerio*)

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DECLARATION

I hereby declare that all the work presented in the thesis entirely my own work. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

High demand of nanonmaterials have produced enormous applications in global industries. However, due to their extensive productions, release of different size of zinc oxide nanoparticles (ZnO NPs) into the environment has raised concerns in which the environment might pose ecotoxicological risk to the aquatic organisms. Presence of natural organic matter (NOM) mainly humic acid (HA) has also gain interest. As HA affect behavior of ZnO NPs which eventually affect the level of toxicity of ZnO NPs. Thus, this study was carried out in order to determine the toxicity level of different size (7.4 nm, 36 nm, and 73 nm) of ZnO NPs with different concentrations (1, 5, 10, 30, 50, and 100 mg/L) in embryonic zebrafish (5 hpf -96 hpf) with and without the presence of HA. This study was also carried out to investigate the behaviour of ZnO NPs in embryo water in terms of aggregation and dissolution. Findings showed that smaller ZnO NPs (7.4 nm) has a higher toxicity than larger NPs (36 nm and 73 nm) in which low survival rate was observed in embryo exposed to smaller ZnO NPs with 0% hatching rate. However, the presence of HA has greatly alleviated the toxicity of three sizes of ZnO NPs as more than 50 % of the survival rates as well as the hatching rates of embryonic zebrafish were observed. Other than that, dissolution and aggregation were found to be one of the most important role that contribute to toxicity. Finding demonstrated that smaller ZnO NPs has high dissolution as compared to larger ZnO NPs in which aggregation has occurred. The presence of HA has surprisingly increased the dissolution of ZnO NPs and stabilized the ZnO NPs in water. Therefore, presence of HA did influence the toxicity and the fate of ZnO NPs in the environment regardless of their particle sizes.

Keywords: ZnO NPs, toxicity, size, dissolution, embryonic zebrafish

***Penilaian Ekoketoksikan Nanopartikel Zink Oksida pada Tahap Awal Ikan Zebra* (Danio rerio)**

ABSTRAK

Bahan nano telah mendapat permintaan yang tinggi dalam menghasilkan produk-produk dalam industri global. Disebabkan oleh pengeluaran yang meluas, penglepasan nano saiz zarah zink oksida (ZnO NPs) ke persekitaran telah menimbulkan kebimbangan dengan risiko ekotoksikologi dalam alam sekitar. Kehadiran bahan organik semulajadi (NOM) terutamanya asid humik (HA) juga mampu mengubah ciri-ciri nano ZnO yang akan memberi kesan kepada tahap ketoksikan ZnO NPs. Oleh itu, kajian ini dijalankan untuk menentukan tahap ketoksikan nano ZnO yang berlainan saiz (7.4 nm, 36 nm, dan 73 nm) dengan kepekatan yang berbeza (1, 5, 10, 30, 50, dan 100 mg/L) ke dalam embrio ikan zebra (5 hpf -96 hpf) dengan kehadiran atau tanpa HA dalam air dan untuk mengkaji perubahan perilaku nano ZnO dalam air. Dapatan menunjukkan bahawa nano ZnO (7.4 nm) yang brsaiz kecil mempunyai ketoksikan yang tinggi daripada nano ZnO yang bersaiz lebih besar (36 nm dan 73 nm) yang menunjukkan kadar hidup yang rendah dalam embrio selepas terdedah kepada nano ZnO yang kecil bagaimanapun tiada penetasan (0 %) berlaku apabila nano ZnO didedahkan kepada embrio. Kehadiran HA telah mampu mengurangkan ketoksikan ZnO NPs kerana lebih daripada 50% embrio ikan zebra mampu untuk hidup dan menetas. Disolusi dan agregasi didapati menjadi salah satu peranan yang penting dalam menyumbang kepada ketoksikan nano ZnO. Keputusan juga menunjukkan bahawa nano ZnO yang lebih kecil mempunyai disolusi yang tinggi berbanding nano ZnO yang lebih besar di mana pengagregatan telah berlaku. Kehadiran HA juga telah meningkatkan kadar disolusi nano ZnO dan menstabilkan nano ZnO dalam air. Oleh itu, kehadiran HA mempengaruhi ketoksikan dan perilaku ZnO NPs dalam alam sekitar tanpa bergantung dengan saiz nano ZnO.

Kata kunci: *ZnO NPs, ketoksikan, saiz, disolusi, embrio ikan zebra*

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LIST OF ABBREVIATIONS

%	Percent
^{o}C	degree celcius
±	Plus minus
Ag	Silver
Ag NPs	Silver nanoparticle
ASC	Ascorbate
cm-1	Per centimeter
CeO_2	Cereum oxide
CuO	Copper oxide
CrO_2	Chromium dioxide
Co_2O_3	Cobalt oxide
CIT	Citrate
DEX	Dextran
ENP	Engineered nanoparticles
FA	Fulvic acid
FT-IR	Fourier transform infrared spectroscopy
g	Gram
GRAS	Generally Recognized as Safe
Hpf	Hour post fertilization
HA	Humic acid
ICP-OES	Inductively coupled plasma optical emission spectroscopy
LC_{25}	25 % lethal concentration

LC_{50}	50 % lethal concentration
mg/L	Milligram per litre
mL	Millimeter
MO NPs	Metal oxide nanoparticles
ng/L	Nanogram per litre
nm	Nanometer
NOM	Natural organic matter
NPs	Nanoparticles
OECD	Organisation for Economic Co-operation and Development
PVP	polymer
ROS	Reactive oxidative stress
TEM	Transmission electron microscope
TiO_2	Titanium dioxide
UV vis	Ultraviolet visible spectroscopy
XRD	X-ray diffraction
ZnO NPs	Zinc oxide nanoparticles

CHAPTER 1

INTRODUCTION

1.1 Background Study

Nanotechnology has become a well-known modern material science research worldwide. The potential application of nanotechnology has the capability to work with a nano scale materials called nanoparticles (NPs) (Jeevanandam *et al*., 2018). Over the past two decades, there has been increasing productions of NPs for different usages such as cosmetics, electronics, catalyst, antibacterial agents, and pharmaceuticals (Kteeba *et al*., 2017). Due to rapid development of nanotechnology, NPs have successfully been applied in numerous applications from energy production, materials usage, antimicrobial agents, to cosmetics products (Lu *et al*., 2015). With the size range of 1-100 nm, NPs can easily penetrate across cell membrane of living organisms (Rana & Kalaichelvan, 2013).

Oxide NPs mainly zinc oxide (ZnO) NPs are widely used in consumer-based industry. Some reports have mentioned the safety of NPs which leads to rapid productions of nano-based products (EU regulation, 2016; Yu *et al.*, 2017). ZnO NPs is one of the common ingredient listed in cosmetic products and in household products such as toothpaste, food colouring, and paints (Julia *et al.*, 2011). Julia *et al.* (2011) reported that there was no evidence that showed negative health effect of using oxide metals NPs when tested on model animals that might consider the safety of NPs in consumer products.

However, the rapid growth of NP-based products has raised a concern on the adverse health impacts in aquatic environment. Baumann *et al.* (2014) also described that nanoscale materials would eventually enter aquatic environment without knowing the potential risk to the organisms when introduced in the environment. Besides, the unique

properties of NPs are not only able to manipulate biological systems, but also be the main factors that might contribute to toxicity. This occurs when rapid productions of NPs-based products are made in which this issue have raised concerns among researchers and the public regarding the potential risks of NPs to the environment (Baumann *et al*., 2014; Jiang *et al.,* 2011).

Advance productions of NPs mainly metal oxide (MO) NPs due to high demands from industries have led most researchers to carry out close monitoring on the adverse effect possessed by the NPs. This is crucial in order to prevent any non-target organisms from being affected or killed. Recently, a major concern was raised as ZnO NPs productions increased all around the globe due to its wide utilization and applications (Kteeba *et al*., 2017; Modunkotuwa *et al.*, 2013). Previous studies have also reported the potential harmful risks of ZnO NPs in microorganisms, plants, and animals. This information might also infer the potential risks for human as NPs exposure getting higher due to the increased commercial products with NPs (Maurer-Jones *et al*., 2013).

The exposure of NPs to human might also lead to the release of NPs to the environment which eventually ends up in ecosystem food web (Jiang*et al.*, 2011). Besides, the presence of natural organic matter (NOM) has gained attention among researchers in the ZnO NPs toxicity studies especially in their fate and behaviours when in contact in water (Akhil *et al.*, 2015; Omar *et al*., 2014; Ong *et al*., 2017). However, there are scarce of ecotoxicological information of ZnO NPs to the organisms especially when HA involved.

Hence, there is a need to assess and evaluate the toxicity of ZnO NPs in organisms due to extensive productions. For this study, embryonic zebrafish was used as a testing organism in order to determine the adverse effect of ZnO NPs when exposed to the

embryonic zebrafish. This might give valuable information mainly on the impacts of ZnO NPs when being released to the environment with or without the presence of HA. ZnO NPs will be synthesized and characterized using transmission electron microscope (TEM). For ecotoxicity study, different sizes of ZnO NPs at different concentrations were tested on the embryonic zebrafish. Besides, comparison between the presence and absence of HA with ZnO NPs were also being determined in embryonic zebrafish. The major outcomes of this study are being able to evaluate toxicity by determining the physiological effects of ZnO NPs in zebrafish based on different size and presence of HA. The behaviour of ZnO NPs in water was also being determined based on the dissolution and aggregation.

This information is essential as this finding might give insights on the alteration of ZnO NPs in term of the behaviour mainly the dissolution in which the amount of zinc(II) ion being released that caused the toxicity and also the aggregation of ZnO NPs that affect the physiological system of test organisms.

1.2 Problem Statement

Rapid growth of NP-based products has raised a concern on the adverse health impacts. Baumann (2014) also described that the potential risk of the aquatic organism might be due to the released of nanoscale materials into the environment. Thus, this becomes an emerging topic in aquatic and environmental studies (Keller *et al.*, 2013; Kteeba *et al.*, 2017). Besides, the unique properties of NPs in terms of size, dissolution and aggregation might need to be considered as they might able to manipulate biological systems and cause toxicity.

This issue has raised concerns among researchers and public regarding the potential risks to the environment (Bai *et al.*, 2010; Jiang *et al.*, 2011; Wehmas *et al.*, 2015).Several

questions regarding to their safety and toxicity have arisen due to numerous novel properties of engineered NPs being produced (Shin *et al.*, 2015). Due to the high capabilities of ZnO NPs in various applications especially in cosmetics and sunscreen products, this might be one of the main prevalence of NPs contamination due to wash-off from individuals into the aquatic system (Akhil *et al*., 2017; Kteeba *et al*., 2017).

There were many toxicity studies of the environmental impacts of ZnO NPs in all taxa from bacteria to higher level organisms such as crustaceans, plants, fishes, and mice. However, there is still lack of information to support the level of toxicity ZnO NPs especially when involve the presence of NOM mainly HA. Domingos *et al.* (2013) also described the importance of the environmental fate of NPs study that highly depended on the physico-chemical properties of NPs under natural condition mainly size, dissolution, aggregation, and the presence of HA.

The toxicity study of ZnO NPs with the presence of other materials and presence of NOM has gained the researchers' interest which most likely alter the properties of NPs. Although there are some research on the properties of ZnO NPs with the presence of HA however, there is still inadequate information regarding the toxicity level of ZnO NPs with presence of HA which might be also affect the behaviour of ZnO NPs in aquatic environment. Akhil *et al*. (2017) stated that environmental factor such as the presence of NOM can be significantly affecting the toxicity of ZnO NPs. There are reports stated that the presence of HA elevated the stability of NPs (Yang *et al*., 2009; Zhang & Gutterman, 2007).

The interaction of humic acid and ZnO NPs that related to the toxicity to aquatic organisms is still lacking thus more data need to be gathered in order to evaluate the potential fate and toxicity of ZnO NPs in aquatic environment.

1.3 Objectives

The study of the ecotoxicity of ZnO NPs in zebrafish consist of three main objectives which are:-

i. To synthesize and characterize ZnO NPs for embryonic zebrafish toxicity assessment.

ii. To determine the toxicity of different sized ZnO NPs at different concentrations and presence of HA in the embryonic zebrafish.

iii. To determine the fate behaviours of ZnO NPs in water with and without the presence of HA after prolonged exposure to embryonic zebrsfish.

CHAPTER 2

LITERATURE REVIEW

2.1 Nanoparticles (NPs)

Nanoparticles (NPs) have existed in the environment for millions of years. Natural occurring NPs include both organic compounds such as proteins, polysaccharides, and viruses and inorganic compounds are iron, oxyhydroxide, and other metals (Hough *et al.,* 2011). Apart from that, NPs can also derive from natural combustion processes such as forest fires, volcano eruptions and deposited into ice core by atmospheric deposition (Murr *et al.*, 2004). Other than that, biological matters degradation produces NPs such as humic acids and fulvic acids (Bhatt & Tripathi, 2011).

Engineered NPs (ENPs) production has gained attention in manufacturing industries worldwide (Milani *et al*., 2017). NPs is defined as a subset of nanomaterials which is materials with at least one external dimension in size range from 1 to 100 nm (ISO, 2008). Salieri *et al.* (2015) stated that nanotechnology was considered as one of the greatest innovations since the beginning of industrial engineering. NPs has the ability to squeeze into spaces that are inaccessible to larger particle (Rana & Kalaichelvan, 2013). Over the last decade, NPs has been used frequently in industrial applications.

Due to high demand of NP based products, various types of engineered nanoparticles (ENPs) are synthesized to be used in wide range of applications (Milani *et al*., 2017; Maurer-Jones *et al*., 2013). ENPs are made into various shapes and sizes depending on the use of NPs in industries including textile, energy, food, cosmetics, and medicines and other characteristics that make them attractive for broad range of application (Hughes, 2015). Most ENPs can be synthesized into both organic and inorganic NPs. Organic NPs include carbon nanotubes and fullerenes while inorganic NPs includes metals NPs (Al, Cu,

Zn), metal oxides (TiO_2, ZnO, CuO, AL_2O_3), and quantum dots (Bhatt & Tripathi, 2010; Rajput *et al*., 2017). Wang *et al*. (2016) has also stated NPs are arguably the most important products of nanotechnology which provide great benefits in this decade.

2.1.1 Productions of NPs

High demand of NPs have produced enormous applications in global industries. Bondarenko *et al.* (2013) has stated that the annual funding for nanotechnology was 17.8 billion USD . Nanotechnology industry was also being projected to reach 7.6 billion USD market in year 2013 according to Business Communications Company (BCC) which has high potential to rise up to 1 trillion USD in year 2020 (Chakraborty *et al*., 2016; Sabourin *et al.*, 2015).

Zhu *et al.* (2012) also stated that more than 1300 nano-products appeared on market. Figure 2.1 shows the NPs growth trends as reported by Pulit-Prociak and Banach (2015). Based on this shown Figure 2.1, there are gradual increment of the nano-productions over time and this will continue to increase until 2025.

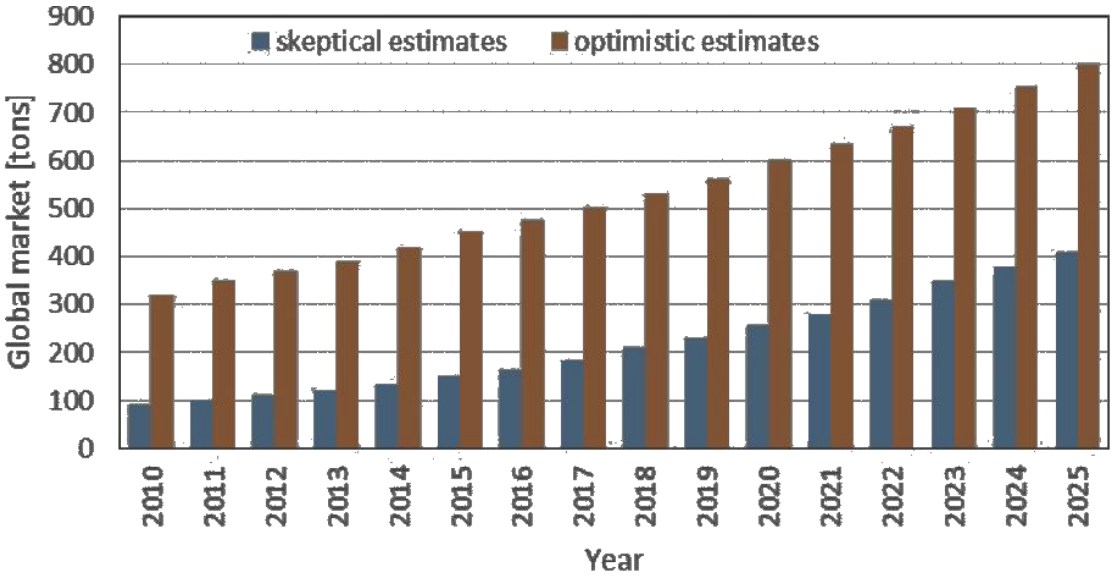


Figure 2.1: Growth trends of NPs productions from 2010 to 2025. Adapted from 'Nanoprticles- A material of the future?' (Pulit-Prociak & Banach, 2016)

Besides, a inventory market done by US-based Woodrow Wilson Centre, has estimated that NPs productions were at 2000 tonnes in 2004 and expected to grow to 58 000 tonnes in period 2011 – 2020 (Arif *et al*., 2016; Maynard *et al.*, 2006; Renzi & Guerranti, 2015). Arif *et al*. (2016) also reported that there are more than 260,000 metric tonnes of NPs produced worldwide in 2010. Productions of nano-based products were estimate to generate up to USD 1 trillion by year 2015 (Kashiwada, 2006; Nel *et al.*, 2006; Zhang *et al*., 2007). As for ZnO NPs productions, 1000 tonnes of ZnO NPs productions was reported annually in 2003 (Ma *et a*l., 2013). UNEP (2007) has predicted that ZnO NPs will increase 58 000 productions from 2011 to 2020. Sun *et al*. (2014) has stated that 16 000 tonnes of ZnO NPs productions were made in European countries in 2012. Furthermore, commercial NPs is already on its way to develop more which nano-products on market has projected to reach USD 100 billion by 2025 (World use of nanotechnology, 2007; Zhu *et al*., 2009).

2.1.2 Applications of NPs

Different types of NPs have been developed for specific applications in various industries. This include metallic NPs, metal oxide NPs, carbon nanotubes, fullerenes, and also quantum dots. Among these NPs, metal oxide NPs productions have widespread applications worldwide.

2.1.2.1 Metal Oxides Nanoparticles (MO NPs)

One of the common types of NPs is metal oxide NPs. This metal containing materials belongs to the second class of ENPs (Bhatt & Tripathi, 2011). Metal oxides (MO) NPs are known to have high specific surface area and more reactive as compared to ordinary sized particles (Auffan *et al.*, 2009). MO NPs are produced in a great amounts and have the highest predicted environment concentrations due to their wide applications including medicine, catalysis, photonics, electronics and environmental remediation (Ding *et al.*, 2015; Sun *et al.*, 2014; Wang *et al.*, 2016a). Titanium dioxide (TiO_2), zinc oxide (ZnO), cerium dioxide (CeO_2), and copper oxide (CuO) are the examples of metal oxide NPs that are commonly used in industries (Bhatt & Tripathi, 2011). Table 2.1 shows the common MO NPs produced for their respective application. TiO2 NPs and ZnO NPs have high photocatalytic properties and ultraviolet (UV) blocking ability thus extensive productions of these NPs to produce household and cosmetic products (Julia *et al.,* 2011; Lu *et al*., 2015). Other than that, CeO_2 NPs are widely used as combustion catalyst in diesel fuels that improves the emission quality (Bhatt & Tripathi, 2011).

Table 2.1: Common MO NPs and their respective applications.

MO NPs	Applications	References
TiO_2	Cosmetics, coating, air purification, solar cells	Tolaymat *et al.* (2010)
ZnO	Cosmetics	Julia *et al.* (2011)
	Paints	Lu *et al*. (2015)
	Household products	Oliviero *et al*. (2017)
	Antimicrobial agents	Dimapilis *et al*. (2017)
Fe_2O_3	Environmental remediation, semiconductors	Fereira da Silva *et al*. (2011)
CuO	Semiconductors, electrical chips, lithium batteries	Bondarenko *et al*. (2013)
CeO_2	Sunscreens	Bhatt & Tripathi (2011)
	Fuel additives, solar cells, gas sensing	Milani *et al.* (2017)

Despite of their massive productions in industries, MO NPs is also well-known for ecotoxicological research (Lopes *et al*., 2014). Among other MO NPs, an extensive production of ZnO NPs has gained more attention in assessing the potential risks in health and environmental impacts (Sharma *et al*., 2009).

2.1.2.2 Zinc Oxide Nanoparticles (ZnO NPs)

Zinc (Zn) is crucial for all living organisms. However, it is toxic in high dosage (Chang *et al.*, 2012). ZnO was also known as zincite exhibits three crystallize structures namely wurtzite, zinc-blende, and rocksalt (Sirelkathim *et al*., 2015; WHO, 2004). ZnO

NPs generally appears as white crystalline powder and nearly insoluble in water (Behera, 2011). Widely utilization of ZnO NPs has a wurtzite crystal structure that attribute to its unique properties (Ma *et al*., 2013). ZnO NPs is known to be a distinctive electronic and photonic wurtzite n-type semiconductor with band gap of 3.37 eV and high exciton binding energy of 60 meV at room temperature. Besides, ZnO is classified as semiconductors in group II – VI in which covalence is on the boundary between ionic and covalent semiconductors (Kołodziejczak-Radzimska & Jesionowski, 2014). This makes ZnO NPs particularly popular for use in commercially available sunscreens and cosmetics to block UV radiation when they are less than 50 nm (Adams *et al.*, 2006; Wehmas *et al*., 2015).

Unites States Food and Drugs Administration (FDA) has also stated that ZnO NPs is recognised as safe materials which is classified as GRAS (Generally recognized as safe) for various applications including food and other commercial products (U.S Department of Health & Human Service, 2015; Yu *et al*., 2017). This recognition leads to the increase of ZnO NPs productions of ZnO NPs across the globe. Worldwide annual productions of ZnO NPs has estimated within range of 550 – 33 400 tonnes hence ZnO NPs was categorised as the third highest productions worldwide (Piccino *et al*., 2012).

Since ZnO NPs are reported as safe to be used, ZnO NPs has become one of the most common ingredients mentioned in consumer-based products especially skin products such as sunscreens as well as household products such as toothpaste, food colouring, and paints (Lv *et al*., 2015; Yu *et al.*, 2017). This is due to the ability of ZnO NPs to reflect UV light (UVA and UVB) which is better than micro-sized ZnO (Chang *et al*., 2012). Besides, Scientific Committee on Consumer (2014) has sreported that 25 % of ZnO NPs (100 nm) concentration is safely used in cosmetic products. Based on Lorenz *et al*. (2011), 40 % of

German population are using sunscreen for 20 days annually. The sufficient amount of sunscreen protection used daily is 36 g (European Commission, 2006).

Apart from that, ZnO NPs possess high optical absorption in UVA and UVB which are also beneficial in antimicrobial products (Gunalan *et al.,* 2012). Among other MO NPs, Salem *et al.* (2015) stated that ZnO NPs is the most recommended for antibacterial agent. Previous studies have proven that the antimicrobial activities of ZnO NPs against pathogenic microorganisms including *Escherichia coli*, *Staphylococcus aureus*, and *Campylobacter jejuni* (Jiang *et al.*, 2011; Salem *et al*., 2015; Vani *et al.*, 2011; Xie *et al.*, 2011). These studies have shown the bacteriostatic or bactericidal effect on the bacteria with low concentration of ZnO NPs.

Other applications of ZnO NPs are also involved in environmental control technology especially for environmental pollutant remediation and medical disinfection (Dimapilis *et al.*, 2017; Ma *et al.*, 2013). Besides, ZnO NPs in powdered forms are commonly used in plastics, ceramics, paints, lubricants, rubbers, batteries, and food (Zn supplement) (Ma *et al.,* 2013). ZnO NPs are also used in polymer fillers and UV absorbent (Han *et al.*, 2014).

Despites its popularity, the possible risks of ZnO NPs to the environment should not be ignored. Advanced development of NPs in nanotechnology industries should also include close monitoring regarding the potential risk of NPs in order to evaluate the risk assessment of NPs before being released into market place. Garner and Keller (2014) also mentioned that the effect of ZnO NPs in environment need to be monitored due to significant increase of ZnO NPs productions to ensure that the productions can prevent the non-target organisms from being affected.

2.2 Ecotoxicity of NPs

Ecotoxicity refers to potential physical, chemical, and biological effect of NPs to organisms in environment (Rana & Kalaichelvan, 2013). Increase productions and utilizations of NPs in consumer and industrial products has aroused global concern regarding their possible risk impact not only to living organisms but also to the environment (El Shemy *et al.,* 2017; Walker, 2012). Nowack *et al.* (2007) has stated that the release of NPs might come from many point sources including production facilities, landfills, wastewater treatment plant, and from non-direct sources such as wearing contaminating NPs products (lotion). This results in a demand for toxicity assessment in the environment. Various studies have been conducted to evaluate ecotoxicological aspects in which most results revealed that NPs have high risk to living aquatic organisms (Bondarenko *et al.*, 2013; Nowack *et al*., 2007). A number of studies were carried out involving the potential risk of NPs to target organisms in environment. In previous research, most toxicity studies were carried out using MO NPs such as TiO_2, SnO_2, and Fe_2O_3 due to their unique properties. However, ZnO NPs has gained interest and become one of the most studied NPs recently. Hence, further studies need to be done in order to monitor closely the potential threats of NPs being released into the environment.

2.2.1 Ecotoxicity of ZnO NPs

Apparently, the increased production of ZnO NPs causes frequent release to environment. Since ZnO NPs was recognized as the third highest in NPs productions thus there might be high potential risk of ZnO NPs to the environment. Table 2.2 shows the concentration of ZnO NPs in aquatic environment that were reported by previous literatures. Boxall *et al.* (2002) studies found that ZnO NPs concentration in environment

in United Kingdom (UK) was less than 100 µg/L in water and expected to increase over time. Meanwhile, studies carried out by Gottschalk *et al.* (2009) in Europe found that ZnO NPs concentration was higher in wastewater treatment (430 µg/l) than in natural surface water (10 µg/L). Besides, Sun et al. (2014) found that 1.7 to 21.0 µg/L of ZnO NPs concentration has caused disruption to surface water and sewage sludge.

Table 2.2: Concentration of ZnO NPs in water samples from literatures.

Wastewater (µg/L)	**Surface water (mg/L)**	**Aquatic environment**	**Reference**
1	-	76	Boxall *et al.* (2002)
430	10	-	Gottschallk *et al.* (2009)
76-760	-	-	Ghosh *et al.* (2009)
			Boxall *et al.* (2002)
1.7- 21.0	-	-	Sun *et al.* (2014)

Many studies have been carried out on ZnO NPs and the results revealed that ZnO NPs caused pernicious effect to the living organisms in ecosystem. For instance, ZnO NPs based products such as sunscreens and paints are utilized which then eventually washed off from the user or rains and find routes to enter the environment (Batley *et al.*, 2012; Baumann, 2014; Kahru *et al.*, 2008). Many studies on toxicology were carried out in aquatic ecosystem since NPs are mostly found in aquatic environment and crustaceans, algae, and fish had been proven to be the most sensitive organisms in NPs studies (Bondarenko *et al.*, 2013; Exbrayat *et al*., 2015). In addition, Han *et al.* (2014) also stated that ZnO NPs are known to cause toxicity against bacteria, plants, and animal cells despites of its use in various applications. For instance, zebrafish has potential to provide findings

in chemical toxicity (Hill *et al.*, 2005). Early studies by Zhu *et al*. (2009) have shown that ZnO NPs to be more toxic to fish life stages. In aquatic environment, one of the exposure routes for NPs uptake in aquatic organisms is via guts. When fishes consume food and water, NPs might enter in the gut cells by diffusing through cell membranes via endocytosis (Chang *et al*., 2012).

Furthermore, the toxicity study of ZnO NPs to aquatic organisms has been recently been the subject of excellent reviews that offer a great scope to the subjects. Mostly, toxicity studies of NPs have focused on three identifications, which are the properties of ZnO NPs that could affect the aquatic organisms, the environmental factors that contribute to toxicity, and the recommended approaches of toxicity testing in order to validate the experiment, which it is necessary for future studies Bundschuh *et al.* (2018). Table 2.3 showed a summary of ecotoxicological studies carried out by previous researchers involving MO NPs. Previous studies carried out by Mansouri *et al.* (2015) on toxicity of cobalt oxide (Co_2O_3) to zebrafish showed that the lethal median concentration (LC50) is 300 mg/L. Mansouri *et al.* (2015) found that Co_2O_3 exposure causes gill injury in zebrafish and there was a presence of aggregation in embryo water after 24 hour NPs exposure.

Besides, studies carried out by Baumann (2014) on iron oxide (Fe_2O_3) NPs which involve different coatings that include ascorbate (ASC), citrate (CIT), dextran (DEX), and polymer (PVP). Result showed that only Fe_2O_3 NPs coated with PVP has lower toxicity.

Baumann (2014) explained that the presence of different coatings has affected the behaviour of NPs. Other than that, there were also studies on the comparison of metal-bearing NPs toxicity tested in zebrafish in which the results also revealed the toxicity of ZnO NPs of two different sizes in which smaller sized ZnO NPs caused more toxicity to the tested organism (Lacave *et al.*, 2016; Wehmas *et al*., 2015). Lacave *et al.* (2016)

explained that the toxicity depended on the chemical composition especially solubility of NPs.

Meanwhile, Table 2.4 showed a summary of ecotoxicological studies of ZnO NPs exposure in different target species. Choi *et al.* (2016) have carried out a toxicity study on ZnO NPs and the results showed that ZnO NPs caused toxicity to the tested organism as compared to the exposure of dissolved Zn that was derived from zinc sulphate ($ZnSO_4$) Zhou *et al.* (2015) have also carried out the toxicity study for coated and uncoated manufactured ZnO NPs. The result has shown that uncoated ZnO NPs generate more toxicity compared to coated ZnO NPs. Zhou *et al*. (2015) described that surface coating of NPs is one of the main factors that generate toxicity. Studies by Chen *et al.* (2014) also revealed that the exposure of ZnO NPs affect the hatching process of the zebrafish especially at increasing concentrations. Previous research by Zhu *et al.* (2009) on the toxicity of ZnO NPs using zebrafish concluded that ZnO NPs were toxic to zebrafish development due to the behaviour of ZnO NPs when in contact with water.

