

A DRUG DELIVERY COMPOSITION

5 **Field of Invention**

This invention relates to a drug delivery composition. In particular, the invention relates to a drug delivery composition for delivering a drug to an aquatic animal. More particularly, but not exclusively, the drug delivery composition is capable of delivering an immunogenic antigen to an aquatic animal to induce an immune response in the aquatic animal.

Background

Worldwide, commercial fishing (catching wild fish) is fast declining and depleting the aquatic life and hence it is no longer sustainable. Nowadays, aquaculture which involves the farming of aquatic organisms such as fish, crustaceans, mollusks and aquatic plants under controlled conditions is an industry that is booming due to its sustainability.

Asia is the largest aquaculture producer with China leading the way. Other third world and developed countries are also favouring aquaculture as a means to provide sustainable vital protein source for their people.

Due to the rapid expansion of aquaculture, many farms are now stocking fish in very high density. The close proximity of the fishes and the occurrence of natural pathogens in the aquatic environment adds to the frequency of disease outbreaks which can wipe out the fish stocks. In doing so, the farm suffers huge losses which may lead them to fold their business.

As such, there is a need for an efficacious immunization of aquatic animals. For example, infectious diseases are common on fish farms, due to intensive fish farming that facilitates the transmission of pathogens in an aqueous environment.

To overcome diseases, in the past, chemotherapy (use of antibiotics) was used extensively. However, inadvertent use of the antibiotics have led to major problems such as development of drug resistance in pathogens and accumulation of undesired chemicals in the fish and environment, leading to its diminishing use nowadays.

As such, alternative solutions like vaccination have been favoured as they can also provide long term protection. In fact, the administration of fish vaccine has prevented the fish farming industry from loss of fish due to disease outbreaks and allowed higher fish yield. Additionally, vaccines may also contribute to the preservation of a healthy and viable wild life stock by minimizing spread of diseases between farmed and wild fish when cultured in the same environment.

Vaccines, delivered to fish, are either oil based or water based. Current routes of vaccine administration are via oral, immersion and injection vaccination.

With oral vaccination, the vaccine is either mixed with the feed, coated on top of the feed (top-dressed) or bio-encapsulated. Oral vaccination has the advantage that it is a very easy vaccine administration method with no stress to the fish, requires little labour, and can be applied at a large scale. However, oral vaccines have a very short term stability once mixed with the feed. Many oral vaccines have been found ineffective as a result of failure to uptake sufficient dosage of antigen, poor antigen delivery and antigen degradation in the digestive tract. In most cases, only limited protection can be obtained and the duration of protection can be rather short. Vaccination via the oral route also suffers from poor potency because of the potential degradation of vaccine components in the gut when administered through fish gastrointestinal tract.

Immersion vaccination works on the ability of mucosal surfaces. When fish are immersed in water containing the diluted vaccine, the suspended antigens from the vaccine may be adsorbed by the skin and gills. Then, specialized cells, such as antibody-secreting cells, present in the skin and gill epithelium will be activated and will protect the fish when fish are exposed to the live pathogen at a later stage. It is an effective method that results in relatively good protection. However, the limitations of immersion vaccination are that the duration of immunity is not very long and a booster vaccination is required when the disease prevails over longer periods. Also, the method is impractical for larger size fish (for example, fish that are bigger and heavier than 10 g) due to cost-effectiveness and the stress that could be induced by vaccination.

Although, current immersion vaccination are sometimes protective, heavy mortalities post vaccination still occurs as not many effective immersion vaccines are currently available for use.

As such, the immersion method suffers from low potency as the efficiency of uptake of vaccine through the gills and skin are limited. Therefore, injection of vaccine directly into individual fish is preferred over oral administration as a defined quantity of vaccine can be administered leading to longer protection.

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Injection vaccines can be administered by intramuscular or intraperitoneal (in the abdominal cavity) injection. Injection vaccination provides for a long duration of protection, i.e., for over a year, and it allows for multiple antigens to be combined in a single vaccine and, therefore, in a single administration. As intraperitoneal injection vaccination involves depositing the vaccine in the abdominal cavity, it is important that the needle should penetrate the targeted abdominal wall of fish by 1 to 2 mm. Higher mortalities indicate incorrect use of anaesthetic, excessive handling/stress of the fish, incorrect needle insertion (e.g., rupturing the spleen), vaccination of diseased or weakened fish, lack of oxygen in the anaesthesia vessel, etc. Short needles might deposit the vaccine in the musculature and cause inflammation and a bad immune response. The right thickness of needle is also important. If the vaccine is viscous, it might pass through the needle with difficulty. In contrast, a needle that is too large can induce sores and secondary infections at the injection site, and allow the vaccine to flow back. The rapidity of the immune response is temperature dependent. It usually takes several weeks before good immune protection is developed as a result of vaccination.

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Despite these advantages, the administration of vaccine via injection suffers from the high cost incurred due to heavy dependence on skilled manpower, and is also prone to accidental injury to the fish and personnel. Furthermore, injection vaccination can only be administered when fish are $\geq 10\text{g}$, hence, smaller fishes cannot be vaccinated and are hence not protected from diseases. Also, formaldehyde inactivated bacterial cells are used as the principle antigen for immunizing fish which is administered via intra-peritoneal injection. This labour and skill intensive process of vaccine administration is the main bottle neck in the vaccination of farmed fish.

Hence, it is highly desirable to develop a drug delivery system, particularly a vaccine delivery system, for small aquatic animals that can reduce the need for injection but still retains the advantages of the injection vaccination method.

Summary of Invention

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In accordance with a first preferred aspect of the present invention, there is provided a drug delivery composition for delivering a therapeutic amount of an active ingredient to an aquatic

animal, the composition comprising (a) a nanoparticle; and (b) the active ingredient contained in said nanoparticle.

Advantageously, the composition of the present invention is useful for delivering drugs to farmed fish. In particular, it is useful for delivering a vaccine for immunizing farmed fish weighing less than 10 g which is difficult by current methodology.

