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## Original Article

# Construction of Influenza A/H1N1 Virosomal Nanobioparticles

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## Abstract

**Background and Aims:** Influenza is one of the main respiratory infections of humans, responsible for 300,000–500,000 annual deaths world-wide. Vaccination is one of the best ways to prevent infections including influenza. Influenza virosomes are virus-like particles, which retain the cell binding and membrane fusion properties of the native virus, but lack the ribonucleoprotein (RNP). A virosomal influenza vaccine has recently been commercially available in Europe (Inflexal V®). The virosome is prepared by membrane solubilization and reconstitution. A new method based on dialyzable detergent has been developed to produce virosomes from an A/H3N2 influenza virus.

**Methods:** In this study attempt was made to construct a virosomal nanobioparticle of influenza A/ PR8 (H1N1). The Madine-Darby Canine kidney (MDCK) cell line was cultured and infected with influenza virus strain A/PR8 and the culture media were harvested and the virus was purified by ultracentrifugation and concentrated by ultrafiltration. Purified influenza virus was treated with 1, 2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC) as a solubilizing detergent to resolve the viral envelope. Ribonucleoprotein was sedimented by ultracentrifugation and the supernatant consisting phospholipids and glycoproteins of influenza virus was reconstituted by removal of DCPC using overnight dialysis against Hank's Buffered Saline (HBS) solution.

**Results:** Finally, the empty influenza virus envelope, called virosome, was observed by transmission electron microscopy (TEM). The size of these particles was estimated to be 50-100 nm.

**Conclusion:** Virosome has been used as a new vaccine formulation and since it is a nontoxic adjuvant carrier it can be used to improve the present commercialized and new vaccines.

**Keywords:** Influenza Virus; Virosome; Nanobioparticles

## Introduction

Influenza is one of the main respiratory infections of humans, responsible for 300,000–500,000 annual deaths world-wide. Vaccination has been a most cost-effective mean to control infectious diseases including influenza infection. Antibodies against hemagglutinin (HA) and neuraminidase (NA) of the virus were shown to prevent infection by the same subtype of influenza A virus since more than three decades ago (1-2) and remain the correlate of protection afforded by inactivated human influenza vaccines (3). The trivalent vaccine contains antigens from two influenza A virus strains and one B strain, as recommended each year by the WHO (4). Current approved influenza vaccines are

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inactivated whole virus, split virus, or subunit antigen, i.e. isolated haemagglutinin (HA) and neuraminidase (NA). The efficacy of the vaccine is significantly compromised when circulating viruses do not have a good match with vaccine strains. A live, attenuated influenza virus vaccine (FluMist®) is also licensed for seasonal influenza and is intended for intranasal administration to people (5-6). Current inactivated vaccines exhibit relatively poor immunogenicity in immunologically naive people, such as children who have not had prior exposure, and overall efficacy of approximately 60% in the elderly, there is substantial room for improvement (7-8). Virosomes represent another novel influenza vaccine strategy. Virosomes in fact are virus-like particles, consisting of reconstituted viral envelopes and structural proteins, lacking the genetic material of the original virus. Functionally reconstituted influenza virosomes preserve the receptor-binding and membrane fusion activity of the viral HA. Since the virosomes, lack the viral RNA, binding and fusion of them to cells does not result in infection (12) and have been shown to be safe and highly immunogenic.

Several approaches have been applied for the production of virosomes. One of the most frequently used methods for the preparation of virosomes is by membrane solubilization and reconstitution. Briefly, the classical procedure for preparation of virosomes from influenza virus or other enveloped viruses generally involves solubilization of the viral membrane mostly with octaethyleneglycol mono(*n*-dodecyl) ether ($C_{12}E_8$), followed by removal of the nucleoproteins by ultracentrifugation. Reconstitution of the viral membrane is accomplished by removal of the detergent by either dialysis or Bio-beads (9-11). Although the $C_{12}E_8$ method is very useful for the production of virosomes, it suffers from some inherent drawbacks. Production according to this method, involves batch processes, often in open systems. This is a challenging situation for industrial processing and requires special attention to obtain and maintain sterility. Moreover, in case virosomes are used as transport vehicles, the compounds to be encapsulated could be adsorbed or inactivated by the hydrophobic resin (19).

A new improved method to produce influenza virosomes based on the solubilization of the influenza virus membrane using short-chain phospholipid dicaproylphosphatidylcholine (DCPC). De Jonge et al. have constructed successfully influenza H3N2 virosome structure using DCPC as solubilizing agent in 2007 (14).

