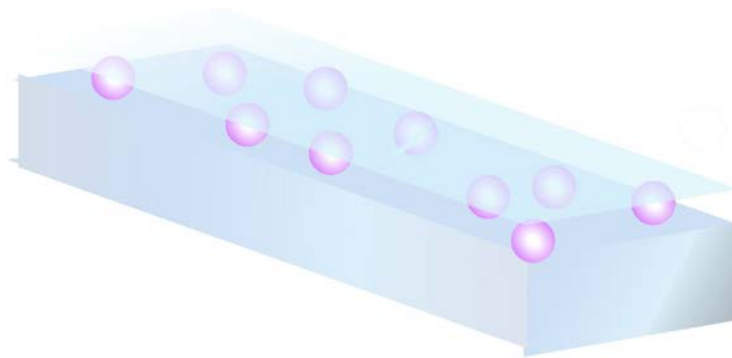# Cellular recognition of nanosized surface enhanced electromagnetic fields

Mastersthesis in Nanotechnology

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Gothenburg, Sweden 2015

MASTERS THESIS 2015

Cellular recognition of nanosized surface enhanced electromagnetic fields

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Abstract

The purpose of this study was to map the interaction mechanism behind nanostructured surfaces interacting with cells. It is widely known that cells behave differently on nanostructured surfaces than on smooth surfaces. What isn't known however is why they do. We tried to isolate one of the possible interaction mechanisms and see if it has any effect on the cells adhesion on a surface. The mechanism in focus was the electromagnetic near fields created in vicinity of a nanostructure due to surface plasmons induced by light. A nanostructured surface was embedded in a very thin film of glass, making the surface flat. The fields created around the particles are not significantly screened by the dielectric glass, and reaches further than the thickness of the glass film. Cells were then cultured on the surface and later stained and analyzed for cell adhesion. No visible response from the specific cell used, A-431, on the 40 nm gold nanoparticles during illumination was observed in this project, suggesting that any observed interaction between these cells and nanoparticles is brought about through a physical contact between the cells and the structure.

Introduction

The field of nanotechnology is a young scientific branch, with its name coined in the seventies. Because of this, still many of the effects caused by nanostructuring are poorly understood. This is one of the reasons nanotechnology is seen by some as rather mysterious. Phenomena that differs greatly from most other things seen around us are possible through nanostructures, and this has the potential to change our lives.

Nanotechnology provides great tools for studying the nature of microbiological entities. By structuring surfaces on a nanoscale, conclusions can be drawn about cell functions for example by observing its behaviour in different environments, which in turn can lead to improvements in biomaterial development. This has been studied before (Hulander, M., Lundgren, A., Faxälv, L., (2013)), and it is well documented that cells interact with periodical structures in interesting ways. The scope of this project is to

draw conclusions about how this interaction occurs, specifically by singling out one possible route of interaction to either confirm or discard it as the cause of the observed trends.

Theory

Plasmonics

Plasmons has gained a lot of attention over the last decades due to their promising potential use in many fields, including photovoltaics and biosensing. They can exhibit unusual properties by strongly interacting with in spite of being confined to a length much smaller than the light wavelength. A plasmon is a collective, coherent wave in the electron plasma of a conductive material (Cademartiri, L., Ozin, G.A., (2012)). All conductive materials carry "free" electrons, or electrons in the conduction band. These electrons are not bound to their original ion, and are thus free to move in the material. Electrons interact with light, as light is electromagnetic radiation, which gives rise to acceleration in charged particles. So the electric (and magnetic) field in light makes electrons move, illustrated in Picture 1.
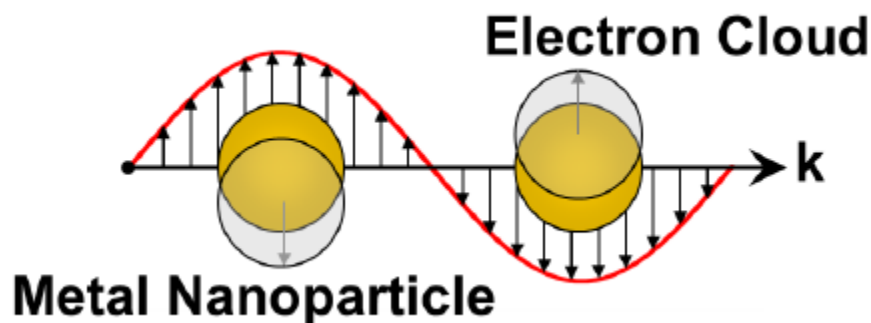
Picture 1: Illustration of an electromagnetic wave exciting oscillatory movement in the electron clouds of gold nanoparticles. (Harrison, R.K., (2012))

Specifically light makes electrons move in the perpendicular direction to the direction of the light, as the nature of electromagnetic radiation is transverse. The quanta, or smallest possible amplitude of a specific oscillation frequency of the electron plasma

due to quantum mechanics, is called a plasmon (Kittel, C., (2005)). They exist in all conductive materials, and can be inside the bulk or confined to the surface. Surface plasmons are the main interest for this project, as they become tunable in resonance frequency for small particles. Just like adjusting the length of a pendulum, the resonance frequency of these electron oscillations can be tuned by controlling the size of the surface that the plasmons is confined to. This resonance frequency is the frequency, or colour, of light that the electrons most strongly interact with.

The phenomenon of surface plasmon resonance on nanoparticles has been used for hundreds of years, most notably in the staining of glass in church windows. Gold has been used to colour windows for centuries, in the form of nanoparticles. Different staining colours has been brought about by unknowingly controlling the size of the gold nanoparticles (NP). Different sized particles shows different plasmon resonance frequency, and thereby interact more strongly with different frequencies of light. The inert nature of gold also insures the staining to be very chemically persistent. The first to hypothesize that this staining made by gold was small species of solid gold was Michael Faraday in 1857 (The birth of nanotechnology (2006)). Following his discovery, important studies were performed on colloidal gold, leading to three different nobel prizes.

Concentrations of gold NPs as low as 10^{-14} M can be detected by the eye due to surface plasmon resonance (Harrison, R.K., (2012)). In the usual case, light interacts very weakly with features smaller than a fourth of the light wavelength, but gold NPs are often smaller than a tenth of the wavelength of the incident light. The optical interaction cross section of the gold NP becomes significantly larger than the particle itself, defining an effective area for the particle when interacting with the light. The particle is effectively attracting light, drawing the light in from other angles, exhibiting a "gravitational pull" on the light. This interaction cross section area is a measure of how efficiently the particle interacts with light. Interaction can be either scattering of the light, redirecting it, or absorption, turning it into another form of energy. The cross sectional area of these two interactions summed up, $c_{scat} + c_{abs}$ divided by the actual cross section on the particle, A_{cs}, is known as the interaction efficiency, Q_{tot}.

$(c_{scat} + c_{abs})/A_{cs}=Q_{tot}$

Gold, and other noble metals, are used effectively for surface plasmon resonances in the visible range. Other metals generally carry resonance frequencies in the ultraviolet spectra. In comparison to, for example, silica NP's, the magnitude of the interaction with light is on the order of 10^4 times smaller than for gold NP's, which decreases with larger wavelengths and is only due to light scattering, without any light absorption. The colour of the sky is a good example of the same type of interaction. The particles in the atmospheres interacts with light weakly, and without plasmonic resonance peaks. Shorter wavelengths are scattered by the atmospheres, while longer wavelengths are transmitted with smaller interactions. Blue light reflected from the earth are scattered back against the atmosphere, while red light is transmitted. The reddish sunset is the same thing, only now the light from the sun travels a longer distance, due to the angle with the earth surface, through the atmosphere, and the blue light are reflected back into space more strongly than light traveling radially towards the earth (Fitzpatrick, R., (2006)). When dealing with a plasmon resonance behaviour however, the picture is not that simple. As the particle absorbs light, it turns the light into another form of energy. The energy is stored within the oscillations of the electron plasma, which if not maintained, relaxes and becomes heat. During the electron cloud oscillation, electric and magnetic fields are created in close vicinity to the particle. These are known as near fields, and can be very complicated to predict. They exist only close to the particle and can be much stronger than the field of the absorbed light, due to the particle being able to store a lot of radiative energy in these fields.

