

# Influenza virus hemagglutinin as a vaccine antigen produced in bacteria

Violetta Sączyńska<sup>✉</sup>

Institute of Biotechnology and Antibiotics, Warsaw, Poland

Recombinant subunit vaccines based on hemagglutinin proteins produced in bacteria (bacterial HAs) are promising candidates for enhancing the supply of vaccines against influenza, especially for a pandemic. Over 20 years after the failure to obtain the antigen with native HA characteristics in the early 1980's, there are increasing data on successful production of HA proteins in bacteria. The vast majority of bacterial HAs have been based on the HA1 subunit of HA expressed separately or as a component of conjugate vaccines, but those based on the ectodomain and the HA2 subunit have also been reported. The most of HAs have been efficiently expressed as insoluble aggregates called inclusion bodies. Refolded and purified proteins were extensively studied for structure, the ability to bind to sialic acid-containing receptors, antigenicity, immunogenicity and efficacy. The results from these studies contradict the view that glycosylation determines the correct structure of the hemagglutinin, as they proved that bacterial HAs can be valuable vaccine antigens when appropriate folding and purification methods are applied to rationally designed proteins. The best evidence for success in bacterial production of protective HA is that vaccines based on proprietary Toll-like Receptor (VaxInnate) and bacteriophage Q $\beta$ -VLPs (Cytos Biotechnology) technologies have been advanced to clinical studies.

**Key words:** recombinant influenza hemagglutinin, recombinant influenza vaccine, subunit influenza vaccine, bacterial hemagglutinin, prokaryotic protein production

**Received:** 29 May, 2014; **revised:** 22 August, 2014; **accepted:** 22 August, 2014; **available on-line:** 08 September, 2014

## INTRODUCTION

Research on subunit vaccines against influenza responds to the demand for technology that would allow the efficient production of the vaccine in a relatively short time, contrary to the traditional egg- or cell culture-based manufacturing (Osterholm *et al.*, 2012). The availability of such technology would be useful for seasonal vaccine production, especially as vaccine antigens need to be matched to circulating strains of influenza virus on an annual basis. This is of special importance in case of a pandemic flu threat, as was highlighted by the emergence of the 2009 H1N1 swine influenza virus pandemic (H1N1pdm09). Currently, the highly pathogenic avian influenza virus (HPAIV) H5N1 poses a permanent pandemic threat as there is still a risk that the virus will acquire the ability of direct transmission between humans. Moreover, emerging disease outbreaks often

of epizootic characteristics are accompanied by high infectivity and mortality among birds, including domestic avian species. Therefore, the development of efficacious influenza vaccines against H5N1 HPAIV is of veterinary and public health significance. An important advantage of the subunit vaccines against HPAIVs is that their usage does not interfere with epidemiological surveillance, as the serological differentiation of naturally vaccinated animals/flocks from vaccinated ones is possible. The so-called DIVA (differentiation of infected from vaccinated animals) strategy is a prerequisite for vaccinations against avian influenza (Suarez, 2005).

The obvious candidates for the production of subunit vaccines against influenza are the major surface glycoproteins of influenza viruses: hemagglutinin (HA) and neuraminidase (NA), since they induce a specific humoral immune response that provides immunity or recovery after the infection (McMurry *et al.*, 2007). Immunity induced by HA and NA varies considerably (Johansson *et al.*, 1989; Sylte & Suarez, 2009) because of different roles they play in the life cycle of influenza viruses. By binding to sialic acid residues of host cell receptors, HA allows internalization of the influenza viruses by endocytosis, and then mediates the fusion of viral and cellular membranes leading to the release of viral RNA to the cytoplasm and virus replication (Skehel & Wiley, 2000). NA is an enzyme that cleaves terminal sialic acid residues from the oligosaccharide chain which determines the release of progeny virus from infected cells and thereby the virus spread (Lamb & Choppin, 1983). Antibodies against HA generated both during infection and vaccination have the ability to neutralize influenza viruses (Wilson & Cox, 1990). In contrast, anti-NA antibodies, referred to as infection permissive, do not prevent a viral infection and act at a later infection stage. Therefore, HA is considered as the main constituent of subunit vaccines, while NA as an additional component enhancing vaccine effectiveness (Johansson & Brett, 2007). HA is also an immunodominant antigen in conventional vaccines against influenza (Sylte & Suarez, 2009).

Hemagglutinin is synthesized as a single polypeptide chain, the so-called precursor HA (HA0), containing a hydrophobic signal sequence, HA1 and HA2 subunits separated by the cleavage site for proteolytic enzymes

<sup>✉</sup> e-mail: saczynskav@iba.waw.pl

**Abbreviations:** HA, hemagglutinin; HA1(HA2), subunit 1(2) of hemagglutinin; VLP, virus-like particle; H1N1pdm09, 2009 H1N1 pandemic; HPAIV, highly pathogenic avian influenza virus; DIVA, differentiation of infected from vaccinated animals; NA, neuraminidase; rHA, recombinant hemagglutinin; Mab, monoclonal antibody; TLR, Toll-like Receptor; APC, antigen presenting cell; gH, globular domain; ssRNA, single stranded RNA; HI, hemagglutination inhibition; HP, highly pathogenic

(Wilson & Cox, 1990). The apolar region of the HA2 subunit adjacent to the cleavage site is referred to as a fusion peptide (Skehel *et al.*, 2001). HA is classified as an integral membrane protein with a large hydrophilic domain on the external surface of the membrane, a small hydrophobic transmembrane domain, and a hydrophilic cytoplasmic domain on the internal side of the membrane (Wilson *et al.*, 1981). The transmembrane and cytoplasmic domains are formed by C-terminal fragments of the HA2 subunit. Posttranslationally, HA undergoes host-cell dependent glycosylation and is cleaved to subunits connected by a disulfide bridge. Cleavage of HA0 is a prerequisite for protein activation, which determines infectivity of influenza viruses (Skehel & Wiley, 2000).

Up to date, crystal structures of several HAs have been solved. All of them share a similar three-dimensional structure. The protein consists of a globular domain formed by the HA1 subunit and the stem domain formed largely by the HA2 subunit, but also by amino acid sequences from the N- and C-termini of the HA1 subunit (Wilson *et al.*, 1981; Ha *et al.*, 2002). The globular domain of HA contains the receptor-binding subdomain, by which the virus binds to the host cell, while the fusion of viral and endosomal membranes is mediated by the stem domain (Wilson *et al.*, 1981; Skehel & Wiley, 2000). The HA2 subunit of the stem is responsible for the formation and stability of the HA homotrimer and its anchorage in the lipid envelope of influenza viruses (Wilson *et al.*, 1981). Cleavage of HA results in structural rearrangements (Chen *et al.*, 1998) such as upon reduction of the pH in the endosome to ~5, the protein adopts a fusogenic conformation in which the fusion peptide is exposed, binds to the endosomal membrane and fusion takes place (Skehel & Wiley, 2000).

The globular domain of HA comprises epitopes, which are targeted by antibodies neutralizing virus infectivity by interference with binding of viruses to the host cell receptors (Wiley *et al.*, 1981; Bizebard *et al.*, 1995; Fleury *et al.*, 1999). Antibodies to the stem domain are able to neutralize the virus infectivity by blocking membrane fusion (Okuno *et al.*, 1993; Ekiert *et al.*, 2009; Sui *et al.*, 2009). Antibodies against the HA1 subunit cross-linking globular domains of the HA trimer could also have the ability to prevent membrane fusion (Barbey-Martin *et al.*, 2002). Due to the variability and susceptibility to mutations (Shih *et al.*, 2007), the globular domain is considered as an immunogenic HA fragment for subtype-specific vaccine production. In contrast, the stem region is relatively well conserved among different HA subtypes (Krystal *et al.*, 1982), and therefore is seen as the target for the universal vaccine development (Pica & Palese, 2013).

## CHALLENGES OF BACTERIAL HEMAGGLUTININ PRODUCTION

The first research on HA protein expression in bacterial cells (the bacterial HA) was undertaken in the early 1980's. Attempts to obtain direct expression of HA proteins failed because of the protein instability in bacterial cells similar to that observed for other complex heterologous proteins expressed bacterially at that time (Davis *et al.*, 1981). For efficient expression, the fragments of influenza virus HA were produced in fusion with bacterial proteins:  $\beta$ -galactosidase (Davis *et al.*, 1981) or trpLE' (Davis *et al.*, 1983). Studies on the antigenic-

ity and immunogenicity of the obtained bacterial HA showed that the protein structures are quite different from those displayed by the native protein (Davis *et al.*, 1981; 1983; Nayak *et al.*, 1985). At the same time, recombinant HAs (rHAs) with biological and structural characteristics similar to native viral HA were obtained in the eukaryotic (animal cell cultures, yeast) expression systems (Nayak *et al.*, 1985).

The potential difficulties of efficient production of properly folded bacterial HA, such as the presence of three hydrophobic regions (signal sequence, fusion peptide, transmembrane domain) and lack of glycosylation, were already seen at the first attempts to obtain the antigen by direct expression in prokaryotic cells (Davis *et al.*, 1981; 1983; Nayak *et al.*, 1985). In bacteria, large hydrophobic regions of proteins are recognized as misfolded and undergo degradation (Baneyx & Mujacic, 2004), and this limits or prevents bacterial expression of transmembrane proteins. Besides, such proteins are usually lethal when expressed in bacterial strains commonly used in the production of biopharmaceuticals. Although the strains of bacteria intended for the production of transmembrane proteins are currently available (Miroux & Walker, 1996; Dumon-Seignovert *et al.*, 2004), hydrophobicity will always decrease expression levels. On the other hand, experience with HA expression in mammalian, insect and yeast cells suggests that soluble HA proteins based on the ectodomain of viral HA can be valuable immunogens (Saelens *et al.*, 1999; Wei *et al.*, 2008; Bosch *et al.*, 2010; Cornelissen *et al.*, 2010; Weldon *et al.*, 2010; Loeffen *et al.*, 2011).