Preferably, the composition of the present invention may include any adjuvants and/or pharmaceutical carriers in order to improve the performance and delivery of the drug delivery system.

Preferably, the nanoparticle is nano-clay. Alternatively, the nanoparticle may be any silica based nanoparticle where the length is above a micron and diameter is in nano range where the aspect ratio is less than 22.

Preferably, the surface of the nanoclay is modified. More preferably, the surface of the nanoclay is modified with aminopropyltriethoxysilane. The surface modification may be carried out by any technique to the skilled person. Other agents, for example polymers such as alginate, starch, glucans, mannoproteins, phosphor-mannans, inulin and the like, may be used to modify the surface of the nanoclay. Advantageously, modification allows for the surface of the nanoclay to be positively charged, allowing it to bind easily to a mucosal surface of the aquatic animal. This, in turn, allows for an increased pay load and a sustained delivery of the active ingredient to the aquatic animals. The modified surface can be investigated and analysed using Fourier transform infrared spectroscopy (FTIR) analysis that will show the presence of specific chemical bonds such as N-H bend and NH₂ groups. Any quantification to arrive at the desired pre-determined characteristics of the present invention can be carried out using differential scanning calorimetry or by thermogravimetry analysis.

Preferably, the nanoclay is halloysite nanoclay. And, more preferably, the halloysite nanoclay is coated with chitosan. The amount of chitosan coated and present in the composition is about 10% wt.

Preferably, the amount of the nanoclay present in the composition is about 10 to 100 mg/L.

Preferably, the active ingredient is any one selected from the group comprising: an immunogenic antigen, a hormone, a nutrient, a mineral, an antibiotic and a therapeutic agent. The immunogenic antigen may be any antigen that is obtained from any one selected

from the group comprising: a virus, a bacteria or parasite. In addition, the antigen may be other immunogenic bacterial, parasitic and viral proteins, whole inactivated virus or viral capsid proteins. Still further, the antigen may be antigens from fungi, or any peptide, protein and/or nucleic acid. The antigens may be of multiple origins, for example one may be from
5 bacterial origin and the other may be from a viral origin or any other suitable combination.

In addition, the immunogenic antigen could also be of viral, bacterial or parasitic subunits, recombinant origin such as recombinant proteins and nucleic acids. Hormones such as growth hormones and gonadotropin can also be used. Nutrients and minerals such as amino
10 acids, vitamins, iron, calcium and phosphates. With regard to antibiotic and therapeutics as active agents, amoxylin and penicillin, outer membrane proteins, bacterial extracellular components or any parasite or any of the parasite's components may be used.

Preferably, the antigen may be any bacterial extracellular component. Alternatively, the
15 antigen may be an outer membrane protein of the bacteria. Preferably, the amount of the outer membrane protein present in the composition is about 1 to 100 mg/L.

In the present invention, an example of the bacteria is *Tenacibaculum maritimum* (TIMA). There are other bacteria from genera of *Streptococcus*, *Vibrio*, *Aeromonas* that may be used
20 to provide the antigen.

Preferably, the composition may comprise more than one active ingredient, for example a plurality of immunogenic antigens. Each immunogenic antigen originating from a different pathogenic entity, for example virus, bacteria and/or parasite.
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Preferably, the aquatic animal is a fish having a weight of less than 10g. The present invention may be useful for delivering an active ingredient to different species of fish, prawns, crab, clams, or any of the cultured aquatic organism.

In accordance with a second preferred aspect of the present invention, there is provided a method for preparing a drug delivery composition for delivering a therapeutic amount of an active ingredient to an aquatic animal, the method comprising (a) providing a nanoparticle; (b) modifying the surface of the nanoparticle; (c) coating the surface of the nanoparticle with a polymer; and (d) loading the active ingredient into the nanoparticle.
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Preferably, the nanoparticle is modified with aminopropyltriethoxysilane.
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Preferably, the polymer may be a natural polymer. More preferably, the natural polymer is chitosan.

5 Preferably, the nanoclay is halloysite nanoclay and present in the composition in the amount of about 10 to 100 mg/L.

Preferably, the loading is carried out by any one of the method comprising: freeze drying, vacuum drying and ultrasonication.

10 Preferably, the drug is any one selected from the group comprising: an immunogenic antigen, a hormone, a nutrient, a mineral, an antibiotic and a therapeutic. The immunogenic antigen may be obtained from the group comprising: a virus, bacteria, and a parasite.

15 In addition, the immunogenic antigen could also be of viral, bacterial or parasitic subunits, recombinant origin such as recombinant proteins and nucleic acids. Hormones such as growth hormones and gonadotropin can also be used. Nutrients and minerals such as amino acids, vitamins, iron, calcium and phosphates. With regard to the use of antibiotics and therapeutics as active ingredient, amoxylin and penicillin, outer membrane proteins, bacterial extracellular components or parasite or any of the parasite's components may be used.

20 Preferably, the antigen obtained from a bacteria is an outer membrane protein of the bacteria, and the step of obtaining the outer membrane protein of the bacteria is carried out prior to loading onto the nanoparticle. The amount of the outer membrane protein present in the composition is about 1 to 100 mg/L.

25 Alternatively, or in addition, the antigen may be an extracellular component of the bacteria.

Preferably, the aquatic animal is a fish having a weight of less than 10g.

30 In accordance with a third preferred aspect of the present invention, there is provided a method of delivering a drug composition to an aquatic animal or inducing an immune response in an aquatic animal, the method comprising administering to the animal an effective amount of the composition in accordance with the first aspect of the present invention.

35 By "inducing an immune response", it is meant to refer to any method that achieves effective vaccination against diseases that may infect the aquatic animal. Effective vaccination

involves the exposure of the immune system of an animal to an "antigen"—a piece of a pathogen or the entire pathogen—and then allowing time for the immune system to develop a response and a "memory" such as antibody, which can prevent infections by the targeted disease-causing organism. In humans, the earlier vaccination is carried out e.g. infant stage, the higher the chances of survival to adulthood. Likewise, in fish, a similar trend is observed.

