Appl Biochem Biotechnol (2010) 162:1996–2007
DOI 10.1007/s12010-010-8975-3

Monitoring of Cell Viability and Proliferation in Hydrogel-Encapsulated System by Resazurin Assay

Jing Xiao · Ying Zhang · Jianzheng Wang · Weiting Yu · Wei Wang · Xiaojun Ma

Received: 17 January 2010 / Accepted: 18 April 2010 /
Published online: 2 May 2010

Abstract Cell microencapsulation is a promising approach for cell implantation, cell-based gene therapy and large-scale cell culture. For better quality control, it is important to accurately measure the microencapsulated cell viability and proliferation in the culture. A number of assays have been used for this purpose, but limitations arise. In this study, we investigated the feasibility and reliability of resazurin as a cell growth indicator in microencapsulated culture system. According to the experiment data, there was a reversible, time- and dose-dependent growth inhibition as observed for resazurin application in encapsulated cells. A positive relationship was observed between reduction of resazurin and CHO cell number in microcapsule. Moreover, the resazurin assay provided an equivalent result to the commonly used MTT method in determining CHO cell proliferation in APA microcapsule with no notable influence on cell distribution and organization pattern. In conclusion, resazurin assay is offered as a simple, rapid and non-invasive method for in vitro microencapsulated cell viability and proliferation measurement.

Keywords Microencapsulated cell · APA · Resazurin · MTT · Non-invasive monitoring of cell proliferation

Introduction

Cell microencapsulation is the process by which cells are physically isolated from the external environment through a semi-permeable membrane. This membrane allows the bi-directional diffusion of oxygen, nutrients and other small molecules for cell growth. Alginate-poly-L-lysine-alginate (APA) microencapsulation is one of the most well-studied

J. Xiao · Y. Zhang · J. Wang · W. Yu · W. Wang · X. Ma (✉)
Laboratory of Biomedical Material Engineering, Department of Science and Technology,
Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road,
Dalian 116023, People's Republic of China
e-mail: maxj@dicp.ac.cn

J. Xiao · J. Wang
Graduate School of the Chinese Academy of Sciences, Beijing 100039, China

microencapsulation technologies, including entrapment of cells in alginate gel beads, formation of alginate-poly-L-lysine membrane and liquefying of the alginate gel core to leave the cells floating in the center of the microcapsule. Compared with conventional culture, cell microencapsulation provides a special microenvironment which affects the cellular behaviors greatly. For example, cells are always formed aggregates and organized into a three-dimensional structure to proliferate, differentiate and function. Cell microencapsulation proved to be a valuable model in the research of gene therapy, artificial cells/organs, large-scale cell culture and drug screening. Thus, effective evaluation of microencapsulated cell viability is highly important for better bioprocess control and quality assurance.

Currently, several methods have been used and each of them has advantages and limitations. Direct visual cell counting requires a rupture of microcapsule and liberation cells from the matrix [1]. The process of cell harvesting is harsh and may yield results that do not accurately represent the in situ state of cells. Hoechst 33258 exhibits fluorescence enhancement upon nucleic acid binding and offers measurement of cell number in microcapsule. Although convenient, this assay cannot achieve sensitivity below 1,000 cells per sample, due to the relatively high background fluorescence and the low extinction coefficients [2]. Additionally, the UV-absorption of some polymers used for encapsulation may lead to false-positive results. The 5-bromo-20-deoxyuridine assay involves the detection of incorporation into replicating DNA in proliferating cells [3]. This method is problematic for the disposal costs and the safety requirements that accompany it. Furthermore, it is less reliable as a bulk measurement, due to the variability in labeling level from sample to sample. Bioluminescence imaging has been used recently, but its application is limited by the needs of complex and expensive apparatus [4, 5]. Aside from methods mentioned above, the 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay proved to be the most commonly used to determine viable cell number in microencapsulated culture system. However, this method presents biohazard to personnel, requires relatively long incubation time for encapsulated cells. In addition, it is destructive to microencapsulation matrix and leads to termination of culture.

Resazruin, the original name for Alamar Blue, is a dye known to act as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor [6]. The reaction is accompanied by a change in color of the culture medium from indigo blue to fluorescent pink, which can be measured easily. Since it is soluble in media, stable in solution, and more importantly non-destructive to cells, continuous monitoring of culture is possible. Mainly for these reasons, this assay has been considered superior to classical tests for cell viability such as MTT [7]. In conventional cell culture, resazurin assay has been used for cytotoxicity determination and cell viability measurement in various cell lines [8–10]. Recently, studies have attempted to demonstrate the use of resazurin in nanofibre mesh scaffold [11] and other cell-polymer constructs [12–14]. Unfortunately, these studies have failed to consider the cytotoxicity of resazurin in long time exposure and no comparison with other cell proliferation techniques was performed. Besides, because the response to resazurin varies in different cells, it is critical to optimize the assay conditions in order to obtain reliable results, which was ignored in all of these studies.

