AM5023- PHYSIOLOGICAL MEASUREMENTS AND INSTRUMENTATION LABORATORY

NANOMOLECULAR- LABORATORY REPORT

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BIOCOMPATIBILITY ASSAY – MTT ASSAY

Aim:

To measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity.

Materials Required:

Cell lines and cell culture reagents

Cells in appropriate medium

Drug/Test Compound

Adjustable pipettes and a repeat pipettor

MTT Reagent

Flat bottom 96-well microtiter plate for culturing the cells

IX PBS (Phosphate Buffered saline)

DMSO (Dimethyl Sulfoxide)

96-well plate reader capable of measuring the absorbance

Theory Of Operation:

This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan. The insoluble formazan crystals are dissolved using a solubilization solution and the resulting coloured solution is quantified by measuring absorbance at 500-600 nanometres using a multi-well spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells.

Procedure:

For the determination of the cytotoxic effect of Test compound on target cells

Preparation of cells:

Always use freshly harvested cells for assay. Seed the cells in a cell culture flask or dish in an amount appropriate for the assay and incubate at 37°C in a 5% CO2 environment. Allow the cells to grow up to 24 hours or till confluence is reached.

Assay controls Include appropriate assay controls i.e.

1. Medium control (medium without cells)

2. Cell control (medium with cells but without the experimental drug/ compound)

3. Vehicle control (medium with the cells and solvent in which the experimental drug or compound is dissolved)

Harvest the cells and use for the assay. (Note: Quantity of the cell suspension to be seeded in the medium depends upon doubling time of individual cell lines and seeding density to be used in assay).

Seed 200µl cell suspension in a flat bottom 96-well plate at the optimized cell density.

Incubate the plate at 370C in a 5% CO2 atmosphere for 24 hrs.

After the incubation period, remove the plates from incubator and treat with test compound at respective concentration and time intervals.

After the incubation period, remove the existing medium from the plate and wash the cells gently with 1X PBS and remove the PBS, add 100 µl of the MTT labelling reagent (final concentration 5mg/ml) to each well.

Return the plates to the incubator and incubate for 2 to 4 hours.

(Note: Incubation time varies for different cell lines. Incubation time should be kept constant while making comparisons. Some cell lines may require incubation for up to 24 hours.)

Remove the plate from incubator after incubation and gently remove the MTT reagent in the plate and add 100 µl of DMSO to each well, provide a gentle shake on the gyratory shaker to enhance uniform dispersion of reagent.

Read the absorbance on spectrophotometer or an ELISA reader by using 570 nm as primary filter.

Subtract the average absorbance values of the blank wells from control wells and corresponding experimental wells.

% Viability - Mean OD (sample)/ Mean OD(Control)x 100

Note: Assay should be done in triplicates.

Results / Observations:

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

Applications: • Cell proliferation: Quantification of changes in proliferative activity of cells caused by trophic factors, cytokines, and growth promoters. Cell cytotoxicity: Evaluation of effects of inhibitors or inducers of apoptosis, cytotoxic reagents, carcinogens and toxins. Drug discovery: High-throughput screening of various anti-cancer drugs

Troubleshooting Points

Problem: Blanks (medium only) give high absorbance readings

Cause	Remedy
The medium is contaminated with cells/bacteria/yeast (visible under microscope).	Discard. Check medium before plating Use sterile technique for cell plating in biological hood. Use sterile 96-well plate.
The medium contains ascorbic acid.	Incubate plate in the dark. Find alternative medium if possible.

Problem: Absorbance readings too high.

Cause	Remedy
Cell number per well too high.	Decrease cell density at plating.
Contamination of culture with bacteria or yeast.	Discard. View wells prior to addition of MTT Reagent to check for contamination.

Problem: Absorbance readings are too low.

Cause	Remedy
Cell number per well is too low.	Increase cell density at plating.
Incubation time for reduction of MTT is too short. No purple colour visible in cells when viewed under microscope.	Increase incubation time with MTT Re-agent until purple color is evident inside cells when viewed under microscope. Longer incubation of up to 24 hours may be required for some cell types.
Incubation time for solubilization of formazan dye too short (intact cells with intracellular dye visible when viewed under the microscope).	Increase incubation time with Detergent Reagent or incubate at 37°C. View under microscope to ensure no crystals remain out of solution.
Cells not proliferating due to improper culture conditions or inadequate time of recovery after plating.	Check that culture conditions (medium, temperature, humidity, CO_2, etc.) are appropriate. View cells periodically to check condition.

