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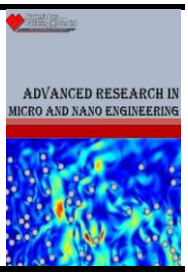

Investigation of The Stability of The Lysozyme in Magnetic Particles for Drug Delivery

Nur Fareeza Zulkifli[1], Siti Amira Othman[1,*]

[1] Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia, Pagoh, Johor, Malaysia

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ABSTRACT

This research investigated lysozyme stability in the presence of magnetic particles for drug delivery. Lysozyme can mostly be found in the hen egg albumin, and it also acts as an essential agent, which is antibodies to fight harmful bacteria or shield the human body. The problems in this research are magnetic particles that can cause toxicity and reduce the therapy's efficiency due to the degradation of carriers (drugs). To minimise this problem, the stability and activity of the lysozyme and Fe_2O_3 were investigated. The structure of lysozyme and Fe_2O_3 before and after adding acid or alkaline was defined, and the suitability of Fe_2O_3 as a drug delivery was determined. The stability of the lysozyme and $Fe_2O_3$was identified using an Ultraviolet-Visible (UV-Vis) Spectrophotometer, and the structure was investigated using a Field Emission Scanning Electron Microscope (FESEM) and Scanning Electron Microscope- Energy Dispersive X-Ray (SEM-EDX). Different concentrations become the parameters of this research as a comparison between the samples. This research found that the combination of the lysozyme alkaline material was suitable for the body at a pH of 12.2. It also found that Fe_2O_3 is ideal for drug delivery in the body when the lysozyme can be carried by Fe_2O_3 and dissolved in the Fe_2O_3.

1. Introduction

Nowadays, the development of the technology affects in every field. It includes health, environment and medication. Research on the stability of lysozyme in magnetic particles for drug delivery is connected to developing technology for magnetic particles. Magnetic particles are essential in drug delivery because they are agents that transport the delivery of medicine to the body. So, it will help the body fight against harmful bacteria that cause pain to the patient.

The lysozyme's history starts with Alexander Fleming, the first person who discovered the lysozyme. He experiments by growing a bacteria culture from his mucus that fell into the culture. He noticed that the bacteria had been killed. From his experiment, lysozyme research has been discovered day by day.

* *Corresponding author.*
E-mail address: sitiamira@uthm.edu.my

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Lysozyme is an enzyme specialising in breaking down certain bacteria's walls. The primary source of the lysozyme can be found in the white egg. Meanwhile, in plants, it can be found in turnip, papaya, and radish, but the structure of the lysozyme is slightly different from that of animals. The human body mostly can be found in saliva, tears, sweat and other body fluids. Lysozyme is known as an antibiotic that can protect the body from harm.

Lysozyme is an antibacterial agent because of the lysozyme's advantage that it can break down the chemical bond in the outer cell wall of the bacteria. In the bacteria cell wall is a layer of peptidoglycan that is the specific site the lysozyme targets. The Peptidoglycan layer contains N-acetylglucosamine and N-acetylmuramic acid, forming a solid glycan chain that acts as a backbone for the cell wall. When the lysozyme breaks the chain, the bacteria is dead. This research uses iron (III) oxide (Fe_2O_3) as the magnetic particle for drug delivery. Fe_2O_3 is the nanoparticle that acts as a targeting drug carrier. Below is one example of the formation of the magnetic particle Fe_2O_3.

Drug delivery is the technology or the system for transporting a pharmaceutical compound in the body. It is needed for the therapeutic effect to achieve safety as it is involved in site targeting within the body. Drug delivery or targeting consists of the drug's chemical formulation and the medical device or drug-device combination products. It is also a concept heavily integrated with dosage form and route of administration.

The lysozyme is used in this research because it is one of the enzymes, and we involved it with the magnetic particle to apply the particle to deliver the drug. The magnetic particle must be nano-sized because the structures and device are smaller than the human cell, which is 10,000 nm in diameter and similar to the size of the lysozyme. Because of their smallest size, the magnetic particles can penetrate the blood-brain barrier and cannot be absorbed by therapeutic and imaging agents.

It has two types of delivery, which are passive targeting and active targeting. Passive targeting is the system that targets systematic circulation. The particle must be circulated in the blood to pass to the target site successfully, and then the particle is coated with the hydrophobic polymer. However, active targeting is the easier binding of the drug carrier to target cells by ligands. It is to increase receptor among the drug and target specific delivery of the drug [2].

One of the major problems of the magnetic particle is the size of the magnetic particle. It brings the problem of toxicity and uncontrolled action as they are interested in the site of action. Their continuous use is used to reduce the efficiency of the therapy due to the degradation of carriers. The toxic cellular effects are observed to manifest in many metabolic disorders. Migration of the Fe_2O_3 can cause the risk of affecting the major organs, which could trigger the body's immunological or inflammatory response.

1.1 Lysozyme

Enzymes are macromolecular biological catalysts. Molecules at the beginning of the process are called substrates, and the enzyme converts them into different molecules called products. Almost all cell metabolic processes need enzymes to occur at rates fast enough to sustain life [5]. Most of the enzymes are proteins.

Lysozyme (N-acetylmuramide glyconohydrolase) is a self-defence enzyme produced in the serum, mucus, and many organs of vertebrates. Its typical applications are an essential index in diagnosing various diseases, including renal disease and leukaemia. It can also be used as a cell-disrupting agent in ophthalmologic preparations, as a food additive in milk products, and as a drug treatment for ulcers and infections [14]. Lysozyme is very important in the human body because it acts as an antigen and helps fight bacteria. In addition, it can break the bacteria cell wall, help restore damage to the body's

tissues by activating fibroblast cells, and help recover diseases such as sinusitis, accumulation of mucus in sinus cavities, and bleeding caused by minor surgery.

