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Proceeding Paper

Investigation the Optical Contrast Between Nanofiber Mats and Mammalian Cells Dyed with Fluorescent and Other Dyes †

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Abstract: Electrospinning can be used to prepare nanofiber mats from diverse polymers and polymer blends. A large area of research is the application of nanofibrous membranes for tissue engineering. Typically, cell adhesion and proliferation as well as the viability of mammalian cells are tested by seeding the cells on substrates, cultivating them for a defined time and finally dyeing them to enable differentiation between cells and substrates under a white light or fluorescence microscope. While this procedure works well for cells cultivated in well plates or petri dishes, other substrates may undesirably also be colored by the dye. Here we show investigations of the optical contrast between dyed CHO DP-12 (Chinese hamster ovary) cells and different electrospun nanofiber mats, dyed with haematoxylin-eosin (H&E), PromoFluor 488 premium, 4,6-diamidino-2-phenylindole (DAPI) or Hoechst 33342, and give the optimum dyeing parameters for maximum optical contrast between cells and nanofibrous substrates.

Keywords: electrospinning; poly(acrylonitrile) (PAN); biopolymers; mammalian cells; fluorescent dyes

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1. Introduction

Cell cultivation is a standard process in biotechnological research and development. Adherent cells are usually grown in well-plates or roller bottles, making the process relatively costly [1]. Alternatively, microcarriers or hollow fibers can be uses as substrates [2,3], while single-use fixed-bed bioreactors use macroporous nonwovens or microfiber membranes [4].

Using nanofiber mats as alternative substrates belongs to the recent research areas [5–7]. The nanofibrous morphology of these substrates can not only improve cell adhesion and proliferation, but even be used to support stem cell differentiation [8].

Producing such nanofiber mats is possible by electrospinning. Different techniques exist, mainly differentiated into needle-based and needleless processes, which enable producing nanofiber mats from diverse polymers, polymer blends as well as with embedded metallic, ceramic or other nanoparticles [9–11]. While the nanofibers in these mats are usually arbitrarily oriented, there are technical possibilities to orient them more or less parallel, which improves the possibilities to guide cell growth [12,13].

One problem, however, is the evaluation of the cell growth on these nanofiber mats, as the usual process of dyeing the cells for microscopic evaluation often leads to dyeing the nanofiber mats, too. This reduced contrast leads to reduced reliability of cell counting or measuring the cell-covered substrate area.

Here we give an overview of dyeing cells on nanofibrous substrates, electrospun from pure poly(acrylonitrile) (PAN) as well as PAN/gelatin, PAN/keratin and PAN/TiO_2. After cultivation for 5 days, the cells were fixed with glyoxal and afterwards dyed with

haematoxylin-eosin (H&E), PromoFluor 488 premium, 4,6-diamidino-2-phenylindole (DAPI) or Hoechst 33342. Examination by white light or fluorescence microscopy, respectively, revealed that especially PAN/gelatin nanofiber mats—which were most advantageous regarding the cell growth—were also colored by the dyes. The paper reports the optimized dyeing parameters to maximize the optical contrast between CHO cells and nanofiber mats.

2. Materials and Methods

The following polymer blends were used for electrospinning nanofiber mats:

- Poly(acrylonitrile) (X-PAN copolymer, consisting of 93.5% acrylonitrile, 6% methylacrylate, and 0.5% sodium methallyl sulfonate, from Dralon, Dormagen, Germany; Mn = 90,000 g/mL, Mw = 250,000 g/mol); 16 wt.% PAN dissolved in DMSO (min. 99.9%, obtained from S3 chemicals, Bad Oeynhausen, Germany)
- PAN (12 wt.%)/gelatin (1 wt.%) in DMSO
- PAN (16 wt.%)/keratin (7 wt.%) in DMSO
- PAN (14 wt.%)/TiO_2 (6 wt.%) in DMSO

Electrospinning was performed by the wire-based electrospinning device Nanospider Lab (Elmarco, Liberec, Czech Republic), applying a voltage of 65–80 kV, using a nozzle diameter of 1.5 mm and a carriage speed of 100 mm/s. The temperature was 21 °C, the relative humidity in the spinning chamber 32%. Spinning was performed for 30 min per sample. Typical nanofiber diameters are around 300 nm for pure PAN [14], approx. 450 nm for PAN/keratin [15], around 100 nm for PAN/TiO_2 nanofibers [16], and approx. 400–800 nm for PAN/gelatin nanofibers [8].

