**Nano-polyplex based on oleoyl-carboxymethy-chitosan (OCMCS) and
hyaluronic acid for oral gene vaccine delivery**

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Abstract

Here we described nano-polyplexes (NPs) made of oleoyl-carboxymethy-chitosan (OCMCS)/hyaluronic acid (HA) as novel potential carriers for oral gene vaccines delivery. Aerolysin gene (aerA) of *Aeromonas hydrophila* as microbial antigen was efficiently loaded to form OCMCS-HA/aerA (OHA) NPs. OHA NPs performed the optimal parameters, i.e. smallest (154.5 ± 9.4 nm), positive charged ($+7.9 \pm 0.5$ mV) and monodispersed system with the N/P ratio of 5 and OCMCS/HA weight ratio of 4. Upon the introduction of HA, OHA NPs was beneficial for the DNA release in intestinal environments in comparison to OA NPs. The mean fluorescence intensity detected in Caco-2 cells incubated with OHA NPs was about 2.5-fold higher than that of OA NPs; however, it decreased significantly in the presence of excess free HA. The OHA NPs and OA NPs decreased the transepithelial electric resistance (TEER) of Caco-2 monolayers obviously and induced increasing the apparent permeability coefficient (P_{app}) of DNA by 5.45–6.09 folds compared with free DNA. Significantly higher ($P < 0.05$) antigen-specific antibodies were detected in serum after orally immunized with OHA NPs than that immunized with OA NPs and DNA alone in carps. These results enable the OHA NPs might resolve challenges arising from gastrointestinal damage to gene antigens, and offer a approach applicable for oral vaccination.

Key words: Nano-polyplex, OCMCS, HA, gene vaccine, oral delivery

1、 Introduction

Aeromonas hydrophila is a ubiquitous bacterial pathogen, which caused infection and several diseases like hemorrhagic septicemia, fin and tail rot in fish, especially carps [1, 2]. The pathogenicity of *A. hydrophila* appeared to involve several extracellular products (ECPs) including aerolysin, hemolysin, enterotoxin, acetylcholinesterase, as well as a surface array protein layer [3, 4]. Carp farming has become a key industry in recent decades over the world. Regarding intensive culture system for carp, boosting the immunity system of carp against stressful circumstances was a good alternative [5, 6].

Vaccine strategies were sought-after because of their low toxicity and success in ameliorating serious diseases [7-9]. First generation whole-cell, and second generation acellular, vaccines both have drawbacks such as side effects and lacking durability [10]. The vaccines were also limited in practical application for the intensive fishing industry because of immunization route and bad effectiveness [11, 12]. To date, oral vaccination based on antigen (non-virulent peptides, proteins or DNA) offered some obvious advantages over parenteral injection routes, for example, the non-invasive nature of this approach, ease of administration and low cost [13-15].

Oral delivery of oral vaccination in fish was encountered to the three major barriers, namely the enzymatic barriers sourced from the host luminal and membrane bound enzymes [16, 17], the immunological cells present within both the enterocytes and underlying connective tissue [18, 19], and the physical barrier of the epithelial cells [20, 21]. Many strategies have been developed to enhance oral bioactive compounds delivery [22, 23]. Among these approaches, nanoparticulate systems have attracted special interest because nanoparticles are able to protect active agents from degradation, improve

biomacromolecules transmucosal transport and provide controlled-release properties for loaded bioactive compounds [24-26].

As a cationic biopolymer obtained from N-deacetylation of chitin, chitosan had excellent features due to its non-toxicity, biodegradability, biocompatibility and bioadhesion [27-29]. Having encapsulated active compounds, chitosan was capable of protecting them from harsh conditions into gastrointestinal (GI) tract and enhances their absorption [30, 31]. In our previous researches, oleoyl-carboxymethyl-chitosan (OCMCS) nanoparticles showed abilities for the sustained release of the ECPs, enhancements to mucosal delivery, and greatly improved antibody responses compared with the free ECPs in carps[32]. However, the effectiveness of OCMCS nanosystem as oral gene vaccine vehicles remained unknown.