Glycosylation is known to play an important role in HA folding during viral multiplication in host cells (Roberts *et al.*, 1993). For this reason, the widely accepted view over the years was that HA glycosylation determines the correct structure of the protein, and the lack of glycosylation is the cause of failures in obtaining valuable vaccine antigens with the bacterial expression system (Davis *et al.*, 1983; Nayak *et al.*, 1985). On the other hand, it is known that oligosaccharides attached to viral HA can modulate protein antigenicity by epitope masking and interfering with their recognition by the respective antibodies (Skehel *et al.*, 1984; Wiley & Skehel, 1987). More recently, experimental data concerning the impact of glycosylation status on conformation, host cell receptor binding activity, antigenicity, immunogenicity, and efficacy of the HA proteins have been published (Bright *et al.*, 2003; Wang *et al.*, 2009; de Vries *et al.*, 2012). Altogether these data justify the view that the removal of structurally nonessential glycans on viral surface glycoproteins (Wang *et al.*, 2009), or avoiding glycosylation (Xuan *et al.*, 2011) may constitute effective approach for vaccine design against influenza. The vaccine strategy relying on non-glycosylated HA protein has been accomplished with bacterially produced antigens. However, it should be noted that obtaining glycosylated proteins by overexpression in bacteria is possible owing to the discovery of the N-glycosylation system in *Campylobacter jejuni* (Wacker *et al.*, 2002). Indeed, glycosylation of HA1 subunit of H1 HA in recombinant *E. coli* containing the glycosylation system of *C. jejuni* has been confirmed (Farahmand *et al.*, 2012).

The main challenge of bacterial HA production is the development of a refolding method, which would allow to obtain the antigen similar to viral HA even though the HA protein can be efficiently produced in bacteria cells as a nonglycosylated protein, in addi-

Table 1. Bacteria-expressed proteins based on HA1 subunit of HA

HA protein	Influenza virus strain		Reference
< H5(1-340 aa) >	A/H5N1/Ck/Hatay	A/Chicken/Hatay/04	Shen <i>et al.</i> , 2008
H5(1-346 aa)*	A/H5N1/VN, HK, THA	consensus sequence of A/Vietnam/1194/04 A/Hong Kong/483/97 A/Thailand/LFPN-2004/04	Chiu <i>et al.</i> , 2009
H1(1-330 aa)** H5(1-330 aa)**	A/H1N1pdm09/CA07 A/H5N1/VN	A/California/07/09 A/Vietnam/1203/04	Khurana <i>et al.</i> , 2010b Khurana <i>et al.</i> , 2011b
H1(1-320 aa)***	A/H1N1pdm09/CA07 A/H1N1pdm09/CA04	A/California/07/09 A/California/04/09	Khurana <i>et al.</i> , 2011a Khurana <i>et al.</i> , 2011b
H5(1-320 aa)***	A/H5N1/VN	A/Vietnam/1203/04	Khurana <i>et al.</i> , 2011a, b Verma <i>et al.</i> , 2012
	A/H5N1/IN	A/Indonesia/5/05	Khurana <i>et al.</i> , 2011a, b
	A/H5N1/WS/Mong	A/Whooperswan/ Mongolia/244/05	Verma <i>et al.</i> , 2012
H7(1-320 aa)*** H3(1-320 aa)***	A/H7N7/NL A/H3N2/WIS A/H3N2/VIC	A/Netherlands/219/03 A/Wisconsin/15/09 A/Victoria/210/09	Khurana <i>et al.</i> , 2011b Khurana <i>et al.</i> , 2011b Khurana <i>et al.</i> , 2011b
H5(28-320 aa)	A/H5N1/VN	A/Vietnam/1203/04	Khurana <i>et al.</i> , 2011b
H1(63-286 aa)*	A/H1N1pdm09/Mex	A/Mexico/4603/09	Aguilar-Yáñez <i>et al.</i> , 2010 DuBois <i>et al.</i> , 2011
H1(63-286 aa)* 2×H1(63-286 aa)*	A/H1N1pdm09	N/A	Sánchez-Arreola <i>et al.</i> , 2013
H1(57-264 aa)* / H1(57-272 aa) / / H1(50-280 aa) /	A/H1N1pdm09/CA04	A/California/04/09	Xuan <i>et al.</i> , 2011
H3(91-261 aa)	A/H3N2/PC	A/Port Chalmers/1/73	Jeon & Arnon, 2002
HA(61-287 aa)	A/H1N1, H3N2, H5N1	H1N1 — consensus seq + H3N2, H5N1 epitopes	Dukhovlinov <i>et al.</i> , 2013

Legend to Table 1: < > purified by electroelution from SDS/PAGE gels, at least partially denatured; \*properly folded monomers; \*\*largely trimers/oligomers from properly folded monomers; \*\*\*more stable oligomers; // refolding efficiency and biochemical properties not satisfactory.

tion devoid of the regions (signal sequence, anchoring peptide) participating in the formation of higher-order structures of HA protein during its biosynthesis in host cells (Wilson *et al.*, 1981). The efforts to preserve the native structure of the vaccine HA are reasonable as most epitopes targeted by neutralizing antibodies are conformational ones (Wiley *et al.*, 1981). Moreover, results from studies on rHAs from eukaryotic expression system indicate that, in addition to a proper conformation of the HA monomer, the oligomerization status of HA antigen has a significant influence on the level and the quality of the immune response (Wei *et al.*, 2008; Weldon *et al.*, 2010). The conclusion is that the trimeric/oligomeric antigens are undoubtedly more immunogenic than monomeric ones, but the oligomerization may also impact on the repertoire of induced antibodies (Wilson & Cox, 1990; Wei *et al.*, 2008; Weldon *et al.*, 2010). In order to obtain the soluble HA proteins forming desired stable oligomeric structures, ectodomain-based antigens produced in mammalian or insect cells have been expressed together with intentionally added foreign trimerizing sequences (Wei *et al.*, 2008; Bosch *et al.*, 2009; Weldon *et al.*, 2010; Loeffen *et al.*, 2011).

## BACTERIAL HEMAGGLUTININS

The increasing data on three-dimensional structures of various-origin HAs in conjunction with the possibilities afforded by *in silico* modeling as well as the development of protein bioengineering and biotechnology have opened up new opportunities for the design of HA proteins to be expressed in bacterial cells. The currently ongoing research is focused on HA1 subunit-, HA2 subunit- and ectodomain-based bacterial HAs. Proteins containing the HA1 subunit or its fragment are intended to provide immunity against influenza viruses of a specified subtype. Despite of the presence of conservative neutralizing epitopes in the HA2 subunit (Okuno *et al.*, 1993; Ekiert *et al.*, 2009; Sui *et al.*, 2009), the longer HA immunogens can also be classified as the subtype-specific, because the globular domain inhibits immune recognition of HA stem region presumably through masking or due to immunodominance (Steel *et al.*, 2010). As highly variable antigenic sites of the HA1 subunit are immunodominant, the problem to provide cross-protection even against related influenza virus strains by vaccination with HA-based vaccines has also been encountered. Attempts to provide intra-subtype cross-protection with

Table 2. Conjugate vaccines with bacteria-expressed HA globular domain

HA protein	Influenza virus strain		Reference
[ H1(101-276 aa) ] { H1(53-324 aa)* } { STF2.H1(53-324 aa)* } H1(62-284 aa)* STF2.H1(62-284 aa)*	A/H1N1/PR8	A/Puerto Rico/8/34	Song <i>et al.</i> , 2008
STF2.H1(62-284 aa)* VAX125	A/H1N1/SI	A/Solomon Island /3/06	Song <i>et al.</i> , 2008 Treanor <i>et al.</i> , 2010 Taylor <i>et al.</i> , 2011
STF2.H1(63-285 aa)* VAX128A STF2R3.H1(63-285 aa)* VAX128B STF2R3.2×H1(63-285 aa)* VAX128C	A/H1N1pdm09/CA07	A/California/07/09	Liu <i>et al.</i> , 2011 Hong <i>et al.</i> , 2013 Taylor <i>et al.</i> , 2012 Liu <i>et al.</i> , 2011 Taylor <i>et al.</i> , 2012 Liu <i>et al.</i> , 2011 Taylor <i>et al.</i> , 2012
STF2.H5(62-284 aa)*   STF2R0.H5(62-284 aa)   STF2R3.H5(62-284 aa)* STF2R3.2×H5(62-284 aa)*	A/H5N1/VN	A/Vietnam/1203/04	Song <i>et al.</i> , 2009 Song <i>et al.</i> , 2009 Liu <i>et al.</i> , 2012; Liu <i>et al.</i> , 2012
STF2R3.H5(62-284 aa)* STF2R3.2×H5(62-284 aa)*	A/H5N1/IN	A/Indonesia/5/05	Liu <i>et al.</i> , 2012
STF2R3.H5(62-284 aa)* STF2R3.2×H5(62-284 aa)*	A/H5N1/AN	A/Anhui/1/05	Liu <i>et al.</i> , 2012
Group A (4 disulfide bridges) gH1_A1* ÷ gH1_A2*  Group B (2 disulfide bridges) gH1_B1* ÷ gH1_B4*  Group C (1 disulfide bridge) [gH1_C1] ÷ [gH1_C5]  Qβ-gH1_A1÷A2 <sup>Vs</sup> Qβ-gH1_B1÷B3 <sup>Vs</sup> Qβ-gH1_B4 <sup>Vins</sup>	A/H1N1/PR8m.a.	mouse adapted (m.a.) A/Puerto Rico/8/34	Jegerlehner <i>et al.</i> , 2013
gH1_A <sup>V</sup> Qβ-gH1_A <sup>V</sup>	A/H1N1pdm09/CA04	A/California/04/09	Jegerlehner <i>et al.</i> , 2013
Qβ-gH5_A <sup>V</sup>  Qβ-gH1_A <sup>V</sup> Qβ-gH3_A <sup>V</sup> Qβ-gH FluB_A <sup>V</sup>	A/H5N1/VN A/H5N1/IN A/H1N1/Bris A/H3N2/Uru B/Bris	A/Vietnam/1203/04 A/Indonesia/5/05 A/Brisbane/59/07 A/Uruguay/716/07 B/Brisbane/3/07	Jegerlehner <i>et al.</i> , 2013
gH1_A* Qβ-gH1_A <sup>V</sup> Qβ(pGlu)-gH1_A <sup>V1</sup>	A/H1N1pdm09/CA07	A/California/07/09	Skibinski <i>et al.</i> , 2013
Gcf(131-230 aa) — H1(62-284 aa)	A/H1N1/PR8	A/Puerto Rico/8/34	Park & Chang, 2012

