A Project Title

BIOLOGICAL NANOPARTICLES AND THEIR MEDICAL APPROACHES: MAGNETITE AND EXOSOMES

A DISSERTATION REPORT

Submitted in Partial Fulfilment for the Degree of MSc

(Biochemistry)

BY

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STATEMENT

I hereby declare that the matter presented in this dissertation entitled, **Biological nanoparticles and their medical approaches: Magnetite and exosomes** is based on the result of investigations carried out by me in the School of Chemical Sciences, Goa University under the supervision of **'Dr. Prachi Torney'** and the same has not been submitted elsewhere for the award of a degree or diploma.

JESCIA L. RODRIGUES

20P0460008

CERTIFICATE

This is to certify that the dissertation entitled, **Biological nanoparticles and their medical approaches: Magnetite and exosomes** is a Bonafide work carried out by '**Miss. Jescia L. Rodrigues'** under my supervision in partial fulfilment of the requirements for the award of the degree of Master of Science in Chemistry at School of Chemical Sciences, Goa University.

Dr. Prachi Torney
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Last but not the least, I would like to thank my parents and my friends for their guidance, support and encouragement.

ABSTRACT

Nanomaterials are in the great demand since past years. They are synthetically obtained by certain formulations but it is also proved to be found naturally as well as biologically. Natural sources that constitute nanomaterials are such as volcanoes, mineral springs, dust, clay etc. Biologically nanoparticles are found as magnetite in magnetotactic bacteria, insects and also human beings. Magnetite acts synergistically to enhance the toxicity of β-amyloid in Alzheimer's disease (AD). Another bionanoparticle is the exosome. Exosomes are extracellular vesicles (EVs) found in plasma, urine, semen, saliva, CSF, breast milk, serum, amniotic fluid, tears, bile, and gastric acid. It has a natural biological purpose as well as can be used for biomedical approaches.

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INTRODUCTION

Nanoscience has gained drastic demand for decades due to the unique size of the particles which ranges from 1 to 100 nm and physicochemical properties. Nanoparticles are often used for various purposes such as in biomedical sciences, medical, imaging, catalyst, technology-based, energy sciences, environmental, and agricultural [23](Sadiq, et al., 2021)

Nanotechnology research has primarily focused on manmade particles, but naturally occurring nanoparticles have been present for millions of years. Naturally occurring nanoparticles include dust, clay, sand, virus and bacteria, while those that are of synthetic origin for example gold, silver, titanium oxide and silica is referred to as the engineered nanoparticles which are normally produced from bulk materials optimized for proper interactions in vivo with biomolecules [23](Sadiq, et al., 2021). A biologically occurring nanoparticle is an assembly of molecules or atoms synthesized in a biological system. These particles include intracellular structures such as magnetosomes and extracellular assemblies such as lipoproteins and viruses [26](Stanley, 2014).

Bionanomaterials are derived from biological origins with a molecular structure in nanoscale dimensions. These bionanomaterials have several advantages such as cost-effectiveness, environmentally friendly, controlled physicochemical properties, longer circulation half-lives, lower toxicity and reproducibility [6](Debnath & Srivastava , 2022).

Lee and Wang (2006) reported the distinctive features of bionanoparticles making them attractive biomaterials compared with synthetic nanoparticles as follows:

— well-organized architectures with a broad selection of sizes at the nanometer scale;
— monodispersed particles with uniform size and shape;
— three-dimensional structures resolved at atomic or near-atomic levels;
— economic large-scale production in gram or even kilogram quantities;
— availability of genomic sequence, through which the composition and surface properties can be controlled through recombinant technology (applicable only in the case of proteins and Viral-like particles (VLPs)) [16](Lee & Wang, 2006).

The potential benefits for bionanoparticles in medical applications include:

1. The large surface area and well-defined reactivity allow the expression of multivalent targeting units which is extremely important for the requirement of weak-binding ligands or the demand for specific geometric arrangement.

2. In-capsid cavity can be used to encapsulate therapeutic chemicals for increased solubility and reduced toxicity as well as for preventing rapid clearance of small molecules.

3. Orthogonal cell-targeting mechanisms can be engineered on one bionanoparticle platform in addition to multiple detection functionalities programmed. [16](Lee & Wang, 2006)

All bionanomaterials have demonstrated less to negligible toxicity. These biomaterials might be metabolized in the body compartment and their by-product may elicit toxic effects in the body. Thus, it is highly essential to study the degradation patterns of these bionanomaterials [6](Debnath & Srivastava , 2022).

Magnetic nanoparticles are abundant in nature and are found in many biological objects. The magnetic nanoparticles found most often in living organisms are magnetite and ferrihydrite (the mineral core of ferritin). Magnetite-containing magnetosomes are rather abundant and highly ordered quasi-one-dimensional chain ensembles of magnetic nanoparticles of iron oxides (Fe_3O_4) are present in the magnetic bacteria Magnetotactic spirillum which plays an important functional role in ensuring the orientation of bacteria in the Earth's magnetic field. This magnetic nanoparticle can be used in the biological systems of targeted transfer of biologically active compounds and drugs in particular for cancer therapy using the hyperthermic effect caused by magnetic heating. These particles are detected, isolated, immobilised and modified for various other approaches [10](Gubin, et al., 2005).

Similarly, exosomes are also found to have useful applications. Exosomes are membrane vesicles that are released by cells upon fusion of multivesicular bodies with the plasma membrane. Their molecular composition reflects their origin in endosomes as intraluminal vesicles. They release by reticulocytes which clear unwanted proteins and allow net loss of the cell surface membrane, thus contributing to red blood cell differentiation. Beyond this clearing function of exosomes, the presence of proteins with adhesion properties may confer the exosome’s other roles. The presence of exosomes in biological fluids such as urine and blood plasma could be exploited as biomarkers for diagnosis purposes [21](Niel, et al., 2006).

The techniques that can be used to characterize nanomaterials include electron microscopy, scanning probe microscopies, atomic force microscopy, X-ray diffraction, neutron diffraction, X-ray scattering, X-ray fluorescence spectrometry, acoustic wave technique, contact angle measurements (Ikhmayies, 2014), cryo-electron microscopy, nuclear magnetic resonance imaging [16](Lee & Wang, 2006).

MAGNETITE

Introduction

Magnetic nanoparticles are nanocrystallites with a narrow size range of 1-100 nm with unique magnetic properties. They have wide applications in nanotechnology. They have been found to have several engineering applications such as ferrofluid technology, high-density data storage, inks, microelectronics, magnetic refrigeration, and batteries. In biomedical, for hyperthermia therapy, bio-sensing, targeted drug delivery, cancer treatment, etc. [22](Pankhurst, et al., 2003). This rise in scope for magnetic nanoparticles is because they obey Coulomb's law, and can be manipulated by an external magnetic field gradient. This property opens up many applications involving the transport and immobilization of magnetic nanoparticles and magnetically tagged biological entities [22](Pankhurst, et al., 2003).

Magnetic materials of nano-size of iron oxide are mainly detected as magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) and are found on the surface of the earth and may also persist in air, water, soil, and biological systems [13](Kaloyianni, et al., 2019). Magnetite nanoparticles (MNPs) are present in many bacteria, insects, and larger animals. Many migratory animals and fishes also have magnetic nanoparticles in their body and use them as biomagnetic compasses for orientation during their migratory travels [28](Walker, et al., 1997).

In biomedical, magnetite nanoparticles have wide applications such as magnetic resonance imaging (MRI) contrast agents, magnetic fluids, hyperthermia therapy, bio-sensing, diagnosis, controlled and targeted drug delivery, cancer treatment, etc. These applications of magnetite are because of its availability, versatility, superparamagnetism, high saturation field, blocking temperature, chemical stability, biocompatibility, and low cost [20](Niculescu, et al., 2021).

Magnetite in an organism is covered by a biological membrane called a magnetosome that contains phospholipids and specific proteins. The core of the magnetosomes is typically composed of magnetite (Fe_3O_4) which can oxidize into maghemite (γFe_2O_3). These are intracellular structures produced by magnetotactic bacteria (MTB) used to navigate the direction of the earth's magnetic field. These fascinating microorganisms were first documented by Salvatore Bellini as early as 1963 [32](Yan, et al., 2012). Magnetosomes are characterized by natural synthesis, good crystallinity, large size, and chain arrangement. This results in pure iron oxide nanoparticles which distribute homogenously in tumours and produce

a large amount of heat under alternating magnetic fields yielding efficient antitumor activity [2](Alphandéry, Edouard, 2020).

The magnetite is also present in the head, thorax, and abdomen of insects like ants and a stingless bee which acts as geomagnetic sensors [5](Bhattacharyya, et al., 2010). The presence of nanoparticles of magnetite has been identified by magnetic analyses of human brain samples [19](Maher, et al., 2016).

The magnetite nanoparticles can be synthesised by physical, chemical or biological methods. Biological methods have greater advantages over physical and chemical methods. The synthesised bionanoparticle, magnetite is characterised by UV-Vis spectroscopy, X-Ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Transmission electron microscopy (TEM), etc.

Morphology

The magnetite chemical formula is Fe_3O_4. It is the most common and utilized form of naturally occurring iron oxides. It is characterized by a crystalline cubic inverse spinel structure (Fig.1), in which ferrous ions (black spheres) occupy half of the octahedral lattice sites and ferric ions (green spheres) occupy the other half of the octahedral lattice sites and all the tetrahedral lattice sites. MNPs were reportedly synthesized as cubes, rods, disks, tubes, plates, hexagons, octahedrons, truncated octahedrons, tetrahedrons, octopods, tetrapods, rings, flowers, concaves, etc., in either solid, hollow, or porous forms. [20](Niculescu, et al., 2021) Figure 2 shows different crystal morphologies and arrangements of magnetosomes as cuboctahedral, bullet-shaped, elongated prismatic and rectangular [32](Yan, et al., 2012).

