

# Chitosan–gelatin scaffolds for tissue engineering: Physico-chemical properties and biological response of buffalo embryonic stem cells and transfectant of GFP–buffalo embryonic stem cells

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## Abstract

The favorable cellular response of newly developed cell line, buffalo embryonic stem (ES) cells to three-dimensional biodegradable chitosan–gelatin composite scaffolds with regard to stem-cell-based tissue engineering is described. Chitosan–gelatin composites were characterized by a highly porous structure with interconnected pores, and the mechanical properties were significantly enhanced. Furthermore, X-ray diffraction study indicated increased amorphous content in the scaffold on the addition of gelatin to chitosan. To develop a transfectant of green fluorescence protein (GFP)–buffalo ES cell, transfection of GFP plasmid to the cell was carried out via the electroporation procedure. In comparison with pure chitosan, cell spreading and proliferation were greater in highly visualized GFP-expressing cell–chitosan–gelatin scaffold constructs. The relative comparison of biological response involving cell proliferation and viability on the scaffolds suggests that blending of gelatin in chitosan improved cellular efficiency. Studies involving scanning electron and fluorescence microscopy, histological observations and flow cytometer analysis of the constructs implied that the polygonal cells attached to and penetrated the pores, and proliferated well, while maintaining their pluripotency during the culture period for 28 days. Chitosan–gelatin scaffolds were cytocompatible with respect to buffalo ES cells. The study underscores for the first time that chitosan–gelatin scaffolds are promising candidates for ES-cell-based tissue engineering.

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**Keywords:** Chitosan; Scaffolds; Buffalo embryonic stem cells

## 1. Introduction

Exploring the possibility of using stem cells for tissue engineering is a potential approach in the field of regenerative and reconstructive medicine because of their ability to renew and to produce specialized cell types. Embryonic stem cells are promising for tissue engineering primarily because of their ability to differentiate into multiple tissue

lineages and their capacity to exhibit pluripotent differentiation [1]. Recent studies have indicated that embryonic stem cells have the necessary potential to provide cells of different types required for tissue replacement [1–3]. The tissue engineering approach is considered promising for repair or regeneration of damaged tissue through replacement with the engineered tissue, in the hope that it will facilitate restoring of the functions during the process of regeneration and subsequent integration with the host tissue [4]. From the perspective of material science and biomedical engineering, tissue can be considered as a cell composite consisting of cells and their extracellular matri-

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ces. The three primary tools in the regeneration of tissue include cells, scaffolds and growth factors.

In general, scaffolds provide the necessary support as artificial extracellular matrices allowing cells to proliferate and maintain their differentiated functions, and serve as a template in guiding the development of new tissue. Different kinds of scaffolds, including natural or synthetic polymer-based and polymer blends, have been considered for tissue engineering [5–10]. Currently, biomimetic natural biopolymer scaffolds have acquired significance as tissue scaffolds for tissue regeneration because of their superior biological response compared with synthetic scaffolds [11–14]. Chitosan is a linear polysaccharide derived from partial deacetylation of chitin [15]. Chitin consists of 2-acetamido-2-deoxy- $\beta$ -D-glucose through a  $\beta$  (1  $\rightarrow$  4) linkage commonly found in the shells of marine crustaceans, insects and the cell walls of fungi. Chitosan is copolymer of (1  $\rightarrow$  4)-2-acetamido-2-deoxy- $\beta$ -D-glucan (*N*-acetyl D-glucosamine) and (1  $\rightarrow$  4)-2-amino-2-deoxy- $\beta$ -D-glucan (D-glucosamine) units randomly or block distributed throughout the biopolymer chain, depending on the processing approach. The ratio of glucosamine to *N*-acetyl glucosamine of chitosan is referred to as the degree of deacetylation (DD) [16]. Chitosan is considered appropriate for biomedical and clinical applications because of its high biocompatibility, biodegradability, non-antigenicity and adsorption properties [17–22].

