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PREPARATION AND CHARACTERIZATION OF NANO-BIO HYBRID BASED MAGNETO LIPOSOME

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ABSTRACT: The biological synthesis of magnetite nano-bio hybrid using plant extracts plays an important role in the field of nanotechnology. The present study demonstrates bio-reductive synthesis of protein coated magnetite (Fe_3O_4) nano-bio hybrid using leaf extracts of *Datura inoxia* plants and its encapsulation into liposome. Liposome is vesicles of varying size consisting of a spherical lipid bilayer and an aqueous inner compartment that are generated in vitro. Magnet-driven liposome used for the targeted delivery of drugs to organs and tissues. These liposome preparations contain encapsulated drug components and finely dispersed magnetic particles inside the liposome. Liposome is useful in terms of biocompatibility, biodegradability, low toxicity and can control bio distribution by changing the size, lipid composition, and physical characteristics. Furthermore, liposome can entrap both hydrophobic and hydrophilic drugs and are able to continuously release the entrapped substrate, thus being useful drug carriers. The nano bio hybrid based magneto liposome has been prepared by reversed phase evaporation (REV) method using oleic and linoleic acid which has been shown to be driven under magnetic field. The magneto liposome was characterized by chemically using potassium thiocyanate (KSCN) and confirmed by UV-Vis spectrophotometer Cary 60, Agilent. Magnetic liposome has an advantage over free magnetic nanocores as various functional groups can be attached to the surface of liposome for ligand-specific targeting. Such aspects of magnetically-driven liposome preparations can be used for the targeted delivery of drugs to organs and tissues.

INTRODUCTION: Liposome represents dominant classes of nanocarriers capable of efficiently encapsulating and delivering a variety of drugs [1]. Hence liposome is vesicular systems that are formed when phospholipids are dispersed in an aqueous solution and self-assemble into one (unilamellar) or more (oligolamellar, multilamellar) concentric bilayers surrounding an aqueous core [2].

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Liposome ensures the targeted delivery of substances encapsulated in the vesicles such as drugs and provide for a prolonged release of such substances. The increasing interest in liposome is related to the unique combination of their physicochemical and biological properties manifested both *in -vitro* and *in- vivo* [3].

Biocompatibility and site specific delivery are the two major issues for magnetic nanoparticle in therapeutic applications [4]. Therefore the green method of synthesis of magnetic nano particles described here is easy, efficient, and eco-friendly in comparison to chemical-mediated synthesis [5]. The chemical synthesis involves toxic solvents, high pressure, energy and high temperature conversion

and microbe involved synthesis is not feasible industrially due to its lab maintenance [6, 7].

The aim of the present study is to demonstrate the bio-reductive synthesis of protein coated magnetic (Fe_3O_4) nano-bio hybrid using leaf extracts of *Datura inoxia* plants and its encapsulation into liposome. The liposomes were characterized by chemical and physical methods using potassium thiocyanate (KSCN) and digital gauss meter respectively. Al last a model along with a theory has been proposed for successfully isolation of magnetic liposome from a mixture of solutions into pure state.

MATERIALS AND METHODS:

Preparation of aqueous leaf extract of *Datura inoxia*

Fresh leaves of *Datura inoxia* were washed under running tap water to remove any debris and dust attached to the leaves and subsequently with millipore water 3 – 4 times [8]. Leaves were air dried for two weeks at room temperature (25°C). The dried leaves were finely powdered through grinding using Lumix grinder.

The extract was prepared by taking 40 g of powdered leaves in a 500 mL round flask with 300 mL of sterile millipore water. Then the above was boiled for 10 min and sieved and filtered twice by using Whatman filter paper No 42. The filtrate was collected and stored at 4 °C and used within a week. Small amount of filtrate was dried at 80^0C and analyzed by FT-IR techniques [9].

Nanoparticle synthesis

Ferric (III) chloride, Ferrous (II) chloride and NaOH were purchased from CDH, and the aqueous leaves extract of *Datura inoxia* was used for the bio reduction process. To synthesize nanoparticles from *Datura inoxia* 0.53 gm of ferrous chloride tetrahydrate ($FeCl_2.4H_2O$, AR) and 1.11gm of Ferric chloride hexahydrate ($FeCl_3.6H_2O$, AR) after weighing is dissolved in 100ml of sterile deionised water in 250ml beaker. The mixture is heated at 80^0C under mild stirring [10].

