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ORIGINAL ARTICLE

Hepatic Cell Encapsulation Using a Decellularized Liver Scaffold

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Abstract
Purpose To improve effect of liver disease treatment, tissue engineering approach such as direct hepatocyte injection has been investigated. Encapsulation, mixing cells and biomaterials to enclose cells within a biomaterial capsule, is commonly used to deliver cells into the body. Many kinds of biomaterials including natural and artificial materials have been used. The capsule must have biocompatibility and microstructure for cell culture, survival and proliferation as well as cell function and therapeutic effects. However, most biomaterials used for encapsulation have low biocompatibility, insufficient constituents and an unsuitable 3-dimensional structure. To solve these problems, we performed encapsulation using a decellularized liver scaffold (DCLS) with a native extracellular matrix (ECM) and natural porous microstructure including vasculature.
Methods DCLS was prepared with 0.1% sodium dodecyl sulfate under agitation and 2 mm^2 sized DCLS pieces were sterilized with peracetic acid (25.6 μl/10 ml) for 24 hours. Histological analysis showed that the DCLS had native ECM, liver specific major architecture and blood vessel structure but no cells. For cell encapsulation, hepG2 cells were injected into DCLS pieces with a syringe and cultured for 5 days.
Results The cells survived and formed a cell mass with a liver ECM microstructure inside the DCLS capsules. The encapsulation status was similar to capsules formed by current encapsulation techniques.
Conclusions DCLS can be used to make an encapsulation cell delivery system.

Keywords Liver disease, Decellularization, Encapsulation, Decellularized liver scaffold, Microstructure

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INTRODUCTION

For end-stage liver disease, liver transplantation is the only curative treatment. However donor organ shortage, high cost, and immune reaction are major limitations. Thus, alternative cell-based liver direct therapies are being investigated [1]. Direct cell injection such as encapsulation is a method to deliver cells into tissues for the treatment or restoration of diseased/damaged liver. Many kinds of biomaterials including collagen, hyaluronic acid and peptides as well as anionic polysaccharides from algae have been used as encapsulation materials [2-5]. These materials should have various characteristics including biocompatibility, biodegradation, physical properties, mechanical stability, permeability, and morphology [6, 7]. The encapsulation materials should reside in tissues for a long time but induce a low degree of inflammation [8].

A common method for encapsulation is a mixture of powder or solution type biomaterials and cells [9-11]. Encapsulation acts to immunoisolate the cells and allows cell transplantation without immunosuppression, which is important for a safe and widely applicable cell therapy [12-14]. In addition, the capsules should allow the free diffusion of nutrients and oxygen, as well as exchange of the therapeutic proteins. Transplanted cell survival, proliferation, and function are very important [15] and therefore, vascularization by cell migration and specific biomaterial properties are required [16-18]. The formation of uniform capsules with repeatability and reproducibility is a challenge, because cell agglutination can occur and empty beads are produced during encapsulation process. Many approaches require a defined stability of the

cell capsules, which has to be controlled by the material properties [19].

Three-dimensional (3D) organ architecture and extracellular matrix (ECM) constituents influence liver cell functions, differentiation and tissue formation as well as diseased liver treatment and reconstruction [20, 21]. The surface properties, including ECM and growth factors (GFs), are important for interactions between the host proteins and cells [22]. If the capsule has an organ 3D structure, GF and ECM, a synergistic effect for liver treatment can be obtained. However, some encapsulation biomaterials do not have native ECM and GFs, and most biomaterials are used as a powder or solution. Therefore, the surface properties and organ 3D structural effects are not available for this type of cell delivery system. Moreover, it is impossible to form a native 3D structure with the currently available tools. Therefore, biomaterials containing ECM, GFs and a native 3D microstructure should be developed for encapsulation.