Large amounts of lysozyme can be found in egg white. For the bird, lysozyme is an antibiotic and a nutrient for early embryogenesis. For humans, it must be found in the tears that act as an anti-bacterial agent by digesting and weakening the rigid bacterial cell wall. Hen egg white lysozyme is often selected as a model protein to understand the underlying tenets of protein structure and function. It is a small globular protein with 129 amino acid residues, containing five α-helices, a three-stranded antiparallel β-sheet, and a large amount of random coil and β-. In addition, its structure is stabilised by four disulfide bonds, with most of the cysteines situated in the α-helices turns [13]. High natural abundance is also one of the significant reasons for selecting hen egg white lysozyme as a model protein for studying. The fact is that lysozyme in hen egg albumin hydrolyses the polysaccharides found in Gram-positive bacteria called walls and is used to treat inflammation, abscess, stomatitis, rheum, etc.

1.2 Magnetic Particle

Various particles with sizes ranging from ten to a hundred nanometers are used for therapeutic purposes. Monosized iron oxide nanoparticles play a dominant role, sometimes called ultra-small superparamagnetic iron oxide nanoparticles. Quantum dots, gold, and recently upconversion nanoparticles have been used less frequently. The main advantages of iron oxide (magnetite Fe_3O_4 or maghemite γ-Fe_2O_3) are their simple preparation and magnetic properties, which are necessary for detection. Moreover, it is convenient that iron oxides are readily metabolised in the body [1].

The nanoparticles have been indicated to enter the human body via inhalation, ingestion, dermal permeation or injection. The small size of the nanoparticles quickly enters the cell and transfers into blood and lymph circulation to arrive at the target sites [11]. Several studies also show that nanoparticles can cross the blood-brain barrier and enter the central nervous system of animals [4]. Today, there is much interest in developing magnetic nanoparticles as new materials, especially for biomedical functions, biomedical tumour treatment, cell labelling and sorting, DNA separation and drug delivery.

The capillaries in the blood are four μm, which is perfect for the nanoparticles to enter [10], and the large particles are usually captured and withheld in the lungs [7]. Particles with larger sizes and aggregations of small particles thus may be trapped, causing blood clots within the capillary bed of the lungs. Most of the nanoparticles tend to aggregate, therefore reducing their surface charge. Table 1 shows the list of the most commonly used biodegradable magnetic particles.

Table 1
List of the most commonly used biodegradable magnetic particles [16]

Matrix of magnetic particles	Biomedical application
Erythrocytes	Drug targeting
	Cell separation
Liposomes	Drug targeting
Phospholipids	Immobilisation of membrane-bound enzymes
Albumin	Drug targeting
	Cell separation
Starch	Drug targeting
	MRI
	Radiotherapy
Poly(lactic acid)	Radiotherapy
Dextran	Cell separation
	Enzyme immobilisation
	MRT
	Local hyperthermia
	Drug targeting
	Immunoenzyme assay
Chitosan	Drug targeting
Polyalkylcyanoacrylate	Drug targeting
Polyethylene imine	Drug targeting

Fe_2O_3 is the perfect particle for biotechnology because it has perfect magnetic responsiveness and easy manipulation of surface alteration. Research on the cooperation between Fe_2O_3 nanoparticles and lysozyme in different fields could help to describe the chemical essences of the interactions between bio-macromolecules and magnetic nanoparticles. The main goal of the research is to identify the thermal stability of lysozyme on Fe_2O_3 magnetic nanoparticles using UV-vis.

1.3 Drug Delivery

A well-designed controlled drug delivery system can overcome some of the problems of conventional therapy and enhance the therapeutic efficacy of a given drug [17]. To obtain maximum therapeutic efficacy, delivering the agent to the target tissue in the optimal amount in the correct period is necessary, causing little toxicity and minimal side effects. High therapeutic efficiency with minimal toxicity and successful application of many therapeutic agents must be improved by many problems [12].

Developments in nanotechnology have shown that nanoparticles have structures smaller than 100nm in size, which have the most significant potential as drug carriers [6]. Due to their smaller size, nanostructures show unique physicochemical and biological properties, making them favourable materials for biomedical applications [18]. The idea of using magnetic micro or nanoparticles to target drug delivery can be traced to the late 1970s [8]. A drug can be covalently attached to the nanocarrier surface, or it can be encapsulated into it. Covalent linking has the advantage of attaching as it controls the number of drug molecules connected to the nano-carrier.

The advantages of drug targeting or drug delivery are that it simplifies the drug administration protocol, reduces the cost of the therapy and reduces drug concentration in the required site, which can be sharply increased without adverse effects on non-target compartments [9]. The properties of the ideal drug carrier are that it must be explicitly recognised and selectively by the target cells, must maintain the specificity of the surface ligands, and the airline should be non-toxic, non-immunogenic and biodegradable particulate.

2. Materials and Methodology

In this experiment, the first thing is to measure the weight of lysozyme, Fe_2O_3 and NaOH. The Electronic weighing scale was used for the dry sample to measure the weight of lysozyme, Fe_2O_3 and sodium hydroxide at different molarities.

The mass of the sample was calculated using this formula

$$\text{mass (g)} = \text{concentration}\left(\frac{\text{mol}}{\text{L}}\right) \times \text{volume (L)} \times \text{formula weight}\left(\frac{\text{g}}{\text{mol}}\right) \quad (1)$$

For the simple wet sample, which is distilled water, a 100 ml measuring cylinder was used. There were usual precautions taken, such as the eye being perpendicular to the scale of the measuring cylinder to remove parallax error.

Hydrochloric acid was calculated using this formula

$$\text{Molarity} = \frac{\text{weight} \times \text{density}}{\text{molecular weight}} \text{ x } 10 \quad (2)$$

The source of the lysozyme was supplied from the company sciencelab.com. Lysozyme was packed in a bottle, and it contains the pure one. The lysozyme was kept in the refrigerator below -4˚C to maintain its quality. The lysozyme was divided into five samples at different masses and molarity, which were 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M (Figure 1 and Table 2).

