Pathogen inactivation through self-cleaning coating materials

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Abstract

Previous research has shown that photocatalysts can inactivate bacteria in different conditions, resulting in possible self-cleaning materials that can help prevent antimicrobial resistance. The mechanisms involved in these interactions are not well known. This report is focused on the creation of such self-cleaning substrates that can be used as possible coatings and have the ability to inactivate pathogens. Different metal oxides were used through the experiments and the most promising were Copper Oxides (I & II). A polymer adhesive was used to keep the substrates compact and uniform throughout the experiments. In order to understand more on the mechanism of bacterial inactivation, experiments were carried out and blue light was used to test for possible photocatalytic activity. Formation of Reactive Oxygen Species (ROS) on the self-cleaning substrates were measured with Fluorescent Spectroscopy. Assays on bacterial growth inhibition were carried out on Gram Negative and Gram Positive bacteria and the results show that Cu_2O can inactivate both of them. Different effects resulting on different possible mechanisms were observed for the two types of bacteria leading to the need for further, more specific and focused research to create a generic assumption on the way these materials interact with pathogens.

Contents:

1. Introduction

Antibiotics created the opportunity for safer clinical procedures for so many years after their original invention. They have been used in so many different fields of medicine and provided multiple positive results. Nowadays, those antibiotics have been used without taking caution and antimicrobial resistance(AMR) starts threatening humanity. Multiple research has been contacted on AMR in different continents and all the results state the same thing. It is important for people to understand the scientific background and also try to effectively prevent it in the next few years. (1) The effects of AMR are not only associated with health. The financial consequences that occur due to lack of effective prevention also account for the constant need to develop new technologies to fight dangerous diseases, leading to a huge money waste. Research around new, promising and improved techniques to prevent AMR is contacted all around the world and results are formed for various new drugs but there is not a visible improvement yet. (2) Indoor spaces such as laboratories or surgical rooms are highly contaminated rooms that need constant sanitation and must always remain clean. Taking into consideration the problem that AMR creates this procedure could be done by other methods such photocatalytically inactivating pathogens inside those rooms. This method is used nowadays with TiO_2 getting enough energy from UV light to catalyze redox reactions and form Reactive Oxygen Species (ROS) that can then disinfect from bacteria or pathogens. (3) For this technique to work all that is needed is UV light that can be provided by a LED lamp in any space and the semiconductor that can catalyze these reactions. (4) This report shows that blue light can also be used to effectively disinfect bacteria and pathogens that exist on top of substrates that contain semiconductors. These semiconductors can be created by combining metal oxides together from copper or iron and by utilizing blue light either from the solar light during the day or by a lamp during the night, those surfaces will be self-cleaning and easily usable as coating materials. Metal oxides are used because of the ability to remain stable at any condition but also due to their safety for use and cost-efficiency. Such materials are easy to find and can be used in high quantities without creating health or environmental problems. The results from this report will provide such photocatalytic materials that could be used as coatings and inactivate pathogens and bacteria using blue light that is provided to every space. (4,5) (20) (21)

2. Scientific Background

2.1 Semiconductors

Semiconductors were used to create substrates that can be photocatalytically activated by blue light energy and catalyze redox reactions. Those semiconductors were created by copper oxides and iron oxides. These materials have separated valence and conduction bands but the band gap between the two is really close which means that electrons can jump from the valence band to the conduction band if the energy that is provided is enough. Blue light contains enough energy to those materials and electrons

get excited. Temperatures also change the way semiconductors work and at room temperature those semiconductors have smaller band gaps. The higher the temperature the smaller the band gap is. That means that inside a laboratory or a surgical room those materials can be used to create substrates that can inactivate pathogens with the use of blue light energy. (6) (7) (15) (16) (19)

2.2 Charge Transfer

When two semiconductors are combined, a process called charge transfer occurs. This process allows the electron to move from the conduction band of one semiconductor to the conduction band of the other and then catalyze certain reactions needing less energy. As can be seen in figure 1. When the two different copper oxides combine, the reduction reaction can happen since the electron from the conduction band of Cu_2O is transferred to the conduction band of CuO which has lower energy. Then the oxidation reaction can also happen with the energy that is provided by blue light since the electron can jump from the valence band of CuO to the valence band of Cu_2O. These two reactions form reactive oxygen species that then react with membrane lipids and proteins of pathogens leading to their inactivation. These materials can be then used as coatings to create self-cleaning surfaces just by using blue light energy. (8) (9) (7) (16) (18) (19)

