Development of a colorimetric metabolic assay and initial testing of Photodynamic therapy photosensitizers for sterilization in the food industry.

by

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Abstract

This thesis implements a colorimetric assay to measure bacterial concentrations to test new photosensitizers. The colorimetric assay was developed from a single point fluorescence to a time kinetic resazurin assay suitable for bacterial concentration measurements.

Listeria monocytogenes were treated with Photodynamic therapy (PDT) as a method to decrease or sterilize the bacterial concentrations in an in vitro sample. PDT test were performed using Methylene Blue as reference photosensitizer, to test the effective behavior of the kinetic resasurin assay. Further PDT tests were useful to determine the bacterial sterilization that new photosensitizers are capable off, this photosenistizers were developed by Karen J. Brewer at the Virginia Polytechnic Institute. Photosensitizers are Mix Metal Supramolecular Complexes (MMSC) that differ from other drugs used in PDT due to the photosensitization process, where oxygen is not the most important element.

Resumen

En esta tesis se emplea un ensayo colorimétrico para medir concentraciones bacterianas, usandose en las pruebas preliminares de fotosensibilizadores. El ensayo colorimétrico se desarrolló apartir de medidas estáticas en el tiempo para ser usado con bacterias, terminando como un esayo con dinámica temporal.

Listeria monocytogenes fue tratando con Terapia fotodinámica (PDT) con la intención de disminuir o esterilizar *in vitro* concentraciones de bacterias.

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THE ONLY EASY DAY, WAS YESTERDAY

Because you are here, next to me

Lorenzo Perez Arenas.
Iktiuh Arenas Heredia.

Thank you, for all

Brenda Ornelas Lilge.

Contents

SYMBOLS

Symbol	*Description*
A	Absorbance
ALA	Amino Clavulinic Acid
$ATCC$	American Type Culture Collection
$BaCl$	Barium Chloride
D	a generalized photosensitizer used as example
$L.\ monocytogenes$	Listeria monocytogenes
$LEDs$	Light Emitting Diodes
$MMSC$	Mix Metal Supramolecular Complexes
MIC	Minimun Inhibitory Concentration
MB	Methylene Blue
NB	Number of Bacterias
$TMPyP$	Meso-tetra (N_methyl_4_pyridyl) Porphine Tetratosylate
P	Radiant power
PDT	Photodynamic therapy
PMH	Princess Margaret Hospital
$PMHCCl$	Princess Margaret Hospital Cell Culture laboratory
$PMHMl$	Princess Margaret Hospital Microbiology laboratory
Rh	Rhodium
H_2SO_4	Sulfuric Acid
S_1	Singlet electronic state
T	Transmittance
TB	Toluidine Blue
T_1	Triplet electronic state
WHO	World Health Organization
FDA	Food and Drug Administration of America

PARAMETERS AND UNITS

Name	*Description*	*Units*
Area	Surface	cm^2
Bacterial concentration	Bacterial density	CFU/ml
CFU	Colony Forming Units	Bacteria capable of forming colonies
Dalton	Atomic mass unit	Dalton
Ligth intensity	The radiant energy flux leaving the source in a given direction	mW
Power density	Power emitted, transfered, or received as radiation or received as radiation	mW/ml
Wavelength	Spatial period of an electromagnetic wave	Nanometers
Water purity	A measure of the components in milliQ water	$M\Omega/cm$
RFU	Photons due to fluorescence	Relative Fluorescent Units
Length	One dimensional distance	cm
g	Earth gravity constant	g
Light dose	Light energy per unit area administered in photodynamic therapy	J/cm^2
Concentration	Chemical concentration of a substance in a solvent	mg/ml
Optical Density	Measure of the attenuation of light in a substance	OD
Molar concentration	Chemical concentration depending on it's molar number	μM
PH	Measure of the acidity	Adimensional

Thesis structure

There is a problematic in the foodborne industry caused by bacterial handling. Such a problematic is based on the need for an assay to measure bacterial concentrations which can be trusted, fast, cheap and low waste producer. A different problem derived from foodborne illnesses is that bacteria have become resistant to antibiotics, therefore new methods to kill bacteria are needed. In this thesis two topics are explained related to possible alternatives to bacterial concentration quantification and sterilization.

The First chapter of this thesis deals with the introductory elements used as a guide for the reader to understand subsequent chapters. Such as basic light interaction with mater, bacterial biology and photodynamic therapy basics. In the second chapter materials, chemicals, bacterial aliquots, machines and techniques used are presented. The chapters three and four contain the results of the performed experiments. Chapter three deals with the kinetic resazurin assay development as a faster, less expensive method to quantify bacterial concentrations. The penultimate chapter shows a photodynamic therapy test for new designed photosensitizers. The last chapter has a compilation of the contributions of this work.

Chapter 1

Introduction

In this thesis two closely related topics are developed. The first one is a colorimetric method to quantify bacterial concentrations. Such a method is used as an alternative tool in treatment used to kill bacteria; it involves the use of light and a photoactivated chemical agent. The basic elements of the two areas of this thesis are shown in this introductory chapter: biological protocols, light mater interactions, and Photodynamic Therapy (PDT) antecedents.

1.1 Motivation

The ingestion of food is an essential part of daily life. Nowadays food is mainly processed in most nations on an industrial scale. If contamination occurs in the food handling industry bacteria are massively spread. Contamination can be caused by three main types of food contaminants: microbiological, chemical and physical [Kastbjerg, 2009]. The vast majority of outbreaks of food-related illness are due to microbial pathogens rather than chemical or physical contaminants.

According to the World Health Organization (WHO) foodborne illnesses are diseases caused by agents that enter the body through the ingestion of food. Over 250 different organisms cause general symptoms such as: diarrhea, abdominal cramps, and nausea [WHO, 2004]. To give a proper treatment, laboratory tests have to be done to identify the microbe, unless the illness is part of a recognized outbreak. Outbreaks of bacterial illness caused by foodborne infections produce enormous public health and economic looses to society all over the world. In USA the cost of medical attention due to foodborne illnesses, together with costs from recall of meat and dairy products can reach as much as 35 billion dollars annually (1997), a similar situation happened to Peru in 1991 with fish and fishery products costing 500 million dollars [Siegman, 2002].

1.1.1 Quantification of bacterial concentration

WHO recommends periodical microbial test of dairy and meat products following the protocols of the International Organization for Standardization (ISO) [Committee et al., 1996]. The policy can be either zero tolerance: were no microorganisms must be found in the samples, or more relaxed policies than allow as much as 1000 cfu/ml or cfu/g (cfu: colony forming units), for each 25 g that have being taken from the sample. The standard test for detection and enumeration of Listeria monocytogenes (L. monocytogenes) is the cfu method [Committee et al., 1996, Charpentier and Courvalin, 1999]. This method requires at least 82 H to be completed, since a set of repetitions has to be made. The need for a faster method that confirms the presence of L. monocytogenes in the industry is of vital importance, not as a substitute for the recommended test, but as an aid to identify it in a time efficient manner. Time efficient detection avoids contaminated samples do not leave the factory and it is avoided that food products to reach the public and cause listeriosis to consumers.

Susceptibility tests are of vital importance to approve new antibacterial techniques or chemicals. This tests require the measurement of cell concentration, in order to find the proper minimal dose to kill bacteria according to the chosen tolerance (for every 25 g taken of the sample, it is expected to find zero or at the most 1000 cfu/ml). The most common test relies on the CFU method, which is a long, tedious, biohazardous waste producer, but yet the most widespread standardized method. In this thesis We develop and optimize a fast colorimetric assay to measure cell concentration by using the capability of metabolically active cells to change Resazurin into a fluorescent compound.

1.1.2 Control of pathogens

L. monocytogenes was discovered by EGD Murray in 1924 following an epidemic affecting rabbits and guinea pigs in animal care houses in Cambridge [Kastbjerg, 2009]. It is the fourth on a list of the ten most destructing bacteria. L. monocytogenes causes human listeriosis with an incidence from 0.3 to 11.3 cases per million persons in the world according to the WHO [WHO, 2004]. A different study shows worldwide nonperinatal listeriosis cases from 1967 to 1999 had a mortality of 413 to over 1149 cases and the perinatal incidence is 0.6 to 4.1 per 10^4 births [WHO, 2004]. According to the Public Health Agency of Canada in 2008 listeriosis outbreak resulted in 23 dead people of 57 confirmed cases.

In a study with 44 patients that were treated with penicillin, the patients mortality rate was of 48% [Appleman et al., 1991], showing that antibiotics does not provide a complete treatment

against bacterial infections. According to WHO until 2004 the minimal number of pathogenic L. monocytogenes cells which must be ingested to cause illness in either normal or susceptible individuals is unknown. However, it has generally estimated to be less than $10^3 cfu/g$ [Kastbjerg, 2009].

Furthermore bacteria in general, and L. monocytogenes in particular, have become resistant to antibiotics. L. monocytogenes strains were first found to be resistant to antibiotics and isolated in France [Charpentier and Courvalin, 1999]. To overcome that problem, an alternative treatment to antibiotics to which bacteria do not develop resistance has to be developed. PDT has been used as bactericidal and proven not to cause resistance on bacteria [Romanova, 2003] for up to seven therapies.

It is important to note that not enough antibiotic for Gram-negative bacteria are being correctly tested (*in vitro*) fewer from the 15 required for 10 years period according to the FDA. Commercialized antibiotics take couple of years to be accepted by health organizations, since bacteria become resistant to them before the FDA testing recommended time soon antibiotics will not be a viable option to cure bacterial infections. Therefore new bactericidal techniques must be implemented, such as the performed tests in this thesis to a set of PDT photosensitizers used as bactericidals.

1.2 Bacteria

Bacteria are prokaryotic microorganisms that have not nuclear membrane. Generally bacteria are divided into Gram-positive and Gram-negative. The difference between them is on the outer membrane (or cytoplasm membrane) and their capability to retain certain dyes as crystal violet and iodine, in figure 1.1 a simple diagram shows the different membranes.

The cytoplasmic membrane is a double layer of lipid molecules (in Gram-negative cells), permeable to small, uncharged or hydrophobic molecules as long as there is an electrostatic potential gradient. A different mechanism for cells to acquire nutrients is by active transport through proteins located usually immerse in the lipid membrane.

Growth and reproduction

Bacteria need a suitable environment to growth and reproduce. For *in vitro* tests, bacteria are in a media that emulates natural environments, such media are called broth and they are prepared depending on bacterial needs such as nutrients, energy, water, oxygen (or the absence of it), appropriate levels of temperature and pH (range of 6 to 8) [Singleton, 2005].

Small populations of bacteria (inoculum) added to culture broth under appropriate temperature conditions result on an increased number of bacteria. The cycle of growth has four stages, and

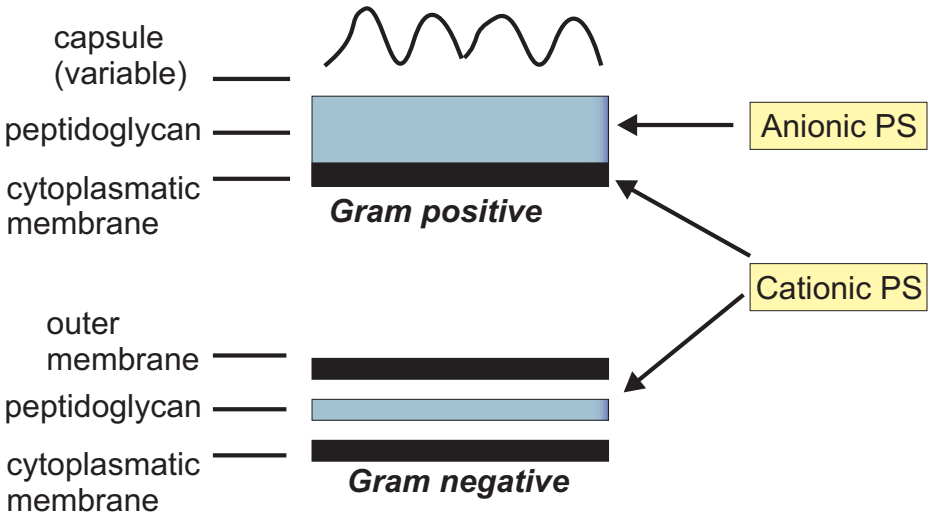

Figure 1.1: Schematic representation of bacterial membranes (modified from [Lihong V.Wang, 2007])

is similar for all bacteria: *Lag phase*: the adjustment period, immediate after inoculum has being transferred to media. *Exponential growth phase*: reproduction period, were growth is balanced and each cell doubles (reproduces) in a geometric progression of the number 2, within and identical units of time, known as doubling time. *Stationary phase* : dead and doubling processes have the same rate. *Death phase*: were bacteria die in a faster rate than they can double (reproduce) [Singleton, 2005, S. Gunsalus, 2007].

Doubling time, as applicable to exponential growth phase, is the time that takes bacteria to double its amount number by cellular reproduction. If there is one organism present at the beginning of the exponential growth phase, the total number present after a defined period of time *t* would be 2^n where *n* is the number of generations. To calculate *n* from the number of bacteria corresponding to different times N_0 and N_t if $N_t = N_0 \times 2^n$ [Singleton, 2005, S. Gunsalus, 2007].

$$DoublingT = \frac{\log 2 \times t}{\log N_t - \log N_0}. \tag{1.1}$$

In a bacterial population only a fraction of bacteria are metabolically active, consuming nutrients from the environment and reproducing. However not all viable cells in a population are metabolically active under all conditions, they have the potential to become activated under favorable circumstances. If so, they are referred to as inactive bacteria [J. Lehtinen, 2003]. One of the conditions is a damaged DNA, reason why bacteria continuously copy and repair DNA. When being under repair reproductive bacterial cycle is altered and they stop or slow down reproduction.

1.3 Interaction of light and matter

Bacteria as other biological compounds are made out of molecules. If molecules interact with light, energy will be transfer to molecules causing physicochemical effects. Some of those effects are absorption, spontaneous and stimulated emission or changes in the molecule itself [Prasad, 2003, Kastbjerg, 2009] . Interactions can be explained at a molecular level or taking mater as a bulk.

1.3.1 Interactions between light and a molecule

Atoms and molecules have quantized energy levels that correspond to electronic configurations. Were the distribution of the electrons of an atom or molecular entity are distributed over a set of one-electron wavefunctions called orbitals, according to the Pauli principle [Setlow, 1962]. Several states with different multiplicities may result from one configuration. Discrete levels are related to intraelectronic structure, vibration of internal atoms (in the case of molecules) and rotations of the molecule. Transitions are processes that take molecules from one stationary state to another by energy exchange. Energy can be absorbed or emitted by the molecule, in the last case in a radiative or nonradiative form [Hetch, 1998, Williams and Becklund, 1972]. Energy absorbed by any material is proportional to the energy of an absorbed photon per molecule. Which depends on the rate of incident photons (light power), molecular concentration of the material and the distance that light travels through the material as stated by the Beers law [Hetch, 1998, Williams and Becklund, 1972]. The amount of energy U that each photon with wavelength λ and frequency ν has is $U = \frac{hc}{\lambda} = h\nu$, where h is planck's constant.

Displacements of the valence electrons in the outer shells of a molecule cause a finite set of possible energy levels. Allowed transitions between levels are regulated by quantum theory, those transitions might be due to the absorption of specific photons giving rice to an absorption spectra of the given molecule or in the case of emitting photons an emission spectra [Hetch, 1998, Williams and Becklund, 1972].

