Chapter 6

General discussion

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The initiation and progression of the aging process depends both on the damaging effects of internal and external environmental conditions and on the efficiency of an organism to neutralize the imposed damage. The random and multi-factorial nature of the mechanisms that are involved in the aging process results in large variations in changes on the molecular and cellular level, even in genetically identical populations (Kirkwood et al., 2005). To understand the interactions and possible synergistic mechanisms of the aging process, and identify the processes that are involved in the early and late stages of aging, the differences that occur in time, so called aging biomarkers, need to be identified. As it is unlikely that one biomarker offers enough information to obtain a high level of specificity and sensitivity, multiple markers are needed (Anderson, 2005). These biomarkers of aging could be used to monitor the effects of aging intervention studies or to determine the biological over the chronological age of an individual.

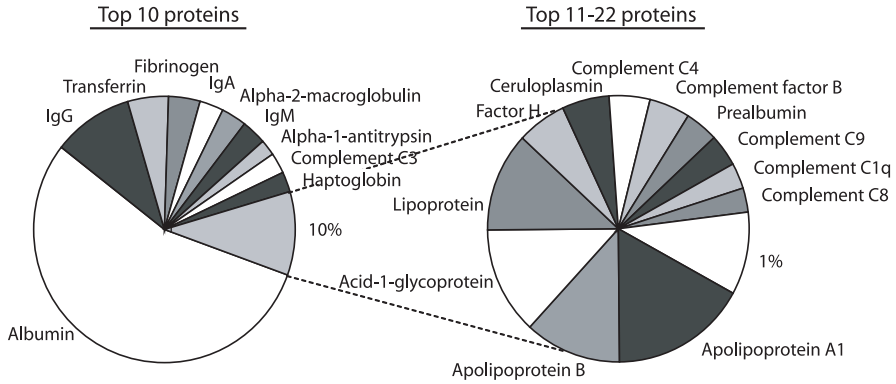

Figure 1: Twenty-two abundant proteins together constitute 99% of the total protein content of blood plasma. Graph depicts the top 10 human plasma proteins that constitute 90% of the total protein content of plasma, followed by the top 11-22 that constitute 9% of the total protein content of plasma. Removal of these proteins would allow the differential analysis of the remaining 1% of the plasma proteins.

Proteomics techniques that have been developed and improved in recent years, allows the simultaneous identification of multiple changes that occur on the protein level. In spite of these efforts, such analyses are still hampered by some unfavorable characteristics of proteins in research. The application of antibodies or their derivatives could provide solutions for some of these challenges. Some favorable characteristics of VHH antibody fragments (Dolk et

al., 2005b; Frenken et al., 2000; Muyldermans, 2001; van der Linden et al., 1999) make them highly suitable for application in this field, especially in combination with phage display technology, which allows selection of VHHs with predefined characteristics (Dolk et al., 2005a; Verheesen et al., 2003), even in an automated setup.

In this thesis, the development of VHH based proteomics techniques is described for identification of protein expression differences in biological samples related to aging.
Studies started with the selection of VHHs against the highly abundant bulk proteins HSA and IgG in human serum. The subsequent application of these antibodies to deplete these proteins from pooled plasma samples of young and old individuals and the comparison of the depleted plasma samples with 2D-DIGE technology resulted in the identification of several differentially expressed proteins. These results indicated an increased pro-coagulant and pro-inflammatory state with increasing age and further suggested an increase in tissue damage with aging. Even though this is in line with previously described data (Aillaud et al., 1986; Ernst and Resch, 1993; Ishikawa et al., 1998; Mackness et al., 1997; Ritchie et al., 1998; Ritchie et al., 1999; Ritchie et al., 2000; Ritchie et al., 2004a; Tofler et al., 2005; Trougakos and Gonos, 2002; Witte et al., 1993), these data do not represent the full nature of the differences that occur in human blood upon aging. Most of the identified differentially expressed proteins are members of the top 22 abundant proteins present in blood, which together represent 99% of the total protein content of plasma (Figure 1). Therefore, data on the differential expression of proteins that are less abundant are missing. This illustrates the limitations of present 2D-technology. Although the reproducibility is increased by the introduction of 2D-DIGE technology and more reliable results are obtained, the sensitivity of this technique is still an issue of concern. Especially the analysis of the plasma proteome offers great challenges in this respect, as it is estimated that the dynamic range, the ratio between the highest and lowest abundant protein, of the plasma proteome is at least greater than 10^{10}. This number is simply based on concentrations of the highest and lowest abundant plasma proteins presently known, such as HSA (~50mg/ml) and the cytokine IL-6 (~1-5pg/ml) respectively (Anderson, 2005). With 2D-technology, protein spots can be

detected with a maximal dynamic range of 10^2-10^3 (Patton, 2002). The sensitivity of spot identification is even lower.