Preferably, administration is carried out by immersing the aquatic animal a medium containing the dispersed drug delivery composition. More preferably, administration is carried out for a fish having a weight of less than 10g and administration is carried out in any type of water such as fresh water, salt water, brackish water or aqueous environment

Preferably, the amount of antigen present in the medium is about 1 to 100 $\mu\text{g/mL}$. The amount of the nanoparticle present in the medium is about 10 to 100 $\mu\text{g/mL}$.

Alternatively, the administration may be carried out by spraying the composition onto the aquatic animal.

Advantageously, the present invention may be used for delivering vaccine to protect fish against viral, fungal and parasite infections/infestations.

Due to the limitations of vaccination via the injection and oral methods, the present invention advantageously provides a nano-delivery platform which can be used to deliver antigens into fish via the immersion (dipping) route.

Preferably, the method comprises inducing an immune response in an aquatic animal against a virus, bacterium or parasite.

Brief Description of Figures

In order that the present invention may be fully understood and readily put into practical effect, there shall now be described by way of non-limitative examples only preferred embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

In the Figures:

Figure 1 show the physico-chemical characterisation of the nanomaterials used in an embodiment of the present invention as a delivery vehicle;

Figure 2 show the cytotoxicity and zebrafish embryo mortality against a variety of nanomaterials used;

Figure 3 shows the efficiency of delivering Green Fluorescent Protein (GFP) into fish cells after surface modification of nanomaterials;

Figure 4 show fluorescence images of the fate of nanoparticle in fish cells and zebrafish embryo;

Figure 5 shows an SDS-PAGE of outer membrane proteins isolated from *Tenacibaculum maritimum* (TIMA), a deadly bacterial fish pathogen;

Figure 6 show images of a method of vaccination of aquatic animals according to an embodiment of the present invention;

Figure 7 is a table showing antibody titres in fish serum immunised with the embodiment of the present invention; and

Figure 8 show results of RT-PCR studies on the immune response in an aquatic animal using a vaccination method according to an embodiment of the present invention.

Detailed Description of the Preferred Embodiments

The present invention relates to a drug delivery composition. Broadly, the drug may be an immunogenic antigen, a hormone, a nutrient, a mineral, an antibiotic or any other therapeutic agent. In particular, it relates to a vaccine delivery composition comprising of a nanoparticle and an immunogenic antigen contained within the nanoparticle.

By “antigen”, it is meant to include any structural substance which serves as a target for the receptors of an adaptive immune response. As such, the immunogenic antigen may be of viral, bacterial, or parasitic agents that elicits or induces an immune response in the immunised organism. In a preferred embodiment of the invention, the immunogenic antigen is an outer membrane protein extracted from a bacterium or any antigenic protein(s).

In a preferred embodiment of the invention, the delivery vehicle is the nanoparticle, and the nanoparticle is nanoclay. The surface of the nanoclay has been modified and coated with chitosan. In a more preferred embodiment of the invention, the nanoclay is halloysite nanoclay such that the immunogenic antigen is loaded within the hollow interior core of the tubular-shaped halloysite nanoclay. In an embodiment of the invention, the immunogenic antigen may be an outer membrane protein of a pathogenic bacteria. As such, in a preferred embodiment of the invention, outer membrane proteins of pathogenic bacteria loaded into chitosan-coated nanoclay for effective immunization of farmed fish using dip vaccination strategy.

Advantageously, the nanoclay delivery vehicle is safe, and may be used to delivery its load in a sustained manner in order to elicit a proper immune response in vaccinated fish. Various nanomaterials of certain composition, shape and dimension were screened with the object of finding one that is inert and harmless for use in aquatic animals – particularly, fish. In the present invention, the halloysite nanoclay was determined to be the best candidate in terms of safety. The fact that halloysite nanoclay has a hollow tubular structure also complements its selection as a suitable material as one can load antigens or drugs into this hollow space, hence, making the nanoclay similar to a syringe and needle. In addition, nanoclay is also readily available, easy to apply and made making it to be a cost-effective vaccine.

In preparing the vaccine delivery composition for delivering a therapeutic amount of a drug to an aquatic animal, the method of preparation comprises providing the nanoclay, modifying the surface of the nanoclay, coating the surface of the nanoclay with a chitosan, and loading an immunogenic antigen into the nanoparticle.

In an embodiment of the invention, the nanoclay is a halloysite nanoclay (NC) and it is initially modified with aminopropyltriethoxysilane (APTES). The APTES modified NC was treated with chitosan to graft chitosan onto the NC surface. The surface functional group can be of others like RGD peptide, fibronectin, chondroitin sulphate, glucosamine PEG, PVP, PEI and the like. In addition, or alternatively, the following may be used to modify the NC: alginate, starch, glucans, mannoproteins, phosphor-mannans, inulin. This enhances the NC's properties in binding to the targeted site such as the gills, skin and gut of the fish, functional modifications were made to the NC particle, which eventually allowed the chitosan-nanoclay (CNC) to strongly bind to the targeted site in fish.

Bacterial antigens such as bacterial outer membrane proteins (OMP) and extracellular components (ECP) proteins meant to be secreted by the bacterial to its surroundings were

extracted and isolated. Basically the protocol involves growing TIMA bacteria in AOA broth for 24h. The bacterial cells from 30 mL was separated out using centrifugation and the supernatant was discarded. Tris + DTT solution was added and the bacterial cells were suspended by pipetting up and down. The tubes were then centrifuged at 6000 rpm for 20 minutes and supernatant was removed. The bacterial pellets were subsequently suspended in buffer containing SDS (1% (w/v) SDS, 0.375M Tris-HCl (pH8.8), 50mM DTT, and 25% (v/v) glycerol). This suspension was boiled at 100°C for 5mins and then transferred into a fresh falcon tube. The mix was sonicated 6 times (10 seconds each time) and then the tube was centrifuged at 6000 rpm for 10 minutes. The supernatant containing isolated OMP were kept in a -80°C freezer till further use. The Bradford protein assay, known to the skilled person, may be used to measure and determine the concentration of the protein content. It is a method that utilizes a dye that stains for protein. The loading of the immunogenic antigen onto the CNC was carried using a vacuum. For this, CNC powder was added to OMP preparation such that 20 mg of protein will be loaded to 100 mg of CNC. The resulting suspension was kept in vacuum drier at 45°C and was dried.

