Journal of Engineering and Natural Sciences
Mühendislik ve Fen Bilimleri Dergisi

Sigma 26
227-246,
2008

Invited Review Paper / Çağrılı Derleme Makalesi

INTERACTION OF GOLD NANOPARTICLES WITH LIVING CELLS

Erdinç SEZGİN, Ömer Faruk KARATAŞ, Dilek ÇAM, İlknur SUR, İsmail SAYIN, Ertuğ AVCI, Kemal KESEROĞLU, Selim SÜLEK, Mustafa ÇULHA*

Yeditepe University, Faculty of Engineering and Architecture, Department of Genetics and Bioengineering, Nanobiotechnology and Molecular Engineering Laboratory, Kayisdagi-ISTANBUL

Geliş/Received: 31.12.2008

ABSTRACT

The interaction of nanoparticles with living systems is under intense investigations due to their increasing use. The unknown effects on human health and vague understanding of their interactions with living systems are the fundamental issues before their use in products consumed by humans. The gold nanoparticles (GNPs) are mostly assumed safe and their use is considered in applications such as drug and gene delivery, photothermal therapy to selectively kill the cancerous cells, and nanoprobes for cellular investigations. This review provides the recent developments in the use of GNPs in medicine and their interactions with living cells along with the basic concepts and techniques used to monitor the interactions such as surface-enhanced Raman scattering (SERS).
Keywords: Nanobiotechnology, gold nanoparticles, SERS, cell.

ALTIN NANOPARÇACIKLARIN CANLI HÜCRELERLE ETKİLEŞİMİ

ÖZET

Nanoparçacıklar, canlı sistemlerde kullanımlarının artması nedeniyle günümüzde oldukça yoğun bir araştırma konusu olmuştur. Fakat nanomateryallerin insanlar tarafından kullanılan tüketim ürünlerinde kullanılmadan önce insan sağlığı üzerinde bilinmeyen etkilerinin olması ve canlı sistemlerle olan ilişkilerinin de tam olarak anlaşılamaması, karşılaşılan ve araştırılması gereken en önemli sorunlar arasındadır. Oysa en yaygın kullanılan nanoparçacıklardan biri olan altın nanoparçacıkların ilaç salınımında, gen aktarımında, kanser hücrelerini öldürme yöntemlerinden biri olan fototermal terapide ve hücresel araştırmalar için nano-probe hazırlanması gibi uygulamalarda kullanımının güvenli olduğu düşünülmektedir. Bu derleme, altın nanoparçacıkların tıptaki ve canlı hücrelerle olan ilişkilerindeki son gelişmeleri ve bu ilişkileri incelemekte kullanılan Yüzeyce Zenginleştirilmiş Raman Saçılması (YZRS) gibi tekniklerle birlikte bu alandaki temel konuları ele almaktadır.
Anahtar Sözcükler: Nanobiyoteknoloji, altın nanoparçacık, YZRS, hücre.

1. INTRODUCTION

Nanotechnology is defined as the creation and utilization of materials, devices, and systems with a control of nanometer-sized structures. [1] With the nanotechnology concept, an intense investigation in several fields such as physics, chemistry, electronics etc. is underway to understand the behavior of nanometer scale structures for their possible novel applications. There is also an enormous effort to use nanotechnology-based tools in biological sciences and medicine in applications such as delivery, diagnosis, treatment and imaging.

*Sorumlu Yazar/Corresponding Author: e-mail / e-ileti: mculha@yeditepe.edu.tr, tel: (216) 578 15 87

The preparation of nanoparticles with different physicochemical properties triggered a remarkable progression in nanotechnology [2-4]. Among them, the noble metal nanoparticles such as gold and silver are finding increasing use in several branches of science and technology. Their use as coloring agents goes back in the human history as old as 4th century (Roman Lycurgus cup), and their use is known in artwork and other materials in 17th century [5]. The recent interest in noble metal nanoparticles is due to the better understanding of their optical properties and their possible applications in many branches of science extending from physical science to medicine and molecular biology. Among the noble metal nanoparticles, the GNPs especially in biological sciences are attracting enormous interest due to their biocompatibility and non-toxicity in addition to its extraordinary optical properties. For a better understanding of the applications of GNPs, it is important to understand the fundamentals behind their unusual optical properties. Therefore, a brief discussion of plasmonics is first introduced here followed by their applications.

1.2. Plasmonics

Surface plasmon plaritons (SPPs) are defined as evanescently and perpendicularly propagating electromagnetic waves generated at a conductor (a noble metal) - metal-dielectric interface via interaction of electromagnetic fields of light with conductor metal's oscillating electron plasma [6]. The generation, propagation, detection and use of SPPs are referred as plasmonics [7]. Although all metals interact with light, only noble metals can support the plasmon formation in the visible region of the spectrum since the requirement is a negative real and small positive imaginary dielectric constant [8].

In 1857, Michael Faraday realized that the bright color of gold colloidal suspension was due to colloidal nanoparticles. However, Gustav Mie was to clarify the origin of the color of colloidal suspension. In his pioneering work, he explained the interaction of light with small spherical particles that is much smaller than wavelength of the interacting light by solving Maxwell's electromagnetic equations in 1908 [9-11]. The detailed theoretical treatment of metal and light interactions for variety of shape and size of silver and gold nanoparticles can be found elsewhere [6, 12, 13]. When the surface plasmons are excited on a thin film of gold or silver nanostructured surface with the help of a prism, it is called surface Plasmon resonance (SPR) [5]. When the excitation takes place on an isolated nanostructure, it is called localized surface plasmon resonance (LSPR). Although both forms have significant uses in biological applications, we will focus on the LSPR because the studies on this report mainly focus on the use of gold nanoparticle GNPs.