Legend to Table 2: [ ] misfolded; { } refolding efficiency not satisfactory; \*properly folded; STF2 — type 2 flagellin of *Salmonella typhimurium*; STF2.H1(H5), STF2R3.H1(H5), STF2R3.2×H1(H5), STF2R0.H5 — STF-HA fusion proteins in C-term, R3, R3.2×HA and R0 formats, respectively; R — replacement of the D3 (R3) or D0 (R0) domain of STF2 with HA protein; R3.2×H1(2×H5) — two HA protein copies per molecule, one in place of the D3 domain, the second one fused to the C-terminus of STF2; | | low TLR5 activity; gH — globular domain; <sup>V</sup>gH proteins covalently linked to *E. coli* produced Qβ-VLPs derived from bacteriophage Qβ with ssRNA inside; <sup>s</sup>soluble; <sup>ins</sup>insoluble; <sup>V1</sup>gH proteins covalently linked to Qβ(pGlu)-VLPs devoid of ssRNA; Gcf — respiratory syncytial virus G protein core fragment.

Table 3. Bacteria-expressed proteins based on HA ectodomain

HA protein	Influenza virus strain		Reference
H1(1-480 aa)* H1 — 50 kDa	A/H1N1pdm09/CA07	A/California/07/09	Khurana <i>et al.</i> , 2010b
H5 — 60 kDa	A/H5N1/VN	A/Vietnam/1203/04	Biesova <i>et al.</i> , 2009
H5 — 63 kDa	A/H5N1/THA	A/Thailand/HA20/05	Horthongkham <i>et al.</i> , 2007
msyB:H5 — 97 kDa	A/H5N1/Ck/Gd	A/Chicken/Guangdong/01	Xie <i>et al.</i> , 2009

Legend to Table 3: \*properly folded monomers

HA1 subunit containing immunogens focus on both the antigen design and adjuvants. Data from studies on immunogenicity of different glycosylation forms of HA proteins indicated that the glycosylation state does not seem to significantly affect the breadth of cross-protection (Bright *et al.*, 2003; Wang *et al.*, 2009; de Vries *et al.*, 2012). The possibility to elicit broader protection by the development of effective vaccine composition was demonstrated with MF59 adjuvant applied to inactivated-H5N1 vaccines (Khurana *et al.*, 2010a).

A quite new approach to obtaining a vaccine which would provide intra- or even inter-subtype protection is engineering the antigen that could focus the immune response to the aforementioned neutralizing epitopes in the HA2 subunit (Okuno *et al.*, 1993; Ekiert *et al.*, 2009; Sui *et al.*, 2009). For this purpose, the cross-reactive HA2 determinants are being unmasked by removal of the immunodominant globular domain from HA proteins, resulting in expression of so-called stalk or stem domain-based immunogens (Steel *et al.*, 2010; Bommakanti *et al.*, 2010; 2012). On the other hand, universal influenza vaccines based on a single or a few conservative epitopes of influenza virus proteins, including HA and produced as proteins consisting of repeated sequences of selected epitopes have also been obtained by expression in bacterial cells (Li *et al.*, 2003; Rudolph & Ben Yedidia, 2011; Atsmon *et al.*, 2012). The epitope-based vaccines are beyond the scope of this review.

### Design

The bacterially produced HA proteins based on the HA1 subunit, expressed separately or as a component of conjugate vaccines as well as those based on the ectodomain and the HA2 subunit of HA are listed in the Tables 1–4 together with the virus strains from which the HA sequences were derived. The fragment of H5 HA containing the signal sequence and the HA1 subunit expressed with (Chiu *et al.*, 2009) or without (Shen *et al.*, 2008) basic amino acids of the cleavage site were produced by overexpression in *E. coli*. The shorter fragments of the HA1 subunit containing the globular domain, expressed in bacteria with the signal sequences, were obtained by Khurana and coworkers (2010b; 2011a; 2011b) and Verma and coworkers (2012). The vaccine project was inspired by results showing that large fragments of the HA1 subunit encompassing the receptor-binding subdomain are bound by broadly neutralizing human monoclonal antibodies (Mabs) from H5N1 recovered individuals, and by polyclonal convalescent sera (Khurana *et al.*, 2009).

Other designs of subtype-specific vaccines against influenza were based on the assumptions that the isolated

globular domain comprises all well-defined antibody-recognizing sites (Xuan *et al.*, 2011), and is able to better expose the receptor-binding region of HA than subunit HA1 or ectodomain (Jeon & Arnon, 2002) and furthermore, has much less surface hydrophobicity than the entire HA ectodomain, so it could be expressed with higher efficiency than larger protein fragments (Aguilar-Yáñez *et al.*, 2010). To obtain a more universal immunogen, the HA1 subunit fragment containing immunogenic epitopes of various HA subtypes was produced (Dukhovlinov *et al.*, 2013).

The first group of conjugate vaccines against influenza produced by overexpression in bacterial cells, listed in Table 2, consists of fusion proteins containing the type 2 flagellin from *Salmonella typhimurium* (STF2), which is the ligand of Toll-like Receptor (TLR) type 5. It was hypothesized that providing the antigen and TLR5 signaling to the same antigen presenting cells (APCs) would lead to enhancement of antigen presentation as well as to the induction of both humoral and cellular responses (Liu *et al.*, 2011). For this purpose, three various-length fragments comprising the globular domain were produced. Thereafter, two of them were fused to the C-terminus of STF2 by a flexible linker (Song *et al.*, 2008). Subsequently, the other formats of conjugated vaccines, denoted R0, R3, R3.2×HA, which differed in the number of copies and the placement of HA molecules in the fusion proteins, were developed (Song *et al.*, 2009; Liu *et al.*, 2011, 2012).

Conjugate vaccines of another group (Table 2) were generated by coupling of bacterial HAs to Q $\beta$ -VLPs i.e. bacterially expressed virus-like particles derived from bacteriophage Q $\beta$  (Jegerlehner *et al.*, 2013; Skibinski *et al.*, 2013). The fragments of the HA1 subunit were chosen for conjugation as they contain the globular domain (gH) comprising most of the protein's neutralizing epitopes, and are expected to fold independently from the rest of the HA molecule (Jegerlehner *et al.*, 2013). It was hypothesized that conjugation of gH protein to Q $\beta$ -VLPs would enhance its immunogenicity and efficacy through a number of mechanisms (Skibinski *et al.*, 2013). Presentation of gH proteins on the surface of VLPs in a repetitive fashion might be a strong activation signal for B cells, and the particulate nature of VLPs makes them likely to be more efficiently taken up by APCs. In addition, the bacterial single stranded RNA (ssRNA), which is incorporated into VLPs during recombinant expression, is a ligand of TLR type 7. Thus, it could activate APCs and B cells upon VLP uptake. Eleven gH proteins containing four (group A), two (group B) or one (group C) disulfide bridge(s) *per* molecule, and comprising intact secondary structure elements were rationally designed and produced (Jegerlehner *et al.*, 2013). Proteins from the A and B groups were subsequently coupled *in vitro*

**Table 4. Bacteria-expressed proteins based on HA2 subunit of HA**

HA protein	Influenza virus strain	Reference
< H5(347-522 aa <sup>1</sup> ) >	A/H5N1/Ck/Hatay	A/Chicken/Hatay/04
(1-185 aa) <sup>x</sup> °	N/A	Kim <i>et al.</i> , 2011
(23-185 aa) <sup>x</sup> °	N/A	Chen <i>et al.</i> , 1999
(38-175 aa) <sup>x</sup> °	N/A	Chen <i>et al.</i> , 1995
H3HA6 <sup>2</sup> M WT +	A/H3N2/HK	A/Hong Kong/2/68
H3HA6 <sup>2</sup> M m1 +		
H3HA6 <sup>2</sup> M m2 +		
H3HA6 <sup>2</sup> M +	A/H3N2/Phil	A/Philippines/2/82
H1HA6 <sup>2</sup> M + °	A/H1N1/PR8	A/Puerto Rico/8/34
H1HA6 <sup>2</sup> M m3 +	A/H1N1/NC	A/New Caledonia/20/99
H1HA6 <sup>2</sup> M m4 +	A/H1N1pdm09/CA07	A/California/07/09
H1HA0HA6 <sup>3</sup> M + •	A/H1N1/PR8	A/Puerto Rico/8/34