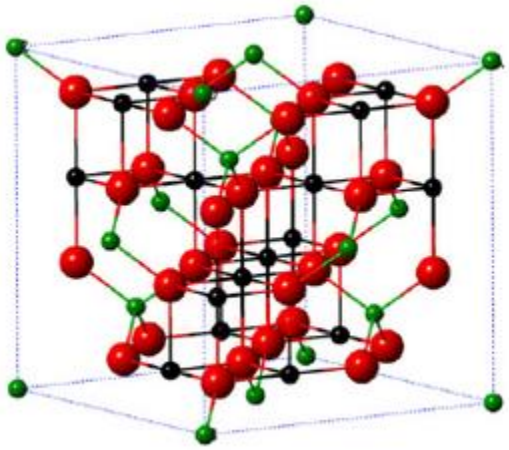

Fig.1: Crystal structure of magnetite. Green spheres, Ferric ions– Fe^{3+}; black spheres, Ferrous ions – Fe^{2+}; red spheres – O^{2-} [20](Niculescu, et al., 2021).

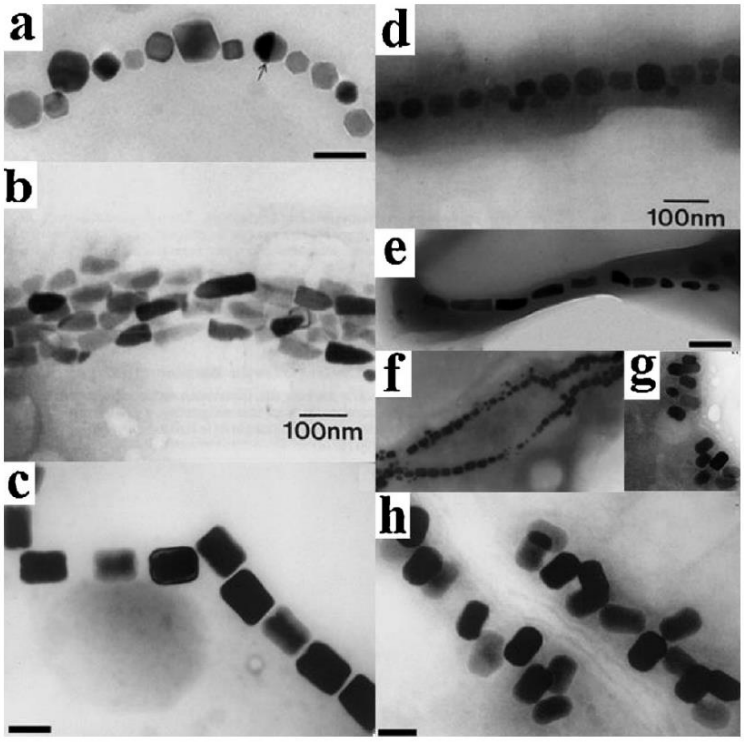

Fig. 2: Crystal morphologies and arrangement of magnetosomes: a) and d) cuboctahedral, b) and e) bullet-shaped, c), f) and g) elongated prismatic and h) rectangular morphologies. a, c, d, e: arranged in one; b: arranged in multiple chains; f: arranged in two; g, h: arranged in irregularly [32](Yan, et al., 2012).

Properties

Magnetite exhibits superparamagnetism when the size of particles is smaller than ~20 nm where the magnetization of nanoparticles is randomized by thermal energy [30](Winsett, et al., 2019). At a certain temperature, magnetite nanoparticles have zero magnetisation on average in the absence of an external magnetic field. Their magnetic susceptibility is very high in this state, allowing them to get magnetised by an external magnetic field. At room temperature, magnetite exhibits ferrimagnetic properties. Magnetite exhibits stronger magnetic properties than maghemite but it is less chemically and physically stable [20](Niculescu, et al., 2021).

Magnetite nanoparticles are biocompatible, biodegradable, non-toxic to humans, dispersible, thermal, chemical, colloidal stable and the possible for functionalization. They are not stable in air, as they have a tendency to oxidize to maghemite and may easily agglomerate after production. Therefore, MNPs used for biomedical purposes usually require corona protection and are either functionalized or surface-coated by polymers, metals, or organic and/or inorganic stabilizing agents. Various biomolecules (e.g., proteins, polypeptides, antibodies) can be bound on the surface of MNPs through a chemical coupling of specific functional end groups.

Moreover, MNPs can be protected by shells of different biocompatible materials, such as natural polysaccharides (e.g., dextran, latex, chitosan, alginate, cellulose), inert synthetic materials (e.g., polyethylene glycol, polyvinylpyrrolidone iodine), and organic acids with different structures (e.g., oleic acid, poly (aspartic acid), heptanoic acid, citric acid). Inorganic shells are made of silver, silica, gold, manganese oxide, or hydroxyapatite [20](Niculescu, et al., 2021).

Magnetosomes are obtained by cultivating magnetotactic bacteria in a growth medium, which is not toxic (for example, ATCC medium 1653 for the AMB-1 species). This contrasts with the use of toxic products often used in chemically synthesized nanoparticles. The magnetosomes can easily be functionalized, due to the presence of various chemical groups at their surface [1](Alphandéry, 2014).

Molecular formula	Fe_3O_4	**Crystallographic System**	Cubic
Colour	Black	**Lattice Parameter**	Å=0.8396 nm
Melting Temperature	1583-1597 ^{0}C	**Structure Type**	Inverse spinel
Type of Magnetism	Ferrimagnetism	**Density**	5.18 g/cm^3

Table 1: A summary of the main physicochemical properties of magnetite [20](Niculescu, et al., 2021).

The magnetosomes are large single magnetic domain nanoparticles arranged in chains inside the bacteria. This arrangement is stable enough to be preserved even after disrupting the bacteria to isolate the magnetosomes. Such arrangement is interesting since it prevents aggregation and yields a high rate of internalization within human cells, two properties that are usually desired for medical applications. The magnetosomes are thermally stable at physiological temperature thereby leading to the magnetic moment. They are covered by biological material made of phospholipids and specific proteins which results in negatively charged magnetosomes with good dispersion in water. By contrast, chemically synthesized nanoparticles are not naturally coated and need to be stabilized by covering with dextran or PEG molecules causing a complex synthesis process [1](Alphandéry, 2014).

Magnetite in organisms

Magnetite in insects

The magnetic material is present in the head, thorax, and abdomen of the insects like an ant, *Solenopsis* substitute and stingless bee *Schwarziana quadripunctata* which acts as geomagnetic

sensors. Nanoparticles isolated from insects have diameters of about 12 and 11 nm in the abdomen and head, respectively. The magnetic material content is slightly higher in heads with antennae than in the abdomen of the ants. The magnetic material in honey bees helps them with orientation, homing, and foraging. Similarly, the ants *Formica rufa* and *Solenopsis invicta* (Buren) (Fig.3) use information from the geomagnetic field for orientation during the foraging process. Also, ferromagnetic material has been detected in *Apis mellifera* abdomens and identified as suitable for magnetic reception. Magnetic nanoparticles within the A. mellifera abdomens are well accepted as it takes part in their magnetoreception mechanism [5](Bhattacharyya, et al., 2010).

Fig. 3: *Formica rufa* (Left) and b) *Solenopsis invicta* (Right)

Magnetite in humans

The presence of nanoparticles of magnetite, a strongly magnetic (ferrimagnetic) mixed Fe^{2+} /Fe^{3+} iron oxide has been identified by Magnetic analyses of human brain samples. The presence of magnetite in the brain is important because it has been linked with potential cellular responses to external magnetic fields (e.g., in MRI studies), ageing, and neurodegenerative disease like Alzheimer's disease (AD). MNPs are directly associated with AD plaques and tangles. In AD there is a formation of senile plaques containing β-amyloid fibrils. When β-amyloid fibrils get associated with redox-active transition metal ions, such as Fe^{2+} ions, β-amyloid can generate damaging reactive oxygen species, directly contributing to oxidative brain damage which is an early key feature of AD. Magnetite acts synergistically to enhance the toxicity of β-amyloid [19](Maher, et al., 2016).

Magnetite in bacteria

Magnetosome is the key component of magnetotactic bacteria (MTB). It is a membrane-bound intracellular magnetic iron-bearing inorganic crystal comprising iron oxide, iron sulphides or both [31](Yan, et al., 2012). MTB take up approximately 100 times more iron than non-magnetic

bacteria [3](Arakaki, et al., 2008). Mature magnetite crystals have a size range of about 35–120 nm which are stable, uniformly magnetized single-magnetic domains (SD). The crystals within the SD size range have the maximum magnetic dipole moment and are permanently magnetic at room temperature. Magnetic crystals smaller than the SD size range do not have persistent remanent magnetizations and are superparamagnetic (SP) at room temperature. However, particles above 120 nm are nonuniformly magnetized because of the formation of domain walls. The domain walls will tend to form multiple magnetic domains of opposite magnetic orientation, thereby reducing the total magnetic remanence of the crystals. Therefore, by controlling particle size, MTB has optimized the magnetic dipole moment per magnetosome [31](Yan, et al., 2012).

The magnetosome membrane consists of proteins, fatty acids, glycolipids, sulfolipids, and phospholipids. The MM-associated proteins are commonly known as Mam (magnetosome membrane) protein or Mms (magnetic particle membrane specific protein). The functional groups in the magnetosome surface are known to be carboxyl, hydroxyl and amino [31](Yan, et al., 2012).

These magnetotactic bacteria (MTB) are commonly found in water columns, freshwater systems, extreme environments and sediments. They can propel through the water by rotating their helical flagella. Different strains of MTB show different morphological properties and adjustments as swimmers in presence of magnetic fields due to the intracellular arrangement of the magnetosomes. The MTB of various morphological types have been found in the freshwater sediments, including bacillus, vibrios, spirilla, cocci, and multicellular (Fig.5). MTB which can produce both iron oxide and iron sulfide is present in marine and lake environments. In the case of the vibrio bacterium, three facultative anaerobic marine vibrios—strains MV-1, MV-2 and MV-4 are from estuarine salt marshes. On the other hand, the *Magnetospirillum magnetotacticum* found in freshwater sediments with strain MS-1 was the first member of the family to be isolated [31](Yan, et al., 2012), [3](Arakaki, et al., 2008). The iron oxide magnetic nanoparticles which are aligned in chains within the bacterium act as biological compass needles that enable the bacterium to migrate along oxygen gradients in aquatic environments, under the influence of the earth's geomagnetic field [3](Arakaki, et al., 2008). These MTB swims to the magnetic north in the northern hemisphere, to the magnetic south in the southern hemisphere, and both ways on the geomagnetic equator [31](Yan, et al., 2012).

