# Poloxamer 188 Supplemented Culture Medium Increases the Vitality of Caco-2 Cells after Subcultivation and Freeze/Thaw Cycles

*Vera Kerleta[1], Isabella Andrlik[1], Susanne Braunmüller[2], Thomas Franke[2], Michael Wirth[1] and Franz Gabor[1]*

[1] Department of Pharmaceutical Technology and Biopharmaceutics, University of Vienna, Austria; [2] Experimentalphysik I, University of Augsburg, Germany

**Summary**

*Cryostorage media containing 1% of the non-ionic surfactant Poloxamer 188 provided full recovery of mammalian cells (González Hernández, 2006), but its effects during thawing of cryostored cells and proteolytic subcultivation are still unknown. In this study, the proliferation and viability of pre-confluent Caco-2 monolayers cultivated in media supplemented with the non-ionic surfactant were investigated. The addition of 0.5% Poloxamer 188 increased proliferation of subcultivated cells 1.5 fold and that of thawed cells about twofold. According to microaspiration experiments the non-ionic surfactant increased the tension of the cell membrane most notably at concentrations ≤ 0.5% owing to adsorption and incorporation into the phospholipid bilayer. Thus, the performance of the cells appears to be improved. Since vitality of cells is a prerequisite for reproducibility and reliability of cell models for absorption studies at early stages of drug development, use of Poloxamer 188 supplemented cultivation media may help to refine cell culturing to further reduce animal trials in preclinical investigations.*

*Keywords: Poloxamer 188, Caco-2 cells, proliferation, cell membrane undulation, micropipette aspiration*

## 1 Introduction

In order to reduce animal trials during preclinical evaluation of APIs (active pharmaceutical ingredient) as well as to circumvent clinical verification of the bioequivalence of certain formulations, the biopharmaceutics classification system (BCS) is recommended by the FDA (Food and Drug Administration) as well as the EMEA (European Medicines Agency) (Kim et al., 2006). The monolayer-forming Caco-2 cell line is an integral part of this biowaiver to assess the permeability of APIs across artificial human intestinal epithelium by mimicking the process of absorption (Artursson et al., 2001). The accuracy of predicting human absorption is about 60% (Sachan et al., 2009).

The reproducibility and reliability of such assays strongly depend on the viability of the cells. Routine cultivation, however, requires procedures which are unfavourable to live cells: Subcultivation of cells might harm or damage the cell membrane in the course of the proteolytic detachment of adherent cells. Even mild manipulation of cells might be injurious because of exposition to shear forces during pipetting. Cryostorage of cells comprises freezing and formation of ice crystals inside and outside the cell, which may disrupt cell membranes. In turn, recrystallisation during thawing can also be detrimental for cell survival (Woods et al., 2004). To overcome these problems and to improve the relevance of cell models, the media that are currently used must be improved.

In terms of cryostorage, all BALB/c myeloma cells stored in medium containing 1% Poloxamer 188 survived freezing/thawing as compared to 80% without a supplement (Gonzáles Hernández and Fischer, 2007). Meanwhile, this cryomedium is commercially available (Filoceth™-media, procryotect GmbH, Ruedlingen, Switzerland), and it was suggested that the surfactant acts by stabilising the cell membrane.

Poloxamer 188 (Pluronic® F-68, PF-68) is a non-ionic surfactant with an average molecular weight of 8400 Da and is composed of EO (polyoxyethylene) and PO (polyoxypropylene) units arranged in a basic triblock structure according to $EO_{76}$-$PO_{30}$-$EO_{76}$. The non-toxic surfactant is approved by the FDA for oral and parenteral application in concentrations ranging from 0.01-10% (http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm, 14.05.2009). It has been reported to exert cytoprotective effects, e.g. higher viability of mammalian cells under high agitation (Al-Rubeai et al., 1993), and multiple protective effects on Tetrahymena cells exposed to various physical and/or chemical stress parameters (Hellung-Larsen et al., 2000). The cytoprotective effects were explained by a complex interaction between the cell membrane and Poloxamer 188 (Al-Rubeai

Received 17th February 2010, received in revised form and accepted for publication 20th May 2010

et al., 1993). However, sometimes contradictory findings were reported in terms of the tension of the cell membrane (Togo et al., 1999; Zhang et al., 1992).

The aim of this work was to elucidate some additional advantageous effects of Poloxamer 188 supplemented media on subcultivated and thawed cells after cryostorage using proliferation and viability as indicative parameters for cytoprotection. Furthermore, to shed some light on the mechanisms of cytoprotection as well as to confirm the explanation of the cryoprotective effects of Poloxamer 188 cell membrane tension measurements were performed.

## 2 Materials and methods

*Chemicals*

Poloxamer 188 was obtained from Sigma-Aldrich Chemie GmbH (Vienna, Austria). All other chemicals in use were of analytical grade.

