2 Results

2.1 Selection of TF-specific antibodies by phage display

The strategies for selecting TF-specific antibodies from phage display repertoires appear straightforward. Different proteins displaying TF such as asialoglycophorin (see chapter 4.1.1), asialofetuin or TF coupled to BSA are commercially available to be used as targets, as well as the corresponding competitors not displaying TF (glycophorin, fetuin and BSA), that could be used to direct the selections toward TF. In addition, a large set of different oligosaccharides conjugated to polyacrylamide has become available (Bovin, 1998), and can be used in both selections and specificity screenings.

The selections were carried out as follows: Asialoglycophorin and TF-PAA were used as bait in normal pannings and TF-PAA-biotin as bait in biopannings (panning on soluble antigen (Hawkins et al., 1992)), in which the phage-TF-PAA-biotin complexes were captured with streptavidin-conjugated Dynabeads. Selections were carried out as three rounds of panning, biopanning or combinations of the two. The combination of traditional panning and biopanning whereby TF is displayed on 2 different carriers is important in order to avoid selection of antibodies toward dominating protein epitopes. In addition, the helper phage KM13 (Kristensen & Winter, 1998) was applied. This helper phage has a trypsin-sensitive site inserted between domain 2 and 3 of the minor phage coat protein III. In a selection set-up, elution with trypsin will simultaneously render non-displaying phage unable to infect and thereby decrease the background by several orders of magnitude. This enables effective selection in fewer rounds of panning, thereby maintaining diversity and avoiding selection bias toward dominating epitopes (Goletz et al., 2002; Jensen et al., 2003; Ravn et al., 2000).

In order to further direct the selections toward the TF moiety, the effect of including soluble competitors in the form of glycophorin or mixtures of irrelevant carbohydrate-PAA conjugates was tested.

Three different types of libraries have been selected as described below. The naïve semi-synthetic libraries, Griffin1, TomlinsonI and TomlinsonJ, offer the advantage that they can be applied in selections of antibodies against most potential targets due to their large diversities . Moreover, the selected antibodies are of human nature, and therefore preferable for therapeutic applications. Patient-derived scFv libraries could yield high affinity binders, and these antibodies would have the same advantages as the binders from the semi-synthetic libraries with respect to human origin. In contrast, antibodies selected from libraries, which are generated from immunised mice, may be immunogenic due to their non-human origin. On the other hand, the immunisation may bias the libraries toward high affinity binders.

2.1.1 Semi-synthetic scFv libraries, Griffin.1, TomlinsonI and TomlinsonJ

The scFv phage display libraries Griffin1, TomlinsonI and TomlinsonJ were selected for binders with affinity for TF. The libraries were selected applying a variety of the above-mentioned strategies. Although weak carbohydrate binders were identified by screening selected clones against asialoglycophorin in ELISA, none of the selected clones showed TF-specificity when tested against a panel of carbohydrate-PAA conjugates (data not shown).

2.1.2 scFv libraries from colorectal carcinoma patients

Serum and PBL samples from 10 colorectal carcinoma patients had been stored at -80°C until testing. The serum samples were tested for a possible antibody response against the TF-carriers asialoglycophorin and TFβα-PAA and the control proteins glycophorin and BSA. Most of the sera showed moderate reactivity to asialoglycophorin and TFβα-PAA at high serum concentration (**Figure 5**/data not shown). An exception was the serum from patient 188, which showed high reactivity to asialoglycophorin. To address the carbohydrate specificity of the antibodies in the sera, it was attempted to compete off the binding to asialoglycophorin by adding high concentrations of TFβα-PAA or *vice versa*. Furthermore, it was attempted to deplete the sera with TFβα-PAA-biotin and streptavidin-conjugated magnetic beads before testing in ELISA. Neither competition nor depletion had any significant effect on the ELISA signals (data not shown). Finally, the antibody responses were tested for their dependence of carbohydrate structures in a binding assay where the carbohydrate structures on asialoglycophorin and TFβα-PAA were disrupted. The assay is exemplified for 2 patient sera in **Figure 5**. Most of the sera bound equally well to antigens independent of the carbohydrate structures, and again all had binding-patterns very similar to that of patient 171. The exception was patient 188, for whom the serum exhibited high sensitivity to the disruption of carbohydrates on asialoglycophorin. In contrast, disruption of the carbohydrate structure on TFβα-PAA had no significant effect. Despite the carbohydrate disruption assay, the presence of truly TFβα-specific antibodies in the sera could not be established in polyclonal ELISA. This can only be addressed if larger amounts of sera are available for affinity purification of the antibodies, and thereby enabling further characterisation of purified fractions as described by Butschak (Butschak & Karsten, 2002).

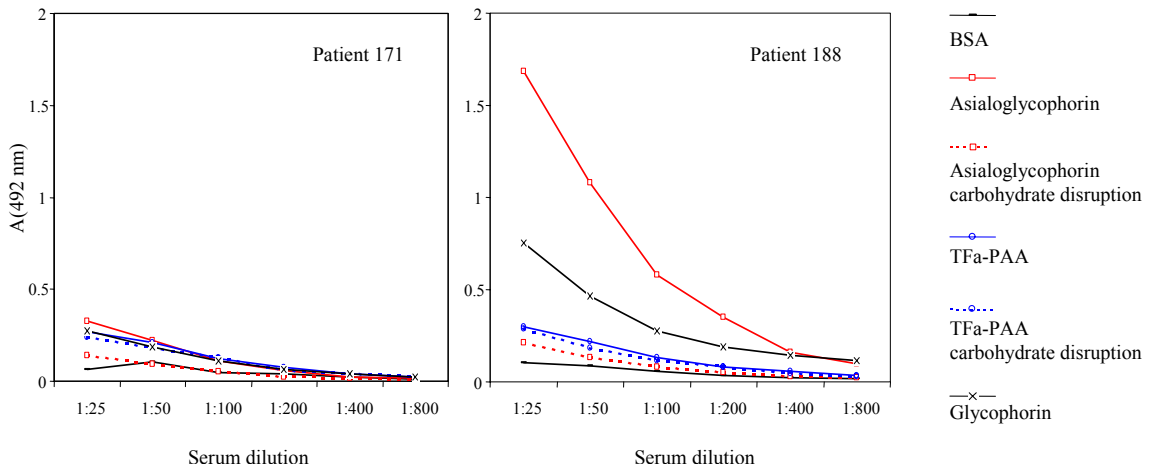

Figure 5 Antibody binding activity of human patient sera
The antibody response of 2 colon carcinoma patients was tested in ELISA in dilution series against two TF-carriers, asialoglycophorin and TFβα-PAA as well as BSA and glycophorin (solid lines). The carbohydrate structures on asialoglycophorin and TFβα-PAA were disrupted by treatment with sodium periodate and sodium borohydride (dashed lines).

2.1.2.1 Generation of scFv libraries from colorectal carcinoma patients

The number of PBLs in the stored samples differed notably among the different patients, ranging from 2×10^4 to 5×10^6. Consequently, all the PBLs were pooled, and mRNA extracted for single-stranded cDNA synthesis. Immobilisation of cDNA on magnetic beads enables retrieval of the cDNA after PCR amplification, allowing multiple PCR amplifications using all the cDNA as template and thus ensures maximal diversity. The quality of the samples was relative poor, probably due to the long storage at -80°C (5-10 years) necessitating extensive PCR amplification to obtain the needed amounts of variable genes. Still, a scFv library was cloned successfully in pHEN2 and transformed into TG-1 resulting in a library of 8×10^5 individual transformations. The insert ratio of the library was tested by PCR and found to be ~90% (N=20).

2.1.2.2 Selection of scFv libraries from colorectal carcinoma patients

Selections were carried out by panning on asialoglycophorin or by biopanning against the tumour-associated carbohydrate-PAA-biotin conjugates: Tn, Lewis^Y, TF, Forssman disaccharide, Sialyl Tn, sialyl Lewis^A and Sialyl Lewis^X. In all cases titre increases were observed in the outputs from the first round of selection (200-500) to the third round of selection (~10^6). Eventhough this is often used as an indicator for successful selection, none of the clones tested in ELISA (N=92 for each antigen after the third round) exhibited antigen-specific binding. Finally, polyclonal phage preparations from the output

of all the panning rounds were tested in ELISA, however again with no indication of binders (data not shown). Consequently, further selection and screening was abandoned.

2.1.3 Generation of scFv libraries from immunised mice

Taking into consideration that TF-specific antibodies in general have been found difficult to obtain, it was decided to investigate an alternative strategy, namely the combination of libraries generated from immunised animals with a new phage display format, display of multivalent scFv. The hypothesis was that since 1aa linkers in free scFvs drives the formation of trimeric complexes, it may also drive the formation of trimeric complexes of scFvs displayed on phage.

2.1.3.1 Generation of scFv libraries from immunised mice

Three Balb/c and two C3H/J mice were immunised with asialoglycophorin in the presence of cyclophosphamide boosted once and sacrificed 4 days after boost. The spleen cells from one Balb/c mouse and one C3H/J mouse were used in a separate attempt to generate hybridomas. Spleen cells from one Balb/c mouse and one C3H/J mouse were used for extracting RNA. Simultaneously, fractions of the splenocytes were cultivated in order to test the culture supernatants for antibody responses in ELISA (Karsten, 1984). The culture supernatant from the C3H/J splenocytes used for RNA extraction indicated the presence of TF-binding antibodies, whereas the supernatants from the other mice were negative (data not shown). The spleen cells from the remaining Balb/c mouse were cultivated for 2 days before RNA extraction. Half of the cells were cultivated in the presence of 2 µg/ml asialoglycophorin for a potential specific boost. The culture supernatants from these cells were not tested for anti-TF antibodies.

Single-stranded cDNA covalently bound to magnetic beads was prepared from the RNA that was extracted from the four splenocyte preparations. The PCR amplification on the cDNA from the C3H/J mouse gave very limited amounts of variable genes, whereas all 3 Balb/c preparations resulted in several micrograms of PCR product.

Two scFv libraries were prepared in parallel from each of the cDNA preparations. ScFv libraries in the standard 18aa-linker format were constructed using the restriction sites in pHEN2, whereas scFv libraries in the 1aa-linker format were constructed as outlined in **Figure 6**.

All the libraries contained more than 5×10^6 individual transformations. PCR on single clones indicated insert ratios of 90 - 95% (N = 30, respectively) while sequencing indicated good diversity of the libraries as no identical clones were identified (N = 16, respectively).

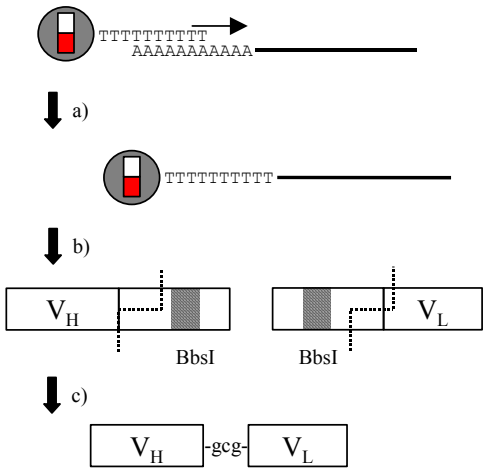

Figure 6 Schematic presentation of library construction.
The mRNA was captured on oligo(dT25) magnetic beads, and single-stranded cDNA synthesised using the beads as template (a). The variable genes were amplified using the whole pool of cDNA as template in subsequent PCR reactions (b). The variable genes were digested with BbsI, and ligated to form scFv genes (c), which were subsequently cloned into pHEN2.

2.1.3.2 Selection of scFv libraries from immunised mice

The generated libraries were selected by one round of panning on asialoglycophorin followed by one round of biopanning using TF-PAA-biotin as bait. From each selection of the 1aa linker libraries, 10^3 to 10^4 clones were retrieved after the second round of panning. Ninety clones from each library were tested in phage ELISA in a primary screening against asialoglycophorin. For the libraries generated in the scFv(1aa) format, this screening indicated 8 binding clones from the library from the non-cultivated spleenocytes, and 16 and 12 binding clones from the two libraries generated from the cultivated splenocytes, the "stimulated" and "non-stimulated" libraries, respectively. A more extensive phage ELISA screening against glycophorin, asialoglycophorin, TF-PAA and BSA revealed 3 binders from the "non-stimulated" library, and 6 from the "stimulated" library. No clone from the libraries from the "non-cultivated" spleen cells was able to bind both asialoglycophorin and TF-PAA without also binding to glycophorin and BSA. The primary screening of selections with the scFv(18aa) libraries indicated 5-10% binders, but all clones failed the following test for specificity. Sequencing of the 9 clones with specificity to asialoglycophorin and TF-PAA revealed the same complementarity-determining regions as well as V_H and V_L framework genes except for some variation in the primer-encoded regions. This strongly implies that the sequence variability for TF-specific antibodies is very narrow.

In contrast, several clones recognising peptide epitopes (asialoglycophorin and glycophorin) or masked peptide epitopes (asialoglycophorin) of glycophorin were selected from the libraries (data not shown; clone 4F1 to be described below).

Table 3 Summary of the first selections

Spleenocytes	Non-cultivated		Cultivated			
			Stimulated		Non-stimulated	
Format	1aa	18aa	1aa	18aa	1aa	18aa
Selection output	~1000	~3500	~10000	~2000	~8500	~1500
1. screen	8	9	16	13	12	14
2. screen	0	0	6	0	3	0

scFv libraries were constructed from spleenocytes isolated from mice. mRNA was extracted directly after isolation (Non-cultivated) or after 2 days in culture. Half the cultivated spleenocytes were stimulated with 2 μg/ml asialoglycophorin. The output after the 2 rounds of selection of the libraries was counted as cfu. In the first screen, 90 clones from each library were picked by random and screened for binding to asialoglycophorin in ELISA. In the second screen, the positive clones were screned for binding to asialoglycophorin and TF-PAA, and no binding to glycophorin and BSA.

In search for additional clones with TF specificity, the "stimulated" and "non-stimulated" scFv(1aa) repertoires were pooled and a new first round panning against asialoglycophorin was performed. This was followed by 2 rounds of biopanning against TF-PAA-biotin using 1 μg/ml in the second round and 100 ng/ml in the third round in order to increase the stringency. Around 1500 clones were retrieved after the third round of which 920 were picked into 96-well microtiter plates. These clones were again screened against asialoglycophorin in phage ELISA indicating 322 binding clones. Subsequently, they were also tested against TF-PAA and BSA leading to the identification of 70 selected clones which were produced in 10 ml cultures and tested simultaneously against asialoglycophorin, glycophorin, TF-PAA, and BSA, resulting in the identification of 25 clones with TF-specific binding. Again, they all encoded the same V_H and V_L framework genes and the same complementarity-determining regions with variations only in the primer encoding regions. A total of 9 primer-sequence combinations were identified, of which one sequence was found in 18 of the 34 clones, and therefore chosen as the main candidate for further investigation. This will be referred to as scFv(1aa).

2.2 Characterisation of different scFv formats on phage particles

2.2.1 Effect of linker length on activity of phage displayed-scFvs

The libraries constructed so far were restricted to the 1aa-linker format or the 18aa-linker format, which may not be the optimal linkers with respect to expression, folding and stability of the scFv when displayed on phage. To address the properties of the repertoires generated in the 1aa-linker format, it was decided to investigate the properties of constructs with other linker length. The selected scFv sequence was subcloned into formats covering all linker lenghts, from 0aa to 9aa, and 18aa, and

inserted in pHEN2, to enable phage production. The phages expressed from clones with linkers of 0aa to 5aa gave higher signals when tested for their binding ability in phage ELISA (**Figure 7**).

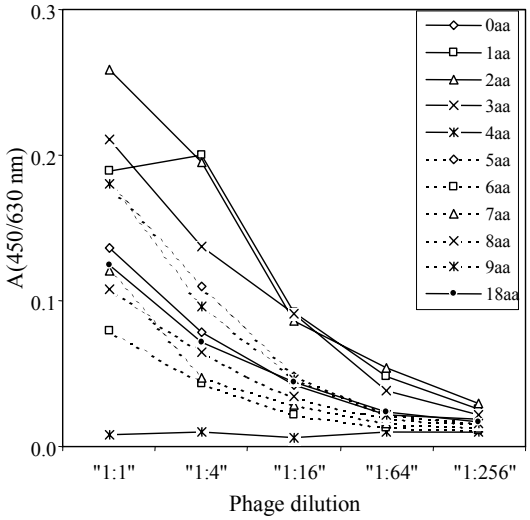

Figure 7 Binding activity of different scFv constructs displayed on phage.
ScFv constructs with various linker length were displayed on phage and tested as cleared culture supernatants in ELISA in the indicated serial dilutions against asialoglycophorin (10 µg/ml) in order to assess the binding capacity; N=2.

2.2.2 Investigation of displayed scFvs

An interesting aspect is whether the displayed scFvs are displayed as multimers of fusion proteins, that is by multimerisation of several full-length scFv-pIII-fusion proteins, or displayed as multimers consisting of a mixture of scFv-pIII-fusion proteins and non-fused scFv. To investigate this, phage particles were produced and precipitated twice with NaCl/PEG. Double precipitation removes proteins not associated with the phage (de Wildt et al., 2002), and any constitutional differences in the displayed complexes should be revealed by Western blot analysis. The concentration of the precipitated phages was normalised by UV measurement. This gives a more precise measure of the amount of phage particles than standard titration, because the method is not dependent on infection efficiency or packing of helper phage genomes. The phage were analysed by Western blots using either detection of the scFv via myc-tag or detection of pIII (**Figure 8**). The blots showed bands of pIII and scFv-pIII fusion proteins for all constructs with no major visual difference in intensity between them. All constructs seemed to be associated with unfused scFv and again with only minor visual differences in intensity. Therefore it appears that there is no significant difference in stoichiometry of scFv to scFv-pIII fusion proteins displayed on the phage. The strength of the association between the un-fused scFv and the phage was probed by washing the phage with up to 4 M urea applying spin filtration. Again free scFv was detected in all constructs, and there seemed to be no significant difference in quantity among them (data not shown). Interestingly, the binding activity of the urea-

washed phages suggested that the scFv(2aa) might be the most stable format for phage display as it was superior in the binding assay after the incubation in 4 M urea (**Figure 9**).

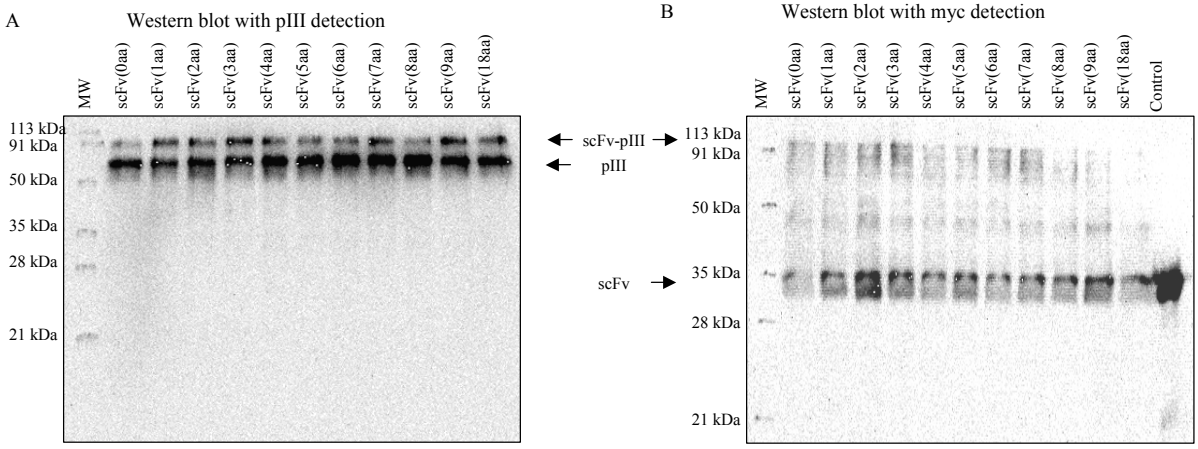

Figure 8 Western blot of phage particles
Phage particles were precipitated and concentrations normalised before analysing on Western blots. Minor phage protein pIII and pIII fusion proteins were detected with the mouse anti-pIII antibody (pSKAN3) (A), while scFv and scFv-fusion proteins were detected with the mouse anti-Myc antibody 9B11(B). Both secondary antibodies were detected with anti-mouse/HRP.

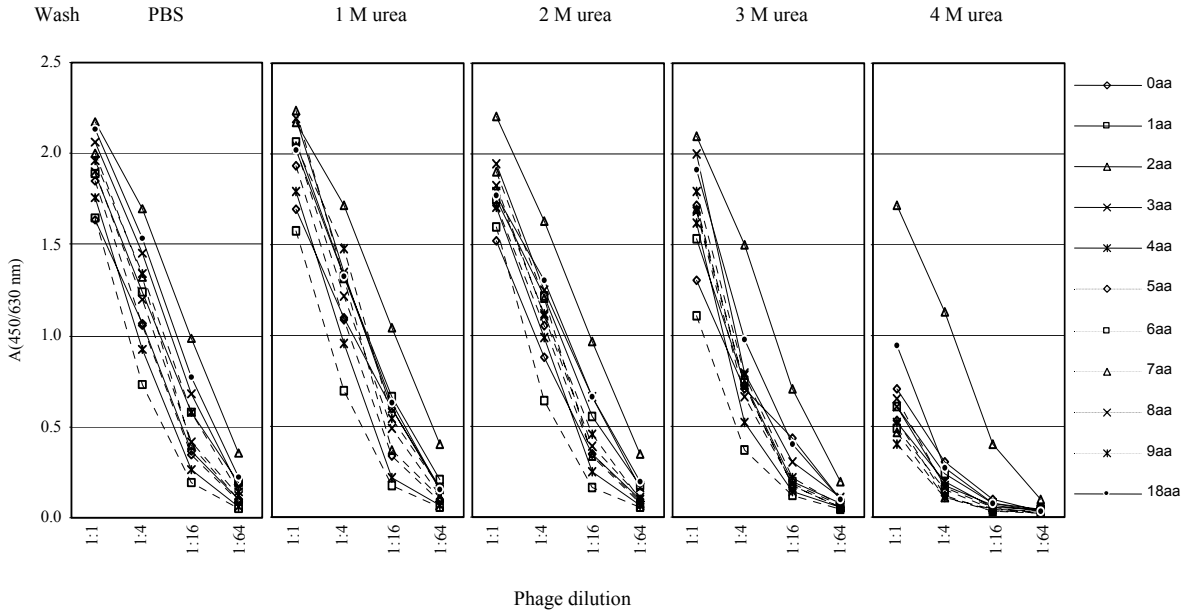

Figure 9 Binding activity of precipitated phage-displayed scFv constructs measured in ELISA.
Phage supernatants were PEG/NaCl precipitated, resuspended in PBS supplemented with increasing concentrations of urea, and incubated at room temperature for 1 h before a second PEG/NaCl precipitation. Phage concentrations were normalised by UV measurement prior to application as serial dilutions in ELISA against asialoglycophorin (5 µg/ml), N=2.