After 10 minutes when the plate of stirrer gets heated up, 5mL of the aqueous solution of leaf extract was added to the above solution drop wise. After few minutes the initial colour of the mixture becomes darker. Further 20 ml of 1M NaOH (0.8gms) was measured and dissolved with sterile deionised water in a beaker and added drop wise to the solution. A change in color of the colloidal solutions and precipitation occurred, confirming green synthesis of Ferric oxide (Fe_3O_4) nanoparticles.

Magnetic Liposome preparation

The aqueous magnetic fluid (1 ml) is first dispersed in 15 ml of chloroform and methanol mixture (2:1 v/v) containing Oleic acid and Linoleic acid mixture in molar ratio of 3:2. The mixture was kept under vigorous magnetic stirring for 15 min at room temperature to obtain unilameller liposomes in water-oil emulsion (W/O). This emulsion is further introduced slowly in an excess of ultrapure water (100 ml) to obtain multiple emulsion W/O/W [11].

Vesicles were obtained by evaporation of chloroform/methanol from the microscopic oil spherules at 50^OC. The flask is kept in warm bath under magnetic stirring to keep the spherules suspended. 0.9% NaCl is added for precipitation of uncapsulated magnetic nanoparticles [12].

Characterization of Magnetic Liposome

Confirmation of magnetic nanoparticles into liposomes was determined by using KSCN as follows: Aliquots of 4 ml of liposomal solutions were mixed with 1 ml of Triton X-100 [1% (v/v) in the final solution] to break the liposomes and release the magnetic nanoparticles. A volume of 1 mL of concentrated HCl (37%) was then added to the samples to ionize the iron oxide crystal core and liberate the iron in its ferric state.

The samples were incubated for a few minutes with 3 mL of a 40 mM KSCN aqueous solution. The product of the reaction between the anion (SCN) and the Fe^{3+} was a red colored complex – pentaaqua (thiocyanate-N) Fe(III) i.e. $[Fe(NCS)(H_2O)_5]^{2+}$ whose absorbance (ABS) at 480 nm was read using a Carry 60 Agilent UV – vis spectrophotometer [2].

Characterization of Nanoparticles

Magnetite (Fe_3O_4) nanoparticles synthesized by

this green method were initially examined using Carry 60 Agilent UV – vis spectrophotometer. FT-IR spectroscopy of *Datura inoxia* leaf extract and magnetite nanoparticles was carried out in the range 4000-400 cm^{-1} by Perkin Elmer FT-IR spectrophotometer which confirmed that the protein present in the extract has the ability to act as reducing agent and stabilizer for Fe_3O_4 nano particles forming protein coated magnetic nano-bio hybrid. Thermogravimetric analysis (TGA) was performed under nitrogen atmosphere at a heating rate of 10℃/min from room temperature up to 700℃.

RESULTS AND DISCUSSION:

The *Datura inoxia* leaves material was collected from the location Latitude: 27N 48' 15.64 and Longitude: 75E 01' 51.36" (FET, Mody University, Lakshmangarh, Sikar district of Rajasthan province of India). The leaves were dried and later finely powdered for extraction of phytochemicals present in it. Bio-reductive green-synthesized Ferric oxide (Fe_3O_4) nano-bio hybrid was produced by treating ferric ions with the leaves extract of *Datura inoxia*. Ferric chloride was taken as the metal precursor in the present experiments whereas leaves extract act as a reducing as well as a stabilizing agent. The color change was noted by visual observation in the Schott Duran beaker which contains Ferric chloride solution with *Datura inoxia* leaves extract.

The color of the Ferric chloride / leaves extract solution changed from light brown to dark brown after 5 min. This color change indicates the formation of Fe_3O_4 magnetic nanoparticles in the solution. The initial pH of the leaf extract was 5.03, whereas that of Ferric and Ferrous chloride was 3. The final pH after the completion of reaction was observed as 10.

The formation of Fe_3O_4 magnetic nanoparticles was confirmed by using UV-visible spectroscopy (UV-vis), Fourier-Transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and transmission electron microscopy (TEM). The formation of the Fe_3O_4 nanoparticles was first monitored using UV-Vis absorption spectroscopy. The UV-Vis spectroscopy revealed the formation of Fe_3O_4 nanoparticles by exhibiting the typical surface plasmon absorption maxima at 290 nm from the UV–Vis spectrum **(Figure 1)**[13].

