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Silk sericin–alginate–chitosan microcapsules: Hepatocytes encapsulation for enhanced cellular functions

Sunita Nayak, Sanchareeka Dey, Subhas C. Kundu*

Department of Biotechnology, Indian Institute of Technology, Kharagpur 721302, India

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ABSTRACT

The encapsulation based technology permits long-term delivery of desired therapeutic products in local regions of body without the need of immunosuppressant drugs. In this study microcapsules composed of sericin and alginate micro bead as inner core and with an outer chitosan shell are prepared. This work is proposed for live cell encapsulation for potential therapeutic applications. The sericin protein is obtained from cocoons of non-mulberry silkworm *Antheraea mylitta*. The sericin–alginate micro beads are prepared *via* ionotropic gelation under high applied voltage. The beads further coated with chitosan and crosslinked with genipin. The microcapsules developed are nearly spherical in shape with smooth surface morphology. Alamar blue assay and confocal microscopy indicate high cell viability and uniform encapsulated cell distribution within the sericin–alginate–chitosan microcapsules indicating that the microcapsules maintain favourable microenvironment for the cells. The functional analysis of encapsulated cells demonstrates that the glucose consumption, urea secretion rate and intracellular albumin content increased in the microcapsules. The study suggests that the developed sericin–alginate–chitosan microcapsule contributes towards the development of cell encapsulation model. It also offers to generate enriched population of metabolically and functionally active cells for the future therapeutics especially for hepatocytes transplantation in acute liver failure.

1. Introduction

Liver dysfunction may clinically lead to hepatic failure due to occurrence of many complications. Additionally due to the diverse functions of the liver it becomes difficult in to construct effective hepatic support system and its replacement. Consequently, new research approach are now focused in designing biological artificial livers by immobilizing or encapsulating primary hepatocytes in variety of scaffolds as a bioreactor for liver support [1–3]. This orthotopic liver transplantation technology can facilitate removal, exchange of oxygen, nutrients and metabolites in the patient body. Microencapsulation involves entrapment or immobilization of biologically active material inside a microsphere with size range of 0.2–3.0 mm and maintained within a semi-permeable support material. Xenogeneic cells can be encapsulated within a microcapsule with semi-permeable membrane. They may be immuno-isolated from the surrounding immune cells, cytokines, immunoglobulins and from other complement factors when transplanted inside body [4]. The presence of semi-permeable membrane avoids requirement of immunosuppressants during transplantation therapy. Various synthetic and natural medicines, enzymes, hormones, proteins, peptides, genes, cells, genetically modified bacterium, fungus and other microorganisms are being used for microencapsulation [5]. In case of cell microencapsulation the fundamental thought is to make direct cell growth, differentiation, support and reconstruction of the liver *in vitro* by mimicking the physiological microenvironment [6]. Predominantly the hydrogel forming materials are of great interest for the formulation of microcapsules owing to their biocompatibility towards soft tissue. They disperse homogeneously the drug and cells throughout the matrix. Moreover control over polymer network formation may be achieved by manipulating the physical and chemical properties of hydrogel polymer used. This is considered to be a decisive factor for permeability to oxygen, nutrients and other molecule. This may facilitate greater viability to the encapsulated cells [7].

Biocompatible materials do not interfere with cell homeostasis, when applied within the capsules and allows survival of the enclosed cells. The most common material used for the preparation of the matrix for cell encapsulation is based on alginate core. Alginate is composed of polysaccharides D-mannuronic acid and L-guluronic acid. It becomes polyanionic in aqueous solution and can be crosslinked by divalent cation, like Ca^{2+} [8]. It is non-toxic and

* Corresponding author. Tel.: +91 3222 283764; fax: +91 3222 278433.
E-mail addresses: sunita013@gmail.com (S. Nayak), sancharika2001@yahoo.co.in (S. Dey), kundu@hijli.iitkgp.ernet.in (S.C. Kundu).

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generally does not elicit an inflammatory response. However, plain alginate microcapsules are incapable of achieving immunoisolation and are hampered by their mechanical instability and high porosity. To overcome this problem, the alginate microcapsules are further enveloped by a polycation layer such as poly-L-lysine (PLL), poly-L-ornithine (PLO), chitosan or lactose modified chitosan [9]. However, polycations are toxic to cells and usually provoke inflammatory reactions. On the other hand chitosan is a copolymer of D-glucosamine and N-acetylglucosamine derived from chitin. Being non-toxic and slow biodegrading polymer, it is widely used in controlled drug delivery. Chitosan can be crosslinked by several reagents like glutaraldehyde, tripolyphosphate and ethylene glycol [10]. However, the synthetic crosslinking agents are considered to be having more or less cytotoxic effects. Hydrogels are fabricated by crosslinking chitosan with genipin, a chemical compound extracted from gardenia fruits producing blue-coloured fluorescent [11]. The crosslinked chitosan is reported to be biocompatible, non-toxic with anti-inflammatory and anti-angiogenesis properties [12]. The genipin crosslinked chitosan micro beads for encapsulation of biological products, living cells, and for controlled release are reported [12–14].

