3 dimensional cell cultures: a comparison between manually and automatically produced alginate beads

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Abstract Cancer diseases are a common problem of the population caused by age and increased harmful environmental influences. Herein, new therapeutic strategies and compound screenings are necessary. The regular 2D cultivation has to be replaced by three dimensional cell culturing (3D) for better simulation of in vivo conditions. The 3D cultivation with alginate matrix is an appropriate method for encapsulate cells to form cancer constructs. The automated manufacturing of alginate beads might be an ultimate method for large-scaled manufacturing constructs similar to cancer tissue. The aim of this study was the integration of full automated systems for the production, cultivation and screening of 3D cell cultures. We compared the automated methods with the regular manual processes. Furthermore, we investigated the influence of antibiotics on these 3D cell culture systems. The alginate beads were formed by automated and manual

procedures. The automated steps were processes by the Biomek[®] Cell Workstation (celisca, Rostock, Germany). The proliferation and toxicity were manually and automatically evaluated at day 14 and 35 of cultivation. The results visualized an accumulation and expansion of cell aggregates over the period of incubation. However, the proliferation and toxicity were faintly and partly significantly decreased on day 35 compared to day 14. The comparison of the manual and automated methods displayed similar results. We conclude that the manual production process could be replaced by the automation. Using automation, 3D cell cultures can be produced in industrial scale and improve the drug development and screening to treat serious illnesses like cancer.

Keywords Life science automation (LSA) · Automated cell culturing · Cancer · Cervix carcinoma cells · 3-D cell culturing · Alginate beads · Alginate matrix

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Introduction

At present, cancer diseases still cause a variety of fatalities. Regarding this, the investigation and treatment of cancer gains in importance (Hanahan and Weinberg 2000). Promoting factors are the age and the influence of environmental factors as well. Today, conventional therapies against cancer cannot promise

a complete cure. But the understanding of the mechanisms of cancer cells can be used to obtain specifically targeted therapeutic strategies. As a result of the limitations of 2D cultures, three dimensional cell culturing (3D) in vitro models have already been used in cancer research. The 3D models provide a nearly physiological environment through their complex structure (Ghidoni et al. 2008). Cells are able to interact with each other and with extracellular matrix (ECM), which is essential for proliferation, migration and apoptosis (Pampaloni et al. 2007; Mountzios et al. 2013). These interactions further support the maintenance of tissue-specific cell functions (Pampaloni et al. 2007). A suitable natural and biocompatible polysaccharide for immobilization of cells in a 3D construct is alginate. Popular 3D in vitro models are alginate beads, which are mainly used for environmental (Cassidy et al. 1996) and basic research like drug development (Rimann and Graf-Hausner 2012), regenerative medicine (Jonitz et al. 2011b; Fischbach et al. 2009; Moshaverinia et al. 2013a) including tissue engineering (Chen et al. 2010) and investigation of cancer (Fischbach et al. 2009). The alginate beads can also be used to encapsulate chondrocytes. The 3D cell culturing in the presence of several growth factors have lead to a differentiation process of the cells, which was shown by the accumulation of ECM components like collagen type II, aggrecan and glycosaminoglycans (Jonitz et al. 2011b). Moreover, the influence of 3D cell cultures associated with growth factors on cancer cell angiogenic capability was investigated with oral squamous cell carcinoma cells (OSCC-3) encapsulated in alginate beads (Fischbach et al. 2009). The interleukin 8 (IL-8) interacts with the vascular endothelial growth factor (VEGF) and is increasingly secreted by stimulated OSCC-3 cells in 3D constructs. The 2D cell cultures with OSCC-3 cells showed the opposite effect. These proangiogenic factors affect the tumor vascularization and confirmed the relevance of alginate beads as a physiological model (Fischbach et al. 2009). The production of 3D cell cultures, especially of alginate beads, is a time-consuming and complex process. Hence, the production process should be simplified by automation, which offers different advantages like a higher reproducibility, sterility, a consistent performance and saving of time and money (Chapman 2003; Carere et al. 2002; Blow 2008; Markin and Whalen 2000). Therefore, ‘The Automation Partnership’

(TAP) developed a fully automated cell culture platform to simultaneously handle different cell lines (Liu et al. 2009). The CompacT SelecT enables incubation, cultivation, splitting and seeding of the cells as well as measuring the viability. The CompacT SelecT cell culture platform was used to compare the manual and automated 2D cultivation of human Caucasian osteosarcoma cells. Due to differences within the protocols, varieties in cell growth emerged (Liu et al. 2009, 2010).