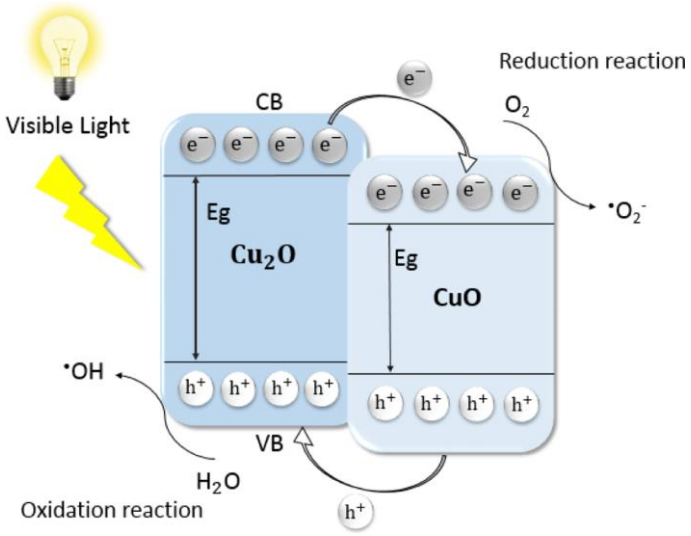


Figure 1. Charge transfer schematic for CuO and Cu_2O

2.3 Reactive Oxygen Species

Reactive oxygen species can be created by reduction and oxidation reactions if the energy that is provided on those semiconductors is enough to cover the energy the reaction needs. In the case of combined semiconductors, the reduction reaction that happens at the conduction band of CuO forms the O_2^- species while the oxidation reaction at the valence band of Cu_2O forms the $OH^{\cdot}$ radicals. These reactive oxygen species can be formed by the energy that blue light provides and this procedure can happen infinitely as long as the oxides remain intact. These reactions can happen on the membrane of pathogens leading to permeabilization of the membrane and as a last step inactivation of the pathogen. (10) (11) (12) (18)

2.4 Membrane Permeabilization

Lipid peroxidation is the main cause of membrane damage that can lead to inactivation of pathogens. Peroxidation can happen when ROS react with the lipids of cell membranes. These photocatalytically produced ROS cause lipid peroxidation and when the membrane is penetrated they oxidize contents at the inside of the cell. Those contents like DNA for example will also leak outside of the membrane causing the inactivation of the pathogen even if ROS are not enough to do it with oxidation. Membrane permeabilization is the main reason for pathogen inactivation but reactive oxygen species may not be the only thing that leads to permeabilization considering that there are short and long lived species and also that there are many different compounds that can react with them before reacting with the membrane. That means that the main tested mechanism is photocatalysis but this report is not only focused on that. (13) (14) (16)

3. Experimental Methods

3.1 Materials

Copper(I) oxide and Ti(IV) were synthesized in former articles. Cu(II) oxide(powder, <10 um, 98%) was obtained from Sigma Aldrich. Mixes of the two copper oxides were created using a molarity ratio of 1:1. Dihydrorhodamine 123 was obtained from Invitrogen™ by Thermo Fischer Scientific. Disodium terephthalate (>99%) and 2-hydroxyterephthalic acid (>98.0%) were obtained from Tokyo Chemical Industry. SYTOX™ Green Nucleic Acid Stain- 5 mM Solution in DMSO was obtained from Invitrogen™ by Thermo Fischer Scientific. BODIPY™ 581/591 C11 (Lipid Peroxidation Sensor) was obtained from Invitrogen™ by Thermo Fischer Scientific. DMSO was obtained from Invitrogen™ by Thermo Fischer Scientific, agar (Ash 2.0-4.5%), LB broth, Escherichia coli (MC4100) were obtained from ATCC and Staphylococcus Carnosus TM300 were obtained from a collaborator. Polymer Adhesive (>99% Transmission and <1% Haze Level) was obtained from THORLABS.

3.2 Substrate Creation

Spin Coating has been used in former studies to create substrates from metal oxides that can be used later as coatings on the wall. This technique did not show the best effect on creating the substrates and the coating was getting off after some uses. A polymer adhesive with high transparency and not chemically active was used in this study to test whether metal oxides would adhere better on top of the glass. A glass used for microscopy is used to create slides of 25mm x 22mm that serve as the main structure that the polymer adhesive is glued on. The polymer adhesive is added with dimensions of 15mm x 15mm. The glass that contains the adhesive on top, is then dipped inside the metal oxides in order to create a visible, compact and uniform surface. All the substrates are then cleaned with distilled water and ethanol and dried using clean air stream.

3.3 Photocatalytic Setup

The Photocatalytic Setup was created by using a 30 W Eurolite LED IP FL-30 SMD blue light source that was fixed at a distance of 20cm from the sample surface using lab lifts. All the samples were illuminated with blue light, while also some measurements were performed using a 30 W Eurolite LED IP FL-30 SMD UV light lamp to act as a control. Cu_2O and/or CuO

were tested mostly under blue light. The setup can be seen at figure 2: Photocatalytic Setup with illumination from above.

Figure 2: Photocatalytic Setup with illumination from

3.4 Oxidation of Dihydrorhodamine 123

Dihydrorhodamine 123 (DHR 123) is used as a fluorescent probe to detect Reactive Oxygen Species that may be produced by the substrates. Oxidation of the probe to Rhodamine 123 which is a fluorescent substance is used to measure these ROS. New substrates are created, cleaned with water and ethanol and then dried with clean air. A solution of 10 uM DHR 123 in PBS is made and 100 uL of this solution is added on every substrate. The substrates are then illuminated under Blue or UV light. A negative control is made by adding 100 uL of DHR 123 in PBS on adhesive glued on the LID and a positive control is created by treating 1 mL of DHR 123 in PBS with Cold Atmospheric Plasma for 5 minutes and then 100 uL are added on top of adhesive glued on the LID. Then the Fluorescence Intensity is measured directly on the LID with CLARIOstar at 487nm excitation wavelength and 535nm emission wavelength.