2.3 scFv expression, purification and characterisation

2.3.1 Optimisation of bacterial protein expression

The production of multimeric scFv was investigated using 3 different strains of *E. coli.* (TG1, BL21(DE3)pLysS and Rosetta™(DE3)pLysS) in combination with 3 different plasmids (pHEN2, pUC119 and pET11a) encoding the scFv(1aa), the PelB leader and the His6 and myc affinity tags. Expression was evaluated in small 50 ml cultures and samples of cells pre-induction, cells post-induction, and TES fractions were evaluated by SDS-PAGE (**Figure 10**).

Based on expression level, the pET11a plasmid in combination with the Rosetta™ clearly had the highest potential, although most of the expressed protein was directed to inclusion bodies. It is noteworthy that induction of expression from the pET11a vector is so strong that it inhibits the growth of the bacteria.

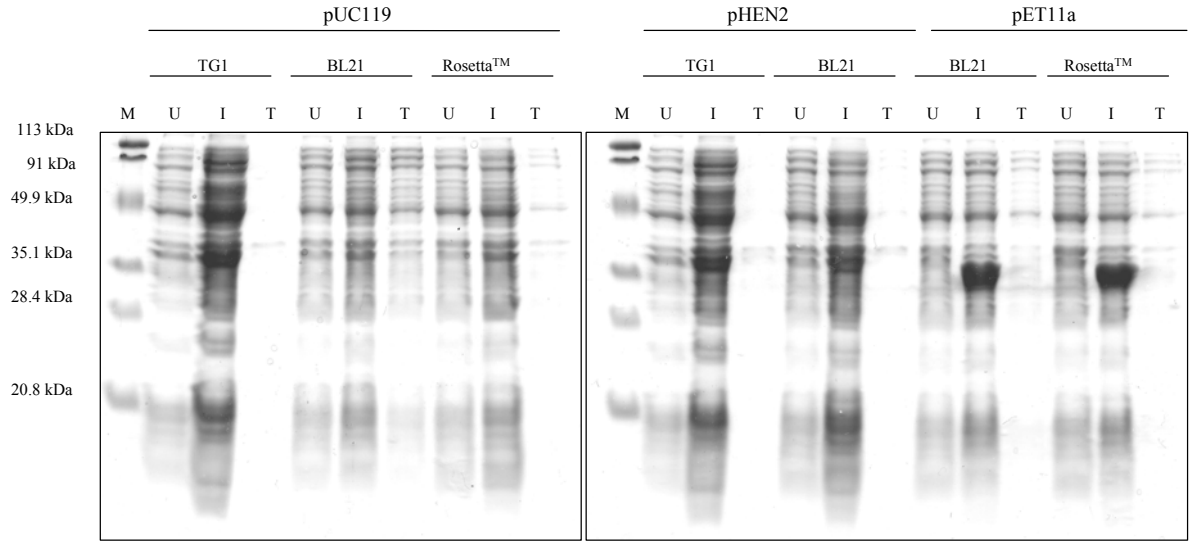

Figure 10 SDS-PAGE of different bacteria and expression vector systems
The scFv(1aa) was cloned into 3 different expression vectors and expression analysed in 3 different *E.coli* strains. Samples were taken before induction (U) after 3-h induction with 1 mM IPTG (I) and after preparing TES fractions (T). The samples were normalised to culture volume before analysing by SDS-PAGE and coomassie staining.

All the different linker length constructs (scFv(0aa) to scFv(9aa) and scFv(18aa)) were cloned into the modified pET11a (chapter 4.1.5) and expressed in Rosetta™. Crude TES fractions were prepared and tested as dilution series in ELISA against asialoglycophorin (**Figure 11a**). The scFv(0aa) and the scFv(1aa) showed the highest binding activity in this assay. The amount of recombinant protein in the TES fractions was compared on Western blots, showing that the higher binding activity of the scFv(0aa) and sFv(1aa) were not due to increased amounts of protein (**Figure 11b**). These data

correlate well with the data from the ELISA testing the binding of the various scFv formats displayed on phage (**Figure 7**).

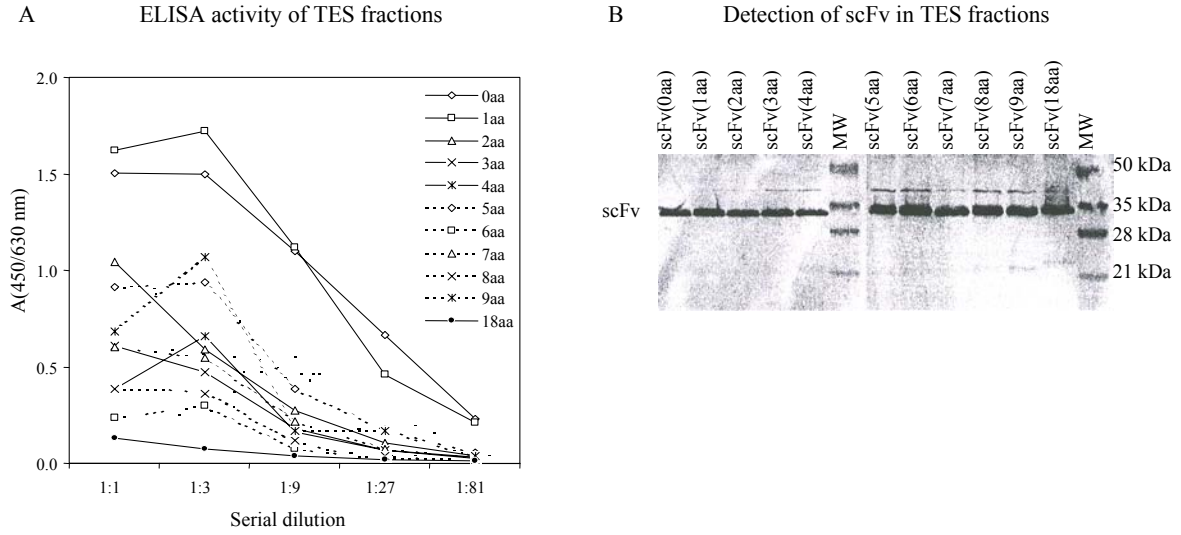

Figure 11 Analysis of soluble protein expressed in bacteria
Crude extracts (TES fractions) from bacteria expressing the different scFv constructs were analysed for binding activity in ELISA against asialoglycophorin (A); N=2, and for the level of scFv expression on Western blots (B).

2.3.1.1 Purification of insoluble protein

Immobilised Metal Affinity Chromatography (IMAC) purification (described in detail below, chapter 2.3.2.1) of the scFv(1aa) using a standard expression and purification protocol yielded 250-500 µg/l culture. However, for large-scale production, it would be highly preferable if the large amounts of recombinant protein directed to the inclusion bodies in Rosetta^{TM} could be retrieved as active protein via refolding (estimated to 2-10 gram/l culture, see **Figure 10**). A number of refolding-strategies have been tested, which were all based on solubilisation of the recombinant protein from inclusion bodies using high concentrations of chaotropic salt such as urea or guanidine hydrochloride. Some of the parameters in the refolding protocols that have been investigated include variable concentrations of chaotropic salt, the presence of a reducing agent (DTT), and refolding during dialysis or chromatographic purification (IMAC). Although the protein was easily purified (**Figure 13**), all attempts to refold the scFv(1aa) have failed so far, and there has been no indication of any binding activity in the "refolded" fractions. Therefore, more priority was given to increasing the amount of soluble protein.

2.3.1.2 Optimisation of cultivation conditions

It is known that cultivation temperature or supplementation of culture medium may have an influence on the amount of active protein expressed, and these parameters were therefore evaluated for their effects. At high cultivation temperature (37°C), most of the recombinant protein produced upon induction is directed to inclusion bodies, whereas at lower cultivation temperatures (30 or 25 °C) the bacteria may be able to process the protein better. A lower cultivation temperature may therefore increase the amounts of protein available in an active and soluble form. This was indeed observed for the scFvs expressed, and led to the conclusion that the optimal cultivation temperature was 25 °C.

Supplementation of the culture medium has been tried with glycerol, ethanol, and sucrose. Glycerol may function as a carbon source but can also have a stabilising effect on the hydrophobic patches of the expressed proteins. However, supplementation with glycerol (1-10%) only resulted in decreased growth rates, presumably due to the increased viscosity of the media. Supplementation with ethanol has already been shown to increase the levels of bacterial chaperones (Thomas & Baneyx, 1997). But with final concentrations between 0 and 10% ethanol, the only effect observed was reduced growth rates. No increase in binding activity was observed for the TES fractions, and the binding activity in the culture supernatant was completely lost.

Kipriyanov and co-workers have investigated the effect of supplementation with sucrose (Kipriyanov et al., 1997; Kipriyanov et al., 1998). They found that supplementation with 400 mM sucrose led to an 80-150 fold increase in soluble protein for shake-flask cultures. For the scFv(1aa) expression in Rosetta^{TM} the observations were slightly different. As with glycerol, higher concentrations of sucrose increased the viscosity of the media, resulting in a slower growth-rate **Figure 12a**. The amount of active protein secreted into the media did increase at higher concentrations, but not to an extent where it justified the large amount of sucrose needed (400 mM) **Figure 12b**. At lower concentrations (50 mM) the amount of active protein in the periplasm did increase, while the binding activity in the media decreased. This indicates that 50 mM sucrose may somehow stabilise the membrane of Rosetta(DE3)pLysS and therefore retain the protein in the periplasm. For low-yield systems, it is profitable to harvest the protein in minimal volumes for easier and faster processing, and it is therefore an advantage to have localised periplasmic expression. The effect of sucrose was also tested at 30 and 37°C, but the effects at higher temperature seemed less significant and less consistent.

Consequently, scFvs were produced in 5L shake-flasks in 2 × TY supplemented with amp, 1% glucose and 50 mM sucrose at an incubation temperature of 25 °C. After overnight induction, the soluble protein was harvested in TES fractions by osmotic shock disruption of the cells.

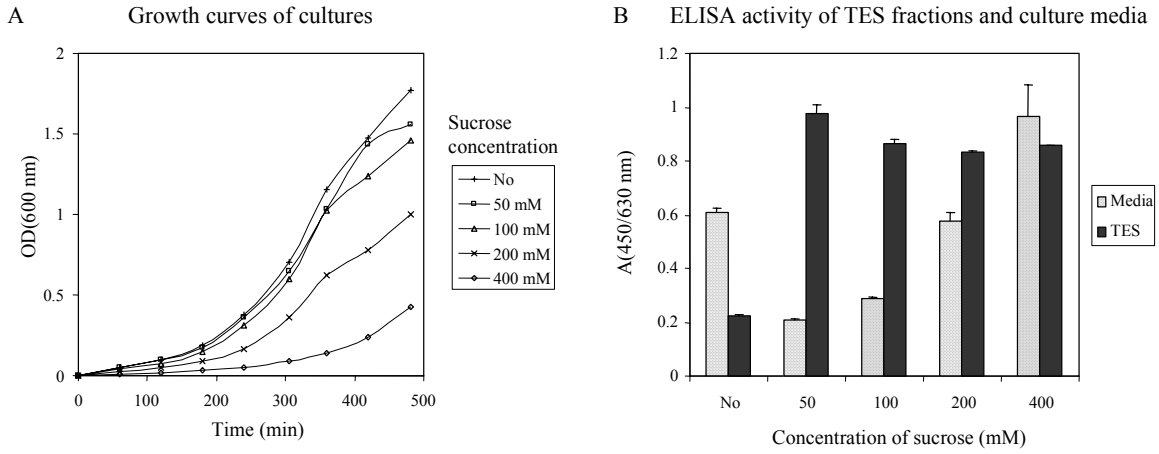

Figure 12 Cultivation at 25°C with supplementation of sucrose
Growth curves at 25 °C in the presence of sucrose. Cultures were inoculated from overnight culture, and incubated at 25 °C. At $OD_{600\ nm}$ ~ 0.6 the cultures were induced with 0.5 mM IPTG and incubated overnight at 25 °C. The optical density of the cultures was measured at $OD_{600\ nm}$ and plotted in (A) as growth curves. Following the overnight induction, the cultures were harvested and TES fractions prepared. The final volumes of the TES fractions were 10% of the culture volume, and the TES fractions were consequently diluted 10 fold before applying in ELISA against coated asialoglycophorin (5 μg/ml). Bound scFv was detected with Tetra-His/HRP (B).

2.3.2 Protein purification

Following expression of the scFvs and harvesting in TES fractions, various purification procedures were established based on 3 different chromatographic strategies. The scFvs are expressed with a hexa-his tag and immobilised metal affinity chromatography (IMAC) was therefore the first purification procedure to be established. Due to the high demands to purity of protein used to develop a candidate for radioimmunotherapy, additional purification procedures were investigated. Purification based on ion exchange chromatography using various matrix materials was investigated (chapter 2.3.3.7) as well as a purification based affinity chromatography using an antigen based matrix (chapter 2.3.3.8).

2.3.2.1 Immobilised metal affinity chromatography (IMAC)

Purification of proteins expressed with a hexa-his tag using immobilised metal affinity chromatography is based on the ability of histidines to coordinate a metal ion that is immobilised to the matrix. The protein is thus retained in the matrix, but can be eluted by eluting the metal ions (addition of EDTA) or by adding imidazole that interrupts the coordination of the lysines. The procedure was first established for the scFv(1aa) because the scFv(1aa) seemed to be the optimal candidate at that time. Soluble scFv(1aa) was purified by IMAC from TES fractions of 4.5 l

expression cultures. An open column system was used because it enabled efficient and easy removal of air bubbles caught in the column resin due to the high concentrations of DNA present in the TES fractions. Optimisation of the washing procedures in order to deplete contaminating proteins was investigated by monitoring the effect of a step-wise increase of the imidazole concentration of the washing buffer. Minor loss of scFv(1aa) was observed after washing with large volumes of buffer (>100 ml) containing up to 50 mM imidazole or/and small volumes of buffer (>10 ml) of up to 70 mM imidazole. Elution of the retained protein was achieved by increasing the imidazole concentration to 300 mM, and the eluted protein was subsequently dialysed into PBS. Samples of the chromatographic steps in a purification of scFv(1aa) have been visualised by SDS-PAGE and coomassie staining in **Figure 13**. The scFvs with other linker lengths were purified according to the same production and purification protocol, and all the scFvs were more than 95% pure according to SDS-PAGE analysis (**Figure 13**).

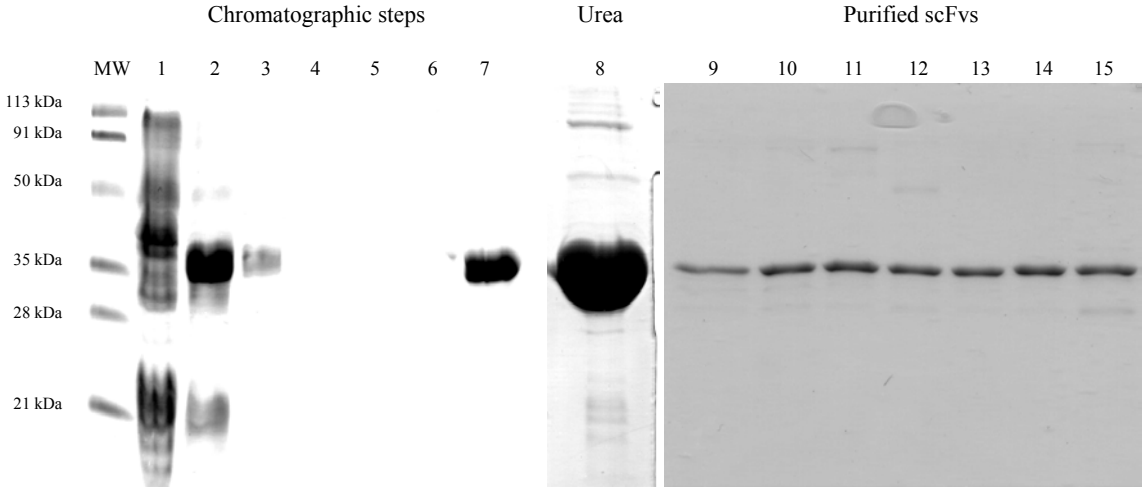

Figure 13 Protein purification of scFv(1aa) from *E.coli*.
Coomassie stained SDS-PAGE of different steps of the protein purification: MW, marker; 1, *E.coli* extract before induction; 2, *E.coli* extract after induction with IPTG; 3, Crude TES fraction before dialysis; 4, TES fraction after dialysis; 5, 70 mM wash of the IMAC chromatography; 6, Fraction in the elution; 7, Eluted scFv(1aa) after PBS dialysis; 8, urea extracted and IMAC purified scFv(1aa); 9-15, purified scFv(0aa), scFv(1aa), scFv(2aa), scFv(3aa), scFv(4aa), scFv(9aa) and scFv(18aa), respectively. The apperant doublebands in lanes 3-7 are probably caused by the salt concentrations, as they are not seen in the dialysed proteins in lanes 9-15.

The production of the scFv(1aa) was tested for reproducibility by assessing the binding activity of individual preparations in ELISA against asialoglycophorin (depicted for 4 consecutive productions in **Figure 14a**) and revealed similar activities for all preparations. It was found that the scFv(1aa) could be stored for at least 5 month at 4°C without detectable change of binding activity. In addition, freezing at -80°C was not associated with any loss of binding activity, and was not dependent on the supplementation of glycerol (**Figure 14b**). The binding activity of the purified scFv constructs with

various linker lengths was tested in ELISA against asialoglycophorin, and revealed the same pattern observed for the crude bacterial extracts **Figure 15** (see **Figure 11** for comparison).

Production of the different scFv(1aa) clones (variations in the primer encoded regions, chapter 2.1.3.2) was investigated, but only minor differences in yield and no significant differences in ELISA activity were observed between the different clones. This indicates that the variations in the primer regions are not vital for the binding activity.

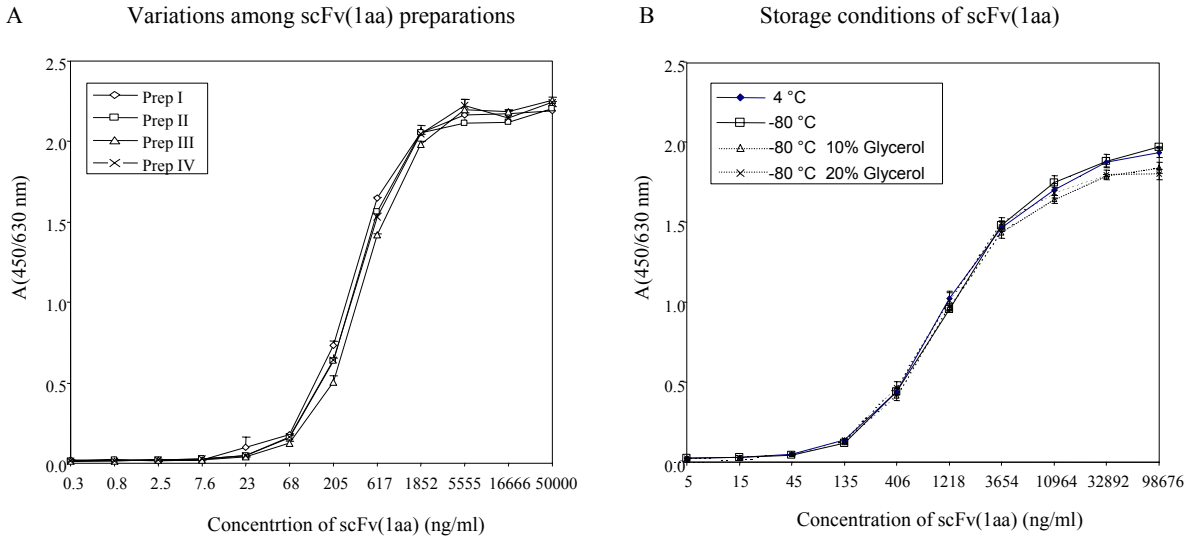

Figure 14 Production variation and storage conditions of the scFv(1aa)
Four consecutive productions of scFv(1aa) were tested in ELISA for binding to asialoglycophorin (5 µg/ml) to demonstrate the reproducibility of the productions (A), N=2. The binding activity of the protein after freezing to -80°C was tested in ELISA against asialoglycophorin (1 µg/ml) (B), N=2

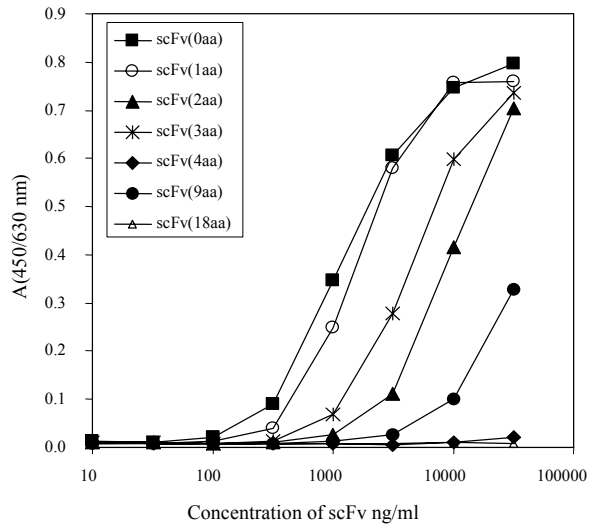

Figure 15 Binding activity of purified scFv constructs in ELISA
The purified scFv constructs, scFv(0aa) to scFv(4aa), scFv(9aa) and scFv(18aa), were tested as dilution series in ELISA for binding to asialoglycophorin (1µg/ml), N=2

2.3.2.2 Summary of yields in scFv preparations

After standardisation of the protein expression and purification, an average of 5.8 mg scFv(1aa) was purified by IMAC from cultures of 4.5 l (N=74). In comparison, the scFv(0aa) expressed and purified with lower yields, 1-1.5 mg pr 4.5 l culture, while the scFv(2aa) expressed and purified well with an average yield of 10 mg pr 4.5 l culture (N=3). The purification procedures using ion exchange chromatography (HiTrap^{TM} Q HP, AEC) and affinity chromatography (TFβα-PAA sepharose, AC) are described in chapter 2.3.3.7 and 2.3.3.8, respectively, but the yields are summarised here to provide an overview (**Table 4**).

