Surface-directed differentiation of embryonic stem cells

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Here, we explore how embryonic stem-cell (ESC) differentiation patterns on surfaces are affected by changes in the processing parameters of plasma-polymerized materials. Mouse ESCs were plated on plasma-polymerized tetraethylene glycol dimethyl ether (tetraglyme) surfaces on glass. Depending on the processing conditions, the plated ESCs precociously express the platelet endothelial cell adhesion molecule and von Willebrand Factor genetic markers which indicate directed differentiation of some ESCs into endothelial cells. The surface properties show that a larger fraction of directed differentiation takes place when the plasma-induced surface chemistry includes a branched ether bond and higher carbon-to-oxygen ratios. © *2008 American Institute of Physics*. [DOI: 10.1063/1.2929387]

Past research has attempted to develop small-scale (<5 mm) artificial blood vessels by fabricating a biocompatible interface between blood, blood components, and the biomaterial. Two approaches have been used. First, a deposited coating on the lumenal surface of a tubular construct is intended to function as a blood delivery system. Plasma-polymerized coatings have often been used for this purpose.[1]

The second approach utilizes tissue engineering whereby transplanted endothelial cells (ECs) are grown on layers of smooth muscle and extracellular matrix proteins which are attached to a scaffolding that comprises the structural support of the artificial vessel.[2] However, such ECs are viable for only several months prior to death.[3]

Alternatively, embryonic stem cells (ESCs) are capable of unlimited, undifferentiated proliferation *in vivo*. To form an artificial blood vessel, vasculogenesis, the formation of blood vessels by *in situ* differentiation of ECs from their hemangioblastic precursors needs to take place.[4] Thus, ESC-derived ECs have the potential to ensure the longevity of an artificial blood vessel if the proper signaling mechanisms to direct the differentiation of these cells into endothelial cells can be controlled.[5]

Differentiation is monitored by genetic markers, which are specific proteins that are expressed by the cells at particular stages. For example, during *in vitro* vasculogenesis, ESCs express platelet endothelial cell adhesion molecule (PECAM) near day 4 of differentiation and express von Willebrand Factor (vWF) near day 11 of differentiation.[4,6]

Past studies have sought to determine the interaction between "standard" polymeric materials and stem cell gene-expression sequences.[7] In this work, we explore how ESC differentiation patterns are affected by their contact with plasma-polymerized surfaces.

ESCs were plated on plasma-polymerized surfaces to compare their adhesion and differentiation properties as a function of plasma-processing conditions. Three classes of surfaces were used: (1) glass cover slips (Corning) onto which a coating of tetraglyme was deposited by plasma polymerization (PP),[8] (2) tissue-culture treated polystyrene[9] (Falcon), and (3) unprocessed glass cover-slips used as a control.

For the PP tetraglyme surfaces, a 13.56 MHz rf plasma, shown in Fig. 1, was used to polymerize the tetraglyme onto flat 22 mm square glass coverslips which were placed in the reactor at a distance of 15 cm from the gas inlet.

The tetraglyme liquid was degassed by freezing it with liquid nitrogen. The flask was then evacuated to a pressure of 40 mTorr. The tetraglyme was allowed to melt to release dissolved gas into the vapor phase. This was repeated three times until the final pressure in the flask reached 10 mTorr to minimize the dissolved gases in the flask. The tetraglyme was heated to 110 °C to supply a constant flow into the plasma chamber. The monomer delivery line was heated to 120 °C to minimize tetraglyme condensation. To assist the tetraglyme flow, argon carrier gas was used.[10] The gas flow rate was 7 SCCM (SCCM denotes cubic centimeter per minute at STP).

All cover slips were pretreated with argon plasma excited at a power of 100 W at a pressure of 100 mTorr for 5 min. The tetraglyme was then allowed to flow into the chamber, and the resulting PP was maintained with an absorbed rf power of 25 W and a reactor pressure of 100 mTorr for 20 min. After PP, the samples were removed and soaked in methanol overnight to remove-PP tetraglyme monomers not bonded to the surface. All surfaces were autoclaved for 30 min at 50 psi at 121 °C, followed by a subsequent 10 min drying period.