Herein, we demonstrated nano-polyplexes (NPs) consisting OCMCS/hyaluronic acid (HA) as novel gene vaccines carriers for oral immunization in carps (Scheme 1). HA was incorporated in NPs as a target ligand attribute to its ability to bind various cellular receptors such as CD44, which was expressed in normal human epithelium cells, chondrocytes and cancerous cells [33, 34]. Aerolysin gene (*aerA*) of *A. hydrophila* was chosen as encapsulating antigen to form OHA NPs. The DNA loading efficiency and release profile from OHA NPs was evaluated *in vitro*. The increased intracellular accumulation of NPs in Caco-2 cells was observed by confocal laser microscopy. The intestinal penetration enhancement properties of NPs were investigated through Caco-2 cell model and *ex vivo* carp intestine model. Subsequently, the serum antibody titer in carps orally immunized by OHA NPs was also investigated. We expect the OCMCS/HA NPs interesting candidates to improve immunity-boosting properties of gene vaccine after

oral administration in water body.

2. Materials and methods

2.1 Materials

Chitosan (Mw = 50 kDa, degree of deacetylation = 93.15%) was made from crab shell and obtained from Laizhou Haili Biological Product Co., Ltd. (Shandong China). Hyaluronic acid, with a molecular weight of 5 kDa, was purchased from C.P. Freda Pharmaceutical Co. Ltd. (Shandong, China). Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum. Cell counting kit assay (CCK-8) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Cy3 and YOYO-1 were obtained from Invitrogen (USA).

pcDNA3.1-aerA was amplified and isolated using a Plasmid Mega Kit (QIAGEN, Valencia, CA, USA). The recovered plasmids were stored at 4 °C in sterile deionized (DI) water. The purity of plasmids was analyzed by gel electrophoresis (0.8% agarose), while their concentration was measured by UV absorption at 260 nm (V-530, Jasco, Tokyo, Japan).

Common carps weighing 200 ± 10 g were used for the experiments. The animal protocol was approved by Shandong Medical Laboratorial Animal Administration Committee. The animal experiment was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996.

2.2 Preparation of the nano-polyplex (NPs): OCMCS /aerA (OA) NPs and

OCMCS-HA/aerA (OHA) NPs

Oleoyl-carboxymethy-chitosan (OCMCS) was synthesized by reaction of chitosan with chloroacetic acid and oleoyl chloride as described in our previous study [35, 36]. Solutions of OCMCS at different concentrations were prepared in phosphate buffer saline (PBS, pH 6.0) in order to achieve N/P ratios of 0.5, 1, 2, 5, 10 and 20 (an amino group to a phosphate group ratio hereafter was defined as charge or N/P ratio). A plasmid DNA solution of 50 µg/ml in 25 mM of sodium sulfate was prepared. An equal volume of chitosan solution (from each concentration) and DNA solution were added (by dropping slowly) together while stirred at various speed (100 – 2000 rpm). The final volume of the mixture in each preparation was limited to below 500 µl in order to yield uniform OA NPs.

OHA NPs were prepared according to the method described by Duceppe and Tabrizian [37]. In brief, 5 mg HA was dissolved in 2 ml of distilled water under magnetic stirring. The OCMCS solution was stirred at a rate of 5000 rpm for 10 min, mixed into the HA solution and stirred for 10 min. Seven different mixtures were prepared with CS:HA weight ratios at 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, and 7:1. The required volume of 50 µg/ml plasmid DNA was gently added to OCMCS/HA solution to form OHA NPs of a selected N/P ratio. The mixture was vortexed rapidly for 3 – 5 s and left for 1 h at room temperature for the OHA NPs to completely form.

2.3 Size and zeta potential measurement

The Z-average hydrodynamic diameter and surface charge of NPs were determined by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at room temperature. The NPs were prepared and analyzed in 1 ml distilled water at

25 °C. All samples were measured in triplicate. The morphological characterizations of the NPs were viewed using scanning electron microscopy (SEM, JSM-330A, Japan) and transmission electron microscopy (TEM, JEM-2010, Japan).

2.4 Gel retarding analysis

OHA NPs were evaluated by agarose gel electrophoresis. The NPs and the naked plasmid were loaded onto a 1% agarose gel containing ethidium bromide in Tris–borate EDTA buffer at pH 8.0. The samples were run on the gel at 120 V for 30 min. The gel was then photographed using a GDS-8000 (UVP, USA).

OHA NPs (N/P ratio 5) prepared with OCMCS: HA weight ratios of 4:1 and naked DNA solution (each containing 1 µg of DNA) were added to 10 U of *Bam*HI and 15 U *Hind*III (Sigma–Aldrich, USA) and kept in a water bath for 30 min at 37 °C. In addition, different concentrations (56–336 ng/µl) of chitosanase (from streptomyces species, activity 18 units/mg, Sigma–Aldrich, USA) were added and the NP–chitosanase solution was maintained in a water bath for 4 h at 37 °C. Agarose gel (1%) electrophoresis was repeated, as described above.