Legend to Table 4: < > purified by electroelution from SDS-PAGE gels, at least partially denatured; <sup>1</sup>full-length HA numbering; <sup>x</sup>low pH, fusogenic conformation; °trimeric; <sup>2</sup>HA2 fragment — linker — <sup>1</sup>peptide from HA1 (N-termini) — linker — <sup>2</sup>peptide from HA1 (C-termini); <sup>M</sup>mutations introduced to remove exposed hydrophobic patches and stabilize the neutral pH conformation; <sup>WT</sup>wild-type; <sup>+</sup>neutral pH, pre-fusion conformation; <sup>m1</sup>disulfide mutant 1; <sup>m2</sup>disulfide mutant 2; <sup>m3</sup>, <sup>m4</sup>additional, sequence related mutations; <sup>31</sup>peptide from HA1 (N-termini) — linker — <sup>2</sup>peptide from HA1 (C-termini) — HA2 fragment, mutation in the cleavage site; •largely aggregated.

to Q $\beta$ -VLPs to form various Q $\beta$ -gH proteins. The HA fragment was also used to design the composite vaccine against both influenza and respiratory syncytial viruses (Park & Chang, 2012).

Although the vast majority of bacterially produced antigens are based on the HA1 subunit, there are also reports on bacterial expression of the proteins containing sequences from HA1 and HA2 subunits of HA (Table 3). Thereby, the resultant immunogens comprise neutralizing epitopes of both HA subunits. The 1-480 aa fragment of the H1pdm09 HA ectodomain, expressed with the signal sequence (Khurana *et al.*, 2010b) as well as full-length ectodomain of H5 HA (Biesova *et al.*, 2009) were produced. Based on the sequences of HA from H5N1 HPAIVs, the 63 kDa immunogen (Hort-hongkham *et al.*, 2007) as well as soluble 97 kDa fusion protein of HA fragment with a chaperonin msyB (Xie *et al.*, 2009) were obtained.

Bacterial expression of HA2 subunit-based proteins (Table 4) for vaccination purposes was reported exceptionally, and the rationale was to obtain the immunogen comprising conservative neutralizing epitopes. Various-length proteins containing exclusively HA2 subunit sequences from the HA ectodomain were bacterially produced (Chen *et al.*, 1995; 1999; Shen *et al.*, 2008; Kim *et al.*, 2011). Alternatively, Bommakanti *et al.* (2010, 2012) rationally designed the stem domain immunogens containing a large portion of the HA2 subunit, and two fragments of HA1 subunit considered to be interacting with HA2 regions in the whole HA molecule. The project resulted in the expression of the prototype constructs, denoted HA6 and HA0HA6.

### Refolding and purification

The vast majority of bacterially produced HA proteins, listed in Tables 1–4, were expressed with affinity 6xHis-tags as insoluble aggregates called inclusion bodies. Besides purification, obtaining of the vaccine with

such a form of HA requires the protein to be dissolved and refolded. Experimental data were published indicating that the majority, but not all of refolded and purified bacterial HAs, adopt the correct conformation. With various procedures aiming to obtain recombinant HA1 subunit (Table 1), it was shown that the effectiveness of protein refolding strongly depends on the applied method (Chiu *et al.*, 2009). In the case of rHAs largely consisting of the globular domain (Tables: 1, 2), it has been proven that the refolding effectiveness and/or its efficiency substantially depends on the HA fragment which has been selected for the vaccine antigen design (Song *et al.*, 2008; Xuan *et al.*, 2011; Jegerlehner *et al.*, 2013). Moreover, these results highlighted the role of the secondary structure elements flanking the globular domain (Song *et al.*, 2008) as well as of the disulfide bond formation (Jegerlehner *et al.*, 2013) for proper folding of the isolated globular domain. It was also shown that some of the globular domain-based proteins (Tables: 1, 2) are able to adopt the proper conformation when they were expressed separately, in a fusion with flagellin (Song *et al.*, 2008) as well as a monomeric or dimeric protein connected by a flexible linker (Sánchez-Arreola *et al.*, 2013).

The structure and/or antigenicity studies showed that some of HA1 subunit-based bacterial HAs, listed in Tables: 1, 2, as well as the 1-480 aa fragment of HA ectodomain (Table 3) display well preserved conformational neutralizing epitopes essential for the induction of the protective immune responses (Song *et al.*, 2008; Chiu *et al.*, 2009; Aguilar-Yáñez *et al.*, 2010; Khurana *et al.*, 2010b; 2011b; DuBois *et al.*, 2011; Xuan *et al.*, 2011; Verma *et al.*, 2012; Hong *et al.*, 2013; Sánchez-Arreola *et al.*, 2013). Comparative studies on antigenicity and TLR5 activity of STF2.H5, STF2R0.H5, STF2R3.H5 fusion proteins (Table 2) showed that both HA protein and flagellin are better presented in the R3 construct than in the C-term one and that decreased antigenicity of the R0 construct is accompanied by the loss of the TLR5

activity (Song *et al.*, 2009). Substantially improved antigenicity of HA protein in the R3 configuration in comparison to the C-term one was also observed in the immunoreactivity studies of STF2:H1pdm09 fusion protein (Liu *et al.*, 2011). However, the strongest immunoreactivity was found for the R3.2×H1pdm09 construct as two HA copies were present in the fusion protein molecule. Screening of the prototype gH constructs (Table 2) produced to be coupled with Q $\beta$ -VLPs revealed that gH<sub>A</sub> and gH<sub>B</sub> proteins bind to the sialic acid on the fetuin glycoproteins indicating that receptor-binding activity of HA was preserved (Jegerlehner *et al.*, 2013).

Taking into account the importance of higher-order structure of the vaccine HAs for induction of protective immune responses, the oligomeric status of bacteria-expressed HA proteins was studied in addition to antigenicity testing. It was reported that refolded and purified HA1 subunit fragments (1-330 aa, 1-320 aa) of HAs from H1N1pdm09 and H5N1 viruses (Table 1), in contrast to the shorter H5 HA fragment (28-320 aa), existed in the majority as trimers and oligomers (Khurana *et al.*, 2010b; 2011a; 2011b; Verma *et al.*, 2012). It has been shown that oligomerization of the HA proteins involves conservative amino acids of the signal sequence and that the 1-320 aa fragments of HAs form more stable oligomers than the 1-330 aa ones. The HA1 subunit-based design was used successfully to obtain properly folded HA proteins from H1pdm09, H5, H7 and H3 HAs (Table 1). All these proteins were shown to form functional oligomers ( $\geq 70\%$ ) which generate rosette-like structures composed of trimers resembling those formed by the native HA isolated from the influenza viruses. The refolded and purified HA1 subunit-based proteins, shorter than that generated by Khurana and coworkers (2010b, 2011a, 2011b) and Verma and coworkers (2012), and expressed bacterially without the signal sequence (Table 1), did not form oligomers and existed as monomers containing the properly folded globular domain of HA (Aguilar-Yáñez *et al.*, 2010; DuBois *et al.*, 2011; Xuan *et al.*, 2011), or as dimers due to the linker peptide (Sánchez-Arreola *et al.*, 2013). In spite of the presence of the signal sequence, the properly folded ectodomain-based HA protein (1-480 aa, Table 3) was monomeric and did not display oligomeric HA activities, such as binding to fetuin and hemagglutination (Khurana *et al.*, 2010b). On the other hand, it was shown that properly folded gH proteins induce agglutination of erythrocytes with comparable efficiency to influenza viral particles upon coupling to Q $\beta$ -VLPs (Jegerlehner *et al.*, 2013).

It was reported that bacterially expressed HA2 subunit-based proteins (Table 4), containing sequences exclusively from the HA2 subunit ectodomain, spontaneously fold into the low pH conformation and trimerize forming stable structures corresponding to that of the native HA in its fusogenic state (Chen *et al.*, 1995; 1999; Kim *et al.*, 2011). By introducing the regions of the HA1 subunit that are in close spatial proximity to the HA2 subunit in the whole HA molecule as well as the rationally designed mutations to the HA2 subunit-based proteins (Table 4), it was possible to obtain soluble immunogens containing the HA2 subunit fragment in the desired neutral pH, pre-fusion conformation (Bommakanti *et al.*, 2010, 2012). This was evidenced by spectroscopic analysis and reactivity studies with broadly neutralizing Mabs. Moreover, it was shown that HA6 design is better than the HA0HA6 one, as the former is trimeric in the majority and the latter is largely aggregated.

## Immunogenicity

Immunization studies showed that bacterial HAs are immunogenic and in animals they elicit HA-specific antibodies. However, more relevant to the evaluation of the protective efficacy of HA proteins are their abilities to induce functional antibodies: inhibiting hemagglutination and neutralizing influenza viruses. Titers of these antibodies measured by the hemagglutination inhibition (HI) and the microneutralization (MN) tests are considered as correlates of protection against influenza. It was demonstrated that proteins based on the HA1 subunit (Table 1) as well as the fragment (1-480 aa) of the HA ectodomain (Table 3), for which conformational integrity was proven by structure and/or antigenicity studies, are capable of inducing antibodies inhibiting hemagglutination by homologous viruses and/or neutralizing these viruses under *in vitro* conditions (Aguilar-Yáñez *et al.*, 2010; Khurana *et al.*, 2010b; 2011a; 2011b; Xuan *et al.*, 2011; Verma *et al.*, 2012). The HI antibody-inducing ability was also shown for recombinant 60 kDa full-length ectodomain protein (Table 3) with not yet demonstrated biochemical properties (Biesova *et al.*, 2009).

