Large-scale production of stem cells utilizing microcarriers: A biomaterials engineering perspective from academic research to commercialized products

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Abstract
Human stem cells, including pluripotent, embryonic and mesenchymal, stem cells play pivotal roles in cell-based therapies. Over the past decades, various methods for expansion and differentiation of stem cells have been developed to satisfy the burgeoning clinical demands. One of the most widely endorsed technologies for producing large cell quantities is using microcarriers (MCs) in bioreactor culture systems. In this review, we focus on microcarriers properties that can manipulate the expansion and fate of stem cells. Here, we provide an overview of commercially available MCs and focus on novel stimulus responsive MCs controlled by temperature, pH and field changes. Different features of MCs including composition, surface coating, morphology, geometry/size, surface functionalization, charge and mechanical properties, and their cellular effects are also highlighted. We then conclude with current challenges and outlook on this promising technology.

1. Introduction
Engineered tissues have great utility for both therapeutic and non-therapeutic applications. Therapeutic covers applications such as regenerative medicine and drug discovery studies. On the other hand, non-therapeutic applications include biochemical and biological sensors for contamination detection, manufactured meat [1] and engineered leather [2]. The key component in these products are billions of living cells, making it paramount to produce large cell quantities which is, unfortunately, challenging by standard laboratories methods.

Conventional monolayer culture of cells could lead to alteration of cell specific ECM secretion, loss of specific morphology and phenotype during passaging [3]. Scaled up manufacturing of the cells using microcarriers in suspension cultures is a promising tool to minimize the limitations of monolayer culture. Three-dimensional (3D) culture of cells allows cells to retain phenotypes and prevent dedifferentiation, during mechanically stimulation [4]. Capitalizing on this finding, various bioreactor systems have been developed to meet an increasing demand for biopharmaceutical product. Currently, the traditional stirred tank bioreactor is the top choice in industry. Some technologies such as airlift and filtration-based bioreactors (i.e., spin, hollow-fibre, acoustic and cross-flow filters) have been utilized infrequently in comparison to the widely used platform such as fixed and fluidized-bed systems (e.g., packed-bed and fluidized-bed bioreactors), disposable bioreactors (e.g., CellCube (Corning), Cell Factory (Nunclon) and the Wave bioreactor (WaveBiotech)), which are widely employed in
Mass production of biopharmaceutical products using microcarriers (MCs) was initially introduced in 1967 by van Wezel who used diethylaminoethyl (DEAE)-Sephadex™ A-50 as a microcarrier [6]. Since then MCs which provide high surface area to volume ratio have been used for large-scale production of anchorage dependent cells, recombinant protein and viral vaccine. For example, 1 g of MCs can potentially provide a surface area equal to fifteen 75 cm² culture flasks [7]. Apart from the great potential for producing large number of cells, MCs are also useful for tissue engineering and drug delivery applications [8,9].

This paper summarises recent advances in the production of MCs for large-scale stem cell manufacturing and tissue engineering applications. We focus on the material engineering viewpoint and discuss how material properties can affect the growth and later fate of stem cells. Finally, stimuli responsive MCs which are gaining popularity are discussed in greater details.

2. The need for large numbers of stem cells

Cell-based therapy and tissue regeneration approaches for human diseases demand a larger number of cells. Stem cells have emerged as promising therapeutic agents owing to their promising traits such as differentiation capacity for treatment of various diseases. Mesenchymal Stem Cells (MSCs) and Pluripotent Stem Cells (PSCs) are two distinct types of stem cells which have been successfully used in human clinical trials [10–17].

MSCs have therapeutic potential for treating heart [18], diabetes [19,20], gastrointestinal [21], liver [22], kidney [17], immune [23,24] and neurodegenerative diseases [25–28] as well as to regenerate bone [29–31] and cartilage [10,32,33]. On the other hand, PSCs including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) currently have limited utility in cell therapy trials due to risk of tumorigenicity [34]. hESCs have been tested for treatment of spinal cord injury [35–37], retinal pigment epithelial (RPE) transplantation [38–40], rare diseases [41] and treatment of stargardt’s macular dystrophy (SMD) [42]. hiPSCs are also used as models for studying diseases such as Parkinson’s [25], Alzheimer’s [43,44], juvenile onset [45], type 1 diabetes mellitus [46] and Duchenne type muscular dystrophy [47].

A review article by Monsarrat et al. [48] comprehensively reports the stem cell-based preclinical and clinical trials registered at ClinicalTrials.gov and diversity in the stem cell field. Fig. 1A shows the estimated cell dose needed per patient for diseases, indicating a requirement of tens of millions to billions of MSCs or PSCs per kg body weight of per patient [10,49,50]. To achieve this clinical quantity of stem cells, appropriate large-scale culture technology is necessary as two-dimensional culture cannot meet the required cell dose for clinical trials.

3. Scaled expansion techniques

There is a variety of methods for large-scale expansion of anchorage-dependent cells, each with its own merits. One common feature of all platforms is to provide sufficient surface area for cells to attach and proliferate. In addition, they should enable efficient gas and nutrients exchange. To determine the throughput of various culture systems and taking into consideration the monetary/labour investment, S-curve plots are proposed in a study by Simaria et al. [51]. These curves, as shown in Fig. 1B, indicate the optimal production ranges of different cell expansion systems (in terms of 10⁹ cells produced per batch) versus required research and development effort to move from a T-flask based process to a large-scale expansion process. The following sections briefly describe the widely used expansion techniques.