In this study influenza A/ PR8 (H1N1) virosome was propagated using a dialyzable short-chain phospholipid DCPC. They were shown by electron microscope.

## Methods

### Virus culture

MDCK (Madin Darbey Canine Kidney) cells were cultured in DMEM medium and optimization of culture conditions for the best propagation of influenza virus was accomplished. Influenza virus (A/PR8/34) was kindly provided by Professor Jan Wilschut (Groningen University, Netherland). Influenza virus inoculated on to MDCK cell line and the virus was harvested after 48 hours. Cell debries were removed by twice centrifugation at 5000 rpm for 5 min. Titration of the viruses in supernatant was performed by hemagglutination assay and dilutions over 1:1024 was assumed as the optimal titer to work with.

### Virus concentration and purification

Viruses in the media were concentrated and purified using two systems. Firstly crude viruses administered to Tangential Flow Filtration (TFF) ultra-filtration set (Millipore, USA) for primitive concentration in 1:10 ratio using a filter with 100,000 Dalton cut off. Further concentration was performed using discontinuous sucrose gradient (10%/60% w/v sucrose in HBS) and centrifugation for 1.5 h (100.000 x g at 4°C at Optima XL-90 Beckman rotor Ti-90, USA ).

The final purified viruses were recovered from the interface of the two sucrose layers, transferred to a 3-12 ml dialysis cassette (Slide-A-Lyzer, Pierce, Rockford, USA) and dialyzed against 2 L of HBS at 4°C for

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overnight to remove the sucrose. The dialyzed influenza viruses were sedimented by ultracentrifugation (100.000 x g for 1 h at 4°C) and the virus pellet was resuspended in 375 µl of HBS with a 1 ml syringe equipped with a 25-gauge needle.

**Solubilization of the viral membrane**

To solubilize the viral membrane, 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DC PC) was used instead of the previously used mono(*n*-dodecyl) ether ($C_{12}E_8$). DCPC is a short-chain detergent-like lecithin with a critical micelle concentration of 14 mM. The viral membrane was dissolved by addition of 375 µl 200 mM DCPC in HBS. The suspension was incubated on ice for 30 min and the nucleocapsid was pelleted by ultracentrifugation (85.000 x g for 30 min at 4°C).

**Reconstitution of virosome**

After Ultracentrifugation, the supernatant consist of the viral glycoproteins and lipids were harvested and the pellet containing ribonucleoprotein of influenza virus was taken separately. Reconstitution of the virus membrane was accomplished by removal of DCPC by means of dialysis cassette with a cut off (MWCO) of 10,000 Dalton. The supernatant containing the viral lipids and membrane proteins was dialyzed against 2 L of HBS overnight at 4°C in a 0.5-3 ml Slide-A-Lyzer (Pierce, Rockford, USA). The buffer was exchanged once and the supernatant was dialyzed for another 12 h.

**Purified virosome preparation**

After dialysis the preparations (termed 'crude virosome preparation') were applied to a discontinuous sucrose gradient (10%/50% w/v sucrose in HBS) and centrifuged for 1.5 h (100.000 x g at 4°C) to separate non-incorporated material from the reconstituted virus membranes. The final preparations (termed 'purified virosome preparation') were recovered from the interface of the two sucrose layers, transferred to a 0.5-3 ml Slide-A-Lyzer and dialyzed against 2 L HBS overnight at 4°C to remove the sucrose.

**Protein assay**

The virosomes were analyzed for protein content by a micro Lowry assay [15]. Briefly : Sodium deoxycholate, TCA, lowry solution and Fulin Cordova solution were used to measure total proteins of the virosomes and the optical density at 750 nm ($OD_{750}$) was measure using Elisa Reader (ANTHOS 2020). Bovine serum albumin (BSA) was used as standard protein to perform the standard curve.

**Hemagglutination assay**

Hemagglutination was performed as confirmatory assay to show whether the reconstituted virosome is able to agglutinate the red blood cells via its surface proteins, haemagglutinin? Serial dilutions of influenza virosome in phosphate buffered saline were set up in a U-shaped 96-wells microtiter plate. Chicken Red blood cells (RBCs) were isolated from whole blood and 1% suspension of freshly isolated erythrocytes in PBS were prepared. An equal volume of 1% red blood cells was added to each well. The plate was gently shaken to mix the contents of each well. After 60 min incubation at 4 °C, sedimentation of the RBCs was visually assessed in each well. The HA was expressed as hemagglutinating units (HAU)/ml; where HAU was defined as the last dilution of the virosome to have hemaglutination (no sedimentation of the RBC).

