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Biocompatibility of Chitin Derivatives *In Vitro*

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Abstract

In this study, the *in vitro* biocompatibility of dibutyrylchitin (DBC) film, non-woven dibutyrylchitin, and non-woven regenerated chitin (RC) was assessed via the functional characteristics of adjoined L929 mouse fibroblasts. The adhesion and proliferation of the cells were observed by scanning electron microscopy (SEM). The results revealed that, among the tested three materials, L929 cells on the DBC film appeared most adhering, spreading, and proliferative. On the non-woven DBC and non-woven RC, the attached cells randomly dispersed along the fiber surfaces. The cells on the non-woven RC were, however, lower in number and mostly existed in a round shape with a lot of blebs on the cell surfaces, compared to those on the non-woven DBC. Furthermore, some of the cells on the non-woven RC also showed the disruption of their plasma membrane.

Keywords: Chitin, Derivatives, Biocompatibility, *In vitro* testing, SEM

Introduction

As an essential step for developing biomedical materials, the assessment of *in vitro* biocompatibility of biomaterials is inevitably performed. Several methods are carried out to determine the biocompatibility of materials, involving biosafety and biofunctionality areas (Kirkpatrick *et al.*, 1998). The principal of biosafety encompasses both cytotoxicity and the complicated field of mutagenesis and carcinogenesis. Cytotoxicity can be assessed by qualitative and quantitative methods (e.g., direct contact test, agar diffusion test, elution test, MTT test, DNA analysis, and membrane integrity test) (Ratner *et al.*, 1996; USP, 2000). Biofunctionality of biomaterials is assessed through functional characteristics of attached cells (e.g., cell adhesion, cell spreading, cell proliferation, and cell biosynthesis function). In general, *in vitro* tests provide rapid and inexpensive data on biological reaction and minimize the use of animal research. However, the results of *in vitro* tests may not be relevant to the implant situation.

Chitin is one of naturally abundant biopolymers. Due to its high molecular weight and crystallinity, chitin is scarcely soluble in common organic solvents, thus it is inapplicably employed. As a result, several chitin derivatives have been developed. Chitin and its derivatives have been proposed for several biomedical applications owing to their biological properties (Muzzarelli, 1977). Dibutyrylchitin (DBC), an ester derivative of chitin, is readily soluble in common solvents and fabricated into both film and non-woven forms. The treatment of DBC under mild alkaline condition yielded regenerated chitin (RC) (Szosland *et al.*, 2001; Muzzarelli *et al.*, 2004). The results on the *in vitro* and *in vivo* investigations of the biological properties of DBC and RC materials revealed that both were biocompatible (Szosland *et al.*, 2003). DBC and RC materials appeared non-cytotoxic to 3T3 mouse fibroblasts. In addition, they could accelerate a wound healing process in albino rabbits (Pielka *et al.*, 2003). The purpose of this study was to evaluate the *in vitro* biofunctionality of DBC film, non-woven DBC and non-woven RC on L929 mouse fibroblasts by scanning electron microscopy (SEM).

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Experimental

Materials

The materials used in this study were dibutyrylchitin (DBC) film, non-woven DBC, and non-woven regenerated chitin (RC), which were prepared according to the method of Muzzarelli *et al.* (2004). Each material was cut into rectangular shape at the size of 0.5 x 1.0 cm^2 before being attached onto a 35mm dish for cell culture and SEM study.

Cell culture

The cell line used in the assay was L929, mouse connective tissue fibroblast cells. The growth medium used was Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml streptomycin (Gibco™, USA). The cells were maintained at 37°C in a 5% CO_2 atmosphere. Once confluence was reached, the cells were plated onto the prepared materials in the culture dishes at the cell density of $2x10^5$ cells/ml. The cultures were maintained for given days. The culture media were changed every 3 days. After 2, 7 and 14 days of the incubation period, the materials were then prepared for the SEM observation. In this study, cover slips were used as controls.

Scanning electron microscopic (SEM) study

At each time point, the materials with the attached cells were removed from the dishes, washed with 0.1 M phosphate buffer (PB) (pH = 7.4), and fixed with 2% (v/v) glutaraldehyde in 0.1 M PB for 4 h at 4°C. The materials were subsequently washed with 0.1 M PB, dehydrated by diethyl ether, critically dried with CO_2, mounted on stubs, and sputter-coated with gold particles. SEM investigation for cell morphology on each material was made at 15 kV emission voltage and the specimen tilt angle of 0 degree on a Jeol JSM-5410 (Jeol, Japan). The experiments were performed in duplicate.

Results and Discussion

The results from the SEM investigation showed that L929 cells were able to adhere to the surfaces of all tested materials with slightly different patterns. Like those on the surface of the cover slip (Figure 1), the cells spreaded well and proliferated on the surface of the DBC film (Figure 2). On the non-woven DBC (Figure 3) and non-woven RC (Figure 4), the attached cells randomly dispersed along the fiber surfaces. The flat surface of the DBC film seemed to provide a better support for the adhesion and proliferation of the cells when compared with the cells on the round fiber surfaces of non-woven DBC and non-woven RC. As seen, numbers of attached cells on the non-woven DBC and non-woven RC were lower than those on the DBC film. At 14-day incubation time, the cells on the non-woven DBC proliferated and formed a sheet-like structure on the fibrillar network (Figure 3). Compared to those on the non-woven DBC, the attached cells on the non-woven RC were lower in number and mostly existed in a round shape. Some of these cells had a lot of blebs on the cell surfaces (Figure 4 e and f). These results suggested that the surface structure as well as the chemical composition of the materials had some effects on the cell adhesion and proliferation.

