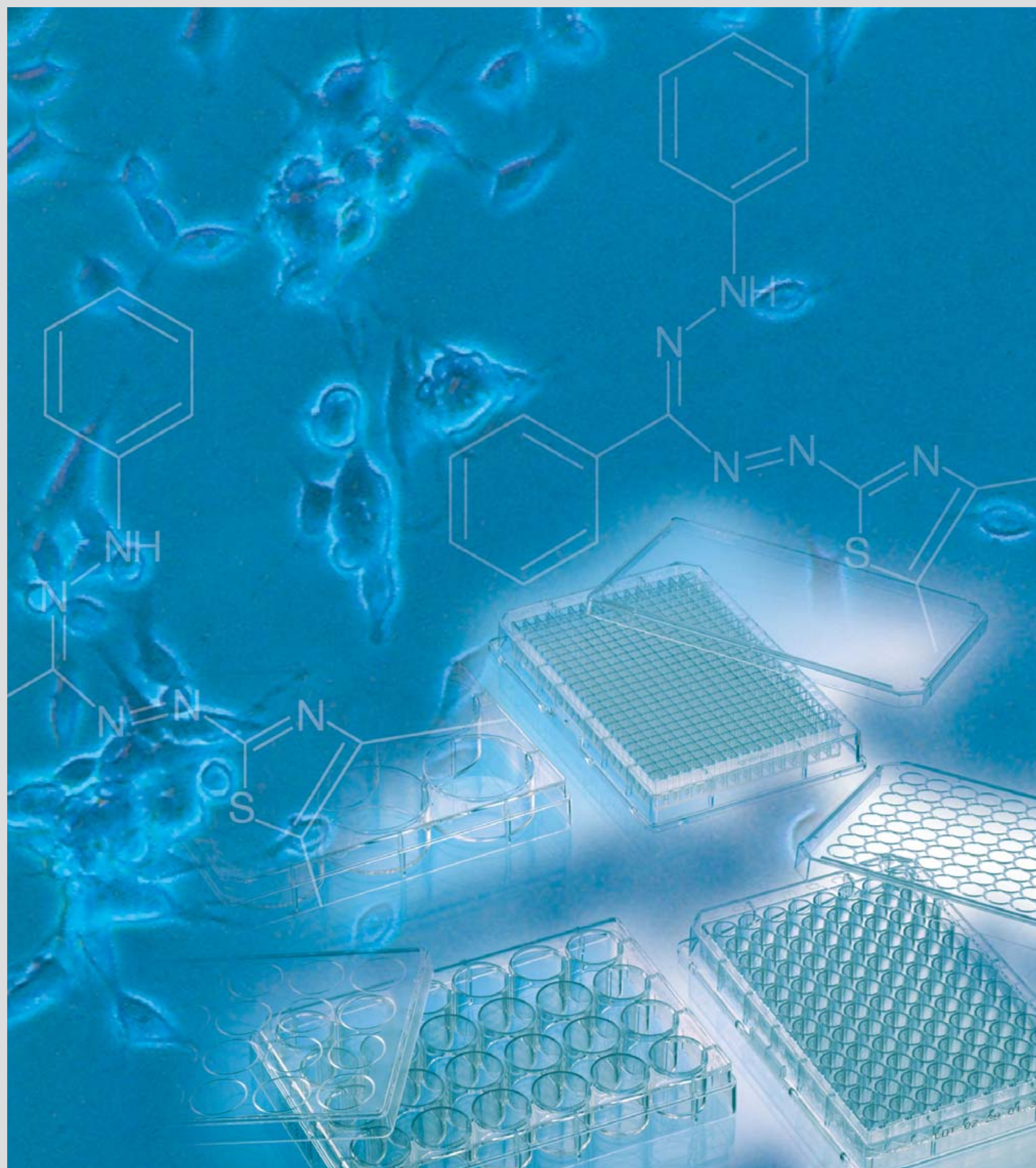
Application Note

Influence of washing steps on cell attachment: Comparison of PDL-coated and cell culture treated microplates



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Introduction

In recent years cell culture has played an increasingly important role in high-throughput screening applications. The impact of cell culture data on drug discovery and subsequently on drug development to the final product is highly significant.

