

Electrosprayed Genipin Crosslinked Alginate-Chitosan Microcarriers for *Ex Vivo* Expansion of Mesenchymal Stem Cells

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Abstract

Mesenchymal stem cells (MSCs) are potential therapeutic candidates, owing to their ability to differentiate into several cell types. However, the gap between availability and demand of MSCs requires alternative expansion methods from 2D culture flasks. Microcarriers are a promising approach for MSC expansion due to their high surface area to volume ratio. However, current commercial microcarriers do not provide the highest cell yield due to low cell attachment efficiencies and difficulty in cell detachment. This study developed a hydrogel-based microcarrier from genipin crosslinked alginate-chitosan beads. Alginate beads were produced by electrospraying before being coated with chitosan and crosslinked in genipin. The degree of crosslinking was determined through fluorescence reading of the genipin-chitosan conjugates. MSCs cultured on these microcarriers had a 26% higher cell attachment and twice the proliferation rate compared to the commercial microcarrier Cytodex 1. Cells easily detached from the hydrogel beads and did not require extended incubation periods or intense agitation during cell harvest. There was no significant difference in gene expression between the two microcarriers for the positive MSC surface markers *CD-90*, *CD-105* and *CD-73* as well as showing either low or no signal for negative MSC surface markers *CD-45* and *CD-34*.

Keywords

Electrospraying, Microcarriers, Mesenchymal stem cells (MSCs), Alginate; Cell Expansion

Introduction

Mesenchymal stem cells (MSCs) are adult stem cells originating from the mesoderm that are free of both ethical concerns and teratoma issues which arise with embryonic stem cells and induced pluripotent stem cells ⁽¹⁾. Like all stem cells, MSCs have two key properties, the first, is self-renewal – the ability to undergo division whilst maintaining their undifferentiated state, while the second is cell potency, which describes the ability to differentiate into several other cell types ⁽²⁾. These properties qualify MSCs as good candidates for cell therapy, involving the transfer of MSCs into the body to restore lost or defective functions within the host ⁽³⁾. This has been extensively researched for treatment of chronic injuries, myocardial infarction, diabetes, stroke etc. The biggest obstacle preventing MSCs from being widely available clinically is the large cell numbers required per therapeutic dose; up to 9 million MSCs per kg patient body weight are needed ⁽⁴⁾. To achieve these numbers, MSC expansion is typically performed through 2D tissue culture flasks. These flasks have several major drawbacks, as they require intensive labour and time consumption during the maintenance of the culture as a result of a poor surface area to volume ratio. Furthermore, they lack the ability to monitor culture parameters such as pH and oxygen levels ⁽⁵⁾.

Microcarriers are small spherical particles (μm - mm) which support cell growth by acting as a surface for the attachment of cells ⁽⁶⁾ and are used to overcome the drawbacks of tissue culture flasks. They offer a large surface area to volume ratio for anchorage dependent cell proliferation within a homogenous suspension culture. This creates more homogenous culture conditions, ease of monitoring and control of the culture parameters ⁽⁷⁾ compared to monolayer cultures such as tissue culture flasks, allowing for easier large scale production of cells ⁽⁸⁾.

Microcarriers of various materials are available commercially such as Cytodex 1, a solid microcarrier based on crosslinked Dextran with DEAE groups ⁽⁹⁾, Cytopore, a porous microcarrier based on cellulose ⁽¹⁰⁾ and solid glass microcarriers ⁽¹¹⁾. Commercial microcarriers are mainly developed for culturing cells to maximise their production of hormones, enzymes, antibodies and other secreted molecules ⁽⁹⁾. These processes do not require cell harvesting as the end product is produced in suspension within the culture medium. Although these microcarriers support cell attachment and growth, it has been proven difficult to detach cells from them at the end of culture, making them unsuitable for culturing cells as therapeutics ⁽¹²⁾. Moreover, the diameter and density of commercial microcarriers such as the Cytodex line have been optimised for use within the traditional stirred tank bioreactor and may not be suitable for use within other types of bioreactors types such as the perfusion or fluidized bed bioreactor ⁽¹³⁾.

More recently, hydrogel-based microcarriers have been developed. These include coating alginate microbeads with gelatin ⁽¹⁴⁾ or collagen ⁽¹⁵⁾ as well as genipin crosslinked gelatin microbeads ⁽¹⁶⁾. It is argued that hydrogel based microcarriers yields a higher attachment efficiency compared to several commercial microcarriers, which have been found to only support up to 60-70% attachment efficiency for stem cells ^{(15),(17)}. Furthermore, through manipulation of production parameters the size of the hydrogel based microcarriers can be optimized for a variety of bioreactors and are not limited by what is available commercially ⁽¹⁵⁾.