A further step for automated cell handling is the production of 3D cell culture systems. The necessary requirements are the biological importance with an effective automated readout plus adequate costs. In detail, there is a configurable and variable system required for automated processes with a high biological importance or application and manageable costs (Mironov et al. 2009). The production of a 3D cell culture was shown with tubeless microfluidic devices. Human mammary fibroblasts were manually suspended in type-I collagen and transferred into a 192 microchannel array with a liquid handler. So far, there are different workstations for 2D cell culturing and a few for the production of 3D cell cultures. However, any automated workstation can realize the production of the commonly used alginate beads (Pampaloni et al. 2007). Some research groups investigated the automated cell encapsulation for different applications. The investigation of the proliferation potential of HepG2 cells embedded in alginate showed an increased proliferation and α -1 antitrypsin concentration in a rotary cell culture system over the time (Coward et al. 2009). Kim et al. (2013) evaluated the encapsulation of human embryonic stem cells on the differentiation potential to dopamine neurons.

A further aspect is that antibiotics are used in regular cell cultivation to prevent contaminations (Lincoln and Gabridge 1998). The routinely application of antibiotics can encourage the emergence of resistance, aseptic working and cryptogenic contaminations (Cohen et al. 2006; Kuhlmann 1995). A main question in automated process is whether antibiotics are required for the automatically cultivation of cells. The most wide-spread antibiotics in cell cultures are a combination of penicillin and streptomycin. The β -lactam penicillin is also a common antibiotic agent, which affects the cell wall syntheses of dividing bacteria (Tomasz 1979). However, streptomycin belongs to the group of amino glycosides and

influences the protein biosynthesis and cell death caused by accumulation of inoperative proteins (Francois 2005).

In the present study, we integrated a fully automated solution for the production, cultivation and screening of alginate beads. These results were compared with the regular manual processes. In concrete terms, this means that we evaluated the manual and automated production process of alginate beads. The automated manufacturing processes were realized with the Biomek[®] Cell Workstation (celisca, Rostock, Germany), which enables the automated cell handling. In addition, we compared HeLa cell-alginate beads (production: manual, automated) by manually and automatically performed bioscreenings (proliferation, cytotoxicity) using a high throughput screening arrangement since only limited studies have addressed the bioscreening and analytical readout of cells encapsulated in alginate matrix (Rimann and Graf-Hausner 2012). Moreover, we examined the question whether antibiotics have an influence on cells embedded in alginate.

Materials and methods

Biomek[®] Cell Workstation

The Biomek[®] Cell workstation is a flexible system for automated cell cultivation (Fig. 1). This system enables the cultivation of different cell types (adherent and

suspension cells) and the production and cultivation of 3D cell cultures (alginate beads, pellet cultures and hanging drops). The basic component is the Biomek[®] NX liquid handler (Beckman Coulter, Brea, CA, USA) combined with steel cannulas and a gripper. This enables the handling of specific cell culture flasks and solutions. A Cytomat incubator (37 °C and 5 % CO₂) (Thermo Fisher Scientific, Waltham, MA, USA), a Vspin[™] centrifuge (Velocity 11, Palo Alto, CA, USA) and a Vi-CELL[™] XR cell counter (Beckman Coulter) are associated with the liquid handler deck. A lift enables the transfer of well plates and cell flasks from the Cytomat (Thermo Fisher Scientific) to the liquid handler deck. These particular automated laboratory positioners (ALPs) are available as placeholder and for specific cell handling. This includes a heatable ALP (Beckman Coulter) for warming solutions, two 3-D tilt racks (AIG, Rostock, Germany) for simulating manual flask handling and a positive positioning ALP (Beckman Coulter) for focused positioning of well plates. The liquid handler is integrated into housing with UV-lights (Vilber, Eberhardzell, Germany) and HEPA filter (Camfil, Stockholm, Sweden) for sterile conditions. The port selection valve combined with a peristaltic pump enables the transfer of six different media with higher volumes (up to 1 l). These media can be stored in a cool box.

The control of the Biomek Cell Workstation is realized by the Biomek[®] NX software (Beckman Coulter, Brea, CA, USA) and consolidated by the management software SAMI EX.

