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Fluorescent imaging of endothelial cells in bioengineered blood vessels: the impact of crosslinking of the scaffold

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Abstract

Fluorescent imaging is a useful tool to monitor and evaluate bioengineered tissues and organs. However, autofluorescence emitted from the scaffold can be comparable or even overwhelm signals generated by fluorescently labelled cells and biomarkers. Using standard fluorescent microscopy techniques, a simple and easy-to-measure signal to noise ratio metric was developed, which can facilitate the selection of fluorescent biomarkers and the respective biomaterials for tissue engineering studies. Endothelial cells (MS1) expressing green-fluorescent protein and red fluorescent protein (mKate) were seeded on poly (epsilon-caprolactone)–collagen hybrid scaffolds that were prepared by crosslinking with glutaraldehyde, genipin and ethyl(dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide. All scaffolds had comparable mechanical properties, which could meet the requirements for vascular graft applications. ethyl(dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide crosslinked scaffolds had a high signal to noise ratio value because of its low autofluorescence in green and red channels. Genipin crosslinked scaffolds had a high signal to noise ratio only in the green channel, while glutaraldehyde crosslinked scaffolds had a low signal to noise ratio in both green and red channels. The signal to noise ratio was independent of the exposure time. The data show that although similar in their mechanical properties and ability to support cell growth, scaffolds crosslinked with different agents have significant differences in causing autofluorescence of the scaffolds. This result indicates that scaffold's preparation method may have a significant impact on direct imaging of fluorescently labelled cells on scaffolds used for tissue engineering.

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Conflict of interest

The authors have declared that there is no conflict of interest.

Keywords

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1. Introduction

Tissue-engineered blood vessels (TEBV) have been developed in recent years as vascular grafts. These bioengineered vessels are usually fabricated from biodegradable polymer scaffolds, seeded with endothelial cells (ECs) and smooth muscle cells (SMCs) and cultured *ex vivo* to allow the tissue to mature before implantation. However, complete maturation of the bioengineered blood vessel, characterized by the formation of confluent EC layer, migration of SMC inside the vessel wall, degradation of the scaffold material over of time and concomitant deposition of extracellular matrix by the implanted cells, usually occurs upon transplantation *in vivo* (Amiel *et al.*, 2006; Kaushal *et al.*, 2001; Lee *et al.*, 2008a, 2008b). Maturation of TEBVs is thus exceptionally complex and detailed information about each step in this process is incomplete because of the lack of appropriate methods to monitor these processes in real time. This leads to the use of destructive methods, such as histology, in order to obtain sufficient sampling during the maturation process.

To address this knowledge gap and to develop better ways to monitor blood vessel maturation, a live-cell fluorescent imaging technology was recently developed to track cells seeded on a scaffold over time (Hofmann *et al.*, 2012a). However, a limiting factor in all fluorescence imaging modalities is the scaffold's autofluorescence (AF). If the scaffold's AF in a spectral window is comparable or even stronger than the fluorescence generated by the fluorescent biomarker, it will not be possible to separate the signal of the fluorescent biomarker from the AF generated by the scaffold. Two methods have been considered to address scaffold's AF. One strategy is to utilize cell labels with fluorescent proteins that have emission wavelengths in the red to far-red spectral window (above 600 nm) such as mKate (Shu *et al.*, 2009) and mNeptune (Lin *et al.*, 2009), where scaffolds and tissues tend to exhibit lower absorption and AF (Weissleder and Ntziachristos, 2003). Another approach is to develop a method that can lower the scaffold's AF, therefore improving the contrast between 'signal' generated by the fluorescent biomarker and the background 'noise' produced by the scaffold.

The intensity and the spectral distribution of the scaffold's AF depend on several parameters such as scaffold's thickness, composition (Sun *et al.*, 2008), and fabrication method, including crosslinking reactions (Chen *et al.*, 2005; Hu *et al.*, 2009). Natural polymers are included in the fabrication of scaffolds in order to improve their biocompatibility; however, high concentrations of natural polymers such as collagen, keratin, and elastin can lead to greater AF (Hagiwara *et al.*, 2011; Roblyer *et al.*, 2009; Viegas *et al.*, 2009). Chemical crosslinking is commonly used to maintain the scaffold's integrity and increase its mechanical strength, but it may alter the scaffold's AF properties because of the incorporation of the crosslinker in the molecular structure of the biomaterial. For example, glutaraldehyde (GA) induces high AF as a result of the presence of aldehyde groups (Tagliaferro *et al.*, 1997) and genipin (GN) induces a strong red AF within collagen hydrogel

(Hwang *et al.*, 2011), which is increased with the crosslinking degree (Sundararaghavan *et al.*, 2008). Although different approaches were developed to reduce AF for histological applications, including light bleaching and chemical quenching agents (Cowen *et al.*, 1985), most of these methods are not applicable to live-cell fluorescent imaging because of cytotoxicity and AF recovery over time (Axelrod *et al.*, 1976). Thus, selecting an appropriate crosslinker to fabricate scaffold is critical in order to obtain a suitable scaffold for the live-cell fluorescent imaging. There have been relatively few studies that quantitatively compare and optimize scaffold fabrication and crosslinking processes for high-contrast fluorescent imaging involving multiple fluorescence biomarkers in cells seeded on scaffolds.

