ReBioStent

Reinforced Bioresorbable Biomaterials for Therapeutic Drug Eluting Stents

Deliverable 8.3

Protocol for optimised pre-seeding of luminal and abluminal stent surface with the two different cell types (HCMEC and HSMC)

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Abstract

D8.3 presents the newly developed pre-seeding protocol of tubular material samples with human primary cells (endothelial and smooth muscle cells). An exemplary evaluation of the pre-seeding technique is shown using PLGA tubes with or without fibronectin coating.

This protocol will be used to evaluate the cytocompatibility of the 8 developed stent prototypes and it will also be facilitated in preparation for experiments in the biomechanical reactor.

This deliverable is of the nature “Other” and this document describes the protocol presented.

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Impressum

Reinforced Bioresorbable Biomaterials for Therapeutic Drug Eluting Stents

ReBioStent

WP8: In vitro assays under simple cell culture conditions and simulated in vivo studies

Task 8.1: Static cytocompatibility tests on the novel materials

Deliverable D8.3: Protocol for optimised pre-seeding of luminal and abluminal stent surface with the two different cell types (HCMEC and HSMC)

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Executive summary

For a good stent biocompatibility complete and swift endothelialisation is a prerequisite for the prevention of stent thrombosis. Thus, the adhesion and proliferation of endothelial and endothelial progenitor cells has to be facilitated by a newly developed stent. In contrast, the proliferation of smooth muscle cells induced by inflammation would lead to stent restenosis. So, smooth muscle cell attachment and proliferation is to be reduced.

D8.3 describes the protocol for pre-seeding of human vascular primary cells into tubular structures thus preparing for the cytocompatibility analysis of the stent prototypes under static (Task 8.1) and flow (Task 8.3) conditions.

List of authors

Company	Author	Contribution
RUB	Dr. Jochen Salber	Biocompatibility testing, WP management
RUB	Dr. Sandra Pacharra	Pre-seeding protocol development and evaluation

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1 Introduction

For the evaluation of the biocompatibility of a new medical device not only the individual base materials but also the final assembly of all components has to be analysed regarding cytocompatibility. Different factors might influence the desired cell behaviour: On the one hand the composition of all base materials together may result in a different cellular response than the one from each individual material. On the other hand the shape of the device – e.g. curved instead of flat – might have an effect on cell adhesion and proliferation characteristics.

In the ReBioStent project a stent will be developed that on the one hand enables easy endothelialisation and on the other hand prevents restenosis. To achieve both aims the luminal and abluminal surfaces will be coated differently. The luminal surface will be modified so that particularly the blood vessel endothelial cells attach to and proliferate on top of the surface. In contrast to that the abluminal surface is supposed to inhibit the adhesion and proliferation of smooth muscle cells in order to prevent stent restenosis.

Here, the protocol for the seeding of human primary cells onto the developed stent prototypes is presented. It will enable the detailed analysis of primary cell behaviour using the same methods also used for flat material samples (see D8.1). As representative cells human cardiac microvascular endothelial cells (HCMECs) and human coronary artery smooth muscle cells (HCASMCs) were used. Cytocompatibility analysis was performed according to DIN EN ISO 10993-5 [1].

2 Protocol for pre-seeding of tubular samples

2.1 Materials

For the protocol development PLGA tubes (Zeus Absorv® - Bioabsorbables) were used.

2.2 Methods

2.2.1 General cell culture

The primary cells human cardiac microvascular endothelial cells (HCMEC) and human coronary artery smooth muscle cells (HCASMC) were obtained from PromoCell.

Primary cells are cultured according to the manufacturer's protocol in endothelial cell growth medium MV (HCMECs) and smooth muscle cell growth medium 2 (HCASMCs). Medium is changed every one to three days while subculturing is performed at 70 to 90% confluence using the detach kit from PromoCell (with seeding densities according to Table 1). An incubator with a humidified atmosphere is used at 37°C and 5% CO_2.

Table 1: Seeding numbers

Cells	Subculture	Tubular pre-seeding
HCMEC	$1\text{-}2\cdot10^4$ per cm^2	$5\text{-}6\cdot10^4$ per ml
HCASMC	$0.75\text{-}1\cdot10^4$ per cm^2	$2.5\text{-}3\cdot10^4$ per ml

2.2.2 Tube preparation

Tube disinfection is achieved by incubation in 70% ethanol for 30 minutes in a 6 well plate followed by drying overnight. The tube is washed 3 times with sterile water and once with D-PBS and equilibrated in respective medium.

