# Concentration and characterization of influenza antigen bulks for potential novel vaccine delivery applications

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## ABSTRACT

As a part of reformulating influenza vaccines by novel delivery technologies it is essential to work with concentrated antigens. This study outlines evaluation of techniques for concentration of seasonal influenza monobulks, characterization of the concentrated antigens in vitro, and immunogenicity of concentrated antigens in vivo. Overall, the individual influenza monobulks were successfully concentrated by the TFF process and the functional activity of the HA was maintained throughout the process. The in vivo immunogenicity of the antigens is retained and is comparable to the unprocessed monobulks. Our demonstrated ability to concentrate current monobulk antigens is an important step towards reformulating influenza antigens for vaccine delivery using new technologies.

***Keywords***: influenza vaccine

## 1 INTRODUCTION

The influenza vaccine is given each year during the flu season to protect against the seasonal influenza virus. Traditionally, an intramuscular injection of antigens from three viruses – A (H1N1), A (H3N2) and B is administered to patients [1]. Today, many companies are evaluating alternative routes of administration for delivery of influenza vaccine, some of them already in market – IDflu by Sanofi/BD a liquid vaccine administered intradermally and Flumist from Medimmune administered intranasally [2,3]. However, the achievement of highly concentrated antigen is a major challenge for reformulating influenza vaccines for administration by novel delivery technologies. The purpose of this study was to concentrate seasonal influenza monobulks, to characterize the concentrated antigens, and to evaluate the concentrated antigens for immunogenicity in vivo.

## 2 MATERIALS AND METHODS

Influenza monobulks for three strains from the 2008/2009 season were obtained from a Novartis manufacturing facility. The strains used were B/Florida/4/2006 (B), A/Brisbane/59/2007 (H1N1), and A/Brisbane/10/2007 (H3N2).

### 2.1 Concentration of influenza monobulks by TFF

The monobulks were concentrated by tangential flow filtration (TFF) using the Spectrum Labs Kros-Flo system equipped with PES hollow fiber filters with a 30kDa cutoff. The antigens were concentrated to different extents depending on the purity of the starting material. Aliquots of the retentate and permeate from the TFF process were collected and used for subsequent characterization.

### 2.2 Characterization of concentrated antigens

The hemagglutinin (HA) content for the monobulks, concentrated antigens, and the permeate was determined by single radial immunodiffusion (SRID) and the total protein content was measured by BCA Assay using the Pierce BCA Protein Assay Kit (Thermo Scientific).

The unprocessed monobulks, concentrated antigens, and the permeate from the TFF process were monitored by size exclusion chromatography (SEC) using a TKSgel column (Tosoh) with UV detection at 215nm (Waters). The mobile phase was 1X PBS (Mediatech) at a flow rate of 1ml/min. A gel filtration standard (Bio-Rad) was run with the samples.

After TFF concentration, the proper amount of NuPAGE sample buffer (Invitrogen) was added to the monobulks, retentates, and permeates, and the samples were vortexed and incubated at 90°C for 10 minutes. The samples were allowed to cool to room temperature and then loaded on a 4-10% Bis-Tris gel (Invitrogen) with a protein ladder (Invitrogen) in Lane 1. The gel was run at 190V for 90 minutes, stained overnight in Coomassie staining buffer (Invitrogen), destained in deionized water for several hours, and imaged.

The Tween80 content in the concentrates after TFF concentration was measured by RP-HPLC using a Jordi DVB 500 A column with refractive index detection (Waters 2414). THF (Sigma) was used as the mobile phase and the retention time for the Tween80 peak was around 4.6 minutes. The Tween80 content for unknown samples was estimated based on a linear regression of Tween80 standards (Sigma).

Cetyl trimethylammonium bromide (CTAB) in the concentrated material was determined by a colorimetric assay based on the formation of a colored complex of bromophenol blue (Acros) and CTAB (Calbiochem) in hydrochloric acid (VWR). The complex was extracted with chloroform (Sigma) and the absorption at 420nm was measured using the Gen5 Biotek Spectrophotometer, and the measured absorption at 420 nm was proportional to the CTAB concentration.

