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Sterilization of PAN/Gelatine Nanofibrous Mats for Cell Growth

Sterilizacija PAN/želatinskih nanovlaknastih kopren za rast celic

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Abstract

Nanofibrous mats can be used as a substrate for eukaryotic cell growth in biotechnology, tissue engineering, etc. Several adherent cells (e.g. human fibroblasts) have been shown to grow well on fine fibres. For most applications, it is necessary to sterilize nanofibrous mats before adding the cells. Another possibility would be the addition of antibiotics and antimycotics to the cell culture medium to prevent microbial infection. However, antibiotics are disadvantageous since they might promote the growth of resistant bacteria in possible future medical applications of nanofibrous mats. Possible sterilization techniques include autoclaving, UV-sterilization, ozone treatment, heat sterilization and other techniques which usually necessitate more expensive equipment, such as gamma irradiation. Systematic examinations of the influence of different sterilization techniques on the cell growth on nanofibrous mats have not yet been reported in the literature. Here, we report on the first experimental investigations of the effect of sterilization with different methods on the properties of polyacrylonitrile (PAN)/gelatine nanofibrous mats, and the resulting growth and adhesion of Chinese hamster ovary cells. While all techniques under investigation yielded sterile nanofibrous mats, autoclaving and heat sterilization change the PAN/gelatine fibre morphology. Ozone, on the other hand, modifies the pH value of the culture medium and partly impedes cell adhesion. UV sterilization also suggests a chemical modification of the nanofibrous mat. Unexpectedly, heat sterilization resulted in the highest amount of adherent Chinese hamster ovary cells grown on PAN/gelatine nanofibrous mats in spite of gelatine melting.

Keywords: polyacrylonitrile/gelatine nanofibrous mats, sterilization, autoclaving, ozone, UV sterilization, heat sterilization, cell growth, adherent cells, CHO cells, tissue engineering

Izvleček

Nanovlaknaste koprene se lahko uporabijo kot substrat za rast celic v biotehnologiji, tkivnem inženirstvu itd. Pokazalo se je, da številne celice, npr. človeški fibroblasti, pritrjene na finih vlaknih, dobro rastejo. Za večino aplikacij je treba nanovlaknaste koprene pred nanosom celic sterilizirati. Druga možnost preprečevanja mikrobnih okužb bi bila dodajanje antibiotikov in antimikotikov v gojišče celične kulture. Vendar pa so antibiotiki manj primerni, saj lahko spodbujajo rast odpornih bakterij v morebitnih prihodnjih medicinskih aplikacijah nanovlaknastih kopren. Mogoče so različne tehnike sterilizacije, vključno z avtoklaviranjem, UV-sterilizacijo, obdelavo z ozonom, toplotno sterilizacijo in druge, ki po navadi zahtevajo dražjo opremo, kot je obsevanje z žarki gama. Pregleda o vplivu različnih tehnik sterilizacije na rast celic na nanovlaknastih koprenah v literaturi še ni. V nadaljevanju predstavljamo prve eksperimentalne raziskave učinka sterilizacije z različnimi metodami na lastnosti poliakrilonitrilnih (PAN)/želatinastih nanovlaknastih kopren in posledično na rast in adhezijo celic. Medtem ko so tehnike, ki so bile raziskane, omogočile

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izdelavo sterilnih nanovlaknastih kopren, pa sta avtoklaviranje in toplotna sterilizacija spremenila morfologijo PAN/želatinastih vlaken. Po drugi strani pa je ozon spremenil vrednost pH-gojišča in delno oviral celično adhezijo. UV-sterilizacija je vodila tudi v kemično modifikacijo nanovlaknaste koprene. Nepričakovano je bilo, da je toplotna sterilizacija vodila v nastanek največje količine celic jajčnikov kitajskega hrčka (celice CHO), ki kljub taljenju želatine rastejo na PAN/želatinastih nanovlaknih.
Ključne besede: PAN/želatinska nanovlakna, sterilizacija, avtoklaviranje, ozon, UV-sterilizacija, toplotna sterilizacija, rast celic, adherentne celice, celice CHO, tkivni inženiring

1 Introduction

Nanofibrous mats with the fibre diameters in the range of some ten to several hundred nanometres can be prepared with electrospinning from diverse polymer solutions or melts. While the easiest technology uses a syringe to extrude the polymer solution through a fine needle into a high electric field [1, 2], needleless technologies allow for higher production output and can often be upscaled from lab to industrial scale [3–5].

Electrospinning from a solution necessitates finding an adequate solvent to dissolve the desired polymer or polymer blend, besides several other prerequisites, such as large enough molecular weight of the polymer to form fibres at all. Several solvents which are often used in electrospinning, however, are toxic or corrosive. While a large number of biopolymers can be electrospun from water [6, 7], only few water-resistant polymers can be spun from low-toxic solvents such as dimethyl sulfoxide (DMSO) [8]. This is one of the reasons why polyacrylonitrile (PAN) is of large interest in electrospinning, apart from its usability as a precursor for carbon nanofibres [9–11].

Preliminary tests in our group showed that animal cells do not grow well on pure PAN nanofibrous mats although serum was added to the culture medium. It is well-known that certain proteins present in the serum promote cell adhesion (e.g. fibronectin [12]). Another possibility to enable cell adhesion is coating the cell culture flasks with collagen, which is an established method to enable cell adhesion [13]. Thus, the effect of adding gelatine to the PAN solution was tested. Commercially available gelatine is derived mostly from porcine skin. It is water-soluble, and consists mainly of a mixture of different denatured and partly hydrolysed collagens. PAN/gelatine nanofibres display slightly increased fibre diameters compared to pure PAN nanofibres [14]. Since the exact distribution of both components in fibres has not been investigated yet, the influence of the fluid culture medium on fibre integrity has to be examined.

