

Investigation of the Photothermal Heating Potential of Small Superparamagnetic Iron Oxide Nanoparticles

by

Kübra Bilici

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Superparamagnetic Iron Oxide Nanoparticles**

Koç University

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This is to certify that I have examined this copy of a doctoral dissertation by

Kübra Bilici

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Havva Yağcı Acar, Ph.D.

Mehmet Kaya, Ph.D.

Uğur Ünal, Ph.D.

Duygu Avcı Semiz, Ph.D.

Mehmet Burçin Ünlü, Ph.D.

Date: _____



Dedicated to my family

ABSTRACT

Superparamagnetic iron oxide nanoparticles (SPIONs) are the most studied nanoparticles for biomedical applications due to its FDA approved formulations and super biocompatibility. They are clinically used as a contrast agent in magnetic resonance imaging (MRI). They have also provided successful results in magnetic dragging and magnetic hyperthermia owing to their strong response to external magnetic field. SPIONs may be prepared with different surface coatings and in various sizes and hence, may be utilized for gene and drug delivery as well as for targeted delivery to diseased sites or cells for specific medical imaging and therapy. SPIONs are also utilized in non-medical fields such as cell sorting, magnetic damping or magnetic refrigeration.

In addition to these application areas, there has been a various number of reports showing that SPIONs may be effective sensitizers in photothermal therapy (PTT). Such potential of SPIONs is very exciting for people working with SPIONs in the field of biotechnology and medicine since this means another way of utilizing SPIONs in PTT which is a local and less invasive treatment for cancer and bacterial infections. SPIONs has an important role in PTT studies because they induce temperature increase when they are irradiated with 808 and 1064 nm laser. Yet, the origin of the photothermal effect in case of SPIONs is still not clearly understood. A systematic study was not ever performed to understand the mechanism. It has been suggested that this effect depends on the absorption and hence to agglomerate sizes; however, SPIONs do not have any strong absorbance at these wavelengths. The agglomerate sizes are not appropriate for in *in vivo* and clinical studies.

In the first part of this thesis, PTT potential of SPIONs was investigated based on irradiation wavelength, laser power and laser intensity. Influence of laser irradiation on the properties of SPIONs were also determined. The dependence of the temperature

increase on the laser irradiation wavelength was tested over 728-838 nm with 10 nm steps and it was found that there is no relation between temperature increase and attenuation within this wavelength range. This result suggested that longer wavelength which provides deeper penetration could not cause any temperature loss. Intensity dependent experiments, where the power was kept constant and the spot size was changed, indicated that the temperature increase does not depend on the laser intensity. But the temperature increase changed with the laser power. Hence, factors affecting the temperature increase were determined. These laser treatments did not provide even 1 °C temperature increase in water within this irradiation wavelength range. This suggested that these laser powers are clinically applicable and safe in the absence of SPIONs. Detailed characterization showed that there is no change on particle properties. Lastly, PTT using small and stable SPIONs were successfully achieved on HeLa cells.

Combination of PTT with other techniques enhanced the therapeutic outcome. Photodynamic therapy, another type of phototherapy, is widely used in clinical applications. Combined PTT and PDT became a very popular in recent years. It is very valuable to perform combined therapy with single irradiation at the same wavelength. In the second part of the thesis, the combined therapy potential of indocyanine green (ICG) and 3-aminopropyltrimethoxysilane (APTMS) coated SPIONs were investigated. ICG was electrostatically loaded to SPIONs and its phototherapy experiments were performed with single irradiation at 795 nm (700 mW) for 10 min. The therapeutic effect of PTT, PDT and combined PTT+PDT was examined on both colon (HT29) and breast (MCF7) cancer cells. PTT experiments with APTMS@SPIONs did not induce any important cell death at these conditions; however, it caused mild hyperthermia. PDT experiments performed with ICG caused more cell death in MCF7 cells compared to HT29 cells. Combined therapy with ICG-APTMS@SPIONs caused near complete cell death on both cell lines with a single laser treatment.

In recent years, phototherapy emerged as an effective option in fight against bac-

terial infections. In the third part of this thesis, the antibacterial activity of free ICG, APTMS@SPIONs, and ICG-APTMS@SPIONs was tested on both planktonic cells and biofilms of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus epidermidis* under laser irradiation. An important growth inhibition was observed on both planktonic cells and biofilms after laser treatment, proving use of SPION based PTT with or without other photosensitizers as an effective antibacterial treatment method.

Chemotherapy is widely used in cancer treatment; however, poor drug accumulation and serious side effects limit the effective therapy. Encapsulation of drug into nanoparticles usually enhances the therapeutic outcome. Further improvement may be achieved via combined chemotherapy and PTT. In the fourth part of this thesis, etoposide (Eto) was electrostatically loaded to BSA@PAA@SPIONs to treat three different prostate cancer cells (PC3, LNCaP, and DU145) with and without laser treatment. High drug accumulation was achieved with nanoparticles and an important cell death was observed as a function of NP dose and laser power.

Overall, this thesis aimed to investigate the factors affecting the photothermal potential of small SPIONs and combine the PTT potential of SPIONs with other therapeutic methods to provide enhanced therapeutic outcomes.

ÖZETÇE

Süperparamanyetik nanoparçacıklar (SPION) klinikte onaylı formülasyonlarının bulunması ve yüksek biyouyumluluğu nedeniyle biyomedikal uygulamalarda en çok çalışılan parçacıklardır. Manyetik sahaya yanıt verebildiği için manyetik rezonans görüntüleme (MRI), manyetik yönlendirme ve manyetik hipertermi uygulamalarında çok başarılı sonuçlar sağlamıştır. SPION'lar farklı yüzey kaplaması ve büyüklükte hazırlanabilmekte, gen ve ilaç gibi terapötik ajanları taşıyabilmekte, hatta ligand konjugasyonu ile spesifik olarak hastalıklı hücre/dokulara medikal görüntüleme ve tedavi için gönderilebilmektedir. SPION'lar *in vivo* uygulama gerektirmeyen manyetik hücre ayırma, manyetik titreşim sönümleme, manyetik soğutma gibi uygulamalarda da çalışılmaktadır.

Bu alanlardaki çalışmalara ek olarak, yakın zamanda SPION'ların fototermal terapi (PTT) uygulamasında da umut vaat edici ve etkili bir ajan olduğunu gösteren çalışmalar yapılmıştır. Son derece biyouyumlu SPION'ların PTT gibi lokal ve sağlıklı dokuya zarar vermeden minimal yan etki ile kanser ve bakteri tedavisinde etkili olabilen bir yöntemde kullanılabilmesi medikal sahada büyük heyecan yaratmıştır. SPION'lar yakın kızılötesi dalga boylarında (808 nm ve 1064 nm) uyarılarak lokal sıcaklık artışı yaratabildikleri için PTT sahasının önemli bir ihtiyacını da karşılamaktadır. Ancak yapılan çalışmalarda fototermal etkinin kaynağı tam incelenmemiş, etkinin incelendiği yayınlarda ise sistematik bir yaklaşım çok benimsenmemiş ve bu etkinin kaynağı tam anlaşılamamıştır. Bu etkinin absorpsiyona bağlı olabileceğinden ve nanoparçacıkların topaklanması ile güçlenebileceğinden bahsedilmiştir. Ancak, bu bahsedilen dalgaboylarında SPION'ların çok güçlü bir absorpsiyonu görülmemekle birlikte topaklaşma da *in vivo* ve klinik çalışmalarda biyodağılımı değiştireceği için istenmeyen bir oluşumdur.

Bu tez çalışmasında öncelikle kararlı, ufak (100nm ve altında) ve yüzey fonksiyonel (amin ve karboksilik asit) SPION'ların PTT potansiyeli uyarı dalgaboyu, lazer gücü ve şiddetine bağlı olarak incelendi. Lazer uygulamasının nanoparçacıklarda herhangi bir değişikliğe neden olup olmadığı incelendi. Sıcaklık artışının dalga boyuna bağımlılığının incelendiği deneyler 10 nm aralıklarla 728-838 nm aralığında yapılmış ve sıcaklık artışının uyarılan dalga boyundaki sönümlenmeden bağımsız olduğu elde edilmiştir. Bu sonuç 728-838 nm aralığında daha yüksek penetrasyon sağlayan ve daha az zararsız uzun dalga boylarının kullanılmasının herhangi bir sıcaklık kaybına neden olmayacağını göstermiştir. Lazer gücü sabit tutulup hüzmeye yarıçapının değiştirilip böylece elde edilen farklı lazer güçlerinde yapılan deneyler meydana gelen sıcaklık artışının uygulanan lazer şiddetinden bağımsız olduğunu göstermiştir. Ancak uygulanan lazer gücü değiştiğinde sıcaklık artışının değiştiği elde edilmiştir. Böylece SPION'lar lazer ile uyarıldığında sıcaklık artışını etkileyen faktörler belirlenmiş ve literatüre SPION PTT mekanizmasının anlaşılmasına katkı bulunacak sonuçlar kazandırılmıştır. Kontrol olarak kullanılan suyun sıcaklığı uyarılan herhangi bir dalga boyu ve gücünde 1 °C bile artmamıştır. Böylece çalışmada kullanılan dalga boylarının ve güçlerin klinik olarak son derece uygun olduğu görülmektedir. Lazer sonrası nanoparçacığın yapısında meydana gelen değişimler farklı karakterizasyon yöntemleri ile incelenmiş ve parçacığın lazer sonrası kararlı yapısını koruduğu görülmüştür. Son olarak hücre çalışması HeLa hücre hattında başarılı bir şekilde gerçekleştirilmiştir.

Tek başına PTT çalışmalarının yanı sıra PTT nin diğer tedavi yöntemleriyle birlikte kullanılması son derece etkili sonuçların elde edilmesine imkan sağlamaktadır. Bir diğer fototerapi yöntemi olan fotodinamik terapi (PDT) klinik uygulamalarda sıkça kullanılmaktadır. Bu iki fototerapi yönteminin birlikte uygulanması son yıllarda popüler bir yöntemdir. Bu kombine tedavinin tek bir dalga boyunda tek bir güçte yapılmasını sağlayacak PDT ve PTT ajanlarının birleştirilmesi çok kıymetlidir. Bu amaç ile tez çalışmasının ikinci aşamasında klinik uygulamada sıkça kullanılan PDT ajanı indosiyanin yeşili (ICG) ile 3-aminopropiltrimetoksisilan kaplı SPION'nun birleş-

tirilerek kombine fototerapi potansiyeli incelendi. ICG elektrostatik olarak SPION'lara (ICG-APTMS@SPION) yüklenmiş ve fototerapi deneyleri 795 nm dalga boyunda tek bir uyarılma ile gerçekleşmiştir. Böylece ICG'nin kararlılık sorunu SPION'lara yüklenmesiyle aşılmıştır. Tek başına PDT, tek başına PTT ve kombine PTT+PDT terapinin başarısı kolon (HT29) ve göğüs kanseri (MCF7) hücrelerinde incelenmiştir. APTMS@SPION kullanılarak yapılan PTT deneylerinde her iki hücre hattında da önemli bir ölüm meydana gelmeyip sadece hafif hipertermi gerçekleşmiştir. Tek başına ICG, PDT deneyleri sonucunda HT29 hücresine kıyasla MCF7 hücre hattında daha çok ölüm meydana getirmiştir. ICG-APTMS@SPION lazer ile uyarılmasıyla yapılan kombine tedavi ile iki hücre hattında da tümör hücreleri hemen hemen tümüyle yok edilmiştir.

Kanser tedavisinin yanı sıra nanoparçacıkların lazer uygulaması bakteri çalışmalarında da önem kazanmıştır. Bu amaçla ICG, APTMS@SPION ve ICG-APTMS@SPION'ın lazer altında antibakteriyel potansiyeli hem planktonik hem biofilm *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* ve *Staphylococcus epidermidis* bakterilerinde test edilmiştir. Lazer uygulaması sonrası hem planktonik hem biofilm seviyede önemli inhibisyonlar gözlemlenmiştir.

Kemoterapi kanser tedavisinde sıkça kullanılmasına rağmen ilacın istenilen bölgede birikiminin az olmasından dolayı istenilen etkili sonuçlar alınamamaktadır. İlaçların bir nano taşıyıcı ile birleşmesi yan etkileri azaltarak daha az dozda daha verimli bir tedaviye imkan sağlayacaktır. Buna ek olarak kemoterapinin PTT gibi lokal bir yöntemle birleşmesi tedaviyi daha etkili kılacaktır. Bu amaçla prostat kanseri tedavisinde sıkça kullanılan etoposide (Eto) elektrostatik bağlanma ile BSA konjuge edilmiş PAA@SPION'lara yüklenmiştir. Üç farklı prostat kanseri hücre hattında (PC3, LNCAP ve DU145) yapılan toksisite deneyleri ilaç taşımının nanoparçacıklarla başarılı bir şekilde gerçekleştiğini göstermiştir. Lazer uygulaması sonrasında ise güce bağlı olarak tümör hücrelerinde önemli ölüm elde edilmiştir.

Özetle, bu tez SPION'ların PTT mekanizmasını etkileyen faktörlerin incelen-

mesini ve farklı kaplamalı SPION'ların PTT uygulamasının diğ er terapi y ntemleriyle birleřtiğinde daha etkili olduğunu g stererek literat re daha etkili ve zararsız tedavi imkanları saėlamayı hedeflemektedir.



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NOMENCLATURE

APTMS	3-aminopropyltrimethoxysilane
B	Magnetic induction
DAPI	4',6-diamidino-2-phenylindole
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DU145	Prostate cancer
EDC	Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EtOH	Ethanol
Eto	Etoposide
FBS	Fetal Bovine Serum
Fe ₂ O ₃	Maghemite
Fe ₃ O ₄	Magnetite
H	Magnetic Field
HeLa	Human Cervical Carcinoma
HT29	Colon cancer
ICG	Indocyanine green
ICP-MS	Inductive coupled-mass spectroscopy
k _b	Boltzman constant
kDa	Kilo Dalton
LNCAP	Prostate cancer
M	Magnetization
MES	2-(N-morpholino)ethanesulfonic acid)
MCF7	Breast cancer
MRI	Magnetic resonance imaging

NH ₄ OH	Ammonium hydroxide
NIR	Near InfraRed
PAA	Polyacrylic acid
PBS	Phosphate buffer saline
PDT	Photodynamic therapy
PTT	Photothermal therapy
PC3	Prostate cancer
SPION	Superparamagnetic iron oxide nanoparticle
Sulfo-NHS	Sulfo-N-hydroxysulfosuccinimide
TEM	Transmission Electron Microscopy
T1	Longitudinal, spin – lattice relaxation
T2	Transverse, spin-spin relaxation
UV	Ultra Violet
VSM	Vibrated sample magnetometry
XPS	X-Ray Photoelectron Spectroscopy
ΔT	Temperature increase
Γ_N	Neel relaxation time
Γ_B	Brownian relaxation time
$\Delta\mu$	Supersaturation
μ	Magnetic dipole moment

Chapter 1

INTRODUCTION

1.1 Magnetism at the Macroscopic Level

Magnetism can be explained as movement of the charged particles through a space or the motion of electrons in an atomic orbital. To understand basics of magnetism, magnetic field should be defined. It is an energy gradient which produces a force detected by the acceleration of an electrical charge moving in the field, by the torque on a magnetic dipole or by the reorientation of the electron spins of atoms. Behaviour of magnetic materials under magnetic field can be explained with the plot of magnetic induction B as a function of applied magnetic field H at various strengths. Magnetic induction is defined as $B = \mu_0(H + M)$ where μ_0 represents the permeability of free space and M is the magnetization of material. Information about the magnetic properties of materials is obtained from M versus H loop which is called as hysteresis loop. Hysteresis loops explain the magnetization behaviour of the magnetic materials under applied magnetic field (Figure 1.1). It contains some important parameters in magnetism such as saturation magnetization, remanence and magnetic susceptibility. The achieved highest magnetization at certain temperature is called as saturation magnetization. Remanence term is used to define the remaining magnetization inside the material after removing magnetic field. The ratio of magnetization over magnetic field represents magnetic susceptibility which determine the magnetization capability of materials. Temperature plays an important role on the magnetization. Demagnetization can occur in magnetically ordered specimen at elevated temperature. This critical temperature is called as Curie temperature [18, 19].

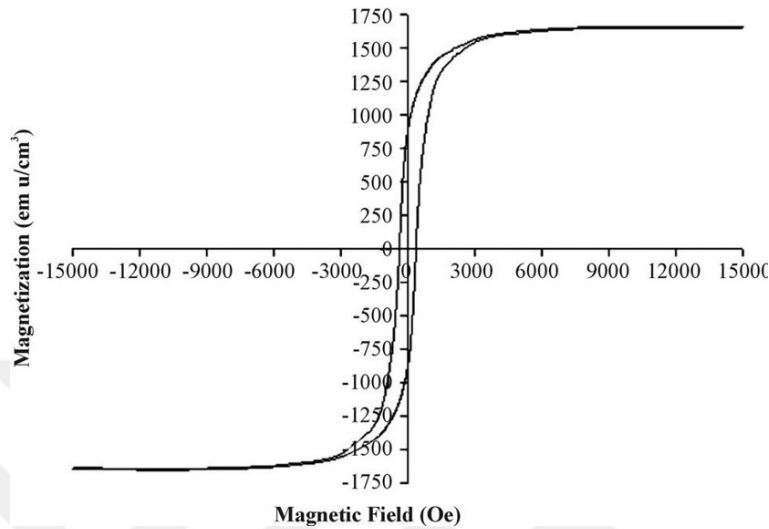


Figure 1.1: Magnetic hysteresis loop of a typical iron film [1].

1.1.1 Classes of Magnetic Materials

Magnetic materials can be classified under five categories based on their susceptibility to magnetic field: diamagnetism, paramagnetism, ferromagnetism, anti-ferromagnetism and ferrimagnetism. Diamagnetism occurs from the non-cooperative orbiting behavior of electrons under magnetic field which can be found in all matters. Applied magnetic field on a single electron orbit causes a reduction on effective current of the orbit which creates an opposing magnetic moment to magnetic field. Diamagnetic materials have a negative susceptibility ($\chi < 0$) and weakly repel an applied magnetic field. In terms of the electronic configuration of the materials, diamagnetism is observed in materials with filled electronic subshells where the magnetic moments are paired and overall cancel each other. All other types of magnetic behaviors are observed in materials that are at least partially attributed to unpaired electrons in their atomic shells, often in the 3d or 4f shells of each atom. In the case of paramagnetism, atomic moments are randomly oriented in the absence of magnetic field. These atomic moments are aligned with the applied field which creates a magnetization in material. However, their susceptibility is weak due to thermal agitations and magnetization disappears when magnetic field is removed. Materials whose atomic

magnetic moments are uncoupled display paramagnetism; thus, paramagnetic materials have moments with no long-range order, and there is a small positive magnetic susceptibility ($\chi \approx 0$). Therefore, magnetization of paramagnetic material is affected by temperature. Ferromagnetic materials possess a large magnetization below critical temperature even in the absence of magnetic field due to strong interaction between electron spins. However, ferromagnetic materials exhibit paramagnetic nature above Curie temperature. Iron, cobalt and nickel are the most common ferromagnetic materials. Antiferromagnetic materials comprise of two or more equivalent sub-lattices which are aligned antiparallel to each other below transition temperature. Oxides, sulfides and chlorides are the typical examples for antiferromagnetic materials. Ferromagnetic material has spontaneous magnetization and it acts as a paramagnet above a certain critical temperature losing its magnetization [19, 20].

1.2 Magnetism at the Microscopic Level

Magnetization depends on the electron-electron interactions in solids at the microscopic level. In bulk magnets, isotropic spin alignment is only present in small portions of the material which is called as magnetic domain instead of the whole material. Magnetic properties of the material is heavily affected by the size of each domain. Each domain generates a magnetic moment by spontaneous magnetization. Ferromagnetic materials consist of a variety of magnetic domains which are i) spontaneously magnetized due to the exchange interaction between spins or ii) aligned parallel to each other under magnetic field.

The important point in the ferromagnetic material, magnetization remains even after the magnetic field is removed and it causes a hysteresis in the B-H loop. In the case of paramagnetic materials, magnetization is weak in when compared to ferromagnetic materials. Due to low number of magnetic dipoles, paramagnetic materials loose its magnetization in the absence of magnetic field which causes no hysteresis in their B-H loop. Overall, these findings suggest that magnetic properties of materials can be totally explained by magnetic domains at the microscopic level [18, 19, 20].

1.2.1 Superparamagnetism

The magnetization capability was affected by two fundamental energy: i) exchange energy arising from Pauli Exclusion Principle and producing ferromagnetic coupling ii) thermal energy producing the spin relaxation [21]. The destruction occurs in ferromagnetic alignment when the exchange energy is not high enough as thermal energy. In the case of a reduction a number of atoms per domain, the interaction energy could reach the level of thermal energy which causes a spontaneous random orientation of electron spins. This means, there is no magnetization at zero field. The important point is the number of atoms per domain here, and it happened when the size changed from bulk to nano. When the remanent magnetization of ferromagnets is cancelled at zero field, materials act as a paramagnetic material. This phenomena is called as superparamagnetism which is achieved with less than 20 nm size of iron oxide or almost 3 nm pure iron [22]. Superparamagnetism possess properties of both paramagnetism and ferromagnetism and is achieved below Curie temperature (Figure 1.2). Size has an important and dominant effect on magnetic behavior of superparamagnet. When the nanoparticle size is less than 20 nm in a superparamagnetic material, there is no any magnetization due to thermally induced spin relaxation. Nanoparticles act as a single domain at 30 nm with some remanent magnetization. In the case of size of 80 nm, nanoparticles reach the highest coercivity possessing multi-magnetic domains [23]. In the presence of magnetic field, magnetic and Stoke's forces act on a magnetic substance. Magnetic force can be written as following which causes the acceleration of the particles:

$$F_m = \frac{4}{3}\pi r_m \nabla(\vec{M}(z)\vec{B}(z)) \quad (1.1)$$

r_m , M , and B represents radius of magnetic part of the particles, magnetization, and magnetic flux, respectively. Stoke's force can be defined by following equation

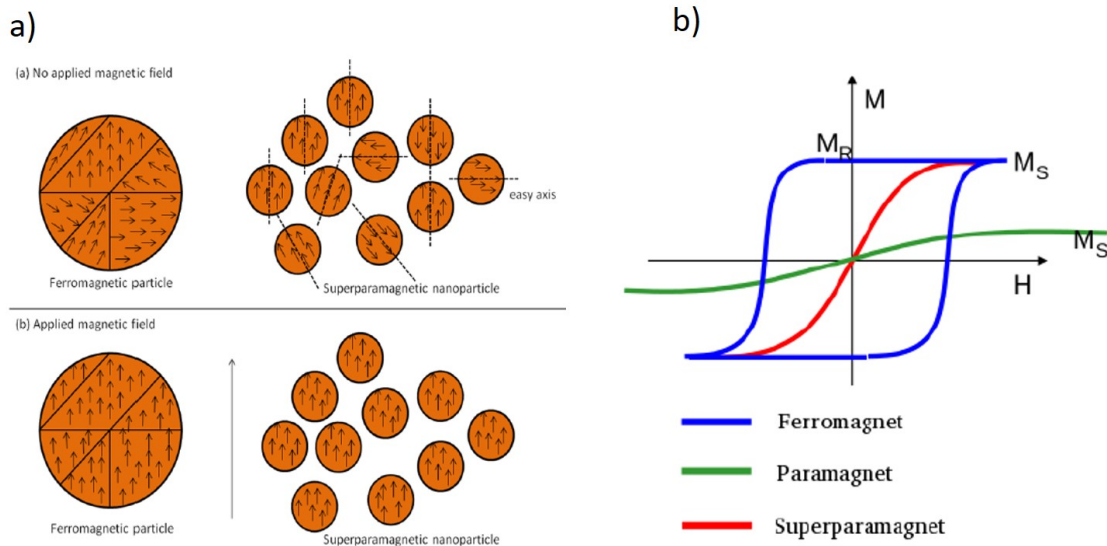


Figure 1.2: a)Magnetic domains in ferromagnets and superparamagnets at applied and zero magnetic field [2] and b)representative magnetization curves for ferromagnetism, paramagnetism and superparamagnetism.

$$F_s = 6\pi\eta r_h \nu \quad (1.2)$$

η , r_h , and ν represents viscosity of liquid, hydrodynamic radius of the particles, and velocity of the particle, respectively. Based on Equation (1.1) and (1.2), hydrodynamic radius of the particle could be larger than magnetic radius when nanoparticles coated with organic ligands. Coating has play an important role in colloidal stability. To prevent the attractive forces and obtain colloidal stability, repulsive forces should be presence in carrier liquid which highly depends on the ionic strength, pH, and coating types.

1.3 Superparamagnetic Iron Oxide Nanoparticles

Studies in the mid-20th century showed that materials switch to single domain phase from multi-domain phase at nanometer scale which causes an increase in the coer-

cive force. SPIONs are small size crystalline iron oxides which consist of a single magnetic domain [24, 25]. Two type of relaxation are presence in colloidal SPIONs: Brownian relaxation and Neel relaxation. Brownian relaxation can be defined as $\Gamma_B = 3\nu\eta_0/kT$ which presents viscous rotation of nanoparticles (ν =volume of the particle, η_0 =viscosity of the liquid, k =Boltzmann constant and T =temperature)[26]. Neel relaxation can be called as necessary time to return equilibrium of the magnetization after perturbation and written in following equation: $\tau_N = \frac{h}{k} \ln 2 \frac{K}{k_B T} \frac{1}{f_0}$ (K=anisotropy constant and f_0 =Larmor frequency [27]. Magnetite and maghemite are the two main crystal structures of SPIONs. In SPIONs, magnetic moment are composed by unpaired 3d electron in Fe^{3+} and Fe^{2+} cations. Electron spin coupling between Fe^{3+} and Fe^{2+} occurs at the octahedral sites and antiparallel couplings happen at tetrahedral sites. Magnetic behavior of SPIONs is correlated with superexchange interactions which is defined as exchange interaction/coupling between these octahedral and tetrahedral sites across oxygen anions. Inclination effect/spin canting effect that is tendency of the surface atomic spins of magnetic nanoparticles to a particular angle destroys superexchange interactions at the surface of nanoparticles. The spin canting effect is occurred by (i) the absence of necessary number of atoms for the formation of single magnetic moment and (ii) lack of organized spins at the surface in comparison with the SPION core. Cationic vacancies, edge roughness, and oxygen vacancies also affect the magnetic behavior of SPIONs.

1.3.1 The Relationship Between Magnetic and Physical Properties of SPIONs

In SPIONs, the decrease in the size causes reduction of saturation magnetization while it increases the spin canting effect. The following equation presents the relationship between the size, spin-canted surface layer and magnetization:

$$m_s = M_s[(r - d)/r]^3 \quad (1.3)$$

M_s and m_s , r , and d describe the saturation magnetization of bulk materials and size reduced particle, the size of nanoparticle and thickness of the disordered surface layer, respectively. Based on the previous reports, spin canted surface layer thickness of maghemite should be 0.5-0.9 nm [28, 29, 30]. Kim et. al showed that 3 nm-sized iron oxide nanoparticles have lower magnetization value than 12 nm-sized iron oxide nanoparticles due to size and spin canting effect. Assuming spin canting layer thickness as 0.9 nm [29], they reported 93.6 % and 38.6 % spins were canted in 3 nm-sized and 12-nm-sized nanoparticles, respectively [31]. Due to reduction in size, 5 nm sized Fe_3O_4 nanoparticles showed low magnetization value which is 27 emu/g [32].

In addition to surface spin canting, volume canting is also induced in magnetic nanoparticles due to Fe vacancies [33, 34]. Baaziz et. al studied the volume and surface canting effects on 5, 8, 11, 15, and 20 nm-sized nanoparticles. They investigated that a surface canting layer thickness of one and two atomic layer is presence for 5 nm and 8 and 11 nm, respectively while canting layer thickness fell rapidly nanoparticles which have size larger than 11 nm. For 8 and 11 nm sized nanoparticles, both surface and volume spin canting occur in spin canting effect [35]. Another study showed that doping with other atoms such as gadolinium occurred both volume and surface spin canting effects in magnetic nanoparticles as well [36].

To prevent agglomeration, organic/inorganic surfactants/capping agents which have different end functional groups such as $-\text{OH}$, $-\text{COOH}$, $-\text{PO}(\text{OH})_2$, $-\text{S}(=\text{O})_2-\text{OH}$ are used as protective layer around SPIONs via electrostatic interactions or covalent bonding [37]. The thickness of this protective layer is between 1-5 nm and more than 100 nm for small organic molecules and large polymers, respectively. When $-\text{OH}$ functional polyoxyethylene (5) nonylohenylether was used as a surfactant, no significant change was observed on the magnetic properties in SPIONs [38]. However, a reduction was occurred in surface spin canting after polyoxyethylene (5) nonylohenylether coating that is confirmed by canting angle measurements of SPIONs. Roca et. al reported that oleic acid which is at the particle surface occurred enhancement in the

surface canting effect in SPIONs making the octahedral positions more susceptible to be canted than tetrahedral positions [39]. Size dependent study with 6-20 nm sized SPIONs by Guardia et. al showed that oleic acid provided high magnetization to nanoparticles causing a reduction in surface spin disorder [40]. Phosphonic acid (-PO (OH) 2) functional groups provided more thermal stability and enhanced binding affinity creating Fe-O-P bond via an extra oxygen atom in their structure [41, 42]. Shape has also an important role to control magnetic properties of SPIONs. The study with solid nanospheres and solid and hollow nanoellipsoids nanoparticles showed that ellipsoids nanoparticles have lower magnetization than spherical nanoparticles due to enhanced surface spin canting effects [43]. Comparative study with different geometries on magnetic behaviors revealed that higher magnetization values were observed for facet, twins, and precipitates than spherical SPIONs which is attributed to increased coupling of atomic magnetic spins [44]. Mixed shaped SPIONs which are comprise of diamond, cubic and triangular-shaped particles have comparative saturation magnetization to bulk sample [40]. Overall, magnetic properties of SPIONs are affected by size, shape and surface coating which can be controlled with synthesis methods. Table 1.1 shows the magnetization values of SPIONs based on size, shape and surface coating from different studies.

Table 1.1: Size, shape, surface coatings and magnetization values of various SPIONs.

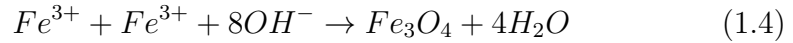
Size (nm)	Shape	Surface coating	Magnetization (emu/g)	Reference
22	Octahedral	Uncoated	80	[45]
100	Trisoctahedral	Gold	20	[45]
13.1 $\pm$ 0.3	Mixed shapes	Mannose	59.1	[46]
9.7 $\pm$ 0.99	Mixed shapes	Maltose	37.4	[46]
3.8 $\pm$ 0.21	Mixed shapes	Lactose	22.02	[46]
12.4 $\pm$ 0.3	Mixed shapes	Galactose	58.08	[46]
14 $\pm$ 4	Spherical	Uncoated	74.9	[47]
18	Spherical	Oleic acid	67	[48]

1.3.2 Synthesis Methods of SPIONs

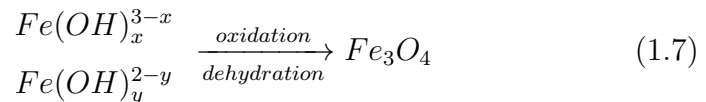
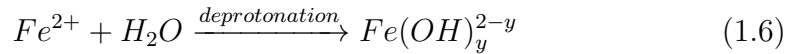
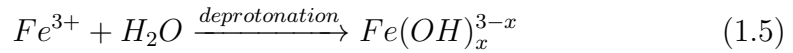
To synthesize SPIONs, physical, wet chemical, and microbial methods have been performed for many years. Wet chemical methods, coprecipitation, thermal decomposition, microemulsion, hydrothermal, and sonochemical methods, are highly preferable for SPION synthesis due to better control on size and surface characteristics [49, 50, 51, 52].

Co-precipitation method

Co-precipitation method is simple and effective approach where nanoparticles are prepared using mixture of ferric (Fe^{3+}) and ferrous (Fe^{2+}) salts and base at moderate temperatures under a non-oxidizing environment [53]. The reaction mechanism can be defined in following equation [54, 55]:

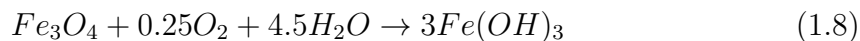


The detailed reaction process can be written in below equations:



Massart et. al optimized pH values (between 9 and 14) and molar ratio of

$[\text{Fe}^{3+}]:[\text{Fe}^{2+}]$ (2:1) in the absence of oxygen to obtain stable aqueous magnetic nanoparticles [56]. Otherwise, Fe_3O_4 could be oxidized as follows:



Synthesis parameters such as reaction time, base molarity, stirring rate and type of base have play an important role in the magnetic properties of SPIONs [57, 58, 59, 60].

Thermal decomposition method

Thermal decomposition method produces highly crystalline and monodisperse SPIONs using surfactants such as oleic acid and oleyl-amine and organic solvents at high boiling points. However, hydrophobic nature of these SPIONs limits their usage in biomedical applications. Therefore, ligand exchange method and bilayer surfactant stabilization method are used to transfer them into aqueous phase [61, 62]. Xu et al. synthesized Fe_3O_4 nanoparticles with hydrophobic coating of oleate and oleyl-amine and then functionalized using combination of polyethylene glycol (PEG) and dopamine via ligand exchange method to perform MRI study [63]. On the other hand, many properties of SPIONs such as magnetic properties and colloidal stability got effected during the phase change [64]. Therefore, Maity et al. suggested one-pot thermolysis method which uses polyol based surfactants to prevent these issues [65].

Hydrothermal method

This method is performed to obtain SPIONs with different shapes and sizes at high temperature and pressure where precursors were dissolved in an aqueous solution through surfactants and capping agents. The obtained SPIONs by this method possess poor crystallinity when compared to thermal decomposition method [66].

Microemulsion method

SPIONs can be synthesized using two immiscible phases (oil and water) with stabilizing agents. Water in oil (W/O) is one of the frequently used microemulsion method which stabilizes agents in continuous oil phase to produce SPIONs [67].

