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Effect of M2e peptide–gold nanoparticle conjugates on development of anti-influenza antibodies

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Abstract

Gold nanoparticles (AuNPs) are successfully used as an adjuvant in the design of effective vaccines and in the preparation of high-affinity antibodies to haptens and complete antigens. Here, we assessed the adjuvant properties of AuNPs conjugated to a synthetic M2e peptide of the influenza A virus capsid. The resulting conjugate, a commercial influenza vaccine, and M2e in combination with different adjuvants were used to immunize laboratory mice. The highest titer was detected in the sera of mice immunized with two adjuvants: AuNPs and AuNP-conjugated CpG oligodeoxynucleotide 1826. With this combination, we also recorded increases in the respiratory activity of splenic lymphocytes, in the respiratory activity of peritoneal macrophages, and in the production of proinflammatory cytokines (IL-6 and IFN-γ). The results indicate that simultaneous immunization of the animals with two conjugates—M2e + AuNPs and CpG + AuNPs—activates antibody development. Therefore, the use of AuNPs as an antigen carrier leads to a complete and coordinated immune response from both cellular and humoral immunity.

Keywords Gold nanoparticles · CpG oligodeoxynucleotide · Influenza A virus · M2e peptide · Immunization · Adjuvant

Introduction

According to the World Health Organization, the annual global incidence of influenza is about 1 billion, with up to 500,000 deaths [1]. The disease is especially severe in children. The most effective means of prophylaxis against influenza and associated complications is vaccination. But seasonal immunity cannot ensure protection in subsequent influenza seasons, mostly because of changes in strain circulation, changes in antigen drift, and decreased immunity. Influenza vaccines are updated annually to include stains predicted to circulate in the coming winter [2, 3].

Currently, different types of vaccines are used, including inactivated, live attenuated, and recombinant subunit vaccines. All of them are mostly trivalent or quadrivalent. Influenza vaccines are given intramuscularly, intracutaneously, or intranasally. The immune system responds mostly to hemagglutinin and neuraminidase, surface glycoprotein antigens of influenza virus [4]. The immunodominant epitopes of these antigens differ greatly between viral strains. For this reason, vaccines protect only against strains included in them.

These limitations have spurred interest in recombinant proteins based on viral antigens. Recombinant vaccines have advantages over live or inactivated ones, because they are usually well purified and characterized; therefore, they are safer and are better suited for large-scale production. Recombinant vaccines also have some drawbacks. For example, an antigen per se is usually weakly immunogenic and requires the use of adjuvants and/or delivery systems (in particular, viruslike particles) [5, 6].

A promising antigen for recombinant vaccines is the surface protein M2e (extracellular domain of Matrix 2 protein). The gene coding for the structure of this protein is preserved unchanged in all influenza A strains; consequently, all viruses can be recognized and eliminated by the immune system of vaccinated persons [7]. Anti-M2e antibodies limit virus replication and viral plaque formation in a cell monolayer in vitro and induce protection against virus subtypes within group A [8]. Anti-M2e antibodies were identified that cross-reacted with seasonal, pandemic H1N1, and highly pathogenic avian

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H5N1 strains [9]. Although anti-M2 antibodies do not neutralize viruses, they ensure antibody-dependent cellular cytotoxicity and are, therefore, important in the immune response to influenza virus [8]. Nonetheless, the application of M2e in vaccine design is restricted by the need for highly immunogenic carriers. There is good reason, therefore, to develop methods for increasing M2e immunogenicity and for using N2e in combination with various stimulants of cellular immune response [10, 11].

Much current interest is in the use of nanoparticles as platforms for vaccines [12–15], including virus vaccines [16–18]. Carriers for experimental influenza vaccines include liposomes [19], ImmunoStimulating COMplexes (ISCOMs) [20], calcium phosphate nanoparticles [21], polymer nanoparticles [22], and silica nanoparticles [23].

Among the most promising antigen carriers used in immunization are gold nanoparticles (AuNPs) [24–26]. This is because, besides being able to carry antigens, AuNPs also have adjuvant properties [27, 28]. AuNP uptake into immune cells activates the production of proinflammatory cytokines, a finding which indicates directly that AuNPs are immunostimulatory. The activation of immune cells by AuNPs may form a basis for the development of new vaccine adjuvants [29].