Table 2
Weight of lysozyme at different concentrations

Concentration (M)	Mass (g)
0.2	0.265
0.4	0.529
0.6	0.794
0.8	1.058
1.0	1.323

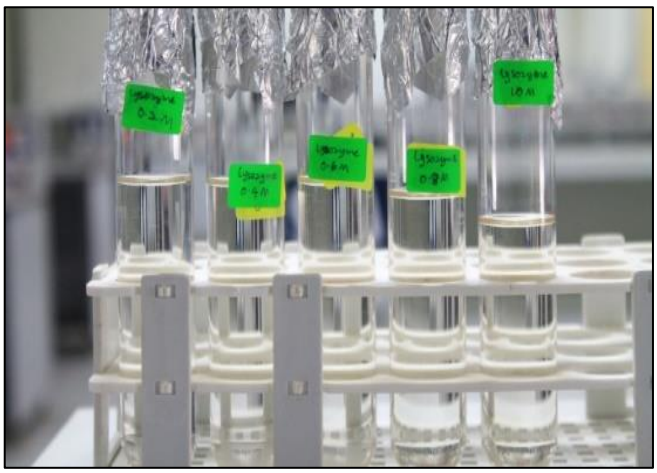


Fig. 1. Lysozyme at different molarities was placed in the test tube and diluted with 50 ml distilled water

Iron (III) oxide or Fe_2O_3 is an inorganic compound that is red-brown and odourless. In the medicine industry, it's used in calamine lotion. The mass of Fe_2O_3 is also divided into different molarities, which are 0.2 M, 0.4 M, 0.6 M, 0.8 M, and 1.0 M (Figure 2). Mass of the Fe_2O_3 was calculated using Eq. (1). The results of the calculation were recorded in the Table 3 below.

Table 3
The mass of the Fe_2O_3 at different concentrations

Concentration (M)	Mass (g)
0.2	1.597
0.4	3.194
0.6	4.791
0.8	6.388
1.0	7.985

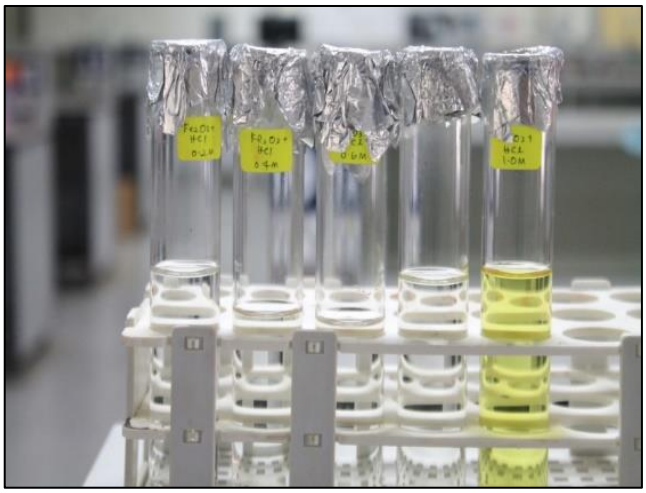


Fig. 2. Solution of Fe_2O_3 adding with HCl

The samples were characterised using the ultraviolet-visible (UV-Vis) spectrophotometer to study absorbance properties; field emission Scanning Electron Microscope (FESEM) was used to analyse the shape and the size of the sample, then the Scanning Electron Microscope- Energy Dispersive X-ray (SEM-EDX) identify the elemental composition of the sample. Analysis of EDX consists of spectra showing peaks corresponding to the element of the accurate composition of the sample. Different molarities affects the activity of the lysozyme. The lysozyme activity was observed and recorded using an ultraviolet-visible (UV-Vis) spectrophotometer.

3. Sample Characterization

3.1 Ultraviolet-visible (UV-Vis) Spectrophotometer

Ultraviolet-visible (UV-Vis) is used to determine the absorbance of the light spectrum. In this research, the wavelength for each sample was fixed at 200 nm until 1000 nm. Figure 3 shows the dilution of the lysozyme at different molarity. The lysozymes were classified with different molarity starting from 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M. Since the unit of the molarity is mol/Liter, which refers to the number of moles of solute per unit litre of solution it also can be defined as the concentration of the solution. So, the graph above refers to the analysis of lysozyme activity at different concentrations using an ultraviolet-visible (UV-Vis) spectrophotometer.

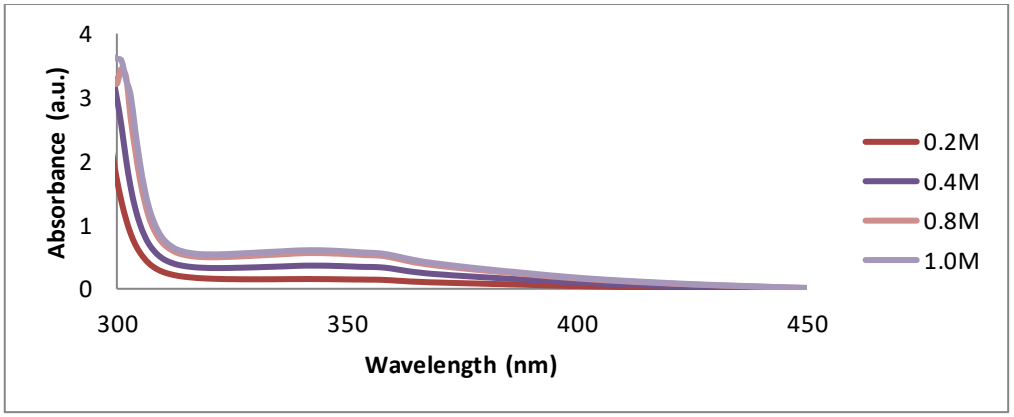
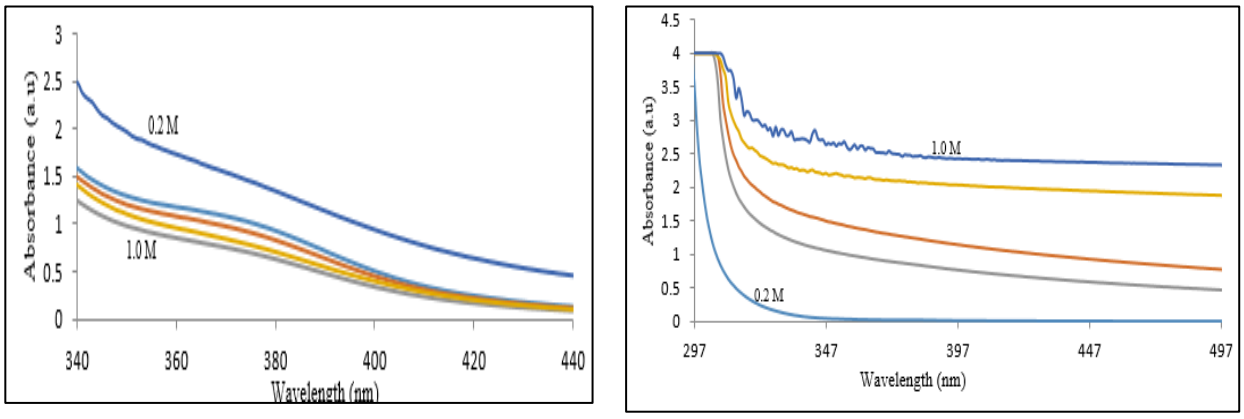


