



Hyaluronan treatment of biotinylated packaging cells increases titre of progeny retrovirus affinity-captured onto paramagnetic particles.

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ABSTRACT

BACKGROUND: The polysaccharide hyaluronan is a major component of the extracellular matrix and has been observed to impact retrovirus infectivity in biological settings. Hyaluronan has also been applied in biotechnology as a non-immunogenic, biocompatible agent to improve control of drug delivery and lentiviral transduction. We carried out a preliminary investigation to ascertain if the presence of hyaluronan influenced titre performance of an engineered retrovirus during the production, capture and infection steps that constitute key metrics for retroviral bioprocess performance.

RESULTS: The PG13.pBabe.puro stable packaging cell line constitutively produces retroviral particles with the gibbon ape leukaemia virus (GaLv) envelope protein and was used here with HeLa cells for retrovirus titration. An established bench-scale retrovirus production procedure was investigated in which packaging cells are chemically biotinylated and progeny retrovirus bound to streptavidin-coated paramagnetic particles (SPMPs) to achieve both retrovirus concentration and enhanced retroviral infection of target cells. Post-biotinylation incubation of PG13.pBabe.puro cells with up to 100µg/mL hyaluronan did not impact the base titre of unconcentrated progeny retrovirus. Incubation of target HeLa cells with up to 100µg/mL hyaluronan did not influence the susceptibility of HeLa cells to infection by retrovirus bound to SPMPs. However, post-biotinylation incubation of PG13.pBabe.puro cells increased titre of progeny retrovirus bound to SPMPs by up to 395%.

CONCLUSION: These observations are consistent with the hypothesis that the presence of haluronan after packaging cell biotinylation increases the efficiency of capture of biotinylated retrovirus by SPMPs. Further work will be needed to confirm if this is indeed the case and if packaging cell incubation with hyaluronan, or related biocompatible carbohydrates, could improve bioprocess performance of other retro- or lenti- viral vectors in therapeutic applications.

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DATE RECEIVED:
December 22, 2015

KEYWORDS:
Retrovirus; hyaluronan; biotin;
viral titre; paramagnetic
particles

INTRODUCTION

Hyaluronan is a major polysaccharide component of the extracellular matrix and is distributed widely throughout the human body in connective, neural and epithelial tissue. The hyaluronan polymer consists of disaccharide monomer units, D-glucuronic acid and D-N-acetyl glucosamine, and can range in size from 5000 Da to in the order of 1 million Da (Cyphert et al. 2015). A dynamic balance between the length of hyaluronan molecules within the extracellular matrix and their binding to cell surface receptors such as CD44 (Zöller 2015) is believed to play a significant role in cell signalling responses to events such as tumour invasion and inflammation. As a non-immunogenic, biocompatible and non-toxic compound, hyaluronan has also been studied extensively in the last decade as an agent for improved drug delivery (Tripodo et al. 2015).

The ability to efficiently introduce DNA of choice into mammalian cells has become more important than ever due to the emergence of powerful genome-editing tools, such as those based on the

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bacterial clustered regularly interspaced short palindromic repeats (CRISPR) / Cas9 nuclease system (Sachdeva et al. 2015), and potent immunotherapeutic strategies, such as engineered T cells (Qasim et al. 2015). These advances present a clear ongoing incentive for boosting the effectiveness of retroviral and lentiviral transduction. We suggest that any and all routes to increasing the efficiency of harvesting retroviral particles and maximising subsequent infectivity of captured virus should be reported where possible in order to develop a diversity of tools and avenues of investigation.

In biological settings the presence of hyaluronan has largely been shown to inhibit or block retro- or lenti-viral infection (Turville 2014). Efforts to deliver lentivirus *in vivo* to tissues have as result been helped by the use of hyaluronan breakdown enzymes such as hyaluronidase (Wanisch et al. 2013). However, in other therapeutic contexts, complexing viral vectors within polysaccharide scaffold mimics of the extracellular matrix has proven advantageous for transduction of target cells (Thomas and Shea 2013, Sun et al. 2014)

Le Doux et al. (1999) previously reported that hyaluronan inhibits retroviral transduction. However, Hughes et al. (2001) showed that paramagnetic particles coated with antibodies to the extracellular matrix component, fibronectin, could capture retrovirus particles secreted from the fibroblast-derived packaging cell line, PG13.pBabe.puro (Miller et al. 1991). Fibronectin and hyaluronan are believed to interact extensively to define extracellular matrix behaviour in normal and cancerous tissues (Evanko et al. 2015). Hughes et al. (2001) also reported that paramagnetic particles coated with the lectin Concanavalin A, which is known to bind the carbohydrate component of cell surface glycoproteins (Pratt et al. 2012), could efficiently capture retroviral particles. However, chemical biotinylation of PG13.pBabe.puro cells allowed capture of progeny virus particles by streptavidin-coated paramagnetic particles (SPMPs) and resulted in the highest retroviral titre increase in the Hughes et al. (2001) study.

