# 行政院國家科學委員會專題研究計畫 期末報告

## 磁性奈米顆粒之細胞毒性評估--利用電化學偵測細胞活性法替代 MTT 法之評估

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報 告 附 件 ：出席國際會議研究心得報告及發表論文

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 102 年 03 月 18 日

中 文 摘 要 ： 磁性奈米顆粒之應用愈來愈廣泛，在生醫方面的應用，如 drug delivery, cellular labeling/cell separation, tissue repair, magnetic resonance imaging 等是大家所熟悉習的, 但這些應用涉及人體或動物, 奈米顆粒對人類或週遭環境是否有毒性或其他影響, 無論有害或無害均應加以探討, 以免重蹈基因改質食品的後路, 引起大眾的恐慌。. 細胞實驗(cell-based assay) 是目前探討物質毒性或生物相容性使用最廣泛的實驗模式(model), -而 in-vitro 的實驗結果與 in-vivo 未必一致, 導致錯誤結論的因素除了不可避免的動物體代謝與細胞培養代謝差異, 尚有一些事實驗藥品上的問題, 如奈米物質可能干擾一般用來測細胞活性(cell viability) 或粒線體功能 (mitochondrial function)的染劑信號之讀取。

本研究利用傳統生化法分析磁性奈米顆粒對酵母菌的影響除了比較 MTT assay 的結果, 也利用 ATP assay 分析磁性奈米顆粒是否進入細胞造成細胞膜破損，結果顯示在兩個小時的暴露時間，10/20 nm 對酵母菌增生有抑制現象，但呼吸代謝增強， ATP 的結果顯示酵母細胞壁沒破損。亦利用電化學方法偵測肝細胞之存活度, 評估於磁性奈米顆粒存在下電化方法替代 MTT assay 偵測細胞活性之可行性。結果顯示 Fe304@polyacrylamide (40/80 nm) 對肝細胞無顯著影響.

中文關鍵詞： 磁性奈米顆粒，細胞毒性， 肝細胞，電化學分析

英 文 摘 要 ： Biomedical application of magnetic nanoparticles such as drug delivery, cellular labeling or cell separation, tissue repair, and magnetic resonance imaging are well known. Since these applications involve use of humans or other animals, it is better safe than sorry. It is important to study their toxicity to humans and environmental.

Cell-based assay is currently major used for all nanotoxicological research, however, data obtained from in vitro experiments could be misleading for a variety reasons, such as the metabolic difference of in-vivo comparing with in-vitro, and certain nanomaterial may interfere with read-out systems of commonly used MTT assays for cell viability and/or mitochondrial function.

This study developed electrochemical method for assessment of cell viability. In order to check the

accuracy of electrochemical detection, more than one assay was done for determining risks of the magnetic nanoparticles, such as the classical dye (MTT) assay, ATP assay for membrane leakage. We evaluated the respiratory effect of several kinds of magnetic nanoparticles on yeasts, and Fe3O4@polyacrylamide magnetic nanoparticles on liver cells using both 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) viability assay and the chronoamperometric method, based on screen-printed carbon electrodes (SPE). Preliminary results show that nanoparticles have no significant effect on the respiration of rat liver cells from these two detection methods. The chronoamperometric method using SPE shows that the current responses are proportional to the ferrocyanide concentrations, with a linear range of 0.03~ 1.0 mM (R2=0.9923), and detection limit (S/N=3) at 32 贡M. The results of paired t-test analysis indicate that assessment of hepatocyte viabilities based on the chronoamperometric method were comparable to those of the MTT viability assay. The chronoamperometric method can be used as a quick alternative method for assessing liver-cell viability.

英文關鍵詞： magnetic nanoparticles, cell toxicity, liver cells, electrochemical detection.

三、報告內容：包括前言、研究目的、文獻探討、研究方法、結果與討論（含結論與建議）等

**前言**

磁性奈米顆粒之應用愈來愈廣泛，在生醫方面的應用，如 drug delivery, cellular labeling/cell separation, tissue repair, magnetic resonance imaging 等是大家所熟悉習的, 但這些應用涉及人體或動物, 奈米顆粒對人類或週遭環境是否有毒性或其他影響, 無論有害或無害均應加以探討, 以免重蹈基因改質食品的後路, 引起大眾的恐慌。.

細胞實驗(cell-based assay) 是目前探討物質毒性或生物相容性使用最廣泛的實驗模式(model), -而 in-vitro 的實驗結果與 in-vivo 未必一致, 導致錯誤結論的因素除了不可避免的動物體代謝與細胞培養代謝差異, 尚有一些事實驗藥品上的問題, 如奈米物質可能干擾一般用來測細胞活性(cell viability) 或粒線體功能 (mitochondrial function)的染劑信號之讀取【1~3】。Monteiro-Riviere 等人【2】的研究發現傳統使用之染劑如 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) 因奈米物質吸附此染劑或粒線體代謝之產物而造成錯誤之評估。 Laaksonen 等人【3】則報告因自發性的氧化還原反應, MTT 無法用來測 porous silica microparticles 的毒性, 因在顆粒表面 MTT 會被還原。**本實驗分兩部分 ( 1) 利用傳統生化方法評估奈米磁性顆粒對酵母菌之生長影響 (2)**應用 99 學年度所開發之赤血鹽(ferricyanide)/succinate 雙介質系統測試奈米磁性顆粒對肝細胞活性之影響， 所以**研究目的、文獻探討、研究方法及結果與討論差異性大，將分兩部分撰寫**

## Part I: 探討奈米磁性顆粒對酵母菌之生長影響

### (一)、 目的: 以酵母菌為實驗模式，探討磁性奈米顆粒是否對環境或人類造成危害

### (二)、 文獻探討

奈米磁性顆粒於各領域應用範疇越趨廣大[4,5,6]，其應用性將因其尺寸、官能基類型、載體以及外層的包覆物有所區別，在此實驗中，皆使用 $Fe_3O_4$ 為載體、化學法合成之奈米顆粒進行探討。在過去研究中發現，奈米粒子會由呼吸、攝食等方式進入生物體中，對組織器官產生毒性，某些金屬類奈米微粒在真菌類試驗中有明顯細胞毒性(cytotoxicity)及基因毒性，使細胞產生氧化壓力(ROS ，relative oxygen species) ，將導致粒線體失去功能，DNA 損傷(DNA damage)及突變(DNA mutation)並造成ＤＮＡ斷裂，最終導致細胞死亡[7,8]。若實際應用於動物細胞、甚至人體上，奈米磁性顆粒是否會產生相同毒性尚不確定，本研究用釀酒酵母菌(yeast)為試驗模式(model)，酵母菌與同爲真核生物的動物和植物細胞具有很多相同的結構及相似之轉譯後修飾作用，具有個體小、代謝快速、容易繁殖等特性，所以酵母菌常被用作研究真核生物的模式生物，遺傳學及分子生物學的研究上，也常用它作為材料。

**酵母菌**英文名稱為 saccharomycete，學名 saccharomyces cerevisiae，是一群圓形或橢圓形的單細胞，為具有細胞核的真核生物，酵母菌在自然界中的分佈非常廣泛，從天寒地凍的極區經溫帶到熱帶的沙漠都可以發現它們的蹤跡。釀酒酵母菌(yeast)是卵形的單細胞個體。具有細胞壁、細胞膜、細胞質和細胞核，細胞質內部有明顯液泡，因其為真菌類，不具葉綠素無法行光合作用，必須利用分解有機質產生的能量來生長。。酵母菌之細胞壁常含甘露糖，喜在含糖量高、酸度較大的水生環境中生長。

酵母菌的生殖方式有無性生殖與有性生殖二種，無性生殖包括出芽生殖與分裂生殖,大多以出芽生殖(budding)的方式來繁殖,細胞直徑大小約 10μm。在良好的營養和生長條件下，酵母生長迅速，細胞上都長有芽體(Figure 1)，在芽體上還可形成新的芽體，以致經常可見到呈簇狀的細胞團。而芽體形成過程為：母細胞形成芽體的部位，由於水解酵素對細胞壁多糖的分解，使細胞壁變薄、大量新的細胞物質---核物質（染色體）和細胞質等在芽體起始部位上堆積，使芽體逐步長大，當芽體達到最大體積時，與母細胞相連部位形成了一塊隔壁，成分是由葡聚糖、甘露聚糖和幾丁質構成的複合物。最後，母細胞與子細胞在隔壁處分離,於是在母細胞處就留下一個芽痕（bud scar），而在細胞上就相應地留下一個蒂痕（birth scar）。

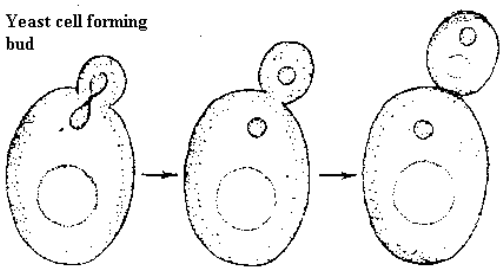



Figure 1. 酵母菌出芽生殖示意圖

在酸度方面,酵母菌能在 pH 值為 3.0-7.5 的範圍內生長,最適合的 pH 為 4.5-5.0。適合之生長溫度在 20℃-30℃,在低於 0℃或者高於 47℃的溫度下,一般酵母细胞無法生長。此外,酵母菌於有氧和無氧環境中都能存活,意即酵母菌是兼性厭氧菌,兼性厭氧型這一類生物在氧氣充足的條件下進行有氧呼吸,將有機物徹底的分解為二氧化碳、大量水和 ATP 以發酵獲得能量;在缺氧或沒有其他體外電子接受者的情形下會將有機物不徹底的分解為乳酸或酒精、少量水和 ATP,亦即利用有機物本身同時作為電子供給者與接受者,以進行氧化還原反應釋放能量。

**(三)、 研究方法:**

本研究從三方面觀察奈米顆粒對酵母菌活性之影響,包括細胞增生量(皆以 biomass 表示)、呼吸代謝活性、ATP 含量,分別針對不同的 marker 及催化酵素進行探討。

**1. 本研究所用之磁性顆粒種類:**

(1) $Fe_3O_4$@$SiO_2$-$NH_2$ (200/400nm)

中心載體為赤鐵礦($Fe_3O_4$),@為包覆(coating 之意),直徑為 200nm,外層包覆厚度為 200nm 之 silica,並接上 $NH_2$ 之官能基,厚度為 400nm。

(2) $Fe_3O_4$@polymer/-$NH_2$ (300nm)

其中 polymer 是指由數十個或數百個單體所聚合而成的物質,而單體則是指化合物例如氯乙烯,乙烯.....等多種有機物質都可當成單體。

(3) $Fe_3O_4$@PAM-$NH_2$(40/80nm)

PAM 為 polyacrylamide(聚丙烯醯胺)之縮寫,結構式如 Figure 2 所示,中心載體為赤鐵礦($Fe_3O_4$),直徑為 40nm,外層包覆厚度為 20nm 之 PAM,並接 $NH_2$ 之官能基,整個磁性顆粒厚度為 80nm。

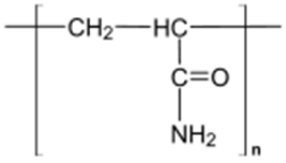


Figure 2 .polyacrylamide 之結構式(來源:維基百科)

(4) $Fe_3O_4$@$SiO_2$(40/100nm)

中心載體為赤鐵礦($Fe_3O_4$)，直徑為 40nm，外層包覆厚度為 60nm 之 silica，厚度為 100nm。

(5) $Fe_3O_4$@$SiO_2$(10/20nm)

中心載體為赤鐵礦($Fe_3O_4$)，直徑為 10nm，外層包覆厚度為 10nm 之 silica，厚度為 20nm。

**2. 細胞活性偵測方法**

實驗中主要目的為希望藉由各活性方法評估磁性奈米顆粒對於酵母菌細胞生長影響，並進一步推測顆粒是否具毒化作用。因此正確的細胞分析法是相對重要的，試劑偵測的時間點、反應的特性、試劑量與化合物處理時間皆是考慮的因素，利用試劑的特性及依偵測的目標marker不同[9]，進一步了解作用的機制以及是否產生毒化作用抑或是產生未知的現象。