3.5 Hydroxylation of Terephthalic acid

Terephthalic acid is a fluorescent probe to measure the creation of hydroxyl radicals specifically. While Terephthalic acid (TA) is not fluorescent its oxidized form 2-Hydroxyterephthalic acid (HTA) is.

New substrates are created, cleaned with water and ethanol and then dried with clean air. The substrates are created on the LID of a COSTAR-12 plate with the use of an adhesive (15mm x 15mm) that is glued on top of glass (15mm x 15mm). A solution of 2,5 mM of Terephthalic acid in PBS is made. 100 uL of this solution is pipetted on top of each substrate. Then the samples are placed at the Photocatalysis setup (Blue or UV light) and are illuminated for a certain amount of time (1hour). Then the LID is placed directly in the CLARIOstar and measured at an excitation wavelength of 320nm and emission wavelength at 435nm. To create positive control 1 ml of TA in PBS is treated with Cold Atmospheric Plasma for 5 minutes and then 100 uL of this solution is added on adhesive on top of the LID. Cold Atmospheric Plasma is used because it is known to produce multiple RONS including hydroxyl radicals.

3.6 Fenton Reaction

Fenton reaction is performed by metal ions that react with H_2O_2 and produce OH^- radicals as shown on figure 3. New substrates are created, cleaned with water and ethanol and then dried with clean air. The substrates are created on the LID of a COSTAR-12 plate with the use of an adhesive (15mm x 15mm) that is glued on top of glass (15mm x 15mm). A solution of 2,5 mM of Terephthalic acid in PBS is made. 100 uL of this solution is pipetted on top of each substrate. A positive control is created using $CuSO_4$-$7H_2O$, while a negative control is made using only 100 uL of TA stored in an Eppendorf tube at dark. Then the samples are placed at the Photocatalysis setup (Blue or UV light) and are illuminated for a certain amount of time (2hours, 4hours). After illumination 100 uL are pipetted in Eppendorf tubes, 10 uL of 1mM H2O2 is added and incubated for 10 min. After incubation, 50 uL of each sample are pipetted in a 96F-Bottom Well twice and measured using CLARIOstar at an excitation wavelength of 320nm and emission wavelength at 435nm.

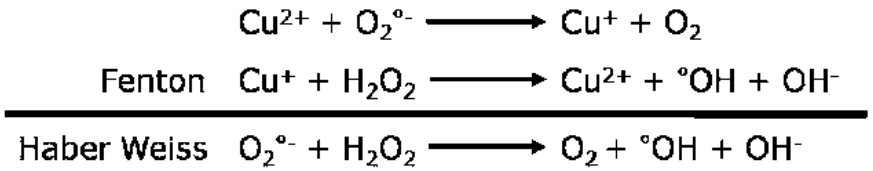


Figure 3: Schematic of a Fenton Reaction. Copper Ions react with H_2O_2 creating OH^- radicals. (17)

3.7 Lipid Peroxidation

3.7.1 Gram Negative Bacteria

New substrates are created, cleaned with water and ethanol and then dried with clean air. A colony of *E. coli* bacteria is added in 5 ml Lysogeny Broth (LB) and placed inside the incubator at 37° C overnight. Then, 200 uL of overnight solution is added in 10 mL fresh LB media and placed back in the incubator for two and a half hours so the bacteria reach their exponential growth phase. After the incubation the Optical Density of the solution is measured at 600nm and then it is washed with 10 ml PBS for 3 times. Washing takes 5 minutes each time at 4000g/rcf. Then the solution is diluted at 0.05 OD in 10 ml PBS. After dilution 110 uL of the solution are added on top of the substrates and are illuminated (Blue light) for a certain amount of time (1 hour). A solution of 10 uM of Bodipy C11 is made in PBS. Then 100 uL of the illuminated solution is transferred in an Eppendorf tube where 150 uL of the PBS/Bodipy C11 solution is added and stored inside an incubator at 37° C for 10 minutes. A washing step is added in order to remove excess of the Bodipy C11 that hasn't reacted (10min centrifuge at 4000g/rcf with PBS). After the washing step, 100 uL of the solution are placed in 96F-Bottom Wells twice. The Fluorescence Spectra is measured from 520 to 590 nm and an Intensity ratio between 520 and 590 nm is calculated.

3.7.2 Gram Positive Bacteria

New substrates are created, cleaned with water and ethanol and then dried with clean air. A colony of *S. carnosus* bacteria and *S. carnosus* expressing the Staphyloxanthin bacteria is added in 5 ml Lysogeny Broth (LB) with 10 ug/ml Tetracycline and 0,5% xylose and placed inside the incubator at 37^{o} C overnight. Then, 200uL of overnight solution is added in 10mL fresh LB media and placed back in the incubator for two and a half hours so the bacteria reach their exponential growth phase. After the incubation the Optical Density of the solution is measured at 600nm and then it is washed with 10 ml PBS for 3 times. Washing takes 5 minutes each time at 4000g/rcf. Then the solution is diluted at 0.05 OD in 10 ml PBS. After dilution 110 uL of the solution are added on top of the substrates and are illuminated (Blue light) for a certain amount of time (1 hour). A solution of 10 uM of Bodipy C11 is made in PBS. Then 100 uL of the illuminated solution is transferred in an Eppendorf tube where 150 uL of the PBS/Bodipy C11 solution is added and stored inside an incubator at 37^{o} C for 10 minutes. A washing step is added in order to remove excess of the Bodipy C11 that hasn't reacted (10min centrifuge at 4000g/rcf with PBS). After the washing step, 100 uL of the solution are placed in 96F-Bottom Wells twice. The Fluorescence Spectra is measured from 520 to 590 nm and an Intensity ratio between 520 and 590 nm is calculated.