	Increase time in culture after plating for cell recovery.

Problem: Replicates have different values.

Cause	**Remedy**
Inaccurate plating or pipetting.	Increase accuracy of cell plating, check accuracy of pipette.

DILUTIONS AND MOLARITY CALCULATIONS/ SAFE LAB PRACTICES

DILUTION

To prepare a fixed amount of dilute solution, we have a formula

C1*V1=C2*V2

Where,

V1 denotes the volume of stock solution needed to make the new solution

V2 is the final volume of the solution

C1= concentration of the stock solution

C2= Final concentration of stock solution

MOLARITY

Molarity (M)= no. of moles of solute/ volume of solution

As we know,

Moles= Mass/molar mass

Molarity(M)= (mass of the solute(W) /molar mass of the solute(M)) *volume of solution (V)

= mass of solute *1000 / (molar mass of the solute * V (ml))

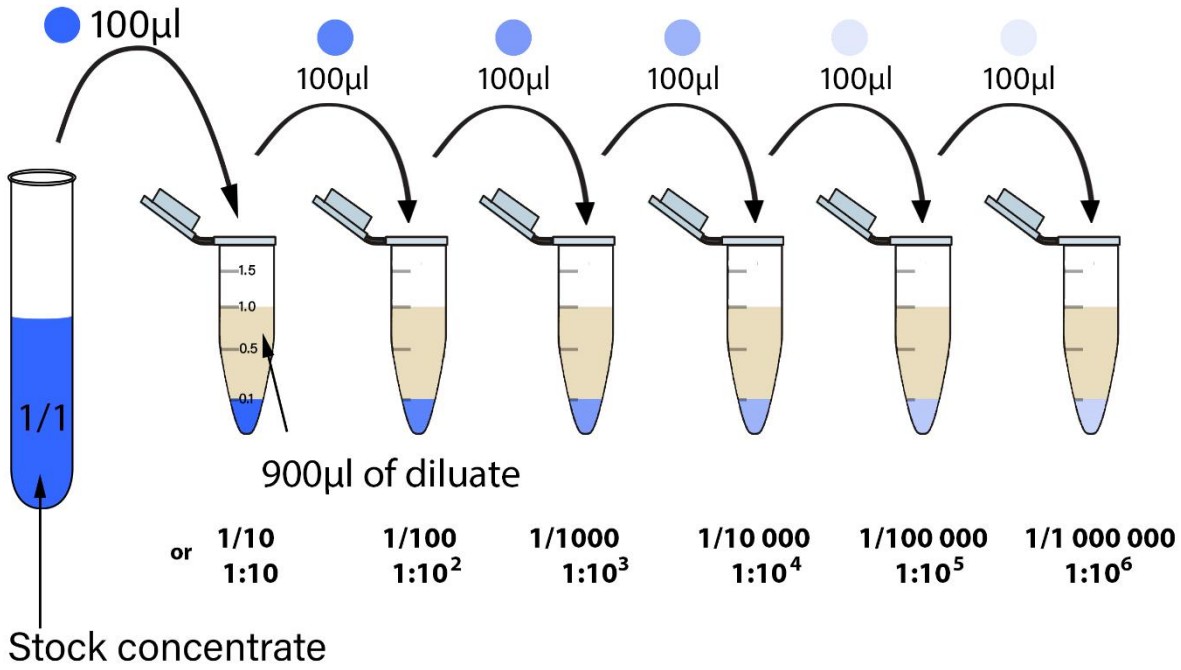
The units of molarity are mol/ L or mol/dm^3

MOLALITY

Molality (m) = No. of moles of solute/ mass of solvent in Kg

m= mass of solute *1000 / (Molar mass of solute * Mass of solvent in g)

SERIAL DILUTION



DILUTION FACTOR

After dilution, the dilution factor (or dilution ratio) represents how much of the original stock solution remains in the entire solution. It's usually expressed as a ratio, although it can also be expressed as an exponent.

The part of the stock solution to the part of the dilutant added (S: D) or the part of the stock solution to the part of the total solution (S: T) is described by the solution factor, which can be expressed as a ratio or an exponent.