2.5 DNA loading and releasing

The OA NPs and OHA NPs (N/P ratio 5 and OCMCS/HA weight ratio 4) were formed according to the method described in Section 2.2. After complex was completely formed at ambient temperature for 30 min, the turbid solution was centrifuged at 20,000×g at 4 °C for 30 min and the supernatant was collected to determine the amount of free DNA. The loading efficiency (LE %) of DNA was calculated by Eq. (1), and the analyses were performed in triplicates.

$$\text{LE\% of DNA} = \left[\frac{A - B}{A} \right] \times 100 \quad (1)$$

Where A was the total concentration of DNA (mg/ml) and B was the concentration of unloaded DNA ($\mu\text{g/ml}$).

The digestive tract of carps was undeveloped and could be divided into buccopharyngeal cavity, esophagus, stomach and intestine (foregut, midgut, and hindgut) [38, 39]. The stomach of carp was immature with weak acidic pH condition around pH 6.0, while the intestine segments were suitable for oral vaccines due to its neutral environment and decreased enzymatic activity [40]. The nano-polyplex were introduced into two different kinds of simulated fluid and kept in a water bath at 25 °C. Acetate buffer (pH 6.0, 0-20 h) and PBS buffer (pH 7.4, 20-50 h) were used as media for the DNA release study, respectively. Dried OA NPs and OHA NPs (3.9 mg) and SDF (1.2 ml) were placed in a micro tube and incubated in a water bath at 25 ± 0.5 °C, respectively. At the sampling time, the incubated dispersing solution was centrifuged, and 400 μl of supernatant was collected for evaluation of the amount of released DNA using a spectrophotometer at 258 nm. An equal volume of fresh buffer was, then, replaced in the solution and the same procedure was repeated for the next sampling time. The accumulative release percentage (Q %) was calculated by Eq. (2), and the analyses were performed in triplicates.

$$Q\% = \frac{C_n V + V_i \sum_{i=0}^{n-1}}{DNA_t \times LE\%} \times 100 \quad (2)$$

where C_n was the DNA concentration at T_n , V was the total volume of release medium, V_i was the sampling volume at T_i , C_i was the DNA concentration at T_i (both V_0 and C_0 were equal to zero), DNA_t was the total amount of DNA used for preparation of the original mixture, $LE\%$ was the loading efficiency of DNA loaded into the nanoparticulate

systems.

2.6 Cytotoxicity assay

2.6.1 Cell culture

Caco-2 cells were cultured in regular growth medium consisting of Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin, and kept in an atmosphere of 5% CO₂ and 95% air at 37 °C.

2.6.2 Evaluation of cytotoxicity

Caco-2 cells were seeded in 96-well plates at 5×10^4 cells/ml in 100 µl of complete DMEM medium, and incubated at 37 °C in 5% CO₂ humidified atmosphere for 24 h. The culture medium was then replaced with 100 µl of freshly prepared culture medium containing OA or OHA NPs. After 24 h incubation, the cell viability was investigated by the CCK-8 assay. Meanwhile, the cytotoxicity of the NPs (200 µg/ml) at different pH values (6.0 and 7.4) was also tested after 24 h of incubation time with Caco-2 cells.

2.7 In vitro cellular uptake of nano-polyplex

Caco-2 cells in DMEM medium were seeded on glass-bottomed dishes (35 mm, kindly provided by MatTek Corporation) for 24 h to form a confluent monolayer. Then the culture medium was replaced by serum-free medium and preincubated at 37 °C for 30 min. After equilibration, the culture medium was changed with 1 ml of freshly prepared Cy3-labeled OHA NPs suspension (100 µg/ml) and incubated at 37 °C for 2 h. To investigate influence of HA on cellular internalization of NPs, free HA as a CD44 receptor blocking agent at concentrations 10 times greater than the HA content of OHA NPs was added to the cells and incubated for 20 min at 37 °C. The experiment was

terminated by washing the cell monolayer three times with PBS and stained with 10 µg/ml Hoechst 33342 for 10 min. The glass-bottomed dishes were viewed by confocal laser scanning microscope (LSM 710 METS, Carl Zeiss Inc.). Cy3-labeled OA NPs performed as a control group. We used MetaXpress and Image J software to quantitatively express intracellular Cy3 fluorescence in the different treatment groups.