M. magnetotacticum MS-1 is first used for molecular genetic study. This bacterium is compatible with *E. coli* in expressing some genes which are necessary for molecular genetic manipulation therefore the recA gene of this organism was cloned and expressed in *E. coli* by Berson et al. (2006). They cloned a 2 kb DNA fragment from *M. magnetotacticum* MS-1 that supplemented iron-uptake deficiencies in *E. coli* indicate that this 2 kb DNA regulated the uptake of iron [31](Yan, et al., 2012).

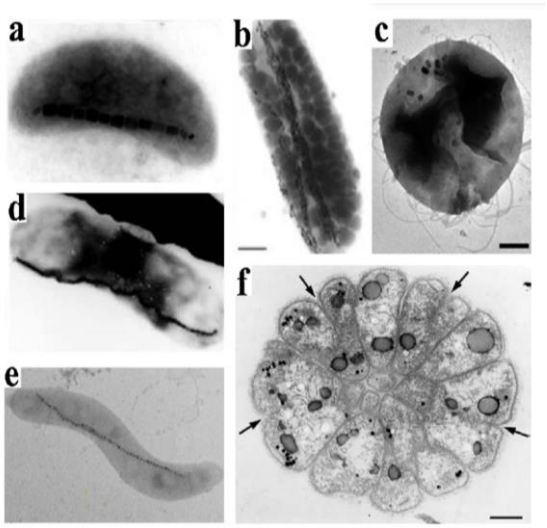

Fig. 4: Various morphology of MTB. a) Vibrios, b) and d) rods, c) coccoid, e) spirilla and f) multicellular organism [31](Yan, et al., 2012).

Mechanism of magnetosome formation

In most bacteria, the magnetosomes are arranged as chains. A Magnetosome membrane (MM) is a lipid bilayer membrane that is formed as vesicles originating from the inner membrane. The genetic formation of the membrane and the mineralization of the magnetite core is known as biomineralization [8](Gorby, et al., 1988). The magnetosome formation comprises three major stages as proposed by Schüler (2002) (Fig. 5). The first step in magnetosome formation is the uptake of extracellular ferric ions via a reductive step. Iron is then thought to be reoxidized to form a low-density hydrous oxide which is dehydrated to form a high-density ferrihydrite. The final step in magnetosome formation is the biomineralization of magnetite, which encompasses all the reactions involving one-third of the ferric ions reduction and further dehydration to form magnetite.

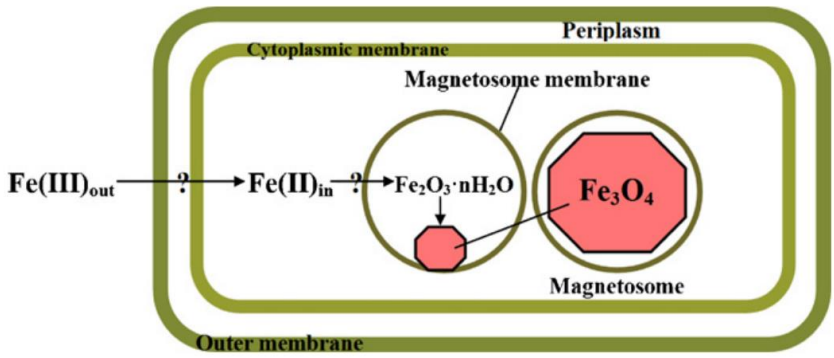

Fig. 5: Model for magnetite biomineralization in *Magnetospirillum* species.

Physiological conditions that favour magnetosome formation

Along with biological and genetic control, the formation of magnetosomes is also very sensitive to changes in chemistry and physical conditions. Oxygen concentration is one of the most important environmental factors which affects the growth of this MTB and also influences the biomineralization of magnetosomes of cultured MTB. It has been reported that cells of *M. magnetotacticum* MS-1 can grow at 0.1–21% oxygen, however, the formation of magnetite magnetosomes in this bacterium is optimal at 1% oxygen and is strongly inhibited at >5% oxygen [31](Yan, et al., 2012).

MTB can take up ferric and ferrous iron, and in some cases the uptake involves siderophores. Siderophores are low molecular weight iron chelators that bind and solubilize ferric iron for uptake. The maximum growth and magnetite formation occurred at an extracellular iron concentration of 100 µM. Iron concentrations above 20 µM iron only slightly increased cell yield and magnetosome content, while iron concentrations higher than approximately 200 µM were growth-inhibiting [31](Yan, et al., 2012).

Nitrogen source is an additional factor for the growth of MTB and magnetite magnetosome biomineralization. The presence of nitrate significantly increased magnetite formation with an optimum concentration of 4 mM. Increased nitrate concentrations (10–20 mM) resulted in reduced magnetite formation but does not affect cell growth. *M. magneticum* can also form magnetite under anaerobic conditions when grown with nitrate [31](Yan, et al., 2012).

Synthesis

Magnetosomes have various applications, especially in biomedicals. They have been extracted from MTB lysing with a detergent, such as NaOH which is followed by magnetic separation of the magnetosomes from bacterial debris. Thus, suspensions containing well-dispersed chains of magnetosomes which are sufficiently stable in water or 5% glucose for injection. Individual magnetosomes constituent of the chains appear to change their composition from magnetite to maghemite during extraction and are surrounded by a biological membrane. Other methods used to extract chains of magnetosomes from whole MTB involve bacterial lysis through sonication or French press, but they appear to be less efficient and convenient to use than NaOH [2](Alphandéry, Edouard, 2020).

To eliminate endotoxins, extra purification steps have to be followed during which MTB are either treated with various organic solvents, such as phenol and chloroform or heated by combustion followed by magnetic separation to isolate pure magnetosome minerals from non-magnetic organic debris. Magnetosome minerals are further autoclaved for sterilization and stabilized under sterile conditions with various coating materials at different pH, temperatures, and the ratios between the masses of magnetosome minerals and the coating [2](Alphandéry, Edouard, 2020).

Nanoparticle fabrication can be performed via two main approaches: top-down and bottom-up. Another classification divides synthesis methods depending on the nature of the processes involved into physical, chemical, and biological methods. Physical methods include top-down techniques, as they employ the fractionation of bulk metal into smaller pieces by mechanical action. By contrast, the bottom-up approach comprises chemical, organic and biological methods [20](Niculescu, et al., 2021).

Biological methods avoid the drawbacks of the physical and chemical methods. They appeared to be a viable alternative to physical and chemical methods because biological methods are eco-friendly, have less energy-intensive syntheses, have no use of toxic chemicals and no production of hazardous by-products. The bacteria-mediated synthetic approach implies magnetotactic and iron-reducing bacteria that can turn out, either intracellularly or extracellularly, single domain MNPs under anaerobic conditions. These bacteria used as reactors multiply rapidly and have ease of cultivation and manipulation therefore they are considered to have main advantages. Bacterial fermentation is scalable, reproducible, and yields high-quality products with lower defects. However, a limitation on large-scale

production is imposed by the relatively small yield, which is not sufficient for commercialization. Moreover, magnetotactic bacteria require a demanding growth, which is difficult to obtain under artificial laboratory conditions, leading to an overall time-consuming and expensive process [20](Niculescu, et al., 2021).

Characterization of magnetite nanoparticles

UV-Vis spectra analysis: UV-Vis spectral analysis is done by using a UV-Vis absorption spectrophotometer. A small aliquot of the sample is diluted in distilled water and measured at a wavelength between 200-600nm. The absorption band can be observed at 316.8nm. The absorbance band in the visible range within 330-450nm indicates the formation of particles of nano diameter [7](Gayatri, et al., 2018).

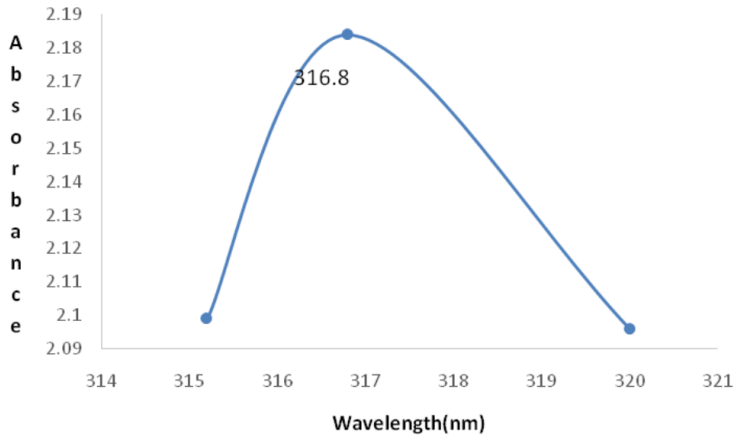

Fig. 6: UV-VIS absorption spectra of magnetite nanoparticles

X-Ray Diffraction (XRD): The crystallographic structural information of the magnetite nanoparticles can be analysed by X-ray diffraction (XRD). The analysis of a sample in diffraction patterns was observed to be from 100 to 700 with a diffractometer. The XRD pattern of Magnetite nanoparticles obtained shows the characteristic peaks at 35.2° (311), 43.1° (400), 53.4° (422), 57.1° (511) and 62.8° (440) as can be seen in Fig. 7, which indicate the crystalline nature and phase purity of the synthesized magnetite nanoparticles [7](Gayatri, et al., 2018).

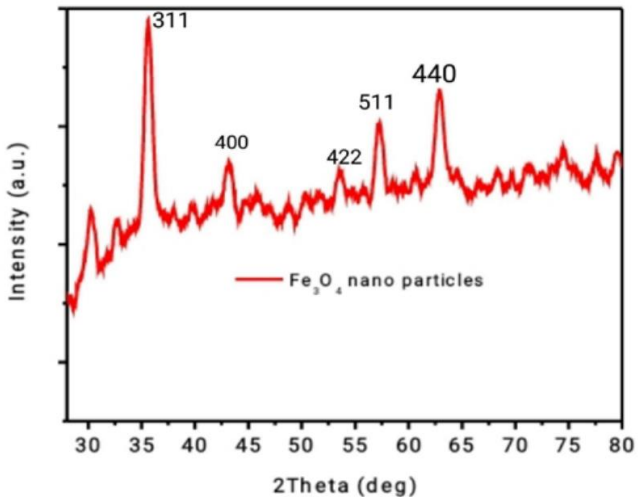

Fig. 7: X-Ray diffraction patterns of magnetite nanoparticle

Scanning Electron Microscopy: The morphology of the magnetite nanoparticles can be observed using SEM. Fig.8 shows the spherical magnetite nanoparticles synthesized. The magnetite nanoparticles agglomerated to a little extent because of their small size and more surface energy. The size is approximate >10nm indicating the synthesis of homogenous nanoparticles [7](Gayatri, et al., 2018).