In this paper, we examined the feasibility and reliability of resazurin for the measurement of cell viability and proliferation in APA microcapsule, and compared that with the MTT assay in extensive culture. Moreover, cytotoxicity of resazurin was also thoroughly tested.

Materials and Methods

Materials

Chinese hamster ovary (CHO) cells and human hepatic carcinoma (HepG2) cells were kindly provided by Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Sodium alginate was purchased from the Chemical Reagent Corp (Shanghai, China), whose viscosity was over 0.02 Pa·s in 1% (*w*/*v*) aqueous solution at 20 °C and whose powder was less than 200 meshes. Poly-L-lysine was purchased from Sigma (St. Louis, MO, USA) (Mw_{vis}=8,200, DP_{vis}=135, MW_{MALLS}=21,500, DP_{MALLS}=103. Here, DP_{vis} and DP_{MALLS} refer to the degree of polymerization measured by viscosity and multi-angle laser light scattering, respectively). All other reagents were purchased from Sigma.

Cell Culture

Cells were maintained under standard culture conditions of 5%CO_2 in air at 37 °C with medium renewal every 2–3 days. The culture media used were MEM for HepG2 cells and RPMI 1640 for CHO cells, all containing 2 mM glutamine, 0.06% (*w*/*v*) penicillin, 0.1% (*w*/*v*) streptomycin and 10% (*v*/*v*) fetal bovine serum. When confluent, cells were detached with 0.05% (*w*/*v*) trypsin/0.02% (*w*/*v*) EDTA and resuspended in the appropriate medium for using the following procedure.

Cell Encapsulation and Culture

Cells were microencapsulated in APA microcapsules as described previously with little modification [15]. Briefly, cells were mixed with 1.5% (*w*/*v*) sterilized sodium alginate to form a cell suspension (2×10^6 cells/mL) and extruded through a 26-gage needle with an electrostatic droplet generator into 100 mM $CaCl_2$ to form calcium alginate gel beads. The microbeads were then suspended in 0.05% (*w*/*v*) poly-L-lysine to form membrane enclosing the alginate gel beads, followed by the addition of 0.15% alginate to neutralize uncoalescent poly-L-lysine. The microbeads were suspended in 55 mM sodium citrate to liquefy the alginate gel core. The diameter of the APA microcapsules was 250–300 μm. The resulted microencapsulated cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere with medium renewal every 2–3 days.

Growth Inhibition by Resazurin on Microencapsulated Cells

The cytotoxicity of resazurin was determined using microencapsulated cells that were inoculated at 5×10^3 cells/well in 24-well plates. A solution of resazurin (2 mM) was prepared and various dilutions were added at 0.1 of the medium volume, i.e. 200 μL for 2 mL of medium. The final concentrations were 25, 50, 100, 125 and 150 μM in growth medium. Control wells were added with equal volume of PBS. Microcapsules were incubated for 24 h, washed with PBS thrice and followed by the addition of fresh medium containing 0.5 mg/mL MTT solution. The plates were returned to the incubator for 24 h, after which the microcapsules were washed and 1 mL dimethyl sulfoxide was added to solubilize the MTT tetrazolium crystal. Absorbance was determined at 570 and 630 nm using a plate reader (Well Scan MK3, Labsystems Dragon, Finland). Cell survival rate was calculated: cell survival(%) = (ODresazurin)/(ODcontrol) ×100%.

To optimize the resazurin assay with regard to growth inhibition and reversibility of inhibition, microencapsulated CHO cells (5×10^3 cells/well) were incubated with 100 μM resazurin for 1, 2, 4, 8 and 24 h. Resazurin-containing medium was removed at different endpoints. The microcapsules were washed and returned to culture with fresh medium for 24 h. After that, MTT assay was performed and cell survival% was calculated.

Resazurin Standard Curve for CHO Cells in APA Microcapsule

When taking into account the inhibitory effect of 100 μM and the need for longer incubation time or higher initial cell concentration of 25 μM, 50 μM resazurin was selected in our following experiments. Microencapsulated CHO cells in serials of numbers were incubated with resazurin for 1, 2, 3, 4, 5, 6, 8 and 10 h, respectively. Then, the absorbance was monitored at 570 and 600 nm. Background absorbance was measured with empty microcapsules at equal volume. An equation was used to calculate the reduction of resazurin [16]. In order to eliminate difference due to medium colour, the experiment was performed using a culture medium without phenol red.

MTT Standard Curve for CHO Cells in APA Microcapsule

CHO cells in serials of numbers were microencapsulated in APA microcapsules, after which the MTT assay was performed to obtain the absorbance of these microencapsulated CHO cells. A standard curve was established by plotting cell number versus absorbance.