Sonochemical method

Ultrasound method generates SPIONs through cavitation of bubbles at ambient temperatures where the size and shapes of SPIONs depend on refluxing time, irradiation time and power [68].

Microwave assisted synthesis

In this method, a highly crystalline and monodisperse nanoparticles are produced in a very short time with less energy consumption due to provided uniform heat by microwaves. SPIONs produced in this method exhibit great stability due to their low surface reactivity. Modification in magnetic properties can be performed depending on concentration of surfactants, microwave power, and reaction time [69, 70].

1.4 SPIONs in biomedical applications

SPIONs are widely used in different biomedical applications including MRI, magnetic hyperthermia, magnetofection, and recently in photothermal therapy. Superparamagnetism [71], small size [72], biocompatibility [73] and functional coatings of SPIONs [74] make them very valuable in biomedicine. Superparamagnetism is one of the biggest advantages since there is no remanent magnetization in the absence of magnetic field [75, 76]. Small sizes (<100 nm) provide high effective surface areas, low sedimentation rates (high stability) and escape from the reticuloendothelial system (RES) [71, 77]. Plasma proteins adsorb on the nanoparticles injected into the bloodstream and causes quick blood clearance, which is called as opsonization. There are ways to limit this process. Reducing the size of nanoparticles below 100 nm is one

of the ways to prevent opsonization [78]. The comparative study with three different size of nanoparticles (80, 170 and 240 nm) showed that the particle with the size of 80 nm provided a significant reduction in density of adsorbed plasma proteins and phagocytic uptake [79].

Coatings also play an important role on blood circulation time and clearance of SPIONs. Plasma components coat hydrophobic particles more effectively in comparison with hydrophilic particles. Therefore, hydrophobic particles are cleared from the blood more rapidly [80]. Polymer coatings such as polyethylene glycol (PEG), dextran, polyethylene oxide (PEO), polyoxamines, and poloxamers are widely used to provide longer circulation times to nanoparticles by limiting opsonization [81, 82, 83]. SPIONs in the form of maghemite and magnetite are preferred over other magnetic materials such as cobalt and nickel due to low toxicity and are the only approved metal oxides in clinical applications [84]. Toxicity of SPIONs depends on dose, size, coating, biodegradability, biodistribution and chemical composition. The toxicity of nanoparticles are different in *in vitro* and *in vivo* studies. Nanoparticles induce more toxicity in *in vitro* studies due to direct exposure of nanoparticles into cells [85].

1.4.1 SPIONs in Magnetic Resonance Imaging

MRI is an imaging technique which uses the water content in body. Change in the alignment of hydrogen nuclei of water molecules under external magnetic field induces MR signals. Nuclei of the waters are excited when radio frequency is applied. The frequency of the radiowaves must be in resonance with the frequency of the hydrogen nuclei. So while returning to its original position, aligned nuclei of hydrogens release energy as radio frequency that can be detected by MRI device. The realignment of hydrogen nuclei represents relaxation times which depends on the tissue types. Both T1 (longitudinal, spin-lattice relaxation) and T2 (transverse, spin-spin relaxation) are called as relaxation times representing necessary times for return into equilibrium positions. To produce contrast in MRI, short T1 and T2 are necessary which depends on the saturation magnetization of nanoparticles and their magnetic interactions with

the protons of water. MRI contrast agents can be categorized as T1 and T2 agents. T1 agents generate bright contrast enhancing spin-lattice relaxation rates while T2 agents produce negative/dark contrast increasing spin-spin relaxations [86]. Even gadolinium (Gd (III)) complexes are widely used as T1 contrast agents, it has many drawbacks including toxicity, short life span, poor cellular uptake and induction of nephrogenic systemic fibrosis. Manganese complexes have disadvantages as well such as low thermodynamic stability, less magnetization, toxicity, and low MRI signal sensitivity [87].

SPIONs has appeared as food and drug administration (FDA) approved T2 MRI contrast (negative contrast) agents. In a short time, SPIONs became highly preferable MRI contrast agent instead of Gd (III) and Mn (II) complexes due to their high saturation magnetization, chemically stable, biocompatible and biodegradable properties [88, 89]. Feidex (Berlex, USA), Resovist (Shering AG, Germany), and Endorem (Guerbet, France) are some of the commercial iron oxide nanoparticles which is developed as T2 contrast agents. Table 1.2 shows some of the currently used SPIONs in MRI.

Table 1.2: Size, shape, surface coatings and magnetization values of various SPIONs.

T2 agent	Relaxivity (mMs) ⁻¹	Side of action	Size (nm)	Reference
Magnetoferritin	R ₂ =218	Blood pool	12	[90]
Sinerem	R ₂ =53	Liver	17-20	[91]
Endorem	R ₂ =107	Liver	58	[92]
Resovist	R ₂ =188	Liver	62	[93]

In addition to commercial agents, many studies have been performed with SPIONs in MRI both in *in vitro* and *in vivo*. For example, both terephthalic acid and 2-aminoterephthalic acid coated SPIONs have high transverse relaxivity values which is 735.3 mM/s and 450.8 mM/s, respectively under 3T MRI [94]. Strable et al. developed highly stable maghemite nanoparticles with the size of 20-30 nm using carboxylated poly(amidoamine) dendrimers which possess high relaxivity values [95].

Chakrawarty et al. developed germanium 69 labelled SPIONs functionalized with a layer of poly (ethylene glycol) for PET and MRI imaging. Results from both PET and MRI images confirmed high uptake of ^{69}Ge -SPION@PEG in *in vivo* experiments. PET/MR dual-modality images reported that the uptake is 7.5, 12.5, and 28 percentage injected dose per gram of tissue (%ID/g) for 0.5 h, 2 h and 20 h in lymph nodes [96]. Wang et. al showed that PEG coated SPIONs and PEG/PEI coated SPIONs

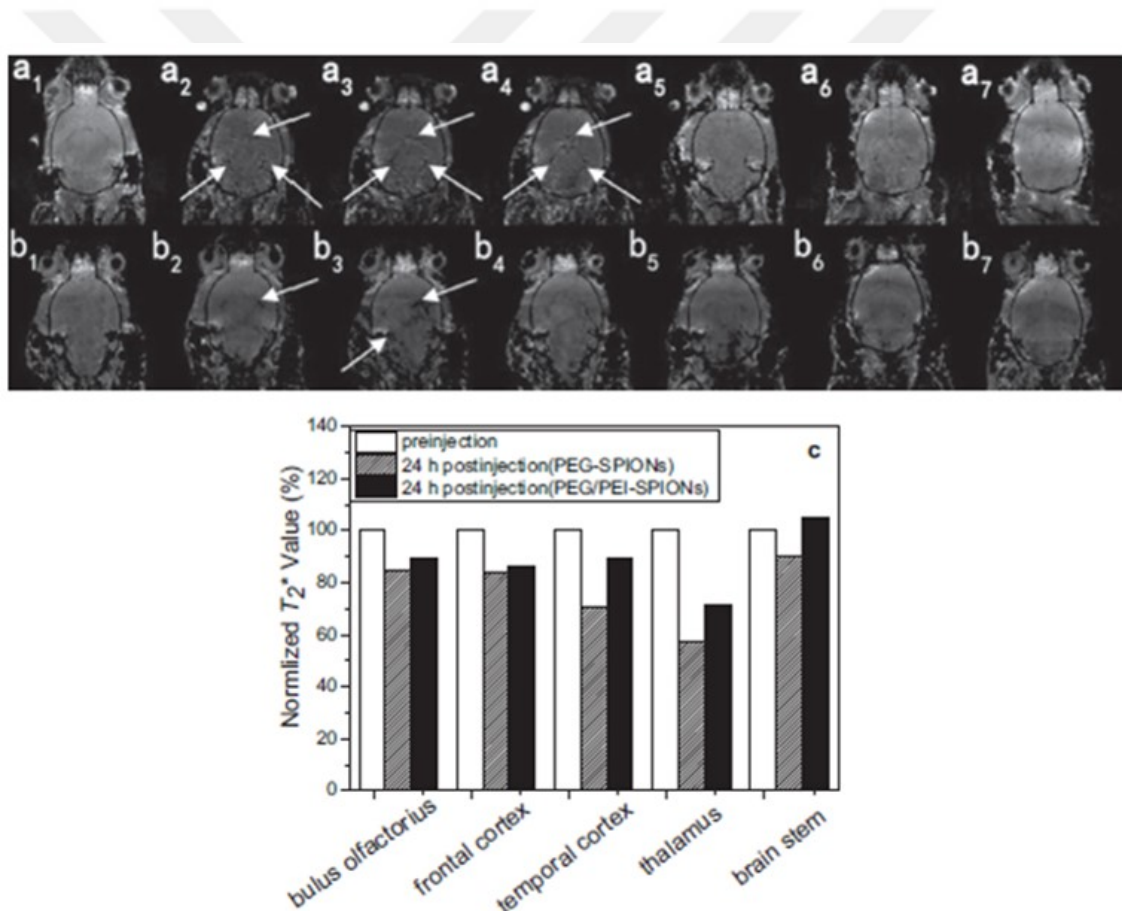


Figure 1.3: T₂ MR images of the mice brains before and after injections of PEG-SPIONs (a₁-a₇) and PEG/PEI-SPIONs (b₁-b₇) and T₂ relaxivity values of various regions in brain before and after injections of PEG-SPIONs and PEG/PEI-SPIONs [3].

exhibited enhanced MRI contrast effects when PEG coated SPIONs and PEG/PEI coated SPIONs are injected at a dose of 10 mg Fe/kg body weight of Kunming mice [3]. Besides, PEG coated SPIONs reduced the MRI signal contrast at bulbus olfacto-

rius, frontal cortex, temporal cortex and thalamus portions of mice after 24 h of its injection in comparison with PEG/PEI coated SPIONs (Figure 1.3).

1.4.2 SPIONs in Drug Delivery

The success of drug therapies are limited due to poor drug accumulation at the desired site. Therefore, it is important to improve the pharmacodynamics and the pharmacokinetics to enhance the drug efficacy and reduce their side effects. Encapsulation of drug into biocompatible nano-carriers such as lipids, polymers, proteins or inorganic nanoparticles are investigated for enhancing blood circulation time and/or for improving the drug accumulation at the diseased sites [97]. SPIONs offer potential to accumulate drugs at the diseased site using an external magnetic field and/or targeting ligands specific for the diseased site [98, 99]. It is really valuable when the drug release is also triggered by the local temperature increase that may be achieved via hyperthermia [100]. Therefore, drug could reach the destination which provide an adjuvant therapy decreasing the side effects. Generally, SPIONs with the size of less than 100 nm is appropriate for magnetic targeting; however, larger particles are preferable for hyperthermia applications [101]. SPIONs can be used as drug delivery agent based on shell around the magnetic core. Hydrophilic and hydrophobic drugs may be loaded to SPIONs by electrostatic interaction, covalent conjugation, nano-precipitation or by nano-emulsion formulations [102, 103]. SPIONs have to possess sufficient magnetic susceptibility to achieve magnetic targeting while applied magnetic field has to be strong enough to deliver them into desired site before drug release. Drug release can arises by diffusion from the matrix around the magnetic cores, cleavage of the drug-nanoparticle bond and the degradation of the matrix triggered with pH, enzymatic reactions or temperature [104]. SPIONs are widely used as drug delivery vehicles in various studies. Studies focused on drug delivery using micron and nano-sized particles has started in the 1970s. The first example of drug (epuribicin) loaded magnetic NPs was performed in animal experiments followed by a Phase I clinical study by Lübke et al [105]. In another study, 164 nm magnetic poly

(ϵ -caprolactone) nanoparticles which is composed of Fe_3O_4 and gemcitabine provided greater therapeutic efficiency in comparison with free gemcitabine in pancreatic tumor of the mice model [106]. PEGylated SPIONs loaded with DOX were reported to accumulate in the lungs in lung carcinoma of the mice model and inhibit tumor growth efficiently at low drug doses compared to free drug [107]. In addition magnetic accumulation studies, specific targeting is a promising method in combination with SPIONs where SPIONs recognize desired tissue with attachment of various ligands. For example, SPIONs conjugated with folic acid which can be recognized from folate receptors in ovarian carcinoma and squamous carcinoma cells was used to deliver anticancer drugs into desired site [108].

1.4.3 SPIONs in Magnetofection

Many gene delivery studies by viral and non-viral carriers (transduction and transfections) had been performed as preclinical and clinical applications. Magnetofection is the transfection method where SPIONs are used to deliver nucleic acids into cells under magnetic field [109]. This idea was first proposed by patent application from Kuehnle and Kuehnle in 1994 (US patent 5516670, issued in 1996). In this method, positively charged magnetic nanoparticles are used to bind negatively charged nucleic acids, deoxyribonucleic acids (DNAs), micro ribonucleic acids (miRNA) or small interfering ribonucleic acids (siRNAs) via electrostatic interactions [110]. Polyethylenimine (PEI) is a highly preferable coating shell used in magnetofection studies. This allows protection of nucleic acid cargo from degradation and provide their release due to 'proton sponge mechanism' of PEI [111]. Castillo et al. synthesized branched PEI and poly (hexamethylene biguanide) (PHMBG) coated SPIONs as siRNA transfection agents and studied their potential on CHO-K1 and HeLa cell lines. PEI-SPIONs provided more transfection efficiency than PHMBG-SPIONs and combination of SPION and PEI caused a reduction on PEI's toxicity [112]. Scherer et al. performed *in vitro* and *in vivo* magnetofection studies with PEI coated SPIONs indicating enhanced transfection efficiencies in the presence of magnetic field [113].

Magnetic cationic liposomes (MCL) consisting 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine and 3s-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol was used in delivery of luciferase reporter gene to human osteosarcoma Saos-2 cells. In the absence of magnetic field, the greater luciferase activity was achieved in 6 h at 1:5 DNA:MCL ratio. On the other hand, the necessary incubation time reduced to 30 min for achievement of maximum luciferase activity with magnetic field [114]. Huang et al. tested magnetofection potential of poly((2-dimethylamino)ethyl methacrylate)-iron oxide nanoparticles/DNA in HEK 293T cells in the absence and presence of magnetic field with and without fetal bovine serum (FBS). Results indicate that higher transfection efficiency compared to PolyMag/pDNA was obtained even at low N/P ratios in the presence of magnetic field using these nanoparticles [115].

1.4.4 SPIONs in Magnetic Hyperthermia

Magnetic hyperthermia is a widely used therapeutic technique in the treatment of cancer which uses alternating magnetic field to oscillate the magnetic moment of SPIONs, converting magnetic energy into thermal energy. The temperature increment depends on size, crystallinity and shape of SPIONs, strength of the magnetic field and frequency of the oscillation. Specific absorption rate (SAR) indicates the efficiency of SPIONs in hyperthermia [87]. In a recent study, SAR for 20 nm magnetic nanocubes and spherical nanocrystals with a deoxychitosan coating were reported as $73.8 \pm 2.3 \text{ W/g}_{Fe}$ and $1.25 \pm 0.12 \text{ W/g}_{Fe}$ at $f=512 \text{ kHz}$ and $H=10 \text{ kA/m}$ [116]. Maity et al. reported compared 500 Watt/g and 135 Watt/g SAR for 44 nm magnetic nanoclusters and 10 nm magnetic nanoparticles, respectively, using 89 kA/m magnetic field at 240 kHz frequency. The solution temperature reached to 42 °C at 0.5, 1, 2, 4 and 8 mg/mL concentration in 7.5, 5.6, 3.8, 2.6 and 1.6 minutes, respectively with magnetic nanoclusters, indicating concentration and time dependence of the temperature increase [117]. In *in vitro* studies conducted with MCF7 cell lines, viability of cells subjected to 1 h magnetic hyperthermia dropped down to 26 % and 38 % with nanoclusters and nanoparticles, respectively. In another study, intraperitoneally in-

jected magnetic nanoparticles caused a reduction in pancreatic tumor growth creating 4 °C temperature increase after 15-20 min treatment at 145 kHz sinusoidal AMF and extended the survival of animals in the *in vivo* studies by 6 days [118]. Wilhelm and coworkers reported SAR values of iron oxide nanocubes (IONC) with different sizes (12, 19, 25, and 38 nm) under various magnetic fields at three different frequencies (320, 520, and 700 kHz). SAR was calculated as 2277 W/g_{Fe} at 700 kHz and 24 kA/m and 1000 W/g_{Fe} at 325 kHz and 22 kA/m for 19 nm IONCs. These particles cause 45-50 % death of KB cancer cells at 2 and 4 g/L of iron concentration after 1 h magnetic hyperthermia treatment and complete cell death was achieved when the concentration increased to > 5 g/L of iron [119]. SPION nanoclusters with polyethy-

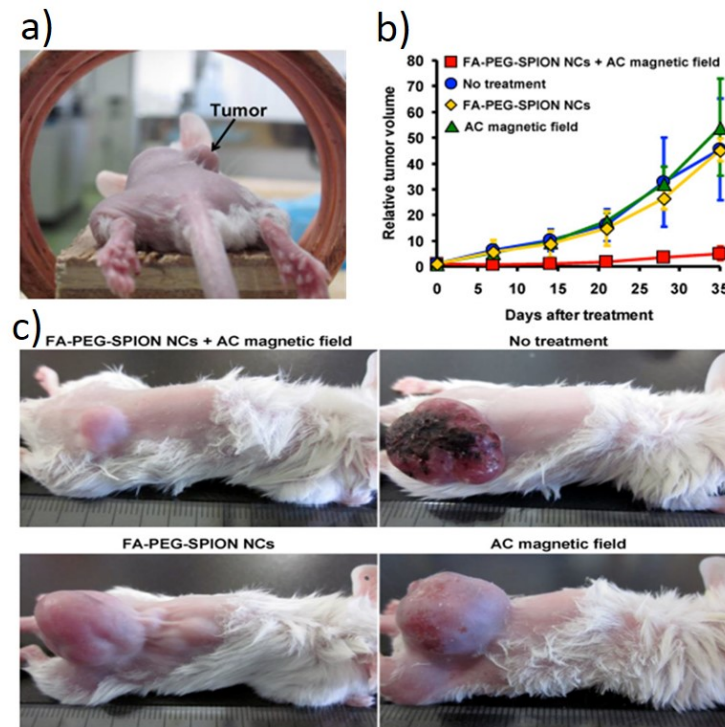


Figure 1.4: a) Image of a mouse during the magnetic hyperthermia treatment, b) tumor growth of mice with and without magnetic field and c) images of mice 35 days with and without treatment [4].

lene glycol-folic acid conjugation (FA-PEG-SPION NCs) provided a SAR value of 248 at H=8 kA/m and f=230 kHz and enhanced accumulation of nanoclusters in folate

receptor overexpressing tumors. Application of these nanoparticles and 10 min magnetic hyperthermia suppressed tumor growth significantly and improved survival of tumor bearing mice tumors derived from a multiple myeloma cell line, over 12 weeks while control animals died in 8 weeks (Figure 1.4)[4]. In another study, Hayashi et al. decorated SPIONs with folic acid as targeting agent and β -cyclodextrin (CD) as drug container and then loaded hydrophobic drug tamoxifen (TMX) [100]. SAR value of TMX loaded FA-CD-SPIONs (20 mg/mL) was 132 W/g when the temperature reached to 42.5 °C at 230 kHz and 100 Oe. They demonstrated controlled release of TMX switching the magnetic field on-off and enhanced accumulation of nanoparticles in the tumor cells more than healthy cells due to folic acid. Ang et al. developed Poly (N-isopropylacrylamide), PNIPA around iron and iron oxide nanoparticles to combine chemotherapy and temperature controlled drug release [120]. The temperature did not increase enough to achieve hyperthermia with PNIPA-Fe where SAR value was only between 0.18-0.67 at different magnetic fields. To achieve drug release, they raised the local temperature to 45 °C under 1.7 kA/m magnetic field in 4 min using PNIPA-Fe₃O₄(2.5 wt %) which had a SAR value of 1.83 W/g under this condition. Hayashi et al. combined MHT and chemotherapy using clustered Fe₃O₄ NPs and DOX [121]. The obtained SAR value was 487 W/g with Fe₃O₄/DOX/PPy-PEG-FA at H=8 kA/m and f=230 kHz for 25 min. Almost 60 % and 90 % DOX release in Fe₃O₄/DOX/PPy-PEG-FA when temperature reached to 44 and 46 °C, respectively. *In vivo* studies revealed that alone MHT and chemotherapy caused a reduction in tumor growth while combined study suppressed tumor totally without any regrowth. The first report on clinical application of MHT showed that aminosilane coated SPION caused an important suppression in prostate tumor growth during 6 weeks treatment [122]. The temperature maximum and minimum temperatures were 48.5 and 40 °C and 42.5 and 39.4 °C for 1st and 6th treatment, respectively. Another important point is that NPs stayed in tumor during the treatment. However, side effects were also occurred such as urinary problems and tiredness. In another study, aminosilane coated SPION was used in thermotherapy to treat glioblastoma multi-

forme [123]. This study showed that thermotherapy using magnetic nanoparticles is safe and promising with no or minor side effects.

1.5 Thermal Therapy

Thermal therapy is a widely used technique in the treatment of cancer, which increases the local temperature above normal level for defined time for many years. The first successful example was reported in the 19th century: Coley toxin was used as a bacterial infection which caused fever to treat a cancer patient [124, 125]. Another successful work was performed using circulating heated water in cervix cancer treatment [125, 126]. However, these therapeutic approaches were not adopted as common clinical practices at that time. Later on, many successful thermal therapy studies as a single treatment method or in combination with other approaches have been reported. Based on the magnitude of temperature increase and heating time duration, thermal therapy can be categorized into three different groups: (i) diathermia, (ii) hyperthermia, and (iii) thermal ablation (Figure 1.5) [5].

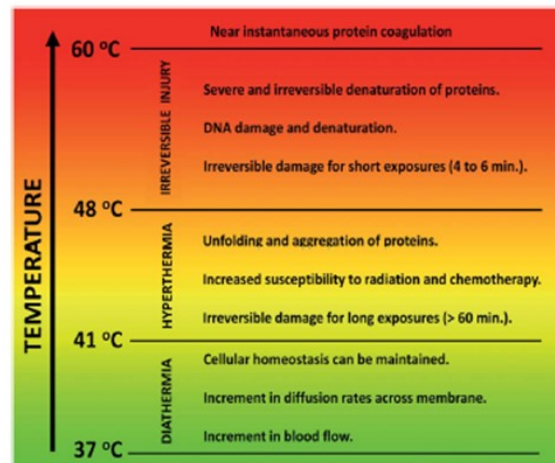


Figure 1.5: Types of the thermal therapy and changes occurred during treatment based on temperature changes [5].

i) Diathermia: The maximum temperature usually reaches to 41 °C for a short time period in this process. Diathermia does not cause a significant damage at the cellular

level but enhances diffusion across cell membrane and increases blood flow [127, 128]. Inducing a couple of degrees temperature increase makes cells more sensitive but does not induce cell death. Diathermia coupled with chemotherapy usually provide successful therapeutic outcome in drug resistant tumors [129]. Besides cancer therapy, diathermia plays an important role in muscle relaxation and pain relief enabling tissues a better stretch [130].

ii) Hyperthermia: Tissue temperature usually reaches to 41-48 °C (clinically relevant range) during hyperthermia treatments [122, 131, 132]. In the clinic, hyperthermia may be applied alone or in combination with other therapeutic modalities such as chemotherapy, immunotherapy or radiotherapy. Cell response to hyperthermia depends on the temperature and duration at that temperature. Protein denaturation may start in mitochondria near 39 °C and makes protein susceptible to aggregation which destructs many macromolecular structures and their functions [133, 134]. When the temperature reaches to 41 °C, cell inactivation is triggered. However, surviving cells may create a resistance against this temperature increment which is called as thermo-tolerance [135]. This thermo-tolerance may be destroyed by increasing the temperature to 43-45 °C, where long term cell inactivation is achieved [136, 137]. Finally, suppressive hyperthermia occurs between 45-48 °C [138, 139].

iii) Thermal ablation: Thermal ablation causes cell death as a result of coagulative necrosis in 2 minutes by increasing the local temperature to 48-60 °C [140, 141, 142, 143, 144]. Thermal ablation was successfully applied for benign prostatic hyperplasia treatment [145] and heart stimulation [146]. Although, it provides efficient results, it can damage adjacent healthy tissues. Irreversible protein denaturation occurs above 60 °C and increasing temperature up to 80 °C causes tissue explosion.

1.5.1 Photothermal Therapy (PTT)

In the case of PTT, irradiation of photosensitizer such as dyes, metallic nanostructures, metal oxide nanoparticles etc. (Figure 1.6a) [6] with near infrared (NIR) laser light creates a local temperature increase which may thermally ablate tumor cells (Figure

1.6b) [7], if these photosensitizers are located within the tumor. This provides a local ‘turn on’ mechanism. NIR light is appropriate for the patient’s safety due to its non-ionizing nature.

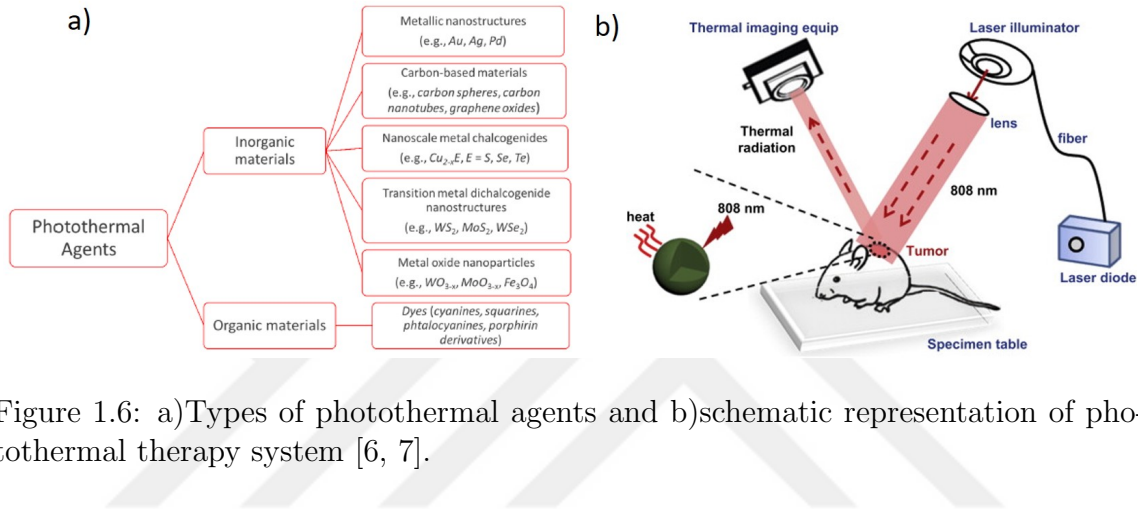


Figure 1.6: a)Types of photothermal agents and b)schematic representation of photothermal therapy system [6, 7].

The wavelength range between 700-980 nm and 1000-1400 nm represents the first and second biological window, respectively (Figure 1.7a). At these wavelengths, absorption and scattering by the tissue are minimized, hence tissue becomes more transparent. Penetration depth in the skin is just 3-4 cm in thermal therapies [147]. However, using laser sources in the medical windows (first and second biological window) can improve the penetration depth [148, 149, 150]. Besides the laser wavelength, na-

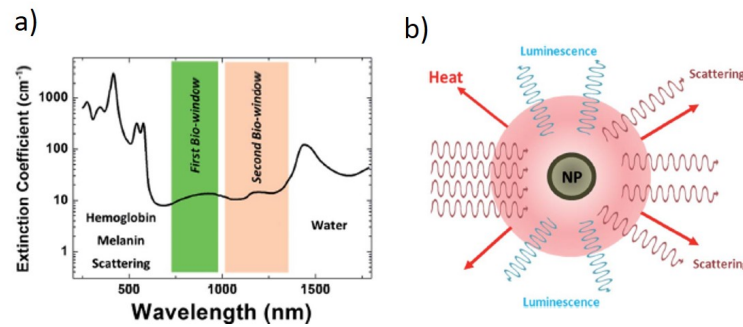


Figure 1.7: Schematic representation of a)biological windows and b)interaction between light and nanoparticles [5].

ture of the nanoparticle as photosensitizer is another important point to achieve safe and efficient PTT. Nanoparticles must satisfy the following criteria: (i) low toxicity; (ii) high absorption in one of the biological windows; (iii) easy functionalization; and (iv) good solubility and stability in different media [151, 152, 153, 154, 155, 156, 157]. Both scattering and absorption process occurs when a tissue is irradiated by laser light. Heat production is created as a result of absorbed incident photons (Figure 1.7b)[5]. Graphene [158], carbon nanotubes [159], metallic nanoparticles (gold [160, 161], silver [162], copper [163]) are widely used in PTT; however, introduction of such nanoparticle-based therapeutic approaches in clinical practice is still limited. SPIONs which are widely used as contrast agents [96, 95] and for magnetic hyperthermia [116, 119] have been recently recognized as PTT agents [164, 14] as well.

Thermal therapies were successfully applied in clinic [165, 8, 166]. Paiva et. al performed Imaging-guided interstitial laser therapy (ILT) using the neodymiumyttrium-aluminum-garnet (Nd:YAG) laser to treat squamous cell carcinoma of the head and neck. The results showed that ILT was applied successfully; however, extra follow-up is required to observe convincing evidence of long-term therapeutic benefits. Figure 1.8a and b shows remission and recurrences after treatment and squamous cell cancer of the lateral tongue before and after laser treatment [8].

a)

TABLE I.
Recurrences and Remissions by Tumor Location.

Tumor Location	Total	Recurred	Remission
Alveolar ridges	6	0	6
Buccal mucosa	3	0	3
Floor of mouth	2	0	2
Lip	3	2	1
Palate	5	1	4
Retromolar trigone	4	0	4
Tongue	1	0	1
Base of tongue	1	1	0
Lateral pharynx	2	0	2
Soft palate	2	1	1
Posterior pharynx	3	1	2
Base of skull	2	1	1
Nasal cavity	3	1	2
Maxillary sinus	3	1	2
Larynx	1	1	0
Neck	28	20	8
Skin	10	6	4
Total	79	36	43

b)

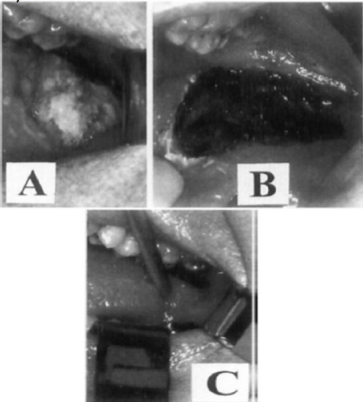


Figure 1.8: a)Remission and recurrences after treatment and b)images of squamous cell cancer of the lateral tongue before treatment(A), directly after treatment(B) and 8 weeks after treatment(C) [8].