*Cell culture techniques*

Caco-2 cells were obtained from DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*, Braunschweig, Germany). The cells were grown in RPMI 1640 (Rosewell Park Memorial Institute) cell culture medium containing 10% foetal bovine serum (FBS), 4 mmol L-glutamine and 150 $\mu$g/ml gentamycine in a humidified atmosphere with 5% $CO_2$ at 37°C. Upon reaching ~80-90% confluence the cells were subcultured with TrypLE® Select and seeded for the proliferation and viability studies.

For cryopreservation 2.5 x $10^6$ cells were suspended in 1 ml cryo-medium (RPMI-medium supplemented as above with additionally 10% FBS and 10% DMSO (dimethyl sulphoxide)). After equilibration in a Nalgene® Mr Frosty Cryobox (Thermo Fisher Scientific, Roskilde, Denmark) for 25 min at -20°C the cryovials were stored at -80°C for at least one week. For thawing, the cryovials were warmed up as fast as possible in water at 37°C. Then, the cell suspension was transferred into 10 ml cell culture medium at 37°C, spun down (1000 rpm, 4°C, 5 min) and the supernatant containing cytotoxic DMSO was discarded. After resuspension in fresh cell culture medium the cells were seeded for proliferation and viability studies.

*Proliferation and viability tests*

The proliferative activity of the cells was determined using the BrdU cell proliferation ELISA test kit (Roche Diagnostics GmbH, Vienna, Austria) according to the manufacturer's instructions. Immediately after splitting or thawing the cells were seeded in a 96-well microplate (Iwaki, Bertoni, Vienna, Austria) at a density of 8 x $10^3$ cells in 200 $\mu$l medium per well and cultivated for 2 or 3 days under cell culture conditions. The medium consisted of 20 $\mu$l sterile-filtered (0.22 $\mu$m pore diameter) surfactant solution in 20 mM isotonic HEPES/NaOH buffer, pH 7.4, or buffer alone and 180 $\mu$l cell culture medium, resulting in 0, 0.2, 0.4, 0.6, 0.8 or 1.0% Poloxamer 188 content. The incorporation of 5-brom-2-desoxyuridine (BrdU) into the DNA of proliferating cells was quantified using a microplate reader (Spectrafluor-reader, Tecan, Grödig, Austria) at 450 nm. The tests were performed at least in triplicate with 6 wells in parallel.

The viability of cells was analysed using the EZ4U test (Easy for you, Biomedica, Vienna, Austria) following the manufacturer's instructions. Briefly, the split cells were seeded in 96-well microplates at a density of 3000 cells/well in 200 $\mu$l medium as above and cultivated for 3 days in a humidified atmosphere with 5% $CO_2$ at 37°C. The tetrazolium salt is reduced by the mitochondria of viable cells to yield water soluble orange coloured formazan, which is quantified at 450 nm. The tests were performed at least in triplicate with 6 wells in parallel.

*Cell membrane tension determination*

The micropipette aspiration technique was applied to determine the stiffness of the cell membrane (Hochmuth, 2000). Micropipettes were pulled from borosilicate glass, filled with PBS, mounted on a syringe and connected with a fluid reservoir. 50 $\mu$l of the cell suspension, containing 2.5 x $10^5$ cells in culture medium, was incubated with 50 $\mu$l 0%, 1% or 5% Poloxamer 188 in 20mM isotonic HEPES/NaOH, pH 7.4, for 60 minutes at 37°C. Under microscopic inspection using a Zeiss Axiovert 200 microscope (Zeiss, Munich, Germany) equipped with a 40x objective and a Hamamatsu camera (Hersching, Germany), the micropipette was moved to touch a cell and a distinct negative pressure was applied to fix the cell at the orifice. The apex of the cell membrane was set as the zero-point for evaluation of the membrane extension. Equal steps of -100 Pa were applied until the cell membrane stopped expanding.

At each pressure change an image was acquired and the position of the apex of the aspirated cell was calculated. These data were fit into equations 1 and 2 to calculate strain and tension (Evans and Rawicz, 1990). The length deformation of an object caused by stretching or compression is called areal strain ($\delta A$) . It has no dimension and can be calculated from:

$$\delta A \cong 2\pi R_p \left(1 - \frac{R_p}{R_c}\right) \Delta L$$

The tension ($\tau$) of the membrane represents the force against the suction pressure and can be expressed as:

$$\tau = \frac{P\, R_p}{2 - 2R_p/R_c}$$

$P$ = suction pressure
$R_p$ = inner diameter of the pipette
$R_c$ = diameter of the cell
$\Delta L$ = length difference.

*Statistics*

Data were exported to the GraphPad Prism statistics software package (GraphPad Prism Software, USA). Statistics were performed using one-way ANOVA with post-hoc Dunnett's tests, comparing the study groups to untreated controls (95% confidence interval). P-values <0.05 were considered significant.

