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An Overview on Applications of Nanoparticles in Biological Systems

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Abstract: Nanotechnology and Nanoscience studies have received much attention in the last decade. These studies involve a wide spectrum of research areas and industrial activities from fundamental sciences to applied sciences on the nanoscale. One of the major developments in nanotechnology studies is the production and application of nanoparticles (NPs) in biological sciences. In general, nanoparticles are smaller than 1000 nm, produced from different materials in different shapes such as spheres, rods, wires and tubes. This assay briefly summarizes the major types of nanoparticles that have been used so far, methods of formulation and discussed the possible applications of these NPs in biological and environmental research and the potential environmental and health impact associated with the use of these NPs.

Key words: Nanoparticle · Biological sciences · Environmental research

INTRODUCTION

Nanotechnology is revolutionizing medicine, particularly in the fields of imaging and drug delivery. For over 30 years, NPs defined as ordered structures with diameters smaller than 1000 nm [1], have been engineered to develop novel diagnostic methods, targeted therapies and vaccine development. Recently, nanoproteomics are used for identification and characterization of biomarkers for cancer and other fatal diseases to aid an early diagnosis and monitor disease progression. Nanoproteomics offers several advantages such as ultralow detection, short assay time, high-throughput capability and low sample consumption [2]. In some of these areas, NPs have delivered effective and scientifically validated solutions, leading to their incorporation into marketable products that are already extending to veterinary species.This assay focused on the basic principles behind the use of NPs for drug delivery, diagnostics and vaccine formulation. Common forms of NPs and their formulation are discussed, along with their clinical applications and limitations, providing the reader with a realistic synopsis of the practical applications of NPs to veterinary medicine at present and in the near future.

Nanoparticle Formulations: This section provides a brief synopsis of the most prominent NPs systems, their applications and limitations, with a view to familiarizing the reader with the basic details of those formulations available for application.

Nanosized Drug Substance: Direct nanosizing of poorly water-soluble drugs enhances their solubility. Micron-size drug particles are milled in a water-based stabilizer solution for 30-60 minutes to generate NPs with unimodal size distribution. The amount of the suspension stabilizer is critical since too little of it is unable to prevent aggregation of small particles and too much of it may accelerate particle growth by Ostwald ripening. Increasing the specific surface area might be useful for formulation of drugs with a low solubility in aqueous environments [3]. Milling or Size reduction is obtained by milling pearls made of steel, glass, zircon dioxide, or polymers such as hard polystyrene. Other milling techniques use rotor-stator colloid mills, or jet mills where particles are accelerated and break upon impaction on either another particle or a wall.Also, several other methods have been described in the literature for nonosizing of drugs such as the use of supercritical fluid technologies principally leading to particles in the size range of 100 to 500 nm for

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griseofulvin [4] or rifampicin [5]. With supercritical fluids like carbon dioxide, particle formation can be controlled by modifying the pressure which governs solubility of the drugs therein. High pressure generally provides for higher drug solubility, so that upon reduction of the pressure the drug precipitates [6]. The higher the drop in pressure is, the faster precipitation occurs and in consequence the smaller the resulting particles become. Another method to prepare amorphous NPs suspension of poorly water-soluble drugs like Cyclosporine A is evaporative precipitation into aqueous solution. Rapid evaporation of a heated organic solution of the drug is followed by its atomization into aqueous solution. This leads to NPs suspension, which can be dried to produce oral dosage forms with low crystallinity and small particle size [7].

Polymeric Nanoparticles: Polymeric NPs are prepared by combining the active substance/drug with a polymer. The active components are dissolved in, entrapped in, or adsorbed to the surface of the polymer NPs. Polymeric NPs exist in a variety of forms ranging from nanospheres to dendrimers and utilize both natural and synthetic polymers. Polymer delivery characteristics, surface properties, morphology and composition can be readily tailored and optimised to achieve the desired drug loading, biocompatibility, targeting, degradation and controlled release kinetics [8].