Silk protein sericin derived from the cocoons of silkworm of different species are now found place in several biomedical applications [15–20]. Sericin is a globular protein with adhesive characteristics, synthesized in the middle silk glands of silkworms. Its molecular weight ranges from 10 to over 400 kDa [15]. The glue protein sericin envelops the fibroin fibres with successive sticky layers helping in the cocoon formation [21]. Sericin usually constitutes 20–30% in mulberry (domesticated) [15,17] and in non-mulberry (wild type) silkworm cocoons the contents of sericin vary based on species, freshness, collection time, storage condition, nature of crop and extraction procedures. However a silkworm race known as 'Sericin Hope' is developed from mulberry species secretes sericin in large quantity as high as 98.5% at the cocoon stage [22]. Sericin is produced during the processing of raw silk fibres in silk industries and is discarded as waste by-product material. Usually it is removed from the silk fibres by the different processes specifically like high alkali, high-pressure steam heating, boiling, and enzymatic treatment [16,23,24]. Sericin promotes proliferation along with attachment of several mammalian cell lines and its use is also recommended in serum free media [25,26]. It is having excellent water-retaining characteristics arising from its high hydrophilic amino acids content [15–17,27]. Its efficacy as a valuable natural ingredient in the food industries and in cosmetics is also reported [24,28–30]. Sericin with its serine-rich residues facilitates as a cryoprotectant for cells [31]. It is antibacterial, antioxidant and resistant to UV light [16,27,32,33]. Sericin is also known to suppress *in vitro* lipid peroxidation [29], and possess antitumour properties [34] with no immunogenicity [35].

The present work deals with the improvement of the functionality of alginate gel micro beads by blending with sericin. Further the beads are coated with chitosan and crosslinked with genipin, a natural crosslinker. The cost effective, abundant and underutilized silk protein sericin from cocoons of non-mulberry tropical tasar silkworm *Antheraea mylitta* is used. The encapsulation of the hepatocytes (liver cells) into this core material of sericin–alginate within chitosan semi permeable membrane is investigated for obtaining alternate microcapsules with improved functional properties.

2. Materials and methods

2.1. Isolation of sericin from cocoons of A. mylitta silkworm

Sericin was isolated by the alkaline degumming method from cocoons of the non-mulberry silkworm *A. mylitta* using the described protocol [32]. In brief, cocoons were cut into finer pieces and were boiled in 0.2 N Na_2CO_3 (Merck) solution for 1 h. The solution was centrifuged and the sericin solution was collected while the pellet was discarded. The supernatant was then dialysed against water for 48 h using 3 kDa dialysis membrane (Pierce, USA) to remove Na_2CO_3 and further solution was concentrated against PEG-6000.

2.2. Preparation of sericin–alginate–chitosan microcapsules

Sericin and alginate (Sigma–Aldrich) in various concentrations were used as the core of microcapsules. They were prepared using extrusion method by droplet formation into gellifying bath containing $CaCl_2$ solution. Basically, the setup consisted of a syringe pump equipped with a syringe, a needle connected with high voltage and the gellifying bath. The needle was connected with the positive end of the high voltage generator, whereas the gellifying bath was grounded. The schematic representation of whole set up is shown in Fig. 1. In brief, firstly the beads were prepared from 2% alginate (w/v) solution in PBS. Alginate solution was pumped out through the needle orifice at different rates (100, 200, 400 and 800 μl/h) and at different voltages (6, 9, 12 and 15 kV). Fine droplets of the solution were collected in the gellifying bath containing 0.2 M $CaCl_2$ solution in a container and left for 15 min. The alginate beads were then coated for 30 min in a 0.5% chitosan solution. Chitosan was dissolved in dilute acetic acid at a pH of 5.2. The chitosan coated microcapsules were quickly treated with 0.01 N NaOH to neutralize the acid producing alginate–chitosan microcapsules. They were cross-linked by immersing the microcapsules in an aqueous genipin solution (2.5 mg/ml at 37 °C for 4 h). The resulting genipin cross-linked alginate-microcapsules were washed and collected. The microcapsules formed were observed under light microscope (Nikon) for the determination of shape and diameter. The microcapsules with optimum size and diameter at optimum voltage were selected for further experimentation. To obtain the preferred size of sericin–alginate beads, different mixtures of sodium alginate 2% (w/v) and sericin (0%, 0.125%, 0.25% and 0.5%) solutions were pumped out through the needle orifice at a predetermine rate and voltage. The similar procedures were followed to coat and crosslink the different beads to produce sericin–alginate–chitosan microcapsules.

2.3. Scanning electron microscopy (SEM)

The surface morphology of the chitosan coated sericin–alginate microcapsules were analysed using JEOL JSM-5800 scanning electron microscope (SEM) at an operating voltage of 20 kV. Briefly, the microcapsules were gradually dehydrated in ethanol of different concentrations and subjected to freeze drying in a lyophilizer. The samples were sputter gold coated prior to analysis.

2.4. Confocal laser microscopy (CLSM)

The morphology of the chitosan coated microcapsules were investigated using confocal laser scanning microscope (CLSM, Olympus) equipped with Argon (488 nm) and HeNe (534 nm) lasers. For image acquisition, the microcapsules in storage solution (deionized H_2O) were directly placed on a cover glass and analysed.

2.5. Swelling characteristics

For determination of swelling behaviour, aliquots of alginate beads without chitosan coating, alginate and 0.125% sericin containing alginate microcapsules were submerged in 2 ml of phosphate buffered saline (PBS, pH 7.4) and 0.9% NaCl at 37 ± 0.5 °C