Growth Curve for CHO Cells in APA Microcapsule

Growth of CHO cells in APA microcapsule was determined via both resazurin and MTT assays. The microencapsulated CHO cells were prepared and cultured in medium without phenol red. At given time points, 50 μM resazurin was added for 2 h. After that, the microcapsules were washed and returned to culture with fresh medium. Meanwhile the old resazurin-containing medium was removed for absorbance measurement. Reduction of resazurin was calculated and cell numbers were computed from the resazurin standard curve.

MTT assay was performed at the same time as described above. A flat oscillator was used to allow the formazan to diffuse out of the microcapsule and completely dissolve in the solvent. Cell numbers were calculated according to the MTT standard curve. All assays were repeated three times.

Cell Morphology

The growth pattern of CHO cells cultured in APA microcapsule with or without resazurin application was observed using a phase-contrast micrography during the culture.

Live/Dead Assay

Microencapsulated CHO cells were washed with PBS and incubated with live/dead staining working solution composed of 2 μM calcein AM and 4 μM ethidium homodimer-1 (ED-1) at 37 °C for 2 h followed by washing in the dark. Samples were observed under a confocal laser scanning microscope (CLSM; Leica, Germany). Live cells were labeled with calcein AM, producing green fluorescence at an excited wavelength of 485±10 nm, and dead cells were labeled with ED-1, emitting red fluorescence at 530±12.5 nm.

Statistical Analysis

Data were analyzed by Student's *t* test or analysis of variance (ANOVA). Differences were considered statistically significant at $P<0.05$.

Results

Growth Inhibition by Resazurin on Microencapsulated Cells

As seen in Fig. 1, cell growth was significantly inhibited by resazurin on encapsualted CHO cells ($P<0.001$). Cell survival for CHO cells began to decline with 100 μM resazurin supplement. The inhibitory effect increased in a dose-dependent manner, and was also observed in HepG2 cells which had reduced cell viability in 100, 125 and 150 μM wells. However, no statistically significant difference was found between the two cell lines, suggesting a similar sensitivity to the growth inhibitory effect of resazurin.

The inhibition was also time dependent as shown in Fig. 2. Microencapsulated CHO cells were incubated with increasing dilutions of resazurin at time intervals spanning 1 to 24 h. At time points more than 4 h, growth inhibition was observed at concentrations higher than 100 μM.

Reversibility of Growth Inhibition by Resazurin

Cell survival began to decline with 100 μM resazurin supplement on both cell lines, meaning the limit of concentration that can be used in our experiments. To investigate the reversibility of growth inhibition, microencapsulated CHO cells were incubated with 100 μM resazurin for different time intervals, washed and cultured with fresh medium. Within the first 4 h, almost no inhibitory effect was observed, which increased for prolonged incubation times (Fig. 3).

Resazurin Assay for Cell Proliferation: Effect of Incubation Time and Cell Number

The two variables which most affect the response of cells to resazurin are length of incubation time and number of cells plated. Therefore, as a first step, the correlation was

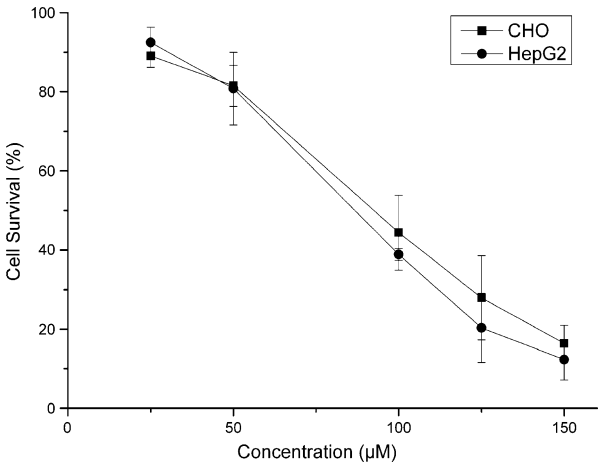

Fig. 1 Growth inhibition of different cell lines depends on the concentration of resazurin in the medium. The *error bars* represent standard deviation of means ($n=3$)

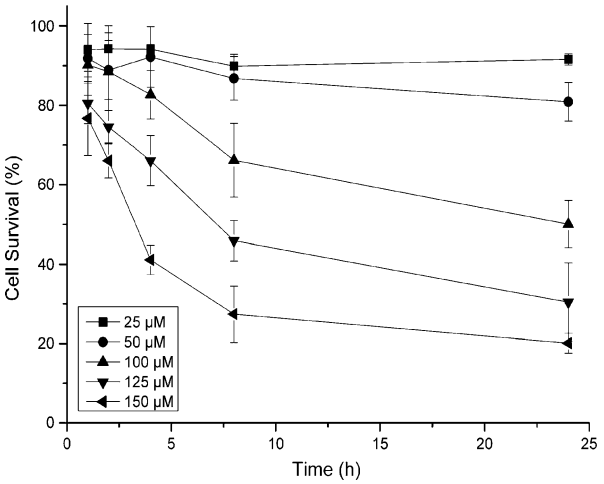

Fig. 2 Growth inhibition of microencapsulated CHO cells depends on the concentration and incubation time of resazurin. The *error bars* represent standard deviation of means (n=3)

established between the cell number of microencapsulated CHO cells and the reduction of resazurin for different incubation times. We found that the present of phenol red did affect the assay (data not shown). Thus, the following experiments were performed with culture medium without phenol red.