偵測化合物對於細胞生長分析大致可分為兩種，細胞存活/增生(cell viability/proliferation)與細胞毒性(cytotoxicity)分析。細胞存活及細胞增生分析為偵測經某些物質處理後的細胞生長或數量增加的情形，主要是偵測健康、活的細胞，亦是在本實驗中主要用來探討細胞活性的所使用的方法; 而細胞毒性分析，則主要偵測物質對於細胞所產生的負面影響，例如細胞死亡，主要是偵測細胞膜的破損，可使用一些非細胞浸透性染劑偵測流失到培養液中的細胞內容物來分析細胞毒性，兩種分析方法皆為具挑戰性的工作。以下將介紹各方法之相關原理與偵測標的:

**(1)光學密度(Optical Density，OD)**

根據 Lambert-Beer 定律，入射光及穿透光具有下列關係:

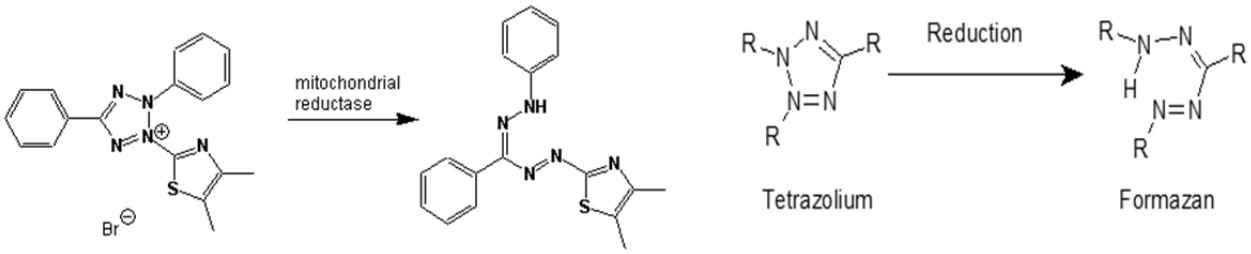
$OD = -\log(I/I_0)$ ( $I_0$：入射光； I：穿透光)

利用 OD 探討酵母菌數量的過程中，當入射光通過細胞溶液時，光線將被溶液中的酵母菌顆粒散射掉，導致穿透光減弱，這個特性呼應了 Lambert-Beer 定律中所提及:「光線進入溶液後被部分介質吸收，導致穿透光減弱」,兩者共通點為光線皆因某種因素減弱，探討穿透光及入射光兩者關係可用於物質定量分析。細菌數目多寡和混濁度成正比，當入射光($I_0$)通過時，被酵母菌散射的光越多，穿透光(I)強度越弱，OD 值相對就越大，可藉此方法了解生長情況及細胞總數。

**(2) MTT assay**

MTT〔3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide〕，透過快速簡便的顏色反應來檢測細胞代謝轉換率，活細胞粒線體內膜中的琥珀酸去氫酶(Succinate dehydrogenase)能夠把MTT結構中tetrazolium代謝還原成藍紫色且不溶於水的formazan，Figure 3為其反應示意圖，接著藉由DMSO幫助formazan溶解，最大吸收在波長570nm。由結果可進一步得知粒線體活性，如果琥珀酸去氫酶還原能力強，表示克氏循環速度很快，可產生較多能量供細胞代謝。由於死細胞的粒線體中不含有琥珀酸去氫酶，所

以MTT assay測結果與活細胞數量或細胞呼吸代謝活性呈正比。




(a) (b)

Figure 3 (a).MTT 試驗作用機制 **(b)** MTT 主要作用部位

### (3) PrestoBlue™ Cell Viability assay

PrestoBlue™ Cell Viability為一均質螢光細胞存活分析試劑、不需前處理。試劑中含有resazurin(alamar blue)偵測細胞的代謝能力(NADH)。細胞中主要代謝途徑都涉及氧化還原反應，其中NADH / NAD的氧化還原態被認為是細胞新陳代謝的控制因子，NAD(nicotinamide adenine dinucleotide)是另一能量儲存之所在，其能量是以還原型態NADH來儲存。當$NAD^+$與一氫原子結合，即形成NADH(Figure 4)。使用深藍色、低螢光性的resazurin受活細胞反應後還原產生粉紅色、高螢光性的resorufin產物，反映出活細胞的代謝能力；死細胞由於喪失了代謝能力，無法將resazurin還原產生螢光。[10,11,12]

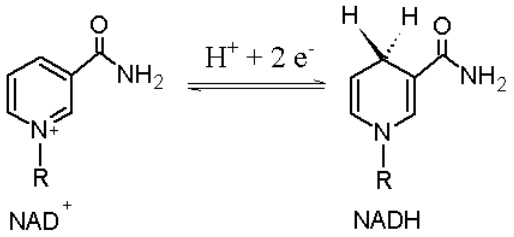
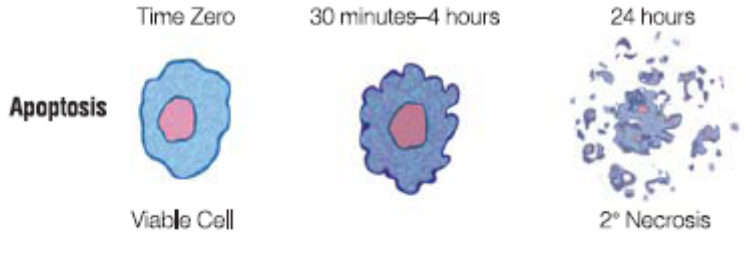


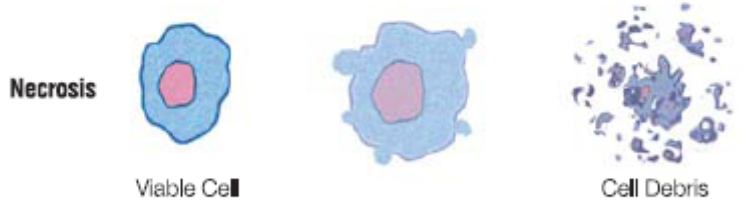

Figure 4. NADH 之氧化態與還原態結構

### (4) BacTiter-Glo® Luminscent cell Viability Assay

BacTiter-Glo™ 是一個均質冷光細菌細胞存活分析試劑。利用冷光酶的化學反應原理偵測活細菌細胞的 ATP，ATP 是極靈敏的細胞存活 marker，為含高能量的磷酸化合物，是儲存能量的地方，因為有太多電荷彼此互相排斥，造成分子的不穩定，當磷酸根之間的鍵結被打斷，能量就被釋放出來。1 莫耳的 ATP 可以產生約 8 仟卡的能量。在細胞破損的數分鐘內，細胞會停止合成 ATP，ATP 會急速降解，且內生的 ATPase 將分解所有殘留的 ATP。BacTiter-Glo® Luminscent Cell Viability Assay 用於偵測培養中的活細胞數。利用冷光酶反應檢視活細胞中的 ATP[13,14]。ATP 含量變化與細胞毒化死亡關係如下表。




| | | | |
|---|---|---|---|
| LDH Release | 0 | + | +++ |
| Caspase | 0 | +++ | ++ |
| ATP | +++ | ++ | 0 |
| MTS | +++ | ++ | 0 |
| Resazurin | +++ | ++ | 0 |




| | | | |
|---|---|---|---|
| LDH Release | 0 | +++ | ++ |
| Caspase | 0 | 0 | 0 |
| ATP | +++ | 0 | 0 |
| MTS | +++ | 0 | 0 |
| Resazurin | +++ | 0 | 0 |

BacTiter-Glo®試劑含可打破細胞壁之藥劑，使細胞釋出 ATP 同時抑制內生的 ATPase，提供 luciferase 催化 luciferin 與 ATP 反應快速產生冷光，產光量與 ATP 量呈正比。此試劑操作簡單，只需加入細胞，反應 5~7 分鐘，即可偵測冷光數值，靈敏度高，10 顆細胞即可偵測，並已證實可用於偵測多種細菌與酵母菌。傳統的 ATP 分析試劑需要細胞裂解(cell lysis)後使用 luciferase 試劑分析。當 cell lysis 步驟時 ATP 降解快速，將造成數據的變異性提高，而此實驗中使用之試劑由於操作不需製備 cell lysate，直接將試劑加入 culture cell 中，cell lysis 與 ATP 反應同時進行，故可減少分析過程中造成 ATP 降解的誤差，提升了數據的再現性與準確度[14,15]。Figure 5 為 ATP 冷光試劑之作用機制，首先 luciferin 經由鎂離子和 luciferase 的催化與 ATP 反應，脫去一分子水與一分子焦磷酸，形成 Luciferyl-AMP。接著又因 luciferase 的催化，$C_4$ 上的質子釋出，形成 lone pair，此 lone pair 又與氧氣形成配位鍵。帶負電之遠端氧原子亦與電負度較大的碳原子結合，即形成不穩定的四圓環構造，脫去 AMP 以及一分子的二氧化碳後就生成氧化態的 oxyluciferin，當激發態的 oxyluciferin 回到 ground state 時，就會發光。

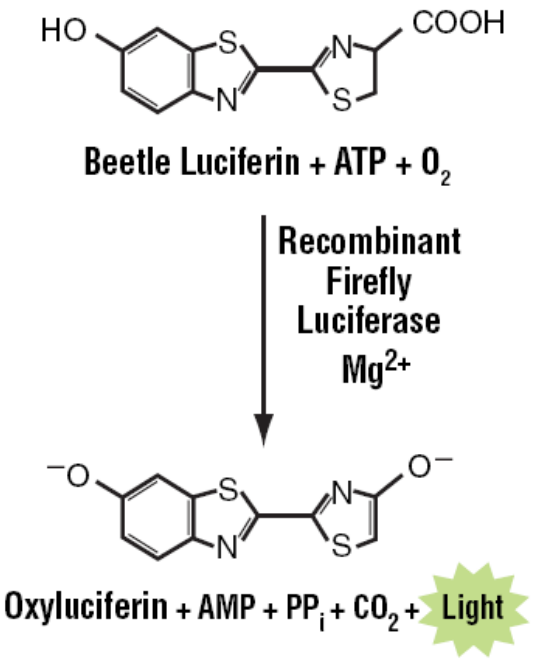



Figure 5.ATP 冷光試劑之作用機制

**(5)細胞計數法:**

直接計數是利用不同的計數器或載玻片（有界線或一般載玻片）在顯微鏡下直接觀察，利用血球計數器直接計數微生物細胞的總數量。計數器上凹槽上每一區域有九大格，其中共分為 25 個中方格（5×5），每一中方格再細分 16 小方格（4×4），1 大格等於 400 小格(5×5×4×4) 如 Figure 6），每大格之長寬各為 1mm，蓋上蓋玻片後液體高度為 0.1mm，總計每大格之液體體積為 $0.1mm^3=10^{-4}cm^3=10^{-4}mL$。計數時將菌液充滿計數器凹槽中，接著以顯微鏡觀察計算 100 小格內菌體數量，如圖，共計算計數盤中反黑的五大區域，並算出平均每小格的酵母菌數 A。最後每毫升總菌數=A×400(共 400 小格)× $10^4$×D(稀釋倍數) 。