AuNPs have been used to develop prototypes of influenza vaccines and generate antibodies against influenza virus antigens. The specific antigens were hemagglutinin [30] and two matrix proteins of influenza virus, M1 [31] and M2 [32, 33] (both synthetic and recombinant).

In 2014, the team led by Harvinder Gill proposed a prototype intranasal influenza vaccine consisting of a synthetic M2e peptide conjugated to AuNPs, with CpG oligodeoxynucleotide (CpG ODN) as the adjuvant [34]. The conjugate induced specific antiviral IgG and protected mice against a lethal dose of PR8 influenza virus. Subsequently, Gill's team reported more detailed results from the use of their prototype vaccine [35, 36].

In recent work, the antibody titer was the highest in the sera of mice immunized simultaneously with antigen–AuNPs and CpG–AuNPs conjugates [37]. Here, we examined the effect of AuNPs conjugated to a synthetic M2e peptide on immune response in intraperitoneally immunized mice.

Materials and methods

Preparation of gold nanoparticle conjugates

The antigen used for immunization was synthetic M2e peptide [acetylated-SLLTEVETPIRNEWGSRSNDSSD-amidated; molecular mass, 2736 Da (Cytokine Co., Russia)].

Spherical AuNPs (average diameter, 15 nm) were made according to Frens [38] by the reduction of tetrachloroauric acid (Sigma-Aldrich, USA) with sodium citrate (Fluka, Switzerland). A 242.5-mL portion of 0.01% aqueous tetrachloroauric acid was heated to 100 °C on a magnetic stirrer in an Erlenmeyer flask fitted with a water-cooled reflux tube. Then, 7.5 mL of 1% aqueous sodium citrate was added and the mixture was boiled for a further 30 min until a bright-red sol formed.

Particle characteristics were measured with a Libra 120 transmission electron microscope (Carl Zeiss), a Specord S 250 spectrophotometer (Analytik Jena), and a Zetasizer Nano-ZS particle size and zeta potential analyzer (Malvern). All measurements were made at the Simbioz Center for the Collective Use of Research Equipment at the Institute of Biochemistry and Physiology of Plants and Microorganisms.

To prepare antigen–15-nm AuNP conjugates, we estimated the "gold number" (minimal amount of antigen that protects the sol against salt aggregation) for the M2e solution. To this end, 20 μl of an antigen solution (initial concentration, 1 mg mL^{-1}) was titrated twofold on a 96-well microtiter plate. Each well-received 200 μL of AuNPs ($A_{520} = 1.0$) and 20 μL of 1.7 M NaCl. The minimal stabilizing concentration for the antigen was 3 μg mL^{-1}. Conjugation was done by simple mixing, and no coupling agents were added. A tenfold excess of the peptide was used, because an excess of a soluble antigen not only does not interfere with immunization but actually facilitates an increase in antibody production [35]. The resultant conjugates are aggregationally stable under conditions close to physiological.

AuNPs (15 nm) were conjugated to 5′-thiolated CpG ODN (Syntol) as described earlier [37, 39]: 100 μl of aqueous ODN was added to 2 ml of AuNP solution. After overnight incubation, the NaCl concentration was increased to 0.1 M with 1 M PBS (pH 7.2). The final mixture was shaken for an additional 24 h, centrifuged at 15000g for 20 min, and resuspended in 0.01 M PBS, containing 0.1 M NaCl.

