Lecture 5

SR Microbeam Analysis at Cellular Level

5.1. Introduction

The advent of micro beam analytical methods opened up new possibilities of studies of biological systems at cellular level. An in situ technique enables analysis of living cells in vitro, and even in-vivo. Electron microprobes and proton microprobes can be used for such studies, but thermal damage to the sample precludes their extensive use. Synchrotron radiation based micro beams provides virtually an ideal tool for this area of study, offering sensitivity for detection of elements at the parts-per-million level comparable to proton microprobes, but without the adverse effect of sample damage. Although the information is limited to only elemental composition, and not the actual compounds, SR also offers additional advantage of enabling chemical state analysis at trace levels. In this chapter a number of applications of SR micro beam analysis at cellular level will be presented to illustrate the power of the method.

In the first section, analysis was carried out on cultured single untreated macrophage cells, and those which have been exposed to solutions containing chromium and vanadium. Images of the distribution of a number of elements, and the chemical state of Cr and Fe were obtained in order to gain better understanding on the mechanism and metabolism of phagocytic activities. In the second section, attempts were made to study single neuron cells.

5.2. Elemental Images of Single Macrophage Cells

5.2.1. Introduction

The interactions and responses of cells (macrophages, neutrophils, fibroblast and endothelial cells) to foreign metal elements have been widely investigated in the past decades. Interaction between cells and metal ions or particles, has been investigated (in-vivo and in-vitro), in the case of wear debris and corrosion in metal implants [1-6]. Phagocytic activity and the inflammatory reaction of macrophages are often discussed from a variety of points of view because of the fact that the macrophage is central to the direction of host inflammatory, immune and phagocytic processes [7]. The problem of cytotoxicity has also been investigated in the interactions between alveolar macrophages and airborne dusts or fly ashes [8-12].

In several previous studies, A.M. Ektessabi et. al. [1-3] applied PIXE, micro-beam PIXE, and SR-XRF (Synchrotron Radiation X-ray fluorescence spectroscopy) to the investigation of human tissues around total hip replacements where SUS316L and Ti-6Al-4V had been inserted into the human body for long periods of time. They showed that Fe, Cr, Ni, and Ti were released and distributed into the tissues around the total hip replacement in the forms of mechanical friction and corrosion products from the implant. Characteristics of metal particles or ions, such as surface chemistry, surface morphology, net charge, porosity and degradation rate, are critical factors in their interaction with cells. These features can result in the initiation of various responses, such as preferential protein adsorption, complement activation, and cell recruitment [13-15]. However, the mechanisms of phagocytosis of macrophages and cytotoxicity to metal ions or particles are extremely complex, and no single model can fully account for the diverse structures and outcomes associated with particle internalization [16,17]. While it is possible for each individual response to be identified and analyzed separately, this approach gives an analysis of only a relatively small component of the many factors controlling the overall host responses [18].

In this section we present an investigation by Kitamura and Ektessabi (2000) of fluctuations in the densities and distributions of intracellular elements simultaneously by using SR-XRF imaging technique, on macrophages cultured in a metallic solution environment under differing conditions. XRF analysis is being increasingly utilized in biomedical science [9, 19] because it is, in contrast to most other elemental analysis techniques, capable of analyzing ultra-trace elements nondestructively. In a single cell, metal ions and cellular nutrient density remain more or less constant. This homeostasis is maintained by the delicate balance of transport activities across the plasma and organelle membranes [20].

In this section, interactions between single cells and metal elements are investigated via the measurement of the density fluctuation and also of the localization of those elements, in single cells that have been cultured in a metallic solution environment.

5.2.2. Culture of macrophages

5.2.2.1. Macrophages

Macrophages are widely distributed throughout the body, displaying great structural and functional heterogeneity. They are to be found in lymphoid organs, the liver, lungs, gastrointestinal tract, central nervous system, serous cavities, bone, synovium, and skin. Macrophage cells play a central role in host inflammatory, immune and phagocytic processes. They are generally large, irregularly shaped cells measuring 25-50 μm in diameter. They often have an eccentrically placed, round or kidney-shaped nucleus with one or two prominent nucleoli and finely dispersed nuclear chromatin [21].