Generally, nanofibrous mats prepared from biopolymers, such as gelatine or alginate, are known to support cell growth for wound healing, tissue engineering or biotechnological purposes [15–19]. Especially for elongated cells, such as neurites or fibroblasts, nanofibres have been shown to support cell growth [20–24].

Unexpectedly, possibilities to sterilize nanofibrous mats before the addition of cells and their potential influences on cell growth are only scarcely mentioned in the scientific literature. Castagna *et al* performed autoclaving and UV irradiation to sterilize polyaniline nanofibrous mats [25]. Arnal-Pastor *et al* used immersion in 70% ethanol for 3 hours, which necessitated careful rinsing with sterile phosphate-buffered saline to remove ethanol residues, making this method very time-consuming [26]. Ethanol was also suggested by Fan *et al* to sterilize regenerated silk fibroin nanofibres [27]. However, absolute ethanol (99.46%) is usually non-sterile since it is known to preserve microbial spores [13]. Therefore, sterilization with ethanol is not a reliable procedure.

In this paper, we report on a comparison of four different sterilization techniques applied for PAN/gelatine nanofibrous mats, and their influence on the growth of adherent CHO cells in the absence of antibiotics and antimycotics. The CHO cells are often used in the production of therapeutic proteins and in diverse biotechnological examinations [28–31]. Hence, they are ideal model cells for preliminary sterility tests.

2 Materials and methods

2.1 Electrospinning

Nanofibrous mats were produced with the wire-based electrospinning machine "Nanospider Lab" (Elmarco, Liberec, Czech Republic). The spinning

parameters were: voltage 70 kV, nozzle diameter 1.5 mm, carriage speed 100 mm/s, static substrate, ground-substrate distance 240 mm, electrode-substrate distance 50 mm, relative humidity 33%, spinning duration 50 min.

The PAN solutions were prepared with a PAN (purchased from Woolworth, Unna, Germany) concentration of 16% in DMSO (min. 99.9%, purchased from S3 Chemicals, Bad Oeynhausen, Germany) by two-hour stirring at room temperature. Gelatine (purchased from Abtei, Marienmünster, Germany) was added in the concentrations of 5% and 10%; higher concentrations were not spinnable.

2.2 Sterilization

Sterilization was performed with the following methods:

A closed box with an ozone generator (CHM GmbH, Amberg, Germany), working according to ISO 105-G03, was used for 15 min, 30 min, 45 min and 60 min. Afterwards, the petri dishes were closed inside the box to avoid contaminations after the sterilization process.

The heat sterilization was performed with an oven UN75 (Memmert, Schwabach, Germany) at the temperatures of 60 °C, 100 °C, 140 °C and 180 °C for 30 min each.

For the UV sterilization, a UV lamp UV-spot 25W/E27 (Eurolite, Waldbüttelbrunn, Germany) was used. Since this lamp was not specified for sterilization, relatively long irradiation times of 1 h, 1.5 h, 2 h, 2.5 h and 4 h were tested. The heat development during the sterilization was monitored with a thermal camera (FLIR E8, FLIR Systems, Wilsonville, OR, USA).

The autoclaving was performed for 20 min at 121 °C with a VX-75 autoclave obtained from Systec (Linden, Germany).

Before the sterilization tests, the nanofibrous mat samples were washed three times with highly purified water. After the sterilization, the samples were placed in the wells of a 24-well cell culture plate (Cellstar®, Greiner, Kremsmünster, Austria) inside a biological safety cabinet Safe 2020 (Thermo Electron LED GmbH, Langenselbold, Germany). Prior to that, 5 ml of mediums was pipetted into the well, using five specimens per experiment. One well was used as a sterility test. The incubation was performed for 4 days (96 h) in a HERAcell 240i incubator (ThermoFisher Scientific, Waltham, MA, USA) at 37 °C and 7.5% CO_2.

2.3 Cell cultivation

For the cell cultivation, Dulbecco's Modified Eagle's medium (DMEM) / Ham's Nutrient Mixture F12 (1 : 1 DMEM/F12) (purchased from SAFC Biosciences, Irvine, UK) was used as a base. The medium was prepared according to the instructions for use. It was supplemented with glucose (Roth, Karlsruhe, Germany) and L-glutamine (Applichem, Darmstadt, Germany) to the final concentration of 4 g/l and 4 mM, respectively. It also contained phenyl red. The pH value was set to 7.4 before the medium was sterile filtered (Sartolab P, 0.45 µm / 0.22 µm, Sartorius, Göttingen, Germany). Finally, 10% sterile donor horse serum (biowest, Nuaillé, France) was added.

The CHO DP-12 cells used in this study were obtained from LGC Standards GmbH, Wesel, Germany (ATCC no. CRL-12445). These Chinese hamster ovary cells grow *in vitro* to form a monolayer if using a serum containing medium.

For the cell cultivation, nanofibrous mat samples of 2 cm × 2 cm were glued with Elastosil E41 RTV-1 silicone rubber (Wacker, Burghausen, Germany) on cover slips (21 mm × 26 mm) (Carl Roth, Karlsruhe, Germany) to keep them inside the medium. After the sterilization, the mats were placed in 6-well plates (Labsolute, Th. Geyer, Renningen, Germany) inside a safety cabinet (HERA Safe 2020, ThermoFisher Scientific, Waltham, MA, USA). 200,000 cells were seeded into each well. In one well, only cells without a nanofibrous mat were cultivated as a control. The cultivation was again performed for 4 days in an incubator at 37 °C and 7.5% CO_2.

Afterwards, the medium was pipetted out of the wells. The cells were dyed with 1 ml methylene blue (euromex, Arnheim, Netherlands; 1 : 16 dilution in PBS). The pretests showed that neither PAN nor gelatine were dyed by it, confirming that all blue objects on nanofibrous mats must belong to cells (not shown).

