Quality Assessment of Primary Human Keratinocyte Cultures with Online Measurement of Oxygen and pH

Abstract

The SDR SensorDish Reader allows precise, non-invasive analysis of the process parameters dissolved oxygen (DO) and pH in cell culture. A correlation between cell proliferation analysed with light microscopy, increase of oxygen consumption, and a decrease in pH value in the culture medium is clearly evident. The oxygen and pH kinetics give valuable information about culture quality and efficiency.

Future Potential for *In Vitro* Tests and Cell Therapy

In vitro tests are standard screening processes in dermatological and cosmetical laboratories. New pharmacological substances, like e.g. against aging of skin, irritation or harmful UV light, can be tested on their efficiency, and quantitatively evaluated under defined conditions in culture vessels. The evaluation systems range from 2D fibroblast cell culture and keratinocyte monolayers up to complex 3D full skin models. *In vitro* cultivated epidermal keratinocytes have become increasingly important in medical research, especially in tissue engineering – e. g. in transplantation of cultivated autologous skin cells for the treatment of burns. The therapeutical reconstruction of destroyed skin after severe burns – in some cases a life-saving measure – shows remarkable success rates. Even successful treatment of non-healing chronic wounds was reported recently. The patient´s own keratinocytes are cultivated *in vitro*, and afterwards stabilised in a fibrin matrix, which is re-transplanted (Kopp et al., 2004).

In this context, it is obvious how important efficient and reliable *in vitro* cell propagation is. Modern monitoring and control systems are very advantageous to ensure reliable quality of cell cultures. They allow monitoring of the important metabolic process parameters – online and non-invasively. Now there is a non-invasive, optosensoric monitoring system for long-time measurement of DO and pH -SensorDish Reader (SDR, Fig. 1). Even minimal changes in any of the two important growth parameters can be detected – in close proximity to the cells. The outstanding performance of this system has already been demonstrated in preliminary studies at Celonic with metabolically active CHO suspension cells for fermentation culture[1]. Different approaches for possible optimisation in biotechnological production of recombinant proteins had been investigated in those tests. This new study by Celonic now focuses mainly on two aspects: first, the sensitivity of the optosensoric SDR measurement system used with cells showing comparatively low metabolic activity; and second, the relation of cell proliferation and oxygen consumption rates or changes in pH values.

Figure 1. Online monitoring of DO and pH value in Kerationcyte cell culture with the SDR SensorDish® Reader by PreSens.

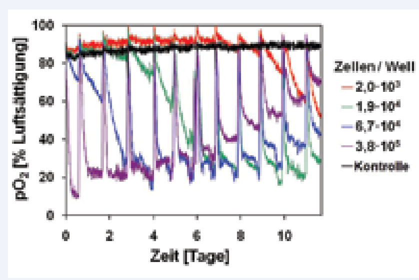
Precise SDR Measurement Technique for Sensitive Cultures

A spontaneously immortalised human keratinocyte cell line (HaCaT-cells; Boukamp et al., 1988) and primary human keratinocytes were used for these experiments. Primary keratinocytes were carefully extracted from biopsy material with dispase and trypsin. A cell preparation with high proliferative potential was chosen. Both cell types have a critical, minimal cell density for inoculation. If this minimal value of cell density is not reached, cell growth is prolonged, or there is no growth at all. Both cell types were cultivated in parallel with four different starting cell densities of 2 x 103, 1.9 x 104, 6.7 x 104, and 3.8 x 105 cells/well. For each cell density one well was analysed online with the SDR. Reference value measurement was performed in cell-free samples. 24-well Oxo- and HydroDishes® by PreSens (Regensburg, Germany) were used as cultivation vessels. The multidishes have integrated chemical optical sensors for DO and pH measurement respectively at the bottom of each well. These sensors are read out non-invasively from the bottom with the 24-channel SensorDish® Reader. The surface of the 24-well dishes was coated with collagen for the experiments. The culture period was 12 days. Cultivation was carried out at standard conditions in 5 % CO_2 atmosphere with defined, serum-free keratinocyte-medium (Gibco, Defined Kerationcyte-SFM). The medium was replaced every day with equilibrated, fresh medium. The measurement results were recorded with the SDR software. The system allows simultaneous monitoring of 10 SDRs in a total of 240 wells.

Oxygen Kinetics and pH Value Measurement in HaCaT-cell Culture

Measurements in epidermal HaCaT-cell culture were used as a reference for primary keratinocytes. These cells are comparatively robust and can be split in cultivation as long as desired. Calf serum was used for generating this cell line. However for experiments in this study the HaCaT-cells were kept in serum-free media. Analysing the oxygen kinetics of HaCaT-cells cultured with different starting cell densities led to several conclusions (Fig. 2). The sensitivity of the SDR system allowed detecting

Figure 2. Oxygen kinetics in long-term cultivation of HaCaT cells.