In immunization studies with the 1-320 aa fragment of H5 HA (Table 1), it was shown that anti-H5 sera are neutralizing not only against the virus from which the vaccine antigen was derived, but also against viruses belonging to other H5N1 HPAIV clades (Khurana *et al.*, 2011a; 2011b). Comparative studies of antisera induced by vaccination with oligomeric (1-320 aa) and monomeric (28-320 aa) H5 HA proteins (Table 1) have documented that induction of high-titer antibodies with broad cross-clade neutralizing activity is related to antigen oligomerization (Khurana *et al.*, 2011b). The significance of oligomerization for immune response quality was further confirmed in vaccination trials with the monomeric and oligomeric forms of the 1-320 aa H5 HA protein (Table 1) obtained by size exclusion chromatography of refolded and affinity purified bacteria-expressed antigen (Verma *et al.*, 2012). Oligomeric protein was superior to the monomeric one in eliciting high-avidity anti-HA and HI antibodies against both the homologous and the heterologous antigens.

The early studies on immunogenicity of flagellin adjuvanted HA proteins carried out with STF2.H1\_SI protein (Table 2) to immunize mice and rabbits, showed the ability of the protein to induce high titers of antibodies inhibiting hemagglutination and neutralizing viruses *in vitro* (Song *et al.*, 2008). Subsequent experiments with various constructs of STF2:HA fusion proteins (Table 2) have proven that placement of the globular domain within flagellin substantially affects the titer of HI antibodies induced by the respective antigens (Song *et al.*, 2009; Liu *et al.*, 2011; 2012). Protein in the R0 configuration, as the exception, was not capable of inducing significant levels of HI antibodies in mice consistently with its low TLR5 activity. Vaccine candidates against H5N1 and H1N1pdm09 viruses in the R3 configuration were more effective in inducing protective HI antibody titers in animals than those in the C-term one. Immunogenicity of STF2R3.H5\_IN and STF2R3.H1\_CA antigens, but not of STF2R3.H5\_AN protein, was further increased by coupling the second HA molecule to the C-terminus of STF2.

Immunogenicity of gH proteins (Table 2) were examined both before and after coupling to Q $\beta$ -VLPs (Jegerlehner *et al.*, 2013; Skibinski *et al.*, 2013). The vaccine candidates when used to immunize mice evoked significantly higher native HA-specific IgG, HI and neutraliz-

ing antibody titers than their unconjugated counterparts. Immunization studies with Q $\beta$ -gH VLPs containing or devoid of ssRNA in the mouse model showed that Q $\beta$ -based vaccines induce a potent T helper cell type 1 (Th1) response and that the presence of ssRNA in Q $\beta$ -VLPs is responsible for an increased Th1 bias (Skibinski *et al.*, 2013).

### Efficacy

The ability of bacteria-expressed H1pdm09 HA immunogens to confer protection against 2009 pandemic influenza was studied in the ferret (Aguilar-Yáñez *et al.*, 2010; Khurana *et al.*, 2010b; Skibinski *et al.*, 2013) and in the mouse (Xuan *et al.*, 2011; Skibinski *et al.*, 2013) models by challenging the vaccinated animals with homologous H1N1pdm09 virus strains which are relatively mild, and thus did not cause severe disease in animals. It was shown that immunization with proteins based on the HA1 subunit (1-330 aa, 57-264 aa, 63-286 aa; Table 1), HA ectodomain (1-480 aa; Table 3) as well as with Q $\beta$ -gH1pdm09 VLPs (Table 2) induce protective immune responses against influenza in mice and ferrets (Aguilar-Yáñez *et al.*, 2010; Khurana *et al.*, 2010b; Xuan *et al.*, 2011; Skibinski *et al.*, 2013). Protective effects of vaccination were evident in that the animals did not get sick from flu or the course of the disease was milder than in unvaccinated controls.

Various formats of STF2:H1pdm09 fusion proteins (Table 2) were examined for vaccination efficacy in the lethal challenge experiment with the mouse adapted H1N1pdm09 virus (Liu *et al.*, 2011). All tested fusion proteins conferred protection against mortality when given to mice at 3  $\mu$ g and even at as little as 0.3  $\mu$ g of antigen *per* dose. However, survival rates in the mice vaccinated at 0.03  $\mu$ g-doses showed consistently with the predetermined immunogenicity rank that the order of the relative effectiveness of the three formats is: R3.2 $\times$ H1 > R3 > C-term.

In most of the challenge experiments testing bacterial H1pdm09 HAs the viral loads in the nasal lavages of ferrets (Khurana *et al.*, 2010b; Skibinski *et al.*, 2013), or in the lungs of mice (Liu *et al.*, 2011; Skibinski *et al.*, 2013) following H1N1pdm09 virus challenge were determined. This led to the conclusion that vaccination leads to substantial reduction of viral titers in both ferrets and mice. Altogether these results demonstrated that vaccination with bacterially expressed vaccine HA could provide control of influenza virus infection and transmission rates in a population under pandemic conditions.

The HA1 subunit-based bacterial HAs originated from H5N1/VN virus (Table 1): largely oligomeric, 1-320 aa, only monomeric, 28-320 aa, (Khurana *et al.*, 2011b) as well as oligomeric and monomeric fractions of the 1-320 aa protein (Verma *et al.*, 2012) were used to immunize ferrets prior to the challenge with homologous (H5N1/VN, clade 1) and/or heterologous (H5N1/WS/Mong, clade 2.2) HPAIVs. Ferrets vaccinated with largely oligomeric rHA were highly protected from lethality against homologous and heterologous challenges, resulting in 100% and 80% survival rates, respectively, while those vaccinated with the oligomeric fraction of rHA were fully protected from both strains of HP H5N1 viruses (100% survival rates). The level of animal protection after immunization with entirely oligomeric rHA was the same as observed in the groups vaccinated with the adjuvanted licensed inactivated-H5N1 subunit vaccine. In contrast, both the 28-320 aa rHA which exists as a monomer and the monomeric fraction of the 1-320 aa protein were not

very protective: only 20% and 33% of animals survived after a homologous challenge, respectively. As few as 17% of animals vaccinated with the monomeric immunogen (1-320 aa) survived after a heterologous challenge. In addition, the challenge experiments revealed that immunization of ferrets with largely or entirely oligomeric rHA (1-320 aa) reduced viral loads in the nasal washes following a challenge with H5N1 HPAIVs. Moreover, viral loads after a heterologous H5N1 challenge were more efficiently controlled in ferrets vaccinated with oligomeric fraction of rHA (1-320 aa) than in ferrets vaccinated with the commercial vaccine against H5N1, and this was related to the induction of higher-avidity antibodies against the HA1 subunit. Therefore, the success of vaccination with oligomeric forms of HA proteins is also due to the capacity to reduce viral replication in the upper respiratory tract. This is important for the prevention of lung infection as well as for the limiting virus transmission.

Four formats (C-term, R0, R3, R3.2 $\times$ HA) of STF2:H5 fusion proteins (Table 2) were examined for efficacy by the lethal homologous challenge experiments in the mouse and/or ferret models (Song *et al.*, 2009; Liu *et al.*, 2012). The protein in R0 configuration failed to induce the protective immune response in mice (0 or 10% survival rates). In contrast, STF2:H5 fusion protein formats other than R0 were highly efficacious in mice and ferrets, resulting in 100% (exceptionally 93%) survival rates for certain immunization schemes. However, immunization of mice at lower-dosage regimens demonstrated that the R3 format of protein is more effective in protecting mice from disease and death than the prototype C-term one and that the protein in the R3.2 $\times$ HA configuration is the most effective, being efficacious even at sub-microgram doses. Importantly, it was shown that STF2:H5 fusion proteins are able to reduce the virus titers in the brains, lungs and nasal wash samples of vaccinated and challenged mice and ferrets, respectively, which could predictably reduce viral transmission.

The challenge experiments with bacterially produced H5 HA immunogens were also performed in chickens, being the main target for vaccination against H5N1 HPAIV. In the high-dose regimen, full protection was achieved with the 97 kDa msyB:H5 protein (Table 3) similar to that obtained with a commercial vaccine against H5N1 HP viruses (Xie *et al.*, 2009). The ability to induce a protective immune response in chickens resulting in high rates of survival in challenge experiments with homologous and heterologous HPAIVs, was shown with bacterial H5 HA designed and produced in the Institute of Biotechnology and Antibiotics (Warsaw, Poland). Currently, the HA protein and the vaccine against influenza are subject to the patent procedure.

Several vaccine candidates based on the H1 and H3 rHAs were examined for efficacy by homologous and/or heterologous challenge experiments with the use of H1N1/PR8 or H3N2 viruses for animal infection. The early STF2:HA immunogen in the C-term configuration - STF2.H1\_PR8 (Table 2) was administered to mice subsequently infected with the mouse adapted H1N1/PR8 virus (Song *et al.*, 2008). All vaccinated animals survived the lethal challenge and showed neither weight loss nor other signs of morbidity.