In order to avoid possible inhibition effect of high-dose and lower signal level due to decreasing concentration of the dye, 50 µM resazurin was used. There was no immediate color change and reduction increase upon addition until 1 h later (Fig. 4). The reaction was optimal after 2 h incubation, where any plating number from 1.56×10^4 to 5×10^5 microencapsulated CHO cells/well could be used and expected to produce a reaction within the linear range (Fig. 5, r^2=0.91). However, if the intent was to incubate for 4 h, the reaction could only be expected to be within the linear range from 6.25×10^4 to 5×10^5 microencapsulated CHO cells/well (r^2=0.87). With high cell number or extended incubation time, the curve reached a point where the resorufin, the deoxygenated product of resazurin, stopped increasing and the reduction level dropped with a corresponding clearing of the resorufin. This was demonstrated when plated at 1.25×10^5 cells/well and incubated for more than 6 h (Fig. 6).

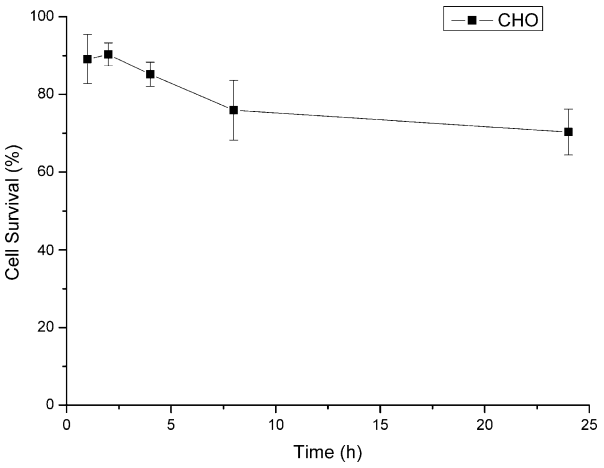

Fig. 3 Growth inhibition of microencapsulated CHO cells can be reversed by medium renewal. The *error bars* represent standard deviation of means (n=3)

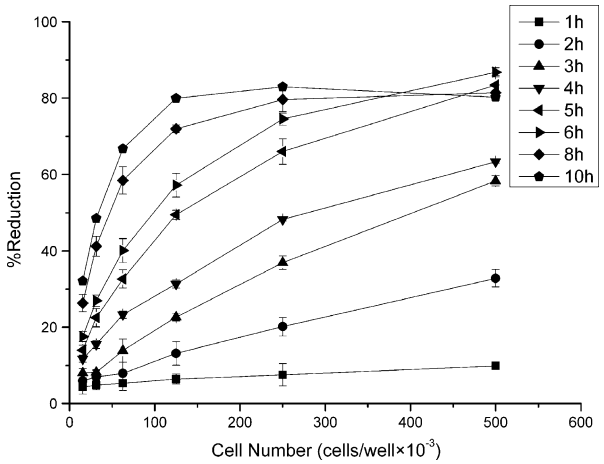

Fig. 4 Effect of initial cell number and incubation time on the %reduction of resazurin. The concentration of resazurin was 50 μM. The *error bars* represent standard deviation of means (n=3)

Comparison of Resazurin with MTT Assay

Parallel experiments involving resazurin and MTT assay were performed to monitor CHO cell proliferation in APA microcapsule. With the resazurin assay, a gradual increase was observed with cell number up to 1.4×10^5 cells/well; thereafter, the growth curve reached a plateau until day 14 (Fig. 7). Upon comparison of the two methods, cells in APA microcapsules followed similar trends. The r^2 values for linear fit were 0.910 and 0.918 by resazurin and MTT assay, suggesting that the resazurin assay can be employed in lieu of MTT to monitor cell viability and proliferation in microencapsulated culture system. However, cells became more aggregated, especially after 10 days of cultivation in the microcapsule, which complicated the MTT assay because of the need to fully dissolve the formazan salt.

