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# Comparison of various types of collagenous scaffolds applied for embryonic nerve cell culture

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ABSTRACT

The purpose of the study was to confirm whether collagen-based scaffolds using different cross-linking methods are suitable elaborate environments for embryonic nerve cell culture. Three 3D sponge-shaped porous scaffolds were composed using collagen alone, collagen with chondroitin sulphate modified by 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride, and collagen cross-linked by 2,3-dialdehyde cellulose (DAC).

Embryonic nerve cells from rats were applied to the scaffolds and stained with bisbenzimide to study cell entrapment within the scaffolds. The metabolic activity of the cells cultured in the scaffolds was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

The majority of cells were differentiated into neurocytes or oligodendrocytes. Collagen and collagen-chondroitin sulphate scaffolds entrapped a low number of cells. The highest cell density was found in the collagen-DAC scaffold. Moreover, in collagen-DAC scaffolds, the metabolic activity was markedly higher than in the other samples.

Although all used scaffolds are suitable for the culture of embryonic nerve cells, the collagen-DAC scaffold properties are the most favorable. This scaffold entraps the highest number of cells and constitutes a favorable environment for their culture. Hence, the Col-DAC scaffold is recommended as an effective carrier for embryonic nerve cells.


## 1. Introduction

Both motor and sensory functions are impaired in patients with spinal cord injury (SCI). The changes are irreversible and are caused by necrosis of neurons and glial cells. Regeneration of axons is impaired [1]. A potential tissue engineering strategy thought to improve the regeneration of the spinal cord is based on the application of cells cultured within scaffolds to the site of injury. Embryonic stem cells are the source of neurocytes and oligodendrocytes in the damaged area which rearrange neural associations and induce remyelination.

Various biomaterials have been tested as scaffolds for the implanted cells. The matrices should support cell growth and the elongation of axons beyond the scaffold, as well as neurocyte reconnection and vascularization of the engrafted tissue [2,3]. Since biomaterials such as collagen are stable, non-toxic and non-immunogenic, collagen scaffolds are recommended for tissue reconstitution. Initially, the scaffold analogs of the extracellular matrix were used only as physical support for the cells [4]. However, molecules of the extracellular matrix are able to regulate the function of cells incorporated within it. The extracellular matrix comprises one-fifth of the central nervous system, but it contains only small amounts of fibrous proteins [5]. Contrary to that, high concentrations of glycosaminoglycans, mainly hyaluronan, heparin sulphate and chondroitin sulphate have been detected in the brain [6–8]. The extracellular matrix plays an important role in the regulation of stem cell differentiation, migration and proliferation during embryogenesis [9]. Integrins, the receptors for the

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extracellular matrix, are involved in the control of the nervous cell function and regulation of nervous cell migration [10]. The attachment of extracellular matrix molecules to the integrins induces signal transmission across the cell membrane, thus influencing cell organization and regulating gene expression. Huang and coworkers used a collagen-glycosaminoglycan matrix for implantation into surgical brain trauma [11]. These scaffolds promoted proliferation, migration and differentiation of endogenous neural precursor cells. The results support the idea of using collagen and glycosaminoglycans for improving nervous tissue regeneration after brain injury. Beneficial effects have been also observed for cellulose grafts in peripheral nerve regeneration [12].

The conditions in the glial scar formed after injury inhibit axon regeneration. Both the growth of the nerve cells and axon regeneration are dependent on environmental stimuli. Our preliminary experiments demonstrate that collagen (Col) scaffolds could be used for embryonic nerve cell culture [13]. The tested scaffolds are believed to serve as carriers for cells that will be implanted in the damaged area. Our previous studies mainly focused on the physicochemical and biological properties of collagen-based scaffolds cross-linked with either 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) or 2,3-dialdehyde cellulose (DAC) [13–15].

The purpose of this study is to select the material with the best biological properties for nerve tissue engineering that could serve as a carrier for the embryonic nerve cells. Therefore, three samples were selected: Col-Col, Col-CS cross-linked with EDC, and Col cross-linked with DAC. The results will indicate which scaffold has the best biological properties for engineering nerve tissue, and which could serve as a carrier for cells which would be implanted to the site of spinal cord injury.

