

Novel llama antibody fusion proteins as deposition aids for particles containing encapsulated actives for use in industrial applications

A thesis submitted to the University of London
for the degree of Doctor of Engineering

by

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"Personally, I like the University. They gave us money and facilities, we didn't have to produce anything. You've never been out of college. You don't know what it's like out there. I've worked in the private sector. They expect results."

Ray, Ghostbusters, 1984

Abstract

The benefits of llama antibodies compared to conventional IgG (most particularly low manufacturing cost and stability) have been well published in literature and this lends them to potential applications in the home and personal care industry. Llama antibody fragments have been fused to a cellulose binding domain moiety and used as a bifunctional protein to aid deposition of particles to cellulosic surfaces. The initial anticipated applications for this technology is in the delivery of actives to fabrics in laundry, however due to the ubiquitous nature of cellulose a number of other possible applications are envisaged.

Efficacy of the protein was first demonstrated in a model particle system using coacervate particles coated with a dye-based antigen using ELISA, Biacore and particle deposition studies. Determined antibody affinities correlated with those in literature. A number of different fusion formats were investigated, and it was found that although addition of a fusion partner adversely affected antibody affinity, a single antibody/double cellulose binding domain format gave enhanced particle deposition, over a particle only control.

The success of the hypothesis in the model system prompted study of a potential commercial equivalent. A particle consisting a melamine urea formaldehyde polymer was chosen. A protein from a range of possible candidates produced by a commercial manufacturer was selected and a purification process was developed that allowed production of protein on the gram scale, with potential for scale-up. The binding, bifunctionality and stability of the protein was then studied using gel densitometry and UV based assays. A substantial increase in particle depositions was demonstrated with a UV microscopy based assay.

Use of the protein to enhance particle delivery to cellulosic surfaces showed promise in both systems studied when compared to particles alone, and has potential applications in product fields such as home and personal care, the agrochemical industry and paper processing.

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1.0 Introduction

1.1 Structure and characteristics of antibodies and antibody fragments

1.1.1 Antibody background

Antibodies are key components of the mammalian immune system. The role of the immune system of mammals is to detect any potentially harmful foreign material or pathogen and elicit an appropriate response against it (Liddel and Weeks, 1995). The immune system can be split into two components: the innate and the adaptive. The innate is a non-specific defense system, used primarily as the first line of resistance to invasion, whereas the adaptive is more specifically designed to elicit an immunological challenge that is highly specific for the target pathogen (Roitt et al., 1998).

The innate system consists of a variety of cells (known as phagocytes and natural killer (NK)) and humoral factors which work in concert to engulf and destroy foreign cells or host cells that have been taken over by the invading body. In some cases, this mechanism is not enough to clear the challenge upon the host, and the adaptive immunity mechanism is needed to overcome it. The innate immune system does not change the nature of its response each time it is challenged; however the adaptive immune system improves with each successive encounter with the same challenge. This confers life long “immunity” to diseases such as measles and diphtheria after the initial infection has been cleared (Roitt et al., 1998). This process makes possible the vaccination of many diseases, using an attenuated or weakened form to promote an initial immune response.

A key component of the adaptive system is the production of specific humoral factors that aid the action of phagocytes in the recognition of foreign particles, known as antibodies (Ab) or immunoglobulins (Ig). At present, antibodies are believed to be the most important part of the immune system (Liddel and Weeks, 1995). The immune system possesses an enormous diversity of immunoglobulins capable of binding almost any foreign invading particle, known as an antigen.

1.1.2 Antibody Structure

Antibodies are glycoproteins secreted by plasma cells derived from β -lymphocytes that have been stimulated to produce them by the presence of the antigen. There are five classes of antibody or Ig molecules, known as IgM, IgG, IgD, IgA, and IgE.

IgG proteins are the most common in the bloodstream (70-75% of the total immunoglobulin pool) and are homodimers, consisting of two identical polypeptide chains of 450 amino acids known as the heavy chains and two identical chains 250 amino acids known as the light chains. The structure of each domain has a characteristic fold that consists of two antiparallel β -sheets with an intramolecular disulphide bond.

As can be seen from figure 1, the molecules consist of variable and non-variable regions. The N-terminal domains of each chain consist of three loops connecting the β -sheets, and these loops vary greatly in length and sequence. The six hypervariable loops are known as complementary determining regions (CDR) and are responsible for binding of the antigen (Maynard and Georgiou, 2000). The area that represents the actual binding site is known as the paratope and this binds to the relevant section of antigen, known as the epitope (Arbabi et al., 1997; Rees et al., 1994). Most antigens have a number of possible binding sites (epitopes) on their surface, and each antibody consequently recognises a specific epitope, rather than the whole antigen.

Although the antibody molecule binds to an antigen specifically, it also plays a further role in the immune system. The basic structure of the antibody molecule is similar in each molecule, and the non-antigen binding section is responsible for interacting with other elements of the immune system, such as phagocytes, or activating the important process of complement (used to lyse the attacking pathogenic cells). Thus the antibody acts as a flexible adaptor molecule, linking various elements of the immune system to recognise specific pathogens and their products (Roitt et al., 1998).

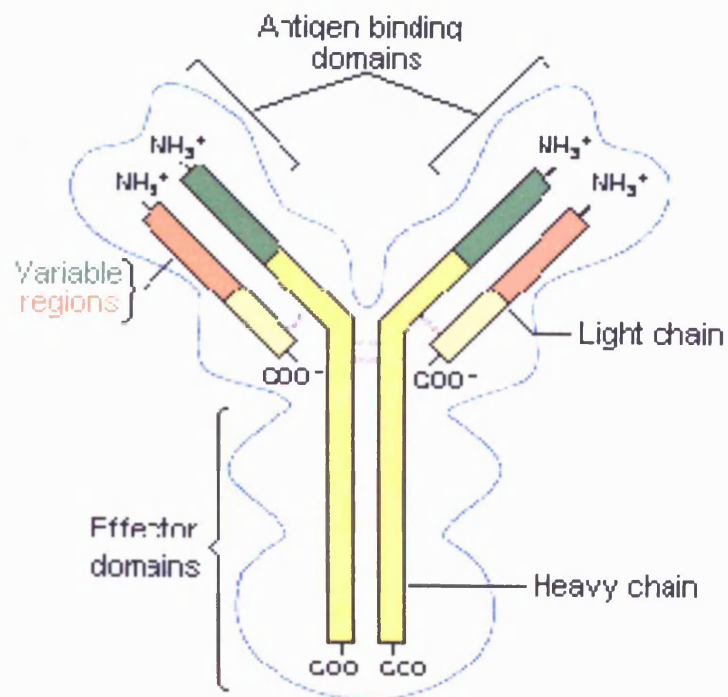


Figure 1: Diagram of a conventional antibody. The disulphide bridges are visible linking the two heavy chains together.

Although the binding of antibodies to antigens is something that happens regularly *in vivo*, it can also occur successfully *in vitro*, opening the door to a host of applications for antibodies in the biotechnology and biomedical industry. The most commonly used immunoglobulin in biotechnology is the IgG molecule (Maynard and Georgiou, 2000).

1.1.3 Antibody Function

As discussed previously, hypervariable regions are clustered at the end of the IgG arms (in a region known as Fab region), and it is these particular residues that interact specifically with the antigen. The framework residues do not usually form bonds with the antigen, although are important in maintaining the structure and integrity of the binding site.

The binding reaction of antibody to antigen involves the formation of a number of non-covalent bonds between amino acids in the antibody and the binding site consisting of a mixture of hydrogen bonds, electrostatic attractions, Van der Waals forces and hydrophobic attractions. Individually these bonds are very weak compared to covalent bonds, but the cumulative effect of a large number of them is considerable (Roitt et al., 1998).

The strength of these bonds is heavily dependent on the distance between the interacting groups, and in order for binding to occur there must be a highly complementary paratope and epitope, both in terms of atomic structure on pairing sides of the antibody and antigen, and also overall shape. If sufficient complementarity is present, then the nature of forces will be strong enough to overcome thermodynamic disruption of the bond. However if the electron clouds of the antigen and antibody overlap, very strong repulsive forces come into play (Roitt et al., 1998).

These forces are responsible for determining the specificity of the antibody molecule for a particular antigen, as any sufficiently different antigen will simply possess too great a total repulsive force, and prevent binding. The sum of the attractive and repulsive forces gives rise to a measure of the strength of the bond, known as the antibody affinity, discussed further in section 1.5. Antibodies also have another measure of strength of binding to an antigen, known as avidity, which is the strength of the binding

i.e. the sum of the binding strength of all domains present. This term allows for multivalent antibody binding (Roitt et al., 1998).

1.1.4 Polyclonal and Monoclonal antibodies

A wide variety of animal hosts have been used to produce antibodies. A response is elicited by inoculation of a selected antigen in sufficient quantities to stimulate production, which is then extracted from the animals blood. Most large molecules such as proteins will elicit a response, for example a human protein will elicit a response in a rabbit, assuming there is no homologous protein already in the rabbit. Obviously the larger the animal, the more antibody can be extracted. Commonly used animals include mice, rabbits and goats.

In animal sera many antibodies of differing structure and configuration will be produced from different β -lymphocytes. These antibodies are known as polyclonal. Early advances in antibody technology were hampered by the need for animal immunization or expression of engineered antibodies in a eukaryotic host (Maynard and Georgiou, 2000), and the amount that could be produced was limited. The fact that antibodies (being proteins) were highly antigenic themselves in non-host organisms also limited their potential.

In 1975, Kohler and Millstein fused myeloma cells with β -cells from an immunized animal. The resultant cells possess both antibody production ability and relative longevity in culture and are called hybridoma cells. Single antibody secreting hybridoma cells can then be selected and cloned so that all progeny produce exactly the same antibody. These identical antibodies are known as monoclonal, and can be created in response to a specific antigen.

Thousands of hybridoma cell lines have now been created using this technology (Maynard and Georgiou, 2000), facilitated by the cloning of relevant antibody expression genes using the polymerase chain reaction (PCR) and subsequent expression in bacterial systems (Pluckthun and Skerra, 1989). In the last ten years techniques that allow high throughput screening of candidates and efficient humanization of antibodies to prevent them eliciting an immune response have further advanced production and understanding.

Currently the principle application for antibodies is in the pharmaceutical industry - there are a number of monoclonal antibodies in production for treatment of cancer and other conditions. There are also a number of antibodies in clinical trials. Other applications are in diagnostics. Both of these applications require a relatively small amount of protein, and allow a high cost due to their market. However, other markets (such as home and personal care and agrochemical) in the biotechnology industry will require a large amount of product at low cost.

1.1.5 Selective protease digestion of the IgG molecule gives antibody fragments

The binding sites for antigen and effector receptors are assigned to different parts of the antibody. The Fc part of the molecule is responsible for the effector functions, and is labelled as the effector domains in fig 1. For some applications this domain can be a hindrance, as many cells possess Fc receptors to which the Fc region will bind regardless of specificity of the molecule. The size of an antibody molecule can also often cause problems when it comes to the application. As such it is often an advantage to remove or cleave off the effector domain (Fc) section of the antibody.

A long established method of cleaving this unwanted portion of antibody is by proteolytic enzyme digestion. A non-specific protease, Papain, splits the antibody in the hinge region, resulting in the formation of two monovalent Fab fragments and an Fc fragment. Bivalent fragments can be made by treatment of the antibody with Pepsin, bromelain or ficin that digest away only the effector region (Liddell and Weeks, 1995).

1.2 Heavy chain only antibody fragments

1.2.1 Making the fragment simpler

The Fv containing the two variable domains (V_H and V_L) known as scFV, is considered the smallest fully functioning molecule of IgG and engineered fragments (see figure 2). A large genetic library has been created with these fragments using PCR, considered a major breakthrough in molecular biology. However the technique is not easy: the cloning of the two correctly spliced gene fragments (V_H and V_L) is an error-prone step; generating a library is time consuming and the constructs are commonly unstable in the bacterial host. Further more the expression, yield, stability and functionality of scFV often turn out to be problematic.

Ward et al. (1989) used an approach whereby the V_H domain in the absence of the V_L domain was isolated and used. This approach avoids the introduction of a peptide linker used in production of scFV constructs that might create additional problems such as reduced affinity, aggregation or proteolytic cleavage. However the insolubility of the isolated V_H domains expressed in bacteria and their reduced antigen affinity relative to the original V_H-V_L combination made them impractical for use.

1.2.2 Naturally simpler antibodies

Certain mammals belonging to the camelid family produce, amongst the normal complement of mammalian antibodies, an unusual type of antibody that has no light chain. In 1993 Hamers-Casterman et al. reported the existence of heavy chain only antibodies in *Camelus dromedaries*. It was later found that these antibodies are found in all camelidae, including llamas and alpacas.

In the Llama (*Lama glama*) six IgG subclasses have been described, two of which are conventional IgG molecules: IgGγ1a and IgGγ1b. The other four are heavy chain only antibodies (IgG2a, IgG2b, IgG2c and IgG3) (Vu, 1997), consisting of only the variable domain of the heavy chain, as demonstrated below, known as VHH. Heavy chain only antibodies lack a C_H1 domain and instead have either a long (29 or 35 amino acids) or short (12 amino acids) hinge region. Figures 2 and 3 illustrate the main differences between the conventional IgG molecule and the VHH molecule.

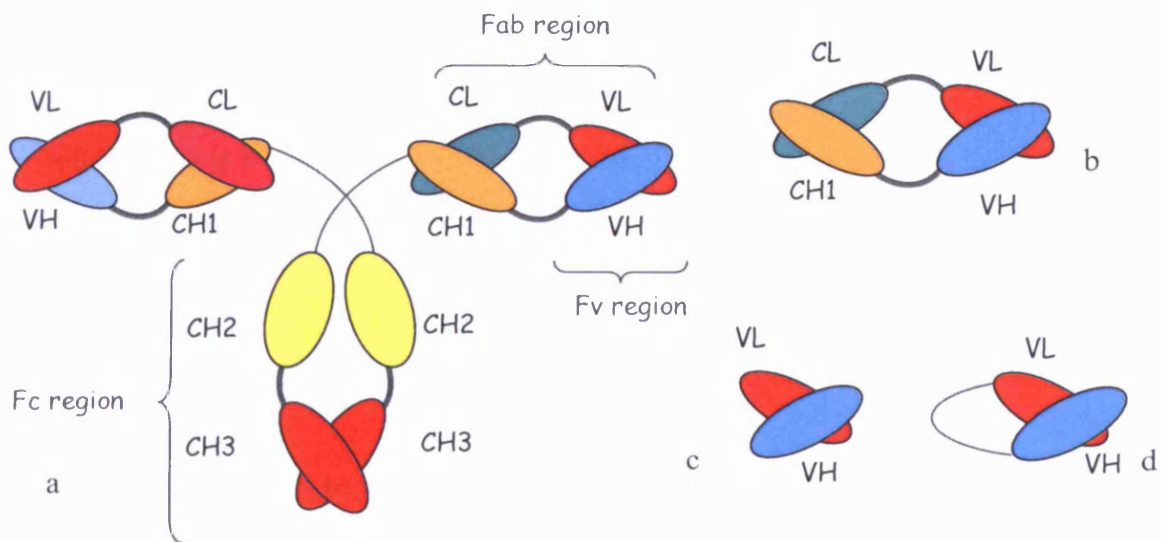


Figure 2: Conventional antibody molecules used in biotechnology:
a: IgG molecule; b: Fab fragment; c: Fv fragment; d: scFv fragment (joined by peptide linker).

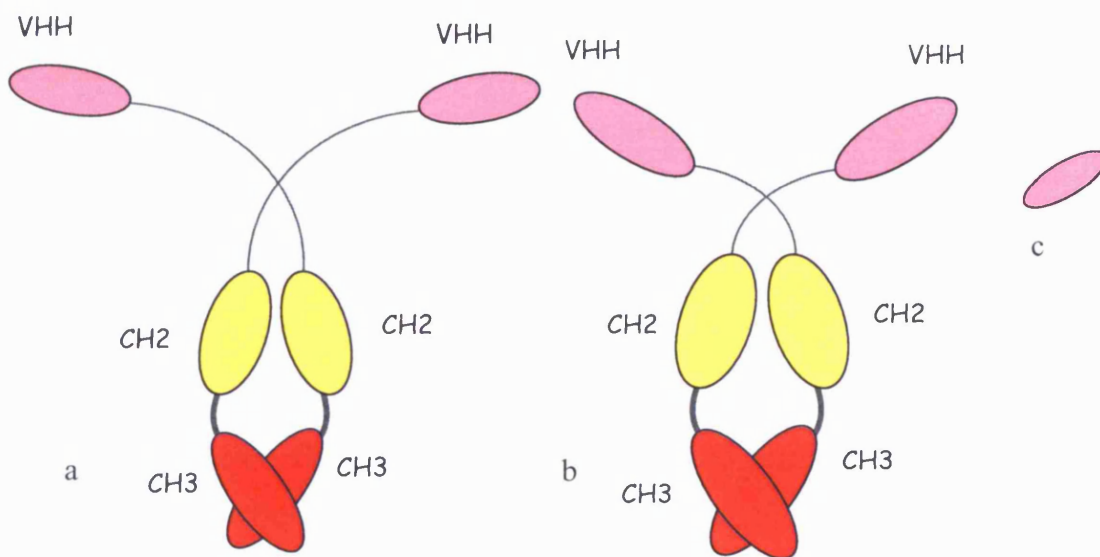


Figure 3: Camelid antibody molecules:
a: Heavy chain IgG molecule (long hinge); b: Heavy chain IgG molecule (short hinge) VHH fragment; c: VHH fragment.

Nguyen et al. (1999) have described heavy chain only antibodies as being the result of mutations in the splice consensus signal of the C_H1 domain of conventional IgG molecules and not somatic hypermutation of the V_H genes. This loss of consensus splice signal is similar to heavy chain disease in humans and mice, but has not resulted in the functional defects that can be observed with the condition. They also have comparable affinities with conventional IgG fragments, Fab and scFV raised to the identical antigens. Nguyen et al. have released further publications (2002) that confirm that these antibodies have evolved from existing conventional antibody genes.

It is likely that these heavy chain antibody genes have been preserved in the camelid species due to a conferred selective advantage. This is believed to be due to the function of the heavy chain only antibodies in recognising novel and unusual epitopes (Nguyen et al. 2003).

Frenken et al. (2000) have described how the simpler VHH molecule fragments can be produced at high levels in *S. cerevisiae* (up to 250mg/l in shake flasks and 1g/l in high density fermentations). These molecules have been found to overcome the folding and aggregation problems associated with more conventional antibody fragments in lower eukaryotes. The binding domain or VHH section is less hydrophobic than the Fv domain of conventional IgG molecules, believed to result in higher secretion efficiency (Thomassen et al, 2002).

It is possible to link two heavy chain only antibodies together to create a bispecific and bivalent antibody. Conrath et al. (2001), tethered two fragments by the structural upper hinge of a conventional IgG molecule to generate bispecific molecules. These molecules are particularly easy to generate, making them ideal for use in many applications.

1.2.3 The source of heavy chain only antibodies used in industry

Llamas produce considerably less heavy chain only IgG molecules than camels (around 45% of total antibody content compared to 75%) and show different binding to Proteins A and G, possibly due to structural or sequence differences. They are however the most common source of heavy chain only antibodies, as they are smaller and easier to keep than camels, and are therefore more practical (van der Linden et al., 1999).

1.2.4 Occurrence in other species

No further heavy chain only antibodies have been identified in other mammals, although some shark species have also been seen to possess an antibody like structure designated new or nurse shark antigen receptor (NAR) (Greenberg et al., 1995). No ligands have been identified as yet for this molecule.

Camelid VHH molecules show about a 75% homology to mammalian V_H sequences. NAR molecules however show only a 25% homology to mammalian V_H sequences and are more similar to V_L and T cell receptor molecules, indicating that they have evolved separately (Greenburg, 1995). Similar antigen binding molecules have been found in the Ratfish known as the Cos5-antibodies (Rast et al., 1998).

1.3 Structure of the VHH molecule and function in relation to conventional IgG molecules

1.3.1 Structure

The antigen-binding domain of the VHH molecule is approximately half the size of that in a conventional IgG molecule. This structural difference has an important effect on antigen recognition. Desmyter et al. (1996) have shown that camel VHH molecules can bind to novel epitopes unrecognized by conventional antibodies, for instance the active sites of certain enzymes. Two out of the six VHH molecules isolated bind to epitopes not recognized by monoclonal antibodies of mouse origin. This is obviously a major advantage to the camelid in terms of additional immune protection.

Desmyter et al. (1996) and Decanniere et al. (1999) note that camelid VHH molecules bind according to the geometric surface of the antigen, and also ionic and hydrophobic interactions. Although conventional IgG molecules prefer to bind to planar surfaces, camelid VHH prefer to bind to grooves or cavities using extended Complementary Determining Regions (CDRs). The affinities found for the heavy chain only antibodies have been found to be in the low nanomolar range, comparable to that of Fab and scFv fragments.

Conventional IgG's recognize large protein antigens through a large antigen-binding surface that is flat, but suitable for high affinity interaction. Small antigens tend to be

bound by conventional IgG antibodies in the groove between the heavy and light chain. Obviously the heavy chain only antibodies are devoid of light chains and hence require the extended CDR to allow binding.

1.3.2 Function

The physico-chemical characteristics of camelid VHHs also differ from conventional IgG molecules. The temperature stability of llama VHH molecules has been shown to be significantly greater than that of conventional IgG. Many llama VHH molecules can be incubated for up to 2 hours at 90°C without significant loss of binding activity (van der Linden et al (1999)). Some have also been shown to bind antigens at temperatures of up to 90°C. This makes them ideal in processes that have environmental stresses (such as pasteurization) without losing the ability of antigen binding.

The extra stability of the VHH molecule has been associated with an additional disulfide bond between CDR 1 and CDR 3 as present in the majority of VHHs (Gharoudi et al., 1997). However although this extra disulphide bond is found in camels it is not so prevalent in llama VHH, which still exhibit the extra stability, indicating that the disulphide bond is not the sole effector.

Not all VHHs exhibit the ability to function at high temperatures, but they do show the ability to function at normal temperatures after exposure to high temperatures. Perez et al. (2001) have noted that this stability is a result of the reversibility of the thermal unfolding process, unlike conventional IgG molecules that exhibit irreversibility upon exposure to heat.

The specificity of llama VHH molecules was examined by van der Linden et al (1999) by examining a fragment specific for the azo dye Reactive Red 6. This antibody was examined for affinity with other, chemically similar azo dyes Reactive Red 120 (a dimer of RR6), Direct Red 80, Reactive Black 6 and Direct Green 26. No affinity was shown for any of these molecules except the Reactive Red 6. The RR-6 llama VHH is therefore highly specific for RR6, demonstrating the ability of llama VHH to discriminate between highly homologous molecules. It was also shown that llama VHH could be raised against different epitopes on the same molecule, for example the alpha and beta subunits of the hCG protein, or the intact protein.

Llama antibodies have also been found that successfully transmute across the human blood brain barrier, raising the possibilities of novel compounds that can be used to target brain diseases. Normal IgG's cannot cross this barrier (Muruganandam et al. 2002).

1.3.3 Applications of llama antibodies

A number of bulk applications for economical antibody fragments exist, as suggested by Frenken et al. (2000) including their use in waste water treatment, industrial scale separations, antibody-enzyme conjugates/fusions, as an ingredient in novel consumer goods with new or improved functionality. The potential also exists to use antibody based technologies to aid in environmental pollution management (Harris, 1999). All these applications require large amounts of inexpensive molecules.

Although production of conventional IgG molecule fragments is becoming more efficient, bulk production at low costs is extremely difficult due to high costs in downstream processing and the fact that large-scale production has not yet been described in detail. Conventional IgG molecules are most commonly used in medical and diagnostic application because of this high production cost. Llama VHH molecules are cheaper to produce and offer a more discriminating product due to their smaller novel target sites.

As well as medical use, VHH molecules can be used in large-scale applications across the biotechnology industry, their principle advantage being a long shelf life due to increased stability. The use of llama antibodies in detergents (for example to control dandruff in shampoos, and as targeted whiteners in laundry detergents) is a good example of where conventional IgG molecules would not be able to withstand the harsh environmental conditions needed (Linden, 2000). Llama antibodies have also been suggested as a possible treatment for prevention of dandruff by inhibiting the growth of organisms which contribute to dandruff. Furthermore they have been found to be stable in shampoos (Dolk et al., 2005).

Furthermore, camelid heavy chain antibodies have been shown to be potent enzyme inhibitors, demonstrated to actively compete for the active site, where conventional antibodies do not bind (Lauwereys et al, 1998). They have been shown to mimic the carbohydrate substrate of lysozyme and thus inhibit enzyme binding (Transue et al., 1998).

Other potential uses for these antibodies include the foods industry. Camelid antibodies have also successfully been used to prevent phage lysis of bacteria used in cheese production by adding 7nM of antibody. As these antibodies are produced in a food grade organism it makes them particularly suitable (Ledeboer et al., 2002). Other uses for antibodies have been demonstrated to be removal of bacteria using an immuno-affinity mechanism (Molloy et al., 1995)

1.4 Antibody fusion proteins and conjugates

Using standard molecular biotechnology techniques it is possible to create a range of multi-functional proteins with the antibody binding domain as part of that function. There are many possible fusion/conjugation partners from tags to aid purification to enzymes for use in diagnostic assays and cytotoxic agents for use in cancer therapy. Of particular relevance to this study is the fusion of the Cellulose Binding Domain (CBD) found in many organisms.

1.4.1 Cellulose Binding Domains

The Cellulose Binding Domain (CBD) was first observed in the fungus *Trichoderma reesei* and the bacterium *Cellulomonas fimi* in the late 1980's (Gilkes et al. 1988 and Van Tilbeurgh et al., 1986 and 1989). CBDs have also been found in some polysaccharide degrading enzymes such as hemicellulases, endo-mannase, acetyl-xylanesterase, xylanase, esterase and pectate lyase (Levy and Shoseyov, 2002). This has led to them being referred to as Carbohydrate Binding Modules (CBMs) in some literature.

They are structurally and functionally independent, noncatalytic modules of the enzymes they are found attached to (Linder et al., 1996). They are commonly between 4 and 20Kda in size and may be found internally or at either terminus of the cellulase enzyme. The two domains are joined by a linking segment of peptide of sufficient length and flexibility to allow the efficient orientation and operation of both (Carrard et al. 2000). Many organisms with a need to degrade cellulose produce cellulases with CBD's. A common feature for all CBD's is that they will bind to cellulose without any hydrolytic activity themselves.

CBDs can be removed from an enzyme by proteolysis or protein engineering. This results in decreased affinity, although the role of the CBD in the enzymes hydrolytic activity remains a matter of debate (Linder et al., 1998). Little or no effect is observed when the substrate is soluble however. They have a typically low content of charged amino acids and a high content of hydroxylamine acids (Reinikainen et al., 1992).

1.4.2 Applications of CBDs

Cellulose consists of linear polymers of thousands of glucose residues linked by β -1,4-glycosidic bonds. It has a rigid structure and is insoluble leading to it being highly chemically inert. This is due to the fact that it is closely packed together and forms long crystals that are stabilized by intermolecular forces (Linder and Teeri, 1996).

The first commercial application (and hence most studied) is the use of CBDs in fusion proteins as tags for affinity purification or immobilization. The reasoning behind this is economically very attractive: CBDs require little or no pretreatment as they adsorb spontaneously to cellulose from most solutions (van der Linden et al., 1998).

Cellulose is also inexpensive and chemically inert rendering it suitable for use in pharmaceutical and food applications. This approach has been used successfully for the purification or immobilization of numerous recombinant proteins such as alkaline phosphatase, Beta-glucosidase streptavidin, blood clotting factor X, protein A, and interleukin (Reinikainen et al., 1992).

1.4.3 Function of CBDs

Early experimentation on CBD mediated protein immobilization has shown very little or no leakage from cellulose leading to the hypothesis that the interaction of CBD's (sourced from *Cellulomonas fimi*) with cellulose is irreversible (Henrissat, 1993, Reinikainen et al, 1997). When considering the function of intact cellulases however, irreversible binding through the CBD seems unlikely as an irreversibly bound CBD would not allow rapid progression of the enzyme to the next substrate molecule (Linder and Teeri, 1998).

Linder and Teeri (1996) have demonstrated that the binding of some CBDs from this organism are reversible and have a relatively fast exchange rate on the cellulose surface. Jervis et al (1997) have shown that in some cases the adsorbed CBD does not stay statically in the same spot, but migrates on the cellulose surface.

One of the first suggestions concerning the role of CBDs was that they might aid in breaking up the cellulose molecule, prior to its actual hydrolysis (Knowles et al., 1987). At least some have been known to penetrate the fibres at surface discontinuities, release non-covalently bound fragments and uncover new cellulose chain ends. This tends only to happen with impure cellulose (such as pure cotton) and not pure crystalline, where there is no evidence for such activity (Linder and Teeri, 1998).

Linder et al., (1996a) have shown that by linking two CBD's together (creating a "double CBD"), the affinity is significantly improved. They hypothesise that linking more CBDs will increase affinity further. This hypothesis is derived from nature itself; cellulases produced by organisms living in extreme conditions can contain up to ten CBDs. Linder et al. (1998) also have shown that the binding of a protein to a CBD to

form a fusion molecule does not affect the activity of the CBD molecule, and is “functionally identical” to the isolated CBD.

Linder et al. (1998) also note the apparent effect of pH on the CBD's studied, noting that the binding is largely unaffected at pH's of 2.5-6.5, but above pH 7.0 there is a clear decrease in affinity. The interaction is estimated to have an approximate K_d of 1×10^{-6} nMol. They also note that although in most cases addition of a fusion protein has no affect, the size of the protein may prevent the CBD from penetrating the substrate as thoroughly as if it was by itself. Addition of another CBD increases the affinity approximately ten fold.

1.4.4 CBD families

Up to 180 different CBDs have been identified (Tomme et al, 1995). The majority of CBDs can be split into three families, see figure 4.

Although most CBDs belong to family I, II or III, there are another 7 families (IV-X). Some CBD's have also been reported to have an affinity towards chitin (Goldstein et al., 1993), suggested to be due to the close structural similarity of cellulose and chitin (Linder and Teeri, 1996). There have been some cases of xylan binding domains (Irwin et al., 1994). Whilst some Family II residues have been reported to enhance physical disruption of cellulose fibres and release small particles of cellulose, all others have no activity other than binding (Din et al, 1991, Tomme et al., 1995).

Family I	Found only in fungi. Contain 32-36 residues. Over 30 members.
Family II	Found only in bacteria. Contain 90-100 residues. Over 30 members.
Family III	Found only in bacteria. Contain 130-172 residues. Over 20 members.

Table 1: The major CBD families described prior to 1995

1.4.5 The structure of CBDs

All CBD structures characterized thus far are based on β -sheet topologies. Studies on the family I CBD from the cellobiohydrolase enzyme found in the organism *T. reesei* (CBHI-CBD) have shown that the CBD folds into a small wedge shaped structure. This is formed by a small irregular triple stranded β -sheet, stabilized by two disulphide bridges.

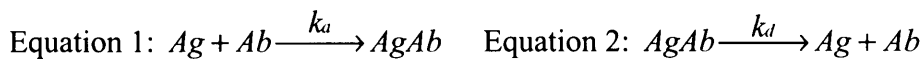
One side of the CBD contains a row of three tyrosines together, and a few potential hydrogen-bonding residues. The spacing of the tyrosines equals the distance of every second glucose moiety in a cellulose chain. Linder et al. (1995) have conducted site directed mutagenesis experiments that establish the importance of these residues in the CBD-cellulose interaction. CBDs found in *C. fimi* and *C. thermocellum* show similar structures (Linder and Teeri, 1997).

The main interaction consists of the stacking of aromatic rings with the glucose rings along a single glucan chain assisted by hydrogen bonding interactions of other CBD residues to the neighboring glucan chains (Tormo et al., 1996). Tormo et al. suggest a binding model implying that the polar residues on three different CBDs form direct hydrogen bonds to cellulose, and these interactions cause a local destabilization in the vicinity of the hydrogen bonded cellulose. For the structure of all known CBDs and similar molecules, an internet resource is available (<http://afmb.cnrs-mrs.fr/CAZY/>).

1.5 Quantifying protein binding

Antibodies form specific complexes via non-covalent interactions with their antigens. The large number of van der Waals interactions, hydrogen bonds, electrostatic interactions and the possible hydrophobic effects lead to very strong binding strengths and high affinities.

The strength of binding of an antibody to its corresponding antigen can be described by the affinity constant. This is a thermodynamic constant that represents the reaction between antigen (Ag) and antibody (Ab); see equations below (Liddell and Weeks, 1995).



$$\text{Equation 3: } [AgAb]/[Ag][Ab]$$

$$\text{Equation 4: } K^A = k_a / k_d$$

Where k_a is the association constant, k_d is the dissociation constant, and K^A is the affinity constant. The affinity constant has been determined by setting up and equilibrium between antigen and antibody and determining the amount of free and bound antigen. Methods of determination, and challenges this presents will be explored later.

Scatchard plots of data are necessary to determine the affinity constant determined by plotting the ratio of bound/free antigen against the concentration of bound antigen in moles per litre.

The negative slope of the Scatchard plot then gives the affinity constant and the X axis (conc. bound antigen) intercept gives the Binding capacity (B_c) (after extrapolation if necessary). The binding capacity represents the effective concentration of binding sites in moles per litre. Both B_c and K^A may change with environmental conditions such as pH and ionic strength of buffers.

1.6 Methods used to quantify binding of antibodies to antigens

There are many available methods to quantify antibody-antigen interaction. Traditional methods include radio-immunoassay (RIA), equilibrium dialysis and Enzyme Linked Immuno Sorbent Assay (ELISA has become the gold standard when dealing with antibodies). More recent advances in binding analysis have led to a range of optical biosensors, most notably the BIAcore and the IAsys Biosensor. Other even more novel techniques include Isothermal Titration Calorimetry (ITC), Atomic Force Microscopy (AFM), Confocal Laser Scanning Microscopy and more recently the Ultrasonic Spectrometer. The more novel the assay, the more delicate, although they potentially offer ways of understanding interaction previously considered impossible. They may also require a large amount of time in assay development; whereas conventional assays may give faster results.

1.6.1 Radio-immunoassay

This is the original method of antibody-antigen analysis, and involves using a known amount of radioactively labeled antigen. Antigen specific antibodies are immobilized on a surface. The radioactively labeled antigen is then allowed to bind to the surface bound antibodies, and the radioactivity is measured. A known amount of unlabelled antigen is then introduced, and the amount of radioactivity measured again. As the labeled and unlabelled antigen will be in competition, the necessary parts can be calculated for a Scatchard plot.

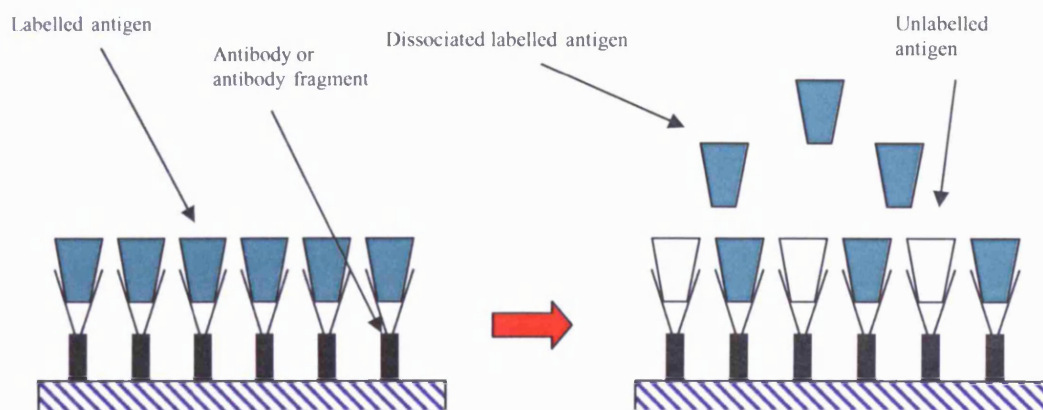


Fig 4: Radioimmuno assay. Labelled antigen competes directly with unlabelled.

RIA allows the analysis of kinetics in real-time at low levels such as those found physiologically, whereas other methods may require large amounts that are unrealistic in nature or application (Banerjee et al., 2002). RIA also allows an accurate study of dissociation, whereas some other methods involve estimating the dissociation constant from the association constant.

Use of radioactivity is becoming less popular with difficulties such as safety considerations and also disposal of contaminated waste. It is however still used as a gold standard assay for investigative assay and not everyday assays such as those used for diagnostics.

1.6.2 ELISA

ELISA (Enzyme linked immuno sorbent assay) is the gold standard analysis method for quantifying antibody reactions. Over the last forty years it has become an established laboratory procedure in the study of physiological, pathological and pharmacological processes (Roitt et al., 1998).

Problems arise with kinetics analysis however as a marker system is employed. The most common method is a simple indirect assay; the substrate is immobilized on a surface. The antibody in question is then allowed to bind to the immobilized substrate. A second antibody is then added which binds to the first antibody. A third antibody conjugated with a marker enzyme is then added and binds to the second antibody. The enzyme substrate is then added and a change in absorbance can be measured. This is directly proportional over a region to the amount of first antibody present (see figure 5). The most common method of performing ELISA's is in a multiwell plate, allowing hundreds of samples to be analysed simultaneously.

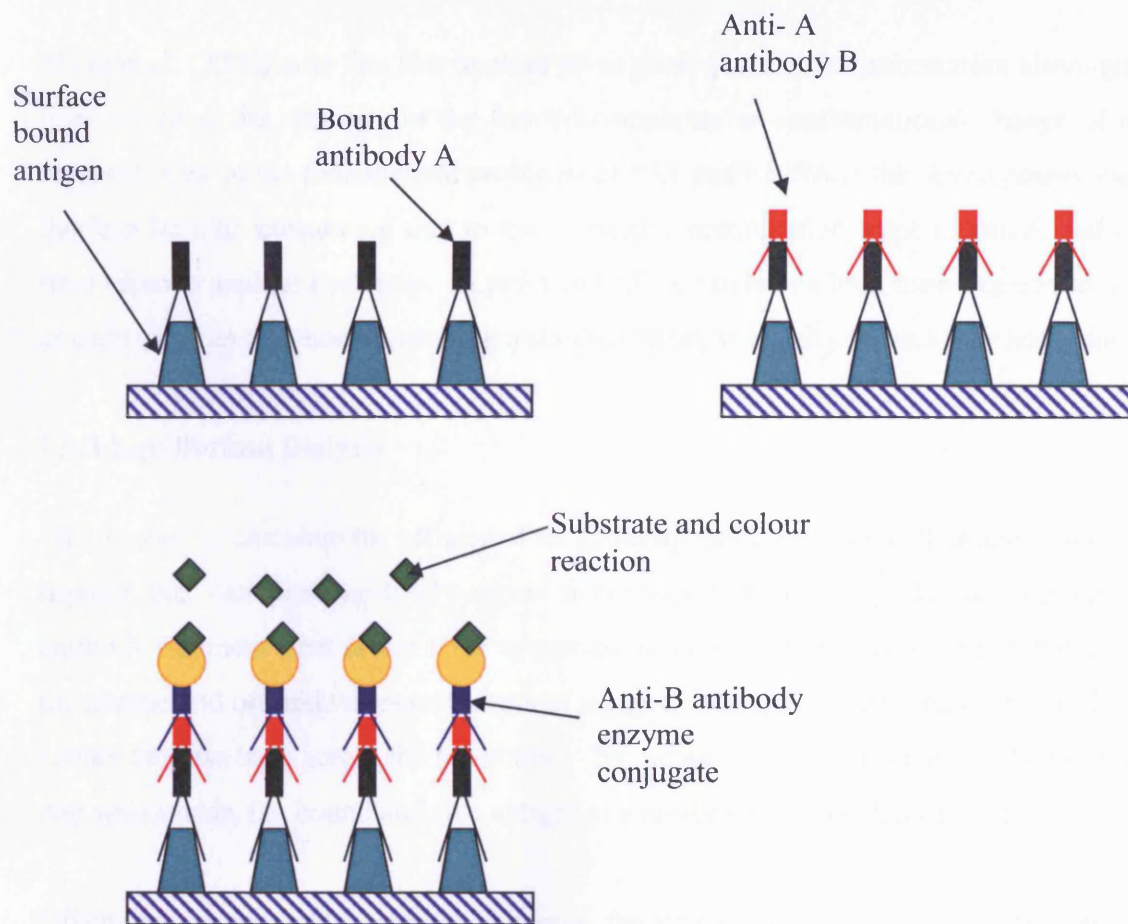


Fig 5: Indirect ELISA. Amount of antibody is measured by a spectra change due to reaction caused by substrate breakdown

Neish et al. (2002) note that this method gives good quantitative information although it does not show the structure of the formed complexes or conformational change of the receptor. One of the fundamental problems of RIA and ELISA is the development stage that can be time consuming due to the numerous optimization steps required, and the time taken to analyse each step. In practice ELISA can take a long time to perform also as each step can take hours, meaning a standard assay is usually at least four hours long.