Electrospinning technology is used extensively to fabricate vascular scaffolds, with fibre diameters ranging from a few nanometres to tens of micrometers (Huang *et al.*, 2003; Naito *et al.*, 2011). To support both cell growth and the mechanical properties of blood vessels, the scaffolds are generally electrospun from composites of biodegradable synthetic polymers and natural polymers, followed by a crosslinking reaction to retain the natural polymers. For the purpose of this study, a poly(epsilon-caprolactone) (PCL)/collagen mix was electrospun and crosslinked with GA, GN and Ethyl(dimethylaminopropyl) carbodiimide (EDC) / *N*-hydroxysuccinimide (NHS) agents. The scaffold's mechanical, vascular and AF properties were compared. In order to quantify the impact of crosslinking agents on the scaffold's AF, a quick and easy-to-implement method was developed to calculate the signal to noise ratio (SNR) using standard fluorescence microscopy. The results showed that scaffold crosslinked with EDC/NHS had low AF and the highest SNR in both green and red channels, which will facilitate live-cell fluorescent imaging of bioengineered blood vessels.

2. Materials and methods

2.1. Materials

Poly(epsilon-caprolactone), with an inherent viscosity of 1.7–1.9 dl/g in CHCl₃ at 30°C, was purchased from Lactel Absorbable Polymers (Pelham, AL, USA). Collagen type I, derived from calf skin, was supplied by Elastin Products Co. (Owensville, MO, USA). Both 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and GA solution (25%) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Genipin was purchased from Wako Chemicals (Richmond, VA, USA). Ethyl(dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

2.2. Preparation of scaffold

Scaffolds were fabricated from a blended solution composed of PCL and collagen with a weight ratio of 1:1 in HFP solution. Electrospinning was conducted using a previously reported method (Lee *et al.*, 2008b), including a high-voltage power supply (Spellman High Voltage, Hauppauge, NY, USA), a syringe pump (Medfusion 2001; Medex, Inc., Carlsbad, CA, USA) and a rotating mandrel (Custom Design & Fabrication, Richmond, VA, USA). The PCL/collagen blended solution was ejected from a blunt needle, through the electrostatic field, and collected onto the stainless steel mandrel (50 mm diameter and 70 mm long for sheet scaffold; 4.75 mm diameter and 125 mm long for tubular scaffold) with a rotating rate of approximately 1000 rpm. The total concentration of PCL and collagen was

5% in HFP. The flow rate of solution, the voltage and the distance between needle tip and mandrel were set to 3.0 ml/h, 20 kV and 10 cm, respectively.

Electrospun scaffolds were crosslinked with GA, GN, or EDC/NHS at room temperature. The GA crosslinking was carried out by placing the scaffold on top of a 2.5% GA solution for 6 h. The GN crosslinking was carried out by using a 1% GN in 70% ethanol solution for 3 days. The EDC/NHS crosslinking was carried out in pH 5.0 phosphate buffered solution for 4 hours and the concentrations of EDC and NHS were both 200 mM. After being washed with deionized (DI)-water to remove un-reacted crosslinking agents or by-products, the crosslinked scaffolds were lyophilized and stored at 4°C.

2.3. Characterization of scaffolds

2.3.1. Microstructure of scaffolds—The ultrastructure of the electrospun scaffolds was observed using a scanning electron microscopy (SEM) (Model S-2260N; Hitachi Co. Ltd, Tokyo, Japan). The dried scaffolds were coated with a thin gold layer using a sputtering system (Hummer 6.2, Anatech Ltd, Denver, NC, USA). The SEM images were acquired at an accelerating voltage of 20–25 kV and a 15 cm working distance. IMAGE-PRO PLUS software (Media Cybernetics, Bethesda, MD, USA) was used to measure the fibre diameters of scaffolds. Three SEM images were taken at different locations for each sample and 20 fibres were randomly selected for measurements. In the current experiments, a 5% PCL/collagen solution was used, resulting in scaffolds with fibres of about 250 nm diameter (see the Supporting Information, Figure S1)

2.3.2. Determination of the degree of crosslinking and collagen content—The degree of crosslinking was determined by comparing the amount of primary amino groups in non-crosslinked scaffolds with that of the crosslinked scaffolds (Bigi *et al.*, 2002; Ofner and Bubnis, 1996). In 2.5% GA vapour the degree of crosslinking of scaffolds was about 25% after 6 h, in GN solution, this was about 85% after 3 days. The EDC/NHS crosslinking reaction rate was much faster than GA and GN, and about 90% of primary amine groups in the scaffold were crosslinked after reaction for 4 h.

As electrospun collagen is easily dissolved, and PCL is insoluble in aqueous solution, the collagen content remaining in the scaffold can be determined by measuring the mass loss of scaffold in DI-water. The percentage of remaining collagen was approximately $90.0 \pm 5\%$, $97.5 \pm 2.5\%$ and $95.0 \pm 5\%$ for GA, GN and EDC/NHS crosslinked scaffolds, respectively.

