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Affinity recovery of Moloney Murine Leukaemia Virus

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Abstract

Lipid enveloped retroviruses such as Moloney Murine Leukaemia Virus (MoMuLV) are commonly used gene therapy vectors. Downstream processing protocols used for their purification are time consuming and a potentially generic, single step capture method for the recovery of retroviral particles is proposed that exploits streptavidin–biotin affinity chromatography. The ability of four conventional adsorbent solid phases, Fractogel®, Sepharose, Magnespheres® and STREAMLINE immobilised with streptavidin, to capture and recover biotinylated Moloney Murine Leukaemia Virus was studied. MoMuLV can be biotinylated whilst retaining infectivity and the biotinylated virus can be adsorbed to Streptavidin Magnespheres yielding a 2298-fold increase in titre. For optimal virus biotinylation purification using Fractogel® streptavidin can yield a 1896-fold increase in cfu/mg of protein and a 1191-fold decrease in DNA/cfu. Infectious virus can be recovered from Fractogel® streptavidin with a maximum recovery of 16.7%.
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Keywords: Moloney Murine Leukaemia Virus (MoMuLV); Affinity chromatography; Gene therapy

1. Introduction

Replication incompetent retroviruses such as Moloney Murine Leukaemia Virus (MoMuLV) are commonly used gene therapy vectors [1] as they allow (i) the stable integration of a therapeutic gene into a host cell genome, (ii) highly efficient gene transfer, (iii) have a wide host range, (iv) low immunogenicity and (v) retroviruses are the only available vectors that selectively infect only proliferating cells [2].

Retroviral vectors are often purified using conventional multi-step downstream processing methods including density gradient centrifugation, utrafiltration and ion exchange chromatography [3]. These are time consuming and some are scale limited and generally result in low recoveries of infectious retroviral particles. The clinical demands on the manufacture of retroviral particles have been reviewed by Braas et al. [4] and Lyddiatt et al. [5].

Affinity chromatography enables a specific separation of the viral vector from contaminating host cell proteins. Recently antibody and heparin-affinity chromatography have been used to purify adeno-associated viruses (AAV) [6] and herpes simplex virus [7]. However, there are potential constraints with both of these techniques that would be problematic in manufacturing. Antibody production, purification and immobilisation are costly at large scale and for each virus type a different antibody would be required [7]. In addition, many cellular proteins are known to associate with heparin, hence the use of a heparin column requires the incorporation of a specific step to remove contaminating heparin-binding proteins [6].

This work demonstrates that affinity chromatography can be exploited as a single step capture method for the generic recovery of retroviral particles exploiting streptavidin and biotin interactions. Hughes et al. [8] described a method for

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Table 1
Physical characteristics of the selected adsorbent solid phases

Adsorbent	Adsorbent configuration	Manufacturer	Adsorbent particle diameter (μm)	Surface area/ml of adsorbent (cm^2)	Ligand density (mg/ml of adsorbent)
Fractogel® streptavidin	Tentacular (80 nm pores)	Merck	40	1504	0.123
Streptavidin Magnespheres®	Non-porous	Promega	1	60385	Unknown
Sepharose streptavidin	Microporous (<100 nm pores)	Amersham Biosciences	100	592	0.105
STREAMLINE streptavidin	Microporous (<200 nm pores)	Amersham Biosciences	200	300	0.31

The relevant manufacturer supplied the physical data for each of the adsorbent solid phases where appropriate. The ligand density data was determined experimental by difference analysis using the Bradford assay subsequent to the coupling protocol.

biotin tagging MoMuLV particles and concentrating these using streptavidin coated paramagnetic particles. Their hypothesis is that retroviral *env* protein residing on the surface of packaging cells can be biotinylated prior to its association with *gag* and *pol*. During continued culture the budding process associates the biotin modified *env* with the retroviral core resulting in the production of biotin labelled retrovirus. Furthermore, they demonstrated that the virus retained infectivity whilst still bound to adsorbent particles [8,9].

Streptavidin is a tetrameric protein derived from *Streptomyces avidinii* that demonstrates a high affinity for biotin ($K_d = 10^{-13}$–10^{-16} M), binding four moles of biotin per mole of protein with a binding site in each protein subunit [10,11]. Three molecular recognition motifs are responsible for the high affinity; (i) one of the surface loops of the streptavidin barrel adopts a closed conformation that covers the binding pocket when biotin is present, (ii) hydrophobic interactions, predominantly between the biotin and four tryptophan residues (Try 79, Try 92, Try 108 and Try 120), contribute to the strong binding and (iii) hydrogen bonding between biotin and streptavidin [12]. Biotin is a water-soluble vitamin and an enzyme co-factor that is synthesized by bacteria and yeast [13].

This paper describes the batch adsorption of exogenously biotinylated MoMuLV particles using conventional chromatography adsorbents (Fractogel®, Sepharose, STREAMLINE and paramagnetic Magnespheres®, Table 1) and methods for the effective desorption of infectious virions.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were purchased from Sigma (Poole, UK) or BDH Ltd. (Hants., UK) as Analar grade or equivalent except where specified in the text. Streptavidin Magnespheres® paramagnetic particles, 1 mg/ml (5×10^8 particles/ml) were supplied by Promega (Southampton, UK). Biotinamindohexanoic acid *N*-hydroxysuccinimide ester (stored at −20 °C in DMSO at 366 mM; BiotinSE) and puromycin were supplied by Sigma (Poole, UK). Rothwell Park Memorial Institute medium (RPMI) and Dulbecco's Modified Eagles Medium (DMEM) cell culture media were supplied by Invitrogen (Paisley, UK).