This study proposes chitosan coated alginate beads, covalently crosslinked with genipin, as an alternative microcarrier for cell culture. The core of the microcarriers is comprised of alginate, a biocompatible hydrogel containing blocks of (1,4)-linked D-mannuroic acid (M) and L-guluronic acid (G) ⁽¹⁸⁾. Divalent cations such as Ca^{2+} bind to G blocks forming ionic interchain bridges, called the egg-box structure, which causes the alginate to gel ⁽¹⁹⁾. Alginate microbeads were

created through electrospraying (Fig 1A) by pumping alginate solution through a nozzle, and subsequently applying a high electric field to the solution, generating mutual repulsion within the electrostatic charges. This surplus charge overcomes the surface tension force and is eventually ejected via the breakup and emission of the microdroplets ^{(20),(21)}.

Alginate discourages cell adhesion due to the lack of surface adhesive properties ⁽²²⁾. As cell adhesion is a requirement for survival, the alginate bead surface is coated with chitosan to promote cell anchorage and interaction with the microcarrier. The polyanionic nature of alginate ⁽¹⁸⁾, allows it to form polyelectrolytic complexes with polycations such as chitosan ⁽²³⁾. Chitosan is a natural polycationic polysaccharide derived from the abundantly available chitin ⁽²⁴⁾. It is biocompatible and resembles glycosaminoglycan in the extracellular matrix ⁽²⁵⁾ and has been shown to support cell adhesion ⁽²⁴⁾. The advantage of chitosan over other gelatin or collagen is that it is not made from mammalian products which have a higher risk of spreading infectious diseases ⁽²⁶⁾.

To provide greater structural integrity and long stability ⁽²⁷⁾, chitosan was covalently bonded to genipin, a natural glucone extracted from ripe *Genipa Americana* fruits ⁽²⁸⁾. Genipin forms bonds with the amine (NH₂) groups of the chitosan creating a tertiary amine bond, following which crosslinking occurs either through polymerization of genipin or the formation of a secondary amide linkage with another chitosan polymer (Fig 1B) ^{(27),(29)}. Genipin is believed to be far more biocompatible compared to synthetic crosslinkers, such as glutaraldehyde which are shown to have cytotoxic effects ⁽³⁰⁾. Past studies have shown that genipin-chitosan conjugates fluoresces under green channel, a property that can be exploited to characterize crosslinking density without the need to add further fluorescence markers ⁽³¹⁾.

In the current study, genipin crosslinked alginate-chitosan microcarriers were developed for MSC expansion. The first objective of this paper was to characterize and optimize the microcarrier production process to create a microcarrier stable within cell culture environments for 2 weeks – the typical amount of time for stem cell expansion ^{(32)–(35)}. The second objective was to investigate the suitability of the created microcarriers for cultivation of MSCs. The properties of interest were the cell attachment, detachment and proliferation rates compared to the popular commercial microcarrier, Cytodex 1.

Materials and Methods

Microcarrier Production

The microcarrier production process is shown in Figure 1A. The alginate microbeads were generated via electrospraying. This is followed by coating and crosslinking of the microbeads with chitosan and genipin respectively.

Electrospraying of alginate microbeads

Sodium alginate (A2033, Sigma Aldrich, USA) was dissolved in a 0.9% (w/v) sodium chloride solution (Sigma Aldrich, USA) to obtain a final concentration of 1% (w/v). The solution was introduced into a 5ml syringe and extruded through a blunt 30G blunt needle. The flow rate was adjusted to 3ml/min, the minimum flow rate at which a constant alginate jet will form. Voltage was applied to the needle via a high voltage power supply (model 73030, Genvolt, UK). A metal ring was placed below the needle and was connected to the ground electrode. Alginate microdroplets generated by the jet breakup gelled within a 0.1M Calcium Chloride (CaCl₂) bath (Sigma Aldrich, USA) for 1 hour. Voltages applied were 3.5kV, 4.5kV, 5.5kV, 6.5kV, 7.5kV and 8.5kV. The distance between the needle tip and the ground ring was set to either 4.5cm or

2.5cm. An optimal voltage and electrode distance was chosen based on the bead diameters obtained. The diameter of 30 beads were measured using ImageJ (National Institutes of Health, USA) software.

Chitosan Coating and Genipin Crosslinking

The alginate beads were then coated in a 1% (w/v) chitosan (Low viscosity, Sigma Aldrich, USA) solution containing 0.1M acetic acid (Fisher Scientific, USA) and 0.1M CaCl_2 for 5 hours. The pH of the chitosan solution was adjusted to 5 beforehand, using 1M sodium hydroxide (Sigma Aldrich, USA). The beads were washed with de-ionised (DI) water 3 times.

The alginate-chitosan beads were crosslinked by immersing in a 1mg/ml genipin (Challenge Bioproducts, Taiwan) solution at 37°C for 24 and 48 hours or 60°C for 4 hours. The resulting microcarriers were collected and washed with DI water 3 times.