Below mentioned are the steps to calculate the dilution factor by hand:

- Find any two of the following three values: stock solution volume (stock), dilutant solution volume (dilutant), and total solution volume (total). This can be done either theoretically (before conducting the experiment) or experimentally (after the experiment)
- With this equation, we can find the third volume using the two volumes: stock + dilutant= total. This step may not be necessary if we know which notation we want to use (S:D or S: T), but it is included for completeness
- Convert the numbers to the same units as each other.
- We decide which notation we require:
    - S:D = set the stock and dilutant amount values as ratio stock: dilutant.

    - S: T = set the stock and total amount values as a ratio- stock: total.
- If necessary, we find the Greatest Common Factor to cancel down the fractions.

EXAMPLES

Note: We divide the final volume by the initial volume.

DF= Vf/Vi

Example 1:

When a 0.1 ml aliquot of a specimen is added to 9.9 ml of diluent, what is the dilution factor?

Solution:

Vf= aliquot volume + diluent volume = (0.1 + 9. 9) ml = 10.0 ml

Df= 10/0.1 = 100

Thus, we have diluted the sample by a factor of 100.

The denominator of a fraction is frequently the dilution factor. A DF of 100, for example, indicates a dilution of 1:100

SAFE PIPETTING PRACTICES:

USAGE

1. Pre-wet tip. Aspirate liquid into the tip and dispense two or three times before taking up the desired volume.
2. Immerse to appropriate depth during aspiration. Immerse the tip below the meniscus. Large volume pipettes should be immersed to 5- 6 mm, while smaller volume pipettes should be immersed by to 2- 3 mm.
3. Pause consistently after aspiration and before removing the tip from the liquid. The liquid will continue moving into the tip after the plunger stops, so this consistent pause before removing the tip from the liquid will ensure the amount of liquid drawn into the pipette is accurate.
4. Use consistent plunger pressure and speed.
5. Aspirate sample at a 90-degree angle and dispense at a 45-degree angle. These angles are optimal for ensuring that all material is drawn up into, and expelled from, the tip.

6. Reduce heat transfer by holding the pipette loosely and setting down when not in use.

STORAGE

1. Store pipettes vertically to avoid contamination.
2. Store pipetted dialed to highest volume setting.
3. Store away from heat and moisture.
4. Pipetted tips should be removed prior to storage.
5. Keep pipettes clean and free of contaminants.
6. When cleaning pipettes with bleach solution, do not saturate as fluid can make its way to the piston and cause corrosion.

CLEANING LABORATORY GLASSWARE

Stir bars, spatulas, funnels, flasks, beakers, and other reusable equipment

1. To remove organic residues, rinse glassware briefly with an organic solvent (acetone or ethanol). The used rinse will then be discarded into the organic waste.
2. Use warm tap water and a brush with soapy water to scrub the inside of curved glassware. This waste water can go down the sink.
3. Remove soapsuds with deionized water to avoid harsh water stains. To expedite the glass- drying process, rinse again with acetone to remove water. The residual acetone will go into the organic waste container.

Practice Questions

1. Determine the number of grams of $NaHCO_3$ that are in one liter of a 2.1 M solution
Molecular wt of $NaHCO_3$ - 84.01g
M = no. of moles / vol in L
2.1M= 2.1. moles / 1L
Moles = mass ÷ molar mass
2.1moles = mass / 84.01
mass = 2.1×84.01mass = 176.421g

2. You have to make 500mL of a 0.5M $BaCl_2$. You have 2.0M barium chloride solution available. Determine how to make the needed dilution.

$C_1V_1=C_2V_2$

$0.5 \times 500 = 2.0 * x$

$x = (0.5 * 500)/(2.0*1000)$

$= 5/40$

$=125ml$

POLYMERASE CHAIN REACTION (PCR)

AIM

Amplify the nucleotide sequence of a desired gene.

MATERIALS REQUIRED

- Sterile distilled water
- 10X Taq buffer
- dNTPs
- Primers (both Forward and Reverse)
- Taq DNA polymerase
- Template DNA

INTRODUCTION

PCR is an in vitro method of enzymatic synthesis of specific DNA fragments developed by Kary Mullis in 1983. It is very simple technique for characterizing, analysing and synthesizing DNA from virtually any living organism (plant, animal, virus and bacteria). The purpose of a PCR is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing or for gene cloning. Any sequence of DNA can be amplified if the flanking sequences are known. Based on the flanking sequence, the primers l and 2 must be designed and used for PCR amplification of the desired gene. PCR amplification is done by the following steps:-

The cycling reactions: There are three major steps in a PCR, which are repeated for 25 or 30 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation at 95°C

During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example: the extension from a previous cycle).