3.1. Multi-tray system

The commonly used T-flasks for cell culture provide a surface area of 25–225 cm². Multi-tray system also known as “cell stacks” or “cell factories” has multiple trays pile above one another and finally generate a skyscraper-like structure, which gives the cells several surfaces to attach. Cell factories can provide surface areas of up to 25,440 cm² much larger than a T-225 flask [49]. There are some worries that environment conditions could vary among units, causing heterogeneity in gas exchange that adversely affect the cell growth. The current system does not allow automatic monitoring or control system, leading to higher labour cost for manual inspection and imprecision from human errors [49].

![Fig. 1. (A) Schematic representation of required amount of cells for some clinical therapies via stem cells (either pluripotent or multipotent); data extracted from Refs. [10,49,50]. (B) Conceptual illustration of a technology S-curve showing the evolution of expansion technologies used in cell therapy manufacture. This curve demonstrates the performance of each technology in terms of billion cells achieved per lot (when using the maximum number of units per lot) against R&D effort/investment. The x-axis represents qualitatively the R&D effort required for a company currently using T-flasks to change to other cell expansion technologies. Cost values are calculated based on the direct costs (material, labor, QC testing) and indirect costs (equipment depreciation only) of the cell expansion process and assuming overheads are spread over 10 lots/year for all scenarios. Graph reproduced with permission from Ref. [51].](image-url)
3.2. Roller bottle

Roller bottles were introduced in the late 1930s and have been used in the biotechnology industry for production of vaccines and various anchorage-dependent cells. They can provide a surface area up to 350,000 cm², which is far superior to that of multi-tray systems. They can also provide mechanical agitation to avoid cell aggregation, a frequent problem in the traditional T-flask or multi-tray systems. This structure can also enable thinner media layer to provide better gas exchange. Nonetheless, it also lacks real-time monitoring and control [49].

3.3. Microcarriers

To overcome existing obstacles in aforementioned systems (i.e., surface area per unit volume, ease of scalability, process monitoring and control capability), Van Wezel described a system where small particles suspended in stirred culture can be used to culture cells inside them or on their surface. The earliest reported particles were positively charged DEAE-Sephadex beads where rabbit embryonic skin cells and human embryonic lung cells successfully proliferated [6]. Since then, microcarrier technology has been used in various large-scale pharmaceutical technologies such as vaccine development, cell therapy bioprocessing, etc. However, there are some differences in product of interest and downstream processing [49].

The most important feature of this technique is that the cells no longer have to attach on the surface of the devices as they can adhere and grow on microcarriers, which form suspension in the bioreactors. The MC technology provides a large surface to volume ratio, which means larger capacity. Apart from economic and flexibility benefits, it has automatic monitoring and control system which requires less human intervention, thus minimizing the risk of contamination [49]. Also, it should be mentioned that compared to microcarrier culture, T-flasks and roller bottles have the advantage of direct microscopic observation of cell conditions (e.g. cell morphology, viability, density, and undifferentiated states or differentiation processes). Moreover, the observation can be automated.

As mentioned earlier, cells in the order of billions to trillions are required for most of cell therapies. Stem cells isolated from donors are initially expanded in 2D culture flasks. Hereafter, the expansion from millions to hundreds of billions in quantity can be realized with microcarriers in bioreactors. Bioreactors play a crucial role in expanding cells in a quasi-physiologic manner, as cells reside and grow in 3D environment in-vitro. However, some major issues such as the exchange of nutrients/metabolites gradients, difficulties in recovery of cells after expansion and induced shear stress caused by moving culture media or rotating shaft must be overcome. The most widely used bioreactor systems are shown in Fig. 2.

For 3D cell culture experiments in laboratory scale, automated benchtop platforms have been developed. BioLevitator™ is a small 3D cell culture system that provides all the functions of a bioreactor placed into an incubator. Lin et al. [52] used this system to study expansion and differentiation of human adipose derived MSCs (hASCs) on two commercial microcarriers (i.e., magnetic microcarrier GEM and cytodex-3). Global Eukaryotic Microcarrier (GEM) is a paramagnetic microcarrier composed of an alginate core where small paramagnetic particles are dispersed in. It is also available with different coatings (gelatin, collagen, fibronectin, etc.). Its magnetic properties facilitate easy control during media exchange, harvesting and assay washes [53]. BioLevitator allows tuning the agitation speed and rotating periods in a programmable manner (Fig. 2G–H).

Numerous types of microcarriers with various properties (i.e., structural form (solid and porous), chemistry, coating, charge etc. are commercially available or are produced for any type of cell expansion or differentiation. In the next section, properties of different types of current commercial and synthetic microcarriers are discussed.

4. Microcarriers classifications

Over the past decades, a broad range of microcarriers with various physicochemical properties have been developed and commercialized. Their properties differ in terms of material composition, size, shape, morphology, surface coating/charge, functional groups, and stiffness (Fig. 3A) which have been shown to affect the final cell expansion/differentiation yield.

Microcarriers can be classified based on the material composition, the substance that cells grow on/in (solid or liquid), surface topography and geometry of carrier. One classification of microcarriers is based on the material (Fig. 3B–ii). Ceramics [56,57] and polymers (i.e., natural and synthetic) are commonly used for fabrication of microcarriers due to their excellent biocompatibility, mechanical properties and reproducibility. Synthetic polymer suffers from poor cell adhesion as compared to natural polymers which are also advantageous as they are inexpensive and can be easily obtained. Ventregel (Ventrex Laboratories, Portland, Maine, USA), Cytodex-3 (Pharmacia) and Cytopore are some examples of commercial microcarriers that composed of gelatin, collagen and cellulose, respectively [4].