Shape and size of particle

In a piece of bulk conductive material, surface plasmons between an insulating region and the material also exists. The reason that the material doesn't show the same optical response typical for surface plasmons, is that the plasmon waves interact with each other and interfere. As light is generally spatially incoherent on a length scale smaller than a bulk piece of material, plasmons with different phase are created along

the surface of the conductor. The waves travel on the surface and interfere with each other, and prohibits radiative energy to be stored in the plasmonic waves. When the materials is broken down to small pieces, this interaction is removed.

For plasmonic NPs larger than a certain size (around 150 nm for gold), more than one plasmon peak can be seen in an absorption spectra. This is due to the electron cloud to oscillate in different modes within the particle. Smaller particles restrict the oscillations of the electrons in such a way that only one mode of oscillation is possible, the dipolar oscillations. For larger particles, for example quadrupolar and octopolar oscillation modes will be obtainable for the electron clouds, manifesting themselves as other absorption peaks, higher in energy than the dipolar oscillation (Harrison, R.K., (2012)).

The shape of the particle also have a large impact on the plasmonic behaviour. Just like the case of larger spherical particles, non-spherical particles can support different modes of oscillation for the free electrons. For example, a nanorod shows two resonant peaks, corresponding to electrons oscillating longitudinally and transversely within the rod. By controlling the shape, the interaction with light can be many times improved, as can the near field strength. Using particles with other geometries than spheres can also be used to freely tune many plasmonic properties, like absorption to scattering ratio. The shape of the particle is oftenly a more important indicator of optical properties than particle size (Cademartiri, L., Ozin, G.A., (2012)).

At pointy ends of non-spherical NP's, fields grow stronger than around the smoother parts. This is because maxwell's equations, which predict electromagnetic behaviour, is a second order differential equations. So when the first order derivative of the charge distribution is not well defined, the field will have a singular characteristic. Of Course the field isn't really singular, because the metal has a limited conductivity, and the radius of curvature is also non zero, but the field around such a geometry can grow strong.

Near fields

As previously mentioned, plasmonic NP's can have a significantly larger light-interaction area than the actual particle cross section. The particle effectively pulls in light from its surroundings. So what happens to the energy absorbed by the particle? The light excites collective, coherent motions in the electron cloud. The electron cloud keeps moving back and forth in the particle, like a pendulum, until the kinetic energy is transformed to some other form of energy, for example heat. The fields created close to this dislocation of charges is called the near field. It is, for a small dipole compared to the wavelength of absorbed light, the near field is effective inside a radius significantly smaller than the wavelength, $r \ll \lambda$ (Wolff, C., (2015)). At the resonance frequency of light, the NP can store a great amount of energy from the light within the motion of the electron cloud, resulting in large oscillation amplitude. The intuitive picture of a pendulum moving back and forth gets problematic when we talk about charges. An accelerating charge radiate energy to its surroundings by emitting photons. When this happens, the vibration of the charge till slow down, losing some energy to the photon. This creates an effective resistance, oftenly called radiative resistance, and is responsible for the process called scattering (Fitzpatrick, R., (2006)). Another phenomena unique to the motion of charged particles is magnetic induction. When a charge moves, so does its electric field. From Maxwell's equations, it is well known that a change in the electric field induces a rotational magnetic field around where this change occurred. If near by conducting materials are able to, they will try to counter this magnetic field by moving electrons in such a way that a magnetic field in the opposite direction is created, eliminating the field created by the change in electric field. This process also disperses energy to its surrounding, however there needs to be means to produce a current in the surroundings for this energy transfer to happen. An oscillating dipole has natural magnetic self induction, and when the oscillating charge distribution changes direction, the magnetic field around the moving charges decreases, in turn creating an electric field helping the electrons to accelerate in the new direction. This energy can be absorbed by the surrounding media.

Because the energy transfer in the near field region of an oscillating dipole has more possible transfer routes than pure em-radiation, it is also harder to understand. While photon absorption of different substances can be well known, near field effects are harder to model. The time and length scales also makes it an extraordinarily hard task to experimentally determine what happens in the near field volume. Some groups have had some success in measuring near field strength using a metal tip in scanning near field optical microscopy. This method is problematic because it can interfere with the plasmonic behaviour of the structure that is studied by the potential of the metal tip. The tip can also collect evanescent waves, and it has a low resolution. Other researchers have used photoelectron emission spectroscopy combined with small angle x-ray scattering. This experimental setup is very complicated which makes it inconvenient. A promising technique however is ultra fast laser plasmonic near field ablation (Harrison, R.K., (2012)). This technique very specifically excited plasmons in particles, without creating heat in the environment. This heating effects can be excluded from possible reasons for observed results. The laser excites plasmons very efficiently, creating very large near fields around them. These fields kicks out electrons from the surrounding area, destroying the material, as shown in picture 2. The holes left in the material can then be studied in scanning electron microscopy to determine the shape and size of the near field.
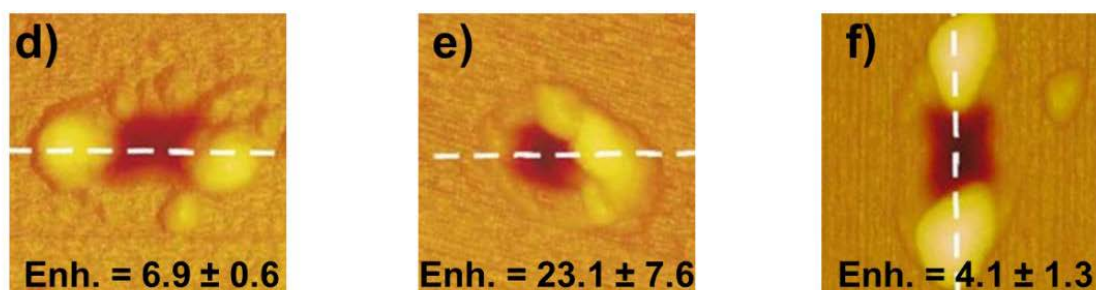
Picture 2: Photos of the ablation sites associated with nanoparticle plasmons excited with a strong laser. (Harrison, R.K., (2012))

Cells

Understanding of cell behaviour is of fundamental importance in design of the next generation biomaterials. This field has been studied thoroughly for centuries, and the field of nanotechnology provides new tools to better understand cells. As most cells are between one and 100 micrometers in size, nanostructures can be substantially smaller and can aid in mechanistic studies of cell functions. The cells used in this project is a human cell, A-431, a skin cell, which is a cell used for research mostly on cancer.

Prior knowledge on cellular interaction with their physical environment concludes that cells responds to changes and structures in their surroundings by altering their cytoskeleton (Geiger, B., Spatz, J.P., Bershadsky, A.D., (2009)). This is also the part of the cell that sends signals for the change to happen. The reconstruction and modification of the cytoskeleton makes the cell move by focal adhesion, which is point where the cell attaches to a surface via the cytoskeleton. Some cells fail to form focal adhesions on some nanostructured surfaces, while the same cells might be able to do this on a different nano surface. The formation of focal adhesion is the formation of a contact point between the cytoskeleton and the extracellular matrix. Cells really use their cytoskeleton to "feel" their surroundings and react to it. Different cellular activity can also be stimulated by different geometries. Concave or convex surfaces can lead to different reactions of the cell sitting on the surface (Vogel, V., Sheetz, M., (2006)), and the cell can sense these geometries through focal adhesion. Studies of human platelets on surfaces with a NP gradient have shown that adhesion can vary as a function of particle distance, and can also show a preference to a certain interparticle distance for maximum adhesion (Hulander M., Lundgren A., Faxälv L., (2013)). Also, it has been shown that in the case of platelets, if the nanostructure details are too small, and under a certain threshold, the platelets can not sense the roughness.
The mechanisms of these interactions with nanotopography are largely unknown, which is the motivation for this study.