Alternatively, the loading may be carried out by freeze drying or ultrasonication. For freeze drying, the suspension was frozen at -80°C for 6 hours and was loaded in freeze drier. Yet another way of loading CNC with OMP is by subjecting the suspension containing OMP and CNC to water bath sonication for 10 min.

Advantageously, an immediate application of this invention is that it can be used to vaccinated fish which are smaller in size e.g. < 10g via an immersion vaccination method, i.e. a "dipping" process as opposed to conventional vaccination via injection which can only be conducted when the fish is of larger size (>10g). As such the outcome is that smaller fishes will be vaccinated at an early life stage thereby protecting them from deadly diseases, resulting in higher fish survival rates in the nursery. In addition to using CNC as a vehicle to deliver to bacterial antigens as vaccine, this platform could also be used for the delivery of other molecules such as viral, parasitic or bacterial recombinant proteins, hormones, nutrients, minerals, antibiotics, and other therapeutics, thus increasing its utility for delivering nutrients and therapeutic agents to the fish. Examples include amino acids, vitamins, iron, calcium, phosphate, amoxylin, penicillin, growth hormone, gonadotropin.

In a preferred embodiment, a vaccine composition consisting of outer membrane proteins of pathogenic bacteria loaded into a nano-composite vaccine delivery system is designed. This composition can be administered via dipping fish in water. Studies using model protein-Green Fluorescent Protein (GFP) showed that the delivery of proteins into cells is several

fold higher when administered by using a nanocomposite consisting of clay nanoclay as the core particle and chitosan as a coating (CNC). CNC was tested for safety and distribution in zebrafish embryo/larvae. Our data showed that the CNC is safe for fish and it is present on the skin, gill and intestine of the fish. These materials were tested for the efficiency to elicit immune response in fish cell line and seabass fish. Data showed that the vaccine composition is superior in eliciting an immune response in fish. In conclusion, the present invention shows that the developed vaccine composition is capable of immunizing fish against pathogenic bacteria and it can be applied by dipping fish in the vaccine composition thereby overcoming the disadvantage of injection vaccination.

A non-limiting example is described below.

EXAMPLE

MATERIALS AND METHODS

1. Preparation of modified Clay-Chitosan NP

Halloysite clay nanoparticle purchased from commercial supplier was washed and dispersed in sterile deionized water and was washed by centrifugation. These particles were then soaked in 1M NaOH overnight at room temperature. Milli Q water and 99.5% ethanol were added to wash the clay halloysite. These clay were treated with APTES by soaking for 2 hours at room temperature and then washed with Milli Q water twice. The mixture was centrifuged and the supernatant was removed. Clay was then left in the oven at 60°C overnight.

Then, 1% (v/v) glutaraldehyde was added to the clay and soaked overnight (37°C). In a separate falcon tube, chitosan was dissolved in (0.05% (w/v)) in 0.1M acetic acid supplemented with 0.25nM sodium azide and to this glutaraldehyde modified clay was added and stirred overnight at 60°C. The functionalized clay chitosan nanoparticles were then washed in deionized water and dried under vacuum for further use.

CHARACTERIZATION OF NANOMATERIALS

1. Measuring Size Distribution and Zeta Potential using ZetaSizer instrument

Each nanomaterials (10mg) was weighed out and transferred into an empty falcon tube. Milli Q water (2mL) was added into the same falcon tube [concentration 5mg/mL]. The mixture was sonicated to ensure nanomaterials were in suspended form. The mixture (100µl) was added into a new falcon tube and 9.9 mL of Milli Q water was added to prepare a working concentration of 50 µg/mL solution. These solutions were used for measuring the hydrodynamic diameter and zeta potential using ZetaSizer (Malvern, USA).

2. Fourier Transformed Infra-Red Spectroscopy (FTIR)

Nanomaterial (1mg) was weighed and transferred to clean empty falcon tube and were added with 5 to 7 drops of dichloromethane (DCM). The mixture was sonicated to ensure that NP material was in suspension form. Using a dropper, 2 to 3 drops of the NP mixture were added onto the KBr disc. The KBr was then was placed in the FTIR (Perkin Elmer, USA) for scanning to obtain the FTIR spectrum.

3. Cytotoxicity and Zebrafish Embryo Toxicity

BF-2 cells were grown in a TC cell culture flask at 28.0 °C in a humidified environment with 5 % CO₂. The growth medium used was MEM. These cells were harvested, counted and resuspended in MEM medium to a cell concentration of 106 cells/mL. 100µl of these cells were seeded into 96 well plates. The plates were incubated at 28.0 °C for 24h. After 24h, the spent medium was replaced with fresh medium containing nanomaterials/nanoparticles. The plates were incubated for another 24 h before the cell culture medium was replaced with medium containing Resazurin reagent. The cell viability was monitored based on the conversion of non-fluorogenic resazurin to a fluorescent dye, resorufin. Cell viability was then expressed as % of control group where the cells were not subjected to any treatment. Silver nanoparticle with reported toxicity to fish and fish cell lines was used as the positive control.

Zebrafish embryos (wild type) at 3 hour post fertilization were used for assessing the toxicity of nanoparticles to zebrafish embryos. Healthy, fertilized zebrafish embryos were selected and one embryo per well were added into 96 well plates. The nanoparticles were suspended in Holtfreter's medium at 100 ppm concentration.

Embryos were added with 100 µl of nanoparticle suspension and were incubated at 28°C for 5 days. At 5dpf, the number of dead and deformed embryos were counted. The mortality rate was calculated and plotted. Zebrafish embryos without exposure to any nanoparticle

was used as the negative control and those which subjected to 100 ppm of silver nanoparticle were used as positive control.