## 3 Results

### 3.1 Influence of Poloxamer 188 on cell proliferation and viability

The proliferative activity of subcultivated Caco-2 cells in the presence of Poloxamer 188 was elucidated by incorporation of BrdU into the DNA of dividing cells in pre-confluent monolayers (Fig. 1). Independent of cultivation time and the concentration range under investigation, the addition of the non-ionic surfactant increased the proliferation. Two days after seeding, the mean Caco-2 proliferation rate increased from 1.25 fold at 0.2% surfactant to a maximum of 1.5 fold at 0.6-0.8% Poloxamer 188 as compared to the control without surfactant. Finally, the effect of 1.0% Poloxamer 188 was found to be similar to that of 0.2% surfactant. Three days post-seeding the proliferation exceeded that of the control 1.26 fold, and the maximum proliferation was observed at 0.4-0.6% Poloxamer 188, amounting to a 1.45 fold increase as compared to the reference. Again, the proliferative activity at 0.8 and 1.0% Poloxamer 188 was similar to that at 0.2%.

Using cells after one freeze/thaw cycle, the same assay revealed that the mean proliferation increases concurrently with the amount of Poloxamer 188 added (Fig. 2). After two days in culture, already 0.2% surfactant enhanced the proliferation 1.2 fold and 1.0% Poloxamer 188 increased it 2.1 fold in comparison to the control. After cultivation for three days this effect was even more pronounced, ranging from a 1.25 fold increase at 0.2% surfactant to a 2.5-fold enhancement at 1.0% Poloxamer 188 as compared to cultivation without surfactant.

In order to assess possible toxic effects of Poloxamer 188, the viability of Caco-2 cells was tested after 3 days of cultivation (Tab. 1). The cells viability was indicated by their mitochondrial activity in reduction of tetrazolium salt to orange coloured formazan. As compared to cultivation without surfactant, the presence of Poloxamer 188 increased the viability of the same number of cells by about 70% in the range of 0.2-0.8% and still 42% at 1.0%. Thus, Poloxamer 188 not only proved to be non-toxic within this period, but it even improved the cells' viability.

### 3.2 Membrane aspiration test

To elucidate the effect of Poloxamer 188 on the physical characteristics of Caco-2 cells, membrane aspiration tests were performed with cells preincubated with culture medium containing 0%, 0.5% or 2.5% surfactant. Basically, when a cell is mounted at the mouth of a micropipette and a negative pressure is applied through the micropipette, the membrane, together with the underlying cytoskeleton, is sucked into the pipette mouth. Thus,