Microcarriers crosslinked at 37°C for 48 hours will henceforth be referred to as ALXL37 while microcarriers crosslinked at 60°C for 4 hours will be referred to as ALXL60.

Microscope Imaging and Fluorescence analysis

The microcarrier diameter and crosslinking density were investigated using a Nikon TiE 2000 (Japan) fluorescence microscope. During image acquisition the alginate and alginate-chitosan microbeads were stored within DI water while genipin crosslinked alginate-chitosan microcarriers were within 1mg/ml genipin solution. Microcarriers were imaged under a green fluorescent channel and a brightfield channel. A green fluorescence intensity profile corresponding to a line across the focal plane of a single bead was acquired using the NIH Elements Advance (Nikon, Japan) under a constant shutter exposure length of 2s. Background fluorescence of the storage solution was subtracted from the total fluorescence of the

microcarriers. The coating layer thickness of each bead was measured and the average fluorescence intensity across the length of the coating layer was calculated. The analysis was performed using NIH Elements Advance. A total of 3 batches of microcarriers were created with the analysis being conducted on 7 microcarriers from each batch (n=21).

Microcarrier Swelling

The microcarriers were immersed in low glucose (1g/l) Dulbecco Modified Eagle's Medium (DMEM) containing 10% Foetal Bovine Serum (FBS) and 0.1% Penicillin/Streptomycin (P/S) for 24 hours at 37°C and 5% CO₂. After this conditioning period, the bead diameters were monitored each day for a period of 2 weeks. A total of 30 microcarriers were measured at each time point. Unmodified alginate beads were measured as a control group.

MSC Culture Studies

Green fluorescent protein (GFP) modified human MSCs-hTERT cell line was kindly provided by the Department of Pediatrics and Adolescent Medicine, LKS Faculty of Medicine, The University of Hong Kong.

The cells were defrosted from frozen and seeded at 5000 cells/cm² using low glucose (1g/l) DMEM with 10% FBS and 0.1% P/S in tissue culture flasks and maintained at 37°C and 5% CO₂ in air. Media exchange was performed every 3 days. MSCs were passaged when 80% confluent using Trypsin/EDTA.

Cell Inoculation

ALXL60 was selected as the most optimal microcarrier and hence used for MSC culture. In order to estimate the total microcarrier surface area, two assumptions were made:

- 1) Microcarriers were perfectly spherical.
- 2) Microcarriers were packed in a face centred cubic arrangement with an atomic packing factor of 0.74.

The total surface area was then estimated as follows:

- 1) The volume and surface area of an individual microcarrier were calculated using the average microcarrier diameter.
- 2) The apparent volume of the microcarrier bed was measured.
- 3) The true volume of the microcarriers was estimated using the atomic packing factor of 0.74.
- 4) The true volume of all the microcarriers was divided by the volume of a single microcarrier yielding the total number of microcarriers.
- 5) The surface area of an individual microcarrier was multiplied by the total number of microcarriers giving the total surface area.

It was essential that an equivalent growth surface area of Cytodex 1 (Sigma Aldrich, USA) was used to ensure a fair comparison between the microcarriers. The required amount of Cytodex 1 was weighed out using the approximate surface area per gram of dry weight, provided by the manufacturer as $4400\text{cm}^2/\text{g}$ ⁽⁹⁾. Cytodex 1 were hydrated in DI water and autoclaved.

22ml glass vials were siliconized using Sigmacote (Sigma Aldrich, USA) to prevent cell adhering to the vessel walls. The required amount of microcarriers was added to the vial and suspended within 10ml of media. Based on the results from assessing microcarrier swelling within a cell culture environment, microcarriers were allowed to condition in media for 2 days. Following this, the media was exchanged and the desired amount of cells was seeded into each

vial. The vials were placed on an orbital shaker for 5 minutes at 90 rpm. Following this the microcarriers were placed in static culture in an incubator at 37° C and 5% CO₂.

Cell Attachment Efficiency

To investigate cell attachment efficiency, cells were seeded at a density of 15000cells/cm² onto microcarriers with an equivalent growth area of 75cm². This seeding density was higher compared to the density used in proliferation studies, as this would provide a sufficient cell count during the assessment of cell attachment and detachment ⁽³⁶⁾.

24 hours after cell inoculation, the supernatant of the microcarrier suspension was sampled and the unattached cells were counted using a Countess cell counter (Invitrogen, USA). The attachment efficiency (A) was determined using equation 1:

$$A = \frac{C_T - C_D}{C_T} \quad (1)$$

Where C_T is the total number of cells seeded, and C_D represents the number of cells that did not attach on the microcarriers. Microcarriers were too large to enter the cell counting plate and hence were always excluded.

