Improved sensitivity of trypan blue dye exclusion assay with Ni^{2+} or Co^{2+} salts

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Abstract

A modified trypan blue dye exclusion assay was developed using Ni^{2+} or Co^{2+} salts to determine the viability of primary and transformed cells. When the cells were preincubated with NiSO_4 or CoCl_2 followed by trypan blue assay, the contrast between stained and unstained cells was significantly increased as compared to the conventional trypan blue dye exclusion assay.

Introduction

Of the various methods for the measurement of cell viability (Freshney, 1994), trypan blue dye exclusion assay (Kaltenbach et al., 1958) is the most commonly used procedure. The assay relies on the alteration in the membrane integrity as determined by the uptake of the dye by dead cells. Though this assay gives a direct measure of cell viability, the lack of sharp contrast often causes difficulty in differentiating between stained and unstained cells. Here we report a modified trypan blue dye exclusion assay which provides a sharp contrast between stained and unstained primary as well as transformed cells.

Materials and methods

Cell lines

Primary cells (mouse thymocytes and bovine skin fibroblasts) and transformed cells (K562 (human leukemic), Sp2/0 (mouse myeloma), EL-4 (mouse lymphoma), and P815 (mouse mastocytoma)) were used. Mouse thymocytes were cultured in RPMI-1640 medium (GIBCO-BRL, USA) supplemented with 10% fetal bovine serum (GIBCO-BRL, USA). Bovine skin

Table 1. Titration of trypan blue with various metal salts in water^a

Metal salt	% Decrease in A_{586}
$\text{Mn}(\text{OAc})_2$	2.5
CoCl_2	3.0
NiSO_4	3.0
$\text{Cr}(\text{NO}_3)_3$	7.9
CuCl_2	14.0
FeCl_3	47.9

^a $[\text{trypan blue}] = 6.4 \times 10^{-5} \text{ M}$, final $[\text{metal}]/[\text{trypan blue}] = 10$

fibroblasts were cultured in IMDM medium (GIBCO-BRL, USA) supplemented with 20% fetal bovine serum and 10 ng/ml recombinant human epidermal growth factor (Boehringer Mannheim, Germany). K562, EL-4, and P815 were separately grown as a suspension in RPMI-1640 medium supplemented with 10% fetal bovine serum. Sp2/0 cells were grown in IMDM supplemented with 10% fetal bovine serum. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 .

Chemicals

Actinomycin D and Trypan blue (Sigma, St. Louis, USA).

Figure 1. Chemical structure of trypan blue.

Figure 2. K562 cells (a, b, c) and mouse thymocytes (d, e, f) were separately cultured in the absence (a and d) or the presence (b, c, e and f) of Actinomycin D. The cell pellet was suspended in fresh medium containing 120 mM NiSO_4 (a, c, d and f) or in medium without NiSO_4 (b and e) for 5 min at room temperature. The cells were then pelleted down and resuspended in 0.1% trypan blue solution for 5 min. An aliquot of the suspension was taken in a hemocytometer and observed under phase contrast microscope to determine the cell viability.

UV-Vis spectrophotometry

All the experiments were done using Shimadzu UV-2100 spectrophotometer. Titrations were done using cuvette of path length 10 mm and difference spectra were recorded using cuvette of path length 1 mm. The concentration of the ligand was 6.4×10^{-5} M for the titrations and 4.5×10^{-4} M for the difference spectra. For the titrations [metal]/[trypan blue] was varied from 0 to 30, whereas for the difference spectra measurements were done at (metal)/(trypan blue) = 0.5 to 10.

Modified trypan blue dye exclusion assay

The various cultures after the treatment with Actinomycin D, the cell death inducer (Kasai et al., 1998),

were separately pelleted down at $1100 \times g$ at room temperature for 5 min. The pellet was resuspended in fresh medium containing 120 mM NiSO_4 or CoCl_2 and incubated for 5 min at room temperature. Cells were pelleted down at $1100 \times g$ at room temperature for 5 min and resuspended in 0.1% trypan blue solution for 5 min. An aliquot of the cell suspension was taken in a hemocytometer, observed under phase contrast microscope and cell viability was determined.