Animal immunization

For immunization, BALB/c white mice were divided into six groups of six in each, and they received injections as follows:

1. "Grippol" vaccine (Microgen, Russia)
2. M2e + CFA (complete Freund's adjuvant; Sigma-Aldrich, USA)
3. M2e + AuNPs
4. M2e + AuNPs + CpG + AuNPs
5. AuNPs
6. PBS

Table 1 Antibody titers obtained with different adjuvants

Immunogen	Antibody titer		
	Maximal titer	Average titer in 6 mice	Log$_2$
"Grippol" commercial vaccine	1:2560	1:1707	10.65 ± 0.85
M2e + CFA	1:5120	1:3840	11.82 ± 0.57
M2e + AuNPs	1:5120	1:3200	11.48 ± 1.2
M2e + AuNPs + CpG + AuNPs	1:20480	1:12800	13.48 ± 1.2
AuNPs	0	0	0
PBS	0	0	0

The dose of M2e in groups 2 to 4 was constant (15 μg). The dose of protein antigens in group 1 was ~ 15 μg. The animals were immunized intraperitoneally by two 50-μL injections with an interval of 10 days in-between. Besides AuNPs, other adjuvants used were CFA and AuNP-conjugated CpG ODN 1826. The animals were killed, and sera were collected on day 42 of the experiment for measurements of antibody titers and interleukin concentrations. In parallel, we measured the respiratory activity of splenic lymphocytes and peritoneal macrophages.

Animal care complied with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, the Guide for the Care and Use of Laboratory Animals, and Russian legislation.

Immunological and toxicological analysis

Peritoneal macrophages and splenic lymphocytes were isolated as described earlier [40]. Antibody titers were estimated by enzyme-linked immunosorbent assay (ELISA) with horseradish peroxidase-labeled antibodies against mouse IgG (Jackson ImmunoResearch, UK). The synthetic peptide was used as the immobilized antigen. The reaction results were obtained on a Multiskan Ascent microplate spectrophotometer (Thermo, USA), as described earlier [37].

To measure the serum interleukin concentration, we used a Plate Screen analyzer (Hospitex Diagnostics, Italy) and reagent kits for IL-1β, IL-6, and IFN-γ (Vector-Best, Russia).

Respiratory activity was measured by the ability of immune cells to reduce nitrotetrazolium blue (MTT; Sigma-Aldrich) to formazan [41]. The concentration of reduced formazan was converted to that per cell [40].

Statistics

Data were statistically processed with Excel 2007 software (Microsoft Corp., USA). The standard error of the mean and its confidence limits were calculated by Student's t test ($p =$ 0.05). The significance of between-samples differences was estimated by a two-sample unpaired Student's t test with unequal variances. Differences were considered significant at $p \leq 0.05$.

Results and discussion

The synthesized Au nanospheres were characterized by transmission electron microscopy, spectrophotometry, and dynamic light scattering. The measured data for the 15-nm particles were as follows: average diameter, 15.2 nm; λ_{max}, 517.1 nm; A_{520}, 1.1; and the number of particles per ml, 1.6×10^{12}. The Au concentration was 57 μg ml^{-1}.

Mice were immunized with "Grippol" commercial influenza vaccine and with the M2e antigen in combination with different adjuvants. Antibody titers were estimated by ELISA and are expressed in the ordinary way and as log$_2$ (Table 1). The highest titer was obtained from the simultaneous use of two adjuvants—AuNPs and AuNPs + CpG (1:12800). This titer was about threefold greater than the titers obtained with AuNPs and CFA and was about sevenfold greater than the titer obtained with "Grippol" vaccine.

Therefore, the production of antibodies can be increased when the animals are immunized simultaneously with M2e + AuNPs and CpG + AuNPs.

The respiratory activity of peritoneal macrophages and splenic lymphocytes was measured by the MTT assay. The concentration of reduced formazan was converted to that per

Table 2 Formazan concentrations for macrophages and lymphocytes

Immunogen	Formazan concentration per cell (pg)	
	Macrophages	Lymphocytes
"Grippol" commercial vaccine	80 ± 4.8	60 ± 3.5
M2e + CFA	90 ± 2.9	50 ± 4.1
M2e + AuNPs	90 ± 3.6	60 ± 4.8
M2e + AuNPs + CpG + AuNPs	100 ± 9.2	80 ± 7.0
AuNPs	70 ± 3.7	50 ± 4.2
PBS	70 ± 4.0	50 ± 3.9

