Fluorescent Labelling of Collagen Production by Cells for Non-Invasive Imaging of Extracellular Matrix Deposition

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Abstract

Extracellular matrix (ECM) is an essential component of tissues and provides both integrity and biological cues for cells. Collagen is one of the major proteins found within the ECM and therefore is an essential component of all engineered tissues. Therefore in this paper we present a method for the online, real-time monitoring of collagen deposition in 3D engineered constructs. This method revolves around the modification of collagen through the addition of azide-L-proline to the cell culture media. The incorporation of the azide-L-proline into the neo-collagen produced by cells can then be detected by reaction with 10 mM of a Click-IT Alexa Fluor 488 DIBO Alkyne. The reaction was shown being specific to the collagen as little background staining was observed in cultures which did not contain the modified proline, and the staining was also depleted after treatment with collagenase and co-localisation of collagen type I staining by immunochemistry assay. Real-time, online staining of the collagen deposition was observed under different culture conditions without affecting ~~the cell metabolism or~~ proliferation. Collagen deposition was observed to be increased under mechanical stimulation; however the localisation was varied across stimulation regimes. This is a new technique for real-time monitoring of cell produced collagen and will be a valuable addition to the tissue engineering field.

Introduction

Tissue engineering aims to regenerate or repair tissues utilising a combination of cells, biomaterials and growth factors to produce tissue-like grafts *in vitro*, which can be transplanted *in vivo* at a site of injury. Currently the monitoring of extra-cellular matrix (ECM) deposition within these three-dimensional (3D) cellular constructs is highly reliant on destructive, end point techniques. Therefore non-destructive, real-time monitoring of ECM deposition would be advantageous within the tissue engineering field allowing for a decrease in the number of samples required for analysis and reducing the reliance on end-point assays.

ECM is a complex network of proteins, which surrounds cells in tissues and is essential for providing mechanical and structural support (1). These molecules are also bioactive playing an important role in fundamental cellular processes such as adhesion, proliferation, differentiation, migration and apoptosis (1, 2). ECM is therefore essential for the production of high quality tissue engineered grafts by providing integrity and ensuring a defined biological function (3). One of the major components of ECM, across all cell types, is collagen which accounts for around 30% of the body's protein. Collagen expression is widespread across a variety of tissues, with 27 types having been identified (4). Due to the abundance of collagen in tissues it has often been used as a marker for tissue quality in the tissue engineering field. Therefore a method of monitoring collagen production by cells non-invasively in tissue engineered constructs would be highly advantageous.

Currently the amount of collagen produced by cells in tissue engineered grafts has be assessed using end point assays, including hydroxyl-proline, gene and immunohistochemistry assays, which require the complete destruction of samples (5, 6). Previous research has also shown that it is possible to utilize modified proline, which makes up around 22% of collagen, as a marker of collagen synthesis in tissue engineering (7, 8). Prolines have previously been modified in a variety of ways including radioactive and non-radioactive isotopes and more recently azide-modified (9-11). The incorporation of azide modified prolines has previously been shown not to affect the formation of the collagen triple helix and can be detected using a fluorescent alkyne which allows for the imaging of collagen deposition (9, 12). However these approaches currently require the destruction of the tissue engineered grafts and therefore do not allow for online, continuous monitoring of tissues *in vitro*.

In this paper we outline a method for the continued monitoring of collagen deposition using an azide modified proline and subsequent fluorescent alkyne for detection of new synthesized collagen. This method is relatively simple and can identify cell produced collagen online, without the need for the destruction of the grafts. This technique will allow for the monitoring of tissue-engineered grafts over time, independent of cell type and target tissue and will therefore be a valuable tool for the tissue engineering community.