The "*quasi-static approximation*", which assumes that the harmonically oscillating electromagnetic field is constant over the volume of the particle, is used to explain the interaction of noble metal nanoparticles with a diameter (d) much smaller than the interacting light's wavelength (λ) [6]. Although this approximation works relatively well for the nanoparticles with diameters smaller than 100 nm, the deviation increases with increased nanoparticle size. Therefore, the approximations based on other mathematical models might be necessary for the nanoparticles larger than 30 nm [14-19]. In the quasi-static limit, $\varepsilon(\lambda) = \varepsilon_1(\lambda) + i\varepsilon_2(\lambda)$ is given as the wavelength-dependent dielectric function and $C_{ext} = C_{abs} + C_{sca}$ is given as the extinction cross section for a nanoparticle. The C_{ext} is

$$C_{ext} = 9\frac{\omega}{c}\varepsilon_m^{3/2}V\frac{\varepsilon_m}{\left[\varepsilon_1 + \chi\varepsilon_m\right]^2 + \varepsilon_2^2} \qquad \text{(Equation 1)}$$

where ε_m is dielectric constant of the surrounding medium, ε_1 and ε_2 are the real and imaginary parts of the dielectric function of the nanoparticle, respectively (6) . The coefficient of χ is 2 and

the resonance condition is $|\varepsilon_1| >> \varepsilon_2$ for a spherical nanoparticle. Upon $\varepsilon_1(\omega) = -2\varepsilon_m$, the condition is fulfilled. The LSPR forms as the equation above has a maximum. The χ value increases with increased aspect ratio, which is defined as the ratio of the long axis to short axis of the nanoparticle (19). The polarization alterations, reflected as the changes in dielectric constant of the medium, at the metal-media interface can be easily recoded as a shift of the resonance position.

The surface plasmons display non-uniformity for rod and triangle shaped nanoparticles. The nanorods shows one strongly red-shifted polarization along its axis and one slightly blue-shifted perpendicular polarization along the long axis (6). These two Plasmon bands are separated clearly as the aspect ratio increases. A similar observation is valid for triangular shaped metal nanoparticles as well. The two resonance peaks, a longitudinal Plasmon peak and a large field enhancement peak at the tips of the triangle, are observed. The variations in size and shape of nanoparticles have enormous impact on their plasmonic properties. For example, the metal nanoparticles larger than 30 nm demonstrates the significant broadening in resonance peak due to retardation effect, which is the result of uneven distribution of electron cloud. This reduced depolarization of the electron cloud around particle center causes dampening and this results with the red shifts and radiative loss.

It is very important to realize that higher order structures can be assembled from nanoparticles. As these structures are prepared with the use of GNPs, the additional shifts due to the interaction of localized modes are also observed. When the assembled structure is constructed from smaller nanoparticles (<30 nm), the interactions can be considered as dipole interactions for an approximation. These electromagnetic interactions can be explained with near-field coupling and far field-coupling [6, 20-22]. It is important to note that the spacing between nanoparticles is critical in colorimetric detection and surface-enhanced Raman scattering (SERS).

Figure 1 illustrates the formation of surface plasmons, where electromagnetic field of light interacts with the oscillating electron cloud of GNPs (a) and LSPR excited on GNPs (b).

When GNPs interact with ordinary white light, they both absorb and scatter the light ($C_{ext} = C_{abs} + C_{sca}$). The absorbed light not only excites the surface plasmons but also is converted to heat [6]. The localized surface Plasmons can be used in sensing [23-25] by monitoring the wavelength shift before and after the interaction of agents with the GNPs and also by monitoring the color change, named as “colorimetric detection” [26]. The other implication of the surface plasmons is the enhancement in Raman scattering. When a molecule or a molecular structure is brought into close contact with the noble metal nanostructures, an extraordinary enhancement, up to 10^{14} times, is observed. This phenomenon is called surface-enhanced Raman scattering (SERS) and there are reports utilizing this technique for single molecule level detection, which rivals fluorescence based detection [27- 29].

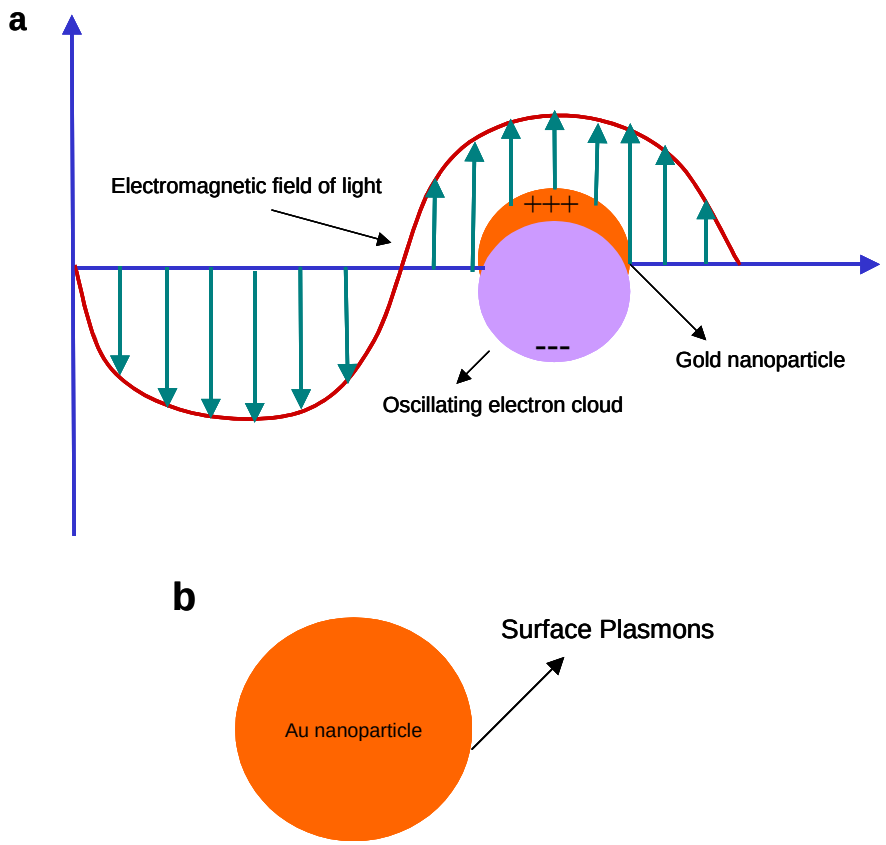


Figure 1. Illustration of interaction of light and metal nanoparticles (a) and localized surface plasmons (LSPR) (b).

2. SYNTHESIS OF GOLD NANOPARTICLES

There are several methods for the synthesis of GNPs, but the methods based on reduction reaction of gold ions are mostly employed. The different methods use different reducing agents and yield nanoparticles with different sizes and shapes. The GNPs prepared with reduction of AuCl (PPh_3) with diborane or sodium borohydride yields 1-2 nm nanoparticles [30] while biphasic reduction of $HAuCl_4$ by sodium borohydride in the presence of thiol capping agents yields 1.5-5 nm GNPs [31,32]. The reduction of $HAuCl_4$ with sodium citrate in water yields GNPs in the range of 10-150 nm sizes [33-35]. To synthesize GNPs in varying sizes, an appropriate trisodium-citrate solution concentration was used to accomplish the reduction of aqueous solution of $HAuCl_4$. Since the surface properties of GNPs are very important, a special attention must be given to the surface properties. Although the reaction media mostly determines the surface properties of GNPs, in some methods capping is carried out during synthesis [32]. However, post-synthesis modifications are commonly employed to optimize the function of GNPs for certain applications. Figure 2 shows the TEM image of the 13 nm GNPs synthesized with citrate reduction method. As it is seen, this method generates quite uniform gold nanoparticles.

