Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 786 (2003) 327–342
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Production, purification and characterisation of genetically derived scFv and bifunctional antibody fragments capable of detecting illicit drug residues

Joanne Brennan*, Paul Dillon, Richard O'Kennedy

Applied Biochemistry Group, School of Biotechnology, Dublin City University, Glasnevin, Dublin 9, Co. Dublin, Ireland

Received 2 July 2002; received in revised form 25 September 2002; accepted 9 October 2002

Abstract

We have generated a single chain antigen binding protein (scFv) recognising morphine. Variable regions of heavy (V_H) and light (V_L) chain antibody genes isolated from a murine immune repertoire were connected via a glycine–serine linker and cloned into the expression vector pAK 400. The scFv was produced in *Escherichia coli* JM83 yielding a functional protein of approximately M_r 30 000. Immunoaffinity chromatography using M3G–BSA–Sepharose column proved most effective for scFv purification. Purity was monitored by SDS–PAGE and Western blotting and the scFv characterised using ELISA and BIAcore™. The scFv was capable of specifically binding free morphine in solution and was applicable to real sample analysis in saliva. In order to express a bivalent ''minibody'' the scFv gene was recloned into a vector containing a gene encoding a helix for dimerisation. The scFv was expressed as a protein of M_r 75 000 and retained its antibody binding capabilities. Cloning the scFv gene into a vector containing the bacterial alkaline phosphatase gene produced a bifunctional molecule, which retained the binding activity of the parental scFv along with the enzymatic activity of alkaline phosphatase.

Keywords: Expression vectors; BIAcore; Purification; scFv

1. Introduction

Heroin was first introduced in 1898 [1] and still remains one of the most frequently abused drugs today. This, along with a steady increase in the abuse of prescription opiates like morphine, has meant that increased attention is being focussed on the development of simple, rapid, sensitive detection methods for drug monitoring in treatment centres, forensic investigations, in the workplace and on the roadside. Traditional methods of detection for these compounds include thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [2]. Although effective, these analytical techniques prove time-consuming, expensive and require skilled personnel to carry out the analysis.

Since the advent of the immunoassay, increased attention has been focussed on using antibodies as detection agents for low molecular mass molecules as an alternative to ''standard'' analytical techniques. Immunoassays have been proven in their sensitivity, specificity, reproducibility and robustness [3] but the

*Corresponding author. Fax: +353-1-7005-412.
E-mail address: joanne.brennan4@mail.dcu.ie (J. Brennan).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00807-3

quality of the assay is entirely dependent upon the availability of high quality antibodies for detection. Previous immunoassays for heroin metabolites have predominantly used polyclonal or monoclonal antibodies [4,5]. However, there is still a demand for simpler, faster detection methods and assay formats that are more amenable to on-site monitoring. Recombinant antibody technology has revolutionised the field of immunoanalysis, allowing the generation of an immortal repertoire of antibody fragments with a defined affinity and specificity towards target molecules [6]. It also offers the possibility of altering the antibody's affinity and specificity [7] if desired. However, although the literature may suggest that obtaining such functional antibodies is straightforward, this is not necessarily the case [8,9]. In order to circumvent some of these problems, Krebber et al. [10], have engineered an optimised phage display system for the generation of single chain antibody fragments (scFvs). The system was optimised for robustness, vector stability, tight control of scFv-Δgene III expression, primer usage for PCR amplification of variable genes, scFv assembly strategy and subsequent directional cloning using a rare single cutting restriction enzyme. A compatible vector series to aid purification, detection, multimerisation and modification is also described. The Krebber system [10] has been employed for the generation of specific scFvs with high affinity for the major metabolites of heroin [11].

The pAK vector series described by Krebber et al. [10] can be used for phage display or for the expression of the antibody in a variety of formats. Each vector consists of a chloramphenicol resistance gene and a tetracycline resistance ''stuffer'' gene, which will be replaced with the genes encoding the variable heavy and light genes of the antibody. The tetracycline resistance casette is flanked on either end with an *Sfi*I restriction site, a restriction site that is very rare in antibody sequences, thus eliminating the possibility of internal digestion. *Sfi*I always cuts two sites at once, leaving a 3-basepair over-hang, to prevent self-annealing and facilitate directional cloning. All vectors contain a *Pel B* leader sequence; a *lac* promoter/operator to allow repression of translation by the addition of glucose; a *lac* repressor gene to ensure independent *lac* promoter repression; and a strong upstream terminator (t_{HP}), which in combination with the *lac* promoter eliminates background expression prior to induction. The primers for the construction of the variable regions encode a shortened version of the FLAG sequence (DYKD), which may be used as a handle for detection/purification [12]. The pAK 400 vector contains a much stronger Shine Dalgarno sequence (SDT7g10) for increased expression and a C terminal hexahistidine tag for purification using immobilised metal affinity chromatography (IMAC) [13–15] or for detection using an anti-his tag antibody. The pAK 500 vector contains a single chain double helix (scdHLX) for dimerisation. This consists of a C-terminal flexible hinge isolated from murine IgG3 and a helix 1-turn-helix 2 followed by a pentahistidine tag. The resultant homodimerised minibody is reported to have increased antigen affinity [16]. The pAK 600 vector encodes the bacterial alkaline phosphatase gene to enable direct detection and facilitate dimerisation by scFv–AP fusions [15,17]. Our aim was to generate novel genetically derived antibody fragments for use in a rapid test for morphine, a metabolite of heroin.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade and purchased from Sigma–Aldrich (Poole, Dorset, UK), unless otherwise specified.

2.2. Morphine-3-glucuronide (M3G) scFv library

A pre-immunised antibody variable domain phage display library was obtained from Dr. Paul Dillon, Dublin City University [11].

2.3. Bacterial strains and plasmids

E. coli XL1-Blue (Stratagene, La Jolla, CA, USA) were used as a host for cloning and *E. coli* JM83 (DCU, Ireland) were used as a host for protein expression. Expression plasmids pAK 400, 500 and 600 were kindly donated by Professor A. Plückthun (Universität Zürich, Switzerland).

2.4. DNA manipulations

Recombinant DNA techniques were performed according to established protocols as compiled by Sambrook et al. [18]. Plasmids were purified using a Wizard Plus Miniprep kit (Promega, Southampton, UK) and digested using *Sfi*I (New England Biolabs, Hertfordshire, UK). Gel purification was carried out using the silica mesh 325 glass beads (Dr. Paul Clarke, DCU, Ireland) according to the Gene Clean method [19]. The scFv fragment was ligated into previously digested pAK 500 and 600 plasmids using T4 DNA ligase (Gibco-BRL, Paisley, UK) according to the manufacturer's recommendations, at a vector to insert molar ratio of 1.8:1. Plasmids were transformed into expression strain *E. coli* JM83 using chloramphenicol to select for recombinant bacteria.

2.5. Soluble expression of scFv fragments

For periplasmic expression of scFv antibody fragments 500 ml of 2×TY media, supplemented with 25 μg/ml chloramphenicol, was inoculated with an overnight culture of *E. coli* JM83 harbouring the expression plasmid pAK 400, 500 or 600 containing the scFv insert. This was incubated with vigorous aeration at 37 °C. Expression was induced when $O.D._{550\ nm}$ reached 0.5–0.6 with 1 m*M* isopropylthiogalactopyranoside (IPTG). After 4 h incubation at 26 °C with vigorous aeration (6 h for pAK 500 and 600 expression), the cells were collected by centrifugation and resuspended in 5% (v/v) the initial culture volume TES (200 m*M* Tris–HCl, pH 8, 0.5 *M* sucrose, 0.5 m*M* EDTA). This was incubated for 1 h on ice and centrifuged at 4000 *g* for 20 min to remove cell debris. The supernatant was dialysed against 50 volumes PBS (phosphate-buffered saline, pH 7.4) (Oxoid, Hampshire, UK), 0.02% (w/v) sodium azide, at 4 °C overnight.