Materials and Methods

Biomaterials

Fluorescent poly(lactic-co-glycolic) acid

Rhodamine b isothiocyanate salt leached scaffolds were prepared as previously described (13). Briefly 2% w/v of poly(lactic-co-glycolic) acid (PLGA) 50/50 (Sigma-Aldrich, UK) was dissolved in chloroform (Fisher Scientific, UK) and solvent cast in a glass dish to produce a PLGA film. This film was then plasma coated to introduce reactive amine groups on to the surface of the PLGA which were further reacted with rhodamine b isothiocyanate to produce a fluorescently labelled PLGA. The films were then lyophilized overnight at -50 °C under vacuum.

3D PLGA scaffolds

Both unlabeled and Fluorescently labelled 3D PLGA scaffolds were produced. PLGA was dissolved in chloroform (10% w/v) and mixed with 50% sodium chloride (Sigma-Aldrich, UK) with particle diameter size of 100–150 μm. The mixture was then placed in an 8 mm diameter mould and the solvent left to evaporate. Once dried the PLGA, salt mix was removed from the mould and cut into discs with a 3 mm thickness. The discs were then placed in distilled water for 1 hour under agitation using a magnetic stirrer at 200 rpm to leach the sodium chloride. The scaffolds were once again lyophilized overnight at - 50°C

under a vacuum. The resulting PLGA scaffolds were observed to have a pore size of between 100-150 µm and a porosity of 80%. Before use the PLGA scaffolds were sterilized in 100% isopropanol (Fisher Scientific, UK) for 30 minutes and washed thoroughly in phosphate buffered saline (PBS; Lonza, UK) to remove residual alcohol**.**

Azide proline preparation

N-Boc-cis-4-azido-L-proline (Sigma-Aldrich, UK) was deprotected through the removal of the tert-butyloxycarbonyl (BOC) group using the strongly acidic cation exchange resin Amberlyst 15 (Sigma-Aldrich, UK) as previously described (14). Amberlyst 15 was initially cleaned with methanol (Fisher Scientific, UK) for 24 hours and subsequently neutralized with 4M ammonia (Fisher Scientific, UK) in 50% methanol. The resin was then re-acidified in 3M hydrochloric acid (Sigma-Aldrich, UK) in 50% methanol (Fisher Scientific, UK) and washed sequentially in methanol, tetrahydrofuran (THF; Sigma-Aldrich, UK) and dichloromethane (DCM; Sigma-Aldrich, UK). 1 g of n-Boc-cis-4-azido-L-proline was dissolved in 20 mL of DCM and added to 2.5 g of Amberlyst 15 and stirred for 14 hours. The resin was then separated from the DCM and washed with hexane (Fisher Scientific, UK), THF and methanol. 10 mL of 4 M ammonia in 50% methanol was then added to the resin and mixed for 30 minutes. 20 mL of THF was subsequently added, the resin removed and the solvents allowed to evaporate. The deprotected azido-L-proline was then resuspended in distilled water, ready for addition to the cell culture media.

Cell Culture

Osteosarcoma cell line, MG63 (passage number 15) and SAOS-2 (passage 10), were utilised for this study. Cells were expanded in basal media: high glucose (4.5 g/L) Dulbecco's Modified Eagle Media (DMEM) (Lonza, UK) supplemented with 10 % foetal calf serum (FCS; Biosera, UK), 2 nM L-glutamine (Lonza, UK), and 25 U Penicillin/25 U Streptomycin (Lonza, UK) at 37°C in humidified air with 5% CO2.

2D Culture

MG63 were cultured in 6 well plates at a density of 5,000 cells/cm^2 and cultured in osteogenic media: low glucose (1 g/L) DMEM supplemented with 10 % FBS, 2 mM L-glutamine, 25 U Penicillin/25 U Streptomycin, 0.1 µM dexamethasone (Sigma-Aldrich, UK), 50 µM ascorbic acid-2-phosphate (Sigma-Aldrich, UK) and 10 mM ß glycerco-phosphate (Sigma-Aldrich, UK). Azido-L-proline was added into the cell culture media at a concentration of 36 µg/mL.