After PBS was again pipetted out of the wells, 1.5 ml glyoxal solution was added to fix the cells [32]. For this, a 3 vol% glyoxal solution, containing 34.02 ml highly purified water, 9.468 ml ethanol (abs., VWR BDH Prolabo, Langenfeld, Germany), 3.756 ml 40% glyoxal (Roth, Karlsruhe, Germany) and 0.36 ml acetic acid p.a. (VWR BDH Prolabo, Langenfeld, Germany), with the pH value between 4 and 5 was used. The well plate was cooled on ice for 30 min and incubated for another 30 min at room temperature before the glyoxal solution was pipetted out of the wells, and the nanofibrous mats were taken out and dried in the air.

2.4 Investigations

An optical examination during cultivation was achieved with an Axiovert 40 CFL microscope (Zeiss, Oberkochen, Germany). After the fixation, confocal laser scanning microscopes (CLSM) VK-9000 and VK-100 (Keyence, Osaka, Japan) were used with the nominal resolution of 2000×.

The number of attached cells on nanofibrous mats was counted with a Neubauer chamber (Hecht Assistent, Sondheim, Germany). The nanofibrous mats were carefully washed with PBS and then treated with a sterile trypsin/EDTA (ethylenediamine tetraacetic acid) solution (biowest, Nuallié, France), digesting the cell-matrix interaction, which results in cell detachment. After 5 min incubation at 37 °C, the trypsinization was stopped by diluting the fluid with a culture medium. A 50 µl cell suspension was mixed with 50 µl trypan blue (a vital stain used to colour the dead cells blue) and the cells were counted with a Neubauer chamber.

3 Results and discussion

The sterilization test (Figure 1) revealed a colour change of the medium in the wells in which the ozone-sterilized specimens were placed. The medium contained phenyl red, a pH indicator. At neutral pH, phenyl red displays a pink colour. When the pH is lowered, the colour of the indicator changes to yellow. In the cell culture technology, this is useful since the change in colour indicates that the nutrients are used up by the cells. Here, no cells were added to nanofibrous mats. Nevertheless, the colour change was observed. This can be attributed to the highly reactive ozone molecules that produced radicals which were able to react with the fibres to change the pH value of the surrounding medium.

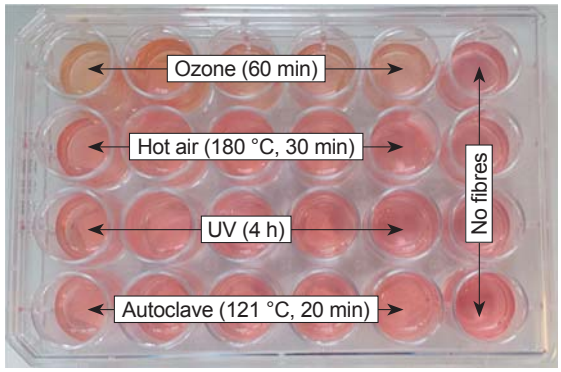


Figure 1: Sterilization test, photograph taken on day 0; four wells were incubated without fibre mats as control ("no fibres")

This effect was only visible directly after the addition of medium; however, on the following day, all wells displayed the same colour as shown here for the not ozone-treated nanofibrous mats, without changing the medium. The medium contained a carbonate buffer system. Since the incubation took place in a CO_2 atmosphere (7.5%), all pH values changed to the same level. It should be mentioned that in the following test series in which all nanofibrous mats were washed with high-purity water before the sterilization, this finding was only slightly visible, indicating that either DMSO or pure gelatine, which could be washed off, was responsible for the effect. Shorter ozone treatment durations also reduced this effect significantly.

Next, the influence of the sterilization processes and the culture medium on the nanofibrous mat morphologies was tested by CLSM imaging. Figure 2 shows a PAN/gelatine nanofibrous mat before the sterilization, with typical fibre diameters in the range 400–800 nm for both gelatine concentrations, with several thicker fibres on the surface, which were dissolved at higher temperatures, indicating that they mostly consisted of gelatine, while former stabilization test series indicated that most fibres contained a blend of PAN and gelatine, which was water-stable [10]. Figure 3 depicts the results after the sterilization with different methods and immersion in a culture medium.

Figure 2: CLSM image of PAN/gelatine nanofibrous mat with 10% gelatine after electrospinning; scale bar indicates 20 µm

Firstly, it was clearly visible that the hot air treatment and autoclaving changed the morphology of