Fig. 1 Biomek[®] Cell Workstation. *Frontal view*: schematic overview of the central components of the arrangement for automated cell cultivation (a), schematic overview of the liquid

handler deck with integrated components and automated laboratory positioner (ALP) (b), realization of the Biomek[®] Cell Workstation (c)

Manual cell cultivation and production of alginate beads

HeLa cells were cultivated in 75 cm² cell culture flasks at 37 °C and 5 % CO₂. Dulbecco's modified eagle medium (DMEM; SIGMA-ALDRICH, Seelze, Germany) was supplemented with (1 %) and without penicillin/streptomycin (SIGMA-ALDRICH) and 10 % fetal bovine serum (FBS; SIGMA-ALDRICH). For the splitting process cells were washed in with 5 ml phosphate buffered saline (PBS) (PAA, Cölbe, Germany) and were treated with 2 ml Trypsin/EDTA (SIGMA-ALDRICH, Seelze, Germany) for 4 min at 37 °C. Trypsin/EDTA was then neutralized with DMEM and the cell suspension was collected into a modular reservoir. After cell count measurement by ViCell and centrifugation, 2×10^6 cells/ml were embedded in alginate (SIGMA-ALDRICH, Seelze, Germany) and the cell/alginate suspension was added dropwise to CaCl₂ (SIGMA-ALDRICH) with a disposable syringe. Alginate beads were washed three times in NaCl (Fluka, Dresden, Germany) and fresh Dulbeccos Modified Eagles medium (DMEM) (SIGMA-ALDRICH) with 10 % fetal bovine serum (FBS), with 2 mM L-glutamine (SIGMA-ALDRICH) and with/without 1 % penicillin–streptomycin (P/S) (SIGMA-ALDRICH) was added (Fig. 2).

Automated production of alginate beads

The automated cell culture process was realized with the Biomek[®] Cell Workstation (Center for Life Science Automation, Rostock, Germany) (Fig. 1). Regarding this, PBS and trypsin were transferred into modular reservoirs. The cell culture media were provided by the port selection valve combined with a peristaltic pump. The Greiner CELLSTAR[®] AutoFlask[™] (Greiner Bio One, Frickenhausen, Germany) were transferred with a lift from the Cytomat to the liquid handler deck. The gripper of the liquid handler robot moved the Auto Flask[™] to different positions within the workstation. HeLa cells were washed with 5 ml PBS. The 3D Tilting-ALP enables the homogenous distribution of PBS through different tilting angles. Cells were detached by incubation with Trypsin/EDTA (SIGMA-ALDRICH) for 4 min using an on deck incubator. The 2 ml Trypsin/EDTA were neutralized with 6 ml DMEM and the cell suspension was collected into a modular reservoir.

Based on the cell suspension after an automated cell culture, the cell count was measured with the Vi-CELL[™] XR (Beckman Coulter) and the cell suspension was transferred into a deep well (Greiner Bio One). After centrifugation the cells were embedded in alginate (2×10^6 cells/ml) and dropped into CaCl₂ (1 bead/well) in a 96 well plate (WP). Alginate beads were washed three times in 200 µl NaCl and 200 µl fresh medium was added (Fig. 2).

Histological staining

Alginate beads were fixed by the incubation with 4 % PFA (Carl Roth, Karlsruhe, Germany), 0.1 M cacodyl buffer (pH 7.4) and 10 mM CaCl₂ at 20 °C for 4 h. Ethylalcohol was used for the dehydration of the samples, which were embedded in paraffin (Institute of Anatomy, University Rostock, Rostock, Germany). After the histological sectioning, the 7 µm samples were transferred on glass slides (Thermo Scientific, Braunschweig, Germany) and incubated on a heating plate (Medax Nagel GmbH, Kiel, Germany). The sections were stored at 37 °C over night. The deparaffinization and staining was followed by a papanicolaous-staining (Morphisto, Frankfurt am Main, Germany). The stained sections were embedded in synthetic resin (Fluka, Dresden, Germany), dried and microscopically documented.

