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# Modification of MTT Assay for Precision and Repeatability and Its Mechanistic Implication

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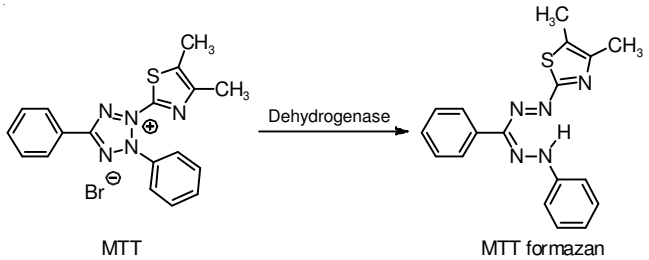
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is an attractive method for antibiotics screening. However, a severe deviation will occur when a strong acidic sodium dodecyl sulfate is used as a lysis buffer in MTT assay and no literatures so far reported the possible reasons. Based on the results of MTT assay and the UV absorption-pH curves of MTT formazan, we putatively attributed this deviation to the presence of the cationic MTT formazan in strong acidic media, which results in a complete disappearance of the peak at 575 nm. Our data suggested that the pH of sodium dodecyl sulfate lysis buffer should be controlled at about 3.5 for more accurate and repeatable results. In addition, the microbial concentration in antibacterial activity test can be directly obtained from the standard curves of microbial concentration against optical density at 600 nm. In comparison with the traditional processes for MTT assay, the above mentioned improvements significantly increase the accuracy and repeatability of this attractive method.

**Keywords: MTT assay, pH of sodium dodecyl sulfate lysis buffer, Mechanistic implication, Structural variation of MTT formazan.**

## INTRODUCTION

The discovery of antibiotics is one of the key achievements of modern medicine. Antibiotic use has contributed enormously to human health. However, microorganisms can evolve and adjust to changes in their environment and have subsequently developed several protective mechanisms to reduce their susceptibility to antibiotics[1]. More and more potent antibiotics lose their efficacy over time, rendering them useless for conditions they could once successfully treat[2-4]. Therefore, there is an urgent need for screening and development of new antibacterial agents effective against resistant strains[5-8]. Screening of active substances plays a crucial role in drug research and development. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay has been widely adopted for low cost, rapidity and high throughput format[9]. In this assay, the yellow and water-soluble MTT is converted to the purple and water insoluble MTT formazan in the presence of mitochondrial dehydrogenase as shown in **Scheme-I**[10]. As a result, the amount of formazan dye produced is directly proportional to the number of metabolically active cells and indicates the reducing potential of the cells[11]. The MTT assay uses frequently, while the reported conditions and parameters of the assay vary widely. Thus attempts to modification of MTT assay are never ceased[12-15]. Incubating cells with MTT, extracting and dissolving the water-insoluble formazan crystals from cells are crucial steps in the MTT assay[16]. However the mechanism of the significant deviation presented in the process of formazan dissolution remains absent. For example, sodium dodecyl sulfate lysis buffer (sodium dodecyl sulfate, isopropanol, HCl) as an acid solvent has been widely used to extract the formazan crystals from the cells, the lysing ability of which is distinctive with different pH values[17]. To our best of knowledge, no literatures reported the possible reasons. In addition, it is well known that the microbial concentration must be carefully controlled within a certain range to achieve reliable results in MTT assay. While the common methods such as plate counting method, weighing method or blood count method take considerable time or financial hit to determine the microbial concentration.




**Scheme-I:** Chemical structure of MTT and its reduced formazan product

In trying to study the mechanism for the deviation of MTT assay caused by the pH of sodium dodecyl sulfate lysis buffer as well as build a count method being more economic and manipulable for antibacterial activity test, the relationships between optical density (600 nm) values and concentrations of microorganism (Gram-positive organisms, Gram-negative organisms and fungus) were explored and effects of a buffer pH on the structure of MTT formazan were disclosed, providing an inference for further modification of the MTT assay.