A decellularized liver scaffold (DCLS) prepared from native liver is an appropriate biomaterial for hepatocyte encapsulation. Decellularization eliminates all cell types from the organ but retains the native 3D organ structure composed of ECM. DCLS has a micro 3D liver structure, vasculature, native ECM, GFs and biocompatibility [23]. In addition, the DCLS maintains glycosaminoglycan (GAG) that has a role in cell adhesion and GFs preservation [24]. DCLS contains many pores (spaces left after the removal of cells) with a size between 10–100 μm, which allows nutrient and oxygen diffusion and is useful for cell culture and blood vessel formation. Previous studies of artificial livers using DCLS indicated that injected or perfused cells diffused into the liver parenchymal region [25-27]. Therefore, cells can be injected into the pores to form a cell mass within the DCLS. This procedure is similar to encapsulation because the wall structures of the DCLS surround the injected cells. Of note, using this method there is no need for mechanical and chemical processes for cell encapsulation and a two-step process, encapsulation and cell injection, is sufficient.

We previously established a DCLS manufacturing method with cultured cells and identified its characteristics [28]. In this study, we used the encapsulation method of applying pieces of DCLS for hepatic cell delivery.

MATERIALS AND METHODS

Manufacture of DCLS pieces

Adult Sprague–Dawley rats (weight, 170–250 g, Samtako BIO Korea Co., Seoul, South Korea) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After the induction of anesthesia, 200 U of heparin (Chungwae Pharma Co., Seoul, South Korea) was systemically administered. The portal vein was cannulated using a 24 or 26-gauge cannula and the liver was perfused with 30 mL of heparinized phosphate-buffered saline (PBS) to clear blood from the liver. The inferior vena cava and superior vena cava were severed and the liver was reperfused with 70 mL of heparinized PBS to remove the remaining blood. The liver was cut into small pieces and decellularized with 0.1% sodium dodecyl sulfate (SDS) under agitation system and then the decellularized liver was chopped into 2 mm^2-sized pieces. The pieces were washed with 10% PBS to remove residual SDS for 120 minutes and sterilized with 0.1% peracetic acid (PAA, 25.6 μl/10ml) for 24 hours. The DCLS pieces were washed again with 10% PBS for 20 minutes to flush out chemical residues before cell injection. Finally, a total of 10 DCLS pieces were made and used for encapsulation.

All animal procedures were conducted in compliance with the guidelines approved by our Institutional Animal Care and Use Committee (Kangwon National University, South Korea).

Histological analysis for DCLS

Liver samples were collected and fixed in 10% neutral buffered formalin for hematoxylin and eosin staining (H&E). The decellularized liver samples were also stained for GAGs using alcian blue and periodic acid–Schiff (PAS) stain, while staining of collagen and elastin was performed by Verhoeff-van Gieson (VVG) staining according to standard protocols.

Scanning electron microscopy (SEM)

Decellularized and native matrices were washed with distilled water, fixed in cold 2.5% glutaraldehyde for 2 hr at 4°C, washed twice in PBS, and dehydrated in an ascending series of ethanol (30, 50, 70, 90, and 100%) for 20–30 min per concentration at room temperature. Samples were transferred to a critical-point dryer and dried with CO_2. Then, samples were gold sputtered and mounted for imaging on a scanning electron microscope (Carl Zeiss, Germany).

HepG2 encapsulation and viability test

HepG2 cells were cultured and proliferated for a few days and then collected with a minimal amount of culture media. HepG2 cells (3×10^5) were injected into DCLS pieces with a 1 mm syringe.

The DCLS injected cells were cultured for 5 days and then cell viability was evaluated using a two-color fluorescence EthD-1/Calcein AM live/dead assay (Molecular Probes, UK).

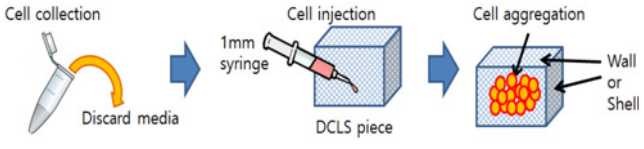

Fig. 1. Preparation procedures.