This result was also supported by Wehmas *et al*. (2015) that carried out similar studies using different metal oxide NPs on zebrafish in which ZnO NPs has showed toxicity than other selected MO NPs. There are also studies that conducted by exposing ZnO NPs in other target organisms such as tilapia fish by Kaya *et al.* (2016), and brine shrimp by Diagloglu *et al.* (2016) and Hanna *et al*. (2013). Marine organism such as mussels and oysters were also tested for ZnO NPs toxicity study as shown in Table 2.4 (Hanna *et al.*, 2013; Trevisan *et al.,* 2014) Therefore, this study highlights the need to assess the effect of ZnO NPs in aquatic environment.

Table 2.3: Summary of ecotoxicological study using MO NPs.

Test organism	Metal oxide (MO) NPs	Size/ nm	Concentration/ mg/L	Outcomes	Reference
Zebrafish (*D. rerio)*	Cobalt oxide (Co_2O_3)	50	10, 40, 70, 100	Co_2O_3 deposited at the bottom Gill injury after 8 days exposure	Mansouri *et al*., 2015
Crustacean (*Daphnia magna*)	Iron oxide (Fe_2O_3)	5.2-6.1	1, 2.2, 5, 7.5, 10, 25, 50, 75, 100	PVP showed lower toxicity PVP-IONP has high colloidal stability	Baumann, 2014
Zebrafish (*D. rerio)*	Ag, Au, Cd, ZnO	24-96, 4.4-40.4, 3.5-4, 20-100	0.001 – 100	Ag NPs is the most toxic Toxicity depends on the solubility of NPs NPs is more toxic to bulk particles	Lacave *et al*., 2016
Zebrafish (*D. rerio)*	ZnO, TiO_2, Ce_2O, SnO_2	8, 10-12, 3, 3	0.08, 0.4, 2, 10	Only ZnO NPs showed toxicity effect Dissolution cause toxicity	Wehmas *et al.,* 2015
Zebrafish (*D. rerio)*	Fe_2O_3	30	0.1, 0.5, 1, 5, 10, 50, 100	50 & 100 mg/L – 75% mortality ≥ 10 mg/L – affect hatching	Zhu *et al*., 2012

Table 2.4: Summary of ecotoxicological study using ZnO NPs.

Test organisms	Size/nm	Concentration/mg/L	Outcomes	Reference
Zebrafish (*D. rerio)*	20 – 30	0.01, 0.1, 1, 10	High ZnO agglomeration at higher concentration Malformations – tail edema(TM), pericardial edema (PE), Yolk sac edema (YSE)	Choi *et al*., 2016
Zebrafish (*D. rerio)*	4.5 – 70	0.06 – 250	Uncoated ZnO NPs cause mortality (24 hpf and 120 hpf) Coated ZnO caused no mortality after 120 hpf Outermost surface chemistry is a primary driver to biological interactions.	Zhou *et al*., 2015
Zebrafish (*D. rerio)*	80	0.1, 0.5, 1, 5, 10	No mortality observed Hatching delay increase at high concentration ZnO sediment at bottom after 3h exposure ZnO partially dissolved in fish water	Chen *et al*., 2014
Zebrafish (*D. rerio)*	<100 <50	0.1, 0.33, 1, 3.3, 10	ZnO NPs present in midgut cells	Santo *et al*., 2014

Table 2.4 continued

Zebrafish (*D. rerio)*	30	1, 5, 10, 25, 50, 100	50 and 100 mg/L killed zebrafish embryo 1-25 mg/L retard embryo hatching ZnO aggregate in embryo water	Bai *et al.*, 2010
Zebrafish (*D. rerio)*	20	0.1, 0.5, 1, 5, 10, 50, 100	Pericardial edema were observed at 50 mg/L & 100 mg/L Aggregation caused toxicity	Zhu *et al.,* 2009
Tilapia (*Oreochromis niloticus*)	10- 30, 100	1, 10	All size caused damage to immune system Affect biological mechanism in gills Damaged organ (gills)	Kaya *et al*., 2016
Brine shrimp (*Artemia salina*)	N/A	0.01, 0.1, 0.5	ZnO aggregation cause toxicity	Daglioglu *et al*., 2016
Oyster *(Crassostera gigas)*	30	50 – 50 ug/L	ZnO accumulate in gills, digestive glands Trigger oxidative stress Cause mitochondrial distruption	Trevisan *et al*., 2014
Mussel (*Mytilus galloprovinciallis*)	20 - 30	0.1, 0.5, 1, 2	ZnO accumulation higher in small mussel	Hanna *et al*., 2013

Table 2.4 continued

Crustaecean (*Daphnia magna*)	30, 80- 100	0.25- 10	ZnO NPs (30 nm) affect feeding activity Dissolution and particle size contribute to toxicity	Lopes *et al*., 2014
i) Cladocaerons (*Ceridaphnia affinis*) ii) Dipteran chironomids *(Chiromonus riparius)* iii) Zebrafish (*Branchydanio rerio*)	15 - 350	0.2, 2, 10, 20	i) Deformed mouth of larvae ii) 100% mortality at 20 mg/L exposure after 120 hpf	Tomilina *et al*., 2014

*N/A - not available

2.3 Factors influencing NPs toxicity

NPs toxicity study have been carried out over the years in order to determine the factors that contribute to the NPs toxicity. Physicochemical properties of NPs are greatly influence the regulation behaviours of ENPs in environment (Ivask *et al*., 2014). These factors include size, surface area, shape, solubility, and aggregation in which these are crucial to determine the possibilities of NPs to cause toxicity to the environment especially when in contact with water (Gatoo *et al.,* 2014).

2.3.1 Factors affecting ZnO NPs toxicity

Since this study only focus on ZnO NPs, it is vital to consider various factors that might affect the toxicity of ZnO NPs. There are many key factors that affect NPs toxicity especially nanosize and nanosurface (Kahru *et al*., 2008). Figure 2.2 shows few factors that affect ZnO NPs toxicity. Bunduschuh *et al.* (2018) has stated that the toxicity of NPs involved at least three mechanisms which are the exposure of NPs produce toxic substance to the exposed media, the surface interactions between NPs with media, and the NPs itself that interact directly causing the biological distruption in molecular state. Thus, understanding the key factors of NPs that lead to change in toxicity is essential in nanotoxicology for further assessment.

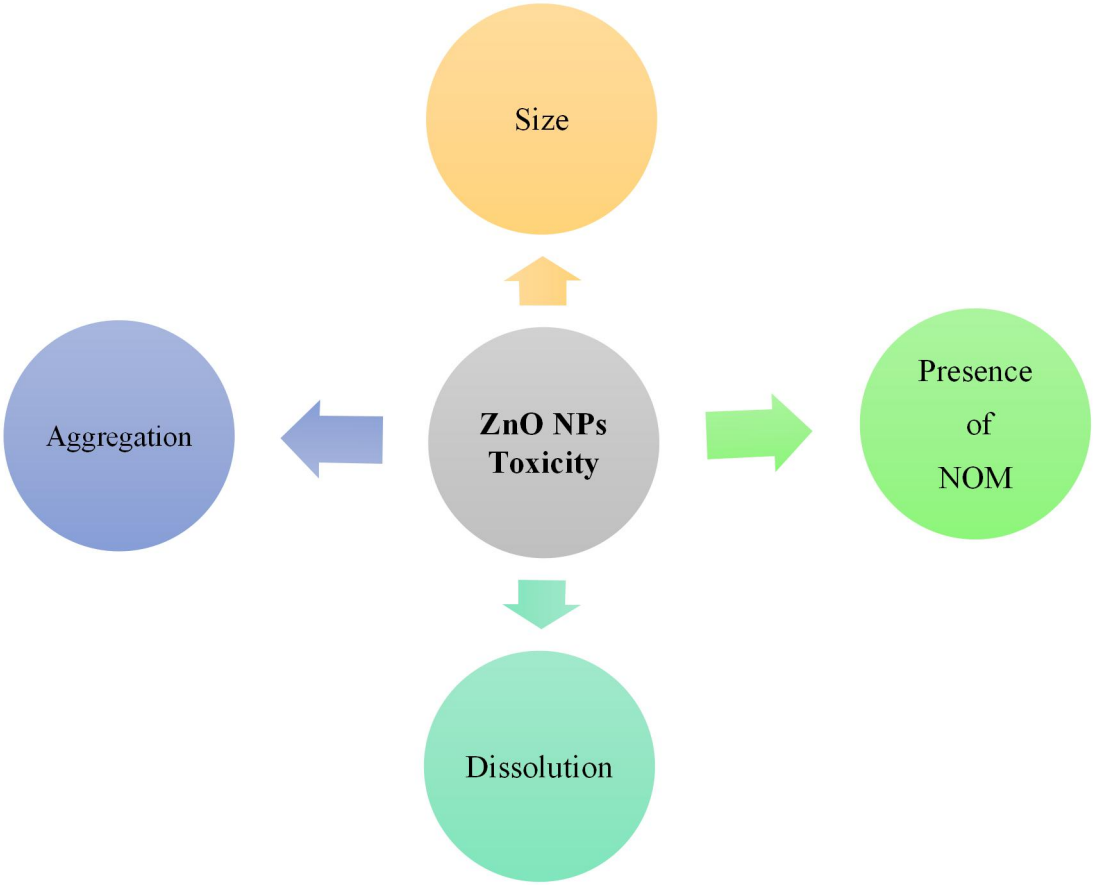


Figure 2.2: Factors affecting ZnO NPs toxicity

2.3.2 Size of ZnO NPs

Apparently, the size of NPs plays crucial roles in determining the toxicity in environment. Unique nanosize of NPs (<100 nm) greatly increase the surface area which affect the solubility (Bhatt & Tripathi, 2011). Nowack *et al*. (2007) has stated that the uptake of NPs is size-dependent which affect the behaviour of NPs. Small NPs are thought to pose a greater risks of toxicity than larger size particle of the same substances (Shin *et al*., 2015). Due to the ability of NPs to penetrate across the cell membrane which might migrate into cells in essentials organ, modify the cell metabolism and cause cell damages (Jiang, 2011).

Studies carried out by Akhil *et al*. (2015) also suggested that particles with smaller size is the main factor causing toxicity to tested organisms. Small particle has large surface area in which this exert toxic responses mainly causing reactive oxygen species (ROS) to be generated that further lead to toxicity in organism (Nowack *et al*., 2007). Tuttle (2012) has stated that the size of ZnO NPs has greatly influenced toxicity that also affect its dissolution rate, amount of reactive surface area, and bioavailability. Braakhuis *et al*. (2016) also stated that decreased size of NPs has increase surface area that was most likely to be the main factor contributed to toxicity of NPs in living organism.

Previous study by Chang *et al.* (2012) stated that ZnO NPs show more toxicity than bulks ZnO. This particular interaction in living cells might lead to cell death which alters the proper functions of biological systems (Jiang, 2011). Lopes *et al*. (2014) has also carried out a research on the effect of different size ZnO NPs to small crustacean Daphnia magna and found that smaller size (30 nm) of ZnO NPs is more toxic as compared to larger size (80 nm and 100 nm). Studies by Sager *et al*. (2008) also stated that toxicity of NPs was induced by the ultrafine and fine particles which has larger surface area. Most of the study that were carried out previously only expose ZnO NPs to zebrafish with range size from 20 – 100 nm (Bai *et al*., 2010; Johnston *et al*., 2010; Yu *et al.*, 2017; Zhu *et al*., 2009).

There are still scarce of information in ecotoxicological studies on the effect of ZnO NPs with size of less than 10 nm to the environment. Bian *et al*. (2011) successfully synthesized smaller ZnO NPs (<10 nm) thus this information might lead manufacturers to produce smaller NPs in industries for a better application in future. Therefore, it is necessary to carry out toxicity test of this particular size of ZnO NPs before commercial products with NPs content being released into marketplace.

2.3.3 Adsorption or Surface Coating to ZnO NPs

On the other hand, different surface coatings on ENPs apparently alter the physicochemical properties of NPs and affect its behaviour in environment (Kahru et al., 2008). NPs are usually coated with inorganic or organic compounds for stability maintenance as colloidal solution (Bhatt & Tripathi, 2011). Thus, adsorption of NPs with other materials is known to be a critical environmental process that might also affect the fate of NPs after being released into the aquatic environments (Akhil *et al*., 2015; Batley *et al.*, 2012; Beegam *et al.*, 2016). Previous study has reported that surface coating also influenced the behaviour of NPs when released into the water (Zhu e*t al.*, 2009). As stated by Bhatt and Tripathi (2011), when NPs are released to the environment, different surface of NPs might cause formation of colloidal solution or aggregation.

A study carried out by Osmond-McLeod *et al.* (2014) using similar size but different surface coating of ZnO NPs resulted in complete protection of tested organisms against ZnO NPs toxicity. Similarly, Zhou *et al*. (2015) has also conducted a study to investigate the toxicity effect of different surface coating of ZnO NPs in zebrafish. This study has shown that uncoated ZnO NPs are more toxic as compared to coated ZnO NPs (Zhou *et al.*, 2015). Based on study by Osmond-McLeod *et al.* (2014), stable surface coating of ZnO NPs gives protective effect against animal cells.

A recent study carried out by Bonfanti *et al.* (2015) to study the toxic effect of different surface coatings such as polymer to ZnO NPs in frog embryo and found that both coatings able to decrease the embryotoxicity of ZnO NPs. Osmond-McLeod *et al*. (2014) has stated that coating that is applied to surface of ZnO NPs is able to reduce the generation of free radicals. The interaction is highly dependent on the the surface charge of NPs. Ghosh *et al.* (2009) have tested Al_2O_3 NPs with the presence of humic acid (HA) and

found that the stability of NPs was enhanced in aqueous condition. This eventually will lead to new information regarding the level of toxicity of ZnO NPs that can be reduced when coating are present in various applications. Hence, the role of surface coating is crucial to determine the toxicity since it involves biological interactions (Konduru *et al.*, 2016) Thus, the interaction of NPs to other molecules might also be the critical components for the survival of organisms.

2.3.3.1 Presence of Natural Organic Matter (NOM)

Recently, study of NPs with the presence natural organic matter (NOM) have gained interest among researchers. NOM is ubiquitous in natural aquatic environment with main components being humic subtances about 50%, polysaccharides, lipids, proteins, and other organic materials (Erhayem & Sohn, 2014; Wang *et al*., 2016a; Zhou *et al.*, 2015). In aquatic environment, humic substance comprises of a mixture of humic acid (HA) and fulvic acid (FA) (Wang *et al*., 2016a).

Due to the abundant presence of NOM in environment, this might causes high potential of the adsorption of NOM onto the NPs surface and eventually formed complexes when being released to the environment (Yang *et al.*, 2009). An article by Peng *et al.* (2017) also mentioned that NOM surface coating to NPs is known to be an alternative 'green' surface coating to enhance NPs stability. NOM usually acts as stabilizer for NPs productions in order to control NPs in environment, control toxicity, and remediate heavy metals in wastewater (Dickson *et al*., 2012). Ong *et al.* (2017) has also mentioned that there were reports that the presence of NOM mitigate the adverse effect exposed by organic chemicals and metals. Previous studies found that the presence of FA

has modulated ZnO NPs by adsorb onto the surface particles thus affect the behaviour of NPs in environment (Miao *et al*., 2010).

The environment process of NPs in aquatic systems have been widely investigated, and most studies discussed the importance NOM in the process. The effects of nanomaterials on the biotic system depend on the abiotic factors in aquatic environments (Kteeba *et al*. 2017). Grillo *et al.* (2015) also stated that the presence of NOM to NPs reduced toxicity to majority of organisms. Previous study by Omar *et al*. (2014) has stated that NOM might influence the biological transformation of ZnO NPs structures that affect microorganism and high level organisms. Edgington *et al.* (2010) has stated that the presence of NOM reduced the particle bioavailability thus reducing the toxicity of particles to the living organisms. Therefore, the presence of HA has gained interest by researchers in the toxicity studies of ZnO NPs in organisms in particular environment especially aquatic environment.

2.3.3.2 Humic acid (HA)

Environment factor including the presence of humic acid (HA) have gained more attention by researchers as it might induce toxicity when interact with NPs. One of the important components in water is HA (Ong *et al*., 2017). Petit (2004) has described that HA comprises of a mixture of weak aliphatic (carbon chain) and aromatic (carbon ring) organic acids which is known to be insoluble in water under acid conditions but soluble under alkaline conditions. According to Akhil *et al.* (2015) and Tang *et al.* (2014), HA is a type of NOM, which can be found in abundance in environment as it is formed due to the decomposition of organic matters. Based on report by Rodrigues *et al.* (2009) and which

was also mentioned by Basumallick *et al*. (2017) that the surface water contains humic substances mainly HA and FA in the range of 0.1 mg/L to 20 mg/L. Previous study by Fong *et al.* (2007) also found that the concentration of HA in water obtained from Sarawak river was determined to be in average of 7.69 mg/L which has within range from previous literature (2.4- 9.5 mg/L).

Reports have been stated that the presence of HA which interact with NPs could increase the NPs stability in aquatic environment (Akhil *et al.*, 2015). Petit (2004) also stated that HA contains mineral elements that are bound to HA which essential to be utilized by living organisms. Grillo *et al.* (2015) described that NOM especially HA that commonly found in water and soil is capable to interact with NPs. Ong *et al.* (2017) also stated that HA might interact with NPs directly, which eventually give effect to the behaviour of NPs when in contact with water. Likewise, the use of HA to coat NPs induces the ability to enhance the colloidal stability (Grillo *et al*., 2015). Han *et al*. (2014) found that the presence of HA has significantly enhanced the dispersion stability of ZnO NPs.

Besides, Maurer-Jones (2013) stated that the presence of humic acid might control the aggregation state of NPs as charged interaction with NPs are formed which improves the NPs stability and influence the mobility in environment. Research carried out by Akhil *et al.* (2015) found that the toxicity of ZnO NPs exposed to bacteria increases with the presence of humic acid. Akhil *et al.* (2015) has stated that the stability of particles is directly proportional to the toxicity of NPs. Most reports have suggested that the presence of HA significantly affect the level of ZnO NPs toxicity (Grillo *et al*., 2015). Therefore, it is necessary to investigate the interaction between ZnO NPs and different concentrations of HA in order to determine the toxicity to aquatic organisms.

2.3.4 Potential fate of ZnO NPs behaviours

The fate of NPs in the environment is significant to the ecosystem and human health. Exposures of NPs to the living organisms especially animal and human are determined by their fate and transport in the environment (Tso *et al.*, 2010). Moreover, the fate of commercial NPs in aquatic environment depends on their physical and chemical properties including size, surface properties of NPs and also the presence of HA in water (Dong *et al*., 2016; Tso *et al.*, 2010). Xu *et al*. (2015) also stated that the stability of NPs mainly depends on the particle properties. There are two main behaviours of ZnO NPs that have been discussed in previous studies which were dissolution and aggregation (Batley *et al*., 2011; Li *et al*., 2012; Lopes *et al.*, 2014; Zhu *et al*., 2009). Figure 2.3 shows the flow of ZnO NPs when being introduced to aquatic environment.

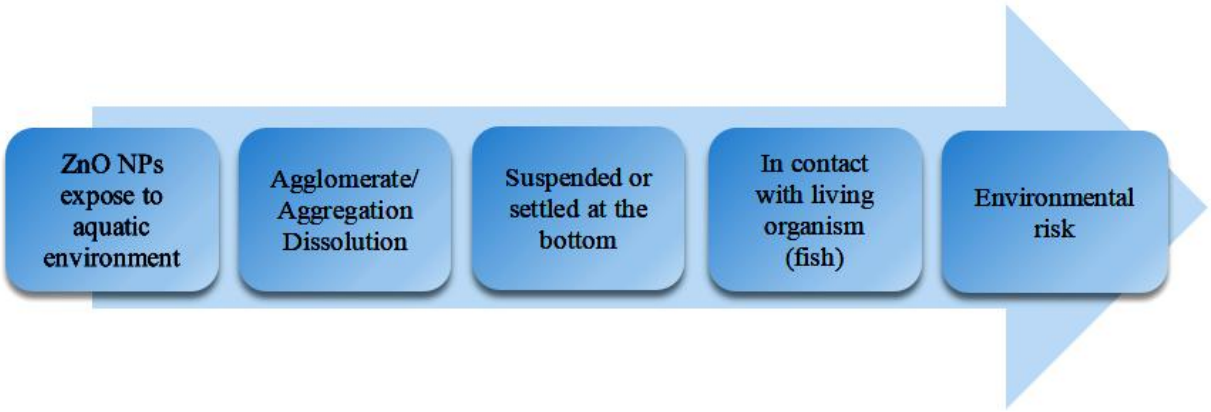


Figure 2.3: Flow of the fate behaviour of ZnO NPs exposure to the aquatic environment.

2.3.4.1 Dissolution

It is well known fact that aquatic organisms are susceptible to free metals (Schultz *et al.* 2014). A major concern that is associated with NPs is the dissolution and release of ions in aquatic environment where by the large surface area for smaller particles results in higher rate of dissolution (Wang *et al*., 2016b). Previous studies have been carried out

mainly on Ag, ZnO, and CuO NPs in which this indicate the extensive productions of NPs in various applications (Misra *et al.*, 2012). Dissolution has become one of the important factors which might affect their abundancy and this is often be a critical step in determining safety of NPs (Misra *et al.,* 2012; Wang *et al.*, 2016b; Zhang *et al*., 2010). Dissolution of NPs is defined as a dynamic process whereby constituent molecules of the solid migrate from the surface to the bulk solution through a diffusion layer (Borm *et al.*, 2006; Misra *et al.,* 2012).

This process is controlled by the solubility of NPs which depends on the size and surface properties of NPs that might give impact to the behaviour of NPs in environment (Misra *et al.*, 2012). Theoretically, solubility increases when the particle size of NPs decreases. The ability of NPs to dissolve can effectively influence their persistence in the environment and act as a critical control on their biological response. Studies by Brunner *et al.* (2006) and Xia *et al.* (2008) have stated that dissolution lead to delivery of highly toxic ions by NPs that has elements that is known to be toxic such as Zn^{2+}, Cu^{2+}, Cd^{2+}, and Ag^{+}. Misra *et al.* (2012) stated that dissolution has greatly affected the uptake pathway, toxicity mechanism, and environmental compartments.

Due to increased awareness of NPs solubility, studies on ZnO NPs in an aquatic environment have been actively conducted to better interpret better biological response of NPs. In such environment, ZnO NPs are known to be dissolved to release Zn^{2+} ions that leads to toxicity in living organisms (Han *et al.*, 2014). Previous studies have compared two different sizes of ZnO NPs to determine the level of dissolution produced and found out that there was no significant difference between nanoparticles and bulk particles of ZnO as summarised by Misra *et al.* (2012). Studies carried out by Franklin *et al.* (2007)

and Xia *et al*. (2008) has concluded that ionic Zn play a major role in triggering toxic responses.

Apart from intrinsic properties of NPs, characteristics of surrounding media mainly the presence of organic components can also affect the suspension stability of NPs in which it further affect the dissolution of NPs (Misra *et al*., 2012). Based on study carried out by Han *et al.* (2014), level of dissolution of ZnO NPs with the presence of humic acid was found to be higher as compared than that the absence of humic acid. Moreover, as the amount of humic acid increase, the level of dissolution of ZnO NPs also increase as stated by Han *et al*. (2014). Studies by Bian *et al.* (2011) also found that the presence of humic acid has enhanced the dissolution of ZnO NPs at high pH. Misra *et al.* (2012) has emphasized the importance of determining the dissolution of NPs when conducting toxicological studies of NPs in order to investigate the biological effects of NPs. Hence, it is crucial to gain information of ZnO NPs dissolution in an aquatic environment in order to gain an insight especially on the stability and the potential toxicity of ZnO NPs (Han *et al*., 2014).

2.3.4.2 Aggregation

The aggregation and agglomeration of NPs also plays an important role in their fate in aquatic environment. According to Schultz *et al.* (2014), this process is dependent of the biotic and abiotic factors. Agglomeration is defined as the reduction of NP actives sites that decrease their stability in water column (Schultz *et al*., 2014). This behaviour was reported to cause exposure reduction where by it might also reduce the toxicity to test organism, however, this behaviour might pose threat to benthic organisms as the particles

have sediment to the bottom of the lakes or river (Batley *et al.,* 2012). In aquatic environment, NPs are known to adsorb molecules to form so called corona which could increase the size of NPs and cause agglomeration to happen (Yung *et al*., 2014). Hence, a close monitoring on the size of NPs agglomerates and aggregates is important in order to predict the distribution of NPs and to determine the bioavailability of NPs to aquatic organisms in the environment.

pH and the ionic composition of water also affect the interaction of NPs in water (Zhang *et al*., 2007; Tso *et al*., 2010). Nowack *et al*. (2007) described that aggregation is particle-size dependent and result in efficient removal of small particles in environmental system. Studies carried out by Tso *et al*. (2010) has stated the occurence of aggregation was due to the increased of NP size when the concentration of exposure increased. that the NPs size has increased when the concentration increased. Tso *et al.* (2010) explained that although same concentration was prepared however smaller NPs has larger diffusion coefficient as compared to larger NPs that aggregate rapidly in water.

Apart from that, the effect of pH also plays an important role on the aggregation of NPs. This is because the pH affects the stability of surface charge and particle interaction. Tso e*t al.* (2010) conducted a study and found that ZnO NPs aggregated rapidly when pH is in acid condition that this explains the existence of dissolved zinc in water. Modunkotuwa *et al.* (2011) has also mentioned that the ZnO NPs tend to aggregate which resulted in the formation of micrometer sized particles at neutral condition (pH 7).

However, the presence of humic substance such as HA has the ability to reduce agglomeration and aggregation of NPs as reported by Xie *et al*. (2008). Studies on ZnO NPs has shown a stable condition when HA was present (Tso *et al*., 2010). O' Connell *et al.* (2002) has carried out similar research and described that the humic polymers and

surfactants could create more thermodynamic favourable surfaces and induce steric and electrostatic stabilization.

2.4 Zebrafish as Test Organism

Most NPs have been tested to most organisms including microorganisms, daphnia, crustaceans, fish, and mammals. Due to high cost and long term assessment of toxicity testing in mammals, zebrafish was commonly used as an alternative testing organism in toxicity studies. Most NPs studies were carried out on zebrafish as the immediate effect can be observed in-vivo after such exposures.

Zebrafish is a small-bodies fish that commonly found in freshwater across Asia. Chakraborty *et al*. (2016) has stated that zebrafish has become a smart vertebrate model for toxicological studies especially in NPs. Nishimura *et al*. (2016) also described the benefit of using zebrafish in toxicological studies. Zebrafish has become one of the most useful model organism for high-throughput development toxicity testing. The development process is highly conserved across vertebrate species, making zebrafish development largely comparable to that mammalians. Using zebrafish for NPs toxicological assessment possess various advantages including their year round prolific breeding, small sizes, high number of eggs productions, rapid development, and the transparency of the embryo (Zhu *et al.*, 2012; Ong *et al*. 2017). Their hatching process only requires 2-days after fertilization thus, reduced the time of exposures of any pollutants for toxicity studies (Meyers, 2018).

Furthermore, Ong *et al*. (2017) has also stated that early development stages are the most sensitive to toxicants. Thus, the embryonic zebrafish are considered to be the most

suitable test organisms to assess aquatic hazards especially involving NPs. Organisation for Economic Co-operation and Development (OECD) (2013) has developed test guidelines (TG236) regarding the use of zebrafish for toxicity testing. The establish guidelines provide more effective protocols when carry out toxicity testing on model organisms such as zebrafish. Therefore, this NPs toxicity study was conducted by referring to OECD protocols with slight modifications.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Zinc acetate dehydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$), humic acid, commercial ZnO NPs were purchased from Sigma-Alrich, absolute ethanol (HmbG), potassium hydroxide (KOH) (Amresco, USA), sodium chloride (NaCl) (Duchefa, Netherland), calcium chloride ($CaCl_2$) (Merck, Germany), magnesium chloride ($MgCl_2$) (Amresco, USA), potassium chloride (KCl) (Duchefa, Netherland), potassium bromide (KBr), distilled water, deionized water, ultrapure water.

3.2 Methodology

3.2.1 ZnO NPs Synthesis

This study was required to compare three different size of ZnO NPs. Commercial ZnO NPs (Sigma-Aldrich, USA) with the size labelled <50 nm and <100 nm were used in this study whereby smaller ZnO NPs which was expected to be <10 nm was synthesized which adopted from Bian *et al.* (2011) with some modifications.

Figure 3.1 shows the schematic diagram of the synthesis process of ZnO NPs via solvothermal method. Briefly, 1.48 g of zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H2O$) was dissolved in 62.5 mL ethanol under 60 ^oC. Next, 0.74 g of potassium hydroxide (KOH) was also dissolved in 32.5 mL ethanol. Then, KOH solution was added dropwise into $Zn(CH_3COO)_2 \cdot 2H_2O$ solution under vigorous stirring at 60 ^oC and left for 3 hours until the reaction is complete. Once the reaction was completed, solution was centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the white product was washed with acetone twice and deionized water once. Lastly, the product was dried at room

temperature and ground to obtain powdered form ZnO NPs. All the samples were then prepared for characterization.

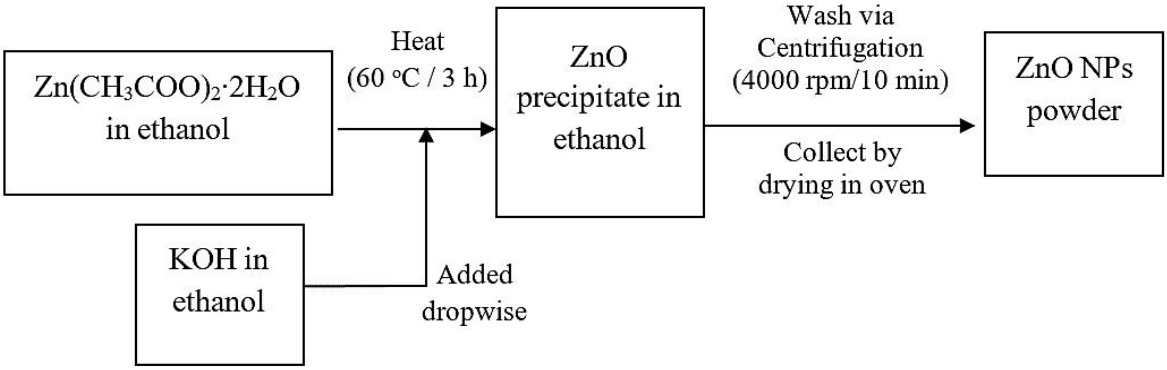


Figure 3.1: Schematic diagram of preparing ZnO NPs via solvothermal synthesis method.