Cell Detachment Efficiency

Following the measurement of attachment efficiency, the microcarriers were allowed to settle and media was removed. Microcarriers were washed with phosphate buffered saline (PBS) twice to remove all unattached cells. Trypsin/EDTA was added to the microcarrier suspension and incubated at 37°C for 5 minutes. Subsequently, the microcarriers were gently pipetted once or twice to achieve a single cell suspension. Culture media was then added to inactivate the trypsin.

The total number of cells harvested was counted by sampling the supernatant. The detachment efficiency (D) was estimated using Equation 2:

$$D = \frac{C_H}{C_T - C_D} \quad (2)$$

Where C_H is the total number of cells harvested using trypsin. The viability of the harvested cells was measured using the Trypan blue assay.

Cell Proliferation

Cell proliferation on microcarriers was investigated for 14 days. Cells were seeded onto the microcarriers at 5000cells/cm² with an approximate growth surface area of 75cm². The amount of media added to each sample was constant at 10ml. Cell Counting Kit 8 (CCK-8) (Sigma Aldrich, USA) assay was performed on day 1, 4, 7, 10, and 14 (with cells being seeded at day 0) to measure cell metabolic activity. WST-8 within the CCK-8 assay was reduced by metabolites in live cells to give rise to a yellow-orange coloured dye which could be measured on an absorbance spectrum ⁽³⁷⁾. According to the suppliers' protocol, the amount of dye generated by cellular activity is directly proportional to the number of living cells ⁽³⁸⁾. Hence, this assay could be used to provide an indication of cell proliferation on the microcarriers. Before each measurement 5ml of culture media was removed. 150µl of CCK-8 solution was added to the microcarriers culture and incubated for 4 hours at 37°C. Following the incubation period, 100µl of media samples were transferred to a 96 well plate. A TECAN (Switzerland) multifunction microplate reader was used to measure the absorbance of the samples at 450nm. The fold increase of the cell number was estimated by the following equation:

$$\text{Cell Number Fold Increase} = \frac{\text{Absorbance value on day of measurement}}{\text{Absorbance value on day 1}} \quad (3)$$

Cell free microcarriers were used to determine the background. On day 6, additional microcarriers with a total surface area of 75cm^2 were added to the microcarrier cultures increasing the growth surface to 150cm^2 . Cell culture media used was doubled to 20ml accordingly and 15ml of media was removed prior to the subsequent addition of CCK-8.

Gene expression analysis

RNA Extraction

MSCs were enzymatically dissociated from the microcarriers using trypsin. Cells were separated from the microcarriers using a $40\mu\text{m}$ cell strainer. The cell number was counted and roughly divided into 500,000 cell batches. Each batch of cells was washed twice with PBS and centrifuged. The cell pellet was stored at -80°C . Cells from the same batch grown in 2D culture but not seeded onto the carriers for the 14 day proliferation were frozen down and used as the control group (referred to as the 2D control).

RNA extraction was performed using RNeasy Plus Microkit (Qiagen, Netherlands), according to the manufacturer's instructions. Briefly, following cell lysis, the lysate was passed through a gDNA eliminator column, and ethanol was subsequently added to the flow through. The mixture was transferred into an RNeasy spin column where RNA binds to the membrane of the column and several washes were conducted to remove contaminants. Finally, RNA was eluted from the column with RNase free water.

To each sample a total of $0.1\mu\text{g}/\mu\text{l}$ of RNA was converted to cDNA by adding 20x reverse transcriptase and 5x cDNA mix from a cDNA synthesis kit purchased from qPCRBIO (UK), adjusting to a final volume of $20\mu\text{l}$ with PCR grade water. According to the suppliers'

instructions, samples were incubated at 42°C for 30 minutes, followed by 10 minutes at 85°C. The cDNA created was stored at -20°C.

Quantitative Polymerase Chain Reaction (qPCR)

cDNA samples were analysed by real-time qPCR. The amplification was performed in triplicates using SyGreen Blue Mix Lo-Rox, qPCRBio (UK). All reactions were performed with 3 biological repeats and 3 technical repeats (n=9). Each sample has a total volume of 20µl with 1µl of cDNA and 400nM of forward and reverse primers. Amplification of cDNA was achieved using a Rotor Gene Q Series, Qiagen (Netherlands), beginning with an initial activation and denaturing step holding at 95°C for 2 minutes. This was followed by 40 cycles with 3 sec at 95°C and 25 sec at 60°C. Each cycle was run for another 25 sec at 72°C during which data was acquired.

