Biomaterials 129 (2017) 163–175

Contents lists available at ScienceDirect

Biomaterials

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

Essential design considerations for the resazurin reduction assay to noninvasively quantify cell expansion within perfused extracellular matrix scaffolds

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ARTICLE INFO

Article history:
Received 22 December 2016
Accepted 11 February 2017
Available online 16 February 2017

Keywords:
AlamarBlue
Bioreactor
Decellularization
Extracellular matrix
Liver
Renal

ABSTRACT

Precise measurement of cellularity within bioartificial tissues and extracellular matrix (ECM) scaffolds is necessary to augment rigorous characterization of cellular behavior, as accurate benchmarking of tissue function to cell number allows for comparison of data across experiments and between laboratories. Resazurin, a soluble dye that is reduced to highly fluorescent resorufin in proportion to the metabolic activity of a cell population, is a valuable, noninvasive tool to measure cell number. We investigated experimental conditions in which resazurin reduction is a reliable indicator of cellularity within three-dimensional (3D) ECM scaffolds. Using three renal cell populations, we demonstrate that correlation of viable cell numbers with the rate of resorufin generation may deviate from linearity at higher cell densities, lower resazurin working volumes, or longer incubation times that all contribute to depleting the pool of resazurin. In conclusion, while the resazurin reduction assay provides a powerful, noninvasive readout of metrics enumerating cellularity and growth within ECM scaffolds, assay conditions may strongly influence its applicability for accurate quantification of cell number. The approach and methodological recommendations presented herein may be used as a guide for application-specific optimization of this assay to obtain rigorous and accurate measurement of cellular content in bioengineered tissues.

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1. Introduction

The increasing use of cellularized three-dimensional (3D) scaffolds in regenerative medicine [1,2] has expanded biomanufacturing, originally centered on using cells to produce recombinant proteins and biological therapeutics [3], to now encompass the cells themselves—either alone or within a tissue scaffold—as a final product for pharmaceutical testing, disease modeling, or direct patient cellular therapy [4]. In response to a shift toward 3D cell culture, often carried out within bioreactors [5–8] or intricate cell-based microfluidic systems [9], there is a need to accurately measure viable cell numbers without disturbing or sacrificing the complex tissue under examination [10], which may be produced from scarce patient-specific cells [11–13] or patient-derived induced pluripotent stem cells [14]. In addition to providing information on cellular growth kinetics within these systems, noninvasive measurement of cell number is necessary to provide an accurate reference point for tissue function and phenotype. Rigorous, reproducible, and noninvasive cell counting measurements are fundamental tools needed to enable evaluation of cell-based products (matrices with cells [8]) and biofabricated

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http://dx.doi.org/10.1016/j.biomaterials.2017.02.015
0142-9612/Published by Elsevier Ltd.

tissues [7,13,15–23]. Measurement assurance of cell counting methods is an important element of process control to accurately reference cell and tissue behavior, measure tissue function on a per-cell basis, evaluate for batch-to-batch variability, and compare data across experiments and between laboratories [24,25].

Resazurin is a blue dye that is internalized by cells and metabolically reduced to the highly fluorescent pink compound resorufin (Fig. 1) that is freely released from cells [26]. The irreversible reduction of resazurin to resorufin is mediated by intracellular diaphorase enzymes [26,27] and generates a strong fluorescent signal that may be measured using a spectrophotometer to non-invasively provide a comprehensive assessment of cellular metabolic activity within a population of cells. The resazurin reduction assay is inexpensive and non-toxic to cells at low concentrations and brief incubation periods (i.e., <4 h [28]), and is therefore a useful method to indirectly measure cell proliferation kinetics. Resazurin has been used to gauge cell number within bioengineered muscle scaffolds [29], lung matrices [30,31], and kidney scaffolds [19]. The calculated viable cell number within a scaffold is determined from a standard curve in which a linear relationship is derived between cell number (on a per-volume basis) and fluorescence intensity (FI), following treatment of the examined cell population with a known volume of resazurin over a specific time-period [26,30,32]. However, the reliability of the calculated cell number using this method is dependent upon (1) a constant average metabolic activity across the cell population that does not change under the desired experimental conditions (i.e., at different evaluation time points) and (2) a constant resazurin reduction rate or, more specifically, a stable rate of FI increase.