The aim of the investigation was to clarify the phenomena that occurred within single cells after interactions with foreign metal elements. To achieve that aim, it is necessary to perform the investigations into the intracellular reactions in-vivo and in-vitro. In particular, the distribution patterns and the densities of the intracellular trace elements are important factors to consider in attempts to understand the functions and defensive mechanisms of the cells against the foreign metal elements. There are only few measurement techniques for investigating the trace elements within single cells. In this study, SR-XRF analysis was employed to measure the trace elements within cultured macrophage single cells. The procedures of the cell culture and the staining, and the morphological observation techniques are described below.

5.2.2.2. Procedures for the cell culture

Mouse macrophages (J774.1) were employed in the experiment reported here. These cells were provided by the RIKEN CELL BANK. The culture medium was RPMI-1640. FBS (Fetal Bovine Serum) was added into the culture medium. The ratio of FBS and RPMI-1640 was 1 to 10. The cells in DMSO (Dimethyl Sulfoxide) and culture medium which were frozen by liquid nitrogen were defrosted in warm water (37 °C). After defrosting, the cells were pipetted into a sterilized tube with the culture medium (5mL). After the centrifugation of the tube, the cells were distributed into a 550 mm diameter dish with 5ml culture medium. The cells were cultured in an incubation box where the temperature was always kept at 37 °C and the concentration

of the carbon dioxide was fixed at 5 %. In this case, the cell divisions were observed approximately every eight hours. When the number of cells had increased enough, a fraction was moved into the new dish with a fresh culture medium.

The metallic solutions were prepared as follows. Pure metal compounds of $CrCl_3$, CrO_3, $FeCl_3$ and VCl_3 still enclosed in their bottles were sterilized in an autoclave for 30 min. The metal powders were then dissolved in sterilized PBS solutions. The metallic solutions were sterilized by using a filter with 0.45 μm pores. Metallic solution for culture is prepared by mixing the culture medium and metallic solution with the ratio of 100 to 1.

5.2.2.3. Histological observation

Macrophages have ultra-structures within their bodies, such as the nucleus, rough endoplasmic reticulum, Golgi complex and mitochondria. For the investigation into the intracellular elements, the ultra-structures were made visible by staining the cells. This is highly desirable since the elemental compositions may be different among the cell organelles. Giemsa's solution, Methyl Green solution, Mayer's Hematoxylin solution and Eosin Y Ethanol solution (0.5 %) were employed in this study for distinguishing the cell nucleus from the cytoplasm.

The staining procedure starts by removing the culture medium from the dish, followed by rinsing by pouring ethanol (100 %) into the dish. After 10 minutes, the ethanol was removed and the dish was dried in air. For Giemsa staining, the Giemsa's solution was diluted with the distilled water (×50) and 5 mL of the diluted solution added into the dish. After half an hour, the Giemsa's solution was removed from the dish. The dish was washed with water and dried. The light microscopic photograph of macrophage cells stained by Giemsa's solution is shown in figure 5.1. It can be seen that the nucleoli in the nucleus were stained purple.

For Methyl Green staining, after rinsing and drying, the dish was wetted by PBS (phosphate buffered saline) solution and methyl green solution (1mL) was added. A small dose of acetic acid was pipetted onto the dish. After 10 minutes, the methyl green solution was removed from the dish followed by washing in distilled water. The light microscopic photograph of macrophage cells stained by the Methyl Green solution is shown in figure 5.2, where it can be observed that the nucleoli in the nucleus of the macrophage were stained green.

Similar procedure was followed for Hematoxylin-eosin staining. After rinsing and drying, the dish was wetted by PBS (phosphate buffered saline) solution and hematoxylin solution added into the dish. After 10 minutes, hematoxylin solution was

removed from the dish and the dish was washed with water for about 10 minutes. Eosin Y Ethanol solution (0.5 %) was diluted with an ethanol solution (80 %). The ratio of Eosin Y Ethanol solution and 80 % ethanol solution was 1 to 3. After hematoxylin staining procedures, Eosin Y Ethanol solution was poured into the dish and a small dose of acetic acid was pipetted onto the dish. After ten minutes, the dish was dehydrated by 70 % ethanol solution twice and by 98 % ethanol solution twice also. The light microscopic photograph of macrophage cells stained by Hematoxylin-eosin solutions is shown in figure 5.3. It can be observed that the nucleoli in the nucleus of the macrophage were stained blue and other parts were stained red.