2.6. Immunoaffinity purification

2.6.1. Preparation of hapten–carrier conjugates

M3G was coupled to either BSA (bovine serum albumin) or OVA (ovalbumin) using standard carbodimmide coupling chemistry. Briefly, a 50 mg/ml solution of M3G was prepared in 50 m*M* HCl and made up to a volume of 5 ml with 0.2 *M* borate buffer, pH 9.0. NHS (*N*-hydroxysuccinimide) and EDC (*N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide hydrochloride) were added to yield final concentration of 0.1 and 0.4 *M* respectively. The solution was incubated at room temperature for 10 min without agitation. Carrier protein (OVA or BSA), at a molar ratio of 1:100 to M3G, was prepared in 0.2 *M* borate buffer, pH 9.0, and added dropwise to the activated solution with stirring. The solution was incubated at room temperature under gentle rotation for 2 h and dialysed against 100 volumes of PBS overnight at 4 °C.

2.6.2. Preparation of immunoaffinity matrix

Cyanogen bromide-activated sepharose was swollen with 1 m*M* HCl for 20 min and sucked dry under vacuum (Millipore sintered glass filtration unit with 0.22 μm pore size filter). Five to 10 mg/ml of hapten–carrier conjugate/ml of wet gel was dissolved in coupling buffer (0.1 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.3) and added dropwise to the gel with stirring. This was incubated overnight with stirring, washed with coupling buffer and incubated with 1 *M* ethanolamine, pH 8, for 2 h under gentle rotation to block unreacted sites. The gel was washed six times alternating between 0.1 *M* acetate buffer, 0.5 *M* NaCl, pH 4, and coupling buffer. The gel was then washed with PBS, 0.02% (w/v) azide and stored in this solution at 4 °C.

2.7. Purification using M3G–BSA–Sepharose affinity column

The affinity column was equilibrated with PBS and 2 column volumes of pAK 400 soluble periplasmic extract were loaded onto the gel. Contaminants were removed by washing with 4 column volumes of PBS, 0.05% (v/v) Tween 20 (PBS/T) and bound antibody fragments were eluted using 0.1 *M* glycine–HCl, pH 2.2. Fractions were immediately neutralised with 200 μl 2 *M* Tris, pH 8.6, pooled and dialysed against 50 volumes PBS at 4 °C overnight.

2.8. Immobilised metal affinity chromatography (IMAC)

Three ml of Ni-NTA resin slurry (Qiagen, West Sussex, UK) was washed twice with wash buffer 1

(50 m*M* $Na_2H_2PO_4 \cdot H_2O$, 300 m*M* NaCl, pH 8). 15 ml of pAK 500 periplasmic extract, 50 μl 1 *M* $MgCl_2$, 200 μl protease inhibitor were added and incubated under gentle rotation at 4 °C overnight. The resin was then centrifuged at 4000 *g* for 10 min and resuspended in 20 ml wash buffer 1, twice. The resin was resuspended in 3 ml wash buffer 2 (50 m*M* $Na_2H_2PO_4 \cdot H_2O$, 300 m*M* NaCl, 10 m*M* imidazole, pH 8) and poured into a polyprep support column (0.8×4 cm). This was washed twice with 600 μl of wash buffer 2. The scFv was eluted using 50 m*M* $Na_2H_2PO_4 \cdot H_2O$, 300 m*M* NaCl, 250 m*M* imidazole, pH 8, and antibody containing fractions were dialysed overnight at 4 °C against 100 volumes PBS 0.02% (v/v) sodium azide.

2.9. Anti-flag M1 monoclonal antibody affinity gel purification

Anti-flag affinity gel purification was carried out according to the manufacturer's instructions. pAK 600 periplasmic extracts and wash buffers were supplemented with 10 m*M* $CaCl_2$ and eluted using 2 m*M* EDTA.

2.10. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

The molecular masses and purity of scFvs was monitored by electrophoresis on a 15% (w/v) SDS–polyacrylamide gel under reducing conditions according to Laemmli [20]. The gels were stained with Coomassie Blue staining solution (0.25% (w/v) Coomassie blue R250 in 45:45:10 methanol:water: glacial acetic acid) and destained under the same solvent system. Alternatively, proteins were transferred to a nitrocellulose membrane by electrophoresis using a Biorad wet blotter. The membrane was blocked using 4% (w/v) non-fat milk powder in Tris-buffered saline (TBS) (50 m*M* Tris, 150 m*M* NaCl, pH 7.4) overnight at 4 °C. The membrane was washed five times with TBS/T and five times with TBS. Bound antibody was detected using monoclonal anti-flag M1 antibody (1 in 400 antibody dilution) in TBS, 1% (w/v) non-fat milk powder, 1 m*M* $CaCl_2$, followed by alkaline phosphatase-labelled anti-mouse IgG antibody (1 in 2000 antibody dilution) in TBS, 1% (w/v) non-fat milk powder. Colour was developed using 5-bromo-4-chloro-3-indolylphosphate–nitro blue tetrazolium chloride (BCIP–NBT) reagent (Pierce, Chester, UK) and the reaction stopped by the addition of 2 m*M* EDTA.

2.11. Immunoblot analysis

One μl of decreasing M3G-OVA concentrations was dotted onto a nitrocellulose membrane and allowed to dry. The membrane was then blocked with 4% (w/v) non-fat milk powder in TBS. Following blocking the membrane was washed three times with TBS and a 1 in 2 dilution of antibody in 1% (w/v) non-fat milk powder was added. Bound phosphatase activity was visualised using BCIP–NBT chromogenic substrate.

2.12. Standard enzyme-linked immunosorbent assay (ELISA)

Microtitre plates (Nunc, Paisley, UK) (immunoplate maxisorb) were coated with 100 μl/well of 50 μg/ml M3G-OVA (6.25 μg/ml for dimeric scFv), 100 μl/well M3G-OVA in PBS at 4 °C overnight and blocked with 150 μl/well 4% (w/v) non-fat milk powder in PBS for 1 h at 37 °C. Wash steps were carried out three times with PBS/T and three times with PBS. All antibody dilutions were made in sterile filtered PBS, 1% (w/v) non-fat milk powder at a volume of 100 μl/well and incubations were preformed at 37 °C for 1 h. Decreasing concentrations of scFv were added to each well, followed by monoclonal anti-flag M1 antibody, peroxidase-labelled anti-mouse IgG antibody and detected using *o*-phenylenediamine (*o*-PD). Absorbances were read after 30 min at 450 nm.

2.13. Competitive studies

These were performed in the same manner as for standard ELISA except decreasing concentrations of free morphine (25 μl) were incubated with scFv (25 μl) on the microtitre plate.

2.14. Direct detection of pAK 600 alkaline phosphatase-labelled antibody

Microtitre plates were coated (3.125 μg/ml M3G-OVA) and blocked as for standard ELISA. Alkaline

phosphatase-labelled scFv (1 in 4 dilution) was incubated with serial dilutions of free drug for 1 h at 37 °C prior to addition to the microtitre plate. Bound antibody was detected using *para*-nitrophenyl phosphate (pNPP) substrate and absorbance at 405 nm read after 45 min at 37 °C.

2.15. BIAcore™ analysis

Analysis was performed using Biacore™ 3000 instrument and data analysis was performed using BIAevaluation 3.0 (BIAcore, St Albans, Hertfordshire, UK). Research grade CM5 sensor chips were employed and Hepes-buffered saline (HBS) (10 m*M* Hepes, 150 m*M* NaCl, 3.4 *M* EDTA and 0.025% (v/v) Tween 20) was used as running buffer. This was filtered (pore size 0.22 μm) and degassed using filtration apparatus (Millipore sintered glass filtration unit) immediately before use. All samples were syringe filtered (0.45 μm pore size) to remove any particulate matter.