1.6.3 Equilibrium Dialysis

This is used to calculate the affinity of an antibody that binds to small antigens such as haptens that can dissolve freely across a dialysis membrane. A known amount of antibody/fragment (that is too large to pass across a membrane) is placed in a dialysis membrane and offered various amounts of antigen. Molecules that bind to the antibody cannot traverse back across the membrane. By measuring the amount of antibody in the bag and outside, the bound and free antigen at equilibrium can be determined.

Given that the amount of antibody is known, the affinity and number of specific binding sites for the antibody can be determined via Scatchard analysis. It was this method that was used to demonstrate that IgG molecules have two identical antigen binding sites.

1.6.4 Optical Biosensors

1.6.4.1 BIAcore

Optical biosensors offer the potential to monitor binding reactions in real time. A major advantage of optical instruments is that there is no need to label the reactants with radioactive or spectroscopic compounds. However one of the reactants must be bound to a surface (Schuck, 1997). Limitations also occur on rapid binding reactions as the binding partner in solution must be transported to the sensor surface in order to bind to its immobilized partner (Wilson, 2002).

BIAcore uses surface plasmon resonance (SPR) technology to monitor the refractive index change as molecules absorb on to or dissociate off of a sensor surface during a binding reaction. The analyte is passed over the sensor surface using a micro flow cell that has been optimized to reduce mass transport effects (Myska et al., 1997). These effects can be further minimized by increasing flow rate and reducing the surface

capacity of the cell, but many reactions occur too quickly and therefore mass transport effects can not be eliminated completely.

SPR occurs when plane polarised light reflected at an interface between media of different refractive index and separated by a thin metal film resonates at a specific angle. This results in a dip in the intensity of the light reflected at that angle. Any increase in mass due to binding events on the cell surface causes the angle to change, and this change can be monitored in real time (Pearson et al., 1998). This process is very sensitive to changes in the media used to conduct the experiment, and care must be taken to minimise change wherever possible. BIAcore has been used to successfully monitor biomolecular interactions including antibody antigen, receptor-ligand and DNA-DNA. Binding studies for monoclonal antibodies have been readily performed (Malmborg and Borrebaeck, 1995).

The BIAcore measures binding in an arbitrary unit known as an RU, one RU being approximately equal to one picogram per square mm of bound protein. The majority of published data on optical biosensors (approximately 90%) involves use of BIAcore (Wilson, 2002).

1.6.4.2 IAsys Biosensor

The IAsys biosensor is a manual biosensor that incorporates Affinity Sensors resonant mirror technology in a single well format. Each cuvette is agitated with a micro stirrer. It has been used for quantification of protein A and human IgG₁ interaction (Lowe et al., 1999). As well as the BIAcore it offers analysis in real time, with multiple data points, not just a beginning and end result.

The main difference between the IAsys and BIAcore is that of the cell – the IAsys uses a stirred cell, making it ideal for particles, but difficult to analyse dissociation, whereas the BIAcore is a flow cell, meaning that particles are liable to clog it up after a maximum size, but ideal for measuring the dissociation. Both machines use an evanescent field for detection of binding, but slightly different methods of generation, the IAsys using a resonant mirror instead of a metal film.

IASys measures binding in Arc seconds, which is approximately four of the BIAcores RU's, or four picograms per square mm (Wilson, 2002). There is some small concern over evaporation from the IAsys stirred cell after prolonged assay use, although the rapid stirring supplies homogenous concentrations of ligand rapidly to the sensor surface, without mass transfer complications.

The IAsys is no longer commercially available, leaving the BIAcore as the sole market instrument.

1.6.5 Atomic Force Microscopy

Atomic force microscopy allows direct measurement of discrete intermolecular forces down to the pico-Newton range, in liquid suspension (Harada et al., 2000). Harada et al point out that common methods only estimate binding strength using antibody affinities; AFM actually gives a direct value. The actual binding strength is calculated by allowing the molecules to bind, then pulling them apart and measuring the forces involved.

A common problem with AFM is that the lack of control of the probing molecule orientation and the conformational changes that proteins undergo when being adsorbed to surfaces may affect the number of binding sites and the forces with which the molecules interact.

Neish et al (2002) describe using atomic force microscopy for visualization of ligand protein interactions. It is possible to bind ligand to a surface and then add receptor and physically pull the complex apart and get force measurements to quantify binding also. It is likely that such visualization may be useful in future research of antibody/antigen interactions. As with most other technologies for analysing antibody/antigen interactions, AFM requires the binding of the ligands to surfaces (Ouerghi et al, 2002).

1.6.6 Isothermal Titration calorimetry

An ITC instrument consists of two identical cells composed of a highly efficient thermal conducting material (Hastelloy or gold) surrounded by an adiabatic jacket. Sensitive thermopile/thermocouple circuits detect temperature differences between the two cells and between the cells and the jacket. Heaters located on both cells and the jacket are

activated when necessary to maintain identical temperatures between all components (Pierce et al., 1999).

The direct observable measured in an ITC experiment is the time-dependent input of power required to maintain equal temperatures in the sample and reference cell. During the injection of the titrant into the sample cell, heat is taken up or evolved depending on whether the macromolecular association reaction is endothermic or exothermic, this heat is proportional to the fraction of bound ligand. Thus, it is of extreme importance to determine accurately the initial concentrations of both the macromolecule and the ligand.

One of the difficulties with this instrument is that extreme care must be taken in all aspects of an ITC experiment, from sample preparation to data analysis, as it is highly sensitive. The concentrations of macromolecule and ligand are critical, especially when one or both partners of a complex are difficult to obtain in large quantities or are of minimal solubility. Both titrant and macromolecule should be exhaustively dialyzed in buffer (preferably in the same flask) to minimize artifacts arising from mismatched buffer components (Pierce et al., 2002).

Although a key advantage of this instrument is its high sensitivity, it is also a double-edged sword – it is often the case that binding being measured are other ancillary effects and not true binding.

1.6.7 Antibody kinetics analysis: conclusion

Many methods exist for antibody kinetics analysis. The majority rely on the equilibrium constant or an estimated or relative equilibrium constant. Fuchs and Gessner (2001) report that more than ten thousand articles are published every year dealing with this constant.

The majority of methods are based on linearization procedures of the Law of Mass Action, most commonly the Scatchard analysis. Methods such as those using optical sensor technologies have computer-supported facilities that allow the calculation of equilibrium constants. However these techniques hold complicated mathematical procedures that can make it difficult to interpret the precision of the results (Fuchs and

Gessner, 2001). All methods are a compromise between ideal conditions, and require an understanding of the limitations of the instrument used.

The necessity to label the proteins in some methods or bind them to a surface is a disadvantage as they may not truly represent real situations, however, these methods are more established. More novel methods may produce data that is hard to understand, and need comparison to these established methods to be truly useful. The best approach where possible is to use a combination of available methods.

1.7 Production of antibodies and antibody fragments

1.7.1 Genetic manipulation in the production of antibodies and antibody fragments: Antibody libraries

Approximately 30% of antibodies in clinical trials in 2002 were estimated to be provided by phage display libraries (Kretzschmar and Ruden, 2002). It is a powerful technology for selection of specific antibodies from a huge library of possible candidates. An alternative technology involves displaying the antibody on the surface of a yeast cell, this is known as yeast display.

Phage display libraries are constructed by cloning large repertoires of genes encoding the antibody or fragment of choice into engineered phage vectors such that they are displayed on the viral capsid protein coat or the yeast cell. A number of enrichment steps are used, to leave colonies of organism that produce specific monoclonal antibodies against the antigen of choice (Kretzschmar and Ruden, 2002).

It is believed that phage display selection is at least as powerful as immune selection. Phages displaying the antibody of choice can be enriched in numerous ways such as binding to antigen coated plates, column matrices, cells, or biotinylated antigen in solution (Winter et al., 1994). The enriched phages can then be grown in bacterial culture and subjected to further rounds of enrichment, increasing the binding strength of candidates obtained. Yeast display is less powerful, but may be used for commercial reasons.

1.7.2 Production in prokaryotic systems

Production of any biological component is most easily done by expression in bacterial systems as bacteria can be easily fermented. This is not always possible; antibodies cannot be produced whole in bacterial expression systems as they lack the necessary glycosylation equipment to produce them. Fragments can be produced in bacteria that are transformed with the genes from hybridomas or β -lymphocytes, and are expressed most commonly in *Escherichia coli* (Skerra and Pluckthun, 1989, Verma et al., 1998). The scFv fragment is the most commonly produced in *E. coli*. Jurado et al. (2002) described an *E. coli* strain engineered to produce scFV in the cell cytoplasm by mutating glutathione oxidoreductase and thioredoxin reductase genes to aid disulphide bridge formation.

Expression levels in bacterial expression systems are variable and hard to predict from case to case. Some of this variation is seemingly dependent on the intrinsic folding capacity and stability of the protein. When folding results in the uncovering of hydrophobic patches, the antibodies form cytotoxic aggregates that kill or damage the *E. coli* cells. Even a single point mutation may have an effect on folding and hence production (Knappik and Pluckthun, 1995).

E. coli has difficulty in forming the intramolecular disulphide bonds of the molecule, as this is carried out far more effectively by the endoplasmic reticulum (ER) and Golgi apparatus of eukaryotic cells. These bonds are essential for the correct three-dimensional structure of the protein. The ER and Golgi are also responsible for folding and secondary protein modifications. As prokaryotic cells do not have these organelles they are therefore unable to perform these important functions.

Eukaryotic cells also contain molecular chaperones, such as Protein Disulphide Isomerase (PDI) that guide and augment the folding of certain antibodies (Buchner et al., 1992). Although chaperone proteins are present in prokaryotes such as *E. coli*, their function is limited when guiding eukaryotic proteins.

A further problem with production in *E. coli* based systems arises down stream. As the antibodies are normally produced intracellularly, the cells must be disrupted to release the product. This causes problems with purification and contamination of biologically

active components, such as endotoxins. These factors contribute to extremely high production costs in *E. coli* vectors meaning that their use is only economically viable for human medicine and diagnostics, and not for large scale production for consumer products (Van der Linden, 1999).

1.7.3 Production in higher eukaryotes

1.7.3.1 Production in mammalian cell culture

Currently, 60-70% of all recombinant protein pharmaceuticals are produced in mammalian cells (Wurm, 2004). Any IgG subclass can be generated in mammalian cells, using the original technology described by Kohler and Millstein (1975). Productivity continues to increase because of improved recombinant expression vectors and enhancement of biomass accumulation (Chadd and Chamow, 2001). Cell culture can now produce yields of 1-2 g unpurified antibody per litre culture media, although this can be decreased on purification. However companies involved in record levels of protein production in cell culture are very reluctant in presenting break-through achievements in this area, or methods to duplicate their success (Wurm, 2004).

As with all cell culture methods, problems can arise over media, which traditionally use about ten percent calf or foetal calf serum. With recent concerns over Bovine Spongiform Encephalitis and similar diseases, raw materials obtained from animal products are becoming increasingly unpopular with regulatory authorities. Although serum free media can be obtained, it can often be substantially more expensive than bacterial simple media. This is because bacteria are capable of synthesising many nutrients that mammalian cells cannot, and must therefore be present in the culture media (Stanbury and Whittaker, 1995). Further more, cell lines used must be prion free, which adds to the production cost.

Wurm (2004) states that mammalian cells cultivated in bioreactors have become superior to microbial systems for the production of clinical products in both levels of production and numbers of products. Although the production of superior proteins in terms of glycosylation is a distinct advantage, often the cost of producing proteins in a cell culture system can be very high.

1.7.3.2 Production in plants

Complete antibodies and fragments have successfully been produced in plants (Franconi et al., 1999 Gibbs, 1997). These have been produced in certain parts of the plant, such as the roots and fruit that may be suitable for mass oral applications. Although yields in plants are high (between 1-5% of plant protein produced) purification is long and difficult (consequently expensive) and the development phase is also time consuming. There is also some concern over incorrect glycosylation of proteins.

1.7.4 Production in Lower eukaryotes

Yeasts, in particular *Saccharomyces cerevisiae* have been used for the successful production of antibody fragments. *S. cerevisiae* has many advantages over less conventional yeasts in that it has Generally Regarded As Safe (GRAS) status, produces high cell densities rapidly in simple media and secretes only few proteins next to the wanted antibody. These organisms can grow in clearly defined media without animal derivatives, can secrete large amounts of recombinant protein (greater than 1g/l) and are easy to scale up, making their use common (Gerngross, 2004). Yeasts and filamentous fungi have been used extensively for the production of recombinant proteins for the enzyme industry, however they are typically unable to provide suitable levels of glycosylation for mammalian proteins.

Production of whole antibodies in *S. cerevisiae* has been reported (Horwitz et al., 1988), although at very low levels (micrograms per litre). A production level of one gram per litre is needed for an economically viable process (Van der Linden, 2000). Another GRAS organism, the methylotrophic yeast *Pichia pastoris* has been successfully used to produce antibodies (Eldin et al., 1997).

Pichia pastoris is a very effective at secreting produced proteins and growing to high cell densities. Excreted proteases can be shown to be inhibited by fermentation at pH 3.0 instead of pH 5.5 (Curvers et al., 2001). Common *P. pastoris* systems use methanol for growth and induction of protein production, meaning that methanol needs to be removed from the product stream. Some systems have been developed which do not use methanol as a carbon source (Chiruvolu et al., 1997). Some *pichia* produced

proteins have recently entered clinical trials, although they have been noted to contain a level of O-mannosylation not present on the native protein (Gerngross ,2004).

1.7.5 Isolation and production of llama heavy chain only antibodies and fragments

Immunisation of llamas to produce llama antibodies is commonly done with the antigens in a water in oil emulsion (Boersma et al., 1992). Llama serum can be fractionated using protein G and protein A chromatography, as described by Hamers-Casterman et al (1993). Fractions containing heavy chain only IgG can then be identified using SDS-PAGE electrophoresis and separated.

The nucleotide sequence of the long and short heavy chain hinges of *Lama glama* were identified and specific PCR primers produced which were used to amplify heavy chain VHH-encoding DNA fragments. This allowed the production of separate VHH libraries that are not contaminated with V_H domains of conventional IgG molecules.

Production of llama heavy chain only antibodies is commonly done in the lower class eukaryotes, such as *Saccharomyces cerevisiae*. Production of conventional IgG fragments in *S. cerevisiae* is not common as the fragments are found to accumulate in the Endoplasmic Reticulum and the vacuole (Frenken et al., 2000). Intact and functional heavy chain antibodies derived from camelids have also been successfully produced in mouse B cells, showing potential for the generation of transgenic single chain antibody/ antibody fragments (Nguyen, 2003).

1.7.6 Conclusion: Antibody production

At the present time, bacterial expression systems are only useful for generation of antibody fragments (Chadd and Chamow, 2001). To make a full-length antibody, mammalian cell culture is the most efficient and cost effective. Other systems such as recombinant plants must be evaluated more thoroughly, but offer a great deal of potential. The main residing problem in all expression systems is the cost of production. This cost is acceptable for medical and diagnostic applications where only a small quantity is used, but for antibodies to realise their potential in the biotechnology industry, large amounts of cheap product are necessary.

The potential to use yeast based productions systems for llama antibody proteins offers a considerable cost advantage over that of conventional antibodies produced in mammalian cell culture.

1.8 Protein Purification

1.8.1 Introduction

It is often not possible to satisfactorily perform detailed analysis of a protein of interest when in a crude form as it may reside within a complex mixture of proteins, nucleic acids, polysaccharides and also the chemicals used to grow the producing organism, even after the cells have been removed. Furthermore pure proteins are often essential in biotechnology for regulatory and safety purposes. It is essential to maintain functionality when purifying proteins for use in a product by retaining secondary, tertiary and quaternary structure, and hence this restricts the use of some damaging techniques. Although it is often possible to renature proteins, the method for this is often expensive and time consuming (Lillehoj and Malik, 1989).

It may not however be essential to obtain levels of absolute purity such as those obtained in the pharmaceutical industry, for example, enzyme purity in washing powders is often satisfactory at 70%, while a therapeutic protein may be needed at 99% (Parry, 2003). Many factors will influence the choice of purification technology to be used, including the cost of the process, the final desired purity, the amount of desired product and the ability to scale-up the process to a larger level.

Down stream processing and purification of proteins often accounts for a large majority of their production cost (50-80%) (Roque et al., 2004). The degree of initial purity has a high level of bearing on the purification cost as the more impure the protein, the higher the number of steps required to purify it satisfactorily. This adds a further cost, and furthermore each step incorporates a loss of product, which becomes cumulative over a number of steps. Doran (1995) quotes an example where a product is 80% recovered on each of five steps. This leaves an overall product recovery of about 33%.

This therefore leads to a need to get the product in as high an initial level as possible by increasing production of the protein by the organism, and also to reduce costs associated with production, either by reducing steps, or increasing efficiency of steps.

1.8.2 Production and Purification of llama antibody – CBD fusion proteins

Production of the proteins of interest is done in three organisms: *Aspergillus niger*, *Pichia pastoris* and *Saccharomyces cerevisiae*. The *Pichia* and *Saccharomyces* produced proteins are supplied in pre-purified form. The proteins produced in *Aspergillus* are in crude form. All proteins are secreted by the production organisms into the supernatant.

A major problem with *Aspergillus* secreted heterologous proteins is their contact with and degradation by extracellular proteases (such as aspergillo pepsin) and this has been shown to affect their production dramatically (Gouka, 1997). Fungal strains deficient in proteases have been isolated or created by molecular genetic approaches and have resulted in significantly improved protein production levels. Gouka et al. (1997) report that protein expression levels have reached 1-2 g/l of fermentation broth.

1.8.3 Stages in purification

Purification of proteins can be seen to have many goals as follows (Roe, 2001):

- Release of protein product into supernatant (If produced intracellularly)
- Removal of bulk material such as cellular matter
- Removal of water to concentrate the protein
- Removal of contaminants
- Stabilisation of protein.

It is usual to use sequential separation techniques that utilise different physicochemical properties of the protein involved (Lillehoj and Malik, 1989). There can be considered 3 main stages in purification: Initial fractionation, purification and polishing. The Initial fractionation is a preparative stage that may involve the first three of the points above. This typically involves techniques such as centrifugation, cell lysis, micro and ultrafiltration.

The first step is often removal of cellular matter from the mixture, although the component carried on to the next step depends on the nature of the protein – extracellular or intracellular (Doran, 1995). This is dictated by the expression system used and the presence of N-terminal signal sequences that allow the translocation through cellular membranes. Each has advantages and disadvantages considered in

regards to a whole process and purification basis by Pierce et al. (2002). The llama VHH fusion protein is extracellular – this means that it is relatively pure as cellular constituents can be removed easily, although it is dilute and susceptible to extracellular proteases. This also offers considerable advantages to the fermentation of the protein.

Separation of cells is most commonly achieved by centrifugation or filtration. These methods are then often followed by precipitation and/or a number of chromatography steps (Bonnerjea et al., 1986) which comprise the purification stage. Three to five chromatography steps may be used in sequence to remove different types of contaminants (Roe, 2001). The polishing stage then comprises removal of the last few contaminants and indeed any breakdown products of the protein. Size exclusion chromatography is often a key step at this stage, although adds a large expense.

It is important to understand the condition in which the protein emerges from each step, crucial factors being the stability of the protein in the conditions at the end of the step and also the need for any preparative work before the next step, for example buffer exchange (Lillehoj and Malik, 1989).

1.8.4 Scale up of process

Often a small scale purification system is the first stage in the development of a large scale commercial process. When designing any purification process it is therefore wise to keep in mind scale-upability (Roe, 2001). This can often mean that high expense, low yield processes that may be suitable for producing pure proteins for detailed analysis may be unsuitable for industrial scale purifications.

Purification goals will also shift as the process is developed – from a low level of purified product where the key is identifying protein and ensuring activity is retained when the process is in the first stages to the final stage of purifying protein in large levels for large scale product testing. It is important that any process to be scaled up is sufficiently robust to cope with any added process parameters (Noble, 2001).

1.8.5 Properties of proteins affect purification

Proteins consist of a large number of amino acids each having different properties. Only a small proportion of these amino acids are exhibited on the surface, and these affect the properties greatly. Any disruption of the protein structure can display a new set of amino acids and a new set of properties.

Amino acids may be hydrophobic, hydrophilic, charged and aromatic. Charged amino acids may be either basic or acidic, giving proteins an amphoteric nature: at low pH proteins are positively charged due to the basic groups and at high pH proteins are negatively charged due to acidic groups. The pH where the net charge is zero is known as the isoelectric point of the protein (Hagel, 2001).

1.8.6 Initial Purification or Clarification

A number of different production mechanisms exist for the production of proteins as discussed previously (section 1.7). The production of proteins from a fermentation stream often leaves the protein at a relatively low level in a complex mixture of contaminants.

Proteins may be located in one of three locations: extracellularly, in the fermentation medium, intracellularly in the periplasmic space or intracellularly as an active protein or an inclusion body. Generally the secreted protein is the easiest to work with as no cellular disruption is needed, although the protein may be very dilute, and susceptible to degradation (Pierce et al., 2002).

Pierce et al. (2002) have studied the production and purification of an enzyme produced extracellularly in *Streptomyces lividans* and periplasmically in *Escherichia coli*. They conclude that three steps are required prior to chromatography with the intracellular protein and the extracellular protein one step (cell removal). Also the use of lysozyme to destroy the cell wall in the intracellular organism process contributes to the protein loading of the system. However the presence of the protein in the periplasm allows a lower level of processing volume. The benefits of secretion of product are also reduced if complex media is used in the fermentation.

In addition, Pierce et al. (2002) note that the presence of cellular debris complicates chromatography of the *E. coli* product stream. It is suggested that production in the periplasm may be better for proteins that require a more oxidising environment for disulphide bond formation and correct folding.

The most frequent first step (after cell disintegration if necessary) is to perform removal of suspended matter using either sedimentation or filtration (Reed and Mackay, 2001). Sedimentation involves separation according to density differences between mixtures. Gravity sedimentation is rarely used as it is too slow, the process can be speeded up by flocculating or coagulating contaminants, and also the application of centrifugal forces. Centrifugation is often the method of choice for yeast and fungal cells as they are relatively large and show a subsequently good sedimentation velocity (Thömmes et al., 2001).

Filtration is also commonly used, the goal of this being to retain any solid matter in a porous filter barrier. A large range of filter equipment is available to separate a whole host of contaminants according to size.

After the solution containing the protein product has been clarified, it may be necessary to concentrate the protein by removing volume of liquid whilst retaining product. A smaller volume of solution is easier to use in subsequent purification steps, such as chromatography (Harris, 2001).

Concentration is achieved by removal of water and other small molecules by addition of a matrix polymer with pores too small for the protein to penetrate, removal of small molecules through a semi-permeable membrane which will not allow the protein of interest to pass or removal of water under a vacuum, for example by freeze drying. Precipitation can also be used to concentrate proteins if the pellet is resuspended in a smaller volume of liquid (Harris, 2001).

However dilution of a sample, as is commonly achieved when using ultrafiltration or performing buffer exchange with a size exclusion column can also be advantageous as in addition to removing aggregates that may interfere with later steps, they may also minimise the effects of proteases (Lillehoj and Malik, 1989).

Deciding which initial step to use in a purification scheme is dependant on the concentration and condition of the protein in the starting solution. The higher the concentration, the easier typically the purification – proteins present in trace amounts are typically very hard to purify. Protein purification is also easier if a good source is available and it is always worth optimising production conditions to ensure a good supply of stable, functional protein (Lillehoj and Malik, 1989). The effect of production of the molecule will not only be present on the initial step, but all subsequent steps (Picket and Hardy, 1984).

1.8.7 Chromatography

1.8.7.1 Chromatography Definition

“Chromatography is based upon the principle of differential partition of a specific component between two phases, the mobile and the stationary phase. The former serves to move the latter in a manner that optimises the physical and chemical interactions of the sample with both phases” (Lillehoj and Malik, 1989). Proteins are usually purified using a system known as liquid chromatography that utilises a liquid mobile phase and a solid stationary phase.

1.8.7.2 High Performance Liquid Chromatography

A system to vastly improve chromatography was developed in the 1970's (and is ubiquitous in protein chromatography today) known as High Performance Liquid Chromatography (HPLC). It has superior resolution and separation to conventional liquid chromatography.

It employs a variety of separation matrices that are strengthened against high pressure, allowing fast flow rates and hence rapid separation (Lillehoj and Malik, 1989). Traditional chromatography methods could not handle high pressures, meaning only low flow rates could be used. Furthermore, advances in matrix development means that column packing is far more efficient, and column void volume reduced. Matrices used are versatile and allow a range of separation techniques to be used.

1.8.7.3 Types of chromatography

There are many types of chromatographic steps available, but they can be separated into four basic groups:

1. Ion Exchange. This is a very common method commonly used early in the process to remove bulk contaminants (Bonnerjea et al., 1986). The protein is adsorbed to a matrix by ionic interaction depending on the conditions in the column. These conditions are determined by the pI of the protein, the protein is then eluted with a high salt concentration. Due to the high reliability on pH and salt levels of this method, the protein often must be desalted/ buffer exchange prior to ion exchange. Furthermore the high levels of salt present in the eluent conditions often require a further desalting step. Further details can be found in section 1.8.7.4.

2. Gel filtration. Molecules are separated by size, which affects the speed at which they travel down the column. Large molecules cannot penetrate the matrix beads, and flow rapidly round the outside. Small molecules can penetrate, and consequently take much longer, therefore big molecules are eluted first, and small ones last (Doran, 1995).

This can serve one of two separation roles. It is often used as a final polishing step to separate a small number of proteins preparatively purified. The disadvantage of this method is that it is relatively expensive, and can only accept a relatively low amount of

protein. Gel filtration can also be used as a buffer exchange/desalting step. Further details can be found in section 1.8.7.5.

3. Affinity Chromatography. This method utilises the binding function of the molecules to be purified. Anything that has a binding function can be purified this way, for example enzymes, hormones, antibodies, receptors and antigens (Doran, 1995). Although capable of giving very high purities very rapidly, commonly elution conditions are harsh, and the protein needs to be exchanged into a more suitable buffer rapidly. Also affinity matrices are often very expensive. Further details are available in section 1.8.7.6.

4. Hydrophobic interaction chromatography. This method utilises the fact that even proteins of hydrophilic nature have an amount of hydrophobic areas on their surface. Although this can work for a high variety of proteins, it needs very high salt solutions and is very temperature dependent (Hagel, 2001). Further details can be found in section 1.8.7.7.

Ion exchange, Affinity and Hydrophobic interaction chromatography can be considered to be adsorptive chromatography (ie the protein binds to the matrix) whereas gel filtration is non-adsorptive (separation occurs due to size exclusion in the matrix)(Hagel, 2001).

Hagel suggests five key phases in an adsorptive chromatography process:

1. Equilibration of the matrix using a mobile phase that allows adsorption of the protein to the matrix.
2. Application of the sample in the mobile phase.
3. Wash using the mobile phase that allows the protein to be retained in the matrix, but removal of contaminants.
4. Desorption of the protein using a mobile phase selected to make elution favourable i.e. by increasing the dissociation constant.
5. Wash, clean and regenerate the matrix ready for the next purification.

1.8.7.4 Ion Exchange

This is a very common preparative, as it has a high throughput and is relatively inexpensive (approximately 5 times cheaper than Protein A media) (www.amershambiosciences.com, 2006). Proteins are bound as mentioned previously by a charge interaction, caused by the molecule of choice adsorbing to a solid phase containing the opposite charge (Lillehoj and Malik, 1989). Two types of ion exchange processes are available: cation and anion exchange. Cation exchangers will bind proteins in a solution of pH below their pI and anion exchangers will bind proteins in a solution of pH above their pI. Matrices can be further separated into weak and strong ion exchangers, a slightly misleading term as this is due to their ionic capacity and not the strength of their binding.

(Levison, 2003) states five stages for development of an ion exchange process:

1. Adjusting the conditions of the crude material such that it encourages binding to the solid phase.
2. Contacting crude material with the solid phase to allow adsorption.
3. Wash the unbound material from the solid phase.
4. Change conditions of the solid phase such that bound molecules desorb and elute.
5. Regenerate solid phase.

The adsorption itself can take place either in a batch process, or a chromatography column. Column chromatography is widely used in industry and will be focused on in this text. Adsorption is commonly done at a low salt concentration and elution at a high salt concentration, although conditions may require a change in pH. The strength of binding between protein and solid phase is dependent upon the solvent pH, ionic strength and selectivity of the counter ion (Lillehoj and Malik, 1989).

Ion exchange can be used in one of two ways: to bind and elute the molecule of interest (known as positive chromatography), or to bind and discard the contaminants, and collect the molecule of interest as it washes straight through the column (known as negative chromatography) (Levison, 2003). The selection of these two methods is dependant on the starting material as well as the end requirement.

For example late on in a process, when the molecule of interest is of higher purity, it may be desirable to remove a particular contaminant, and negative chromatography achieve that. However ion exchange is commonly used as a preparative step, and in this case it is often desirable to use positive chromatography. Also positive chromatography often concentrates the molecule of choice, where negative does not. Ion exchange is a very good method for maintaining biological functionality (Lillehoj and Malik, 1989).

1.8.7.5 Affinity Chromatography

A large market for successful purification of proteins to a very high purity in the pharmaceutical industry has led to many systems being developed for aiding purification of a protein product from a crude process stream. Affinity chromatography methods are amongst the most powerful and versatile of chromatography methods (Lillehoj and Malik, 1989).

Proteins perform their biological function through one or more binding events, for example enzyme and substrate, inhibitor and co-factor, and antibody and antigen. This can often be ideal for adsorption chromatography. Key stages in affinity chromatography are choosing a suitable ligand, immobilising the ligand to a matrix, binding the protein of choice to the affinity matrix, removal of non-specifically bound proteins and then removal of the protein of choice (Angal and Dean, 2001). The method depends on a highly specific interaction between the protein of interest and a molecule immobilised on a chromatographic matrix, and the molecules subsequent release under elution conditions (Verheesen et al., 2003).

Affinity chromatography is one of the most powerful techniques for protein purification since it is highly selective, allowing purification in a single step from a complex mixture. Also, along with all adsorbent chromatography systems, it offers the opportunity to concentrate the protein of choice. Contaminants present after affinity chromatography are generally few and in low concentration although may comprise the matrix constituents (Lillehoj and Malik, 1989).

However problems with affinity chromatography may arise in obtaining a suitably specific, stable matrix ligand that exhibits reversible binding (Angal and Dean, 2001).

Problems may be encountered with the high associated cost of the affinity resin, difficulties in scale up and leaching of bound ligand from the column (Trabbic-Carlson et al., 2004).

Affinity purification of a protein can be achieved either by utilising the native properties of the protein (for example purification of IgG molecules using Protein A chromatography (Lindmark et al., 1983)) or by adding a fusion “tag” to the protein using molecular biology techniques. This involves producing the protein of interest as a fusion protein with a protein or peptide tag which binds reversibly to a ligand immobilised on a chromatography matrix (Trabbic-Carlson et al., 2004). The fusion tag is selected to have properties that allow binding of the protein to a corresponding affinity matrix (such as His tag and Nickel NTA matrix (Terpe, 2003)). This method often allows one-step purification of the desired protein to a very high level of purity.

1.8.7.6 Affinity chromatography of antibodies

Antibodies are commonly purified using Protein A or G as a ligand. Protein A binds specifically to the Fc region of immunoglobulins from a number of species and is found natively on the cell wall of the bacterium *Staphylococcus aureus*. The matrix is however expensive and requires low pHs to elute antibody. Protein G is found on *Streptococcus*. Protein G matrices are also expensive and require low pHs to elute (Angal and Dean, 2001).

Although both proteins bind to antibodies, they are subtly different in binding to antibodies of different species, for example, Protein A binds to human, rabbit, mouse and guinea pig antibodies, while Protein G binds to all these, and also goat, sheep, cow and horse.

Antibodies bind to these proteins with very high affinity meaning that the conditions necessary to elute proteins are very harsh, and may even lead to denaturation. Lillehoj and Malik (1989) quote that a 30 minute incubation at pH 3.0 or 10.0 can result in up to a 65% reduction in antibody titre, meaning that the eluent must be neutralised to a suitable pH as rapidly as possible after elution. Often the antibody can be irreversibly inactivated by the harsh conditions used in protein A and G chromatography (Huse et al. 2002).

It is also possible to use immobilised antigen to purify antibodies, however similar problems remain in elution and there exists the chance of leaching of the antigen into the product (Huse et al. 2002).

1.8.7.8 Affinity Chromatography using an engineered fusion tag

Addition of a fusion tag to a protein can provide a good way of purifying them using affinity chromatography. Addition of a short peptide tag for example the hexa-histidine tag to a recombinant protein can allow their simple purification using immobilised metal affinity chromatography (IMAC) (Paramban et al., 2004). Furthermore addition of a 6xHis tag has been reported to enhance activity and stability of recombinant proteins. The affinity tag can be removed after purification with a site-specific protease, and has been used in the purification of many proteins of biological interest (Mohanty and Wiener, 2004).

Positioning of the tag is important, Mohanty and Wiener (2004) have investigated the use of an N-terminal 6-histidine, N-terminal 10-histidine, C-terminal 6-histidine and C-terminal 10-histidine. They have shown that while solubilisation of the protein is largely unaffected, the N-terminal 10 histidine protein binds significantly more strongly to the chromatographic matrix. Length of the tag is also shown to affect yield of protein, the 10-histidine tags reducing the yield significantly.

1.8.7.9 Use of Cellulose Binding Domains as fusion tags in affinity chromatography

(Shpigel et al., 2000) have used Cellulose Binding Domains to create a fusion protein based affinity purification system for IgG molecules. The fusion protein consists of a CBD used to immobilise the protein on a cellulose matrix and staphylococcal protein A, used to purify the IgG molecules using standard Protein A affinity procedures.

All other affinity methods rely on the use of an expensive matrix to hold the affinity ligand. Cellulose has ideal physical properties and is very economical, especially for large-scale purification.

Shpigel et al., (2000) purified their CBD fusion protein using a cellulose column. They bound the protein to the column in PBS pH 7.4 and eluted with high pH (12.5). The high pH was then neutralised with HCl and the protein lyophilised. Many proteins have been expressed with CBD affinity tags and still retain high specific activity (Levy and Shoseyov, 2002). Extremes of pH used may cause damage to the protein.

1.8.7.10 Gel Filtration

Separation according to size has been used since the mid 1950s (Porath and Flodinm, 1959) when it was termed gel filtration. More recently it has been generally accepted that the term size exclusion chromatography is more suitable. (Cutler, 2001). It was the first of the matrices to be adapted to HPLC (Lillehoj and Malik, 1989).

The matrix consists of beads of known porosity which are packed into the column. The mobile phase can penetrate into the beads, and also flow around them in what is known as the voids volume. Separation occurs as molecules of differing molecular weights can penetrate to different degrees within the matrix. This means that different sized proteins have different volumes to flow through within the column, dictating when the time they take to reach the end of the column.

In an ideal situation large proteins elute first as they gain little penetration into the matrix, and small proteins elute last, as they penetrate further into the matrix (Cutler, 2001). However in reality there is usually some interaction between the protein and the size exclusion support, and this can change the elution order. This effect is commonly minimised with use of salts or detergents (Lillehoj and Malik, 1989).

The main limitations of size exclusion are that all proteins elute in one column volume or less. This offers low capacity relative to other techniques and also means that separation of complex mixtures is difficult, rendering size exclusion most suitable for a polishing step (Cutler, 2001). The polishing step is aimed at removing distinct contaminants, in particular low molecular weight contaminants including salts and may also be used to change the mobile phase (Rojas et al., 2004).

Proteins with a molecular weight difference of less than 10 % can commonly not be separated satisfactorily. Resolution can be enhanced by addition of columns in series containing the same size pore support, although this obviously increases run time and

cost (Lillehoj and Malik, 1989). A good advantage with size exclusion chromatography however is the ability to choose a variety of mobile phases, reducing the need for desalting procedures, making it ideal for a final polishing step.

1.8.7.11 Hydrophobic Interaction Chromatography

Hydrophobic Interaction Chromatography (HIC) relies on hydrophobic interaction between protein sample and the adsorbent (Lillehoj and Malik, 1989). Although the exact mechanism of HIC is unclear, the interaction is increased by high ionic strength and high temperature. Increasing the ionic strength does increase the chance of precipitation and increasing the temperature may affect the stability of the protein. It is thought best to use the most hydrophobic ligand that is compatible with the protein to allow use of low ionic strengths in the process (Hagel, 2001).

1.8.8 Other methods

Trabbic-Carlson et al. (2004) have reported a novel method of purifying proteins using a fusion tag that selectively aggregates the product of choice depending on a narrow temperature range. By sequential steps of aggregation centrifugation and resolubilisation, a method described as inverse transition cycling allowed the purification of proteins chloramphenicol acetyltransferase, blue fluorescent protein, thioredoxin and calmodulin to high purity and maintained functionality.

1.8.9 Determination of Yield and Purity

Several methods are available for the determination of yield and purity in a protein purification process, however the selection of these methods will be dependent on a number of factors, such as the amount of protein available, the nature of the protein to be analysed, assay accuracy and sensitivity and the presence of any contaminants which may affect assay results (Baines, 2001).

A number of techniques are available to determine total protein concentration in samples including UV absorbance at 280nm, Lowry assay, Bradford assay and BCA assay. Each of these methods is subject to inherent limitations. Often a standard curve

is determined using a level of a highly pure protein, commonly Bovine Serum Albumin. (Baines, 2001).

Determination of purity is the more difficult procedure. When a protein has an inherent determinable activity, such as in the case of enzymes, the change in either an added component, or the protein in question can be determined. Many substrates and reaction products have an absorbance measurable using a standard spectrophotometer (Baines, 2001). Proteins that have no recognised biological activity, for example structural proteins require an alternative method for analysing purity (Lillehoj and Malik, 1989).

Assaying other proteins is often done using Enzyme Linked Immunosorbent Assays (ELISAs) as described in section 1.6.2. This is particularly common in the case of an antibody product. However this requires the presence of antibodies to the proteins to be assayed and the ability to immobilise the antigen in a standard level on a surface to be analysed (Baines, 2001). This can often cause a situation where no antiserum to a protein is available until the protein is pure, and recognised purification can not be performed until a suitable antiserum is developed (Lillehoj and Malik, 1989).