1.5.2 SPIONs containing hybrid nanoparticles in PTT

Combination of therapeutic methods with imaging modalities offer tremendous advantages. Combination of imaging with PTT is especially interesting since it is possible to allocate the site of photosensitizers, which would be activated locally with the laser. This is called as image guided PTT and highly desired in the clinic. Combination of nanoparticles traditionally used for PTT such as gold with SPIONs not only provide opportunity for MRI tracking but also for magnetic transfection of the cells/tumors with PTT agent under magnetic field. Hence, combination of photosensitizers with SPIONs provide multifunctional nanomaterials for combined action of magnetic targeting, MRI and PTT. In previous years, many hybrid gold-iron oxide nanoparticles composed of Fe_3O_4 nanoparticles with a gold nanoshells or gold nanospheres to combine MRI and PTT. For example, targeted delivery and effective PTT was reported

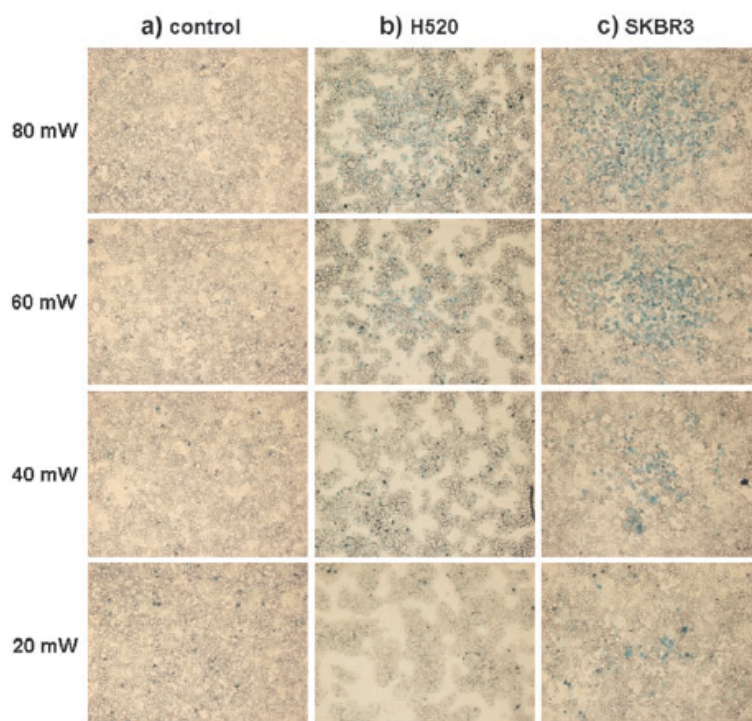


Figure 1.9: Optical images of a)control SKBR3 cells, b)H520 and c)SKBR3 cells incubated with hybrid nanoparticles after 10 s irradiation with a femtosecond-pulse laser [9].

by Fe_3O_4 nanoparticles containing a gold nanoshell functionalized with anti-Her2/neu when tumor cells treated with these nanoparticles were exposed to 10 s laser at 800 nm using a femtosecond-pulsed laser with 20-80 mW laser power [9]. Due to receptor targeting, killing of SKBR3 cells were achieved even at 20 mW laser power but required 60 mW for H520 cancer cells (Figure 1.9). Femtosecond-pulse laser (10 s) caused cell death in short exposure time compared to continuous-wave laser (2-7 min) [9, 167, 168, 169]. In another work, hybrid nanoparticles were developed with iron oxide nanoparticle (ION) cores and gold shells for the combination of MRI/optical imaging and PTT applications [170]. These hybrid nanoparticles were conjugated with an antibody, which can specifically recognize epidermal growth factor receptor (EGFR). EGFR is a common biomarker for many epithelial cancers. *In vitro* studies revealed that cells treated with these nanoparticles caused an appreciable cell death upon single irradiation with nanosecond pulsed laser at 700 nm with 400 mJ/cm² pulse. No significant cell mortality was observed with unlabeled nanoparticles even after 600 pulses indicating the safety of the laser irradiation in the absence of photosensitizer. A monodispersed Fe_3O_4 @SiO₂@GNSs-PEG (PMGNSs) containing Fe_3O_4 inner core, silica as the midlayer and gold nanoshells at the outside with the size of 90 nm as MR/CT dual imaging and PTT agent [171]. The temperature of aqueous solution of NPs were increased with increasing NP concentration and laser power density. A significant cell death was observed with 3 min 808 nm laser irradiation at 2 W/cm² in the *in vitro* studies. In the presence of magnet, almost complete cell death was achieved under same conditions, pointing out the possibility of magnetic transfection as means to locate photosensitizers at the site of interest. The PTT potential of a single chain antibody conjugated Dumbbell-like Au- Fe_3O_4 NPs was tested on SW122 cells under 808 nm continuous wave laser using 3.2-100 W/cm² intensity [172]. An appreciable cell mortality was induced at 5.1 W/cm² which was determined as the threshold for apoptotic cell death. At higher intensities (31.5 and 100 W/cm²), necrosis-related cell death was reported. Complex magnetic hollow gold nanoshell nanoparticles in 40-80 nm sizes were developed in three steps [173]. First, IONPs

were conjugated on the 3-mercaptopropyltrimethoxysilane (MPTMS) functionalized Ag cores. Then, IONPs were encapsulated in a silver structure. As a last step, gold salt was added to form hybrid NPs. To investigate its *in vivo* PTT potential, mice bearing melanoma tumors were irradiated with a continuous 808 nm laser at 4 W/cm² for 30 seconds. The intravenous injection (IV) of NPs (1012 particles in 100 μ L) provided a reduction on tumor growth while intratumoral injection (IT) totally ablated the tumor. A rattle-shaped IONP@shell-in-shell structure consisting of IONP core and a double shell of Au/Ag alloy exhibited stronger absorbance around 1000 nm in comparison with IONP which gives an opportunity to use 1064 nm laser in PTT experiments [174]. In the *in vitro* studies conducted by human glioblastoma cell (U87), cell mortality was 47.4 % and 83.7 % with and without magnetic field, respectively, after 10 min laser irradiation at 3 W/cm² where Fe concentration was 16.4 ppm and Au concentration was 100 ppm. Presence of magnetic field enhanced the accumulation of NPs at the tumor site which caused suppression of tumor growth. Some studies have been performed with gold nanorod-IONPs. Wang et. al performed PTT study on SKBR-3 cells with Au-rod-Fe₃O₄ and Au-rod-Fe₃O₄-Herceptin using 785 nm laser with 4.53 W/cm² laser power [175]. They reported that appreciable cell death was observed with Au_{rod}-Fe₃O₄-Herceptin due to receptor mediated uptake of nanoparticles by SKBR-3 cells while there was no change without Herceptin modification. Recently, magnetic gold-nanorod/PNIPAAmMA nanoparticles (MPGNRs) were synthesized for combination of photoacoustic (PA) imaging and PTT [10]. In the presence of an external magnet, most of the cells died under 2 W/cm² 808 nm laser irradiation in 2 min (Figure 1.10a). With gold nanorods (PGNRs) at 2 W/cm² for 3 min irradiation 12 °C temperature increase but with (MPGNRs) under magnetic field and laser irradiation 63 °C temperature increase were reported (Figure 1.10b and c). Therefore, tumor growth was slowed during the treatment for 10 days; the volume only increased by 284 $\pm$ 79 for MPGNRs-MT-NIR treated group. On the other hand, the volume increased by 1137 $\pm$ 90 for control group and 915 $\pm$ 75 for PGNRs-MT-NIR treated group (Figure 1.10d). Multifunctional pDNA/Au@pDM/Fe₃O₄ nanocompos-

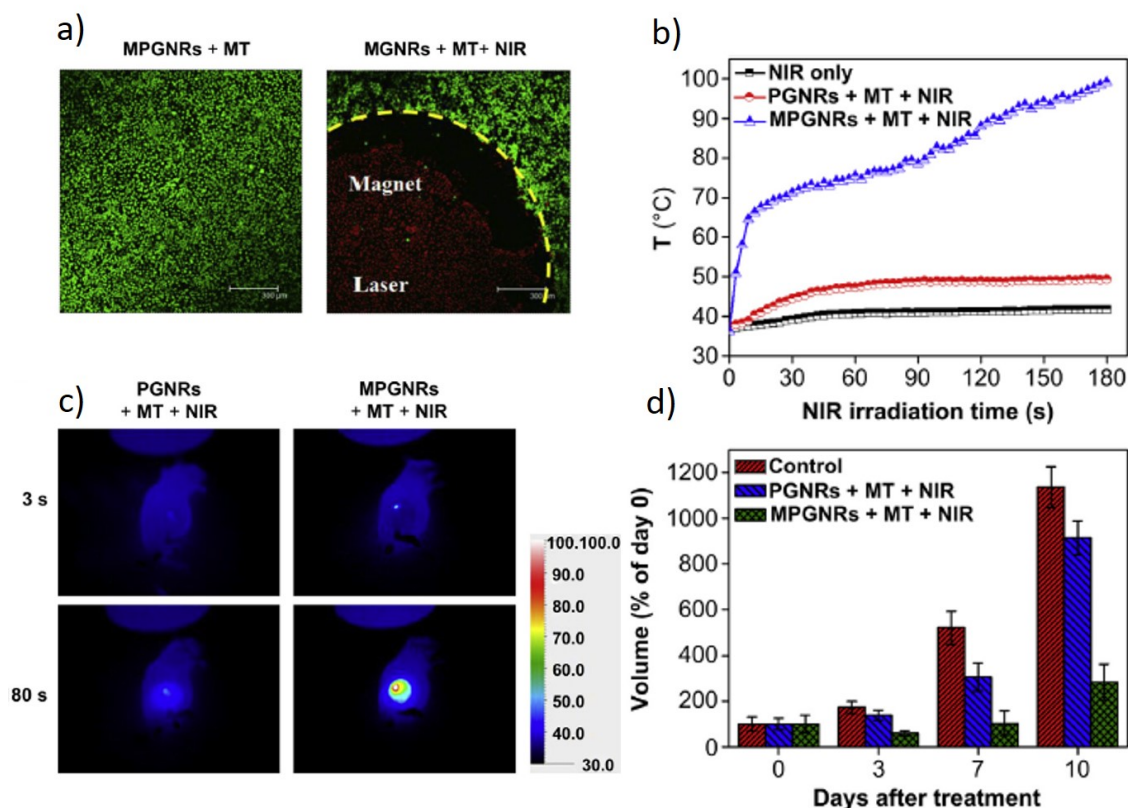


Figure 1.10: a) Fluorescence micrographs of cells treated with hybrid nanoparticles with (right) and without (left) laser irradiation under magnetic field, b) temperature increase in *in vivo* as a function of irradiation time, c) image of thermal map after laser irradiation and d) tumor volume during 10 days after treatment [10].

ites that were used as trimodal imaging and combined photothermal/gene therapy agents showed enhanced absorbance at 750 nm combining the NIR absorbance properties of Fe_3O_4 nanoparticles and Au nanorods hu2016multifunctional. Therefore, it showed great photothermal performance producing 50.1°C temperature increase after 5 min 2 W/cm^2 808 nm laser irradiation. Effective combination of gene therapy and PTT was reported in the *in vitro* studies using C6 cells. In *in vivo*, tumor ablation was achieved with p53/Au@pDM/ Fe_3O_4 after laser irradiation as a result of obvious tumor apoptosis.

Combinations of IONPs with other inorganic nanoparticles such as graphene oxide, copper sulfide, manganese and molybdenum sulfide were also used for photothermal

therapy. For example, PEG functionalized graphene oxide-iron oxide nanoparticles with stronger absorbance than graphene oxide were effectively used in multimodal imaging guided photothermal therapy [176]. As the result of, total tumor elimination after PTT applied at a low power density of 0.5 W/cm^2 for 5 min at 808 nm was observed by tracking the tumor volume change by MRI with no regrowth. In another study, graphene oxide (GO-IONP-Au-PEG) was decorated with both iron oxide nanoparticles and gold nanoparticles for PTT combined with MRI and X-ray dual mode imaging [177]. The aqueous solution of GO-IONP-Au-PEG induced 17°C temperature increase under 808 nm laser irradiation at 1 W/cm^2 in 5 min while GO-PEG and GO-IONP-PEG created much lower temperature increases at the same GO concentration ($10 \mu\text{g/mL}$). Cancer cells were almost completely killed by GO-IONP-Au-PEG at a power density of 2 W/cm^2 while GO-PEG and GO-IONP-PEG provided only limited cell death under same conditions. In the presence of an external magnetic field, significant cell death was observed with GO-IONP-Au-PEG even at 1 W/cm^2 . In the *in vivo* experiments, complete tumor ablation was achieved with GO-IONP-Au-PEG NPs at 0.75 W/cm^2 in 5 min by increasing the local temperature to 55°C . Tian et. al synthesized ultrasmall $\text{Fe}_3\text{O}_4@\text{Cu}_{2-x}\text{S}$ core-shell nanoparticles as a dual-modal mode imaging and PTT agent [178]. The temperature increase of aqueous solutions were controlled by changing Cu content of NPs. For example, the temperature increment was 17°C at 50 ppm Cu content under 980 nm laser irradiation at 0.6 W/cm^2 reaching 16 % photothermal conversion efficiency. Photothermal ablation was induced on HeLa cells after 4 min laser irradiation. In the *in vivo* studies, histological images revealed cell shrinkage and nuclear damage. The percentage of necrosis was 38 % and 27 % for cells treated with $\text{Fe}_3\text{O}_4@\text{Cu}_{2-x}\text{S}$ and for control cells, respectively. In six heating/cooling cycles, $\text{Fe}_3\text{O}_4@\text{Cu}_{2-x}\text{S}$ NPs induced same temperature increase after each cycle while there was some loss in temperature increase with Au nanorods. A theranostic MSIOs nanocomposite was developed by attaching ultra-small Fe_3O_4 NPs on the surface of the multilayer MoS_2 nanoflakes [179]. Its photothermal conversion efficiency was calculated as 43.93 % at concentration of 170

ppm after 10 min 1 W/cm² 808 nm laser irradiation. Combination of laser+MSIOs showed more toxic effect on HeLa than HepG2 cells where the cell viability was 5.3 % and 28 % for HeLa and HepG2 at 200 µg/mL concentration, respectively, after laser irradiation. In the presence of magnet, a significant cell death was achieved on HeLa cells even at 10 µg/mL. In the *in vivo* studies, 17 °C and 22 °C temperature increase were reported after laser irradiation (0.6 W/cm²) with and without magnet, respectively. Only laser+MSIOs reduced the tumor growth significantly while magnet+laser+MSIOs ablated tumor totally after 9 days and no reoccurrence was reported within 15 days. In another study, theranostic manganese doped iron oxide (MnIO) nanoparticles modified with denatured bovine serum albumin (MnIO-dBSA) were developed for T1-weighted MRI and PTT combination [180]. It showed concentration dependent PTT potential at 2 W 808 nm laser irradiation by killing majority of the cancer cells at 200 µg/mL concentration without any destruction on control cells. In the *in vivo* studies, total tumor ablation was achieved with 5 min laser treatment at 1.5 W which increased the surface temperature to 70 °C. No tumor re-growth was observed within the following 19 days. The black area on tumor after laser irradiation healed in a week.

1.5.3 SPIONs as photosensitizers in PTT

Historically, SPIONs were not used as PTT agents but rather used to provide MRI modality and/or magnetic transfection ability to more known photosensitizers as described above. However, recently it was recognized that SPIONs may be used for PTT by themselves. To understand the photothermal mechanism of SPIONs, many studies have been performed based on different nanoparticles and laser parameters such as size, shape, concentration and intensity. Shen et al. showed that Fe₃O₄ nanoparticles can be used as PTT agents alone with comparable temperature increases with gold nanospheres [164]. Fe₃O₄ NPs coated with carboxymethyl chitosan induced 54.2 °C temperature increase at 300 µg/mL concentration using 808 nm laser with 2 W/cm² in 5 min. There was no appreciable difference between the temperature increase of

CMCTS@Fe₃O₄ and hollow gold nanospheres which points out that Fe₃O₄ NPs can be used as promising PTT agents. Besides, CMCTS@Fe₃O₄ were more biocompatible than hollow gold nanospheres. In the *in vitro* studies viability of KB and MCF7 cell lines decreased below 10 % after 3 min laser irradiation at 75 μ g/mL CMCTS@Fe₃O₄ NPs. In the *in vivo* studies, tumor xenografts were totally ablated in 10 days under a tumor-targeted magnetic field with post-irradiation. Chu et al. studied photothermal

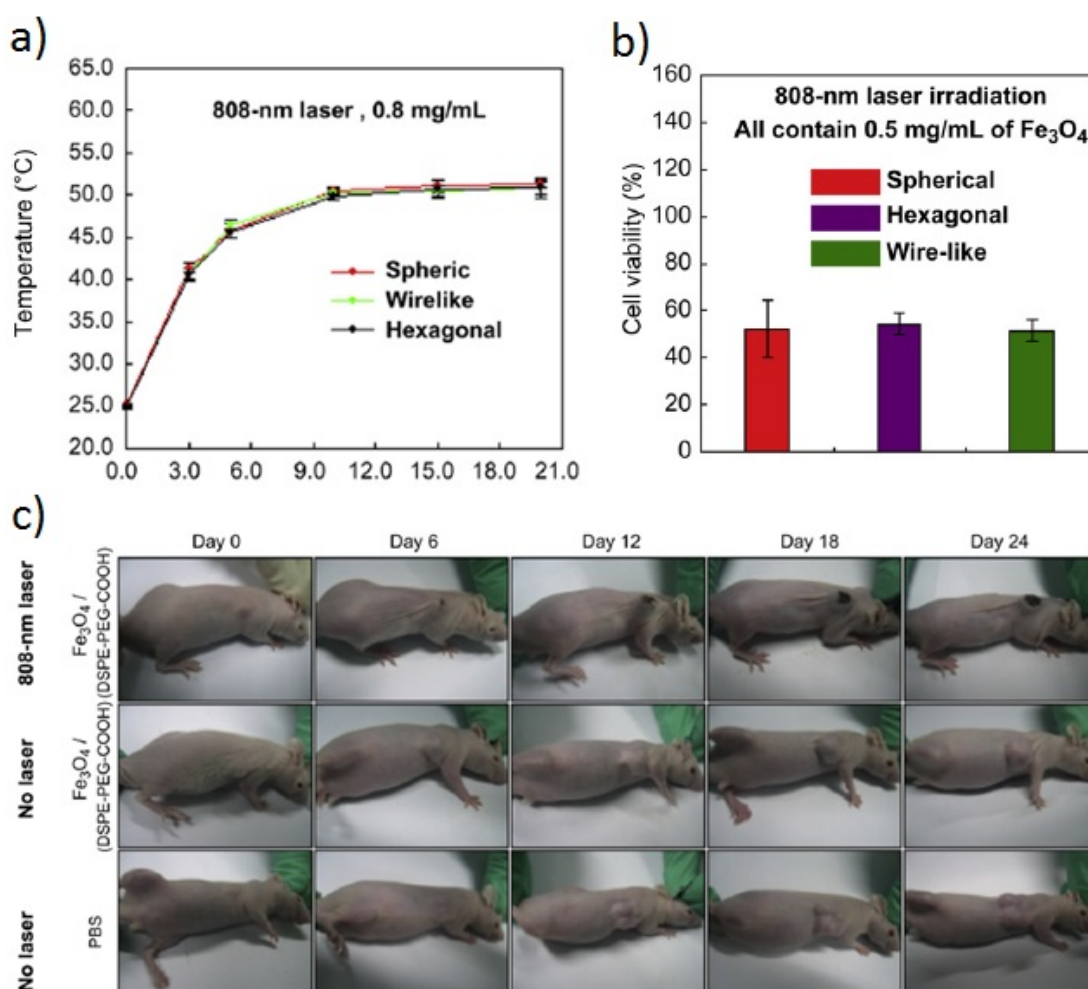


Figure 1.11: a) Temperature increase of spherical, hexagonal and wire-like Fe₃O₄ under 808 nm laser irradiation, b) cell viability of esophageal cancer cell treated with various shapes of Fe₃O₄ after laser treatment and c) representative photos of tumors with and without laser irradiation [11].

effect of spherical, hexagonal and wire-like Fe₃O₄ using red (655 and 671 nm) and

near infrared (808 nm) lasers in *in vitro* and *in vivo* experiments [11]. Under 808 nm laser irradiation, 200 μL aqueous solution of spherical Fe_3O_4 induced 13, 25, and 28 $^\circ\text{C}$ temperature increase after 10 min at 0.08, 0.8, and 8 mg/mL nanoparticle concentrations, respectively. Same temperature increase was observed with hexagonal and wire-like Fe_3O_4 under same conditions which was attributed to similar absorbance of these nanoparticles at the irradiation wavelength (Figure 1.11a). On the other hand, spherical Fe_3O_4 created 2-4 $^\circ\text{C}$ more temperature increase at shorter wavelengths which was explained with a slightly stronger absorbance of NPs at this region. In the *in vitro* studies, there was limited cell death after 3 min irradiation at 37 $^\circ\text{C}$ with 0.5 mg/ml of spherical, hexagonal and wire-like Fe_3O_4 while cell viability was dropped to almost 50 % after 20 min irradiation (Figure 1.11b). In the *in vivo* experiments, laser irradiation at 808 nm was applied in every 24 h for 24 days at 8 mg/mL concentration of spherical Fe_3O_4 . After 4 days, tumor tissue was suppressed with nanoparticle + laser irradiation while the tumor growth was continued during 24 days on controls (Figure 1.11c). Shen et. al compared the photothermal efficiency of individual and clustered Fe_3O_4 nanoparticles using 5 W/cm^2 under 808 nm laser irradiation [12]. Clustered NPs induced greater temperature increase than individual NPs due to 3.6 fold stronger absorbance at 808 nm (Figure 1.12a and b). For example, 26.4 $^\circ\text{C}$ temperature increase was achieved with clustered NPs while 17 $^\circ\text{C}$ increase was obtained with individual NPs at 50 g/mL NPs concentration. Clustered NPs caused significant cell death on A549 cells as a result of apoptotic cell death, but individual NPs showed limited cell death. In the *in vivo* study, there was no appreciable suppression of tumor growth with individual or clustered nanoparticles after 180 s and 120 s laser irradiation, respectively (Figure 1.12c). An effective tumor suppression was observed with clustered nanoparticles after 180 s laser irradiation where tumor volume decreased from 955.3 mm^3 to 222.8 mm^3 (Figure 1.12d). Chen et. al studied the PTT potential of highly crystallized iron oxide nanoparticles (HCIONPs) under 885 nm laser irradiation at 2.5 W/cm^2 and compared their results with the commercial iron oxide nanoparticles [181]. At 0.5 mg Fe/mL, HCIONPs induced 33 $^\circ\text{C}$ temperature increase,

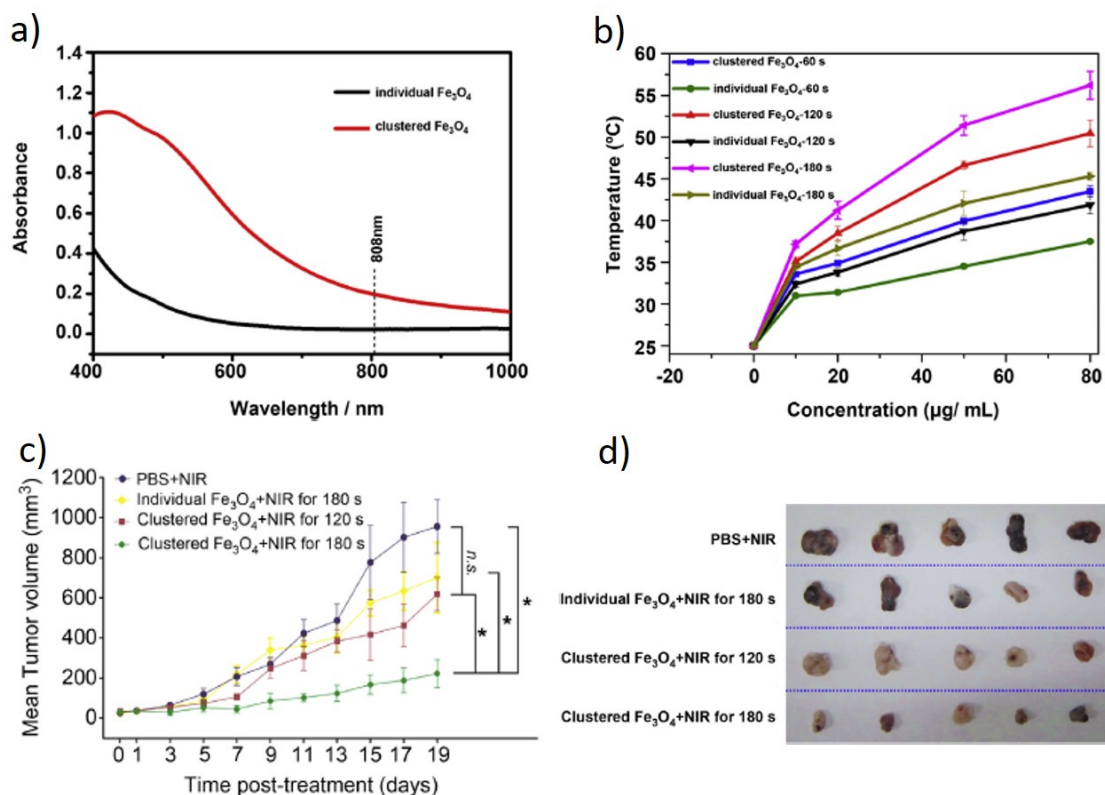


Figure 1.12: a) Absorbance spectrum of individual and clustered Fe₃O₄ NPs, b) the temperature increase of individual and clustered NPs with various irradiation time as a function of concentration, c) tumor growth of different groups after treatment and d) photograph of tumors after excision in the presence of laser irradiation. [12].

while commercial NPs created only 7 °C under the same conditions. This enhancement was attributed to highly crystalline nature and preferred lattice orientation of prepared HClONPs. This nanoparticle was able to ablate tumor totally after 3 weeks post irradiation increasing the temperature from 35 °C to 60 °C at tumor surface. A PEGylated Fe₃O₄ with a mean diameter of 30 nm was used as an effective PTT agent on C6 cells [182]. It showed enhanced PTT efficiency with increased nanoparticle concentration and intensity. Almost complete cell death was achieved at 2 W/cm² after 5 min laser irradiation. Zhou et. al synthesized PEGylated iron/iron oxide nanoparticles (Fe@Fe₃O₄ NPs)[183]. The temperature increase of aqueous Fe@Fe₃O₄ NPs was tested as a function of NPs concentration and laser power density under

808 nm laser irradiation. Higher temperature increase was observed with increasing NP concentration and laser power density. The comparison of Fe@Fe₃O₄ NPs with Au nanorods showed that they have comparable PTT conversion efficiency under the same conditions. Besides that, Fe@Fe₃O₄ NPs revealed greater photostability than Au nanorods inducing same temperature increase after each laser on/off cycle for 5 cycles. There was no difference in TEM images and absorption spectra of NPs after laser irradiation, as well. *In vitro* studies were performed on HeLa cells using different NP concentrations and laser power densities in the absence and presence of an external magnet. The cell viability was dropped to 85 % and 13 % at 0.08 and 0.38 W/cm², respectively. At low power density, cell death was occurred as a result of apoptosis; however, both apoptosis and necrosis were observed at high power density. In the *in vivo* studies, tumor xenograft was totally ablated after 14 day of 5 min irradiation per day using 0.31 W/cm² power density in magnetic field. Dunn et al. compared the photothermal potential of relatively small poly(acrylic acid)/Fe₃O₄(40 nm) and large uncoated Fe₃O₄(310 nm) under 785 nm (38.5 kW/m²) laser irradiation of NPs [184]. Poly(acrylic acid) (PAA) coating provided better stability and reduced the innate toxicity on MDA-MB-231 cells. The PAA/Fe₃O₄ prepared by a hydrothermal route started to show significant dark toxicity at about 1 mg/mL, but effective photothermal ablation was achieved after 20 min of irradiation. Guo et. al synthesized four different sizes of Fe₃O₄ nanoparticles (60, 120, 200, and 300 nm) for MRI, photoacoustic tomography, and photothermal therapy [185]. In aqueous PTT measurements, each nanoparticle induced almost same temperature increase under 808 nm laser irradiation at 1.5 W/cm² for 10 min. The cell viability of MCF7 cells dropped to 50.9 %, 65.6 %, 70.8 %, and 81.4 % with Fe₃O₄-60, Fe₃O₄-120, Fe₃O₄-200, and Fe₃O₄-310 nanoparticles, respectively, after 3 min laser irradiation and 24 h incubation. Small nanoparticles caused more cell death due to higher internalization than larger nanoparticles. On the other hand, large nanoparticles caused significant reduction on tumor growth in comparison with other nanoparticles where the apoptosis index is 33.3 %, 11.2 %, 9.2 %, and 4.2 % for Fe₃O₄-310, Fe₃O₄-200, Fe₃O₄-120,

and Fe_3O_4 -60, respectively. Han et. al prepared Fe_3O_4 NPs with electricall charged surfaces for cancer cell biomarkerless targeting and photothermal killing [186]. The temperature was raised above 55°C after 5 min 2 W/cm^2 808 nm laser irradiation with both 0.15 mg/mL positively (MNCs+) and negatively (MNCs-) charged NPs. MNCs + and MNCs – did not bind onto normal cells due to neutral or weakly charged surface of normal cells. MNCs + occurred cell death on HeLa cells after laser irradiation where it bound to cells strongly due to negatively charged surface of cancer cells. A hyaluronan (HA) modified – SPIONs were used as effective MRI and PTT agents on CD44 HA receptor-overexpressing breast cancer in both *in vitro* and *in vivo* studies [187]. Effective death of HA-SPION treated MDA-MB-231 cells was observed after 10 min irradiation with 808 nm laser with 2 W/cm^2 laser intensity. Complete tumor suppression was observed after PTT in the *in vivo* experiments without any reoccurrence. Hai et al. prepared human serum albumin (HAS) coated maghemite nanoparticles to perform targeted PTT experiments [188]. HSA was labeled with rhodamine and the high internalization was observed on HeLa cells. The SAR was calculated as 1870 W/g with 9.2°C temperature increase and 1340 W/g with 14.1°C temperature increase at 2.5 and 6.5 mM iron concentrations, respectively. They have compared the PTT with magnetic hyperthermia using the same nanoparticles and reported a SAR value of 76 W/g with 5.6°C temperature increase even at 55 mM iron concentration. In the *in vitro* study conducted with HeLa and fibroblast cells treated with 2.5 mM iron concentration, temperature increase to 41.5°C for HeLa cells and 40°C for fibroblast cells were reported after 1 W/cm^2 laser irradiation at 808 nm. Methylene blue assay showed that HSA-maghemite NPs with laser treatment created an important cell death as a result of apoptosis; however, no damage was observed on fibroblast cells. Huang et. al developed clustered Fe_3O_4 nanoparticles (CNPs) for optical coherence tomography cellular imaging and photothermal therapy [189]. The temperature increased up to 59°C with 380 mW/cm^2 laser intensity under 1064 nm laser irradiation at 375 ppm [Fe]. The viability of HeLa cells decreased to 59 % and 18 % after 1 day and 3 days incubation after laser irradiation, respectively. In the

presence of a magnet, 65 % cell mortality was observed after 1 day of incubation after laser treatment.

1.6 SPIONs in combined photothermal therapy and photodynamic therapy

Combination of PTT with other therapeutic modalities such as photodynamic therapy (PDT), chemotherapy and magnetic hyperthermia (MHT) increases the therapeutic outcome. Especially in case of PDT and chemotherapy combinations, nanoparticles does provide stability to such therapeutic reagents, in addition to PTT potential or ability to direct them magnetically to the site of interest. Indocyanine green (ICG) is an FDA approved fluorescent dye with low stability, bioavailability and fair PDT/PTT performance. However, SPION based nanostructures loaded with ICG (SPION@DSPE-PEG/ICG) both improved the stability of ICG and enhanced the temperature increase in experiments performed with aqueous suspensions of such particles [190]. For example, the temperature increment was 24.2, 19.1, 17.2, and 16.7 °C for SPION@DSPE-PEG/ICG NPs and 20.4, 8.6, 6.8, and 4.6 °C for free ICG after consecutive laser on/off experiments. In the *in vitro* studies performed with HeLa cells, more than 95 % cell mortality was observed at 7.2 $\mu\text{g/mL}$ ICG (50 $\mu\text{g/mL}$ SPION) concentration after 10 min irradiation with 808 nm laser with 8 W/cm^2 laser intensity. In the *in vivo* experiments, total tumor ablation was achieved at 0.64 W/cm^2 reaching 58 °C during 10 min laser irradiation without any re-growth. Poly(lactic-co-glycolic acid) nanoparticles encapsulating SPIONs, ICG and perfluoropentane ($\text{Fe}_3\text{O}_4/\text{ICG@PLGA/PFP}$) with average diameter of 290 nm were reported to provide higher temperature increase than free ICG or Fe_3O_4 NPs but in comparable amounts to mixture of ICG and Fe_3O_4 when irradiated at 808 nm (Figure 1.13)a [13]. *In vitro* studies performed with MCF7 cells showed that majority of cells were killed after 5 min 808 nm laser irradiation at 1 W/cm^2 (Figure 1.13b). Total tumor ablation was obtained by increasing the temperature from 32.2 °C to 52.3 °C as a result of coagulative necrosis in the *in vivo* experiments and all mice were alive 48

days after laser irradiation (Figure 1.13c).

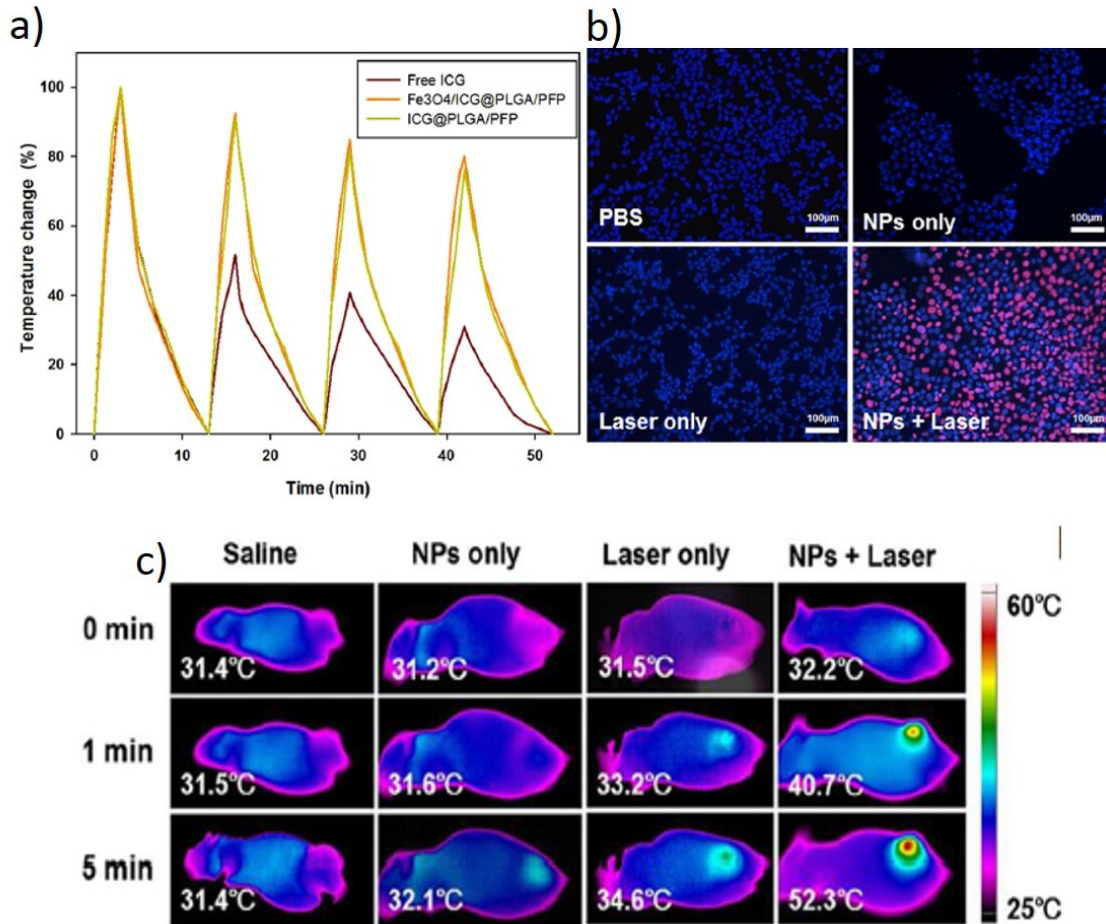


Figure 1.13: a) Temperature changes of free ICG, ICG@ PLGA/PFP NPs and Fe₃O₄/ICG@PLGA/PFP NPs after laser on/off cycles, b) fluorescence images of MCF7 cells treated with Fe₃O₄/ICG@PLGA/PFP NPs under 808 nm laser irradiation and c) thermal images of mice at different treatment times [13].