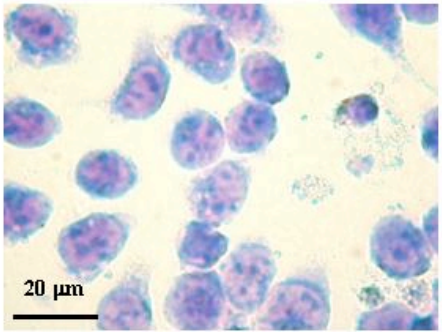


Figure 5.1.

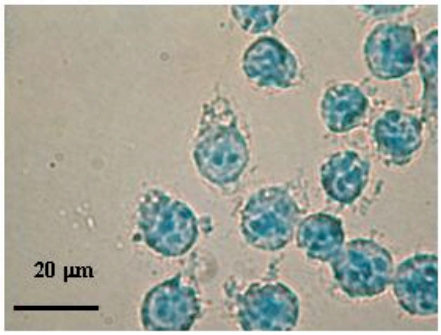


Figure 5.2.

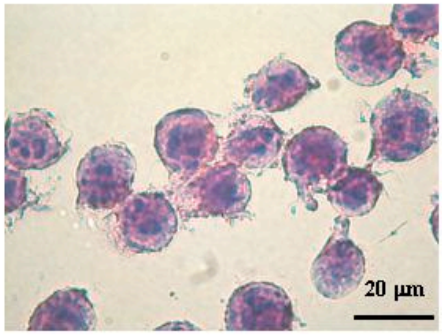


Figure 5.3.

Figure 5.1, 5.2 and 5.3. Light micrsocopic photographs of macrophage cells stained with Gimsa's solution (figure 5.1), Methyl green solution (figure 5.2) and Hematoxylin-Eosin solution (figure 5.3).

By performing the staining procedures, many of the cell organelles can be visualized and recognized. The staining solutions do not contribute any contaminants that can interfere with the measured elements, and thus SR-XRF analysis can be carried

out precisely on selected cell organelles.

The staining procedures described above were employed in order to make the cell organelles visible. Another staining procedure, the trypan blue, is used to distinguish the dead cells from the living ones. If the cells are alive, they are not stained by trypan blue solution, but if they are dead the solution enters the cells and the cells are stained blue.

For this investigation, the cells were cultured in solutions containing different concentrations of chromium chloride ($CrCl_3$), chromium oxide (CrO_3), iron chloride ($FeCl_3$) and vanadium chloride (VCl_3). The dosing solutions were prepared by dissolving $CrCl_3$ (0.04 and 0.4 g/L), CrO_3 (0.04 and 0.4 g/L), $FeCl_3$ (0.04 and 0.4 g/L) and VCl_3 (0.04 and 0.4 g/L) in the culture medium. After exposure times of 0.17, 0.5, 4, 8, 12, 24, 48 and 72 h., the cells were stained by trypan blue solution. In order to evaluate the strength of the cytotoxicity of the metallic elements, it is necessary to define a parameter: "death rate", defined as the ratio of the number of the stained cells divided by the total cells sampled. If the membrane and intracellular structures of the cell are broken and it is impossible to judge the color, the death rate is regarded as 100 %.

The results for culture in chromium chloride ($CrCl_3$) solutions (0.04 and 0.4 g/L) under different conditions are shown in table 5.1 and 5.2. In these cases, the death rate of the cells was about zero under all conditions. It is probable that the macrophage cells might be all alive in a Cr solution under these concentrations. However, it cannot be concluded that all of the macrophage cells survived in these Cr solutions due to the fact that the trypan blue dye exclusion test cannot judge the death of the cells perfectly.

Table 5.1. Rate of death in macrophage cells cultured in $CrCl_3$ solution (0.04 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	1	295	0.34
0.5	0	200	0.00
4	0	200	0.00
8	0	200	0.00
12	0	200	0.00
24	0	200	0.00
48	0	200	0.00
72	0	200	0.00

Table 5.2. Rate of death in macrophage cells cultured in $CrCl_3$ solution (0.4 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	0	200	0.00
0.5	0	200	0.00
4	0	200	0.00
8	0	200	0.00
12	0	200	0.00
24	0	200	0.00
48	0	200	0.00
72	0	200	0.00