Polymers used for parenteral delivery have to be biodegradable and are mostly based on polyacrylates (e.g., polycyano-acrylates) [9, 10] or polyesters (e.g., polylactides) [11, 12]. A number of different polymers have been evaluated for the development of oral vaccines, including naturally occurring polymers (e.g., starch, albumin, chitosan, alginates and gelatin) and synthetic polymers (e.g., polylactide-co-glycolides (PLGA), polyanhydrides, polycyanoacrylates and phthalates).

Chitosan is a deacetylated chitin that is of great interest as a functional material that can increase the paracellular permeability of intestinal epithelia. Because of low production costs, biocompatibility and very low toxicity, chitosan is a very interesting excipient for vaccine delivery research [13].

Polymer chains are cleaved by hydrolysis, leading to water soluble and physiological lactic acid as metabolite.synthetic polymers may be less advantageous due to their limited solubility in physiologically compatible liquids. They are often soluble only in organic solvents and depending on their structure, most synthetic polymers are highly lipophilic and require additional excipients.

Lipid Based Colloidal Systems: They resemble oil-in water emulsions, but with the internal phase being small in size and in many cases of solid consistency. Another lipid based colloidal system are liposomes, vesicular structures akin to cell membranes.

Solid lipid nanoparticles SLN can be prepared by rapidly injecting a solution of solid lipids in a water miscible solvent mixture into water to get particles of 80-300 nm [14, 15].

SLN often require surfactants for their stabilization or prevent aggregation and to enable a nanosized dispersion being generated during processing. Also, these surfactants lead to more round particles, whereas plain lipids generally form cubic crystal-like particles. SLNs exhibit several advantages over polymeric NPs. For example, they have comparatively higher drug entrapment efficiency and can be administered by multiple routes (orally, topically and IV). Moreover, hydrophobic drugs are stable in their lipid matrix. They protect sensitive drugs from the external environment. They have minimal toxicity and they do not require the use of organic solvents in their production (which can be easily scaled up to commercial level [16, 17]. Additionally, SLNs can provide controlled release formulations lasting up to several weeks. They adhere to mucosal surfaces, promote the absorption of orally administered drugs and have particular potential for drug delivery to the brain as they are capable of transporting pharmaceuticals across the blood brain barrier [18, 19].

Liposomes are vesicular carriers comprising a hydrophilic drugs in the core surrounded by one or more lipid bilayer membranes that consists typically of phospholipids (lecithins), cholesterol and glycolipids and having a thickness of about 5 nm. Liposomes can be produced in sizes from below 50 nm up to several μm depending on the composition and the manufacturing process [20].

Liposomes are suitable for topical, IV and IM administration, but because they are susceptible to degradation in the gastrointestinal tract they are rarely suitable for oral use. They have been investigated for targeted drug, imaging agent, vaccine and gene delivery with promising results [21, 22].

Nanoemulsions are dispersions of oil and water where the dispersed droplets are stabilised with a surface film composed of surfactant and co-surfactant. Most commonly, drugs are loaded into the dispersed phase where the droplet size is typically 20- 200 nm. An oil-in-water emulsion consists of dispersed oil droplets within an aqueous solution. Water-in-oil emulsions and water-in oil- in water emulsions have also been formulated for biomedical application. Low-cost, solvent free nanoemulsions have been produced for use in veterinary field [23] and promising results have been achieved using nanoemulsions for drug delivery, particularly via the oral and transdermal routes [24-26]. However, nanoemulsions are relatively new nanoparticles and a considerable amount of fundamental work needs to be performed to fully establish their physiochemical behaviour. Additionally, the high concentrations of solvents, surfactants and co-surfactants in some nanoemulsion formulations can be toxic to the tissues where they accumulate or are applied, resulting in haemolysis, cellular damage and tissue inflammation [27, 28].

Polymeric Micelles: Polymeric micelles have a unique structural composition characterized by a hydrophobic core sterically stabilized by a hydrophilic shell or corona to be highly water soluble. The hydrophilic shell may be one of four compounds like phospholipid or hydrophilic polyethylene oxide and hydrophobic polypropylene oxide blocks or poly (L-amino acid), or finally polyester that is composed of biocompatible polymers [29]. Polymeric micelles have long circulation times ensue from the steric hindrance awarded by the presence of a hydrophilic shell and the small size (l0- 100 nm) [30].