Type A and B gH proteins (Table 2) were examined in challenge experiments before and after coupling to Q $\beta$ -VLPs (Jegerlehner *et al.*, 2013). Mice immunized with gH1\_B\_PR8 proteins quickly succumbed to H1N1/PR8 virus infection, and those immunized with gH1\_A\_PR8 and gH1\_A\_CA were only partially protected. In con-



trast, all mice immunized with gH proteins conjugated to QB-VLPs at the same dosage regimen survived after infection with challenging viruses. Importantly, immunization of mice with the most promising prototype influenza vaccines: QB-gH\_A1\_PR8 and QB-gH\_A2\_PR8 resulted in complete protection against lethal infection not only with the homologous, but also with highly drifted viral strains. The broad protective potential of candidate vaccines based on Q $\beta$ -gH VLPs was further strongly confirmed with Q $\beta$ -gH1\_A\_CA protein applied for mouse vaccination before infection with H1N1/PR8 virus. Moreover, it was shown that protection from mortality and largely from morbidity against both homologous and heterologous influenza virus strains can be achieved with low, even sub-microgram doses of antigen.

The HA6- or HA0HA6-like stem domain immunogens based on the HA sequences of various H1N1, H1N1pdm09 and H3N2 influenza virus strains (Table 4) successfully protected mice from homologous viral challenge, resulting in 100% survival rates at certain dosage regimens. High survival rates were also observed in mice immunized with H3HA6\_Phil protein and infected with heterologous A/HK/68(H3N2) virus (80%) as well as those immunized with H1HA6\_NC and H1HA6\_CA proteins, and infected with highly drifted H1N1/PR8 viral strain (90%). However, H3HA6\_HK failed to confer protection against a heterosubtype H1N1/PR8 influenza virus. These results led to the conclusion that stem domain immunogens are able to provide intra- but not inter-subtype protection.

### Clinical studies

VaxInnate has already generated clinical data for its bacterially produced influenza vaccines based on proprietary Toll-like Receptor technology. The first one was a monoavalent seasonal vaccine (VAX125) containing H1N1/SI virus HA protein genetically fused to flagellin in the initial C-term configuration (Table 2). With the VAX125, the seroconversion rates: 64% in healthy young adults (18–49 years old) at 0.5  $\mu$ g to 2  $\mu$ g doses and 75% in elderly subjects (65–84 years old) at 5  $\mu$ g and 8  $\mu$ g doses were achieved resulting in high seroprotection rates: 91% and 98%, respectively (Treanor *et al.* 2010; Taylor, *et al.* 2011). Importantly, the vaccine was generally well tolerated, although a few instances of flu-like symptoms at doses 3–8  $\mu$ g were noticed. Altogether, these results showed that the new vaccine is safe and highly immunogenic even in the elderly population, a group that is typically less responsive to influenza vaccines. Moreover, the efficacious doses of VAX125 were substantially lower than those used for conventional vaccines against influenza e.g. Fluzone owing to the adjuvant activity of flagellin which, on the other hand, was also responsible for the improved immune response in the elderly.

STF2:H1pdm09 fusion proteins in C-term, R3 and R3.2 $\times$ HA configurations contained in VAX128A, VAX128B and VAX128C, respectively (Table 2), and were evaluated for safety and immunogenicity successively in rabbits and in humans (Taylor *et al.*, 2012). In both models, it was found that replacing the HA antigen in the D3 position of flagellin in VAX128B and VAX128C produces a vaccine that was at least as immunogenic and better tolerated at higher doses than the C-terminal fusion construct (VAX128A). VAX128C selected for the second study caused high seroconversion (79%) and seroprotection (92%) rates in subjects 18–64 years old at the doses of 1.25  $\mu$ g and 2.5  $\mu$ g.

High levels of immune responses at low doses of antigen, and the relative ease of the production associated with bacterial expression suggest that VaxInnate's technology can represent an effective strategy for enhancing the global influenza vaccine supply. VaxInnate announced that it can generate at least 50 million doses within 5–6 months, with first doses becoming available as soon as after 12 weeks. In both a rapidly emerging pandemic and in seasonal virus mismatch events, such speed is a distinct advantage. Currently, VaxInnate's active pandemic programs focus on H5N1 and H7N9 candidates and research on seasonal influenza vaccine is still continuing. In March 2014 VaxInnate began a phase I clinical trial to evaluate a quadrivalent recombinant vaccine for prevention of seasonal flu — VAX2012Q.

Another bacterially produced HA-based immunogen advanced into clinical study is the H1N1 influenza vaccine candidate based on Cytos' proprietary bacteriophage Q $\beta$ -VLP technology. Started in May 2013, the first phase I clinical trial was conducted by the Agency for Science, Technology and Research (A\*Star) and Cytos Biotechnology. A\*Star reported that the novel vaccine was safe, well tolerated and induced immune responses which were comparable to those of approved seasonal influenza vaccines.

### CONCLUSIONS

The technology for producing large quantities of proteins in bacteria, including biopharmaceuticals, has been practiced for over two decades. Exploitation of this technology for the production of the HA-based vaccines against influenza seems to be hampered for a long time by the widely accepted view that glycosylation determines the correct structure of the protein. More recently, experimental data for both considerable independence of HA folding from the glycosylation status, and the successful production of bacterial HAs have been published. In contrast to full-length or ectodomain-based HAs produced in eukaryotic expression system, the vast majority of bacterially produced HA proteins have been based on the HA1 subunit and only a few examples of bacterial HAs based on the ectodomain or the HA2 subunit have been reported so far. The recombinant proteins, derived from the 2009 H1N1 pandemic, highly pathogenic H5N1, seasonal H1N1 and H3N2 as well as prototype H1N1/PR8 influenza viruses, have been efficiently expressed in the form of inclusion bodies.

The studies of refolded and purified HA proteins proved that bacterial HAs can be valuable vaccine antigens. Although devoid of the regions participating in the formation of higher-order structures of HA during its biosynthesis in host cells, bacterial HAs can adopt the conformation similar to that displayed by the native protein. In addition, some of these proteins were shown to form functional oligomers, what has a proven beneficial effect on both protective efficacy and dose sparing. Consistently with the biochemical characteristics, immunization studies clearly demonstrated the potential of the bacterially produced vaccine candidates to confer intra-subtype protection against influenza viruses, including highly drifted ones as well as to provide control of virus infection and transmission rates in animals. Moreover, clinical studies with vaccines based on proprietary Toll-like Receptor (VaxInnate) and bacteriophage Q $\beta$ -VLPs (Cytos Biotechnology) technologies generated positive data.

The data so far published indicate that the success in the bacterial production of the vaccine antigens strongly depends on the HA fragment chosen for expression as well as on the applied refolding method. For short fragments of HA, it was well documented that careful selection of the peptides from the protein sequence to be expressed with the target HA regions, such as the globular domain or the HA2 subunit, is prerequisite to obtain properly folded proteins with satisfactory efficiency. It was also shown, that the expression of specified-length fragments from HA1 subunit with the signal sequences, which are absent in the mature viral HAs, is essential for protein oligomerization. In some vaccine projects, one should consider the introduction of rationally designed mutations to obtain protein adopting the desired conformation, as it was evidenced for the stem domain immunogens. The determinations of the HA sequence for the antigen production in bacteria, especially those based on the short HA fragments, were often preceded by the analyses of the HA structure *in silico*. Experience with bacterial HAs shows that *in silico* modeling is helpful in the selection of protein sequences to be expressed bacterially, but does not guarantee success of the design. The value of refolded and purified rHAs needs to be verified through extensive studies of structure, the ability to bind to sialic acid-containing receptors, antigenicity, immunogenicity and finally efficacy in the challenge experiments and clinical trials. Once the HA protein is validated, the precise selection of the sequence from HA of various influenza A virus strains seems to be possible simply through structural alignment of the prototype HA fragment with the target HA proteins.

The vast majority of valuable, bacterially produced, vaccine antigens have been expressed with His-tags and purified by metal affinity chromatography, which is both expensive and poses the safety problem related to the presence of even trace amounts of metal in the final products. Thus, the development of appropriate purification procedures is needed to take advantage of bacterial cell expression technology for enhancing the supply in vaccines against influenza, especially for a pandemic. The research on subunit vaccines against influenza based on bacterial HAs is still continuing. The exemplification is the patent pending HA protein designed and bacterially produced in the Institute of Biotechnology and Antibiotics (Warsaw, Poland).

## Acknowledgements

This work was funded by European Funds Portal Innovative Economy "Centre of medicinal product biotechnology. Package of innovative biopharmaceuticals for human and animal therapy and prophylactics." POIG.01.01.02.-14-007/08-06.