2.3.3. Mechanical property of the scaffolds—Dumbbell-shaped samples, 4-mm wide at the narrowest point with a length of 18 mm, were punched from electrospun scaffolds. Samples were immersed in Dulbecco's Phosphate Buffered Saline (DPBS) for 10 min and then the mechanical properties of the wet samples were measured using a uniaxial load test machine (Instron5544; Instron Corporation, Issaquah, WA, USA) equipped with a 10 N load cell. The experiment was carried out at an extension rate of 10.0 mm/min. The scaffold's stress-strain curves were recorded, according to which the Young's modulus and the tensile strength and strain at break were obtained. Three to five samples were measured for each scaffold, allowing the mean and standard deviation to be calculated.

2.3.4. Wettability measurements—Approximately 5- μ m thick scaffolds were prepared by electrospinning PCL/collagen blend solution directly on plastic coverslips, and then crosslinking with GA, GN or EDC/NHS. The scaffold's wettability was evaluated by measuring its surface contact angle with water. Briefly, 2.5 μ l of DI-water was dropped on the scaffold's surface and the droplet's profile was imaged at specific time-points using a contact angle meter (Cam 100; KSV Instruments Ltd., Espoo, Finland). Based on the images, the water contact angle was calculated by the instrument's software. For each type of scaffold (non-crosslinked (NCL), GA, GN or EDC/NHS crosslinked), eight measurements were taken at different locations, and the mean and standard deviations were calculated.

2.3.5. Suture retention strength—The suture retention of the scaffolds was determined with a uniaxial loaded test machine (Instron) as previously described (Lee *et al.*, 2008b). Briefly, the rectangular scaffold was soaked in DPBS at room temperature for 24 h, one end was fixed to the stage clamp of Instron, and the opposite end was connected to the other clamp of Instron by a suture (5-0 Prolene; Ethicon Inc., Piscataway, NJ, USA). The scaffolds were tested with a crosshead speed of 10 mm/min until the scaffold was completely torn off; the suture retention was then recorded.

2.3.6. Compliance and burst pressure strength—To check the compliance and burst pressure, one end of the tubular scaffold was sealed with a suture and the other end was connected to an inflation device (Cook Medical Inc., Bloomington, IN, USA), through which water was injected into scaffold. The luminal pressure was measured by a sphygmomanometer, which was connected to the inflation device and the diameter change of the scaffold was recorded using a digital camera. Compliance is defined according to Eqn (1) and its value was calculated within the pressure range from 80 to 120 mmHg. The luminal pressure was increased gradually until failure occurred and the burst pressure strength was recorded.

$$\text{Compliance}(\%) = [(D_{120}/D_0) - (D_{80}/D_0)] / (120 - 80) \times 100\% \quad (1)$$

here D_0 , D_{80} and D_{120} are external diameters of tubular scaffold at 0, 80 and 120 mmHg of luminal pressure.

2.3.7. Fluorescence spectrum analysis—A fluorescence spectrophotometer (FluoroMax-4; Horiba Scientific, Edison, NJ, USA) was used to obtain the fluorescence spectrum of the scaffolds. Scaffolds (approximately 300 μ m thick) were soaked in DI-water at room temperature for 24 h and then fixed on a solid sample holder and the sample was set up at a 30° angle to the excitation, to minimize reflection of the excitation light into the emission detector. For excitation scans, the emission wavelength was fixed at 450, 509, 560 and 633 nm. For emission scans the excitation wavelength was set at 350, 488, 532 and 588 nm. The slits for excitation and emission were set at 5 nm.

2.4. Cell culture

Murine MS1 ECs (American Tissue Culture Collection) were grown in Dulbecco's Modified Eagle Medium with low glucose (DMEM, purchased from Thermo Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (P/S), and maintained in a humidified 5% CO₂ incubator at 37°C.

2.5. Fluorescent imaging

MS1 cells were fluorescently labelled to express either green-fluorescent protein (GFP-MS1) or red-fluorescent protein (mKate-MS1) using a lentivirus vector. The peak of GFP absorption and emission spectra are located at 488 nm and 507 nm, respectively. mKate is red-shifted relative to GFP and has absorption and emission peaks at 588 nm and 635 nm, respectively (Shaner *et al.*, 2005; Wang *et al.*, 2008). The GFP-MS1 and mKate-MS1 cells were observed using a fluorescence microscope (DMI4000B; Leica Microsystems GmbH, Wetzlar, Germany), which was mounted with light filters (Chroma Technology Corp, Rockingham, VT) for the blue channel [hybrid 'ET' 4',6-diamidino-2-phenylindole (DAPI)/Hoechst filter set; excitation filter 307–388 nm, emission filter 435–485 nm], green channel ['ET' GFP/fluorescein isothiocyanate (FITC)/AF488 filter set; excitation filter 450–490 nm, emission filter 500–548 nm] and red channel ['ET' tetramethylrhodamine isothiocyanate (TRITC)/Cy3/DsRED filter set; excitation filter 527–558 nm, emission filter 590–652 nm]. Two imaging methods were performed and compared: one was based on traditional histological sections and the other imaged cells directly on whole scaffolds without destruction of the sample.

2.5.1. Histological analysis—Fluorescently labelled MS1 cells were seeded on scaffolds with a cell density of 4000 cells/cm². After 5 days in culture, the cell-seeded scaffolds were fixed with 4% paraformaldehyde (PFA) and embedded in optimal cutting temperature compound (OCT). Sections 8-μm thick were cut and stained with DAPI (Vector Laboratories, Burlingame, CA, USA) to visualize nuclei.