An essential requirement for culturing cells *in vitro* is a surface substrate which provides a basis for firm attachment of anchorage-dependent cells under *in vitro* culture conditions. Besides physically treated culture vessels, surfaces coated with biomolecules are an additionally powerful tool for these applications. In high-throughput screening (HTS) the need for automation often results in experimental setups which are stressful for the cells. These applications require a more effective surface for cell attachment. High injection pressures of buffers during washing steps are often unavoidable and may lead to significant loss of cells using standard tissue culture treated vessels and classic culture conditions. Therefore, cell culture experiments using non-coated surfaces for cultivation are sometimes not feasible and have to be replaced by protein coated culture vessels.

Greiner Bio-One offers coated surfaces with natural proteins and synthetic polypeptides – like Collagen Type I and Poly-Lysine – for the cultivation of specific cell lines and various experimental procedures. Poly-Lysine enhances the attachment of cells by generating a positive charge on the surface. The applications in Poly-Lysine coated culture vessels include the integration of washing steps in an experimental setup, the cultivation of cells under serum-free or serum-reduced conditions[1] or the transfection of cells. It has been proven that Poly-D-Lysine (PDL)-coated surfaces provide beneficial advantages when culturing neuronal cell lines with respect to both viability and proliferation.

Two forms of Poly-Lysine, Poly-L-Lysine (PLL) and Poly-D-Lysine (PDL) which differ in the steric configuration of the molecule, are available on the market (**Fig. 1**). These molecules are the mirror image of each other, termed enantiomers. While both coatings are widely used, PDL unlike PLL is not degraded by cellular proteases. Therefore, it may be the preferred coating for some applications and cell lines.

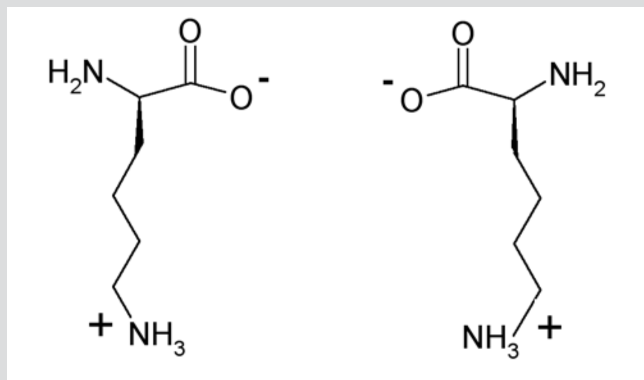

Figure 1: Steric configuration of L- and D-Lysine

As a synthetic protein, Poly-Lysine increases cell attachment without influencing specific signalling pathways in the cell. Therefore, the numbers of different parameters influencing the cells are minimised and more specific conclusions on the drugs under test may be obtained. Another advantage of this specific coating is that Poly-Lysine is a synthetically produced polypeptide and as such cannot introduce any animal derived impurities into the cell culture system.

Greiner Bio-One also offers culture vessels coated with Collagen Type I. Collagen Type I is one of the major proteins found in the extracellular matrix and in connective tissue like skin, tendon and bone. *In vivo*, the cellular interaction with Collagen Type I is mediated largely through integrin receptors[2]. *In vitro*, Collagen Type I influences the differentiation, morphology[3] and growth of a variety of cell lines including endothelial[4], epithelial, hepatocytes and muscle cells as well as other transfected cell lines.

The following application will focus on the impact of washing steps in an experimental setup by using cell culture treated and PDL-coated (CELLCOAT®) microplates.