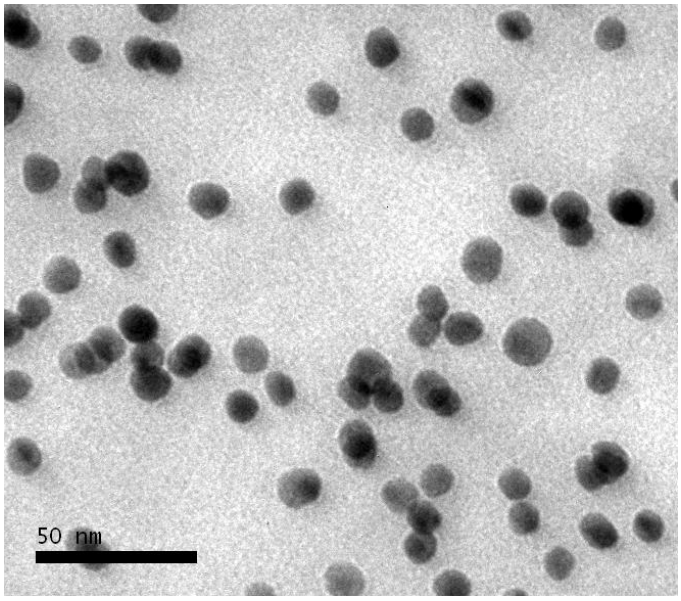


Figure 2. TEM image of citrate reduced gold nanoparticles.

3. UPTAKE OF GOLD NANOPARTICLES INTO LIVING CELLS

It is hypothesized that the GNPs are taken into the cell upon nonspecific binding of serum proteins on the surface of gold nanoparticles, which in turn mediates uptake of nanoparticles. [2] The surface characteristics of nanoparticles effect their internalization into living cells. The surface properties of gold nanoparticles are mostly predicted by synthesis procedure if the chemical surface modification with a ligand is not pursued. For instance, gold nanospheres and gold nanorods synthesized in the presence of reducing agents such as (Cetyl trimethylammonium bromide) CTAB, a cationic micellar surfactant, show cytotoxicity. [35] In addition, the internalization of GNPs is also effected. For example, CTAB coated gold nanorods are internalized by KB cells at a high number density and migrated toward the perinuclear region over a 24 hour period, which shows nonspecific internalization of gold nanoparticles that can result in the production of interference during site directed imaging and damage on healthy cells in a thermal therapy application. Exchanging CTAB with different hydrophilic surfactants such as bis(p-sulfonatophenyl)phenylphosphine (BSP) or methylated poly(ethylene glycol)-dithiocarbamate (mPEG-DTC) decreased the nonspecific internalization of gold nanorods within the cell [36]. Besides, the modification of the gold nanoparticles with thioglucose increased the internalization of gold nanoparticles in cancer cells, which have higher metabolism compared to healthy cells. In the same study, it was demonstrated that modification of gold nanoparticles with cysteamine AET and citric acid ligands TGS which resulted in positively charged nanoparticles by using cysteamine as the capping agent, and modification with thioglycolic acid TGA which resulted in negatively charged nanoparticles effected the uptake of gold nanoparticles to the living cells. Uptake of TGA-GNPs was negligible, but AET-GNPs and TGS-GNPs were internalized effectively. Although both AET-GNPs and TGS-GNPs were positively charged, AET-GNPs were showed to attach heavily to the outer membrane of target cell and TGS-GNPs were showed to be effectively uptaken. Therefore, it was outlined that changing the charge of the surface of gold nanoparticles doesn't effectively assist the internalization process [37].

The reports indicate that the uptake of GNPs is showed to be cell dependent and optimal size of GNPs that are taken inside the cell differs from cell to cell. MCF7 cells internalize GNPs up to 100 nm that is the maximum size for receptor-mediated endocytosis (RME), which is needed for targeted delivery of gold nanoparticles. The same pattern in size dependence was demonstrated in HeLa cells with 50 nm optimum internalization size. Although it is a known idea that smaller GNPs should be internalized easily through pores and channels in the cell membrane, when they are compared to larger ones, GNPs without surface modification are thought to be taken inside through endocytosis [37]. The concentration of the GNPs in the medium that living cells exposed is also effective in the uptake of gold nanoparticles. The more the concentration of GNPs, the more GNPs are internalized [38].

The spherical and rod shaped GNPs are extensively used in biomedical applications and they differ in pattern of uptake into living cells. The gold nanorods with 74 x 14 nm in size showed to be internalized into the cell less effectively than spherical nanoparticles which has 74 and 14 nm sizes. This is speculated to stem from the CTAB amount on the surface of the nanoparticles, which prevents the interaction of serum proteins with gold nanorods, which have more CTAB on their surfaces. Also binding of serum proteins nonhomogenously on the surface of gold nanorods may result in diminishing of ligand receptor interaction that ultimately decreases the uptake of gold nanorods. [2] As a result, it is clear that surface properties, size and shape of GNPs determine the uptake of gold nanoparticle process.

The study of toxicity of gold nanoparticles is essential due to the fact that GNPs are commonly used in imaging, diagnostics, delivery and therapy applications in medical world. There are various results, which describe the GNPs as toxic [39] or non-toxic [40] because of their surface chemistry and size.

The surface modifications with molecules such as cysteine, biotin, citrate, glucose and cetyltrimethylammonium bromide (CTAB) are diversed as cationic, neutral or modifications. Goodman et al. found out that cationic (ammounium-functionalized) GNPs are more toxic than anionic (carboxylate-substituted) ones within the same size [41]. They demonstrated that it is due to interactions of cationic gold particles with the negatively charged cell membrane. In another study, the cytotoxicity effect of CTAB, which is an essential molecule while preparing gold nanorods [35], was shown [42]. Since some of bound CTAB molecules are then solubilized in the solution, the CTAB coated gold nanoparticles are needed to be washed in order to get rid of the cytotoxicity effect. In addition, the toxicity effect was reduced also by coating the gold nanoparticles with polyethyleneglycol (PEG) [43]. Furthermore, Takahashi et al. showed that phosphatidylcholine (PC) passivated gold nanorods have less toxicity compared with the CTAB-coated gold nanorods [44].