For immunostaining, the cell-seeded scaffolds were fixed with 4% PFA, rinsed twice with DPBS, dehydrated through a series concentration of ethanol/water solutions, and then embedded in paraffin. Sections 8-μm thick were stained with a GFP antibody (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the reaction was developed with 3,3'-diaminobenzidine (DAB) (Vector Laboratories).

2.5.2. Imaging cells on scaffolds—Fluorescently labelled MS1 cells were seeded on scaffolds at a cell density of about 15 000 cells/cm², and cultured overnight. Subsequently, the cell-seeded scaffolds were placed on a glass slide and sealed with a glass coverslip. For each scaffold, eight representative images were taken at different locations using the fluorescent microscope. The intensity of the fluorescence emitted by the scaffold and/or cells was determined by analysing the grey level of corresponding positions in the fluorescent microscope images using IMAGE-PRO PLUS software. The value of grey level at the cell site is defined as 'signal', and the grey level of scaffold's AF is regarded as the background 'noise'. Thus, the SNR was obtained according to Eqn (2):

$$\text{SNR} = \text{grey level at the cell location} / \text{grey level of the scaffold} \quad (2)$$

From each of the eight images five to 10 cells were chosen at random to determine the 'signal', and the scaffold's background around the cells was chosen as the 'noise'. Statistical analysis was obtained from 40 to 80 individual events.

2.6. Statistical analysis

All quantitative data were presented as mean $\pm$ standard deviation (SD). Statistical analysis (Microsoft Excel™) was carried out based on Student's *t*-test and the differences were considered significant at $p < 0.05$.

3. Results

3.1. Mechanical properties and surface wettability of crosslinked scaffolds

Polycaprolactone/collagen composite scaffolds were fabricated by electrospinning and then chemically crosslinked with GA, GN or EDC/NHS (See the Supporting Information, Figure S1), as described in the Materials and methods section and previous publication (Lee *et al.*, 2008b). Stress–strain curves were generated for the crosslinked scaffolds (Figure 1a) and the Young's modulus was calculated. The results indicated similar modulus values for all 3 crosslinking agents, about 11 MPa, suggesting that the scaffolds have similar stiffness. However, crosslinking with GN and EDC/NHS resulted in higher tensile stress values and tensile strain at break when compared to GA crosslinking scaffold (Fig. 1a), suggesting that GN and EDC/NHS crosslinked scaffolds have stronger mechanical strength.

All scaffolds had a similar wettability, as determined by the water's contact angle ($35 \pm 5^\circ$), 5 s after water placement (Figures 1b and S2). The contact angle of GA crosslinked scaffolds decreased quickly from 35° to 0° within 3 min, indicating that GA crosslinked scaffolds are the most hydrophilic and most easily wetted. The GN crosslinked and NCL scaffolds had a similar surface wettability and their contact angle decreased from 35° to 10° within 3 min. The EDC/NHS crosslinked scaffolds were the most hydrophobic and their contact angle changed only 5° within 3 min. It is possible that the EDC/NHS crosslinked scaffolds had a more compact surface (Figure S1) and thus, the water-spreading rate is slower than in the other scaffolds.

3.2. Suture retention strength, burst pressure and compliance of vascular scaffold

Suture retention was compared between scaffolds of different thickness and crosslinked with different agents. The results showed that scaffolds crosslinked with GA, GN or EDC/NHS had similar suture retention values of about 0.5 N and 1.0 N for $240 \pm 20 \mu\text{m}$ and $500 \pm 50 \mu\text{m}$ thickness, respectively (Figure 1c), which were much lower than that of fresh pig carotid artery ($700 \pm 100 \mu\text{m}$ with $3.1 \pm 1.4 \text{ N}$). However, previous studies indicated that PCL/collagen scaffolds could meet the requirement of implantation in a rabbit model (Tillman *et al.*, 2009).

The burst pressures of hydrated scaffolds showed that EDC/NHS and GN crosslinked scaffolds $240 \pm 20 \mu\text{m}$ thick had burst pressures greater than 2000 mmHg, which was slightly higher than that of the GA crosslinked scaffold (Figure 1d). This difference may be result from the fact that scaffolds crosslinked with EDC/NHS had a greater degree of crosslinking than that of scaffold crosslinked with GA. The burst pressures of scaffolds $500 \pm 50 \mu\text{m}$ thick exceeded 4000 mmHg, with no significant difference between the scaffolds, but higher than a fresh carotid artery obtained from pig. The PCL/collagen scaffolds, crosslinked with the different crosslinking agents, showed a moderate diameter increase of about $6.0 \pm 2.0\%$ as pressures increased from 0 to 200 mmHg (Figure 1e). In comparison, a greater diameter increase was observed in fresh pig's carotid artery ($31 \pm 12\%$) when pressures increased to 200 mmHg. The compliance, calculated for values between 80 and 120 mmHg, showed no significant differences between the different scaffolds (Figure 1f). The values were about 0.065%/mmHg and 0.045%/mmHg for scaffolds $240 \pm 20\mu\text{m}$ and $500 \pm 50\mu\text{m}$ thick, respectively, which was significantly lower than that of the fresh pig's carotid artery ($0.3 \pm 0.1\%$ / mmHg). Despite the lower compliance values, previous studies indicated that vascular scaffolds made from PCL/ collagen kept about 85% patency over 1 month in a rabbit model (Tillman *et al.*, 2009).