2.2. Preparation of affinity adsorbents

2.2.1. Fractogel®

Streptavidin was immobilised onto Fractogel® EMD Azlactone following the manufacturers' guidelines (Merck, Poole, UK). Streptavidin (2 ml of a 10 mg/ml solution) was added to pre-swollen gel (2 g dry weight ≡ 7 ml of gel) swollen in phosphate buffer (50 mM, pH 8.0). The reaction was continued for 24 h at room temperature after which the adsorbent was washed with 0.2 M glycine at pH 8.0, 0.2 M glycine at pH 3.0 and phosphate buffered saline (PBS, 0.1 M, pH 7.4), respectively. The concentration of streptavidin bound/ml of adsorbent was determined by mass balance exploiting the Bradford assay.

2.2.2. Sepharose and STREAMLINE

Activation of Sepharose CL-6B and STREAMLINE was completed as described following a protocol adapted from McCreath and Chase [14]: a sample of adsorbent (4 ml settled volume) was washed (two times) with ice-cold NaOH (0.17 M, 46 ml). The adsorbent was then incubated with ice-cold NaOH (0.17 M, 46 ml) for 1 h at 4 °C. Once the particles had settled the residual NaOH was removed and replaced with 2 ml of fresh NaOH (0.17 M, ice-cold). A suspension of ice-cold acetone containing 250 mM cyanuric chloride (400 μl) was added to the adsorbent slurry and mixed for 6 min at 4 °C. The activated adsorbent was then washed in ice-cold acetone (46 ml for 5 min), 50% acetone (46 ml for 5 min) and PBS (46 ml for 5 min). After the adsorbent had settled the PBS was removed and replaced with 3.5 ml of fresh PBS. A solution of streptavidin was added to the activated adsorbent (1 ml of a 10 mg/ml solution) and agitated by end over end mixing for 3 h at room temperature. The supernatant was removed and the adsorbent washed (3 × 10 ml PBS). The concentration of streptavidin bound/ml of adsorbent was determined by difference analysis exploiting the Bradford assay. The remaining reactive groups were blocked by overnight incubation with 0.2 M ethanolamine/HCl (4 ml, pH 8.5) at room temperature.

2.2.3. Batch adsorption

Fractogel® streptavidin and Sepharose streptavidin were sanitised with 0.25 M NaOH (30 min at room temperature) then exhaustively washed with sterile Dulbecco's phosphate buffered saline (DPBS, pH 7.4) to equilibrate the adsorbent solid phases. Streptavidin Magnespheres® were supplied

sterile and prepared by washing (3 × 1 ml) in DPBS. Adsorbent (50 μl) was added to 3 ml of the harvested retrovirus containing cell culture supernatant and mixed at room temperature for 2 h using a rotary mixer. The adsorbent was sedimented under gravity and the virus cfu/ml was determined. The adsorbent was washed (2 × 1 ml) with DPBS. At this stage the Streptavidin Magnespheres® were plated to determine the cfu/ml adsorbed to the adsorbent, therefore they were not used in the desorption studies. In this study the biotinylated retrovirus was desorbed using 0.6 mM d-biotin. After the adsorbents were washed and 1 ml of 0.6 mM d-biotin (suspended in DPBS) was added to the adsorbent sample and mixed for 2 h at room temperature using a rotary mixer. After desorption the cfu/ml of supernatant was determined.

2.2.4. *Cell lines*

HeLa adherent epithelial cells (human cervical cancer cells) were routinely grown in RPMI medium supplemented with 10% foetal calf serum (FCS, LabTech International), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. PG13 pBabe.puro producer cells were generated as previously described in [9]. The cells are NIH 3T3 based retroviral packaging cells with the Gibbon Ape Leukaemia virus envelope gene that produce MoMuLV transferring puromycin resistance. PG13 packaging cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Both cell lines were passaged by trypsinisation.

2.2.5. *Biotinylation of retroviral particles*

Retroviral particles produced from BiotinSE treated Murine fibroblast-derived PG13 packaging cells (Moloney Murine Leukaemia Virus) acquired biotinylated envelope proteins as they bud from the PG13 cell membrane as described in detail in Hughes et al. [8,9]. Briefly, PG13 producer cells were trypsinised and plated at 1×10^6 cells/90 mm petridish. After 72 h of culture, the medium was thoroughly aspirated and replaced with 10 ml of freshly prepared BiotinSE reagent (10 ml of PBS, pH 8.0, 0.75 mM $CaCl_2$ and 0.48 mM $MgCl_2$ with 500 μM BiotinSE). The reagent was contacted with the PG13 cells for 30 min at room temperature after which the reagent was thoroughly aspirated and replaced with 12 ml of DMEM supplemented with 10% FCS and the cells returned to 37 °C. After a further 3–4 h the medium was replaced and the cells returned to the incubator for 18 h after which the retrovirus was aspirated. Non-biotinylated retroviral particles were produced following the same protocol omitting the BiotinSE.

2.2.6. *Determination of viral titre*

Adherent HeLa cells were trypsinised and adjusted to 1×10^5 cells/ml of cell suspension and 0.5 ml plated into each well in a 24 well plate. The plates were placed into a 37 °C incubator for 24 h after which 0.5 ml of RPMI containing 8 μg/ml polybrene was added to each well, and the plates returned to the incubator for 1–2 h. Preparations of freely suspended or Streptavidin Magnesphere® bound virus were titred as described by Hughes et al [8]. Briefly, samples were diluted serially 1:10 in RPMI with 10% FCS and 100 μl was added to triplicate wells with mixing. After 48 h the medium in the wells was replaced with 1 ml of RPMI with 10% FCS containing 5 μg/ml puromycin. The plates were regularly checked and fresh RPMI containing 5 μg/ml was added when required. The plates were returned to culture for a further 2 weeks after which the colony forming unit number was determined. The concentration of cfu/ml was calculated as the number of colonies/well multiplied by the dilution factor.