## REFERENCES

- Aguilar-Yáñez JM, Portillo-Lara R, Mendoza-Ochoa GI, García-Echauri SA, López-Pacheco F, Bulnes-Abundis D, Salgado-Gallegos J, Lara-Mayorga IM, Webb-Vargas Y, León-Angel FO, Rivero-Aranda RE, Oropeza-Almazán Y, Ruiz-Palacios GM, Zertuche-Guerra MI, DuBois RM, White SW, Schultz-Cherry S, Russell CJ, Alvarez MM (2010) An influenza A/H1N1/2009 hemagglutinin vaccine produced in *Escherichia coli*. *PLoS One* **5**: e11694.
- Atsmon J, Kate-Ilovitz E, Shaikovich D, Singer Y, Volokhov I, Haim KY, Ben-Yedidia T (2012) Safety and immunogenicity of multimeric-001 — a novel universal influenza vaccine. *J Clin Immunol* **32**: 595–603.
- Baneyx F, Mujacic M (2004) Recombinant protein folding and misfolding in *Escherichia coli*. *Nat Biotechnol* **22**: 1399–1408.
- Barbey-Martin C, Gigant B, Bizebard T, Calder LJ, Wharton SA, Skehel JJ, Knossow M (2002) An antibody that prevents the hemagglutinin low pH fusogenic transition. *Virology* **294**: 70–74.
- Biesova Z, Miller MA, Schneerson R, Shiloach J, Green KY, Robbins JB, Keith JM (2009) Preparation, characterization, and immunogenicity in mice of a recombinant influenza H5 hemagglutinin vaccine against the avian H5N1 A/Vietnam/1203/2004 influenza virus. *Vaccine* **27**: 6234–6238.
- Bizebard T, Gigant B, Rigolet P, Rasmussen B, Diat O, Bösecke P, Wharton SA, Skehel JJ, Knossow M (1995) Structure of influenza virus haemagglutinin complexed with a neutralizing antibody. *Nature* **376**: 92–94.
- Bommakanti G, Citron MP, Hepler RW, Callahan C, Heidecker GJ, Najar TA, Lu X, Joyce JG, Shiver JW, Casimiro DR, ter Meulen J, Liang X, Varadarajan R (2010) Design of an HA2-based *Escherichia coli* expressed influenza immunogen that protects mice from pathogenic challenge. *Proc Natl Acad Sci USA* **107**: 13701–13706.
- Bommakanti G, Lu X, Citron MP, Najar TA, Heidecker GJ, ter Meulen J, Varadarajan R, Liang X (2012) Design of *Escherichia coli*-expressed stalk domain immunogens of H1N1 hemagglutinin that protect mice from lethal challenge. *J Virol* **86**: 13434–13444.
- Bosch BJ, Bodewes R, de Vries RP, Kreijtz JH, Bartelink W, van Amerongen G, Rimmelzwaan GF, de Haan CA, Osterhaus AD, Rottier PJ (2010) Recombinant soluble, multimeric HA and NA exhibit distinctive types of protection against pandemic swine-origin 2009 A(H1N1) influenza virus infection in ferrets. *J Virol* **84**: 10366–10374.
- Bright RA, Ross TM, Subbarao K, Robinson HL, Katz JM (2003) Impact of glycosylation on the immunogenicity of a DNA-based influenza H5 HA vaccine. *Virology* **308**: 270–278.
- Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC (1998) Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. *Cell* **95**: 409–417.
- Chen J, Skehel JJ, Wiley DC (1999) N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil. *Proc Natl Acad Sci USA* **96**: 8967–8972.
- Chen J, Wharton SA, Weissenhorn W, Calder LJ, Hughson FM, Skehel JJ, Wiley DC (1995) A soluble domain of the membrane-anchoring chain of influenza virus hemagglutinin (HA2) folds in *Escherichia coli* into the low-pH-induced conformation. *Proc Natl Acad Sci USA* **92**: 12205–12209.
- Chiu FF, Venkatesan N, Wu CR, Chou AH, Chen HW, Lian SP, Liu SJ, Huang CC, Lian WC, Chong P, Leng CH (2009) Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus. *Biochem Biophys Res Commun* **383**: 27–31.
- Cornelissen LA, de Vries RP, de Boer-Luijtz EA, Rigter A, Rottier PJ, de Haan CA (2010) A single immunization with soluble recombinant trimeric hemagglutinin protects chickens against highly pathogenic avian influenza virus H5N1. *PLoS One* **5**: e10645.
- Davis AR, Bos T, Ueda M, Nayak DP, Dowbenko D, Compans RW (1983) Immune response to human influenza virus hemagglutinin expressed in *Escherichia coli*. *Gene* **21**: 273–284.
- Davis AR, Nayak DP, Ueda M, Hiti AL, Dowbenko D, Kleid DG (1981) Expression of antigenic determinants of the hemagglutinin gene of a human influenza virus in *Escherichia coli*. *Proc Natl Acad Sci USA* **78**: 5376–5380.
- de Vries RP, Smit CH, de Bruijn E, Rigter A, de Vries E, Cornelissen LA, Eggink D, Chung NP, Moore JP, Sanders RW, Hokke CH, Koopmans M, Rottier PJ, de Haan CA (2012) Glycan-dependent immunogenicity of recombinant soluble trimeric hemagglutinin. *J Virol* **86**: 11735–11744.
- DuBois RM, Aguilar-Yáñez JM, Mendoza-Ochoa GI, Oropeza-Almazán Y, Schultz-Cherry S, Alvarez MM, White SW, Russell CJ (2011) The receptor-binding domain of influenza virus hemagglutinin produced in *Escherichia coli* folds into its native, immunogenic structure. *J Virol* **85**: 865–872.
- Dukhovlinov I, Al-Shekhdat R, Fedorova E, Stepanova L, Potapchuk M, Repko I, Rusova O, Orlov A, Tsybalova L, Kiselev O (2013) Study of immunogenicity of recombinant proteins based on hemagglutinin and neuraminidase conservative epitopes of influenza A virus. *Med Sci Monit Basic Res* **19**: 221–227.
- Dumon-Seignovet L, Cariot G, Vuillard L (2004) The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3). *Protein Expr Purif* **37**: 203–206.
- Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA (2009) Antibody recognition of a highly conserved influenza virus epitope. *Science* **324**: 246–251.
- Farahmand B, Khodabandeh M, Mahboudi F, Fotouhi F, Saleh M, Barkhordari F, Tabatabaian M, Nasab FP, Kheiri MT (2012) Influenza virus hemagglutinin: a model for protein N-glycosylation in recombinant *Escherichia coli*. *Intervirology* **55**: 219–224.
- Fleury D, Barrère B, Bizebard T, Daniels RS, Skehel JJ, Knossow M (1999) A complex of influenza hemagglutinin with a neutralizing an-

