

Review Article

A Review on the Development of Microcarriers for Cell Culture Applications

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ABSTRACT

Microcarrier-based cell culture systems have gained significant attention and popularity in tissue engineering and regenerative medicine. In this culture system, tissue cells are grown as a monolayer on the surface of small solid particles called microcarriers (100 to 300 μm), kept suspended in the culture medium by stirring. This technology has paved the way for creating engineered tissues, one of the cutting-edge topics in tissue engineering and regenerative medicine. Microcarrier-based approaches have been proposed for three-dimensional (3D) cell culture in which cellular morphology and functions are maintained *in vivo*. This paper provides an overview of the optimal characteristics such as microcarriers' size, shape, density and porosity. Various methods of preparation of microcarriers and surface modification techniques have been elaborated. Recent advances and applications of microcarriers in biotechnology fields, like the production of viral vaccines and recombinant proteins, culture and expansion of stem cells (SC), are described.

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INTRODUCTION

Cell culture technology has recently played a vital role in producing biological products such as vaccines, hormones, antibodies,

interferons, and clotting factors. Wezel first put forward the concept of a microcarrier-based culture system (1967). He proposed to culture cells as monolayers on the surface of small beads called microcarriers to support the attachment of adherent cells in bioreactors. This technology was believed to be used to produce inactivated poliomyelitis vaccines in 1972. Pharmacia Biotech AB (Sweden) collaborated with Wezel and developed Cytodex microcarriers, which are now commercially available (Badenes et al., 2016). The first microcarriers used were diethylaminoethyl (DEAE)-Sephadex A-50 resin beads (cross-linked dextran polymers), commonly used for column packing in ion-exchange chromatography (Badenes et al., 2016). Microcarriers based on biopolymers are preferred because of their superior biocompatibility and biodegradability. They can be made from natural or synthetic polymers. Alginates, chitosan, cellulose, and collagen are examples of natural polymers. Synthetic polymers include polycaprolactone (PCL), polyethylene glycol (PEG), polyglycolic acid (PGA), and polylactic acid (PLA). Several types of microcarriers are available commercially, like Cytodex (1, 2 and 3), Cytopore (1 and 2), CultiSpher (G, S and GL), Hillex II-170, ProNectin F, FACT III, and CGEN 102-L factors (Chen et al., 2020; Zhou et al., 2019).

Earlier cells were cultured on static surfaces such as T-flasks, multi-tray systems, and roller bottles. However, these conventional methods have a smaller surface area for cell culture, thus decreasing productivity (Merten, 2015). Microcarriers, on the other hand, can provide a surface area to volume ratio that is up to 10 times higher (Cytodex can offer a ratio of $30 \text{ cm}^2/\text{cm}^3$ in 1 mL medium) than T-flasks (ratio of $3 \text{ cm}^2/\text{cm}^3$ in 1 mL medium) (Clapp et al., 2018). Another limitation of the static culture system is its inefficiency in establishing multidirectional cellular interactions as they occur in the microenvironment *in vivo*. It may alter the morphology of cells and the expression of genes (Azahar et al., 2023; Tavassoli et al., 2018). Microcarriers have been proposed as a strategic alternative as they can facilitate cell attachment, have higher cell yields, and provide a more efficient environment for transporting gases and nutrients (Silva et al., 2015). These benefits have led to extensive research into the microcarrier for the 3D cell culture in cell therapy applications, tissue repair and regeneration.

This paper aims to discuss the different properties of microcarriers, such as size and shape, concentration, density, porosity, elasticity, and polymers, that are suitable for their preparation. Efforts to improve microcarrier surface properties are also discussed, and various techniques used for surface modification and biomolecule immobilization are discussed. An overview of the methods used for preparing microcarriers, namely emulsion-solvent evaporation and suspension polymerization, is given. In addition, applications of microcarriers in cell culture technology for producing vaccines, recombinant proteins, and stem cells are elaborated.

PROPERTIES OF MICROCARRIERS

Size and Shape

Microcarriers should have a uniform size distribution and be within the 100 to 300 μm diameter range, enabling them to remain suspended during stirring (Clapp et al., 2018). Uniform size distribution enables even distribution of cells and ensures a homogeneous culture (Chen et al., 2013; Merten, 2015). The proliferation of cells on microcarriers is affected by their size. Small-sized microcarriers may aggregate, leading to decreased cell viability due to insufficient nutrition and growth factors. At the same time, large-sized microcarriers provide a larger surface area to volume ratio on which large volumes of cells can be cultured (Clapp et al., 2018).

The size and shape of microcarriers are known to affect cell behavior. Hence, it is important to investigate their impact on cell expansion and proliferation (Clainche et al., 2021). The existing microparticles have different shapes, such as spherical and cylindrical shapes. Spherical microspheres are particularly interesting due to their ease of fabrication, injectability and large proportion among commercial microcarriers. Small-size microspheres can be injected directly into the targeted tissue defect or tumor sites with minimally invasive surgical procedures (Chen et al., 2013). The high sphericity of these microspheres facilitates improved and direct delivery of cells to the target site and lowers inflammatory responses associated with foreign body implantations (Chen et al., 2020; Hossain et al., 2015). Besides, spherical microcarriers can generate higher cell yield by producing open aggregates with thinner cell layers compared to cylindrical microcarriers with compact aggregates (Ornelas-González et al., 2021). They have been applied in biomedical applications such as controlled-release vehicles for vaccines, drug encapsulation, and hormone and therapeutic agent carriers.

