Biomaterials 35 (2014) 8065–8077

Contents lists available at ScienceDirect

Biomaterials

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

Reporter cell activity within hydrogel constructs quantified from oxygen-independent bioluminescence

Dennis Lambrechts [a, c], Maarten Roeffaers [b], Greet Kerckhofs [a, c], Johan Hofkens [d], Tom Van de Putte [e], Jan Schrooten [a, c, **], Hans Van Oosterwyck [c, f, *]

[a] Department of Materials Engineering, KU Leuven, Kasteelpark Arenberg 44 – Box 2450, 3001 Leuven, Belgium
[b] Center for Surface Chemistry and Catalysis, KU Leuven, Kasteelpark Arenberg 23, 3001 Leuven, Belgium
[c] Prometheus, Division of Skeletal Tissue Engineering Leuven, KU Leuven, Herestraat 49 – Box 813, 3000 Leuven, Belgium
[d] Molecular Imaging and Photonics, KU Leuven, Celestijnenlaan 200F, 3001 Leuven, Belgium
[e] TiGenix NV, Haasrode Researchpark 1724, Romeinse Straat 12 box 2, 3001 Leuven, Belgium
[f] Biomechanics Section, KU Leuven, Celestijnenlaan 300C – Box 2419, 3001 Leuven, Belgium

ARTICLE INFO

Article history:
Received 8 May 2014
Accepted 1 June 2014
Available online 21 June 2014

Keywords:
Hydrogel
Bioluminescence
Spatial distribution
Model
Luciferase
Oxygen

ABSTRACT

By providing a three-dimensional (3D) support to cells, hydrogels offer a more relevant *in vivo* tissue-like environment as compared to two-dimensional cell cultures. Hydrogels can be applied as screening platforms to investigate in 3D the role of biochemical and biophysical cues on cell behaviour using bioluminescent reporter cells. Gradients in oxygen concentration that result from the interplay between molecular transport and cell metabolism can however cause substantial variability in the observed bioluminescent reporter cell activity. To assess the influence of these oxygen gradients on the emitted bioluminescence for various hydrogel geometries, a combined experimental and modelling approach was implemented. We show that the applied model is able to predict oxygen gradient independent bioluminescent intensities which correlate better to the experimentally determined viable cell numbers, as compared to the experimentally measured bioluminescent intensities. By analysis of the bioluminescence reaction dynamics we obtained a quantitative description of cellular oxygen metabolism within the hydrogel, which was validated by direct measurements of oxygen concentration within the hydrogel. Bioluminescence peak intensities can therefore be used as a quantitative measurement of reporter cell activity within a hydrogel, but an unambiguous interpretation of these intensities requires a compensation for the influence of cell-induced oxygen gradients on the luciferase activity.

1. Introduction

Bioluminescence is the light that is generated through the enzyme-catalysed oxidation reaction of luciferase on its substrate, luciferin. This reaction enables the quantitative measurement of reporter cell activity via stable and sufficient integration of the luciferase reporter gene under control of a suitable promoter [1]. Most often the luciferase enzyme from the firefly, *Photinus pyralis*, is used as a reporter [2]. Activity of the firefly luciferase requires the presence of oxygen, ATP and Mg^{2+} to catalyse the conversion of luciferin into oxyluciferin accompanied by the release of a photon [3]. Although oxygen and ATP are frequently assumed to be readily present within the bioluminescent reporter cell assay, possible shortage in their supply may lead to an erroneous interpretation of the emitted bioluminescence intensities [4]. An approach in which possible shortages of these components can be quantified and that is able to compensate for their influence on the bioluminescence reaction, is therefore strongly required.

