Chitosan Microcarriers in Mammalian Cell Culture

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***Abstract*—**Chitosan is a naturally-occurring biomaterial with many advantages, including being non-toxic, biocompatible and biodegradable. It has a wide range of applications due to its numerous properties, and thus finds uses in wound dressings, biopesticides, tissue engineering, and pharmaceuticals. Chitosan microspheres have been produced via a variety of techniques that exploit the different properties of Chitosan, especially its gelling properties, and do so with the intended applications in mind. These techniques are often discussed in light of their use as drug carriers. As such, Chitosan has not received much attention as a microcarrier substrate for cell culture. This review provides an insight into the various reported techniques of production of Chitosan microspheres for use in mammalian cell culture and highlights recent findings of the evaluation of Chitosan as a microcarrier substrate.

***Keywords*—** Biomaterials Chitosan, mammalian cell culture, microcarriers.

I. Introduction

CHITOSAN, also known as Poliglusam, Deacetylchitin, and Poly-(D) glucosamine, is one of the naturally-derived polysaccharide co-polymers. It is an amino-polysaccharide, consisting of glucosamine and N-acetyl glucosamine units, and is derived by de-acetylation processes from chitin found in crustacean exoskeletons and is also found in some fungal cell walls and insect cuticles.

Chitosan is also considered to be one of the most abundant biodegradable materials. Industrially, Chitosan is produced from crustacean shells such as those from shrimp and crab. Recently, interest had sparked in producing Chitosan from fungi, such as *A. niger* and *M. rouxi*, as it is a major structural biopolymer in the cell walls of fungi from the genera Mucor, Absidia, and Rhizopus.

Chitosan has various uses which range from water filtration, self-healing paint coating, soluble dietary fiber, biopesticide, to those in pharmaceuticals as an excipient, as an antibacterial [1], in hemostatic agents [2], dental materials, and even in gene delivery [3]. Chitosan can be used as a drug carrier [1], as a delivery platform for parenteral formulations, disintegrant [4] and in tablet coating. Chitosan is also used to mask bitter-tasting substances [5]; and is thought to be a promising bioadhesive at physiological pH values [6]. Recently, interest in Chitosan has been more focused on Chitosan nanoparticles and microspheres for drug delivery, especially those targeting the respiratory epithelium and for drugs that will be sprayed through the nasal or buccal cavities. In regards to this, Chitosan nanoparticles were found to be compatible with respiratory epithelium [7], and controlled release of incorporated drugs was achieved when Chitosan was used as a drug carrier. Chitosan also shows mucoadhesive properties [4] and antimicrobial properties, further enhancing its potential in the respiratory epithelium. Some of the characteristics that determine these applications can be attributed to its strong hydrogen bonding, strong charges, high molecular weight, sufficient chain flexibility; and surface energy properties [4].

Figure 1 below shows the structure of Chitosan.

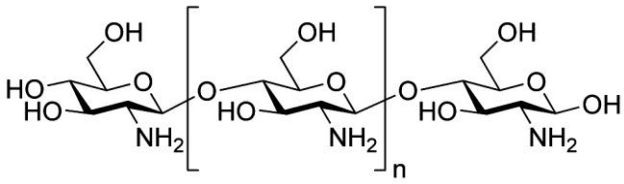

Figure 1 Structure of Chitosan

Chitosan is polycationic, where the surface groups of Chitosan are mainly cationic, with a few acetyl groups. These properties can be enhanced by surface treatments such as UV treatment.

II. Some Properties of Chitosan

One important parameter that determines many biological and physiochemical properties of Chitosan is its degree of deacetylation (DDA). It determines, for example, crystallinity, rate of degradation, cell-to-substrate adhesion and response, and hydrophilicity. Molecular weight (MW) is another important factor that determines Chitosan properties such as tensile strength, rate of degradation, and moisture content. However, the effects of these two factors are not so straightforward. For instance, Cao et al., [8] prepared six kinds of Chitosan films with similar MW but various DDAs. They measured factors such as film crystallinity, swelling and

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mechanical properties, protein adsorption, and primary rat Schwann cell proliferation on the films and found that DDA had a marked effect on the physicochemical properties and Schwann cell affinity of Chitosan films. Higher DDA Chitosan films showed a greater crystallinity, a higher elastic modulus and tensile strength and a lower swelling index than those with lower DDA. On the other hand, Hamilton et al., [9], and Chatelet, Damour, and Domard, [10], found no relationship between DDA, MW, or growth of cells (Osteoblasts and keratinocytes). Thus, we cannot generalize about the effects of DDA and MW of Chitosan on these properties.