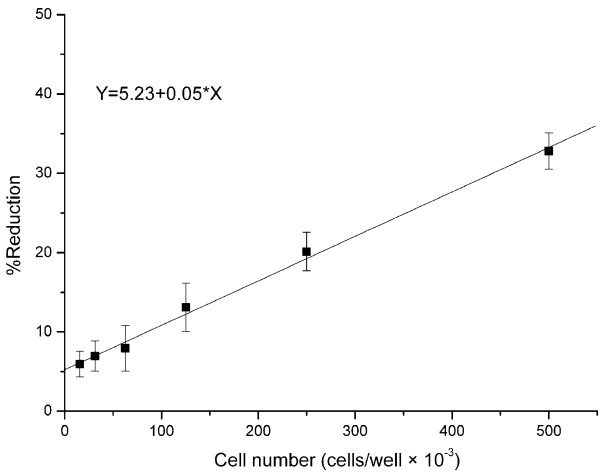

Fig. 5 Quantitative correlation between the cell number of microencapsulated CHO cells and the %reduction of resazurin. Microcapsules were incubated with 50 μM resazurin for 2 h. The *error bars* represent standard deviation of means (n=3)

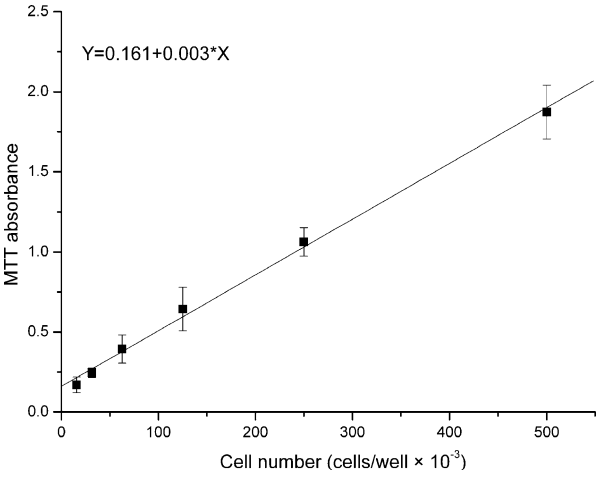

Fig. 6 Quantitative correlation between the cell number of microencapsulated CHO cells and the MTT absorbance. The *error bars* represent standard deviation of means (n=3)

Growth Pattern of CHO Cells in APA Microcapsule

Morphological characteristic of the CHO cells cultured in APA microcapsule was analyzed by light microscopy. No obvious adverse effect was observed on cell aggregation and distribution in both groups (Fig. 8). In the control group, cells distributed separately after the APA microcapsule was liquefied with sodium citrate (Fig. 8a). After 1 day of cultivation, cells began to aggregate separately (Fig. 8b) and proliferated to form clumps with a diameter about 100 μm on day 7 (Fig. 8c). Larger spheroids with a diameter about 200 μm formed on day 14 (Fig. 8d). Microencapsulated cells with resazurin supplement showed similar growth pattern as the control (Fig. 8e–f).

Cells were also examined by live/dead staining and observed under the confocal laser scanning microscope during the culture period, where the green fluorescence showed the calcein AM-labeled viable cells and the red fluorescence showed the ED-1-labeled nonviable cells. Most of the cells were alive, and viable cells mainly localized in the periphery of the cell spheroids (Fig. 9). The strong green fluorescence manifested that cells in both groups maintained high viability after growing in the microcapsule for 7 days.

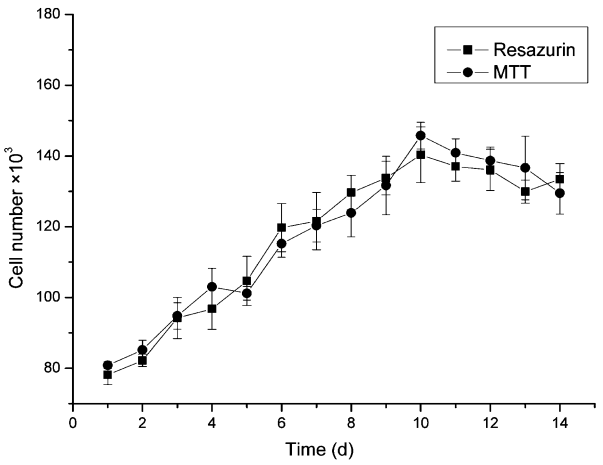

Fig. 7 Comparison of resazurin assay and MTT assay for detection microencapsulated CHO cell proliferation in 2 weeks. The *error bars* represent standard deviation of means (n=3)

Discussion

Cell microencapsulation provides a special microenvironment through a biocompatible semipermeable membrane. This membrance allows the bi-directional diffusion of nutrients, oxygen, secreted therapeutic product and metabolic waste, but prevents the high molecular weight substances from the cells, such as antibodies and immunocytes. This model is regarded as a promising strategy for tissue engineering and cellular therapy. Thus, biologically essential parameters reflecting cell viability and proliferation have to be defined for better application.

A number of cell viability and proliferation assays have been developed more specifically for use in monolayer cell population, but limitations may arise when applied to the microencapsulated culture system. The main disadvantage is the destruction to microencapsulation matrix and cell aggregates. Besides, they are all labor-intensive, time-consuming and require sufficient samples in the context of assay.