Principles for controlling particle shape

When considering the near fields around plasmonic particles, not only the size but the shape very much influences the field created by plasmonic excitations. A pointy particle has larger fields around the pointy parts than around the flatter parts. More plasmonic oscillation modes also becomes available in a non spherical particle, resulting in multiple peaks in the absorption spectra. The particle size can be adjusted for the application. For examples, nanorods are quite commonly used in a variety of fields, including photovoltaics (Cademartiri, L., Ozin, G.A., (2012)). If the purpose of the particle is to enhance the near field strength, many pointy parts of the particle is desired. According to (Personick, M.L., Langille, M.R., Zhang, J., (2011)), concave cubical particles can be synthesized by silver underpotential deposition. Silver ions are electrodeposited on a gold surface through the fact that the reduction potential at the gold surface is lower than the potential for the silver to deposit on itself, resulting in preferential attachment to the gold surface rather than creation of silver complexes. Different facets of the face centered gold crystal structure have different surface energies (Personick, M.L., Langille, M.R., Zhang, J., (2011)). Silver can stabilize these facets, and by increasing the silver ion concentration, more facets on the gold NP can be stabilized, and the growth of the particle can thus only occur in selected directions. The concave cube is bound by twenty four {7 2 0} planes, where the silver ions has adhered to, and stabilized facets of lower surface energy than the {7 2 0} facet.

Methods

Formulating the product

The goal of this project was to investigate whether the complex near field around the plasmonic NP's could affect cells trying to adhere to the surface. It is thus important to isolate this mechanism from any other potential interaction mechanism. Steric interaction is a possibility when cells try to bind to a surface, therefore the nanostructure needs to be embedded in a dielectric medium, without free charges that screens electric

fields. The dielectric needs to be transparent to visible light to allow the particles to absorb the light. Surfaces needs to fit the microscope used, and the cell-growth template. If cells can be cultured under illuminated conditions, on a surface with a nanostructure covered by a thin film, they can later be stained and studied in a microscope. To study the potential dependence of cell behaviour with field density, a gradient concentration across the surface could be used. If the concentration of particles would change across the length of the surface, so would the strength of the near field on the surface. To further isolate this effect, the same experiment should be conducted in dark conditions as well. A control surface without the shallow nano structure but with the same chemical composition should also be studied as a control surface.

The route to developing such a surface starts at creating the NP's, then they must adhere to a surface, and then the surface must be coated by a suitable thin layer of material.

Particle synthesis

Gold NP's was synthesized using a seed and growth solution, according to (Park, Y.K., Park, S., (2008)).

The 12 nm gold NP seed solution was made by first mixing 100 ml deionized water with 100 ml 1mM $HAuCl_4$ solution. This solution was heated until boiling, then 10 mL 38,8 mM sodium citrate was added, and left to boil for 20 minutes. The seed solution was cooled and left until needed.

Growth solution was created by mixing 0,4 ml 10mM $AgNO_3$, 4 ml 20 mM $HAuCl_4$ and 170 ml deionized water. To this, 6 ml seed solution was added to create 40 nm particles. The reaction was initiated by adding 30 ml 5,3 mM ascorbic acid at a rate of 0,6 mL/min during stirring. The particle solution was shelved until usage.

Gradient fabrication

40 ml particle solution were centrifuged, after which the particles were transferred to 20 ml deionized water, giving the particles a non-ionic environment necessary for binding the NPs in a concentration gradient by ionic diffusion. Glass surfaces were cut to size (), to fit into plastic cells for cell growth. Due to the design of the surface holder used later, a guiding slit were scratched onto the surface, dividing it in two along the short side. This slit is used later to break the surface in the right place after particle treatment, fitting it into the plastic cell for cell growth. The surfaces were cleaned for 10 minutes in TL1 solution, consisting of 15:4:4 water, ammonium and hydrogen peroxide, heated to 70 C°. The surfaces were dried carefully, and placed standing in a beaker together with a 200 ml 1:1 mixture of methanol and 3-aminopropyldimethylethoxysilane, $C_7H_{19}NOSi$, and sealed for two hours. This solution vaporizes easily, and binds to the surface. The silanol part of this molecule binds to the glass substrate, while the amino group provides an electrostatic binding site for the gold NP's. After two hours, the glass surface were mounted in a holder, custom made to hold five surfaces vertically into the particle solution. The particle/water solution was brought into a glass beaker with a conical bottom. The surface holder, designed to fit this very beaker perfectly, was then placed on top of the beaker, with the surfaces pointing down into the solution, shown in picture 3. A mixture of citric buffer and 1% wt Ficoll was then injected, 16,5 ml at a rate of 2 ml/min. This created a sharp division between the buffer solution and the NP solution, with the edge of the glass surfaces placed on this division. When the buffer solution diffuses through the NP solution, it screens the electrostatic repulsion between the NPs, reducing the average binding site distance on the surface, and thus creating a concentration gradient along the surface, with higher concentration further down the surface. The surfaces were kept in this beaker undisturbed for 90, 75 and 60 minutes, giving different concentration profiles. After NP treatment, the surfaces were cleaned in milliQ and kept in water until the spin coating process.
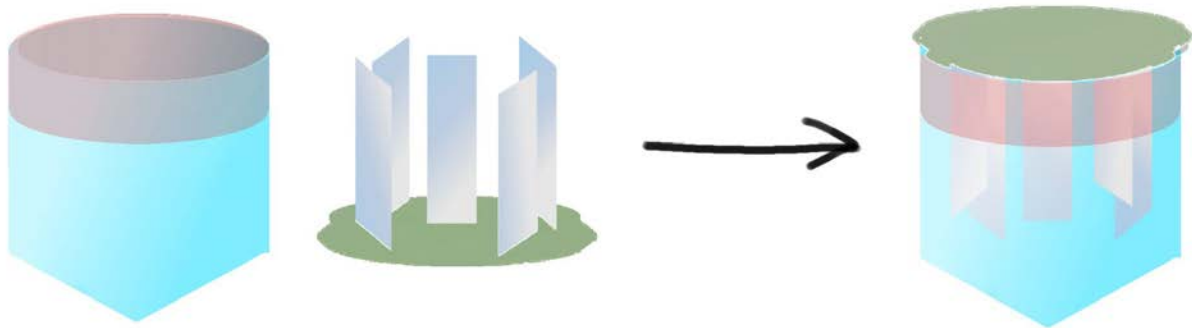
Picture 3: The surface holder was placed into a beaker with NP solution. The arrow shows the contraption after injection of citrate buffer.

Spin coating

A spin on glass, called HSQ was used to cover the surface. The chemical formula of the HSQ monomer is $H_8Si_8O_{12}$, and it is a cubical molecule. These molecules crosslinks during post-bake, and forms a silicon dioxide layer. The surfaces were spin coated with HSQ. After drying the gradient surfaces, they were broken along the guiding slit made earlier. A 2% HSQ solution was used, and was spun onto the surfaces for 60 seconds, using a 2500 rpm velocity. According to (HSQ Thickness vs. Spin Speed Curves), the thickness of the film in these conditions is close to the particle size, completely and shallowly covering the nanostructure. The surfaces was then post baked at 100 C for 1 hour. The particle gradient should now be sufficiently covered in glass, as depicted in picture 4.
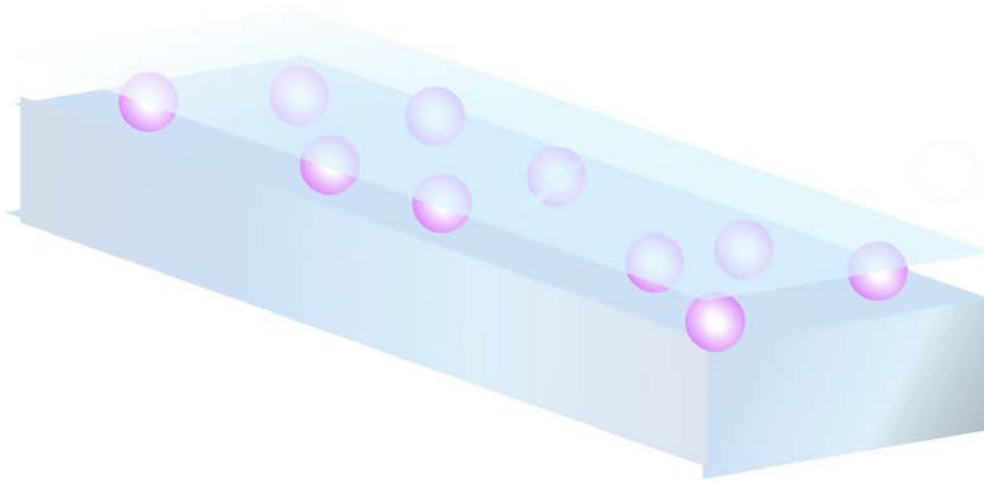
Picture 4: Nanoparticles, represented by purple spheres, embedded in a thin layer of glass.

