

Here's the patent which features the application of graphene oxide. (Not the Moderna patent, but the original Chinese one.) The text below is a formal English translation. The formatting was good but not perfect, so I improved it just a tiny bit in one or two places.

<https://patents.google.com/patent/CN112220919A/en>

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## **Nano coronavirus recombinant vaccine taking graphene oxide as carrier**

### **Abstract**

The invention belongs to the field of nano materials and biomedicine, and relates to a vaccine, in particular to development of 2019-nCoV coronavirus nuclear recombinant nano vaccine. The invention also comprises a preparation method of the vaccine and application of the vaccine in animal experiments. The new corona vaccine contains graphene oxide, carnosine, CpG and new corona virus RBD; binding carnosine, CpG and neocoronavirus RBD on the backbone of graphene oxide; the CpG coding sequence is shown as SEQ ID NO 1; the novel coronavirus RBD refers to a novel coronavirus protein receptor binding region which can generate a high-titer specific antibody aiming at the RBD in a mouse body, and provides a strong support for prevention and treatment of the novel coronavirus.

### **Classifications**

**A61K39/12** Viral antigens

**A61K39/385** Haptens or antigens, bound to carriers

**A61K39/39** Medicinal preparations containing antigens or antibodies characterised by the immunostimulating additives, e.g. chemical adjuvants

**A61P31/14** Antivirals for RNA viruses

**C07K14/005** Peptides having more than 20 amino acids; Gastrins; Somatostatins; Melanotropins; Derivatives thereof from viruses

**C07K17/14** Peptides being immobilised on, or in, an inorganic carrier

**A61K2039/55561** CpG containing adjuvants; Oligonucleotide containing adjuvants

**A61K2039/60** Medicinal preparations containing antigens or antibodies characteristics by the carrier linked to the antigen

**A61K2039/627** Medicinal preparations containing antigens or antibodies characterised by the link between antigen and carrier characterised by the linker

**C12N2770/20022** New viral proteins or individual genes, new structural or functional aspects of known viral proteins or genes

**C12N2770/20034** Use of virus or viral component as vaccine, e.g. live-attenuated or inactivated virus, VLP, viral protein

### **Description**

Nano coronavirus recombinant vaccine taking graphene oxide as carrier

### Technical Field

The invention belongs to the field of nano materials and biomedicine, and relates to development of a vaccine development platform. In particular to the development of 2019-nCoV coronavirus nuclear recombinant nano-vaccine. The invention also includes the use of the vaccine in animal testing.

## Technical Field

The vaccine is an ultimate weapon for eliminating major infectious diseases, has the advantages of lowest cost and more advantages of prior enemy than other therapies, undoubtedly becomes hopeful to the public, the smallpox is eliminated by human beings through vaccination, the poliomyelitis cases are reduced by 99 percent, the infectious diseases such as diphtheria are rare, and the incidence rate of diseases such as measles, neonatal tetanus and the like is remarkably reduced. The effect of vaccines on human health is not excessive, and the birth of each new vaccine is a great victory for human beings to overcome an infectious disease! To date, no medical treatment has been able to have such an important, lasting and profound effect on human health as a vaccine; nor is any therapeutic available to eliminate a disease from the earth at the very least cost of a vaccine.

After the occurrence of SARS-CoV-2 epidemic, different laboratories in China have completed the isolation of virus strains, and in order to make a big step forward in vaccine development, we believe that we will soon have a final weapon for the eradication of SARS-CoV-2, however, until now there is no approved vaccine or drug for the treatment of CoV infection, and there is a great need to develop an effective drug for the treatment or prevention of coronavirus infection and outbreak.