2.2.7. *Retroviral stability studies*

The stability of the biotinylated MoMuLV particles in various desorption reagents was assessed using 0.6–10 mM d-biotin [15], 6 M guanidine–HCl, pH 1.5 [10] and 6 M urea [16]. MoMuLV was added to the relevant desorption buffer and mixed at room temperature for 120 min using a rotary mixer. Samples (100 μl) were taken before and after the contact time to determine viral titre. The 6 M guanidine–HCl and 6 M urea samples were dialysed against PBS, pH 7.4 (3 ml: 100 ml PBS) for 120 min at room temperature before the titre was determined by assay. MoMuLV in cell culture supernant containing no additives was used as the control. No significant loss in titre was observed after 120 min with the control samples (results not shown). All other samples were assayed without prior dialysis.

2.2.8. *Total protein determination*

Total protein concentrations were determined using a commercially available assay reagent supplied by PERBIO (Cheshire, UK) that is based upon a colourimetric method proposed by Bradford [17]. A standard curve was generated using bovine serum albumin (BSA) at concentrations between 0 and 30 μg/ml in DPBS. The standards were incubated in an equal volume of assay reagent for 5 min at room temperature (0.5 ml of sample + 0.5 ml of assay reagent). The absorbance of the standards was determined at 595 nm against a buffer blank. Experimental samples diluted to a suitable concentration were prepared using the same method as the standards and the total protein determined from the standard curve.

2.2.9. *SDS-PAGE analysis*

Samples were analysed by SDS-PAGE using NuPAGE Novex Bis-Tris 4–12% Gels and the XCell Mini-Cell according to the manufacturer's instructions (Invitrogen, UK). The gel was photographed using the Kodak (US) EDAS 290 utilising a visible light illuminator.

2.2.10. *Total DNA determination*

DNA concentrations were determined by fluorometric assay using the intercalating dye PicoGreen (Cambridge Bioscience, Cambridge, UK) according to the manufacturers

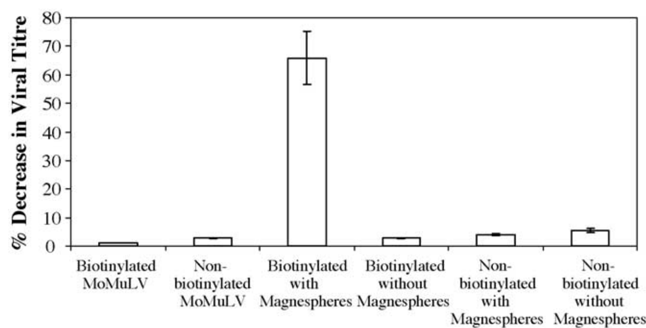

Fig. 1. Capture of biotinylated and non-biotinylated MoMuLV using Streptavidin Magnespheres®. After the Streptavidin Magnespheres® were washed (2 × 1 ml DPBS) to equilibrate the adsorbent, 3 ml of crude cell culture supernatant was added containing either biotinylated or non-biotinylated virus (1.77×10^3 cfu/ml and 1.80×10^3 cfu/ml, respectively). The samples were mixed at room temperature for 120 min using a rotary mixer. Samples (100 μl) were taken at 0 min and 120 min and the cfu/ml of the supernatant measured and the percentage of viral particles removed from the supernatant was determined. All viral titre assays were conducted in triplicate and the error bar represents the standard error of the three assays.

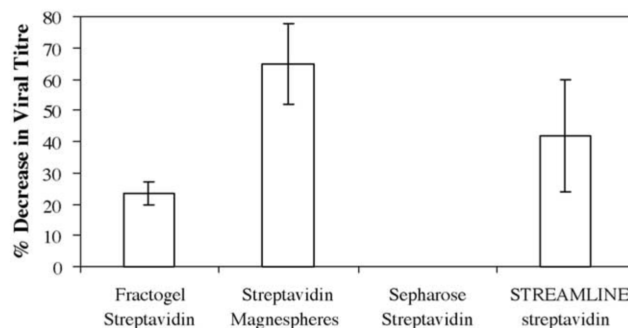

Fig. 2. Affinity capture of biotinylated MoMuLV. Each adsorbent (i) Fractogel® streptavidin, (ii), Streptavidin Magnespheres (iii) Sepharose streptavidin ® and (iv) STREAMLINE streptavidin was sterilised before use with 1 ml of 0.25 M NaOH and equilibrated in DPBS at pH 7.4. Adsorbent (50 μl) was added to 3 ml of crude cell culture supernatant containing biotinylated retroviral particles and contacted at room temperature for 120 min using a rotary mixer. Samples of the supernatant (100 μl) were taken before and after adsorption and the percentage of viral particles removed from the supernatant determined. All viral titre assays were conducted in triplicate and the error bar represents the standard error of the three assays.

instructions. A standard curve was prepared using DNA concentrations up to 1 μg/ml. TE buffer was used as the diluent for both the DNA standards and the DNA determinations.

3. Results and discussion

3.1. Biotinylation of retroviral particles

Preliminary experiments with Streptavidin Magnespheres® indicated that biotinylated MoMuLV adsorbed successfully with approximately 65% (±9.71%) of the infectious retroviral particles removed from the supernatant (Fig. 1). There was no significant adsorption of non-biotinylated MoMuLV and the retroviral supernatant was observed to have no significant loss in titre over a 120 min time period at room temperature. It was concluded that the decrease in viral titre over time when contacted with Streptavidin Magnespheres® was attributable to the affinity interaction with biotin on the surface of the retroviral particles. Biotinylation of the retroviral particles did not affect the viral titre as compared with non-biotintylated MoMuLV, where the initial titres were 1.77×10^3 cfu/ml and 1.80×10^3 cfu/ml, respectively.