2.2.3 Tube coating with fibronectin

For fibronectin coating disinfected tubes are covered with 10 µg/ml fibronectin solution (Promokine) in PBS and incubated at 37°C for 30 minutes. Tubes are then placed into a fresh sterile polystyrene 6 well plate and equilibrated in respective medium.

The coating procedure was adapted from [2].

2.2.4 Tubular pre-seeding

After removal of equilibration medium 1 ml cell suspension is added to the tube (cell number as indicated in Table 1). To allow cell attachment the tube is incubated at 37°C,

5% CO_2 for 30 minutes. Then, the tube is rotated, 1 ml cell suspension is added and again incubated for 30 minutes. This process is repeated two more times so that the tube is completely immersed in cell suspension. The tube is rocked from time to time for the next 3 hours. Finally, the tube is incubated at 37°C, 5% CO_2 for the desired time period under static conditions.

The tube pre-seeding protocol was adapted from [3].

2.2.5 Live/dead cell staining

Staining is achieved using the Live/Dead Cell Staining Kit II (PromoKine) according to the manufacturer's protocol. PromoKine's Live/Dead Cell Staining Kit II provides a two-colour fluorescent staining of live (green) and dead cells (red) using two probes and is suited for animal live and dead cells. Calcein-AM stains live cells green, while EthD-III stains dead cells red.

Fluorescence microscopy is done with the IX 51 (Olympus) using the FITC and TRITC filter sets.

2.2.6 Other analysis techniques applicable to pre-seeded tubes

In order to determine the number of cells attached to a tube it is carefully washed and cells are detached using the Detach Kit (PromoCell). The resulting cell suspension is centrifuged at 200x g for 5 minutes. After cell resuspension in 50 µl respective medium 50 µl 0.4% Trypan Blue solution is added and cells are counted using a Neubauer chamber (adapted from [4]).

To visualise cell morphology in more detail cells are fixed using 4% paraformaldehyde in D-PBS, permeabilised with 0.4% Triton-X-100 in D-PBS and stained using desired dyes. Nuclei are visualised using DAPI, F-actin is stained with a phalloidin-coupled dye and the presence of focal adhesion points is shown using an anti-vinculin antibody.

Another visualisation technique applicable to the tubes is scanning electron microscopy (SEM). Cells are fixed using 4% paraformaldehyde in D-PBS and gradually dehydrated using an ethanol gradient. Sputter-coated samples are analysed by SEM.

2.3 Results and discussion

In order to prove the feasibility of the developed pre-seeding protocol untreated and fibronectin-treated PLGA tubes were seeded with the two human primary cell types used in the ReBioStent project.

2.3.1 Endothelial cells (HCMECs)

The pre-seeding with endothelial cells is important in the stent development in order to evaluate if the stent allows attachment and proliferation of endothelial cells. The complete and swift endothelialisation of a stent in turn is a prerequisite for the reduction of neointimal hyperplasia and stent thrombosis [5].