For cellular proteomes this situation is less extreme, with the most abundant proteins only constituting around 5% of the total protein content as is described for yeast (Futcher et al., 1999). However, irrespective of the dynamic range, the sheer number of different proteins complicates the analysis of any proteome, considering the total number of genes, differential RNA splicing, differential protein translation initiation and numerous different post-translational modifications (Anderson, 2005). Among other things, such as the heterogeneity in an aging population as discussed in chapter 5, this greatly limits the analysis of the full spectrum of protein changes that occur during aging.

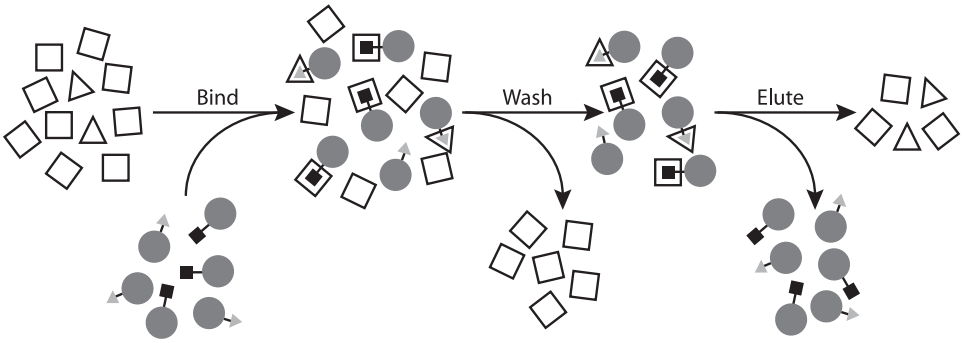

Figure 2: Schematic representation of protein equalizer beads (Ciphergen). Random peptide libraries are immobilized on beads, which are added to a biological sample containing high (square) and low abundant proteins (triangle). In theory, an equal amount of each peptide is present in this library. Depending on the amount of beads used, an under-capacity of peptides, which bind to the high abundant proteins, and an over-capacity of peptides, which bind to the low abundant proteins can be obtained. The high abundant proteins that are not bound will be washed away, thereby concentrating the low abundant proteins. Ultimately, the ratio between the amount of beads used and the sample volume determines the concentration of the low abundant over the high abundant proteins.

In proteomics research, this recognition has led to further development of affinity chromatography based techniques that reduce the complexity of proteomes and enable the analysis of low abundant proteins. For the plasma proteome, one of these strategies involves an extension to the in this thesis described depletion of the high abundant proteins HSA and IgG. At present, techniques are available that enable the depletion of the 22 most abundant proteins in human plasma (Schuchard et al., 2005) (Figure 1), which offers

the potential analysis of the remaining 1% total protein content in plasma. Furthermore, techniques have been developed for the enrichment of minor abundant proteins, such as the use of protein equalizer beads (Righetti et al., 2005) (Figure 2). This approach is based on random peptide libraries in which each peptide has a certain affinity for a specific protein. In theory, this library contains comparable amounts of each peptide. Consequently, depending on the amount of beads used, low abundant proteins can be enriched while the bulk of high abundant proteins will be lost during the washing steps (Figure 2). Both approaches would be beneficial in respect to the enormous dynamic range found in the plasma proteome. However, the beneficial aspects of these approaches have to be verified in proteomics studies that are aimed at identification of protein expression differences, such as the reproducibility of these techniques. Furthermore, large amounts of biological samples are needed to analyze the minor abundant proteins.

Fractionation or analyzing a confined set of proteins is another popular approach for reducing the complexity of a proteome. This can be achieved by separating proteins based on their charge, hydrophobicity, solubilization and size (Fountoulakis et al., 1999; Pieper et al., 2003a; Pieper et al., 2003b; Rothemund et al., 2003), or by fractionation of cellular organelles (Snape et al., 1990). In addition, fractionation can also be achieved by using antibodies that recognize conserved amino acid epitopes, as described for the KDEL sequence in chapter 4, or that recognize post-translational modifications, as has been shown for phosphorylation (Gronborg et al., 2002; Maguire et al., 2002). However, the separation potential of these techniques often lacks in specificity as several proteins are usually found in multiple fractions and because of unwanted protein-protein interactions during sample preparation. This hampers the interpretation and reduces the reproducibility of these techniques.