Concentration of Microcarriers

The concentration of microcarriers affects the hydrodynamic environment of the culture. Theoretically, cell concentration should be increased with the microcarrier concentration due to the greater surface area available for cell adhesion. However, studies showed that increasing microcarrier concentration might be deleterious as higher microcarrier concentration may cause higher collision frequency (Maillot et al., 2022; Tsai et al., 2020). It has been proved by Luo et al. (2021) and Croughan et al. (1998) that a higher concentration of Cytodex-1 microcarrier resulted in lower cell growth rate and lower cell expansion fold during culture of Chinese Perch Brain cell (CPB) and FS-4 fibroblast cells. Croughan et al. (1998) discovered that the impact of microcarrier concentration varied according to the degree of agitation. In a laboratory-scale vessel, microcarrier concentration had no adverse effect during mild agitation. However, interactions between microcarriers and eddies at

high degrees of agitation caused hydrodynamic damage. The interaction increases the collision frequency, resulting in cell damage. A higher agitation rate will also decelerate cell attachment on the microcarrier due to loss in contact between cells and microcarriers (Luo et al., 2021). Besides, higher microcarrier concentrations may be accompanied by cytotoxicity of the culture system as increased cellular metabolites are produced, which leads to faster consumption of the culture medium and can be detrimental to cell viability. Due to high cell density, a more frequent medium change is required to overcome nutrient limitations and metabolite accumulation (Tsai et al., 2020). Thus, various factors such as agitation speed, availability of culture media, labor cost and time consumption need to be considered to obtain the optimal microcarrier concentration for maximum cell concentration (Luo et al., 2021).

Density of Microcarriers

Researchers have comprehensively investigated the relationship of microcarrier density on cell adherence and proliferation. Their density should be slightly higher than that of the culture medium to facilitate the separation of cells from the medium. Microcarriers with a density close to the culture medium can remain suspended with mild stirring, reducing the chances of shear stress and collisions (Zhou et al., 2019). A density range of 1.02–1.04 g/cm³ is regarded as optimal for microcarriers; most commercially available microcarriers have densities falling within this range (Chen et al., 2020). Cell adhesion efficiency of PCL microcarriers with a density range of 1.05–1.06 g/cm³ is up to 80%, whereas adhesion efficiency decreases to 35% with higher density range of 1.10–1.14 g/cm³. Microcarriers with high densities (~1.14 g/cm³) are difficult to suspend in stirred bioreactors and form large aggregates with cells leading to necrosis (Li et al., 2017).

Porosity of Microcarriers

Depending on their porosity, microcarriers are classified as non-porous, microporous, and macroporous. Microcarriers like Plastic Plus and FACT III from SoloHill Engineering are non-porous, providing a smooth cell adhesion and proliferation surface. The transfer of nutrients and the elimination of toxic products occur efficiently (Pörtner, 2015). Microporous microcarriers like Cytodex and SoloHill have small pores, which might limit cell infiltration to microcarrier surfaces only. Cells continue to proliferate under sufficient nutrients, but an arrest in proliferation is seen once confluence is reached as the microcarrier surfaces are fully attached to cells. There are no more surfaces available for cell proliferation. In the case of macroporous microcarriers such as Cytoline, CultiSpher and Fibracell, cells embed themselves within large pores and proliferate inside. These large pores shield the embedded cells from shear stress generated in a bioreactor and enhance productivity by offering a larger surface area for attachment and proliferation of

cells (Clapp et al., 2018). These macroporous microcarriers provide a multilayer culture system that resembles the *in vivo* cellular environment and facilitates multidirectional cellular interactions (Huang et al., 2018). The pore spaces can harbor a variety of cells like skeletal myoblasts, hepatocytes, articular chondrocytes, preosteoblasts, and mouse gastric stem cells (mGSCs) (Kankala et al., 2019).

The porous microcarriers are prepared with different porogens such as camphene, paraffin, sodium chloride, water and gelatin (Ravikumar, 2016; Samsudin et al., 2018; Zhou et al., 2019). Fabrication of porous microcarriers can be achieved by a two-step procedure of porogen leaching and freeze-drying (Zhou et al., 2019). Other methods involving microfluidic technology have also been successful in generating highly porous microcarriers (Kankala et al., 2019). Although porous microcarriers have been extensively explored for their applications in cell culture, currently, there are no studies to establish the optimum pore size for different types of cells.

Elasticity of Microcarriers

The elastic modulus of microcarriers is evaluated using the atomic force microscopy (AFM)--based nanoindentation technique. The elasticity of microcarriers plays a critical role in providing anchorage to cells. It ensures that the adherent cells remain undisturbed while experiencing turbulence or shear stress while stirring the culture medium (Huang et al., 2018). It is crucial in cellular differentiation and designing microcarriers with ideal mechanical properties for tissue regeneration. Soft matrix (1 kPa) promotes adipogenic, neuronal, and chondrogenic cell differentiation. Muscle cell differentiation is promoted by matrix having intermediate stiffness (11 kPa), while stiffer surfaces (34 kPa) promote osteogenic differentiation (Huang et al., 2018). The concentration and degree of cross-linking of polymers can be adjusted to control the elastic properties of microcarriers. Fabricated microcarriers have different elastic modulus ranging from 33.93 kPa to 132.68 kPa by blending different ratios of gelatin and chitosan (Ding et al., 2022).