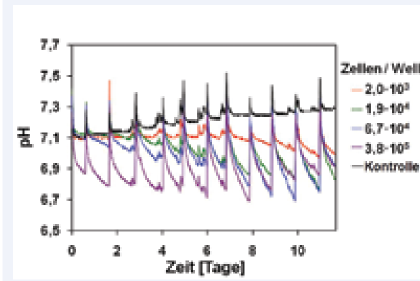
even smallest changes in the hardly metabolically active HaCaT-cell culture – even with starting cell densities of only 1.9 x 104 cells/well. Lower starting cell densities showed no difference to values measured in the cell-free control wells. Starting cell densities of 2 x 103 cell/well are too low for HaCaT-cells to grow. Microscopic analysis (Fig. 3) verified

Figure 3. Microscopic analysis of HaCaT cells seeded at different cell densities after 3 (above) and 12 (below) days

that even single cells that had been adherent at the beginning were gone by day 12 of cultivation time. It could be observed that with continuing cultivation time the minimum DO value evened out at approximately 22 – 30 % air sat. in all cultures independently from starting cell density. Remarkably, in the culture with the highest cell density of 3.8 x 105 cells/well the metabolically available DO concentration dropped to 5 % air sat. at the beginning of cultivation, but decreased to only 25 % air sat. after medium change. This is probably caused by removing non-adherent cells during medium change that could not grow onto the surface after inoculation because of the high cell density. On the other hand a continually increasing oxygen consumption rate could be measured for the cell culture inoculated at 6.7 x 104 cells/well. On day 6, this culture reached the same DO values as the culture seeded with highest cell density. Because of its oxygen consumption rate and the correlation with microscopic analysis (Fig. 3), cultures seeded at this cell density lie within the optimal detection range of the testing system. The cells can proliferate optimally with maximum growth rate. Starting cell concentrations of 1.9 x 104 cells/well can be considered not very usable. Only after 5 days could a continual decrease of the DO level in the medium be detected. Therefore the ideal starting cell density for HaCaT culture is 6.7 x 104 cells/well. High proliferation potential and metabolic activity can be deduced from the rapidly decreasing DO level to minimum values within a few hours after media change.

Similar results could be observed for the simultaneously recorded pH values (Fig. 4). The decrease in pH

Figure 4. pH kinetic in long-term cultivation of HaCaT cells.

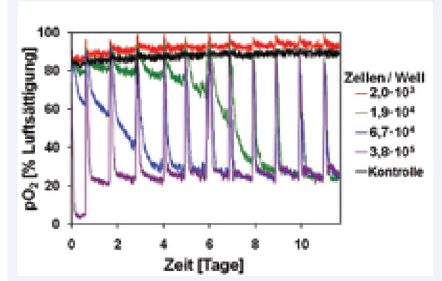


values correlated closely with the respective starting cell density. For the culture with highest inoculation density, relative pH decrease was highest after each medium change – and linked to cell number and metabolic activity. Different from DO, the pH values continually decreased over the whole culture period of 12 days. Cultures with highest starting cell density show a pH value of 6.8 after 12 days of cultivation. Contrary to DO kinetics, differences caused by starting cell density could still be clearly detected towards the end of the culture period.

Surprising Difference: DO and pH Value Kinetics in Primary Keratinocytes

Oxygen consumption in primary keratinocytes (Fig. 5) is similar to that of HaCaT-cells at the beginning of the

Figure 5. Oxygen kinetics in long-term cultivation of primary human keratinocytes



cultivation. In long-term cultivation there are noticeable differences though. Cultures with high starting cell densities reach a minimum DO level of 22 % after only 3 days. After an initial minimum of 10 % an upward trend can be detected on day 1. Different from HaCaT-cell culture, this increase continues after day 5. DO values stabilise at a higher level after each medium change. Towards the end of the cultivation period DO reaches a value of 70 % air sat. There is a significant difference to the measured values in HaCaT-cell cultures.

It can be concluded that primary keratinocytes gradually reduce their metabolic activity. However, the noticeable decrease in oxygen consumption is not related to reduced viable cell numbers (Fig. 6).

