Tailor-made surface coatings for cell cultivation in a closed plastic bag system


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Abstract: Cultivation of adherent living cells is usually performed in open systems which are sensitive to microbial contamination. To overcome these problems a disposable closed bag system was developed and the growth of adherent cells is realized by a coating of the inside of the bag. Plasma polymer films obtained from (3-aminopropyl)-trimethoxysilane (APTMS) at atmospheric pressure in a dielectric barrier discharge directly in the closed bags turned out as promising coatings to improve adherent cell growth. The investigation of the long term stability of primary amino groups on the surface of the coating lead to group densities of 1.8 nm⁻² after 24 weeks of storage.

Keywords: cell cultivation, atmospheric-pressure plasma, plasma-polymerization APTMS

1. Introduction

Human mesenchymal stem cells, i.e. from human bone marrow aspirates, are currently intensively investigated for their promising clinical application in hematopoiesis, immunoregulation as well as for tissue engineering purposes. The cultivation of living cells is very sensitive to microbial contamination when the cultivation takes places in open systems or in systems, which have to be opened for filling. We have developed a disposable closed bag system to exclude contamination during cultivation of cells for therapeutic applications. However, the plastic surfaces in the bags have to be modified to enable growth of adherent cells such as above mentioned mesenchymal stem cells.

From the literature it is known that functionalization of polymer surfaces can easily be achieved by plasma treatment using oxygen or nitrogen containing gas-mixtures [1-3]. In order to ignite a discharge exclusively in the inside of a closed volume, which is surrounded by dielectric walls, but not in the immediately adjacent gas (normally air), to create functional groups on the interior walls, it is necessary to use process gases with smaller breakdown field strengths than the gas in the environment. For this purpose a new efficient atmospheric-pressure plasma process has been developed, utilizing an organic film forming agent in combination with helium as process gas in order to create an amino group containing coating in the inside of the bag.

As wet-chemical silanization with (3-aminopropyl)-trimethoxysilane (APTES) is a frequently used method to improve the adhesion of biomolecules [4-6], due to the creation of primary amino groups onto the surface of the substrate, the analogous (3-aminopropyl)-trimethoxysilane (APTMS) was used to modify the plastic surfaces. APTMS was used instead of APTES because it exhibits a higher vapor pressure, providing a higher amount of the monomer in the gas phase.

2. Experimental

Gas permeable and transparent bags made of polyolefin suitable for suspension cell culture (Miltenyi Biotec) were used for coating and adherent cell cultivation. APTMS (> 97 %) and 4-trifluoromethylbenzaldehyde (TFBA, > 98 %) were supplied by Sigma-Aldrich. The purity of helium as process gas was > 99.999 % (Air Liquide).

Fig. 1: Arrangement for the plasma treatment of the cell cultivation bag.

The plastic bags were filled automatically with helium and the corresponding film forming agent (APTMS) in an automatically filling system developed at Fraunhofer IST. Gaseous APTMS was accomplished by passing helium through a bubbler containing liquid APTMS at room temperature. To ensure virtually oxygen-free conditions the bags were emptied and refilled several times with the gas mixture. The plasma treatment was performed using a DBD arrangement with electrodes located above and be-
low the bag, so that the discharge was ignited inside the bag upon application of an alternating voltage of sufficient magnitude [7].

As a high voltage electrode an ITO-electrode (i.e., a 0.7 mm thick glass plate with a sputtered indium tin oxide layer) with a diameter of 16.5 cm was used, a thin steel plate (10 x 15 cm$^2$) served as a ground electrode. Typical parameters used, include a sinusoidal mid-frequency (30 kHz) voltage supply providing a power of approx. 30 W and a treatment duration of 20 s working under pulsed operating conditions (1 ms plasma on, 10 ms plasma off).

The modified interior bag surfaces were analyzed using FTIR-ATR spectroscopy (FTIR Nicolet 5700, Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with an MCT detector and a DuraSampIR single-reflexion 45° diamond ATR crystal using unpolarized light and a spectral resolution of 4 cm$^{-1}$ for the determination of the homogeneity and a spectral resolution of 1 cm$^{-1}$ for the measurements of the amino group density on the derivatized samples.

The number of primary amino groups per square nanometer was determined by chemical derivatization with 4-trifluoromethylbenzaldehyde (TFBA) followed by quantitative FTIR-ATR measurements (CD FTIR-ATR) of the absorption band area due to vibrations of the C-CF$_3$ moieties in the resulting 4-trifluoromethylbenzaldimines. A detailed description of the method is given in [8].

To determine the suitability of the modified bags for adherent cell culture, cultivation of human mesenchymal stem cells in the cell culture bags was investigated at the HZI. Cells were isolated from human bone marrow aspirates supplied by the Klinikum Celler Str., Braunschweig, Germany. The cells were cultivated on APTMS modified surfaces and compared to adhesion and growth on the unmodified surface. Cells were expanded for 14 days in mesenchymal stem cell expansion medium (Miltenyi Biotech) and proliferation was analyzed microscopically after 3, 7, 10 and 14 days.

3. Results

3.1 Plasma-polymerization

The success of the inner coatings of the bags was investigated by FTIR-ATR measurements. In order to bring out the absorption spectra of the coatings, difference spectra were obtained by subtracting a spectrum of an untreated polymer bag from the spectra obtained after plasma treatment.