## EXPERIMENTAL

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Chem Great Wall (China). Other chemicals (reagent grade) used were purchased from Aldrich (U.S.A) and Sinopharm Chemical Reagent Co., Ltd (China). Absorbance was determined on a microplate reader (SpectraMax® Plus384 Molecular Devices, USA) and optical density was determined on UV-visible spectrophotometer (UV-2450, Japan). *E. coli* ATCC 35218, *S. aureus* ATCC 29213, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 90028 were obtained from School of Life Science and Technology (University of Electronic Science and Technology of China, China) and mildews (CFCC 5336, CFCC 88528 and CFCC 6382) were purchased from China Forestry Culture Collection Center (Beijing, China).

**Standard curves of bacterial concentration against optical density:** Firstly, microbial samples were subcultured on appropriate mediums. *E. coli* ATCC 35218, *S. aureus* ATCC 29213, *B. subtilis* ATCC 6633 and *C. albicans* ATCC 90028 were subcultured on nutrient agar while other microbial samples were subcultured on potato dextrose agar (PDA). Two Gram-positive bacterial strains (*S. aureus* ATCC 29213, *B. subtilis* ATCC 6633) and two Gram-negative bacterial strains (*E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853) were incubated at 37 °C. Other fungal samples, plates were incubated at 28 °C. Then the microbial samples at exponential growth phase were diluted into different concentrations using normal saline. The optical densities (OD) at 600 nm of microbial concentrations were measured with UV-visible spectrophotometer. The microbial concentrations were determined by spread plate counting method. The standard curves of microbial concentration against optical density are depicted in Fig. 1.

With the purpose of verifying correctness of standard curves, the $MIC_{50}$s of ciprofloxacin, kanamycin B and amphotericin B against Gram-positive bacterial strain (*S. aureus* ATCC 29213), Gram-negative bacterial strain (*E. coli* ATCC 35218) and a fungus (*C. albican* ATCC 90028) were tested by a colorimetric method using the dye MTT, respectively. Excepting for the suspension of the microorganism was prepared to contain approximately $10^5$ cfu $mL^{-1}$, according to the standard curves and the pH of the sodium dodecyl sulfate lysis buffer was controlled about 3.5, all other conditions were same as the previously reported method[18]. The obtained $MIC_{50}$s together with the data reported by previous workers[19-21] were presented in Table-1.

**Effects of pH of sodium dodecyl sulfate lysis buffer on inhibition rates:** Except of sodium dodecyl sulfate lysis buffer being set at different pH values (1.5, 2.0, 2.5, 3.0, 3.5 and 6.0), all other conditions were the same as those for the determination of $MIC_{50}$s. The effects of pH of sodium dodecyl sulfate lysis buffer on inhibition rates were presented in Fig. 2 (A).

**Effects of pH of sodium dodecyl sulfate lysis buffer on the structures of MTT and MTT formazan:** MTT formazan was obtained by reference method[13]. The resulted MTT formazan and MTT were respectively dissolved in sodium dodecyl sulfate lysis buffers with different pH (pH = 1.0, 2.5, 3.5, 6.0, 11.0). The absorption spectra of MTT and MTT formazan were obtained using a UV-visible spectrophotometer.

TABLE-1
THE OBSERVED $MIC_{50}$S AND THE DATA REPORTED BY REFERENCES

| Microorganisms | *S. aureus* | *E. coil* | *C. albicans* | References |
|---|---|---|---|---|
| Ciprofloxacin | 0.43 μM | - | - | 0.39 μM [Ref. 21] |
| Kanamycin B | - | 7.6 μM | - | 6.40 μM [Ref. 20] |
| Amphotericin B | - | - | 0.25 μM | 0.30 μM [Ref. 19] |