According to the research of coronavirus vaccines such as SARS and MERS, the main target point of the existing coronavirus vaccine is the S protein of coronavirus. Vaccines need to induce not only humoral and cellular immune responses, but also mucosal immune responses, and with the aid of adjuvants to induce balanced Th1 and Th2 pathways to produce truly effective vaccines. At present, the research of more SARS and MERS vaccines mainly focuses on viral vector vaccines and subunit vaccines, and a large number of researches show that the difficulty of SARS and MERS lies in that long-term memory B cells cannot be stimulated to generate, the long-term memory cells in the healed SARS and MERS patients can only last for 2-3 years, immunological memory cannot be generated, and the vaccine development failure is caused, and only 6 potential coronavirus vaccines enter the clinical research stage at present, but no 1 effective vaccine is approved to be marketed.

## Disclosure of Invention

The invention aims to provide a coronavirus recombinant vaccine.

Another purpose of the invention is to provide a preparation method of the virus recombinant vaccine.

It is still another object of the present invention to provide the use of the recombinant vaccine of the virus.

In view of various problems of the conventional vaccines at present, how to change the problems of the existing vaccines and enhance immune response is a problem which is always considered, in order to improve the immunocompetence of the immunogen and enhance the immune response capability of the body, the most basic method is to mix the immunogen with an adjuvant, and the immune adjuvant is a promoter capable of enhancing the immune response of the body to the immunogen. CpG Oligodeoxynucleotide (ODN) is a very promising adjuvant discovered in recent years. CpG ODN has been shown to have better adjuvant activity in vivo, in vitro and clinical studies in animals, and the best studies are CpG7909 and CpG 1018. 11/9.2017, the hepatitis B vaccine approved by Dynavax Technologies of the United States of America FDA and using CpG1018 as an adjuvant is on the market, is the first approved CpG ODN adjuvant vaccine in the world and is used for preventing HBV infection of adults 18 years

old and older, and a plurality of different types of CpG ODN are used as adjuvants in a plurality of clinical trials. CpG is combined with TLR9 to activate immature pDC cells and induce natural and adaptive immune response, but a single CpG structure has limited activation effect on immune cells and is easy to be rapidly hydrolyzed by exonuclease, so that the stability of the CpG in vivo is insufficient, and side effects are also caused; CpG Oligodeoxynucleotide (ODN) synthesized in the sequence can also enhance the stimulation effect, and after the CpG is coupled with other proteins such as antigen and the like, the CpG oligodeoxynucleotide is combined, so that the CpG oligodeoxynucleotide has a very obvious immune activation effect.

Graphene is a two-dimensional carbon nanomaterial consisting of carbon atoms in sp hybridized orbitals in a hexagonal honeycomb lattice. The basic structural unit of the material is the most stable benzene six-membered ring in the organic material, and the material is the most ideal two-dimensional material at present. Graphene Oxide (GO) is a Graphene oxide derivative, and is a exfoliated product. Due to the characteristics of unique SP2 hybridization, a perfect two-dimensional structure and high reactivity of the edge, the treatment platform based on the hybrid structure can be used as an ideal load and grafting carrier in design and development, and plays an important role in aspects of nano-drug delivery systems, biological detection, tumor treatment, cell imaging and the like.

The present invention has been completed based on the above-mentioned studies.

The invention discloses a brand-new vaccine development method based on a graphene oxide material serving as a framework for loading CpG molecules and recombinant proteins. Based on the technical platform, a new nano new crown vaccine is prepared by combining the recombinant protein of the RBD region of the Spike protein of the SAR-CoV-2. The prepared nano new corona vaccine has stronger immunogenicity in mouse experiments and can generate high-titer antibodies.

In one aspect, the invention provides a coronavirus vaccine comprising graphene oxide, carnosine, CpG, and RBD. In a preferred embodiment of the invention, the vaccine is named GO-Car-carnosine-CpG-RBD vaccine.

Graphene Oxide (GO) is an oxide of graphene, and after oxidation, oxygen-containing functional groups on the graphene oxide are increased, so that the graphene oxide is more active than graphene. For example, hydroxyl groups and epoxy groups are randomly distributed on a graphene oxide monolith, while carboxyl groups and carbonyl groups are introduced at the edge of the monolith. Common commercial products of graphene oxide are in the form of powder, flakes and solutions, and are brown-yellow in color.