3.2.2 ZnO NPs and HA Stock Preparation

Stock solutions for three sizes ZnO NPs with concentration of 100 mg/L were prepared by adding 0.05 g of ZnO NPs powder into 0.5 L Schott bottle containing 0.5 L ultrapure water prior for characterization. All three solutions were then sonicated using Ultrasonic cleaner (Elma, Germany) for 30 minutes that was also described by Oliviero *et al.* (2017). All the samples were then sent for characterization. All the steps were repeated for HA preparation.

3.2.3 HA-ZnO NPs Preparation

Since this study also determine the interaction of ZnO NPs with presence HA, a mixture of ZnO NPs (100 mg/L) with HA was prepared. Three different concentration of HA were used in this study mainly 5 mg/L, 20 mg/L, and 50 mg/L. All the solutions were

sonicated for 30 minutes and then were left overnight. The solutions were then centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the product formed at the bottom of the tube was collected and dried prior to further analysis by using FT-IR according to Yang *et al.* (2009).

3.2.4 ZnO NPs, HA and HA-ZnO NPs Characterization

Prior to physical characterization, ZnO NPs powder was dispersed in absolute ethanol which was based on procedure by Bian *et al.* (2011) in which TEM (JOEL model 1230) was used in order to measure the particle size and observe the primary shape of ZnO NPs. Basically, copper grid coated with fomvar solution was used for TEM analysis. 4 µl of sample was loaded onto each of the copper grid prepared and were allowed to dry for 10 minutes in the oven at 50^{o}C before being sent for TEM observation. All the images were collected and the shape was determined as well as the size of 200 particles were measured randomly. All the sizes were recorded and the mean size of ZnO NPs were calculated to determine the particle size distribution.

Apart from physical characterization, chemical characterizations were also carried out using Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), and ultraviolet visible (UV-vis) spectroscopy.

For XRD analysis, characterization process was only applied to ZnO NPs only. This method was used to determine the crystal structure phase and primary crystal size pf ZnO NPs. The XRD pattern was obtained using X-ray diffractometer with Cu-Kα radiation of 40 kV and 30 mA with step size of 0.017^{o}. The crystal primary size was calculated according to Debye-Scherrer's formula :-

Scherrer's Equation:

$$\text{Particle size } (D_p) = \frac{0.89\ \lambda}{d \cos\theta}$$

Where 0.89 is Scherrer's constant, λ is a wavelength of X-rays, θ is Bragg diffraction peak, and d is full width at half maximum (FWHM) of diffraction peak.

Besides, Brunauer-Emmett-Teller (BET) (Quantachrome, US) was used to analyse the surface area of the synthesized ZnO NPs. About 0.3 g of ZnO NPs powder were placed in the tube and was allowed to degas at 175^{o}C for 2 hours as referred to Zhou et al. (2015) in flowing nitrogen. The N_2 absorption-desorption isotherms of samples were then be measured. Energy-dispersive X-ray spectroscopy (EDX) was also being used for ZnO NPs characterization. EDX (JOEL 6390LA, Japan) was used in order to determine the purity of synthesized ZnO NPs.

UV-vis spectrophotometer (Perkin Elmer Lamda 25) was also used for ZnO NPs and HA characterization. ZnO NPs and HA solutions were analysed using UV-vis spectrophotometer within frequency range from 100 nm to 600 nm. The absorption peak obtained from the spectrophotometer were recorded and then compared with the findings from previous literatures for confirmation purposes.

Meanwhile, FT-IR was used to determine the functional group of ZnO NPs, HA, and HA-ZnO NPs, respectively. 0.01 g of sample was mixed with 0.09 g of potassium bromide (KBr) as described by Yang *et al.* (2009). The mixture was ground gently using pestle and mortar before being put into a metal hole. The content inside the metal hole was pressed until a thin disc shaped layer formed before being analysed via FT-IR. The spectra obtained was recorded from 400 to 4000 cm^{-1}. The adsorption of HA-ZnO NPs was also

being analysed via this method as this method was published previously by Yung *et al.* (2011).

3.3 Zebrafish Husbandry and Maintanence

Adult zebrafish was purchased from Beau-fish Worldwide Sdn. Bhd., Kuala Lumpur, Malaysia. The fish was maintained in 25 L aquarium tank at approximately 28 ^{o}C of dechlorinated water with 14 h and 10 h light and dark condition of respectively. Fish were monitored on daily basis and fed with dry flakes twice a day. The water was also changed once every month depending on the quality of water condition. This protocol was referred from Nishimura *et al.* (2016).

3.3.1 Zebrafish Breeding Process and Embryo Collection

Breeding process was carried out in the late evening by placing a pair of male and female adult fish (1:1) into 2 L tank separated by a net and was filled with dechlorinated water as shown in Figure 3.2. Female zebrafish was identified to have larger white, round belly whereas male zebrafish was smaller, have flat belly, and more active as compared to female zebrafish as shown in Figure 3.3. Male and female fish were left overnight in the dark and spawning was triggered on the next morning once the light present. After spawning was triggered, these fish were put back into their original large tank and all the embryos were then collected.

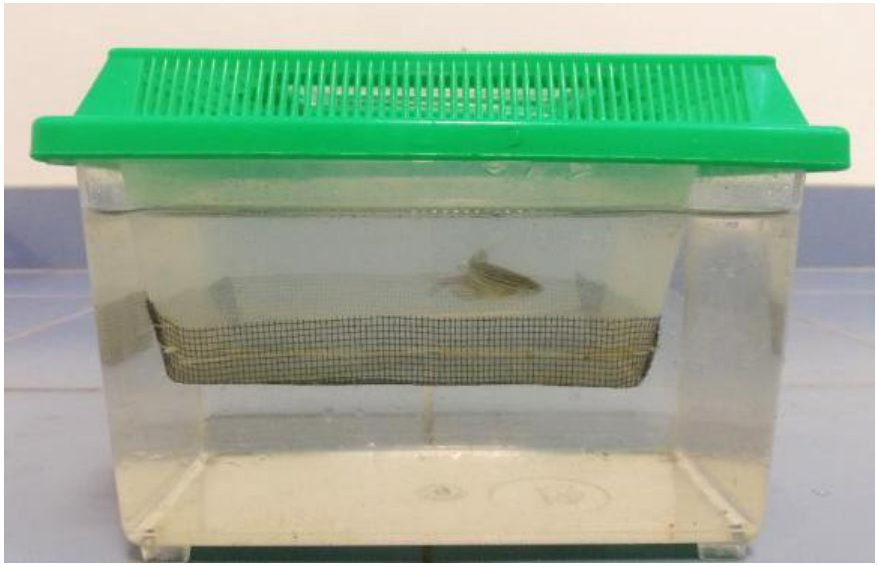


Figure 3.2: Breeding set-up of adult zebrafish.

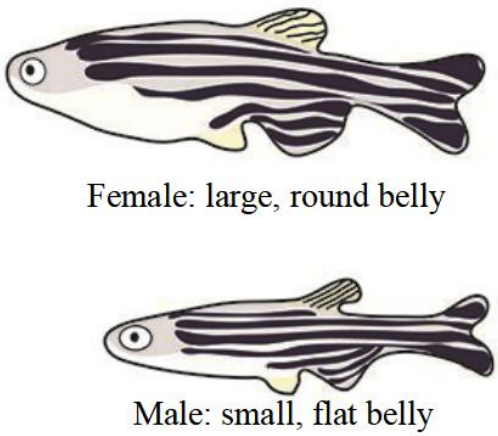


Figure 3.3: Zebrafish morphology between male and female.

All the embryos were collected after 45 minutes after spawning by filtering the fish water using strainer, gently washed with tap water to remove all the impurities, and put into a petri dish containing embryo water. The embryos water was prepared according to zebrafish module by Westerfield (2000). 10 µl of methylene blue was added into freshly

prepared 1 L embryo medium to reduce bacterial and fungal growth in the medium. Selection of healthy embryos were done by observing the embryos under microscope (Olympus SZ2, Japan). All unfertilised and dead embryos were removed. The healthy embryos were then incubated in dark under 27 ^{o}C in the incubator (Thermo Scientific) for further experiments (Kteeba *et al.*, 2017; Wehmas *et al*., 2015).

3.2.7 Toxicity Assessment in Embryonic Zebrafish

Different concentrations of three sizes ZnO NPs were exposed to the embryonic zebrafish for the toxicity assessment. This study was carried out according to OECD protocol (2013). 100 mg/L of ZnO NPs solution was prepared and diluted into respective concentrations (1, 5, 10, 30, 50, and 100 mg/L). The solutions were adjusted to pH 6.5- 7.5 as referred to Lacave *et al.* (2016).

What differs from other previous studies was this toxicity assessment was carried out using petri dish instead of using 24-well plate. The illustrated diagram of a set-up toxicity experimental procedures was shown in Figure 3.4. The experiment was carried out using petri dish by placing 20 embryos at gastrula stage (5-6 hpf) into each dish containing 20 ml of 100 mg/L ZnO NPs solution. For this treated group, one concentration of ZnO NPs (100 mg/L) was exposed to the embryonic zebrafish. Triplicates were prepared resulting 60 embryos were exposed to one concentration. Meanwhile, another 20 embryos were placed into another separate petri dish that only contain embryo medium which act as control group.

All the treatments were kept in dark in incubator (Thermo Scientific) under fixed temperature at 27 ^{o}C. Steps were repeated by replacing 100 mg/L to another five different

ZnO NPs concentrations (50, 30, 10, 5, and 1 mg/L). Exposure of ZnO NPs solutions were replaced every 24 hours. The test was considered valid only when the survival rate in control group was more than 90 % based on study conducted by Lacave et al. (2016). The exposure of ZnO NPs was carried out until the embryos reached 96 hpf.

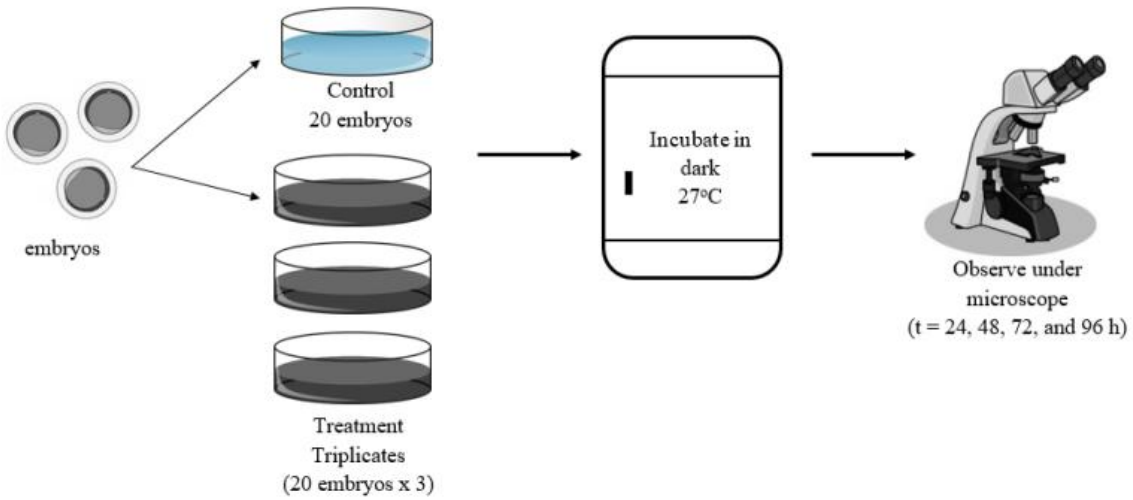


Figure 3.4: Set-up experiment for ZnO NPs toxicity assessment in embryonic zebrafish.

The endpoints including the survival rate and the hatching rate were observed under stereoscopic microscope (Olympus SZ2, Japan) and recorded every 24 h until the embryonic zebrafish reached 96 hpf. All the effects of exposed especially the morphology of embryos, survival rate, and hatching rate were observed and compared with the normal embryonic zebrafish based on Kimmel *et al.* (1995). The reference of the development of embryonic zebrafish at each stages was shown in Appendix A.

Similar exposure of ZnO NPs was repeated to embryonic zebrafish however, HA was added. Different concentrations of HA were used to mimic the concentration in the

environment. Specifically, 50 mg/L, 20 mg/L, and 5 mg/L of HA were added to six concentrations ZnO NPs before exposing to the embryonic zebrafish. Control group for this part of experiment was using embryo medium containing HA. Similar endpoint observations were determined and were compared with the exposed group of ZnO NPs without the presence of HA.

3.2.8 Dissolution and Aggregation of ZnO NPs analysis

This study also determines the possible factor that might affect the toxicity to embryonic zebrafish. Dissolution of ZnO NPs was determined by collecting water samples from the exposed embryos at 0 h and 24 h. The embryo medium were centrifuged at 3000 rpm for 10 minutes before being analysed by using ICP-OES (Perkin Elmer 8000). The dissolution of embryo medium were compared between 0 h and 24 h. ZnO NPs with the addition of HA were also being analysed using the same methods. The solutions between three sized ZnO NPs and also between HA-ZnO NPs were also being compared in order to determine the amount of Zn^{2+} ions released after 24 h and also to determine the dissolution of the solution that might be affected by the size and the presence of HA.

Besides, physical characterization was also being conducted after 24 h of exposures in order to determine the morphology of ZnO NPs that might lead to aggregation with and without the presence of HA. About 4 µl of the exposed embryo medium was loaded onto coated copper grid for TEM analysis. This analysis was carried out to observe the aggregation behaviors of ZnO NPs in the water.

3.2.9 Statistical Analysis

All the toxicity experiments were repeated three times independently, and the data were recorded as mean with standard deviation (SD). LC50 after 96 hpf exposure was calculated using probit analysis was performed to determine the significant difference between control and treated groups via Minitab17 software.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of ZnO NPs and Humic Acid (HA)

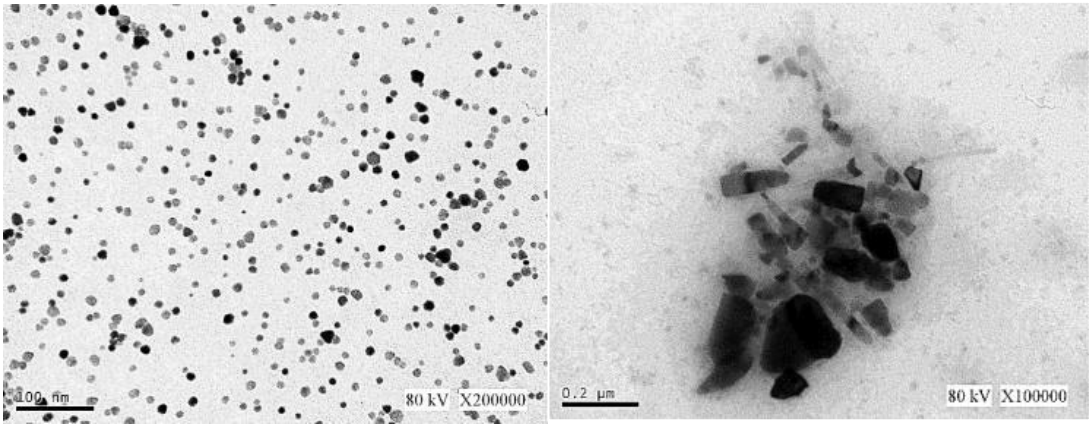
Characterization was carried out on both ZnO NPs and HA using few techniques including XRD, TEM, FT-IR, and UV-Vis spectroscopy. Laborda *et al.* (2016) has stated that characterization of NPs and HA are an important preliminary step prior to determine the properties of ZnO NPs and HA that might potentially affect the behavior of NPs and the toxicity.

4.1.1 ZnO NPs

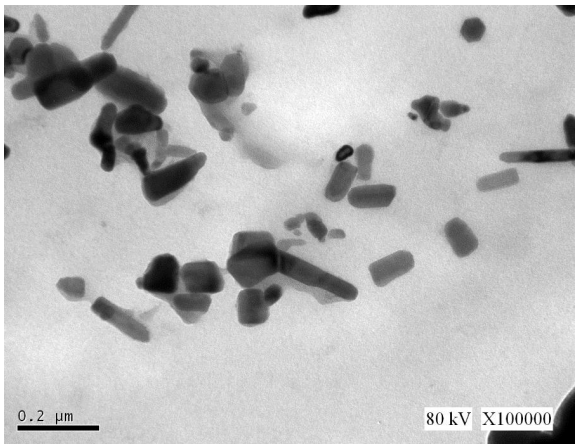
Physical characterization was carried out on three different sizes of ZnO NPs by using TEM (JOEL 1230). 200 particles from each types of ZnO NPs were measured randomly in order to obtain the particle size distribution. Table 4.1 summarised the mean particle size of three different types of ZnO NPs. Figure 4.1 shows the TEM images of ZnO NPs. The mean particle size of three different ZnO NPs were determined to be 7.4 ± 1.2 nm, 36.2 ± 10.8 nm, and 73.2 ± 11.8 nm.

Table 4.1: Mean particle size and range of ZnO NPs

Mean particle size (nm)	Range (nm)
7.8 ± 1.2	4.4 – 9.8
36.2 ± 10.8	20 – 52
73.2 ± 11.8	41 – 98



a. 7.4 nm **b. 36 nm**



c. 73 nm

Figure 4.1: TEM images of ZnO NPs. a) 7.4 nm, b) 36 nm, c) 73 nm

Besides, the graph for particle size distribution was plotted and it was found that more than 30% of the ZnO NPs have the size of 7 nm, 30 nm, and 70 nm respectively as shown in Figure 4.2. The shape was determined to be near spherical for 7 nm synthesized ZnO NPs while both commercial ZnO NPs exhibited irregular shape. Since this study was focusing on the toxicity of embryonic zebrafish due to the size and the behaviours of ZnO NPs in water thus, the shape of ZnO NPs was neglected as described by Ong *et al.* (2014) which has stated that the shape of ZnO NPs does not affect the toxicity of the tested organisms.

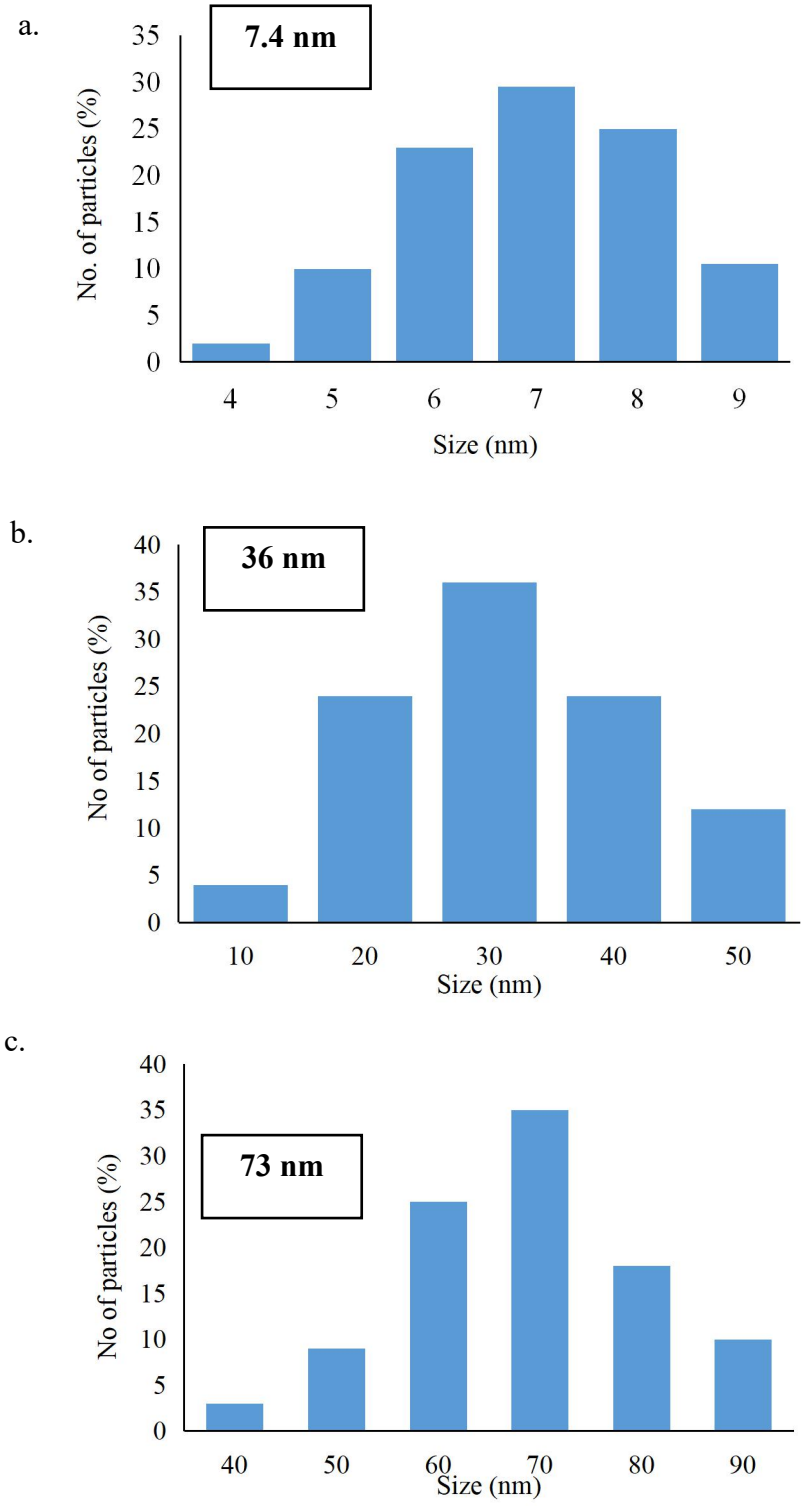


Figure 4.2: Particle size distribution of ZnO NPs (n= 200). a) 7.4 nm, b) 36 nm, c) 73 nm

Apart from that, chemical characterizations were also carried out such as FT-IR, XRD, BET, EDX, and UV-Vis spectroscopy. These characterization methods were

commonly used in order to characterize the properties of NPs (Šarić *et al.*, 2015). Figure 4.3 shows the spectrum of FT-IR of three different ZnO NPs. FT-IR was used to determine the functional group of each ZnO NPs.

FT-IR was performed in order to study and determine the functional groups of prepared synthesized ZnO NPs. Based on Table 4.2, hydroxyl (-OH) group of all three ZnO NPs were attributed to the water content from the samples that were analysed by FT-IR. This finding were commonly reported by previous literatures on the characterization of NPs using FT-IR (Bian *et al*., 2011; Yang *et al.*, 2009) . Meanwhile, the transmission peak of less than 500 cm^{-1}which are 414.87 cm^{-1}, 461.98 cm^{-1}, and 417.82 cm^{-1} respectively were attributed to Zn-O stretching vibration which confirmed the presence of ZnO NPs in accordance to the analysis done by Yang *et al.* (2009). All the observed peaks were referred from previous literatures in order to confirm the findings (Bian *et al*., 2011; Modunkotuwa *et al.*, 2011; Yang *et al*., 2009). This obtained data was also being described from previous studies regarding FT-IR spectrum of ZnO NPs properties that was also observed within range of less than 500 cm^{-1} (Lavand *et al*., 2015; Phoohikong *et al.*, 2017; Yang *et al*., 2009; Zak *et al*., 2011).

Similar findings were also found from previous studies related to ZnO NPs synthesis and characterization. The peaks at 1339 and 1556 cm^{-1} were symmetric and asymmetric O-C-O stretching vibration of adsorbed carbonate anion respectively. Meanwhile, the peaks at 1047 cm^{-1} that indicate the lattice vibration of carbonate generated absorption peaks. Besides, hydroxyl group stretching can be seen at the absorption peak of 3417 cm^{-1}. Apart from that, peaks of 1402 cm^{-1} and 1339 cm^{-1} indicate the presence of $Zn(CH_3COO)_2{\cdot}2H_2O$ that associate with CH_3 bending modes similar with result obtained by previous literature (Wu *et al*., 2011). Many ZnO NPs has been made using different

types of synthesis method however the obtained FT-IR spectrum regarding ZnO NPs synthesis have shown similarities (Jayaseelan *et al.,* 2012; Yang *et al*., 2009). Therefore, FT-IR result has shown to be high purity of synthesized ZnO NPs. Wu *et al*. (2011) has stated that this technique provides information about surface functional group that are present on surface that give a useful description of surface speciation.

Apart from that, XRD was also conducted for ZnO NPs characterization. XRD was used in order to determine the crystalline phase of ZnO NPs. Figure 4.4 shows the XRD pattern of three different sized ZnO NPs.

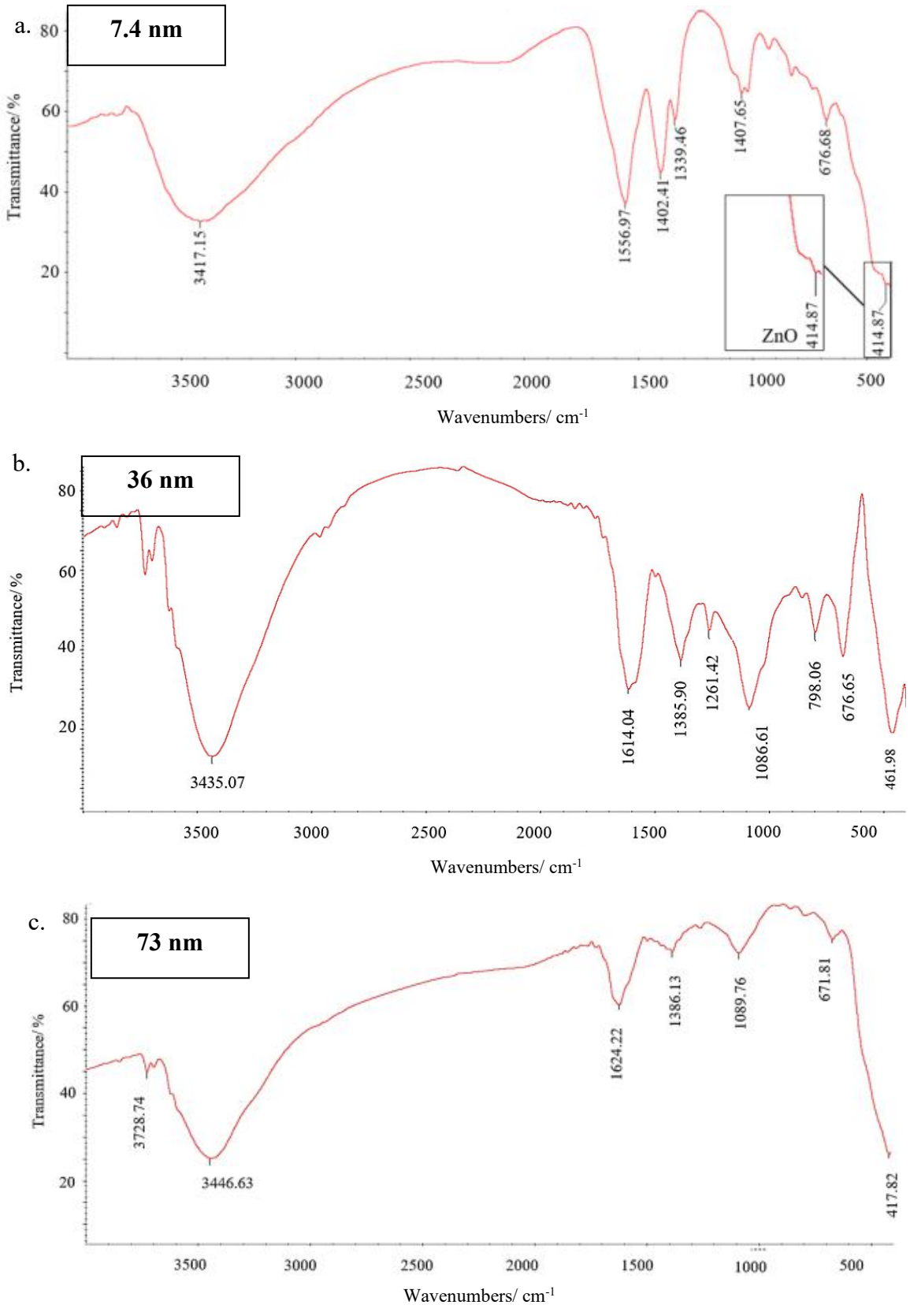


Figure 4.3: FT-IR spectrums of ZnO NPs a) 7.4 nm b) 36 nm c) 73 nm

Table 4.2: FTIR Spectra of commercial and synthesized ZnO NPs.

Size	7.4 nm	36 nm	73 nm
Hydroxyl group (-OH)	3417.15	3435.07	3446.63
COO- asymmetric stretching	1556.97	1614.04	1624.22
COO- symmetric stretching	1402.41	1385.90	1386.13
Zn-O stretching vibration	414.87	461.98	417.82

Based on Figure 4.4, all three types of ZnO NPs have shown similar broad peaks that represent ZnO. The primary crystal size of ZnO NPs was determined by using Debye-Scherrer's formula as mentioned in the methodology. The crystal size of three different ZnO NPs were determined to be 10.08 nm, 26 nm, and 61 nm respectively. Besides, XRD also determine the phase of the ZnO NPs. Based on the pattern obtained, all three ZnO NPs have similar peaks as the XRD pattern from standard reference of zinc shown in Appendix C. Thus the similarities also revealed that all three ZnO NPs exhibited wurtzite phase. Apart from that, XRD data also revealed the percentage of zinc content in all three ZnO NPs.

The obtained data showed that all three ZnO NPs have more than 95 % zinc content. This finding is very important especially to the synthesized ZnO NPs (7.4 nm) as this has proven that the synthesis process was carried out accordingly and no contamination from other substances was being detected. This also shows that the synthesized ZnO NPs (7.4 nm) is in high purity (99%). Hence, XRD analysis is one of essential preliminary steps to characterize ZnO NPs as not only able to determine the crystal size but also able to determine the phase and the purity of the ZnO NPs that are important especially in ZnO NPs productions in industries.