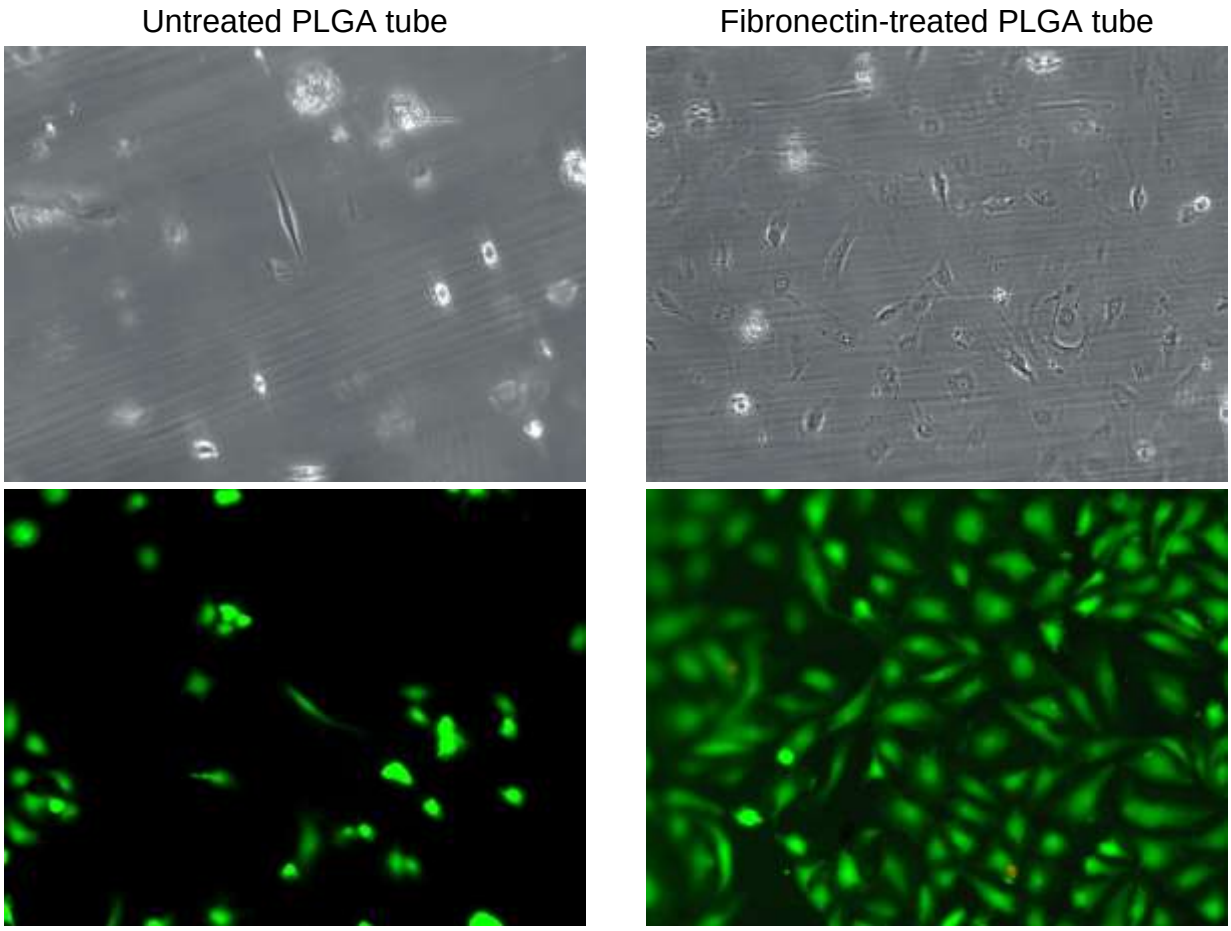

Figure 1: Microscopic images of HCMECs on tubular samples. Bright field and Live/Dead staining images of HCMECs seeded onto untreated or fibronectin-treated PLGA tubes after 48 hours of incubation.

Figure 1 proves that the developed pre-seeding protocol works well for HCMECs if the surface is suitable for endothelial cell attachment: Bare PLGA is not suitable for HCMEC adhesion while fibronectin-coated PLGA serves as a good adhesion substrate.

Cells attach to the surface during the initial four 30 minute adhesion steps as indicated by a good tube interior coverage (data not shown) while normal cell morphology is reached

after three hours at the most. Furthermore, a two-day incubation time is sufficient to reach 80% to 90% confluence (see Figure 1) which allows for all desired analyses to be performed (cell staining followed by microscopy, cell counting, SEM). Also, this cell density will be sufficient for the transfer into the biomechanical reactor followed by prolonged cytocompatibility experiments under flow conditions.

2.3.2 Smooth muscle cells (HCASMCs)

Not only the endothelial cell analysis but also the evaluation of smooth muscle cell proliferation on a stent also is crucial. Local inflammation resulting from stent placement stimulates vascular smooth muscle cell proliferation which in turn leads to neointimal thickening and restenosis [6]. Thus, to analyse if a stent is able to subdue smooth muscle cell adhesion and proliferation pre-seeding experiments have to be conducted.