Another method for determining purity of protein in question is to use Sodium Dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This technique, combined with Western blot procedures, can determine the molecular weight of the protein in question, the presence of contaminating proteins and using gel densitometry technology, the approximate purity. Further more, SDS-PAGE and Western blot technology is readily available in commercial formats (Goetz et al., 2004). Two-dimensional electrophoresis is also a powerful method for determining purity; this involves separating proteins according to net charge on an IEF gel strip, and then running the strip on a conventional SDS gel. This has the ability to separate and identify over 100 proteins on a single gel (Lillehoj and Malik, 1989).

Proteins are commonly visualised using one of two stains: Coomassie Blue, which can detect 25 ng protein (in most cases) and gives a good quantifiable result, and silver staining which is a more sensitive method (0.25ng) but less suitable for determining protein concentration. Both stains are also readily commercially available in differing formats (www.piercenet.com and www.invitrogen.com).

1.9 Intellectual property and antibodies

The Intellectual Property (IP) situation surrounding the production of heterologous proteins is complex and must be considered and issues dealt with before any product development can occur. A number of patents exist for key areas of antibody and protein production and a suitable approach must be taken to licensing or circumventing appropriate technology.

A number of key technologies must be used in the production of antibody based proteins including but not limited to: molecular libraries, vector, lead/expression sequences, phage or yeast display, library panning techniques, expression system, protein tag sequences, and purification techniques. All of these are covered by different patents, for example a patent search (Micro Pat) yields 143 patents with phage display in the title. Issues such as the expression system may be solved by use of a novel organism on which no patents exist; these however require a great deal of development and such systems often do not have GRAS status (Punt et al., 2002).

1.10 Aims of the research

The goal of this research project was to investigate the function of a range of Llama antibodies and Llama antibody-Cellulose Binding Domain (CBD) fusions produced to a range of surfaces and particles with potential commercial relevance.

The potential application of this technology is the targeted delivery of capsules containing a benefit agent payload to cellulosic materials (for example paper and cotton). Possible generic uses for CBDs are explored in Levy and Shoseyov (2002) and allow for uses in agrochemical, bioprocessing, home and personal care and diagnostic industries. One of the applications in the home and personal care industry is as a delivery mechanism for perfume/fragrance to laundry in the laundry wash cycle.

Currently, Unilever spends approximately \$160m per year on perfume for fabrics in its detergents, 95% of which gets rinsed straight off the clothes and wasted. The other 5% of the perfume commonly lasts a maximum of twenty-four hours (Unilever internal data). The key problem is that washing and deposition are conflicting processes. There is a need for better delivery, deposition and retained benefit of fragrances in the wash to give more cost-efficiency and a greater consumer benefit.

The objective is to use a slow release capsule to retain perfume benefit for longer periods of time, addressing the retained benefit issue. However as perfume encapsulation is obviously a more expensive technology (approximately 3 times) than simple addition of perfume the added benefit brings an increased cost. The idea is to use an antibody fusion protein targeting system to improve deposition of capsules and therefore save costs by reducing the amount of capsules needed.

The aims of this project are:

1. Research the characteristics of the fusion proteins binding function in a model system to a range of surfaces in order to determine proof of principle. Although many systems exist for monitoring antibody-antigen interactions, this often involves coating a substrate or the antibody itself onto a surface in order to measure the reaction. This limitation makes the measuring of the CBD-cellulose interaction very difficult, due to the inert nature of cellulose. Derivation of a suitable assay will be necessary, both for fusion protein to particle, and for particle to cellulosic surface.
2. Apply the findings to a more commercially relevant system. Difficulties encountered with binding of protein to cellulose will make the measurement of binding to some surfaces very difficult also: the inertness which is a benefit in the particle application is a hindrance in the quantification of binding. It is possible new assays will have to be developed.
3. Develop a purification system for specific proteins. A further challenge will involve the purification of the commercial proteins; these are deliberately designed without any affinity fusion tags and as such will not be amenable to simple affinity purifications and it is unlikely they will bind protein A and G.
4. Characterise binding to relevant surfaces and other characteristics of pure proteins. Assessment of binding functionality and stability will be essential to determine any potential commercial relevance of chosen candidate proteins.

2.0 Materials and Methods

2.1 Molecules used

The following molecules were used in this study and kindly provided by Unilever Research and Development, Bedfordshire:

Reactive Red 6 dye (ICI chemicals, product discontinued).

Llama VHH Anti-RR6 – Lyophilised Cellulose Binding Domain fusion proteins A, B, C, D. For details on production and structure see Lewis et al. (2006) and Davies and Parry (1997).

Llama VHH Anti-Melamine – Cellulose Binding Domain fusion proteins MelA – MelK.

Melamine particles containing fluorescent dye (Nile red).

All other chemicals were obtained from Sigma (Dorset) unless otherwise stated. All solutions were made in milliQ ultrapure water (Millipore, Hertfordshire) water unless otherwise stated.

2.2 Manufacture of RR6-BSA conjugate and determination of amount of RR6 bound to BSA

The RR6 molecule was conjugated with BSA for ease of study and binding to surfaces according to the following protocol:

A 1gL^{-1} RR6 solution (10mL) was made in borate buffer (0.1M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.05M NaCl, pH 8.5). A 0.1gL^{-1} BSA solution (10 mL) was also made in borate buffer. The two solutions were combined and placed on a rotary mixer overnight at 25°C .

The solution was then washed repeatedly with phosphate buffered saline (PBS, 85gL^{-1} NaCl, 9.81gL^{-1} $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 4.53gL^{-1} $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$, pH 7.15) using an Amicon stirred cell concentrator system (Millipore, Hertfordshire). Excess dye was removed until the colour ran clear. The conjugate was then blocked in Tris-HCl buffer

(50mMol, pH 8.0) for 16 hours, and then washed further with PBS. It was then stored at 4°C.

The RR6-BSA conjugate was analysed using MALDI-TOF mass spectrometry (analysis performed by M-Scan limited, Wokingham, UK). MALDI-TOF mass spectrometry was performed using a Voyager STR Biospectrometry Research Station Laser-Desorption Mass Spectrometer coupled with Delayed Extraction. An aliquot of BSA and of BSA-RR6 was dissolved in 0.1% aqueous TFA to produce approximately 1mgmL⁻¹ solutions. Aliquots (1µL) were analysed using a matrix of Sinapinic acid. BSA (Fluka) was used as an external calibrant.

2.3 Standard ELISA protocol

Greiner 96 well Microton 600 High Binding plates (E and K scientific, UK) were coated overnight at 4°C with 200 µL 0.25mg/ml RR6-BSA conjugate (manufactured as above) in carbonate binding buffer (0.018 Mol Na₂CO₃, 0.032 Mol NaHCO₃, pH 9.8 (made fresh on day of use). They were then washed once with PBS-t buffer (as above, with 0.15% tween-20).

Each plate was then blocked using a blocking buffer solution comprising 1% BSA in PBS-tm (PBS containing 0.15% v/v Tween-20 and 0.1% non-azide antibacterial agent) for 30 minutes at room temperature on a plate shaker. The plate was then washed three times in PBS-t and gently shaken dry. A sample of 200µL was added to the relevant wells and incubated for one hour at room temperature on a plate shaker. The plate was then washed three times in PBS-t buffer using a plate washer.

A polyclonal rabbit anti-llama antibody serum (kindly provided by Unilever Research and Development, Vlaardingen) was then added to each cell (200 µL of 1/2000 dilution in PBS-t) and incubated for one hour at room temperature on a plate shaker. The plate was then washed three times in PBS-t buffer using a plate washer and shaken dry. Goat anti-rabbit alkaline phosphatase conjugate in PBS-t (200µL of 1µgml⁻¹ solution, Pierce, Illinois) was added to each well and incubated for an hour at room temperature.

The plate was then washed three times in PBS-t and shaken dry. Alkaline phosphatase Substrate p-Nitrophenyl phosphate (pNPP) was diluted in substrate buffer at a final

concentration of 1mg/ml (1mol Diethanolamine, pH 9.6 and 1mMol MgCl_2 in milliQ H_2O) at was added to each well in 200 μL aliquots and incubated for 15 minutes at room temperature. The absorbance was then read at 410nm using a Thermo labs MRXTC revelation multi-well spectrophotometer (Thermo labs, Basingstoke, UK).

2.3.1 BSA-RR6 ELISA

A stock solution of llama anti-RR6 molecules A, B, C and D was made. Each molecule was prepared in a 1 mgmL^{-1} solution in PBS-t and agitated thoroughly until dissolved. Stock solutions were stored at 4°C for a maximum of 5 days until use. Each sample was serially diluted in PBS-t to the desired concentration directly before use. Each sample was then used in the ELISA protocol described above.

2.3.2 Competition ELISA

For each plate a serial dilution of antibody solutions A-D was prepared as above. This was used to prepare a standard dilution curve. A set of samples were also prepared by using a standard working stock solution of sample (1 mgmL^{-1}) and adding a serially diluted amount of BSA-RR6 conjugate (as described above). This was then incubated at room temperature for 30 minutes before being used in the ELISA protocol above.

2.4 BIAcore

RR6-BSA conjugate was immobilized on a CM5 Biacore chip (Carboxymethyl Dextran surface, BIAcore ltd., Uppsala, Sweden) using a BIAcore 2000 and standard BIAcore EDC/NHS binding procedures (approximately 2000 Biacore Response units). One flow cell was kept as a blank control, and subjected to the immobilization procedure in the absence of the protein conjugate.

TBS-t was used as a running buffer and wash solution (12.1 gL^{-1} Trizma base, 8.7 gL^{-1} NaCl and 0.15% Tween-20 in milliQ, pH 7.4), and the chip was regenerated with 50mMol HCl after each use. All solutions were kept as similar as possible for each experiment, to reduce buffer effect errors.

Serial dilutions of sample in TBS-t were added to each flow cell for ninety seconds and then washed off with TBS-t using the KINJECT command of the BIAcore and the

association and dissociation response measured. The bound antibody was removed from the BSA-RR6 chip using 50mMol HCl. The chip was then washed and re-equilibrated with TBS-t before addition of a new sample.

A COINJECT command was used to improve dissociation constants by injecting buffer containing blocked RR6 (blocked in 10mMol Diethylacetate on a roller mixer overnight) and exchanged to a TBS-t buffer using a pd10 buffer exchange column (GE Healthcare, Buckinghamshire) at the dissociation phase.

Initial experiments to determine affinity in running buffer were performed. The affinity of the fusions in the presence of chemical components used at concentrations used in a popular commercial detergent brand (Alkaline silicate, Sodium Carbonate, Sodium Sulphate and Sodium tri-poly-phosphate (STPP)) was then determined. This level of detergent component was also included in the running buffer for each experiment to minimise addition effects.

The analysed molecules were diluted to between 40 and 200 nMol concentrations in the running buffer and detergent component and passed through the cell at $30 \mu\text{Lmin}^{-1}$ for 60 seconds before being washed in 0.76 mgL^{-1} blocked RR6 in running buffer.

2.5 Particle binding experiments

Cotton swatches were prepared by using a pressure-operated hole-punching device to give uniform circles of cotton (5mm diameter). Plain cotton sheet was used for the swatches. Cotton strands were prepared by pulling a strand from sections of territowelling and cutting them to a two centimetre length.

Co-acervate particles were prepared according to internal Unilever protocols and a fluorescent dye (either Nile blue or red) was added (See appendix 1). Solutions of gum arabic and gelatin were made (3% w/v, Sigma) and heated to approximately 50°C on a stirrer hotplate for 4 hours. An oil suspension of Nile Blue was made ($50\mu\text{l}$ in 3mls sunflower oil). A mixture was then made of 50 ml of heated gum arabic and 50 ml heated gelatin and the oil suspension added.

The pH of the mixture was then adjusted to between 3.8 and 3.9 with a stock solution of 10% glucono-D-lactone. The particles created were then washed in PBS before hardening with 1% v/v glutaraldehyde for 3 hours on a rolling mixer in a fume cupboard. The particles were then washed in PBS and incubated overnight with high pH borate buffer containing 0.1% v/v RR6 (100mMol borate, 50mMol NaCl).

The particles were then washed in Tris-HCl (50mMol) twice for 30 mins on a rolling incubator, spun down at 5000 rpm and the supernatant discarded. The particles were then washed further in PBS until the supernatant was clear. The particles were then sieved to remove any greater than 50µm in diameter.

2.6 Determination of binding method

Sixteen hole-punched swatches of woven cotton were prepared by washing twice in PBS for 10 minutes in 100mls PBS. Experimentation was performed in 8ml glass vials.

The control vial (Vial 1) contained 50µl of particles and 4 swatches in 1 ml PBS and was incubated for 30 minutes on a rotating incubator. In Vial 2 200ug VHH-CBD fusion and 50µl particles in 1 ml PBS were pre-incubated for 30 minutes and then 4 swatches added and the mixture incubated for 30 minutes. In vial 3 200ug VHH-CBD fusion and the swatches in 0.95ml PBS were pre-incubated for 30 minutes and then 50µl particles incubated for 30 minutes on a rotating incubator. In vial 4, 200µg VHH-CBD fusion, 50µl particles and 4 swatches were incubated in 1ml PBS for 30 minutes on a rocking incubator.

Each batch of differently treated swatches was then washed in 5mls PBS in a new 8ml glass vial for 15 minutes. The swatches were then removed and mounted on a microscope slide. Particles visible on each swatch were then counted under x100 magnification.

2.7 Comparison of bifunctionality of fusion proteins

Using the method chosen as the most ideal in section 2.6, the four different fusion proteins were incubated with cotton swatches as a direct comparison between fusions.

2.8 Initial Characterisation of the melamine system

As mentioned in section 2.1 the following molecules and particles were kindly provided by Unilever:

Fusion protein MelA: Manufactured in house at small scale using *Pichia pastoris* expression system. This protein is a research protein and contains a hexa-histidine fusion tag. It is purified in house using metal affinity chromatography. Due to licensing problems with the purification and expression system, and also inability to scale up at Unilever, this molecule is a purely experimental one, used to test the efficacy of the system. After purification, the molecule is freeze dried into vials and stored at room temperature.

Fusion protein MelB: This is the MelA molecule manufactured and purified by Unilever in house in *Saccharomyces cerevisiae*. Differences in the production system may lead to differences in structure and functionality. This protein has also been freeze dried into vials and stored at room temperature.

Fusion proteins MelC - MelK: were manufactured by a different commercial manufacturer in *Aspergillus niger*. These proteins are secreted and hence provided in frozen (-20°C) crude clarified supernatant format. They were stored at this temperature prior to use.

Cellulose surfaces studied were Crystalline cellulose (Sigmacell, Sigma), cotton (Woven polycotton, woven cotton, terry towel, kindly provided by Unilever), microscope tissue (Fisher Scientific, Leicestershire) and tissue (Kimberly Clark, Kent).

2.9 Determination of protein concentration

Concentration of the proteins was predetermined and kindly provided by Unilever using band densitometry on SDS-PAGE gel and total protein assay (BCA) (Sapan et al., 1999).

2.10 Comparison of proteins by SDS gel and Western blot

All proteins were diluted to approximately 0.5 mgmL⁻¹ fusion protein. Aliquots of 60µL were placed into 0.5mL volume eppendorfs and 20µL loading buffer was added

(4x stock solution: 40% v/v glycerol, 4% w/v SDS, 0.04% bromophenol blue in pH 8 100mMol Tris.Cl). The samples were denatured by boiling for five minutes. Samples were then pulsed at 5000 rpm in a lab centrifuge to force the sample to the bottom of the eppendorf. The samples were then loaded onto two ready-gels (12.5 % gel, Ready gel, Bio-rad, Hertfordshire, UK): 40 μ L of sample on one gel, and 10 μ L for the other gel.

Both gels were run until the loading buffer band was about to run off the base of the gel at 70mV using the mini-protean II gel kit (Bio-rad). Rainbow markers (GE Healthcare, Buckinghamshire, UK) were used in the first lane. The gel with the greater amount of sample was removed from the cassette and washed in milliQ water for ten minutes on a rocking incubator, and then incubated with Gelcode Blue (Pierce, Illinois, USA) for 2 hours on a rocking incubator. The gel was then destained overnight in milliQ on a rocking incubator, and mounted in acetate to dry.

The gel with the lesser amount of sample was removed from the cassette and placed in a Protean II Western cassette with Nitrocellulose paper according to supplied protocols, and run overnight at 30mV. The Nitrocellulose paper was then extracted from the cassette and blocked for 1 hour in 100 mls PBST containing 1% w/v Marvel milk powder on a rocking incubator at 25°C. The nitrocellulose paper was then washed in 100 mLs PBSt for 3 x 5 minutes, and then incubated with 0.05% v/v rabbit anti-llama serum in PBSt containing 1% Marvel for 1 hour on a rocking incubator at 25°C. A further wash step (as above) was performed and the nitrocellulose paper incubated in 0.1% v/v goat-anti rabbit alkaline phosphatase conjugate in PBSt containing 1% Marvel milk powder.

A further wash step (as above) was then performed and the substrate added in 20 mls milliQ water (5-Bromo-4-Chloro-3-Indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) tablets, BCIP 0.15mg/mL, NBT 0.3mg/mL in 100 mMol tris buffer pH 9.5) and the colour allowed to develop (approximately 10 minutes). The nitrocellulose was then washed repeatedly in milliQ to stop the reaction, and allowed to dry on tissue paper.

2.11 Determination of amount of fusion bound to particles

Fusion protein MelA was identified on gels using derived western blot information. A 20% w/v suspension of melamine particles in PBS was made. A 500µg/mL solution of MelA fusion in PBS was made. A 10x working stock solution of a two popular commercial laundry detergent bases (detergents without perfume and enzymes) OMO France and Saturn Silver was made in PBS.

A total reaction mixture of 280µl in incubation buffer was used, containing 120µL 20% particles and 5µg - 55µg fusion. Controls contained no particles. Each reaction mixture was placed in a 2 ml eppendorf. The reaction mixture was incubated for 1 hour at 25°C temperature on a rotary mixer. After incubation, the reaction mixture was centrifuged at 13000 rpm for 5 minutes in a bench-top centrifuge.

Supernatant (30 µL) was then extracted out of each reaction mixture and placed in a 0.5 ml eppendorf. 10µL of loading buffer (as in 2.10) was added. Each 0.5mL eppendorf was then placed in a boiling water bath for 5 mins to denature the protein.

After denaturation, 30 µL of each sample was loaded onto a ten-lane ready gel (as above). The gels were then washed and destained as above. After drying the gels were analysed using gel densitometry equipment (Bio-rad Gel doc 1000) and corresponding image analysis software.

Further analysis was performed on the binding of the fusion to gelatin particles to investigate possible cross-functionality. A similar method to the one described above was used with the 20% w/v melamine particle solution being replaced with 20 % w/v gelatin particles. Analysis of the fusion binding to melamine particles was also performed in the presence of two laundry detergents bases (Omo France and Saturn Silver) at a level of 6.8 gL⁻¹ in 27°F water (controlled hardness water containing 2.16mMol Ca and Mol 0.054 mMol Mg) (level typical of average tap water).

2.12 Binding of protein to cotton in the presence of detergents

Cotton threads were extracted from a piece of Terry Towelling and cut into lengths of 5mm. One thread was placed into each well in a 96-well 0.45µm filter plate (MAHV 0.45µm, Millipore). A 2x stock of detergent bases (no enzymes or perfume) of 7 commercially available detergent powders were made using the levels recommended for use. PBST and 27°F water were included as a control.

Aliquots of 100µL of each detergent base or control were added into triplicate wells. Aliquots of 100µL 27°F water or PBST containing 20µg fusion were then added to the relevant wells, and aliquots of 27°F water or PBST added to the control wells. The filter plate was incubated on a plate shaker for 1 hour at 25°C.

After incubation the plate was washed 20 times with 200 µL PBST buffer, (the buffer being extracted by a vacuum from underneath the plate after each wash using a plate vacuum manifold (Millipore)) and 200µL of 1/1000 v/v diluted Rabbit anti-llama IgG added (as in 2.3). The plate was then incubated for an hour at 25°C on an orbital shaker.

A further 20 washes were performed as described above, and 200µl of 1/1000 v/v diluted goat anti-rabbit alkaline phosphate conjugate added. The plate was then incubated for 1 hour at 25°C. The plate was then washed 10 times as described above. Each thread was then removed and placed in the corresponding well in a new filter plate (as above). The new filter plate was then washed ten times and 200µL of pNPP substrate added in di-ethanolamine buffer (as in 2.3). The plate was allowed to develop for approximately 20 minutes, and the newly coloured buffer sucked through into a convention 96 well plate. The absorbance was then read at 405 nm.

2.13 Visual demonstration of binding using fluorescently labeled melA.

Fusion melA was labelled fluorescently using a fluorescent protein conjugation kit (Alexafluor labelling kit, Invitrogen, Paisley) and supplied protocols. The newly fluorescent protein was then stored in PBS at 4°C in the dark. A microscopy flow cell was created in house at Unilever that allowed a standard microscope slide to have liquid

flowed over it at speeds dictated by a connected syringe pump. Due to the thickness of the flow cell however, maximum obtainable resolution was x100.

Fabric microscope slides were prepared for the flow cell by attaching a thread of fabric (Cotton, Nylon or Polyester) to the slide and fixing the ends to the back of the slide with adhesive tape. Melamine slides were prepared by partially dissolving the melamine particles in pure ethanol (99.5%), placing the mixture on the slide and allowing the ethanol to evaporate at 37°C in an incubator.

A fusion solution was made (50mg l⁻¹) in a 6.8g l⁻¹ solution of Shakespeare Rational base. This mixture was flowed over each slide at 20mls per minute. A competition study was also performed on the melamine cell, the cell being washed with a non-fluorescent fusion, and then washed with a fluorescent fusion. Pictures were taken at specific time intervals of 1 minute to show effect of fluorescent fusion with time. Resolution and measurement variables were kept identical in a bid to allow suitable comparison.

2.14 Particle incubation experiments

A screening experiment was performed with available fusion proteins to determine the best candidates for further study. Lens cleaning tissue was hole punched into swatches using (using the pressure operated hole punching device). All incubations were performed in 15ml tubes. Into 2.6 mls PBS 25 µg of fusion protein and 10µl 10% w/v capsules were added and mixed for 30 minutes on a rota-mixer.

Following pre-incubation, 5 lens tissues swatches were added and further 2.4mls PBS added, and mixed for 1 hour on a rota-mixer. The liquid was then removed and 5mls PBS added, and mixed for 5 minutes on a rota-mixer. This wash cycle was then repeated a further 2 times.

After washing the swatches were removed and mounted on a microscope slide. A photograph was taken of the approximate center of each swatch under UV light at x100 magnification. The numbers of particles visible under the x100 magnification circle were then counted at the top, bottom, left and right of the swatch.

2.16 Purification of llama antibody fragments

All chromatography buffers were degassed using a 0.2µm Nylon filter under vacuum.

All columns were cleaned according to proscribed protocols and stored in 20% ethanol (degassed).

2.16.1 Identification of commercial candidates using SDS-PAGE and Western blot

SDS-PAGE and Western blot was performed on samples as described in section 2.10.

2.16.2 Concentration of proteins

Dilute proteins were concentrated using a stirred cell concentrator (Amicon) containing a 10KDa membrane. Various concentrator volumes were used, from 10 to 150mls and the protein concentrated to the desired volume for buffer exchange (2.5mls to 15mls for small scale or 100mls for large scale buffer exchange). Nitrogen was passed under pressure into the stirred cell to push the filtrate through the membrane and the cell stirred using a magnetic stirrer set to approximately 120 rpm.

Both filtrate and concentrate was collected and stored at 4°C.

2.16.3 Small scale buffer exchange of proteins

Small scale buffer exchange was performed by using up to 6 PD10 desalting columns in parallel (GE Healthcare). The columns were equilibrated in 10 column volumes (25mls) of buffer to be exchanged into. A column volume of sample (2.5 mls) was then added to each column and allowed to enter the matrix. The wash was then discarded. The protein was eluted in 3.5 mls desired buffer and the eluate collected and pooled with other column eluates.

2.16.4 Large scale buffer exchange of proteins

Large volumes of protein solution were buffer exchanged using a 1L column containing Sephadex G-25.

2.16.5 Protein A chromatography

A Hi-trap protein A column (1ml volume) was used to test the potential of protein A for the purification of the fusion protein melC. Protein was buffer exchanged as above (small scale) into a 20mMol pH 7.0 sodium phosphate buffer. The protein was then loaded onto the column at 1ml/min using an Amersham FPLC (GE Healthcare) and washed in the 5 column volumes of loading buffer.

Bound protein was then eluted in 0.1Mol glycine-HCl pH 2.7, collected into 2 ml fractions that were immediately neutralized in 100mMol Tris-HCL pH 8.0. Peaks were then concentrated if necessary as described above.

2.16.6 Anion Exchange Chromatography

Anion exchange chromatography was performed using a MonoQ column (5 ml volume (GE healthcare). Samples were buffer exchanged (small scale) into pH 9.2 20mMol Ethanolamine as determined by the pI of the first protein to be purified, Jal 625, 8.2 (estimated using a Deleage and Roix (1987) based model using the computer program DNASTar (<http://www.dnastar.com/>).

Sample was loaded onto the column at 1 ml/min and the column washed in 5 column volumes. Bound proteins were then eluted in pH 9.2 20mMol Ethanolamine containing 1Mol NaCl at varying concentrations in the loading buffer to give a salt gradient.

Fractions were collected in 2 ml aliquots in 5ml tubes and stored at 4°C for analysis.

2.16.7 Cation Exchange Chromatography

Cation exchange chromatography was performed using a MonoS column (5 ml volume). Samples were buffer exchanged (small scale) into pH 7.2 20mMol phosphate as determined by the pI of the first protein to be purified, Jal 625, 8.2 (estimated using the method described in section 2.16.6).

Sample was loaded onto the column at 1 ml/min and the column washed in 5 column volumes. Bound proteins were then eluted in pH 7.2 20mMol phosphate containing 1Mol NaCl at varying concentrations in the loading buffer to give a salt gradient.

Fractions were collected in 2 ml aliquots in 5ml tubes and stored at 4°C for analysis.

2.16.8 Freeze drying of pure proteins

Pure proteins obtained were buffer exchanged as above into pH 7.15 PBS and aliquoted into vials for freeze drying. The samples were then frozen at -70°C for 2hrs before being placed in a freeze drier for 48 hours. Freeze dried samples were sealed under vacuum and capped to preserve them. They were then stored at room temperature.

2.17 Large scale purification of proteins: sampling

Samples were taken at each stage of large-scale purification. All samples were frozen and stored at -20°C in aliquots for later analysis.

2.17.1 Analysis of samples

2.17.2 Gel electrophoresis and Western blot

Samples were analysed by gel electrophoresis and Western blot as described in 2.10. For more sensitive analysis of protein samples Silver stain was used. Gels were fixed in 40% ethanol, 10% acetic acid in milliQ for 30 minutes on a rocking incubator, prior to following supplied protocols (Silver Quest, Invitrogen, Paisley UK).

2.17.3 Total protein assay

Total protein content of each sample was assessed using BCA assay (Pierce) in a 96 well plate format. A standard curve was produced using BSA and used to calculate the concentration of protein present. All samples were done in triplicate and accepted variance below 5%.

2.17.4 Cellulose Binding

An approximate measure of activity to cellulose was determined using a UV based method. Protein samples were diluted to 1.5 mg/ml in PBS as determined by BCA assay (above) and 1ml added to a 2ml eppendorf. 0.5ml PBS or Avicel (2%w/v) was then added and the mixture incubated on a rotary incubator for 1 hour at 25°C.

They were then centrifuged at 15000 rpm in a lab centrifuge for 5 minutes. The absorbance of the supernatant was then read, and the percentage decrease from the original calculated to give a relative cellulose binding activity.

2.18 Assays with purified protein candidate

2.18.1 Stability of protein

Protein degradation in crude and pure form was assessed using a SDS-PAGE based method. Proteins were diluted to approximately 2 mg/ml protein in PBS and stored at 37°C in an incubator, with samples being taken at 0, 2, 7, 14 and 31 days.

All samples were then run on SDS-PAGE and stained according to section 2.10. Where visible breakdown of proteins was apparent, the subsequent sample was diluted less to visualize protein present.

2.18.2 Binding of pure protein to crystalline cellulose and melamine capsule material

Binding of the pure protein to crystalline cellulose and melamine capsule material was determined in an adaptation of the basic cellulose activity assay in section 2.18.4. A concentration gradient of fusion protein (8 replicates) was created in 1 ml aliquots in 2 ml eppendorfs. The eppendorfs were then split into 2 batches, each of four replicates. Into the first batch 0.5 ml PBS was added, and into the second batch 0.5 ml of cellulose mixture (avicel in PBS). The experiment was repeated with three concentrations of avicel, 2.5 % w/v, 7.5% w/v and 10% w/v.

The eppendorfs were then incubated on a rotary incubator for 1 hour at 25°C. They were then centrifuged at 1500 rpm in a lab centrifuge for 5 minutes. The absorbance of

the supernatant was then read, the results from the control concentration gradient being used to create a standard curve.

The standard curve was then used to calculate the concentration of the unbound fusion protein.

3.0 Results and discussion: Investigation and characterisation of the function of llama antibody fusion proteins and potential for use in a novel micro-particle delivery system

3.1 Introduction

In order to assess the potential of a llama antibody-CBD fusion to aid deposition of micro-particles a model system was devised for study. This involved three stages: development of an antibody-CBD fusion to a known antigen that could be attached to the particle surface, and attachment of said antigen to the particle surface, and delivery of particles to cellulosic surfaces in the presence and absence of the derived fusion protein.

The objective of this work is to conduct proof of principle experiments on the model particle delivery system, including characterizing the antibody binding to its antigen, manufacturing particles containing antibody binding sites, and to investigate the effect of the conformational differences of the proteins on the delivery mechanism.

The antigen selected for use is the azo-dye Reactive Red 6 (RR6). Reactive Red 6 is a hapten azo dye with a molecular weight of about 765 (see fig. 6). This molecule was selected as it has been shown previously that it is possible to raise an antibody to the molecule (van der Linden et al., 1999) of comparable specificity and affinity to mouse monoclonal antibodies for the RR6 molecule. The protein raised by van der Linden was specific enough that it did not show cross reactivity to the dimer of the RR6 dye known as RR120.

Unilever research and development, Colworth has created and produced a number of multi-functional proteins by fusing a protein with known binding towards the RR6 moiety and a fungal CBD to give proteins with 1 or 2 VHH “hands” and 0,1 or 2 CBD “feet” (see figure 7). These molecules provide the basis for a system to potentially deliver particles coated in RR6 to cellulosic surfaces and were designed to prove the concept of such a system.

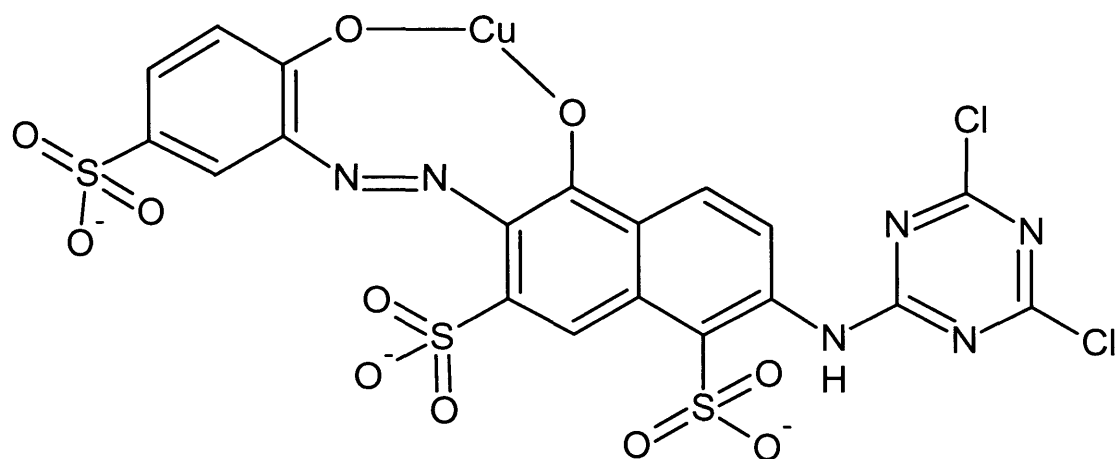


Figure 6: The RR6 dye molecule - a hapten azo-dye, approximate molecular weight 765. The dye will link covalently and irreversibly with amine groups present on a surface. The surface then requires repeated washing and blocking, to prevent further non-specific interactions.

Further more, the nature of the RR6 molecule is such that it is ideal for coating surfaces irreversibly, as it is a dye that attaches covalently. As it is a relatively small molecule, it should provide a large number of binding sites for the raised fusion protein. Methods for production of gelatin-gum arabic particles (Co-acervate) have been previously described in literature (Tolstuguzov et al., 1997). Particles can also be loaded with benefit agent or a marker in order to assess functionality.

Llama heavy chain fragments offer considerable advantages compared to other more conventional antibody fragments including ease of production in yeasts, extra stability and small size whilst maintaining binding function similar to that of IgG molecules (van der Linden et al., 1999). The binding moiety chosen has been fused in a variety of configurations using standard molecular biology techniques to a cellulose binding domain (Family 1) from *Trichoderma reesei*. Exact configurations of the fusion proteins studied are explored in table 2.





Molecule Schematic		Molecule Information
A		Anti-RR6 single VHH molecule. Molecular weight: 14167.86 Length: 130 amino acids
B		Anti RR6 single VHH fused with a single CBD molecule. Molecular weight: 19505.69 Length: 180 amino acids
C		Anti RR6 two VHH molecule fused with a single CBD molecule. Molecular weight: 33656.55 Length: 310 amino acids
D		Anti RR6 single VHH molecule fused to two CBD molecules. Molecular weight 2442.80 Length: 228 amino acids

Table 2: The anti-RR6 CBD fusion model system. Protein A is the single VHH anti-RR6 and is used as a control. Proteins B-D consist of VHH anti-RR6-CBD fusions in varying formats.

3.2 Determination of amount of RR6 bound to BSA

The RR6 molecule is conjugated with BSA for ease of study and binding to surfaces. This creates a protein molecule presenting known antigens available for binding, and allows the use of EDC/NHS binding chemistries to bind the antigen onto the necessary surface for analysis (conjugation chemistry is explored in Hermanson, 1996). It also inactivates the dye, meaning that a soluble antigen presenting molecule that does not conjugate with any available chemistries can be used.

Spectroscopic analysis (using MALDI-TOF spectrometry) shows that between 1 and 10 RR6 molecules were attached to each molecule of BSA, with 60% of BSA molecules having 1 RR6 molecule attached (data not shown).

Consequently calculations that involved the amount of RR6 molecules attached to the BSA was performed twice, assuming a minimum (1 per molecule) and a maximum (10 per molecule) being used. This allowed a range to be given, and also the result based upon the mode of the data, which can be assumed to be 1 RR6 per molecule of BSA.

3.3 Binding of fusion proteins to RR6 BSA conjugate.

Two methods of investigating the binding of fusion proteins to RR6 were used – simple and competition ELISA. In the first instance a simple ELISA was performed on the proteins to give a direct comparison of the binding of the different proteins.

RR6-BSA conjugate was coated to the surface of a 96 well plate. Binding of fusion was then measured, and results shown in figure 7. The absorbance of the curve is directly proportional to the amount of antibody bound. Each result is the mean of duplicate samples. Negative controls also studied (see table 3) and demonstrated the assays reliability by showing no response.

A key assumption is that the secondary antibody (a rabbit anti-llama protein) binds to each fusion protein equally, and hence allows direct comparison between all the fusions. This is not necessarily the case however, as the structure of the fusion proteins, i.e. the presence of the CBD/CBDs and also a second VHH, may cause the anti-llama to bind differently.

Protein A (the single VHH fragment) shows similar binding to protein B (the VHH RR6-CBD fusion) and protein C (the VHH RR6-VHH RR6-CBD) in respect to the RR6-BSA conjugate. This shows that the addition of a single CBD has no seriously detrimental effect on the binding of the protein to the substrate.

Furthermore addition of a second VHH to the VHH-CBD molecule has no determinable effect on the binding to the substrate. However it could be that no added benefit is noticed because the assay only shows how the amount of binding changes with fusion concentration. This means that other benefits such as increased strength of binding or increased rate of binding will not be shown with this method.

Also, due to the density of the RR6-BSA conjugate being tightly packed on a surface, an element of steric hindrance maybe occurring. This steric hindrance may be in the fusion depositing onto the substrate, or the anti-llama protein binding to the llama regions on the fusion.

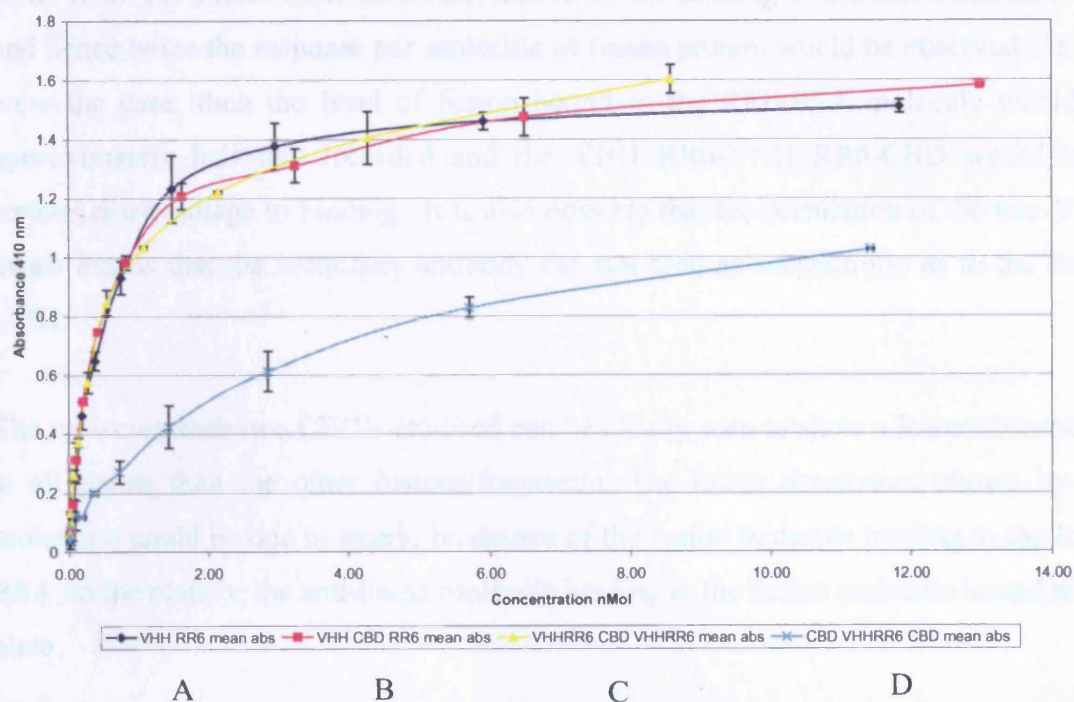


Figure 7: Binding of fusion proteins to RR6-BSA coated well plates. Fusions A-C show similar curves, with fusion D showing a substantially lower response.

Control	Description
1	No RR6-BSA coated to well
2	No incubation with VHH fusion
3	No incubation with secondary antibody
4	No incubation with tertiary antibody
5	No incubation with enzyme substrate

Table 3. Controls used in ELISA assay.

It would be expected also that with twice the available llama protein domains in the VHH RR6-VHH RR6-CBD molecule, that twice the binding of the anti-llama antibody and hence twice the response per molecule of fusion protein would be observed. If this were the case, then the level of fusion bound to the RR6-BSA molecule would be approximately half that recorded and the VHH RR6-VHH RR6-CBD would be a serious disadvantage to binding. It is also possible that the orientation of the two VHH heads means that the secondary antibody can not bind as successfully as to the single VHH.

The molecule with two CBD's attached can be clearly seen to show a lower absorbance at all points than the other fusions/fragments. The lower absorbance shown by the molecules could be due to steric hindrance of the fusion molecule binding to the RR6-BSA on the plate or the anti-llama molecule binding to the fusion molecule bound to the plate.

