Tracking DNA double-strand breaks
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CHAPTER 4

COATING OF COVERSLEIPS WITH GLOW-DISCHARGED CARBON PROMOTES CELL ATTACHMENT AND SPREADING PROBABLY DUE TO CARBOXYLIC GROUPS

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ABSTRACT

Background: For high-resolution microscopy, cells have to be analyzed through thin glass coverslips. Therefore, it is necessary to culture cells on coverslips for preservation of cell morphology. We found cell attachment and spreading to be relatively slow processes, even when cells were plated on coated coverslips. This slowness presents a problem, particularly when synchronized cell populations are used.

Methods: In this paper, we present a method that is based on glow-discharged carbon coating of coverslips which promotes rapid attachment and spreading of cells, enabling rapid analysis of cells after plating. Results obtained with carbon-coated coverslips were compared with those of other types of coating. Two fibroblast lines, an epithelial cell line, and a carcinoma cell line were tested.

Results and Conclusions: All cell lines showed a rapid adhesion on carbon-coated coverslips. With fibroblasts we found the carbon coating to be superior to other coatings tested, mainly because the carbon did not influence cell morphology. Using synchronized or irradiated cells produced similar results. The superior performance of carbon coating is probably due to carboxylic groups on the glow-discharged carbon layer. The carbon layer does not interfere with microscopy or immunocytochemical staining procedures.

Key terms: fluorescence microscopy; confocal microscopy; fibroblasts; coating; carbon; cell attachment; cell spreading; cell synchronization

INTRODUCTION

Fluorescence and confocal microscopy are indispensable tools for the identification and characterization of macromolecules in tissues and cells. These techniques are particularly useful for studying spatial relationships between different cellular components. Visualization of macromolecules requires objectives with high magnification and numerical aperture. Because of the short working distance of these objectives, cells are usually observed through thin glass coverslips. For this purpose, cells are usually spun down on glass slides and observed through coverslips. In many cases, this procedure leads to bad morphology. Therefore, culturing followed by fixation of the cells on coverslips is a better option; for the analysis of living cells, it usually is the only option.

Unfortunately, most anchorage-dependent cell lines do not attach readily to glass surfaces without coating to promote cell attachment and spreading. Even with coated coverslips, we found attachment and spreading to be relatively slow processes. When studying cell-cycle dependent processes, this
slowness can present a significant problem. For instance, synchronization of cell populations is lost when the cells are not properly attached and spread within 3 h after plating. In addition, there is considerable cell loss due to cell death and clumping of cells when attachment is too slow.

In this paper, we present results obtained with a method that is based on carbon coating of coverslips, which promotes rapid attachment and spreading of asynchronously growing and synchronized cells. Populations of synchronized cells were obtained by flow-cytometric sorting or by mitotic shake-off (1). One epithelial cell line, one carcinoma line, and two fibroblast lines were tested, and the effects of carbon coating of coverslips were compared with those of other frequently used coatings.

MATERIALS AND METHODS

Cell Cultures. V-79 Chinese hamster lung fibroblasts and RUC rat ureter carcinoma cells were cultured in Eagle’s Minimum Essential Medium (MEM) with Hank’s salts (Gibco BRL, Life Technologies, Rockville, MD) containing 10% fetal calf serum (FCS). The cells were incubated in a 37°C incubator in an atmosphere of 2% CO₂ in air. HSF-7 human skin fibroblasts (kindly provided by Dr. D. J. Chen, Los Alamos National Laboratories, Los Alamos, NM) were cultured in Dulbecco’s MEM (Gibco BRL) supplemented with 10% FCS and incubated in an incubator in an atmosphere of 10% CO₂ in air. MDCK dog kidney epithelial cells (kindly provided by Dr. E. Pinelli, University of Utrecht, Utrecht, The Netherlands) were cultured in RPMI (Gibco BRL) supplemented with 10% FCS in an incubator with an atmosphere containing 5% CO₂. Cells were grown as monolayers in culture flasks (Costar, Cambridge, MA).