POLYMERS USED IN THE PREPARATION OF MICROCARRIERS

Microcarriers have been produced using a variety of natural and synthetic polymers due to their chemical flexibility, biocompatibility, and biodegradability. They can also be immobilized with desired biomolecules. Natural polymers are the preferred candidates for biomedical applications since they are biodegradable with inherent bioactivity, biocompatibility, and bioresorbability (Reddy et al., 2021). They are non-toxic as they have biochemical similarities with human extracellular matrix (ECM) components and are thus safe for human use. Natural polymers are plant origin (cellulose, starch, dextran, and pectin) or animal origin (chitosan, collagen, chondroitin, and gelatin). Microcarriers made from synthetic polymers can be categorized into biodegradable or non-biodegradable

microcarriers. Biodegradable polymers are susceptible to enzymatic and/or chemical deterioration associated with living organisms and are easily decomposed. The demand for synthetic biodegradable polymers has grown exponentially over the years because of their excellent degradation ability and biocompatibility. Various polymers are used extensively for biomedical engineering and as microcarriers for drug delivery. Some examples of the most widely used polymers are PCL, PGA, PLA and PLGA (Tavassoli et al., 2018; Zhou et al., 2019). Non-biodegradable polymers are comprised of long chains of carbon and hydrogen atoms and are resistant to environmental degradation. Polycarbonate (PC), polyethylene terephthalate (PET), and polystyrene (PS) are examples of non-biodegradable synthetic polymers involved in cell culture (Tavassoli et al., 2018).

Some disadvantages of natural polymers are weak structural integrity, poor mechanical strength, lack of industrially acceptable processability and economic viability. These factors have restricted its application in tissue engineering. Conversely, synthetic polymers are versatile, have strong mechanical properties, desired flexibility and stability, and resist chemical degradation. However, synthetic polymers can induce inflammatory reactions and are considered toxic. They often lack cell adhesion and require chemical modifications (Reddy et al., 2021). These constraints can be overcome by introducing natural polymers on the surface of synthetic polymers or by creating a combination of natural and synthetic polymers (Reddy et al., 2021). Natural-synthetic polymer blends have enhanced cell adhesion and mechanical properties. Blended polymeric materials were created by grafting cellulose on polylactide (PLLA) polymers, which exhibited improved adhesion of hepatocellular liver carcinoma cells (HepG-2) as opposed to microcarriers fabricated solely from PLLA (Yang et al., 2016). Blending PCL with hydroxyapatite (HA), an essential element required for bone regeneration, has shown promising results in bone tissue engineering applications (Zheng et al., 2017).

SURFACE MODIFICATION OF MICROCARRIERS

Surface properties are fundamental for the good design and functioning of microcarriers. Surface energy and hydrophilicity are crucial in regulating cell-polymer interactions in culture systems (Omrani et al., 2020). However, it has been observed that polymers often lack the desired surface properties required for specific applications. Hence, surface properties are modified to create an advanced multifunctional product. Hydrophilic surfaces are more favorable for cell adhesion as adhesion molecules on the cell membrane surface tend to adhere to the hydrophilic surfaces compared to the hydrophobic surfaces. The high hydrophobicity and low bioabsorption of synthetic polymers make it difficult for cells to adhere to their surfaces (Shahrifi et al., 2020). Numerous research has led to the development of various surface modification methods that enhance the physical and chemical characteristics of polymer microcarriers without changing their bulk properties.

Physical methods like surface abrasion and chemical methods like wet chemical oxidation are commonly used (Omrani et al., 2020).

Additionally, high-energy methods such as ultraviolet-ozone treatment (UVO) and plasma treatment can enhance wettability and immobilization of biomolecules by incorporating functional groups on microcarrier surfaces (Recek et al., 2016). The chemical nature of the groups is dependent on the gases used. For instance, nitrogen-containing groups (C-N, N-C=O) are from nitrogen and ammonia plasma, oxygen-containing groups (C-CO₂, C=O, O-C=O) are from oxygen plasma, and fluorine-containing groups (C-CF_n, C-F, CF₂, CF₃) are from sulfur hexafluoride plasma (Minati et al., 2017). Omrani et al. (2020) demonstrated increased adhesion of mouse embryonic fibroblasts onto the plasma-treated polyether ether ketone (PEEK) surface. The general objectives of microcarrier surface modification are presented in Table 1.

Table 1
Objectives of microcarrier surface modification (Minati et al., 2017; Recek et al., 2016)

Microcarrier surfaces are usually modified to:
<ul style="list-style-type: none"> • Microcarrier surfaces are usually modified to: • Introduce random or specific functional groups • Improve hydrophilicity • Improve surface energy • Enhance surface conductivity • Facilitate adsorption of biomolecules • Facilitate adhesion of microorganisms • Eliminate contaminants • Change surface morphology and roughness • Change chemical or biological reaction kinetics

TECHNIQUES FOR SURFACE MODIFICATION OF MICROCARRIERS

Plasma Treatment

Surface modification using plasma is a highly productive technique in regenerative medicine and tissue engineering, such as stem cell generation, wound healing, and skin tissue engineering. (Mozaffari et al., 2021). It is done to improve the hydrophilic properties of the microcarrier surface, thereby enhancing the materials' biocompatibility. Plasma is an ionized gas; the gas in the cell ionizes to a plasma state and emits UV light. The formed ions will collide with the neutral gas molecules to produce numerous reactive species. The interactions between the reactive species and the polymer surfaces placed inside the chamber will result in surface modification by forming functional groups derived from gas particles introduced to the polymer surface. Examples of gases include oxygen, ammonia, sulfur dioxide, nitrogen and argon (Laput et al., 2022; Minati et al., 2017). Recek et al. (2016) have treated PCL surfaces with oxygen, ammonia, and sulfur dioxide plasma and concluded that surfaces treated with oxygen and ammonia exhibited better cell adhesion. The proliferation of HUVEC increased by more than 60% for oxygen and ammonia plasma-treated PCL compared to sulfur dioxide plasma-treated PCL, which showed a 40% increase in cell viability. Besides, the sulfur dioxide plasma-treated PCL also demonstrated poorer cell adhesion through SEM analysis. However, Syromotina et al. (2016) demonstrated that ammonia plasma-treated poly(3-hydroxybutyrate) surfaces showed better adhesion

and proliferation of mouse embryonic fibroblasts as compared to oxygen-treated surfaces. Hence, the efficiency of different plasma treatments needs further investigation to ensure their applicability and biocompatibility.