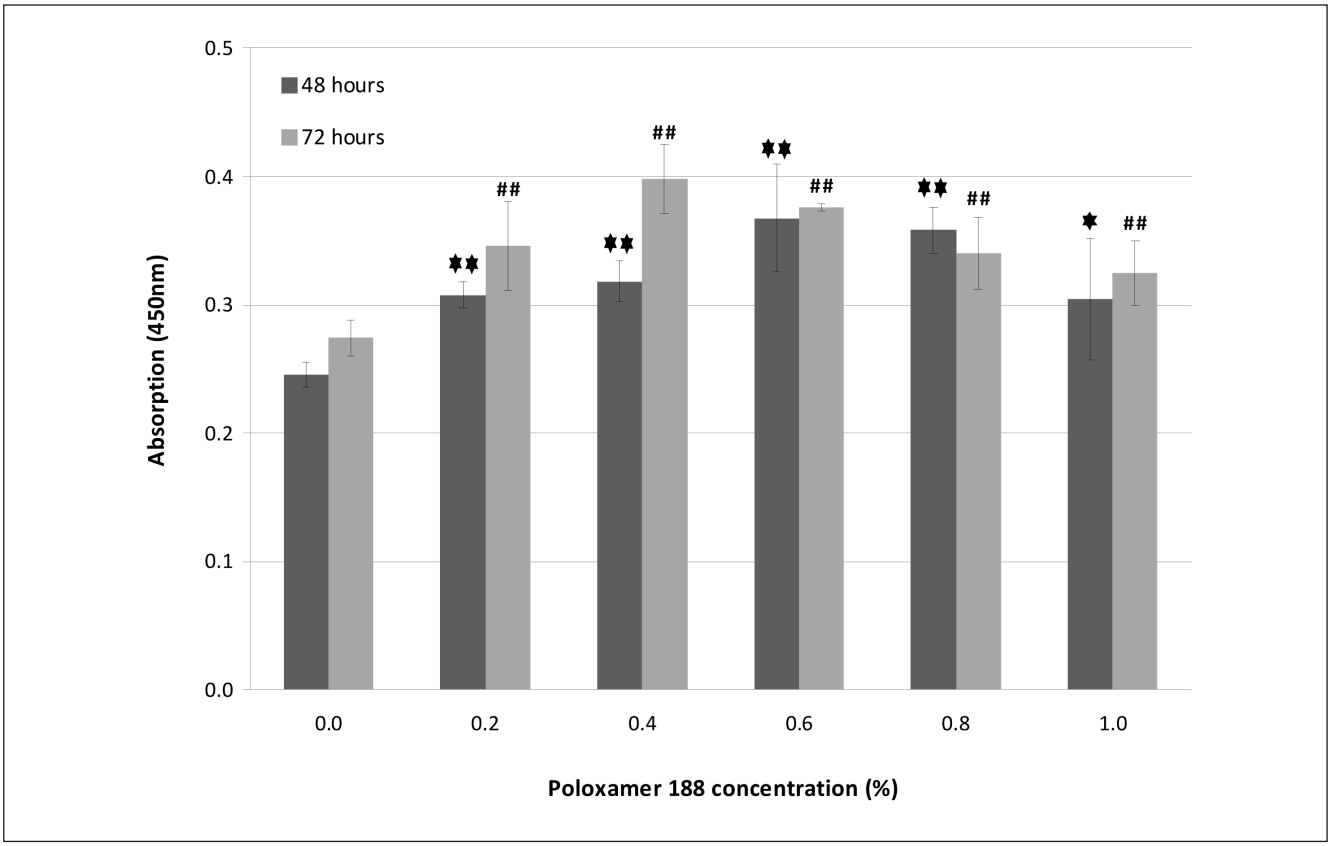


**Fig. 1: Proliferative activity of Caco-2 cells after subcultivation and propagation in presence of Poloxamer 188 on day 2 and 3 post seeding (n=6, mean ±SD).**

* indicate significant differences with respect to the untreated control at 48 h (*p<0.05; **p<0.01); # indicate significant differences with respect to the untreated control at 72 h (##p<0.01).

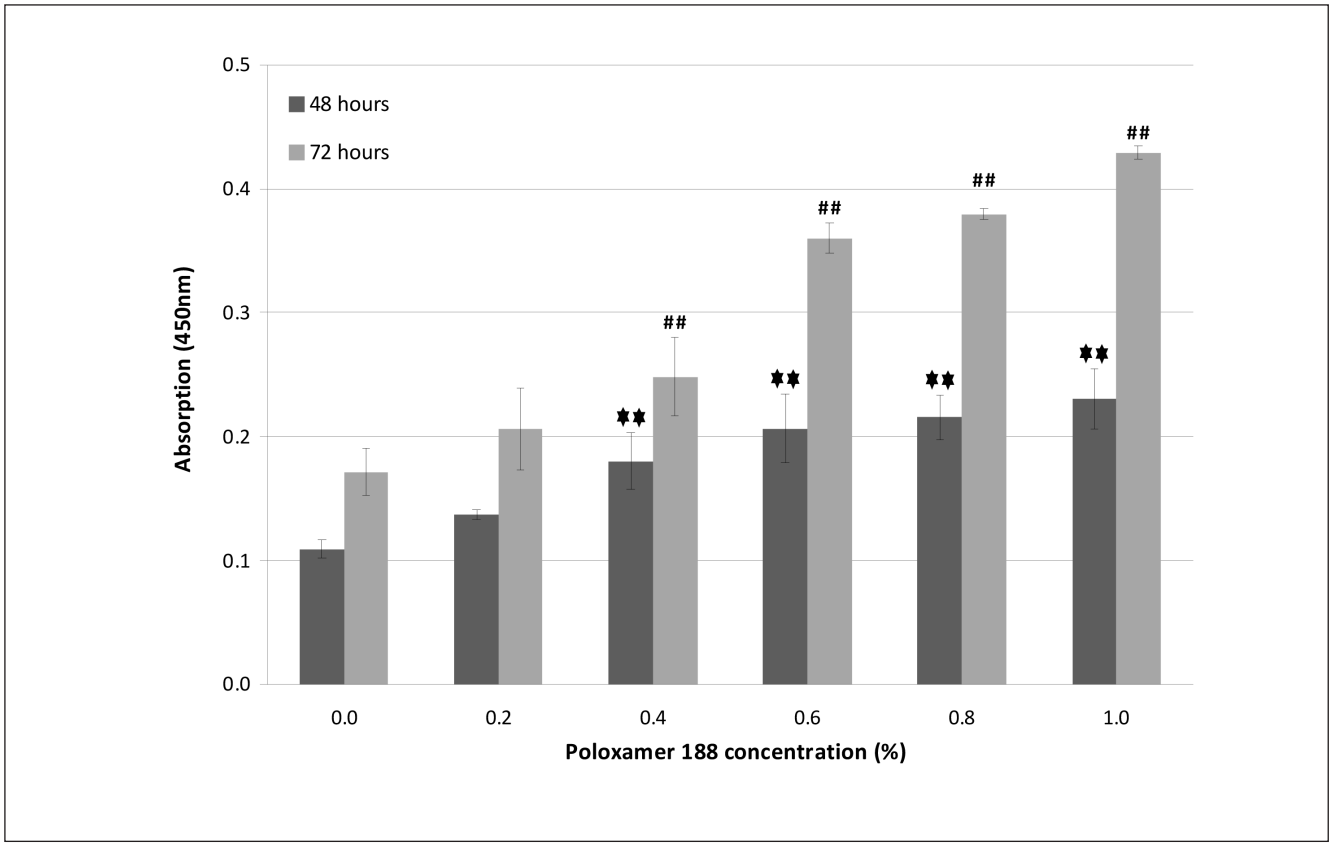


**Fig. 2: Proliferative activity of Caco-2 cells after one freeze/thaw cycle and cultivation in presence of Poloxamer 188 on day 2 and 3 post seeding (n=6, mean ±SD).**

* indicate significant differences with respect to the untreated control at 48 h (*p<0.05; **p<0.01); # indicate significant differences with respect to the untreated control at 72 h (##p<0.01).