1.3.2 Absorption and scattering

When an electromagnetic wave reaches mater two process can occur, either its energy is absorbed, or its direction of propagation is modified (reflection or scattering if the substance is composed of multiple objects with different refractive index). If energy is absorbed by mater, the process is ruled by the Beer Lambert Law: A beam with spectral radiant power P_0 and wavelength λ passing through a volume of mater (sample) of slice l (optical path length, cm, strictly should be per unit infinitesimal path length) and the absorption coefficient μ_a, is exponentially attenuated

[Prasad, 2003, Smith and King, 2000, Born and Wolf, 1987].

$$P(\lambda) = P_0(\lambda)e^{-\mu_a(\lambda)l} \tag{1.2}$$

The scattering coefficient μ_s is defined as the probability of photon scattering in a medium per unit path length. Optical scattering originates from light interacting with biological structures which range from cell membranes to whole cells. Photons are scattered most strongly by a structure whose size matches the optical wavelength and whose refractive index mismatches that of the surrounding medium.

The extinction coefficient μ_t, also referred to as the total interaction coefficient is given by $\mu_t = \mu_a + \mu_s$. The reciprocal of μ_t is the mean free path between interaction events. The Beer Lambert Law can then be represented in terms of the μ_t coefficient

Transmittance can be defined as the ratio of the transmitted spectral radiant power$P(\lambda)$ to that incident on the sample due to absorption and/ or scattering [Prasad, 2003, Smith and King, 2000, Born and Wolf, 1987]. Transmittance $T = \frac{P}{P_0}$
Percentage Transmittance: $\%T = 100\,T$

Absorbance is defined as:

$$A = log_{10}(\frac{P}{P_0}) = log_{10}(\frac{1}{T}) = -log_{10}(T) = log_{10}(\frac{100}{\%T}) = 2 - log_{10}(\%T) \tag{1.3}$$

Absorbance depends linearly on the total quantity of the absorbing compound in the light path through the sample since: $A(\lambda) = e(\lambda)lc$. Since transmittance obeys an exponential relationship with power P_0, it is convenient to use absorbance or Optical Density (OD) as standard measurement, OD is a measure of the attenuation given by absorption and scattering, given by the Beer Lambert Law with the total extinction coefficient. [Prasad, 2003, Smith and King, 2000, Born and Wolf, 1987].

Excited state relaxation processes to reach ground state

If a photon or photons are absorbed by a molecule with an energy that matches an exited state, an electron of the molecule will be elevated from the ground state to such an excited state. Excitation can also be caused by other mechanisms, like mechanical, or chemical. Molecules will return to it's ground state by three possible pathways: non-radiative, radiative and photochemical changes to the molecule. Lifetime is defined as the average time that an excited molecule spends in the excited state before returning to the ground state. A list of the possible relaxation process is illustrated in figures 1.2, 1.3 and [Banzo, 2010, S.E. Braslavsky and Verhoeven, 1996], each process is explained as follows:

1. Non radiative (figure 1.2):

   Relaxation to ground state takes place by transforming electronic and vibrational states into different electronic or vibrational states.

   a. Vibrational relaxation:
      The excited molecule decreases its vibrational energy within a single electronic state.

   b. Internal conversion:
      It is the isoenergetic transition between two electronic states with the same spin multiplicity, generally followed by vibrational relaxation.

   c. Intersystem crossing:
      It is the isoenergetic transition between two electronic states with different spin multiplicity, generally followed by vibrational relaxation.

2. Radiative relaxation (figure 1.2):
   Relaxation to ground state takes place by converting the energy excesses into bigger wavelength electromagnetic radiation.

   a. Fluorescence:
      Spontaneous emission of radiation upon transition between two electronic states with the same spin multiplicity.

   b. Phosphorescence:
      Spontaneous emission of radiation upon transition between two electronic states with different spin multiplicity.

3. Photochemical changes (figure 1.3):

   In photochemical changes typically two molecules must interact. One that after absorbing light (photosensitizer P) exchanges energy with the other molecule “A ”by a photochemical or photophysical action.

   a. Photochemical:
      The excited photosensitizer P^* exchanges energy with the molecule A reacting to form another exited complex B^*.

b. Photophysical:

The exited photosensitizer P^* exchanges energy with the molecule "A ", as a result the molecule achieves an exited state A^*.

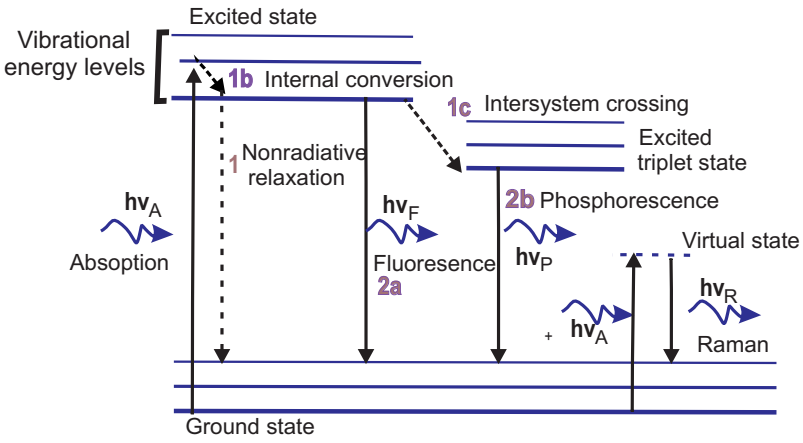

Figure 1.2: Jablonski energy diagram showing excitation and relaxation process: radiative and non-radiative (modified from [Lihong V.Wang, 2007])

Molecules (organic) in the ground state have paired electrons with total spin number $S = 0$ and spin multipicity $2S + 1 = 1$, named as singlet state. As an exception Oxygen molecule in ground state total spin number is $S = 1$, therefore oxygen spin multipicity is 3, named as triplet state [Prasad, 2003]. Chemical reactions occur only between biomolecules with the same spin multiplicity. When a molecular ground state is in a singlet state, the excitation of an electron to an excited state can produce either a state where the two electrons are still paired (singlet state) or where the two electrons are unpaired (a triplet state). Usually energy of the excited triplet state has less energy than that of singlet state, energy excesses are converted into heat [Prasad, 2003].

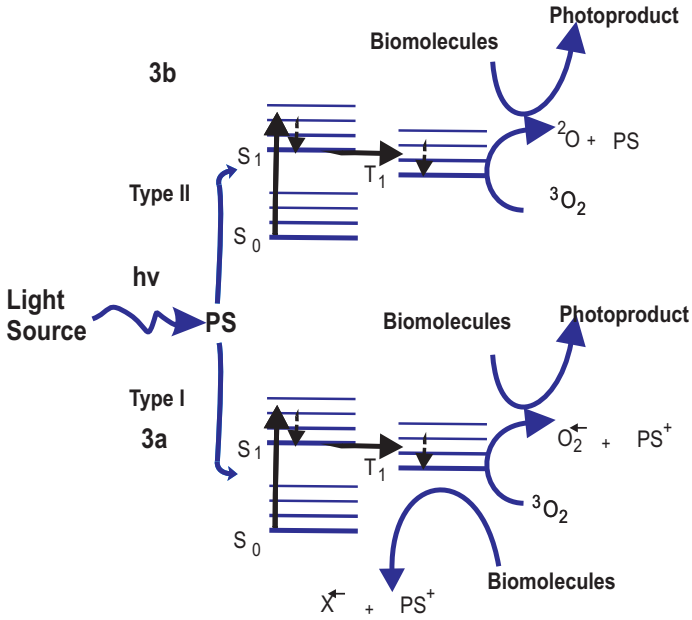

Figure 1.3: Jablonski energy diagram showing photochemical and photofisical changes, related to PDT photooxidation types (modified from [Lihong V.Wang, 2007])

1.4 Assays to measure cell concentration

To measure PDT reflectivity as a bactericidal it is necessary to quantify the bacterial cell number, or concentration previous and post bactericidal treatments. This quantification can be direct or indirect. Methods discussed in this section are direct (CFU) and indirect (colorimetric) [Singleton, 2005]. According to O. Toole direct methods are those were the organisms are dispersed either in or on an agar gel medium and incubated to permit the formation of visible colonies which are then counted [Toole, 1983].

1.4.1 Viable cell count: colony forming unit

In the CFU method an inoculum of 10 μl is spread or smeared over the surface of a sterile plate, the plate is incubated at 37 $^{\circ}C$ and the viable cell count is estimated from a) the number of colonies b) the volume of inoculum used c) the degree of dilution of the sample [Singleton, 2005, S. Gunsalus, 2007].

A dilution is achieved by inoculating 100 μl of the sample into 900 μl of broth (dilution 1), mixing it and plating it into an agar plate that will be incubated for 24 hours. From dilution 1 the process is repeated by taking 100 μl of dilution 1 and adding it to 900 μl of broth, mixing,

plating and continuing with the process. After 24 h colonies formed in the agar plates will be counted as representation of the bacteria transferred to the agar plates from the original inoculation. Some plated bacteria will have a confluent growth, some will have colonies too numerous to be counted and those more diluted will have too few colonies to be statistically valid. So the proper dilution has to be found to determine the bacterial concentration on the original sample, such that the formed colonies are in a range of (100 to 300) for a 10 cm diameter agar plate [Singleton, 2005, S. Gunsalus, 2007].

1.4.2 Optical density

If bacteria grows in liquid media, bacteria act as scatters, producing a turbid media. Turbidity increases with bacterial number, so a way to measure bacterial concentration is to measure absorption. Turbidity is a quick repeatable method that does not affects cells but it is limited to suspensions with low orders of bacterial concentration having as a lower boundary 10^7 cell per ml [Singleton, 2005, S. Gunsalus, 2007], furthermore the dynamic range is only one order of magnitude.

McFarland Standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with the McFarland Standard. A McFarland Standard is a chemical solution of barium chloride ($BaCl_2$) and sulfuric acid (H_2SO_4); the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial suspension of known concentration as indicated in the table [Singleton, 2005, S. Gunsalus, 2007].

McFarland Scale	(CFU) $(\times 10^6/mL)$	1% $BaCl_2$/1% H_2SO_4 (mL)
0.5	<300	0.05/9.95
1	300	0.1/9.9
2	600	0.2/9.8
3	900	0.3/9.7
4	1200	0.4/9.6
5	1500	0.5/9.5
6	1800	0.6/9.4
7	2100	0.7/9.3
8	2400	0.8/9.2
9	2700	0.9/9.1
10	3000	1.0/9.0

Table 1.1: *McFarland standars*

An alternative is the use of spectrophotometers, basing the results on McFarland optical den-

sity, for example for McFarland tube number 0.5 the turbidity corresponds to an Optical Density of $OD_{\lambda=600nm} = 0.123$, measuring the same OD for a culture in liquid media indicates that bacterial culture are equivalent to 2.8 $\times 10^8 CFU/ml$. It is important to consider that the OD of the bacterial sample has to be taken subtracting that of the broth where it suspended, which is easily accomplished in a dual beam spectrophotometer.

1.4.3 Colorimetric metabolic assays

An alternative to CFU and optical density techniques to count bacterial concentrations are colorimetric methods. Based on the use of fluorescent substances or genetically engineered microorganisms with a fluorescent marker that depend on the metabolic development of cells. The last one requires the incorporation of specific genes into the chromosomes of bacterial cells, this genes encode fluorescent proteins, that will be produced by active cells, therefore a quantification of fluorescent light leads to a cell concentration measurement. Examples of genetically modified cells are gfp-luxAB markers [J. Lehtinen, 2003], carboxil-X-rhodamine and Lucifer Yellow [Drevets and Elliott, 1995]. This techniques show an increased relative fluorescent signal with increasing metabolically active bacterial number and so increased levels of fluorochrome. If bacteria are exposed to a starvation process, some markers (luxAB) do not show the actual bacterial concentration due to their dependence on energy used by the cells. Being an efficient method it requires bacteria to be previously labeled with a fluorochrome producer gene. It is important to remark that in foodbone industry, contamination occurs by unlabel bacteria.

Alternative colorimetric assays are based on the use of dyes that once they interact with bacteria are changed into substances with other optical properties than those of the original dye. An example is Nitrate Reduction Assay, based on the ability of some bacteria to reduce nitrate to nitrite. The presence of nitrite can easily be detected with specific reagents, which produce a color change that can be measured spectroscopically [A. Martin, 2009]. A different example is the use of 33 % acetic acid which is changed and the result can be measured at 590 nm with an spectrophotometer. A further example is given by an oxidation reduction indicator called Resazurin, it is a blue nonfluorescent and non-toxic dye that becomes pink with fluorescent properties after being reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncolored and nonfluorescent) [S. D. Sarkera and Kumarasamyc, 2007]. It have being used for antimicrobial activity as an indicator of bacterial cell number present in milk [Fang et al., 1995]. The reducing activity of milk is multifactorial involving the activities of bacteria and somatic cells, but it can be caused by oxygen tension, ascorbic acid and sulfhydryl contents [Fang et al., 1995]. At [Mariscal et al., 2009] standard growth, curves were performed for different bacterial biofilm

concentration, and fluorescence was used as a relative measurement of the bacterial activity since resazurin is rapidly reduced in the presence of living cells. In general [Mariscal et al., 2009] work shows that for each standard growth curve the line comprised of a first phase with a high slope line, an intermediate phase of stability and a final phase of decline. This behavior is due to resazurin being transformed by the inoculum, when resasurin resources are over the decline phase starts. In bacteria biofilms it was shown that for different bacterial concentrations the first phase started at longer times for smaller concentrations independent of the strains used [Mariscal et al., 2009].

Resazurin has being used as a method to measure bacterial concentration on disinfectant action against bacteria in liquid media, the protocol used by [S. D. Sarkera and Kumarasamyc, 2007] only accepts false or positive measurements. They incubate bacteria with the disinfectant allowing it to act on bacteria for a period of time, then a fix concentration of resazurin is added, and bacteria culture are further incubated for 24 H. If resazurin change to resorufin (showing a pink color) then the disinfectant is inefficient. If the substance is blue regardless of the amount of resorufin, bacteria are not resistant to that dose of disinfectant. If the sample contains the disinfectant and regardless of the amount of resasurin concentration the color changes the bacterial strain is call to be resistant to that disinfectant [S. D. Sarkera and Kumarasamyc, 2007]. No spectrometric measurements were performed to discriminate resorufin quantities. This resazurin protocol does not allows the user to quantify the amount of bacterial kill.

1.5 Photodynamic therapy (PDT)

PDT uses a photoactivated drug (photosensitizer) that is not toxic if light is not applied, that is activated by low intensity light (in the visible or uv range), to initiate a physical or chemical reaction called photosensitization and creates highly reactive cytotoxic molecules. Such molecules are singlet oxygen or free radicals that interact and damage organic compounds that are part of biological structures.

After light irradiation, photosensitizer molecules absorb energy and are excited from ground state to an exited state (S_1 electron spins paired). Relaxation to the ground state can occur by fluorescence, internal conversions, heat loss or it may undergo a transition to the triplet exited state (T_1). By inter system crossing a further decay to the ground state by a photochemical or photophysical process due to intersystem crossing trough type II photodynamic action, creating oxygen reactive species like singlet oxygen, or alternative directly from S_1 to T_1 state creating hydrogen peroxide, superoxide or hydroxyl radical (Type I).

The application of PDT depends on the biological target, as targets abnormal cells like cancer

cells [Prasad, 2003, Banzo, 2010, Wilson and Patterson, 2008]. Or the destruction of healthy but unwanted cells as in cosmetic dermatology, in particular with Amino Clavulinic Acid (ALA). It is used to treat acne, remove hair, re-model tissue, skin damaged by the sun (actinic keratosis). In macular degeneration abnormal growth of leaky blood vessels in the choriocapillaris cause loss of central vision, it is treated by PDT to slow the vision loss [Wilson and Patterson, 2008]. Another possible target are infections due to bacteria, where PDT is used as a bactericidal technique in vivo [Romanova, 2003, Tardivoa et al., 2005, Wilson and Patterson, 2008].