Bioscreening

EZ4U-cell proliferation assay

The EZ4U Proliferation assay (Biomedica, Wien, Austria) is based on the reduction of tetrazolium salt to colored formazan by vital cells. The proliferation was evaluated at two time points (14d, 35d). Alginate beads were transferred into a new 96 well microtiter plate and 250 µl fresh medium as well as 25 µl of the EZ4U reagent were added. After an incubation period of 3 h at 37 °C, 200 µl of the supernatant was transferred into a new well and the absorbance was measured at 450 nm by the PHERAstar (BMG Labtech, Ortenburg, Germany).

Adenylate kinase—cytotoxicity assay

The adenylate kinase cytotoxicity kit (PromoKine, Heidelberg, Germany) is based on the detection of ubiquitous adenylate kinase from cells with damaged membrane. The alginate beads were cultivated in

Fig. 2 Overview of the overall process for alginate bead production. The alginate bead production is divided in the regular cell cultivation and the alginate bead production. The regular process steps are similar for the manual and automated bead manufacturing

200 µl medium/well. After preparing the reagent working solution, 100 µl of the supernatant of the alginate beads were transferred into a new well. 100 µl of the reagent working solution were added, followed by an incubation of 5 min at 20 °C. The luminescence was measured with the PHERAstar (BMG Labtech).

Automated high throughput bioscreening

The automated screening combined the proliferation and toxicity screenings in one method associated with

the high throughput screening arrangement (Fig. 3). This includes the Biomek[®] NX liquid handler (Beckman Coulter) and Biomek FX (Beckman Coulter) liquid handler for specific handling of solutions outside the housing. Within a housing are a barcode reader (Beckman Coulter), plate sealer (HJ-Bioanalytik GmbH, Mönchengladbach, Germany), peeler cover (HJ-Bioanalytik GmbH), lid station, wash station (Bio-TEK Instruments Inc., Bad Friedrichshall, Germany), centrifuge (Sigma, Osterode, Germany), Cytomat (Thermo Fisher Scientific), Cytomat

Fig. 3 High throughput screening arrangement. The automated bioscreening was realized by a high throughput screening system. The system with all integrated devices is schematically visualized (a), realization of the Biomek® Cell Workstation in the laboratory of celisca (Center for Life Science Automation) (b)

hotel (Kendro, Langensfeld, Germany) and PHERAstar, NOVOstar, and FLUOstar galaxy readers (BMG Labtech) for the detection of absorbance, luminescence, and fluorescence. The whole devices are connected with a rail-mounted Motoman robot (Yaskawa, Fukuoka, Japan) as system integrator.

The processes are controlled by the Biomek software and the SAMI EX management software.

For the automated bioscreening the disposables were stored in the Cytomat-Hotel. The well plates with the alginate beads were incubated in the Cytomat (37 °C, 5 % CO₂). For automated bioscreening the Motoman transfers disposables and the well plates with beads to the shuttle of the Biomek® FX liquid handler. The shuttle transfers the labware to the liquid handler

deck and the gripper is positioning the labware. The required reagents were provided in low volume reservoirs. Generally, the automated liquid handling was performed with the Biomek® FX combined with a 96 well pipetting head and disposables.

The automated bioscreenings were performed in one method. The cytotoxicity assay uses the supernatant and the proliferation assay the vital cells in fresh medium. For the automated toxicity screening 100 µl/well supernatant of the beads and 100 µl/well reagents were transferred into a white well plate by the Biomek® FX. After 5 min of incubation (20 °C), luminescence was detected using the PHERAstar (BMG Labtech). Parallel cell proliferation was evaluated by the transfer of the rest of 100 µl medium

into the waste and 250 µl fresh medium to the beads plus addition of 25 µl reagent per well with the Biomek® FX (Beckman Coulter). After 3 h of incubation (5 % CO₂, 37 °C), absorbance was detected with the PHERAstar (BMG Labtech) at 450 nm.

Statistical analysis

Statistical analysis was performed by calculating the mean values and the standard errors. Significances were calculated by the one way ANOVA test. Differences were regarded as significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)

Results

Microscopic evaluation

The manually and automatically produced alginate beads were evaluated by a microscopic investigation 1 day after manufacturing. The size of the alginate beads was analyzed using the Image-Pro Plus software (Media Cybernetics, Warrendale, PA, USA).

The size (diameter) of the manually produced alginate beads amounted to an average of 3127.6 µm compared to an average of 3190.5 µm for the automatically produced beads 1 day after manufacturing (Table 1).