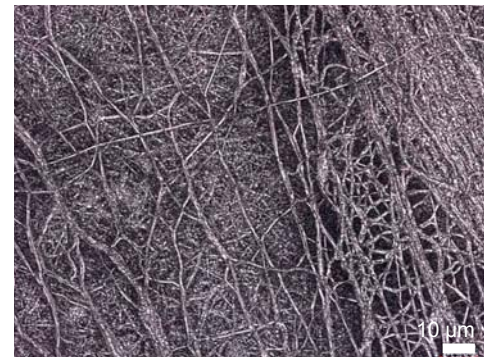
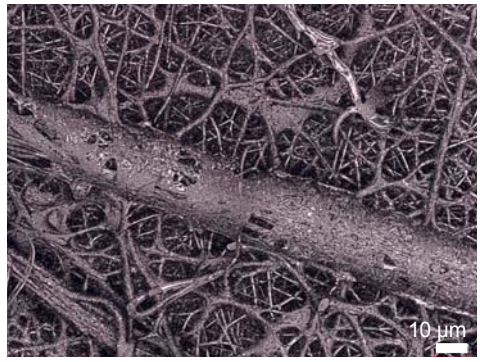
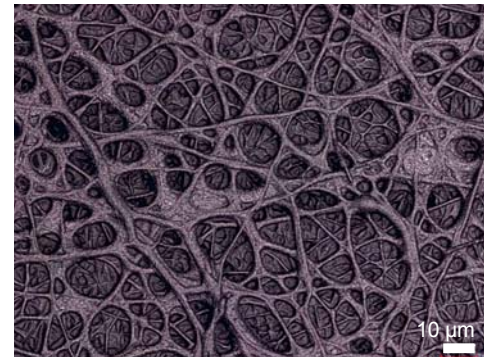
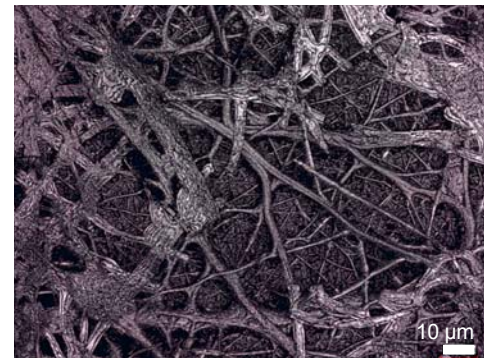
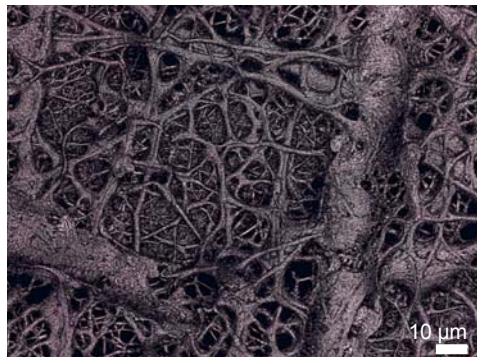
Sterilization technique	After sterilization	After culture medium
Ozone (60 min)		
Hot air (180 °C, 30 min)		
UV (4 h)		
Autoclave (121 °C, 20 min)		

Figure 3: CLSM images of nanofibrous mats taken after sterilization and after following immersion in culture medium; scale bars indicate 10 µm

fibres. In both cases, gelatine was molten and afterwards solidified again, as described above. A higher temperature used for autoclaving seemed to slightly reduce the viscosity of the molten gelatine and correspondingly increased its spreading over the near environment before the re-solidification.

This could be expected since gelatine was shown to start melting at the temperatures around 32–40 °C [33], the temperature range which was exceeded in both processes. It should be mentioned that during the cell cultivation at 37 °C, gelatine could also be assumed to be molten. In addition, similar changes were visible in parts with UV-sterilized nanofibrous mats, which could be attributed to the hot UV lamp positioned very close to the samples, resulting in the temperature on the sample surface of about 32–36 °C, as measured with a thermal camera.

After the ozone treatment, the relatively thick, long gelatine fibres could clearly be distinguished from the PAN or PAN/gelatine fibres. Previous research revealed that in most fibres, PAN and gelatine were mixed [14]; however, in all cases, pure gelatine fibres were also found in PAN/gelatine nanofibrous mats as visible in the confocal laser scanning microscope images after the stabilization of fibre blends. In these former test series, PAN/gelatine nanofibre mats were stabilized at different temperatures and heating rates, resulting in a clear colour differentiation between the afterwards brown PAN and the silvery gelatine. This enabled distinguishing between both materials optically, instead of using chemical microscopic methods. The morphology of these nanofibrous mats was not different from the one of the original mat.

After the incubation of the ozone treated nanofibrous mat in a culture medium, the thickest fibres were still visible, underlining that they contained PAN as well, since pure gelatine fibres would be expected to dissolve in an aqueous culture medium.

After the general proof that these sterilization methods were applicable to PAN/gelatine nanofibrous mats, shorter treatment times (in the case of ozone and UV sterilization) or lower temperatures (in the case of hot air sterilization) were investigated. In the following cases, contaminations occurred, namely at 15 min of ozone treatment (1 of 5 samples was contaminated), 60 °C heat treatment (6 of 10 samples), 2.5 h of UV treatment (2 of 10 samples), 2.0 h of UV treatment (1 of 10 samples), 1.5 h of UV treatment (1 of 10 samples) and 1 h of UV treatment (2 of 10 samples).

These numbers of contaminated samples exhibited that only for the heat treatment at 60 °C, the sterilization did not work for most samples. In other cases, nanofibrous mats were sterilized at high percentage. While this clearly shows that autoclaving – as the standard method – is still preferable in terms of sterility, the ozone and UV treatments resulted in significantly better maintaining of the nanofibre structure and they can thus be assumed to be more eligible with respect to cell growth. While the sterility in the range of 90% is inacceptable for medical purposes, it may be tolerable for basic research on cell cultures. For the special application of nanofibrous mats for cell growth and cell adhesion, the statistical sterilization success and the dimensional stability of nanofibrous mats have to be balanced. Future investigations will examine the statistical sterilization success of these milder treatments in detail.

For the final cell culturing experiments, the original sterilization temperatures and durations, respectively (cf. Figure 3), were adopted. Figure 4 depicts exemplarily one experiment in which CHO DP-12

a) b)

Figure 4: 6-well plate with ozone-sterilized nanofibrous mats (10% gelatine) in culture medium (only medium in lower right well as reference) with CHO DP-12 cells, (a) start of cultivation, (b) end of cultivation at day 5

cells were cultivated on ozone-sterilized nanofibrous mats containing 10% gelatine. Similar results were gained with 5% gelatine content; a quantitative comparison is given later on.