2.8 Enhanced permeation measurements *in vitro*

2.8.1 Excised intestinal tissues permeation measurements

The nano-polyplexes, suspended in Hank's Balanced Salt Solution (HBSS) buffered at pH 6.5, were subjected to permeation measurement by means of Franz diffusion cells with a 2 cm² orifice area thermostated at 25 °C. The segments of carp intestine laying on a filter paper, was placed between the donor and the acceptor chambers of the Franz diffusion cell. The receptor chamber of the cell was filled with 25 °C phosphate buffer (pH 6.8). YOYO-1 was used to label the plasmid DNA. 500 µl of YOYO-1-labeled OHA or OA NPs in phosphate buffer (pH 6.0) at a concentration of 2 mg/ml were placed on the excised carp intestine at 25 °C and protected from light for 3 h. At fixed time intervals, 500 µl samples of the acceptor phase were withdrawn and replaced with fresh buffer. YOYO-1 labeled DNA was assayed by means of spectrofluorimetric detection. As control, the permeation test of YOYO-1 labeled free DNA solution in phosphate buffer (pH 6.0), at the same concentration of nanoparticulate systems was performed. The apparent permeability coefficient (P_{app}) was calculated using the following Eq. (3):

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A} \cdot C_0 \cdot 60 \quad (3)$$

Where P_{app} was the apparent permeability (cm/s), dQ/dt was the permeability rate, A was the diffusion area of the membrane (cm²), and C_0 was the initial concentration of the

DNA.

2.8.2 Caco-2 cell monolayer permeability studies

Caco-2 cells, with a passage number 25 – 30, were cultured on polycarbonate membrane filters (pore size 0.3 μm , area 0.33 cm^2) in Corning Transwell 24-well plates (Corning Incorporated, Europe) at a seeding density of 3×10^4 cells/ml. The culture medium was added to both the apical and the basolateral compartments and was changed every second day for 21 days. Afterwards, the cells were post-fixed with 1% osmium tetroxide in 0.1 M NaCac buffer (pH 7.4). The cells were then dehydrated and embedded in epoxy resin. Ultrathin sections (60 nm) were cut parallel to the coverslip, post-stained with uranyl acetate and lead citrate, and examined with a TEM FEI Tecnai 10 (Philips) at 120 kV.

The transepithelial electrical resistance (TEER) across cell monolayers was monitored by means of a volt ohmmeter with a pair of chopstick electrodes (Millipore Corp. Bedford, MA, USA) during cell culture to investigate the integrity of the prepared monolayer (Sadeghi et al., 2008). Only monolayers with TEER values higher than 450 $\Omega \text{ cm}^2$ were used in the permeation study. For the transport research, YOYO-1 labeled DNA was used in free form and in the form of NPs suspensions. Consequently, 2.0 ml of 0.25 mg/ml final concentration of free DNA and 0.25 mg/ml NPs were added to the apical side of the cell culture dish. The transport experiments were performed in HBSS at pH 6.5 (apical compartment) at 37 °C using an orbital shaker (50 rpm). The solution in the basolateral compartment was kept at pH 7.4 in all the experiments. Samples of 200 μl were withdrawn from the basolateral part at predetermined time of 15, 30, 45, 60, 120 and 240 min and replaced with equal volumes of fresh HBSS. The samples were

analyzed for the YOYO-1 labeled DNA content using a fluorescence microplate reader (Polarstar, Germany). The P_{app} was calculated according to the Eq. (3). After 2 h, the samples were carefully removed from the apical part and the cell monolayers were rinsed and replaced with fresh culture medium for evaluation of TEER recovery. The monolayers were allowed to incubate for 24 h at 37 °C in regular cell culture conditions. The TEER was monitored at predetermined time intervals of 0, 0.5, 1, 2, 4, 8 and 24 h. The experiments were done in triplicate.

2.9 In vivo transfection assay

2.9.1 Transfection study of nano-polyplex in vivo

In vivo nano-polyplex transfection was evaluated by measuring the serum antibody titres after oral administration in carps. Carps (n = 4) were immunized orally with OHA NPs (N/P ratio of 5 and OCMCS: HA weight ratio of 4:1), 500 µl of OA NPs (N/P ratio of 5) or free pCDNA3.1-aerA DNA concentration 5 µg/carp by using a feed syringe. Immunization was repeated three times at 2-week intervals. Sera were collected 2 weeks after the primary, secondary and last immunization, respectively, for antibody assay. Furthermore, an additional set of carp (n = 4) were acted as non immunized control. Sera from non-immunized control carp was also used for antibody assay.