Material and Methods

Item	Manufacturer	Cat.-No.
DMEM medium	Biochrom AG	F0435
Fetal calf serum	Invitrogen Life Technologies	10270-106
Penicillin Streptomycin	Biochrom AG	A 2213
PBS	Biochrom AG	L1825
MTT reagent	Sigma Aldrich GmbH	M5655
Isopropanol	Carl Roth GmbH	9866.2
Hydrochloric acid	Fluka	84420
96 well microplates, cell culture treated	Greiner Bio-One GmbH	655 090
96 well CELLCOAT® microplates, PDL-coated	Greiner Bio-One GmbH	655 946

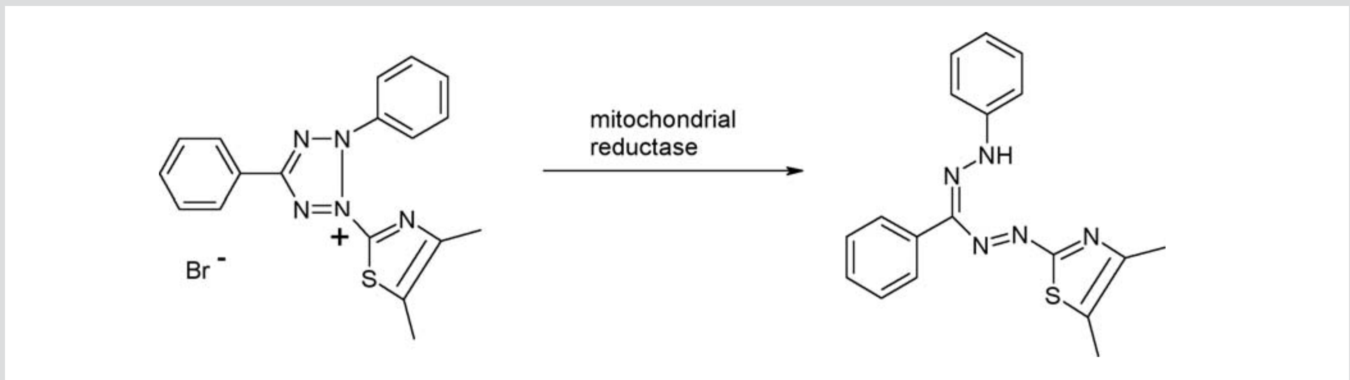

Figure 2: MTT is reduced to Formazan by a mitochondrial reductase

Cell culture

Using an MTT assay, the effect of washing steps on cell attachment is demonstrated in the following experiment. The MTT assay is a laboratory test for measuring cell vitality by mitochondrial activity. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) is reduced to purple Formazan by a mitochondrial reductase (**Fig. 2**).

The amount of purple Formazan formed is indicative of the vitality of the cells. The Formazan is dissolved in acidic Isopropanol afterwards and then quantified by measuring the absorption at 590 nm.

To demonstrate the effect of washing steps on cell attachment, a Neuroblastoma cell line was seeded on cell culture treated and PDL-coated microplates in densities of 50,000 cells/well; 100,000 cells/well and 200,000 cells/well.

DMEM medium supplemented with 20 % FCS and 1 % Penicillin-Streptomycin was used for cultivation.

After an incubation of 45 min at 37° C and 5 % CO_2, the supernatant was discarded by aspiration. Then, the cells were washed by pipetting. The washing solution was aspirated and replaced by medium containing MTT in a concentration of 1 mg/ml. The microplates were incubated for 1 hour at 37° C and 5 % CO_2. Pictures were taken before the washing step and after adding the MTT.

Finally, the Formazan formed by living cells was dissolved in 150 µl acidic Isopropanol and evaluated by measuring the optical density at 590 nm.