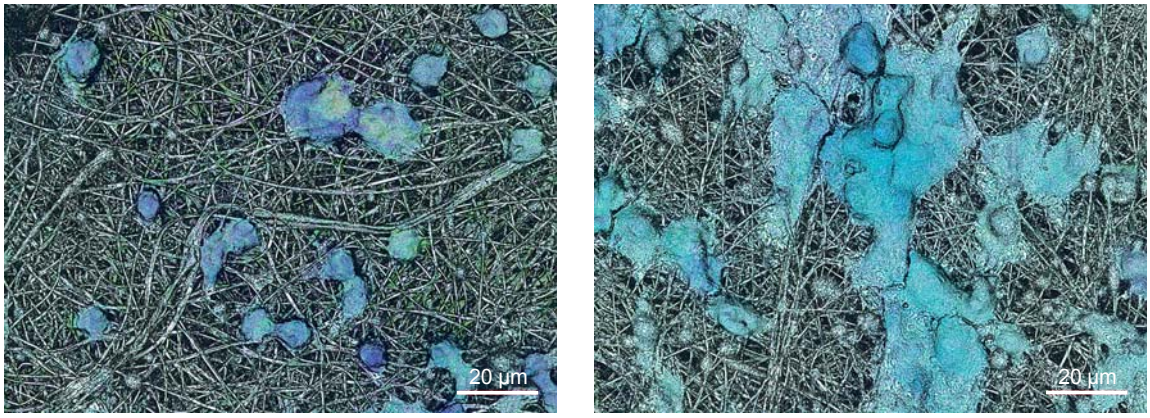


Figure 5: CLSM images of CHO DP-12 cells on different areas of ozone-sterilized PAN/gelatine nanofibrous mats; scale bars indicate 20 µm

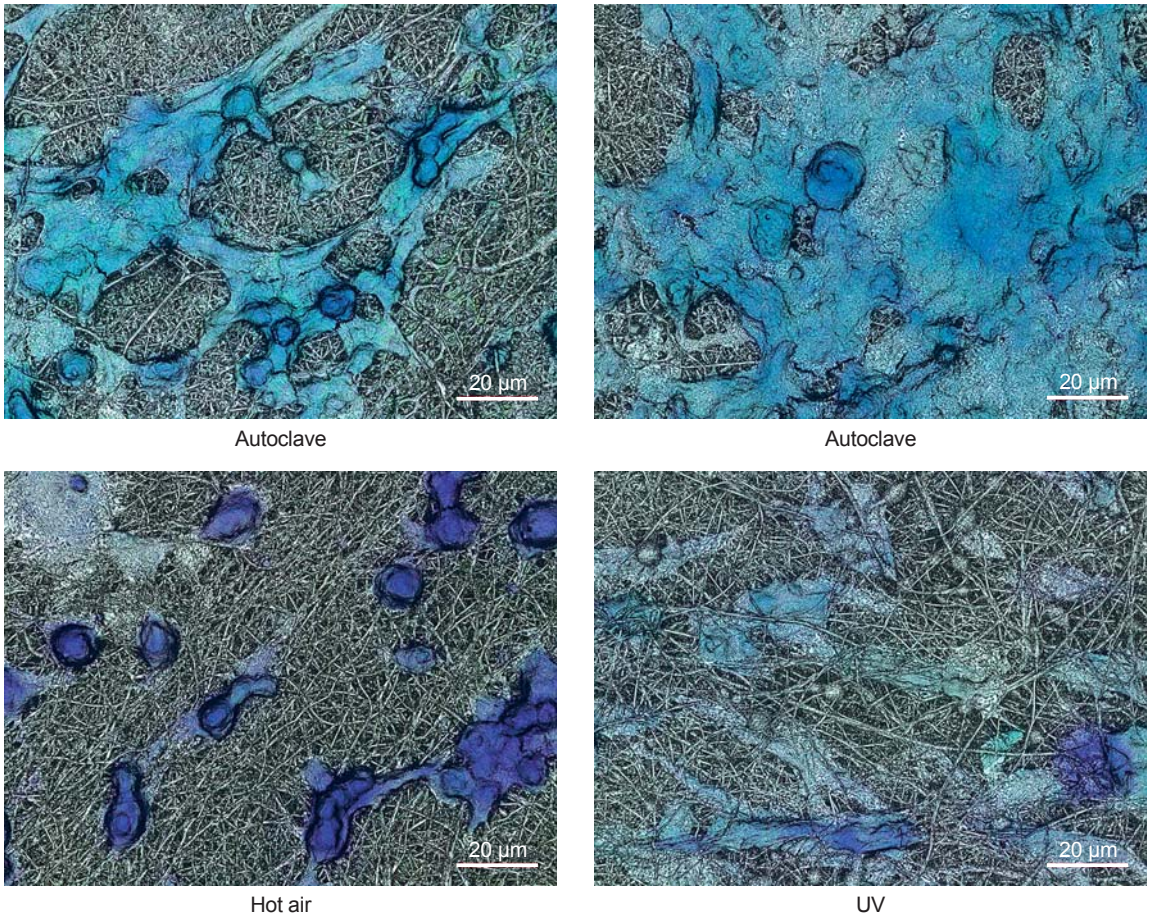


Figure 6: CLSM images of CHO DP-12 cells on different areas of PAN/gelatine nanofibrous mats sterilized with different methods; scale bars indicate 20 µm

Firstly, different colours at the end of cultivation indicate that the cell growth occurred unequally in different wells. The colour change is based on the change in the pH value, as discussed before in the case of ozone-treated samples. Here, however, the nutrient consumption of cells resulted in this pH change, indicating that the wells where the colour of the medium changed to yellow will show the highest cell concentration. Comparing the reference well (lower right) with the others, the cell growth seems to be reduced on ozone-sterilized nanofibrous mats. Figure 5 depicts the CLSM images of CHO DP-12 cells on ozone-sterilized nanofibrous mats with 10% gelatine. The small, round cells seem to be only punctually adhered, while the larger bluish zones show flattened adherent cells where the cytoplasm spread over a larger area. Non-adherent and punctually adhered CHO DP-12 cells have dimensions of approximately 12–17 μm, while the adhered cells in the control wells were measured to have the lengths of approx. 65 μm due to their fibroblast-like morphology (not shown).