Kang et al. produced SPION/Prussian blue (SPION/PB) nanoparticles coated with polyetylenimine (PEI) and loaded with ICG (FPPI) for combined effect of PB and ICG in photodynamic and photothermal therapy [191]. The temperature increment was over 20 °C with 200 g/mL nanoparticle concentration upon irradiation at 808 nm (2 W/cm²) and the PTT conversion efficiency was reported as 51.53 %. Viability of nanoparticle (8 μg/mL ICG)+laser treated cells dropped down to 19.3

% in the absence and to 10.8 % in the presence of external magnetic field. Significant tumor suppression was reported in the *in vivo* experiments, as well. ICG loaded magnetic carbon nanoparticles (ICG@MCNPs) was used for infrared fluorescence/magnetic resonance dual-mode imaging and photothermal therapy [192]. After 5 min laser irradiation at 808 nm (2 W/cm^2), the temperature increment was recorded as 19.5 and 39.9 °C at 3 and 25 $\mu\text{g/mL}$ ICG concentration, respectively, while this laser treatment increased the temperature of PBS control by 9.4 °C. ICG@MCNPs caused more cell death (4T1 cells) than free ICG and MCNPs after 3 min laser irradiation. In the *in vivo* experiments, 9.37, 7.5, and 7.7 fold increase was observed in tumor volume with PBS, PBS+laser irradiation, and only ICG@MCNPs, respectively during 18 days. However, the combination of laser and ICG@MCNPs was able to ablate tumors totally with single irradiation at 5 mg/kg of ICG without any apparent necrosis. A core-shell magnetite nanocluster@poly(dopamine)-PEG@ICG nanobead acted as an efficient MR imaging and PTT agent with great stability [193]. At 3 $\mu\text{g/mL}$ ICG concentration, 60 % and 89 % cell mortality was observed in the absence and presence of magnetic field, respectively, when irradiated 5 min at 808 nm (2 W/cm^2). In *in vivo*, NPs irradiation under magnetic field showed a significantly tumor growth inhibition during one week; however, reoccurrence occurred after one week. ICG and doxorubicin loaded Fe_3O_4 @ Polydopamine/PEG nanoparticles (FPPI-D NPs) were used in combination of PTT/PDT and chemotherapy under magnetic guidance [194]. FPPI-D NPs induced higher temperature increase under 808 nm laser irradiation at 2 W than Fe_3O_4 or Fe_3O_4 @PDA NPs. It also showed great stability during laser on/off experiments indicating just 4.9 % loss in temperature increase between first and fourth cycle. ROS generation produced by free ICG and FPPI NPs was tested on HeLa cell and more ROS was observed after laser treatment of FPPI NPs. Under laser irradiation and magnetic field, almost complete cell death was achieved with FPPI NPs at 50 $\mu\text{g/mL}$ NP concentration after 10 min irradiation. Another example for combined chemotherapy and PDT/PTT via single agent is DOX loaded Fe_3O_4 @ MnO_2 @polypyrrole (Fe_3O_4 @ MnO_2 @PPy) nanocomposite [195].

To enhance the generation ability of singlet oxygen during laser treatment which enables to obtain enhanced PDT/PTT, PPy was first combined with MnO₂ to coat Fe₃O₄. Combined chemotherapy and enhanced PDT/PDT was achieved by loading DOX on the Fe₃O₄@MnO₂@PPy nanocomposite. DOX loaded Fe₃O₄@MnO₂@PPy caused 91.6 % and 76.2 % cell mortality with and without magnetic field, respectively at 1 W/cm². Another hybrid nanoparticle designed for combination of chemo and PTT/PDT is HMCuS/DOX@IONP-PEG [196]. It showed great PTT efficiency possessing 42 % conversion efficiency. There was a significant ROS generation after irradiation of HMCuS/DOX@IONP-PEG. In *in vitro*, cell viability decreased to 7.8 % with HMCuS/DOX@IONP-PEG +laser irradiation while it dropped to 42. 2 % and 61.7 % with free DOX and HMCuS/DOX@IONP-PEG. A significant reduction was observed in tumor growth with irradiation of HMCuS/DOX@IONP-PEG. Almost total tumor ablation and necrosis was reported if irradiation of nanoparticles were combined with magnetic field. DOX loaded porous hollow Fe₃O₄ was developed as MRI and combined PTT/chemotherapy agent [197]. In *in vitro*, alone hyperthermia with Fe₃O₄ and chemotherapy with DOX did not effectively kill tumor cells; however, combined PTT and chemotherapy with DOX loaded Fe₃O₄ induced 75 % cell death at 150 μ g Fe/mL when irradiated with 808 nm laser (1.5 W/cm²). Wilhelm and coworkers showed an effective photothermal heating from iron oxide nanocubes and an additive effect when magnetic hyperthermia was applied simultaneously (Figure 1.14) [14]. For example, the temperature increase of aqueous solutions at [Fe]=12 mM concentration was 17 °C, 9 °C, and 8 °C with the DUAL, laser and MHT mode (Magnetic field= 25 mT at 250 kHz frequency and laser power=0.3 W/cm²), respectively. They performed the same experiment with spherical, 9 nm iron oxide nanoparticles; however, iron oxide nanocubes provided 2-3 times more efficient results. In the *in vitro* studies, laser irradiation induced the same temperature increase as in the aqueous solution while MHT failed to do so. DUAL-mode caused more cell death (viability decreased to 14 %) than only laser (viability decreased to 74 %) or only MHT (viability decreased to 36 %) application at 12 mM iron concentration. DUAL-mode

caused complete tumor regression as a result of apoptotic cell death without any abnormalities.

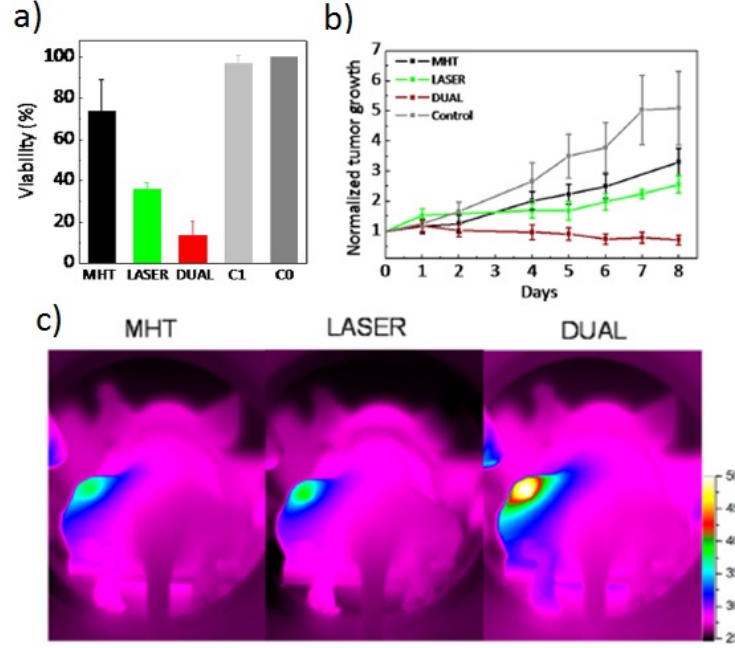


Figure 1.14: a) Cell viability of SKOV3 cells after 600 s each treatment, b) average tumor growth during 8 days and c) thermal image of mice during the treatment [14] .

Wilhelm and coworkers performed a comparative study based on composition of material (gold, iron oxide, and cobalt ferrite), shape of material (cubes, rods, spheres, stars and shells) and heating method (MHT and PTT) [198]. Probably this is the most elaborate work investigating the PTT characteristics of SPIONs so far. In these experiments, rock-like 9-11 nm maghemite nanoparticles (IONPs) and cobalt ferrite nanoparticles (CFNPs), 20 nm magnetite nanocubes (IONCs), and 25 nm maghemite nanoflowers (IONFs) as magnetic nanoparticles and 10 nm gold nanorods (AuNR), 150 nm nanoshells (AuNS) and 25 and 85 nm gold nanostars (AuNST) as plasmonic nanoparticles were used at a concentration of $1.4 \text{ g}_{Fe}/\text{L}$ and $0.15 \text{ g}_{Au}/\text{L}$. PTT and MHT experiments were performed at $0.3 \text{ W}/\text{cm}^2$ and $1 \text{ W}/\text{cm}^2$ and 18 mT-470 kHz and 24 mT-900 kHz. For PTT experiments, 808 nm laser irradiation was used for 85 nm AuNST, AuNS, and AuNR which has plasmon maxima at 800 nm and mag-

netic nanoparticles while 680 nm laser irradiation was applied for 25 nm AuNST which exhibits plasmon maxima at 650 nm. The temperature increase of these aqueous solutions showed that plasmon nanoparticles induced more temperature increase than magnetic nanoparticles at lower molar concentration. Second important finding is that IONFs induced less temperature increase in PTT experiments compared to MHT due to low absorbance of maghemite NPs in NIR. Nevertheless, magnetite and cobalt ferrite showed good PTT effect which caused by their absorbance. With all magnetic nanoparticles, the heating loss was observed in *in vitro* MHT experiments due to inhibition of Brownian relaxation or magnetic dipolar interactions by intra-endosomal aggregation. On the other hand, IONCs showed comparable SAR value (1200 W/g_{Fe}) with 25 nm AuNST (7500 W/g_{Au}) in PTT experiments at 1 W/cm^2 . *In vivo* experiments revealed that IONCs induced more temperature increase with PTT in comparison with MHT at same concentration. It also showed that AuNST provided more therapeutic effect at low doses than magnetic nanoparticles. As a final step, they compared the heating efficiency at the day of injection and 3 days after injection and reported that the efficiency decreased at day 3 for MHT and AuNST PTT while it enhanced for IONCs PTT.

1.7 Thermal Therapy in Bacteria Studies

The antibiotic-resistant bacteria not only result from the gene mutation or structural transformation of bacteria, but also arise from the formation of bacterial biofilms. It is estimated that up to 80 % of clinical infections, such as tooth decay, catheters and fracture fixation infection, are one of the most important and increasing public health problems is the antibiotic-resistant bacterial infections. According to Center for Disease Control and Prevention, at least 2 million people are infected by an antibiotic-resistant bacteria, and at least 23000 of these people die every year in USA. The an estimated economic burden of this is above 35 billion/year [199]. European Centre for Disease Prevention and Control reported that 33000 people die every year in EU due to infections with antibiotic-resistant bacteria and the cost of this is about

1 billion Euro/year. They also report a serious number of health-care related infections and state that 1 out of every 3 patients are infected every day, reaching to approximately 8.9 million cases per year in both hospitals and long-term care facilities. Antibiotic resistance is usually due to genetic mutation of bacteria but there is solid evidence that excessive and unnecessary use of antibiotics accelerated the development of drug resistance. Biofilm formation is a major hurdle in the fight against such infections. According to the report of National Institutes of Health (NIH), 65 % of microbial and 80 % of chronic infections, are associated with biofilm formation [200]. The close contact between bacteria and formation of protective matrix overcoating prevents drug penetration into the biofilm. Biofilm formation is reported on breast implants, prostheses, heart valves, pacemakers, defibrillators, catheters, contact lenses, etc [200]. There are serious non-device related ones such as periodontitis which is a gum infection and osteomyelitis which is a bone disease. Considering the increasing anti-bacterial resistance, number of patients infected and died due to such infections and the economic burden on countries, quick, practical and effective solutions to eradicate drug resistant biofilms and defeat antibiotic resistant bacterial infections are extremely urgent.

In recent years, many studies proposed that SPIONs as themselves, combination of PTT and iron oxide nanoparticles or MHT and iron oxide nanoparticles showed great antimicrobial activity. Javanbakht et al. investigated the effect of bare (14 nm), negatively charged (20 nm) and positively charged (21 nm) SPIONs on a bacterial biofilm (*Streptococcus mutans*) [201]. At higher SPIONs concentration, positively charged nanoparticles induced more bacteria killing than SPIONs while the effect was almost same for lower concentrations. When compared to negatively charged SPIONs, positively charged killed bacteria greater at all concentrations. Leuba et al. evaluated the reduction in growth of *Staphylococcus aureus* (*S. aureus*) using various functionalized SPIONs without any antibiotics after 3, 10 and 24 hours treatment [202]. A decrease was 33.5 %, 31.1 %, and 28.1 % with carboxylate, isocyanate, and amine functionalized SPIONs, respectively. Geilich et al. developed biocompatible

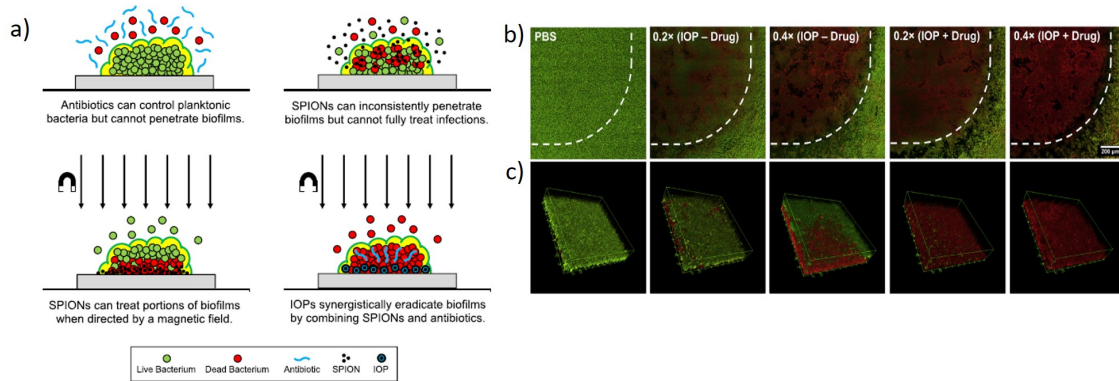


Figure 1.15: a) Treatment of biofilms using SPIONs and antimicrobials, b) Live/dead staining of biofilms under treatment with SPIONs magnetic field and c) 3-D reconstructions of the biofilm thickness under magnetic field [15].

nanocarriers which contain SPION and antibiotic methicillin to treat medical-device-related infections under magnetic field [15]. Due to applied magnetic field, SPIONs were able to penetrate into *S. epidermis* RP62a biofilm which show high resistance to methicillin. An important reduction was observed in volume of biofilm while complete eradication was achieved in the presence of both SPIONs (40 $\mu\text{g}/\text{mL}$) and methicillin (20 $\mu\text{g}/\text{mL}$) (Figure 1.15). Darwish et al. synthesized SPIONs with coating of oleic acid (132 nm), PEI (174 nm) and PEI-methyl cellulose (242 nm) on single gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) and biofilms [203]. The temperature reached 55.8, 75 and 65.4 $^{\circ}\text{C}$ with OA-MNP, PEI-MNP, PEI-Mc-MNP, respectively under 1 kW of magnetic field for 5 min. Positive results were observed with all NPs; however, best result which is 10 % growth inhibition with <150 mg/l was achieved with PEI-Mc-MNP. Park et al. investigated the SPION efficiency on destruction of *Pseudomonas aeruginosa* (*P. aeruginosa*) PA01 biofilm under magnetic field [16]. The temperature reached 55, 60, and 62.3 $^{\circ}\text{C}$ at 40, 50, and 60 mg/mL SPION concentration under 3 kA/m magnetic field with the frequency of 492 kHz. A dramatic destruction (more than 4 log) occurred with 60 mg/mL SPION concentration in 8 min in the presence of magnetic field. On the other hand, SPION at a concentration of 60 mg/mL did not produce any change on biofilm volume

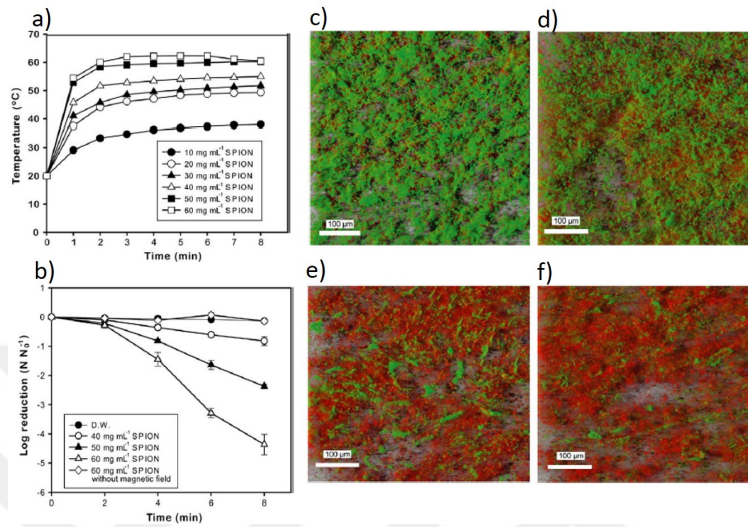


Figure 1.16: a) Temperature increase of SPIONs at different concentrations on the PA01 biofilm under magnetic field, b) quantitative assay of the reduction in PA01 biofilm treated with SPIONs under magnetic field and c) microscopy images of biofilms after hyperthermia treatment at different concentrations (c-f)[16].

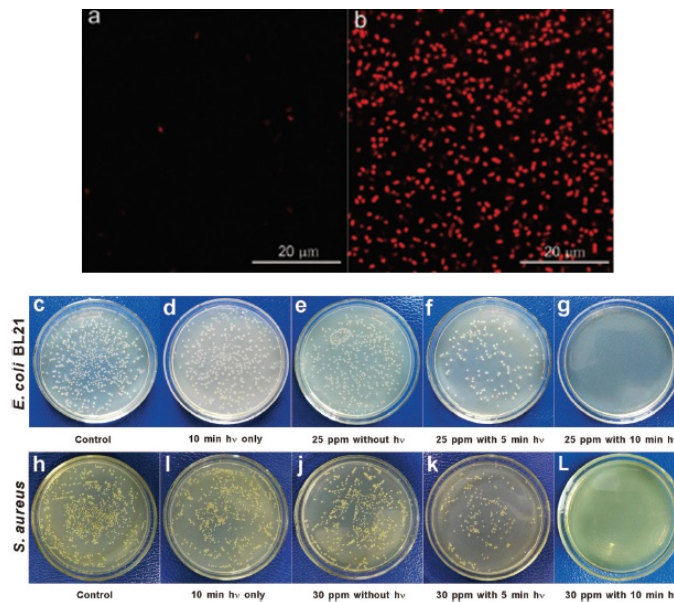


Figure 1.17: Confocal images of E.coli a) before and b) after laser treatment and c) growth of E.coli and S.aureus treated with nanoparticles before and after treatment [17].

without magnetic field(Figure 1.16). The photothermal potential of Ag@Fe₃O₄-PEI NPs was tested on E.coli BL21 (gram-negative bacteria) and S. aureus (gram-positive bacteria)under 808 nm laser irradiation at 2 W/cm² for 10 min [17]. Ag@Fe₃O₄-PEI NPs caused total inhibiton for E.coli BL21 and S. aureus at 25 ppm and 30 ppm concentration,respectively(Figure 1.17).

1.8 The Proposal and the Aim of the Thesis Work

SPIONs of various sizes, shapes and coatings are being used and investigated for contrast generation in MRI for diagnostic, magnetic dragging of nanoparticles to a site of interest or for capturing magnetic nanoparticles, delivery of drugs/genes in the absence or presence of magnetic field and magnetic hyperthermia where thermal ablation of tumors are aimed. This multifunctional nature allowed combination of many of these tasks in a single particle such as magnetic imaging, dragging, drug/gene delivery combined with magnetic hyperthermia. Yet, they are considered as theranostic (therapeutic+diagnostic) nanoparticles. While SPIONs were being investigated for the delivery of photosensitizers for photodynamic therapy, it was recognized that without the presence of any photosensitizer, SPIONs themselves may provide local temperature increase when irradiated with near-infrared (NIR) laser, providing photothermal therapy. Although SPIONs are known for magnetic hyperthermia, the set up for such procedure is far from practical. On the other hand, laser treatment may be much more local and easier considering surface applications and combination of lasers with fibers, endoscopes, etc. This is also extremely exciting since many photosensitizers, organic or inorganic, mostly require the UV, and few, the visible wavelengths. UV is not safe, visible light and UV have limited penetration depth. Use of NIR laser is safer for the unsensitized tissue and provide better penetration depth (few cm) but requires more complex structures such as gold nanorods, upconverting nanoparticles or some special quantum dots, which are not FDA approved, yet. Amongst all inorganic photosensitizers SPIONs does have an upper hand since there are FDA approved examples and are considered safe. In a way, these findings made researchers

to repurpose an existing material. Also, unlike alternative nanoparticles that may be excited at the NIR, synthesis of SPIONs are relatively low cost, reproducible and more controllable. However, most successful examples of SPIONs, which were only few at the beginning of this thesis work, report wavelength dependence and better results with clustered or cubic nanoparticles of iron oxide. Clustered particles are not good for real clinic applications since large particles quickly clear from blood and it would be difficult or impossible to target them to diseased sites with molecular recognition. Cubic particles are more difficult to make and keep stable. Hence, this thesis is devoted to investigate the PTT potential of small, stable, spherical, aqueous SPIONs, which are easy to make and modify if needed, and understand its potential in cancer therapy via *in house* experiments.

The first goal of the thesis work is to investigate the PTT mechanism of SPIONs and the differences in the properties of SPIONs before and after the laser treatment. To achieve this, the effect of three major-laser dependent parameters: i) wavelength, ii) intensity and iii) power was studied on the photothermal heating induced by small, stable and highly biocompatible poly(acrylic acid) coated SPIONs (PAA@SPIONs) which are known in the literature and used very frequently in our group. This particle is safe, easy to make in small sizes (less than 100 nm hydrodynamic size) and has a very long self-life (years). To determine wavelength dependence of SPION based photothermal heating, temperature increase was measured between 728-838 nm with 10 nm steps using 260 mW a tunable, green-pumped continuous-wave Ti^{3+} :sapphire laser. Intensity and power dependence were studied between 1.85-9.76 W/cm² intensity keeping the power constant with different spot sizes and at various power values between 105-280 mW keeping the spot area constant, respectively. Laser on/off experiments and detailed characterization of SPIONs before and after laser irradiation was performed to understand if the interaction of light with SPIONs under these conditions cause and fundamental change in the properties of SPIONs, which may influence to re-usability of nanoparticles. PTT potential was tested *in vitro* based on optimized laser parameters using tumor cells.

The second goal of the thesis work is to combine photothermal therapy and photodynamic therapy via a single laser treatment, where instead of thermal ablation sensitization of tumor to enhance the PDT outcome may be achieved. For this purpose, an FDA approved fluorescent dye, Indocyanine green (ICG), with reported PDT/PTT potential was used. As the SPION composition, trimethoxy aminosilane coated SPIONs (APTMS@SPIONs), which are again small, stable and easy to make nanoparticles, well known in the literature were chosen, mostly because of their cationic character. Anionic ICG was loaded electrostatically to cationic APTMS@SPIONs. This combination aim to provide major advantages: i) prevent the instable structure of ICG, ii) enhance bioavailability of ICG, iii) excite both SPIONs and photosensitizer at the same wavelength (795 nm) to achieve a synergy with a single laser treatment, iv) achieve dual mode imaging (MRI and optical) where florescence may be utilized easily during the laser treatment allowing image guided therapy. Solution heating experiments were used to investigate the photothermal heating potential of ICG, APTMS@SPION and ICG loaded APTMS@SPIONs. In the *in vitro* tests conducted with breast and colorectal tumor cells, the combined PTT/PDT effect, cell death mechanism and optical imaging capabilities were examined.

The third goal of the thesis work is to adopt this approach to biofilm eradication. Biofilms and antibiotic resistant bacteria are extremely difficult problems awaiting urgent solution. Thermal ablation or even mild hyperthermia may be advantages to enhance the sensitivity of bacteria to antibiotics and penetrate into the biofilms. Indeed, in the last two years, there is an enormous activity in the field of phototherapies directed to biofilms. Indeed, this may be an area where nanoparticle based phototherapies may be practically used much sooner than in clinic for cancer therapy. In order to determine the antimicrobial activity SPION based PTT, both APTMS@SPIONs and ICG-APTMS@SPIONs were tested on planktonic and sessile cells of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus epidermidis*. Antimicrobial activity of these nanoparticles were compared with the antimicrobial activity of free ICG. Dark toxicity of all three reagents were also investigated.

The fourth goal of this thesis is to extend this work to combined chemotherapy and PTT. For this purpose Etoposide, a hydrophobic chemotherapeutic agent, was chosen. As a carrier, PAA@SPION was modified with bovine serum albumin (BSA) with an inspiration from Abraxane, which is a crosslinked BSA nanoparticle loaded with hydrophobic Paclitaxel. Etoposide was loaded to BSA@PAA@SPION. PTT potential of BSA@PAA@SPIONs was investigated in aqueous solutions and the therapeutic effect of the new nanostructure was tested *in vitro* on three different prostate cancer cell lines (PC3, LNCaP, and DU145).

Chapter 2

PHOTOTHERMAL POTENTIAL OF PAA@SPIONS

2. INVESTIGATION OF THE FACTORS AFFECTING THE PHOTOTHERMAL THERAPY POTENTIAL OF SMALL IRON OXIDE NANOPARTICLES OVER THE 730-840 nm SPECTRAL REGION

2.1 Introduction

Chemotherapy and radiotherapy are the most widely used therapeutic approaches for cancer treatment and are often used in combination. However, they both suffer from serious side effects and damage healthy tissue. In recent years, great efforts have been made in the development of local therapies which include targeted drug delivery [204, 205] and alternative treatment methods such as photodynamic therapy (PDT) [206, 207] and photothermal therapy (PTT) [168].

PTT emerged as a local and promising therapeutic method in cancer treatment, where irradiation of sensitive nanoparticles with near infrared (NIR) laser creates a local temperature increase (hyperthermia) at the tumor site. Usually gold nanoparticles [160, 161], C-nanotubes [159], and graphene [158] are investigated for PTT. However, there are issues limiting their clinic application such as limited biodegradability and photostability as well as long-term toxicity. Recently, superparamagnetic iron oxide nanoparticles (SPIONs) which are used as contrast agents in MRI and for magnetic hyperthermia have been recognized as PTT agents as well. Some SPION formulations are already in clinical use and magnetic hyperthermia has been investigated for a while but its clinic use may be limited. Hence, PTT potential of SPIONs are very exciting in the field of medicine. PTT, on the otherhand, has still some drawbacks limiting its clinical applications. These include necessity of high particle concentra-

tion and high laser power. In the case of SPIONs, although most of the studies relate the photo-thermal heating effect of SPIONs to absorbance, the mechanism underlying this effect is not well understood yet. Comparison of the reported data is not so easy either, due to the differences in the experimental setup or protocols, including differences in the sample volume, container and laser power used. The influence of size was considered as a variable and studied in the literature. The reported data favour clustered particles. However, small (less than 150 nm) or ultra-small (less than 50 nm) sizes are usually preferred for many *in vivo* studies with the consideration of biodistribution and ability to utilize receptor mediated targeting.

Here, we studied the photothermal heating effect of small, highly biocompatible poly(acrylic acid) coated superparamagnetic iron oxide nanoparticles (PAA@SPIONs) prepared via an aqueous precipitation method wherein the growing crystals were coated with biocompatible PAA *in situ*. The dependence of photothermal heating on laser wavelength over the 728-838 nm range along with the attenuation was investigated for the first time in the literature. In addition, the influence of laser intensity and power (relatively low power compared to those reported in the literature) was investigated in well-controlled experiments. In order to further investigate the origin of the heating and the effect of laser irradiation on the nanoparticle properties, a detailed characterization of PAA@SPIONs, before and after laser irradiation, was performed. Lastly, the applicability of these small nanoparticles for PTT was tested on the HeLa cell line with a biomedically acceptable wavelength and laser power.

2.2 Experimental

2.2.1 Materials

Iron(II) chloride and iron(III) chloride were purchased from Merck (purity levels 99 %, Darmstadt, Germany). Poly(acrylic acid) (Mw 2100 kDa) and dimethyl sulfoxide Hybri-MaxTM were obtained from Sigma (MO, USA). Vivaspin 20 centrifugal filters (30 kDa MW cut-off) were obtained from Sartorius (Goettingen, Germany). Roswell Park Memorial Institute 1640 medium (RPMI 1640, 1×), trypsin EDTA and

penicillin–streptomycin solutions were obtained from Multicell, Wisent Inc. (QC, Canada). Fetal bovine serum (FBS) was provided by Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Phosphate-buffered saline (PBS) tablets and thiazolyl blue tetrazolium bromide (MTT) were obtained from Biomatik Corp. (ON, Canada). 96-well plates were obtained from Nest Biotechnology Co., Ltd (Wuxi, China). Only ultra-pure water (18.2 M Ω , RephiLe Bioscience and Technology, Shanghai, China) was used when needed. All reagents were of analytical grade or of the highest purity.

2.2.2 Iron oxide nanoparticle synthesis

Poly(acrylic acid) coated SPIONs were synthesized by a coprecipitation method. Briefly, FeCl₃·6H₂O and FeCl₂·4H₂O were first dissolved in deoxygenated water at a mole ratio of Fe³⁺/Fe²⁺ of 2/1. PAA (2 g) was then added under continuous nitrogen flow. The suspension was heated to 85 °C and 4.63 mL of 4.5 M NH₄OH was injected under vigorous stirring. The solution was maintained at this temperature for 30 min and cooled to room temperature. The solution was left on a magnet overnight to remove any precipitated particles. Finally, PAA@SPIONs were washed several times with DI water using Vivaspin 20 centrifugal filters (30 kDa MW cutoff) and stored at room temperature.

2.2.3 Characterization

The absorbance spectra of the PAA@SPIONs were obtained using a Shimadzu UV-Vis-NIR spectrometer in the 500–900 nm range. Hydrodynamic size measurements were performed by using a Malvern Zetasizer Nano ZS. Particle shapes and morphologies were recorded with a transmission electron microscope (TEM-Philips-FEI-Tecnaï G2 F30) operating at an accelerating voltage of 200 kV. A drop of aqueous suspension was added on a carbon coated Cu grid. The iron content of nanoparticles was measured using an Agilent 7700XICP-MS (inductively coupled plasma-mass spectrometer). The samples were etched with nitric acid and sulphuric acid. Magnetic characterization was performed on a Quantum Design SQUID VSM at 300 K. X-ray

photoelectron spectroscopy (XPS) was performed on a Thermo Scientific K- α XPS with Al K $_{\alpha}$ monochromatic radiation.

2.2.4 Cell culture and conditions

HeLa cells were cultured in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum and 1 % (v/v) penicillin–streptomycin and incubated in a 5% CO₂ humidified incubator at 37 °C.

2.2.5 Cytotoxicity assay

The cytotoxicity of the prepared nanoparticles on HeLa cells was measured with an MTT (thiazolyl blue tetrazolium bromide (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl-tetrazolium bromide)) assay. HeLa cells were seeded at a density of 1×10^4 cells per well into 96-well plates with culture medium and incubated in a 37 °C, 5 % CO₂ humidified incubator for 24 h. On the second day, the medium was changed to a fresh culture medium, containing PAA@SPIONs and incubated further for 24 h. Afterwards, the cells were washed with PBS(pH = 7.4, 1 $\times$) to remove uninternalized nanoparticles and incubated for 4 h after the addition of 50 μ L of MTT solution(5 mg/mL⁻¹ in PBS) and 150 μ L of culture medium into each well. Purple formazan crystals formed by the viable cells were dissolved with DMSO : ethanol (1 : 1 v/v) by gentle shaking for 15 min. The absorbance intensities were recorded at 600 nm with a reference reading at 630 nm using a BioTek ELX800 absorbance microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Relative cell viability was calculated by applying the following formula with the assumption of 100 % viability of control cells which did not receive any treatment:

$$Cellviability = [Absorbance_{sample}/Absorbance_{control}] \times 100 \quad (2.1)$$

2.2.6 Photothermal heating procedure for colloidal PAA@SPION solutions

To investigate the wavelength dependence of photothermal heating caused by PAA@SPIONs, a tunable, green-pumped continuous-wave Ti^{3+} :sapphire laser, operating in the 728-838 nm wavelength region, was employed.

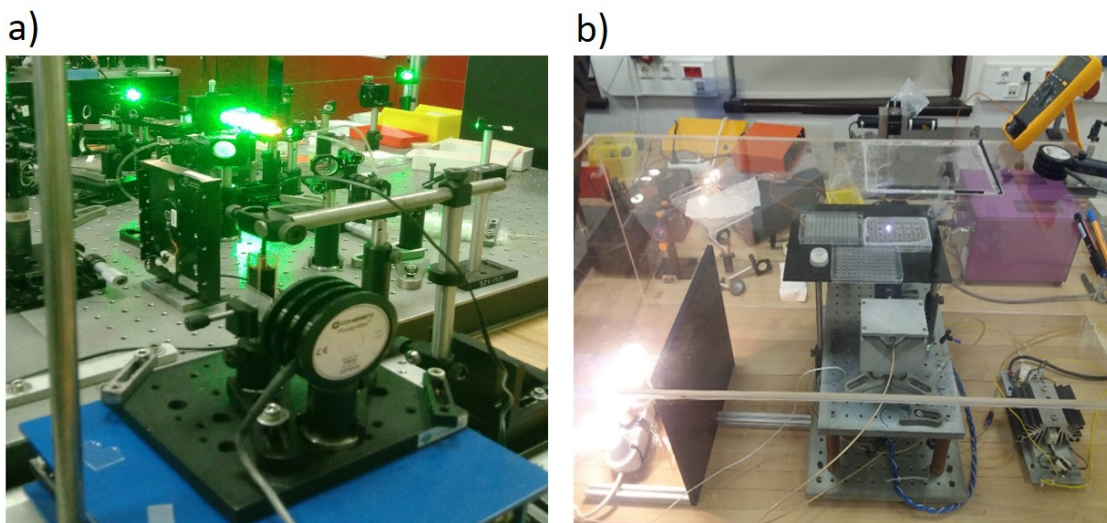


Figure 2.1: Our laser setup for a) solution and b) *in vitro* PTT experiments.

Our laser set up for solution and *in vitro* experiments is shown in Figure 2.1. An SF-10 dispersing prism inserted into the resonator of the Ti^{3+} :sapphire laser enabled the tuning of the output wavelength in steps of 10 nm in the 728 to 838 nm range. Intensity/power-dependent measurements were further performed by using a continuous-wave diode laser at 785 nm and a fiber-coupled laser diode array at 795 nm. For the wavelength dependent measurements, the aqueous solution of PAA@SPIONs (0.75 mL) at 38 mM Fe concentration was placed in a polystyrene cuvette and irradiated at different laser wavelengths (728-838 nm with 10 nm steps) for 20 minutes. The incident power at each wavelength was kept constant at 260 mW, corresponding to an intensity of 3 W/cm². A new sample was used for each measurement and each measurement was performed in triplicate. The intensity and power-dependent measurements were performed by following the same procedure with a 785 nm laser

diode. The temperature increase was measured with a thermocouple. The experimental setup scheme is shown in Figure 2.2a.