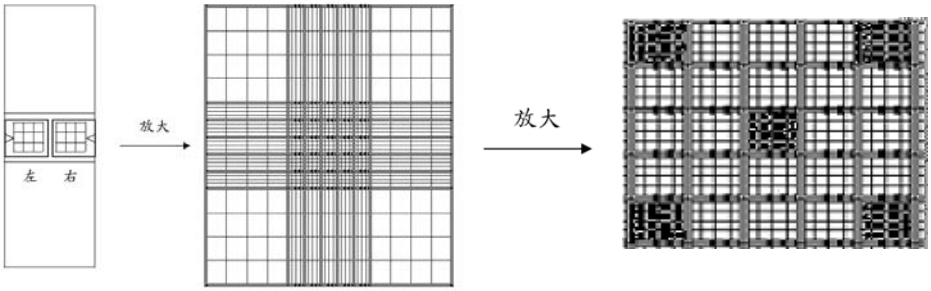
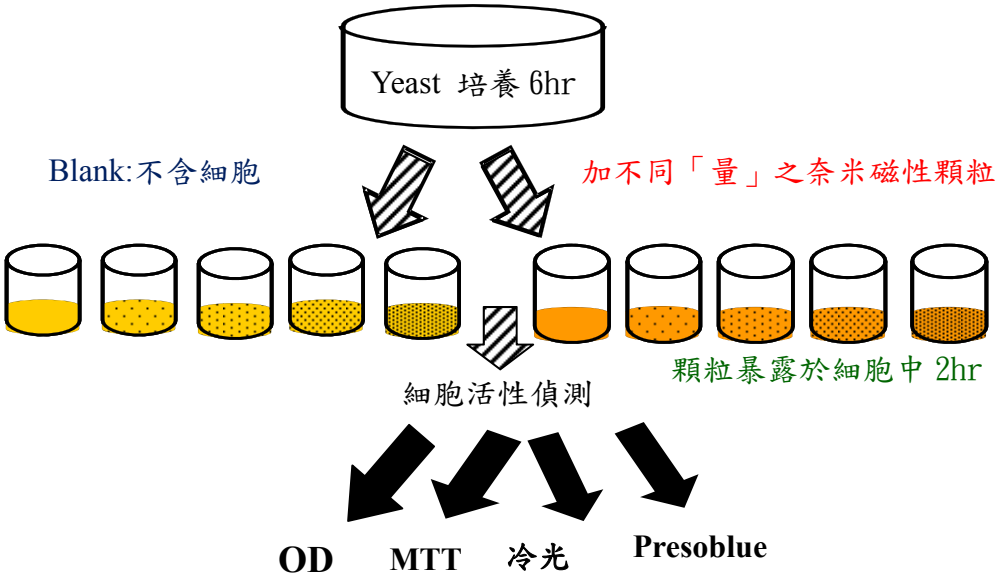
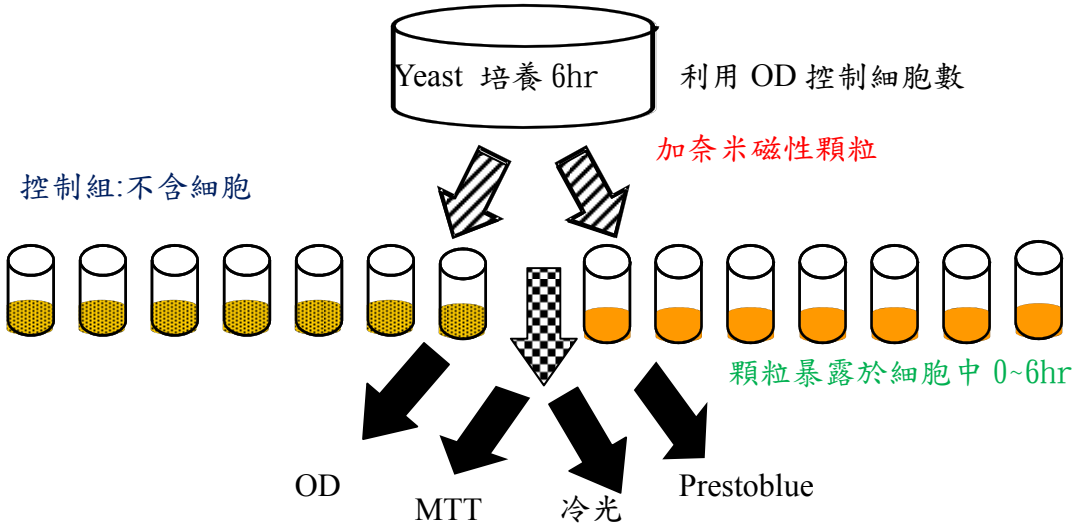



Figure 6.血球計數器細部劃分情形

**( 6) 磁性顆粒「量」對細胞活性影響**




**(7) 暴露於磁性顆粒的「時間」對細胞活性影響**



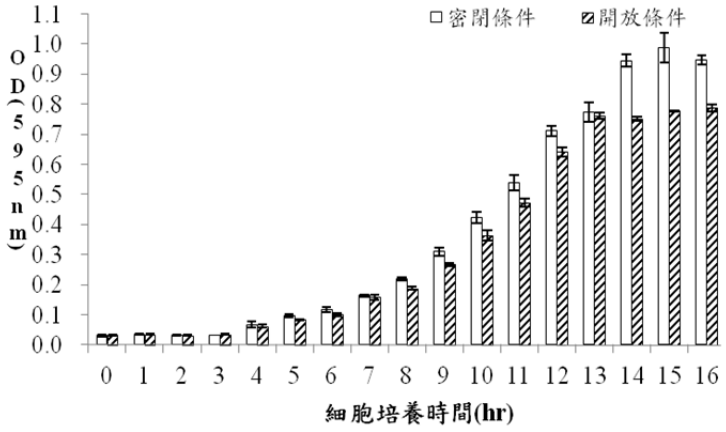

**(四) 結果與討論**

**4-1 探討「生長環境」對酵母菌生長情況影響**

實驗中所使用之培養基為 Malt Extract $R_2$(麥芽萃取物)，內含有還原醣成分，測量之 pH 值平均為 4.9，為低蛋白培養基。影響細胞生長有許多種因素，包括外在環境、培養基、溫度、培養方法…等，在探討磁性顆粒對於細胞活性影響前，觀察酵母菌細胞基本的生長曲線是必要的，了解其生態變化及影響生長之因素，將會更容易掌控實驗變因。

Figure 7 為酵母菌細胞生長的最適化條件及影響其生長之變因探討結

果，酵母菌喜好的生長溫度為 30℃，觀察同樣為平面 shaking、30℃培養箱進行「密閉」及「開放」環境之酵母菌生長情形觀察，在 OD 及 MTT 兩種活性偵測方法結果下可得知在氧氣較稀疏的密閉情況下，酵母菌生長較為快速，隨時間增長可明顯觀察到酵母菌積累於試管底部，且會產生發酵的氣味;於開放環境、氧氣充裕的環境下反而生長較緩慢，這個結果呼應前半部原理中提及之酵母菌為兼氧厭氧性細菌之一的特性，重點是在開放環境下，培養基容易被蒸發，導致生長期間細胞濃度失調，增加變因。由於目的是尋找細胞生長之最適化條件，避免未知因素干擾，往後酵母菌培養皆於平面 shaking、30℃、密閉系統下進行。




(a)

(b)

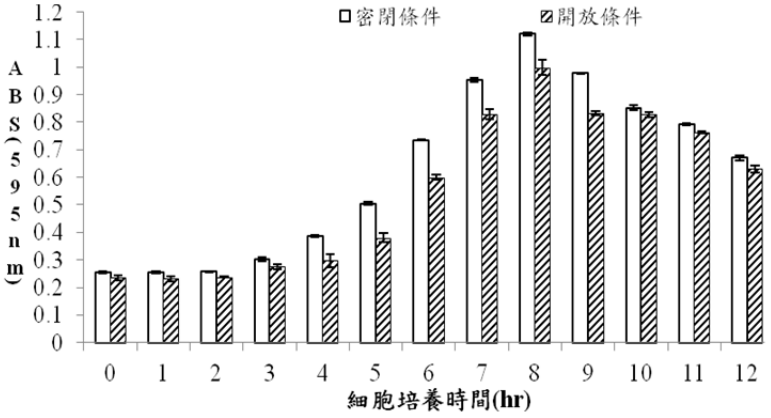



Figure 7.(a)OD 法偵測密閉與開放環境下之酵母菌生長情況
(b)MTT 試驗偵測密閉與開放環境下之酵母菌生長情況

Figure 8 為培養方法對酵母菌生長之影響，結果皆顯示平面 shaking、轉速 200rpm 條件下生長結果最佳，其中 shaking 及 rotating 方法下，細胞間的碰撞機會較多，生長情況較好;靜置培養可能因細胞沉降堆疊生長較緩慢。開始生長的 0 到 4 小時，不同培養方法對酵母菌的生長是沒有顯著影響的($P>0.05$)，但約 5 小時後，細胞開始大量分裂，不同方法培養對細胞的生長產生了顯著的影響($P<0.05$)。而細胞類型大致可分為三類: 活細胞、失活細胞與死細胞，活細胞具有完整細胞壁且可行呼吸作用;失活細胞指仍有完整之細胞壁但無法行呼吸作用;死細胞指細胞壁遭破壞且無法行呼吸作用。

可發現生長約 10 小時後 OD 值是隨時間而漸漸趨平，但 MTT 法之吸收則呈下降趨勢，原因為培養過程並無更換培養基，酵母菌會因養分不足失去活性，由原理得知，OD 偵測的是完整的 particle，只要細胞壁無遭受破壞，就仍是一個完整的 particle、依舊可測得其散光，所以此方法無法分辨活細胞與失活細胞，才會有趨平現象，

MTT 法則是利用活細胞粒線體中獨有的琥珀酸去氫酶(SDH)和 MTT 轉換，且細胞加入 MTT 後仍繼續培養 4 小時，對照其下降趨勢之結果得知細胞代謝轉換率隨時間降低，應是無更換培養基導致細胞所需養分不足，導致細胞代謝活性降低。

**4-2 探討各種奈米磁性顆粒「暴露時間」對酵母菌之影響**

分別於靜置、旋轉、平面 shaking 進行探討，由此可知磁性顆粒作用是否會因外力干擾而影響。為避免開放系統下可能導致培養基蒸發等現象產生濃度改變之干擾，將統一於密閉系統下操作。

**4.2.1 靜置 shaking 培養，培養箱溫度 30℃**

探討的顆粒包括 $Fe_3O_4$@$SiO_2$-$NH_2$(200/400nm)、$Fe_3O_4$/polymer/$NH_2$(300nm)，酵母菌生長曲線於培養後 0~4 小時生長情況不明顯，於此期間加藥，即使有影響效果也不容易觀察到，甚至可能被誤認為人為誤差。第 5~6 個小時開始快速分裂，進入對數期(log phase)，細胞數幾乎呈倍數增加，此時間點加入顆粒將使作用效果更明顯。0 小時為不加顆粒，由此可了解初始點是否相同。藉 OD 法觀察其生長趨勢，但並無法充分說明顆粒是否造成細胞死亡亦或失去活性，所以利用 MTT 法輔助結果。

同時作一為不含顆粒之控制組，了解無藥物作用下細胞的生長趨勢。其中 OD 為即時偵測，以 relative biomass yield 呈現。而 MTT 於加入試劑後需等待 3 至 4 小時方能進行偵測，以 relative MTT converting 呈現，由兩種方法互相比較評估顆粒之影響。

$$\text{relative biomass yield}=\frac{\text{Exp(實驗組)yeast 增生率}}{\text{Control (控制組)yeast 增生率}}$$

$$\text{relative MTT converting (ie. 單位細胞對 MTT 之轉換率)}=\frac{\text{Exp 單位細胞轉換率（MTT/OD)}}{\text{Control 單位細胞轉換率（MTT/OD)}}$$

由結果(figure 9)得知 $Fe_3O_4$@$SiO_2$-$NH_2$(400nm)在加藥 1 hr 後快速增生，但呼吸代謝活性減弱。$Fe_3O_4$/polymer/$NH_2$(300nm)對酵母菌之增生沒有影響，但對呼吸代謝活性有明顯的抑制作用。因兩者顆粒尺寸均大於 100nm，不屬奈米級，往後探討將著重奈米級磁性顆粒的部分。

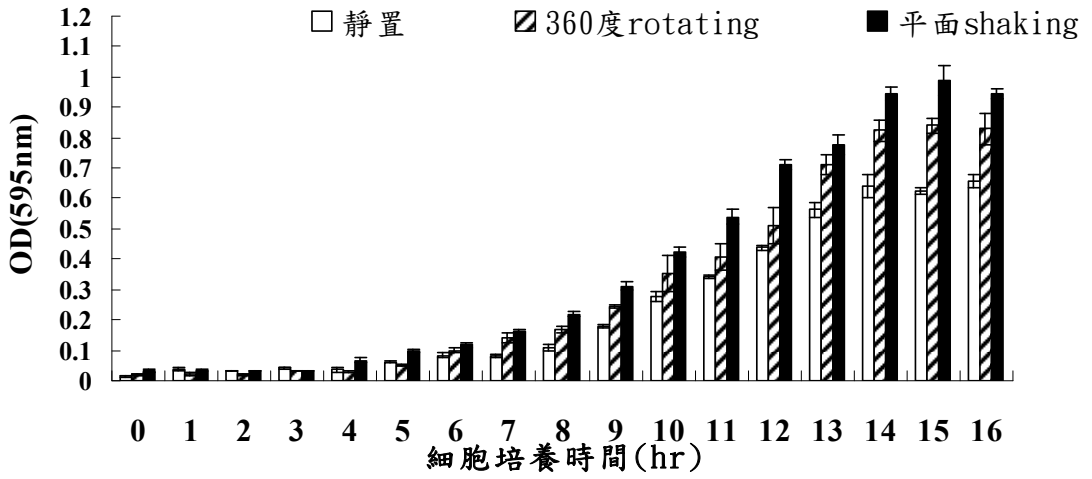
**4-2.2 密閉條件下 360°旋轉培養、rotator 轉速為 12rpm、溫度 30℃**