In light of observations by Hughes et al. (2001), that the extracellular matrix components of packaging cell appear to co-associate with their progeny retroviruses, we sought to test the hypothesis that the presence of an additional, exogenous extracellular matrix component, hyaluronan, could enhance the capture of biotinylated progeny virus from PG13.pBabe.puro cells. Possible mechanisms for such an enhancement of capture include cross-linking of streptavidin-bound virus particles to non-bound particles via hyaluronan-based interactions.

MATERIALS AND METHODS

Hyaluronan preparation. Hyaluronic acid sodium salt from rooster comb (product code H5388, Sigma-Aldrich, Munich, Germany) of MW = 1.3 million Da was dissolved to 5mg/mL in a hyaluronan storage buffer (100mM sodium acetate, 100mM sodium chloride). Purified hyaluronidase enzyme (provenance not recorded) was dissolved in hyaluronidase storage buffer (20mM sodium phosphate, 0.45 % w/v sodium chloride, 0.01 % w/v bovine serum albumen) to a concentration of 2mg / mL = 10 U / μ L. 30 μ L of this hyaluronidase solution was mixed with 1.2mL of 5mg/mL hyaluronan and incubated at 37°C over night in anticipation of achieving partial cleavage of hyaluronan into a mixture of different molecular weight polysaccharides. Digested hyaluronan was then stored at 4°C and used for all subsequent experimentation.

Standard virus production, capture and titration. The procedure reported by Hughes et al. (2001) was followed as summarised in Figure 1. Briefly, on Day 1 of a given experiment 1×10^6 PG13.pBabe.puro cells were seeded onto a 9cm diameter round plate in 15mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 μ g/mL streptomycin, and 100 U/mL penicillin. 5-8 plates were routinely seeded in order that 4 plates with matching cell densities could be selected for further experimentation. Typically by Day 5 the PG13.pBabe.puro cells reached 80-90% confluence, approximately $1-2 \times 10^7$ cells per 9cm plate. At this stage growth media was removed and replaced with 10mL of pH 8.1 phosphate buffered saline (PBS) solution containing 0.5mM biotin amido caproate N-hydroxy succinimide ester (BSE) from Sigma-

Aldrich (product code B2643) and 0.75mM of CaCl₂ and MgCl₂. Cells were incubated at room temperature for 30 min, then the BSE solution removed and replaced with growth medium and the cells returned to 37°C. After 2 hours at 37°C that medium was discarded and replaced again with fresh medium for overnight (16 hours) incubation. Also on Day 5 HeLa cells were plated in wells of a 24 well plate at 5x10⁴ cells per well.

At the start of Day 6 of the procedure polybrene was added to HeLa cell growth medium to a final concentration of 4.4µg / mL 4 hours prior to their infection with retrovirus. 1.25 x10⁹ SPMPs in 2.5mL PBS was then transferred to a 15mL Falcon tube and a Dynal® MPC-6 magnetic particle concentrator (Invitrogen, Carlsbad, California, USA) used to remove liquid from beads, as described by Hughes et al. (2001), leaving only a bead pellet. Over night growth medium from PG13.pBabe.puro cells was removed from cells, passed through a 45µM filter and 5mL added to the SPMP pellet. This SPMP slurry was then placed on a roller for 90 mins at 4°C for virus capture. An MPC was then used to reduce the volume of the bead slurry to 20µL. Aliquots of this concentrated virus:SPMP suspension were serially diluted for titration purposes and a volume of 100µL always used to infect HeLa cells. HeLa cells were subjected to puromycin selection at 5µg / mL on Day 8 and monitored for cell death and emergence of resistant colonies over Days 9-20. Puromycin-resistant cell colonies were stained with Coomassie, as described by Hughes et al. (2001), and counted.