Annealing at 50-65°C

Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension at 72°C

This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to

the primer on the 3' side the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).

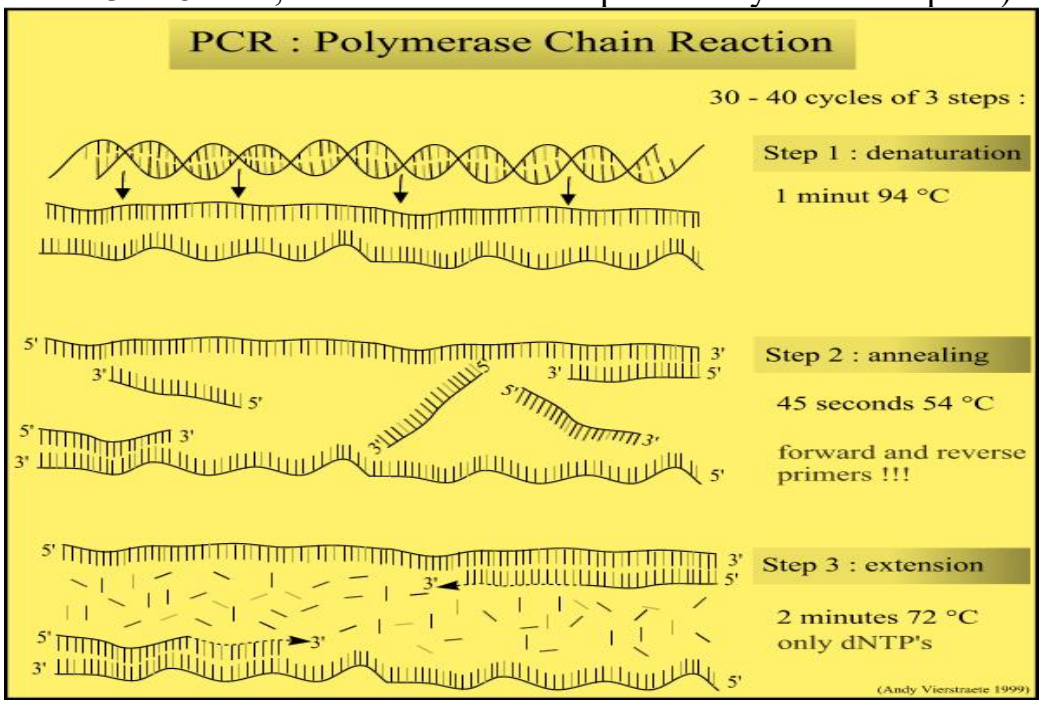


Fig.01-Different steps in PCR

EXPONENTIAL AMPLIFICATION

Both strands are copied during PCR, so there is an exponential increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, and three cycles will result in 8 copies and so on.