4. Testing for the Delivery of Green Fluorescence Protein (GFP)

The nanomaterials (10 mg) – clay, chitosan, HA and MSNP, were transferred into individual cryotubes. The appropriate volume of DI H₂O were added to the cryotubes to form stock solutions of 10 mg/ml for each nanomaterial. The stock solutions were then vortex mixed and sonicated.

GFP was prepared by adding PBS (30 µl) to GFP (20 µl). The NP stock solutions (10 µl) and GFP (10 µl) were added into individual eppendorf tubes. The eppendorf tubes were subjected to vacuum for 30 minutes. MEM (1 ml) was added and the eppendorf tubes were vortex mixed for 10 seconds. The eppendorf tubes were centrifuged at 15 000 rpm for 30 minutes and the supernatant (800 µl) was discarded. MEM (800 µl) was added and the eppendorf tubes were centrifuged again. Supernatant (800 µl) was discarded and MEM (800 µl) was added and the eppendorf tubes were vortex mixed. More eppendorf tubes were prepared – GFP (in MEM), clay (in MEM), MSNP (in MEM), chitosan (in MEM), HA (in MEM). The eppendorf tubes were sonicated at 40 Amp. for 15 seconds. Spent medium in the 96 well-plate was removed and the individual solutions containing nanoparticles+GFP (100 µl) were added to the respective wells. The 96 well-plate was then incubated for 3 hours (28.0°C, 5% CO₂). Spent medium was removed from the 96 well-plate and the 96 well-plate was rinsed twice with PBS (100 µl to each well). Hoechst 33342 dye was prepared in a falcon tube (15 ml) by adding Hoechst (10 µl) to PBS (10 ml). The Hoechst dye solution was added (100 µl to each well) and the 96 wellplate was placed in the incubator for 10 minutes. The 96 well-plate was brought for plate reading at 395 nm (excitation) and 508 nm (emission). Image analysis of the 96 well-plate was taken by HCS.

5. Synthesizing Clay with Fluorescein isothiocyanate (FITC)

FITC labelled clay nanoparticles were synthesized to study the in vitro and in vivo distribution of nanoparticles. 10mg of halloysite clay nanoparticles were weighed and was added with 2 mL 5% APTES in water. The solution was mixed on a shaker for 30 minutes. After that the nanoparticles were centrifuged three times (once on its own and twice by DI water) at 20000 rpm for 5 minutes and 30 seconds. Two mL of 0.1mg/ml FITC was added to each of the nanoparticles and shaken overnight. The excess FITC was then removed by

washing the nanoparticles solution three times (once on its own and twice by DI water) at 20000 rpm for 5 minutes.

6. Testing the ability of Clay-FITC to bind to BF2 cells

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A 96-well black well plate was seeded with BF2 cells using MEM as the diluent. The cells were incubated overnight. The next day a working solution of clay-FITC was prepared to a dilution of 5mg/ml to 200ug/ml using MEM as the diluent. From the BF-2 cell plate, spent medium was then replaced with the various clay-FITC diluted medium and incubated overnight again. On the third day, Hoechst reagent in PBS was prepared and the spent medium was replaced with this solution. The images of the cell were then taken by the high content screening instrument.

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7. Imaging of Zebra fish embryos with Clay-FITC

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Healthy zebrafish embryos were seeded in 96-well black plate and were incubated at 28°C for 24 hours post fertilization. The medium surrounding the eggs was replaced with Holtfreter's medium containing 100ug/ml concentration solution of the FITC labelled Clay nanoparticle, Clay-nanoparticle alone or Holtfreter's medium alone and incubated for 3h. The eggs were then washed and then imaged using high content screening.

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A total of 12 newly hatched zebrafish was placed in a 96-well blackplate in egg wash medium. The egg wash medium was then replaced with 100ug/ml of Clay-FITC in 5% APTES in Holtfreter's medium. The fish were then exposed for 3h to the nanoparticles. After 3h, the medium from 6 fish were discarded and washed twice with Holtfreter's medium and images were taken by high content screening. The remaining 6 fish were incubated in the nanoparticle solution overnight and then washed and imaged by high content screening.

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8. Extraction of Outer Membrane Protein of TIMA

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From a flask that containing TIMA bacteria grown in AOA broth for 24h, 30mL was transferred into a sterile falcon tube and bacteria was pelleted down at 6000 rpm for 20 minutes and the supernatant was discarded. Tris + DTT solution was added and the bacteria was suspended by pipetting up and down. The tubes were then centrifuged at 6000 rpm for 20 minutes and supernatant was removed. The bacteria pellet was suspended in SDS sample solution (1% (w/v) SDS, 0.375M Tris-HCl (pH8.8), 50nm DTT, and 25% (v/v) glycerol). The bacteria mixture was boiled 100°C for 5mins and then transferred into a fresh

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falcon tube. To this mix, sonication was done 6 times (10 seconds each time) and then the tube was centrifuged at 6000 rpm for 10 minutes and the supernatant was harvested and kept in a -80°C freezer till further use. For short term storage, the supernatant may be stored at -20°C. The protein content in the extract was analyzed by Bradford assay.

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The protein profile of outer membrane protein extracted from TIMA was assessed by resolving on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Prior to loading, samples were thawed and mixed with 2% SDS solubilisation buffer. Each sample was heated in a water bath at 100°C for 10 mins and then centrifuged at 16000 g for 5 mins before aliquoting the supernatant to load into gel. The SDS-PAGE was run at 90V for the initial 20 minutes and subsequently run at 120V for 1 hour and 20 minutes. The gel was stained using Coomassie blue.

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Figure 5 shows the SDS-PAGE of OMP of *Tenacibaculum maritimum* stained with Coomassie Blue R. Greyscale analysis of gels was used to establish the contrast. M- Marker, R- Reference, OMP- OMP extracted. It was observed that a high degree of match between protein profile of OMP extract and that of extracellular components reported in literature, suggesting that the present protocol could extract the major OMPs to be used as antigen.