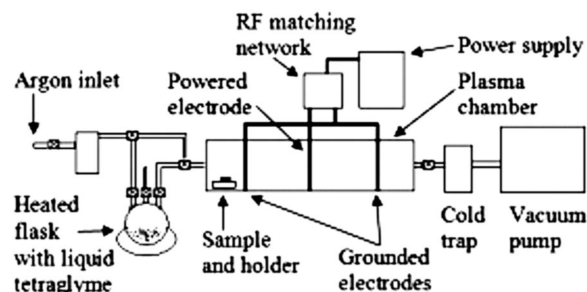

FIG. 1. Plasma polymerization reactor. The glass cover slips were placed on the bottom of the cylindrical vacuum chamber as shown. The plasma was excited with a 13.56 MHz power supply.

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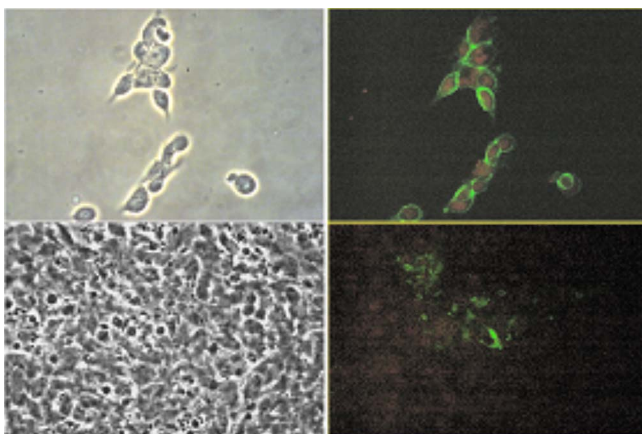

FIG. 2. (Color online) R1 ESCs differentiating on plasma polymerized tetraglyme-coated glass cover-slips precociously express endothelial lineage markers. The left column shows the phase-contrast images of the fixed R1 mouse ESCs. The right-hand column shows staining of the CD-31 or vWF markers with the Alexa 488 secondary antibody. These images are overlayed with PI staining of all cell nuclei. The first row shows groups of CD-31+ cells on the plasma-polymerized tetraglyme surface 3 days after plating. The second row shows groups of vWF+ cells on the plasma-polymerized tetraglyme surface 7 days after plating.

Before plating, the cells were cultured and grown on a mouse fibroblast feeder layer [STO; American Type Tissue Collection (ATCC)] to inhibit premature differentiation. The cells were grown in Dulbecco's modified eagle media with 15% fetal calf serum. The ESCs were isolated from the STO fibroblast layer by preplating, and suspensions of (5×10^4) ESCs were prepared in 2 ml of media and plated on 22 mm^2 surfaces. The media was changed daily, but the media composition was not altered, nor were growth factors added when the R1 ESCs were removed from the fibroblast feeder layer and plated on the surfaces.

After a number of preliminary screening experiments using antibody-linked immunofluoresence, we determined that the ES cells after 3 days expressed PECAM and after 7 days expressed vWF. This is compared with 4–5 days for PECAM and 11–13 days for vWF when the cells were not in contact with the PP surface. Triplicate samples were fixed and stained using a standard antibody staining technique.[11] We used two primary antibodies, PECAM (BD) at 3 days and vWF (Sigma) at 7 days. Since these primary antibodies are not conjugated to a fluorophore, we incubated the cells with a secondary antibody that is conjugated with the ALEXA-488.[12] This allows us to observe either PECAM or vWF using an epifluorescence microscope with filters to detect the ALEXA-488 fluorescent signal. We quantified the ALEXA-488 (Invitrogen) fluorescence. A secondary antibody only control allowed us to distinguish signal that comes from the cells and not from surface artifacts or autofluorescence.