Table 4 Summary of purification yields

	IMAC (mg/4.5 litre)	AEC (% retrieved)	AC (% retrieved)
scFv(0aa)	1-1.5 mg (N=22)	>15% (N=7)	~50% (N=5)
scFv(1aa)	~5.8 mg (N=74)	50-60% (N=22)	~15% (N=32)
scFv(2aa)	~10 mg (N=3)	30-40% (N=1)	<5% (N=2)

Yields of various purifications. For the IMAC-purification, the yield is correlated to culture volumes, whereas the yield for the anion exchange chromatography (AEC) and affinity chromatography (AC) purifications are expressed as percent of input (IMAC-purified).

As seen, the scFv(1aa) and the scFv(2aa) were purified with relative high yield by anion exchange chromatography, whereas the highest yield in the affinity chromatography with an immobilised TF matrix was achieved with the scFv(0aa) (this difference will be further elaborated in chapter 2.3.3.7 and 2.3.3.8)

2.3.3 Protein characterisation

The purified scFv(1aa) was characterised using a range of different methods. The binding activity and specificity was assessed in ELISA and subsequently tested in immunocytochemistry or immunohistochemistry. Finally, the constructs have been evaluated with respect to valency by size exclusion chromatography and affinity by surface plasmon resonance.

2.3.3.1 Extensive analysis of the fine-specificity of the scFv(1aa) in ELISA

The first step of protein characterisation, namely the binding activity in ELISA, has already been described above as a quality-control step in the protein production (**Figure 15**). In the ELISA, the antigens are coated on a plastic surface, and the scFvs subsequently probed for binding. Finally, the bound antibodies are detected with enzyme-conjugated secondary antibodies. Asialoglycophorin was chosen as antigen due to its high content of TF (see chapter 4.1.1). The TF-specificity of the purified scFv(1aa) was tested in ELISA for binding to asialoglycophorin with glycophorin as a negative control. Furthermore, the fine-specificity was determined by probing for binding to an extensive array

of commercially available carbohydrate-PAA conjugates (**Figure 16**). Binding in this ELISA was detected with an anti-His-tag antibody, tetra-His/HRP. Strong binding was detected towards asialoglycophorin and TFβα (the natural core-1 structure) conjugated to PAA via a flexible linker (-$OCH_2CH_2CH_2NH$-) or a more constrained linker (-OC_6H_4NH-). Furthermore, minor binding was observed to core-2 (GlcNAcβ1-6[Galβ1-3]GalNAcα) and Galα1-4GlcNAcβ. The binding activity toward TFβα and core-2 was confirmed in a similar specificity test where the scFv was detected by the second affinity tag, using the anti-myc antibody, 9E10 (data not shown). In contrast, no binding to Galα1-4GlcNAcβ was observed, and a cross-reactivity of the Tetra-His/HRP antibody towards this antigen was subsequently demonstrated.

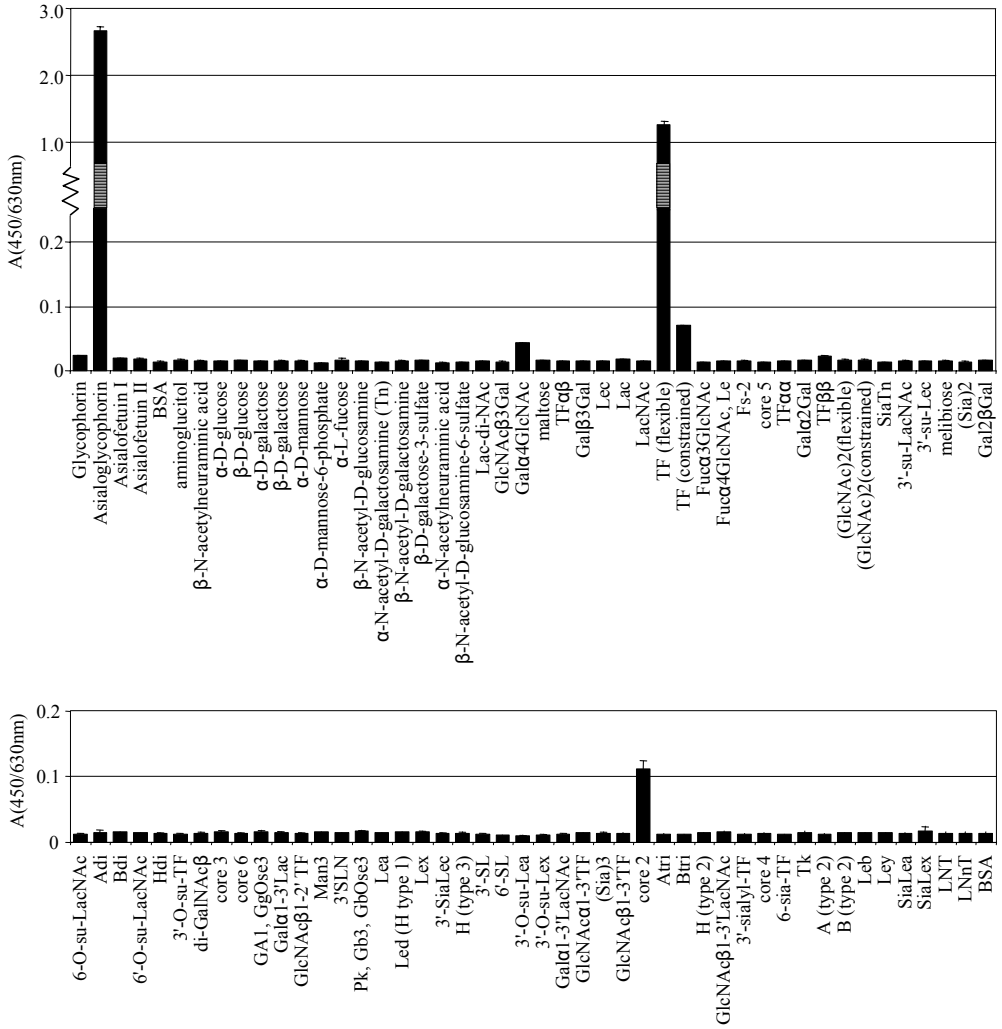

Figure 16 Fine-specificity of scFv(1aa)
ScFv(1aa) was tested in ELISA for binding to a large panel of antigens to access the fine specificity. Proteins and carbohydrate-PAA conjugates were coated at the solid phase and probed for binding with the scFv(1aa). Detection of the scFv(1aa) was performed with tetra-His/HRP. N=2

2.3.3.2 Analysis of the specificity of clone 4F1

The clone 4F1 was selected from the "stimulated" library using asialoglycophorin as bait (chapter 2.3.3.2). The 4F1 was demonstrated to bind asialoglycophorin, but not glycophorin nor a panel of carbohydrate-PAA conjugates including TF (**Figure 17**), indicating that 4F1 binds a carbohydrate dependent epitope or a masked peptide epitope. Sequencing revealed that the V_L of the 4F1 is identical to the V_L of the scFv(1aa) whereas the V_H sequences were slightly different (74% identical residues, with 13 differences in the framework regions, 4 differences in HCDR1, and 4 in HCDR2, and a completely different HCDR3). Sequences are not shown to protect patent filing.

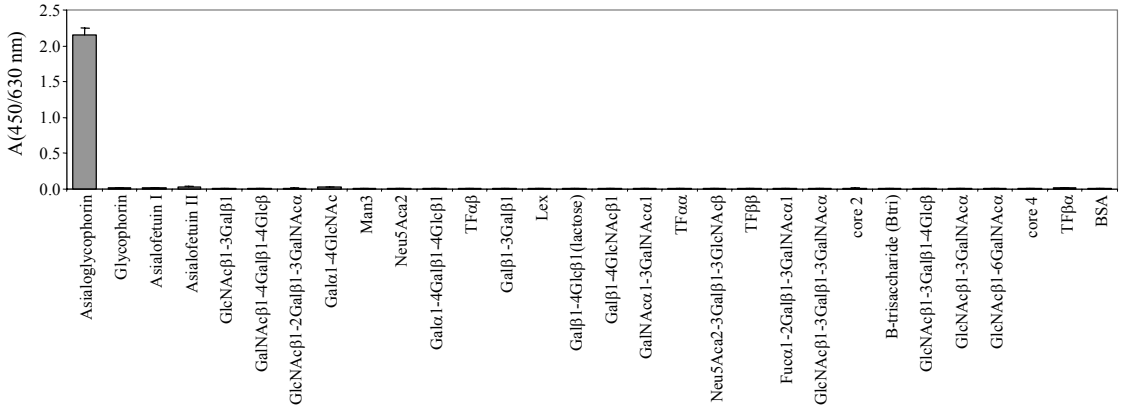

Figure 17 Fine-specificity of 4F1
The 4F1 antibody (scFv(1aa)-format) was produced and purified to testing the fine-specificity in ELISA similar to that in **Figure 16**. Selected antigens from the test are depicted. N=2.

2.3.3.3 Immunocytochemistry with the scFv(1aa)

Targeting of natural cell-surface displayed TF with the scFv(1aa) was tested in immunocytology with a set of TF-positive and negative cell lines. Bound scFv(1aa) was detected with an anti-myc antibody (9B11) and subsequently with an anti-mouse-Cy3 antibody. The binding pattern to the cell line KG-1 (acute myelogeneous leukemia) revealed approximately 50% positive cells (varying from strong to weak) with a punctiform pattern of signals (**Figure 18B)**. This is an identical staining pattern compared with the mouse mAb, A78-G/A7 shown in **Figure 18A** (Karsten et al., 1995). Very few stained cells (<<0.1%) were observed for the K-562 (malignant erythropoitic cells from a chronic myelogenous leukaemia), while the two glycoengineered cell lines NM-D4 and NM-F9 (see chapter 4.1.4) both contained very high levels of stained cells (80-100%). Good staining was also observed with the adherent ZR-75-1 breast cancer cell line, while the non-adherent ZR-75-1 cell line showed low staining percentage (<<0.1% of the cells were stained). This is again very similar to the binding pattern of the A78-G/A7 (data not shown).

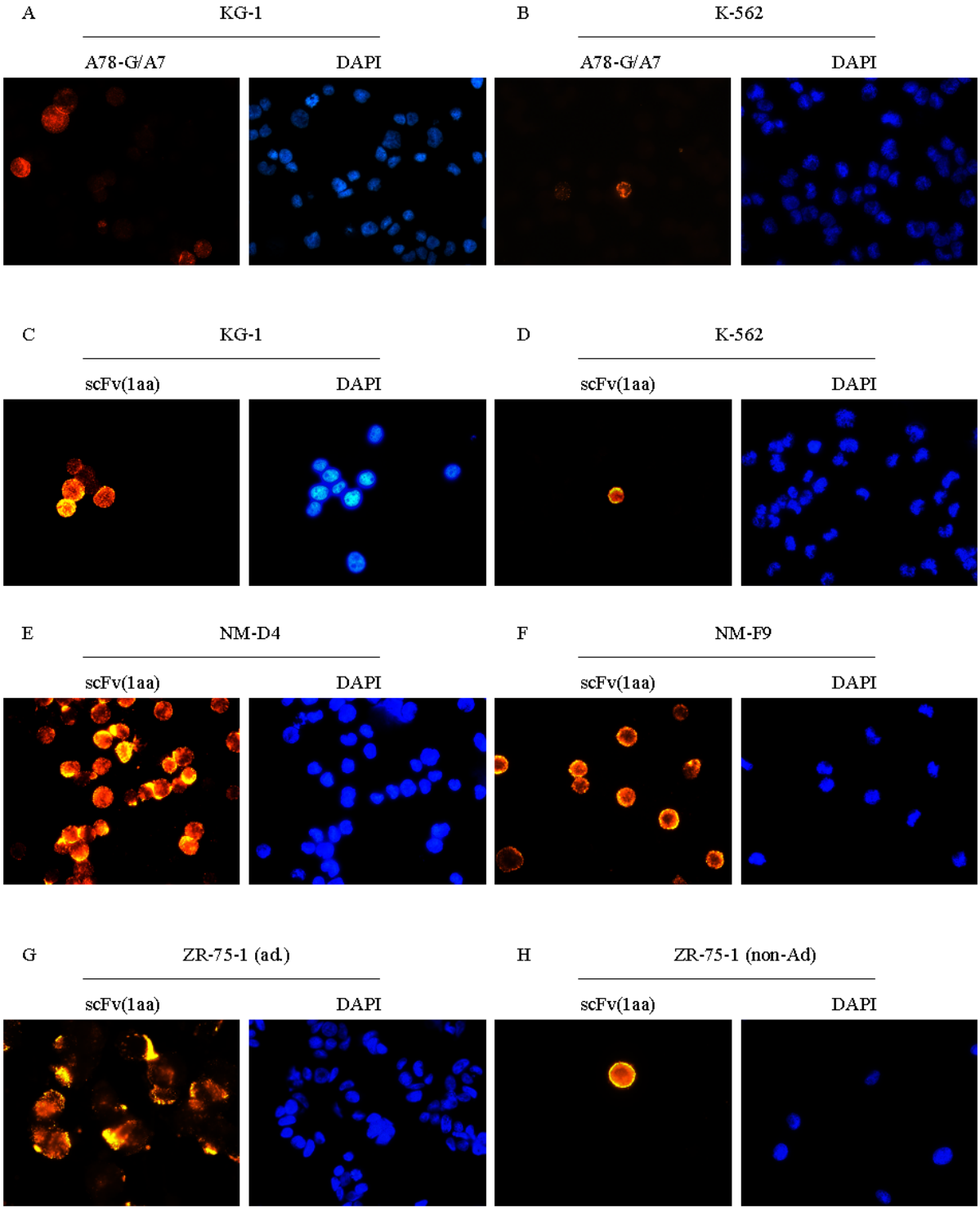

Figure 18 Immunocytochemistry with scFv(1aa)
The scFv(1aa) was applied in immunocytochemistry for detection of cell-surface displayed TF. All cell lines were stained with scFv(1aa), A78-G/A7 (positive control) and scFv(L36) (negative control). Two stainings are shown with the A78-G/A7 to illustrate the positive controls (A and B), while C to H show stainings with the scFv(1aa). All cell lines exhibited similar staining patterns for the A78-G/A7 and the scFv(1aa). None of the cell lines were stained with scFv(L36) (Sanz et al., 2003; Sanz et al., 2002; Sanz et al., 2001) (not shown). Positive control stainings with the A78-G/A7 were detected with anti-mouse-Cy3 antibody, while the scFv stainings were

detected with anti-Myc antibody 9B11 and subsequently anti-mouse-Cy3 antibodies. Counterstaining of nuclei was performed with DAPI.

2.3.3.4 Immunohistology of human colon carcinoma with the scFv(1aa)

Immunohistology was performed on cryosections from human colon carcinoma patients. The sections were stained with the scFv(1aa) and detected with mouse anti-myc 9B11 and subsequently with anti-mouse/POD. Brown staining revealed TF-positive structures (**Figure 19A** and **B**). No staining of the normal colon sections was observed (**Figure 19C** and **D**).

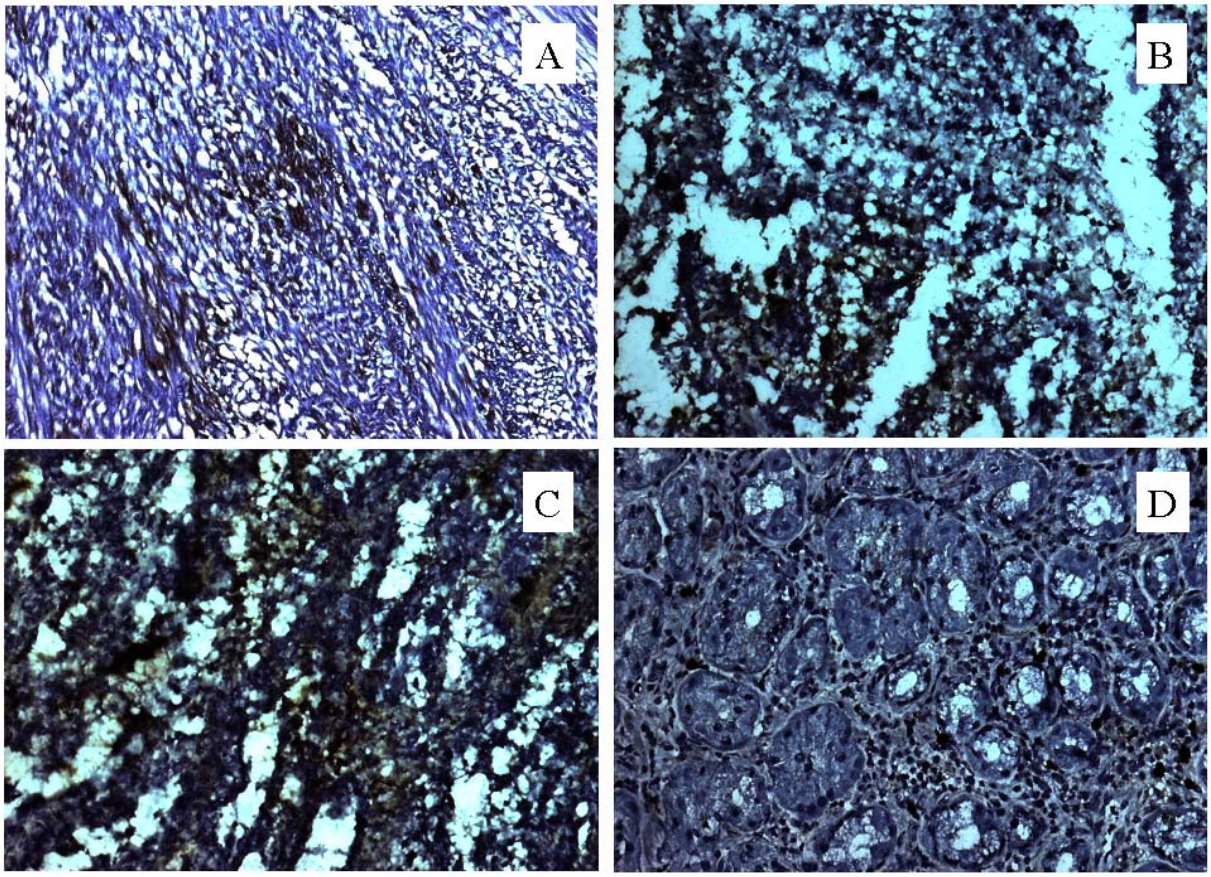

Figure 19 Immunohistology of human colon carcinoma
Cryosections of human colon carcinomas were stained for TF with A78-G/A7 (A) and scFv(1aa) (B and C). D shows normal colon sections treated with the scFv(1aa). Sections stained with A78-G/A7 were treated directly with rabbit anti-mouse-Ig POD conjugate, while sections stained with the scFv(1aa), were treated with the mouse anti-myc 9B11 prior to the rabbit anti-mouse-Ig POD conjugate.

2.3.3.5 Size exclusion chromatography

The multimerisation grade of the scFvs with different linker length was analysed by size exclusion chromatography on a custom-made G200 column that was calibrated in PBS with 5 marker proteins, chymotrypsinogen A (25 kDa), chicken albumin (45 kDa), bovine albumin (68 kDa), aldolase (158

kDa) and katalase (240 kDa). Representative elution profiles are shown in **Figure 20**. The scFv(18aa) elutes with two peaks located at ~28 kDa and ~60 kDa, which correlates well to a mixture of monomers and dimers (28 and 56 kDa, respectively) with the majority in the dimeric fraction. The scFv(2aa) elutes in an almost symmetric peak at 60 kDa correlating to the formation of dimers (56 kDa), while the elution profile of the scFv(1aa) shows a peak located at ~70-80 kDa, which could indicate that the scFv(1aa) forms a mixture of dimeric and trimeric complexes (56 and 84 kDa, respectively), or that the trimers (84 kDa) disintegrate on the column to form dimers. The latter assumption is supported by the observation that the "dimer fraction" of the peak re-runs as a dimer, whereas the re-run of the "trimer fraction" also runs as a dimer (data not shown). The shift from trimeric to dimeric complexes of the scFv(1aa) may be catalysed by the column, because a mixture of trimeric and dimeric complexes was not affected by long-term incubations at 37°C in dilute concentrations. It was concluded that the scFv(1aa) forms trimeric complexes, and that these may not withstand the gelfiltration treatment.

The elution profile of the scFv(0aa) shows a peak at ~120 kDa indicating the formation of larger multimeric, presumably tetrameric complexes. The tetrameric complexes of scFv(0aa) withstands the chromatographic analysis and the peak re-runs as a single peak with the same location, in contrast to the observations with the scFv(1aa) (data not shown). In addition the scFv(0aa) also survived 26 h incubation at 37 °C, after which no changes were detected in the elution profile (**Figure 20b**). The elution profiles of the scFv(3aa), scFv(4aa), scFv(9aa) all formed peaks at ~60 kDa, corresponding to the formation of dimers (not shown). Therefore it can be expected for the remaining scFv constructs will behave similarly.