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9. Loading TIMA outer membrane proteins (OMP) in chitosan clay nanoparticle

The suspension containing OMP proteins of TIMA extracted were aliquoted into Eppendorf tubes such that the total protein is 100 µg/mL. The protein suspension subjected to freeze-drying overnight to ensure all excess water had completely evaporated. The CNC nanoparticle suspension in deionized water was aliquoted into these tubes so that the concentration will be 100 µg/mL in the final solution. The suspension was sonicated and subjected to vacuum loading. Adequate amount of water was added and sonicated to get the protein loaded into the CC nanoparticle.

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10. Exposing fish fry to vaccine preparations

Figures 6A and 6B show the immersion vaccination of the test fish carried out in various beakers.

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Six weeks old barramundi (seabass) fish fries were selected for treating with the developed vaccine composition. Sea bass fries were acclimated to fresh water by gradually reducing the salt water content. Ten fish fry (barramundi fingerlings) (>10g) were acclimatized in fresh water were placed in a beaker containing 300 mL of water. Outer membrane protein isolated from *Tenacibaculum maritimum* – the bacterial pathogen causing tenacibaculosis in seabass. Clay and clay-chitosan nanoparticles with or without the loaded antigen, OMP that is directly added to the water and formaldehyde inactivated bacteria were used for the tests.

To this water, 40mg of CNC nanoparticles loaded with TIMA OMP was added. Control experiments were also performed, OMP alone (20 mg) (i.e. OMP in water alone), CNC nanomaterial alone (40 mg), TIMA vaccine (1 mL of OD 10). The following is a description of the various beakers shown in Figure 6:

1. Inactivated bacteria
2. OMP proteins alone
3. Clay nanoparticle loaded with OMP
4. Nanoclay alone
5. CNC loaded with OMP
6. Clay chitosan (CC) alone
7. Control containing PBS only

Aeration was accomplished by bubbling air into the water to help keep the nanomaterials suspended in the water and the fish were kept in the treatment tanks for 18-24h. After the treatment, the fish were transferred into aerated fish tanks containing fresh water.

With reference to Figure 7, bacterial agglutination tests with fish serum were carried out. Six weeks old seabass fry were subjected to vaccine made from formalin inactivated *Tenacibaculum maritimum* (TIMA), TIMA outer membrane protein (OMP), clay-chitosan nanomaterials (CC), and nanomaterials loaded with OMP (CC-OMP). Blood from euthanized fish were collected seven days post treatment. The separated serum was diluted in a 96 well plated and to each well a fixed amount of inactivated TIMA bacteria was added followed by incubation at 30°C for 24h. The level of agglutination in the wells were recorded and shown in Table Y. Results showed that fish treated with clay (C)+OMP and clay-chitosan (CC)+OMP showed the highest antibody titer (1:640) which is comparable to that produced in fish treated with the TIMA bacterial pathogen. Comparing clay and clay-chitosan to the C+OMP and CC+OMP, the antibody titers were 2-4 times higher, indicating a significant stimulation of the fish immune system to the new vaccine formulation.

11. RNA extraction and RT-PCR

RNA from the excised tissues (gill, liver, kidney and spleen) was extracted using TRIzol reagent. The lysate with TRIzol Reagent were incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes and was then transferred into a Eppendorf tube. Chloroform (500µl) was added and mixed vigorously by hand for 15 seconds. The tube was then incubated at room temperature for 2-3 minutes and was then into a fresh clean Eppendorf tube. The tubes were centrifuged at 12 000g for 15 minutes (4°C) and the colourless, upper phase containing the RNA (400µl) was transferred into an RNase-free Eppendorf tube. An equal amount of 70% Ethanol (400µl) was added to dilute down the concentration to 35%. The tube was then mixed by inverting to disperse any visible precipitate that may form after adding Ethanol. This mix was further subjected to purification on spin-cartridge and subjected to DNase to remove DNA contamination. The purity of RNA was ensured by checking the Absorbance ratio at 260/280 nm.

For RT-PCR, a standard 20µl PCR reaction size was set up in ice as detailed elsewhere. Each PCR tube/plate were capped/sealed and gently mixed and briefly centrifuged to keep the solution at the tip of the Eppendorf tube and then placed into the Real-Time PCR instrument (ABI). RT-PCR data was then collected and analysed.

RESULTS AND DISCUSSION

1. Physico-chemical characterization of nanomaterials to be used as the delivery vehicle

Nanoparticles of Clay and Clay-Chitosan were observed under FESEM which showed the tubular morphology of Halloysite clay nanoparticles. The stock solution prepared in deionised water was aliquoted and diluted, and a drop was dried on a clean Scanning Electron Microscope (SEM) grid. These are shown in Figure 1. Figure 1A shows clay while Figure 1B shows clay-chitosan. The tubular morphology of halloysite clay particles are evident. The tubular morphology of halloysite clay particles are evident, where the length of chitosan modified clay nanoparticle was lower than the original clay particles. The zeta-potential measurement of particles suspended in deionized water showed change in the negative surface charge of clay to a positive surface charge in chitosan modified clay nanoparticle (tabular data in Figure 1C). The evidence of chitosan modification is evident

from the appearance of peaks corresponding to NH stretch in the FTIR spectrum (Figure 1D).

The internal dimension of clay nanoparticles varied from 60-120 nm and the length of tubes varied from 300-2000 nm. The chitosan modified clay nanoparticles were shorter in length compared to the unmodified clay nanoparticles and had a slightly bigger external diameter.

The analysis of hydrodynamic diameter (HDD) and zeta-potential also suggested the successful modification of clay nanoparticles by chitosan. The chitosan modified clay nanoparticles were positively charged and the overall HDD also increased.

The peak at $\sim 1030\text{ cm}^{-1}$ is assigned to the stretching mode of apical Si-O and the band at $\sim 900\text{ cm}^{-1}$ is caused by the stretching vibrations of Si-O-Si. The band observed at $\sim 540\text{ cm}^{-1}$ is the deformation vibration of Al-O-Si. The deformation vibration of the inner-surface hydroxyl (OH) groups at $\sim 900\text{ cm}^{-1}$ and Si-O-Si at $\sim 460\text{ cm}^{-1}$ can further confirm the existence of corresponding group.