If it is caused by steric hindrance of the protein depositing on the RR6 substrate, then it is representative of the protein being less efficient at binding the substrate. However limitations of the assay such as the indirect nature, and the possible existence of steric hindrance in the secondary antibody stage mean that the protein may be just as effective at binding RR6, and it is not shown. A further assay, the competition ELISA should give more data to help understand the results of the simple ELISA.

3.4 Ability of free substrate RR6-BSA conjugate to compete for fusion binding in ELISA

Figure 8 demonstrates the ability of free RR6-BSA to compete with RR6-BSA bound to the 96 well-plate for the four fusion proteins. Each point is the mean of a set of quadruplicates, with error bars showing 2 standard errors. Each curve is done on different 96 well plates and consequently the graph is normalised data for a competition ELISA. Each point is shown as a percentage of the mean of the final four sets of quadruplicates for the single VHH molecule.

The X-axis shows dilution factor of the competing RR6 in parts per ml. Negative blanks comprised of individual components of the assay were also performed to show

validity of data. All negative blanks showed no absorbance at 510 nm, demonstrating that the individual components of the ELISA such as the secondary and tertiary antibodies do not affect the assay by binding the plate or RR6-BSA non-specifically.

As with the simple ELISA, the VHH RR6, VHH RR6-CBD and VHH RR6-VHH RR6-CBD have very similar responses. It can be seen there is significant difference between the molecule with two CBD's and the rest of the proteins.

Competition ELISA shows the amount of free substrate required to inhibit binding of the protein to the substrate attached to the plate. The more dilute the free substrate that competes with the antibody binding to the substrate attached to the plate, the more efficient the binding. This graph shows that the molecule with two CBD's appears to exhibit better binding strength than the other molecules, which are similar in strength.

This figure appears to directly contradict the data in section 3.1.3, which shows that the 2-CBD fusion protein is the weakest binder. It would however agree with the data if it was assumed that the protein in fact had less available binding sites per mole of protein, due to steric hindrance by the presence of the 2 CBDs. If there were less binding sites per mole of protein, then it would be expected that it would take less free RR6 to inhibit the binding.

It would also be expected that the fusion protein with 2 VHHs would require twice the amount of free RR6 to inhibit, as there are 2 binding sites per molecule. However this is clearly not the case, as shown by the graph, as it takes the same amount of BSA-RR6 to inhibit the protein binding as it does the VHH RR6-CBD and The VHH molecule. This could be due to the protein being able to use only one VHH binding site at a time, perhaps due to steric hindrance of the second site by the bound RR6 BSA.

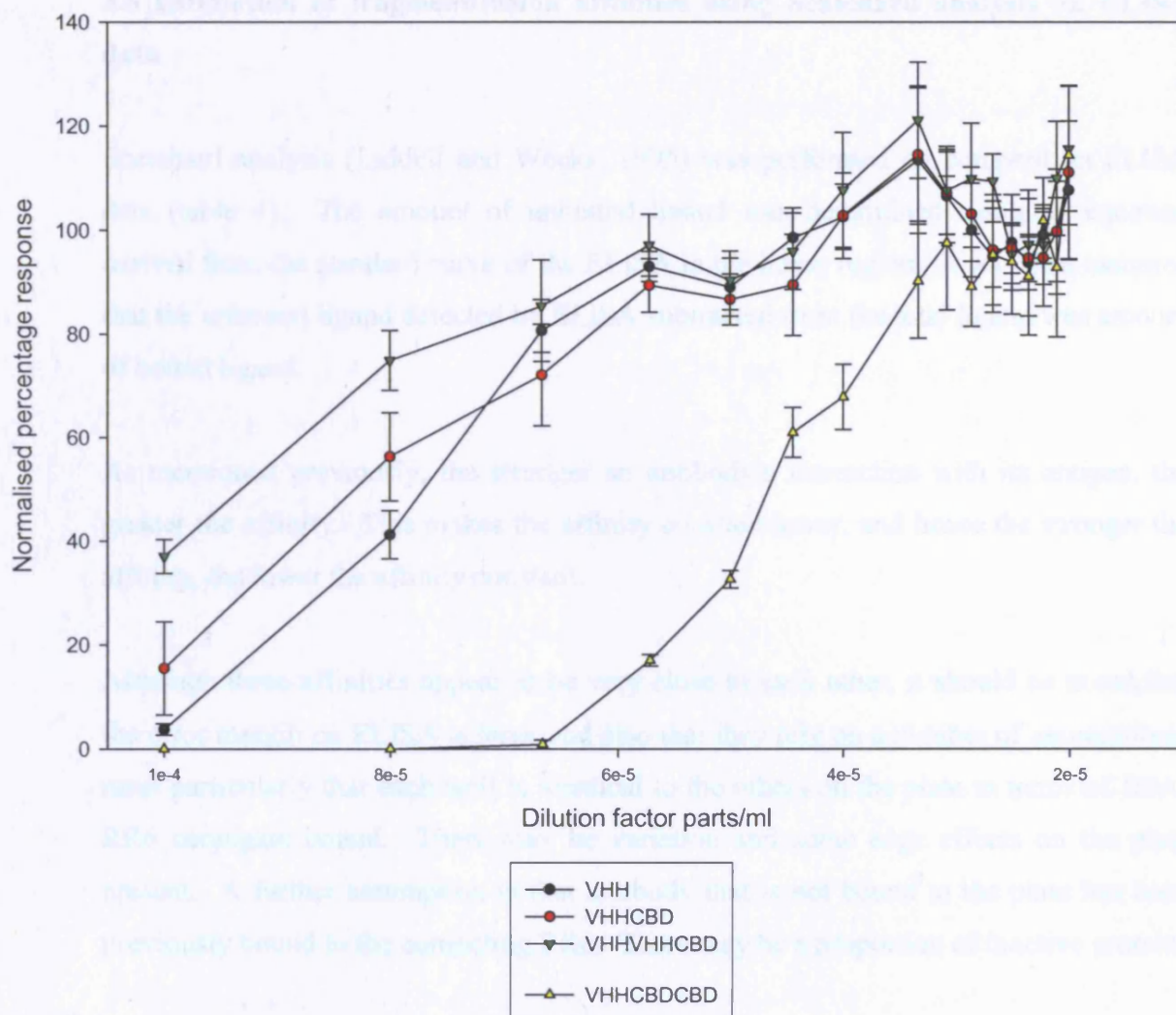


Figure 8: Competition ELISA for model system fusions

Minimum RR6 present per BSA (1RR6/BSA molecule)				Maximum RR6 present per BSA (10RR6/BSA molecule)			
VHH	VHH CBD	VHH CBD	VHH CBD CBD	VHH	VHH CBD	VHH CBD	VHH CBD CBD
3.30×10^{-12}	5.00×10^{-12}	5.00×10^{-12}	1.00×10^{-11}	1.10×10^{-10}	2.00×10^{-10}	2.00×10^{-10}	2.00×10^{-10}

Table 4: Calculated affinities of fusion molecules using Scatchard analysis on ELISA data.

3.5 Estimation of fragment/fusion affinities using Scatchard analysis on ELISA data

Scatchard analysis (Liddell and Weeks, 1995) was performed on competition ELISA data (table 4). The amount of unbound ligand was determined using an equation derived from the standard curve of the ELISA in the linear region. It was then assumed that the unbound ligand detected by ELISA subtracted from the total ligand was amount of bound ligand.

As mentioned previously, the stronger an antibody's interaction with its antigen, the greater the affinity. This makes the affinity constant lower, and hence the stronger the affinity, the lower the affinity constant.

Although these affinities appear to be very close to each other, it should be noted that the error margin on ELISA is large and also that they rely on a number of assumptions, most particularly that each well is identical to the others on the plate in terms of BSA-RR6 conjugate bound. There may be variation and some edge effects on the plate present. A further assumption is that antibody that is not bound to the plate has been previously bound to the competing RR6. There may be a proportion of inactive protein.

It is believed that the sensitivity of this particular assay is not good enough to draw definitive conclusions from the data, as all experimental errors were magnified in the calculation stage. Furthermore, as discussed in section 1.6.2, inherent issues with the indirect nature of ELISA only cloud the issues further.

It was hence decided to research a more direct approach, BIAcore. BIAcore uses a technique known as Surface Plasmon resonance to directly measure the association and dissociation of a ligand with its binding partner in real time (Malmborg and Borrebaeck, 1995). As mentioned previously (section 1.6.4.1) the binding partner must be bound to the surface of a BIAcore chip for measurement, this may alter the binding parameters.

3.6 BIAcore analysis

A box plot was chosen to represent the data to accurately show the range of results obtained (figure 9). The X-axis represents 1/affinity constant in order to show that the stronger the affinity, the higher on the X-axis the box is found. The box section of the graphs shows the area in which 75% of the data points lie and is taken as the range of affinities the protein exhibits. In some cases this range is quite large. Error bars show the 5th/95th percentiles, and once again vary between samples, with the single VHH molecule showing the smallest error bars.

The graph shows that the single VHH has an affinity of approximately 4.5nMol. Published affinities for IgG antibodies are in the nMol range (0.5 to 350) (Jansson M et al., 1997, Beresford et al., 1999 and Quinn and O’Kennedy, 2001). As the protein falls into this range, it is deemed to show sufficient affinity and functionality towards its target antigen. VHH antibody affinities are commonly slightly lower than corresponding IgG’s (van der Linden, 1999), however, other advantages as described previously more than compensate.

The addition of a CBD to the VHH molecule reduced the binding affinity to 23 nMol. However, this drop in affinity appears to be counteracted by the addition of a second VHH domain in the fusion protein as this VHH-VHH-CBD demonstrated an affinity of about 10 nMol. The CBD-VHH-CBD molecule format shows an affinity of about 20 nMol, indicating that adding a further CBD did not have a significant negative effect.

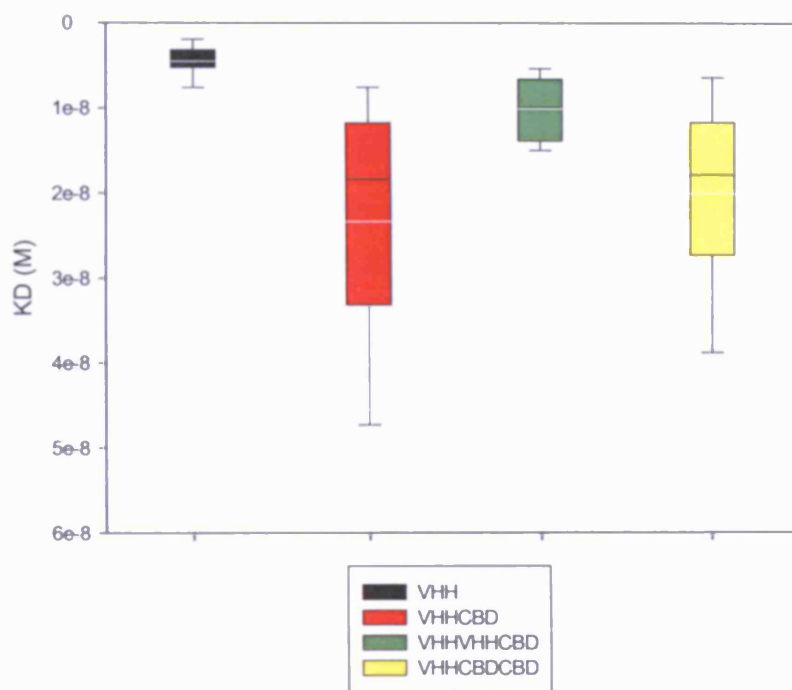


Figure 9: BIAcore affinities for model system fusions. The box plot shows the interquartile range, with the error bars demonstrating the 5th/95th percentiles. The white bar shows the mean of the data. The Y-axis is inverted to demonstrate the strongest affinities at the top of the graph.

Previous work on a range of llama VHH anti-RR6 fragments has demonstrated affinities of 22 to 83 nM as determined by IAsys biosensor (Spinelli et al., 2004). This difference could be due to a difference in protein structure and consequently function or the difference in method of affinity determination used. As discussed in section 1.6.5, IAsys uses a similar method of affinity constant determination to BIAcore.

It is postulated that the reduction in affinity caused by the presence of the CBD is caused by a greater steric hindrance due to the increased size of the molecule. This effect was apparently limited, as the addition of another CBD had no greater detrimental effect than that of the single CBD. This may suggest that optimising the molecular structure between the CBD and the first antibody fragment may return the antibody binding strength back to its single domain counterpart. The addition of another VHH, as indicated in the VHH-VHH-CBD protein, reduced this hindrance by offering another binding site.

Addition of two CBD's shows no discernible difference from addition of one – perhaps indicating that the detrimental effect is limited. The molecule with two VHH heads and one CBD shows increased binding over the molecule with one VHH head and one CBD, indicating that addition of another VHH goes part of the way to correct the problem caused by the CBD. The increased binding of the two VHH molecule could be caused by both heads binding to the ligand or just one head binding and the other holding the CBD section out of the way, reducing steric hindrance.

3.7 Binding of fusion in the presence of industrial chemical components

Figure 10 shows the effect of chemical components on the fusion protein affinities. These chemicals are used as key components in the laundry industry. Binding affinities were calculated using BIAevaluation analysis software. The effect of the components plotted in Figure 10 varies with fusion molecule. The most stable of the fusion proteins was the protein C (VHHRR6-VHHRR6-CBD) that maintained an affinity of about 10 nM in the presence of each component.

STPP, which significantly reduced the affinity of the single VHH molecule to 48 nM, appeared to have little or no effect on the other fusion molecules. It is plausible that the presence of the CBD conveys an extra stability to the molecule, perhaps by affecting the charge layer around the molecule, or by physically preventing the STPP from interacting with the VHH component.

Figure 11 shows the affinities of the various fusions in the presence and absence of alkaline silicate. It can be seen that alkaline silicate has a severely detrimental affect on the fusion molecules to a large extent, in particular with the single VHH molecule, reducing the affinity to approximately 550nMol. The VHH-CBD and VHH-VHH-CBD affinities were reduced to approximately 250nMol, and the CBD-VHH-CBD to approximately 300nMol. This appears to suggest that the presence of the CBD, whilst having a minor negative effect on antibody affinity under an idealised condition, convey an additional stability in more extreme reaction conditions.

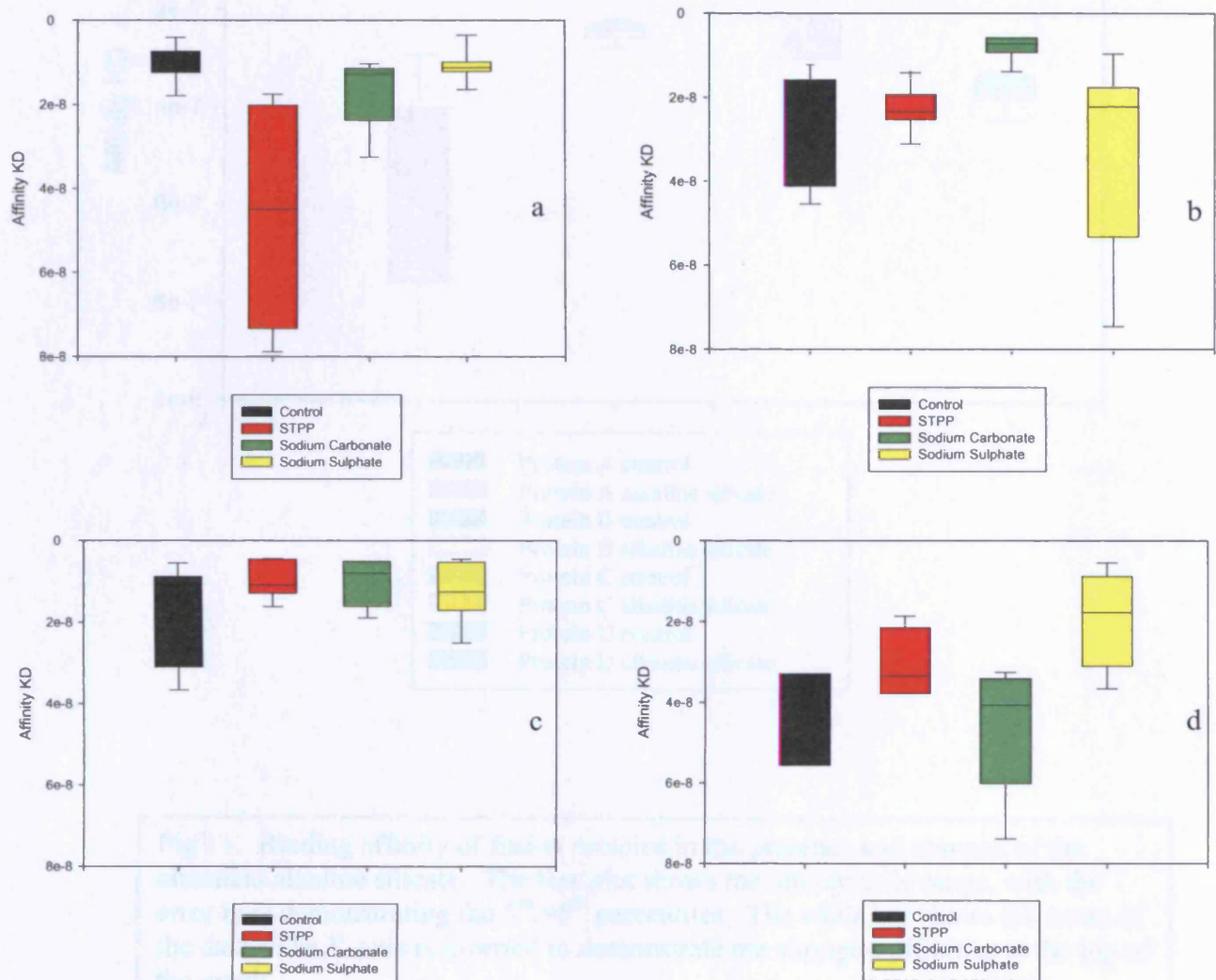


Figure 10 a-d: demonstrate the effects of some commercial chemical components on fusion protein binding affinity, with the letter corresponding to that of the proteins in table 2. The box plot shows the interquartile range, with the error bars demonstrating the 5th/95th percentiles. The white bar shows the mean of the data. The Y-axis is inverted to demonstrate the strongest affinities at the top of the graph.

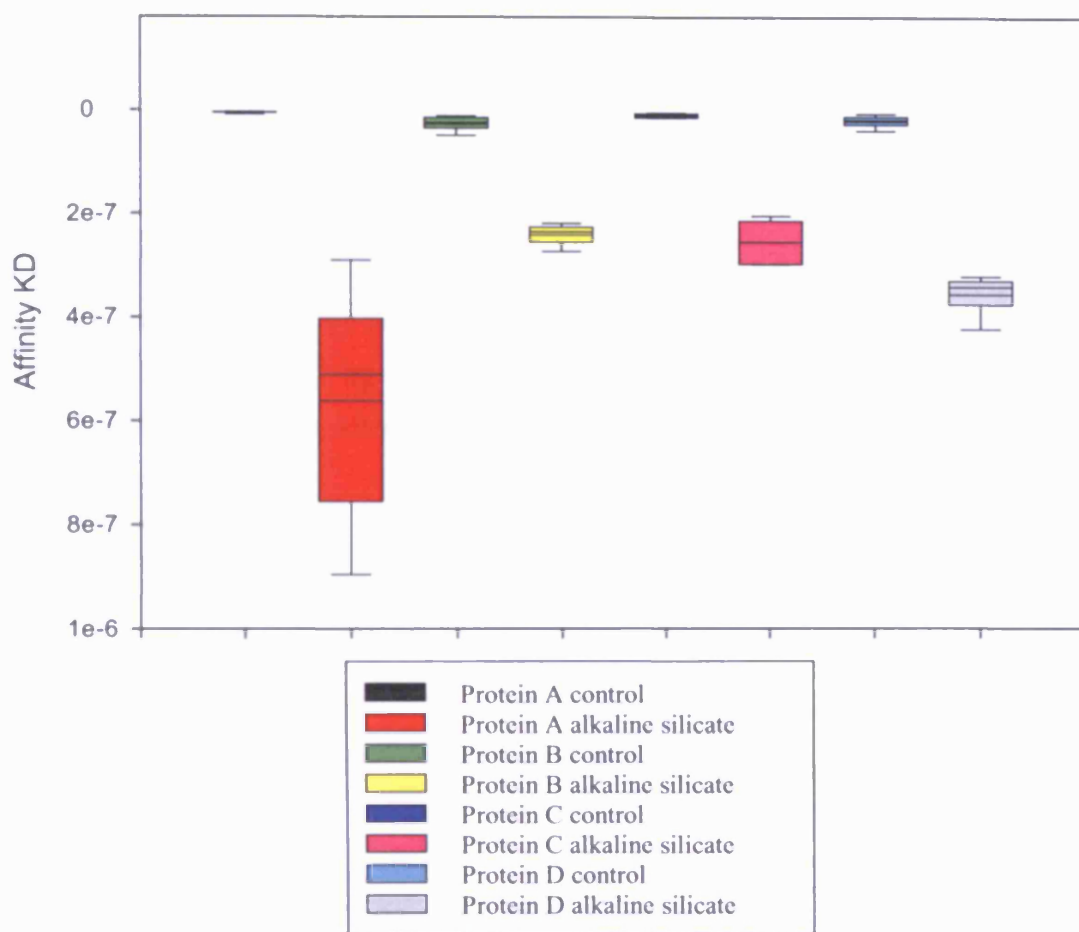


Fig 11. Binding affinity of fusion proteins in the presence and absence of the chemical alkaline silicate. The box plot shows the interquartile range, with the error bars demonstrating the 5th/95th percentiles. The white bar shows the mean of the data. The Y-axis is inverted to demonstrate the strongest affinities at the top of the graph.

3.8 Fluorescent Co-acervate Particle experiments

Coacervate particles represent the potential end use for the fusion. As described previously RR6 can be bound to the particles conveying a binding site for the antibody fusion. Experiments that involve counting particles on cotton samples are useful as they represent both binding events in an end use situation: the antibody to the RR6 and the CBD to the cotton, and the effect of the proteins on particle delivery. The particles can be fluorescently labeled by including a fluorescent dye in a manufacturing step as demonstrated in the appendices (see section 10.1).

3.9 Particles bound to cotton

Further experiments studied the binding of particles to cotton threads, fragments and swatches. Photographs (figures 12 and 13) were taken to show the technology qualitatively, and counts were performed for quantitative analysis.

The particles are naturally “sticky” and will bind to cotton by themselves. Although these photographs show the bound particles, there is no guarantee that the presence of the antibody fusion is affecting the binding. As such a quantitative method was required.

It was investigated whether the presence of the fusion molecule increased the number of particles bound to the cotton.

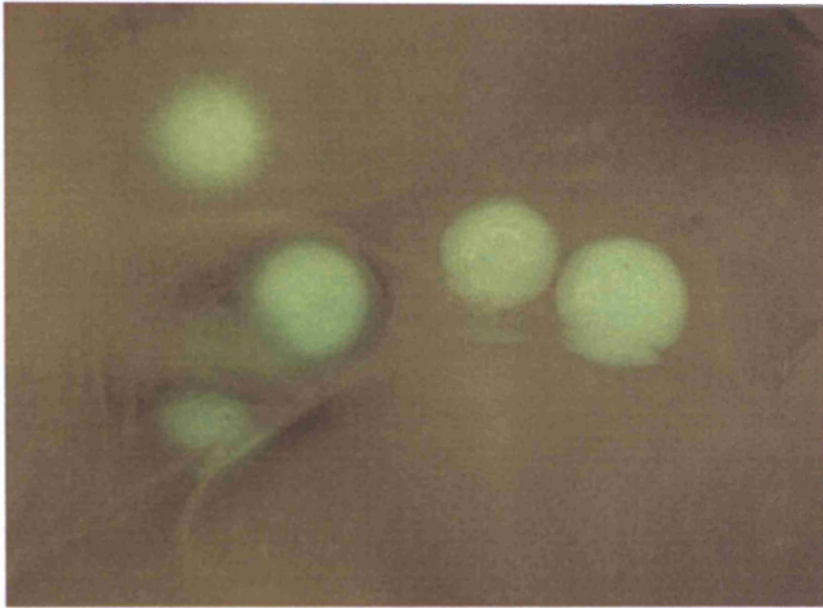


Fig 12. Particles bound to cotton swatch x100 magnification

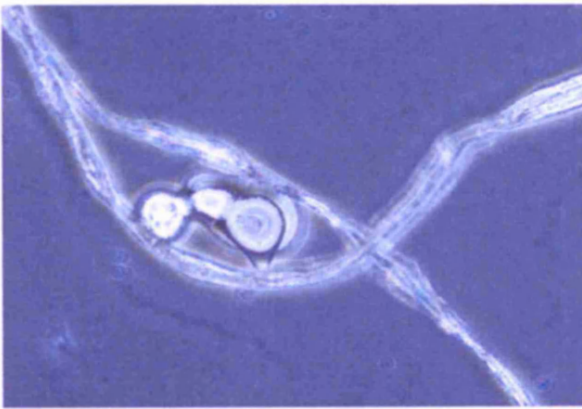


Fig 13. Particles bound to cotton thread x100x magnification

3.10 Determination of ideal binding method

As can be seen from figure 14, the presence of the VHH -RR6 CBD has a large impact on the deposition of particles to the cotton surface if applied in the right manner. It is clear that if the protein is prebound to the particles prior to addition of the cotton there is a distinct benefit in deposition. It is interesting that this benefit is only noticed using the pre-binding to particles method and not the pre-binding to cotton or the self-assembly approach.

It is possible that because in the other methods the fusion gets spread too thin to have a noticeable effect, however in the pre-binding to particles method the fusion is focused specifically on the particles activating them into specific cellulose binders.

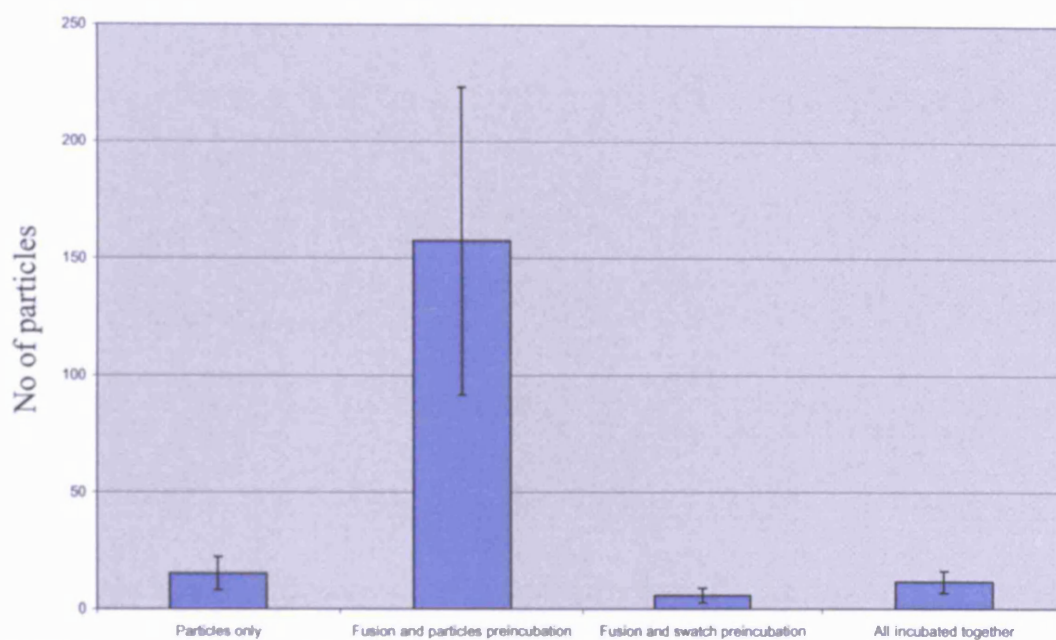


Figure 14. Determination of ideal binding method. The Y axis represents the number of particle visible under x100 magnification on a randomly selected spot on the cellulose swatch (approx radius of area 0.2 mm).

3.11 Comparison of effect of different fusions on bifunctionality

Figure 14 shows the effect of the fusion protein on the deposition of particles to a cotton surface. The single VHH with no CBD attached has no effect on the deposition of the particles to the surface, and is very similar to that of the control. The addition of a single CBD to the VHH significantly increases the deposition of the particles to the surface. This is further accentuated by use of either a double VHH or CBD, with the double CBD giving the highest deposition.

It is interesting that the molecule with 2 CBD's has the greatest level of bifunctionality, yet the lowest affinity for the RR6. It is likely that the addition of the extra CBD gives the particle an increased chance of tethering strongly to the cellulose, and by pre-binding the fusion to the particle; it has a chance to convey the extra binding ability.

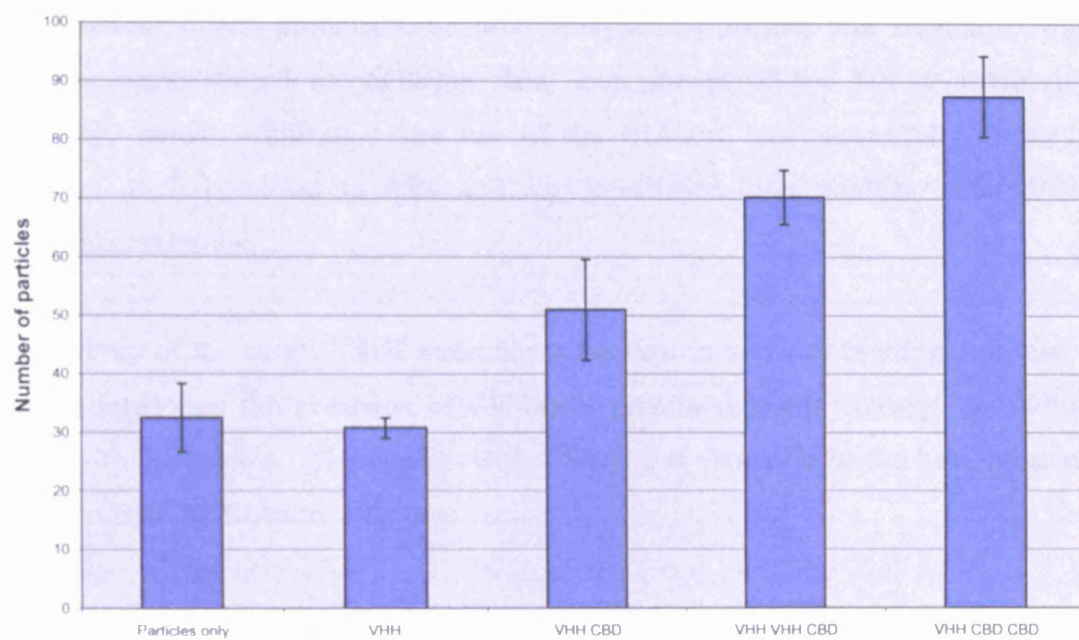


Figure 15. Comparison of effect of different fusions using pre-binding method. The Y axis represents the number of particle visible under 20000 times magnification on a randomly selected spot on the cellulose swatch.

3.12 Summary

The different fusion proteins have been analysed by ELISA and BIAcore. ELISA methods demonstrated inconclusive data, and proved of too low a sensitivity to accurately render affinities. The use of the BIAcore has successfully defined the affinities of the proteins to RR6 and also confirmed their stability under different chemical conditions.

The affinity of the single VHH molecule is the best in terms of binding, however it is demonstrated that the presence of additional protein domains conveys an additional stability to the protein. The protein with 2 VHH s is shown to be the best, most stable binder to RR6 by BIAcore analysis.

RR6 coated particles have been successfully manufactured containing sunflower oil as a neutral component to represent what eventually would be the particle active load. The load can be seen clearly in microscope pictures of the particles, and can be seen bound to cotton fragments in the presence of the fusion qualitatively and backed up quantitatively with particle counts of particles and fusion compared to a particle only control.

Use of the fusion proteins and particles in bifunctionality assays shows that binding is enhanced in the presence of the fusion protein when the particles have been pre-incubated with the fusion protein prior to addition to cellulosic substrates. The fusion protein containing 2 CBDs appears to convey the best bi-functionality.

These initial “proof of principle” experiments confirm that the proteins bind successfully to the RR6 substrate and that they successfully aid the deposition of particles containing RR6 to cotton surfaces. These results were sufficient to encourage further research in this area, and further work will be in the investigation of proteins raised directly to a particle surface.

4.0: Initial Characterisation of the Melamine Particle System and selection of suitable candidate proteins

4.1 Introduction

A number of problems are foreseeable with using the reactive red 6 system in a commercial application. Sources of reactive red 6 are very limited as it is no longer manufactured; and stored supplies are at the gram level, never able to meet a commercial need. Also the use of what is essentially a red dye in clothing applications raises some issues.

The coacervate particles are problematic as they contain gelatin, which is an undesirable component in commercial formulations due to concerns over the bovine source and the presence of disease causing prions (Silveira et al., 2004). Furthermore the Coacervate particles have a very poor release profile, as shown in figure 16.

It can be seen that although the initial free perfume level is low, the coacervate particles release the perfume rapidly, within 30 minutes at 25°C and 60°C. This level is then comparable to free perfume. In fact a slow, steady release profile over a period of days is the desired effect.

Three other main types of particle were considered: latex, melamine urea formaldehyde polymer and silica particles. Latex particles were not chosen, as although they have good release profiles, the allergy of latex is well documented in literature (Warshaw, 2003). Similarly silica particles were discounted due to fears over silicosis (Merget et al., 2002 and Ding et al., 2002). Due to this, the melamine urea formaldehyde polymer particle was chosen, as this has good release profiles. Melamine is a reactive compound which forms highly inert polymers (the structure of melamine is given in figure 17).

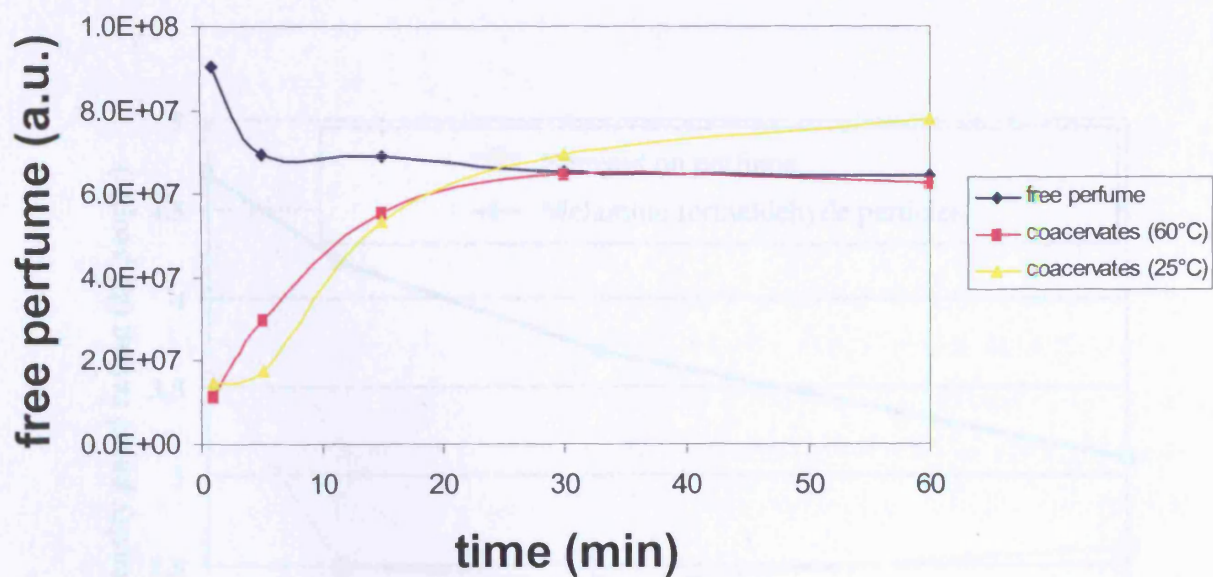


Figure 16. Release profile of perfume from coacervate particles compared to that of free perfume. Free perfume is detected by Solid-Phase Micro Extraction Gas-Chromatography Mass Spectrometry (SPME-GCMS) of headspace above particles/perfume (Source: Unilever Research, Vlaardingen).

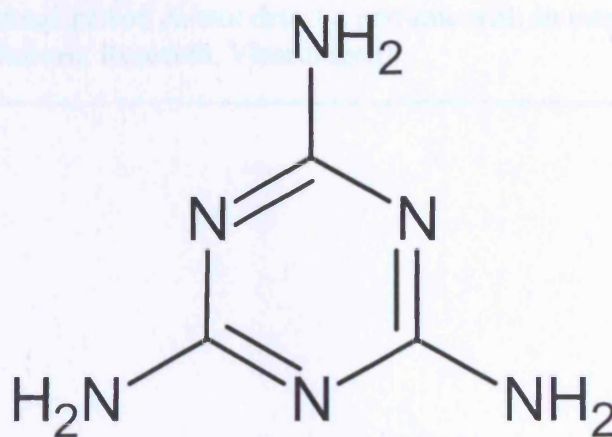


Fig 17. Chemical structure of melamine.

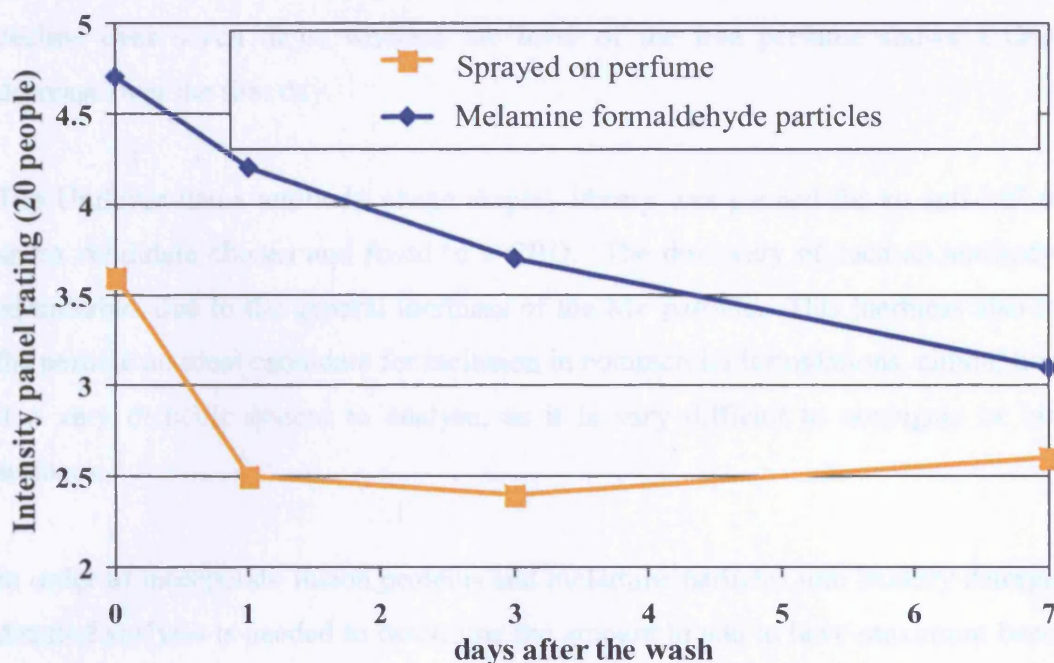


Figure 18. Release profile of perfume from melamine formaldehyde particles as determined by a panel of twenty experts. Topnote is the name of a very volatile yet popular perfume used in laundry. An average person cannot detect a perfume with an intensity panel rating of below 2.5. (Source:Unilever Research, Vlaardingen).

As can be seen from figure 18, the melamine-formaldehyde (MF) particles have a slower release profile. The perfume level of the MF particles shows a slow steady decline over seven days, whereas the level of the free perfume shows a dramatic decrease over the first day.