## 2. Materials and methods

### 2.1. Materials and methods

Collagen type I was derived from porcine tendons (Euroimplant, Poland). Bi-component 3D scaffolds consisting of collagen (Col) and chondroitin sulphate (CS) were synthesized using a two-stage process: multiple freeze-drying followed by 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) cross-linking according to the methods given in our earlier work [14]. EDC has been used because it is a zero-length, non-toxic and biocompatible cross-linker that conjugates carboxylates −COOH to primary amines $-NH_2$ of collagen. Furthermore, Col was modified by a biocompatible 2,3 dialdehyde cellulose (DAC) in a reaction given earlier by Pietrucha [15]. The synthesis of the collagen-dialdehyde (Col-DAC) scaffold was performed in three steps: (i) preparation of 2,3 DAC, (ii) construction of 3D Col sponge, (iii) modification of Col sponge by solution of DAC and lyophilisation.

Comprehensive spectroscopic (FTIR), biological (collagenase degradation) and thermal (DSC) profiles, the micro-architecture (mercury porosimetry) and the amine group composition of the Col, Col-CS and Col-DAC sponge-like scaffolds were examined. A detailed description of the methods for the preparation and assessment of scaffolding has been given previously [13–15]. Some results of the physicochemical and biological characterization of the Col scaffold with and without immobilized CS, as well as the Col-DAC scaffold, are summarized in Table 1.

### 2.2. Isolation and culture of embryonic nerve tissue

Euthanized pregnant rats were used for the study. Embryos at day 17 of gestation were placed into sterile cold PBS after dissection of the uterus. Brains were removed from the embryos and rinsed with cold PBS. The meninges and blood vessels were removed. To remove the connective tissue, the samples were incubated with 1 ml of enzymatic solution composed of collagenase (1 mg/ml) and dispase (2 mg/ml) at room temperature for about 5 min. The samples were incubated with 2 ml trypsin for 5 min at 37 °C. The trypsin was neutralized with 5 ml of medium and the sample was centrifuged at 1000 rpm.

The cells were resuspended with 100 μl of medium (MACS NeuroBrew-21, Milteny Biotec, Bergisch Gladbach, Germany) the supernatant discarded and the cells gently triturated (3-5X). Cells were seeded on laminin coated dishes. The cells were cultured in MACS NeuroBrew-21 containing gentamycin (25 μg/ml) and fungizone (2.5 μg/ml).

### 2.3. Flow cytometry experiments

The cells were fixed (30 min) by incubation with Fixation Buffer (BD Cytofix) and then permeabilized by incubation with BD Phosflow Perm Buffer III. The samples were washed and centrifuged (5 min, 1000 rpm).

The permeabilized cells were stained at a temperature of 4 °C–8 °C for 30 min in Stain Buffer (BD Pharmingen), washed and centrifuged for 10 min at 2000 rpm. The cells were stained with the following antibodies: mouse anti-MAP2B conjugated with Alexa Fluor 488, mouse anti-MBP conjugated with PE (Abcam), mouse IgG2b isotype control conjugated with PE (BD Pharmingen), and mouse IgG1 k isotype control conjugated with Alexa Fluor 488 (BD Pharmingen). The stained samples were then analysed using a FACScan analytical flow cytometer (Becton Dickinson) and the results were compared with the isotype control.

### 2.4. Cells entrapment study

Cells were seeded on the scaffolds at increasing cell densities of $10^4$/well, $3 \times 10^4$/well, $9 \times 10^4$/well, and incubated for 24 h in medium (37 °C, 5% $CO_2$). The application of different cell densities allows the highest cell number that can settle within the samples to be identified. An optimal density of cells ensures closer contact between the cells and allows the formation of intercellular connections. After washing, the scaffolds containing the cells were incubated for 30 min (37 °C, 5% $CO_2$) with bisbenzimide (1 μg/ml, Sigma). After incubation, the scaffolds were washed with PBS and the stained nuclei were counted under the microscope at 100× magnification. The experiments were carried out in 96-well plates [13].

### 2.5. MTT experiments

To study the cytotoxicity of the tested samples (Col, Col-CS, Col-DAC), the cells were applied to the scaffolds in 96-well plates at a density of $5 \times 10^4$/well. The optimal conditions of the experiment were established earlier [13]. The cells were divided into four groups: control cells (seeded on laminin), cells applied to Col or Col-CS scaffolds, and cells cultured within Col-DAC materials. The cells were cultured for five or ten days. After this time, the samples were washed with PBS. MTT reagent (5 mg/ml) was applied to each well at 50 μl and incubated for 4 h at 37 °C in a humid atmosphere containing 5% $CO_2$. After removal of MTT solution, 200 μl DMSO was added to each well and incubated for 10 min. Sorensen buffer was then added to each well and the absorbance was determined at 570 nm.

### 2.6. Statistical analysis

As the data distribution was not normal, checked by Lilliefors