Fig. 2 shows two difference spectra of an APTMS coated plastic bag, measured on the upper (blue curve) and the lower side (red curve) of the bag, respectively. The measurements, which were similarly repeated for many other treatment runs show, that there are no significant differences between the coating structures on both sides. However, the coating on the lower side, adjacent to the steel electrode, is significantly, albeit only a few percent, higher. The peak at 1100 cm$^{-1}$ corresponds to the absorption of O-Si-C and the peaks between 1500 cm$^{-1}$ and 1700 cm$^{-1}$ can be attributed to the absorption of amines and amides. The broad band at wavenumbers above 3000 cm$^{-1}$ belongs to –N-H and –O-H vibrations.

The optimization of the plasma treatment leads to a homogenous distribution of the coating within the plastic bag with a thickness variation of less than 8 % over the central 90 % of one side of the bag.

A plasma treatment duration of 20 s was chosen in order to prevent thermal effects on the plastic bags. Smaller treatment duration may result in inhomogeneous coatings, hampering adherent cell growth over the complete surface, as the effective plasma treatment will be diminished simultaneously.

As it will be shown below the obtained film thickness is sufficiently large in order to achieve adherent cell cultivation. If it is necessary to deposit thicker coatings it will be recommended to perform the process twice instead of...
prolonging the treatment, in order to avoid depletion of the film-forming agent in the gas mixture as well as the just mentioned detrimental thermal effects on the plastic bags.

The homogeneity of the films made it possible to measure the long-term stability of the coating and the accessible functional groups. Measurements concerning the long-term stability were performed every eighth week after the plasma treatment within a time period of 24 weeks in total. The coated plastic bag was filled with nitrogen and stored at room temperature. For every measurement a small stripe was clipped from the bag and measured an FTIR spectrum was taken from it. The rest of the bag was closed and refilled with nitrogen. The infrared absorption of the O-Si-C peak at ~1100 cm\(^{-1}\) did not change significantly within this time period, hence it can be concluded that the silicon-organic coating is stable under the described conditions. Results of these measurements are summarized Fig. 3 which shows the area values for the O-Si-C peak from the FTIR-ATR spectra. The analysis indicates that the films thickness is a little bit higher at the side of the steel electrode, as already shown in Fig. 2, due to the asymmetric configuration of the deposition arrangement, with the thick glass plate serving as a dielectric barrier at the upper electrode.

To determine the density of primary amino groups, a chemical derivatization reaction with TFBA was performed, followed by FTIR-ATR measurements (spectral resolution 1 cm\(^{-1}\), 128 Scans). The results of the investigation are summarized in Fig. 4. Immediately after the plasma treatment approx. 5 primary amino groups per nm\(^2\) were detected by FTIR-ATR measurements. The long term stability shows an exponential decrease of the amount of primary amino groups \(y\) to a virtually constant level (\(y = 3 \cdot \exp(-x/3.76) + 1.78; R = 0.999\)). Within 16 weeks the density of these functional groups has reached a constant value of ~1.8 nm\(^2\). As the plastic bag is permeable for oxygen, oxidation processes are assumed to be the major initiator for the decrease in primary amino groups. The FTIR-ATR spectra are not sensitive enough to point out changes in the absorption region of amides, which should confirm this assumption, because immediately after film formation there are already amides present in the coating. The formation of additional amides during ageing may be determined by XPS (X-ray photoelectron spectroscopy), which will be part of future investigations.

3.2 Cell cultivation
Cultivation of human mesenchymal stem cell was performed at the HZI. Fig. 5 demonstrates that the surface was rendered suitable for cell adhesion and proliferation by the APTMS coating (left) while no initial attachment and growth was observed on the untreated surface (right).

Analyses after 7, 10 and 14 days show similar results, which leads to the conclusion that the APTMS coated bags provide a promising and non-toxic surface modification for adherent cell cultivation.

Further investigations should take into account the long-term stability of the APTMS coating in different culture expansion media.

4. Conclusions
The results show that it is possible to achieve biocompatible coatings in the inside of a plastic bag using helium as process gas enriched with silicon-organic film-forming agents in a DBD arrangement. The homogeneity of the coating is realized by statical plasma treatment between two electrodes, which makes it possible to investigate long-term stability of the functional groups.

The primary amino groups show a remarkable long-term stability after 24 weeks of storage, as 1.8 amino groups per nm\(^2\) can still be detected. Oxidation processes of the amines could not be detected by the analytical method used, but will be part of future investigations, e.g. by XPS analyses, as well as the determination of the film thickness of the coating achieved in the closed bag system.

Fig. 4: Long-term decay of the density of primary amino group incorporated into the APTMS film.

Fig. 5: Human mesenchymal stem cell cultivation in the closed bag system. Left: Adhesion and proliferation of cells on the APTMS modified surface. Right: Aggregated nonadherent cells on the unmodified surface after three days in culture (100x).
Cultivation of adherent cells was successfully performed at the HZI and confirms that plasma-polymerized APTMS films promote the adhesion of living cells.

Future investigations will include the extension of the inner coating to other film-forming agents and first experiments in which plastic bags will be coated locally.

References

[7] German patent DE102006036536 B3