2.16. Immobilisation of hapten–carrier on sensor surface

Immobilisation of conjugate was carried out according to standard amine coupling. Briefly, the carboxymethylated dextran surface was activated by mixing equal volumes of 0.1 *M* NHS (*N*-hydroxysuccinimide) and 0.4 *M* EDC (*N*-ethyl-*N*-(dimethylaminopropyl) carbodiimide hydrochloride) and injecting the mixture over the sensor chip surface for 7 min at a flow-rate of 5 μl/min. One hundred μg/ml of M3G-OVA in 10 m*M* acetate buffer, pH 4.3, was injected over the activated surface, at a flow-rate of 5 μl/min, for 40 min. Unreacted sites were capped by an injection of 1 *M* ethanolamine, pH 8.5, for 7 min. The surface was regenerated five times with 10 m*M* NaOH prior to use.

2.17. Inhibition assay

Neat antibody was mixed with decreasing concentrations of free morphine in PBS and allowed to reach equilibrium at 37 °C for 2 h prior to use. Twenty μl of each sample was injected over the surface, at a flow-rate of 5 μl/min for 4 min, in random order. The surface was regenerated with a 1 min pulse of 10 m*M* NaOH prior to further injections.

3. Results

3.1. Construction of expression plasmids for production of novel scFv fragments

The 800-base pair insert encoding the variable heavy and light chain variable regions, connected via a $(gly_4ser)_4$, was isolated from the expression plasmid pAK 400 using the *Sfi*I restriction sites, flanking the region. This was ligated into previously digested plasmids pAK 500 (4094 base pairs), encoding a double helix for dimerisation and pAK 600 (5269 base pairs), encoding the bacterial alkaline phosphatase gene (Fig. 1). The ligated plasmids were transformed into calcium competant *E. coli* JM83 cells, and recombinant bacteria were identified using chloramphenicol resistance as a selection marker.

3.2. Expression of novel scFvs

A single recombinant colony was grown in 2×TY and scFv expression induced with IPTG. The expression media and length of induction time were optimised for monomeric scFv production (Fig. 2). Induction time needed to be increased for expression of larger proteins (dimers and fusion proteins). Antibody fragments were isolated from the periplasmic lysate; however, if large-scale cultivation was required, secretion of scFv into the supernatant could be achieved simply by increasing induction time further. Monomeric scFvs were purified using affinity chromatography employing the antigen immobilised onto sepharose. The dimeric scFvs produced had a high functional affinity for the antigen of interest, and therefore, could not be easily eluted from the affinity resin. For this reason, the pentahistidine tag on the secreted antibody was employed as a handle for purification on a Ni-NTA resin. The dimer showed increased affinity for the resin compared to the monomeric scFv, due to the presence of two histidine tags on each scFv (data not shown). Several attempts were made to purify pAK 600 lysate on an anti-flag M1 agarose resin. However, the affinity of the resin for the scFv proved too weak to bind the antibody sufficiently. There are several possibilities

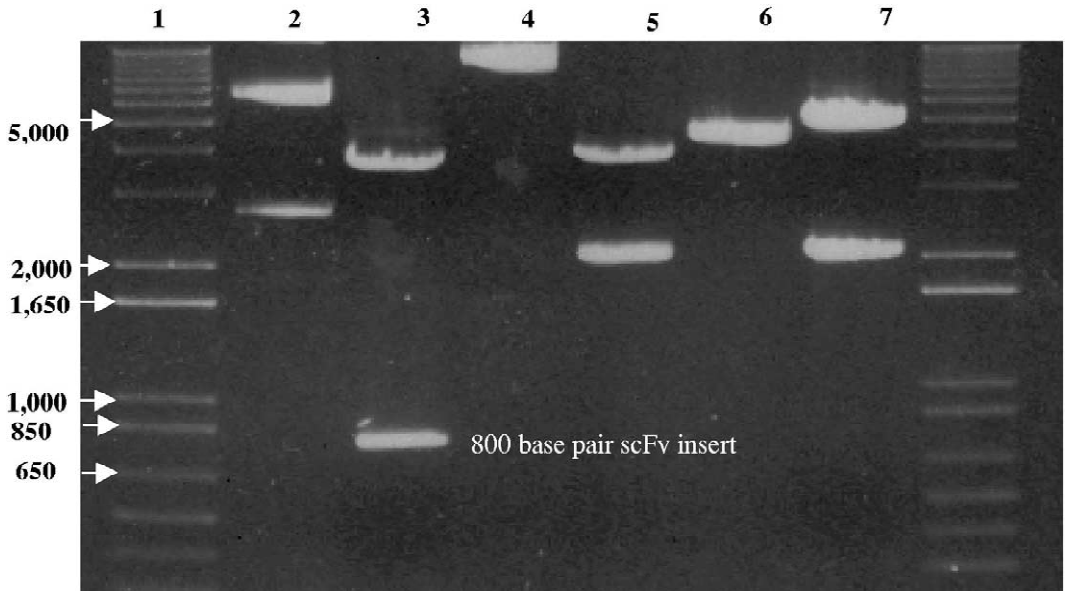

Fig. 1. Gel showing: (1) DNA ladder (Gibco-BRL); (2) pAK 400 vector containing scFv insert; (3) digested pAK 400 and 800-base pair scFv insert; (4) undigested pAK 500; (5) digested pAK 500 and 2000-base pair tetracycline insert; (6) undigested pAK 600; and (7) digested pAK 600 with 2000-base pair tetracycline insert.

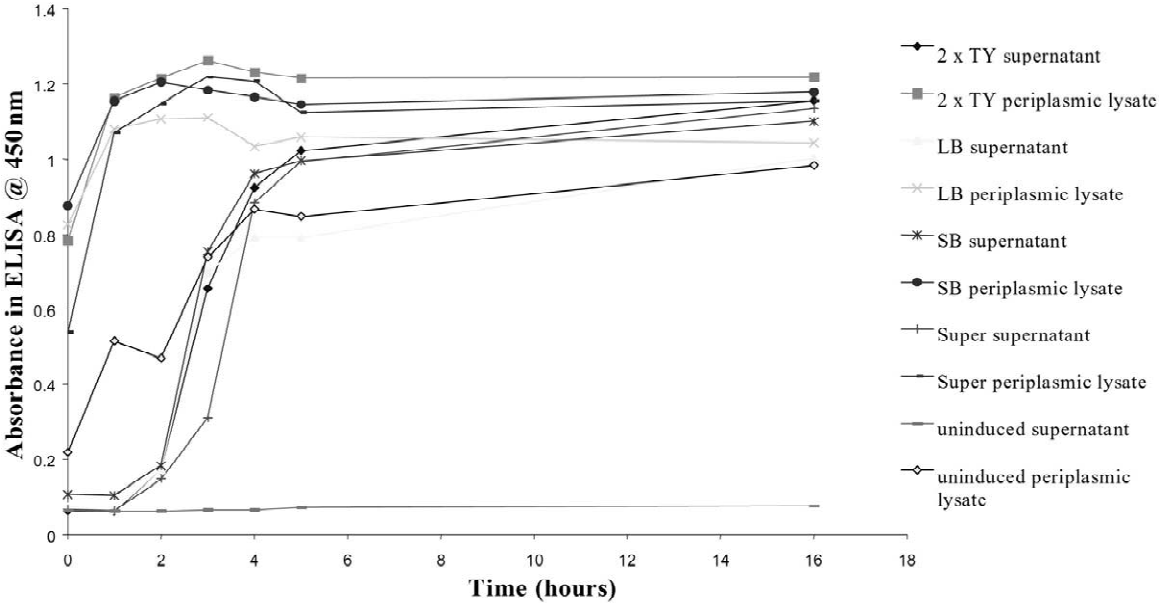

Fig. 2. Determination of optimal conditions for scFv expression. 2×TY (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl), LB (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaC1), SB (20 g/l tryptone, 10 g/l yeast extract, 10 g/l NaC1, 50 m*M* K_2 HPO_4) and Super (25 g/l tryptone, 15 g/l yeast extract, 5 g/l NaC1) were employed. 2×TY media with an induction time of 3–4 h was chosen as optimal for periplasmic expression.

for this. Firstly the FLAG tag may have been inefficiently processed or cleaved during lysis of the periplasm. Secondly the antibody recognition site maybe inaccessible for binding due to steric hindrance by the enzyme. However, this is not the case as the anti-flag M1 antibody was capable of binding the scFv fusion protein in an ELISA situation (data not shown). Using the FLAG protein as a standard it was observed that there were variations in the binding capabilities of the resin supplied and therefore it was not possible to further optimise it for purification.