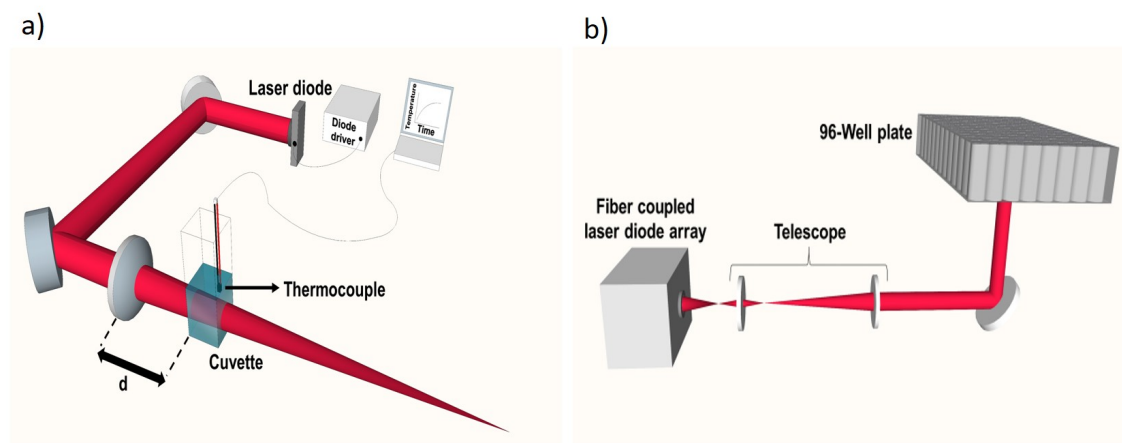


Figure 2.2: Schematic of the experimental setup for (a) solution measurements and (b) *in vitro* measurements. The initial temperature is set at 37 °C for *in vitro* measurements.

2.2.7 *In vitro* PTT procedure

The HeLa cells were seeded in a 96 well plate and incubated with three different concentrations (500, 1000 and 2000 $\mu\text{g/mL}$). Then, the cells were irradiated with a fiber-coupled laser diode array (795 nm) for 10 min from the bottom of the plate (Figure 2.2b). The initial temperature was kept constant at 37 °C for *in vitro* measurements ($n = 4$, number of replicates). The cell viability was measured by MTT assay after 24 h of incubation.

2.2.8 Statistical analysis

For toxicity tests, statistical significance was determined using Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparison test of GraphPad Prism software package (GraphPad Software, Inc., USA). p-Value 0.05 was considered as the indication of statistical significance. All data were reported as mean $\pm$ standard deviation (SD).

2.3 Results and Discussion

2.3.1 Photothermal effect of superparamagnetic iron oxide nanoparticles in solution

Colloidally stable PAA@SPIONs were prepared via a simple aqueous route (Figure 2.3). The resulting nanoparticles were spherical with an average crystal size of 4 ± 1 nm based on the TEM image analysis (Figure 2.4a). It is important to note that these particles are quite small compared to those reported in previous studies (9-20 nm ([14, 11]) or 225 nm ([12])). Intensity-based and number-based hydrodynamic sizes of these particles are 101.8 nm and 14.22 nm, respectively, according to the DLS measurements (Figure 2.4a and b).

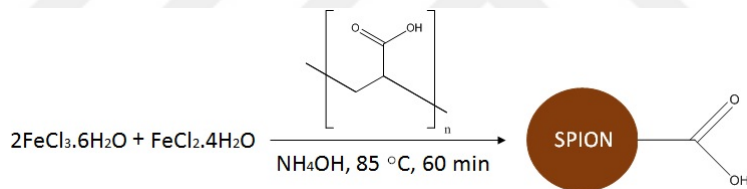


Figure 2.3: Aqueous synthesis of SPION@PAA

The influence of three major parameters ((i) laser irradiation wavelength, (ii) laser intensity and (iii) laser power) on photothermal heating obtained from colloidal PAA@SPIONs in aqueous solutions was investigated at a constant iron concentration of 38 mM. Figure 2.5a shows the temperature increase (ΔT) induced by PAA@SPIONs upon excitation at 785 nm with 260 mW (3 W/cm^2) of laser power for 20 minutes. The major increase in the temperature, which is about 5°C , was almost completed in the first 5 minutes (Figure 2.5, blue column).

When DI water was used as a control, no temperature increase was detected under identical conditions, indicating that the source of temperature increase is the SPIONs in water (Figure 2.5a, red column, and 2.6). If the laser power was increased to 800 mW (2 W/cm^2), a 5.9°C temperature increase was achieved in 100 s and a 12.8°C increase was obtained after 20 minutes (Figure 2.5a, green column). First, the amount of temperature increase after 100 s is comparable to those reported for 9 nm

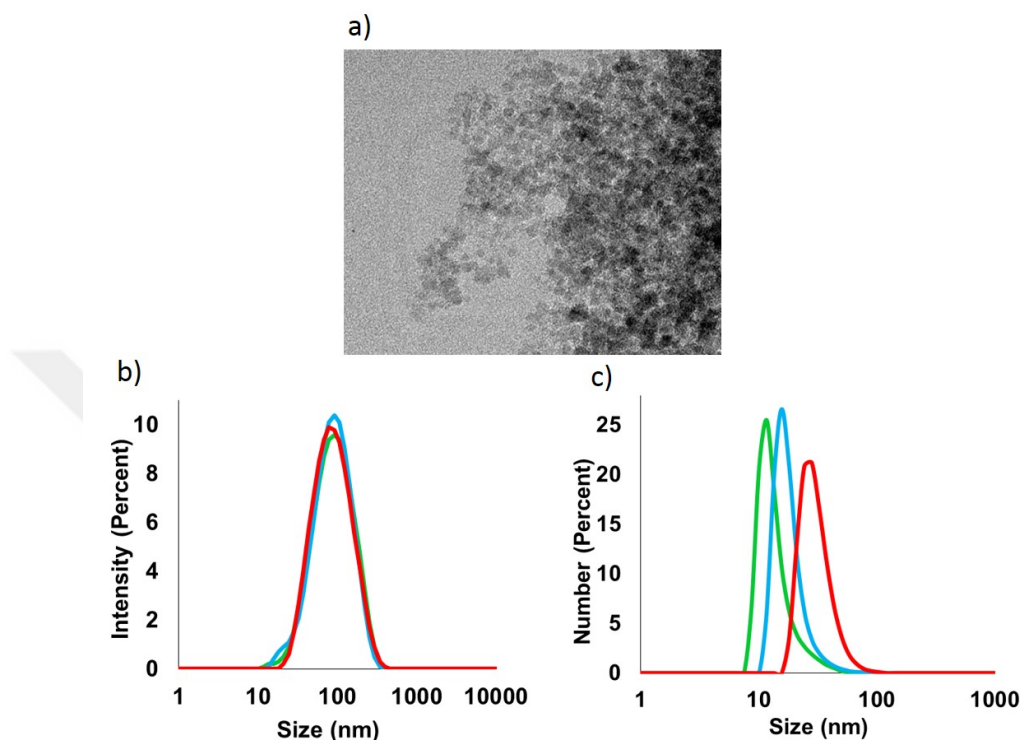


Figure 2.4: a)Transmission electron microscopy (TEM) image, b)intensity based hydrodynamic size, and c)number based hydrodynamic size of PAA-SPIONs.

iron oxide crystals at a comparable irradiation power and intensity by Wilhelm et al. In many reports, the laser spot size is not given, which makes it impossible to calculate the real power used. However, in most of the examples found in the literature, there is usually a couple of degrees increase in the temperature of water which is used as a control, even at low laser irradiation intensities. This is possibly due to the high value of the actual power. In our study, no temperature increase was observed in water in the control experiments. This indirectly indicates that relatively lower laser power levels are used here in comparison with many of the reported studies. Also note that although the intensity during 800 mW excitation is lower, a higher temperature increase was observed. This is because photothermal heating in solutions depends predominantly on the total incident power rather than the intensity, as discussed further below.

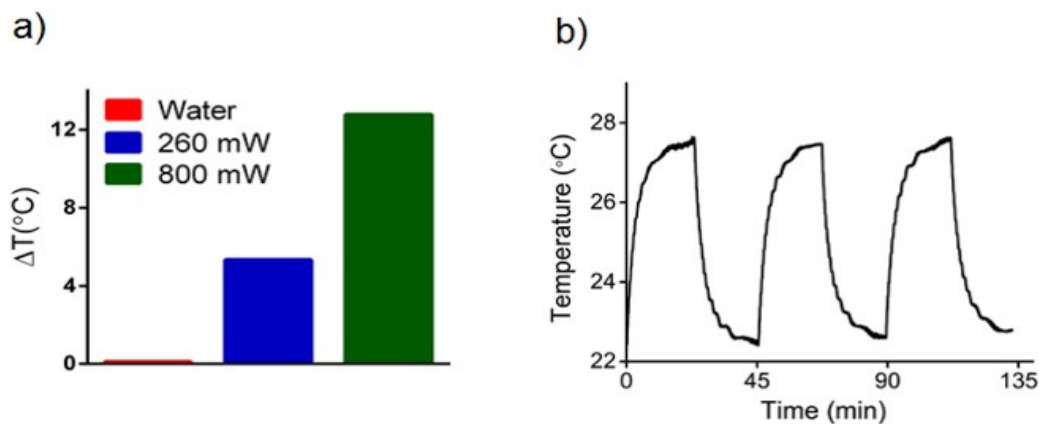


Figure 2.5: a) Measured temperature increase of colloidal PAA@SPIONs and water after 20 min of laser irradiation and b) laser on/off experiments under 260 mW (3 W/cm^2) of incident power. In all experiments, the irradiation wavelength was 785 nm except for the 800 mW (2 W/cm^2) experiment which was performed at 795 nm.

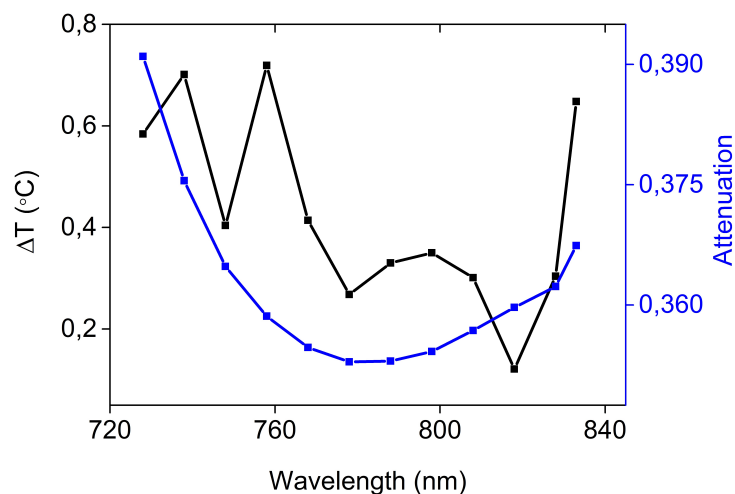


Figure 2.6: Temperature increase of water at 728-833 nm region after 20 min irradiation with 260 mW laser power (spot size 1.68 mm).

When nanoparticles were subjected to four cycles of laser on/off irradiation under the same conditions, the same ΔT was observed after each cycle, indicating that the origin of such a mechanism is not eliminated during the heating or laser irradiation, and the same photothermal effect may be obtained from the same solution after

several heating/cooling cycles (Figure 2.5b). The same result was observed when the nanoparticles were irradiated at 728 and 808 nm (Figure 2.7).

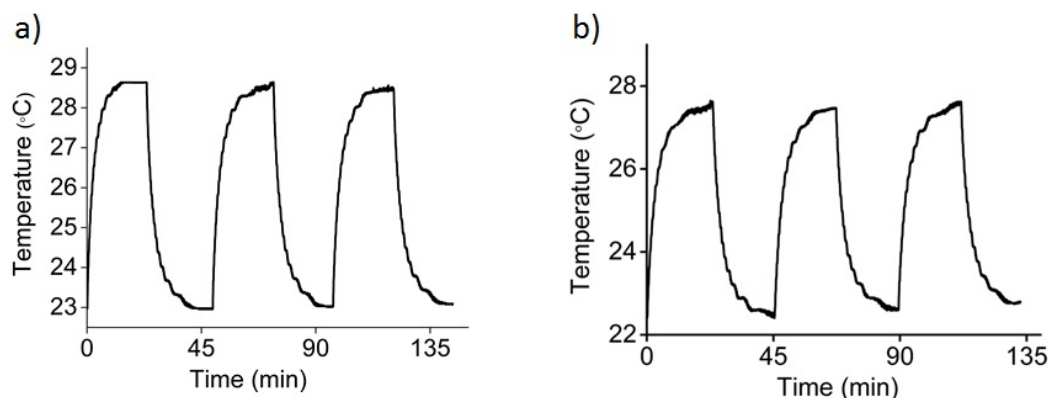


Figure 2.7: Laser on/off experiments of colloidal PAA@SPION solution. Irradiation wavelength, power, spot size: (a) 728 nm, 260 mW, 1.68 mm and (b) 808 nm, 260 mW, 1.68 mm.

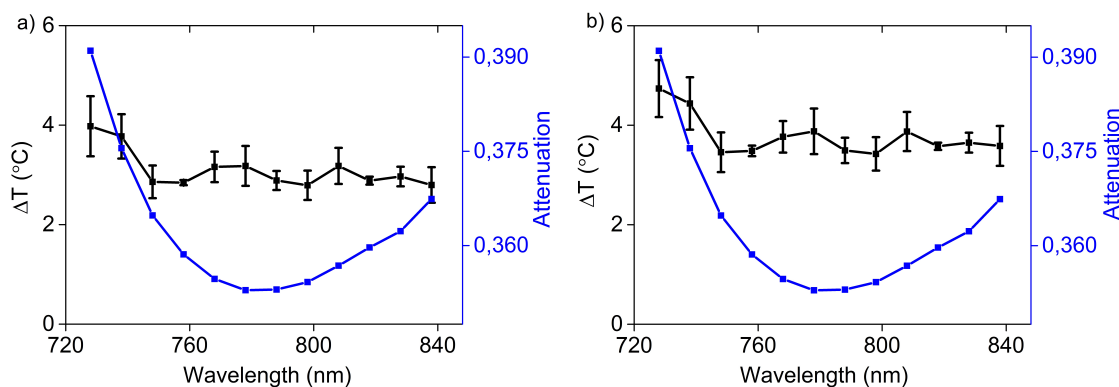


Figure 2.8: Temperature increase (ΔT) recorded for colloidal PAA@SPION irradiated (a) 5 min and (b) 10 min at different wavelengths (728-838 nm, power 260 mW, spot size 1.68 mm).

Next, the dependence of the temperature increase of the colloidal PAA@SPION solutions on the laser irradiation wavelength was tested over the 728-838 nm wavelength range. The temperature increase was measured after 5, 10 and 20 minutes of irradiation at these wavelengths (Figure 2.8a,b and 2.9). Since most of the literature

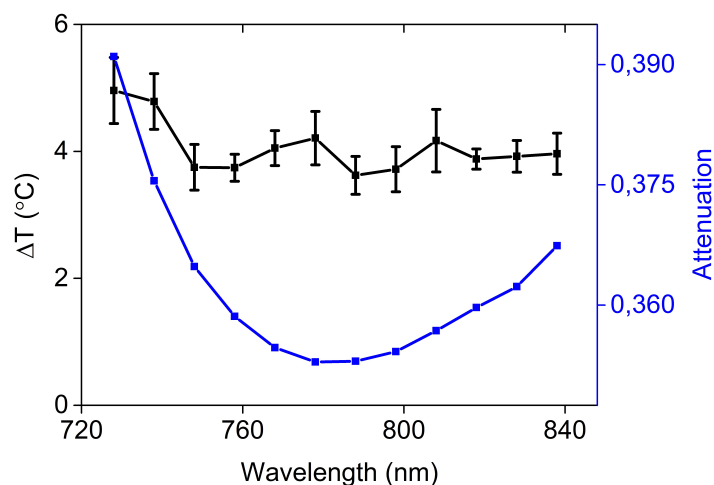


Figure 2.9: Temperature increase measured for colloidal PAA@SPIONs at different irradiation wavelengths (728-838 nm with 10 nm steps) with 260 mW (3 W/cm^2) laser power after 20 min of irradiation.

related photothermal heating of SPIONs to the absorbance at the excitation wavelength, we have checked the attenuation at these wavelengths, as well. Figure 2.9 also shows the measured attenuation of the PAA@SPIONs in this wavelength range, determined from the measured transmission (T) by using the formula: 'attenuation = $-10 \log(T)$ '. Note that there appears to be no strong correlation between the measured attenuation and the strength of the photothermal effect. This is mainly because transmission losses result from both absorption and scattering in the medium, and the strong wavelength dependence in the attenuation (see Figure 2.9) below 780 nm is probably due to scattering (due to its typical λ^{-4} dependence) and not absorption. To recognize the fact that transmission losses can be in general due to both scattering and absorption, the vertical scale in Figure 2.9 was labelled as 'attenuation'. The data shown in Figure 2.9 suggest that although one expects to see a strong correlation between ΔT and absorption, a simple transmission measurement may not reveal this relationship since both scattering and absorption contribute to losses in the transmission. The slight temperature increase below 740 nm may be due to an increase in the absorption of SPIONs at lower wavelengths. This requires further investigation.

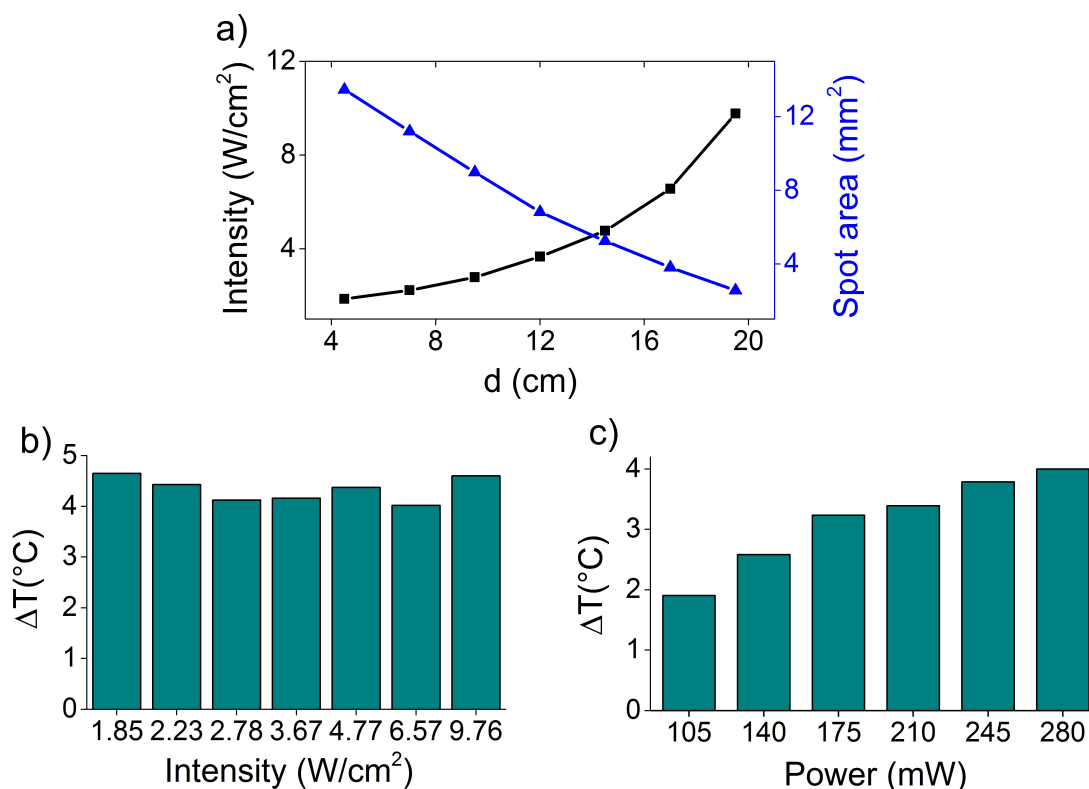


Figure 2.10: a) Intensity and spot area as a function of distance from the lens (d), temperature increase obtained with PAA@SPIONs at b) different laser irradiation intensities (at constant power, different spot sizes) and c) different laser powers at 785 nm laser irradiation (at different powers, constant spot size).

Another important parameter in PTT is the total laser power incident on the nanoparticles. In the literature, experimental procedures typically specify the incident intensity/fluence in units of W/cm^2 . This is because the total required power to attain a specific temperature rise can then be determined from the knowledge of the surface area of the tissue. In particular, in the case of photothermal heating in solution, it is further important to explore whether the extent of photothermal heating depends on the power, intensity or both. This is especially important if the absorption mechanism of the nanoparticles has saturation behaviour, which typically leads to an intensity-dependent absorption coefficient, as is the case in several solid-state bulk absorbers. In such a case, one would expect a change in the absorption

and hence heating characteristics of the nanoparticles when the effective beam area is varied, while maintaining the same power on the sample. To explore such saturation behaviour, the experimental setup shown in Figure 2.2a was used. The output beam (spot area of 1.98 mm × 2.97 mm) from the 785 nm diode laser was focused with a converging lens of 25 cm focal length to produce a converging beam whose measured spot size varied as a function of the distance (d) from the lens, as shown in Figure 2.10a. The resulting variation in the incident beam intensity at a constant beam power of 260 mW is further plotted in Figure 2.10a. To explore whether the temperature increase depends on the power or the intensity, two sets of measurements were done. In the first set, the temperature increase was recorded for seven different cuvette positions along the focused beam axis, while keeping the input power constant at 260 mW. Figure 2.10b shows the measured ΔT for the seven different intensities between about 2 to 10 W/cm². In the second experiment, the cuvette was placed at the largest beam area position and the temperature was measured as a function of input power between 105 and 280 mW (0.75 W/cm², 1 W/cm², 1.25 W/cm², 1.5 W/cm², 1.75 W/cm², and 2 W/cm²) at this location (Figure 2.10c). As can be seen from these measurements summarized in Figure 2.10, the temperature increase of the solution depends on the power and not on the intensity, indicating that there are no intensity-dependent saturation effects in the absorption mechanism of SPIONs up to the maximum incident intensity investigated in our experiments. As noted above, specifying the incident laser radiation in the units of W/cm² is important for *in vitro/in vivo* PTT applications, where the total required incident power can then be determined from the knowledge of the tissue surface area. In addition to these measurements, several particle properties were investigated before and after the laser irradiation to detect any possible changes as a result of laser irradiation which may be pinned down as the origin of the photothermal effect in SPIONs. No significant change in attenuation, or saturation magnetization was observed after 20 minutes of laser irradiation (Figure 2.11). No change in the magnetization is very valuable for possible medical applications. XPS could give information on the Fe states but

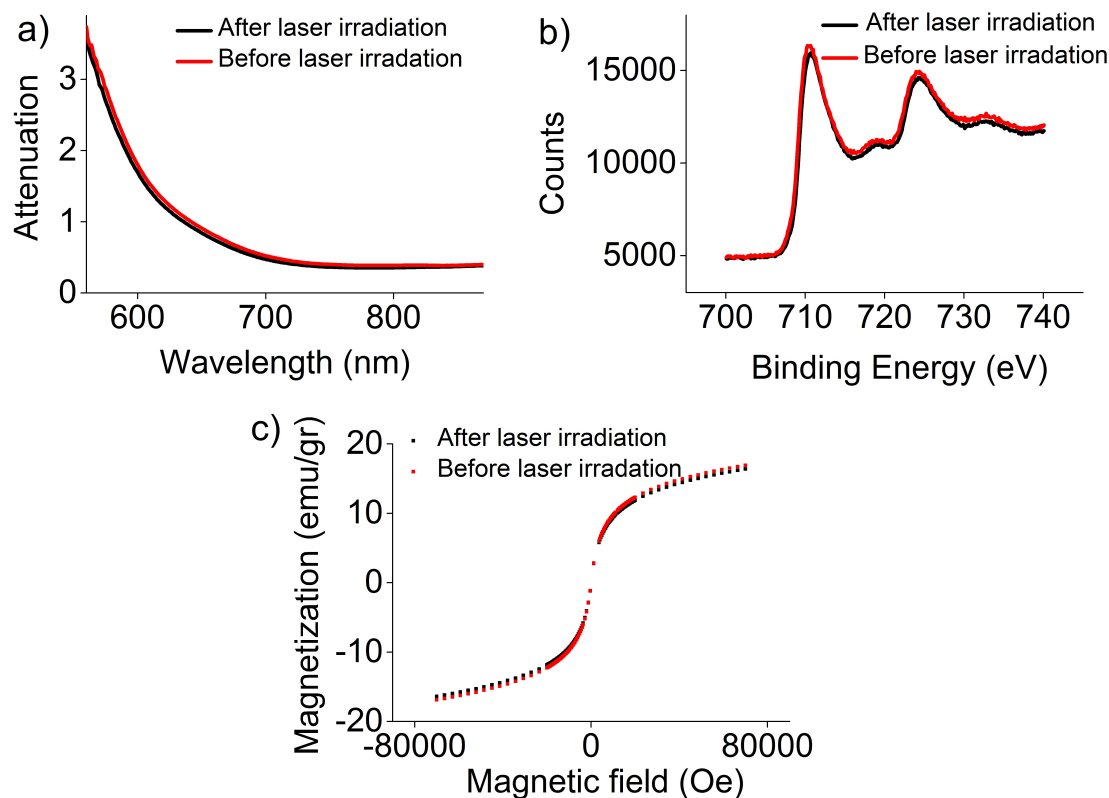


Figure 2.11: (a) Absorption spectra of PAA@SPIONs before and after laser irradiation, (b) magnetization spectra of PAA@SPIONs before and after laser irradiation, and (c) binding energy of Fe (2p) before and after laser irradiation.

showed no significant difference before and after irradiation which at least partially agrees with the maintained magnetic properties. The intensity based hydrodynamic size of these nanoparticles was 101.8 nm and 102.5 nm before and after laser irradiation, respectively. No change in the hydrodynamic size indicates that there is no loss of the coating and related aggregation during the irradiation of nanoparticles, which is valuable in combination with the repeatability of the PTT effect.

2.3.2 *In vitro* PTT potential of small PAA@SPIONs

In vitro cytotoxicity of PAA@SPIONs was evaluated on HeLa cells using the MTT assay. Initially a dose-dependent study was carried out to determine the safe dose to proceed with. As can be seen in Figure 2.12, PAA@SPIONs did not cause any

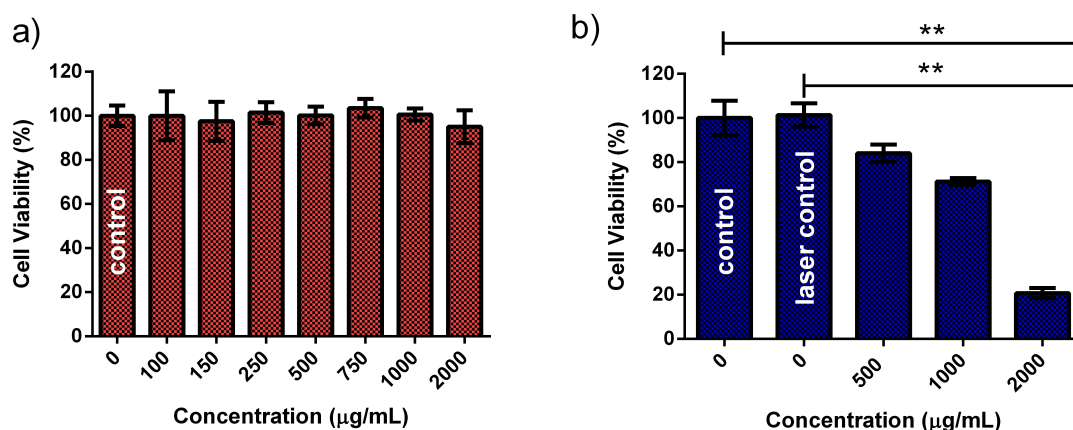


Figure 2.12: a) Toxicity of PAA-SPIONs on the HeLa cell line with different concentrations ($n = 5$) and b) the effect of photothermal therapy of PAA-SPIONs on HeLa cell viability with different concentrations at 795 nm laser irradiation ($n = 4$). Data are expressed as mean $\pm$ SD. ** $p < 0.01$

significant decrease in the viability of HeLa cells even at 2000 $\mu\text{g/mL}$ concentration (95 % cell viability) after 24 h of incubation (Figure 2.12a). After this, the cells were treated with 2 mg/mL PAA@SPIONs for 24 h and then irradiated with a 795 nm laser using 800 mW power (2 W/cm^2) for 10 minutes to assess the PTT efficiency of the nanoparticles. Untreated cells were used as a general control. A set of HeLa cells which are not treated with the nanoparticles were also subjected to laser irradiation, following the same protocol to evaluate the laser irradiation on the cells. As a result, no photothermal destruction was observed when cells without nanoparticles were exposed to laser irradiation while a significant reduction in cell viability which increases with the nanoparticle dose was seen in irradiated cells: the viability dropped down to 84.10 %, 71.25 % and 20.75 % when treated with 500 $\mu\text{g/mL}$, 1000 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$ nanoparticles even at such a low laser power (Figure 2.12b). These findings imply that PAA@SPIONs are promising PTT agents.

2.4 Conclusion

In this study, small, stable and highly biocompatible PAA@SPIONs were synthesized and fully characterized to investigate the influence of NIR laser irradiation on these nanoparticles and to study the influence of laser-dependent variables and attenuation on the magnitude of photothermal heating obtained with these small SPIONs. A series of controlled experiments indicate that the temperature increase obtained from the NIR irradiation of PAA@SPIONs is not dependent on the excitation wavelength within the 728-838 nm range, sample attenuation or intensity of the laser irradiation but on the laser power. This is quite important since aggregation, large size and higher attenuation at the excitation wavelength were usually attributed to the stronger photothermal effect in the case of SPIONs. Hence, this work provides a new insight into the photothermal effect of PAA@SPIONs. Different characterization methods showed that there is no significant change in the critical properties of nanoparticles such as magnetization, absorbance, hydrodynamic size or composition after laser irradiation. Combined with the repeatability of the temperature increase, this is very valuable for medical applications. An important point to highlight here is that the SPIONs used in this study are quite small in terms of the crystal size and the hydrodynamic size. The ability to achieve hyperthermia with such small particles is very critical for future *in vivo* experiments to adjust biodistribution and allow the conjugation of targeting moieties. In the *in vitro* measurements, excellent biocompatibility of these PAA@SPIONs as well as their dose dependent PTT effect was demonstrated in HeLa cells as a proof of concept with 800 mW (2 W/cm^2) laser power and a short irradiation period (10 min) where no cell death in control cells was observed. The low laser power and short treatment period coupled with the small size and biocompatibility of the SPIONs demonstrate a very promising composition and procedure for further practical hyperthermia applications.

Chapter 3

COMBINED PHOTOTHERMAL THERAPY AND PHOTODYNAMIC THERAPY

3. INDOCYANINE GREEN LOADED APTMS COATED SPIONs FOR DUAL PHOTOTHERAPY OF CANCER

3.1 Introduction

Phototherapies, such as photodynamic (PDT) and photothermal therapy (PTT), are highly promising and relatively safe therapeutic approaches compared with other techniques for cancer treatment, due to their locality and "on command" activity. PDT, which is an approved phototherapeutic modality, generates reactive oxygen species such as singlet oxygen to induce tumor death via excitation of photoactivable molecules/dyes at a specific wavelength absorbed by that molecule [208, 209, 210]. However, clinical applications still suffer from major drawbacks including hydrophobic nature of photosensitizers [211], quick clearance coupled with low local concentration at the tumor site and the need for activation at short wavelengths[212].

Indocyanine green (ICG) is a well-known FDA approved, water soluble fluorescent dye used in medical diagnostic with the advantage of fluorescence in the near-infrared region, considered as the medical imaging window [213, 214]. Recently, it has been recognized as a photosensitizer for PDT, which is indeed very promising for image-guided phototherapy. However, aqueous solutions of ICG lack stability and also suffer from rapid clearance [215, 216]. Therefore, it is really valuable and necessary to combine ICG with nanoparticles to overcome these issues and there are some successful examples of such an approach [217, 218, 219, 220, 221, 191, 222]

Superparamagnetic iron oxide nanoparticles (SPIONs) have been used in magnetic

hyperthermia and magnetic resonance imaging for many years and recently, its PTT potential has also been discovered. The combination of PTT with other techniques such as chemotherapy, magnetic hyperthermia, and PDT was found to increase the therapeutic outcome.

In this work, we investigated another popular SPION composition, 3-aminopropyltrimethoxysilane(APTMS)coated, small, cationic SPIONs for their PTT potential and synthesized ICG loaded APTMS@ SPIONs for combined PTT and PDT effect with a single laser treatment at 795 nm. Such an approach has the potential to provide a valuable agent for dual mode imaging as well as image guided therapy. In the *in vitro* studies, we have shown the synergistic effect of combination of photosensitizers on production of reactive oxygen species (ROS) and reducing cell viability using HT29 and MCF7 cell lines after 10 minutes of laser irradiation at 795 nm with an intensity of 1.8 W/cm^2 .

3.2 Experimental

3.2.1 Materials

Iron (II) chloride and iron (III) chloride were purchased from Merck (purity levels 99%, Darmstadt, Germany). 3-aminopropyltrimethoxysilane (APTMS) was obtained from Alfa Aesar (MA, USA). Cardiogreen (indocyanine green) was provided by Sigma-Aldrich (MO, USA). Suprapur nitric acid (65%) and Suprapur sulphuric acid (96%) were obtained from Merck Milipore (MA, USA). Vivaspin 20 centrifugal filters (10 kDa MW cut-off) were purchased from Sartorius (Goettingen, Germany). Dimethyl sulfoxide Hybri-MaxTM was obtained from Sigma (MO, USA). Roswell Park Memorial Institute 1640 (RPMI 1640) and McCoy's 5A media, trypsin EDTA, and penicillin streptomycin were provided by Multicell, Wisent Inc. (QC, Canada). Fetal bovine serum (FBS) was obtained from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Phosphate-buffered saline (PBS) tablets and thiazolyl blue tetrazolium bromide (MTT) were purchased from Biomatik Corp. (ON, Canada). 96-well plates were obtained from Nest Biotechnology Co., Ltd (Wuxi, China). In all experiments, only

ultra-pure water was used (18.2 MΩ, Rephile Bioscience and Technology, Shanghai, China).