Three conventional adsorbent solid phases Fractogel®, Sepharose and STREAMLINE, commonly used for the purification of biological products [18–20] were studied. Streptavidin was covalently attached to each adsorbent (Table 1). Batch adsorption studies (Fig. 2) indicated that Fractogel® streptavidin and STREAMLINE streptavidin could effectively capture biotinylated MoMuLV with 23.4% (±3.8%) and 41.9% (±18.3%) of virus adsorbed, respectively, from the crude cell culture supernatant. The retroviral capture on the Streptavidin Magnespheres® was greater than that achieved by STREAMLINE streptavidin, Fractogel® streptavidin or Sepharose streptavidin (Table 2). This was attributed to the diameter of the adsorbent solid phases where Streptavidin Magnespheres® have a greater specific surface area available for adsorption (Table 1). The MoMuLV particles have an estimated diameter of 100 nm and the pore sizes of the selected adsorbents are such that there would be little penetration of virus into the internal volume of the adsorbent beads [21]. Therefore, the external surface area available for adsorption is an important consideration when selecting adsorbents suitable for the recovery of retroviral particles. However, Streptavidin Magnespheres® may well not be a viable adsorbent at process scale for reasons of cost and further work was done with conventional process materials. Fig. 2 also indicates that within the sensitivity of the viral assay there was no observable capture of biotinylated MoMuLV particles by Sepharose streptavidin. This may again be attributable to the

Table 2
The viral loading for each adsorbent included in the study calculated from the decrease in viral titre over a 120 min contact period

Adsorbent	Adsorbent loading (cfu/ml of adsorbent)	Percentage of virus removed from the supernatant (%)
Fractogel® streptavidin	3.9×10^5	23.4
Sepharose streptavidin	0.0	0
Streptavidin Magnespheres®	8.9×10^5	65.3
STREAMLINE streptavidin	2.9×10^5	41.9

Virus containing cell culture supernatant (3 ml with 2.5×10^4 cfu/ml) was added to equilibrated adsorbent (50 μl) and mixed at room temperature for 120 min using a rotary mixer. Samples (100 μl) were taken before and after the contact time and assayed to determine cfu/ml of supernatant.

adsorbent design (Table 1) as this adsorbent has small pores (approximately 100 nm in diameter) coupled with a low surface area and ligand density for virus adsorption.

3.2. Retroviral stability studies

Successful desorption of infectious viral particles was investigated. Three different desorption protocols were selected; (i) desorption with biotin [15], (ii) 6 M guanidine–HCl, pH 1.5 [10] and (iii) 6 M urea [16]. The retroviral vectors needed to be recovered in an infectious state and studies were therefore conducted to investigate the effect of the desorption reagents on the infectivity of the desorbed MoMuLV particles.

Batch desorption was studied over a 120 min time period and it was observed that the viral titre in non-biotinylated cell culture supernatants decreased by approximately 3.2% (±2.9%) over 120 min (Table 3). Guanidine–HCl and urea are commonly used desorption reagents for biotinylated products bound to streptavidin [10,16], however the results indicate that these reagents inactivate the retroviral particles and render them non-infectious (Table 3). d-Biotin is another common desorption reagent and concentrations between 0.6 and 10 mM d-biotin were studied (Table 3). The virus was more stable in the presence of 0.6 mM d-biotin than any of the other concentrations, having a decrease in virus titre of approximately 12.2% (±3.1%). Therefore 0.6 mM d-biotin dissolved in DPBS, pH 7.4 was selected as the desorption buffer for the study.

3.3. Desorption of biotinylated MoMuLV

The effectiveness of 0.6 mM d-biotin to desorb biotinylated MoMuLV was studied with Fractogel® streptavidin and STREAMLINE streptavidin (Fig. 3). Sepharose streptavidin was not studied further due to the poor adsorption performance of the beads (Fig. 2).

Table 3
Stability of biotinylated MoMuLV contacted with various desorption reagents

Desorption condition	Initial viral titre (cfu/ml of supernatant)	% Decrease in viral titre
Retroviral supernatant	2.5×10^4 ($\pm 6.7 \times 10^3$)	3.2 (±2.9)
10 mM d-biotin	4.3×10^4 ($\pm 2.1 \times 10^3$)	65.6 (±5.8)
7.5 mM d-biotin	5.7×10^4 ($\pm 4.9 \times 10^3$)	52.3 (±6.4)
5.0 mM d-biotin	6.2×10^4 ($\pm 6.9 \times 10^3$)	45.6 (±4.7)
2.5 mM d-biotin	4.8×10^4 ($\pm 7.5 \times 10^3$)	35.9 (±5.3)
1.0 mM d-biotin	4.25×10^4 ($\pm 8.0 \times 10^3$)	20.8 (±2.5)
0.6 mM d-biotin	7.25×10^4 ($\pm 1.5 \times 10^3$)	12.2 (±3.1)
6 M guanidine-HCl, pH 1.5	5.12×10^4 ($\pm 3.8 \times 10^3$)	100 (±5.6)
6 M urea	4.98×10^4 ($\pm 4.2 \times 10^3$)	100 (±7.4)

Virus containing supernatant (3 ml) was prepared in each desorption reagent and mixed at room temperature for 120 min using a rotary mixer. Samples (100 μl) were taken before and after the contact time and the viral titre of supernatant determined.