於此狀態下觀察活性影響作用的包括 $Fe_3O_4$@PAM-$NH_2$(40/80nm) $Fe_3O_4$/polymer/$NH_2$(300nm)、$Fe_3O_4$@$SiO_2$-$NH_2$(200/400nm)及 $Fe_3O_4$@$SiO_2$ (10/20nm)。Figure 10 結果顯 $Fe_3O_4$@$SiO_2$ (10/20nm)於 MTT 結果的部分，明顯於加藥後第 2 個小時活細胞數目大量減少，對照 OD 結果細胞總數仍有增加狀態，意即死細胞數目增加，但第 3 個小時候活細胞數目反而隨時間增加，整體趨勢為先降後升，往後將針對此現象詳細探討。

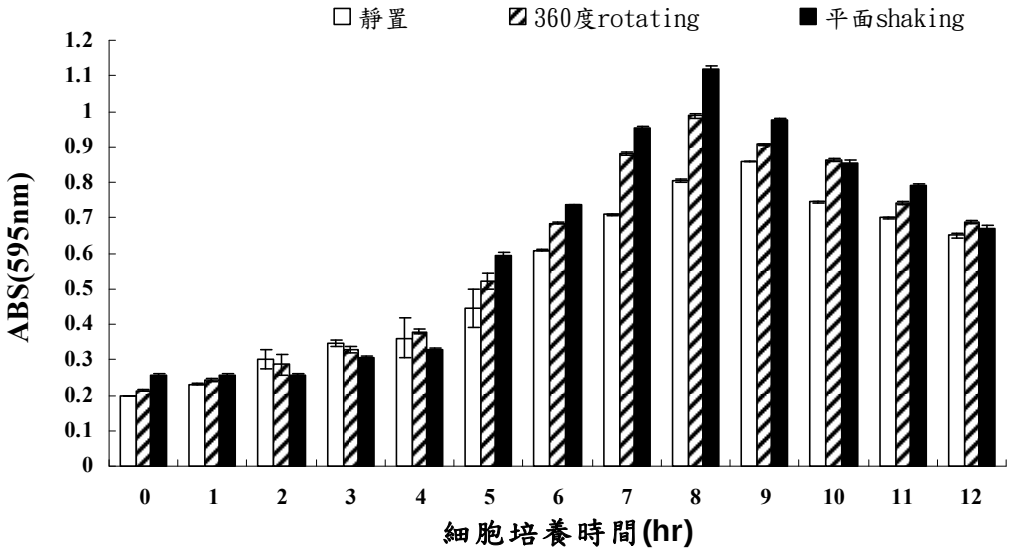
$Fe_3O_4$@PAM-$NH_2$(40/80nm)之 MTT 結果亦呈先降後升趨勢。反觀與 $Fe_3O_4$@$SiO_2$-$NH_2$(200/400nm)尺寸較大顆粒作用促使酵母菌生長，與靜置培養之結果相似。$Fe_3O_4$@$SiO_2$-$NH_2$(200/400nm)為包覆較厚之顆粒，於 OD 幾乎呈持平狀態，趨近 100%，而尺寸大的顆粒於 MTT 試驗結果皆無明顯規律。初步推斷可能原因為:尺寸小之顆粒易進入細胞中與之產生作用，進一步影響細胞活性。大顆粒不易分散，短時間內聚集並沉降於底部，減少細胞碰撞機會。

**4-2.3 平面 shaking 培養，轉速為 200rpm，培養箱溫度 30℃**

於此狀態下觀察活性影響作用的顆粒包括 $Fe_3O_4$@PAM-$NH_2$(40/80nm) $Fe_3O_4$/polymer/$NH_2$(300nm)、$Fe_3O_4$@$SiO_2$- (40/100nm) $Fe_3O_4$@$SiO_2$ (10/20nm)。 Figure 11 顯示 $Fe_3O_4$@PAM-$NH_2$(40/80nm)較特別的部份仍為 MTT 結果，明顯於加藥後第 2~3 個小時細胞活性減少，第 5 個小時後反而隨時間而增加。$Fe_3O_4$@$SiO_2$(40/100nm)之細胞總數隨時間增加，其 MTT 則呈下降趨勢，相同點為其趨勢皆漸漸減緩，由此判斷，過程中細胞總數增加，但細胞活性隨時間減少，即死細胞或失去活性之細胞增加。 $Fe_3O_4$/polymer/$NH_2$(300nm)之 OD 及 MTT 結果幾乎呈持平狀態，初步評估此培養方法下，$Fe_3O_4$/polymer/$NH_2$(300nm)對於磁性顆粒並無毒性影響。 $Fe_3O_4$@$SiO_2$(10/20nm)之 OD 結果明顯的隨時間大幅下降，但其 MTT 試驗表示與顆粒作用後，生長相對較佳，與 $Fe_3O_4$@$SiO_2$(40/100nm)有很相似的影響結果。

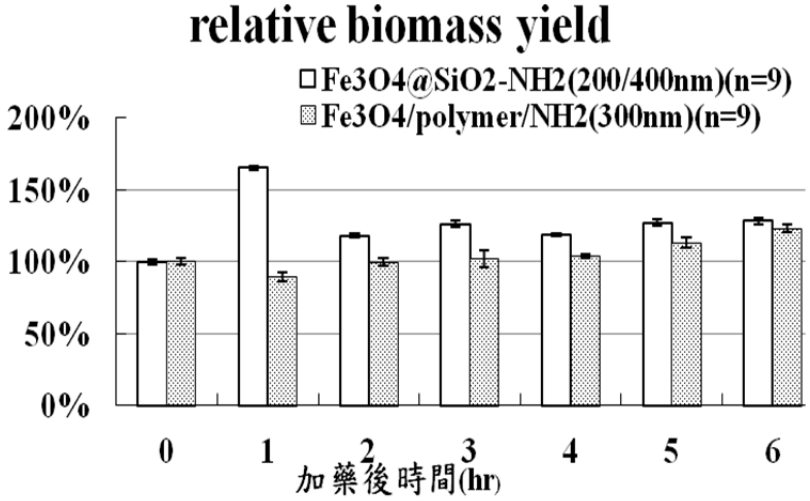



(a)

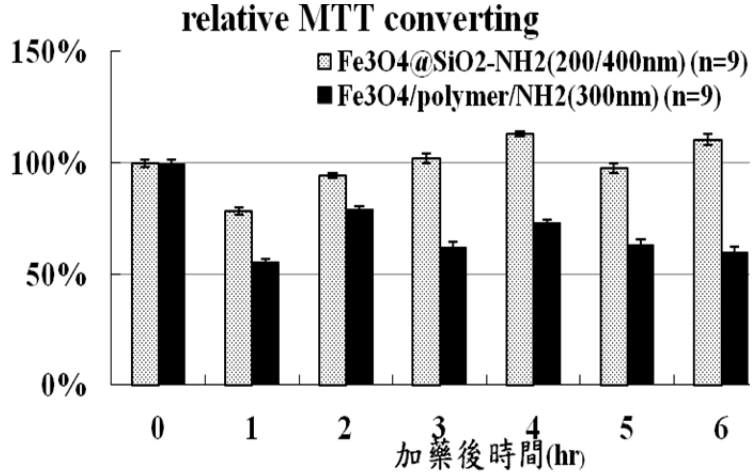



(b)

Figure 8. (a)OD 觀測不同外力培養方法下之酵母菌生長曲線
(b)MTT 法觀測不同外力培養方法下之酵母菌生長曲線

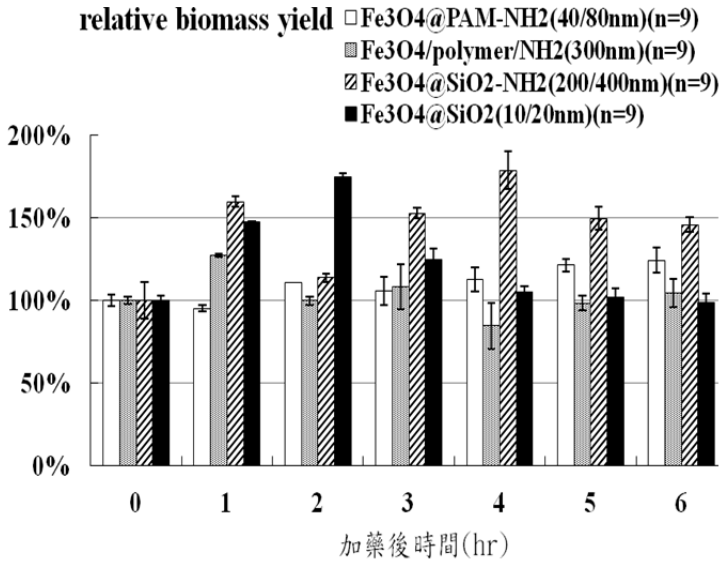



(a)

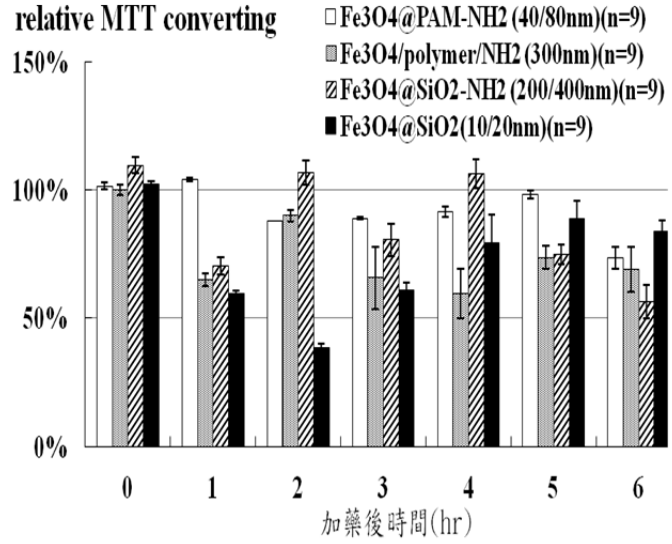



(b)

Figure 9.分別以 OD 及 MTT 觀察各時間點下 $Fe_3O_4$@$SiO_2$-$NH_2$(200/400nm) 、$Fe_3O_4$/polymer/$NH_2$(300nm) 對酵母菌生長影響，靜置 shaking 培養，培養箱溫度 30℃。

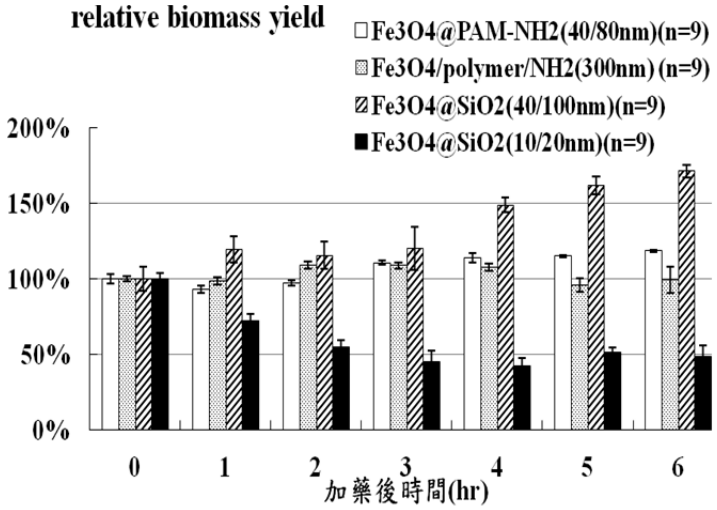



(a)