Furthermore, the alginate beads were evaluated by microscopy from the first to fifth week. The alginate beads were cultivated in medium with antibiotic (Fig. 4) and medium without antibiotic. Alginate beads in both cultivation media visualized the same results. In the first weeks embedded cells appear isolated with a consistent cell distribution. In the second week, the growth of the cells is visualized by a

formation of cell aggregates. From the third until the fifth week the cell-aggregates developed and sizes increased. The cells embedded in alginate are cultivated in a stable three dimensional form (Fig. 4).

Histological staining

The three dimensional cell cultures were histologically evaluated on day 14 and day 35 cultivated in medium with and without antibiotics. The papanicolaous-staining is used for cytological investigation of gynecologic samples especially for cervix carcinoma cells. The cell nuclei are dark violet after dying. The amount of cell aggregates within the alginate beads increased from day 14 until day 35. Furthermore, on day 35 the size and compactness of the cell aggregates was increased (Fig. 5).

Furthermore, the area of the cell aggregates of histological sectioned and stained (papanicolaous) slides of alginate beads was evaluated at both time points of cultivation (day 14, day 35) (Fig. 6). The area of the aggregates was quantified using the Image Pro Plus software.

The cell aggregate areas were increased non-significantly by one-third from day 14 to day 35 for both cultivation forms (+P/S, -P/S; manual, automated). The cell aggregates cultivated in medium with antibiotic (+P/S) exhibited larger aggregates (day 14, 35). Regarding this, the aggregates of the manual produced beads (+P/S) have a 20 % larger area compared to aggregates in medium without antibiotic (day 14, 35). The differences of aggregate areas of automatically produced beads were less than 6 % (day 14, 35). Furthermore, the areas of cell aggregates of manual beads are about 9 % larger compared to automatically produced beads cultivated in medium with antibiotic (day 14, 35). In contrast, aggregate

Table 1 Diameter (in µm) of automatically and manually produced alginate beads cultivated in medium with (+P/S) and without (-P/S) antibiotic (manual, +P/S: $n = 9$; manual, -P/S: $n = 7$; automated, +P/S: $n = 10$; automated, -P/S: $n = 6$)

Antibiotic	Diameter (µm) of alginate beads	
	Manually manufacturing	Automatically manufacturing
+P/S	3083.8 µm (± 10.1)	3195.6 µm (± 51.2)
-P/S	3171.4 µm (± 58.4)	3185.4 µm (± 46.2)

Data are presented as mean value ± standard error. Statistical analysis was done with one way ANOVA test. No significances are observed

Fig. 4 HeLa cells embedded in alginate matrix for about 5 weeks. Development of automatically and manually manufactured HeLa cells encapsulated in three dimensional alginate matrix in medium with penicillin/streptomycin for about 5

weeks of cultivation ($n = 6$). Pictures were taken with an optical microscope using an objective with a fourfold magnification ($\text{bar} = 200 \mu\text{m}$)

Fig. 5 Papanicolaous-stained histological sections of HeLa cells embedded in alginate matrix. Histological analysis of HeLa cells embedded in alginate after 14 and 35 days of incubation with antibiotics ($n = 6$). Pictures were taken with an optical microscope using an objective with a 4- and 20 fold magnification ($\text{bar} = 100 \mu\text{m}$)

areas (–P/S) of automatically produced beads were larger about 11 % (day 14) and 2 % (day 35).

Proliferation screenings

In order to analyze the proliferation in three dimensional alginate matrices, proliferation was evaluated

by the EZ4U assay (Fig. 7). Regarding this, tetrazolium salt develops colored formazan by living cells. The automated screenings have been performed on a high throughput screening system (Fig. 3).

Generally, the results (manual and automated screening) displayed the reduction of the proliferations from day 14 until day 35 for cells cultivated in media

Fig. 6 Area (μm) of cell aggregates consisting of alginate-encapsulated HeLa cells. The area of automatically and manually produced alginate beads containing the HeLa cells embedded in alginate matrix cultivated in media with and without penicillin/streptomycin on day 14 and day 35 (manual, +P/S: $n = 18$; manual, -P/S: $n = 9$; automated, +P/S: $n = 14$;

automated, -P/S: $n = 10$). Statistical analysis was done with one way ANOVA test. Data are presented as mean value $\pm$ standard error. No significances are observed. The manually manufactured alginate beads in medium with antibiotics were chosen as control