The sizes of the nanoparticles are similar to the sizes of the cellular components and proteins. Thus, they can pass the natural barriers and cause tissue reactions. Water soluble $Au_{55}[Ph_2PC_6H_4SO_3H(Na)]_{12}Cl_6$ with B-DNA showed that 1.4nm Au_{55} cluster cores selectively bound to the major groove of the DNA which can blockade the transcription. But the gold nanoparticles larger or smaller then the 1.4nm could not bind the major groove so stable [45].

It is found that size of the gold nanoparticles affects the kinetics and the saturation inside the mammalian cells [2]. Parnodet and his colleagues found that 13 nm gold nanoparticles inside the cells cause disappearance of the actin stress fibers and loss of viability [39]. Also it has shown that lysine and poly-l-lysine capped 3.5nm GNPs are non-toxic [46]. Citrate- biotin- and cetyltrimethylammonium bromine capped 18 nm GNPs, glucose reduced 12nm gold nanoparticles and cysteine- and citrate- capped 4 nm GNPs were all biocompatible [40]. Studies from Simon and coworkers demonstrated that the cytotoxicity of the triphenylphosphine monosulfate and triphenylphosphine trisulfate capped GNPs depend mainly on the particle size and not on ligand chemistry. They also found that 1-2nm sized GNPs were highly toxic and both smaller gold compounds and larger 15nm gold colloids were comparatively non-toxic [47].

The shapes of the GNPs also affect the cytotoxicity. A few groups studied the silica core coated gold nanoshells. The study by Hirsch et al. suggested that the exposure of the cells to the nanoshells did not cause cell death [48]. Also in a study by Loo et al., breast cancer cells are exposed to anti- HER2 bio-conjugated gold nanoshells and no difference was observed in their viability compared to the control group [49, 50]. The cytotoxicity of the gold/copper nanoshells showed that the cell viability decreased by %15 with the increasing concentration [51]. Also a few groups studied cytotoxicity of the gold nanorods. A study demonstrated that the viability of the cells after the nanorod incubation with nanorods did not decrease significantly [52]. More recently it has been found that the chemicals involved in the preparation of the nanorods cause the cytotoxicity [53].

The GNPs show different cytotoxicity for different cell types. Thomas et al. used monkey kidney (COS-7) cells to be transfected by polyethylenimine(PEI)-conjugated GNPs and found ≈ 20% decrease in cell viability [53]. Tkachenko et al. showed four-different peptide-BSA coated nanoparticles caused different toxicity for different cell lines [54]. The peptide-BSA-gold nanoparticles conjugated with adenovirus fiber protein caused 20% cell death in HeLa cells while only 5% in the 3T3/NIH cells. Goodman et al. also tested the effect of gold nanoparticle exposure in three different cell lines (COS-1 mammalian cells, mammalian red blood cells, and *E.Coli* cultures) using 2nm sized cationic (ammonium-functionalized) and anionic (carboxylate-functionalized) gold nanoparticles and found similar toxic effects for all three cell lines [41]. This contradicts the findings of Tkachenko et al. as they found a difference between cell lines; however, this could be due to the use of different surface coatings.

The similar cell specific differences were observed between the studies of Connor et al. and Patra et al. In their work, Connor et al. examined a series of gold nanoparticles with surface modifiers including biotin, CTAB, cysteine, citrate and glucose for uptake and acute toxicity in human leukemia (K562) cells [42]. While citrate, biotin, and CTAB coated gold nanoparticles were not toxic, CTAB and the gold salt ($AuCl_4$) precursor solution showed severe toxicity. On the other hand, citrate-capped gold nanoparticles were found to be cytotoxic to a human carcinoma (A549) cell line at certain concentrations in the findings of Parta et al. [55].

Due to the complexity of the problem and ambiguity of the reported results, we have also investigated the toxicity of GNPs without surface modifications, which provides the starting point for our gene delivery and SERS studies. Figure 3 shows a white light image of HeLa cells incubated with GNPs. The aggregates of GNPs are visible in the living cells. Figure 4 shows the cytotoxicity of the GNPs in different cell types at increased GNP concentrations. As it is seen, the toxicity of the GNPs depends on the cell type and increased GNP concentration. It was found that the 13 nm size nanoparticles at eight times diluted concentration (represented as 25x on Figure 3) influenced the viability of the cancerous cells (A) while it had no effect on the viability of the healthy L929 cells (C). The further cell culture studies with the control cells showed the influence of the GNPs on the morphology of cancerous cells while no detectable influence was observed on the immortalized healthy mouse fibroblast cells (L929 cells). The cancerous A549 cells started to form neurites that are the sign of the slowing down of the proliferation and the death. The sudden decrease in the viability of the cancerous A549 cells with the treatment of 41 nm GNPs was also observed (Figure 4 B). However, during the consecutive days, the number of viable cells continued to proliferate. On the other hand, the treatment of 41 nm GNPs with the healthy L929 cells resulted with decreased proliferation (Figure 4 D). As it is seen, the results are not very conclusive and it seems that there are possibly many factors contributing the response of the cells.

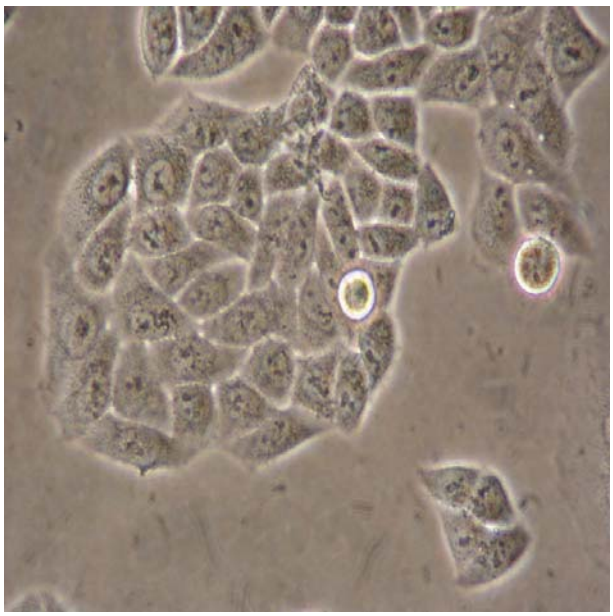
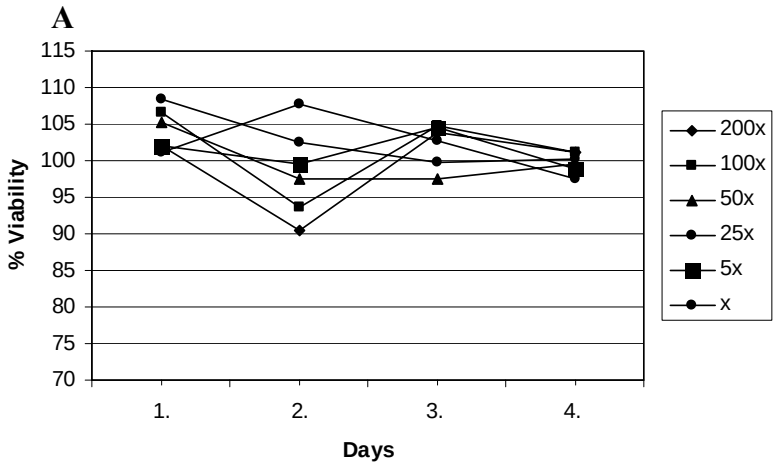