3.3. Assessment of scaffold's autofluorescence

A scaffold's AF has a significant impact on fluorescence imaging of scaffolds seeded with fluorescently labelled cells. We first measured the influence of crosslinking agents on the scaffold's AF using a standard fluorescent microscope. Figure 2 shows the grey level values for PCL/collagen scaffolds crosslinked with GA, GN or EDC/NHS. The intensity of the scaffold's AF is proportional to the brightness of each image pixel, represented by an integer in the range of 0–255. Specifically, 0 indicates that the image is completely dark (no AF is emitted) and a value of 255 indicates that the pixel is saturated. An exposure time of 250 ms was used in order to avoid saturation of the CCD camera by the scaffold's AF. The NCL scaffolds had low AF in the blue, green and red filters (channels). Glutaraldehyde crosslinking produced low AF in the blue channel, but very strong AF in the green and red channels (about 12.5- and 6.7-fold higher, respectively) compared with NCL scaffolds. Genipin crosslinked scaffolds had low AF in the blue and green channels with high AF in the red channel (similar to the GA crosslinked scaffold). Compared with the other crosslinking agents, EDC/NHS crosslinked scaffolds displayed low AF in all channels, similar to NCL scaffolds.

To examine the scaffold AF in detail, the excitation and emission spectra from the scaffolds were obtained with a fluorescence spectrophotometer. Figure 3 shows the excitation spectra: i.e. the fluorescence intensity as a function of the excitation wavelength using a fixed emission wavelength at 450 nm, 509 nm (for GFP maximal emission), 530 nm and 633 nm (close to mKate maximal emission). The 509 nm and 530 nm emission wavelengths are in the range of the green channel of fluorescence microscope (excitation filter 450–490 nm; emission filter 500–548 nm). The GA crosslinked scaffold displayed high fluorescence intensity at 509 nm and 530 nm emission wavelength, with excitation wavelengths between 450 nm and 490 nm. The other scaffolds had similar fluorescence intensity in this range of excitation wavelengths. For 633 nm emission (to simulate the red channel of fluorescence

microscope; excitation filter 527–558 nm and emission filter 590–652 nm), GN crosslinked scaffolds had the highest fluorescence intensity, between 525 nm and 560 nm, while NCL and EDC/NHS crosslinked scaffolds showed the lowest fluorescence intensity in this range. These results are consistent with the fluorescence microscope autofluorescence analysis (Figure 2). For the 450 nm emission wavelength, which is within the blue channel of the fluorescence microscope (excitation filter 307–388 nm; emission filter 435–485 nm), all crosslinked scaffolds displayed a higher intensity of fluorescence than NCL scaffolds between 307 nm and 388 nm excitation wavelength. However, GN crosslinked scaffolds had the lowest intensity of fluorescence, as measured by the fluorescence microscope, but this is likely due to the difference in the configurations between the fluorescence microscope and spectrophotometer.

Figure 4 shows the fluorescence emission spectra of the scaffolds, measured by a fluorescence spectrophotometer, at four fixed excitation wavelengths: 350 nm, 488 nm (for GFP maximal excitation), 532 nm and 588 nm (for mKate maximal excitation). These excitation wavelengths approximate the centre of the excitation filter for the standard fluorescence microscope channels. At an excitation wavelength of 350 nm, GA crosslinked scaffolds showed the highest intensity of fluorescence between 435 nm and 485 nm (corresponding to the emission filter of the fluorescence microscope blue channel: 435–485 nm), GN crosslinked scaffold displayed lower intensity of fluorescence, while NCL and EDC/NHS crosslinked scaffolds had similar intensities and the lowest intensity of fluorescence in this range. At a 488 nm excitation wavelength, GA crosslinked scaffold showed a strong emission peak around 525 nm and much higher intensity of fluorescence across the range between 500 nm and 550 nm (corresponding to the emission filter of the fluorescence microscope green channel: 500–548 nm), and the other three scaffolds displayed similar and a lower intensity of fluorescence within this range. At 532 nm excitation wavelength, GA crosslinked scaffolds had a strong fluorescence at emission under 600 nm, GN crosslinked scaffolds had a peak intensity emission at 610 nm, while NCL and NHS scaffolds showed weak fluorescence between 590 nm and 652 nm (corresponding to the emission filter of the fluorescence microscope red channel: 590–652 nm).