Results and discussion

Because of its unique structural features, trypan blue (Figure 1) provides multiple sites for metal ion bind-

ing (Buckingham and Clark, 1987). In the presence of a suitable metal ion the resulting metal-ligand complex might show a change in the color intensity which could provide a sharp contrast between the stained and unstained cells. To ascertain the effects of various transition metal salts on trypan blue, an aqueous solution of the ligand was titrated with increasing concentrations of the metal ion and monitored by UV-Vis spectrophotometry at 586 nm. As seen from Table 1 the percent decrease in A_{586} is in the order of $\text{Fe}^{3+} > \text{Cu}^{2+} > \text{Cr}^{3+} > \text{Co}^{2+} = \text{Ni}^{2+} > \text{Mn}^{2+}$. As a next step the cell suspension was preincubated with different concentrations of the metal ion and the cell viability assay was performed. In presence of 120 mM NiSO_4 or CoCl_2 a sharp increase in the intensity of the dye in the dead cells was observed (Figure 2). Similar results were obtained with all the cell cultures used in the present study. In the assay with NiSO_4 at concentrations above 120 mM precipitation of hydroxide was observed during the preincubation step. There was no improvement in the staining of dead cells with $\text{Mn}(\text{OAc})_2$ even at 200 mM, whereas the dead as well as the live cells got stained intensely even with 25 mM of $\text{Cr}(\text{NO}_3)_3$. The studies with 10–120 mM of FeCl_3 or CuCl_2 could not be performed due to the precipitation of their hydroxides at the physiological pH, whereas at a lower concentration (<10 mM) no improvement in the staining was observed. We, therefore, believe that due to the loss of membrane integrity of the dead cells, intracellular concentration of metal ions increased resulting in the formation of a complex with the ligand. Due to the presence of multiple binding sites on trypan blue it may be difficult to ascertain the exact nature of the complex(s) formed. The difference spectra (Figure 3) showed the presence of isosbestic point which implies a dynamic equilibrium between the metal and the ligand (Tsukube et al., 1996).

Conclusion

In summary, the sensitivity of trypan blue dye exclusion assay for primary and transformed cells was significantly improved by preincubating the cells with NiSO_4 or CoCl_2 . No toxic effect was observed with either of the metal salts. It is noteworthy to mention that the trypan blue concentration used in the present assay is 0.1% instead of 0.4% in the conventional assay.

Figure 3. Titration of trypan blue with Ni^{2+} , represented as the first derivative of the difference spectra. $[\text{trypan blue}] = 4.5 \times 10^{-4} \text{ M}$; $[\text{Ni}^{2+}]/[\text{trypan blue}] = 0\text{--}10$. The isosbestic point is at 635.6 nm. Isosbestic point for Co^{2+} is at 635.4 nm as obtained from a similar experiment.

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References

- Buckingham DA & Clark CR (1987) For a general review on the coordination compounds of Co^{2+} and Ni^{2+} salts see: Cobalt. In: Wilkinson G (ed.) *Comprehensive Coordination Chemistry*. Vol. 4 (pp. 635–900) Pergamon, Oxford.
- Sacconi L, Mani F & Benzi A (1987) Nickel. In: Wilkinson G (ed.) *Comprehensive Coordination Chemistry*. Vol. 5 (pp. 1–347) Pergamon, Oxford.
- Freshney RI (1994) *Culture of Animal Cells: A Manual of Basic Technique*, (pp. 287–307) Wiley-Liss, NY.
- Kaltenbach JP, Kaltenbach MH & Lyons WB (1958) Nigrosin as a dye for differentiating live and dead ascites cells. *Exp Cell Res* 15: 112–117.
- Kasai T, Ohguchi K, Nakashima S, Ito Y, Naganawa T, Kondo N & Nozawa Y (1998) Increased activity of oleate-dependent type phospholipase D during actinomycin D-induced apoptosis in Jarkat T cells. *J Immunol* 161: 6469–6474.
- Tsukube H, Furuta H, Odani A, Takeda Y, Kudo Y, Inoue Y, Liu Y, Sakamoto H & Kimura K (1996) Determination of stability constants. In: Atwood JL, Davies JED, MacNicol DD, Vögtel F and Lehn J-M (eds.) *Supramolecular Chemistry*, Vol. 8 (pp. 425–482) Pergamon, Oxford.