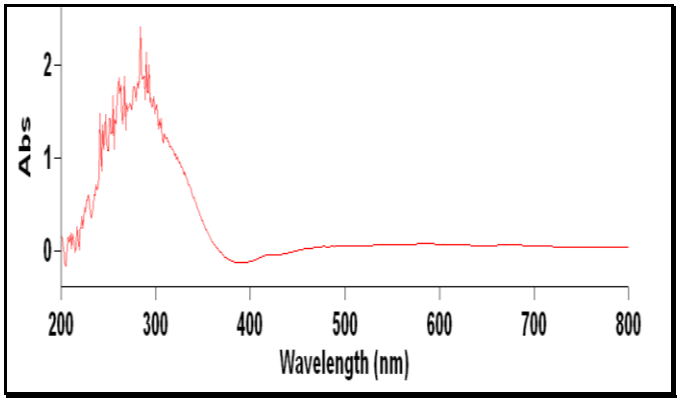


FIGURE 1: UV – VISIBLE SPECTRUM OF SOLUTION AFTER TREATMENT WITH LEAF EXTRACTS. THE CHARACTERISTIC PEAK AROUND 290 NM WAS OBTAINED; CONFIRMING THE SYNTHESIS OF MAGNETITE NANOPARTICLES.

FTIR spectroscopy was used to identify the functional groups of the active components based on the peak value in the region of infrared radiation. The strong band at 1640.11 cm^{-1} and the shoulder peak at 1411.06 cm^{-1} are identified as the amide I and amide II of the protein, which arise due to -C=O and -NH stretching vibrations in the amide linkage of the protein. The shift of the band from 1,640.11 cm^{-1} to 1,639.21 cm^{-1} was attributed to the binding of a -C=O group with the nanoparticles. The presence of magnetite nanoparticles can be seen by two strong absorption bands at around 583.45 and 449.69 cm^{-1} which, corresponding to the Fe-O stretching band of bulk magnetite (Fe_3O_4). These results revealed that the C=O groups were bonded on the magnetite particle surface. Overall the observation confirms the presence of protein in leaf extract, which acts as a reducing agent and stabilizer for magnetite nanoparticles **(Figure 2)**.

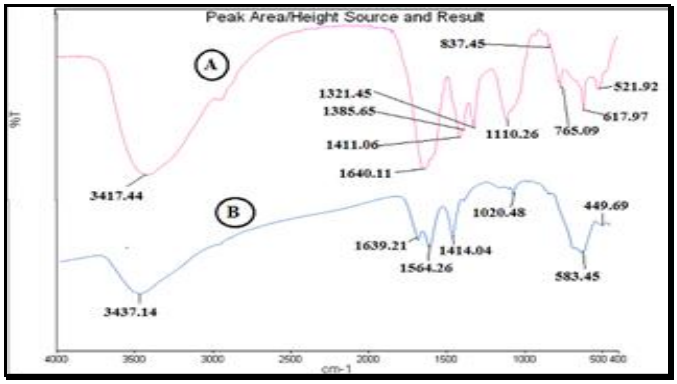


FIGURE: 2. (A) FTIR SPECTRUM OF DRIED AQUEOUS EXTRACT OF *DATURA INOXIA* LEAVES. (B) FTIR SPECTRUM OF SYNTHESIZE FE_3O_4 MAGNETIC NANOPARTICLES.

Figure 3 revealed the TGA results for these magnetic nanoparticles. An initial weight loss of 6.11% around 120^{0}C was occurred, followed by 9.66%, 2.36% and 5.45% at 340^{0}C, 510^{0}C and 699^{0}C respectively. The final residual left was 76.39%, thus confirming that the protein was conjugated to the magnetic naoparticles forming the nano-protein hybrid. The initial weight loss of magnetite nanoparticles powder under 100^{o}C is likely to be caused by the contained water.