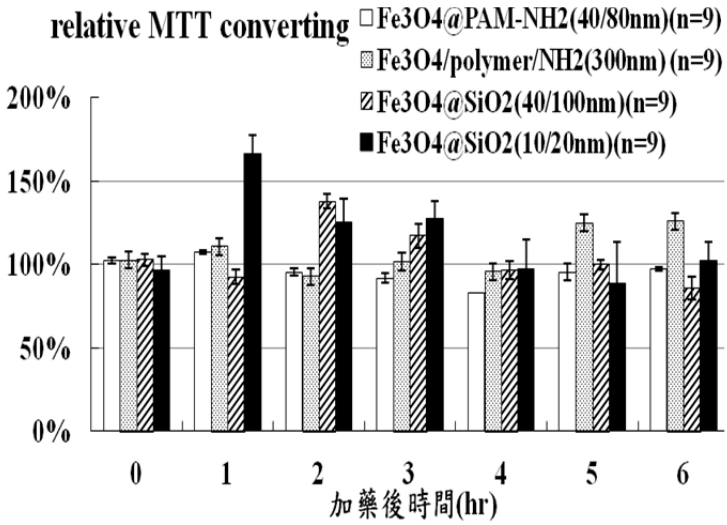



(b)

Figure 10.以 OD 及 MTT 觀察各時間點下 $Fe_3O_4$@PAM-$NH_2$(40/80nm) $Fe_3O_4$/polymer/$NH_2$(300nm)、$Fe_3O_4$@$SiO_2$-$NH_2$(200/400nm)及 $Fe_3O_4$@$SiO_2$ (10/20nm)對酵母菌生長影響，培養於密閉條件下 360° 旋轉培養、rotator 轉速為 12rpm、溫度 30℃。




(a)




(b)

Figure 11.以 OD 及 MTT 觀察各時間點下 $Fe_3O_4$@PAM-$NH_2$(40/80nm)、$Fe_3O_4$/polymer/$NH_2$(300nm)、$Fe_3O_4$@$SiO_2$- (40/100nm) 及 $Fe_3O_4$@$SiO_2$ (10/20nm)對酵母菌生長影響，於平面 shaking 培養，轉速為 200rpm，培養箱溫度 30℃。

### 4-2.4 各顆粒於不同方法培養下之影響差異

將同一種顆粒於不同方式培養之結果統整，由結果可知在不同外力的施加下，磁性顆粒對酵母菌活性影響也隨之不同。針對顆粒較小 $Fe_3O_4$@PAM-$NH_2$(40/80nm) 結果，shaking 與 rotating 培養大多無顯著差異，超過奈米級的顆粒影響皆有顯著的不同(Figure 12)。

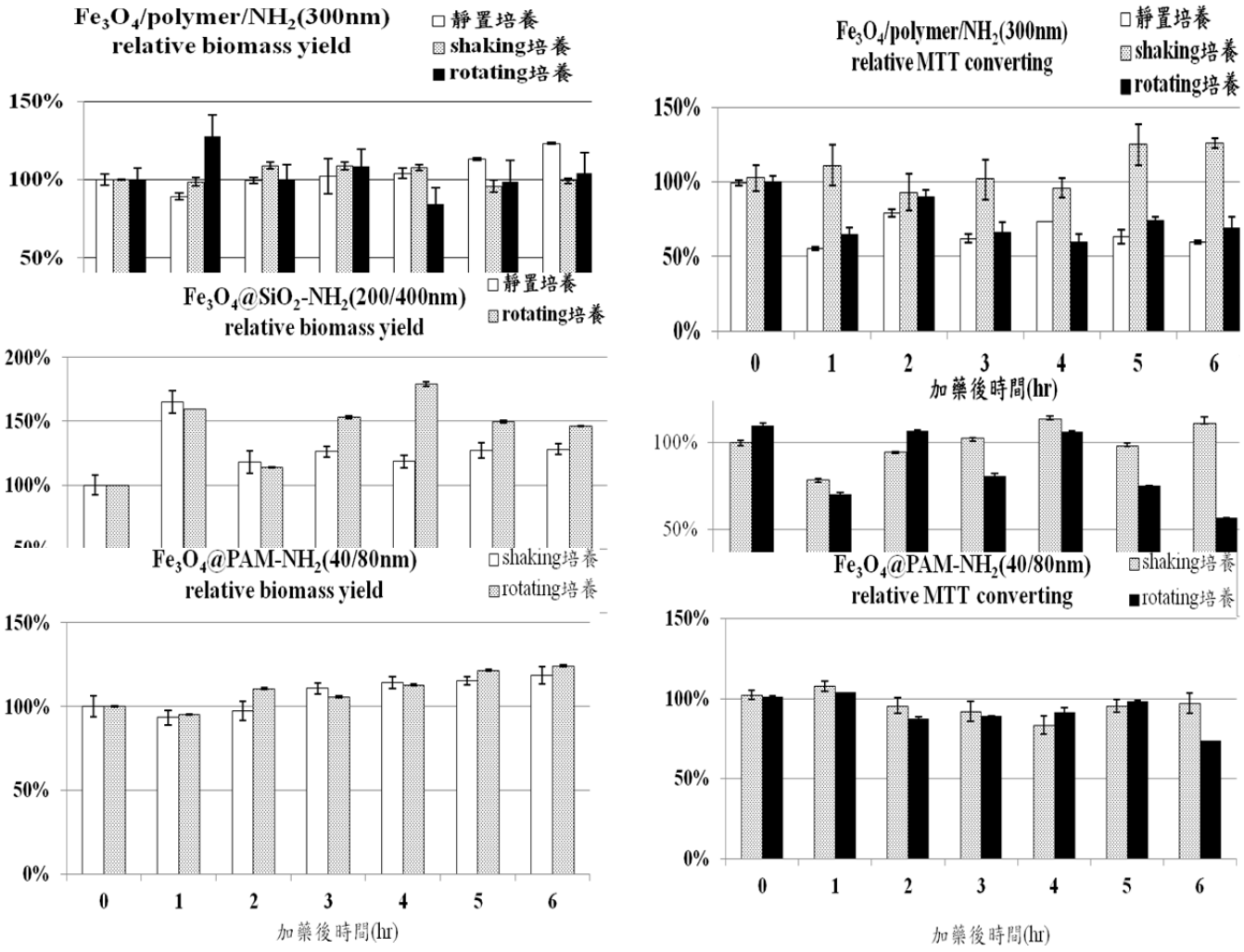



Figure 12.觀察同一顆粒於不同方法培養下，暴露時間對細胞影響差異探討相同顆粒是否因不同外力影響下，對酵母菌活性影響差異。

4-2.5　比較細胞活性與 ATP 含量

$Fe_3O_4$@$SiO_2$ (10/20nm)在不同培養條件下對酵母菌影響， 有顯著差異且有增加細胞呼吸代謝之現象， 此與 Laaksonen 等人[3]的報告一致，因自發性的氧化還原反應, MTT 無法用來測 porous silica microparticles 的毒性, 因在顆粒表面 MTT 會被還原，故進一步利用 prestoblue 分析代謝活性及細胞 ATP 之變化。 Figure 13 結果顯示， 酵母菌單位細胞之 ATP 含量不受 $Fe_3O_4$@$SiO_2$ (10/20nm) 的影響， 但在 100~200 ug/mL 的 $Fe_3O_4$@$SiO_2$ (10/20nm)條件下，細胞增生數度下降但細胞呼吸代謝活性增加。 MTT 及 prestoblue 均是測細胞呼吸代謝活性之指標， 雖二者變化量不相同， 但變化趨勢一致。

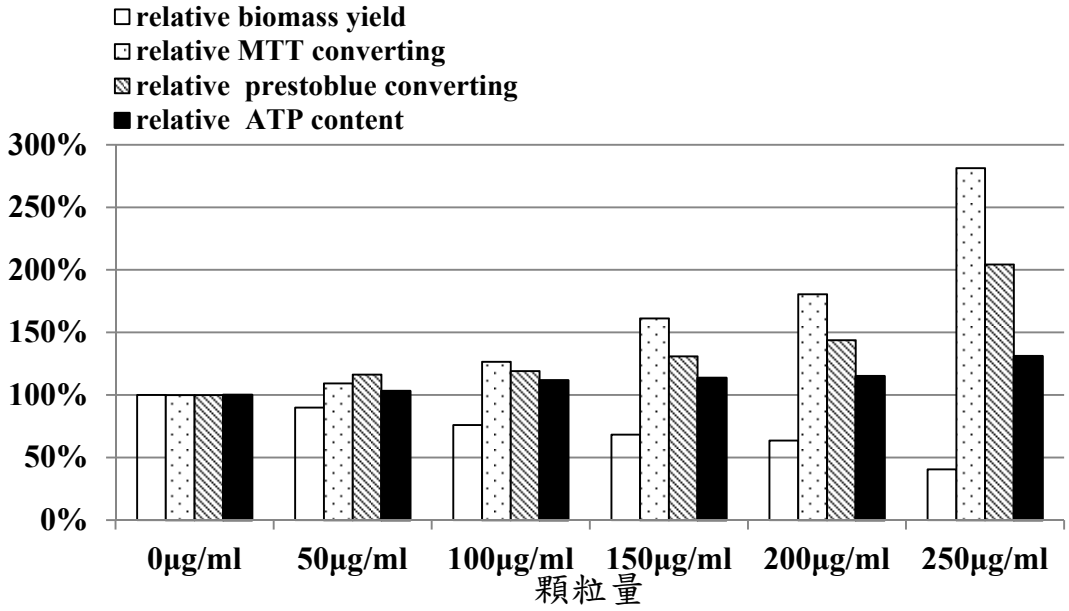



Figure 13 酵母菌與不同量 Fe3O4@SiO2 (10/20 nm) 於平面 shaking 培養，轉速為 200rpm，培養箱溫度 30℃，培養 2 hr。

(五) 結論

1. 將同一種顆粒於不同方式培養之結果統整，由結果可知在不同外力的施加下，磁性顆粒對酵母菌活性影響也隨之不同。針對顆粒較小 $Fe_3O_4$@PAM-$NH_2$(40/80nm) 結果，shaking 與 rotating 培養大多無顯著差異，超過奈米級的顆粒影響皆有顯著的不同。
2. 顆粒越小對細胞活性影響越顯著。
3. **針對磁性奈米顆粒導致細胞轉換率先降後升初步原因評估:**

根據酵母菌生長特性， 酵母菌對環境適應能力強，可馴化後適應外來的衝擊，磁性奈米顆粒於第 2 個小時毒化效果達最大，往後毒化作用效果隨時間降低。 由 ATP 的結果即可驗證。加入磁性顆粒會延遲細胞進入快速分裂期的時間，初期細胞仍處於適應環境階段；後半階段可能藉由改變基因、生長模式迎合新環境繼續生長。

## Reference


1. N. Lewinski, V. Colvin, R. Drezek, Small, 4 (2008) 26.
2. Monteiro-Riviere, A.O. Lnman,, L.W. Zhang, , Toxicol. Appl. Pharmacol. 234, (2009) 222.
3. T. Laaksonen., H. Santos, H. Vihola, J. Salonen, Chem. Res. Toxic, 20 (2007),1913.
4. N.A. Monteiro-Riviere, A.O Inman, L.W.Zhang, Toxicology and Applied Pharmacology 234 (2009) 222-235
5. Po-Chiao Lin, Shu-Hua Chen, Kai-Yi Wang, Mu-Lin Chen, Avijit Kumar Adak, Jih-Ru R Hwu, Yu-Ju Chen, Chun-Cheng Lin. Anal. Chem. 81(2009)8774–8782
6. Jing Ding, Ke Tao, Jiyu Li, Sheng Song, Kang Sun. Colloids and Surfaces B: Biointerfaces 79(2010)184-190
7. Hui Shi, Xiaoxiao He, Yuan Yuan, Kemin Wang, Dan Liu. Anal. Chem. 82(2010)2213-2220
8. O. Treeck, G. Pfeiler, F. Horn, B.Federhofer, H. Houlihan, A. Vollmer, and O. Ortmann. Mol. Cell. Endocrinol 264(2007)50-60
9. C. Cecchi, A.Pensalfini, S. Baglioni, C. Fiorillo, R. Caporale, L. Formigli, G. Liguri, M. Stefani. FEBS J.273(2006)2206-2222
10. C.A.Piantadosi, H.B. Suliman.. J. Biol Chem. 281(2006)324-333
11. Feng Shi, Shigeyuki Kawai, Shigetarou Mori, Emi Kono, Kousaku Murata. FEBS Journal 272(2005)3337-3349
12. Kai-Uwe Frohlich, Hans-Werner Fries, Jan-Michael Peters, Dieter Mecke. Biochimica et Biophysica Acta 1253(1995)25-32
13. Štefan Fujs, Martina Ekert, Janez Ščančar, Peter Raspor. Journal of Basic Microbiology 47(2007)301-308
14. Ludwig k. Limbach, Yuchun Li, Robert N. Grass, Tobias J. Brunner, Marcel A. Hintermann, Martin Muller, Detlefgunther, Wendelin J. Strak. Environ. Sci. Technol 39(2005)9370-9376
15. 張振平、陳春萬，”不同型態奈米微粒之製作與細胞株或動物暴露系統開發與測試研究”，行政院勞工委員會勞工安全衛生研究所，IOSH97-H323，2009 年 3 月

## Part II 磁性奈米顆粒對肝細胞呼吸代謝活性之影響

### I. INTRODUCTION

The biomedical applications of magnetic nanoparticles are well known in drug delivery, cellular labeling, cell separation, tissue repair, and magnetic-resonance imaging. Since these applications involve humans or animals, it is essential to study their toxicity to humans and the environment.