Carnosine, known by the scientific name  $\beta$ -alanyl-L-histidine, is a crystalline solid composed of a dipeptide consisting of two amino acids,  $\beta$ -alanine and L-histidine. Carnosine has strong antioxidant ability, and can scavenge Reactive Oxygen Species (ROS) and alpha-beta unsaturated aldehydes, which are formed by over-oxidation of fatty acids in cell membranes during oxidative stress.

CpG motifs have the effect of activating the body's immune system and can be used as adjuvants. Preferably, the CpG coding sequence is shown as SEQ ID NO 1.

RBD (spike receptor binding domain), specifically a coronavirus protein (S protein) Receptor Binding Domain (RBD) in the present invention. For example, the RBD protein can be selected as follows:

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PNITNLCPFGEVFNATRFASVYAWNRRKRISNVCVADYSVLVNSASFSTFKCYGVSPTKLNLDLCTNVYA  
DSFVIRGDEVRQIAPGQTGKIADYNYKLPDDF
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TGCVIAWNSNNLDSKVGGNYNLYRLFRKSNLKPFFERDISTEIQAGSTPCNGVEGFNCYFPLQSYG  
FQPTNGVGYQPYRVVLSFELLHAP (SEQ ID NO 2)。

the coronavirus vaccine disclosed by the invention is obtained by combining carnosine, CpG and a novel coronavirus RBD on activated graphene oxide.

The dosage of GO in the coronavirus vaccine provided by the invention is used as a framework base, the dosage is usually excessive, and the dosage of carnosine can be about twice of that of GO. CpG and new coronavirus RBD are used as biological macromolecules, and the dosage of the CpG and the new coronavirus RBD is less, and is usually one ten thousandth of that of GO in mass ratio. And RBD is used in an amount more than 2 times that of CpG, such as CpG: RBD = 1: 2-10, preferably, the dosage of RBD is 3-6 times of that of CpG.

In another aspect, the present invention provides a method for preparing the coronavirus vaccine, the method comprising the steps of:

- obtaining CpG, RBD recombinant protein and carnosine;
- adding freeze-dried GO powder into a phosphate buffer solution, and carrying out ultrasonic treatment;
- adding EDC and NHS to activate GO solution, removing excess EDC/sulfo-NHS in the reaction solution through ultrafiltration, and adjusting the pH of the reaction solution to be neutral;
- adding carnosine, CpG, and RBD recombinant proteins to the reaction solution, incubating with activated GO;

Excess unconjugated protein was removed from the reaction solution and sterilized for use.

Preferably, the duration of the ultrasound is 2 to 3 hours. The ultrasonic conditions were 200W, 40 kHz.

Preferably, the phosphate buffer has a neutral pH, e.g., 6.8 to 7.6, more preferably 7.0 to 7.4, or 7.2.

Preferably, the method of removing excess EDC/sulfo-NHS or unconjugated protein is ultrafiltration.

In a preferred embodiment of the present invention, the ratio of graphene oxide, carnosine, CpG, and RBD is: 26 mg: 40 mg: 1.2.  $\mu\text{g}$ : (3-6)  $\mu\text{g}$ .

Preferably, the reaction temperature is 20-28 °C. For example, room temperature is used.

In a preferred embodiment of the present invention, the GO-Car-carnosine-CpG-RBD vaccine is prepared by the following method: GO was coupled to carnosine using a modification of the EDC-NHS reaction, 26mg of GO lyophilized powder was added to 5.20 mL of phosphate buffer (PBS, pH = 7.4) and sonicated (200W, 40 kHz) at 25 °C for 3 h. The GO solution was activated by the addition of 6.82 mg EDC (N1- ((ethylimino) methyl-ene) -N3, N3-dimethyl propane-1,3-diamine, Chinese: 1- (3-dimethylaminopropyl) -3-ethylcarbodiimide) and 7.73 mg NHS (N-N-hydroxysuccinimide) at 25 °C. Excess EDC/sulfo-NHS was removed from the reaction solution by ultrafiltration and the pH of the solution was then adjusted to 7.4. Then, 40mg of carnosine, 1.2 $\mu\text{g}$  CpG, and various concentrations of RBD recombinant protein