Inorganic Nanoparticles: In early studies, inorganic NPs demonstrated great potential as nanocarriers for therapeutic agents, vaccines and imaging agents. However, their clinical application is limited by concerns over toxicity, lack of biodegradability and persistent tissue accumulation. Therefore relatively few inorganic nanoparticles have progressed to clinical application [31].

Ceramic Nanomaterials: Ceramic NPs made of materials such as silica, alumina and titania, have several advantages over polymeric Nps systems in that they are easy to prepare and engineer to a desired shape, size and porosity. Also, they protect the adsorbed particles against denaturation induced by extreme pH and temperature.

However, titania NPs appear to possess considerable *in vivo* toxicity [31].

Carbon Nanomaterials: Carbon nanomaterials such as carbon nanotubes and carbon nanohorns have been investigated as drug carriers. They also have potential for vaccine delivery as they amplify the immunological response [32]. However, single walled carbon nanotubes trigger oxidative stress and are cytotoxicin cultured cell lines [31].

Metallic Nanomaterials: Various metals have been used to prepare NPs. Gold,silver and copper are most commonly used, with gold Nps being the most intensively studied [33, 34]. The main applications of metal nanoparticles lie in biosensing/imaging and cancer thermotherapy, although they are also being explored for targeted drug delivery [35, 36]. However, they have a range of toxic effects which, combined with their prolonged retention in tissues [37].

Quantum Dots: Quantum dots are comprised of an inorganic core, an inorganic shell and an aqueous coating to which biomolecules can be conjugated. It measures approximately 2-10 nm and fluoresce when stimulated by light so that its biomedical applications are primarily focused on imaging [38] and used as biomarkers providing a highly sensitive diagnostic and research tool [39]. However, clinical application of quantum dots is limited by their potential cytotoxicity and slow elimination [40, 41].

Magnetic Nanoparticles: Magnetic nanoparticles are commonly composed of iron oxide due to its high in vivo degradability. They have been investigated for use as biosensors, for imaging and for drug delivery where it can be pulled out of suspension in the blood stream and into localised disease sites by application of a high gradient magnetic field over that tissue [42, 43]. Concerns over toxicity and the accumulation of metal-based particles are a significant barrier to the clinical application of magnetic nanoparticles [44] at the present time.

Formulation of Some Magnetic Nano Particles: To synthesise Fe_3O_4 nanoparticles, 10.4 g of $FeCl_3 \cdot 6H_2O$ and 4.0 g of $FeCl_2 \cdot 4H_2O$ were dissolved in 100 mL of deionised water, degassed with nitrogen gas for 15 min and heated to 80°C. Then, 15 ml of NH_4OH (32% solution) was added dropwise to the solution as precipitating agent. After 15 min the solid was separated by a magnet and washed three times with 0.1 mol L^{-1} NaCl solution [45].

Magnetite Silica (SiO2/Fe3O4) Nanopartices: As it is possible to attach drugs or enzymes to the surface of the