3.8 Membrane Permeabilization

SYTOX Green is an impermeant to live cells dye that when it comes into contact with nucleic acids it reacts and shows >500-fold fluorescence intensity. It is a good indicator of cell death for both gram positive and gram negative bacteria and provides a clear view of the living and dead cells using a fluorescence microplate reader. After incubating bacteria 100 uL of a bacterial solution of 0,05 OD is added inside an Eppendorf's tube. Then 100 uL of PBS are added and 0,5 uL of SYTOX Green stain. 100uL of this solution are transferred to 96 well plates and measured using the CLARIOstar fluorescent reader. Measurement is taken at an excitation wavelength of 488 nm and emission wavelength at 560 nm. After the measurement 1 uL of Triton X-100 0,1 % $^{w}/_{v}$ is added in each well and a second measurement is performed. Triton X-100 is a surfactant that is used to destroy membranes and inactive bacteria. At the end the two measurements exhibit the amount of bacteria killed from the substrates compared to the total amount inside the well.

3.9 Bacterial Inactivation

3.9.1 Gram Negative Bacteria

New substrates are created, cleaned with water and ethanol and then dried with clean air. A colony of *Escherichia coli* bacteria is added in 5 ml Lysogeny Broth (LB) and placed inside the incubator at 37^{o} C overnight. Then, 200uL of overnight solution is added in 10mL fresh LB media and placed back in the incubator for two and a half hours so the bacteria reach their exponential growth phase. After the incubation the Optical Density of the solution is measured at 600nm and then it is washed with 10 ml PBS for 3 times. Washing takes 5 minutes each time at 4000g/rcf. Then the solution is diluted at 0.05 OD in 10 ml PBS. After dilution 100 uL of the solution are added on top of the substrates and are illuminated (Blue light) for a certain amount of time (30 minutes, 1hour, 2hours, 4hours). Serial dilutions on Agar plates are done after illumination. Samples are diluted 5 times (80 uL PBS + 20 uL from the previous

Eppendorf tube) each dilution for 6-7 dilutions in Eppendorf tubes. Each spot inside the Agar Plates contains 5 uL from the Eppendorf tubes that contain the bacteria and PBS. The Agar plates are stored in the incubator at 37^{o} C overnight and scanned the next morning.

3.9.2 Gram Positive Bacteria

New substrates are created, cleaned with water and ethanol and then dried with clean air. A colony of *Staphylococcus carnosus* (PTx 15) bacteria and *Staphylococcus carnosus* expressing the Staphyloxanthin (PTx Sx) bacteria is added in 5 ml Lysogeny Broth (LB) with 10 ug/ml Tetracycline and 0,5% xylose and placed inside the incubator at 37^{O} C overnight. Then, 200uL of overnight solution is added in 10mL fresh LB media and placed back in the incubator for two and a half hours so the bacteria reach their exponential growth phase. After the incubation the Optical Density of the solution is measured at 600nm and then it is washed with 10 ml PBS for 3 times. Washing takes 5 minutes each time at 4000g/rcf. Then the solution is diluted at 0.05 OD in 10 ml PBS. After dilution 100 uL of the solution are added on top of the substrates and are illuminated (Blue light) for a certain amount of time (1hour). Serial dilutions on Agar plates are done after illumination. Samples are diluted 5 times (80 uL PBS + 20 uL from the previous Eppendorf tube) each dilution for 6-7 dilutions in Eppendorf tubes. Each spot inside the Agar Plates contains 5 uL from the Eppendorf tubes that contain the bacteria and PBS solution. The Agar plates are stored in the incubator at 37^{o} C overnight and scanned the next morning.

4. Results and discussion

4.1 Comparison of materials created and coating quality.

Comparing figures 4 & 5, a big difference can be seen on the creation of coating materials. The polymer adhesive clearly provided a better solution of making more potent and more compact substrates that can later be tested for bacterial inhibition or production of Reactive Oxygen Species. Though the substrates were easily reusable and can be cleaned quite firmly with MiliQ-water and ethanol, each experiment was contacted with a different set of new substrates. All the experiments showed a sign of either oxidation or reduction of the Cu_2O substrates when a solution was treated on top of them for a period of time. In total 5 different materials were tested for their ability to self-clean and those were Cu_2O, CuO, a mix of Cu_2O and CuO, TiO_2 and Fe_2O_3. These materials could be used to create coatings that are self-cleaning but the amount of time that they can be used for is limited unless a new more improved design is created.