Over the past few years, the resazurin reduction method has been used by the bioengineering and tissue engineering community for indirect measurement of total viable cell numbers present within 3D ECM scaffolds containing living cells (i.e., recellularized scaffolds) [6,30,33]. By design, perfusion bioreactor culture systems provide a technological means to deliver nutrient-rich culture medium to cells deep within 3D scaffolds that would normally be prohibited by the diffusible distance of oxygen and nutrients from the scaffold surface that interfaces with the culture medium in traditional stagnant culture. The penetration of media, and nutrients or metabolic indicators carried within it, into scaffolds within perfusion bioreactors is afforded by tiny embedded conduits within synthetic biomaterials or the retained vasculature within decellularized organ scaffolds [5,6,34]. By taking advantage of direct media delivery to growing cells within these scaffolds, via the infiltrating vasculature, resazurin may be directly delivered to cells and its reduced product, resorufin, then sampled within the recirculated media to measure metabolic activity and indicate cell number [5,6,19]. Our laboratory [6] and others [30,31] have used this approach to estimate the number of growing cells within perfusable 3D ECM scaffolds. Importantly, this noninvasive method provides valuable data to compare scaffold seeding methods or gauge the efficiency of scaffold recellularization [6], evaluate the effects of cytotoxic drugs on cell viability within scaffolds [30,31], and analyze cell proliferation during extended, continuous culture over multiple days [5,19,30].

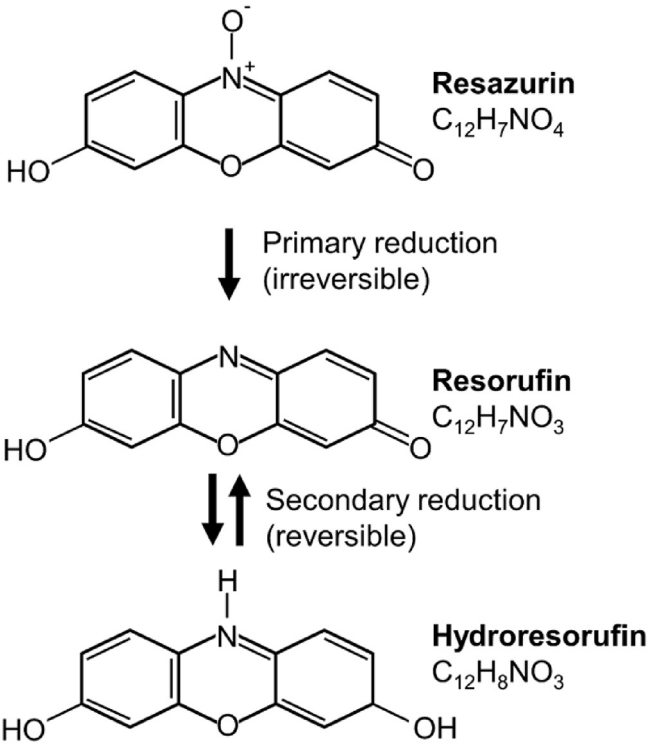

Fig. 1. Stepwise conversion of resazurin to resorufin and hydroresorufin. Resazurin (blue) diffuses into cells where it is irreversibly reduced by diaphorase enzymes to a highly fluorescent (pink) compound, resorufin. In a subsequent, reversible reaction, resorufin may be further reduced to colorless, non-fluorescent hydroresorufin [26,27].

While prior studies demonstrate that total viable cell number can be accurately correlated with FI generated over a specific time period [26,32], the resazurin reduction assay has important limitations that are often overlooked. We found that large numbers of cells and extended assay times may cause the observed reduction rate to slow at longer incubation periods by depleting the resazurin pool. This is particularly important for applications involving engineered tissues or biomaterial scaffolds with high cell densities due to the layered geometry of 3D cell culture. Moreover, while the primary reduction of resazurin to resorufin is irreversible, an important, but lesser-known, byproduct may be generated from a secondary reaction that depletes resorufin and produces the colorless and non-fluorescent hydroresorufin (Fig. 1) [26,27]. These factors may cause the resazurin assay to greatly underestimate the actual number of cells present in cell-dense biomaterial scaffolds. Therefore, the purpose of this study was to provide a comprehensive evaluation of the resazurin reduction assay, specifically to noninvasively measure cell number during growth within thick 3D ECM scaffolds. With this investigation, we share valuable insights not given elsewhere, and provide guidelines for more accurately quantifying cell number during extended cell proliferation within perfusion bioreactor systems for tissue engineering.

2. Materials and methods

2.1. Cell culture

Immortalized Madin-Darby Canine Kidney (MDCK) epithelial cells were cultured in DMEM/F12 medium with L-glutamine (Life Technologies #11320033) supplemented with 10% fetal bovine serum (FBS; Corning #35-010-CV) and 1% Penicillin-Streptomycin (Mediatech #30-002-CI). Immortalized human renal proximal tubule epithelial (RPTE; ATCC #CRL-4031) cells were cultured in Clonetics renal epithelial growth medium (Lonza #CC-3190) supplemented with an additional 10% FBS. Immortalized TK188 human fibroblasts derived from fibrotic human kidneys [35] were cultured in low-glucose DMEM with sodium pyruvate (Thermo Fisher Scientific #11885-084) supplemented with 10% FBS, 1% MEM non-essential amino acids (Corning #25-025-Cl), 1% L-glutamine (Thermo Fisher Scientific #25030-081), and 1% Penicillin-Streptomycin. Human HepG2 hepatocarcinoma cells (ATCC #HB-8065) were cultured in MEM (Gibco #11095) supplemented with