CHAPTER 4
CONCLUDING DISCUSSION

Diseases are characterised by the interaction of molecules in one way or another, whether the disease occurs in humans, animals or plants. At least one of these molecules will be a macromolecule like protein or DNA. During poisoning, the responsible toxin usually resembles the normal substrate or ligand and subsequently binds and inactivates the macromolecular target. Infectious diseases are normally precipitated by the expression of a macromolecule on the surface of a pathogen that binds to a host macromolecular target. In auto-immune diseases the body produces anti-self immunoglobulins that recognize and bind to macromolecules on normal cells and subsequently cause damage. This dissertation concerns the sophisticated biomolecular interaction analysis (BIA) biosensor technology with which the interaction of molecules with one another can be measured if and when they take place. The dissertation demonstrates the particular advantages of this technique to better our understanding of the interactions that take place in the pathogenesis of two diseases, an infectious disease (tuberculosis) and an auto-immune disease (Guillain-Barré syndrome)

One of the key molecular interactions that take place during the pathogenesis of tuberculosis (TB) is the recognition of *Mycobacterium tuberculosis* surface antigens by the host's immune cells and immunoglobulins. Vaccinating new-born individuals with *M. bovis* Bacillus Calmette Guerrin (BCG) effectively prevents infection, but complicates diagnosis of TB, since antibodies against BCG cross-react with purified protein derivative (PPD) used world-wide in skin-tests for TB diagnosis (Mustafa, 2002). Human immune deficiency virus (HIV) co-infection also hampers TB diagnosis as HIV patients have almost immeasurably low titres for antibodies against these protein antigens. However, antibodies against waxes (mycolic acids) on the surface of *Mycobacterium* species are still produced in measurable quantities, even in immunocompromised HIV positive patients (Schleicher *et al.*, 2002). The interaction of these mycolic acids (MA) with anti-MA antibodies may be exploited for a more reliable diagnostic technique. Unfortunately these antibodies are of low affinity and are easily washed away during enzyme-linked immunosorbent assay wash steps. Lacking wash steps, a biosensor is able to measure such low affinity antibodies in real-time. J.A. Verschoor and D.G.R. Siko

adopted this technique for TB serodiagnosis by immobilizing MA contained in liposomes on non-derivatised surfaces of a cuvette for measurement in a resonant mirror biosensor (Siko, 2002). This dissertation shows how this technique was optimised so that interactions between closely related structures can be measured and used to discriminate between them. This resolution is essential since MA of *M. tuberculosis* consist of three closely related subclasses that have significantly different binding properties to antibodies (Pan *et al.*, 1999). The interactions of these individual MA subclasses with host immunoglobulins can be particularly helpful to determine the specific mechanism of infection. It has been suggested that one of these subclasses (methoxy-MA) mimics the structure of cholesterol, abundantly found in all animal cell membranes. The most important evidence to support this hypothesis was obtained with direct interaction analysis of cholesterol-containing liposomes with MA-containing liposomes on the biosensor (Siko, 2002). Since the presence of the oxygenated MA (keto- and methoxy-MA) are associated with virulence (Pan *et al.*, 1999), the understanding of how these molecules interact, may be crucial for understanding virulence of, and immunity to TB.

Before the individual subclasses of MA can be compared, they must be separated. In this study it was shown how this separation could be achieved using thin layer chromatography. For proper separation the MA had to have its carboxylic acid group methylated. This methylation might influence its immunogenicity and has consequences for molecular recognition by antibodies in the biosensor method. Structures of the methylated and separated compounds wait to be determined by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), a better technology than EI-MS for this purpose (Laval *et al.*, 2001). It is expected that if preparatively separated MA are immobilized using the novel biosensor method, a difference in affinity of binding between antibodies and the different subclasses of MA will be demonstrated much more clearly than was demonstrated by Pan *et al.*, (1999), who used ELISA for this purpose.