For stability studies, aliquots of the concentrated antigens were stored in glass vials at 4°C and analyzed for protein content by BCA and HA content by SRID at each time point for up to five weeks.

## 2.3 Immunogenicity of concentrated antigens

The concentrated influenza antigens were evaluated for their immunogenicity in vivo and compared against original monobulk vaccine material. The angitens were administered intramuscularly in Balb/C, and serum samples were collected and evaluated for functional anti-influenza antibody titers by hemagglutination inhibition (HI). The HI assay was carried out on individual sera taken 2 weeks after the second immunization. Briefly, 25 µl of two-fold serially diluted samples were incubated with 25 µl of strain-specific influenza antigen (whole virus, containing four hemagglutinin units) for 60 minutes at room temperature. A 0.5% v/v suspension of red blood cells obtained from adult turkeys were added and the mixture was incubated for another 60 minutes. Reactions were followed through visual inspection: a red dot formation indicates a positive reaction (inhibition) and a diffuse patch of cells a negative reaction (hemagglutination). As a negative control and to determine the background values of the assay, serum samples of mice immunized with buffer were tested in parallel. Serum response to vaccine antigens was considered positive if a rise in antibody titers > 4-fold compared to background was detectable. All sera were run in duplicate. The HI titer is defined as the serum dilution in which the last complete agglutination inhibition occurs. The antibody concentration corresponds to the reciprocal value of the titer.

# 3 RESULTS AND DISCUSSION

## 3.1 Concentration of antigens and characterization

The influenza monobulks from strains B, H1N1, and H3N2 were concentrated to HA levels of 29, 28, and 20 mg of HA per ml respectively by SRID (Table 1). SRID results for the concentrated antigens and the permeate from the TFF process compared to the unprocessed monobulk indicated that concentrated antigens remain intact by SRID, and that there is little or no HA present in the permeate. H3N2 was the most challenging to concentrate because it had the lowest starting HA concentration. The ratio of HA/Total Protein is approximately equivalent for the unprocessed monobulks and concentrated material. The highest concentration-fold achieved by TFF was for H1N1, which was concentrated 82-fold.

Table 1: Total protein and HA content for influenza antigens concentrated by TFF

| Strain | Total Protein Content (mg/ml) | HA Content (mg/ml) | HA/Total Protein | Conc. Fold |
|---|---|---|---|---|
| B Monobulk | 0.543 | 0.493 | 0.91 | 59 |
| B TFF Concentrate | 37.516 | 29.0 | 0.77 | |
| H1N1 Monobulk | 0.515 | 0.344 | 0.67 | 82 |
| H1N1 TFF Concentrate | 32.215 | 28.07 | 0.87 | |
| H3N2 Monobulk | 0.344 | 0.249 | 0.72 | 74 |
| H3N2 TFF Concentrate | 26.53 | 18.52 | 0.70 | |

The molecular weight of the HA protein is approximately 76kDa, and aggregates of trimers may be present in the monobulk. This is evident in the SDS-PAGE gel for the unprocessed monobulk, where there is a band at ~76kDa corresponding to the monomer, a band just above 160kDa corresponding to the dimer, and a band around 260kDa corresponding to trimers or aggregates. The protein bands for concentrated B/Florida/4/2006 were consistent with unprocessed monobulks by SDS-PAGE analysis, as demonstrated in Figure 2, and similar results were obtained for the other two antigens. The absence of protein in the permeate further confirms that little or no HA is lost to the permeate during the TFF process.