Cell growth

Cells were cultured on 15 gradient surfaces, and six reference surfaces. Six of the gradients were cultured in illuminated conditions and nine were cultured in the dark. Three reference surfaces were grown cells on in the dark, and three in bright conditions. The Light source were placed so that the main light beam hit the samples perpendicular to the surface, illustrated in picture 6, promoting near fields with the largest part going up through the glass substrate. Cells were grown on the samples with a density of 10 000 cells/cm^2 for six hours.

The composition of the light was analyzed and the wavelength/intensity plot is represented in Picture 5. This picture shows that the LED light source used had an emission peak around 460 nm, which was significantly larger in intensity than any other frequency. This emission spectra did not match the plasmon resonance of the gold NP's, which is around 530 nm (Harrison, R.K., (2012)), and is thus not an optimal light source for our purpose. However, this measurement was performed in a late stage of

the project, and there were no time to resolve the issue.
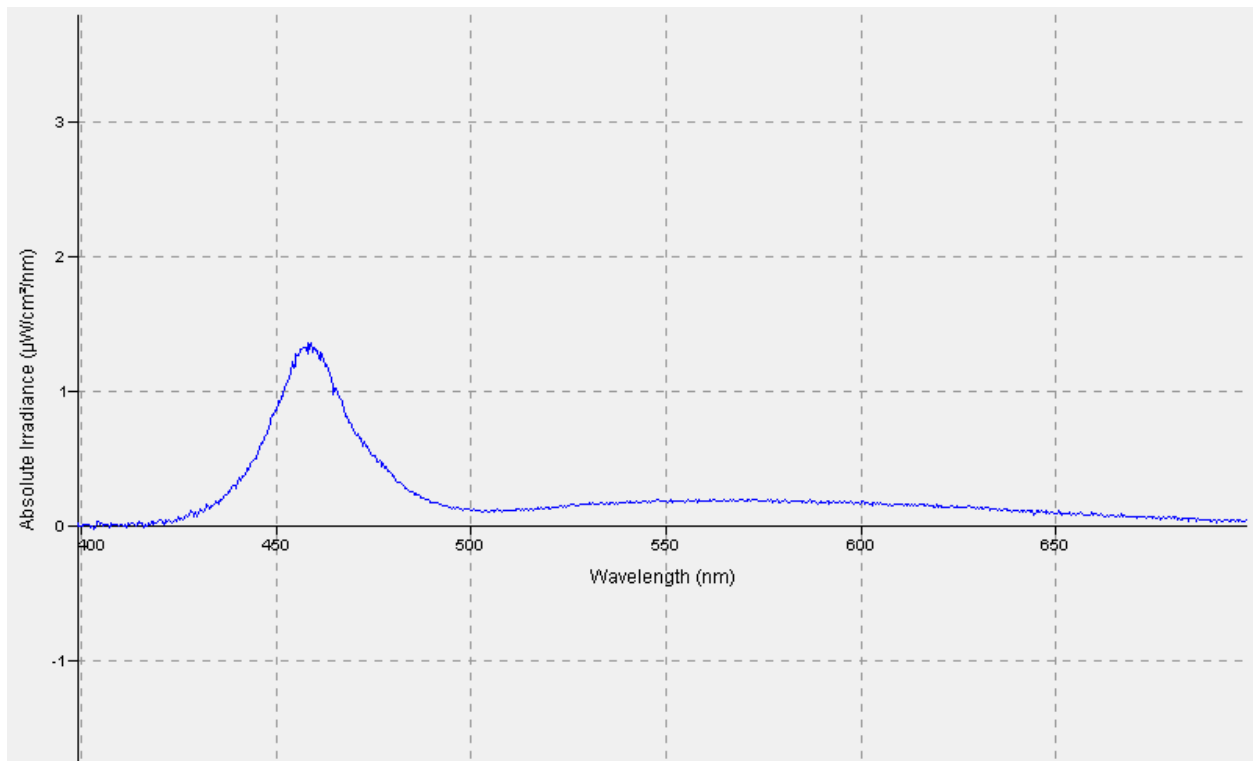
Picture 5: The emission spectra of the LED light used to illuminate the samples. The peak emission does not coincide with the resonance frequency of the NP's.
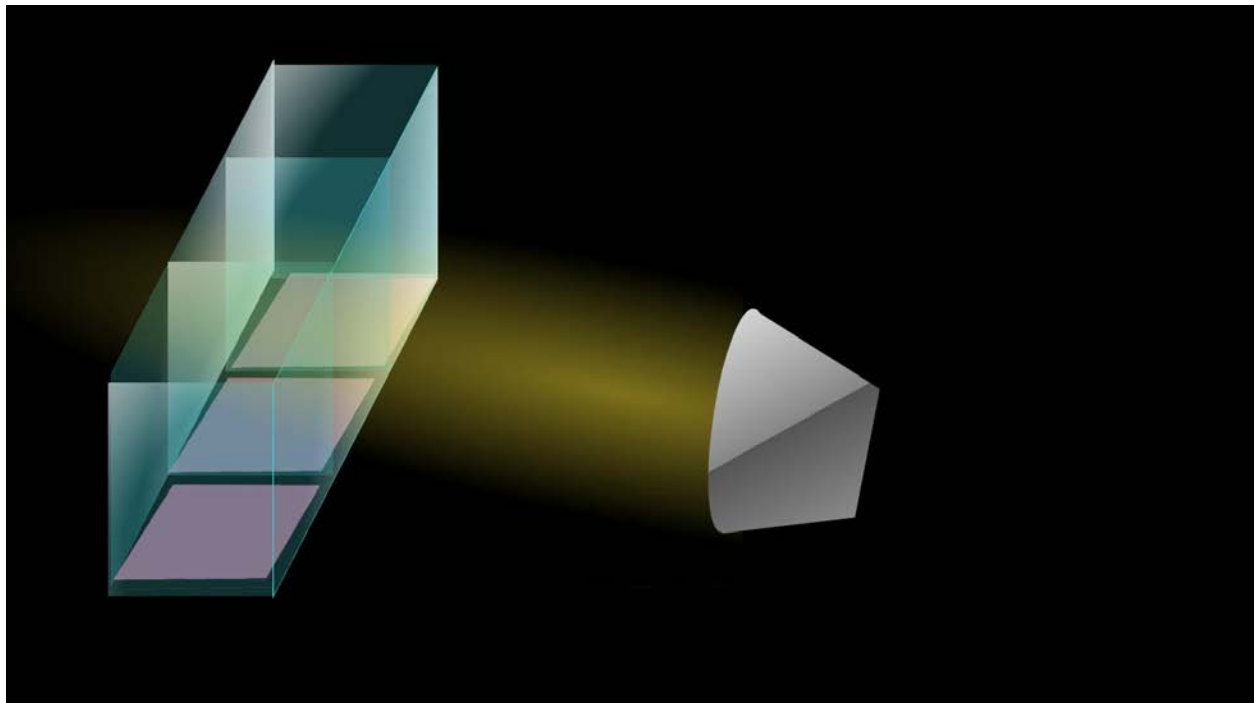
Picture 6: The cells containing the surfaces and the cell solution in the bright conditions was illuminated from the side to maximize the near field from the particles pointing up through the surfaces.