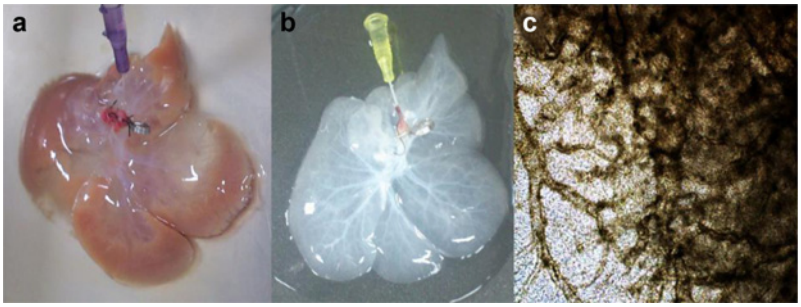

Fig. 2. Decellularization process. (a) Decellularization process. Cells were come out from rat liver by 0.1% SDS solution and some parts were begun to be transparent. (b) After finishing decellularization process, the completely transparent DCLS was made. (c) Complicated vascular structure of DCLS was observed. (X40)

Briefly, 20 μL of the supplied 2 mM EthD-1 stock solution was added to 10 mL of sterile, tissue culture–grade D-PBS to make an approximate 4 μM EthD-1 solution. The reagent was combined by transferring 5 μL of the supplied 4 mM calcein AM stock solution to the 10 mL EthD-1 solution. The resulting solution was vortexed to ensure thorough mixing. One ml of final solution was added to the culture dish and following incubation, images were captured within 30 min by microscopy.

Histological analysis for encapsulation

The DCLS pieces containing HepG2 cells were fixed in 10% neutral buffered formalin for H&E staining. Then cell images within DCLS pieces were obtained for microscopy.

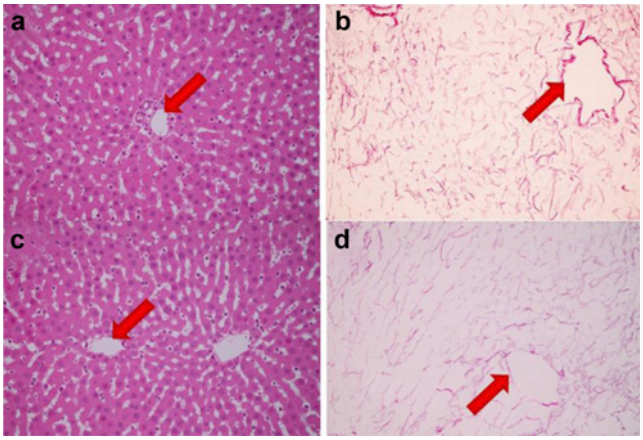

Fig. 3. H/E images of native and decellularized liver. (a and c) Native liver structure has parenchymal region and blood vessels. (a: portal triad and c: central vein, red arrows, X200) (b and d) Decellularized liver has dispersed parenchymal structure and blood vessels. (red arrows are blood vessels, X400)

RESULTS

Production of DCLS

All kinds of liver cells were removed from the rat liver during the 0.1% SDS solution treatment, leaving empty spaces surrounded a connective tissue network, producing a completely decellularized liver scaffold. The appearance of the DCLS was transparent and the internal vascular structure could be observed (Figs. 2a and 2b) with a blood vessel network of various diameters (Fig. 2c).

A comparison of H&E staining and SEM images of native liver and DCLS indicated obvious differences before and after decellularization. The native liver had many cells and normal blood vessel structure including portal triads composed of the portal vein, arteries and bile duct (Figs. 3a and 3c).