Primers were used to detect *CD-90 (THY1)*, *CD-105 (ENG)*, and *CD-73 (NT5E)*, representing positive MSC markers, as well as *CD-45 (PTPRC)* and *CD-34*, representing negative MSC markers. Primer information, accession numbers and amplicon sizes are shown in Table 1. Gene expression levels were determined using the comparative Ct method. Delta Ct (ΔCt) values were obtained after normalization to the reference gene *GAPDH*. Relative gene expression was calculated by normalizing the ΔCt values for ALXL60 and Cytodex 1 to the 2D control group and the resulting delta-delta Ct ($\Delta\Delta Ct$) values were used in equation 4:

$$\text{Relative Gene Expression} = 2^{-\Delta\Delta Ct} \quad (4)$$

Statistical Analysis

An unpaired t-test was used to compare significance between cell attachment and detachment between ALXL60 and Cytodex 1 as well as comparing the average bead diameter of ALXL37 and ALXL60.

A one way ANOVA test with Post-Hoc Tukey analysis was performed to compare the fluorescence intensity and coating layer values to test for significance.

In order to determine significance between cell proliferation on ALXL60 and Cytodex 1, a two way ANOVA with Post-Hoc Tukey analysis was performed. The same procedure was used for analysing CD marker gene expressions for cells grown on the microcarriers and also characterizing bead size following electrospraying of alginate beads.

For all analysis $p < 0.05$ was deemed significant.

Results

Electrospraying of Alginate microbeads

The correlation between voltage and bead diameter is shown in Figure 2A, with the bead size from 200-300 μm . In general the bead size decreased with increasing voltage. Post Hoc analysis revealed significant increase in all the microbead diameters as the voltage was increased. The only exception was at an electrode distance of 4.5cm where there was no significant difference in microbead diameter as the voltage was increased from 7.5kV to 8.5kV.

Electrode distance has a strong influence on the diameter of alginate beads, with the bead size decreasing significantly as the electrode distance was reduced from 4.5cm to 2.5cm.

Coating and Crosslinking of Beads

Chitosan interacts with the surface of alginate microbead resulting in a buildup of chitosan on the bead surface. This can be seen in Fig 2E where a clear coating layer surrounds the bead core as oppose to uncoated alginate beads in Fig 2C.

Under brightfield, genipin crosslinked alginate-chitosan microcarriers (Fig 2G) appear similar to alginate-chitosan beads (Fig 2E). However the microcarriers, transformed from white (Fig 2Bi) to a blue-green colour due to the pigmentation formed (Fig 2Bii). Moreover, when viewed in green channel, the coating layer expressed fluorescence (Fig 2H). In contrast fluorescence was not observed in both alginate beads without chitosan coating within a genipin solution (Fig 2D) and uncrosslinked alginate-chitosan beads in DI water (Fig 2F).

In this study, we crosslinked the beads at 60°C, in contrast to 37°C used by most studies ^{(31),(39),(40)}. The fluorescence intensities of ALXL60 was comparable to that of ALXL37 (Fig 3A). As the rate of reaction is higher at elevated temperatures crosslinking at 60°C for 4 hours (ALXL60) achieved similar crosslinking density compared to crosslinking at 37°C for 48 hours (ALXL37). Both ALXL60 and ALXL37 displayed higher fluorescent intensities compared to beads crosslinked at 37°C for 24 hours. The thickness of the coating layer was non-significant between ALXL37 and ALXL60 despite differences in crosslinking temperature (Fig 3B).

Microbead Swelling

It is essential for microcarriers to remain stable in a cell culture environment to ensure their suitability for MSC expansion. Figure 4 depicts the diameter change for alginate based microbeads over a period of 2 weeks in DMEM. Alginate microbeads swelled significantly from 220µm to 342µm following 24 hours incubation in cell culture media (day 0 – day 1). The bead diameter on day 1 was significantly lower compared to bead diameters measured at other time

points from day 2-14. Following this, the beads stabilized from day 2-14 with no significant changes in bead diameter.

ALXL37 and ALXL60 microcarriers displayed significantly lower swelling on day 1 compared to alginate beads with the diameter increasing from 220 μ m to around 270 μ m. From day 1-2 further swelling was observed in both microcarriers from 270 μ m to around 290 μ m. The microcarriers were stable from day 2-14 with no significant changes in bead diameter. A t-test yielded no significant difference in diameter between ALXL37 and ALXL60 at every time point.

Attachment Efficiency of hMSCs on microcarriers

After 24 hours of incubation of MSCs with ALXL60 and Cytodex 1, cells attached and flattened out into spindle-like morphology on both microcarriers (Fig 5A). This can be clearly seen under green channel with GFP labelled cells (Fig 5B). The attachment efficiency for MSC (presented in Figure 5C) on ALXL60 was found to be 76%. On the other hand, Cytodex 1 had a significantly lower attachment efficiency of 50%.

hMSC Detachment from Microcarriers

Cell harvesting using trypsin/EDTA yielded a detachment efficiency of 55% from ALXL60 (Fig 5D). The viability of the cells harvested was 96%. On the other hand, Cytodex 1 had a significantly lower detachment efficiency at only 38% (Fig 5D) while the viability of the cells was 92%.