Fig. 3. Lysozyme at different concentrations

The absorbance of light shown at 1.0 M was the higher peak reading at 0.6 au, with the wavelength at 320 nm until 360 nm. At the 0.8 M concentration, the absorbance is 0.55 au while the wavelength is between 340 nm and 360 nm. The absorbance of light is detected at 0.5 au, which is for 0.6 M of concentration, and the wavelength is between 327 nm and 367 nm. The 0.4 M concentration shows the absorbance result was at 0.4 au, and the wavelength was at 328 nm to 364 nm, while the lowest result of the absorbance was at 0.13 au. The wavelength was at 359 nm to 342 nm, which for the lowest concentration of dilution lysozyme at 0.2 M. Tryptophan and tyrosine, two aromatic residues found in lysozyme, are photooxidized, causing this. This can break down disulfide bridges in the protein and produce photochemical compounds, including kynurenine and tyrosine.



(a) (b)

Fig. 4. Lysozyme was added with (a) NaOH and (b) HCl

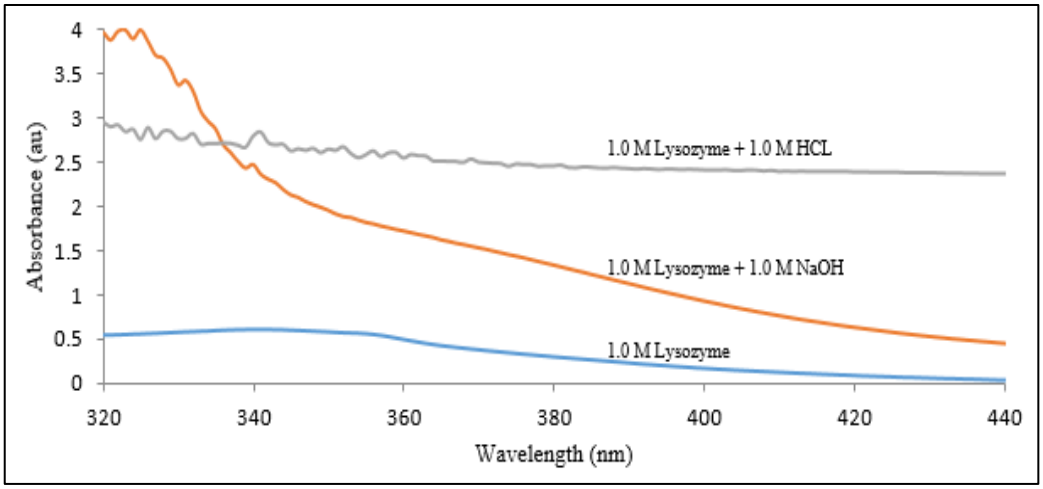


Fig. 5. The graph for different types of lysozyme at fixed molarity

Hydrochloric acid and sodium hydroxide were added in the 1.0 M concentration of lysozyme, as shown in Figure 4(a) and 4(b). It is to determine the activity of the lysozyme in the presence of acid and base. In this research, the suitable concentration of the acid and base was determined by the absorbance of light using an ultraviolet-visible (UV-Vis) spectrophotometer. The highest peak of the absorbance at a specific wavelength was chosen. 1.0 M of hydrochloric acid was added to the lysozyme solution. The result of the absorbance of light using a UV-Vis spectrophotometer is 2.91 au, and the wavelength is 320 nm to 380 nm. Because HCl breaks down the three-dimensional structure of lysozyme, it loses its enzymatic function. It may be impacted by changes in its secondary and tertiary structures brought about by the acidic environment.

Lysozyme solution mixed with 1.0 M concentration of NaOH showed the highest peak, while the lysozyme solution was at the lowest peak, and the acidic lysozyme solution was at the top, as shown in Figure 5. The absorbance of light in the mixture of NaOH and lysozyme solution was at 4.0 au, with the wavelength at 320 nm to 330 nm. NaOH can lead to denaturation of the protein by disrupting hydrogen bonds and other interactions that preserve the three-dimensional structure of lysozyme. So, we can conclude that the activity of the lysozyme is suitable in the alkaline state at pH 12.2 [3].