3D and bioreactor culture

MG63 were statically seeded on to either fluorescent or non-fluorescent PLGA scaffolds. Briefly 1 x10^6 MG63 were suspended in 50 µL of basal media and evenly pipetted on to scaffold (Ø 8 mm; depth 3 mm). The cells were allowed to attach for 90 minutes at 37°C in

humidified air with 5% CO_2 before osteogenic media was added to the scaffold. Once again 36 µg/mL of azido-L-proline was added to the culture media.

24 hours after seeding on to the PLGA the scaffolds were moved to bioreactor culture systems with control scaffolds cultured statically in 24 well plates. Two bioreactor conditions were utilised in order to assess collagen deposition under different culture conditions (Figure 1). Cyclic hydrostatic pressure was applied to the MG63 seeded PLGA scaffolds using a custom-made bioreactor designed and built in a collaboration between Professor Alicia El Haj (ISTM, Keele University, UK) and Tissue Growth Technologies (Minnetonka, MN, USA), as described previously (15). Cyclic hydrostatic was applied at a frequency of 1 Hz between a pressure range of 0 and 279 kPa, for 1 hour daily over the 10 day differentiation period. A Quasi-vivo bioreactor (Kirkstall Ltd, UK) was used to apply shear stresses to the constructs. A flow rate of 0.5 mL/minute was used to apply shear forces to the MG63 on the scaffolds. In all systems complete media changes were performed every 2 days.

Detection of azido-L-proline modified collagen.

Detection of the azido-L-proline modified collagen was performed using a commercially available alkyne by a modified protocol based on that described by Amgarten *et al* and can be seen in Figure 2 (9). Constructs were removed from culture as washed in phosphate buffered saline (PBS) to remove any FBS from the constructs. Constructs were then placed in serum

free media: High glucose DMEM supplemented with 2 mM L-glutamine and 25 U Penicillin/25 U Streptomycin. 10 µM of Click-IT Alexa Fluor 488 DIBO Alkyne (Life Technologies, UK) was then added to the media and the constructs were incubated for 1 hour at 37°C in humidified air with 5% CO_2 in the dark. The constructs were then washed four times with PBS and then placed back into culture media. Confocal laser scanning microscopy (CLSM) was employed to visualize the fluorescently tagged collagen. 3D visualisation of samples was achieved by scanning sections of 10 µm intervals, over a range of 500 µm from bottom to top (z-axis) with a x10 long working distance, air lens (Olympus, UK). Individual imaging parameters used for the analysis, i.e. confocal aperture, photomultiplier tube (PMT), offset and gain, were maintained throughout the experiment and across groups.

Staining Specificity

Collagen quantification

Collagen quantification was performed on pre-stained, papain digested constructs. Before the collagen assay was performed the fluorescence of the digest was assessed at Ex: 480 nm/Em: 520 nm. 50 µL of picrosirius red was added to 50 µL of digest and left to react for 10 minutes. The solution was then centrifuged at 12,000 rpm for 10 minutes, and the resulting supernatant removed. The pellet was then washed in distilled water and centrifuged again. This washing process was repeated until the supernatant was clear. 50 µL of 1 M NaOH (Sigma-Aldrich,

UK) was then added to the pellet and left for 30 minutes to dissociate the picrosirius dye from the collagen. The optical density of the resultant solution was then analysed at 490 nm. A rat tail collagen type 1 standard curve ranging from 1 µg/mL to 1 mg/mL (First Link, UK) was also prepared.

Collagenase treatment

The 2D samples were treated with 1 mg/mL of collagenase b (Sigma-Aldrich, UK) for 30 minutes before staining with Click-IT Alex Fluor 488 DIBO Alkyne. Staining then proceeded as previously described and samples were fixed and counterstained with 50 µg/mL 4,6-Diamidino-2-phenylindole (DAPI) (Sigma Aldrich, UK).