The Unilever llama antibody phage display library was panned for an anti-MF fusion and a candidate chosen and fused to a CBD. The discovery of such an antibody was remarkable, due to the general inertness of the MF particle. This inertness also makes the particle an ideal candidate for inclusion in commercial formulations, although makes it a very difficult system to analyse, as it is very difficult to conjugate or bind to surfaces.

In order to incorporate fusion proteins and melamine particles into laundry detergents a detailed analysis is needed to determine the amount to add to have maximum benefit at minimum cost. As such it is important to find a level where the melamine particles are saturated with fusion protein to determine the maximum binding, and the point where further addition becomes unnecessary.

Butler et. al (1992) investigating antibody mono-layers on polystyrene-latex surfaces determined a maximum possible surface coverage of $0.5 \mu\text{gcm}^{-2}$ of a random monolayer of antibodies. This figure is a maximum and is highly dependant on pH, salt concentration and the nature of the surface. It is likely that surface coverage can commonly be 5-10 times less than $0.5 \mu\text{gcm}^{-2}$.

Internal Unilever data suggests a maximum coverage of $0.3 \mu\text{gcm}^{-2}$ of IgG (Badley, 1993). This figure can be used to model particle coverage of antibody and potential amounts to use and these calculations can be found in the appendix (section 10.2).

Using this data it can be estimated that approximately 10 mg of protein per kg of washing powder assuming 0.2% loading of perfume (loading varies between detergent types, but 0.2% is an average (Parry, 2003)) is needed. This is comparable to dosing of other proteins such as enzymes. It is important to assess whether this level is achievable, and what percentage of the protein actually binds to the particle, in order to understand the commercial potential of this technology.

4.2 Quantification of level of fusion bound to melamine particles

Figure 19 shows an example of the SDS PAGE gels used to determine disappearance assay data. The melA protein can be observed to be approximately 20 KDa in size, which corresponds closely with the derived value (18759 Da) (as described in section 2.16.6). A second band can be seen at approximately 15KDa, in about 50% abundance. It is likely that this is the antibody section - VHH M1E7 or part of VHH M1E7 as it shows disappearance in the presence of the particles, and also shows positive on a Western blot labeled with an anti-llama antibody (data not shown).

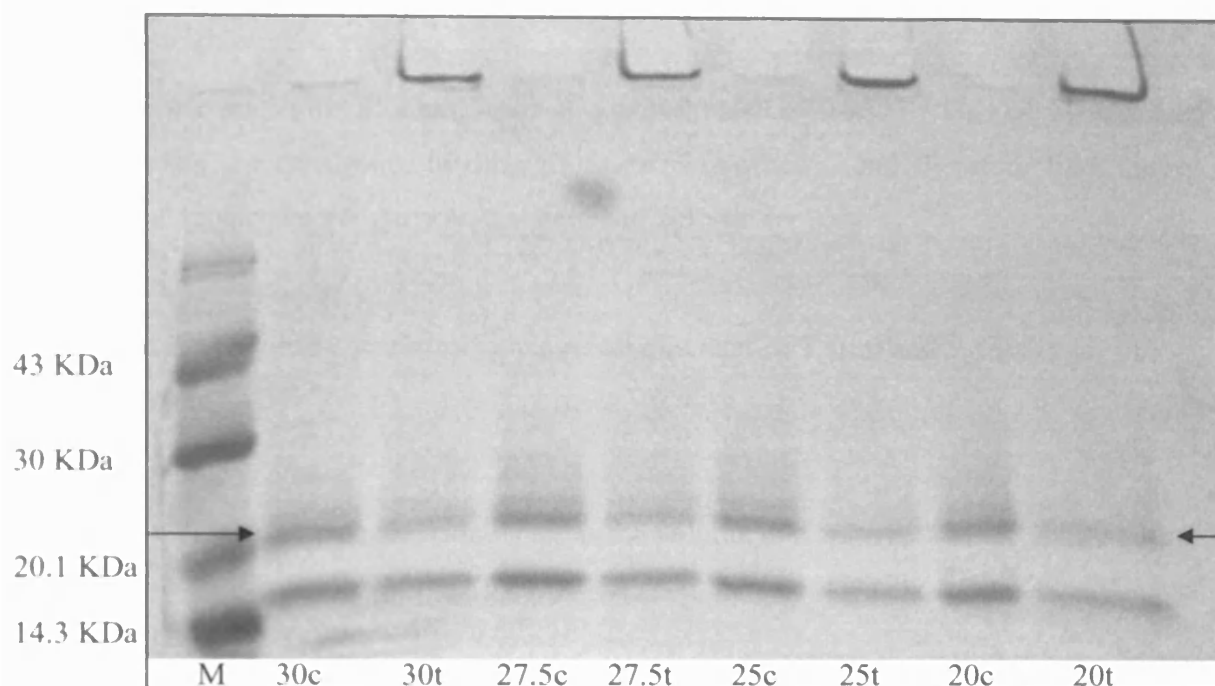


Figure 19: Example Gel used to calculate disappearance assay data below. The arrows identify the fusion used. Key: M: Marker, c: Control, t: Test. The number represents the concentration of the fusion used (μg). The lowest band is believed to be a fusion protein fragment.

Figure 20 shows the binding of fusion melA to blue melamine particles in PBS buffer. The line of best fit is derived from the Weibull 5-parameter sigmoidal curve function available on Sigma plot and has a R-squared value of 0.9805. This curve was used to calculate the maximum loading of fusion to particles, and therefore the number of fusion molecules per particle, as described below.

The blue melamine particles have a mean diameter of 7.5 μm and a density of 1.51 gcm^{-3} .

The volume of 1 particle, calculated as the volume of a sphere, $4/3\pi r^3$ is 220.89 μm^3 .

The total volume of melamine particles in 1 cm^3 20% suspension is calculated using the density of melamine to be 0.155 cm^3 .

The number of particles is therefore equal to the total volume of melamine divided by the volume of one particle, 7.01×10^8 .

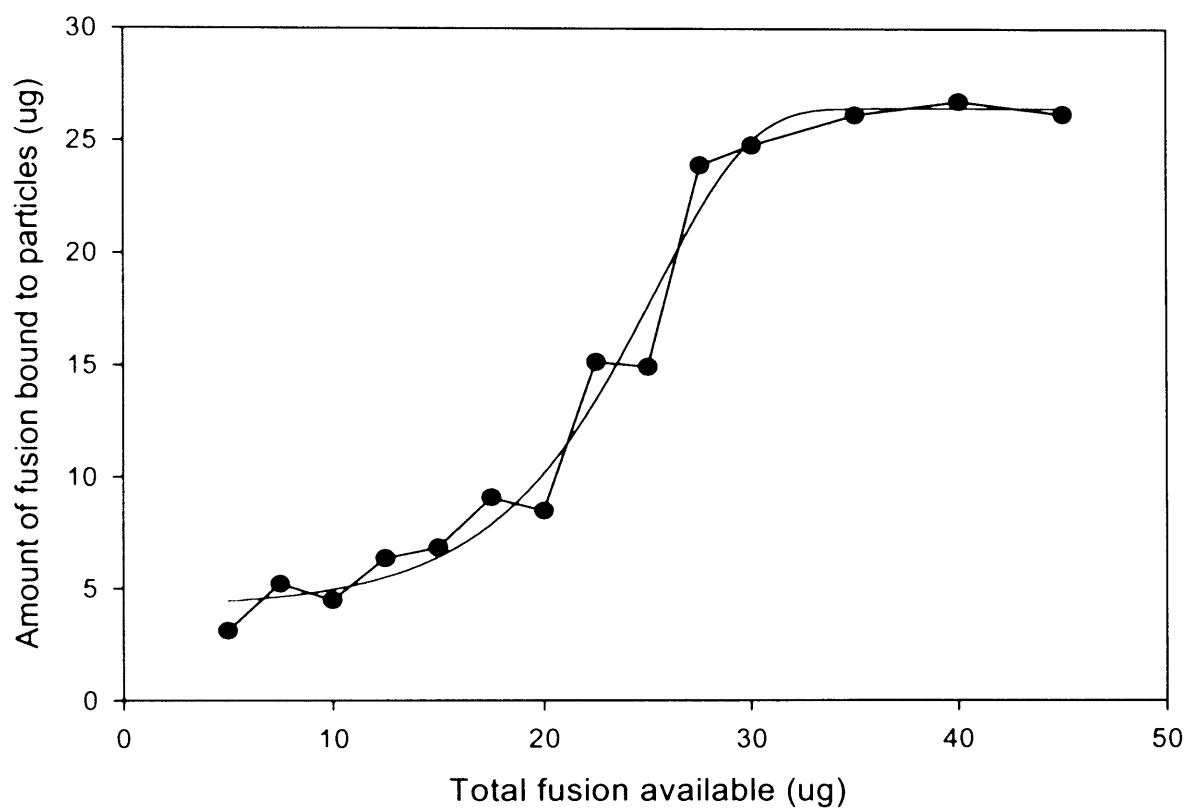


Figure 20: Graph of mclA fusion bound to melamine particles in PBS with increasing concentration of fusion.

The number of particles therefore in the reaction mixture (120µl of the 20% suspension) is 8.40×10^7 .

The maximum amount of fusion that binds to 120µl of 20% suspension of particles is derived from figure 2 to be 26.4311 µg.

The amount of fusion binding to each particle is calculated at 3.15×10^{-7} µg fusion per particle. Using the molecular weight of the fusion (18579) it is determined that there were 1.70×10^{-17} Moles of fusion per particle.

Multiplication of the number of moles per particle by Avogadro's number (6.022×10^{23}) gives the number of fusion molecule bound per particle: 1.024×10^7 .

The surface area of one particle is calculated previously as 1.77×10^{-6} cm². The total surface area available for binding in the reaction mixture can therefore be calculated as 148.68 cm² using the number of particles as calculated previously.

The amount of fusion bound per cm² can be calculated as:

$$2.643112 \times 10^{-6} / 148.68 = 1.78 \times 10^{-7} \text{ g per cm}^2$$

This gives a maximum value of 0.178 µg fusion M1E7 per cm² or 0.944 mg fusion per gram of capsule. To obtain this level of binding in the reaction mixture requires 34µg fusion, of which 26.4311µg binds, giving a percentage binding of 77.7%. Therefore to achieve 0.178µg per cm² binding requires 0.228µg fusion in total.

4.3 Cross functionality with coacervate particles

Figure 21 demonstrates that the anti-melamine fusion shows some binding to coacervate, but only a third to a half of the binding to the melamine particles. The difference is to be expected, as the M1E7 antibody domain found on melA was raised to the melamine surface of the particles and should demonstrate little cross-functionality. The specificity of llama VHHs is well known, and commented on by van der Linden et al., (1999) who compares VHHs with binding affinities for reactive dyes as being highly specific and not binding to dimers of the antigen dye, or indeed chemically closely related dyes.

This is a measure of the specificity of the protein and indicates that the protein has a specific binding functionality towards the melamine particle as desired.

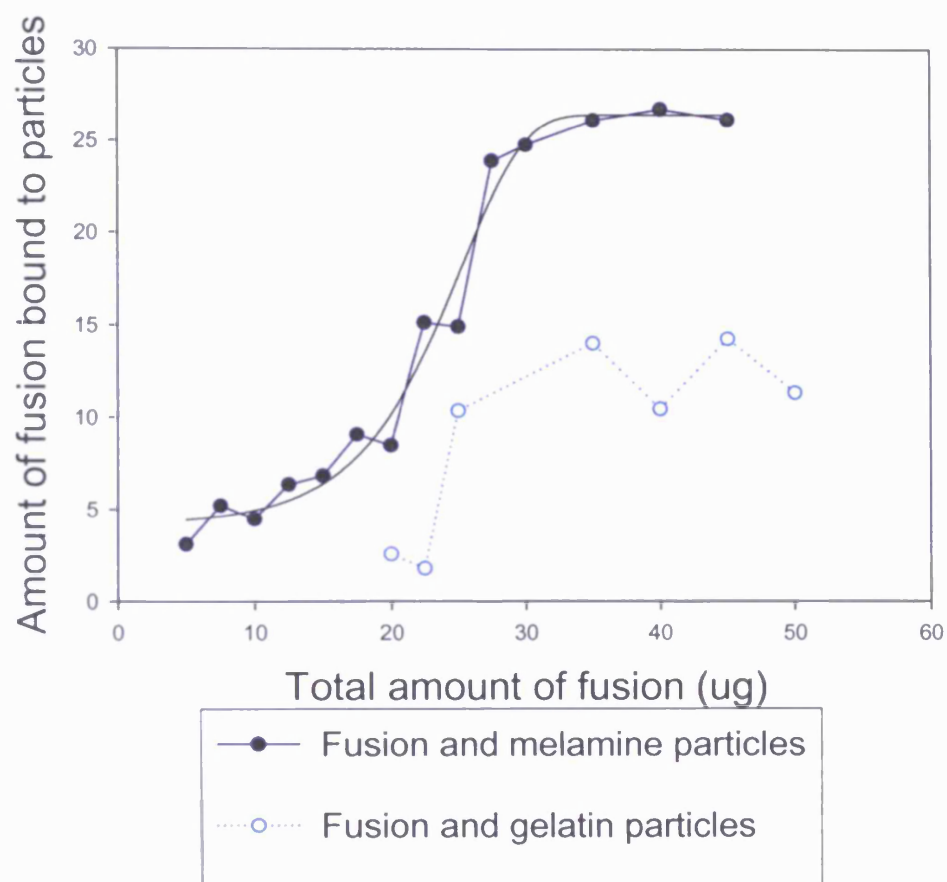


Figure 21: Binding of the fusion mela to melamine and co-acervate particles.

4.4 Anti-melamine binding in the presence of detergent bases

Figure 22 demonstrates the binding of the fusion in the presence of two laundry detergent bases, OMO France and Saturn Silver. As described previously a laundry base is a complete detergent formulation without the presence of perfume and enzymes. As can be seen from the graph, the fusion shows little change when in the presence of OMO France, still reaching the same level of deposition, but just at a slightly higher amount of total fusion - 43 μ g instead of 37.5. Saturn Silver however has a massively detrimental effect, and within the range studied there were only initial signs of binding at the higher end of total fusion concentration. As a consequence, further work with this protein will not include Saturn silver.

It is important that the fusion works in available detergent bases, and the above information would seem to indicate that OMO France is a better choice for formulation than Saturn Silver. Llama antibodies that bind in the presence of surfactants have also been selected by Dolk et al, (2005), with an aim of treating scalp complaints such as dandruff by placing the antibodies in shampoo. Dolk et al. add a further comment on the potential of these antibodies to be able to work in a wide variety of conditions.

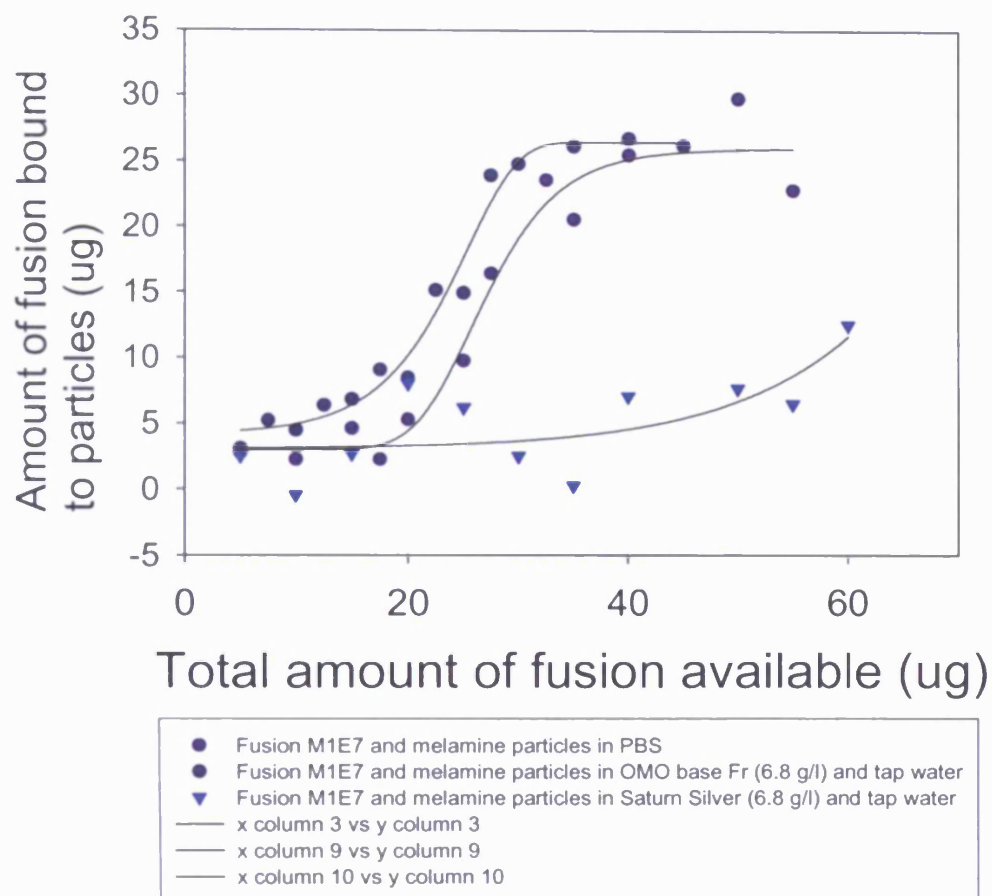


Figure 22: Binding of melaA fusion protein to particles in the presence of detergent bases OMO France and Saturn Silver.

4.5 Binding of fusion to cotton in the presence of detergent

Fortunately binding of the fusion to cotton is easily measured using a simple filter-plate ELISA protocol. This method is not practical for measuring of the proteins binding to particles as the particles were observed to totally block the filters. There is also a measure of non specific binding of the protein to the plate. This binding is counteracted by the removal of the cotton swatch/thread at the final step into a new filter plate; this is not possible with particles without suffering loss to the filter.

Figure 23 demonstrates the binding of the fusion molecule to cotton in the presence of detergent bases. As can be seen a level of binding comparable to that of the positive controls 27°F water (A controlled hardness water designed to represent standard tap water) and PBST can be observed in the majority of detergent bases, with Shakespeare rational apparently the strongest binder. Although OMO MA has the most detrimental effect on binding, clear evidence of binding is observed over the control. It is interesting that in the presence of PBST the error bars for both the control and the test were at their largest, this could be due to a surfactant effect of the tween in the PBST. This chart shows that all the available detergent bases have potential to be used in this system.

In this experiment the detergent base Saturn Silver had been eliminated from study due to its poor performance in section 4.4. OMO France can be seen to have an active CBD and antibody functionality to the particle and cellulose surfaces. Unfortunately due to the time necessary to perform the assay detailed in section 4.4 it was not possible to perform detailed analysis of protein-capsule binding in the presence of each detergent base.

4.6 Visual demonstration of binding Fabrics and biofilms

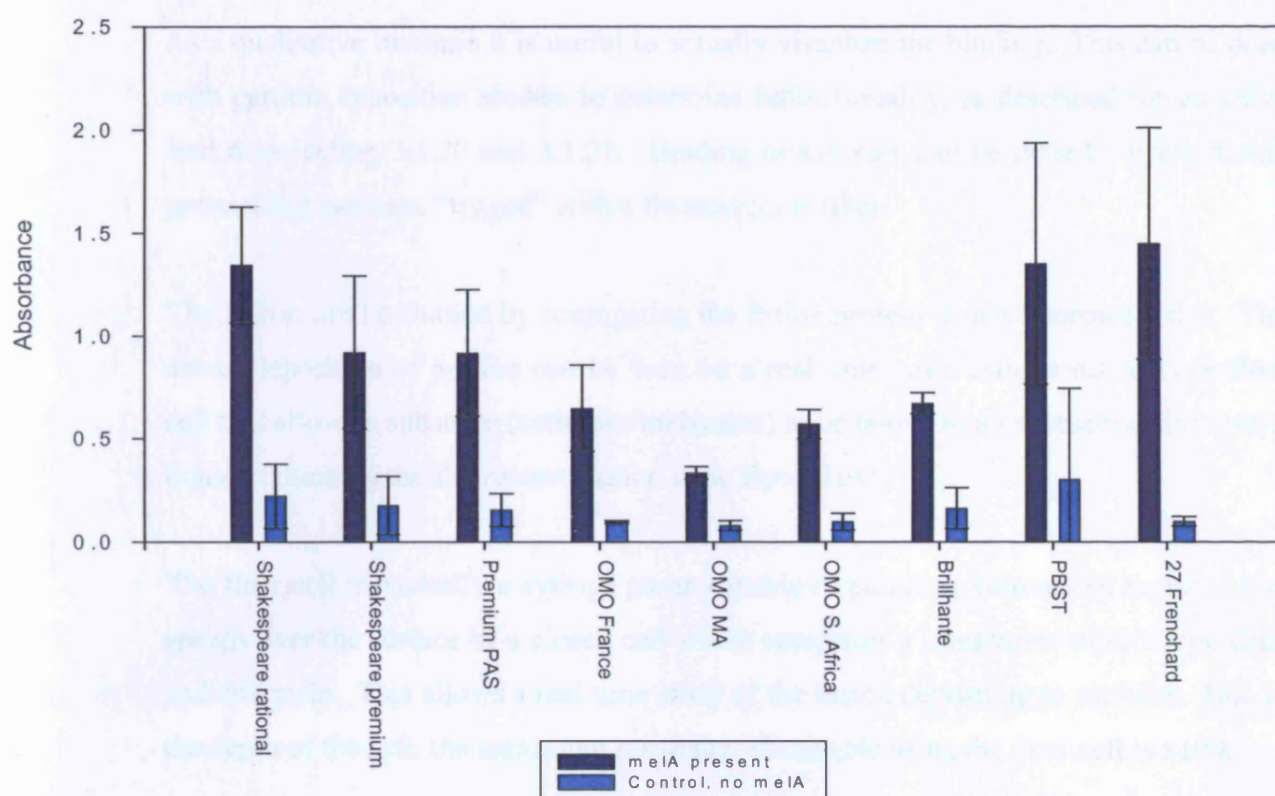


Figure 23. Binding of the fusion to cotton strands in the presence of laundry detergents. 27°F water and PBST are include as a control. The Error bars represent the standard error and the bars the mean of the triplicate results obtained.

4.6 Visual demonstration of binding Fabrics and Melamine

As a qualitative measure it is useful to actually visualize the binding. This can be done with particle deposition studies to determine bifunctionality, as described for Reactive Red 6 in section 3.1.20 and 3.1.21. Binding assays can also be done by using fusion protein that has been “tagged” with a fluorescent marker.

The fusion can be studied by conjugating the fusion protein with a fluorescent dye. The actual deposition of protein can be seen on a real time basis using a microscope flow cell that allows a substrate (cellulose/melamine) to be bound to a microscope slide and a liquid containing the fluorescent fusion to be flowed over it.

The flow cell is basically a syringe pump capable of pumping volumes of liquid at low speeds over the surface of a closed cell which comprises a transparent microscope slide and coverslip. This allows a real time study of the fusion depositing to surfaces. Due to the depth of the cell, the maximum resolution obtainable using the flow cell is x100.

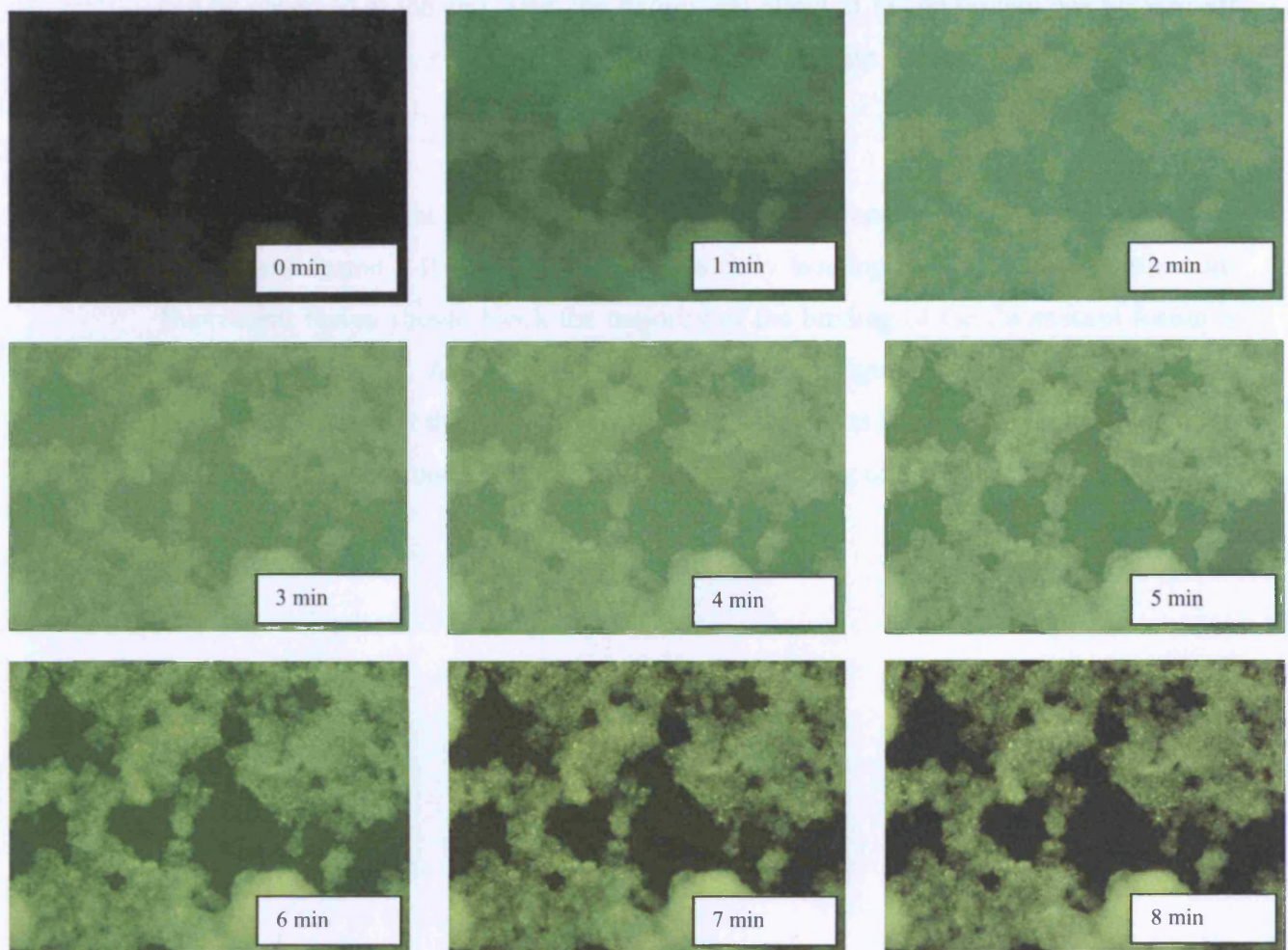


Figure 24. Fluorescently labelled fusion flowing over a melamine slide. The fusion can be seen to adhere to the melamine, making it fluoresce.

Figure 24 shows the deposition of the fluorescent fusion onto the melamine surface, and the consequent fluorescing of the melamine. A substantial increase in the fluorescence can be observed at the end, after the fluorescent cloud of fusion protein has washed off the slide. It is also retained over the time and appears not to wash off easily (not shown).

Figure 25 shows another melamine slide, but this one is pre-washed with non-fluorescent fusion. If the fusion is successfully binding to the melamine, the non-fluorescent fusion should block the majority of the binding of the fluorescent fusion is subsequently added. As can be seen when comparing figures 24 and 25, figure 25 is a lot less fluorescent at the end of the cycle than 24. This is because it is believed that the binding of the non-fluorescent fusion inhibits the binding of the fluorescent fusion.

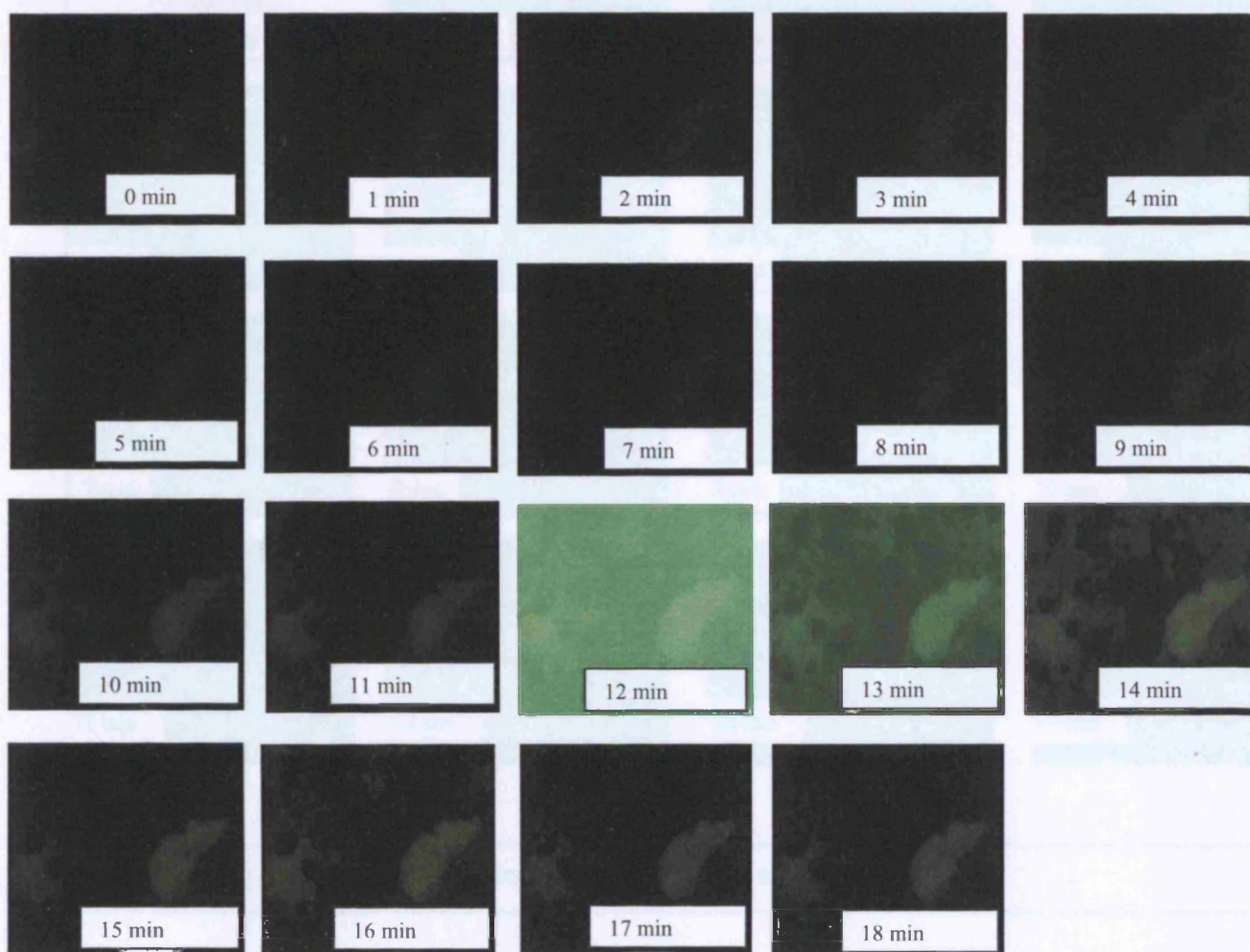


Figure 25. Binding of fluorescent labelled fusion to pre blocked melamine slide. Non fluorescent fusion is added at 0 minutes and fluorescent added at 12 minutes. The binding of the non-fluorescent fusion inhibits the binding of the fluorescent fusion.

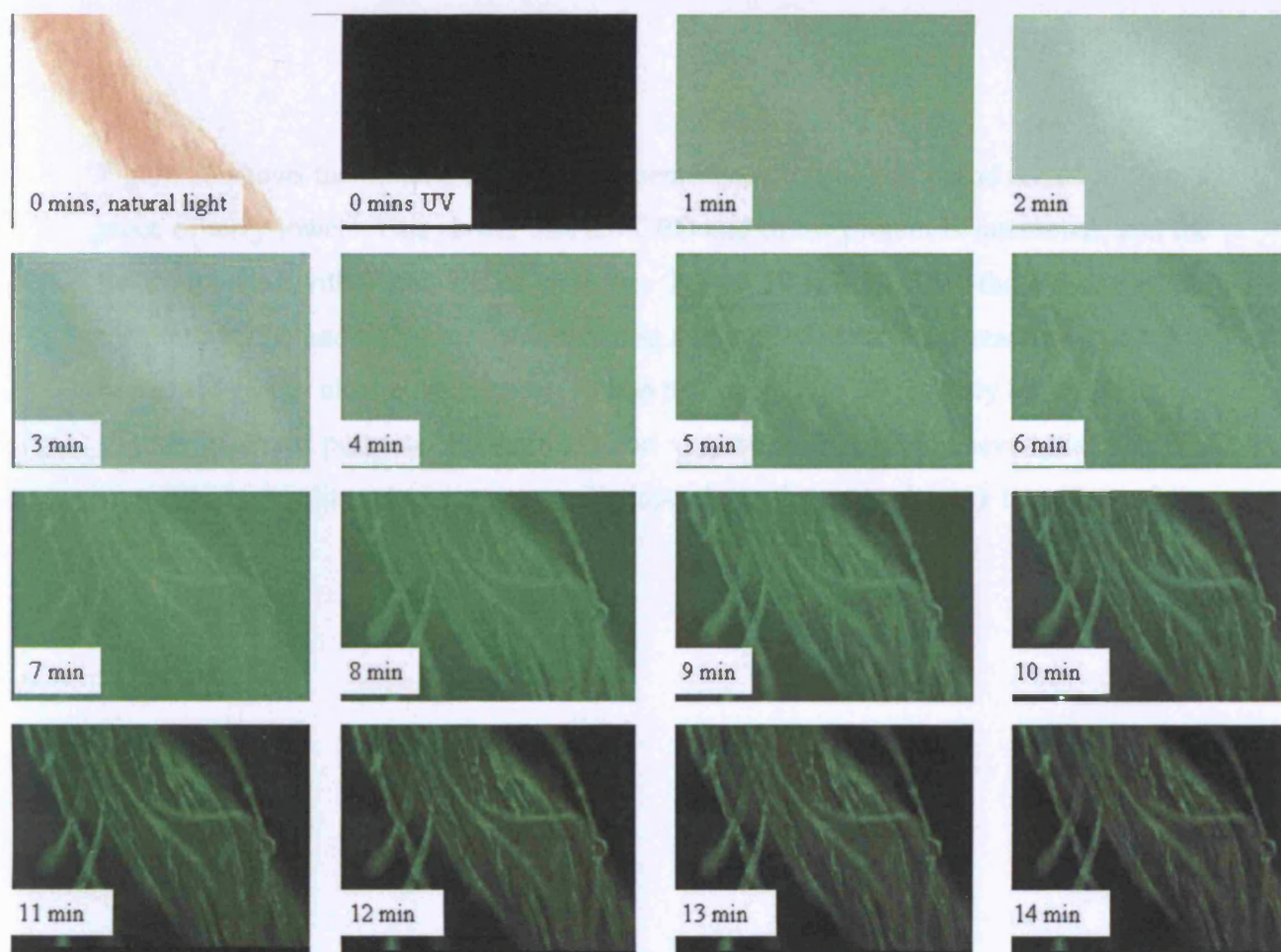


Figure 26 Binding of fluorescent labelled fusion to a cotton strand.

Figure 26 shows the binding of the fluorescent fusion to a cotton strand removed from a piece of terry-towel. This shows that the CBD end of the protein is functional, and the fusion remains with 5 minutes of washing. Figure 27 and 28 show the binding of the fusion to Nylon and Polyester demonstrating a degree of some cross-reactivity, possibly brought about by charge interactions. It can be noticed that Nylon only binds the fusion slightly, whereas polyester binds the fusion very strongly. It is believed that this is a non-specific binding, as it is more pH dependent (data not shown) than that of the cellulose binding.

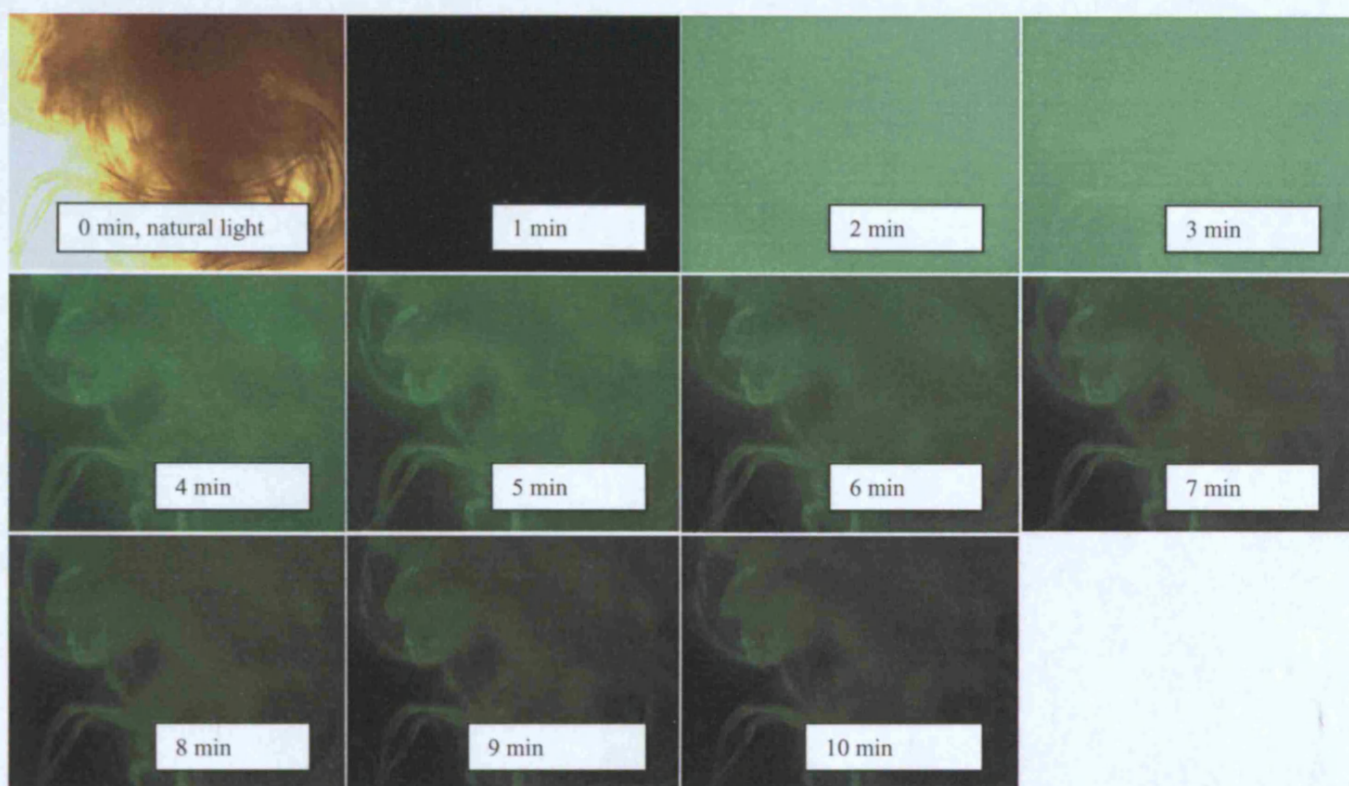


Figure 27 Binding of fluorescent labelled fusion to Nylon.