**Electron Microscopy**

Influenza virus and virosome were dialyzed against ammonium acetate buffer (75 mM ammonium acetate, 2.5 mM Hepes, pH 7.4) overnight at 4°C. The virosome suspensions were applied to glow-discharged 200 mesh grids covered with a Formvar film. Virosomes were stained with freshly prepared 3% ammonium molybdate, pH 7.2 and examined under electron microscope.

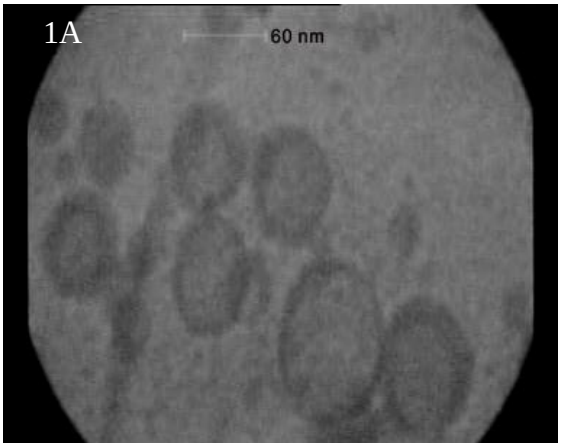
## Results

**Biochemical and biophysical characterization of purified virosome**

Total proteins of reconstituted influenza virosome as well as the whole virus pre and post concentration were determined by lowry assay. The average of our experiments showed purified virosomes contained approx. 37.5% of the initial membrane proteins (Table 1).

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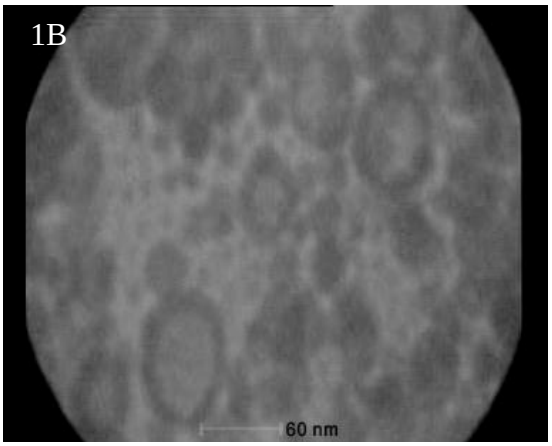




**Fig. 1A & B.** Analysis of influenza virosomal nanobioparticles by negative stain transmission electron microscopy.

**Table 1.** Protein concentration of the virus and virosome.

| Total viral protein | Viral surface protein | Reconstituted virosomes |
|---|---|---|
| 2000 µg | 400 µg | 150 µg |

**Table 2.** Hemagglutination titer of the virus and virosome.

| Before concentration | After concentration | Virosome |
|---|---|---|
| 20480/ml | 2097152/ml | 655360/ml |

**Hemagglutination assay**

The functional feature of hemagglutinin incorporated in virosomes was determined by HA assay. Influenza virosomes exhibited a high agglutination activity (655,360 HAU/ml) (Table 2).

**Electron Microscopy**

Virosomes produced in this study resemble native virus, as revealed by negative stain electron microscopy (Fig. 1 A and B). Virosomes purified on a discontinuous sucrose gradient had an average diameter of 50-100 nm.

## Discussion

The production of influenza virosomal candidate has been described elsewhere. It was produced by extracting haemagglutinin (HA) and neuraminidase and fatty acids from influenza virus and reassembling them to produce a liposome-like structure (16-20). The influenza virus enters cells through interaction of the viral envelop glycoprotein HA with sialic acid residues on the target cell surface. This is followed by entry of the virus particles into the endocytic pathway and subsequent low pH-dependent fusion of the viral envelop with the endosomal membrane (17-18). By desirable quality of the viral membrane glycoproteins, virosomes combine to cellular receptors and fuse with the target cell membrane, thus actively deliver encapsulated compounds to the cytosol of the target cells. Virosomes provide a platform that has the capacity to meet both requirements as a candidate vaccine. First, virosome acts as an antigen presentation system to activate both the humoral and the cellular arm of the host immune system, to induce antibody responses as well as cell-mediated immunity. Second, virosomes provide the opportunity to incorporate lipophilic or amphiphilic adjuvants as a vaccine to enhance the antibody response against the viral haemagglutinin (12).