Immunohistochemistry

Immunohistochemistry was used to counterstain collagen type I deposition. Collagen expression was investigated using a commercial antibody, sheep anti-collagen type I (5 µg/mL) (R&D systems, UK), mouse anti-osteocalcin (10 µg/mL, AbCam, UK) and detection was carried out with a complementary secondary TRITC conjugated antibody (R&D Systems, UK).

Short interfering RNA experiments

Short interfering RNA (SiRNA) (Qiagen, UK) was used to knockdown the expression of collagen type I, V and X expression in SAOS-2 cells, as these are the most prominent

collagens expressed within this cell line (16). SAOS-2 cells were chosen for knockout experiments due to the relatively low numbers of collagens expressed by this cell line when compared to MG63 . SAOS-2 cells were seeded in 12-well plates ($4x10^4$ cells/cm^2) after 24 hours media was changed and 5 nM of each collagen SiRNA was added per test well with 3 µL of HiPerfect transfection agent (Qiagen, UK). SiRNA death and scrambled control wells were also run in parallel. When cell death was observed in the death control (after 48 hours), 36 µg/mL of azido-L-proline to the cells and incubated for 72 hours for collagen to be deposited. The collagen deposition by the cells was imaged fluorescently using Click-IT Alexa Fluor 488 DIBO Alkyne.

To assess gene down regulation RNA was extracted using a RNeasy mini Kit (Qiagen, UK) and cDNA was synthesised using a high capacity RNA to cDNA master mix (Life Technologies, UK) and 100 ng of RNA per reaction. Real time PCR was performed using QuantiTect primers (Qiagen, UK) for collagen type 1A1 (QT00037793), collagen type 5A1 (QT00044527) and collagen type 10A1 (QT00096348). Results were analysed using the ΔΔct method in relation to the housekeeping gene, 18S (QT00199367; Qiagen, UK) expression. The ΔΔct value was then normalised to levels of expression in untreated SAOS-2 cells.

Quantification of cellular proliferation ~~and metabolism~~

DNA content was quantified using PicoGreen at day 10 both in untreated and treated samples. Samples were digested overnight at 60 °C in 300 µL of papain digestion solution (5

U/mL papain from papaya latex (Sigma-Aldrich, UK), 6 mM n-acetyl-L-cysteine (Sigma-Aldrich, UK) and 1 mM EDTA in 200 mM phosphate buffer pH 6.8). Before PicoGreen quantification 50 µL of digested sample was placed in a 96 well plate and the background fluorescence read at Ex: 480 nm/Em: 520 nm, to assess the fluorescence from the collagen staining. 50 µL of digested constructs were reacted with 50 µL of PicoGreen reagent and the fluorescence read again at Ex: 480 nm/Em: 520 nm the background was then subtracted from the PicoGreen samples. A standard curve of Lambda DNA was prepared ranging from 15.625-1000 ng/mL.

Cell proliferation and viability were quantified by the Alamar Blue™ assay at day 3, 5 and 7. Culture media was removed and both stained and unstained samples were washed in PBS. 300 µL of DMEM containing 10 % Alamar Blue™ reagent was added and incubated with the cells for 2 h at 37°C in a humidified atmosphere. After which, 100 µL of solution was removed and the fluorescence was quantified at 530/590 nm (excitation/emission) (BioTek Synergy II). After quantification, samples were thoroughly washed in PBS to remove any residual fluorescence from the Alamar Blue™ assay.

Statistical Analysis

All experiments were run with 5 replicates in each group. Statistical analysis was performed using ANOVA, with a Tukey T post-hoc test. All error bars are presented as a standard error of the mean. Results were considered statistically different at a significance level of 5%.