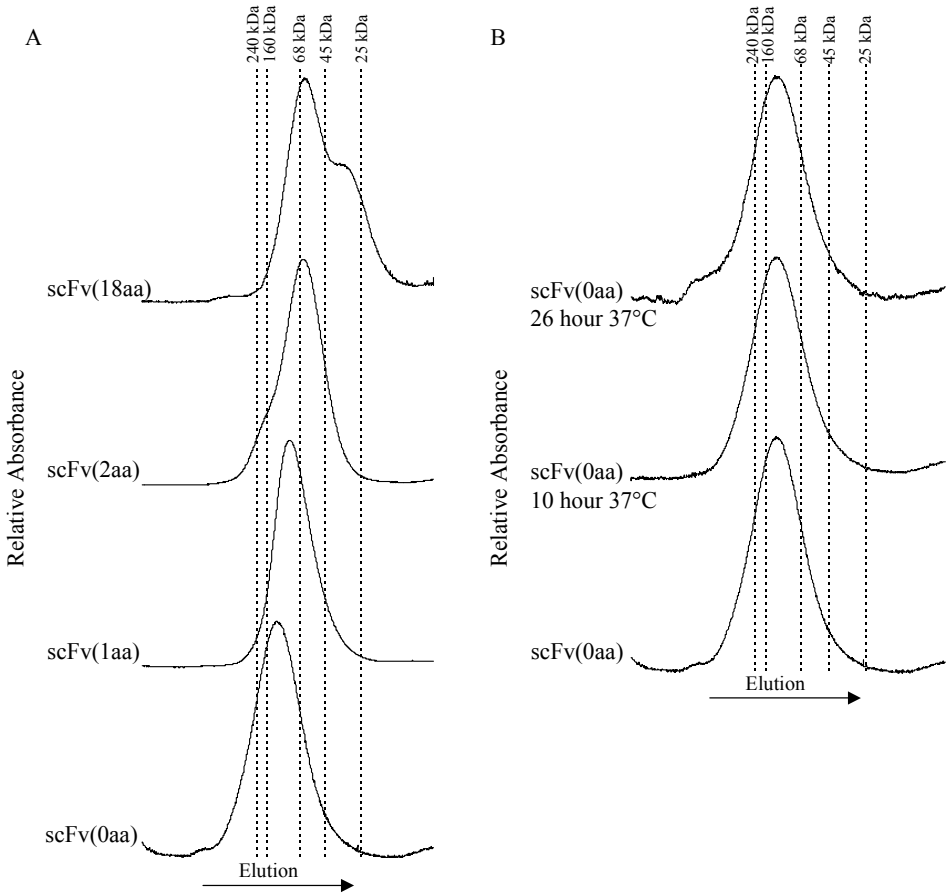

Figure 20 Size exclusion chromatography
IMAC-purified scFv(18aa), scFv(2aa), scFv(1aa) and scFv(0aa) constructs were analysed on a calibrated G200 column equilibrated in PBS (pH 7.0) at a flow rate of 0.1 ml/min giving rise to the elution profiles shown in (A), while (B) depicts the elution profiles of the scFv(0aa) after incubation at 37°C for 0, 10 and 26 h.

2.3.3.6 Real time binding kinetics

The binding kinetics of the different constructs were evaluated by real time surface plasmon resonance (Biacore). Experimental conditions of high density of asialoglycophorin immobilised on the sensor-chip were used to allow multivalent binding of the constructs to TF. Examples of the sensorgrams are presented in **Figure 21**, and the kinetic data are summarised in **Table 5**. The scFv(18aa) exhibited very poor binding (maximal binding within the experimental settings was 125 resonance units), and could not be evaluated with the Biacore software, but illustrates the low affinity of this construct. The assumed dimeric constructs (scFv(2aa) – scFv(9aa)) all showed significantly improved binding kinetics in comparison to the scFv(18aa). Differences were observed between the dimeric constructs obtained with different linkers. The scFv(3aa), which has a similar elution profile as the scFv(2aa), scFv(4aa), and scFv(9aa) in the size exclusion chromatography, performed better in both ELISA and Biacore (compare **Table 5** and **Figure 15**). This shows that the mere formation of dimeric complexes does not automatically ensure the best kinetics, but that optimisation of the linker can lead to

improvements in both binding activity and stability. As expected, the best binding kinetics were observed for the presumed trimeric scFv(1aa) and the presumed tetrameric scFv(0aa). It is intriguing that there were only minor differences observed between the scFv(0aa) and the scFv(1aa). The scFv(1aa) seems to have a slightly slower dissociation rate than the scFv(0aa), although the latter has a higher valency, better overall binding (max RU), and appears to be more stable when analysed by size exclusion chromatography. In general, the obtained sensorgrams revealed a very good correlation between the real time binding kinetics and the direct ELISA analysis (compare **Table 5** and **Figure 15**). It is often reported that lower dissociation constants are caused by slower dissociation rates, which is clearly seen in the overlay plot of the sensorgrams **Figure 21** (correlate with **Table 5**).

An interesting observation was done when the dissociation rates were recorded under conditions of added soluble antigen. Soluble TF (200 mM) had no significant effect on the dissociation rates of the scFv(0aa), scFv(1aa) and scFv(2aa), whereas TF displayed on either asialoglycophorin or PAA (~50 μM calculated TF concentration) did moderately increase the dissociation rates (**Figure 22**). It indicates a dependency of multivalent interaction. This is in contrast to the observation with a Lewis^Y IgM antibody, where the dissociation rate was dramatically increased by the addition of soluble Lewis^Y (Rheinnecker et al., 1996).

Table 5 Summary of binding kinetics

	ka (1/Ms)	kd (1/s)	KD (M)	RU (Max)[a]
ScFv(0aa)	2.3×10^4	2.1×10^{-3}	8.8×10^{-8}	9434
ScFv(1aa)	6.8×10^3	1.5×10^{-3}	2.2×10^{-7}	6566
ScFv(2aa)	1.4×10^4	2.7×10^{-3}	2.0×10^{-7}	3656
ScFv(3aa)	6.7×10^3	2.3×10^{-3}	3.4×10^{-7}	5521
ScFv(4aa)	7.5×10^3	3.8×10^{-2}	5.1×10^{-6}	860
ScFv(9aa)	1.1×10^4	4.7×10^{-3}	4.3×10^{-7}	1918
ScFv(18aa)[b]	ND	ND	ND	125

Sensorgrams were obtained at 5 different concentrations (28 μg/ml, 17 μg/ml, 11 μg/ml, 7 μg/ml and 4 μg/ml) for all the constructs, and the binding kinetics evaluated with the Biacore evaluation software. Calculations were based on tetrameric formation of scFv(0aa) ~ 112 kDa, trimeric formation of scFv(1aa) ~ 84 kDa, and dimeric formation of scFv(2aa), scFv(3aa), scFv(4aa) and scFv(9aa) ~ 56 kDa.

[a] RU (max) is the maximal bound antibody in resonance units after injecting a concentration of 28 μg/ml for 400 sec.

[b] The kinetics of the scFv(18aa) was not evaluated (ND) as the sensorgrams did not enable fitting with the software.

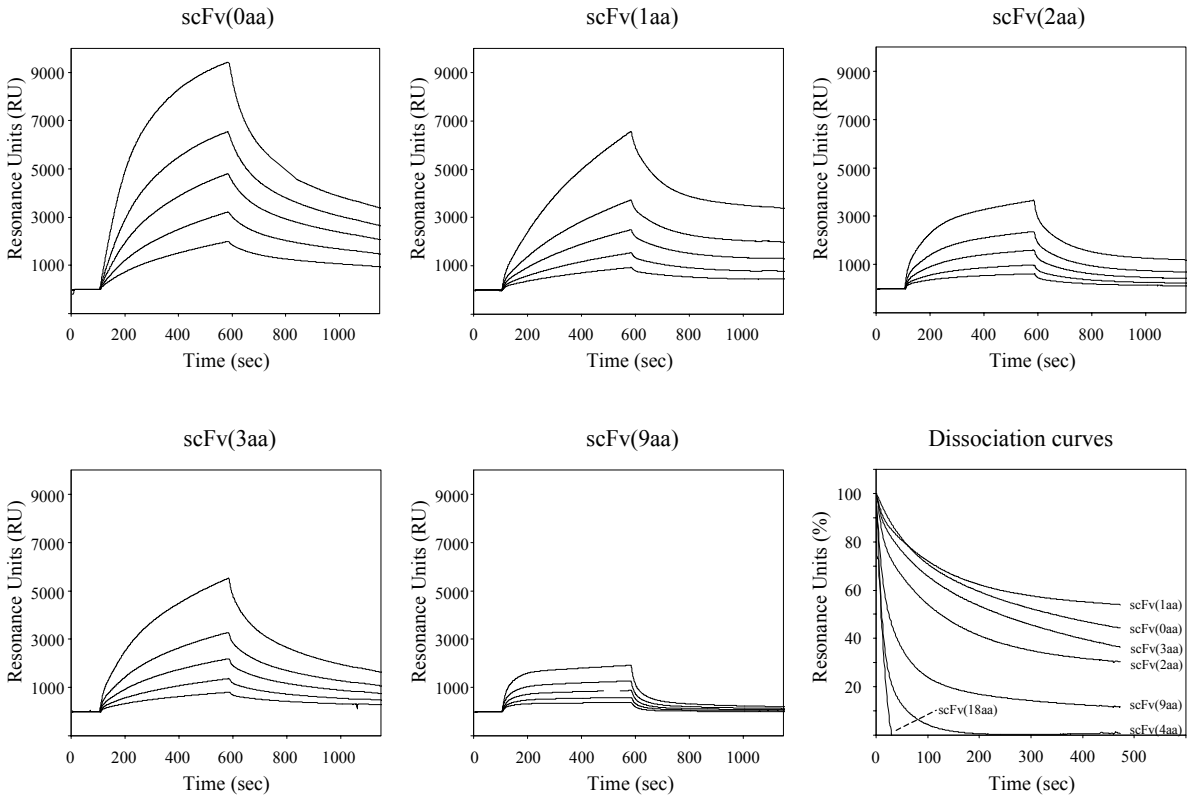

Figure 21 Real time binding kinetics
Real time binding kinetics were investigated by surface plasmon resonance on a Biacore2000 instrument. Shown are overlay plots of sensorgrams obtained for the scFv(0aa), scFv(1aa), scFv(2aa), scFv(3aa), and scFv(9aa) at 5 different concentrations (4, 7, 11, 17 and 28 µg/ml) against immobilised asialoglycophorin. An overlay plot of dissociation curves obtained for scFv constructs at 17 µg/ml against immobilised asialoglycophorin demonstrates the differences in dissociation rates.

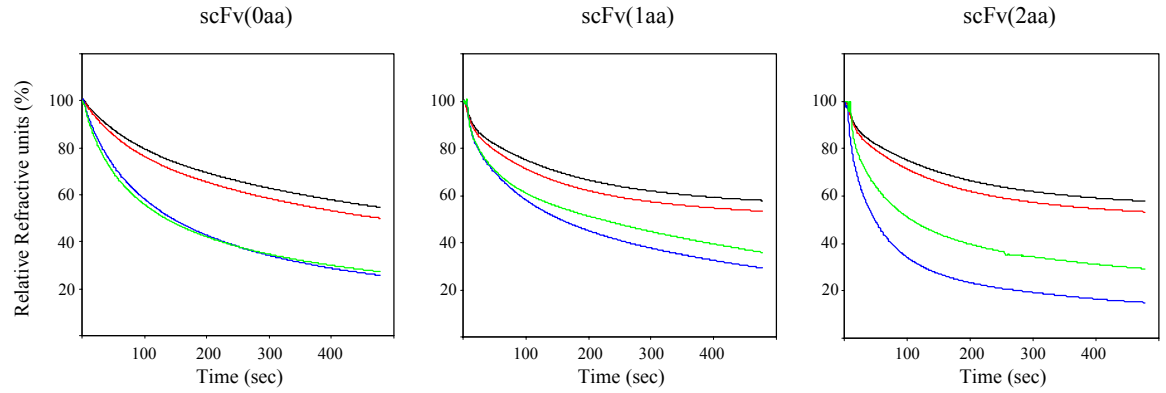

Figure 22 Competition in real time binding kinetics
The dissociation of scFv(0aa), scFv(1aa) and scFv(2aa) from asialoglycophorin coupled to the chip was investigated as simple dissociation (black curve) and under the influence of TF as either soluble oligosaccharide (red curves; [TF] = 200 mM) or displayed on either asialoglycophorin (green curves; [TF] ~50 µM) or PAA (blue curves; [TF] ~50 µM).

2.3.3.7 Anion exchange chromatography (AEC)

Ion exchange chromatography was investigated as a means of further purification of the IMAC-purified scFv(1aa). A relative large panel of different matrix materials were tested in either open columns or pre-packed columns. The different columns have various characteristics, and they may differ in elution profiles, and some of them may separate contaminating proteins or elute in more concentrated fractions. Finally, ion exchange chromatography could offer the means of fast and efficient depletion of imidazole or divalent cations present after the IMAC purification, but unacceptable for the conjugation of a chelator (Chapter 2.6)

At first, open column systems were investigated applying either 30-Q sepharose or 30-S sepharose. The 30-Q sepharose could be used in combination with an ethanolamine buffer (pH 9.5) in which the scFv(1aa) could be loaded at 100 mM NaCl, and eluted by increasing the NaCl concentration. Unfortunately, the scFv(1aa) was not eluted with a sharp peak, but leaked off the column continuously when raising the NaCl concentration to 500 mM over a linear gradient. At 400 to 425 nM NaCl a contaminating protein of ~ 80 kDa was eluted. Alternatively, "starting-state-elution" application of the 30-Q column applying the protein in the elution buffer from the IMAC purification enabled binding of the contaminating protein, while pure scFv(1aa) passed the column (**Figure 23**). In the final purification protocol, this purification step was omitted as the optimised IMAC protocol also removes the contaminating protein (see chapter 2.3.2.1). The cation exchange matrix 30-S sepharose was also tested, but was found to severely inactivate the protein.

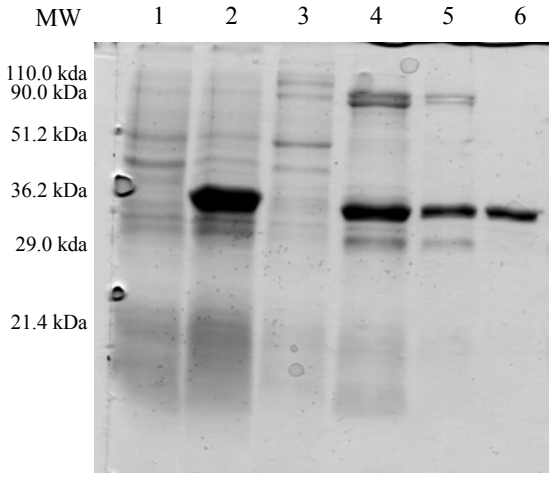

Figure 23 SDS-PAGE of samples from the chromatographic steps in "starting-state-elution"
SDS-PAGE analysis of protein purification of scFv(1aa) by "starting-state-elution". Samples as follows: MW, Molecular weight; 1, Cells pre-induction; 2, Cells post-induction; 3, TES fraction; 4, IMAC elution; 5, Dialysed IMAC elution; 6, Protein after "starting-state elution".

Secondly, A range of commercially available pre-packed ion exchange columns was investigated for their applicability in the protein purification. All the cation exchange columns (HiTrap™ SP Sepharose™ FF, HiTrap™ CM Sepharose™ FF and HiTrap™ SP Sepharose™ XL) caused partial or complete inactivation of the scFvs. Several of the anion exchange columns had the same effect (HiTrap™ Q Sepharose™ FF, HiTrap™ DEAE Sepharose™ FF, HiTrap™ ANX Sepharose™ (high sub) FF and HiTrap™ Q Sepharose™ Q XL), except for the HiTrap™ Q HP and the HiTrap™ Q FF columns that could be applied without detectable inactivation of the scFv(1aa). Of these the HiTrapTM Q HP was superior with respect to both separation in fractions with high homogeneity, and elution in fractions with high protein concentration. Consequently, this procedure was established as a standard secondary purification procedure. The IMAC purified scFv(1aa) could be loaded on the HiTrap™ Q HP column after simple dilution into a buffer with final concentration of 50 mM $NaCo_3$, pH 8.7, and 50 mM NaCl. After washing, the protein could be eluted by increasing the NaCl concentration to 300 mM (**Figure 24A**). Binding activity was detected in the eluted fraction, while no binding activity was detected in the run-through or in the column strip with 1M NaCl (data not shown).

The physical properties of the HiTrap™ Q HP-purified scFv(1aa) was tested using the established assays and compared to the IMAC-purified scFv(1aa).The binding activity of the HiTrap™ Q HP purified scFv(1aa) in ELISA was equal to that of the IMAC-purified scFv(1aa)(**Figure 24B**). Analysis of the eluted fraction by size exclusion chromatography showed that the HiTrap™ Q HP chromatography purified scFv(1aa) forms trimeric complexes (**Figure 24C**), again in agreement with the IMAC purified scFv(1aa). Finally, the real time binding kinetics were analysed by Biacore (**Figure 24D**), yielding the constants summarised in **Table 6**, which are also in good agreement with the data obtained for the IMAC-purified scFv(1aa).

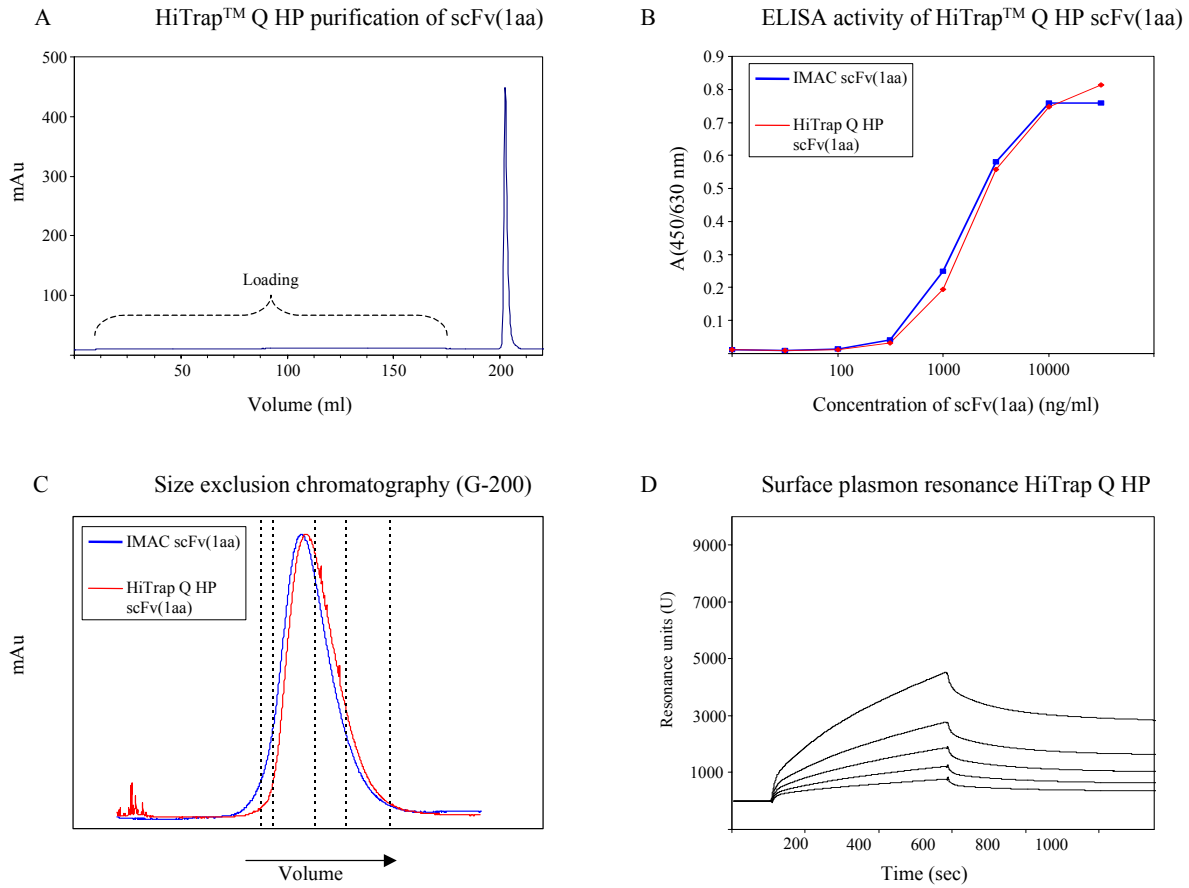

Figure 24 Anion exchange chromatography (AEC) HiTrap™ Q HP
The IMAC purified scFv(1aa) was further purified on a HiTrap™ Q HP column using a carbonate buffer system (pH 8.7). Protein was loaded at low NaCl concentration (50 mM) and eluted at 300 mM as shown on the chromatogram in A. The HiTrap™ Q HP-purified scFv(1aa) exhibited conserved binding activity in ELISA against asialoglycophorin (B), and had a conserved profile when analysed by size exclusion chromatography on an equilibrated G-200 column (C). Real time binding kinetics were analysed by surface plasmon resonance on an asialoglycophorin chip with a Biacore2000 (D).

2.3.3.8 Affinity chromatography (AC)

As for the ion exchange chromatography, the affinity chromatography was investigated with respect to its potential application in the further purification of the IMAC-purified scFvs.