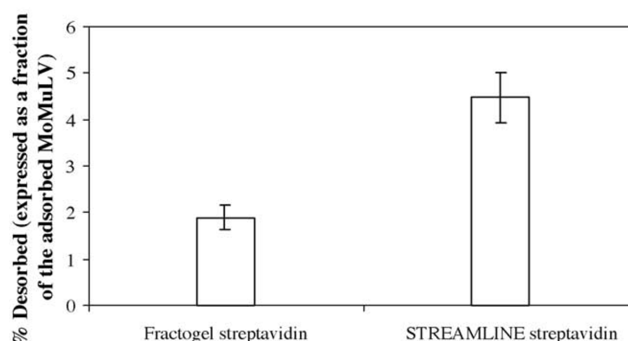

Fig. 3. Desorption of biotinylated MoMuLV in 0.6 mM d-biotin from Fractogel® streptavidin and Sepharose streptavidin. After adsorption the supernatant was removed and the adsorbent (50 μl) washed in DPBS at pH 7.4 (2 × 1 ml). The biotinylated virus was desorbed using 1 ml of 0.6 mM d-biotin (suspended in DPBS, pH 7.4). The adsorbent was mixed for 120 min at room temperature using a rotary mixer. Samples (100 μl) were taken and assayed to determine the concentration of desorbed virus expressed as a fraction of the adsorbed MoMuLV. All viral titre assays were conducted in triplicate and the error bar represents the standard error of the three assays.

Initial experiments on MoMuLV desorption were conducted with virus prepared using biotinylation reagent containing 500 μM BiotinSE, when less than 5% of the adsorbed virus was desorbed from both Fractogel® streptavidin and STREAMLINE streptavidin. Mass balances on the supernatant biotin concentration before and after incubation with the cells suggests that multiple biotin molecules were present on the surface of the retroviral particles. Multi-point attachment of each retroviral particle to the streptavidin ligands was therefore possible, resulting in strong binding of the biotinylated virus and the adsorbent resulting in the observed poor recoveries.

Fig. 4a and b shows the specific titre (cfu/mg of protein) and specific DNA content (μg DNA/cfu), respectively, for the recovered MoMuLV following batch purification by adsorption to Fractogel® streptavidin or STREAMLINE streptavidin. Notwithstanding the low recovery of infectious virus, the purity of the eluted product, in terms of total protein and DNA contents per virus, is significantly improved upon that of the crude cell culture supernatant. For Fractogel® streptavidin there was an 11-fold increase in the specific titre (cfu/mg of protein) in addition to a six-fold decrease in the specific DNA content (μg of DNA/cfu). STREAMLINE streptavidin demonstrated a 21-fold increase in the specific titre alongside a 1.2-fold decrease in specific DNA content. The poor specific DNA content for STREAMLINE streptavidin purified virus was attributed to the large pore structure of this adsorbent (Table 1), since its 200 nm pores would permit DNA oligonucleotides to enter the adsorbent and become trapped. Fractogel® streptavidin has much smaller pores (80 nm in diameter) with the ligands on tentacles surrounding the adsorbent beads reducing the potential for particle entrapment. It was therefore concluded that Fractogel® streptavidin was the superior adsorbent for the recovery of biotinylated retroviral particles from crude cell culture supernatants.

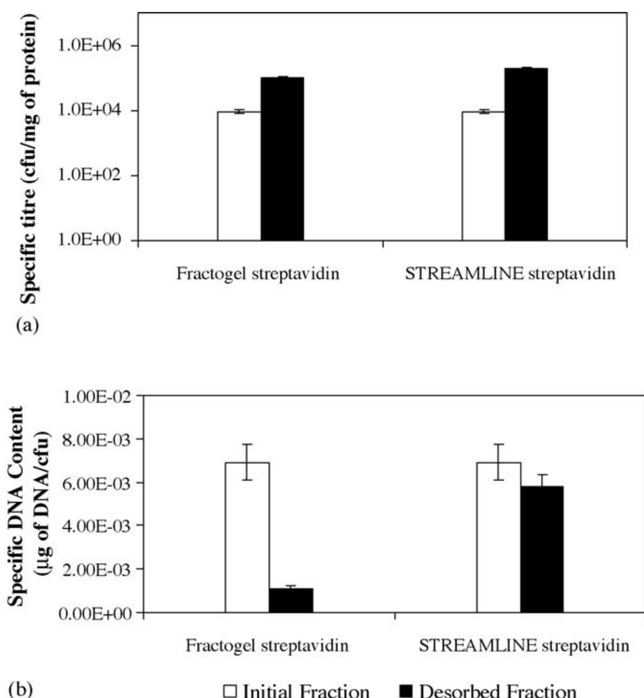

Fig. 4. Purity data of the recovered MoMuLV particles from batch adsorption and desorption with Fractogel® streptavidin and STREAMLINE streptavidin. The adsorbent (50 μl) was contacted with 3 ml of virus containing crude cell culture supernatant and mixed for 120 min at room temperature. The adsorbent was then washed (4 × 1 ml) with DPBS and the virus was desorbed by adding 1.0 ml of 0.6 mM d-biotin to the adsorbent and mixing, using a rotary mixer, for 120 min at room temperature. Samples were taken to determine the viral titre (cfu/ml), protein content (to determine specific titre; cfu/mg of protein) and the DNA content (to determine specific DNA content; μg of DNA/cfu). All viral titre assays were conducted in triplicate and the error bar represents the standard error of the three assays.

3.4. Optimisation of the biotinylated MoMuLV to enhance virus recovery

Based upon these observations the number of potential interactions between the streptavidin and the biotin on the surface of the retroviral particles was reduced to minimise the possibility of multi-point binding. This was achieved by lowering the concentration of BiotinSE that was contacted with the PG13 packaging cells during the biotinylation step. For reference purposes higher BiotinSE concentrations were also included in the study.