Recent research demonstrated that highly deacetylated chitosan scaffolds are cytocompatible with buffalo embryonic stem cells [7,23]. Interestingly, the pluripotent characteristics of buffalo ES cells could be maintained on chitosan scaffolds when cell–scaffold constructs were studied for 28 days. Furthermore, physico-chemical and biological properties of the scaffolds were promising for use as scaffolds in tissue engineering. Based on these positive findings, the present paper describes the development of constructs of buffalo ES cells and chitosan–gelatin scaffolds with the aim of improving the biological and mechanical properties compared with pure chitosan scaffolds. Gelatin is a soluble protein obtained by hydrolysis of naturally occurring collagen. Interestingly, it has a number of attributes that make it suitable as a biomaterial for tissue engineering. They include low cost, good biocompatibility, biodegradability, low immunogenicity, increased cell adhesion, migration, differentiation and proliferation. Gelatin is blended with chitosan to improve its biological activity, because it contains an Arg-Gly-Asp (RGD)-like sequence, which promotes cell adhesion, migration and forms a polyelectrolyte complex [24,25]. The backbone of gelatin has free carboxyl groups, enabling it to blend with cationic chitosan to form a network by hydrogen bonding.

The objective of the present study was to synthesize chitosan–gelatin composite scaffold, investigate the physico-chemical and mechanical properties, and study the biological response of scaffolds to buffalo ES cells. The primary aim was to develop a coherent understanding of the process–structure–functional property relationship and biological response of the scaffolds. Thus, a focused study that combines the different aspects including microstructure,

functional and mechanical properties, biodegradation profile and biological response is described here to advance understanding further in the effort to develop three-dimensional (3-D) cell-composite constructs. Buffalo embryonic cells are a newly isolated ES cell line which was previously described in Refs. [26,27]. An accompanying objective is to develop a transfectant green fluorescence protein–buffalo ES cell model, in which the cell spreading and proliferation process in 3-D scaffolds can be monitored with high sensitivity by fluorescence microscopy. Green fluorescence proteins (GFP) observed from the jellyfish *Aequorea victoria* could be used as a marker of gene expression and protein localization in living and fixed tissues [28]. In this study, the electroporation method was applied and optimized to develop GFP transfected–buffalo ES cells. Furthermore, a method is described of observing cellular responses using those highly visualized GFP-expressing buffalo ES cells. The cytocompatibility of transfected buffalo embryonic stem cells with chitosan–gelatin scaffolds is examined in anticipation of possible tissue engineering and gene therapy applications. The synthesis and experimental procedure are described in detail because the potential utility and successful application of scaffolds depends on the process adopted.

## 2. Materials and methods

### 2.1. Materials

Chitosan obtained from shrimp shell with 88% DD and  $810 \pm 20$  kDa molecular weight were provided by Bioprocess Technology Laboratory, Asian Institute of Technology, Thailand. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA (0.25% trypsin/0.53 mM EDTA) in Hank's buffered salt solution and phosphate buffer saline (PBS) without calcium and magnesium were obtained from GIBCO, Invitrogen Corporation, USA. The embryonic stem cell marker kit was obtained from Chemicon International, CA. Gelatin, acetic acid, NaOH, dimethyl sulfoxide (DMSO), lysozyme and trypan blue (0.4%) were of analytical or cell culture grade obtained from Sigma, St Louis, MO.

### 2.2. Synthesis of pure chitosan and chitosan–gelatin scaffolds

Chitosan and chitosan–gelatin scaffolds were individually fabricated by freezing and lyophilization. Two per cent (w/v) of chitosan and gelatin solution was prepared separately by dissolving in 1% (v/v) acetic acid aqueous solvent and deionized water at 37 °C, respectively. The homogenous mixture (w/w%) with different ratios of chitosan and gelatin was transferred to polystyrene Petri dishes, refrigerated at 4 °C, frozen at  $-20$  °C for 24 h and lyophilized in a freeze dryer. The resulting scaffolds (area  $2\text{ cm}^2$ , thickness 3.0 mm) were neutralized to remove acetate by immersing them in 10% NaOH followed by washing with water until neutralized, followed by lyophilization. The scaffolds are referred to from now on as C, CG21, CG11, CG12 for pure chitosan scaffold

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