2.9.2 Collection of antigen and ELISA study

Aerolysin of *A. hydrophila* were chosen as antigen and purified on the basis of the method of Rodriguez et al[41]. Protein content of aerolysin was assayed spectrometrically at 595 nm using the Bradford protein assay. Specific antibodies against *A. hydrophila* were determined by enzyme-linked immunosorbent assay (ELISA)[42]. Antibody titres were expressed as the log₂ of reciprocal of endpoint dilution exhibiting

A₄₅₀ more than three times that of background.

2.9.3 Histopathology assays

The toxicity of the nano-polyplex was evaluated *in vivo*. Carp (n = 4) were reared in 10 L plastic aquaria that were filled with dechlorinated tap water (water temperature 25 °C) and aerated. Animals were randomly divided into four groups: the experimental groups were fed orally with OHA NPs, OA NPs and free DNA solution (DNA concentration 10 µg per carp) by using a catheter for 7 days; Carps orally treated with PBS acted as a control group. Animals were observed carefully for the onset of any signs of toxicity. After sacrificed, internal organs of each animal were harvested and observed grossly. For histological examinations, the kidney, liver, spleen, and intestine were collected from each animal. Tissues were fixed with formalin, and tissue sections cut and processed using routine histological methods in an automated system. Sections of 5 µm were prepared after paraffin embedding. The slides obtained were stained with routine hematoxylin and eosin staining method in an automated stainer.

2.10 Statistical analysis

All experiments were repeated at least three times. All data were presented as mean $\pm$ standard deviation ($\pm$ SD). A two-way analysis of variance (ANOVA) for multiple comparisons, followed by post-hoc test was used to analysis the data. A *P* value of less than 0.05 was considered statistically significant. Calculations were done using the software Origin 8.0.

3. Results and discussion

3.1. Physicochemical characterization of nano-polyplex

The *nano-polyplex* was formed as a result of complex coacervation. The size of

nano-polyplex was important for cellular uptake and could be affected by the N/P ratio and OCMCS: HA weight ratio as demonstrated in Fig. 1. The effect of charge ratio on the particle size and surface charge was examined at N/P ratios of 0.5–20. As shown in Fig. 1A, OHA NPs with N/P ratios of 5 had the smallest sizes (154.5 ± 9.4 nm). The zeta potential of OHA NPs increased slightly with the N/P ratio (ranging from -2.4 to $+15.8$ mV). At N/P ratio below 1, OCMCS was unable to condense DNA which resulted in negative zeta potential. The increase of surface charge with N/P ratio over 1 could be result from the exclusion of redundant positive charge provided by OCMCS inside NPs.

The influence of the OCMCS/HA ratio (w/w) on the size of NPs was shown in Fig. 1B. The size and zeta potential of the NPs were dependent on the composition, with an increase in size and a decrease in the surface charge with increasing HA content. The NPs showed the smallest particle size (167.9 ± 11.3 nm) with OCMCS/HA weight ratio of 4. At OCMCS/HA weight ratio below 4, significant decreases turned up in particle sizes with increasing ratios, however, it only varied slightly with N/P ratio over 4. The zeta potential becomes more positive as the amount of OCMCS within the polyelectrolyte complex increases (from $+7.1$ to $+16.3$ mV).

The surface charge should be considered when the complex was introduced into the body. Although positively charged NPs could be attached to the cell membrane and enter the cell more easily, NPs with high positive charge should be avoided because it might cause toxic side effects on cells, nonspecific interactions between complex and cells, and also exchange reactions of DNA with negatively charged macromolecules existing in the body during delivery. The experimental conditions were maintained for further analyses, because the OHA NPs with N/P ratio 5 and OCMCS/HA ratio (w/w) 4 presented the

desired features in terms of size and zeta potential.

The morphological characterizations of the OHA NPs (N/P = 5, OCMCS/HA = 4) were evaluated by SEM (Fig. 2A) and TEM (Fig. 2B). The NPs had similar nanometric dimension and showed round shaped particles with a size around 150 nm, which was in accordance with the diameter obtained by DLS.