Based on the spectrum for Clay-Chitosan, the peaks were similar to the peaks in the spectrum of clay. However, due to the presence of chitosan, the peak at $\sim 1600\text{ cm}^{-1}$ indicates the NH bend for NH_2 due to the presence of the NH group and the doublet peaks at ~ 3400 to 3600 cm^{-1} indicates the NH stretch for NH_2 . Also, in the Clay-Chitosan and Clay-APTES spectrum it showed amplified peaks such as the C-H, C=N and Si-O-Si. This is further proves that APTES and Chitosan had successfully attached to the surface of Clay (Halloysite).

2. Cytotoxicity and zebrafish embryo mortality

With reference to Figure 2, blue gill fry (BF2) cells cultured in 96 well plates were subjected to increasing concentration of nanoparticles prepared in MEM medium. The cell viability was measured using resazurin assay. The result was normalized to negative control. Silver nanoparticles were used as a positive control. Similarly, zebrafish embryos at 1000 cell stage were subjected to $100\text{ }\mu\text{g/mL}$ of nanoparticles and the mortality rate was recorded.

Data showed that except for silver, none of the tested nanomaterials induced a significant cytotoxic effect. Although the bare nanoclay showed $\sim 20\%$ loss in cell viability at the highest tested dose, surface modification with chitosan improved its biocompatibility. Similar

observations were observed in zebrafish embryos where none of the tested nanomaterials, except for silver were toxic.

3. Delivery of Green fluorescent protein (GFP)

The efficiency of different nanomaterials in delivering green fluorescent protein (GFP) to BF2 cell line was assessed. In this experiment, 100 µg of nanomaterials were loaded with GFP using vacuum assisted loading which were washed to remove the surface associated GFP. The cells grown in 96 wells were exposed to GFP loaded in various nanomaterials. After 3 hours incubation, the cells were washed with PBS for three times and were imaged using high content screening. Cells treated with nanomaterials alone served as the control. The images were analysed using a software to assess the efficiency of the protein delivery. Figure 3A show fluorescence microscopic images of BF2 cells with/without GFP delivery using different nanomaterials. The nucleus is stained with Hoechst 33342 and presence of GFP in cytoplasm is seen as green fluorescence. The average fluorescent intensity was calculated and the quantitative fluorescence intensity is plotted as shown in Figure 3B. The fluorescence intensity from cells treated with nanoparticle without GFP loading was subtracted in order to remove the background noise. Our data showed that GFP delivery is highest using chitosan coated clay nanoparticles.

Results showed that the cells treated with clay-chitosan produced highest level of fluorescence intensity suggesting that among the nanoparticles tested, this is the most effective one in delivering GFP to cells. There was no green fluorescence from cells treated with GFP alone which showed little uptake of protein when applied directly to the cells and that the increased fluorescence observed in cells treated with nanoparticle loaded GFP is because of the cellular entry and delivery of GFP by nanoparticles.

4. Fate of nanoparticle in cells and zebrafish embryo

In order to determine if the clay nanoparticles are taken up into cells and in to various organs of fish, clay nanoparticles coupled with FITC (green fluorescence) were used. The results of which are shown in Figure 4A to 4C. BF2 cells seeded in 96 well plates were exposed to 100 µg/mL of FITC labelled clay nanoparticles. The media containing nanoparticles was removed and cells were washed with PBS three times. Hoechst 33342 in PBS was used to counter stain the cell nucleolus. Fluorescence images using the blue and green channels were captured using epifluorescence microscopy. The presence of nanomaterials in fish cells was demonstrated by the presence of FITC- green signals (see Figure 4A).

FITC coupled clay nanoparticles were also tested in zebrafish embryos and larvae to determine its uptake and tissue distribution. The zebrafish embryos/larvae were exposed to 100 µg/mL of FITC labelled clay nanoparticles for 3 hours in Holtfreter's medium. After the treatment, the embryo and larvae were washed with PBS and the images were taken using epifluorescence microscopy. The images showed that at the embryo stage, the nanoparticles were sticking to the chorionic membrane (see Figure 4B). The larger image showed fluorescent signals binding tightly to the chorionic membrane, whereas the hatched embryo is devoid of any fluorescence signal. Image of zebrafish embryo in the inlet shows the fluorescence signals confined to the exterior surface of embryo. However, when the fish larvae are treated with nanoparticles, fluoresce signals could be detected in areas of the skin, gill, mouth, abdomen and intestine of the fish (see Figure 4C).

The assessment of localization of clay nanoparticles in cellular compartments showed that clay particles could be internalized into fish cells. In the case of zebrafish embryos, nanoparticles are stuck to the chorionic membrane. The image showed fluorescent signals on chorionic membrane where the hatched embryo is devoid of any fluorescence signal. However, when the fish larvae are treated with nanoparticles, fluoresce signal can be detected in areas of skin, gill, mouth, abdomen and intestine. These data suggested that nanoparticles could gain entry to fish tissue via gill, skin and digestive tract. While, the presence of nanoparticles in these tissues can be seen via imaging, more studies are warranted in understanding the long term persistence of nanoparticles in these tissues.

5. Efficiency of vaccination

The serum from fish exposed to different vaccine modalities were separated and tested for its efficiency to cause agglutination of bacterial cells. The degree of agglutination of bacterial cells could be correlated to the level of antibody produced in fish. It was noticed that fish treated with clay-chitosan and clay loaded with OMP elicited a strong antibody response, antibody titre of 1:640, which is comparable to the response observed using the whole pathogen, TIMA. The data is shown in Figure 7. Interestingly, clay-chitosan (CC) alone also managed to induce a significant increase in antibody response in fish compared to clay alone. Thus, the clay-chitosan nanoparticle may be inferred to have an adjuvant effect.

These data highly suggest that nanoparticles of either clay+OMP (C+OMP) or clay-chitosan + OMP (CC+OMP), could mimic bacterial pathogens and could elicit a proper immune response in fish <10g.