Cell-based assay is currently an important method used for all nanotoxicological research. Since all drugs are, in the end, metabolized in the liver, drug-induced liver injury has been the biggest single cause of safety-related drug-marketing withdrawals in the last 50 years[1]. Liver-cell models are increasingly used to evaluate chemical hepatotoxicity, and these now play an important role in the drug-development process [2]. The MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay is one of most commonly used methods for the assessment of cell viability.

MTT measures the activity of mitochondrial succinate dehydrogenase, which reduces MTT to purple formazan in living cells. Since reduction of MTT can only occur in metabolically active cells, the MTT assay allows the assessment of the viability and proliferation of cells. The quantity of formazan formed is proportional to cellular viability. Forming formazan in living cells is a time-consuming process, however, since cells must be raised with MTT for 3 to 4 h. Formazan is a water-insoluble chemical, and is generally solubilized using DMSO or isopropyl alcohol. These organic solvents are not environmental friendly.

Electrochemical measurements of respiratory chain activity have been used to evaluate cellar viability and used to study the cytotoxicity of chemicals. Mediated electrochemistry based on a single-mediator or double-mediator system has been used for studying redox activity of Saccharomyces cerevisiae cells (yeasts)[3-7], and cancer cells, such as Hep G2[8, 9] and HL-60 [10]. Different cell types require different mediators [7]. Certain mediators can dramatically improve the performance by acting as an electron shuttle between an intracellular reducing center and an external electrode[7]. To our knowledge, electrochemical detection has not yet been demonstrated as a method for probing the viabilities of primary liver cells. In this study, we aim to evaluate the respiratory effect of magnetic core/shell ($Fe_3O_4$@polyacrylamide) nanoparticles, and identify optimal conditions for a simple and rapid electrochemical method as a substitute for MTT assay in assessing the viability of hepatocytes.

### II. MATERIALS AND METHODS

#### *A. Reagents and apparatus*

Dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), potassium ferricyanide (BioUltra, ≥99.0%, containing hexacyanoferrate (II) ($[Fe(CN)_6]^{4-}$) ≤200 mg $kg^{-1}$), potassium ferrocyanide (BioUltra, ≥99.5%), and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, a tetrazole), were purchased from Sigma (St. Louis, MO, USA). William's E medium, antibiotics mixture (50 μg $mL^{-1}$ of penicillin, 50 μg $mL^{-1}$ of streptomycin, and 10 μg $mL^{-1}$ of neomycin), glutamine, and non-essential amino acids (NEAA) were from Gibico (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Culture dishes and microplates were from Nunc (Roskilde, Denmark). Three screen-printed electrodes (SPE) consisting of a carbon

working electrode, a carbon counter electrode and a pseudo-silver reference electrode, were from Zensor R&D Co. (Taichung, Taiwan). EmStat, an electrochemical sensor interface with PSTrace software (PALM instruments BV, Houten, Netherlands) were used for electrochemical measurements. Flex Station 3 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) was used to measure the absorbance of the MTT assay.

The preparation of magnetic and core/shell nanoparticles ($Fe_3O_4$@polyacrylamide) have been described in previous study [11]. The diameters of the iron oxide and core/shell nanoparticles were approximately 40nm and 80nm, respectively.

### B. *Cell culture*

Normal rat liver cells were provided by professor Hwang at Department of Applied Chemistry, Chung Shan Medical University. Primary hepatocytes were isolated from male Wistar rats (weighing 200~250g), as described by Hwang et al. [12]. Rat hepatocytes were cultured in William's E medium supplemented with 10% FBS, 2 mM glutamine, and 1% antibiotic mixture at 37 $^0$C in a humidified atmosphere with 5% $CO_2$ and 95% air. Primary rat hepatocytes were then seeded in 24-well culture plates or in 10-cm culture dishes. Cells were allowed to attach for 12h, then the cultured medium was replaced with fresh culture medium (control samples) or with medium supplemented with magnetic nanoparticles for cytotoxicity study (test samples).

### C. *MTT-assay assessment of cellar viability*

After incubating with the magnetic nanoparticles, we removed the cultured supernatants and added fresh medium with MTT (0.5mg mL$^{-1}$) to incubate at 37$^0$C for 4 h. After removing the MTT medium, DMSO was added to dissolve formazan crystals. The absorbance of formazan was measured at 550 nm. The percentage of viability was calculated using the following equation:

$$\text{viability}\,\% = \frac{A_{\text{test}} - A_{blank}}{A_{control} - A_{blank}} \times 100\%$$

Where $A_{test}$ is the absorbance of the cells cultured with magnetic nanoparticles, $A_{control}$ is the absorbance of normal cultured cells, and $A_{blank}$ is absorbance of culture medium.

### D. *Electrochemical assessment of cellular viability*

After incubating with the magnetic nanoparticles, we removed the cultured supernatants and added fresh medium with ferricyanide and succinate. After 20 minutes, 50 μL of the medium was withdrawn, and applied to the surface of SPE for measuring the oxidation current *(i)* of ferrocyanide.

The percentage of viability was calculated using the following equation:

$$\text{viability}\,\% = \frac{i_{test} - i_{blank}}{i_{control} - i_{blank}} \times 100\%$$

### E. *Statistical analysis*

All experiments were repeated in four replicates. Data in the figures were expressed as the mean±standard deviation. We determined statistical significance of the data using one-way ANOVA or t-test by Microsoft Excel®. Differences were considered significant at the level of $p<0.05$.

## III. RESULTS AND DISCUSSION

### A. *Optimization of electrochemical detection*

We used a flow-injection analysis system with amperometric detection in the hope of speeding up electrochemical detection. The cultured medium contained a high concentration of proteins, however, and quickly caused electrode fouling (data not shown). We used chronoamperometry with disposable screen-printed electrodes as an alternative method to solve this problem. The optimized potential at +0.4 V was applied for 60 seconds. The chronoamperogram showed that the limiting current of diffusion control was reached at 40 seconds. Figure 1 shows the plot of the limiting currents at 40 seconds versus ferrocyanide concentrations. The results show that the current responses are proportional to the ferrocyanide concentrations with a linear range of 0.03~ 1.0 mM ($R^2$=0.9923), and detection limit (S/N=3) at 32 μM. The disposable SPE solves the protein-adsorbing problem, as well as the problem of cross contamination from well to well. The limiting current with minimized nonfaradic current improved the reproducibility of detection.

Figure 2 shows the mediator-assisted effect of ferricyanide concentration on electrochemical detection. We performed the mediator-assisted assessment using 5 mM succinate and various concentrations of ferricyanide. The respiratory cycle of cultured cells produced a measurable amount of ferrocyanide when cultured medium was supplemented with 10 mM ferricyanide and 5 mM succinate, and reacted for more than 20 min. In regard to sensitivity, these conditions were chosen for later experiments.

### B. *Cell counts assessed by electrochemical detection and MTT assay*

Figures 3a show the current response versus cultured density of rat hepatocytes. The oxidation currents were linearly increased with the cultured cell density ($R^2$=0.9944). Figure 3b shows a good correlation of normalized current with normalized absorbance of MTT assay. The absorbance reached a plateau for the cell density greater than 3 x$10^5$ cells $mL^{-1}$, because a limited amount of MTT reagent was used in the assay. The linear range of the electrochemical method is wider than that of the MTT assay under this condition.

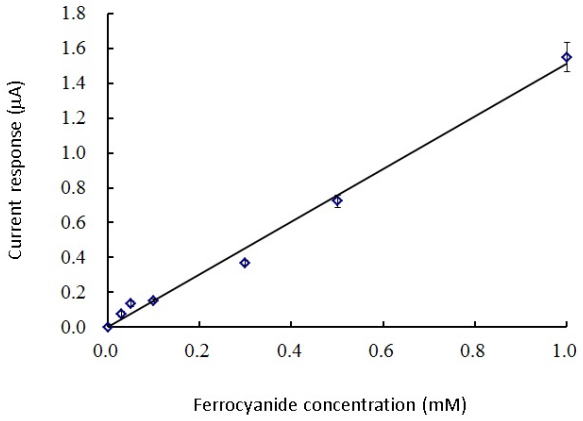



Figure 1. Chronoamperometric current response versus ferrocyanide concentration at time of 40 s after applying +0.4 V vs. pseudo-Ag. The ferrocyanide was prepared in a culture medium with 10% FBS, 10.0 mM ferricyanide and 5.0 mM succinate.

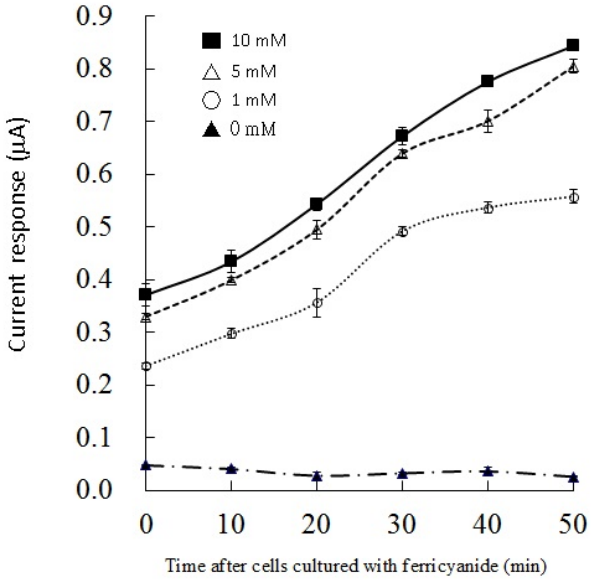



Figure 2. The effect of ferricyanide concentration on electrochemical detection at various culture time. Rat hepatocytes (3 x$10^6$) were seeded in 10 cm culture dishes with 5 mL culture medium.

### C. *Respiratory effect of $Fe_3O_4$@polyacrylamide magnetic nanoparticles on hepatocytes*

We used the chronoamperometric method and MTT assay to evaluate the respiratory effect of $Fe_3O_4$@polyacrylamide magnetic nanoparticles on the viabilities of primary rat hepatocytes. The results are shown in Figure 4.

The cell viabilities assessed by either chronoamperometry or MTT assay were analyzed by ANOVA using Microsoft Excel®. The viabilities of cells cultured with magnetic nanoparticles at a concentration range of 0 to 100 ug $mL^{-1}$ showed no

significant difference. The preliminary results show that $Fe_3O_4$@polyacrylamide magnetic nanoparticles have no significant effect on liver-cell respiration.