3.2.2 Synthesis of 3-aminopropyltrimethoxysilane (APTMS) coated SPIONs

APTMS coated SPIONs were synthesized by a co-precipitation method from iron salts ($[\text{Fe}^{3+}]: [\text{Fe}^{2+}] = 2:1$) in basic pH at 85 °C under argon atmosphere. Briefly, iron salts were dissolved for 20 min. The mixture of APTMS (5.86 mL) and NH_4OH (15.78 mL) was degassed and injected into the solution at 85 °C. After 1 hour of reaction, colloidal solution was cooled to the room temperature and left on a magnet to remove any precipitated particles. Finally, it was washed several times with DI water by using 10 kDa centrifugal filters and stored at room temperature.

3.2.3 ICG loading on APTMS@SPIONs

ICG at a concentration of 0.5 mg/mL was added dropwise to NPs solution (5 mg/mL, 10 mL) and mixed at 750 rpm for 2 h at room temperature. ICG-APTMS@SPIONs were washed using 10 kDa centrifugal filters to remove free ICG. The drug loading amount was calculated from standard calibration curve prepared from the absorbance of free ICG at 780 nm (Figure 3.1). Drug encapsulation efficiency (EE) and loading efficiency (LE) were calculated by using following formulas:

$$EE(\%) = [\text{Weight of loaded drug} / \text{Weight of drug input}] \times 100 \quad (3.1)$$

$$LE(\%) = [\text{Weight of loaded drug} / \text{Weight of drug loaded SPIONs}] \times 100 \quad (3.2)$$

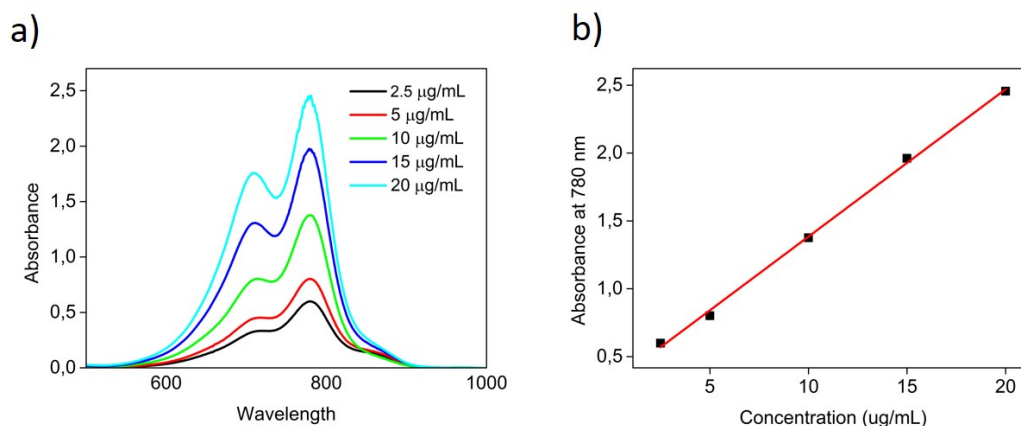


Figure 3.1: a) Absorption spectrum of free ICG at different concentrations and b) concentration dependent absorbance (at 780 nm) calibration curve for free ICG.

3.2.4 Characterization methods

Hydrodynamic sizes and zeta potential were measured using a Malvern Zetasizer Nano ZS. Iron contents of nanoparticles were determined by inductively coupled plasma-mass spectrometer (Agilent 7700XICP-MS) after samples were etched with nitric acid and sulphuric acid. Absorbance spectra were obtained using a Shimadzu UV-VIS-NIR spectrophotometer. Magnetic characterization was performed on a Vibrating Sample Magnetometry System QuantumDesign PPMS9T) at 300 K.

3.2.5 Cell culture and conditions

MCF7 (human breast adenocarcinoma cell line) and HT29 (human colon adenocarcinoma cell line) cells were seeded at 37 °C under 5 % CO₂ humidified incubator in RPMI 1640 and McCoy's 5A medium, respectively, supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin.

3.2.6 In vitro imaging of ICG and ICG-APTMS@SPIONs

Cells were seeded at a density of 175000 cells/well in glass bottom petri dish and incubated at 37 °C for 24 h. ICG and ICG-APTMS SPIONs were added on the

following day at 10 $\mu\text{g}/\text{mL}$ ICG concentration to cells with fresh culture medium. After 24 h incubation, medium was removed and 4 % paraformaldehyde solution was used to fix cells. After 20 min incubation, cells were washed three times with PBS. Cells were treated with 10 $\mu\text{g}/\text{mL}$ DAPI, a nuclear staining dye, for 15 min and then washed three times with PBS. Cells without ICG and ICG-APTMS@SPIONs were used as controls. Images were obtained by Zeiss fluorescence microscope using filters for DAPI (λ_{exc} : 358 nm and λ_{em} : 461 nm) and fluorescence (λ_{exc} : 782 and λ_{em} : 800 nm), respectively.

3.2.7 Intracellular uptake of ICG-APTMS@SPIONs

The quantitative uptake of ICG-APTMS@SPIONs was determined from Fe concentration by ICP-MS. Both MCF7 and HT29 cell lines were seeded at a density of 175000 cells/well in 6-well plates and incubated for 24 h at 37 °C. Then, cells were treated with 10 $\mu\text{g}/\text{mL}$ ICG or 183 $\mu\text{g}/\text{mL}$ Fe concentration. After 24 h incubation, cell medium was removed and cells were washed 3 times with 2 mL of PBS (pH 7.4). After detaching cells by trypsin/EDTA, precipitated cells were transferred into 10 mL volumetric flasks. The cell precipitates were dried on a hot plate and treated in 1 mL acid mixture (ultrapure H_2SO_4 and HNO_3 (1:9 v/v)) for 5 days. The cell lysates were diluted to 10 mL volume using deionized water and Fe concentration was determined using a freshly prepared Fe standard curve.

3.2.8 Cytotoxicity assay

The cytotoxicity of nanoparticles was evaluated on HT29 and MCF7 cells using MTT (Thiazolyl blue tetrazolium bromide (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide)) assay. Cells were seeded at a density of 1.25×10^4 cells per well and incubated for 24 h. Next day, NPs with different concentrations were added to cells with fresh medium. After 24 h incubation, cells were incubated with 50 μL of MTT (5 mg/mL in PBS) solution and 150 μL medium for 4 h. DMSO:ethanol solution was then added to dissolve purple formazan. A microplate reader was used to

read (BioTek Instruments Inc., Winooski, VT, USA) the absorbance at 600 nm with a reference reading at 630 nm. Following formula was used to calculate the relative cell viability:

$$Cellviability = [Absorbance_{sample}/Absorbance_{control}] \times 100 \quad (3.3)$$

3.2.9 NIR irradiation of aqueous solutions

The temperature increase of aqueous solutions of free ICG and aqueous colloidal ICG@APTMS solutions was measured at 5 $\mu\text{g}/\text{mL}$ ICG or 91 $\mu\text{g}/\text{mL}$ Fe concentration using a 795-nm fiber-coupled laser diode array (FAP-79-25C-800-B, Coherent, Inc.) at a laser intensity of 1.8 W/cm^2 for 3 min. The laser diode array was driven with around 8.5 A of current. The coupling fiber had a diameter of 800 micron. Furthermore, the linewidth of the diode laser output was 2.8 nm. Laser on/off experiments were performed for 3-7 min (heating/cooling) and 3-16 min (heating/cooling) for ICG and ICG-APTMS@SPIONs, respectively. For APTMS@SPIONs, the temperature increase upon NIR irradiation was measured as a function of time over 20-min windows, for different laser intensities (in the range of 0.8-2.1 W/cm^2) and nanoparticle concentrations (in the range of 1-20 mg/mL total NPs concentration). In this case, laser on/off experiments were performed using 20 mg/mL NP concentration at an intensity of 1.3 W/cm^2 (incident power of 500 mW and spot radius of 3.5 mm). In all of the experiments, the temperature of the solutions was measured with a thermocouple.

3.2.10 In vitro NIR irradiation

Cells were seeded in a 96-well plate, cultured as described above and then treated with free ICG, APTMS@SPON and ICG-APTMS@SPION, separately. After 24 h incubation, the cell medium was replaced with 200 μL fresh medium and cells were irradiated using a fiber-coupled laser diode array at 795 nm, with an irradiation intensity of 1.8 W/cm^2 for 10 minutes from the bottom of the plate. After 24 h post-irradiation

incubation, MTT assay was used to determine the cell viability. During laser irradiation, the ambient temperature was kept at 37 °C with a homemade incubator. Cells which were not treated with NPs or free ICG were also subjected to the same irradiation procedure. Cells which were not treated with ICG, NPs or laser were used as a control.

3.2.11 *Live/Dead Cell Viability assay*

For qualitative analysis, live/dead cell viability assay (The LIVE/DEAD Viability/Cytotoxicity Kit, ThermoFisher Scientific) was utilized based on manufacturer's instructions. Briefly, cells were treated with ICG-APTMS@SPION (2.5g/mL ICG-45.5 $\mu\text{g/mL}$ Fe and 5 $\mu\text{g/mL}$ ICG-91 $\mu\text{g/mL}$ Fe) and an equivalent amount of APTMS@SPION and free ICG for 24 h, and then were irradiated as described above. After 24h incubation, cells were stained in a 96-well plate with calcein AM and ethidium homodimer-1 and fluorescent images were taken using Nikon, ECLIPSE ts100 microscope.

3.2.12 *ROS generation*

To determine ROS level that is produced due to the photo therapy, a ROS kit (Cellular ROS assay kit (Orange) ab186028, abcam) was utilized based on manufacturer's instructions. Briefly, cells that were treated with the test materials with different ICG or Fe concentrations and treated with the laser as described above, were stained in a 96-well plate with 100 μL /well ROS orange solution. This solution contains a cell permeable dye which luminesces in the orange region when it reacts with ROS. Fluorescence intensities were read at Ex/Em=540/570 nm using microplate reader (Synergy H1, Biotek Instruments).

3.2.13 *Statistical analysis*

The level of statistical significance was determined using t-test of GraphPad Prism software package (GraphPad Software, Inc., USA). p-value <0.01 was considered for

statistical significance. All data were reported as mean standard deviation (SD).

3.3 Results and Discussions

3.3.1 Synthesis and characterization of ICG-APTMS@SPIONs

Cationic SPIONs were synthesized by a co-precipitation method as described in our previous work. These APTMS@SPIONs have number-based hydrodynamic sizes of 18 nm, a ζ potential of 37.7 mV and increasing magnetization with increasing magnetic field (Figure 3.2a). Then, anionic ICG was loaded to these cationic nanoparticles via electrostatic interaction (Figure 3.3). This resulted in full charge reversal to -44 mV and slight increase in the hydrodynamic sizes of the nanoparticles to 34.8 nm.

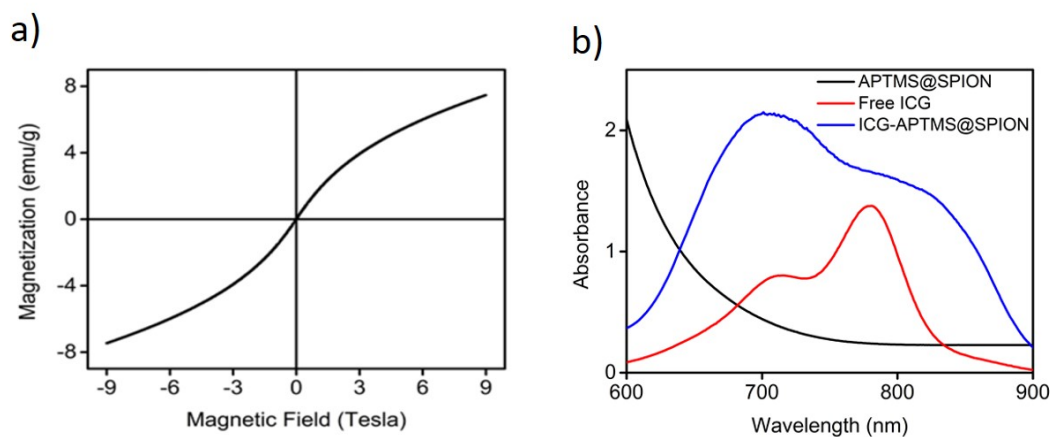


Figure 3.2: a) Field dependent magnetization of APTMS@SPIONs and b) UV-vis-absorption spectra of free ICG, APTMS@SPIONs and ICG loaded APTMS@SPIONs. ICG concentration of ICG-APTMS@SPION and free ICG are identical ($10 \mu\text{g ICG/mL}$).

Figure 3.2b shows UV-vis absorbance spectrum of free ICG, APTMS@SPIONs and ICG-APTMS@SPIONs. Free ICG has an absorbance peak at 710 nm and a stronger one at 780 nm; however, APTMS@SPIONs had no significant absorbance peak in that region, but a decreasing absorbance with wavelength from 600 to 800 nm. Absorbance spectrum of ICG-APTMS@SPIONs shows the two peaks of ICG with a

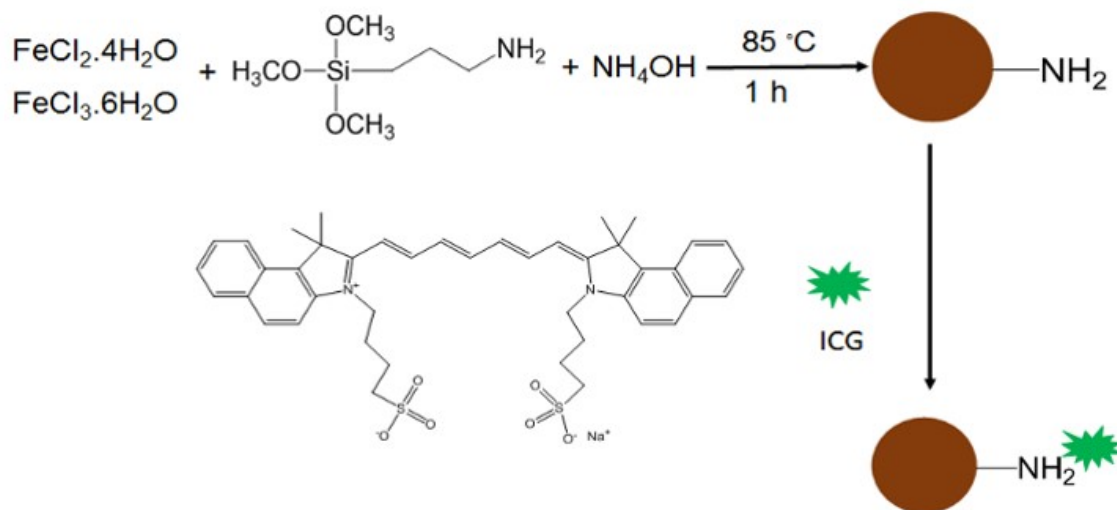


Figure 3.3: Preparation of ICG loaded APTMS@SPIONs.

red shift at the long wavelength region and enhanced intensity at 710 nm, confirming the electrostatic interaction between ICG and NPs. Encapsulation efficiency and loading efficiency were determined as 15 % and 1.5 wt %, respectively from standard UV calibration curve of ICG at 780 nm.

3.3.2 Temperature increase in aqueous solutions

Photothermal heating potential of APTMS@SPIONs were investigated as a function of nanoparticle concentration, irradiation time and laser intensity at 795 nm. While 1 mg NP/mL provided a very small temperature increase ($1.7\text{ }^\circ\text{C}$), 5 mg/ml increased the temperature by approximately $8\text{ }^\circ\text{C}$ at $1.3\text{ W}/\text{cm}^2$ laser intensity, which is quite attractive for mild hyperthermia (Figure 3.4a). Increasing concentration of NPs increased ΔT up to $17.4\text{ }^\circ\text{C}$ at 20 mg NP/ml concentration. At this highest concentration, when the laser intensity was increased to $2.1\text{ W}/\text{cm}^2$, the peak temperature reached $50\text{ }^\circ\text{C}$, which can be effectively used for damaging target cells (Figure 3.4b). Alternating heating and cooling experiments show no loss in the heating potential of the APTMS@SPION nanoparticles with ΔT of 17°C at every cycle, indicating very high and desired photothermal stability (Figure 3.4c). Next, the

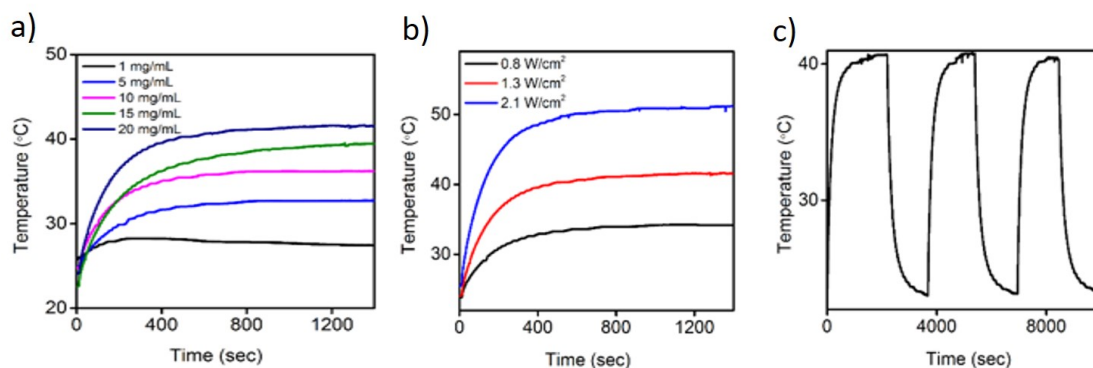


Figure 3.4: Time dependent change in temperature of APTMS@SPIONs solutions a) at different NPs concentrations (1 mg NP contains 35 μg Fe) irradiated at 1.3 W/cm², b) irradiated at different intensities (20 mg NP/mL), and c) after laser on/off cycles (20 mg NP/mL).

photothermal heating potential of ICG-APTMS@SPIONs was investigated in comparison with free ICG. At 5 $\mu\text{g}/\text{mL}$ ICG concentration, a temperature increase of 11.5 °C and 20 °C was observed with 1.8 W/cm² laser intensity in 3 min with free ICG and ICG-APTMS@SPIONs (5 $\mu\text{g}/\text{mL}$ ICG, 91 $\mu\text{g}/\text{mL}$ Fe, 2.6 mg/mL nanoparticle), respectively (Figure 3.5a), indicating a combined effect of the SPION and ICG in increasing the solution temperature. These concentrations and laser intensity levels were designed to achieve mild hyperthermia and PDT combined effect in further *in vitro* and *in vivo* studies. Under the same laser conditions, no temperature increase was detected in plain water, which is very crucial from a practical point of view, when medical applications are considered. To determine the photostability of ICG and ICG-APTMS@SPIONs, three cycles of laser on/off experiments were performed. In all experiments, irradiation heating cycle was 3 min, but the cooling cycle was 7 min for free ICG and 16 min for ICG-APTMS@SPIONs, since in the latter case, the temperature increase was higher and required more time to cool down the solution. Peak temperatures were 36.6, 35.3 and 33.4 °C for free ICG (Figure 3.5b) but 44.6, 44.6 and 43 °C for ICG-APTMS@SPIONs (Figure 3.5c) after each irradiation. Overall, ICG-APTMS@SPIONs showed dramatically more stable structure than free ICG, as expected, since APTMS@SPION was quite stable (Figure 3.5c) and ICG as a dye

is prone to photodegradation/bleaching.

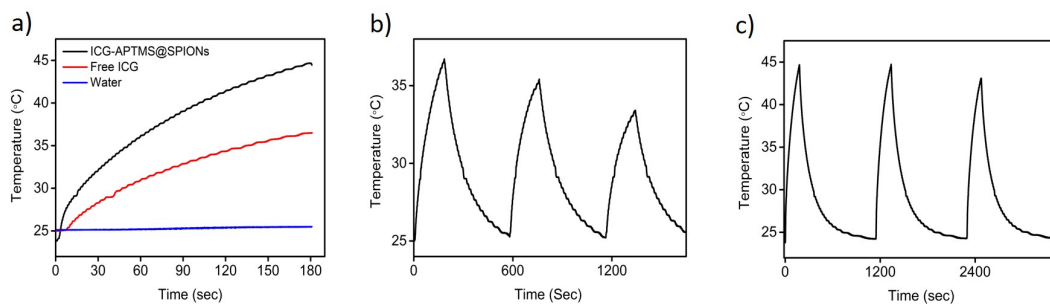


Figure 3.5: a) Measured temperature increase of water, free ICG and ICG loaded APTMS@SPIONs after 3 min laser irradiation (795 nm, 1.8 W/cm²), b) laser on/off experiments of free ICG and c) ICG loaded APTMS@SPIONs ICG (5 μ g/mL ICG, 91 μ g/mL Fe).

3.3.3 *In vitro* evaluation of APTMS@SPIONs with and without photoexcitation

Dose dependent cytotoxicity of cationic APTMS@SPIONs ([Fe]:18-275 μ g/mL) with (795 nm laser at 1.8 W/cm², 10 min) and without laser irradiation was evaluated on two different cell lines, MCF7 (breast cancer) and HT29 (colon cancer) using standard MTT assay. In the whole concentration range, nanoparticles were found as statistically non-toxic in either cell line without laser irradiation. In the case of MCF7 cells, laser irradiation did not reduce viability either (Figure 3.6a). In the case of HT29, although some reduction in viability is observed after laser irradiation, values are above 80 %, indicating non-significant toxicity (Figure 3.6d). To confirm MTT results, cells treated with 91 μ g/ml Fe containing nanoparticles with and without laser treatment were stained with live/dead cell viability assay kit. Cells that were not treated with nanoparticles or laser were used as controls, cells treated only with laser were used for laser control to determine the effect of laser irradiation on cell viability, and cells treated with nanoparticles but not with laser were used for the determination of NP toxicity (Figure 3.6b and e). No dead cells were observed if cells were treated with only APTMS@SPION or only with laser, which confirms

that the nanoparticle dose and laser intensity and irradiation procedure are safe. After laser exposure, APTMS@SPIONs did not cause a significant thermal ablation in MCF7 (Figure 3.6b), but a limited damage (red dots) on HT29 (Figure 3.6e). The influence of APTMS@SPIONs on ROS levels was investigated at different concentrations after laser irradiation. Neither the laser procedure itself nor treatment of cells with APTMS@SPIONs+laser caused any important increase in the ROS level of the treated cells (Figure 3.6c and 3.6f) which is in agreement with the cytotoxicity data.

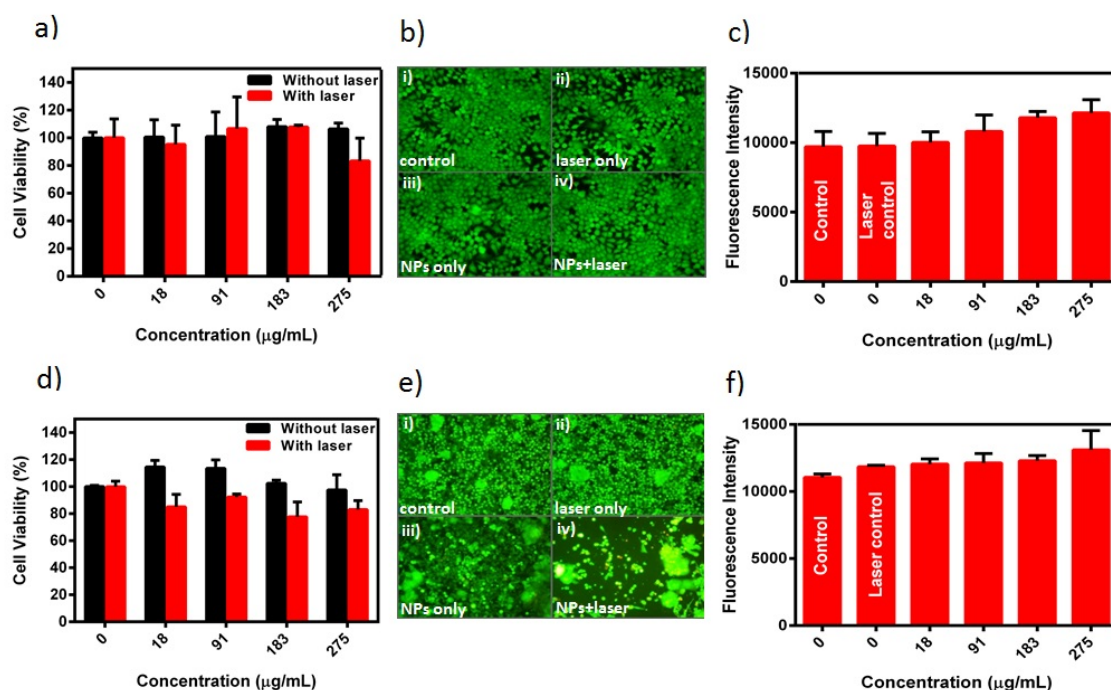


Figure 3.6: Influence of APTMS@SPIONs on a)MCF7 and d)HT29 cell viability with and without laser irradiation at different Fe concentrations, images of stained b)MCF7 and e)HT29 with live/dead assay kit (i) control, (ii) laser treatment only, (iii) APTMS@SPIONs only (91 $\mu\text{g/mL}$ Fe) and (iv) laser treatment at 91 $\mu\text{g/mL}$ Fe, and ROS generation by APTMS@SPIONs in c)MCF7 and f)HT29 after laser irradiation (All laser experiments were performed at the laser wavelength of 795 nm and intensity of 1.8 W/cm² for 10 min).

3.3.4 Photodynamic therapy with free ICG

Cytotoxicity of free ICG with and without laser irradiation on HT29 and MCF7 cell lines was studied between 1-15 μg ICG/mL concentration and 795 nm laser at 1.8 W/cm^2 intensity for 10 min irradiation. ICG is completely safe in the studied range, but causes significant toxicity after laser irradiation in a dose dependent manner after 5 $\mu\text{g}/\text{mL}$ dose and is more toxic to MCF7 than HT29 (Figure 3.7a and d). The

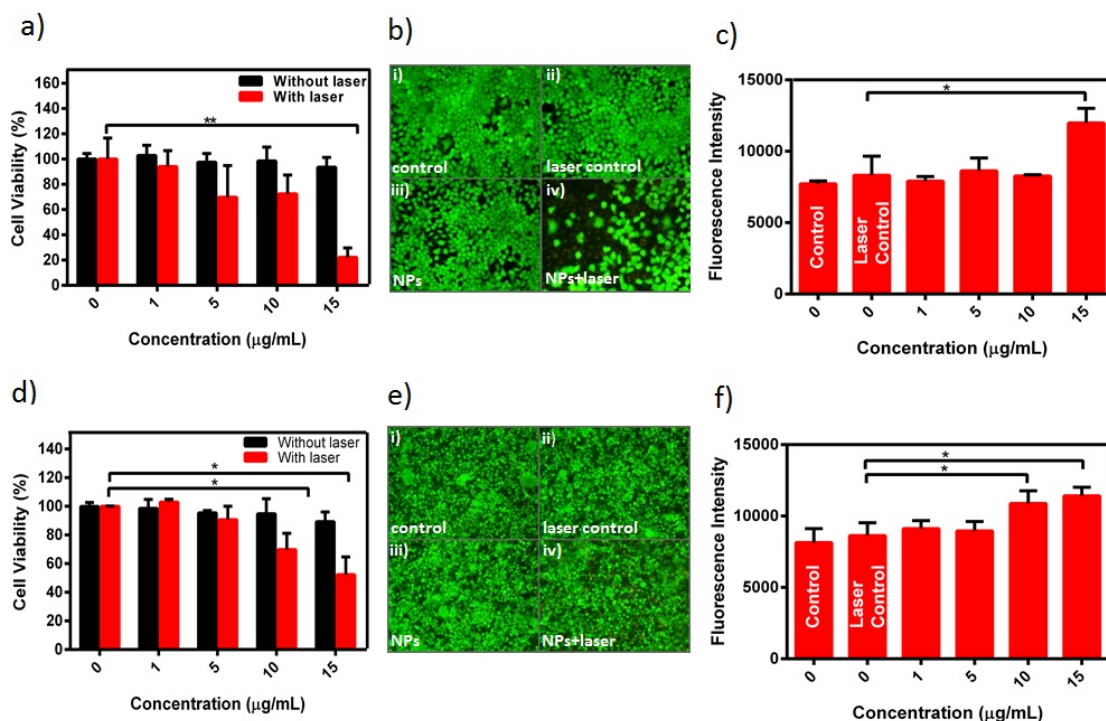


Figure 3.7: Influence of free ICG on a)MCF7 and d)HT29 cell viability with and without laser irradiation at different ICG concentrations, images of stained b)MCF7 and e)HT29 with live/dead assay kit (i) control, (ii) laser treatment only, (iii) ICG only (5 $\mu\text{g}/\text{mL}$ ICG) and (iv) laser treatment at 5 $\mu\text{g}/\text{mL}$ ICG, ROS generation by ICG in c)MCF7 and f)HT29 after laser irradiation (All laser experiments were performed under 795-nm laser irradiation at 1.8 W/cm^2 for 10 min).

viability of MCF7 cells dropped to 22 % and the viability of HT29 cells to 52 % at 15 μg ICG/mL concentration, indicating an IC_{50} higher than 15 μg ICG/mL for HT29 but between 10-15 $\mu\text{g}/\text{mL}$ for MCF7. Fluorescence microscopy images of cells treated with 5 $\mu\text{g}/\text{mL}$ ICG stained with live/dead cell kit, showed very few dead

cells (red) in MCF7 and even less in HT29, in agreement with MTT results (Figure 3.7b and e). Red fluorescence from ethidium homodimer-1 indicates cells that lost their plasma membrane integrity. Live cells were detected based on esterase activity, which is tracked by green fluorescent calcein-AM staining. Further analysis of ROS generation after laser irradiation indicates a significant increase only at 15 $\mu\text{g/mL}$ ICG concentration (1.55 fold increase) and only in MCF7 cells, supporting the MTT and live/dead assay results (Figure 3.7c and f). This suggests that ICG+Laser increases the ROS level at high concentration in MCF7 cells, which drives cells to death as a result of PDT and some degree of PTT.

3.3.5 Dual photodynamic and photothermal therapy with ICG-APTMS@SPIONs

APTMS@SPION nanoparticles are proven safe in this study. Under the selected laser irradiation procedure, they did not produce a significant cell death either. In the solution studies, it was shown that under the laser irradiation procedure adopted for the cell studies above, APTMS@SPIONs can cause a mild hyperthermia. Hence, they can be considered as safe delivery vehicles for therapeutic agents, which can provide a PTT effect at least in the form of mild hyperthermia enhancing the drug release and drug efficacy in potential therapy of cancer. Therefore, dual therapy experiments were performed on both cell lines with the same laser irradiation protocol using ICG-APTMS@SPIONs. ICG-APTMS@SPIONs are highly biocompatible in a concentration range of nanoparticles carrying 1-15 μg ICG/mL (18-275 μg Fe/mL) according to MTT assay performed on both cell lines. When irradiated for 10 min at 1.8 W/cm^2 , the viability of MCF7 cells dropped to 18, 11 and 6 % at 5, 10, and 15 $\mu\text{g/mL}$ ICG concentrations, respectively, while there was no reduction in viability at 1 $\mu\text{g/mL}$ or in control cells (Figure 3.8a). In the case of HT29 cell line, approximately 9, 3.7 and 3.6 % cell viability was observed at 5, 10, 15 μg ICG/mL, again with no significant death in control cells or at 1 μg ICG/mL (Figure 3.8d). The results indicate that this dual therapy approach induces a greater efficiency compared to single PTT

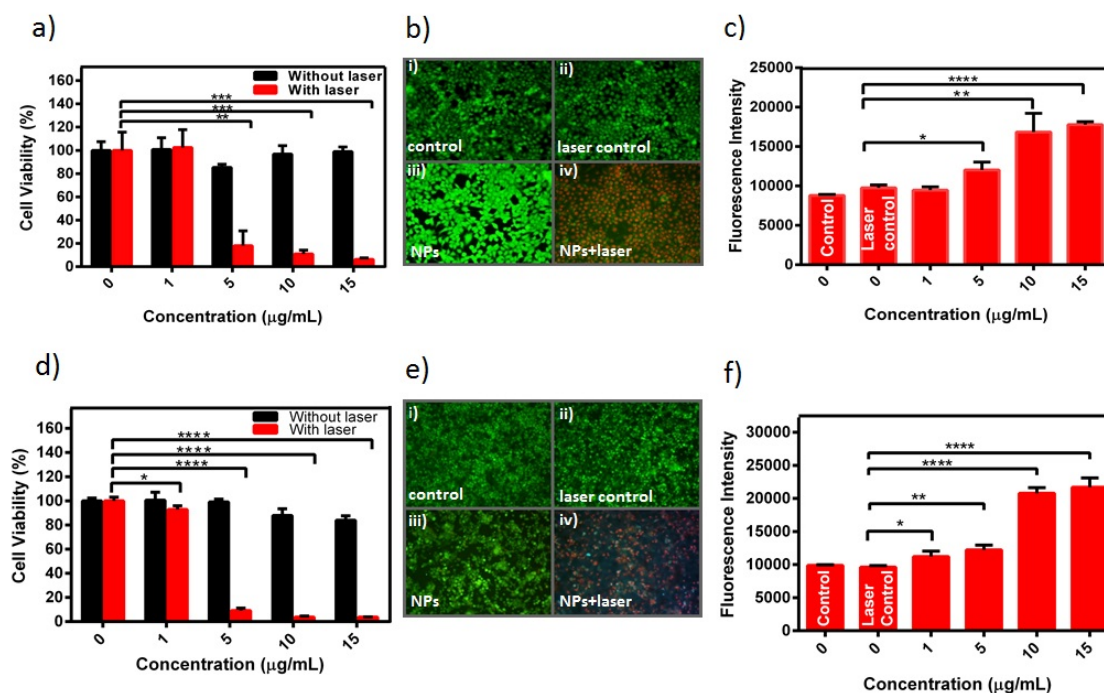


Figure 3.8: Effects of ICG-APTMS@SPIONs on a)MCF7 and d)HT29 cell viability with or without laser irradiation at different ICG concentrations; images of b)MCF7 and e)HT29 cells stained with live/dead assay kit (i) control (untreated cells), (ii) laser treatment only, (iii) ICG-APTMS@SPIONs (5 μg/mL ICG or 91 μg/mL Fe) only and (iv) ICG-APTMS@SPIONs (5 μg/mL ICG or 91 μg/mL Fe) + laser treatment; ROS generation by ICG in c)MCF7 and f)HT29 after laser irradiation (All PTT experiments were performed with 795- nm laser irradiation at 1.8 W/cm² for 10 min)

(mild hyperthermia) generated by SPIONs or PDT obtained by ICG. This caused a tremendous reduction in the IC₅₀ values of ICG in both cell lines to a value between 1-5 μg/mL. Live/dead assay and the corresponding fluorescence images of the treated cells confirmed successful cell killing at 5 μg/mL (Figure 3.8b and e) in MCF7 and HT29 in a dose dependent manner. ICG-APTMS@SPION+laser increased the ROS level significantly reaching 2.02-fold and 2.19-fold increase in the case of MCF7 and HT29, respectively (Figure 3.8c and f), supporting the observed cell toxicity. Initially, we have seen that ICG+laser was more influential on MCF-7 at only 15 μg/mL with increased ROS level, while APTMS@SPION+laser caused no significant cell death or ROS increase in either cell line. However, ICG-APTMS@SPION+laser combination

is highly effective in killing both tumor cells at IC50 values less than 5 $\mu\text{g/mL}$ ICG via a dual therapy. These results suggest that almost complete tumor ablation may be achieved via such DUAL therapy with a single laser treatment at a safe dose.