Based on the substance that cells grow on it, microcarriers are divided into two categories, i.e., solid and liquid (Fig. 3B–ii). Examples of solid microcarriers are Cytodex-3 (dextran matrix coated with thin layer of denatured collagen) and Cytopore (the macroporous carrier). While the cultured cells on solid microcarriers could be potentially damaged by shear stress and carrier collisions during stirring, porous microcarriers provide internal spaces for cells attachment, thus protecting cells from stresses generated inside a bioreactor. However, one challenge of a porous system is poor system oxygen/nutrient transportation and accumulation of waste products inside the carrier. Collection and detachment of cells from microcarriers are usually performed via proteolytic enzyme
Microcarriers could be classified based on i) material, ii) the substance that cells grow either solid or liquid, iii) surface topography of carrier and iv) geometry. Then the microcarriers with tissue engineering capabilities, including different types and recent advances are discussed (D). Lastly, the final part covers the stimulus responsive MCs as various stimuli such as temperature, pH, light, chemicals and electric or magnetic field could be used to trigger a specific cell behaviour (E). Inset mages in the panel (D) are adapted from Ref. [62].

In the next section, microcarriers with tissue engineering capability will be highlighted. The final part covers the stimulus responsive MCs as various stimuli such as temperature, pH, light, chemicals and electric or magnetic field could be used to trigger a specific cell behaviour and also facilitating the cell detachment from the carrier.

4.1. Commercial microcarriers

Characteristics of various microcarriers available in the market are presented in Table 1. Commercial microcarriers are available in various shapes and properties. As mentioned earlier, a wide range of MCs are made in a spherical shape. Spherical MCs could be either solid or porous. Fig. 4 A–B displays Cytodex 1 and Cytopore as two examples of spherical MCs. 2D microhex is an example of hexagon shape carriers produced by Nunc™ (Fig. 4C). Fibra-cell® is a product of New Brunswick™ that has a disc shape (Fig. 4D). Lens-shape MCs, i.e. Cytoline 1, 2 (Fig. 4E–F) are produced via GE Healthcare. Lastly, DE-52 and DE-53 are available cylindrical MCs in the market, which are produced by Whatman™ (Fig. 4G–H).

From the perspective of materials used as the microcarriers core, polymers i.e., natural and synthetic polymers are most common. Synthetic polymers such as poly-hydroxyethyl-methacrylate, polystyrene, polyacrylamide, polyurethane and glucose have been reported by different studies. However, a lack of cell recognition sites with consequent low cell growth rates swayed researchers to use natural polymers such as collagen and other ECM protein or polysaccharides to coat microcarriers instead. Other materials in the laboratory scale have been used, but they are not yet introduced as commercial products. PLGA, Alginate and recently decellularized adipose tissue (DAT) and micronized acellular dermal matrix (MADM) [45] facilitated successful proliferation of cells. These materials have the benefit of removing cell-harvesting step by a proteolytic enzyme or mechanically scraping of cells, as the cells could be directly used via injection or as cell-seeded scaffold constructs for tissue engineering applications.

The surface of microcarrier plays a critical role in cell attachment and proliferation, as the cells usually attach to surface curvature sites (in solid microcarriers). Hence, functionalization of

treatment (trypsin) or mechanical scraping. Cell membrane integrity may be compromised by these methods. In this regard, another cultivation system based on liquid/liquid interface was developed. In a liquid microcarrier system, cells grow at the interface of culture media and a hydrophobic liquid. Pilarek et al. reported culture of three kind of mammalian cells, namely epithelial A431 cells, BHK-21 fibroblasts, and C2C12 myoblasts at the interface of cell culture media and hydrophobic perfluorodecalin (PFD) which are two immiscible liquid [58]. Recently, Hanga and colleagues [59] introduced Fluorinert FC40/DMEM system interface for culture of human mesenchymal stem cells (hMSCs). Presence of cells on the hydrophilic side of the perfluorocarbon (FC40)/growth medium interface has been demonstrated as a promising way for scalable enzyme-free harvesting method.

Microcarriers are also categorized based on their surface topography (Fig. 3B–iii) i.e. from smooth, microporous to macroporous, each with its benefits and challenges. Smooth microcarriers are ideal for cells not affected by shear. Microporous microcarriers (e.g., Cytodex) have small pores on the surface, which provides increased surface area for better cell anchorage. Cells could attach on the surface and/or within macroporous microcarriers (e.g., Cultispher and Cytopore) which have internal pores with a very large surface area [60].

In terms of geometry, microcarriers are available with spherical, cylindrical, lens and hexagon shapes (Fig. 3B–iv). Due to the variety of shapes of microcarriers and mechano-sensitivity of stem cells to mechanical forces and properties of cultured substrates, design and selection of microcarriers for proliferation and differentiation could be different, but the most common shape is spherical particles with diameter size of 100–300 μm [10]. MSCs which are large cells that grow as a monolayer require about 150 μm spheres for good spreading, whereas hPSC which grow as aggregates prefer spheres of less than 100 μm to self-aggregate as clusters of cells and microcarriers [10, 61].

In this paper, we review the microcarriers in three main subjects. First, we introduce the microcarriers that have been commercialized and widely used for laboratory and pre-clinical purposes. Different properties of each product will be discussed.
accurate structural analysis of microcarriers is essential to predict properties of commercially available microcarriers used for the large-scale manufacturing of SCs.