Resazurin is a water-souble extracellular redox indicator that can be reduced by living cells, generates a soluble fluorescent pink reaction product. This property provides a sensitive means to detect changes in cell viability. Several lines of evidence indicated that the resazurin assay was a valid cell growth measure in microencapsulated cell culture system: (1) resazurin was minimally toxic to cells; (2) reduction of resazurin was directly proportional to the viable cell number in the microcapsule over a wide range; and (3) resazurin assay was comparable with the MTT assay to quantify encapsulated cell proliferation.

Previous studies have reported the non-toxicity of resazurin to various cell lines [6, 17, 18]. In thepresent study, we examined the cytotoxicity of resazurin at concentration from 25 to 150 μM in microencapsulated culture system. There was a significant reduction in cell survival for microencapsulated CHO and HepG2 cells at concentrations higher than 100 μM.

This growth inhibitory effect showed in a manner of dose- and time-dependent, but no significant difference was found in different cell lines. This is accordant with other results for Swiss 3T3 fibroblast cells [19] and epithelial ovarian carcinoma cells [20]. Apart from the sensitivity differences of cell types against resazurin, experiment protocols varying in the concentration and the incubation time have to be considered. Higher concentration or longer incubation time of resazurin may become toxic to cells or alter their natural metabolism. However, this kind of inhibition can be reversed with medium refreshment. After resazurin removal, cells can be cultured further as demonstrated for CHO cells.

A significantly positive correlation was established between the cell number of microencapsulated CHO cells and the reduction of resazurin after 2 h incubation with 50 μM resazurin. But the reduction appeared to reach a plateau at durations longer than or equal to 4 h, especially for higher cell numbers. These results suggest that the staining technique could be used as a quantitative assessment of cell proliferation in cell microencapsulation, but assay parameters should be optimized depending on specific experimental requirements prior to the assay.

The usefulness of resazurin assay for microencapsulated cell proliferation quantitation was also evidenced by comparison with MTT assay, which is based on the conversion of yellow tetrazolium salt, MTT, to purple formazan crystals by metabolically active cells. Although the MTT assay has been predominantly used in microencapsulated culture system, there are still some disadvantages: (1) presents biohazard to personnel; (2) it is destructive and not suitable for precious seed cells; and (3) the insolubility of formazan crystals generated by aggregated cells is a technical problem often encountered, which can lead to high well-to-well variability.