A virosomal influenza vaccine has been commercially available for a number of years (Inflexal V®). Clinical studies have

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demonstrated that intramuscular administration of it induces haemagglutination–inhibition (HI) titers in human, similar to those induced by conventional whole virus or subunit vaccines. The virosome-adjuvanted hepatitis A virus (HAV) vaccine induced seroconversion in 100% of vaccines with high and long-lasting HAV-specific antibody titers. The vaccine is marketed in several countries under the name Epaxal®. Application of influenza virosome as a delivery system for protein\peptide and nucleic acids was investigated in previous studies. Like native influenza virus, influenza-derived virosomes enter cells through receptor mediated endocytosis and subsequently fuse with the endosomal membrane (12-13).

Influenza virus has been used for the generation of virosomes for more than a decade. Preparation of virosomes from influenza virus or other enveloped viruses generally involves solubilization of the viral membrane, followed by deletion of the viral RNAs and proteins by ultracentrifugation. Then, reconstitution of the viral membrane is accomplished by deletion of the detergent by dialysis (9-11).

In conclusion, in this study we describe and characterize the production of influenza A/PR8 (H1N1) virosome using a dialyzable short-chain phospholipid DCPC as a membrane solubilizer. Since the virosome has a virus like structure having HA glycoproteins on the surface, it could mimic the hemagglutination capability on the virus. Hemagglutination assay result confirmed that the glycoprotein HA was truly incorporated into virosomes retaining its biological activity. Future studies may be needed to evaluate capability of these virosome to induce immune responses against influenza virus in animal models.

## Acknowledgment

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## References

1. Brian R, Julius A, Robert M, Chanock MD. Association of serum anti-neuraminidase antibody with resistance to influenza in man. N Engl J Med, 1972;286(25):1329-32.
2. JL V. Host defenses against influenza virus: the role of antihemagglutinin antibody. J Immunol. 1975;115:434-439.
3. Cox RJ, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol 2004;59(1):1-15.
4. MR H. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. Vaccine. 2002;20(25-26):3068-87.
5. Block SL, Hayden FG, Ambrose CS, Zeng W, Walker RE. Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5–49 years of age. vaccine, 2008 ;26(38):4940-6.
6. Cintra OA. Safety, immunogenicity and efficacy of influenza vaccine in children. J. Pediatr, 2006;82: S83-90.
7. KL N. The efficacy, effectiveness and cost-effectiveness of inactivated influenza virus vaccines. vaccine. 2003;21:1769–75.
8. Greenberg HB. Immunization against viral respiratory disease: a review. Pediatr Infect Dis J. 2004;23:254-61.
9. Uchida T, Yamaizumi M, Miyake Y, Okada Y. Reconstitution of lipid vesicles associated with HVJ (Sendai virus) spikes. Purification and some properties of vesicles containing nontoxic fragment A of diphtheria toxin. J. Cell Biol, 1979;80(1): 10–20.
10. Stegmann T, Booy FP, Van Breemen JZ, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. EMBO J. 1987;6(9):2651–59.
11. Paternostre M, Meyer O, Ghanam M, Ollivon M, Blumenthal R. Solubilization and reconstitution of vesicular stomatitis virus envelope using octylglucoside. Biophys. J. 1997;72(4):1683–1694.
12. Huckriede A, Stegmann T, Daemen T, Medema J, Palache AM. The virosome concept for influenza vaccines. vaccine. 2005;1:26-38.
13. Paternostre H. Short-chain phospholipids as detergents, Biochim. Biophys. Acta. 2000;1508(1-2):164-81.
14. De Jonge J, Holtrop M, Schoen P, Scherrer P, Cullis PR, Wilschut J, Huckriede A. Cellular gene transfer mediated by influenza virosomes with

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[ DOI: 10.21859/isv.5.2.13 ]

encapsulated plasmid DNA. Biochem. J. 2007;1: 41–49.

15. Peterson GL. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. 1977;83: 346–356.

16. Earp LJ, Park HE, White J. The many mechanisms of viral membrane fusion proteins. Curr. Top. Microbiol. Immunol. 2004;285: 25-66.

17. Jardetzky TS. Virology: a class act. Nature. 2004; 427:307-308.

18. Bron R, Wilschut J. Cellular cytoplasmic delivery of a polypeptide toxin by reconstituted influenza-virus envelopes (virosomes). Biochemistry. 1994; 33(31):9110-7.

19.Kheiri MT. Protective immunity in mice following immunization with the cochleate-based subunit influenza vaccines. Iranian Biomedical Journal. 2001;5(1):33-38.

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