UVO TREATMENT

UVO treatment is a photosensitized oxygenation process introducing oxygen-based functional groups to the treated surface. It has been demonstrated to have potential in cell culture, such as the generation of skin and stem cells. (Samsudin et al., 2018; Suzuki et al., 2021). During the treatment, UV-driven ozone-oxygen synthesis and dissociation cycle produce atomic oxygen continuously (Özçam et al., 2014). UV rays with wavelengths between 184.9 nm and 253.7 nm are emitted from low-pressure mercury lamps. Atomic oxygen is a very reactive oxygen species, which may interact with the polymer chain, ambient oxygen molecules, ozone, and water vapor in various reaction pathways to form oxygen-containing functional groups such as C-O, COO-, C=O, -OH. The atomic oxygen also reacts with the carbon atoms of the polymer chain, producing alkyl radicals that may react with the molecular oxygen, producing peroxy radicals and then forming peroxide intermediates. The hydroxyl, peroxy, and hydroperoxide groups may oxidize, producing carbonyl compounds with ketone, ester, or carboxylic acid groups (Arifin et al., 2022; Sia et al., 2023).

In recent years, this method has effectively improved the surface chemistry of polymers (with respect to surface energy and hydrophilicity). The surface energy of polyurethane was increased to ~38.8% by using UVO treatment (Kuang & Constant, 2015). In another study, the hydrophilicity of polymer surfaces, namely poly(dimethyl siloxane) (PDMS) and poly(vinylmethylsiloxane) (PVMS), were significantly improved by UVO treatment (Özçam et al., 2014). This technique is cost-effective, feasible, offers a high degree of control, and does not leave residues or contaminants. Additionally, this treatment is suitable for heat-unstable materials as it can be easily carried out at room temperatures with different gases, solvents and solutions (Yusilawati et al., 2010).

Wet Chemical Method

The wet chemical-based method is a classical approach to microcarrier surface modification. This technique uses liquid reagents to generate reactive functional groups on the surfaces (Govindarajan & Shandas, 2014). Introducing functional groups (such as amino, carboxyl, hydroxyl, and sulfate) modifies and enhances the hydrophilicity of the treated surfaces (Tham et al., 2014). Wet chemicals penetrate porous surfaces deeper than high energy-based modification approaches, producing a more stable and noncorrosive immobilization surface. It is an attractive alternative due to its affordability and high-yield production (Govindarajan & Shandas, 2014). However, monitoring the concentration of liquid reagents

is crucial in preventing bulk degradation of the polymers (Tham et al., 2014). Wet chemical modification is achieved by aminolysis and hydrolysis.

During aminolysis, amine groups are introduced on microcarrier surfaces. Certain polymer surfaces are hydrophobic and lack interaction with biomolecules, with the introduction of amine groups on polymer surfaces. Subsequent grafting onto the biomolecules is achieved through conjugation (Holmes & Tabrizian, 2015). 1-6-hexanediamine and ethylenediamine are common diamines used during the aminolysis of polymers (Holmes & Tabrizian, 2015; Shi et al., 2019). In general, aminolysis increases the roughness and wettability of polymer surfaces and hence improves the interactions of the protein with the surfaces. However, it should be noted that aminolysis is a non-specific method, and there is a possibility of surface degradation of polymers, so reactions should be carried out under controlled conditions to avoid adverse effects on the bulk properties of polymers (Holmes & Tabrizian, 2015).

During hydrolysis, hydroxyl and carboxyl groups are introduced on polymer surfaces. These are produced due to hydrolysis of ester linkages, which are the backbones of polymers. PGA, PLA, PLGA, and PCL are examples of polymers used during hydrolysis (Holmes & Tabrizian, 2015; Tham et al., 2014). Hydrolyzed microcarrier surfaces allow the grafting of biomolecules, thus promoting cell adhesion and proliferation. Tham et al. (2014) subjected PLA microcarriers, which are relatively hydrophobic and lack adequate cellular interaction, to alkaline hydrolysis. Post-treatment, the hydrolyzed PLA microcarriers showed enhanced hydrophilicity. In another study, Zhou et al. (2019) hydrolyzed PCL microcarriers in an alkaline solution before conjugating them with HA to produce a hydrophilic substrate more conducive to human fibroblasts' growth and proliferation. However, this method could cause irregular topography of the surface, which might affect the bulk properties of polymers (Holmes & Tabrizian, 2015).

The research was performed simultaneously during aminolysis and hydrolysis to analyze the influence of each reaction on material biocompatibility. Shi et al. (2019) conducted aminolysis and hydrolysis of PLA microcarriers. Then, they compared the end products of each reaction for their potential to induce the growth of human osteoblast-like cells. Both methods showed good cell adhesion and growth, but hydrolyzed PLA proved to be a better substrate for cell cultivation.