3.3.6 *In vitro* imaging of ICG and ICG-APTMS@SPIONs

Fluorescence imaging potential gained by the incorporation of ICG to SPIONs is a valuable asset for image guided therapy. Hence, the imaging potential of ICG and ICG-APTMS@SPIONs were investigated on both HT29 and MCF7 cells at 10 $\mu\text{g/mL}$ ICG concentration after 24 h incubation (Figure 3.9 and 3.10). Both cell lines showed

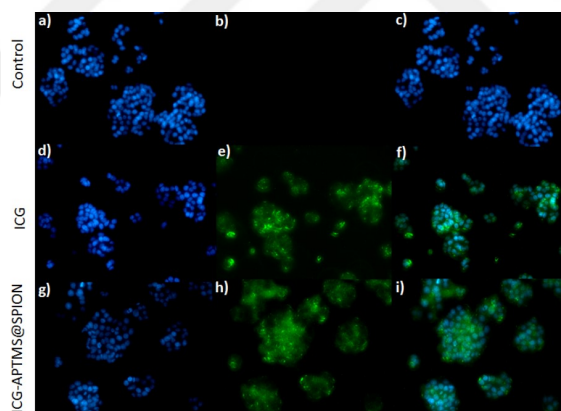


Figure 3.9: Fluorescence microscopy images of ICG and ICG-APTMS@SPIONs by HT29. DAPI (a,d,g), fluorescence (b,e,h), and merged (c,f,i) images of control cells, ICG treated cells, and ICG-APTMS@SPIONs treated cells.

strong fluorescence of ICG delivered freely or on SPIONs, indicating high internalization of both by these cell lines. This also supports a synergistic therapeutic effect of ICG-APTMS@SPIONs, since free ICG was internalized by the cells in comparable amounts based on the images seen in Figure 3.9 and 3.10. Internalization of ICG-APTMS@SPIONs was investigated from the intracellular Fe concentration by ICP-MS in both MCF7 and HT29 cell lines. Cells were treated with ICG-APTMS@SPIONs for 24 h. HT29 cells showed more uptake than MCF7 cells (Figure 3.11).

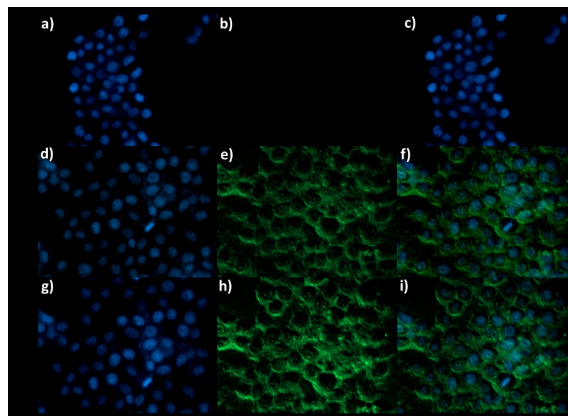


Figure 3.10: Fluorescence microscopy images of ICG and ICG-APTMS@SPIONs by MCF7. DAPI (a,d,g), fluorescence (b,e,h), and merged (c,f,i) images of control cells, ICG treated cells, and ICG-APTMS@SPIONs treated cells

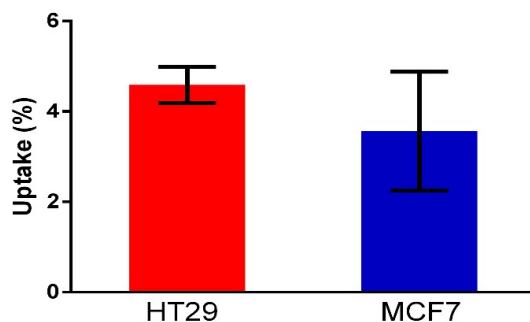


Figure 3.11: Intracellular quantification of ICG-APTMS@SPIONs in MCF7 and HT29 cell lines by inductively coupled plasma-mass spectroscopy (ICP-MS). Cells were incubated with 10 $\mu\text{g}/\text{mL}$ ICG concentration for 24 h.

3.4 Conclusions

In this study, we reported therapeutic efficiency of ICG-APTMS@SPIONs in dual therapy on breast and colon cancer cells using a single laser treatment. We developed stable and biocompatible cationic APTMS@SPIONs by a co-precipitation method and loaded ICG via electrostatic interactions to produce ICG-APTMS@SPIONs. APTMS@SPIONs, although widely used for SPION-based drug delivery/imaging studies, were investigated for the first time for their behavior under NIR irradiation.

A dose and laser intensity dependent increase in the solution temperature was observed at 795 nm irradiation in aqueous solutions of the APTMS@SPIONs. Here, it is important to note that small and spherical SPIONs which are usually preferred for *in vivo* studies can provide PTT. Anionic ICG, an FDA approved dye, which has potential for PDT but needs improved stability and efficiency, was electrostatically loaded to these cationic SPIONs. ICG-APTMS@SPIONs irradiated at 1.8 W/cm² provided significantly higher temperature increase than either of its components. About 20 °C local temperature increase was achieved in 3 min irradiation while no increase in nanoparticle-free water was detected. This is important to prevent damage to healthy tissue in the future *in vivo* studies. In addition, ICG-APTMS@SPIONs showed better photo-stability, in terms of generating the same temperature increase after each cycle, compared to free ICG, which is a positive impact of SPIONs. In the *in vitro* studies, both APTMS@SPION and ICG-APTMS@SPION are found safe in a broad concentration range in MCF-7 and HT-29 cell lines in the absence of NIR irradiation. Fluorescence microscopy images of cells treated with ICG-APTMS@SPION demonstrated high intracellular loading of nanoparticles and strong intracellular fluorescence originating from ICG, which is indeed quite valuable for potential optical-image guided local laser treatment. Irradiation for 10 min at 1.8 W/cm² was set as a safe procedure for cells that lack a photosensitizer. APTMS@SPION+laser showed no significant cell death or ROS generation, hence shown as a safe delivery vehicle for ICG. Free ICG+laser demonstrated some cell death and ROS generation, which is more effective in MCF7 cells. However, under the developed mild laser treatment protocol, ICG-APTMS@SPION+laser combination showed dramatically enhanced cell death with a single laser treatment in both cell lines, reducing viability below 20 and 10 % in MCF7 and HT29 cells, respectively, with more than 2-fold increase in ROS generation, compared to free ICG. Indeed, considering the low sensitivity of HT29 cells to ICG+laser treatment, near complete death of HT29 cells after ICG-APTMS@SPION+laser treatment points out a significantly enhanced therapeutic outcome by the use of this dual phototherapy approach: sensitization of cells via mild hyperthermia with SPIONs for

more effective PDT via ICG. Overall, the novel approach investigated in this work combines SPIONs and ICG, which can be both activated with the same NIR laser at a single wavelength, at the same time, and at the same laser intensity, offering a unique opportunity for the combination of diagnosis and image-guided therapy as well as a great practical advantage in future *in vivo* and clinical studies.



Chapter 4

COMBINED ANTIBIOTIC AND PHOTOTHERMAL TREATMENT OF BACTERIA

4.1 *Introduction*

Many studies have been performed to find a way to treat bacterial infections especially in case of multidrug-resistant bacterial strains. Biofilms possess high innate resistance to antimicrobial agents. More than 80 % of human infectious diseases belong to biofilms. Therefore, it is extremely urgent to find new strategies to exterminate biofilm related infections. Antibiotics are not effective to destroy biofilms due to protective polymeric coating of biofilms preventing drug penetration. Antibiotic loaded nanoparticles provided some efficient results in treatment of biofilms. Very recently, phototherapies, especially PDT against biofilm or bacterial infections became very exciting due to some promising results reported in the literature. Combination of photosensitizers with nanocarriers are very recent but a highly promising approach which increases the stability, bioavailability and therapeutic outcome of the phototherapy. For example, ICG loaded nanospheres coated with chitosan (ICG-Nano/c) were irradiated with 0.5 W, 805 nm laser for the elimination of *Porphyromonas gingivalis* [223]. ICG-Nano/c provided 2-log₁₀ bacterial reduction while there was no change with only laser irradiation or free ICG treatment of bacteria. Combination of NIR photothermolysis and photodynamic therapy was performed on *S. aureus* and *S. epidermidis* using gold nanorod + ICG under 808 nm laser irradiation (50 mW/cm²)[224]. Such combination provided significantly more growth inhibition than only ICG or gold nanorod.

In this work, ICG loaded APTMS@SPION nanoparticles were developed for com-

bined PDT-PTT of bacterial cells and biofilms. This construct was designed to increase the stability and bioavailability of the PDT agent ICG by attaching it to a small, stable and well known APTMS@SPION and provide a synergistic effect via mild hyperthermia or may be thermal ablation that will be created with the laser irradiation of SPIONs at NIR wavelengths. Here, the antimicrobial activity of free ICG, APTMS@SPIONs and ICG loaded APTMS@SPIONs under 795 nm NIR laser irradiation was investigated on planktonic and sessile cells of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus epidermidis*. There was no growth inhibition in either planktonic or sessile cells treated with free ICG, APTMS@SPIONs, and ICG-APTMS@SPIONs without laser irradiation. A significant inhibition of growth was observed at low ICG or NP doses when 1150 mW laser exposure was applied for 10 min but the response was cell dependent as expected.

4.2 Materials and Methods

Bacteria types, *Pseudomonas aeruginosa* (ATCC® 700829TM), *Klebsiella pneumoniae* (ATCC® 700831TM), *Escherichia coli* (ATCC® 25922TM) and *Staphylococcus epidermidis* (ATCC® 35984TM) were purchased from ATCC®.

795 nm laser with 1150 mW output power (3W/cm²) was used for the experiments.

Phototherapy procedure on planktonic cells: This work was performed by Nazlı Ataç at Koc University Hospital. Planktonic cells were incubated in 96-well plates with ICG, APTMS@SPIONs or ICG-APTMS@SPIONs at 25 µg/mL ICG or 284 µg/mL Fe concentration overnight at 37 °C. After 10 min laser irradiation, 20 µL of each bacterial suspension was serially diluted in 180 µL PBS and 100 µL of diluted sample was spread on Tryptic Soy Agar (TSA) in order to record colony forming units/mL (CFU/mL) on the following day.

Phototherapy procedure on biofilms: Biofilms were statically formed in 96-well plates with Tryptic Soy Broth supplemented with 0.2 % glucose after 24 hours incubation at 37 °C. Afterwards, non-adherent bacteria were removed by washing two times with PBS and 100 µL of ICG, APTMS@SPIONs or ICG loaded APTMS@SPIONs

were added onto biofilm in corresponding wells at 25 $\mu\text{g/mL}$ ICG or 284 $\mu\text{g/mL}$ Fe concentration. Following the static overnight incubation, biofilms were irradiated for 10 min. After laser irradiation, biofilm was scraped from the wells into 180 μL PBS and homogenized via IKA homogenizer for 20 seconds. Homogenized cells were further diluted in PBS to be spread on plates and CFU/mL values were recorded after an overnight incubation.

Microscopy studies: Confocal microscopy was used to investigate the uptake of free ICG and ICG-APTMS@SPIONs utilizing the fluorescence of ICG. Once 24h-biofilm was formed on 12 mm glass slides, free ICG or ICG-APTMS@SPIONs were added at 25 $\mu\text{g/mL}$ ICG concentration for overnight incubation. Slides were then washed with PBS for several times and fixed with 3.5 % formaldehyde solution for one day. On the following day, glass slides were washed several times and stained with nuclear stain, DAPI.

4.3 Results and Discussions

The antimicrobial activity of free ICG, APTMS@SPIONs and ICG-APTMS@SPIONs at 25 $\mu\text{g/mL}$ ICG or 284 $\mu\text{g/mL}$ Fe concentrations under 795 nm laser irradiation with 1150 mW laser output power and 3 W/cm^2 laser intensity on *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus epidermidis* was investigated. Sensitivity of planktonic and sessile cells to this laser irradiation procedure in the absence of photosensitizers were also tested and no significant impact on colony formation/growth was observed suggesting that in the absence of a photosensitizer this laser procedure is safe. Toxicity of the ICG, APTMS@SPION and ICG loaded APTMS@SPION were also tested in the absence of laser irradiation to test the dark toxicity of these agents on the tested cells.

Pseudomonas aeruginosa: None of the reagents created a dark toxicity either in planktonic or biofilm form of *Pseudomonas aeruginosa*. Photosensitization + laser treatment created more dramatic growth inhibition of planktonic cells than biofilms, as may be expected due to the well known difficulty of penetration into biofilms and

increased drug resistance (Figure 4.1).

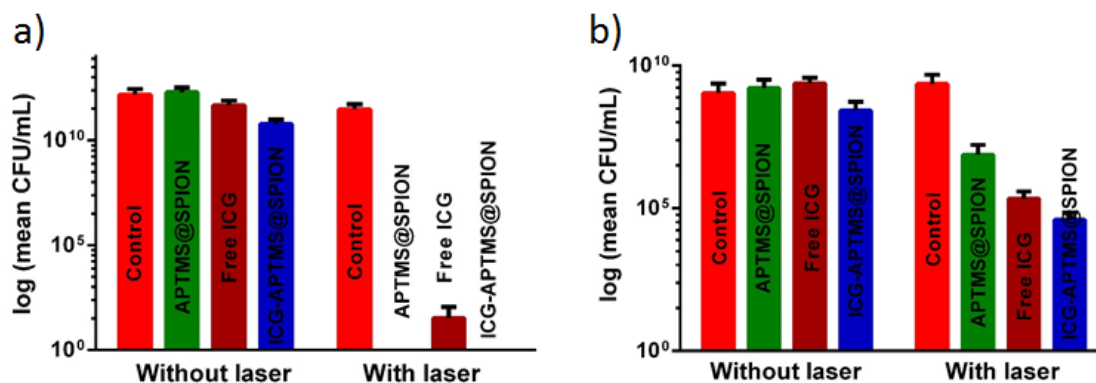


Figure 4.1: Inactivation of the planktonic (a) and biofilm (b) of *Pseudomonas aeruginosa*.

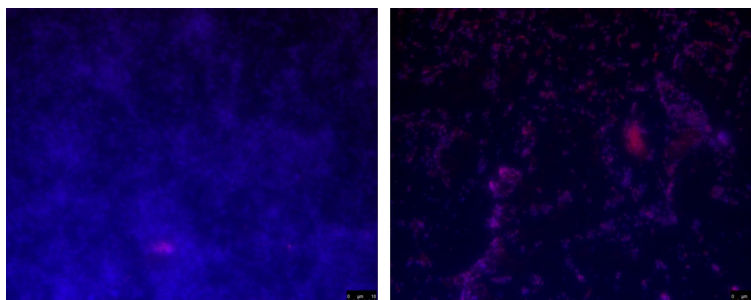


Figure 4.2: Fluorescence microscopy images of ICG (a) and ICG-APTMS@SPIONs (b) by *Pseudomonas aeruginosa*.

Free ICG provided a significant growth reduction but APTMS@SPIONs and ICG-APTMS@SPIONs caused a total inhibition after laser irradiation at this ICG and NP concentration (25 and 284 $\mu\text{g/mL}$). In the biofilms of *Pseudomonas aeruginosa*, APTMS@SPIONs, free ICG and ICG-APTMS@SPIONs provided an important decrease in colony number after laser irradiation, in the given order, reaching to about 50 % reduction with ICG-APTMS@SPIONs. Confocal microscopy images indicate better internalization of ICG when delivered with NPs (Figure 4.2).

Klebsiella pneumoniae: Dark toxicity was not observed with none of the reagents

in planktonic or biofilm form of *Klebsiella pneumoniae*. Photosensitization + laser treatment induced greater growth inhibition of planktonic cells than biofilms (Figure 4.3). Free ICG and ICG-APTMS@SPIONs caused a total inhibition in planktonic cells; however, APTMS@SPIONs did not provide any important change after laser irradiation at 25 $\mu\text{g/mL}$ ICG and 284 $\mu\text{g/mL}$ Fe concentration. In the biofilms,

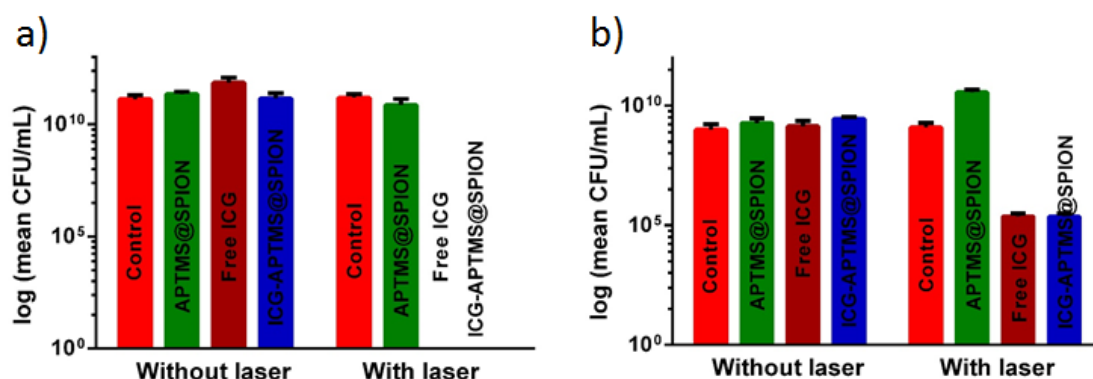


Figure 4.3: Inactivation of the planktonic (a) and biofilm (b) of *Klebsiella pneumoniae*.

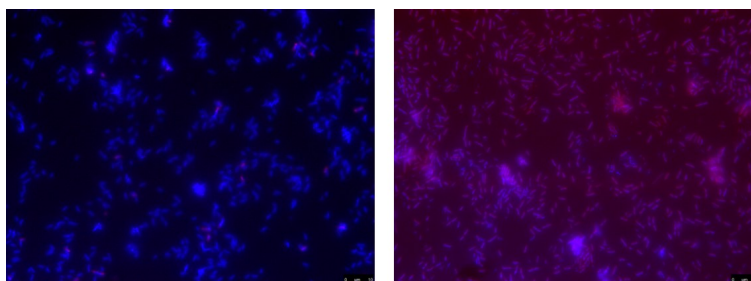


Figure 4.4: Fluorescence microscopy images of ICG (a) and ICG-APTMS@SPIONs (b) by *Klebsiella pneumoniae*.

an important reduction in colony number that was 50 % was observed with both free ICG and ICG-APTMS@SPIONs. Confocal images showed that there was better internalization of ICG-APTMS@SPIONs in comparison with free ICG (Figure 4.4).

Escherichia coli: None of the reagents created a dark toxicity either in planktonic or biofilm form of *Escherichia coli*. Laser irradiation of APTMS@SPIONs did

not create any inhibition on either planktonic or biofilm form of *Escherichia coli* at 25 $\mu\text{g/mL}$ ICG and 284 $\mu\text{g/mL}$ Fe concentration. Free ICG provided an important growth reduction in planktonic cells; however, ICG-APTMS@SPIONs caused a complete inhibition after laser irradiation. In the biofilms of *Escherichia coli*,

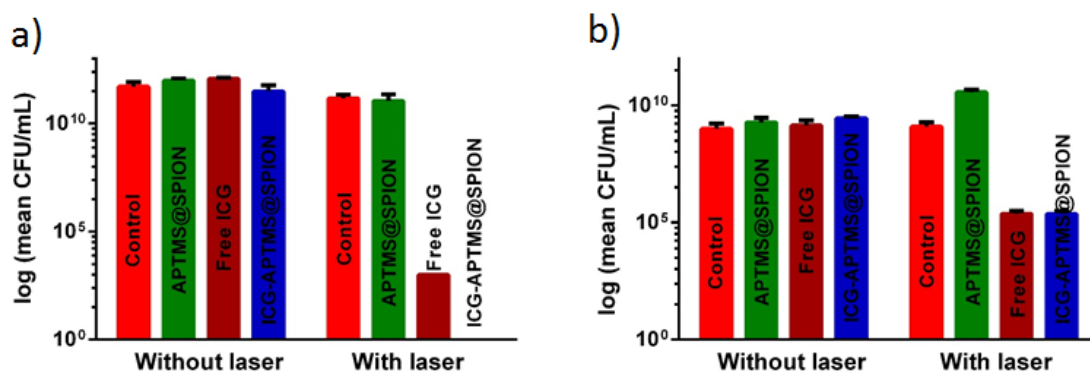


Figure 4.5: Inactivation of the planktonic (a) and biofilm (b) of *Escherichia coli*.

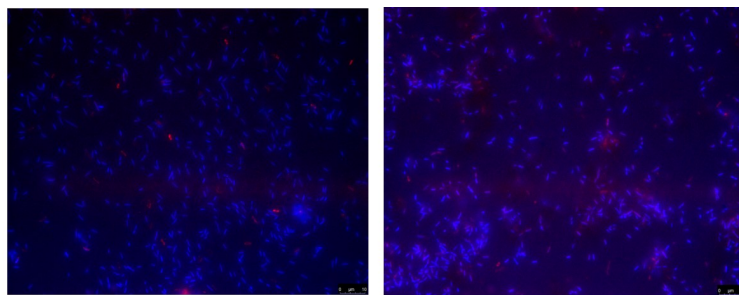


Figure 4.6: Fluorescence microscopy images of ICG (a) and ICG-APTMS@SPIONs (b) by *Escherichia coli*.

free ICG and ICG-APTMS@SPIONs caused an important decrease in colony number after laser irradiation, in the given order, reaching to about 40 % reduction with ICG-APTMS@SPIONs (Figure 4.5). Confocal microscopy images exhibited better internalization of ICG when delivered with NPs (Figure 4.6).

Staphylococcus epidermidis: Dark toxicity was not observed with free ICG, APTMS @SPIONs and ICG-APTMS@SPIONs in planktonic or biofilm form of *Staphylococcus*

epidermidis. Laser irradiation of APTMS@SPIONs did not provide any inhibition on both planktonic and biofilm of *Staphylococcus epidermidis* at 25 $\mu\text{g/mL}$ ICG and 284 $\mu\text{g/mL}$ Fe concentration. ICG and ICG-APTMS@SPIONs provided a significant growth reduction in the colony number of biofilm of *Staphylococcus epidermidis*; however, they caused a total inhibition in planktonic cells after laser irradiation (Figure 4.7).

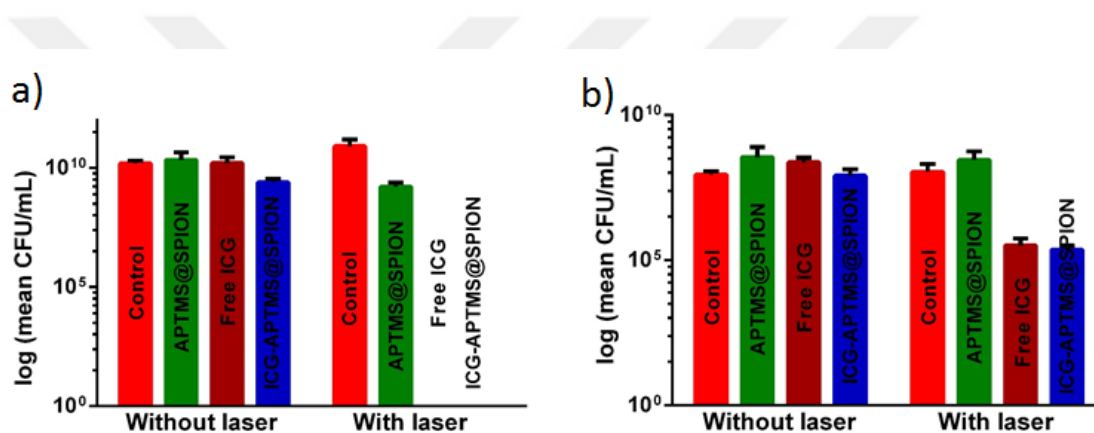


Figure 4.7: Inactivation of the planktonic (a) and biofilm (b) of *Staphylococcus epidermidis*.

4.4 Conclusions

In this study, the microbial activity of ICG-APTMS@SPION under laser irradiation on both planktonic and sessile cells of different bacteria types were investigated. None of the reagents created a dark toxicity either in planktonic or biofilm form of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus epidermidis*. More significant growth inhibition was observed in planktonic cells than biofilms with photosensitization+laser treatment. In the case of planktonic *Pseudomonas aeruginosa*, APTMS@SPIONs and ICG-APTMS@SPIONs created a total inhibition but free ICG provided 4 fold reduction after laser irradiation at 25 $\mu\text{g/mL}$ ICG or 284 $\mu\text{g/mL}$ Fe concentration. An important decrease in colony number of biofilm of *Pseudomonas aeruginosa* was observed with free ICG,

APTMS@SPIONs, and ICG-APTMS@SPIONs. APTMS@SPIONs did not provide any important growth inhibition in either planktonic or biofilm of *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus epidermidis*. In *Klebsiella pneumoniae*, free ICG and ICG-APTMS @SPIONs created a total inhibition in planktonic cells, but a significant reduction was occurred in the colony number of biofilm after laser irradiation. In *Escherichia coli*, a complete inhibition was achieved with ICG-APTMS@SPIONs in planktonic cells while a significant growth inhibition was observed with free ICG after laser irradiation. In biofilm of *Escherichia coli*, both free ICG and ICG-APTMS@SPIONs caused a reduction in colony number after laser irradiation. Both free ICG and ICG-APTMS@SPIONs created a total inhibition in planktonic cells of *Staphylococcus epidermidis* while they caused an important decrease in the colony number of biofilm. Confocal microscopy images exhibited better internalization of ICG when delivered with NPs in *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. Internalization of ICG and ICG-APTMS@SPIONs should be investigated using confocal microscopy as well. Lower doses to study the minimum inhibitory concentration and higher doses for total eradication of biofilms need to be studied. Also, antibiotic conjugation and addition of targeting moieties may increase the therapeutic outcome dramatically.

Chapter 5

COMBINED CHEMOTHERAPY AND PHOTOTHERMAL THERAPY

5.1 Introduction

Chemotherapy is one of the widely used therapeutic methods practiced for the treatment of various cancer types. However, it has many serious drawbacks including low drug accumulation at the desired site, poor bioavailability, side effects and development of multidrug resistance. Encapsulation of drugs into nanoparticles can at least minimize these problems. In addition, combined chemotherapy with other techniques such as radiotherapy or phototherapies usually improves the therapeutic outcome.

Etoposide, podophyllotoxin anticancer drug, is widely used for the treatment of various cancer types including lung, ovarian, and prostate [225, 226]. However, drug resistance, poor water solubility, myelosuppression, and metabolic inactivation limits the efficiency of therapy [227, 228, 229]. Although the development of targeted drug delivery systems provided some advantages, multidrug resistance is still major obstacle for an efficient chemotherapy [230, 231, 232]. However, combination of chemotherapy with other techniques such as photothermal therapy can help to destruct multidrug resistance [233].

In this study, Eto-PTT combination therapy is suggested as a potentially effective method to treat tumors. In order to achieve this, considering water insolubility of Eto, BSA conjugated PAA@SPIONs were developed and Eto was loaded to these BSA@PAA@SPIONs. *In vitro* dark toxicity of nanoparticles, effect of Eto, PTT and Eto+PTT on the viability of PC3, DU145, and LNCaP prostate cancer cells were investigated. Prostate cancer is the second leading cause of cancer death among the

men globally. Especially, methastatic ones are difficult to treat. Here, a promising approach and promising theranostic nanoparticles are being suggested for the treatment of methasthetic prostate tumor.

5.2 Experimental

5.2.1 Materials

Iron (II) chloride and iron (III) chloride were purchased from Merck (Darmstadt, Germany). Poly (acrylic acid) (Mw, 100 kDa) and dimethyl sulfoxide Hybri-MaxTM were provided by Sigma (MO, USA). Bovine serum albumin was obtained from Gen-Depot. Etoposide was purchased from Kocak. Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (sulfo NHS) were purchased from Thermo Scientific (USA). Vivaspin 20 centrifugal filters were obtained from Sartorius (Goettingen, Germany). Roswell Park Memorial Institute 1640 medium (RPMI 1640), trypsin EDTA and penicillin-streptomycin solutions were purchased from Multicell, Wisent Inc. (QC, Canada). Fetal bovine serum (FBS) was provided by Capricorn Scientific GmbH (Ebsdorfergrund, Germany).

5.2.2 Iron oxide nanoparticle synthesis

Poly(acrylic acid) coated SPIONs were synthesized by a coprecipitation method. The experimental procedure was explained in Chapter 2.

5.2.3 Bovine serum albumin conjugation to PAA@SPIONs

Bovine serum albumin (BSA) was conjugated to PAA@SPIONs via EDC/NHS chemistry. PAA@SPIONs were activated with 24.3 mg EDC and 27.6 mg Sulfo-NHS in MES buffer (pH=6) for 20 min. Then, Sulfo-NHS active SPION was exchanged to PBS (pH=7.4) using centrifugal filters (MW cut-off 10 kDa) for 30 min. 1.40 gr BSA was added to nanoparticles and mixed for 48 h at 4 °C. As a final step, the reaction mixture was quenched with excess hydroxylamine and purified with 100 kDa

centrifugal filters.

5.2.4 Determination of BSA concentration on SPIONs by Bradford Assay

As a reference standard curve (Adj. $R^2=0.9930$), bovine serum albumin protein was prepared in PBS at 0, 2, 4, 6, and 8 $\mu\text{g/mL}$ concentration. Washing product of

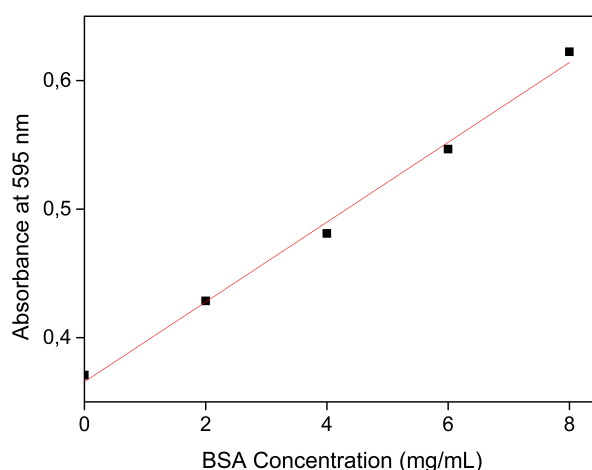


Figure 5.1: Bradford assay to determine the amount of BSA on SPION. A standard curve of BSA concentration ($\mu\text{g/mL}$) versus absorbance at 595 nm.

BSA@PAA@SPION was measured as analyte. 1 mL of each reference and analyte were added into 1 mL of Bradford Reagent and shaken for 10 min at room temperature. Then absorbance of each reference and analyte at 595 nm was recorded using UV-Vis spectroscopy (Figure 5.1).

5.2.5 Etoposide loading to BSA@PAA@SPION

ETO was added dropwise to NPs solution and mixed at 750 rpm for 24 h. ETO-BSA@PAA@SPION were washed using 10 kDa centrifugal filters to remove free ETO. The drug loading amount was calculated from standard calibration curve prepared from the absorbance of free ETO at 285 nm (Figure 5.2). Drug encapsulation efficiency (EE) and loading efficiency (LE) were calculated by using following formulas:

$$EE(\%) = [Weightofloadeddrug/Weightofdruginput] \times 100 \quad (5.1)$$

$$LE(\%) = [Weightofloadeddrug/WeightofdrugloadedSPIONs] \times 100 \quad (5.2)$$

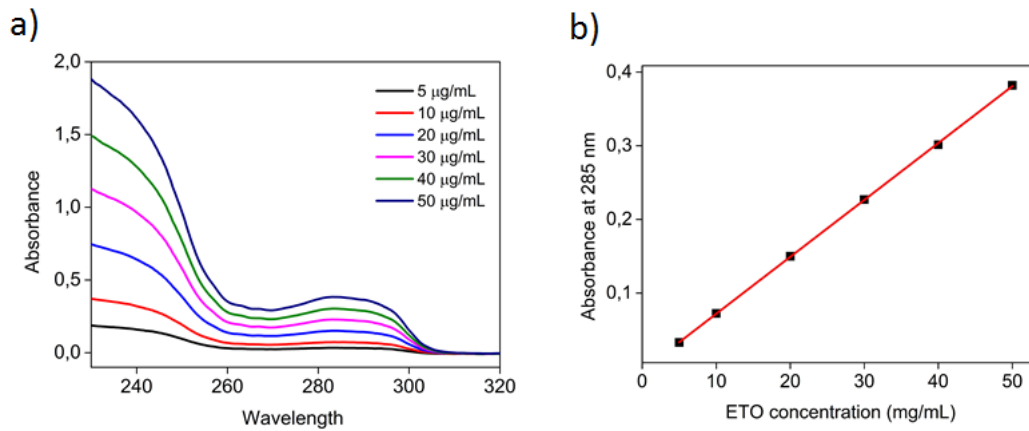


Figure 5.2: a) Absorption spectrum of free Etoposide at different concentrations and b) concentration dependent absorbance (at 285 nm) calibration curve for free Eto.

5.2.6 Characterization

Absorbance measurements were performed on a Shimadzu UV-Vis-NIR spectrometer. The hydrodynamic size and potential of nanoparticles were measured by Malvern Zetasizer Nano-ZS. The iron content of nanoparticles was measured using an Agilent 7700XICP-MS (inductively coupled plasma-mass spectrometer). The samples were etched with nitric acid and sulphuric acid.

5.2.7 Cell culture

PC3, DU145, and LNCaP cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 1% penicillin-streptomycin and incubated in a 5 % CO₂ humidified incubator at 37 °C.

5.2.8 In vitro PTT procedure

Cells were seeded at a density of 5×10^3 in 96-well plates and NPs were added directly at different concentrations at same time. After 48 h incubation, cells were irradiated using a fiber-coupled laser diode array (795 nm) for 10 min. The initial temperature was kept constant at 37 °C. After laser irradiation, cells were incubated further 24h at 37 °C in 5 % CO₂ atmosphere. The cell viability was measured by ATP and SRB assay.

5.2.9 ATP and SRB assay

Cells were seeded at a density of 5×10^3 in 96- well plates and NPs and free Eto were added to the cells at the same with different concentrations. After 48 h incubation, ATP content in the living cells was determined according to manufacturer's protocol (ATP Bioluminescence Assay, Sigma, USA). Luminescence signal was recorded using a Luminometer (FLx800 Microplate Fluorescent Reader, Bio-Tek, USA) with a count integration time of 1 s.

For SRB assay, cells were fixed using 50 % (w/v) ice-coldtrichloroacetic acid (TCA; 50 μ l) at 4 °C for 1 h. Cells were washed with deionized water to remove TCA and then stained with 50 μ l of SRB solution (0.4 % (w/v) in 1 % aqueous acetic acid) for 30 min at room temperature. The excess dye was washed out using 1 % (v/v) acetic acid. To dissolve protein-protein dye, the plate was subsequently air-dried prior to addition of 10 mM Tris base solution (pH=10, 150 μ l). Then the absorbance was read using a spectrophotometer (FLASH Scan S12,Analytik Jena) at 564 nm.

5.3 Results and Discussions

5.3.1 Synthesis and characterization of ETO-BSA@PAA@SPIONs

Eto loaded BSA@PAA@SPIONs were successfully prepared as shown in Figure 5.3 for combined chemotherapy and photothermal therapy.

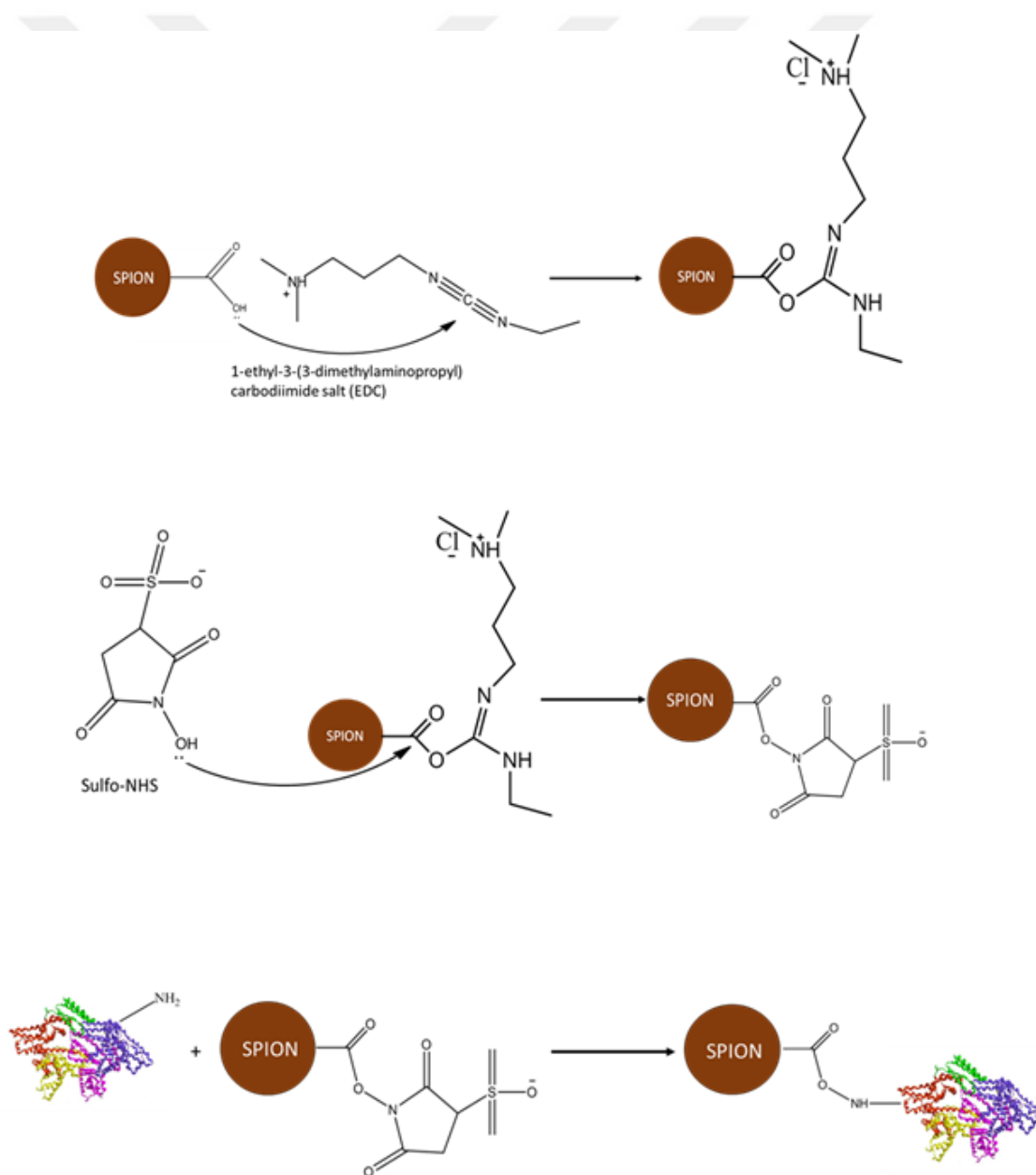


Figure 5.3: Synthesis mechanism of BSA@PAA@SPION

PAA@SPIONs were synthesized by a co-precipitation method. To conjugate BSA to the nanoparticles, the carboxylic group of PAA@SPION was activated by EDC/NHS in MES buffer and reacted with amine groups of the BSA. Then, Eto was loaded to these nanoparticles via electrostatic interaction. PAA@SPIONs have

Table 5.1: Hydrodynamic size and zeta potential of prepared SPIONs

Sample name	Intensity based size (nm)	Number based size (nm)	ζ potential (mV)
PAA@SPION	137.9	8.3	-23.2
BSA@PAA@SPION	145.2, 14.83	8.4	-18
Eto-PAA@SPION	122, 12.42	7.27	-19.5

number-based hydrodynamic sizes of 8.3 nm and a η potential of -23.2 mV. After BSA conjugation, charge was -18 mV which confirms successful conjugation reaction. Eto loading produced nanoparticles with a similar hydrodynamic size (7.27 nm) but reduced the ζ potential to -19.5 mV. EE and LE were determined as 93.3 % and 9.33 wt %, respectively, from the absorbance of Eto at 285 nm.

5.3.2 Dark toxicity of the agents

In the *in vitro* studies, PC3, LNCaP, and DU145 prostate cancer cells were used. Cytotoxicity of free Eto, PAA@SPION, BSA@PAA@SPION, and Eto-BSA@PAA@SPION was evaluated using both ATP and SRB assays to provide reliable evaluation of toxicity. ATP assay, which is a bioluminescence assay, determines the level of cellular ATP in viable cells by recombinant firefly luciferase. ATP is more sensitive than SRB assay. Cells were incubated with free Eto or NP compositions at a concentration range between 0.16-10 $\mu\text{g/mL}$ Eto or 20 $\mu\text{g/mL}$ Fe for 48 h. Based on the both SRB and ATP assay, PAA@SPION and BSA@PAA@SPION were totally biocompatible on all three cell lines. In the case of DU145 cells, IC₅₀ of free Eto and Eto-BSA@PAA@SPION were 10 and 1.3 $\mu\text{g/mL}$ based on ATP assay. IC₅₀ value were not reacted with free Eto in the studied dose range while it was 5 $\mu\text{g/mL}$ for

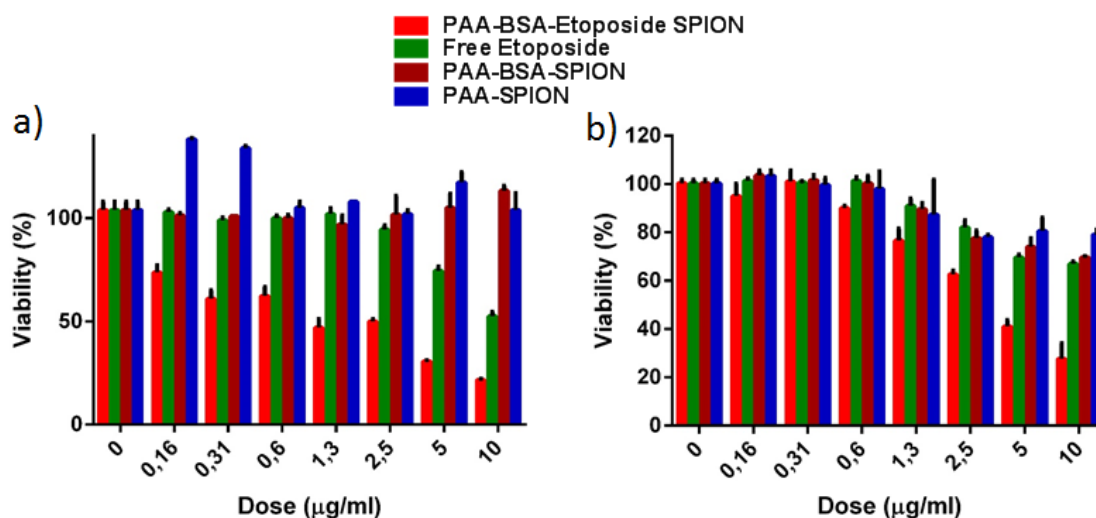


Figure 5.4: Viability of DU145 treated with PAA@SPIONs, BSA@PAA@SPIONs, Eto-BSA@PAA@SPIONs and free Eto after 48-h incubation assessed by a) ATP and b) SRB assays.

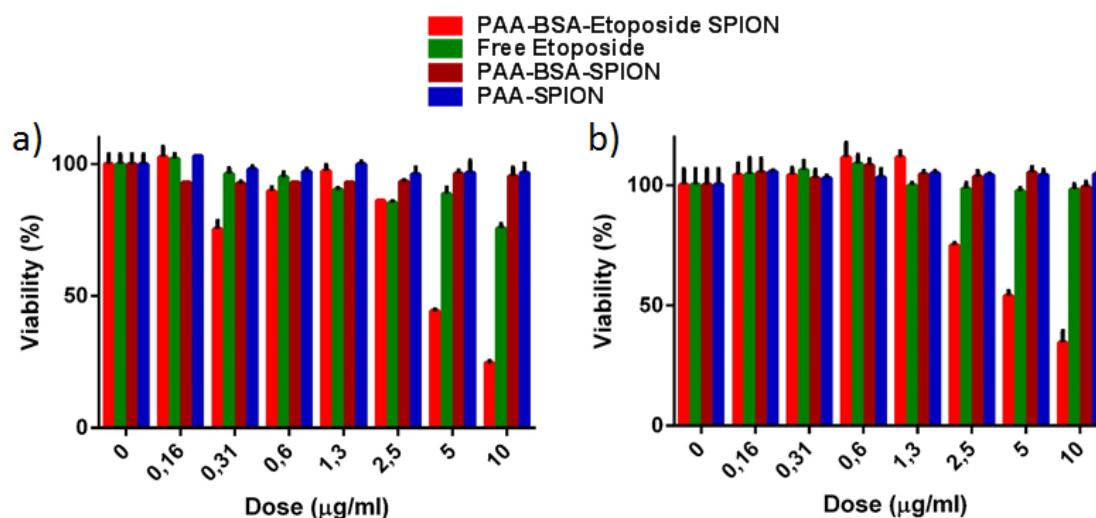


Figure 5.5: Viability of PC3 treated with PAA@SPIONs, BSA@PAA@SPIONs, Eto-BSA@PAA@SPIONs and free Eto after 48-h incubation assessed by a) ATP and b) SRB assays.

Eto-BSA@PAA@SPION as determined by SRB assay (Figure 5.4). In PC3 case, IC₅₀ was between 2.5-5 $\mu\text{g/mL}$ and 5-10 $\mu\text{g/mL}$ based on ATP and SRB assays, respec-

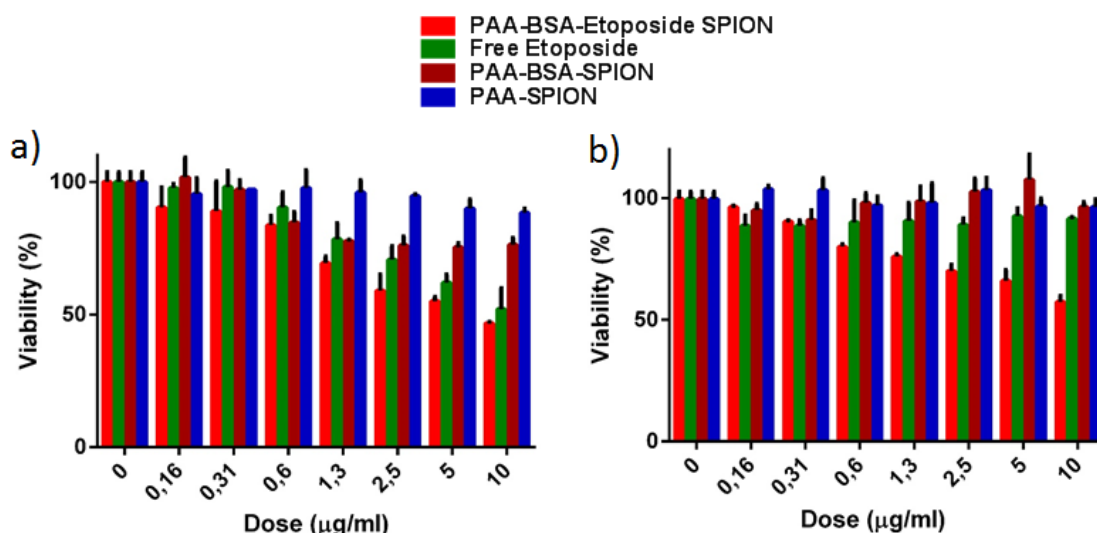


Figure 5.6: Viability of LNCaP treated with PAA@SPIONs, BSA@PAA@SPIONs, Eto-BSA@PAA@SPIONs and free Eto after 48-h incubation assessed by a) ATP and b) SRB assays.

tively for Eto-BSA@PAA@SPIONs. Nevertheless, IC₅₀ could not be determined for free Eto by either assay (Figure 5.5). LNCaP was the most robust cell line compared to PC3 and DU145 where IC₅₀ values could not be found for free Eto and Eto-BSA@PAA@SPION (Figure 5.6). Results provided by two different assays on each cell line exhibited that drug was delivered more effectively to tumor cells due to NPs reducing the IC₅₀ dramatically.

5.3.3 Photothermal toxicity of Eto-BSA@PAA@SPION

Photothermal therapy experiments were performed on DU145 and PC3 cell lines at 800 mW and 1000 mW laser power for 10 min laser irradiation at 808 nm using Eto-BSA@PAA@SPION. Viability of cells treated with 800 mW laser power was no different than their dark toxicity (Figure 5.7). DU145 was more sensitive than PC3 to combination therapy (Figure 5.8a). Even at low concentrations (0.3 and 0.6 $\mu\text{g/mL}$ Eto concentration), cell viability decreased to 24 % with laser irradiation, showing 37 % more cell death than its dark toxicity. The cell death difference was 20 % between

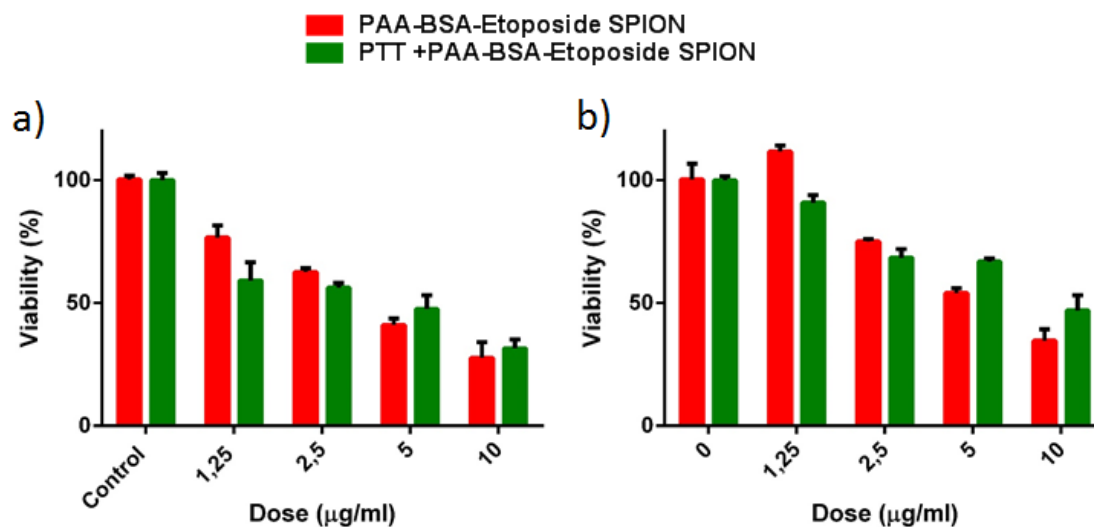


Figure 5.7: Viability of a)DU145 and b)PC3 treated with Eto-BSA@PAA@SPIONs after 800 mW laser irradiation assessed by SRB assay.

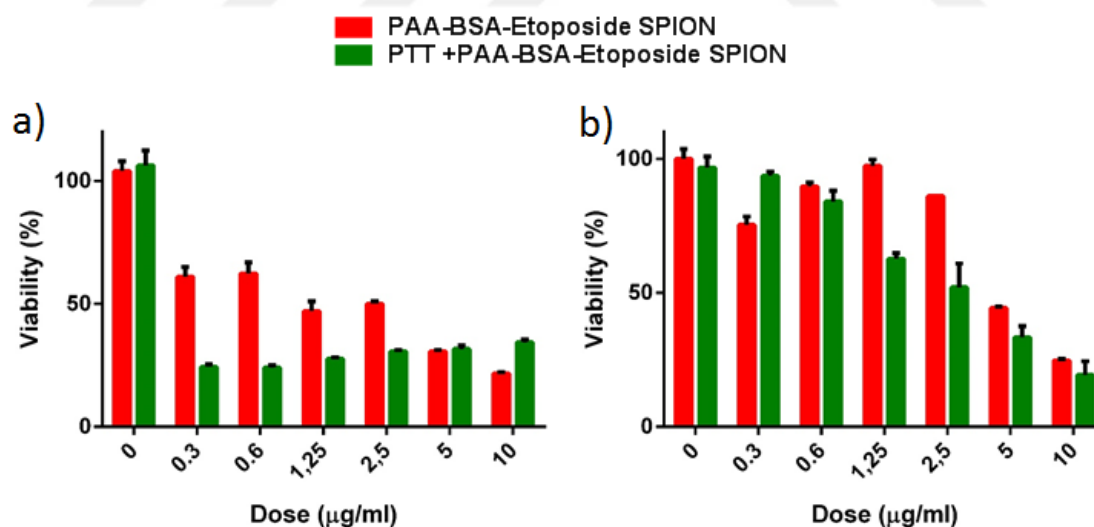


Figure 5.8: Viability of a)DU145 and b)PC-3 treated with Eto-BSA@PAA@SPIONs after 1000 mW laser irradiation assessed by ATP assay.

before and laser irradiation for Eto-BSA@PAA@SPIONs at 1.25 and 2.5 µg/mL. Toxicity at higher concentrations were comparable to their dark toxicity. In PC3 cell line, no significant enhancement in cell death was observed at 0.3 and 0.6 µg/mL Eto concentration with Eto-BSA@PAA@SPION compared to its dark toxicity after laser

irradiation. However, 35 % more cell death was achieved after laser irradiation at 1.25 and 2.5 $\mu\text{g/mL}$ Eto concentration. Viability was reduced by 11 % and 5 % at 5 and 10 $\mu\text{g/mL}$ Eto (Figure 5.8b). Concentrations with Eto+PTT compared to dark toxicity.

5.4 Conclusions

In this study, Eto was successfully loaded to ultrasmall BSA conjugated SPIONs in therapeutically effective doses. Drug internalization by cells were enhanced with drug loaded nanoparticles. Therefore, Eto-BSA@PAA@SPION caused more dark toxicity compared to free Eto on PC3, DU145, and LNCaP cells. LNCaP was the more robust cell line in comparison with DU145 and PC3 cells. To achieve further increase in toxicity, DU145 and PC3 cells were irradiated for 10 min using 800 mW and 1000 mW laser powers. Photothermal therapy experiments caused an important cell death based on laser power. There was no change on cell death with 800 mW; however, a significant reduction occurred under 1000 mW irradiation, reducing the required effective dose of Eto dramatically. DU145 cell line was more sensitive to laser irradiation compared to PC3 cell line. This study demonstrated a successful combination of SPION-PTT with chemotherapy and reduced the effective dose of Eto dramatically.

Chapter 6

CONCLUSIONS

SPIONs which are widely used as MRI contrast agents and for magnetic hyperthermia, gene delivery and drug delivery are now being investigated for photothermal therapy (PTT). It is really important and valuable to use these highly biocompatible agents in local phototherapy wherein NIR lasers are utilized. This does not only transform magnetic hyperthermia to a more practical and local therapy area but also provide a practical particle that may be addressed at NIR, which is one of the major needs of existing phototherapies both for the safety of the unsensitized tissue and for deeper penetration of light. In addition, ability of creating photothermal effect with SPIONs allows combination therapies and possibility for image guided therapy, which are well within the future scope of phototherapies and nanoparticle based diagnostics and therapy. However, PTT mechanism of SPIONs are not still well understood, yet needs further investigation and improvement for clinical translation. One of the issues seen in the *in vivo* studies is the harm on the healthy tissue of the animals due to high laser powers that heat up the unsensitized cells, as well. Another clinic problem of organic small molecule photosensitizers are low accumulation in the target and long lasting photosensitization which requires patients to stay in dark for a while. Hence, SPION-based PTT may provide dramatic improvement in all these issues. At the beginning of this thesis work, the factors that affect the photo-induced temperature increase and the influence of laser irradiation on nanoparticle properties were investigated in detail.

In Chapter 2, the effect of three different laser variables, wavelength (between 728 and 838 nm), intensity (1.85-9.76 W/cm²) and power (105-800 mW) were investigated on photothermal heating potential of PAA@SPIONs which are 4–1 nm (in-

tensity based=14.22 nm), colloiddally stable and highly biocompatible nanoparticles. These experiments showed for the first time in the literature that there is no strong correlation between the temperature increase and irradiation wavelength/attenuation and laser intensity but with the laser power. The major increase in the temperature of SPION solutions was almost 5 °C at 260 mW under 785 nm irradiation and was achieved quickly in 5 minutes, while no temperature increase was recorded in DI water, not even at 800 mW power. This is an extremely important and a notable improvement over literature, indicating that this laser procedure is indeed mild and possibly will not harm healthy tissue in the absence of nanoparticles. Laser on/off experiments confirmed the photothermal stability of nanoparticles where same temperature increase was observed after each irradiation cycle. This would allow single dose administration to the patient but potentially multiple laser excitation, if nanoparticles would remain in the site of interest, tumor, biofilm, etc, for the desired period of time. Detailed characterization of SPIONs was performed before and after laser irradiation for the first time again in the literature. Hydrodynamic size measurements exhibited that no agglomeration or coating loss occurred during the laser irradiation. No significant change was observed on the Fe states by XPS exhibiting maintained magnetic properties. Overall, there was no change in the size, chemistry, attenuation or saturation magnetization of SPIONs after laser irradiation which is valuable for medical applications and agrees with the reproducibility of the photothermal heating over and over again with the same particle. Use of such photothermal heating in thermal ablation of tumor cells were studied as the first step of bioevaluations. The cell viability of HeLa cells dropped to 20.75 % at 2 mg/mL NPs concentration under 795 nm laser irradiation at 800 mW (2 W/cm²) without any damage on control cells. In brief, significant cell death was achieved using a clinically safe laser power/intensity at safe nanoparticle doses using small, spherical SPIONs which lacks dark toxicity for the first time. These results were published in Photochemical and Photobiological Sciences.

In Chapter 3, photothermal therapy was combined with photodynamic therapy

which combines different cell death mechanisms utilizing ICG loaded APTMS@SPIONs. MRI is a very effective pre-operative method, but optical imaging may be quite practical in the operation room for image-guided phototherapy where the operator could see the area that needs to be irradiated. Hence, amongst different PDT agents, ICG was used in this study. ICG is an FDA approved florescent dye and a recently acknowledged PDT agent that is sensitive to desired NIR wavelength just as SPIONs. Aqueous solutions of small (18nm), spherical cationic APTMS@SPIONs provided a great temperature increase as a function of nanoparticle concentration and laser intensity with high photothermal stability. For example, the temperature reached 50 °C at 20 mg NP/mL when irradiated at 795 nm at 2.1 W/cm². ICG-APTMS@SPIONs provided higher temperature increase than both free ICG and APTMS@SPION. About 20 °C temperature increase was recorded after 3 min laser irradiation of ICG-APTMS@SPIONs at 1.8 W/cm² and same temperature increase was observed after each irradiation cycle. Therefore, combining ICG and APTMS@SPIONs provided two positive and important outcomes: i) higher temperature increase than free ICG and APTMS@SPIONs and ii) greater photostability than free ICG. Later is one of the most important drawbacks of free ICG in the clinic in addition to poor bioavailability. Therapeutic potential of ICG-APTMS@SPIONs was tested *in vitro* on breast (MCF7) and colon (HT29) cancer cells. Initial toxicity studies indicated that there is no dark toxicity of the nanoparticles or ICG at the studied doses in either cell line. PTT studies were performed with 10 min 795 nm laser irradiation at 1.8 W/cm². APTMS@SPIONs did not cause a significant cell death or ROS generation, pointing out that it may be a safe delivery vehicle for ICG. In the case of free ICG, ROS generation and a reduction on cell viability were observed at and above 10 µg ICG/mL, and much more effectively on MCF7. Combined PTT and PDT therapy with ICG-APTMS@SPIONs via a single laser treatment decreased the cell viability below 20 and 10 % in MCF7 and HT29, respectively, and caused 2- fold increase in ROS generation compared to free ICG. This synergistic effect is attributed to mild hyperthermia created by SPIONs, which made cells more vulnerable to PDT. Also,

nanoparticles were tracked successfully in the cells using a confocal microscope in NIR region. These particles consist of ICG, which is well accepted in the clinic, commercial, FDA approved dye/sensitizer and small, stable SPION, which may be relatively easily approved since there are FDA approved SPION contrast agents in the market. System combines dual imaging providing pre-post operative and intra-operative imaging in medical window with improved bioavailability of ICG and finally operation in NIR wavelength with a safe laser treatment procedure. These are notable advantages for commercialization and results are highly promising suggesting great potential of these SPIONs in clinic. These results are submitted to Journal of Photochemistry and Photobiology B: Biology for publication.

In Chapter 4, the success that was recorded in cancer cells with SPIONs in Chapters 2 and 3 were adopted to the treatment of planktonic and sessile bacterial cells, which is an urgent need indeed considering the increasing number of antibiotic-resistant bacterial infections. The antimicrobial activity of APTMS@SPIONs, free ICG and ICG-APTMS@SPIONs was investigated on both planktonic cells and biofilms of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus epidermidis* under laser irradiation. None of the reagents created a dark toxicity either in planktonic or biofilm form of each bacteria type. Cationic APTMS@SPIONs were studied here due to expected favorable interaction with the negative bacterial cell wall. It caused total inhibition of planktonic cell growth and a significant growth reduction only in biofilms of *Pseudomonas aeruginosa* after 10 min laser irradiation at 1150 mW (3 W/cm²) at 284 µg/mL Fe concentration. However, it did not provide any important change on both the growth of planktonic cells or biofilms of *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus epidermidis*. Free ICG induced total growth inhibition of planktonic *Klebsiella pneumoniae* and *Staphylococcus epidermidis* and noticeably reduced the growth of planktonic *Pseudomonas aeruginosa* and *Escherichia coli* under same laser irradiation procedure at 25 µg/mL ICG concentration. An important growth inhibition was also observed on biofilms with ICG, as well. ICG-APTMS@SPIONs created total growth inhibition in planktonic

cells of *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Escherichia coli* after laser irradiation at 25 $\mu\text{g/mL}$ ICG or 284 $\mu\text{g/mL}$ Fe concentration. It also caused an important reduction in the colony number of biofilm. This is indeed the first report of such antibacterial activity of APTMS@SPIONs and enhanced activity of combined PDT/PTT on biofilms in the literature. In deed, this part of the thesis work is supported by TUBITAK project no 118S547 and being conducted in collaboration with Prof. Sennaroğlu, Prof. Can, Abdullah Muti and Nazlı Ataç.

In Chapter 5, another and more frequently used combination of therapies, chemo- and photothermal therapy was demonstrated based on small, spherical SPIONs under mild laser irradiation procedure. Both SPIONs described here are hydrophilic and hence not suitable for the loading of hydrophobic/non-water soluble drugs. However, most popular chemotherapy agents are not water soluble so, BSA was conjugated covalently to PAA@SPION then loaded with Etoposide. Cytotoxicity of free Eto, PAA@SPION, BSA@PAA@SPION and Eto-BSA@PAA@SPION were evaluated by both SRB and ATP *in vitro* assays on DU145, PC3, and LNCaP prostate cancer cells. No significant toxicity was observed with PAA@SPIONs and BSA@PAA@SPIONs on either cell line. Both ATP and SRB results showed that drug was delivered more effectively to tumor cells via NPs. LNCaP found out as the most resistant cell line. The IC₅₀ value was determined as 1.3 $\mu\text{g/mL}$ and 2.5-5 $\mu\text{g/mL}$ for DU145 and PC3 cell lines, respectively. Photothermal therapy experiments were performed using 800 mW and 1000 mW laser power on DU145 and PC3 cell lines. There was no significant cell death on either cell lines at 800 mW laser irradiation. DU145 was more sensitive to laser irradiation compared to PC3 cell line. The cell viability of DU145 cell decreased to 24 % even at 0.3 and 0.6 $\mu\text{g/mL}$ Eto concentrations, indicating superior therapeutic efficiency of combination therapy and enhanced delivery of Eto to cancer cells. Detailed study of cell-death mechanism are being investigated by Prof. Ulukaya and Merve Erkisa at İstinye University.

As a conclusion, this thesis provided important findings to understand the factors

that affect the photothermal heating and the origin of this phenomenon in SPIONs. It was shown that at least in the region between 728-838 nm, it is not wavelength dependent as was suggested before, and cubes or clusters are not necessary to have a therapeutic efficiency. On the contrary, small SPIONs with functional surfaces, which are highly suitable for *in vivo* applications may be effectively utilized along with laser powers in the range of 105-800 mW without causing any damage to unsensitized cells, again an important improvement to the literature. The therapeutic efficiency and applicability of PTT, combined PTT + PDT, and combined chemotherapy+PTT was investigated on different cancer cell lines at clinically compatible NP doses and laser powers. As a last step, the antimicrobial activity of SPION based PTT and PTT/PDT combination were tested on different bacteria types and biofilms, which is one of the most critical areas requiring quick and efficient therapeutic solutions.

Based on the information acquired in this thesis work and materials that were prepared, couple more studies can be suggested for the future. From the fundamental perspective, EPR study under laser irradiation may be suggested to understand the interaction of light with SPIONs. From the practical perspective, the most important study is probably transfer of these results to animal studies to prove safe PTT and combination therapies as well as image guided PTT using these SPIONs. Investigation of photoacoustic imaging potential of these SPIONs has a great value as well in combined diagnosis and therapy and image guided local phototherapy. *In vivo* application will be studied as a part of TUBITAK project which was accepted in January 2019. In deed, preliminary investigation of this is being currently conducted by Assoc. Prof. Mehmet Burçin Ünlü at Bogaziçi University. In case of adaptation of these findings to drug resistant bacterial infections and biofilms, anionic nanoparticles, molecular targeting, photo+drug combination will be studied as a part of the Project no 118S547 funded by Scientific Research Council of Turkey(TUBITAK).

Another utility of SPION based PTT may be in treatment of cancers with drug-resistance. Hence, a new research proposal to study thermal ablation potential as well as mild hyperthermia+chemotherapy combination in treatment of drug-resistant

cancer types was submitted to TUBITAK in March 2019.

Combination of clinically used photosensitizers with SPION-based PTT may be used for different cancer types such as melanoma, breast, colon, pancreas and prostate as well as in hard to treat infections. A joint proposal with UCL and Prof. Steve Periera was submitted to TUBITAK, as well.

One final suggestions of this thesis should be development of appropriate laser systems that may be attached to catheters for practical *in vivo* and clinical applications.

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