Fig. 1 Concentration of IFN-γ in sera of animals immunized with M2e in combination with different adjuvants
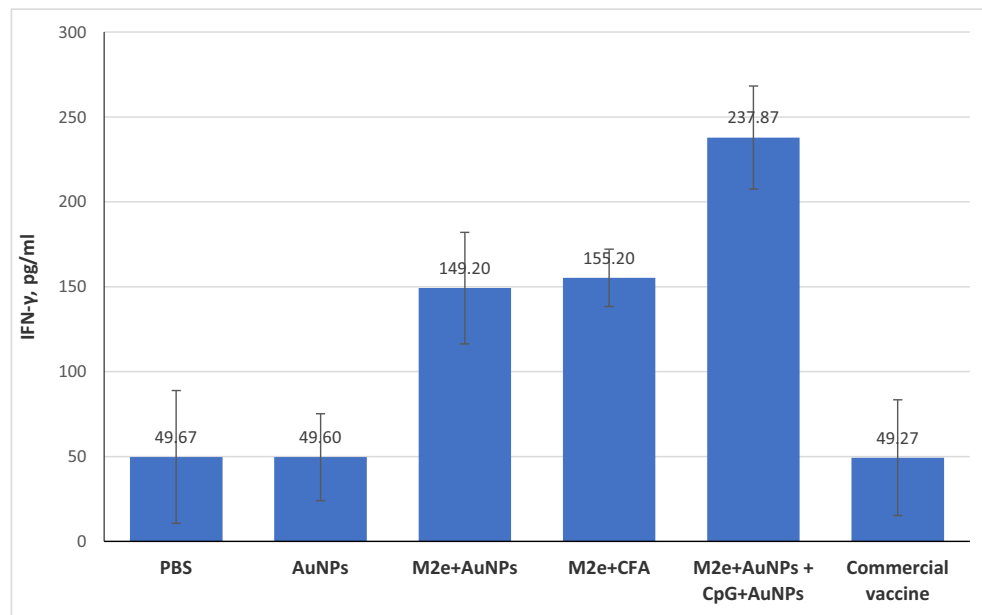
cell (Table 2). The respiratory activity of peritoneal macrophages was highest in the animals given M2e + AuNPs + CpG + AuNPs, increasing by about 40% ($p = 0.0042$) relative to PBS and by about 25% ($p = 0.039$) relative to the commercial vaccine. M2e + AuNPs and M2e + CFA were almost equal in their effect on dehydrogenase activity in peritoneal macrophages ($p = 0.73$). Similar results were obtained for the splenic lymphocytes. These findings could be explained by the more effective penetration of AuNP conjugates into phagocytic cells, with improved antigen presentation to the antibody-producing cells.

We also measured the production of the proinflammatory cytokines IL-1β, IL-6, and IFN-γ in sera from the immunized animals (Figs. 1, 2, and 3). The content of IFN-γ obtained with M2e + AuNPs + CpG + AuNPs was 1.5-fold greater than that obtained with M2e + AuNPs ($p = 0.0022$) or with M2e + CFA ($p = 0.0008$). It also was 5.6-fold greater than the IFN-γ content obtained with the commercial vaccine. The production of IL-1β did not differ significantly among the groups of mice. The production of IL-6 increased significantly with M2e + AuNPs, M2e + CFA, and M2e + AuNPs + CpG + AuNPs, as compared with the use of the commercial vaccine.