It was shown here that the novel method of immobilizing liposomes on a non-derivatised cuvette surface is problematic due to two main shortcomings. The first is that the two cells of the binary cuvette are often not equally responsive to mass accumulation from identical solutions. Calibrating the cells with a dilute serum solution prior to the actual measurement has been shown to overcome this problem (Thanyane, 2003), but it is expected that

automated pipetting may solve the problem to a large extent. The second is that, despite the cuvette surfaces being made of hafnium-oxide, a corrosion resistant compound, the surface of these cuvettes deteriorate during serial cycles of liposome immobilization, interaction analysis and regeneration. Cuvettes may last for up to forty regenerations but usually last for less than twenty (not shown). The integrity of cuvette surfaces is measured by the quality of the resonance scan, which was monitored throughout experiments, and was used to determine when a cuvette has become defect or exhausted. Since similar studies reported that up to fifty regenerations are possible with similar cuvettes (Altin *et al.*, 2001), the current number of regenerations possible is less than ideal and adds to the expensiveness of the technique. The harsh potassium hydroxide regeneration step has been blamed for this deterioration but was found to be a crucial step in the regeneration of the cuvette surface. It is expected that the re-design of the biosensor configuration to especially suit TB serodiagnosis may result in cuvettes that are either cheaper or less prone to deterioration.

Future work aims at using the biosensor to measure and calculate the quantitative binding properties of TB patient antibodies to the MA subclasses and the binding properties of the MA subclasses with cholesterol. The importance of cholesterol in the uptake of *M. tuberculosis* by macrophages has been illustrated by Gatfield & Pieters (2000). They indicated that cholesterol depleted macrophages were inhibited from infection by *M. tuberculosis* and also that *M. tuberculosis* binds cholesterol directly. Since complement receptor type 3 (CR-3) is responsible for receptor-mediated uptake of *M. tuberculosis* (Cywes *et al.*, 1997) and its activity mediated by the presence or absence of cholesterol, it was further suggested that the accumulation of cholesterol into cholesterol-rich microdomains, or lipid rafts, may be crucial to *M. tuberculosis*'s infectivity (Gatfield & Pieters, 2000). Many protein receptors are located in lipid rafts (Stulnig *et al.*, 2001) and their co-localisation with the lipid rafts is crucial for function (Simons & Toomre, 2000). Lipid rafts have been implicated in immune cell activation (Cherukuri *et al.*, 2001) and it is known that they play a role in the infectivity of HIV-1 (Campbell *et al.*, 2001). The importance of determining the molecular interaction affinities and mechanisms of the different MA-subclasses with cholesterol and their different antibodies, can therefore not be overstated.

Auto-immune diseases like the Guillain-Barré syndrome (GBS) are triggered by a specific kind of molecular interaction manifested by molecular mimicry (Moran, 1997). Guillain-

Barré syndrome is most commonly triggered by prior infections with *Campylobacter jejuni* (Winer *et al.*, 1988; Jacobs *et al.*, 1998) because the bacterial surface lipopolysaccharides (LPS) closely resemble gangliosides in the membranes of the host's peripheral nerves. This leads to cross-reaction of anti-LPS antibodies with the gangliosides, triggering the onset of GBS. In this study it was shown that the interaction of the antibodies with these gangliosides (glycolipids) could be followed with a biosensor method very similar to that used for determining the interactions of anti-MA antibodies with MA in TB patients. Although both gangliosides and MAs are lipids, gangliosides have a carbohydrate moiety that necessitated a different biosensor technique to measure the interaction between gangliosides and antibodies. It was discovered that the surface activation with a cationic detergent, cetyl pyridinium chloride, used for immobilisation of MA-liposomes, is not required when coating with G_{M1}-liposomes. This was explained by the absence of the long hydrophobic MA tails protruding from the MA-liposomes as opposed to the presence of the hydrophilic sugars coating the surfaces of G_{M1}-liposomes. Ganglioside-liposomes could spontaneously associate with the polar surface of non-derivatised cuvettes. Blocking of hydrophobic sites with saponin to prevent non-specific interactions was determined to be redundant as well. This was based on the following: the cationic detergent enables MA-liposomes to build up in layers on the cuvette surface, probably by insertion of the hydrophobic detergent tail in the liposome membrane. These non-uniform, unblocked surfaces are vulnerable to non-specific associations. If the surfaces are blocked, more specific binding can be measured. In G_{M1}-liposomes however, it is expected that the liposomes form a uniform bilayer on the surface. This theory is supported by observations with atomic force microscopy where it was shown that liposomes spontaneously flatten on a smooth glass surface, break open, slide over the bottom layer and fill available space until a uniform bilayer is created (Fisher & Tjärnhage, 2000; Deleu *et al.*, 2001). Excess liposomes are washed away. It is further supported by observations by Altin *et al.* (2001) who included a dilute sodium hydroxide washing step to lyse unbound liposomes for easy aspiration. Athanassopoulou *et al.* (1999) also noted that blocking of heterogeneous lipid bilayers containing G_{M1} and PC, formed on a hydrophobic surface, is unnecessary.