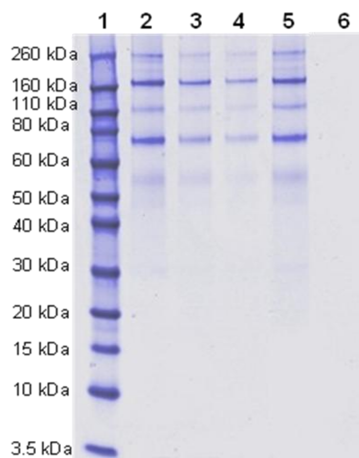
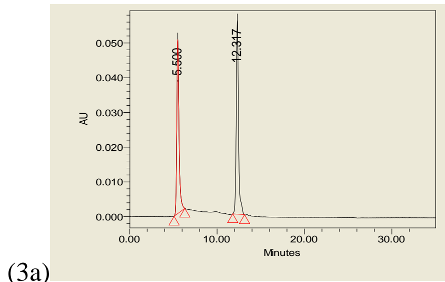
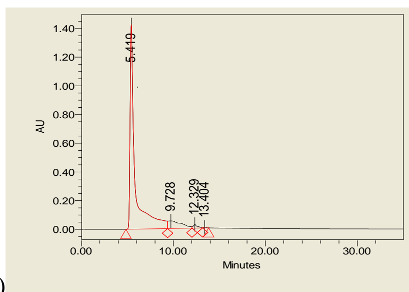



Figure 2: SDS-PAGE for aliquots from TFF concentration of B/Florida/4/2006. Lane 1: Molecular weight ladder. Lanes 2,3,4: B/Florida/4/2006 monobulk 0.5ug, 1.0ug, 2.0ug loaded respectively. Lane 5: B/Florida/4/2006 TFF concentrated to 24mgHA/ml, 1.0ug loaded. Lane 6: B/Florida/4/2006 TFF permeate.

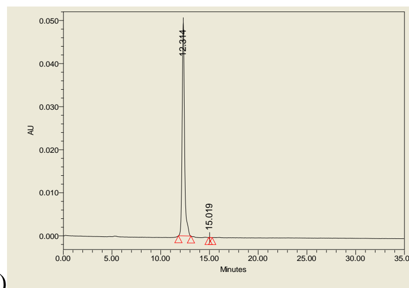
SEC chromatograms for B/Florida are shown in Figure 3. The early peak around 5 minutes for rosettes in the monobulk (corresponding to proteins > 670kDa) is retained in the concentrated material, while the peak around 12 minutes (corresponding to proteins < 2kDa) is present in the permeate. Similar results were seen for the other two strains.




(3a)




(3b)




(3c)

Figure 3: SEC profile for (a) the unprocessed monobulk, (b) concentrated antigen retained during TFF, and (c) permeate collected during TFF concentration for A/Brisbane/59/2007 (H1N1).

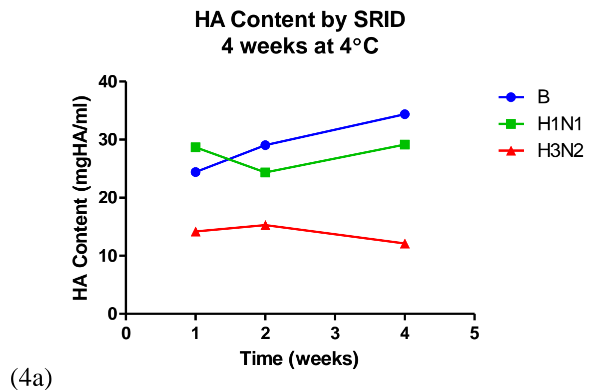
Tween80 and CTAB are used in the purification of subunit antigen from the virus grown in eggs and present in the influenza monobulk material. The monobulks contain 0.2-0.3µg Tween80 per µg HA and less than 0.2µg CTAB per µg HA. As seen in Table 2, Tween80 is concentrated along with the antigens during TFF and the Tween80/HA ratio is comparable to that of the unprocessed monobulks. CTAB is not concentrated during the process and the CTAB/HA ratio is lower for the concentrated material as compared to the monobulk.

Table 2: Tween80 and CTAB content for influenza antigens concentrated by TFF.