The retroviral particles became biotinylated by the incorporation of the biotinylated cell membrane of the packaging cells as the virions bud out of the cells. By reducing the concentration of BiotinSE contacted with the cell surface the number of biotin molecules attached to each retroviral particle should be reduced.

Streptavidin Magnespheres® were used to investigate the influence of reducing the biotin concentration on the capture of the MoMuLV as this was the benchmark adsorbent. Fig. 5a shows the capture of biotinylated MoMuLV expressed as a percentage of the retroviral particles adsorbed from the supernatant after a contact time of 120 min. To determine capture efficiency the virus bound to the Streptavidin Magnespheres® was titred directly. The results indicated that the most efficient capture of biotinylated MoMuLV was achieved when the PG13 packaging cells were contacted with 500 μM BiotinSE reagent (Table 4) having a 2298-fold increase in viral titre for a 30-fold decrease in volume (see Fig. 5b). The reduction in capture efficiency at 1 and 2 mM was attributed to an increase in interaction sites for each viral particle as each retroviral particle could potentially interact with more binding sites on the streptavidin ligands resulting in fewer overall available binding sites. For Streptavidin Magnespheres®, reducing the concentration of the BiotinSE contacted with the packaging cells also reduced the capture efficiency and this was interpreted as being caused by a reduction in the number of interactions between the biotin and streptavidin ligands.

The influence of degree of viral biotinylation on the performance of Fractogel® streptavidin was also examined and the results are shown in Fig. 6a and b. In contrast with the trend seen with Streptavidin Magnespheres® the initial observation was that reducing the concentration of BiotinSE contacted with the packaging cells did not similarly reduce the Fractogel® adsorption capacity. Desorption of MoMuLV from Fractogel® streptavidin was attempted using 0.6 mM d-biotin (Fig. 6b). Here, as the concentration of contacted

Table 4
The increase in viral titre due to the concentrating effect of Streptavidin Magnespheres®

Concentration of contacted BiotinSE (μM)	Initial MoMuLV concentration (cfu/ml of supernatant)	Concentration of plated Magnespheres® (cfu/ml supernatant)	Increase in virus concentration (for a 30-fold decrease in volume)
0	0.82×10^3 ($\pm 1.0 \times 10^4$)	1.14×10^4 ($\pm 7.8 \times 10^4$)	13.9
50	1.03×10^3 ($\pm 1.25 \times 10^2$)	1.27×10^4 ($\pm 6.7 \times 10^2$)	12.3
100	2.18×10^3 ($\pm 1.42 \times 10^2$)	2.13×10^4 ($\pm 2.4 \times 10^2$)	9.8
250	7.0×10^2 ($\pm 1.22 \times 10^2$)	9.83×10^4 ($\pm 5.6 \times 10^2$)	140
500	3.86×10^4 ($\pm 1.13 \times 10^4$)	8.87×10^7 ($\pm 1.0 \times 10^4$)	2298
1000	8.43×10^4 ($\pm 1.34 \times 10^4$)	7.0×10^7 ($\pm 1.67 \times 10^4$)	830
2000	6.0×10^4 ($\pm 1.0 \times 10^4$)	8.93×10^6 ($\pm 9.5 \times 10^4$)	149

Virus containing supernatant (3 ml) from cultures of PG13 cells biotinylated with reagent containing a range of BiotinSE concentrations was contacted with 50 μl of Streptavidin Magnespheres®. The adsorbent was mixed at room temperature for 120 min using a rotary mixer and the initial cfu/ml and non-bound cfu/ml of supernatant determined. After adsorption the Streptavidin Magnespheres® were washed in DPBS (2 × 1 ml) then 50 μl of adsorbent was resuspended in 1400 μl of DMEM (supplemented with 10% FCS, penicillin/streptomycin and L-glutamine). The number of viral particles on the Streptavidin Magnespheres® was determined using a titration assay. The increase in viral titre is expressed as the increase in viral titre with respect to the decrease in volume of the system (i.e. from 3 ml down to 100 μl, a 30-fold decrease).

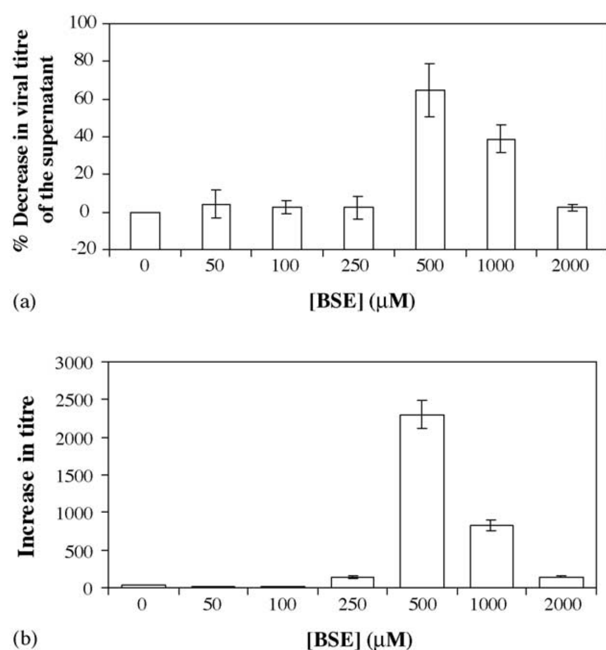

Fig. 5. The influence of the concentration of BiotinSE contacted with the PG13 producer cells upon the subsequent capture and concentration of biotinylated MoMuLV on Streptavidin Magnespheres®. Virus containing supernatant (3 ml) from cultures of PG13 cells biotinylated with reagent containing a range of BiotinSE concentrations was contacted with 50 μl of Streptavidin Magnespheres® for 120 min at room temperature using a rotary mixer. Samples (100 μl) were taken before and after the contact period and assayed to determine cfu/ml of supernatant and the percentage adsorbed from the supernatant was calculated (a). In addition, the Streptavidin Magnespheres® were washed with DPBS, pH 7.4 (2 × 1 ml) and the 50 μl of adsorbent resuspended in 100 μl of RPMI. The Streptavidin Magnesphere® bound virus was then plated in the same manner as the virus samples and the cfu/ml determined (see [8]) (b). All viral titre assays were conducted in triplicate and the error bar represents the standard error of the three assays.