### Properties of commercially available microcarriers

#### Solid Microcarriers

<table>
<thead>
<tr>
<th>Microcarrier</th>
<th>Manufacturer</th>
<th>Matrix/Coating</th>
<th>Shape &amp; Dimension (μm)</th>
<th>Charge, Surface area (cm²/g), Density (g/ml)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytodex 1</td>
<td>GE Healthcare/Amersham Biosciences</td>
<td>Crosslinked Dextran/DEAE (Tertiary amine)</td>
<td>Spherical/D 190 ± 58</td>
<td>+, 4400, 1.03</td>
<td>[63]</td>
</tr>
<tr>
<td>Cytodex 2</td>
<td>Amersham Biosciences</td>
<td>Dextran matrix with N,N,N-trimethyl-2-hydroxyaminopropyl groups</td>
<td>Spherical/D 135-200</td>
<td>NA, 1.04</td>
<td>[4, 5]</td>
</tr>
<tr>
<td>Cytodex 3</td>
<td>GE Healthcare</td>
<td>Crosslinked Dextran/denatured porcine-skin collagen</td>
<td>Spherical/D 175 ± 36</td>
<td>No charge, 2700, 1.04</td>
<td>[10, 63]</td>
</tr>
<tr>
<td>Hillex®</td>
<td>Solohill</td>
<td>Dextran/Cationic trimethyl ammonium</td>
<td>Spherical/D 160-180</td>
<td>+, NA, 1.11</td>
<td>[4, 70]</td>
</tr>
<tr>
<td>Biosilon Nunc</td>
<td>Polystyrene/None</td>
<td>Spherical/D 160-300</td>
<td>No charge, 255, 1.07</td>
<td>[63]</td>
<td></td>
</tr>
<tr>
<td>Collagen Solohill</td>
<td>Polystyrene/Type I porcine collagen (gelatin)</td>
<td>Spherical/D 125-212</td>
<td>No charge, 480, 1.02</td>
<td>[63, 71]</td>
<td></td>
</tr>
<tr>
<td>Glass Solohill</td>
<td>Cross-linked polystyrene/High silica glass</td>
<td>Spherical/D 125-212</td>
<td>No charge, 360, 1.02</td>
<td>[63]</td>
<td></td>
</tr>
<tr>
<td>Plastic Solohill</td>
<td>Polystyrene/None</td>
<td>Spherical/D 125-212</td>
<td>No charge, 480, 1.02</td>
<td>[63]</td>
<td></td>
</tr>
<tr>
<td>Plastic Plus Solohill</td>
<td>Polystyrene/None</td>
<td>Spherical/D 125-212</td>
<td>No charge, 360, 1.02</td>
<td>[63]</td>
<td></td>
</tr>
<tr>
<td>SyntheMAX® II</td>
<td>Corning</td>
<td>Polystyrene conjugated with peptide/carboxylic acylate conjugated with vitronectin peptide</td>
<td>Spherical/D 125-212</td>
<td>No charge, 360, 1.02</td>
<td>[63]</td>
</tr>
<tr>
<td>FACT III (FACT 102-L)</td>
<td>Solohill</td>
<td>Polystyrene/Type I porcine collagen (gelatin)</td>
<td>Spherical/D 169 ± 44</td>
<td>+, 480, 1.02</td>
<td>[63, 71]</td>
</tr>
<tr>
<td>CGEN 102-L</td>
<td>Thermo Scientific</td>
<td>Polystyrene/Type I porcine collagen</td>
<td>Spherical/D 169 ± 44</td>
<td>1.02</td>
<td>[10]</td>
</tr>
<tr>
<td>Hillex® CT NuncTM</td>
<td>Polystyrene/Cationic trimethyl ammonium</td>
<td>Spherical/D 160-180</td>
<td>+, Na, 1.12</td>
<td>[4, 62]</td>
<td></td>
</tr>
<tr>
<td>P Plus 102-L</td>
<td>Thermo Scientific</td>
<td>Polystyrene/None</td>
<td>Spherical/D 169 ± 44</td>
<td>+, Na, 1.02</td>
<td>[10]</td>
</tr>
<tr>
<td>ProNectin® F (Pro-Solohill (Thermo Scientific)</td>
<td>Polystyrene/Recombinant fibronectin</td>
<td>Spherical/D 169 ± 44</td>
<td>No charge, 480, 1.02</td>
<td>[72]</td>
<td></td>
</tr>
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<td>Hillex® CT NuncTM</td>
<td>Polystyrene/Triethylamine</td>
<td>Spherical/D 170 ± 10</td>
<td>+, NA, 1.12</td>
<td>[4, 63]</td>
<td></td>
</tr>
<tr>
<td>2D MicroMatrix</td>
<td>Polystyrene/Tissue culture treated</td>
<td>Hexagon/L 125 × W 25</td>
<td>Unspecified, 360, 1.05</td>
<td>[73]</td>
<td></td>
</tr>
<tr>
<td>SphereCol® Advanced BioMatrix</td>
<td>Polystyrene/Type I human collagen (VitroCol®)</td>
<td>Spherical/D 125–212</td>
<td>No charge, 1.03</td>
<td>[72]</td>
<td></td>
</tr>
<tr>
<td>Tosoh 65 PR</td>
<td>Tosoh Bioscience</td>
<td>Hydroxylated methylacrylate/Promtamine sulfate (cationic peptide)</td>
<td>Spherical/D 65 ± 25</td>
<td>NA, 4200, 1.05</td>
<td>[63]</td>
</tr>
<tr>
<td>Tosoh 10 PR</td>
<td>Tosoh Bioscience</td>
<td>Hydroxylated methylacrylate/Promtamine sulfate (cationic peptide)</td>
<td>Spherical/D 10</td>
<td>NA, 9000, 1.04</td>
<td>[63]</td>
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</table>