In the same way, the other three sterilization methods were examined. Figure 6 shows the CHO DP-12 cells on some of these sterilized nanofibrous mats. While the hot air treatment mostly showed small and thus poorly adhered cells, good cell attachment was achieved by autoclaving.

Interestingly, in the case of UV sterilization, partly only a very fine blue layer was visible which may be the cell residues or cell products which are embedded in the nanofibrous mat and fixed by the glyoxal solution.

These CLSM images suggest that autoclaving is the best sterilization technology for the growth of CHO DP-12 cells on PAN/gelatine nanofibrous mats.

To quantitatively examine this question, adherent cells on PAN/gelatine nanofibrous mats with 5% and 10% gelatine were detached by trypsin and subsequently counted using a Neubauer chamber. The results are depicted in Figure 7. It should be mentioned that the cell counting with a Neubauer chamber is usually assumed to have the statistical uncertainty of 20%; this value was used for the error bars.

Firstly, on the nanofibrous mats with 10% gelatine, slightly higher cell concentrations were found. However, as the error bars indicate, this tendency is not significant.

For both gelatine concentrations, ozone gave the worst results, which might be related to the observed pH change in the culture medium, suggesting chemical changes in the nanofibrous mat, which apparently suppressed the cell growth. This finding fits well with the observation in Figure 5, showing only few cells on ozone-treated nanofibrous mats. While Figure 6 suggested that autoclaving would be the ideal sterilization method, the cell counting revealed that the hot air sterilization worked even better. Comparing the respective images in Figure 6, one possible explanation is that during the cell counting with a Neubauer chamber, larger and smaller cells are counted in the same way, while the adherent cells cover larger areas on nanofibrous mats and thus give the impression that more cells are grown on autoclaved nanofibrous mats. Another explanation could be that the cells were not fully detached by trypsinization on autoclaved nanofibrous mats due to tighter binding.

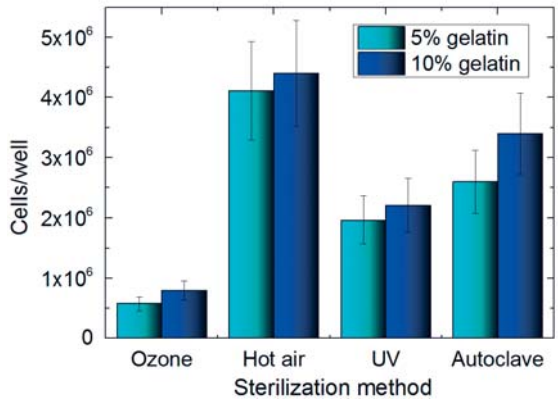


Figure 7: Cell numbers after 4 days of cultivation on differently sterilized nanofibrous mats, counted with Neubauer chamber trypsinization (N = 2)

Combining all findings presented in this study, it can be concluded that the heat treatment methods, i.e. hot air and autoclaving, are preferable to chemical methods, i.e. ozone treatment, or physical methods, i.e. UV irradiation. One possible explanation for this finding is the partial melting of gelatine during the heat treatment that creates larger areas in which more gelatine is exposed on the surface of nanofibres, making it accessible to cells for attachment; an effect which would even be visible to a certain extent in UV-treated nanofibrous mats due to the heat development of the UV source, as described above. This idea corresponds to the tendency towards slightly larger cell concentrations on nanofibrous mats with higher gelatine content and

previous findings of a student project (unpublished) that the CHO DP-12 cells do not grow at all on pure PAN nanofibrous mats.

4 Conclusions and outlook

PAN/gelatine nanofibrous mats were prepared by needleless electrospinning and sterilized using ozone, hot air, UV irradiation and autoclaving. While the hot air treatment, even at only 60 °C, and autoclaving melted gelatine and thus changed the morphology of nanofibrous mats, the ozone sterilization suggested a change in the nanofibrous mat chemistry. Unexpectedly, the degree of gelatine melting at high temperatures seemed to correlate with the cell attachment, resulting in the hot air treatment showing the best cell growth, while the ozone sterilization (the only method without a thermal component) resulted in the poorest cell growth. For all sterilization methods, the cell growth and attachment on PAN/gelatine blends is possible, opposite to pure PAN nanofibrous mats, as shown by previous experiments.

These findings suggest further chemical examinations, especially of the ozone treated samples, as well as an optimization of the electrospinning process to include more gelatine in nanofibrous mats. Finally, different temperature treatments in a broader temperature range will be investigated to optimize the cell growth conditions.

