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Current methodology of MTT assay in bacteria – A review

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ABSTRACT

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay is a popular tool in estimating the metabolic activity of living cells. The test is based on enzymatic reduction of the lightly colored tetrazolium salt to its formazan of intense purple-blue color, which can be quantified spectrophotometrically. Under properly optimized conditions the obtained absorbance value is directly proportional to the number of living cells. Originally, the MTT assay was devised for use in eukaryotic cells lines and later applied for bacteria and fungi. As the mechanism of MTT reduction was studied in detail mostly considering eukaryotic cells, the lack of information resulted in generating a vast variety of MTT based protocols for bacterial enzymatic activity evaluation. In the presented article the main aspects of the MTT assay applicability in bacterial research were summarized, with special emphasis on sources of inaccuracies and misinterpretation of the test results.

1. Introduction

Microbiology practice utilizes a variety of indicators of bacterial physiological state, and therefore numerous definitions of the viability of cultured cells have been established. Most common techniques are based on reproductivity estimates, which assess viability of the population as the percentage of cells able to divide. The influence of factors that reduce microbial vitality but not necessarily affect multiplication can only be ascertained by detection of alterations in cells structure and function. These encompass observations of general morphology and specific membrane characteristics (membrane potential, integrity e.g. ability to exclude dyes, and cell motility) and biochemical profiling of specific molecules, including enzymes (Li and Song, 2007). In the context of this article, the prerequisite of culture viability is measurable enzymatic activity. Among numerous methods that associate the level of enzymatic activity with the condition of microbial population, assays based on biotransformations of tetrazolium salts have gained much popularity.

Over the years, tetrazolium salts have been incorporated in a variety of experimental protocols such as oxidoreductase activity measurements, subcellular localization of oxidoreductases, detection of superoxide radicals, *Mycoplasma* screening and – above all – microbial viability and growth estimation (Bernas and Dobrucki, 2000). Novel methods that utilize the reaction of tetrazolium salts reduction were invented and optimized in the late 20th and early 21st century. This research was accelerated due to growing knowledge of tetrazolium salts reduction mechanisms and synthesis of new compounds of this class.

One of the most common examples of tetrazolium salts used in bioassays is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). MTT-based procedures are widely applied to mammalian cell lines, bacteria and fungi. Notably, the reported protocols differ drastically in nearly all aspects of assay conditions (Young et al., 2005). Such incoherence is substantial especially when a novel set of experiments needs to be optimized. In the presented article, a summary of the most important aspects of the MTT assay of bacterial viability is reported.

2. General background

The term *tetrazolium* derives from the salts' chemical structure, as they are heterocyclic compounds with four atoms (*tetra*) of nitrogen (*aza*) in the tetrazole ring, defining this group of organic salts. The additional 1*H* or 2*H* in the nomenclature of tetrazolium salt describes the formal location of hydrogen atom (Fig. 1).

In general, tetrazolium salts in solutions are colorless or lightly colored compounds. The tetrazole ring (Fig. 2) can udergo enzymatic

Abbreviations: DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; MDR, multidrug-resistant (bacterial strain); MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SDS, sodium dodecyl sulfate; TTC, 2,3,5-triphenyl-2*H*-tetrazolium chloride; TV, tetrazolium violet, 2,5-diphenyl-3-(1-naphthyl)tetrazolium chloride; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt; WST-5, 2,2′-dibenzothiazolyl-5,5′-bis[4-di(2-sulfoethyl)carbamoylphenyl]-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)ditetrazolium, disodium salt; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt; XTT, 2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide inner salt

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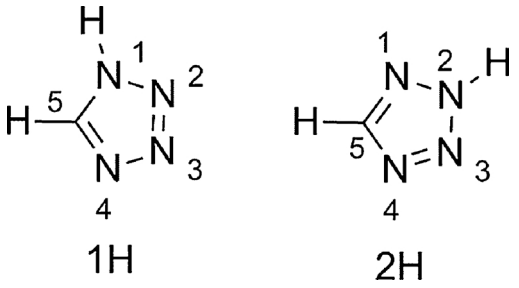

Fig. 1. Structure of the tetrazole rings characteristic for tetrazolium salts.

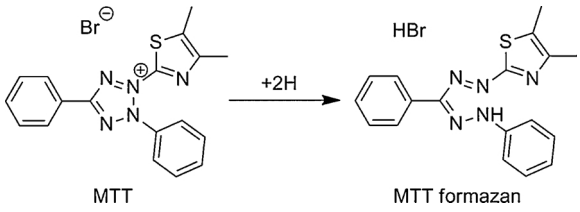

Fig. 2. Reduction of MTT bromide to its formazan.

reduction resulting in its cleavage. The product of the reaction is a corresponding formazan of intense color (Berridge et al., 2005).

Tetrazolium salts were first described in 1894. 2,3,5-triphenyl-2*H*-tetrazolium chloride (TTC) (Table 1) was the initial, prototypic compound that formed a water-insoluble formazan upon reduction. The subsequently developed tetrazolium salts, including MTT, were based on its structure (Altman, 1976; Tsukatani et al., 2008).