A column was packed with TFβα-PAA-Sepharose, which should be applicable as the scFv(1aa) was found to bind immobilised TFβα-PAA (chapter 2.3.3.1). The IMAC purified scFv(1aa) could be further purified by affinity chromatography on the TFβα-PAA-Sepharose column simply by loading the protein on the column at neutral conditions (PBS, pH 7.0). Subsequently, the bound scFv(1aa) could be retrieved from the column by basic elution (PBS, pH 11). An example of a chromatogram is depicted in **Figure 25A**. Approximately 15% of the IMAC-purified scFv(1aa) could be retrieved from the affinity column. After neutralisation or re-buffering into PBS, all the AC-purified scFv(1aa) could rebind on the column, thereby demonstrating that it was not irreversibly denatured by the elution

conditions (data not shown). The AC-purified scFv(1aa) was tested in ELISA against asialoglycophorin (**Figure 25B**), where it was found approximately 6 fold more active than the IMAC-purified scFv(1aa). No ELISA activity was found in the run-through.

Size exclusion chromatography analysis of the AC-purified scFv(1aa) gave a peak correlating to a higher molecular weight that the IMAC-purified scFv(1aa), which indicated that the affinity chromatography enriches for higher multimeric complexes, presumably tetrameric (**Figure 25C**). Biacore analysis of the AC-purified scFv(1aa) showed that it has a higher dissociation constant (Kd: 2.0×10^{-8} M) caused by a higher ka (~2.5 fold) and a lower kd (~4 fold) (**Figure 25D and Table 6**).

Furthermore, the AC-purified tetrameric complexes proved stable, and exhibited the same profile by chromatography on the G-200 column after 2 weeks of storage at 4°C or after re-chromatography of the peak fraction. The ELISA profile was conserved after storage at 4°C (>11 days) or storage at -80°C (>8 month) in PBS (pH 7), but also at 4°C (>2 weeks) in PBS (pH 11) at ~ 250 µg/ml.

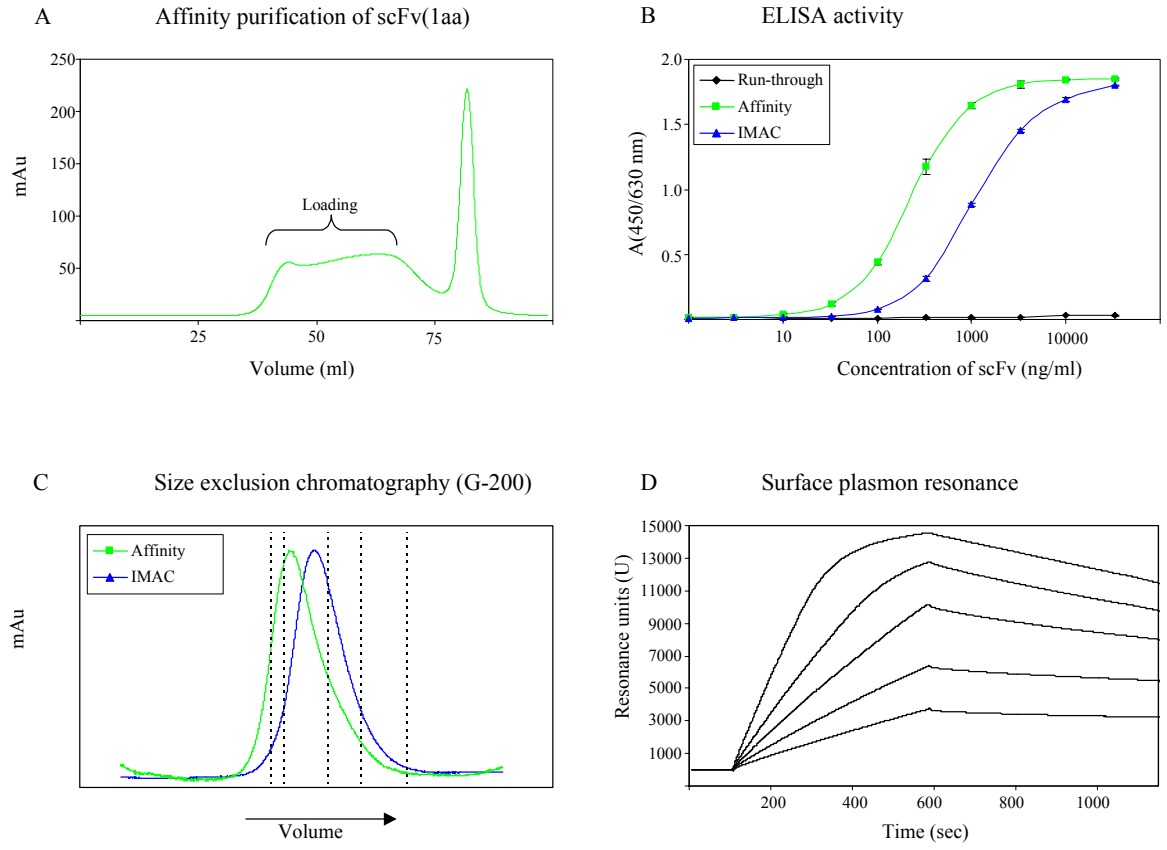

Figure 25 Affinity Chromatography (AC)
The IMAC-purified scFv(1aa) can be further enriched using an affinity column. The protein was loaded in PBS pH 7 and could be retrieved by increasing the pH to 11 as shown on the chromatogram in A. The AC-purified scFv(1aa) had an increased binding activity in ELISA towards asialoglycophorin (~6 fold) (B). Size exclusion chromatography of the AC-purified scFv(1aa) indicates that the eluted protein is enriched for tetrameric complexes (C). Finally, the binding kinetics were investigated by surface plasmon resonance (D).

Affinity purification of the scFv(0aa) gave a higher yield on the affinity column (30-50%) than the scFv(1aa), while the scFv(2aa) gave very low yields. These observations are in good agreement with the note that the affinity purification seems to enrich for tetrameric complexes (refer **Figure 20**). This is also in agreement with the observation that less than 2-5% of the HiTrap^{TM} Q HP purified scFv(1aa) bound on the affinity column.

Finally, the affinity column was used to investigate the different scFv(1aa) clones (differences in the primer encoding regions), revealing that the differences had no effect on the percentage of retrieval, which further indicates that the clones will also have similar profiles in size exclusion chromatography (data not shown).

Table 6 Summary of binding kinetics

Preparation	ka (1/Ms)	kd (1/s)	KD (M)
IMAC-purified scFv(1aa)	6.8×10^{3}	1.5×10^{-3}	2.2×10^{-7}
AEC-purified scFv(1aa)	4.9×10^{3}	8.7×10^{-4}	2.0×10^{-7}
AC-purified scFv(1aa)	2.0×10^{4}	4.1×10^{-4}	2.0×10^{-8}

The binding activity of the AC-purified scFv(1aa) was further assessed in a competition ELISA, which was based on detection of the mouse antibody A78-G/A7 binding to asialoglycophorin in the presence of increasing concentrations of scFv(1aa). The competition IC_{50} of the AC-purified scFv(1aa) was lower than that of the Ni-NTA-purified scFv(1aa) (**Figure 26**). The competition with A78-G/A7 is important because this antibody is a well-established TF-specific antibody, and has been applied in a range of tumour immunohistochemistry studies of TF. The competition results show that the scFv(1aa) binds the same "epitope", which is assumed to be clusters of TF at certain densities, and therefore validates the scFv as a therapeutic candidate with a similar antigen profile.

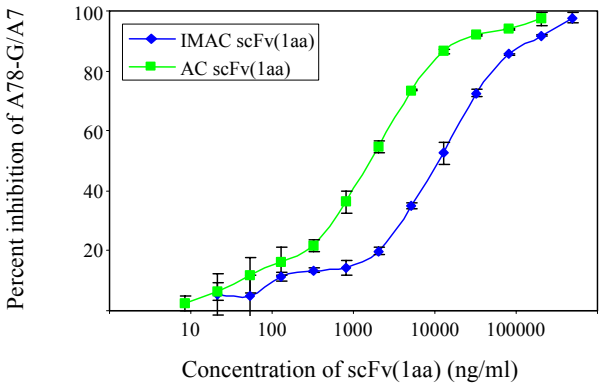

Figure 26 Competition ELISA
Binding of A78-G/A7 to asialoglycophorin in ELISA was inhibited by the scFv(1aa) in a concentration dependent mannar, N=2.

2.4 Alternative formats for multimerisation of scFvs

As described in the introduction (referring to **Figure 4**) there are many alternatives for generating multivalent recombinant antibodies. It is impossible to predict which format will possess the best properties with respect to stability, valency, affinity, and size, as well as good production characteristics. Therefore a few of the formats that can be expressed in bacteria were tested, such as scDiabody and different formats based on fusion to domains that multimerise.

2.4.1 Generation of scDiabody repertoire

As described in the Introduction, the scDiabody format has some appealing properties with respect to stability and potential generation of dimeric tetravalent complexes. A lot of speculation has gone into the design of the linkers connecting the variable domains, but it may also be possible to select the optimal linkers using phage display. Consequently a library was designed in which all the three linkers connecting the variable domains were randomised. The cloning strategy applied circumvented the problematic deletion of restriction sites and enabled library cloning in 3 steps (**Figure 27**).

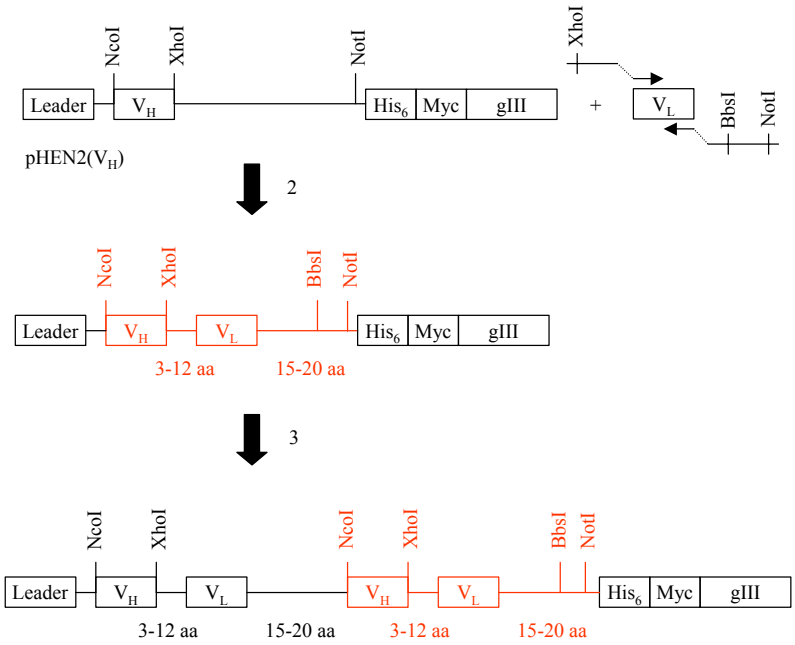

Figure 27 Cloning strategy for the generation of a scDiabody library
In the first step the V_H of the selected scFv was inserted into an empty pHEN2 to establish a preliminary vector (pHEN2(V_H)). PCR amplification of the V_L introduced diversity into the linkers on both sides. In step 2), the V_L library was inserted into pHEN2(V_H) using restriction sites XhoI and NotI, thereby generating a scFv library in pHEN2 in which a BbsI site is located upstream of the NotI site. In step 3), the scFv library was excised with NcoI and NotI to generate a library insert, and BbsI and NotI to generate a library vector. The BbsI is a non-palindromic enzyme generating a 4-base overhang, which in this design is compatible with the NcoI overhang. Consequently, the generated library insert fits into the generated vector library and results in the scDiabody library.

Two independent libraries were prepared and transformed into TG-1 resulting in 1.5×10^6 and 3.2×10^6 individual transformations with insert ratios of 80 and 90% (PCR screen). Library diversities were not tested by sequencing, as it was assumed that the cloning strategy would ensure the diversity in the linkers. Phages produced from the libraries were selected both by panning using asialoglycophorin coated to immunotubes and by biopanning using TF-PAA-biotin and subsequent capture with streptavidin-conjugated magnetic beads. Titers from the selections are summarised in **Table 7**

Table 7 Selection of scDiabody repertoires.

	scDiabody repertoire I		scDiabody repertoire II	
	Panning	Biopanning	Panning	Biopanning
1. Round	400	8	1400	2
2. Round	12000	-	50000	-

Output of the different panning rounds in cfu.

Twenty clones were picked from each round of panning plus the 10 clones from the biopannings, and tested in an expression and binding assay (**Figure 28**). Expression of clones 5, 15 17, and 72 was tested in 1 litre cultures followed by IMAC purification, but the yield was very low (~50-100 µg/l). ELISA activity of the purified constructs was in the range 5-20% of the scFv(1aa), and it was therefore decided not to prioritise these clones for further pursue.

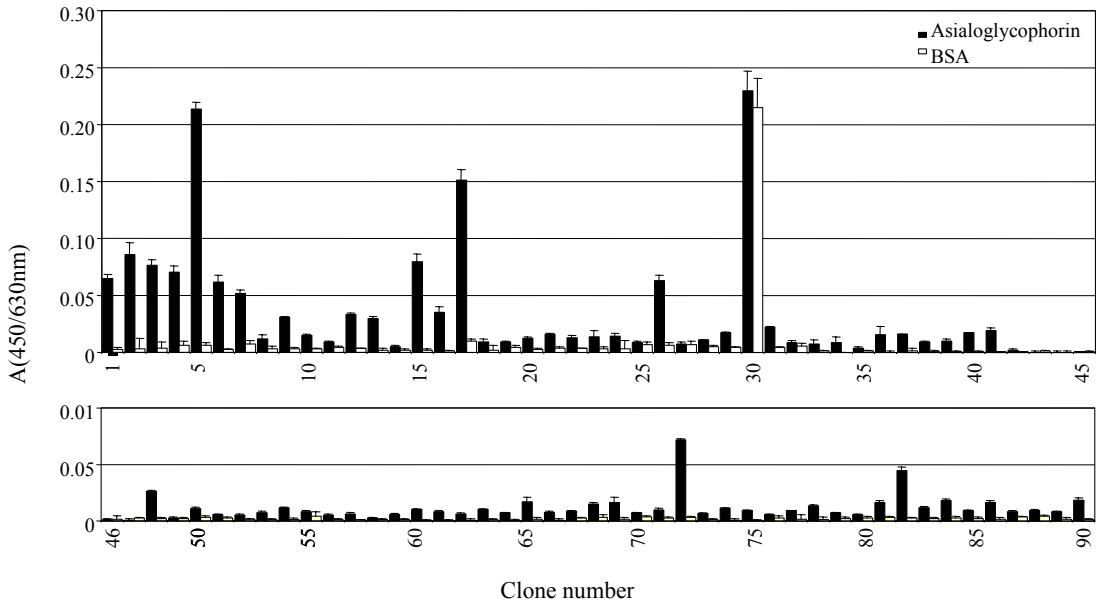

Figure 28 screening of scDiabody clones
Clones from biopanning (1-10) and from panning (11-90) were tested for ELISA activity in TES fractions from 10 ml overnight cultures in a set-up similar to the test of the TES fractions of the different scFv linker constructs.

2.4.2 Multimerisation strategies by domain fusion

The simplest fusion constructed was the addition of a C-terminal cystine to the scFv(18aa). The scFv(18aa) was chosen because of the multimer formation of the other scFv constructs, that easily could drive the formation of larger aggregates when combined with the disulfide-bridge coupling. Even with the scFv(18aa) the results were very disappointing with no detectable binding activity of IMAC-purified protein (data not shown). A possible explanation is the odd number (3) of cysteines in the construct, which sometimes hampers the folding of the protein.

As described in the introduction, the secretory tailpiece of an IgM can drive the formation of multimeric constructs when fused to C_{H3} (Olafsen et al., 1998). Whether the tailpiece could also drive the formation of multimeric constructs when fused directly to a scFv was tested with the scFv(18aa). Minor binding activity was detected in ELISA, but both activity and yield of this construct was very low (data not shown), and this was therefore not persued further.

Finally, the scFv(18aa) has been fused to the multimerisation domain of the human Papillomavirus type 1 E1^E4 protein (Ashmole et al., 1998) the trimerisation driving helix of mannan binding lectin (Kilpatrick, 2002; Tan et al., 1996), but again only minute binding activity of the constructs were detected in ELISA (data not shown).

2.5 Affinity maturation

There are several reasons for persuing an affinity maturation of the selected scFv (discussed in detail in chapter 3). Phage display offers the means of performing this *in vitro*, simply by generating secondary libraries and selecting these under increased selection pressures as descriebed by Gram, Hawkins, Marks, Kjaer and many more (Gram et al., 1992; Hawkins et al., 1992; Kjaer et al., 2001; Marks et al., 1992). In brief, most strategies are based on randomisation of selected regions of the scFv gene (most offen the CDRs) thereby generating a repertoire of limited diversity. Subsequently, the selection protocol is designed to enrich for higher affinity clones.

2.5.1 Generation of secondary libraries

A panel of degenerate primers were designed to enable a restricted randomisation of CDR in the scFv. The localisations of the primers are indicated in **Figure 29**. Randomisation of the heavy chain CDR2 (HCDR2), HCDR3 and light chain CDR3 (LCDR3) were introduced by partial randomisation of the nucleotide sequence of the CDR so that at each position there was a 70% bias for the original base, and 10% for each of the three others. Four libraries were generated by SOE-PCR and cloned in pHEN2. The details are summarised in **Table 8.**

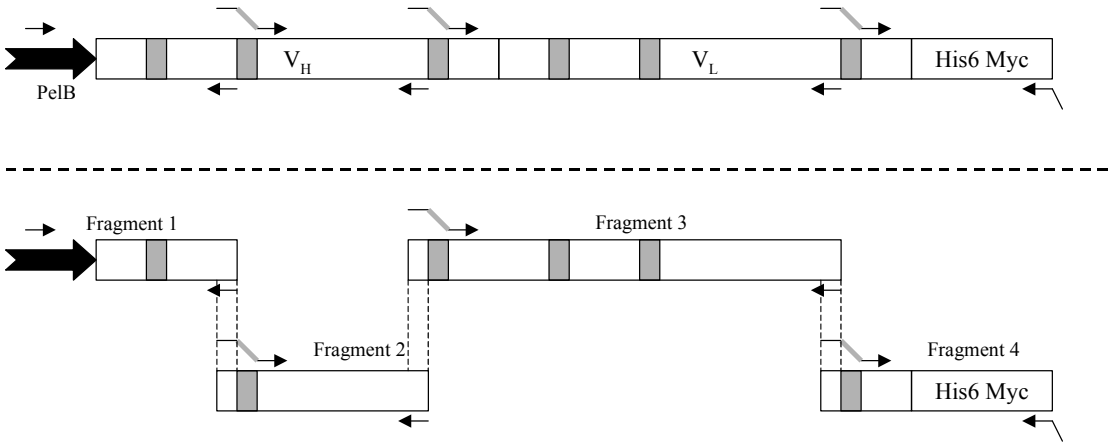

Figure 29 Primers for affinity maturation
The localisation of the annealing sequences for the primers for the affinity maturation-library construction have been illustrated schematically with the gene for the scFv. The grey areas represent the CDRs. The primer regions that were targeted for randomisation are depicted in grey.
Below the line, the fragments produced by PCR have been illustrated, including the overlapping regions necessary for the SOE PCR.

Table 8 Summary of secondary repertoires.

Repertoire	Randomised	Format	Size	Insert ratio
Library 1	HCDR3-70%	ScFv(18aa)	9×10^6	100% (N=20)
Library 2	HCDR3-3-16	ScFv(18aa)	2.5×10^8	90% (N=20)
Library 3	HCDR3-70%	ScFv(1aa)	2.0×10^7	95% (N=20)
Library 4	CDR-70%	ScFv(0aa, 1aa)	2.5×10^6	85% (N=20)
Library 5	CDR3-70%	ScFv(1aa)	5.2×10^6	90% (N=20)

HCDR3-70%: 70% bias for original sequence in HCDR3
HCDR3-3-16: Complete randomisation of HCDR3 ranging from 3aa to 16aa
CDR-70%: 70% bias for original sequence in HCDR2, HCDR3 and LCDR3
CDR3-70%: 70% bias for original sequence in HCDR3 and LCDR3.

Sequencing of clones from the unselected libraries was performed to test the diversities of the libraries.

2.5.2 Selection of secondary libraries

Although all the libraries have been selected at least twice, the overall outcome has been somewhat disappointing, with a few exceptions.

Library 1 and 2 were selected both by biopanning using TF-PAA-biotin and by normal panning using asialoglycophorin-coated immunotubes. During the 3 rounds of selection, single clones were tested for binding in ELISA against asialoglycophorin. As seen in the earlier selections against TF, no binders

were identified from libraries in the 18aa linker format, and sequencing of clones from the respective rounds revealed no consensus sequence (data not shown).

Because of the lack of success with the scFv(18aa) format, it was again decided to change to the scFv(1aa) format. Consequently, library 3 was generated, and selected by 3 different selection strategies: panning on asialoglycophorin, panning on asialoglycophorin in the presence of glycophorin as competitor, and biopanning using TF-PAA-biotin. All 3 selection strategies were successful, as binding clones were identified in ELISA against asialoglycophorin (data not shown). Sequencing revealed that all clones with specificity toward TF encoded the same HCDR3 sequence as that of the founder clone (**Figure 30**). This result might raise the suspicion that the library was contaminated with the founder clone. However, many of the clones encoded point mutations outside HCDR3, which are most likely introduced by the Taq-polymerase, and indicate that the presence of the sequence may be an effect of the overlapping PCR assembly strategy used for the library construction and not a contamination.