Figure 3. White light image of HeLa cells incubated with GNPs.



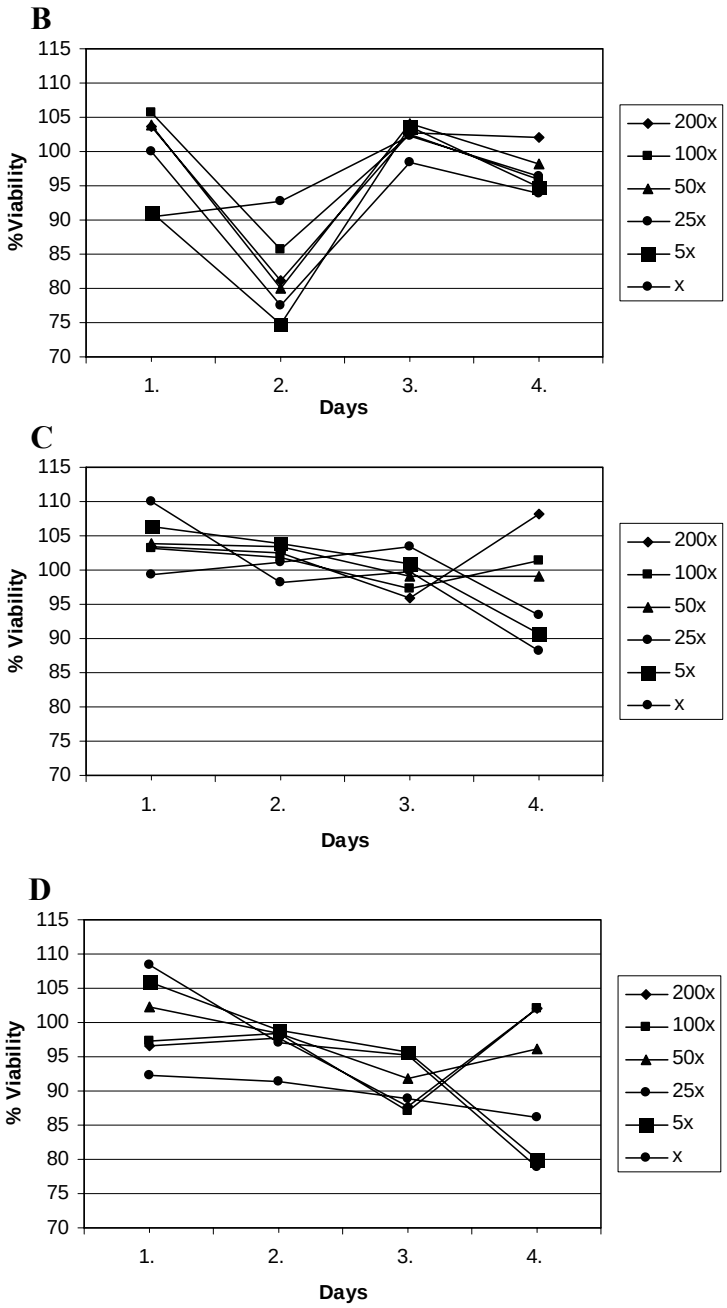


Figure 4. The effect of GNPs at increased concentration on the viability of the cancerous A549 lung cancer cells at the sizes of 13 nm (A) and 41 nm (B) GNPs and the healthy L929 mouse fibroblast cells at 13 nm (C) and 41 nm (D).

4. DELIVERY

The targeted delivery of therapeutics is highly desirable. However there are major problems with delivery applications. These problems are selectivity, specificity, uptake of particles and activity of the particles in the cell. The GNPs are currently under investigation for delivery purposes and there are several reasons why they are considered as possible delivery agent such as their suitability for surface modifications, which facilitates the binding of different molecules such as nucleic acids, proteins, sugars or some drugs, assumed low toxicity, and plasmonic properties, which may be used for the release of drug agent upon reaching the target site. The surface modifications enhance the efficiency of specificity and selectivity of GNPs, also affect the uptake of GNPs into the cell . There are many kinds of modification on GNPs.

For the cellular uptake of bio-molecules into the cell, poly-cationic materials such as liposomes are generally used. The surface properties of GNPs can chemically be modified with several biological molecules such as peptides and oligonucleotides. When the GNPs are modified with oligonucleotides, the interaction of extracellular proteins with DNA-GNP is the main factor for their intracellular uptake. There is a direct proportion for the DNA loading and cellular uptake due to extracellular proteins loading on DNA-GNP complex [56]. The real mechanism behind this is not completely understood yet.

Another important modification type is glutathione (GSH) modification, which mediates the release of thiol modified biomolecules from the nanoparticles [57]. The mechanism could be related to the concentration differences of GSH in cytoplasm and extracellular matrix [58-60].

The circulation time in blood is another important point in delivery applications. The increased hydrophilic surface properties of GNPs to provide stealth properties for the nanoparticles causing longer circulation times and the positively charged surfaces enhance endocytosis of the complex [41].