Tables 5.3 and 5.4 show the results for culture in Cr oxide (CrO_3) solutions (0.04 and 0.4 g/L) under different conditions. In table 5.3, after 8 h. exposure, the number of the cells stained blue increased. The death rate of the cells reached 100 % after 72 h. In table 5.4, the cells started to be stained after 4 h. The death rate of the cells reached 100 % after 24 h. Therefore, it can be concluded that the cells died in shorter time and the death rate of the cells reached 100 % rapidly when the concentration of the Cr oxide solution is higher, implying that the concentration of Cr oxide solution is related to the strength of the toxicity to the cells. Furthermore, the rapid increase of the death rate in high concentration Cr solution may be explained by the phenomenon that the cell divisions can be interrupted by excessive uptake of Cr. In these cases, the cells were severely affected by the cytoxicity of Cr oxide. Compared with the results of exposure to Cr chloride, it can be concluded that the valence state (+3 or +6) of Cr in the solution is related to the toxicity to the macrophage cells.

Table 5.3. Rate of death in macrophage cells cultured in CrO_3 solution (0.04 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	3	303	0.99
0.5	0	200	0.00
4	0	200	0.00
8	54	239	22.59
12	67	220	30.45
24	147	227	64.76
48	358	423	84.63
72	200	200	100.00

Table 5.4. Rate of death in macrophage cells cultured in CrO_3 solution (0.4 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	0	200	0.00
0.5	0	200	0.00
4	28	310	9.03
8	49	270	18.15
12	29	195	14.87
24	200	200	100.00
48	200	200	100.00
72	200	200	100.00

Tables 5.5 and 5.6 show the results for culture in V chloride (VCl_3) solutions (0.04 and 0.4 g/L) under different conditions. All of the cells were alive when the dosing solution concentration was 0.04 g/L. For the 0.4 g/L concentration solution, the death rate of the cells started to increase after 12 h. Under this high dose of V solution, the cells were injured by the toxicity of V.

For the exposure to Fe chloride ($FeCl_3$) solutions (0.04 and 0.4 g/L) the results are shown in tables 5.7 and 5.8. All cells were alive when the concentration of the dosing solution was 0.04 g/L.

Table 5.5. Rate of death in macrophage cells cultured in VCl_3 solution (0.04 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	0	200	0.00
0.5	0	200	0.00
4	0	200	0.00
8	0	200	0.00
12	0	200	0.00
24	0	200	0.00
48	0	200	0.00
72	0	200	0.00

Table 5.6. Rate of death in macrophage cells cultured in VCl_3 solution (0.4 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	0	200	0.00
0.5	0	200	0.00
4	0	200	0.00
8	0	200	0.00
12	0	200	0.00
24	69	200	26.54
48	200	200	100.00
72	200	200	100.00

Table 5.7. Rate of death in macrophage cells cultured in $FeCl_3$ solution (0.04 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	0	200	0.00
0.5	0	200	0.00
4	0	200	0.00
8	0	200	0.00
12	0	200	0.00
24	0	309	0.00
48	0	200	0.00
72	0	200	0.00

Table 5.8. Rate of death in macrophage cells cultured in $FeCl_3$ solution (0.4 g/L).

Time (h)	Num. of dead cells	Num. of sample cells	Death rate (%)
0.17	2	200	1.00
0.5	3	200	1.50
4	0	200	0.00
8	13	192	6.77
12	25	200	12.50
24	0	200	0.00
48	200	200	100.00
72	200	200	100.00

The graphs of the death rate according to the exposure time are shown in figure 5.4 and 5.5. Based on the trypan blue dye exclusion test, the cytotoxicity of metallic elements varies for different metals. Furthermore, there is evidence that the valence state of the metallic element affects cytotoxicity. However, considering that the trypan blue dye exclusion test does not indicate dead or alive cells perfectly, it is probable that the cytotoxicity of metallic elements may be more complex. This necessitates application of other analytical techniques for further investigations into the intracellular phenomena at a single cell level.