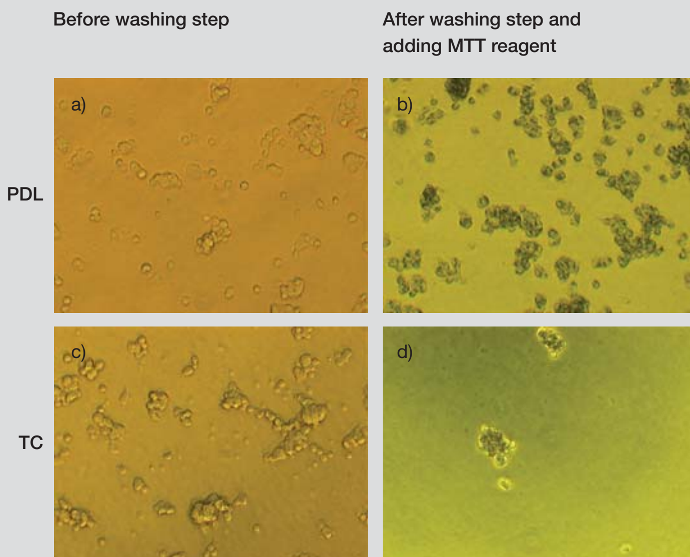

Figure 3: Cell density of 50,000 cells/well

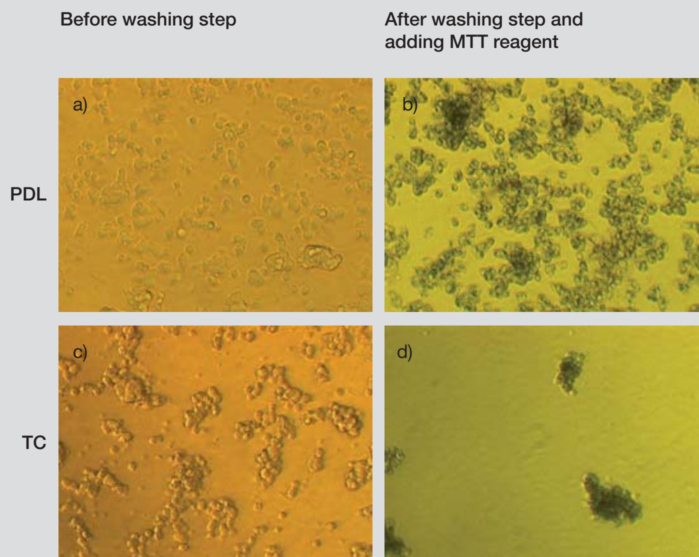

Figure 4: Cell density of 100,000 cells/well

Results

Microscopical observation

In order to determine the optimal cell concentration, various cell densities were seeded in 96 well microplates in triplets. Cell densities of 50,000 cells/well; (**Fig. 3**) 100,000 cells/well (**Fig. 4**) and 200,000 cells/well were used (**Fig. 5**).

After incubation of one hour at 37° C and 5 % CO_2, cells were microscopically observed. The attachment of the cells on the PDL-coated microplates is significantly better than on the cell culture treated microplates. On the PDL-coated microplates, cells had already started to spread out and showed the first signs of adherence (**Fig. 3a, 4a and 5a**). In contrast, cells on the cell culture treated surface had a round morphology and formed clusters of floating cells (**Fig. 3c, 4c and 5c**).

To demonstrate the impact of specific experimental conditions, like washing steps in automated systems, the cells were washed several times by pipetting. On the cell culture treated surfaces nearly all cells were washed away. Only a few cells remained as floating clusters on the surface (**Fig. 3d, 4d and 5d**). Compared to these observations, the cells cultivated on PDL-coated surfaces attach firmly to the surface and remain completely on the microplate bottom even after extensive washing of the wells (**Fig. 3b, 4b and 5b**). These observations were reproducible for all seeded cell densities and pipetted triplets.

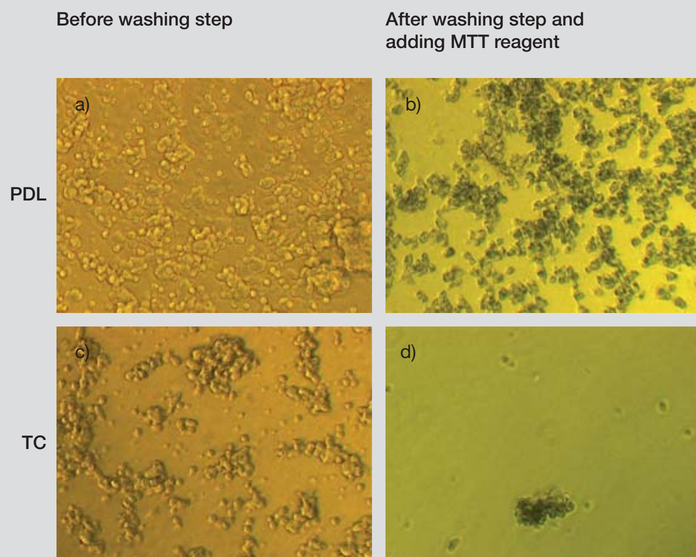

Figure 5: Cell density of 200,000 cells/well

Figures 3, 4 and 5: Microscopical observation of cells before and after adding the MTT reagent and the included washing step