Cell Proliferation

MSCs attached onto ALXL60 and proliferated over a course of 2 weeks, Fig 6A and 6B show the microcarrier culture on day 7 and day 14 respectively. CCK-8 assay revealed a steady cell

fold increase with respect to day 1 (24 hours following cell seeding) for both ALXL60 and Cytodex 1, as illustrated in Figure 5E. However, there was a significantly higher fold increase throughout the culture for cells grown ALXL60 compared to Cytodex 1. The final fold increase at the end of the 14 day period was 4.6 and 2.3 respectively.

qPCR and Gene Expression of CD Markers

There was no significant difference in the relative gene expression levels of cells cultured on ALXL60 and Cytodex 1 compared to the 2D control, which was normalised to 1 (Figure 7). In addition there was no significant difference in the relative gene expression levels between ALXL60 and Cytodex 1 (Figure 7).

No expression for *CD-45* could be detected for all the groups tested indicating absence of leukocytes ⁽⁴¹⁾. The threshold cycle (CT) values obtained for *CD-34*, a marker for hematopoietic and endothelial cells ⁽⁴²⁾, were significantly higher compared to the 3 positive makers (Data not shown).

Discussion

Electrospraying Produces Homogenous Microbeads of Required Size

Statistical analysis showed that, at an electrode distance of 4.5cm, there was no significant difference in the bead diameter between 7.5kV and 8.5kV. This phenomenon, where bead size remains constant at higher voltages, has been observed in several other studies ^{(43)–(45)}. The electrostatic force counteracts the surface tension and when a critical electrostatic force is reached, the surface tension reaches a minimum and no further decrease in diameter is achieved ⁽⁴⁵⁾. However, lowering the electrode distance to 2.5cm led to a continued decrease in microbead diameter as the voltage was increased from 7.5kV to 8.5kV. This suggests that the critical

electric force was yet to be reached but the relationship between the electrical force and surface tension was beginning to plateau. Therefore at higher voltages, only a decrease in electrode distance would increase the electrical force sufficiently to produce a significant effect on bead diameter.

Based on these results, the most optimal parameters to produce the microcarriers was 7.5kV and 2.5cm electrode distance. The diameter created was close to 200 μ m, which is within the range of the preferred size of spherical microcarriers from 90-300 μ m ^{(46),(47)}. Within this range the microcarriers would have a sufficient growth surface per bead to support cell proliferation while providing a high total surface area to volume ratio ^{(48),(49)}.

The relative standard deviation of the diameter of the alginate beads was less than 10% which was lower than that of Cytodex 1 which was measured to be 20%. This shows uniformity within the beads produced.

Genipin Crosslinking Density Characterized by Fluorescence

Fluorescence intensity generated by the chitosan genipin reaction provides an indication of the degree of crosslinking in the beads. Higher fluorescence intensity, indicates more conjugates are formed, and as a result stronger microcarriers ⁽⁴⁰⁾. There are two key factors to take into consideration when analysing the fluorescent intensity and coating layer thickness. Firstly, the fluorescent intensity and coating layer thickness values obtained was only an approximation, as the resolution of the fluorescent microscope would not be high enough to determine the boundary between the coating layer and the alginate core. Secondly, the coating and crosslinking would not be uniform across all beads as these processes were not performed under a good mixing environment such as a stirred tank vessel or a fluidization chamber ⁽⁵⁰⁾.

Genipin Crosslinked Alginate-Chitosan Microcarriers Stable in Cell Culture Media

Although ALXL37 and ALXL60 displayed no significant difference in terms of fluorescence intensity or coating layer thickness, high crosslinking temperatures of the latter could alter rheological properties due to denaturation or depolymerisation of the hydrogel ^{(51),(52)}. This would lead to potential weakening of the gel structure and hence the stability of the microcarriers. Therefore, to ensure stability in cell culture conditions, bead swelling behaviour in media was assessed.

Alginate beads swell and eventually degrade in the presence of monovalent ions such as Na^+ ^{(53),(54)} within cell culture media. The Na^+ present in the external solution undergoes ion-exchange with Ca^{2+} binding to the M groups. As a result, electrostatic repulsion between COO^- groups relaxes the chain, causing increased bead swelling. In the later stages of swelling, Na^+ ions begin interchanging with Ca^{2+} in G groups causing the disintegration of the egg box structure, leading to dissolution of the bead. Alginate beads demonstrated no swelling in DI water thus confirming that swelling was due to ion exchange rather than osmosis ⁽⁵⁵⁾.