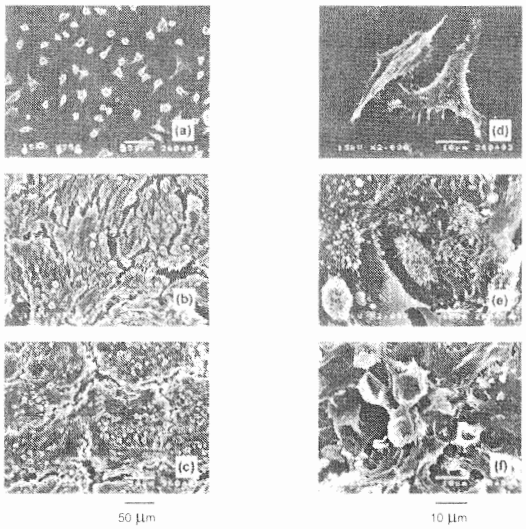

Figure 1. SEM micrographs of L929 cells on the cover slip after different incubation times: (a) 2 days, x350; (b) 7 days, x350; (c) 14 days, x350; (d) 2 days, x2000; (e) 7 days, x2000; and (f) 14 days, x2000.

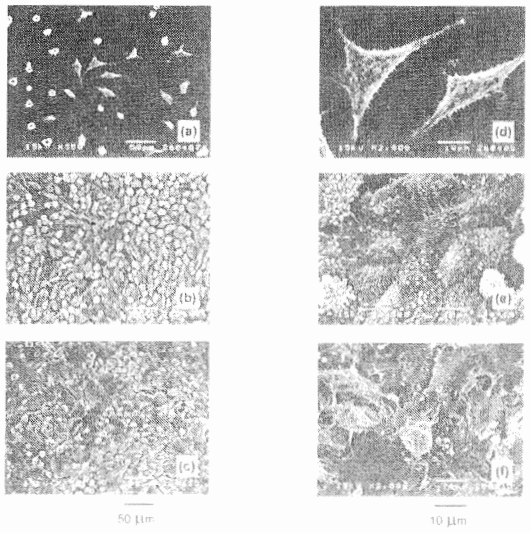

Figure 2. SEM micrographs of L929 cells on the dibutyrylchitin film after different incubation times : (a) 2 days, x350; (b) 7 days, x350; (c) 14 days, x350; (d) 2 days, x2000; (e) 7 days, x2000; and (f) 14 days, x2000.

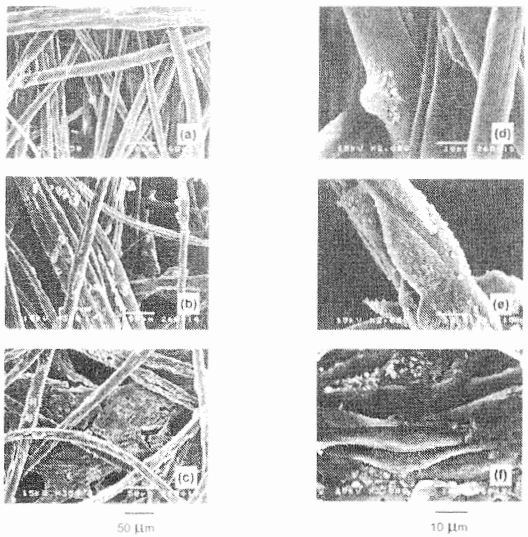

Figure 3. SEM micrographs of L929 cells in the non-woven dibutyrylchitin after different incubation times : (a) 2 days, x350; (b) 7 days, x350; (c) 14 days, x350; (d) 2 days, x2000; (e) 7 days, x2000; and (f) 14 days, x2000.

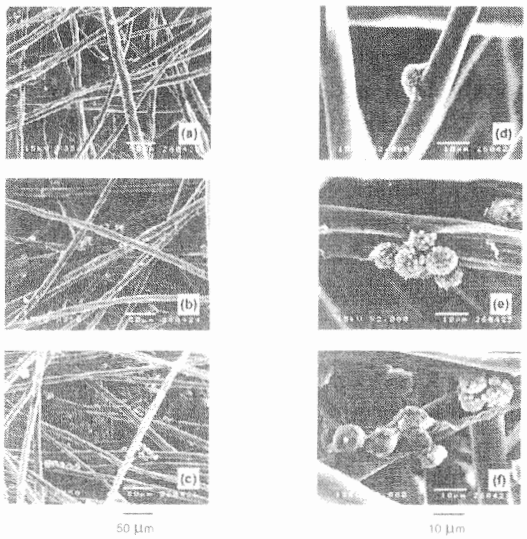

Figure 4. SEM micrographs of L929 cells on the non-woven regenerated chitin after different incubation times: (a) 2 days, x350; (b) 7 days, x350; (c) 14 days, x350; (d) 2 days, x2000; (e) 7 days, x2000; and (f) 14 days, x2000.

Intriguingly, most of the cells on the non-woven RC showed not only a lot of blebs, but also the disruption of their plasma membranes as well as collapsed filopodia. This result was probably attributed to the chemical composition of the non-woven RC, and it was worsened by diethyl ether used as a substitution for ethanol in the dehydration step. Diethyl ether obviously damaged the cell membrane and the subplasmalemmal proteins. Cells with blebs were rarely observed on the materials dehydrated by ethanol (data not shown). In this study, diethyl ether was employed as a dehydrating agent since the DBC film and non-woven DBC dissolved in ethanol.

In conclusions, the L929 cells on the DBC film appeared most spreading and grew with the highest rate of proliferation, among the tested three samples. According to Szosland et al. (2003), non-woven DBC and non-woven RC were found to be biosafe, non-cytotoxic. Consequently, it can be concluded that DBC film, non-woven DBC, and non-woven DBC were biocompatible and suitable for biomedical applications, although non-woven RC seemed to possess the least biofunctionality.

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