Results

Modification of collagen and Subsequent Detection

Initial experiments were carried out in both 2D and 3D, fluorescent PLGA cultures (Figure 3 and Figure 4 respectively). Fluorescence was observed in the cultures which were incubated with the azide-L-proline after secondary staining with the Click-IT, suggesting that the collagen produced by the cells had been modified with the azide-L-proline. The control group cultured without the addition of the modified proline were shown to exhibit little background staining, suggesting that the staining of natural azdies within the culture was negligible.

Staining Specificity

The specificity of the staining was assessed in three ways to ensure that the Click-IT was bound to the azide modified collagen and not to other azide containing molecules within the cultures (Figure 5). The fluorescence within the constructs and the total collagen concentration was also correlated using digested 3D constructs. This allowed for an excellent correlation between the collagen content and the amount of fluorescent staining with a Pearson's R of 0.8434 (Figure 5a).

.After collagenase treatment it was observed that the fluorescence within the cultures was observed to be decreased. DAPI staining showed no difference in staining suggesting that the

cells were still attached to the plate and that the collagenase treatment had not disrupted the cells (Figure 5b).

Secondary collagen type I staining was also performed to investigate co-localisation between both the collagen tagging technique and the expression of collagen type I. The collagen type I antibody was co-localised with the FITC tagged modified proline, showing a high specificity of the modification (Figure 5c).

Finally SiRNA experiments allowed for the knockdown of collagen I, V and X in Soas-2 cells (Supplementary data 1). This resulted in a decrease in fluorescent staining using Click-IT Alexa Fluor 488 DIBO Alkyne (Figure 5d). This therefore highlights that the level of fluorescence observed is due to the amount of collagen deposited by the cells.

Online Monitoring Collagen Deposition in Bioreactor Systems

It was observed that the collagen deposition could be monitored online through the use of repeated secondary Click-IT staining steps. The repeated staining was not shown to affect the metabolic activity of the cells in static conditions using Alamar Blue^{TM}, with no significant differences being observed between unstained and stained cells (Figure 6a). It was also observed that the DNA content in the stained constructs, while varied between bioreactor systems, was not affected by the staining process after 10 days. (Figure 6b). This indicates

that the online monitoring of the collagen deposition using modified proline and subsequent Click-IT detection had no effect on the metabolic activity or proliferation of the cells.

It was observed that the localisation of the deposition of cell produced collagen was varied between the bioreactor systems. Both of the bioreactor systems were shown to have increased collagen deposition over that of the static controls, which exhibited both lower collagen content and levels of fluorescence at Day 10 (Figure 6c and Figure 6d respectively). While there was no significant change in the quantity of collagen produced by cells cultured in the hydrostatic and flow regimes, differences in the localisation of the collagen were observed (Figure 6d). Culture within the flow bioreactor was shown to exhibit a collagen shell, with higher deposition being observed on the periphery of the construct, whereas cyclic hydrostatic pressure allowed for a more even dispersion of the collagen deposition throughout the construct (Figure 6d).

Discussion

The online monitoring of tissues during culture remains a challenge in the tissue engineering field and there is a high reliance on endpoint analysis of the cultured tissues. This paper presents a method for real time monitoring collagen deposition within tissue engineered constructs and allows for not only the quantification of collagen deposition but also the localisation of the cell produced collagen within tissue engineered constructs. This research has shown that collagen production can be continuously monitored using the azide-proline and Click-IT detection method, without affecting the viability or proliferation of the cells. This allows for the online monitoring of ECM deposition overtime on a single sample, which is an essential addition to the tissue engineering field.

Previous research has shown that azide modified proline was incorporated into collagen using HPLC and infrared spectrometry (9). It has also been shown that the formation of the triple helix is not affected by the addition of the azide modification (12). Previously azide modified prolines have been used for the imaging of collagen production however this was performed in 2D and only performed on fixed cells (9). This paper has shown that the collagen deposition can be monitored over time within the same sample, therefore allowing for a reduction in end point analysis and a single sample to be continuously monitored. The specificity of the reaction was high, as confirmed through counter immunohistological

staining and the depletion of the stain after collagenase treatment, which is essential to ensure that the fluorescent staining observed is actually the modified collagen.