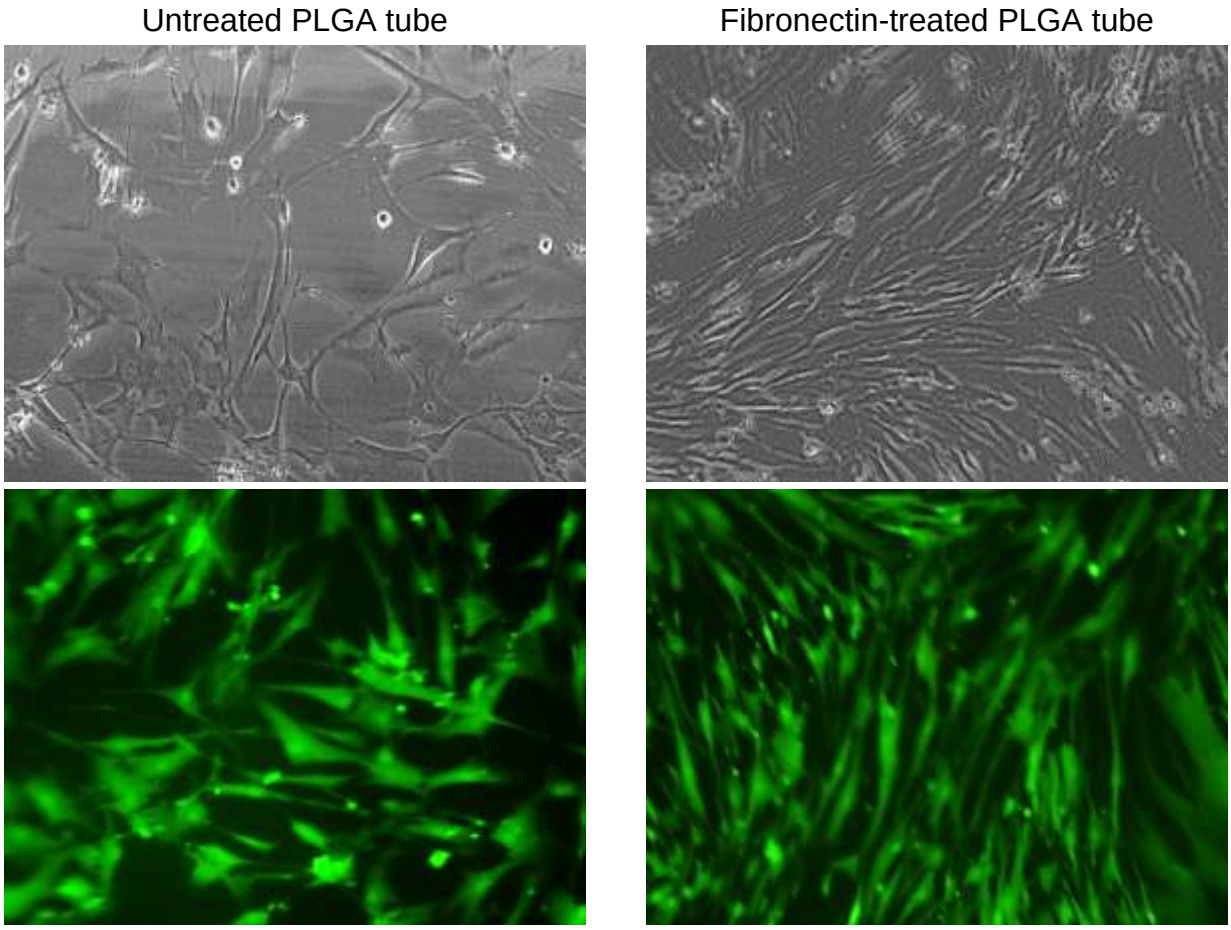

Figure 2: Microscopic images of HCASMCs on tubular samples. Bright field and Live/Dead staining images of HCMECs seeded onto untreated or fibronectin-treated PLGA tubes after 48 hours of incubation.

Figure 2 proves that the developed protocol can be used for the pre-seeding of tubular samples with primary smooth muscle cells. HCASMCs were able to attach to untreated and fibronectin-treated PLGA tubes while cell adhesion and proliferation was improved on the fibronectin-coated sample.

3 Abbreviations

DAPI	4',6-diamidino-2-phenylindole
DIN	Deutsches Institut für Normierung (German Institute for Standardisation)
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate buffered saline
EN	European standard
HCASMC	Human coronary artery smooth muscle cells
HCMEC	Human cardiac microvascular endothelial cells
ISO	International Organisation for standardisation
PLGA	Poly-L-lactic-co-glycolic acid
PS	Polystyrene
SEM	Scanning electron microscopy
TCPS	Tissue culture-treated polystyrene

4 References

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