#### Macro Porous Microcarriers

<table>
<thead>
<tr>
<th>Microcarrier</th>
<th>Manufacturer</th>
<th>Matrix/Coating</th>
<th>Shape &amp; Dimension (μm)</th>
<th>Pore size (μm)</th>
<th>Charge, Surface area (cm²/g), Density (g/ml)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE-52</td>
<td>Whatman®</td>
<td>Cellulose/DEAE (Tertiary amine)</td>
<td>Cylindrical/L 130 ± 60 × D 35 ± 7</td>
<td>+, 6800, 0.9</td>
<td>[63]</td>
<td></td>
</tr>
<tr>
<td>DE-53</td>
<td>Whatman®</td>
<td>Cellulose/DEAE (Tertiary amine)</td>
<td>Cylindrical/L 130 ± 60 × D 35 ± 7</td>
<td>+, 6800, 1.1</td>
<td>[63]</td>
<td></td>
</tr>
<tr>
<td>QA-52</td>
<td>Whatman®</td>
<td>Cellulose/Quaternary ammonium</td>
<td>Cylindrical/L 130 ± 60 × D 35 ± 7</td>
<td>+, 6800, 1.2</td>
<td>[61]</td>
<td></td>
</tr>
<tr>
<td>CM52</td>
<td>Whatman®</td>
<td>Cellulose/Carboxymethyl</td>
<td>Cylindrical/L 130 ± 60 × D 35 ± 7</td>
<td>+, 6800, NA</td>
<td>[61]</td>
<td></td>
</tr>
<tr>
<td>RapidCell</td>
<td>MP Biomedical</td>
<td>Glass/None</td>
<td>Spherical/L 150-210</td>
<td>No charge, 325, 1.04</td>
<td>[63]</td>
<td></td>
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<tr>
<td>G2767</td>
<td>Sigma Aldrich</td>
<td>Glass/Uncoated</td>
<td>Spherical/D 180 ± 30</td>
<td>NA, NA, 1.03</td>
<td>[10]</td>
<td></td>
</tr>
<tr>
<td>G2517</td>
<td>Sigma Aldrich</td>
<td>Glass/Uncoated</td>
<td>Spherical/D 120 ± 30</td>
<td>NA, NA, 1.03</td>
<td>[10]</td>
<td></td>
</tr>
<tr>
<td>G2892</td>
<td>Sigma Aldrich</td>
<td>Glass/Uncoated</td>
<td>Spherical/D 120 ± 30</td>
<td>NA, NA, 1.04</td>
<td>[10]</td>
<td></td>
</tr>
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#### Weighted

<table>
<thead>
<tr>
<th>Microcarrier</th>
<th>Manufacturer</th>
<th>Matrix/Coating</th>
<th>Shape &amp; Dimension (μm)</th>
<th>Pore size (μm)</th>
<th>Charge, Surface area (cm²/g), Density (g/ml)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoline 1, 2</td>
<td>GE Healthcare/Amersham Biosciences</td>
<td>Polyelethylene and silica/None</td>
<td>Lens-shape/L 2.1 ± 0.4 mm, W 0.75 ± 0.35 mm</td>
<td>10–400</td>
<td>–, NA, 1.32</td>
<td>[10, 72]</td>
</tr>
</tbody>
</table>

Surface with ECM proteins (laminin, vitronectin, fibronectin etc.), applying RGD motifs or growth factors and positive charging the surface (usually slight positive charge) is used to promote the attachment and proliferation of cells.

While material composition of some microcarriers are similar, accurate structural analysis of microcarriers is essential to predict the proliferation and fate of the attached stem cells. For example, dextran, gelatin and cellulose are among the most prevalent materials used for commercially available microcarriers. CultiSpher-S and Cytodex-3 are two gelatin-based microcarriers (Fig. 4). The surface characterization of CultiSpher-S shows a homogeneous distribution of gelatin, while the surface of Cytodex-3 surface is the proliferation and fate of the attached stem cells.
composed of a heterogeneous pattern of gelatin i.e. a mixture of polysaccharides and gelatin. This difference in the gelatin distribution pattern greatly change the proliferation, shape and lineage-specific differentiation of MSCs and PSCs [63]. In terms of modulus and stiffness, CultiSpher® possesses a higher stiffness compared to Cytodex-3 and the homogeneous gel of the same material. X-ray photon-electron spectroscopy (XPS) analysis and atomic compositions of these two microcarriers are compared in Fig. 4 J-i. The ratio of atomic composition and functional groups for N and C=O groups (gelatin) is about 1.70 (ratio of CultiSpher® to Cytodex-3), also this ratio for C=O groups (dextran) is about 0.20. This ratio is 1.00 for equal amount of analysed groups (Fig. 4 J-ii). Accordingly, MSCs on CultiSpher® displayed high degree of spreading and a well-organized actin distribution while a lower degree of spreading was observed on Cytodex-3 carrier (Fig. 4 J-iii,iv) [64]. Expansion and fate decision of stem cells (PSCs and MSCs) on these two microcarriers were also influenced by different surface chemistry, gelatin distribution, and stiffness differences. The macroporous structure of CultiSpher®. CultiSpher® and Cytodex-3 also encouraged adhesion and proliferation of mouse ESCs [65], and promoted osteogenic differentiation of MSCs [66].

Stem cell shape and organization can be effectively modulated by microcarrier materials properties. For instance, it has been shown that −NH2 groups promote MSC proliferation, spreading and osteogenic commitment, while lowered MSC spreading and enhanced chondrogenesis has been observed on the surfaces with increased concentration of −COOH groups [67]. Stem cells sense the matrix stiffness and respond differently with their shapes and fates. Rigid surfaces (stiffness of 34 kPa) promoted spindle-like shape and osteogenic differentiation of MSCs, while softer substrates (i.e., 1 kPa) encouraged chondrogenic, adipogenic or neuronal differentiation. Intermediate stiffness promoted muscular lineage [68]. Another physical cue is surface topography of microcarriers. Schmidt et al. [69] showed that microscale curvature of microcarriers could promote intracellular tension and osteogenic differentiation of MSCs.