All samples were sterilized by incubation with 1 mol. HCl for 1 h before they were rinsed with phosphate buffered saline (PBS) buffer solution 3–5 times.

Cell cultivation was performed with the Chinese hamster ovary cell line CHO-DP12 (ATCC CRL-12445) (LGC Standards GmbH, Wesel, Germany). As the cultivation medium, Dulbecco's Modified Eagles Medium (DMEM F12 +10% FCS) was used, to which $NaHCO_3$, D-glucose and L-glutamine were added. The pH value was set to 7.4, before the medium was sterile filtered in an autoclaved 1 L bottle, and finally 100 mL sterile donor horse serum were added.

To prepare the substrates for cell growth, they were glued on cover glasses (21 mm × 26 mm) to avoid floating in the medium. 6-well plates were used for cultivation. Cell suspensions of 5 mL per well were pipetted into the wells with a concentration of 3.8×10^4 cells/mL (i.e., 2×10^4 cells/cm^2). The well plates were cultivated at 37 °C and 5% CO_2 for 5 days in an incubator.

Fixation of the cells on the substrates was performed by glyoxal solution (2 mL per well) after the medium was pipetted off the wells and the substrates were washed with ultra-pure water. After removing the glyoxal solution, the wells were washed with PBS buffer and twice with ultra-pure water.

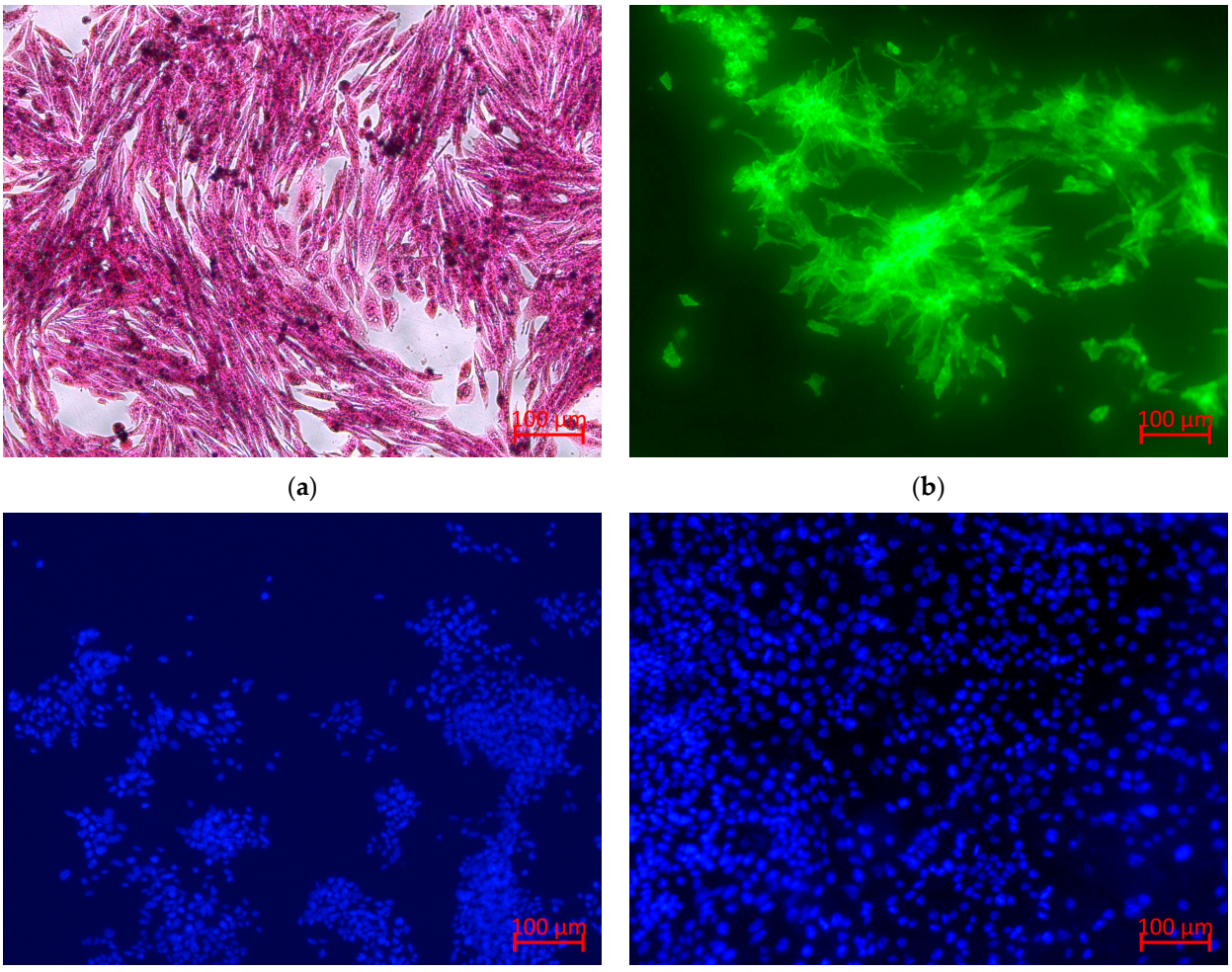
Dyeing the cells on the substrates after fixation was performed with four different fluorescent dyes:

- Hematoxylin and eosin-Y (H&E Fast Staining Kit from C. Roth, Karlsruhe, Germany) (hematoxylin solution 2 mL/well for 6 min, then rinsed with water, differentiated by 0.1% HCl, blued for 6 min in water and finally stained with eosin-Y for 30 s)
- PromoFluor 488 premium (1 mL/well of 0.1% Triton X-100 in PBS for 5 min, washed with PBS, dyed with 1 mL of 1% bovine serum albumin in PBS + 2.5 µL PromoFluor for 20 min, washed with PBS)
- 4′,6-Diamidin-2-phenylindol (DAPI) (3 drops DAPI solution Roti-Mount FluorCare per sample)
- Hoechst 33342 (16 mM Hoechst dye solution dissolved 1:2000 in PBS pipetted into the wells for 10 min, washed with PBS)

The cells were investigated by an inverse microscope Axiovert 40 CFL (Carl Zeiss, Göttingen, Germany) during cultivation and by an inverse fluorescent microscope Zeiss Axio Observer after dyeing.

3. Results

As a reference, Figure 1 depicts cells grown on the pure well ground with different dyes. Generally, the cells are well visible on the well ground with all dyes. The H&E dye (Figure 1a) stains cell nuclei in purple-blue by hematoxylin and the extracellular matrix and cytoplasm in pink by eosin [17], so that all parts of the cells are visible. PromoFluor stains the cytoplasm in green (Figure 1b), while DAPI stains cell nuclei (Figure 1c) and Hoechst binds to DNA (Figure 1d). Combinations of DAPI or Hoechst with PromoFluor can thus be used to receive images of blue cell nuclei and green cytoplasm.