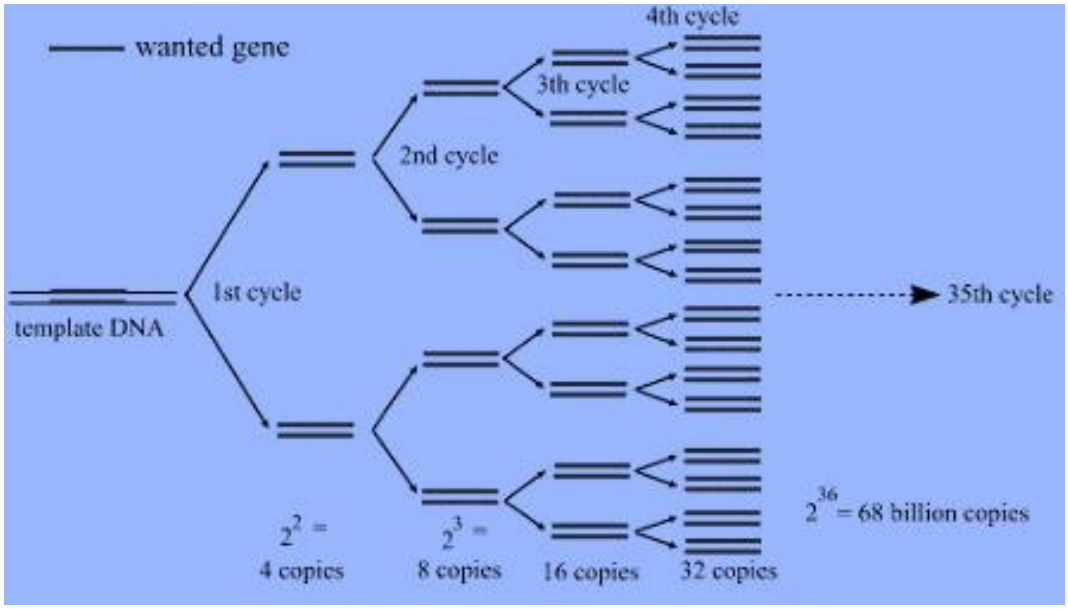


Fig.02-Exponential amplification of the gene in PCR

PROCEDURE

• Gently vortex and briefly centrifuge all solutions after thawing.

• To a 0.2 ml PCR microfuge tube add the following contents

Content	Volume
Sterile distilled water	12.8 µl
Taq buffer (10X)	2.0 µl
2 mM dNTPs mix	2.0 µl
Forward Primer (10 to 15 pM)	1.0 µl
Reverse Primer (10 to 15 pM)	1.0 µl
Taq polymerase (1.25 U/50 µl)	0.2 µl
Template DNA	1.0 µl
Total volume	20.0 µl

- Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube.
- Overlay the sample with half volume of mineral oil or add an appropriate amount of wax. This step may be omitted if the thermal cycler is equipped with a heated lid.
- Place the tubes into the slots of thermocycler and run for 35 to 40 cycles with the following PCR condition:-

Step 1: 95° C for 10 mins
Step 2: 95° C for 1 min
Step 3: Annealing temp (55-60) for 1 min 3 0)
Step 4: 72°C for 1 min
Step 5: 35 time's step 2
Step 6: 72 °C for 10 mins
Step 7: 4 °C forever
Step 8: End

RESULT

Familiarised various steps involved in PCR reaction and the related equipment.

SYNTHESIS OF HEMATITE NANOPARTICLES AND CHARACTERIZATION

INTRODUCTION

Hematite (α-Fe_2O_3) is a stable iron oxide nanoparticle with low cost, high corrosion resistance, thermal stability, conductivity, n-type semiconducting and magnetic properties. Owing to varying particle size, shape, magnetic and physical properties, α-Fe_2O_3 nanoparticles have been considered as a polymer reinforcement.

AIM

To synthesize spherical hematite nanoparticles and undergo characterization of nanoparticles

MATERIALS REQUIRED

Iron Nitrate ($Fe(NO_3)_3$)

Barium Chloride ($BaCl_2$)

Polyvinyl Pyrolydine (PvP)

THEORY OF OPERATIONS

Formation of the uniform α -Fe_2O_3 ellipsoidal particles are obtained by forced hydrolysis, which is obtained by precipitation of iron (III) perchlorate in the presence of urea. Urea ($CO(NH_2)_2$) liberates hydroxide ions which is used to promote the precipitation of metal oxides when heated in aqueous solutions. The addition of phosphate resulted in ellipsoidal α -Fe_2O_3 particles whose axial ration increased with the relative phosphate to iron amount. The particle shape from the tiny spheres evolved to ellipsoids could be explained through the aggregation of the initially precipitated particles. This led to a reduction in the observed particle count as aging time increased. The growth of particles occurs through the aggregation of much smaller primary particles or through the diffusion of solutes from the solution to the particle's surface.