**Tab. 1: Viability of Caco-2 cells**

Viability of Caco-2 cells after splitting and cultivation in cell culture medium containing Poloxamer 188 for 3 days (n=6, mean ±SD).

| Poloxamer 188 conc. (%) | 0.0 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
|---|---|---|---|---|---|---|
| Absorption (450 nm) | 1.07 ±0.01 | 1.74 ±0.04 | 1.68 ±0.06 | 1.68 ±0.05 | 1.71 ±0.09 | 1.53 ±0.02 |

the length of the apex depends on the cell's rigidity, i.e. the stiffer the cell the shorter is the extended part of the cell (Fig. 3).

For quantitative description of the results, the strain was set at 1.0 (Equation 1) and the tension was calculated from Equation 2. Accordingly, the mean tension, meaning the negative pressure necessary to extend the cell volume to the same point in the micropipette as with untreated cells, was 49 mN/cm$^2$ (0.5% Poloxamer 188) or 70 mN/cm$^2$ (2.5% Poloxamer 188) (Tab. 2). Thus, the stiffness of the cell membrane increases with the concentration of Poloxamer 188.

## 4 Discussion

According to the literature and the marketed Filoceth™-media, the non-ionic surfactant Poloxamer 188 is useful in cryopreservation media to increase viability of cells after thawing (González Hernández and Fischer, 2007). In order to detect further effects on the cultivation of human cell lines, varying amounts of the surfactant were added to commonly applied cell culture media and used during cultivation of Caco-2 cells. Proliferation as well as viability of cells served as decisive parameters.

In the presence of Poloxamer 188, the proliferative activity of cells stressed by either proteolytic subcultivation or freezing/thawing was considerably higher than without it. For subcultivated cells, the most pronounced effect on pre-confluent monolayers was observed upon addition of 0.4-0.6% Poloxamer 188, as represented by a 1.5 fold increase (Fig. 1). In case of Caco-2 cells after one freeze/thaw cycle, the proliferative activity steeply increased with the Poloxamer 188 concentration, exceeding that of the Poloxamer-free medium 2.1-2.5 fold (Fig. 2).

Interestingly, the proliferation of cells after one freeze/thaw cycle in cell culture medium without Poloxamer 188 is 56% lower than that of non-frozen cells, most probably due to cy-