Synchronization of Cells. Synchronized cell populations were obtained by mitotic shake-off (1) or flow-cytometric sorting. For flow-cytometric sorting, Hoechst 33342 (Calbiochem, San Diego, CA) was added to cell cultures in a final concentration of 5 mg/ml (2). After incubating for 60 min at 37°C, cells were trypsinized, collected in medium supplemented with Hoechst 33342 (5 mg/ml), and concentrated by centrifugation. G1 cells were sorted with a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). Synchronized cells were plated on coverslips at a concentration of 20,000 cells/cover slip. Synchronization was checked by rerunning part of the sorted sample through the flow cytometer.

Irradiation of Cells. V-79 cell cultures in plateau phase were irradiated using a ¹³⁷Cs source, yielding a dose rate of approximately 1 Gy/min (3). After irradiation with 0 Gy, 5 Gy, or 10 Gy, cells were trypsinized, diluted, and plated on coverslips coated with carbon or on coverslips coated with fibronectin.

Coating of Coverslips. Two different brands of round coverslips (diameter, 1.5 cm) were tested: Assistant (Hecht, Sondheim, Germany) and Menzel (Braunschweig, Germany). Coverslips were cleaned with a solution of 0.4 g K₂Cr₂O₇ per 10 ml sulfuric acid.
Table 1. Characteristics of fibroblasts after plating on coverslips with various coatings to promote rapid attachment and spreading. The x symbol indicates the attachment time (time between plating and spreading) of 80% or more of the plated cell population on the various substrates. When more than 80% of the plated cell population was not spread within 7 h, a large variation in attachment time was observed: some cells were well spread after 7-10 h, while others spread only at 12-16 h after plating. Experiments were performed at least 3 times.

<table>
<thead>
<tr>
<th>Coating</th>
<th>Time between plating and spreading (&gt;80% of the plated cell population)</th>
<th>Confluency within 5 days after plating</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: Costar culture flasks</td>
<td>x</td>
<td>Yes</td>
<td>Poor optical quality and strong autofluorescence</td>
</tr>
<tr>
<td>Uncoated coverslips</td>
<td>x</td>
<td>Yes</td>
<td>Discussed in the text</td>
</tr>
<tr>
<td>Carbon</td>
<td>x</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Fibronectin (BD)</td>
<td>x</td>
<td>Yes</td>
<td>At 5 h after plating 37% of the cells were 3-4 times larger than cells plated in culture flasks</td>
</tr>
<tr>
<td>Poly-L-lysine (BD)</td>
<td>x</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Poly-L-lysine (Sigma)</td>
<td>x</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Permanox</td>
<td>x</td>
<td>Yes</td>
<td>Poor optical quality and strong autofluorescence</td>
</tr>
<tr>
<td>Collagen type 5</td>
<td>x</td>
<td>No</td>
<td>At 16 h after plating, all cells were dead</td>
</tr>
<tr>
<td>Gelatin 2%</td>
<td>x</td>
<td>No</td>
<td>At 16 h after plating, 50% of the cells were dead</td>
</tr>
<tr>
<td>Gelatin 1%</td>
<td>x</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Gelatin 0.1%</td>
<td>x</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Alcian blue</td>
<td>x</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Cell residues (cells removed using a syringe)</td>
<td>x</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>
Coverslips were coated in one of the following ways:
- with Alcian Blue (4)
- with poly-L-lysine (5)
- with collagen type 5: coverslips were covered with a solution of 50 mg collagen type 5 (Sigma, St. Louis, MO) per milliliter acetic acid and air dried
- with gelatin: coverslips were covered with a thin layer of a solution of gelatin (Merck, Darmstadt, Germany) in aqua dest (concentration: 0.1, 1, or 2% w/v); after 30 min incubation at 37°C, the gelatin solution was removed and the coverslips were air dried
- with cell residues: cells were plated on uncoated coverslips; after the cultures had reached confluency, cells were removed by trypsinization or by spraying the conditioned culture medium using a syringe with a 21-gauge needle
- with carbon: coverslips were etched in a Balzers SC-040 (Balzers Instruments, Liechtenstein) in an argon atmosphere (0.1 mbar) for 2.5 min and a current of 3–5 mA to prevent detachment of the carbon layer from the coverslips; after etching, the coverslips were transferred to a Balzers MED-010 and coated with 5–10 nm carbon (10-5 mbar); the carbon layer was hydrophilized and negatively charged by glow discharge (1 min, 30 mA) in air (6); the equipment required for this procedure can usually be found in laboratories with electron-microscopy facilities.