were added to the solution and reacted with activated GO at 25 °C for 2 h. Subsequently, excess unconjugated protein was removed from the reaction solution by ultrafiltration. The prepared product is marked as GO-Car-carnosine-CpG-RBD vaccine. Finally, GO-Car-carnosine-CpG-RBD vaccine solution was contacted with sterile filter (0.22 μm) and stored in sterile containers at 4 °C for subsequent experiments.

The invention establishes a nano recombinant protein vaccine preparation technical platform capable of quickly exciting the human immune system, and can quickly produce a large amount of preventive vaccines after infectious viruses are confirmed. The technical platform fully utilizes the characteristic that the surface of graphene oxide is provided with COOH, hydroxyl and other groups, and utilizes the interaction between pi-pi bonds to assemble the screened RBD recombinant protein, CpG molecules and carnosine together to prepare the nano recombinant protein vaccine based on the graphene oxide as the framework. The vaccine can stimulate an organism to generate a high-titer RBD neutralizing antibody aiming at SAR-CoV-2, and lays a technical foundation for preventing and treating coronavirus infection and future large outbreaks of similar epidemics.

In another aspect, the invention provides an application of the GO-Car-carnosine-CpG-RBD vaccine, namely an application of the GO-Car-carnosine-CpG-RBD vaccine in preparation of a medicine for preventing a new coronavirus.

Preferably, the application of the composition can improve the immunity of organisms to the new coronavirus.

Preferably, the GO-Car-carnosine-CpG-RBD vaccine can generate specific antibodies aiming at RBD, and the specific antibody titer is high. In the embodiment of the invention, the nano neocorona vaccine realizes stronger immunogenicity in a mouse test and can generate high-titer antibodies.

The invention has the beneficial effects that:

A brand-new vaccine technical platform is developed for a framework loaded CpG molecule and recombinant protein based on a graphene oxide material and combined with the recombinant protein of the RBD region of the Spike protein of SAR-CoV-2 to prepare a novel nano coronavirus vaccine, a high-titer specific antibody aiming at the RBD can be generated in a mouse body, and a strong support is provided for prevention and treatment of novel coronavirus.

### Drawings

In order to more clearly illustrate the technical solutions in the embodiments of the present application, the drawings needed to be used in the embodiments will be briefly described below, and it is obvious that the drawings in the following description are only some embodiments of the present application, and it is obvious for those skilled in the art to obtain other drawings without creative efforts.

FIG. 1 is a schematic diagram and time schematic representation of GO-Car-carnosine-CpG-RBD vaccine mouse immunization;

FIG. 2 shows the change of specific RBD antibody in serum 28 days after the mice were immunized and the change of cytokine production by spleen cells 42 days after the mice were immunized.

### Detailed Description

The technical solutions in the embodiments of the present application will be described clearly and completely below, and it should be understood that the described embodiments are only a part of the embodiments of the present application, and not all embodiments. All other embodiments, which can be derived by a person skilled in the art from the embodiments given herein without making any creative effort, shall fall within the protection scope of the present application. Methods and techniques not specifically described may be performed using techniques conventionally known in the art. For example, refer to molecular cloning handbook of cold spring harbor.