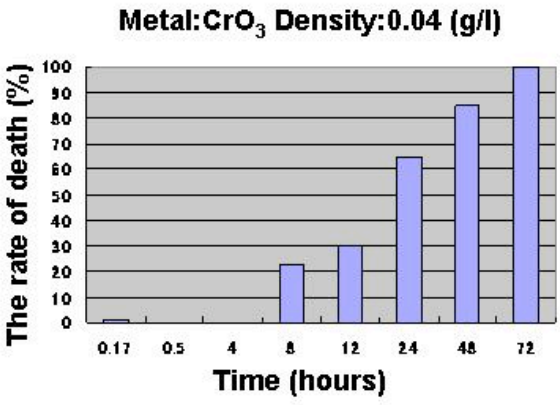


Figure 5.4

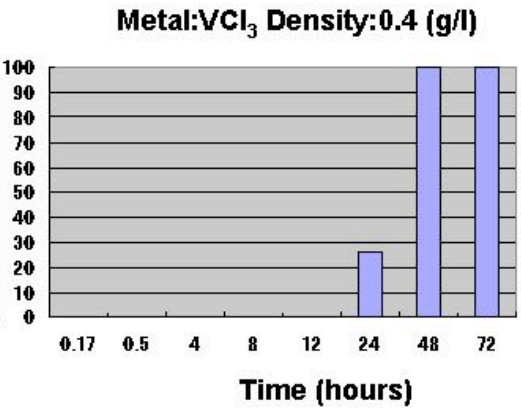


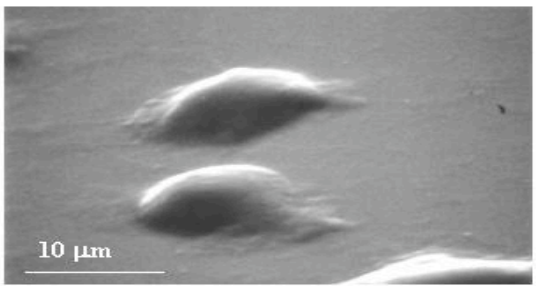
Figure 5.5

Figure 5.4 and 5.5. Graphic of the death rate vs. time in macrophage cells cultured in 0.04 g/L CrO_3 (figure 5.4) and 0.4 g/L VCl_3 (figure 5.5).

5.2.2.4. Morphological observation

When macrophage cells internalize the foreign bodies, many ruffles are observed on the surface of the cells. It is possible that the surface changes with the process of cell death due to excessive accumulation of metallic element within their bodies. In order to observe the differences of the morphological states of the single cells after the culture in the normal and metallic solution environment, SEM (scanning electron microscope) analysis was employed.

Silicon plates were employed as substrate because of their good electrical conductivity. The plates were immersed in ethanol solution and sterilized by ultraviolet ray for 24 h. Macrophage cells were cultured on these silicon plates and fixed by a formalin solution for 24 h. The fixed cells on the silicon plate were then coated with a thin layer of carbon. The selection of the fixation solution is important to visualize the fine structures on the surface of the cells clearly. For comparison, other fixing techniques were tried, namely by ethanol and methanol. The results are shown in figure 5.6, 5.7 and 5.8. When the cells were fixed by methanol or ethanol solutions, the fine structures on the surface of the cell were lost. However, with formalin the fine structure of the cell was distinct as shown in figure 5.8.



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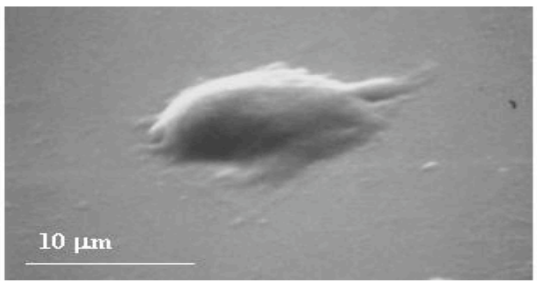

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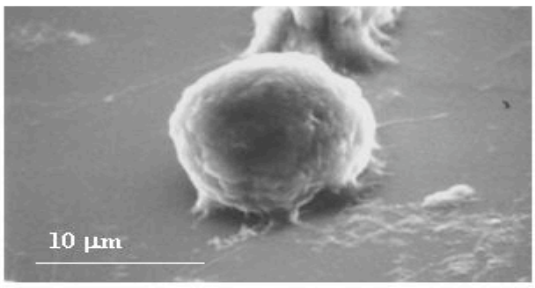


Figure 5.8

Figure 5.6, 5.7 and 5.8. In order to compare formalin fixation technique with other fixation solution, macrophages were fixed with ethanol (figure 5.6), methanol (figure 5.7) and formalin (figure 5.8). It can be seen that the fine structure of the cells was lost when the cells were fixed with ethanol or methanol. However, it is clear that the fine structure of the surface was distinct in figure 5.8

