CHAPTER I
INTRODUCTION

Influenza viruses are negative sense RNA viruses. They are human respiratory tract pathogens causing a highly contagious disease, **"Influenza"** that may be in the forms of sporadic cases throughout the year, annual epidemics, and occasional devastating pandemics (Wright and Webster, 2001). The viruses can also infect several other mammals as well as avian species (Keawcharoen *et al*., 2004; Kuiken *et al*., 2004; Songserm *et al*., 2006). They are classified into three types, *i.e.,* types A, B, and C, based on their structural proteins, epidemiologic patterns, host range, and pathogenicity (Kurstak *et al.*, 1990). Influenza A viruses are further subdivided into 16 hemagglutinin (H) and 9 neuraminidase (N) subtypes (please see details in the literature review; chapter III). Multiple combinations of any H with any N subtypes are possible. The H1N1, H2N2 and H3N2 subtypes used to cause pandemics (Johnson and Mueller, 2002; Horimoto and Kawaoka, 2005). Subtypes H5, H7 and H9 are virulent subtypes among avians (Alexander, 2000; Li *et al*, 2003). Nevertheless, all H and N subtypes could be isolated from water fowl reservoirs (Fouchier, 2005). The recent emergence of the highly pathogenic avian influenza (HPAI) virus strains belonging to H5N1 subtype which ravaged million of poultry, their ability to mediate interspecies transmission to infect mammals as well as humans, and the high mortality rates among the infecting hosts create a global threat of influenza (Chotpitayasunondh *et al*., 2005; Tiensin *et al*., 2005; Ungchusak *et al*., 2005).

Currently available influenza vaccines elicit only limited immunity among the vaccines. They protect only against infections that are caused by strains and/or subtypes that matched with the vaccine strains. One of the current vaccine obstacles is the continuous antigenic change of the circulating influenza viruses either gradually (antigenic drift) or entirely (antigenic shift); thus the vaccine strains have to be adapted regularly. The vaccine protective duration is relatively short and frequent immunization, *e.g.,* annually, is required to sustain the protective antibody level (Schwartz and Wortley, 2006). Most vaccines confer low protective rates among the elderly (Jefferson *et al*., 2005).

Currently, there are only two families of anti-influenza virus drugs. One is the amantadanes or the ion channel (M2) blockers (Pales and Compans, 1976) and another is the neuraminidase inhibitors (Hay *et al*., 1985). Nevertheless, drug resistant influenza strains have emerged (de Jong *et al*., 2005; Le *et al*., 2005).

For the influenza pandemic preparedness, it is necessary that a vaccine that confers broad protection against influenza and new anti-influenza drugs should be developed. Their supply should also meet the demand during the disease crisis. Alternatively, a therapeutic antibody that can interfere with the virus pathogenicity should be sought.

The influenza virus RNA-dependent RNA polymerase complex, consisting of three subunits, *i.e.,* acid polymerase protein (PA), and two basic polymerase proteins, PB1 and PB2, is a pivotal structure of the influenza viruses. The PB1 subunit contains the conserved motifs characteristic of RNA-dependent RNA polymerase (Biawas and Nayak, 1994; Argos, 1998) and binds to the virus RNA (vRNA) and cRNA promoter (Cianci *et al.,* 1995; Li *et al*., 1998; Gonzáles and Ortín, 1999). It also has nucleae activity required to snatch the capped primers from the host's pre-mRNA for the viral RNA transcription (Li *et al*., 2001). The PB2 is responsible for recognition and binding the cap structure of host mRNAs (Fodor *et al*., 2002; Lee *et al*., 2002; Fechter *et al*., 2003). The exact role of the PA is less well understood, but it has been shown to be essential for both viral transcription and replication (Fodor *et al*., 2002, Fodor *et al*., 2003; Huarte *et al*., 2003; Kawaguchi *et al*., 2005). The interactions between the individual polymerase subunits have been identified. The N-terminal region of the PB1 interacts with the C-terminal region of the PA, while the C-terminal region of the PB1 interacts with the N-terminal of the PB2 subunit. No direct interaction has been demonstrated for PB2 and PA (Gonzáles *et al*., 1996; Toyoda *et al*., 1996; Zűcher *et a*l., 1996; Perez and Donis, 2001; Ohtsu *et al.,* 2002). Recently, it was found that the peptide corresponding to PA-binding domain of the PB1 could block polymerase activity of the influenza A and inhibited the viral spread (Ghanem *et al*., 2007). Therefore, targeting the viral polymerase subunit(s) should provide a novel strategy for developing an anti-viral compound against not only the influenza viruses but also other RNA viruses.

Antibodies have been used for treatment of infections and intoxication as early as 1890s (cited by Stiehm, 1998). After the discovery of antibiotic penicillin by Sir Alexander Flemming in 1928, however, most infectious diseases have been treated with chemotherapeutic agents. To date, the passive antibody therapy (used to be called serum therapy) still retains its niche for the treatment of viral infections, such as rabies, cytomegalovirus, hepatitis (Kelier and Stiehm, 2000), envenomation, such as snake bites (Warrell, 1999), and intoxications, such as diphtheria, tetanus, pertussis, botulism (Kelier and Stiehm, 2000). The therapeutic antibodies may be from heterologous sources: from immune horse/ovine serum or murine monoclonal antibodies (Stiehm, 1998; Wilcox, 1998). Nevertheless, the therapeutics often elicit hypersensitivity reaction, *i.e.* immediate reaction such as anaphylaxis or delayed reaction such as serum sickness caused by the anti-isotypic response of the recipient to the foreign immunoglobulins. Human convalescing serum is the best source of the therapeutic antibodies. However, the supply is limited and the ethical issue is of concern. Alternatively, monoclonal human antibodies specific to antigen of interest were produced in transgenic animals carrying human immunoglobulin transgenes (Nicholson *et al*., 1999) or by using Epstein-Barr virus transformed immune B lymphocytes from either immunized or disease convalescing individuals (Traggiai *et al*., 2004). Obstacles of these technologies are: the former requires expensive, well equipped animal facilities and high skill personnel. Also the animal ethics are of particular concern. The latter is not only requires the tissue culture facilities, the prolonged human immunization against a particular antigen which is incompatible with the ethics and not possible for the low immunogenic or toxic molecules (in the case of using immune B cells from immunized individuals), but also is hazardous to the laboratory personnel because the Epstein-Barr virus must be used.

In this study, recombinant N-terminal PA, PB1 and PB2 proteins of highly pathogenic influenza A virus, H5N1 subtype, have been successfully produced using prokaryotic protein expression system. The recombinant proteins were used in the phage bio-panning process to select phage clones displaying human single chain antibody fragments (HuScFv) from a large repertoire human antibody phage library constructed in own laboratory from multiple Thai blood donors. The phage clones were used individually to infect appropriate *E. coli* strains and the HuScFv were

induced to express by the *E. coli*. The HuScFv binding specificities were determined using several immunological assays. The HuScFv specific to the polymerase proteins of influenza virus in their cell penetrating version have high potential as anti-influenza therapeutics.