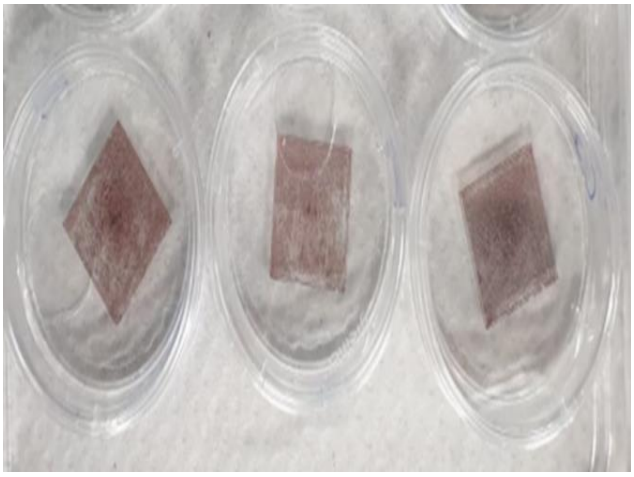


Figure 4: Development of a photocatalyst for inactivating pathogens using visible light
M. C. Heijnen, Dr. E.M. Hutter, Dr. J.H.F.F. Lorent, Dr. D. Osadchii, July 2021

Figure 5: Use of polymer adhesive and creation of more potent substrates.

4.2 Formation of Reactive Oxygen Species with Dihydrorhodamine 123

The oxidation of Dihydrorhodamine 123 to measure the formation of ROS showed no effect on every substrate except the TiO_2 one. Both at Blue light and UV light (which acts as a control) there were no signs of ROS formation for the substrates that contain copper oxides. Dihydrorhodamine 123 is not a very specific dye but it is useful when measuring different species of radicals. This experiment proved that the substrates are not creating radicals while illuminated except of the TiO_2 which is known to have this mechanism. Other fluorophore probes should be used to be sure that this effect is repeatable and the substrates are not photocatalytically active.

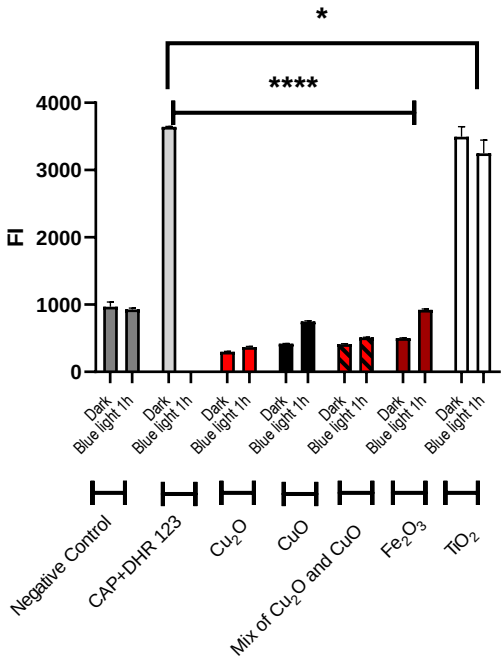


Figure 6: Formation of ROS in Blue light for an illumination time of 1 hour. Dihydrorhodamine 123 was oxidized only by TiO_2

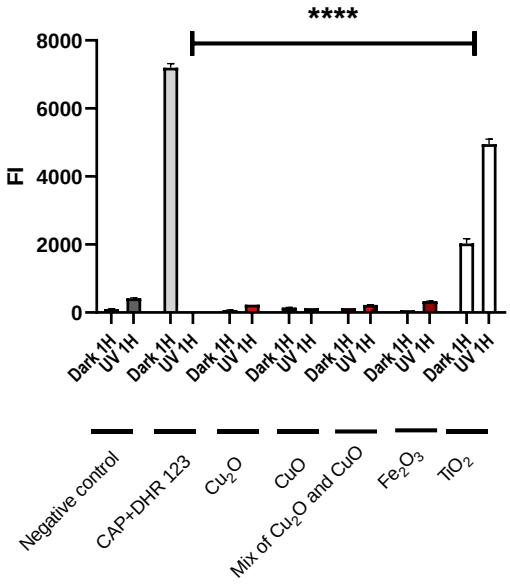
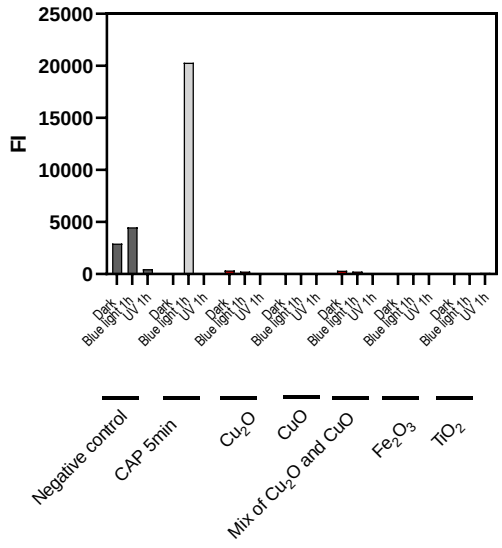


Figure 7: Formation of ROS in UV light for an illumination time of 1 hour. Dihydrorhodamine 123 was oxidized only by TiO_2

4.3 Hydroxylation of Terephthalic acid



Hydroxylation of Terephthalic acid is a more specific assay to measure formation of OH^- radicals. There radicals would show if the substrates work with a photocatalytic mechanism using the energy provided by the Blue light.