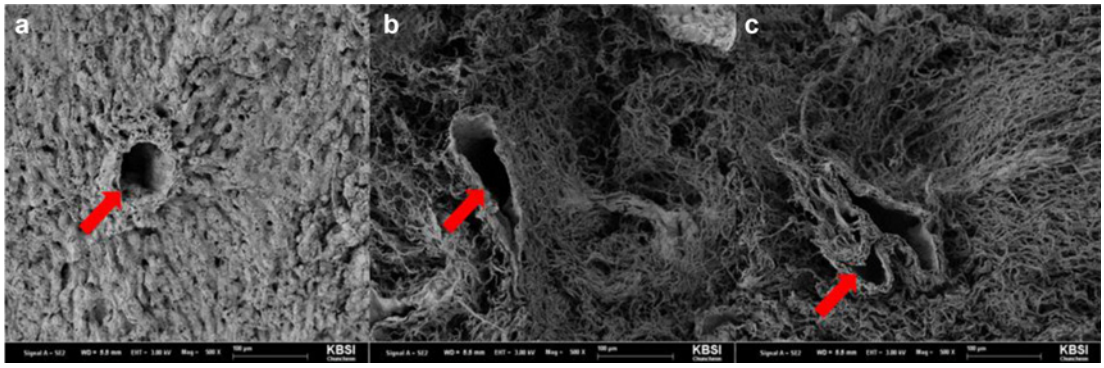

Fig. 4. SEM images of native and decellularized liver. (Red arrows are blood vessels, Bar = 100 um). (a) Native liver has dense and definite structure and cells. (b and c) Decellularized liver scaffold has disarranged net structure, pores and blood vessels. (b: central vein and c: portal triad)

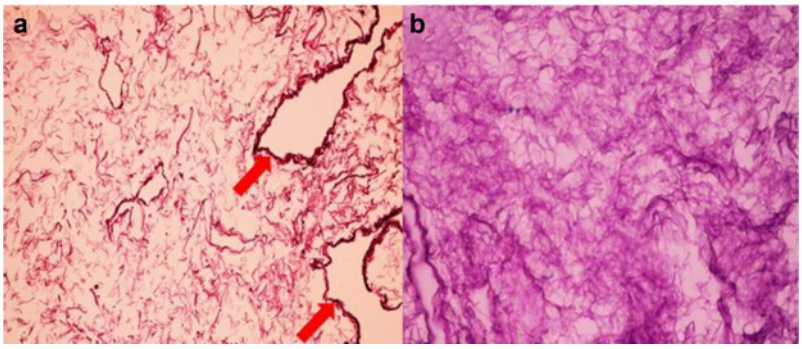

Fig. 5. ECM components of decellularized liver. (X400). DCLS has collagen and elastin (a, red arrows are blood vessels) and glucosaminoglycan (GAG, b). These are main components of liver ECM.

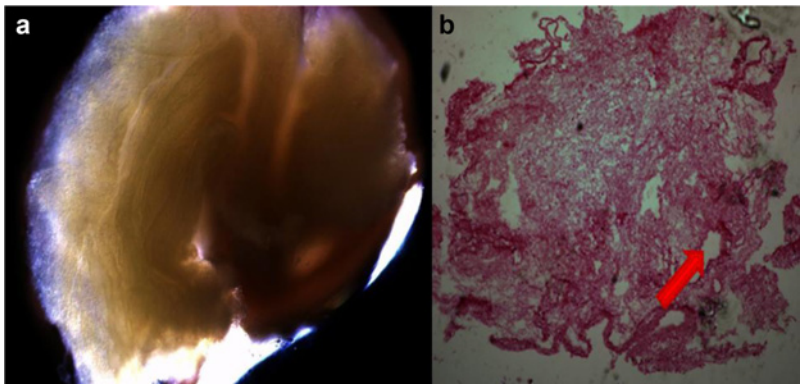

Fig. 6. DCLS piece (X40). DCLS piece with microstructure, (a) under microscopy and (b) H/E staining (red arrow is blood vessel).

Portal triad structures were also identified by SEM (Fig. 4a). The DCLS contained no liver cells but retained the micro liver structure. The empty spaces and ECM structure of DCLS indicated the scaffold porous structure (Figs. 3b and 3d). All the empty spaces were connected. Blood vessels had thick walls and large ducts and were identified easily. The SEM images of DCLS showed network structures of the ECM and the plain faces of blood vessel walls and the portal triad (Figs. 4b and 4c). The DCLS retained collagen, elastin, and GAG, the main components of the native liver ECM. Specific staining showed that elastin was only present on blood vessel walls but that collagen and GAGs were present throughout the whole DCLS structure (Figs. 5a and 5b).