Fig. 7 Proliferation of alginate-encapsulated HeLa cells. Proliferation of HeLa cells embedded in automatically and manually produced alginate beads and cultivated in medium with and without penicillin/streptomycin on day 14 and day 35 compared with manual (a) (manual, +P/S: $n = 18$; manual, -P/S: $n = 13$; automated, +P/S: $n = 15$; automated, -P/S: $n = 12$, in eight replicates) and automated (b) bioscreening

(manual, +P/S: $n = 9$; manual, -P/S: $n = 6$; automated, +P/S: $n = 10$; automated, -P/S: $n = 7$; in eight replicates). Statistical analysis was done with one way ANOVA test. Data are presented as mean value $\pm$ standard error. Significances are based on the manually produced beads cultivated in medium with antibiotic on day 14: * $p < 0.05$, ** $p < 0.01$

with (+P/S) and without (-P/S) antibiotics. Certainly, the signal strength of automatically screened beads was in the middle one-third increased compared to the manually screening. On all detection dates (day 14, 35), the proliferation of cells encapsulated in alginate and

cultivated in medium without antibiotic were almost higher compared to cells cultivated in medium with antibiotic in both detection forms (manual, automated). The proliferation of automatically encapsulated cells (+P/S) was significantly decreased on day 35.

On day 14, the comparison of manually screened beads cultivated in different media (with antibiotic, without antibiotic) visualized an increased proliferation of cells in medium without antibiotic (−P/S) about 23 % (manually produced) and 19 % (automatically produced) compared to cells in medium with antibiotic. The automatically screened beads (−P/S) showed an increased proliferation of about 3 % (manual produced) and 2 % (automated produced) compared to encapsulated cells in medium with antibiotic on day 14. The proliferation of manually produced beads (−P/S) was higher by about 7 % (manual screening) and 4 % (automated screening) compared to cells in medium with antibiotic (+P/S) on day 35. However, the proliferation of the automatically produced beads cultivated in medium without antibiotic (−P/S) was increased by about 15 % (manual screening) and 20 % (automated screening). But the proliferation of encapsulated cells in alginate matrix cultivated in medium with antibiotic (+P/S) was decreased.

The proliferation of the automatically produced beads (+P/S, −P/S) was lower compared to the manual manufactured beads (8 % on day 14, 30 % on day 35). In contrast on day 14 and 35, the proliferation of automatically produced beads compared to manually manufactured beads was in most cases higher (day 14: manual 2 %; day 35: manual 4 %, automated 30 %) and similar in medium without antibiotic on day 14.

Toxicity screenings

The toxicity was directly evaluated by the determination of the adenylate kinase that is released of the plasma membrane of damaged cells in the supernatant (Fig. 8). This detection of the screenings was manually and automatically evaluated with a high throughput screening system (Fig. 3).

Generally, the toxicity decreased from day 14 until day 35. The cytotoxicity of beads with encapsulated cells cultivated in medium with antibiotic (+P/S) was higher compared with the toxicity of beads in medium without antibiotic (−P/S; Fig. 8).

The signal strength of automatically screened toxicity was two-third increased compared to the manual screening on day 14.

On day 35, both screening forms (manual, automated) visualized a significant decrease of cell cytotoxicity from HeLa-alginate beads cultivated in

media without antibiotic to the control on day 14 (manual, +P/S) plus automated screened beads with antibiotic.

The cytotoxicity of beads (−P/S) was reduced in the average of 12 % (day 14, manually and automatically manufacturing) and 30 % (day 35, manually manufacturing) to beads cultivated with antibiotic. However, the toxicity of beads without antibiotic was reduced by about 53 % (manually screening) and 2 % (automatically screening).

The comparison of the manually and automatically manufactured beads shows almost similar toxicities of alginate beads in media with and without antibiotic on day 14 plus for beads cultivated in medium with antibiotic (+P/S) on day 35 (manually and automatically screening). The cell toxicity of manually manufactured beads cultivated in medium without antibiotic was increased about 30 % to automatically produced beads (manually screening). In contrast to the automatically screening, the toxicity of manually manufactured beads (−P/S) was decreased by about 45 % to automatically produced beads.