Infections other than that of *C. jejuni* or immunocompromising events like surgery or vaccinations may also trigger GBS (Ang, 2001). If the onset of GBS is so well explained by molecular mimicry between LPS and gangliosides, how can other infections or events trigger the disease? It is believed that the adaptive immune system constitutes a delicate

equilibrium of idiotypes (Bona, 1987). A disturbance in this so-called idiotype network causes an imbalance, which the body will try to correct. Failure to do so may have significant effects on the entire immune system. The disturbed idiotype network may then provide the pathway towards susceptibility to various infections and to immune compromise, triggering the onset of GBS or other auto-immune diseases. Idiotypes of complementary and opposing sets of antibodies in the idiotype network will be closely related and affinities for the same idiotype or antigen may be very similar. Although attempts have been made to give structure to the idiotype network concept (Perelson, 1989; Rossi *et al.*, 1989; Kazatchkine & Coutinho, 1993), it is not easily generalised and remains poorly understood. The biosensor technique developed and optimised in this study may be ideally suited to distinguish between closely related antibodies and provide crucial detail of how the idiotype network functions. To distinguish between idiotypes it is necessary that differences in affinity between antibodies can be measured. Using cholera toxin B subunit, a natural ligand for G_{M1} as a receptor, it was demonstrated that the affinity constant derived from the IAsys biosensor method performed here, correlated well with affinity constants generated by other biosensor methods (Athanassopoulou *et al.*, 1999) as well as isothermal calorimetry studies (Masserini *et al.*, 1992).

Initial studies with control and GBS patient sera showed sensorgrams for binding to G_{M1}. Prior calibration of the two cells in the IAsys cuvette by association analysis of diluted control serum to the immobilized G_{M1}-liposome coat allowed the semi-quantitative characterization of the sera. The technique was shown to be amenable to determine the anti-G_{M1} antibody activity in GBS patients at the peak of the disease and after their recovery. Comparing antibody levels during different stages of the disease may allow the elucidation of some of the complexities of the regulation of the idiotype network. If these idiotype antibodies (Ab1) can be isolated and immobilised on the biosensor cuvette surface and then used to assess affinities of the anti-Id antibodies (Ab2), even more of the idiotype network may be understood. The biosensor is currently the only technique that can be applied to elucidate Id-anti-Id interactions without the need for labelling one of the ligands.

It has already been shown that idiotypes can be immobilized on biosensor surfaces and the affinities of anti-Id antibodies against this accurately determined (Haimovich *et al.*, 1998). Understanding the idiotype network better should enable us to understand how seemingly unrelated infections and events can precipitate an auto-immune disease like GBS. It might

also be the explanation for observations that anti-HIV-1 antibodies increase HIV-1 infection of monocytes and lymphocytes (Robinson *et al.*, 1988) and that cross-reactivity exists between lymphocyte antigens and Id-anti-Id immune complexes (Root-Bernstein, 1995). Süsal *et al.* (1996) has already hinted at the auto-immune characteristics of acquired immune deficiency syndrome (AIDS) by demonstrating how different opposing sets of idiotypes or mimics of idiotypes can disturb the idiotype network. This has serious consequences for current Phase III clinical trials on vaccines based on a surface antigen like gp120 (Johnston, 2003). Introducing a self-antigen to generate antibody proliferation, the standard aim of a vaccination, may disturb the sensitive idiotype network equilibrium of immunocompromised individuals in such a way that it cannot recover. In the case of AIDS vaccines, where the antigen mimics MHC-structure of the host, this may lead to the progression to full-blown AIDS. Using GBS as a model, a better understanding of HIV/AIDS and other auto-immune diseases, in the context of the idiotype network, may lead to novel therapies to substitute or supplement existing drugs.

The expectation of the discovery of fine-specificity between antibodies and lipid antigens in auto-immune aspects of infectious diseases has been brought close to realisation with this study. Cross reactivity of antibodies between MA and cholesterol in TB, and ganglioside specificity of auto-antibodies in GBS can be characterised by biomolecular interaction analysis with biosensor technology. Immobilisation methods for lipid antigens and characterisation of antibody binding to these ligands have been amply demonstrated. The next focus level will be the characterisation of anti-MA antibodies to the individual members of MA subclasses and relating the binding properties of anti-G_{M1} and anti-anti-G_{M1} antibodies at different stages of GBS. This has all the potential of extending the current naive understanding of auto-immunity in infectious diseases to a universal comprehension of Id-anti-Id relationships. This may launch a new appreciation of the value of idiotype vaccination in seemingly incurable diseases – even a cure for AIDS.