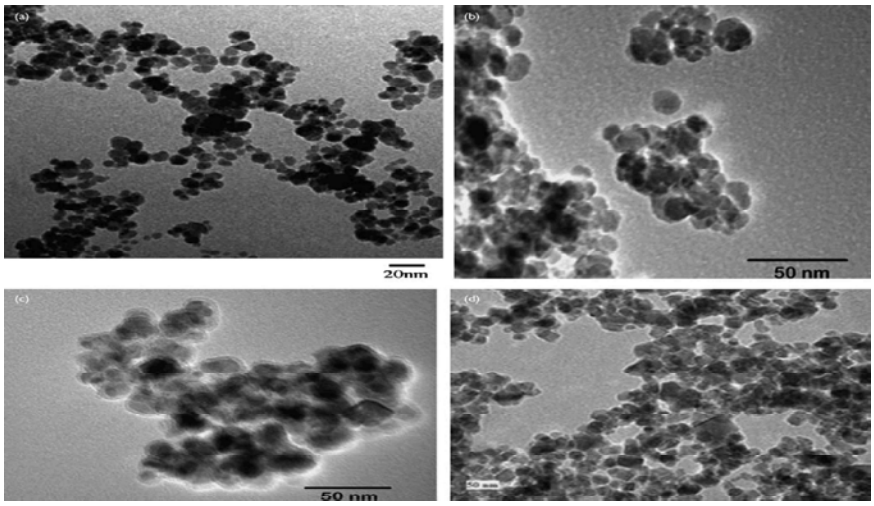

Fig. 1: El-Hady *et al*. [46] a) Fe3O4 nanoparticles, (b) magnetite silica nanoparticles, (c) magnetite silica NPs with immobilized lipase and(d) magnetite silica nanoparticles without lipase

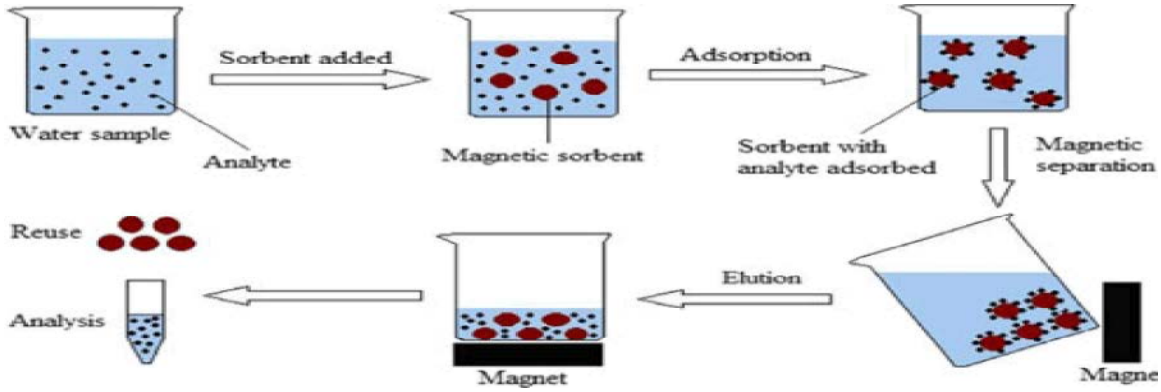

Fig. 2: Magnetic solid-phase extraction procedure Chen *et al*. [48]

magnetic particles and use magnetic fields to hold them at the site where it is needed, El-Hadi *et al.* [46] used silica coated magnetite nanoparticles (SiO_2/Fe_3O_4) for immobilization of lipase enzyme. During the magnetite preparation, they added drops of TEOS into the reaction mixture of iron during agitation. After homoginzation for 15 minutes followed by sonication for 15 minutes then adding NH4OH dropwise with continuous stirring, the silica coated magnetite particles were finally separated from the liquid using a permanent magnet, washed with distilled water several times and allowed to dry in air. Lipase immobilization was carried out by treatment of the lipase solution with the nanoparticles directly. and Fe_3O_4/SiO_2/enzyme were evaluated under the transmission electron microscope (TEM) as shown Figure 1.

Magnetite Chitosan Fe_3O_4-CS Nanoparticles: Chitosan solution was prepared by dissolving 1 g of CS powder in 100 ml of 1% v/v hydrochloric acid (HCL, 38%), after which 25 ml of 10 mg/ml sodium tripolyphosphate (TPP) solution was added to cross-link the CS [47].After stirring for 10 min, 15 ml of 0.1 ml/mol $Fe(NH_4)_2(SO_4)_2$ solution was added into the mixture, under the protection of nitrogen and a controlled flow of oxygen (0.5% v/v). Then twenty five millilitre of 1 N NaOH was added slowly to the suspension to precipitate the coated nanoparticles. The resulting Fe_3O_4-Cs nanoparticles were recovered from the suspension by applying a magnet. They were washed with deionized water several times until the pH reached 7.0, resuspended in 50 ml of deionized water and stored at 4°C until use.

Immunomagnetic Nanoparticles: Immunomagnetic separation employs magnetic particles with bound antibodies specific against the target (micro) organism or virus to be separated. Recently, immunomagnetic separation has been successfully used in water virology, microbiology and parasitology to detect important viral,

microbial and parasitic contamination. Several immunomagnetic beads are commercially available.