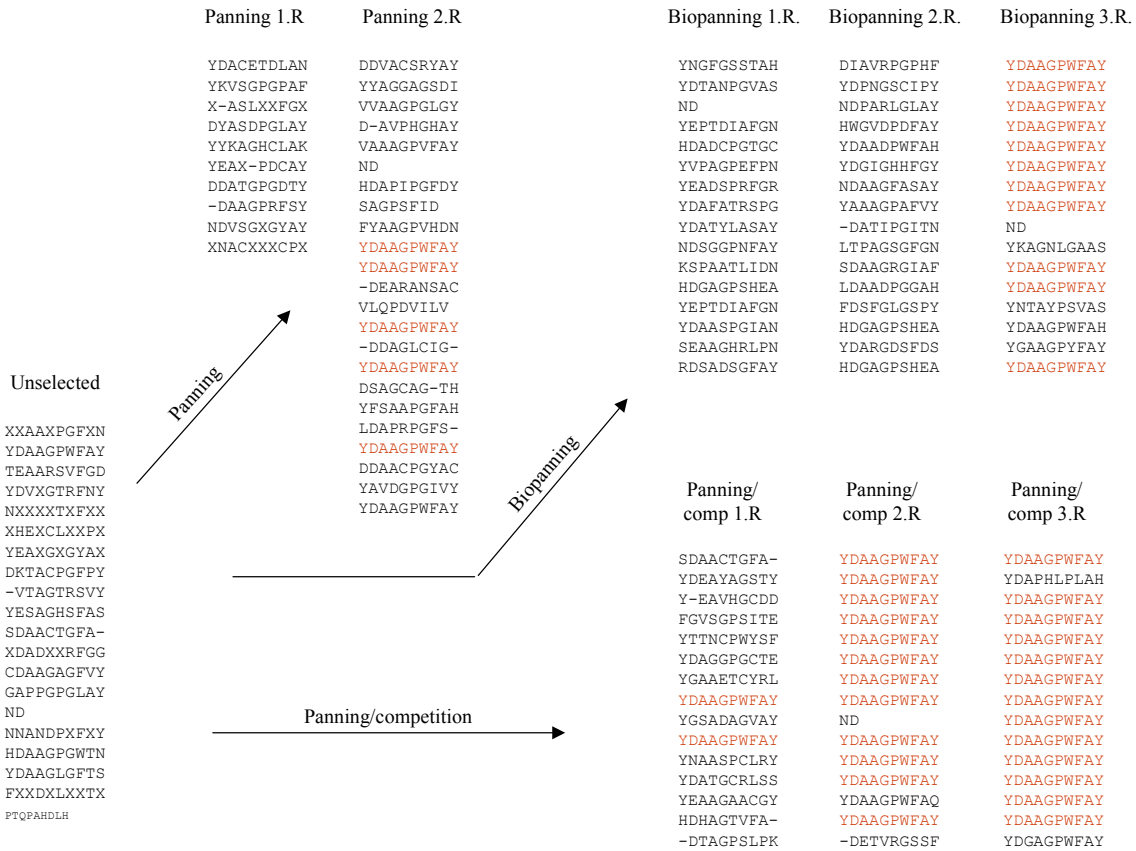

Figure 30 Selection of Library 3

Sequences of the HCDR3 regions from clones picked during the different selection schemes. The sequences in red are identical to the sequence of the founder clone. The panning selection was performed on asialoglycophorin immobilised in immunotubes (1 µg/ml). The competitive selection was performed as the panning selection, but with the addition of 50 µg/ml glycophorin. Biopanning selection was performed with TF-PAA-biotin (0.1µg/ml).

Sequencing of clones from library 4 showed that the library was biased toward stop-codons in HCDR2. The clones with stop-codons were not eliminated during the selection, but seemed to dominate as described by Carcamo *et al.* (Carcamo et al., 1998). No clones with affinity for TF were identified from this library.

Library 5 was designed to simultaneously optimise HCDR3 and LCDR3. This library was selected by one round of panning on asialoglycophorin followed by two rounds of biopanning on TF-PAA-biotin. Clones with affinity for asialoglycophorin were identified in ELISA, but in the control ELISA they all bound to glycophorin with a similar or higher signal (**Figure 31**). This was unexpected because the biopanning should direct the selection toward TF. On the other hand, these resluts demonstrate the good randomisation of the library.

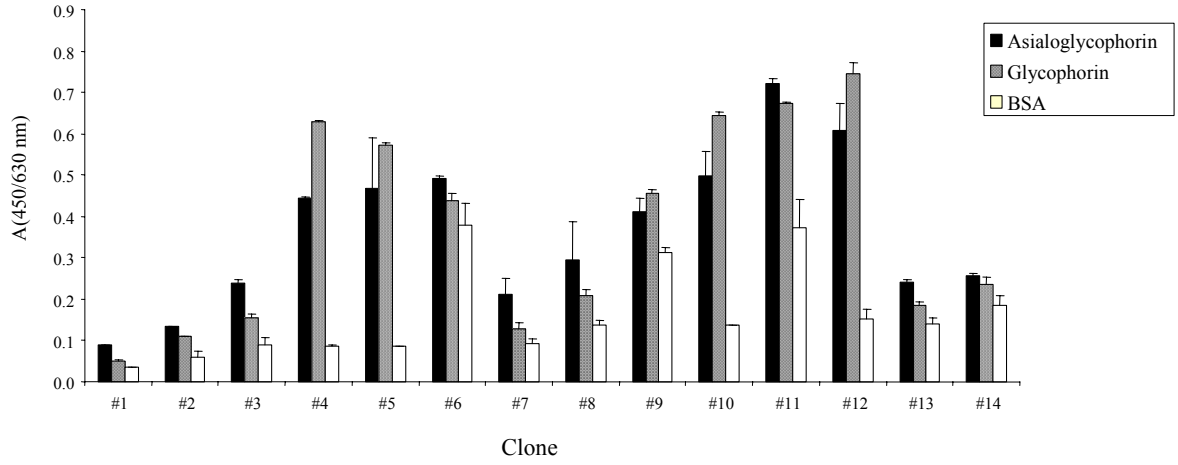

Figure 31 Specificity of clones selected from Library 5
Clones selected from Library 5 were screened against asialoglycophorin, glycophorin and BSA in phage-ELISA.

2.6 Generation and evaluation of a radiotherapeutic candidate

A few guidelines for choosing the best therapeutic candidate can be derived from the literature. Small molecules with high binding affinity have been preferred, because a small molecular size is associated with good tumour penetration and fast serum clearance thereby ensuring good tumour to normal tissue ratios. A high affinity should provide good tumour retention (Kd in the nanomolar range is often mentioned as a guideline). Recently, studies with antibody fragments conjugated to chelators such as DTPA or DOTA have revealed problematic uptake and retention in the kidneys (Rogers et al., 1995; Tsai et al., 2001). The studies suggest that chelate-conjugated antibodies smaller than the kidney cut-off (~60-80 kDa) are internalised by the proximal tubulus cells, and subsequently degraded to chelator-lysine in the lysosomal compartments, and that the chelator-lysine clears slowly from these compartments (Tsai et al., 2001).

Based on these guidelines, it was decided to focus on the scFv(1aa) due to its apparent formation of trimeric complexes (84 kDa). In addition, the affinity of the scFv(1aa) was shown to be higher than the scFv constructs with longer linkers (which form dimers). Theoretically, the dimeric scFvs should have an advantage in respect to tumour penetration, but the binding activity of these clones was inferior in the *in vitro* assays. A relative good expression and purification yield was achieved for the scFv(1aa) which further supported the choice as a primary candidate for the pioneering pre-clinical investigations of the targeting potential of TF and the format of the antibody.
In extension of these experiments, preliminary investigations of the scFv(0aa) and the scFv(2aa) have also been conducted to obtain information on dimeric and tetrameric constructs, using the same protocols that were established for the scFv(1aa)

2.6.1 Conjugation of diethylenetriaminepentaacetic acid (DTPA) to scFvs

^{111}In and ^{90}Y are radiometals of clinical interests with respect to diagnosis and therapy, respectively. Labelling of antibodies with these radiometals involves two steps; conjugation of chelator and radiolabelling.
Diethylenetriaminepentaacetic acid (DTPA) is a well characterised chelator for labelling antibodies with radiometals (suggested reviews (Batra et al., 2002; DeNardo et al., 1999; Goldenberg, 2003; Postema et al., 2001; Trikha et al., 2002; von Mehren et al., 2003). However, the conjugation procedure of DTPA to different antibodies needs optimisation with respect to stoichiometry, pH, time and temperature in order to avoid loss of binding activity.
For optimisation of the production of a radioactively labelled antibody, the effect of the stoichiometry of the chelator conjugation reaction, and the effect of the applied buffers were investigated before an optimised conjugation strategy was designed.

2.6.1.1 Titration of DTPA in conjugation of the scFv(1aa)

The most applied strategy for conjugation of a chelator to a monoclonal antibody is based on standard coupling chemistry. Isothiocyanatobenzyl DTPA is mixed with the antibody under slightly basic reaction conditions (carbonate buffer, pH 8.7) at a reaction temperature of 37°C. The isothiocyanatobenzyl reacts with primary amines on accessible lysines. For the scFv(1aa) the incubation at 37°C should be avoided for extended periodes, because the protein looses binding activity in the carbonate buffer (chapter 2.6.1.2). Moreover, the conjugation grade could hamper the binding activity as 5 lysines were identified in the CDR regions. Consequently, the effect of chelator to antibody ratio was titrated at 3 different temperatures, 37°C, 30 °C and 25 °C, covering a range

from a 3-fold excess of scFv(1aa)-polypeptide to a 27-fold excess of the chelator (**Figure 32**). No loss of binding activity was observed when the ratio of the chelator was increased. In contrast, a larger loss of binding activity was observed at 30 °C and 37°C as compared to 25 °C, similar to the loss of binding activity observed in testing the buffer effects (chapter 2.6.1.2). Test-labelling of the different conjugate preparations with ^{111}In, and investigations using thin layer chromatography (TLC) showed that all the preparations were labelled. The preparations were not investigated further with respect to their conjugation stoichiometry, but as no loss of binding activity was observed, it was decided to perform further conjugations at room temperature for 1 h with high excess of chelator (~27 fold).

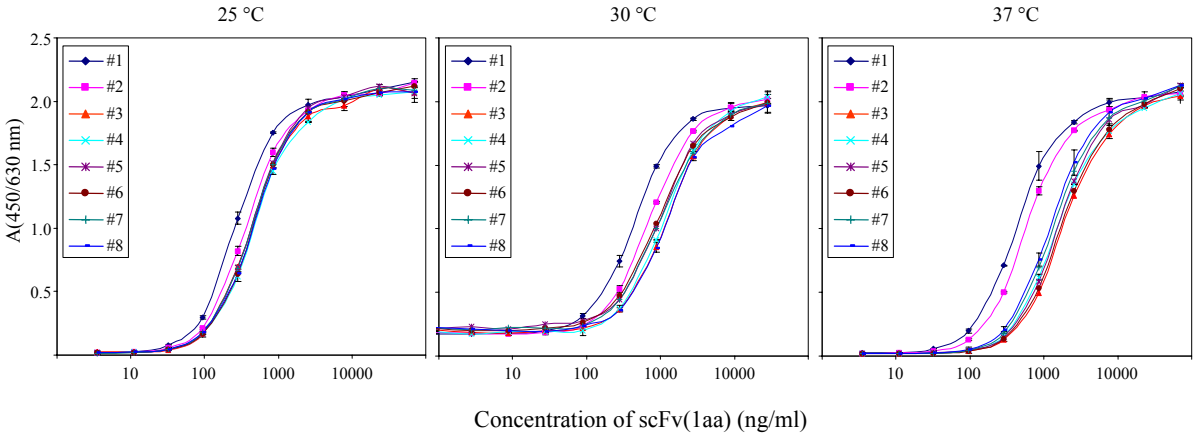

Figure 32 Titration of chelator
Test conjugation of chelator DTPA to scFv(1aa) was followed by binding-activity test in ELISA against asialoglycophorin (1 µg/ml). Buffer changes were performed on mini-gel-filtration-spin-columns, which enabled efficient separation for samples less than 25 µl. Conjugation was performed at 25, 30 and 37 °C, and samples contained: #1, Postive control (IMAC-purified); #2, Carbonate buffer change; #3, Carbonate buffer change and 1 h incubation at given temperature; #4-#8, Carbonate buffer change and incubation at given temperature with different ratios of chelator, 1/3, 1, 3, 9 and 27 fold, respectively. N=2

2.6.1.2 Binding activity of the scFv – effect of different buffers

The binding activity of the scFv(1aa) was carefully investigated with respect to the different buffers needed for conjugation of chelator (carbonate buffer, pH 8.7) and subsequent labelling with radioactivity (acetate buffer, pH 4.3). The binding activity was assayed in direct ELISA, where the amount of active protein was correlated to a well-established standard curve. The results of the stability test is depicted in **Figure 33** and summarised in **Table 9**. The binding activity of the scFv(1aa) in the carbonate buffer seems especially troublesome, and necessitates fast and efficient buffer changes. Furthermore, a method was desired that could deplete any residual imidazole and divalent cations such as Ni^{2+}. Buffer exchange applying Centricon Y-30 is usually a straightforward procedure, however, it is rather time-consuming, and the membrane often disrupts applying the carbonate buffer. As an alternative solution, ion exchange chromatography was investigated (chapter

2.3.3.7). A procedure based on the HiTrap™ Q HP pre-packed column was found superior, and was consequently applied for the buffer exchange. (Note that the procedure is not applicable for the scFv(0aa), as scFv complexes larger than trimers are retained in the column.)

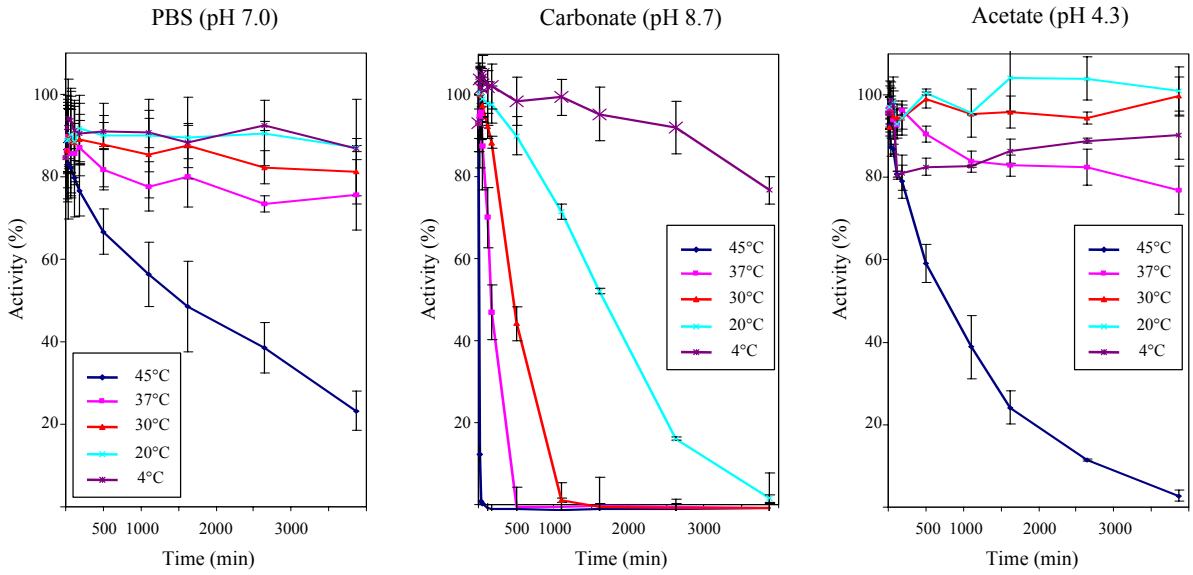

Figure 33 Antibody binding activity in different buffers
The binding activity of the scFv(1aa) was measured at different time points to investigate the time and temperature dependence. The binding activity was measured in ELISA, and correlated to a standard curve of the scFv(1aa) in order to calculate the percentage of active protein at the different time points. N=2

Table 9 Binding activity in different buffers as binding-activity halftimes $T_{1/2}$

Temperature	PBS, pH 7.0	Carbonate buffer, pH 8.7	Acetate buffer, pH 4.3
4 °C	>3780[b]	>3780[a]	>3780[b]
21 °C	>3780[b]	1650	>3780[b]
30 °C	>3780[b]	470	>3780[b]
37 °C	>3780[a]	170	>3780[a]
45 °C	1620	7	780

[a] >75% of binding activity after 3780 min. [b] >85% of binding activity after 3780 min

2.6.1.3 Preparation of DTPA-conjugated scFvs

From the experiments described above, the following protocol for the preparation of DTPA-conjugated scFvs was developed for the scFv(1aa). It consists of 6 steps;

i) Expression and harvesting of the scFv as described in chapter 2.3.1

ii) Purification by IMAC as described in chapter 2.3.2.1

iii) Purification by AEC in order to deplete imidazole, divalent cations and potential contaminations and to exchange the buffer as described in chapter 2.3.3.7

iv) Conjugation of DTPA using a high molar excess of chelator as described in chapter 2.6.1.1. Reaction conditions were as follows: room temperature, 1 h incubation time, protein concentration ~ 0.5 mg/ml (higher protein concentrations may result in a higher conjugation stoichiometry, but the scFv(1aa) had a tendency to precipitate at concentrations higher than 0.5 mg/ml)

v) Buffer exchange into acetate buffer, pH 4.3, using spin filtration. This was achieved by first diluting the conjugation-reaction mixture 10 fold with PBS, followed by extensive washing with the acetate buffer, pH 4.3.

vi) Storage of preparations at –80 °C. This was preferred as freezing of the proteins had no effect on the binding activity (measured in ELISA or on the Biacore).
Furthermore, storage in the acetate buffer was very convenient because the protein could be thawed and labelled directly (very convenient for coordinating trials with respect to protein, ^{111}In and mice).

DTPA was conjugated to four individual preparations of scFv(1aa) (termed scFv(1aa)$_{PR1}$ to scFv(1aa)$_{PR4}$), one of scFv(0aa) and one of scFv(2aa). Preparation data are summarised in **Table 10**. For preparations scFv(1aa)$_{PR1}$ and scFv(1aa)$_{PR2}$ the protein concentrations in the conjugation reaction was lower (200 µg/ml and 250 µg/ml, respectively), and the protein was also stored at a lower concentration than the other preparations. The lower concentrations seem to slow down the labelling kinetics as described below in chapter 2.6.1.4. Besides, all four scFv(1aa) preparations were comparable with respect to similar binding activity in ELISA, RIA, and in cell binding assays described below (chapter 2.6.2.2 and chapter 2.6.2.3, respectively).

Table 10: Chelated preparations

Preparation	Yield[a]	Concentration	Stoichiometry (DTPA : scFv)
ScFv(0aa)	2.3 mg (75%)	520 µg/ml	1:20
ScFv(1aa)$_{PR1}$	0.8 mg (44%)	120 µg/ml	ND
ScFv(1aa)$_{PR2}$	0.3 mg (42%)	300 µg/ml	ND
ScFv(1aa)$_{PR3}$	6.0 mg (69%)	550 µg/ml	1:5
ScFv(1aa)$_{PR4}$	1.6 mg (55%)	550 µg/ml	ND
ScFv(2aa)	8.8 mg (76%)	525 µg/ml	1:6

[a] Yield: amount of protein obtained and stored at –80 °C. The scFv(1aa)$_{PR1}$ to scFv(1aa)$_{PR4}$ are four individual preparations. The scFv(1aa)$_{PR1}$ and scFv(1aa)$_{PR2}$ were applied in the conjugation reaction at lower protein concentration, and they were stored at lower protein concentrations than the other preparations. The scFv(1aa) and scFv(2aa) were prepared strictly according to the procedure outlined above, while step *iii*) was omitted for the scFv(0aa) and was replaced by dialysis into the carbonate buffer at 4°C over 32 h (4 changes) because the scFv(0aa) could not be purified on the HiTrap^{TM} Q HP column. ND; Not Determined

The multimerisation grade of the DTPA-conjugated scFvs was investigated by size exclusion chromatography. The DTPA-conjugated proteins exhibited profiles very similar to the profiles of the IMAC-purified founder scFvs with minor shoulders for the scFv(0aa) and scFv(2aa) (**Figure 34**). The binding activity of the DTPA-conjugated proteins was tested in ELISA against asialoglycophorin with the founder scFvs as internal controls. All three DTPA-conjugated scFvs had higher binding activity in ELISA compared to the IMAC-purified controls, although the conjugated DTPA should increase the absorbance due to the benzyl group and therefore underestimate the protein concentration. Finally, the binding kinetics of the scFv(1aa)$_{PR3}$ were investigated by Biacore, resulting in the following constants: ka: 5.1×10^{3} 1/Ms ,kd: 3.6×10^{-4} 1/s , Kd: 7.0×10^{-8} M, revealing small differences to the IMAC-purified scFv(1aa) (ka: 6.8×10^{3} 1/Ms ,kd: 1.5×10^{-4} 1/s , Kd: 2.2×10^{-8} M), but correlating well with the ELISA observations.