It is known that cell shape dependent changes regulate stem cell signalling and fate decisions [74–76]. Substrates with high modulus of stiffness have been found to enhance the secretion of proangiogenic factors (such as vascular endothelial growth factor (VEGF) and interleukin-8) which led to activation of RhoA/ROCK signalling [63]. RhoA/ROCK activation stimulated osteogenesis but inhibited adipogenesis; therefore, manipulating microcarrier surface with controlled physical, mechanical and chemical properties could potentially modulate cell shape, and consequently, regulate signalling pathways. This will affect proliferation and differentiation of stem cells [77,78].

In the last decade, several studies compared different commercial microcarriers for large-scale cell expansion. In a study by Chen et al. [61], expansion, growth and pluripotency of human embryonic stem cells (hESC) on 10 different microcarriers (i.e., DE53, DE52, QA52, CM52, Cytodex 1, 3, CultiSpher® G and Cytopore 2, Tosoh 65 PR and Tosoh 10 PR) with 7 types of ECM coating (Matrigel, hyaluronic acid, heparin, vitronectin, fibronectin and laminin) were investigated. The highest cell number was achieved by positively charged, cylindrical and spherical microcarriers and it was found that Matrigel or laminin coatings was essential for long-term stable cultivation of hESC (Fig. 4 i).

Schop et al. also evaluated nine different microcarriers (i.e., Cytodex-1 and -3, ProNectin®, Hillex®, Glass, Plastic, Plastic plus,
FACT III, and Collagen microcarriers) for adhesion efficiency of BM-MSCs to selected microcarriers. Cytodex-1 showed the highest adhesion efficiency [70]. Recently, evaluation of 17 types of microcarriers (Collagen, Cultispher®-G, Cytodex-1 and 3, FACT III, SphereCol®, ProNectin®F, Cytopore 1 and 2, Enhanced Attachment, Glass, Hillex®, MicroHex, Plastic, Plastic Plus, PVA and SyntheMAX II®) for culture of hBM-MSCs was performed [72]. The criteria considered in this study were the proliferation rate on microcarriers, amenability for processing using Xeno-Free conditions and maintaining multipotency of MSCs after detaching the cells from microcarriers. Eventually, SoloHill plastic microcarriers were determined as the optimal choice.

In another study [101], the effects of a star-shaped PLLA’s molecular structure and functional groups on controlling the structure at micro and nano scale was studied. By varying arm numbers and arm lengths of star-shaped PLLA, microspheres which are either smooth or fibrous at the nanoscale, and either nonhollow, hollow, or spongy at the microscale, could be produced (Fig. 5-D). Different routes for implementation of drugs and cells delivery capability into microcarrier are reviewed in two recently published review articles [89,102].

Shekaran and colleagues [97] developed biodegradable polycaprolactone microcarriers coated with ECM proteins (namely poly-I-lysine (PLL) or fibronectin (FN) or multiple layers consisting of both). In vitro expansion and in vivo bone formation in a mouse subcutaneous ectopic model, confirmed the applicability of this microcarriers (Fig. 6A–D). Hydroxyapatite and hybrid gelatin/hydroxyapatite-microcarriers [98] and poly-ε-caprolactone (PCL) microspheres with tunable porous structures and hollow inner core were also successfully developed for tissue engineering purposes (Fig. 6E–F) [99].

The design of tissue specific microcarriers is useful for regenerating different tissues including bone, cartilage, skin, central nervous system, and liver [5]. For instance, Turner et al. [103] developed a method to fabricate microcarriers from decellularized adipose tissue (DAT) that mimic the composition of the native ECM for tissue-specific applications. Using this microcarrier, the culture of hASC was successfully scaled-up in a spinner flask system and tests results confirmed its clinical potential as an injectable cell delivery vehicle (Fig. 6G(I–V)).

In recent years, considerable interest has grown in the development of microcarriers without animal-derived components. Fan and co-workers [105] reported production of xeno-free microcarriers in which polystyrene beads were coated with recombinant human vitronectin and human serum albumin where UV irradiation was applied during preparation. Results showed successful growth of hPSCs up to more than 20-fold and maintenance of their pluripotency.

More recently, an injectable macroporous microcarrier based on a collagen I-based recombinant peptide (CellInnetstm) was developed using a double emulsification technique and different cross-linking methods [104]. The injectable macroporous microcarrier with 20 µm pore interconnection diameter supported higher cell ingrowth and proliferation into the MC compared to a commercially available equivalent (CultiSpher) and the osteogenic lineage of hBMSCs was preserved when cultured in differentiation medium.

5. Stimulus responsive microcarriers

There is also a rising interest in employing stimulus responsive materials with properties that can be controlled by environmental changes in scale up systems and cell therapy applications. The physical and chemical characteristics of biomaterial used for preparation of microcarriers can be altered by external stimuli, such as electric filed, light irradiation, temperature changes, pH shifts, shear stress forces, and the addition of small biochemical molecules [106]. In the tissue engineering, the greatest benefit of stimulus responsive carriers is their potential in designing the next generation of delivery systems.

During the cell harvesting, proteolytic enzyme treatments are widely used for detaching cells from microcarriers. Employing proteolytic enzymes for detachment can damage cell membrane proteins, decrease cell viability, and consequently decrease the grafting efficiency in transplantation. Furthermore, proteolytic enzymes are animal derived and harbour associated risks with disease transmission and cell phenotype alteration [108]. To
overcome these difficulties, stimuli responsive materials sensitive to environmental changes have been recently explored [109].