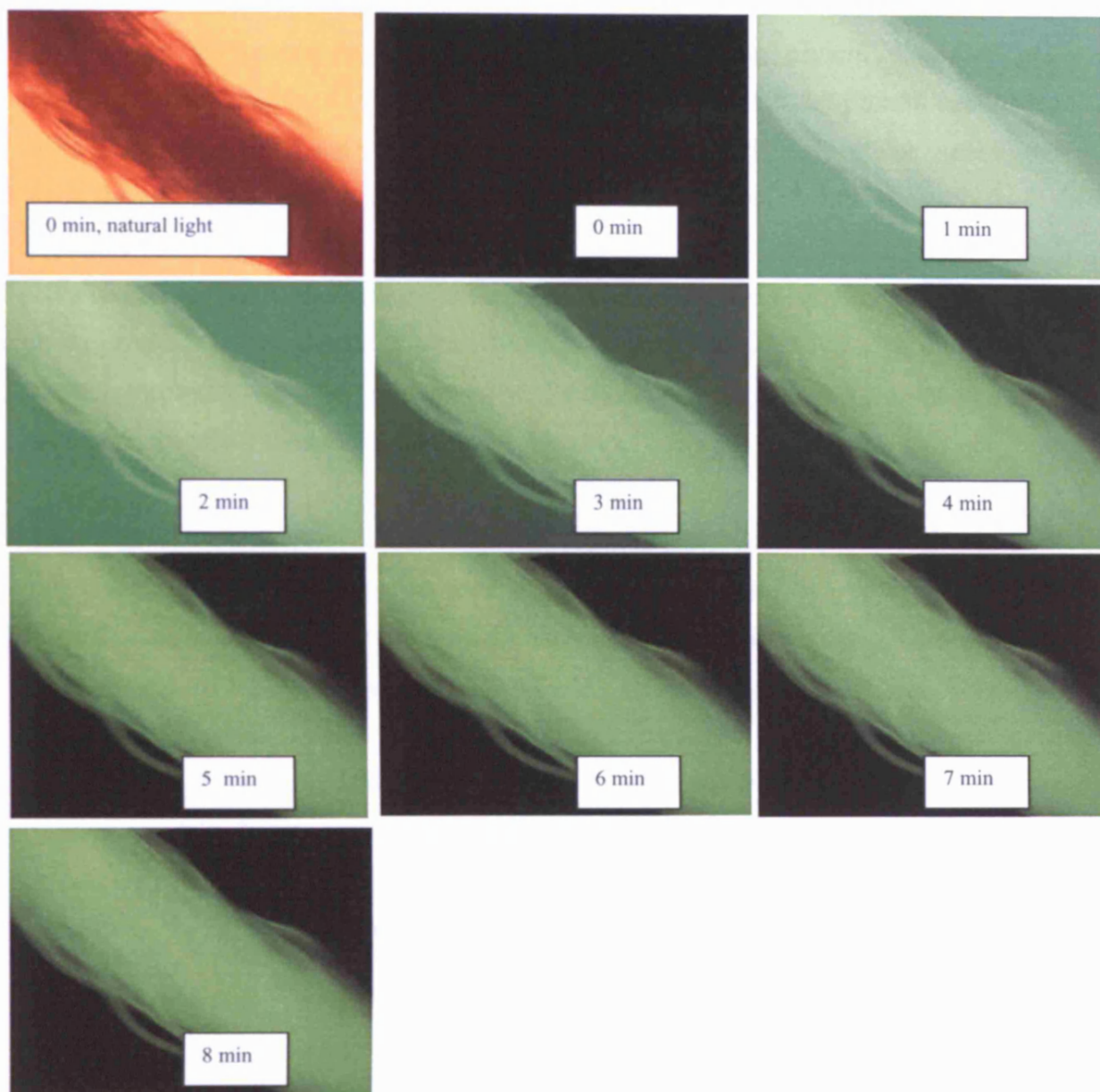


Figure 28 Binding of fluorescent labelled fusion to Polyester.

4.7 Potential commercial anti-melamine candidate proteins

Due to the successful function of the melamine binding protein discussed and characterised in section 4.1-4.6, a number of potential commercial protein candidates have been produced by a contract manufacturer for study. These proteins were based upon the anti-melamine melA protein although have key structural differences.

This is necessary as Unilever does not have the capabilities for large scale production of these proteins. This signals a move towards a commercial product, as the contract manufacturer has the facilities necessary to produce the proteins on a pilot and then commercial scale if the need arises.

The contract manufacturer uses a different expression system which will likely alter their functional characteristics. In order to anticipate functional variability a number of potential candidates with structural differences been made to screen. These contain varying types of VHH and also one or two CBD's of varying types in varying formats.

The proteins also exhibit different production characteristics which will affect selection further. An initial screening assay has been used to select candidates for purification (if necessary), further analysis and eventual selection of a lead candidate. Protein candidates are explored in table 5.

Protein name	Structure	Expression	Notes
melA	M1E7::CBD _{Tr}	Good	Produced in <i>Pichia pastoris</i> Research lab-scale protein; not producable large scale
melB	M1E7::CBD _{Tr}	Good	Produced in <i>Saccharomyces cerevisiae</i> Research lab-scale protein; not producable large scale Appears highly glycosylated
melC	CBD _{C315} ::M1E7::CBD _{EGV}	variable	Produced in <i>Aspergillus niger</i> Batch to batch variability. Very impure.
melD	CBD _{C315} ::KR::M1E7::CBD _{Tr}	Good	Produced in <i>Aspergillus niger</i> Pure due to KR region
melE	CBD _{C315} ::KR::M1E7::L27::CBD _{Tr}	Good	Produced in <i>Aspergillus niger</i> Pure due to KR region
melF	CaLipBprepro::M1C8::CBD _{EGV}	Good	Produced in <i>Aspergillus niger</i> Pure due to CaLipBprepro region
melG	CBD _{C315} ::KR::M1C8::CBD _{EGV}	Medium	Produced in <i>Aspergillus niger</i> Impure
melH	CBD _{C315} ::KR::M1C8::L27::CBD _{EGV}	Medium	Produced in <i>Aspergillus niger</i> Impure
melI	CBD _{C315} ::M1G11::CBD _{EGV}	Good	Produced in <i>Aspergillus niger</i> Impure
melJ	CBD _{C315} ::M1C8::CBD _{EGV}	Good	Produced in <i>Aspergillus niger</i> Impure
melK	CBD _{C315} ::M1E7::L27::CBD _{EGV}	Good	Produced in <i>Aspergillus niger</i> Impure

Table 5: Structure of available proteins for testing. The KR or CaLipBprepro regions allow affinity purification of the protein by selective adsorption followed by cleavage at that site.

4.8 Initial screening of commercial candidate proteins

Due to the crude nature of some the proteins produced, they need purification to allow more detailed study and also for the end-use application. The screening procedure was used to determine suitable candidates for purification.

Figure 29 shows digital microscope pictures (x100 magnification) of the particles and their interaction with cellulosic tissue. Crude demonstrates where the protein has been used in its impure form, a clarified fermentation supernatant. The tissue that was selected is very strong and thin and hence ideal for incubation in liquids and then viewing under a microscope. The particles were “activated” by pre-incubating with the fusion protein or PBS in the case of the control.

It can be seen that some of the fusions show a noticeable increased particle deposition over control, equivalent or higher than that of a 100 fold increase in particle concentration alone (eg proteins melA, melC and melK). Some fusions showed no added binding effect and were removed from the study.

Further considerations must be taken into account when selecting fusion proteins to continue studies with, such as the production characteristics of the protein. As such, fusion proteins melA and melB are not available for commercial purposes (they can only be produced in house and at small scale) and were retained only for benchmarking studies. In-house proteins were expressed in *Saccharomyces cerevisiae* or *Pichia pastoris*, while proteins produced by the contract manufacturer were expressed in *Aspergillus niger*.

Concerns over batch to batch variability of protein melC were highlighted in the apparent difference between binding exhibited. The low quantity of batch 1 means that only simple studies can be conducted with it, and there is a limited amount available for purification.

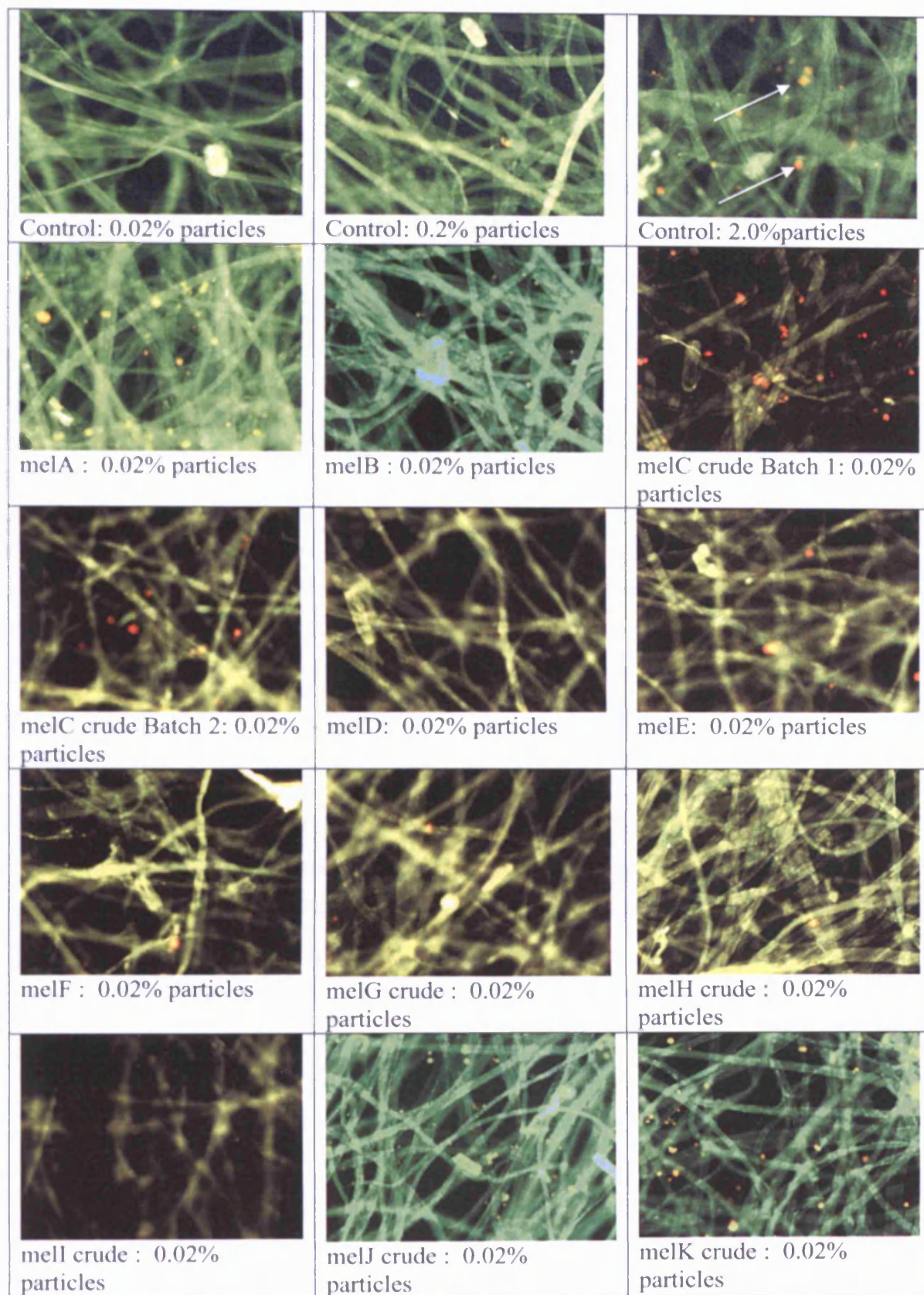


Fig 29. Screening experiment to determine suitable candidates for further study and purification. Particles are shown as orange spheres (demonstrated with arrows) as they contain the red fluorescent dye Nile Red. The auto-fluorescence of the cellulose is also shown.

Furthermore problems with the production of melC raise doubts as to its commercial relevance, although its strong functionality means it was chosen as a candidate for further study. Proteins melJ and melK were much more easily produced reproducibly, and were chosen for purification and further study.

It is interesting to see that the proteins that demonstrate successful binding from *Aspergillus niger* are closely related structurally (Table 5), suggesting a move towards an ideal protein structure. They all contain one VHH and two CBDs in the same orientation. Protein melK is structurally very similar to melC, just containing a shortened linker.

Therefore, the following fusion proteins were chosen for further study, and where appropriate, purification:

- melA
- melB
- melC
- melJ
- melK

As mentioned previously, proteins melA and melB were produced at a small scale and are used for initial benchmarking studies. Only low quantities were available for use. Proteins melC, melJ and melK need purification prior to use.

Proteins melC, melJ and melK have no affinity tags such as the histidine tag to facilitate purification for commercial reasons and also it is believed that the presence of such a tag may inhibit the expression of the protein in the *Aspergillus* production system. As such a purification system needs to be developed that uses either the binding domains incorporated into the protein, or a generic purification system.

4.9 Summary

Initial characterisation of the protein has been performed. The binding of the protein melA to the particle surface has been successfully researched using a gel densitometry based method and subsequent calculation done as to the possible loading of the protein. It is possible to use this data in future cost calculations for the system.

The protein is able to bind successfully to the particle surface in the presence of certain detergent bases suggesting their commercial relevance. Little cross reactivity with co-acervate particles seems to be observed, as expected due to the specific nature of the protein.

Further studies on the binding of the protein to the cotton surfaces in the presence of different detergents suggest suitable detergent bases for the proteins successful operation. Visual demonstration of the protein binding to both melamine and cotton surface is performed using a fluorescent fusion protein. Some binding of the protein is confirmed with Nylon and Polyester.

The protein melA shows definite potential for use in this system. However it is produced on a small scale on site, and hence is not a viable commercial prospect in its current format. Proteins melC, melJ and melK showed potential but need further purification prior to further analysis and use.

5.0 Purification and analysis of suitable protein candidates

5.1 Introduction

Different applications for antibody based proteins require different degrees of purity. For example, in the bio-pharmaceutical industry, very high levels of purity are necessary, and the downstream process used may include up to 9 steps (not including buffer exchange steps). The process encompasses a number of chromatography and virus inactivation/clearance steps (Sommerfeld and Strube, 2005) demonstrated in figure 30, leading to purification contributing to up to 80% of the total manufacturing cost. These high process costs are acceptable due to the relatively low amounts of antibody needed and the high profit margins associated with pharmaceutical products (Roque et al., 2004).

Although some of the high process costs associated with antibody production are eliminated with the use of yeast expression (Muyldermans et al, 2001), a large percentage of process costs are in down stream processing. In other sectors of the biotechnology industry (such as the target market for this product) however the converse is often true; a large amount of product is needed relative to a low profit margin. There is therefore an essential need to employ alternative down-stream processing options to reduce cost.

A potential for affinity chromatography exists utilising the binding domains present in the fusion protein i.e. the anti-melamine particle fragment and the cellulose binding domains present. These were chosen to be strong binders, and hence were likely to need harsh chemical elution conditions, which will affect yield. The suitability of the particles is questionable as they are designed to release their contents under shear or damage such as that that may be incurred in such a process.

As mentioned previously, the use of cellulose binding domains in affinity chromatography is common (and the cost of cellulose is very low compared to that of other adsorbents), however the cellulose binding domains present have been specifically chosen to bind at a very high strength, and would require extreme elution conditions, which were likely to inactivate the protein.

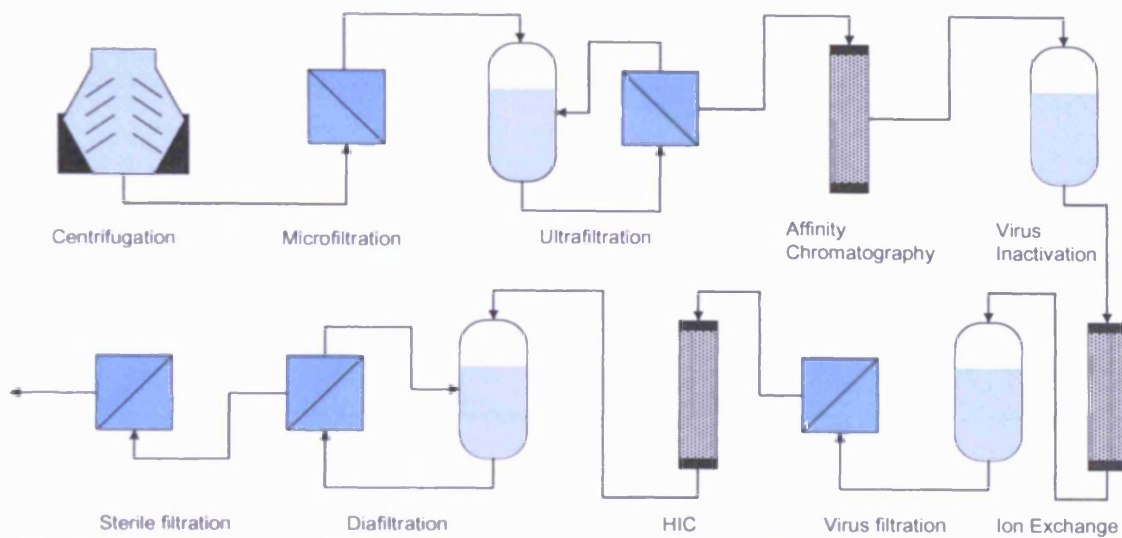


Fig 30 A generic purification process for a biopharmaceutical monoclonal antibody protein produced using mammalian cell culture. The many steps are shown, including virus inactivation and removal and three different chromatography steps

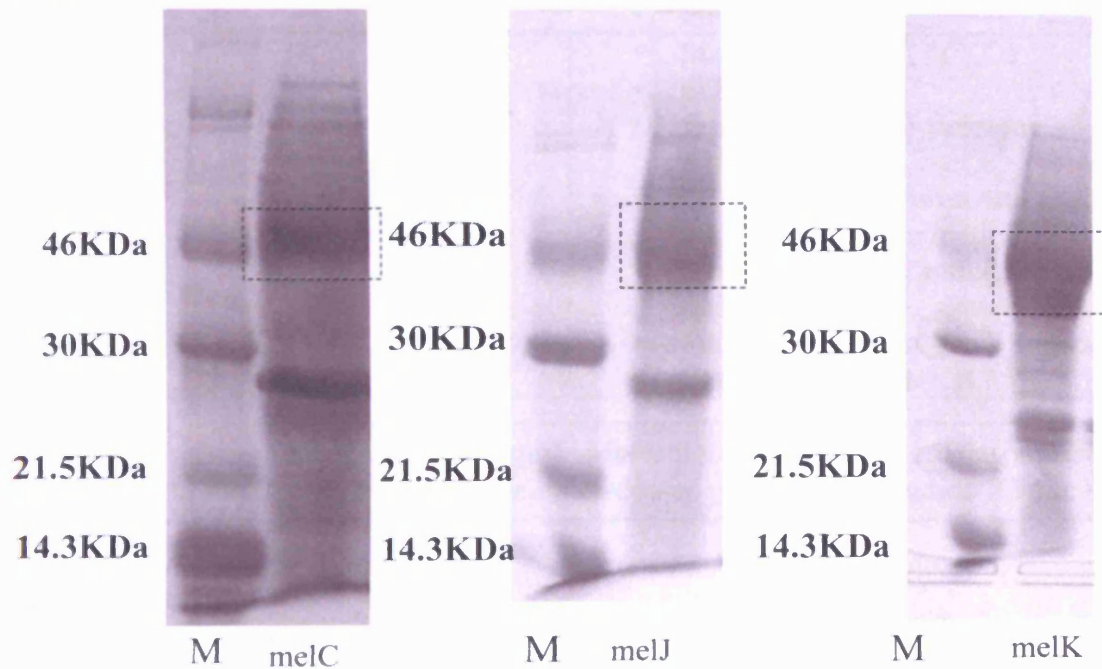


Fig 31 The three candidate proteins chosen for purification. They are all approximately the same size (45-46KDa). The band is also quite broad, this may be caused by their varied glycosylation.

melC	<ul style="list-style-type: none"> • 46 Kda M.W. • pI 8.2 • Fusion of 2 CBD Domains and 1 VHH domain • Most promising protein (as demonstrated in figure 29), but one of most impure (about 5%) • Suffers with batch to batch variability
melJ	<ul style="list-style-type: none"> • 46 Kda M.W. • pI 8.2 • Fusion of 2 CBD Domains and 1 VHH domain. • Same protein as melC but with different antibody head. May suffer similar problems with batch to batch variability • Purity varies
melK	<ul style="list-style-type: none"> • Molecular Weight 46KDa • pI 8.18 • Fusion of 2 CBD domains and 1 VHH domain • Incorporates a shorter linker than melC, to aid in production. • Similar structure and function to melC. • Purity varies from batch to batch: Batch A 10%, Batch B 55%.

Table 6. The three candidate proteins in detail. Molecular weight and pI are derived from the available model in the DNASTar programme, as discussed previously in section 2.16.6.

In keeping with the application of this technology being in the home and personal care sector a final level of 60-70% purity was chosen. This is similar to purity level of laundry enzymes in commercial detergents (Parry, 2003), and is believed to represent a good compromise between cost and purity.

As mentioned previously a degree of pure protein is required to allow detailed characterisation of function and also for regulatory and safety reasons within the final product. The need to purify is also exemplified by the presence of fungal/bacterial proteases in the fermentation product stream which may degrade the protein product (Gouka et al, 1997).

The lower level of required purity means that step number can be reduced, which reduces cost. Also the ability to successfully produce the protein in GRAS yeast means that the safety steps associated with cell culture lines and endogenous virus populations are circumvented.

Figure 31 demonstrates the starting conditions of the crude fusion protein derived by gel electrophoresis. As can be seen, MeIC is the most impure protein, and hence the most difficult to purify. However due to its high functionality it is an important candidate. The structure of the proteins is explored further in table 6.

As mentioned previously, the expression of these proteins in a fungal based system gives many advantages, and one of these is in the downstream processing area. The product stream is not contaminated by endotoxins as found in bacterial expression systems, or the possible presence of mammalian viruses as found in cell culture systems (Verma et al, 1998).

5.2 Small scale purification of candidate proteins

Whole VHH antibodies have been previously purified by protein A and G chromatography (Muyldermans 2001), however the VHH fragments have no Fc region necessary for this method of purification. Lack of adsorption was confirmed by initial experiments using protein A/G which showed no binding affinity. Due to the modelled pI of the candidate proteins being relatively high, cation exchange was chosen as a suitable method (Amersham biosciences guidebook, 11-0004-21).

Recommended protocols for cation exchange (GE Healthcare instructions 71-5107-89 AE) involve the loading of the crude mixture at 1pH below the pI of the protein and elution with a salt gradient (up to 1 mol) at the same pH. This is usually sufficient to separate proteins according to varying surface charges (Amersham biosciences guidebook, 11-0004-21).

The recommended protocol (as described above) was tried; however, an adaptation was required to give satisfactory purification and recoveries. The adaptation was made taking into account the known structural properties of the protein, and the best ideal pH conditions for separation.

The failure of the suggested approach was perhaps due to the modelled pI being inaccurate, or the multi-domain nature of the protein affecting the charge distribution on the protein. Experiments to determine ideal pH for binding determined a pH of 6.7 (1.5 pH units below the estimated pI). However, up to a 2M salt solution did not elute this protein, and a pH shift and salt was needed to elute the protein successfully (data not shown). The loading buffer used was 50mMol MES pH 6.7 and elution buffer used was 50mMol HEPES pH 8.2, 1M NaCl. The elution pH is the calculated pI of the protein.

Figure 32 demonstrates successful small scale purification of melC. As can be seen, the protein appears to be extremely pure after the ion exchange step, and this result precipitated a further study using a greater amount of protein. However, during the second run, and subsequent runs it can be observed that a second band purifies with the protein melC as well. It is believed that this is a breakdown product of the protein and demonstrates questions marks over the proteins stability, and therefore whether this is a suitable candidate.

A larger scale purification run was attempted with the melC demonstrated in figure 33.

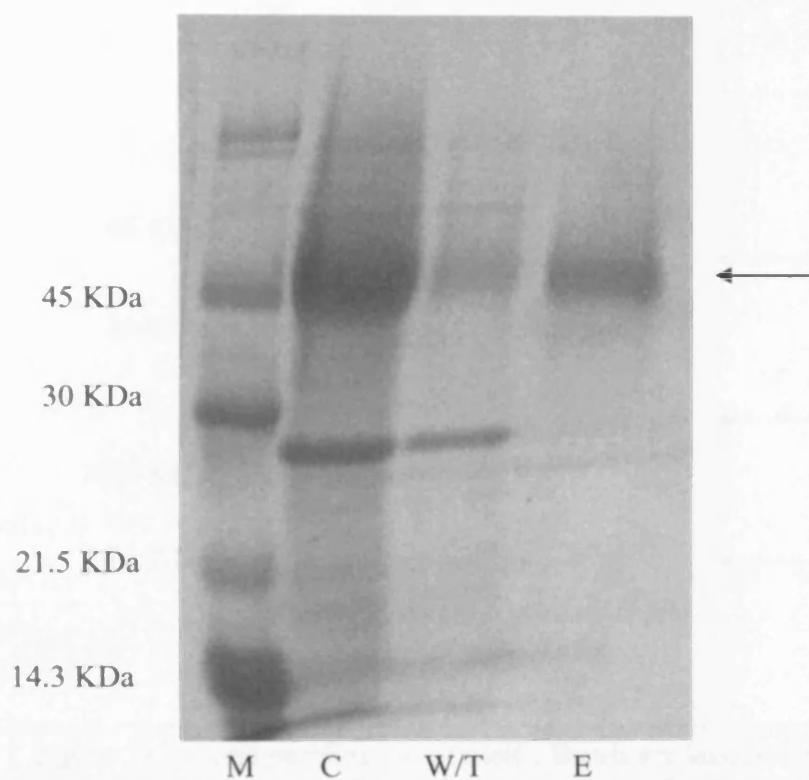


Figure 32. Initial purification of protein melC. The protein can be seen to be successfully purified, the contaminant bands removed in the wash through. The arrow shows the location of the band representing melC.

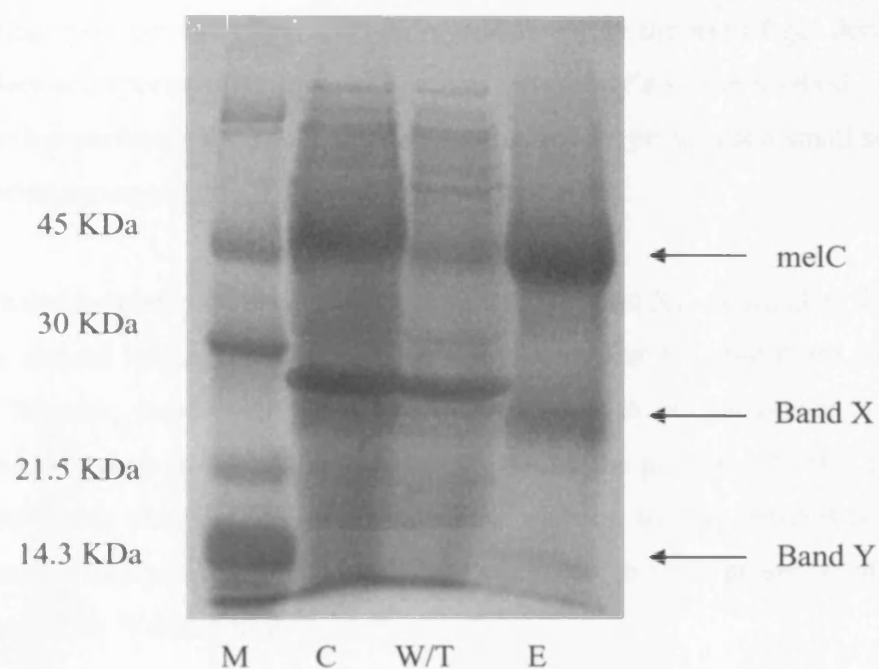


Figure 33 Second purification of melC. Bands are labelled with arrows. The second band can be seen to purify at this stage at approximately 23KDa, the lower band 14.3 KDa

Step	%Purity	Total %Recovery
Process input	6	
After PD 10 column	9	Approx 98%
After mono S	89	Approx 90%

Table 7. Purity and recovery of protein in the lab scale process

Percentage purities and recoveries were calculated by the use of gel densitometry. As such they are approximate, due to inherent variability's in the method. It can be seen that the ion exchange method successfully purifies the protein at a small scale, with high recoveries demonstrating the suitability of this method.

The second band can be seen clearly in figure 33 (band X). A smaller, third band (band Y) can also be seen. The longer the crude is stored at 4°C, the more substantial these bands become, indicating that some factor, likely to be the presence low levels of secreted proteases in the crude mixture degrading the protein. As this protein purifies with the fusion protein it has similar ionic properties, so may perhaps be a fragment of the llama VHH protein. This is further suggested by the presence of llama protein determined by Western blot.

This rapid degradation of the protein, coupled with inherent instability of the production process which could often lead to low quantities of product (due to cleavage of the protein during fermentation) or inactive product mean that further research on melC was stopped. The contract manufacturer attempted to correct this problem by insertion of a shorter linker to add stability, and this gave the protein melK, that could be produced easily at high concentrations and appeared relatively stable during fermentation. Although initial studies have shown it is not as active as protein melC, it is the most suitable candidate, offering a compromise between stability and function.

As such it was necessary to develop the process further for protein melK and also another protein of interest melJ. As discussed previously protein melJ is similar to melC, containing a different VHH antibody clone, but identical structure and CBDs. An initial study was performed with the method developed for melC due to the similarity of the proteins structure and their similar pI's. The results of that study can be seen in figure 34.

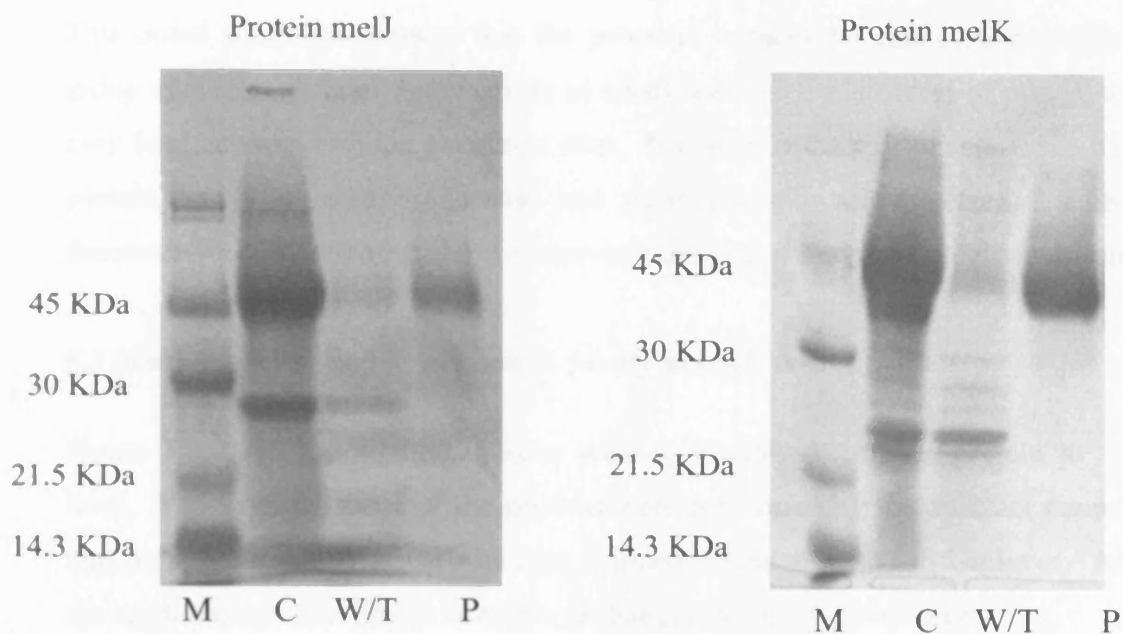


Figure 34: Scouting purification of proteins melJ and melK. As can be seen both are purified successfully to similar levels of purity to that of melC. C: crude, W/T: wash/through, P: Purified.

This initial study has showed that the potential remains for this system to purify this group of fusion proteins successfully at small scale, to a high level of purity, with one easy (and economical) ion exchange step. It is then necessary to select one candidate protein for pilot scale production and purification to allow accurate studies into functionality and determine the commercial potential of the particle delivery system.

5.3 Scale up of developed process to purify gram-levels of protein

Figure 35 shows the method used to scale up purification of the protein to the gram level. The first two steps of the process were performed by the contract manufacturer that produces the protein. It was then shipped frozen at -20°C to Unilever. After this the crude supernatant filtrate is buffer exchanged before ion exchange.

This is a simple and easily up-scaleable approach. Fig 36 shows a gel of each of the purification steps involved in the process stained with Coomassie blue. The total protein content of each well is identical (determined through BCA assay). As can be seen the protein is purified to a high level by the ion exchange step, demonstrating successful scale up from the small-scale system. The major contaminants can also be seen to be removed by the ion exchange step and were present in the wash through.

It can be shown that the buffer exchange steps do not affect the purity visible in the 5-50kDa range. In order to demonstrate the presence of minor contaminants, a more sensitive stain was also employed. Silver stain is not quantifiable like coomassie blue, but is much more sensitive (Fig 37).

The silver stained gel clearly shows more contaminating bands in the crude mixture than the gel stained with Coomassie, demonstrating its sensitivity. It is noticeable that these bands were not present in the purified protein mix, showing that the system is removing the majority of both major and minor contaminating proteins. The Western blot is used to determine the presence of llama proteins. It shows that contaminating proteins were not of llama origin. Although it is demonstrates presence of llama proteins in all wells, it is very sensitive and also not quantifiable.

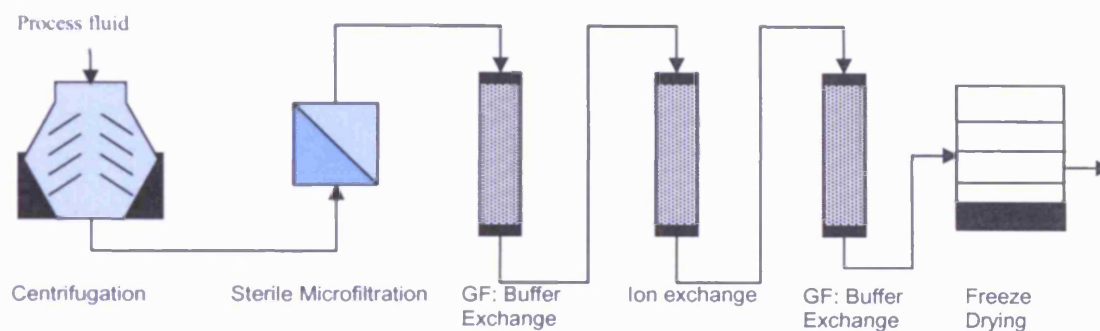


Figure 35 Flow Diagram for scaled up purification of protein melK.

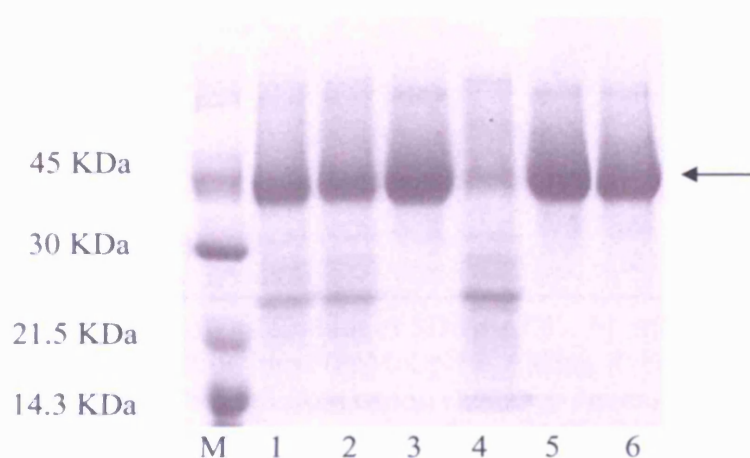


Figure 36: SDS-PAGE stained with Coomassie Blue. M: marker, 1: Crude fusion protein, 2: Protein after desalting into 50mMol pH 6.7 MES, 3: Protein eluted from Cation exchange column, 4: Wash-through from cation exchange column, 5 Protein after desalting into pH 7.15 50mMol PBS, 6 Protein after freeze-drying (re-suspended). The bulk of the protein of choice can be seen to be eluted in the cation exchange step to a very pure level. Likewise the contaminant proteins can be seen to be removed in the wash.

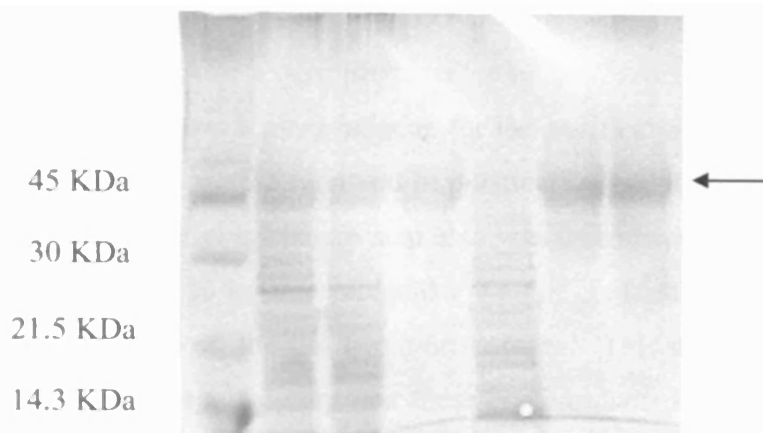


Figure 37: SDS-PAGE stained with Silver stain. M: marker, 1: Crude fusion protein, 2: Protein after desalting into 50mMol pH 6.7 MES, 3: Protein eluted from Cation exchange column, 4: Wash-through from cation exchange column, 5 Protein after desalting into pH 7.15 50mMol PBS, 6 Protein after freeze-drying (re-suspended). The higher sensitivity of the silver stain shows no additional bands in the eluted section. The wash through sample shows many contaminating bands.



Figure 38: Western blot of SDS-PAGE. M: marker, 1: Crude fusion protein, 2: Protein after desalting into 50mMol pH 6.7 MES, 3: Protein eluted from Cation exchange column, 4: Wash-through from cation exchange column, 5 Protein after desalting into pH 7.15 50mMol PBS, 6 Protein after freeze-drying (re-suspended). The band is labelled initially with a rabbit anti-llama polyclonal and then a goat anti-rabbit alkaline phosphatase conjugate. The colour is developed by addition of BCIP-NP. The band can be seen to be present in all samples, but seemingly more weakly in the wash through stage of ion exchange.

Table 8 shows a mass balance for the purification process. This has clearly shown the bulk of the protein removed in purification is during the cation exchange step, although the first buffer exchange step also removes some contaminating proteins as well. This is believed to be low molecular weight contaminants that elute very slowly and were left behind on the gel filtration column. This effect is not present in the second gel filtration step, showing that these contaminants have been removed by this stage. It also shows, as expected, that freeze drying has no effect on the concentration of the protein present.

Figure 39 demonstrates the successful removal of total protein across the process and subsequent increase in percentage cellulose binding activity. Total protein was calculated using a BCA assay as described above and the total protein added to each step calculated. It can be seen that approximately 20% of the original total protein content is removed in the first buffer exchange. It is believed that this reduction is due to the long retention time of small proteins/protein fragments in the buffer exchange column, and their subsequent removal from the process. A further 50% of the total protein is removed in the ion exchange step, and can be seen to wash through the column (data not shown). Activity is increased by approximately 40%.

A small amount of protein is lost in the final desalting column, but it is only 2-3 percent, this is acceptable for the column, and no protein is lost in the freeze drying step. The decrease in total percentage protein is followed by a close negative correlation with the percentage binding to avicel, representing percentage purity.

Over the whole process the percentage binding to avicel increases by approximately 50%, while the percentage protein is reduced by 70%. This difference could be due to the percentage cellulose binding being approximate, as it is only measured as a percentage reduction in absorbance at 280nm, or the presence of another cellulose binding protein in the crude mix, that skews the assay.