As a positive control to ensure that both vWF and PECAM immunofluorescence assays were functional, rat aortic endothelial cells (Cell Applications, Inc.), which are always positive for both markers, were plated on plasma-polymerized tetraglyme surfaces and fixed and stained several days after plating.

Figure 2 shows images of two representative plasma-polymerized tetraglyme surfaces. The phase-contrast microscope images in the left-hand column show the cells in the field of view. The right-hand column shows the antibody-specific staining with propidium iodide stained-cell nuclei images overlayed.

TABLE I. R1 mouse ESCs plated on plasma polymerized tetraglyme surfaces express PECAM on day 3 after plating and vWF on day 7 after plating. R1 mouse ESCs plated on tissue-culture treated polystyrene and control glass cover slips did not express PECAM on day 3 after plating nor vWF on day 7 after plating.

Surface	Antibody	Days postplating	Positive samples/ total samples tested
Polystyrene	PECAM	3	0/7
Polystyrene	vWF	7	0/9
Glass cover slips	PECAM	3	0/27
Glass cover slips	vWF	7	0/32
Tetraglyme	PECAM	3	38/54
Tetraglyme	vWF	7	47/63

In the first row of Figure 2, staining for PECAM (CD-31) showed that PECAM-positive cells were observed from R1 mouse ES cells that had differentiated for 3 days on the tetraglyme-treated surfaces. These CD-31 positive cells are early endothelial cells.

The second row shows the results for staining with the vWF antibody. This was done 7 days after plating on the tetraglyme-treated surfaces.

Thus, in contrast with work previously reported,[4] ESCs that were plated on plasma-polymerized tetraglyme surfaces showed precocious expression of both PECAM after 3 days and vWF after 7 days of being plated on the surfaces (see Table I). However, ESCs that were plated on the tissue-culture treated polystyrene and the control glass cover-slip surfaces did not express PECAM until *after* day 4 and vWF until *after* day 14. This difference suggests that surface interactions between the differentiating R1 ESCs and the plasma-polymerized tetraglyme coating were present and resulted in precocious differentiation.

To determine how plasma-processing parameters affect the yield of ECs when plated on the plasma-polymerized tetraglyme coating, a two-level, four variable factorial experimental design was conducted. Four processing variables (1) power, (2) reactor pressure, (3) plasma-processing time, and (4) sample radial position were chosen. Triplicates of the sixteen conditions were fabricated and plated cells were immunostained for vWF after 7 days of plating on each surface according the procedures described above.

The results of this analysis are summarized as follows. Of the four main effects, only the reactor pressure and the plasma-processing time are significant. Furthermore, increases in these two variables decrease the EC percentages. Of the interactive effects, combinations between pressure, processing time, and radial position must also be considered. We thus conclude that the fraction of differentiating cells can be significantly modified depending on the processing parameters which must modify the surface properties of the plasma-polymerized surfaces.

To investigate this, it was determined by x-ray photoelectron spectroscopy (XPS) and contact-angle measurements that surface coatings produced under reduced pressures (100 mTorr) exhibit higher cross linking and increased hydrophobicity. By combining XPS with mass spectrometry and immunofluorescence staining of vWF after 7 days of differentiation, it was found that increased cross-linking and

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higher ratios of carbon-to-oxygen increase the effect of ESC-directed differentiation on ESc. From Fourier transform infrared (FTIR) analysis of plasma-polymerized tetraglyme surfaces, carbonyl functional groups were found that were not present in the tetraglyme monomer. In addition, by combining FTIR with immunofluorescence staining, it was found that the directed-differentiation effect of ESCs into ESc is likely to depend upon the presence of a branched ether bond[13] on the plasma-polymerized tetraglyme surfaces.

These results indicate that the plasma-polymerized coating chemistry is significantly different than the input vapor monomer alone and, more importantly, this chemistry can be modified by varying the processing parameters which changes the fraction of differentiated EC from 0% to 4%. However, directing the differentiation of ESCs via cell-surface interactions involves activation and repression of signaling and regulatory pathways that are not yet fully elucidated.

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