a) Cells cultivated on PDL-coated CELLCOAT® microplates before washing step

b) Microscopical MTT assay results of cells cultivated on PDL-coated CELLCOAT® microplates after washing step

c) Cells cultivated on cell culture treated microplates (TC) before washing step

d) Microscopical MTT assay results of cells cultivated on cell culture treated microplates (TC) after washing step

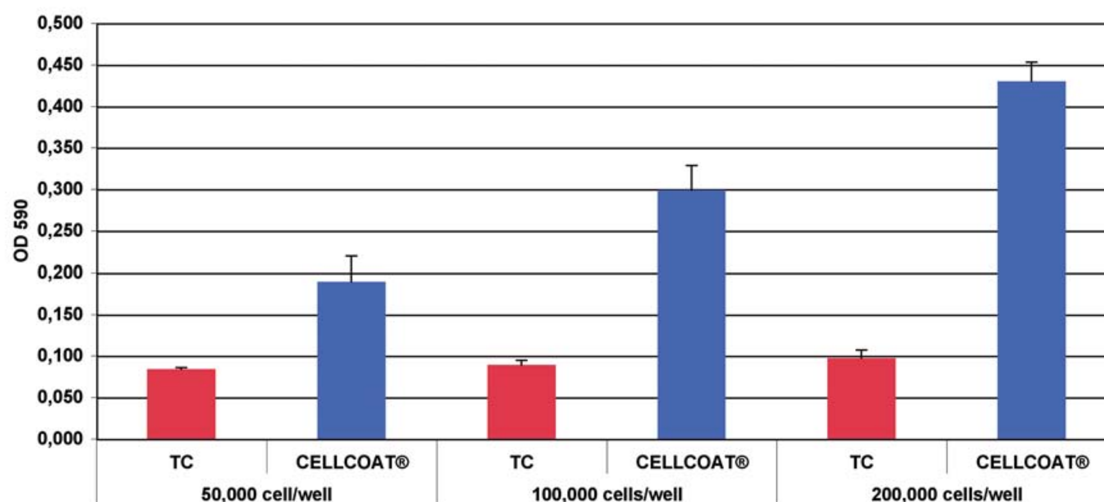

Figure 6: Spectroscopic evaluation of the dissolved Formazan at 590 nm

Spectroscopic evaluation

To verify the microscopic observations, the blue Formazan formed by living cells in the MTT assay was dissolved in acidic Isopropanol and absorption was evaluated at 590 nm in a Tecan Safire spectrometer (**Fig. 6**).

The absorption of Formazan in the cell culture treated (TC) wells did not exceed the baseline of 0.1 units. These results support the observation that most of the cells of the used Neuroblastoma cell line on the cell culture treated surface were washed away during the washing step included in this experiment.

The absorption of the Formazan on the PDL-coated surface (CELLCOAT®) is proportional to the seeded cell density. The higher the cell density, the higher the measured signal. These results support the conclusion that all cells were attached on the PDL-coated surface and were not detached by the washing step.

Conclusion

The illustrated experiment demonstrates the suitability of PDL-coated microplates for the cultivation of sensitive cell lines *in vitro*. In addition, PDL-coated microplates greatly enhanced the adherence of cells to the surface compared to cell culture treated microplates, when experiments included washing steps. Based on these facts, PDL-coated CELLCOAT® microplates are a valuable tool in high-throughput screening and academic applications. The enhanced attachment of the cells to the various protein-coated surfaces enables experiments to be conducted which would be impossible to achieve without these innovative products.

References

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