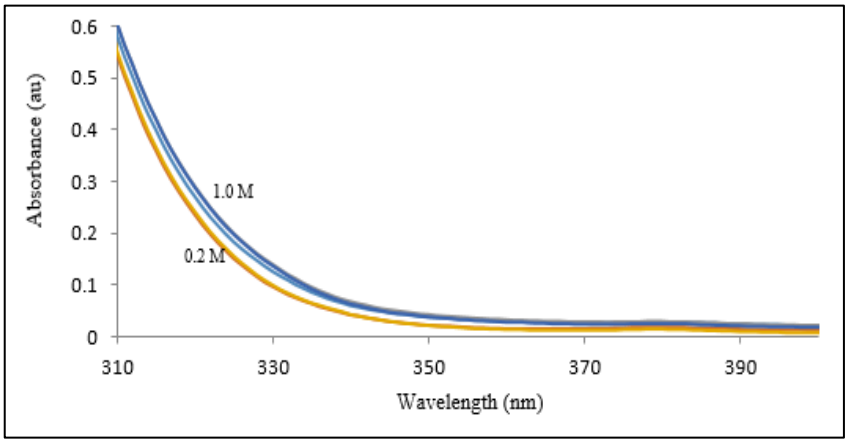
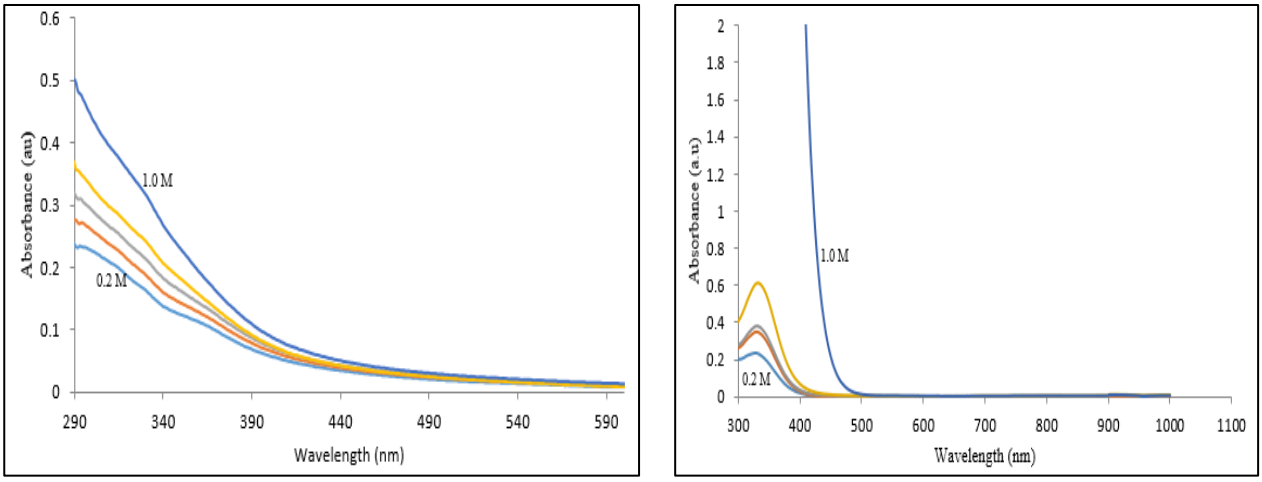


Fig. 6. The graph absorbance of Fe_2O_3



(a) (b)

Fig. 7. Fe_2O_3 adding with (a) NaOH (b) HCl

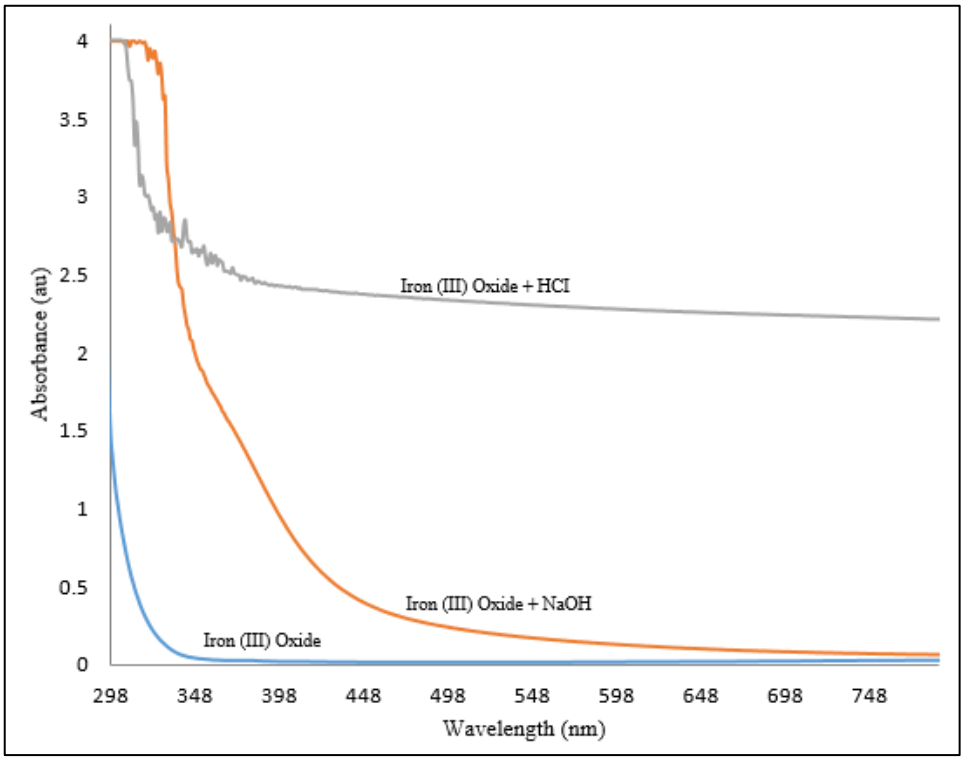


Fig. 8. Graph absorbance against wavelength of different type solutions of Fe_2O_3

The graph absorbance Fe_2O_3 against wavelength was characterised using an Ultraviolet-Visible (UV-Vis) spectrophotometer, as shown in Figure 6. Visible light can be effectively absorbed by iron oxide. Under UV light, iron oxide can demonstrate photocatalytic activity. This indicates that when they are exposed to UV light, they can produce reactive oxygen species (ROS). Figure 7(a) and 7(b) shows the graph absorbance of Fe_2O_3 added with sodium hydroxide and hydrochloric acid.