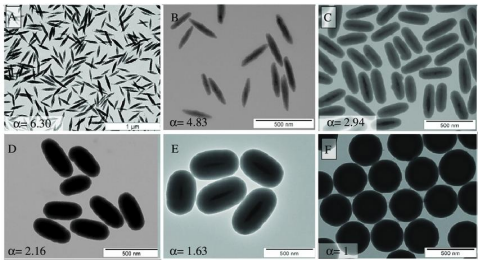


Fig. 1 : Ellipsoidal hematite nanoparticles

PROCEDURE

Synthesis of hematite nanoparticles (Here hematite acts as a targeted drug delivery carrier; a cargo)

1. Ellipsoidal hematite nanoparticles are produced by combining 0.5388 mmoles of $Fe(ClO_4)3.6H_2O$, 2.497 mmoles of NH_2CONH_2, and 0.13 mmoles of NaH_2PO_4 in 25 mL of Milli-Q water.
2. The solution is placed in a hot air oven and incubated at 100±2°C for 24 hours.
3. After incubation, the solution should change color to reddish-brown, confirming hematite nanoparticle synthesis.
4. Next, the solution is centrifuged at 8,000 rpm for 30 minutes.
5. The resulting pellets are washed repeatedly with Milli-Q water until the supernatant becomes clear.
6. Finally, the pellets are redissolved in Milli-Q water to create a nanoparticle suspension.

CHARTERIZATION OF HEMATITE NANOPARTICLES

UV-V is spectroscopy

The UV-Visible absorption spectra of the hematite nanoparticles were acquired by subjecting the HNP suspension to a scan within the wavelength range of 200 to 1100 nm using a UV spectrophotometer.

Scanning electron microscopy

The process of sample preparation involved depositing a droplet of the HNP solution onto a 10x10 mm^2 glass slide. To enhance conductivity, a 120s gold sputter coating was applied. High-resolution scanning electron microscopy (SEM) was then utilized for imaging.

PARTICLE SIZE HISTOGRAM AND CALCULATION

Calculation

To determine the mass of each compound when combined in a 25 ml solution, we'll use the given quantities in moles and their respective molar masses.

Given

- Fe(CIO4)3.6HO: 0.5385 mmoles

- NH2CONH2: 2.497 mmoles
- NaH2PO4: 0.13 mmoles

Solution

Molar masses:

- Fe (CIO4)3.6HO

Molar mass of Fe = 55.845 g/mol
Molar mass of Cl = 35.453 g/mol Molar
mass of O = 15.999 g/mol Molar mass of
H2O = 18.015 g/mol

Fe (CIO4)3.6HO = Molar mass of Fe + 3 * Molar mass of Cl + 6 * Molar massof O + 6 * Molar mass of H2O

Molar mass of Fe (CIO4)3.6HO = 55.845 + 3 * 35.453 + 6 * 15.999 + 6 * 18.015 = **419.32 g/mol.**

- NH2CONH2

Molar mass of N (Nitrogen) = 14.007 g/molMolar
mass of H (Hydrogen) = 1.008 g/molMolar mass
of C (Carbon) = 12.011 g/mol Molar mass of O
(Oxygen) = 15.999 g/mol

Molar mass of NH2CONH2 = Molar mass of N + 2 * Molar mass of H + Molarmass of C + 1 * Molar mass of O

Molar mass of NH2CONH2 = 14.007 + 2 * 1.008 + 12.011 + 15.999

Molar mass of NH2CONH2 **= 60.056 g/mol**

- NaH2PO4.

Molar mass of Na (Sodium) = 22.99 g/mol Molar
mass of H (Hydrogen) = 1.008 g/mol
Molar mass of PO4 (Phosphate group) = **94.971 g/mol**

Molar mass of NaH2PO4 = Molar mass of Na + Molar mass of H2 + Molar massof PO4 Molar mass of NaH2PO4 = 22.99 + 2.016 + 94.971

Molar mass of NaH2PO4 = **119.976 g/mol**

1. Mass of Fe (CIO4)3.6HO:

Mass = Number of moles × Molar mass
Mass = 0.5385 mmoles × 419.32 g/mol = **225.919 mg**

2. Mass of NH2CONH2:

Mass = Number of moles × Molar mass
Mass = 2.497 mmoles × 60.056 g/mol = **149.899 mg**

3. Mass of NaH2PO4:

Mass = Number of moles × Molar mass
Mass = 0.13 mmoles × 119.976 g/mol = **15.597 mg**

PARTICLE SIZE CALCULATION FROM IMAGE J

The image is loaded in Image J and the scale is set according to the scale bar in the image. Then the image is filtered using band pass filtered and then threholded to get the edges. Then The particle analysis is performed.