Figure 6. Microscopic analysis of primary human keratinocytes seeded at different cell densities after 3 (above) and 12 (below) days

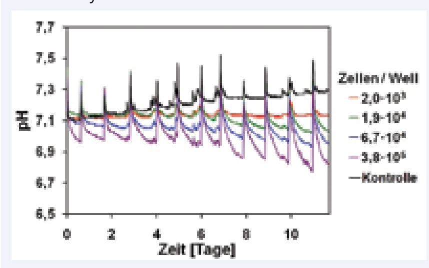
Primary keratinocytes show distinct contact inhibition compared to the HaCaT cells, and after forming a monolayer cells stop proliferating. In this state of cultivation metabolism is reduced respectively. Another reason for reduced metabolic activity can be the limiting culture conditions caused by high cell density. Some of the cells might have been damaged by this, which could have caused the reduced oxygen consumption during the following days.

Microscopic analysis shows an increased number of small, dark-appearing cells after 12 days – probably with reduced vitality. Comparing this to the HaCaT-cell cultures after 12 days, cell complexes of the primary keratinocytes seem looser. This demonstrates that primary keratinocytes react more sensitively under limited conditions than HaCaT cells.

Cultures with cell densities of 6.7 x 104 and 1.9 x 104 cells/well show similar kinetics and tendencies to the above described highest cell density. In both cases a decrease of the DO level could be recorded with a respective postponement of 3 days. Kinetics of cell growth and microscopic analysis suggest that initial cell concentrations of 6.7 x 104 cells/well seem to be optimal conditions for fast, unrestricted growth and high metabolic activity in the cultures. After an initial minimum value the DO level reaches higher values with every medium change. The DO value for the sample with 6.7 x 104 cells/well is approximately 45 % air sat., and for the sample with 1.9 x 104 cells/well approximately 30 % air sat. respectively – in both cases with an upward trend. The sample with lowest starting cell densities of 2 x 103 cells/well shows distinctive differences. Unlike the HaCaT cells, this culture reveals significant metabolic activity from day 7 on. Primary keratinocytes can tolerate low initial cell densities better than the HaCaT cells. The serum-free culture system was optimised for the cultivation of primary keratinocytes by Celonic. It is obvious that HaCaT

cells adapted to serum containing media cannot adapt to the serum-free environment as well as primary cells. Microscopic analysis during cultivation confirmed a significant increase of cell density in cultures with starting cell densities of 2 x 103 cells/well (Fig. 6). On day 12 the culture vessel surface was almost completely overgrown with cells.
pH kinetics (Fig. 7) for different

Figure 7. pH kinetics in primary keratinocyte cultivation



inoculation sizes show the same tendency as DO kinetics. In cultures with higher initial cell density, pH values decrease from 7.2 to 6.7 after each media change. This recurrent decrease lessens from day 8 on. Cultures with lower initial cell density also show a decrease of pH value between media changes in the beginning of the cultivation period. In cultures with the second and third highest cell density this decrease lessens after day 10 and 11 respectively. Even in the culture with lowest starting cell density a pH value decrease could be detected after day 7, which became larger till the end of the cultivation period. These effects are associated with the cell number and activity of the primary keratinocytes, and are most obvious toward the end of the cultivation period. In the same way as for the kinetics of cultures with highest cell density, the decrease in pH of the cultures with 6.7 x 104 and 1.9 x 104 cells/well with reduced oxygen consumption is lesser – which indicates reduced cell activity in the older cultures.

Insight into the Milieu of Sensitive Keratinocyte Cultures

Precise insights into metabolic conditions within the culture vessels allow for new and promising perspectives in optimisation of cell culture processes. The optosensoric measurement system SDR does not require invasive intervention to measure inside the culture. This method is safe and reliable, especially in long-term cultivation experiments. The cultures can be kept under sterile and constant environmental conditions. This reduces risks as well as workload. Online monitoring allows for continuous recording even of unexpected or unwanted conditions inside the incubator. Optimal culture conditions can be realised by non-invasive measurement at any time. Any interferences or unwanted conditions can be detected in time and adapted.

The recorded kinetics give proof that the SDR system is ideally suited for monitoring of sensitive, primary keratinocyte cultures. Even at low initial cell densities DO and pH kinetics can be clearly determined. With these graphs detailed statements about culture development can be deducted. Measurement data allows identifying the timespan – dependent on the initial cell density – in which cultures will reach maximum metabolic activity and highest proliferation rates. The data also reveal periods of static metabolism or even degenerative processes. This way defined cultivation times can be set, according to process objective, in which cultures show optimal physiological conditions, as for example for the further use in transplantation or for pharmacological screening tests.

Future up-scaling processes may be based on the results gathered in the 24-well format. The SensorDish Reader is not only a monitoring system but also a very sensitive sensor system for characterisation and adjustment of ideal physiological culture conditions. These conditions can be optimised methodically by specific modification of the parameters, as e.g. the addition of growth regulators like cytokines. The whole research project profits from more economically designed processes and can be transferred into a commercial application faster.

References

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