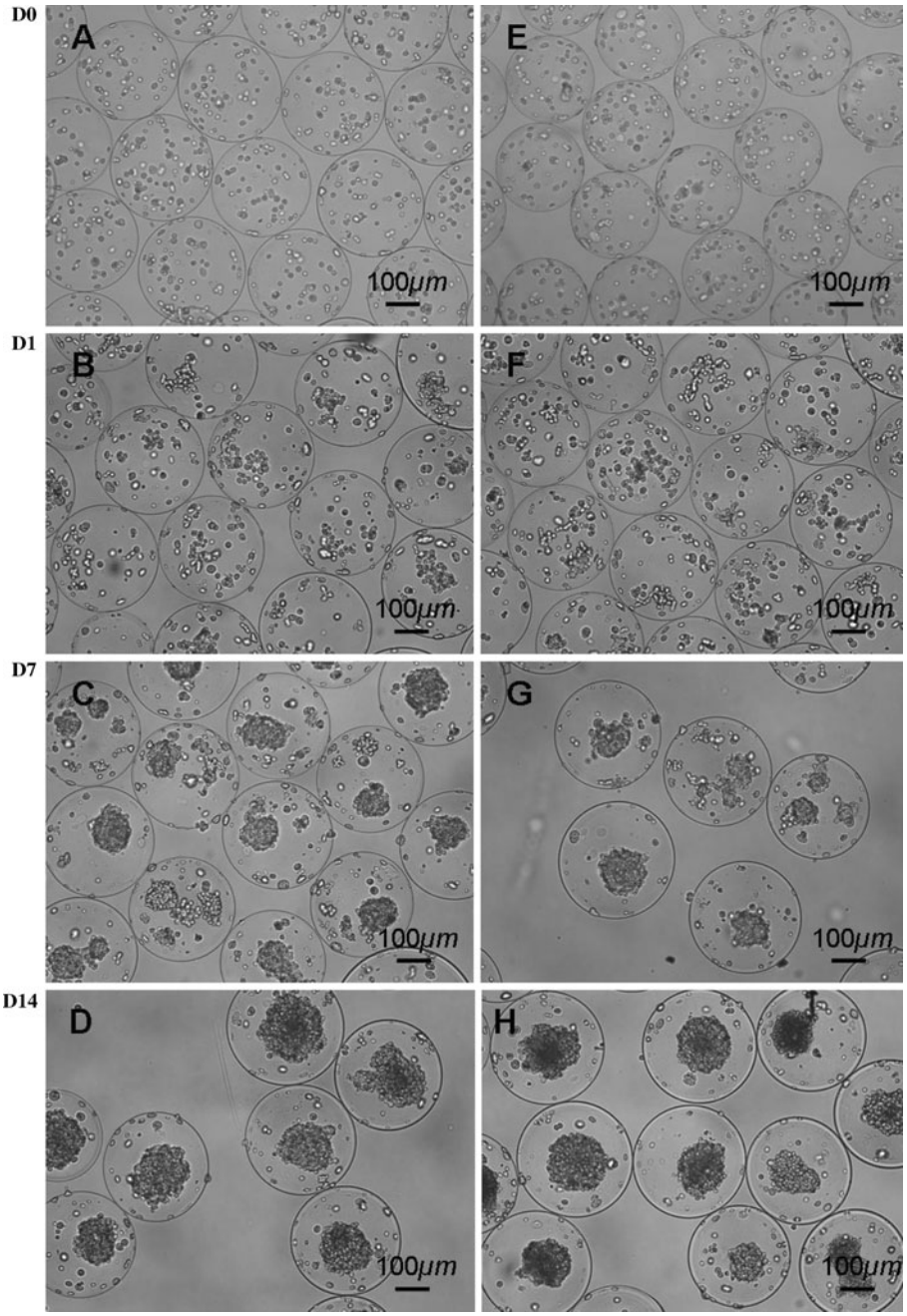

Fig. 8 Morphological characteristic of CHO cells cultured in APA microcapsules with or without resazurin application: **a**–**d** Microencapsulated CHO cells without resazurin application; **e**–**h** Microencapsulated CHO cells with resazurin application (×10 bars=100 μm)

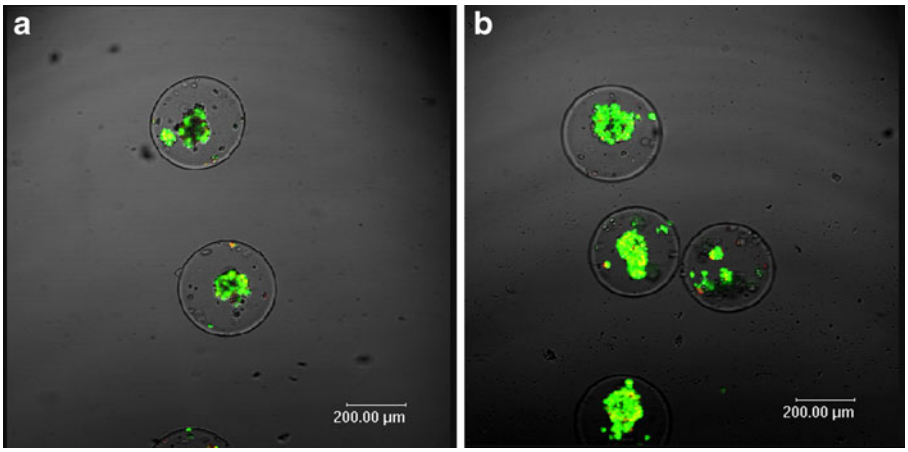

Fig. 9 Images of fluorescence labeled CHO cells cultured in microcapsule for 7 days revealed by confocal laser scanning microscopy. Viable cells were labeled in green by calcein AM and nonviable cells were labeled in red by ED-1. **a** Microencapsulated CHO cells without resazurin application; **b** Microencapsulated CHO cells with resazurin application (×10 bars=200 µm)

Our data indicate that the resazurin assay and the MTT assay are comparable means to monitor cell growth in APA microcapsule. Additionally, resazurin offers several distinct advantages. Firstly, it allows choice of detection method. Unlike MTT can only be detected colorimetrically, resazurin offers extra versatility in that results may be determined qualitatively by visual color change or quantitatively using absorbance/fluorescence-based instrumentation. Moreover, the reaction product is stable for hours at room temperature and can be stored at 4 °C, reading within 1–3 days. Secondly, since the oxidation-reduction potential of resazurin (E_0=+380 mV) is intermediate between cytochrome, a cytochrome oxidase complex (E_0=+290 mV) and molecular oxygen (E_0=+820 mV), resazurin can substitute for molecular oxygen as an electron acceptor in the electron transport chain [21]. Therefore, resazurin assay can preserve cell viability, which is much more practical for precious tissue engineering seed cells. Thirdly, it is water soluble, and thus, makes it possible to measure cell number in situ, without microencapsulation matrix rupture or deformation. Samples can be processed at fewer steps and shorter incubation time, which helps minimize the variability and easily adaptable to automation. This feature provides advantages in terms of undisturbed culture and less labour-intensive. Fourthly, with resorufin the only byproduct, this assay is non-toxicity to personnel.