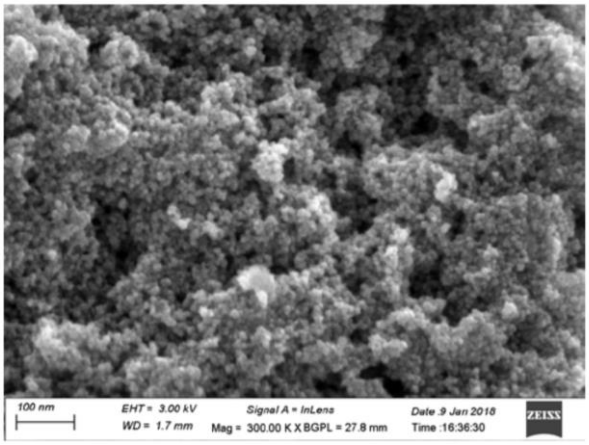

Fig. 8: SEM image of magnetite nanoparticles

Transmission electron microscopy: TEM analysis confirms the size of the magnetite nanoparticles to be below 10nm [7](Gayatri, et al., 2018). Figure 9 shows the transmission electron microscopy (TEM) images obtained. TEM analyses the size, distribution, and shape of the particles. The typical size of observed NPs is 10±5 nm. The addition of starch decreases the amount of agglomerated nanoparticles as 0.5% starch stabilized magnetite NPs [25](Soshnikova, et al., 2013).

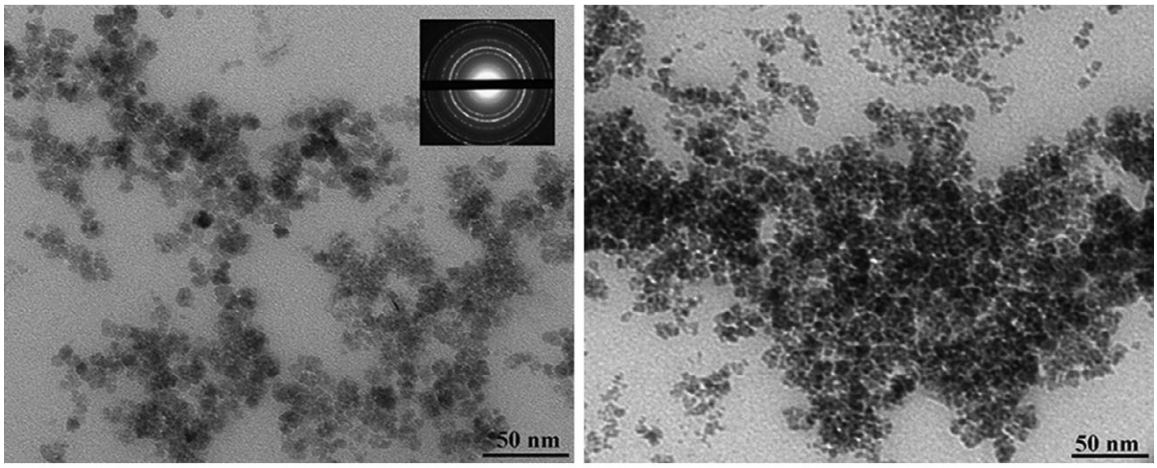

Fig. 9: TEM images of starch-modified (Left) and control nonmodified magnetite NPs (Right).

Applications of magnetite

Magnetite has magnetic properties, biocompatibility, biodegradability, non-toxicity to humans, thermal, chemical, colloidal stability, dispersibility, and possibility for functionalization. These features of magnetite grab a great interest in various applications such as magnetic resonance imaging (MRI) contrast agents, hyperthermia therapy, bio-sensing and diagnosis, controlled and targeted drug delivery, cancer treatment, etc. Moreover, MNPs have intrinsic antimicrobial properties that can act in synergy with other substances, such as antibiotics or natural products. Hence, these iron-based nanoparticles represent promising materials for developing unconventional antimicrobials that can coat medical devices, carry specific and time-eluting drugs to a target tissue, and minimize side effects due to the small number of antibiotics they can deliver [20](Niculescu, et al., 2021). To use these particles for biotechnological applications, it is important to consider the surface modification of magnetic particles with functional molecules such as proteins, antibodies, peptides and DNA [3](Arakaki, et al., 2008).

Bacterial magnetic particles have been employed in numerous purification procedures such as the extraction of mRNA and DNA from biological samples such as tissues, blood, and bacterial cells or for the detection of different cyanobacterial DNA with genus-specific probes. The immobilization of proteins, peptides, and enzymes on magnetic particles facilitates the selective separation and reuse of immobilized enzymes such as glucose oxidase and uricase [14](Lang, et al., 2006).

Magnetic resonance imaging

Magnetotactic bacteria used in MRI have a natural tendency to target tumours in humans when they are administered intravenously. MTB was visualized in tumours using MRI [1](Alphandéry,

2014). The interest in using MTB is based on their ability to propel themselves with their flagella at high speeds and, hence, they can overcome a strong flow of fluid as a counter-current, as observed for *M. magneticum*. Given that MTB might have to swim against the bloodstream in the body to reach their target, this property is exploited to enable MTB navigation in a human [2](Alphandéry, Edouard, 2020).

Magnetosomes extracted from MTB for cancer treatment

Magnetotactic bacteria could target tumours through passive targeting, also known as the enhanced permeability and retention (EPR) effect. In this case, MTB diffuses through the holes of angiogenic blood vessels. On the one hand, it was suggested that modified magnetosomes with the hydrodynamic size of ~200 nm and positive zeta potential of ~50 mV favoured their accumulation in the tumour through the EPR effect. Living MTB (MC-1) can be used for cancer treatment [2](Alphandéry, Edouard, 2020).

On the other hand, MTB can be directed using their aerotaxis sensorial system, which permits them to swim toward oxygen-depleted areas. Such a property is particularly interesting for cancer treatment because it promotes MTB targeting hypoxic tumour areas. Thus, MC-1 cells can penetrate hypoxic regions of colorectal tumours following their injection in a peritumoral region of these tumours. Magnetosome magnetic moment can align parallel to a low-intensity magnetic flux, orientating MTB in the direction of an applied magnetic field [2](Alphandéry, Edouard, 2020).

Although whole MTB and magnetosome chains extracted from MTB have been reported to yield antitumor activities through various mechanisms of action, the presence of endotoxins in these suspensions makes it difficult to foresee their use in humans. Therefore, a purification technique has been urged to get rid of endotoxins from the magnetosome surface to yield nonpyrogenic magnetosome minerals, which are stabilized by an added synthetic coating [2](Alphandéry, Edouard, 2020).

Magnetic hyperthermia

Magnetosomes are also sensible applicants to treat cancers using magnetic hyperthermia. Magnetic hyperthermia is a technique in which magnetic nanoparticles are administered to tumours and then heated under the application of an alternating magnetic field. The heat induces anti-tumour activity. To be efficient for magnetic hyperthermia, the nanoparticles need to produce a large amount of heat. For ferrimagnetic nanoparticles, the quantity of heat

generated under the application of an alternating magnetic field is proportional to the area of their hysteresis loop, which will increase with increasing nanoparticle sizes [1](Alphandéry, 2014).

The heating mechanisms of the magnetosomes have aimed to determine which type of magnetosomes between the magnetosomes contained in whole magnetotactic bacteria, the chains of magnetosomes isolated from magnetotactic bacteria and the individual magnetosomes detached from the chains by heat and SDS treatments, are the most suitable candidate for the magnetic hyperthermia treatment of tumours. There are essentially two mechanisms that can produce heat once magnetosomes are exposed to an alternating magnetic field. They are either due to the reversal of the magnetosome magnetic moment or to the physical rotation of the magnetosomes under the application of an alternating magnetic field. To eliminate the contribution of the rotation to the heating mechanism of the magnetosomes, suspensions of whole magnetotactic bacteria that do not produce heat by rotation are exposed to an alternating magnetic field [1](Alphandéry, 2014).

Drug delivery

Due to the presence of various chemical groups at the surface of the magnetosomes, it is the potential to conjugate drugs such as doxorubicin to the magnetosome surface. Magnetosomes to which doxorubicin is conjugated have been tested as anti-tumour agents against hepatic cancer. It has been shown that by conjugating doxorubicin to the magnetosomes, it was possible to slightly increase the anti-tumour activity from 79% for doxorubicin alone up to 87% for doxorubicin bound to the magnetosomes. The advantage of using magnetosomes is mainly due to the decrease in toxicity. While doxorubicin is highly toxic when it is used with a mortality rate of 80%, doxorubicin bound to the magnetosomes is much less toxic with a mortality rate of 20%. Therefore, there is a large increase in the benefit to risk ratio when doxorubicin is conjugated into the magnetosomes, showing the potential of drugs conjugated to magnetosomes for cancer treatments [1](Alphandéry, 2014).

Magnetosome mechanisms of action

The large magnetosome surface area can be used to attach and carry various molecules, such as drugs, targeting agents, RNA, and immune entities, improving their delivery to the tumour and in some cases, controlling their activity under the application of an external stimulus. Magnetosome cellular internalization with human cervix epithelial (HeLa) cells has a role in antitumor activity, although it cannot be concluded whether it increases or decreases such

activity. Indeed, on the one hand, internalization might destroy or dissolve magnetosomes, notably in lysosomes. On the other hand, it could yield magnetosome–drug complexes close to cellular organelles, such as cell nuclei or mitochondria, and favour cellular death. Full tumour destruction could be achieved when magnetosomes only partly cover the tumour (i.e., ~10% of it). This advises that indirect mechanisms of tumour destruction occur, either through the recruitment of immune cells or apoptosis, where both mechanisms could yield bystander effects (i.e., the death of tumour cells at some distance from the magnetosomes). In some cases, immune mechanisms may be concerned with antitumor activity through the accomplishment of immune cells, such as T lymphocytes or polynuclear neutrophils. Using a system of NPs exposed to the alternating magnetic field (AMF) possibly enables activating and reactivating immune cells on demand by applying the AMF several times. For treatments involving AMF or laser application, heat production is necessary to induce cellular death, because the antitumor activity is only observed above a certain level of tumour temperature elevation [2](Alphandéry, Edouard, 2020).