3.4. Fluorescent imaging of fluorescent cells seeded on vascular scaffolds

Once the autofluorescence characteristics of each scaffold were determined, MS1 cells labelled with GFP and mKate were seeded on these scaffolds. MS1 is a microvascular endothelial cell line that maintains stable fluorescent labelling over many passages. The authors (Arbiser *et al.*, 2000) and others (Li *et al.*, 2000; Kumaran *et al.*, 2005; Hong *et al.*, 2013) have successfully used the MS1 cells in various studies and observed typical endothelial behaviour and preservation of endothelial properties, including endothelium morphology and physiology. In contrast, other primary endothelial cells, such as human umbilical vein endothelial cells (HUVECs), have limited passaging capabilities and do not have a stable fluorescence and cell morphology (Bala *et al.*, 2011; Prigozhina *et al.*, 2011). The seeded scaffolds were harvested after 5 days and visualized in the blue, green and red channels (Figure 5). To facilitate detection of the cells, the nuclei were stained blue with DAPI and imaged in the blue channel to confirm that the cells grew well on all four scaffolds. In the green channel, GFP-MS1 cells were clearly visible on NCL and EDC/NHS

crosslinked scaffolds, but were hard to detect on GA and GN crosslinked scaffolds. In the red channel, mKate-MS1 cells seeded on NCL and EDC/NHS crosslinked scaffolds were clearly visible, but mKate-MS1 cells could not be detected on GA and GN crosslinked scaffolds because of the high AF of the scaffold in the red channel. To confirm the presence of GFP-positive cells on the scaffold, sections were immunostained with an anti-GFP antibody. A single layer of positively stained GFP-expressing cells was detected (Figure S3).

3.4.1. Evaluation of fluorescence signal to noise ratio of cells seeded on scaffolds

Histological examination of cells and scaffold fluorescence, as shown in Figure 5, allows for qualitative but not quantitative analyses of the data. To better quantify the fluorescence results, the grey level of the scaffold was defined as background or 'noise' and the grey level of the cells as 'signal'. In this way, the SNR can be used to quantify cell-specific fluorescence and analyse the imaging data (Hofmann *et al.*, 2012b). Similar size areas of the scaffold, with (signal) and without (noise) cells, were selected, as shown in Figure 6a, and grey level values were calculated. Using GN crosslinked scaffolds as an example, it was found that the grey level values of the cells and the scaffold were increased in a linear manner, with longer exposure times (Figure 6b), but the SNR remained the same for different exposure times (Figure 6c). Similar results were obtained with the other three scaffold types (Figure S4 and data not shown). These results indicate that the SNR was independent of the exposure time, allowing comparison of the SNR of different scaffolds using images that were obtained with different exposure times. The SNR values were further quantified from images of cells seeded on scaffolds crosslinked with different agents, similar to the image shown in Figure 6a. The NCL and EDC/NHS crosslinked scaffolds have similar SNR values – about 1.55 in both green and red channels (Figure 6d) – confirming the histological results in Figure 5. The GN crosslinked scaffold had a high SNR in the green channel and low SNR in the red channel (Figure 6d). The SNR of GA crosslinked scaffold was close to 1.05 (cell fluorescence is similar to the scaffold's AF) in both channels (Figure 6d). In addition, the effect of other parameters, such as the scaffold thickness and the ratio of PCL to collagen was tested. It was found that EDC/NHS crosslinked scaffolds had the lowest AF and highest SNR in both green and red channels when compared with the other two crosslinkers (data not shown), similar to the results shown in Figures 2 and 6.

4. Discussion

A central obstacle in tissue engineering is the inability to determine the maturity level of the bioengineered tissue without sacrificing the tissue construct for histological analyses. A major goal of many studies was to use fluorescent imaging for tracking cells seeded on a scaffold without disturbing the sample (Wang *et al.*, 2008). However, one potential obstacle for such studies is the inherent AF of many scaffold biomaterials, which interferes with fluorescence imaging of cells seeded on these scaffolds (Georgakoudi *et al.*, 2008; Sun *et al.*, 2008). In addition, preparation of scaffolds for tissue engineering may require the use of a crosslinking reaction, which may further increase the AF owing to the incorporation of the crosslinker molecules into the scaffold's molecular structure. A major goal of the current study was to quantify and compare the effects of different crosslinking agents on the AF of scaffolds. Specifically, GA, GN and EDC/NHS were used to crosslink collagen to a

polymeric scaffold, prepared by electrospinning (Lee *et al.*, 2008b). An additional objective of the current study was the development of a simple and easy-to-measure metric that can facilitate the selection of appropriate fluorescent molecules for tissue engineering studies using fluorescently labelled cells and a standard fluorescent microscope. The results of this study can be applied to other scaffold parameters in order to determine the feasibility of fluorescent molecular imaging in complex bioengineered tissues.

The peaks of GFP excitation and emission spectra are at 488 nm and 507 nm, and those of mKate are at 588 nm and 635 nm, respectively (Shaner *et al.*, 2005; Wang *et al.*, 2008). The results of the present study show that SNR values display no obvious difference between GFP-and mKate-labelled cells for EDC/NHS crosslinked scaffolds (Figure 6D), despite the fact that PCL/collagen scaffolds have lower AF in far-red than in the green region (Shu *et al.*, 2009). This can be explained by a small mismatch between the excitation filter of the red filter set (527–558 nm) and the mKate excitation peak (588 nm), resulting in an inefficient mKate-labelled cell excitation compared with an efficient excitation of the scaffold's AF. In addition, fluorescent proteins with different emission wavelengths can exhibit significant differences in brightness (Shaner *et al.*, 2005), which may become comparable to the variations in scaffold AF in different spectral channels. It was also found that the SNR of fluorescently labelled cells seeded on different scaffolds is independent of the exposure time, as long as the microscope CCD camera is not saturated (Figure 6). This result is to be expected, as the SNR metric characterizes the relative strength between scaffold AF and cell fluorescence intensities, which should be independent of the imaging instruments.