Most of the above described techniques are usually combined with 2D-gelelectrophoresis and/or mass spectrometry. This enables the discovery of novel biologically relevant target proteins. However, because of the labor-intensive protocols, the use of these techniques seems less suitable for use in clinical laboratories. Furthermore, it should be noted that results obtained with all of these proteomics studies should be verified with an additional technique as observed differences can be caused by the variety of different steps that are applied during sample preparation. Furthermore, sample handling is

another issue that should be treated with care as it has been shown that prolonged storage of samples may result in the modification of some proteins, which can lead to misleading results and conclusions (Franzen et al., 1993). This further necessitates the confirmation of the obtained results in additional experiments with preferably fresh biological samples.

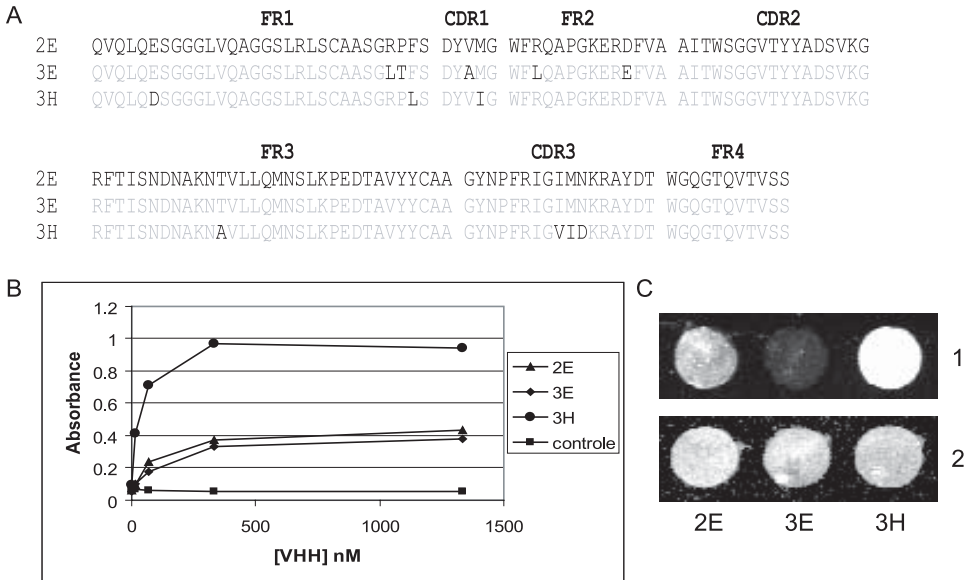

Figure 3: Non-specific covalent immobilization of antibody fragments can decrease antibody activity. (A) Three VHH antibody fragments, clone 2E, 3E and 3H, selected against the same antigen revealed highly homologues amino acid sequences. (B) Differences in binding characteristics were revealed in an ELISA with 500ng/ml coated antigen, detected with different concentrations of purified VHH. Clone 2E and 3E showed similar binding characteristics, indicating comparable binding affinities, while clone 3H showed superior binding characteristics over the other clones, indicating a higher binding affinity. These differences in binding characteristics are most likely caused by differences in the CDR regions, especially CDR3. (C) Upon non-specific covalent immobilization on an aldehyde glass-slide and subsequent incubation with Cy3 labeled antigen, the highest signals were obtained with clone 3H (panel 1), which was in agreement with the ELISA. In contrast to the results obtained with ELISA, clone 3E revealed significantly lower signals compared to clone 2E when immobilized on the aldehyde glass-slides, while similar amounts of VHH were immobilized (panel 2). These differences were therefore most likely caused by differences in preferential coupling of specific amino acid residues between clone 2E and 3E.

A proteomics technique that could enable the expression analysis of low abundant proteins, without high abundant protein depletion or sample fractionation, is the application of antibody micro-arrays, in which antibodies

are immobilized on a surface, each in a separate spot, to capture their respective antigen (Haab et al., 2001). Furthermore, antibody micro-arrays offer several additional advantages, such as the small amount of biological sample needed, and the fully automated manner in which these experiments can be performed, allowing the analysis of large numbers of samples.

Although this technique provides the simultaneous analysis of several target proteins with high sensitivity, several obstacles still have to be overcome. This approach requires the generation of numerous monoclonal antibodies that have to be tested extensively on antigen specificity and sensitivity, which makes it a far from straight forward approach. To enable the generation of such antibodies, fully automated selection and screening protocols are required, which cannot be realized for hybridoma technology as it depends on animal immunization and cell culture maintenance. The application of recombinant antibody fragments in combination with phage display technology could offer a solution for generation of a wide variety of antibodies with different specificities (Amstutz et al., 2001). However, the issue of specificity and sensitivity of the selected antibodies is still a matter of concern.