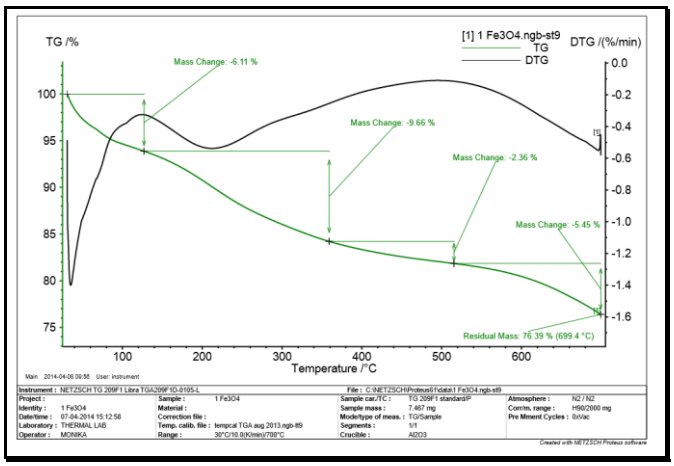


FIGURE 3: THERMOGRAVIMETRIC ANALYSIS (TGA) RESULTS OF MAGNETIC NANOPARTICLES

The protein of *Datura inoxia* leaf extract decomposes completely at temperature higher than 600℃ and the residual weight of magnetite nanoparticles is 76.39 % at 700℃. The results of TGA illustrated that there is amide I and amide II in *Datura inoxia* leaf extract in the magnetite nanoparticles with weight is around 34%. Overall the TGA demonstrated that *Datura inoxia* leaf extract existed on the surface of magnetite nanoparticles [5].

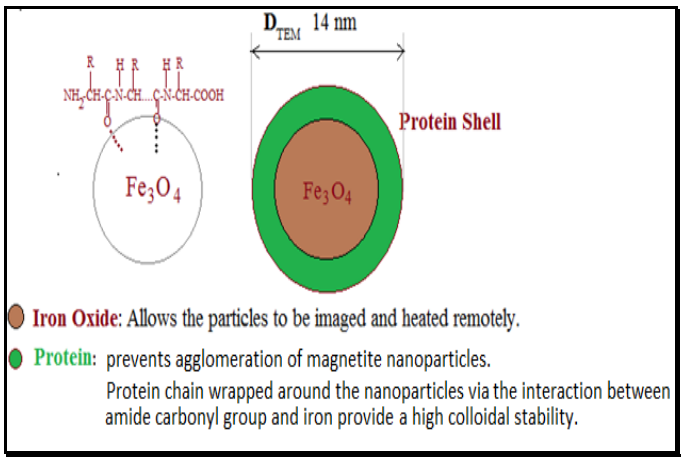


FIGURE 4: CORE-SHELL STRUCTURE OF PROTEIN-COATED MAGNETITE NANOPARTICLES

The results of FTIR and TGA confirm that protein was coated onto the magnetite nanoparticle surface forming nano-bio hybrid. In the ferrofluid, protein was chemisorbed onto the magnetite nanoparticle surface through their amide carbonyl groups by forming interactions with the Fe atoms as shown in **Figure 4.**

Magnetic hysteresis loops of the prepared magnetic nanoparticles synthesized by leaf extracts method displayed the magnetization at different temperatures such as 10 K, 100 K and 300 K (Figure 5). The magnetic properties were measured by SQUID which shows the magnetic behaviour in that magnetization and demagnetization curves completely overlapped[14]. In addition, particles also demonstrated almost identical magnetic susceptibility and saturation magnetization at different temperature, suggesting that the applied synthesis strategy did not alter the magnetic properties.

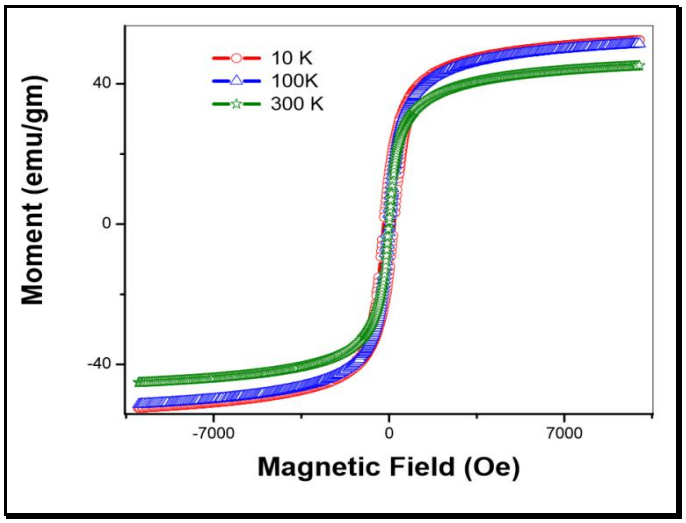


FIGURE 5: MAGNETIC HYSTERESIS LOOPS DISPLAYING THE PHYSICAL CHARACTERIZATION OF MAGNETIC NANOPARTICLES SYNTHESIZED BY BIO REDUCTIVE METHOD.