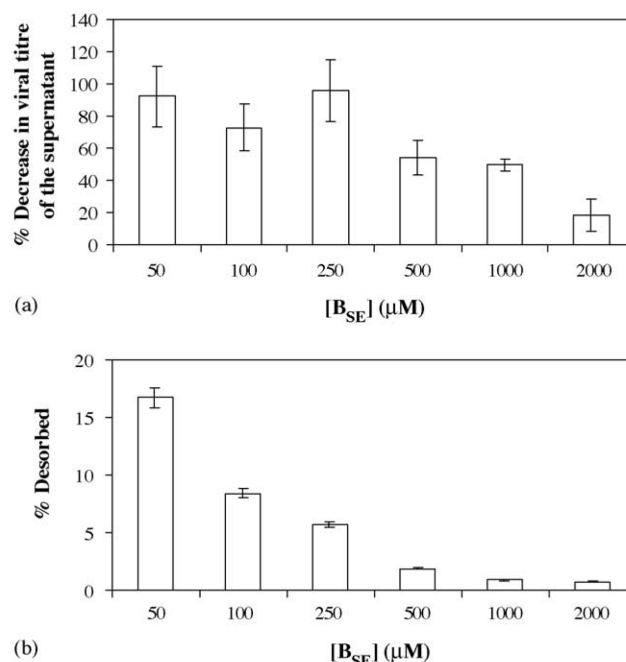

Fig. 6. The influence of the concentration of BiotinSE contacted with the PG13 producer cells upon the subsequent capture and release of biotinylated MoMuLV on Fractogel® streptavidin. Virus containing supernatant (3 ml) from cultures of PG13 cells biotinylated with reagent containing a range of BiotinSE concentrations was contacted with 50 μl of adsorbent. The adsorbent was sterilised with 0.25 M NaOH then equilibrated with DPBS, pH 7.4. Virus containing cell culture supernatant was added (3 ml) and the adsorbent mixed for 120 min at room temperature using a rotary mixer. The adsorbent was then washed (4 × 1 ml) with DPBS and the virus was desorbed by adding 1.0 ml of 0.6 mM d-biotin to the adsorbent and mixing, using a rotary mixer, for 120 min at room temperature. Samples (100 μl) were taken before and after the adsorption and elution phases and the cfu/ml of supernatant estimated using a titration assay, from which data the percentage decrease (a) and recovery (b) were calculated. All viral titre assays were conducted in triplicate and the error bar represents the standard error of the three assays.

BiotinSE was decreased the recovery of infectious retro viral particles increased from 0.74% (±0.04%) at 2 000 μM to 16.7% (±1.8%) at 50 μM (see Table 5). Furthermore, as the BiotinSE concentration was decreased the specific DNA content of the recovered viral particles also decreased. The retrovirus recovered using 50 μM BiotinSE for biotinylation showed a 1896 times increase (6.2×10^6 cfu/mg of protein) in specific titre and a 1191 times decrease (1.15×10^{-6} μg of DNA/cfu) in the specific DNA content (Fig. 7a and b). This sample was also analysed using SDS-PAGE (Fig. 8) that illustrates that the recovered MoMuLV contains less protein contaminants than the virus containing cell culture supernatant. This indicates a significant protein clearance and it is noteworthy that protein will be present in the viral preparation as 62% of a retrovirus is comprised of protein [3]. It is

Table 5
The adsorbent loading and the percentage biotinylated virus desorbed from Fractogel® streptavidin

Concentration of contacted BiotinSE (μM)	Adsorbent loading (cfu/ml of adsorbent)	Concentration of eluted MoMuLV (cfu/ml of adsorbent)	Fraction of eluted MoMuLV (%)
2000	0.23×10^6 ($\pm 1.43 \times 10^4$)	1.7×10^3 ($\pm 1.1 \times 10^2$)	0.74 (±0.04)
1000	0.56×10^6 ($\pm 1.42 \times 10^4$)	1.1×10^4 ($\pm 1.5 \times 10^3$)	0.89 (±0.05)
500	0.89×10^6 ($\pm 2.15 \times 10^4$)	5.1×10^4 ($\pm 4.9 \times 10^3$)	1.9 (±0.23)
250	1.8×10^6 ($\pm 1.74 \times 10^4$)	0.8×10^3 ($\pm 1.17 \times 10^3$)	5.7 (±0.98)
100	0.72×10^6 ($\pm 1.17 \times 10^4$)	1.2×10^5 ($\pm 3.2 \times 10^3$)	8.4 (±1.26)
50	2.5×10^6 ($\pm 1.73 \times 10^4$)	2.1×10^5 ($\pm 1.29 \times 10^3$)	16.7 (±1.8)

Before use the adsorbent was equilibrated with DPBS, pH 7.4. The adsorbent (50 μl) was added to 3 ml of virus containing supernatant and mixed at room temperature for 120 min. The adsorbent capacity was determined as the total cfu/ml of adsorbent. After adsorption the adsorbent was washed in DPBS (2 × 1 ml) then added to 1 ml of 0.6 mM d-biotin and mixed at room temperature for 120 min. The percentage recovery was determined as the percentage of the adsorbed cfu/ml of adsorbent.