Discussion

Cancer diseases are combined with high therapeutic costs without warranties of healing (Bogenrieder and Herlyn 2003). In this respect, three dimensional cell cultures are an important strategy for compound screenings (Rimann and Graf-Hausner 2012), tissue engineering (Coward et al. 2009) and investigation of tumors (Rokstad et al. 2012). Three dimensional cell cultures enable the mimic of in vivo conditions. Especially, cells embedded in sodium alginate display a suitable source for this investigations (Kumachev et al. 2011; Pampaloni et al. 2007; Xu et al. 2013). The manual manufacturing is an established process in research (Jonitz et al. 2011b; Fischbach et al. 2009). The automated alginate bead production close to the manual manufacturing steps in combination with bioscreening is new and established in this paper.

We addressed these topics by the evaluation of a fully automated solution for alginate bead manufacturing combined with high throughput screening. The cervix carcinoma cells were manually and automatically embedded in 1.2 % sodium alginate matrix and compared by manually and automatically proliferation and toxicity screening methods.

Fig. 8 Cytotoxicity of HeLa cells encapsulated in alginate matrix. Adenylate kinase of HeLa cells embedded in automatically and manually produced alginate beads and cultivated in medium with and without penicillin/streptomycin on day 14 and day 35 combined with manual (**a**) (manual, +P/S: $n = 22$; manual, -P/S: $n = 16$; automated, +P/S: $n = 22$; automated, -P/S: $n = 19$, in eight replicates) and automated **b** bioscreening

(manual, +P/S: $n = 9$; manual, -P/S: $n = 9$; automated, +P/S: $n = 9$; automated, -P/S: $n = 6$; in eight replicates). Statistical analysis was done with one way ANOVA test. Data are presented as mean value $\pm$ standard error. Significances are based on the manually produced beads cultivated in medium with antibiotic on day 14: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

The established method is a down-screened process of the manual alginate bead production. The cells are likewise embedded in 1.2 % sodium alginate (2×10^6 cells/ml) and dropped in 100 mM CaCl_2 solution for bead formation. In the manual procedure 10–15 beads were pooled into a 48 well plate (Jonitz et al. 2011b). Instead of this, one bead/well is produced in the adapted automated process for further individual tests by high throughput screening systems. This new down-scaling process is combined with a reduction of required solutions and media and consequently costs. The comparison of the manual and automated manufacturing process visualized equivalent results related on the size, proliferation and toxicity to HeLa cells encapsulated in alginate matrix.

The microscopic evaluation in combination with the Image pro plus software displayed the size of 3127.6 μm (manual production) and 3190.5 μm (automated production) rounded shape HeLa-cell alginate beads produced by this new method. However, the published automatically manufactured alginate beads are μ -constructs. The sizes of these constructs are more than threefold smaller. The encapsulated dental derived mesenchymal stem cells were automatically generated scaffolds with a size of 1000 $\mu\text{m} \pm 100 \mu\text{m}$ (Moshaverinia et al. 2013a). Moreover, even smaller alginate beads were automatically produced with a size of 500 μm (Chen et al. 2010; Kim et al. 2013) and 80 μm (Tagler et al. 2013). But, we suggest that an increased size of the cancer constructs better simulates in vivo conditions and

cancer tissues for further investigations by the cell density and the environmental conditions. Certainly, if beads are needed for defect repairs by transplantation processes lower sizes are more useful.

The cell accumulation is increased over the time in cell-alginate beads (Fischbach et al. 2007; Xu et al. 2013). In our studies, the development of cell-aggregates within the alginate beads was confirmed. The microscopic investigations and histological sections visualized cells aggregated with increased size from day 14. However, cell-aggregates ($< 10^4 \mu\text{m}^3$) showed already an amplified size from day 3 onwards (Xu et al. 2013). These are necrotic and apoptotic niches located in the center of tumors (Cassidy et al. 1996). We are confident that these aggregates are essential for the development of in vitro tumor tissue and further investigations.