Fig. 2 Concentration of IL-1β in sera of animals immunized with M2e in combination with different adjuvants
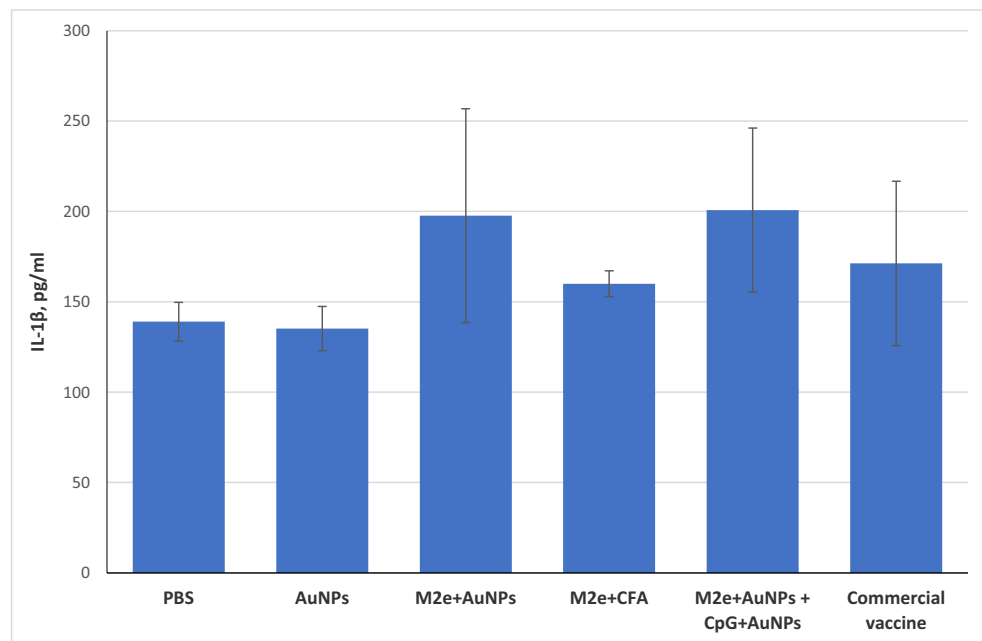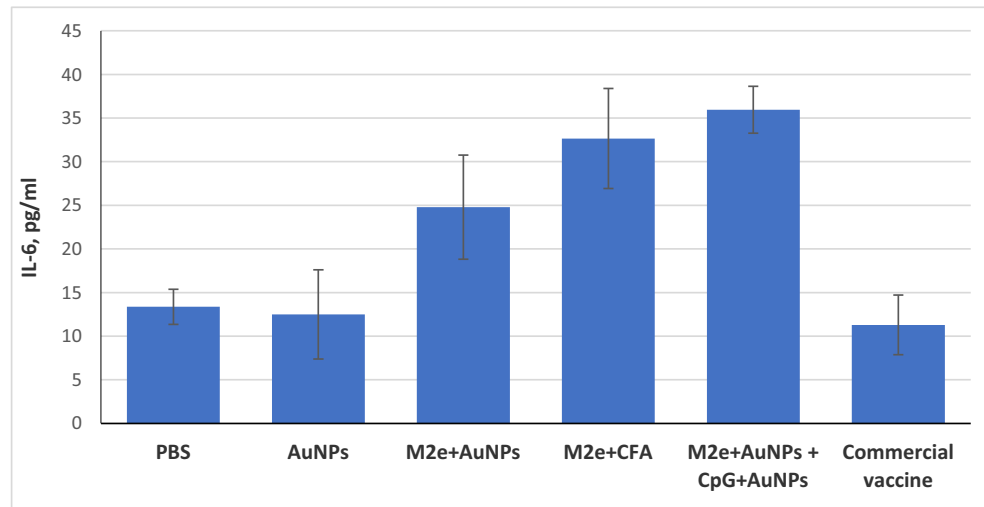
Fig. 3 Concentration of IL-6 in sera of animals immunized with M2e in combination with different adjuvants

IFN-γ, which mediates inflammation in viral infections, inhibits virus replication, promotes the expression of MHC II, and activates NK cells and macrophages [42]. IL-6 helps B lymphocytes mature into antibody-producing cells [42]. Therefore, the increased concentration of IL-6 indicates that immunization with M2e + AuNPs activated the polyclonal production of Ig.

Conclusions

Thus, of the immunogens tested in this study (including a commercial vaccine), M2e + AuNPs + CpG + AuNPs was most effective, producing antibodies with the highest titer. It also was better at increasing the respiratory activity of lymphoid cells and the production of proinflammatory cytokines. The immunostimulatory (adjuvant) effect of AuNPs may be due to the more effective penetration of conjugates into phagocytic cells, which leads to improved antigen presentation to the antibody-producing cells. Consequently, the use of AuNPs as an antigen carrier leads to a complete and coordinated immune response from both cellular and humoral immunity.