Fig. 1. V-79 fibroblasts visualized with phase-contrast microscopy 3 h after plating. A: Cells plated on uncoated coverslips are not spread within 3 h after plating. B: morphology of cells on carbon-coated coverslips is similar to the morphology of cells plated in culture flasks. C: A larger number of abnormally large cells was found in cultures plated on fibronectin-coated coverslips. Bar = 10 µm.
Cells were also cultured on the following “ready-to-use” coverslips: coverslips coated with fibronectin or poly-L-lysine (Becton Dickinson Labware, Bedford, MA) and Permanox coverslips (Nunc, Roskilde, Denmark).

**Analysis of Cell Attachment.** All coverslips were placed in 25-well “replica dishes” (Greiner, Frickenhausen, Germany). Cells plated on coverslips were monitored with a phase-contrast microscope (Diavert, Leica, Wetzlar, Germany). Cell attachment was assessed by measuring the time between plating and spreading. Spreaded cells could be recognized easily by visual observation (Fig. 1B). Photographs were made with a 35-mm camera (Orthomat W, Leica).

## RESULTS AND DISCUSSION

The delay between plating and spreading of cells and the capacity of the fibroblast cultures (V-79 and HSF) to reach confluency on the different coverslips are summarized in Table 1. No differences were observed between Menzel and Assistant coverslips. Synchronized V-79 cells, obtained by mitotic shake-off or flow sorting and plated on coverslips coated with either carbon or fibronectin, produced similar results. Moreover, irradiation of V-79 cells before plating on either carbon- or fibronectin-coated coverslips had no effect on the period between plating and spreading.

The results show that coating of coverslips with gelatin (1% and 2%), collagen, poly-L-lysine, and fibronectin presented problems. Gelatin and collagen coatings resulted in cell death, and cultures on poly-L-lysine-coated coverslips did not reach confluency. We found a higher number of abnormally large cells on fibronectin-coated coverslips (Fig. 1). It is known that (cellular) fibronectin can have an effect on cell morphology (7,8). Coating with Alcian Blue, cell residues, or gelatin (0.1%) did not cause any of these negative effects but did not promote rapid attachment and spreading. Permanox appeared to be unsuitable because of its poor optical qualities. Only on carbon-coated coverslips

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**Fig. 2.** V-79 fibroblasts fixed at 3 h after plating on a carbon-coated coverslip. Replication patterns in cell nuclei are visualized by immunocytochemical staining of IdUrd incorporated in replicating DNA. Bar = 10 μm. (Photograph was taken with a CCD camera, coupled to a fluorescence microscope (Leica DM RA HC).
were attachment and spreading of fibroblasts as fast as in culture flasks, and negative side effects were not observed.

Epithelial and carcinoma cells were cultured on uncoated coverslips, culture flasks, and coverslips coated with carbon, fibronectin (“ready-to-use”; Becton Dickinson) or poly-L-lysine (Becton Dickinson). Comparing the results with those obtained using the fibroblasts demonstrated two differences: there was no increase in the number of abnormally large cells on fibronectin-coated coverslips, and MDCK epithelial cells did not attach to uncoated coverslips and eventually died. On carbon- and fibronectin-coated coverslips, attachment of the epithelial and carcinoma cells was as rapid as in culture flasks. Cell culture on carbon-coated coverslips, subsequent fixation (with paraformaldehyde or glutaraldehyde), and immunocytochemical staining of the cells was feasible (Fig. 2). Problems due to autofluorescence or decreased fluorescence did not occur (data not shown).

Glow, or electrical plasma discharge, the method we used to hydrophylize the carbon-coated coverslips, is also a basic treatment given to polystyrene to produce “tissue culture grade” culture vessels (9). With this treatment, there is an increase in the amount of (hydrophilic) carboxylic acid groups of the polystyrene surface (10,11). The amount of surface carboxylic acid groups has been correlated with cell adhesiveness (11). Therefore, the superior performance of the carbon-coated coverslips may well be due to an increased amount of carboxylic groups in the carbon layer. It is not likely that carbon itself promotes cell adhesion. Li et al. (12) found that diamondlike carbon coating decreased cell adhesion.

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