References

1. GREINER, Andreas, WENDORFF, Joachim H. Electrospinning: a fascinating method for the preparation of ultrathin fibers. *Angewandte Chemie International Edition*, 2007, **46**(30), 5670–5703, doi: 10.1002/anie.200604646.
2. LI D., XIA, Y. Electrospinning of nanofibers: reinventing the wheel? *Advanced Materials*, 2004, **16**(14), 1151–1170, doi: 10.1002/adma.200400719.
3. YALCINKAYA, Fatma, YALCINKAYA, Baturalp, JIRSAK, Oldrich. Influence of salts on electrospinning of aqueous and nonaqueous polymer solutions. *Journal of Nanomaterials*, 2015, **2015**(5), 134251, doi: 10.1155/2015/134251.
4. MILASIUS, Rimvydas, RAGAISIENE, Audrone, RUKUIZIENE, Zaneta, MIKUCIONIENE, Daiva. Possibilities of manufacturing an electrospun web with baltic amber. *FIBRES & TEXTILES in Eastern Europe*, 2015, **5**(113), 42–47.
5. MOLNAR, Kolos, NAGU, Zsombor K. Corona-electrospinning: Needleless method for high-throughput continuous nanofiber production. *European Polymer Journal*, 2016, **74**, 279–286, doi: 10.1016/j.eurpolymj.2015.11.028.
6. GROTHE, Timo, GROßERHODE, Christina, HAUSER, Thomas, KERN, Philip, STUTE, Kai, EHRMANN, Andrea. Needleless electrospinning of PEO nanofiber mats. *Advances in Engineering Research*, 2017, **102**, 54–58, doi: 10.2991/icmmse-17.2017.9.
7. GRIMMELSMANN, Nils, HOMBURG, Sarah Vanessa, EHRMANN, Andrea. Electrospinning chitosan blends for nonwovens with morphologies between nanofiber mat and membrane. *IOP Conference Series: Materials Science and Engineering*, 2017, **213**(1), 012007, doi: 10.1088/1757-899X/213/1/012007.
8. WORTMANN, Martin, FREESE, Natalie, SABANTINA, Lilia, PETKAU, Richard, KINZEL, Franziska, GÖLZHÄUSER, Armin, MORITZER, Elmar, HÜSGEN, Bruno, EHRMANN, Andrea. New polymers for needleless electrospinning from low-toxic solvents. *Nanomaterials*, 2019, **9**(1), 52, doi: 10.3390/nano9010052.
9. MADDAH, Bozorgmehr, SOLTANINEZHAD, Mostafa, ADIB, Korosh, HASANZADEH, Mahdi. Activated carbon nanofiber produced from electrospun PAN nanofiber as a solid phase extraction sorbent for the preconcentration of organophosphorus pesticides. *Separation Science and Technology*, 2017, **52**(4), 700–711, doi: 10.1080/01496395.2016.1221432.
10. PANTHI, Gopal, PARK, Soo-Jin, CHAE, Su-Hyeong, KIM, Tae-Woo, CHUNG, Hea-Jong, HONG, Seong-Tshool, PARK, Mira, KIM, Hak-Yong. Immobilization of Ag_3PO_4 nanoparticles on electrospun PAN nanofibers via surface oximation: Bifunctional composite membrane with enhanced photocatalytic and antimicrobial activities. *Journal of Industrial and Engineering Chemistry*, 2017, **45**, 277–286, doi: 10.1016/j.jiec.2016.09.035.
11. GROTHE, Timo, WEHLAGE, Daria, BÖHM, Tobias, REMCHE, Alexander, EHRMANN, Andrea. Needleless electrospinning of PAN nanofibre mats. *Tekstilec*, 2017, **60**(4), 290–295, doi: 10.14502/Tekstilec2017.60.290-295.

12. RICHARDSON, Sarah A., RAWLINGS, Thomas M., MUTER, Joanne, WALKER, Marc, BROSENS, Jan J., CAMERON, Neil R., EISSA, Ahmed M. Covalent attachment of fibronectin onto emulsion-templated porous polymer scaffolds enhances human endometrial stromal cell adhesion, infiltration and function. *Macromolecular Bioscience*, 2019, **19**(2), e1800351, doi: 10.1002/mabi.201800351.
13. GSTRAUNTHALER, Gerhard, LINDL, Toni. Zell- und Gewebekultur. 7th edition. Berlin : Springer, 2013.
14. SABANTINA, Lilia, WEHLAGE, Daria, KLÖKKER, Michaela, MAMUN, Al, GROTHE, Timo, GARCÍA-MATEOS, Francisco José, RODRÍGUEZ-MIRASOL, José, CORDERO, Tomás, FINSTERBUSCH, Karin, EHRMANN, Andrea. Stabilization of electrospun PAN/gelatin nanofiber mats for carbonization. *Journal of Nanomaterials*, 2018, **2018**, 6131085, doi: 10.1155/2018/6131085.
15. PAMESHBABU, Arun Prabhu, DATTA, Sayanti, BANKOTI, Kamakshi, SUBRAMANI, Elavarasan, CHAUDHURY, Koel, LALZAWMLIANA, V., NANDI, Samit K., DHARA, Santanu. Polycaprolactone nanofibers functionalized with placental derived extracellular matrix for stimulating wound healing activity. *Journal of Materials Chemistry B*, 2018, **6**(42), 6767–6780, doi: 10.1039/C8TB01373J.
16. SHAKIBAIE, Mehdi, TABANDEH, Faterneh, SHARIATI, Parvin, NOROUZY, Amir. Synthesis of a thin-layer gelatin nanofiber mat for cultivating retinal cell. *Journal of Bioactive and Compatible Polymers*, 2018, **33**(4), 371–381, doi: 10.1177/0883911518776337.
17. ZHU, Caihong, ZHU, Junhui, WANG, Chengwei, CHEN, Ruihua, SUN, Lining, RU, Changhai. Wrinkle-free, sandwich, electrospun PLGA/SF nanofibrous scaffold for skin tissue engineering. *IEEE Transactions on Nanotechnology*, 2018, **17**(4), 675–679, doi: 10.1109/TNANO.2018.2801569.
18. ALIPPILAKKOTTE, Shebi, SREEJITH, Lisa. Benign route for the modification and characterization of poly(lactic acid) (PLA) scaffolds for medicinal applications. *Journal of Applied Polymer Science*, 2018, **135**(13), 46056, doi: 10.1002/app.46056.
19. VIGANI, Barbara, ROSSI, Silvia, SANDRI, Giuseppina, BONFERONI, Maria Cristina, MILANESI, Giulia, BRUNI, Giovanna, FERRARI, Franca. Coated electrospun alginate-containing fibers as novel delivery system for regenerative purposes. *International Journal of Nanomedicine*, 2018, **13**, 6531–6550, doi: 10.2147/IJN.S175069.
20. ZINE, Rashtrapal, SINHA, Mukty. Nanofibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/collagen/graphene oxide scaffolds for wound coverage. *Materials Science & Engineering C, Materials for Biological Applications*, 2017, **80**, 129–134, doi: 10.1016/j.msec.2017.05.138.
21. CHARERNSRIWILAIWAT, Natthan, ROJANARATA, Theerasak, NGAWHIRUNPAT, Tanasait, OPANASOPIT, Praneet. Aligned electrospun polyvinyl pyrrolidone/poly epsilon-caprolactone blend nanofibrous mats for tissue engineering. *International Journal of Nanoscience*, 2016, **15**(1–2), 1650005, doi: 10.1142/S0219581X16500058.
22. OKTAY, Burcu, KAYAMAN-APOHAN, Nilhan, ERDEM-KURUCU, Serap, SÜLEYMANOGLU, Mediha. Fabrication of collagen immobilized electrospun poly(vinyl alcohol) scaffolds. *Polymers for Advanced Technologies*, 2015, **26**(8), 978–987, doi: 10.1002/pat.3512.
23. CHEN, Honglin, HUANG, Jin, YU, Jiahui, LIU, Shiyuan, GU, Ping. Electrospun chitosan-graft-poly(ε-caprolactone)/poly(ε-caprolactone) cationic nanofibrous mats as potential scaffolds for skin tissue engineering. *International Journal of Biological Macromolecules*, 2011, **48**(1), 13–19, doi: 10.1016/j.ijbiomac.2010.09.019.
24. KLINKHAMMER, Kristina, SEILER, Nadine, GRAFAHREND, Dirk, GERARDO-NAVA, José, MEY, Jörg, BROOK, Gary A., MÖLLER, Martin, DALTON, Paul D., KLEE, Doris. Deposition of electrospun fibers on reactive substrates for *In Vitro* investigations. *Tissue Engineering: Part C*, 2009, **15**(1), 77–85, doi: 10.1089/ten.tec.2008.0324.
25. CASTAGNA, Rosella, TUNESI, Marta, SAGLIO, B., DELLA PINA, Cristina, SIRONI, Annalisa, ALBANI, Diego, BERTARELLI, C., FALLETTA, E. Ultrathin electrospun PANi nanofibers for neuronal tissue engineering. *Journal of Applied Polymer Science*, 2016, **133**(35), 43885, doi: 10.1002/app.43885.
26. ARNAL-PASTOR, M., MARTÍNEZ RAMOS, C., PÉREZ GARNÉS, M., MONLEÓN PRADAS, M., VALLÉS LLUCH, A. Electrospun adherent-antiadherent bilayered membranes based