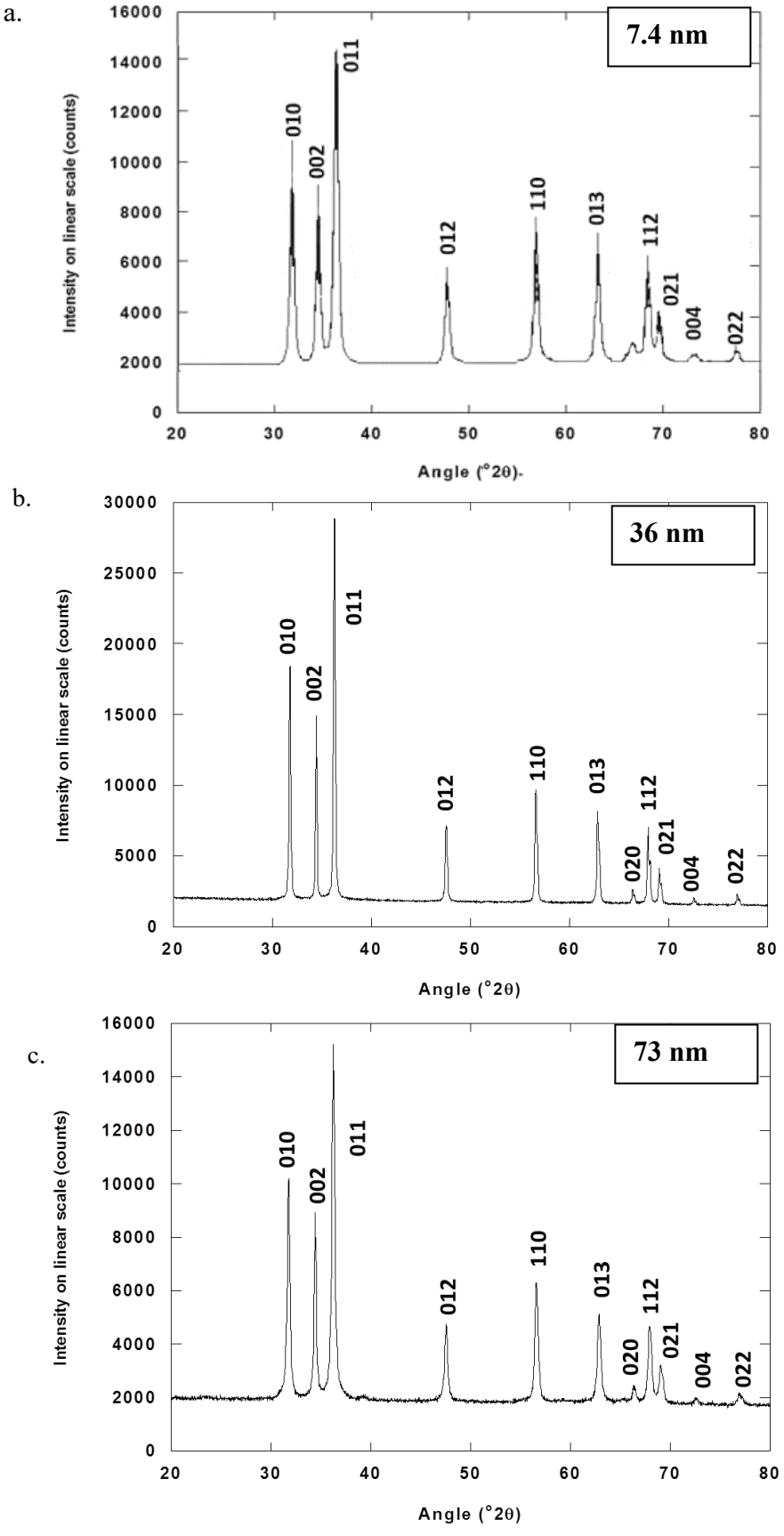


Figure 4.4: XRD spectra of three different sized ZnO NPs. a) 7.4 nm, b) 36 nm, c) 73 nm

Other than XRD analysis, all three ZnO NPs samples were also being characterized via Brunauer-Emmett-Teller (BET) analysis. BET was carried out in order to determine the specific surface area for three different sizes of ZnO NPs. Figure 4.5 shows nitrogen (N_2) adsorption-desorption isotherms of ZnO NPs obtained from BET analysis (Quantachrome, US). This figure shows the typical N_2 sorption isotherms of ZnO NPs where by the graph represent type IV adsorption in low pressure region. The isotherm relative was observed to be relative flat and similar result was also obtained by Zhou *et a*l. (2009). Zhou *et al.* (2009) has described that the adsorption isotherm was completely under superposition which usually occurs in micropores. Hwang and Barron (2011) has stated that type IV adsorption generally has mesoporous materials which the size range between 2 nm to 50 nm based on the IUPAC classification.

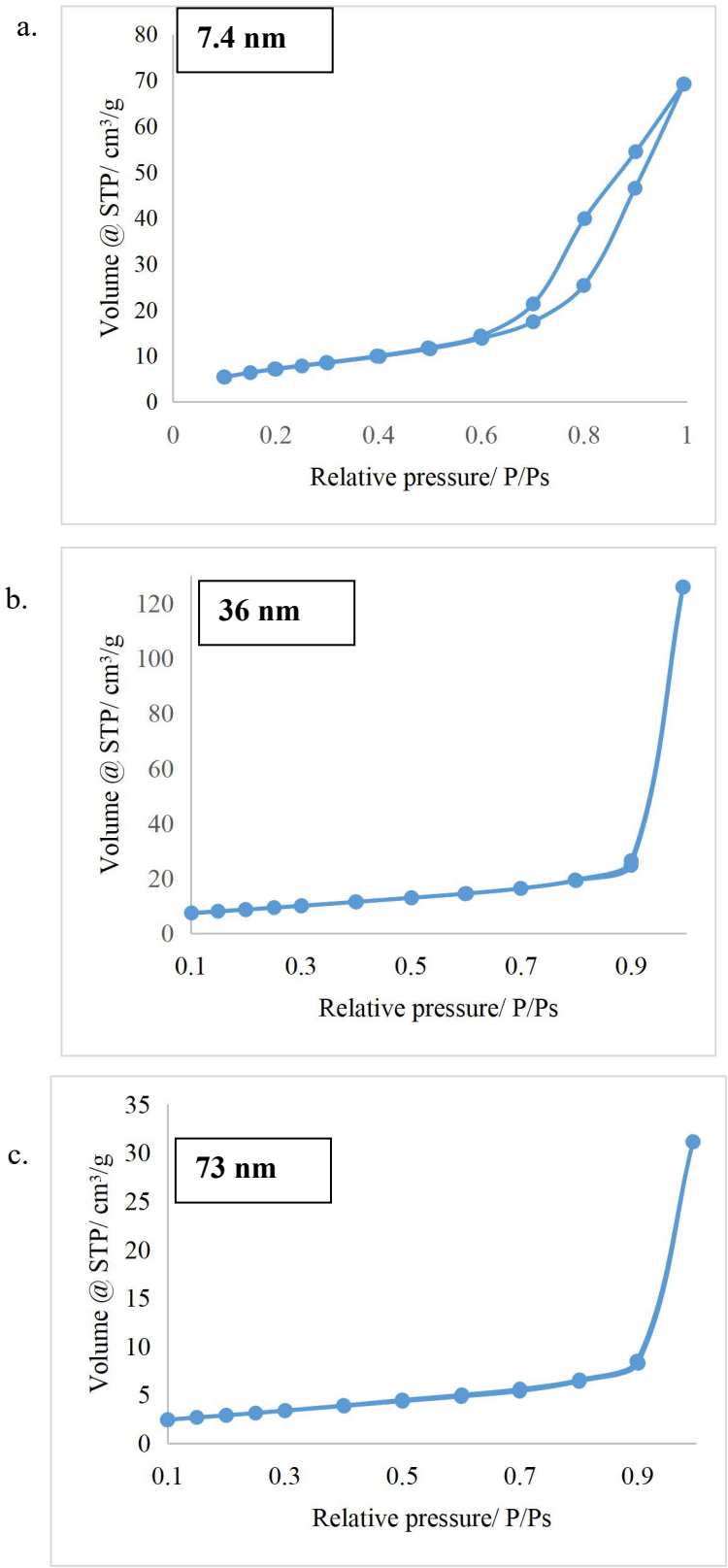


Figure 4.5: NPs adsorption and desorption isotherms of ZnO NPs

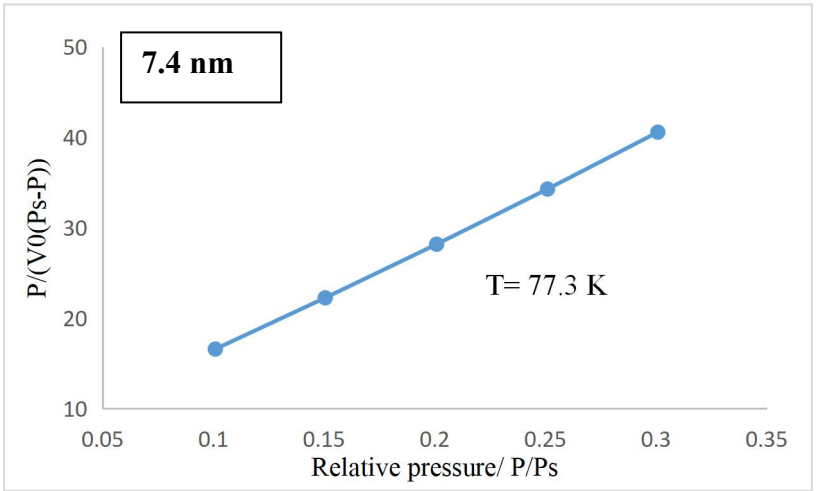
BET was carried out in order to determine the specific surface area for three different sized of ZnO NPs by N_2 adsorption temperature of 77 K as shown in Figure 4.6. The specific surface area was also determined to be 101.32 m^2/g. Similar finding was also found from previous literature by Bian *et al*. (2011) which obtained 105 m^2/g for 4 nm ZnO NPs as measured by TEM. This shows that smaller NPs attribute to high surface area. Furthermore, the average particle can also be calculated from BET data. Since the shape of ZnO NPs was determined to be in spherical shape, average particle size can be calculated based on the equation $D_{BET} = 6000/ \rho \cdot Sw$ in which D_{BET} is the average particle size, ρ is the theoretical density of the sample which was 6.11 g cm^{-3}, and Sw is the obtained surface area as referred to Zhou *et al.* (2009) and Ghasemzadeh *et al.* (2015). Table 4.3 summarised the BET results of ZnO NPs. The size of ZnO NPs obtained from BET is in agreement with the size obtained from TEM and XRD. Thus, this confirms that the size of synthesized ZnO NPs was in nanoscale which is approximately 10 nm.

Table 4.3 shows the surface area obtained from BET. This revealed that smaller NPs has higher surface area than larger NPs. Figure 4.6 shows the BET plots of ZnO NPs. The specific surface area for three sized ZnO NPs were determined that smaller ZnO NPs (7.4 nm) attribute to high surface area which is 31.32 m^2/g as compared to larger ZnO NPs (36 nm and 73 nm) which obtained 28.04 m^2/g and 10.48 m^2/g respectively. The size of ZnO NPs that were determined via TEM and XRD also in agreement with the specific surface area data obtained from BET analysis in which smaller NPs exhibit greater surface area as compared to larger NPs.

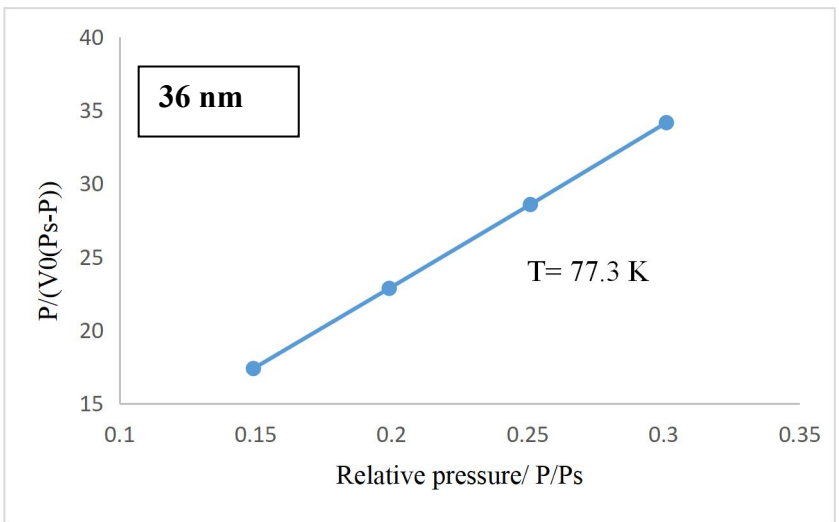
Table 4.3: BET surface area of ZnO NPs

ZnO NPs	Surface area/ m^2/g	Size/ nm
Synthesized (7.4 nm)	101.32	9.7
Commercial (36 nm)	28.04	35.0
Commercial (73 nm)	10.48	93.7

a.



b.



c.

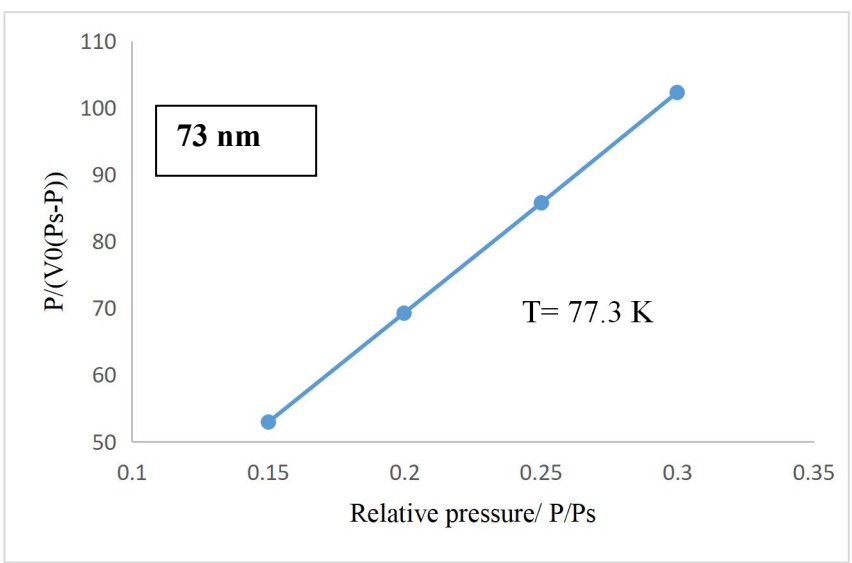


Figure 4.6: BET plot of ZnO NPs.

Meanwhile, the purity of ZnO NPs was determined via EDX analysis. Figure 4.7 shows the EDX spectrum of ZnO NPs. EDX was used in order to determine the element composition that present in the samples. Table 4.4 shows the summarised data of the element content of ZnO NPs from EDX analysis.

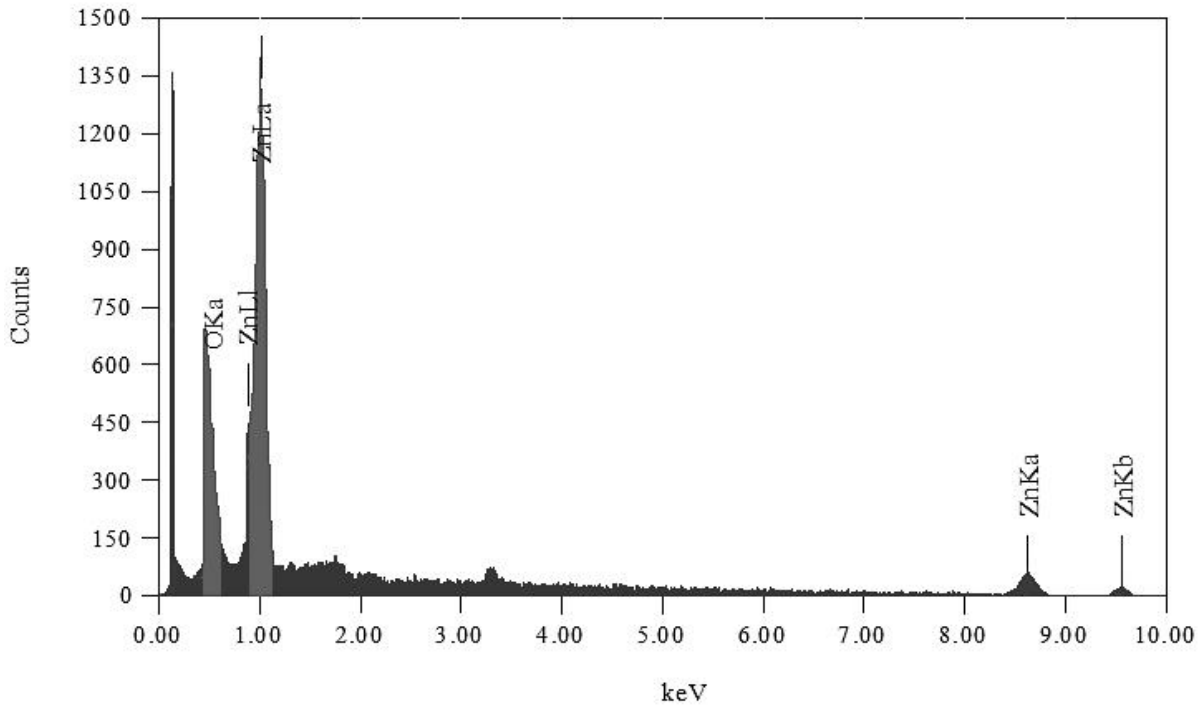


Figure 4.7: EDX pattern of ZnO NPs

Table 4.4: EDX result of ZnO NPs

ZnO NPs	**Element composition / %**	
	Zn	**O**
7.4 nm	76.3	23.7
36 nm	72.5	27.5
73 nm	71.2	28.8

Result revealed that the EDX data for ZnO NPs with the size of 7.4 nm was composed of two elements which are Zn (76.3 %) and O (23.7 %). Besides, ZnO NPs with 36 nm comprise of 72.5 % of Zn and 27.5 % of O. ZnO NPs with 73 nm also have similar element content which were 71.2 % and 28.8 %. These result has confirmed that the ZnO NPs has high purity. Similar finding was also found in previous studies by Brintha and Ajitha (2015) that obtained the mass percentage of Zn and O were 73.9 % and 26.1 % respectively. Meibner *et al.* (2104) also obtained the elemental peak of ZnO NPs mainly contain 75 % of Zn and 24 % of O element. Hasnidawi *et al.* (2016) has stated that the theoretical expected mass percent of Zn and O were 80.3 % and 19.7 %. Thus, the EDX result revealed that the synthesized ZnO NPs was in high purity which contain high Zn and O element composition.

Further characterization was also carried out where by UV-vis spectroscopy was used for ZnO NPs characterization. Figure 4.8 shows the UV-vis spectra of synthesized ZnO NPs.

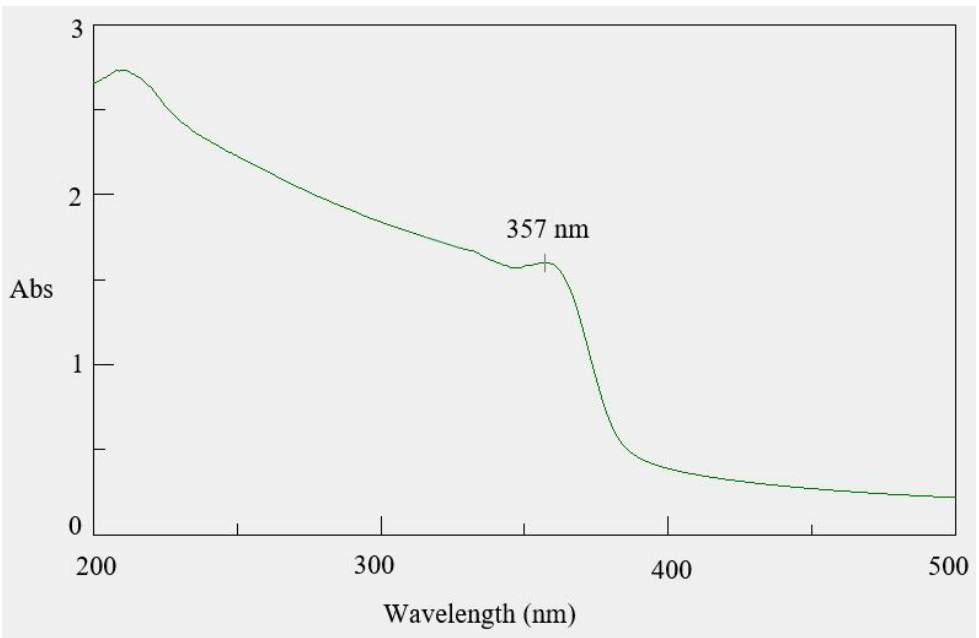


Figure 4.8: UV spectrum of ZnO NPs

ZnO NPs was dispersed in ultrapure water and then the solution was used to perform UV-Vis measurement. The absorption peak was observed at 357 nm which can be assigned to the intrinsic band-gap absorption of ZnO. For larger ZnO NPs, the absorptions were also observed at range between 300 nm and 370 nm. Similar result of absorption band that represent ZnO NPs was also obtained from previous research in which the range of absorption band were from 355 to 380 nm (Akhil *et al.,* 2017; Bian *et al.,* 2011; Lavand *et al*., 2015; Talam *et al.,* 2012; Zak *et al*., 2011).

These supporting data confirmed the presence of ZnO NPs as the absorption band obtained are similar. Wang *et al*. (2011) also obtained similar findings which deduced that the obtained peak showed a better UV absorption for ZnO NPs. Furthermore, the absorption peak of ZnO NPs also confirmed the properties of ZnO NPs, which is known for UV protections in sunscreens products (Yung *et al.,* 2014). Hence, good absorption of ZnO NPs within the UV region was one of the factor that most commercial products especially beauty care and household products contain ZnO NPs.

4.1.2 Humic Acid (HA)

Previous studies have reported the abundancy of HA present in the environment and the ability to interact with ZnO NPs. Thus, this study also focused on the presence of NOM specifically humic acid (HA) to be interacted with ZnO NPs for toxicity studies. Commercial HA was being characterized before going further to determine the interaction and the toxicity of ZnO NPs. Based on Figure 4.9, the obtained TEM image of HA shows the complex structure of HA. So far, there is no recent image of humic acid under TEM from previous literatures however, Kim *et al*. (2009) and Drodz (1978) has shown similar morphological structure of humic acid as shown in the appendix D. Figure 4.9 shows the

morphology of HA that appeared to be in clustered form. This was likely due to the existence of polymeric structures in nature as described by many researchers. This characterization is important not only to determine its own morphology but also to determine the alteration of ZnO NPs in terms of the behavior when HA is added which might affect the toxicity to aquatic environment.

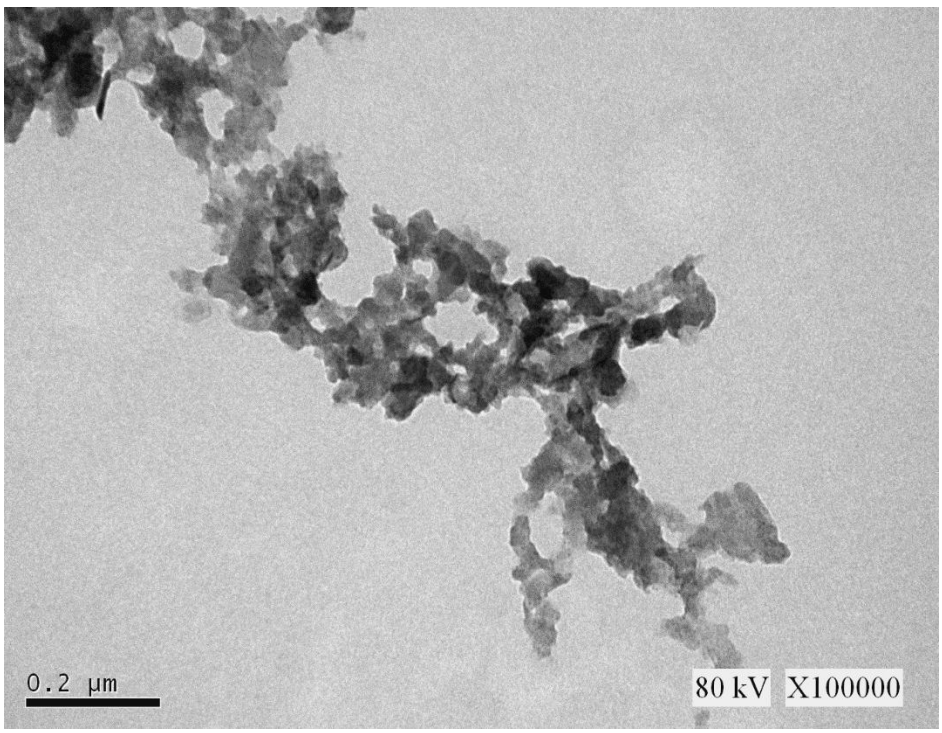


Figure 4.9: TEM image of HA

Besides, UV vis spectrum of HA was also obtained as shown in Figure 4.10. Previous studies have found that the absorption peak of NOM was located at 254 nm (Akhil *et al.,* 2015). The peak obtained was located at 254 nm that closely correlated to the previous published article (Akhil *et al.,* 2015; Bian *et al.,* 2011).

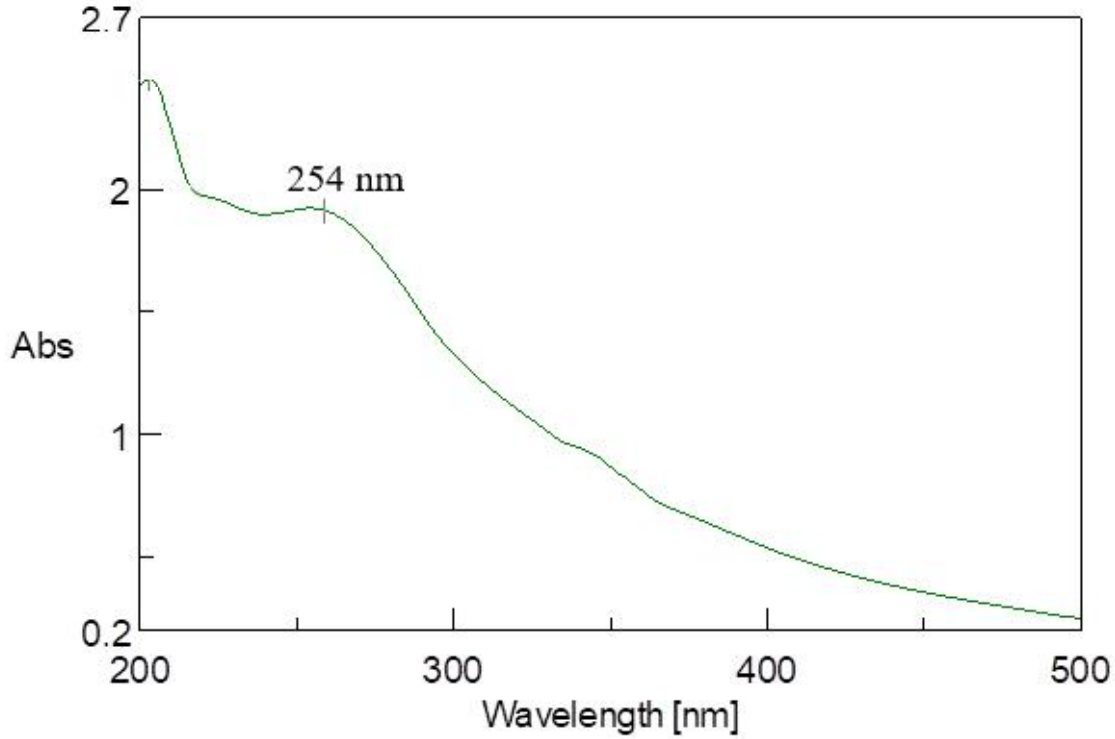


Figure 4.10: UV spectrum of humic acid (HA)

4.2 Interaction of humic acid (HA) and ZnO NPs

In this study, the interaction of HA-ZnO NPs was only determined via FT-IR spectroscopy. Previous study by Yang *et al.* (2009) has also determined the interaction via FT-IR. Figure 4.11, Figure 4.12, and Figure 4.13 show the FT-IR spectra of the ZnO NPs in the presence of humic acid. After two days of incubation, there was an addition of peaks which can be observed for ZnO NPs in the presence of HA when analysed using FT-IR. The existence of peaks can be seen despites of any concentration of HA.