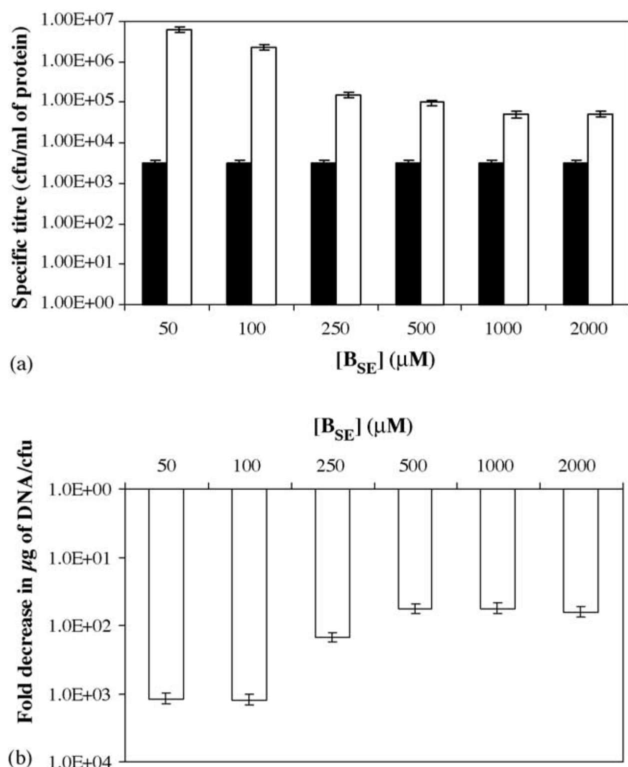

Fig. 7. The influence of the concentration of BiotinSE contacted with the PG13 producer cells upon the subsequent purity of virus following batch adsorption and desorption of MoMuLV on Fractogel® streptavidin. Virus containing supernatant (3 ml) from cultures of PG13 cells biotinylated with reagent containing a range of BiotinSE concentrations was contacted with 50 μl of adsorbent and mixed for 120 min at room temperature. The adsorbent was then washed (4 × 1 ml) with DPBS and the virus was desorbed by adding 1.0 ml of 0.6 mM d-biotin to the adsorbent and mixing, using a rotary mixer, for 120 min at room temperature. Samples were taken prior to adsorption and following elution to determine the viral titre (cfu/ml), protein content (cfu/mg of protein, a) and the fold decrease in μg DNA/cfu of the recovered fractions relative to the initial fraction (b).All viral titre assays were conducted in triplicate and the error bar represents the standard error of the three assays.

assumed that the protein present in lane 6 is serum albumin from the foetal calf serum. Individual viral proteins are not visible on this gel. The data compares favourably with the work of Kuiper et al. [22] who demonstrated specific titres of 18.1×10^4 cfu/mg of protein with a specific DNA content of 2.2×10^{-4} μg/cfu following purification of MoMuLV from crude cell culture supernatants using hydroxyapatite chromatography.

Finally, we note that Hughes et al. [8] show that the efficiency of biotin dependent capture is similar for the amphotropic envelope as well as for the feline endogenous virus envelope RD114. In recent work, we have developed novel metabolically biotinylated 293T derived cells and have shown the lentiviral vectors derived from these cells to be superior to chemical biotinylation, providing greater capture efficiency. These bio-lentiviral vectors have been demonstrated to efficiently complex with streptavidin paramagnetic particles, resulting in the most efficient purification and concentration

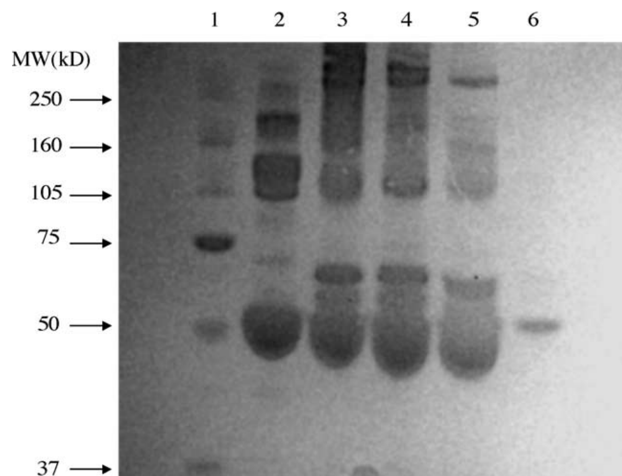

Fig. 8. SDS-PAGE analysis of 20 μl samples of the 50 μM biotinylated MoMuLV supernatant loaded and purified by adsorption to Fractogel® streptavidin with desorption in 0.6 mM d-biotin. Lane 1, molecular weight marker; Lane 2, 10% foetal calf serum diluted in PBS; Lane 3, complete cell culture medium; Lane 4, MoMuLV containing supernatant; Lane 5, MoMuLV containing supernatant after adsorption; and Lane 6, recovered fraction.

method yet described for lentiviral vectors. This work will be published in due course.

4. Conclusions

The data demonstrates that MoMuLV can be successfully biotinylated by chemical treatment and the resulting virus captured exploiting streptavidin activated chromatography adsorbents, providing a potential generic recovery process for retroviral particles. Fractogel® streptavidin displayed a superior performance compared to other adsorbent solid phases included in the study (Sepharose, STREAMLINE and Magnespheres®). Modifying the number of biotin molecules on the surface of the retroviral particle improved the recovery of infectious viral particles and significantly increased the contaminating protein and DNA clearance. The overall purity of the recovered retroviral particles was increased by more than 1000-fold.

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