Various sources for the ambiguity in bioluminescent signal interpretation have already been identified and can for example be related to the positioning of the animal or sample [5], the direct effects of luciferase enzyme inhibitors (such as anaesthetics) or their indirect effects on the cardiovascular condition [6]. The latter effect would mainly influence the transport of luciferin to the reporter cells leading to a change in bioavailability of substrate for the bioluminescence reaction [4]. Not only the transport of luciferin via the blood has been suggested as possible mechanism for the

* Corresponding author. Celestijnenlaan 300C – Box 2419, 3001 Leuven, Belgium.
** Corresponding author. Kasteelpark Arenberg 44 – Box 2450, 3001 Leuven, Belgium.
E-mail addresses: Jan.Schrooten@kuleuven.be (J. Schrooten), Hans.VanOosterwyck@kuleuven.be (H. Van Oosterwyck).

http://dx.doi.org/10.1016/j.biomaterials.2014.06.002
0142-9612/

modification in bioluminescence signal dynamics, also changes in geometry of the multi-cellular tissue model [7] or the introduction of a hydrogel carrier [8] could strongly interfere with the observed dynamics.

Application of a hydrogel provides a 3D structural support for the cells which is more suitable for mimicking a physiologically relevant micro-environment to the cells [9,10]. To create such a compatible environment, a multitude of different features (degradation, bioactivity, mechanical properties) can be engineered into the hydrogel [11]. Patterning approaches have thereby enabled independent integration and fine spatiotemporal control of these features *in situ*, such as the covalent photopatterning of thiol-containing functional groups within poly(ethylene glycol) (PEG) hydrogels that are produced via click chemistries [12]. Owing to the complexity of the 3D cell micro-environment and the numerous cues that exist, multifactorial methods are necessary to investigate these features and their combinations in high-throughput, e.g. by using robotic technology to simultaneously control type and concentration of spotted biomolecules and substrate stiffness [13,14]. Hence hydrogels have been proposed as screening platforms to identify the instructive and regulatory mechanisms of various biochemical and biophysical cues on the fate of encapsulated cells [8,15,16]. Although their 3D micro-environment can be controlled through various modification and integration strategies, they come with some major design challenges that are associated with the adequate supply of oxygen and nutrients [17]. Unfortunately these limitations are also applicable to the availability of luciferin substrate, therefore strongly complicating the analysis of the bioluminescent screening assay [18].

Motivated by the challenges of unambiguous bioluminescent signal interpretation and the significant variability that exists in performing bioluminescence measurements, several authors have explored the use of additional measurement parameters and assays to compensate for these effects. These measurements should then be performed in addition to the standard quantification of bioluminescence peak intensities. Among the reported analyses are the integrated signal intensities (or 'Area Under the Curve', AUC), peak times, parameters related to signal dynamics, and parallel orthogonal assays. Integrated intensities and AUCs have been identified as more suitable read-outs to match data obtained from anaesthetized with unanaesthetized conditions [6]. However, this parameter could not entirely compensate for the inhibitory effect on the luciferase activity, and in addition prolonged acquisition times would be required to obtain a value for this parameter. Peak times have been correlated with the size of multi-cellular spheroids [5]. Pharmacokinetic modelling approaches combined with bioluminescent signal dynamics has allowed for a better appreciation of the bio-distribution and availability of luciferin substrate within different cell culture systems [7,19]. And finally, orthogonal assays have been proposed to assess specific interference of chemical compounds with luciferase reporter activity or to show reporter-independent activity [2,20].

These strategies are better to deal with the variability in bioluminescent assays, but are based on the assumptions of a uniform presence and homogeneous distribution of reporter cells within the multi-cellular structure and the assumption of an easy accessibility to oxygen and luciferin. This study therefore aims at investigating the effects of cell spatial distribution (cells seeded uniformly or in monolayer configuration), hydrogel dimensions (diffusion distance), and cell-mediated oxygen gradients on the emitted bioluminescent light within an agarose hydrogel. We test the hypothesis that oxygen-independent bioluminescence intensities can be used as a quantitative measure of bioluminescent reporter cell activity within a hydrogel. Our strategy is based on the use of an experimentally validated mathematical model that we previously developed to describe the average photon flux emitted from bioluminescent reporter cells embedded within an agarose hydrogel [18]. We will further validate this model for the different hydrogel dimensions and seeding distributions. Finally, we will demonstrate how oxygen-independent, model-based bioluminescence intensities can be obtained that can be correlated to viable cell numbers.