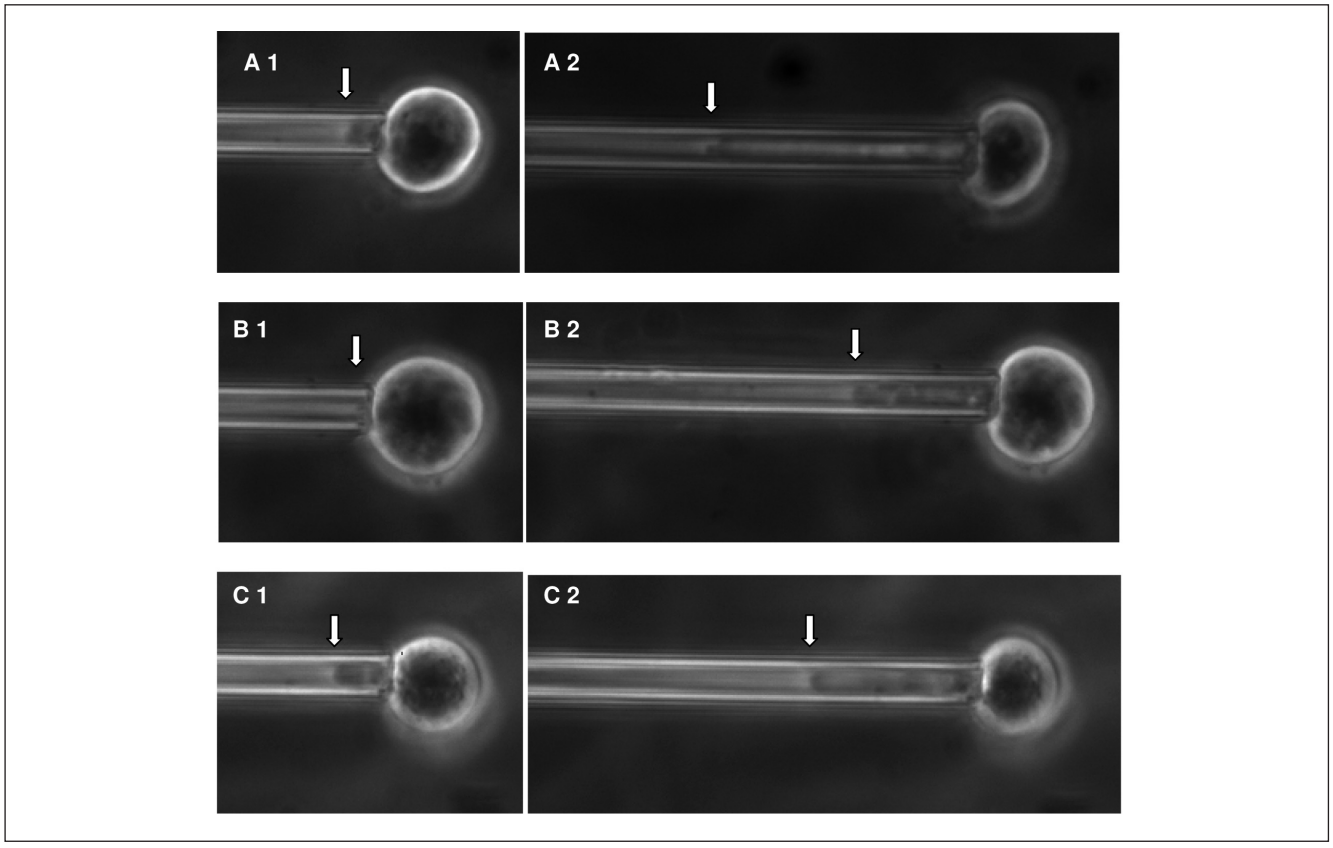


**Fig. 3: Microaspiration of Caco-2 cells in the absence (A) or presence (0.5% B and 2.5% C) of Poloxamer 188 without applying pressure (A1, B1, C1) and at a suction pressure of 1 kPa (A2, B2, C2).**
The arrows indicate the apex shift of the cell membrane in response to the applied pressure and presence of Poloxamer 188.

totoxic effects of DMSO (Guell et al., 2009) and damaging ice crystals (Fig. 1, 2). Thus, the steeper increase in proliferation rates post thawing, especially at surfactant concentrations higher than 0.4%, might be attributed in part to the lower number of seeded viable cells as well as a considerable number of cells which would have been damaged upon cryostorage and thawing but could be rescued by the resealing properties of the surfactant (Togo et al. 1999). In addition, the enhanced viability of the cells after cultivation in the presence of surfactant for three days confirmed the cytoprotective effect of 0.2-0.8% Poloxamer 188 (Tab. 1). These results are in line with reports that Poloxamer 188 protects cells against chemical and physical stress (Hellung-Larsen et al., 2000) and facilitates cell membrane resealing after wounding by decreasing the cell surface tension (Togo et al., 1999). In contrast, another study revealed that the mean membrane bursting tension as well as the mean elastic compressibility modulus of cells increases in the presence of Poloxamer 188 as a consequence of a supposed increase in membrane tension (Zhang et al., 1992).

In order to get an idea of the impact of Poloxamer 188 on the membrane tension of the single cell, the micropipette aspiration technique was applied. For this assay two surfactant concentrations were chosen: (i) 0.5% Poloxamer 188, as it exerted an optimum impact on cell proliferation and considerably increased viability, and (ii) 2.5% Poloxamer as an extreme, exceeding the most useful investigated concentration for post-thaw proliferation (1.0%).

**Tab. 2: Cell membrane tension**
Tension (set at strain 1) of Caco-2 cell membranes after incubation for 60 min at 37°C without and with 0.5% or 2.5% Poloxamer 188 (n=9, mean ±SD).

| Poloxamer 188 conc. (%) | Tension (mN/cm$^2$) |
|---|---|
| 0.0 | 194.81 ±53.10 |
| 0.5 | 243.95 ±38.73 |
| 2.5 | 264.28 ±58.09 |

The micropipette aspiration assays revealed that the tension of the plasma membrane concurrently increased with the amount of surfactant added (Tab. 2, Fig. 3). Interestingly, the difference in tension between 0% and 0.5% Poloxamer 188 was 49 mN/cm$^2$ as necessary to achieve strain 1, whereas only 21 mN/cm$^2$ was measured upon increasing the Poloxamer 188 concentration from 0.5% to 2.5%. Additionally, as depicted in Figure 3, the horizontal shift of the cell's apex between 0% and 0.5% Poloxamer 188 is 42%, while further increasing the

Poloxamer content from 0.5% to 2.5% provoked only a shift of 8% in comparison to the untreated cell (100%). Consequently, the effect of Poloxamer 188 is more pronounced in the range below 0.5%, implying that marked changes in membrane rigidity occur already in the presence of low amounts of the non-ionic surfactant.