AuNPs can be used as an adjuvant to improve the effectiveness of vaccines, stimulate antigen-presenting cells, and provide controlled release of antigens. In addition, the immunogenicity of AuNPs is determined by their physicochemical properties, such as size, shape, charge, and surface functionalization. Studying the immune response from the use of AuNPs as a carrier and adjuvant in antibody preparation will allow evaluation of their potential for use in vaccine design [43, 44]. Such nanotechnology would improve vaccine safety, potency, and availability, offering compelling platforms toward addressing many public health threats. More comprehensive studies of AuNPs are expected to reveal further applications. With increasing knowledge in immunology

that uncovers the profound immune responses needed for effective defense, AuNPs are poised to attract growing attention in vaccine development.

References

1. Ghebrehewet S, MacPherson P, Ho A (2016) Influenza. BMJ 355: i6258. https://doi.org/10.1136/bmj.i6258
2. Trombetta CM, Montomoli E (2016) Influenza immunology evaluation and correlates of protection: a focus on vaccines. Expert Rev Vaccines 15:967–976. https://doi.org/10.1586/14760584.2016
3. Sano K, Ainai A, Suzuki T, Hasegawa H (2017) The road to a more effective influenza vaccine: up to date studies and future prospects. Vaccine 35:5388–5395. https://doi.org/10.1016/j.vaccine.2017.08.034
4. Treanor JJ (2016) Influenza vaccination. N Engl J Med 375:1261–1268. https://doi.org/10.1056/NEJMcp1512870
5. Seth A, Ritchie FK, Wibowo N, Lua LHL, Middelberg APJ (2015) Non-carrier nanoparticles adjuvant modular protein vaccine in a particle-dependent manner. PLoS One 10:e0117203. https://doi.org/10.1371/journal.pone.0117203
6. Quan F-S, Lee Y-T, Kim K-H, Kim M-C, Kang S-M (2016) Progress in developing virus-like particle influenza vaccines. Expert Rev Vaccines 15:1281–1293. https://doi.org/10.1080/14760584.2016.1175942
7. Deng L, Cho KJ, Fiers W, Saelens X (2015) M2e-based universal influenza A vaccines. Vaccines 3:105–136. https://doi.org/10.3390/vaccines3010105
8. Kolpe A, Schepens B, Fiers W, Saelens X (2017) M2-based influenza vaccines: recent advances and clinical potential. Expert Rev Vaccines 16:123–136. https://doi.org/10.1080/14760584.2017.1240041

9. Ozawa T, Jin A, Tajiri K, Takemoto M, Okuda T, Shiraki K, Kishi H, Muraguchi A (2011) Characterization of a fully human monoclonal antibody against extracellular domain of matrix protein 2 of influenza A virus. Antivir Res 91:283–287. https://doi.org/10.1016/j.antiviral.2011.06.012

10. Lee Y-N, Kim M-C, Lee Y-T, Hwang HS, Lee J, Kim C, Kang S-M (2015) Cross protection against influenza A virus by yeast-expressed heterologous tandem repeat M2 extracellular proteins. PLoS One 10:e0137822. https://doi.org/10.1371/journal.pone.0137822

11. Esmagambetov IB, Alekseeva SV, Sayadyan KS, Shmarov MM (2016) Current approaches to universal vaccine against influenza virus. Russ J Infect Immun 6:117–132. https://doi.org/10.15789/2220-7619-2016-2-117-132

12. Dobrovolskaia MA, McNeil SE (eds) (2013) Handbook of Immunological Properties of Engineered Nanomaterials. World Scientific Publishing, Singapore

13. Zaman M, Good MF, Toth I (2013) Nanovaccines and their mode of action. Methods 60:226–231. https://doi.org/10.1016/j.ymeth.2013.04.014

14. Prashant CK, Kumar M, Dinda AK (2014) Nanoparticle based tailoring of adjuvant function: the role in vaccine development. J Biomed Nanotechnol 10:2317–2331. https://doi.org/10.1166/jbn.2014.1991

15. Liu Y, Xu Y, Tian Y, Chen C, Wang C, Jiang X (2014) Functional nanomaterials can optimize the efficacy of vaccines. Small 10:4505–4520. https://doi.org/10.1002/smll.201401707