Chemical modifications of basic tetrazolium salts resulted in diversity of chemical properties and possible applicability of the newly generated compounds (Fedotcheva et al., 2017). Nowadays, generally used tetrazolium salts are MTT, inner salt MTS, NBT, XTT and a variety of WSTs (Water Soluble Tetrazolium salts) – WST-1, WST-5, WST-8 (Table 1). Tetrazolium salts with water-soluble formazans were developed in order to enable more reliable, continuous measurements of the progress of their reduction. They are, however, not obviously superior to salts that yield water-insoluble products - e.g. XTT and MTS require incorporation of an additional intermediate electron acceptor to advance production of their formazans (Goodwin et al. 1995). Also, cell lines and microbial strains may differ in their capability to reduce tetrazolium salts (Tachon et al., 2009). In microbial viability estimations MTT seems to be a more reliable choice, as it produces less background absorbance comparing to WST-5, WST-8 and XTT (Wang et al., 2010).

The first report on the possible application of MTT in estimating the viability of living cells was written by Mosmann (1983), who described the possible evaluation of mammalian cell lines response to cytotoxic compounds. This methodology was optimized for a mouse lymphoma cell line and was afterwards applied by researchers in countless experiments involving eukaryotic as well as prokaryotic cells. The application of an optimized MTT protocol allowed to measure culture growth and – in general - enzymatic activity. For many years, the MTT assay was one of the most frequently conducted tests of cells susceptibility (Liu et al., 1997).

Prior to Mosmann work (Slater et al., 1963) it had been long accepted, despite the lack of biochemical understanding of tetrazole enzymatic reduction, that catalysis of the reaction might occur only in living cells, as MTT salt could not be reduced by dead cells or erythrocytes. Early works of Slater et al. (1963), Altman (1976) and Burdon et al. (1993) indicated that the main enzymes capable of MTT reduction were mitochondrial dehydrogenases, their nonmitochondrial isoenzymes and even flavin oxidases. Recently, major role of eukaryotic NADH-dependent oxidoreductases in the reduction process was evidenced (Li and Song, 2007; Stockert et al., 2012). It was supposed that in eukaryotic cells MTT was reduced only in active mitochondria (Mosmann, 1983). Recently, this approach was questioned as mitochondria are generally not characterized with reducing properties and MTT salt has higher affinity to other organelle, especially the endoplasmic reticulum (Stockert et al., 2012).

The specification of the cellular site engaged in the MTT reduction was a crucial step towards the unraveling of the possible mechanism. In mixtures with eukaryotic cells, MTT is transported through the membrane by endocytosis (Lü et al., 2012). The net positive charge of MTT facilitates its cellular uptake (Berridge et al., 2005). Once in the cytoplasm, the MTT bromide is transported and accumulated in the preferable anionic reduction site - endoplasmic reticulum (Stockert et al., 2012). Here, accumulation of MTT salt has no short-term cytotoxic effect (Lü et al., 2012) and the reduction rate is dependent on glycolytic NADH production. Accumulation of the produced formazan is unfavorable, as microcrystals precipitation in the cytoplasm may increase cell permeability, induce the apoptosis or even mechanically disrupt the cell envelope (Lü et al., 2012). Detailed mechanism of formazan excretion remains uncertain (Stockert et al., 2012). Crystals depletion also may lead to mechanical cell injuries (Lü et al., 2012). Exocytosed formazan can form extracellular deposits and crystals may continue to build up due to extracellular MTT reduction (Berridge et al., 2005). Formation of formazan deposits on the outer cell surface may as well be facilitated by membrane-bound NADPH oxidases, which are known to produce metabolites that reduce other tetrazolium salts (Honoré et al., 2003).

Scientific literature provides the information mostly on the mechanism of MTT reduction in eukaryotic cell, briefly summarized above. As far as prokaryotic reduction of MTT is concerned the available research is scarce, however, interesting reports on other water-soluble tetrazolium salts can be found. In the work on tetrazole reduction in lactic acid bacteria it was proven using mutagenesis approach in *Lactococcus lactis*, that in growing cells menaquinones were necessary for electron transfer from intracellular NADH to tetrazolium violet (TV) substrate, whereas in resting cells TV was reduced directly by the membrane NADH dehydrogenases (Tachon et al., 2009). This determined the final location of formazan deposits either outside the cell or in the inner part of the plasma membrane, respectively.

The reduction efficiency depends on the kind of tetrazolium salt, even in a single bacterial strain – e.g. in the same work by Tachon et al. (2009) *Lactococcus lactis* strain inactivated for NADH dehydrogenase was unable to reduce tetrazolium violet (TV) but it was still active regarding MTT reduction. It is probably the effect of engaging separate electron transport chains (often simultaneously) for biotransformations of structurally different tetrazolium salts.

Thus, biochemical characteristics of tetrazole reduction in procaryota is influenced by structure- diffusibility characteristics of the tetrazolium salt, culture conditions, microbial species and the phase of strain growth. All these factors need to be taken into consideration as they may contribute to misestimating of assay results.

3. MTT in microbiological assays

Although studies of enzymatic tetrazolium salts reduction were first applied in eukaryotic cell research, this type of assays are now widely used for viability estimations of microbial cells. The mechanism of MTT reduction by bacteria is, however, still poorly understood (Tachon et al., 2009) and that creates a danger of its misinterpretation. MTT assay results indeed often do not agree with data obtained by other cell-growth or viability estimation methods. Nevertheless, numerous reports on bacterial strains tested for viability and respiratory activity with MTT reduction-based techniques were released throughout the years. Predictably, basic microplate susceptibility estimations are the most common practice (Table 2). In addition, MTT is applied in other protocols e.g. for multidrug-resistant bacteria determination (Foongladda et al., 2002; Montoro et al., 2005; Mshana et al., 1998), biofilm formation evaluation (Brambilla et al., 2014) or even indirect