There are three types of solution in Figure 8: acidic Fe_2O_3, alkaline Fe_2O_3 and Fe_2O_3 only. The fixed variable in the Fe_2O_3 experiment is the concentration of the solution, which is 1.0 molarity. The acidic solution is the mixture of Fe_2O_3 and 4.1 ml of hydrochloric acid diluted with 50 ml of distilled water, while the alkaline solution, 20 grams of sodium hydroxide, was added. In alkaline solutions, iron's oxidation state can fluctuate. For instance, in an alkaline environment, as opposed to an acidic one, iron(II) can be oxidised to iron(III) more easily. This is because iron hydroxides are formed, and iron

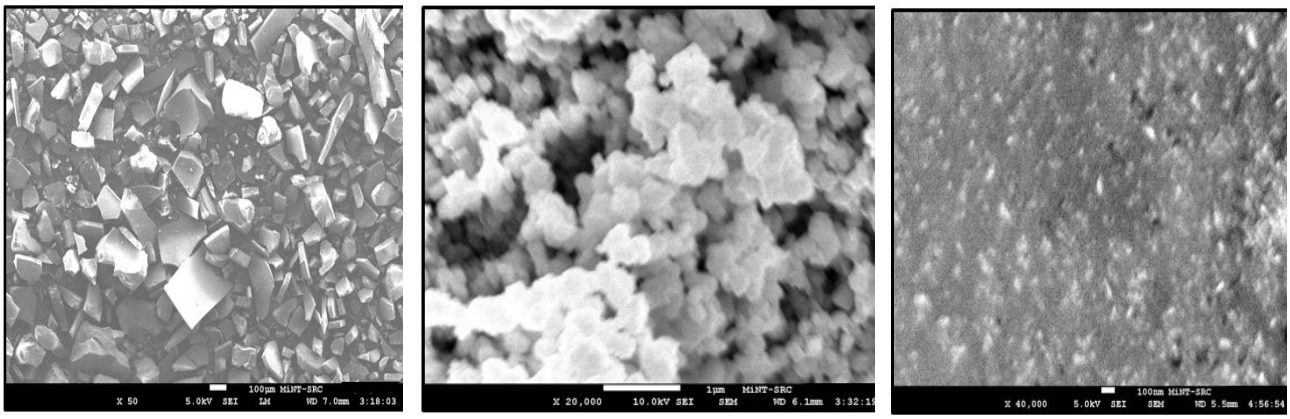
oxides can be produced by additional oxidation. In alkaline solutions, iron's oxidation state can fluctuate. For instance, in an alkaline environment, as opposed to an acidic one, iron(II) can be oxidised to iron(III) more easily. This is because iron hydroxides are formed, and iron oxides can be produced by additional oxidation. In contrast to alkaline solutions, iron oxide exhibits distinct behaviour in acidic ones. Iron oxide dissolves easily in solid acids like hydrochloric acid (HCl) but is insoluble in water. By binding to the iron ions, these substances increase their solubility.

The highest wavelength peaks are acidic solution and alkaline solution at the 4au absorbance of light. The wavelength of the acidic Fe_2O_3 was 307 nanometers, and the wavelength of alkaline Fe_2O_3 was 322 nanometers. The lowest peak of wavelength was the dilution of Fe_2O_3. Figure 8 contains unsmooth lines because of some error. The reason for the problem could be:

i. Light sources in the UV are weak. The UV lamp must be checked to ensure it is on and not broken.
ii. The detector was not sensitive to the UV. Usually, it happens at long wavelengths.
iii. the spectrophotometer was not selected in the right filter when scanning through UV wavelength.
iv. The blank or reference samples have deficient UV transmission.

3.2 Scanning Electron Microscope- Energy Dispersive X-ray (SEM-EDX)

The samples were characteristic of using FESEM to study the structure of the pure lysozyme, Fe_2O_3, and the mixture of lysozyme and Fe_2O_3 with NaOH. The thin film method was used. The sample was dry at the temperature of 100˚C on the glass slide.



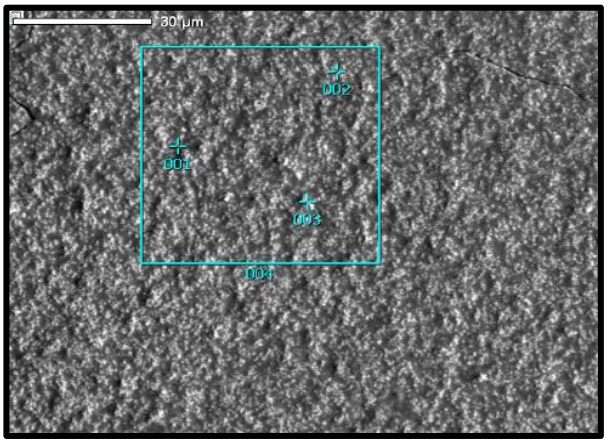
(a) (b) (c)

Fig. 9. Morphology of (a) lysozyme (b) Fe_2O_3 (c) lysozyme adding with NaOH and Fe_2O_3.

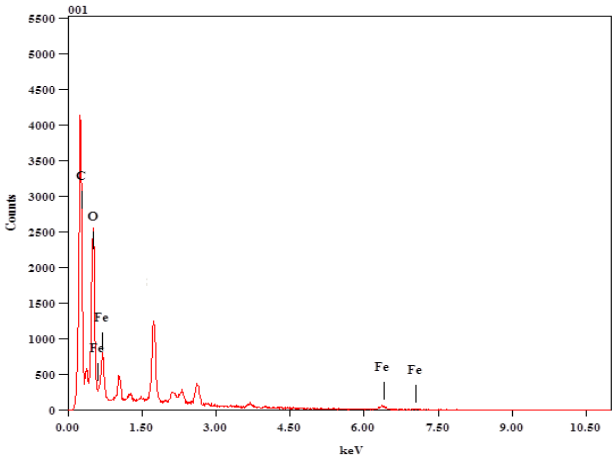
FESEM was used to analyse the minor structures from 1µm of the diameter on the surface of cells. Figure 9 shows the pure structure of lysozyme without adding sodium hydroxide and Fe_2O_3 on the FESEM at magnification 50. The structure of the lysozyme can be seen in the form of a crystal, and the size was not fixed. This is because the pure lysozyme was diluted with distilled water and dried in the furnace at 100˚C, affecting the lysozyme's size and structure. Figure 9 shows the structure of pure Fe_2O_3. The image shows that the particles are closely together in a chain and an irregular shape. The image's magnification is at 20 000 because the Fe_2O_3 were at the smallest particle or can be said that at the nano size. The magnifications of each image were different because the particle size of each sample was different. It also depends on the sample's solution. When the 1.0M dilution of the lysozyme was added with 1.0M of Fe_2O_3 and sodium hydroxide, the particle dissolved in the