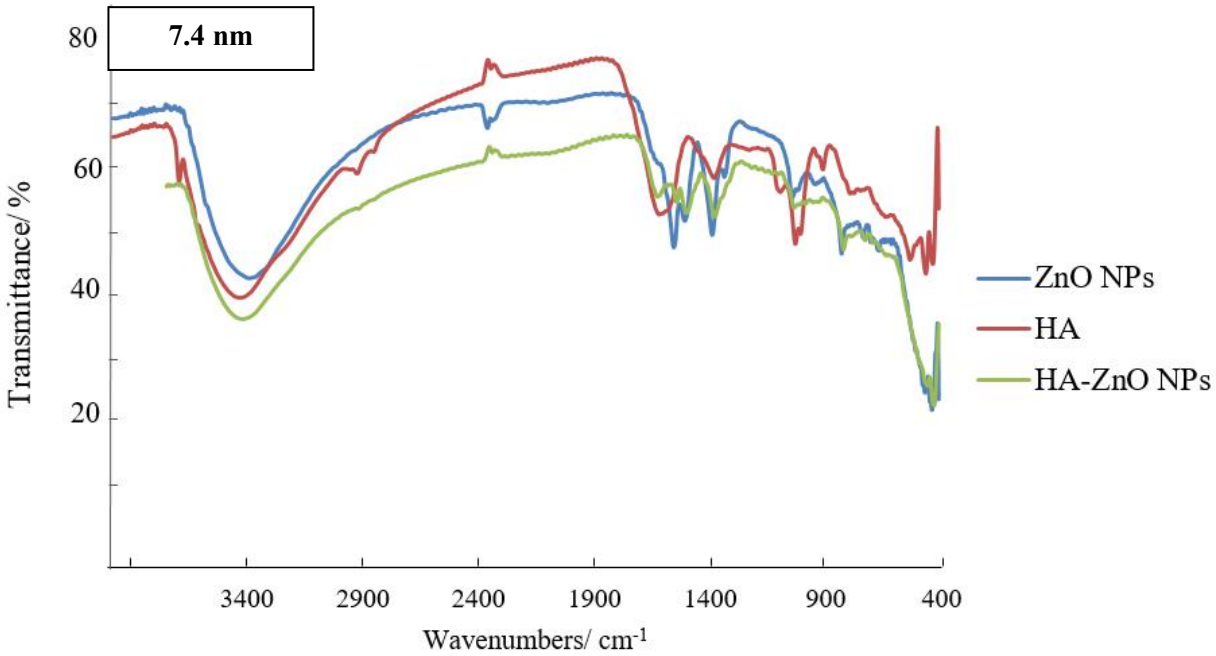


Figure 4.11: FT-IR spectra of HA-ZnO NPs a) HA b) ZnO NPs (7.4 nm) c) 50 mg/L

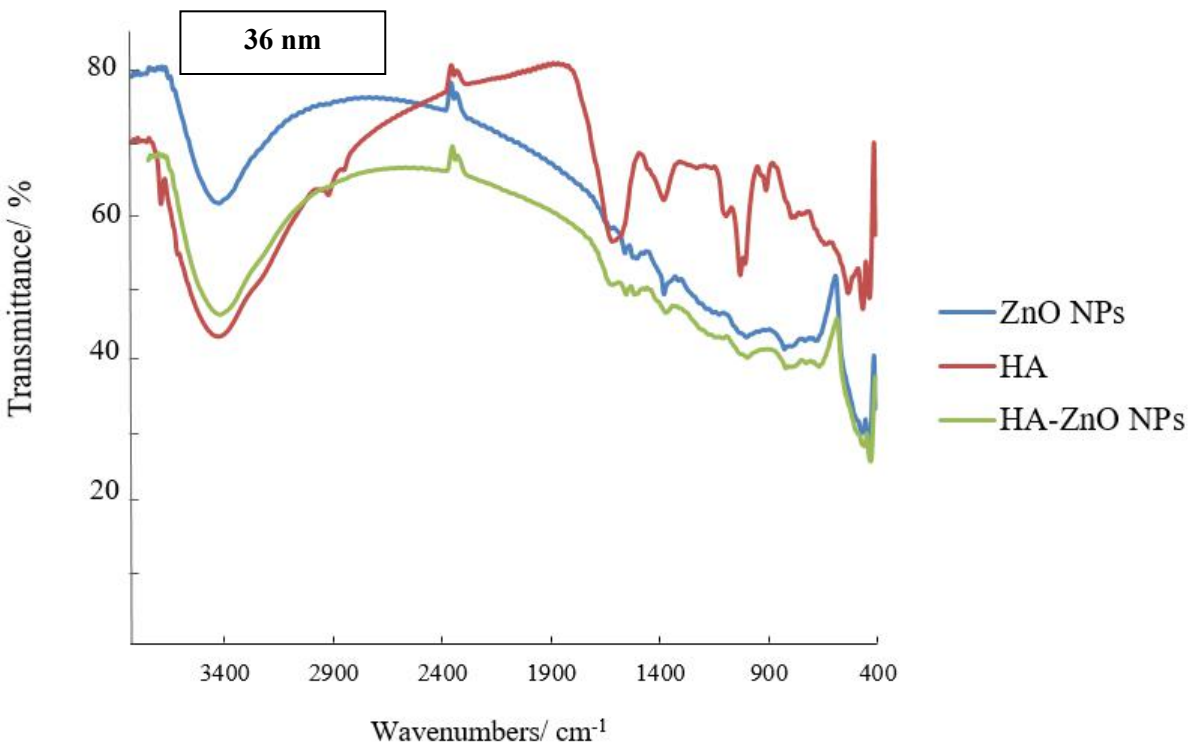


Figure 4.12: FT-IR spectra of HA-ZnO NPs a) HA b) ZnO NPs (36 nm)
c) 50 mg/L HA-ZnO

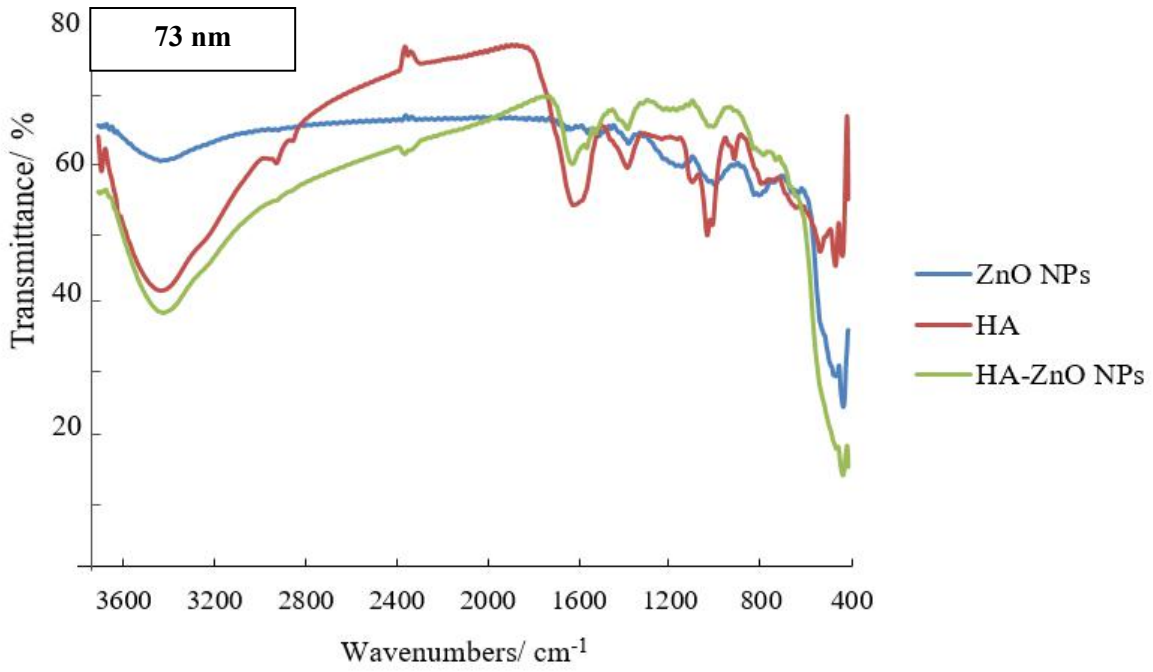


Figure 4.13: FT-IR spectra of HA-ZnO NPs a) HA b) ZnO NPs (73 nm) c) 50 mg/L HA-ZnO

All three sized of ZnO NPs have shown a similar patterns of peak when interact with HA. For instance, there were absence of peak at 1200 cm^{-1} which represent the carbonate group for all HA-ZnO NPs in which ZnO NPs do not have peak at 1200 cm^{-1} whereas HA has peak at 1200 cm^{-1}. Studies carried out by Yang *et al.* (2009) has also found the absence of carbonate group when HA presence in ZnO NPs. Furthermore, there are also absence of carboxyl group (COOH) peak diminished at 1700 cm^{-1}. Kang *et al.* (2008) andYang *et al*. (2009) explained that there was a strong interaction occurred between COOH and ZnO NPs surface that eventually cause the peak to disappear. This study also found that even at low concentration of HA, ZnO NPs can still be interacted with HA and might transform the behaviour of ZnO NPs in aquatic environment. This data

has also revealed one of important information that the HA are able to adsorb with ZnO NPs which might also explain the alteration of ZnO NPs behaviour in aquatic environment.

4.3 Embryonic toxicity of different sized ZnO NPs

4.3.1 Control group

Embryonic zebrafish were exposed with ZnO NP with and without presence of HA. As the embryos were collected, all the dead or unfertilized embryo were removed. Dead embryo was appeared to be opaque as shown in Figure 4.14 as referred from previous literature by Kimmel *et al.* (1995).

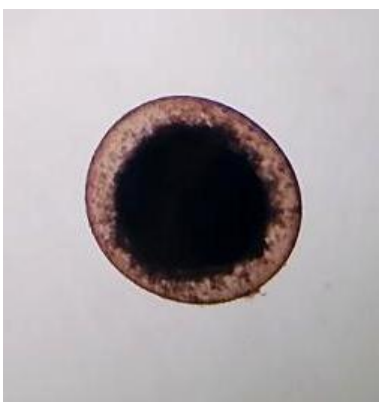


Figure 4.14: Dead embryonic zebrafish at 4 hpf.

Control group was prepared in order to observe the normal development of embryos, which was further compared with the development of embryos that exposed to ZnO NPs and HA-ZnO NPs especially in terms of the survival rate and the hatching rate after 96 hpf. Healthy embryonic zebrafish at gastrula stage (5-6 hpf) was selected for this study. The embryo water was prepared according to studies by Ong *et al*. (2017) which containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM $MgCl_2$, and 0.33 mM $CaCl_2$. Few drops

of methylene blue was added into the embryo water in order to reduce the growth of fungi and also prevent contamination (Westerfield, 2000). The experiment was considered valid if the survival of embryonic zebrafish was more than 90% which was referred to previous studies by Lacave *et al*. (2016). The development was observed until 96 hpf which was accordance to OECD (2013).

The exposure periods were until 96 hpf as this period was also being described by previous literatures mainly regarding the exposure of ZnO NPs to the embryonic zebrafish (Bai *et al*., 2010; Kteeba *et al*., 2017; Lacave *et al*., 2016; Lavand *et al*., 2016; Ong *et al*., 2013; Ong *et al*., 2017; Tomilina *et al*., 2014). Figure 4.15 shows the normal development of embryonic zebrafish that was observed from gastrula stage (5 hpf) until larvae stage (96 hpf) which act as control in further toxicity experiment. The morphology of the development embryonic zebrafish was observed according to well-known published article by Kimmel *et al.* (1995).

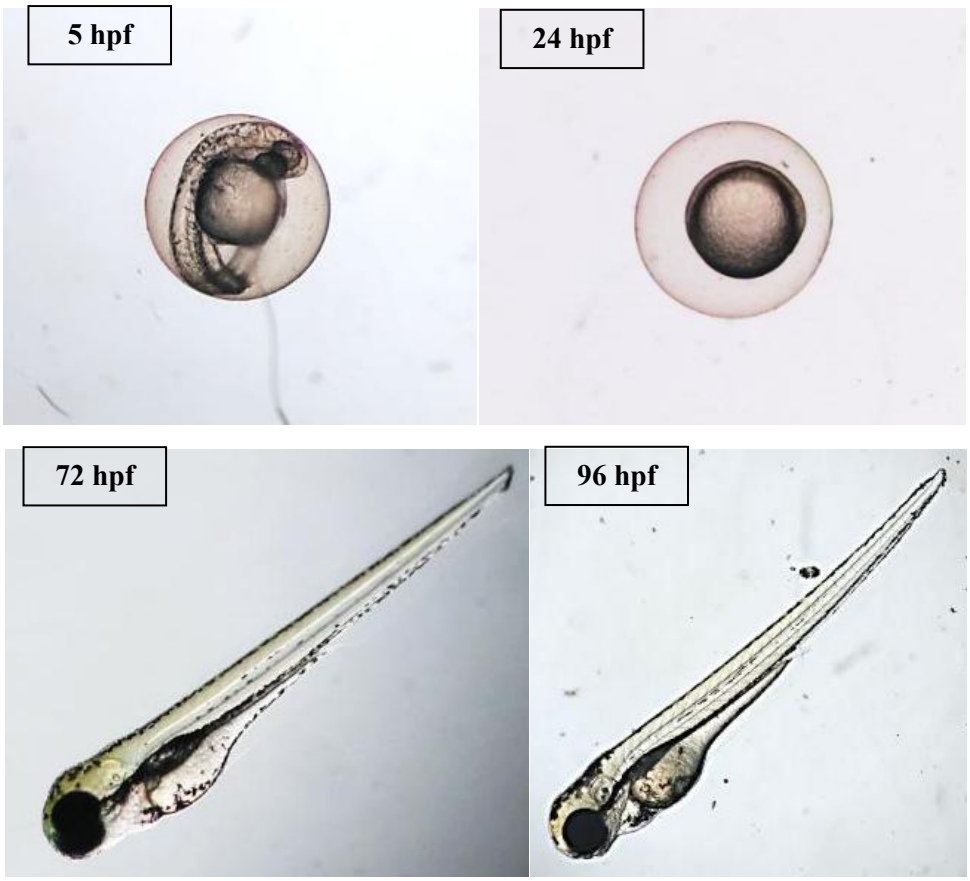


Figure 4.15: Normal development of embryonic zebrafish at 5 hpf until 96 hpf

4.3.2 ZnO NPs exposure to embryonic zebrafish

In this study, embryonic zebrafish was assessed to three different size ZnO NPs exposures at different concentrations (1, 5, 10, 30, 50, and 100 mg/L). Based on Figure 4.16, the graph has shown the significant reduction of survival rate when the concentration of ZnO NPs exposed to the embryonic zebrafish over 96 hpf increased. For all three sized of ZnO NPs, higher concentration mainly 50 mg/L and 100 mg/L have demonstrated high toxicity whereby more than 70% of embryonic zebrafish were observed died after 96 h of exposure. Among three size of ZnO NPs (Figure 4.16), smaller ZnO NPs (7.3 nm) has shown high toxicity which only 3.9 ±1.9 % was able to survive as compared to both larger ZnO NPs (36 nm and 73 nm) which 16.7 ±

3.3 % and 27.2 ± 10.8 % were survived respectively after being exposed to 100 mg/L of ZnO NPs after 96 hpf. This finding has also been mentioned in previous literatures that stated that smaller ZnO NPs has higher toxicity than larger ZnO NPs (Wehmas *et al*., 2015).

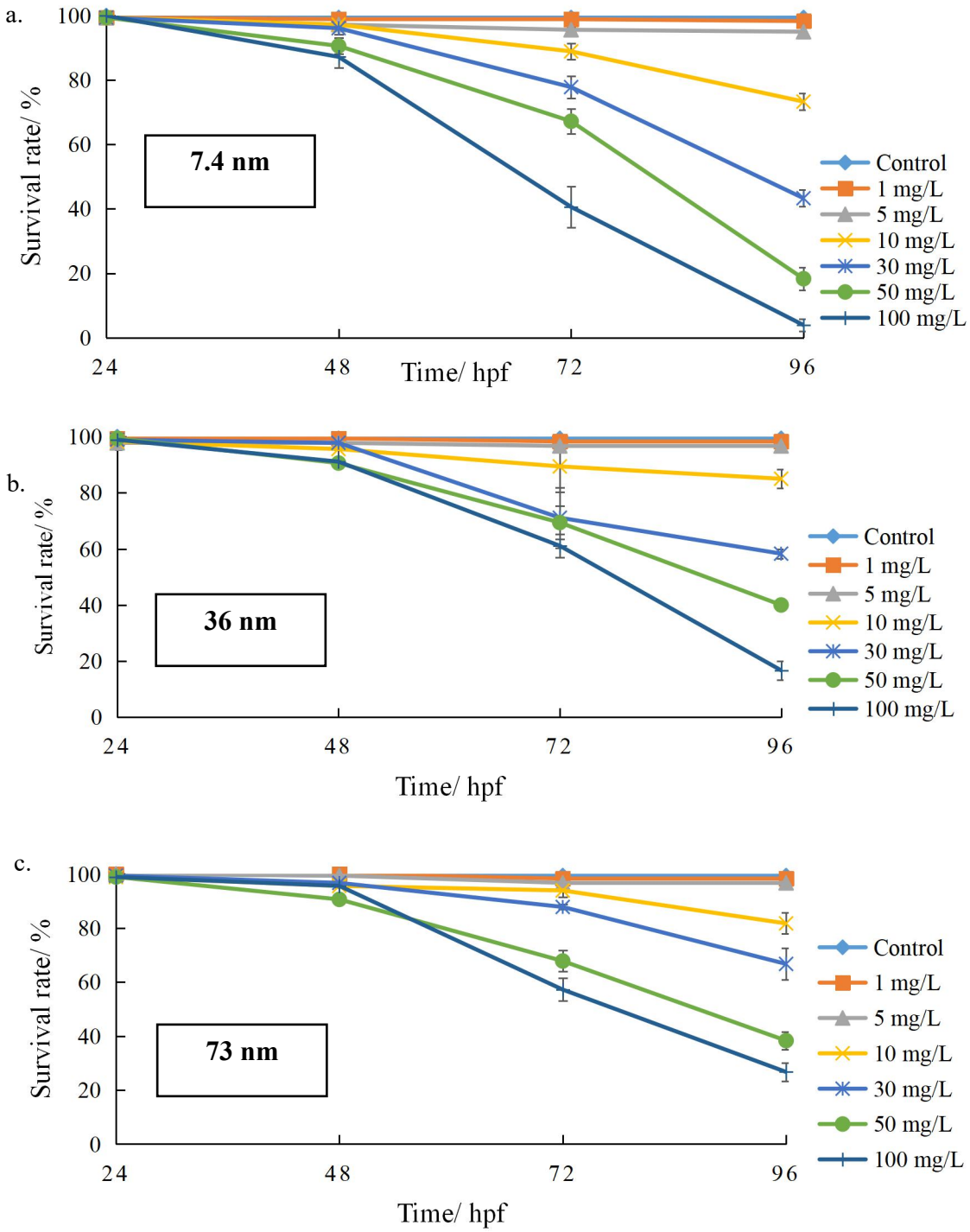


Figure 4.16: Survival rate of embryonic zebrafish exposed to different concentrations of three different sized ZnO NPs over 96 hpf. Error bars represent ± one standard deviation from the mean of three replicates.

The high level of toxicity of smaller ZnO NPs mainly due to the smaller size itself that give great surface area which able to penetrate into the chorion of the embryo with the pore size of 500 to 700 nm as described by Felix *et al.* (2013) and cause the death of the embryo. The severe reduction of survival rate exposed to ZnO NPs has revealed that the smaller size of ZnO NPs (7.3 nm) was able to penetrate through the chorion pores and affect the embryos inside the chorion. The penetration of ZnO NPs into the embryonic zebrafish was also found in this study, as shown in Figure 4.17 that the impurities observed was ZnO NPs. This eventually has affected the physiological process of the embryonic zebrafish and eventually have caused death. Santo *et al*. (2014) also found that the smaller size of ZnO NPs is more toxic as it is able to penetrate into the chorion which justified the toxicity of ZnO NPs.

However, at lower concentration of ZnO NPs mainly 5 mg/L and 1 mg/L, all three sizes ZnO NPs have shown similar pattern of the survival rate as the control group. More than 90 % of the embryos were able to survive after 96 h exposures. This finding was also found by Bai *et al.* (2010) in which about less than 10 % of mortality being observed on embryonic zebrafish when exposed to less than 5 mg/L of ZnO NPs. This finding revealed that at lower concentrations (< 5 mg/L) of ZnO NPs cause no effect to the embryonic zebrafish in terms of the survival rates.

In addition, Figure 4.17 shows the images of morphology of embryonic zebrafish in control group and treated group which was exposed to 100 mg/L at 24 hpf. In control group, there was no presence of particles attached to the surface of the embryonic zebrafish. However, for treated group after 24 h of higher concentration of ZnO NPs exposures, ZnO NPs has started to accumulate onto the surrounding of the surface of the embryonic zebrafish. Besides, the ZnO NPs was also observed at the bottom of the plate after 24 h of

exposure. This finding also correlate to previous studies by Bai *et al.* (2010), Tomilina *et al*. (2014), and Zhu *et al*. (2009) in which more ZnO NPs has attached on the surface of embryonic zebrafish when exposed to larger ZnO NPs. Further explanation was discussed in the next part of study which is the hatching rate of embryonic zebrafish exposed to ZnO NPs.

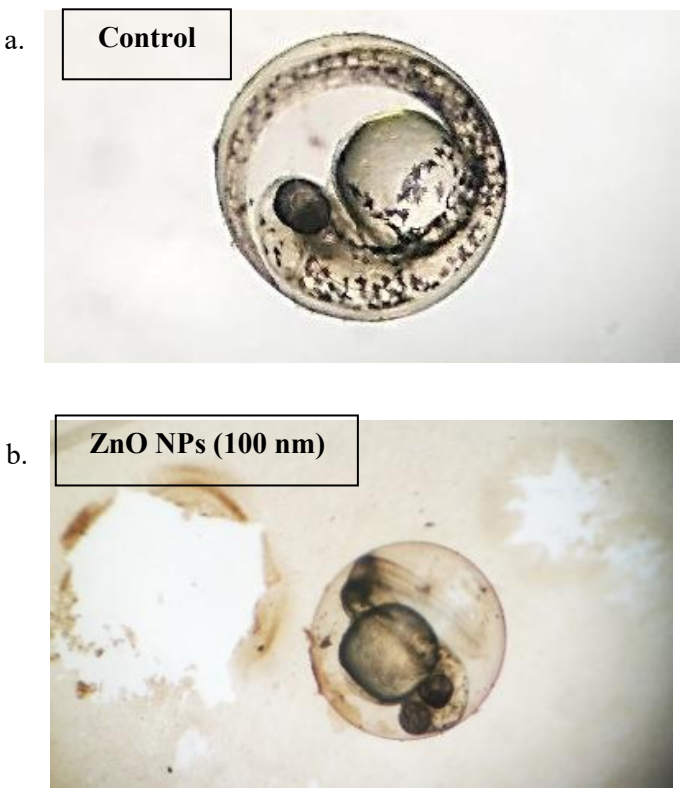


Figure 4.17: Embryonic zebrafish at 24 hpf. a) Control (Embryo water) b) 100 mg/L ZnO NPs (100 nm)

Further observation on the morphology of embryonic zebrafish was conducted after 96 hpf. Figure 4.18 has showed the morphology of the embryonic zebrafish that was exposed to ZnO NPs at 96 hpf. At higher ZnO NPs exposure (100 mg/L), the surface of the embryo has been fully covered with ZnO NPs which this ultimately causing the death of

embryos over 96 hpf. The death of embryo was appeared to be opaque and no heartbeat and movement were detected after 96 hpf. In contrast, control group has shown that the embryo has hatched and became larvae.

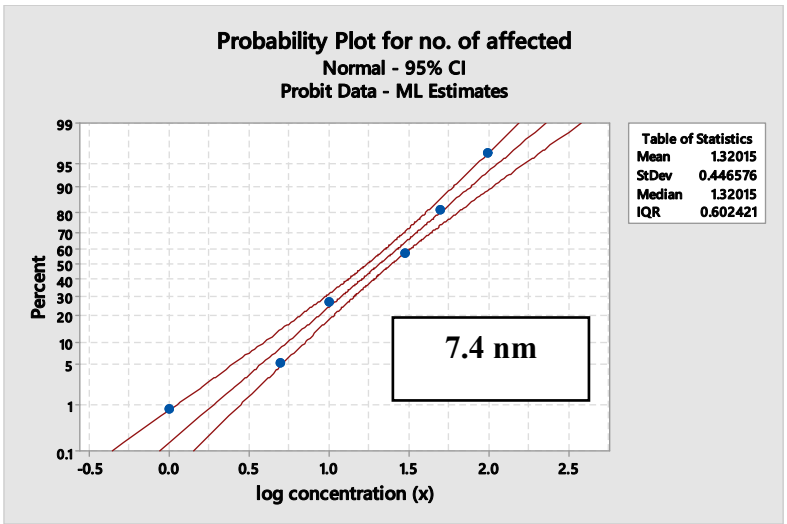
Figure 4.18: Morphology of embryonic zebrafish at 96 hpf exposed to 100 mg/L ZnO NPs (100 nm).

The adsorption of ZnO NPs onto the surface of the embryos that caused the death of embryos was mainly due to the lack of nutrient intake and oxygen exchange from the surrounding. This finding was also found by previous studies by Bai *et al.* (2010), Tomilina *et al.* (2014), and Vicario-pares *et al*. (2014) which also explained the attachment of ZnO NPs onto the surface of embryos has caused blockage that resulted in reduced nutrient intake and eventually lead to death to occur. This study has shown the toxicity of ZnO NPs is dose dependent which higher concentrations mainly more than 50 mg/L ZnO NPs has caused severe toxicity to the embryonic zebrafish especially for smaller size ZnO NPs.

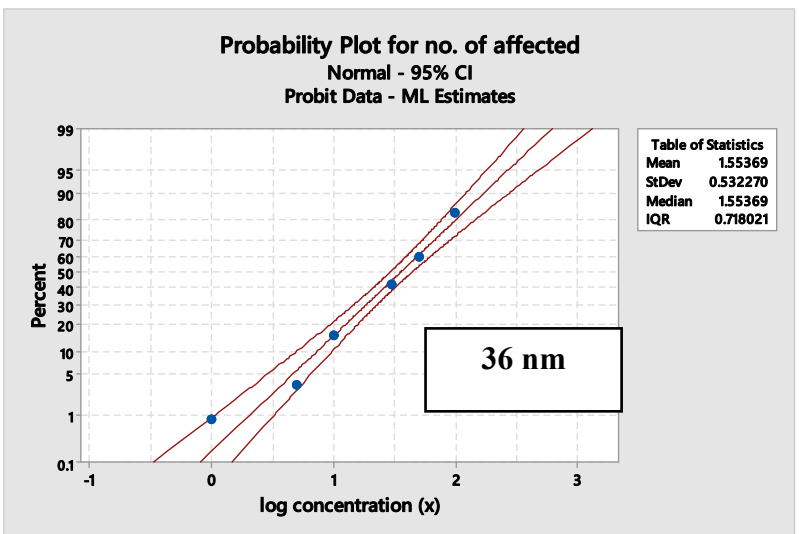
Besides, further toxicity assessment was carried out by determine the acute toxicity of three different size of ZnO NPs. The acute toxicity was determined by calculating the

median lethal concentration (LC_{50}) for three different sized ZnO NPs. Figure 4.19 shows the data obtained for 96 hpf LC_{50} of three different sized ZnO NPs by using Minitab17 software. High concentration of ZnO NPs exposed to embryonic zebrafish has caused the increase in mortality. The dots in the figure represent the concentrations of ZnO NPs that were used in this study and the percentage are the number of mortality of exposed embryonic zebrafish.

a.



b.



c.

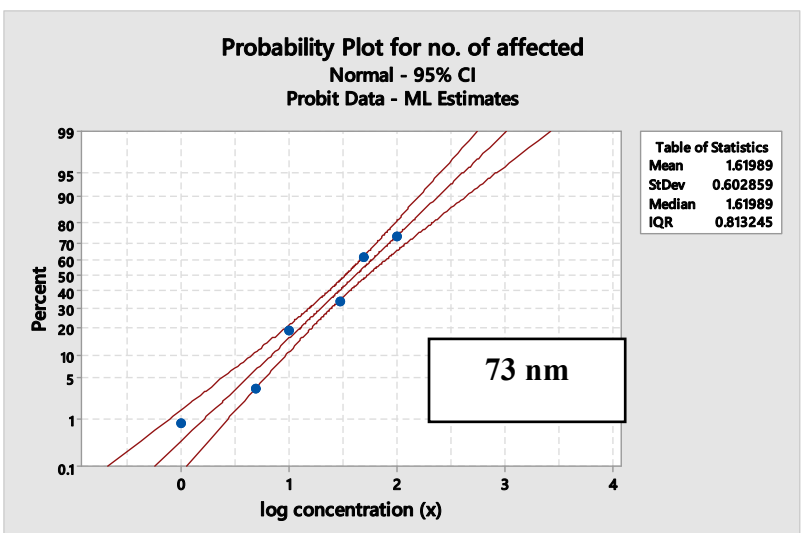


Figure 4.19: Probit analysis of ZnO NPs. a) 7.4 nm, b) 36 nm, c) 73 nm

Meanwhile, Table 4.5 is the summarised of 96h-LC_{50} of three different sized ZnO NPs exposed to the embryonic zebrafish that were calculated from the probit analysis. The LC_{50} for larger ZnO NPs (73 nm) was determined to be 41.6 mg/L where as ZnO NPs with the size of 36 nm and 7.4 nm were determined to be 35.7 mg/L and 20.9 mg/L respectively. When comparing the size and the level of toxicity, probit analysis also revealed that smaller ZnO NPs has higher toxicity compare than that ZnO NPs with larger size.

Table 4.5: LC_{50} of ZnO NPs exposed embryonic zebrafish.

Particle size (nm)	**96h-LC_{50}**
7.4	20.9
36	35.7
73	41.6

Little concentration of smaller ZnO NPs was able to kill half of the exposed embryonic zebrafish. Although the exposure of ZnO NPs are dose-dependent as described by Bai *et al*. (2010) however, size of ZnO NPs might also contribute to the toxicity according to the data obtained. Thus, at low dosage of ZnO NPs exposure, there will be a high possible risk might be posed by the organisms in the aquatic environment.

Apart from the survival rate determination, the endpoint for this study also determine the hatching rate of embryonic zebrafish over 96 hpf. Apart from causing death to the embryos, the exposure of ZnO NPs has caused a severe hatching delay to the embryonic zebrafish especially when exposed to higher ZnO NPs concentrations. Generally, Kimmel *et al*. (1995) has described that the hatching process began to occur as early as 48 hpf for the normal embryonic zebrafish. Based on the control group prepared, over 60 % of the embryos have hatched at 48 hpf and near 100 % (99.4 ± 1.0 %) of

embryos hatched at 72 hpf in embryo water. An apparent observation was seen when the embryos were exposed to different concentration of ZnO NPs. Figure 4.20 shows the hatching rate of embryonic zebrafish that were exposed to different concentrations of three sized ZnO NPs over 96 hpf.

Based on Figure 4.20, at higher concentration regardless of the size, near 100 % of embryos were unable to hatch. Only 3.9 ± 1.9 % of hatching rate was observed at higher concentration of smaller ZnO NPs (7.4 nm). Unlike the survival rate, the hatching rate for larger size ZnO NPs exposure has shown a significant effect of the embryonic zebrafish. ZnO NPs with the size of 36 nm has caused no hatching (0 ± 0%) at higher concentrations (50 mg/L and 100 mg/L) where as ZnO NPs with the size of 73 nm also caused no hatching when exposed to 30 mg/L to 100 mg/L.