III. Chitosan Microcarriers

Cell culture techniques have become vital to the study of animal cell structure, function and differentiation and for the production of many important biological materials. A support matrix of microcarriers facilitates attachment and growth of anchorage-dependent and even suspension-type cells, in cell culture processes due to various surface properties.

According to Malda and Frondoza, [11], 'the microcarrier bioreactor culture system offers an attractive method for cell amplification and enhancement of phenotype expression'. In addition to being substrates for the anchorage-dependent cells, microcarriers can also be used to deliver the expanded cells to the site of the defect- thus acting as scaffolds, and this applies to Chitosan [12]. For cell cultures where the goal is an extracellular product, microcarriers are again an advantage since they trap the cells while the product can be harvested with the media.

Microcarriers can be solid or porous, depending on the intended application. Porous microcarriers have the advantage of a large surface area to volume ratio as they can have cells growing inside the pores. Several types of microcarriers are available commercially including Dextran-based (e.g. Cytodex), Collagen-based (e.g. Cultispher), and Polystyrene-based (e.g. SoloHill Engineering) microcarriers. They differ in their porosity, specific gravity, optical properties, presence of animal components, and surface chemistries.

Studies, such as those described in the next section, have shown that both porous and non-porous (smooth) Chitosan microcarriers can be successfully fabricated and several cell lines were grown on them.

IV. Methods Of Producing Chitosan Microcarriers

Chitosan microspheres can be produced through a number of techniques. Sinha, [4], reviews these techniques of producing Chitosan microspheres with the intended use of Chitosan microspheres as carriers for drugs. The size of these microspheres was in many cases, therefore, significantly smaller than those needed for microcarrier cell culture.

A classification of these techniques, is described in details in [4]; where the processes for the production of chitosan microspheres are classified into 6 main ones namely interaction with anions, thermal cross-linking with citric acid, solvent evaporation, interfacial acylation, coating on preformed microparticles and cross-linking with chemicals. Under the interaction with anions, such as sulphates, hydroxides and molybdates, there are 3 processes: emulsification and ionotropic gelation, wet phase inversion and co-acervation which encompasses precipitation, precipitation chemical cross-linking and complex co-acervation. Cross-linking can be single or multi-emulsion glutaraldehyde cross-linking, formaldehyde cross-linking or genipin cross-linking.

To date, Chitosan microcarriers have been produced for the purpose of cell culture, and these microcarriers are of various properties e.g. smooth or porous and of various sizes and pore diameters.

Whilst many of these techniques are applicable, with modification, in production of microcarriers for cell culture, of the above-mentioned techniques, the water-in-oil (W/O) emulsion crosslinking technique, solvent evaporation, spray-drying and ionic gelation are the main techniques that are often seen in the production of these Chitosan microcarriers.

Xuan et al., [1], describe a W/O emulsification cross-linking method and the parameters of this method were optimized for the production of smooth Chitosan spheres, with a 132 µm particle size distribution. The characterization of the microspheres included size, morphology, and FTIR spectra. The methodology involves Chitosan solution (2% Chitosan in acetic acid) in a mixture of toluene, span-80 (surfactant), emulsified for 60 minutes and then cross-linked using formaldehyde for another 60 minutes. Volume of toluene and concentration of Chitosan solution were found to be key factors in this study. Dehydration was achieved by ethanol and ether.

In Lu et al., [13], an anti-phase suspension method with temperature-controlled freeze extraction was used to produce porous 180-280 µm Chitosan microspheres on a large-scale. The method combines the emulsification with freezing the microspheres in liquid nitrogen and then dehydration in a mixture of absolute ethanol and sodium hydroxide. This latter technique makes use of the fact that Chitosan gels in an alkaline environment [14] and this prevents its re-dissolution when extracting out the solvents. Ethanol was used to reduce the freezing point. A limitation of this method is its use of carbon tetrachloride, amongst other organic solvents, which has adverse health effects and is no longer commonly used.

By avoiding crosslinking with glutaraldehyde which is difficult to completely remove, and by avoiding freeze-drying which is time-consuming, energy-consuming, and forms surface skin [14], the technique by Lu et al. [13] produced Chitosan microcarriers that were also shown to support attachment and proliferation of chondrocytes.

Whilst Chitosan shows potential as a scaffold for bone and cartilage tissue engineering, Chitosan was also studied for liver regeneration. In Wu et al., [15], porous Chitosan microcarriers were produced for hepatocyte culture in a very simple technique where microdroplets of Chitosan solution are generated and frozen directly in liquid nitrogen and then vacuum freeze-dried. The study cites that apart from its biocompatibility and biodegradability, chitosan was also a suitable choice due to its structure being similar to glycosaminoglycans that form a part of the liver extracellular matrix. The microcarriers produced by this technique were shown to be safe, stable, and generated high density and

activity of liver cells. One of its advantages is that the technique does not involve a mixture of toxic organic solvents that would have been difficult to remove completely.