The selectivity and specificity are the other important points that should be considered. The selectivity of the delivery complex can be enhanced with certain modification to the GNPs. Feldheim et al. highlighted the of existence the three barriers or obstacles for achievement of delivery. These are outer cell membrane, endosomal escape and nuclear membrane. The conjugation of special nuclear localization sequences (NLS) from different viruses with BSA and GNP could be route for safe and efficient delivery [54]. This goal can also be achieved with conjugation of special peptides from viruses with GNPs [61]. A new approach to overcome the delivery associated abovementioned issues is to use different peptide sequences simultaneously attached to the same GNP. Wang et al. prepared a multifunctional GNP with combining CALNN AND CALNNRRRRRRRR. While arginine reach-rich peptide sequence can enable the cellular internalization, the combined sequence can enable the nuclear and endoplasmic reticulum localization [62].

As it is stated earlier, changing the surface charge properties with some ligands enhance the cellular uptake with increasing the selectivity [43]. The use of antibodies is another solution for enhanced selectivity and specificity [63]. The folic acid-GNP complex was reported to target the cancer cells [64]. This complex is recognized by folate receptor that is over expressed in tumor cells, thus drug carrier nanoparticles can find the cancerous cells in tissue by folic acid-folate interaction and release drug specifically to the tumor cells. Therefore, targeted therapy prevents the potential damage of drugs to healthy cells. Another type complex is hyaluronic acid-GNP complex. Similarly hyaluronic acid is recognized by the receptor that is over-expressed in cancer cells and nanocomplex is able to enter into the cell [65].

The other interesting approach is to stop the activity of the expressed gene product by interfering with the expression machinery. Mirkin et al demonstrated the gene regulation with the antisense oligonucleotide conjugated GNPs that is stable against enzymatic digestion [66].

There are also other encouraging and novel studies utilizing GNPs for the delivery applications. For example, Rotello et al. modified GNP with special peptide that resembles to the histone protein in size, shape and surface properties [67] to enhance the activity of delivered DNA. Nagasaki et al. prepared siRNA GNP complex that contains siRNA with thiol group bound to pegylated GNP [68].

5. PHOTOTHERMAL THERAPY

The GNPs can be an alternative for thermal therapy because the sensitivity of one nanoparticle can be up to a million fluorescence molecules that are currently used for the medical applications [69]. The GNPs are more photo-stable, do not suffer from photobleaching, and much less laser power needed to increase the local temperature to kill the cancer cells when compared to the laser power employed in molecular photothermal therapy. Therefore, cancer cells can be easily killed next to the healthy cells.

The plasmonic properties of GNPs can be tuned by changing the shape and size of the nanoparticles. For example, as mentioned earlier, the rod shaped nanoparticles have two absorption maximum, a strong one in NIR region due to longitudinal oscillation of electrons and a weaker maximum in visible region, due to transverse electronic oscillation [70-72]. The longitudinal Plasmon resonance wavelength can be shifted into NIR region by increasing the aspect ratio. Figure 5 shows the LSPR shift as the aspect ration of a gold nanoparticle containing suspension increases and the TEM images of gold nanoparticles.

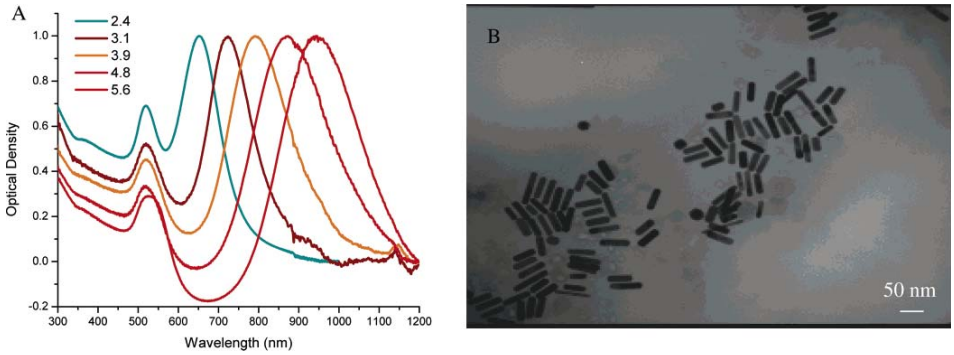


Figure 5. Shift of resonance wavelength of gold nanorods as aspect ratio increases (A), TEM image of gold nanorods (B). Reprinted with permission from Ref. 71. Copyright @American Chemical Society.

The applicability of photothermal therapy was demonstrated in several reports [48, 50, 73-76]. Huang et al. reported one of the most important studies for the photothermal therapy [71]. In that study, the anti- epidermal growth factor receptor (anti-EGFR) were chemically attached to the gold nanorods to target epidermal growth factor receptor (EGFR) on the cellular membrane of cancer cells. The healthy HaCaT (human Keratinocytes) cells, and two malignant epithelial cell lines of human oral squamous cell carcinoma, HSC and HOC malignant cells, were incubated with the gold nanorods attached to coated with anti-EGFR. Figure 6 shows the comparison of anti-EGFR attached spherical (A) and rod shaped gold nanoparticles (B). The cells incubated with anti-EGFR attached gold nanorods are exposed to a 800 nm wavelength light using a continuous wave Ti:sapphire laser. Figure 7 demonstrates the effectiveness of the therapy as the laser power increased. The cancer cells were damaged at 80 mW while healthy cells were

damaged at 120 mW. This demonstrates the possibility of destroying cancer cells next to health cells.

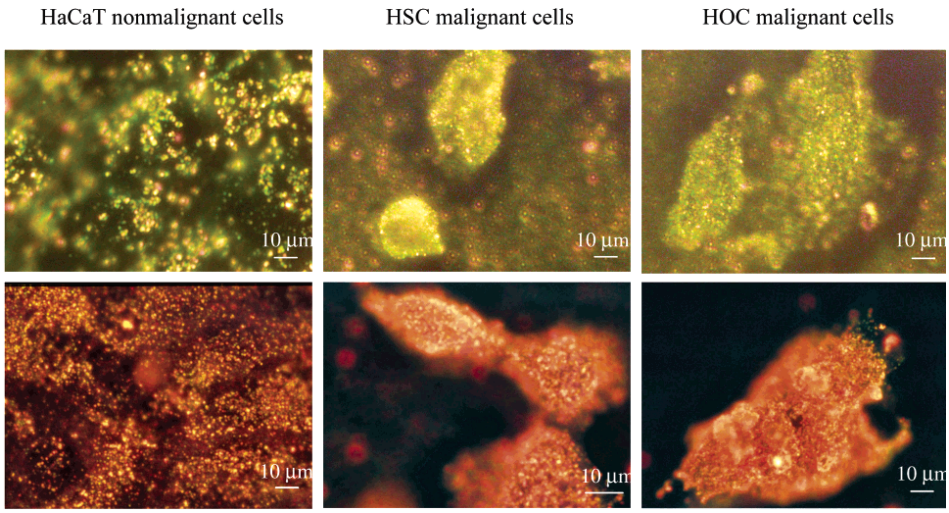


Figure 6. Optical microscopy images of malignant and healthy living cells after their treatment with spherical GNPs (A) and gold nanorod (B) conjugated anti-EGFR antibody. Reprinted with permission from Ref. 71. Copyright @American Chemical Society.