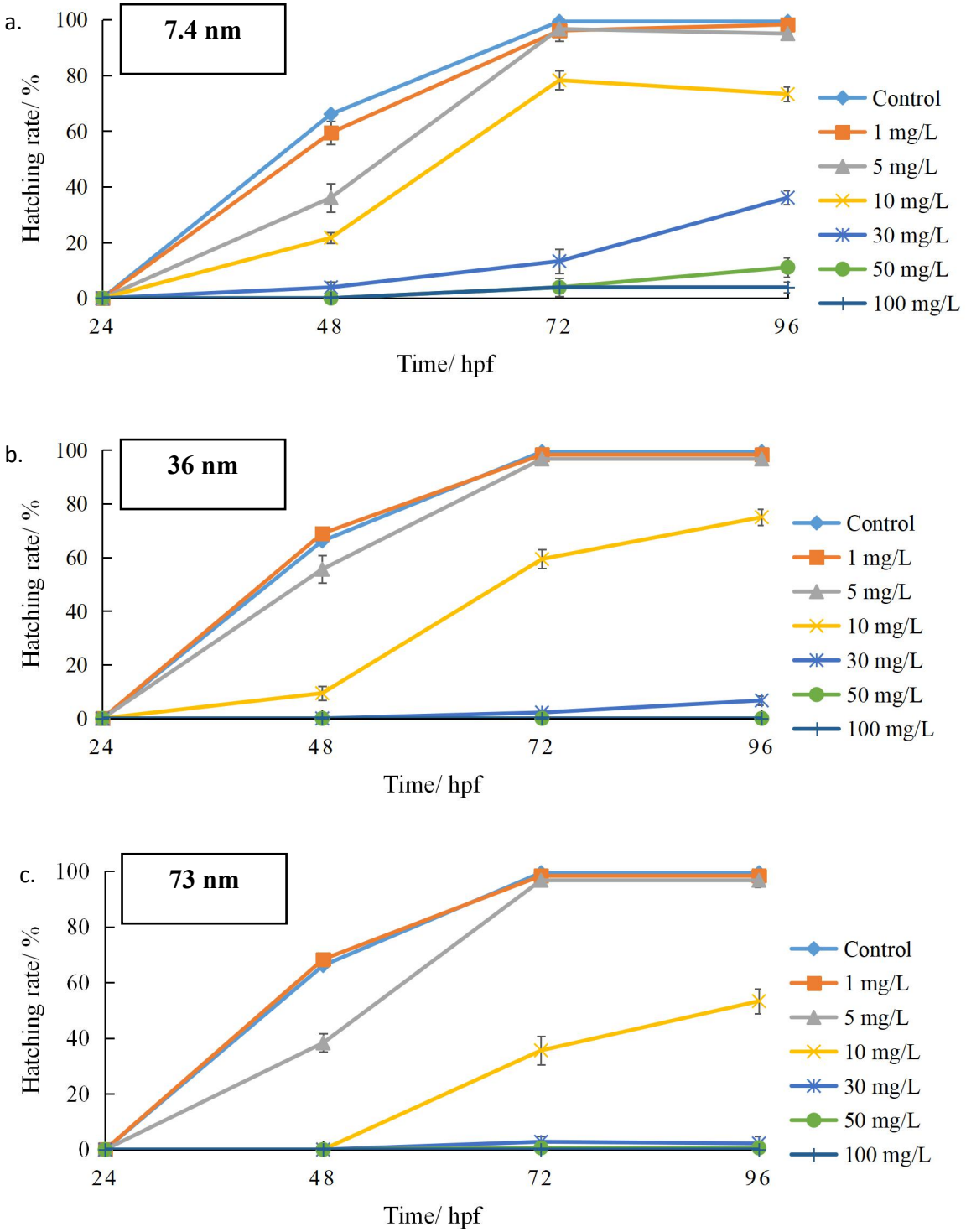


Figure 4.20: Hatching rate of embryonic zebrafish at different concentrations of ZnO NPs. a) 7.4 nm, b) 36 nm, c) 73 nm

For embryos exposed to 10 mg/L ZnO NPs (7.4 nm), more than 30 % of the embryos were able to hatched becoming to larvae after 48 hpf whereas there was less than 20 % of embryos able to hatch after 48 hpf when exposed to 10 mg/L of 36 nm ZnO NPs. However, embryos exposed to 73 nm ZnO NPs (10 mg/L) has caused a hatching delay in which at 48 hpf, there was no hatching observed. At 96 hpf, more than 50 % of all embryos exposed to three sizes ZnO NPs managed to hatced. Although the embryonic zebrafish managed to survive inside the chorion based on the body movement and the observed heartbeat however, the prolonged exposure of ZnO NPs and the delay of hatching over 96 hpf might eventually lead to the death of embryos.

This finding also correspond to the previous studies by Lin *et al.* (2012) and Vicario-pares *et al.* (2014) in which there were apparent reduced hatching rate of embryonic zebrafish at 72 hpf. Studies by Vicario-pares *et al*. (2014) have stated that there was adverse effect on the hatching rate of embryonic zebrafish when exposed to 10 mg/L ZnO NPs. Studies by Lacave *et al.* (2016) has shown that over 120 h of ZnO NPs has caused 100 % death of embryonic zebrafish. On the other hand, as mentioned previously, lower concentration of ZnO NPs for all three sized ZnO NPs (1 mg/L and 5 mg/L) has also shown similar hatching rate as the control group which is more than 90% of hatching rate after 96 hpf.

One of the main factors that contribute to the hatching delay was the accumulation of ZnO NPs on the surface of embryonic zebrafish. The opaque appearance was an indication that the embryo was died at 96 hpf as shown in Figure 4.21b and Figure 4.21c as no body and heartbeat movement was being observed. As mentioned previously, the lack of nutrient intake from the surrounding over time caused the decrease of oxygen inside the chorion has delayed the hatching process thus causing the death of the embryo occurred.

Studies by Lee *et al.* (2007) and Kteeba *et al.* (2017) has also revealed that the presence of ZnO NPs attached onto the surface of embryos has interfered the regular transport of nutrient through the chorion pores.

a. Control

b. 100 nm ZnO (Embryo)

c. 100 nm ZnO (Larva)

Figure 4.21: Embryonic zebrafish at 96 hpf. a) Control (Embryo water) b-c) 100 mg/L ZnO NPs (100 nm)

Apart from the lacking of nutrient intake, the disruption of hatching enzyme was also being the main factor that cause the delay of hatching process. No data was presented in this study however the disruption of hatching enzyme was explained by previous studies where by hatching enzyme called metalloproteinase (Tomilina *et al.,* 2014; Vicario-pares *et al*., 2014). This enzyme has been interrupted when ZnO NPs was present in which the characteristic of the metal ion released from the ZnO NPs can fit to the active site of the zebrafish hatching enzyme which responsible for chorion degradation after 48 hpf (Vicario-pares *et al.*, 2014).

Other than that, Bai *et al.* (2010) and Ong *et al.* (2013) have stated that the exposure of ZnO NPs to the embryonic zebrafish have caused the hatching delay by directly affecting the physiological process in which the ZnO NPs adsorbed onto the embryos surface directly result in more brittle shell and disrupt the hatching enzyme. Thus, this explain the factor of the hatching delay that caused by the high exposure of ZnO NPs to the embryonic zebrafish. Despites of higher exposure concentration, lower ZnO NPs concentration mainly 10 mg/L and 30 mg/L also show some hatching delay where by not all embryos were able to hatch. Thus, direct contact of ZnO NPs to the larvae might affect the physiology of larvae and eventually causing death due to lack of nutrient and oxygen intake.

4.4 Effect of Humic Acid on different sized ZnO NPs induced toxicity

On the other hand, the other part of this study involves the presence of humic acid (HA). Various studies have reported that the presence of NOM reduced the toxicity in aquatic organisms in terms of the survival rate (Chen *et al*., 2012; Edgington *et al*., 2010;

Fabrega *et al*., 2009; Gao *et al*., 2009; Gao *et al*., 2012; Ouyang *et al*., 2017). Most recent study has more interest on HA regarding the potential of NOM to alter the behaviour of ZnO NPs in aquatic environment (Akhil *et al.,* 2015; Kteeba *et al.,* 2017; Yang *et al*., 2009). Based on this study, results revealed that there are major differences in terms of the ZnO NPs toxicity with and without the presence of HA. The toxicity of ZnO NPs to the embryonic zebrafish was greatly alleviated when there was presence of HA. Figure 4.22 shows the survival rate of the embryonic zebrafish when exposed to the different concentrations of three different sized ZnO NPs with the presence of 50 mg/L of HA over 96 hpf.

Based on Figure 4.22, all three sized of ZnO NPs exposure have shown high survival rate when 50 mg/L of HA was added. Surprisingly, at high concentration of ZnO NPs (100 mg/L), the survival rate of the embryonic zebrafish was observed to be more than 60 % at 96 hpf, specifically 63.1 %, 63.9 %, and 69.4 % survival rate exposed to 7.4 nm, 36 nm, and 73 nm of ZnO NPs respectively. Furthermore, ZnO NPs concentrations of 50 mg/L and 30 mg/L also showed high survival rate as more than 75 % was observed. Meanwhile, at lower concentration, there are no significant effect of ZnO NPs toxicity as the survival rate were similar to HA control group. Previous finding of ZnO NPs exposure without the presence of HA also revealed the same result in which no toxicity effect was observed when exposed to <10 mg/L of HA.

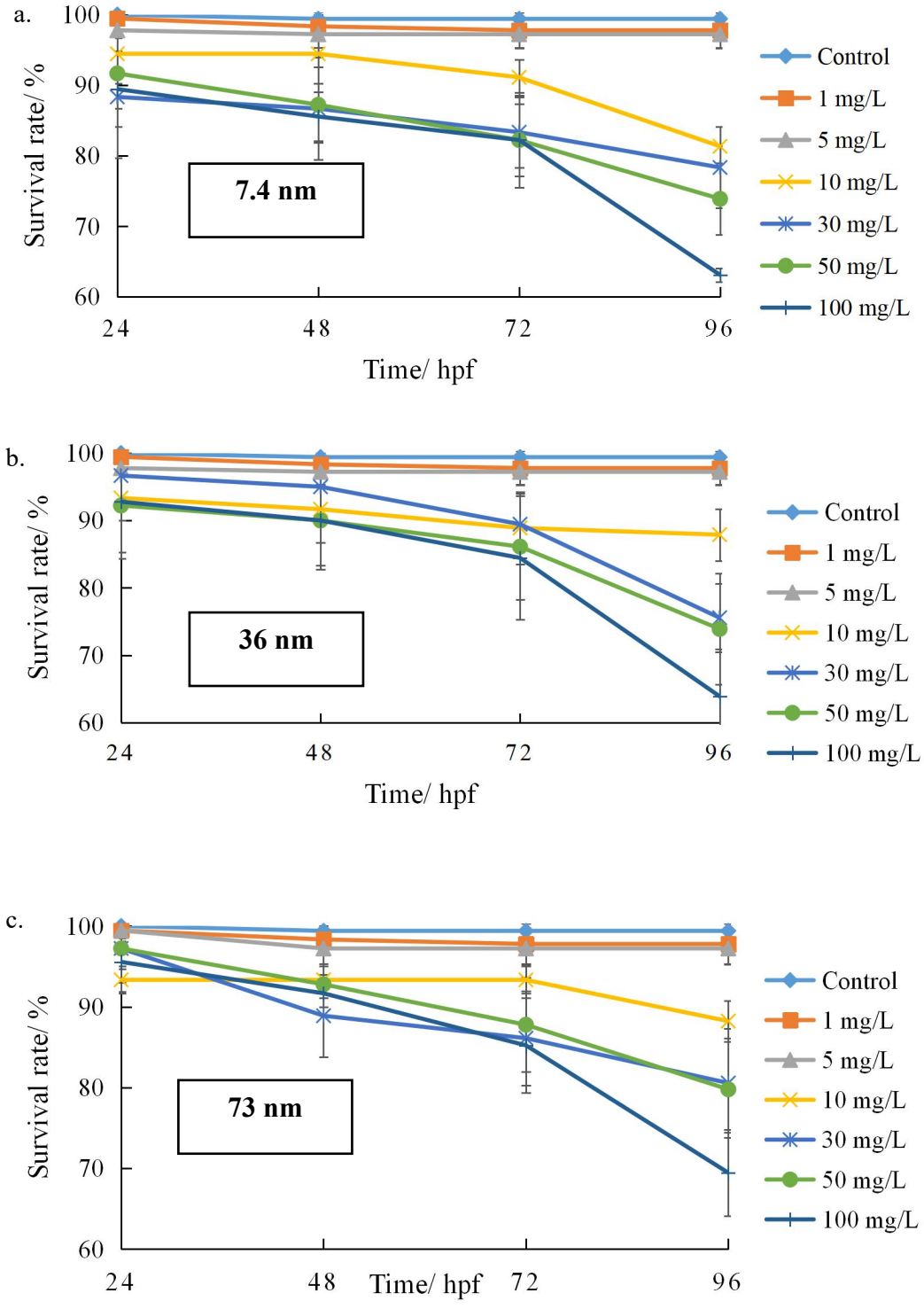


Figure 4.22: Survival rates of embryonic zebrafish exposed to different concentrations of ZnO NPs with the addition of 50 mg/L of humic acid.
a) 7.4 nm, b) 36 nm, and c) 73 nm

So far, there was less study on the toxicity effect of ZnO NPs with the presence of 50 mg/L HA exposed to higher organisms. Previous studies by Akhil *et al.*, (2015) have carried out study on the toxicity effect of ZnO NPs with the presence of 50 mg/L HA in bacteria. Results revealed that high concentration of HA has exhibited antimicrobial effect to the tested bacteria namely *Escherichia coli* and *Pseudomonas putida* (Akhil *et al.*, 2015). In addition, this result has shown that the presence of HA has lifted the toxicity of ZnO NPs in bacteria which also deduced that the presence of HA has antimicrobial effect that benefit in antimicrobial agent products.

However, in this study 50 mg/L of HA concentration added to ZnO NPs has tremendously reduced the toxicity in embryonic zebrafish. The use of embryonic zebrafish in this study mimic the aquatic organisms that settle down at the bottom of water column which high interaction of ZnO NP and NOM could occur (Zhu *et al.*, 2012). Apart from adding 50 mg/L of HA, this study has also added lower concentration of HA. Generally, there is no fixed concentration of HA being described however, Basumallick *et al.* (2017) has described the range of HA in environment was approximately 0.1 mg/L to 20 mg/L. Hence, 5 mg/L and 20 mg/L of HA was used in this study mimicking the environment HA concentration.

Based on Figure 4.23 and Figure 4.24, results showed that the presence of HA have also reduced the ZnO NPs toxicity regardless of the NPs sizes as compared to the toxicity of ZnO NPs without the presence of HA. Similar studies have also been carried out by Kteeba *et al.* (2017) and Ong *et al.* (2016). Kteeba *et al.* (2017) has conducted the toxicity effect of ZnO NPs with the presence of selected NOM including HA. Results found that HA has shown the best mitigation effect on ZnO NPs toxicity. Meanwhile, Ong *et al.*, (2016) has also found that more than 50 % of embryonic zebrafish survived after 96 hpf

when exposed to 100 mg/L of ZnO NPs with presence of 10 mg/L HA. Luo *et al.* (2016) has described that the adsorption of HA to NPs may be caused by the electrostatic interaction as its mechanism that might explain the decrease level of ZnO NPs when HA present.

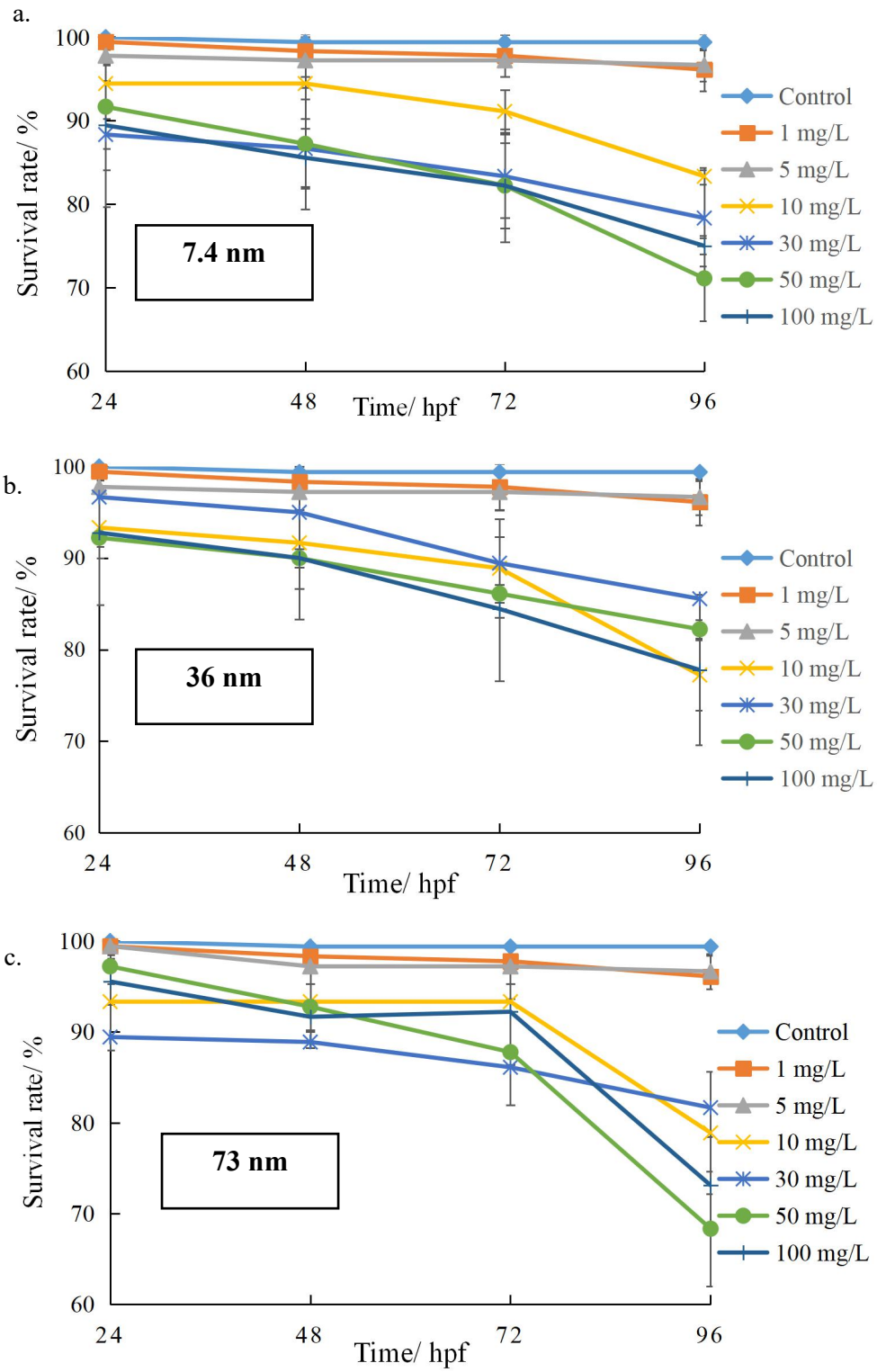


Figure 4.23: Survival rates of embryonic zebrafish exposed to different concentrations of ZnO NPs with the addition of 20 mg/L of humic acid.
a) 7.4 nm, b) 36 nm, and c) 73 nm

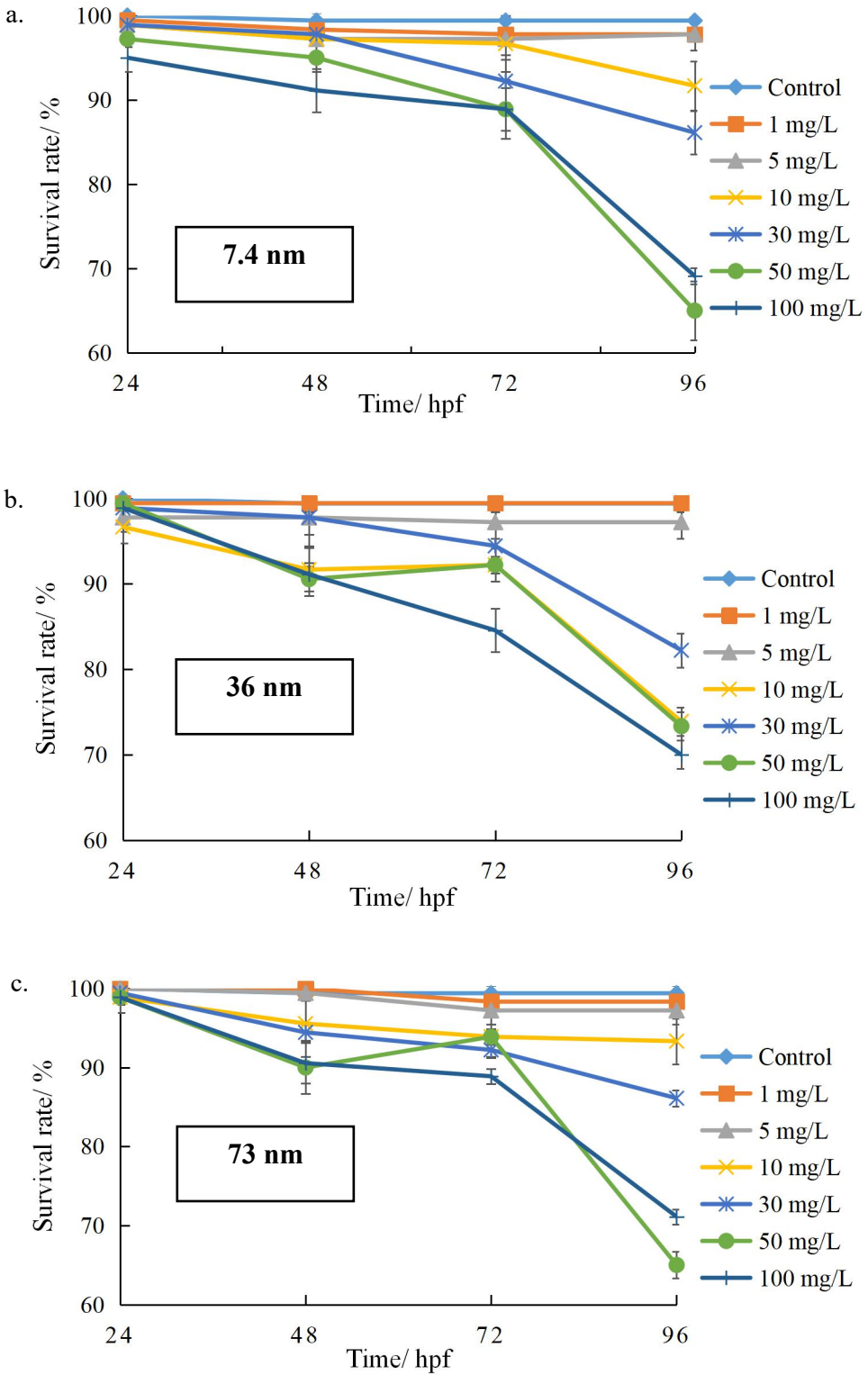


Figure 4.24: Survival rates of embryonic zebrafish with exposed to different concentrations of ZnO NPs with the addition of 5 mg/L of humic acid. a) 7.4 nm, b) 36 nm, and c) 73 nm

Furthermore, all the embryos were still able to survive despite of the different HA concentrations being exposed to the embryos. Figure 4.25, Figure 4.26, and Figure 4.27 have shown similar patterns of results of the hatching rate of embryonic zebrafish exposed to different concentrations of three different sized of ZnO NPs with the presence of different concentrations of HA. The result has showed that in control group where by only HA being exposed to the embryonic zebrafish, more than 90 % of them were survived whereas the treated group, more than 50 % of embryonic zebrafish were able to survive as compared to the exposure of ZnO NPs without the presence of HA.

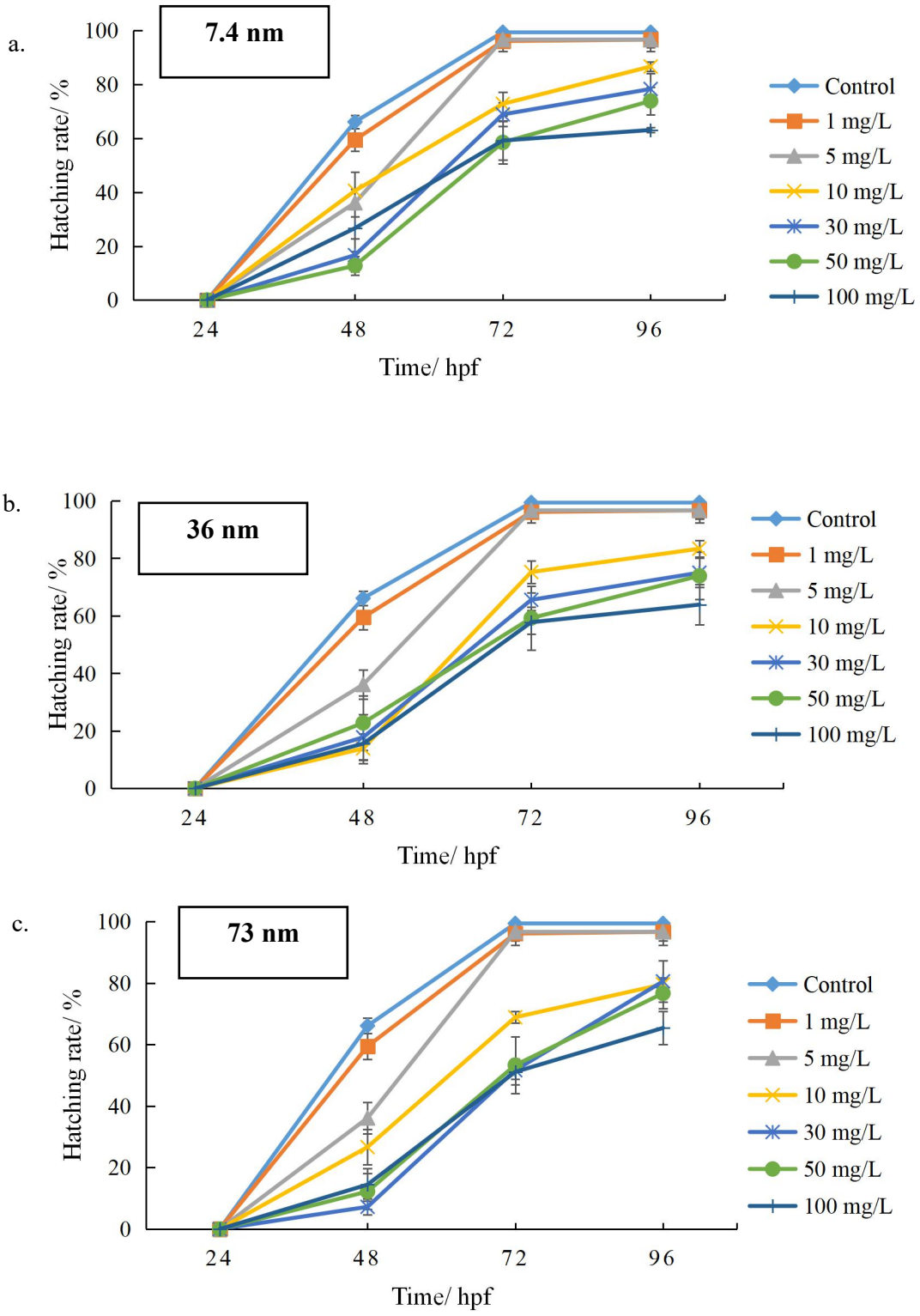


Figure 4.25: Hatching rate of embryonic zebrafish exposed to different concentrations of ZnO NPs with the addition of 50 mg/L of humic acid.
a) 7.4 nm, b) 36 nm, and c) 73 nm

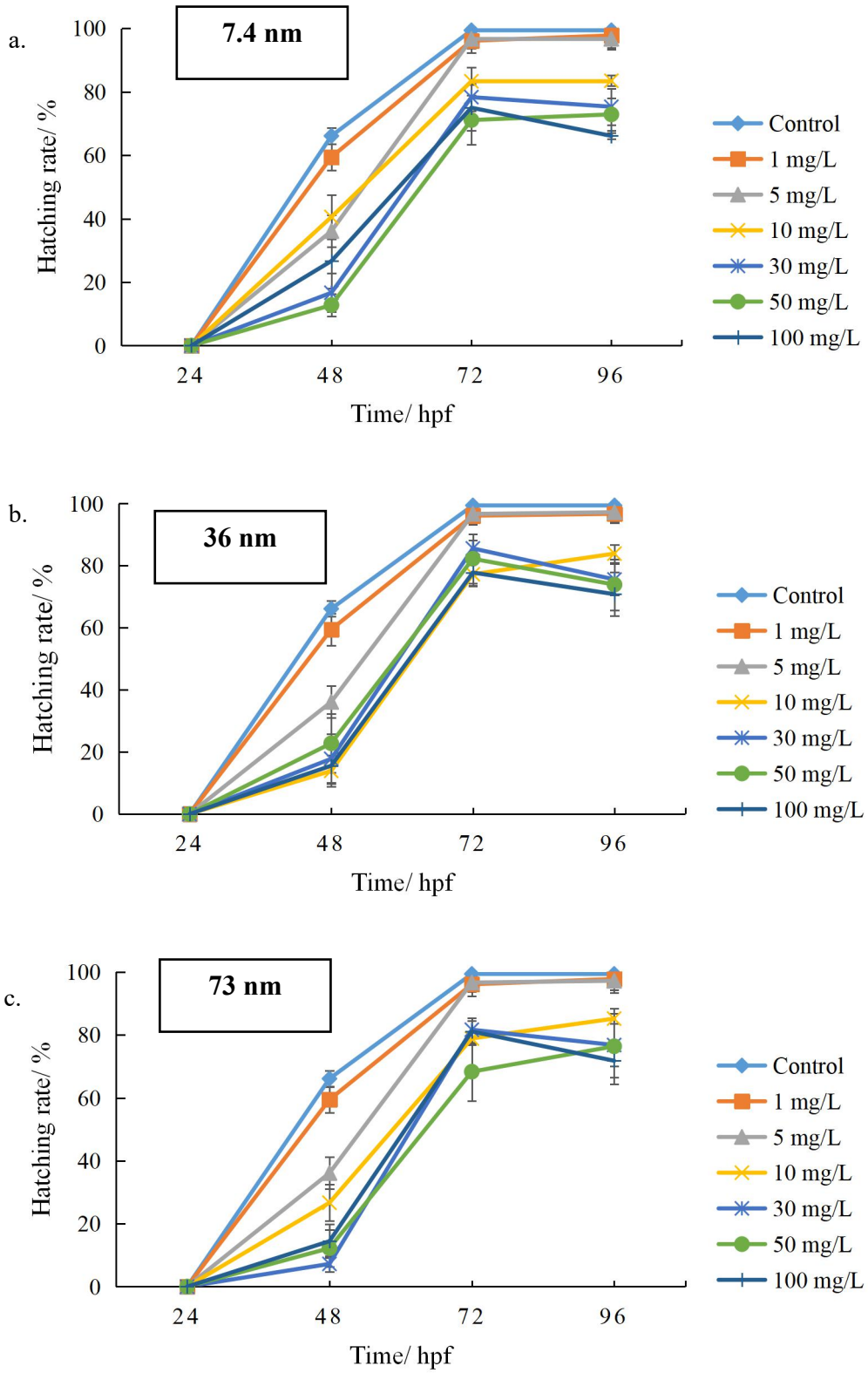


Figure 4.26: Hatching rate of embryonic zebrafish exposed to different concentrations of ZnO NPs with the addition of 20 mg/L of humic acid.
a) 7.4 nm, b) 36 nm, and c) 73 nm

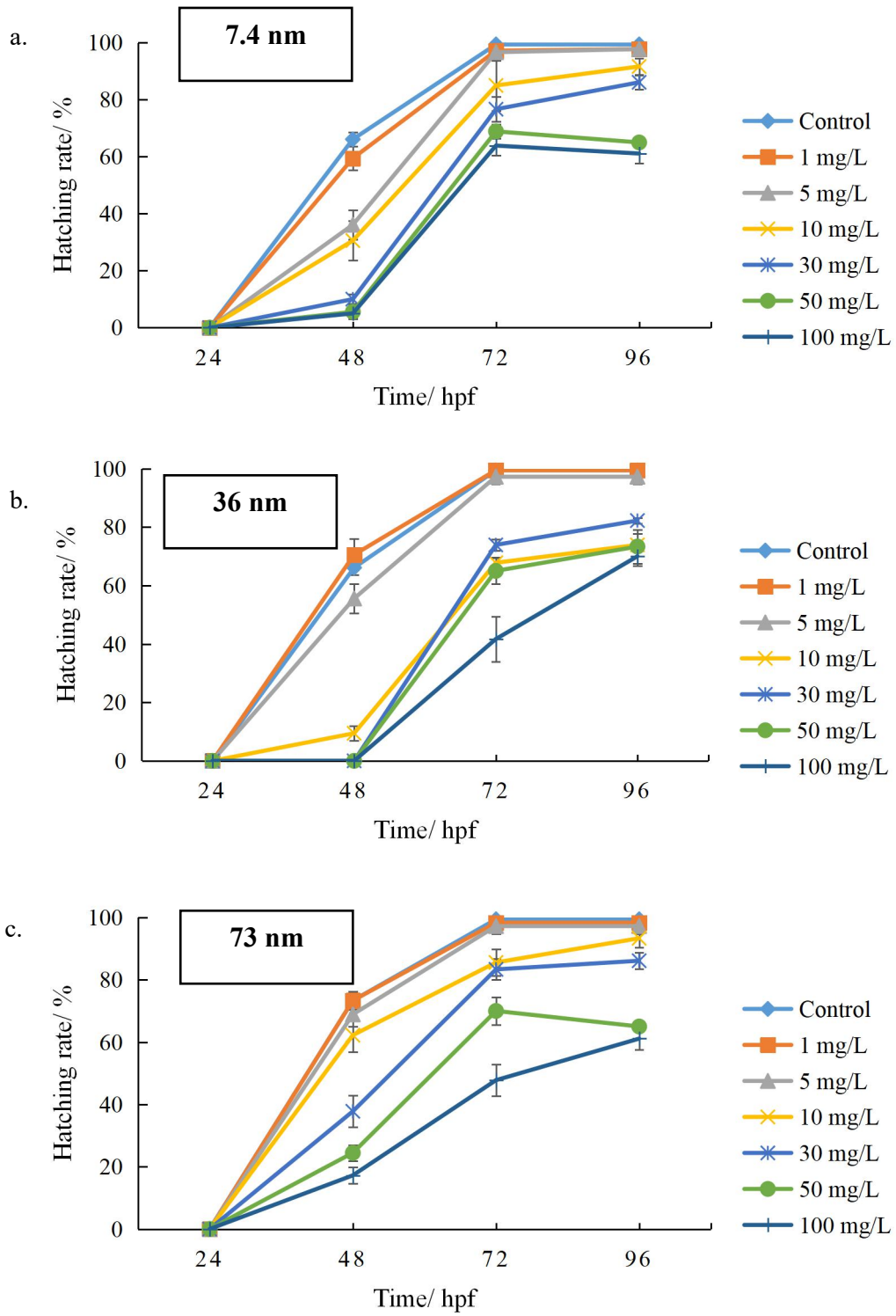


Figure 4.27: Hatching rate of embryonic zebrafish exposed to different concentrations of ZnO NPs with the addition of 5 mg/L of humic acid.
a) 7.4 nm, b) 36 nm, and c) 73 nm

Besides, without the presence of HA, the morphological appearance of embryos were not able to be observed clearly as most of the ZnO NPs have accumulated onto the surface of embryos.