mixed solution, making it the smallest, and the magnification was at 40 000 when characterised under FESEM.

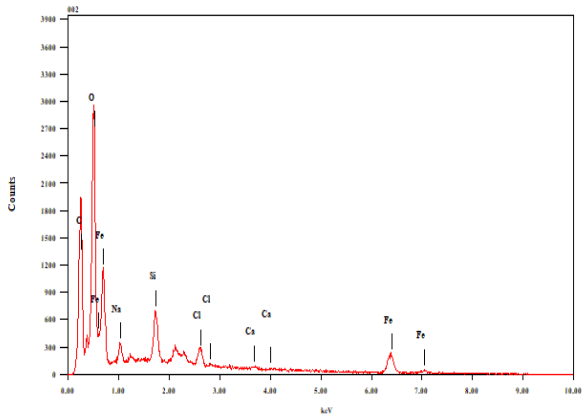
In acidic situations, lysozyme can show notable morphological alterations. The lengthy, thread-like formations known as amyloid fibrils are typically formed by lysozyme in acidic environments. Lysozyme molecules can partially unfold in acidic environments, revealing hydrophobic areas facilitating aggregation. The production of fibrils characterises amyloid structures as a result of this aggregation. These fibrils comprise the whole lysozyme and can range in length. The lysozyme protein may partially unfold in an alkaline environment, exposing hydrophobic areas that encourage aggregation. In contrast to those generated under acidic circumstances, aggregates formed in alkaline solutions are often more diverse in size and shape and less organised.



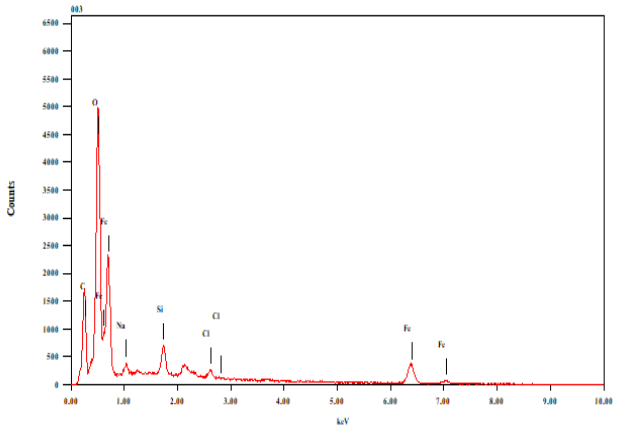
(a)



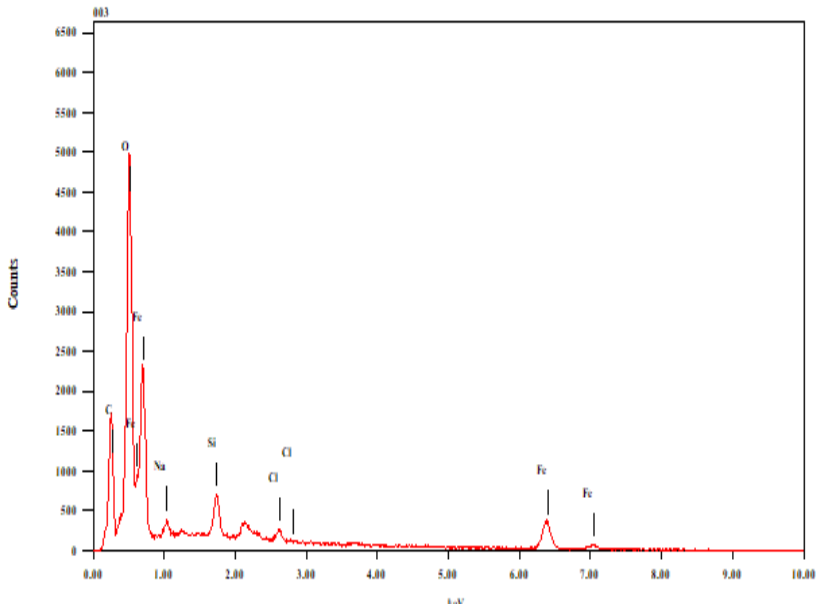
(b)



(c)



(d)



(e)

Fig. 10. Structure elements of Fe_2O_3 at point (a) 001 (b) 002 (c) 003 (d) 004

Figure 10 shows the structure of the Fe_2O_3. Each graph in Figure 10(a)-10(d) represented each point that pointed at the structure surface. The analysis of the sample was carried out using an energy-dispersive X-ray that was attached to the SEM. The sample structure was complex to explain due to the low magnification of the image.

Table 4
The mass percentage of the elements at points 001 to 004

	Carbon (%)	Oxygen (%)	Iron (%)
Point 001	47.28	28.66	7.84
Point 002	24.54	28.86	34.22
Point 003	16.00	31.79	46.33
Point 004	39.36	32.31	18.70

From the data in Table 4, iron and oxygen were the main elements in the dilution of Fe_2O_3. The highest percentage of oxygen can be found at point 004, while the lowest percentage is at the point; meanwhile, the lowest percentage of oxygen was at point 001. At point 003, it was shown that the highest percentage of iron and the lowest percentage of iron was at point 001, but in this case, the mass percentage of the iron must be higher than oxygen, following their molecular weight. In conclusion, at point 003, the percentage of iron was the highest and followed their molecular weight [15].