on cross-linked hyaluronic acid for advanced tissue engineering applications. *Materials Science and Engineering: C, Materials for Biological Applications*, 2013, **33**(7), 4086–4093, doi: 10.1016/j.msec.2013.05.058.

27. FAN, Lin-peng, ZHANG, Kui-hua, SHENG, Xiao-yue, WU, Chun-chen, LI, Jun, MO, Xiu-mei, WANG, Hong-sheng. Electrospinning and post-spin treatment of regenerated silk fibroin nanofibers. In *International Forum on Biomedical Textile Materials : proceedings*. 2010, 96–100.
28. MÖLLER, Johannes, KORTE, Katrin, PÖRTNER, Ralf, ZENG, An-Ping, JANDT, Uwe. Model-based identification of cell-cycle-dependent metabolism and putative autocrine effects in antibody producing CHO cell culture. *Biotechnology and Bioengineering*, 2018, **115**(12), 2996–3008, doi: 10.1002/bit.26828.
29. KRAHN, Natalie, SPEARMAN, Maureen, MEIER, Markus, DORION-THIBAUDEAU, July, MCDOUGALL, Matthew, PATEL, Trushar R., DE CRESCENZO, Gregory, DUROCHER, Yves, STETEFELD, Jörg, BUTLER, Michael. Inhibition of glycosylation on a camelid antibody uniquely affects its FcγRI binding activity. *European Journal of Pharmaceutical Sciences*, 2017, **96**, 428–439, doi: 10.1016/j.ejps.2016.09.040.
30. WIPPERMANN, Anna, RUPP, Oliver, BRINKROLF, Karina, HOFFROGGE, Raimund, NOLL, Thomas. The DNA methylation landscape of Chinese hamster ovary (CHO) DP-12 cells. *Journal of Biotechnology*, 2015, **199**, 38–46, doi: 10.1016/j.jbiotec.2015.02.014.
31. HAREDY, Ahmad M., NISHIZAWA, Akitoshi, HONDA, Kohsuke, OHYA, Tomoshi, PHTAKE, Hisao, OMASA, Takeshi. Improved antibody production in Chinese hamster ovary cells by ATF4 overexpression. *Cytotechnology*, 2013, **65**(6), 993–1002, doi: 10.1007%2Fs10616-013-9631-x.
32. RICHTER, Katharina N., REVELO, Natalia H., SEITZ, Katharina J., HELM, Marin S., SARKAR, Deblina, SALEEB, Rebecca S., D'ESTE, Elisa, EBERLE, Jessica, WAGNER, Eva, VOGL, Christian, LAZARO, Diana F., RICHTER, Frank, COY-VERGARA, Javier, COCEANO, Giovanna, BOYDEN, Edward S., DUNCAN, Rory R., HELL, Stefan W., LAUTERBACH, Marcel A., LEHNART, Stephan E., MOSER, Tobias, OUTEIRO, Tiago F., REHLING, Peter, SCHWAPPACH, Blanche, TESTA, Ilaria, ZAPIEC, Bolek, RIZZOLI, Silvio O. Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy. *The EMBO Journal*, 2018, **37**(1), 139–159, doi: 10.15252/embj.201695709.
33. MICHON, C., CUVELIER, G., RELKIN, P., LAUNAY, B. Influence of thermal history on the stability of gelatin gels. *International Journal of Biological Macromolecules*, 1997, **20**(4), 259–264, doi: 10.1016/S0141-8130(97)00024-X.