The final magnetic fluid was washed thrice with distilled water resulting in a colloidal solution of magnetic nanoparticle conjugated with protein **(Figure 6).** The solution is kept under 4^{0}C until further use. Ferric chloride $FeCl_3.6H_2O$ and ferrous chloride $FeCl_2.4H_2O$ and *Datura inoxia* leaf extract are in one aqueous phase in the reaction system. The C=O of amide group in *Datura inoxia* leaf extract chelated with Fe^{3+} and Fe^{2+} to form ferric and ferrous Protein.

With heating, OH^- of NaOH would be involved in the reaction. A competition between of C=O….Fe^{3+} and C=O....Fe^{2+} bonds and the formation of HO^- …Fe^{3+} and OH^-…Fe^{2+} …bonds and a result of formation of ferric hydroxide, $Fe(OH)_3$ and ferrous hydroxide, $Fe(OH)_2$.

The formation of ferric hydroxide and ferrous hydroxide form a shell core structure with Protein chain of *Datura inoxia* leaf extract as core. Ferric hydroxide and ferrous hydroxide in core dehydrated ($-H_2O$) forming protein coated magnetite (Fe_3O_4) nano-bio hybrid crystals.

The shell of Protein of *Datura inoxia* leaf extract chains attached on Fe_3O_4 surface through chelation of C=O…Fe^{3+} and C=O…Fe^{2+} at the end of the reaction, Fe_3O_4 nano-bio hybrid crystals were capped and stabilized by Protein chain of *Datura inoxia* leaf extract. The formation mechanism has been adopted from the earlier published article [5].

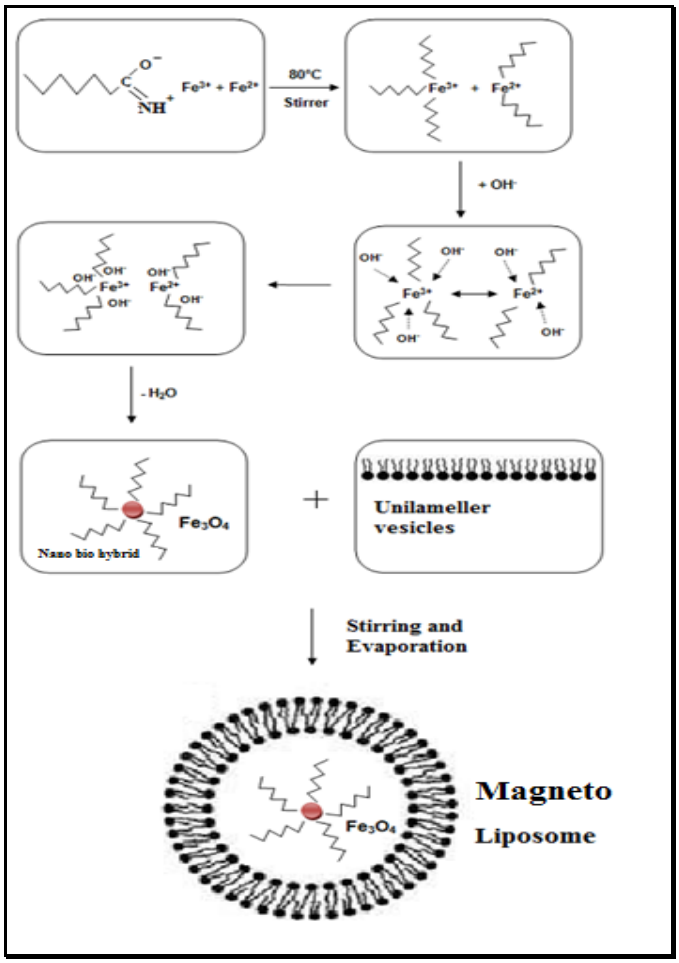


FIGURE 6: MECHANISM FOR SYNTHESIS OF PROTEIN CONJUGATED Fe_3O_4 NANOPARTICLES ENCAPSULATED IN LIPOSOMES.

Liposomes are just hollow spheres of lipids, i.e. some lipids form membranes that close on themselves forming liposomes.