Current online collagen production monitoring is based on genetic modification of cells using either a fluorescent or luciferase reporter (17-19). The advantage of these methods is it allows for the determination of the type of collagen which is expressed when compared to the proline modification method which only gives an assessment of total collagen independent of type. However genetic modification also has disadvantages as each individual plasmid or vector is species and cell dependent. There may also be addition complications if the reporter system is only transiently transfected into the cells, therefore not allowing for long term monitoring of gene expression (20). The azide proline method however can be used with any cell type or tissue to monitor collagen deposition in long term cultures. Determination of the type of collagen deposited can then be identified using immunohistochemical counterstaining as shown in Figure 5b. This technique also allows for the use of different fluorophores and utilized a commercially available Click-IT alkyne from Life Technologies and therefore allows for some rigor to the technique as this is a reproducible chemical.

This method also allows for us to monitor collagen deposition in 3D and within bioreactors using confocal microscopy. This paper has shown that it is possible to monitor the effect of

different mechanical stimulation regimes on the localization of collagen deposition on a porous 3D scaffold in real time. It is well known that cells deposit collagen in response to mechanical stimulation and that is regulated by both the magnitude and regime (21-23). The use of the perpendicular flow, Quasi-vivo bioreactor showed the formation of a collagen shell in response to the application of the shear stresses. The Quasi-vivo bioreactor did not perfuse media through the scaffold material and therefore allowed for the application of shear stress on the exterior of the scaffold as seen in spinner flask bioreactors, which exhibited matrix deposition on the periphery of the scaffolds (24). The application of cyclic hydrostatic pressure however led to a more homogeneous deposition of collagen throughout the scaffold, which is consistent to previous reports in that cells deposited collagen in response to cyclic hydrostatic pressure, also as the mechanical stimulation was observed throughout the scaffold this deposition was more uniform (25, 26). The method presented in this paper therefore allows for the visualization of collagen deposition, which is essential in tissue engineering as the formation of ECM shells or the formation of non-homogeneous ECM deposition could affect the quality of the tissue and possible lead to the formation of a necrotic core. Therefore it is essential that these conformational changes can be observed over time so as not to rely on end point analysis.

As well as allowing of the visualisation of the collagen deposition this method can also be used to semi-quantify the amount of collagen in the constructs in relation to controls, through the digestion of the constructs and the measurement of fluorescence in the digest. It was shown that there was a good correlation between collagen content and fluorescence within the digest with a Pearson's R of 0.8434. This high correlation between fluorescence and collagen content means that relative collagen deposition can be determined using azide proline incorporation. Currently total collagen content is quantified using either hydroxyl-proline assays or picrosirus red de-staining, however this cannot be achieved concurrently with visualisation due to the required destruction of the sample (5, 27). Our method however allows for visualization and subsequent relative quantification of collagen simply using the same sample and protocol. In this paper we have shown that relative quantification is possible down to 10 µg of collagen after sample digestion, this was based on a correlation between a picrosirius red de-staining protocol and the fluorescence of the PLGA digest. While lower collagen concentrations have not been assessed it can be assumed that the sensitivity of this method both for visualisation and relative quantification will be based on the sensitivity of the fluorescent equipment used during analysis.

This method therefore has many advantages and can be utilised across cell types and target tissues and does not require any specialized equipment. It also allows for online monitoring is which is an essential tool for the progression of the tissue-engineering field. This technique

has several future directions for development. Previous research has used modified prolines *in vivo* to monitor collagen production, for example Positron Emission Tomography was used to investigate collagen deposition in rats using cis-[18F]fluoro-proline (11). Therefore this technique may well be able to be utilised to assess the turnover of collagen *in vivo* and also to monitor graft remodeling *in vivo* after implantation through the reduction in fluorescence.

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