Polycaprolactone/collagen blends have been proposed to be used as vascular scaffolds because the addition of the natural polymer into the synthetic polymers can promote cell attachment and proliferation on the scaffold (Lee *et al.*, 2008b). Chemical crosslinking methods are used to attach the collagen to the synthetic polymer in order to improve its mechanical properties and avoid rapid release. Comparable mechanical properties were observed for scaffolds crosslinked with different agents, which meet the requirements for blood vessels. Cell growth on scaffolds crosslinked with GA, GN or EDC/NHS was also similar, with slightly better growth on scaffolds crosslinked with GN and EDC/NHS. However, as in most cases GA is not completely inactivated upon reaction with collagen, some unreacted aldehyde groups may be left in the scaffolds (Collins and Goldsmith, 1981) and thus may be toxic to cells. The toxicity of GA was much higher than that of GN (data not shown), supporting a previous observation. In addition, upon degradation there may be a release of unreacted crosslinking agents, which may further cause cytotoxicity. Indeed, GN crosslinking is more stable than GA crosslinking. Ethyl(dimethylaminopropyl) carbodiimide/ *N*-hydroxysuccinimide crosslinking is a zero-length reaction and the molecules will not be incorporated into the scaffolds and unreacted EDC/NHS or their byproducts are easily removed by rinsing. As expected, EDC/NHS crosslinked scaffolds showed slight better cell growth rates compared with GA scaffolds (data not shown).

Through quantitative analysis, the effects of different crosslinking agents on the scaffold's AF were measured and the signal-to-noise ratios (SNR) determined between the fluorescently labelled cells and the scaffold's AF. It was found that although GA is the most commonly used crosslinker, in most cases GA crosslinked scaffolds exhibited high green

and red AF (Figure 2), most likely caused by residual aldehyde groups (Collins and Goldsmith, 1981; Tagliaferro *et al.*, 1997). Genipin crosslinking includes reaction with amine groups of proteins and the ring-opening polymerization within GN molecules, a pH dependent reaction (Mi *et al.*, 2005). The GN crosslinking reaction was performed in 70% ethanol, during which no aldehyde groups would be expected to form. Instead, a π -conjugated structure was formed, containing four double bonds per GN molecule. This may explain why GN-crosslinked scaffolds displayed a deep blue colour and the optical absorption and scattering within GN-crosslinked scaffolds are highly wavelength dependent. The AF of GN crosslinked scaffolds exhibited significant variation between different spectral channels, which may pose significant difficulties for multilabel tissue engineering studies. It has been reported that the green AF of elastin and collagen could be reduced and shifted to a red emission after treatment with blue dyes, such as pontamine sky blue (Cowen *et al.*, 1985) or toluidine blue. Ethyl(dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide crosslinking is a 'tight' reaction, coupling carboxyl and amine groups of collagen, respectively (Olde Damink *et al.*, 1996). Unreacted EDC/NHS, or their byproducts, can be easily removed by rinsing. The EDC/NHS crosslinking causes an increase in the amide content in scaffolds but in the present study it did not increase the AF of the scaffold in either the green and red channels, suggesting that a single or unconjugated amide group did not cause a significant increase in the AF. Together, these data indicate that crosslinking of PCL/collagen scaffolds with EDC/NHS is superior to GA or GN in terms of the scaffold AF.

Fixation of tissue specimen for histology with EDC/NHS caused lower AF than that of GA or PFA (Robinson 1987; Schmid *et al.*, 2009). The results of the present study are also applicable to other methods used to assess the growth of cells on different scaffolds, such as histology and immunostaining. As shown in Figure 5, EDC/NHS crosslinked scaffolds had a comparably low background in both green and red channels so the fluorescently labelled cells could be easily observed. The GN crosslinked scaffolds had a low background in the green channel, but very high autofluorescence in the red channel, while GA crosslinked scaffolds had high AF in both the green and red channels. As such, the high autofluorescence may hinder the ability to detect the epifluorescence of the cells for histological applications.

5. Conclusions

Crosslinking of electrospun PCL/collagen scaffolds with EDC/NHS resulted in the lowest AF of the scaffold and its tensile strength was comparable to that of GA and GN crosslinked scaffolds. A simple SNR assessment method was developed to quickly evaluate the quality of fluorescent imaging of cells, using standard fluorescent microscopy. The data show that SNR values of GFP- and mKate-labelled cells were better on EDC/NHS crosslinked scaffolds compared with other crosslinking agents. The method presented here can be easily adapted to other imaging methods as well as tissue models, where it can offer quantitative guidance on how to select the scaffold's materials, chemical modification such as crosslinking and the fluorescent labelling schemes that are optimal for specific tissue-engineering studies. In addition, this method may also serve as a quantitative framework for the selection of fluorescent 'tags' for tissue-engineering studies. Such a quantitative