Staining cells

The cells were studied by staining with Millipore Actin Cytoskeleton and Focal Adhesion Staining Kit and subsequently looked at in a fluorescence microscope. Phosphate buffered saline (PBS) buffer is used throughout the protocol to mimic the environment within the human body, with similar osmotic and ionic concentrations. After culturing, cells were fixed to the surface by 4% buffered paraformaldehyde treatment for 20 minutes at room temperature. This fixes cells to the location that they adhered to, and minimizes risk of cells slipping away during treatment of the surface. A wash buffer was used in between segments of the staining, made from 0.05% Tween 80 in PBS. The surfaces was washed twice inside their plastic containers. To permeabilize the cells, the surfaces was treated with a solution of 0.1% Triton X-100 in PBS for 5 minutes. This penetrates the cell walls, leaving it open for staining agents. Surfaces was washed, and blocking solution was applied for 30 minutes. The primary antibody, Vinculin monoclonal antibody, binding to specific parts of the cells, was diluted in blocking solution to 4 ug/mL in blocking solution and incubated for one hour at room temperature. After this, surfaces was washed three times for 5 minutes each, after which the secondary antibody, Gt x Ms, FITC-conjugated, and TRITC conjugated phalloidin was diluted to 2 ug/mL each in PBS and incubated for 45 minutes. This was followed by washing the surfaces three times for five minutes each once again. The surfaces was treated with DAPI, 4 ug/mL in PBS, then washed three times again and stored until microscopic study. The primary and secondary antibody was used to detect focal contacts of the cells, with an emitted light wavelength of ca 520 nm. DAPI, emitting light at ca 455 nm, stains the cell nuclei and TRITC conjugated phalloidin, emitting 600 nm light, attaches to the F-actin of the cell. This gives a picture of the cell where different parts has different colours.

Shape particles

Non-spherical geometries on gold NP's is available through silver underpotential deposition (Personick, M.L., Langille, M.R. , Zhang, J., (2011)). Seeds were created by, while rapidly stirring a solution of 0.25 ml HAuCl4 (10 mM) and 10 ml CTAC (100 mM) quickly injecting ice cold NaBH4 (10 mM), and then after one minute of stirring, this solution was left undisturbed for two hours.

Growth solution was then prepared by mixing 0.50 ml HAuCl4 (10 mM), 100 µL of AgNO3 (10 mM), 0.20 mL of HCl (1.0 M), and then slowly injecting 0.10 mL of ascorbic acid (100 mM) into 10.00 mL of 100 mM CTAC.

The seed solution was diluted to 1/1000 concentration, and 0.1 mL of this solution was added to the growth solution. After mixing the solution, it was left undisturbed overnight.

Mathematical predictions

The diffusion process was modelled to investigate the ionic strength across the beaker where the NP gradient binding was conducted. The transport process of ions by brownian motion can be understood through Fick's law. This law provides a differential equation, which when solved gives a complete description of the buffer concentration profile. Fick's law states

$$J = -D \frac{dC}{dx}$$

Where J is the molar flux, x is the position in x direction, C is the local concentration and D is the diffusivity. By the law of mass conservation, the species flux into a volume element minus the flux out of it must equal the time rate of change of that species inside of that element plus any creation or destruction of that species. This gives the expression

$$\frac{dC}{dt} = \frac{d}{dx}\left(D \frac{dC}{dx}\right)$$

If D is assumed to have no concentration dependence, it can be taken out of the derivative, yielding

$$\frac{dC}{dt} = D\frac{d^2C}{dx^2}$$

This differential equation requires transformations to be solved, and is most conveniently solved by a laplace transform in t and a fourier transform in x, with the initial value being Heaviside's function, with the step located at the interface between the solutions. x is said to range from minus to plus infinity. Performing a laplace transformation we arrive at:

$$s\tilde{C}(x,s) - C_0 H(-x) = D\frac{d^2\tilde{C}(x,s)}{dx^2}$$

with s being the transformed time variable and H the Heaviside step function, defined at H(x) =1 if x>0, and H(x)=0 otherwise. Transforming the spatial variable x through Fourier transform leads to the expression:

$$s\hat{C}(\xi,s) - C_0\frac{1}{2}\left(-\frac{1}{i\pi\xi} + \delta(\xi)\right) = -(2\pi\xi)^2 D\hat{C}(\xi,s)$$

with ξ being the transformed spatial variable, with the fourier transform of the Heaviside function as

$$F(H(X)) = -\frac{1}{i\pi\xi} + \delta(\xi)$$

So consequently

$$\hat{C}(\xi,s) = \frac{C_0}{2}\frac{\left(-\frac{1}{i\pi\xi}\right) + \delta(\xi)}{s + D(2\pi\xi)^2}$$

Now, by the following inverse transform identities,

$$F^{-1}\left(\delta(\xi)\hat{f}(\xi)\right) = \hat{f}(0)$$

$$F^{-1}\left\{\frac{1}{2\pi i\xi}\hat{f}(\xi)\right\} = \int_{-\infty}^{x} f(z)\,dz$$

$$F^{-1}\left\{\frac{2a}{a^2 + (2\pi\xi)^2}\right\} = e^{-a|x|}$$

we get an expression for the Laplace transformed function:

$$F^{-1}\{\hat{C}(\xi,s)\} = \frac{C_0}{2}\left(\frac{e^{-\sqrt{\frac{s}{D}}x}}{s}\right)$$

Inverse laplace transform yields

$$L^{-1}\{\tilde{C}\} = \frac{C_0}{2}\,erfc\left(\frac{x}{2\sqrt{Dt}}\right)$$

This expression gives the ionic strength at an x value at a certain time for x and t >0. To calculate the average interparticle distance, DLVO theory can be used. This method combines the attractive van der waals forces with the ionic repulsion between the particles. Approximating the surface coverage needs an additional assumption, namely random sequential adsorption (RSA), meaning that the particles approaches the surface from random directions, with average kinetic energy kbT. RSA coverage of physical plates on a surface that does not allow overlapping is known to be c = 0.547 [][][] Now approximating the electrodynamic interaction between the particles by adding to the particle radius, and thus using the RSA approximation with larger spheres, the real surface coverage can be calculated from the ionic strength of the surrounding media through DLVO theory. A simplified expression for the effective radius of the particles

was derived through second order iteration by (Semmler, M., Ricka, J., Borkovec, M., (2000)), which expresses the effective particle radius as:

$$a_p^{eff} = \frac{1}{2\kappa} \ln \left(\frac{A}{\ln(A)} \right)$$

with

$$A = \Lambda Z^2 \kappa L_B \frac{2\kappa a_p}{\left(1 + \kappa a_p\right)^2}$$

where

$$L_B = \beta e^2 / 4\pi\varepsilon\varepsilon_0$$

$$\kappa^2 = 4\pi L_B n$$

$$\beta = \frac{1}{k_B T},$$

a_p is the real particle radius, epsilon is the dielectric constant, kb is the boltzmann constant and n is the ionic strength.

Now, the new surface coverage when assigning particles a new radius, must be the old coverage times the percentage of cross sectional area still available for the particles, resulting in the expression:

$$\theta_{max} = \theta_{jam} \left(a / a_{eff} \right)^2$$

where

$$\theta_{jam} \approx 0{,}547$$

is the coverage with real hard spheres.
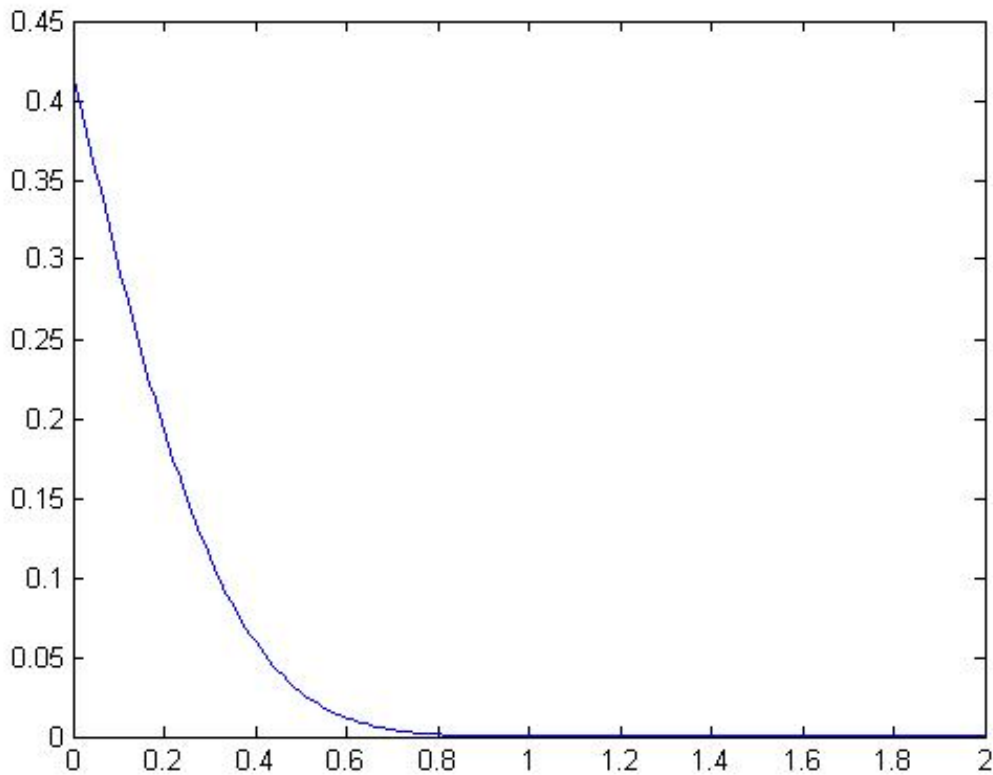
Graph 1: Surface coverage in percentage on the y axis, surface position in centimeters on the x-axis, with the value for $D = 6,904*10^6$ cm^2/min, $n_0 = 0,147$ M, $a_p = 20$ nm, $L_b = 0.72$ nm (value for water and room temperature) over 2 cm with 90 minutes diffusion gradient.

