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# Extracellular matrix-enriched polymeric scaffolds as a substrate for hepatocyte cultures: in vitro and in vivo studies

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**Abstract**

Tissue engineering is a promising approach to developing hepatic tissue suitable for the functional replacement of a failing liver. The aim of the present study was to investigate whether an extracellular cell matrix obtained from fibroblasts-cultured within scaffolds of hyaluronic acid (HYAFF$^{TM}$) could influence the proliferation rate and survival of rat hepatocytes both during long-term culture and after in vivo transplantation. Cultures were evaluated by histological and morphological analysis, a proliferation assay and metabolic activity (albumin secretion). Hepatocytes cultured in extracellular matrix-enriched scaffolds exhibited a round cellular morphology and re-established cell–cell contacts, growing into aggregates of several cells along and/or among fibers in the fabric. Hepatocytes were able to secrete albumin up to 14 days in culture. In vivo results demonstrated the biocompatibility of HYAFF-11$^{TM}$ implanted in nude mice, in which hepatocytes maintained small well-organised aggregates until the 35th day.

In conclusion, the presence of a fibroblast-secreted extracellular matrix improved the biological properties of the hyaluronan scaffold, favoring the survival and morphological integrity of hepatocytes in vitro and in vivo.


*Keywords:* Scaffold; Cell morphology; Hyaluronic acid; Biomaterial; Cell culture; Fibroblast; Hepatocyte

## 1. Introduction

Treatment of hepatic diseases has been greatly improved by the advent and evolution of liver transplantation [1]. Yet as demand for donor organs continues to increase beyond availability, the need for alternative therapies becomes evident [1]. Several approaches, including extracorporeal devices, cell transplantation and tissue-engineered constructs, have been proposed as potential adjuncts or even replacements for transplantation [1,2].

Tissue engineering of implantable cellular constructs is an emerging cellular therapy for hepatic disease. This approach remains largely experimental and must overcome a number of significant hurdles before becoming a viable clinical modality. Hepatocytes, known to be anchorage dependent, are immobilized on scaffolds, encapsulated in aggregates, or cultured ex vivo to form liver "organoids" that are then surgically transplanted [1–3]. Hepatocytes have been implanted in many sites including the peritoneal cavity and mesentery, as well as the spleen, liver, pancreas, and subcutaneous tissues [1,4–6]. These constructs have utilized scaffolds of various chemical compositions both of synthetic and biologic composition. Synthetic scaffolds are derived from biodegradable polyesters and polysaccharides;

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biologic scaffolds from hyaluronic acid, collagen, and more complex biomatrices [1,7–12]. Alternatively, hepatocytes have been encapsulated to promote cell aggregation and liver-specific function as well as provide immuno-isolation. Encapsulation models include alginate, alginate–polylysine composites and fibers. Spheroidal hepatocyte aggregates, heterospheroids of hepatocytes and non-parenchymal cells, and co-cultures formed on in vitro templates have also been proposed as tissue organoids for implantation [1,13,14].

In recent years, tissue engineering has supplied new instruments for the in vitro reconstruction of a natural cellular environment. It is now well known that extracellular matrices like collagen layers play an important role in preventing dedifferentiation of hepatocytes in long-term culture experiments [15].

For many years, this laboratory has investigated hyaluronic acid-derived biomaterials for the creation of tissues such as epidermis, dermis and cartilage reconstructed by means of primary cell culture in a three-dimensional non-woven fabric, made from benzyl esters of hyaluronic acid (HA) (HYAFF$^{TM}$). Under these conditions, cells proliferate within the interstices of the scaffolds, maintaining their original phenotype. In particular, fibroblasts deposit extracellular components such as types I, III, and IV collagen, and adhesion molecules such as laminin and fibronectin [16–20].

Recently, the use of HYAFF$^{TM}$ as a scaffold for hepatocyte culture has been proposed by Catapano et al. [35]. In the present study, our goal was to investigate whether the extracellular matrix produced by fibroblasts cultured in HYAFF$^{TM}$ non-woven fabric constructs could influence hepatocyte morphology and metabolism. In addition, we investigated hepatocyte transplantation in the rat subdermis using the extracellular matrix enriched scaffold (EMES) as a carrier.