1.5.1 Photodynamic therapy stages

Photodynamic therapy (PDT) has tree main stages, 1) application and absorption of the drug (period for selective retention), 2) light exposure and excitation of the photosensitizer 3) Degradation of biological compounds. Absorption and light exposure times variate depending on the specific application [Banzo, 2010, Wilson and Patterson, 2008, Maisch, 2007, O. and et all, 2006, Wilson and Patterson, 2008].

Oxidation of relevant biomolecules or photodynamic action happens throw two different mechanisms: Type I where the photosensitizer reacts with molecules as H_2O and forms free radicals and peroxides and type II where singlet oxygen is involved. Type II has being the most used mechanism to explain biological damage to cells, although bouth process (types I and II) occur simultaneously [Prasad, 2003, Banzo, 2010]. On reported tests, the used photosensitizers mainly undergo trough a type II and the presence of oxygen is required, becoming an important part of PDT. In type II reaction the photosensitizer acts as a catalysis. Good Oxygenation of cells and their surroundings becomes a problem to solve in order to optimize PDT effects, an alternative to it are drugs that use type I process as main oxidation path.

1.5.2 Mechanisms of photooxidation of biological compounds

Oxygen radicals and singlet oxygen damage lipids, proteins and nucleic acids that lead to severe cellular damage (like membrane rupture and leakage of cellular components) resulting in necrosis or activation of passive cell death: apoptosis [Prasad, 2003, Banzo, 2010, Zeina B, 2010]. It is usually accompanied by a loss of membrane integrity and associated with characteristic morphological changes such as organelle and cell swelling, malformation, loss of integrity of mitochondrial, lysosomal and plasma membranes and eventual breakdown of the cell, leading to release of its contents into the surrounding area [Banzo, 2010]

Damage to proteins occurs at specific sites, fragmenting peptide chains, altering electrical charge, aggregating cross-linked reaction products [A. Arora and Srivastava, 2002]. Damage to

Lipids (peroxidation) involves three steps: initiation, propagation and termination. The initiation is the result of the abstraction of an hydrogen from an unsaturated fatty acid and a hydroxyl radical, creating a carbon radical product that is capable of reacting with ground state oxygen in a chain reaction, known as the propagation step. Damage to nucleic acids appears due to the oxidation of nucleic acid, sugars or bases, degenerating nucleotides or sugar cleavage, giving rice to mutations or replication inhibition.

1.5.3 Photosensitizers

Photosensitizers can be delivered exogenous (direct aplication of the drug) or endogenous. In endogenous pathways a precursor is aplied and it is converted by a metabolism of the cells into a photoreactive compound called photosensitizer (an example is 5-ALA that is converted into protoporphyrin IX (PPIX)) [Singleton, 2005, Luksiene and Zukauskas, 1998].

There are a wide range of photosensitizers, some are synthetase by plants naturally used against microbial or eukaryotic organism. Commercial photosensitizers are in general cationic azine photosensitizers, cyanines, macrocyclic porphyrines and pthalocyanines [Wainwright, 1998].

Among azine photosensitizers some dyes can be found, such as Methylene blue (MB), Toluidine blue (TB), Neutral red, Proflavine, Acridine orange, Aminacrine, Ethacridine. Some organisms involved in dental and oral infections have been successfully treated with MB and TB, such as Streptococus spp. Candida albicans [Wainwright, 1998]. As well as L. monocytogenes [A. Arora and Srivastava, 2002]

Macrocyclic photosensitizers have being use to disinfect open wounds as well as treating cancer. Porphyrin compounds were the first to get approval to be used in clinical trials by de Food and Drug Administration (FDA). Its use on bacteria shows efficiency on Gram-positive bacteria only [Tardivoa et al., 2005]. Phthalocyanines have a high yield of singlet oxygen production, reason why they have been included in cancer PDT assays, as well as in blood disinfection due to their effective virus photoinactivation. In vitro tests were performed with bacteria such as Streptococcus sanguis and methicillin resistant Staphylococcus aureus and other Gram-positive and Grand-negative bacteria [Wainwright, 1998].

Certain physical and chemical characteristics are required for an ideal photosensitizer, such as high absorption at wavelengths where light can penetrate tissue but yet not damage it, for applications where tissue is involved in the range of $\lambda = [630, 900]$ preferably longer wavelengths for maximum light penetration in tissue [Wilson and Patterson, 2008]. Other features might be high chemical stability, the use of pure molecular species or molecules made out of subunits with specific and different properties. Ease and low cost of synthesis, as well as solubility in wa-

ter facilitates handling and administration, no toxicity should be found before light irradiation [Wilson and Patterson, 2008]. One important factor to consider is a selective uptake in critical biological targets like subcellular components. Due to the intrinsic PDT nature a long-lived triplet state at the activation time is the most important characteristic of a photosensitizer.

1.5.4 Dark toxicity and minimum inhibitory concentration

When performing a PDT experiment two aspects are important to consider, light and photosensitizer dose. If any of them affects the selected target by itself (no light irradiation in the case of the photosensitization) then PDT is not considered to be useful, since photosensitization has to be due to the conjunct effect. If light is not present and cells are killed then the photosensitizer used is considered to have dark toxicity.

The minimum dose needed to kill 99% of targeted cells is known to be the minimum inhibitory concentration. In PDT not only the drug dose needs to be found but the combination of it with a reasonable short light irradiation time.

1.5.5 PDT as a bactericidal technique

Tappeiner reported that toxic effects in the presence of light are not due to heat, Tappeiner's student Oskar Raab reported for the first time a PDT effect against Paramecium [O. and et all, 2006], it was the dependence on light of a toxic effect of acridine hydrochloride against Paramecia caudatum (Paramecium) [Luksiene and Zukauskas, 1998]. There are a wide variety of combinations of photosensitizers and bacteria tested in literature, reviews as [Luksiene and Zukauskas, 1998, Maisch, 2007, Wainwright, 1998]. In general photosensitizers undergo throw the type II process of photodynamic action. Bacteria are tested in concentrations close to $\times 10^7 cfu/ml$, with periods of incubation from 20 to 60 min for exogenous photosensitizers and 2 to 4 h for endogenous photosensitizers. Illumination periods for Minimum Inhibitory Concentration (MIC) are 30 to 60 min.

Bacteria are separated into Gram-positive and Gram-negative, their characteristics differ on the shape and permeability of the cell membrane [Maisch, 2007]. Neutral photosensitizers or negatively charged ones sterilize Gram-positive bacteria, due to the permeability of the membrane as they cannot permeate the more negatively charged Gram-negative outer membrane.

For Gram-negative bacteria positively charged photosensitizers or anionic photosensitizers linked to cationic polypeptides have shown an effective kill, but the use of precursors that open the protein channels allow photosensitizers to enter the cell and act against organelles or DNA [Luksiene and Zukauskas, 1998].

Photosensitizer advantages as antimicrobial agents in conjunction with PDT are: treatment efficiency is independent of the antibiotic resistant pattern of the strain. Bacteria have not show to develop resistance to photosensitization. Inactivation of bacteria can be achieved without causing harmful effects to surrounding cells of up to 5 logarithms of bacterial concentration reductions.

Amino clavulanic acid 5-ALA has being used on Gram-positive and Gram-negative bacteria, being more successful on Gram-positive ones and is approved to be use on patients by the FDA [Maisch, 2007]. *In vitro* test of some photosensitizers may not prove to be as successful in experimental models since there are interactions with tissue that change the actual PDT dose, like light attenuation or absorption and oxygen accessibility. Many of the studies using Staphylococcus reported that some strains may be quite resistant to photodamage compared to other closely related strains [K. O'Riordan, 2005]. It is essential that photosensitizer and light combinations reproducibly demonstrate good antimicrobial activity at least for some strains within a given species [K. O'Riordan, 2005].

1.5.6 Photosensitizers used with Listeria monocytogenes

L. monocytogenes has being treated with photosensitizers as Methylene Blue [Romanova, 2003] and ALA [I. Buchovec and Luksiene, 2010]. Three different photosensitizers were used by Romanova [Romanova, 2003] to inactivate L. monocytogenes: meso_tetra (N_methyl_4_pyridyl) porphine tetratosylate salt (TMPyP), toluidine blue (TB), and methylene blue trihydrate (MB), illuminated with 660 nm and an intensity of 7.6 mW/cm^2. All photosensitizers were incubated for 60 min and illuminated 60 min. TMPyP MIC is 6.25 ($\mu g/ml$) achieved 6 log of kill, MB MIC at 3.125 ($\mu g/ml$) resulted in 5 log of kill, the minimum inhivitory concentration MIC at 3/125 ($\mu g/ml$) also 5 log of kill.

It was reported that L. monocytogenes was inactivated decreased their viability in vitro (4 log) by endogenous photosensitizer 5-aminolevulinic acid (ALA 7.5 mM), after incubation for 0 to 2 h in the dark, followed by illumination with light at $\lambda = 400$ nm at an energy density of 20 mW/cm^2 for a period from 0 to 20 min for a total energies of 0 to 24 J/cm^2 [I. Buchovec and Luksiene, 2010].

Chapter 2

General Techniques, Materials and Methods

This chapter describes all biological and chemical compounds as well as optical techniques employed for the development of this project.

2.1 Bacterial aliquots

Bacterial cultures must be standardized, and obtained from type culture collections (as ATCC) to guaranty genetically pure and well defined bacterial strains. Bacteria need enriched media to live and grow. Culture media supply microorganisms with nutrients such as proteins and peptides. Depending on the particular bacterial strains a specific recommended media culture should be used, when comparing results to other studies.

2.1.1 Culture media

Broth is a nutritious ph-buffered culture medium that contains protein, peptones and other nutrients necessary to support the growth of fastidious (capable of infect other organisms) and nonfastidious microorganisms.

Bacteria for our experiments were growth in two different enriched broth infusion (culture media). All culture media were prepared by the Culture Media Facility of the Princes Margaret Hospital in Toronto Ontario Canada. They are prepared according to standard procedures. Culture media can be prepared as a liquid solution or mix with agar (1:1) to create a solid media appropriate for bacteria to form colonies, as used to determine the CFU.

Brain Hearth Infusion media (BHI)

Brain hard infusion is an enriched media, contains infusions of brain and heart tissue as well as peptides and salts. It is recomended by the American Type Culture Colection (ATCC) for the growth of L. monocytogenes. The mix of BHI components comes in a powder that has to be dissolved in deionized water by heating to the boiling point and maintain heat for 10 minutes while boiling. The mix is subsequently autoclaved to ensure sterility.

Luria Bertani media (LB)

Luria Bertani media (LB) contains a lower amount of rich materials compared to BHI, it contains yeast and bactotryptone as well as salts. LB media is not recommended by the ATCC to grow L. monocytogenes but it prevents bacteria to get into an inactive metabolic stage.

2.1.2 Listeria monocytogenes (L. monocytogenes)

The bacteria used for the experiments is Listeria monocytogenes and it was purchased from the ATCC, catalog number 7644. It is a genetically pure strain, bacteria does not show resistant to any antibacterial and belong to a single strain. L. monocytogenes is a Gram positive, aerobic bacilli bacteria that does not form spore, it exhibits a motile structure when grown at $27°C$, and can grow at low temperatures as low as $-4°C$. L. monocytogenes is a food-borne human pathogen that grows intracellularly. Bacteria are moderately susceptible to disinfectants such as a mix of 70 % alcohol and to antibiotics: penicillin, ampicillin, aminoglycosides, tetracyclines (resistance has been observed), chloramphenicol [D. and Marshall, 1993].

2.2 Resazurin

Resazurin sodium salt is a non-fluorescent, water soluble, non-toxic dye with an absorption peak at $\lambda = 600\ nm$. The molecular formula is $C_{12}H_6NNaO_4$ and its average molecular weight is 251.17 dalton. Resazurin used by us was purchased from Sigma-Aldrich, catalog number R7017.

Resazurin is an oxidation-reduction indicator dye. Metabolically active cells reduce resazurin to resorufin and dihydroresorufin by an intracellular mechanism. Resazurin was diluted in distilled water and purified water, purification was achieved by distillation, water was not autoclaved so it might contain microorganisms as bacteria. For of purified water the process involves a filter that eliminates bacteria, usually less than $100\ cfu/ml$ concentration of bacteria are left [1].

[1] Mili-Q water system user manual (Advantage A10,Millipore)

2.3 Photosensitizers

2.3.1 Methylene Blue

Methylene Blue (MB) have being used as a photosensitizer. Its principal PDT action path is by converting triplet state singlet oxygen into reactive singlet state oxygen [Appleman et al., 1991, Romanova, 2003]. MB as used in our experiments was purchased from Sigma-Aldrich, catalog number: $7220-79-3$. MB's average molecular formula is $C_{16}H_{18}C_lN_3S3H_2O$ and it's average molecular weight is 373.90 dalton.

2.3.2 Mixed Metal Supramolecular Complexes (MMSC)

As part of this project new photosensitizers were tested to see if they can be efficiently used in photodynamic therapy. Such photosensitizers are Mix Metal Supramolecular Complexes (MMSC), arranged as supramolecular assemblies of coupled subunits that perform different tasks.

MMSC are assembled such that individual tasks can be combined into more complex functions. Subunits can be multimetalic complexes, reactive cites and site-specific targeting all joined by covalent bonds. Other subunits can be attached without modifying physical properties as absorption band. Complexes were synthesized by Karen J. Brewer at the department of Chemistry, Virginia Polytechnic Institute and Blacksburg State University, [B. Storrie and Brewer, 2006].

MMSC were synthesized as oxygen-independent photosensitizers by coupling two Rhodium (Rh) or Os light absorbing units through polyazine bridging ligands to a Rh center. The presence of the Rh site introduces low lying metal-to-metal charge transfer states that are capable of visible light induced photocleavage of DNA via an oxygen independent pathway [B. Storrie and Brewer, 2006, Boerner and Zaleski, 2005].

- D1
  Molecular Formula: $C_{68}H_{52}N_{16}Ru_2Cl_7$
  Molar Mass: $1646.6g/mol$

- D2
  Molecular Formula: $C_{58}H_{42}N_{14}Ru_2RhCl_7P$
  Molar Mass: $1489.344g/mol$

- D3
  Molecular Formula: $C_{58}H_{42}N_{14}Os_2RhCl_4P_3F_{18}$
  Molar Mass: $1994.4g/mol$

- D4
  Molecular Formula: $C_{72}H_{56}N_{14}Ru_2RhCl_4P_3F_18$
  Molar Mass: $1998.2g/mol$

- D5
  Molecular Formula: $C_{68}H_{52}N_{16}Ru_2RhBr_2Cl_5$
  Molar Mass: $1735.6g/mol$

- D6
  Molecular Formula: $C_{48}H_{34}N_{10}RuRhCl_5$
  Molar Mass: $1131.5g/mol$

2.4 Power density measurements: Thermopile

The power density of the light sources were measured using a thermopile operating on the principle of sensing thermal radiation of the light sources Thermopiles are made of an array of thermocouples that measure a gradient of temperature, by measuring the change on the thermoelectric potential.

The thermopile detector has a circular area with a diameter of $D = 1.6\ cm$, dividing by the area of the detector.

$$Area = \pi(\frac{D}{2})^2 = \pi(\frac{1.6}{2})^2 = 2cm^2$$

The average of nine measurements was taken over the area of the light source. Two light sources were tested, a green light source with $46 \pm 2\ mW/cm^2$ at $\lambda = 530\ nm.$ and a red light source with $7.6 \pm 2\ mW/cm^2$ at $\lambda = 630\ nm.$ Both had arrays of LEDs with a bandwidth of 20 nm.

2.5 Spectrometric measurements

Various samples were prepared to measure absorption and fluorescence. Some experiments required the use of different volumes of L. monocytogenes in broth, so, samples were placed either in a cuvette or a 96 well plates. The appropriate spectrometer was used depending on the experiment since spectrophotometers were different for cuvettes or 96 well plates.