Side by side liposomes preparation was carried out in which aqueous magnetic fluid was mixed with Oleic acid and Linoleic acid mixture resulting in complete miscible of the oils in organic solvent. During the initial stage of addition of water causes the oil layer to settles down at the bottom of the flask. Once the volatile liquid evaporates completely the oil emulsion starts floating on the surface of the flask forming granules of different sizes attaching to the walls of the flask **(Figure 7).**

(a) (b)

FIGURE 7: (a) PREPARATION OF MAGNETIC LIPOSOMES. (b) VESICLES CONTAINING PROTEIN CONJUGATED Fe_3O_4 NANOPARTICLES.

Assay of magnetite nanoparticles encapsulated into liposome was assayed using KSCN. Detailed mechanism has been presented in **Figure 8**. Confirmation of magnetic nanoparticles into

liposomes was determined by using KSCN. Each step is magnified in the figure for clear understanding of the process carried out at the molecular level.

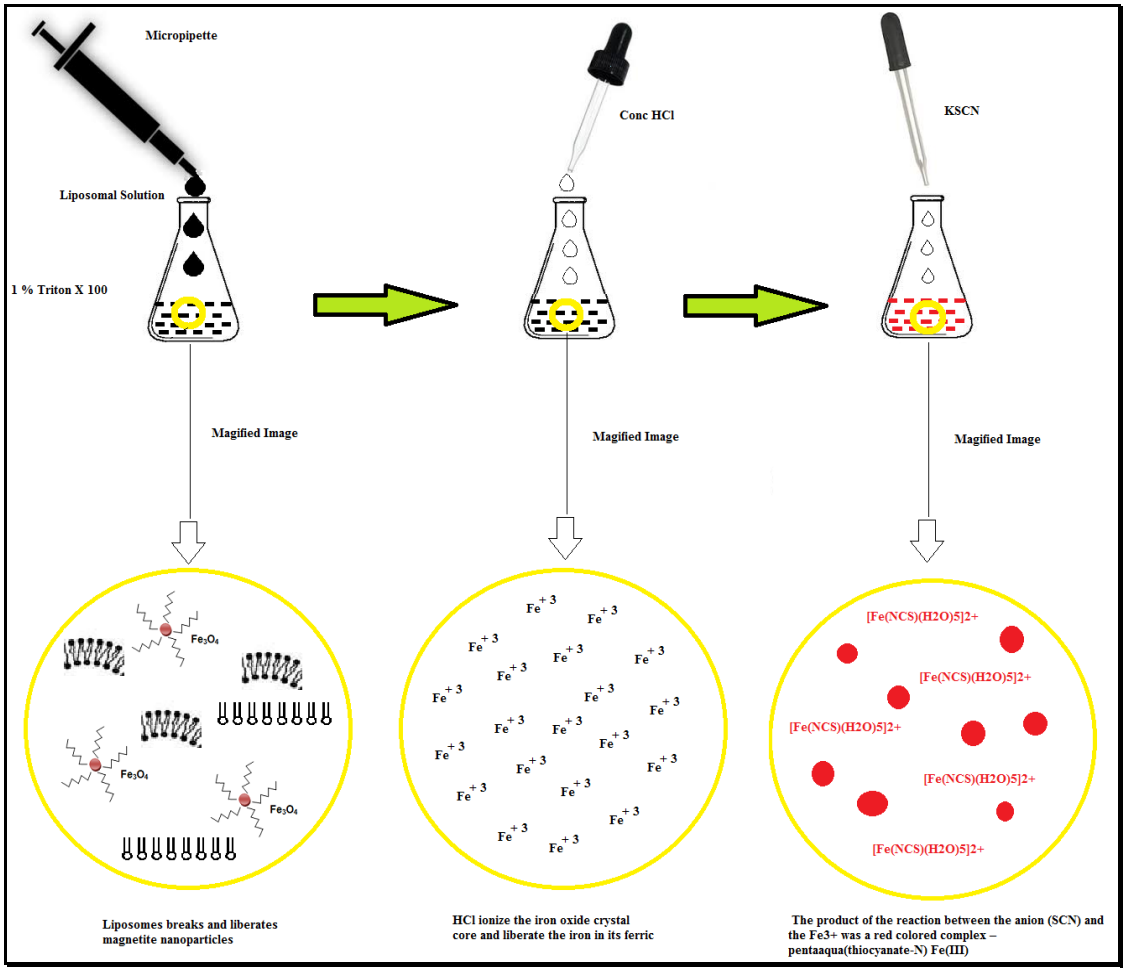


FIGURE 8: ASSAY OF MAGNETITE ENCAPSULATED INTO LIPOSOME

Addition of liposomal solutions into Triton X-100 causes the disruption of lipid bilayer into small pieces and unilameller structures. Concentrated HCl ionize the iron oxide crystal core and liberate the iron in its ferric state. The KSCN aqueous solution results into a red coloured complex – pentaaqua (thiocyanate-N) Fe (III) whose absorbance (ABS) at 480 nm was read using a Carry 60 Agilent UV – vis spectrophotometer **(Figure 9)**.