However, the presence of HA have reduced the accumulation of ZnO NPs onto the chorion and the embryos still can be observed under the microscope. This finding was shown in Figure 4.28 and Figure 4.29.

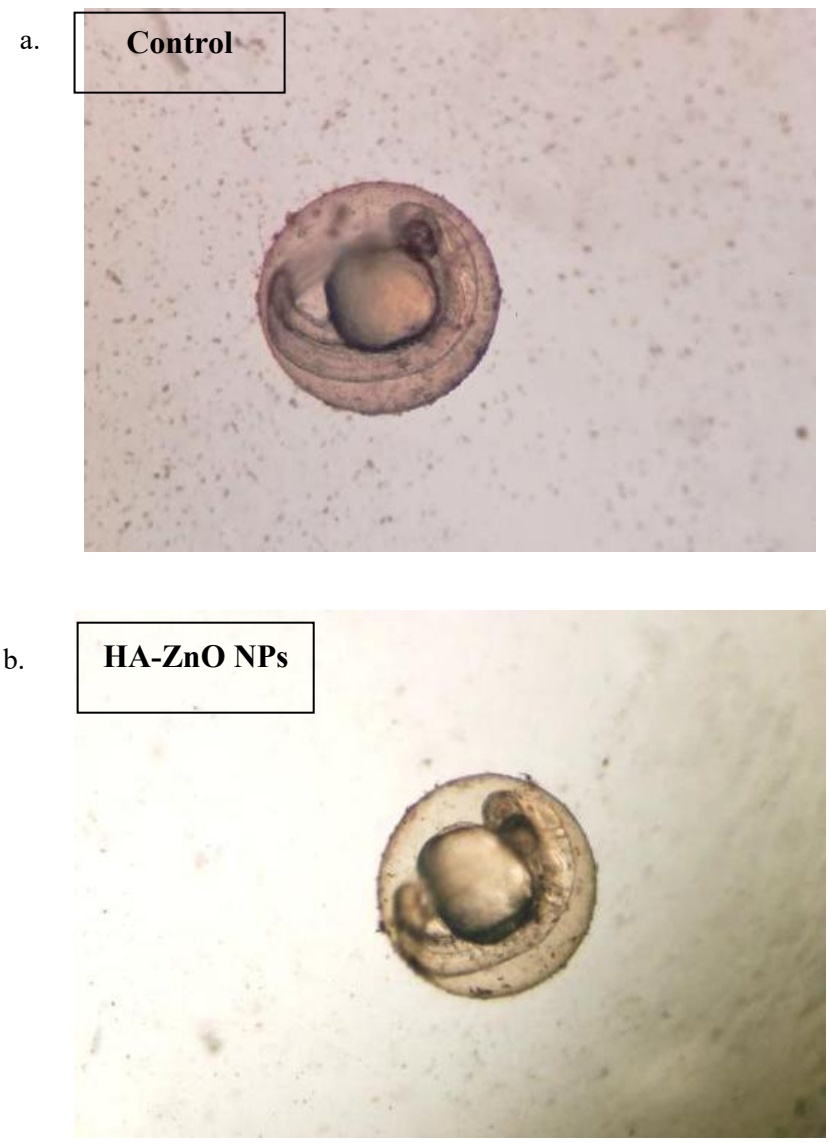


Figure 4.28: Embryonic zebrafish at 24 hpf. a) Control (Embryo water with HA) b) 100 mg/L ZnO NPs with HA

a.

b.

Figure 4.29: Embryonic zebrafish at 96 hpf. a) Control (Embryo water with HA) b) 100 mg/L ZnO NPs with HA

Similar finding was also being described by Kteeba *et al.* (2017) in which, the presence of NOM such as HA has reduced the adsorption of the ZnO NPs onto the surface of embryonic zebrafish. This finding has revealed the ability of the embryonic zebrafish to survive when there was presence of HA.

Unlike the hatching rate of embryonic zebrafish exposed to ZnO NPs only, the presence of HA has also reduced the hatching delay after 96 hpf. The control group which

contain only HA has shown more than 90 % of embryonic zebrafish hatched whereas more than 60 % of the embryonic zebrafish hatched after 96 hpf.

4.5 Effect of HA on the Fate Behaviour of ZnO NPs

4.5.1 Dissolution

Most studies have mentioned that dissolution was one of the main factor that contribute to ZnO NPs toxicity. Oliviero *et al.* (2017) has also stated that the dissolution process is crucial mainly for metal-bearing NPs due to the involvement of metal ions released which has potential risk of toxicity in organisms. Odzak *et al.* (2017) also stated the importance of determining the dissolution process in order to study the fate and the bioavailability of NPs in environment. Based on the toxicity study mentioned earlier, result showed that the smaller sized of ZnO NPs has shown great toxicity effects as compared to larger ZnO NPs. Therefore, the behaviour of ZnO NPs mainly its dissolution was determined in term of the dissolution of ZnO NPs via ICP-OES.

Figure 4.30 shows the graph of the amount of zinc (Zn^{2+}) ions released from three different sizes of ZnO NPs at different ZnO NPs concentrations. The result showed that the amount of Zn^{2+} ions released from ZnO NPs with larger size (73 nm) decrease at increasing concentration after 24 h of exposure. Similar trend of decrement was also observed for ZnO NPs with the size of 36 nm when the concentration of ZnO NPs increased. However, smaller ZnO NPs (7.4 nm) has shown significant increase of Zn^{2+} ions at increasing ZnO NPs concentrations after 24 h exposures.

Furthermore, Figure 4.31 shows the amount of Zn ions released by three different size of ZnO NPs at 100 mg/L (0 h and 24 h). Significant difference of the amount of Zn^{2+}

released from ZnO NPs were determined as smaller ZnO NPs (7.4 nm) has released more Zn^{2+} ion after 24 h (23.4 ± 0.2 mg/L) as compared to large sized ZnO NPs (36 nm and 73 nm) which decrement of Zn^{2+} ions have occurred at 24 h which were 16.9 ± 0.2 mg/L and 10.0 ± 0.1 mg/L respectively ($p < 0.05$). This finding was supported by previous literatures in which have mentioned that the properties for smaller ZnO NPs have tendency to release more Zn ions as compared to the larger size of ZnO NPs (Bian *et al*., 2011; Lopes *et al*., 2013; Tso *et al*., 2010). Study by Xia *et al*. (2011) also found that 25 % of Zn^{2+} ions released from 50 mg/L of ZnO NPs with the size of 158 nm. Franklin *et al.* (2009) also mentioned that approximately 1 mg/L of Zn^{2+} was released from 10 mg/L of ZnO NPs. Therefore, the properties of having smaller in size have greatly increase the tendency of ZnO NPs to release more zinc ions as compared to larger size of ZnO NPs.

The release of more Zn^{2+} ions from smaller ZnO NPs also correlate with the endpoint data (96h LC_{50}) obtained in which at higher concentration, the lethality of embryonic zebrafish after 24 h exposures were observed to be higher as compared to larger ZnO NPs exposure.

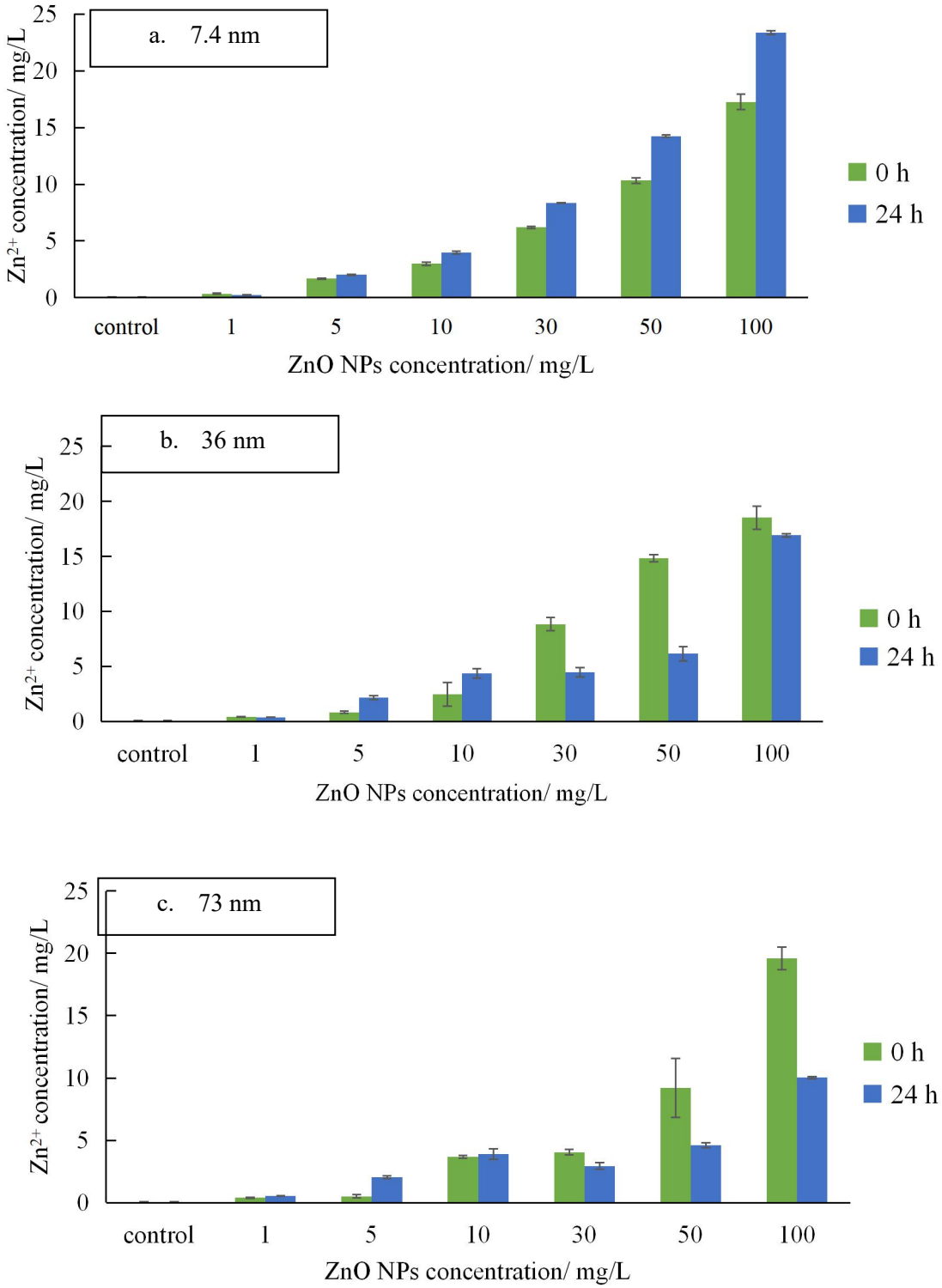


Figure 4.30: Zn^{2+} ion released from different concentration of three different sized ZnO NPs in embryo water at 0 h and 24 h via ICP-OES. a) 7.4 nm, b) 36 nm, and c) 73 nm

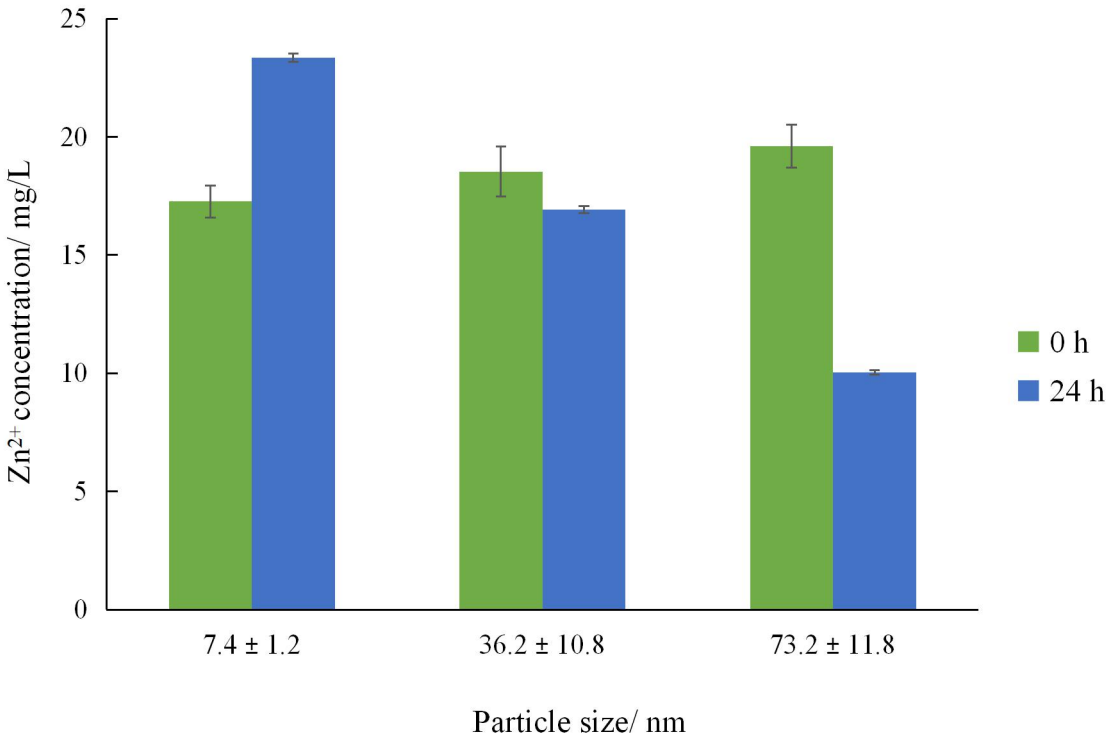


Figure 4.31: Zn^{2+} ion released from 100 mg/L of three different sized ZnO NPs at 0 h and 24 h via ICP-OES.

Apart from that, the dissolution of ZnO NPs was also determined with the presence of different concentration of HA in embryo water. Previous studies have suggested that the presence of NOM such as HA affect the behaviour of ZnO NPs in water. Thus, this study was carrie out to determine the dissolution of three different ZnO NPs with the presence of HA. Figure 4.32 shows the amount of Zn^{2+} ions released from three different sized ZnO NPs with the addition of 50 mg/L HA.

Although the literature has reported the NOM concentration in aquatic environment was from 1 mg/L to 20 mg/L as mentioned by Basumallick *et al.* (2017) however, the addition of 50 mg/L HA was carried out in order to observe any significant different in terms of the dissolution rate of ZnO NPs after 24 h regardless of the size of ZnO NPs. Based on Figure 4.32, all three sizes of ZnO NPs has demonstrated the increase

of Zn^{2+} ions released after 24 h exposure ($p > 0.05$). At higher concentration which is 100 mg/L, ZnO NPs with the size of 73 nm (Figure 4.32c) has released 18.79 ± 0.6 mg/L at 0 h to 22.24 ± 0.4 mg/L at 24 h of Zn^{2+} ions. Similar result was also obtained by ZnO NPs with the size of 36 nm. 15.09 ± 0.8 mg/L of Zn^{2+} ions was obtained at 0 h and increase to 22.90 ± 0.4 mg/L at 24 h of exposure. Meanwhile, smaller ZnO NPs with the size of 7.4 nm also released 16.48 ± 1.2 mg/L of Zn^{2+} ions at 0 h to 28.32 mg/L Zn^{2+} ions at 24 h of exposure. Thus, there are no significant difference between the dissolution among three different sizes of ZnO NPs when 50 mg/L HA was present.

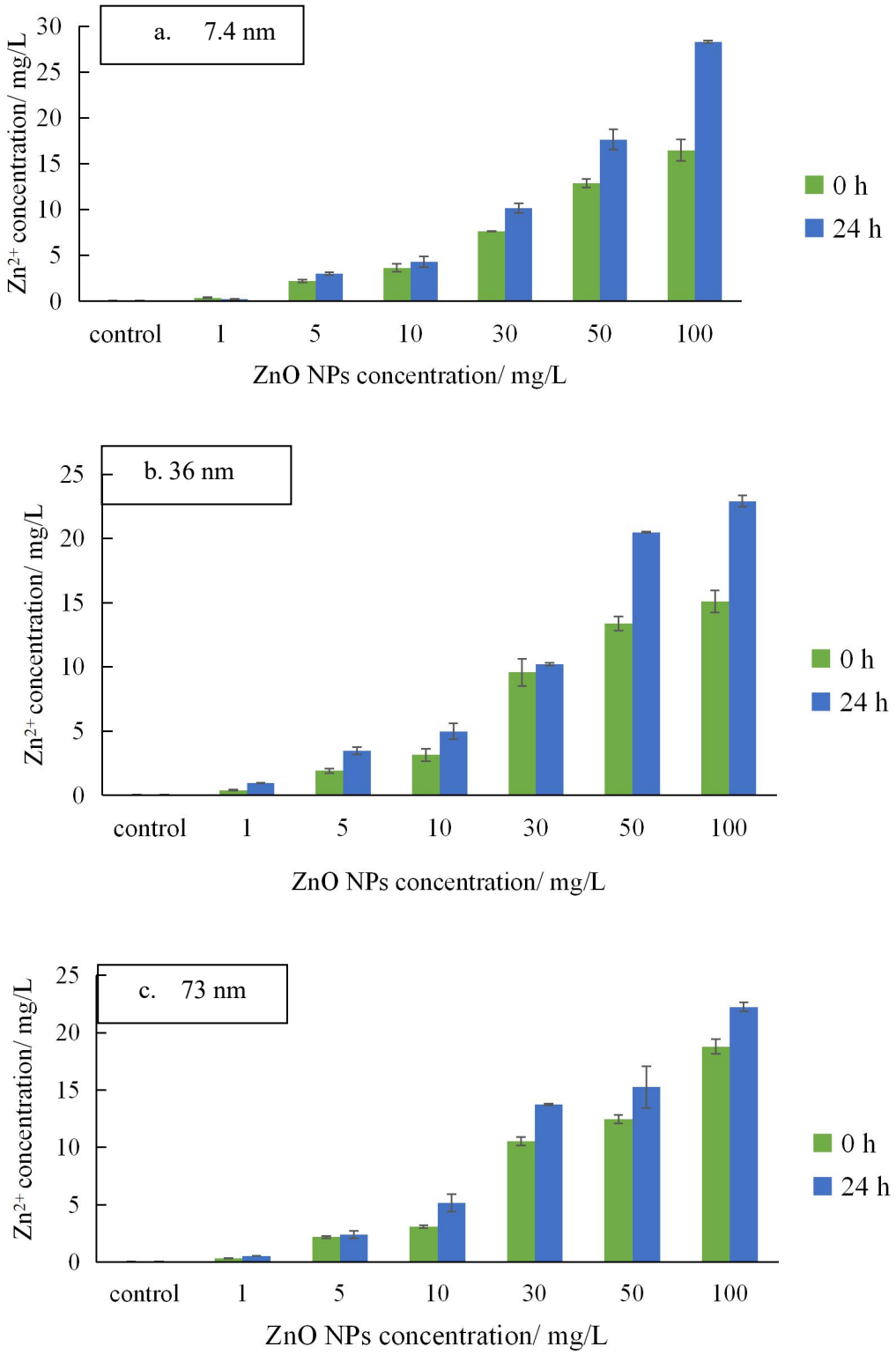


Figure 4.32: Zn^{2+} ion released from different concentration of three different sized ZnO NPs in embryo water containing 50 mg/L HA at 0 h and 24 h via ICP-OES. a) 7.4 nm, b) 36 nm, and c) 73 nm

Furthermore, the dissolution of ZnO NPs was also determined with the addition of 20 mg/L and 5 mg/L of HA in which within the range of the reported concentration of HA in aquatic environment. Figure 4.33 and Figure 4.34 show the amount of Zn^{2+} ions released from three different size of ZnO NPs at 0 h and 24 h with the addition of 20 mg/L and 5 mg/L of HA respectively. Both figures have shown similar result patterns as there were increase of Zn^{2+} ions concentration from 0 h to 24 h at increasing ZnO NPs concentrations ($p > 0.05$).

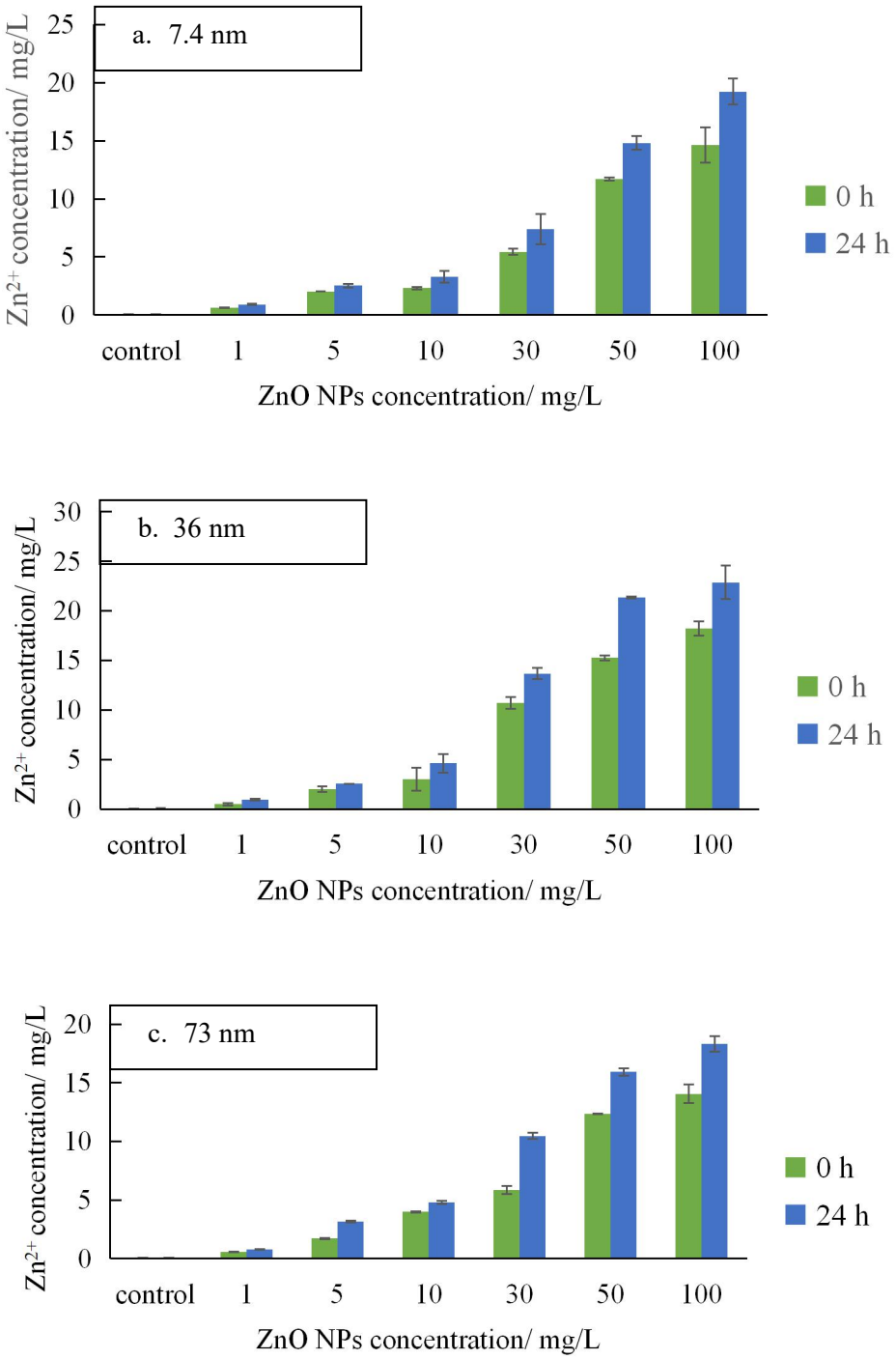


Figure 4.33: Zn^{2+} ion released from different concentration of three different sized ZnO NPs in embryo water containing 20 mg/L HA at 0 h and 24 h via ICP-OES.
a) 7.4 nm, b) 36 nm, and c) 73 nm

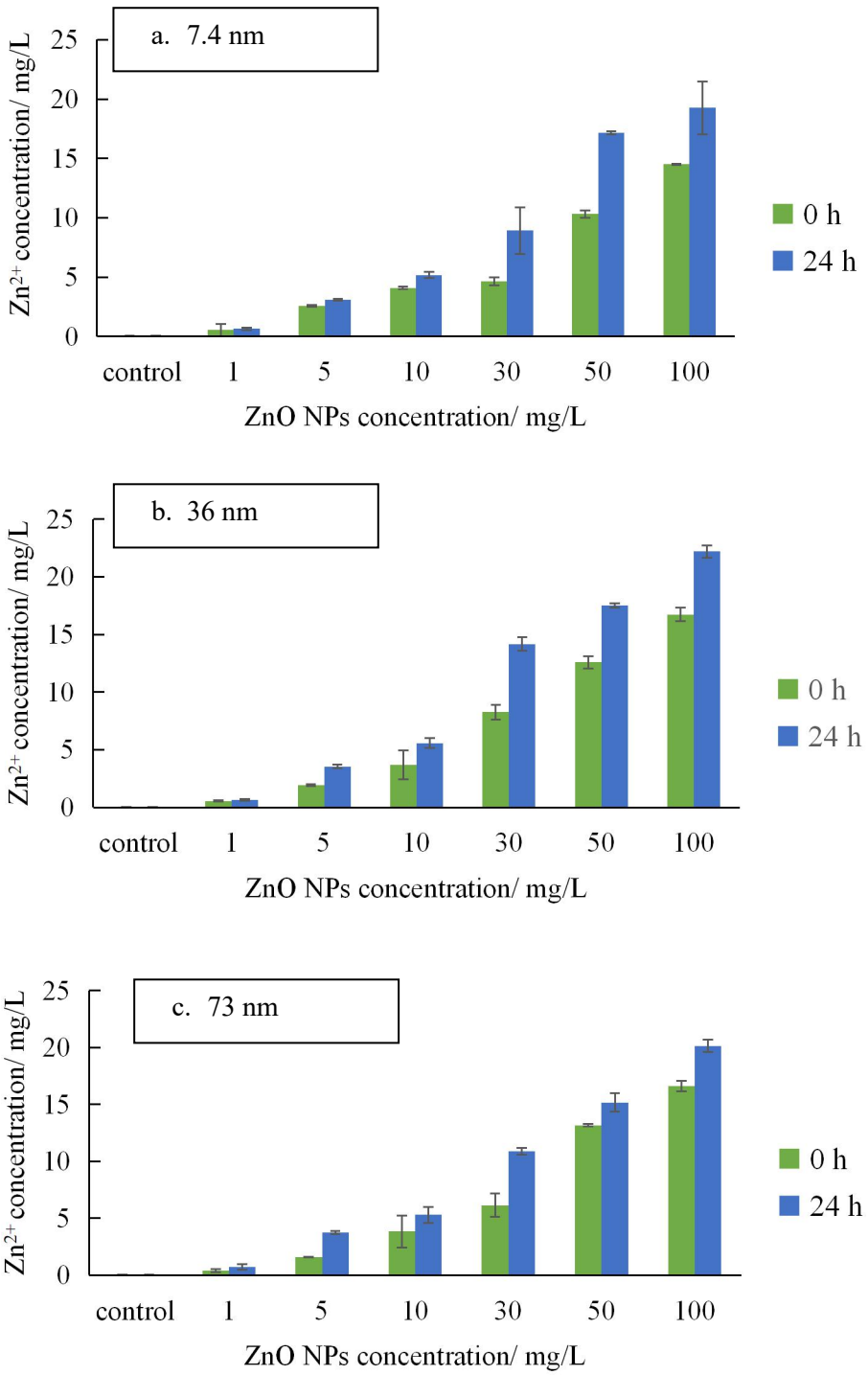


Figure 4.34: Zn^{2+} ion released from different concentration of three different sized ZnO NPs in embryo water containing 5 mg/L HA HA at 0 h and 24 h via ICP-OES. a) 7.4 nm, b) 36 nm, and c) 73 nm

Meanwhile, Figure 4.35 shows the comparison of the amount of Zn^{2+} ions released from three different sizes of ZnO NPs at high concentration (100 mg/L) with the presence and absence of HA. Result shows that the amount of Zn^{2+} ions released by larger size ZnO NPs without the presence of HA have decreased after 24 h of exposure. However thethe dissolution to increase after 24 h when HA present. This might be due to the aggregation process that occur especially larger size of ZnO NPs with high concentration were in contact with water after prolonged exposure. Tso *et al.* (2010) has described that the presence of HA has prevented the ZnO NPs from aggregated in water.