### Example 1

Preparation process of Graphene Oxide (GO) -carnosine-CpG-RBD recombinant protein vaccine preparation

Selecting a TLR9 receptor nucleic acid sequence CpG ODN M362 which has cross reaction to both human and mouse, wherein the specific sequence is as follows: 5'-TCGTCGTCGTTTC: GAACGACGTTGAT-3' (25 mer, SEQ ID NO 1), coupling GO with carnosine using a modification of the EDC-NHS reaction, 26mg of a lyophilized powder of GO was added to 5.20 mL of phosphate buffer (PBS, pH = 7.4) and sonicated (200W, 40 kHz) at 25 °C for 3 h. The GO solution was activated by the addition of 6.82 mg EDC (N1-((ethylimino) methyl-ene) -N3, N3-dimethyl propane-1,3-diamine, Chinese: 1-(3-dimethylaminopropyl) -3-ethylcarbodiimide) and 7.73 mg NHS (N-N-hydroxysuccinimide) at 25 °C. Excess EDC/sulfo-NHS was removed from the reaction solution by ultrafiltration and the pH of the solution was then adjusted to 7.4. Then, 40mg of carnosine, 1.2µg CpG, and various concentrations of RBD recombinant protein were added to the solution and reacted with activated GO at 25 °C for 2 h. Subsequently, excess unconjugated protein was removed from the reaction solution by ultrafiltration. The prepared product is marked as GO-Car-carnosine-CpG-RBD vaccine. Finally, GO-Car-carnosine-CpG-RBD vaccine solution was contacted with sterile filter (0.22 µm) and stored in sterile containers at 4 °C for subsequent experiments.

### Example 2

Test of Graphene Oxide (GO) -carnosine-CpG-RBD recombinant protein vaccine immunized mice

6-week-old female BALB/c mice were immunized by subcutaneous injection at 0, 14, and 28 days, respectively, for 28 days and 42 days according to the schedule shown in FIG. 1, blood was collected by drawing blood at , and serum was separated and tested for specific antibodies against RBD. Mice were sacrificed at 42 days, splenocytes isolated, and tested for specific T cell immune responses and cytokine secretion.

Grouping and dose determination of immunized mice:

1. (graphene oxide + carnosine) + 1.2 $\mu$ g cpG +3 $\mu$ g RBD
2. (graphene oxide + carnosine) + 1.2 $\mu$ g cpG +6 $\mu$ g RBD
3. Aluminum hydroxide +6 $\mu$ g RBD (1: 1)
4. 6 $\mu$ g RBD
5. Liposome (lipo) +6 $\mu$ g RBD group

Mouse strains: BALB/c mic (n = 6).

The schedule for immunization of mice with the GO-Car-carnosine-CpG-RBD vaccine is: blood was collected and first immunized as starting point for immunization of mice. And (5) collecting blood for the second time on the 7 th day, and inspecting a new corona virus adding system to master the principle. Collecting blood for the third time on day 14, and enhancing immunity. Collecting blood for the fourth time on day 28, enhancing immunity, detecting antibody in serum, and if positive, preparing to collect spleen cells. The fifth blood collection on day 42, after which the blood was sacrificed and splenocytes were isolated and subjected to cytokine experiments.

The test results show that 3 $\mu$ g and 6 $\mu$ g groups of the GO-Car-carnosine-CpG-RBD vaccine generate high-titer specific antibodies for RBD after mice are immunized, and the GO-Car-carnosine-CpG-RBD vaccine is significantly different from the traditional adjuvant group, the RBD group and the liposome group (figure 2). Further analyzing the specific immune response of the T cells separated from the spleen, the result shows that the GO-Car-carnosine-CpG-RBD vaccine can stimulate the organism to generate specific IFN-gamma cytokines, improve the immunity of the organism and resist the epidemic situation of new coronavirus.

The above description is only for the specific embodiments of the present application, but the scope of the present application is not limited thereto, and any changes or substitutions that can be easily conceived by those skilled in the art within the technical scope of the present disclosure should be covered within the scope of the present application. Therefore, the protection scope of the present application shall be subject to the protection scope of the claims.

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The original is here, in Chinese, this document containing the illustrations.

The application was filed by Shanghai National Engineering Research Center for Nanotechnology Co Ltd on 27 September, 2020.

<https://patentimages.storage.googlea...112220919A.pdf>