Analytica Chimica Acta 890 (2015) 83–90

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Electrochemical evidence for asialoglycoprotein receptor – mediated hepatocyte adhesion and proliferation in three dimensional tissue engineering scaffolds

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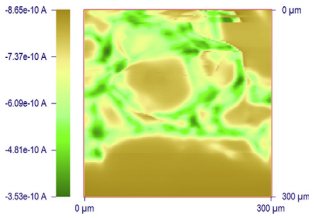
HIGHLIGHTS

- A methodology for electrochemical imaging of polymer scaffolds is proposed.
- The new methodology allows quantification of live cell activity in scaffolds.
- Hepatoblastoma cells and primary hepatocytes are studied electrochemically.
- Asialoglycoprotein receptors play a major role in hepatocyte adhesion.
- Galactose-modified scaffolds are found to promote hepatocyte proliferation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 January 2015
Received in revised form
30 April 2015
Accepted 30 June 2015
Available online 10 August 2015

Keywords:
Electrochemistry
Hepatocyte
Scaffold
Tissue engineering
Receptor

ABSTRACT

Asialoglycoprotein receptor (ASGPR) is one of the recognition motifs on the surface of hepatocytes, which promote their adhesion to extracellular matrix in liver tissue and appropriate artificial surfaces. ASGPR-mediated adhesion is expected to minimize trans-differentiation of hepatocytes *in vitro* that is generally observed in integrin-mediated adhesion. The aim of the present study is to verify the role of ASGPR in hepatocyte adhesion and proliferation in scaffolds for hepatic tissue engineering. Scanning Electrochemical Microscopy (SECM) is emerging as a suitable non-invasive analytical tool due to its high sensitivity and capability to correlate the morphology and activity of live cells. HepG2 cells and rat primary hepatocytes cultured in Polyvinyl alcohol (PVA)/Gelatin hydrogel scaffolds with and without galactose (a ligand for ASGPR) modification are studied using SECM. Systematic investigation of live cells cultured for different durations in scaffolds of different compositions (9:1 and 8:2 PVA:Gelatin with and without galactose) reveals significant improvement in cell–cell communication and proliferation on galactose incorporated scaffolds, thereby demonstrating the positive influence of ASGPR-mediated adhesion. In this work, we have also developed a methodology to quantify the respiratory activity and intracellular redox activity of live cells cultured in porous tissue engineering scaffolds. Using this methodology, SECM results are compared with routine cell culture assays viz., MTS ((1-Oxyl-2,2,5,5,-tetramethyl-Δ3-pyrroline-3-methyl) Methanethiosulfonate) and Albumin assays to demonstrate the better sensitivity of SECM. In addition, the present study demonstrates SECM as a reliable and sensitive tool to monitor the activity of live cells cultured in scaffolds for tissue engineering, which could be used on a routine basis.

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http://dx.doi.org/10.1016/j.aca.2015.06.057
0003-2670/

1. Introduction

The liver is the largest organ of the body with excellent regenerative capacity, which is responsible for a wide range of functions including albumin secretion, urea synthesis and other multitude of functions. Toxins, viral infections, alcohol consumption and other diseases affect the liver. Once affected, it starts losing its function, which in turn affects the functioning of many other organs ultimately resulting in mortality in severe cases. The most successful therapeutic approach to liver diseases has been liver transplantation. However, with the incongruence between demand and supply arising due to limited availability of donors, alternative approaches are being explored. Tissue engineering is one of the promising approaches for liver regeneration, in which biocompatible polymer scaffolds are used to mimic the native extracellular matrix for hepatocyte adhesion and proliferation [1]. Further, the three-dimensional architecture of the scaffolds is expected to facilitate effective regeneration of the damaged liver eventually *in vivo*. For the method to be fruitful, hepatocytes should adhere, communicate and proliferate after seeding them in the scaffold *in vitro* along with the necessary nutrients and growth factors. However, these processes do not occur as expected due to the tendency of primary hepatocytes to trans-differentiate *in vitro* and lose their phenotype [2].