However, immunomagnetic particles can also be prepared in the laboratory using appropriate magnetic beads and an appropriate antibody. In this procedure, a magnetic adsorbent (either a general one, such as magnetic charcoal, or a magnetic affinity adsorbent) is added to a solution or suspension containing the target analyte(s). The analyte is adsorbed on to the magnetic adsorbent and then the whole complex is recovered from the suspension using an appropriate magnetic separator. The analyte is consequently eluted from the recovered adsorbent and analysed [48].

Nanoparticles Improve the Therapeutic Index: There are over 200 Nps drug delivery systems in development, with at least 30 nanoparticle based therapeutic products approved for clinical use in humans and a similar number in clinical trials [49, 50]. Many of these preparations are prohibitively expensive for veterinary use, but, several nanoparticle formulations are already available on the veterinary market and as NPs production facilities are scaled up for commercialisation,these preparations will increasingly become more affordable for veterinary application.

Nps improve the therapeutic index of the pharmaceutical agents. They carry and enable the use of drugs that would otherwise be insoluble or unstable. Because of preferential accumulation at target sites, NPs increase the concentration of pharmaceutical at its intended site of action, resulting in increased efficacy and lower systemic toxicity and drug concentration in healthy tissues.

Lastly, NPs have reduced clearance compared to the parent drug and thus provide a method of sustained controlled release over a period of days or even weeks [51-53]. As a consequence of these mechanisms, NPs formulations require a reduced dose compared to free drug. This is particularly pertinent to veterinary medicine as it may allow the use of expensive human pharmaceuticals whose application has previously been precluded by the cost of dosing and reduce the levels in carcasses leading to lower environmental impact and lower residues in food. NPs can be loaded with drugs via encapsulation within the particle or via surface attachment [54]. The method of drug loading depends upon the type of nanoparticle as well as the drug type and the target. Targeting of NPs to specific sites is achieved passively via the enhanced permeability and retention effect [55]. This effect relies on the ability of intravenous (IV) nanoparticles to extravasate at sites of increased vascular permeability, but otherwise be retained in the circulation. This results in accumulation of NPs at sites of increased vascular permeability (e.g. tumours, infections and areas of inflammation), hence targeting of the agents they carry to these sites [56]. Opsonisation and subsequent uptake of NPs by the reticuloendothelial system reduces the number remaining in circulation and able to extravasate [57]. To overcome this, nanoparticles can be coated with hydrophilic substances, the most commonly used being polyethylene glycol, which reduces opsonisation and prolongs circulation time [58]. In fact, omission of a hydrophilic coating results in rapid uptake of NPs by cells of the mononuclear phagocyte system, rendering them ideal for targeting intracellular parasitic, bacterial, fungal and viral infections [59]. Passive targeting is often less expensive than active targeting and ispotentially more useful for application to veterinary medicine.In addition to passive targeting, active targeting may be necessary to increase the interaction between NPs and target tissues. This is achieved via attachment of a targeting moiety to the nanoparticles, causing them to adhere to a particular receptor/cell type so increasing their concentration at the site of interest [60].

The use and development of antibodies and antibody fragments to target NPs to a particular tissue or cell type can be expensive. However, targeting NPs to specific tissues simply by altering their charge, or coating them in a substance that is naturally taken up by that tissue is a more cost-effective approach in developing targeted NPs for veterinary use. This method has been successfully used to enhance NPs adherence to and uptake across the blood-brain barrier for treatment of neurological diseases [61, 62] at the target site the next essential step is drug release. A myriad of mechanisms for NPs uptake may occur based on the properties and surface characteristics of the nanoparticle in question [63]. Possible mechanisms of drug release are liberation due to NPs disintegration, or enzymatic breakdown; diffusion from the intact NPs; release from the surface of the nanoparticle; fusion of the NPs with the cell surface membrane and subsequent release of contents into the cell; endocytosis of the NPs with subsequent release of contents into the endoplasmic reticulum and triggered release initiated by application of an external factor, such as a magnetic field or a change in temperature or pH [64, 65]. Often a combination of these processes coexists and particles can be engineered to have optimal and controllable release kinetics that target them to specific intracellular pathways.

For more understanding to the concept we give a model such as the recently developed liposome-encapsulated hemoglobin/silica nanoparticle (LEHSN) as an oxygen carrier. It provides an alternative for blood transfusion, which effectively solves some problems such as limited number of donors and the potential risk brought by unmatched blood or virus infection like human immunodeficiency virus (HIV). As Hb molecule is a tetramer that rapidly dissociates in to two dimers, which results in renal failure [66]. LEHSN was fabricated by a water-in oil-in-water (W/O/W) double emulsion approach.

Briefly, Silica NPs(SNs~*10 nm)* was added Intobovine Hb aqueous solution to obtain a complex of Hb/SNs. Which was added into water in which acetyl trimethyl ammonium bromide (CTAB) was dissolved, followed by an ultrasonic dispersion for 5 min. The CTAB aqueous applications. Solution containing Hb/SNs served as the water phase for the following emulsion. Under stirring, the water phase was mixed withthe oil phase composed of chloroform and lecithin to get a water-in-oil (W/O) emulsion. Then additional water (acted as the outer water phase) was added into the W/O emulsion to form a W/O/W double emulsion. The final emulsion system was vacuum evaporated to remove the organic solvent and to obtain LEHSN. SNs served as rigid core provide a supported framework for lecithin membrane and enhance the stability of liposomes that formed a cell membrane-like environment for the controlled release of Hb.

In comparison with liposome-encapsulated Hb (LEH), LEHSN shows substantially enhanced stability and improved release property of Hb *in vitro* [67].

Nanoparticle Based Vaccine Delivery: Vaccines, designed to stimulate a long lasting and protective antibody response to a pathogen, are comprised primarily of antigen and adjuvant. Traditionally, inactivated microorganisms providedthe antigen, but recently there has been a shift towards the use of safer synthetic peptides and recombinant proteins [68]. Alone, these new vaccine candidates are often poorly immunogenic and sensitive to degradation and they require an optimised adjuvant that improves immunogenicity [68]. Conventional adjuvants are not tuneable, but with the advent of nanotechnology a plethora of novel antigen carrying strategies are now available. These novel Nps based adjuvants are highly tuneable and can be engineered for reduced dosage frequency via a convenient administration route in order to provoke a specific immune response, e.g. the intranasal route to better target mucosal immunity [69-71]. This makes them highly amenable to engineering for veterinary field where large numbers of animals may need to be treated at once in a commercial unit, or when vaccination by conventional means is inconvenient due to extensive management systems or poor accessibility (e.g. wildlife). NPs adjuvants increase the immunogenicity of a vaccinein five (potential) ways [68]. Firstly, by mimicking pathogen-associated molecular patterns they can activate pattern recognition receptors, such as Toll-like receptors and trigger intracellular signalling cascades that initiate the innate immune response, resulting in enhancement of the adaptive immune response. Secondly, by upregulating co-stimulatory molecules on antigen presenting cells. Thirdly, NPs adjuvants can control the residence time, location and dose of antigen released so as to maintain immunity levels and enhance translocation of antigen to lymph nodes. Fourthly, they act as a depot to provide prolonged delivery of antigens. Finally, NPs canbe engineered to produce virus like particles that have similar morphology to virus capsid and stimulate immune responses without the infectious genetic material that is responsible for host infection [68]. Nanoparticles are γ compounds that include amino acid - poly γ-glutamic acid (γ-PGA) [69, 72] poly lactic acid (PLA) [73], poly lactic-co-glycolic acid (PLGA) [74], chitosan [75], gelatin [76], calcium phosphate (CaP) [77], silica [78], gold [79], magnetite [80], strontium phosphate [81], magnesium phosphate and manganese phosphate [82]. NPs adjuvants that are approved for veterinary use (or in clinical trials) include emulsions, liposomes, polystyrene nanobeads, immune-stimulating complexes (ISCOMs) and in organicparticles [68, 83, 84]. For practical veterinary use, nanoparticle adjuvantsneed to be inexpensive, stable, easy to administer and biodegradable in species used for human consumption [85]. To date, more than 40 diseases of animal species including equine influenza and Streptococcus equi var. equi infection in horses [86, 87], foot-and-mouth disease, bovine virus diarrhoea virus and *Toxoplasma gondii* in ruminants [88-90]; Newcastle disease and H5N1 influenza in poultry [91, 92], enterotoxigenic *E. coli* and atrophic rhinitis in swine [93] and parvovirus and atopic dermatitis in dogs [69, 84, 94] have nanoparticle vaccine delivery systems that are either successfully developed or under development.