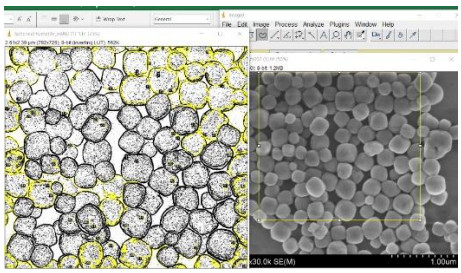
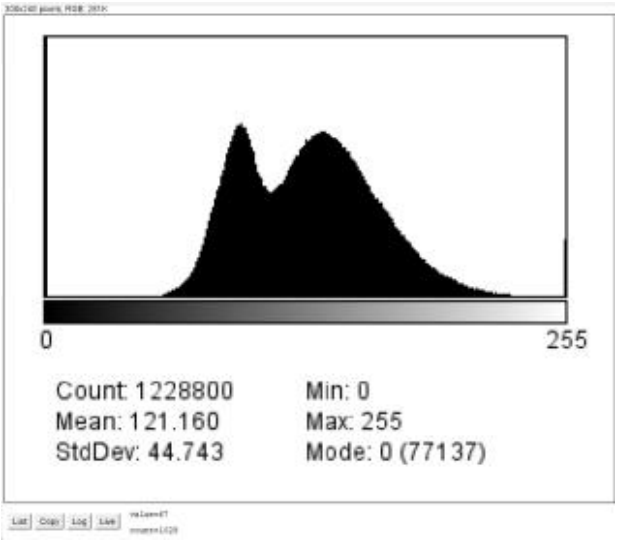


Fig. 2. Getting particle area from Image J

The area obtained from the Image J was used to calculate the particle diameter and then the particle size distribution was plotted in Origin as shown in the figure below.

	Area	Mean	Min	Max	Angle	Length
1	9.734E-4	156.619	131.209	202	18.838	0.295
2	5.883E-4	164.095	114.000	178.409	36.027	0.178
3	8.023E-4	181.130	125.333	223.018	14.036	0.243
4	0.002	157.629	117.000	212.000	7.289	0.281
5	0.002	157.629	117.000	212.000	7.289	0.281
6	0.001	148.608	120.208	212.333	16.750	0.350
7	8.986E-4	150.558	130.307	187.514	7.549	0.273

	Area	Mean	Min	Max	Angle	Length
1	0.001	127.485	102	155.149	16.587	0.321
2	0.001	131.789	109.154	162.000	14.859	0.331
3	7.689E-4	162.233	123.000	192.795	-8.130	0.231
4	8.117E-4	157.349	123.500	193.154	-27.937	0.243
5	0.001	146.565	115.353	203.667	-2.291	0.329
6	8.010E-4	154.523	117.500	198.770	19.654	0.242



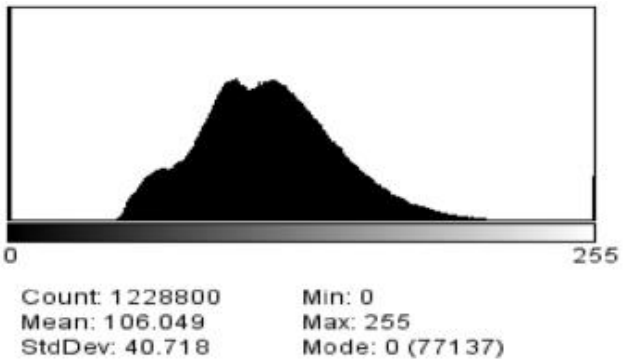


Fig 3. Particle Size Distribution

PARTICLE MORPHOLOGY

The precise calculation and control of Fe(ClO4)3.6H2O, NH2CONH2, and NaH2PO4 masses are pivotal in tailoring particle morphology. These meticulous measurements directly influence nanoparticle size, shape, and surface characteristics, crucial in crafting effective drug delivery systems. Such control ensures reproducibility and consistency, essential for optimizing nanoparticles' properties for targeted therapeutic applications.

RESULTS/ OBSERVATION

In summary, ellipsoidal hematite nanoparticles, serving as targeted drug carriers, are successfully synthesized through a meticulous process involving precise quantities of Fe(ClO4)3.6H2O, NH2CONH2, and NaH2PO4 in a Milli-Q water solution. The controlled incubation at 100±2oC for 24 hours yields a distinct reddish-brown color, confirming nanoparticle formation. Centrifugation and thorough washing result in purified pellets, later redissolved in Milli-Q water, ultimately producing a nanoparticle suspension ready for potential applications in targeted drug delivery systems.