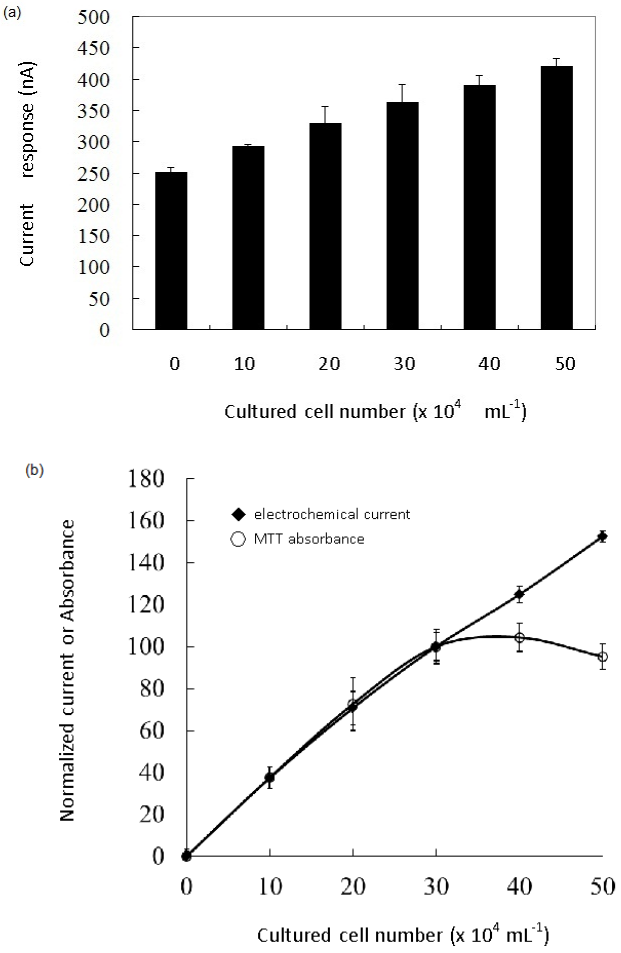



Figure 3. (a) Current response versus rat hepatocyte density. The error bar represents a standard deviation for n=4. (b) The correlation of normalized current produced by oxidation of ferrocyanide and normalized absorbance of formazan. The normalized responses are based on the responses of 3 x $10^5$ cells $mL^{-1}$.

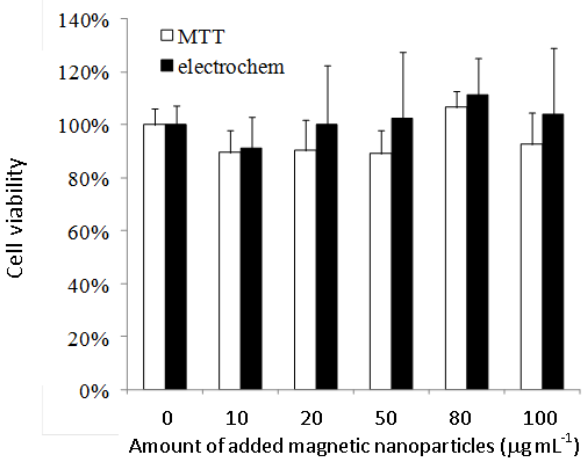



Figure 4. The viability effect of Fe4O3@polyacrylamide magnetic nanoparticles. Primary rat hepatocytes (3x105 cells/well) were treated with magnetic nanoparticles for 30 h.

## IV. CONCLUSION

We compared the viabilities of cells treated with same amount of nanoparticles and measured by chronoamperometry with those of the MTT assay by t-test. Again, the result showed no statistically significant difference. The chronoamperometric method can be used as a quick alternative method for assessing liver-cell respiratory activity.

## REFERENCES

1. F.D.A. Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research, USA, "Guidance for industry drug-induced liver injury: premarketing clinical evaluation," in Book Guidance for industry drug-induced liver injury: premarketing clinical evaluation, Series Guidance for industry drug-induced liver injury: premarketing clinical evaluation, Editor ed.^eds., City, 2009.
2. A. Guillouzo, "Liver Cell Models in in Vitro Toxicology," Enviromental Health Perspectives, vol. 106, pp. 511-532, April 1998.
3. J. Zhao, Z. Wang, C. Fu, M. Wang, and Q. He, "The mediated electrochemical method for rapid fermentation ability assessment," Electroanalysis, vol. 20, (no. 14), pp. 1587-1592, 2008.
4. E.E. Krommenhoek, J.G.E. Gardeniers, J.G. Bomer, X. Li, M. Ottens, G.W.K. Van Dedem, M. Van Leeuwen, W.M. Van Gulik, L.A.M. Van Der Wielen, J.J. Heijnen, and A. Van Den Berg, "Integrated electrochemical sensor array for on-line monitoring of yeast fermentations," Anal. Chem., vol. 79, (no. 15), pp. 5567-5573, 2007.
5. J.-L. Roustan and J.-M. Sablayrolles, "Feasibility of measuring ferricyanide reduction by yeasts to estimate their activity during alcoholic fermentation in wine-making conditions," Journal of Bioscience and Bioengineering, vol. 96, (no. 5), pp. 434-437, 2003.
6. J. Zhao, M. Wang, Z. Yang, and Z. Yang, "Measurement of inhibitory effects of furfural and furfural alcohol using coupled redox mediators," Enzyme Microb. Technol., vol. 37, (no. 2), pp. 246-253, 2005.
7. J. Zhao, M. Wang, Z. Yang, Z. Wang, H. Wang, and Z. Yang, "The different

behaviors of three oxidative mediators in probing the redox activities of the yeast Saccharomyces cerevisiae," Analytica Chimica Acta, vol. 597, (no. 1), pp. 67-74, 2007.
8. H.Y. Ju and J.K. Park, "Cytotoxicity test based on electrochemical impedance measurement of HepG2 cultured in microfabricated cell chip," Anal. Biochem., vol. 341, (no. 2), pp. 308-315, 2005.
9. R.M. Pemberton, J. Xu, R. Pittson, N. Biddle, G.A. Drago, S.K. Jackson, and J.P. Hart, "Application of screen-printed microband biosensors to end-point measurements of glucose and cell numbers in HepG2 cell culture," Anal. Biochem., vol. 385, (no. 2), pp. 334-341, 2009.
10. H.N. Li and Y.X. Ci, "Electrochemical method for analyzing intracellular redox activity changes of the etoposide-induced apoptosis in HL-60 cells," Anal. Chim. Acta, vol. 416, (no. 2), pp. 221-226, 2000.
11. H.Y. Tsai, J.R. Chan, Y.C. Li, F.C. Cheng, and C.B. Fuh, "Determination of hepatitis B surface antigen using magnetic immunoassays in a thin channel," Biosensors & Bioelectronics, vol. 25, (no. 12), pp. 2701-2705, 2010.
12. J.-M. Hwang, T.-H. Tseng, Y.-Y. Tsai, H.-J. Lee, F.-P. Chou, C.-J. Wang, and C.-Y. Chu, "Protective effects of baicalein on tert-butyl hydroperoxide-induced hepatic toxicity in rat hepatocytes," J. Biomed. Sci., vol. 12, (no. 2), pp. 389-397, 2005.

總結論:

從酵母菌實驗發現愈小的磁性奈米顆粒對細胞影響愈大，雖然因酵母菌有適應環境的特色， 長期培養對其生長沒有大影響， 但人類細胞並無此能力， 所以操作奈米顆粒應採取適當的防護措施。

# 國科會補助專題研究計畫出席國際學術會議心得報告

日期：101 年 12 月 10 日

| | | | |
|---|---|---|---|
| 計畫編號 | NSC　100－2113－M－040－002－ | | |
| 計畫名稱 | 磁性奈米顆粒之細胞毒性評估--利用電化學偵測細胞活性法替代 MTT 法之評估 | | |
| 出國人員<br>姓名 | 蔡惠燕 | 服務機構<br>及職稱 | 中山醫學大學 (教授) |
| 會議時間 | 101 年 10 月 21 日<br>至<br>101 年 10 月 24 日 | 會議地點 | 中國 昆明 |
| 會議名稱 | (中文) 2012 第十一屆中國國際奈米科技研討會<br>(英文) The 11th China international conference on the NanoScience and Technology | | |
| 發表題目 | (中文) 各種包覆材質之磁性奈米顆粒之生物毒性初步探討<br>(英文) The prelimainary evaluation of various encapsulated magnetic nanoparilces on biological toxicity | | |

一、參加會議經過

原本計畫要參加每年三月在美國舉行的大型國際分析化學研討會(Pittcon)，但其摘要接受截止日在前一年的八月，本計畫剛開始做根本來不及參加，後來在三月份投稿 2012 international conference on biomedical engineering and biotechnology,conference paper 被接受， 可收錄 proceeding 中並至 conference 做口頭報告(澳門)，後來受邀到在中國昆明

舉行的 the 11th China international conference on nanoscience and technology 做口頭報告。

二、與會心得

第一次參加非分析化學的研討會，與不同領域的人交流，學會了不同角度看事物。 五月分參加 2012 international conference on biomedical engineering and biotechnology，感覺非常不好, 第一天的 keynote speech 有 thermography measurement 的主題， speaker 一直 show 一些臨床上的影像然後告訴大家"這是 xx 疾病" 看著一些噁心的相片一個小時，差點吃不下午餐。 第二天分組我們的分在 Biology science section, 三個小時有 24 個 speaker， 本來還在納悶要如何進行，一個人要講多久，結果連我們只有 5 個 speaker 出現， 怎會有這樣子的國際研討會!

八月份受邀到大陸昆明參加 11th China international conference on nanoscience and technology，該大會就好多了。第一天有來自美國工程院的院士 Prof. Baughman， 美國科學促進會的院士 Prof. Yury Gogotsi，中國百人計畫特聘教授/研究員等人 ，大家報告的內容主要在石墨烯，奈米碳管、硅、TiO2 等之製備技術，與特性探討。少數幾位報告與醫學相關的較吸引我的興趣， 如上海交大王瑾曄教授"conductive polymers with nanostructure as coating material for neural prosthesis"，華中師範大學楊旭教授講的 nano-medicine，提醒大家如何在有 benefits 進一步做

risk assessment，讓第二天我報告”探討磁性顆粒對細胞活性的影響及開發的電化學偵測方法”，引起眾多迴響，讓很多從事材料開發者注意到材料安全問題。

三、發表論文全文或摘要

**The respiratory effect of magnetic core/shell($Fe_3O_4$@polyacrylamide) nanoparticles, assessed using both MTT viability assay and chronoamperometric method**

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[3] Department of Applied Chemistry, National ChiNan University, Natu, Taiwan

**Abstract**

We evaluated the respiratory effect of $Fe_3O_4$@polyacrylamide magnetic nanoparticles using both the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) viability assay and the chronoamperometric method, based on screen-printed carbon electrodes (SPE). Preliminary results show that nanoparticles have no significant effect on the respiration of rat liver cells, according to both detection methods. The chronoamperometric method using SPE shows that the current responses are proportional to the ferrocyanide concentrations, with a linear range of 0.03~ 1.0 mM ($R^2$=0.9923), and detection limit (S/N=3) at 32 μM. The results of paired t-test analysis indicates that assessment of hepatocyte viabilities based on the chronoamperometric method were comparable to those of the MTT viability assay. The

chronoamperometric method can be used as a quick alternative method for assessing liver-cell viability.

Keywords: Chronoamperometry, screen-printed electrodes, hepatotoxicity, viability assay, magnetic nanoparticles

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## Introduction

The biomedical application of magnetic nanoparticles, for example in drug delivery, cellular labeling or cell separation, tissue repair, and magnetic-resonance imaging, are well known. Since these applications involve humans or animals, it is important to study their toxicity to humans and the environment.

Cell-based assay is currently an important method used for all nanotoxicological research. Since all drugs are, in the end, metabolized in the liver, drug-induced liver injury has been the biggest single cause of safety-related drug-marketing withdrawals in the last 50 years(Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research 2009). Liver-cell models are increasingly used to evaluate chemical hepatotoxicity, and these now play an important role in the drug-development process (Guillouzo 1998). The MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay is one of most commonly used methods for the assessment of cell viability. MTT measures the activity of mitochondrial succinate dehydrogenase, which reduces MTT to purple formazan in living cells.

Since reduction of MTT can only occur in metabolically active cells, the MTT assay allows the assessment of the viability (cell counting) and proliferation of cells. The quantity of formazan formed is proportional to cellular viability. Forming formazan in living cells is a time-consuming process, however, since cells must be raised with MTT for three to four hours. Formazan is a water-insoluble chemical, and is generally solubilized using DMSO or isopropyl alcohol. These organic solvents are not environmental friendly.

Electrochemical measurements of respiratory chain activity have been used to evaluate cellar viability and used to study the cytotoxicity of chemicals. Mediated electrochemistry based on a single-mediator or double-mediator system has been used for studying redox activity of Saccharomyces cerevisiae cells (yeasts)(Krommenhoek et al 2007; Roustan & Sablayrolles 2003; Zhao et al 2007; Zhao et al 2005; Zhao et al 2008), and cancer cells, such as Hep G2(Ju & Park 2005; Pemberton et al 2009) and HL-60 (Li & Ci 2000). Different cell types require different mediators (Zhao et al 2007). Certain mediators can dramatically improve the performance by acting as an electron shuttle between an intracellular reducing center and an external electrode(Zhao et al 2007). To our knowledge, electrochemical detection has not yet been demonstrated as a method for probing the viabilities of primary liver cells. In this study, we aim to evaluate the respiratory effect of magnetic core/shell ($Fe_3O_4$@polyacrylamide) nanoparticles, and identify optimal conditions for a simple and rapid electrochemical method as a substitute for MTT assay in assessing the viability of hepatocytes.