	Protein in (mg)	Protein Eluted (mg)	Protein waste (mg)	Total protein out
Desalting 1	7470	6025.8	46.7	6072.5
Cation exchange	5386.7	2337.1	2637.7	4974.8
Desalting 2	1926	1784.22	0	1784.22
Freeze Drying	1702.5	1700	0	1700

Table 8: Mass Balance of total protein through out the developed process

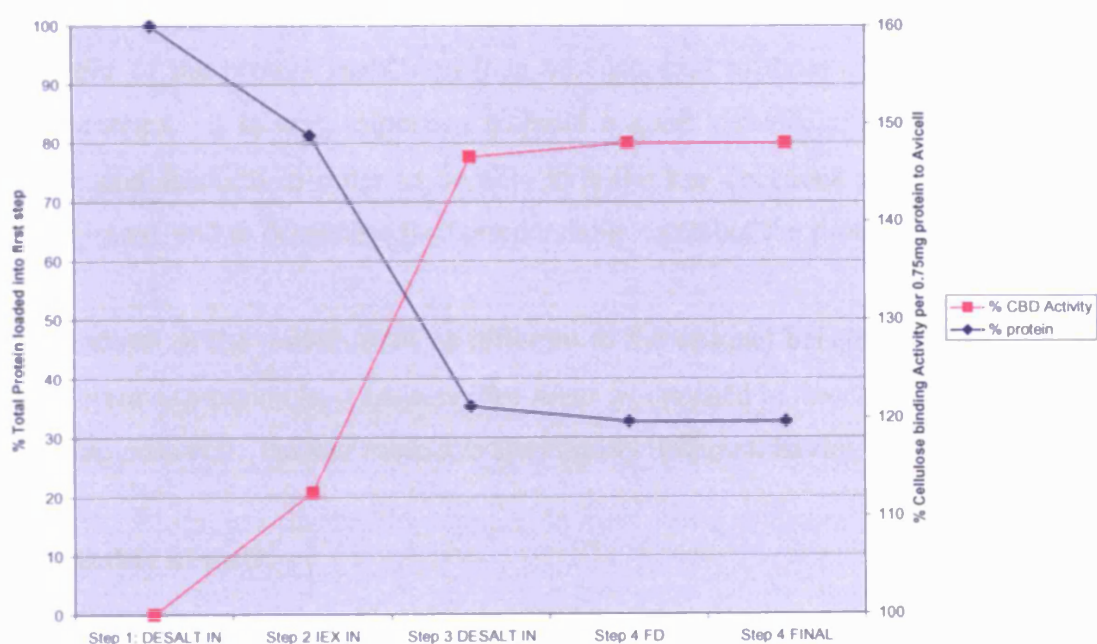


Figure 39: Percentage decrease in total protein as detected by BCA assay versus percentage increase in cellulose binding to Avicell. It can be seen that the ion exchange step decreases the total protein by approximately 50%, whilst increasing cellulose binding activity by about 40%.

Alternatively, it could just be due to non-binding of the protein to the ion exchange matrix, which would correlate with an approximate recovery of 80%. This indicates an increase in percentage specific activity, and quantifiably demonstrates purification of the protein.

The purification of the protein melK to the gram level provided suitable quantities for detailed analysis. As mentioned previously the selection of MelK is a compromise between functionality and ease of production and purification. The process developed proved very successful in purifying the protein to a high level of purification.

The next step is to determine certain properties of the protein – such as stability in solution, binding to melamine and cellulose, and the bifunctionality of the protein. The properties of the protein melK can then be compared to those of the benchmark lab scale proteins. It is very important to build a good knowledge base of this proteins activity and function in order to be able to make key decisions at a later stage in its development, and to determine the commercial potential of the protein.

The function of the protein may be different to the original benchmark proteins due to its different expression host (*Aspergillus niger* as opposed to *Saccharomyces cerevisiae* or *Pichia pastoris*). Further more it is structurally different, having two VHH heads.

5.4 Stability of melK

The overall protein stability is very important in both product formulation and shelf life. Whilst freeze-drying has proved very successful at preserving both crude and pure proteins for a very long time, it is very expensive on a large scale. As such, it is likely that in final product formulation, the protein will be stored in an alternative manner. An important consideration then is how stable the purified protein is over the crude in a liquid.

As can be seen from figure 40 the purified version of the protein offers considerable stability benefits over the crude in the conditions shown. The crude protein can be seen to breakdown rapidly at 37°C, and needs substantially less and less dilutions to make the protein visible on the gel.

The pure protein stays intact across the 31 days at 37°C demonstrating the advanced stability of the llama fusion protein. This is very important in terms of final product formulation, demonstrating that the protein has the robustness to be suitable commercially.

As can be seen from Lane A, the fusion protein that has been stored at 4°C for three weeks prior to this study is already demonstrating signs of breakdown, with three bands visible around the 45KDa region. It is believed that these were initial breakdown products of the fusion protein.

As the time course continues, the other crude protein samples (b-c) begin to show degradation products by 2 days (40x dilution), noticeable as a darkening under the 45KDa region. By 7 days, the fusion protein in crude form is not visible at all at 20x dilution, and by 14 days is not visible at 1x dilution. This corresponds with a denser band at around 20 KDa, which may be due to the higher concentration used and consequently does not get broken down, or is an advanced breakdown product of the fusion protein.

Protein F (crude batch 2 of melK) shows a similar degradation to that of crude batch 3 – although it is noticeably a lot less pure than that of batch 3. It is interesting that all proteins in protein F show obvious degradation. This is less noticeable in the crude form of batch 3 (proteins a-c), perhaps due to its purer nature.

It is believed that the degradation observed in the crude protein mix is caused by extracellular proteases secreted by the expression host. As the fusion protein is secreted it makes purification easier by meaning that it is not in contact with a large number of intracellular proteins, however it means that the protein will come into contact with these extracellular proteases.

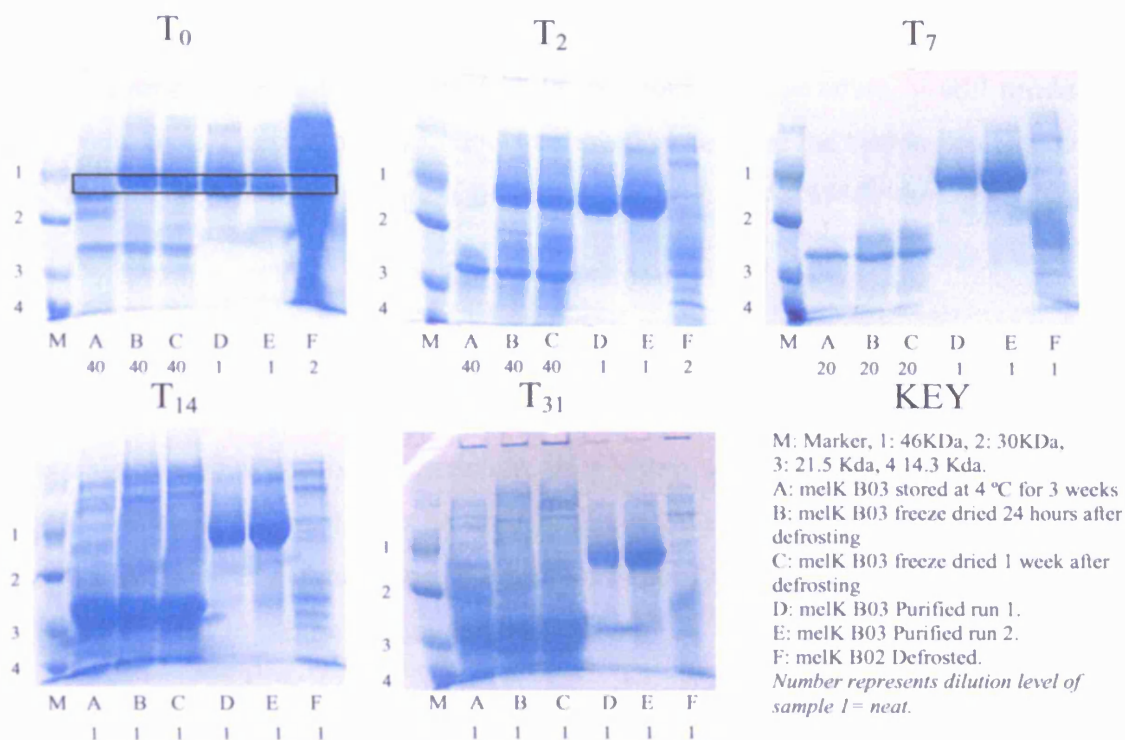


Fig 40 Stability of fusion protein melK crude versus pure as demonstrated by SDS-PAGE. The fusion is highlighted by the box on the first gel. Time is represented in days at 37°C.

Aspergillus species are known to secrete a number of extracellular proteases – although the strain used in the fermentation of melK is a low protease strain, it still produces a low level. Over a period of time, this is believed to degrade the fusion protein, even at 4°C, as observed by the condition of protein A at T_0 . At 37°C, this degradation is accelerated.

The pure protein contains either no protease, or protease at a substantially lower level. This makes the pure protein much more stable and resistant to degradation. This would be essential for final product formulation, as it is impractical for the protein to be stored frozen or even at 4°C if it is to be used in the home and personal care industry. This also means that it is essential that the protein is purified as rapidly after production as possible, in order to minimise loss in yield.

The stability of the llama antibody based protein at 37°C is also observed in a bifunctional llama antibody construct produced for research by Conrath et al. (2001). The protein is seen to be stable for over 72 hours at 37°C in either a PBS or mouse plasma preparation. However it is shown to have lost approximately 20% in that time, suggesting that the protein melK is substantially more stable.

5.5 Binding of the pure protein to crystalline cellulose

Fusion protein binding to crystalline cellulose has been investigated, as demonstrated in figure 41. The newly purified melK was compared at three different levels of cellulose (avicell) suspended in PBS buffer– 2.5% w/v, 7.5% w/v and 10%. As is expected, the 10% w/v avicell showed higher binding than the 7.5%, and the 7.5% higher than the 2.5 %.

All graphs were fitted with a Weibull 5 parameter curve as detailed in the computer program Sigmaplot. The R^2 value of the 2.5 % curve is low (around .8), perhaps as the majority of the reaction appears finished even at the lowest fusion level, and any added increase in fusion has no further effect. Consequently this curve was not used any further.

The other two R^2 values were high (0.99) showing the goodness of fit of the curve. It is interesting that the curve fitted is sigmoidal, and identical to that fitted for the protein melA to melamine. This suggests that they share similar binding characteristics, even though the substrates and binding domains were different.

At 10% w/v avicell, the maximum binding protein per ml using the derived equation is 0.76 and at 7.5 % avicell it is 0.56. Using these two numbers it is possible to derive a maximum binding of 7.6 and 7.4 mg fusion protein per gram of cellulose respectively. This gives an average of 7.5 mg fusion protein per gram of Avicell. This is a very high level compared to that derived previously for the protein melA to melamine (0.948mg per gram of capsule), nearly 8 times higher.

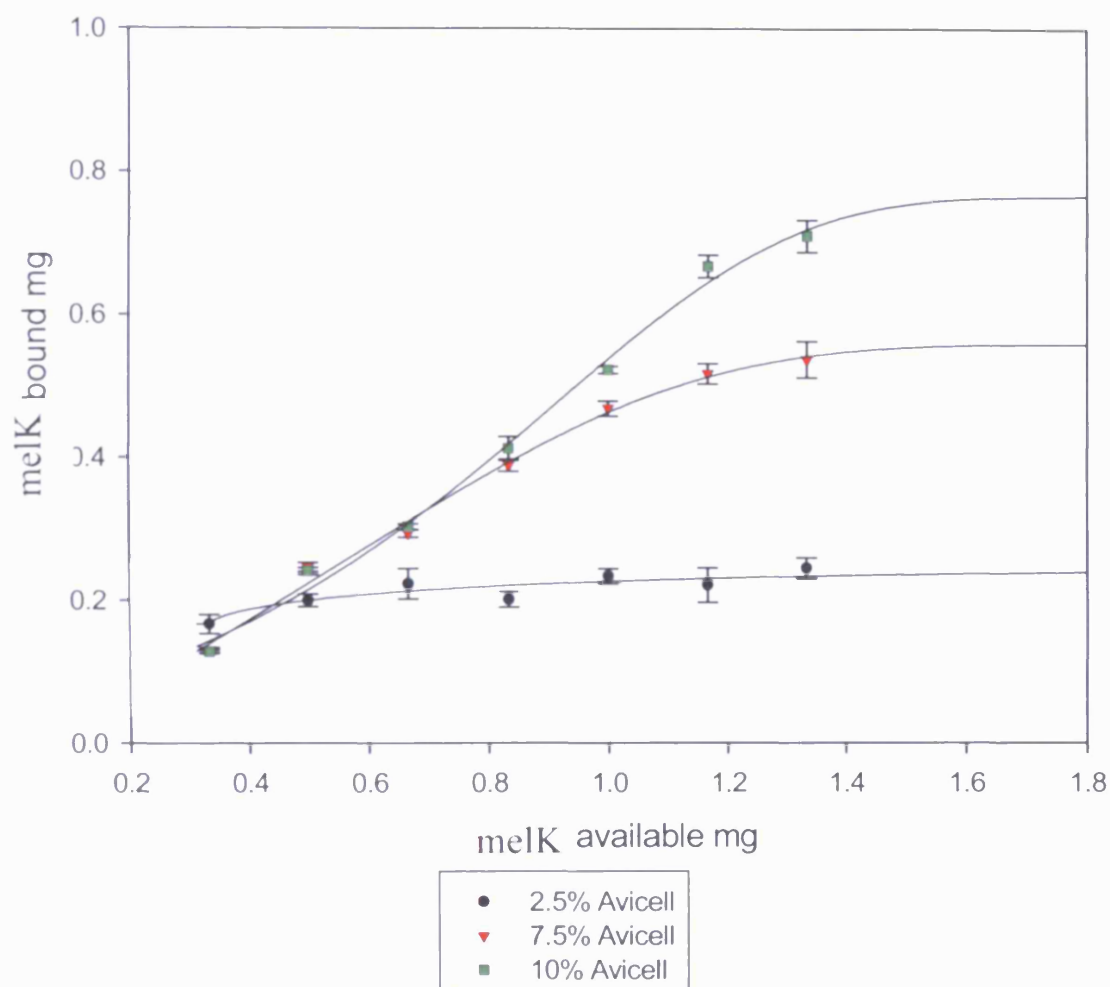


Figure 41. Binding of fusion protein MelK to different quantities of crystalline cellulose. The data is fitted with a Weibull 5-parameter sigmoidal line of best fit.

5.6 Binding of the fusion protein to melamine

In order to derive the binding of the protein to melamine capsules, it was essential that they pellet strongly upon centrifugation. The density of melamine is approximately 1.5 so they should pellet; however due to the encapsulated active volatile ingredients being in a carrier oil, the density of the capsules varies greatly.

It can be observed upon centrifugation that three fractions of capsules form: those that pellet, those that stay in suspension and those that float. It was postulated therefore that those that pelleted have lost the majority of their encapsulated material (it is obviously of no use to have capsules so strong that they do not release their ingredients). Those that were in suspension have some of their encapsulated actives present, and those that float were high in encapsulated actives. It is also possible that the amount of material encapsulated varies capsule to capsule on formulation.

It is known that the use of certain solvents destroys the capsule integrity and is capable of removing the encapsulated material (internal Unilever data), indeed such a method is used to quantify the amount of encapsulate present. Consequently it was decided to employ a solvent washing step to remove the capsule contents and attempt to make the capsules pellet – if the hypothesis was correct, then this should happen.

Capsules were washed repeatedly with Acetone and spun down. It was observed by the third acetone wash that all capsules pelleted, and they were then washed repeatedly with PBS to remove all traces of the solvent.

Fig 42 demonstrates the binding of the protein MelK to the melamine capsule. It is noticeable that more capsules were required (25%) to produce a similar response to that noted in avicell (10%). This demonstrates a lower level of binding to the melamine capsule surface than to that of cellulose, likely due to a higher number of binding sites available on the cellulose surface.

The pure protein has shown positive binding to melamine and cellulose, and hence a bifunctionality assay was performed comparing it to other proteins.

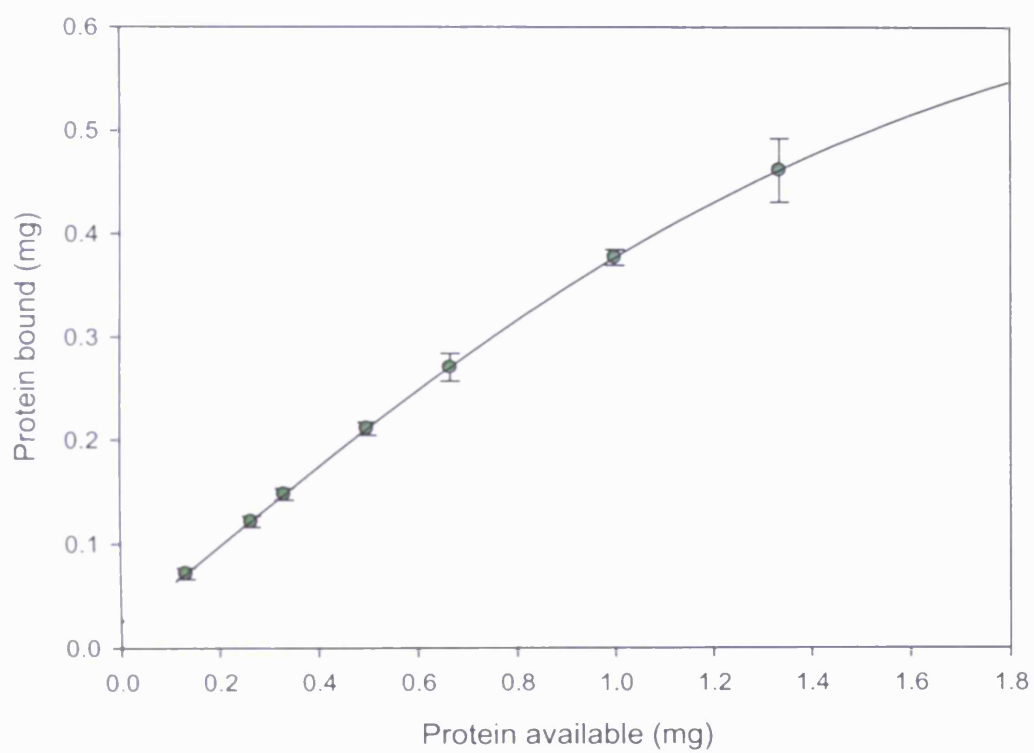


Figure 42: Binding of the protein melK to melamine particle surface.

5.7 Bifunctionality of purified melK compared to that of other mel fusion proteins

The bifunctionality of purified melK protein is demonstrated as comparable to that of the other candidate proteins in figure 42. The protein demonstrates an increase in binding equivalent to that of a 100 fold increase in particles alone.

In this study the ratio of fusion protein to particle was kept at a constant level. The amount of particle/fusion protein mix was then adjusted to a suitable level to conduct particle counts. The control (particle only) showed no particle deposition at the level at which the particle and fusion protein test demonstrated deposition (0.02% v/v capsules). Consequently, higher levels of the control were selected (0.2 and 2% particles) in order to determine a level of control that gave equivalence of bifunctionality to that of the test mix.

Although this assay shows relatively high variability as demonstrated by the error bars, there were still some significant differences. The two fusion proteins melA and melC demonstrate similar bifunctionality at a high level, and then the proteins melB and melK, and then the protein melJ.

As discussed previously, the proteins melA and melC demonstrate superior bifunctionality to that of melK. However melK was selected as a compromise between functionality and ease of production. As can be seen the protein melJ demonstrates inferior bifunctionality to melK confirming melK as the lead candidate.

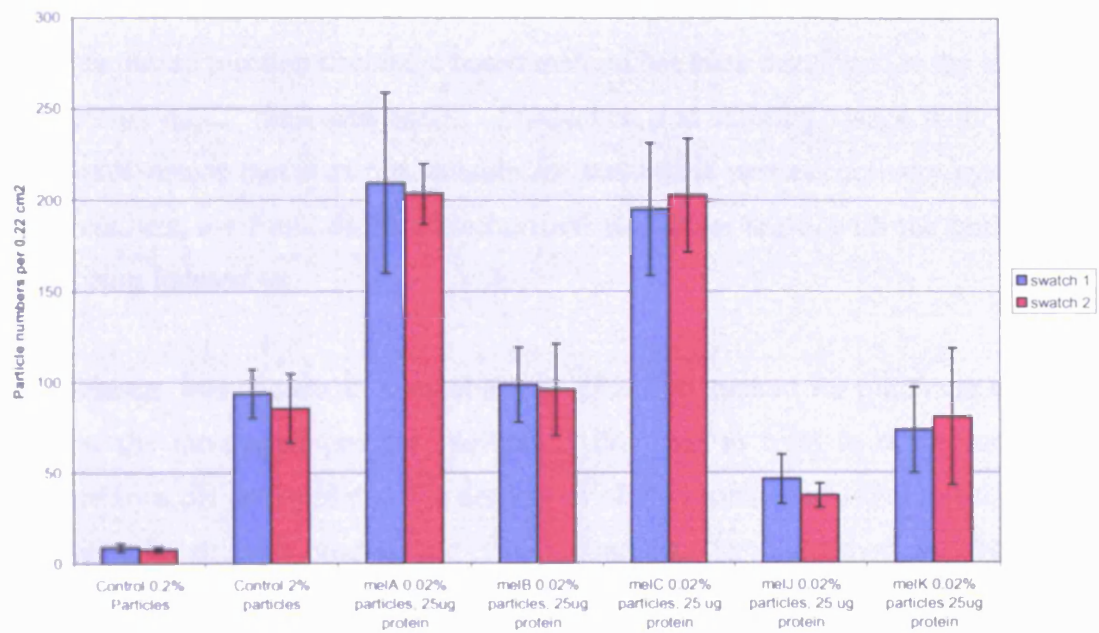


Figure 43: Bifunctionality of fusion proteins

5.8 Summary

A simple one step cation exchange based method has been developed at the lab scale for the proteins melC, melJ and melK. Production and stability issues with the protein melC have meant that it is not suitable for use in the particle delivery system. Two close relatives, melJ and melK were purified at a larger scale, with the purification of melK being focused on.

Ion exchange was chosen as a suitable cost effective method for purifying the protein, however the developed process required a low pH to bind to the column matrix, followed by a pH equivalent to the derived pI of the protein and 1Mol salt to remove it. This was a far stronger binding and elution condition than published proteins; however it appeared to give very high purity.

The fusion protein melK was purified to a high level of purity with good recoveries, and then freeze dried prior to further use. Stability of the fusion protein was assayed using a SDS-PAGE method to visualize break-down products of the protein. Binding of the protein to cellulose and melamine particles was also assayed prior to use of the protein in a bifunctionality deposition study.

6.0 Conclusions

6.1 Investigation and characterisation of the function of llama antibody fusion proteins and potential for use in a novel micro-particle delivery system

A model system based on a llama antibody fragment showing a binding moiety to the azo-dye reactive red 6 has been developed and fused to a cellulose binding domain from *Trichoderma reesei*. The objective was to study this protein and determine its capability for use to deliver micro-particles to cellulosic surfaces.

A number of different formats of the fusion protein have been researched using ELISA based assays and surface Plasmon resonance (BIAcore) to determine functionality towards the azo-dye. Although ELISA data was interesting, variability proved too high to conclusively determine binding affinities. BIAcore data however allowed binding affinities to be calculated and these tallied with published data, in the low nMol region.

This research demonstrated that all proteins could successfully bind the azo-dye RR6, however the presence of an additional antibody fragment conferred an extra stability when the protein was binding in the presence of some commercial detergent components. Binding affinities were calculated, and the presence of the CBD shown to have little effect on binding to RR6.

Increased deposition of particles to cellulose surfaces was demonstrated using a particle deposition assay, determining bifunctionality of the protein. Use of bifunctionality assays shows that binding is enhanced in the presence of the fusion protein when the particles have been pre-incubated with the fusion protein prior to addition to cellulosic substrates. The fusion protein containing 2 CBDs appears to convey the best bifunctionality.

Issues with the particles source and release profile meant that this system was only suitable as a model system for study, however the research supported the further development of a potential commercial system utilising a more suitable melamine based particle.

6.2 Initial Characterisation of the Melamine Particle System and selection of suitable candidate proteins

A protein raised to the surface of melamine particles was created for study. This protein, known as melA is a proof of principle protein produced at a lab scale. The melamine particles show the stability and release profile suitable for cellulose binding applications.

Initial characterisation of the fusion protein binding to the particle surface has been studied using a gel densitometry based method. This allowed calculations to be done as to the possible loading of the protein, and provided data suitable for use in cost feasibility studies.

The protein has been proven able to bind successfully to the particle surface in the presence of certain detergent bases demonstrating commercial relevance. Little cross reactivity with co-acervate particles has been observed, as expected due to the specific nature of the binding of the protein.

Further studies on the binding of the protein to the cotton surfaces in the presence of different detergents suggest suitable detergent bases for the proteins successful operation. Visual demonstration of the protein binding to both melamine and cotton surface is performed using a fluorescent fusion protein. Some binding of the protein is confirmed with Nylon and Polyester.

The success observed with the protein melA led to the creation of a number of commercial candidates from commercial protein suppliers. These have been screened for suitability, giving rise to a number of potential candidates comprising a similar structure – two CBD's and 1 VHH. The suitable commercial candidates (melC, melJ and melK) required further purification prior to use.

6.3 Purification and analysis of suitable protein candidates

Due to the impure nature of the candidate proteins produced by the commercial manufacturers it was necessary to purify selected candidates for storage and further analysis. A cation exchange chromatography based method was developed which successfully purified the protein melC to a high level of purity.

This method utilised an optimised binding condition for the protein to the chromatography matrix, and elution conditions utilising a pH shift and high salt. The fusion protein melC was however proved to be unsuitable for further development due to production issues. As such, the method was applied to proteins melJ and melK.

Both fusion proteins were successfully purified at microgram scale prior to scale up to the gram scale. The method developed used only one ion exchange step, and consequently is substantially more cost effective than other antibody purification techniques such as those used in the pharmaceutical industry.

Due to the apparent generic nature of the developed process and its simplicity it shows great promise. The production of gram levels of protein also allowed a more detailed analysis of the process. Fusion protein was followed through the process, and after ion exchange, the majority of contaminant proteins were observed in the wash through – down to silver stain levels of detection.

The purified protein was then studied for stability using a gel densitometry based method to look for degradation of the fusion protein after timed incubations at 37°C. Pure fusion protein was shown to be stable after 31 days at 37°C, where the crude was seen to have totally degraded after 7 days, and partially after just 2 days. This demonstrated the potential of the protein to be stored as a product without the need for expensive freeze-drying.

Due to the higher levels of pure protein available, it was possible to use an assay that required more protein allowing a detailed study of the protein binding to crystalline cellulose and melamine capsule materials. A binding curve of the protein was generated for 3 different levels of cellulose demonstrating a good relationship between cellulose available for binding, and total protein bound.

Binding of the protein to melamine was studied and the binding curve shown. The bifunctionality of the pure protein was then shown and compared to that of the other crude candidate proteins. It was shown to be an effective aid to deposition of the particle to cellulosic surfaces.

6.4 Future work

The work done in this project has focused on the development of the protein for use in the particle delivery system. As such it is possible to perform more detailed analysis in the area of the protein characterisation of both the RR6 base protein and the melamine based protein. Studies into the structure of the proteins utilising techniques such as x-ray crystallography would be beneficial.

Further work into ideal binding conditions would also be beneficial. There are two areas which can be further optimised: the ratio and conditions of binding of the protein to the capsule and the ratio and conditions of binding of the activated capsules to the cellulosic surface. The type of cellulosic surface can also be researched, as applications can be envisaged in other areas such as paper manufacture, wood treatment and the agrochemical industry. It is also worth studying other similar cellulosic surfaces such as chitin.

Further work in the area of purification should focus on the use of new matrices that can handle salt level such as the Capto MMC matrix from Amersham and also the removal of the two buffer exchange chromatography steps from the process. This could either be done with ultrafiltration or by loading fermentation crude onto the matrix after primary clarification, and by adapting the elution buffer to the final desired conditions.

Further research into the area of particle surface chemistry should also be performed. The fusion protein has been selected according to ability to bind the particle, however it may be possible to further increase binding, and subsequent bifunctionality by comparison and selection of new melamine particles.

Pilot studies using initially scaled down washing machines and appropriate conditions, followed by full scale washing machine trials to determine efficacy of the system under wash conditions including water hardness and detergent type will also be performed. If

this is successful, storage and consumer trials will be undertaken to determine perceived benefit, prior to any consideration of formulation into a product.

Development of the system for other applications also has good potential. The concept of using these fusion proteins to deliver capsules has been demonstrated in this thesis, and a logical progression would be transfer of the system for use with other cellulosic surfaces: such as the delivery of particles to paper for the diagnostics industry, delivery of encapsulated actives to plants and untreated wood to prevent pest infestation.

6.5 Summary

A novel llama fusion protein has been studied and used successfully in a system to deliver particles to cellulosic surfaces. Proof of principle was demonstrated with a model system and then further in a system designed to have commercial relevance. Due to the success of the protein in the commercially relevant system a range of proteins were produced which were screened for bifunctionality prior to development of a one-step ion-exchange based purification system for the lead candidates. The pure protein was then assayed for stability and functionality to the capsule surface and crystalline cellulose, and also bifunctionality.

7.0 Bioprocess project management and validation of bioprocesses at Unilever

7.1 Bioprocessing at Unilever

Unilever is a diverse company featuring many arms which have products in the following areas: home and personal care, frozen foods and ice cream, teas and other beverages and also processed foods. As such its research needs are constantly changing and adapting to meet the market requirements in specific areas.

Historically the majority of Unilevers research in the home and personal care area has focussed on chemical formulation; indeed they used to own chemical industries now part of ICI. Use of biotechnology was limited to enzyme technology in the laundry detergent industry.

Although a great deal of expertise has been developed in the area of laundry enzyme production, Unilever therefore has limited experience of developing biotechnology products and in particular their production.

With the pressures of a competitive market comes the need to adapt, and Unilever is embracing the opportunities biotechnology has to offer. This has required a paradigm shift in the core thinking of Unilever: it has to think increasingly like a pharmaceutical company in respect to its product development. However it cannot afford some of the luxuries that pharmaceutical companies have in regards to bioprocessing.

The use of biotechnology brings new challenges in all areas of research, and process to product must be considered and acted upon from the earliest stages of development. As such the route from research candidate to product must be managed carefully.

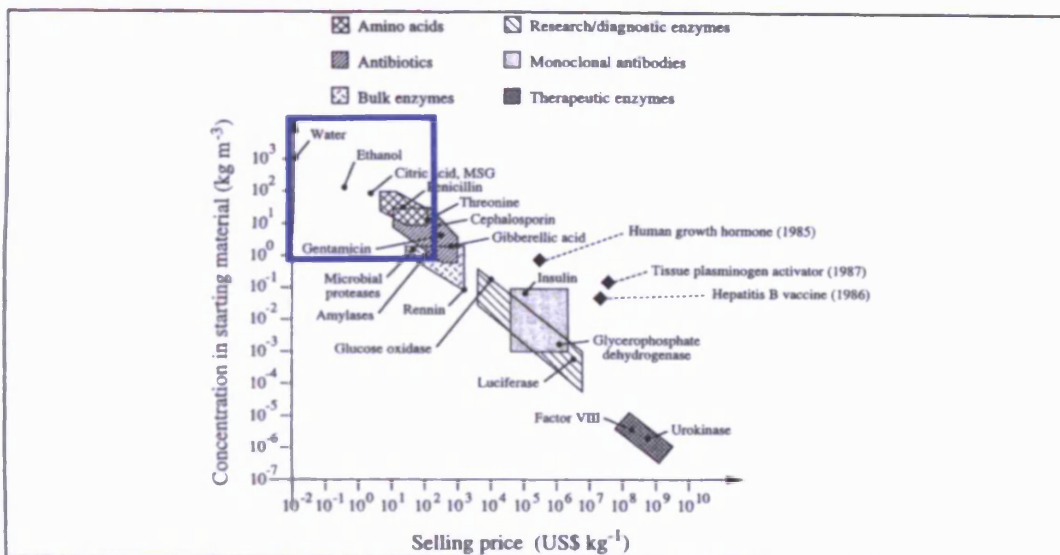


Figure 44: Yield of fermentation products compared to selling cost. As can be seen as yield decreases, selling price increases. Unilever must operate in the area demonstrated by the blue box, hence high yield, low cost bio-processes are necessary.

Whilst pharmaceutical companies can operate in a low yield – high cost environment though it is the opposite for Unilever, as demonstrated in figure 43, the selling cost of Unilevers products can not approach that of pharmaceutical companies such as Pfizer or Merck. Products must be delivered cheaply and in large quantities. However this can be partly achieved due to the lower specification of product; for example purity requirements are often much lower.

Biotechnology production options are also limited in Unilever. Manufacturing capabilities focus on the processing of food products and the formulation of chemicals. Fermentation capabilities are limited, and hence sourcing products from contract manufacturers is often the method of choice.

Use of contract manufacturers allows Unilevers research and development group to focus on its area of expertise, namely production of small scale candidates using molecular biology, and allow scale-up development to be performed by the manufacturer. The early aim is simply a production of a low-level of protein for analysis and proof of principle experiments and then passing of the technology to the manufacturer who is then under pressure to produce the protein as rapidly as possible in order to maximize profit margins.

Production of the antibodies for use in laundry products was at the late developmental stage at the time of completion of this thesis. The development of an *Aspergillus* production system with the contract manufacturer and their expertise in such systems should allow a relatively easy transition to a much larger scale if necessary.

A close liason with the manufacturer is necessary to define acceptable process parameters based on cost and regulation. Regulation for the product is similar to that of laundry enzymes – not nearly as strict as those for that of the pharmaceutical industry, making the cost substantially lower.

7.2 Validation of the antibody purification process

Initial research into the purification of the antibody using ion exchange proved successful. If the process were to be scaled up a number of measures would need to be taken to validate its efficacy, robustness and repeatability. The successful repeated use

of each piece of process equipment also needs to be validated and sufficient cleaning and sanitization processes developed. Removal of contaminants from the process equipment needs to be demonstrated, as indeed does removal of any cleaning chemicals.

As the equipment is already in place in the contract manufacturers, it will already be qualified for use, and capable of being used for generic purification. The system will need to be thoroughly tested with the candidate protein using methods similar to those used in section 5, such as SDS-PAGE (with silver staining sensitivity). Detailed and repeated mass-balances need to be performed to demonstrate that productivity and recovery is not lost over time. Studies into suitable lifetimes for all perishable equipment need to be determined such as filter and chromatography matrix usage.

The contract manufacturer will have many protocols in place and standard operating procedures for validations of their processes, it is just a case of adapting them to suit the antibody product. This will involve supply of suitable assays in order to monitor protein concentration etc.

Once a suitable validation method is in place the process can be tightly monitored and maintained.

7.3 Conclusion

The structured path and transition of early development at Unilever research and development to scale up and production at a contract manufacturer is the most suitable method for production of a biotechnology protein product. The contract manufacturers' expertise and plant capabilities in bio-processing and validation of bioprocesses is essential for production; whilst the ability to retain ownership of the science behind the product is essential to Unilevers interests.

Abbreviations

Ab	Antibody
AFM	Atomic force microscopy
BSA	Bovine serum albumin
CBD	Cellulose binding domain
CDR	Complementary determining regions
ELISA	Enzyme linker immuno sorbent assay
ER	Endoplasmic reticulum
GRAS	Generally regarded as safe
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
IMAC	Immobilised metal affinity chromatography
ITC	Isothermal titration calorimetry
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
RIA	Radio-immunoassay
RU	Response unit
RR6	reactive red 6
SDS-PAGE	SDS poly-acrylamide gel electrophoresis
SPR	Surface Plasmon resonance
STPP	Sodium tri-poly phosphate
UV	Ultra-violet

Appendix 1: Production of particles

Particles were examined at two stages in their manufacture: After initial production and after hardening.

As can be seen from the photographs, the fluorescence is concentrated in the small globules inside the particles after initial production. The globules consist of sunflower oil that is used as a carrier base for the benefit agent. The particles at this stage are quite soft and susceptible to degradation. Detrimental osmotic conditions in particular may lead to lysis of particles.

As can be seen from the above pictures the hardening process has the effect of spreading the fluorescence labeling (Nile Blue) throughout the particle. The individual globules can still be seen indicating that they are intact, but it is likely that physico-chemical changes have drawn some, or all of the fluorescence out of the oil carrier. Indeed the globules may exhibit no fluorescence and only seem to fluoresce because of the particle casing behind them.

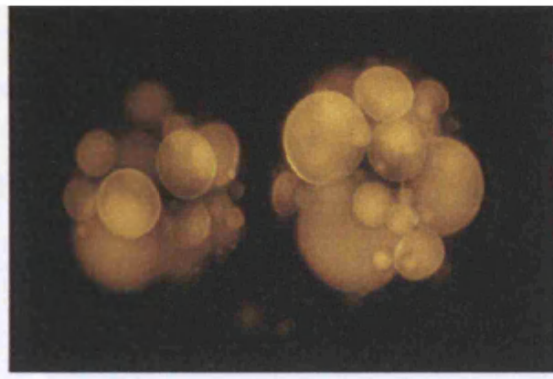
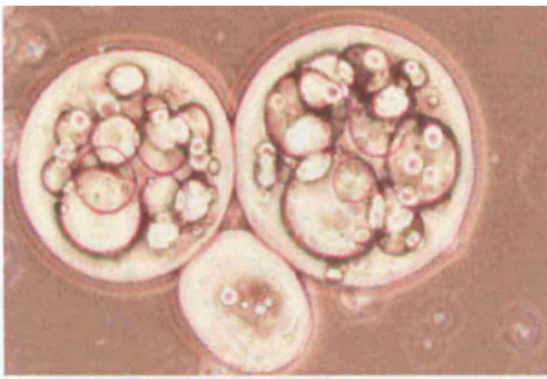


Figure 45: Particles after initial production x200 magnification. Identical pictures, the one on the right showing the Nile Blue labeling.

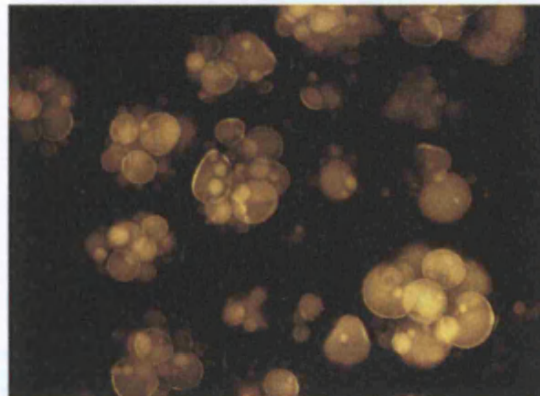
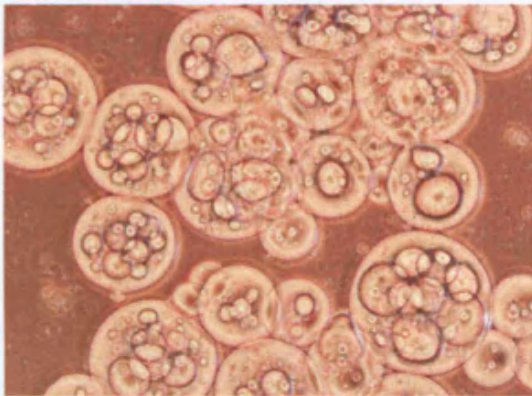


Figure 46: Particles after initial production x100 magnification. Identical pictures, the one on the right demonstrates the fluorescent labeling with Nile Blue.