It can be seen in Graph 1 that the coverage quickly falls from its maximum value, and it goes to zero with zero ionic concentration. This does not agree with reality, as parts of the gradient surface with no ionic strength still gets covered with particles. This is due to the ideal theoretic case of the approximation, where the van der waals force has been taken out of the equation and the effective particle radius resulting from coulomb interaction is singular when n = 0. This expression works for ionic concentrations that is not too close to zero.

The near fields around gold NP's are very localized. To a good approximation, they can be said to not interact with other particles if the particles are spaced wider than the length of the particle diameter. This is the case throughout this study, and this approximation is thus valid. The total field on the surface is therefore the sum of fields from individual particles. As the plasmon is confined to a surface between a metal and a dielectric, both these materials affect the plasmon properties. It can be seen when comparing a non-covered surface covered in 40 nm NP's in water and air, and also a surface covered in spin-on glass that the intensity of the purple-reddish colour changes, indicating a stronger plasmon-light coupling in some media than others.

The nature of near fields are hard to predict mathematically. This is due to the different component of the oscillatory dipole field. In close vicinity to the field, the Coulombic part of the field is of importance, where charges near by feel the direct charge distribution within the NP. This field is inversely proportional to the distance to the power of two.

A little further away from the source, the field has dipole characteristics, by the fact that the two charged nodes of the NP is approximately the same distance from all points in space. The field strength is inversely proportional to the distance to the power of three.

Both of these fields transfer energy to its surrounding reactively, meaning the transfer itself affects the dipole directly. Of there is no receiver of the energy, the energy remained stored within these fields,

There is also a region in space where magnetic induction is a significant energy transfer mechanism. This happens through inducing currents in the surrounding via changing magnetic fields.

The complicated picture of the near field makes this a mathematically challenging task to express. Concerning this project, the fact that we know nothing about how the cells interact with oscillating fields of this type and frequency makes the real field strength less important, as few conclusions can be drawn from the data. Expressing the actual strength of the near field is by this reasoning deemed unnecessary.

Results

Film thickness

The HSQ thickness was measured through ellipsometry on two coated silica wafers and one clean silica wafer. Each covered surface was measured in three different spots, four times for each spot, and an average was calculated. The values are presented in the table below:

nM	1	1	1	1	2	2	2	2	3	3	3	3
Empty	2,3	2,5	2,3	1,4	2,4	2,6	2,4	2,3				
Surf. 1	40	39,8	39,8	40	40,4	40,3	40,3	40,3	39,5	39,7	39,5	39,6
Surf. 2	37,5	37,4	37,6	37,3	37,7	37,5	37,9	37,7	37,6	37,4	37,3	37,6

The oxide layer due to oxygen diffusion into the silica wafer is represented by the first row. The mean value of this data is 2,4 nm, which is subtracted from the value of the film thickness. The mean value for data for the film thickness is 38,7, and 36,3 after the oxide layer has been accounted for. This thickness is thinner than required to totally cover the particles, and not optimal for the experiment. The parts of the spheres that is not covered however can be assumed to be quite flat, and probably have neglectable interaction with the cells. This measurement was performed at such a late stage in the project that there was no time to redo the surface.
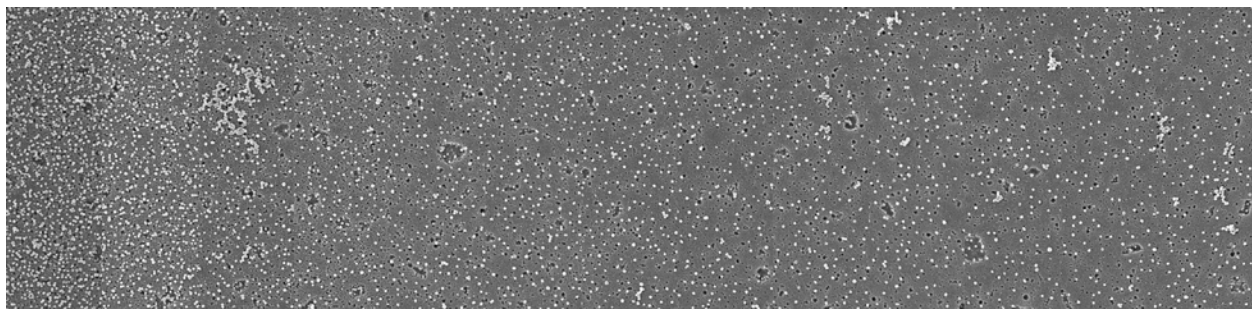
Gradient SEM

Gradient were looked at in an SEM microscope, to determine the surface coverage and to confirm that a gradient had indeed been produced. HSQ covered surfaces were also looked at with different electron voltages, to try to resolve the NP's embedded within the surface.

Picture 6 shows a spin coated surface with NP's shallowly embedded. The voltage of the electrons in the SEM pictures can be adjusted to see more of the surface, or to let the electrons penetrate the surface and scatter on the NP's. Both the smooth surface of the spin-on glass and the coverage of gold NP's can be seen here.
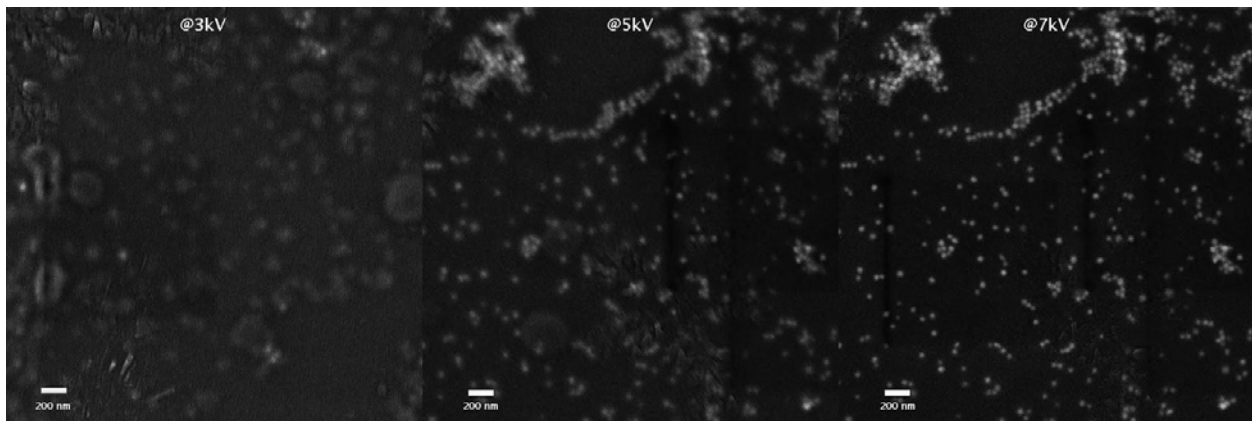
Picture 7: Three consecutive pictures of embedded particles with increasing electron energy, resulting in better resolver NP's to the right, and better HSQ surface structure to the left.