3.2. Integrity of the associated DNA

Integrity of the associated plasmid DNA and protection against nuclease degradation by nanoparticles were determined by agarose gel electrophoresis. Gel electrophoresis indicated when the N/P ratio was above 1, the DNA was totally retained within the gel loading well, which illustrated the complete coalesce of plasmid with OCMCS (Fig. 2C).

As revealed in Fig. 2D, NPs with OCMCS-HA protected DNA from degradation by *Bam*HI and *Hind*III (lane 5) while naked DNA was completely degraded (lanes 2 and 3). The integrity of the associated DNA was confirmed after degradation of the NPs with chitosanase (lane 4). The NPs with OCMCS-HA (lane 7) protected the DNA against degradation when chitosanase was added following *Bam*HI and *Hind*III. On the contrary, DNA was completely degraded (lane 6) when chitosanase was added previously. The results indicated that OCMCS-HA was capable to load DNA efficiently and preserve its integrity.

3.3. DNA loading and release in vitro

The LE of DNA into the complex was calculated based on the total amount of DNA added in the NPs preparation step. The HA introduced into the NPs did not interfere with DNA loading, and the LE of DNA in OA NPs and OHA NPs was $96.23 \pm 5.22\%$ and $92.31 \pm 3.21\%$, respectively (Table 1).

The releasing profiles of DNA from the OA NPs and OHA NPs were investigated in different pH environment at 25 ± 0.5 °C. The amount of released DNA was measured by a spectrophotometer at 258 nm. Two kinds of NPs showed similar release tendency in acetate buffer (pH 6.0). There was an initial burst in the first 2 h and then a sustaining release up to around 10% of the total DNA from OA NPs and OHA NPs after incubation for 20 h at pH 6.0 (Fig. 3A). Afterwards, the release medium was replaced to PBS (pH 7.4). It should be pointed out that OA NPs and OHA NPs exhibited quite different releasing profiles within 20 h - 50 h of incubation in SIF. The cumulative release of DNA from OA NPs was about 31% in SIF due to partial amino deprotonation causing the weaker electrostatic interaction between OCMCS and DNA. However, much more release of DNA was observed when OHA NPs were incubated at pH 7.4, and over 77% of cumulative release was detected under the identical conditions. Because of the existence of negative charged HA, the electrostatic interaction between these oppositely charged macromolecules-OCMCS and DNA was seriously weakened, resulting in the fairly fast release of DNA.

The corresponding TEM images of were taken to visualize the morphology of OHA NPs simulating the environment in the GI tract. As shown in Fig. 3B, no significant differences were detected in the sizes of OHA NPs pH 6.0 in SDF. At pH 7.4 in SIF, the structure of OHA NPs had gotten loosened and disintegrated. These results were consistent with *in vitro* DNA release data.

3.4 Cytotoxicity analysis

In order to develop OHA NPs as novel gene delivery carriers for GI system, an assessment of any potential toxic interaction with intestinal epithelia is significant. The

cytotoxicity of OA NPs and OHA NPs to Caco-2 cells was estimated using the CCK-8 assay. As depicted in Fig. 3C, compared with untreated cells, the Caco-2 cells retained more than 90% viability when treated with various concentrations (100 – 1000 µg/ml) of OA NPs or OHA NPs. Meanwhile, blank NPs (1000 µg/ml) exhibited no obviously cytotoxicity for Caco-2 cells at different pH values (6.0 and 7.4) (Fig.3D).

3.5 Cellular uptake assay

To estimate the cellular uptake efficiency of OHA NPs and OA NPs, Caco-2 cells were subjected to confocal laser scanning microscope (CLSM). The fluorescent intensity in Caco-2 cells treated with Cy3 labeled OHA NPs was 2.5-fold higher ($P < 0.05$) than that of Cy3 labeled OA NPs at 4 h observation period (Fig. 4A and 4B). HA was known for its implication in several processes, such as the regeneration of corneal and conjunctival epithelial cells, through an interaction with the CD44 receptor[43]. With the objective of elucidating whether CD44 has been responsible for the receptor-mediated uptake[44], we blocked the receptor by the addition of excess free HA. As expected, the cellular uptake of OA NPs varied negligibly after the receptor blockage. On the contrary, the excess free HA caused a significant decrease ($p < 0.01$) of mean fluorescence intensity when the cells were treated with OHA NPs. Consequently, we concluded that the targeting ligand HA incorporated in OHA NPs was capable to enhance cellular internalization.