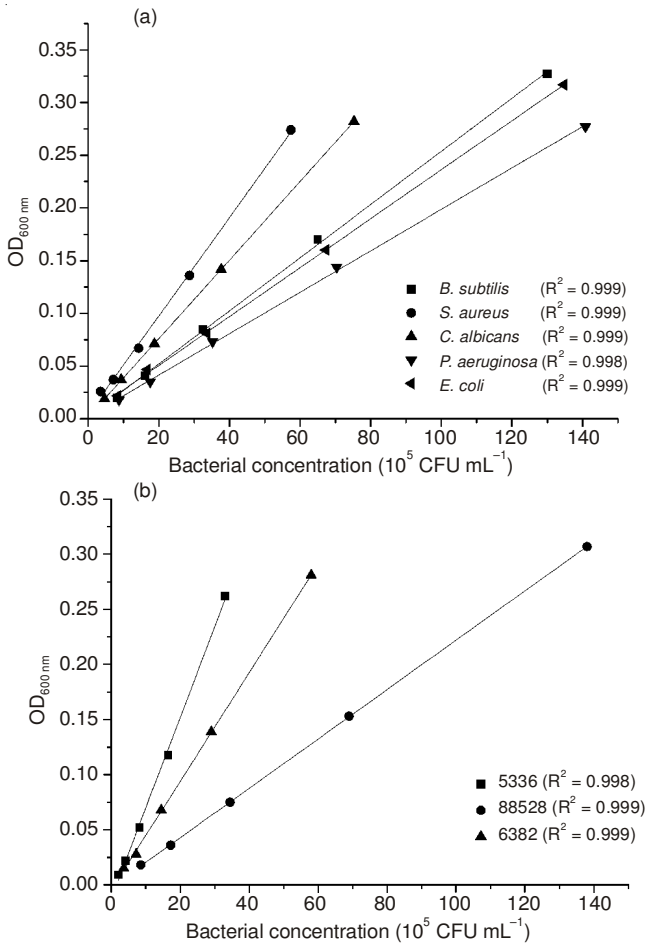



Fig. 1. Standard curves of bacterial concentration against optical density

## RESULTS AND DISCUSSION

**Standard curves of bacterial concentration against optical density:** These data disclosed that there is a significant positive correlation between microbial concentrations and optical density values at 600 nm (Fig. 1, $R^2 > 99.9$ %). The concentration of all microorganisms is approximate to $10^6$ cfu $mL^{-1}$ at optical $density_{600\ nm}$ being about 0.1, which was transferred to 96-well plates after 10 times dilution by appropriate

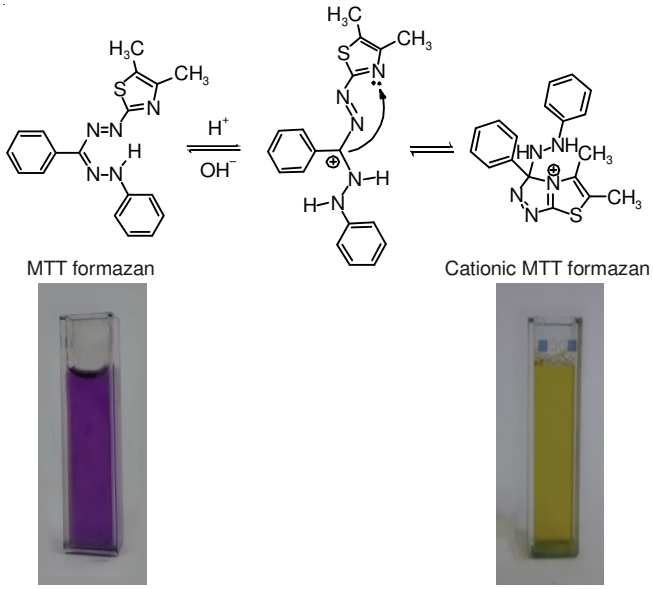
liquid medium for determination of $MIC_{50}$. The resulted $MIC_{50}$s of three clinical antibiotics were consistent with those reported data, which strongly suggested the accuracy of this quick counting method. In comparison with the traditional spread plate method, the suspensions of the microorganism for MTT assay could be prepared easily and quickly with the help of standard curves.