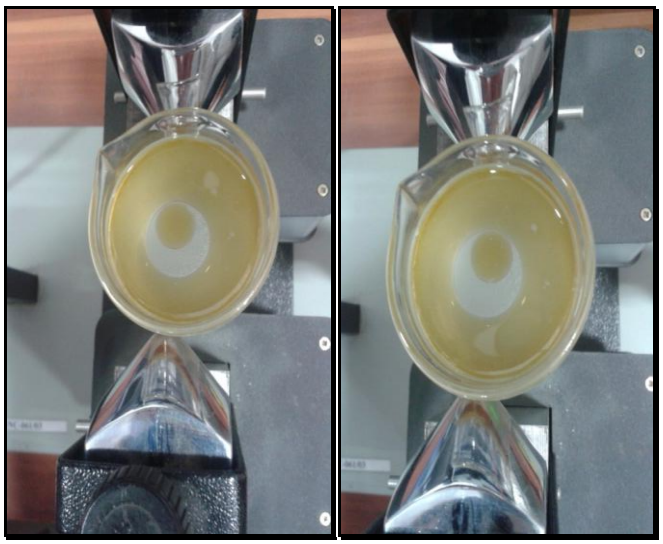
Magnetic nanoparticles have gained significant interest in recent years for various biomedical applications.

Their intrinsic magnetic properties have been explored in cell labelling/cell separation, developing bioassays, in drug delivery, to induce local hyperthermia in response to an external alternating magnetic field and to selectively destroy cancer cells [10].

FIGURE 9: PRODUCTION OF RED COLOURED COMPLEX – PENTAQUA (THIOCYANATE – N) Fe (III)

The confirmation of encapsulated magnetic nanoparticles into liposome was determined by using high power magnetic field. The liposomal solution was placed between the poles of digital

gauss meter under the influence of current. 4 A of current was supplied for obtaining strong magnetic field. A liposomal solution without magnetic nanoparticles was taken as a blank or reference **(Figure 10).**



(a) **(b)**

(c)

FIGURE 10: (a) BLANK LIPOSOMAL SOLUTION BEFORE APPLICATION OF MAGNETIC FIELD. (b) BLANK LIPOSOMAL SOLUTION AFTER APPLICATION OF MAGNETIC FIELD. (c) INSTRUMENT MEASUREMENT OF MAGNETIC FIELD UNDER THE CONTROL OF VOLTAGE.

The deflection of liposomal fluid containing magnetic nanoparticle was then studied under magnetic field **(Figure 11)**. A magnetic field of 498 gauss was observed in blank solution and a reading of 512 gauss was revealed in case of magnetic liposomes. Availability of such easily prepared magnetic liposomes is an aid in expanding the therapeutic application of liposomes.

(a) **(b)**

(c)

FIGURE 11: (a) MAGNETIC LIPOSOMAL SOLUTION BEFORE APPLICATION OF MAGNETIC FIELD. (b) MAGNETIC LIPOSOMAL SOLUTION AFTER APPLICATION OF MAGNETIC FIELD. (c) INSTRUMENT MEASUREMENT OF MAGNETIC FIELD UNDER THE CONTROL OF VOLTAGE.

Their magnetic properties allow rapid and focussed delivery of drug, before they are deleteriously affected by the blood system or otherwise. Besides, due to their unique specificity, use can be made of external vibrating magnetic fields in their deliberate, on site, rupture and immediate release of their contents [15, 16].

A novel method for complete purification of liposomal entrapped magnetic nano-bio hybrid has been performed as mentioned in **Figure 12**. A syringe was filled with the fresh prepared liposomal solution. The syringe is then connected to a flexible plastic tube. The tube is then placed between the poles of a high magnetic field. The other end of the tube is placed in the collector.