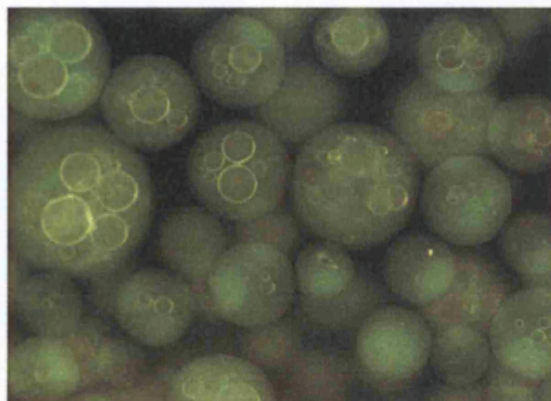
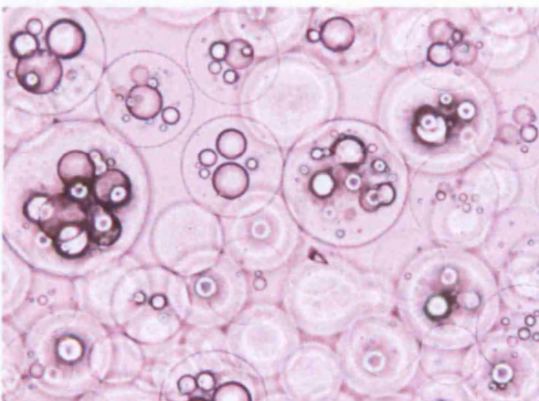


Figure 47: Particles after hardening x100 magnification, picture on the right shows fluorescence with Nile Blue labeling.

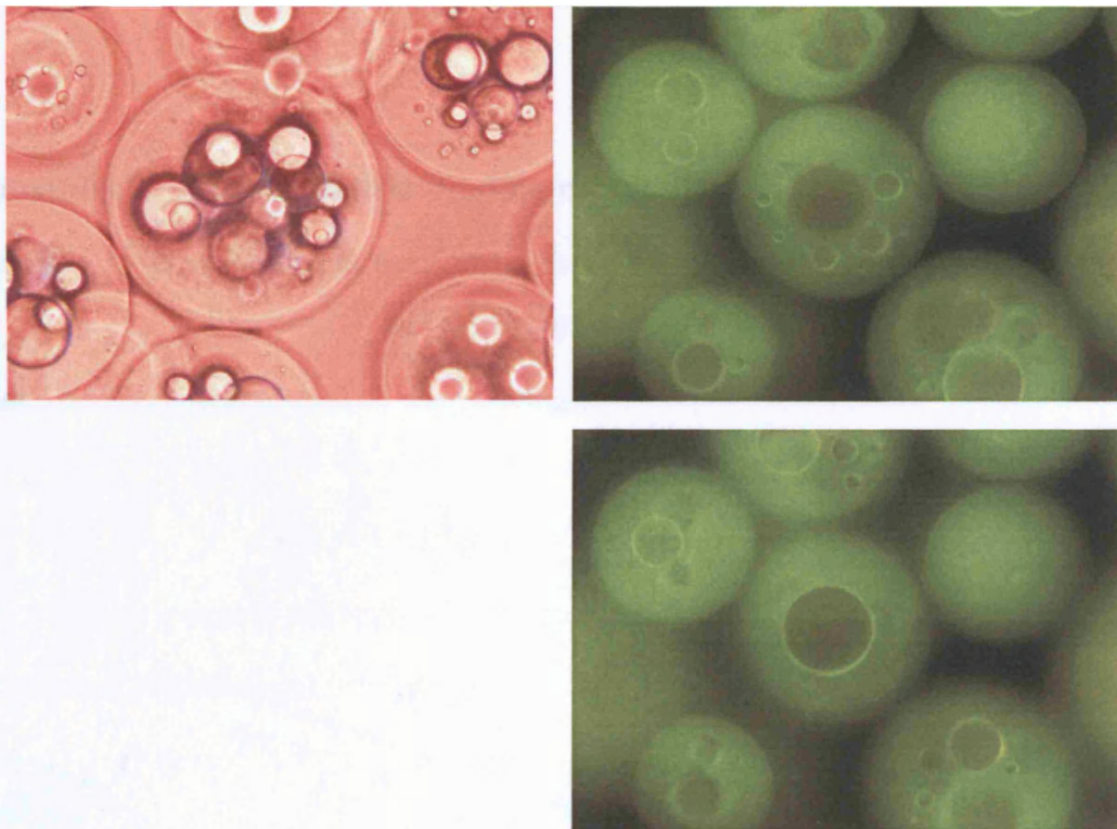


Figure 48: Particles after hardening 20000x magnification, pictures on the right shows fluorescence with Nile Blue labeling at different depths of focus.

Although the

The estimated particles

for 0.2% of particles

calculated as

$$1.1 \times 10^{-10} \times 42.28 \times 10^3 = 4.65 \times 10^{-6} \text{ g}$$

The desired levels of particles are 0.2%, 0.4% and 1.0% in library powder. This requires 0.4%, 0.44% and 2.2% particles respectively, or 1 kg of library powder is 4.7g, 9.3 and 33.5 g particles. Taking 1kg of 0.2% perfumed powder, the number of particles can be calculated from the density

$$4.7 \times 10^{-6} = 3.64 \times 10^{-6}$$

The volume of one particle calculated as a sphere as given by the eqn

$$\frac{4}{3} \pi r^3 = 2.36 \times 10^{-10} \text{ m}^3$$

Therefore the number of particles needed for 0.2% perfume in 1kg of powder is

or

$$2.36 \times 10^{-10} \times 1.1 \times 10^3 = 2.59 \times 10^{-7} \text{ particles per kg powder}$$

Appendix 2: Fusion particle binding calculations

The surface area of one particle is calculated as a sphere of diameter 7.5 μm as:

$$\pi d^2 = 1.77 \times 10^{-6} \text{ cm}^2$$

Therefore in a monolayer of 0.3 $\mu\text{g cm}^{-2}$ there are

$$0.3 \times (1.77 \times 10^{-6}) = 5.31 \times 10^{-7} \text{ } \mu\text{g fusion per particle}$$

$$5.31 \times 10^{-13} \text{ g fusion per particle}$$

The molecular weight of the fusion molecule is 18579 therefore the amount of fusion molecule per particle can be calculated as

$$(5.31 \times 10^{-13}) / 18579 = 2.86 \times 10^{-17} \text{ Moles of fusion per particle}$$

The number of fusion molecules bound to each particles can then be calculated using Avogadros number

$$(2.86 \times 10^{-17}) \times (6.02 \times 10^{23}) = 17205555 \text{ fusion molecules per particle}$$

Although this figure is a maximum and the actual figure may be as 5-10 times less. The melamine particles consist (by weight) of 42.3% “Topnote” perfume of a density 1.0 and 57.7% of melamine polymer with a density of 1.51. The overall density is calculated as

$$(57.7 \times 1.51) + 42.3 \times 1.0 / 100 = 1.29 \text{ g cm}^{-3}$$

The desired levels of perfume are 0.2%, 0.4% and 1.0% in laundry powder. This requires 0.47%, 0.95% and 2.35% particles respectively, in 1kg of laundry powder, 4.7g, 9.5 and 23.5 g particles. Taking 1kg of 0.2%-perfumed powder, the total volume of the particles can be calculated from the density

$$4.7 / 1.29 = 3.643 \text{ cm}^3$$

The volume of one particle calculated as a sphere is given by the equation

$$\frac{4}{3} \pi r^3 = 2.21 \times 10^{-10} \text{ cm}^3$$

Therefore the number of particles needed for 0.2% perfume in 1kg of powder would be

$$3.643 / 2.21 \times 10^{-10} = 1.65 \times 10^{10} \text{ particles per kg powder}$$

The mass of fusion molecules per particle was previously calculated at 5.31×10^{-13} g. Therefore for 1kg powder

$$(1.65 \times 10^{10}) \times (5.31 \times 10^{-13}) = 8.76 \times 10^{-3} \text{ g fusion per kg powder}$$

Percentage perfume	Fusion required per kg powder (mg)
0.2	8.76
0.4	17.7
1.0	43.8

Table 9. Estimated amount of fusion required per kg washing powder according to internal data.

So it is estimated that approximately 10 mg of protein per kg of washing powder at 0.2% loading of perfume is needed. This is comparable to dosing of other proteins such as enzymes. It is important to assess whether this level is achievable, and what percentage of the protein actually binds to the capsule.

References Cited

- Angal, S., and P. Dean, 2001, Purification by exploitation of activity, in S Roe ed., *Protein Purification Techniques*: Oxford University Press, p. 213-240.
- Arbabi, G. M., A. Desmyter, L. Wyns, R. Hamers, and S. Muyldermans, 1997, Selection and identification of single domain antibody fragments from camel heavy-chain antibodies: *FEBS Lett.*, v. 414, no. 3, p. 521-526.
- Badley, A. 1993. Internal Unilever report.
- Baines, D., 2001, Analysis of purity, in S Roe ed., *Protein Purification Techniques*: Oxford University Press, p. 27-50.
- Banerjee, A., N. S. Srilatha, and G. S. Murthy, 2002, Real-time kinetic analysis of hCG-monoclonal antibody interaction using radiolabeled hCG probe: presence of two forms of Ag-mAb complex as revealed by solid phase dissociation studies: *Biochim.Biophys.Acta*, v. 1569, no. 1-3, p. 21-30.
- Boersma, W. J., W. J. Bogaerts, A. T. Bianchi, and E. Claassen, 1992, Adjuvant properties of stable water-in-oil emulsions: evaluation of the experience with Specol. *Res.Immunol.*, v. 143, no. 5, p. 503-512.
- Bonnerjea, J., S. Oh, M. Hoare, and P. Dunnill, 1986, Protein purification: The right step at the right time: *BIO/TECHNOLOGY*, v. 4, p. 954-958.
- Buchner, J., I. Pastan, and U. Brinkmann, 1992, A method for increasing the yield of properly folded recombinant fusion proteins: single-chain immunotoxins from renaturation of bacterial inclusion bodies: *Anal.Biochem.*, v. 205, no. 2, p. 263-270.
- Butler, J. E., L. Ni, R. Nessler, K. S. Joshi, M. Suter, B. Rosenberg, J. Chang, W. R. Brown, and L. A. Cantarero, 1992, The physical and functional behavior of capture antibodies adsorbed on polystyrene: *J.Immunol.Methods*, v. 150, no. 1-2, p. 77-90.
- Carrard, G., A. Koivula, H. Soderlund, and P. Beguin, 2000, Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose: *Proc.Natl.Acad.Sci.U.S.A*, v. 97, no. 19, p. 10342-10347.
- Chadd, H. E., and S. M. Chamow, 2001, Therapeutic antibody expression technology: *Curr.Opin.Biotechnol.*, v. 12, no. 2, p. 188-194.
- Chiruvolu, V., J. Cregg, and M. Meagher, 1997, Recombinant protein production in an alcohol oxidase-defective strain of *Pichia pastoris* in fedbatch fermentations: *Enzyme and Microbial Technology*, v. 21, no. 4, p. 277-283.
- Chu, L., D. Robinson. Industrial Choices for protein production by large scale cell culture. *Current Opinion in Biotechnology* 12, 180-187. 2001.
- Conrath, K. E., M. Lauwereys, M. Galleni, A. Matagne, J. M. Frere, J. Kinne, L. Wyns, and S. Muyldermans, 2001, Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae: *Antimicrob.Agents Chemother.*, v. 45, no. 10, p. 2807-2812.

Cumming, R., and G. Icton, 2001, Cell Disintegration and extraction techniques, in S Roe ed., Protein Purification Techniques: Oxford University Press, p. 83-111.

Curvers, S., P. Brixius, T. Klauser, J. Thommes, D. Weuster-Botz, R. Takors, C. Wandrey. Human Chymotrypsinogen B production with *Pichia pastoris* by Integrated Development of Fermentation and Downstream Processing. Part 1. Fermentation. Biotechnology Progress 17, 495-502. 2001.

Cutler, P., 2001, Chromatography on the basis of size, in S Roe ed., Protein Purification Techniques: Oxford University Press, p. 187-211.

Das, K., R. Doley, and A. K. Mukherjee, 2004, Purification and biochemical characterisation of a thermostable, alkalophilic, extracellular alpha-amylase from *Bacillus subtilis* DM03, a strain isolated from the traditional fermented food of India: Biotechnology and Applied Biochemistry, v. 40, p. 291-298.

Davis, P., N. Parry. Detergent composition comprising benefits. [WO0146357]. 2001.

Decanniere, K., A. Desmyter, M. Lauwereys, M. A. Ghahroudi, S. Muyldermans, and L. Wyns, 1999, A single-domain antibody fragment in complex with RNase A: non-canonical loop structures and nanomolar affinity using two CDR loops: Structure., v. 7, no. 4, p. 361-370.

Deleage, G., and B. Roux, 1987, An algorithm for protein secondary structure prediction based on class prediction: Protein Eng, v. 1, no. 4, p. 289-294.

Desmyter, A., T. R. Transue, M. A. Ghahroudi, M. H. Thi, F. Poortmans, R. Hamers, S. Muyldermans, and L. Wyns, 1996, Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme: Nat.Struct.Biol., v. 3, no. 9, p. 803-811.

Ding, M., F. Chen, X. Shi, B. Yucesoy, B. Mossman, and V. Vallyathan, 2002, Diseases caused by silica: mechanisms of injury and disease development: Int.Immunopharmacol., v. 2, no. 2-3, p. 173-182.

Dolk, E., d. van, V. H. D. Lutje, G. Vriend, H. de Haard, S. Spinelli, C. Cambillau, L. Frenken, and T. Verrips, 2005, Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo: Appl.Environ.Microbiol., v. 71, no. 1, p. 442-450.

Dolk, E., M. van der Vaart, D. Hulsik, G. Vriend, H. de Haard, S. Spinelli, C. Cambillau, L. Frenken, T. Verrips. Isolation of Llama Antibody Fragments for Prevention of Dandruff by Phage Display in Shampoo. Applied and Environmental Microbiology 71[1], 442-450. 2005.

Doran, P., 1995, Doran, P. Bioprocess Engineering Principles: Academic Press Inc, US.

Eldin, P., M. E. Pauza, Y. Hieda, G. Lin, M. P. Murtaugh, P. R. Pentel, and C. A. Pennell, 1997, High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*: J.Immunol.Methods, v. 201, no. 1, p. 67-75.

Fassina, G., M. Ruvo, G. Palombo, A. Verdoliva, and M. Marino, 2001, Novel ligands for the affinity-chromatographic purification of antibodies: *Journal of Biochemical and Biophysical Methods*, v. 49, no. 1-3, p. 481-490.

Franconi, R. et al., 1999, Functional expression in bacteria and plants of an scFv antibody fragment against tospoviruses: *Immunotechnology*, v. 4, no. 3-4, p. 189-201.

Frenken, L. G. J., R. H. J. van der Linden, P. W. J. J. Hermans, J. W. Bos, R. C. Ruuls, B. de Geus, and C. T. Verrips, 2000, Isolation of antigen specific Llama VHH antibody fragments and their high level secretion by *Saccharomyces cerevisiae*: *Journal of Biotechnology*, v. 78, no. 1, p. 11-21.

Fuchs, H., and R. Gessner, 2001, The result of equilibrium-constant calculations strongly depends on the evaluation method used and on the type of experimental errors: *Biochem.J.*, v. 359, no. Pt 2, p. 411-418.

Galaev, I. Y., and B. Mattiasson, 1999, 'Smart' polymers and what they could do in biotechnology and medicine: *Trends in Biotechnology*, v. 17, no. 8, p. 335-340.

Gerngross, T. U., 2004, Advances in the production of human therapeutic proteins in yeasts and filamentous fungi: *Nature biotechnology*, v. 22, no. 11, p. 1409-1414.

Gibbs, W. W., 1997, Plantibodies. Human antibodies produced by field crops enter clinical trials: *Sci.Am.*, v. 277, no. 5, p. 44.

Gilkes, N., R. Warren, R. Miller, D. Kilburn. Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. *Journal of Biological Chemistry* 263, 10401-10407. 1988.

Goetz, H., M. Kuschel, T. Wulff, C. Sauber, C. Miller, S. Fisher, and C. Woodward, 2004, Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination: *J.Biochem.Biophys.Methods*, v. 60, no. 3, p. 281-293.

Goldstein, M. A., M. Takagi, S. Hashida, O. Shoseyov, R. H. Doi, and I. H. Segel, 1993, Characterization of the cellulose-binding domain of the *Clostridium cellulovorans* cellulose-binding protein A: *J.Bacteriol.*, v. 175, no. 18, p. 5762-5768.

Gouka, R. J., P. J. Punt, and C. A. van den Hondel, 1997, Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects: *Appl.Microbiol.Biotechnol.*, v. 47, no. 1, p. 1-11.

Greenberg, A. S., D. Avila, M. Hughes, A. Hughes, E. C. McKinney, and M. F. Flajnik, 1995, A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks: *Nature*, v. 374, no. 6518, p. 168-173.

Hagel, L., 2001, Separation on the basis of chemistry, in S. Roe ed., *Protein Purification Techniques*: Oxford University Press, p. 155-185.

Hamers-Casterman, C., T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. B. Songa, N. Bendahman, and R. Hamers, 1993, Naturally occurring antibodies devoid of light chains: *Nature*, v. 363, no. 6428, p. 446-448.

Harada, Y., M. Kuroda, A. Ishida. Specific and Quantized Antigen-Antibody Interaction Measured by Atomic Force Microscopy. *Langmuir* 16, 708-715. 2000.

Harris, B., 1999, Exploiting antibody-based technologies to manage environmental pollution: *Trends Biotechnol.*, v. 17, no. 7, p. 290-296.

Harris, E. L. V., 2001, Concentration of the extract, in S. Roe ed., *Protein Purification Techniques*: Oxford University Press, p. 27-50.

Henrissat, B., 1993, Hidden domains and active site residues in beta-glycanase-encoding gene sequences? *Gene*, v. 125, no. 2, p. 199-204.

Horwitz, A. H., C. P. Chang, M. Better, K. E. Hellstrom, and R. R. Robinson, 1988, Secretion of functional antibody and Fab fragment from yeast cells: *Proc. Natl. Acad. Sci. U.S.A.*, v. 85, no. 22, p. 8678-8682.

Houdebine, L. M., 2002, Antibody manufacture in transgenic animals and comparisons with other systems: *Current Opinion in Biotechnology*, v. 13, no. 6, p. 625-629.

Hunt, B., C. Goddard, A. P. J. Middelberg, and B. K. O'Neill, 2001, Economic analysis of immunoadsorption systems: *Biochemical Engineering Journal*, v. 9, no. 2, p. 135-145.

Huse, K., H. J. Bohme, and G. H. Scholz, 2002, Purification of antibodies by affinity chromatography: *Journal of Biochemical and Biophysical Methods*, v. 51, no. 3, p. 217-231.

Irwin, D., E. D. Jung, and D. B. Wilson, 1994, Characterization and sequence of a *Thermomonospora fusca* xylanase: *Appl. Environ. Microbiol.*, v. 60, no. 3, p. 763-770.

Jervis, E. J., C. A. Haynes, and D. G. Kilburn, 1997, Surface diffusion of cellulases and their isolated binding domains on cellulose: *J. Biol. Chem.*, v. 272, no. 38, p. 24016-24023.

Jones, C., A. Patel, S. Griffin, J. Martin, P. Young, K. O'Donnell, C. Silverman, T. Porter, and I. Chaiken, 1995, Current trends in molecular recognition and bioseparation: *Journal of Chromatography A*, v. 707, no. 1, p. 3-22.

Jurado, P., D. Ritz, J. Beckwith, L. de V., and L. A. Fernandez, 2002, Production of functional single-chain Fv antibodies in the cytoplasm of *Escherichia coli*: *J. Mol. Biol.*, v. 320, no. 1, p. 1-10.

Jurado, P., D. Ritz, J. Beckwith, V. de Lorenzo, and L. A. Fernandez, 2002, Production of Functional Single-Chain Fv Antibodies in the Cytoplasm of *Escherichia coli*: *Journal of Molecular Biology*, v. 320, no. 1, p. 1-10.

Kaltenbrunner, O., C. Tauer, J. Brunner, and A. Jungbauer, 1993, Isoprotein analysis by ion-exchange chromatography using a linear pH gradient combined with a salt gradient: *Journal of Chromatography A*, v. 639, no. 1, p. 41-49.

Kaltenbrunner, O., and A. Jungbauer, 1997, Simple model for blending aqueous salt buffers: Application to preparative chromatography: *Journal of Chromatography A*, v. 769, no. 1, p. 37-48.

Kaltenbrunner, O., and A. Jungbauer, 1996, Adsorption isotherms in protein chromatography combined influence of protein and salt concentration on adsorption isotherm: *Journal of Chromatography A*, v. 734, no. 1, p. 183-194.

Kaltenbrunner, O., A. Jungbauer, and S. Yamamoto, 1997, Prediction of the preparative chromatography performance with a very small column: *Journal of Chromatography A*, v. 760, no. 1, p. 41-53.

Keller, K., T. Friedmann, and A. Boxman, 2001, The bioseparation needs for tomorrow: *Trends in Biotechnology*, v. 19, no. 11, p. 438-441.

Knappik, A., and A. Pluckthun, 1995, Engineered turns of a recombinant antibody improve its in vivo folding: *Protein Eng*, v. 8, no. 1, p. 81-89.

Knowles, J., P. Lehtovaara, M. Penttila, T. Teeri, A. Harkki, and I. Salovuori, 1987, The cellulase genes of *Trichoderma*: *Antonie Van Leeuwenhoek*, v. 53, no. 5, p. 335-341.

Kohler, G., and C. Milstein, 1975, Continuous cultures of fused cells secreting antibody of predefined specificity: *Nature*, v. 256, no. 5517, p. 495-497.

Kretzschmar, T., T. v. Ruden. Antibody discovery: phage display. *Current Opinion in Biotechnology* 13, 598-602. 2002.

Lauwereys, M., G. M. Arbabi, A. Desmyter, J. Kinne, W. Holzer, E. De Genst, L. Wyns, and S. Muyldermans, 1998, Potent enzyme inhibitors derived from dromedary heavy-chain antibodies: *EMBO J.*, v. 17, no. 13, p. 3512-3520.

Ledeboer, A. M. et al., 2002, Preventing phage lysis of *Lactococcus lactis* in cheese production using a neutralizing heavy-chain antibody fragment from llama: *J.Dairy Sci.*, v. 85, no. 6, p. 1376-1382.

Levison, P. R., 2003, Large-scale ion-exchange column chromatography of proteins. Comparison of different formats: *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.*, v. 790, no. 1-2, p. 17-33.

Levy, I., and O. Shoseyov, 2002, Cellulose-binding domains: Biotechnological applications: *Biotechnology Advances*, v. 20, no. 3-4, p. 191-213.

Lewis, W., E. Kesharvarz-Moore, J. Windust, D. Bushell, N. Parry, 2006, Construction and evaluation of novel fusion proteins for targeted delivery of micro particles to cellulose surfaces: *Biotechnology and Bioengineering*, v. 94, no 4, p. 625-632.

Liddell, E., and I. Weeks, 1995, Antibody Technology (Introduction to Biotechniques Series): Bios Sicientific Publishers Ltd..

Lillehoj, E, V Malik. Protein Purification. Advances in Biochemical Engineering 40, 19-71. 1989.

Linder, M., M. L. Mattinen, M. Kontteli, G. Lindeberg, J. Stahlberg, T. Drakenberg, T. Reinikainen, G. Pettersson, and A. Annala, 1995, Identification of functionally important amino acids in the cellulose-binding domain of *Trichoderma reesei* cellobiohydrolase I: Protein Sci., v. 4, no. 6, p. 1056-1064.

Linder, M., and T. T. Teeri, 1996, The cellulose-binding domain of the major cellobiohydrolase of *Trichoderma reesei* exhibits true reversibility and a high exchange rate on crystalline cellulose: Proc.Natl.Acad.Sci.U.S.A, v. 93, no. 22, p. 12251-12255.

Linder, M., I. Salovuori, L. Ruohonen, and T. T. Teeri, 1996, Characterization of a double cellulose-binding domain. Synergistic high affinity binding to crystalline cellulose: J.Biol.Chem., v. 271, no. 35, p. 21268-21272.

Linder, M., T. Nevanen, L. Soderholm, O. Bengs, and T. T. Teeri, 1998, Improved immobilization of fusion proteins via cellulose-binding domains: Biotechnol.Bioeng., v. 60, no. 5, p. 642-647.

Lindmark, R., K. Thoren-Tolling, and J. Sjoquist, 1983, Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera: J.Immunol.Methods, v. 62, no. 1, p. 1-13.

Lowe, C. R., 2001, Combinatorial approaches to affinity chromatography: Current Opinion in Chemical Biology, v. 5, no. 3, p. 248-256.

Lowe, P. A., T. J. Clark, R. J. Davies, P. R. Edwards, T. Kinning, and D. Yeung, 1998, New approaches for the analysis of molecular recognition using the IAsys evanescent wave biosensor: J.Mol.Recognit., v. 11, no. 1-6, p. 194-199.

Malmborg, A. C., and C. A. Borrebaeck, 1995, BIAcore as a tool in antibody engineering: J.Immunol.Methods, v. 183, no. 1, p. 7-13.

Mason, A. B., Q. Y. He, T. E. Adams, D. R. Gumerov, I. A. Kaltashov, V. Nguyen, and R. T. A. MacGillivray, 2001, Expression, Purification, and Characterization of Recombinant Nonglycosylated Human Serum Transferrin Containing a C-Terminal Hexahistidine Tag: Protein Expression and Purification, v. 23, no. 1, p. 142-150.

Maynard,J, G Georgiou, 2000, Antibody Engineering. Annual Review of Biomedical Engineering 02, 339-376.

Merget, R., T. Bauer, H. U. Kupper, S. Philippou, H. D. Bauer, R. Breitstadt, and T. Bruening, 2002, Health hazards due to the inhalation of amorphous silica: Arch.Toxicol., v. 75, no. 11-12, p. 625-634.

Mesta, L., A. Heyraud, J. P. Joseleau, and P. R. Coulet, 2003, Catalytic properties of endoxylanase fusion proteins from *Neocallimastix frontalis* and effect of

immobilization onto metal-chelate matrix: *Journal of Biotechnology*, v. 101, no. 3, p. 253-265.

Mohanty, A. K., and M. C. Wiener, 2004, Membrane protein expression and production: effects of polyhistidine tag length and position: *Protein Expression and Purification*, v. 33, no. 2, p. 311-325.

Molloy, P., L. Brydon, A. J. Porter, and W. J. Harris, 1995, Separation and concentration of bacteria with immobilized antibody fragments: *J.Appl.Bacteriol.*, v. 78, no. 4, p. 359-365.

Muruganandam,A, J Tanha, S Narang, D Stanimirovic. Selection of phage-displayed llama single-domain antibodies that transmigrate across human blood-brain barrier endothelium. *FASEB journal* , 240-243. 2002.

Muyldermans, S., 1 A.D., Camel Single-domain Antibodies as Modular Building Units in Bispecific and Bivalent Antibody Constructss: *Journal of Biological Chemistry*, v. 276, no. 10, p. 7346-7350.

Muyldermans, S., 2001, Single domain camel antibodies: current status: *J.Biotechnol.*, v. 74, no. 4, p. 277-302.

Nakamura, K., and K. Takeo, 1998, Affinity electrophoresis and its applications to studies of immune responsel: *Journal of Chromatography B: Biomedical Sciences and Applications*, v. 715, no. 1, p. 125-136.

Neish, C. S., I. L. Martin, R. M. Henderson, and J. M. Edwardson, 2002, Direct visualization of ligand-protein interactions using atomic force microscopy: *Br.J.Pharmacol.*, v. 135, no. 8, p. 1943-1950.

Nguyen, V. K., R. Hamers, L. Wyns, and S. Muyldermans, 1999, Loss of splice consensus signal is responsible for the removal of the entire C(H)1 domain of the functional camel IGG2A heavy-chain antibodies: *Mol.Immunol.*, v. 36, no. 8, p. 515-524.

Nguyen, V. K., C. Su, S. Muyldermans, and L. W. van der, 2002, Heavy-chain antibodies in Camelidae; a case of evolutionary innovation: *Immunogenetics*, v. 54, no. 1, p. 39-47.

Nguyen, V. K., X. Zou, M. Lauwereys, L. Brys, M. Bruggemann, and S. Muyldermans, 2003, Heavy-chain only antibodies derived from dromedary are secreted and displayed by mouse B cells: *Immunology*, v. 109, no. 1, p. 93-101.

Noble, J., 2001, Scale-up considerations, in S Roe ed., *Protein Purification Techniques*: Oxford University Press, p. 241-253.

Ouerghi, O., A. Touhami, A. Othmane, H. B. Ouada, C. Martelet, C. Fretigny, and N. Jaffrezic-Renault, 2002, Investigating specific antigen/antibody binding with the atomic force microscope: *Biomol.Eng*, v. 19, no. 2-6, p. 183-188.

Paramban,R, R Bugos, W Su. Engineering Green Fluorescent Protein as a Dual Functional Tag. *Biotechnology and Bioengineering* 86[6], 687-697. 2004.

Parry,N. 2003. Personal communication.

Perez, J. M., J. G. Renisio, J. J. Prompers, C. J. van Platerink, C. Cambillau, H. Darbon, and L. G. Frenken, 2001, Thermal unfolding of a llama antibody fragment: a two-state reversible process: *Biochemistry*, v. 40, no. 1, p. 74-83.

Picket,A, K Hardy. Comparison of hosts for rDNA. *Applied Molecular Genetics 3rd European Congress Biotechnology* 4, 309-313. 1984.

Pierce, J. J., S. C. Robinson, J. M. Ward, E. Keshavarz-Moore, and P. Dunnill, 2002, A comparison of the process issues in expressing the same recombinant enzyme periplasmically in *Escherichia coli* and extracellularly in *Streptomyces lividans*: *Journal of Biotechnology*, v. 92, no. 3, p. 205-215.

Pierce, M. M., C. S. Raman, and B. T. Nall, 1999, Isothermal titration calorimetry of protein-protein interactions: *Methods*, v. 19, no. 2, p. 213-221.

Pluckthun, A., and A. Skerra, 1989, [34] Expression of functional antibody Fv and Fab fragments in *Escherichia coli*, *Methods in Enzymology*: Academic Press, p. 497-515.

Pollock, D. P., J. P. Kutzko, E. Birck-Wilson, J. L. Williams, Y. Echelard, and H. M. Meade, 1999, Transgenic milk as a method for the production of recombinant antibodies: *Journal of Immunological Methods*, v. 231, no. 1-2, p. 147-157.

Porath, J., 1959, Fractionation of polypeptides and proteins on dextran gels: *Clin.Chim.Acta*, v. 4, p. 776-778.

Punt, P.J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus, and C. van den Hondel, 2002, Filamentous fungi as cell factories for heterologous protein production: *Trends in Biotechnology*, v. 20, no. 5, p 200-206.

Przybycien, T. M., N. S. Pujar, and L. M. Steele, 2004, Alternative bioseparation operations: life beyond packed-bed chromatography: *Current Opinion in Biotechnology*, v. 15, no. 5, p. 469-478.

Rast, J. P., C. T. Amemiya, R. T. Litman, S. J. Strong, and G. W. Litman, 1998, Distinct patterns of IgH structure and organization in a divergent lineage of chondrichthyan fishes: *Immunogenetics*, v. 47, no. 3, p. 234-245.

Reed, I., and D. Mackay, 2001, Clarification Techniques, in S Roe ed., *Protein Purification Techniques*: Oxford University Press, p. 51-81.

Rees, A., D. Staunton, D. Webster, S. Searle, H. A. , and J. Pedersen, 1994, Antibody Design:beyond the natural limits: *Journal of Chromatography B: Biomedical Sciences and Applications*, v. 715, no. 1, p. 125-136.

Reinikainen, T., L. Ruohonen, T. Nevanen, L. Laaksonen, P. Kraulis, T. A. Jones, J. K. Knowles, and T. T. Teeri, 1992, Investigation of the function of mutated cellulose-binding domains of *Trichoderma reesei* cellobiohydrolase I: *Proteins*, v. 14, no. 4, p. 475-482.

Riechmann, L., and S. Muyldermans, 1999, Single domain antibodies: comparison of camel VH and camelised human VH domains: *Journal of Immunological Methods*, v. 231, no. 1-2, p. 25-38.

Roe, S., 2001, Purification Strategy, in S Roe ed., *Protein Purification Techniques*: Oxford University Press, p. 1-10.

Roitt, I., J. Brostoff, and D. Male, 1998, *Immunology*: Mosby.

Rojas, E., J. dos Reis Coimbra, L. Minim, A. Zuniga, S. Saraiva, V. Minim. Size-exclusion chromatography applied to the purification of whey proteins from the polymeric and saline phases of aqueous two-phase systems. *Process Biochemistry* 39, 1751-1759. 2004.

Roque, A. C., C. R. Lowe, and M. A. Taipa, 2004, Antibodies and Genetically Engineered Related Molecules: Production and Purification: *Biotechnology Progress*, v. 20, p. 639-654.

Sapan C.V., R.L. Lundblad and N.C. Price, 1999, Colorimetric protein assay techniques: *Biotechnology and Applied Biochemistry*, v. 29, no. 2, p. 99 -108.

Schuck, P., 1997, Use of surface plasmon resonance to probe the equilibrium and dynamic aspects of interactions between biological macromolecules: *Annu.Rev.Biophys.Biomol.Struct.*, v. 26, p. 541-566.

Shpigel, E., A. Goldlust, A. Eshel, I. K. Ber, G. Efroni, Y. Singer, I. Levy, M. Dekel, and O. Shoseyov, 2000, Expression, purification and applications of staphylococcal Protein A fused to cellulose-binding domain: *Biotechnology Progress*, v. 31, p. 197-203.

Silveira, J. R., B. Caughey, and G. S. Baron, 2004, Prion protein and the molecular features of transmissible spongiform encephalopathy agents: *Mad Cow Disease and Related Spongiform Encephalopathies*, v. 284, p. 1-50.

Singh, P. C., and R. K. Singh, 1996, Choosing an appropriate bioseparation technique: *Trends in Food Science & Technology*, v. 7, no. 2, p. 49-58.

Stanbury, and Whittaker, 1995, *The Principles of Fermentation Technology*. Pergamon Press.

Terpe, K., 2003, Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems: *Appl.Microbiol.Biotechnol.*, v. 60, no. 5, p. 523-533.

Thomassen, Y., W. Meijer, L. Sierkstra, and C. Verrips, 2002, Large-scale production of VHH antibody fragments by *Saccharomyces cerevisiae*: *Enzyme and Microbial Technol*, v. 30, p. 273-278.

Thommes, J., M. Halfar, H. Gieren, S. Curvers, R. Takors, R. Brunschier, and M. R. Kula, 2001, Human chymotrypsinogen B production from *Pichia pastoris* by integrated development of fermentation and downstream processing. Part 2. Protein recovery: *Biotechnol.Prog.*, v. 17, no. 3, p. 503-512.

Tolstoguzov, V., 1997, Protein Polysaccharide interactions, Food Proteins and their applications: Marcel Dekker Inc, New York, p. 171-199.

Tomme, P., D. P. Driver, E. A. Amandoron, R. C. Miller, Jr., R. Antony, J. Warren, and D. G. Kilburn, 1995, Comparison of a fungal (family I) and bacterial (family II) cellulose-binding domain: J.Bacteriol., v. 177, no. 15, p. 4356-4363.

Tormo, J., R. Lamed, A. J. Chirino, E. Morag, E. A. Bayer, Y. Shoham, and T. A. Steitz, 1996, Crystal structure of a bacterial family-III cellulose-binding domain: a general mechanism for attachment to cellulose: EMBO J., v. 15, no. 21, p. 5739-5751.

Trabbic-Carlson, K., L. Liu, B. Kim, and A. Chilkoti, 2004, Expression and purification of recombinant proteins from *Eschericia coli*: Comparison of an elastin like Polypeptide fusion with an oligohistidine fusion: Protein Science.

Transue, T. R., E. De Genst, M. A. Ghahroudi, L. Wyns, and S. Muyldermans, 1998, Camel single-domain antibody inhibits enzyme by mimicking carbohydrate substrate: Proteins, v. 32, no. 4, p. 515-522.

van der Linden, R. H. et al., 1999, Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies: Biochim.Biophys.Acta, v. 1431, no. 1, p. 37-46.

van der Linden R. H. 1999, Unique characteristics of llama heavy chain only antibodies. PhD thesis, Utrecht university (The Netherlands).

van der Linden, R. H., B. de Geus, G. J. Frenken, H. Peters, and C. T. Verrips, 2000, Improved production and function of llama heavy chain antibody fragments by molecular evolution: J.Biotechnol., v. 80, no. 3, p. 261-270.

van der, Linden. R., B. de Geus, W. Stok, W. Bos, D. van Wassenaar, T. Verrips, and L. Frenken, 2000, Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of Lama glama: J.Immunol.Methods, v. 240, no. 1-2, p. 185-195.

Van Tilbeurgh,H, P Tomme, M Claeysens, R Bhikhabhai. Limited proteolysis of the cellulohydrolase I from *Trichoderma reesei*. Separation of functional domains. FEBS Letters 1986[204], 223-227. 1986.

van Tilbeurgh, H., G. Pettersson, R. Bhikabhai, H. De Boeck, and M. Claeysens, 1985, Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Reaction specificity and thermodynamics of interactions of small substrates and ligands with the 1,4-beta-glucan cellobiohydrolase II: Eur.J.Biochem., v. 148, no. 2, p. 329-334.

van Tilbeurgh, H., F. G. Loontjens, Y. Engelborgs, and M. Claeysens, 1989, Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Binding of small ligands to the 1,4-beta-glucan cellobiohydrolase II and influence of glucose on their affinity: Eur.J.Biochem., v. 184, no. 3, p. 553-559.

Verheesen, P., M. R. ten Haaf, N. Lindner, C. T. Verrips, and J. J. W. de Haard, 2003, Beneficial properties of single-domain antibody fragments for application in

immunoaffinity purification and immuno-perfusion chromatography: *Biochimica et Biophysica Acta (BBA) - General Subjects*, v. 1624, no. 1-3, p. 21-28.

Verma, R., E. Boleti, and A. J. George, 1998, Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems: *J.Immunol.Methods*, v. 216, no. 1-2, p. 165-181.

Vu, K. B., M. A. Ghahroudi, L. Wyns, and S. Muyldermans, 1997, Comparison of llama VH sequences from conventional and heavy chain antibodies: *Mol.Immunol.*, v. 34, no. 16-17, p. 1121-1131.

Ward, E. S., D. Gussow, A. D. Griffiths, P. T. Jones, and G. Winter, 1989, Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*: *Nature*, v. 341, no. 6242, p. 544-546.

Warshaw, E. M., 2003, Latex allergy: *Skinmed.*, v. 2, no. 6, p. 359-366.

Wilson, S. 2002, personal communication.

Winter, G., A. D. Griffiths, R. E. Hawkins, and H. R. Hoogenboom, 1994, Making antibodies by phage display technology: *Annu.Rev.Immunol.*, v. 12, p. 433-455.

Wurm, F. M., 2004, Production of recombinant protein therapeutics in cultivated mammalian cells: *Nature biotechnology*, v. 22, no. 11, p. 1393-1398.

Yamamoto, S., P. K. Watler, D. Feng, and O. Kaltenbrunner, 1999, Characterization of unstable ion-exchange chromatographic separation of proteins: *Journal of Chromatography A*, v. 852, no. 1, p. 37-41.

Zhou, X., B. Xue, S. Bai, and Y. Sun, 2002, Macroporous polymeric ion exchanger of high capacity for protein adsorption: *Biochemical Engineering Journal*, v. 11, no. 1, p. 13-17.