Hyaluronan treatment of target cells. At the stage in the above procedure where polybrene is added to HeLa target cells, digested hyaluronan was also added to some HeLa cell wells, to a final concentration of 1µg/mL, 10µg/mL or 100µg/mL and always to give a final growth medium volume of 1mL.

Hyaluronan treatment of packaging cells. Hyaluronan was added, to the indicated final concentrations, to the fresh medium used for overnight (16 hour) incubation of PG13.pBabe.puro cells at the end of Day 5.

RESULTS AND DISCUSSION

Previous work by Hughes et al. (2001) demonstrated that infectious retroviral particles are secreted continuously from the stable packaging cell line PG13.pBabe.puro into the surrounding growth media. Hughes et al. (2001) also reported that biotinylation of PG13.pBabe.puro cells by chemical means enabled capture of progeny retrovirus by SPMPs. SPMP binding enabled concentration of retroviral particles and a concomitant increase in infectivity likely due to enforced settling of virus particles onto target cells by the action of gravity or directed electromagnetic attraction.

We repeated the procedures reported by Hughes et al. (2001) for cultivation and chemical biotinylation of PG13.pBabe.puro packaging cells, SPMP-based capture of progeny retrovirus and infection of HeLa cells for retroviral titration (Figure 1). We also modified these procedures by either incubating PG13.pBabe.puro packaging cells with hyaluronan after their biotinylation (Figures 2 and 4) or by incubating the target HeLa cells with hyaluronan prior to their infection (Figure 3).