In a method that combines foaming by mechanical stirring then followed by liquid hardening by sodium hydroxide crosslinking [16], 3D microporous scaffolds of Chitosan. While this technique does not produce microspheres, this is one of the few studies that apply chitosan directly into 3D scaffolds (instead of composites). Perhaps, the same technique could be employed with minor modifications to the mechanical foaming, to produce chitosan microspheres.

Porous Chitosan beads can also by produced by electrospraying of Chitosan solution and collection in liquid nitrogen followed by freeze-drying. A novelty in the method was presented in [17] where the problem of maintaining the pore size was overcome by a thaw-refreeze method; where the frozen microspheres were thawed by shaking at room temperature for 7 minutes, and they are then refrozen at -20 degrees Celsius before finally being lyophilized. The microspheres were evaluated by growth of human mesenchymal stem cells.

Similarly, Chitosan microspheres with an open-pore structure can be produced by a 25 gauge needle extrusion and air-entrainment, followed by placing the microcarriers in a beaker of 0.1 M sodium hydroxide, freezing in liquid nitrogen and then freeze-drying. Madihally et al., [18], report the effect of freezing temperature and chitosan concentration on pore diameter of bulk chitosan scaffolds, where the highest pore diameter, at -20°C is achieved with the lowest chitosan concentration in the experiment (1%); and further that the degree of shrinkage of lyophilized material decreased with chitosan concentration.

Porous Chitosan microcarriers were produced by a method of suspension crosslinking using glutaraldehyde and lyophilization [19]. As mentioned previously, a drawback of the glutaraldehyde crosslinking is the difficulty in its complete removal while maintaining the desired microsphere structure and characteristics. Other explored alternatives are sodium sulphate and sodium citrate as crosslinking agents [4].

Each of the techniques cited above were selected, by their respective researchers, amongst other methods due to certain features that were important for the respective applications of the Chitosan. Apart from these differences, the Chitosan used itself was of different properties and this important difference makes the task of comparing and contrasting the different methods of Chitosan microcarrier production more complex.
Table I below illustrates a difference in the property of the chitosan itself that was used in some of these references. Some properties of the resulting Chitosan Microcarriers (CMCs) are also shown. The point being, has the DDA affected the CMCs in any way? Why were these DDAs chosen to be used in these experiments? It is noted that, in chitosan microcarrier studies, emphasis is put on the process conditions and factors, rather than the initial source of Chitosan itself. Without a deeper understanding of chitosan's properties, the task of choosing or designing a method to produce chitosan microcarriers becomes elusive.

TABLE I
DDA VALUES FOR CHITOSAN USED IN SOME REFERENCES

Reference	DDA	Resulting CMCs
[1]	83.7-85%	Smooth 132 µm
[13]	85.1%	Porous 35-60 µm mean pore size
[15]	75-85%	Porous
[16]	80%	Porous
[17]	66%	Porous
[18]	>75%	Porous
[19]	90%	Porous 50 µm pore size

Similarly, apart from the Chitosan itself, the choice and design of methodology should show a consideration of the effects on the environmental effects of the chosen reagents. As the Chitosan microcarriers would be competing with other existing microcarriers in the market for both functionality and price, it is wise to include this in selecting a Chitosan microcarrier production method.

V. Modification of Chitosan microcarriers

UV-irradiation can be considered as good method for molecular weight modification as well as for modification of the surface of Chitosan films by formation of Hydroxyl and Carbonyl groups. Studies on Chitosan films show that the surface roughness of Chitosan films is reduced, though porosity increased with exposure time, as a result of UV exposure [20].

VI. Conclusion

To sum up, chitosan is a marvelous natural polymer that is very promising in the field of tissue engineering, as it is biodegradable and biocompatible, and therefore it should be further evaluated in a variety of mammalian cell culture applications. To date, chitosan microcarriers have yet to be explored in depth for lung and skin cells as well as in light of immunoisolation. This is probably due to this area being overshadowed by the potential of chitosan as a 3D scaffold for bone regeneration. A major limitation of the work on Chitosan microcarrier production thus far is the lack of clear, systematic multivariate analysis and it thus appears that the methodologies cannot be compared and contrasted easily. Overall, research on Chitosan microcarriers appears to be promising and there is a lot to be explored on the subject.

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