3.6 In vitro permeability studies

3.6.1 TEER measurements

The transepithelial electrical resistance (TEER) across Caco-2 monolayers was measured to evaluate the integrity of the Caco-2 cell monolayers used in the permeation

experiments 21 – 28 days after the seeding. The initial TEER values were in the range of $734.7 \pm 52.3 \Omega \text{ cm}^2$ with no monolayer less than $450 \Omega \text{ cm}^2$, indicating integrity of the prepared monolayers, which was able to mimic the intestinal epithelium (Fig. 5A). In order to further explore the integrity of the Caco-2 cell monolayers, the ultrathin sections were detected by TEM. The microvilli (a) and the tight junction (b) were visible in Fig. 5B and Fig. 5C.

The variations in TEER of Caco-2 cell monolayers were measured to evaluate the effect of the different formulations on the paracellular permeation of DNA (Fig.5D). Compared with the group treated with free DNA, a remarkable TEER decrease was observed within the first 30 min, after the apical addition of the OHA NPs or OA NPs suspensions were added to the monolayers. The lowering of the TEER % profiles implicated that the nanoparticulate systems were responsible for the modulation of the cell junction integrity. The possible mechanisms for the NPs facilitating opening of tight junctions seemed to be based on the positive charges of the OCMCS, which interact with the cell membrane resulting in a structural reorganization of tight junction-associated proteins. To evaluate TEER-reversibility, the monolayers were rinsed and allowed to regenerate in fresh medium after 2 h incubation period with the different samples. The cell monolayers displayed a transient and reversible decrease in TEER. Approximately complete recovery of the observed effects was achieved within 24 h, indicating that changes in TEER were not mediated through the damage of tight junctions or permanent changes of membrane function.

3.6.1 Permeation enhancement in vitro

The permeation of YOYO-1 labeled DNA across excised carp intestine and Caco-2

cell monolayers was evaluated by fluorescence microplate reader, respectively. Compared to free DNA, both OHA NPs and OA NPs increased the permeation of DNA as revealed in Fig. 6A. It was found that the apparent permeability coefficient (P_{app}) varied negligibly with incorporation of HA in NPs. The P_{app} values of DNA across excised carp intestine in OHA NPs and OA NPs were 5.45 and 5.87 folds higher than that in the free DNA solution, which were $5.89 \pm 0.27 \times 10^{-7}$ cm/s and $6.35 \pm 0.43 \times 10^{-7}$ cm/s, respectively. For Caco-2 cell monolayers, the P_{app} values of DNA loaded in OHA NPs and OA NPs were 6.82 ± 0.41 cm/s $\times 10^{-7}$ and 7.12 ± 0.39 cm/s $\times 10^{-7}$, respectively, which was significantly higher by 5.82 folds and 6.08 folds as compared to that of free DNA. These findings supported the TEER measurement results and indicated that NPs had proved as an effective penetration enhancer of hydrophilic and/or high molecular weight molecules across the intestinal epithelium even in neutral environments by means of the mechanism (tight junctions opening and widening of paracellular pathway).

3.7 ELISA study

ELISA studies were carried out to examine the effect of oral immunization of carps. Serum antibody titres in carps orally fed with OHA NPs, OA NPs, free DNA or PBS were measured 2 weeks after primary, secondary and third immunization (Fig. 6B). Antibody titers increased significantly 14 days after secondary immunization (28 days after primary immunization) when fish were immunized with OHA NPs. 14 days after the last immunization (42 days after primary immunization), the highest antibody responses were observed. OA NPs treatment group demonstrated significant lower ($P < 0.01$) antibody titres compared with that immunized with OHA NPs and slightly higher than

free DNA. There was no detectable antibody in serum of carps treated with PBS. These data indicated OHA NPs could effectively protect the vaccine from degradation when passing through the fish GI tract and delivered DNA to transport across the intestinal epithelium to induce adaptive immune response. The introduction of HA in OHA NPs undertook intracellular pathways that favored the gene expression.

3.8 Histopathology assays

The systemic toxicity of the nanoparticulate delivery system was investigated by histopathology assays (Fig. 6C). No significant differences in pathological signs were detected between the mice received OHA NPs and the untreated control group. All above results meant that OHA NPs did not cause the unexpected side effects and could be used as safe drug carriers.