3.3. Characterisation

The quality of the purified antibody fragments was monitored by SDS–PAGE analysis and subsequent Western blotting of proteins onto nitrocellulose membranes (Fig. 3). Monomeric scFv yielded a specific band at M_r 29 000 in both purified and unpurifed samples, indicating that the antibody can be specifically detected even in crude periplasmic lysate. The dimerised scFv yielded three bands, one at M_r 29 000 representing monomeric scFv, a predominant band at M_r 35 000, representing the reduced monomeric fusion protein and a faint band at and M_r 75 000 representing the dimeric version of the scFv. This demonstrates the stability of the aliphatic helix as a dimerisation domain, as a small proportion still remains intact, even under the denaturing conditions of SDS–PAGE. The scFv–alkaline phosphatase fusion protein yields a strong, specific band at M_r 85 000.

3.4. The scFv–alkaline phosphatase fusion protein is bifunctional

The bifunctional nature of the scFv–AP fusion protein was analysed by immunoblot (Fig. 4). The antibody was seen to specifically detect 200, 100, 50, 25 and 12.5 μg/ml of M3G-OVA conjugate. Monomeric scFv gave no detectable reaction product, whereas the scFv–AP fusion protein exhibited a decreasing reaction product with decreasing M3G-OVA concentration. This demonstrates that the scFv–AP fusion protein is bifunctional, retaining its enzymatic activity while bound to the M3G-OVA conjugate.

3.5. Binding characteristics

The functionality of monomeric, dimeric and alkaline phosphatase-labelled single chain antibody fragments was characterised by their ability to bind

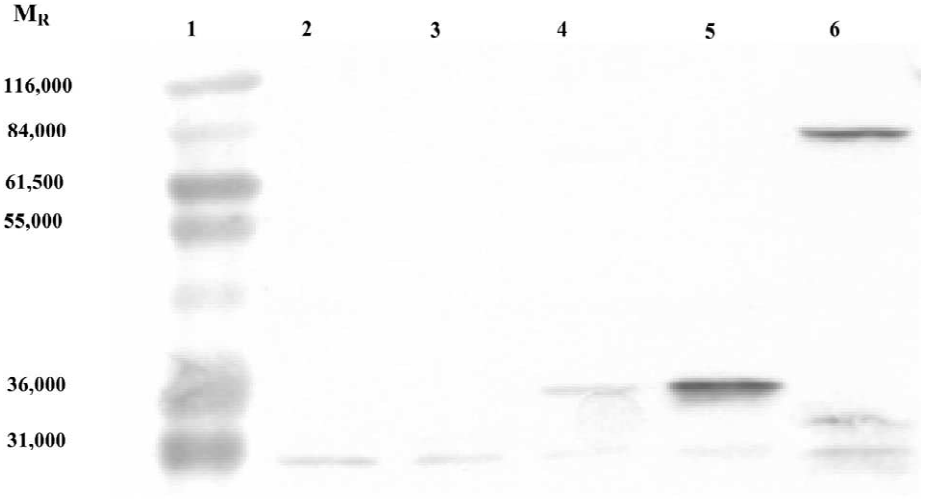

Fig. 3. Western Blot analysis of scFvs. Bound antibody was detected using anti-flag monoclonal Ml antibody and alkaline phosphatase-labelled anti-mouse antibody, followed by visualisation with BCIP/NBT substrate. Lanes: (1) molecular mass markers; (2) pAK 400 periplasmic lysate; (3) affinity purified pAK 400; (4) pAK 500 periplasmic lysate; (5) IMAC purified pAK 500; (6) pAK 600 periplasmic lysate.

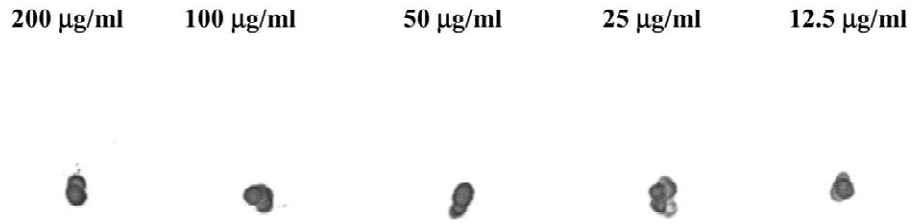

Fig. 4. Dot blot of scFv–AP fusion protein. The scFv–AP fusion protein could specifically and directly detect 200, 100, 50, 25 and 12.5 μg/m1 of M3G-OVA in an immunoblot analysis. M3G-OVA was dotted onto a nitrocellulose membrane in decreasing concentrations. Strips were incubated with scFv–AP fusion protein and monomeric scFv. Bound phosphatase activity was visualised using BCIP/NBT. No detectable reaction product could be visualised for monomeric scFv (data not shown), whereas bound scFv–AP fusion protein retained enzyme activity while bound to the conjugate.

free morphine in solution. This was assessed using ELISA and BIAcore™ analysis.

3.6. ELISA characterisation

ELISA conditions for each scFv were optimised for maximal sensitivity. Monomeric scFv was used at a 1 in 8 dilution on plates coated with M3G-OVA at a concentration of 50 μg/ml. The antibody was shown to have a detection range from 6 to 1563 ng/ml (Fig. 5). The antibody was also proved to be applicable to real sample analysis in saliva as a detection matrix, showing the same range of detection (Fig. 6). It was subsequently used to analyse real samples and results obtained correlated well with findings using the standard polyclonal immunoassay technique [21]. Dimeric scFv showed an increase in antigen binding capabilities due to the

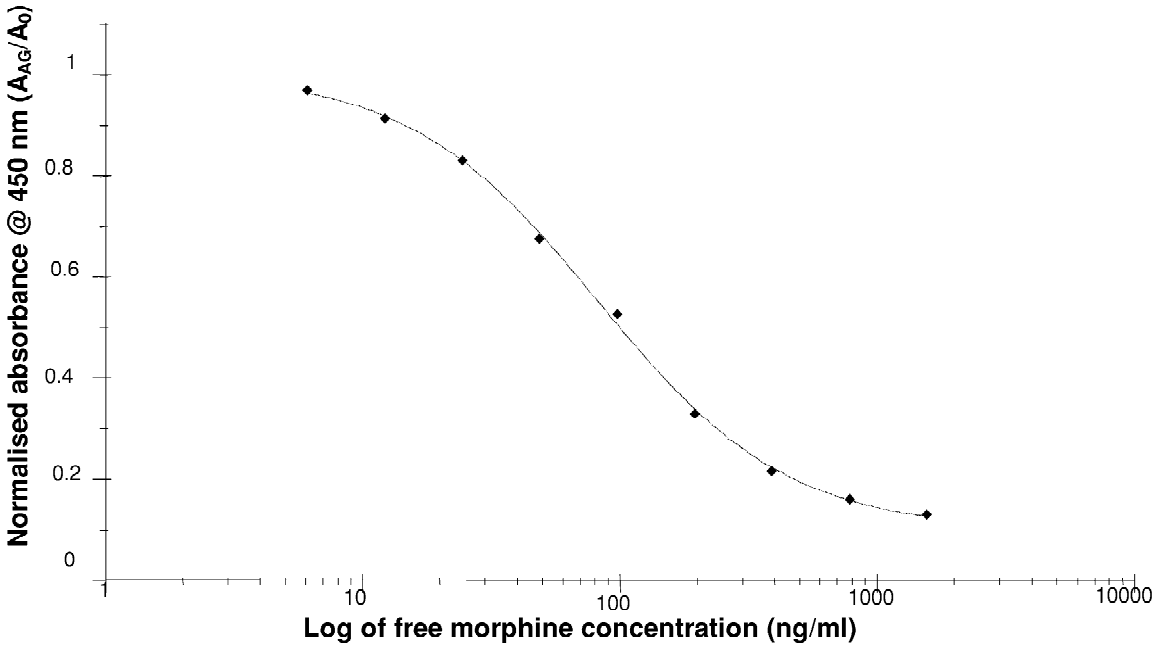

Fig. 5. Competitive assay curve for free morphine in PBS solution using monomeric scFv antibody at a 1 in 8 dilution with a M3G-OVA coating concentration of 50 μg/m1. Five replicates were performed and coefficients of variation were all less than 10%. The range of detection for free morphine was found to be from 6.1 to 1562.5 ng/ml. The normalised absorbance readings were obtained by dividing the absorbance at each free morphine concentration (A_{AG}) by the absorbance at zero free morphine concentration (A_0).