The gradients fabricated were also studied in an SEM before the spin-on process, and pictures were taken along the gradient with 0,5 mm intervals. The collected pictures shows a clear NP density gradient along the surface, shown in Picture 8.

Cell adhesion

Cells were studied in a microscope with the appropriate filters for the light frequencies needed for the fluorescence dye. One of the staining agents did not stain the cells in a satisfactory manner, and its frequency was thus filtered out of the pictures. The dye that did not work, TRITC- conjugated phalloidin, was supposed to stain the cell focal adhesion points. As the focal adhesions can not be seen, attention is directed at the form of the cell, to see if it spreads out on the surface or not. Pictures 9a-d shows cells on a surface in light and with a gradient embedded beneath the surface. In comparison with pictures 11 a and b which shows cells in dark conditions with no NP's present in the substrate, the adhesion of the cell to the surface can not be seen to differ from pictures 9. This suggests that the cell adhesion to the surface is not affected by the near fields around the NP's at these conditions. Also, pictures 10 a-d shows cells cultured on a surface in darkness with NP's embedded in the wafer, and the adhesion looks the same also here. The same goes for the plain substrate in lighted conditions (11 c-d). The cells are not affected by the near fields around these particles in these circumstances.
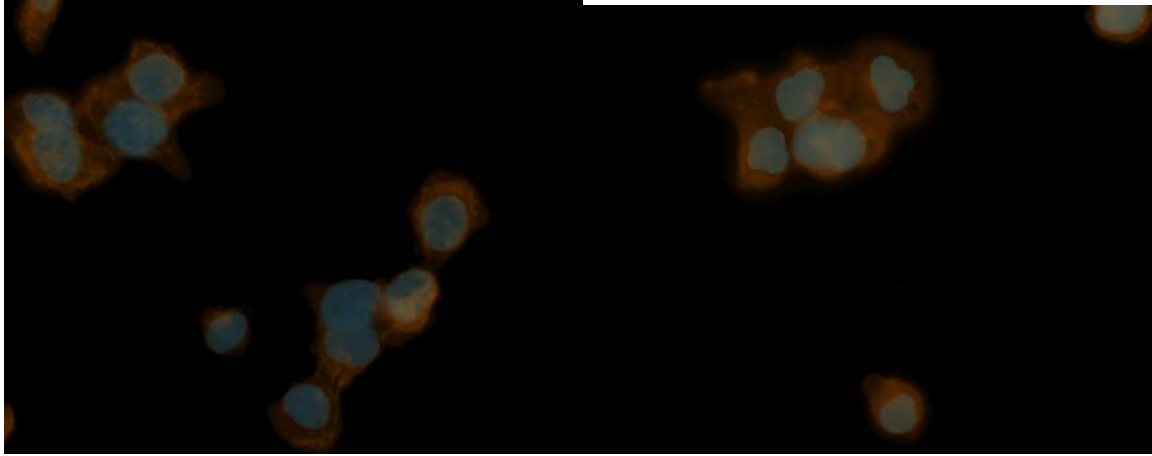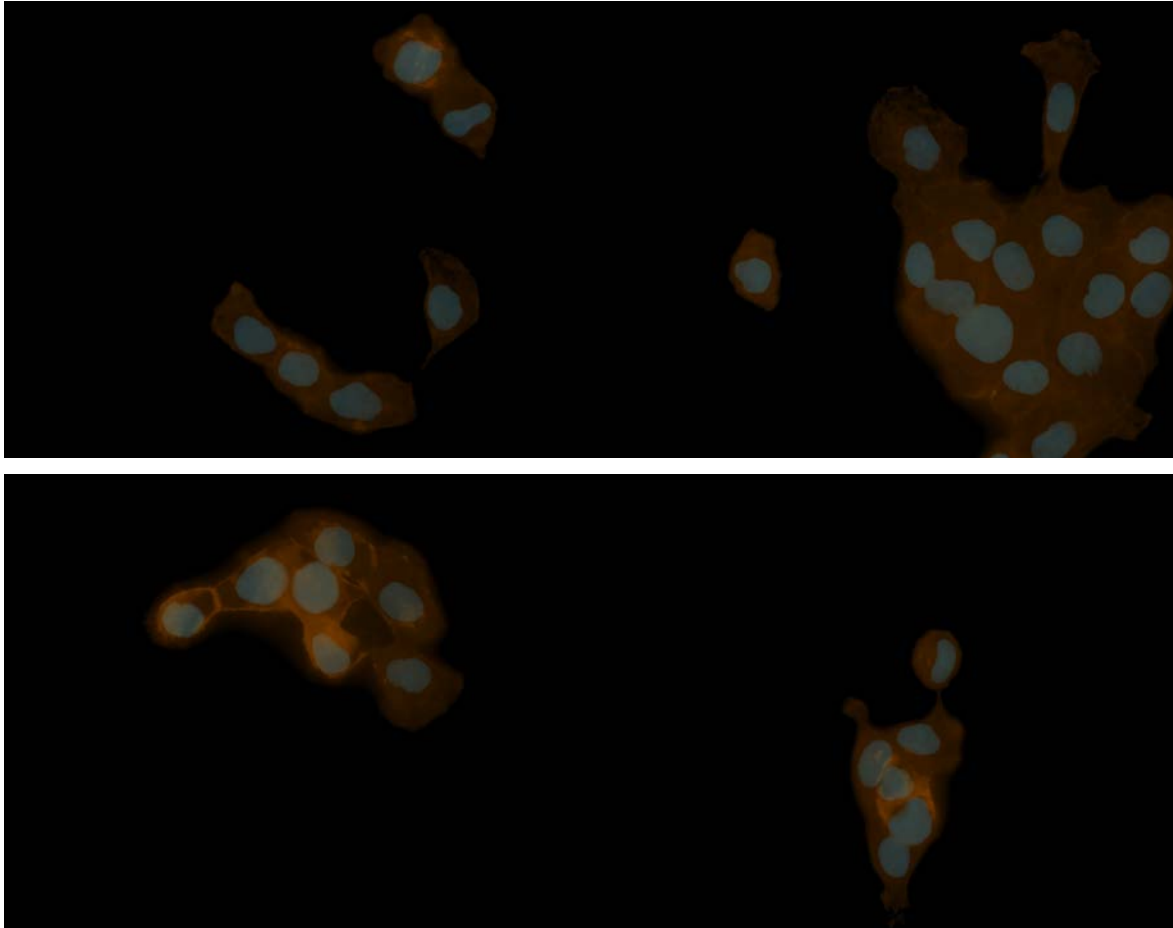
Picture 9, a-d, shows fluorescence microscopy of surfaces with embedded NP's with cells cultivated onto the surface during illumination.
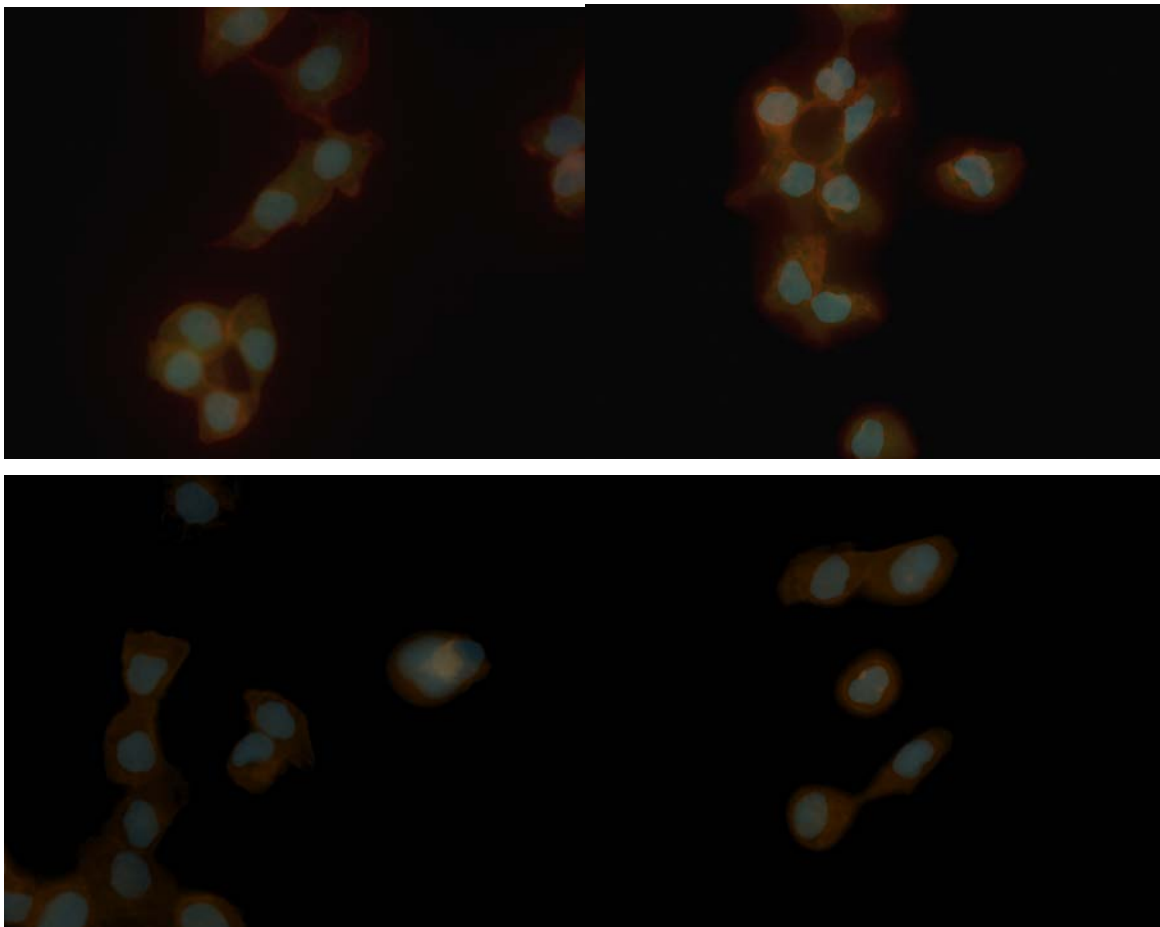