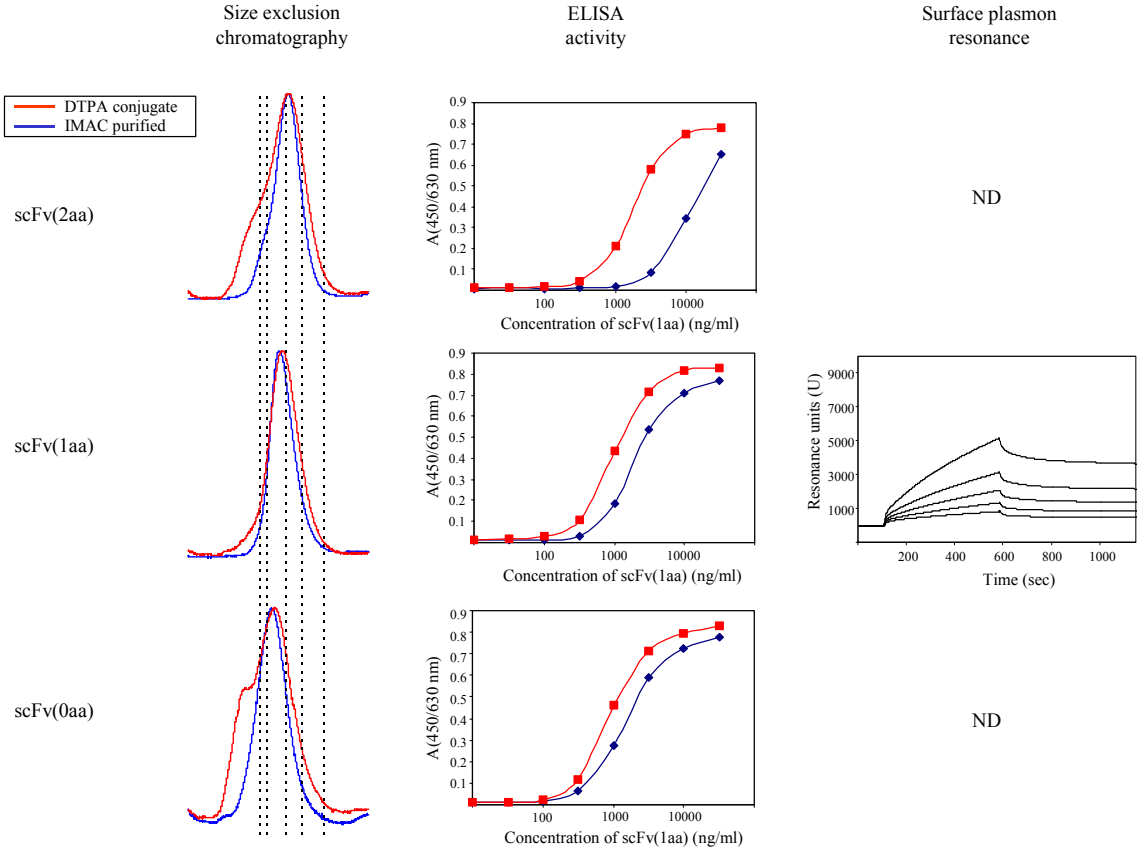

Figure 34 Quality control of DTPA conjugated scFvs
The multimerisation grade of the DTPA conjugated scFvs was investigated by size exclusion chromatography using an equilibrated G-200 column with a flowrate of 0.1 ml/min. The binding activity of the constructs were tested in ELISA by binding to immobilised asialoglycophorin (0.1 µg/ml), with the different IMAC-purified constructs as controls, and finally the real time binding kinetics were investigated by surface plasmon resonance using a chip with immobilised asialoglycophorin on a Biacore2000 (for the scFv(1aa)).

2.6.1.4 Labelling

The conjugation of DTPA to the scFvs enables labelling with radiometals such as ^{111}In and ^{90}Y. Of these the preferred radiometal for investigating the *in vitro* characteristics and the pharmacokinetics is ^{111}In because it is a γ-emitter, while ^{90}Y as a β-emitter is applicable for therapeutic trials. The labelling kinetics of several of the preparations were investigated by thin layer chromatography (TLC) as shown in **Figure 35** and elaborated in the figure legend. The kinetics seem to depend on the protein concentration and the stoichiometry of conjugation, the slowest labelling was thus observed for the preparations scFv(1aa)$_{PR1}$ and scFv(1aa)$_{PR2}$ (lower protein concentration), and the scFv(0aa) (lower stoichiometry, 1:20) when compared to the kinetics of the scFv(1aa)$_{PR3}$. After labelling, the preparations were washed with PBS using spin filtration (Centricon Y-30), resulting in a buffer change and ensuring that the majority of the radioactivity in the preparation was bound to the antibodies (>95%).

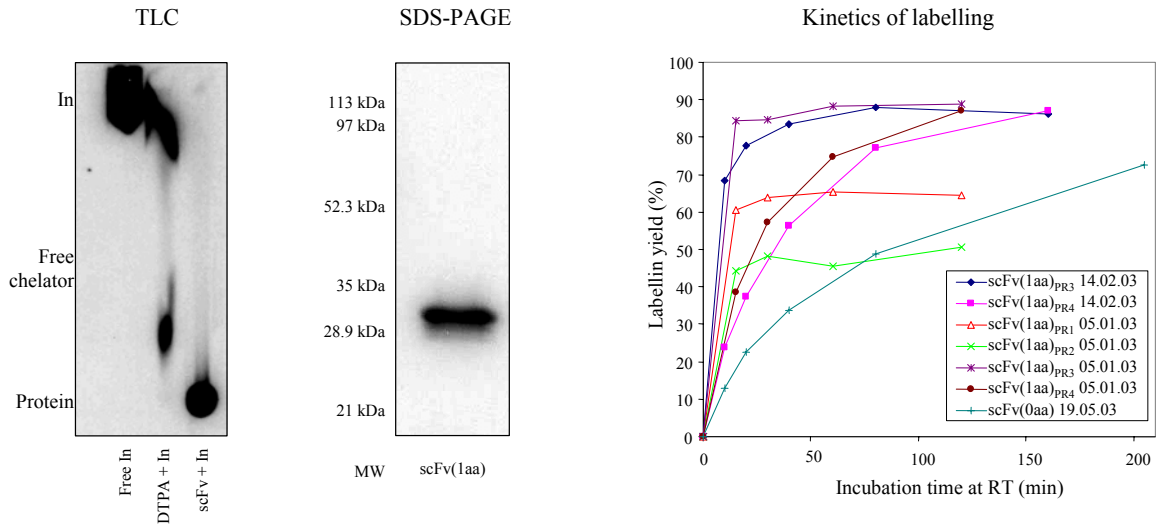

Figure 35 Labelling of scFvs
Labelling yield of the scFvs with ^{111}In was investigated by thin layer chromatography (TLC). The left panel shows a radiograph of a TLC showing a clear separation of free, DTPA-bound and scFv bound ^{111}In, with high, moderate and low mobility, respectively. Cutting the thin layer chromatography plate and measuring the radioactivity enables a quantification of the labelling and thereby analysis of the kinetics as shown in the right panel. Labelling was performed at an antibody to ^{111}In molar ratio of >1000. The plot of the data indicates a correlation between the kinetics and the protein concentration or chelator concentration when correlated to **Table 10**. The purity/stability of the labelled scFv was assessed by SDS-PAGE as illustrated for the scFv(1aa) showing a single band.

2.6.2 Analysis of the ^{111}In labelled scFvs

The ^{111}In labelled scFvs were first analysed for size, stability, and activity in binding assays against solid-phase immobilised TF and cell-surface exposed TF.

2.6.2.1 Size exclusion chromatography

The size of the ^{111}In labelled scFv(1aa) was investigated by size exclusion chromatography using 10 ml mini-columns packed with G-50, G-75, G-100 and G-150 as depicted in **Figure 36**. The scFv(1aa) is eluted in the void volume on columns with G-50 or G-75, indicating that the molecules are larger than 80 kDa. In addition, scFv(1aa) migrates slower on the columns with G-100 and G-150, indicating that the molecules may be smaller than 150 kDa. Analysing the scFv(1aa) by size exclusion chromatography on a Superdex 200 10/30 column equilibrated with 1% BSA-PBS shows a single peak at an expected lower molecular mass than that of the labelled control IgG_1 (150 kDa) (**Figure 36**). In summary, the size exclusion chromatography of the ^{111}In-labelled scFv(1aa) indicates a conserved formation of trimeric complexes.

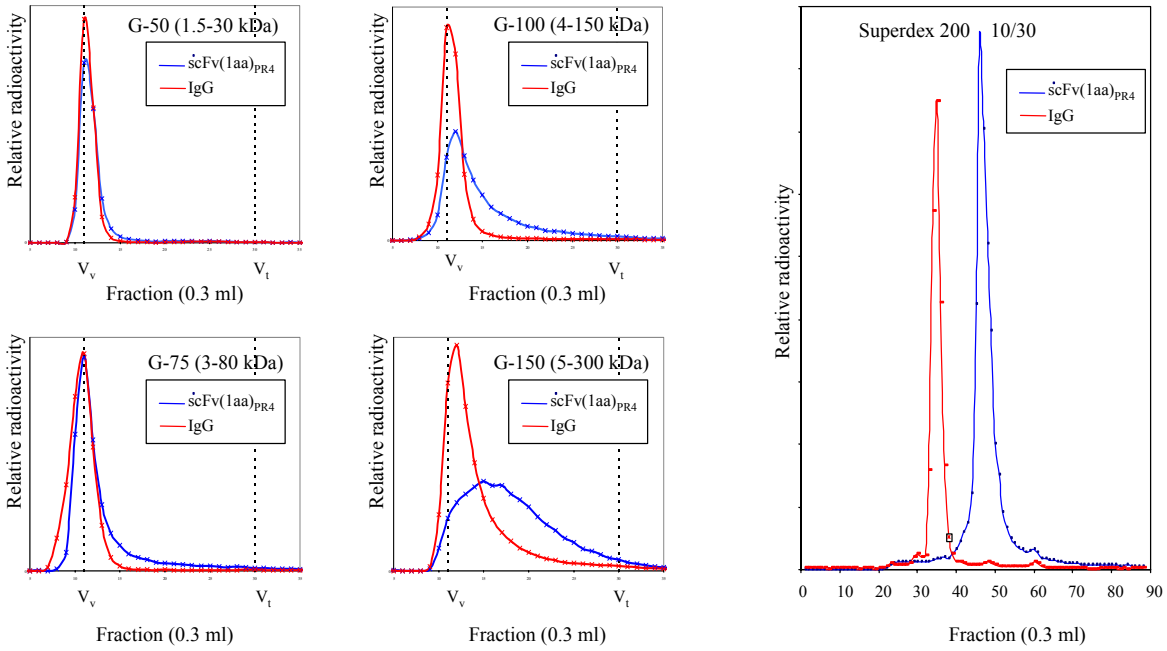

Figure 36 Size exclusion chromatography of labelled scFv(1aa)
^{111}In-labelled scFv(1aa) and a control IgG were passed over 10 ml columns packed with either G-50, G-75, G-100 or G-150 in order to address the molecular size of the antibody (A) All columns were equilibrated with 1% BSA-PBS and run by gravity flow. The relative size of the scFv(1aa) was demonstrated by size exclusion chromatography on a Superdex 200 10/30 column equilibrated with 1% BSA-PBS and run with a flow-rate of 0.1 ml/min. The scFv(1aa) runs as a single peak and slower than the peak of the control IgG_1 (150 kDa).

2.6.2.2 Radioimmunoassay (RIA)

Radioactive labelling of the antibodies enables tracing of the antibodies in a binding assay by detection of radioactivity. The amount of bound antibody and the amount of free antibody can subsequently be calculated.

The ^{111}In labelled scFvs were targeted to solid-phase immobilised asialoglycophorin or TF-PAA in *in vitro* binding assays (RIA). Scatchard analysis of the data from RIA of scFv(0aa) and scFv(1aa)

against different concentrations of asialoglycophorin and TF-PAA are exemplified in **Figure 37,** and a summary of data is presented in **Table 11**. The dissociation constants for the scFv(0aa) and scFv(1aa) are very similar to the constants obtained by the Biacore analysis. Again the scFv(0aa) has a better affinity than the scFv(1aa).

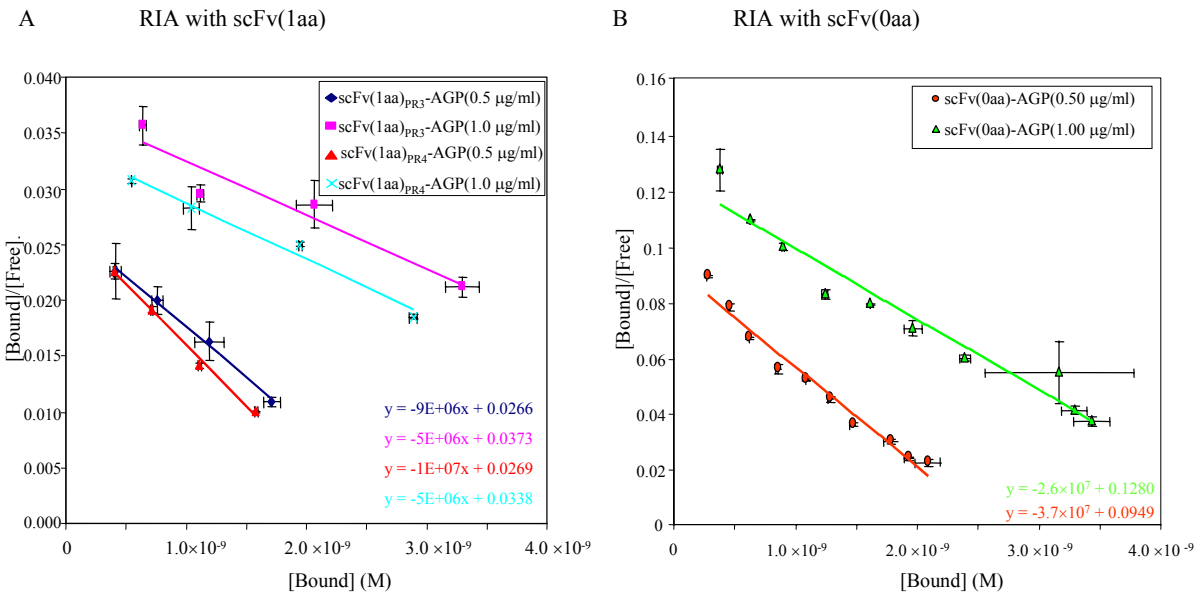

Figure 37 Scatchard analysis of RIA
Immobilised asialoglycophorin or TF-PAA was targeted with ^{111}In labelled scFv(0aa) or scFv(1aa) in a radioimmunoassay (RIA). The obtained data on amounts of bound antibody and non-bound antibody (free) can be processed in a Scatchard plot in which the slope of the trendline equals the negative association constant (Ka) (see appendix A for explanation). Data from RIA with the scFv(1aa)$_{PR3}$ and scFv(1aa)$_{PR4}$ are processed in (A), while data from RIA with the scFv(0aa) are processed in (B). The calculated dissociation constants are summarised in **Table 11**.

Table 11 Dissociation constants measured by RIA

RIA	Antigen conc.	Kd (M) ScFv(0aa)	Kd (M) ScFv(1aa)
	0.5 µg/ml	2.7×10^{-8} (N=1)	$1.0\times10^{-7} \pm 1.1\times10^{-8}$ (N=4)
	1.0 µg/ml	3.9×10^{-8} (N=1)	$1.7\times10^{-7} \pm 4.3\times10^{-8}$ (N=8)
Biacore	Antigen	Kd (M) ScFv(0aa)	Kd (M) ScFv(1aa)
	-	8.8×10^{-8} (N=1)	$2.2\times10^{-7} \pm 1.1\times10^{-8}$ (N=4)

Dissociation constants Kd for scFv(0aa) and scFv(1aa) have been calculated by Scatchard plots from data obtained in radioimmunoassays against asialoglycophorin as antigen (N) = number of individual experiments.

2.6.2.3 Cell bindings

The results of the *in vitro* cell binding assays may reflect more relevant data of the antibody binding to natural occurring antigens compared to the artificial RIA conditions, and cell binding can help elucidate several interesting aspects such as the percentage of antibodies able to bind, the dissociation constant (Kd) for binding to the cells, and the number of antibody binding sites on the cells. Binding

assays were performed with ^{111}In-labelled preparations of the three constructs, scFv(0aa), scFv(1aa) and scFv(2aa). The percentage of antibodies able to bind can be addressed in consecutive cell bindings, where the unbound antibodies from one round of cell binding are allowed to associate with new cells. Theoretically, all active antibodies will bind to cells, and the percentage of non-active antibodies can be measured in the last supernatant. Consecutive cell-binding with scFv(1aa) on NM-D4 cells indicate that more than 50% of the scFv(1aa) can bind to the cells using a standard experimental set-up (2×10^6 cells in 200 µl) **Figure 38**. In addition, it was observed that the binding percentage was increased when the cell concentration was increased (10^7 cells in 200 µl) **Figure 38**. The consecutive binding with the four different preparations of scFv(1aa) (PR1-PR4) shows an exceptional similarity, demonstrating the reproducibility of the preparation protocol. Alternatively, the binding activity level can be investigated by titrating a given amount of antibody with increasing amounts of cells in a given volume (binding sites in excess). Extrapolation of the plot of percent bound versus the amount of cells indicates a maximum binding percentage as seen in **Figure 38**. For the scFv(1aa) the Lindmo plot indicates a binding activity of approximately 50%. Lindmo and co-workers investigated this in further detail. If the law of mass action is applied on the equation of the association constant, and the correlating for the fraction (r) of active antibody, equation {1} is found (see appendix 2).

{1} $$[Ab_{Total}]/[Ab_{Bound}] = 1/r + 1/rKa[Ag_{Free}]$$

Assuming that the concentration of free antigen is significantly higher than the concentration of bound antigen, the concentration of free antigen is approximately equal to the total antigen concentration (or cell concentration). Thus in a blot of $[Ab_{Total}]/[Ab_{Bound}]$ versus inverse cell concentration, extrapolation of a straight line to the interception with the ordinate gives the value of 1/r. For the scFv(1aa) preparation shown in Figure 38, r was thus calculated to 48%.

In conclusion, the consecutive bindings and the Lindmo analysis indicate a good level of binding activity. It should be noted that the cells are biological systems with potential variations between cultures, and no possibility for controlling antibody internalisation, antigen shedding or cell rupture. Finally, the equilibrium kinetics may be to slow to reach equilibrium in the cell binding, and may have a significant effect on the Lindmo analysis (Lindmo et al., 1984). Based on these results and the size exclusion chromatography, it was assumed that the majority of the scFvs were active.

The non-specific association of scFv to cells was addressed in a high cell concentration cell binding of scFv(1aa) with either K-562 or NM-D4 (2×10^7 cells in 200 µl). The non-specific binding to the K-562 cells was less than 0.9% while 36.2% bound to the NM-D4 cells. It should be noted that the 0.9% binding activity was close to the detection limit.

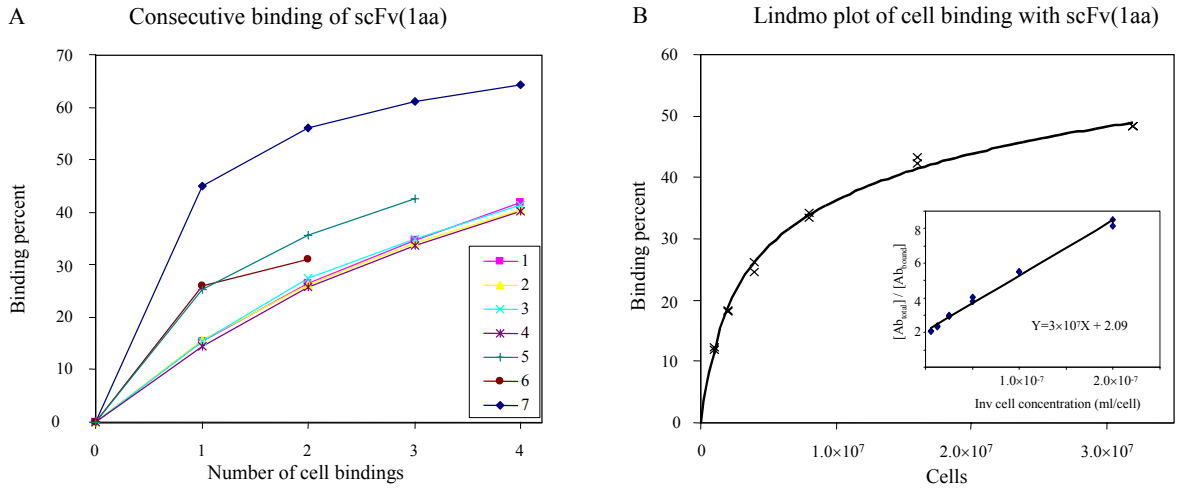

Figure 38 Proportion of active antibodies in the labelled antibodies
The proportion of active scFv in the preparations was investigated by consecutive binding to NM-D4 (A) or in a Lindmo plot (B). The consecutive bindings were performed in 200 µl with 2×10^6 cells at 4°C. After 1 h of incubation, cells were spun, and the supernatant transferred to fresh cells for a new binding incubation. Four simultaneous consecutive bindings were performed with the four different preparations of scFv(1aa) (PR1-PR4), curves 1-4 shows exceptional similarity. Data for curves 5 and 6 were obtained individually with $scFv(1aa)_{PR3}$, and data for curve 7 was obtained with $scFv(1aa)_{PR3}$ but performed with 10^7 cells in 200 µl. For the Lindmo plot, increasing concentrations of NM-D4 cells were incubated in 200 µl with a constant concentration of labelled scFv(1aa). In the inserted plot in B, the data has been depicted according to the extended Lindmo plot, which indicats an r value of 48% (see appendix 2).

The association constant for the cell binding was determined by another experimental set-up. Measurements of the binding level in a series of antibody concentrations with a constant number of cells were processed in a Scatchard plot, in which the slope of the trendline equals the negative association constant (Ka), and an extrapolation to the abscissa reveals the number of binding sites (see appendix). An example of a Scatchard plot analysis for the binding of two different preparations of scFv(1aa) to NM-D4 cells are shown in **Figure 39**, and a summary of the cell binding data is presented in **Table 12**.

Different cell lines had different numbers of binding sites, and the antibodies bound with different affinities to the cell lines. Again, the scFv(0aa) had a lower dissociation constant than the scFv(1aa) or the scFv(2aa) in good agreement with the other *in vitro* analysis (Chapter 2.3.3.1, 2.3.3.6, and 2.6.2.2). The binding percentage observed for the scFv(1aa) binding was generally lower than the binding percentage obtained for the consecutive binding with the scFv(1aa) which was expected since the experiments were made with higher concentrations of scFv(1aa), which lowers the binding percentage due to the equilibrium constants. Although the standard deviations are relatively high, it is possible to compare the binding percentage of the scFvs to the different cell lines. This supports the observation that the scFv(0aa) is more affine than the scFv(1aa) and scFv(2aa), as it has higher binding percentages for the different cell lines.