It is most unlikely that micellar effects contribute to changes in membrane tension, since the critical micelle concentration of Poloxamer 188 is about 1 mM (Batrakova et al., 1998). However, Poloxamer 188 not only adsorbs to the cell membrane, as confirmed by rheogoniometric investigations (Al-Rubeai et al., 1993), but it also incorporates into the phospholipid-bilayer and decreases the fluidity of the cell membrane, as indicated by fluorescence polarisation experiments (Ramirez and Mutharasan, 1990). Thus, it is supposed that the increase in membrane tension is due to adsorption and incorporation of Poloxamer 188 into the cell membrane.

Apparently, this gain in stiffness also reduces the "Brownian" shape low amplitude and high frequency fluctuations of large areas of the cell membrane, the so-called undulations (Partin et al., 1989; Pierres et al., 2009). Most probably, this decrease in undulatory movements of the cell membrane facilitates the contact of cell adhesion molecules with the growth support and finally results in a higher number of adherent and proliferating cells, saving time to reach confluence as required for high throughput studies. Moreover, the cell volume increases by 5% in the presence of Poloxamer 188 and the cell rounds up (Raucher and Sheetz, 1999), which additionally increases the contact area of the cell membrane with the support (Hellung-Larsen, 2005).

Altogether, the observed and reported effects of Poloxamer 188, including the gain in stiffness, are thought to enhance attachment of adherent Caco-2 cells and so to increase the proliferation of subcultivated and thawed cells. These findings are in accordance with the results of the viability assay relying on the overall mitochondrial activity. Therefore, 0.5% Poloxamer 188 might be added to cell culture media to exploit its stabilising effects during cell cultivation. Interestingly, 1% Poloxamer 188 is already a cryoprotective compound of a marketed freezing medium.

In addition, the viability assays confirm the integrity of the monolayer and the reliability of the results obtained. As Poloxamers can inhibit function of P-gp (Kabanov et al., 2002) and cell lines differ with regard to absorption as well as intracellular accumulation of the surfactant (Gigout et al., 2008), the medium applied for transport studies should not contain Poloxamer 188. Nevertheless the beneficial effect of Poloxamer 188 supplement on improved cell adhesion was confirmed for Caco-2 cells. Since this adhesion enhancement seems to rely on a rather general mechanism, future work will focus on whether this effect translates also to other adherent and non-adherent cell cultures as well as its impact over different passages.

All in all, the non-ionic surfactant Poloxamer 188 represents a valuable cytoprotective supplement for cell culture media, not only for cryoprotection of cells but also to improve cell viability and proliferation after subcultivation and freezing/thawing procedures. That way, the reproducibility and the reliability of cell culturing at early stages of drug and formulation development might be improved, hence reducing and consequently at least partly replacing animal and human experiments as required for research and approval of drug formulations by legal authorities.

## References


Al-Rubeai, M., Emery, A. N., Chalder, S. and Goldman, M. H. (1993). A flow cytometric study of hydrodynamic damage to mammalian cells. *J. Biotechnol. 31*, 161-177.

Artursson, P., Palm, K. and Luthman, K. (2001). Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Del. Rev. 46*, 27-43.

Batrakova, E. V., Han, H. Y., Alakhov, V. Yu. et al. (1998). Effects of Pluronic block polymers on drug absorption in Caco-2 cell monolayers. *Pharm. Res. 15*, 850-855.

Evans, E. and Rawicz, W. (1990). Entropy-driven tension and bending elasticity in condensed-fluid membranes. *Phys. Rev. Lett. 64*, 2094-2097.

Gigout, A., Buschmann, M. D. and Jolicoeur, M. (2008). The fate of Pluronic F-68 in chondrocytes and CHO cells. *Biotechnol. Bioeng. 100*, 975-987.

González Hernández, Y. and Fischer, R. W. (2007). Serum-free culturing of mammalian cells – Adaptation to and cryopreservation in fully defined media. *ALTEX 24*, 110-116.

Guell, I., Alves, P. M., Gabor, F. and Wirth, M. (2009). Viability of the human adenocarcinoma cell line Caco-2: Influence of cryoprotectant, freezing rate and storage temperature. *Scientia Pharmaceutica 77*, 133-141.

Hellung-Larsen, P. (2005). Cloning of Tetrahymena cells in a chemically defined medium is possible in the presence of surfactants or at reduced temperature. *J. Biotechnol. 115*, 167-177.

Hellung-Larsen, P., Assaad, F., Pankratova, S. et al. (2000). Effects of Pluronic F-68 on Tetrahymena cells: protection against chemical and physical stress and prolongation of survival under toxic conditions. *J. Biotechnol. 76*, 185-195.

Hochmuth, R. M. (2000). Micropipette aspiration of living cells. *J. Biomech. 33*, 15-22.

Kabanov, A. V., Batrakova, E. V. and Alakhov, V. Yu. (2002). Pluronic® block copolymers for overcoming drug resistance in cancer. *Adv. Drug Del. Rev. 54*, 759-779.