Free Morphine Concentration (μg/ml) in saliva	Calculated Mean ± Standard Deviation	Coefficients of variation (CV's) %
1562.5	0.07700 ± 0.00279	3.63
781.25	0.09650 ± 0.00279	2.99
390.625	0.14650 ± 0.0021	1.48
195.3125	0.20750 ± 0.00687	3.31
97.65625	0.324000 ± 0.01268	3.91
48.82813	0.417000 ± 0.01958	4.69
24.41406	0.52000 ± 0.01466	2.82
12.20703	0.57750 ± 0.02230	3.86
6.103516	0.59650 ± 0.01482	2.48

Fig. 6. Monomeric scFv as used in ELISA analysis for morphine in saliva. Five sets of nine morphine standards from 6.10 to 1562.5 ng/ml were analysed.

presence of two binding sites. This led to a decrease in sensitivity for free morphine as the gain in valency increased the chance of one arm binding to the immobilised conjugate. In order to increase sensitivity M3G-OVA was immobilised at the lower concentration of 6.25 μg/ml and the scFv concentration used was a 1 in 8 dilution. The antibody exhibited a detection range from 195 to 50 000 ng/ml (Fig. 7). The pAK 600 vector expresses the alkaline phosphatase enzyme at the carboxy terminus of the antibody causing the formation of dimerised scFv–AP fusions. This results in the binding characteristics of this fusion protein being similar to that of the dimeric scFv, when used in a competitive format (data not shown). However, if this antibody is used in an inhibition format, allowing antibody and free drug to reach equilibrium before addition to the immunoplate, a more sensitive assay can be developed. Using the antibody in this format gave a detection range from 6.1 to 12 500 ng/ml, with % C.V.s less than 11% (Fig. 8). The production of such a labelled scFv offers the possibility of reduced assay times, eliminating the need for the use of secondary enzyme-labelled antibodies and thereby reducing the number of steps in the assay.

3.7. BIAcore™ characterisation

scFv binding was analysed in ''real-time'' using a surface plasmon resonance based biosensor (BIAcore™). In order to optimise immobilisation of the hapten–protein conjugate onto the dextran surface, the conjugate was prepared in a buffer with a range of low ionic strengths and injected over an inactivated surface. Optimal binding of the conjugate was achieved in 10 m*M* sodium acetate buffer, pH 4.3. Approximately 6500 RU of conjugate binding could be achieved at this pH, corresponding to a protein concentration on the chip surface of approximately 6.5 ng/mm^2 (Fig. 9). Non-specific binding analysis was carried out by injecting the antibody over an OVA and an underivatised surface. This was found to be negligible (Fig. 10). Regeneration of the monomeric scFv antibody from the sensor chip surface could be achieved using the relatively mild conditions of a 1 min pulse of 10 m*M* NaOH. This

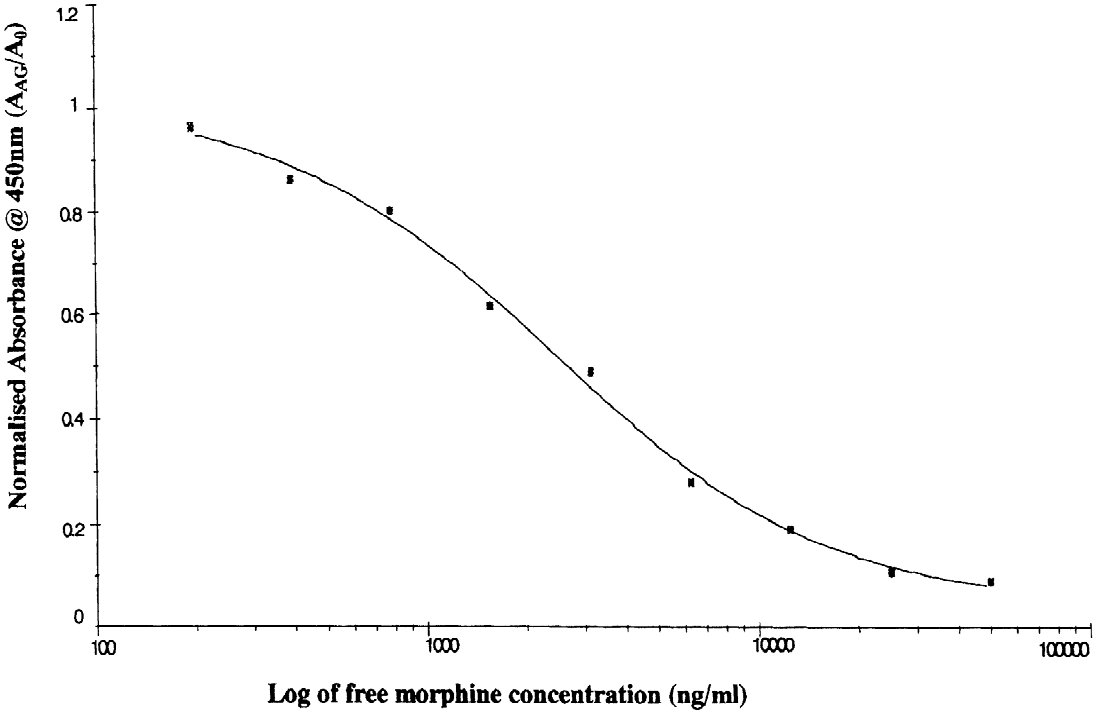

Fig. 7. Competitive assay curve for morphine in PBS using dimeric scFv antibody at a 1 in 8 dilution with a M3G-OVA coating concentration of 6.25 μg/m1. The range of detection for free morphine was found to be from 195 to 50 000 ng/ml.

solution had a minimal effect on the immobilised surface, as 50 regenerations could be performed without any significant loss in antibody binding (Fig. 11). However, variations in surface binding ability were observed during the first five cycles, and therefore it was necessary to regenerate a new surface five times prior to use in an assay format. Regeneration of dimeric scFvs is currently under optimisation.

A BIAcore™ inhibition assay was developed to determine of the range of detection of monomeric scFv for free morphine. A range of decreasing morphine concentrations were mixed with purified scFv and allowed to reach equilibrium at 37 °C. The samples were then injected over a M3G-OVA chip in random order, followed by regeneration. The range of detection was found to be from 381.5 to 781 250 pg/ml (Fig. 12). Coefficients of variation (C.V.s) were between 0.81 and 7.25%. The binding response of dimeric and AP–scFv fusion proteins is currently being optimised.

4. Discussion

Heroin is probably the best known of the opiates due to its high level of abuse worldwide. Heroin is the diacetyl derivative of morphine. Upon administration, it is rapidly metabolised to 6-acetylmorphine and then to morphine. Morphine undergoes glucuronidation in the liver prior to excretion and eliminated from the body mainly as morphine-3-glucuronide (60%) and morphine-6-glucuronide, respectively.