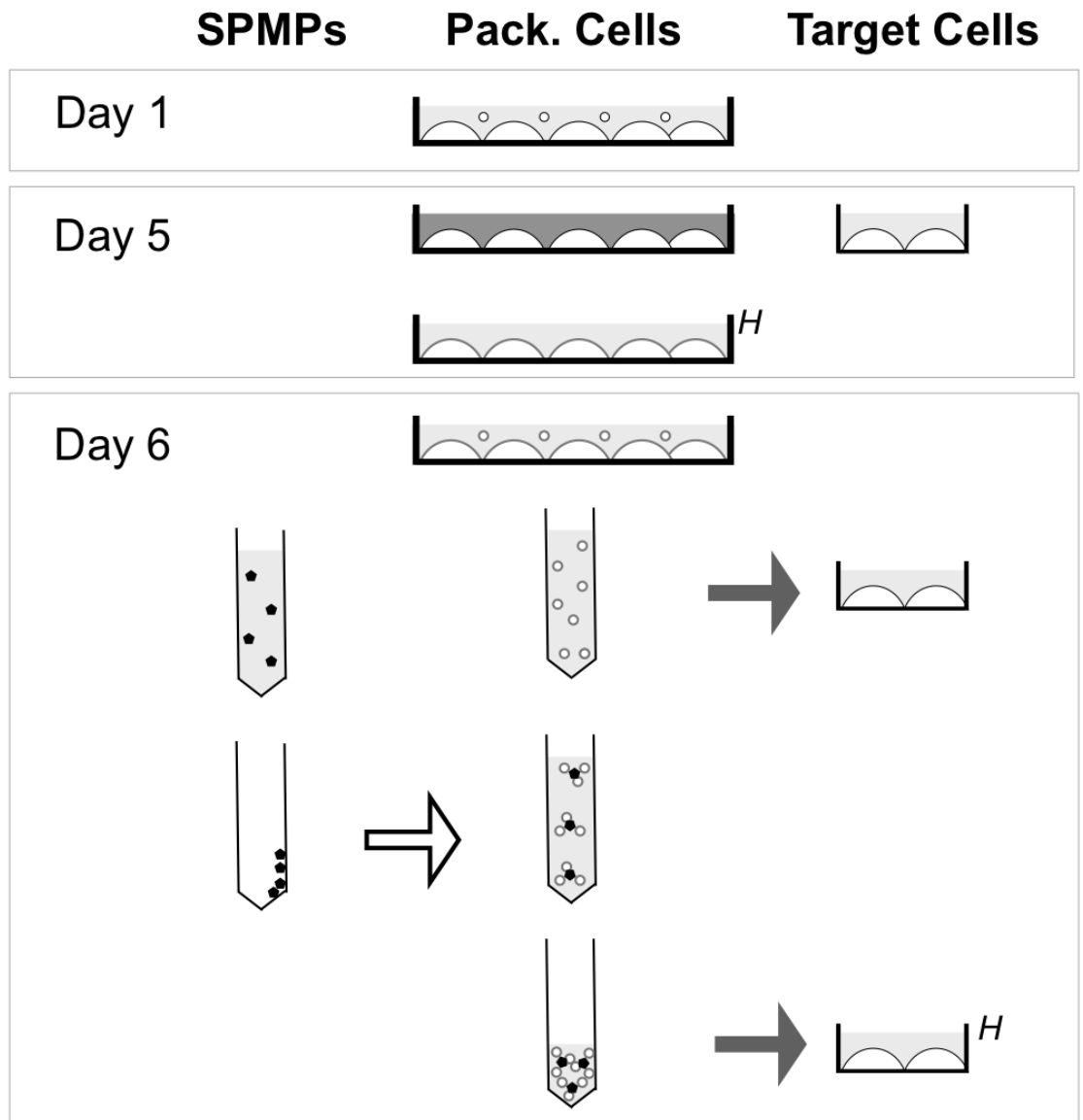


Figure 1: Capture and concentration of biotinylated retrovirus by streptavidin-coated paramagnetic particles (SPMPs) and titration by infection of HeLa cells. Summary of procedure detailed in Materials and Methods. The PG13.pBabe.puro cell line (Pack. Cells) constitutively produces retroviral particles and was plated on Day 1. On Day 5 HeLa cells (Target Cells) were plated for titration and PG13.pBabe.puro growth medium was replaced with a biotinylation solution (dark medium) that was then replaced with fresh medium (light medium) overnight. On Day 6 HeLa cells were incubated with polybrene 4 hours prior to infection with unconcentrated, virus-containing supernatant from PG13.pBabe.puro cells (uppermost dark arrow). Also on Day 6 SPMPs (pentagons) were concentrated by complete liquid removal then resuspension (open arrow) in virus-containing supernatant. Virus-bound SPMPs were then concentrated and used to infect HeLa cells for titration (lowermost dark arrow). H indicates steps in which a hyaluronan incubation was added as detailed in Materials and Methods.

Post-biotinylation hyaluronan treatment of PG13.pBabe.puro cells did not influence infectivity of progeny retroviral particles in solution. After the biotinylation of PG13.pBabe.puro packaging cells we incubated the cells with hyaluronan at the concentrations indicated in Figure 2, for 16 hours (see also the uppermost 'H' in Figure 1). We then removed and retained the PG13.pBabe.puro cell

growth medium and used it to infect HeLa cells for titration of infectious viruses following the procedure of Hughes et al. (2001). We anticipated that using such a relatively low titre, unconcentrated retrovirus solution would best reveal any increase in retrovirus infectivity or production resulting from the incubation with hyaluronan.

The presence of hyaluronan during post-biotinylation incubation of PG13.pBabe.puro cells had no influence on the infectivity of progeny virus particles (Figure 2). This suggest that hyaluronan, unlike agents such as sodium butyrate and caffeine (Ellis et al. 2011), does not act to stimulate additional virus productivity of packaging cells. This result is also consistent with the hypothesis that any hyaluronan that may remain associated with progeny virus particles after their release from packaging cells does not act to increase the frequency of subsequent infection events.