16. Sokolova V, Westendor AM, Buer J, Überla K, Epple M (2015) The potential of nanoparticles for the immunization against viral infections. J Mater Chem B 3:4767–4779. https://doi.org/10.1039/C5TB00618J

17. Gupta A, Das S, Schanen B, Seal S (2016) Adjuvants in micro- to nanoscale: current state and future direction. Wiley Interdiscip Rev Nanomed Nanobiotechnol 8:61–84. https://doi.org/10.1002/wnan.1354

18. Shen Y, Hao T, Ou S, Hu C, Chen L (2018) Applications and perspectives of nanomaterials in novel vaccine development. Med Chem Commun 9:226–238. https://doi.org/10.1039/C7MD00158D

19. Mann JF, Shakir E, Carter KC, Mullen AB, Alexander J, Ferro VA (2009) Lipid vesicle size of an oral influenza vaccine delivery vehicle influences the Th1/Th2 bias in the immune response and protection against infection. Vaccine 27:3643–3649. https://doi.org/10.1016/j.vaccine.2009.03.040

20. Coulter A, Harris R, Davis R, Drane D, Cox J, Ryan D, Sutton P, Rockman S, Pearse M (2003) Intranasal vaccination with ISCOMATRIX adjuvanted influenza vaccine. Vaccine 21:946–949. https://doi.org/10.1016/S0264-410X(02)00545-5

21. Knuschke T, Sokolova V, Rotan O, Wadwa M, Tenbusch M, Hansen W, Staeheli P, Epple M, Buer J, Westendorf AM (2013) Immunization with biodegradable nanoparticles efficiently induces cellular immunity and protects against influenza virus infection. J Immunol 190:6221–6229. https://doi.org/10.4049/jimmunol.1202654

22. Rieger J, Freichels H, Imberty A, Putaux J-L, Delair T, Jerome C, Auzely-Velty R (2009) Polyester nanoparticles presenting mannose residues: toward the development of new vaccine delivery systems combining biodegradability and targeting properties. Biomacromolecules 10:651–657. https://doi.org/10.1021/bm801492c

23. Neuhaus V, Chichester JA, Ebensen T, Schwarz K, Hartman CE, Shoji Y, Guzman CA, Yusibov V, Sewald K, Braun A (2014) A new adjuvanted nanoparticle-based H1N1 influenza vaccine induced antigen-specific local mucosal and systemic immune responses after administration into the lung. Vaccine 32:3216–3222. https://doi.org/10.1016/j.vaccine.2014.04.011

24. Salazar-González JA, González-Ortega O, Rosales-Mendoza S (2015) Gold nanoparticles and vaccine development. Expert Rev Vaccines 14:1197–1211. https://doi.org/10.1586/14760584.2015.1064772

25. Carabineiro SAC (2017) Applications of gold nanoparticles in nanomedicine: recent advanced in vaccine. Molecules 22:857. https://doi.org/10.3390/molecules22050857

26. Dykman LA, Khlebtsov NG (2017) Immunological properties of gold nanoparticles. Chem Sci 8:1719–1735. https://doi.org/10.1039/C6SC03631G

27. Dykman LA, Sumaroka MV, Staroverov SA, Zaitseva IS, Bogatyrev VA (2004) Immunogenic properties of colloidal gold. Biol Bull Russ Acad Sci 31:75–79. https://doi.org/10.1023/B:BIBU.0000014358.98422.9c

28. Dykman LA, Staroverov SA, Bogatyrev VA, Shchyogolev SY (2010) Adjuvant properties of gold nanoparticles. Nanotechnol Russia 5:748–761. https://doi.org/10.1134/S1995078010110029

29. Dykman LA, Khlebtsov NG (2018) Gold nanoparticles in biomedical applications. CRC Press, Boca Raton

30. Wang C, Zhu W, Wang B-Z (2017) Dual-linker gold nanoparticles as adjuvanting carriers for multivalent display of recombinant influenza hemagglutinin trimers and flagellin improve the immunological responses in vivo and in vitro. Int J Nanomedicine 12:4747–4762. https://doi.org/10.2147/IJN.S137222