4. Conclusions

The present study demonstrated the potential of oral immunization for induction of humoral immune responses in carp. OHA NPs were successfully prepared as novel, nonviral vectors for antigen gene delivery. The optimum N/P ratio was found to be 5 with the ideal OCMCS/HA weight ratio of 4 which resulted in the smallest nanoparticles. Involving HA in OHA NPs was beneficial for the DNA release as well as cellular uptake. As shown in permeation studies on Caco-2 monolayer and freshly collected carp intestinal mucosa, the OHA NPs had a strong permeation-enhancing effect for DNA. Biocompatibility assessment revealed OHA NPs were low toxicity. Oral administration of OHA NPs induced a significant higher antibody response compared to OA NPs under the same conditions. We envision that the nano-polyplex based on oleoyl-carboxymethyl-chitosan (OCMCS) and hyaluronic has the potential to be

developed as a oral gene vaccine delivery platform for protective immunity in carps.

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Table 1: Loading efficiency (LE) and *in vitro* release amount of DNA in OA NPs and OHA NPs (N/P ratio 5 and OCMCS/HA weight ratio 4).

Samples	LE of DNA (%)	Release amount of DNA (%)	
		Acetate buffer	PBS
		(pH 6.0)	(pH 7.4)
OA NPs	96.23 $\pm$ 5.22	8.5 $\pm$ 1.7	31.4 $\pm$ 2.7
OHA NPs	92.31 $\pm$ 3.21	11.4 $\pm$ 1.9	77.4 $\pm$ 2.7

Scheme 1. Illustration of preparation of nano-polyplexes (NPs) consisting of OCMCS/hyaluronic acid (HA) as novel gene vaccines carriers and *in vitro* release of DNA in PBS.

Figure 1. Effect of N/P ratio (A) and OCMCS/HA ratio (w/w) (B) on particle size and zeta potential of OHA NPs.

Figure 2. SEM (A) and TEM (B) imaging of OHA NPs. C) Gel retarding analysis of OHA NPs; Lane 1, DNA ladder; Lane 2, naked DNA; Lanes 3–8, OHA NPs prepared at N/P ratios of 20:1, 10:1, 5:1, 2:1, 1:1 and 1:2, respectively. D) Electrophoresis of OHA NPs to determine the integrity of plasmid; NPs were digested with chitosanase and DNase after synthesis. Lane 1, DNA ladder; Lane 2, naked DNA; Lane 3, naked DNA+ *Bam*HI and *Hind*III; Lane 4, OHA NPs + chitosanase; Lane 5, OHA NPs + *Bam*HI and *Hind*III; Lane 6, OHA NPs + chitosanase+ *Bam*HI and *Hind*III; Lane 7, OHA NPs + *Bam*HI and *Hind*III + chitosanase.

Figure 3. A). *In vitro* releasing profile of DNA at 25 ± 5 °C from OHA NPs and OA NPs in acetate buffer (pH 6.0) and PBS (pH 7.4), respectively. B). TEM images of OHA NPs in acetate buffer (pH 6.0) and PBS (pH 7.4). C) *In vitro* cytotoxicity of OA NPs and OHA NPs on Caco-2 cells at different concentrations from 100µg/ml to 1000µg/ml. D) *In vitro* cytotoxicity of OA NPs and OHA NPs on Caco-2 cells at different pH environments. Data represent the mean $\pm$ SD (n = 6).

Figure 4. A) CLSM images of the internalization of Cy3 labeled OA NPs and OHA NPs in Caco-2 cells after incubation for 2 h in the presence and absence of free HA as an inhibitor. Cells were counter-stained with Hoechst 33,342 (blue) for nuclei. B) Quantitatively express intracellular fluorescence in the different treatment groups. Asterisks (*) or double asterisks (**) denoted statistical significance; *, $P < 0.05$; **, $P < 0.01$.

Figure 5. A) The variation of TEER of Caco-2 cell monolayers during the culture of 21 d. B) and C) TEM images of flat embedded ultrathin sections of Caco-2 polarized cell monolayers (red asterisk: brush border; blue asterisk: tight junction); (D) Effect of free DNA solution, OA NPs or OHA NPs on the TEER values across Caco-2 cell monolayers.

Figure 6. A). Apparent permeability coefficient (P_{app}) of free DNA, OA NPs or OHA NPs through excised carp intestine and Caco-2 monolayer. B). Serum antibody titers in carps orally immunized with free DNA, OA NPs or OHA NPs. Data represented the mean $\pm$ SD ($n = 4$). Asterisks (*) denoted statistical significance; *, $P < 0.05$. C). Representative photomicrographs of the liver, spleen, intestine and kidney sections (H&E staining) of carp orally treated with OHA NPs and control group.

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