In addition to antibody generation, antibody activity has to be maintained after immobilization on the array surface. A large portion of classical antibodies has been shown to lose their functionality upon immobilization (Haab et al., 2001), which can also occur with recombinant antibodies. VHHs with highly homologues amino acid sequences can reveal large differences in antigen binding when covalently immobilized on an array surface (Figure 3). This is most likely caused by the preferential coupling of specific amino acid residues in or close to the binding domain of the antibody. Improvement of antibody functionality can be obtained by specific immobilization via the C- or N-terminus of the antibody molecule. Especially recombinant antibodies are suitable for such an approach, as they can be genetically modified with specific tags fused to their C- or N-terminus designed for directed immobilization, which can significantly increase their activity (Figure 4). Ideally, a tag should be used that enables specific covalent immobilization on an array surface, which has low background characteristics, without losing antibody functionality.

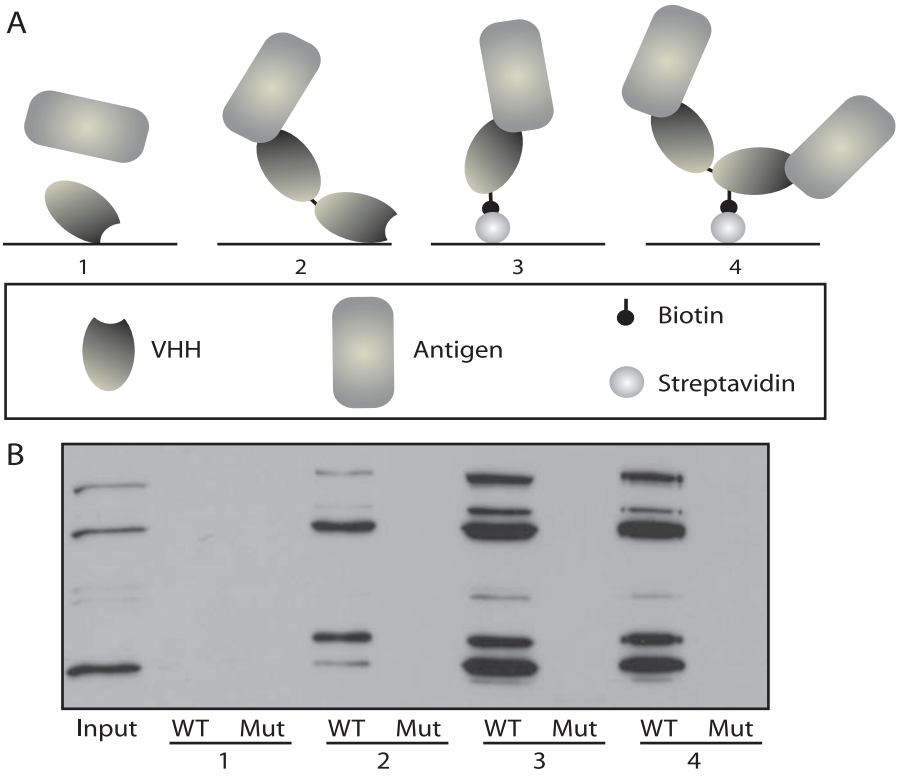

Figure 4: Directed immobilization of antibody fragments or application of antibody fragments with two binding domains can increase the binding activity significantly compared to non-specific covalently immobilized antibody fragments. (A) Schematic representation of non-specific covalent immobilization of monovalent (panel 1) and bivalent (panel 2) VHH antibody fragments, and directed immobilization on immobilized streptavidin via a biotin label attached to a biotinylation sequence at the C-terminus of monovalent (panel 3) and bivalent (panel 4) VHHs. (B) Immuno precipitation of ER-resident proteins from a HeLa cell lysate with the anti-KDEL VHH clone 5 (WT) and a binding-negative mutant of this antibody fragment. Non-specific covalent immobilization of the monovalent VHH on CNBr-sepharose did not result in antigen binding (1). Binding activity was improved upon use of bivalent VHHs (2). The best binding activity was observed upon immobilization of monovalent (3) or bivalent (4) VHHs to streptavidin via a biotin tag at the C-terminus.

Despite these challenges, the application of antibody micro-arrays seems to offer great potential for analyzing protein expression differences because of the high specificity and sensitivity that can be obtained. As it is quite unlikely that the first generation of antibody arrays will contain antibodies to recognize all the proteins from higher eukaryotic organisms, arrays should be constructed to study a predefined set of proteins in a biologically relevant

setting. Indeed, such arrays have been developed to study differences in expression of several cytokines (http://www.raybiotech.com).

Arrays containing antibodies directed against the potential aging biomarkers described in chapter 3 and 5 could be exploited to develop an antibody micro-array for aging research. Such an array could be used to study the effect of aging intervention studies or to determine the biological over the chronological age of an individual. This requires the selection of antibodies that specifically recognize unique epitopes on a specific isoform of a single gene product, which is one of the challenges that affinity ligand based proteomics is confronted with.