It has been documented that high serum in the culture medium may affect the result [6, 12]. No influence from serum was found, but there was a shift of reduction curve caused by phenol red in the medium. Although we did not consider the reversibility of growth inhibition by resazurin at concentrations higher than 100 µM, and only APA microcapsule was used, our study can clearly indicate the feasibility and reliability of resazurin for the measurement of cell viability and proliferation in microencapsulated culture system.

Up to now it is the first report in standard reduction of resazurin to viable cell number and compares it with the MTT assay in microencapsulated culture system. In summary, reduction of resazurin, detectable as a change in absorbance/fluorescence, provides a non-invasive, simple and effective measure of cell viability and proliferation in microcapsule. In comparison with other methods as performed by our laboratory, the resazurin assay offers a continuous cell growth monitoring and kinetic study. Such detection is anticipated to be

valuable both alone and in combination with other assays in the research of cell microencapsulation. We are presently modifying the resazurin assay to expand its utility to other hydrogel-encapsulated systems.

Acknowledgements This work was supported by the National Natural Science Foundation under Grant number 30970885 and National Key Sci-Tech Special Project of China under Grant number 2008ZX10002-019. We thank the many colleagues and collaborators who have contributed to the development of this work.

References

1. Kung, I. M., Wang, F. F., Chang, Y. C., & Wang, Y. J. (1995). *Biomaterials, 16*, 649–655.
2. Jones, L. J., Gray, M., Yue, S. T., Haugland, R. P., & Singer, V. L. (2001). *Journal of Immunological Methods, 254*, 85–98.
3. Zhang, X. L., Wang, W., Xie, Y. B., Zhang, Y., Wang, X. L., Guo, X., et al. (2006). *Applied Biochemistry and Biotechnology, 134*, 61–76.
4. Kedzlorek, D. A., Walczak, P., Fu, Y., Azone, N., Arepally, A., Bulte, J. W., et al. (2008). *Circulation, 118*, S519–S519.
5. Kedzlorek, D. A., Walczak, P., Fu, Y., Azone, N., Arepally, A., Bulte, J. W., et al. (2009). *Journal of the American College of Cardiology, 53*, A453–A453.
6. Page, B., Page, M., & Noel, C. (1993). *International Journal of Oncology, 3*, 473–476.
7. Hamid, R., Rotshteyn, Y., Rabadi, L., Parikh, R., & Bullock, P. (2004). *Toxicology in Vitro, 18*, 703–710.
8. Perrot, S. B., Dutertre, C. H., Martin, C., Warnet, J. M., & Rat, P. (2003). *Cytometry Part A, 55A*, 7–14.
9. Shahan, T. A., Siegel, P. D., Sorenson, W. G., Kuschner, W. G., & Lewis, D. M. (1994). *Journal of Immunological Methods, 175*, 181–187.
10. Nasiry, S., Geusens, N., Hanssens, M., Luyten, C., & Pijnenborg, R. (2007). *Human Reproduction, 22*, 1304–1309.
11. Francois, S., Chakfe, N., Durand, B., & Laroche, G. (2009). *Acta Biomaterialia, 5*, 2418–2428.
12. Galler, K. M., Cavender, A., Yuwono, V., Dong, H., Shi, S., Schmalz, G., et al. (2008). *Tissue Engineering Part A, 14*, 2051–2058.
13. Tuschl, G., Hrach, J., Walter, Y., Hewitt, P. G., & Mueller, S. O. (2009). *Chemico-Biological Interactions, 181*, 124–137.
14. Wilson, C. G., Sisco, P. N., Gadala, F. A., Murphy, C. J., & Goldsmith, E. C. (2009). *Biomaterials, 30*, 5639–5648.
15. Ma, X. J., Vacek, I., & Sun, A. (1994). *Artificial Cells Blood Substitutes and Immobilization Biotechnology, 22*, 43–69.
16. Goegan, P., Johnson, G., & Vincent, R. (1995). *Toxicology in Vitro, 9*, 257–266.
17. Ahmed, S. A., Gogal, R. M., & Walsh, J. E. (1994). *Journal of Immunological Methods, 170*, 211–224.
18. Larson, E. M., Doughman, D. J., Gregerson, D. S., & Obritsch, W. F. (1997). *Investigative Ophthalmology and Visual Science, 38*, 1929–1933.
19. Voytikharbin, S. L., Brightman, A. O., Waisner, B., Lamar, C. H., & Badylak, S. F. (1998). *In Vitro Cellular and Developmental Biology-Animal, 34*, 239–246.
20. Squatrito, R. C., Connor, J. P., & Buller, R. E. (1995). *Gynecologic Oncology, 58*, 101–105.
21. Back, S. A., Khan, R., Gan, X. D., Rosenberg, P. A., & Volpe, J. J. (1999). *Journal of Neuroscience Methods, 91*, 47–54.