The cells cultured under normal condition are shown in figure 5.9 and 5.10. The cells cultured in a Cr chloride solution (0.4 g/L) for 1.5 h. are shown in figure 5.11 and 5.12. The cells cultured in V chloride solution (0.04 g/L) for 1.5 h. are shown in figure 5.13 and 5.14. The cells cultured in Cr oxide solution (0.04 g/L) for 4 h. are shown in figure 5.15 and 5.16. From the results of trypan blue dye exclusion test, it can be concluded that the toxicity of a Cr oxide solution is the strongest and that of the Cr chloride solution is the weakest of all solutions employed in that experiment. It can be seen that the structure on the surfaces of the cells cultured in a Cr oxide solution (0.04 g/L) for 4 h. were different from the cells cultured in the other metallic solutions. The cells shown in figures 5.15 and 5.16 may be injured within their bodies because of the unusual activities originated from the uptake of the excessive toxic metal. When some foreign bodies are internalized in macrophage cells, the ruffle structures are observed on the surface of the cells. However, in this case (figure 5.15 and 5.16), the change of the shape of the cell may be different from that of the cell internalizing some foreign bodies. The states of the surface of the cells were almost identical when the cells were cultured

in Cr chloride, V chloride for 1.5 h. and non-metallic solutions.

It can be concluded that the structure of the surface of macrophages approximately reflects the intracellular activities against foreign metal elements. Morphological observation thus will assist the investigations into cell functions and intracellular activities.

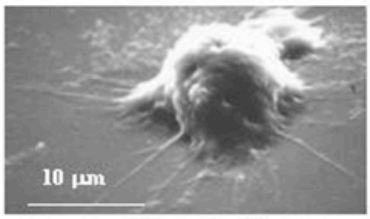


Figure 5 - 9

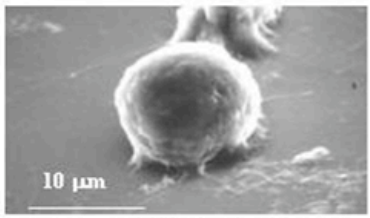


Figure 5 - 10

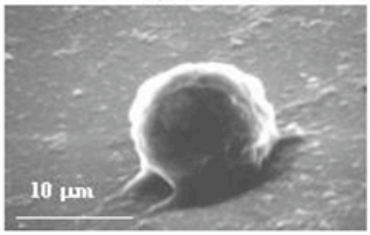


Figure 5 - 11

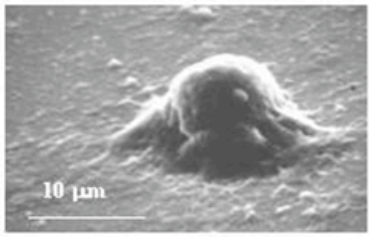


Figure 5 - 12

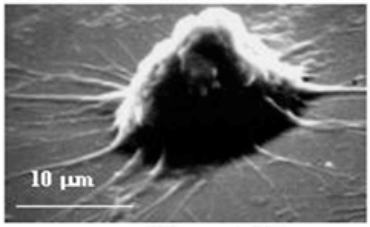


Figure 5 - 13

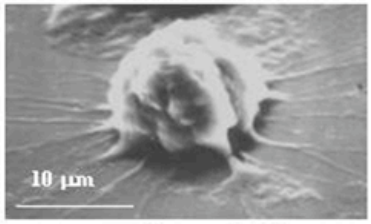
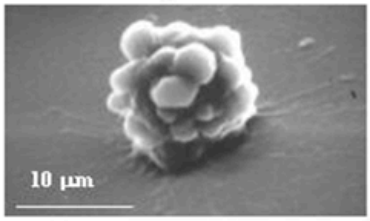


Figure 5 - 14



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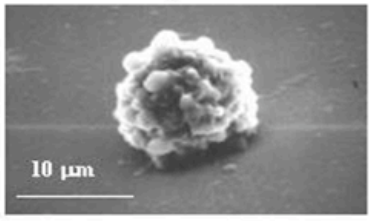

Figure 5 - 16

Figure 5.9-5.16. Figure 5.9 and 5.10 show cells cultured under normal conditions. The cells cultured in 0.4 g/L CrO_3 solution for 1.5 h. are shown in figure 5.11 and 5.12. The cells cultured in 0.04 g/L VCl_3 solution for 1.5 h. are shown in figure 5.13 and 5.14. And the cells cultured in 0.04 g/L CrO_3 solution for 4 h. are shown in figure 5.15 and 5.16.

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