approach is particularly important for studies involving multiple cell types, where each cell type is labelled with a unique fluorescent marker (Shaner *et al.*, 2005). In this scenario, it is very important to maintain sufficiently large SNR over desired spectral channels as well as minimal crosstalk between different fluorescent markers. The quantitative approach developed here may also find applications in other fluorescence-intensity-based methods, such as fluorescence-quenching-based oxygen sensing (Young *et al.*, 1996).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mechanical and physical properties of crosslinked vascular scaffolds. Polycaprolactone (PCL)/collagen-based scaffolds were fabricated by electrospinning and further cross-linked with glutaraldehyde (GA), genipin (GN) and ethyl(dimethylaminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS), as described in the Materials and methods section. (a) Stress-strain curves of wet samples were generated using a uniaxial load test machine. GN and EDC/NHS crosslinked scaffolds have higher tensile strain and tensile stress values at break than GA crosslinked scaffolds. (b) Water contact angle was measured for up to 3

minutes for each scaffold. The contact angle for scaffolds crosslinked with EDC/NHS changed very slowly, probably owing to the compact structure of these scaffolds. Suture retention strength in Newtons (N) (c) and burst pressures in mmHg (d) were determined for scaffolds with 0.24 mm and 0.5 mm thickness and for porcine carotid artery, as described in the Materials and methods section. Progressive diameter change of the vascular scaffolds (0.5 mm thickness), as a function of luminal pressures (e), was used to determine the compliance of the vascular scaffolds (f). All vascular scaffolds had lower suture retention and compliance values compared with native porcine carotid artery; however, burst pressures of 0.5-mm thick scaffolds were higher than that of the porcine carotid artery

Figure 2.

Measurements of the scaffold's autofluorescence using a standard fluorescent microscope. Scaffolds crosslinked with glutar-aldehyde (GA), genipin (GN) and ethyl(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS), as well as non-crosslinked (NCL) scaffolds (300 μm thick), were imaged (exposure time 250 ms) in the blue, green and red channels, as described in the Materials and methods section. GA cross-linked scaffolds have high autofluorescence (AF) in the green and red channels and lower AF in the blue channel. GN crosslinked scaffolds have low AF in the blue and green channels, but high AF in the red channel. In contrast, EDC/HNS crosslinked scaffolds have low AF in the blue, green and red channels, similar to NCL scaffolds. * $p < 0.05$ (compared with NCL scaffold)

Figure 3.

Excitation spectra of the vascular scaffolds at fixed emission wavelengths. Fluorescent intensity for scaffolds crosslinked with glutaraldehyde (GA), genipin (GN) and ethyl(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS), at 450 nm, 509 nm, 530 nm and 633 nm emission wavelengths, was measured as described in the Materials and methods section. Excitation peaks were at 360 nm, 390 nm and 460 nm for scaffolds crosslinked with EDC/NHS, GN and GA, respectively. Excitation peak for non-crosslinked (NCL) scaffolds was 360nm

Figure 4.

Emission spectra of the vascular scaffolds at fixed excitation wavelengths. Fluorescent intensity for scaffolds crosslinked with glutaraldehyde (GA), genipin (GN) and ethyl(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) at 350 nm, 488 nm, 532 nm and 588 nm wavelengths, was measured as described in the Materials and methods section. The emission peak was at 420 nm with 350 nm excitation wavelengths for scaffolds crosslinked with GA and EDC/NHS and NCL scaffolds. GA crosslinked scaffolds had another emission peak at about 525 nm with 488 nm excitation wavelength. The emission peak for GN crosslinked scaffolds was at 610 nm with 488 nm or 532 nm excitation wavelengths

Figure 5.

Fluorescent imaging of histological sections of fluorescent cells seeded on scaffolds. Green fluorescent protein (GFP)-MS1 (a) and mKate-MS1 cells (b) were seeded on non-crosslinked (NCL) scaffolds or scaffolds crosslinked with glutaraldehyde (GA), genipin (GN) and ethyl(dimethylaminopropyl) carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS), as described in Materials and methods section. The 8-μm thickness sections of cell-seeded scaffolds were imaged in the blue, green and red channels, using exposure times of 2 s, 6 s and 6 s, respectively, as described in the Materials and methods section. Labelled cells were clearly visible in all channels on scaffolds cross-linked with EDC/NHS and on NCL scaffolds (scale bar = 50 μm)

Figure 6.

Image analysis of fluorescent cells seeded on scaffolds. Green fluorescent protein (GFP)-MS1 and mKate-MS1 cells were seeded on scaffolds cross-linked with glutaraldehyde (GA), genipin (GN) and ethyl(dimethylaminopropyl) carbodiimide (EDC)/ *N*-hydroxysuccinimide (NHS) and non-crosslinked (NCL) scaffolds, as described in the Materials and methods section. (a) A representative image of GFP-MS1 cells on a 300-μm thick scaffold crosslinked with GN. (b) Gray level values of cell and scaffold fluorescence as a function of exposure times. (c) Signal-to-noise ratio (SNR) was calculated for three separate locations, showing that the exposure time does not change the SNR. (d) The SNR for GFP-MS1 and mKate-MS1 cells seeded on scaffolds crosslinked with GA, GN and EDC/ NHS and NCL scaffolds, measured in the green and red channels. * $p < 0.05$ (compared with GA crosslinked scaffold)