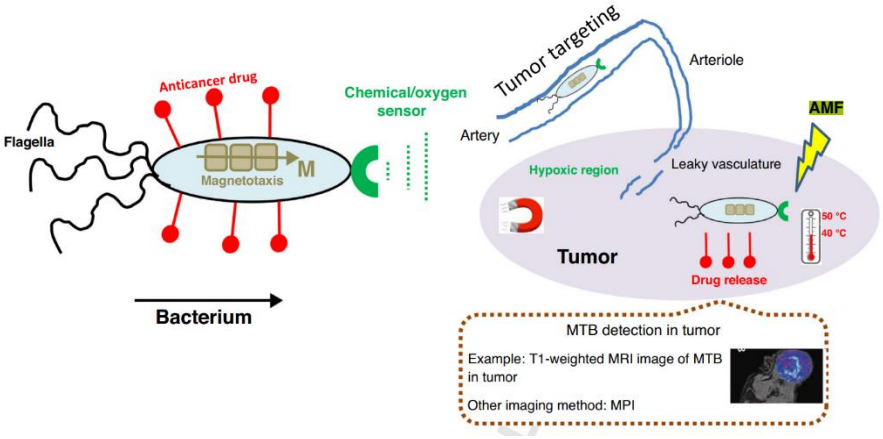

Fig. 10: How whole magnetotactic bacteria (MTB) can target, destroy, or image a tumour.

Other medical applications of bacterial magnetosomes

Magnetosomes can be used for other applications, for example, to detect nucleotide polymorphism, which is useful to diagnose diseases such as cancer, hypertension, or diabetes, to separate cells or to detect DNA. To separate cells, magnetic beads or SPION have been used. However, these two types of magnetic materials present drawbacks. Magnetic beads are large and therefore stop cells from dividing and proliferating correctly. SPION on the other hand is only weakly magnetic due to their unstable magnetic moment at physiological and room temperatures, which makes them poorly efficient to separate cells. In contrast, the magnetosomes are of smaller sizes than the magnetic beads and are more strongly magnetic

than the SPION because of their ferrimagnetic properties. This makes them ideal candidates for applications in cell separation [1](Alphandéry, 2014).

Magnetosomes have also been used for immunoassays, for example, to detect small molecules such as pollutants, hormones, or toxic detergents. These molecules are hooked up to the magnetosome surface exploitation antibodies that specifically bind to them. The complex formed by the magnetosomes and these molecules has then been detected [1](Alphandéry, 2014).

Magnetosomes are used to extract DNA. For that, they have been modified and covered with layers of amino silanes that link DNA. The complex formed by the magnetosomes and DNA has been bound to a magnetic column and DNA has been collected by elution with a phosphate buffer [1](Alphandéry, 2014).

EXOSOMES

Introduction

Extracellular vesicles (EVs) are the vesicles secreted by cells into the extracellular space. The three main subtypes of EVs are microvesicles (MVs), exosomes, and apoptotic bodies, which can be differentiated based on their biogenesis, release pathways, size, content, and function. Exosomes are small vesicles of size 50–150 nm which are secreted by almost cells through the endosomal pathway [15](Lee, et al., 2021). They are also referred to as intraluminal vesicles (ILVs) [29](Wang, 2019). Since they are nanosized and carry cell surface molecules, they have a high capacity for penetrating the interstitial organs as well as a natural targeting capacity [12](Jang, et al., 2013). They are enclosed within a single outer membrane and are secreted by all cell types. They have been found in plasma, urine, semen, saliva, bronchial fluid, cerebral spinal fluid (CSF), breast milk, serum, amniotic fluid, synovial fluid, tears, lymph, bile, and gastric acid [29](Wang, 2019). They are rich in macromolecules [9](Gould, 2019).

They were initially considered as cellular waste resulting from cell damage or by-products of cell homeostasis and have no significant impact on neighbouring cells but recently these extracellular vesicles are studied as functional vehicles that carry a complex cargo of proteins, lipids and nucleic acids and are capable of delivering these cargos to the target cells. Thus, exosomes represent a novel mode of intercellular communication, which may play a major role in many cellular processes, such as immune response, signal transduction and antigen presentation [32](Zhang, et al., 2019).

Exosomes are the internal vesicles formed by the inward budding of the cellular compartments known as multivesicular body (MVB). When MVB fuse with the plasma membrane, these internal vesicles are released as exosomes, which can transfer the DNA, RNA and proteins to the distant recipient cells, and influent various aspects of cell behaviour and physiology [18](LUAN, et al., 2017).

Exosomal composition

Exosomes usually contain transmembrane proteins, lipid-anchored membrane proteins, peripherally associated membrane proteins, and soluble proteins of the exosome lumen (Figure 12). They commonly have membrane transport and fusion proteins (GTPases, Annexins, flotillin), tetraspanins (CD9, CD63, CD81, CD82) and heat shock proteins (Hsc70, Hsp 90), proteins involved in multivesicular body biogenesis (Alix, TSG101), lipid-related proteins and

phospholipases. In addition to proteins, exosomes are enriched in certain raft-associated lipids such as cholesterol, ceramide, other sphingolipids, and phosphoglycerides with long and saturated fatty-acyl chains. Similarly, exosomes contain common components such as chaperones (Hsc70 and Hsp90); subunits of trimeric G proteins; cytoskeletal proteins (e.g., actin, tubulin, moesin); ESCRT proteins (Tsg 101, Alix); clathrin; proteins involved in transport and fusion (Rab 7, Rab 2, Annexins); several enzymes and elongation factors [21](Niel, et al., 2006).

Exosomes additionally hold saccharide groups on their outer surface. These are enriched in mannose, polylactosamine, α-2,6 sialic acid, and complex N-linked glycans [27](Vlassov, et al., 2012).

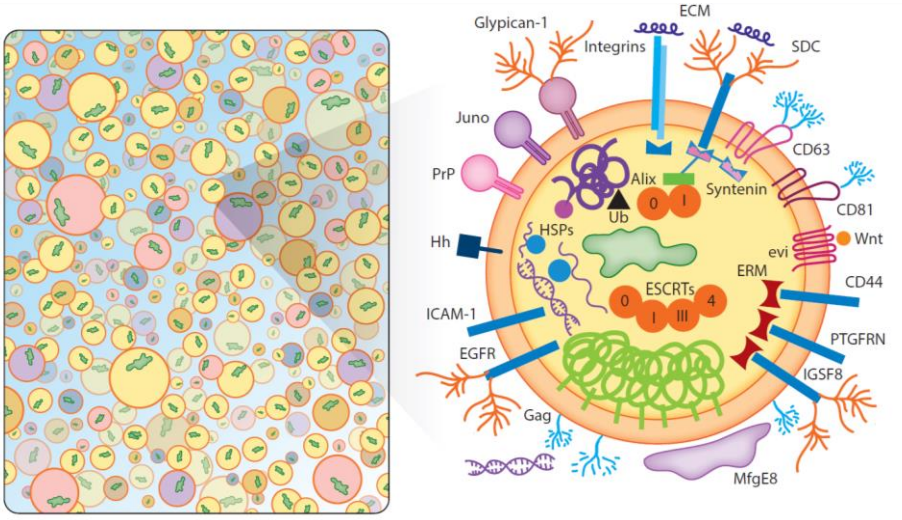

Fig.11: Exosomes are heterogeneous in composition and enriched in protein complexes, adhesion molecules, RNAs, DNAs, and complex glycans [9](Gould, 2019).

Integral Exosomal Membrane Proteins

In exosomes, tetraspanin proteins (CD81, CD82, CD37, and CD63) are highly enriched and CD81 is the most highly enriched protein in exosomes whereas CD63 protein is the least enriched protein in exosomes. Tetraspanins facilitate the trafficking, function, stability, and oligomerization of other membrane proteins. Exosomal tetraspanins also play a role in the exosomal secretion of virus-encoded membrane proteins [9](Gould, 2019).

Lipid-Anchored Outer Membrane Proteins

The exosome surface contains an array of lipid-anchored proteins. These include the ectonucleotidases CD39 and CD73, the sperm receptor Juno; the complement-inhibiting

proteins CD55 and CD59, glypican-1, and both the cellular prion protein (PrPC) and its amyloidogenic conformer, PrPSC [9](Gould, 2019).

Peripheral Surface Proteins

Exosomes also carry peripherally associated surface proteins which are also involved in signalling. These consist of many wingless (Wnt) proteins and their integral membrane cargo receptor/sorting chaperone. Exosomes as multiplexed signalling platforms able to transmit complex autocrine and paracrine signals. The exosome surface is also rich with extracellular matrices (ECM) proteins such as fibronectin, tenascin C and ECM1 which also have important roles in signalling and adhesion [9](Gould, 2019)**.**

Lipid-Anchored Inner Membrane Proteins

The exosome inner membrane is rich in acylated, lipid-anchored proteins. These include prenylated small GTPases (Rabs, Ras, Rho, etc.), myristoylated signalling kinases (e.g., Src) and palmitoylated membrane proteins [9](Gould, 2019).

Exosomal Enzymes

Exosomes secret osteogenic vesicles loaded with enzymes involved in bone formation. These include CD39, CD73, phosphatases, pyrophosphatases, calcium-binding annexins, and phosphate transporters. Exosomes also contain RNA editing enzymes, lipases, proteases, glycosyltransferases, glycosidases, and metabolic enzymes, many of which have the potential to modify exosome content raising the possibility that exosomes represent a reservoir for generating macromolecules that are chemically distinct from their cellular forms. Cancer cell-derived exosomes have an even greater enzymatic range owing to their exosomal packaging of mutant Ras proteins, receptors, and hyaluronan synthase-3 and their RNA interference processing activities [9](Gould, 2019).