HepG2 culture within DCLS pieces

Small DCLS pieces made from the whole DCLS maintained the liver ECM structure including blood vessel structures (Figs. 6a and 6b). HepG2 cells injected into the DCLS pieces formed a cell mass within each DCLS piece. Each cell mass was about 2 mm in diameter (Fig. 7a). A cell viability assay indicated green fluorescence was present throughout the whole DCLS piece and red fluorescence was not observed in any cell mass; therefore, most of the injected HepG2 cells had survived (Figs. 7b-7d). H&E staining showed that the HepG2 cells formed a dense cell mass and that the ECM

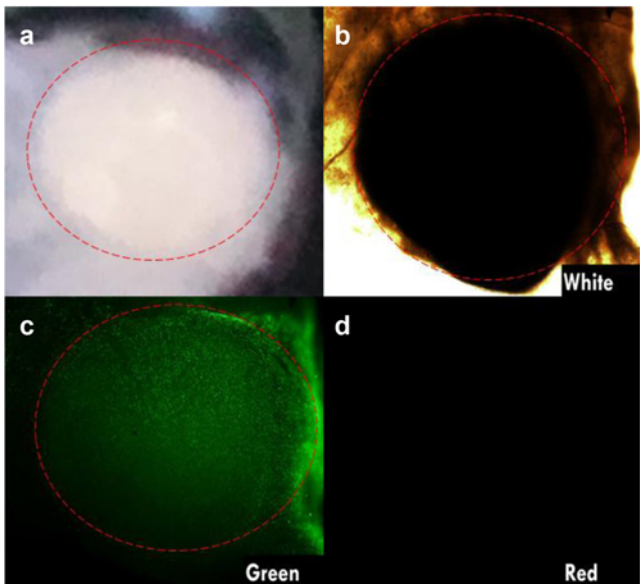

Fig. 7. HepG2 cells injection. The red doted circles are cell masses (X40). (a) Optical image of HepG2 cells injection into DCLS piece. (b-d: microscopy image) (b) Cell viability assay was applied on the DCLS piece. (c) Alive cells showed green fluorescence but (d) dead cells showed red fluorescence (red fluorescence was not detected).

structure including blood vessels was maintained between the cells (Figs. 8a-8d). This indicated that cell injection formed

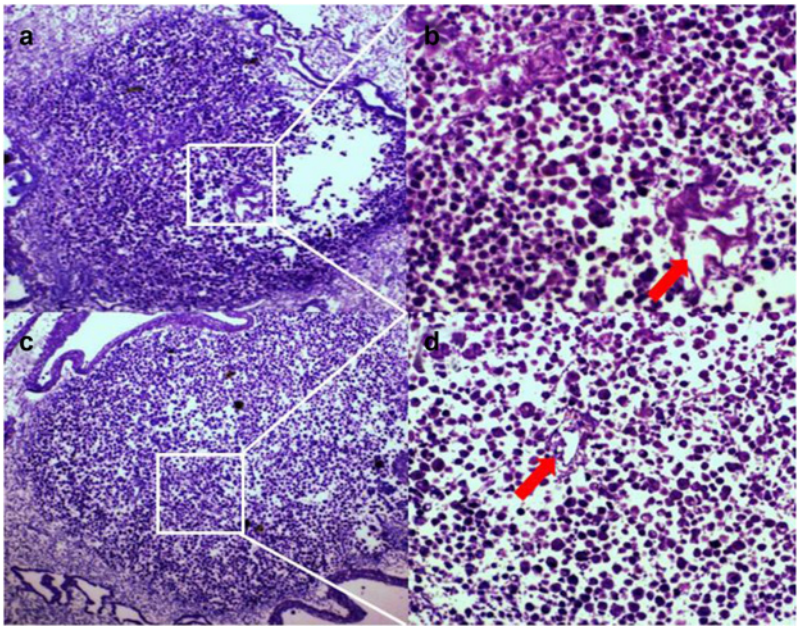

Fig. 8. Cell mass inside of DCLS pieces. (a and c, X40) The injected HepG2 cells formed cell mass inside of DCLS piece. (b and d) There are blood vessel structures (red arrows, X200) and ECM architecture.

a cell mass that was mixed within the liver ECM structure, which resembled common encapsulation structures.