2.5.1 Spectrophotometers and spectrofluorometers

The machines used for cuvets were a dual beam spectrophotometer Cary 300 Bio UV-visible, Varian and a spectrofluorometer FluoroLog-3, Horiba. For the spectrofluorometer parameters were set as: Emission Spectra, integration time $0.1\ s$, slits $5\ nm$, $\lambda_{ex} = 560\ nm$, $\lambda_{em} = [565, 700]\ nm$. In the case of 96 well plates the microplate reader used was SpectraMax M5, Molecular Devices. Options used were Emission Spectra, integration time $0.1\ s$, slits $5\ nm$, $\lambda_{ex} = 560\ nm$, $\lambda_{em} = [565, 700]\ nm$.

2.5.2 Optical density of L. monocytogenes samples

L. monocytogenes in LB media was placed in one arm of the spectrophotometer (Cary 300), in the reference arm a cuvette with only LB media was used. The spectrophotometer was employed in absorbance mode, recording optical density in the rage of $\lambda = [400, 800]\ nm$. It is worthwhile to note that due to the particle nature of bacteria we measure attenuation and as seen on figure 2.1, it decreases as a function of increasing wavelength. The corresponding spectra was recorded for different concentrations of L. monocytogenes.

To achieve different concentrations OD measurements were made to the same sample letting bacteria to reproduce, concnetration increases with the time samples were left in the cuvette. As it can be seen on figure 2.1 the spectra of different concentrations ranging from a bacterial concentration $BC = [0.5, 9] \times 10^8 cfu/ml$ are similar in shape but as the concentration increases the spectra gets displaced on the positive vertical (OD) axes. Difference in the shape of the spectra can be seen with one order of magnitude of difference in the bacterial concentration.

When the bacterial concentration is lower than $9 \times 10^6 cfu/ml$ OD signals from the L. monocytogenes cuvette and LB cuvette are not statistically significantly different and the spectrophotometer can no longer distinguish scattering caused by bacteria as a function of wavelength, since OD values are the same for $5 \times 10^5 cfu/ml$ and $3 \times 10^4 cfu/ml$.

Including bacterial concentrations ranging from $2 \times 10^7 cfu/ml$ to $3 \times 10^8 cfu/ml$ a linear regression was computed for the correspondent Optical density (OD) values measured in the spec-

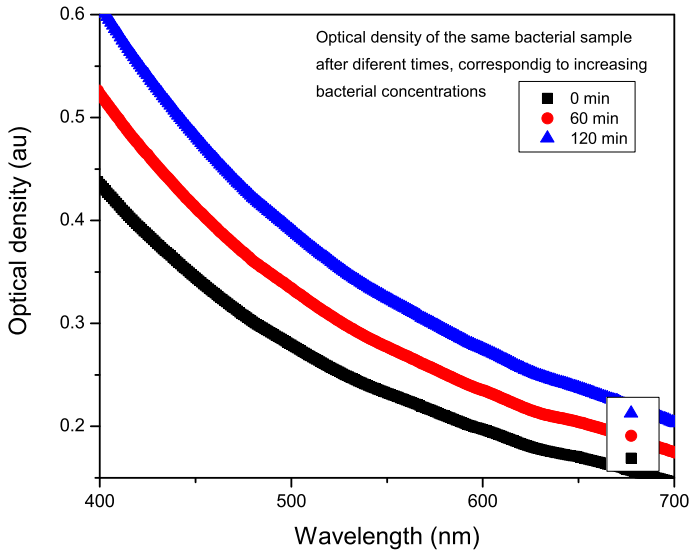

Figure 2.1: L. monocytogenes optical density spectra

trophotometer using a $\lambda = 600\ nm$ wavelength. In figure 2.2 experimental data were arranged in a OD dependent number of bacteria, a linear regression with a 95% of confidence interval is shown. The linear fit indicates that data follow an slope of 2.3 $\pm$ 0.16 NB/OD, with a $R^2 = 0.956$ indicating a good fit. Using figure 2.2 it is possible to calculate the number of bacteria interpolating or extrapolating the linear regression.

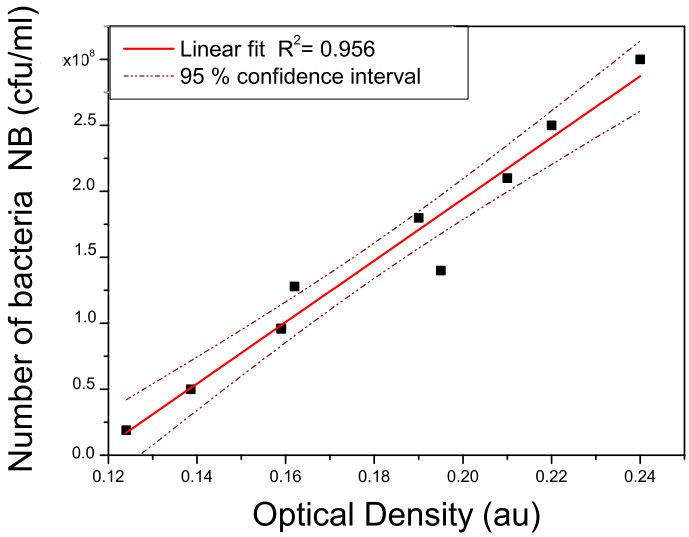

Figure 2.2: L. monocytogenes optical density linear relationship with bacterial number

2.5.3 Resorufin

Resorufin is a fluorophore that has an excitation wavelength at $\lambda_{ex-max} = 560\ nm$ and an emission maxima in the range of $\lambda_{em-max} = [580, 600]\ nm$, when dissolved in water. Fluorescence spectra were recorded for two samples with the same concentration, dissolved in purified and distilled water. Figure 2.3 shows a plot of fluorescence emission as a function of wavelength of resazurin at 0.01 mg/ml . Black and red lines correspond to a solution of resazurin that was dissolved in distilled water. The blue line indicates a dilution in pure water (Mili-Q water, $18.3 M\Omega cm$). Spectra show two fluorescent peaks, one at $584\ nm$ corresponding to Resorufin fluorescence maxima, indicating that the mix might have resorufin on it. The second peak and resazurin maxima is localized at $682\ nm$.

Resazurin was converted to resorufin by allowing it to be in distilled water for seven days before performing a third measurement. This process can be observed in figure 2.3 were the spectra shows two picks that are inverted with respect to those from the previous measurements.(black line in figure 2.3).

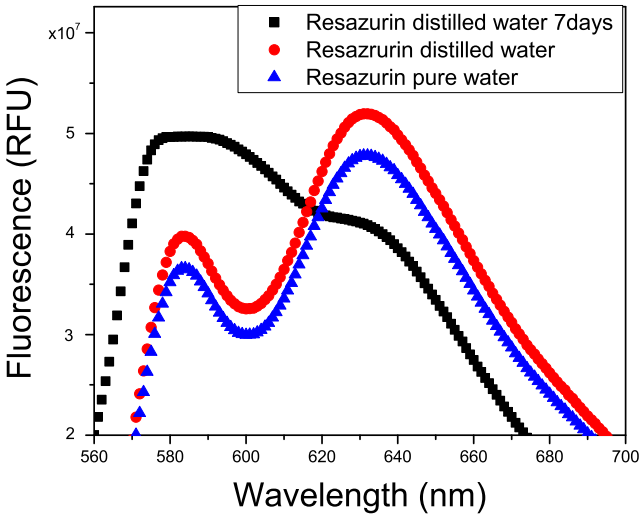

Figure 2.3: Fluorescence spectra of Resazurin and resorufin dissolved in water

2.5.4 Photosensitizers

Methylene Blue absorption spectra was acquired by diluting MB in autoclaved purified water $3.14\ \mu M$. An absorption spectra is show in figure 2.4, OD maxima is located at $\lambda = 665\ nm$.

Absorption spectra of the MMSC photosensitizers was acquired by diluting each photosensiticer as show in the next table using the spectrophotometer Cary 300. Absorption spectra are show in figure 2.5. Absorption maxima are located in the range of $\lambda = [500, 550]\ nm$ corresponding to a green section of the visible electromagnetic spectra.

- $D1 : 6.7\ \mu M$
- $D2 : 3.1\ \mu M$
- $D3 : 25\ \mu M$
- $D4 : 7.24\ \mu M$
- $D5 : 2.8\ \mu M$
- $D6 : 5\ \mu M$

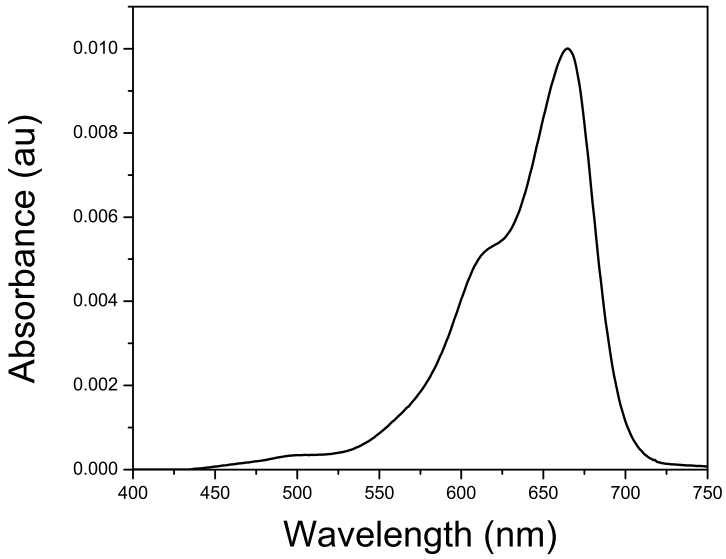

Figure 2.4: Absorption spectra of Methylene Blue diluted in water

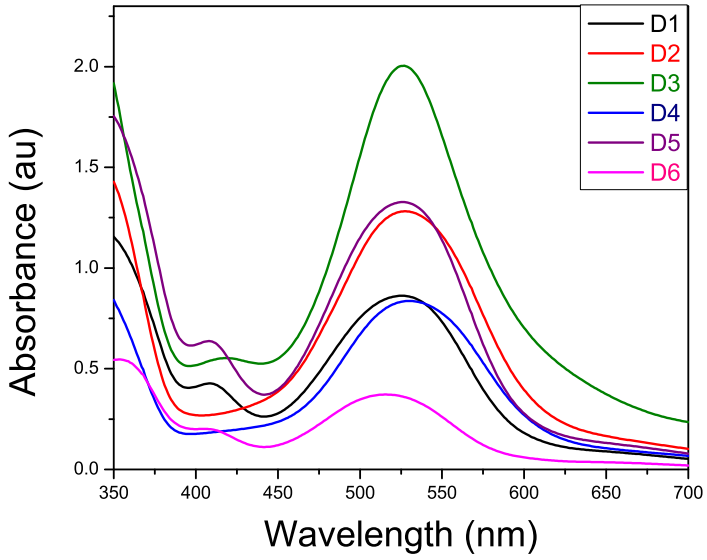

Figure 2.5: Absorption spectra of MMSC photosencitizers

Chapter 3

Kinetic Resazurin Assay

3.1 Introduction

As shown in section 2.5.2 L. monocytogenes aliquots were suspended in liquid media, and expose to visible light, becoming an example of a scattering sample. A method to detect the presence of bacteria in a liquid solution is the OD measurements of different concentrations of bacteria with concentrations from $0.01\ to\ 9\ \times 10^{9} cfu/ml$. If lower concentrations need to be measured a different method has to be used such as a colorimetric assays. Colorimetric assays such as resazurin have being used to measure cell concentrations of eukaryote cell lines [Anoopkumar and Carey, 2005].

By measuring resorufin fluorescent signal due to resazurin conversion as a part of the cellular metabolism, we developed a method to quantify bacterial concentrations.

3.2 Optimization

3.2.1 Fluorescence: excitation and emission wavelengths

In this section it is discussed the difference of the OD and fluorescence of resazurin mixed with 2 different samples: bacteria in media and media only (for LB and BHI).

The absorption spectra of resazurin was measured for two different mixtures, one for Luria Bertani (LB) broth and a second one for brain Hearth Infusion (BHI).

Resasurin was diluted in LB broth and L. monocytogenes in LB broth; OD values were measured using a two-arm spectrophotometer Cary 300. $3.5\ ml$ of LB media were used as the reference to eliminate spectral absorption effects of the media.

d c A sample of L. monocytogenes was prepared in LB media with an $OD_{\lambda=600} = 0.17$ corresponding to a cell density of 2×10^{8} cfu/ml (counted by cfu method). A cuvette with $3.5\ ml$ of

L. monocytogenes was used to reduce resazurin into resorufin by placing the mix into an incubator for 20 min. The concentration used was 0.01 mg/ml of resazurin with L. monocytogenes in LB broth. In figure 3.1 it is labeled as LmLB. Another cuvette was filled with 3.5 ml of LB media and Resazurin, using a dilution of 0.01 mg/ml of resazurin (labeled as LB in figure 3.1).

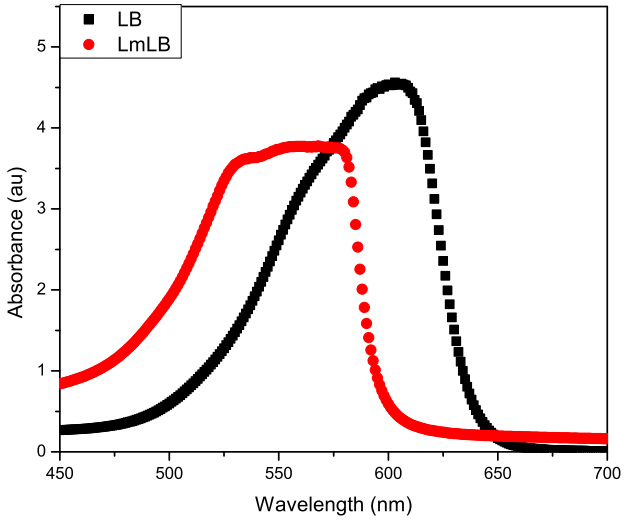

Figure 3.1: Absorption spectra: mix of resazurin in LB media (LB) and resazurin in L. monocytogenes and LB (LmLB).

The maximum peaks of absorption for LB-Resazurin is $\lambda_{max-abs} = 615\ nm$, and for LmLB $\lambda_{max-abs} = [550, 575]\ nm$. In the region between 600 and 630 nm the relative difference between the two spectra is bigger, than at the rest of the measured wavelengths, this part of the spectra could be used to differentiate samples by measuring OD. But the linearity and the dynamic range of the OD as a function of bacterial concentration was poor for all the possible wavelengths. Hence this method could not be directly applied, we change to a colorimetric assay based on a 96 well plate from which absorption (OD) or fluorescence measurements can be performed using a spectrofluorometer (SpectraMax M5, Molecular Devices).

Our goal is to quantify the number of bacteria in a sample, to do so an alternative approach to absorbance measurements is required such as quantifying the sample's fluorescent intensity. The fluorescent response of resazurin and resorufin were obtained by using broth media and L. monocytogenes with resazurin after 20 min. When recording signals that correspond to a concentration

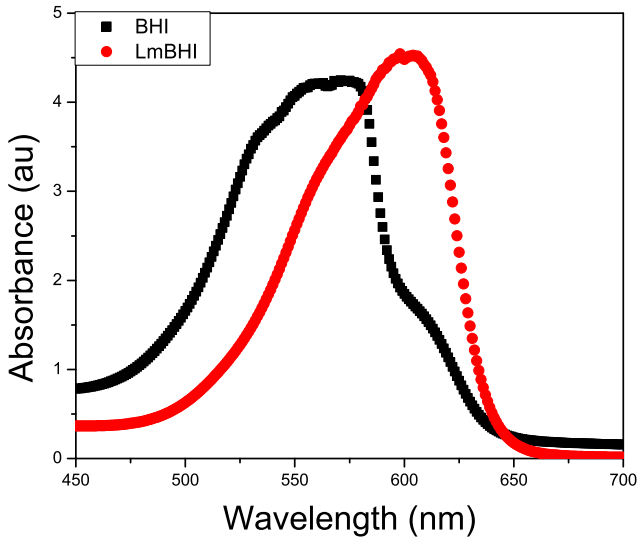

Figure 3.2: Absorption spectra: mix of resazurin in BHI media (BHI) and resazurin in Listeria m. and BHI (LmBHI).

of L. monocytogenes aliquots and resazurin the background signal is given by broth and its resazurin fluorescence. Since resazurin can be converted into resorufin by reduction in broth, it is necessary to find a broth were resazurin conversion is minimized.