Exosome glycoconjugates and lipids

The outermost surface of the exosome consists of a glycan covering attached to surface proteins and certain outer leaflet lipids. The exosome membrane contains phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositols (PIs), phosphatidic acid (PA), cholesterol, ceramides, sphingomyelin, glycosphingolipids, and several lower abundance lipids. The exosome bilayer is that it has PE and PS in its outer leaflet, whereas PE and PS are normally depleted from the outer leaflet of the plasma membrane by

phospholipid flippases. Exosome membranes are also enriched in lysophospholipids. cholesterol, gangliosides, sphingolipids, and ceramides [9](Gould, 2019).

Exosomal RNAs

Exosomes contain RNAs and can transfer these extracellular RNAs (exRNAs) in the functional form to other cells and tissues. Gag and Gag-like proteins also impact exosomal RNA content, as Gag proteins bind their genomic RNA and other RNAs and transfer them into nascent exosomes [9](Gould, 2019).

Exosomal DNA

Exosomes contain DNA, including single-stranded DNA, double-stranded DNA, genomic DNA, mitochondrial DNA, and even reverse-transcribed complementary DNAs.

Protein category and description	Examples
Tetraspanins	CD9, CD63, CD81, CD82, CD37, CD53
Heat shock proteins (HSP)	HSP90, HSP70, HSP27, HSP60
Cell adhesion	Integrins, Lactadherin, Intercellular Adhesion Molecule 1
Antigen presentation	Human leukocyte antigen class I and II/peptide complexes
Multivesicular body Biogenesis	Tsg101, Alix, Vps, Rab proteins
Membrane transport	Lysosomal-associated membrane protein 1/2, CD13, PG regulatory-like protein
Signaling proteins	GTPase HRas, Ras-related protein, furloss, extracellular signal-regulated kinase, Src homology 2 domain phosphatase, GDP dissociation inhibitor, Syntenin-1, 14-3-3 Proteins, Transforming protein RhoA
Cytoskeleton components	Actins, Cofilin-1, Moesin, Myosin, Tubulins, Erzin, Radixin, Vimentin
Transcription and protein synthesis	Histone1, 2, 3, Ribosomal proteins, Ubiquitin, major vault protein, Complement factor 3
Metabolic enzymes	Fatty acid synthase Glyceraldehyde-3-phosphate dehydrogenase Phosphoglycerate kinase 1 Phosphoglycerate mutase 1 Pyruvate kinase isozymes M1/M2 ATP citrate lyase ATPase Glucose-6-phosphate isomerase Peroxiredoxin 1 Aspartate aminotransferase Aldehyde reductase
Trafficking and membrane fusion	Ras-related protein 5, 7 Annexins I, II, IV, V, VI Synaptosomal-associated protein Dynamin, Syntaxin-3
Antiapoptosis	Alix, Thioredoxine, Peroxidase
Growth factors and cytokine	Tumor Necrosis Factor (TNF)-α, TNF Receptors, Transforming growth factor-β
Death receptors	FasL, TNF-related apoptosis inducing ligand
Iron transport	Transferrin receptor

Table 2: Common protein components of exosomes [32](Zhang, et al., 2019).

Exosome biogenesis

Exosomes can arise by vesicle budding into endosomes with the plasma membrane. Plasma membrane exosomal budding is also observed for glioblastoma exosomes. Exosome biogenesis occurs in three modes:

1. vesicle budding into discrete endosomes that mature into multivesicular bodies, which release exosomes

2. immediate release by direct vesicle budding from the plasma membrane

3. delayed release by budding at intracellular plasma membrane–connected compartments (IPMCs) (Figure 13) [9](Gould, 2019).

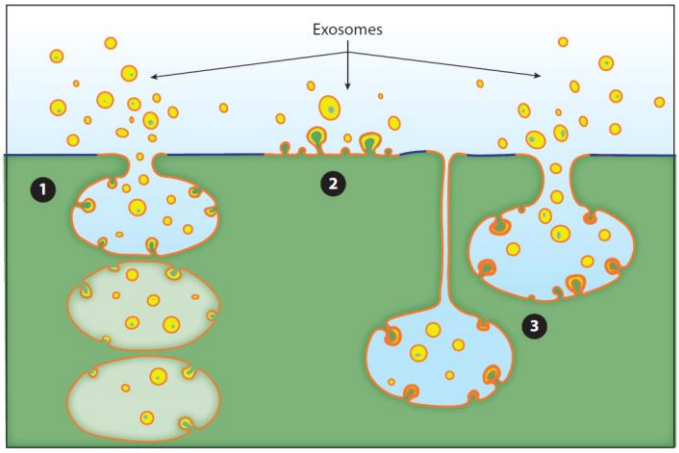

Fig. 12: Shows Exosomes bud formation from endosome and plasma membranes by three modes [9](Gould, 2019).

Lipids are crucial in exosome biogenesis, especially those able to form a cone and inverse cone shapes. Exosomes are rich in outer leaflet PE and PS. Other phospholipids that may promote exosome biogenesis include PA, a cone-shaped lipid produced by PLD2, which has been implicated in the budding of certain exosomal cargoes, and lysophospholipids, which are generated by exosomal phospholipase A2 enzymes. Also, ceramide abundance has been positively associated with an increase in exosome biogenesis [9](Gould, 2019).

Rab35 also contributes to exosome biogenesis. Rab35 localizes primarily to the plasma membrane, where it also contributes to the regulation of plasma membrane PIP2 levels. Loss of Rab35 deprives the plasma membrane of its molecular identity, impairing the rapid recycling of plasma membrane proteins and lipids back to the cell surface. Rab11 is also implicated in exosome biogenesis. Rab11 appears to influence exosome biogenesis via a calcium-induced homotypic fusion/maturation of MVBs, upstream of exosome release [9](Gould, 2019).

Exosome uptake takes place by multiple mechanisms, including macropinocytosis, phagocytosis, clathrin-dependent endocytosis, and clathrin-independent endocytosis [9](Gould, 2019).

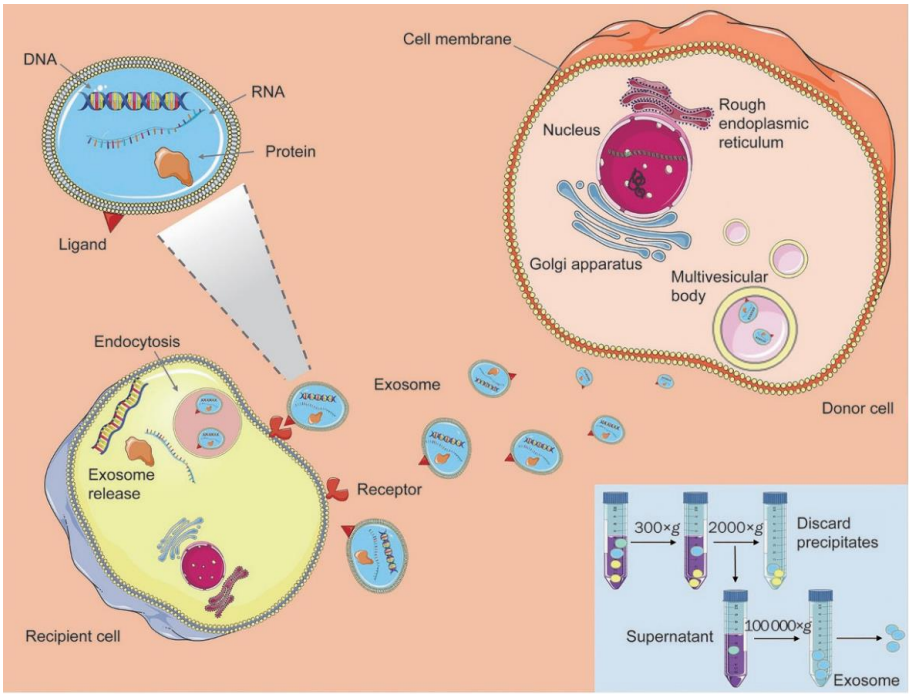

Fig. 13: Exosome generation, secretion and cargo transfer from the donor cells to the recipient cells. The inset shows an ultracentrifugation protocol [18](LUAN, et al., 2017).

Isolation

Ultracentrifugation-based isolation techniques

Ultracentrifugation is one of the most commonly used techniques in exosome isolation [17](Li, et al., 2017). They are of two types – differential ultracentrifugation and density gradient ultracentrifugation. Differential ultracentrifugation for the isolation of exosomes generally consists of a series of centrifugation cycles of different centrifugal forces and duration to isolate exosomes. This isolation is based on the density and size differences of exosomes from other components. The centrifugal force used for ultracentrifugation typically ranges from ~100,000 to 120,000 × g. Cleaning of human plasma or serum is carried out before the start of isolation of exosomes to get rid of large bioparticles in a sample. The sample is loaded with protease inhibitors to prevent the degradation of exosomal proteins. In consecutive rounds of centrifugation and pouring off, the RCF (g) and the centrifugation time are increased to pellet smaller particles. After the first 200×g and 2000×g centrifugations, pellets that contain dead cells and cell debris are discarded and the supernatant is kept for the next step. In later steps, the pellets are resuspended in phosphate buffered saline (PBS) for further analysis [18](LUAN, et al., 2017) Finally, the isolated exosomes are again re-suspended and stored at -80ºC until

further analysis. This method of isolating exosomes is also known as the pelleting method or simple ultracentrifugation method [17](Li, et al., 2017).

For density gradient ultracentrifugation, there are two types of density gradient ultracentrifugation, namely isopycnic ultracentrifugation and moving-zone ultracentrifugation. The density gradient ultracentrifugation has become popular in the isolation of exosomes. Here separation of exosomes is accomplished based on their size, mass, and density in a pre-constructed density gradient medium in a centrifuge tube with progressively decreased density from bottom to top. Upon applying a centrifugal force, solutes including exosomes in the sample move as individual zones through the density gradient medium towards the bottom, each at its specific sedimentation rate, thus leading to discrete solute zones. The separated exosomes can then be conveniently recovered by simple fraction collection [17](Li, et al.2017).