Several approaches have been proposed to minimize or control trans-differentiation of primary hepatocytes *in vitro*. One such approach is to modify the adhesion mechanism of hepatocytes on tissue engineering scaffolds in order to improve their phenotype stability. It has been found that if hepatocyte adhesion on scaffolds is mediated by integrin receptors on cell surface, there is more chance for trans-differentiation [3]. The hypothesis was verified by promoting cell adhesion through Asialo Glycoprotein Receptors (ASGPR) present on hepatocyte cell surface. More specifically, rat hepatocytes cultured on galactose (ASGPR-ligand) modified scaffolds show superior performance over unmodified scaffolds in terms of cell attachment, albumin synthesis and cytochrome P-450 function [4]. There are few other reports correlating galactose modification of substrates with hepatocyte adhesion, proliferation and functional integrity [5]. Despite the systematic attempts to verify the effect of ASGPR on hepatocyte regeneration *in vitro*, the research is yet to arrive at a consensus owing to functional dependence of hepatocyte on topological and biochemical cues of the scaffold. For instance, certain functions like urea synthesis do not correlate with ASGPR-mediated enhancement of other functions like albumin synthesis [4]. Nevertheless, appreciable differences in hepatocyte spheroid morphology were observed between galactose-modified and unmodified scaffolds. Hence analytical techniques and methodology to correlate morphology with metabolic activity of hepatocytes could provide deeper insight of the scenario.

Scanning Electrochemical Microscopy (SECM) is a sensitive analytical technique capable of correlating cell morphology with its activity *in vitro*. The technique employs an ultra-microelectrode probe, which scans adherent live cells in culture plate immersed in a suitable liquid medium (electrolyte solution with redox species) and acquires a reactivity map of the live cells in a non-invasive manner by monitoring intracellular oxidation–reduction processes [6]. SECM has been demonstrated to monitor respiratory activity, oxidative stress, intracellular activity, cytotoxicity, multi-drug resistance and membrane permeability of live cells cultured on flat surfaces. We have employed SECM to understand redox processes in intact plant tissues [7,8]. Quantitative applications reported for SECM include measurement of cholesterol [9], dopamine [10] and reactive oxygen species [11]. Correlation of cellular activity with cell morphology has been achieved by integrating the SECM tip positioner with the sample stage of an inverted optical microscope [12]. In the above report, cytotoxicity of menadione to hepatoblastoma (HepG2) cells has been verified using SECM by following the concentration of thiodione released by the liver cells during the cellular detoxication process. However, this methodology to correlate morphology and cellular activity of live cells is applicable only to transparent substrates but not for opaque polymer scaffolds used in tissue engineering. In addition, most of the previous reports on SECM of live cells have focused on single cell studies except a few in which the viability of isolated pancreatic islets before transplantation using glucose-induced respiratory activity was assessed [13]. For tissue engineering applications, cumulative information on viability and functional activity of live cells grown on scaffolds could provide more insights to the cell–matrix interactions. To the best of our knowledge, application of SECM for imaging live cells cultured on three-dimensional porous polymer scaffolds is yet to be reported. However, SECM has been applied to characterize surface conductivity and diffusion of redox species through porous scaffolds [14]. The fewer number of reports available on this topic could be due to the interference of scaffold parameters and morphology with cell morphology and activity during SECM measurement. In this context, we report here an appropriate SECM methodology for studying and quantifying activity of live cells cultured in porous tissue engineering scaffolds made of PVA and Gelatin. The methodology is validated by comparing the influence of PVA/Gelatin scaffolds of different composition on the behavior of live hepatocytes and hepatoblastoma (HepG2) cells cultured in them. The SECM results are then compared with cell culture assays reported independently on the scaffolds [15]. Thus the new SECM methodology is tested and validated using known scaffolds of reported behavior in order to demonstrate the sensitivity of SECM to the behavior of live cells on 3D substrates like scaffolds.

2. Experimental section

2.1. Materials

Poly(vinyl alcohol) (PVA, M_w 186 kDa) and Gelatin from Bovine skin, Type B (M_w 50 kDa) were procured from Sigma Aldrich. D-Galactose was obtained from Himedia Ltd., India, HepG2 cells was obtained from the National Centre for Cell Science (NCCS), Pune, India and primary rat hepatocytes along with thawing and maintenance medium from Invitrogen, USA. HepG2 cells were cultured in Dulbecco's modified Eagles media (DMEM, Sigma Aldrich, India) and supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotics (penicillin-streptomycin, Gibco, USA). CellTiter^{96} aqueous was purchased from Promega, USA. The albumin ELISA kit was procured from Bethyl Laboratories, USA. HGF was procured from PeproTech, USA. Ferrocenemethanol and Potassium ferrocyanide and Dulbecco's modified PBS (DPBS) were procured from Sigma Chemicals Ltd., USA). SECM measurements were performed using CH Instruments Model 920 C Scanning Electrochemical Microscope and 10 μm dia. Pt disk ultramicroelectrode (RG = 10) was purchased from CH Instruments, USA.

2.2. Methods

2.2.1. Scaffold fabrication

The hydrogel scaffolds were prepared by freeze/thaw technique with two different ratios of PVA and gelatin viz., 9:1 and 8:2 ratio. The thickness of the scaffolds was 1.00 ± 0.10 mm. The scaffolds were characterized for mechanical properties, wettability (contact