Biomolecule Immobilization

An effective approach to enhance polymer-based microcarriers' biological functionalities is immobilizing biomolecules on their surfaces. High hydrophobicity and low bioadsorption of synthetic polymer-based microcarriers pose difficulty for cell adhesion (Shahrifi et al., 2020). The attachment of natural hydrophilic polymers like cellulose, collagen or gelatin improves the surface energy and hydrophilicity of the microcarriers, making

them suitable for cell adhesion and proliferation. Ma et al. (2002) immobilized gelatin or collagen on PLLA and proved that immobilization improved surface wettability. In another study, arginine was immobilized on polyetherimide films. These surface-modified films showed approximately 85% increase in cell adhesion in less than two hours as opposed to untreated films (Sengupta & Prasad, 2018). Examples of ECM proteins, like collagen, gelatin, and fibronectin, as well as short peptide sequences, can be incorporated to create biomaterials/biological systems that closely mimic the natural cell environments. Apart from providing structural support to cells, these biomaterials can also regulate cellular morphology, attachment, differentiation, migration, and immune responses (Nikolova & Chavali, 2019). This biomimetic approach has been applied in biomedical applications like tissue engineering, device implantation, diagnostic assays, and drug delivery systems (Nikolova & Chavali, 2019).

Techniques for Biomolecule Immobilization

Albumin and heparin are two common biomolecules immobilized using different techniques (Frey et al., 2020). The method used depends on the type of biomolecule involved and the surface properties of polymers (Mohamad et al., 2015). Surface properties like the presence of functional groups, hydrophobicity, and surface charge influence the efficiency of biomolecule immobilization. Surface modification techniques such as plasma and UVO treatment tend to introduce polar and hydrophilic functional groups on the polymers. These functional groups decrease the hydrophobicity of polymer surfaces and are often applied in protein immobilization with a coupling agent such as EDAC (Arifin et al., 2022; Guo et al., 2020). Besides, the charges on the polymer surface also affect biomolecule immobilization, as shown in a previous study that showed higher efficiency of biomolecule immobilization between oppositely charged polymer surfaces and biomolecules (Guo et al., 2020).

Physical and chemical methods immobilize biomolecules. Physical methods are immobilization by weaker, mono-covalent interactions such as hydrophobic interactions, hydrogen bonding, van der Waals forces, ionic binding, and mechanical entrapment of biomolecules within polymer matrices. Covalent bonds are formed in chemical methods through amide, ether or thio-ether linkages between the biomolecules and polymer surfaces. These bonds confer stronger and lasting immobilization (Mohamad et al., 2015). Three main methods exist to immobilize biomolecules: physical adsorption, entrapment, and covalent binding (Frey et al., 2020).

Physical Adsorption

The physical adsorption technique is the simplest approach to introduce biomolecules on microcarrier surfaces. Polymer samples are soaked in a dissolved biomolecule solution and incubated for immobilization. Alternatively, the process can be carried out by drying

the biomolecule solution on polymer surfaces and then rinsing away the non-adsorbed molecules with an appropriate buffer solution (Mohamad et al., 2015). The adsorption of biomolecules occurs spontaneously via non-specific intermolecular forces such as hydrogen bonding, hydrophobic interactions, van der Waals forces, and ionic binding (Frey et al., 2020). These intermolecular forces are highly influenced by environmental conditions like ionic strength, pH, or polarity of the solvent; hence, fluctuations in these environmental conditions may potentially reverse the adsorption process (Wieland et al., 2020).

Guo et al. (2020) found that the charge of the attached molecules influences immobilization efficiency. In a previous study, heparin molecules were immobilized by ion attraction on aminolyzed PCL surfaces. These heparin molecules were then used to adsorb lysozyme (LZM) and bovine serum albumin (BSA) protein molecules. It was observed that the negatively charged heparin molecules absorbed more of the positively charged LZM. Clara-Trujillo et al. (2019) created a biomimetic environment for cell culture by using magnetic microspheres. The microspheres were prepared by immobilizing acrylates and acrylic acid and coated with fibronectin (FN) (Clara-Trujillo et al., 2019). Cell adhesion of porcine mesenchymal stem cells (pMSCs) onto these magnetic microspheres was observed. These microspheres provided a flexible 3D environment for cells to remodel and reorganize during cell development and homeostasis. Levato et al. (2015) observed that physical adsorption allowed rapid and uncontrolled release of adhered cells. Using a combination of physical adsorption and covalent bonding could control the release rate of cells, and this approach could be used to design efficient biomaterials for controlled cell delivery.

It is a simple and inexpensive strategy for immobilizing biomolecules without toxic or complicated linker chemistry, which may have a larger commercial potential due to its ability to preserve the activity and integrity of biomolecules (Guo et al., 2020). However, there is progressive leaching of the immobilized biomolecules as the intermolecular forces holding them together are relatively weak and cannot control the orientation and conformation of biomolecules. The leaching of immobilized biomolecules will slowly revert the surface properties of polymers to initial hydrophobic properties, which is not conducive to cell adhesion (Mohamad et al., 2015). Besides, this method's poor reproducibility and low operational stability have only limited its applications in laboratory procedures and preliminary studies.

Covalent Bonding

Covalent bonding is the most popular method for immobilizing biomolecules on microcarrier surfaces (Mohamad et al., 2015). Growth factors, proteins, and ECM components are a few examples of biomolecules covalently grafted on microcarriers. The formation of strong covalent bonds between functional groups of biomolecule microcarriers carries out immobilization. Cross-linking reagents link biomolecules directly

to the immobilization surfaces (zero-length crosslinkers) or indirectly work by introducing molecular spacers between the cross-links. Carbodiimides, Woodward's reagent K (N-ethyl-3-phenylisoxazolium-3'-sulfonate), and N,N'-carbonyldiimidazole are the most widely used zero-length crosslinkers. Among the carbodiimides, 1-ethyl-3-(3dimethylaminopropyl) carbodiimide hydrochloride (EDAC/EDC) is the most popular reagent. To obtain more stable conjugates, EDC is often cross-linked with N-hydroxysuccinimide (NHS) or Sulfo-NHS (water-soluble analog of NHS). A few research that have used EDAC with NHS (or Sulfo-NHS) for bioconjugation are listed in Table 2.

The activity of covalently bonded biomolecules is largely dependent on the shape and composition of polymer surfaces and the nature of the coupling method used (Mohamad et al., 2015). The main advantage of this technique is that there is no leaching of biomolecules into the environment, although solutions or substrates of high ionic strength are present (Wieland et al., 2020). This technique is more robust, provides uniform immobilization, has higher surface coverage, and is able to increase the immobilized biomolecules' thermal stability compared to physical adsorption (Mohamad et al., 2015). However, this technique has disadvantages such as longer incubation time, toxic and complex linkage chemistry applications, tedious protocols, and expensive operational costs (Wieland et al., 2020).

Table 2

Researches that used EDAC/NHS (Or Sulfo-NHS) reagents for bioconjugation

Title of Paper	Conjugation	References
"Ocular biocompatibility of gelatin microcarriers functionalized with oxidized hyaluronic acid."	Oxidized hyaluronic acid onto gelatin microcarriers	Lai & Ma, 2017
"Surface Modification of Microporous of Polycaprolactone (PCL) Microcarrier to Improve Microcarrier Biocompatibility"	Gelatin onto PCL microcarriers	Samsudin et al., 2018
"Biomimetic microspheres for 3D mesenchymal stem cell culture and Characterization"	Hyaluronic acid onto acrylic acid incorporated ethyl acrylate and ethyl methacrylate copolymer microspheres	Clara-Trujillo et al., 2019
"Droplet-based vitrification of adherent human induced pluripotent stem cells on alginate microcarrier influenced by adhesion time and matrix elasticity"	Matrigel onto alginate microcarriers	Meiser et al., 2021

METHODS FOR PREPARATION OF MICROCARRIERS

Emulsion-solvent Evaporation Method

Emulsion methods used to produce nanosized microcarriers (size < 10 µm) are commonly classified as single-emulsion methods and double-emulsion methods. Single emulsion methods such as oil-in-water (O/W), water-in-oil (W/O), oil-in-oil (O/O); double emulsion methods such as water-in-oil-in-water (W/O/W). In O/W emulsions, oil droplets are

dispersed in an aqueous phase, whereas in W/O emulsions, water droplets are dispersed in an oil phase. The O/O emulsion system consists of two immiscible oils (the first oil phase dispersed as droplets in a continuous second oil phase). The W/O/W emulsion system extends the W/O emulsion method in which the W/O emulsion is dispersed in the second aqueous phase (Campos et al., 2013).

O/W emulsions are the simplest emulsion systems used to prepare microcarriers. The method of O/W emulsion-solvent evaporation comprises four steps (Figure 1): (1) The dissolution of polymers in a suitable organic solvent followed by dispersion of the active compound in the organic phase, (2) Emulsification of the organic phase in an immiscible aqueous phase, (3) Evaporation and subsequent removal of the solvent, with consequent hardening of the dispersed phase into solid microspheres, and (4) Harvesting of the microspheres by filtration or centrifugation, followed by drying (Campos et al., 2013). PCL and PLGA microcarriers have been fabricated using an O/W emulsion system (Samsudin et al., 2018). Keratin-based microcarriers, fabricated using W/O emulsion systems, act as good substrates for bone marrow-derived MSC (BM-MSC) (Thompson et al., 2020).

Although emulsion-solvent evaporation methods, especially single emulsion, have been widely practiced for microcarrier production due to ease of fabrication, this method has several shortcomings. The main disadvantages faced are the difficulty in controlling the evaporation rate, low encapsulation efficiency, uneven distribution of particles, and

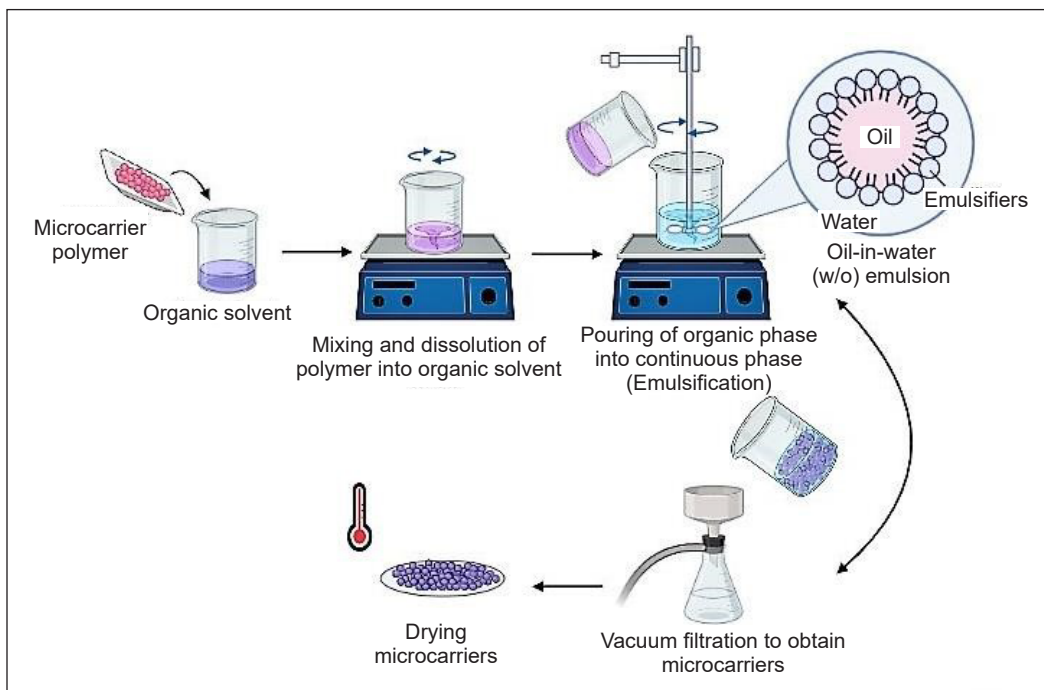


Figure 1. Steps involved in the oil-in-water emulsion-solvent evaporation technique (Lagreca et al., 2020; Campos et al., 2013)

unpredictable release of drugs or growth factors (Dashtimoghadam et al., 2020). Hence, double emulsion is emerging as an attractive microcarrier due to its high level of sample isolation and convenient cargo loading, which gives it a high potential for controlled release. However, this technique is more complicated to ensure on-demand core release and trajectory control during application (Zhang et al., 2022). Besides, microfluidic-assisted technology has been used with the emulsion-solvent evaporation technique to produce microcarriers with uniform shapes and sustained release ability. Dashtimoghadam et al. (2020) encapsulated vascular endothelial growth factor (VEGF) into PLGA microcarriers using a microfluidic-assisted double emulsion-solvent evaporation technique. The microcarriers produced exhibited a biphasic release pattern of VEGF, with rapid release followed by sustained release due to slow diffusion from the matrix.

Suspension Polymerization

This method produces 5 to 1000 μm particles and larger than those produced during emulsion-solvent evaporation (May 2016). In suspension immobilization, the dispersed phase consisting of monomers and monomer-soluble initiators is added to an immiscible solvent, which forms the continuous phase in the presence of surfactants or stabilizers. The solution is then heated to activate the initiator for radical immobilization. The microspheres are then collected and washed to remove the stabilizer. A major shortcoming of this approach is that the microcarriers produced have high polydispersity; micro-sieving is required to obtain well-defined particles, which reduces the yields (Saralidze et al., 2010). Cer et al. (2007) used suspension immobilization to design PEG-based microcarriers, which exhibited high cell adhesion and proliferation rates and could be used as a potential alternative to commercial microcarriers. Thermosensitive microcarriers have also been prepared by suspension immobilization. Gümüřdereliođlu et al. (2013) first prepared poly(2-hydroxyethyl methacrylate) (PHEMA) beads using this technique, then grafted N-isopropylacrylamide (NIPAAm) onto the PHEMA beads leading to the production of thermosensitive PHEMA-gPNIPAAm microcarriers.

APPLICATIONS OF MICROCARRIERS

Production of Viral Vaccines

One of the earliest and major uses of animal cell culture is the replication of viruses in culture medium to produce vaccines. Vaccines are being produced industrially using continuous or immortalized cell lines for viral replication. The most used cell lines are Vero, Chinese hamster ovary (CHO), BHK-21, Madin-Darby Bovine Kidney (MDBK), and human fetal lung fibroblasts (Verma et al., 2020). These cell lines are preferred as they are susceptible to infections, extremely resistant to genetic modifications, and can be grown

frequently *in vitro*. Initially, cells were cultivated in T-flasks, roller bottles, and cell factories; now, they are cultivated in microcarrier cultures to produce inactivated vaccines (Ismail et al., 2021). Microcarrier-based cell culture systems have demonstrated great potential in vaccine production and are good alternatives to egg-based vaccine production processes.

Stainless steel stirred-tank bioreactors equipped with marine impellers are the most typical bioreactors used for vaccine production (Silva et al., 2015). According to reports, the maximum size of stirred-tank bioreactors used to culture cells on microcarriers is 6,000 L, which Baxter Biosciences has used for producing influenza vaccines using Vero cells grown on Cytodex microcarriers (Eisenkraetzer, 2014). Wave-mixed bioreactors have also shown promising results for the culture of cells on microcarriers and have been employed to produce mink enteritis vaccines (Silva et al., 2015). The conditions within the bioreactors need to be monitored constantly throughout the culture process. For instance, temperature is mostly maintained at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for mammalian cells, and pH is mostly maintained within a narrow range at 7.2 ± 0.1 . Oxygen partial pressure $p\text{O}_2$ needs to be optimal with approximately 20-50% of air saturation, and carbon dioxide partial pressure $p\text{CO}_2$ needs to be varied to control the pH of the medium used containing sodium bicarbonate (Pörtner, 2015). Table 3 enlists various vaccines produced by the microcarrier culture system.