A Cell binding on NM-D4

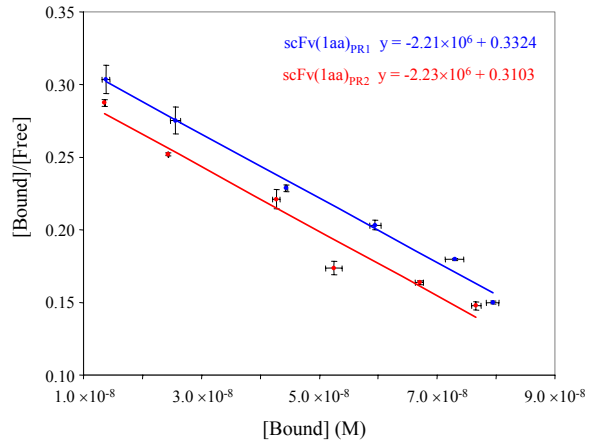

Figure 39 Cell binding with scFv(1aa)
Two individual preparations of scFv(1aa) were labelled with ^{111}In, and applied in cell binding studies against cell lines NM-D4 (A) and KG-1 (B). Binding was performed in final volumes of 200 µl applying 2×10^6 cells and incubating 1 h at 4°C. Data was obtained by tracing the radioactivity after a brief washing and centrifugation. [Bound] corresponds to the indirect concentration scFv(1aa) associated with the cell pellet while [Free] corresponds to the concentration of scFv(1aa) in the supernatant.

Table 12 Summary of data from cell bindings with the ^{111}In labelled scFvs

	Cell type	Ka (M^{-1})	Binding sites	% binding	Kd (M)
ScFv(0aa)					
N=4	NM-D4	$1.1\times10^7 \pm 3.6\times10^6$	$4.8\times10^6 \pm 3.3\times10^6$	37.8 ± 6.2	$1.0\times10^{-7} \pm 4.6\times10^{-8}$
N=3	ZR-75-1	$2.6\times10^6 \pm 9.5\times10^5$	$2.5\times10^6 \pm 2.0\times10^5$	10.3 ± 2.9	$4.2\times10^{-7} \pm 1.6\times10^{-7}$
ScFv(1aa)					
N=9	NM-D4	$2.1\times10^6 \pm 7.3\times10^5$	$8.1\times10^6 \pm 5.5\times10^6$	17.2 ± 7.5	$5.3\times10^{-7} \pm 1.8\times10^{-7}$
N=12	KG1	$1.3\times10^6 \pm 1.8\times10^6$	$1.6\times10^7 \pm 1.0\times10^6$	11.6 ± 8.2	$2.5\times10^{-6} \pm 1.9\times10^{-6}$
N=3	ZR-75-1	$7.8\times10^5 \pm 3.1\times10^5$	$2.2\times10^6 \pm 1.2\times10^6$	2.3 ± 0.6	$1.5\times10^{-6} \pm 7.4\times10^{-7}$
ScFv(2aa)					
N=1	NM-D4	1.2×10^6	9.2×10^6	14	8.3×10^{-7}
N=2	ZR-75-1	$3.0\times10^5 \pm 2.5\times10^5$	$1.2\times10^7 \pm 8.6\times10^6$	3.5 ± 0.7	$5.2\times10^{-6} \pm 4.4\times10^{-6}$

2.6.2.4 Stability of the ^{111}In labelled scFvs

The stability of the ^{111}In-labelled scFvs was investigated in serum incubation studies addressing two different aspects. Firstly, the integrity of the ^{111}In labelling was investigated by TLC showing that no indium was released from the protein, and by spin-filtration, applying Amicon YM-100 tubes with a cut-off around 100 kDa, showing that the protein is not proteolytically degraded (data for the YM-100 filtrations are presented in **Table 13)**. The data clearly show that the protein is not proteolytically degraded. In addition, it is interesting that less radioactivity can be spun through after the incubation in FCS.

Secondly, the binding activities of the ^{111}In-abelled scFvs were investigated in cell binding following an overnight incubation in BSA-PBS or in human serum (data summarised on **Table 14**). The data show that the binding activity of the scFv(0aa) is unaffected by the incubation in human serum, which is also indicated for the scFv(1aa) and scFv(2aa).

Table 13 Percent radioactivity spun through YM-100 after incubation in FCS

	scFv(0aa)	scFv(1aa)	scFv(2aa)
PBS	54%	73%	93%
FCS	16%	12%	17%

Samples of the ^{111}In-labelled scFv(0aa), scFv(1aa) and scFv(2aa) were incubated 24 h at 37°C in either PBS or in FCS. Subsequently, the proteins were filtered using Amicon YM-100 tubes (cut off 100 kDa). The radioactivity in the spin through was measured and the percentage of the total calculated. (N=2)

Table 14 Cell binding with scFvs after incubation in FCS

0 H	scFv(0aa)	scFv(1aa)	scFv(2aa)
	37.8 ± 6.2%	17.2 ± 7.5%	14%
1 h	scFv(0aa)	scFv(1aa)	scFv(2aa)
PBS	38%	11%	15%
FCS	40%	14%	14%
24 h	scFv(0aa)	scFv(1aa)	scFv(2aa)
PBS	44%	13%	20%
FCS	29%	12%	16%
72 h	scFv(0aa)	scFv(1aa)	scFv(2aa)
PBS	44%	12%	24%
FCS	47%	4%	14%

Samples of the ^{111}In-labelled scFv(0aa), scFv(1aa) and scFv(2aa) were incubated at 37°C in either BSA-PBS or in human serum. Cell binding was performed with 2×10^6 NM-D4 cells in 200 µl at 4°C and with 500 ng scFv(0aa), 1 µg scFv(1aa) and 2 µg scFv(2aa), respectively.

2.6.3 Biodistribution

2.6.3.1 Biodistribution in healthy mice

The first biodistribution experiment was carried out in healthy nude mice in order to test the targeting of normal organs. Three animals were injected with doses of 5 µg ^{111}In-labelled scFv(1aa), and sacrificed after 24 h in order to measure the radioactivity in the different organs. Results are summarised in **Table 15**. The radioactivity uptake in the kidney was in the range of 50% ID/g, while all other organs were in the range of 0 - 2.6% ID/g.

Table 15 Biodistribution of scFv(1aa) in healthy nude mice

Study (NMD)	Dose (µg)	Time (h)	ScFv type	Serum (%ID/ml)	Tumour (%ID/g)	Liver (%ID/g)	Kidney (%ID/g)	Lung (%ID/g)	Heart (%ID/g)	Spleen (%ID/g)	Brain (%ID/g)	Bone marrow (%ID/g)
29	5	24	1aa	1.9 (0.15)	-	2.6 (0.13)	52.5 (2.69)	1.3 (0.02)	1.2 (0.07)	2.2 (0.25)	0.7 (0.01)	0.31 (0.07)

^{111}In-labelled scFv(1aa) was injected into 3 healthy nude mice to investigate the biodistribution. Uptake in organs is expressed as percent injected dose per gram (%ID/g). Standard deviations are shown in parentheses. NMD is the Nemod nomenclature for mouse trials

2.6.3.2 Biodistribution in nude mice bearing colorectal carcinoma xenografts

A mouse model based on xenotransplants of human colorectal carcinoma in nude mice was established at EPO GmbH. Prior to the trial, the tumour tissue had been tested positive for TF by immunohistochemistry of tumour cryosections (Karsten, personal communication). Tumour targeting with the scFv(1aa) was investigated in this model in two studies (NMD 36 and NMD 40) addressing dose and time course, respectively. The data have been summarised in **Table 16**. There are several important observations. Tumour uptake was quantified to 5.6 %ID/g and 3.7 %ID/g (5 µg dose at 24 h) for the NMD 36 and NMD 40, respectively. The difference could be caused by the differences in average tumour size, which were 42 and 376 mg, respectively. Secondly, the tumour targeting did not seem to be dose dependent and the scFv(1aa) bound to the tumour was residing there. The radioactivity (%ID/g) in the tumour decreased over time but not more significant than from other organs. The high uptake in the spleen in NMD 36 was not observed in NMD 40, or in NMD 29, and is probably caused by unknown individual conditions in NMD 36. The high uptake in the kidneys was measured in both trials and decreased only with the same rate as for the tumour over time.

Table 16 Biodistribution in nude mice bearing human colorectal carcinoma xenografts

Study (NMD)	Dose (µg)	Time (h)	ScFv type[a]	Serum (%ID/ml)	Tumour (%ID/g)	Liver (%ID/g)	Kidney (%ID/g)	Lung (%ID/g)	Heart (%ID/g)	Spleen (%ID/g)	Brain (%ID/g)	Bone marrow (%ID/g)
36	1.7	24	1aa	2.3 (0.21)	7.1[b] (3.76)	6.2 (0.55)	54.4 (5.96)	2.4 (0.43)	1.9 (0.28)	13.2 (2.26)	0.4 (0.03)	1.0 (0.13)
36	5	24	1aa	2.1 (0.37)	5.6[b] (0.83)	7.0 (2.32)	50.2 (10.4)	2.7 (0.13)	2.0 (0.27)	15.5 (6.67)	0.1 (0.01)	1.3 (0.07)
36	15	24	1aa	2.3 (0.11)	5.2[b] (1.11)	7.5 (1.87)	42.4 (2.42)	2.3 (0.21)	1.7 (0.23)	18.1 (5.99)	0.1 (0.01)	1.0 (0.21)
40	5	24	1aa	0.7 (0.15)	3.7[c] (0.43)	4.8 (0.57)	77.5 (12.9)	2.2 (0.18)	1.6 (0.1)	4.3 (0.54)	0.1 (0.11)	0.9 (0.12)
40	5	48	1aa	0.2 (0.13)	2.6[c] (0.30)	3.6 (0.11)	55.4 (7.73)	1.0 (0.12)	1.0 (0.03)	3.4 (0.23)	0 (0.01)	0.8 (0.13)
40	5	72	1aa	0.1 (0.04)	1.9[c] (0.26)	3.3 (0.33)	36.2 (4.56)	0.7 (0.05)	0.8 (0.09)	2.8 (0.46)	0 (0)	0.6 (0.16)

^{111}In-labelled scFv(1aa) was injected into nude mice bearing human colorectal carcinoma xenografts. Groups of 3 mice were investigated in the biodistributions. Uptake in tumours and organs is expressed as percent injected dose per gram (%ID/g). Standard deviations are shown in parentheses.

[a]; the antibody tested: 1aa, scFv(1aa).

[b,c]; The average tumour size in the NMD 36 was 42 mg, while the average tumour size in NMD 40 was 376 mg.

2.6.3.3 Biodistribution in nude mice bearing ZR-75-1 xenografts

A second mouse model based on xenotransplants of ZR-75-1 cells (subcutaneous injection of 10^7 cells) in nude mice was established at EPO GmbH and used to investigate biodistribution of the scFvs. Immunofluorescence (chapter 2.3.3.3) and cell binding experiments (chapter 2.6.2.4) demonstrated the TF exposure on these cells. Futhermore, immunohistochemistry on cryosections of the tumours with a chimeric IgG1 encoding the variable genes from the scFv(1aa) (cloned and expressed by Dr. Antje Danielczyk) indicated TF expression. **Figure 40** shows two sections stained with the chimeric IgG. The upper panel shows scattered staining of TF-positive tumour cells (brown) interspersed TF-negative cells (blue). In the lower panel, the distribution of the TF-positive cells seems to be more dense but also less intense. Necrotic areas are also seen on the sections (white). Immunohistochemistry was performed with the chimeric IgG1 because the mice unexpectedly expressed high levels of IgG, causing high levels of background due to the mouse origin of the available secondary antibodies for detection of the scFv(1aa) (not shown).

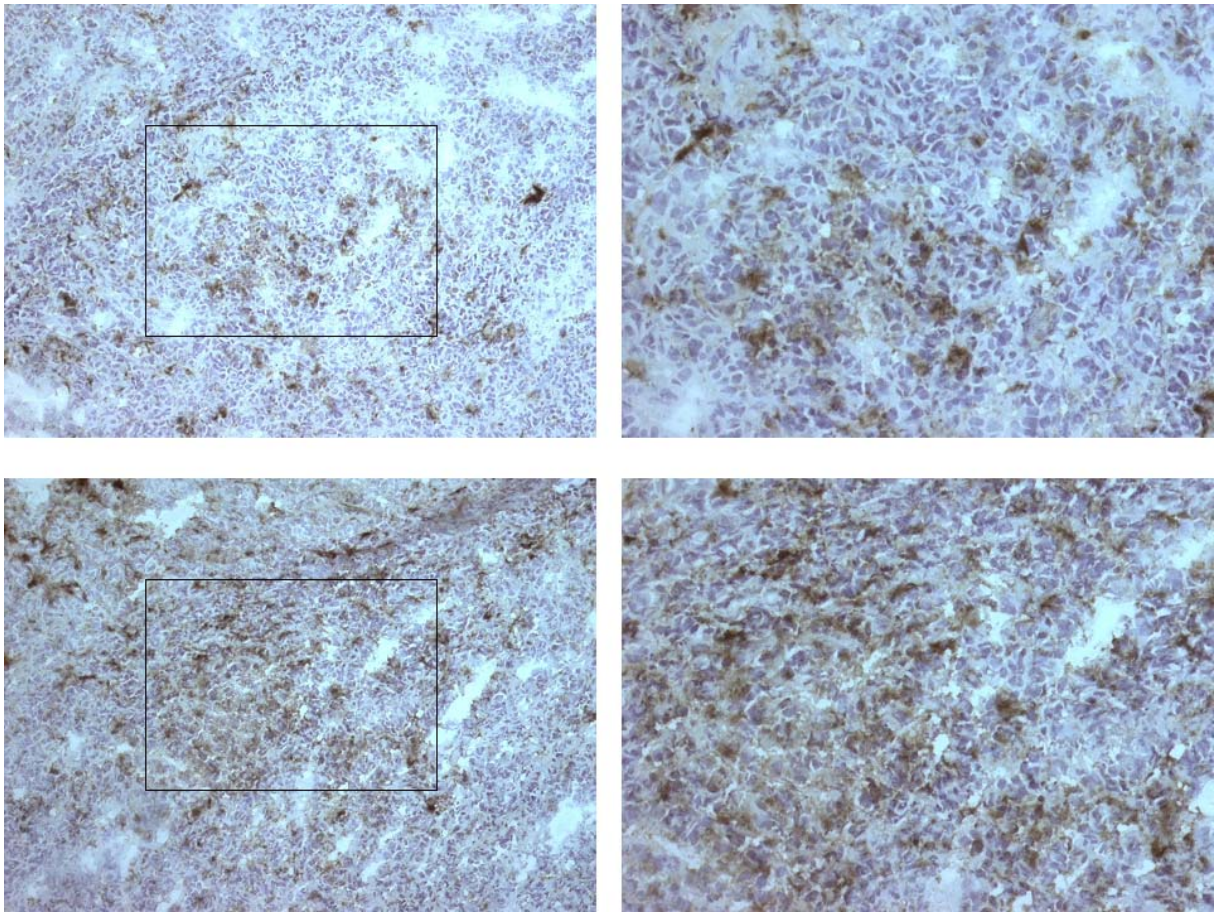

Figure 40 Immunohistochemistry of tumour from ZR-75-1 mouse model
Cryosections of tumours from the ZR-75-1 mouse xenotransplant model were stained with a chimeric IgG1 encoding the variable genes of the scFv(1aa). Photos were taken with 10x lens (left panel) or 20x lens (right panel), respectively.

The purpose of the first biodistribution trial in the ZR-75-1 mouse model (NMD41) was to compare the distribution of the scFv(1aa) in this model with the distribution in the colorectal carcinoma model (chapter 2.6.3.2). Hence, groups of 4 mice were injected with doses of 5 and 15 µg ^{111}In-labelled scFv(1aa), and the distribution evaluated after 24 h (**Table 12**). A higher tumour uptake was observed for the ZR-75-1 mouse model, especially for the 5 µg dose, resulting in ~ 10 %ID/g, while all other organs had low uptakes. Included in NMD41 was also an investigation of the potential effect of free lysine with respect to a possible reduction of the kidney burden. However, in contrast to other studies, no effect of free lysine were observed (Behr et al., 1995; Behr et al., 1997; Tsai et al., 2001; Yazaki et al., 2001).

If the high kidney burden observed for the scFv(1aa) was caused by the size of the scFv(1aa) molecule, which is close to the cut-off size for the kidney filtration, then the larger scFv(0aa) should result in a less significant kidney burden. Consequently, the biodistribution of the scFv(0aa) was investigated in NMD43, in which a group of 3 mice were injected with a dose of 5 µg ^{111}In-labelled scFv(0aa), and the distribution investigated after 24 h **(Table 17)**. Compared to the scFv(1aa), the kidney burden of the scFv(0aa) was indeed decreased 7 fold, while no increase was seen for tumour targeting. It should be noticed that the average tumour size in NMD 43 was significantly larger than the average tumour size in NMD 36 and NMD 41, but more comparable to the average tumour size in NMD 40, where tumour uptake was considerably higher. The serum clearance of the scFv(0aa) was slightly slower than the serum clearance of the scFv(1aa) as illustrated in **Figure 41.**

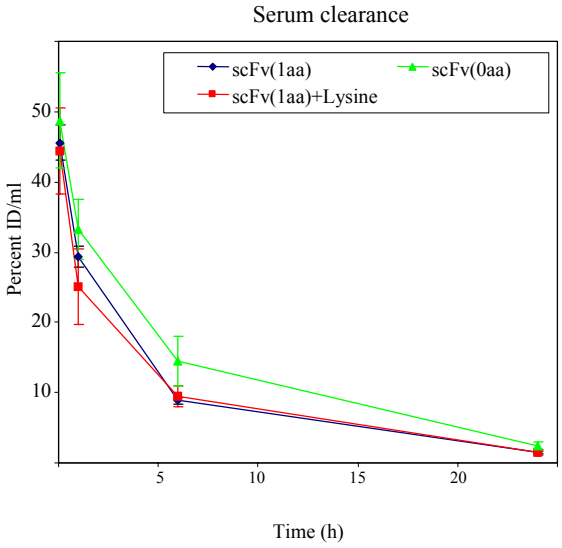

Figure 41 Serum clearance in the ZR-75-1 mouse model
Serum clearance rates of the ^{111}In-labelled scFv(0aa) and scFv(1aa) in nude mice bearing human colon carcinoma or ZR-75-1 tumour xenografts

These results led to the last biodistribution trial (NMD 4), which was a short time course study of the scFv(0aa) and scFv(1aa) investigating the distribution at 4 and 24 h. As seen in **Table 17**, the overall tumour uptake in this trial was lower than that in the previous trials for both the scFv(0aa) and the scFv(1aa). Another trial with mice from the same cohort where another antigen was targeted revealed a similar drop in tumour targeting (Stahn, personal communication). Still, the data reveal the same trend as before for the kidney burden, namely that the burden is significantly less for the scFv(0aa) than for the scFv(1aa). In addition, there were only minor differences between the distribution at 4 and 24 h, indicating that the majority of the tumour targeting occurs shortly after injection, and thus in contrast to the general observations for IgGs, where tumour targeting increases during several days.

Table 17 Biodistribution in nude mice bearing ZR-75-1 xenografts

Study (NMD)	Dose (µg)	Time (h)	scFv type[a]	Serum (%ID/ml)	Tumour (%ID/g)	Liver (%ID/g)	Kidney (%ID/g)	Lung (%ID/g)	Heart (%ID/g)	Spleen (%ID/g)	Brain (%ID/g)	Bone marrow (%ID/g)
41	5	24	1aa	1.4 (0.16)	10.8[d] (2.88)	3.7 (0.15)	56.7 (2.33)	1.7 (0.11)	1.5 (0.06)	5.4 (0.75)	0.1 (0.01)	1.0 (0.16)
41	5	24	1aa[L]	1.5 (0.37)	7.8[d] (2.61)	3.6 (0.89)	54.9 (10.9)	2.2 (0.74)	1.6 (0.27)	5.2 (1.37)	0.1 (0.02)	0.9 (0.20)
41	15	24	1aa	1.5 (0.40)	5.6[d] (1.52)	3.7 (0.28)	57.4 (8.43)	2.0 (0.41)	1.7 (.026)	6.2 (0.77)	0.1 (0.03)	0.9 (0.20)
43	5	24	0aa	1.0 (0.24)	8.1[e] (1.45)	5.3 (0.92)	11.6 (0.34)	1.9 (0.19)	1.9 (0.19)	6.7 (1.07)	0.1 (0)	1.7 (0.90)
45	5	24	0aa	-	2.6[f] (0.37)	9.5 (1.90)	13.7 (1.46)	8.9 (0.83)	4.5 (0.40)	5.9 (0.55)	0.5 (0.08)	1.4 (0.28)
45	5	48	0aa	-	4.2[f] (1.41)	5.4 (0.82)	11.9 (0.18)	2.3 (0.35)	1.9 (0.12)	8.2 (0.96)	0.1 (0.01)	1.9 (0.77)
45	5	72	1aa	-	2.7[f] (0.43)	4.7 (0.91)	37.5 (5.23)	5.7 (0.88)	3.2 (0.52)	7.3 (2.07)	0.3 (0.02)	0.5 (0.24)
45	5	72	1aa	-	2.5[f] (0.31)	3.2 (0.78)	40.7 (4.52)	1.4 (0.10)	1.2 (0.03)	6.4 (0.21)	0.1 (0)	0.8 (0.25)

[111]In-labelled scFv(1aa) was injected into nude mice bearing human colorectal carcinoma xenografts. Groups of 4 mice were investigated in NMD41 and NMD45, while a group of 3 mice were investigated in NMD43. Uptake in tumours and organs is expressed as percent injected dose per gram (%ID/g). Standard deviations are shown in parentheses.

[a]; the antibody tested: 1aa, scFv(1aa); 0aa, scFv(0aa); [L] Co-injection of 50 mg lysine

[d,e,f]; The average tumour size in the NMD41 was 32 mg, while the average tumour size in NMD43 was 260 mg, and , the average tumour size in NMD45 was 324 mg.