The results show that none of the substrates formed OH^- radicals under UV or Blue light illumination for 1 hour. That effect differs from the one with the Dihydrorhodamine 123, were TiO_2 showed formation of ROS for both UV and Blue light. That means that this current probe is probably not suitable for contacting those experiments because it either gets degraded by the substrates or maybe there is a photo-quenching effect.

Figure 8: Hydroxylation of Terephthalic acid on both Blue and UV light. There is no formation of ROS for any of the tested substrates.

4.4 Fenton Reaction

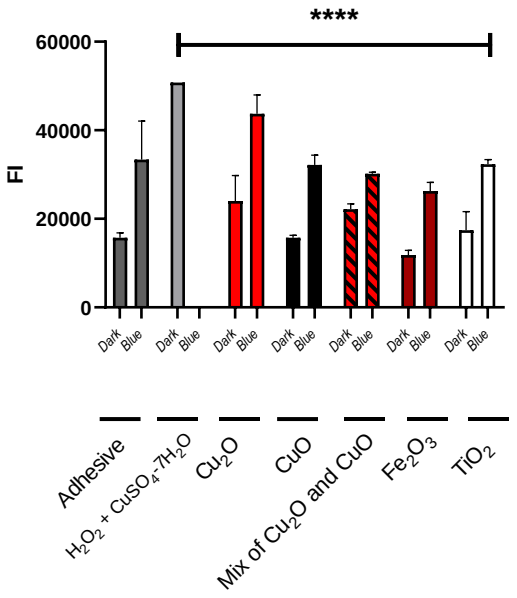


Figure 9: Fenton reaction induced by Blue light. Illumination time is 1 hour.

Fenton reaction assay is performed to realize whether metal ions would form reactive oxygen species. The substrates should not contain any metal ions but this experiment can prove whether reduction of the oxide happened and if there are impurities. The results showed that there was no formation of ROS by the substrates themselves. As shown in figure 9. Comparing the substrates to the polymer adhesive which acts as a negative control, there is no difference in the Fluorescence Intensity while at Dark. There is a difference though between Dark and Blue light showing that this reaction is induced by Blue light. The graph also shows that the positive control created far more ROS than the substrates. This positive control was made purely to induce Fenton reaction through Cu^+ provided by $CuSO_4$.

4.5 Lipid Peroxidation

4.5.1 Gram Negative

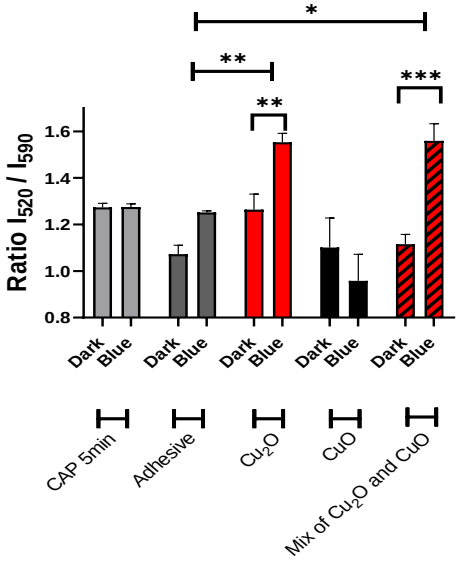


Figure 10: Membrane Lipid Peroxidation of *Escherichia coli*

Using Bodipy C11 as a fluorophore probe it is easy to measure membrane lipid peroxidation. A ratio between the fluorescence intensity at 520 nm and 590 nm was calculated and as shown in figure 10. the different substrates that contain Cu_2O showed an effect of Blue light on the peroxidation of the membrane lipids. This effect was only induced at those two substrates meaning that it is probably connected to Cu_2O and the effect it has on membrane lipids of gram negative bacteria when it gets energy from Blue light.

4.5.2 Gram Positive

A ratio between the fluorescence intensity at 520 nm and 590 nm was calculated and as shown in figures 11. and 12. for gram negative bacteria all of the substrates containing the copper oxides have the same pattern where blue light induces the peroxidation of membrane lipids. This effect can also be seen at the ones expressing the Staphyloxanthin meaning that this antioxidant does not make a difference at lipid peroxidation. Substrates containing TiO_2 didn't show any membrane lipid peroxidation showing that the mechanism of copper oxides and titanium oxide differs. Copper oxides most probably use the energy provided by blue light to damage the membranes by oxidizing the membrane lipids.