Most studies have examined bead swelling within PBS and saline ^{(55)–(59)}, however very few have examined swelling in cell culture media ⁽⁶⁰⁾. Despite the ionic composition of media being similar to PBS there are slight differences, notably the trace of Ca^{2+} present in the media. Furthermore, there is a possibility that growth factors and amino acids in the media and FBS added could interact with alginate beads and its coating layer ⁽⁶¹⁾, affecting the swelling characteristic.

Applying a coating layer around alginate beads have been previously shown to provide resistance to the alginate core swelling hence increasing bead stability and lowering the swelling

ratio ^{(58),(62)}. This behaviour was also observed in this study by the significantly lower swelling of the chitosan coated ALXL37 and ALXL60 within DMEM compared to unmodified alginate beads.

The bead diameter of both ALXL37 and ALXL60 was stable in DMEM throughout the entire culture period with the exception of day 1, where the beads had yet to reach equilibrium. This could be circumvented through increasing the conditioning period from 24 hours to 48 hours.

The stability throughout the culture and non-significant differences in diameter between ALXL37 and ALXL60 demonstrates the swelling behaviour and stability of the ALXL60 within cell culture conditions were not compromised by the high temperatures used during crosslinking. Due to the decreased production time, ALXL60 was the microcarrier of choice for the cell culture work.

Superior Cell Culture Properties on ALXL60 Compared to Cytodex 1

A key issue with commercial microcarriers is their difficulty in cell detachment following trypsin treatment ⁽¹²⁾. ALXL60 proved to have significantly higher detachment efficiency compared to Cytodex 1. However, the genipin crosslinked alginate-chitosan microcarriers also maintained a superior attachment efficiency. The poor attachment and detachment efficiency displayed by Cytodex 1 could be due to the fact that the carriers were not initially designed for stem cell expansion but rather for the production of biomolecules ^{(9),(17)}.

In order for the cell numbers to increase continuously throughout the 14 day culture, additional microcarriers were added on day 6. Preliminary experiments without the addition of extra carriers, showed that cell growth rate slows around day 7 (data not shown) due to the cell numbers reaching the confluency. This was seen in the microcarrier culture on day 7 (Fig 6A)

showing a proportion of fully confluent microcarriers (indicated by green arrows), while several empty microcarriers represent the freshly added microcarriers (indicated by red arrows). By day 14, MSCs migrated to the growth surfaces of the empty microcarriers resulting in a confluent culture (Fig 6B).

Similar to cell passaging in 2D culture, the addition of extra microcarriers ensured sufficient growth surface area was available throughout the culture and ensured that cell confluency was not reached. This was achieved through bead-to-bead transfer of cells to the newly added microcarriers. It was shown in a previous report by Schop et al that the additional surface area provided would prevent the stationary growth phase from being reached during microcarrier culture ⁽⁷⁾. Unlike cell passaging, addition of extra microcarriers does not utilize proteolytic enzymes such as trypsin which degrades the extracellular matrix and its cell receptors leading to reduced viability ^{(63),(64)}. Moreover, the process is simple and less labour intensive process compared to cell passaging saving running costs and time.

qPCR and Gene Expression Displayed No Changes to MSC Phenotype

qPCR was performed to ascertain whether the harvesting procedure or the growth surface affected the phenotypic properties of hMSCs. The surface markers: *CD-90*, *CD-105* and *CD-73* should be present on hMSCs as defined by the International Society for Cellular Therapy ⁽⁴²⁾. The defined negative hMSC markers are used to mainly exclude the presence of monocytes, hematopoietic cells, leukocytes, B cells and lymphocytes within the hMSC culture ⁽⁶⁵⁾. These cells are within the heterogeneous cell population obtained during MSC isolation ⁽⁴²⁾. However, the cells used in this study were immortalised commercial cell lines therefore contamination needs to be kept to a minimum. Hence only 2 negative markers, *CD-45* and *CD-34* were investigated for qPCR. Due to the immortality of the cell line, little or no changes in cell

phenotype were expected with an increasing passage number ⁽⁶⁶⁾. Hence, any potential changes to MSC phenotype would likely be due to the culture process and material.

It has been shown previously that culture on Cytodex 1 does not alter the hMSC surface markers ⁽⁶⁷⁾. Therefore due to the similar expression levels of CD markers between ALXL60 and Cytodex 1, it is likely that MSCs cultured on ALXL60 retained their phenotypic properties. Moreover, the lack of significant difference in relative gene expression for the cells harvested from ALXL60 compared to the 2D control further confirmed that the phenotype of the cells did not change during the 14 days of culture.

Conclusions

In this study we have successfully created alginate-chitosan microcarriers which support the growth of MSCs for cell expansion. MSC proliferation was found to be significantly higher on ALXL60 compared to the commercial product Cytodex 1 over a 2 week expansion period. Furthermore, the cells easily detached from the ALXL60 following trypsin/EDTA treatment, overcoming one of the main drawbacks associated with commercial microcarriers, where long incubation and high agitation in proteolytic enzyme solution are required during cell harvest, lowering cell yield.