L. monocytogenes preferably grows in liquid Brain Hard Infusion media (BHI). If another media, for instance LB media, is used to incubate L. monocytogenes, bacteria metabolism and grow rate will be slower than that in BHI. This affects resazurin conversion into resorufin, thus, BHI and LB media were tested to find which one has the highest fluorescent dynamic range as funtion of bacterial concentration when mix with resazurin. To do so, two cultures of L. monocytogenes (one per broth) were prepared and resazurin was added at a $0.01\ mg/ml$. Fluorescence spectra were acquired using dilutions of the samples previously described, in such a way that absorbance at $\lambda_{ex} = 560\ nm$ had an optical density of $OD_{max-560} < 0.1$.

The optimized fluorescence wavelengths, excitation and emission, of resorufin signals have to be found. Considering that resazurin in autoclaved broth converts it into resorufin, acting as black noise. A broth with a lower signal needs to be found, so two broths are tested in this section.

Fluorescence emission spectra of both broths in figure 3.3 have $\lambda_{max} = 583\ nm$ with a secondary peak at $\lambda = 637\ nm$ (labels BHI-Resazurin and LB-resazurin). For samples with

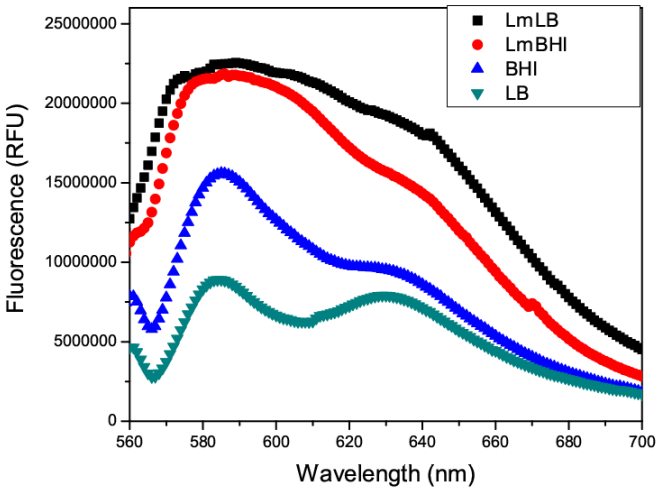

Figure 3.3: Fluorescence spectra: mix of resazurin in: LB media (LB) , L. monocytogenes and LB (LmLB), BHI media (BHI) and L. monocytogenes and BHI (LmBHI).

Listeria the peak is located at $\lambda_{max} = 585\ nm$ with a range from $\lambda = [575, 605]\ nm$, the signal slowly decreases at longer wavelengths. The ratio of the difference between listeria and broth over broth can be used as an indicator of the dynamic range of the signal in the media used. In figure 3.4 the computed ratio is presented. Further measurements will be restricted at the global ratio maxima that corresponds in LB media at $\lambda_{max} = 606\ nm$. Figure 3.3 shows relative fluorescence units (RFU).

As it can be observed in figure 3.4 the ratio of the difference between Listeria and LB broth over broth is at most 2.5 (corresponding to $\lambda = 606\ nm$), this can only allow detecting 2.5 orders of magnitude of difference for bacterial concentrations. Being this quantifications not sufficient for sterilization tests since at list 4 orders of magnitude are required to be tested.

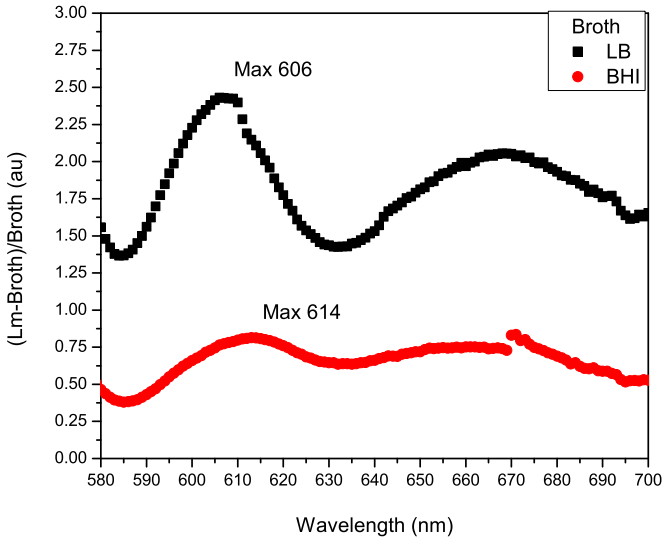

Figure 3.4: Ratio of Fluorescence spectra: $(Listeria - media)/media$, for BHI and LB broth.

3.2.2 Concentration of Resazurin

Different resazurin concentrations were continuously monitored while the resazurin conversion is in process the mixture is expose to standard resorufin excitation wavelength, and the fluorescence signal will be increased with time. This was recorded for 120 min for different concentrations of resazurin in the mixture as seen on figure 3.5.

To be able to chose the optimal resazurin concentration two aspects are important: maximize the time required for the fluorescent signal before it reaches a saturated stage, as well as minimize the concentration of resazurin. With the tested concentrations the smallest concentration with the longer time before saturation is 0.02 mg/ml, therefore it is the appropriate concentration to be used. As seen on figure 3.5 after 90 min for the lower resazurin concentration (0.01 mg/ml) fluorescent signal saturates the detector. In the case of 0.02 mg/ml saturation a change in the slope is observed at 120 min. For the chosen resazurin concentration the process of signal saturation for the given L. monocytogenes density $2.4 \times 10^8 cfu/ml$ starts after 150 min, in figure 3.6 marked in a box $\boxminus$ with horizontal lines.

A reliable assay must be representative as well as a good dynamic range. A single time point fluorescent signal is a prefered low labored effort measurement for bacterial concentration. How-

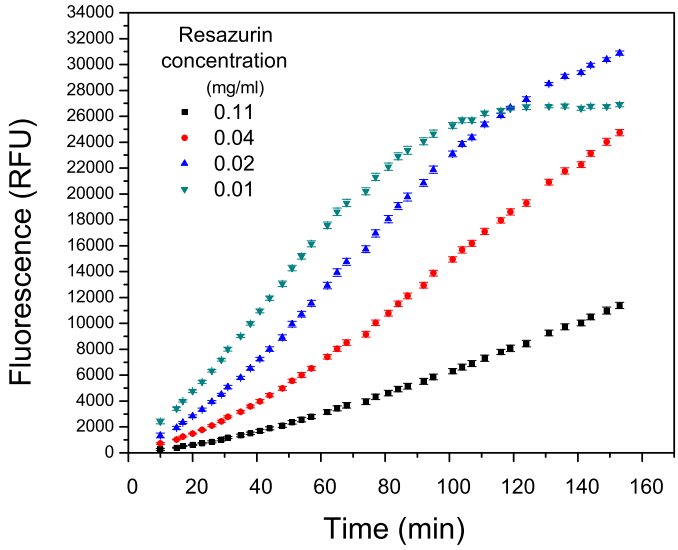

Figure 3.5: Fluorescence as a function of time for different concentrations of resazurin with $2.4 \times 10^8 cfu/ml$, $\lambda_{ex} = 560\ nm,\ \lambda_{em} = 600\ nm$.

ever the dynamic range of our resazurin assays seems to be compromised for concentrations lower than $1 \times 10^7 cfu/ml$.

As it can be seen on figure 3.6 fluorescent signals for concentrations lower than $1 \times 10^7 cfu/ml$ are to close to be distinguished, marked with a box □. In the region where the signal of bacterial concentrations is higher than $\times 10^8 cfu/ml$ is not saturated, signals from lower concentrations does not have a good dynamic range, nor are they linear $[\times 10^5, \times 10^6, \times 10^7, \times 10^8]\ cfu/ml$.

In figure 3.6, for bacterial concentration of the order of magnitude $10^7\ cfu/ml$ a change in the slope can be seen, marked with a box filled with squares ⊞. Such a change is due to the bacterial exponential grow, suggesting a doubling of the bacterial population at this point.

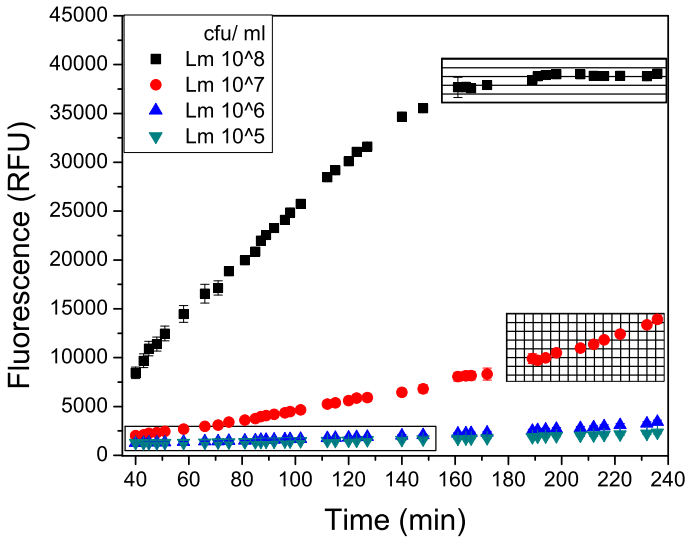

Figure 3.6: Fluorescence as a function of time for different values of bacterial density, resazurin concentration $0.02\ mg/ml$. Saturated section ⊟, exponential growth ⊞, data from two different bacterial concentrations that present similar values inside the error range □

3.2.3 Time kinetics

Resazurin change into resorufin is due to bacterial metabolic consumption of nutrients as well as due to a change in the population. When fluorescence increases due to metabolic consumption, only the slope of a linear regression of a time dependent fluorescence is constant if bacteria are still in exponential growth phase. If bacteria double their amount while fluorescent measurements are being performed a change in slope takes place. This change can be visualized in figure 3.6 for a concentration initially of $\times 10^7$ after 160 min changes the slope (in a box filled with squares ⊞). To identify if the change in slope is due to a higher bacterial concentration four rows of a 96 well plate were filled up with L. monocytogenes in LB media with a concentration of $2.5 \times 10^8 cfu/ml$. Resazurin was added on each row with a difference of one hour between them. Fluorescence was measured every five minutes over 240 min. In figure 3.7 results are plotted against time for four different series corresponding to different bacterial concentrations. It can be seen that for all series there is a time when all resazurin is converted into its product. It was found that the corresponding slope was higher for rows where resazurin had being added later. Since bacteria could multiplied in a one hour period, a higher number of bacteria were present when resazurin was added therefore

the slope is higher.

To be able to identify the point where fluorescent signal starts to plateau, a plot of the data shown in figure 3.7 reseting time to 0 every time resazurin was added to a new sample.Locating them with the time after which resazurin was added to the correspondent series is taken as 0, as shown in figure 3.8.

For the initial bacterial concentration the computed slope is $m_{0H} = 239 \pm 2\ RFU/s$ (RFU: relative fluorescent units), saturation is reached after 150 min. After one hour the slope increased to 1 hour $m_{1H} = 411 \pm 6\ RFU/s$ with a saturation point at min 100 min, the slope further increased to $m_{2H} = 526 \pm 13\ RFU/s$ after two hours and the saturation point move to 75 min, and finally reaching a slope of $m_{3H} = 1190 \pm 36\ RFU/s$ after three hours but the saturation starts at early as 45 min. For 3H the slope is steep and it reaches a maxim of bacterial concentration that the assay can resolve. Aspects to be consider are the fact that slopes can not be undefined as they are for vertical lines, moreover a statistically significant amount of data need to be computed. If the saturation point is closer to 0 min less and less points will be available. A reasonable data set can be achieved using 75 min as a limit.

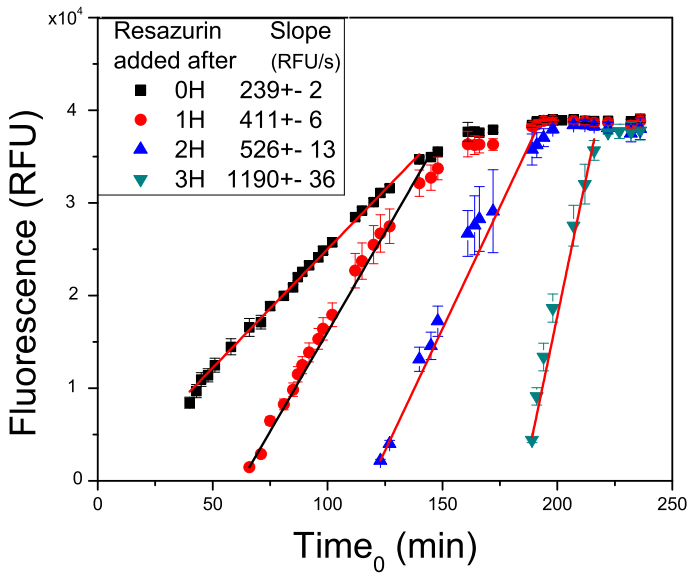

Figure 3.7: Kinetic resazurin $0.02mg/ml$ with different slopes for bacterial density of an order of magnitude of $\times 10^8$, using an absolute time scale

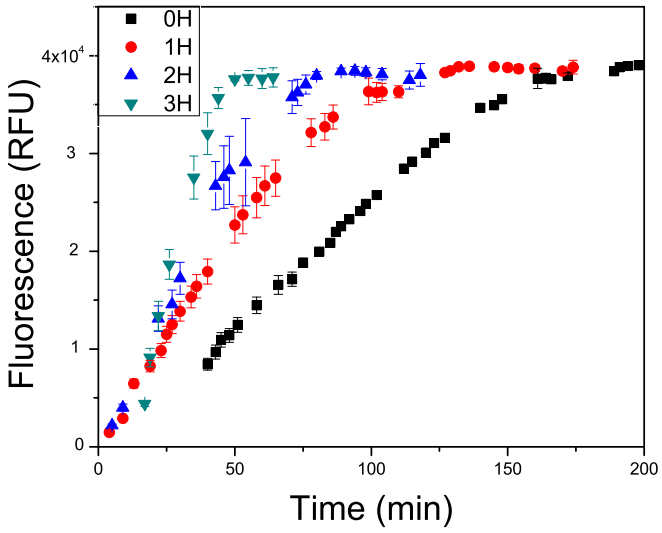

Figure 3.8: Kinetic resazurin $0.02mg/ml$, time scale corresponds to the moment when resazurin was added to the sample

3.2.4 Bacterial concentration as a function of time kinetic fluorescence

In the previous section we show that a single fluorescent reading does not have a proper dynamic range since fluorescent signal from low concentrations have small differences between each other in the rage where signals and bacterial concentrations higher than $2.5 \times 10^8 cfu/ml$ are saturated. Therefore fluorescent readings as a function of time ranging from 0 up to 120 min are appropriate for further analysis. A different approach to single point fluorescence is a time kinetic monitoring. Since the signal is linear for all the concentrations tested with orders of magnitude of: $[\times 10^5, \times 10^6, \times 10^7, 6 \times 10^8]$ cfu/ml, a trend was performed and the correspondent linear slope was recorded for different concentrations of bacteria. An example is shown on figure 3.9, and time scaled was restricted to a maximum of 90 min. The scale of the data in figure 3.9 do not show properly the data for concentrations $[\times 10^5, \times 10^6] cfu/ml$, a different plot is used to show details of those linear regressions, shown in figure 3.10. Bacterial number was determined by a CFU assay founding for this data the concentrations $[6.4 \times 10^5, 6 \times 10^6, 5.9 \times 10^7, 9.8 \times 10^8] cfu/ml$.