Current immunoassay techniques for the detection of heroin focus mainly on polyclonal or monoclonal antibodies as the detection agent for heroin metabolites in urine and plasma. However, the use of these detection matrices suffers from some inherent drawbacks. Urine sample collection is an invasion of privacy and requires supervision, analysis requires sample preparation, and the ability of the patient to provide a specimen on demand is reduced by chronic drug abuse (personal observation). The collection of

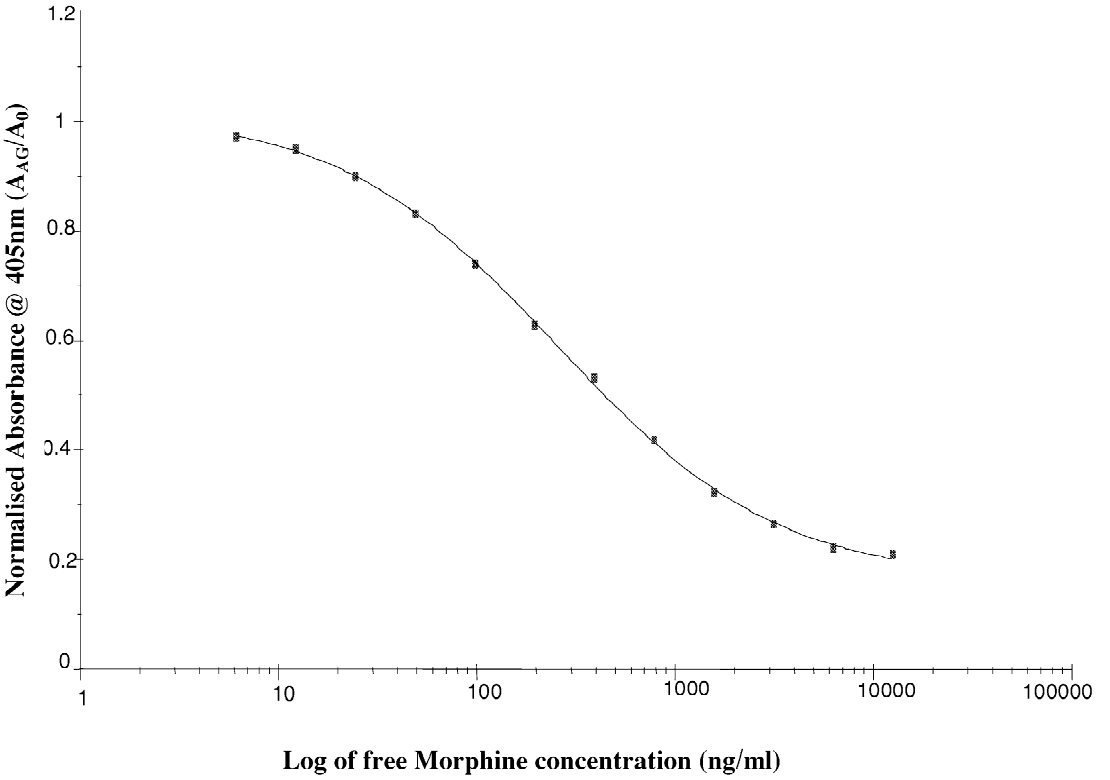

Fig. 8. Inhibition assay curve for free morphine in PBS using dimeric seFv–alkaline phosphatase fusion protein at a 1 in 4 dilution and a M3G-OVA coating concentration of 3.125 μg/m1. The detection range for free morphine was found to be from 6.1 to 12 500 ng/ml.

plasma samples also proves invasive, requiring trained professionals to collect samples. Due to the potential health risks associated with plasma and the extensive sample preparation required before analysis, it is a less preferable matrix. Saliva as an alternative detection matrix overcomes many of these disadvantages. The collection procedure is non-invasive and sample preparation is minimal. Studies have shown morphine levels to be as high as 20 μg/ml in saliva shortly after administration [21,22]. We have investigated the use of novel recombinant antibody fragments for the detection of morphine, the main metabolite of heroin found in saliva.

Soluble scFvs were produced using the expression vector pAK 400, exploiting the strong Shine Dalgarno sequence for increased levels of protein expression. Sufficient levels of scFv could be expressed on a laboratory scale for assay development. However, production could be easily scaled-up by increasing incubation times so scFv is secreted into the media (500–1000 ml). An affinity column employing M3G-BSA immobilised on a Sepharose resin was used as a simple, rapid and economical purification method. ELISA analysis showed that the antibody could be used to specifically detect morphine, even when used directly in periplasmic lysate. Western blot analysis demonstrated that the specificity of the secondary and tertiary antibodies, with no non-specific binding to any proteins in the lysate.

ELISA analysis of the purified antibody proved to be sensitive and specific in the detection of morphine. Levels as low as 6 ng/ml in a sample volume as small as 25 μl could be detected, even in a biological matrix such as saliva. Results were extensively characterised with regards to their reproducibility over an extended time period with C.V.s all below 9%.

Biosensor-based assays were also developed using

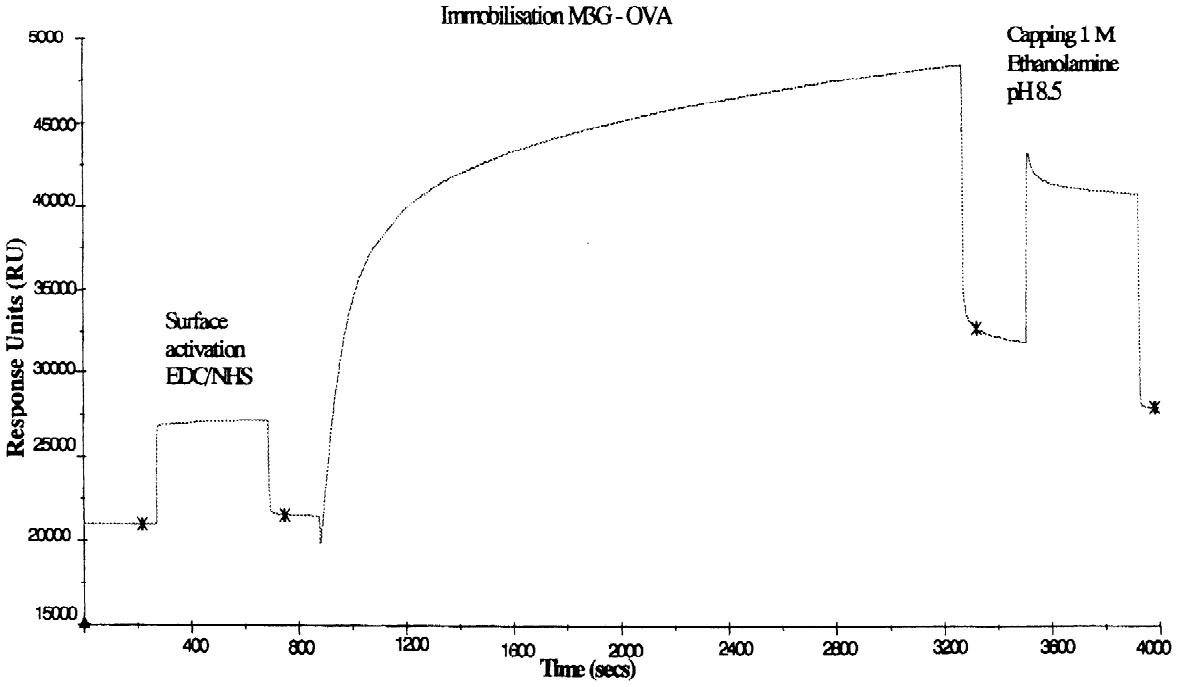

Fig. 9. Immobilisation of 100 μg/ml M3G-OVA in 10 m*M* sodium acetate buffer, pH 4.3. The dextran surface is activated with EDC/NHS. 100 μg/ml of M3G-OVA in 10 m*M* sodium acetate, pH 4.3, is passed over the activated surface, for 40 min, at a flow-rate of 5 μl/min. The surface is then capped using 1 *M* ethanolamine, pH 8.5, to block any unreacted sites. Approximately 6500 response units of conjugate binding can be seen.

this antibody on BIAcore™, an optical-based biosensor. BIAcore™ works on the principle of surface plasmon resonance, which allows visualisation of biomolecular interactions in ''real-time''. This technology allows rapid, automated detection of small molecules with high sensitivity and specificity, and limited sample consumption. Binding characteristics of the scFv were monitored using this technology. The inhibition assay format employed proved to be more sensitive than ELISA for the detection of morphine with a detection range of 381.25 to 781 250 pg/ml achieved in PBS, and C.V.s below 8%. This represents a 20-fold increase in assay sensitivity.