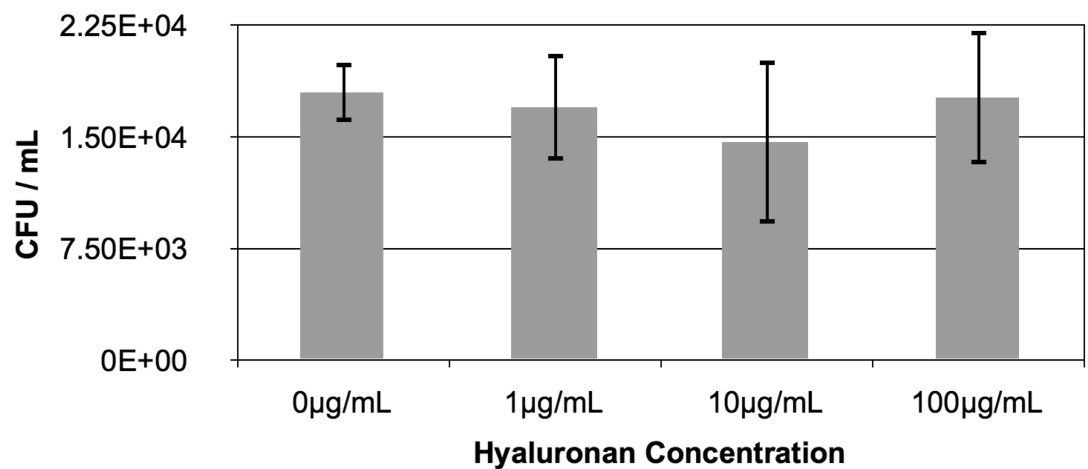


Figure 2: Titre of free retrovirus produced from biotinylated packaging cells incubated with hyaluronan. After chemical biotinylation PG13.pBabe.puro packaging cells were incubated with 0µg/mL, 1µg/mL, 10µg/mL or 100µg/mL hyaluronan (as indicated in axis labels) for 16 hours. 100µL of this PG13.pBabe.puro packaging cell medium was then added to target HeLa cells and resultant puromycin-resistant colony numbers counted. Error bars indicate standard deviation over n=3 biological repeats.

Incubating HeLa cells with hyaluronan did not enhance their susceptibility to infection by retroviral particles bound to SPMPs. We next sought to determine directly if hyaluronan treatment of target cells can increase the frequency of infection events achieved by a given amount of retroviral material. To do this we tested whether a 4-hour incubation of HeLa cells with hyaluronan influenced their infection by SPMP-bound retroviral particles derived from PG13.pBabe.puro cells. Figure 3 indicates that incubating HeLa cells with up to 100 µg / mL hyaluronan had no effect on their infection by SPMP-bound retrovirus.

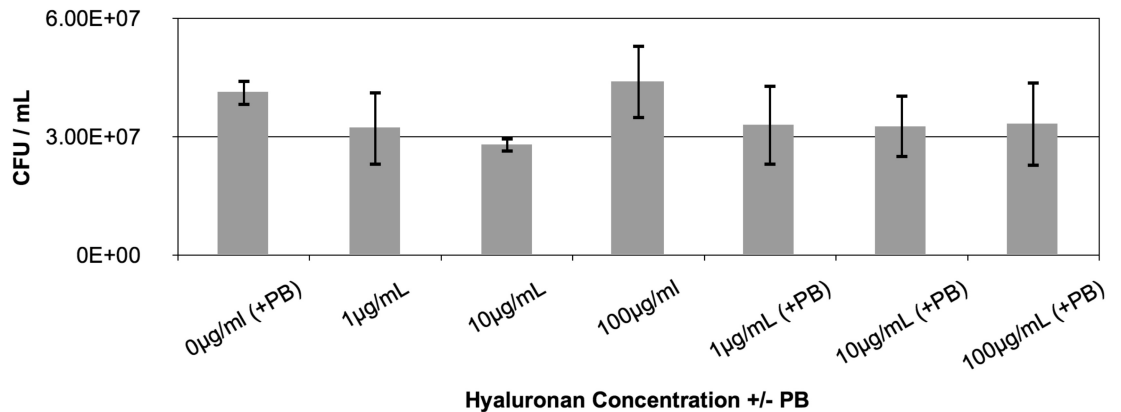


Figure 3: Titre of SPMP-bound retrovirus used to infect HeLa cells pre-incubated with hyaluronan. HeLa cells were seeded in 24 well plate as targets for retroviral infection (as in Figures 2 and 4) and 24 hours later growth medium was supplemented either with additional growth medium (indicated by '0µg/mL' in x axis labels), polybrene to 4µg/mL (indicated by 'PB' in x axis labels), hyaluronan (to 1µg/mL, 10µg/mL or 100µg/mL, as indicated in axis labels) or with both polybrene and hyaluronan. Cells were then incubated for 4 hours before a 100µL slurry of biotinylated retrovirus bound to SPMPs was added for retrovirus titration. Error bars indicate standard deviation over n=3 biological repeats.

Post-biotinylation hyaluronan treatment of PG13.pBabe.puro cells enhanced SPMP-based capture of progeny retrovirus. The previous data are consistent with the hypothesis that hyaluronan does not stimulate either retrovirus production by packaging cells (Figure 2) or infection of target cells by SPMP-bound retrovirus (Figure 3). As such we finally asked the question, could hyaluronan influence the biophysical process of retrovirus capture by SPMPs? To answer this question we incubated PG13.pBabe.puro packaging cells as previously for 16 hours with up to 100 µg / mL hyaluronan after their chemical biotinylation. However this growth medium was now incubated with SPMPs for virus capture and concentration. Figure 4 shows that this hyaluronan treatment increased the average titre of subsequently SPMP-bound retrovirus 395% in the case of 100 µg / mL hyaluronan.