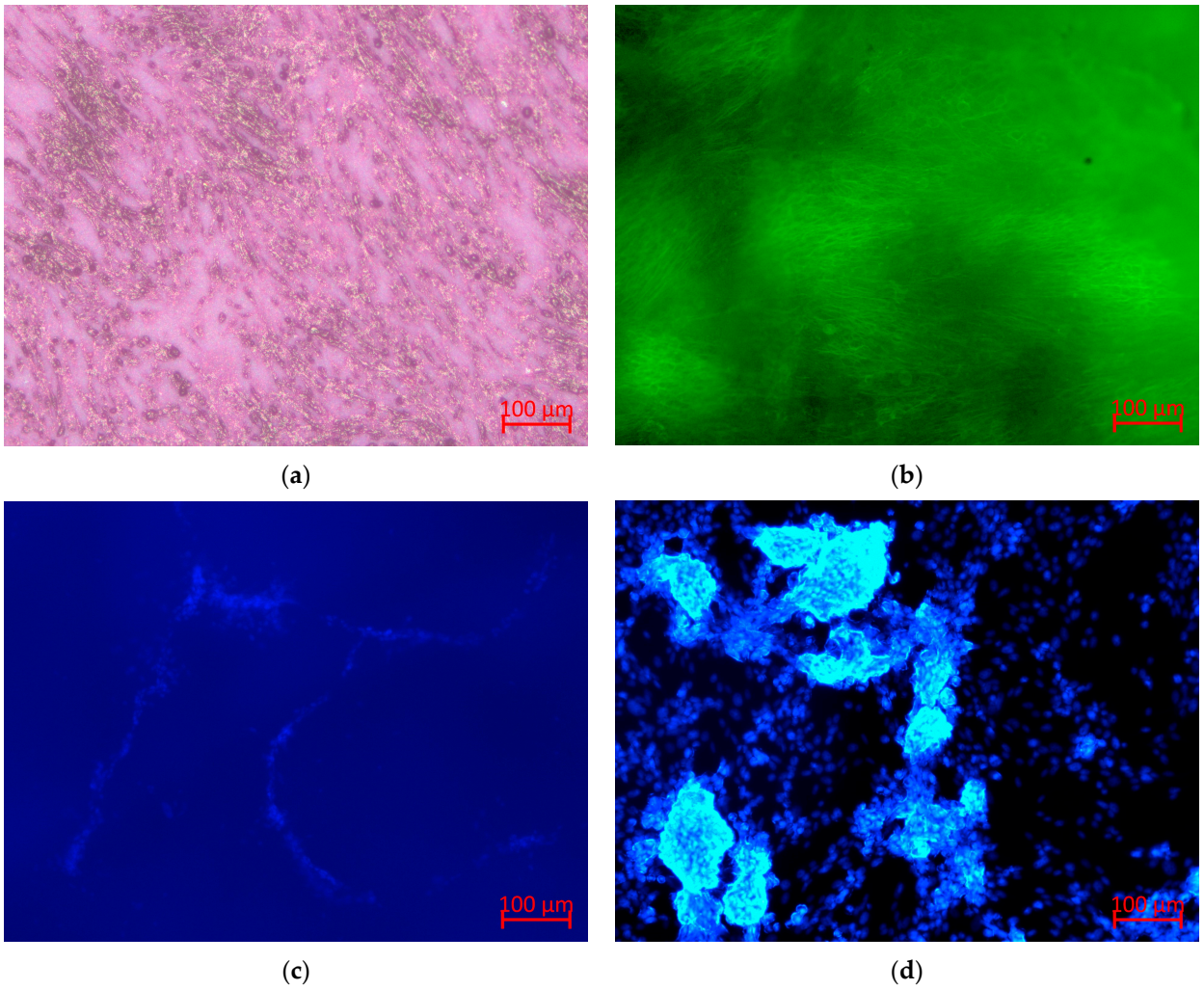


(**a**) (**b**)

(**c**) (**d**)

Figure 1. Cells grown on the well ground, dyed with (**a**) H&E; (**b**) PromoFluor; (**c**) DAPI; and (**d**) Hoechst.

While all four dyes enable staining the cells on the well ground without problems, differentiating between cells and substrate is much more complicated on nanofiber mats. As an example, Figure 2 depicts CHO cells grown on a PAN/gelatin nanofiber mat, stained with different dyes. In all cases, the cells are less well visible than on the well grounds. The sample stained with Hoechst reveals some bright cell agglomerations, but also shows the cell nuclei in the best way.



(**a**) (**b**)

(**c**) (**d**)

Figure 2. Cells grown on a PAN/gelatin nanofiber mat, dyed with (**a**) H&E; (**b**) PromoFluor; (**c**) DAPI; and (**d**) Hoechst.

DAPI was found to strongly stain the PAN/gelatin nanofiber mats, making it not ideal for optical evaluation of cells on these substrates. H&E enables seeing cell nuclei as well as cytoplasm, although with relatively low contrast to the substrate. Hoechst has a good contrast, but needs several washing steps which may lead to rinsing cells off the substrate. PromoFluor, finally, stained the gelatin too strongly to enable seeing cells.

4. Conclusions

Depending on the nanofiber material, different dyes are preferable. The different amounts of staining the nanofiber mats depends on chemical reactions between the dyes and the different materials in the nanofiber mats under investigation, which are usually not described in the scientific literature, but must be taken into account if cells are grown on substrates other than the usual well grounds.

On PAN nanofiber mats, all four dyes led to suitable dyeing results. Similarly, all four dyes were well suitable for PAN/keratin. For PAN/gelatin, H&E and Hoechst should be preferred. PAN/TiO_2 nanofiber mats were too strongly stained by DAPI, Hoechst and PromoFluor to enable seeing cells, making H&E the only suitable dye.

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