Although there was high amount of Zn^{2+} ions being detected, when HA present however, level of toxicity of ZnO NPs to the embryonic zebrafish has reduced significantly. Akhil *et al*. (2015) and Tso *et al*. (2010) have reported that the presence of NOM mainly HA has increase the stability of ZnO NPs in water. Other explanation would be the properties of ZnO NPs mainly the zeta potential was altered when HA present. Tso *et al.* (2010) described that the zeta potential of ZnO NPs has changed to negative thus stabilize the ZnO NPs in water. Garner *et al*. (2014) also explained that the presence of HA resulted in the more negatively charged particles which help to stabilize ZnO NPs via electrosteric stabilization mechanism as well as the steric hindrance.

Hence, the dissolution was affected by the particle size of ZnO NPs where by ZnO NPs with the particle size smaller than 10 nm tend to release more Zn^{2+} ions after prolonged exposure and large ZnO NPs has lower amount of Zn^{2+} ions released after prolonged exposure which might be caused by another ZnO NPs behaviour which is aggregation.

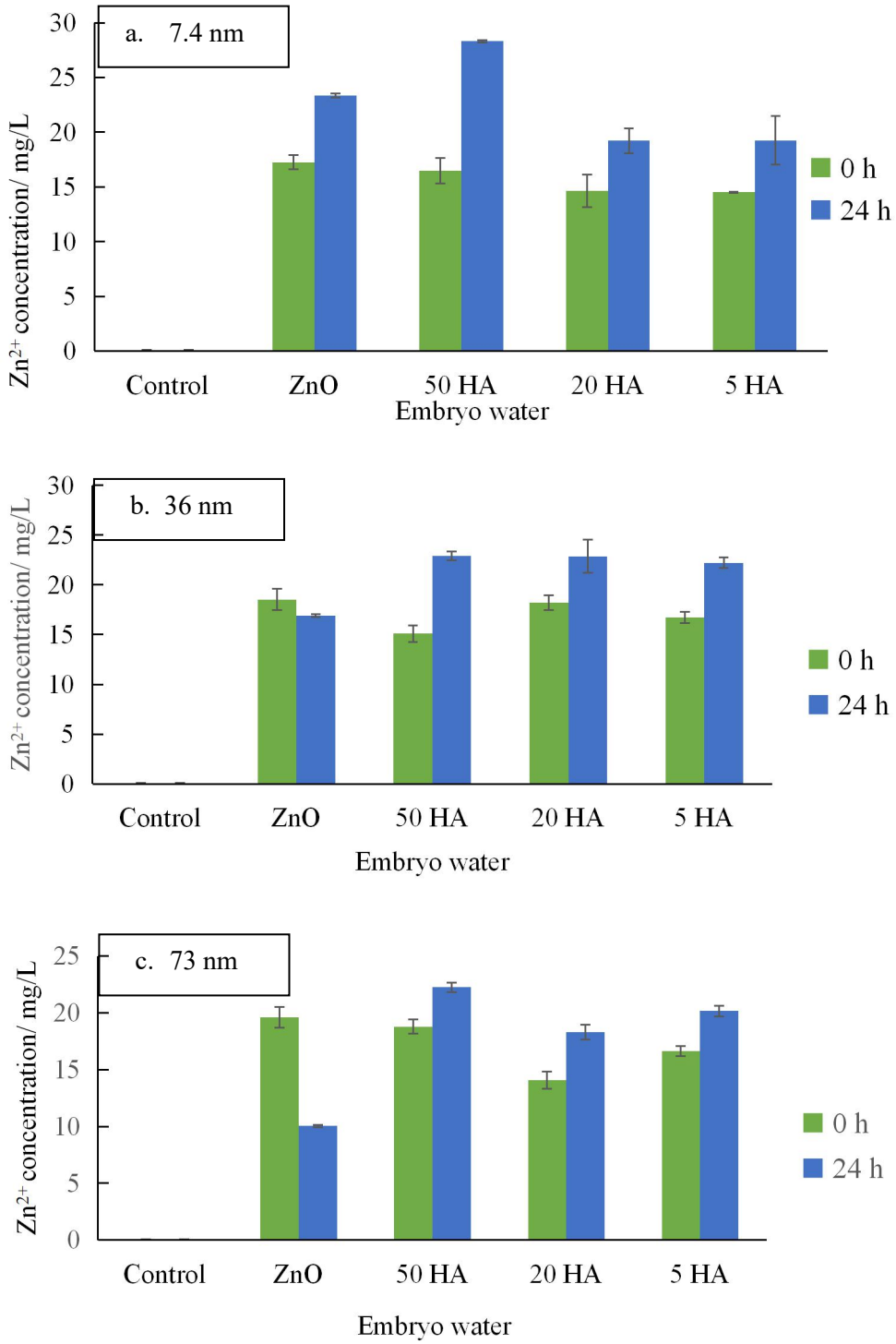


Figure 4.35: Zn^{2+} ion released from 100mg/L of three different sized ZnO NPs in embryo water containing different concentrations of HA at 0 h and 24 h via ICP-OES.
a) 7.4 nm, b) 36 nm, and c) 73 nm

4.5.2 Aggregation

Apart from dissolution, the ability of ZnO NPs to aggregate was also being considered to be the factor to cause the death of the embryonic zebrafish as well as the disruption of the hatching process. Bai *et al.* (2010) has stated the aggregation factor was also need to be considered in order to evaluate the toxicity of NPs. Previous literatures has also mentioned regarding the ability of NPs aggregate over time (Griffith *et al*., 2007; Lopes *et al*., 2013; Yu *et al*., 2011).

Figure 4.36 shows the image of ZnO NPs from the embryo medium after 24 hours of incubation via TEM analysis. It can be observed that the particles tend to stick to each other and form into larger size of particles. The figure also show that larger size ZnO NPs tend to aggregate more which can be also seen on the surface of embryos. Nowack *et al.* (2007) has stated that NPs tend to interact among themselves in the environment. Thus, this explained the aggregation that occur rapidly in the environment.

Besides, Bai *et al*. (2010) has also stated that the aggregation cause change in size of ZnO NPs. There was no data obtained for the size changes however, the TEM image has shown the aggregation of ZnO NPs in embryo water after 24 h of exposure mainly for larger ZnO NPs. Furthermore, this aggregation of ZnO NPs has caused the accumulation of ZnO NPs onto the surface of embryos had caused a severe hatching delay which has contributed to the toxicity of ZnO NPs.

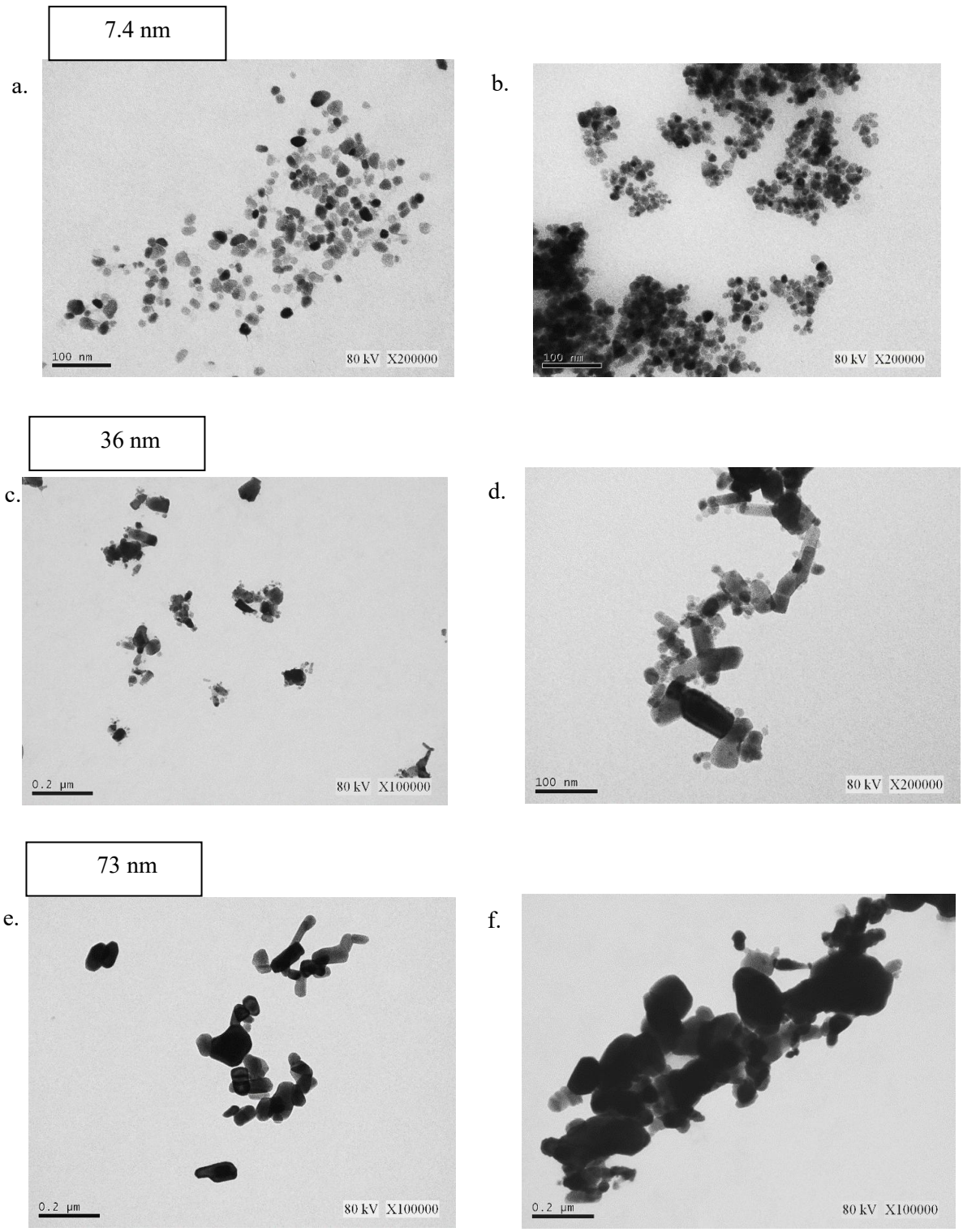


Figure 4.36: TEM images of 100 mg/L ZnO NPs at 0 h and 24 h in embryo water. a-b) 7.4 nm, c-d) 36 nm, e-f) 73 nm

On the other hand, the presence of HA in ZnO NPs exposure has caused the increase of Zn ions released in water. This shows that HA has the ability to reduce the aggregation of ZnO NPs in water (Akhil *et al*., 2015). Figure 4.37 shows the TEM images of ZnO NPs with the presence of 50 mg/L HA at 0 h and 24 h.

Based on Figure 4.37, the primary particles of ZnO NPs can still be seen when HA was added as compared to the absence of HA. Keller *et al.* (2010) has carried out studies and also found out that the NOM that adsorbed onto the ZnO NPs has significantly reduced the aggregation thus increase the stability of ZnO NPs in aquatic environment. Besides, the presence of HA also reduced the size of ZnO NPs in aqueous suspension. There is no data obtained in this study however, study by Ong *et al*. (2017) has found that there was a decrease of ZnO NPs particle size when HA was presented which might explain the increase of dissolution rate and the increase of stability in water.

Hence, the presence of NOM especially HA has greatly affected the properties of ZnO NPs when in contact with water in terms of dissolution and aggregation. This finding also correlated with the toxicity assessment of ZnO NPs in which the presence of HA has alleviated the level of ZnO NPs towards the test organisms regardless of the particle size of ZnO NPs.

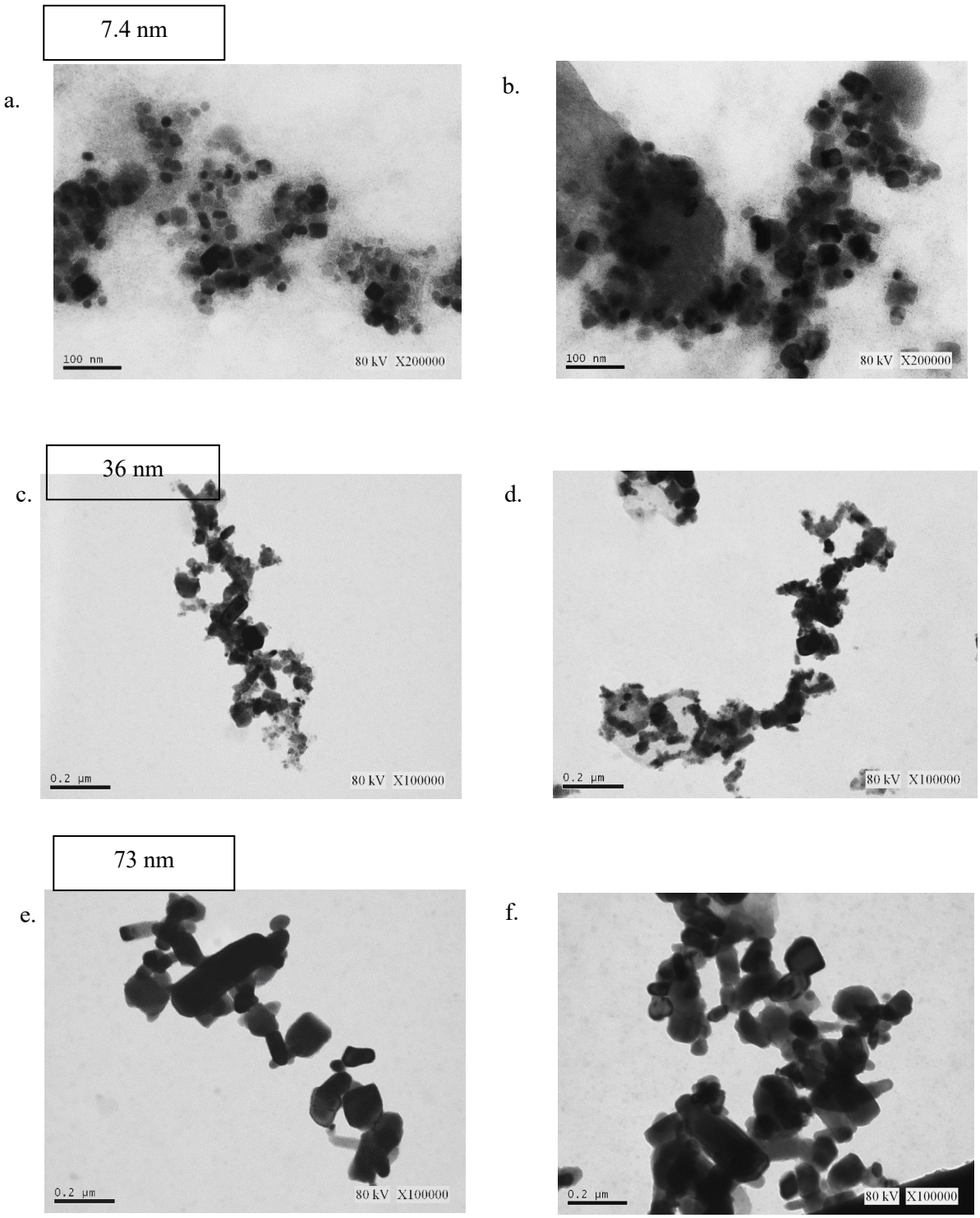


Figure 4.37: TEM images of ZnO NPs at 100mg/L with the presence of 50 mg/L HA at 0 h and 24 h. a-b) 7.4 nm, c-d) 36 nm, e-f) 73 nm

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

ZnO NPs is a crucial material especially in numerous applications in industries nowadays. In this study, result have shown that significant toxicity of ZnO NPs to the embryonic zebrafish when exposed to three different particle size of ZnO NPs. The survival rates have shown a significant decrease at increasing ZnO NPs concentrations. In term of size of NPs, smaller ZnO NPs (7.4 nm) has demonstrated to have high level of toxicity as compared to larger ZnO NPs (36 nm and 73 nm). Other than that, the hatching rates of the embryonic zebrafish has shown similar level of toxicity when exposed to three different sizes of ZnO NPs which apparently due to the accumulation of ZnO NPs onto the surface of the chorion thus preventing the nutrient uptake by the embryo inside the chorion.

However, exposure of ZnO NPs to the embryonic zebrafish with the presence of HA has greatly reduced the level of ZnO NPs toxicity where by more than 50 % of the exposed embryonic zebrafish were able to survived and hatched regardless of the size of ZnO NPs. This deduced that the particle size do not significantly affect the toxicity of ZnO NPs to the test organism especially when HA was present. Regardless of their sizes, the toxicity effect was reduced when HA was present. Furthermore, the dissolution effect was also determined and result demonstrated smaller ZnO NPs has higher dissolution as compared to larger ZnO NPs after 24 h of exposure which might due to the rapid aggregation especially at high ZnO NPs concentration.

On the other hand, the presence of HA in ZnO NPs has caused the increase amount of Zn^{2+} ions released in water regardless of the ZnO NPs sizes. The presence of HA has strongly altered the properties of ZnO NPs and kept the ZnO NPs stabilized in aqueous

environment as well as reduce the toxicity of ZnO NPs to the organisms.

Therefore, this study has highlighted the necessities of collecting data of the potential toxicity of ZnO NPs in order to carry out a close monitoring especially in aquatic environment as more ZnO NPs based products are being produced more over time. It is recommended to carry out more studies involving other mechanisms of ZnO NPs, the potential interaction of ZnO NPs to other NOM, and the risk assessment of ZnO NPs. This also includes the molecular study on the ability of HA that help in reducing the level of ZnO NPs toxicity. Hence, the information obtained might add more insights regarding the risk posed by ZnO NPs in future.

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APPENDICES

Appendix A

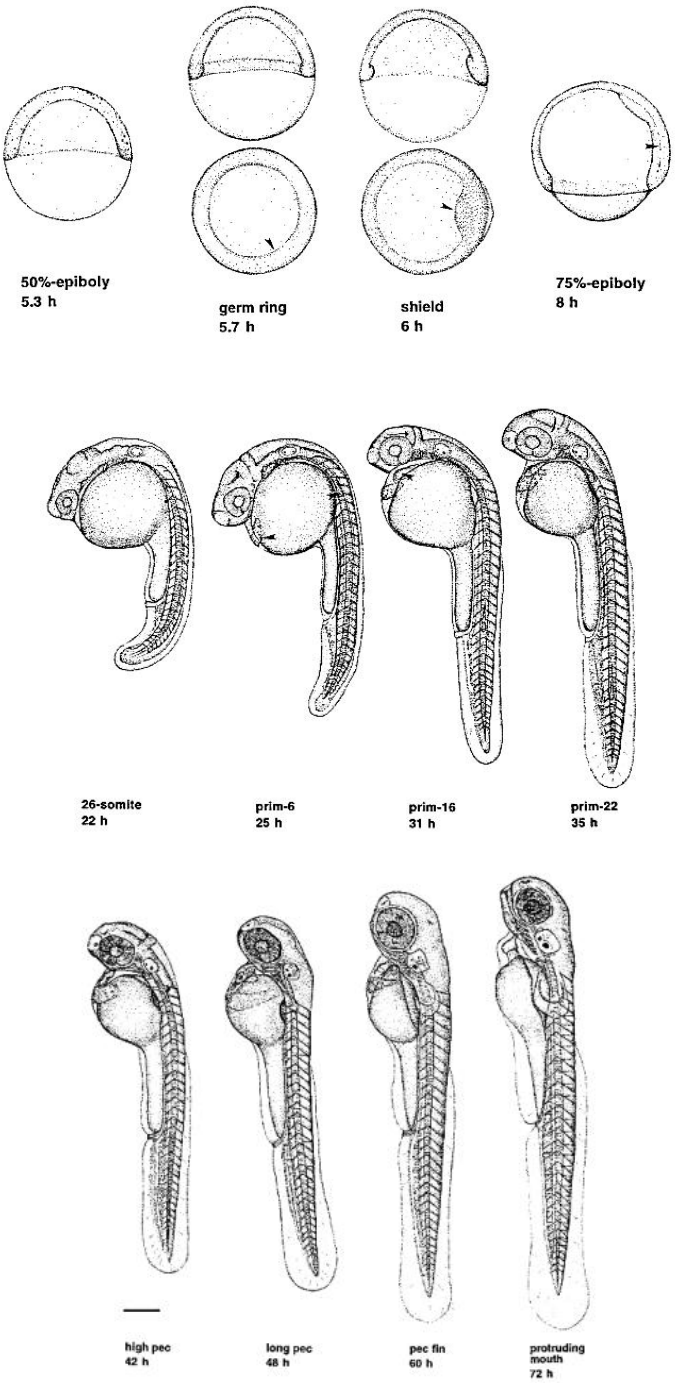


Figure 1: Development of embryonic zebrafish (Kimmel *et al*., 1995)

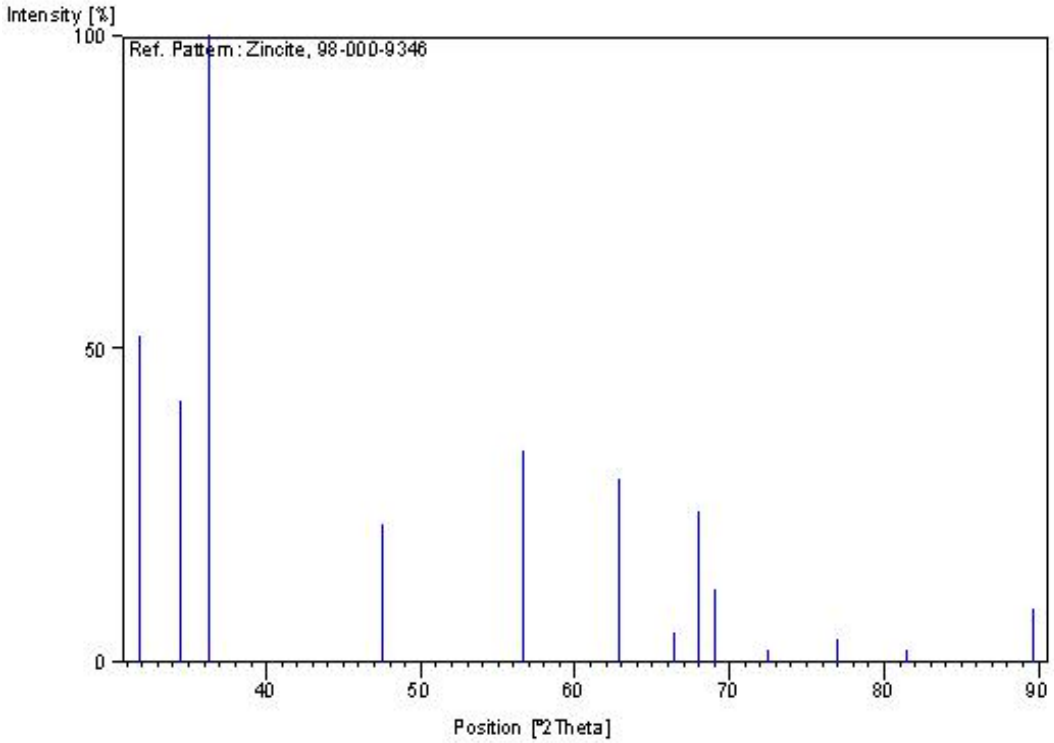
Appendix B

Table 1: FT-IR spectra of ZnO NPs from previous literatures.

Author	Yang *et al.* (2009)	Bian *et al.* (2011)	Modunkotuwa *et al*. (2012)
Size (nm)	20	4	15
Hydroxyl group (-OH)	3480	n.a	n.a
COO- asymmetric stretching	1510	1586	1500
COO- symmetric stretching	1390	1400	1389
Zn-O stretching vibration	456	n.a	n.a

*n/a not available

Appendix C



PDF code: 00-036-1451
Primary reference: Bernstein, J.L. Abrahams, S.C., *Acta Crystallographica B (24,1968-38,1982)*, **25**, 1233, (1969)

Figure 2: ZnO reference for XRD analysis

Appendix D

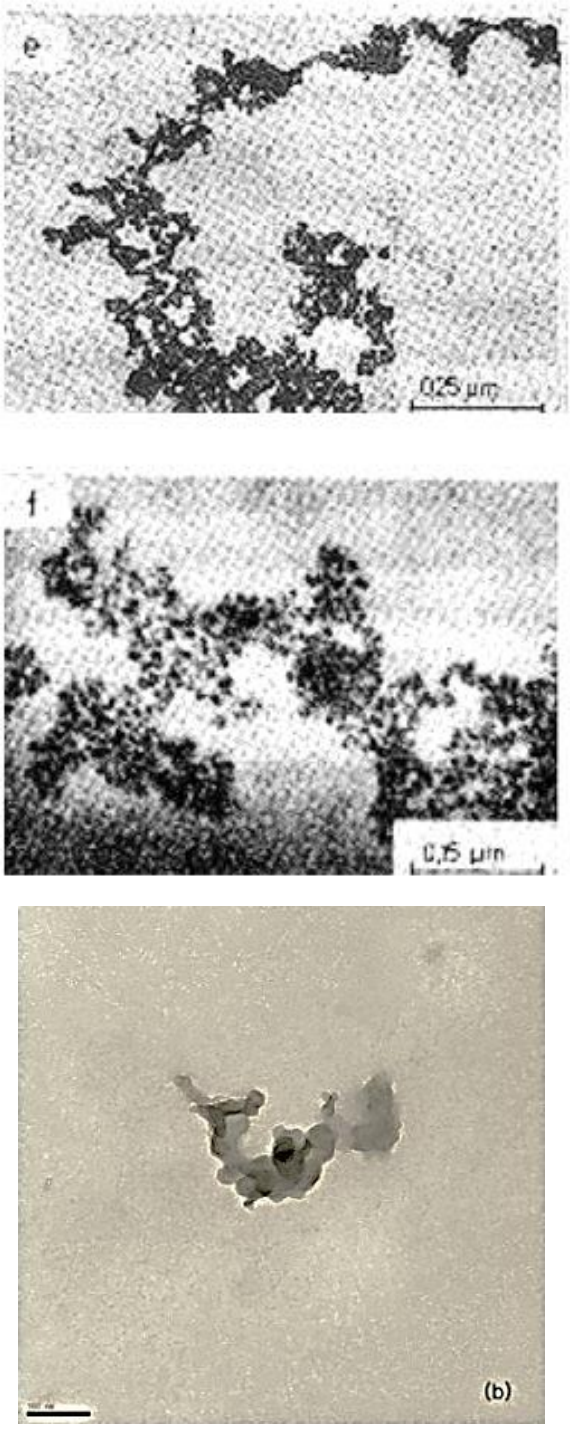


Figure 3: TEM images of HA from previous studies by Drodz (1978) and Kim *et al*. (2009).

Appendix E

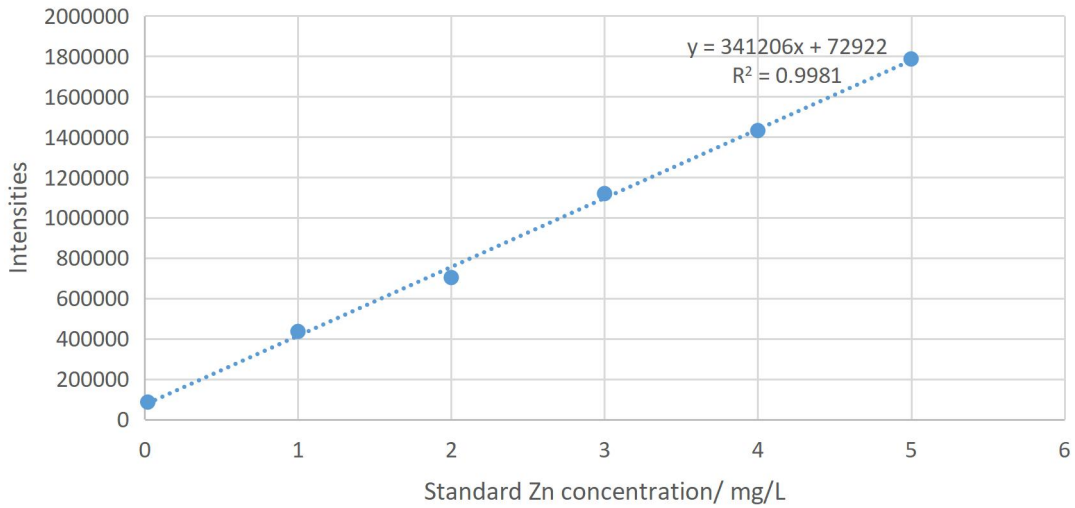


Figure 4: Calibration curve of ICP-OES

Appendix F

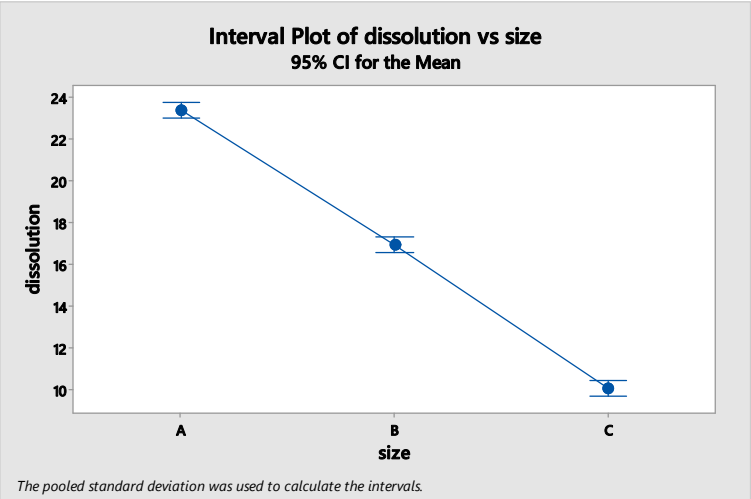


Figure 5: One-way ANOVA result with Tukey's multiple comparison between size of ZnO NPs and dissolution.