DISCUSSION

Encapsulation is a mixing method that fills capsules with cells and materials through chemical and mechanical processes. Different to other encapsulation methods, cell encapsulation using DCLS pieces in this study was very simple, requiring only a chemical treatment step for decellularization, DCLS chopping and cell injection. Reduced chemical treatment and mechanical processes increases the viability and number of high quality cells for cell delivery that is important for successful encapsulation because the state of the encapsulated cells might influence the capsule properties [29].

All liver cells were eliminated from the liver tissue after decellularization, and empty spaces connected to each other were formed. However, the native liver architecture including blood vessels was maintained. The spaces left by the eliminated cells resembled pores of a scaffold and were suitable for cell injection. The natural 3D microstructure of DCLS aided cell survival, growth, and proliferation. Pores sized between 20–60 μm have an impact on vascularization and fibrotic tissue formation and lead to improved hepatocyte engraftment and sufficient oxygenation [30-32]. Thus, it might be possible to induce cell migration from native liver through blood vessels to the DCLS pieces. Previous studies have reported the diffusion distance of oxygen and nutrients *in vivo* is less than 2 mm. Therefore, we produced DCLS pieces of 2 mm^2 for *in vivo* transplantation. However, if capillary networks are present to deliver oxygen and nutrients, the diffusion distance of oxygen and nutrients can be over 2 mm [33]. The connection of blood vessels to the DCLS aids the survival of transplanted cells and the treatment and restoration of the diseased liver. Although a pathway for blood flow is not required, the results of treatment will be better if blood vessel reconstruction within DCLS pieces is obtained by through coculturing vascular endothelial cells and smooth muscle cells.

The constituent of the encapsulation material is an important factor because it influences cell survival, toxicity, and therapeutic effects. DCLS capsules contained native ECM including collagen, elastin and GAG, and some GFs [23]. These natural elements are highly biocompatible and can be incorporated, dissolved and absorbed into the liver tissue. Interactions between hepatocytes, non-parenchymal cells, and ECM and GFs leads to the rapid restoration of liver mass and regeneration, predominantly through the proliferation of the adult hepatocyte population [34-41]. GAGs are also important and form a bridge for cell attachment and GFs reservation.

Recent studies reported a decellularized material applied as a powder to encourage tissue specific remodeling and to enhance biocompatibility. Its ECM components and GFs promoted cell penetration into scaffolds [42,43]. However, when using a powder type of decellularized material additional chemical and mechanical processes are required causing a loss of bioactivity during manufacturing. Therefore, we used a simple process and the DCLS structure to encapsulate cells.

A very high cell density was used to mimic the *in vivo* cell

density and prevent numerous cell loss by large volumes of culture media. The injected cells could traffic into the spaces in the DCLS pieces and interacted with the ECM structure to form a cell mass. Histology results showed the cell mass mimicked encapsulation. Green fluorescence of the cell mass by a cell viability assay showed that the encapsulation method, cell density, and 2 mm^2 sized-DCLS pieces were appropriate for making capsules containing living cells. Although the HepG2 cells were well mixed within the DCLS architecture and encapsulated well, there is a need to innovate advanced techniques to form a stable cell mass inside the DCLS structure to diminish cell loss by leakage.

CONCLUSION

DCLS was shown to be useful for cell encapsulation as it contained native ECM, blood vessels and a native 3D microstructure. Hepatocyte encapsulation using DCLS pieces was easily formed using a one-step chemical process (decellularization) and cell injection culture. The injected cells survived within the DCLS piece and formed a cell mass mixed with the native liver ECM architecture. In conclusion, DCLS might be a beneficial material for cell encapsulation.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENTS

Jinn Hoon Ghim declares that he has no conflict of interest in relation to the work in this article. Kamal Hany Hussein declares that he has no conflict of interest in relation to the work in this article. Park K-M declares that he has no conflict of interest in relation to the work in this article. Heung Myong Woo declares that he has no conflict of interest in relation to the work in this article.

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