## 2. Materials and methods

Institutional Animal Care of the University of Padova approved the animal protocol employed in this study. Hepatocytes were harvested from 2- to 3-month-old male Wistar rats. To study in vivo survival rates, we used 15 SCID mice purchased from Charles River Laboratories (Boston, MA) and maintained in our animal facilities under pathogen-free conditions.

### 2.1. Biomaterials

Biomaterials used in the present study were derived from the total esterification of hyaluronan (synthesized from 80–200 kDa sodium hyaluronate) with benzyl alcohol, and are referred to as HYAFF-11$^{TM}$. The final product is an uncross-linked linear polymer with an undetermined molecular weight; it is insoluble in aqueous solution yet spontaneously hydrolyzes over time, releasing benzyl alcohol and hyaluronan. HYAFF-11$^{TM}$ was used to create non-woven meshes (NW) (50 μm-thick fibers; specific weight of 100 g/m$^2$). Properties of these substrata have been described in detail elsewhere (20). These devices were obtained from Fidia Advanced Biopolymers (FAB, Abano Terme, Italy).

### 2.2. Cell cultures

#### 2.2.1. Isolation

*Human dermal fibroblasts* were prepared according to a modified version of the Rheinwald & Green protocol [21]. After epithelial sheet dispase removal, dermis was cut into small pieces (2–3 mm$^2$) and fibroblasts were isolated by sequential trypsin and collagenase digestion. These cells were then cultured in monolayer conditions with DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) (Bidachem) plus 2 mM L-glutamine and (100 U/ml)/(100 μg/ml) penicillin/streptomycin (cDMEM). Medium was changed twice a week and cells harvested by trypsin treatment.

*Hepatocytes* were harvested from 2- to 3-month-old male Wistar rats. Animals were anesthetized in a chamber containing 1.5% isoflurane and 2 l of oxygen; 1 ml of pentothal 0.05 mg/ml was injected intraperitoneally. A midline laparotomy was performed, and after sectioning the vena cava, a silicon catheter was inserted in the portal vein. The liver was perfused for 9 min with 30 ml/min of calcium-free buffer solution (37 °C, pH 7.4) equilibrated with 2 l/min $O_2$ 30 min prior and throughout the procedure. Excision of the liver was completed with increasing perfusion rates up to 50 ml/min. The liver was subsequently perfused for 6 min with a buffer solution containing 0.05% collagenase and 5 mmol/l of calcium chloride equilibrated with $O_2$ at 50 ml/min. The perfusion was accelerated by 10 ml/min every minute for 9 min. Thereafter, the swollen liver was gently stirred and the resulting cells resuspended in ice-cold wash medium. This suspension was filtered through a nylon mesh (grid size: 100 μm). The cell pellet was collected by centrifugation at 50 g for 4 min, and resuspended in 50 ml of wash medium. Further purification was performed by a modified Kreamer [22]: to 12.5 ml of cell suspension, 12.5 ml of Percoll and 1.5 ml of 10 × HBS were added. The cell pellet was collected by centrifugation at 500 g for 5 min, resuspended, and washed twice before seeding into the scaffolds.

### 2.3. Cell culture in HYAFF-11$^{TM}$ scaffolds and into EMES

Pieces (1 × 1 cm) of HYAFF-11$^{TM}$ non-woven material were fixed to the bottom of the culture plates by means of a fibrin clot. The following cells were then seeded:

*Human dermal fibroblasts*: After detachment from culture plates, fibroblasts were cultured in HYAFF-11$^{TM}$ scaffolds at a density of $10^5$ cells/cm$^2$. These were cultured in DMEM supplemented with 10% FBS; bFGF (10 ng/ml) and ascorbic acid (50 μg/ml) were added fresh. After three weeks, these dermal like structures were subjected to osmotic shock (1 h in sterile $H_2O$ at 37 °C) to eliminate cells and not extracellular matrix forming the EMES.

*Hepatocytes:* Cell suspensions were collected by centrifugation at 500 g for 4 min, resuspended in 1 ml of DMEM and seeded at a density of $2 \times 10^6$ cells/cm$^2$ onto: