A first step towards detection of biofilm using fluorometry

A Master's Thesis by
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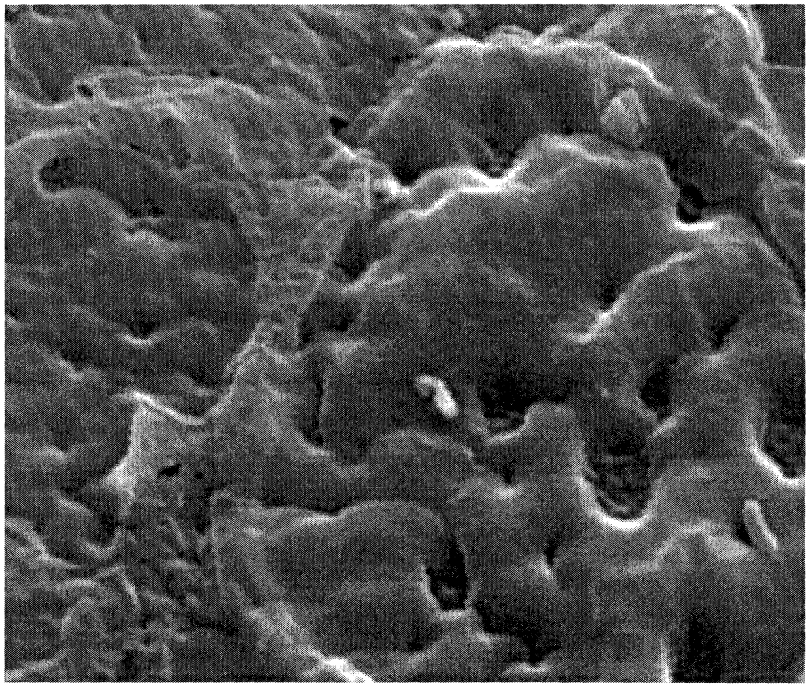
Lund Reports on Atomic Physics, LRAP-278
Lund, November 2001



Abstract

This Master's Thesis is an evaluation of whether it is possible to use an optical method to detect biofilm in dialysis machines. Biofilm is an organic layer containing bacteria and polysaccharides that attaches to surfaces.

Several optical methods were considered, but fluorometry was chosen since certain compounds (pyridoxine, riboflavin and NAD(P)H), present in all organisms, are fluorophores. A conventional detection method for evaluation of the presence of biofilm is to examine samples with electron microscope.

When detecting free-floating bacteria the detection limit, with the system used in this study, is 10^7 CFU/ml. The fluorescence signal increased with ~200% when circulating cell suspension in a system for six days. After a disinfection procedure with hot water the fluorescence decreased, but not to the initial level. This result indicates that biofilm still is present and that a disinfection procedure of this type is not sufficient to remove biofilm. Pieces of drainage tubes from dialysis machines were examined using electron microscopy, and the presence of biofilm was confirmed.

Further work is needed to increase the sensitivity and to assess a relationship between the amount of bacteria and the fluorescence signal.

Contents

1 Introduction

The aim of this Master's Thesis is to investigate if an optical detection method can be used to detect biofilm in dialysis machines. Biofilm is a thin organic layer consisting of bacteria and polysaccharides, which is formed on any surface where bacteria and nutriment are present. Presence of biofilm in the machines could be a nuisance, or even a serious threat to the dialysis patients' health.

Since biofilm build-up could constitute a serious health risk, it is very important to be able to detect it as early as possible. Once biofilm has formed, it is virtually impossible to get rid of, due to the bacteria's use of polysaccharides to build protective layers around themselves. The resistance to chemicals for bacteria in a biofilm environment is of the order of 1000 times better than for free-floating bacteria. This implies that very concentrated chemicals must be used to kill the bacteria in the biofilm. Such extensive use of chemicals could damage the dialysis equipment, and would most certainly cause an unwanted environmental stress, and is therefore not an option.

Gambro's vision is to have an optical device that detects the biofilm formation at an early stage, indicating when disinfection and cleaning are necessary. Such a device would ideally raise the already high standards when it comes to the safety and comfort of the patients.

2 Background & theory

2.1 The dialysis machine

Dialysis is a life-saving treatment for people with no, or only a very limited, kidney function. According to Gambro basics[1] the kidneys have the important functions to regulate the water level, the electrolyte balance, as well as the acid-base balance in the body. They are also responsible for the removal of waste products from the blood. The waste products consist mostly of urea, a nitrogen-containing substance that is formed when proteins are metabolised, and creatinine, a substance formed during muscle metabolism.

Dialysis can be divided into two groups: haemodialysis (HD) and peritoneal dialysis (PD).

- In HD an artificial kidney, a so-called dialyser, is used for the removal of excess fluid and waste products. This dialyser is part of an HD machine, which runs and controls all processes involved in a dialysis treatment. A treatment usually takes place at a dialysis clinic or at a hospital, and is performed three times a week, taking about 4-5 hours each time.
- In PD the peritoneum, i.e. the membrane around the abdominal cavity, is used as a dialyser, and a catheter is placed in the patient's lower abdomen for insertion and draining of dialysis fluid. PD could be performed in the patient's own home using a special PD machine. The dialysis fluid has to be exchanged 3-10 times every 24 hours, depending on the patient's needs. This treatment procedure can only be used for a limited period (approximately 5 years) since the peritoneum wears out with time, and it is normally used by patients waiting for a kidney transplantation.

The HD machine has two separate circuits: a blood circuit and a fluid circuit. In the blood circuit the patient's blood is led out of the body, through a dialyser, and then back into the patient again. In the fluid circuit water is warmed up (to 36-40°C) and mixed with a dialysate concentrate called BiCart. The concentrate consists of sodium, potassium, calcium, magnesium, and chloride ions. Bicarbonate, and in some cases glucose, is also added to the mixture before it is ready to use, see Figure A. The mix of ingredients in the dialysis fluid is chosen so that it will balance the levels of water, electrolyte and pH in the body. Interaction between the two circuits occurs in the dialyser, where the renal and regulation processes take place through osmosis and diffusion.

The waste products and excess fluid from the patient, is transported to the drain with the dialysis fluid. Any bacteria present, coming either from the patient or the dialysis fluid, would have a very good nutriment supply and a nice and warm environment, giving almost perfect conditions for biofilm (see 2.2) formation in the drainage tube.

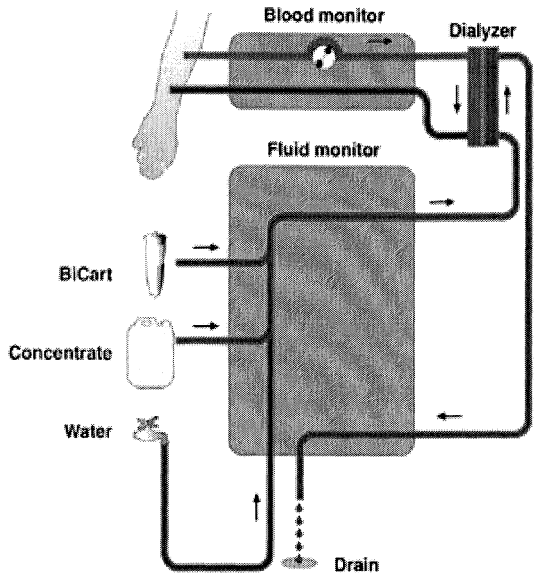


Figure A. The principles of a dialysis machine.

Since it is not practically possible to replace the tubes in the fluid circuit very often, the biofilm will at regular intervals be provided with more nutriments. This could result in an explosive growth and spread of the biofilm, if there had not been strict disinfection routines before and after every patient. After each treatment the machine is disinfected with hot water (95°C) for about 45 minutes. This process will kill practically all bacteria, since very few organisms can survive at these temperatures. Additional disinfection and cleaning processes are to flush the machine with solutions of hot water and citric acid once a week (to remove calcium and iron precipitates), and sodium hypochlorite every 14 days for removal of proteins and fatty substances.

2.2 Biofilm

Biofilm is a thin organic layer consisting mainly of bacteria and polysaccharides. A biofilm can form on any surface, provided that there are bacteria and a some nutriment present. In the early 1990s it was discovered that bacteria in biofilms are living as in a community, helping each other to live and prosper.[2]

According to websites at Montana University and Edstrom Industries[2, 3] there are two kinds of bacteria: planktonic bacteria, which are floating around freely in a fluid, and sessile bacteria, which are attached to a surface causing biofilm. Before the discovery of biofilm, bacteria were considered being solitary organisms, but now it is assumed that about 99% of the bacteria in the world live in biofilm communities.

The biofilm research has for a long time been affected by two false assumptions about bacterial behaviour:[3]

- Planktonic and sessile bacteria act in the same way.
- Biofilm consists of disorderly chunks of bacteria.

These assumptions are wrong, since when bacteria stick to a surface, they switch on to using a different set-up of genes, they give up their bachelor-life and start living together in well-organised communities. In these "slime cities" the bacteria cooperate about matters concerning food supply, waste disposal, and defence against attackers.

2.2.1 Biofilm formation

Biofilm formation can be divided into three steps (Figure B):[3]

1. Planktonic bacteria settle on a surface.
2. A layer of bacteria is formed, the bacteria start producing polysaccharides, and a shield of polysaccharides as protection is produced.
3. The biofilm develops by forming cell aggregates, interstitial pores, and conduits.

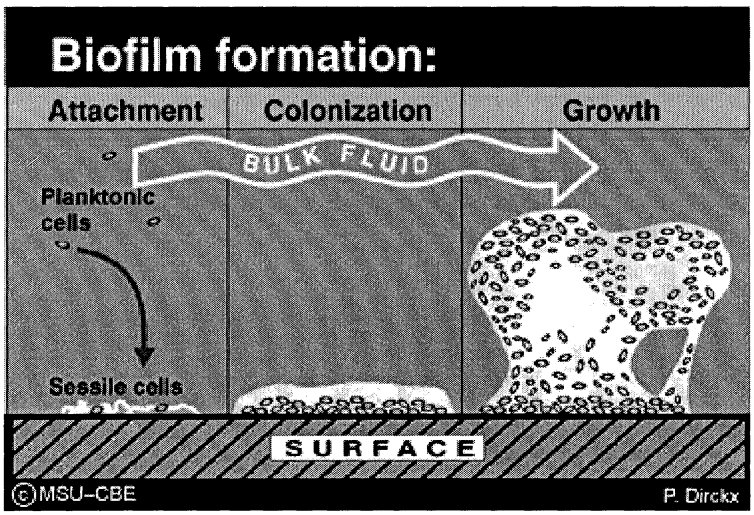


Figure B. Biofilm formation.

Almost immediately after a surface gets in contact with a fluid containing bacteria, some of the bacteria will form an organic layer on the interface between the fluid and the substrate. Some species of bacteria have been found to attach to a surface already after 30 seconds of fluid/substrate contact.[3] It is not yet known exactly how bacteria settle on surfaces, but one theory is that the organic substances neutralize the electrostatic energy of the surface, thus making it more accessible to bacteria. Yet another reason might be that in a tube with a flowing fluid, the flow is close to zero alongside the tube walls. This results in very low flowing forces and therefore some bacteria will get stuck on the walls.[2]

As soon as bacteria attach to a surface their "personality" changes, i.e. their genetic set-up is changed. The pioneering bacteria on the surface start forming a protective layer of polysaccharides. This layer also acts as glue, helping the bacteria to stick to the surface. The first bacteria also make other preparations for staying longer by forming structures, where water can pass by and they can exchange nutriments. They set up an ion exchange system, with which they catch nutrients from the fluid passing by outside their polysaccharide shield. The bacteria have now given up their bachelor life, and are fully adapted to a life in a community.[2]

When this community is built, the supply of nutriments has been secured, the bacteria start reproducing; the growth phase has started. As the bacterial society is expanding, they also try to initiate communication and collaboration with surrounding societies. The cooperation leads to a rapid expansion of the bacteria layer, and the forming of a biofilm. The biofilm keeps on growing until it reaches a thickness of the order of 10 μm.[5] At some places the growth conditions might be better than at other places, which could result in local mushroom-like structures with a thickness of about 100-200 μm. These are however more sensitive to flowing forces and more likely to be torn away. As this happens, the build-up of new biofilm is immediately initiated, and there will soon be no sign of the incident.

The biofilm is divided into layers with different levels of metabolism. The upper parts are very active, and do therefore have a high metabolism, whereas the lower parts have significantly lower metabolism. A mature biofilm could have anaerobic conditions in the parts closest to the surface which it is attached to, while the parts closest to the fluid are aerobic. Nourishment is transported to the lower layers with fluids floating through the internal conduit network. Actually, most of the biofilm is just plain water. Only 5-25% of a mature biofilm consists of bacteria, whereas water constitutes 95-99% of the remaining part. This leaves, amazingly enough, only a very small percentage for the polysaccharide defence system, on which the entire biofilm society rely.[2]

A fully developed biofilm acts as living tissue; all organisms cooperate to get rid of the waste products, to protect themselves against chemicals, and to make the most use of the supply of nutriments. In fact, in an environment with several different sorts of bacteria, substances that some just dispose as waste, others might find nutritious. This results in the rather amazing ability of the biofilm to break down compounds that no single kind of bacteria can break down.[2]

To summarize, there are a number of advantages for sessile bacteria, as compared to the planktonic bacteria:[2]

- Trace organics, such as biocides and other potentially destructive substances, will be concentrated to the surface, having trouble to penetrate the polysaccharide shield.
- Sticky polymers concentrate nutrients from the fluid.
- Neighbouring colonies could take care of waste products.

By using different enzymes of the different species of bacteria, almost any organic substances could be broken down.

2.3 Biofilm eradication

The only way to completely remove a biofilm is to combine a chemical attack with a mechanical cleaning process. Using only either of these ways to erase the biofilm will result in a failure. The physical destruction of the biofilm would make the organisms in the generally well-organized society much more exposed and susceptible to the biocides, but without the addition of chemicals some rests of biofilm will always be left behind, and soon a new biofilm will be under construction.

Biofilms can recover very fast to its initial state after an incomplete eradication. Reasons for this fast recovery are:[2]

- There are still enough viable bacteria in the remains of the biofilm, which makes the recovery faster than initialisation of a new biofilm.
- The residual biofilm makes the surface rougher, and this makes it easier for other bacteria to attach to it.
- The surviving organisms will soon start producing a protective slime shield consisting of polysaccharides.

The resistance against chemicals is about 1000-1500 times better for bacteria in biofilm than for planktonic bacteria. If the goal is to kill every single bacterium in the biofilm by the use of chemicals only, *too* much chemical products would have to be used. Such an extensive use of strong chemicals have many drawbacks, e.g. an increased risk for the staff that handle and use the chemicals on a daily basis, additional wear and tear on the dialysis equipment, and also an increased environmental stress.[5]

There are a number of reasons for the power of resistance of biofilm to biocides, and they could be divided into two groups: chemical/physical resistance, and biological resistance.

2.3.1.1 Chemical and physical resistance

Some biofilms have substances in the surface layer of the biofilm that neutralizes an antimicrobial agent by a chemical reaction. This resistance mechanism reduces the bulk fluid concentration of the agent to ineffectual levels, and the biofilm will therefore be unaffected by the attack. The efficiency of this defence system depends on the concentration of the antimicrobial agent, as well as its ability to react with the biofilm substance.

As previously mentioned, a biofilm is equipped with a shield of polysaccharides that prevents chemicals from getting in contact with the enclosed bacteria. At first, this was assumed to be a physical barrier only, but measurements of effective diffusion coefficients in biofilms indicate that a number of antimicrobial agents, especially those with a low molecular weight, are not very efficiently prevented from entering the biofilm. The diffusive transport coefficients for some antimicrobial agents are of the same order of magnitude as some non-toxic substances, e.g. pure water.

A more recent theory claims that the polysaccharide shield work closely together with the rest of the biofilm, and this combination constitutes a physical shield as well as a chemical shield. As an antimicrobial agent penetrate the polysaccharide shield, an interaction between the agent and some substance in the biofilm neutralize the antimicrobial activity. Thus, for an agent to be harmful to the biofilm, the rate of diffusive penetration must exceed the rate of deactivation. This theory has been proven experimentally several times using the antimicrobial agent hypochlorite, and is believed to be valid for other biocides as well.[2]

2.3.1.2 Biological resistance

Even though the biofilm is too thin, or insufficiently reactive with the antimicrobial agent, to be able to rely on either of the preceding resistance mechanisms, it has turned out that the biofilm still can survive a biocide attack. The reduced susceptibility of biofilm microorganisms could be explained with two types of biological resistance.

The first type of resistance is based on that nutriments are not evenly distributed throughout the entire biofilm, and some parts will therefore exist in a slow-growing or starved state. Organisms in such slow- or non-growing states are less susceptible to many antimicrobial agents, than the rapidly growing parts, due to their low metabolism.

The second type of biofilm susceptibility limitation assumes that there are local types of distinct and relatively resistant biofilms. This state of the biofilm is not a result of a nutrient limitation, but is more a result of other stimulus, e.g. contact with a solid surface, or attainment of a threshold cell density.[2]

2.3.2 Biofilm formation prevention

2.3.2.1 Surface smoothness

There are yet no materials discovered that biofilm cannot attach to, but during the first hours of contact between bacteria and a surface, the biofilm formation depends slightly on the surface smoothness. The smoother the surface, the less attachment of biofilm. But in the long run, smoothness does not appear to significantly effect the total amount of biofilm that will attach to a surface. Experiments with electropolished stainless steel and PVC plastics have been done, and after about six months of use they had the same amount of microorganisms growing on each surface.[3]

2.3.2.2 Flow velocity

High flow velocity will not prevent attachment of bacteria to tube surfaces, but it will make the biofilm denser and less thick. The thickness of the biofilm could be estimated by calculating the theoretic thickness of the layer with laminar flow closest to the tube wall, also called the boundary layer or the laminar sublayer. Regardless of the turbulence and flow velocity in the centre of the tube, there will almost always be a laminar sublayer and a flow close to zero along the tube wall.[3]

2.3.2.3 Nutriment supply

Like all living creatures, bacteria require a certain amount of nutriments for growth and reproduction. If these amounts could be kept low enough, bacteria growth could be limited. There are, however, at least two facts that make this method inapplicable when trying to reduce the bacterial growth:[3]

- Bacteria in biofilm can live on very low nutriment levels. Even in the purest of water systems they can find enough nutriments to form a biofilm.
- A dialysis treatment can never be sterile, and the dialysis fluid together with the waste products from the patient will make an excellent supply of nutriments.

2.4 Conventional biofilm detection methods

Despite very little information about site, extent or composition of biofilm in a system can be obtained by examining water samples, this is still one of the most frequent approaches for biofilm detection in the industry today. To get the desired information of the biofilm the surface has to be examined. This could be done in a number of different ways, but only two will be discussed here, namely Electron Microscopy and CFU-counting.

2.4.1 Electron Microscopy (EM)

The great advantage of an electron microscope, as compared to an ordinary optical microscope, is above all the resolution of the images obtained. An optical microscope uses wavelengths around 500 nm, and due to diffraction effects it cannot resolve objects smaller than a few hundred nanometers.[18]

Electrons also have wave properties, and their wavelengths could very well be several thousand times smaller than those of visible light. The resolution of an electron microscope is therefore a lot better, and samples could be studied in much greater detail. This gives that the surface of the biofilm can be examined. There are two different kinds of electron microscopes: transmission electron microscopes, and scanning electron microscopes.

2.4.1.1 Transmission electron microscope (TEM)

Electrons are emitted from a cathode and accelerated by a potential difference. The electrons are formed to a parallel beam by a magnetic field before they pass through the very thin sample (usually in the range 10-100 nm) to be examined. The sample scatters the electrons that are passing through it, and then magnetic lenses focus them onto a fluorescent screen for viewing. The denser regions appear darker in the image, since these regions scatter more electrons and therefore give rise to fewer electrons reaching the screen.

Intuitively, one might think that when the electron wavelength is about 0.01 nm, the resolution would be of the same order of magnitude. It is, however, seldom better than about 0.5 nm due to a number of reasons, e.g. the difficulty of having the correct focal lengths of the magnetic lenses. That depends on the current in the coils, and the speed of the electrons, which is never the same for all electrons in the beam.[18]

2.4.1.2 Scanning electron microscope (SEM)

An important variation is the scanning electron microscope, where the electron beam is focused to a very thin line that is swept across the sample. As the beam scans the sample, electrons will be emitted from the surface of the sample. These secondary electrons will be collected and produce an image of the surface of the sample.

A big advantage of SEM, as compared to TEM, is that the sample can be thick, since it is only the surface that is examined. Also, the secondary electron production depends on the angle of incidence on the surface of the electron beam, which results in SEM images having a more three dimensional appearance than a conventional optical microscope. Regarding the resolution, it is typically of the order of 10 nm. Not as good as for TEM, but still much better than optical microscopes.[18]

2.4.2 CFU-counting

A very common method to estimate the amount of bacteria in biofilm is CFU-counting, where CFU stands for Colony Forming Units, i.e. gatherings of bacteria that form growing colonies. The method is very simple; the biofilm is scraped off the surface to be examined, and the pulp is dispensed in water. A small sample from this solution is put to grow on a so-called TGEA plate, (tryptone glucose extract agar) and the number of CFUs is counted.

It could be difficult to correlate the CFU-value to the fluorescence signal for at least two reasons. At first, *all* biofilm has to be mechanically removed from the surface, and secondly, the biofilm has to be *completely* dispensed in the water. Especially the latter could be difficult to accomplish, since there could be a tendency of the biofilm to get lumpy when it is scraped off the surface. This could result in that a lump of several microorganisms will be counted as one CFU. Despite the roughness of this method and its problems, it is today one of few methods that can estimate the amount of bacteria in biofilm.

2.5 Optical detection methods

To be able to choose which optical biofilm detection method to use in the practical experiments, a number of alternatives were investigated and evaluated. Among the parameters that affected the choice was the theoretical sensitivity of the system, which is important since the biofilm is very thin and will only give very weak signals. It is also important that it is a non-destructive method, so that the dialysis equipment will not be damaged. Another requirement is that the detection system could be made small and inexpensive This gives that the surface of the biofilm cat be examined.

2.5.1 Optical rotation

Sugar is an optically active compound, which means that it has the ability to rotate the plane of vibration of plane-polarized light. Since biofilm partly consists of polysaccharides, a comparison of the planes of vibration of the illumination light and the transmittance light could, in theory, indicate the presence of biofilm. This would be an inexpensive and non-destructive method.

However, the thickness of the polysaccharide layer in the biofilm is only of the order of 10 μm, so this would only result in a very slight angular rotation.[6] As a rough estimate, say that the rotatory power of the polysaccharide layer is 10°/mm,[7] then the angle difference to be detected would be about 0.01°. To be able to detect such small polarization rotations, very precise and well-linearized equipment has to be used. In addition, other optically active compounds, which may be present in bulk fluid, could contribute to the detected polarization rotation, thus making it, with the technique available today, impossible to extract the part of the rotation coming from the polysaccharide layer.

2.5.2 Infrared absorption spectrometry

Almost all molecules have the ability to absorb infrared light. The more there is of a certain molecule, the larger the absorption will be. There are two different approaches to practical use of this method; either a monochromatic light source could be used, and a reduction of the transmitted light will indicate presence of biofilm, or a broad-banded light source could irradiate the sample of interest, and dips at specific wavelengths in the transmittance spectrum could be used as biofilm markers.[8]

This method offers an inexpensive and non-destructive detection method but it might, however, not be the best choice, since the biofilm layer is so thin that it will only slightly affect the transmitted light. The signal difference between a clean and a befouled system might therefore fall within the noise variations, thus making this technique difficult to use for our purpose.

2.5.3 ATR-FTIR spectroscopy

Attenuated Total Reflection Fourier Transform InfraRed spectroscopy is another, more sensitive, method based on absorption of IR-light, which can provide valuable information related to the chemical structure of a compound. It utilizes that absorption of radiation in the mid-infrared region is related to fundamental vibrations of the chemical bonds. An investigation of this kind will result in an absorption spectrum, where peaks at different wavelengths represent the presence of certain chemical bonds in the examined compound.[9]

An ATR-FTIR spectroscopic measurement uses a so-called Internal Reflection Element (IRE), usually made of zinc selenide (ZnSe) or germanium (Ge), which is put in contact with the substance of interest. IR radiation is focused onto the end of the IRE, and is reflected down the length of the crystal. At each internal reflection, the IR radiation actually penetrates a short distance (~1 μm) from the surface of the IRE into the substance (Figure C).[9]

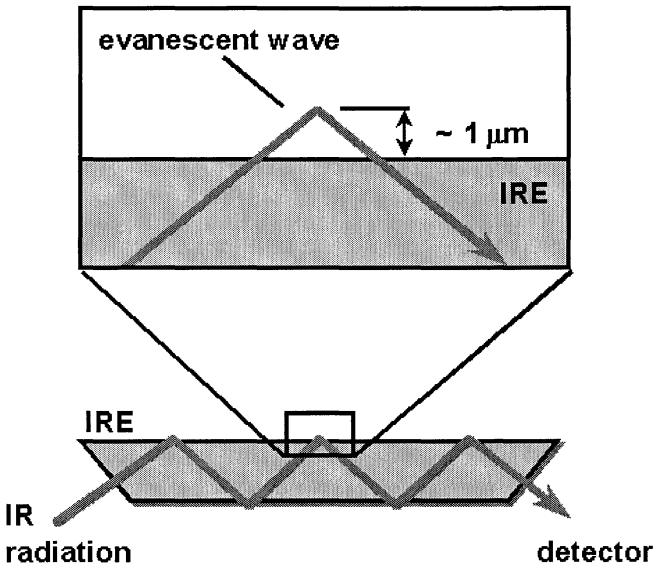


Figure C. Total internal reflection at the interface of an internal reflection element.[9]

If the IRE was to be put in contact with the bulk fluid, a biofilm build-up would start taking place, thus making it accessible for the penetrating IR light. This only allows a fraction of the biofilm to be investigated. This might not be a very important drawback, since characteristic carbon related bond peaks from organic compounds in the biofilm most likely will appear in the absorption spectrum anyway. Such carbon related peaks could be used as markers of biofilm, since the IRE does not contain any carbon. On the other hand, a more significant drawback is that the needed equipment is rather expensive, and could not possibly be installed in each single dialysis machine.

2.5.4 Raman spectroscopy

When light passes through a transparent medium, the present compounds scatter some of the light in all directions. A fraction of the scattered light has a slightly different wavelength from that of the incident light. This shift in wavelength, depends on the chemical structure of the molecules responsible for the scattering.[8]

Raman spectra are acquired by irradiating a sample, in this case a biofilm, with powerful visible or near-infrared laser light. A rather powerful laser must be used, since the intensities of the Raman lines almost never exceed 0.001% of the laser intensity.[8] There are today commercially available diode lasers that emit light with a power of a few tens of watts that could be powerful enough to be used, and small enough to fit inside a dialysis machine. These

lasers are however not very cheap, but they will probably be more affordable in a few years, thus making Raman spectroscopy an interesting method to use for biofilm detection.

2.5.5 Fluorometry

When a molecule absorbs a photon, it will be excited to a more energetic and instable state. After a very short time (about 10^{-5} to 10^{-8} s), the excess energy of the molecule could be emitted in the form of another photon, slightly less energetic than the previously absorbed one. This phenomenon is referred to as fluorescence, and measurement of the fluorescence light is called fluorometry.

Many organic molecules have the ability to fluoresce when excited with light in the UV or blue wavelength region. The fluorescence light will often be visible, and could be detected by sensitive detectors. The detection limits are usually one to three orders of magnitude smaller than those encountered in absorption spectroscopy.[8] This makes the fluorescence method seem like a good choice for the purpose of detecting biofilm.

There has also been some research done on different bacteria, and their fluorescence in different environments. A number of fluorescing compounds have been identified in the bacteria[7-10], and these are therefore present in biofilm as well. Accordingly, the fluorescence method seems like the best choice, based on the criteria stated earlier.

The available fluorometry equipment chosen to be used in this project is neither small nor cheap, but the vision is that diode lasers could be used to generate the light in the future, making the equipment substantially smaller. It might also be possible to make the equipment fairly cheap, if it turns out that only a simple detector would be needed for the recording of the fluorescence light. Therefore, use of fluorometry seems like a good starting point in the search of an optical method to detect biofilm in dialysis machines.

3 Fluorescence

3.1 Basic Fluorescence Theory

The fluorescence process starts with the absorption of a photon by a molecule, and subsequently another photon, the so-called fluorescence photon, will be emitted. For an incoming photon to have a chance to be absorbed, there has to be an energy match between the energy of the photon and the difference in energy between an excited state and the ground state of the molecule.[16]

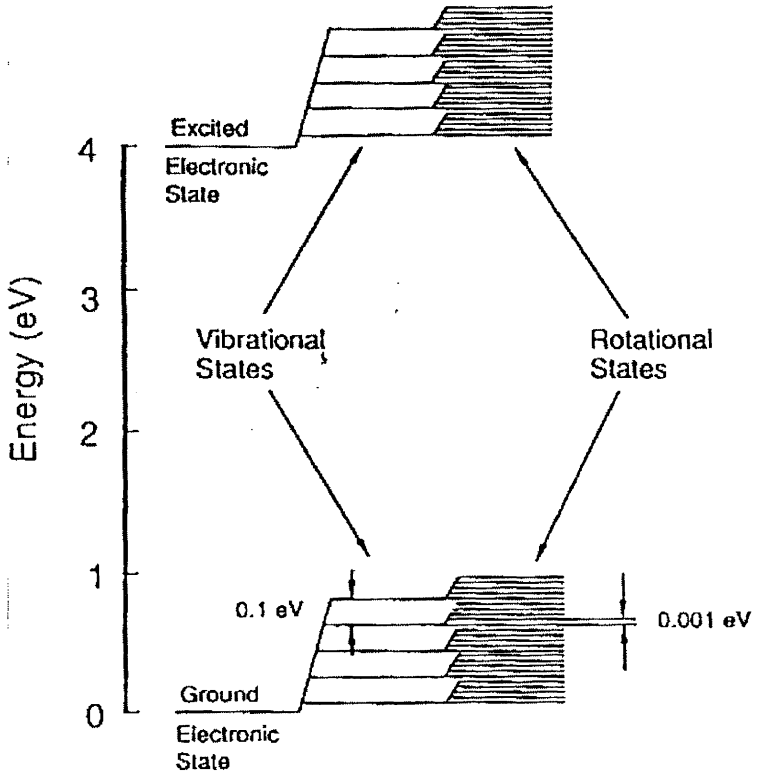


Figure D. Schematic molecular energy level diagram with electronic, vibrational, and rotational levels.[17]

The electronic states of a molecule are divided into vibrational levels, which in turn are divided into rotational sublevels (Figure D). Due to the very complex structure of organic molecules, there will be an enormous amount of vibrational and rotational levels within each electronic level, which leads to substantial overlapping of energy levels, and so-called energy bands will be formed. The band structure makes it possible for photons in a wide wavelength interval to become absorbed. The Full Width at Half Maximum, FWHM, of the excitation profiles for organic compounds could therefore be of the order of tens of nm. Since the band structure is involved in the deexcitation process as well, this light will also have rather broad wavelength distributions.

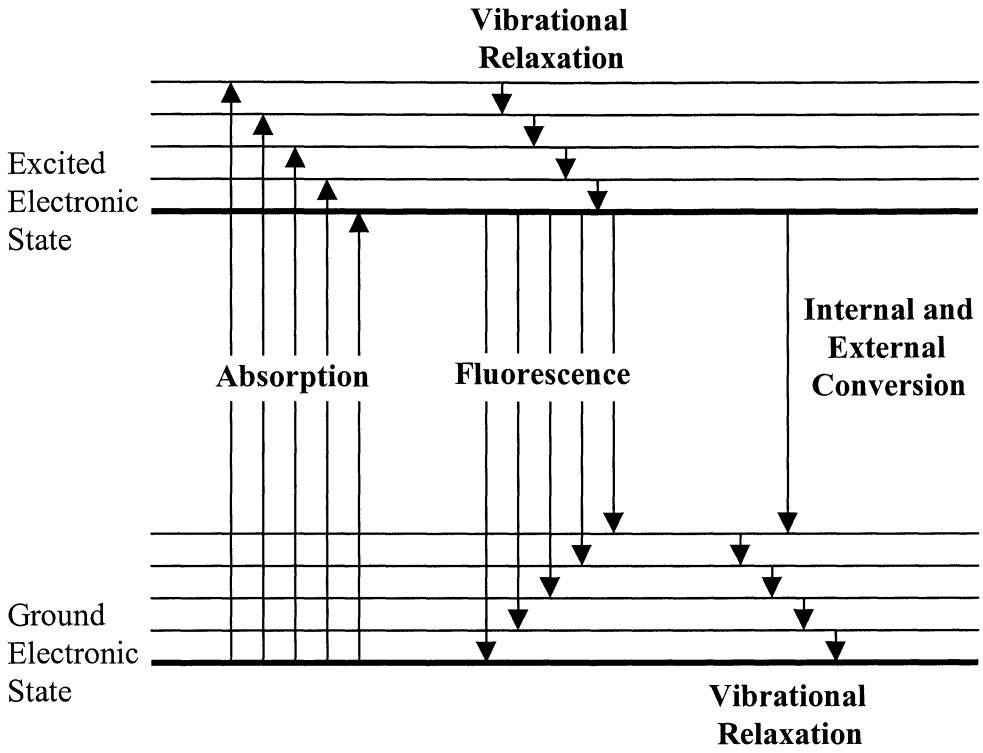


Figure E. Diagram showing absorption of a few photons, and some of the possible relaxation processes.

The fluorescence photon is less energetic than the absorbed photon, and it does therefore have a longer wavelength. This is because of loss of energy due to non-radiative internal processes in the molecule, e.g. vibrational and rotational relaxation within the electronic state (Figure E). All the absorbed photon energy will not be sent back as fluorescence light. Instead, some fraction of it will be lost to internal and external conversion of the stored energy into, e.g. heat, or perhaps induction of a chemical reaction.

3.2 Fluorescence from Organic Compounds

The biofilm, being examined in this project, was produced by bacteria and yeast cells that can be found in dialysis machines. A number of different fluorescing compounds, so-called fluorophores, have been found in yeast cultures and in other bacterial environments. In fact, some of the most thoroughly examined organic fluorophores are important vitamins or amino acids, or important substances for the metabolism of the cell, and are therefore present in living organisms of all kinds. This makes it seem possible that their fluorescence signals might reveal something about biofilm composition and metabolism.

To be able to determine the amount of biofilm on the inside of the tubes in the dialysis machine, it would be desirable if the fluorescence signal from a certain fluorophore was proportional to the biomass of the biofilm. This is rarely the case though. Taking a certain mass of biofilm, some fluorophore levels can vary quite a bit from case to case, due to the enormous variations in biofilm composition, see 2.2.

3.2.1 NAD(P)H

Previous studies[10-13] have proven that detection and analysis of fluorescence light is a very useful technique when monitoring and controlling fermentation processes. Many organic compounds fluoresce in the UV or blue wavelength region. Two very common fluorophores in the cells are NADH (reduced nicotinamide adenine dinucleotide) and NADPH (reduced nicotinamide adenine dinucleotide phosphate). Their task is to produce useful energy for the organism in the metabolic processes in the cells. NADH and NADPH, further called NAD(P)H since their biological and optical properties are very much alike, are therefore present in all living organisms.

In 1957 the first experiments measuring the NAD(P)H fluorescence were performed. Duysens and Amesz[14] found that the fluorescence spectra from two different kinds of yeast and one kind of algae, respectively, were very similar to that from NAD(P)H. This discovery might have raised some hope among contemporary researchers that the intensity of the NAD(P)H fluorescence signals could be a good indicator of the presence and amount of living organisms. However, further experiments during the following years proved that the NAD(P)H signal alone is not a very reliable measurement of microorganism amounts.

The amount of NAD(P)H in living organisms, such as yeast and other bacteria, is controlled in a balance reaction, a so-called redox reaction. Experiments have shown that NAD(P)H fluorescence is very sensitive to the concentration of dissolved oxygen.[11] A low oxygen level shifts this balance towards higher levels of NAD(P)H and lower levels of $NAD(P)^+$, i.e. from right to left in the equation below, and vice versa.[3, 11, 13]

$$\underset{\text{low oxygen level: to the left}}{\underset{\text{high oxygen level: to the right}}{NAD(P)H \quad \Delta \quad NAD(P)^+ + e^-}}$$

Due to a separation of the excitation peak maximums for the two compounds by some 50 nm ($NAD(P)^+$: λ_{ex}<300 nm, and NAD(P)H: λ_{ex}~350 nm)[11], the level of free oxygen in the solution has a big influence on the intensity of the NAD(P)H fluorescence signal.

3.2.2 Other Fluorophores

After these first examinations were performed several other fluorophores for detection of microorganisms have been identified. Some of the most commonly used fluorophores in yeast and bacterial cultures are tryptophan (an amino acid), riboflavin (vitamin B2), pyridoxine (vitamin B6), and the above mentioned NAD(P)H. The excitation and emission wavelength profiles for these compounds can be seen below (Figure F and Figure G). There are of course other fluorophores as well, but these particular four compounds have proven to be the most interesting for a number of reasons:[12]

- They are all key metabolic components, and are therefore present in all kinds of environments with microorganisms.
- An Hg arc lamp could very well be used as an excitation light source, since it has suitable spectral lines (vertical lines in Figure F) in the wavelength region of interest.
- They fluoresce in fairly separate and distinct regions, without severe overlap of the curves (Figure G).

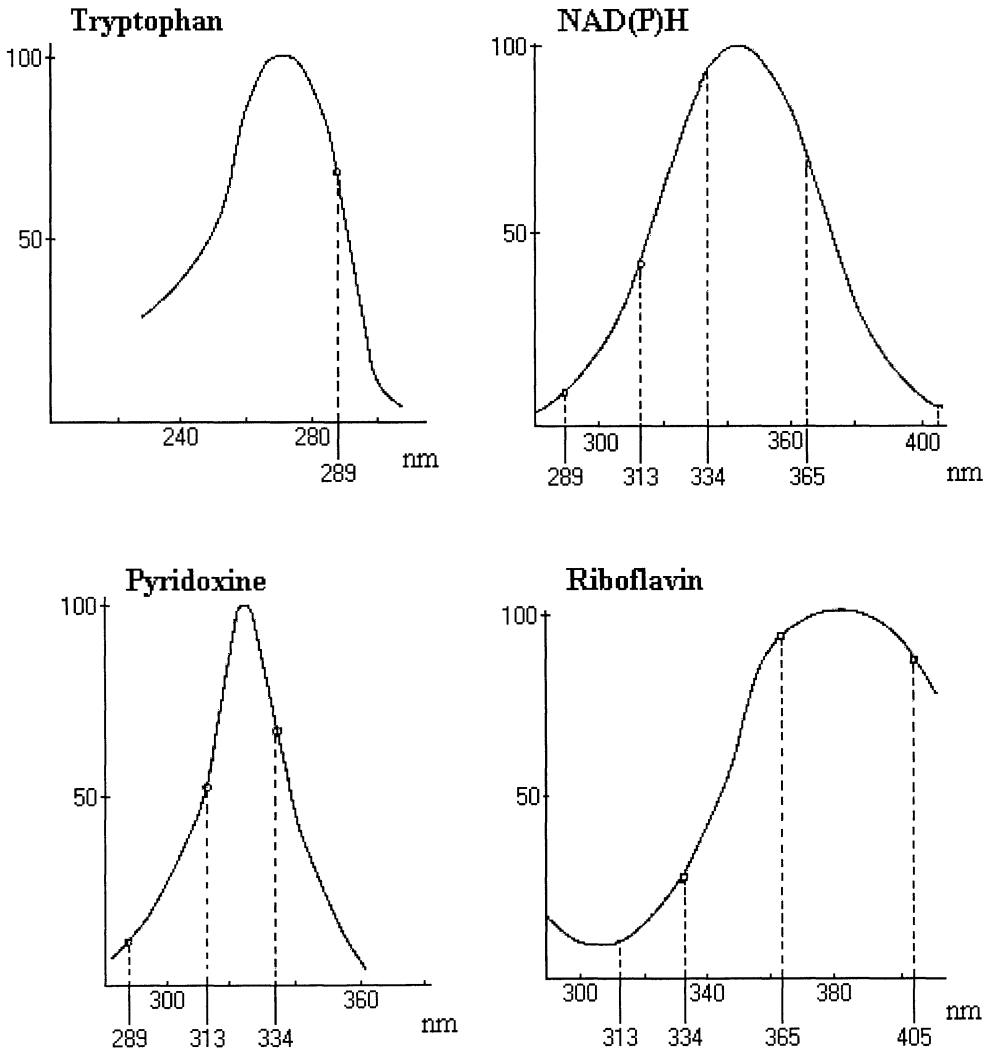


Figure F. The percent of maximum fluorescence output of the four major cellular fluorophores at various excitation wavelengths. The vertical lines are the spectral lines of the Hg-lamp.[12]

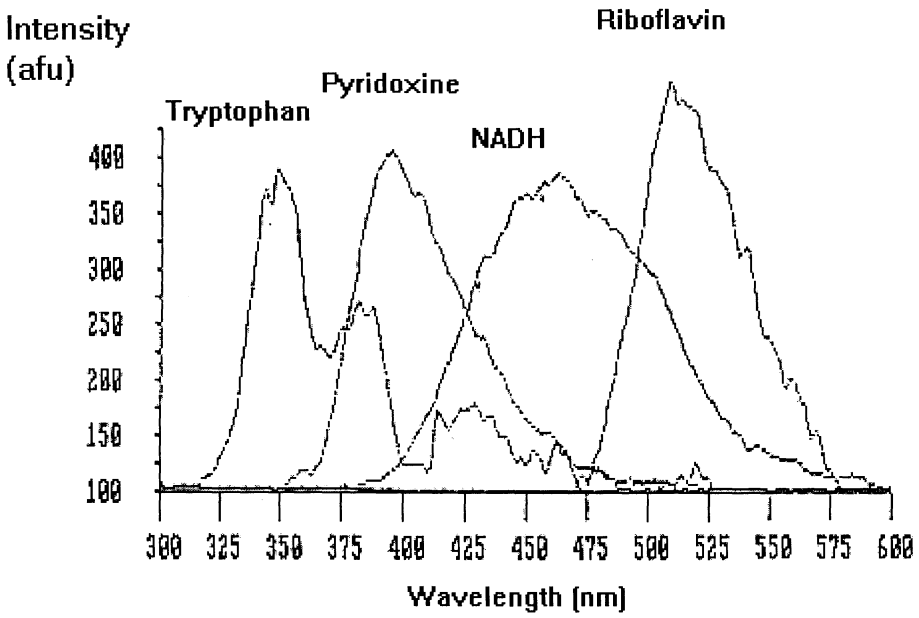


Figure G. Fluorescence emission spectra of the four fluorophores excited by Hg arc lamp spectral lines.[12]

3.2.3 Interpretation of Fluorescence Signals

These four compounds are also interesting because the fluorescence signal of each compound give some piece of information about either the metabolic state of the bioprocess, or the amount of biomass. One should keep in mind though, that translation of the fluorescence signals into useful information is not at all a straightforward process. Depending on circumstances such as what kind of bacteria that are present, and if the cellular system is in growth, in steady-state, or perhaps even declining, the fluorescence signals could be more or less informative. However, a few examples of how signals from the different fluorophores could be interpreted can be seen below.

3.2.3.1 Tryptophan

In yeast cultures (e.g. *Candida utilis*[13] and *Saccharomyces cerevisiae*[11], i.e. baker's yeast), the tryptophan signal has turned out to be rather proportional to the dry mass of the yeast. Since yeast has a high protein concentration in the cells, tryptophan, being an amino acid, is a good indicator of the total protein concentration of the culture. In fact, all organisms, living and dead, contain some amount of tryptophan, accordingly making this fluorescence signal a general measure of biomass concentration.

3.2.3.2 NAD(P)H

NAD(P)H fluorescence is a reasonable indicator of the cellular metabolic state. For example, a drop of the NAD(P)H fluorescence signal could indicate a switch from a phase with steady increase of cell mass, to a phase with low metabolic activity.[11] This switch will lead to an increase of the level of dissolved oxygen in the bulk fluid surrounding the culture, since when aerobic microogranisms (e.g. *S. cerevisiae*) multiply they consume oxygen. As stated earlier, a higher oxygen level will give a weaker NAD(P)H signal. As the cell growth stops, the oxygen level will go up, and therefore the NAD(P)H signal will drop.

3.2.3.3 Pyridoxine

Experiments have shown that in cellular systems (*S. cerevisiae*) containing glucose, the pyridoxine fluorescence signal closely follows the concentration of glucose in the bulk fluid.[10, 12] As the glucose is being used up, and turned into e.g. ethanol, in the fermentation process the pyridoxine fluorescence signal is strongly reduced. Therefore, in fermentation processes consuming glucose, the pyridoxine signal could be considered an inverse measure of metabolic activity.

3.2.3.4 Riboflavin

Riboflavin gives the strongest fluorescence signal of the four biofluorophores at similar concentrations and monitoring conditions.[13] When a system of yeast cells is growing exponentially, riboflavin, as well as the other three fluorophores, show a wide linear relationship between the logarithm of its fluorescence intensity and its concentration in the culture.

3.3 Biofilm Fluorescence Measurements

Early instruments for study of a culture's biological parameters and processes, such as cell concentration, cell activity, and environment conditions, were usually limited to measuring (what was thought to be) the NAD(P)H fluorescence only. These instruments was not always to such big help, since it is virtually impossible to excite NAD(P)H without also exciting pyridoxine and/or riboflavin. Because of an overlap in the fluorescence spectra of the three compounds, the recorded signal was not only due to NAD(P)H, and that led to interpretation mistakes. In the last decade or so, it has been found that by simultaneously monitoring the intensity of the fluorescence signal from tryptophan, pyridoxine, riboflavin, as well as NAD(P)H, a much better understanding of the state of the culture and ongoing processes can be obtained.[12]

If the biofilm grows thick enough, it may develop an anaerobic environment in the biofilm layers closest to the pipe wall (Figure H), creating better conditions for NAD(P)H fluorescence. However, how thick the biofilm has to be to ensure anaerobic conditions is probably impossible to say. It is most likely dependant on the composition of bacteria in the biofilm, as well as its structure.

There is an obvious risk that a weak NAD(P)H fluorescence signal makes you draw the erroneous conclusion that the amount of biofilm is low, whereas the real reason for it might be a high level of free oxygen. The risk for the corresponding error to appear when measuring tryptophan, riboflavin or pyridoxine fluorescence is close to non-existent, since they are present in all organisms, aerobic and anaerobic.[11]

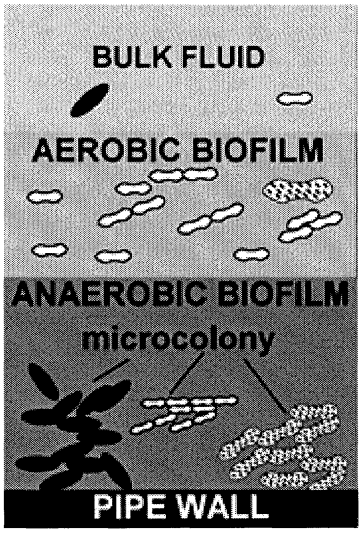


Figure H. An anaerobic biofilm may develop underneath the aerobic layer.[3]

3.3.1 Variables that affect fluorescence signals

Both molecular structure and chemical environment influence whether a substance will or will not fluoresce. These factors also determine the intensity of the signals when fluorescence does occur.[8, 13, 15] When dealing with such complex media as organic compounds there is usually some uncertainty involved in how the fluorescence signals will appear. Some of the major parameters are listed below, along with a short discussion about each one.

3.3.1.1 Quantum yield

The quantum yield, or quantum efficiency as it is sometimes called, is defined as the ratio of the number of molecules that fluoresce to the total number of excited molecules. There are compounds that do not fluoresce at all, and do therefore have a quantum yield of zero, but there are also examples of molecules that fluoresce very efficiently, and have efficiencies approaching unity.

It has been found empirically that fluorescence is favoured in molecules that have rigid structures. Lack of rigidity in a molecule probably causes an enhanced internal conversion rate, and as a consequence there will be an increase in the likelihood for radiationless deactivation.

3.3.1.2 Temperature

An increased temperature will result in an increased frequency of collisions between molecules. This will in turn increase the probability for deactivation by external conversion, resulting in fewer molecules that are likely to emit fluorescence light. The result of a higher temperature is therefore a reduced fluorescence light intensity.

3.3.1.3 pH-value

The fluorescence properties of some compounds is dependent on the pH-value of the surrounding media. As the pH of the media is changed from acetic to being more basic, the fluorescence sometimes get shifted towards longer wavelengths. This behaviour is most commonly observed when dealing with pH-value changes of aromatic compounds, i.e. molecules with a ring structure, with acidic or basic ring substituents, whereas for other types of molecules it might be difficult to observe any shift.

In theory, there would be an increase of NAD(P)H fluorescence when the fluid surrounding the bacteria gets more acidic, because of the balance reaction below:

$$\underset{\text{more basic: to the right}}{\text{NAD(P)H}} \quad \overset{\text{more acidic: to the left}}{\Delta} \quad \text{NAD(P)}^{+} + e^{-}$$

This is not necessarily the case in practice though, since a lowered pH-value very well could lead to a less pleasant environment for living organisms. A reduced number of living bacteria could give less fluorescence light, unless it was fully compensated for by an increase of the NAD(P)H level.

3.3.1.4 Quenching agents

The amount of fluorescence light could become reduced if there are molecules present that cause deexcitation of the fluorophore by colliding with it. This effect, known as quenching, makes the fluorophore go from the excited state to the ground state without emitting any light. Instead the energy might be transformed into heat, lead to ionisation, dissociation of the fluorophore, or perhaps induce a chemical reaction of some sort.

Certain compounds are known to be particularly effective quenchers of fluorescence. Usually these compounds form short-lived collisional complexes with the fluorophore in an excited state, and then the energy of the excited state is dissipated as heat. The extent of quenching

does not only depend on the nature of the quencher, but also on the extent to which the quencher can interact with the fluorophore.

For instance, free oxygen is a good quencher of tryptophan fluorescence. The small molecule can penetrate into small crevices in the structure of tryptophan, and by that quench its fluorescence.[15] So, when there is a good NAD(P)H signal, due to the low level of free oxygen, a good tryptophan signal could probably be found as well.

3.3.1.5 Concentration

It could be shown mathematically that the fluorescence intensity from a solution is proportional to the concentration of the fluorophore in the solution, as long as this concentration is low.

As the concentration increases the linearity will be lost due to self-quenching and self-absorption. Self-quenching is the result of collisions between excited molecules of the same kind. Logically, this factor is expected to increase with increasing concentration. Self-absorption occurs when the wavelength of emission overlaps with an absorption peak. If this is the case, the probability for loss of fluorescence light in the expected wavelength interval will increase.

3.3.1.6 Dissolved oxygen

As previously discussed (see 3.2.3.2), the presence of dissolved oxygen could reduce the fluorescence intensity, if a balance reaction is driven away from the fluorescing state. This is not the only fluorescence reducing effect of high levels of dissolved oxygen. It could also lead to increased quenching, as was discussed in the previous section (3.3.1.4).

3.3.1.7 Medium viscosity

Medium viscosity can change the collisional quenching of fluorescence. In general, increase of viscosity increases fluorescence intensity as it reduces the collisional quenching. Among the four major fluorophores, NAD(P)H is the most viscosity sensitive. Since biofilm viscosity is difficult to control, it might be necessary to develop a mathematical model if a complete and correct compensation is wanted for its effect.

3.3.1.8 Metabolic state

The cellular metabolic state is also very important. Several investigations have shown that the fluorescence signals are different, for the same amount of fluorophore, depending on which state the bacteria are in, i.e. if they are in the exponential growing phase, the steady-state phase, or perhaps in a state where it is trying to defend itself against a biocide or an attacking fluid, such as hot water, a chemical, etc. The exact behaviour of the registered signal is hard to predict, and varies with the fluorescence light of the compound that is being studied.

4 Materials & methods

4.1 Optical components

To be able to detect biofilm in a dialysis machine using fluorometry, there has to be an optical window somewhere in the machine, or placing an optical fibre in the flow path. As a first step the choice was to have an optical window. An optical window has to transmit wavelengths in the range 250-600 nm, in order to fully cover the excitation and emission wavelengths of the four interesting fluorescing compounds in the microorganisms (see 3.2). To be able to detect the very small fluorescence signals from the organisms, it is desirable that the window itself is not fluorescent. The chosen material does also have to withstand harsh physical and chemical environments, such as hot (~95°C) liquids, detergents, high as well as low pH-values, etc.

Blood leak detector (PSU)
The blood leak detector is a cylindrical, yellowish plastic unit (made of Poly Sulphone Plastic, PSU), which is detecting the leakage of blood into the fluid circuit of the dialysis machine. Since the blood leak detector already is a part of the machine, it has proven to withstand the physical and chemical strains necessary. However, an optical investigation showed that it does not transmit any light below 350 nm (see Appendix A, Figure 1), and it exhibits strong fluorescence emission when illuminated with UV and violet light. That makes this device rather useless for bacterial fluorometry.

Drainage tube
The drainage tube from the dialysis machine was dismissed for pretty much the same reasons as the blood leak detector, i.e. its strong fluorescence and low transmission of light below 300 nm (see Appendix A, Figure 2).

Glass
There are several different kinds of glass, but only one was examined in this study. This was a weaker fluorophore than the blood leak detector and the drainage tube, but was still not ideal for fluorescence measurements. For transmission properties see Appendix A, Figure 3.

Plastic cuvette (PMMA)
PMMA-plastic, i.e. Plexiglas, has good optical properties, being non-fluorescent, and transmitting light down to about 250 nm (see Appendix A, Figure 4). On the other hand, it does not withstand hot liquids, and it is therefore not an option.

Quartz
Quartz is a common material in optical environments because of its very good optical properties: it can transmit light in the range 200-2500 nm, and is non-fluorescent. It did also pass the physical and chemical tests, and was therefore chosen to be included in the experimental set-up for the biofilm examinations.

4.2 Excitation wavelengths

To maximize the intensity of the emission signal, the best choice of excitation wavelengths would be to hit the peak of the excitation profiles of the four fluorophores, see 3.2.2. Unfortunately, no such light sources were at our disposal. Instead, a so-called OMA-system (Optical Multichannel Analyser) was used, which was equipped with a pulsed 337 nm nitrogen laser, with a possibility to change the wavelength to 405 nm with the dye DPS (4,4-diphenylstilbene).[20, 21]

The 337 nm light excites three (pyridoxine, NAD(P)H, and riboflavin) of the four interesting fluorophores, and is therefore a very useful wavelength. 405 nm could only be used to excite riboflavin, but to substantially higher degree than the 337 nm light. A drawback for the 405 nm light is that a great deal of the power is lost in the conversion process. Altogether, it almost evens itself out, so the 405 nm light was mainly used to confirm the results from the 337 nm measurements.

To be able to study the fluorescence signals from tryptophan, excitation light with a shorter wavelength (typically 250-290 nm) would have to be used. An external lamp emitting a strong 254 nm Hg spectral line was supposed to fill this void, but to detect the tryptophan signal the OMA-system had to be slightly modified. This turned out to be easier said than done, and after a number of unavailing attempts to get it to work the vision of recording the tryptophan signal was given up on.

The same lamp could also emit light in the wavelength region 365-380 nm. These wavelengths excite NAD(P)H and riboflavin, and could therefore possibly give confirming and/or supplementary information about these compounds. The recorded fluorescence signals did however not provide any useful information at all. It is hard to see why this is the case, since there are no limitations in the OMA-system for these wavelengths. Anyhow, the lamp did not work in a satisfactory way, and was therefore not used in any further experiments.

4.3 Fluorescence wavelengths

To be able to analyse the fluorescence signals from the different fluorescent compounds (pyridoxine, NAD(P)H, and riboflavin), their signals were integrated over the following wavelength intervals: pyridoxine 385-415 nm, NAD(P)H 450-480 nm, and riboflavin 500-530 nm.[13]

4.4 Fluorometry equipment

An OMA-system with a pulsed 337 nm nitrogen laser, and the possibility to get 405 nm light by the use of a dye, was used.[20, 21] The excitation light was focused onto an optical fibre, which guided the light to the examination object. The same fibre guided the fluorescence light, via some lenses and filters, into a spectrograph and finally to a CCD-camera. The CCD-camera was connected to a computer, which was equipped with suitable software for spectrum analysis.

The precision of the equipment was experimentally examined by repeatedly measuring the fluorescence from an object. It was found to give a maximal variation of ±3 %.

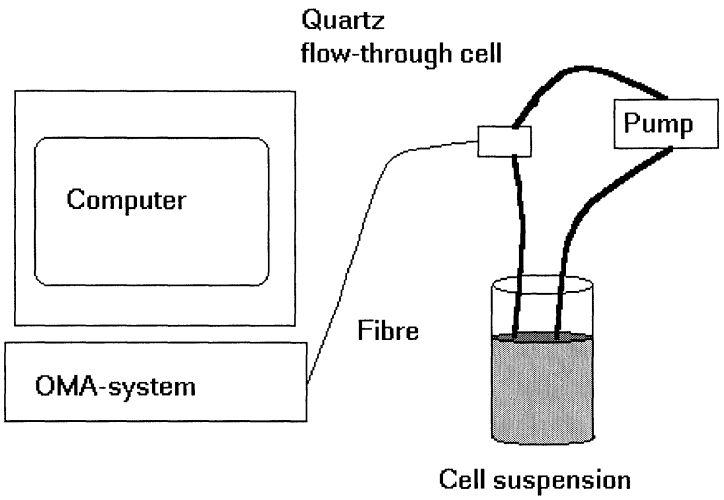


Figure I. The fluorometry equipment.

4.5 Microorganisms

The cell suspension used in the different experiments, except the yeast measurement, was a mixture of the following bacteria and yeast cells suspended in sterile water: *Pseudomonas aeruginosa, Staphylococcus aureus, Burkholderia cepacia*, and *Candida albicans* (yeast).

These microorganisms were chosen since they are among the most commonly present ones in dialysis machines. *P. aeruginosa* and *B. cepacia* are bacteria that can be found everywhere in our environment, including water. *S. aureus* usually live on the human skin and in the gastrointestinal tract, and *C. albicans* constitutes a normal part of the flora from warm-blooded animals, but is also known to be water bacteria.

These microorganisms are used to having low levels of nutriments available, thus they are reacting quite slowly to changes in the environment, compared to bacteria used having a better supply of nutriments.

The concentration of the microorganisms was (in CFU/ml): *P. aeruginosa* 7.9×10^7, *S. aureus* 7.3×10^7, *B. cepacia* 7.7×10^7, and *C. albicans* 3.2×10^6. This resulted in a total microorganism concentration of 2.3×10^8 CFU/ml.

4.6 Fluorescence of cell suspension as a function of concentration

In order to examine how the fluorescence signal varies with different concentrations of the cell suspension, a number of suspensions were prepared. Starting with the suspension described in 4.5, which was then stepwise diluted by a factor of 10, until a suspension with a concentration of 230 CFU/ml was prepared. Samples from each of these suspensions were put in separate test tubes, and an optical fibre connected to the OMA-system was immersed in the fluid when measuring the fluorescence.

4.7 Fluorescence of yeast as a function of fermentation time

The cell suspension consisted of 200 g of baker's yeast (*Saccharomyces cerevisiae*) dissolved in two litres of water at 40°C and there was a continuous rotation on the fluid. An optical fibre, connected to the OMA-system, was placed in the middle of the suspension, several centimetres from the walls of the container, an Erlenmeyer flask (see Figure J). To create advantageous growth conditions, 80 ml of a glucose solution (500 mg/ml) was added to the cell suspension, giving an initial glucose concentration of 2%. Measurements were done every five minutes for about two hours.

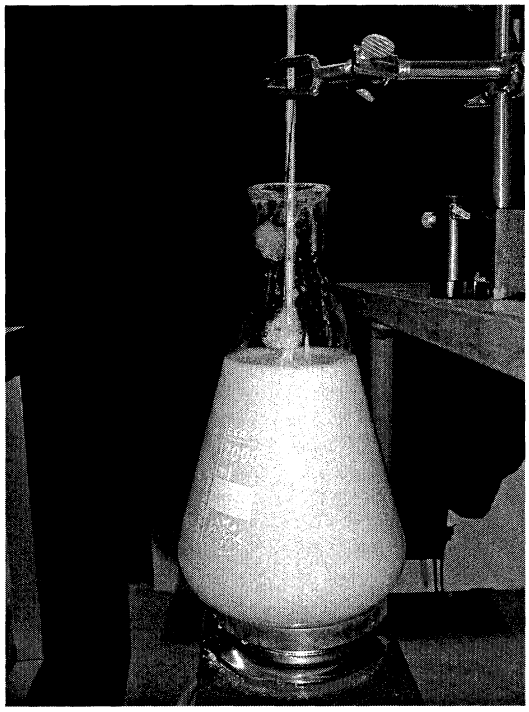


Figure J. The experiment set-up for the yeast measurement.

4.8 Fluorescence of cell suspension as a function of pH

To investigate the connection between the pH-value of the cell suspension and its fluorescence signal, six different solutions with pH-values from 3 to 8 were prepared. The mixture and concentration of microorganisms were the same as described in 4.5.

The pH-value was measured with a pH electrode and the index of pH was adjusted by adding very small amounts of solutions of either hydrochloric acid (HCl) or sodium carbonate (Na_2CO_3). Samples of the suspensions with adjusted pH-values were put in separate sealed test tubes, and were left alone for 24 hours to adapt to their new environment. The fluorescence was measured in the same way as the concentration measurements above.

4.9 Fluorescence of water as a function of temperature

The aim of this experiment was to examine the fluorescence signal of water as a function of temperature. About two litres of RO-water (cleaned in an Reversed Osmosis process, i.e. water with very low levels of microorganisms) was slowly heated on a hotplate. An optical fibre, properly immersed into the water, connected to the OMA-system registered the fluorescence signal with about 10°C spacing, starting at 21°C and ending at 91°C.

4.10 Fluorescence of biofilm

This experiment was done to see how the fluorescence changes when biofilm is growing on a surface, as well as how the biofilm fluorescence is affected when the surface is disinfected by a similar routine as the one performed after each dialysis treatment.

A cell suspension with the same mixture and concentration as described in 4.5 above was used. This suspension was circulated in a system with a quartz flow-through cell, and a pump that was set at a flow rate of 500 ml/min, which is about the same flow as typically used in a dialysis machine. To record the fluorescence signals, an optical fibre connected to the OMA-system was placed perpendicular to the quartz wall of the flow-through cell (see Figure K).

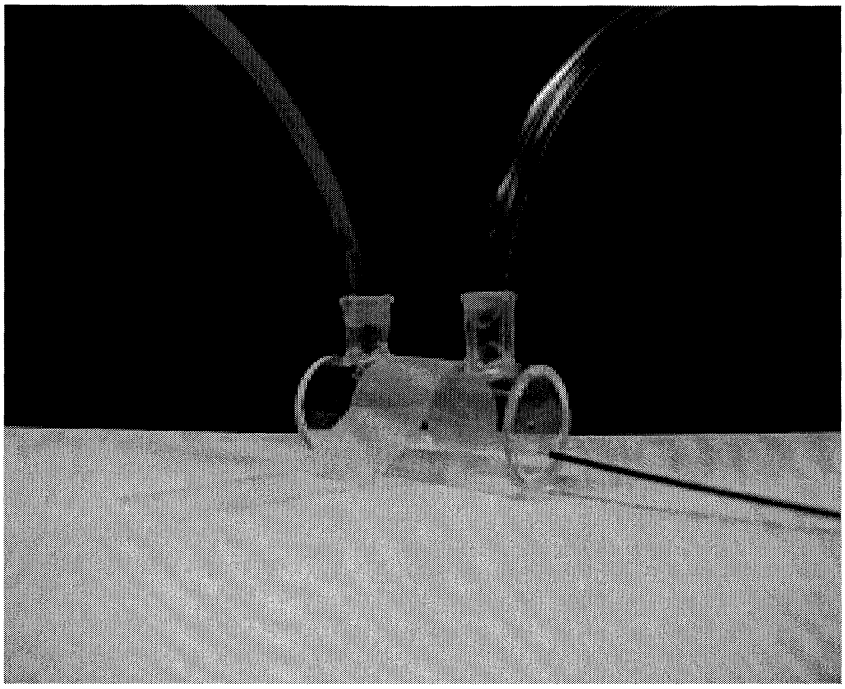


Figure K. The quartz flow-through cell and the fibre.

Three different reference signals were recorded: fluorescence from a clean flow-through cell containing water at 22°C, water at 95°C, or a cell suspension. These reference signals were recorded to study the influence of other fluorophores to be able to distimguish the biofilm fluorescence from the recordings. Two measurements cannot otherwise be compared to each other, since the fluorescence contribution from the bulk fluid would be unknown.

After six days of circulation, the cell suspension was removed, and a disinfection treatment was carried out. In the same manner as after a dialysis treatment, hot water (95°C) was circulated in the system for 45 minutes, and finally it was filled with water at 22°C. The fluorescence was measured during the circulation of the cell suspension, as well as before and after the disinfection routine, with water at 22°C and 95°C in the system. All fluorescence signals obtained were then compared with their respective reference signal.

4.11 Biofilm in drainage tube

In order to evaluate whether there is a biofilm build-up or not in the drainage tubes of dialysis machines, parts of such tubes were cut off and analysed with an electron microscope. The pieces were cut off close to the outlet. Two tubes were analysed: the first was almost new (only used during one treatment), while the second had been in use for several years. Both tubes were disinfected with a standard post-patient disinfection procedure before being removed from the machine.

5 Results & discussion

This thesis aims at taking the first couple of steps towards making optical detection of biofilm in dialysis machines possible. As part of this process, mapping of how the fluorescence signals depend on environmental parameters, e.g. temperature and pH-value, has to be performed. Another interesting task is to find some kind of correlation between the fluorescence signals of different compounds and the metabolic state of the bacteria. For these reasons some preparatory experiments have been performed before an actual biofilm measurement was carried out.

5.1 Fluorescence of cell suspension as a function of concentration

Cell suspensions with different concentrations were examined in order to evaluate how low concentrations of free-floating bacteria that can be detected using this technique and equipment. The fluorescence signal is expected to be proportional to the concentration of the cell suspension[11-13], but as can be seen in figure L this is not valid over the entire range of concentrations with our equipment.

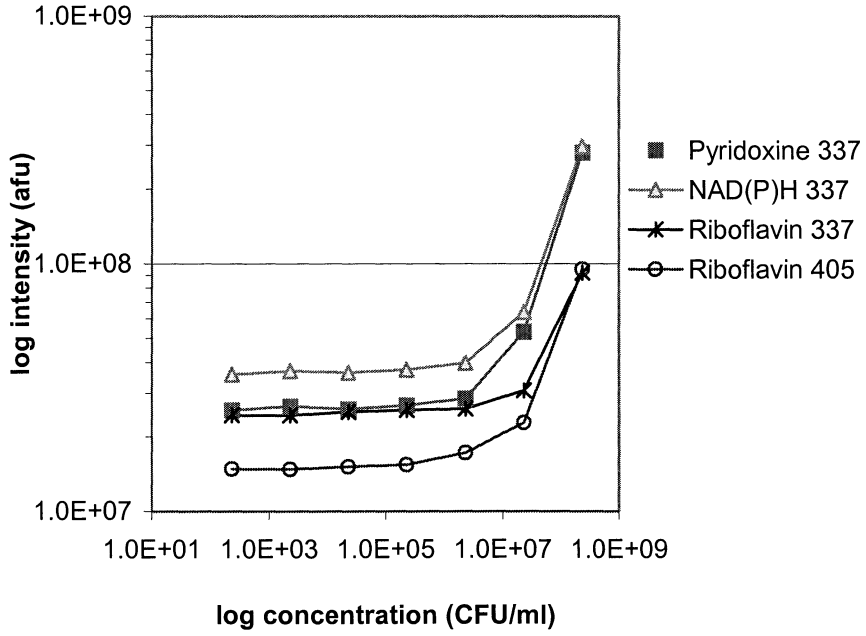


Figure L. The fluorescence from different suspension concentrations. Excitation wavelengths 337 and 405 nm.

The concentration has to exceed 10^7 CFU/ml for an increase of the fluorescence to be registered. That is several orders of magnitude worse than the desired limit. For comparison, tap water in Lund has about 10^2 CFU/ml, and people having their own well have been found to have a few thousand CFU/ml in their water. As a guideline, it would be desirable if a

detection method of this kind could make a difference between the tap water and the well water.

To check the linearity of the fluorescence signals, a number of suspensions with concentrations around 10^6-10^7 CFU/ml were prepared. This recording shows a very good linearity (see Figure M), and could be said to be in accordance with the theory. The R^2- values for the respective curves are: NAD(P)H 0.99, riboflavin 0.98, and pyridoxine 0.99. The R^2-value is a statistical measure of how well two curves are in accordance to each other. In this case the recorded curve is compared with a linear curve. These values indicate a good linearity.

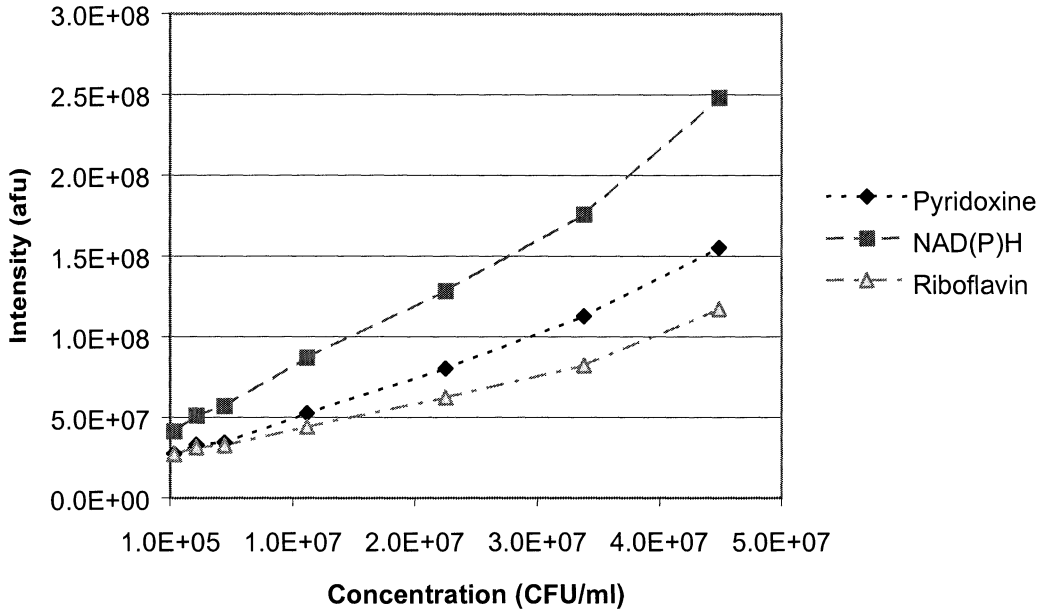


Figure M. The fluorescence from different suspension concentrations. Excitation wavelength 337 nm.

5.2 Fluorescence of yeast as a function of fermentation time

As glucose is added to a yeast culture, the glucose could be fermented to ethanol, C_2H_5OH, and carbon dioxide, CO_2, in a process called alcoholic fermentation. This does not happen instantly though, but to avoid very long measurement sessions baker's yeast (*S. cerevisiae*) has been used, since it reacts faster to an addition of glucose than most other kinds of yeast.

The object of this experiment was to see if increased metabolic activity during the fermentation process in some way reflects on the fluorescence signals. About ten minutes after the addition of glucose to the yeast suspension, foam started to form on the surface. The production of foam indicates that the glucose is being fermented into ethanol and carbon dioxide. This production of foam peaked about 30 minutes after the glucose addition, and another ten minutes later it had almost completely stopped.

During the liveliest part of the fermentation, i.e. when the metabolic activity is high, the level of NAD(P)H is believed to be somewhat raised.[19] In figure N it can be seen that the recorded signal does not significantly increase. From the time when glucose was added, to the time when the foam-forming process stopped, the NAD(P)H signal has gained about 5 %.

To judge from the vigorous foaming a much more noticeable peak was expected somewhere between 10 and 40 minutes after the glucose addition. This could mean one of two things: there is not any significant increase of the NAD(P)H level during the foaming process, or the equipment was simply unable to detect the increase. The somewhat raised levels of fluorescence about 45 minutes after glucose was added to the suspension is believed to be due to an increase of the number of yeast cells.

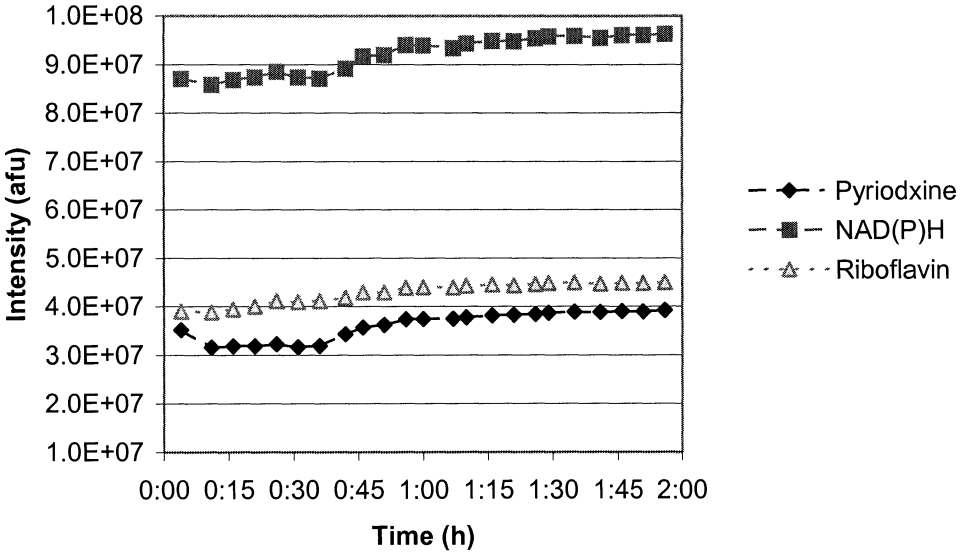


Figure N. Yeast cell fluorescence after the addition of glucose. Excitation wavelength 337 nm.

5.3 Fluorescence of cell suspension as a function of pH

To investigate the dependence of the fluorescence signal of the pH-value of the cell suspension, several mixtures with different pH-values were prepared. The time required for the microorganisms to fully adapt to their environment depends on the type of microorganism, but as an estimation they could need a few hours. Since the organisms used for this experiment are normally living in water, they are used to exist in nutriment-poor surroundings. Therefore they might need a little more time for the adaptation, and the measurements were performed 24 hours after the mixtures were prepared.

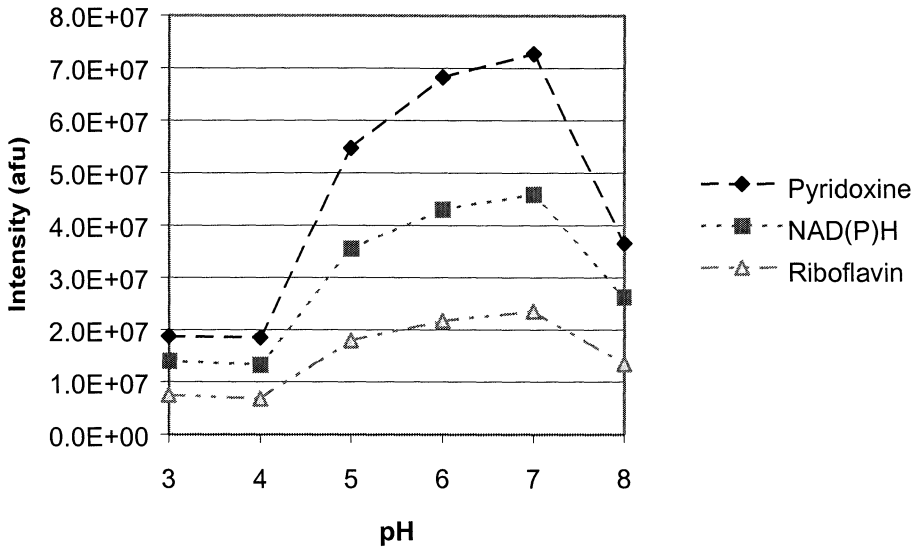


Figure O. The fluorescence as a function of pH. Excitation wavelength 337 nm.

As can be seen in figure O above, the fluorescence signals for all fluorophores are peaking at a pH-value of about 7, which indicates that the cell concentration is as highest there. This was expected since the pH-value of pure water is 7.0, and these microorganisms have become very well adapted to that environment during the evolution.

When the pH-value falls below 4 the bacteria in the cell suspension are expected to be dead, whereas the yeast cells are almost unaffected by the acidic environment. This is very well illustrated in figure O by the flat line between pH-value 3 and 4. If the pH-value is increased above 7.0 the fluorescence signal declines. This is also according to the theory, since these surroundings are more basic than the bacteria desire, and fewer bacteria can survive.

5.4 Fluorescence of water as a function of temperature

As a preparation for the biofilm experiments, the variation of the water fluorescence with changing temperature had to be examined. The results, which can be seen in figure P, agreed very well with what was predicted in 3.3.1.2. The graph indicates a linear dependence of the fluorescence signal for all three fluorophores. The R^2-values for the respective curves, when compared to linear curve, are: NAD(P)H 0.90, riboflavin 0.95, and pyridoxine 0.81.

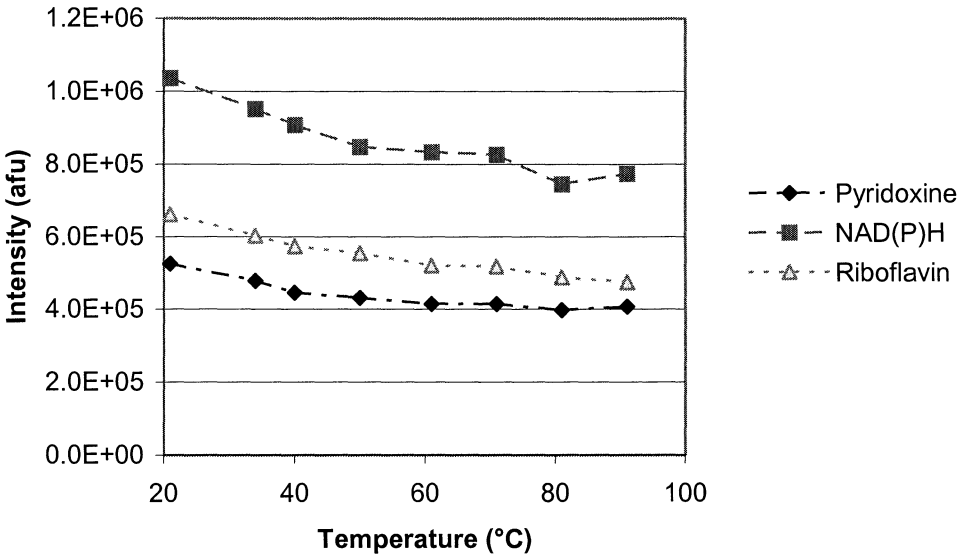


Figure P. The fluorescence of water as a function of the temperature. Excitation wavelength 337 nm.

5.5 Fluorescence of biofilm

The fluorescence results from the biofilm experiment system could be divided into three parts: a study of the biofilm build-up phase, the effects of the disinfection routine as measured with water at 22°C as bulk fluid, and the same effects with water at 95°C as bulk fluid.

To clarify, the disinfection routine is in both cases performed by circulating hot (95°C) water for 45 minutes. The only difference between the two ways of measuring the effects of the cleaning routine is that in one case it is measured with the hot water still in the system, whereas in the other case room-tempered water has replaced the hot water as bulk fluid.

5.5.1 Biofilm build-up phase

The cell suspension (described in 4.5) was circulated in the system for six days, and during this period three measurements were performed. The first was done shortly after the circulation was started (assumed to be free from biofilm), and the second and third after two and six days, respectively (see Figure Q).

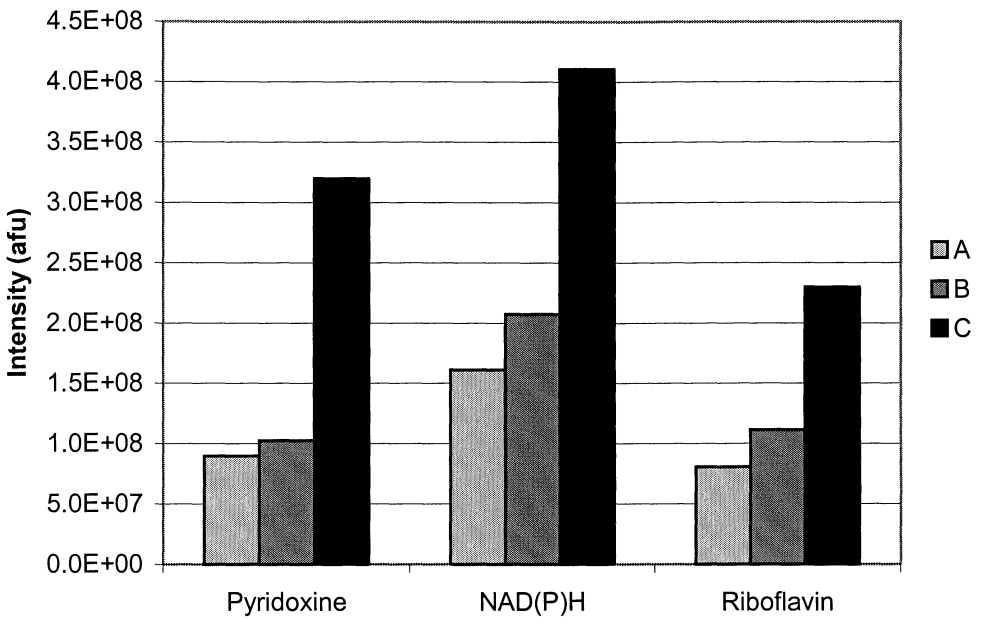


Figure Q. The fluorescence from biofilm. Excitation wavelength 337 nm. A=clean flow-through cell with cell suspension, B=after two days of circulation with cell suspension, and C= after six days of circulation of cell suspension.

The increase in the fluorescence signals after two days were in the span 15-40% for the three fluorophores, four days later, the signals had increased by 150-250% as compared to the initial measurement. If these results in a tolerable way reflect the growth of the biofilm, they imply that when the bacteria have established a firm connection to a surface they can grow unimpededly.

An objection to this conclusion could be that the increase in fluorescence is due to an increase in the cell concentration in the suspension. This is not very likely though, since there are no nutriments added to the suspension.[19] It is therefore more likely that the cell concentration is somewhat reduced during the six days in the system. This means that the fluorescence signal at the sixth day should be even greater, if bulk fluids with the same cell concentration were to be used for all measurements. This indicates that some kind of biofilm has been established on the walls of the flow-through cell.

5.5.2 Effects of a disinfection routine

The reason for doing two measurements on the effect of the disinfection routine just varying the bulk fluid temperature, is to compare them with each other. It could be interesting to see if the pyridoxine, NAD(P)H, and riboflavin signals react in the same way regardless of the temperature. They might be reflecting the metabolic state of the cells in some way. This is something that needs to be further investigated, but other experiments[7-10] have shown that one or more of the fluorescence signals from the discussed compounds could be used as a marker of a metabolic state of a specific cell type.

5.5.2.1 Room-tempered (22°C) water as bulk fluid

For starters, with room-tempered water in the system, the fluorescence signals from the different fluorophores are increased with 430-550% when comparing the system at initiation, and the same system six days later (see Figure R).

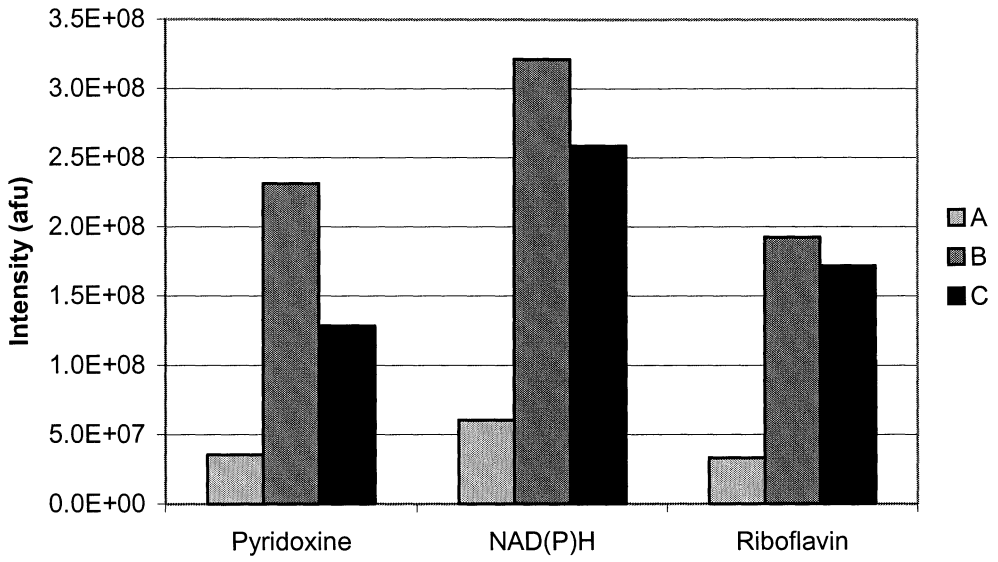


Figure R. The fluorescence of water (22 °C) in a quartz flow-through cell. Excitation wavelength 337 nm. A=clean flow-through cell, B=after the circulation of cell suspension, and C=after the disinfection procedure with hot water.

After the disinfection process all fluorophores indicate a reduction of the amount of biofilm. The largest reduction (53%) is indicated by the pyridoxine signal, and the smallest by riboflavin (13%), whereas NAD(P)H lies somewhere in between (24%). Noticeable is that the metabolic state of the biofilm seems to be of importance for the fluorescence signal. If that was not the case, the reduction of the fluorescence signals is expected to be about the same for each fluorophore.

It is also obvious that the disinfection process does not remove the biofilm to a very large extent. The fluorescence from what is left after the disinfection exceeds the fluorescence from the clean system with 260-420%. This confirms what have been stated earlier (see 2.3); biofilm cannot be removed using only hot water, a combined mechanical and chemical process is needed.

5.5.2.2 Hot (95°C) water as bulk fluid

In many ways the results with hot water (see Figure S) resembles the ones for room-tempered water as bulk fluid. Firstly, there is a significant difference between the clean system and the same system six days later. Secondly, since the reduction of the signals from the fluorophores clearly differs, a strong indication of dependence of the metabolic state of the biofilm could be assumed. Thirdly, the pyridoxine signal is reduced the most during the cleaning process, and the riboflavin signal the least. And lastly, the figure leaves no room for doubt that the disinfection process does not remove very much of the biofilm.

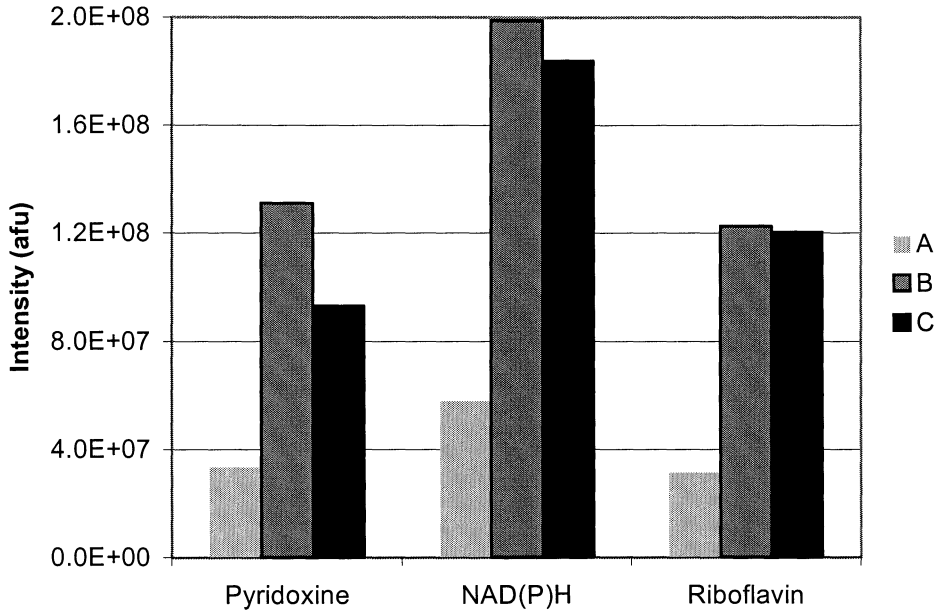


Figure S. The fluorescence of water (95 °C) in quartz flow-through cell. Excitation wavelength 337 nm. A=clean flow-through cell, B=after the circulation of cell suspension, C=after the disinfection procedure with hot water.

One aspect of the results differs: the reduction of the fluorescence signals with room-tempered water as bulk fluid (13-53%) was larger than for hot water (2-39%). Especially the reduction of the riboflavin fluorescence signal (2%) is of interest, since it implies that there is virtually no reduction of the amount of biofilm at all.

Another interesting result from these experiments is that the temperature dependence of the fluorescence phenomenon is very clear. The registered fluorescence signals from the room-tempered system are almost twice as strong as from the hot water system, which corresponds to the results in 5.4.

What should also be remembered is that the concentration of the cell suspension used in these experiments is several orders of magnitude above what could ever be expected in a dialysis machine. Nor would a dialysis machine go continuously for six days straight without being disinfected.

5.6 Biofilm in drainage tube

To get close-up images of the surface of a drainage tube from a dialysis machine, a Scanning Electron Microscope was used (see 2.4.1.2). Two different tubes were investigated; one had been used for only one treatment (Figure T), and the other one had been installed in the machine for many years and had therefore experienced several hundred treatments (Figure U).

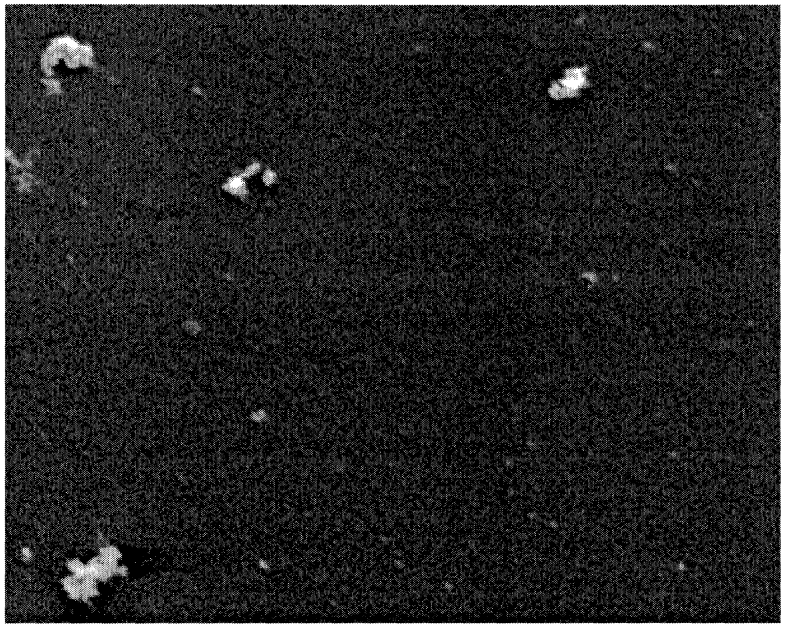


Figure T. The drainage tube from a dialysis machine used only during one treatment, after the disinfection process. 2000 times magnified. The width of the figure is approximately 20 μm.

The white particles in figure T have not been positively identified, but their very irregular shapes reveal that they are *not* bacteria. More likely is that those pieces are some kind of precipitates from the dialysis fluid. It is however clear that no biofilm has been formed.

The surface of the old tube looks very much different from the new one. Figure U shows the surface of a biofilm. The vast majority of bacteria in the biofilm cannot be seen since they have hidden themselves underneath the polysaccharide layer. There are however a few bacteria that are yet not fully covered with slime. One possibility is that these bacteria attached to the biofilm during the last treatment, and have not yet had enough time to cover up.

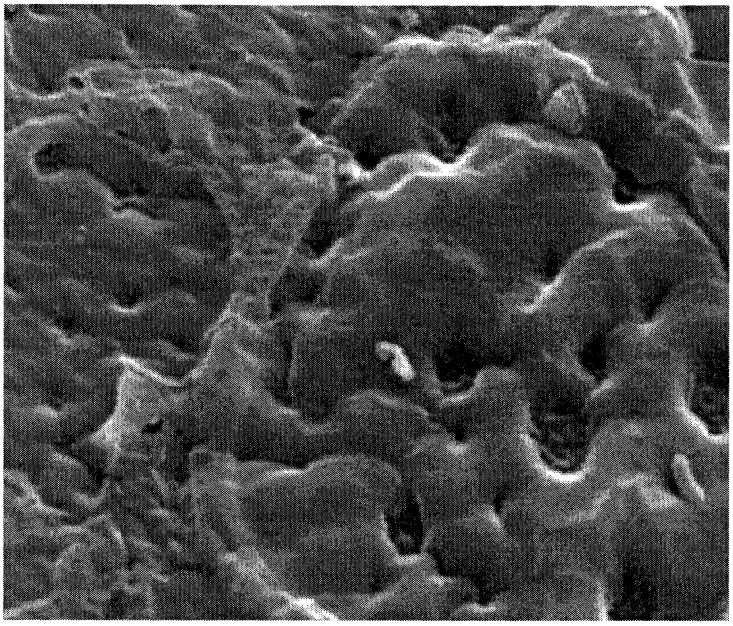


Figure U. An old drainage tube from a dialysis machine, after the disinfection process. 2000 times magnified. The width of the figure is approximately 20 μm.

A simple experiment was performed to finish this series of experiments. A cell suspension with the same microorganisms as described in 4.5 was circulated in a closed system for 10 hours. The concentration of the suspension was 1.8 x 10^7 CFU/ml. Figure V above shows that no biofilm has yet been formed, but there are small colonies of bacteria on the surface. If these are not properly removed they will most likely start to build protective layers around themselves i.e. forming a biofilm, forming a biofilm. Further research has to be done, to establish how quickly the biofilm is formed.

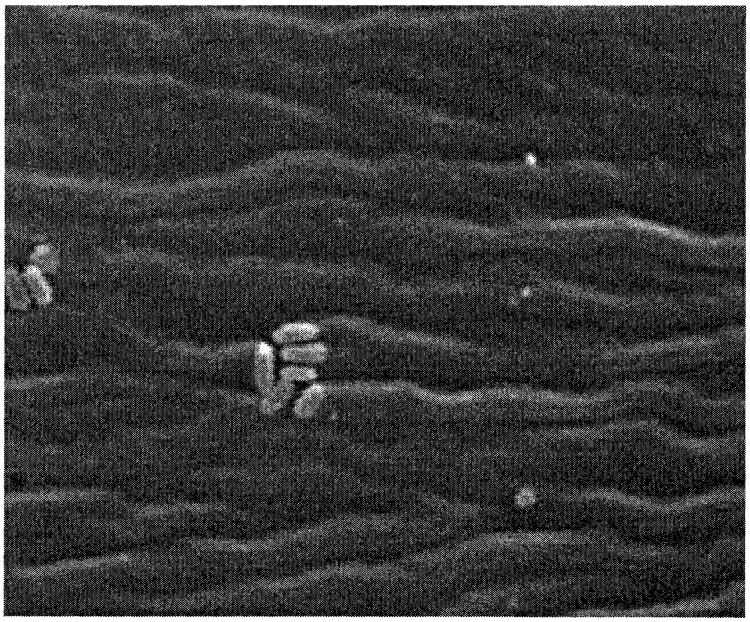


Figure V. After 10 hours of circulation with cell suspension. 2000 times magnified. The width of the figure is approximately 20 μm.

6 Further research & development

The investigation of how useful optical methods are for detection of biofilm in dialysis machines is in no way finished with this report. There are a number of possible ways to further develop and improve the method that has been used in this project. Some of them could be found in the list of suggestions below.

Build a system for the sole purpose of detection of the optical parameters sought for.
The OMA-system, which was used for the experiments in this project, is not at all built for our purposes. The optical filters and dichroids in the equipment could not be changed, which resulted in a drastic reduction of the detected pyridoxine fluorescence signal. The system was also limited to two excitation wavelengths, 337 nm and 405 nm. If another light source in the wavelength region 250-290 nm could be added, it would be possible to study the tryptophan fluorescence signal as well, which could give more information about the biofilm.

Use a container for the cell suspension that can control physical and chemical variables.
Parameters like temperature, pH, aeration rate, glucose level, oxygen and nitrogen levels, all affect the biofilm in some way, and could therefore have some influence on the optical signals as well.[13] It would be important to be able to control these variables online in the further research on how the optical signals are affected by physical and chemical changes. In our investigations we did not have access to such sophisticated equipment, but had to control these parameters in very work- and time-consuming ways.

Use the same optical fibre for both the excitation light and the fluorescence light.
In the quest for light sources that could provide new information about the behaviour of the microorganisms, a lamp emitting light in the wavelength region 365-380 nm was tested (see 4.2). The recorded fluorescence signals did however not provide any useful information at all. Our belief is that if both the excitation light and the fluorescence light are guided through the same fibre, it will be more photon efficient and therefore give better results.

Investigate what amounts of the different fluorophores that are present in the biofilm.
If it is possible to find out, it would be of utmost interest to know what amounts of fluorophores there really are in the biofilm under examination. Perhaps the four fluorophores discussed in this report (tryptophan, pyridoxine, NAD(P)H, and riboflavin) are not the most abundant. There could very well be other compounds worthy of some further research. Marose, Lindemann, and Scheper[10] have studied the fluorescence from a number of other organic compounds, and that could perhaps be a good starting point in the search for other fluorophores.

Investigate the possibility to use a fluorescing compound that attaches to bacteria.
The detection limit of the fluorometry system used for this project is several orders of magnitude too high. This limit would be significantly lowered if some kind of strongly fluorescing compound could be flushed through the system, which was followed by a detection of the fluorescence signal. This fluorescing compound should have the quality that it easily attaches to bacteria, and preferably fluoresce in different wavelength regions depending on some property of the bacteria, such as if they are dead or alive.

Study the time-resolved fluorescence signals from different bacteria and biofilm.
A time-resolved study of the fluorescence signals could reveal more about a sample. All fluorophores have a specific decay time, and if a recorded signal has a good match with the decay time of a certain fluorophore a stronger connection has been established. It might however not be a simple task to apply this technique to biofilm detection, since there are so many different compounds that fluoresce in biofilms. The fluorescence signal is a mix of several fluorophores, and it could be impossible to extract the contribution from every single compound.

Investigate other optical detection methods.
This thesis investigates experimentally how useful fluorometry could be for biofilm detection, but that does not mean that there are no alternatives. There could very well be other methods that are more suitable for the purpose. The exclusion of some alternative methods in 2.5 is based on a number of criteria that might be modified in the future.

7 Conclusions

Optical detection of biofilm is a difficult and rather complex task. It is also complicated by the fact that it requires knowledge of two very different areas: physics and microbiology.

If this detection problem could be solved it would simplify biofilm discovery very much, since there are not any really good methods in use today. Electron microscopy and CFU-counting are not ideal since they require sample preparations, and the measurements give too inaccurate results. These methods are also destructive, i.e. the biofilm samples have to be removed from its habitat to be examined, which makes continuous study of the growth more difficult.

This Master's Thesis is a first step towards using fluorometry to detect biofilm in dialysis machines, and it has been shown that the fluorescence signal is affected by the amount of biofilm. It has also been stated that when trying to eradicate the biofilm with flushing hot water through the system, the fluorescence signal decreases but never returns to its initial value, indicating that there still is biofilm present. This outcome is supported by biofilm research where it is stated that the removal of biofilm is an almost impossible task.

The sensitivity of the system is not good enough, but that could most likely be improved. Further research and improvements are needed to make a commercial product based on fluorometry for biofilm detection. A deeper understanding of how the fluorescence from the bacteria in a biofilm is affected by physical and chemical variations in the surroundings is also required.

8 Abbreviations

AFU = arbitrary fluorescence unit

ATR-FTIR = attenuated total reflection fourier transform infrared

CCD = charged coupled device

CFU = colony forming unit

DPS = 4,4-diphenylstilbene

EM = electron microscopy

FWHM = full width half maximum

HD = haemodialysis

IR = infrared

IRE = internal reflection element

NADH = reduced nicotinamide adenine dinucleotide

NADPH = reduced nicotinamide adenine dinucleotide phosphate

OMA = optical multichannel analyser

PD = peritoneal dialysis

PMMA = polymethylmethacrylate

PSU = poly sulphone

RO = reverse osmosis

SEM = scanning electron microscopy

TEM = transmission electron microscopy

9 Acknowledgements

We would like to thank our supervisor Thomas Hertz, Gambro, for helping us with this very interesting and challenging project, for his never-ending enthusiasm and curiosity, and for giving us free reins trying to solve this problem.

We would also like to thank Stefan Andersson-Engels, our supervisor at the Division of Atomic Physics at Lund Institute of Technology, and the personnel at the division.

Helena Jeppsson has given us invaluable help and information regarding the world of microorganisms, and has also explained their behaviour with words that physicists can understand. Furthermore, she has read and corrected the microbiology chapters in this work, and supported us with cell suspensions, and for that we give her our thanks.

Bengt Jonasson should also be thanked for repeatedly providing us with dialysis fluid on very short notice.

Finally we would also like to express our gratitude to everybody at Gambro Treatment Systems Research, for their support during the setbacks, for their patience with all our questions, for their help with all the practical problems we have encountered, and for all the good discussions and laughs at the coffee breaks.

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11 Appendix

A. Transmission measurements

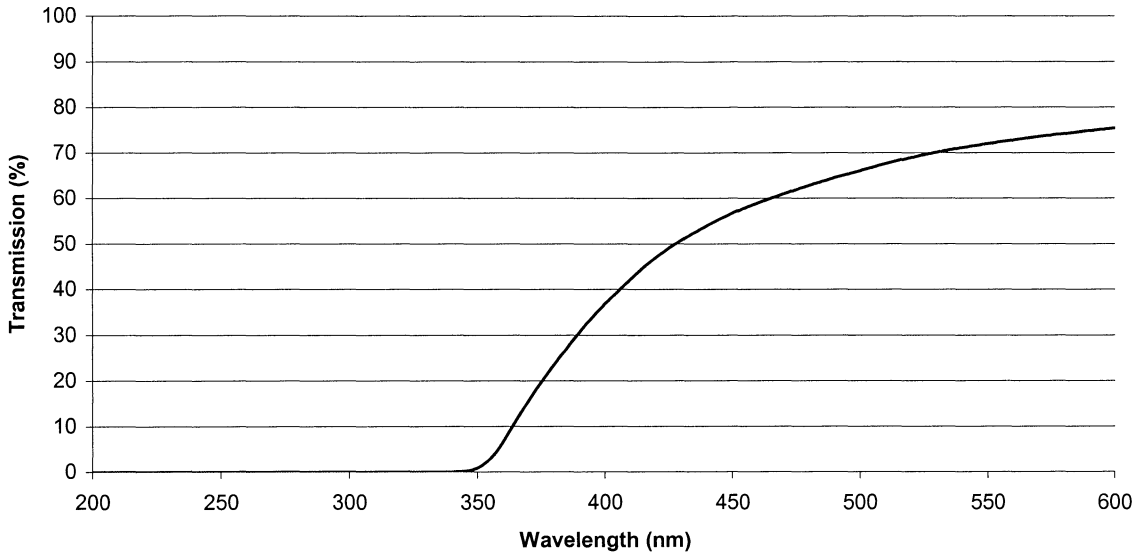

Figure 1. Transmission blood leak detector.

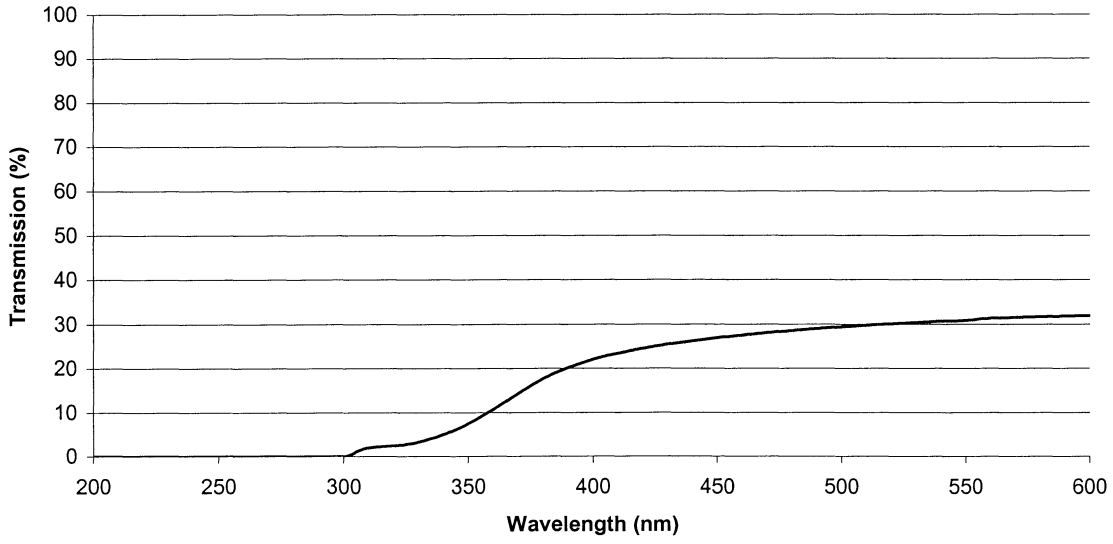

Figure 2. Transmission drainage tube.

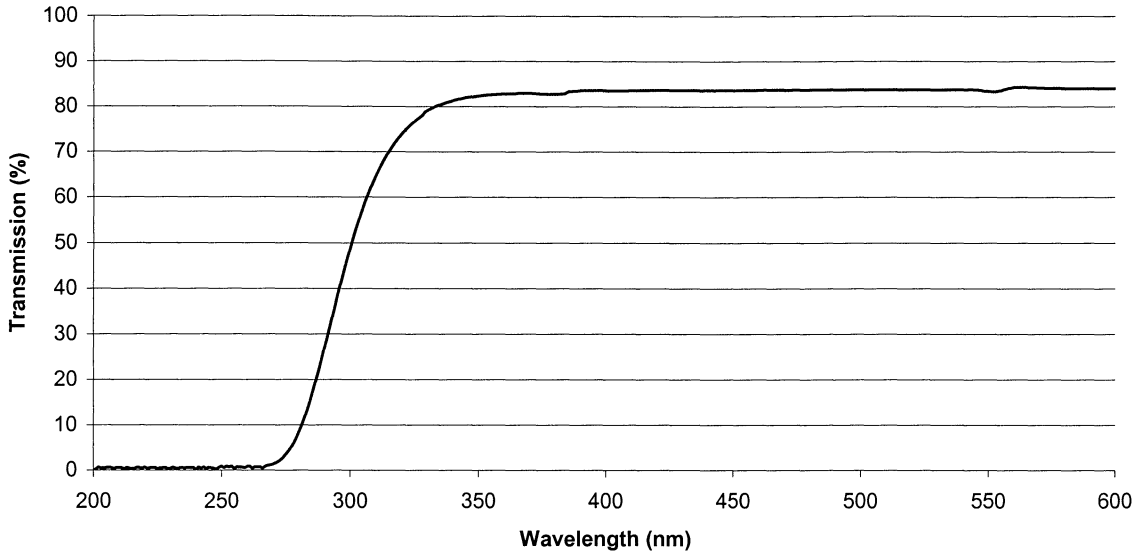

Figure 3. Transmission glass.

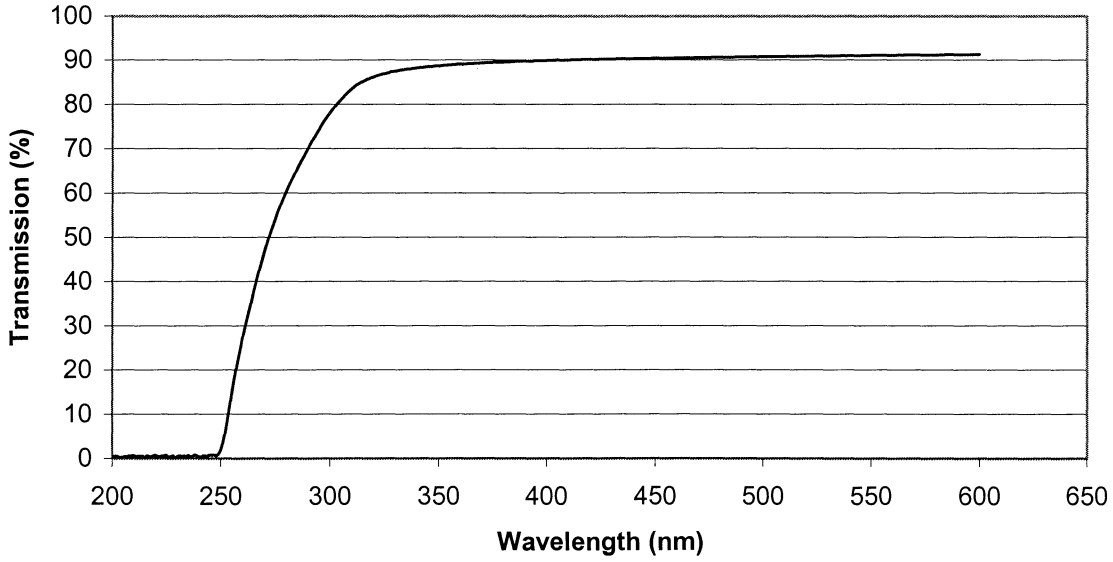

Figure 4. Transmission PMMA- plastic cuvette.