The correspondent bacterial concentration are plotted against the slopes of the kinetic re-

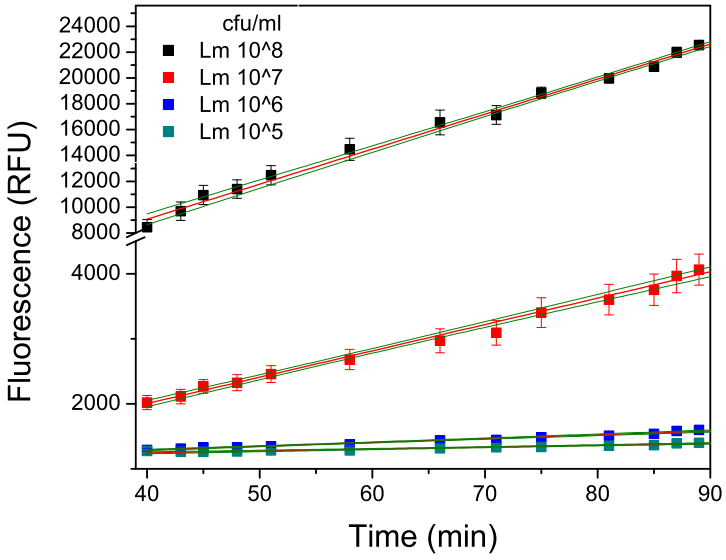

Figure 3.9: Kinetic resazurin assay for different bacterial concentrations using a resazurin concentration of $0.02mg/ml$, bacterial concentrations $[\times 10^5, \times 10^6, \times 10^7, 6 \times 10^8]cfu/ml$

sazurin assay (KRA), the plot in figures 3.9 and 3.10 (a zoom of figure 3.10 are of mayor importance due to it's implication in bacterial concentration measurements. Bacterial concentrations were measured using the CFU method and the KRA performed for each concentration, with this the slope of the line is used as a parameter to quantify the bacteria present in a liquid media. From figure 3.9 an increase in the slope can be appreciated as a function of increasing bacterial number. The relationship of the quantity of aliquots and the computed slope of the KRA is shown on figure 3.11.

It can be seen in figure 3.11 that the slopes are shallow for low bacterial concentrations, and for concentrations near $\times 10^7\ cfu/ml$ and higher changes in the slope increase compared to smaller bacterial concentrations. Being a point in bacterial concentration were our assay can not longer measure and so it seem that a stationary stage of bacterial number against slope is reached. Bacterial number dependence on the slope of the resazurin assay is not linear.

Figure 3.11 has a semi-log scale and data are distributed linearly, with a positive slope. From this is derived an exponential dependence of the bacterial concentration whit the kinetic resazurin assay slope. The relationship is shown on equation 3.1, were BC stands for bacterial concentration and SKRA for slope of kinetic resazurin assay.

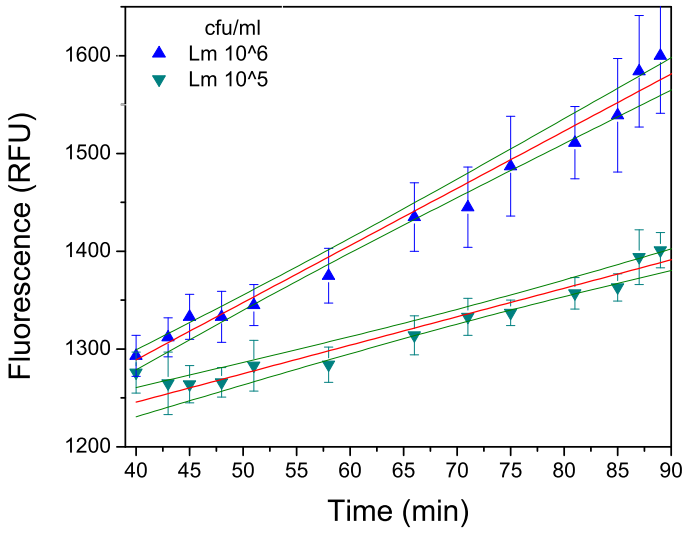

Figure 3.10: Kinetic resazurin assay for different bacterial concentrations using a resazurin concentration of $0.02\ mg/ml$, bacterial concentrations $[10^5, 10^6]\ cfu/ml$

$$BC = (1.438 \times 10^7)(e^{\frac{SKRA}{62.67}}) \tag{3.1}$$

By the aid of equation 3.1 an unknown sample can be calculated and the bacterial concentration computed.

3.3 Kinetic Resazurin assay protocol (KRA)

Independent of the specific format of the container used to keep L. monocytogenes in liquid media and the ml of it used, the protocol of the kinetic resazurin assay (KRA) will have fourth parts. Introducing resazurin at a fix concentration ratio to the media used, measuring fluorescence over time with given excitation $\lambda = 560\ nm$, and emission wavelength $\lambda = 600\ nm$ and their bandwidth, processing recorded data to be finally compared with those reported in figure 3.11 and using equation 3.1.

Resazurin was optimized for its use with L. monocytogenes in an initial concentration of $0.02\ mg/ml$, fluorescent measurements have to be taken every 10 min for as long as 90 min. Fluorescent excitation wavelength is $\lambda = 560\ nm$ and emission light is measured at $\lambda = 600\ nm$ by the use of a plate reader in fluorescence spectra bottom mode (spectrophotometer SpectraMax

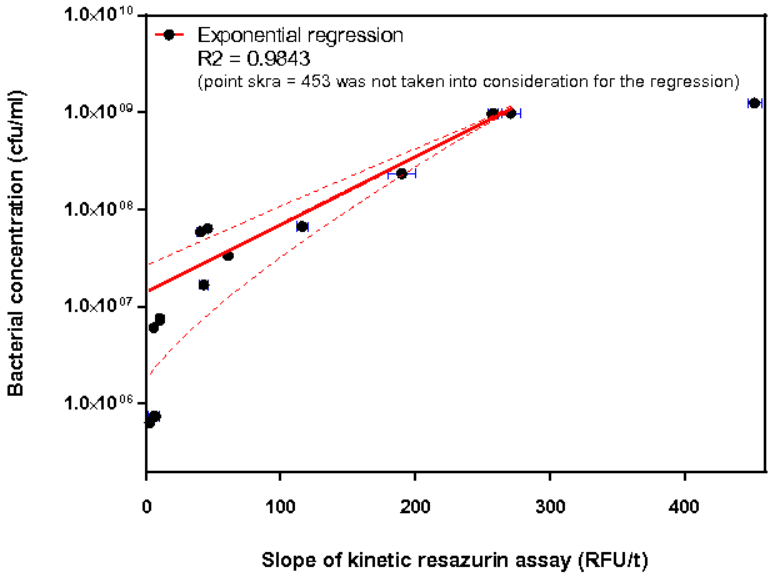

Figure 3.11: Bacterial concentration as a function of kinetic resazurin assay slope

M5, Molecular Devices). After the data acquisition a postprocessing period involves two steps: fitting data to a linear trend, and performing a comparison of the computed slope with the established values in section 3.2.4. To obtain the bacterial concentration of the samples equation 3.1 is used that relates the slope of KRA with bacterial concentrations as shown on figure 3.11.

3.4 Discussion

The useful Resazurin conversion into resorufin has being used to measure bacterial concentrations. An important characteristic of the developed assay is that it performs a time kinetic analysis of the fluorescence response of the resazurin conversion due to bacterial metabolism and overcomes the limited dynamic range of the static measurement. Optimum values of excitation and emission

wavelengths and the proper biological media associate with them were determined as defined in the last section.

It is important to state that in figures 3.9 and 3.10 there is a shift in the vertical axis, meaning that a background fluorescent signal is present. As presented in chapter 1 [1]. resazurin sellers state that the powder has some resorufin present. This lack of purity might explain resorufin fluorescence peak present in the resazurin fluorescent spectra as explained in section 3.2.1.

Bacterial concentrations as a function of slope of the kinetic resazurin assay are shown in figure 3.11, an exponential dependence was derived from it. In a different approach we can say that the slope (SKRA) changes whit the logarithm of the bacterial concentration. When resazurin is added to a bacterial sample the resorufin conversion will be limited for concentrations close to $5 * 10^8 cfu/ml$. Bacteria are still in the logarithm growth stage, so the metabolism and so the NDA+ production is not compromised. Therefore the limiting factor might be related to the diaphorases production and availability of components in the broth needed for resazurin reduction. when the concentration of bacteria increases in a media the biomass increases too, such as the products that bacteria liberate into the broth. Figure 3.11 shows that the dynamic assays KRA allows up to 4 logs of cell kill to be detected.

3.5 Conclusions

Colorimetric assays have being used as faster methods to quantify cell concentrations. In this chapter we developed a method based on the conversion of resazurin into resorufin a fluorescent dye, by metabolically active cells, in particular bacteria. Bacterial cells having a higher metabolism than eucaryotic cells converting resazurin with a higher rate. This made one point fluorescence measurements difficult to be achieved therefore a time kinetic approach was used. In such a way that the speed of the fluorescence is the factor to be consider to measure bacterial concentration. A compilation of different bacterial concentrations is shown on figure 3.11, same that is used as reference to compare unknown samples.

[1] Cell Biology study -proves: Reactive Oxigen study of the company Interchim

Chapter 4

Photodynamic therapy as a bactericidal technique

4.1 Introduction

Photodynamic therapy (PDT) has being used mainly for cancer treatments, as mentioned in section 1.5, however it can also be used to kill bacteria. Either if the photosensitizer in PDT is used as bactericidal element or to treat tumors, it is important to achieve a minimum or no harm to the surrounding tissue. In vitro tests have shown that porphyrin-based photosensitizers killed efficiently multiresistant staphylococcal strains and Estherichia colie without harming human keratinocytes and dermal fibroblasts [T. Maisch and Abels, 2005]. A different in vitro study involving the use of Toluidine Blue (TB) against Vibrio vulnificus was the preceding stage for an in vivo experiment were mice infected with the same bacteria show 53% survival after being treated with PDT, the dose was $100\ \mu g$ of TB/ml exposed to $150\ J/cm^2$ at $80\ mW/cm^2$, in vitro light dose was $100\ J/cm^2$ [Tak Wah Wong and Chuang, 2005]. It has being shown that PDT as a bactericidal technique can be implemented with low harm in the host tissue [T. Maisch and Abels, 2005] .

Besides from a non toxic effect on eukaryotic cells another important characteristic of PDT as a bactericidal technique is that bacteria have not shown resistance to it [T. Maisch and Abels, 2005, F. Giuliani and Molteni, 2010]. Some resistance was found when bacteria were incubated only with photosensitizer after 10 passages, no light exposure was performed and remained constant after 20 cycles. If bacteria were subject to photosensitizer and light exposure in between passages they presented the same susceptibility as the wild type strain to the first PDT treatment [F. Giuliani and Molteni, 2010].

This section uses the PDT protocol developed at Princes Margaret Hospital as in two separated

PDT experiments, the first experiment with a known photosensitizer and the second one is a test of recently developed photosensitizers.

An example of PDT as bactericidal application was shown by [Romanova, 2003] using Methylene Blue (MB) as photosensitizer. Demonstrating 5 log of bacterial kill, for $[3.125\ \mu g \backslash ml]$, L. monocytogenes was incubated with MB 50 min, exposed to 60 min to $6\ J \backslash cm^2$ at $1.67\ mW \backslash cm^2$. An experiment was performed based on the doses used in [Romanova, 2003] and compared to results derived by our Resazurin assay and CFU assay. To do so, PDT light exposure characteristics in [Romanova, 2003] were matched for our light source to determine equal effective excitation of the photosensitizer. Experiments were performed at Princes Margaret Hospital Microbiology laboratory (PMHMl). The effective light dose was calculated by multiplying the total light source power by the inner product of MB absorption spectra and the light source emission spectra (section 4.3.2).

Upon completion of this equivalence demonstration experiment an initial test of Mixed Metal Supramolecular Complexes (MMSC) mediated Photodynamic Therapy (PDT) efficacy as bactericidal with photodynamic therapy was performed at PMHMl . MMSC were developed by Karen J. Brewer, from the Department of Chemistry at the Virginia Polytechnic Institute and State University [B. Storrie and Brewer, 2006].

4.2 PDT protocol

This protocol is applicable for any compound used as photosensitizer: “D”. Incubation time of D and Light exposure can be variated according to D and the biological target. The bacterial strain used here is Listeria monocitogenes, and the presented preparation of the aliquots is specific for L. monocytogenes.

In this thesis the bacterial concentration used as reference is 1.5 $\times 10^8 cfu/ml$ for Photodynamic therapy (PDT) to inactivate Listeria monocytogenes (see section 2.1.2). Four samples using the same bacterial concentration were used, one as a reference, and the others for susceptibility test of light alone, drug alone to determine dark toxicity, and PDT therapy combining drug and light.

4.2.1 Standard amount of bacteria

A culture of Listeria monocytogenes (L. monocytogenes) was grown. 2 colonies from the stock agar media were taken and left overnight at $37°C$ in Brain Hard Infusion media (BHI) $10\ ml$. The period varied between twelve to seventeen hours. The culture was transferred to a new environment by taking L. monocytogenes in BHI and centrifugating over 5 min (Centrifuge: r = 5 cm, gravity 2500g). $3\ mL$ of the supernatant (BHI broth) were withdrawn and $3\ mL$ of Luria-Bertani media (LB) were added to the pellet bacteria, and the solution was toughly mixed. Listeria suspended in LB media was kept in the dark at $37°C$ for one hour, to let bacteria adapt into the new media.

After incubation in LB media, aliquots of Listeria were diluted with LB media until the solution reaches a standard optical density of $OD_{600} = 0.132 \pm 0.01$, equivalent to a cell density of $1.5x10^8 cfu/ml$ (cfu: colony forming units). OD values were measured using the dual beam spectrophotometer (Cary 300 Bio UV-visible, Varian). 3.5 ml of LB media in a plastic cuvette were used as the reference for 3.5 ml of bacterial concentration solutions.

Bacteria, LB media controls and drug concentration gradients in the 96 well plates are as follows: two different sterile, flat, clear polystyrene 96 well plates (Falcon 353072) were filled following the distribution of figure 4.1 using a multi channel pipette. 96 well plates columns 1 to 6 were filled with $100\ \mu l$ of Listeria in LB media per well. Columns 7 to 12, were filled with $100\ \mu l$ of LB media per well. Each row will be used for a different compound “D”or concentration gradient of the same compound “D”.

In the microplates, $600\ \mu l$ of Listeria (rows 1 to 6) and $600\ \mu l$ of LB media (rows 7 to 12) will be deposited per well. Depending on the total samples to be tested (n), a total of $C = n \times 600 \mu l$

of LB media were placed in a reservoir, from which LB media is pipetted into the corresponding wells with a multi channel pipette with 6 tips. C ml of Listeria were then placed into the same reservoir, and the corresponding wells were inoculated with $100\ \mu l$ each.

If different concentrations of a photosesitizer "D"were used, each concentration occupies a different row of the 96 well plate. $100\ \mu l/well$ of drug solution were added. As an example in figure 4.1, c1 stands for drug concentration 1, c2 for concentration 2 (a 2x dilution of c1). In this exemplification 2.5 ml of each drug concentration to be pipetted were placed into the same reservoir in stages, starting with the most dilute concentration c3.