- tibody that binds outside the virus receptor binding site. *Nat Struct Biol* **6**: 530–534.
- Ha Y, Stevens DJ, Skehel JJ, Wiley DC (2002) H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. *EMBO J* **21**: 865–875.
- Hong M, Lee PS, Hoffman RM, Zhu X, Krause JC, Laursen NS, Yoon SI, Song L, Tussey L, Crowe JE Jr, Ward AB, Wilson IA (2013) Antibody recognition of the pandemic H1N1 Influenza virus hemagglutinin receptor binding site. *J Virol* **87**: 12471–12480.
- Horthongkham N, Srihrakul T, Athipanyasilp N, Siritantikorn S, Kantakamalakul W, Poovorawan Y, Sutthent R (2007) Specific antibody response of mice after immunization with COS-7 cell derived avian influenza virus (H5N1) recombinant proteins. *J Immune Based Ther Vaccines* **5**: 10.
- Jegerlehner A, Zabel F, Langer A, Dietmeier K, Jennings GT, Saudan P, Bachmann MF (2013) Bacterially produced recombinant influenza vaccines based on virus-like particles. *PLoS One* **8**: e78947.
- Jeon SH, Arnon R (2002) Immunization with influenza virus hemagglutinin globular region containing the receptor-binding pocket. *Viral Immunol* **15**: 165–176.
- Johansson BE, Brett IC (2007) Changing perspective on immunization against influenza. *Vaccine* **25**: 3062–3065.
- Johansson BE, Bucher DJ, Kilbourne ED (1989) Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. *J Virol* **63**: 1239–1246.
- Khurana S, Chearvae W, Castellino F, Manischewitz J, King LR, Honorkiewicz A, Rock MT, Edwards KM, Del Giudice G, Rappuoli R, Golding H (2010a) Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. *Sci Transl Med* **2**: 15ra5.
- Khurana S, Larkin C, Verma S, Joshi MB, Fontana J, Steven AC, King LR, Manischewitz J, McCormick W, Gupta RK, Golding H (2011a) Recombinant HA1 produced in *E. coli* forms functional oligomers and generates strain-specific SRID potency antibodies for pandemic influenza vaccines. *Vaccine* **29**: 5657–5665.
- Khurana S, Suguitan AL Jr, Rivera Y, Simmons CP, Lanzavecchia A, Sallusto F, Manischewitz J, King LR, Subbarao K, Golding H (2009) Antigenic fingerprinting of H5N1 avian influenza using convalescent sera and monoclonal antibodies reveals potential vaccine and diagnostic targets. *PLoS Med* **6**: e1000049.
- Khurana S, Verma S, Verma N, Crevar CJ, Carter DM, Manischewitz J, King LR, Ross TM, Golding H (2010b) Properly folded bacterially expressed H1N1 hemagglutinin globular head and ectodomain vaccines protect ferrets against H1N1 pandemic influenza virus. *PLoS One* **5**: e11548.
- Khurana S, Verma S, Verma N, Crevar CJ, Carter DM, Manischewitz J, King LR, Ross TM, Golding H (2011b) Bacterial HA1 vaccine against pandemic H5N1 influenza virus: evidence of oligomerization, hemagglutination, and cross-protective immunity in ferrets. *J Virol* **85**: 1246–1256.
- Kim CS, Epand RF, Leikina E, Epand RM, Chernomordik LV (2011) The final conformation of the complete ectodomain of the HA2 subunit of influenza hemagglutinin can by itself drive low pH-dependent fusion. *J Biol Chem* **286**: 13226–13234.
- Krystal M, Elliott RM, Benz EW Jr, Young JF, Palese P (1982) Evolution of influenza A and B viruses: conservation of structural features in the hemagglutinin genes. *Proc Natl Acad Sci USA* **79**: 4800–4804.
- Lamb RA, Choppin PW (1983) The gene structure and replication of influenza virus. *Annu Rev Biochem* **52**: 467–506.
- Li H, Ding J, Chen YH (2003) Recombinant protein comprising multi-neutralizing epitopes induced high titer of antibodies against influenza A virus. *Immunobiology* **207**: 305–313.
- Liu G, Song L, Reiserova L, Trivedi U, Li H, Liu X, Noah D, Hou F, Weaver B, Tussey L (2012) Flagellin-HA vaccines protect ferrets and mice against H5N1 highly pathogenic avian influenza virus (HPAIV) infections. *Vaccine* **30**: 6833–6838.
- Liu G, Tarbet B, Song L, Reiserova L, Weaver B, Chen Y, Li H, Hou F, Liu X, Parent J, Umlauf S, Shaw A, Tussey L (2011) Immunogenicity and efficacy of flagellin-fused vaccine candidates targeting 2009 pandemic H1N1 influenza in mice. *PLoS One* **6**: e20928.
- Loeffen WL, de Vries RP, Stockhofe N, van Zoelen-Bos D, Maas R, Koch G, Moormann RJ, Rottier PJ, de Haan CA (2011) Vaccination with a soluble recombinant hemagglutinin trimer protects pigs against a challenge with pandemic (H1N1) 2009 influenza virus. *Vaccine* **29**: 1545–1550.
- McMurry JA, Johansson BE, De Groot AS (2008) A call to cellular & humoral arms: enlisting cognate T cell help to develop broad-spectrum vaccines against influenza A. *Hum Vaccin* **4**: 148–157.
- Miroux B, Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* **260**: 289–298.
- Nayak DP, Davis AR, McQueen NL, Bos TJ, Jabbar MA, Sivasubramanian N, Lionelli G (1985) Biological and immunological properties of haemagglutinin and neuraminidase expressed from cloned cDNAs in prokaryotic and eukaryotic cells. *Vaccine* **3** (3 Suppl): 165–171.
- Okuno Y, Isegawa Y, Sasao F, Ueda S (1993) A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J Virol* **67**: 2552–2558.
- Osterholm MT, Kelly NS, Manske JM, Ballering KS, Leighton TR, Moore KA (2012) The compelling need for game-changing influenza vaccines: an analysis of the influenza vaccine enterprise and recommendations for the future. University of Minnesota Center for Infectious Disease Research and Policy.
- Park MH, Chang J (2012) Immunogenicity and protective efficacy of a dual subunit vaccine against respiratory syncytial virus and influenza virus. *Immune Netw* **12**: 261–268.
- Pica N, Palese P (2013) Toward a universal influenza virus vaccine: prospects and challenges. *Annu Rev Med* **64**: 189–202.
- Roberts PC, Garten W, Klenk HD (1993) Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. *J Virol* **67**: 3048–3060.
- Rudolph W, Ben Yedidia T (2011) A universal influenza vaccine: where are we in the pursuit of this “Holy Grail”? *Hum Vaccin* **7**: 10–11.
- Saelens X, Vanlandschoot P, Martinet W, Maras M, Neiryck S, Contreras R, Fiers W, Jou WM (1999) Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. *Eur J Biochem* **260**: 166–175.
- Sánchez-Arreola PB, López-Urriarte S, Marichal-Gallardo PA, González-Vázquez JC, Pérez-Chavarría R, Soto-Vázquez P, López-Pacheco F, Ramírez-Medrano A, Rocha-Piñaña MR, Alvarez MM (2013) A baseline process for the production, recovery, and purification of bacterial influenza vaccine candidates. *Biotechnol Prog* **29**: 896–908.
- Shen S, Mahadevappa G, Oh HL, Wee BY, Choi YW, Hwang LA, Lim SG, Hong W, Lal SK, Tan YJ (2008) Comparing the antibody responses against recombinant hemagglutinin proteins of avian influenza A (H5N1) virus expressed in insect cells and bacteria. *J Med Virol* **80**: 1972–1983.
- Shih AC, Hsiao TC, Ho MS, Li WH (2007) Simultaneous amino acid substitutions at antigenic sites drive influenza A hemagglutinin evolution. *Proc Natl Acad Sci USA* **104**: 6283–6288.
- Skehel JJ, Cross K, Steinhauer D, Wiley DC (2001) Influenza fusion peptides. *Biochem Soc Trans* **29**: 623–626.
- Skehel JJ, Stevens DJ, Daniels RS, Douglas AR, Knossow M, Wilson IA, Wiley DC (1984) A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. *Proc Natl Acad Sci USA* **81**: 1779–1783.
- Skehel JJ, Wiley DC (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69**: 531–569.
- Skibinski DA, Hanson BJ, Lin Y, von Messling V, Jegerlehner A, Tee JB, Chye de H, Wong SK, Ng AA, Lee HY, Au B, Lee BT, Santoso L, Poidinger M, Fairhurst AM, Matter A, Bachmann MF, Saudan P, Connolly JE (2013) Enhanced neutralizing antibody titers and Th1 polarization from a novel *Escherichia coli* derived pandemic influenza vaccine. *PLoS One* **8**: e76571.
- Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, Jacobs A, Liu G, Huang Y, Desai P, Maksymiuk G, Takahashi V, Umlauf S, Reiserova L, Bell R, Li H, Zhang Y, McDonald WF, Powell TJ, Tussey L (2008) Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. *PLoS One* **3**: e2257.
- Song L, Zhang Y, Yun NE, Poussard AL, Smith JN, Smith JK, Borisevich V, Linde JJ, Zacks MA, Li H, Kavita U, Reiserova L, Liu X, Dumuren K, Balasubramanian B, Weaver B, Parent J, Umlauf S, Liu G, Huleatt J, Tussey L, Paessler S (2009) Superior efficacy of a recombinant flagellin: H5N1 HA globular head vaccine is determined by the placement of the globular head within flagellin. *Vaccine* **27**: 5875–5884.
- Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, Haye K, García-Sastre A, Palese P (2010) Influenza virus vaccine based on the conserved hemagglutinin stalk domain. *MBio* **1**: e000818–10.
- Suarez DL (2005) Overview of avian influenza DIVA test strategies. *Biologicals* **33**: 221–226.
- Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA (2009) Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol* **16**: 265–273.
- Sylte MJ, Suarez DL (2009) Influenza neuraminidase as a vaccine antigen. *Curr Top Microbiol Immunol* **333**: 227–241.
- Taylor DN, Treanor JJ, Sheldon EA, Johnson C, Umlauf S, Song L, Kavita U, Liu G, Tussey L, Ozer K, Hofstaetter T, Shaw A (2012) Development of VAX128, a recombinant hemagglutinin (HA) influenza-flagellin fusion vaccine with improved safety and immune response. *Vaccine* **30**: 5761–5769.
- Taylor DN, Treanor JJ, Strout C, Johnson C, Fitzgerald T, Kavita U, Ozer K, Tussey L, Shaw A (2011) Induction of a potent immune response in the elderly using the TLR-5 agonist, flagellin,

- with a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125, STF2.HA1 SI). *Vaccine* **29**: 4897–4902.
- Treanor JJ, Taylor DN, Tussey I, Hay C, Nolan C, Fitzgerald T, Liu G, Kavita U, Song L, Dark I, Shaw A (2010) Safety and immunogenicity of a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125) in healthy young adults. *Vaccine* **28**: 8268–8274.
- Verma S, Dimitrova M, Munjal A, Fontana J, Crevar CJ, Carter DM, Ross TM, Khurana S, Golding H (2012) Oligomeric recombinant H5 HA1 vaccine produced in bacteria protects ferrets from homologous and heterologous wild-type H5N1 influenza challenge and controls viral loads better than subunit H5N1 vaccine by eliciting high-affinity antibodies. *J Virol* **86**: 12283–12293.
- Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, Panico M, Morris HR, Dell A, Wren BW, Aebi M (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. *Science* **298**: 1790–1793.
- Wang CC, Chen JR, Tseng YC, Hsu CH, Hung YF, Chen SW, Chen CM, Khoo KH, Cheng TJ, Cheng YS, Jan JT, Wu CY, Ma C, Wong CH (2009) Glycans on influenza hemagglutinin affect receptor binding and immune response. *Proc Natl Acad Sci USA* **106**: 18137–18142.
- Wei CJ, Xu L, Kong WP, Shi W, Canis K, Stevens J, Yang ZY, Dell A, Haslam SM, Wilson IA, Nabel GJ (2008) Comparative efficacy of neutralizing antibodies elicited by recombinant hemagglutinin proteins from avian H5N1 influenza virus. *J Virol* **82**: 6200–6208.
- Weldon WC, Wang BZ, Martin MP, Koutsonanos DG, Skountzou I, Compans RW (2010) Enhanced immunogenicity of stabilized trimeric soluble influenza hemagglutinin. *PLoS One* **5**: e12466.
- Wilson DC, Skehel JJ (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem* **56**: 365–394.
- Wilson DC, Wilson IA, Skehel JJ (1981) Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* **289**: 373–378.
- Wilson IA, Cox NJ (1990) Structural basis of immune recognition of influenza virus hemagglutinin. *Annu Rev Immunol* **8**: 737–771.
- Wilson IA, Skehel JJ, Wiley DC (1981) Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* **289**: 366–373.
- Xie QM, Ji J, Du LQ, Cao YC, Wei L, Xue CY, Qin JP, Ma JY, Bi YZ (2009) Preparation and immune activity analysis of H5N1 subtype avian influenza virus recombinant protein-based vaccine. *Poult Sci* **88**: 1608–1615.
- Xuan C, Shi Y, Qi J, Zhang W, Xiao H, Gao GF (2011) Structural vaccinology: structure-based design of influenza A virus hemagglutinin subtype-specific subunit vaccines. *Protein Cell* **2**: 997–1005.