## Materials and Methods

### *Reagents and apparatus*

We used the following reagents and apparatus in this study:

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, a tetrazole), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), potassium ferricyanide

(BioUltra, ≥99.0%, containing hexacyanoferrate(II) ($[Fe(CN)_6]^{4-}$): ≤200 mg $kg^{-1}$), and potassium ferrocyanide (BioUltra, ≥99.5%) from Sigma (St. Louis, MO, USA). We used these without further purification. William's E medium, antibiotics mixture (50 μg $mL^{-1}$ of penicillin, 50 μg $mL^{-1}$ of streptomycin, and 10 μg $mL^{-1}$ of neomycin), glutamine, and non-essential amino acids (NEAA) from Gibico (Grand Island, NY, USA). Fetal bovine serum (FBS) from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Culture dishes and microplates from Nunc (Roskilde, Denmark). Three screen-printed electrodes (SPE) consisting of a carbon working electrode, a carbon counter electrode and a pseudo-silver reference electrode, from Zensor R&D Co. (Taichung, Taiwan). EmStat, an electrochemical sensor interface with PSTrace software (PALM instruments BV, Houten, Netherlands). We used this for potential control and for data acquisition during electrochemical measurements. Flex Station 3 microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). We used this to measure the absorbance of the MTT assay.

Magnetic core/shell ($Fe_3O_4$@polyacrylamide) nanoparticles were provided by professor Fuh at Department of Applied Chemistry, National Chi Nan University. The properties of magnetic nanoparticles have been described in a previous study (Tsai et al 2010). The diameters of the iron oxide and core/shell nanoparticles were approximately 40nm and 80nm, respectively.

*Cell culture*

Normal rat liver cells were provided by professor Hwang at Department of Applied Chemistry, Chung Shan Medical University. We isolated primary hepatocytes from male Wistar rats (weighing 200~250g), as described by Hwang et al. (Hwang et al 2005). Rat hepatocytes were cultured in William's E medium supplemented with 10% FBS, 2 mM Glutamine, and 1% antibiotic mixture at 37 $^0$C in a humidified atmosphere with 5% $CO_2$ and 95% air. We then

seeded primary rat hepatocytes in 24-well culture plates or in 10cm culture dishes. Cells were allowed to attach for 12 hours before we replaced the culture medium with fresh culture medium (control samples) or with medium supplemented with magnetic nanoparticles for cytotoxicity study (test samples).

*MTT-assay assessment of cellar viability*

After incubating with the magnetic nanoparticles, we removed the cultured supernatants and supplemented fresh medium with MTT (0.5mg $mL^{-1}$), before incubating at $37^0$C for a further 4 hours. After removing the MTT medium, we added DMSO, in order to dissolve the formazan crystals. The absorbance of formazan was measured at 550nm. We calculated the percentage of viability using the following equation:

$$\text{viability}\,\% = \frac{\mathrm{A}_{\text{test}} - A_{blank}}{A_{control} - A_{blank}} \times 100\%$$

*Electrochemical assessment of cellular viability*

After incubating with the magnetic nanoparticles, we removed the cultured supernatants and supplemented fresh medium with ferricyanide and succinate. After 20 minutes, we withdrew 50 μL of the medium and applied it to the surface of the SPE. We then measured the oxidation current of the ferrocyanide.

We calculated the percentage of viability using the following equation:

$$\text{viability}\,\% = \frac{i_{test} - i_{blank}}{i_{control} - i_{blank}} \times 100\%$$

*Statistical analysis*

All experiments were repeated in four replicates. Data in the figures were expressed as the

mean±standard deviation. We determined statistical significance of the data using one-way ANOVA or Student's t-test by Microsoft Excel®. Differences were considered significant at the level of $p<0.05$.

## Results

*Optimization of electrochemical detection*

We used a flow-injection analysis system with amperometric detection in the hope of speeding up electrochemical detection. The cultured medium contained a high concentration of proteins, however, and quickly caused electrode fouling (data not shown). We used chronoamperometry with disposable screen-printed electrodes as an alternative method to solve this problem. The optimized potential at +0.4 V was applied for 60 seconds. The chronoamperogram showed that the limiting current of diffusion control was reached at 40 seconds. Figure 1 shows the plot of the limiting currents at 40 seconds versus ferrocyanide concentrations. The results show that the current responses are proportional to the ferrocyanide concentrations with a linear range of 0.03~1.0 mM ($R^2$=0.9923), and detection limit (S/N=3) at 32 μM. The disposable SPE solves the protein-adsorbing problem, as well as the problem of cross contamination from well to well. The limiting current with minimized nonfaradic current improved the reproducibility of detection.

Figure 2 shows the mediator-assisted effect of ferricyanide concentration on electrochemical detection. We performed the mediator-assisted assessment using 5.0 mM succinate and various concentrations of ferricyanide. The respiratory cycle of cultured cells produced a measurable amount of ferrocyanide when cultured medium was supplemented with 10.0mM ferricyanide and 5.0mM succinate, and reacted for more than 20 minutes. In regard to sensitivity, these conditions were chosen for later experiments.

*Cell counts assessed by electrochemical detection and MTT assay*

Figures 3a and 3b show the current response versus cultured density of rat hepatocytes. The oxidation currents were linearly increased with the cultured cell density ($R^2$=0.9944). Figure 3c shows a good correlation of normalized current with normalized absorbance of MTT assay. The absorbance reached a plateau for the cell density greater than 3 x$10^5$ cells $mL^{-1}$, because a limited amount of MTT reagent was used in the assay. The linear range of the electrochemical method is wider than that of the MTT assay in the current condition.

*Respiratory effect of $Fe_3O_4$@polyacrylamide magnetic nanoparticles on hepatocytes*

We used the chronoamperometric method and MTT assay to evaluate the respiratory effect of $Fe_3O_4$@polyacrylamide magnetic nanoparticles on the viabilities of primary rat hepatocytes. The results are shown in Figure 4.

**Discussion**

In the present study we evaluated the respiratory effect of magnetic core/shell($Fe_3O_4$@polyacrylamide) nanoparticles on rat liver cells using both MTT viability assay and chronoamperometric method. The cell viabilities assessed by either chronoamperometry or MTT assay were analyzed by ANOVA using Microsoft Excel®. The viabilities of cells cultured with magnetic nanoparticles at a concentration range of 0 to 100 ug $mL^{-1}$ showed no significant difference. The preliminary results show that $Fe_3O_4$@polyacrylamide magnetic nanoparticles have no significant effect on liver-cell respiration.

We compared the viabilities of cells treated with same amount of nanoparticles and measured by chronoamperometry with those of the MTT assay by t-test. Again, the result showed no statistically significant difference. The chronoamperometric method can be used as a quick alternative method for assessing liver-cell viability.

**Acknowledgments**

The Taiwan Grains and Feeds Development Foundation and the National Science Council, Taiwan supported this work. The authors wish to acknowledge Mr. W.J. Ting for the technique assistant with cell cultures.

四、建議

一個好國際研討會的辦理，可增加國家的曝光度並建立國家形象，但參加澳門舉辦的會議就覺得好像小孩開大車,且主辦單位學校高層完全不重視，只有一個系主任及一個承辦秘書小姐負責招呼所有與會者。 參加昆明的會議就感覺很有秩序很有條理， 原來該研討會一直由幾個奈米技術學會的助理負責事物性的工作，學會中有固定的學術委員幫忙邀請與安排參加學者，所以對辦理會議之工作內容非常熟悉，然後每年到不同的地方辦理，由當地學校協助當地的事宜。此作法值得化學會參考。

五、攜回資料名稱及內容

2012 international conference on biomedical engineering and biotechnology 只發了一本 conference program guide, proceeding 出刊時會寄給與會者，但我寄了 email 要了，還是沒收到（又是一個很不好感覺）

the 11th China international conference on nanoscience and technology 帶回 conference program booklet， nanosceience and nanotechnology 期刊一期(2012, vol 9, serial No. 52)，science china 期刊一期（ 2013, vol. 56, No.1）

## 六、其他

無

# 國科會補助計畫衍生研發成果推廣資料表

日期:2012/12/12

| | |
|---|---|
| 國科會補助計畫 | 計畫名稱: 磁性奈米顆粒之細胞毒性評估--利用電化學偵測細胞活性法替代 MTT 法之評估 |
| | 計畫主持人: 蔡惠燕 |
| | 計畫編號: 100-2113-M-040-002- 學門領域: 電化學 |

無研發成果推廣資料

# 100 年度專題研究計畫研究成果彙整表

| 計畫主持人：蔡惠燕 | 計畫編號：100-2113-M-040-002- |
|---|---|
| 計畫名稱：磁性奈米顆粒之細胞毒性評估--利用電化學偵測細胞活性法替代 MTT 法之評估 | |

| 成果項目 | | | 量化 | | | 單位 | 備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等） |
|---|---|---|---|---|---|---|---|
| | | | 實際已達成數（被接受或已發表） | 預期總達成數(含實際已達成數) | 本計畫實際貢獻百分比 | | |
| 國內 | 論文著作 | 期刊論文 | 0 | 0 | 100% | 篇 | |
| | | 研究報告/技術報告 | 1 | 1 | 100% | | |
| | | 研討會論文 | 1 | 1 | 100% | | |
| | | 專書 | 0 | 0 | 100% | | |
| | 專利 | 申請中件數 | 0 | 0 | 100% | 件 | |
| | | 已獲得件數 | 0 | 0 | 100% | | |
| | 技術移轉 | 件數 | 0 | 0 | 100% | 件 | |
| | | 權利金 | 0 | 0 | 100% | 千元 | |
| | 參與計畫人力（本國籍） | 碩士生 | 2 | 2 | 100% | 人次 | 增加兩位大專生 |
| | | 博士生 | 0 | 0 | 100% | | |
| | | 博士後研究員 | 0 | 0 | 100% | | |
| | | 專任助理 | 0 | 0 | 100% | | |
| 國外 | 論文著作 | 期刊論文 | 1 | 1 | 100% | 篇 | |
| | | 研究報告/技術報告 | 0 | 0 | 100% | | |
| | | 研討會論文 | 2 | 1 | 100% | | |
| | | 專書 | 0 | 0 | 100% | 章/本 | |
| | 專利 | 申請中件數 | 0 | 0 | 100% | 件 | |
| | | 已獲得件數 | 0 | 0 | 100% | | |
| | 技術移轉 | 件數 | 0 | 0 | 100% | 件 | |
| | | 權利金 | 0 | 0 | 100% | 千元 | |
| | 參與計畫人力（外國籍） | 碩士生 | 0 | 0 | 100% | 人次 | |
| | | 博士生 | 0 | 0 | 100% | | |
| | | 博士後研究員 | 0 | 0 | 100% | | |
| | | 專任助理 | 0 | 0 | 100% | | |

| | |
|---|---|
| 其他成果<br>(無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。) | 1. 獲得 2012 中國化學會年會壁報論文獎<br>2. The 11th China international conference on the NanoScience and Technology, Kunming（昆明）2012, 10, 21-24. 特邀口頭報告 the prelimainary evaluation of various encapsulated magnetic nanoparilces on biological toxicity. |

| | 成果項目 | 量化 | 名稱或內容性質簡述 |
|---|---|---|---|
| 科教處計畫加填項目 | 測驗工具(含質性與量性) | 0 | |
| | 課程/模組 | 0 | |
| | 電腦及網路系統或工具 | 0 | |
| | 教材 | 0 | |
| | 舉辦之活動/競賽 | 0 | |
| | 研討會/工作坊 | 0 | |
| | 電子報、網站 | 0 | |
| | 計畫成果推廣之參與（閱聽）人數 | 0 | |

# 國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限)

利用電化學方法偵測細胞活性, 並利用於探討奈米磁性顆粒之細胞毒性。電化學偵測細胞活性可在 20~30 min 內知道結果, 相較於傳統 MTT 方法需 3~4 小時快速.目前使用螢光染劑方法雖也可在 10~30 min 得到結果, 但螢光染劑昂貴且不穩定, 偵測時需昂貴的螢光光譜儀，相較之下電化學方法在成本上便宜許多。