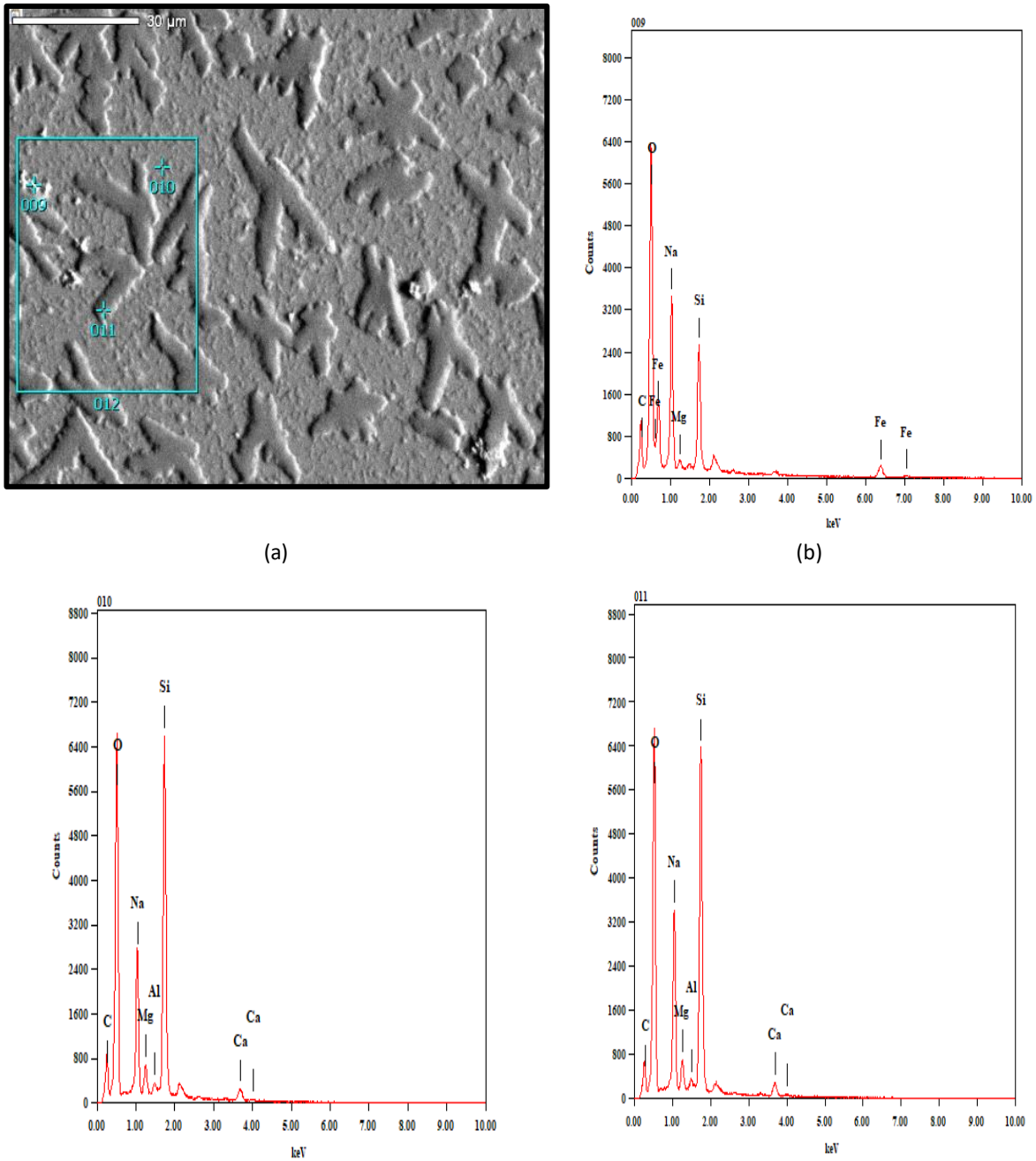


Fig. 11. List of elements at points 009, 010 and 011 in the structure of dilution Fe_2O_3 synthesised with lysozyme and sodium hydroxide

Figure 11 is the final sample characterised using a Scanning Electron Microscope. 4 points were marked at the structure to analyse elements in each end. The magnification of the sample was at 30µm. The sample structure shows the T shape image but is not fixed. The image also shows that the structures are rare and have a gap between each other. The percentage of mass at each point was recorded in Table 5 below.

Table 5
The mass percentage of the elements in the structure of Figure 11

	Carbon (%)	Oxygen (%)	Iron (%)	Sodium (%)
Point 001	9.31	34.06	24.85	18.03
Point 002	10.05	40.73	0	11.58
Point 003	7.74	39.97	0	14.54
Point 004	12.38	39.12	0	21.02

The highest percentage of carbon atoms can be found at point 004, while the lowest rate was at point 003. The range mass percentage of oxygen was between 34.06 and 40.74 %. Iron atoms can only be found at point 001, and the highest rate of sodium was at point 004.

4. Conclusion

In conclusion, the most stable lysozyme was at the concentration of 1.0 M, and the Fe_2O_3 was at 0.6 M. The sample's stability depends on the energy transition to produce the highest peak absorbance and wavelength. Moreover, at an alkaline state, the lysozyme shows the highest absorbance reading compared to HCl, which means that lysozyme can react faster in that state than HCl.

When the sample dilution of lysozyme and Fe_2O_3 was characterised under FESEM, the structure of the lysozyme was crystal form while Fe_2O_3 was irregular form. After the solution was mixed at a 1.0 M concentration of NaOH, the particles came in the smallest size and blended; we can assume that the magnetic particle is suitable for carrying lysozyme in the body as drug delivery. Investigation of the element properties using SEM-EDX makes the statement above much more potent—the chemical properties of the solutions found in each dotted at higher atomic percentages.

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