The results from this work showed that genipin crosslinked alginate-chitosan based microcarriers can act as a potential alternative to commercial microcarriers for MSC expansion.

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Figure Legends

Fig 1. Microcarrier manufacture protocol and chitosan genipin reaction chemistry. A) Production of genipin crosslinked alginate-chitosan microcarriers. Alginate microbeads were created through electrospraying. Following this, the beads were coated in a chitosan and CaCl_2 solution causing a chitosan coat to build up on the microbead surface. Finally, the beads were added to a genipin solution, crosslinking the chitosan coating layer. B) Two possible crosslinking reactions between genipin and chitosan. (Left) The formation of a tertiary amine group as well as a secondary amide. (Right) Polymerization of genipin following the formation of a tertiary amine group with chitosan.

Fig 2. Alginate microbeads and genipin crosslinked alginate-chitosan microcarriers. A) Comparison of microbead diameter vs voltage for different electrode distances. There was no significant difference between bead diameter for 7.5kV and 8.5kV for beads created at an electrode distance of 4.5cm. Error bars show standard deviation. B) i) Alginate microbeads following electrospraying. ii) Microcarriers turn blue after successful genipin crosslinking. C) Alginate microbeads created using electrospraying at 7.5kV with an electrode distance of 2.5cm. D) Incubation of alginate microbeads within a genipin solution displayed no fluorescence under the green channel. E) Alginate-chitosan microbeads prior to crosslinking with genipin under brightfield channel. F) Alginate-chitosan microbeads under green channel. No fluorescence was observed. G) Genipin crosslinked alginate-chitosan microcarriers under brightfield channel. H) Genipin crosslinked alginate-chitosan microcarriers displaying fluorescence under green channel. All scale bars represent 500 μm .

Fig 3. (A) Fluorescence intensity and (B) coating layer thickness of microcarriers under various crosslinking conditions. * denotes the fluorescence intensity of microcarriers crosslinked at 37°C

for 24 hours was significantly lower compared to microcarriers crosslinked at 37°C for 48 hours (ALXL37) and microcarriers crosslinked at 60°C for 4 hours (ALXL60). Error bars represent standard deviation.

Fig 4. Microcarrier diameter within DMEM. Day 0 denotes the diameter prior to addition of DMEM. All beads display significant swelling when submerged in media from day 0 to day 1. (Green) Alginate microbeads. (Black) ALXL37 (Microcarriers crosslinked with genipin at 37°C). (Red) ALXL60 (Microcarriers crosslinked with genipin at 60°C). For all 3 groups, bead diameter on day 1 was significantly smaller compared to diameters measured at certain days between days 2-14. These are denoted by numbers with the corresponding colour above the day 1 plot. Microcarrier diameter remained stable from day 2-14 for all 3 groups. Error bars represent standard deviation.

Fig 5. MSC growth properties within microcarrier culture. A) Microcarrier culture 24 hours following cell seeding under brightfield channel. B) MSCs expressing green fluorescence within microcarrier culture under green channel. C) MSC attachment efficiency on ALXL60 (Microcarriers crosslinked with genipin at 60°C) and Cytodex 1. Error bars represent standard deviation. * denotes significance ($p < 0.05$) between the attachment efficiency of the two microcarriers. D) MSC detachment efficiency on ALXL60 and Cytodex 1. Error bars represent standard deviation. * denotes significance ($p < 0.05$) between the detachment efficiency of the two microcarriers. E) Cell proliferation on ALXL60 and Cytodex 1 over a 14 day period. Error bars show standard deviation. * denotes a significantly higher fold increase on ALXL60 compared to Cytodex 1 from Day 4 to Day 14. CY: Cytodex 1.

Fig 6. MSC culture of ALXL60 (Microcarriers crosslinked with genipin at 60°C). A) Cells growth on the microcarriers at day 7, 24 hours following addition of fresh microcarriers. The

existing microcarriers were confluent with MSCs (indicated by green arrows) while the fresh microcarriers added were empty or had few cells attached (indicated by red arrows). B) MSCs on ALXL60 on day 14, most microcarriers were confluent with cells following bead-to-bead transfer to the fresh microcarriers. Scale bar represents 500µm.

Fig 7. Relative gene expression compared to the 2D control (normalised to 1) of *CD-90*, *CD-105*, *CD-73*, *CD-34* and *CD-45* cell surface markers for cells harvested from ALXL60 (Microcarriers crosslinked with genipin at 60°C) and Cytodex 1 following a two week culture period. Error bars show standard deviation. There was no significant difference between the gene expressions of all 3 growth surfaces (ALXL60, Cytodex 1 and 2D) for the CD surface markers tested. CY: Cytodex 1.