In order to screen alginate beads, we evaluated the proliferation of cells embedded in alginate and toxicity in the supernatant. The bioscreenings were evaluated in manual and automated procedures. The low density alginate beads (0.5×10^6 cells/ml alginate) showed an increased proliferation until day ten. The higher proliferation rates of cells in three dimensional cell culture systems is probably be caused by lower shear stress (Coward et al. 2009). However, the proliferation of high density alginate bead cultures (2×10^6 cells/ml alginate) was decreased till day 14 (Moshaverinia et al. 2013b). Our bioscreening confirmed these results. The EZ4U assay also visualized the partly significant reduction of the proliferation of our high

density alginate beads (2×10^6 cells/ml alginate) from day 14 till day 35. Thus, low density alginate beads might be better for long term cultivations. The stagnation and reduction of the proliferation seems to be associated with increased compactness and size of cell aggregates in the beads, which reflects *in vivo* tumor tissue. Additionally, the reduction of the proliferation rates can be explained by the development of cell-aggregates and proliferation resistance caused by limited space in the beads (Xu et al. 2013). Furthermore, the reduced cell growth is also combined with the accumulation of metabolic end products and hypoxia within three dimensional cell culture systems (Malda et al. 2007; Jonitz et al. 2011a). Moreover, any influence of penicillin and streptomycin on proliferation and influence of lactate in HeLa cells in 2D cultures could not be verified (Düwelhenke et al. 2006). But, our screenings showed contrary results by partly significantly decreased proliferation of cells in alginate beads in medium with antibiotic in contrast to cell-alginate beads in medium without antibiotic on day 35. The difference of the toxicity was evaluated through the detection of adenylate kinases released by the damaged plasma membrane of dead cells using a high throughput screening system. However normally, the live/dead assay in combination with fluorescence microscopy is used for the differentiation of living and death cells and visualisation of toxicity (Xu et al. 2013). Certainly, the quantification of the cytotoxicity by the bioscreening of cells in alginate beads is not a common screening form but could improve the investigations in a high throughput manner. Our screening methods confirmed the reduction of the toxicity. The results showed a decreased cytotoxicity of alginate beads from day 14 until day 35. The toxicity of beads in medium without antibiotic were significantly decreased on day 35 (manual and automated screening). Furthermore, the automatically screened beads cultivated in medium with antibiotic (+P/S) were also significantly decreased on day 35. Regarding this, the literature supported these results (Xu et al. 2013; Vemmer and Patel 2013). The reduced toxicity over the time seems to be connected with the reduced proliferation rate caused by proliferation resistance (Xu et al. 2013) and the protection of embedded cells against biotic and abiotic factors resulting in an extended longevity (Vemmer and Patel 2013).

The manual and automated 2D cell cultivation by the CompacT SelecT cell culture platform was

compared. The input and output of human Caucasian osteosarcoma cells was evaluated. The cell number and proliferation of automatically handled cells was decreased by about 41.7 % (study 1) and 29.1 % (study 2) compared with the manual treated cells (Liu et al. 2010). However, the Biomek® Cell Workstation enabled the mainly increased cell proliferation of automatically handled (production, screening) cells by more than 1.8 %. The automatically produced beads showed also a decreased proliferation at the manual evaluation of the proliferation. Certainly, the reduced proliferation was 8.5 % (day 14). Regarding this, the Biomek® Cell Workstation enabled more gentle cell cultivation processes.

The absorbance and luminescence signal of the automatically performed screenings is increased compared to the manual screenings. Thus, the automated high throughput bioscreening needed assays with stable reagents. Nevertheless, the tendencies of the different screening forms (manual, automated) are similar.

With these findings, we suggest that cultivation of high density alginate beads until 35 days enable the development of constructs similar to cancer tissue by the formation of cell aggregates over the time. This is associated with a decreased proliferation and toxicity due to the limited space in alginate beads, arrest of the cells and the cell growth.

Conclusion

In summary, we successfully established a new method for the automated alginate bead production. The automatically produced beads are comparable with the manually produced beads based on the size (average 3 mm) and the decrease of the proliferation as well as the cytotoxicity from day 14 until day 35. This new automated manufacturing method is close to the manual alginate bead production. Furthermore, we are able to perform a non-published fully automated process, which includes the manufacturing, cultivation and bioscreening of three dimensional alginate bead cultures. We recommend the cultivation without antibiotic for a better simulation of *in vivo* conditions. Regarding this, the proliferation of beads in media without antibiotic is higher and the toxicity is lower compared to the beads in media with antibiotic. Nevertheless, to verify the proliferation rates, we will quantify the DNA amount. Further investigations will

also include compound screenings with alginate beads and subsequent bioscreening. The automated solution is a stable and reproducible method to replace the manual process.

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Conflict of interest The authors confirm that there are no conflicts of interest.

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