5.1. Temperature responsive microcarriers

Temperature sensitive materials (mostly polymers) undergo phase transition around the lower critical solution temperature (LCST). Some thermoresponsive polymers become insoluble in temperatures above the LCST when they are soluble in temperatures below LCST and other thermoresponsive systems become soluble in temperatures above UCST, i.e., upper critical solution temperature. In biomedical application of thermoresponsive polymers, aqueous solvent systems can optimize the hydration state in the phase transition step [107,110].

Among thermoresponsive polymers, poly (N-isopropyl acrylamide) (pNIPAAm) shows soluble/insoluble behaviour in aqueous media below and above LCST of 32 °C [109]. Sharp phase transition of pNIPAAm of about 32 °C in aqueous media and the hydrophilic to

![Fig. 5. A) Three typical strategies for cell delivery and tissue regeneration. i) cell seeded 3D porous scaffolds, ii) cell-mixed hydrogel and iii) Cell-laden microcarrier. B) Three distinct types of microcarrier that are used for controlled encapsulation and release. Image is adopted from Ref. [88]. C) Schematic of linear and star shaped PLLA nanofibrous microspheres and nanofibrous hollow microspheres. i: SEM image of a nanofibrous microsphere, showing the nanofibrous architecture on the microsphere surface. ii: SEM image of a nanofibrous hollow microsphere, showing the nanofibrous architecture and a hole of approximately 20 μm on the microsphere shell. iii: A 2D cross-section confocal image of the nanofibrous hollow microspheres. iv: A high-magnification image of the microsphere in ii, showing the nanofibers, which have an average diameter of about 160 nm. Images are reproduced from Ref. [91]. D) Dissipative particle dynamics simulations and SEM images of 16-arm star shaped PLLA. By varying arm numbers and arm lengths the structures undergo a transition from: (top row) nonhollow; to (middle row) hollow; to (bottom row) spongy. Images in panels A and D are reproduced from Ref. [89].](image-url)
hydrophobic alteration of polymer above 32 °C can be exploited for cell attachment and detachment. Above 32 °C cell attachment on pNIPAAm occurs while cell detachment happens when temperature drops below 32 °C in hydrophilic state of pNIPAAm, eliminating the need for enzymatic treatments [108].

In 2012, thermally sensitive pNIPAAm hydrogel microcarriers conjugated with a cell adhesive motif, GRGDY, were prepared and utilized as cell culture substrate for chondrocytes culture and detachment [112]. This was one of the early works reporting the utility of thermo-responsive polymers for surface coating of microcarriers demonstrating a reversible swelling and deswelling behaviour around the LCST, which enabled the enzyme-free chondrocytes attachment and detachment. Later in 2010, Yang et al. reported a novel thermo-responsive polymeric nanofilm for controlling cell adhesion and growth by synthesizing a copolymer, poly[(N-isopropylacrylamide-co-hydroxypropyl methacrylate-co-3-(trimethoxysilyl)propyl methacrylate) (abbreviated pNIPAAm copolymer) using free radical polymerization [113]. The group demonstrated the effective control of adhesion, growth, and detachment of HeLa and HEK293 cells using their copolymer nanofilm. A separate study using similar nanofilm testified that the best range of contact angle for cell culture is between 40° and 70° [115]. Similar group also reported successful coating of Cytodex-3 microcarriers with pNIPAAm, allowing human bone marrow-derived mesenchymal stem cells (hBM-MSCs) to adhere, spread, and grow successfully (>82%) on the microcarriers inside a spinner flask. They have reported a significant reduction of the apoptosis and cell death using their proposed approach compare to the trypsin treated groups [116].

The same strategy has been applied on other type of microcarriers such as dextran, glass, alginate, poly (styrene) and hydroxyethyl methacrylate (HEMA) beads by optimizing the grafting density of pNIPAAm or combining with other materials (e.g., positively charged quaternary amine monomer, 3-aminopropyl triethoxysilane (APTES), and hydrophobic monomer N-tert-butyl acrylamide (tBAAm)) for culture and expansion of other cell types such as Chinese hamster ovary (CHO-K1), mouse fibroblasts (L929) and epithelial human keratinocytes (HS2) cells [53,111,115,117,118]. Using these new strategies, various studies have reported higher cell detachment efficiency, proliferation and productivity (Fig. 7A) [114,119].

Thermo-sensitive pNIPAAm-coated microcarriers may also be produced from water-in-oil (W/O) single emulsions at a temperature below the LCST of pNIPAM as shown by Cheng et al. in 2007 [120]. They reported production of microcapsules with hollow structures and thin membranes, high monodispersity, excellent reversible thermo-sensitivity and fast response to environmental temperature suitable for cell or drug encapsulation. Besides cell adhesion on the microcarriers surface, cell (or bioactive materials) encapsulation in the inner compartment of the particles may also be considered using combination of pNIPAM and gelable materials. The encapsulation of pancreatic cells into a pNIPAM-based microcapsules has been reported by Lu and colleagues using a mixture of pNIPAAm-PEG-pNIPAAm tri-block copolymer [83]. MTT assays showed undetectable cytotoxicity of the polymers towards pancreatic cells, indicating the potential use of these thermo-responsive polymers as cell encapsulation membranes.
polymers with different features have been widely investigated to satisfy the growing clinical demands. Although numerous types of microcarriers with different features have been used in recent years, the development of new controlled polymerization methods such as atom transfer radical polymerization (ATRP), and reversible addition fragmentation chain transfer (RAFT) polymerization [127].