In fact, the number of microorganism in a given sample is usually too great. A series of gradiently diluted samples (generally 0.1 mL) therefore must be plated on series of agar surface. After 8 h or longer incubation, the plates with 30 to 300 CFUs (colony forming units) are selected for CFU counting. Obviously, the spread plate method will take a lot of time and efforts to accomplish all these operations.

**Effects of pH of sodium dodecyl sulfate lysis buffer on inhibition rates:** The effects of pH on inhibition rates by sodium dodecyl sulfate lysis buffers (pH = 1.5, 2.0, 2.5, 3.0, 3.5, 6.0) were evaluated. The sodium dodecyl sulfate lysis buffers produce very close inhibition rates if the pH of sodium dodecyl sulfate lysis buffer is set at a range from 2.5 to 6 (Fig. 2A). The observed inhibition rates significantly changed, however, provided the pH is lower than 2.0. Furthermore, the color of the cavities is normally deep purple with buffer having pH of 2.5 to 6.0, whereas a yellowish color observed when the pH is lower than 2.0, indicating that a sudden change occurs in the buffer with pH in the range from 2.0 to 1.5.

To explore the effect of pH on color, 100 µL of deep purple solution was added to a centrifugal tube from a 96-well plate, which was then added 100 µL HCl (0.1 mol $L^{-1}$). The color changed to yellowish immediately and the color returned to purple when 0.1 mol $L^{-1}$ of NaOH was subsequently added. This clearly indicated that the structure of MTT formazan is reversibly changed in strong acid (**Scheme-II**).

**Effects of pH of sodium dodecyl sulfate lysis buffer on the structures of MTT and MTT formazan:** As shown in Fig. 2B, two main UV absorption peaks are observed at about 250 and 380 nm, which are nearly not changed with pH, indicating that MTT is stable in the condition of pH 1-11 and apparently it is not a source of the major deviation in the MTT assay (measured at about 575 nm). To the contrary, in UV absorption spectra of the MTT formazan (Fig. 2C), absorption peaks at 575 nm strongly influenced by pH values of sodium dodecyl sulfate lysis buffers and reverse changes with absorption peaks were observed at about 400 nm. When the pH is less than 2.5, the absorption peaks at 575 nm are almost disappeared. The possible reason for absorption peaks (575 nm) decreasing and shifting to high frequency (400 nm) is