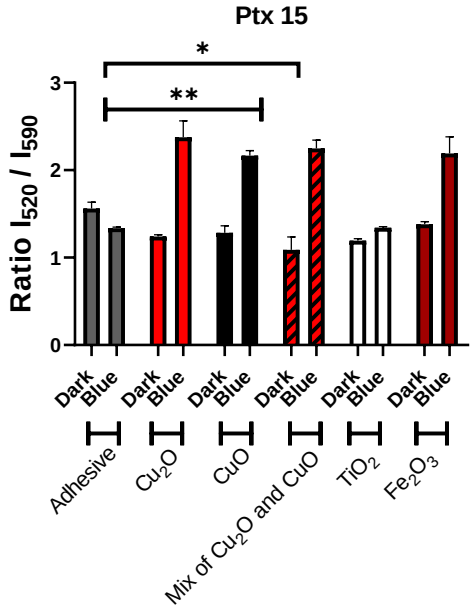


Figure 11: Membrane Lipid Peroxidation of *Staphylococcus carnosus*

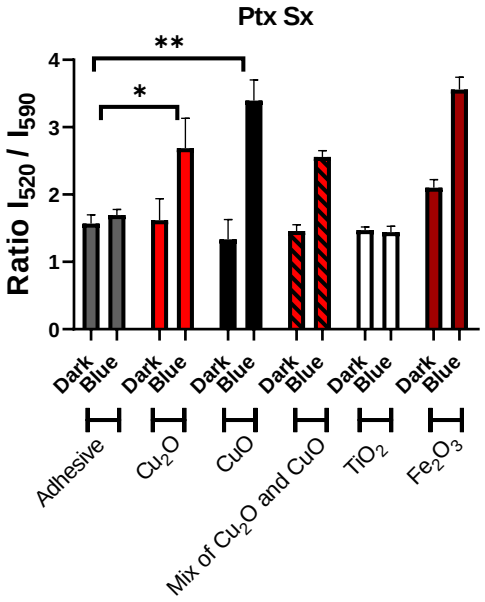


Figure 12: Membrane Lipid Peroxidation of a *Staphylococcus carnosus* strain that expresses the Staphyloxanthin.

4.6 Membrane Permeabilization

Membrane permeabilization was only tested on gram negative bacteria. Figure 13. shows that the effect of the permeabilization on gram negative bacteria is not light dependent and the biggest effect is created by CuO. When adding Triton X-100 there is a clear difference on the amount of membranes that get permeabilized but it is also shown that CuO has permeabilized all of the membranes before adding the Triton X-100. The results differ for other oxides but substrates that contain Cu_2O show that there is permeabilization while not illuminating the substrates.

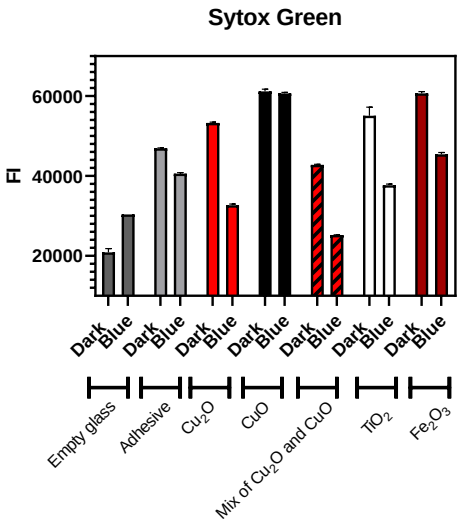


Figure 13: Membrane Permeabilization of Gram negative bacteria *E. coli.* SYTOX Green is used as the fluorophore.

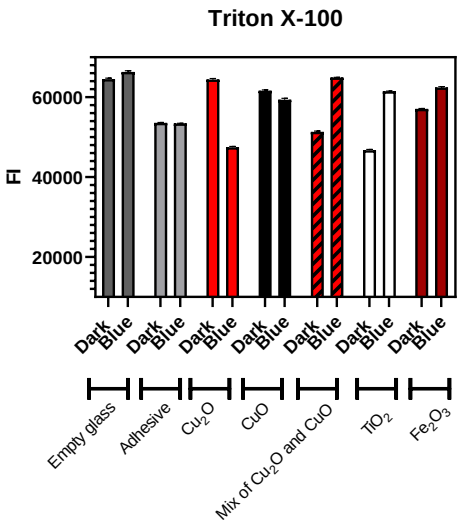


Figure 14: Triton X-100 is used to permeabilized all the bacteria in the solution and then compare with the first measurement.

4.7 Bacterial inactivation

4.7.1 Gram Negative

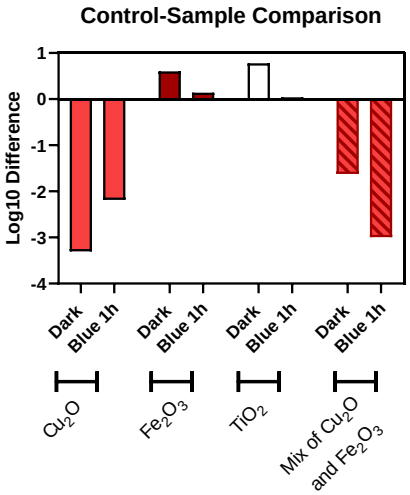


Figure 15: Logarithmic difference between control (adhesive) and substrates for *E. coli*

Bacterial inactivation by photocatalytic substrates was tested on the MC4100 E. coli strain in log phase. Bacteria were diluted to an OD of 0.05. The dilution factor was 5x meaning that 20 uL of the bacterial solution was added in 80 uL of Buffer solution (PBS) and it is done 6 times. For each dilution then 5uL are pipetted on top of the agar plates creating a spot which is visible as bacteria grow overnight. At the end by measuring the amount of bacteria left on each spot and comparing that to the spots of the same dilution factor but from the negative control, a graph that contains the logarithmic difference between control-sample can be created. As shown in Figure 15. both of the substrates containing the Cu_2O show a strong effect on destroying bacteria after just one hour of treatment. While TiO_2 and Fe_2O_3 substrates contain more bacteria than the control group. It is deducted that the effect is not light dependent considering the inactivation of the bacteria on the Cu_2O containing substrates happens at dark as well.