A large body of works employed photo-sensitive materials (photo-responsive or photodegradable) to coat substrates such as glass for cell culture applications. The most extensively studied photo-responsive polymers in the literature are made from azobenzenes, spiropyrans and spirooxazines which are light responsive moieties [127]. For example, Barille and colleagues developed a photo-controlled scaffold comprising an azobenzene based methacrylic copolymer for facile light-induced patterning and guidance of neuron cells [128]. In another work, Hong et al. [129] cultured mouse calvaria derived pre-osteoblastic (MC3T3-E1) cells on TiO2 nano dot-coated quartz substrate. By applying UV 365, more than 90% of the cells detached with high cell viability. In another work, Griffin and co-workers synthesized a series of PEG-hydrogels to encapsulate and release human MSCs, upon UV irradiation, without compromising the cell viability [130]. Furthermore, selective cell detachment based on plasmatic substrate by embedding gold nano particles on the surface of culture support can be obtained through laser radiation [131]. Similar to the light/photo-responsive polymers, electricity-responsive polymers [132] have been also utilized for cell harvesting applications. With the electricity-induced method, cells are normally cultured on a thin film of thiol layer, which is an electricity-responsive polymer, and then get detached upon applying an electrical field. Despite great advances in development and usage of light/photo and electric-responsive polymers, we couldn’t find any work in the literature reporting their utility for production of smart microcarriers.

In the magnetism-induced method for culture of cells [133], a large magnet is typically placed under the culture flask to immobilize magnetic nanoparticles on which to form a thin film layer. Then, the cells are cultured on this layer and detached upon removal of the magnet. A commercially available stimulus responsive microcarrier is the Global Eukaryotic Microcarrier (GEM) which is shown in Fig. 7B. GEM is designed to support large-scale cell culture in BioLevator as described in the earlier sections. GEM is a magnetic microcarrier with alginate core and protein coating on the surface. During medium exchange and culture harvesting, the presence of magnetic core in GEM microcarrier allows better control over samples for dispersion by magnetic forces [135].

6. Concluding remarks and future outlook

Great hopes and expectations are linked to stem cells as a tool for cellular therapy and tissue engineering. Although several products have made it to commercial stage, most of the research is still performed in small scales using conventional systems such T-flasks. Over the past decades, large production of stem cells (i.e., mainly anchor-dependent cells) using microcarriers in bioreactor systems has become one of the most popular technologies to satisfy the growing clinical demands. Although numerous types of microcarriers with different features have been used, there is still challenges, which represent opportunities for future application. Manipulating microcarrier features including composition, coating, morphology, geometry/size, surface functionalization, charge and stiffness with controlled physical, mechanical and chemical properties could potentially affect proliferation and differentiation of stem cells and regulate signalling pathways. Therefore, based on the desired application, an ‘ideal’ microcarrier could be designed.

To control stem cells differentiation fate, specific features could be manipulated to design fate dependent carriers. For example, by altering surface functional groups, e.g. adding –NH2 groups MSC proliferation, spreading and osteogenic commitment will promote...
...tion is required in order to develop application-specific biomechanical properties of microcarriers, and also standardization of applications, the number of works reporting the utility of them has been emerged. Although they have been employed for a wide range of polymers ranging from linear and graft polymers to polymer gels and even hybrid organic-inorganic composites have been emerged. While numerous works have been devoted toward development of new generation, of polymeric microcarriers in the lab environment, i.e., a bioreactor environment. The biocompatibility and potential biodegradability of these materials have not been adequately addressed and warrant more research. More importantly, the physicochemical (and functionality) changes that can be induced to the cells in parallel by the applied stimulus, such as pH and temperature changes must be thoroughly investigated.

In conclusion, microcarrier technology holds a great promise for cell therapies and regenerative medicine. To achieve success, more focus research is required to develop new generation of low-cost thermoresponsive microcarriers based on pNIPAAm for the purpose of cell harvest development have been conducted but a more refined method of polymerization is required. One major issue is the cost of these materials which must be reduced drastically for mass manufacturing of polymeric microcarriers. In addition, more effort is required to master the properties-function relationship and use these materials in stem cell research and within the bioreactor environment. The biocompatibility and potential biodegradability of these materials have not been adequately addressed and warrant more research. More importantly, the physicochemical (and functionality) changes that can be induced to the cells in parallel by the applied stimulus, such as pH and temperature changes must be thoroughly investigated.

While numerous works have been devoted toward development of new generation, of polymeric microcarriers in the lab environment (mainly T-flasks), we recognize that limited studies examined their microcarriers in an actual working environment, i.e., a bioreactor. Cultivation systems such as T-flasks (and spinner flasks) are widely used in stem cell research providing viable and simple methods for cultivation. However, they are limited in terms of control (pH, oxygen tension, metabolic activity, etc.) and scalability. One important issue which has been overlooked is the presence of particulates in the final product. It has been reported that breakage of microcarriers during the production cycle can be one of source of particulates, representing a quality challenge and may pose safety concerns. As such, more detailed studies are required to fully characterise polymeric microcarriers in terms of particulate generation in order to meet the expectations of quality, safety, efficacy and commercial viability.

In conclusion, microcarrier technology holds a great promise for cell therapies and regenerative medicine. To achieve success, more focus research is required to develop new generation of low-cost...
microcarriers (i.e., smart microcarriers) with perfectly tuned structural properties to best support sensitive stem cells within the dynamic environment of a bioreactor for expansion and differentiation of cells for therapy.

Conflicts of interest
There are no conflicts of interest to declare.

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