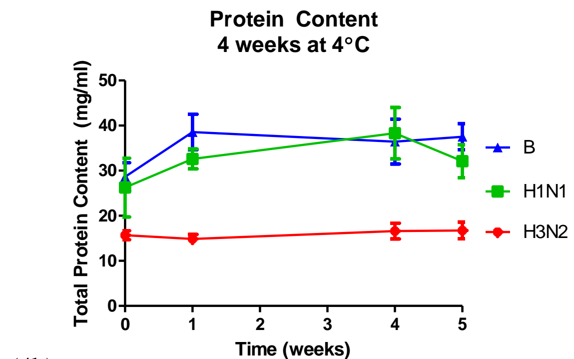
| Strain | µgTween80/ µgHA | µgCTAB/ µgHA |
|---|---|---|
| B Monobulk | 0.21 | ≤ 0.2 |
| B TFF Concentrate | 0.17 | 0.01 |
| H1N1 Monobulk | 0.23 | ≤ 0.2 |
| H1N1 TFF Concentrate | 0.16 | 0.01 |
| H3N2 Monobulk | 0.20 | ≤ 0.2 |
| H3N2 TFF Concentrate | 0.09 | 0.01 |

## 3.2 Stability of concentrated antigens at 4°C

The antigens concentrated by TFF were found to be stable for 3-4 weeks in solution at 4°C by SRID analysis (Figure 4). The stability and aggregation behavior of the antigens was also confirmed by SEC and SDS-PAGE over the stability period. Beyond 4 weeks, the antigens are visibly aggregated and HA is not detected by SRID.




(4a)




(4b)

Figure 4: Stability of concentrated antigens. (a) HA Content by SRID for 4 weeks at 4°C. (b) Protein content for concentrated antigens for 5 weeks at 4°C.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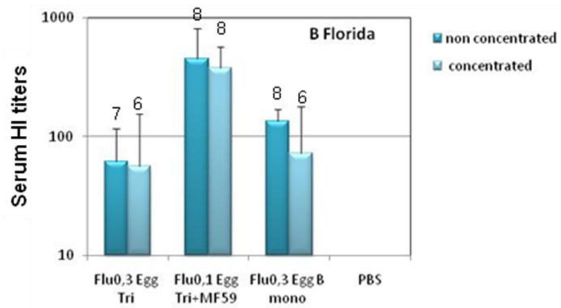
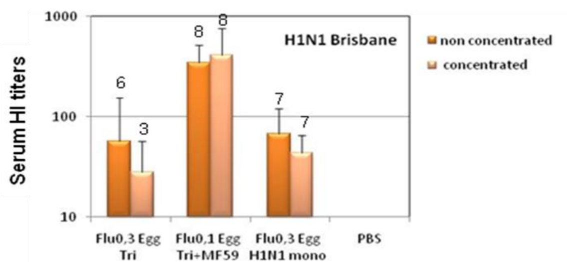
## 3.3 Immunogenicity of concentrated antigens

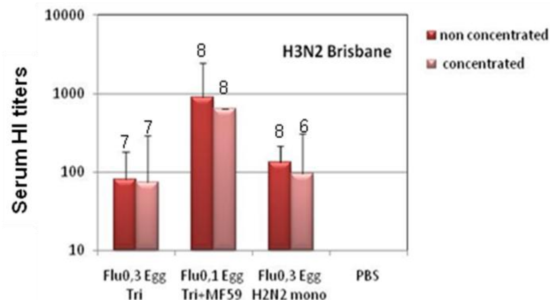
Serum HI titers for the concentrated antigens administered individually and as a trivalent vaccine with and without MF59 adjuvant were found to be comprable to that of unprocessed monobulks formulated with and without MF59 (Figure 5). This indicates that the antigens maintain their immunogenicity through processing by TFF.




(5a)




(5b)




(5c)

Figure 5: Serum HI titers for the concentrated antigens (a) B/Florida, (b) H1N1, and (c) H3N2 administered individually and as a trivalent vaccine with and without MF59 adjuvant.

# 4 CONCLUSIONS

Overall, the individual influenza monobulks were successfully concentrated by TFF, with concentration levels between 59 - 82-fold achieved for the different strains. The functional activity of HA was maintained throughout the process as demonstrated by SRID. The concentrated antigens were stable in solution for 3-4 weeks at 4°C. The immunogenicity of the antigens is retained during the process.

Our demonstrated ability to concentrate the representative monobulk antigens is an important step towards reformulating influenza antigens for vaccines delivery using new technologies. Future studies looking at the concentration of other strains will be necessary in order to evaluate the robustness of the process for strain changes from year to year. In addition, it will be necessary to optimize the TFF process to achieve higher yield.

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