These detection ranges compare favourably with previously published immunoassay techniques. Beike et al. [23] have reported an immunoaffinity-based extraction method for the detection of morphine in blood with a detection limit of 3 ng/ml and a quantitation limit of 10 ng/ml for morphine. A radioimmunoassay developed by Steiner and Spratt [24] exhibits a sensitivity of 500 ng/l of serum for morphine and a similar technique reported by Spector [25], exhibits a detection limit between 50 and 100 pg of morphine in serum. Usagawa et al. [5], have reported an immunoassay using a monoclonal antibody directed against morphine capable of detecting concentrations as low as 100 pg/ml. Speckl et al. [26], have employed GC–MS to monitor the presence of opiates in the saliva and urine of subjects participating in a drug withdrawal programme. The detection limits for opiates in saliva were found to be 10 ng/ml. The assay developed in our lab for morphine in saliva, using recombinant assay techniques, compares favourably with these detection limits, with minimal sample preparation, requiring only a simple freeze–thaw step, followed by centrifugation.

The main advantage of recombinant antibody technology over traditional monoclonal and polyclonal antibody technology is the possibility of altering the antibody's affinity, specificity and phys-

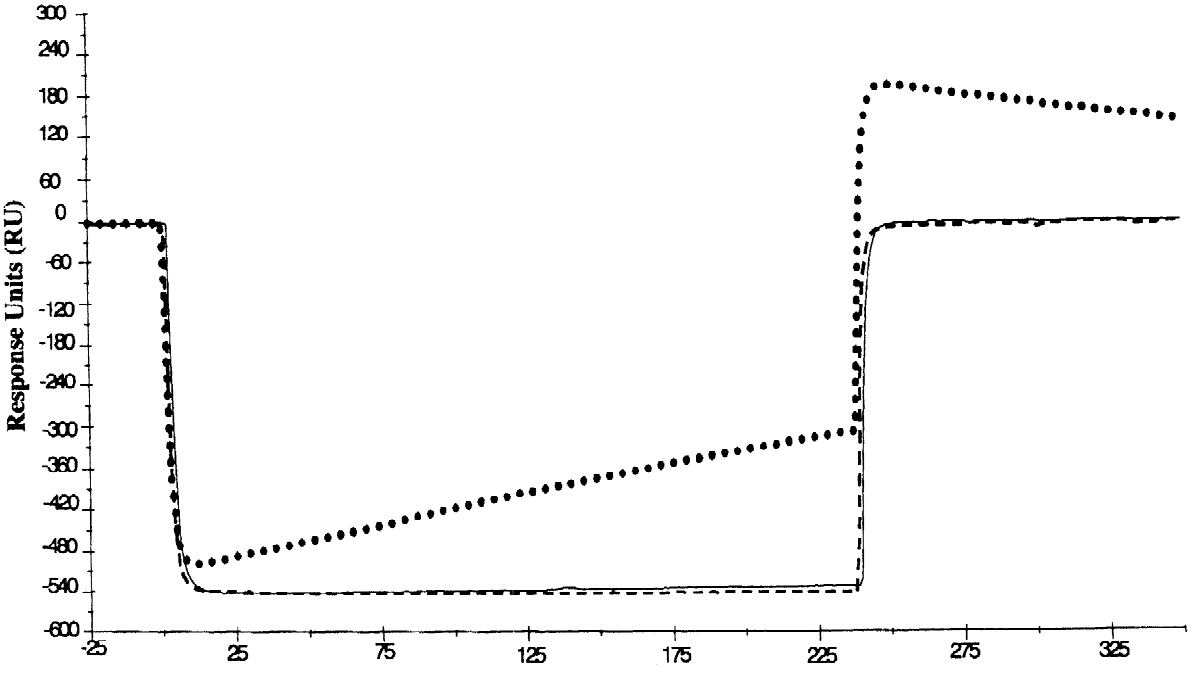

Fig. 10. Overlay plot demonstrating binding of affinity purified monomeric scFv to immobilised M3G-OVA, OVA and an unactivated dextran surface. This resulted in negligible binding to the control OVA (—) and dextran surfaces (– –), however, 200 response units can be seen binding to the immobilised M3G-OVA (• •) surface. This indicates that the scFv is specific to the M3G portion of the conjugate.

ical characteristics with relative simplicity. Multivalency is a phenomenon that occurs in many naturally occurring biological structures, such as antibodies, thereby increasing the interaction energy between biomolecules [7]. The dimerisation of an scFv results in increased functional affinity and thermodynamic stability of the antibody. For this reason bivalent antibodies are gaining increasing recognition for their potential role in immunotherapy. Aside from this, bivalent antibodies may prove an invaluable tool in immunological detection systems. The use of scFvs in a sandwich ELISA format has so far been hampered by the denaturation of the fragments upon immobilisation to a solid support, resulting in a loss of binding activity. In contrast, upon immobilisation of a dimeric scFv, one binding site will usually remain functional.

Western blot analysis confirmed the dimeric nature of the scFv, with a band at M_r 35 000, representing the reduced monomeric fusion protein, and a faint band at M_r 75 000, representing the unreduced dimeric scFv. This molecular mass is in agreement with the published reports by Rheinnecker et al. [27], who used the same helix to dimerise an scFv with a theoretical molecular mass of M_r 31 400 and reported a molecular mass of M_r 60 000 for the dimeric version. A similar helix was employed by Kerschbaumer et al. [28], who reported a molecular mass of M_r 70 000 for their fusion protein and Pack et al. [16] reported a similar molecular mass for their scdHLX antibody with only an insignificant amount of monomeric scFv produced.

The dimeric scFv exhibited an increase in avidity that was clearly evident in the inability to elute functional antibody from the antigen affinity column using extreme pH. For this reason the carboxy terminal pentahistidine tag was employed as a purification handle in IMAC chromatography. Although there are many references to this purification technique cited in the literature [13–15], this does not prove as straightforward as is first thought, and may require extensive optimisation.