In isopycnic ultracentrifugation, during centrifugation, exosomes sediment along with the density gradient medium to where they have the same density as the medium that is at the isopycnic position. After the exosomes have reached their isopycnic position, the centrifugal force further focuses the exosomes into a sharp zone and upholds them there, implying that isopycnic ultracentrifugation is static. Alternatively, a sample containing exosomes can be uniformly mixed with a gradient medium in the case of self-generating gradient materials such as caesium chloride. During centrifugation, the exosomes move to their isopycnic position while a density gradient of caesium chloride is generated. Exosomes can then be extricated from the density region of interest between 1.10 and 1.21 g/ml, where they are concentrated [17](Li, et al., 2017).

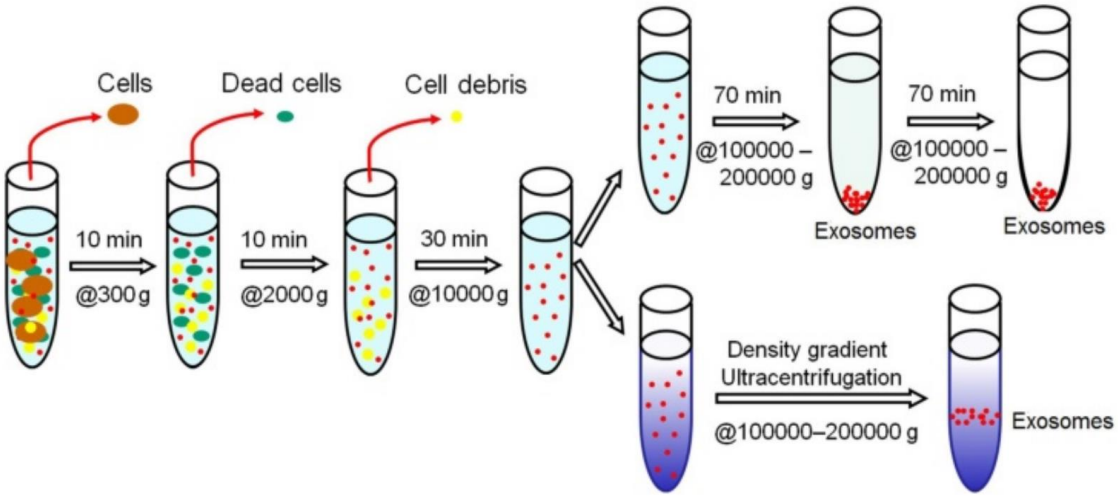

Fig. 14: Schematic representation of isolating exosomes by differential ultracentrifugation. All centrifugations are carried out at 4 ^{0}C [17](Li, et al., 2017).

In moving-zone ultracentrifugation, a sample containing exosomes is loaded as a thin zone on top of a gradient density medium having a lower density than that of any of the solutes. The exosomes in the sample are separated based on their size and mass instead of density. This allows the separation of extracellular vesicles with similar densities but different sizes. Because the densities of the solutes including exosomes are greater than the density of the gradient medium, moving-zone ultracentrifugation is dynamic rather than static. In other words, all solutes will eventually pellet at the bottom of the centrifuge tube when a prolonged period of centrifugation is executed. Therefore, the duration of centrifugation must be carefully optimized. In addition, to prevent exosomes from pelleting out, a high-density cushion is often layered at the bottom of the centrifuge tube. In contrast, exosomes will never sediment to the bottom of the centrifuge tube in isopycnic ultracentrifugation irrespective of the duration of centrifugation [17](Li, et al., 2017).

Ultracentrifugation is often coupled with isopycnic or moving-zone techniques to allow the exosomes of relatively low densities to float and to further purify the exosomes. This technique has been known to be able to improve the number of exosomes isolated. After characterization, the fraction of interest containing the exosomes is diluted in PBS and subjected to another round of ultracentrifugation at ∼100,000 × g to yield pure exosomes for further analysis [17](Li, et al., 2017).

Size-based isolation techniques

One of the popular size-based exosome isolation techniques is ultrafiltration. Therefore, exosomes can be isolated based on their size using membrane filters with defined molecular weight or size exclusion limits. Ultrafiltration is faster than ultracentrifugation and does not require special equipment. However, the use of force may result in the deformation and breaking up of large vesicles which may potentially skew the results of downstream analysis [17](Li, et al., 2017).

Western blot was engaged to detect exosomal biomarkers and electron microscopy was employed to examine the typical features of exosomes. For cell-free samples like urine, serum, cerebrospinal fluid, and cell culture medium, a commercial exosome isolation kit for exosome isolation and RNA extraction from isolated exosomes have been developed [17](Li, et al., 2017).

Sequential filtration is applied to isolate exosomes from cell culture supernatants. Large and rigid components are eliminated, but large flexible components can pass through the filter.

Sequential filtration allows the isolation of exosomes with high purity and functional integrity as a result of low manipulation forces [17](Li, et al., 2017).

Another size-based separation technique applied to exosome isolation is size exclusion chromatography (SEC). In SEC, a porous stationary phase is utilized to sort macromolecules and particulate matters out according to their size. Components in a sample with small hydrodynamic radii can pass through the pores, thus resulting in late elution. Components with large hydrodynamic radii including exosomes are excluded from entering the pores [17](Li, et al., 2017).

Immunoaffinity capture-based techniques

The presence of plenty of proteins and receptors in the membrane of exosomes offers an excellent opportunity to develop highly specific techniques for the isolation of exosomes by tapping on immunoaffinitive interactions between those proteins (antigens) and their antibodies, and specific interactions between the receptors and ligands. For example, a microplate-based enzyme-linked immunosorbent assay (ELISA) was developed for capturing and quantifying exosomes from plasma, serum, and urine. Plasma samples were pre-cleaned by a brief round of low-speed centrifugation to eliminate cellular debris and large bioparticles and to concentrate extracellular vesicles. Moreover, the RNA yield achieved from the captured exosomes using the microplate was higher than that obtained by ultracentrifugation [17](Li, et al., 2017).

Exosome precipitation

Exosomes can be settled out of biological fluids. For this purpose, water-excluding polymers by altering their solubility or dispersibility such as polyethylene glycol (PEG) is engaged. The water-excluding polymers tie up water molecules and force less soluble components out of the solution. Generally, samples are incubated with a precipitation solution containing PEG with a molecular weight of 8000 Da. After incubation at 4 °C overnight, the precipitate containing exosomes is isolated employing either low speed centrifugation or filtration. Exosome precipitation is easy to use and does not require any specialized equipment. This allows easy integration into clinical usage by exploiting existing technologies and is scalable for large sample sizes. Afterwards, the exosomes can be quantified by CD9 ELISA. The purity of exosomal proteins can be evaluated by Western blot and RNA quantified by qRT-PCR. Exosomes were sized and quantified employing nanoparticle tracking analysis [17](Li, et al., 2017).

The main disadvantage of the polymer-based exosome precipitation is the co-precipitation of other non-exosome contaminants, such as proteins and polymeric materials. The pre-isolation step removes subcellular particles such as lipoproteins, while the post-isolation step removes the polymeric materials using a Sephadex G-25 column [17](Li, et al., 2017).

Isolation Technique	Isolation principle	Potential Advantage	Potential Disadvantage
Ultracentrifugation-based techniques	Density, size, and shape based sequential separations of particulate constituents and solutes	Reduced cost and contamination risks with separation reagents, Large sample capacity and yields large amounts of exosomes	High equipment cost, cumbersome,, long run time, and labor intensive low portability – not available at point-of-care, high speed centrifugation may damage exosomes thus impeding downstream analysis.[95]
Size-based techniques	Exosome isolation is exclusively based on the size difference between exosomes and other particulate constituents	Ultrafiltration: Fast, does not require special equipment, good portability, direct RNA extraction possible. SEC: high-purity exosomes, gravity flow preserves the integrity and biological activity; superior reproducibility, moderate sample capacity.	Ultrafiltration: low equipment cost, moderate purity of isolated exosomes, shear stress induced deterioration, possibility of clogging and vesicle trapping, exosomes loss due to attaching to the membranes. SEC: Moderate equipment cost, requires dedicated equipment, not trivial to scale up, long run time.
Exosome Precipitation	Altering the solubility or dispersibility or exosomes by the use of water-excluding polymers	Easy to use, does not require specialized equipment, large and scalable sample capacity	Co-precipitation of other non-exosomal contaminants like proteins and polymeric materials. Long run time, Requires pre-and post-cleanup.
Immunoaffinity capture-based techniques	Exosome fishing based on specific interaction between membrane-bound antigens (receptors) of exosomes and immobilized antibodies (ligands)	Excellent for the isolation of specific exosomes, Highly purified exosomes – much better than those isolated by other techniques, high possibility of subtyping.	High reagent cost, exosome tags need to be established, low capacity and low yields, only works with cell-free samples, tumor heterogeneity hampers immune recognition, antigenic epitope may be blocked or masked.[54]

Table 3: Comparison of exosome isolation techniques [17](Li, et al., 2017).

Characterisation

Fluorescence microscopy can detect exosomes, provided that the exosomes are labelled with fluorescent probes specific for the exosome bilayer, exosome proteins, nucleic acids, or carbohydrates. Single-particle interferometric reflectance (SPIR) imaging can visualize individual exosome sizes in the 50 to 200 nm range, and when combined with conventional fluorescence microscopy can also detect the presence of specific lipids, proteins, and nucleic acids, and carbohydrates [9](Gould, 2019).