Table 3
Vaccines produced by microcarrier culture system

Vaccines against	Cell Line	Microcarrier	References
Yellow fever 17DD virus	Vero cells	Cytodex™ 1	Mattos et al., 2015
Enterovirus A71(EV-A71)	Vero cells	Cytodex™ 1	Chia et al., 2018
Louis Pasteur 2061 (LP2061) rabies virus	Vero cells	Cytodex™ 1	Trabelsi et al., 2019
Recombinant vesicular stomatitis virus–Zaire Ebola virus (rVSV-ZEBOV)	Vero cells	Cytodex™ 1	Kiesslich et al., 2020
Human parainfluenza virus type 3 (PIV3), respiratory syncytial virus (RSV), Severe acute respiratory syndrome coronavirus (SARS-CoV), and varicella-zoster virus (VZV)	Primary human bronchiotracheal cells (HBTCs), BEAS-2B cells, normal human neural progenitor (NHNP) cells	CultiSpher-G	Goodwin et al., 2015

Production of Recombinant Proteins

Recombinant proteins are widely used in pharmaceuticals for therapeutic treatments and prevention of diseases such as diabetes, cancers, and infectious diseases. They are exogenous proteins encoded by recombinant DNA cloned in a production organism (Burnett & Burnett, 2020). Microcarrier-based cell culture technology serves as a promising tool for producing a variety of recombinant proteins that are being used in basic research as well as pharmaceutical development. Over the last two decades, mammalian cell protein

expression has become the dominant recombinant protein production system for clinical applications; about 60-70% of all recombinant proteins are produced in mammalian cells. The most used mammalian cell is the immortalized CHO cell. Other cell lines like BHK, mouse myeloma (NS0), and human retinal cells have also gained regulatory approval to produce recombinant proteins (Fliedl & Kaisermayer, 2014).

These cell lines are cultured on microcarriers in stirred tanks and fluidized-bed bioreactors. Compared to normal suspension cultures, microcarriers show increased cell growth and excellent harvest yields (Tharmalingam et al., 2011). Chevalot et al. (1994) observed higher cell densities and higher amounts of human gamma-glutamyl transferase (GGT) when recombinant CHO cells were cultured on microcarriers. Similarly, Shirokaze et al. (1995) showed that the growth of recombinant CHO cells with microcarriers gave about double the yields interleukin-4 compared to the amount obtained through suspension culture. Table 4 lists commercially available recombinant proteins manufactured using microcarrier-based cell culture technology.

Table 4

Commercially available recombinant proteins produced using microcarrier-based cell culture technology

Product	Cell line	Microcarrier	Manufacturer
Aldurazyme™ (recombinant human α L-iduronidase or rhIDU)	CHO cells	Cytopore™	Biomarin
Myozyme™ (recombinant human acid alpha-glucosidase)	CHO cells	CytoPore™	Genzyme
Cerezyme™ (imiglucerase)	CHO cells	CytoPore™	Genzyme
GONAL-F® (recombinant human follicle stimulating hormone)	CHO cells	Cytodex™ 3	Merck
Luveris® (recombinant human luteinizing hormone)	CHO cells	Cytodex™ 3	Merck

Expansion of SCs

SCs have recently made significant progress in targeting the modulation of immune responses and tissue and organ regeneration. They are used to treat a myriad of diseases and conditions of the blood and immune system. This requirement of SCs for future cell therapy has evoked interest in applying microcarriers as the preferred platform for SC expansion. These cells have a remarkable capacity to self-renew and differentiate into other cell types (Mattiasson, 2018). SCs for clinical use are mostly produced through conventional static adherent cultures unsuitable for large-scale production. Conventional static adherent culture models, such as T-flask roller bottles, have smaller surface areas compared to microcarriers. Besides, 2D cell culture cannot provide an environment for multidirectional interactions between cells and the extracellular matrix, which might cause cellular morphology and gene expression changes. In contrast, microcarriers enable the scaling up of cell production in small volumes of the medium by supplying a large

surface area for cell growth in suspension cultures and providing a 3D environment for multidirectional cell interactions (Huang et al., 2020; Tavassoli et al., 2018). Numerous strategies (using different bioreactors, microcarriers and culture mediums) are being applied to shift the production to microcarrier suspension cultures to fulfill the future demand for quality-assured SCs (Kumar & Starly, 2015). Table 5 lists various research studies that have successfully cultured SCs using microcarrier-based culture systems.

Table 5
Culturing of different types of stem cells using microcarriers

Cell type	Microcarrier	Reactor type	Purpose	References
Human embryonic stem cells (hESCs)	Cytodex 1 and Cytodex 3	Spinner flask	Expansion and differentiation	Park et al., 2014
Mesenchymal stem cells (MSCs)	Corning®Synthemax®II polystyrene and CELLstart™coated SoloHill plastic microcarriers	Spinner flask	Expansion	Silva et al., 2014
	Corning® Enhanced Attachment, Corning® Low Concentration Synthemax®II, Corning® Collagen, SoloHill® Plastic Plus, SoloHill® Glass Coated, SoloHill® Collagen Coated, SoloHill® Pronectin F	Spinner flask	Expansion and differentiation	Heathman et al., 2018
	SoloHill® Plastic P102L, Cytodex-3 and Hillex	Spinner flask	Expansion	Rafiq et al., 2018
	Cytodex-1, Corning, GhaterDisc-1, GhaterDisc-2 and GhaterDisc-3	Spinner flask	Expansion	Clainche et al., 2021

CONCLUSION

Microcarriers are gaining considerable attention due to their overwhelming potential in numerous fields, especially regenerative medicine and bioengineering. Morphology, physical and chemical properties of microcarriers have been deeply studied to determine the suitability of microcarriers in cell cultures. Modifying surface properties of microcarriers and immobilizing biomolecules on their surfaces have successfully enhanced their biocompatibility and made them more conducive for cell growth and differentiation. Surface modification methods like plasma treatment, UVO treatment, and wet chemical methods can improve the hydrophilicity and wettability of microcarriers without altering their bulk properties. Microcarrier-based cell culture technology has appeared as a robust platform for tissue engineering. This review concludes that microcarriers have great potential in cell culture technology; the availability of a large surface area enables mass cultivation of cells. More detailed studies on microcarriers, including clinical studies, are required to understand

the mechanism of interactions of microcarriers with various biological molecules and cells for greater development in medical and bioengineering fields.

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