Kim, J. S., Mitchell, S., Kijek, P. et al. (2006). The suitability of an in situ perfusion model for permeability determinations: Utility for BCS Class I Biowaiwer requests. *Mol. Pharmaceutics 3*, 686-694.

Partin, A. W., Schoeniger, J. S., Mohler, J. L. and Coffey, D. S. (1989). Fourier analysis of cell motility: Correlation of motility with metastatic potential. *Proc. Natl. Acad. Sci. U.S.A. 86*, 1254-1258.

Pierres, A., Monet-Corti, V., Benoliel, A.-M. and Bongrand, P. (2009). Do membrane undulations help cells probe the world? *Trends Cell Biol. 19*, 428-433.

Ramirez, O. T. and Mutharasan, R. (1990). The role of the plasma membrane fluidity on the shear sensitivity of hybridomas grown under hydrodynamic stress. *Biotechnol. Bioeng. 36*, 911-920.

Raucher, D. and Sheetz, M. P. (1999). Membrane expansion increases endocytosis rate during mitosis. *J. Cell Biol. 144*, 497-506.

Sachan, N. K., Bhattacharya, A., Pushkar, S. and Mishra, A. (2009). Biopharmaceutical Classification system: A strategic tool for oral drug delivery technology. *Asian J. Pharm. 3*, 76-81.

Togo, T., Alderton, J. M., Bi, G. and Steinhardt, R. A. (1999). The mechanism of facilitated cell membrane resealing. *J. Cell Sci. 112*, 719-731.

Woods, E. J., Benson, J. D., Agca, Y. And Critser, J. K. (2004). Fundamental cryobiology of reproductive cells and tissues. *Cryobiology 48*, 146-156.

Zhang, Z., Al-Rubeai, M. and Thomas, C. R. (1992). Effect of Pluronic F-68 on the mechanical properties of mammalian cells. *Enzyme Microb. Technol. 14*, 980-983.

**Acknowledgment**

We kindly thank Dr. Stefan Toegel for helping with statistical analysis.

**Correspondence to**

Mag. pharm. Vera Kerleta
Department of Pharmaceutical Technology and Biopharmaceutics
University of Vienna
Vienna, Austria
e-mail: vera.kerleta@univie.ac.at

# Calendar of Events

**» APHIS/AWIC Workshop: Considering Alternatives; Making a Difference**
**September 23-24, 2009**
**Kansas City, Missouri, USA**
http://awic.nal.usda.gov/nal

**» 16th Annual Congress on Alternatives to Animal Testing/ 13th Annual Congress of EUSAAT/ 16th International Congress on In Vitro Toxicology**
**September 2-4, 2010**
**Linz, Austria**
http://www.eusaat.org/index.php/congresses

**» International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Vaccine Safety Testing: State of the Science and Future Directions**
**September 14-16, 2010**
**Natcher Conference Center**
**NIH, Bethesda, MD, USA**
http://iccvam.niehs.nih.gov/meetings/schedule.htm

**» Symposium on Alternative and Supplementary Methods to Animal Experiments**
**October 4-5, 2010 (in German)**
**University of Veterinary Medicine Hannover**
**Hannover, Germany**
e-mail: Ulrike.Oberjatzas@tiho-hannover.de

**» Workshop on Reduction and Refinement: Combining Excellence in Science and Animal Welfare**
**October 4-5, 2010**
**Brussels, Belgium**
http://ec.europa.eu/enterprise/epaa/3_3_workshops.htm

**» 2010 In Vitro Alternatives Forum**
**October18-19, 2010**
**Old Town Alexandria, VA, USA**
http://www.iivs.org/documents/282.pdf

**» Animal Welfare and Scientific Research Symposium**
**October 24-26, 2010**
**Bethesda, MD, USA**
http://grants.nih.gov/grants/olaw/seminar/index.html

**» Workshop: Teaching and Education of 3Rs Approaches**
**October 25-27, 2010**
**Konstanz, Germany**
e-mail: caat-eu@uni-konstanz.de

**» CAAT-Europe Information Day**
**October 28, 2010**
**Konstanz, Germany**
e-mail: caat-eu@uni-konstanz.de

**» Toxicity Testing in the 21st Century and Alternative Methods**
**November 26, 2010**
**Milan, Italy**
e-mail: gianpaolo.rossini@unimore.it

**» 2010 EPAA Conference**
**November 30, 2010**
**Brussels, Belgium**
http://ec.europa.eu/enterprise/epaa/index_en.htm

**» ICCVAM Workshop Series on Best Practices for Regulatory Safety Testing**
**January 19+20, 2011**
**William H. Natcher Conference Center**
**Bethesda, MD, USA**
http://iccvam.niehs.nih.gov/meetings/schedule.htm

**» 8th World Congress on Alternatives & Animal Use in the Life Sciences**
**August 21-25, 2011**
**Montréal, Canada**
http://www.wc8.ccac.ca/