Feature	Exosome	Apoptotic body	MV
Size	Homologous 30–100 nm	Heterogeneous 1–5 μm	Heterogeneous 100–1000 nm
Markers	Membrane impermeable (PI negative) CD63, TSG101, Alix, flottilin	Membrane permeable (PI positive) Annexin V, DNA, histones	Membrane impermeable (PI negative) integrin, selectin, flotillin-2
Density	1.13–1.19 g/mL	1.16–1.28 g/mL	1.25–1.30 g/mL
Contents	Protein, lipid, different RNA species, and DNA	Cytosolic content (protein, RNAs, fragmented DNA) and cellular organelles	Protein, lipid, different RNA species, and DNA
Determinant of controlled contents	The cellular origin and physiological state of the cell	The cellular origin and stimuli	No direct correlation
Lipids	A major sorting of lipidic molecules from the parental cells (include BMP)	Characterized by phosphatidylserine externalization	The lipid contents are primarily derived from plasma membrane, and resemble the parental cells (without BMP)
Origin	Multivesicular bodies fusion with plasmatic membrane	Cellular debris, plasma membrane blebbing during cell apoptosis	Direct outward budding or blebbing from the plasma membrane
Mechanism of release	Constitutive or inducible, depending on the cell type of origin	Rho-associated kinase I and myosin ATPase activity	Relocation of phospholipids to the outer membrane, cytoskeleton rearrangements, generation of membrane curvature, and vesicle release
Detection methods	Electron microscopy, Western blot for exosome enriched markers	Flow cytometry, electron microscopy,	Flow cytometry, electron microscopy
Isolation methods	Ultracentrifugation (100,000–200,000×*g*) filtration, density gradient Immunoprecipitation, Immune affinity capture and ExoQuick precipitation methods	Ultracentrifugation (10,000–20,000×*g*)	No standardized methods
Size determination and quantification	Dynamic light scattering Nanoparticle tracking analysis Surface plasmon resonance		

Table 4: Characterisation of main extracellular vesicles [32](Zhang, et al., 2019).

Applications

A common interest in exosomal research is in studying their ability to act as carriers of biomarkers for diseases. For example, exosomes in both plasma and CSF have been found to contain alpha-synuclein, a protein associated with Parkinson's disease. The use of exosomes as carriers of biomarkers is ideal because these vesicles are found in bodily fluids, such as blood and urine, which allows for minimally to non-invasive "liquid biopsy" type methods to diagnose and even monitor a patient's response to treatment. exosomes can be used in vaccine development and for other immunological purposes. Further, exosomes have a long-circulating half-life, are well tolerated by the human body, capable of penetrating cellular membranes and potentially targeting specific cell types, which makes their best use for such immunological applications. Also, because of these inherent advantages of exosomes, they are ideal for the development of drug delivery systems [29](Wang, 2019).

Finally, it has been demonstrated that the mesenchymal stem cell exosomes themselves can act as a therapeutic entity to help reduce tissue injury. Exosomes have been found to stimulate immune responses by acting as antigen-presenting vesicles. In the nervous system, exosomes have been found to help promote myelin formation, neurite growth, and neuronal survival, thus playing a role in tissue repair and regeneration. At the same time, exosomes in the central nervous system (CNS) have been found to contain pathogenic proteins, such as a beta amyloid peptide, superoxide dismutase, and alpha synuclein that may aid in disease progression. [29](Wang, 2019) Exosomes act as mediators of intercellular communication. The messages transmitted by this intercellular communication may include those for growth, division, survival, differentiation, stress responses, apoptosis, etc. [27](Vlassov, et al., 2012)

It also appears that these vesicles are not ingested by macrophages leukocytes, presumably because they are recognized as 'self' by the immune system, as there seems to be a very long half-life in the bloodstream enabling communication between remote anatomical locations [27](Vlassov, et al., 2012).

Exosomes as Components and Remodelers of the Extracellular Matrix

Once released from the cell, exosomes can become integral components of the ECM. As ECM components, exosomes provide a mechanism by which cells can manipulate ECM composition and function. Exosomes can also serve as templates for producing a wide array of diffusible signalling molecules, such as adenosine, prostaglandins, PA, and lysophospholipids. Other instances of exosome-mediated ECM modulation include their contribution to the formation,

growth, and spread of amyloid-rich aggregates, plaques, and tangles in neurodegenerative disorders [9](Gould, 2019).

Exosome-mediated intercellular communication

Traditionally, cells communicate with neighbouring cells through direct cell-cell contact including gap junctions, and cell surface protein/protein interactions, while communicating with distant cells through secreted soluble factors, such as hormones and cytokines, to facilitate signal propagation. The bioactive molecules contained in exosomes have been shown to impact target cells via the following mechanisms:

(1) direct stimulation of target cells via surface-bound ligands

(2) transfer of activated receptors to recipient cells and

(3) epigenetic reprogramming of recipient cells via delivery of functional proteins, lipids, and RNAs.

In the immune system, exosomes have an important function in immunoregulation, including antigen presentation, immune activation, immune suppression, and immune tolerance via exosome-mediated intercellular communication. It has been reported that exosomes derived from dendritic cells (DCs) pulsed with tumour peptides can eradicate or suppress the growth of established murine tumours via the presentation of class II-peptide complexes to naive T lymphocytes and the priming of specific cytotoxic T lymphocytes in vivo. The selective transmission of exosomal genetic information makes them attractive candidates for the diagnosis and treatment of diseases [32](Zhang, et al., 2019).

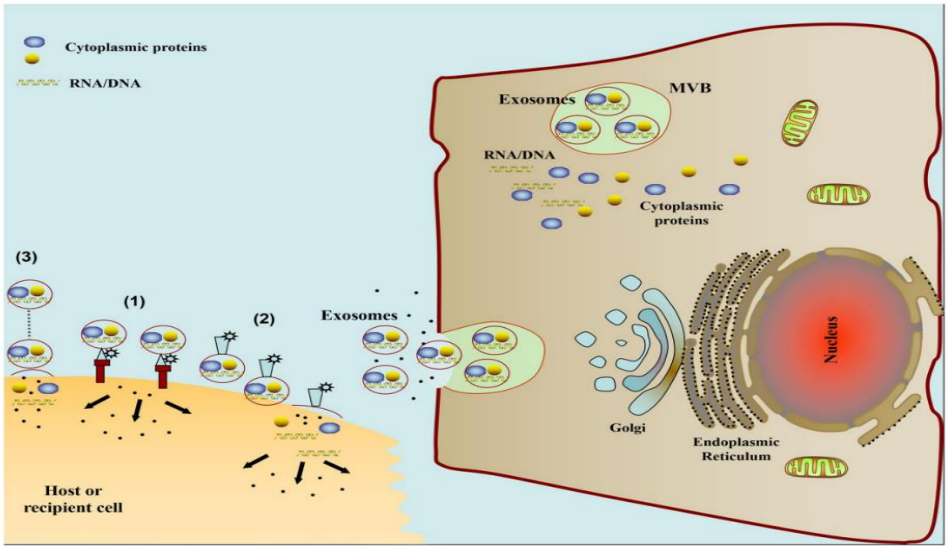

Fig. 15: The schematic diagram of pathways involved in exosome mediated cell-to-cell communication [32](Zhang, et al., 2019).

Exosomes in diagnostics

Over the past few years, exosomes have been discovered in almost all body fluids, including blood, urine, saliva, breast milk, cerebrospinal fluid, semen, amniotic fluid, and ascites. These exosomes with a specific profile of miRs, proteins and lipids can mirror the cellular origin and its physiological state, like a "fingerprint" or a "signature" of the donor cell. Therefore, exosomes and their cell- or condition-specific cargos may better reflect the cellular processes and be used as biomarkers of various diseases. For example, the serum level of exosomal miR-21 has been found to robustly distinguish patients with oesophagal squamous cell cancer from patients who have benign diseases. The diagnostic value of salivary exosomes, and found that their cargos of miR-1246 and miR-4644 were significantly higher in cancer patients [32](Zhang, et al., 2019).

Urine could be a very useful source of exosomal markers of urogenital diseases. Urinary exosomes were reported to contain the mRNA encoding two molecules known to be overexpressed in prostate cancer, PCA3 and the TMPRSS2: ERG fusion. Aquaporins 1 and 2 have been also characterized as markers of renal ischemia/reperfusion injury and anti-diuretic hormone action. Saliva is another fluid easily obtained by non-invasive means. Exosomes have also been found in human saliva, and these contain nucleic acid and protein that may serve as disease biomarkers [27](Vlassov, et al., 2012).

Exosomes as drug delivery vehicles

Exosomes derived from dendritic cells (Dex) pulsed with tumour peptides were actively investigated as clinical cell-free cancer vaccines. Dex was found to possess molecules necessary for antigen presentation, such as MHC class I, MHC class II, and costimulatory adhesion molecules, each of which facilitates the functionality of Dex in vivo. To further improve the efficacy of Dex immunotherapy, engineered Dex was developed to carry tumour antigen-associated (TAA) proteins or mRNAs, thereby inducing CTL response, leading to inhibition of tumour growth [32](Zhang, et al., 2019).

The first one used vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived exosomes (DEX). DEX was generated containing functional MHC/ peptide complexes capable of promoting T cell immune responses, including tumour rejection. They have established a GMP (Good Manufacturing Practice, necessary for manufacturing prescriptiongrade drugs) process to produce pharmaceutical-grade exosomes on a large scale. These exosomes were then used as vaccines [27](Vlassov, et al., 2012).

CONCLUSION

In the last decades, nanotechnologies and biotechnologies regarded as apart from each other. The combination of these two scientific fields founded the new interdisciplinary science of nanobiotechnology. This new approach corresponds to the current scientific urge for improving the existing strategies for nanoparticle synthesis and inventing new ones. There have been many studies done over the past few years on the structure, synthesis and physiological roles of naturally occurring nanoparticles. In nature, nanoparticles are found in clay, dust, mineral springs etc. Whereas including humans, nanomaterials are found in insects and bacteria. Biologically produced nanoparticles are highly diverse but also offer features that make them attractive for biomedical uses. The uniformity of their structure, low toxicity, ability to evade the immune system and capacity for modification have shown interest in bionanoparticles. The bionanoparticles in this study include magnetite and exosome but there are also ferritin, lipoproteins and virus-like particles (VLPs). The magnetite biomedical purposes are magnetic hyperthermia, cancer treatment, drug delivery, MRI contrast agent, etc. The exosome diagnostics, intercellular communication, drug delivery, etc. As our knowledge of the biology of naturally occurring nanoparticles expands and challenges related to the synthesis of such particles are overcome, it is likely that the biomedical applications of natural nanoparticles will expand further. The most important clinical applications of currently available nanotechnology are in the areas of biomarker discovery, cancer diagnosis, and detection of infectious microorganisms. Nanomedicine promises to play an important role in the future development of diagnostic and therapeutic methods.

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