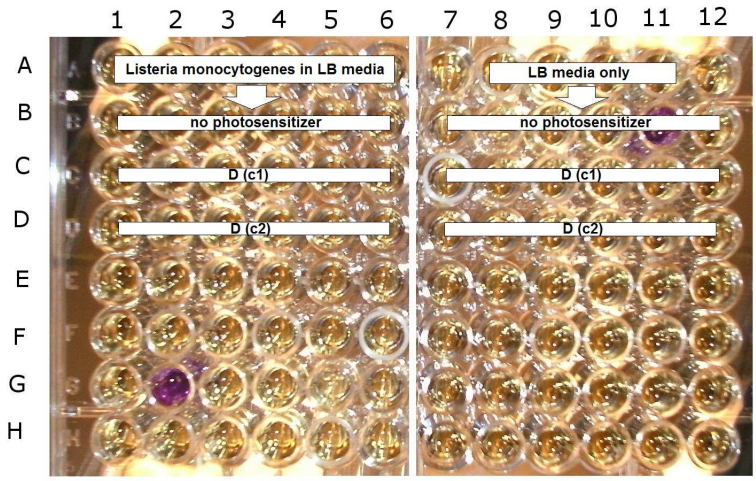

Figure 4.1: distribution of bacteria (Lis), Culture media (LB),photosensitizer (D) and dilutions of D (c1,c2) in a microplate

Drug "D" has to be absorbed by the bacterial cells in a so called absorption time (varied depending on "D"and bacterial strain). After LB broth, bacteria aliquots, and drug "D"were placed in the 96-well plates, they were taken to the incubator for 30 min at $37°C$.

4.2.2 PDT samples

A pair of 96 well plates has identical distribution of L. monocytogenes in LB media and LB media only with and without drug "D", according to figure 4.1. One plate was exposed to light wile the other was incubated in the dark. The reference sample has not being exposed to drug or light (ND NL). Samples that were incubated with drug "D"or its dilution series c, were labeled as D. If not drug was applied then the label is ND.

4.2.3 Light exposure

One of the 96 well plates was exposed to light (“L ”Label for samples) for “30 min ”wile the other plate was left in the dark (“NL ”Label for samples). Light sources were an array of diodes providing a fairly homogenous power density across the plate an a characteristic center wavelength. Two different light sources were used a green and a red one. The red light source with $27.2 \pm 2.2\ mW/cm^2$ applied at $\lambda = 630\ nm$. The green light source with $46 \pm 2\ mW/cm^2$ emitted at $\lambda = 530\ nm$. Power density was measured as indicated on section 2.4.

Bacteria handling takes place in the microbacteriology laboratory (PMHMl, Room 7_305) of Princes Margaret Hospital (PMH). Light exposure was perform at PMH in the Cell Culture laboratory (PMHCCl, Room 8 _ 305). And microplates have to be transported from PMHMl to PMHCCl. For transport, microplates were covered with a paper towel soaked with Virox 5 and inserted in a plastic bag which in turn is placed inside a closed plastic container cleaned with Virox 5. To prevent contamination of surfaces of PMHCCl with L. monocytogenes all surfaces were clean before and after microplates had contact with them.

Photosensitizers can be activated by sun light or white light, hence to prevent undesired exposure microplates remain covered with paper towels when not being manipulated or keep them in the dark.

4.2.4 Colony form Unit assay

After light exposure the surviving number of bacteria was quantified using a colony forming unit assay. The sample volume was pipetted up and down in order to achieve an homogeneous distribution of bacterial aliquot. $10\ \mu l$ of the bacterial aliquot were taken from each of the wells with Listeria with a pipette and were mixed with $990\ \mu l$ of LB media. From the resulted in additional dilution steps. Seeding on agar plates was performed by smearing lines with the loop on the BHI agar plate. Exposed agar plates were left in the incubator overnight and the colony forming units were counted (CFU) after 24 hours. The number of bacteria is equivalent to $CFU \times \frac{1}{dilution\ steps} \times \frac{1}{0.01}\ ml = CFU \times 10000$.

4.2.5 Metabolic assay: Kinetic Resazurin assay

Dilutions of Resazurin were prepared to arrive at a final dilution of $\frac{1}{24}\ mg/ml$ in each well of the 96 well plate. Using a reservoir and a multi-channel pipette, $50\ \mu l$ of resazurin were pipetted into all wells of both plates, first the wells with LB media only and then the wells containing Listeria. Both the non-irradiated and irradiated 96 well plates were placed in the incubator. Fluorescence

is measured every 10 min for 90 min after resazurin was added and mix with L. monocytogenes in LB. Excitation light was $\lambda = 560\ nm$ and emission light was measured at $\lambda = 600\ nm$ with a bandwidth of 9 nm. Fluorescence measurements were made using a spectrofluorometer (SpectraMax M5, Molecular Devices). Wavelength selection is by monochromators that were tunable in 1 nm increments. Parameters were set as: spectrum read mode, bottom well reading, 3 readings per well, speed normal and PMT low. Transportation procedure of the samples to the spectrophotometer is as for the light exposure section.

4.2.6 Drug and Resazurin concentrations

All solutions were prepared in the dark. Stock solutions were prepared the day before the experiment and the proper dilutions achieved when Listeria is being incubated in LB media.

Resazurin Stock was prepared with a concentration of $1\ mg/ml$. 10 ml of water (MiliQ water, $18.3\ M\Omega cm$) was used to disolve 10 mg of resazurin. The solution was sterilized by filtering it with a $0.22\mu m$ membrane filter (MillexGV), due to pipetting and filter absorption the final volume was in the range of 8 ml to 9.5 ml. A $\frac{1}{24}\ mg/ml$ dilution was reached by two series dilutions.

Photosensitizer were prepared with a stock concentration (c_0) from which dilutions were made depending on the the molar mass of the compound $(M_{(compound)})$ and the desired concentration; a solution is prepared and three dilutions of half the previous concentration were prepare for pipetting. Every dilution is achieved by mixing half of the last concentration with the same volume of LB media.

Stock solution were produced by $\frac{1}{100}$ of the molecular weight of the drug diluted in 10 ml of milliQ H_2O.

4.3 Methylene Blue: a case to compare Resazurin and CFU assays

4.3.1 Characteristics of the experiments

An experiment using Methylene Blue as photosensitizer was performed as referenced in a previous PDT assay described by [Romanova, 2003].

[Romanova, 2003] used Listeria monocytogenes Lm 353 (Canadian Research Institute for Food Safety) which was grown in brain heart infusion broth (BHI) at 30° C, to a concentration of $1 \times 10^6 cfu/ml$. Photosensitizer administration was performed at room temperature, while gentle shaking was provided.

In PMHMl Listeria monocytogenes Lm 7644 (American Type Culture Collection ATCC) was grown in brain heart infusion broth (BHI) at 37° C. When aliquots were in a logarithm growth stage they were transferred from BHI to LB broth, L. monocytogenes in LB broth was left to adjust for 1 H, and the bacterial stock solution was mixed with LB media to a concentration of $10^8 cfu/ml$. Bacterial suspension was inoculated into a pair of 96 well plates. Six different wells per sample were tested by adding $100\ \mu l$ of the final L. monocytogenes solution per well. Photosensitizer was added in $100\ \mu l$ solution per well. Incubation period was maintained at 37° C. Plates were not agitated during that time.

To test dark toxicity, one of the plates was not exposed to light and left in the incubator at 37° C while the other was irradiated. Measurements of bacterial concentration were performed by two methods. The colorimetric assay developed at PMHMl (see chapter 3) and one dilution with the CFU assay as a general reference. Resorufin Fluorescence after PDT was measured by addition of $50\ \mu l$ of resazurin per well. Multiple readings for a period of 90 min were used to compute a linear regression slope [RFU/min] of fluorescence time kinetics. The slope of the linear regression corresponds to a specific concentration of bacteria as shown in the calibration experiments. Results of Photodynamic therapy units were expressed as cfu/ml.

For a direct comparison with [Romanova, 2003], we wanted to administered the same PDT effective light dose. Table 4.1 shows characteristics of both systems. With our equipment permiting only the adjustment of the irradiation time, to deliver the same PDT light dose. Available data are optical power density, useful optical power density and proper energy dose to derive the required exposure time.

geometrical approach. It was found that the percentage of light used from each source was

22 % for 4.2 and 10 % for UHN of the original optical power density. Since the optical power density of the light boxes is 7.6 (mW/cm^2) for [Romanova, 2003] and 27.2 for the UHN light source, the corresponding useful power density are 1.67 (mW/cm^2) for [Romanova, 2003] and 7.3 (mW/cm^2) for our light source.

Characteristics of light sources		
Parameters	a)	b)
$\lambda(nm)$	660	630
Optical power density (mW/cm^2)	7.6	27.2
Percentage of light	29	10
Useful power density (mW/cm^2)	1.67	7.4
Irradiation time (min)	60	13, 50*
Light dose (J/cm^2)	6	6, 26*
MB concentration (μM)	8.4	16.9
Initial bacterial concentration (cfu/ml)	10×10^6	10×10^8
Effective pdt dose $(\mu MmJml/cfucm^2)$	6.66×10^-3	1.34×10^-4

Table 4.1: *Characteristics of the light sources a) [Romanova, 2003] and b) used at PMHMl (* Second PDT administration)*

Light dose adjustments depend on the optical properties of the photosensitizer, and the available light source of the correspondent experiment. Absorption spectra of the photosensitizer correlated with the emission spectra of the light source give the effective excitation wavelengths. The light dose is calculated by multiplying the PDT effective power density by the irradiation time in seconds. If seen as vectors this operation can be easily computed as a dot product, resulting in a scalar number named as efficacy of the excitation. PDT efficacy of the excitation is defined as the superposition in wavelength from the absorption spectra of the photosensitizer and the emission spectra of the light source. For [Romanova, 2003] a total PDT light dose of $1.67\ mW/cm^2 \cdot 3600\ s = 6\ J$ was reported. If the same Light dose has to be applied with our light source the resultant irradiation time is $13.5\ min$. Figure 4.2 shows normalized light source emission spectra of [Romanova, 2003] our sources as well as methylene blue absorption spectra. Diode arrays were considered as light sources with a spectral Full Width Half Max of $11\ nm$; for [Romanova, 2003] centered at wavelength $\lambda = 660\ nm$, and $\lambda = 630\ nm$ for the PMHMl light box. In figure 4.2 such light sources are represented together with methylene blue absorption spectra with it's normalized spectral area.

Attention must be focused at the wavelengths were methylene Blue can be exited by a given PDT light source. In order to determine a light source's PDT activation efficacy and with this the useful power density, an inner product needs to be computed of the normalized spectra of

photosensitizer absorption and light source emission. The result is the percentage of the total emitted power that can be used to activate the photosensitizer (useful power density). Thus the total light dose is calculated by multiplying the irradiation time in seconds by the useful power density.

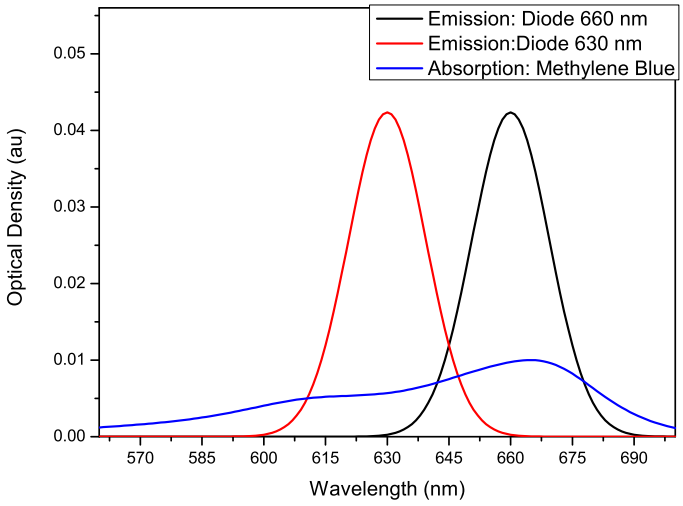

Figure 4.2: Absorption spectra of Methylene Blue. Emission spectra of two light sources with maxima of emission at $\lambda = 660\ nm$ for [Romanova, 2003] and $\lambda = 630\ nm$ used at PMHMl

In a separate analysis for each emission spectra a correlation with MB was performed using an inner product of the vectors. Calculating the correspondent percentage of light from the light box that effectible exited MB. As analogy from the projection of a vector "a" into a vector "b" in a

4.3.2 Comparison of results

Romanova et al. found that using a Methylene Blue concentration of 3.125 $\mu g/ml$ and 793 mJ/cm^2 resulted in five logs of bacterial inactivation [Romanova, 2003].

Reproducibility of the conditions used by [Romanova, 2003] was performed using the light dose calculated in section 4.3.1 and the same bacterial concentration of 2.5×10^6. Les than 1 log of bacterial kill was achieved, that resulted in a concentration close to 1×10^5, same that is outside of the valid range for our assay, as explained in section 3.2. Due to this restriction a modification on the bacterial concentration had to be made to 2.5×10^8 . An initial test show no effective kill,

the parameter that could be modified was the irradiation time which was extended to 50 min with the corresponding results shown in figure 4.3 and table 4.2.

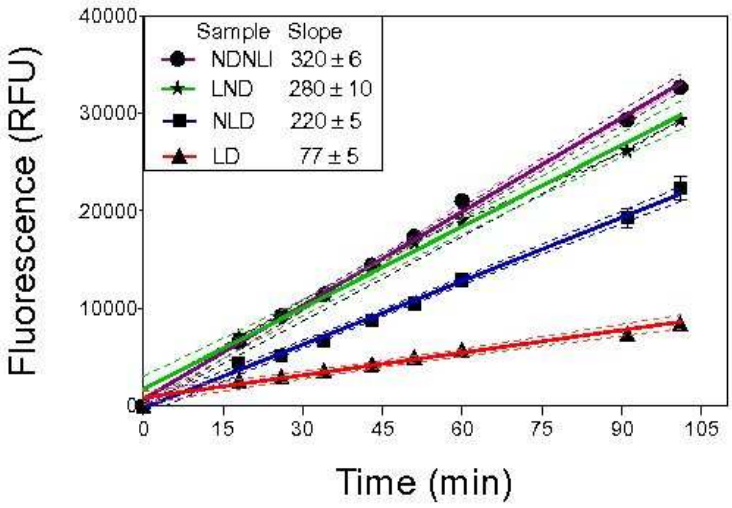

Figure 4.3: Fluorescence kinetics for Methylene Blue PDT experiment, Control NDNL, light toxicity LND, dark toxicity NLD, PDT toxicity LD

The figure 4.3 and table 4.2 shows results of a PDT experiment with Methylene Blue and characteristics listed on b) part of table 4.1. In figure 4.3 the fluorescent change as function of time is plotted with the correspondent slope for each sample. From the slope, bacterial concentration was calculated (refer to section 3.2.4). As separate control, a CFU assay was performed using one dilution only as indicated by colorimetric assay.

	NDNL	DNL	NDL	DL
Slope $(\frac{RFU}{min})$	320± 6	220±5	280±10	77±5
r^2	1	1	0.99	0.97
Kinetic Resazurin assay $(\frac{cfu}{ml})$	1.3×10^9	9.6×10^8	1.4×10^9	6.4×10^7
CFU $(\frac{cfu}{ml})$	1.6×10^8	1.5×10^8	1.4×10^8	1.3×10^7

Table 4.2: Comparison of CFU and kinetic Resazurin (chapter 3) assays of PDT experiment with MB performed at PMHMl. The slope of the linear regressions are shown $RFU = \alpha time$

Table 4.2 shows a comparison of the CFU and Resazurin assays. If Metylene Blue was in the solution it is marked as “D ”, if no photosensitizer was applied then “ND”. Samples that were not

exposed to light are labeled with “NL” otherwise “L” is used. Reference sample (NDNL) was not exposed neither to light nor to photosensitizer. Light effect (NDL) and dark toxicity (DNL) were not significant, as opposed to pdt treatment (DL) that show one and a half log of kill.