**Scheme-II:** Possible conjugated structures of MTT formazan in different pH sodium dodecyl sulfate lysis buffers

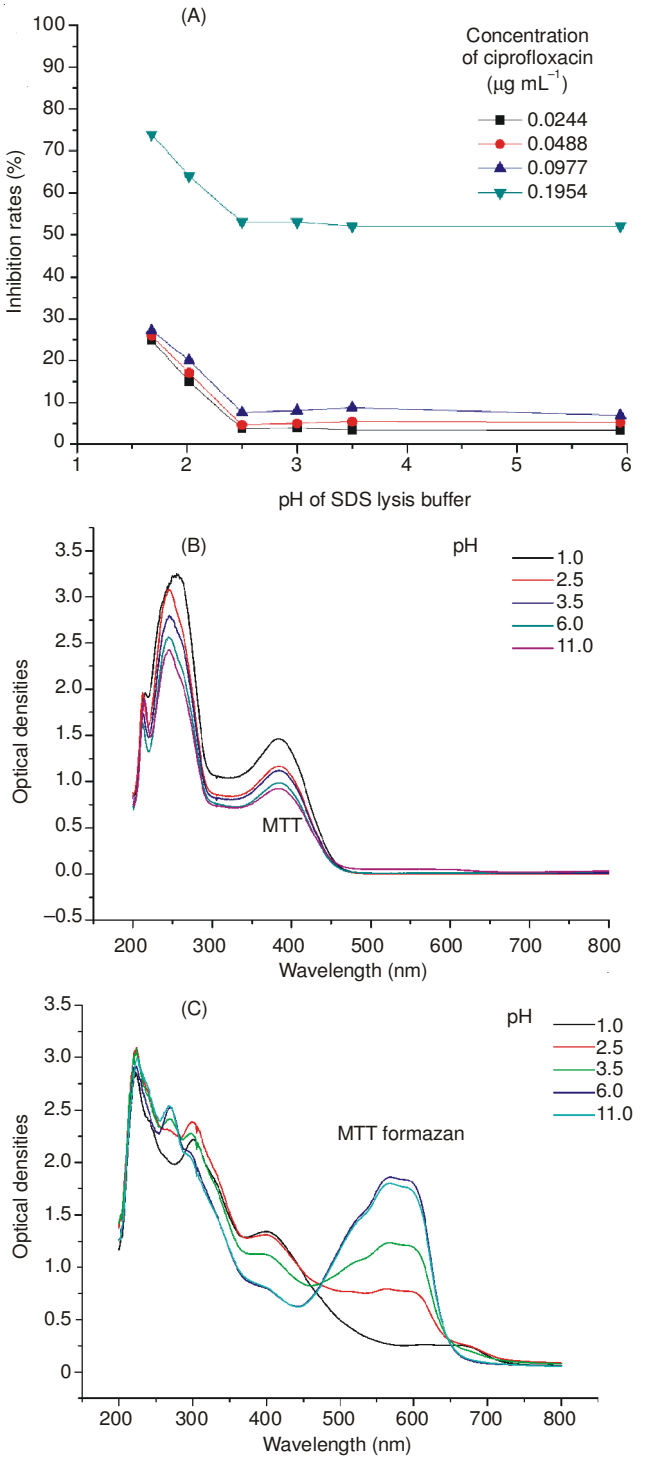



Fig. 2. (A) The inhibition rates with different pH of sodium dodecyl sulfate lysis buffers; (B) Absorption spectra of MTT in different pH sodium dodecyl sulfate lysis buffers; (C) Absorption spectra of MTT formazan in different pH sodium dodecyl sulfate lysis buffers

that the basic nitrogen atom in MTT formazan is reactive with HCl, yielding a cationic MTT formazan (**Scheme-II**). The conjugated system of MTT formazan is therefore broken. The change of conjugated system with pH value also provides a reasonable explanation for the above mentioned color change (yellowish at pH = 2.0 and deep purple at pH > 2.5). When the pH is at 6.0 or 11.0, the absorption peak (575 nm) of MTT formazan at the same concentration is much higher than others. To avoid overranging the span (0-4) of microplate reader, a lower concentration of microbial suspension must be set than normal assay. It is well known that lower concentration of microorganism would bring larger experimental errors. The mechanism of structural variation of MTT formazan suggested that for achieving the best accuracy in MTT assay, the pH of sodium dodecyl sulfate lysis buffers should be controlled at about 3.5.

## Conclusion

A quick counting method based on the curves of microbial concentration (including Gram-positive organisms, Gram-negative organisms and fungus) against optical density for MTT assay was established, with the help of which the suspensions of the microorganism can be prepared easily and quickly. Furthermore, the effects of pH on inhibition rates and the structure of MTT formazan were studied. The results from mechanism study disclosed that the structure of MTT formazan changed reversibly into a cationic form in a strong acidic sodium dodecyl sulfate lysis buffer, which causes a severe deviation in MTT assay. For the purpose of avoiding this deviation, the pH of sodium dodecyl sulfate lysing buffer should be set at about 3.5. The above mentioned modifications greatly improve the accuracy and manipulability of MTT assay.

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