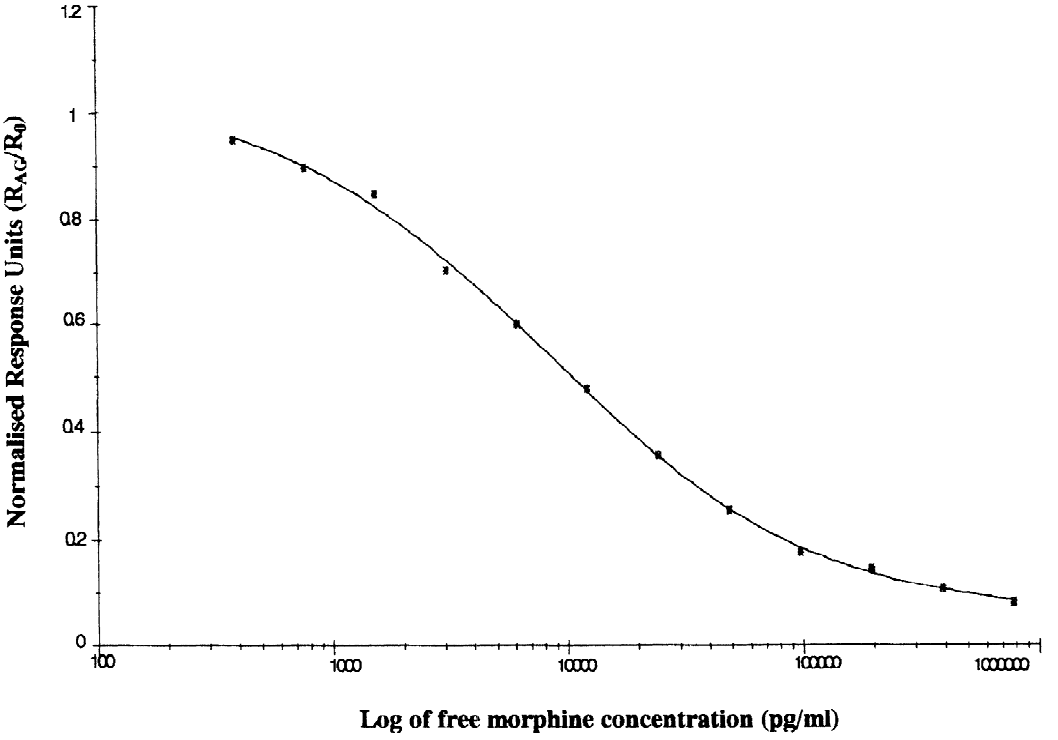

Fig. 12. BIAcore binding response curve for inhibition assay for free morphine in PBS solution using monomeric scFv antibody. Three replicates were performed and C.V.s were all below 10%. M3G-OVA was immobilised on the sensor chip surface. The range of detection was found to be from 381.5 to 781 250 pg/ml. The binding response at each free morphine concentration (R_{AG}) was divided by the antibody response in the absence of free morphine (R_0) to give a normalised binding response.

of the scFv–alkaline phosphatase fusion protein. However, sufficient binding to the anti-flag resin could not be achieved to separate the antibody from impurities in the lysate. Upon further investigation, it was observed that the column had less than 20% of the specified binding activity for pure flag protein. Einhauer and Jungbauer [30] have carried out kinetic analysis on the binding of the anti-flag antibody in the presence and absence of calcium and have revealed no difference on association and dissociation rates was observed. As this technology relies on calcium dependence for efficient purification, this may explain the lack of affinity of the resin for the antibody, even with the presence of two flag tags per antibody. However, immunoblot and Western blot analysis shows non-specific binding of contaminating proteins in the periplasmic lysate to be negligible. This compares to similar findings published by Lindner et al. [15], who have reported that the *E. coli* lysate itself acting in conjunction with standard blocking agents to prevent non-specific binding, eliminating the need for further purification of this protein.

The binding capabilities of the scFv–alkaline phosphatase fusion protein were analysed by inhibition ELISA. The detection range of the antibody (6.1–12 500 ng/ml) was greatly increased through dimerisation. Although the sensitivity was not increased, the speed in which the assay can be performed, makes the antibody particularly suited to drug monitoring in situations where a rapid yes/no response is required, for example in road-side testing. Current limits of detection for confirmatory screening employed by the Regional Laboratory of Toxicology, UK, is 300 μg of morphine per litre of urine. The sensitivity of this antibody would easily

meet these criteria and could be potentially applicable to road-side monitoring on a novel detection device in a matter of minutes [21].

Acknowledgements

The support of Dublin City University Educational Trust, Enterprise Ireland and The National Centre for Sensor Research is gratefully acknowledged.

References

[1] R. Dams, T. Benijts, W.E. Lambert, D.L. Massart, A.P. De Leenheer, Forensic Sci. Int. 123 (2001) 81.
[2] R.A. Braithwaite, D.R. Jarvie, P.S. Minty, D. Simpson, B. Widdop, Ann. Clin. Biochem 32 (1995) 123.
[3] J. Fitzpatrick, L. Fanning, S. Hearty, P. Leonard, B.M. Manning, J.G. Quinn, R. O' Kennedy, Anal. Lett. 33 (13) (2000) 2563.
[4] T.A. Gough, in: The Analysis of Drugs of Abuse, 1st edition, Wiley, Chichester, UK, 1991.
[5] T. Usagawa, Y. Itoh, E. Hifumi, A. Takeyasu, Y. Nakara, T. Uda, J. Immunol. Methods 157 (1993) 143.
[6] S.J. Daly, P.P. Dillon, J. Brennan, L. Dunne, J. Fitzpatrick, R. O'Kennedy, Anal. Lett. 34 (11) (2001) 1799.
[7] A. Pluckthun, P. Pack, Immunotechnology 3 (1997) 83.
[8] A.J. Killard, Ph.D. thesis, Dublin City University, 1998.
[9] M.J.C. Alcocer, C. Doyen, H.A. Lee, M.R.A. Morgan, J. Agric. Food Chem. 48 (2000) 4053.
[10] A. Krebber, S. Bornhauser, J. Burmester, A. Honneger, J. Willuda, H.R. Bosshard, A. Plückthun, J. Immunol. Methods 201 (1997) 35.
[11] P.P. Dillon, B.M. Manning, S.J. Daly, A.J. Killard, R. O'Kennedy, J. Immunol. Methods (2002) in press.
[12] K.S. Prickett, D.C. Amberg, T. P Hopp, BioTechnques 7 (6) (1989) 580.
[13] R. Janknecht, G. de Martynoff, J. Lou, R.A. Hipskind, A. Nordheim, H.G. Stunnenberg, Proc. Natl. Acad. Sci. USA 88 (1991) 8972.
[14] K.M. Muller, K.M. Arndt, K. Bauer, A. Plückthun, Anal. Biochem. 259 (1998) 54.
[15] P. Lindner, A. Bauer, A. Krebber, L. Nieba, E. Kremmer, C. Krebber, A. Honegger, B. Klinger, R. Mocikat, A. Plückthun, BioTechniques 22 (1997) 140.
[16] P. Pack, M. Kujau, V. Schroeckh, U. Knüpfer, R. Wenderoth, D. Riesenberg, A. Plückthun, Bio/Technology 11 (11) (1993) 1271.
[17] W. Wels, I. Harwerth, M. Zwickl, N. Hardman, B. Groner, N.E. Hynes, Bio/Technology 10 (1992) 1128.
[18] J. Sambrook, E.F. Fritsch, T. Maniatis, in: Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[19] M.W. Mather, J.A. Keightley, J.A. Fee, Methods Enzymol. 218 (1993) 695.
[20] U.K. Laemmli, Nature (London) 227 (1970) 680.
[21] L. Fanning, Ph.D. thesis, Dublin City University, 2002.
[22] R.K. Leute, E.F. Ullman, A. Goldstein, J. Am. Med. Assoc. 221 (1972) 1231.
[23] J. Beike, H. Kohler, B. Brinkman, G. Blaschke, J. Chromatogr. B 726 (1999) 111.
[24] M. Steiner, J.L. Spratt, Clin. Chem. 24 (2) (1978) 339.
[25] S. Spector, J. Pharmacol. Exp. Ther. 178 (2) (1971) 253.
[26] I.M. Speckl, J. Hallbach, W.G. Guder, L.V. Meyer, T. Zikler, Clin. Toxicol. 37 (1999) 441.
[27] M. Rheinnecker, C. Hardt, L.L. Ilag, P. Kufer, R. Gruber, A. Hoess, A. Lupas, C. Rottenberger, A. Plückthun, P. Pack, J. Immunol. 157 (1996) 2989.
[28] R.J. Kerschbaumer, S. Hirschl, A. Kaufmann, M. Ibl, R. Koening, G. Himmler, Anal. Biochem. 249 (1997) 29.
[29] A. Einhauer, A. Jungbauer, J. Chromatogr. A 921 (2001) 25.
[30] A. Einhauer, A. Jungbauer, J. Biochem. Biophys. Methods 49 (2001) 455.