6. SURFACE-ENHANCED RAMAN SCATTERING (SERS)

Raman spectroscopy is a vibrational spectroscopic technique that provides chemical information about a molecule or molecular structure. As different from IR spectroscopy, its selection rule is based on the change in the polarizability of bonds. Therefore, it provides detailed information about the functional groups rich in electron in the molecular structure. The other advantage of Raman spectroscopy is the immunity to water that is always constituted in biological materials. As a powerful mode of Raman spectroscopy, SERS can be utilized for the probing the cellular environment of the GNPs after their introduction into the living cells. The entry of GNPs into living cells is mostly assumed to be through the endocytosis. As discussed above, the efficient entry of GNPs is size, shape and surface characteristics dependent.

Kniepp et al. reported the first attempt to utilize the GNPs as nanoprobes in living cells [77]. The same group reported their later studies demonstrating the possibility of the approach by providing the molecular level information from the inside of a single living cell [78]. These early studies demonstrated the possibility of using SERS for cellular studies.

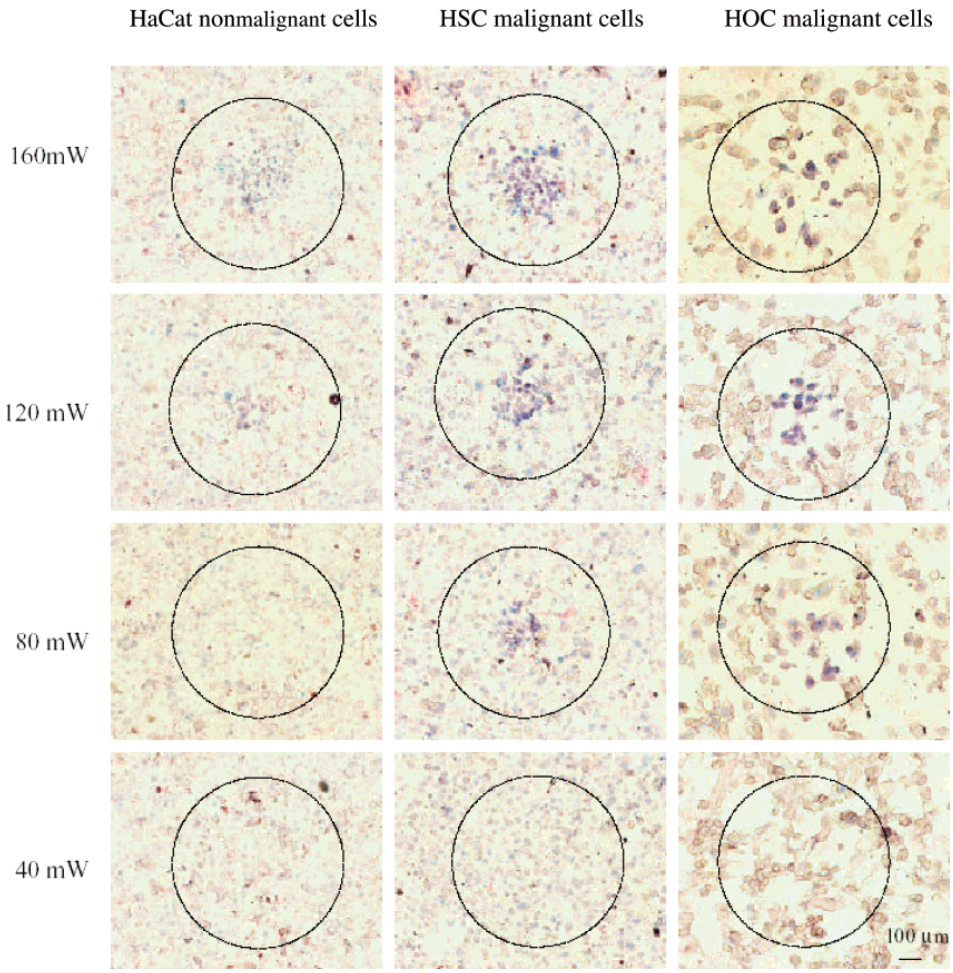


Figure 7. Cells exposed to laser light at increasing power. Reprinted with permission from Ref. 71. Copyright @American Chemical Society.

In this report, we discuss some of our results as an example. The nanoparticles used in the SERS study were 13 nm GNPs and their uptake by living cells was adequate. The SERS enhancement of the 13 nm GNPs are not satisfactory due to the poor SPs formation at this size. However, the reasonably good SERS spectra from the GNPs introduced cells were obtained. This can be explained with the formation of loosely bound gold nanoparticles inside the living cells. As the concentration of GNPs is increased in the media, the entry of more GNPs into the cells was observed. The careful inspection of the image on Figure 2 reveals that the GNPs are indeed forming aggregates in the cell. This must be dependent on the concentration of GNPs in the media. Because of its ultra-high sensitivity, SERS is a potential technique for biological analysis. [79]. There are several applications of SERS in cellular activities such as intracellular pH monitoring [80], identification of microorganisms [81-84], enzyme-drug interactions [85] or all cellular component analysis [77]. One of the important questions arising as the GNPs enters into

the living cells is their possible destinations and the molecular structures in contact with them. It is our ongoing effort to understand the localization of the gold nanoparticles and their contact structures in the cell. The first structure we are interested in is mitochondria that might come into contact with GNPs due to their vital function in many biochemical processes in the cell. Although there is no indication that GNPs has vital influence on living cells *in vitro* experiments, there is a recent study indicating more than thirty genes are activated upon interaction of GNPs with living cells [68]. Along with SERS, we have utilized Confocal Laser Microscopy to understand the localization of GNPs and their possible contact structures. Figure 8 shows the confocal microscopy image of the GNPs and stained mitochondria. Although the aggregates of GNPs do not seem in contact with the mitochondria, the imaging method may not show single nanoparticles in contact with mitochondria membrane. Since the SP formation wavelength shift to longer wavelength as the aggregates formed, a laser at 633 nm was used for the visualization of GNP aggregates. The surface plasmon excitation wavelength for a colloidal suspension containing 13 nm GNPs is 520 nm. This means that the imaging methodology used here does not give information about the GNPs widely separated in the cell.