Calcium phosphate nanoparticles provide safe and easily manufactured vaccine adjuvant and delivery system for DNA vaccines. Recently, FMDV “O”P1-3CD DNA vaccine was encapsulated in calcium phosphate nanoparticles of size 50-100 nm diameters. *In vitro*

transfection efficiency of these calcium phosphate nanoparticles was found to be as good as commercial transfecting reagent lipofectamine. In vivo analysis of the calcium phosphate nanoparticle P1-3CD(CaPNP1-3CD) FMDV "O" vaccine in mice and guinea pigs could induce significant cell mediated and humoral immune response. Also, immunized mice and guinea pigs were protected against the challenge virus [95]. A detailed review of nanoparticle based veterinary vaccines is provided byScheerlinck and Greenwood [83]. Also, calcium phosphate (CaP) particles were coupled with inactivated Newcastle disease virus (NDV) vaccine.The surface morphology of CaP particles coupled to NDV was found to be spherical, smooth and with a tendency to agglomerate. The humoral and cell mediated immune responses induced by CaP coupled NDV vaccine were assessed in comparison to a commercial live vaccine (RDV 'F') and showed prolonged haemagglutination inhibition (HI) and enzyme linked immunosorbent assay (ELISA) titres in the serum even at fourth and fifth week post-vaccination (PV), unlike RDV 'F' inoculated chickens whose titres declined to insignificant levels by this time [96, 97].

Nanoparticles as Diagnostic Tool: Nps have been widely used as a signal reporters to detect biomolecules in DNA assay, immunoassay and cell bioimaging. Gold NPs based probe have been used in identification of pathogenic bacteria in DNA-microarray technology [98]. The highly sensitive biomolecule detection is due to the huge surface area that facilitateefficient macromolecularinteractions compared to bulk solvent; and the effective signalamplification [99, 101]. Few sensing methods based on NPs were reported for the detection of Hepatitis B and C viruses [102], Respiratory Syncytial Virus [103], *E. coli* in water [104], *Staphylococcus aureus* [105].

Although there are fewer application of nano particles in environmental studies than in biomedical studies, the use of quantum dots (QD) as a fluorescence labeling system in microbial detection has been successfully demonstrated when QD conjugated wheat germ agglutinin binds to sialic acid residues on bacterial cell wall. QD can be further conjugated with specific antibodies to detect C*ryptosporidium parvum and Giardia lambli* in water samples which was time and effort consuming if investigated by routine concentration method [39].

We can conclude that metallic and inorganic nanoparticles exhibit unique properties in terms of particle aggregation, photoemission, electrical and heat conductivity.

Immunoassays can benefit from the application of magnetic nanoparticles or microparticles. In general, magnetically responsive particles can be used in an enormous number of applications, ranging from molecular biology to waste water treatment [106, 107]. Magnetic particles exhibit important properties where it has the power of selective separation and removal of magnetically responsive nanoparticles and microparticles and other relevant materials from complex samples using an external magnetic field (e.g. an appropriate magnetic separator, permanent magnet, or electromagnet). Also it has the ability of targeting and localization of magnetic particles to the desired place using an external magnetic field [108].

The potential toxic effect of nanoparticles on organisms in natural environments is still unknown. However with the current practice of discharging nanoparticle waste, nanoparticles will find their way into waste water and eventually to different aquatic environments. Thus there is a need to understand the potential hazard of nanoparticles on human health and on other organisms, while the use of nanoparticles in various scientific researches and medical applications continues.

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