The RT-PCR results shown in Figure 8 also confirmed that superior immune response was elicited when the fish were treated with OMP loaded nanoparticles. Six weeks old sea bass fries were subjected to inactivated TIMA bacteria (TIMA), outer membrane protein (OMP), clay-chitosan nanomaterials (CC), and nanomaterials loaded with OMP (CC-OMP). Different tissues from euthanized fish were collected seven days post treatment and the RNA was extracted for RT-PCR. Clay chitosan loaded with OMP showed significant increase in the expression level of immunoglobulin heavy chain (IGH) and complement component 1q subcomponent-like 4 like (CLQL) in gills, spleen, and liver, thus, suggesting an antigen specific response in these organs. Heat inactivated bacteria showed a significant increase in expression level of CLQL in these tissues while OMP alone showed significant HepC response in kidney and spleen + liver tissues. Based on the results obtained from the experiments, we are able to conclude that Clay NP and Clay-Chitosan are both effective as nanomaterials for fish vaccine delivery via the immersion (dipping) route of administration. However, comparing the nanoclay (NC) to Clay-Chitosan NP, the latter is a more effective nanomaterial for drug delivery. The reason for choosing Chitosan the outer layer coating is because it exerts a positive surface charge unlike the Clay NP's surface negative charge, thus allowing the clay-chitosan NP to bind more strongly to mucosal surfaces such as the gills, skin and gut of the fish. In doing so, this newly developed modified nanomaterial is superior as a carrier for antigens for use in immunising fish or other aquatic organisms.

Whilst there has been described in the foregoing description preferred embodiments of the present invention, it will be understood by those skilled in the technology concerned that many variations or modifications in details of design or construction may be made without departing from the present invention.

CLAIMS

1. A drug delivery composition for delivering a therapeutic amount of an active
5 ingredient to an aquatic animal, the composition comprising:
 - (a) a nanoparticle; and
 - (b) the active ingredient contained in said nanoparticle.
2. The composition according to claim 1, wherein the nanoparticle is a nanoclay and the
10 surface of the nanoclay is modified.
3. The composition according to claim 2, wherein the surface of the nanoclay is
modified with aminopropyltriethoxysilane.
- 15 4. The composition according to any one of the preceding claims, wherein the nanoclay
is coated with chitosan.
5. The composition according to claim 3, wherein the amount of chitosan coated and
present in the composition is about 10% wt.
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6. The composition according to any one of the preceding claims, wherein the nanoclay
is halloysite nanoclay.
7. The composition according to any one of the preceding claims, wherein the amount
25 of nanoparticle present in the composition is about 10 to 100 mg/L.
8. The composition according to any one of the preceding claims, wherein the active
ingredient is any one selected from the group comprising: an immunogenic antigen, a
hormone, a nutrient, a mineral, an antibiotic and a therapeutic agent.
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9. The composition according to claim 8, wherein the immunogenic antigen is any
antigen obtained from the group comprising: a virus, a bacteria and a parasite.
10. The composition according to claim 9, wherein the antigen obtained from a bacteria
35 is an outer membrane protein of the bacteria.

11. The composition according to claim 10, wherein the amount of the outer membrane protein present in the composition is about 1 to 100 mg/L.

12. The composition according to any one of claims 8 to 11, wherein the active ingredient comprises a plurality of immunogenic antigens.

13. The composition according to any one of the preceding claims, wherein the aquatic animal is a fish having a weight of less than 10g.

14. A method for preparing a drug delivery composition for delivering a therapeutic amount of an active ingredient to an aquatic animal, the method comprising:

- (a) providing a nanoparticle;
- (b) modifying the surface of the nanoparticle;
- (c) coating the surface of the nanoparticle with a polymer; and
- (d) loading the active ingredient into the nanoparticle.

15. The method according to claim 14, wherein the nanoparticle is a nanoclay and the surface of the nanoclay is modified with aminopropyltriethoxysilane.

16. The method according to any one of claims 14 or 15, wherein the polymer is chitosan.

17. The method according to any one of claims 14 to 16, wherein the nanoclay is halloysite nanoclay and present in the composition in the amount of about 10 to 100 mg/L.

18. The method according to any one of claims 14 or 17, wherein the loading is carried out by any one of the method comprising: freeze drying, vacuum drying and ultrasonication.

19. The method according to any one of claims 14 to 18, wherein the active ingredient is any one selected from the group comprising: an immunogenic antigen, a hormone, a nutrient, a mineral, an antibiotic and a therapeutic agent.

20. The method according to claim 19, wherein the immunogenic antigen is any antigen obtained from the group comprising: a virus, a bacteria and a parasite.

21. The method according to claim 20, wherein the antigen obtained from a bacteria is an outer membrane protein of the bacteria, and the step of obtaining the outer membrane protein of the bacteria is carried out prior to loading onto the nanoparticle.
- 5 22. The method according to claim 21, wherein the amount of the outer membrane protein present in the composition is about 1 to 100 mg/L.
23. The method according to any one of claims 14 to 22, wherein the aquatic animal is a fish having a weight of less than 10g.
- 10 24. A method of delivering a drug composition to an aquatic animal or inducing an immune response in an aquatic animal, the method comprising administering to the animal an effective amount of the composition according to claim 1.
- 15 25. The method according to claim 24, wherein the administration is carried out by spraying the composition onto the aquatic animal.
26. The method according to claim 24, wherein administration is carried out by immersing the aquatic animal in a medium containing the dispersed composition.
- 20 27. The method according to any one of claims 24 to 26, wherein the method comprises inducing an immune response in an aquatic animal against a virus, bacteria or parasite.
28. The method according to claim 27, wherein the amount of antigen present in the medium is about 1 to 100 µg/mL.
- 25 29. The method according to claim 28, wherein the amount of the nanoparticle present in the medium is about 10 to 100 µg/mL.
- 30 30. The method according to any one of claims 24 to 29, wherein the aquatic animal is a fish having a weight of less than 10g and administration is carried out in any type of water selected from the group: fresh water, salt water, brackish water and any aqueous environment.