Picture 10 a-d, shows fluorescence microscopy of surfaces with embedded NP's with cells cultivated onto the surface without lighting.

Picture 11, a-b, shows fluorescence microscopy of reference surfaces with no embedded NP's, and with cells cultured in light, while c-d shows the same surfaces with cells grown in darkness.

Shape particle

An SEM study were performed on silica wafers covered with concave cubical NPs through the same vaporization protocol performed with spherical particles. A SEM picture of this is shown in picture 11
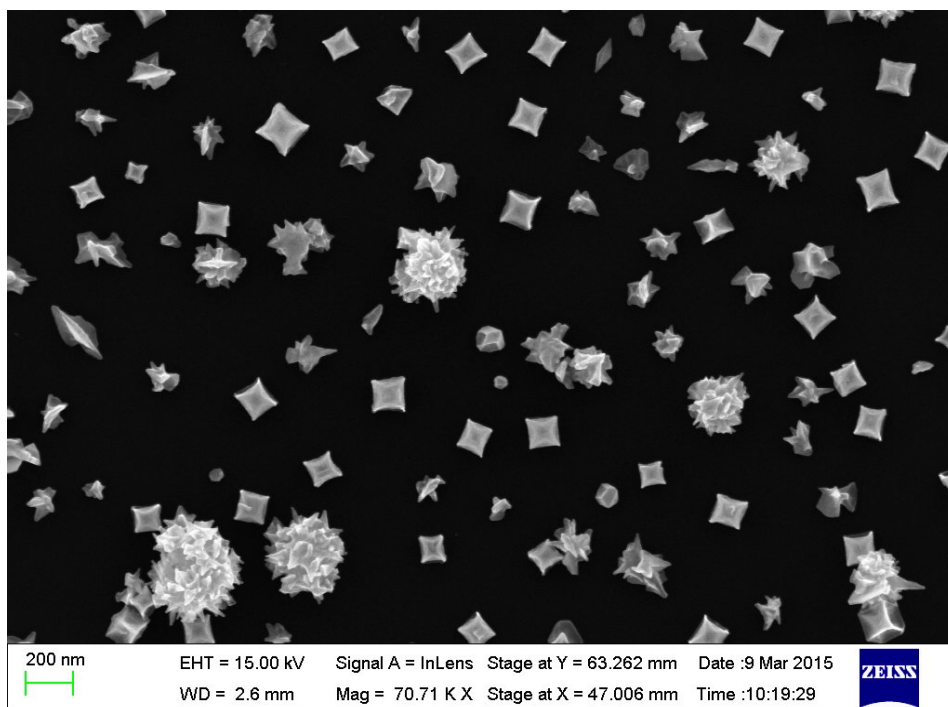
Picture 12: SEM image of the particles created according to (Personick, M.L., Langille, M.R. , Zhang, J., (2011)) on a silica substrate. The image shows the distorted fields around the corners of the NP's.

Picture 12 shows a polydisperse mix of different shaped particles, mainly concave cubes around 100 nm in length, but also larger gold clusters of starlike character. The particles are also quite evenly spaced out with distances of around 200 nm, suggesting a significant electrostatic repulsion in effect. The strong edge effects can also be seen in this image, as brighter fields around the pointy parts on the particles. Here, the electrons in the SEM are scattered more strongly due to this effect. These particles are suitable for applications where strong near fields are desirable, as in this project, but the time limitations prohibited experimental use with embedding and cell culturing.

Discussion

The obtained results do not indicate on any cell interaction with near fields around plasmonic NP's. There would beyond no doubt be an effect at the right field strength, as this energy stored in the near field ultimately ablates the material around it by kicking

out it's electrons (Harrison, R.K., (2012)). The threshold for any easily resolvable interaction was not reached in this project. One reason for the lack of cell response could be the short culturing time. The cell might not have time to bind to the surface, which also could explain the lack of TRICT seen in the picture, as it stains the focal adhesion points.

There could have been a lot of improvements in the experimental execution of this project. Different cell types could have been studied, which might be more or less sensitive to the fields in vicinity to the NP's. The non-spherical particles could have been embedded in a film in the same way, producing larger near fields than the spherical NP's. A suggested protocol for creating a similar surface with these more polydisperse particles is to bind these particles to a polymeric surface by vaporization. This surface could later be embedded in epoxy, whereafter the plastic surface is dissolved in toluene. The new, epoxy surface could then be spin coated in HSQ, in the same way as earlier to eliminate any surface chemistry differences. This would ensure that all the shape particles are situated close to the surface, without contributing to any surface roughness. Another possible route of improving the method is to make sure the right frequency light is used to excite the specific plasmones. Also, the near field strength are mostly in the oscillatory direction of the electrons, so illuminating the surface from the side would bring the largest part of the near field to the cells covering the surface. Near fields are however notoriously hard to measure, making the optimization process hard.

Another way to study the phenomena is to not cover the surfaces with glass, and then study weather illuminating the samples during cell culturing has any effect on the cells adhesion. This would require reference surfaces in light and darkness, and it would make sure that the HSQ layer does not absorb the near field energy.

One time consuming aspect of this project has been to find a reliable way to produce the surface on which to culture cells. The first idea was to have a thin gold film on a glass substrate, cover this with gold NP's and embed in epoxy. The glass would then be etched away and cells would be grown on the gold film. The problem with this would be though the electron screening of near fields in the gold film. An advantage however would be in the binding process of the NP's, which is not an easy task in general. When this idea was abandoned, the same thing were tried but now with a

sputtered layer of SiO2 on a polymeric surface, which was covered with a gradient of NP's and embedded in epoxy. The plastic would later be dissolved in toluene. It was only in the later stage of the project that the solution involving HSQ was brought up, which was considered a more time and cost efficient way to perform the same experiments.

Conclusions

We have seen that for A-431 cells under the light conditions used in this project, the near fields around 40 nm gold NP's does not impact the cell behaviour notably. Gradients of 40 nm gold NP's were successfully manufactured using a citrate buffer and a vapourization functionalizing. We have also seen that NP's, bound to a glass substrate by the protocol presented in methods, is not dislocated during the spinon process of HSQ for one minute and 2500 rpm speed.

Acknowledgements

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