2. Materials and methods

2.1. 293T cell culture

Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Invitrogen, Merelbeke, Belgium) supplemented with 10% irradiated foetal bovine serum (Gibco), and 1% antibiotic-antimycotic (A/A) solution (100 units ml^{-1} penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Invitrogen). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Medium was refreshed every 2–3 days and cells passaged when sub-confluent.

2.2. Cell transduction

293T cells were transduced with a lentiviral vector (pCH-EF1a-3flag-fLuc-T2A-eGFPIres-Bsd, 3.1×10^8 TU ml^{-1}), which was a kind donation from Dr. Greetje Vande Velde (MoSAIC, KU Leuven). The day before transduction, cells were seeded in a 96-well plate at 1×10^4 cells per well. On the day of transduction, medium was replaced by DMEM containing serial dilutions of the vector and incubated for 24 h. After 24 h, medium was replaced with DMEM containing 1 μg ml^{-1} blastidicin for antibiotic selection of the stably transduced cell population, and was continued for 2–3 weeks. Transduction efficiencies were analysed by flow cytometric analysis (FACS).

2.3. Hydrogel screening setup

Low melting point agarose hydrogels (2% in DMEM, Invitrogen) containing stably transduced 293T cells were prepared in wells of a 96-well black plate (MicroWell 96-well optical-bottom plates, Nunc, Thermo Scientific). Cells were either uniformly distributed within the hydrogel or contained as a monolayer underneath the hydrogel (Fig. 1). Three different hydrogel heights (2 mm, 4 mm, and 6 mm) were prepared for both monolayer and uniformly seeded conditions. Cell were seeded at a density of 1×10^6 cells ml^{-1} for the uniform distribution, which resulted in a total cell number of 6.4×10^4 (2 mm), 12.8×10^4 (4 mm), and 19.2×10^4 (6 mm). Total cell numbers and seeding densities were equal for the monolayer distribution, which resulted in cell surface densities of 2, 4, and 6×10^5 cell cm^{-1}, respectively. For the monolayer configuration, cells were seeded in cell culture surface coated wells and allowed for cell attachment during a 3 h period. After this period the hydrogel was poured into the well and gelation was continued for 5 min. Upon completion of the gelation process, a total amount of 100 μl DMEM was transferred to the wells and the well plate was incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Oxygen measurements

Measurements of oxygen concentration were performed with sensor spots (PSt3, PreSens, Regensburg, Germany) mounted to the coverglass bottom of the optical well plate. Signals obtained from the sensor spot were transmitted via a polymer optical fibre to a fibre optic oxygen transmitter (Fibox 3, PreSens,

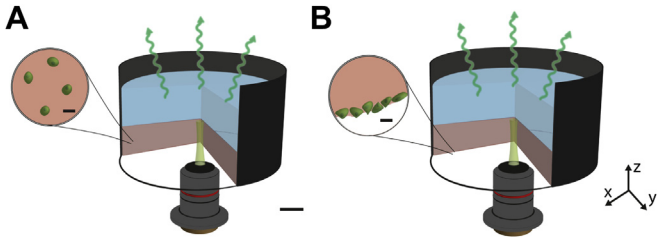

Fig. 1. Illustration of the implemented geometries for the hydrogel screening setup. Luciferase reporter cells (in green) are contained within the agarose hydrogel (in pink) either in a uniformly distributed configuration (A) or as a monolayer (B). Hydrogels are radially confined by the well borders (in black) of a 96-well plate. At the bottom position, hydrogels are confined by a coverglass base. These geometries depend on passive diffusion from the medium (in blue) that is located on top of the hydrogels as the main transport mechanism for supply of oxygen and luciferin to the reporter cells. Exogenous luciferin is added to this medium at the start of the reporter assay. Scale bar, 1 mm. Scale bar figure insets, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)