The data in Figure 4 are consistent with hyaluronan acting to favour increased capture of biotinylated retrovirus by SPMPs. However these data do not rule out other possibilities, for instance that hyaluronan could be utilised as a nutrient by PG13.pBabe.puro cells to effect an increase in cell growth and/or virus production. However, it is worth considering that chemical biotinylation of PG13.pBabe.puro cells is terminated by both removal of the BSE reagent and a 2-hour incubation with ultimately discarded growth medium prior to addition of hyaluronan in fresh medium. As such any significant post-biotinylation increase in cell or virus numbers compared to non-hyaluronan treated cells would also likely dilute the level of biotinylation of the additional cells and virus particles. Another possibility is that any residual hyaluronan associated with virus-bound SPMPs (Figure 4) may favour HeLa cell infection events in a manner that does not manifest itself when free virus particles infect HeLa cells in the presence of hyaluronan (Figure 2).

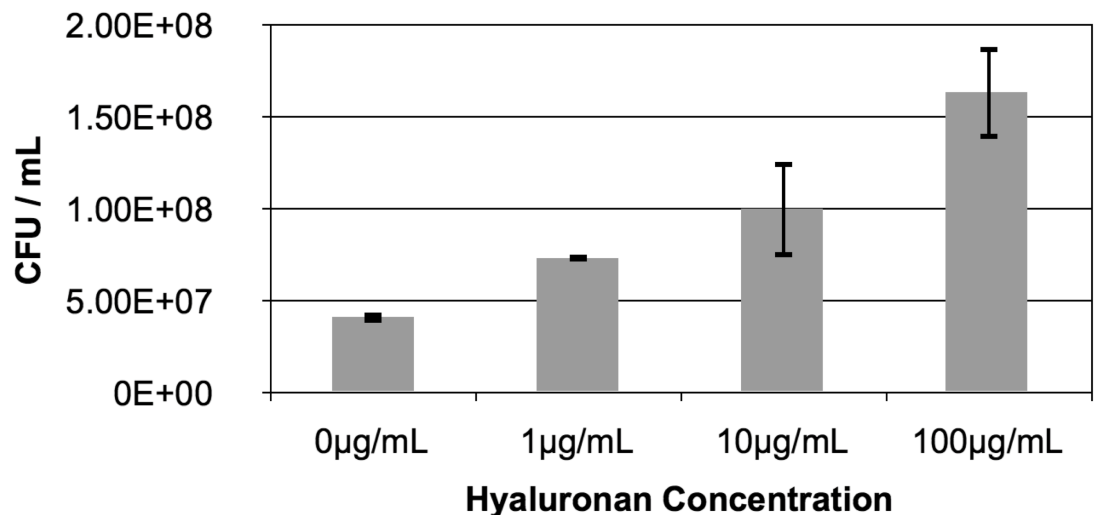


Figure 4: Titre of SPMP-bound retrovirus produced from biotinylated packaging cells incubated with hyaluronan. After chemical biotinylation, PG13.pBabe.puro packaging cells were incubated with hyaluronan for 16 hours as described in Figure 2. These packaging cell growth media with different hyaluronan concentrations were each then mixed with SPMPs and the resultant virus-bound SPMPs concentrated using an MPC. The bead slurry was then titrated using HeLa target cells. Error bars indicate standard deviation over n=3 biological repeats.

CONCLUSIONS

In conclusion, we have shown that post-biotinylation incubation of a retroviral packaging cell with hyaluronan increased the titre of subsequently paramagnetic particle-bound virus by up to 395%. We suggest this increased titre was due to enhanced virus capture. The success of this preliminary study indicates that further investigation is warranted into addition of exogenous agents to enhance lenti- or retrovirus production and capture.

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COMPETING INTERESTS

There are no competing interests of any nature associated with this work.

ACKNOWLEDGEMENTS

Valuable advice and input from David Darling, Farzin Farzaneh, Lucas Chan, Nicola Hardwick, Joop Gaken and Kevin Ford is warmly acknowledged. The support of the BBSRC is also gratefully acknowledged.