31. Mezhenny PV, Staroverov SA, Volkov AA, Kozlov SV, Laskavy VN, Dykman LA, Isayeva AY (2013) Construction of conjugates of colloidal selenium and colloidal gold with the protein of influenza virus and the study of their immunogenic properties. Bull Saratov State Agrarian University 2:29-32 (in Russian)

32. Chen Y-S, Hung Y-C, Liau I, Huang GS (2009) Assessment of the in vivo toxicity of gold nanoparticles. Nanoscale Res Lett 4:858–864. https://doi.org/10.1007/s11671-009-9334-6

33. Mardanova ES, Kotlyarov RY, Kuprianov VV, Stepanova LA, Tsybalova LM, Lomonosoff GP, Ravin NV (2015) Rapid high-yield expression of a candidate influenza vaccine based on the ectodomain of M2 protein linked to flagellin in plants using viral vectors. BMC Biotechnol 15:42. https://doi.org/10.1186/s12896-015-0164-6

34. Tao W, Ziemer KS, Gill HS (2014) Gold nanoparticle-M2e conjugate coformulated with CpG induces protective immunity against influenza A virus. Nanomedicine (London) 9:237–251. https://doi.org/10.2217/nnm.13.58

35. Tao W, Gill HS (2015) M2e-immobilized gold nanoparticles as influenza A vaccine: Role of soluble M2e and longevity of protection. Vaccine 33:2307–2315. https://doi.org/10.1016/j.vaccine.2015.03.063

36. Tao W, Hurst B, Shakya AK, Uddin MJ, Ingrole RS, Hernandez-Sanabria M, Arya R, Bimler L, Paust S, Tarbet EB, Gill HS (2017) Consensus M2e peptide conjugated to gold nanoparticles confers protection against H1N1, H3N2 and H5N1 influenza A viruses. Antivir Res 141:62–72. https://doi.org/10.1016/j.antiviral.2017.01.021

37. Dykman LA, Staroverov SA, Fomin AS, Khanadeev VA, Khlebtsov BN, Bogatyrev VA (2018) Gold nanoparticles as an adjuvant: Influence of size, shape, and technique of combination with CpG on antibody production. Int Immunopharmacol 54:163–168. https://doi.org/10.1016/j.intimp.2017.11.008

38. Frens G (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature Phys Sci 241:20–22. https://doi.org/10.1038/physci241020a0

39. Bogatyrev VA, Dykman LA, Khlebtsov BN, Plotnikov VK, Khlebtsov NG (2005) Optical properties of colloidal gold–oligothymidine conjugates and their variations on hybridization with polyadenylic acid. Colloid J 67:413–421. https://doi.org/10.1007/s10595-005-0112-6

40. Dykman LA, Staroverov SA, Mezhenny PV, Fomin AS, Kozlov SV, Volkov AA, Laskavy VN, Shchyogolev SY (2015) Use of a synthetic foot-and-mouth disease virus peptide conjugated to gold nanoparticles for enhancing immunological response. Gold Bull 48: 93–101. https://doi.org/10.1007/s13404-015-0165-1

41. Bernas T, Dobrucki JW (2000) The role of plasma membrane in bioreduction of two tetrazolium salts, MTT, and CTC. Arch Biochem Biophys 380:108–116. https://doi.org/10.1006/abbi. 2000.1907

42. Abbas A, Lichtman AH, Pillai S (eds) (2014) Cellular and Molecular Immunology, 8th edn. Saunders, Philadelphia

43. Chattopadhyay S, Chen J-Y, Chen H-W, Hu C-MJ (2017) Nanoparticle vaccines adopting virus-like features for enhanced immune potentiation. Nanotheranostics 1:244–260. https://doi. org/10.1002/adhm.201701395

44. Tazaki T, Tabata K, Ainai A, Ohara Y, Kobayashi S, Ninomiya T, Orba Y, Mitomo H, Nakano T, Hasegawa H, Ijiro K, Sawa H, Suzuki T, Niikura K (2018) Shape-dependent adjuvanticity of nanoparticle-conjugated RNA adjuvants for intranasal inactivated influenza vaccines. RSC Adv 8:16527–16536. https://doi.org/10. 1039/c8ra01690a