As table 4.2 shows, light had no considerable effect on L. monocytogenes, since the slopes from reference sample; NDNL and NDL are similar. No considerable effect can be reported due to dark toxicity. From the results of table 4.2 we can see that resazurin assay overestimates bacterial concentration in respect to the CFU assay. CFU shows in general a PDT kill of one log more than our assay.

The Initial bacterial concentration used by us 2.5×10^8 and for [Romanova, 2003] 2.5×10^6 affected the effective pdt dose in $(\mu MmJml/cfucm^2)$ were the difference has one order of magnitude: 6.66×10^{-3} [Romanova, 2003] and 1.34×10^{-4} UHN. A lower bacterial kill can be expected since the PDT dose per bacterial concentration is lower for the experiments at PMH. A factor that was not considered is the shift in wavelengths of the light sources, MB might produce more efficiently singlet oxygen or reactive species for some wavelengths independently of the absorption spectra. A possibility is that MB might be highly absorbing at a wavelength that can produce low reactive species compared to another wavelength were MB has low absorption but high reactive species production. L. monocytogenes used by us is a wild strain that have not being genetically altered nor is it antibiotic resistant. In [Romanova, 2003] it is not specified if some alteration has occurred, since L. monocytogenes was isolated from a different source.

4.4 Photosensitizer Mixed Metal Supramolecular Complexes for inactivation of bacteria

This section discuses the initial test of Mixed Metal Supramolecular Complexes (MMSC) as bactericidals with photodynamic therapy.

Tested drugs D were incubated with Listeria monocytogenes for 30 min after incubation in LB media. One 96 well plate was exposed to light for 30 min, the other microplate was left inside the incubator. Using CFU and Resazurin assays the effectiveness of PDT, dark toxicity and light effect were tested. PDT experiments were performed by separated blocks of tree drugs that shared the same drug concentration. Table 4.3 (shown at the end of the chapter) shows results from drugs 1, 2 and 3 ($250\ \mu M$). Table 4.4 (shown at the end of the chapter) shows results from drugs 4,5 and 6 ($50\ \mu M$) [1]. Each experiment was performed on different days therefore the samples used as control are different in tables 4.3 and 4.4.

Results of bacterial concentration are based on the kinetic resazurin assay, as mentioned in chapter 3. The slope of the linear regression of the fluorescent signal has to be correlated to standard measurements. How well the linear regression approaches the given data depends on the slope. Some of the variables that affect the slope of the kinetic resazurin assay (skra) are the wavelengths use to excite the photosensiticer, saturated signal, as well as changes in the slope due to bacterial growth like exponential growth . When data presented saturation and exponential behaviors that part was not included in the linear regression range (see optimization section in chapter 3).

Effectiveness of MMSC used as photosensitizers for sterilization would be expected not to have dark toxicity, since one of the goals that PDT has, is not to affect the target unless the combined light drug effect is given. As a test a low dark toxicity can be allowed in order to find compounds that have higher logs of bacterial kill compared to dark toxicity logs, to get the highest photosensitization and so a proper sterilization efficacy as possible. Logarithms of bacterial concentration are shown in separated bars per drug Dx in figure 4.5 corresponding to data on tables 4.3 and 4.4. The Difference from irradiated “L” and not irradiated “NL” samples is the effective killing achived by PDT, shown in figure 4.5 also represented numerically in each bar.

[1] In experiments made with cellular lines at PMH unpublished data

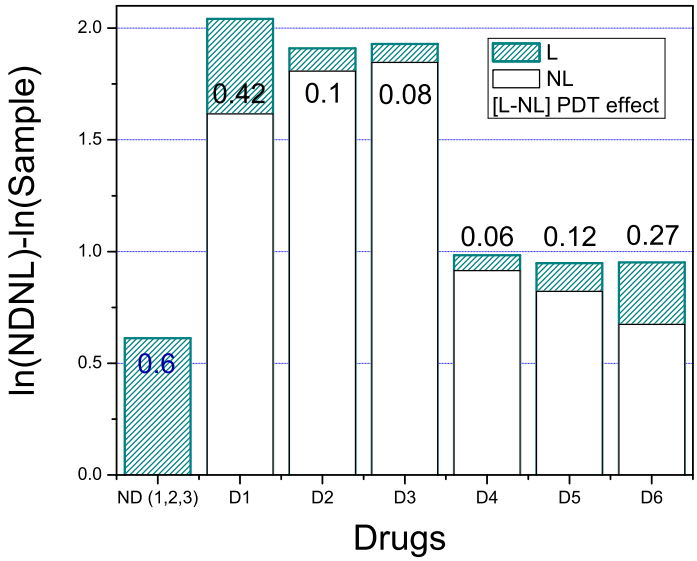

Figure 4.4: Log of kill based on kinetic Resazurin assay: PDT for MMSC, data shown on Logarithm scale.

Data shown on tables 4.3 and 4.4 can be separated by those exposed to light and not exposed. A comparison of the light efficacy compared to the no light no drug reference was performed for two different experiments, the one corresponding to drugs 4,5 and 6 show no statistical difference (table 4.3). Opposed to the ones corresponding to drugs 1, 2 and 3 were light only, affected L. monocytogenes normal growth (table 4.4).

Under our selected conditions, a PDT effect is caused if the kra linear regression of drug and light sample is sadistically significant compared to that of drug only. Drugs 1,2,3,5 and 6 show pdt effect reported in graph 4.4. Drug 4 show no PDT effect, since there was no statistical significant change between light and no light experiments with that drug.

4.5 Discussion

Two different PDT experiments were performed, one of them to test the use of the developed kinetic resazurin assay. The second to test new compounds. The methyelene blue tests had a difference with each other in the total PDT doses of one order of magnitude. However, the bacterial strains do not come from the same L. monocytoges culture used but they were isolated from different places, small differences like antibiotic resistant or mutations are unknown. The log

of kill difference was of 2 as opposed to the 5 logs of kill achieved by [Romanova, 2003]. A possible cause is the inhomogeneous distribution of the photosensitizer during the MB incubation period, as well as the actual free radical and oxygen production of the photosensitizer as a function of wavelength independent of the absorption spectra, due to different wavelength maxima of the light sources. Comparing results from kinetic resazurin assay and CFU assay, an over estimation of the bacterial concentration was found for this particular case.

About kinetic resazurin assay an undesired process was found wile analyzing complexes. If bacteria have a mayor change in population (doubling their amounts) during time kinetic fluorescent measurements the linear regression associate to the bacterial concentration will be affected. To overcome this situation, multiple repetitions have to be made of the drug tests. But another important step might be the use of multiple repetitions within the same experiment changing the initial resazurin addition time.

From graph 4.5 it can be seen that bacteria have a growth that is not completely linear. Fluorescence in the kinetic resazurin assay is due to resorufin conversion same that is related to bacterial clonogenic and metabolic stages. When bacteria have a normal clonogenic reproduction they experiment an exponential growth. If DNA is damaged the clonogenic activity will be stopped or slowed down, as mentioned in chapter 1, bacteria suffer an arrest. In a different perspective we can say that fluorescence in the KRA has two components, a linear and an exponential. Being the linear component the most predominant, since it is related to the metabolic activity.

Changes in the KRA are related to the linear and exponential components of the bacterial growth, one related to clonogenic activity and the other to bacterial metabolism. If any of those parts is affected or modified, changes in the slope or in the exponential will be modified. For metabolic consumption, changes would be caused by a bacteria in a different metabolic stage than that used by us (exponential growth) or for bacteria with a reasonably longer or shorter doubling time than that of L. monocytogenes. If the reproduction cycle of bacteria is affected the exponential growth will lose strength and might become linear as in the case exposed by us with MMSC, meaning that bacteria were damaged and the reproduction cycle affected by the MMSC.

Fluorescence signal in the Kinetic Resazurin Asay can be modeled using equation 3.1 , that contemplates the metabolic action by a linear approximation and the clonogenic reproduction with an exponential function. To have a complete model it would be required to be test under 2 sets of conditions, one were only the linear part is affected and so the exponential part can be constant, and so derive from the experiment, that is, testing different bacterial strengths. In the other hand to find the linear component bacteria have to be stress due to environmental factors such as being exposed to conditions that damage the external bacterial layers, or directly the reproductive cycle

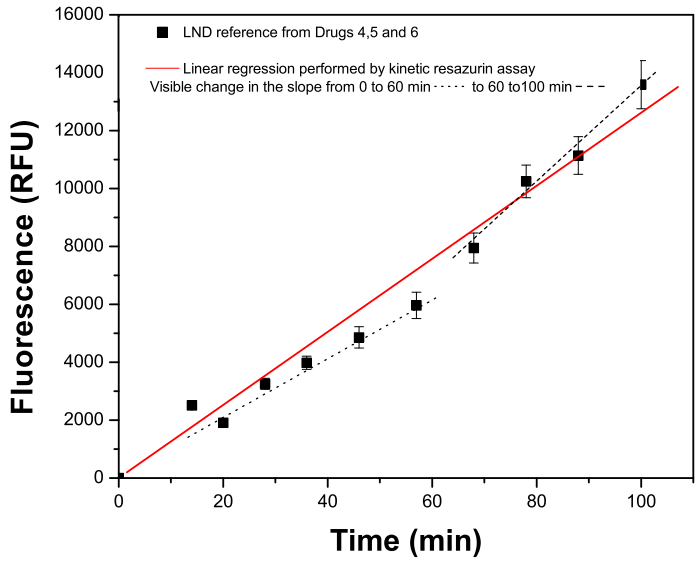

Figure 4.5: Linear regression of one particular data (LND) showing a change in slope within the range of the kinetic resazurin assay.

by corrupting the DNA. While testing MMSC we use Photodynamic Therapy to cause that stress.

In figure 4.4 PDT effect for drugs 1, 2, 3, 5 and 6 is shown, photosensitizers had a fixed concentration, changing the concentration dark toxicity can be further reduced, as well the minimum inhibitory PDT dose can be found.

4.6 Conclusion

The current chapter deals with in vitro Photodynamic Therapy applied to L. monocytogenes, and the efficacy of its eradication for different compounds as photosensitizers.

Methylene Blue was used as a reference to compare two assays CFU and Resazurin. Test show that a higher bacterial concentration per PDT dose causes lower bacterial kill, as expected. Factors that could affect this besides the lower doses are the lower power delivered in the bulk of the substance (L. monocytogenes in LB) due to a higher scattering or absorption of the LB media. A difference in the singlet oxygen and free radicals produced by MB due to the used of a differentiated wavelength. Another possible difference in the experiments is that a higher concentration of bacteria is reproduced faster, and a part of them will cooperate with the metabolic consumption of resazurin while for a lower concentration this process is less notorious.

It was found that the presented kinetic Resazurin assay is an effective method to measure bacterial concentrations between $[1x10^5, 9x1^9](\frac{cfu}{ml})$. Being more effective in terms of the short time and low effort needed to perform the KRA assay (2 hours), compared to the 24 to 36 hours that CFU requires as well as for the cost of materials used for each assay and the lower production of biohazarous waste, saving on sterilization and disposal mechanisms for biohazardous material required in the CFU method.

Six different MMSC were tested with L. monocytogenes as a first attempt to indicate the effectiveness of their use as sterilizing photosensitizers. It was found that D4 $(125\ \mu M)$, D1 $(50\ \mu M)$ and D6 $(125\ \mu M)$ as initial concentrations show about one Log of bacterial kill due to PDT. D6 Shows the lowest dark toxicity of all compounds. PDT Minimum inhibitory concentration has yet to be found, and further experiments, variating drug concentration as well as light exposure are required.

	Reference ND		125 μM D1		125 μM D2		125 μM D3	
	No light	Light	No light	Light	No light	Light	No light	Light
slope ($\frac{RFUml}{cfu}$)	296 ± 9	208 ± 5	63 ± 2	1.9 ± 0.1	35.7 ± 0.9	21 ± 0.7	30 ± 0.5	18.3 ± 0.8
r^2	0.9942	0.9967	0.9926	0.9625	0.9959	0.9918	0.9983	0.9878
Resazurin ($\pm 2 \times 10^4 \frac{cfu}{ml}$)	1.63×10^8	3.99×10^7	3.96×10^6	1.48×10^6	2.54×10^6	1.48×10^6	2.33×10^6	1.93×10^6
CFU ($\frac{cfu}{ml}$)	2×10^8	11×10^8	_	$< 1 \times 10^5$	_	$< 1 \times 10^5$	_	$< 1 \times 10^5$

Table 4.3: PDT experiment with drugs 1, 2 and 3 performed at PMHMl

	Reference ND		125 μM D4		125 μM D5		125 μM D6	
	No light	Light	No light	Light	No light	Light	No light	Light
slope ($\frac{RFUml}{cfu}$)	142 ± 27	133 ± 8	10 ± 1	0.9 ± 0.06	24 ± 2	6 ± 0.3	45 ± 5	5.3 ± 0.2
r^2	0.7937	0.9699	0.9347	0.9648	0.9291	0.9797	0.9037	0.9835
Resazurin ($\pm 2 \times 10^4 \frac{cfu}{ml}$)	1.22	1.4×10^7	2.12×10^6	1.58×10^6	2.12×10^6	1.58×10^6	2.97×10^6	1.57×10^6
CFU ($\frac{cfu}{ml}$)	2×10^8	11×10^8	-	$< 1 \times 10^5$	_	$< 1 \times 10^5$	_	$< 1 \times 10^5$

Table 4.4: PDT experiment with drugs 4, 5 and 6 performed at PMHMl

Chapter 5

Conclusions

5.1 Summary

In this thesis two studies were performed, the first part involves the development of a time kinetic resazurin assay to quantify bacterial concentrations. In the second the colorimetric assay was used as a tool in Photodynamic therapy (PDT) experiments. Mix Metal Supramolecular Complexes (MMSC) were used as bactericidal photosenitizers, and their effectiveness was tested.

Resazurin change into fluorescent resorufin is due to bacterial metabolic consumption of nutrients as well as for a change in the bacterial population. When fluorescence increases due to metabolic consumption only the growth rate is constant, therefore the slope of a linear regression is constant. A different approach to single point fluorescence quantification is a time kinetic monitoring. A Kinetic Resazurin Assay with optimized parameters was developed for the used with L. monocytogenes in Luria Bertani media. The fluorescent signal of resorufin was monitored in time. Linear regression of the fluorescence changes in time are taken for different bacterial concentrations within a range were the fluorescence signal is not saturated and there is no sign of exponential growth. A graph showing the relationship of the slope of the kinetic resazurin assay and the bacterial concentration was used to find unknown concentrations of bacteria.

Mixed metal supramolecular complexes MMSC were tested with one concentration, and against one bacterial strain. There are six MMSC from those D4, D1 and D6 have more than 0.85 log of bactericidal effect. Dark toxicity was found to be smaller than the bactericidal effect, which is a positive aspect for drugs used as photosenitizers. With respect to drugs D2, D3 and D4 dark toxicity was higher than one but bactericidal effect was in the range of 0.42 and 0.59 log.

5.2 Future work

With respect to the kinetic resazurin assay, tests have to be performed with different bacterial strains. Depending on the doubling time of the bacterial strain the slope of the kinetic resazurin assay might be modified. If doubling times are higher than that of L. monocytogenes in LB media the slope of kinetic resasurin assay will be higher for the standard bacterial concentrations compared to those reported in this thesis. Modifications to the assay might be necessary, an example of it is a saturation of the resazurin fluorescent signal in the time kinetic domain, then resazurin concentration could be adjusted lowering the initial concentration used. Sensibility test to resazurin affecting bacteria are an important requirement for new strains.

PDT with MMSC requires further measurements to determine de minimum inhibitory concentration of the MMSC. Photosensitizers were useful against L. monocytogenes as a first approach, other bacteria should be tested, including gram negative ones. Since MMSC use photosensitization path I, it would be interesting to test them with anaerobic bacteria, due to the low effectiveness of other photosenitizers that require oxygen.

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