4.7.2 Gram Positive

Bacterial inactivation by photocatalytic substrates was tested on two different *S. carnosus* strains in exponential phase. Bacteria were diluted to an OD of 0.05. The same method as for gram negative bacteria was used and the results can be seen at figure 16. for the normal S. carnosus strain and figure 17. for the strain expressing the Staphyloxanthin. Figure 15. Shows a clear effect of substrates that contain Cu_2O in bacterial inactivation. This effect is related to blue light as shown by the difference in the graph bars between blue light and dark. Figure 16. shows a similar effect while also CuO seems to inactivate bacteria when illuminated with blue light for one hour. Taking those two results into consideration, it is safe to assume that the mechanism of bacterial inactivation on gram positive bacteria is light dependent and probably driven by photocatalysis. That means that reactive oxygen species are formed and disinfect the substrates from bacteria making them good materials for self-cleaning coatings against gram positive bacteria. Another interesting thing is that even though Staphyloxanthin is an antioxidant that should protect bacteria from oxidation, results show that in the case of copper oxides there is no difference on the effect that is observed.

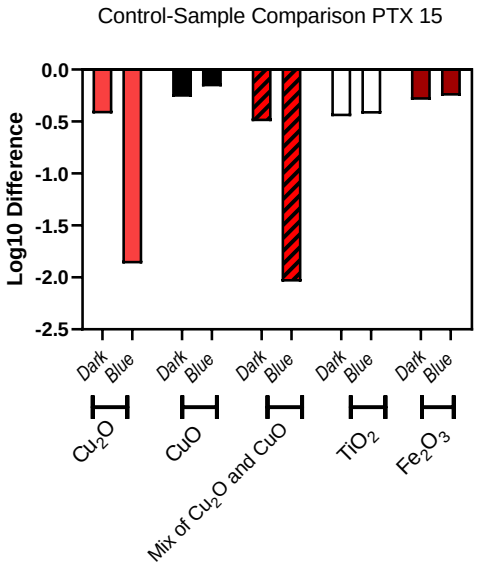


Figure 16: Logarithmic difference between control (adhesive) and substrates for *S. carnosus*

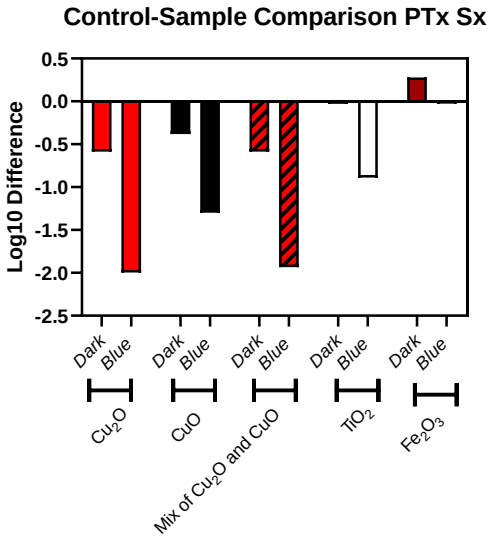


Figure 17: Logarithmic difference between control (adhesive) and substrates for *S. carnosus* expressing the Staphyloxanthin

4.8 Light Dependence and possible mechanism

Results of the experiments carried to understand the possible mechanism of those semiconductors show that there is not a simple mechanism involved in the inactivation of pathogens. Even though there were not any reactive oxygen species formed from the substrates, bacterial inactivation assays on gram positive bacteria showed a clear light dependent effect after only one hour of blue light illumination. Lipid Peroxidation on both gram negative and gram positive bacteria showed that peroxidation of membrane lipids

happens when pathogens come in contact with copper oxide substrates. This means that there is formation of reactive oxygen species that is also induced by blue light illumination as shown in the graphs. Bacterial inactivation assay and permeabilization of membranes on gram negative bacteria showed that the effect is not light dependent as first noted. That means that there may be another underlying mechanism that happens before or in parallel with photocatalysis.

5. Conclusion

Substrates created to be used as coatings were created using metal oxides such as copper and iron. The tested copper oxides were found to have an effect on the inactivation of pathogens. Experiments that were performed to test for formation of reactive oxygen species did not show any measurable results. New fluorophore probes or different techniques to measure the formation of ROS might be more resourceful and need to be carried out. Even though formation of reactive oxygen species was not measured membrane lipids peroxidation happened on both gram negative and gram positive bacteria and the effects were enhanced under blue light illumination for one hour. That means that photocatalysis is taking place in this reaction and ROS are formed.

Bacterial inactivation assays and permeabilization of membrane assays on gram negative bacteria showed that the mechanism behind their inactivation is not light dependent. That means that an underlying mechanism is also happening while photocatalysis forms all those ROS. The effects of the second mechanism are clear on gram negative bacteria while on gram positive there is again this enhanced effect when blue light energy is involved. Taking those into consideration, there are two different mechanism working in parallel, when inactivation of pathogens on those self-cleaning substrates is happening and those mechanisms are more or less dependent on the type of pathogen they are trying to inactivate.

The creation of self-cleaning coating materials was successful but these materials are not yet ready to be mass produced or applied in labs or surgical rooms. More research needs to be done on the materials to get a full view of those mechanisms and also be able to apply these materials to create coatings of a larger scale.

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