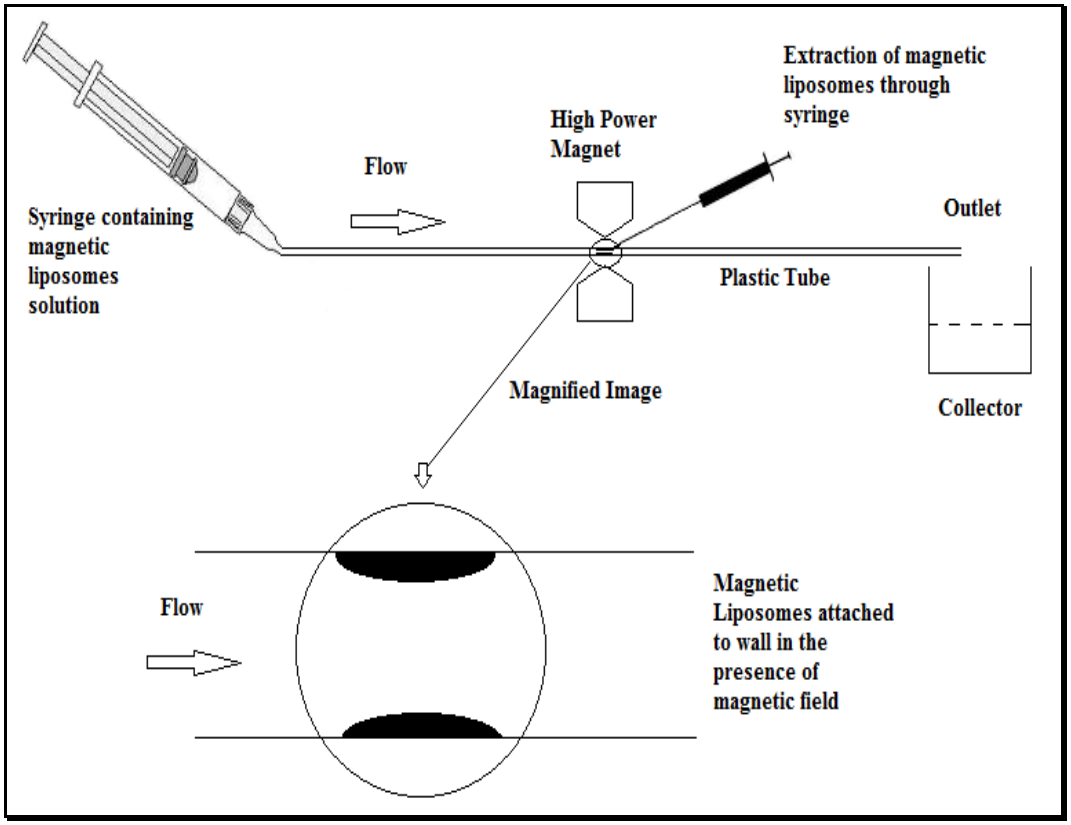


FIGURE 12: EXTRACTION OF MAGNETIC LIPOSOMES

Now with a constant rate the liposomal solution is allowed to pass in the syringe. When the magnetic nano particles entrapped in liposomes reaches at the magnetic field. The magnetic liposomes get attached to the wall of the flexible plastic tube. The solution containing liposomes devoid of magnetic nanoparticles are drained into the collector. Now with the help of another syringe the purified magnetic liposomes were extracted by piercing into the flexible plastic tube.

A major advantage in using such magnetic liposomal nanoparticles for chemotherapeutic applications is to prevent systemic drug toxicity by encapsulating the drug [10]. In addition, the particles can allow for sustained drug release over time, potentially lowering the dosing frequency and improving patient compliance.

CONCLUSIONS: In the present study we report encapsulation of plant protein mediated green synthesis of magnetite nano-bio hybrid into liposomes. This involves a green approach for the synthesis of protein coated Fe_3O_4 magnetic nano-bio hybrid using leaves extracts of *Datura inoxia* containing protein which have been found to be very effective stabilizing agent by forming a coating on the surface of the nano particle besides being acting as reducing agent for the formation of magnetite nano particles.

A theory and the mechanism have also been proposed for isolation of pure magnetic liposomes. This is the base of our future investigation where drug will be entrapping into magnetic liposome for target delivery. Such hybrid magnetic liposomes can be a robust drug delivery platform with high drug encapsulation yield, tuneable and sustained drug release profile, excellent serum stability, and potential for differential targeting of cells or tissues.

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