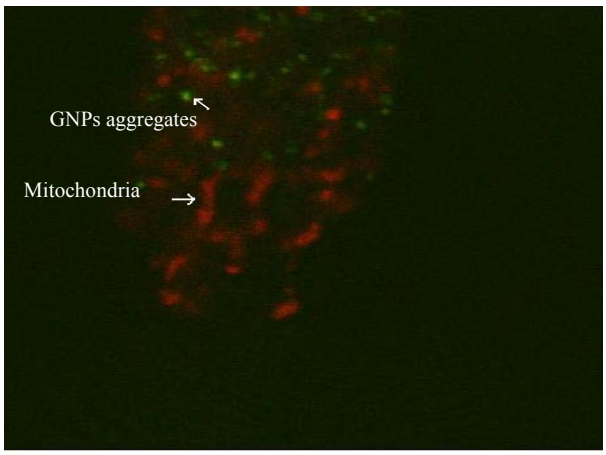


Figure 8. The localization of the nanoparticle aggregates with respect to mitochondria.

Figure 9 shows the SERS spectra obtained from three different cell lines. Each band on the spectrum belongs to a bond vibration originating from a macromolecule contact in contact with GNPs in the cell. It should be noted that each band could be originating from a number similar bond in the same molecule or different molecules. For example, the origin of the band at 649 cm^{-1} could be amino acid side chain containing thiol group or C-C twisting of a protein. The band at 1129 cm^{-1} is possibly the result of C-C stretch in fatty acids that is a part of cellular membrane lipids. The band at 1349 cm^{-1} can be assigned to cholesterol and again to fatty acids. Considering the passage of the GNPs through the cell membrane, it is evident that the GNPs are strongly interacting with membrane components. As it is seen, a careful inspection of SERS spectra obtained from cells give some clues about the molecules and molecular structures in contact with the GNPs.

Since SERS can provide information about the cellular environment of gold nanoparticles, we have further investigated whether the SERS can differentiate cell types or cancer cell lines based on their molecular level differences. Figure 9 represents the SERS spectra of three different cancer cell lines; A549 lung cancer, HeLa, cervix cancer, and AB, brain tumor cells. The spectra were acquired from whole cells using a lower magnification objective to

generate a large size of laser spot to cover as large as possible area on the cell. The spectrum for each cell is the average of the ten spectra. The spectral differences are marked on the figure. Although more study is necessary on the concept, the preliminary data shows that it is possible to distinguish cell types easily.

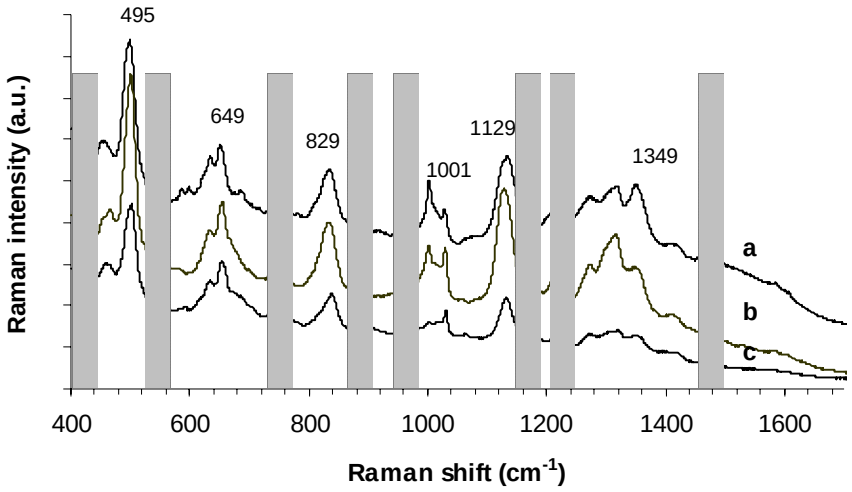


Figure 9. SERS spectra of different cell lines. a) A549 lung cancer, b) HeLa, cervix cancer, c) AB, brain tumor.

4. CONCLUSIONS

The use of GNPs in medical applications is finding increasing use due to their assumed non-toxicity, easy surface chemistry and plasmonic properties. They can be used for delivering drugs or genes, selective killing of cancerous cells, and probing the cellular processes at molecular level. The first issue for their use in medical applications is the toxicity concerns and their effect in long term. As a general problem for all kinds of nanoparticles and nonostructures, there are several questions regarding their possible effects on living organisms from cellular level to higher organizations such as human being. In this extent, there is an increasing worldwide effort to clarify the issues related GNPs' use in many medical related fields.

In order to gain information about the distribution of nanoparticles in the cell, the use of fluorescence imaging and visualization of SPs of GNPs and their aggregates can be utilized. When the images of mitochondria and SPs are overlaid, the localization of GNP aggregates with respect to mitochondria is visible. Due to the fact that only certain size of clusters can be observed, the smaller aggregates formed from a few nanoparticles or individual nanoparticles are not easily observed. As could it be concluded from the dilution and size studies, that only the size of GNPs are small enough and its concentration low enough to prevent extensive aggregation has an influence on the cellular biochemical activities. This supports the idea that, in addition to the surface charge, the size of the nanoparticles is critical for their interference with the biochemical processes. The degree of aggregation of the GNPs in cancerous and healthy cells could be different due to the significantly different content of ionic species in both cells. This may be another effect on the cancerous cells. The localization of the nanoparticles were examined and found that they form aggregates around or close to mitochondria in the cells. Because single nanoparticles couldn't be observed with confocal microscopy under these experimental

conditions, the specific interaction of nanoparticles with mitochondria cannot be examined. Additionally, the cell culture studies with control cells showed the influence of nanoparticles on the morphology of cancerous cells while no detectable influence was observed on morphology of immortalized healthy mouse fibroblast cells. A549 cells started to form neurites that are the sign of slowing down of growth and apoptosis. Therefore, the GNPs are showed to be effective against A549 small cell carcinoma cells. In further experiments different cancerous cultures will be used to study the influence of gold nanoparticles on cancer cells. SERS studies show that not only molecules and molecular structures in contact with gold nanoparticles can be elucidated but also the cell types can be differentiated.

Acknowledgement

The financial support provided by Yeditepe University and TUBITAK is gratefully acknowledged for the work partly presented here.

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